INTERACTIONS OF β-BUNGAROTOXIN AND DENDROTOXIN WITH NERVE TERMINALS

by

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ABSTRACT

Little information is available on the molecular mechanism for the release of neurotransmitters or on the properties of macromolecular component(s) involved in this process. However, a number of neurotoxins have been identified that perturb specifically the release of neurotransmitter(s) and, thus, may be useful as probes for biochemical studies on release mechanism(s). This project entailed a study of the interactions with neuronal macromolecule(s) of two such presynaptically-acting neurotoxins, β-bungarotoxin (β-BuTx) and dendrotoxin (DTX), that exhibit inhibitory and facilitatory action, respectively, on neurotransmitter release.

β-BuTx, a dichain protein containing Ca^{2+}-dependent phospholipase A_2 activity, was purified to homogeneity from the venom of *Bungarus multicinctus* by ion-exchange chromatography followed by preparative isoelectric focussing or chromatofocusing. It was radiolabelled to high specific radioactivity (80-100 Ci/mmol) with N-succinimidyl (2,3-^{3}H) propionate. A di-^{3}H-propionylated derivative (^{3}H-β-BuTx) was obtained which was free from unlabelled toxin and devoid of phospholipase A_2 activity; nevertheless, it was highly neurotoxic and inhibited the release of neurotransmitter in rat brain slices.

Examination of the binding of ^{3}H-β-BuTx to rat cerebrocortical synaptosomes revealed a single class of non-interacting sites (150 fmol/mg protein) of high affinity (K_d = 0.60 nM) with association and dissociation rate constants of 7.8 X $10^5 M^{-1}s^{-1}$ and 5.6 X $10^{-4}s^{-1}$ at 37°C,

respectively. The binding site of ^{3}H-β-BuTx was inactivated totally by heating and trypsinization, indicating its proteinaceous nature. The binding was blocked by unlabelled β-BuTx with a K_i of 0.29 nM, indicating that tritiation did not lower significantly the affinity of the toxin. Protease inhibitor homologues, toxin I and DTX from *Dendroaspis polylepis* and *angusticeps* inhibited completely the specific binding of ^{3}H-β-BuTx with K_i values of 0.07 nM and 0.50 nM, respectively.

DTX, purified from *Dendroaspis angusticeps* by chromatography on Sephadex G-50 and Bio-Rex 70 was found to potentiate neurotransmitter release in rat and guinea-pig hippocampal slices. It was radioiodinated to high specific radioactivity (250 Ci/mmol) using a modification of the chloramine-T method. The resultant derivative (^{125}I-DTX) retained complete biological activity and was shown to bind with high affinity (K_d = 0.50 nM) to a single class of non-interacting sites (600 fmol/mg protein) on rat cerebrocortical synaptosomes. Treatment of membranes with HCl, heat, trypsin and proteinase K destroyed completely the binding activity, indicating the binding component is a protein. Unlabelled DTX and toxin I blocked totally the binding of ^{125}I-DTX with K_i values of 0.47 nM and 0.06 nM, respectively. β-BuTx inhibited the binding partially and with low potency.

Using light microscope autoradiography, the binding sites of ^{3}H-β-BuTx and ^{125}I-DTX in rat cerebellar and hippocampal sections were localized in synapse-rich regions, with lower densities of sites in other regions containing cell-bodies and myelin. Ultrastructural locali-

zation of both these toxins using electron microscope autoradiography revealed binding sites on synaptosomal plasma membrane consistent with their possible association with neurotransmitter release.

The possible interactions of β-BuTx and DTX on nerve terminals are discussed in relation to their different modes of action and the possible association of their binding component with neurotransmitter release.

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LIST OF PUBLICATIONS

Some of the data shown in this thesis have previously been presented in the following publications:

1. Biochemical and electrophysiological demonstrations of the actions of β-Bungarotoxin on synapse in brain. Halliwell, J.V., Tse, C.K., Spokes, J.W., Othman, I. and Dolly, J.O. (1982). *J. Neurochem. 39*, 543-550.

2. Preparation of neurotoxic ^{3}H-β-Bungarotoxin: Demonstration of saturable binding to brain synapses and its inhibition by toxin I. Othman I.B., Spokes, J.W., and Dolly, J.O. (1982). *Eur. J. Biochem. 128*, 267-276

3. Tritiation of β-Bungarotoxin and its saturable binding to membranes of cerebrocortical synaptosomes. Othman, I.B. and Dolly, J.O. (1982). *Biochemical Soc. Trans. 10*, 386-387.

4. Excitatory effects of dendrotoxin on the hippocampus *in vitro*. Docherty R.J., Dolly, J.O., Halliwell, J.V. and Othman, I. (1983). *J. Physiol. (Lond.) 336*, 58P.

5. Synaptic binding sites in brain for (^{3}H)-β-Bungarotoxin - A specific probe that perturbs transmitter release. Othman, I.B., Wilkin, G.P. and Dolly, J.O. (1983). *Neurochem. Int. 5*, 487-496.

6. Identification and location of a neuronal binding component for the facilitatory snake protein, Dendrotoxin. Dolly, J.O., Othman, I.B. and Halliwell, J.V. (1983). *J. Neurochem. 41*, S148.

ABBREVIATIONS

ACh	Acetylcholine
AChR	Acetylcholine-receptor
β-BuTx	β-Bungarotoxin
BV-PLA_2	bee venom phospholipase A_2
CNS	central nervous system
DTX	Dendrotoxin
EOFA	ethoxyformic anhydride
epps	end-plate potentials
^{3}H-β-BuTx	di-^{3}H- propionylated β-BuTx
^{125}I-β-BuTx	Iodinated β-BuTx
^{125}I-DTX	Iodinated DTX
mepps	miniature end-plate potentials
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TTX	Tetrodotoxin

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To my *ANI*

who cares and shares

CHAPTER I

GENERAL INTRODUCTION

1.1 NEUROTRANSMITTER RELEASE

The study of the process of transmitting messages from one part of the body to another has been a major physiological investigation for a very long time. Most of these studies have been carried out at peripheral synapses such as at vertebrate motor end-plates and the giant synapses in the stellate ganglion of the squid. These two examples of peripheral synapses form some experimental basis for the understanding of the essential features of neurotransmission in the central nervous system (CNS). Essentially, the same neuronal processes go on in the peripheral and central synapses but those in the brain are more elusive when it comes to the precise investigation, an indication of the complexities of the system in the latter.

The universal means of communication in the nervous system is the impulse or electrical signals which are transmitted and relayed from one neuron to another. The nature of the nerve impulse has been known since the 1930s and has been extensively studied especially by Hodgkin and Huxley (1952) on squid giant axons. Nerve cell maintains high internal concentrations of K^+ and low concentrations of Na^+ relative to external medium, by means of energy-dependent ion pumps; at rest, the potential difference across the plasma membrane is $\sim$ -60 mV. As an impulse is propagated along the nerve, transient changes in the permeability for Na^+ and later K^+ ions, occur in sequential portions of the membrane (Hodgkin and Huxley,1952), resulting in depolarising current (or action potential) and

repolarising currents, which are propagated along the membrane until they reach the nerve terminal. The arrival of action potentials at the axon terminal initiates a series of events that affect neurotransmission. Of these, the most important is an influx of Ca^{2+} into the nerve terminal down its electrochemical gradient (Stinnakre, 1977), which results in the release of a chemical transmitter. It has now been well established, at the vertebrate neuromuscular junction that the chemical transmitter is acetylcholine (ACh) which is released in the form of large numbers of discrete packages or quanta (del Castillo and Katz,1954). Released ACh diffuses away from the nerve terminals across the synaptic cleft towards the postsynaptic membrane, and interacts with acetylcholine receptors (AChRs) thereon. The role of this AChR is to recognize the neurotransmitter in a manner that leads to an increase of muscle membrane conductance for Na^+ and K^+ ions; these give rise to a local depolarisation (end-plate potential or epp), which will elicit an action potential in the muscle membrane after a certain threshold is reached. When the action potential is initiated, the whole muscle membrane is thus excited and contraction of muscle ensues. Meanwhile the ACh molecules remaining in the synaptic cleft are rapidly hydrolysed by the enzyme acetylcholinesterase, and an active recharging of the depolarised muscle membrane follows. Spontaneous release of ACh from a single vesicle may take place without any nerve impulse, and the depolarization of end-plate thus elicited is called a miniature end-plate potential (mepp). Details of this current concept of this event responsible for carrying the impulse from nerve to muscle

can be found in reviews as by Gage (1976), Hubbard and Quastel (1973) and Hubbard (1973).

The mechanism by which the chemical transmitter is released from the presynaptic terminal into the synaptic cleft is not fully understood at present. In 1956, del Castillo and Katz proposed that the quanta of ACh were stored in the synaptic vesicles and released from the vesicles directly into the synaptic cleft. They originally suggested that the release of ACh occurred when a specific region (reactive centre) on the vesicle membrane contacted a specialized region on the axolemma of the nerve terminal. The contact between the two specialized regions resulted in localized increases in the permeability of both membranes to ACh and the release of the vesicle content of ACh ensued. In a later model (del Castillo and Katz,1956; Katz,1962), they proposed that after alignment of the reactive centres, the membranes of the synaptic vesicles fused transiently with the axolemma, so that the interior of the vesicle was connected directly with the synaptic cleft and the ACh content was lost through diffusion. Despite the acceptance of this vesicular hypothesis by many investigators, it has been challenged by others who have suggested that the secreted ACh comes directly from the cytoplasm and that quanta arise because the membrane of the nerve terminals contains ACh channels that open for a relatively fixed period of time. The main objections to the vesicular hypothesis can be found in articles by Marchbanks (1975, 1978) and review by Tauc (1982).

To date, a large body of evidence has been reported which is in favour of the vesicular hypothesis and makes the cytoplasmic hypothesis less attractive. Firstly,

electron microscopy showed that nerve terminals contain large numbers of synaptic vesicles (Birks *et al.*,1960; De Robertis and Bennett,1954; Palade,1954; Palay, 1954, 1956; Robertson,1956; Sjostrand,1953). Since it was first proposed (del Castillo and Katz,1956), the quantal nature of transmitter release has been established at numerous vertebrate (Kuno,1971; Martin.1966) and invertebrate (Bittner and Harrison,1970; Dudel and Kuffler,1961) synapses, and vesicles have been found at virtually all chemical synapses (Elfvin,1976). Furthermore, synaptic vesicles isolated from nervous tissue by cell fractionation have been shown to contain high concentration of ACh (De Robertis *et al.*,1961, 1963; Israel *et al.*,1970; Whittaker,1959, 1965; Whittaker and Sheridan,1965; Whittaker *et al.*,1964, 1972) and isolated nerve endings have been shown to transport choline and to synthesize ACh (Dowdall,1975; Dowdall *et al.*, 1976; Fonnum,1975; MacIntosh and Collier,1976).

Recent results using the freeze fracture technique have provided further evidence that synaptic vesicles fuse with the axolemma when nerve terminals are stimulated to secrete quanta of ACh; the results with rapid freezing show that the fusion of the vesicle and the release of a quantum are closely linked in time (i.e. they occur within a few milliseconds of each other) (Ceccarelli and Hurlbut,1980). However, it has not been demonstrated that the ACh secreted as a quantum is released from a vesicle that has fused with the axolemma; this final proof of the vesicular hypothesis has not yet been forthcoming. Until this happens, the most profitable course is to adopt the vesicular hypothesis as a working hypothesis and examine the questions raised by

its acceptance.

In the CNS, there is a great deal more diversity in the types of synapses, particularly in the chemical transmitter substances used, but they do possess many common features to those at the neuromuscular junction. Most obvious of all is their dependency on Ca^{2+} for the release of neurotransmitters in response to the action potential Quantal release of transmitter has been observed at synapses in vertebrate spinal cord (Kuno,1964), and autonomic ganglia (Blackman *et al.*,1963). Furthermore, the ultrastructure of nerve terminals in the CNS is very similar to that of the vertebrate neuromuscular junction, having synaptic vesicles, intraterminal mitochondria and presence of active zones (Akert *et al.*,(1975)

1.2 EFFECTS OF SNAKE VENOM ON THE NERVOUS SYSTEM

From the current concept of neurotransmission, it is obvious that it can be impaired at any point either presynaptically or postsynaptically (Hubbard and Quastel, 1973) by various macromolecules. Even before the establishment of the mode of neuromuscular transmission, the site of action of curare was thought to be at the nerve endings (Bernard,1856). It is now beyond doubt that this agent blocks transmission by competing at the AChR with the nerve-impulse released ACh. Another group of macromolecules which have been known for centuries to produce symptoms relevant to the nervous system in the envenomed subject are snake venoms. The most characteristic of the symptoms is the paralysis of voluntary muscle which results in

respiratory paralysis. The term 'Neurotoxin' frequently used in the literature to describe a venom or a venom fraction which produces paralysis or convulsions before death in envenomed animal. This is believed to be the major causative factor of intoxication by snake venoms.

The pharmacological actions of many of these venoms, especially those of the *Elapidae* and *Hydrophidae* families, and their purified toxins have been elucidated in recent years. The sites of action when injected peripherally are mostly localized within the neuromuscular junction, a site most vulnerable to the actions of chemical agents. However, the pharmacological actions of whole venoms are in general very complicated since other constituents of the venom such as cardiotoxins, phospholipases and other related membrane toxins, may affect the nerve and muscular structure specifically or non-specifically. In order to avoid such confusion due to the multiplicity of actions of the whole venom, the latter is fractionated into its various components and the purified neurotoxins are used for pharmacological studies on the nervous systems. The actions of various types of neurotoxins at neuromuscular junction are now known to be very specific, and some of them have been currently used as powerful tools for physiological and chemical studies of the acetylcholine receptor (Dolly,1979; Conti-Tronconi and Raftery,1982).

1.3 CLASSIFICATION OF TOXINS ISOLATED FROM SNAKE VENOM

On fractionation of snake venoms, the purified toxins can be conveniently grouped into the following categories, on the basis of their pharmacological properties

(Lee,1979) :

Group 1 Postsynaptic neurotoxins

Consisting of both 'short' and 'long' curaremimetic neurotoxins.

Group 2 Cardiotoxins

Cytotoxins, direct lytic factors and membrane-active toxins.

Group 3 Toxins affecting Na^+ channels

Crotamine and related basic polypeptides of low molecular weights, mainly from rattlesnake venoms.

Group 4 Myonecrotic phospholipases A_2

Basic phospholipase A_2, notexin and taipoxin.

Group 5 Neurotoxins that inhibit neurotransmitter release

β-bungarotoxin, crotoxin, notexin and taipoxin.

Group 6 Neurotoxins that facilitate neurotransmitter release

Protease inhibitor homologues from mamba venoms; dendrotoxin, toxin I and Toxin K.

Groups 1 and 2 have been shown to exhibit sequence homology in their primary structure but they have different pharmacological actions. Neurotoxins in groups 4 and 5 may be classified together as toxins with a phospholipase A_2 structure. Only toxin groups 5 and 6 will be extensively reviewed as they are more pertinent to the current studies. Toxins in other groups will be discussed

very briefly, and for further detailed discussion of these toxins, the following reviews and articles should be referred to: Karlsson,1979; Eaker, 1975; Tu,1973, 1977; Yang,1974, 1978; Low,1979; Lee,1972 , 1979.

1.3.1 Postsynaptic (curaremimetic) neurotoxins

Postsynaptic neurotoxins produce a non-depolarizing neuromuscular blockade by binding specifically to the nicotinic ACh receptors (AChR) on the postsynaptic membrane of the motor end-plate (Lee,1970, 1972). The release of ACh from the motor nerve terminal into the synaptic cleft is not inhibited, but its binding to the receptor is blocked (Chang and Lee,1963; Lee and Chang,1966). To date, more than 40 of these toxins from the venoms of both land and sea snakes in the families of *Elapidae* and *Hydrophidae* have been sequenced (Tu,1977; Karlsson,1979), representing one of the largest homologous series of sequenced proteins. No curaremimetic toxins have been isolated from venoms of other snake families.

Postsynaptic neurotoxins can be classified into two distinct size groups called 'short' and 'long' neurotoxins (Tu,1977). Short neurotoxins (Type I) contain 60 to 62 amino acids and four disulfides bridges, whereas the long neurotoxins (Type II) have 71 to 74 residues and five disulfide bonds. The tertiary structures of these molecules have been investigated by X-ray diffraction (Low *et al.*,1976; Low,1979; Tsernoglou and Petsko,1976; Tsernoglou *et al.*, 1976) and nuclear magnetic resonance (Endo *et al.*,1979; Fung *et al.*,1979). The postulated structures for these neurotoxins have allowed speculation about the nicotinic AChR active-site conformation.

Many of these postsynaptic neurotoxins have been purified and labelled to high specific radioactivity with ^{125}I-iodine or tritium (Dolly *et al.*,1981b;Fulpius, 1976) with little loss of activity. For this reason, together with the availability of toxin-horseradish peroxidase conjugate (e.g. Lentz *et al.*,1977), and fluorescent derivatives (e.g. Andersen and Cohen,1974), these neurotoxins have proved invaluable probes for the identification, quantitation, localization, purification and characterization of the nicotinic AChR of both the peripheral (Heidmann and Changeux,1978; Dolly,1979; Karlin,1980) and central (Barnard and Dolly,1982; Norman *et al.*,1981; Morley *et al.*,1981) nervous systems.

1.3.2 Cardiotoxins

Membrane-active toxins cause a toxin-induced permeability change of cell membrane and consequently impair both the structure and functions of cells (Lee, 1971, 1972). This group consists of cardiotoxin (Lee *et al.*, 1968), the direct lytic factor (DLF) (Condrea *et al.*,1964; Aloof-Hirsch *et al.*,1968), cobramines (Larsen and Wolff, 1968), cytotoxin (Braganca *et al.*,1967) and other membrane-active polypeptides (Karlsson,1979). More than twenty of these toxins have been sequenced (Karlsson,1979) and are found to be either identical or homologous to one another. They consist of 60 to 61 amino acid residues in a single-chain cross-linked by four disulfide bonds. The main distinct characteristic of these toxins is the large number of lysines and hydrophobic residues in the amino acid composition. In addition, the invariance of the

disulfide positions in the sequence also characterizes the homology in this group (Karlsson,1979). Furthermore, their primary structures show some homologies with postsynaptic neurotoxins; the locations of four disulfide bridges are identical in all these toxins and they share some other structural or conserved residues (Gly-44, Pro-50, Asn-69 and Tyr/Phe-25). However, in spite of their strong resemblance to curaremimetic neurotoxins, they possess no postsynaptic activity.

The mode of action of these toxins is still unclear. It is assumed that the initial binding is caused by an electrostatic interaction between the positively charged toxins and the negatively charged surface of the membrane. Two hypotheses were put forward for the subsequent process. In the first case, the hydrophobic parts of the toxin penetrate into the hydrophobic layer of membrane and disrupt its structure (Klibansky *et al.*,1968) resulting in leakiness and membrane disorder. This renders the membrane phospholipid accessible to attack by phospholipase A. The hydrolysis products, lysophosphatides, are lytic and will cause further damage. In the second hypothesis, the membrane is disrupted by an interchange reaction between disulfides in the toxin and the sulfhydryl groups in the membrane (Vogt *et al.*,1970). However, subsequent reports produced evidence against this disulfide interchange mechanism (Vogt *et al.*,1970; Mollay and Kreil,1974; Fryklund and Eaker,1975).

The toxin-induced permeability change has many biochemical, pharmacological and pathological consequences such as hemolysis, cytotoxicity, depolarisation of

excitable membranes, effects on transport of anions, amino acids and glucose and effects on membranal enzymes. Details of these studies on membrane toxins can be found in various reviews (Jimenez-Porras,1968, 1970; Lee,1979, 1972; Tu, 1973; Condrea,1974, 1979).

1.3.3 Toxins affecting sodium channels

Laure (1975) isolated a strongly basic polypeptide containing 42 amino-acid residues and three disulfide bonds from the venom of *Crotalus durissus terrificus* variety *crotaminicus*. It was called crotamine and the primary sequence shows no apparent similarities with the sequences of post-synaptic neurotoxins, membrane toxins or phospholipase (PLA_2). This protein was found to produce immediate contracture followed by spontaneous and irregular twitching of rat hemidiaphragm. The effect can be completely antagonized by tetrodotoxin, Ca^{2+}, Mg^{2+} and K^+, but only partly by denervation or previous treatment with curare (Cheymol *et al.*,1971a, 1971b; Brazil and Excell,1971). Recent evidence suggests that crotamine may act at a regulatory site of the Na^+ ionophore by competing with K^+ rather than in the activation mechanism of the Na^+-channel activity (Chang and Tseng,1978).

Similar polypeptides with close resemblance to crotamine have also been isolated from other species of *Crotalus*. These include *Crotalus viridis helleri* (Maeda *et al.*,1978), *C.adamanteus*, *C.horridus horridus* and *C.viridis viridis* (Bonilla *et al.*,1971; Lee *et al.*,1972a). Peptide C isolated from the venom of *C.viridis helleri* (Maeda *et al.*,1978) contains 43 amino acid residues and

three disulfide bonds. Its sequence shows a high degree of homology with crotamine. One of the main characteristics of crotamine is that most of the basic amino acid residues are distributed in the N-terminal and C-terminal parts, leaving the middle part rather hydrophobic (Laure,1975).

1.3.4 Myonecrotic phospholipases A_2

These toxins and the inhibitory neurotoxins may also be classified together as they contain a phospholipase A_2 structure in their primary amino acid sequences. In fact, some of the presynaptic neurotoxins also display myotoxicity and the classification of such toxins as neurotoxins or myonecrotic toxins indicates only their most conspicuous characteristics.

Notexin is a potent myonecrotic toxin causing necrosis of muscle fibres with edema and infiltration of lymphocytes within 14 hours after subcutaneous injection (Harris *et al.*,1975). Taipoxin is also known to exhibit similar myotoxicity to notexin, but in a much less potent manner. In addition, notexin exhibits postsynaptic and direct musculotrophic effects, decreasing the sensitivity of the motor end plate to ACh and producing a contracture of muscle (Lee *et al.*,1977). Another neurotoxin, ceruleotoxin, an acidic protein isolated from the venom of *Bungarus caeruleus* (Bon and Changeux,1975, 1977), also possesses both presynaptic and musculotrophic actions on skeletal muscle similar to notexin (Lee and Ho,1979).

1.3.5 Neurotoxins that inhibit neurotransmitter release

This group of neurotoxins produces a neuro-

muscular blockade by acting primarily, if not exclusively, on the motor nerve endings, inhibiting the release of ACh from the presynaptic membrane; intoxicated animals die from respiratory paralysis (Chang,1979). Since the discovery of β-bungarotoxin (β-BuTx), isolated from the Formosan krait, *Bungarus multicinctus*, as a presynaptic neurotoxin (Chang and Lee,1963), several other snake neurotoxins with similar pharmacological properties have been isolated from other elapid venoms. Among those that are well characterized are notexin and notechis II-5 from the venom of the Australian tiger snake, *Notechis scutatus scutatus* (Karlsson *et al.*,1972), toxin III-A and III-B from the Indian krait, *Bungarus caeruleus* (Lee *et al.*,1976b; Lee and Tsai,1977), taipoxin isolated from the venom of *Oxyuranus scutellatus* (Fohlman *et al.*,1976) and crotoxin from the South American rattlesnake, *Crotalus durissus terrificus* (Slotta and Fraenkel-Conrat,1938). Crotoxin was the first crystalline snake toxin isolated (Slotta and Fraenkel-Conrat,1938).

Each of these neurotoxins contains phospholipase A_2 activity (Table 1-1) which is greatly enhanced in the presence of an emulsifying agent, such as sodium deoxycholate. The enzymic activity is contributed by the presence of a common polypeptide, which has extensive homology in its primary structure with phospholipase A_2 from porcine pancreas and other snake venoms (Kondo *et al.*,1978a). All these toxins except for notexin and notechis-II have at least one other subunit with differing amino acid sequences. Despite this difference in the overall structure and size, these neurotoxins have a similar pattern of presynaptic effects on acetylcholine release at motor end

Table 1-1 Neurotoxins that inhibit neurotransmitter release

	LD_{50} µg/g mouse	Molecular Weight (M_r)	Molecular weight of subunits (M_r)	PLA_2† activity µmol fatty acid/ min./mg protein	Reference
β-Bungarotoxin	0.01	20.5 K	13.5 K + 7 K	130 (63)*	1
Crotoxin	0.05	22.0 K	14.0 K + 8 K	70-75∞	2
Notexin	0.025	13.6 K	13.6 K	600	3, 4
Taipoxin	0.002	46.0 K	18 K + 14 K + 13 K	1	5

1. Kondo *et al.*,1978
2. Habermann and Breithaupt, 1978
3. Karlsson, 1973
4. Halpert and Eaker, 1975
5. Fohlman *et al.*,1976
* Strong *et al.*,1976 (Spokes and Dolly,1980)
∞ Hendon and Fraenkel-Conrat,1971
† Phospholipase A_2 , PLA_2

plates (Chang,1979) though distinct differences are apparent in their actions.

1.3.5.1 Crotoxin

Crotoxin,the main neurotoxin of the South American rattlesnake, *Crotalus durissus terrificus*, was isolated in a crystalline form as early as 1938, by Slotta and Fraenkel-Conrat (1938). It consists of a complex of at least two components; a basic phospholipase A_2 known as crotoxin B and an acidic protein, crotapotin or crotoxin A (Breithaupt *et al.*,1974, 1975; Hendon and Fraenkel-Conrat,1971; Rubsamen *et al.*,1971). The basic phospholipase A_2 is moderately lethal (540 µg/kg mouse) whereas crotapotin alone shows neither toxicity nor enzymic activity, but on recombination of both subunits practically full lethality can be restored (Hendon and Fraenkel-Conrat,1971; Rubsamen *et al.*,1971; Breithaupt *et al.*,1975). The phospholipase A_2 is a single chain of 140 amino acid residues cross-linked by eight disulfide bridges and its partial amino acid sequence has been reported (Omori-Satoh *et al.*,1975).

Crotapotin consists of three peptide chains held together by disulfide bridges; chain A contains 40 amino acid residues, chain B contains 34 residues and chain C, 14 resiudes (Breithaupt *et al.*,1974). Crotapotin not only increases the toxicity of the basic phospholipase but also suppresses its enzymic activity by as much as 90% (Breithaupt,1976). A contrary result has, however, been obtained by Fraenkel-Conrat *et al.* (1976), who reported that crotoxin A and crotoxin B have equal catalytic activities. When crotoxin B, which has one histidine residue,

was reacted with p-bromophenacyl bromide, a protein which is devoid of both lethality and catalytic activity was obtained. This derivative can combine with crotapotin, but the complex is inactive (Fraenkel-Conrat *et al.*,1976). Crotoxin has a triphasic effect on spontaneous and evoked release of ACh at the neuromuscular junction, typical of that seen with snake toxins which have phospholipase activity (Chang and Lee,1977; Hawgood and Smith,1977; Chang,1979). However, it has also been reported to have myotoxic effects (Breithaupt,1976) and to reduce the sensitivity of end plates to ACh (Vital-Brazil and Excell, 1971) and electroplaque (Bon *et al.*, 1979) to carbamyl choline.

1.3.5.2 Taipoxin

Taipoxin is the most lethal neurotoxin that has been isolated from a snake venom. The intravenous LD_{50} is only 0.002 µg/g mouse, which is even lower than β-BuTx (Fohlman and Eaker,1977). Taipoxin is a non-covalent ternary complex of three subunits-α, β and γ- which can be dissociated at neutral pH by 6M-guanidine hydrochloride. This dissociation is completely reversible with complex reformation and full toxicity restored upon removal of the guanidium hydrochloride. The three subunits have differing isoelectric points (< 2.5, ∿ 7, > 10) indicating that electrostatic forces are important in holding the complex together (Fohlman and Eaker,1977). The α-subunit is a basic phospholipase A_2 with an LD_{50} of 300 µg/g mouse, the β-subunit is a neutral phospholipase A_2 analogue but practically non-toxic. The γ-subunit is

an acidic glycoprotein with four residues of sialic acid and is also non-toxic.

Taipoxin was shown to act presynaptically by Kamenskaya and Thesleff (1974) in a similar manner to other presynaptic toxins, and has not been reported to affect the postsynaptic response. However, the α-subunit, potentiated by the γ-subunit causes muscle necrosis, similar to that produced by notexin following subcutaneous injection into rats (Harris *et al.*,1975).

1.3.5.3 Notexin and Notechis II-5

The Australian tiger snake, *Notechis scutatus scutatus* has both curaremimetic and presynaptic neurotoxins. Three homologous proteins have been isolated from the tiger snake venom. Two of them, notexin (Notechis toxin) and notechis II-5, have presynaptic effects whilst the third, notechis II-1, shows neither toxicity nor enzymic activity (Karlsson *et al.*,1972; Halpert and Eaker,1975, 1976). Both notexin and notechis II-5 are single chain polypeptides containing 119 amino acid residues and seven disulfide bonds.

A weak phospholipase A_2 activity was detected when the toxins were assayed against egg yolk (Karlsson,1973), which is greatly enhanced in the presence of sodium deoxycholate (Halpert *et al.*,1976). Notexin and notechis II-5 show great homology with porcine pancreatic phospholipase A_2 (Haas *et al.*,1970) and phospholipases from other elapid venoms. The phospholipase A_2 activity of notexin appears to be essential for its presynaptic action; upon treatment with p-bromophenacyl bromide (Volwerk *et al.*,1974; Harris *et al.*,1975), notexin loses practically all of its lethality,

phospholipase A_2 activity and myotoxicity. Like most phospholipases notexin and notechis II-5 require Ca^{2+} for activity. Notexin differs from notechis II-5 quantitatively with notechis II-5 being less toxic but with higher phospholipase A_2 activity.

Notexin acts primarily on the presynaptic site by inhibition of ACh release. The frequency of miniature end plate potential (mepps) is reduced although the amplitude distribution is widened, and the epp is reduced to a quantal size. Like β-BuTx (see below), the action of notexin needs a latent period and is accelerated by nerve impulses. The changes in fine structure of nerve terminals of the diaphragm from notexin-treated mice at death are almost identical with those induced by β-BuTx except that the depletion of synaptic vesicles is not marked (Cull-Candy *et al.*,1976). In contrast to β-BuTx, notexin causes degenerative necrosis of muscle, 12 to 24 hours after subcutaneous injection (Harris *et al.*, 1975). In isolated muscle preparations, notexin not only produces contracture but also depresses the ACh response of muscle at higher concentrations (Lee *et al.*,1976a).

1.3.5.4 β-Bungarotoxin

β-Bungarotoxin (β-BuTx) is one of the many toxins obtained from the fractionation of *Bungarus multicinctus* venom on the cation-exchanger, Sephadex CM-50 (Lee *et al.*,1972b;Kelly and Brown,1974 ; Abe *et al.*,1977; Hanley *et al.*,1977; Eterovic *et al.*,1975; Spokes and Dolly,1980). It has a molecular weight of 21000 daltons and consists of two subunits, the A and B chains, linked by disulfide bridges (Kondo *et al.*,1978a; Kelly and Brown,1974; Hanley *et al.*,1977).

The larger of the subunits, the A-chain (M_r 14000), has a considerable degree of sequence homology to pancreatic and other snake venom phospholipases A_2, whilst the sequence of the B-chain (M_r 7000 daltons) is similar to that of certain protease inhibitors and their homologues (Kondo *et al.*,1978a;Strydom and Joubert,1981; Table 1-2).

β-BuTx exhibits phospholipase A_2 activity probably due to the A-chain, which shows sequence homology with other phospholipases A_2. This enzymic activity can be inactivated by chemical modification of the toxin with p-bromophenacyl bromide (Abe *et al.*,1977; Kondo *et al.*,1978b;Halliwell *et al.*,1982) by replacement of Ca^{2+} in the medium with Sr^{2+} (Strong *et al.*,1976). Although this enzymic activity is required for both whole animal toxicity and full expression of the neuromuscular blocking action at nerve terminals (Abe *et al.*,1977), it is not sufficient to account for the potency and specificity of action of β-BuTx (Strong *et al.*,1976). This is substantiated further as inactivated β-BuTx can produce residual, transient inhibition of ACh release at the vertebrate neuromuscular junction (Chang and Su,1982). These results suggest that β-BuTx interacts with specific macromolecule(s) at the nerve terminal to cause an inhibition of transmitter release, probably mediated by its B-chain.

A protein called β-ceruleotoxin has been isolated from *Bungarus caeruleus* (Abe *et al.*,1977; Lee *et al.*, 1976b). It has similar subunit composition to β-BuTx, shows phospholipase activity in the presence of Ca^{2+} and deoxycholate and, although much less potent, produces a triphasic effect on ACh release at the neuromuscular junction like that of β-BuTx (see below). Two other toxins have been isolated

from *Bungarus fasciatus* (Bon and Saliou,1982; Bon and Changeux,1977) and *Bungarus multicinctus* (Tobias *et al.*, 1978; Livengood *et al.*,1978) venoms; these display some 'β-BuTx-like' pharmacological actions but each contains a single polypeptide with molecular weights of 12000-14000 and 11500, respectively.

1.3.5.5 The action of β-BuTx at peripheral and central synapses

Inhibitory neurotoxins have many common pharmacological actions and probably have the same mode of action on neurotransmitter release (Chang *et al.*,1977; Chang,1979). They inhibit the evoked release of ACh from the terminals of motor neurones (Lee,1972 ; Karlsson,1973; Howard,1977 ; Habermann,1978; Howard and Gundersen,1980; Chang,1979), and some but not all cholinergic neurones of the autonomic nervous system (Chang and Lee,1963; Kato *et al.*,1977; Ziegler,1978; Harris and Zar,1978; Lullmann-Rauch and Thesleff,1979). However, despite these similarities in the mode of action of these neurotoxins at motor end plates, recent pharmacological evidence showed that β-BuTx, crotoxin and taipoxin bind to separate sites thereon (Chang and Su, 1980). These neurotoxins are not known to affect non-cholinergic terminals in the peripheral nervous systems (Kato *et al.*,1977). However, notexin was shown to block motor transmission in the guinea pig vas deferens and seminal vesicles but the mechanism of this effect is unclear (Harris and Zar,1978). Amongst these presynaptic neurotoxins, β-BuTx is the most extensively studied and thus will be described in detail.

The neuromuscular blockade induced by crotoxin, notexin or taipoxin has similar characteristics to β-BuTx but these toxins differ in their sensitivity towards different species (Chang *et al.*,1977; Cull-Candy *et al.*,1976; Kamenskaya and Thesleff,1974; Chang and Lee,1977). For example, crotoxin increases mepps frequency in mouse diaphragm but not in rat diaphragm (Chang and Lee,1977) and the converse is true for taipoxin (Cull-Candy *et al.*,1976; Chang *et al.*,1977).

The inhibition of neurotransmission by β-BuTx at the neuromuscular junction was first reported by Chang and Lee (1963). Since this study, detailed electrophysiological analysis on isolated nerve-muscle preparations has revealed that the blockade occurs in three phases (Abe *et al.*,1977; Kelly *et al.*,1979a;Caratsch *et al.*,1981; Chang *et al.*,1973). Within 5 to 10 min. of the addition of β-BuTx, there is a decrease in the amplitude of end plate potentials (epps) (first phase). This is followed by an increase in evoked epp amplitude over the subsequent 30 to 60 minutes. Finally, the third phase ensues, with a decrease in the evoked epps amplitude until epps are undetectable when the nerve is stimulated. At this stage the frequency of mepps also decreases gradually until they are barely detectable when complete neuromuscular blockade is observed. Temporary recovery of the blockade was reported by repetitive stimulation or by increasing external Ca^{2+} concentration (Chang *et al.*, 1973a). The mechanism involved in causing the first phase of β-BuTx action is not known. However, replacement of Ca^{2+} with Sr^{2+} in the incubation medium (Strong *et al.*,1976)

or modification of the phospholipase active site with p-bromophenacyl bromide (Abe *et al*.,1977; Kondo *et al*.,1978b) causes only the first phase of the blockade (Abe *et al*., 1977; Kelly *et al*.,1979a). It was suggested that the toxin's phospholipase A_2 activity is required for the second and third phases (Abe *et al*.,1977; Kelly *et al*.,1979a). During the second phase, in addition to the increase in the frequency of mepps, there seems to be an increase in quantal content (Chang *et al*.,1973a;Oberg and Kelly.1976a) together with delayed release. This suggests that the second stage was caused by an increase in the level of free Ca^{2+} in the cytosol of the nerve terminals (Oberg and Kelly,1976a; Abe *et al*.,1976).

In the CNS, β-BuTx is more lethal when administered in the ventricular spaces of rat brain, than at the periphery (Hanley and Emson,1979). Even sublethal amounts of β-BuTx caused widespread neuronal damage without any selectivity towards cholinergic synapses (Hanley and Emson, 1979). Electrophysiological analysis shows that β-BuTx blocks irreversibly the release of neurotransmitter in rat olfactory cortex (Dolly *et al*.,1980) and hippocampal (Halliwell and Dolly,1982a, 1982b) slices. Similar to its effect at the periphery, phospholipase A_2 activity is required for neurotransmitter blockade; however, in the absence of enzymic activity, appreciable (20%) inhibitory activity was observed. Inactivation of the phospholipase A_2 activity of the toxin by substitution of Sr^{2+} for Ca^{2+} (Halliwell *et al*.,1982) or by modification of an essential histidine residue with p-bromophenacyl bromide greatly reduces its ability to block neurotransmission but does not prevent the ability of toxin to bind to its

component(s) at the nerve terminal. Other evidence indicating the requirement of the enzymic activity for the full expression of its action is the lack of neurotoxicity in the brain when β-BuTx was modified with N-bromosuccinimide (Hanley and Emson,1979). β-BuTx also shows a lack of neurotransmitter specificity in the central nervous system; its action on rat olfactory cortex and hippocampal slices is on areas where the neurotransmitters are predominantly amino acids (White *et al.*,1979; Collins,1979; Harvey *et al.*,1975). However, β-BuTx did not inhibit in a triphasic manner similar to that observed at the neuromuscular junction (Abe *et al.*,1976; Kelly *et al.*,1979a). It is not known whether the lack of triphasic effect is due to the measurement of aggregate synaptic responses of many cells whose accessibility to the neurotoxin varies greatly or that such a complex pattern is not exhibited in the CNS (Halliwell *et al.*,1982).

The ultrastructural changes of motor nerve terminals induced by β-BuTx and the other presynaptic toxins are identical (Chen and Lee.1970; Abe *et al.*,1976; Landon *et al.*, 1980). Rat nerve terminals blocked by β-BuTx showed a slight decrease in the number of synaptic vesicles but an increase in the invagination of plasma membranes (Chen and Lee,1970; Landon *et al.*,1980). This morphology has been attributed to a toxin-induced inhibition of the endocytosis that allows recycling of vesicles after transmitter release (Chen and Lee.1970; Lassignal and Heuser,1977).

The effects of β-BuTx on synaptosomes prepared from rat brain and *Torpedo* have been a subject of great interest in recent years. β-BuTx has been shown to produce a variety of effects on rat brain synaptosomes. In this

system, β-BuTx causes a partial inhibition of the uptake of several neurotransmitters and non-transmitter compounds including choline, deoxyglucose, γ-aminobutyrate (GABA), norepinephrine, serotonin, glutamate, aspartate and other amino acid neurotransmitters (Tse *et al.*,1980; Wernicke, *et al.*, 1974, 1975; Sen *et al.*,1976). It also causes an increase in the release of ACh, glutamate, aspartate and GABA (Spokes and Dolly,1980; Tse *et al.*,1980; Smith *et al.*,1980; Wernicke *et al.*,1974, 1975) and a decrease in plasma membrane potential, as measured with fluorescent dyes (Sen and Cooper,1978; Ng and Howard,1978). It was suggested that the initial effect of β-BuTx on synaptosomes is to decrease the synaptosomal membrane potential, thereby producing the secondary effects such as decreasing ATP levels utilized by the synaptosomal Na^+ /K^+ ATPase to re-establish the original membrane potential (Ng and Howard,1978) and inhibition of various synaptosomal transport process. The toxin does not depolarize synaptosomes by the opening of Na^+-channels of the plasma membrane since its activity on synaptosomes is independent of extracellular Na^+ concentration (Ng and Howard, 1978) and tetrodotoxin insensitive (Halliwell *et al.*,1982). In synaptosomes and nerve terminal sacs prepared from *Torpedo* electric organs, β-BuTx and other presynaptic neurotoxins block choline transport in a similar manner to that observed in rat brain synaptosomes (Dowdall *et al.*,1977; Dowdall,1977).

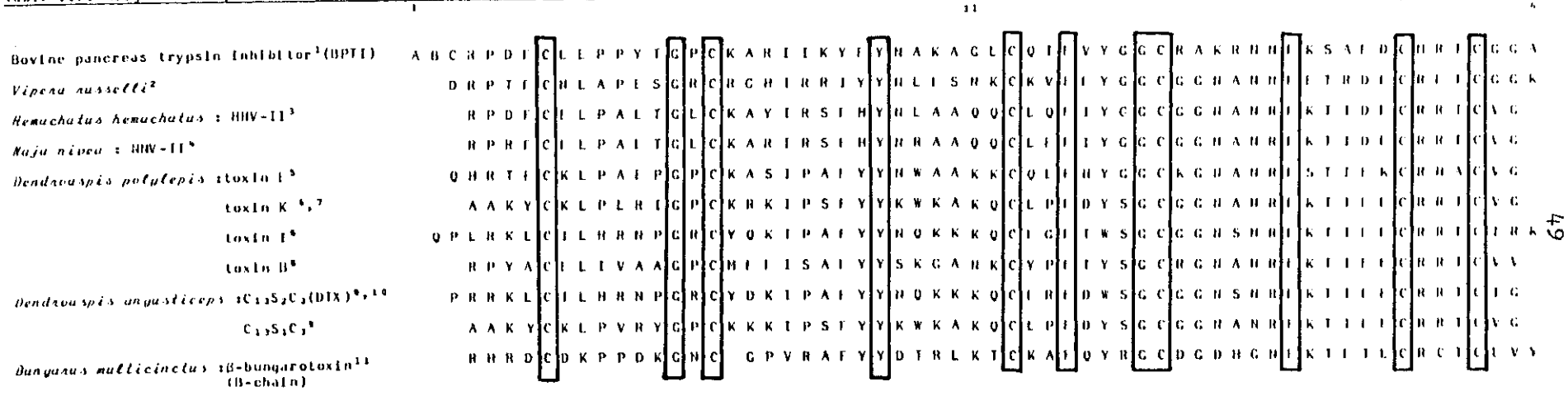
β-BuTx has also been shown to inhibit the uptake of Ca^{2+} by mitochondria from rat brain (Wagner *et al.*,1974) and by sarcoplasmic reticulum vesicles prepared from mammalian skeletal muscle (Ng and Howard,1980; Lau *et al.*,1974). However, the ability of β-BuTx to inhibit mitochondrial Ca^{2+}

uptake is lost when the toxin is converted to a non-toxic phospholipase by treatment with ethoxyformic anhydride (EOFA) in the presence of dihexanoyllecithin (DiC_6)(Ng and Howard,1980; Howard and Truog,1977). These effects may also be mechanistically relevant to the toxicity of the toxin. It has been proposed that β-BuTx and the other presynaptic neurotoxins act *in vivo* by entering the cytoplasm of the affected cell and exerting their effect on some internal structure (Kamenskaya and Thesleff,1974). If this were indeed the case, the Ca^{2+} transport systems of nerve terminal mitochondria might be the actual targets *in vivo* of PLA neurotoxins. The pathological effect produced by this toxin is consistent with this mechanism. However, β-BuTx could also produce this effect by acting at and altering Ca^{2+} extrusion across the plasma membranes of nerve terminals. At this stage, the exact mechanism of β-BuTx cannot be ascertained; nevertheless, the latter hypothesis is made more feasible by the report that β-BuTx binds to the exterior of nerve terminal membranes (Howard and Wu,1976).

1.3.6 Neurotoxins that facilitate neurotransmitter release

Initial pharmacological studies of venom from the Eastern green mamba, *Dendroaspis angusticeps*, indicate that it has several effects on nerve-muscle preparations at low concentration it facilitates neuromuscular transmission, at higher concentration, it reduces ACh sensitivity and at still higher concentration, it reduces muscle contractility (Barrett and Harvey,1979). Fractionation of the venom by chromatographic methods into its constituents

Table 1.2. Alignment of protease inhibitors from various sources.



[1] Kassell et al.,(1965), [2] Takahashi et al.,(1974), [3,4]Hokama et al.,(1976), [5] Joubert & Strydom (1978), [6] Strydom (1973), [7] Strydom (1977), [8] Strydom & Joubert (1981), [9] Joubert & Taljaard (1980), [10] Harvey & Karlsson (1982), [11] Kondo et al., (1978).

showed that the neuromuscular facilitation observed was produced by one polypeptide named dendrotoxin (DTX) (Harvey and Karrlson,1980). A number of other facilitatory neurotoxins have been isolated and sequenced from another mamba venom, *Dendroaspis polylepis*, including toxin I and toxin K (Strydom,1973b). Toxins in this group are chemically different from postsynaptic neurotoxins or cardiotoxins; they consist of 57-60 amino acid residues in a single chain with three disulfide bridges. They form the 'inactive' protease inhibitor homologues on the basis of amino acid sequence homologies with other protease inhibitors from snake venoms and other sources (Table 1-2); but the former has little or no protease inhibitory activities either on trypsin or chymotrypsin.

Interestingly, the facilitatory activity was only observed with polypeptides that exhibit close homology with DTX (Harvey and Karlsson,1982). Toxin I and toxin K differ only in 5 and 20 amino acids residues compared with DTX, respectively, and can cause the facilitation of neurotransmitter release similar to DTX. In contrast, protease inhibitor homologues from *Hemachatus hemachatus* (HHV-II), and *Naja nivea* (NNV-II) (Hokama *et al.*,1976) which have considerable more differences (30 and 31 residues respectively) in their amino acid sequences from DTX, did not exhibit any facilitatory activity. Trypsin inhibitor from bovine pancreas also has no facilitatory effects on neuromuscular transmission (Harvey and Karlsson,1982).

DTX contains 59 amino acid residues in a single chain, cross-linked by three disulfide bonds, and has no enzymic activity (Harvey and Karlsson,1980; Joubert and

Taljaard,1980).At a concentration of 0.5 µg/ml (7 X 10^{-8}M) and above, it causes an irreversible increase in responses to indirect stimulation by 200-250%; high concentrations of KCl, carbacol, ACh or direct stimulation have no effect on the observed increase, suggesting that the augmentation is caused by an increase in the amount of ACh released by nerve stimulation (Harvey and Karlsson,1980). Using a partially purified form of DTX, fraction DA3 from gel-filtration (Harvey and Karlsson,1980), it has been shown that it also causes a slow progressive increase in the quantal content of the end plate potential (epp) up to 40 times the control value (Harvey and Gage,1981). The spontaneous release of ACh was affected in a biphasic manner; firstly by reducing the frequency of mepps to 20-50% of the control during the first 40 min. after addition of toxin, followed by an increase to about four times the control. This biphasic effect was accompanied by very large spontaneous epps (giant potentials) which could be 10 times the amplitude of control mepps. Removal of Ca^{2+} from the medium containing 1 mM EGTA did not abolish the giant potentials, therefore suggesting that the latter did not result from localized action potentials in the nerve terminal (Harvey and Gage,1981). Such giant potentials have also been observed in the presence of vinblastine (Pecot-Dechavassine,1976), crotoxin (Hawgood and Santana de Sa,1979) and 3,4 diaminopyridine (Durant and Marshall, 1980). Pecot-Dechavassine (1976) suggested that the giant-potential can be a result of a multi-modal amplitude distribution induced by macromolecule(s) such as vinblastine; no evidence from the amplitude histograms was obtained for DA3,

to suggest that such a mechanism was operating for the latter (Harvey and Gage,1981).

The precise mechanism by which this group of neurotoxins facilitate neurotransmission is yet to be established. The suggestion that they cause the release of larger or fused synaptic vesicles (Harvey and Gage,1981) awaits further experimentation.

1.4 THE PRESENT STUDY

It is evident that snake venom neurotoxins may be useful as tools for studying components involved in neurotransmission. The availability of neurotoxins that specifically interfere with neurotransmitter release provides useful tools for trying to elucidate the mechanism of release at nerve terminals. Among these neurotoxins, β-BuTx and DTX have a unique specificity in that they solely affect the presynaptic nerve membrane (Harvey and Karlsson,1980; Chang and Lee,1977), with resulting effects on release of neurotransmitter. The biochemical and electrophysiological interaction of β-BuTx with peripheral and central synapses has been well documented over the recent years (Abe *et al.*, 1976; Halliwell and Dolly,1982a, 1982b). Furthermore, attempts to identify the binding component of β-BuTx on nerve membrane have been reported but the specificity of its binding site still remains to be demonstrated (Oberg and Kelly,1976b;MacDermott *et al.*,1978).

In the present study, the availability of a neurotoxic derivative of β-BuTx, radiolabelled to high specific activity with N-succinimidyl (2,3-^{3}H) propionate

provides an excellent opportunity to identify and characterize the binding sites for β-BuTx on nerve terminals. The identity of these binding sites in relation to other presynaptically acting neurotoxins including taipoxin, botulinum neurotoxin, α-latrotoxin, toxin I and DTX was investigated. The effect of other phospholipases A_2 from bee venom and *Naja melanoleuca* on ^{3}H-β-BuTx binding to cerebrocortical synaptosomes was also measured. Particular attention was focussed on the effect of the protease inhibitor homologues, toxin I and DTX isolated from the mamba venoms. These toxins inhibited completely the binding of ^{3}H-β-BuTx to synaptosomes. In contrast to β-BuTx, DTX and toxin I are very much less toxic when injected intraperitoneally and cause facilitation of neurotransmitter release at neuromuscular junction.

Following these observations, DTX was subsequently purified to homogeneity, and its effects on central synapses were investigated on rat hippocampal slices. Its effects on the uptake of $^{45}Ca^{2+}$ in synaptosomes were measured The binding properties of DTX to cerebrocortical synaptosomes was determined, facilitated by radioiodination of the toxin to high specific activity with a modification of the chloramine-T method. The effects of other presynaptically acting neurotoxins and phospholipases A_2 including β-BuTx, crotoxin, phospholipase A_2 from bee venom and *Naja melanoleuca*, on the binding of ^{125}I-DTX were also measured.

Finally, using light and electron microscope autoradiography, the binding sites of β-BuTx and DTX were localized on brain slices and synaptosomal membranes.

CHAPTER 2

PURIFICATION OF β-BUNGAROTOXIN

2.1 INTRODUCTION

β-Bungarotoxin (β-BuTx) from the venom of *Bungarus multicinctus* was the first protein among the presynaptic neurotoxins (Section 1.5) shown to exhibit a presynaptic action at nerve terminals of motor endplates (Chang and Lee,1963). Together with α-bungarotoxin (α-BuTx), they constitute the two major components obtained from the fractionation of the crude venom. Since the first report of its isolation (Chang and Lee,1961), various other methods have been employed in the purification procedures. Table 2-1 summarises these procedures and the evidence for the purity and identification of the resultant β-BuTx preparations. Clearly, some of the early evidence for homogeneity was weak or absent and, in some cases, persistent contaminants were still detectable. The incomplete purification of the toxin could result in incorrect characterization of its biological action, which could diminish its usefulness as a probe for biochemical studies.

The original designation of β-BuTx and γ-bungarotoxin for the two presynaptic neurotoxins isolated from the venom on starch-zone electrophoresis (Chang and Lee,1963) was abandoned when it was later shown that the crude venom contained at least three other presynaptic neurotoxins (Lee *et al.*,1972b). These fractions were separated by ion-exchange chromatography on CM-Sephadex C50 ; the proteins were eluted with ammonium acetate buffer of increasing ionic strength and pH. The

Abbreviations used in Table 2.I

AAA, amino acid analysis; CAE, cellulose acetate electrophoresis; CM, carboxymethyl : IEF, isoelectric focussing ; NH_4OAc, ammonium acetate ; SP, sulpho-propyl ; SDS, sodium-dodecyl sulphate .

References quoted in Table 2.I

1, Chang and Lee, (1963) ; 2, Lee *et al.*,(1972);
3, Kelly and Brown ,(1974) ; 4, Wernicke *et al.*,(1974);
5, Abe *et al.*, (1977) ; 6, Kato *et al.*, (1977) :
7, Hanley *et al.*, (1977) ; 8, Kondo *et al.*, (1978) ;
9, MacDermott *et al.*, (1978a) ; 10, Spokes and Dolly ,(1980)

Table 2-1 Purification methods for β-BuTx

Ref.		Procedure	Criteria of purity
1	(a)	Starch-zone electrophoresis	None
2	(a)	CM-Sephadex C50 gradient elution (0.05 M, pH 5.0 - 1M NH_4OAc, pH 6.8)	CAI (pH 7.4) AAA
	(b)	Rechromatography on CM-cellulose (Gradient elution with 0.1-0.3 M NH_4OAc)	
3	(a)	As 2 (a)	Gel-filtration SDS-PAGE Analytical IEF AAA
4	(a)	CM-cellulose gradient elution (0.05 M, pH 5.0-1.0 M, pH 6.8)	CAI (pH 7.4) AAA
	(b)	Gel-filtration on Sephadex G-75	
	(c)	As step (a) except gradient elution with 0.5-0.7 M NH_4OAc	
5	(a)	CM-Sephadex C25 gradient elution (0.05-0.4 M NaCl)	Native-PAGE (pH 4.3)
	(b)	Gel-filtration on Sephadex G-50	SDS-PAGE (pH 7.6)
	(c)	Rechromatography as (a) (Gradient elution with 0.1-0.3 M NaCl)	
6	(a)	Gel-filtration on Sephadex G-50	Native-PAGE (pH 4.3)
	(b)	SP-Sephadex gradient elution (0.35-1 M NaOAc)	
7	(a)	As 2 (a)	SDS-PAGE
	(b)	Gel-filtration on Sephadex G-50	Analytical IEF
	(c)	Rechromatography on Bio-Rex 70 (Gradient elution with 0-1 M NaCl)	AAA
8	(a)	As 2 (a)	Native-PAGE (pH 4.3)
	(b)	Gel-filtration on Sephadex G-75	SDS-PAGE (pH 7.6) Analytical IEF AAA
9	(a)	CM-Sephadex C25 gradient elution (0.05-1 M NH_4OAc, pH 5.6-pH 7.0)	CAI (pH 7.4) Native-PAGE (pH 7.0)
	(b)	Rechromatography on SP-Sephadex C25 (Gradient elution with 0.21-0.5 M NaCl)	SDS-PAGE (pH 7.2)
10	(a)	As 2 (a)	Native-PAGE (pH 4.3)
	(b)	Preparative isoelectric focussing on Sephadex G-75	SDS-PAGE (pH 7.6) Analytical IEF

most abundant of the presynaptic neurotoxins was designated β-BuTx (Lee *et al.*,1972b)and this designation was retained in other preparations of the toxin by other workers (Table 2-1). Furthermore, nearly all subsequent preparations of β-BuTx have involved chromatography on an ion-exchanger similar to that used by Lee *et al.*,(1972b) but this was followed by a further fractionation step. Although a few of the early reports claimed that a pure preparation of β-BuTx was obtained using only these procedures (Kelly and Brown,1974; Abe *et al.*,1977), other workers found that such preparations were heterogeneous; at least two contaminating proteins were found (Lee *et al.*, 1972b;Wernicke *et al.*,1974; Abe *et al.*,1980). Despite these difficulties in obtaining pure toxin in the early preparations, Spokes and Dolly (1980) have isolated homogeneous β-BuTx using preparative isoelectric focussing on Sephadex G-75, after the initial fractionation on CM-Sephadex C50.

In addition to β-BuTx, four other β-type neurotoxins have also been isolated and their primary sequences determined (Kondo *et al.*,1978a,1982a, 1982b). These β-type bungarotoxins are related to β-BuTx by having one homologous subunits, either the A or the B-chain. They share some common physical properties including an estimated molecular weights of 21000; each has two subunits, the A-chain ($M_r \sim 13000$) and the B-chain ($M_r \sim 7000$) linked by disulfide bridges. However, they exhibit quantitative differences in their toxicity and phospholipase A_2 activity (Kondo *et al.*,1982a, 1982b). Alignment of the sequences of β-BuTx and the β-type neurotoxins (identified as β_2, β_3

β_4 and β_5-bungarotoxins) revealed the following homology: i) the B-chain of β_3 and β_5-bungarotoxins were identical to the B-chain of β-BuTx and were designated B-1 type. β_2-toxin has an identical B-chain sequence to β_4-toxin and this was termed B-2 type. The amino acid substitutions between the B-1 and B-2 chains were found in 22 residues. ii) the A-chains of the five β-toxins can be classified into 3 types : A-1 denotes the A chains of the β-BuTx and β_2-toxins, A-2 comprised those of β_3 and β_4 toxins, and A-3 referred to that of β_5-toxin. In these cases, the amino acid displacements between A-1 and A-2, A-1 and A-3, and A-2 and A-3 chains were 5, 9 and 12 residues respectively.

Dissimilarity in the B-chains of the β-type bungarotoxins seem not to change significantly their neurotoxicity and phospholipase activities (Kondo *et al.*,1982a, b). On the other hand, substitution of some of the amino acid residues in the A-chains, particularly by consecutive displacement in residues 55-60 of the A-3 chain, markedly decreased the neurotoxicity of the protein (Kondo *et al.*, 1982b). It was noteworthy the two types of B-chains and the three types of A-chains had homologous amino acid sequences to proteinase inhibitors (Strydom and Joubert, 1981) and phospholipases A_2 (Kondo *et al.*,1982b) respectively.

In this chapter, purification of β-BuTx to complete homogeneity from the crude venom of *Bungarus multicinctus* will be described using preparative isoelectric focussing or chromatofocusing. It has been shown previously, that by using preparative isoelectric focussing (Spokes and Dolly, 1980), it is possible to separate four

different contaminants in peak 5 over a narrow range pH-gradient on Ultrodex. Similar criteria to that used by these workers will be employed to determine the purity of the β-BuTx obtained in this study. These criteria were also used to determine the homogeneity of β-BuTx isolated by chromatofocusing, a new column chromatography offerring high resolution in the separation of proteins based on differences in their isoelectric points, with the added advantage of the high capacity of the ion-exchange resins. The advantages of this method over preparative isoelectric focussing will be discussed. In addition, chromatofocusing will also be employed to separate the different components of peak 4 and 6 obtained from fractionation of the venom of *Bungarus multicinctus* on CM-Sephadex C50, in attempts to isolate the other β-type neurotoxins present in the venom; these components were characterized with respect to their molecular size and charge.

2.2 MATERIALS

Bungarus multicinctus venom was obtained from Miami Serpentarium Laboratories, Florida, USA. Pharmalytes (pH 8-10.5) and Ampholines (pH 7-9, and pH 9-11) carrier ampholytes were obtained from Phamacia and LKB, respectively. Polybuffer PBE 118, Sephadex G-25, G-50 and CM-Sephadex C50 were purchased from Pharmacia. Egg-yolk phosphatidylcholine (grade 1) and Ultrodex were obtained from Lipids Products, Surrey, UK. and LKB respectively. Sodium deoxycholate, α-chymotrypsinogen A, trypsin inhibitor (soya bean),

cytochrome C, myoglobin and bovine serum albumin were from Sigma. All other chemicals and reagents were of analytical grade unless otherwise specified.

2.3 METHODS

2.3.1 Ion-exchange chromatography of *Bungarus multicinctus* venom

Chromatography on CM-Sephadex C50 was performed by a modification of the procedure used by Lee *et al.*, (1972b). CM-Sephadex C50 was equilibrated with 0.05M ammonium acetate (NH_4OAc) buffer, pH 7.0 at 4°C and then packed into a column (2.5 X 100cm). The venom (0.5g) dissolved in 8ml of the same buffer was centrifuged at 39000 X g for 15 min, to remove any insoluble materials. The supernatant was applied to the column and the latter was washed with 0.05M NH_4OAc, pH 7.0 until the A_{280}nm on the UV monitor had returned to baseline. Adsorbed protein was eluted with a convex gradient using 400ml of 0.05M NH_4OAc, pH 7.0 and 0.9M NH_4OAc, pH 7.4; 4ml fractions were collected. Protein concentration was determined from A_{280}nm and NH_4OAc concentration calculated from conductivity measurements. Fractions in peaks 4, 5 and 6 were pooled and desalted on Sephadex G-25 (coarse) column (2.5 X 50cm) equilibrated with 0.05M NH_4OAc pH 7.0. Proteins were lyophilized and stored at 4°C until required for further purification.

2.3.2 Preparative isoelectric focussing

Fraction 5, separated by the ion-exchange chromatography, was subjected to isoelectric focussing on a

flat bed of Ultrodex (specially purified Sephadex G-75 [superfine] for electrophoresis) on a LKB 2117 Multiphor apparatus as described by Winter *et al.* (1975). Ultrodex (1.6g) was mixed with deionized water (38ml) and 2ml of Pharmalyte pH 8-10.5. Three filter paper wicks, soaked in a solution of ampholyte of the same concentration as in the slurry, were placed at each end of a glass-bottomed trough (2.8 X 23cm). The trough was weighed and after degassing, the slurry was poured evenly into it. The bed was reweighed and the weight of the slurry determined; the latter was then air-dried to a predetermined evaporation limit of 65% set by the manufacturer (the weight of the slurry was reduced by 35%) to produce a bed of the correct consistency. The trough was placed on a cooling plate through which ice-cold water was circulated. Electrical contact with electrodes was made with a further wick at each end, soaked with 1M NaOH and 1% Pharmalyte pH 8-10.5 at the cathode and anode ends, respectively. After prefocussing for 30 min at 8W, constant power, the sample ($\sim$ 25mg dissolved in 0.2ml of water) was applied in a narrow band towards the cathode end of the bed. The gel was focussed for 4 hours at 10W constant power. When focussing was complete, a print of the bed was made with strips (3mm) of filter paper (Whatman No.1), and this was used to locate the protein bands; the paper print was fixed in 10% trichloroacetic acid (w/v) and stained for protein with 0.04% Coomassie brilliant blue R-250 in methanol/water/acetic acid (5:5:1 by volume) and destained in the latter solvent mixture. The proportion of resin bed containing the major protein band was cut out and

layered on a column of Sephadex G-50 (superfine) equilibrated in 0.05M NH_4OAc, pH 7.0, to remove the Pharmalytes. The protein peak was lyophilized.

2.3.3 Chromatofocusing

This was performed on Polybuffer PBE 118 according to the method detailed in Pharmacia Handbook (1980). The resin was equilibrated with 0.025M triethylamine-hydrochloric acid (TEA-HCl) pH 11 and packed into a column (1 X 30cm). A freeze-dried sample ($\sim$ 25mg) of fraction 5 material was dissolved in 2ml of eluent buffer consisting of 2.2% (v/v) Pharmalytes (pH 8-10.5) pH 8; 5ml of the latter buffer was applied to the column prior to loading of proteins. All buffer used was degassed before use. Adsorbed proteins were eluted with the eluent buffer; approximately 10-12 column volumes were required for elution of proteins with the pH gradient used. Protein was monitored by A_{280nm}; pH of the fractions was measured using a microelectrode. Protein peaks were pooled and lyophilized before removal of Pharmalytes by gel filtration on Sephadex G-50 (superfine) in 0.05M NH_4OAc buffer, pH 7.0. Chromatofocusing was also performed on peaks 4 and 6 in a similar manner described above for peak 5, in an attempt to separate the other β-type bungarotoxins.

2.3.4 Characterization of protein peaks: Analytical methods

2.3.4.1 Analytical isoelectric focussing

This was performed for 2h at 8W in slabs gel of polyacrylamide prepared as described in Table 2-2.

Table 2-2 Composition of polyacrylamide gel for isoelectric focussing

	Volume (ml)
Acrylamide (29.1%; w/v)	10.0
Bisacrylamide (0.9%; w/v)	10.0
Ampholine pH 9-11 (40%)	2.4
Ampholine pH 7-9 (40%)	0.6
Glycerol (87%; v/v)	7.0
Distilled water	30.0
Ammonium persulfate (1%; w/v)	2.0

Electrical contact between the platinum wire electrodes and the gel surface was made through filter paper wicks soaked in 2% Ampholine pH 7-9 and 1 M sodium hydroxide for the anode and cathode, respectively. Samples (∿ 50 µg) were applied to the surface of the polyacrylamide gel in 5 µl aliquots on a small (2 X 8 mm) filter paper (Whatman No. 1). Focussing was taken to be completed when cytochrome C, used as a marker and applied to a separate track, was focussed into a sharp band. The protein bands on the gel were stained with 0.04% (w/v) Coomassie brilliant blue G-250 in 3.5% (w/v) perchloric acid and destained in the latter solution.

2.3.4.2 Polyacrylamide gel electrophoresis (PAGE)

This was carried out at pH 4.3 in the β-alanine/acetate slab system described by Reisfeld *et al.*(1962), in gels consisting of 10% acrylamide and 0.25% bisacrylamide. Bromocresol green was used as a tracking

dye and the protein bands were stained with the staining solution described above for the isoelectric focussing gels (Section 2.3.4.1).

Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Swank and Munkres (1971) using 12.5% (w/v) acrylamide, 1.25% (w/v) bisacrylamide and 8M urea in the final gel. Samples were analysed under non-reducing and reducing [10% (v/v) β-mercaptoethanol in sample buffer] conditions. After electrophoresis the gels were fixed with 25% isopropanol/10% acetic acid, stained with 0.04% Coomassie brilliant blue R-250 in 45% methanol/9% acetic acid. Chymotrypsinogen A, myoglobin, trypsin inhibitor (soya bean) and cytochrome C were used as molecular weight markers; 10-50 µg of each protein were applied to the same track. Under reducing conditions, proteins in sample buffer containing β-mercaptoethanol were boiled for 3 min. prior to loading to gel tracks.

2.3.4.3 Assay of phospholipase activity

This enzymic activity was measured by a titrimetric method using a Radiometer autotitrator as a pH-stat. After evaporation of the chloroform/methanol mixture under a stream of nitrogen, the egg phosphatidyl choline (grade 1) was dispersed by sonication in 100 mM NaCl/10 mM $CaCl_2$, pH 8.0, solution. Aliquots (5ml) of this solution containing 10 µmol of substrate was added to a 5ml beaker, followed by the addition of 10 µmol of the emulsifying agent, sodium deoxycholate (unless otherwise specified); the mixture was equilibrated to pH 8.0. When a steady baseline was obtained,

toxin (in distilled water) was introduced to initiate the reaction. Fatty acid liberated in the incubation mixture was titrated with 1 mM NaOH to pH 8.0, by the autotitrator. The rate of hydrolysis of phosphatidyl choline was linear for at least the initial 5 min. Activities were calculated from the initial slope within this time period and expressed as μmol of H^+ liberated min^{-1} mg of $protein^{-1}$. Duplicate assays were reproducible within 6-8%.

2.3.4.4 Lethality determination

This was determined following administration of toxin by two different routes:-

i) Peripheral toxicity: This was carried out by intraperitoneal injection of toxin samples (dissolved in 0.85% NaCl/0.01% bovine serum albumin) into 15-20 g mice; at least 4 animals were used for each dosage tested.

ii) Central toxicity: This was determined by intraventricular administration of toxin samples into rat brain. Sprague-Dawley rats (∿ 200 g) were anaesthetised with fluorothane and positioned on a stereotaxic apparatus. Toxin samples (10 μl) dissolved in 0.9% saline were injected into the cerebral intraventricular space at coordinates lateral (L) 1.7 mm, sagittal (S) -0.2 mm, and depth (D) 4.0 mm, according to the streotaxic atlas of Koenig and Klippel (1963); 4 animals were again used at each dosage tested.

2.4 RESULTS

2.4.1 Fractionation of *Bungarus multicinctus* venom

Initial chromatography of the venom of *Bungarus multicinctus* on CM-Sephadex C50 gave an elution profile as shown in Fig. 2.1, which was similar to that obtained by Spokes and Dolly (1980). A small amount of non-proteinaceous material (Peak 1), tentatively identified as guanosine (Lee *et al.*, 1972b),was removed by washing the column. Adsorbed proteins were eluted using a convex gradient of 0.05 M to 0.9 M NH_4OAc, pH 7.4. Using the major peak positions for alignment, comparison of the elution profile with earlier published figures (Spokes and Dolly, 1980; Lee *et al.*,1972) identify the following: fraction 2 consists of α-BuTx, fraction 5 comprises the pre-synaptically-acting β-BuTx components, fractions 4 and 6 include the other β-type neurotoxins. Fractions containing peaks 4, 5 and 6 were pooled, desalted on Sephadex G-25 (coarse) and freeze-dried for further purification steps.

When the material from peak 5 was subjected to PAGE at pH 4.3 under native conditions, 2 protein bands were obtained (Fig.2.2A). On analytical isoelectric focussing of the material on a pH range of 8-11, at least four components were visible by protein staining with Coomassie G-250 (Fig. 2.3A). This result indicated that this preparation was not homogeneous. Furthermore, the impurities observed on analytical isoelectric focussing were not revealed by SDS-PAGE which gave a single protein band in the absence of a reducing agent (Fig. 2.4A) and two bands when the sample was treated with 10% β-mercaptoethanol (Fig. 2.4B). This, it would appear

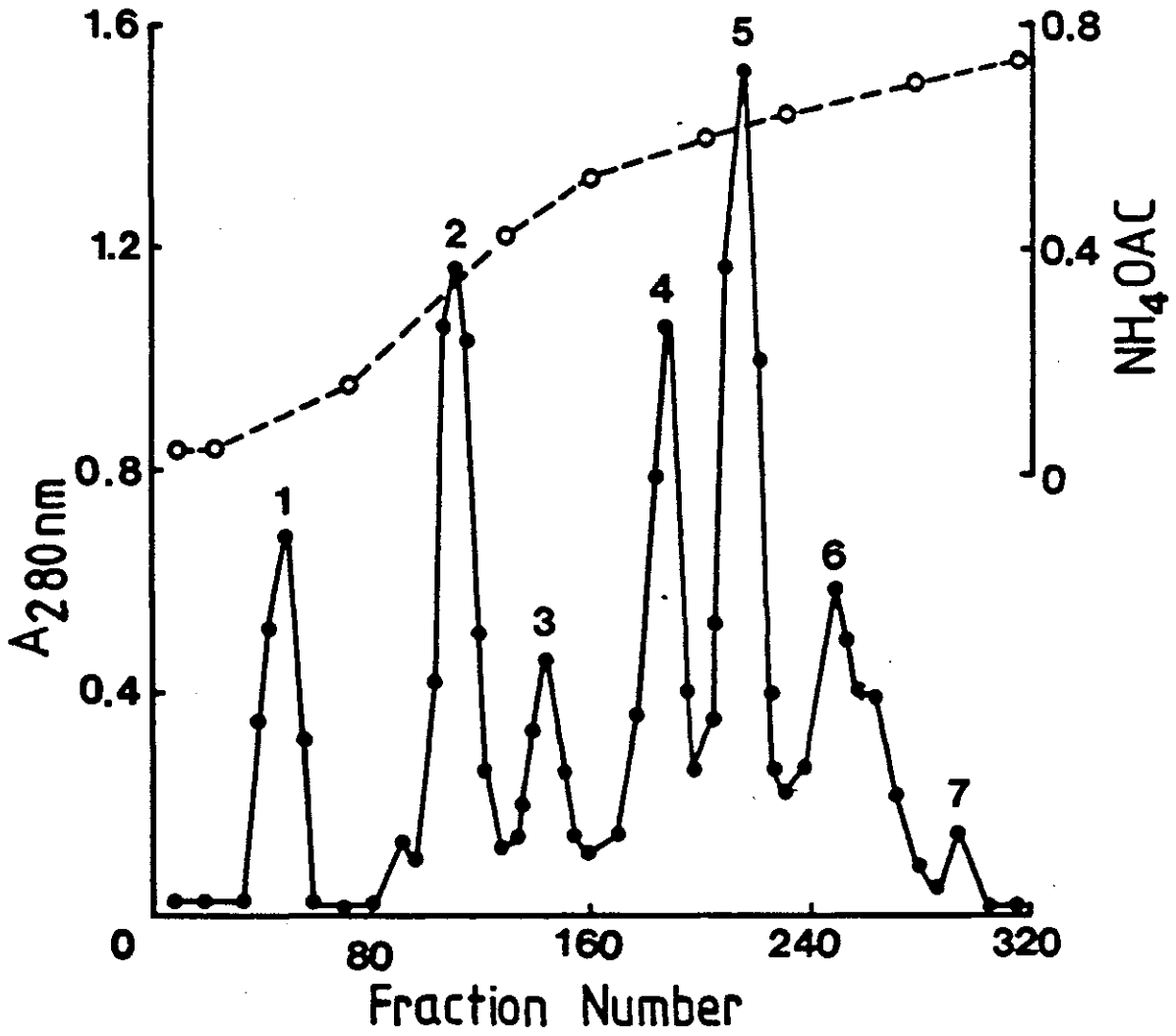
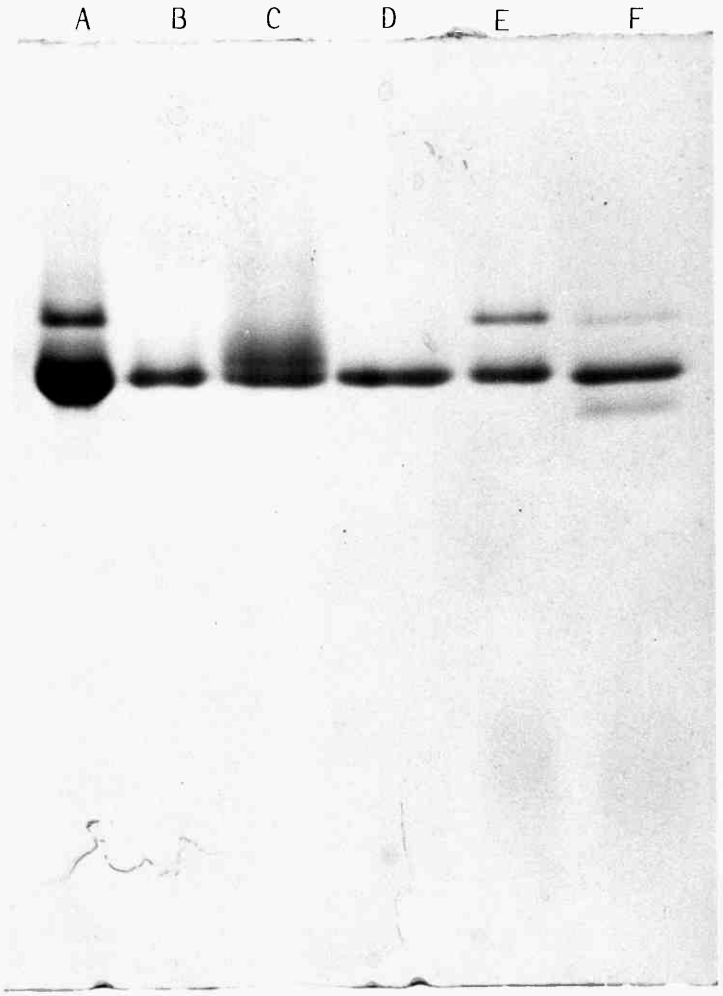


Fig. 2.1 Ion-exchange chromatography of *Bungarus multicinctus* venom

Chromatography of venom (500 mg) was carried out as described in Section 2.3.1. Elution from a carboxyl Sephadex C50 column was performed using a convex gradient of ammonium acetate concentration (○); 4 ml fractions were collected and the protein concentration measured by A_{280} nm (●).

Fig. 2.2 Polyacrylamide gel electrophoresis of β-BuTx under non-denaturing conditions



Native-PAGE at pH 4.3 was carried out as described in Section 2.3.4.2. Samples (∿ 25 µg) were applied at the anodic end of the gel; protein bands were stained in Coomasie G-250 (0.04%; w/v) in perchloric acid (3.5%; w/v)

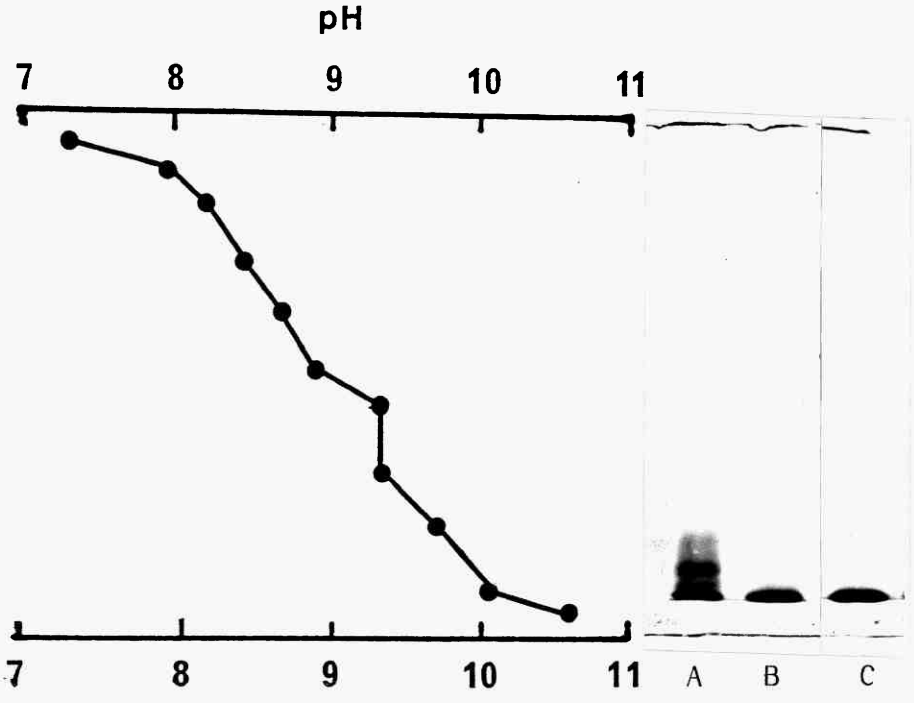
A Fraction 5 from Sephadex C50 column (Fig. 2.1)

B Major component from preparative isoelectric focussing of fraction 5 material

C, D, and E are fractions 5-1, 5-2 and 5-3 respectively, from chromatofocusing of fraction 5 material (Fig. 2.6)

F Fraction 6-2 of fraction 6 from CM-Sephadex C50 column (Fig. 2.1)

Fig. 2.3 Analytical isoelectric focussing of β-BuTx



Focussing of material from peak 5 following ion-exchange chromatography of initial venom fraction (50 µg) (A), pure β-BuTx from preparative isoelectric focussing (B), and from chromatofocusing (C). After focussing the pH gradient (●) was measured with a micro-electrode. The proteins were stained in G-250 (0.04 w/v) in perchloric acid (3.5 w/v).

Fig. 2.4 SDS-PAGE of β-BuTx preparations

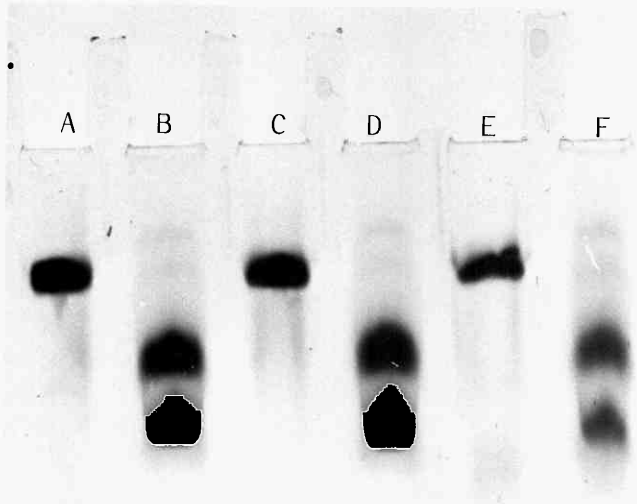
Electrophoresis was carried out in a gel containing 12.5% acrylamide, 1.25% bisacrylamide and 8 M urea as described in Section 2.3.4.2.

1. A Impure β-BuTx under non-reducing conditions
   B Impure β-BuTx under reducing conditions
   C & D Pure β-BuTx from preparative isoelectric focussing under non-reducing and reducing conditions, respectively
   E & F Pure β-BuTx from chromatofocusing under non-reducing and reducing conditions, respectively

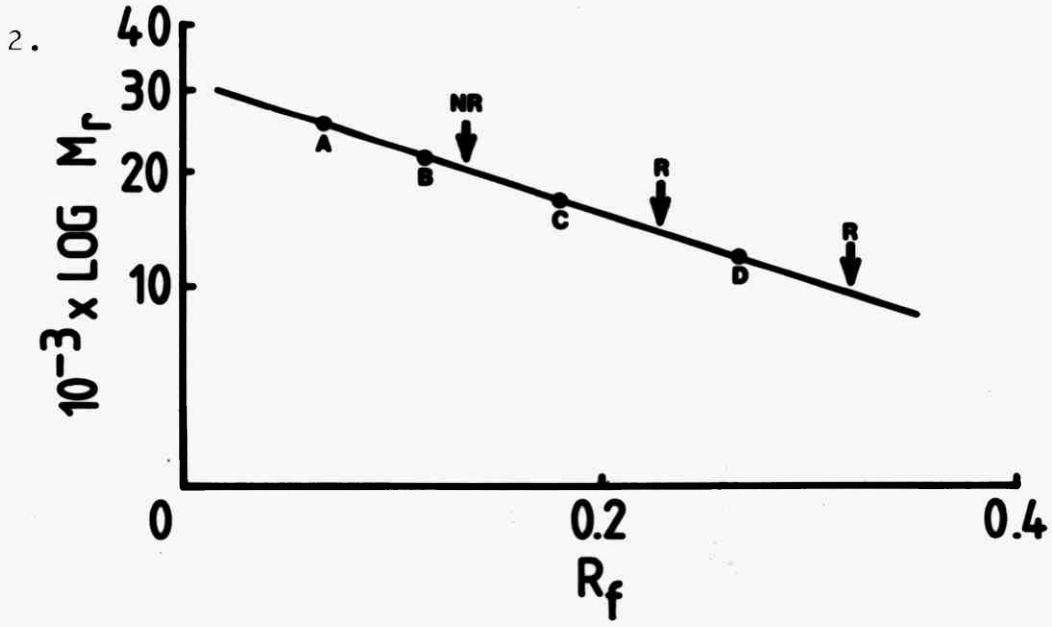
2. Semilogarithm plot of the relative mobilities and molecular weights of protein standards.
   A Chymotrypsinogen A
   B Myoglobin
   C Trypsin inhibitor (soya bean)
   D Cytochrome C

Arrows indicate the position of toxin bands under non-reducing (NR) and reducing conditions (R).

1.



2.



that the contaminating proteins have similar molecular size and subunits as β-BuTx.

2.4.2 Further purification of β-BuTx

2.4.2.1 Preparative isoelectric focussing

As shown above, when the material from peak 5 of the venom fractionation was subjected to analytical isoelectric focussing, one major and three minor components were observed (Fig. 2.3A). This result indicated that the impurities could be separated by isoelectric focussing on a preparative scale. Using Pharmalyte pH 8-10.5 as the amphoteric carrier, preparative isoelectric focussing of fraction 5 was carried as described in Methods. Paper prints of the focussed proteins (Fig. 2.5) showed the separation of three contaminants which were more acidic than the major component (pI 10.5); the former were stained at a rather low intensity.

The protein eluted from the major band was shown to be homogeneous on PAGE at pH 4.3 (Fig. 2.2B) and analytical isoelectric focussing (Fig. 2.3B); henceforth, it will be referred to as β-BuTx.

2.4.2.2 Chromatofocusing

Preparation of pure β-BuTx by preparative isoelectric focussing gave homogeneous toxin but normally the yield was low (Table 2.3; ∿ 23% of starting material). The availability of resins for chromatofocusing made it possible for the purification of β-BuTx to be carried out using the latter method. Chromatofocusing of peak 5

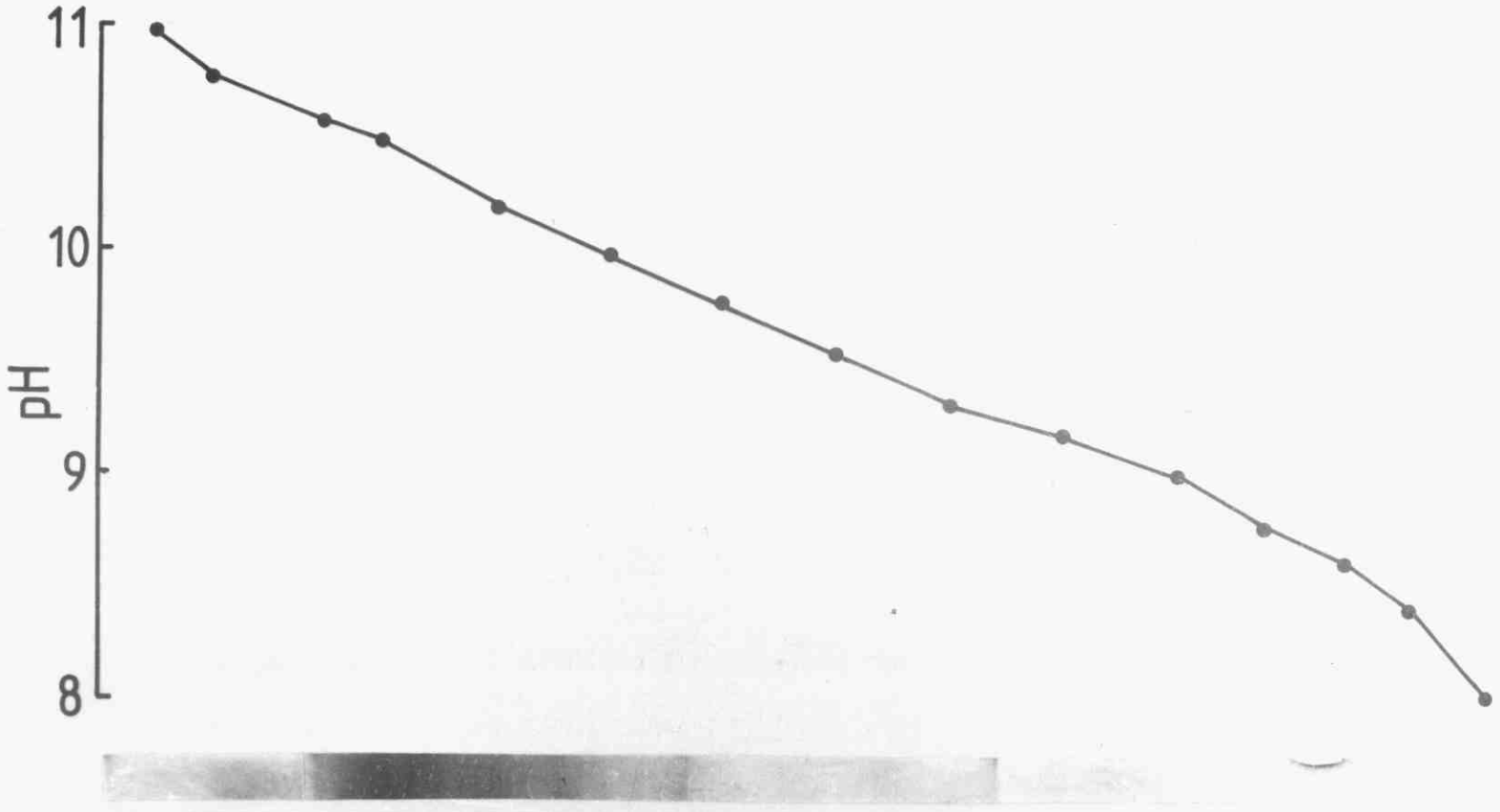


Fig. 2.5 Preparative isoelectric focussing of β-BuTx

Material from peak 5 from CM-Sephadex C50 (Fig 2.1) column was focussed in a flat bed of Ultrodex as described in Section 2.3.2. After focussing, the pH gradient (●) was measured with a microelectrode and a paper print of the bed was fixed with trichloroacetic acid and stained with Coommasie R-250 as described in Section 2.3.2 ; 24 mg of peak 5 material was used with 2 % (w/v) Pharmalyte pH 8-10.5 carrier ampholytes.

on Polybuffer PBE 118, which has high resolving capacity for basic proteins, was carried out with 2.2% (v/v) Pharmalyte (pH 8-10.5), pH 8, as the elution buffer. Elution profile of peak 5 (Fig. 2.6) showed 3 peaks; the major one will be referred to as fraction 5-2. Protein from this peak was gel-filtered to remove the carrier ampholytes, lyophilized and characterized in similar manner to the β-BuTx obtained from preparative isoelectric focussing. Table 2.3 shows the total amount of protein recovered from each peak fraction of the chromatofocusing profile. It is clear that the recovery of protein from chromatofocusing was much higher than from preparative isoelectric focussing. Furthermore, it provided the further advantage of allowing the other protein peaks to be obtained in addition to the major component.

Fraction 5-2 was shown to be homogeneous on PAGE at pH 4.3 (Fig. 2.2D) and analytical isoelectric focussing (Fig. 2.3C); it exhibited identical electrophoretic mobilities as the major band obtained from preparative isoelectric focussing and, henceforth, will be referred to as β-BuTx. Fractions 5-1 and 5-3 exhibited the presence of more than one protein band (Fig. 2.2C,E) indicating that the impurities were still present in these fractions; they were not characterized any further.

2.4.3 Properties of purified β-BuTx

β-BuTx purified by preparative isoelectric focussing and chromatofocusing was characterized with

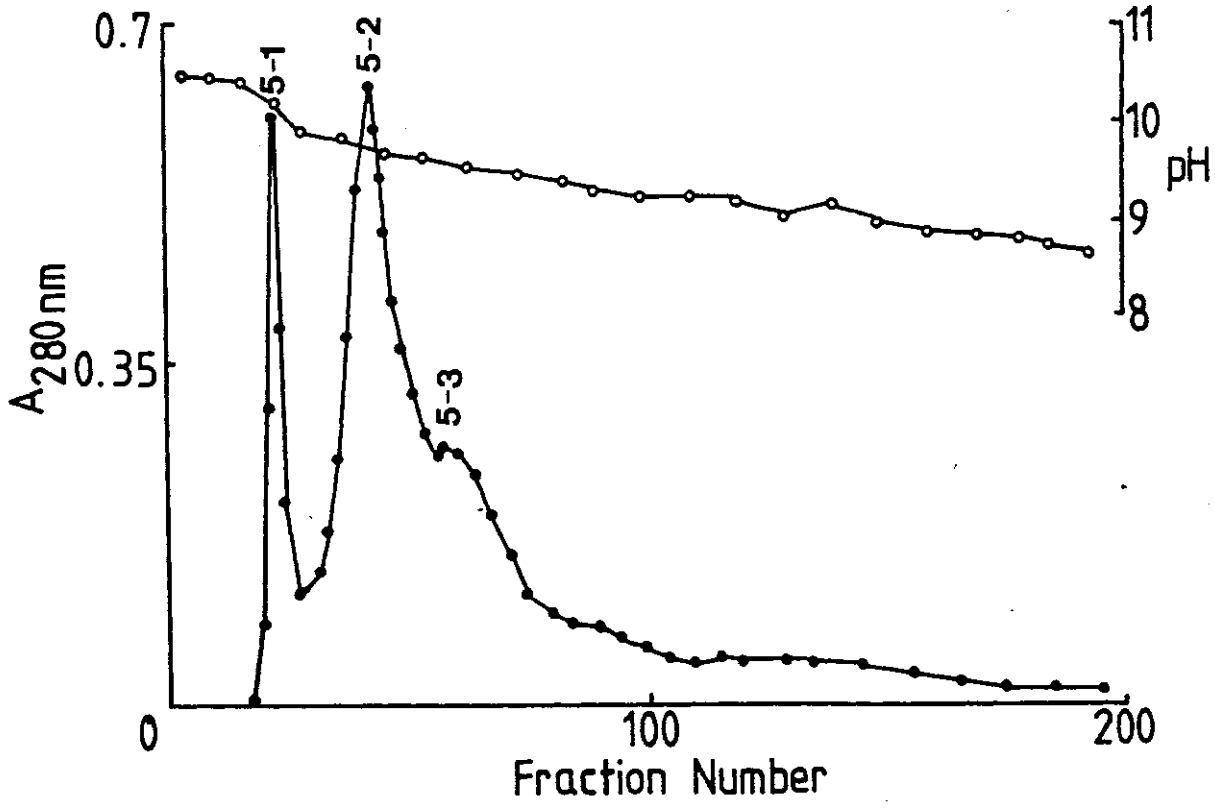


Fig. 2.6 Chromatofocusing of peak 5 material obtained from CM-Sephadex C50 chromatography

This was carried as described in Section 2.3.2. Proteins were eluted using 2.2% (v/v) Pharmalyte (pH 8-10.5), pH 8, from polybuffer PBE 118 column. 500 µl fractions were collected, protein concentration (●) was measured by A_{280} nm (●), and pH (○) measured using a microelectrode; 24.4 mg of peak 5 material was used.

Table 2-3 Recovery of protein following isoelectric focussing and chromatofocusing of β-BuTx

METHODS	COMPONENTS	AMOUNTS (mg)
Preparative isoelectric focussing (24 mg of Peak 5)	Major band (β-BuTx)	5.4
Chromatofocusing (24.4 mg of Peak 5)	5-1	2.5
	5-2 (β-BuTx)	8.4
	5-3	5.9

Amount in parenthesis indicate the initial amount of protein material from peak 5, obtained from CM-Sephadex C50 chromatography, used.

respect to molecular size and charge. The toxin exhibited a single band on native-PAGE at pH 4.3 (Fig. 2.2,B, D) and had an isoelectric point of 9.9 (±0.04; n=5) (Fig. 2.3, B, C). The pure toxin from both preparations also gave a single band on SDS-PAGE, with an apparent molecular weight of 20000 (±1000; n=4) under non-reducing condition (Fig. 2.4C, E); after reduction with β-mercaptoethanol, two protein bands corresponding to molecular weights of 11400 (±500; n=4) and 9000 (±750; n=4) were obtained (Fig. 2.4D, F). These preparations of β-BuTx appeared homogeneous with respect to size as well as charge. These results are in close agreement with those reported for β-BuTx (Spokes and Dolly,1980; Kondo *et al.*,1975a; Hanley *et al.*,1977), although some of these authors used only the novel chromatographic methods for β-BuTx purification (Kondo *et al.*, 1975a; Hanley *et al.*, 1977).

2.4.4 Lethality and phospholipase A_2 activity of β-BuTx

The peripheral lethality of β-BuTx from both preparations was tested over a range of 0.5-0.005 μg/g body weight in 15-20 g mice. As previously observed (Chang and Lee,1963; Spokes and Dolly, 1980) the toxin took at least one hour to kill the mice even at the highest dose. The data shown in Table 2-4A show that the minimum lethal dosage (MLD) of β-BuTx after intraperitoneal injection into mice was estimated to be 10 ng/g body weight for both preparations of the toxins.

The lethality of β-BuTx was also monitored by direct administration of the toxin into the intraventri-

Table 2-4 A Whole animal toxicity of β-BuTx purified by preparative isoelectric focussing (PIEF) and chromatofocusing (CHROM)

Route of administration	Dose (µg/g body wt)	β-BuTx from PIEF		β-BuTx from CHROM	
		No. of animals injected	No. of animals killed	No. of animals injected	No. of animals killed
Intraperitoneal injection	0.005	6	0	4	0
	0.01	6	5	4	3
	0.05	6	6	4	4
	0.1	6	6	4	4
	Dose (ng/g body wt)				
Intraventricular injection	10	4	4	2	2
	5	4	4	2	2
	1	4	4	2	2
	0.5	4	4	2	2
	0.05	4	3	2	2
	0.025	4	0	2	0
	0.005	4	0	2	0

B. Whole animal toxicity of bee venom phospholipase A_2 (BV-PLA_2)

	Dose (µg/g body wt)	BV-PLA_2	
		No. of animals injected	No. of animals killed
Intraperitoneal injection	0.1	2	0
	1	2	0
	2	2	2
	5	2	2
	Dose (ng/g body wt)		
Intraventricular injection	10	2	2
	5	2	2
	1	2	0
	0.5	2	0
	0.05	2	0

cular spaces of rat brain, using a stereotaxic apparatus. By this route, the minimum lethal dose of the toxin was about 200 times lower than for the peripheral toxicity (0.05 ng/g vs 10 ng/g body weight, respectively; Table 2-4A), indicating that the toxin is more potent in the central than in the peripheral nervous system. Animals injected intraventricularly with a lethal dose of the toxin, developed significant symptoms of intoxication after one hour. These symptoms included salivation, hyperactivity such as tremor and repetitive fore and hind limb movements, followed by spasmodic convulsive seizures of increasing frequency until death occurred. Since β-BuTx possessed intrinsic phospholipase activity (see below) the question arose whether the neurotoxicity observed was contributed by the enzymic activity, causing non-specific physical disruption in the brain. A relatively non-toxic phospholipase A_2 purified from bee venom was injected in a similar manner and its MLD determined. It was found that bee venom phospholipase A_2 (BV-PLA_2) was 100 times less toxic than β-BuTx (5 ng/g vs 0.05 ng/g body weight, respectively; Table 2-4B). The toxicity of BV-PLA_2 agrees with the central neurotoxicity of phospholipase A_2 isolated from *Naja naja atra* venom (Lee and Chen, 1977). These results imply that, it is the combination of enzymatic and toxic activities present in β-BuTx that makes it such a lethal protein in the CNS. Also, BV-PLA_2 was relatively non-toxic compared to β-BuTx ($\sim$ 200 fold less) when administered peripherally (Table 2.4B).

The intrinsic phospholipase A_2 activity of

Table 2-5 Phospholipase activity of β-BuTx [purified by preparative isoelectric focussing (PIEF) and chromatofocusing (CHROM)] and bee-venom phospholipase A_2 (BV-PLA_2)

	Phospholipase activity (μmol $min.^{-1}$ {mg of protein}$^{-1}$)		
	β-BuTx		BV-PLA_2
	PIEF	CHROM	
No addition	0	0	ND
$CaCl_2$	0.8	1.5	325
Sodium deoxycholate	1.3	1.4	ND
$CaCl_2$ + Sodium deoxycholate	65.6	69.7	1536

Assays were carried out at 37°C using the titration method described in Section 2.3.4.3. The reaction mixture (vol. 5.0 ml) contained 10 μmol of dispersed egg-yolk lecithin and NaCl (100 mM). Additions of $CaCl_2$ (10 mM) and sodium deoxycholate (0.18 mM) were made as indicated.
ND - not determined.

β-BuTx was assayed using egg-phosphatidyl-choline as substrate. The rate was linear for the first 5 min. at pH 8.0 and 37°C. Negligible phospholipase A_2 activity was observed in the absence of detergent or Ca^{2+} (Table 2-5). In the presence of both Ca^{2+} and sodium deoxycholate, β-BuTx from both preparations (preparative isoelectric focussing and chromatofocusing) exhibited similar phospholipase A_2 activity but the values obtained were much lower than that observed for BV-PLA_2 (Table 2-5). These results are consistent with the finding of Spokes and Dolly (1980) who examined β-BuTx made by preparative isoelectric focussing only. Also, the results showed that the major fraction, 5-2, obtained from chromatofocusing exhibited similar properties with respect to toxicity (both in peripheral and central nervous system), phospholipase activity and its molecular size as well as charge to β-BuTx obtained from preparative isoelectric focussing.

2.4.5 Chromatofocusing of fractions 4 and 6 from CM-Sephadex C50 ion-exchange chromatography

Fractions 4 and 6 obtained from the initial fractionation of the venom on CM-Sephadex C50 showed multiple components on native-PAGE at pH 4.3 (Fig. 2.7, 1D; 2.7, 2A). When fraction 4 was chromatofocused, two peaks, referred to as 4-1 and 4-2, were obtained (Fig. 2.8). On native-PAGE at pH 4.3, fraction 4-2 was homogeneous but it did not have similar mobility to β-BuTx (Fig. 2.7,1C). Fraction 4-1, however, was still heterogeneous; it contained additional impurities that were seen in the impure material of fraction 4 (Fig. 2-7,1B). The reason for this is not known and it is

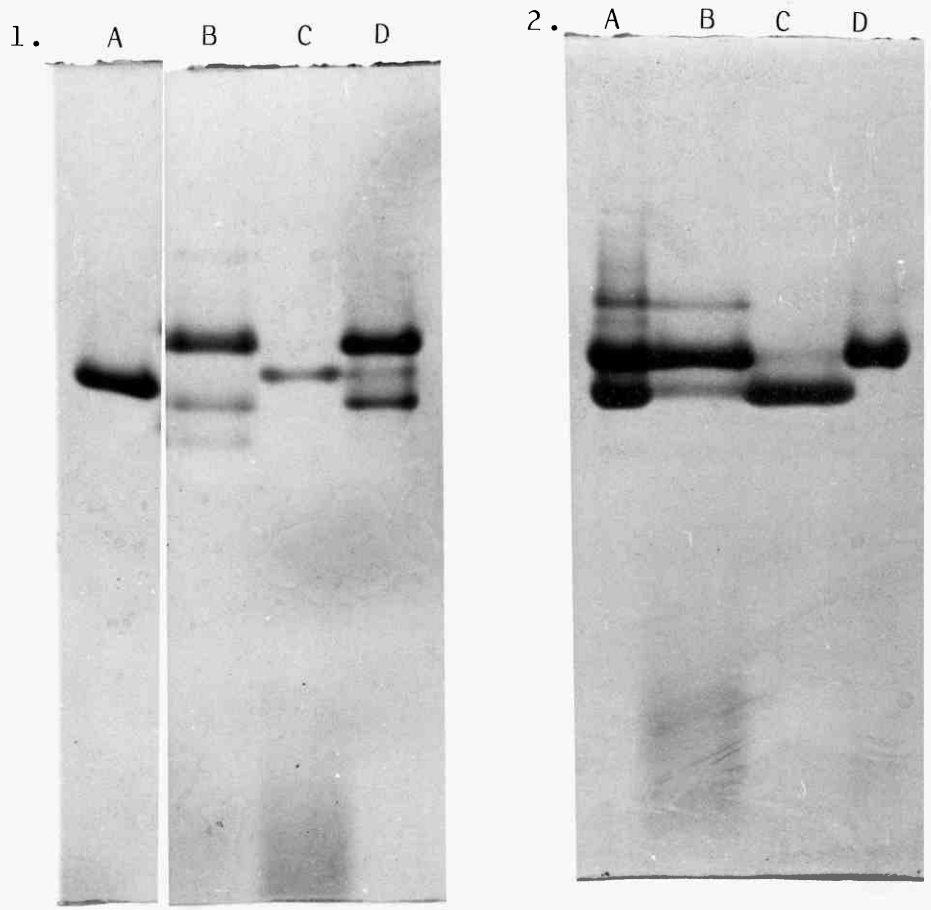


Fig. 2.7 Polyacrylamide gel electrophoresis of various peaks from chromatofocusing of peak 4 and peak 6

Native-PAGE at pH 4.3 was carried out as described in Section 2.3.4.2. Gels were stained as described in Fig. 2.2

1. A Pure β-BuTx from preparative isoelectric focussing (Fig. 2.2B)
   B & C are fractions 4-1 and 4-2 respectively, from chromstofocusing of peak 4 from CM-Sephadex C50 column (Fig. 2.1)
   D Peak 4 from CM-Sephadex C50 column (Fig. 2.1)
2. A. Peak 6 from CM-Sephadex C50 column (Fig. 2.1)
   B & C are fractions 6-2 and 6-1 respectively from chromatofocusing of peak 6
   D Pure β-BuTx from preparative isoelectric focussing (Fig. 2.2B)

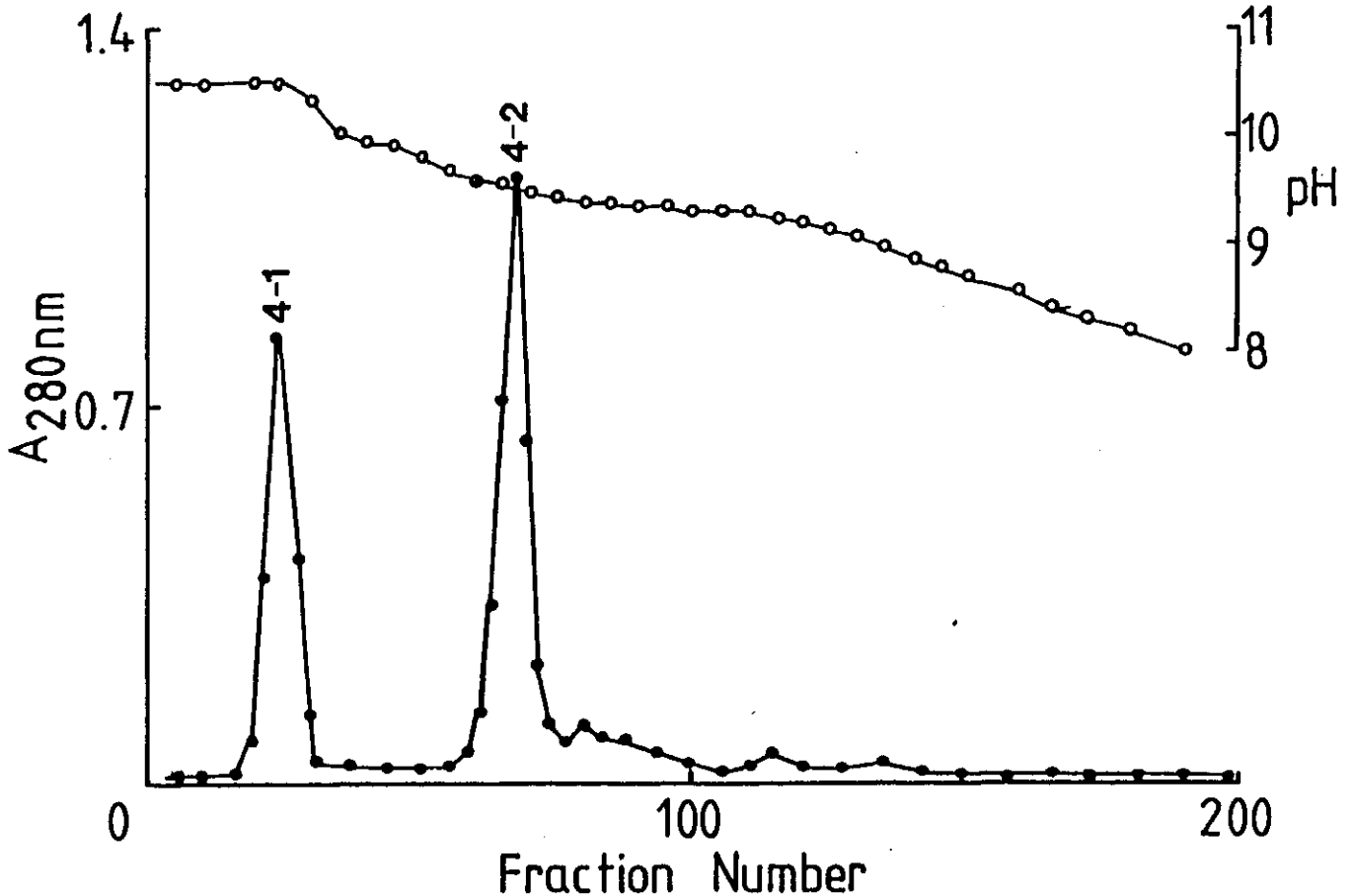


Fig. 2.8 Chromatofocusing of peak 4 material from CM-Sephadex C50 chromatography

This was carried out as described in Section 2.3.2, on a column of PBI 1:8 Protein was eluted as previously described in Fig. 2.8. After measurement of protein concentration by A_{280} nm (●), the pH gradient (○) was measured with a microelectrode.

possible that some proteins in the major band undergo some forms of modification resulting in proteins which exhibit different electrophoretic mobilities than those observed in fraction 4. Since fraction 4-2 was homogeneous on native-PAGE at pH 4.3, (Fig. 2.7, 1C), it was tested for its toxicity and enzymic activities. Table 2-6A showed that it has toxicity of 0.05 µg/g body weight and exhibited enzymic activity equivalent to 50.7 µmol H^{+} min^{-1} mg $protein^{-1}$.

When fraction 6 was chromatofocused, 2 peaks were obtained (Fig. 2.9) and these were designated fractions 6-1 and 6-2. When samples obtained from the peaks were subjected to native-PAGE at pH 4.3, the two major components of fraction 6 were found to have been separated by the chromatofocusing (Fig. 2.7,2B,C). However, the two components still contained a small degree of impurities which seemed impractical to separate even using this method, though they represented only a small percentage of the total protein present (Fig. 2.7,2B,C). Interestingly, it would appear that fraction 6-2 exhibited similar electrophoretic mobility as β-BuTx (Fig. 2.7,2B,D). Determination of MLD of fractions 6-1 and 6-2 showed toxicities of 0.05 and 0.01 µg/g body weight, respectively; however, their lethality in the CNS were not determined. Examination of their enzymic activities showed that fractions 6-1 and 6-2 have phospholipase activity of 49.2 and 51.2 µmol H^{+} $min.^{-1}$ mg $protein^{-1}$ (Table 2-6B). Collectively, these results indicate that it was possible to isolate some of the other β-neurotoxins components by using this method.

Although the different purification schemes do

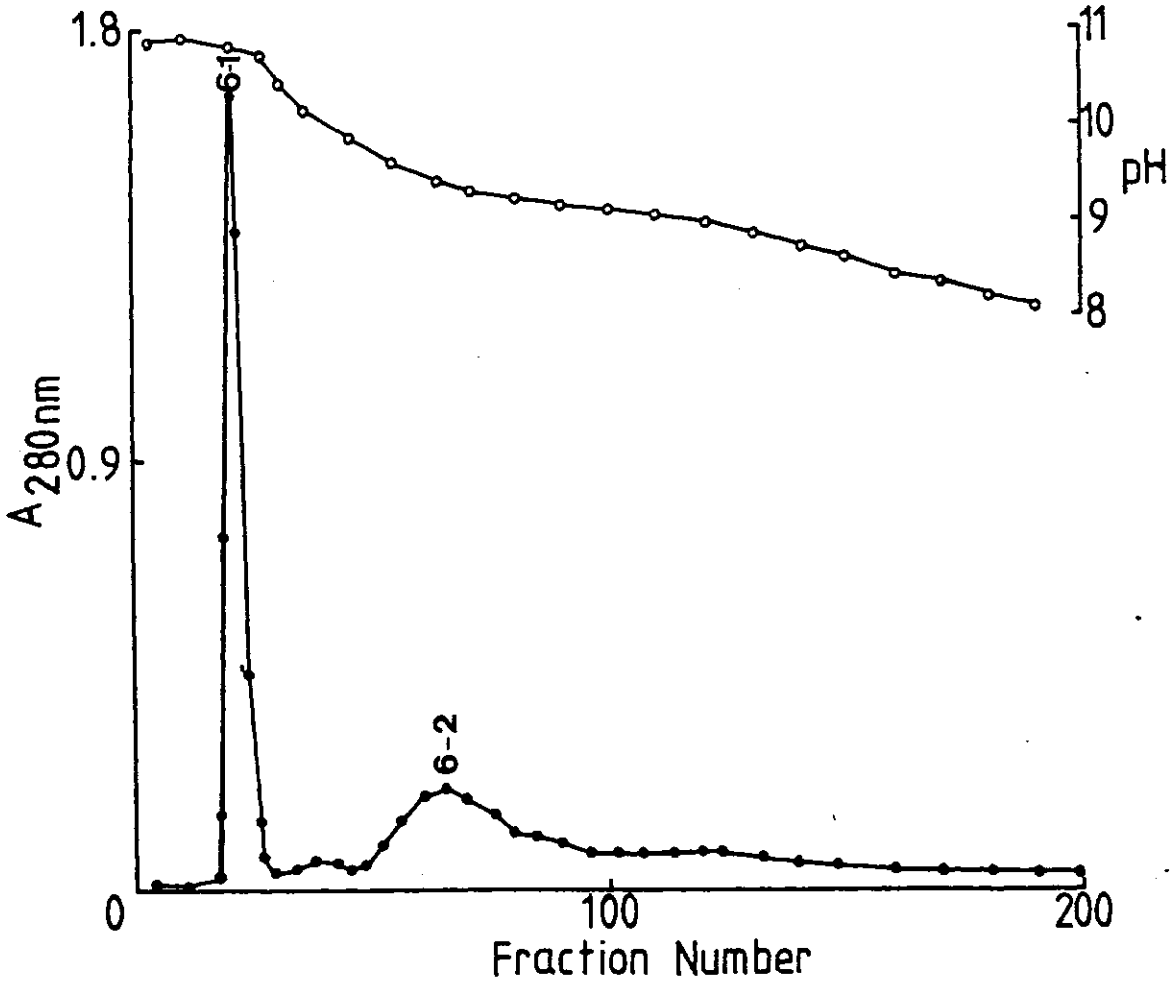


Fig. 2.9 Chromatofocusing of peak 6 material obtained from CM-Sephadex C50 chromatography

Peak 6 obtained from CM-Sephadex C50 chromatography (Fig. 2.1) was chromatofocused on a column of PBE 118 (1 X 30 cm), at 22°C as described in Section 2.3.2. Elution of proteins was carried out using 2.2% (v/v) Pharmalyte (pH 8-10.5), pH 8. Protein concentration in the fractions (500 µl) collected was determined by A_{280} nm (●) and pH (○) measured by using a micro-electrode.

Table 2-6 A Whole animal toxicities of fractions 4-2, 6-1 and 6-2

Fractions	Dose (µg/g body wt)	No. of animals injected	No. of animals killed
4-2	0.01	2	0
	0.05	2	2
	0.10	2	2
6-1	0.005	2	0
	0.01	2	2
	0.05	2	2
	0.10	2	2
6-2	0.005	2	0
	0.01	2	0
	0.05	2	2
	0.10	2	2

B. Phospholipase activities of peak fractions

	Fractions		
	4-2	6-1	6-2
Phospholipase A_2 activity (µmol H^+/min./mg protein)	50.7	49.2	51.2

Intraperitoneal injections of toxins samples into 15-20 g mice was as described in Methods. Activities were determined as described in Section 2.3.4.3 using egg-yolk lecithin in 100 mM NaCl/10 mM $CaCl_2$, pH 8.0 and in the presence of sodium deoxycholate. Fatty acid liberated was titrated with 1 mM NaOH by an autotitrator, at 37°C.

not allow an unambiguous correlation between the isotoxins described here with those reported by Kondo *et al* (1982a, b), nevertheless, from the enzymic activities and toxicities, it can be postulated that fraction 4-2 was β_5-neurotoxin, fractions 6-1 and 6-2 were β_3 and β_2 neurotoxins respectively. However, further analysis such as determination of amino acid compositions of each fraction would be necessary to support this postulation.

2.5 DISCUSSION

Several purification schemes have been used to obtain the predominant component called β-BuTx, that has a prejunctional blocking activity on isolated nerve-muscle preparation, from the venom of *Bungarus multicinctus* (Table 2-1). The first separation on starch zone electrophoresis could only detect 3 major components called α, β and γ bungarotoxins based on their electrophysiological actions (Chang and Lee, 1963). The availability of newer techniques such as ion-exchange chromatography on various types of resins, makes it possible to obtain higher resolution in fractionation of this venom. Subsequent purification on CM-Sephadex C25 or C50, separated 6 (Spokes and Dolly, 1980; Lee *et al*,1972b)and 13 components (Hanley *et al.*,1977;Kondo *et al.*,1978a) depending on the types of salt-gradient used. However, to obtain homogeneous β-BuTx preparation after this fractionation further steps were found necessary (Spokes and Dolly,1980; Kondo *et al.*,1978 a).

Peak 5 in the elution profile (Fig. 2.1) is equivalent to peak V obtained in a similar profile by

Spokes and Dolly (1980). In this study, further purification of peak 5 on preparative isoelectric focussing or chromatofocusing revealed a very basic protein which is composed of two, covalently linked, polypeptide chains whose apparent molecular weights are 11400 and 9000 , as measured by SDS-PAGE (Fig. 2.4). The molecular weights obtained were similar to those reported for other β-BuTx preparations (Spokes and Dolly,1980; Abe *et al.*,1977; Hanley *et al.*,1977; Kelly and Brown,1974). However, they are slightly different to those calculated from their amino acid sequences of 13500 and 7000 (Kondo *et al.*,1978a); this discrepancies in the molecular weight may be due to a difference between the unreduced toxin and its constituent polypeptide chains in their interactions with the detergent. Also, at such low molecular weights, the non-ideal shapes of molecules may be an important factor in their relative mobilities in SDS gels (Swank and Munkres,1971). The lethalities (Table 2-4) and phospholipase activities (Table 2-5) of these toxins preparations were consistent to those reported earlier (Spokes and Dolly,1980; Lee *et al.*,1972b).

Most workers have carried out the fractionation of *Bungarus multicinctus* on carboxymethyl ion-exchange columns using linear gradients of NH_4OAc concentration and pH (Table 2-1). The differences in the number of proteins peaks, which vary between six and thirteen, may be due to the variation in the slopes of the gradients used. This was followed by a further step, either by ion-exchange chromatography (Wernicke *et al.*,1974; Lee *et al.*,1972b; Strong *et al.*,1976; Hanley *et al.*,1977; Kato *et al.*,1977; MacDermott *et al.*,1978a), gel filtration (Kondo *et al.*,1978a)

or a combination of these techniques (Abe *et al.*,1977) to obtain pure β-BuTx. However, only two of these β-BuTx preparations reported, give single band on electrophoresis at acid pH (Abe *et al.*,1977; Kondo *et al.*,1978a). Furthermore, only one of these two preparations was reported as giving a single band on a narrow range isoelectric focussing (Kondo *et al.*,1978a). The β-BuTx prepared by Abe *et al.*, (1977) was later shown to contain at least two components on two-dimensional narrow range isoelectric focussing and SDS-PAGE (Abe *et al.*,1980). Spokes and Dolly (1980), however, used neither of the above techniques but instead employ preparative isoelectric focussing to purify β-BuTx. Homogeneous β-BuTx was reported; it exhibited similar toxicity and enzymic activity to that reported by Kondo *et al.*,(1978a). In this study, this method was adapted to obtain homogeneous β-BuTx from peak 5. In addition, β-BuTx was also obtained by chromatofocusing in the later part of this study. Chromatofocusing offers an additional advantage in the purification procedure of β-BuTx, over preparative isoelectric focussing, in that it is much easier to set up and needs no specialized equipment as in the case of the latter. It can be performed in a similar manner to column chromatography. It is a new technique first described by Sluyterman *et al.*, (1977; 1978a, b) using a stabilising medium and formation of a pH gradient within that medium by way of amphoteric buffering compounds such as Pharmalytes pH 8-10.5, used in this preparation. Unlike preparative isoelectric focussing, chromatofocusing uses a titration reaction rather than an electric field to create a pH gradient and thus need no specialized equipment.

In addition, the yield of protein was much higher than preparative isoelectric focussing, as shown in Table 2-3. However, despite these advantages, it was impractical to separate the contaminants of β-BuTx in peak 5 in pure form as shown in fractions 5-1 and 5-3. The reason for this is not known and it is possible that these fractions undergo deamidation as they are eluted from the column of the PBE 118, resulting in proteins exhibiting different electrophoretic mobilities as the original fractions.

The presence the β-type isotoxins in the crude venom was detected by various workers (Abe *et al.*,1977; Hanley *et al.*,1977; Spokes and Dolly,1980); the amino acid compositions of four of these isotoxins are very similar with minor variations in their amino acid sequences (see Introduction; Kondo *et al.*,1982a,b). The contaminants separated by narrow range isoelectric focussing and chromatofocusing in this study may represent a few of these 'isotoxins' and therefore it is necessary to remove them completely for studies on the mode of action of β-BuTx and before radiolabelling of the toxin for binding studies with nerve terminals (Chapter 3). These contaminants although invariably present, appeared to vary in their relative abundance from one preparation to another of crude venom.

In the CNS, β-BuTx is about 200 times more potent than in the peripheral nervous system making it one of the most potent macromolecules therein. Similar finding has also been reported (Hanley and Emson,1979), in addition to its degenerative lesioning of neurons, affecting both cell-bodies and nerve terminals, without any neurotransmitter specificity. This

lesioning was accompanied with the decline in the levels of marker enzymes such as choline acetyltransferase (ChAT; EC 2.3.1.6) and glutamate decarboxylase (GAD; EC 4.1.1.15) over a 4-day period, indicating that the toxin could damage neuronal structures (Hanley and Emson,1979). These effects of β-BuTx were not attributed totally to its phospholipase A_2 activity, since β-BuTx modified with N-bromosuccinimide did not cause the lesioning (Hanley and Emson,1979). Furthermore, non-toxic phospholipase A_2 isoenzyme from *Vipera russelli* did not produce this degenerative lesioning when injected in a similar manner to β-BuTx (Hanley and Emson,1979).

CHAPTER 3

TRITIATION OF β-BUNGAROTOXIN WITH RETENTION OF BIOLOGICAL ACTIVITY AND ITS BINDING TO NERVE TERMINALS IN THE CENTRAL NERVOUS SYSTEM

3.1 INTRODUCTION

The characterization of the nicotinic AChR was greatly facilitated by the discovery of α-neurotoxins (Section 1.3.1), a group of highly specific probes which bind irreversibly to receptor in both its membrane-bound and soluble states. Similarly, another group of presynaptically acting neurotoxins that specifically inhibit neurotransmitter release (Section 1.3.5) can be used as tools in elucidating the mechanism of the release process(es), which is poorly understood at present. Although the various neurotoxins in this group produce a similar pattern of presynaptic effects on ACh release at motor nerve terminals, pharmacological studies suggest that each has a distinct target thereon (Chang and Su,1980). With the exception of β-BuTx, all the others have been shown to exhibit postsynaptic actions to varying degrees (Chang *et al.*,1977; Bon and Changeux,1977; Hanley,1978). Hence, this unique specificity of β-BuTx in affecting solely the presynaptic nerve membrane (Chang *et al.*,1977), makes it an ideal probe for the study of neurotransmitter release.

The pharmacological actions of β-BuTx at the neuromuscular junction have been well documented (Section 1.3.5.5; reviewed by Howard and Gundersen,1980). It also affects the release of several neurotransmitters (Section 1.3.5.5; Spokes and Dolly,1980; Tse *et al.*,1980; Wernicke *et al.*, 1974; Smith *et al.*,1980) from purified brain nerve terminals. More recently, it has been demonstrated electrophysiologically, that this toxin blocks neurotransmission at central synapses in slices of rat olfactory cortex (Halliwell and

Dolly,1982b). As in the case of peripheral synapses (Abe *et al.*,1977), its action on the central nervous system is decreased, but not abolished (Halliwell *et al.*, 1982), when the phospholipase activity is inhibited by replacement of Ca^{2+} with Sr^{2+} or modification of a histidine residue in its catalytic site with p-bromophenacyl bromide (Dolly *et al.*,1980; Halliwell and Dolly,1982a). Moreover, in rat hippocampal slices it was shown to act presynaptically and to be most potent in terminal-rich areas (Halliwell and Dolly,1982b). Thus, as in the case of the neuromuscular junction, β-BuTx probably binds specifically to functional sites on the presynaptic membrane (Dolly *et al.*,1980; Halliwell and Dolly,1982a, 1982b) thereby amplifying the subsequent effects of its phospholipase activity. It appears, therefore, that this neurotoxin could be used effectively for identification of nerve membrane components concerned with transmitter release in the central nervous system. As a prerequisite for such investigation, a biologically active derivative of radiolabelled β-BuTx must be obtained.

There were three previous reports of preparations of radiolabelled β-BuTx. Using the chloramine-T method, Oberg and Kelly (1976b)labelled β-BuTx to specific radioactivities corresponding to an incorporation of 0.1 to 0.28 mol of ^{125}I-per mol of toxin with both subunits being labelled. The toxin retained 85% of its phospholipase A_2 activity but its lethality was not determined. This iodinated-derivative showed saturable binding to brain membranes with a dissociation constant of 1.7 nM. However, this preparation of iodinated β-BuTx was also

shown to bind saturably to other membranes from liver and blood cells with similar affinity, indicating the lack of specificity of this derivative for nerve terminals. After completion of this part of the thesis, a preliminary report on the iodination of the toxin with N-succinimidyl 3-(4-hydroxy-5-(^{125}I)iodophenyl) propionate was made by Summers and Thompson (1980). However, it did not include the determination of its lethality and phospholipase activity; binding data obtained with the preparation was not reported.

Radiolabelling with tritium was reported by MacDermott *et al.* (1978) using pyridoxal 5'phosphate to form a Schiff base with an amino group on the protein, which was then stabilised by reduction using NaB($^{3}H_4$). (^{3}H)-Pyridoxalation of β-BuTx with this method, produced a labelled derivative with specific radioactivity within the range of 1.4-8.4 Ci/mmol but its peripheral toxicity was lowered 10-fold. Unfortunately, the phospholipase activity of this preparation was not reported (MacDermott *et al.*,1978). Binding of (^{3}H)-pyridoxalated-β-BuTx to synaptosomes was measured by a centrifugation assay; saturable binding was observed with a high dissociation constant of 0.21-0.37 μM. Competition experiments with increasing concentrations of unlabelled toxin demonstrated that the native toxin has a K_i of 25 nM; this indicates that the binding affinity was drastically reduced by pyridoxalation. MacDermott *et al.*(1978) also attempted to iodinate β-BuTx using a more gentle method than chloramine-T, employing the enzyme lactoperoxidase but the resultant derivative was inactive.

In this chapter, the preparation of ^{3}(H)-propiony-

lated β-BuTx of high specific radioactivity is described; it was free from unlabelled β-BuTx and exhibited appreciable biological activity. The procedure selected used N-succinimidyl (2,3-^{3}H)propionate that introduces tritiated propionyl groups of high specific radioactivity; it had been already used successfully to label α-neurotoxins (Dolly *et al.*,1981a;Sugiyama and Yamashita,1981). Despite abolition of its phospholipase A_2 activity by tritiation, examination of the toxin's action at synapses in rat olfactory cortex indicates that it is able to block neurotransmission. The kinetics of its saturable binding to rat cerebrocortical synaptosomes are investigated and the specificity of this binding component is examined by assessing the effect of other presynaptically-acting neurotoxins and phospholipase A_2 on the binding of the tritiated toxin.

3.2 MATERIALS

N-succinimidyl (2,3-^{3}H)propionate and (9,10-^{3}H) oleic acid with specific radioactivities of 48 and 5.7 Ci/mmol,respectively,were supplied by the Radiochemical Centre (Amersham, Bucks,UK). N,N-Dicyclohexyl carbodiimide and N-hydroxy-succinimide (crystalline) were obtained from Sigma Chemical Co. Materials used for SDS-PAGE, titrimetric phospholipase assay, gel-filtration and ion-exchange chromatography, were as described previously in Section 2.2. All chemicals were of analytical grade unless otherwise stated.

Sources of neurotoxins and phospholipase A_2: Homogeneous

β-BuTx and DTX were purified as previously described in Chapter 2 and 3, respectively. Botulinum neurotoxin, purified to homogeneity as described previously (Tse *et al.*, 1982), was provided by R.Williams of this laboratory. Phospholipases A_2 purified from venoms of bee (*Apis mellifera*)(Shipolini *et al.*,1971) and *Naja melanoleuca* were gifts from Dr. R.Shipolini. Toxin I purified from *Dendroaspis polylepis* was purified by Dr. E.Karlsson and characterized electrophysiologically by Dr. A.Harvey (Harvey and Karlsson,1980). Tityustoxin was isolated from *Tityus serrulatus* by Dr. C.Diniz (Gomez and Diniz,1966). α-latrotoxin purified from black widow spider venom (Grasso,1976) and taipoxin from venom of *Oxyuranus scutellatus* (Fohlman *et al.*,1976) were kindly provided by Drs. A.Grasso and D.Mebs, respectively.

3.3 METHODS

3.3.1 Synthesis of N-succinimidyl propionate

This was achieved using a modification of the method of Rappoport and Lapidot (1974). N-hydroxy succinimide (320 mg; 2.13 mmol) was dissolved in 12 ml of ethyl acetate (dehydrated by passing through a molecular sieve type 4A. Aluminium sodium silicate) followed by the addition of 0.2 ml of propionic acid (2.13 mmol). In another container, dicyclohexyl carbodiimide (580 mg; 2.81 mmol) was dissolved in 1 ml of dried ethyl acetate. The reaction was initiated by adding the latter solution to the N-hydroxy-succinimide/propionic acid mixture; the reaction mixture

was stirred overnight at 22°C. After filtration to remove the dicyclohexyl urea (white precipitate), the filtrate was lyophilized and redissolved in benzene before fractionation of components by high-performance liquid chromatography (HPLC) with hexane:ethyl acetate (3:1 v/v) solvent system on Porasil (Silica) columns; 1 ml fractions were collected and monitored by absorbance at 254 nM. After crystallization under vacuum, the absorption spectra of the materials from the 3 major peaks separated were obtained on a UNICAM Spectrophotometer. Absorption at wavelengths of 251 and 254 nm was shown by fraction C3, while C2 absorbed at 249 nm; these values were very close to to the value obtained for N-hydroxy succinimide, which absorbed at 254 nm(Fig. 3.2). Thus C2 and C3 were further analysed for their chemical compositions and melting points. In a separate experiment, an aliquot (5 µl) of the tritiated N-succinimidyl propionate obtained from Amersham Radiochemical centre, was analysed on HPLC under similar conditions to that of the prepared compound. Fractions obtained were measured for their radioactive contents by scintillation counting.

3.3.2 Radiolabelling of β-BuTx to high specific activity

Tritiation of β-BuTx with N-succinimidyl (2,3-^{3}H) propionate was performed by modification of the method of Dolly *et al.*,(1981a) described for α-BuTx. The reagent, N-succinimidyl (2,3-^{3}H) propionate (specific activity 48 Ci/mmol; 2.1 ml; 99.9 nmol) was transferred to a plastic microfuge tube in aliquots of 300 µl, followed by evaporation of the solvent under a stream of nitrogen.

β-BuTx (0.7 mg; 33.3 nmol) was added in 350 µl of 0.02 M phosphate buffer, pH 7.4, to the dried reagent giving a ratio of 3:1 for tritiated reagent to toxin. After thorough mixing, the reaction was allowed to continue with constant mixing for 2 hours at 22°C. Before the reaction was terminated by gel-filtration, on a Sephadex G-50 (superfine) column (44 X 1) equilibrated with 0.02 M phosphate buffer, pH 7.4, an aliquot (5 µl) of the incubation mixture was removed for determination of total radioactivity recovered from the walls of the microfuge tube. Fractions (500 µl) obtained were analysed for protein concentration measured by A_{280} nm; their radioactive content was determined by scintillation counting using Soluene-based scintillant. Labelled β-BuTx, eluted in the void volume was pooled and protein accurately determined using the method of Lowry *et al.*(1951) using unlabelled toxin as standard.

3.3.3 Radiolabelling of β-BuTx to low specific activity

This was carried out by a modification of the above method (Section 3.3.2). Instead of using radiolabelled N-succinimidyl (2,3-^{3}H) propionate, a mixture of radiolabelled and unlabelled derivatives in the ratio of 1:19 was used.

A mixture of reagent (19 parts N-succinimidyl propionate to 1 part N-succinimidyl {2,3-^{3}H} propionate; total 99.9 nmol) was transferred to a plastic microfuge tube After evaporation of solvent as described above, β-BuTx (0.7 mg; 33.3 nmol) was added in 0.35 ml of 0.02 M phosphate buffer, pH 7.4. The ratio between reagent and toxin was still maintained at 3:1. Subsequent steps were similar to

those described in Section 3.3.2.

3.3.4 Characterization of ^{3}H-propionylated β-BuTx: Analytical Methods

3.3.4.1 Polyacrylamide gel electrophoresis

Urea-SDS-PAGE was performed according to the procedure of Swank and Munkres, described in Section 2.3.4.2. After destaining the gel, it was processed for fluorography using the method of Bonner and Laskey (1974). Directly after destaining, the gel was soaked in about 20 times its volume dimethyl sulfoxide (Me_2SO) for 30 min., followed by a second 30 min. immersion in fresh Me_2SO. The gel was immersed in 4 volumes of 20% (w/w) PPO in Me_2SO (22.2%, w/v) for 3 hours, and then 20 volumes of distilled water for 1 hour. It was then dried under vacuum on a Bio-Rad gel-drier, and exposed to FUJI X-ray film which was held in contact with the dried gel. The fluorogram was exposed for 4 days at -70°C prior to development through Kodak-O-Mat. The dried gel was sliced into 2 mm sections, and its radioactive contents determined by scintillation counting in Soluene-base toluene scintillant.

3.3.4.2 Isoelectric focussing

Preparative isoelectric focussing of tritiated toxin was performed in a flat bed (11 X 6 cm) of Ultrodex containing 1.5% Ampholines (pH 7-9 and pH 9-11) and 10% (w/w) sucrose for 3-5 hours at 4 W constant power. Aliquots of each 2 mm of the focussed gel were analysed for radio-activity; sections containing the major radioactivity peak were eluted with 50 mM NH_4OAc, pH 7.4, and gel-filtered on

Sephadex G-50 (superfine) to remove the Ampholines. The tritiated toxin was also subjected to analytical isoelectric focussing; the gel was then sliced into 2 mm sections for determination of radioactivity. In a separate track, unlabelled toxin was focussed simultaneously and fixed with 40% trichloroacetic acid (TCA); it was washed with 10% (w/v) TCA, stained with 0.2% Coomassie brilliant blue R_{200} in 5% methanol/10% acetic acid and destained in the latter solution.

3.3.4.3 CM-Sephadex chromatography of ^{3}H-propionylated β-BuTx and β-BuTx

CM-Sephadex C25 was equilibrated with 50 mM NH_4OAc/50 mM NaCl, pH 7.4 (starting buffer) and packed into a 2 ml syringe-column. Pure β-BuTx (1 mg) was added to ^{3}H-propionylated β-BuTx (5 µl, 20.3 pmol) in starting buffer and loaded on to the column. After washing through with 10 bed volumes of starting buffer, the adsorbed protein was eluted with a linear salt gradient of 50 - 400 mM NaCl. Fractions (450 µl) were collected; protein concentration monitored at A_{280} nm, and radioactive content determined by scintillation counting in Soluene-base toluene scintillant.

3.3.4.4 Determination of biological and chemical specific activities of ^{3}H-propionylated β-BuTx

Lethalities of ^{3}H-propionylated toxin following peripheral and central administration were performed as described in Section 2.3.4.4. The chemical specific radioactivity of labelled toxin was determined from its radio-

active and protein contents; the latter was measured colorimetrically by the method of Lowry *et al.* (1951), using unlabelled toxin as standard (Section 3.3.2).

3.3.4.5 Phospholipase A_2 assay

Radiolabelled toxin was analysed for phospholipase A_2 activity with a titrimetric assay using egg-yolk lecithin dispersed in deoxycholate, as described in Section 2.3.4.3. In addition, phospholipase activity towards radiolabelled membrane phospholipids of synaptosomes was measured essentially as described by Sen and Cooper (1978) with the following modifications. Purified synaptosomes of 5-week-old rats were used rather than synaptic plasma membranes from 16-day animal; 400µCi of ^{3}[H]Oleic acid complexed with BSA (80 nmol) were injected intracerebrally using stereotaxic apparatus positioned at L-4.0, S-(-0.2) and D-2.5 mm (Köenig and Klippel,1963). Animals were killed 90 min. after injection and cerebrocortical synaptosomes prepared as outlined below. The procedure of Gatt and Barenholz (1969) was used for extraction of the tritiated fatty acids produced in the incubation. The activity observed was proportional to toxin concentration (up to ≈ 50 nm) and linear with time within the periods used to measure the rate of hydrolysis, in agreement with published results (Sen and Cooper,1978).

3.3.5 Electrophysiological recordings of the action of ^{3}H-propionylated β-BuTx on rat olfactory cortex slices

Extracellular recordings were made from surface

slices of rat olfactory cortex (Dolly *et al.*,1980; Halliwell and Dolly,1982). The lateral olfactory tract (LOT) runs across the surface of these slices and fibres from this form synapses with the dendrites of cortical cells just below the surface. Following decapitation and rapid removal of the brain, slices of rat olfactory cortex 400-500 µm thick were prepared by cutting off the outer layers of the cortex with a bow cutter and guide (McIlwain and Rodnight,1962). After a period of at least 1 hour preincubation in a holding bath containing oxygenated Krebs solution at room temperature, slices were transferred to a recording chamber (Brown and Halliwell,1981) which was constantly perfused (3-4 ml/min) with modified Krebs solution at 28°C. The Kreb's buffer consisted of (mM); Na^+, 118; K^+, 6; Ca^{2+}, 2.5; Mg^{2+}, 1.2; Cl^- 125; HCO_3^-, 25; $H_2PO_4^-$, 1.0; D-glucose, 11; pH 7.4, and saturated with 95% O_2/5% CO_2. The slices were kept constantly submerged in the Krebs solution which could be changed for media containing different ionic composition and toxin concentration. Conventional stimulating and recording techniques were employed for the electrophysiological investigation of the slices. When the anterior end of the LOT was stimulated, a field potential with both pre- and post-synaptic components could be recorded from the surface of the prepiriform cortex some little distance from the macroscopically visible tract (Dolly *et al.*,1980).

3.3.6 Preparation of synaptosomes

Rat cerebrocortical synaptosomes were prepared by a modification of the method of Gray and Whittaker (1962)

as described by de Belleroche and Bradford (1977). Whole cerebral cortex was rapidly removed from adult female Sprague-Dawley rats (150-200 g), previously killed by stunning, and placed in ice-cold 0.32 M sucrose. Using a glass-perspex homogenizer, a 10% (w/v) homogenate was prepared followed by centrifugation in a Beckman Type 30 rotor at 1000 X g for 10 min., in a Beckman L565 Ultra-centrifuge. Of the two fractions obtained, the pellet (P_1) was discarded while the supernatant (S_1) was retained and recentrifuged in the type 30 rotor for 20 min. at 20000 X g to yield the P_2 and S_2 fractions. The crude mitochondrial pellet (P_2) was resuspended in 0.32 M sucrose, and 15 mls of this suspension was carefully layered onto a discontinuous sucrose density-gradient which consisted of 20 ml of 0.8 M sucrose above 20 ml of 1.2 M sucrose. The sucrose gradients were then centrifuged at 76000 X g for 1 hour in a Beckman SW 25.1 rotor, to yield three fractions; P_2A, P_2B, and P_2C. The synaptosomal (P_2B) fraction was collected at the 0.8 M and 1.2 M sucrose interface, in approximately 1.0 M sucrose. This was diluted to 0.45 M with dropwise addition of cold deionized water. The synaptosome suspension was finally sedimented at 54000 X g for 20 min. in a Type 30 rotor to yield synaptosomal pellets; which were resuspended in the required volume of Krebs phosphate medium for subsequent experiments.

3.3.7 Measurement of the binding of ^{3}H-propionylated β-BuTx to synaptosomes

Aliquots (150 µl) of the bulk synaptosomal suspension (∿ 3 mg protein/ml) in Krebs-phosphate buffer,

containing (in mM):NaCl, 124; KCl, 5; Na_2HPO_4, 20; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $CaCl_2$, 0.75; glucose, 10; pH 7.4, saturated with 95% O_2 : 5% CO_2, and 1 mg/ml bovine serum albumin (BSA) (reaction buffer) were placed in plastic microfuge tubes and equilibrated at 37°C for 5 min. before addition of tritiated toxin (30 µl in reaction buffer). The final volume of the incubation was 250 µl. Following incubation for a standard period of 30 min., the reaction was terminated by dilution with 1 ml of reaction buffer at 4°C, followed by centrifugation at 9000 X g for 2 min in an Eppendorf centrifuge. The pellet was washed twice with the same buffer and dissolved in Soluene (100 µl). The bottom half of each microfuge containing the dissolved synaptosomal pellet was then cut-off and counted at 33% efficiency, in a Soluene-based toluene scintillation cocktail. Non-specific binding was measured similarly except that a 100-fold molar excess of unlabelled β-BuTx was added to the synaptosomes prior to the tritiated toxin; this was substracted from the total binding to give the amount of specifically bound toxin. Synaptosome pellets from identical samples to those used in the binding assay except that they were suspended in incubation medium without the inclusion of BSA, were digested in 0.1 M NaOH and assayed for protein by the method of Lowry *et al.*, (1951), using BSA as standard. Results are expressed as fmol of toxin bound per mg protein, and are the mean of four separate experiments.

The initial rate of association of toxin with its binding sites was determined by using 5 nM ^{3}H-β-BuTx, at 37°C, and 0.4-0.7 mg/ml of synaptosomal suspension. Binding

was terminated at various times by dilution with 1 ml of ice-cold reaction buffer and centrifugation as above. Non-specific binding was calculated as detailed before. For measurement of dissociation rates, the binding sites were labelled to saturation under standard conditions. Specific and non-specific binding was measured prior to addition of excess unlabelled toxin to equivalent samples. The rate of dissociation was calculated by measuring the remaining amount of specifically bound toxin after further periods at 37°C.

Lysed synaptosomes were prepared by incubation of synaptosomal suspensions with 50 mM Tris-HCl, pH 8.0 for 45 min. at 0°C. The membrane fraction was sedimented and resuspended in reaction buffer from which aliquots were taken for use in binding experiments. For trypsinization of synaptosomes, the latter were incubated with trypsin (0.5 mg/ml at 37°C for 60 min.) followed by the addition of excess trypsin inhibitor (1 mg/ml). The trypsinized membranes were sedimented, washed and resuspended in reaction buffer before being assayed for binding of toxin. Synaptosomal membranes were also treated with 1 M HCl or heated at 95°C for 15 min., washed and resuspended in reaction buffer prior to the binding assay.

In competition experiments, the amount of toxin binding to synaptosomes was measured similarly to the equilibrium experiments, in the absence or presence of various concentrations (0.01 nM-1μM) of test substances added simultaneously with the tritiated toxin (final concentration used, 5 nM). For binding measurements in Ca^{2+}-free solution, Ca^{2+} in the reaction buffer was replaced with

equimolar amounts of Sr^{2+} or EGTA.

During the latter part of this study, a filtration assay was developed for the binding of ^{3}H-β-BuTx to synaptosomes. The assay used Whatman GF/C glass fibre filters. Prior to application of samples, the filters were pre-wetted with 0.1% polylysine in reaction buffer (100 µl). Aliquots (0.1 ml) of test and control incubations were applied to the pre-soaked filter, and dried under suction. Following two washes with 4 ml of reaction buffer, the filters were dried with an infra-red lamp and counted in Soluene-base scintillation cocktail.

3.4 RESULTS

3.4.1 Preparation of N-succinimidyl propionate

To facilitate studies on the radiolabelling of β-BuTx and to allow propionylated β-BuTx to be prepared in large quantities for electrophysiological studies, N-succinimidyl propionate (NSP) (Fig. 3.1) was synthesised and purified. The reaction between N-hydroxysuccinimide and propionic acid was initiated by addition of dicyclohexyl carbodimide to produce dicyclohexyl urea and imido-esters. The reaction was allowed to go to completion by stirring overnight at 22°C. After removal of the insoluble dicyclohexyl urea by filtration, the filtrate was lyophilised and redissolved in benzene. The concentrated solution was fractionated using HPLC on a silica column. Four components were obtained and will be referred to as A-1, A-2, A-3 and A-4 (Fig. 3.2). After lyophilization and resuspension in toluene, absorbance of samples from the three major peaks A-1, A-2 and A-3 was measured in the UV range. Only A-3 showed

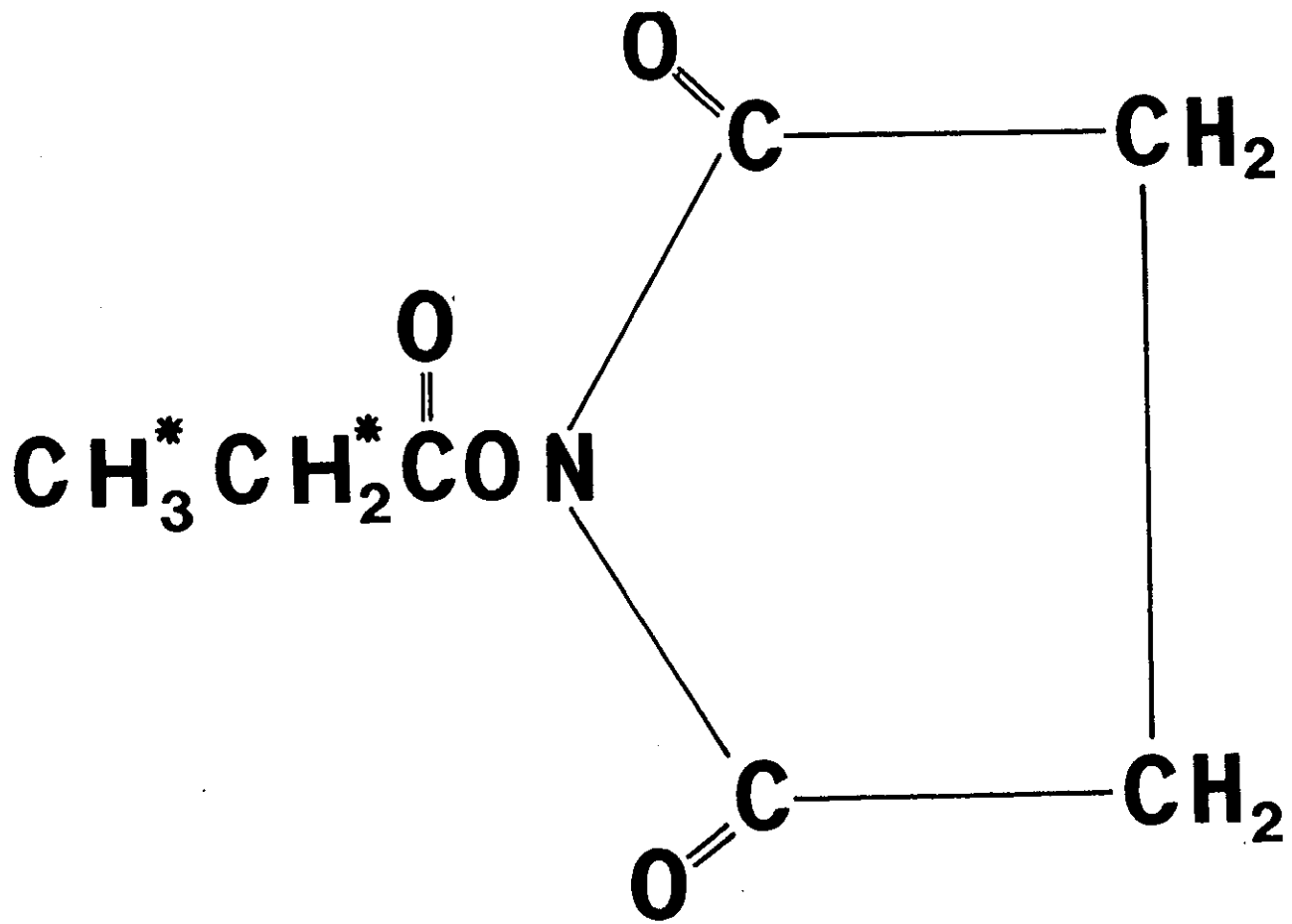
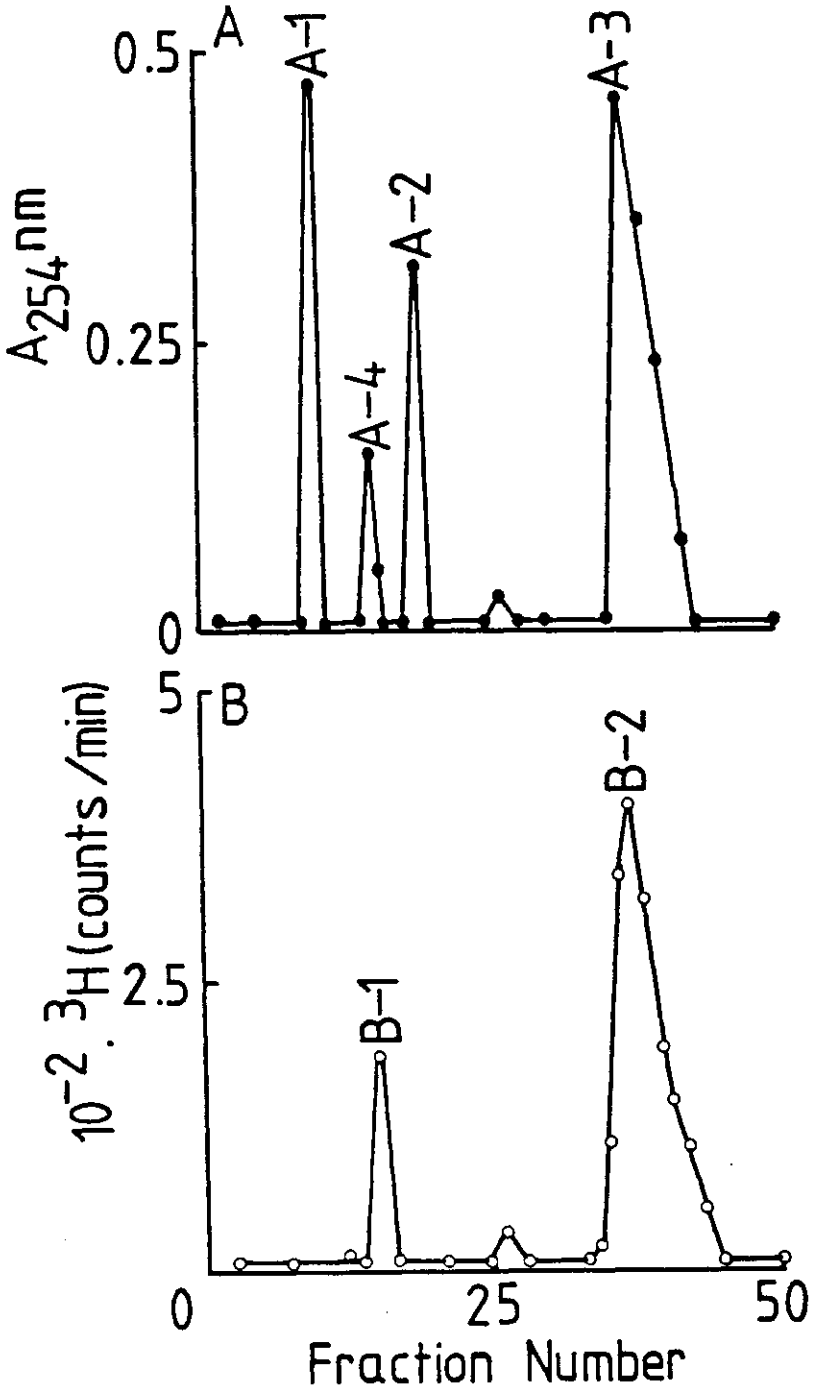


Fig. 3.1 N-succinimidyl (2,3-^{3}H) propionate

* indicates hydrogen being replaced by tritium

Fig. 3.2 Synthesis of N-succinimidyl propionate



A. High-performance liquid chromatography (HPLC) of filtrate (Section 3.3.1) obtained from the reaction mixture. This was carried out on a silica column as described in Methods using hexane:ethyl acetate (3:1 v/v) solvent system.

B. HPLC of N-succinimidyl (2,3-^{3}H) propionate (Fig. 3.1) obtained from Amersham Radiochemical Centre, under identical conditions used in A.

absorbance at 254 nm corresponding to the heterocyclic succinimide group; this absorbance at 254 nm was also exhibited by N-hydroxysuccinimide (Fig. 3.3). A-2 showed an absorbance at 249 nm only, indicating that the compound is not NSP, and is probably one of the imido-esters present as a by-product of the reaction. Further analysis of the melting point and element compositions of A-2 and A-3 indicated that the two compounds possessed different physical and chemical properties. The data in Table 3-1 showed that the percentage element composition of A-3 was very similar to the calculated values for NSP (Fig. 3.1); also the melting point measured for A-3 was identical to that of NSP. These results proved that A-3 was NSP, and it will thus be referred to as the latter. It is noteworthy that, to facilitate the final crystallization of the reagent obtained, it was necessary to 'dry' all solvents used, and that all glassware was free from any moisture. Further evidence was obtained to show that A-3 is indeed NSP, when the tritiated derivative obtained from Radiochemical Centre, Amersham, was fractionated on the HPLC system under identical conditions as used for the filtrate; one major radioactivity peak referred to as B-2, corresponding to peak A-3, was obtained. In addition to fraction B-2, a smaller peak was also obtained, referred to as fraction B-1; this was probably one of the by-products of the breakdown of the reagent. This peak was also observed in the fractionation of the filtrate (fraction A-4, Fig. 3.2).

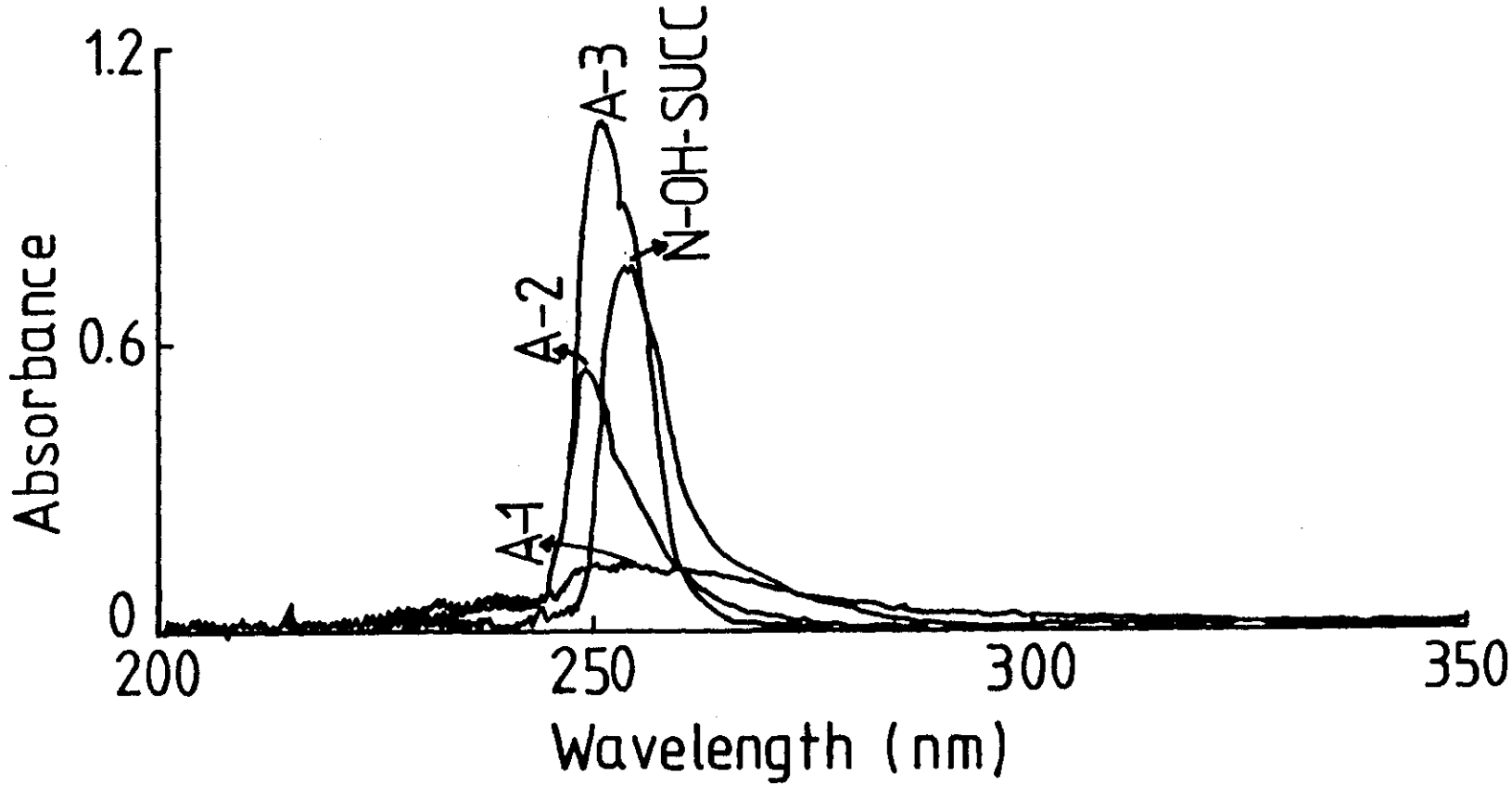


Fig. 3.3 Absorption spectra of fractions A-1, A-2 and A-3, obtained after HPLC, and N-hydroxy succinimide (N-OH-SUCC). Measurements were made on a UNICAM spectrophotometer. Similar absorption spectra was obtained when measurements were made with fractions dissolved in hexane: ethyl acetate (3:1) mixture. In the latter the peaks were shifted to 257nm and 263 nm.

Table 3-1 The melting point and percentage element composition of fractions A-2 and A-3 (N-succinimidyl propionate) from HPLC

FRACTION	MELTING POINT	% ELEMENT COMPOSITION			
		CARBON	HYDROGEN	NITROGEN	OXYGEN
A-2	129-131 °C	68.2	9.88	9.82	
A-3	43-45 °C	48.77	5.21	7.97	
N-succinimidyl propionate (NSP) (calculated from structure shown in Fig. 3.1)	45 °C	49.12	5.26	8.18	37.44

Micro-analysis of fractions A-2 and A-3 (N-succinimidyl propionate) was carried out by the Department of Chemistry, Imperial College of Science and Technology.

3.4.2 Radiolabelling of β-BuTx with N-succinimidyl (2,3-^{3}H) propionate

Two types of preparations of radiolabelled β-BuTx derivatives were carried out, namely of high and low specific radioactivities. The former was performed using tritiated N-succinimidyl (2,3-^{3}H) propionate while the latter was performed using a mixture of unlabelled and labelled N-succinimidyl propionate.

In the preparation of the high specific activity derivative of β-BuTx, the amount of N-succinimidyl (2,3-^{3}H) propionate (48 Ci/mmol) added to the reaction tube was calculated to give a 3-fold molar excess of the tritiated reagent over β-BuTx, which was added in 350 µl of reaction buffer. After 2 hours of constant agitation at 22°C, three aliquots of 5 µl were taken and their radioactive content determined; it was found that 78% of the total radioactivity was present in solution. Thus, the actual ratio of toxin to labelling reagent was 1:2.3. The reaction mixture was applied to a Sephadex G-50 (superfine) column (Fig. 3.4) from which the recovery of radioactivity was 73%; 63% of this was eluted in the void volume, corresponding to (^{3}H)-propionylated β-BuTx (^{3}H-β-BuTx) and 37% was eluted in the second peak, as the unreacted reagent.

On analytical isoelectric focussing, (^{3}H)-β-BuTx showed one sharp peak of radioactivity (Fig. 3.5A) indicating that a single species of modified β-BuTx had been formed. This derivative had a pI of 9.6 and focussed at a point quite separate from native toxin (pI = 9.9), in an adjacent tract on the same gel (Fig. 3.5A). A large amount (∿ 1 nmol) of ^{3}H-β-BuTx was subjected to isoelectric focussing on a

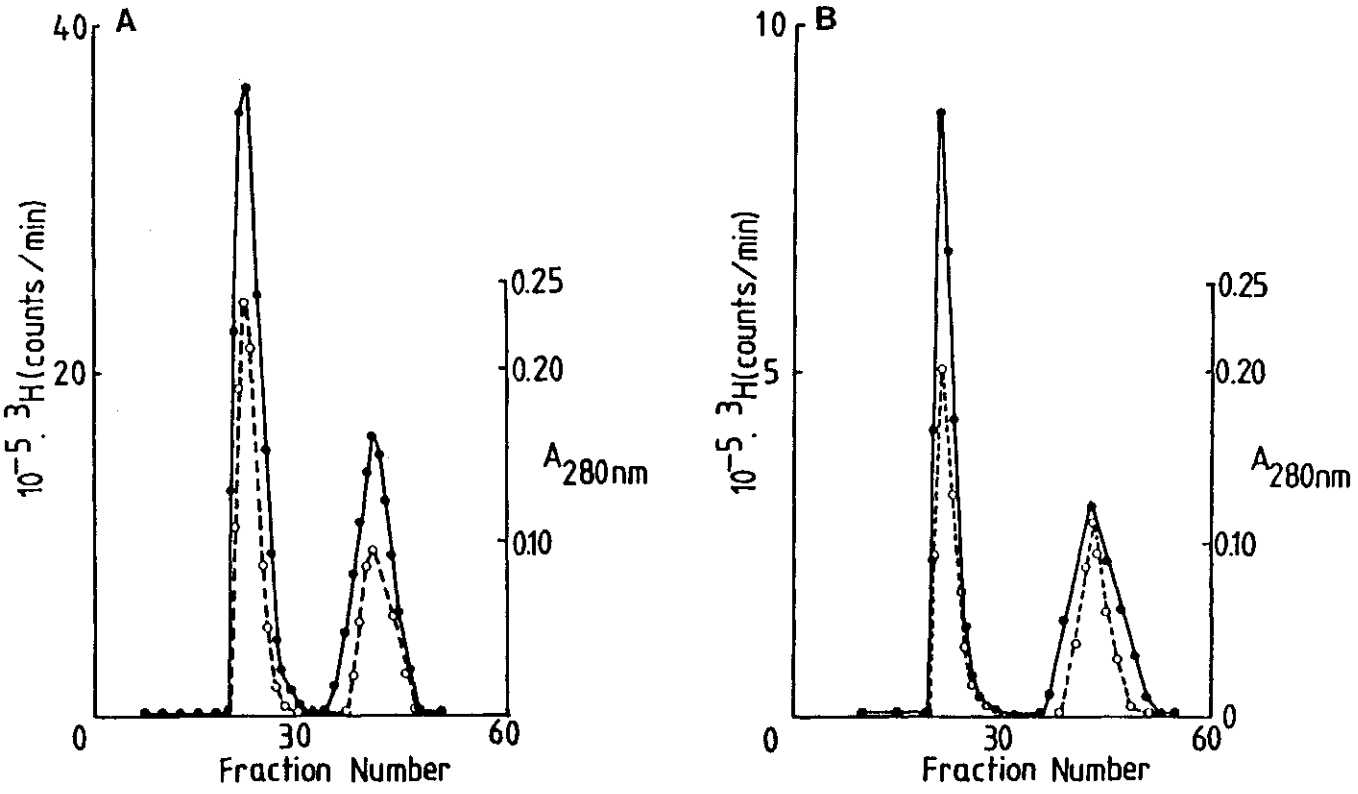


Fig. 3.4 Separation of ^{3}H-propionylated β-BuTx from excess reagent by gel-filtration

A High specific activity preparation of β-BuTx: The reaction between the toxin and N-succinimidyl (2,3-^{3}H) propionate was terminated by loading the reaction mixture to a Sephadex G-50 column (46 X 1 cm), equilibrated with 50 mM ammonium acetate, pH 7.4. Radioactivity (●) and absorbance (O) of the fractions (0.5 nm) were determined.

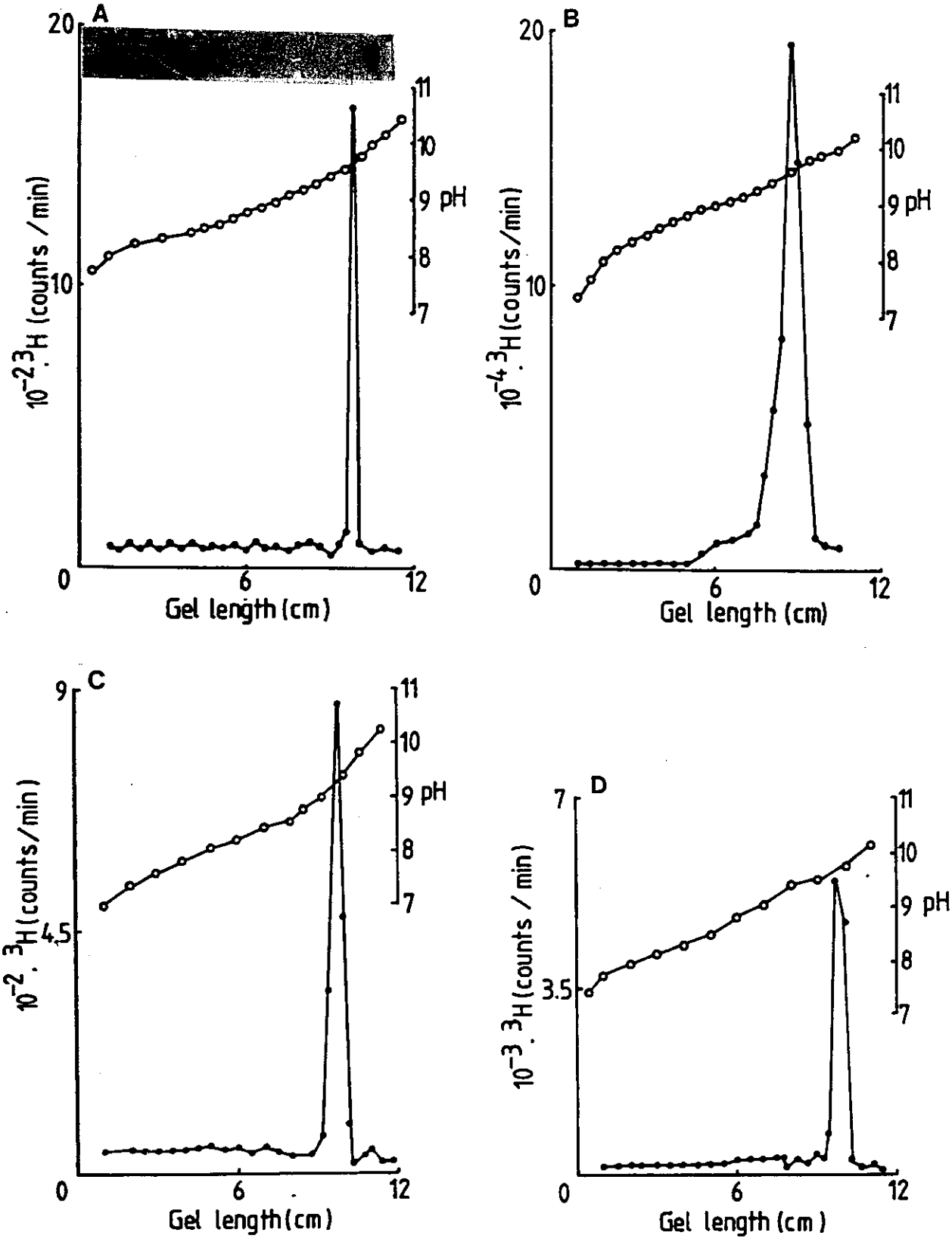
B Low specific activity preparation of β-BuTx: A mixture of labelled and unlabelled derivatives of N-succinimidyl propionate was reacted with β-BuTx. Reaction was terminated as described above (A). Radioactivity (●) and absorbance (O) of the fractions (0.5 ml) were determined as described in Methods.

Labelled toxin was eluted in the void volume (1st peak)

Fig. 3.5 Isoelectric focussing of ^{3}H-propionylated β-BuTx

A and C : Analytical polyacrylamide gel electrophoresis focussing of high and low specific radioactivity preparation of β-BuTx, respectively. Focussing was performed as described in Methods. The pH gradient (O) and radioactive content (●) of gel slices (2mm) were measured. A also showed focussing of unlabelled β-BuTx in a separate track of the gel (upper) which was stained with Coommasie G-250 after fixation and washing. The unlabelled toxin was focussed at 0.3 pH (pH 9.3) higher than the tritiated derivatives (pH 9.6)

B and D: Preparative focussing in a flat bed of Ultrodex of the same samples used in A and C respectively. Focussing was performed as described in Methods. The pH gradient was measured (O), the gel was sectioned and each section was eluted in buffer ; radiactive contents (●) of aliquots of these eluates were measured.


A
20
10
0
6
12
Gel length (cm)
10^{-2}. ^{3}H (counts / min)
11
10
9 pH
8
7
B
20
10
0
6
12
Gel length (cm)
10^{-4}. ^{3}H (counts/min)
11
10
9 pH
8
7
C
9
4.5
0
6
12
Gel length (cm)
10^{-2}. ^{3}H (counts/min)
11
10
9 pH
8
7
D
7
3.5
0
6
12
Gel length (cm)
10^{-3}. ^{3}H (counts / min)
11
10
9 pH
8
7

preparative scale in a bed of Ultrodex (Fig. 3.5B); again, a single sharp peak of radioactivity was obtained (pI = 9.6) and the central fraction of this was pooled. When this fraction was tested for lethality in mice, there was little change observed in the neurotoxicity (see Section 3.4.3) relative to the radioactive contents (Table 3-2) before and after separation of any native toxin present, by electrofocussing. In fact, from the slightly lower toxicity of the focussed sample, it was calculated that the unfocussed tritiated sample contained less than 4% unlabelled toxin. Additional evidence for this was obtained from the absence of phospholipase A_2 activity (Table 3-2B) indicating that the labelled toxin contained negligible amounts of unlabelled toxin, prior to electrofocussing (see Section 3.4.3).

The presence of a single species of modified toxin was also supported by the observation that the radioactive material was eluted as a single peak following ion-exchange column chromatography on CM-Sephadex C25 (Fig. 3.6), with a NaCl gradient (0.05 M - 0.4 M), before the peak of absorbance at 280 nm which corresponds to the unlabelled β-BuTx. However, this chromatographic method could only resolve the two toxin derivatives partially, since there was a high degree of overlap between these two peaks, indicating that it was not possible to separate completely any unlabelled-toxin from the labelled derivative using this technique. It would seem that a more satisfactory method in resolving these derivatives of β-BuTx would be preparative isoelectric focussing as described before (Section 3.3.4.2).

In the preparation of β-BuTx tritiated to low specific activity, a mixture of radiolabelled and unlabelled

Lethality tests (A) were performed for both preparations of high and low specific radioactivity as described in Section 3.3.4.4. A toxicity of 0.06 µg/g body wt. was obtained for ^{3}H-β-BuTx after preparative isoelectric focussing and was calculated using the specific radioactivity of the unfocussed material. Phospholipase A_2 activities (B) of both derivatives of ^{3}H-propionylated β-BuTx were determined using a titrimetric assay *(a)*, described in Section 2.3.4.3, and radiolabelled membrane phospholipids of synaptosomes *(ℓ)* as described in Section 3.3.4.5. The enzymic activity was assayed in the absence (-DOC) or presence (+DOC) of sodium deoxycholate at 37°C. Control samples containing no addition in the presence and absence of sodium deoxycholate (DOC) exhibited activities of 150 and 173 dpm/min/mg protein, respectively.

Table 3-2 Whole animal toxicity and phospholipase activity of ^{3}H-propionylated β-BuTx

A.

Route of administration	Dose (μg/g body wt)	High specific activity derivative (^{3}H-β-BuTx)		Low specific activity derivative	
		No. of animals injected	No. of animals killed	No. of animals injected	No. of animals killed
Intraperitoneal injection	0.005	3	0	3	0
	0.01	3	0	3	0
	0.05	3	3	3	2
	0.10	3	3	3	3
	Dose (ng/g body wt)				
Intraventricular injection	0.02	2	0	2	0
	0.05	2	0	2	0
	0.10	2	0	2	0
	0.25	2	2	2	1
	0.50	2	2	2	2
	1.0	2	2	2	2

B.

Substrate		High specific activity derivative (^{3}H-β-BuTx)	Low specific activity derivative	β-BuTx
Lecithin[a] (μmol H^{+}/min./mg protein	+ DOC	0	0	68.5
	- DOC	0	0	0.9
Radiolabelled[b] synaptosomes (dpm/min./mg protein	+ DOC	5800	6700	450000
	- DOC	450	300	790

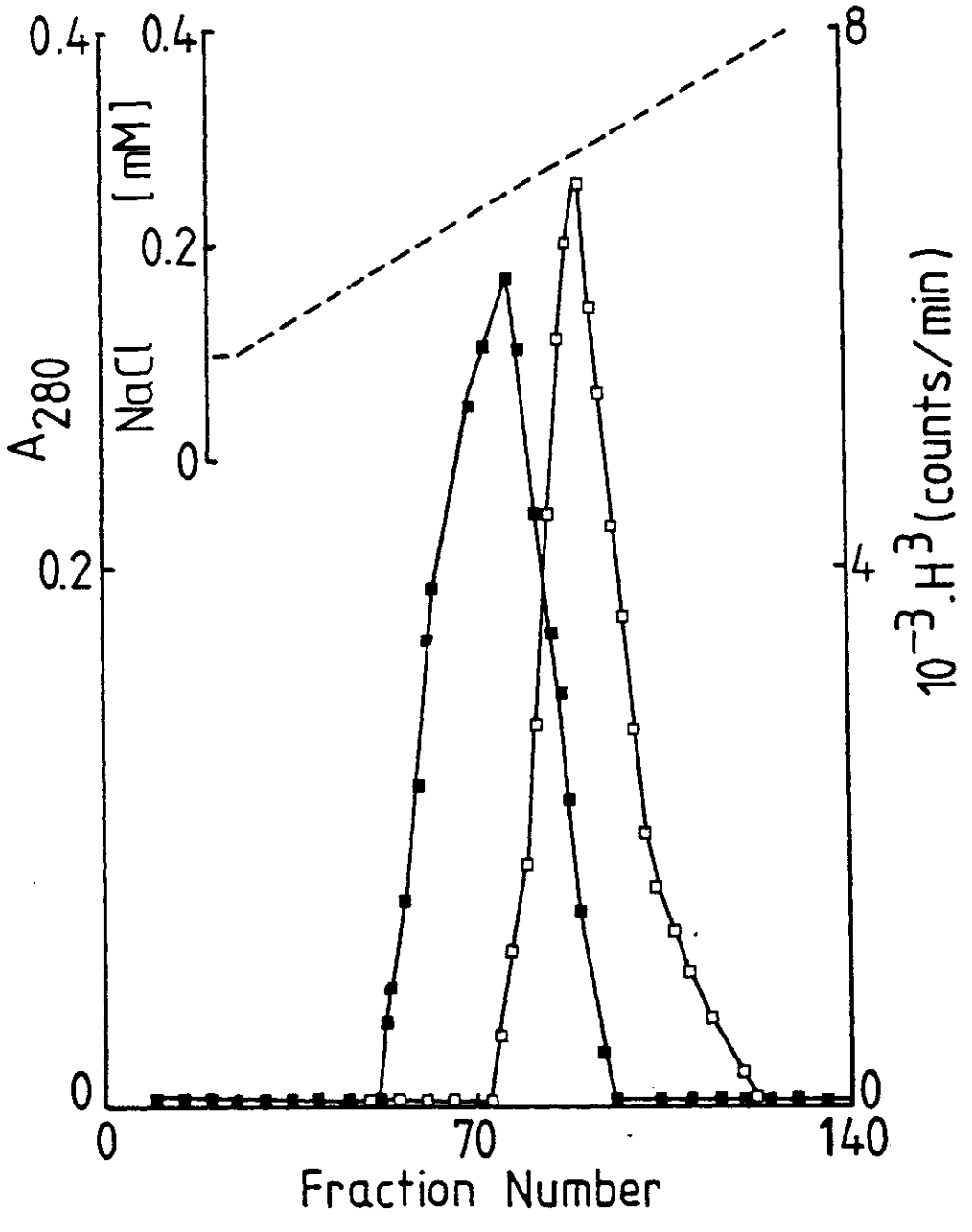


Fig. 3.6 Ion-exchange chromatography of native and ^{3}H-propionylated β-BuTx

Radiolabelled toxin from the high specific activity labelling was applied to a CM-Sephadex C25 column (0.4 X 10 cm) and eluted with linear gradient of NaCl (50-400 mM) (----) as described in Methods. Radioactivity (■) was determined by counting 10 µl aliquots in xylene-based scintillation cocktail containing Triton X-100 (10% v/v). Protein (□) of the fractions at 280 nm.

N-succinimidyl propionate in the ratio of 1:19 was used but the ratio between reagent and toxin was still maintained at 3:1. After separation of the labelled toxin on Sephadex G-50 (superfine) equilibrated with 50 mM NH_4OAc pH 7.4 (Fig. 3.4B), the labelled derivative obtained (referred to as low-specific activity derivative) was subjected to analytical isoelectric focussing as described in Methods. As in the case of ^{3}H-β-BuTx, the labelled derivative obtained in this preparation was focussed as a sharp single peak, with an identical pI as the former (Fig. 3.5C). On preparative isoelectric focussing this derivative was also focussed as a single band (Fig. 3.5D). When the neurotoxicity and enzymic activity were measured for the low-specific activity derivative, similar results were obtained as for ^{3}H-β-BuTx, indicating that this modified toxin preparation exhibited similar properties to the latter (Table 3.2).

3.4.3 Characterization of radiolabelled derivatives of β-BuTx

The chemical specific radioactivity of ^{3}H-β-BuTx and the low-specific activity derivative were determined by measurements of protein and radioactive contents by colorimetric method and liquid scintillation counting, respectively. ^{3}H-β-BuTx had a specific radioactivity of 102 Ci/ mmol which is very close to twice that of the tritiated reagent used indicating the presence of two moles of label per mole of toxin. Moreover, the sharpness of the peak of ^{3}H-β-BuTx on isoelectric focussing and the clear shift of 0.3 in its pI from that of native toxin (Fig. 3.5A) strongly suggests

that all the toxin was di-^{3}H-propionylated. This was consistent with the nearly equal labelling of both the toxin's subunits (1:1.1) for the larger and smaller polypeptides, respectively, demonstrated by counting the gel slices after electrophoresis (Fig. 3.7A); tritiation of the two chains was confirmed by fluorography.

The low-specific activity derivative had a specific radioactivity of 5.2 Ci/mmol. Since the radiolabelled N-succinimidyl (2,3-^{3}H) propionate was diluted twenty times in the reaction mixture with the unlabelled derivatives, and assuming that both derivatives have identical rates of labelling of proteins, a stoichiometry of 2:1 of reagent to toxin would be obtained. On SDS-PAGE and under reducing conditions, equal labelling was observed of both subunits (Fig. 3.7B). These results were consistent with that of ^{3}H-β-BuTx.

Examination of the phospholipase activity of native toxin using the titrimetric assay described in Section 2.3.4.3, showed that the toxin hydrolysed 68.5 μmol lecithin min^{-1} mg $protein^{-1}$ in the presence of deoxycholate (Section 2.3.4.3, Table 2-3 and 3-2); in contrast, no phospholipase activity was detectable in ^{3}H-β-BuTx and the low-specific activity derivatives of β-BuTx with this assay either in the presence or absence of deoxycholate (Table 3-2B). Phospholipase A_2activity was also determined using the putative natural substrate, cerebrocortical synaptosomes, quantifying the amount of tritiated fatty acid released by ^{3}H-β-BuTx and the low-specific activity derivative; again both derivatives contained negligible activity (1.3% and 1.5% of native toxin, respectively) in the presence of

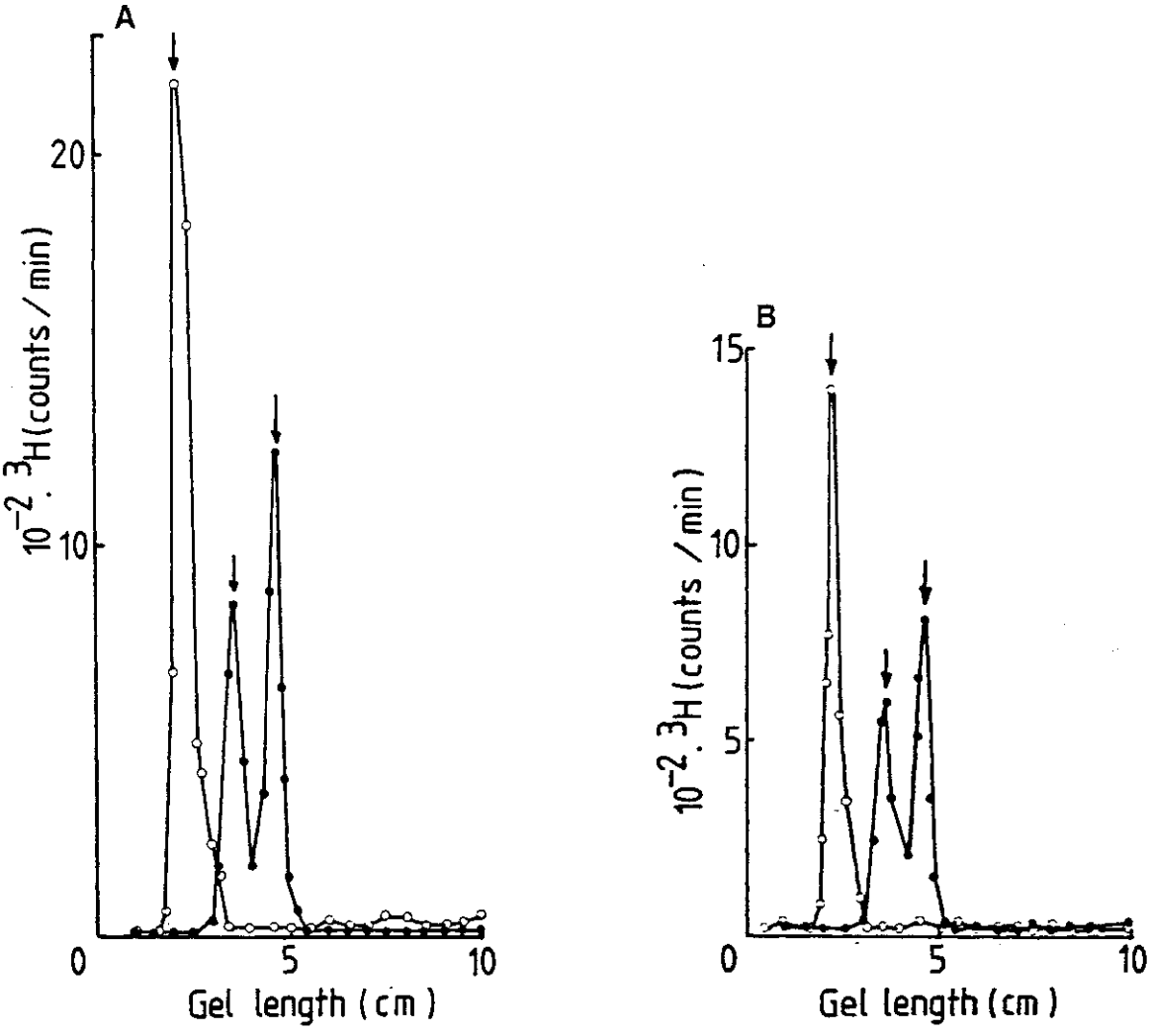


Fig. 3.7 Sodium-dodecyl sulphate/urea gel electrophoresis of ^{3}H-propionylated β-BuTx

A ^{3}H-β-BuTx from radiolabelling of β-BuTx to high specific activity

B β-BuTx derivative from radiolabelling to low specific activity of β-BuTx

SDS-UREA PAGE was performed as described in Methods, under reducing (●) and non-reducing (○) conditions. After electrophoresis, the gel was processed for fluorography: tracks were sliced into 2-mm sections and their contents of radioactivity determined by scintillation counting. Arrows indicate the position of unlabelled toxin run under similar conditions in different tracks, fixed, then stained with Coomassie R_{250} and destained. Integration of the peaks areas of the two subunits of the tritiated toxin gave a ratio of 1.1 : 1.0 labelling for the A and B chains respectively.

deoxycholate (Table 3-2). Similarly, no activity was detectable in the absence of deoxycholate. The lack of significant phospholipase A_2 activity must be due to the propionylation of a residue, presumably in the larger polypeptide, which is essential for enzymic activity. It is assumed but not proven, that lysine groups are labelled as demonstrated for propionylated α-BuTx (Dolly *et al.*, 1981a). This assumption is supported by the demonstration of an essential residue, presumably a lysine amino group, in the phospholipase A_2 active site of β-BuTx which can be modified by ethoxyformic anhydride (EOFA) (Howard and Truog,1977). Recent X-ray crystallographic analysis of the active site of bovine pancreatic phospholipase A_2, which is homologous to the A-chain of β-BuTx, showed the presence of 4 lysine residues distributed in a ring surrounding the entrance to the active site of the enzyme (Dijkstra *et al.*,1981); modification of any one of these residues could prevent the correct alignment of substrate for the subsequent ester hydrolysis. Modification of β-BuTx with EOFA resulted in the loss of both the enzymic activity and neurotoxicity, thus indicating the modifications of essential residues on the toxin molecule. Hence the loss of phospholipase A_2 activity could be due to the modification of one or more of the lysine residues described above.

The lethality of radiolabelled β-BuTx derivatives, as expected, was reduced in the absence of phospholipase activity; nevertheless the radiolabelled toxin derivatives retained considerable biological activity. When administered intraperitoneally, the toxicities of the labelled toxin derivatives were decreased five-fold relative to the

native toxin (Table 3-2). Similarly, this five-fold decrease in neurotoxicity was also observed when ^{3}H-β-BuTx and the low specific activity derivative were injected into the cerebral ventricular spaces of rat brain by stereotaxic administration. The neurotoxicity of β-BuTx was 200 times higher in the intraventricular spaces of rat brain than when it was administered intraperitoneally (Table 2-3A). From these results, the radiolabelled β-BuTx derivatives are highly toxic when compared to native toxin and to the phospholipase A_2 from bee-venom (Table 2-3B). Both derivatives are stable on storage for a period of several months at -20°C in 50 mM NH_4OAc, pH 7.4.

3.4.4 Electrophysiological analysis of the action of ^{3}H-propionylated β-BuTx on rat olfactory cortex slices

The action of β-BuTx in the CNS has been reported only recently by Dolly and coworkers (Dolly *et al.*, 1980; Halliwell and Dolly,1982a; 1982b); analysis of the action was carried out on rat olfactory cortex and hippocampal slices. On rat olfactory cortex, β-BuTx causes a rapid blockade of the synaptically-evoked N-wave followed by a slower depression and subsequent abolition of the presynaptic volley; this effect was found to be irreversible (Dolly *et al.*,1980; Halliwell and Dolly,1982a). Abolition of the intrinsic PLA_2-activity of the toxin by the substitution of Sr^{2+} for Ca^{2+} (Strong *et al.*,1976) or modification of a histidine residue at the active enzymic site with p-bromophenacyl bromide (Abe *et al.*,1977), markedly reduced the effectiveness of β-BuTx in blocking neurotransmission in

the olfactory cortex (Dolly *et al.*,1980); the half-times for the blockade were extended (from 25 min. in Ca^{2+}) to 102 min. and 65-150 min. respectively. It was therefore, important to determine the action of ^{3}H-β-BuTx, which lacks enzymic activity, in the central nervous system. For this purpose, the low specific activity derivative prepared in a similar manner to the high-specific activity toxin, and shown to possess similar properties to the latter (see above), was tested on rat olfactory cortex slices. When 230 nM of this toxin was added to the preparation, it caused a decline in the N-wave amplitude leading to a complete blockade (Fig. 3.8A) of neurotransmission. The presence of ^{3}H-β-BuTx also caused the presynaptic action potential to decline steadily but at a slower rate than the postsynaptic response, as was observed with native β-BuTx (Fig. 3.8A) (Dolly *et al.*,1980; Halliwell and Dolly, 1982a). The ratio between the postsynaptic and presynaptic response, regarded as a measure of relative synaptic efficiency, showed a steady decline following a latent period of 20 min. after addition of ^{3}H-β-BuTx, to produce a complete blockade of neurotransmission after about 60 min. (Fig. 3.8B). The half-time obtained for the blockade was about 32 min., indicating that propionylation did not significantly affect the electrophysiological actions of the toxin.

3.4.5 Binding of ^{3}H-β-BuTx to rat cerebrocortical synaptosomes

The binding of ^{3}H-β-BuTx to synaptosomes from rat cerebral cortex was saturable (Fig. 3.9A); on Scatchard

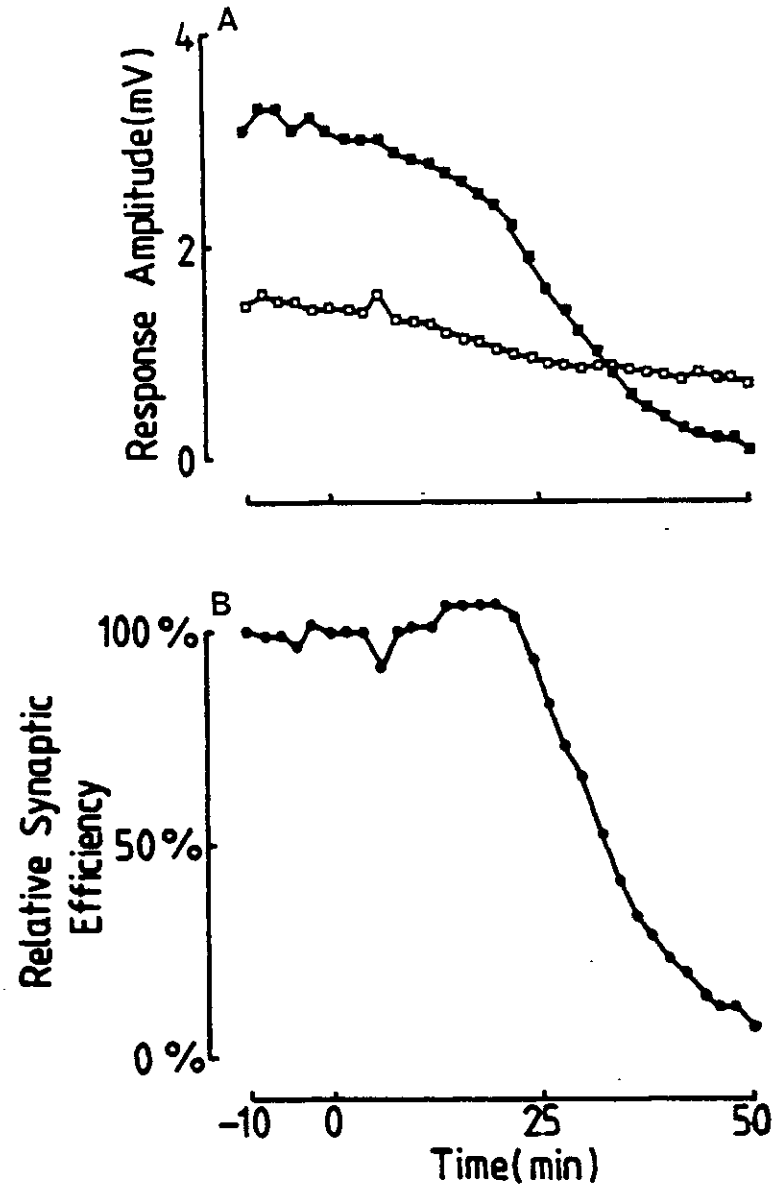
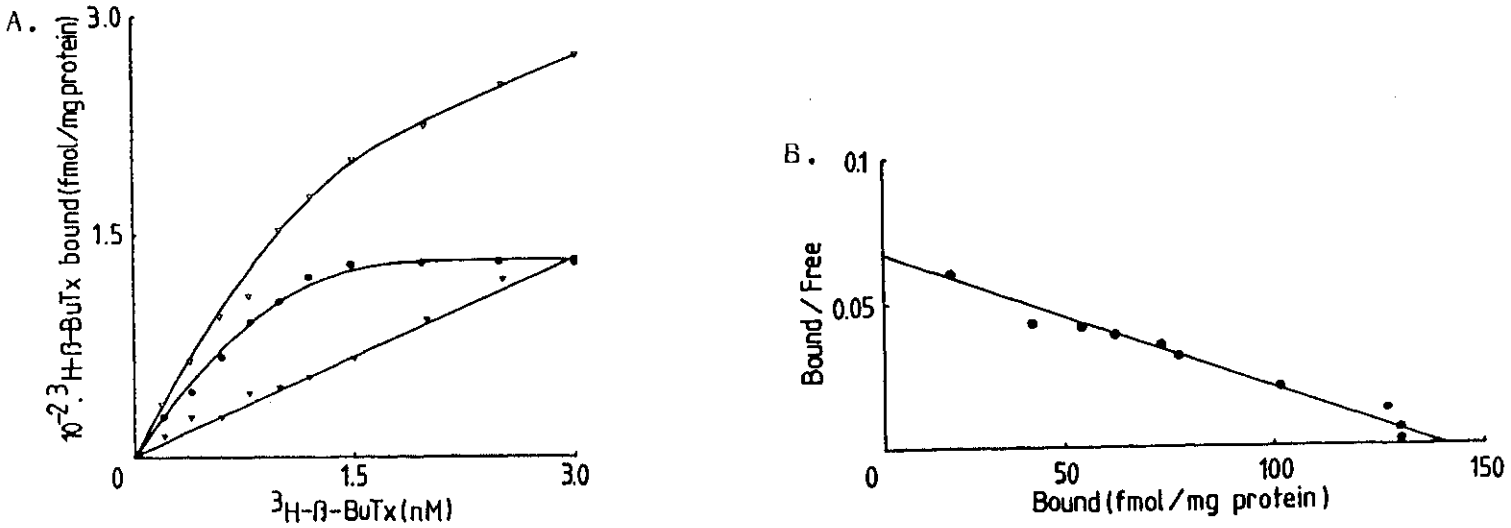


Fig. 3.8 Effect of ^{3}H-propionylated β-BuTx on neurotransmission in rat olfactory cortex

Field potentials, initiated by stimulation of the anterior end of the lateral olfactory tract (LOT) (100 μA, 100 μs pulse), were recorded in the piriform cortex and measured to determine the amplitude of the presynaptic action potential volley and the negative wave (post-synaptic response) at a fixed latency of ∿ 3 ms on the falling phase. In **A**, the effect of 230 nM of low specific activity derivative of ^{3}H-β-BuTx is shown. Presynaptic (□) and post-synaptic (■) response amplitude are plotted against time; labelled toxin was added at time zero. In **B**, synaptic efficiency, the ratio of the amplitude of post-synaptic to presynaptic responses, was calculated at each time point and expressed as a percentage of the value at zero time.

(These experiments were performed by Dr.J.V.Halliwell, Dept. of Pharmacology, School of Pharmacy,London University)

Fig. 3.9 Binding of ^{3}H-β-BuTx to synaptosomes



A. Synaptosomes (0.6 mg protein/250 µl) were preincubated at 37°C in the absence and presence of unlabelled toxin for 5 min. Different concentrations of ^{3}H-β-BuTx were then added and after a further 30 min. incubation, the total amounts of bound radioactivity were measured by a centrifugation assay as described in Methods. The values obtained for non-specific binding (▼) were subtracted from the total (▽) to give the amount of specifically bound (●) ^{3}H-β-BuTx. Average values observed with duplicate samples are expressed relative to the amount of measurable protein in the equivalent samples. It should be noted that the concentrations of free toxin were measured in the supernatant of the reaction mixture after dilution and sedimentation. To check the accuracy of these values, they were remeasured for 2 concentrations in the undiluted supernatants after sedimentation of the reaction mixtures; the values obtained were 20% and 18.6% for 0.5 nM and 5 nM, respectively, lower than those used in the plot shown above. Attempts were made to measure the specific binding by the modification of the standard assay, in which the dilution step was omitted and the pellet was rinsed but not resuspended during one immediate rapid wash. This was found to give an unacceptable higher value of non-specific binding which increased dramatically with higher toxin concentrations and this prevented accurate measurement of specifically-bound toxin. Hence, extrapolation of a more accurate values for the K_d and B_{max} was not feasible.

B. Scatchard analysis of the data in A.

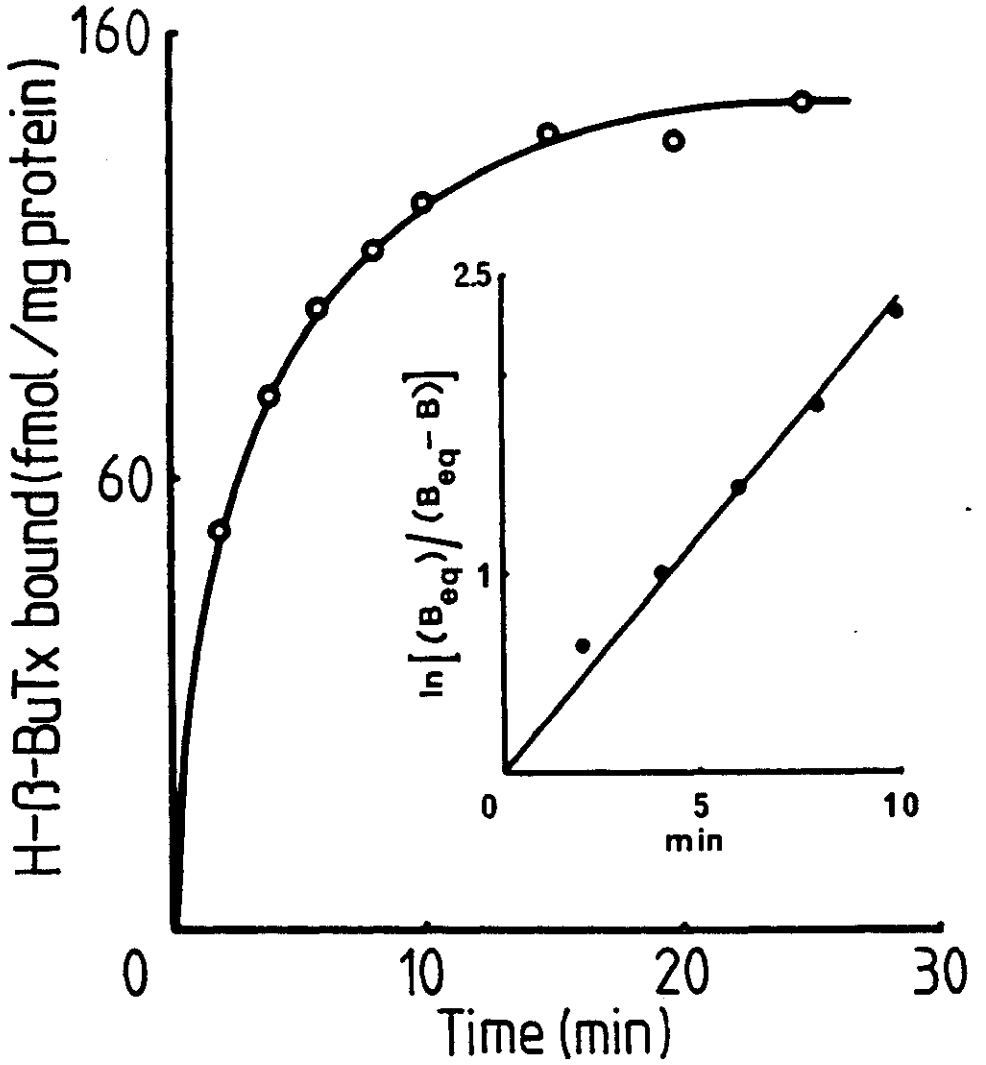
analysis (Fig. 3-9B), a single component was observed with a maximum capacity of 135-160 fmol/mg protein, and a K_d of 0.6 nM. The binding was blocked by a 100-fold excess of unlabelled β-BuTx with non-specific binding representing an acceptable fraction of the total (≃ 30%). Furthermore, no saturable binding was detectable to myelin, isolated during purification of the synaptosomes.

At a concentration of 5 nM ^{3}H-β-BuTx, this binding was rapid with a $t_{\frac{1}{2}}$ of 12.5 min. at 37°C; it reached a plateau after 20 min. and thereafter there was a slight increase in the non-saturable binding only (Fig. 3-10). This association of ^{3}H-β-BuTx with synaptosomes can be analysed as a pseudo-first order reaction using the equation:

$$\ln \frac{\{B_{eq}\}}{\{B_{eq}\} - \{B\}} = (\{L\}k_1 + k_{-1})t \quad \ldots\ldots \text{ Equation 3-1}$$

where $\{B_{eq}\}$ is the concentration of bound ligand at equilibrium, $\{B\}$ is the concentration of bound ligand at time t, $\{L\}$ is the concentration of ligand used, k_1 and k_{-1} are the rate constants of association and dissociation, respectively. When $\ln \frac{\{B_{eq}\}}{\{B_{eq}\} - \{B\}}$ was plotted as a function of time (Fig. 3-10), a straight line was obtained with a slope $(\{L\}k_1 + k_{-1})$ equalling 4.1×10^{-3} s^{-1}. The rate of dissociation (k_{-1}) of ^{3}H-β-BuTx from its binding site was determined by adding unlabelled toxin (500 nM) to a synaptosomal suspension which had been previously incubated with ^{3}H-β-BuTx (5 nM). The subsequent rate of decrease in specifically bound radioactivity (Fig. 3-11A) had a $t_{\frac{1}{2}}$ of 25 min. at 37°C, and was linear when plotted according to the rate equation

Fig. 3.10 Time-course of binding of ^{3}H-β-BuTx to cerebrocortical synaptosomes



Suspensions of synaptosomes (0.5 mg protein/ 250 µl) were preincubated with and without 500 nM unlabelled toxin for 5 min. at 37°C prior to addition of 5 nM of ^{3}H-β-BuTx. The amounts of total and non-specific binding of ^{3}H-β-BuTx at the times indicated were measured as described on Fig. 3.9. The non-specific binding was subtracted from the total to give specific binding (O) shown for duplicate samples. Insert: Pseudo-first order plot of the data where B_{eq} and B represents the amounts of ^{3}H-β-BuTx bound at equilibrium and at time t, respectively.

which described a first order reaction:

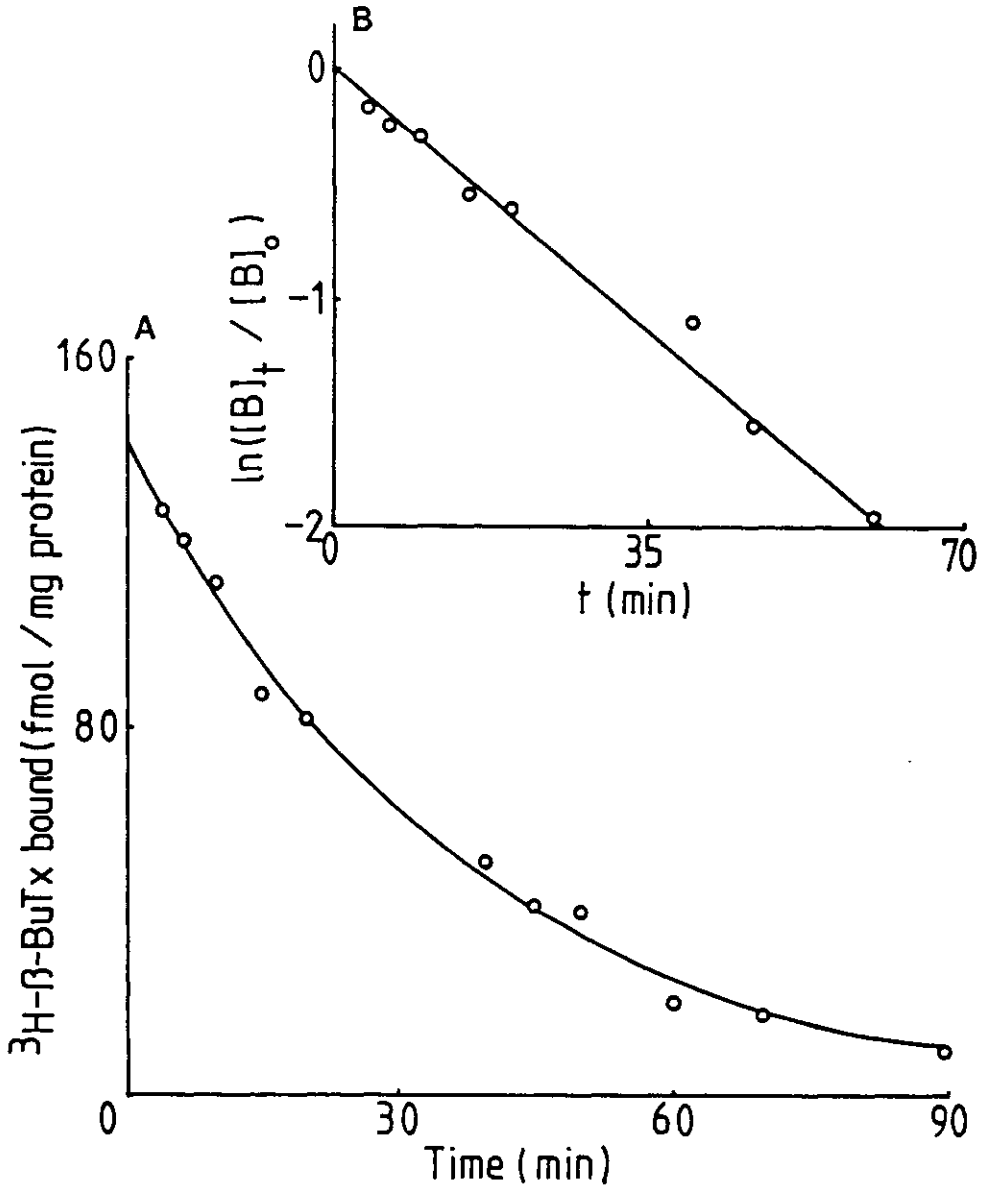
$$\ln \frac{\{B\}}{\{B_0\}} = -k_{-1}t \quad \text{..... Equation 3-2}$$

where $\{B_0\}$ and $\{B\}$ are the concentrations of bound toxin at time zero, and time t, respectively. From the slope of the linear plot (Fig. 3-11B), k_{-1} was found to be $5.6 \times 10^{-4} s^{-1}$. By substitution of this value into Equation 3-1, k_1 was shown to be $7.8 \times 10^5 M^{-1} s^{-1}$; this is similar to values reported for mono-^{3}H-propionylated α-BuTx reacting with acetylcholine receptors (Dolly *et al.*,1981a). The dissociation constant, K_d, calculated from these values of k_1 and k_{-1} (taken from this and two similar experiments) was 0.57 (± 0.015) nM; the latter agrees with the value of 0.6 nM obtained from Scatchard analysis above (Fig. 3-9B). Competition of ^{3}H-β-BuTx binding with increasing concentrations of unlabelled β-BuTx (Fig. 3-12A) demonstrated that the native toxin had a K_i of 0.29 (± 0.07; n = 6) nM; this indicates that the binding affinity is not decreased appreciably by tritiation. Collectively, the kinetic data is consistent with a single class of non-interacting sites of high affinity.

3.4.6 Lack of involvement of the phospholipase active site with the binding of ^{3}H-β-BuTx to cerebrocortical synaptosomes

In view of the intrinsic phospholipase A_2 activity of β-BuTx, the effects of the latter on ^{3}H-β-BuTx binding were examined; although ^{3}H-β-BuTx lacked significant catalytic activity, it could conceivably bind substrate. As

Fig. 3.11 Time-course of dissociation of ^{3}H-β-BuTx from its binding component on synaptosomes



A ^{3}H-β-BuTx (5 nM) was incubated at 37°C with synaptosomes (0.5 mg protein/250 µl) for 30 min. and the amount of specifically bound toxin was determined in Fig. 3.9. Unlabelled toxin (500 nM) was added and the amount of specifically bound toxin (O) was measured thereafter at the times indicated. Non-specific binding was determined simultaneously in another sample by inclusion of unlabelled toxin (500 nM) in the initial incubation mixture; it was subtracted from the total to give the amount of specific toxin remaining (O).

B First-order plot of the data in A.

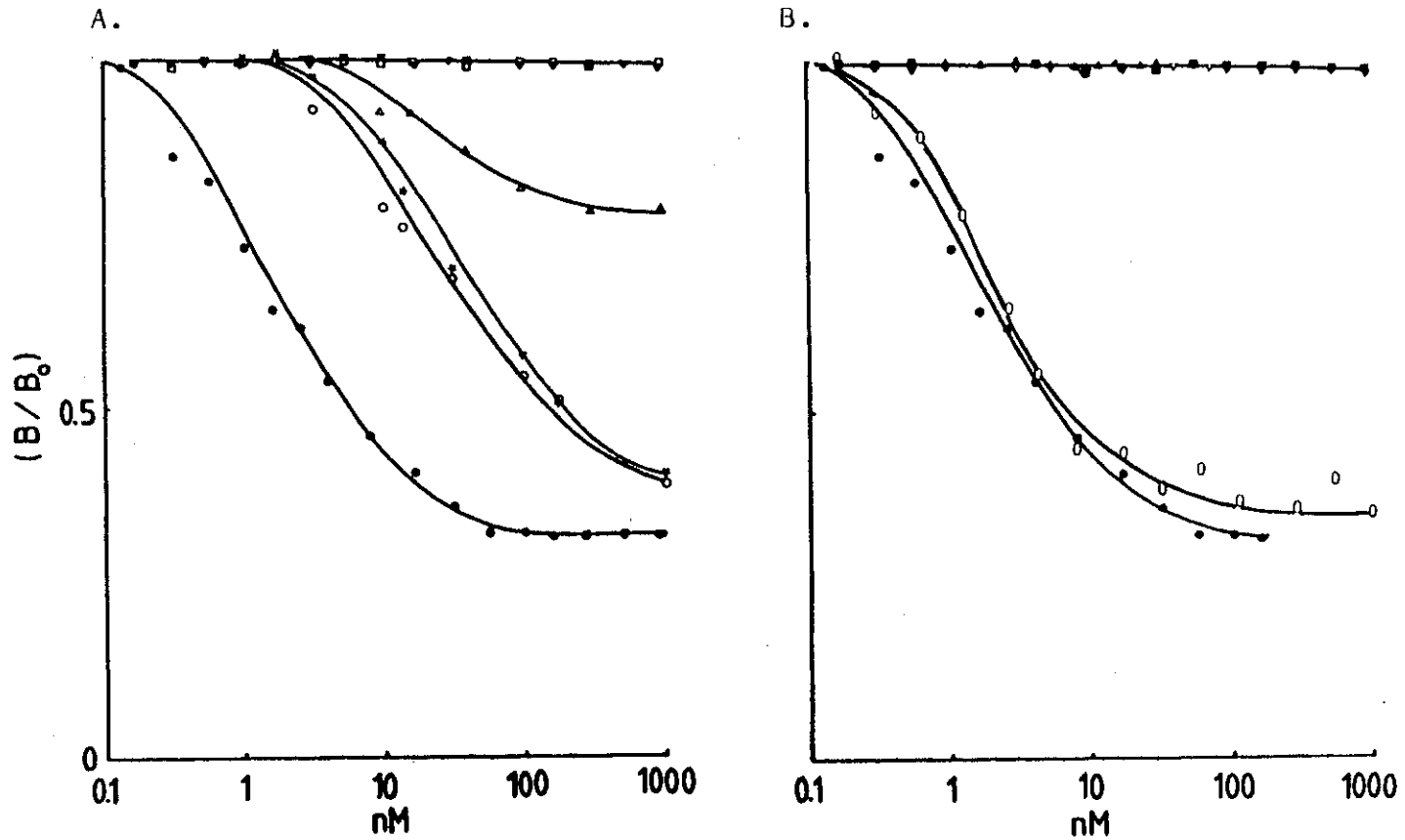


Fig. 3.12 ^{3}H-β-BuTx binding to synaptosomes in the presence of other presynaptic neurotoxins and phospholipases A_2

A. Synaptosomes (0.6 mg protein/250 µl) were incubated for 30 min. at 37 °C in the presence of 5 nM ^{3}H-β-BuTx with different concentrations of unlabelled toxin (●). BL_0 and BL represent the amount of ^{3}H-β-BuTx binding in the absence and presence of competing protein: p-bromophenacyl-bromide-labelled β-BuTx (Δ), BV-PLA_2 (○ and □, in the absence of Ca^{2+}), tityustoxin (▼) and taipoxin (★).

B. Similar experiments as in A were performed using unlabelled β-BuTx (● and °, in the absence of Ca^{2+}) botulinum neurotoxin (■), α-latrotoxin (▲) and phospholipase A_2 from *Naja melanoleuca* (▽).

Each K_i was calculated using the observed IC_{50} value, according to the equation:

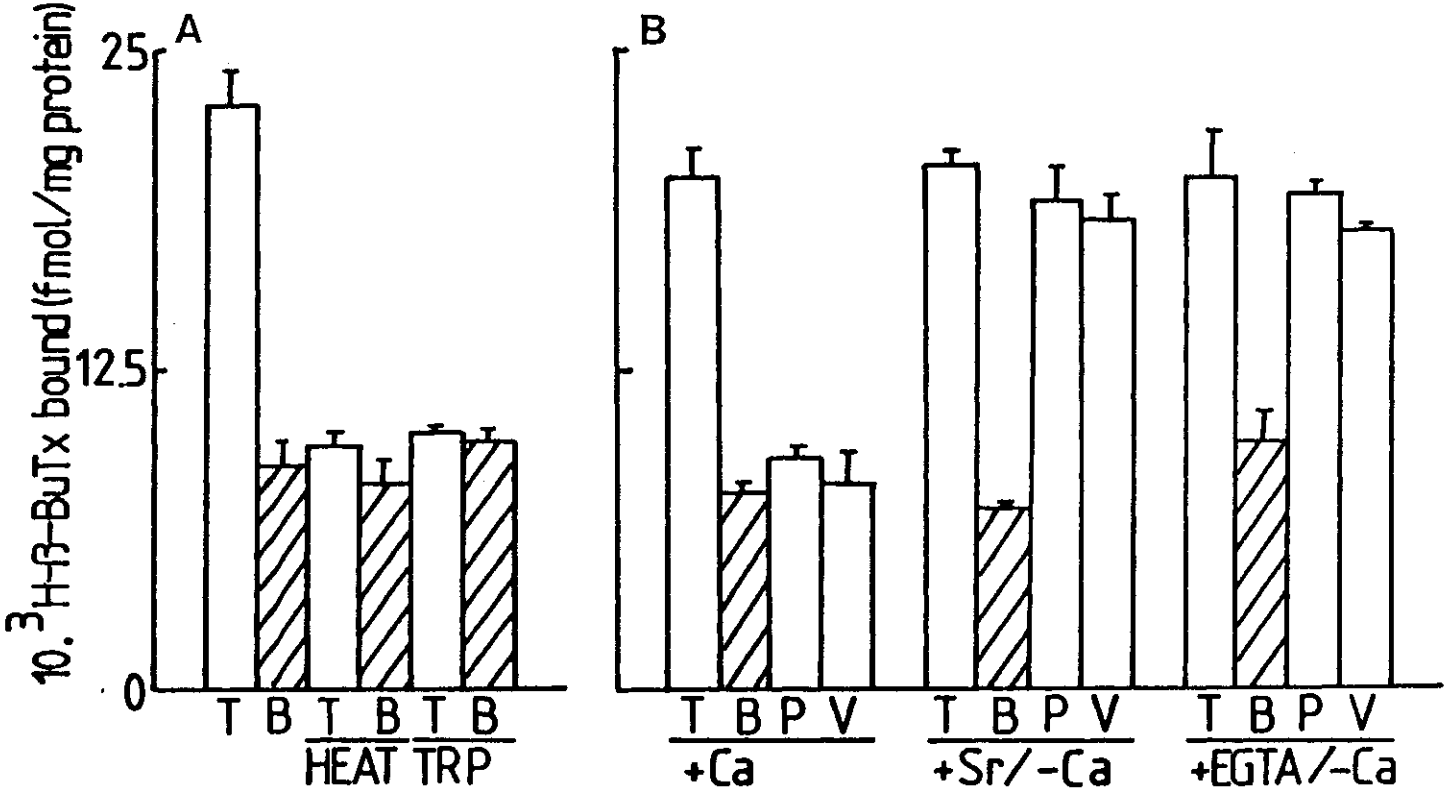
$$K_i = IC_{50} / \left(1 + (\{L\}/K_d)\right)$$ where {L} is the ^{3}H-β-BuTx molar concentration.

phospholipase A_2 requires Ca^{2+} for the binding of monomeric phospholipid substrate (Volwerk *et al.*,1974), the effect of Ca^{2+} removal on the binding was examined. The extent of ^{3}H-β-BuTx binding was not altered by removing Ca^{2+} (Fig. 3-13B); similar conclusions were drawn from electrophysiological measurements on olfactory cortex with native toxin (Dolly *et al.*,1980). Phospholipase A_2 from the snake venom *Naja melanoleuca* had no effect on the binding (Fig. 3-12A) while phospholipase A_2 from bee venom, which is considerably more active but much less neurotoxic than β-BuTx (Table 3-2), antagonised the binding of ^{3}H-β-BuTx but with a K_i value of 5.9 (± 0.16; n = 4) nM; the latter value, calculated from the IC_{50} (Fig. 3-12A), was 10-fold higher than the toxin's K_d. Furthermore, the apian enzyme was ineffective in Ca^{2+}-free medium, with Ca^{2+} substituted with Sr^{2+} or in the presence of EGTA (Fig. 3.13B). These findings suggest that the binding was not due to enzyme-substrate interaction; similar results were reported for the binding to electroplax membranes of another snake neurotoxin, crotoxin (Bon *et al.*,1979), that contains a homologous phospholipase chain (Lee,1979). Moreover, a derivative of β-BuTx whose phospholipase activity was removed by labelling with p-bromophenacyl bromide (Halliwell *et al.*,1982), inhibited ^{3}H-β-BuTx binding to synaptosomes (Fig. 3.12A) albeit with low potency.

3.4.7 Nature of ^{3}H-β-BuTx binding sites on synaptosomal membranes

Treatment of the synaptosomes with trypsin for 60 min. at 37°C destroyed the binding component responsible

Fig. 3.13 A. Effect of heat treatment and trypsinization on ^{3}H-β-BuTx binding to synaptosomes
B. Effect of divalent cations on ^{3}H-β-BuTx binding to synaptosomes



A. Synaptosomes were heat-treated (HEAT) or preincubated in Kreb's-phosphate buffer in the the absence or presence of trypsin (1 mg/ml) (TRP) using the conditions detailed in Methods. After termination of treatment by dilution and centrifugation, synaptosomal pellets were suspended in reaction buffer and then incubated with 5 nM ^{3}H-β-BuTx for 30 min. at 37°C. Total (T) and non-specific (B) was quantitated in the absence and presence of 500 nM unlabelled toxin, respectively, by the centrifugation assay described in Fig. 3.4. The data given were obtained from 4 determinations.

B. The binding of ^{3}H-β-BuTx was determined in the presence or absence of Ca^{2+}; in the latter Ca^{2+}, was substituted with either Sr^{2+} or EGTA. Total binding (T) and non-specific binding (B) was determined as described in A. The effect of BV-PLA$_2$ (V) and taipoxin (P) on ^{3}H-β-BuTx was also investigated under these conditions.

for saturable binding of the toxin, indicating that these binding sites are proteinaceous in nature (Fig. 3.13A). Similarly, when the synaptosomes were heated at 95^{0}C for 15 min., the specific binding of ^{3}H-β-BuTx was destroyed giving further evidence for the proteinaceous nature of the binding sites. Measurements of the binding of ^{3}H-β-BuTx to lysed synaptosomes, showed saturable binding, indicating that the binding component was membrane-bound, which is consistent with results from sub-cellular fractionation studies described in Section 6.3.3.

3.4.8 Specificity of ^{3}H-β-BuTx binding sites on synaptosomal membranes

To assess the specificity of the binding sites, the effect of a number of other presynaptic neurotoxins on the binding was investigated in competition experiments, as described in Methods. Although taipoxin has a similar mode of action to β-BuTx at motor end-plates (Fohlman *et al.*, 1979), it showed only weak antagonism of ^{3}H-β-BuTx binding, with a K_i of 6 nM, calculated from its IC_{50} (Fig. 3.14A). Also, with the latter, no inhibition was apparent on inactivation of the toxin's phospholipase A_2 activity by removal of Ca^{2+} or its substitution by Sr^{2+} (Fig. 3.13B). Another neurotoxin with a unique potency, botulinum neurotoxin, produced by *Clostridium botulinum* is known to specifically inhibit evoked and spontaneous release of ACh at the rat neuromuscular junction (Tse *et al.*,1982). In addition, it partially blocks resting and K^+-stimulated efflux of ACh from rat cerebrocortical synaptosomes (Dolly *et al.*,1981b). However, a purified preparation of this potent neurotoxin, at concentra-

tions of to 1 μM, had no detectable effect on synaptosomal binding of ^{3}H-β-BuTx, indicating that these two neurotoxins have distinct sites (Fig. 3.12A). This is consistent with the observation that the actions of β-BuTx on neurotransmitter accumulation and efflux from synaptosomes are not significantly affected by botulinum nuerotoxin (Dolly *et al.*, 1981b).

Likewise, α-latrotoxin, purified from black widow spider venom, which causes a large increase in neurotransmitter release from brain synaptosomes (Grasso *et al.*,1978) and at the neuromuscular junction (Frontali *et al.*,1976), displayed no ability to alter the binding of ^{3}H-β-BuTx; at 0.1-50.0nM concentration (Fig. 3.12B). In a similar experiment, tityustoxin, known to bind to a voltage-sensitive component of sodium channels (Catterall,1980), did not affect the binding of ^{3}H-β-BuTx (Fig. 4-12A). In contrast, purified and biologically active preparations of toxin I and dendrotoxin (DTX) isolated from *Dendroaspis polylepis* and *angusticeps* respectively, were found to inhibit totally the binding of ^{3}H-β-BuTx with K_i values of 0.07 (± 0.01; n = 4) nM and 0.5 (± 0.06; n = 4) nM, respectively (Fig. 3.14A, 3.14B). These results are of great interest, as toxin I and DTX have been shown to facilitate the release of neurotransmitter in nerve-muscle preparations (Harvey and Karlsson,1980; 1982). DTX has also been shown to cause the facilitation of neurotransmitter release in rat hippocampal slices (Chapter 4; Docherty *et al.*,1983). The observed antagonism of ^{3}H-β-BuTx binding is in accordance with the report (Harvey,1982; Harvey and Karlsson,1982) that toxin I and DTX antagonise the irreversible inhibition of neurotransmitter release produced by

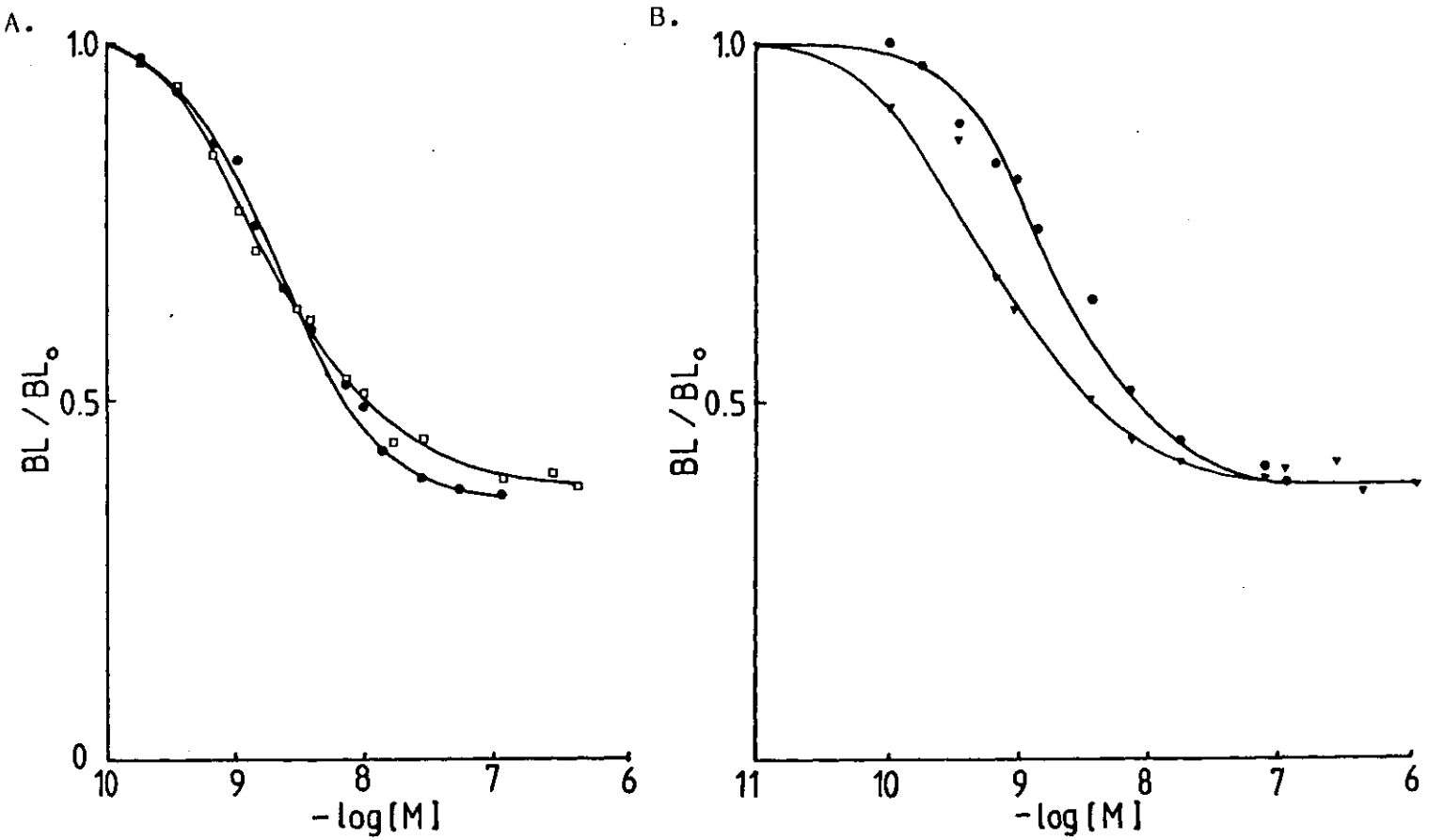
β-BuTx. Collectively, these results indicate that the binding sites for ^{3}H-β-BuTx, at least on these synaptosomal membranes,are distinct from the binding components of most other presynaptic neurotoxins. Moreover, the observation that two other physiologically active neurotoxins, toxin I and DTX, can inhibit specifically the binding of β-BuTx with very high potency emphasises the functional specificity of the binding component involved.

3.5 DISCUSSION

3.5.1 Tritiation of β-BuTx with N-succinimidyl-(2,3-^{3}H) propionate

A method has been developed for the labelling of β-BuTx with N-succinimidyl-(2,3-^{3}H) propionate and the isolation of di-^{3}H-propionylated species of high specific radioactivity; it was fully characterized with respect to toxicity (both in the peripheral and central nervous systems), phospholipase activity and its saturable binding to rat cerebrocortical synaptosomes (Othman *et al.*,1982). The conditions of tritiation are very gentle and efficient since such a mild method of labelling is essential to prepare radiolabelled derivative which is biologically active. Furthermore, the extent of propionylation (Dolly *et al.*,1981a) can be controlled by varying the ratio of protein/reagent used. A preliminary study of the various conditions used for radiolabelling β-BuTx with this reagent have been described (Spokes,1982). Using the conditions described in this Chapter, a stable, di-^{3}H-propionylated preparation of toxin that does not undergo inactivation by self-irradiation was obtained (Othman *et al.*,1982). A change in charge

Fig. 3.14 Inhibition of ^{3}H-β-BuTx binding by DTX and toxin I



A. Competition experiments were carried out as described in Fig. 3.12, using increasing concentration of unlabelled β-BuTx (●) or Dendrotoxin (□) purified from *Dendroaspis angusticeps*.

B. Similar experiments as in A were performed in the presence of unlabelled β-BuTx (●) or toxin I isolated from *Dendroaspis polylepis* (▼).

The K_i was calculated using the observed IC_{50} value as described in Fig. 3.12

introduced into the labelled species allows it to be separated from native toxin (Fig. 3.5B) by preparative isoelectric focussing but not satisfactorily by ion-exchange chromatography on CM-Sephadex C25 (Fig. 3.6). The measured specific radioactivity of 102 Ci/mmol, which is consistent with the presence of di-propionylated species, is high relative to that obtainable by other methods of tritiation and is adequate for most studies. The absence of unlabelled toxin excludes the possible presence of more highly labelled molecules. It cannot be ruled out that a number of di-substituted species are present in which residues at different positions have been modified. However, the sharpness of the focussed band, the equal labelling of its two subunits (Fig. 3.7A), the absence of phospholipase activity and the monophasic kinetics of its interaction with synaptosomal membranes, all argue against this.

Characterization of ^{3}H-β-BuTx indicated that the derivative was free from phospholipase activity, and retained about 20% of its neurotoxicity as measured both in the peripheral and central nervous systems with lethality tests. The substantial biological activity demonstrated for ^{3}H-β-BuTx, even though the intrinsic phospholipase activity is absent or negligible, suggests that its neurotoxicity can be exhibited independently of phospholipolysis, with the enzymic activity amplifying the former. This is in accord with the electrophysiological demonstration of toxin-induced blockade of transmitter release in the olfactory cortex by ^{3}H-β-BuTx (Fig. 3.8).

An investigation of the relationship between the neurotoxicity and phospholipase A_2 activity of β-BuTx using

several agents that modify proteins in various ways was reported by Howard and Truog (1977). Among the reagents tested was ethoxyformic anhydride (EOFA) which was thought to modify lysine residues; derivatives of toxin labelled with these reagents were biologically inactive as demonstrated by the lack of neurotoxicity. This could be due to differences in the extent of modification: particular groups labelled or their positions in the toxin molecule.

Likewise, ^{3}H-pyridoxalation of β-BuTx, using pyridoxal 5' phosphate and NaB($^{3}H_4$), to low specific radioactivity (8.4 Ci/mmol), lowered its peripheral toxicity by 10-fold. These changes could be due to the differences in the extent of labelling of sites when compared to ^{3}H-propionylation used in this study. Furthermore, pyridoxalation introduces a much bulkier group which could decrease the affinity of the toxin to its binding sites (see below). ^{125}I-Iodinated toxin prepared by the chloramine-T method was shown to retain phospholipase activity but its lethality was not determined (Oberg and Kelly,1976b). In this laboratory, β-BuTx radioiodinated using the above procedure yielded a preparation which was non-toxic when injected intraperitoneally into mice at a concentration of 0.4 µg/g body weight; also, an exceptionally high content of binding sites (B_{max} = 1.8 pmol/mg of protein) of low affinity (K_d = 2 X 10^{-8}M) on rat cerebrocortical synaptosomes was observed (A. Pelchen and J.O.Dolly, unpublished observations). However, when β-BuTx was labelled with ^{125}I-iodine to a specific radioactivity of 500 Ci/mmol by a modification of the chloramine-T procedure that employed mild conditions which have been used successfully with botulinum neurotoxin (Williams *et al.*,1983),

a high affinity binding to synaptosomes was observed (K_d = 5 X 10^{-10}M) with the expected content of sites of 120 fmol/mg protein. However, its lethality in mice was reduced 2-fold relative to ^{3}H-β-BuTx and the extent of non-specific binding was much higher than the latter (A.Pelchen and J.O.Dolly, unpbulished observations).

An initial report on the iodination of β-BuTx using lactoperoxidase indicates that this treatment renders it non-toxic (MacDermott *et al.*,1978). However, more recently, during the course of this study, successful radioiodination using this enzymic method was reported (Rehm and Betz, 1982). Although this toxin derivative was not fully characterized in terms of its neurotoxicity and enzymic activity, it was shown to bind to a single class of high affinity binding sites (K_d = 0.47 nM; B_{max} = 50 fmol/mg of protein) on chick synaptic membranes. Even though the ^{125}I-β-BuTx exhibited similar affinity to that observed with the ^{3}H-β-BuTx reported in this study, the maximum number of binding sites for the former was much lower; the discrepancy may be partly due to species differences. In contrast to the binding of ^{3}H-β-BuTx, Rehm and Betz (1982) showed that the binding of the ^{125}I-β-BuTx derivative obtained by the lactoperoxidase method occurred in a Ca^{2+}-dependent manner; the latter can be substituted by Co^{2+} or Sr^{2+} but not Mg^{2+}. As the phospholipase activity of the radioiodinated derivative was not examined, it is not possible to make a direct comparison with ^{3}H-β-BuTx, which does not contain any enzymic activity (Table 3-1). However, it is highly likely that the ^{125}I-β-BuTx retained a substantial degree of enzymic activity upon radioiodination which could account for its Ca^{2+}-dependency. Rehm and Betz (1982) also

postulated that the specific binding is mediated by the A-chain of the toxin molecule. The binding data presented in this study provide arguments against this hypothesis. Firstly, the ability of the protease inhibitor homologues, toxin I and DTX to inhibit the binding of ^{3}H-β-BuTx with high potency (Fig. 3. 14A, B). These toxins have been shown to facilitate neurotransmitter release at peripheral (Harvey and Karlsson,1980; 1982) and central (Docherty *et al.*,1983; Chapter 4) synapses and contain homologous polypeptides to the B-chain of β-BuTx (Table 1-2). Secondly, removal of Ca^{2+} by EDTA or substitution by Sr^{2+} has no effect on the binding of ^{3}H-β-BuTx (Table 3-13) indicating the lack of involvement of phospholipase activity in its binding. Evidence in support of this hypothesis have been reported by electrophysiological studies in rat olfactory cortex (Dolly *et al.*,1980) and at the neuromuscular junction (Kelly *et al.*,1979a;Caratsch *et al.*,1981).

Preliminary studies on the radioiodination of β-BuTx with N-succinimidyl-3-(4-hydroxy-5-^{125}I-iodophenyl) propionate (Bolton and Hunter reagent) were reported (Summers and Thompson,1980). The reagent should modify the same groups as N-succinimidyl (2,3-^{3}H) propionate but does introduce a much bulkier group that could be detrimental to the biological activity of the toxin; on the other hand, higher specific radioactivity could be obtained but with a short half-life. No further studies have since been reported, and the preliminary study did not include quantification of the toxin's lethality and phospholipase activity.

3.5.2 The binding of ^{3}H-β-BuTx to rat cerebrocortical synaptosomes: its nature and specificity

The binding of ^{3}H-β-BuTx to synaptosomal membranes is saturable and of high affinity (K_d = 0.57 nM); it was blocked by unlabelled β-BuTx with a K_i of 0.29 nM indicating that no significant change (within experimental error) occurred on propionylation. Consequently, it can be concluded that a non-essential residue was modified on the toxin (probably the B-chain), which is likely to be a lysine (see Results). In general, it is difficult to equate directly the binding parameters of a ligand to its efficacy *in vivo*; however with ^{3}H-β-BuTx, the K_d value obtained herein is similar to the approximated concentration shown to kill rats after intraventricular injection (Table 3-2) and to cause neuronal degeneration in the brain (Hanley and Emson, 1979). Likewise, the content of saturable binding sites is in the range expected for a potent neurotoxin. Interestingly, the value obtained is also similar to the number of binding sites for the ^{125}I-labelled preparation of a single-chain presynaptic toxin which exhibits a similar mode of action to β-BuTx isolated from the same snake venom (Donlon *et al.*,1979). However, the fraction of this latter binding which can be blocked by the unlabelled toxin and the biological activity of the iodinated derivative were not described. Comparison of the K_d values reported for synaptosomal binding of ^{3}H-pyridoxalated β-BuTx (K_d = 0.29 μM) and ^{3}H-β-BuTx indicates that the affinity of the former derivative for the binding sites is 100-fold lower than that obtained in the present study. Thus the very much higher content of binding sites observed for ^{3}H-pyridoxalated toxin (1.4 pmol/mg protein) could be

due to the presence of different sites displaying weaker affinity (K_d = 0.29 μM). This could be explained by the bulky pyridoxal moiety decreasing the toxin's neurotoxicity and affinity for the primary target site. Similar findings were also reported for pyridoxalated α-BuTx (Jones and Thompson,1980) which exhibited lower affinity for ACh receptors than the propionylated derivative (Dolly *et al.*,1981a). Radioiodinated derivatives of β-BuTx obtained by the chloramine-T method showed high affinity binding (0.7 - 1.7 nM) to brain synaptosomes and also to membranes from erythrocytes and liver, albeit in lower amounts (Oberg and Kelly,1976b). The content of these sites in brain is far higher than reported here in this study; as the biological activity of the radioiodinated toxin was not quantified directly, and the displaceability with unlabelled toxin and the specificity of binding were not examined, a meaningful comparison of its binding sites with those in this study is difficult.

The rate constant of 7.8 X 10^5 $M^{-1}s^{-1}$ obtained for the association of ^{3}H-β-BuTx to synaptosomal membrane at 37°C shows that binding is rapid and it is unlikely to be a rate-limiting step in the toxin-induced inhibition of neurotransmission. This would be consistent with the suggestion that the initial specific binding of toxin is followed by one or more steps that result in the final blockade of transmitter release (Abe *et al.*,1977; Kelly *et al.*,1979a). In view of the apparent irreversible interaction of β-BuTx with olfactory cortex and hippocampus measured electrophysiologically (Dolly *et al.*,1980; Halliwell and Dolly,1982a; 1982b), it was surprising to discover that the dissociation rate (k_{-1} = 5.6 X 10^4s^{-1}) was so fast. It must be emphasised that

this was measured in cerebral cortex synaptosomes at 37°C compared with electrophysiological recordings performed at 24°C with the toxin in olfactory cortex and hippocampus slices over relatively short-time periods.

The lack of involvement of phospholipase A_2 activity, in the actions of ^{3}H-β-BuTx was demonstrated by the high affinity saturable binding to synaptic membranes in the absence of Ca^{2+} (Fig. 3.13). With the absence of significant phospholipase activity towards lecithin or synaptosomal membranes (Table 3-2), the Ca^{2+}-independent binding must occur other than via an enzyme-phospholipid link; this idea has been postulated previously for binding of β-BuTx at the neuromuscular junction (Abe *et al.*,1977; Kelly *et al.*,1979a). Furthermore, the interaction of pancreatic phospholipase A_2, which shows sequence homology with the larger chain of β-BuTx (Kondo *et al.*,1978a),with phospholipid vesicles is of low affinity with a K_d of 10 μM (Volwerk,1974) indicating that such binding via the catalytic site is too weak to account for the high-affinity binding and known specificity of β-BuTx for nerve terminals. Investigation of the specificity of ^{3}H-β-BuTx binding indicated that β-BuTx modified on the A-chain with p-bromophenacyl bromide could antagonise the specific binding, albeit with low potency. This can be reconciled with the proposal that acylation changed the conformation of the toxin site involved in the specific binding despite the chemical modification being on the larger chain. This would account for its low potency (Fig. 3-12A) and for its feeble ability (Abe *et al.*,1977) to protect peripheral synapses from the action of native toxin; also it explains its much lower neurotoxicity relative to ^{3}H-β-BuTx. The inability

of the relatively non-toxic phospholipase A_2 from *Naja melanoleuca* to antagonise ^{3}H-β-BuTx binding argues for a specific binding locus on the toxin for synaptic membranes that must be separate from the catalytic site. However, BV-PLA_2 did inhibit the binding of ^{3}H-β-BuTx but with much lower potency (K_i = 5.9 nM) than unlabelled β-BuTx; the Ca^{2+}-dependency of this inhibition indicates that it is due to the enzymic activity. This phospholipase A_2 had the same effect on the binding components for ^{125}I-botulinum neurotoxin (Williams *et al.*,1983) and ^{125}I-DTX on synaptosomal membranes (see Chapter 5). This enzyme is capable of protecting motor nerve endings from the subsequent action of β-BuTx (Abe and Miledi,1978); such protection was observed when the toxin was applied after removing the phospholipase from the incubation medium. Its effect on ^{3}H-β-BuTx binding may, therefore, be the result of perturbation of neuronal membranes by phospholipolysis, rather than direct competition for the same binding site. The substrate specificity of the apian enzyme might explain why it is more potent than other phospholipases A_2 in its action. However, without further experimentation, the basis of the inhibitory effect cannot be established with any certainty.

The toxin's binding component was sensitive to trypsinization and heat treatment, suggesting that it is a protein. It seems specific for β-BuTx in the sense that no competition of binding was observed with the presynaptically-acting proteins, botulinum neurotoxin, taipoxin (in the absence of Ca^{2+}) and α-latrotoxin. The failure of botulinum neurotoxin, that inhibits transmitter release, to modify

^{3}H-β-BuTx binding accords with other evidence (Dolly *et al.*, 1981b; Chang *et al.*, 1973b) for distinct sites for this neurotoxin and β-BuTx. In addition, the existence of separate binding sites for these different neurotoxins accords with recent pharmacological evidence that β-BuTx, crotoxin and taipoxin exert their inhibitory effects on neuromuscular transmission by binding to separate sites (Harvey and Karlsson, 1982; Chang and Su, 1980). If, as suggested (Simpson, 1976), β-BuTx interacts with sites exposed by the exocytotic process, α-latrotoxin which facilitates transmitter release (Grasso *et al.*, 1978) might be expected to increase the binding, but this was not the case; moreover, binding occurs to lysed synaptosomes. When the effect of tityustoxin, which is thought to interact with the modulatory component of sodium channels, on ^{3}H-β-BuTx binding was tested, it was found not to antagonize it specific binding to synaptosomal membranes. When synaptosomes were treated simultaneously with tityustoxin and β-BuTx, the amount of neurotransmitter release was very much smaller than the sum of the amounts released with each of these toxins. The binding data reported here (Fig. 3.14A) make it unlikely that this phenomenon could be explained by both these toxins binding to the same site; thus, an indirect mechanism may be operative and the reported (Okamoto, 1980) ability of β-BuTx to inhibit the binding to electroplaque membranes of a related scorpion toxin must, therefore, be the result of its phospholipolytic action.

The ability of toxin I and DTX from mamba venoms to inhibit the binding of ^{3}H-β-BuTx with very high potency means that a physiologically active antagonist has been found for the latter. Both these protease inhibitor homologues,

cause an increase in the release of ACh at the neuromuscular junction, in contrast to β-BuTx which blocks neurotransmitter release. The importance of this finding provides strong evidence for the proposal that β-BuTx interacts with a nerve membrane component concerned with the process of transmitter release. This result could also explain the unique toxicity of toxin I and DTX when administered intraventricularly into rat-brain (Chapter 4) and the electrophysiological demonstration that toxin I and DTX can antagonise the inhibitory action of β-BuTx on neurotransmitter release in chick nerve-muscle preparations (Harvey,1982; Harvey and Karlsson,1982). Furthermore, toxin I and DTX are very much less neurotoxic than β-BuTx following peripheral injection into mice (Harvey and Karlsson,1980; Strydom,1976; Chapter 4); nevertheless, when injected with β-BuTx, they greatly reduce the lethality of the latter (Chapter 4). This antagonism may be due as much to the opposing effects of these toxins as to their common binding sites at nerve terminals.

CHAPTER 4

PREPARATION AND CHARACTERIZATION OF HOMOGENEOUS DENDROTOXIN FROM *DENDROASPIS ANGUSTICEPS*: ITS EXCITATORY ACTION ON CENTRAL SYNAPSES

4.1 INTRODUCTION

There are four species of snakes in the genus *Dendroaspis*. Over the recent years, fractionation of venoms from these species has yielded a number of short and long neurotoxins (Strydom,1972a; 1972b; 1973a; Shipolini *et al.*,1973; Banks *et al.*,1974) and a number of low molecular weight polypeptides exhibiting little or no toxicity (Strydom,1973b; 1976; Shipolini *et al.*,1973; Viljoen and Botes,1973; Joubert,1975). The latter include the *angusticeps*-type proteins, trypsin inhibitor and trypsin inhibitor homologues.

A number of the postsynaptic neurotoxins have been sequenced from 3 of the species (Shipolini *et al.*,1973; Banks *et al.*,1974; Strydom.1976); the green eastern mamba, *Dendroaspis angusticeps*, does not appear to contain either short or long neurotoxins but instead possesses other types of proteins called *angusticeps*-type proteins (Viljoen and Botes,1973; 1974). These proteins are structurally homologous to the short neurotoxin and cytotoxin groups but completely distinct in their immunochemical properties and have little or no toxicity (Viljoen and Botes,1973; 1974). These *angusticeps*-type proteins are also present in the other three species of *Dendroaspis* venoms (Strydom,1976; 1977; Joubert and Taljaard,1979; Joubert *et al.*,1978; Joubert and Viljoen,1979; Shipolini and Banks,1974). Although toxins of this group have low toxicity when tested individually, when combined with other components of the same or other mamba venom, a synergistic lethal effect was observed (Strydom.1976; 1977; Joubert and Taljaard,1979; Joubert *et al.*,1978; Joubert

and Viljoen,1979).

The presence of protease inhibitor homologues in *Dendroaspis* venoms was first reported by Strydom (1973b) in his primary structure studies of low-molecular weight components from *Dendroaspis polylepis polylepis* venom. The sequences of two of its components, toxin I and toxin K, were found to be homologous to the basic trypsin inhibitor of bovine pancreas (BPTI) (Kassell *et al.*,1965; Table 1-2) and cow colostrum (Chechova *et al.*,1971) but these homologues exhibit no activity against the action of chymotrypsin or trypsin (Strydom,1976); and thus have been referred to as 'inactive' protease inhibitor homologues. *Dendroaspis polylepis* also contained active protease inhibitor homologues, toxin B and toxin E (Strydom,1976, Strydom and Joubert,1981) which are homologous to BPTI (Table 1-2). In addition to toxin I and toxin K, the inactive protease inhibitor homologues also include two other proteins $C_{13}S_2C_3$ and $C_{13}S_1C_3$, isolated from *Dendroaspis angusticeps* (Joubert and Taljaard,1980). A high degree of homology in the amino acid sequence is observed between these four polypeptides (Table 1-2).

At the time when these protease inhibitor homologues were isolated and sequenced (Strydom,1973b; 1976; 1977; Joubert and Taljaard,1980), their pharmacological action was not known. However, recently, Harvey and Karlsson (1980) described the isolation of a neurotoxin called dendrotoxin (DTX) that enhances ACh release at the neuromuscular junction, from *Dendroaspis angusticeps*. Its sequence was found to be identical with $C_{13}S_2C_3$

obtained by Joubert and Taljaard (1980) (Harvey and Karlsson,1982). This was followed by the demonstration that the other protease inhibitor homologues, including toxin I and toxin K, can also cause the facilitation of neurotransmitter release in chick biventer-cervices nerve-muscle preparations (Harvey and Karlsson,1982). It would appear, however, that only polypeptides which contain a high degree of sequence homology with DTX can cause this augumentation (Section 1.3.6; Harvey and Karlsson,1982). Among the other proteins tested for the facilitatory activity were protease inhibitors from bovine pancreas (BPTI) and the venoms of other species of snakes in the *Viperadae* and *Elapidae* families (Harvey and Karlsson,1982), which have shown to contain protease inhibitor homologues (Takahashi *et al.*,1974). Those from the venoms of snakes include components from *Vipera russelli* (Takahashi *et al.*,1974), *Hemachatus hemachatus* and *Naja nivea* (Hokama, *et al.*,1976). However, in contrast to the protease inhibitor homologues, no facilitation was observed with any of these protease inhibitors (Harvey and Karlsson,1982).

DTX and its closely-related proteins, toxin I and toxin K, were found to be effective at a concentration as low as 0.5 µg/ml (70nM), causing a 200% augumentation of muscle twitch in the nerve-muscle preparation (Harvey and Karlsson,1980; 1982). This result suggests that DTX and cogeners are binding to specific macromolecules on the presynaptic membranes which may be directly or indirectly involved in the process of neurotransmitter release. This suggestion is substantiated further by the

demonstration that they can also antagonize the action of β-BuTx which has been established to act presynaptically and inhibit the release of neurotransmitter (Section 1.5) in nerve-muscle preparations, and to have a specific binding component on neuronal membranes (Chapter 3; Othman *et al.*,1982). The results obtained in the latter also suggests that the inhibitor homologues may be sharing some common sites with β-BuTx, which again could be involved in the process of neurotransmitter release. Collectively, these findings clearly showed that the protease inhibitor homologues or facilitatory neurotoxins as they have been referred to, can be very useful tools for the study of the mechanism of neurotransmitter release.

As a prerequisite for such studies, neurotoxin free from any contaminant is necessary. This Chapter describes the isolation and characterization of one of these facilitatory neurotoxins, DTX, from *Dendroaspis angusticeps*. To date, all electrophysiological analysis of this toxin have been carried out at peripheral synapses (Harvey and Karlsson,1980; 1982) and no report is yet available on its effect at central synapses. Thus, the effect of DTX in the latter was investigated on hippocampus slices from rat and guinea pig. From these studies, it was found that the effect of DTX on the slices involved Ca^{2+}-channel activity; the latter was also investigated on isolated nerve terminals, where its effects on the $^{45}Ca^{2+}$-flux across the plasma membrane was measured.

4.2 MATERIALS

Dendroaspis angusticeps venom (Lot 42C-2100) was obtained from Sigma Chemical Co.. Bio-Rex 70 was supplied by Bio-lab. Chymotrypsin, Myoglobin, Cytochrome C and trypsin inhibitor were obtained as previously indicated in Section 2.2. All other chemicals used were analytical grade and as described in Section 2.2.

4.3 METHODS

4.3.1 Chromatography of *Dendroaspis angusticeps* venom

This was carried out using a modification of the method of Harvey and Karlsson (1980).

4.3.1.1 Gel-filtration of crude venom

Sephadex G-50 (superfine) resin was swollen, packed into a column (2.5 X 150 cm) and equilibrated with NH_4OAc buffer (0.1 M, pH 4.7). The crude venom ($\sim$ 500 mg) was dissolved in 8 ml NH_4OAc (0.1 M, pH 4.7). Insoluble materials were removed by centrifugation at 10000 rpm for 20 min. The clear supernatant was applied to a column of Sephadex G-50 (superfine) already equilibrated with the acetate buffer. Fractionation was carried out at 4^{0}C in the same buffer at a flow rate of 24 ml h^{-1}; 4 ml fractions were collected on a Gilson fraction collector with protein concentrations monitored at A_{280} nm. Peak fractions were pooled and freeze dried.

4.3.1.2 Ion-exchange chromatography of peak 5

Bio-Rex 70 cation-exchanger was packed into

a column (2 X 42 cm) after equilibrated with 0.2 M NH_4OAc buffer, pH 7.3. Peak 5 (672 mg) was dissolved in 8 ml of 0.1 M NH_4OAc pH 7.3 and applied to the column. After washing with the latter buffer (250 ml), a linear 2-step gradient of 0.10-0.35 M (800 ml) and 0.35-1.4 M (1200 ml) of NH_4OAc, pH 7.3, was applied. Elution was carried out at a flow rate of 16 ml h^{-1} (at room temperature) and protein concentration monitored at A_{280} nm as described above. Peak fractions were pooled and freeze dried to reduce the total volume prior to desalting. The latter was carried out on a column (0.9 X 14 cm) of Sephadex G-10 equilibrated with 0.01 M NH_4OAc, pH 7.3. Protein peaks were pooled, lyophilized and stored at 4 °C until further analysis.

4.3.2 Characterization of protein peaks: Analytical methods

4.3.2.1 SDS-PAGE

This was performed according to methods of Laemli (1970). Slab gels containing increasing concentrations of acrylamide in the direction of migration of proteins were used. A linear gradient of 15%-20% (w/v) acrylamide, with the ratio of 20:1 (w/v) acrylamide to bisacrylamide, was cast as the separating gel, overlaid with a 5% (w/v) stacking gel. 10-50 µg of the proteins were applied under non-reducing and reducing conditions; the sample buffer in the latter contained 5% (v/v) β-mercaptoethanol and was pretreated by boiling at 90°C for 3 min. prior to loading. After electrophoresis, gels were stained in 0.035% Coomassie brilliant blue G-250 in 3.5% (w/v)

perchloric acid and destained in the latter. Molecular weight of proteins in samples was determined by comparing their electrophoretic mobilities with known molecular weight markers, run simultaneously on a different track in the same gel slab. The molecular weight markers were chymotrypsinogen, trypsin inhibitor (soya bean), myoglobin and cytochrome C.

4.3.2.2 Analytical isoelectric focussing

Focussing at a narrow pH-range (pH 7-11) was performed in slab gels of polyacrylamide described in Section 2.3.4.1.

4.3.2.3 Lethality determination

Peripheral and central toxicity of protein was determined as previously described for β-BuTx in Section 2.3.4.4.

4.3.3 Electrophysiological recordings of the action of DTX on hippocampal slices

This was performed according to the method of Halliwell and Dolly (1982a; 1982b). Following decapitation and rapid removal of rat or guinea-pig brain, slices were cut transverse to the axis of a freshly dissected hippocampus by means of a home-made chopper. The latter allowed a weighted razor blade to fall through the brain tissue and after each chop, the position of the hippocampus was advanced by 400 μm on the specially-constructed cutting table. This gave a series of slices which were incubated and examined electrophysiologically.

In the hippocampus structure, the afferent fibre systems impinge on well localized regions of separate pyramidal and granule cell populations (Andersen *et al.*,1971; Lomo,1971; Jefferys,1979). When one such fibre system in the stratum radiatum of the hippocampus, synapsing with the basal dendrites of CA 1 group of pyramidal cells is stimulated, intra- and extracellular responses may be recorded. Extracellular activity was recorded through electrodes filled with 10% (w/v) NaCl; intracellular recordings were made with electrodes filled with either 3 M KCl or 4 M potassium acetate (50 - 100 MΩ). Electrical stimuli (constant current 10 - 200 μA, 100 μs, rectangular pulses) were delivered to stratum radiatum of CA 1 through fine (∿ 35 μm) teflon-coated platinum/iridium wires.

4.3.4 Determination of the influx of $^{45}Ca^{2+}$ into synaptosomes

4.3.4.1 Preparation of synaptosomes

Rat cerebrocortical synaptosomes were prepared by a modification of the method of Gray and Whittaker (1962) described by deBelleroche and Bradford (1977). Details of this method have been described in Section 3.3.6.

4.3.4.2 Uptake of $^{45}Ca^{2+}$ into synaptosomes

This was measured by using a modification of the ion-exchange chromatography method described by Gastro *et al.*,(1976). The DOWEX AG-50 WX-8 (50 - 100 mesh) ion-exchanger was converted from its acidic to a salt form by stirring in 1 M Tris-base until it reached alkaline pH.

This was followed by extensive washing with distilled water until the pH returned to neutral. The equilibrated resin was packed in a series of columns (0.6 X 15 cm); twelve of these prepacked columns were used for each set of experiments. Before starting the experiment, the column was equilibrated with Tris-phosphate buffer containing (mM): Tris, 20; NaCl, 120; KCl,5; $CaCl_2$, 1.0; $NaHPO_4$, 1.2; $MgCl_2$, 1.3; Glucose, 10; saturated with 95% : 5%, O_2 : CO_2, and titrated to pH 7.4. Purified synaptosomes resuspended in the latter buffer were kept on ice throughout the duration of the experiment; 1 ml aliquots of this suspension are diluted 1:1 (v/v) with the same buffer and preincubated at 37°C for 10 min. This was followed by the addition of 1.0 µCi $^{45}Ca^{2+}$; appropriate amounts of test protein (where specified) and buffer to give a final volume of 3 ml was added. Aliquots (200 µl) were sampled at the specified intervals, and quickly washed down the ion-exchanger column with 2 ml of Tris-phosphate buffer containing 2 mg/ml BSA. Radioactive $^{45}Ca^{2+}$ trapped inside the synaptosomes was eluted off the column with the void volume; 200 µl aliquots of the eluate (total volume 2.2 ml) was added to xylene-Triton scintillation cocktail for determination of radioactivity. An aliquot (200 µl) of the reaction mixture was retained and used for determination of protein concentration using the method of Lowry *et al.*,(1951).

4.4 RESULTS

4.4.1 Fractionation of *Dendroaspis angusticeps* venom

A total of 2.99 g of crude venom of *Dendroaspis angusticeps* (from the same Lot 42c-2100) was fractionated on a Sephadex G-50 (superfine) column as described in Methods. Fig. 4.1 shows a typical elution profile for the six fractionations carried out. The relative weights of materials obtained from the 8 fractions resolved are shown in Table 4-1A. The components in each of these fractions were analysed on SDS-PAGE (Fig. 4.2). As expected, the crude venom contained a mixture of proteins of various sizes, ranging from 94000 to less than 14400 (Fig. 4.2) which can be separated by gel preparation (Fig. 4.1). It would appear that fractions 4, 5, 6 and 7 have molecular weights 14400 (Fig. 4.2); this is probably due to these components having dissimilar conformation to the standard markers used, which could affect the mobilities of these proteins. Furthermore, accurate low molecular weight determination is difficult to obtain on gels having such a wide gradient (5-30%) of acrylamide concentration. More precise molecular weight determination for these proteins can be obtained on a system having a narrow gradient of acrylamide concentration (see below).

The elution profile of the gel-filtration (Fig. 4.1) is very similar to the profile previously obtained by Harvey and Karlsson (1980), except for the slightly higher resolution of peak III obtained in the former. Alignment of the two profiles revealed that fractions 4, 5, 6 and 7 obtained in this study correspon-

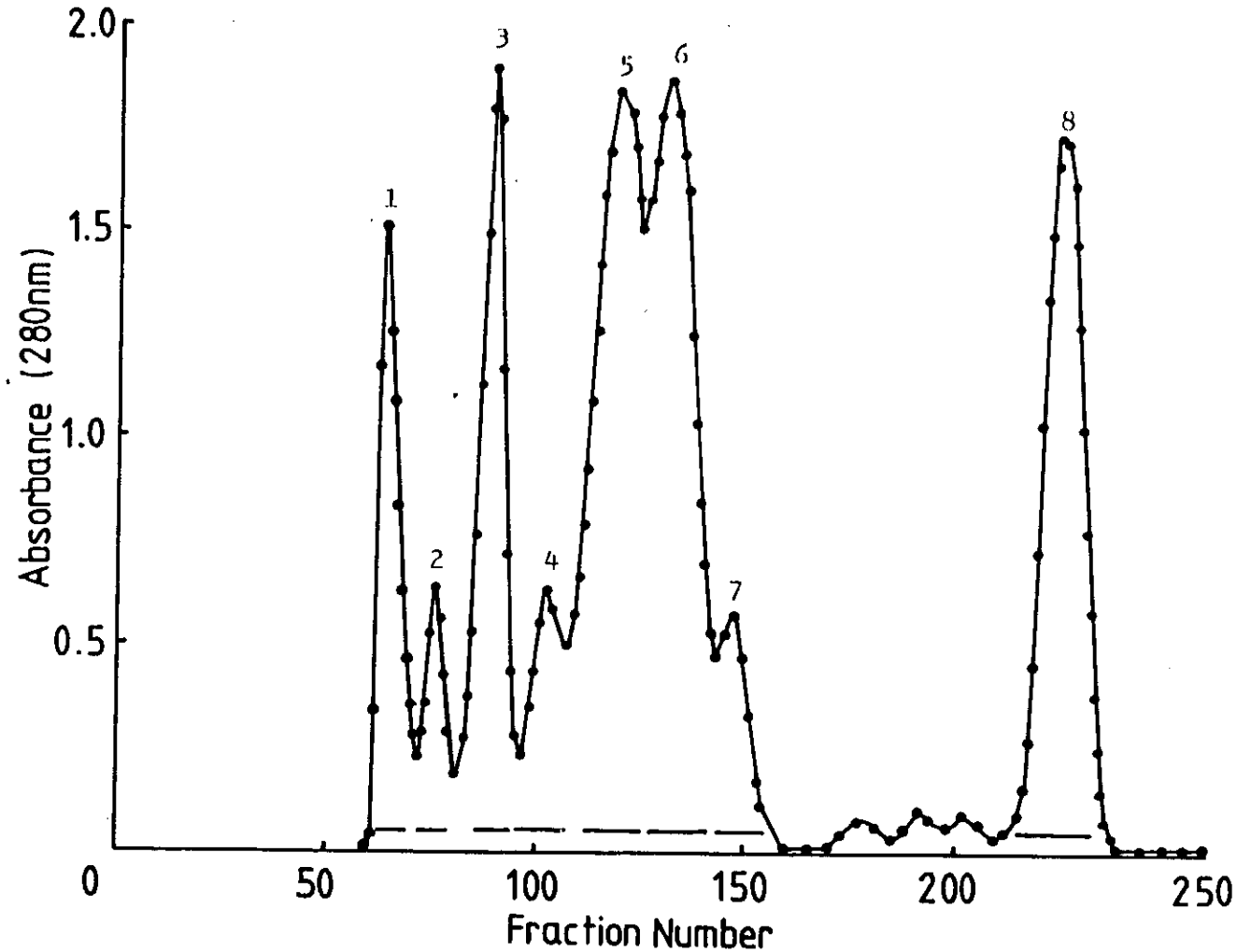


Fig. 4.1 Elution profile for chromatography of crude venom on Sephadex G-50 (superfine) resin in 0.1 M NH_4OAc buffer pH 4.7. The proteins were pooled as indicated by horizontal bars.

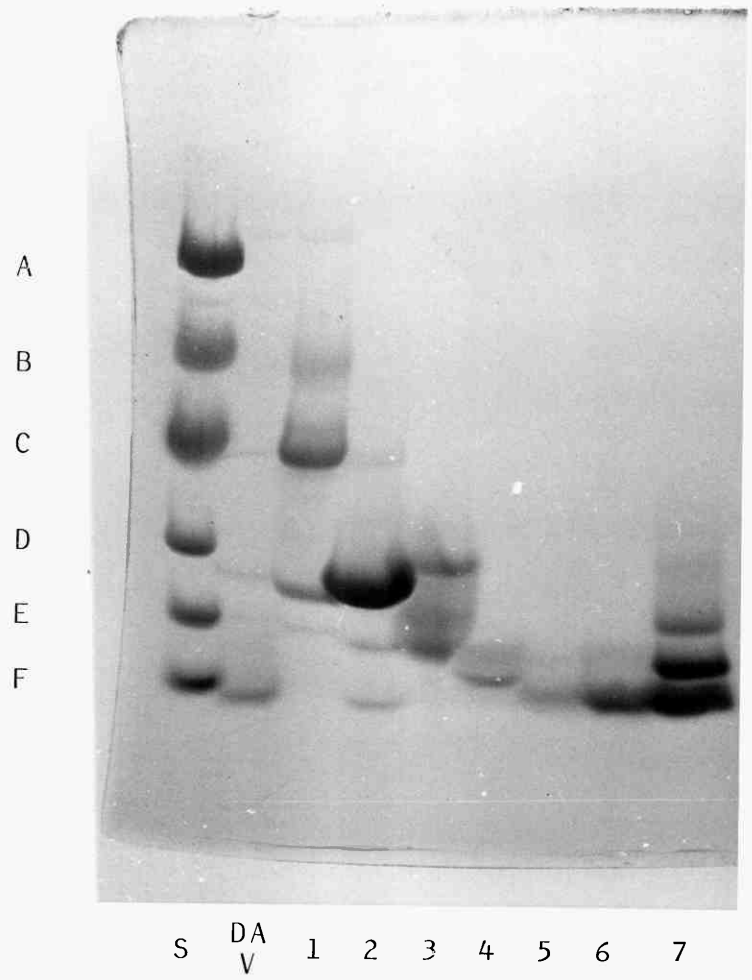


Fig. 4.2 Characterization of fractions separated on Sephadex G-50

SDS-gradient pore gel electrophoresis of fractions isolated from crude venom of *Dendroaspis angusticeps*.
DA V crude *Dendroaspis angusticeps* venom; 1-7 refers to fraction numbers of protein peaks obtained from gel-filtration.
Track S contained the protein standards used: Phosphorylase b (A), Albumin (B), Ovalbumin (C), Anhydrase (D), Trypsin Inhibitor (E) and α-lactalbumin (F)

ded to peak III of the previous report (Harvey and Karlsson,1980). Since peak III has been shown to contain DTX, it was necessary to examine which of the four fractions obtained in this fractionation, contained the facilitatory neurotoxin. To allow this differentiation, the inhibitory property of DTX on the specific binding of ^{3}H-β-BuTx with high potency (Chapter 3) was used as a convenient criterion for the presence of the former in the different fractions. Fractions 4, 5, 6 and 7 were screened for their inhibitory activities on the binding of ^{3}H-β-BuTx to synaptosomes. Using a standard concentration for each fraction (assuming that each has a molecular weight of 7000), competition experiments were carried out similar to that described in Section 3.3.7. Fig. 4.3 shows the effect of the fractions on ^{3}H-β-BuTx binding to synaptosomal membranes. Fractions 4 and 5 inhibited totally the specific binding of ^{3}H-β-BuTx similar to native β-BuTx (Fig. 4.3). The slight difference observed in the potency of fractions 4 and 5 in inhibiting ^{3}H-β-BuTx binding to synaptosomes may be due to the relatively lower amount of DTX present in the latter fraction. Further examination of the elution profile (Fig. 4.1) showed considerable degree of cross-contamination between these four fractions; thus the lower potency of fractions 6 and 7 in inhibiting the binding of ^{3}H-β-BuTX (Fig. 4.3) could be due to the presence of DTX cross-contaminated from fraction 5.

From the total amount of protein recovered (Table 4-1), fractions 4 and 5 accounted for 4.7% and 23% of the whole venom respectively, and due to the

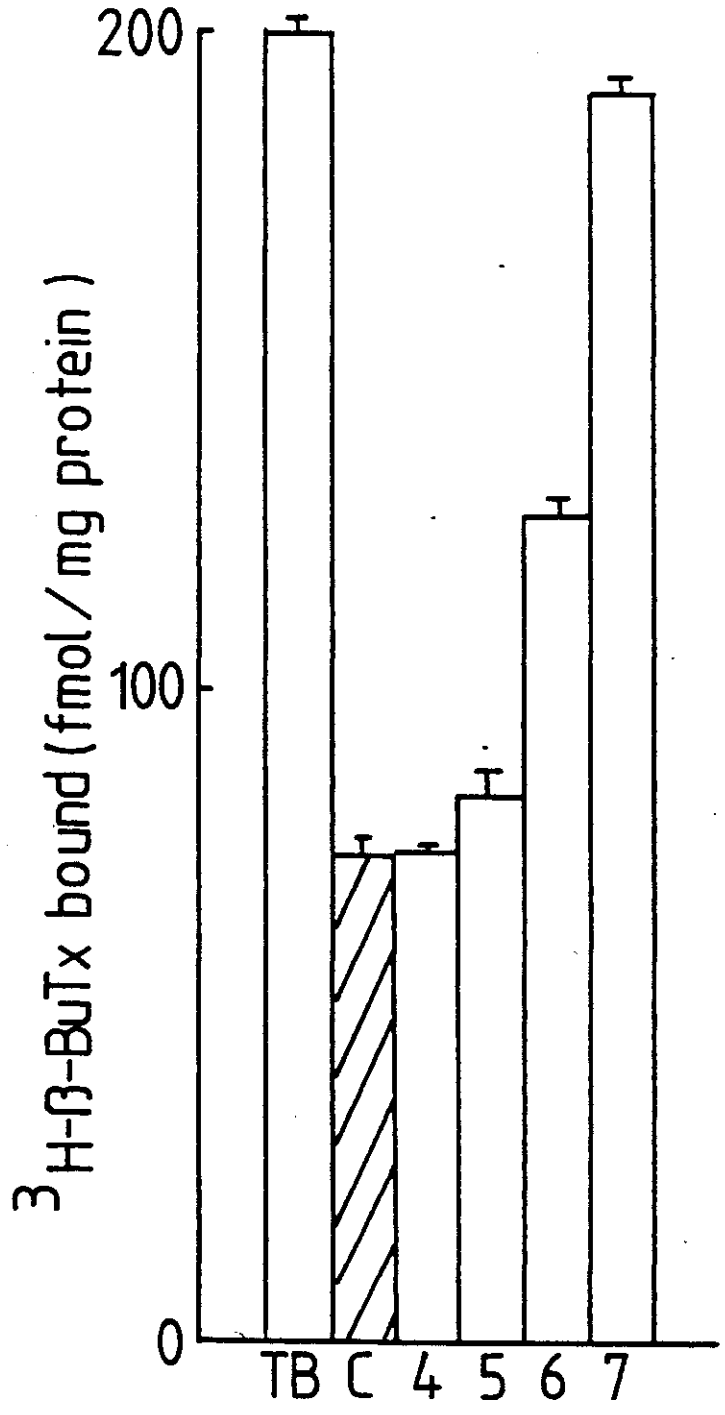


Fig. 4.3 Effect of fractions 4, 5, 6 and 7 on the specific binding of ^{3}H-β-BuTx to synaptosomes

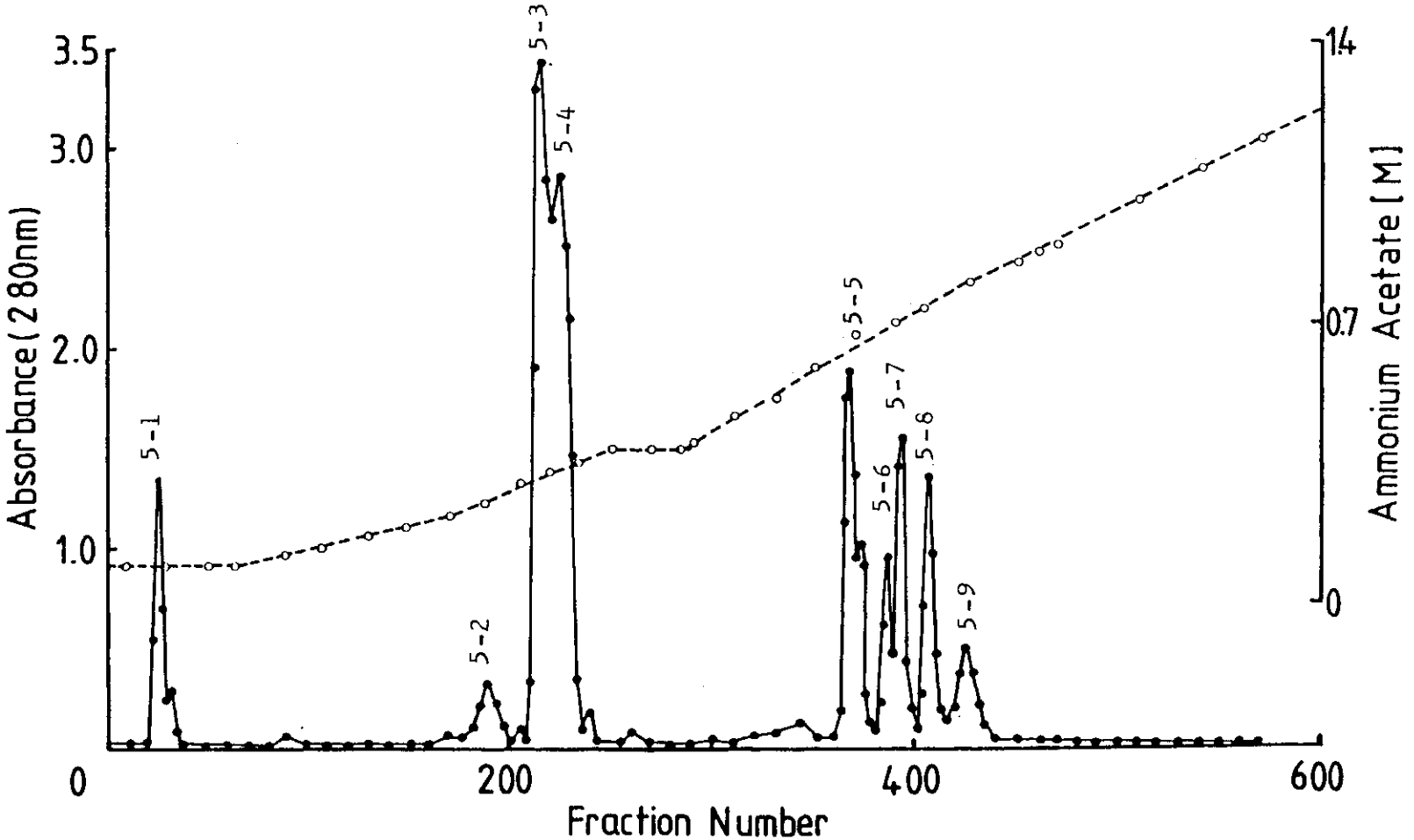
The binding of ^{3}H-β-BuTx to synaptosomes was determined in the presence ( ) or absence (TB) of 500 nM β-BuTx as described in Section 3.3.7. Incubations were also performed in the presence of 1 μM fractions 4, 5, 6 and 7, obtained from gel-filtration of *Dendroaspis angusticeps* venom (Fig. 4.1). Values given were obtained from 4 separate determinations.

higher amount of fraction 5, this was fractionated further on Bio-Rex cation-exchanger. Ion-exchange chromatography of fraction 5 was carried out using a modification of the method of Harvey and Karlsson (1980). A two-step linear salt gradient was used instead of the convex gradient used by the latter; this modification did not seem to impair the separation of the proteins, especially in the last part of the gradient. Fig. 4.4 shows the profile for elution of proteins from the ion-exchanger; more than 10 components were resolved. Alignment of the profile obtained (Fig. 4.4) with that reported by Harvey and Karlsson (1980) indicated that they were similar, except for the absence of several other components in the former which are probably present in fraction 4, 6 and 7 obtained in this study. Comparison of the two profiles showed that the fraction designated 5-5 corresponded to peak 9 (DTX) obtained by Harvey and Karlsson (1980); the former was pooled, desalted on Sephadex G-50 as described in Methods, and lyophilized. Other fractions were also treated in a similar manner to that of fraction 5-5. Table 4-1B shows the amount of each fraction obtained from the fractionation. Fraction 5-5 accounted for about 6% of fraction 5 or about 1% of the whole venom. It would also appear from Table 4.1B that fractions designated 5-3 and 5-4 accounted for the major components in fraction 5 (33.5% and 37.1% respectively).

4.4.2 Characterization of fraction 5-5

The homogeneity of fraction 5-5 was investigated using SDS-PAGE, under reducing and non-reducing

Fig. 4.4 Ion-exchange chromatography of fraction 5 (Fig. 4.1) on a column of Bio-Rex 70 (2.0 X 42 cm) equilibrated with 0.2 M NH_4OAc, pH 7.3



After application of peak 5 (dissolved in 0.1 M NH_4OAc, pH 7.3), the column was washed with 0.1 M NH_4OAc, pH 7.3, followed by a linear 2-step gradient of 0.1-0.35 M (800 ml) and 0.35-1.4 M (1200 ml) of NH_4OAc, pH 7.3. Elution was carried out at a flow rate of 16 ml h^{-1} ; 4 ml fractions were collected. Protein concentration was determined at A_{280} nm (●); broken lines (----) indicates NH_4OAc concentration measured by conductivity measurements. The numbered peaks were pooled, desalted and lyophilized.

Table 4-1 A. Yields of various peaks separated from the venom of *Dendroaspis angusticeps* on Sephadex G-50

B. Recovery of protein materials following ion-exchange chromatography on Bio-Rex 70 of fraction 5 obtained from Sephadex G-50

A.

Fraction Number	Freeze-dried weights (mg)
1	251
2	76
3	346
4	134
5	678
6	854
7	63
8	59

B.

Fraction Number	Freeze-dried weights (mg)
5-1	19
5-2	16
5-3	176
5-4	196
5-5	32
5-6	16
5-7	10
5-8	25
5-9	14
5-10	24

conditions, and analytical isoelectric focussing with a narrow pH range of 7 - 11. Under non-reducing condition on SDS-PAGE containing 15% - 20% (w/v) acrylamide concentration, fraction 5-5 showed a single band with a molecular weight of 7000 (±500; n = 3); (Fig. 4.5) chymotrypsinogen, cytochrome C, myoglobin and trypsin inhibitor (soya bean) were used as molecular weight markers (Fig. 4.5A,). When fraction 5-5 was reduced with β-mercaptoethanol and run on the same gel, on a different track, it appeared to contain a single band with identical molecular weight as in the unreduced form of the protein; this indicates that fraction 5-5 consists of a single polypeptide with a molecular weight of 7000 (±500; n = 3) daltons consistent with the finding of Harvey and Karlsson (1980) for DTX. Fraction 5 was also analysed on the same gel system; under non-reducing condition, two protein bands with molecular weights of about 10500 (±1000; n = 3) and 7000 (±500; n = 3) were obtained (Fig. 4.5). From the staining pattern, the larger molecular weight component appeared to be the major species of fraction 5, however, on reduction with β-mercaptoethanol only one band with a molecular weight of 7000 (±650; n = 3) was obtained (Fig. 4.5). This result suggests that fraction 5 probably consists of a dimer and monomer and the former can be separated in the presence of a reducing agent into its monomer form (Fig. 4.5).

Analytical IEF on a polyacrylamide gel with a narrow pH range of 7-11 of fraction 5-5 showed a single sharp band focussed with a pI of 10.5 (±0.2; n = 3), indicating the very basic nature of the protein molecule

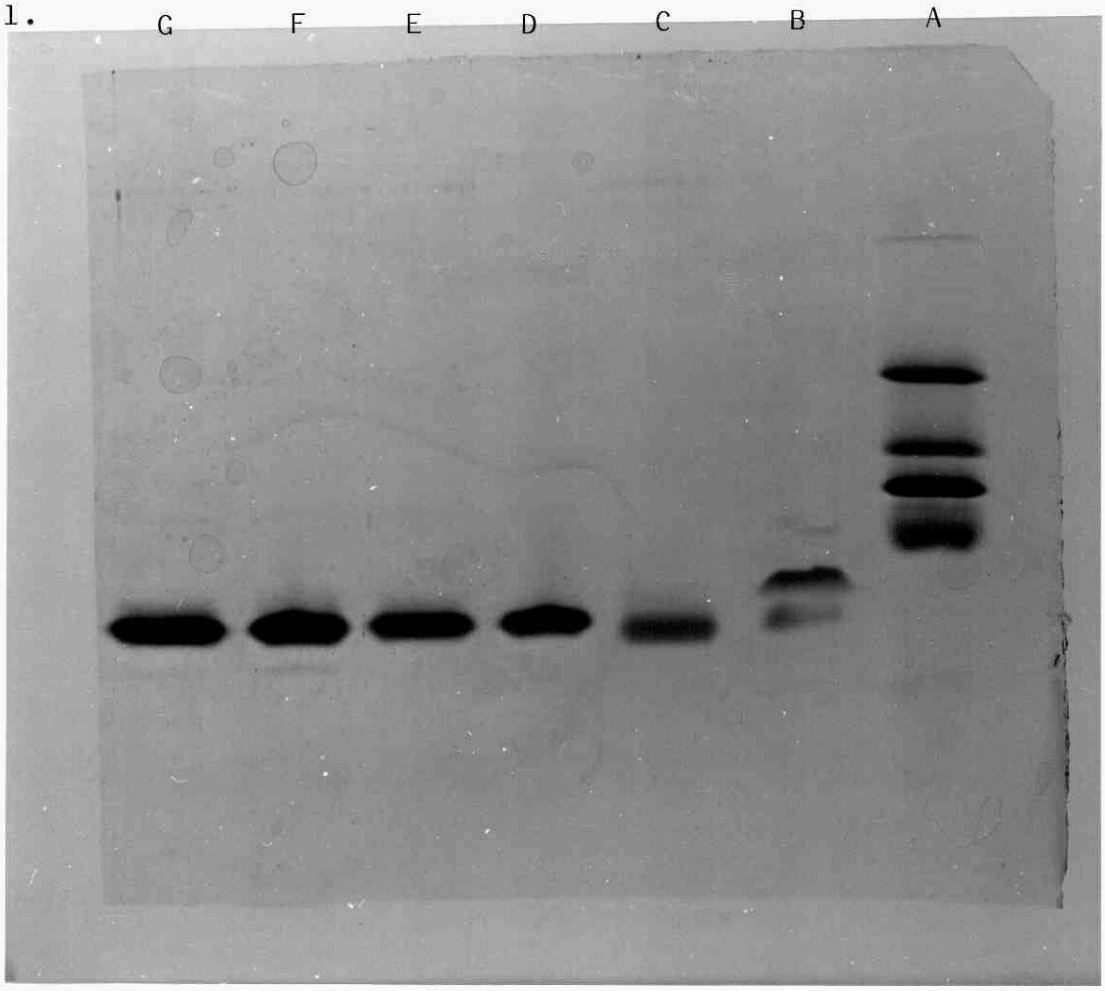
Fig. 4.5 Polyacrylamide gel electrophoresis of fraction 5-5 obtained from ion-exchange chromatography on Bio-Rex 70

SDS-gradient pore gel, containing 15%-20% (w/v) acrylamide, electrophoresis was performed as described in Methods:

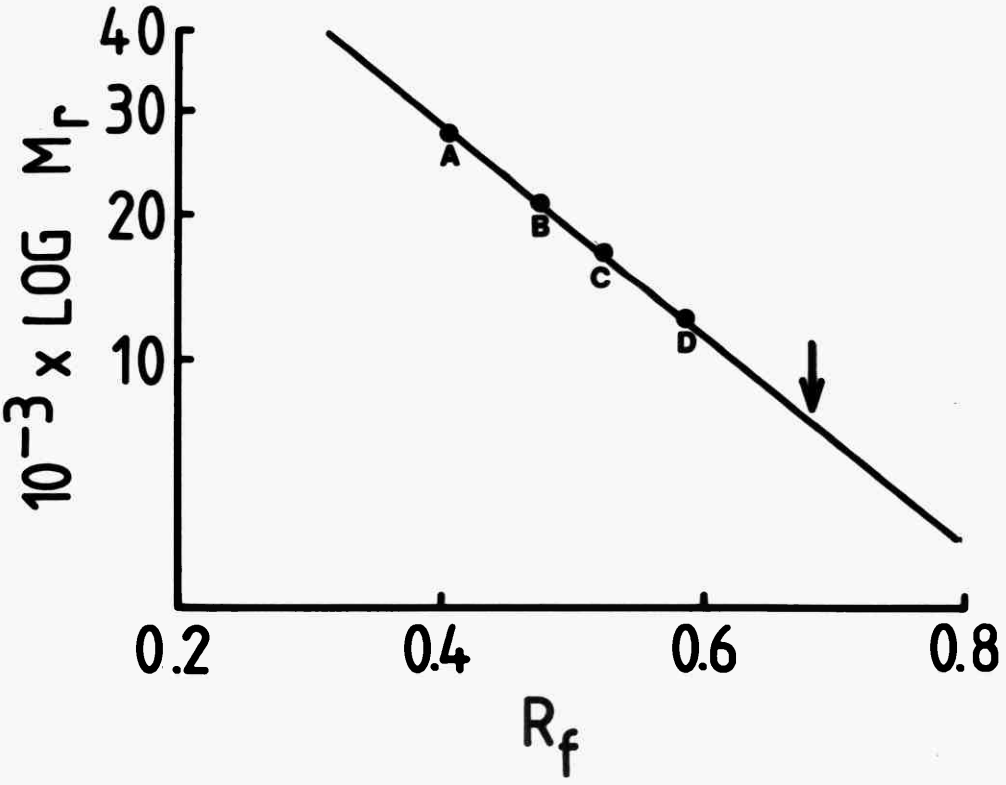
1. A Consist of molecular weight markers as indicated below (2.)
   B & C Fraction 5 from gel-filtration on Sephadex G-50 (superfine) (Fig. 4.1) under non-reduced and reduced conditions respectively.
   D & F Different amount (10 and 25 µg respectively) of fraction 5-5 (DTX) from ion-exchange chromatography (Fig. 4.4) of fraction 5 under non-reducing condition.
   E & G Different amounts (10 and 25 µg) respectively) of fraction 5-5 (DTX) from ion-exchange chromatography (Fig. 4.4 of fraction 5 under reducing condition.
2. Semilogarithm plot of the relative mobilities and molecular weights of protein standards (1-A above).
   A. chymotrypsinogen B. myoglobin
   C. trypsin inhibitor (soya bean D. cytochrome C

Arrows indicate the position of protein bands for fraction 5-5 (DTX) under non-reducing and reducing conditions.

1.



2.



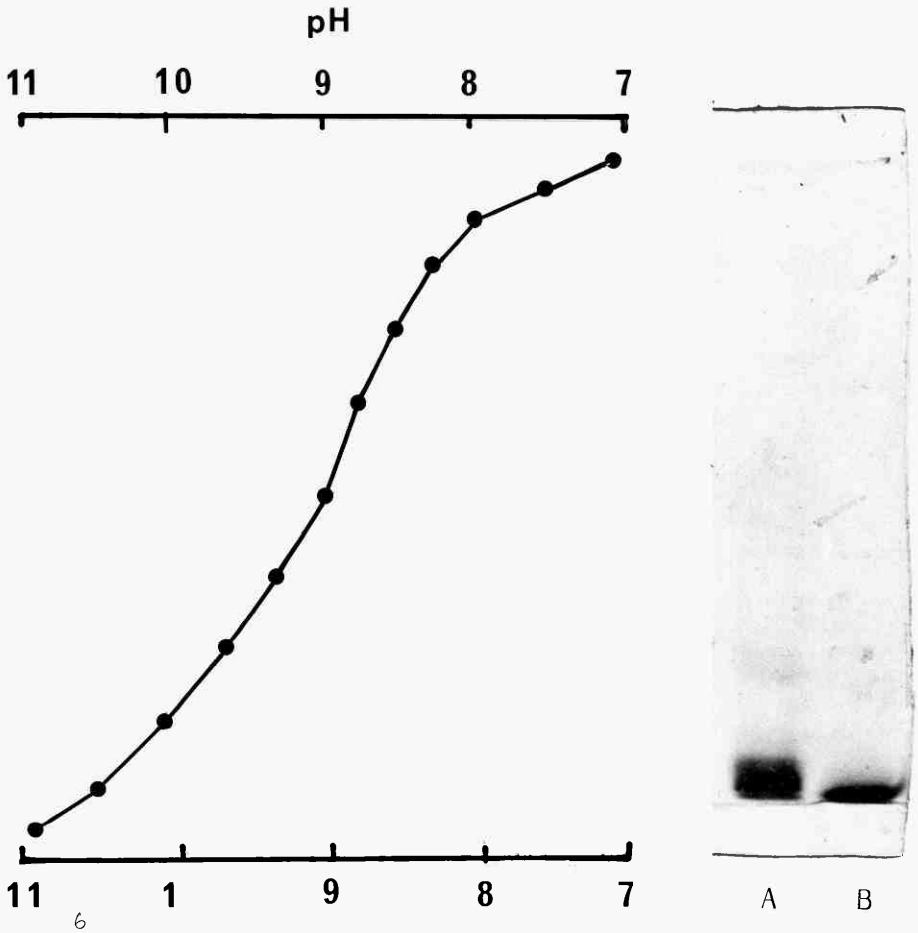


Fig. 4.6 Analytical isoelectric focussing of fraction 5.5 and fraction 5

This was performed as previously described in Section 2.3.4.1. 50 µg of fraction 5 (A) and fraction 5.5 (B) was applied to the polyacrylamide gel. After focussing, the pH gradient (●) was measured with a micro-electrode. The proteins were stained in G-250 (0.04%; w/v) in perchloric acid (3.5%; w/v).

(Fig. 4.6). This fraction appeared homogeneous with respect to charge. However, it was rather surprising to find that fraction 5 was also focussed as a single band at around the same pH as fraction 5-5; this suggests that in addition to the latter, fraction 5 also contains other components that are very basic, and that they cannot be separated on the basis of their net charge alone.

It has been reported that proteins belonging to the protease inhibitor homologues group, including DTX, are relatively non-toxic when administered by intraperitoneal injection into mice (Strydom,1976; Harvey and Karlsson,1980). When the lethality of fraction 5-5 was determined, 700 µg was needed to kill a mouse (∿ 20 g) (Table 4-2). This result is consistent with the previous findings by other workers (Harvey and Karlsson,1980) for the lethality of DTX. Table 4-2 indicates that the MLD of fraction 5-5, i.e. the minimum lethal dose, after intraperitoneal injection into mice, could be estimated as 35 µg/g body weight. This result, together with the observation that it is a single-chain polypeptide of 7000 (±500; n = 3) molecular weight, is consistent with the findings of Harvey and Karlsson (1980) for DTX; henceforth fraction 5-5 will be referred to as the latter.

Table 4-2 also shows that DTX was much more potent when injected into the ventricular spaces of rat brain. The central neurotoxicity was determined at about 2.5 ng/g body weight, which makes it 10^4-times more toxic than in the periphery. The symptoms of envenomation were observed within 15 min. after animals had reco-

Table 4-2A Whole animal toxicities of DTX and toxin I

Route of administration	Dose (µg/g body wt)	Fraction 5.5 (DTX)		Toxin I	
		No. of animals injected	No. of animals killed	No. of animals injected	No. of animals killed
Intraperitoneal injection	0.5	2	0		
	2.5	2	0		
	5.0	2	0		
	10.0	2	0		
	35.0	2	2		
Intraventricular injection	0.025	4	0	4	0
	0.050	4	0	4	0
	0.25	4	0	4	0
	0.50	4	0	4	2
	2.5	4	2	4	2
	5.0	4	2	4	2

B. Effect of Fraction 5.5 and toxin I on the neurotoxicity of β-BuTx following intraperitoneal injection into mice

Toxin(s)	Dose (µg/g body wt)	No. of animals injected	No. of animals killed	Mean survival time (h)
β-BuTx (1.19 pmol)	0.025	2	2	3-4
Fraction 5.5 (7.1 pmol)	0.05	2	0	-
Fraction 5.5 (71.4 pmol)	0.50	2	0	-
β-BuTx (1.2 pmol) + Fraction 5.5 (7.1 pmol)	0.025 + 0.05	2	2	10-11.5
β-BuTx (1.2 pmol) + Fraction 5.5 (71.4 pmol)	0.025 + 0.5	2	2	10-13
Toxin I (8.3 pmol)	0.058	2	0	-
Toxin I (59.4 pmol)	0.416	2	0	-
β-BuTx (1.2 pmol) + Toxin I (8.3 pmol)	0.025 + 0.058	2	2	12-16
β-BuTx (1.2 pmol) + Toxin I (59.4 pmol)	0.028 + 0.416	2	2	14-18

vered from the anaesthetic. Hyperactivity with rapid tremors of hind and fore limbs were observed followed by death. Similar findings were obtained for toxin I (Table 4-2B) except that toxin I was five-times more potent than DTX in the CNS. This difference in the central toxicity could probably be due to the different species from which these protease inhibitor homologues were isolated (Harvey and Karlsson,1980; Strydom,1973b).

Since these proteins were reported to antagonize the pharmacological action of β-BuTx at peripheral synapses (Harvey and Karlsson,1982), their effect on the neurotoxicity of the latter, which has been shown to be highly neurotoxic when injected intraperitoneally (Chapter 2), was investigated. In the presence of 7.1- or 71.4-fold-molar excess of DTX, the mean survival time for a lethal dose of β-BuTx (0.025 µg/g body wt.) was extended by more than 3-fold (Table 4-2C). With toxin I, similar findings were obtained (Table 4-2C). These results suggest that DTX and toxin I bind to the same site as β-BuTx, decreasing the affinity for the latter to bind; as a result the time to death, which is dependent on the dosage of toxin, was prolonged. This was clearly demonstrated on both occasions, when β-BuTx was injected together with excess of either one of the protease inhibitor homologues (Table 4-2C)

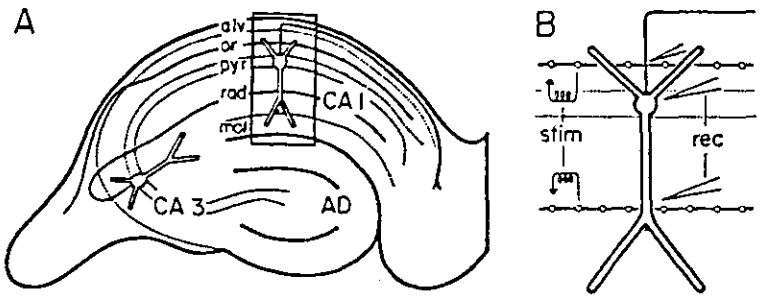
4.4.3 Electrophysiological recordings of the action of DTX on hippocampal slices

To date, all pharmacological studies of DTX have been carried out with nerve muscle preparations

(Harvey and Karlsson,1980; 1982) and its action on central synapses is yet to be reported. In this study, the action of DTX on the latter was investigated on transverse slices of rat and guinea pig hippocampus. Extracellular potentials were recorded from the zone activated fibres in the dendritic layers in stratum radiatum, while intracellular recordings were made from the soma of CA1 pyramidal cells (Fig. 4.7). The fibres were stimulated by small rectangular pulses of electrical current of constant 10-100 μA at 100 μs intervals which were delivered through fine teflon-coated platinum/iridium wires.

When monitored extracellularly, the evoked field potential in the dendrites consisted of an initial di- or triphasic deflection (Fig. 4.8A) followed by a later negative wave (asterisk). The latter is the extracellular counterpart of the intracellularly recorded excitatory postsynaptic potentials (EPSP) (Andersen *et al.*,1978). DTX produced a small facilitatory effect on the initial synaptic component of the extracellular field potential in stratum radiatum:9 of 11 slices displayed enhanced (∿ 120% of control) synaptic activity after one hour's exposure to 250 nM DTX, with no change in the number of presynaptic fibres recruited by the stimulus, as judged by the size of the fibre volley recorded (Andersen *et al.*,1978; Fig. 4.8A, B). More marked was the development of entirely spontaneous activity (2 slices; Fig. 4.8A1) or, more commonly, (n = 7) regenerative synaptic activity longer than 100 ms (see Fig. 4.8B), where previously a single synaptic negative wave (∿ 20 ms) was obtained. A concomitant increase in the amplitude and frequency of synaptically-driven population

Fig. 4.7 Transverse section of a hippocampal slice and position of electrodes for electrphysiological measurements



A. Diagram of the transverse hippocampal slice with the studied CA1 region boxed in. The abbreviations mark the histological layers: alv, alveus or stratum oriens; pyr, stratum pyramidale; rad, stratum radiatum; mol, stratum moleculare; AD, area dentata; CA1 and CA3, nomenclature according to Lorente de No' (1934).

B. Diagram showing the position of stimulation (stim) and recording (Rec) electrodes. The employed unmyelinated fibres in stratum radiatum with their enpassage boutons symbolised by lines and circles.

(Illustrations adapted from Andersen *et al.*,1978; Fig. 1)

Fig. 4.8 Electrophysiological analysis of the action of DTX on hippocampal slices

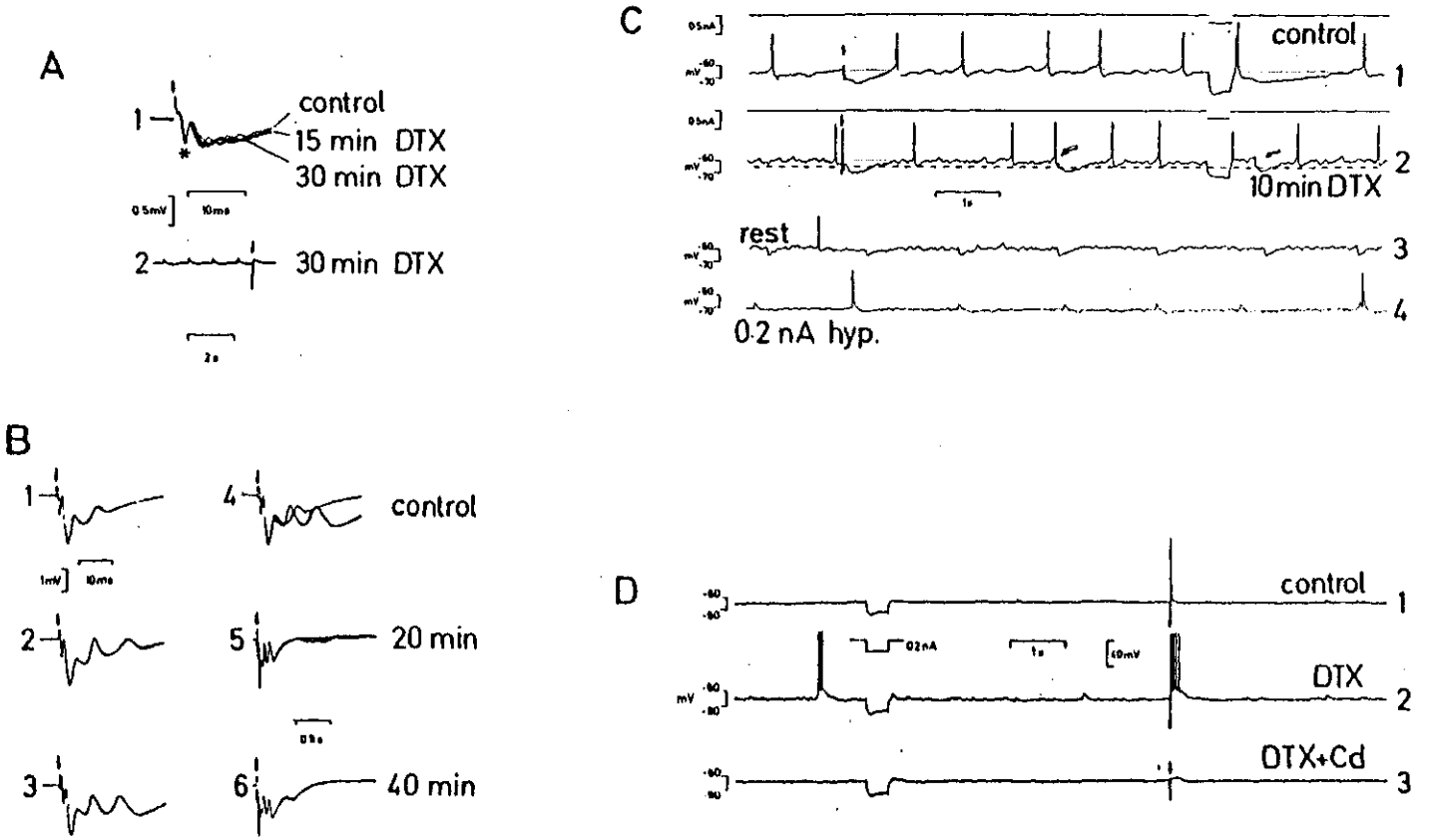
A & B Recordings in stratum radiatum 2 mm from the stimulation site in rat slices. 100 µA, 100 µs shocks were presented at the indicated times after administration of 240 µM DTX in the medium. In A1, the presynaptic volley is shown (*) and is followed by the postsynaptic negative wave at this dendrite recording site. Note the spontaneous activity and contracted time scale in A2. In B, traces 1-3 show radiatum responses before, and at 20 and 40 min. after DTX. Traces 5 and 6 are longer sweeps and trace 4 comprises a superimposition of 1 and 3 for comparison. Note the change in the number and amplitude of the positive-going population spikes induced by the toxin.

C Intracellular records from a rat CA1 neuron impaled with an acetate filled electrode. Traces 1 and 2 comprise of current (upper) and voltage (lower) records, before and 10 min. after 250 nM DTX respectively. Shocks to radiatum afferents (120 µA, 100 µs) were given (straight arrows) as were hyperpolarization current injections of 0.3 µA (deflection in current trace). The dashed line in C2 denotes mean resting potential prior to exposure to DTX. Note the toxin-induced membrane noise and the spontaneous ipsps, either preceded by a spike (open curved arrow) or not (filled curved arrow). The spikes have been attenuated in these records by the low frequency response (DC-100 Hz) of the writing chart recorder. C3 and C4 were recorded following a washout for 20 min. of the toxin after a half-hour total exposure time. C3 shows the resting potential of the neuron; C4 shows cell activity during passage of a constant 0.2 nA hyperpolarizing current.

D Recordings from a guinea pig CA1 cell impaled with a chloride containing electrode. D1 control record showing electrotonic potential to 0.2 nA hyperpolarizing current and synaptic response to deliver of 50 µA, 100 µs stimulus to stratum radiatum (arrow). D2: same but after 20 min. in 950 nM DTX. D3: same but 15 min. after the addition of 200 µM Cd^{2+} as well as toxin.

These experiments were carried out by Dr.J.V.Halliwell, Dept. of Pharmacology, School of Pharmacy, London University.

FIG. 4.8


A
1
control
15 min DTX
30 min DTX
2
30 min DTX
B
1
4
control
2
5
20 min
3
6
40 min
C
control
1
10min DTX
2
rest
3
4
0.2 nA hyp.
D
control
1
DTX
2
DTX+Cd
3

spikes (Andersen *et al.*,1971) occurred (Fig. 4.8B) and the latency of these shortened. All these changes were irreversible.

To allow a more detailed analysis of the action of the toxin at a cellular level, intracellular recordings were carried out using microelectrodes filled with 3 M KCl or 4 M K acetate. The results obtained were based on recordings made from 24 CA1 cells having mean membrane potentials of -71 ±6 mV, action potential amplitudes of 97 ± 15 mV (± SD) and input resistance of 67 ± 29 mΩ. Using the standard concentration of DTX employed for extracellular analysis, it alone did not induce any systematic change in either the resting membrane potential or input resistance of 18 cells. Sixteen cells displayed depolarizations which are associated with an increase in resistance and an enhancement of the frequency of the action potential, either singly or as bursts (Fig. 4.8C, D). Bursting was observed in all the cells impaled with KCl filled electrodes and in 3 out of 7 neurones impaled with acetate-filled electrodes.

A more striking and consistent effect of DTX on the neurones was its facilitation of spontaneous synaptic activity (Fig. 4.8C, D). It would appear that much of this activity was inhibitory since the reversal potential for many of the spontaneous events observed with acetate-filled electrodes was close to -70 mV (Fig. 4.8C). The concomitant increase in spontaneous depolarising potentials in neurones impaled with KCl-filled electrodes is in accord with Alger and Nicholl's interpretation of this activity as mainly ipsps, reversed because of chloride

ion loading. In their report, they have shown that these potentials are associated with a conductance increase, are chloride (Cl^-) dependent and are affected by drugs known to act primarily on GABA-mediated transmission; furthermore, these potentials can be abolished by tetrodotoxin (TTX) and Mg^{2+} ions (Alger and Nicholls,1980).

In view of the facilitatory activity of DTX at neuromuscular junctions where the transmitter is ACh (Harvey and Karlsson,1980; 1982), the effect of cholinergic antagonists on the action of DTX was investigated in hippocampal slices. Neither mecamylamine (up to 100 µM) nor atropine (up to 1 µM), administered either before or after the addition of DTX to the preparation, had any effect on the excitatory activity of DTX. This result suggests that DTX-evoked release of ACh alone cannot cause the toxin's excitatory effects. Furthermore, transmitter release was required for full expression of the convulsant action of DTX since bursting behaviour was not observed when Ca^{2+} was replaced in the medium with 7-15 mM Mg^{2+}; in the presence of Cd^{2+} (200 µM), a known Ca^{2+}-channel blocker, the increase in synaptic noise was also blocked suggesting the involvement of Ca^{2+}-channels in the action of DTX. When tetrodotoxin (0.5 µM) was added to the incubation medium, the convulsant action of DTX was also blocked; however, in the presence of the former, the toxin induced a pronounced membrane depolarization (of up to 20 mV, $n = 3$) and threshold for initiating TTX-insensitive spikes appeared to be lowered.

4.4.4 Effect of DTX on the influx of $^{45}Ca^{2+}$ into synaptosomes

Synaptosomes showed the ability to accumulate external $^{45}Ca^{2+}$, and the time course of this process was relatively fast (Fig. 4.9A). In this system, the rate of $^{45}Ca^{2+}$-influx was found to be biphasic; an initial rapid uptake in the first minute followed by a slower influx over the subsequent 4-min. period examined. In the absence of any depolarising agents, synaptosomes exhibit a basal calcium-influx which presumably represents a continual cycling of calcium-ions across the synaptosomal plasma membrane, probably via the Ca-Ca or Ca-Na-exchange mechanism operating under these conditions. It was also found that synaptosomes stored for more than 2 h in suspension in the Tris-phosphate buffer, exhibited a rise in the resting calcium-influx (data not shown). This is probably due to the synaptosomes' diminishing capacity to maintain ion-gradients across the plasma membrane, either by virtue of decreasing plasma membrane integrity, or of metabolic collapse and consequent inefficiency of metabolically-driven pumps such as Na^+/K^+ATPase. Due to this reason, experiments were performed within 1 h after the final centrifugation step in the preparation of synaptosomes.

When synaptosomes were exposed to DTX; the rate of $^{45}Ca^{2+}$-uptake was increased 2 to 3 fold above control (Fig. 4.9A). This increment was dependent on the the concentration used, with minimal and maximal changes being observed at 1 nM and 1 µM, respectively (Fig. 4.9A). Inclusion of Cd^{2+}, a Ca^{2+}-channel blocker

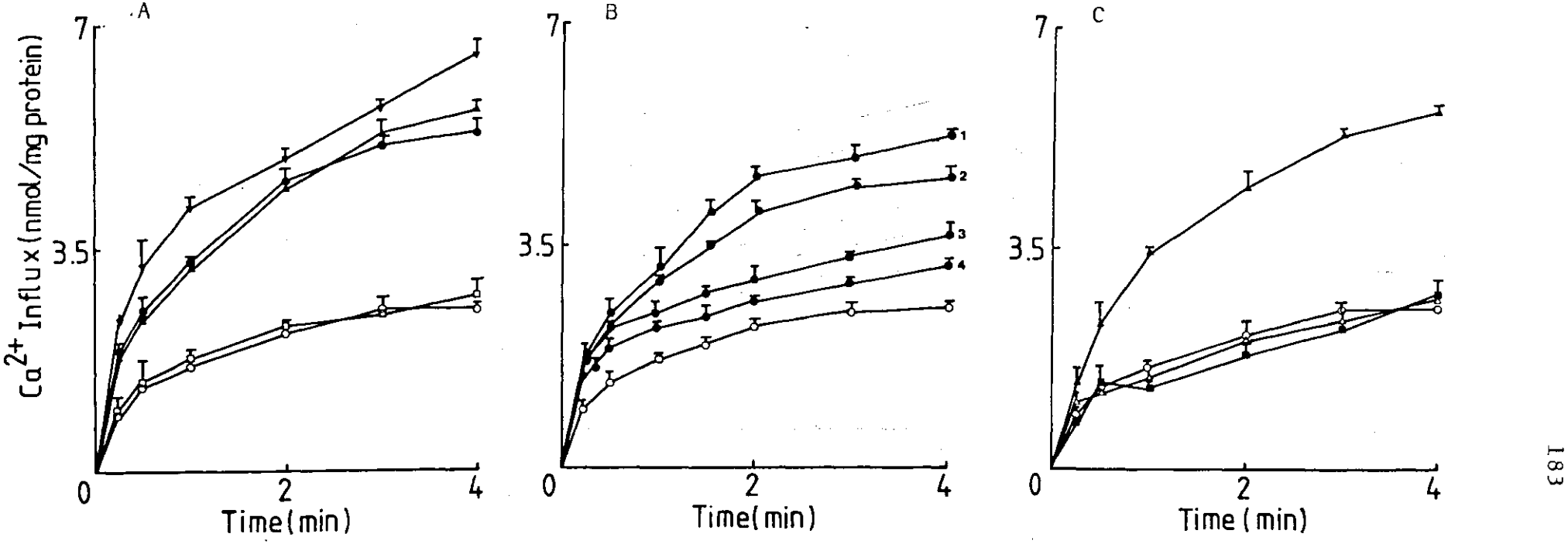


Fig. 4.9 Influx of $^{45}Ca^{2+}$ into synaptosomes

The rate of Ca^{2+} influx was measured by a cation exchange chromatography method as described in Methods. At zero time, $^{45}Ca^{2+}$ (final specific activity 1.0 µCi/mmol) was added into synaptosomal suspensions that had been pre-equilibrated at 37°C in Tris-phosphate medium containing 1 mM $CaCl_2$, together with various test proteins. Aliquots of these suspensions were sampled at various time intervals.

A In addition to control sample (O), 1 µM TsTx in the absence (▼) or presence of 1 µM TTx (□), 1µM DTX in the absence (●) or presence of 0.1 µM TTX (▲) were tested.

B Concentration dependence on the uptake of $^{45}Ca^{2+}$ 1,2,3, and 4 (●) correspond to 1.0,0.1,0.01 and 0.001 µM DTX respectively.

C Effect of Ca^{2+}-channel antagonist on the uptake of $^{45}Ca^{2+}$. Samples incubated with DTX (1 µM) in the presence (■) or absence of 1 mM Cd^{2+} (▲). Values obtained for Cd^{2+} (1 mM)(△) alone, are similar to control (O) which did not contain any addition.

in the medium suppressed the increased influx of $^{45}Ca^{2+}$ seen in the presence of 1 μM DTX (Fig. 4.9B). In the presence of depolarising agents such as tityustoxin, which extends the open-time of Na^+-channels, a large increase in the rate of $^{45}Ca^{2+}$-influx was observed (Fig. 4.9) which is consistent with previous findings on other scorpion toxins (Blaustein,1975). This increase can be blocked totally by tetrodotoxin, an inhibitor of Na^+-channels, with the resting rate being observed (Fig. 4.9). However, when tetrodotoxin was added together with DTX, the increase in Ca^{2+}-flux was still observed (Fig. 4.9), suggesting that the effect of DTX was not mediated through the Na^+-channels. DTX also increased the influx of $^{45}Ca^{2+}$ into synaptosomes treated simultaneously with tityustoxin, but under these conditions, depolarization reduced its effectiveness and additivity in the effects of the two toxins was not observed. These results suggest that these toxins are causing increased $^{45}Ca^{2+}$-influx into synaptosomes through a separate mechanism.

4.5 DISCUSSION

While a complete study of the low-molecular weight constituents of the venom of *Dendroaspis angusticeps* has not been attempted, following the protocol of Harvey and Karlsson (1980), DTX was isolated and characterized. Its pharmacological actions at central synapses of rat and guinea pig hippocampus slices were investigated. In view of the involvement of Ca^{2+}-channel activity in the action of DTX on the hippocampal slices, its effects on the

influx of $^{45}Ca^{2+}$ into cerebrocortical synaptosomes was also measured. Peaks 4, 5, 6 and 7 in the elution profile shown in Fig.4.1 are equivalent to peak III in a similar profile obtained by Harvey and Karlsson (1980). Of these 4 fractions, fraction 5 contained a very basic, single-chain polypeptide whose molecular weight (7000 ± 500; n = 3) as measured by SDS-PAGE, was similar to that reported for other DTX preparations (Harvey and Karlsson,1980; Joubert and Taljaard,1980). DTX has an isoelectric point of 10.5 (Fig. 4.6) and exhibited low toxicity when administered intraperitoneally (Table 4-2A); the latter result was similar to that reported for DTX (Harvey and Karlsson,1980). However, in the central nervous system, DTX was highly neurotoxic (Table 4-2B); it is about 10^4 times more neurotoxic than at the periphery (Table 4-2A, B).

Two different procedures have been reported for the isolation of DTX, both being reported about the same time (Harvey and Karlsson,1980; Joubert and Taljaard, 1980). In the procedure described by Joubert and Taljaard (1980), two protease inhibitor homologues, referred to as $C_{13}S_2C_3$ and $C_{13}S_1C_3$ were isolated. However, except for the ion-exchange chromatography on CM-cellulose, no further details for the purification procedures of the two proteins have been reported; thus comparison between the procedures adapted for the purification of DTX (in the former case known as $C_{13}S_2C_3$) in this study and that employed by Joubert and Taljaard (1980) is rather difficult. On establishing the amino acid sequences of these proteins, a high degree of homology of their sequences was observed with that of toxin I and toxin K isolated from the venom of *Dendroaspis*

polylepis (Strydom,1973b; Table 1-2). On closer analysis, the sequence of $C_{13}S_1C_3$ differs from that of toxin K in only three positions, while that of $C_{13}S_2C_3$ (DTX) differs from that of toxin I in only five positions.

Even though DTX is relatively non-toxic, it can provide some degree of protection against the action of β-BuTx, when injected simultaneously in the periphery (Table 4-2C); this phenomenon was also exhibited by its closely-related homologue, toxin I (Table 4-2). These results are in accordance with the antagonism observed between these two types of neurotoxins in nerve-muscle preparations (Harvey,1982; Harvey and Karlsson,1982) and on synaptosomes (Chapter 3). These results give further support for the postulation that they may have at least some common binding sites(s) (see Section 5.5) which are involved in the process of neurotransmitter release.

At central synapses, DTX induces a permanent facilitation of neurotransmission in areas using excitatory amino acids as transmitters; thus this showed that DTX can also affect other types of synapses in addition to the cholinergic synapses as observed at the neuromuscular junction. The spontaneous activity of DTX seems to require Ca^{2+} and can be blocked by tetrodotoxin, suggesting that it is synaptic in nature. It also acts like a potent convulsant whose direct actions require involvement of Ca^{2+}-channel activity (Fig. 4.8D) which can be blocked by Ca^{2+}-channel antagonists, such as Cd^{2+}. However, from these electrophysiological experiments, it cannot be ascertained whether the effect of DTX observed was a result of the latter's direct interaction with the Ca^{2+} -channel; an

indirect mechanism may be operative in eliciting this spontaneous synaptic activity.

On isolated nerve terminals, DTX caused an increase in the influx of $^{45}Ca^{2+}$ (Fig. 4.9) which cannot be blocked by tetrodotoxin, suggesting that the observed effect on Ca^{2+}-influx is not mediated via the Na^{+}-channels. This increase in $^{45}Ca^{2+}$-influx in synaptosomes can be blocked by the divalent antagonist of the Ca^{2+}-channel, Cd^{2+}, suggesting the involvement of channel activity on the toxin's action. However, from the results obtained in this study, it cannot be ascertained whether its action on the latter is direct or indirect, but it does establish the involvement of Ca^{2+}-channel activity in the action of DTX on synaptosomes. After this preliminary investigation, further studies on the latter using the method described in this Chapter together with a centrifugation assay described by Scott *et al.* (1980) have been carried out; similar results were obtained (A. Breeze and J.O.Dolly, unpublished observation) with both assay systems showing an absolute increase in the uptake of Ca^{2+} in the presence of DTX. In addition, in Na^{2+}-free medium, DTX gives an additional increase in Ca^{2+}-influx when applied to synaptosomes equilibrated in the former medium, suggesting that DTX acts to stimulate Ca^{2+}-influx into the synaptosomes rather than block its efflux through the Na^{+}-Ca^{2+} exchange system (A.Breeze and J.O.Dolly, unpublished observations), which has been postulated to be the predominant efflux mechanism (Blaustein 1975). In addition to Cd^{2+} (Fig. 4.9), other Ca^{2+}-channel antagonists including nifedipine, D-600 and verapamil can

decrease the DTX-induced influx of Ca^{2+} into synaptosomes, albeit with low efficacy (A.Breeze and J.O.Dolly, unpublished observation).

Despite these additional observations, it is still not possible to ascertain the mechanism of action of DTX on Ca^{2+}-channel activity; thus one can only postulate the various ways in which DTX can elicit the increase in Ca^{2+}-influx observed. Two possible mechanisms by which DTX might enable Ca^{2+} to enter the synaptosomes are : i) activation of physiologically operating voltage-dependent Ca^{2+}-channels or ii) induction of new channels in the plasma membrane. Transition metals and verapamil (Norris *et al.*,1983) can inhibit fully K^{+}-induced Ca^{2+}-influx into synaptosomes; when these organic and divalent-cations antagonists of Ca^{2+}-channel were tested in the presence of toxin, such full inhibition was not observed. The reduction in Ca^{2+} entry observed at high Cd^{2+} concentration might be due to Cd^{2+} permeating in place of $^{45}Ca^{2}$. The feeble antagonism observed with the organic antagonist such as verapamil, D-600 and nifedipine argues against the first mechanism being operative.

DTX could also induce new channel formation in the plasma membrane, as postulated for α-latrotoxin from black widow spider venom (Grasso *et al.*,1982; Finkelstein *et al.*,1976). This might involve DTX acting through two distinct mechanisms, induction of 'specific' channels and a secondary contribution from voltage-sensitive Ca^{2+}-channels, which perhaps are activated by the concomitant depolarization caused by these 'specific' channels. However, the relatively short period of time of incubation

makes this hypothesis very unlikely. Furthermore, the total amount of Ca^{2+}-influx caused by DTX is not comparable to that obtained from known Ca^{2+}-ionophores such as A_{23187} (Akerman and Nicholls,1981). Other possible modes of action include depolarization of the synaptosomal membrane by an unspecified mechanism or inhibition of the K^{+}-rectifying current after membrane depolarization as indicated from pharmacological evidence, as the response is similar to that produced by 3,4-diaminopyridine (Harvey and Karlsson,1980).

CHAPTER 5

RADIOIODINATION OF DENDROTOXIN WITH RETENTION OF BIOLOGICAL ACTIVITY AND ITS BINDING TO BRAIN SYNAPTOSOMES

5.1 INTRODUCTION

Homogeneous DTX from *Dendroaspis angusticeps* has been shown to affect neurotransmission at both peripheral (Harvey and Karlsson,1980; 1982) and central synapses (Chapter 4; Docherty *et al.*,1983); furthermore, it inhibits totally the specific binding of ^{3}H-β-BuTx to cerebrocortical synaptosomes (Chapter 3). The potentiation of neurotransmission observed in the hippocampal slices accords with the facilitation observed at the neuromuscular junction with DTX and its homologues (Harvey and Karlsson,1982). These low-molecular weight proteins form a new class of venom neurotoxins with the ability to facilitate the release of neurotransmitter in the peripheral and central nervous systems (see Section 1.3.6; Chapter 4; Docherty,1983). Even though some of these proteins were purified nearly a decade ago; it was only recently that their pharmacological actions have been studied on a nerve-muscle preparation (Harvey and Karlsson, 1980; 1982). It was found that not all known active and inactive protease inhibitor homologues (Table 1-2) can potentiate neurotransmitter release; only those which are closely homologous to DTX can cause this facilitation (Harvey and Karlsson,1982).

It has been shown that pretreatment of nerve-muscle preparations with DTX or toxin I, followed by the addition of β-BuTx caused a reduction in the blocking activity of the latter (Harvey,1982; Harvey and Karlsson, 1982). When this sequence was reversed, with β-BuTx added in Sr^{2+}-containing medium to the preparation followed by

either DTX or toxin I, no potentiation of neurotransmitter release was observed (Harvey and Karlsson,1982). These results were interpreted to suggest that β-BuTx, toxin I and DTX may share some common binding sites, which are involved in neurotransmitter release. However, from their pharmacological actions, it must be postulated that the actions of β-BuTx and the facilitatory neurotoxins involve different mechanisms in exerting opposing effects on neurotransmitter release. Interestingly, other inhibitory neurotoxins such as notexin, crotoxin and taipoxin did not exhibit similar antagonism to that observed with β-BuTx. DTX did not appear to inhibit the binding of notexin. In fact, the blocking activity of notexin was enhanced by DTX pretreatment, suggesting that notexin binds to nerve terminal at sites distinct from the DTX and β-BuTx sites, although there seems to be some interaction between these sites. Similar results were obtained with crotoxin. In contrast, taipoxin appears to act independently through a third site, since no interactions between the former and DTX could be demonstrated (Harvey and Karlsson,1982).

DTX and toxin I can inhibit ^{3}H-β-BuTx binding to cerebrocortical synaptosomes with very high potencies (K_i of 0.5 nM and 0.07 nM, respectively; Chapter 3); this provides direct evidence for the interactions of these neurotoxins with a nerve terminal component involved with the process of transmitter release. This suggestion is supported by the demonstration that DTX can also induce permanent facilitation of neurotransmitter release in rat and guinea pig hippocampal slices (Chapter 4; Docherty

et al.,1983). Collectively, these results indicate that DTX and toxin I can be used as an additional probe for the study of neurotransmitter release. As for such study, DTX offers several advantages over β-BuTx; it has no enzymic activity and it comprises a single polypeptide rather than two subunits as present in β-BuTx. Since the characterization of the binding component for β-BuTx has been described in Chapter 3, it is of great interest to see whether the DTX acceptor protein exhibits similar kinetic properties to the former.

This chapter describes the preparation of a radioiodinated derivative of DTX, which retained complete biological activity (an important prerequisite for meaningful binding studies), and the demonstration of its saturable binding to rat cerebrocortical synaptosomes. The nature of the binding component and its specificity with regard to other presynaptically-acting neurotoxins and phospholipase A_2 was also investigated.

5.2 MATERIALS

$Na^{125}I$ carrier-free, was obtained from Radiochemical Centre, Amersham. Chloramine-T and L-Tyrosine were purchased from Sigma Chemical Co. Purified crotoxin (Chang and Lee,1977) from the venom of *Crotalus durissus cascavella* was kindly provided by Dr. B. Hawgood. The pure preparations of taipoxin, phospholipase A_2 from bee venom and *Naja naja melanoleuca* are detailed in Section 3.2. All other materials were as in Section 2.2.

5.3 METHODS

5.3.1 Radioiodination of DTX

Radiolabelling with ^{125}I-iodine was routinely performed with a modification of the chloramine-T method (Williams *et al.*,1983) employing milder conditions to yield a neurotoxic-DTX derivative of high specific radioactivity. DTX (100 µg) in a maximum volume of 50 µl was added to 5 mCi (50 µl) carrier-free Na^{125}I, and the reaction was initiated by the addition of chloramine-T (5 µl, final concentration 0.22 mM) in 100 mM sodium phosphate buffer, pH 7.4. After 30 s, the reaction was quenched by the addition of an excess of L-tyrosine (50 µl;1 mg/ml) in 100 mM sodium phosphate buffer, pH 7.4 containing 150 mM NaCl (elution buffer). ^{125}I-Labelled DTX (^{125}I-DTX) was separated from tyrosine and free iodine by gel-filtration on a Sephadex G-25 (superfine) column (12 cm X 1.0 cm), pre-equilibrated with the elution buffer. The amount of free radiolabel present in a labelled-toxin sample (5 - 10 µl) was determined by precipitation with trichloroacetic acid (10% (w/v)) using bovine serum albumin as a carrier protein.

5.3.2 Characterization of ^{125}I-DTX

5.3.2.1 Analytical isoelectric focussing

^{125}I-DTX was subjected to analytical focussing as described in Section 2.3.4.1. After focussing, the gels were sliced into 2 mm sections and counted for radioactivity in a γ-counter. A separate track of the gel containing unlabelled toxin was stained with 0.4% (w/v) Coomassie brilliant blue G_{250} in 3.5% (w/v) perchloric acid and

destained in the latter solution.

5.3.2.2 Determination of biological and chemical specific activities

Lethality of ^{125}I-DTX was measured by intraventricular injection of samples (10 µl in elution buffer) into rats (∿ 200 g body wt) using stereotaxic equipment as described in Section 2.3.4.4. For control, animals were injected with 10 µl of the elution buffer in a similar manner as test samples. The chemical specific-radioactivity of labelled toxin was determined from its radioactive and protein contents; the latter was measured colorimetrically (Lowry *et al.*,1951) using unlabelled toxin as standard.

5.3.3 Demonstration of saturable binding of ^{125}I-DTX to rat cerebrocortical synaptosomes

5.3.3.1 Preparation of synaptosomes

Rat cerebrocortical synaptosomes were prepared as described in Section 3.3.6 with the following modifications; 2 mM HEPES, pH 7.5 was used in the 0.3 M sucrose and the discontinuous density gradients were made using 8% (w/v) and 13% (w/v) Ficoll dissolved in the former solution, instead of sucrose solutions.

5.3.3.2 Binding of ^{125}I-DTX to synaptosomes

This was measured in a similar manner to the method described for ^{3}H-β-BuTx binding to rat cerebrocortical synaptosomes (Section 3.3.7). Purified synaptosomes prepared by discontinuous Ficoll-gradient centrifugation were resuspended in Krebs-phosphate buffer, pH 7.4

(Section 3.3.7) and diluted into the same buffer containing 1 mg/ml bovine serum albumin (reaction buffer).

Saturability of binding was ascertained by the incubation of aliquots of this suspension (final protein concentration 0.3-0.5 mg/ml) with increasing concentrations of ^{125}I-DTX for 30 min. at 37^{0}C; the reaction was terminated by dilution with 1 ml of reaction buffer at 4^{0}C followed by centrifugation at 10000 X g for 2 min. The pellet was washed twice at 4^{0}C (total time of $\sim$ 6 min.) with the reaction buffer before γ-radiation counting. Non-specific binding was measured similarly in the presence of 100-fold molar excess of DTX over the highest concentration of ^{125}I-DTX used; this was subtracted from the total to give the amount specifically bound toxin.

The rate of association of toxin with its binding sites at 37^{0}C was determined by using 2.5 nM ^{125}I-DTX and 0.3-0.5 mg/ml of synaptosomal suspension. Binding was terminated at various intervals by dilution with 1 ml of ice-cold reaction buffer and centrifugation as above. Non-specific binding was calculated by incubation in the presence of 100-fold molar excess of native DTX; it was subtracted from the total to give the amount specifically bound.

The rate of dissociation of ^{125}I-DTX from synaptosomal membrane was studied at 37^{0}C, after preincubation of synaptosomes (0.5 mg/ml) under saturating incubation conditions with ^{125}I-DTX (2.5 nM final concentration) for 30 min. at 37^{0}C; specific and non-specific binding were measured as above prior to addition of excess unlabelled DTX to equivalent test incubations. The rate of dissociation was

calculated by measuring the remaining amount of specifically bound toxin after further periods at 37°C.

The effect of other presynaptically-acting neurotoxins and phospholipase A_2 on ^{125}I-DTX binding was studied in competition experiments. ^{125}I-DTX (2.5 nM) was incubated with synaptosomes in the presence of increasing concentrations (10 pM-1 μM) of test proteins for 30 min. Binding was terminated by dilution and centrifugation as above.

Lysed synaptosomes were prepared as described in Section 3.3.7. After resuspension of membrane fractions in reaction buffer, aliquots were taken for use in the binding experiments. Synaptosomes were also treated with either trypsin (0.5 mg/ml, 30 min., followed by trypsin inhibitor (1 mg/ml)) or proteinase K (0.5 mg/ml for 30 min. followed by treatment with phenylmethylsulphonyl fluoride; 2.5 mM) and subsequent washing of the sedimented membranes. For heat or acid treatments, synaptosomes were heated at 95°C for 15 min. or resuspended in 1 M-HCl for 30 min. at 37°C, followed by centrifugation. Treated synaptosomes were washed, resuspended in the reaction buffer and used in the binding assays.

5.4 RESULTS

5.4.1 Radiolabelling of DTX

Homogeneous DTX was radiolabelled with ^{125}I-iodine using a modification of the chloramine-T method that employs mild conditions which has been used successfully to label botulinum neurotoxin (Williams *et al.*,1983). After separation of labelled protein from free ^{125}I and

tyrosine by gel-filtration on Sephadex G-25 (Fig. 5.1), the specific activity of ^{125}I-DTX was determined accurately from the radioactive and protein contents. A high specific activity (250 Ci/mmol) was obtained for ^{125}I-DTX and it retained complete biological activity, as assessed by central neurotoxicity. When the labelled protein was administered by intraventricular injection into the brain using a stereotaxic apparatus, the lethality observed (2.8 ng/g body wt.; n = 3), was almost identical to the neurotoxicity of native DTX (2.5 ng/g body wt.; Section 4.4.2). It proved impractical to separate ^{125}I-DTX from any unlabelled toxin present by isoelectric focussing (see below), hence, the contribution of the latter to the toxicity observed could not be quantified. However, other preparations of ^{127}I-DTX, which gave an equimolar ratio of ^{127}I-iodine to protein using the same method but employing a mixture of labelled and unlabelled iodine, showed no significant change in its toxicity (A. Black and J.O.Dolly, unpublished observations). Furthermore, another preparation of ^{125}I-DTX with much higher specific activity ($\sim$ 1250 Ci/mmol) behaved identically to this less radioactive material in binding studies. As shown in Section 4.4.2, native DTX was found to be relatively non-toxic when administered intraperitoneally (> 700 µg per mouse i.e. $\sim$ 35 µg/g body wt.), thus the labelled toxin was not tested for its peripheral toxicity.

The majority of the radioactivity ($\sim$ 95%) in the fraction containing ^{125}I-DTX was precipitated with trichloroacetic acid (10%: w/v). On analytical isoelectric focussing, ^{125}I-DTX gave a major radioactive peak with an isoelectric point of 10.5, similar to unlabelled neurotoxin (Fig. 5.2);

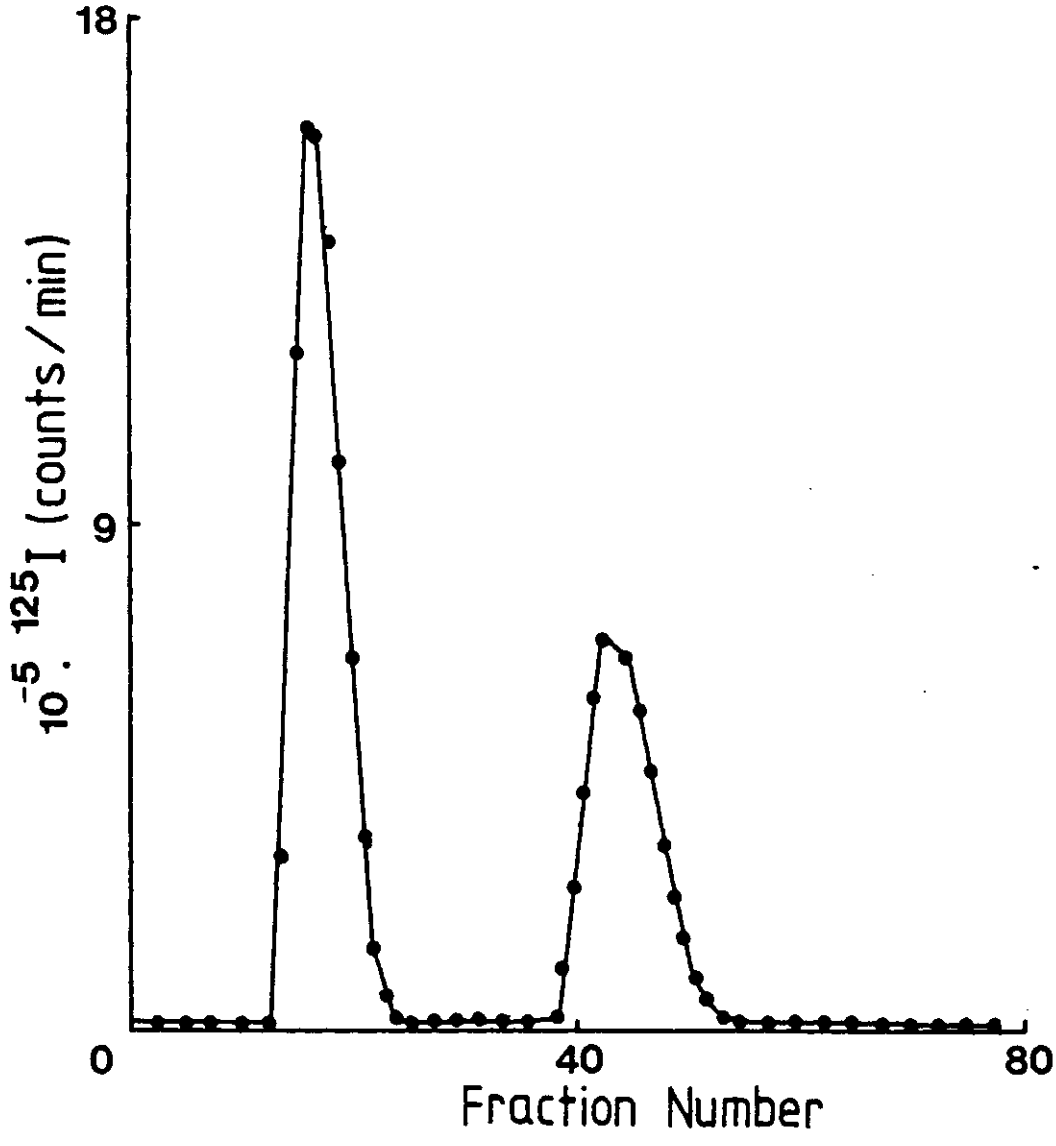


Fig. 5.1 Separation of ^{125}I-DTX from free ^{125}I-iodine and tyrosine by gel filtration

Radioiodination of DTX using a modification of chloramine-T method was carried out as described in Methods. After termination of the reaction by addition of tyrosine, the mixture was applied to a column of Sephadex G-25 (superfine) (1 X 12 cm) equilibrated with 100 mM sodium phosphate buffer; pH 7.4 containing 150 mM NaCl. Radioactivity (●) of the fractions (0.45 ml), was determined by γ-counting.

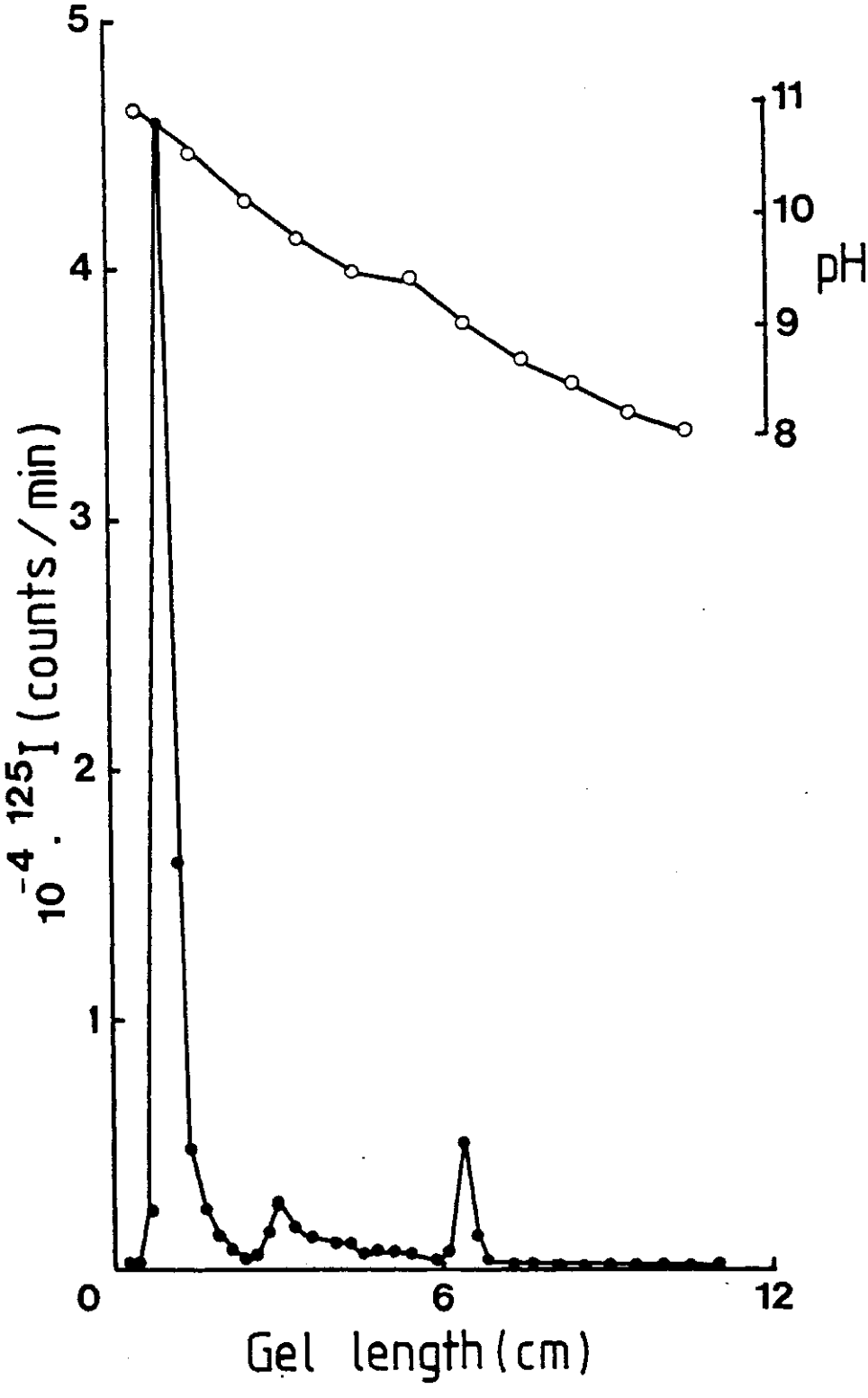


Fig. 5.2 Isoelectric focussing of ^{125}I-DTX

Analytical polyacrylamide gel isoelectric focussing of a sample of ^{125}I-DTX after gel filtration of the labelling mixture (Fig. 5.1). Focussing was performed as described in Section 2.3. The pH gradient (o) and radioactive content (●) of gel slices (2 mm) were measured.

this indicated that radioiodination did not alter significantly the net charge of the protein molecule and thus could not be separated from any unlabelled toxin present, either by ion-exchange chromatography or chromatofocusing. Two other radiolabelled species, probably di- and tri-labelled, were separated by IEF but they represented negligible amounts of the total radioactivity (7.4% and 7.8%, respectively).

5.4.2 Saturable binding of ^{125}I-DTX to rat cerebrocortical synaptosomes

The binding of ^{125}I-DTX was investigated using rat cerebrocortical synaptosomes. It has been shown that DTX can inhibit the binding of ^{3}H-β-BuTx to the latter with high potency, thus, characterization of its binding component in synaptosomes would permit comparison of the acceptors for both toxins.

On incubation of synaptosomal suspensions with increasing amounts of ^{125}I-DTX (0.01 - 4 nM), a defined amount of binding was observed; its saturability was demonstrated by the ability of 100-fold molar excess of unlabelled DTX to prevent the binding of labelled toxin (Fig. 5.3A); 25% (at 2.5 nM) non-specific binding was observed. Scatchard analysis of this data showed that the labelled neurotoxin binds specifically to a single class of non-interacting sites of high affinity with a K_d of 0.51 (± 0.08; n = 8) nM (Fig. 5.3B); the content of binding sites was 600 (± 51.8; n = 8) fmol/mg protein (Fig. 5.3B). Lysis of the synaptosomes did not affect the value observed for the maximum binding of ^{125}I-DTX indicating that the interaction is not

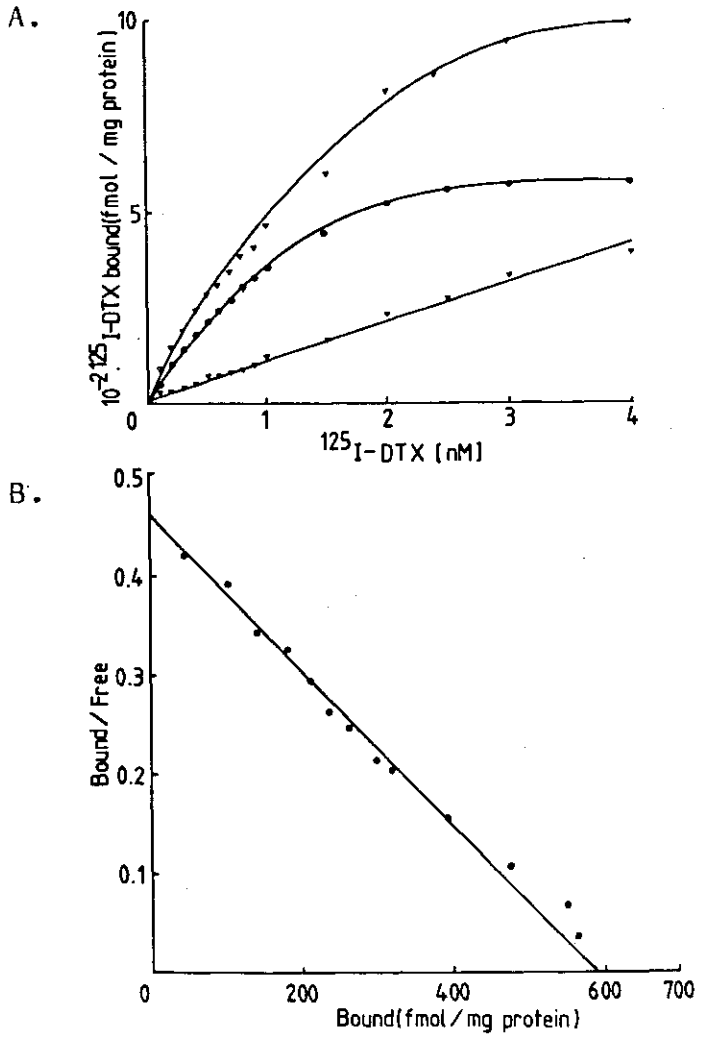


Fig. 5.3 Binding of ^{125}I-DTX to synaptosomes

A. Synaptosomes suspensions (0.5 mg/ml) were preincubated at 37 °C in the absence (▽) and presence (▼) of unlabelled DTX for 5 min. Different concentrations of ^{125}I-DTX were then added and after a further 30-min. incubation, the total amounts of bound radioactivity were measured by a centrifugation assay, as previously described in Section 3.3.7. The values obtained for non-specific binding (▼) were subtracted from the total (▽) to give the amount of specifically bound (●) ^{125}I-DTX. Average values observed with triplicate samples are expressed relative to the amount of measurable protein in the equivalent sample. It should be noted that the concentrations of free ^{125}I-DTX were calculated using the values of total and bound toxin. To check the accuracy of these, they were determined for two concentrations (0.5 and 2.5 nM) in the undiluted supernatants, after sedimentation of the reaction mixtures; as the values were lower from those calculated previously by an average of 15%, the corrected K_d is taken to be 0.43 nM rather than 0.5 nM. Also, the total specific binding was remeasured by a modification of the standard assay, in which the dilution step was omitted and the pellet was rinsed but not suspended during one rapid immediate wash; this gave a new value of B_{max} (766 fmol/mg protein) which was 25% higher than indicated above. This new rapid assay is, therefore, preferable as the extent of non-specific binding is only slightly higher than that obtained with the prolonged standard washing, as it minimises the dissociation of the specifically bound toxin.

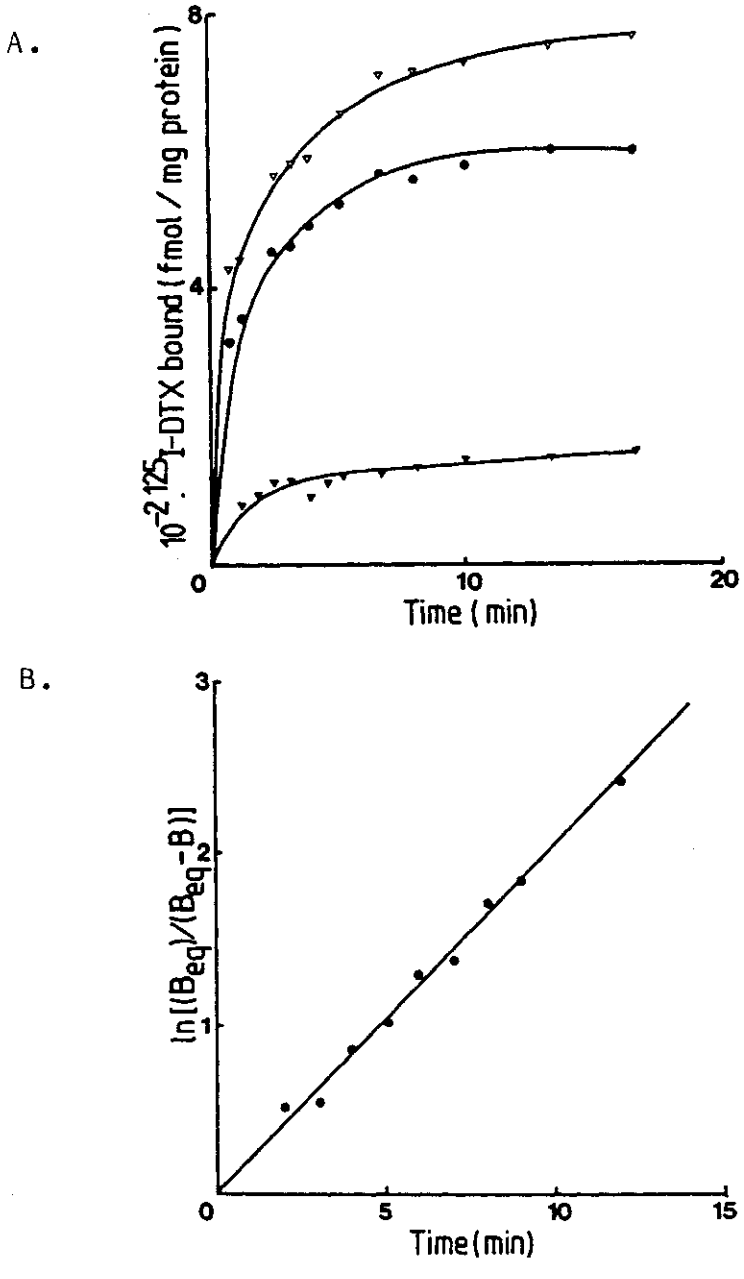
B. Scatchard analysis of the data in A.

due to internalization of the toxin.

Kinetics of the interaction of ^{125}I-DTX with its acceptor site were studied by methods detailed in Section 3.3.7. The binding of ^{125}I-DTX was found to be rapid, reaching a plateau after 15 min. at 37°C, using 2.5 nM ^{125}I-DTX; there was a slight increase in the non-specific binding thereafter (Fig. 5.4A). Analysis of the association of ^{125}I-DTX with synaptosomes using pseudo-first order reaction (Equation 3-1; Section 3.4.5) gave a slope of 3.36 X $10^{-3}s^{-1}$, which is equal to $\{L\}k_1 + k_{-1}$, with L, k_1, and k_{-1} as previously defined in Section 3.4.5 (Fig. 5.4B). The rate of dissociation (k_{-1}) of ^{125}I-DTX from its binding site was determined by adding an excess of unlabelled toxin to a synaptosomal suspension in which the binding sites had been previously saturated with ^{125}I-DTX. The subsequent rate of decrease in specifically bound radioactivity (Fig. 5.5A) had a $t_{\frac{1}{2}}$ of 45 min. at 37°C; a straight line was obtained when the data was plotted according to the rate-equation which describes a first-order reaction (Equation 3-2, Section 3.4.5) (Fig. 5.5B). The slope of the line showed that the dissociation rate constant, k_{-1}, is 2.86 X $10^{-4}s^{-1}$. Using this value for k_{-1}, together with the slope obtained from the association rate plot (Fig. 5.4B), the association rate constant, k_1, was calculated; its value was found to be 6.15 X 10^5 $M^{-1}s^{-1}$. The dissociation constant, K_d, calculated from these values of k_1 and k_{-1} was 0.47 (± 0.07; n = 8) nM; the latter is very close to the value of 0.51 nM obtained from Scatchard analysis above.

Competition of ^{125}I-DTX binding with increasing

Fig. 5.4 Time course of ^{125}I-DTX binding to rat cerebrocortical synaptosomes



A. Suspensions of synaptosomes (0.5 mg/ml), prepared as described in Methods were incubated with 2.5 nM ^{125}I-DTX for the times indicated at 37°C. For measurement of the non-specific binding 250 nM of unlabelled toxin was added 5 min. prior to addition of ^{125}I-DTX. This non-specific binding (▼) was subtracted from the total (▽) to give specific binding (●), shown for duplicate samples.

B. Pseudo-first-order plot of the data in A, where B_{eq} and B represent the amounts of ^{3}H-β-BuTx bound at equilibrium and at time t, respectively. The slope obtained for this line, 3.3 X $10^{-3}s^{-1}$, equals $\{L\}k_1 + k_{-1}$ where L is the concentration of toxin used. k_1 and k_{-1} are the association and dissociation rate constants. respectively.

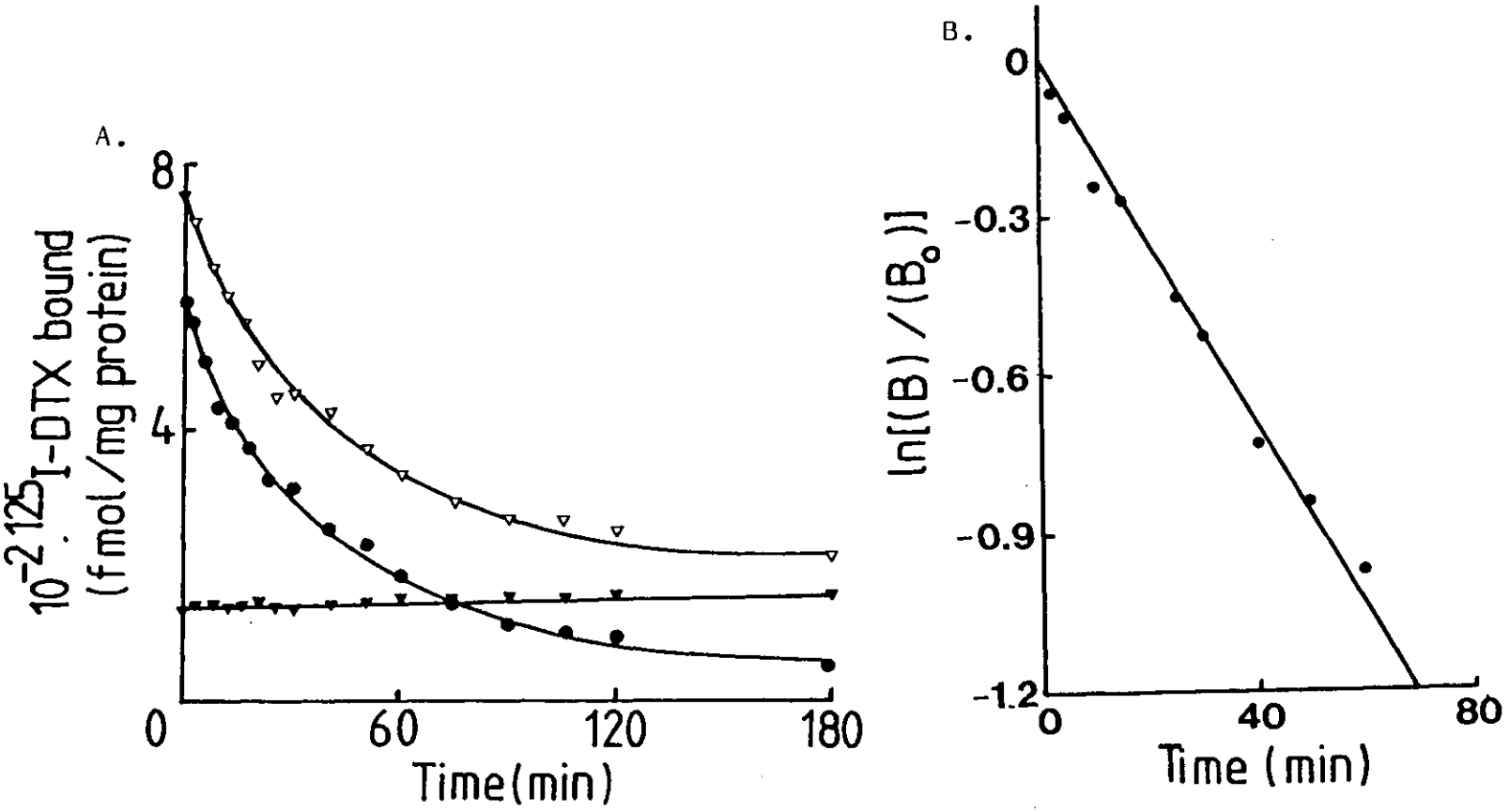


Fig. 5.5 Dissociation of ^{125}I-DTX from rat cerebrocortical synaptosomes

A. Synaptosomes prepared as described under Methods (0.5 mg of protein/ ml) were incubated with 2.5 nM ^{125}I-DTX for 30 min. at 37°C and the amount of specifically bound (●) was determined as in Fig. 5.4. Unlabelled DTX (250 nM) (▽) was added and the amount of specifically bound toxin was measured thereafter at the time indicated. Non-specific (▼) was determined simultaneously in another sample by inclusion of unlabelled DTX (250 nM) in the initial incubation mixture and subtracted to give the amount of specifically bound toxin remaining.

B. First-order plot of the data in A

concentrations of unlabelled DTX (Fig. 5.6) demonstrated that the native toxin had a K_i of 0.47 (± 0.06; n = 8) nM; this indicates that there was no change in the toxin's affinity on radioiodination. Removal of Ca^{2+} from the incubation medium did not affect the inhibitory activity of unlabelled DTX (Fig. 5.7) indicating that its binding was not dependent on Ca^{2+}. Collectively, these data are consistent with ^{125}I-DTX binding to a single class of non-interacting sites of high affinity on rat cerebral synaptosomes.

5.4.3 Nature of the saturable binding component for ^{125}I-DTX on synaptosomes

This was investigated on synaptosomes which had been subjected to one of the following treatments: protease digestion either with trypsin or proteinase K, heat or acid (HCl). Samples which had been preincubated with trypsin followed by an excess of trypsin inhibitor and subsequent washing of the membranes prior to incubation with ^{125}I-DTX, failed to show any specific binding when 2.5 nM of ^{125}I-DTX was used (Fig. 5.8). Furthermore, increasing concentrations of native toxin did not inhibit ^{125}I-DTX binding to trypsinized synaptosomes, indicating that trypsin totally destroyed the component responsible for saturable binding of the toxin (Fig. 5.8). Similar results were obtained when synaptosomes were treated with proteinase K (Fig. 5.8). The binding of ^{125}I-DTX was also inactivated either by heating the synaptosomal suspension for 15 min. at 95°C or treating it with 1 M HCl at 22°C (Fig. 5.8).

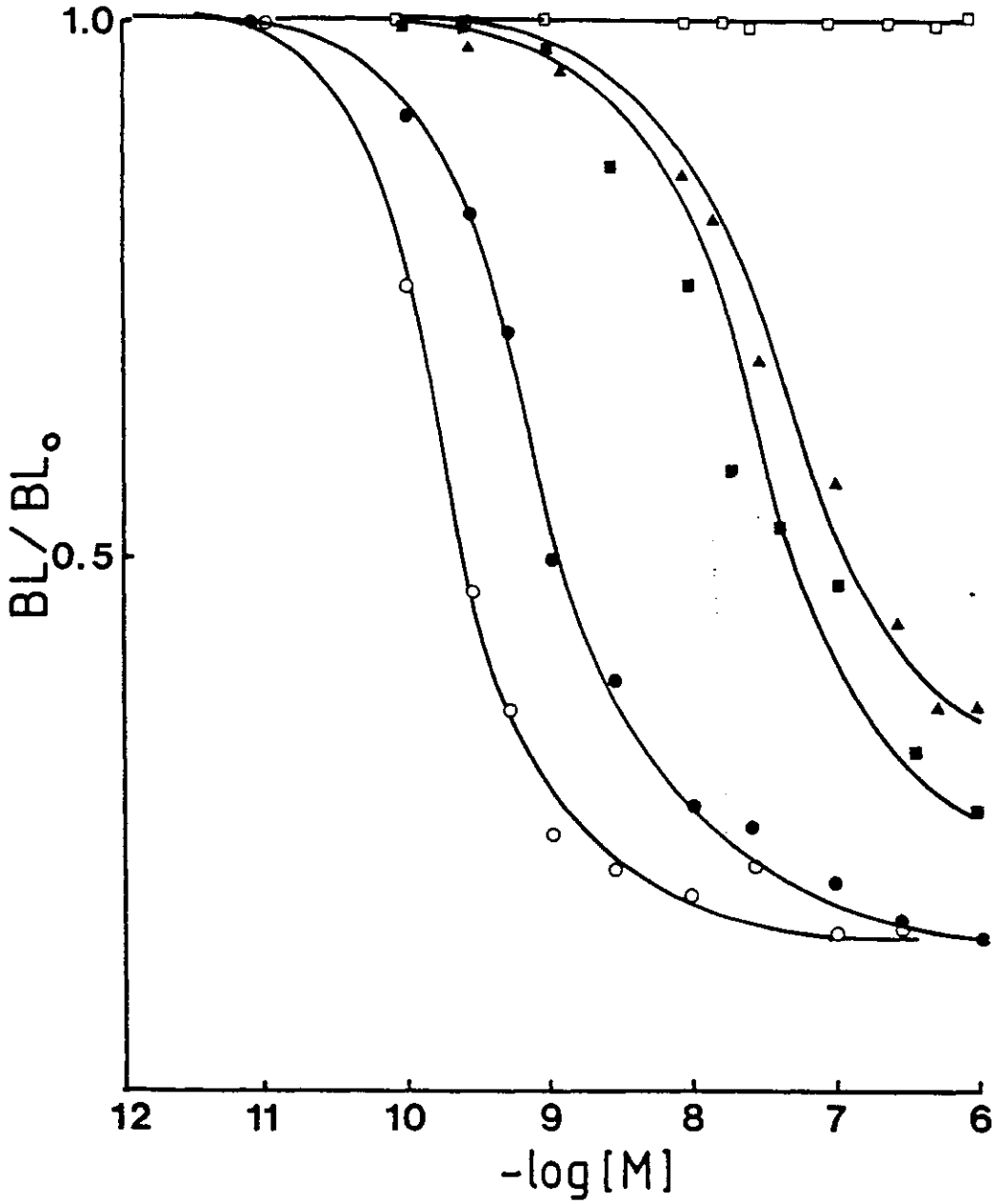


Fig. 5.6 ^{125}I-DTX binding to synaptosomes in the presence of unlabelled DTX, toxin I, taipoxin and phospholipases A_2 from bee venom and *Naja melanoleuca*

Synaptosomes (0.5 mg protein/ml) were incubated for 30 min. at 37°C with 2.5 nM ^{125}I-DTX in the presence of increasing concentrations of unlabelled DTX (●). BL_0 and BL represent the amount of ^{125}I-DTX binding in the absence and presence of competing protein: toxin I (○), taipoxin (▲), BV-PLA_2 (■) and phospholipase A_2 from *Naja melanoleuca* (□). K_i was calculated using the observed IC_{50} value according to the equation $K_i = IC_{50} / 1 + (\{L\}/K_d)$ where {L} is the ^{125}I-DTX molar concentration used.

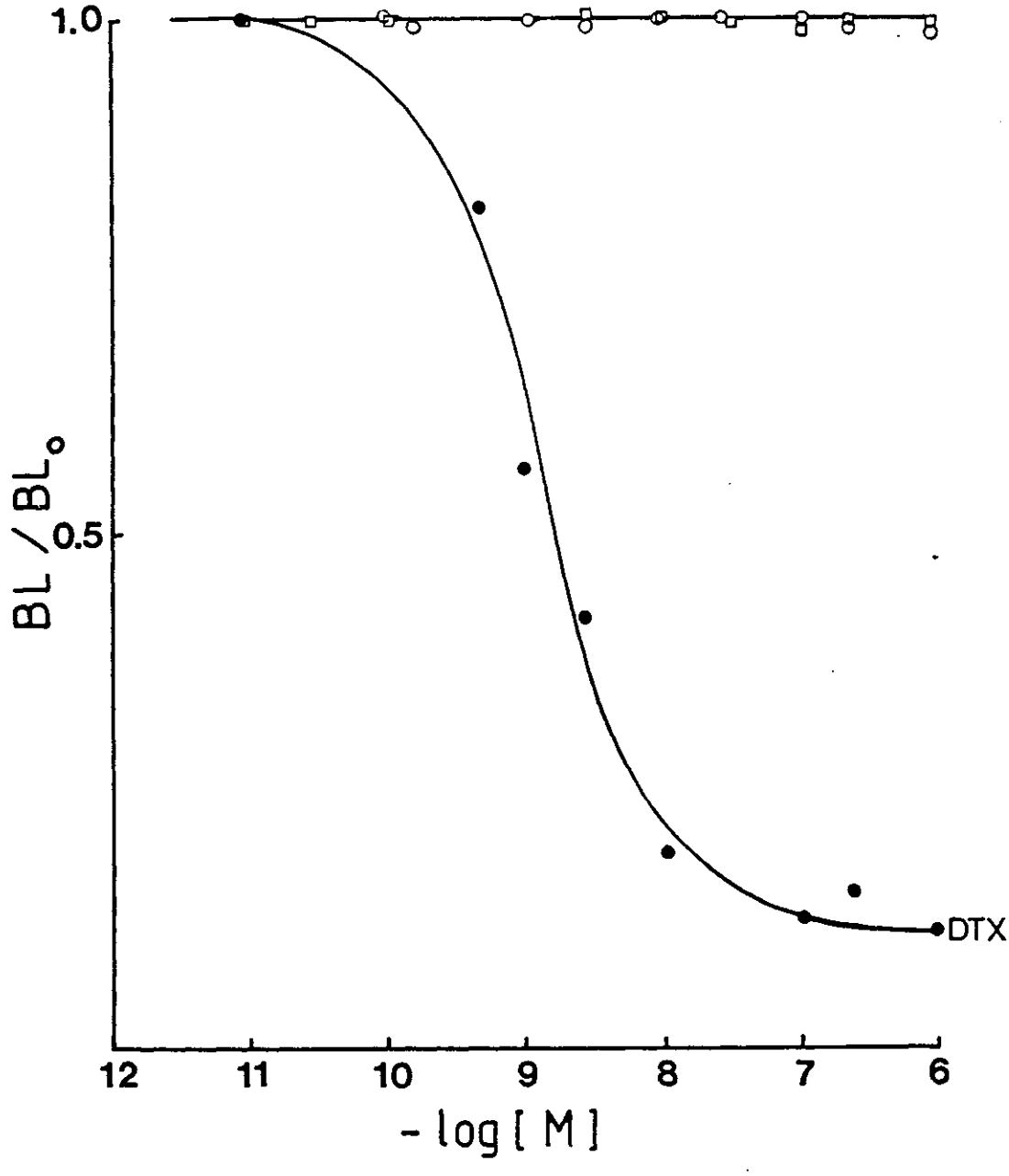


Fig. 5.7 ^{125}I-DTX binding to synaptosomes in Ca^{2+}-free medium: Effect of native DTX, taipoxin and BV-PLA_2

Experiments were performed as described in Fig. 5.6 except that Ca^{2+}-free medium was used; the latter was replaced by EGTA. BL_0 and BL represent the amount of ^{125}I-DTX binding in the absence and presence of competing protein: native DTX (●), taipoxin (○) and BV-PLA_2 (□).

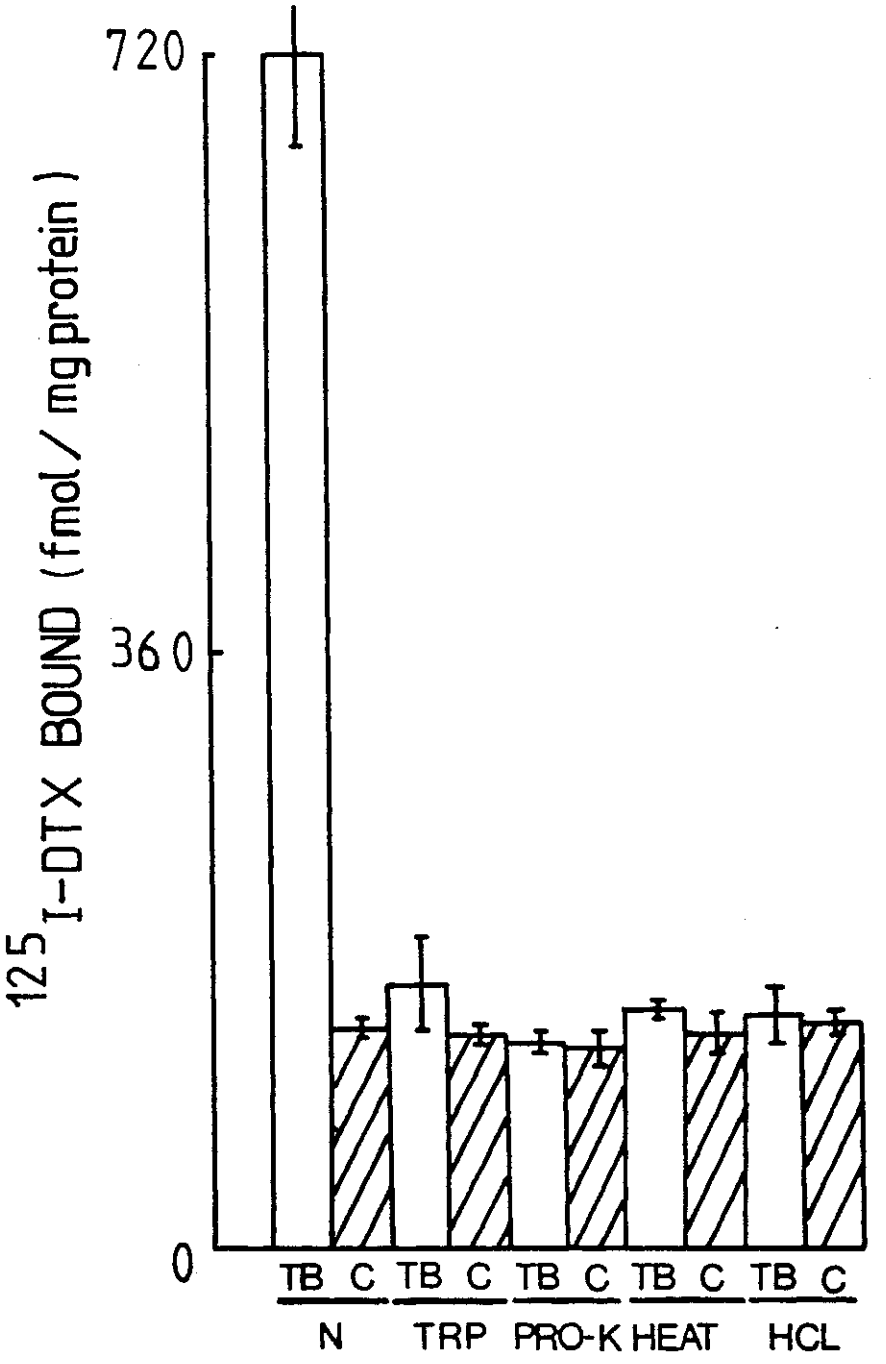


Fig. 5.8 Effect of various treatments on ^{125}I-DTX binding to synaptosomes

Synaptosomes were treated with one of the followings: Heat, acid (HCl), trypsin (TRP) and proteinase K (PRO K) using conditions detailed in Methods. After termination of the preincubation by dilution and centrifugation, synaptosomal pellets were resuspended in reaction buffer and then incubated with 2.5 nM ^{125}I-DTX for 30 min. at 37^{0}C in the absence (TB) or presence (C) of 250 nM unlabelled DTX. Binding was quantified by the centrifugation assay described in Section 5.3. Average values of triplicate samples were given.

5.4.4 Specificity of the acceptor sites for ^{125}I-DTX on synaptosomal membranes

To assess the specificity of the DTX acceptors, the effects of a number of pure phospholipase A_2 and presynaptic neurotoxins on the binding were investigated. Phospholipase A_2 from the venom of the snake *Naja melanoleuca* had no effect on the binding (Fig. 5.6). In contrast, phospholipase A_2 from bee-venom antagonized the binding of ^{125}I-DTX in the presence of Ca^{2+}; the K_i was 9.3 nM, calculated from IC_{50} (56 nM) (Fig. 5.6), which is about 20 fold higher than the toxin's K_d. On removal of Ca^{2+} from the incubation medium; no inhibition was apparent with this phospholipase A_2 (Fig. 5.7) suggesting a non-specific interaction of the enzyme.

Of all the presynaptic neurotoxins tested, only taipoxin showed inhibition of ^{125}I-DTX binding (Fig. 5.6) but again this was attributed to its phospholipase A_2 activity as in the case of bee-venom phospholipase A_2; no inhibition was apparent on inactivation of the phospholipase activity by Ca^{2+} removal (Fig. 5.7) or its substitution by Sr^{2+} (data not shown). In contrast, another presynaptic neurotoxin which has a similar mode of action at motor end-plates as taipoxin, called crotoxin, purified from the snake venom of *Crotalus durissus cascavella*, did not inhibit the binding of ^{125}I-DTX in the presence (Fig. 5.9) or absence (Fig. 5.7) of Ca^{2+}. When β-BuTx (up to 1 mM) was tested for its ability to inhibit ^{125}I-DTX binding, it was found to be capable of displacing only about 26% of the total specific binding of ^{125}I-DTX, with an IC_{50} of 266 nM (obtained from the 26% inhibition) and a K_i of 44.3 nM (Fig. 5.9).

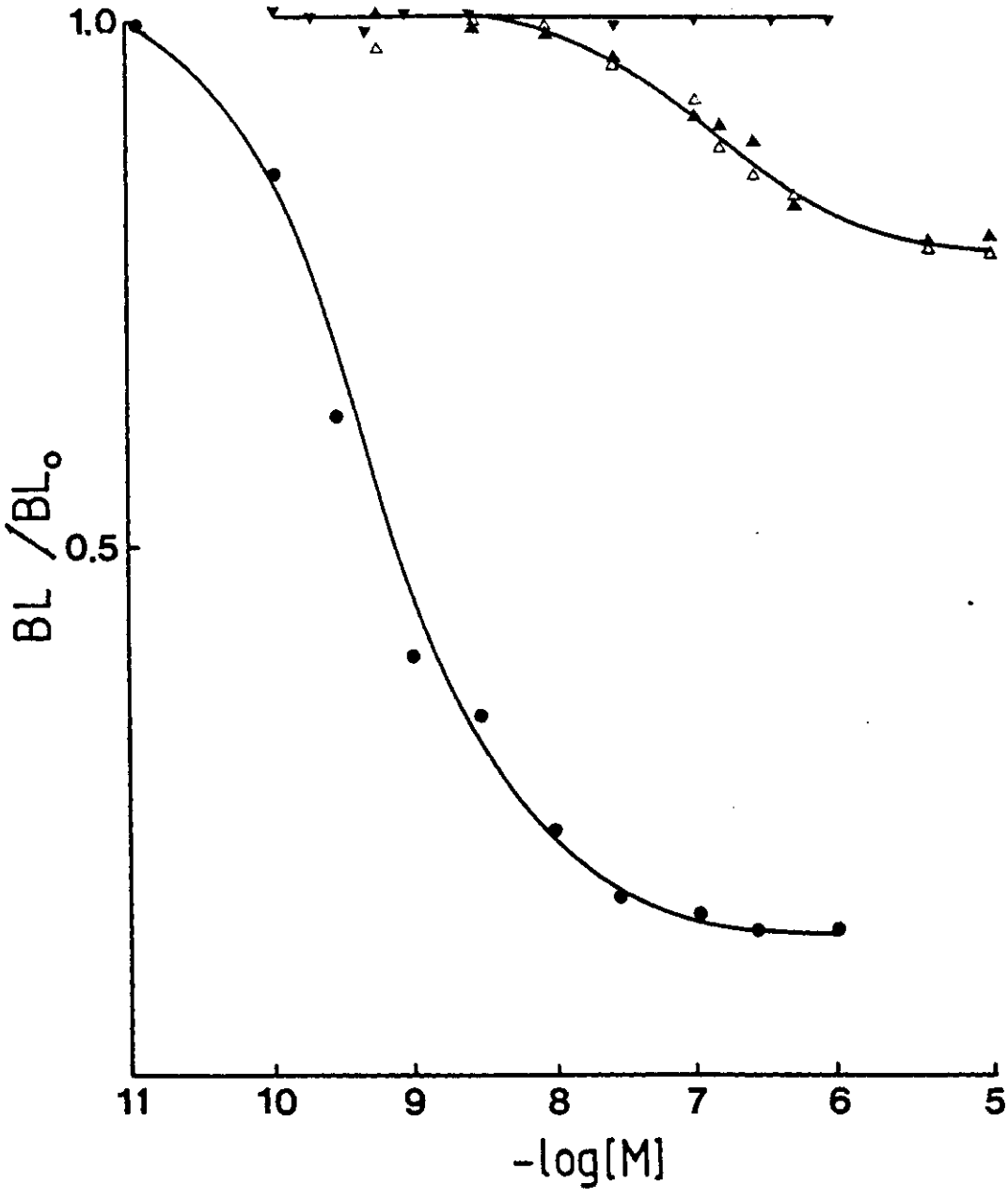


Fig. 5.9 ^{125}I-DTX binding to synaptosomes in the presence of native DTX, β-BuTx and crotoxin

Competition studies were performed as described in Fig. 5.6 with different concentrations of unlabelled DTX (●), crotoxin (▼) and β-BuTx (△ and ▲ , in the absence of Ca^{2+}). K_i value for β-BuTx was calculated using the IC_{50} value obtained from the 26% inhibition observed; the former was calculated using the equation described in Fig. 5.6.

This partial antagonism was still observed in Ca^{2+} -free medium (Fig. 5.9) suggesting that it was not caused by the non-specific interaction of its intrinsic phospholipase A_2 activity.

In a similar experiment, toxin I from *Dendroaspis polylepis*, which is closely related to DTX (Table 1.2) was found to inhibit totally the binding of ^{125}I-DTX with a K_i of 0.06 nM (Fig. 5.6). This antagonism observed between these two toxins was expected on the basis of homologous amino acid sequences and the similarity in their pharmacological effects observed at the neuromuscular junction (Harvey and Karlsson,1982). However, toxin I was more potent in inhibiting ^{125}I-DTX binding than unlabelled DTX (K_i = 0.47 nM) (Fig. 5.6) and this accords with the findings that toxin I is five-times more toxic than DTX in rat brain (see Section 4.4.2).

In summary, these results indicate that the binding site for ^{125}I-DTX, at least on synaptosomal membranes, is distinct from the binding component of the presynaptic neurotoxins, crotoxin and taipoxin. However, β-BuTx inhibited, to some extent, the binding of ^{125}I-DTX, but with low efficacy, indicating an indirect mechanism is operative in the interactions of these toxins. The ability of toxin I to inhibit the binding of ^{125}I-DTX with high potency suggests that it is binding to the same site as the latter.

5.5 DISCUSSION

The ability of toxin I (Othman *et al.*,1982; Chapter 3) and DTX (Chapter 3) to antagonize the binding of ^{3}H-β-BuTx with high potency on rat cerebrocortical synaptosomes, and also the pharmacological action of β-BuTx on chick biventer cervices nerve-muscle preparations (Harvey,1982; Harvey and Karlsson,1982) initiated this investigation in characterizing the DTX binding component (s). DTX was chosen as the crude venom was cheaper and more readily available; also its purification procedure has been documented (Harvey and Karlsson,1980; Joubert and Taljaard,1980; Chapter 4). It is intriguing that toxin I and DTX could totally inhibit β-BuTx binding since they exert opposing pharmacological actions at nerve terminals.

The experiments in this Chapter established a method for radiolabelling DTX. The resultant ^{125}I-DTX derivative obtained was biologically active in the CNS, as indicated by its neurotoxicity, which at 2.8 ng/g body wt., was still extremely toxic. Since the contribution of unlabelled toxin in the preparation to the toxicity cannot be quantified directly, another preparation of iodinated toxin which gave an equimolar ratio of ^{127}I-iodine to protein was tested for its toxicity. The result obtained for the latter was similar to ^{125}I-DTX (A.Black and J.O.Dolly, unpublished observations), indicating that radioiodination did not affect significantly the biological activity of the toxin. The ^{125}I-DTX preparation also contained negligible amounts of di- and tri- labelled species

represented by two smaller peaks of radioactivity on isoelectric focussing (Fig. 5.2); the extent of the involvement of such minute quantities in binding studies should be negligible.

The presence of saturable binding sites for ^{125}I-DTX on brain synaptosomes has been demonstrated by both equilibrium and kinetics measurements. The labelled neurotoxin binds specifically to a single class of non-interacting sites of high-affinity (K_d = 0.51 nM); the content of sites is 600 fmol/mg protein. This binding was inhibited by unlabelled DTX with a K_i of 0.47 nM which demonstrate that radioiodination did not alter the affinity of the toxin. The rate constant of 6.10 X $10^5 M^{-1}s^{-1}$ obtained for the association of ^{125}I-DTX to synaptosomal membrane at 37^{0}C shows that the binding is rapid and is, therefore, unlikely to be a rate-limiting step in the toxin-induced augmentation of neurotransmission. The dissociation rate constant (k_{-1}), 2.86 X $10^{-4}s^{-1}$ at 37^{0}C was somewhat faster than expected from the apparent irreversibility of the toxin at the neuromuscular junction (Harvey and Karlsson,1982) and in hippocampal slices (Chapter 4; Docherty *et al.*,1983). Binding of ^{125}I-DTX occurred equally well to lysed as to intact synaptosomes, indicating that the binding sites are membrane bound and the toxin is not internalized. The binding component(s) is thought to be of a proteinaceous nature since its activity is completely removed by trypsinization, proteinase K, heating or acid treatment of the synaptosomes.

The specific binding was unaffected by the relatively non-toxic phospholipase A_2 from *Naja melanoleuca*

although that from bee-venom inhibited the binding with a potency (K_i = 9.3 nM) 20-fold lower than the unlabelled DTX. However, the latter was ineffective in Ca^{2+}-free buffer. Similarly, taipoxin was able to antagonize ^{125}I-DTX binding in the presence of Ca^{2+}; removal of the latter from the incubation medium also removes the inhibition. These results indicate that the antagonism observed is mediated by the enzymic activity of the neurotoxin and phospholipase A_2, suggesting that the binding of ^{125}I-DTX to nerve-terminals may have some dependence on the membrane-lipid environment. The failure of phospholipase A_2 from *Naja melanoleuca* to have an effect on the binding of ^{125}I-DTX to synaptosomes may be a reflection of the substrate specificities of the phospholipases. Interestingly, in the presence of Ca^{2+},bee-venom phospholipase A_2 and taipoxin can also inhibit the binding of ^{3}H-β-BuTx (Othman *et al.*,1982; Chapter 3) to synaptosomes with similar affinities to that observed with ^{125}I-DTX. These observations suggest that the binding components of these presynaptic neurotoxins may be located very close to one another and in a phospholipid environment which may be present only on the presynaptic membrane of nerve terminals.

With regards to the specificity of the toxin binding component(s), it seems specific for DTX and its homologue, toxin I. No competition was observed with crotoxin and taipoxin (in the absence of Ca^{2+}) suggesting that their binding sites are distinct from that of DTX. The existence of separate binding sites for these neurotoxins accords with the pharmacological evidence obtained from their interactions with protease inhibitor homologues

including DTX and toxin I, at chick nerve-muscle preparation (Harvey and Karlsson,1982). The existence of separate binding sites for these neurotoxins has also been demonstrated by other workers (Chang and Su,1980). The inefficiency of β-BuTx in antagonizing ^{125}I-DTX binding was rather surprising. It was capable of displacing only 26% of the total bound ^{125}I-DTX, and has a K_i of 43 nM indicating that the potency is low. It was initially assumed that this was caused by the non-specific action of its intrinsic phospholipase A_2 activity. However, removal of Ca^{2+} from the medium, thus inactivating the enzymic activity, did not abolish the partial inhibition observed ((Fig. 5.7); this data suggests that the antagonism was not caused by enzyme-substrate interaction. On the other hand, DTX was capable of displacing ^{3}H-β-BuTx binding with a K_i of 0.5 nM (Chapter 3), which is equipotent with the affinity of ^{3}H-β-BuTx for its binding sites. These results can be interpreted in a number of ways : a) β-BuTx may be exhibiting greater selectivity to the different types of neurons in the CNS than DTX; this suggestion is supported by the autoradiographic localization study at light microscope level (see Chapter 6). On the hippocampal sections, β-BuTx exhibits the highest level of binding on the molecular layer of the dentate gyrus, moderate binding to the stratum oriens and stratum radiatum, and very low level to the granule cell layer. DTX, on the other hand, showed high density of sites in the molecular layer of the dentate gyrus, on the stratum oriens and stratum radiatum (Chapter 6); b) examination of the total number of binding sites for DTX and β-BuTx on synaptosomes

indicates that the former has 4-times more sites than β-Butx. As 25% of these sites are displaceable by β-BuTx (Fig. 5.6) which corresponds to the total number of β-BuTx sites on synaptosomes (Chapter 3), it would appear that DTX is capable of binding to additional sites other than the binding component for β-BuTx. It cannot be ascertained at this stage of the study, whether they they are binding to the same site on the binding component or not; however, on the basis of the sequence homology between the B-chain of β-BuTx and DTX (Kondo *et al.*,1978a;Strydom and Joubert,1981; Table 1.2), it is probable that they can share at least some common binding sites. Pharmacological evidence (Harvey and Karlsson,1982) also gives support to this hypothesis. It was not surprising to find that toxin I can totally block the binding of ^{125}I-DTX to synaptosomes (Fig. 5.6) as their primary structures differ only by 5 residues (Table 1-2) and they exhibit a similar mode of action in potentiating neurotransmitter release at the neuromuscular junction (Harvey and Karlsson,1982). However, with a K_i of 0.06 nM, toxin I exhibits greater affinity for the binding sites than unlabelled DTX itself; this can be reconciled with the demonstration than the latter is more neurotoxic than DTX when administered in rat brain. (Chapter 4). As previously shown, toxin I also exhibits greater affinity for ^{3}H-β-BuTx binding sites than native β-BuTx and DTX (Chapter 3). The reason for the greater affinity is not known, and further experiments with toxin I would be necessary to clarify the observed differences.

Despite this demonstration of the existence of specific binding sites for DTX, their function and the toxin's

mechanism of action remain obscure. It is likely that the binding component(s) observed is responsible for the toxin's high potency when injected intraventricularly and in inducing spontaneous excitatory activity on hippocampal slices (Chapter 4). This postulation is supported by the discrete localization of ^{125}I-DTX on the synapse-rich regions of the hippocampus (Chapter 6).

CHAPTER 6

AUTORADIOGRAPHIC LOCALIZATION OF THE BINDING SITES FOR β-BUTX AND DTX IN RAT BRAIN

6.1 INTRODUCTION

Biochemical and electrophysiological studies on β-BuTx and DTX have shown that these neurotoxins are highly active at central synapses (Othman *et al.*,1982; Halliwell and Dolly,1982a; 1982b; Chapter 3; Chapter 4; Docherty *et al.*,1983). The first evidence obtained to indicate their potency in the CNS was their extreme toxicity when administered in the ventricular spaces of rat brain (Othman *et al.*,1982; Chapter 2 and 4). It has been shown that β-BuTx is 200 times more toxic in the CNS than at the periphery (Chapter 2), and causes extensive non-selective neuronal degeneration (Hanley and Emson,1979). With a minimum lethal dose of 0.05 ng/g body wt., β-BuTx is one of the most potent proteins ever isolated from snake venoms. Moreover, electrophysiological studies on rat olfactory cortex (Dolly *et al.*,1980) and hippocampal slices (Halliwell and Dolly,1982a; 1982b), revealed that β-BuTx is an effective blocker of neurotransmission in areas of the CNS using excitatory amino acids as neurotransmitters (White *et al.*,1979). Furthermore, in hippocampal slices, β-BuTx acts preferentially in terminal rich-areas (Halliwell and Dolly,1982b). The non-selectivity of β-BuTx for specific neurotransmitter terminals in the olfactory cortex and hippocampal slices was also observed in rat brain synaptosomes. At these isolated nerve terminals, it causes the release of several inhibitory and excitatory neurotransmitters (Tse *et al.*,1980; Spokes and Dolly,1980; Smith *et al.*, 1980; Sen and Cooper,1976; Wernicke *et al.*,1974). Kinetic studies on the binding of ^{3}H-β-BuTx to rat cerebrocortical

synaptosomes demonstrate that the toxin binds to a single class of non-interacting sites (B_{max} = 150 fmol/mg protein) of high affinity (K_d = 0.6 nM) (Othman *et al.*,1982; Chapter 3). This binding can be antagonized by the facilitatory neurotoxins, toxin I and DTX (K_i's of 0.07 and 0.5 nM, respectively) (Othman *et al.*,1982; Chapter 3), which further suggests that the binding component(s) of β-BuTx is involved in the process of neurotransmitter release. Collectively, these results strongly suggest that β-BuTx binds specifically to functional sites in the presynaptic membranes of nerve terminals.

In contrast to β-BuTx, DTX is relatively non-toxic when administered intraperitoneally (Chapter 4; Harvey and Karlsson,1980); however, in the CNS, it has a toxicity of 2.5 ng/g body wt., which is 10^4 orders of magnitude more toxic than the periphery (Chapter 4). To date, no morphological study similar to that reported for β-BuTx (Hanley and Emson,1979) has been carried out on the effect of DTX in the brain. DTX has been shown to cause the facilitation of neurotransmitter release at the neuromuscular junction (Harvey and Karlsson,1980; 1982) and on hippocampal slices of rat and guinea pig (Docherty *et al.*,1983; Chapter 4). In the latter, it induces a permanent facilitation of synaptic transmission and acts like a potent convulsant, whose direct actions require the involvement of Ca^{2+}-channel activity. On synaptosomes, it causes an increase in the total amount of $^{45}Ca^{2+}$ accummulated, which can be inhibited by a Ca^{2+}-channel blocker, such as Cd^{2+} (Chapter 4). Furthermore, its effect on $^{45}Ca^{2+}$-influx is insensitive to tetrodotoxin, which suggests that the effect is not caused by

depolarization mediated through the Na^+-channels (Chapter 4). In view of these electrophysiological and biochemical effects of DTX on brain slices and synaptosomes, the kinetics of its interaction with cerebrocortical synaptosomes were measured. Binding studies of biologically active ^{125}I-DTX derivative indicated that the binding is saturable and of high affinity (K_d = 0.5 nM; Chapter 5). A single class of non-interacting sites was observed, and the binding could be inhibited by toxin I and β-BuTx; the latter with low efficacy (Chapter 5).

In this chapter, using the biologically-active ^{3}H-β-BuTx and ^{125}I-DTX derivatives, previously used for the characterization of their respective binding components on rat cerebrocortical synaptosomes (Chapters 3 and 5), the localization of their binding sites on rat-brain cerebellar and hippocampal sections was investigated at the light microscope level. Employing the light-microscope autoradiographic techniques described by Young and Kuhar (1979), the distribution of their relative grain densities on cryostat sections of cerebellum and hippocampus was quantitated. In addition, the ultrastructural localization of the binding components which have been characterized (Chapters 3 and 5) for these toxins on rat cerebrocortical synaptosomes was carried out using electron-microscope autoradiography.

6.2 MATERIALS

^{3}H-β-BuTx and ^{125}I-DTX were prepared as previously described in Chapters 3 and 5. Nuclear emulsions (K5 and L4) and films (HP5 and FP4) were purchased from Ilford Ltd.,

Essex. Pyronin Y and methyl green were obtained from Hopkins and Williams. D-19 developer and Ficoll were obtained from Kodak Eastman and Pharmacia, respectively. All other chemicals used were of analytical grade unless otherwise stated.

6.3 METHODS

6.3.1 Light-microscope autoradiography

This was carried out essentially following the procedures of Young and Kuhar (1979). It involves preparation of tissue sections, mounting on slides, binding of radiolabelled neurotoxins, and attaching emulsion-coated coverslips on top of these slides for autoradiography.

6.3.1.1 Preparation of tissue sections

Female Sprague-Dawley rats (180-250 g), were anaesthetised with either ether or pentabarbitone (60 mg/kg), and perfused with 200 ml of 0.1% (w/v) paraformaldehyde in phosphate buffer saline, pH 7.4 over 10-15 min. prior to removal of the brain. This lightly fixed tissue has been found to show improved preservation of its structural features but yet retains receptor binding activities for various ligands that have been tested (Young and Kuhar,1979; 1981). After removal of the brain, slices were prepared from the appropriate parts and quickly frozen in isopentane, initially at -40°C, followed by immersion in liquid N_2 at -170°C. A whole brain area containing hippocampus or cerebellum was mounted in a Bright cryostat microtome (Model OTF/AS/M/V) and 10 µm sections were thaw-mounted onto warm subbed (dipped into a solution of 0.5 g gelatin and 50 mg chrome alum in

100 ml water) slides. Sections (2-4) were mounted onto slides and air-dried at 22°C for at least 1 h. These slides were used immediately or stored at -20°C for later use. For hippocampal sections, sections corresponding to A 3180 µm - A 4890 µm of Koenig and Klippel (1963) were used.

6.3.1.2 Binding of radiolabelled-neurotoxins (^{3}H-β-BuTx or ^{125}I-DTX)

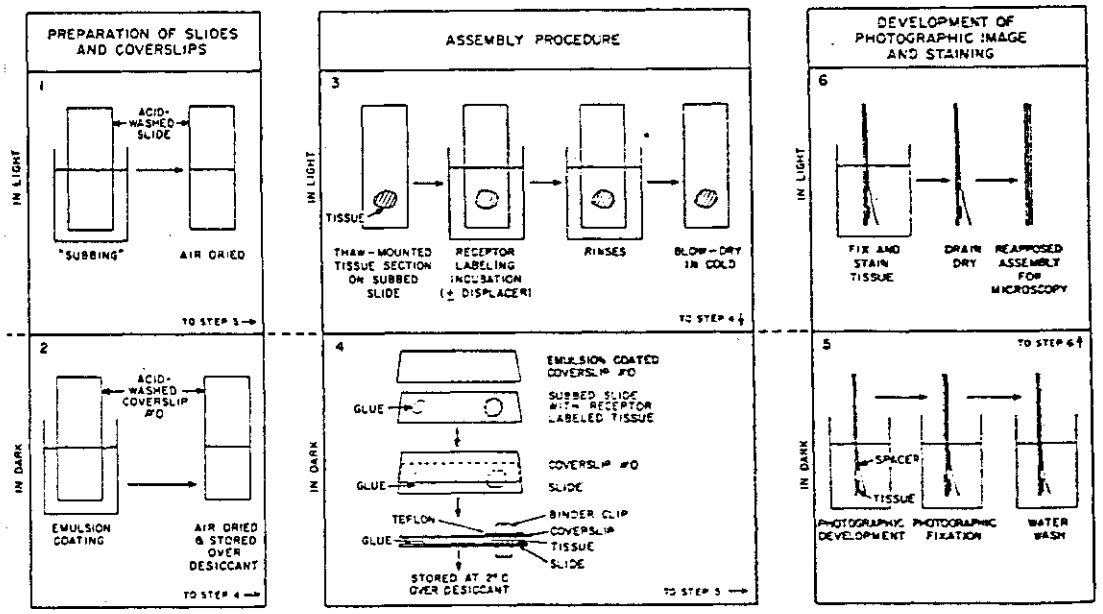
The slides containing mounted-tissue sections were brought to room temperature prior to preincubation in Kreb's-phosphate buffer, pH 7.4 containing 1 mg/ml bovine serum albumin (reaction buffer; see Section 3.3.7) for 5 min. For optimization of labelling conditions, these slides were incubated at 22°C for up to 30 min. with different concentrations (0.1-5.0nM final concentration in 100 µl) of labelled neurotoxins in reaction buffer containing 0.1% polylysine. After incubation, the slides were rinsed quickly in reaction buffer at 4°C (usually three 2-min. rinses in ∿ 30 ml of reaction buffer) and dried at 70-80°C on a hot-plate. For determination of their radioactive contents, the sections were scraped from the slides with a razor blade and placed in scintillation cocktail (Othman *et al.*,1982). With this method, it was shown that saturable binding occurred with 5 nM ^{3}H-β-BuTx or 2.5 nM ^{125}I-DTX used. Non-specific labelling, measured likewise in the presence of a 100 fold-molar excess of unlabelled neurotoxin, was found to represent less than 30% of the total radioactivity on cerebellar and hippocampal sections. For light microscope autoradiography, tissues labelled in this manner were dried in the cold (4°C) after labelling, and processed for light micros-

cope autoradiography as described below.

6.3.1.3 Autoradiography

Fig. 6.1, present a schematic illustration of the *in vitro* labelling technique adapted from Young and Kuhar (1979).

Fig. 6.1 Schematic illustration of *in vitro* labelling technique



Coverslips (22 X 70 mm) were coated by dipping into Ilford K5-Nuclear emulsion (diluted 1:1.5 with distilled water at 43°C), air dried for 3 h. and stored over dessicant at 4°C. The emulsion-coated coverslips were attached to the slides with tissue sections in the dark with glue (Loctite super-glue) which was placed on one end of the slide. After the glue set, slides were assembled and held together with bulldog clips. The assemblies were stored dessicated at 4°C for varying lengths of time. After exposure, the binder clips

were removed in the dark and the coverslips gently bent away from tissue section with a rubber spacer. The emulsion was developed at 16°C in D-19 developer (3 min.) , hardened (3 min.) with potassium chrome alum and sodium sulphate (7.5 g + 15.0 g in 250 ml water) and fixed in 25% sodium thiosulphate (4 min.). After rinsing the slides in distilled water (3 times for 5 min. each), they were dried over silica gel overnight and fixed the next day with Carnoy's solution as described below.

6.3.1.4 Fixing and staining of tissues

The tissues were fixed in CH_3CH_2OH : $CHCl_3$: CH_3COOH (60:30:10) (Carnoy,1887) for at least 30 min.

To differentiate the different cell-layers, tissues were stained with pyronin Y/methyl green solution (Trevan and Sharrock,1951); the procedure for this process is shown in Table 6.1.

Table 6-1 A) Staining with pyronin Y/methyl green

i. Wet sections with distilled water
ii. Stain for 20-30 min. in the working solution of methyl-green and pyronin Y (Soln C)
iii. Rinse in distilled water for a few seconds, and blot lightly until almost dry
iv. Dehydrate in acetone for not more than 1 min.
v. Rinse in equal parts of acetone and xylene; clear in pure xylene
vi. Mount in DEPEX medium

B) Notes

i. All steps are carried out at room temperature
ii. DNA (chromatin) stains green or blue-green
iii. RNA (nucleoli) stain rose red; (granules) dark rose red;

and (plasma cell cytoplasm) stains purple

C) Stock solutions

Solution A:

2% aqueous methyl green (chloroform washed)	10 cm^3
5% aqueous pyronin Y	17.5 cm^3
Distilled water	250 cm^3

Solution B: Acetate buffer, pH 4.8

0.1 M Sodium acetate	119 cm^3
0.1 M Acetic acid	81 cm^3

Solution C: Working solution

Equal volumes of solution A and B were mixed prior to staining

6.3.1.5 Photography

Photographs were taken using FP4 (Asa 125) or HP5 (Asa 400) Ilford black and white film with a camera mounted on a Reichert Microscope. To visualize the different cell layers, the stained brain sections were viewed using bright field optics. The distribution of the silver grains on the slices was viewed under dark field optics on the microscope. Grain densities were obtained from photographs taken at high magnification of the specific areas viewed under dark field optics.

6.3.2 Electron-microscope autoradiography

6.3.2.1 Preparation of synaptosomes

Rat cerebrocortical synaptosomes were prepared using Ficoll-sucrose density-centrifugation as described in Section 5.3.3.

6.3.2.2 Binding of ^{3}H-β-BuTx to synaptosomes

In these experiments, di-^{3}H-propionylated β-BuTx (^{3}H-β-BuTx) prepared in Chapter 3, which had a specific radioactivity of 102 Ci/mmol and free from unlabelled toxin and phospholipase A_2 activity was used. Binding of ^{3}H-β-BuTx (5 nM) to synaptosomes was measured following essentially the procedure described in Section 3.3.7, using a centrifugation assay. The extent of non-specific labelling was assessed by inclusion of a 100-fold excess of unlabelled β-BuTx in similar incubations.

6.3.2.3 Autoradiography

After the final washing in the reaction buffer (Section 3.3.7) at 4°C, the labelled synaptosomal pellet was fixed in 2% glutaraldehyde/Krebs phosphate buffer for 1 h. and post-fixed in 1% OsO_4 in the same buffer. This was followed by dehydration carried out through 25%, 50%, 75% and 100% (v/v) ethanol/water, and embedding in Spurr's resin. Sections (500-600 Å) were cut on an LKB ultramicrotome and processed for electron-microscope autoradiography following essentially the method of Fertuck and Salpeter, (1976). The ribbons of sections were placed on a glass slide covered with a film of formvar, which was then dipped in Ilford L4 emulsion diluted 1:2.4 , and exposed up to 4 months in light-tight boxes at 4°C. After development in D-19 for 2 min. and fixation in 25% sodium thiosulphate for 4 min., the membrane (formvar) was removed from the slide by flotation on distilled water and a grid was placed on the ribbon of sections. The formvar was recovered by touching it with a piece of parafilm to which it adhered, and lifting it out

of the water. After drying at 22°C, the grid was detached from the parafilm and stained in 12% uranyl acetate for 15 min. prior to viewing in a Hitachi electron microscope.

6.3.2.4 Binding of ^{125}I-DTX to synaptosomes

This was carried out essentially following the procedures described in Section 5.3.3, using 2.5 nM ^{125}I-DTX. For electron microscope autoradiography, pellets labelled with ^{125}I-DTX were treated in a similar manner to that described for ^{3}H-β-BuTx-labelled synaptosomes above. Following 6 weeks exposure, the sections were developed in D-19 and viewed as described above.

6.3.3 Sub-cellular fractionation of synaptosomes treated with ^{3}H-β-BuTx

For lysis of synaptosomes, suspensions were incubated in 5 mM-Tris HCl pH 8.0 for 40 min. on ice. After washing and resuspension of the pellet in the reaction buffer (see Section 3.3.7), ^{3}H-β-BuTx binding was carried out with 5 nM ^{3}H-β-BuTx for 30 min. at 37°C. Non-specific binding was measured simultaneously in similar incubation conditions with the presence of a 100-fold molar excess of unlabelled toxin. After the third washing, the pellet was resuspended in 0.5 ml of reaction buffer; 2 vol. of 48% (w/v) sucrose-HEPES (2 mM) buffer, pH 7.4 was mixed with the synaptosomal pellet (total vol. ∿ 1.8 ml) in a SW60 tube (5 ml). This was overlaid with 28% (2 ml) and 10% (0.5 ml;(w/w)) sucrose-HEPES buffer, pH 7.4, and centrifuged at 34000 rpm for 38 min. After fractionation of the gradient, the specific binding of ^{3}H-β-BuTx to myelin, synaptosomal plasma membrane and mitochondria (ultrasynapto-

somal) was determined by scintillation counting; protein was determined by the colorimetry method of Lowry *et al.*,(1951).

6.4 RESULTS

Before undertaking the autoradiographic studies, the optimal binding conditions for the labelled-neurotoxins (at the concentrations used) were established in the slide-mounted tissue sections. Each slide contained either four cerebellar or two hippocampal sections from the same rat brain.

6.4.1 Localization of β-BuTx sites on brain-tissue sections and synaptosomes

6.4.1.1 Binding sites for ^{3}H-β-BuTx and ^{125}I-β-BuTx on cerebellar and hippocampal sections

As a prerequisite for the localization study, it must be ascertained that the experiments were carried out under saturating conditions. To test for specific binding, a time course of ^{3}H-β-BuTx binding to the slide-mounted tissue-sections was examined. Rat cerebellar and hippocampal sections were incubated with 5 nM ^{3}H-β-BuTx for various times at 22°C. The sections were then rinsed in the reaction buffer for 6 min. (3 X 2 min. rinses) at 4°C, and radioactivity on these tissue sections was determined by scintillation counting as described in Methods. Under these conditions, the specific binding of ^{3}H-β-BuTx increased with time and was saturable after 20 min. (Fig. 6.2). The non-specific binding, determined in the presence of a 100-molar excess of unlabelled β-BuTx, was at a level of 30% of the total binding. Under these conditions,

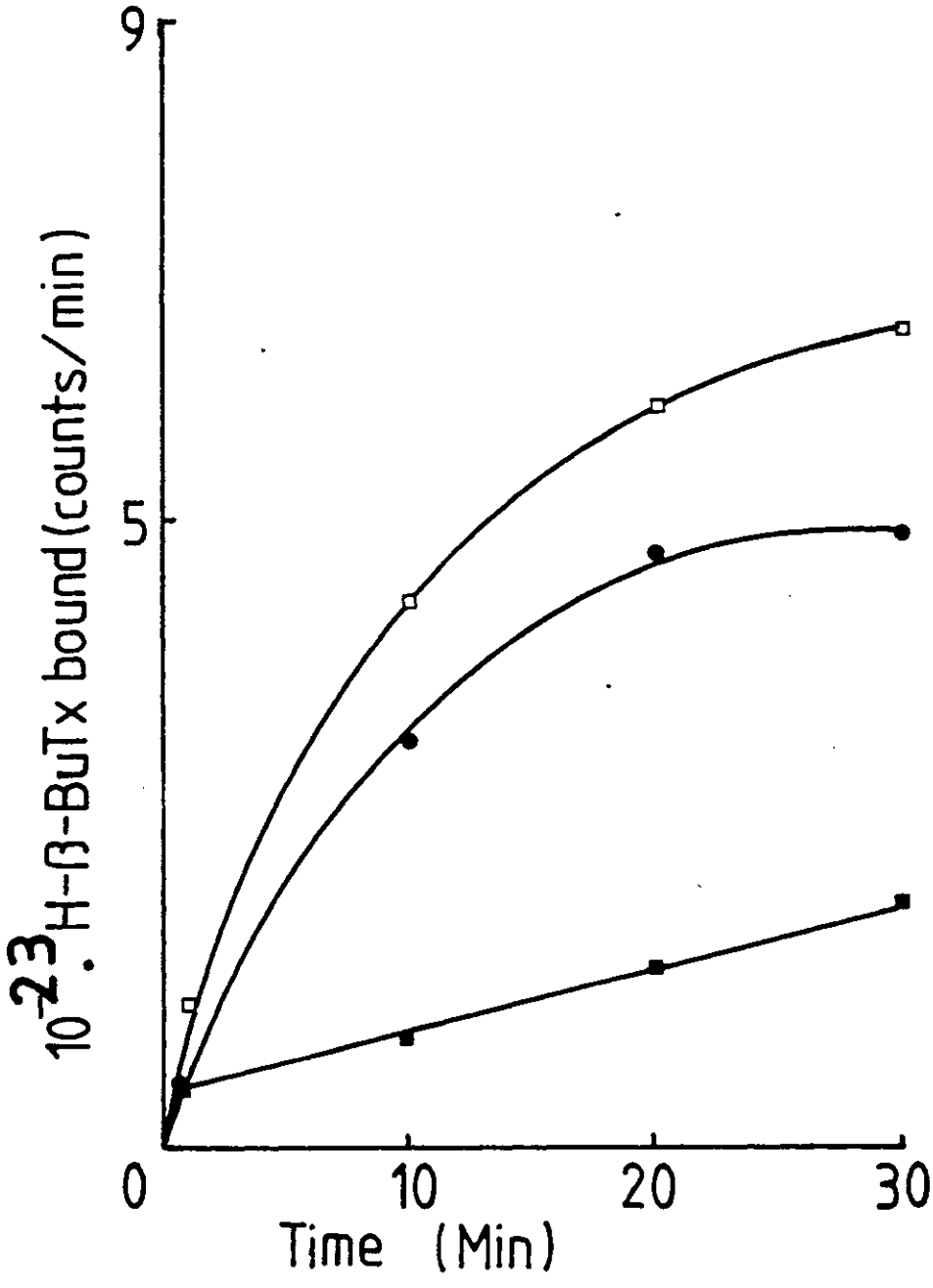


Fig. 6.2 Time course of ^{3}H-β-BuTx binding to rat cerebellar sections

Sections (10 μm) were incubated with 5 nM ^{3}H-β-BuTx in the presence and absence of 100-fold molar excess of unlabelled toxin; for various lengths of time, washed twice for 2 min. each, and processed for biochemical analysis as described in Methods. The non-specific (■) binding was subtracted from the total (□) to give the specifically bound toxin (●). Values are the means of triplicate determinations.

maximal binding of ^{3}H-β-BuTx with the concentration used was observed on slide-mounted-tissue sections. As it was not intended to characterize the kinetics of ^{3}H-β-BuTx binding to these sections, no further biochemical experiments were carried out. Hence, with a standard concentration of 5.0 nM ^{3}H-β-BuTx and an incubation time of 30 min., the tissue sections were labelled and processed for light-microscope autoradiography as described in Methods.

Labelled cerebellar sections were processed for autoradiography and, after 3 weeks exposure, significant numbers of silver grains were visualized (Fig. 6.3A). Areas representative of the specific cell layers were viewed at high magnification, photographed and silver grains quantitated (Table 6-2). The autoradiograms of test samples revealed a localised pattern of labelling; in contrast, few grains were visible in the control specimens (Fig. 6.3C; Table 6-2) which were treated with an excess of unlabelled toxin, indicating that the binding was saturable as expected from the radioactive measurements using scintillation-counting (Fig. 6.2).

To visualize the different cell-layers, tissues were fixed with Carnoy's solution and stained with pyronin Y and methyl green, which stains nucleic acids (Trevan and Sharrock,1951). Examination of the autoradiograms revealed that the majority of the silver grains were deposited over the molecular layer, which is known to have a high content of synapses (Palay and Chan-Palay,1974). A much lower content of grains was observed in the granule cell-layer which consists mainly of cell-bodies. The grain densities in the white matter and all areas of control samples (Fig. 6.3C; Table 6-2)

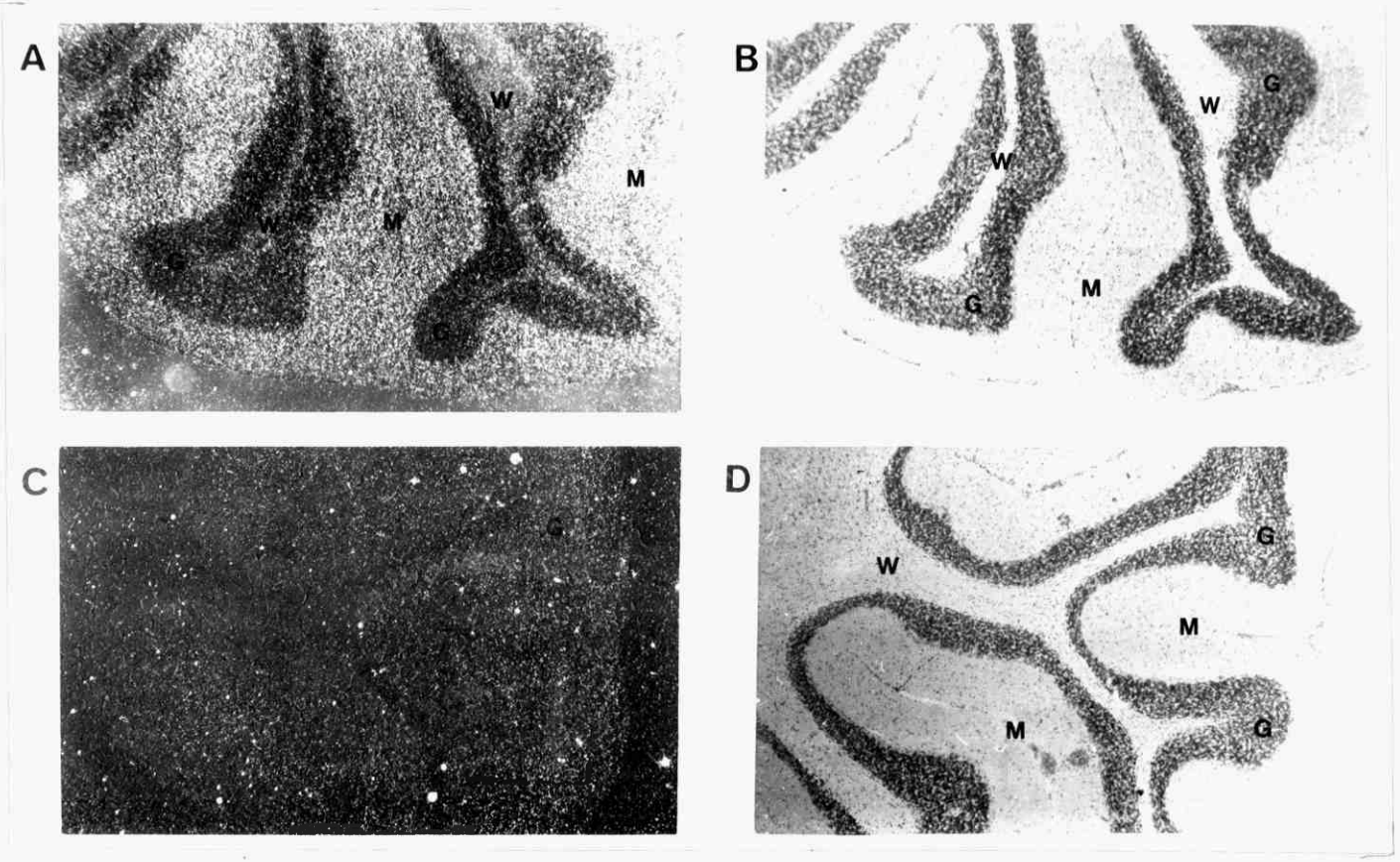


Fig. 6.3 Distribution of ^{3}H-β-BuTx binding sites in rat cerebellum

Sections were prepared, labelled and process for autoradiography described in Methods.Following exposure for 3 weeks, the specimens were developed in Kodak D-19 developer and stained with pyronin/ methyl green solution.

A and C : dark field visualizatin of test and control samples, respectively.

B and D : bright field visualization of test and control samples respectively, showing the molecular layer (M),granular cell layer (G) and white matter (W).

Table 6-2 Relative distributions of grains in cerebellar sections labelled with ^{3}H-β-BuTx and ^{125}I-β-BuTx

Areas	Grain densities* / 100 μm²			
	^{3}H-β-BuTx		^{125}I-β-BuTx	
	Test	Control	Test	Control
Molecular layer	16.3 (± 1.8)	3.2 (± 0.7)	21.3 (± 6.3)	9.9 (± 1.6)
Granule cell layer	4.3 (± 0.2)	2.4 (± 0.6)	9.5 (± 0.6)	10.1 (± 1.9)
White matter	3.0 (± 0.9)	2.1 (± 0.5)	10.8 (± 1.3)	10.1 (± 1.7)

* Mean values (± S.D) shown were obtained from counting a minimum of 12 areas from two separate sections using photographs taken at high magnification. The extent of non-specific binding observed in the control samples for ^{3}H-β-BuTx accords with that measurable by scintillation counting (Fig. 6.2).

were similar and at a level 5-6 fold lower than that in the molecular layer of test samples (Fig. 6.3A; Table 6-2).

The localisation of β-BuTx on cerebellar sections was also carried out with a ^{125}I-β-BuTx derivative which has a specific radioactivity of 500 Ci/mmol and was prepared using a modification of the chloramine-T method described by Williams *et al.*,(1983) (A.Pelchen and J.O.Dolly, unpublished observation). The binding properties of this derivative to synaptosomes have been described in Section 3.5. Incubation of the cerebellar sections with 1 nM ^{125}I-β-BuTx over a period of time showed saturable binding analogous to that observed with ^{3}H-β-BuTx. Using 1 nM ^{125}I-β-BuTx and an incubation time of 30 min. as the standard concentration and time, cerebellar and hippocampal sections were treated for light-microscope autoradiography as described in Methods.

After 3 weeks exposure, the autoradiograms were developed in Kodak D-19 developer and stained with pyronin Y/methyl green, as described in Methods. Examination of the distribution of ^{125}I-β-BuTx binding on cerebellar sections revealed a similar pattern of labelling to that of ^{3}H-β-BuTx. The molecular layer contained the highest grain densities, with less binding occurring in the granule cell layer and the white matter. However, there was one apparent difference to the binding of ^{3}H-β-BuTx observed (Fig. 6.3A) the level of non-specific binding of the iodinated derivative was much higher, both in the granule cell layer and the white matter (Fig. 6.4A; Table 6-2). Control samples also demonstrated this high level of non-specific binding in all areas (Fig. 6.4C; Table 6-4). These results are consistent with the

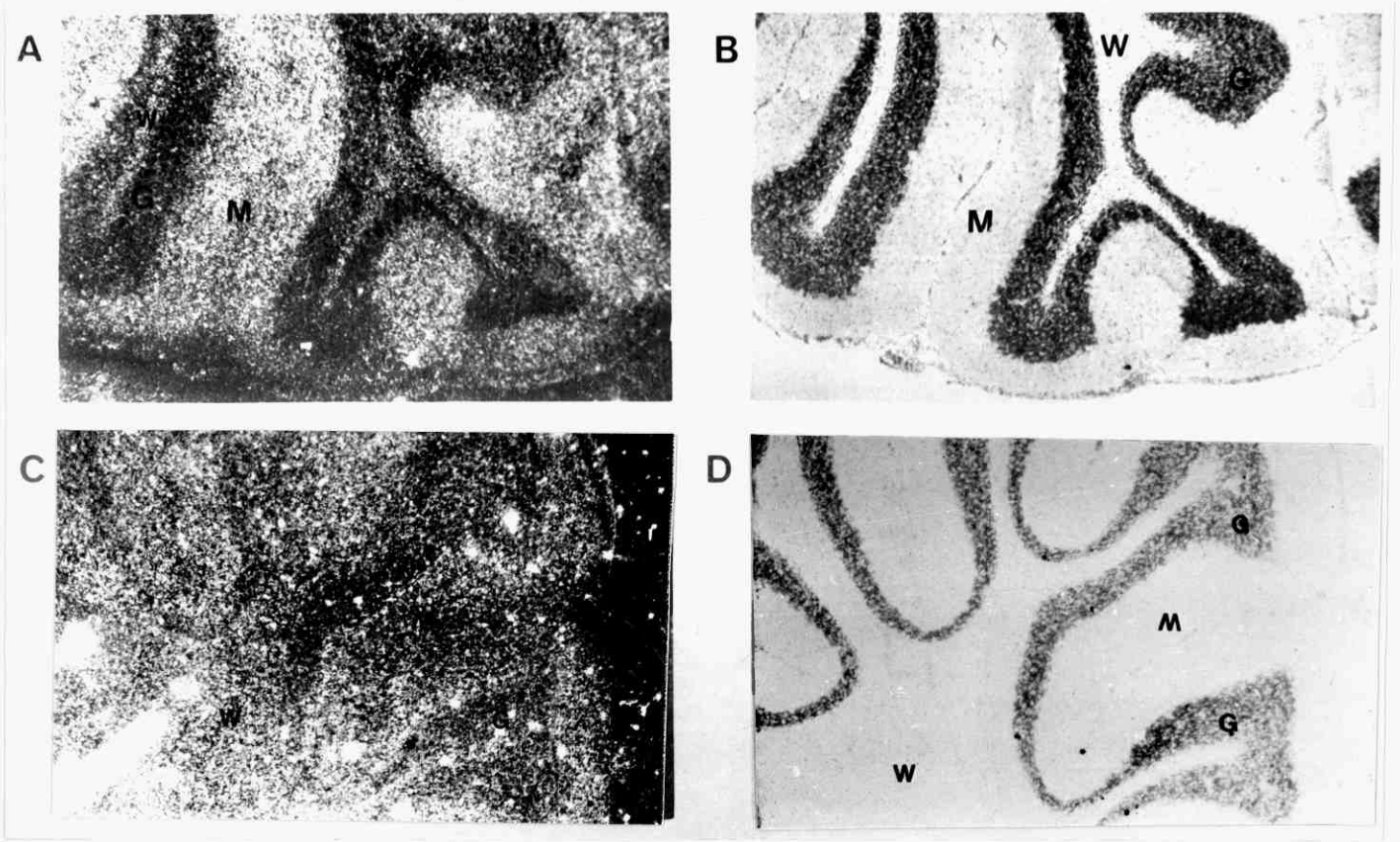


Fig. 6.4 Distribution of ^{125}I-β-BuTx binding sites in the rat cerebellum

Sections were treated as Fig. 6.3, except ^{125}I-β-BuTx was used.

A and B : dark fields visualization of test and control samples respectively, showing the distribution of the silver grains.

C and D : bright field visualization of test and control,respectively, showing the different cell layers :- molecular layer (M), granule cell layer (G), and the white matter (W). (Mag. X 28.7)

kinetic data obtained for ^{125}I-β-BuTx binding to synaptosomes; this also showed a high level of non-specific binding (∿ 45-50%) under similar conditions (A.Pelchen and J.O.Dolly, unpublished observations). From this observation, it is apparent that radioiodination alters the binding properties of β-BuTx more dramatically than the gentle tritiation of β-BuTx by ^{3}H-propionylation.

A comparable pattern of ^{3}H-β-BuTx binding was observed on hippocampal sections (Fig. 6.5A); saturable binding of ^{3}H-β-BuTx was obtained in biochemical experiments, with control samples having a relatively low level of radioactivity, as determined by scintillation counting (data not shown). Few grains were found in those regions containing cell-bodies (the granular and pyramidal cell layers) and myelin (Table 6-3). Terminal-rich regions exhibited a high content of silver grains, most pronounced in the dentate gyrus regions (Fig. 6.5A). Here, the molecular layer contained 3-4 times the total grain densities found in the controls, suggesting preferential binding of ^{3}H-β-BuTx to this area. The stratum oriens and stratum radiatum contained approximately 60% of the silver grains observed in the molecular layer (Table 6-3) and were uniformly labelled. In control specimens, the grain densities were uniformly low and, as a result, photography in dark-field at low-magnification was found to be difficult to obtain. However, at high magnification, quantitation of the grain densities indicated that the non-specific binding was at a level of about 30% of the total binding (Table 6-3).

The distinct distribution of ^{3}H-β-BuTx binding on hippocampal sections was also observed with the

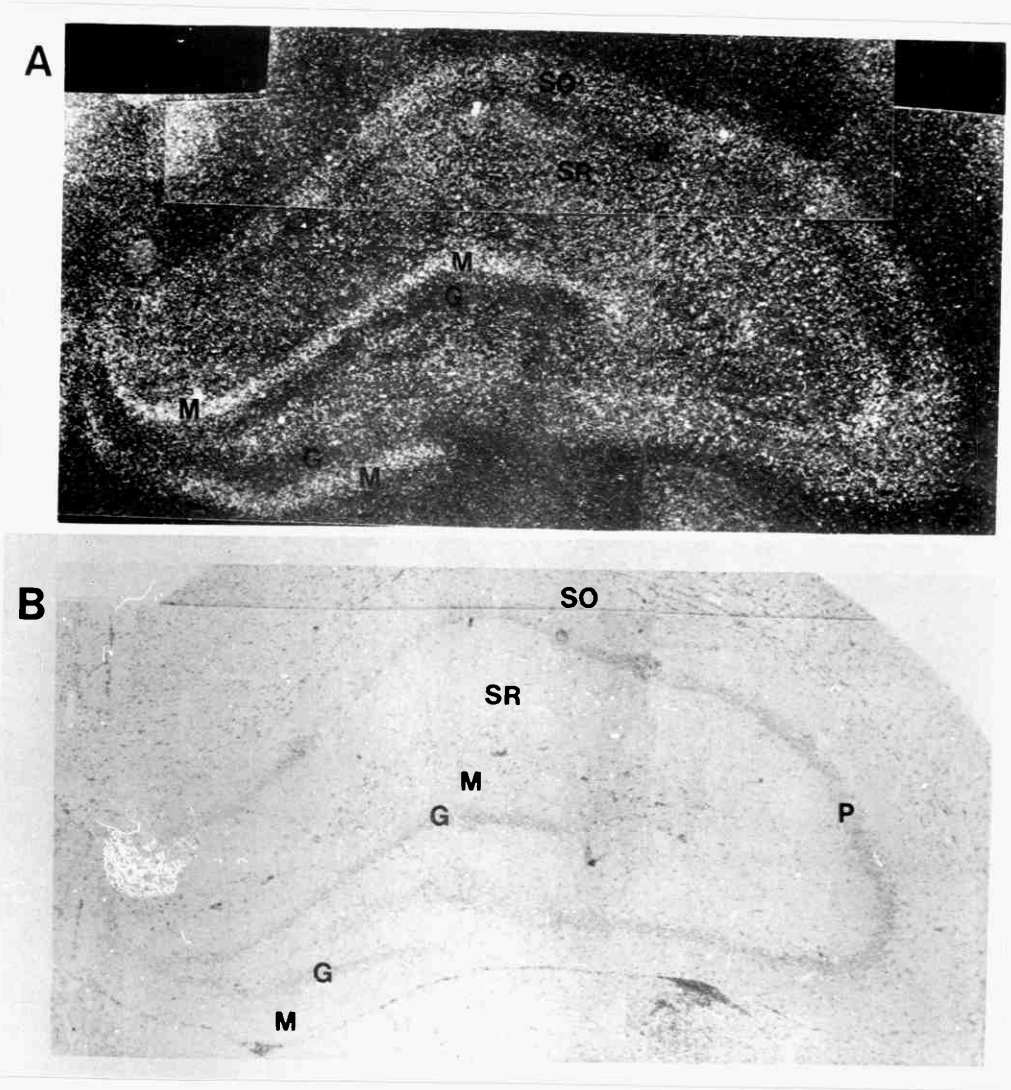


Fig. 6.5 Autoradiographic localization of ^{3}H-β-BuTx binding sites in rat hippocampal sections

Whole brain was used for the preparation and labelling of cryostat sections by procedures described in Methods. Photographs are shown of samples incubated with ^{3}H-β-BuTx alone which were viewed in the microscope under dark (A) and bright (B) field optics. Sections (B) stained with pyronin Y/methyl green solutions show the pyramidal cell (P), granule cell (G), molecular layer (M), stratum oriens (SO) and stratum radiatum (SR).

Table 6-3 Relative distributions of grains on hippocampal sections labelled with ^{3}H-β-BuTx and ^{125}I-β-BuTx

Areas	Grain densities* / 100 μm²			
	^{3}H-β-BuTx		^{125}I-β-BuTx	
	Test	Control	Test	Control
Molecular layer	16.5 (± 1.5)	4.7 (± 0.9)	37.9 (± 2.2)	13.6 (± 1.7)
Granule cell layer	3.7 (± 0.7)	3.3 (± 0.9)	14.4 (± 1.3)	13.9 (± 1.3)
Stratum oriens	10.7 (± 1.6)	3.8 (± 0.9)	21.9 (± 2.5)	13.6 (± 1.7)
Stratum radiatum	10.0 (± 1.1)	3.0 (± 0.9)	21.8 (± 2.9)	12.9 (± 1.2)
Pyramidal cell layer	5.6 (± 0.8)	4.1 (± 0.7)	16.3 (± 1.5)	13.2 (± 1.4)

* Mean values (± S.D) shown were obtained from counting a minimum of 12 areas in each layer specified, from the same section of whole brain.

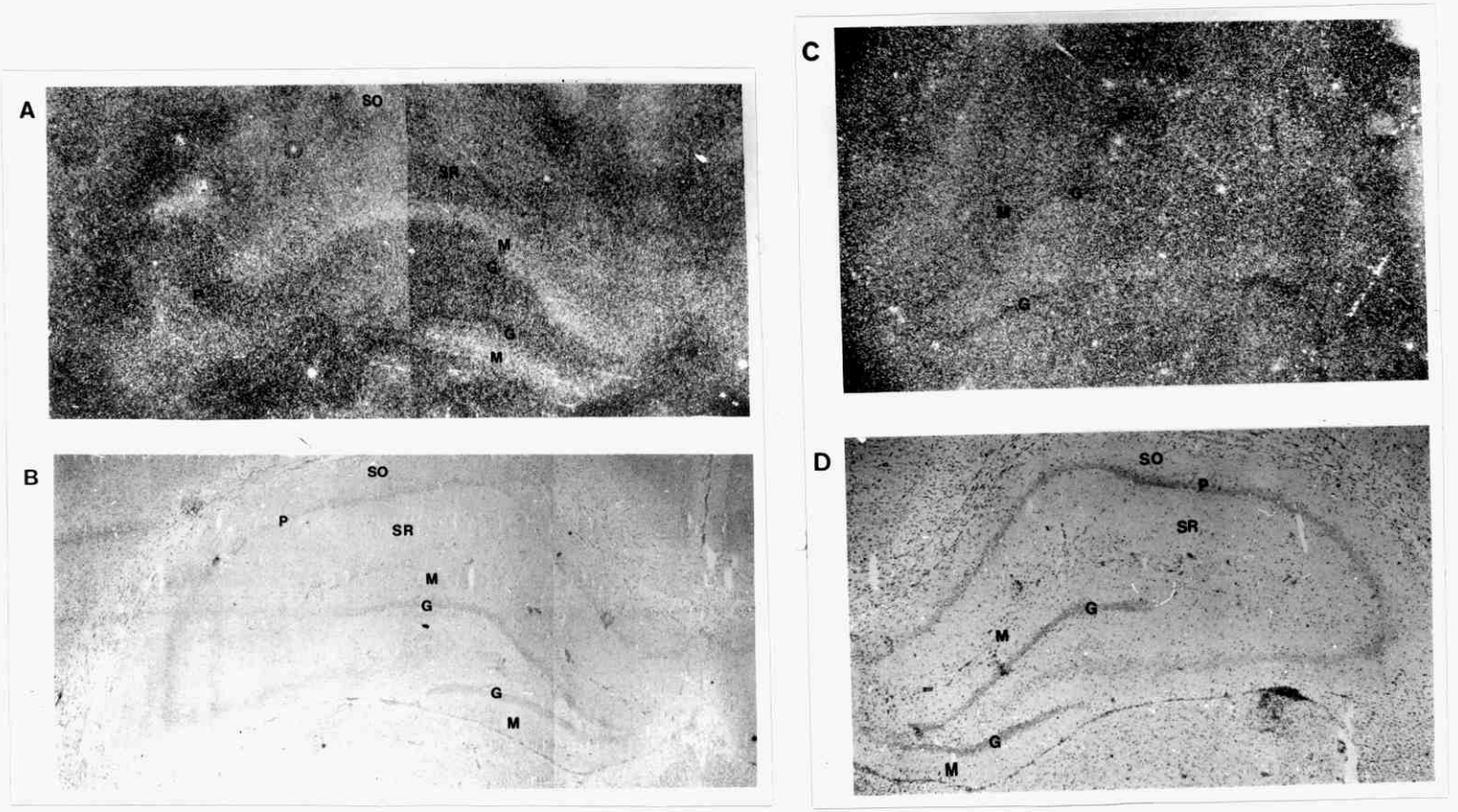


Fig. 6.6 Light-microscope autoradiography of ^{125}I-β-BuTx binding sites in rat hippocampus

Whole brain was used for the preparation and labelling of cryostat sections by procedures described in Methods. Photographs are shown of samples incubated with ^{125}I-β-BuTx in presence (C & D) or absence (A & B) of 100-fold molar excess of unlabelled DTX, viewed under dark (A & C) and bright (B & D) fields. Sections (B & D) stained with pyronin Y/ methyl green show the pyramidal cell (P), granule cell (G), molecular layer (M), stratum radiatum (SR) and stratum oriens (SO).

^{125}I-β-BuTx derivative (Fig. 6.6A). Again, the molecular layer contained the highest level of ^{125}I-β-BuTx binding compared to the stratum oriens and stratum radiatum. However, no clear distinction was observed between the other layers containing the cell-bodies (granule cell-layer and pyramidal cell-layer) and the stratum oriens and stratum radiatum as seen with ^{3}H-β-BuTx since the level of non-specific binding of ^{125}I-β-BuTx to these sections was rather high, similar to that observed on cerebellar slices (Fig. 6.6A; B; Table 6-3).

6.4.1.2 Localization of β-BuTx binding sites on synaptosomes

For the ultrastructural localization of the binding component on rat cerebrocortical synaptosomes, the latter were labelled with 5 nM ^{3}H-β-BuTx and treated for electron microscope autoradiography as described in Section 6.3.2. As the biochemical characterization of the binding component has been extensively described in Chapter 3, its precise localization on the synaptosomes was, therefore, of great interest.

Despite the relatively high specific radioactivity of the ^{3}H-β-BuTx used, very few grains were obtained after 2 months exposure of autoradiograms, and thus longer exposure was found to be necessary to obtain a significant number of grains. After 4 months exposure, the autoradiograms showed that specific of ^{3}H-β-BuTx was observable to synaptosomes in test sample (Fig. 6.7A; B); grains were visually absent from the control samples incubated in the presence of 100-fold molar excess unlabelled β-BuTx (Fig. 6.7C). The saturable binding observed is consistent with

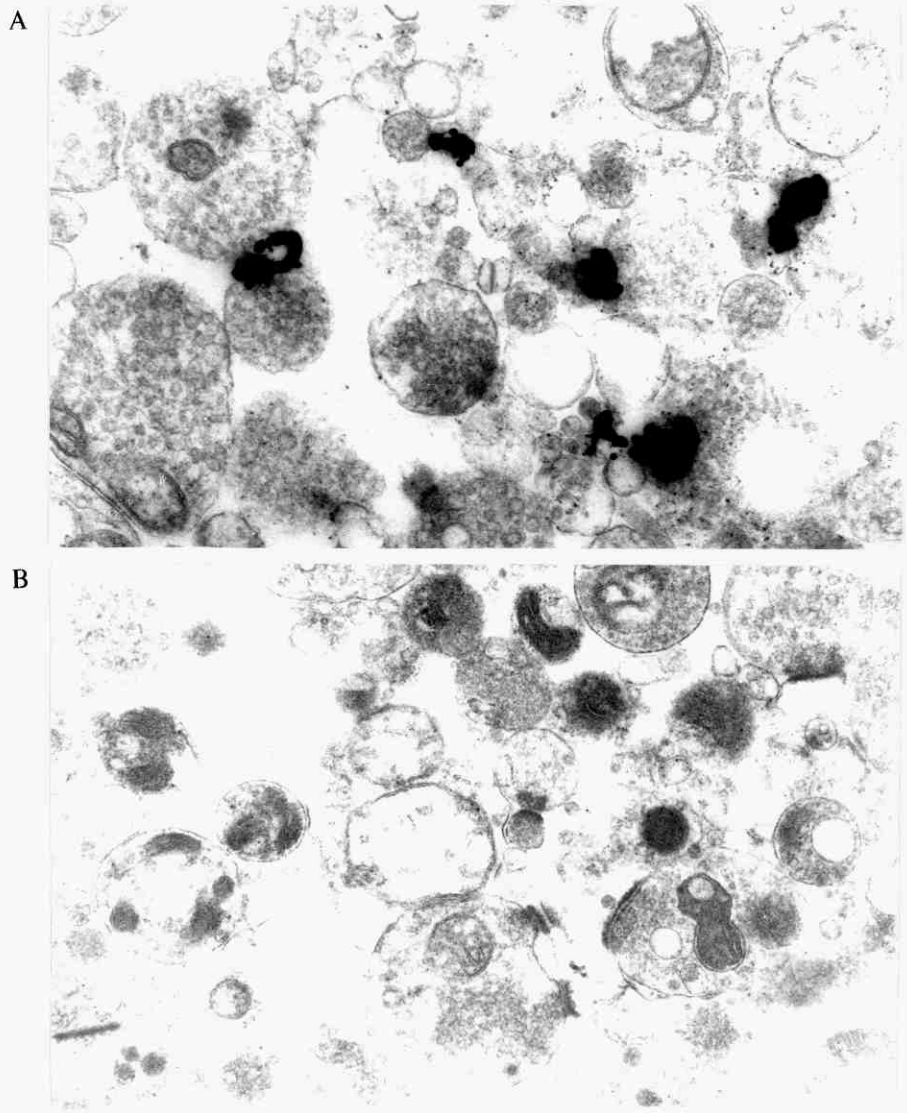


Fig. 6.7 Electron microscope auradiographic localization of ^{3}H-β-BuTx binding component in rat cerebrocortical synaptosomes

Density centrifugation in Ficoll was used to purify the synaptosomes; they were suspended (2.5 mg protein/ml) in Kreb's/polylysine/serum albumin medium and labelled with 5 nM ^{3}H-β-BuTx with (B) and without (A) the inclusion of 100-fold molar excess β-BuTx. After rapid washing, the synaptosomes were fixed with glutaraldehyde, treated with OsO_4, dehydrated in ethanol and embedded in Spurr's resin. Sections (∿ 600 A) were cut on a LKB ultramicrotome; a monolayer of Ilford L4 emulsion was applied these samples by the dipping technique. After 4 months' exposure, the specimens were developed in Kodak D-19 developer, stained with 12% uranyl acetate examined in a Hitachi electron microscope and photographed (A, X 16642 ; B, X 16642).

the more detailed kinetic studies on synaptosomes (Chapter 3). However, it was difficult to ascertain the exact sites of binding, as the synaptic junction was much smaller than the silver grains formed from the emulsion used. In most cases, the silver grains were present in the vicinity of synaptic junctions (Fig.6.7B); the latter are characterised by the presence of synaptic vesicles in the synaptosomes and thickening of the plasma membrane on the postsynaptic side. Attempts to increase the resolution were made by developing the autoradiograms with phenidone-ascorbic acid developer (Lettre and Paweletz ,1966), rather than using Kodak D-19 developer. Using the former, the silver grains formed were visualized as black hexagonal spots, much smaller but greater in number than the silver grains formed with D- 19 developer. Autoradiograms processed in this manner also showed specific binding in the vicinity of the synaptic junction. However, despite this modification it was still not possible to determine the exact location of the binding sites on the synaptosomal membranes. Investigation on the relative distribution of ^{3}H-β-BuTx binding to lysed synaptosomes established by sub-cellular fractionation of the latter, using the floatation-sedimentation-gradient procedure (Jones and Matus, 1974) described in Methods, showed significant enrichment of toxin binding activity to synaptic plasma membranes. The values obtained for the specific binding of ^{3}H-β-BuTx to the synaptic plasma membranes, the mitochondrial and myelin fractions separated in this manner were 310, 102 and 0 fmol/mg prrotein, respectively. This demonstration of 2-3 fold enrichment (relative to intact synaptosomes) together with the high

content of sites recovered therein, indicates that the binding component is located on the neuronal membrane, as contamination of this isolated fraction with other membrane material has been shown to be minimal (Jones and Matus, 1974). This suggestion is consistent with the finding on the autoradiographic localisation of ^{3}H-β-BuTx binding to synaptosomes (see above), where the silver grains were localised in the vicinity of the synaptic junction.

A similar pattern of labelling was also observed when ^{125}I-β-BuTx was used on synaptosomes ; however, as expected, the level of non-specific binding indicated by the number of silver grains present in control samples were relatively high.

6.4.2 Localization of ^{125}I-DTX binding sites in brain sections and on synaptosomes

6.4.2.1. Distribution of ^{125}I-DTX binding sites on cerebellar and hippocampal sections

As in the case of ^{3}H-β-BuTx, the optimal conditions for ^{125}I-DTX binding were initially investigated before any autoradiography was carried out. The time course of ^{125}I-DTX binding to slide-mounted tissue sections was examined as described previously in Methods. It was shown that binding of 2.5nM ^{125}I-DTX reached a maximum after 20 min.

incubation (Fig. 6.8). Using this concentration and an incubation time of 30 min., cerebellar and hippocampal tissue sections were labelled with ^{125}I-DTX before being processed for autoradiography as described in Methods.

After exposure for varying periods of time, the autoradiograms were developed in Kodak D-19 and cell layers visualized after staining with pyronin Y/methyl green. The distribution of the silver grains was visualized under dark-field; grains were quantified. Using cerebellar sections, the autoradiograms of test samples showed a localized pattern of labelling with grains present in discrete areas (Fig. 6.9). The synapse-rich molecular layer contained the highest grain density; this result was analogous to that obtained for the distribution of β-BuTx binding on similar sections. Control samples, which had been treated with a 100-fold molar excess of unlabelled toxin, showed low levels of grain density in all areas, indicating that the binding observed was saturable (Fig. 6.9). When grain densities in control samples were quantified in areas representing the molecular layer, granule cell layer and white matter (Table 6-4), the level of binding observed was about 20% of test samples. Furthermore, a uniform distribution of silver grains throughout the control samples were observed.

When hippocampal sections were labelled with ^{125}I-DTX, saturable binding was initially apparent from biochemical studies; control samples had a much lower radioactive content; the time course of ^{125}I-DTX binding to hippocampal sections was similar to that obtained on cerebellar sections, reaching a plateau at 20 min. The labelling pattern obtained for ^{125}I-DTX showed discrete areas of toxin

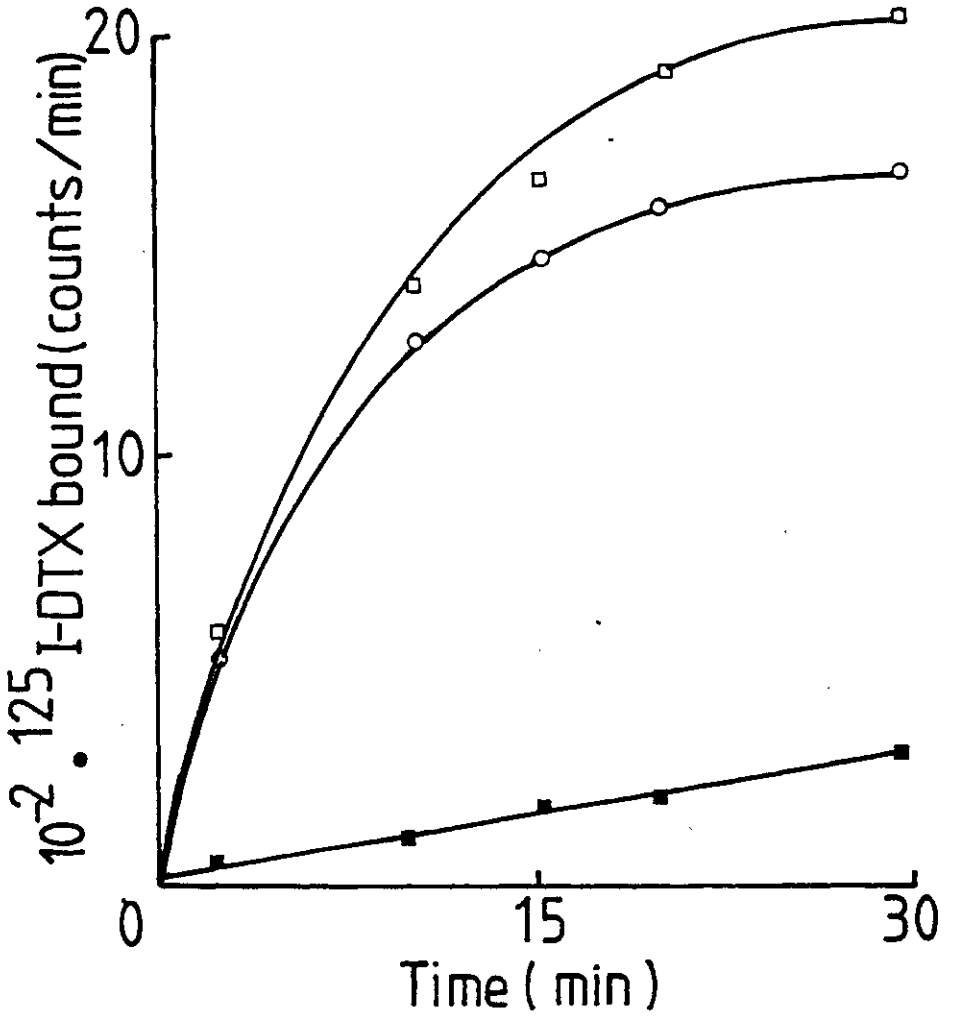


Fig. 6.8 Time-course of ^{125}I-DTX binding to rat cerebellar sections

Cryostat sections (10 µm) were preincubated with and without 250 nM unlabelled DTX for 5 min. at 22°C prior to addition of 2.5 nM ^{125}I-DTX. The amounts of total and non-specific binding of ^{125}I-DTX at the times indicated were measured as described in Fig. 6.2. The values obtained for non-specific (■) binding were subtracted from the total (□) to give the amount of specifically bound (○) ^{125}I-DTX. Values are the means of triplicate determinations.

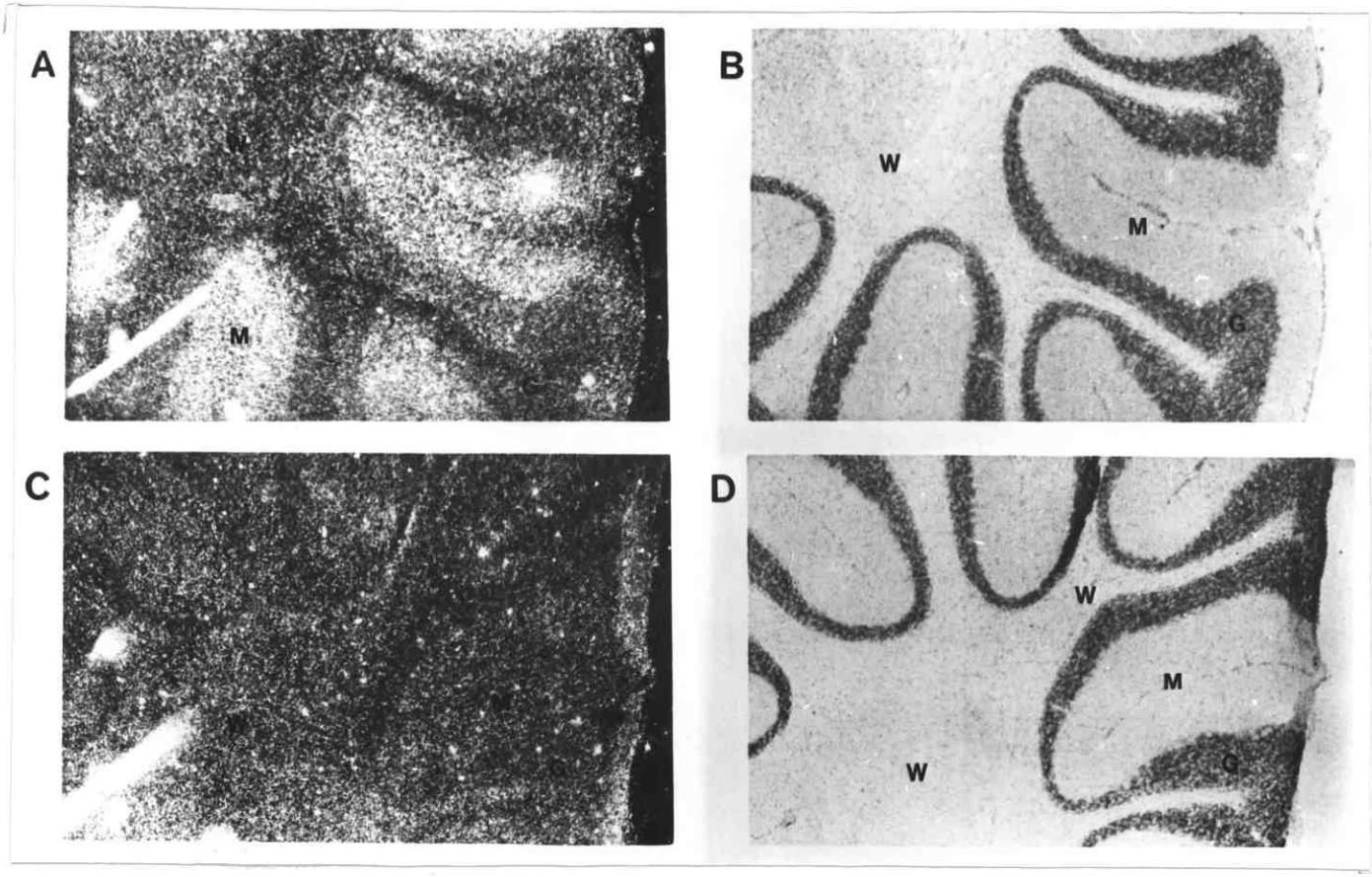


Fig. 6.9 Light-microscope autoradiography of ^{125}I-DTX binding sites in rat cerebellum

Rat cerebellum sections were labelled with 2.5 nM ^{125}I-DTX and processed for light-microscope autoradiography as described in Methods. Following 3 weeks exposure, specimen were developed in Kodak D-19 developer, and stained with pyronin/methyl green. Autoradiograms were visualized on a light microscope under dark (A & C) field and bright (B & D) field. A & B are test samples, C & D are control samples in the presence of 100-fold molar excess of unlabelled DTX.

Table 6-4 Relative distributions of grains on cerebellar (A) and hippocampal (B) sections labelled with ^{125}I-DTX

A

Areas	^{125}I-DTX Grain densities/100 μm²	
	Test	Control
Molecular layer	48.2 (± 13.5)	9.9 (± 1.5)
Granule layer	9.9 (± 1.7)	10.9 (± 1.9)
White matter	9.3 (± 0.9)	9.1 (± 1.7)

B

Areas	^{125}I-DTX Grain densities/100 μm²	
	Test	Control
Molecular layer	54.7 (± 5.9)	15.2 (± 1.3)
Granule layer	12.2 (± 2.2)	12.6 (± 1.6)
Stratum oriens	52.1 (± 2.2)	13.0 (± 1.8)
Stratum radiatum	49.7 (± 7.6)	14.7 (± 3.2)
Pyramidal cell layer	14.2 (± 3.5)	14.4 (± 1.8)

A the mean values (± S.D) were obtained as previously indicated in Table 6-2. The extent of non-specific binding observed in control samples (∿ 20%) was in accordance with the results obtained by γ-counting (Fig. 6.9)

B the mean values (± S.D) were obtained as previously described in Table 6-3

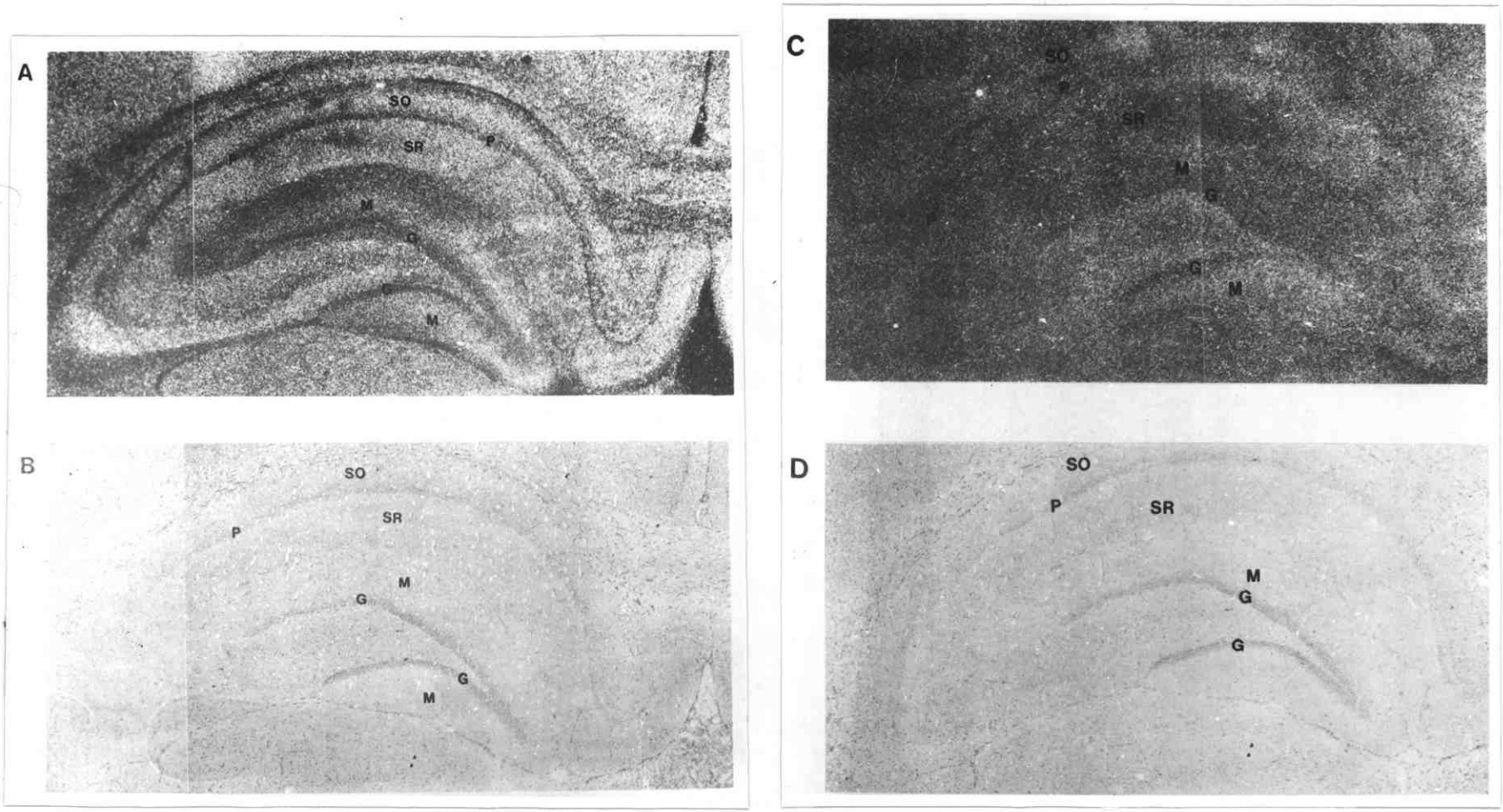


Fig. 6.10 Light-microscope autoradiography of ^{125}I-DTX binding sites in rat hippocampal

Whole brain was used for the preparation and labelling of cryostat sections by procedures described in Methods. Photographs are shown of samples incubated with ^{125}I-DTX in presence (C & D) or absence (A & B) of 100-fold molar excess of unlabelled DTX, viewed under dark (A & C) and bright (B & D) fields. Sections (B & D) stained with pyronin Y/methyl green show the pyramidal cell (P), granule cell (G), molecular layer (M), stratum radiatum (SR) and stratum oriens (SO).

binding. Examination of the test samples, after differentiation of the cell layers by staining with pyronin Y/methyl green, demonstrated that the majority of the silver grains were deposited in the terminal-rich areas, with a lower density of grains found in regions containing cell-bodies including the granule cell layer and the pyramidal cell layer (Fig. 6.10). When the distribution of the silver grains were quantified on representative areas of the section, the molecular layer, stratum oriens and stratum radiatum contained high grain densities consistent with their high content of synapses (Table 6-4B). This result showed that DTX is less selective than β-BuTx, which has been shown to bind preferentially to the molecular layer of the hippocampus. Values obtained for the control samples were similar for all observed (Table 6-4B) and these represented less than 20% of those in regions of higher grain density. It was also observed that the cerebral cortex showed a fairly uniform high-density grain distribution similar to that of the stratum oriens and stratum radiatum. It is noteworthy that the part of the hippocampus exhibiting the highest content of binding sites, especially the stratum radiatum, has been used for electrophysiological measurements; the latter showed the spontaneous excitatory activity which can be blocked by TTX indicating its synaptic nature (Chapter 4).

6.4.2.2 Localization of ^{125}I-DTX binding sites on synaptosomes

As in the case of ^{3}H-β-BuTx, the ultrastructural localization of ^{125}I-DTX binding sites on synaptosomes was carried out after it had been established that the toxin exhibited saturable binding with high affinity (K_d = 0.5 nM)

to a single-class of non-interacting sites on isolated nerve-terminals (Chapter 5). Using synaptosomes purified by Ficoll-sucrose gradient centrifugation, the binding of ^{125}I-DTX was performed as described in Section 5.3. The synaptosomal pellets were processed for electron-microscope autoradiography as described in Methods.

After exposure of autoradiograms for 9 weeks, they were developed with Kodak D-19 developer and visualized in a Hitachi-601 electron microscope. The majority of the grains were found within the vicinity of synaptic junctions on the synaptosomes (Fig. 6.11). This was in accord with the electrophysiological studies of DTX in the CNS, which indicated its effect on neurotransmitter release. The affinity and maximal binding of DTX observed in either intact or hypotonically lysed synaptosomes (Chapter 5) were very similar, indicating that the majority of the binding occurred to the plasma membrane of the synaptosomes. In control samples, in the presence of a 100-fold molar excess of unlabelled DTX, few grains were observed (Fig. 6.11) The electron microscope autoradiograms also showed higher grain densities than those of ^{3}H-β-BuTx binding (Fig. 6. 7); this is consistent with the iodinated DTX having a higher specific activity and that it binds to more sites (∿ 4 times) than ^{3}H-β-BuTx on synaptosomal membranes. In addition. a significant number of silver grains was observed after only 9 weeks with ^{125}I-DTX. However, despite this demonstration of ^{125}I-DTX binding to synaptosomes, it is not possible to identify their precise ultrastructural location on these membranes.

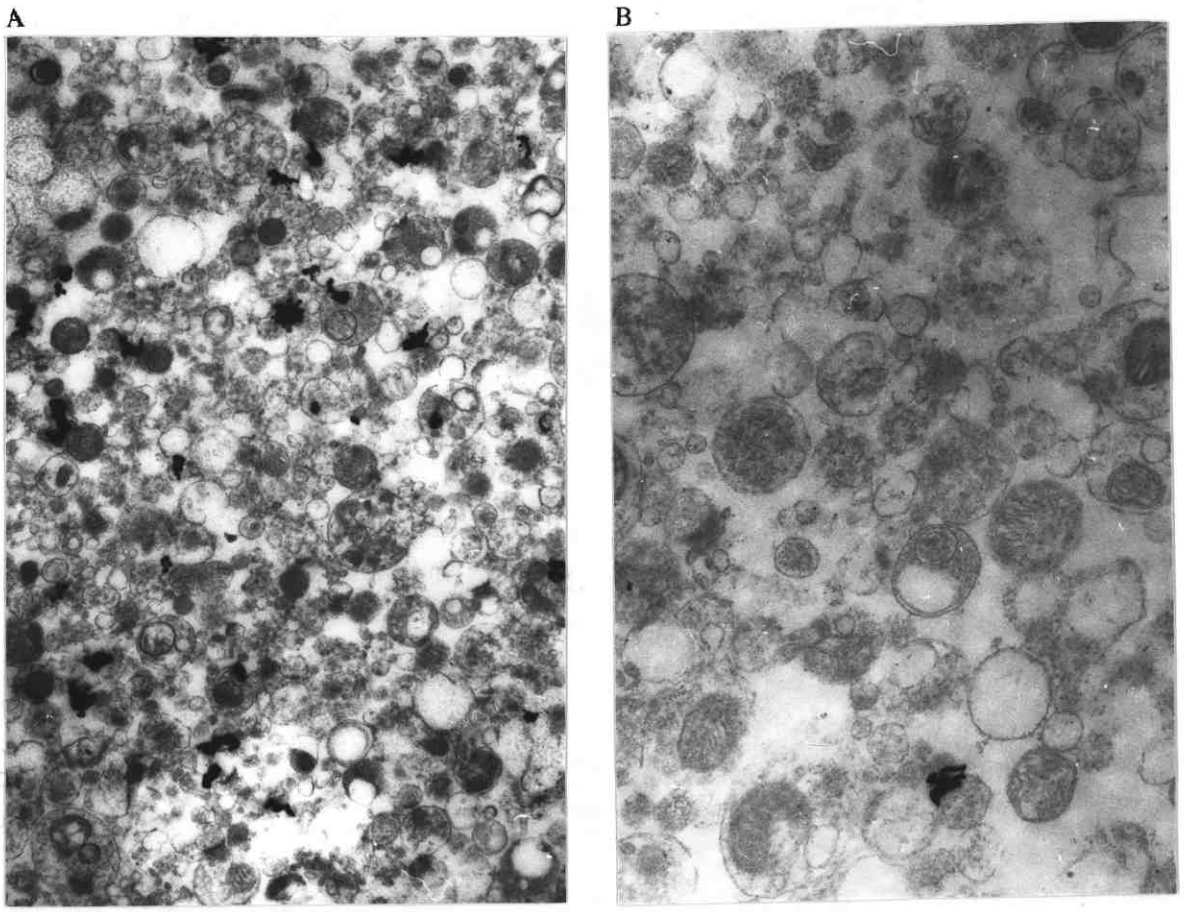


Fig. 6.11 Electron microscope autoradiographic localization of the ^{125}I-DTX binding component on rat cerebrocortical synaptosomes

After 84 days exposure, specimens were developed with Kodak D-19 developer, stained with 12% Uranyl acetate examined on a Hitachi electron microscope. A. Test samples B. Control in the presence of 100-fold molar excess unlabelled DTX (A 6594 X ; B 11304 X).

6.5 DISCUSSION

The present study demonstrate that the binding sites for β-BuTx and DTX are distributed throughout the CNS in well-defined anatomical layers. Using the light-microscope autoradiographic techniques described by Young and Kuhar (1979), which have been successfully used for the binding of other ligands to their receptor sites (for example, Young and Kuhar,1979; 1981; Warmsley *et al.*,1982; Monaghan and Cotman,1982; Palacios *et al.*,1981), it has been shown that β-BuTx and DTX bind to discrete areas in rat-cerebellar and hippocampal sections.

The cerebellar cortex of rat brain displays a uniform and simple structure (Fig. 6.3B, D). It consists simply of a highly folded sheet of cells and their processes, everywhere arranged into the same three layers; the molecular layer, granule cell layer, and white matter (Fig. 6.3, B, D). Within the centre of each folium lies a thin lamella of white matter, composed of the the myelinated afferent and efferent fibres connecting the cortex with their centres. Details of the arrangement and interconnection of neurons in three layers have been clearly described by Palay and Chan-Palay (1974). However, it can be generalised that the molecular layer consists mainly of nerve endings of various types of cells including the granule cells in the granular layer, Purkinje cells which form a layer one-cell thick lying on the upper margin of the granular layer, and the basket-cells which are located above the Purkinje-cell bodies. The granule cell layer consists mainly of the cell bodies of granule cells and has

very few synapses when compared to the molecular layer. These cells send their axons up into the molecular layer and these fibres make up the bulk of the molecular layer. The Purkinje cell bodies form a layer one-cell thick lying on the upper margin of the granular layer. They also extend an elaborate dendritic arborization into the molecular layer. Just above the Purkinje cell bodies is a cell of intermediate size - the basket-cell which also radiates dendrites through the molecular layer of the cerebellum.

In contrast, the hippocampal formation of rat-brain is more complex than that of the cerebellum; it consists of more cell layers and has more complex neuronal inter-connecting pathways. Fig. 6.12 shows a schematic drawing of a cross-section of the rat hippocampal formation and some of the neuronal interconnection between the layers. The main input is via the perforant path (PP) from area entorhinal to the dentate and hippocampal molecular layers, and this may receive information from essentially all parts of the cortex (van Hoesen *et al.*,1972). A large proportion of the synapses in the dentate molecular layer arise from the perforant path terminals, and from the terminals of the mossy fibres of the granular cells in the granule cell layer (Nafstad,1967; Matthews *et al.*,1976). These synapses are excitatory (Lomo, 1971a) and were among the first synapses described to show potentiation in response to stimulation (Lomo,1971b; Bliss and Lomo.1973). The stratum oriens and stratum radiatum contain synapses arising mainly from the axons of the pyra-midal cells (in the pyramidal cell layer) of the CA3 region. Again, these pyramidal cell projections are all excitatory (Andersen *et al.*,1971). These projections are arranged in

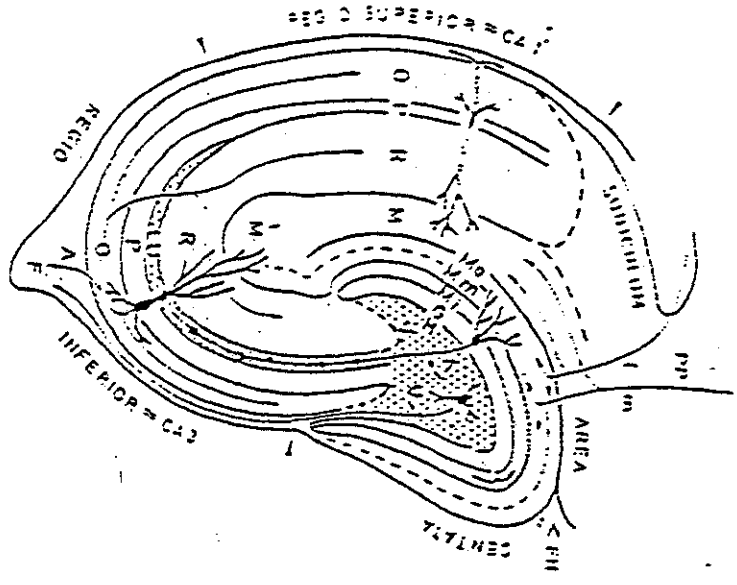


Fig. 6.12

Schematic drawing of a cross-section of the rat hippocampal formation. The systems of excitatory nerve terminals are arranged in *laminae* parallel to the curved cortical surface and the pyramidal (P) and granular (G) cell layers. The axons giving rise to these terminals run in *lamellae* perpendicular to the laminae and parallel to the plane of the section shown, but there is also a considerable *longitudinal spread* of axons perpendicular to the lamellae.

The chief input of the region is the perforant path (PP) from the area entorhinalis to the fascia dentata and CA3. It comprises a medial (m) and lateral (l) part, exciting the middle (Mm) and outer (Mo) parts of the granular cell dendrites, respectively. This laminated projection extends to the molecular layer (M) of CA3 and is mostly ipsilateral. A separate set of cells in area entorhinalis sends PP fibres (temporo–ammonic fibres) bilaterally to the molecular layer of CA1.

The axons of the granular cells, the mossy fibres, give rise to characteristic giant boutons exciting dendrites of cells in hilus fasciae dentatae (H,CA4) and in stratum lucidum (LU) of hippocampus CA3 (coarse stippling). These pyramidal cells have axons synapsing with dendrites in the finely stippled area in hippocampus and area dentata ipsilaterally and contralaterally. Ipsilateral axons from CA3 to the superficial part of stratum radiatum (R) of CA1 are known as Schaffer collaterals. For references, see text.

Symbols: A, alveus; F, fimbria; G, stratum granulare of area dentata; H, hilus fasciae dentatae; FH fissura hippocampi; LU, stratum lucidum (mossy fibre layer); M, stratum lacunosum-moleculare of hippocampus; Mo, Mm, and Mi, outer, middle, and inner parts of stratum moleculare of area dentata; O, P, and R, strata oriens, pyramidale, and radiatum of hippocampus; PP, perforant path from area entorhinalis with lateral (l) and medial (m) parts; ▼, borders between cortical sub-fields

(Adapted from Storm-Mathisen, 1981)

a lamellar fashion resulting in the preservation of the three-synapse chain - perforant path-granular cell, mossy fibre-CA3 pyramid, CA3 pyramid axon-CA1 pyramid - in a 400 µm transverse section of the hippocampus (Skrede and Westgaard,1971) which was used for electrophysiological analysis (Chapter 4; Docherty *et al.*,1983).

Prior to the autoradiographic experiments, the time course of binding of the radiolabelled toxins, using a known concentration, was determined on the slide-mounted tissue sections. The binding observed for both radiolabelled toxins was specific as it can be blocked on each occasion by excess of the respective unlabelled toxin. Autoradiograms prepared from these labelled tissue sections showed clearly the distribution of the binding of these neurotoxins in the CNS. As their binding kinetics have been extensively described (Chapters 3 and 5), their localization on cerebellar and hippocampal sections, and on cerebrocortical synaptosomes is a considerable step forward in determining their sites of action; to date, no presynaptically-acting neurotoxin isolated from snake venoms, has been localized.

The distribution of ^{3}H-β-BuTx binding sites in the CNS of rat is anatomically selective. In cortical structures (cerebellar cortex and hippocampus), the density of β-BuTx binding sites exhibits variations among layers as well as among regions. The cerebellum exhibited a distinctive pattern of silver grain distribution (Fig. 6.3). The molecular layer which is rich in synapses (see above), had the highest level of silver grains, which is consistent with β-BuTx affecting neurotransmitter release. The granule cell

layer, containing mainly cell-bodies, and the white matter region, had negligible levels of silver grains; similarly, in the control samples which displayed the non-specific binding, no anatomical specificity was observed. Comparable binding was also obtained in the hippocampus labelled with ^{125}I-β-BuTx; silver grains were most pronounced in the molecular layer; moderate levels on the stratum oriens and stratum radiatum, and background level on the pyramidal cell layer and granule cell layer (Fig. 6.4). Collectively, these results showed that the binding sites of β-BuTx are localized on synapse-rich regions of the cerebellum and hippocampus of rat brain. Interestingly, these molecular layers of the cerebellum (Levi *et al.*,1982) and the hippocampus (Storm-Mathisen,1981; White *et al.*,1979) have been shown to consist predominantly of glutamatergic neurons, suggesting that β-BuTx may be binding selectively to amino-acid nerve terminals in rat brain.

Given the selective distribution of β-BuTx, it was predicted that certain regions would be more sensitive to the inhibitory action of β-BuTx. This was supported by the electrophysiological analysis carried out on rat hippocampal sections (Halliwell and Dolly,1982a; 1982b) where β-BuTx was shown to affect preferentially the neuronal excitability in terminal regions of the hippocampal fibre pathway (Halliwell and Dolly,1982a; 1982b). Furthermore, ^{3}H-β-BuTx used in these autoradiographic studies, has been shown to inhibit neurotransmission in the CNS with equal potency to native toxin (Chapter 3). From competition experiments (Chapter 3), it was found that the labelled neurotoxin had a similar affinity to native toxin; thus, the regional distribution

of ^{3}H-β-BuTx binding might be expected to be responsible for the high potency of the toxin when administered intraventricularly by stereotaxic injection (Chapters 2 and Chapter 3).

Ultrastructural localization of ^{3}H-β-BuTx binding indicates that the binding sites are located mainly on the synaptosomal plasma membrane of the synaptosomes; grains were visually absent from the control samples incubated in the presence of native toxin (Fig. 6.7). Despite the relatively high specific radioactivity of ^{3}H-β-BuTx, 4 months exposure was necessary for appearance of significant numbers of grains. Thus attempts were made to increase the sensitivity of the detection of this apparently low density of toxin binding sites and thereby facilitate a statistical analysis of grain distribution with respect to the plasma membrane. β-BuTx was labelled with ^{125}I-iodine to a specific radioactivity of 500 Ci/mmol (A.Pelchen and J.O.Dolly, unpublished observation) by a modification of the Chloramine-T procedure that employed milder conditions and has been successfully used for labelling botulinum neurotoxin (Williams *et al.*,1983). Physical properties of the ^{125}I-β-BuTx obtained by this method, and its binding to rat cerebrocortical synaptosomes have been discussed in comparison to ^{3}H-β-BuTx binding in Chapter 3.

Specific binding sites for ^{125}I-β-BuTx were observed in rat cerebellar and hippocampal sections, the localization pattern was similar to that obtained with ^{3}H-β-BuTx. The molecular layers of the cerebellum and the hippocampus were the prime target of ^{125}I-β-BuTx binding, analogous to ^{3}H-β-BuTx. Ultrastructural localization of

the binding sites for the radioiodinated derivatives of β-BuTx on synaptosomes indicates that they reside mainly on the synaptosomal membrane, similar to ^{3}H-β-BuTx. During the course of its study, ultrastructural localization of β-BuTx labelled with ^{125}I-iodine using the chloramine-T method as described previously by Oberg and Kelly (1976b) was reported on *Torpedo* electric organ synaptosomes (Esquerda *et al.*,1982). Since the kinetics of the radioiodinated β-BuTx binding to these peripheral nerve-terminals have not been characterized, it was difficult to ascertain whether the autoradiographic localization of its binding sites in these tissues was a result of high or low affinity binding. Furthermore, our results using radioiodinated β-BuTx labelled in a similar manner to that of Esquerda *et al.*,(1982) in this laboratory showed that the labelled-derivative was relatively non-toxic (MLD of 0.4 µg/g body wt.) and binds to rat brain synaptosomes with low affinity (B_{max} = 1.8 pmol/mg protein; K_d = 2 X 10^{-8} M) (A.Pelchen and J.O.Dolly, unpublished observations). Thus, it is possible that the observed localization on *Torpedo* electric organ synaptosomes is due to low affinity binding sites. It is also noteworthy that the iodinated β-BuTx derivative was reported to have an exceptionally low specific activity of only 5.8 Ci/mol and yet grains were visualized after only 6 days exposure of the autoradiograms; this is consistent with a high content of sites, probably of low affinity in view of the high concentration of labelled toxin used.

Autoradiographic studies of DTX on cerebellar and hippocampal sections revealed comparable results to those obtained with β-BuTx. ^{125}I-DTX binding sites in the rat CNS were

also anatomically selective with grain density variations among the layers and regions. The molecular layers of the cerebellum and the hippocampus were the main target areas for ^{125}I-DTX binding. In addition, the stratum oriens and stratum radiatum of the hippocampus were equally labelled by DTX. These regions contained high grain densities, similar to those obtained for the molecular layer of the hippocampus (Table 6-4B). As mentioned earlier, axons originating from various sources of the neuronal pathways terminate in these areas making them highly rich in synapses. Thus, these results accord with DTX affecting neurotransmitter release in these regions as has been measured electrophysiologically (Chapter 4). In view of its high potency when administered intraventricularly in rat brain (Chapter 4), the distribution of labelling observed could possibly account for the neurotoxicity. Using electron microscope autoradiography, the binding sites, which have been characterized by kinetic experiments (Chapter 5), have been localized; they reside mainly on the synaptic plasma membrane and in the vicinity of synaptic junctions.

Collectively, these autoradiographic results indicate a selective distribution of the binding sites of β-BuTx and DTX in terminal-rich regions in the CNS. Ultrastructural localization demonstrates that such binding occurs on the synaptic plasma membranes, and most probably on the presynaptic side. It would appear, however, that the binding of DTX to these regions is less selective than that of β-BuTx; this is more obvious in the hippocampus (Fig. 6.4; 6.10) where in addition to the molecular layer, high density of binding sites were observed in the stratum oriens and stratum

radiatum, indicating that DTX binds less selectively than β-BuTx in these regions. As both toxins contain high densities of binding sites in the molecular layer, it is highly possible that they share at least some common binding sites in these regions. However, at this stage, it is difficult to ascertain whether the antagonism observed between DTX and β-BuTx is a result of a direct or indirect interaction of the binding sites with the toxins. Furthermore, it is difficult to assess whether these neurotoxins bind to sub-population of synapses in the CNS. However, investigations of the effects of β-BuTx on transmitter release from cerebrocortical synaptosomes (Wernicke *et al.*, 1975; Sen and Cooper,1978; Tse *et al.*,1980) and its degenerative action on neurons in muscle, brain and retina (Hirokawa,1978; Hanley and Emson,1979; Rehm *et al.*,1982) indicate that the toxin affects most of the synapses tested. However, it is noteworthy that the intrinsic phospholipase activity of the toxin preparations used almost certainly contributed to some degree to these effects, a complication which is absent from the localization studies of β-BuTx in this chapter. In addition, at the periphery, β-BuTx affects only nicotinic cholinergic synapses (Kato *et al.*,1977) whilst the CNS experiments, to date, have been restricted to measurement of its effects in regions of olfactory cortex and hippocampus (Halliwell and Dolly,1982a; 1982b) where glutamate and aspartate are predominant putative neurotransmitters. Therefore, with the limited information available, it can only be stated at this stage that the demonstrated binding of β-BuTx accords with the known distribution of amino-acid releasing terminals in cerebellum and hippocampus

(Levi *et al.*,1982; Storm-Mathisen,1981) and the toxin's inhibitory actions on transmitter release in the latter (Halliwell and Dolly,1982a, b). In contrast, DTX has been shown to cause facilitation of neurotransmitter release at these amino acid releasing terminals in hippocampus (Docherty *et al.*,1983); this is in accordance with the demonstrated localization in the CA1, CA2 and CA3 regions in the hippocampus. No transmitter selectivity has been reported for DTX either at peripheral or central synapses.

In summary, the localization of the binding sites of ^{3}H-β-BuTx and ^{125}I-DTX in synapse-rich regions of rat brain gives support to the postulation that these sites may be involved either directly or indirectly, with the process of neurotransmitter release and thus, could be useful probes for the study of the latter.

CHAPTER 7

GENERAL DISCUSSION

7.1 INTRODUCTORY REMARKS

β-BuTx and DTX have been shown to be extremely neurotoxic when administered intraventricularly into rat brain (Chapters 2 and 4, respectively). The high potency of these neurotoxins, together with their observed electrophysiological actions on rat hippocampal slices (Halliwell and Dolly,1982a, 1982b; Docherty *et al.*,1983; Chapter 2), strongly suggests that they interact specifically with functional macromolecules on the presynaptic plasma membrane of nerve terminals in the CNS. This thesis describes the binding properties and specificities of proteinaceous binding components for both β-BuTx and DTX on synaptosomal membrane (Chapters 3 and 5). The kinetic studies were facilitated by radiolabelling these neurotoxins to relatively high specific radioactivities with radioisotopes such as tritium and ^{125}I-iodine without appreciable loss of biological activity (Chapters 3 and 5, respectively). With the availability of these radiolabelled derivatives, the localization of the binding sites on rat cerebellar and hippocampal sections has been shown using light-microscope autoradiography. Ultrastructural localization of ^{3}H-β-BuTx and ^{125}I-DTX on rat cerebrocortical synaptosomes using electron-microscope autoradiography showed that the binding of these toxins is in close association with the synaptic junction of the nerve terminal (Chapter 6).

7.2 DI-^{3}H-PROPIONYLATED β-BUTX AND ITS USEFULNESS AS A PROBE

In this study, β-BuTx has been successfully radiolabelled with N-succinimidyl (2,3-3H) propionate, a useful labelling reagent (Dolly *et al.*,1981b; Sugiyama and Yamashita,1981). A stable, di-^{3}H-propionylated species of high specific radioactivity free from unlabelled material was obtained. The labelled derivative was fully characterized with respect to toxicity (both in the peripheral and central nervous systems), phospholipase activity, blockade of neurotransmitter in rat olfactory cortex and saturable binding to rat cerebrocortical synaptosomes (Chapter 3). The conditions of tritiation were very gentle and efficient, with the resultant derivatives exhibiting similar affinity to native toxin for its binding sites, indicating that modifications occurred on unessential residues in the toxin molecule. The availability of this radiolabelled derivative enabled the characterization of the nature, specificity and kinetic properties of the toxin's binding sites (see below).

To date, several reports on the radiolabelling of β-BuTx have appeared in the literature. The derivatives described therein were either inadequately characterized with respect to their biological and enzymic activities, or did not exhibit specificity for nerve terminals. Table 7.1 summarizes these previous studies and compares them to the labelled derivative used in this study. Previous tritiation of β-BuTx using the pyridoxalation method lowered its peripheral toxicity 10-fold and also its binding affi-

Table 7.1 Radiolabelling of β-BuTx

Radio-isotope	Labelling procedure	Specific activity (Ci/mmol)	Neuro-toxicity (% of native)	Enzyme activity (% of native)	K_d (nM)	B_{max} (source of membrane) (pmol/mg protein)	Inhibition by native β-BuTx (K_i)	Ref.
^{125}I	Chloramine-T	220-610	ND	85%	1-2 nM	13.0 (rat brain) 2.4 (liver) 0.25 (ery*)	ND	1
^{3}H	Pyridoxalation	1.4-8.4	10%	ND	210 nM	1.43 (rat brain)	25 nM	2
^{125}I	Lactoperoxidase	13.6	0	ND	ND	ND	ND	2
^{125}I	Bolton and Hunter Reagent	> 800	ND	ND	ND	ND	ND	3
^{3}H	N-succinimidyl (2,3,^{3}H) propionate	102	20%	0	0.6	0.150(rat brain)	0.29 nM 0.5 nM DTX 0.07 nM Tx I†	4
^{125}I	Lactoperoxidase	1000-1200	50-90%	ND	0.47	0.050(chick)	ND	5

ND - not determined. K_d was calculated from data obtained in equilibrium experiments using Scatchard analysis.

1 Oberg and Kelly (1976b)
2 MacDermott *et al.*, (1978)
3 Summers and Thompson (1980)
4 This work (Othman *et al.*, 1982)
5 Rehm and Betz (1982)

* erythrocytes
† Toxin I

nity (K_d = 0.21 M) to rat synaptosomal membranes. Furthermore, pyridoxalation of β-BuTx decreases the affinity of the toxin to its binding site about 10-fold (K_d 0.21 μM vs K_i 0.025 μM; the latter was determined from competition experiments). This showed a significant loss in affinity which is probably due to conformational changes of the toxin molecule as a result of the labelling process (MacDermott *et al.*,1978). After iodination using the chloramine-T method, the labelled toxin was shown to retain 85% of its phospholipase activity, but its lethality was not reported (Oberg and Kelly,1976b). Although the dissociation constant determined by these authors (K_d 0.7-2.0 nM) was similar to that reported in this study, the number of binding sites was about 10-fold higher (Table 7.1) and, in addition to neuronal membranes, the iodinated toxin was also shown to bind to liver and red-blood cell membranes. Furthermore, the displaceability of the labelled-toxin from its binding sites by unlabelled β-BuTx, as well as the nature and specificity of the binding were not examined. Work carried out in this laboratory showed that β-BuTx labelled with ^{125}I-iodine, using the chloramine-T method as described by Oberg and Kelly (1976b) is relatively non-toxic (0.4 μg/g body wt.) and exhibits low-affinity binding (K_d = 2 X 10^{-8} M) to a high content of binding sites (B_{max} = 1.8 pmol/mg protein) (A.Pelchen and J.O.Dolly, unpublished observations). However, using a modification of the chloramine-T method that employs milder conditions and which has been successfully used to label another presynaptically-acting protein, botulinum neurotoxin (Williams *et al.*,1983), a biologically active

derivative was obtained. This iodinated β-BuTx exhibited specific binding with high affinity (K_d = 0.5 nM; B_{max} ∿ 125 fmol/mg protein) to rat cerebrocortical synaptosomes, which was comparable to the binding properties of ^{3}H-β-BuTx. In addition, the iodinated toxin showed comparable localization of binding sites on rat cerebellar and hippocampal sections to that obtained by the latter. However, despite these similarities, the iodinated-β-BuTx was found to be very unstable, probably due to self-iodination, and showed a higher degree of non-specific binding.

Preliminary studies on the iodination of β-BuTx with N-succinimidyl 3-(4-hydroxy-5-{^{125}I}iodophenyl) propionate have been reported during the course of this work (Summers and Thompson,1980). The method described should yield a biologically active product as it modifies similar residues to the reagent used in this study. However, this reagent introduces a much bulkier group to the protein molecule which could be detrimental to the biological activity of the toxin. This iodinated reagent offers the advantage of being available at much higher specific radioactivities than N-succinimidyl (2,3-^{3}H) propionate. The preliminary report (Summers and Thompson,1980) did not describe the biological and enzymic activities nor the nature and specificity of the binding component(s) for the radioiodinated toxin.

In contrast to the initial report of MacDermott *et al.*,(1978), in which β-BuTx labelled using the lactoperoxidase method was shown to be non-toxic, Rehm and Betz (1982) have recently shown that the ^{125}I-β-BuTx derivative

obtained by this method could retain 50-90% of its neurotoxicity and exhibited similar binding affinity (K_d = 0.47 nM) to chick synaptic membranes as ^{3}H-β-BuTx. However, the total number of binding sites identified on these membranes was three-fold lower than that shown in this study; this difference may be due to the different animal species from which the synaptic membranes were prepared. Furthermore, it was also found that the binding of the radioiodinated β-BuTx was strongly dependent on divalent-cations especially Ca^{2+}; substitution of Ca^{2+} with Mg^{2+} inhibited the binding of β-BuTx (Rehm and Betz,1982). However, since the phospholipase activity of this derivative was not determined, it is difficult to ascertain whether the Ca^{2+} required is directly involved in the binding or in the enzymic activity of the toxin.

From these studies, it can clearly be seen that a stable preparation of radiolabelled β-BuTx derivative, fully characterized with respect to its physical and binding properties was needed. The ^{3}H-β-BuTx obtained in this study offers a number of advantages over the other radiolabelled derivatives (Table 7.1) for use as a probe for its binding component on nerve terminals. It has a relatively high specific radioactivity and can, therefore, be detected at very low levels in any sample by liquid scintillation counting, or by nuclear emulsion in autoradiographic studies (Chapter 6). This is particularly useful when low amounts of binding sites for toxin are present at the nerve terminal. The substantial biological activity (retention of 20% lethality when injected by both peripheral and central routes) demonstrated for ^{3}H-β-BuTx suggests that its neuro-

toxicity can be exhibited independently of phospholipolysis as the ^{3}H-β-BuTx is free from any phospholipase activity. The ^{3}H-β-BuTx obtained was stable and can be stored at -20^{0}C over a period of several months and does not undergo inactivation by self-irradiation. In rat olfactory cortex, ^{3}H-β-BuTx has been shown to block neurotransmission with similar potency to native toxin. Furthermore, from competition studies, it has been demonstrated that propionylation did not alter significantly the affinity of the toxin. The relatively long half-life of the radioisotope also constitutes an important practical advantage of this derivative; together with its stability, it makes repeated preparation unnecessary.

7.3 THE MODE OF ACTION OF DTX IN THE CNS

Previous reports of the pharmacological actions of DTX have been concerned mainly with nerve-muscle preparations where the neurotransmitter involved is ACh (Harvey and Karlsson,1980, 1982). DTX's specificity for other types in the peripheral nervous system is yet to be investigated. However, in the CNS, DTX can affect not only cholinergic synapses but also other types of synapses as well. This was demonstrated by its action on rat hippocampal slices where the predominant putative neurotransmitters are glutamate and aspartate (Storm-Mathisen,1981 ; Levi *et al.*,1982). In contrast to β-BuTx, which causes blockade of neurotransmission in slices of rat olfactory cortex (Dolly *et al.*,1980; Halliwell and Dolly,1982a) and hippocampus (Halliwell and Dolly,1982b), DTX induces a permanent

facilitation of synaptic transmission in rat and guinea pig hippocampal slices, and acts like a potent convulsant whose direct action involves Ca^{2+}-channel activity (Chapter 4). When monitored extracellularly, an enhancement of the amplitude of the initial synaptic wave was observed; however, the number of presynaptic fibres recruited by the stimulus remained unaffected (Section 4.4.3; Docherty *et al.*,1983). Intracellular recordings from the CA1 neurons showed that DTX causes irreversible enhancement of spontaneous synaptic activity. Furthermore, the resting potentials were not systematically altered under the action of DTX, which indicates that it does not induce a tonic depolarization in the postsynaptic membrane but it facilitates the release of neurotransmitter, thereby inducing incessant trains of EPSP-like potentials in the CA1 neurons. These appeared to be synaptic in origin since they can be blocked or severely reduced by omitting Ca^{2+} from the medium (with 15 mM Mg^{2+} as replacement), or by inclusion of TTX (0.5 µM) or Cd^{2+} (200 µM) therein.

Interestingly, similar spontaneous excitatory activity was also exhibited by black widow spider venom (BWSV) of the *Lactrodectus lesperus* species (Yamamoto and Matsui,1982). This demonstration of its excitatory action on guinea pig slices is of great interest, as venoms from this family of spiders have been shown to be potent activators of neurotransmitter secretion in a number of vertebrate preparations such as the neuromuscular junction (Frontali *et al.*,1976), rat brain slices (Tzeng and Siekevitz, 1978) and synaptosomes (Grasso *et al.*,1978; Grasso and Senni,

1979). α-latrotoxin, which is the major component of BWSV, have been shown to be responsible for the effects observed in these preparations. This toxin consists of a single polypeptide chain with a molecular weight of 130K. The mechanism of action of this toxin is not known with certainty; however, it has been widely speculated that its actions involve the formation of ion-channels across the plasma membrane, which make the membrane permeable to ions such as Na^+, K^+, Li^+, Ca^{2+}, Mg^{2+} and the organic ions methylamine and choline (Hurlbut and Ceccarelli,1979; Finkelstein *et al.*,1976). The neurotoxicity of α-latrotoxin has been ascribed to its ability to act as an ionophore for Na^+ and Ca^{2+} in nerve terminal membranes (Pumplin and Reese,1977; Finkelstein *et al.*, 1976). It has also been proposed that BWSV activates a contractile network at the presynaptic terminal, thereby stimulating exocytosis (Tzeng and Siekevitz,1979). In addition, it has also been suggested that BWSV might interact with the presynaptic plasma membrane at sites near the large intra-membranous particles that line the active zone and then induce release by allowing circumvention of the Ca^{2+}-dependent step of normal secretion (Ceccarelli *et al.*,1979).

Comparison of DTX with α-latrotoxin shows that the two toxins have little in common except for the observed facilitation of neurotransmitter release at peripheral and central synapses. (Harvey and Karlsson,1980; 1982; Frontali *et al.*,1976; Docherty *et al.*,1983; Yamamoto and Matsui,1982). However, despite this similarity at this stage of our study it is difficult to speculate on

the mechanism of action of DTX, since little information is available on its biochemical effects on nerve terminals.

7.4 THE LACK OF INVOLVEMENT OF PHOSPHOLIPASE ACTIVITY IN THE BINDING OF ^{3}H-β-BUTX TO NERVE TERMINALS

The effect of β-BuTx at the motor end plate and in rat brain slices is shown to be a complex (Section 1.3.5.5) and depends, in part, on the toxin's endogeneous phospholipase activity (Abe *et al.*,1977, 1976; Strong *et al.*,1976, 1977; Chang *et al.*,1977; Howard and Truog, 1977; Caratsch *et al.*,1981; Dolly *et al.*,1980; Halliwell and Dolly,1982a, 1982b). In addition, a large body of evidence exists for a toxin-induced inhibition of transmitter release in the peripheral (Abe *et al.*,1977; Chang *et al.*,1977; Abe and Miledi,1978; Kelly *et al.*,1979a; Caratsch *et al.*,1981) and central (Dolly *et al.*,1980; Halliwell *et al.*,1982) nervous systems, which is independent of its phospholipase activity. The typical triphasic effect of β-BuTx on evoked transmitter release from motor nerve terminals involves the early inhibition of transmitter release which is independent of its enzymatic activity, followed by a transient increase of release and a subsequent complete blockade which is dependent on the latter (Abe *et al.*,1977; Abe and Miledi,1978; Kelly *et al.*,1979a). Thus, the complex sequence of events caused by β-BuTx can be conveniently ascribed to one action which is independent of the toxin's endogeneous phospholipase activity and another which is dependent on it.

In the CNS, the situation appears to be more

complicated than that observed at the neuromuscular junction. When the enzymic activity of β-BuTx is destroyed by chemical modification with p-bromophenacyl bromide (Halliwell *et al.*, 1982; Dolly *et al.*,1980) or N-succinimidyl (2,3- H)-propionate (Chapter 3), the toxin can still cause comples blockade of neurotransmission in slices of rat olfactory cortex (Chapter 3; Dolly *et al.*,1980; Halliwell *et al.*,1982), although in the former case, its rate of action is reduced 2-3 fold (Dolly *et al.*,1980; Halliwell *et al.*,1982). In addition, both derivatives were toxic when injected intraventricularly into rats, although higher doses than native toxin were required (Othman *et al.*,1982; Chapter 3). Similarly, when the enzymic activity of β-BuTx is dramatically reduced by replacing Ca^{2+} with Sr^{2+}, β-BuTx still blocks neurotransmission in rat olfactory cortex at a rate 2-3 fold lower than in the presence of Ca^{2+}. Thus, in the CNS, it would appear that phospholipase-independent action of β-BuTx, possibly as a result of its specific binding, is sufficient to block neurotransmission. This may mean that the binding sites for β-BuTx in the peripheral and central nervous systems are different, or that the sites are part of complexes which perform similar functions in the two systems but which differ in their sensitivity.

In this study, biochemical evidence was presented to indicate that the specificity of binding of β-BuTx to nerve terminals is not conferred by its enzymic activity i.e. by the binding of substrate to the enzyme active site. This is based on the finding that ^{3}H-β-BuTx, which has no enzyme activity and retains considerable biological activity in the CNS, binds specifically and with high affinity to

rat cerebrocortical synaptosomes (Chapter 3; Othman *et al.*,1982). Furthermore, the binding is not inhibited in Ca^{2+}-free medium; under these conditions the ability of the toxin to bind to and catalyse the hydrolysis of monomeric phospholipid substrates is inhibited (Strong *et al.*,1976). The inefficiency of β-BuTx modified with p-bromophenacyl bromide, which has no phospholipase activity in antagonizing the specific binding of ^{3}H-β-BuTx is further evidence that the binding is occurring through a mechanism which does not involve its enzymic activity (see below). Phospholipase A_2 from *Naja melanoleuca* has no effect on the binding; however, BV-PLA_2 was able to inhibit ^{3}H-β-BuTx binding to synaptosomes (Chapter 3; Othman *et al.*,1983; Rehm and Betz,1982) and also to protect against the action of β-BuTx at the neuromuscular junction (Abe and Miledi,1978). This is probably due to perturbation of the membrane by its phospholipolytic action since in Ca^{2+}-free medium, it had no inhibitory effect on ^{3}H-β-BuTx binding.

From the present study, evidence was also obtained to indicate that the binding of β-BuTx is mediated by the B-chain of the molecule. This is based on the finding that protease inhibitor homologues, DTX and toxin I, can inhibit the binding of ^{3}H-β-BuTx with very high potency. As previously discussed (Section 1.3.6; Table 1-2), these inactive protease inhibitor homologues showed a high degree of sequence homology with the B-chain of β-BuTx, and also to other active protease inhibitor homologues (Table 1-2). It would appear, however, that only inactive protease inhibitor homologues which facilitate neurotrans-

mitter release can exhibit this antagonism for ^{3}H-β-BuTx binding (Chapter 3; Othman *et al.*,1982). Active trypsin inhibitors obtained from bovine pancreas and soya-bean do not inhibit the binding of β-BuTx (Rehm and Betz,1982).

7.5 NATURE OF THE BINDING SITES FOR ^{3}H-β-BUTX AND ^{125}I-DTX ON SYNAPTOSOMAL MEMBRANES

Despite the demonstration of the existence of specific binding sites for β-BuTx and DTX on rat cerebrocortical synaptosomes, their function and the toxins' mechanisms of action remain obscure. Binding of ^{3}H-β-BuTx and ^{125}I-DTX occurred equally well to lysed and intact synaptosomes (Chapter 3; Othman *et al.*,1982; Chapter 5) indicating that the binding sites are membrane bound. Binding of these toxins to specific membrane proteins unique to nerve terminals would be consistent with their potent presynaptic actions in central synapses (Docherty *et al.*, 1983; Othman *et al.*,1982; Chapters 2, 3, 4 and 5). The phospholipase activity might also act to best effect on the surrounding lipid annulus associated with the specific membrane proteins and fluid lipid layer. This phospholipid environment may be unique to presynaptic membrane terminals, and can be affected by BV-PLA$_2$ and taipoxin in the presence of Ca^{2+}. The failure of phospholipase A$_2$ from *Naja melanoleuca* to inhibit the binding of ^{3}H-β-BuTx and ^{125}I-DTX to synaptosomal membrane may be a reflection of the different substrate specificities of the phospholipases.

As the mechanisms of the facilitatory and inhibitory actions of DTX and β-BuTx, respectively, are both

unknown, it is difficult to predict the exact function of the binding component(s) of these toxins in affecting the release process of neurotransmitter(s). Thus, the nature of the proteins which might be involved in β-BuTx and DTX binding remains a matter for speculation.

Changes in Ca^{2+}-influx into the terminal have been implicated in the blockade of neuromuscular transmission by β-BuTx (Kelly *et al.*,1979a; Gundersen *et al.*, 1980). Similar changes in Ca^{2+}-flux are observed in brain mitochondria (Ng and Howard,1980; Wagner *et al.*,1974). However, neither Co^{2+} nor D600, both known Ca^{2+}-channel antagonists, have any effect on β-BuTx binding (Rehm and Betz,1982) on chick synaptic membranes. Furthermore, voltage-dependent Ca^{2+}-channels in vertebrate and invertebrate preparations are also insensitive to β-BuTx (Abe *et al.*,1976; Livengood *et al.*,1978). There is also strong evidence indicating that the depolarization of nerve terminals by β-BuTx is not mediated by the Na^{+}-channel (Halliwell *et al.*,1982), contrary to previous reports (Abdul-Ghani *et al.*,1981; Smith *et al.*,1980). It seems unlikely for these channels to be the toxin's binding sites as they are not confined to nerve terminals. Furthermore, tityustoxin, a scorpion toxin which extends the open-time of Na^{+} channels, fails to antagonize the binding of ^{3}H-β-BuTx to synaptosomes (Chapter 3; Othman *et al.*,1982). Thus, the reported (Okamoto,1980) ability of β-BuTx to inhibit the binding of a related scorpion toxin from *Leiurus quinquestriatus* to electroplaque membranes, may be the result of the phospholipolytic action of β-BuTx. It has also been suggested (Simpson,1976; Howard and Gundersen,

1980) that β-BuTx interacts with sites exposed by the exocytotic process; thus, α-latrotoxin which facilitates neurotransmitter release (Grasso *et al.*,1978; Howard and Gundersen,1980) might be expected to increase the binding, but this was not the case (Chapter 3; Othman *et al.*,1982).

As in the case with β-BuTx, the possible involvement of Ca^{2+}-channels has been implicated in the action of DTX on synaptosomes (Chapter 4). DTX causes an increase in $^{45}Ca^{2+}$ accummulation in synaptosomes which can be inhibited by Ca^{2+}-channel antagonists such as Cd^{2+} and to a lesser extent verapamil, D600 and nifedipine (A.Breeze and J.O.Dolly, unpublished observations). Electrophysiological measurements in hippocampal slices showed that it causes potentiation of neurotransmitter release and acts as a potent convulsant whose direct action requires Ca^{2+}-channel activity. These results would also be consistent with its facilitatory effect at peripheral synapses. It has been well established that the release of chemical transmitter must be preceded by an influx of Ca^{2+}ions via the Ca^{2+}-channels into the nerve terminals down their electrochemical gradient (Stinnarke,1977; Baker *et al.*,1971). However, at this stage of investigation, it cannot be ascertained whether the effect of DTX on the voltage-dependent Ca^{2+}-channel activity is direct or indirect. Also it is not known whether the binding component (s) demonstrated is directly responsible for the observed increase in Ca^{2+}-influx. Recent studies (A.Black and J.O.Dolly, unpublished observations) showed that none of the Ca^{2+}-channel antagonists including Cd^{2+}, verapamil, D600 and nifedipine can inhibit the ^{125}I-DTX binding to

synaptosomes, thus eliminating the possibilities of direct action of DTX on the Ca^{2+}-channel ot of the binding component being directly associated with the latter.

A second possibility for the nature of the binding component observed for ^{125}I-DTX is the Na^{+}-channel. However, it has been shown that TTX has no effect on the increase of Ca^{2+}-flux caused by DTX, thus indicating that the Na^{+} channel is not involved. Other possibilities include the K^{+} channel as pharmacological studies (Harvey and Karlsson,1980) showed that the effect observed with DTX in nerve-muscle preparations is very similar to that produced by 3,4 diaminopyridine. Direct proof of this hypothesis is still being investigated.

7.6 SPECIFICITY OF THE ACCEPTOR SITES FOR ^{3}H-β-BUTX AND ^{125}I-DTX ON SYNAPTOSOMAL MEMBRANES

This study has demonstrated the existence of specific binding sites for ^{3}H-β-BuTx and ^{125}I-DTX on rat synaptosomal membranes. The binding sites for the former have been shown to be specific for β-BuTx and the facili tatory neurotoxins, toxin I and DTX. Other presynaptically-acting neurotoxins including botulinum neurotoxin, taipoxin (in the absence of Ca^{2+}) and α-latrotoxin failed to inhibit the binding of ^{3}H-β-BuTx indicating that these neurotoxins are binding to separate sites on the synaptosomal membrane. This suggestion is supported by the demonstration that β-BuTx was unable to inhibit the binding of radioiodinated botulinum neurotoxin (Williams *et al.*,1983). Moreover, from electrophysiological measurements at the neuromuscular

junction, it was apparent that β-BuTx can still bind after chronic treatment with botulinum neurotoxin; however, under these conditions, the expression of β-BuTx's action was suppressed (Tse *et al.*,1982). Similarly, the existence of separate sites for taipoxin accords with recent pharmacological evidence that β-BuTx and taipoxin exert their inhibitory effects on neuromuscular transmission by binding to separate sites (Chang and Su,1980).

The ability of DTX and toxin I to inhibit the specific binding of ^{3}H-β-BuTx on synaptosomes, accords with the pharmacological evidence for the antagonism between these toxins in chick-biventer preparations; the latter also suggests that these toxins are binding to the same sites (Harvey,1982; Harvey and Karlsson,1982). DTX and toxin I can inhibit the binding of ^{3}H-β-BuTx with high potency (K_i's of 0.50 nM and 0.07 nM, respectively); in fact, toxin I exhibited even greater affinity for the binding sites than native β-BuTx (K_i = 0.29 nM). The antagonism observed between these neurotoxins also provides evidence for the possible involvement of the binding component(s) of β-BuTx in the process of transmitter release.

Investigation of the specificity of the binding component(s) demonstrated for ^{125}I-DTX showed that only toxin I can inhibit its specific binding with high potency. Its K_i of 0.06 nM indicates that it has greater affinity (∿ 10-fold) for the binding sites than DTX itself. These higher affinity is also demonstrated by its central neurotoxicity in rat brain; toxin I is at least 5-times more toxic than DTX (Section 4.4.2). The antagonism observed indicates that these toxins may be sharing a common binding

site; furthermore, their primary amino acid sequences show a high degree of homology (Table 1-2). In fact, they differ in only 5 residues in their polypeptide chains. However, despite these similarities, it is not known why toxin I exhibits a higher affinity than DTX; it is possible that substitution of the 5 amino acid residues could cause conformational changes in the ternary structure of the protein molecule which could alter the affinity of the toxin's active site.

Crotoxin and taipoxin (in the absence of Ca^{2+}) did not have any effect on ^{125}I-DTX binding, indicating that their binding sites are separate from that of the latter on the nerve terminals. These findings accord with recent pharmacological evidence obtained from nerve-muscle preparations (Harvey and Karlsson,1982); the inhibitory action of crotoxin was enhanced by pretreatment of the preparation with DTX suggesting that the former was interacting with sites distinct from those for DTX. Addition of taipoxin to a preparation that had previously been treated with the facilitatory neurotoxin caused no significant changes in the time taken for blockade of neurotransmitter release, which suggests that taipoxin is binding to a separate site (Harvey and Karlsson,1982).

In competition experiments, β-BuTx showed partial inhibition of ^{125}I-DTX binding; it can only displace ∿ 25% of the ^{125}I-DTX bound to the nerve membrane. This low potency of β-BuTx in inhibiting ^{125}I-DTX binding will be discussed (see below) in relation to the possible interactions of their binding sites, as made evident by the observed binding kinetics and localization studies.

7.7 POSSIBLE INTERACTIONS BETWEEN THE BINDING SITES OF β-BUTX AND DTX AT NERVE TERMINALS

Comparison of the kinetics of binding of β-BuTx and DTX to their acceptor proteins on synaptosomal membranes (Table 7-2) strongly suggests that these toxins are binding to the same binding component(s). β-BuTx and ^{125}I-DTX exhibit high affinity binding (K_d's of 0.6 nM and 0.5 nM, respectively) to a single class of non-interacting sites on these membrane preparations (Table 7-2). The association rate constants of the binding proteins for both toxins are almost identical and in the order of $10^5 M^{-1}s^{-1}$, indicating that the binding is rapid and is unlikely to be a rate-limiting step in their actions at nerve terminals. In view of the apparent irreversibility of the interactions of β-BuTx (Halliwell and Dolly, 1982a, 1982b; Dolly *et al.*,1980) and DTX (Docherty *et al.*, 1983; Chapter 4) on hippocampal and rat olfactory cortex slices as measured electrophysiologically, it was somewhat surprising that the dissociation rates (in the order of $10^{-4}s^{-1}$) were so fast. This discrepancy may be due to the different conditions in which these experiments were carried out (Section 3.3.5; Section 3.3.7; Section 4.4.3; Halliwell and Dolly,1982a; Othman *et al.*,1982; Docherty *et al.*,1983). The similar dissociation constants for the bindings of these toxins (K_d's of 0.5 nM and 0.6 nM for ^{125}I-DTX and ^{3}H-β-BuTx respectively; Table 7-2) also indicate that they are binding to the same component(s). Furthermore, the binding component(s) for both toxins exhibits some dependency on the membrane-phospholipid environ-

Table 7-2 Properties of the specific binding protein(s) for β-BuTx and DTX on synaptosomal membranes

	B_{max} (fmoles/mg protein)	(a) $k_1 \times 10^{5}$ ($M^{-1}s^{-1}$)	(a) $k_{-1} \times 10^{-4}$ (s^{-1})	(ℓ) $K_d \times 10^{-10}$ (M)	$K_1 \times 10^{-10}$ (M)
^{3}H-β-BuTx	150	7.8	5.6	6.0	2.8 (β-BuTx) 3.1 (DTX) 0.7 (Toxin I)
^{125}I-DTX	600	6.15	2.9	5.0	4.0 (DTX) 0.6 (Toxin I) ∿ 450 (β-BuTx)*

* Incomplete inhibition

(a) at 37°C

(ℓ) From Scatchard plot

ment as they are both affected by the phospholipase A_2 activity of BV-PLA_2 and taipoxin, another presynaptically -acting neurotoxin. Also, the binding of these toxins to their binding components was not dependent on divalent -cations, including Ca^{2+}. Interestingly, toxin I can inhibit the binding of ^{3}H-β-BuTx and ^{125}I-DTX with equal potency, which further suggests that the latter toxins are binding to the same component(s).

However, despite the overwhelming evidence to suggest that these toxins are binding to the same component(s), another line of evidence was obtained to suggest that these toxins may be sharing only some but not all binding sites. This is shown firstly from the total number of binding sites of DTX on synaptosomal membranes; it was found that ^{125}I-DTX binds to 4-times the number of binding sites as ^{3}H-β-BuTx. Further evidence was obtained from localization studies in rat hippocampal sections; incubation of radiolabelled toxins with tissue sections under identical conditions showed that these toxins have different distribution of binding sites. In addition to the molecular layer, which is the prime target of ^{3}H-β-BuTx binding, ^{125}I-DTX exhibited equally high level of binding sites in the stratum oriens and stratum radiatum of the hippocampus (Fig. 6.5 and 6.11). It would appear from these studies that greater selectivity of binding was exhibited by β-BuTx than DTX in rat hippocampus sections. However, at this stage of the study, it is not known whether ^{125}I-DTX binds to the same sites as ^{3}H-β-BuTx in the molecular layer of the hippocampus, but from the data obtained from competition studies on synaptosomes, which demonstrate the ability

od DTX to antagonize ^{3}H-β-BuTx binding, it is pertinent to suggest that DTX can also inhibit the binding of ^{3}H-β-BuTx in the molecular layer. Direct proof of this suggestion requires further autoradiographic studies of both radiolabelled toxins in the presence of unlabelled DTX and β-BuTx.

At this stage of investigation, the nature of the interaction of βBuTx and DTX with these binding sites is not yet known ; the kinetic measurements and localization studies suggest that the interaction of these toxins is complex and indirect mechanism may be operative for the antagonism observed. To elucidate the mechanism that may be involved in the interactions of β-BuTx and DTX with nerve terminals, further molecular characterization of their binding component(s) must be carried out (see below).

7.8 SUGGESTIONS FOR FURTHER STUDIES

As the structural expression of function in biological systems is three-dimensional at all levels of resolution, the study of structure-function relationships of β-BuTx and DTX can be greatly facilitated by the knowledge of their-dimensional structures. These can be achieved by using X-ray crystallographic studies which have been used successfully to establish the three-dimensional

structure of erabutoxin b, a postsynaptic neurotoxin obtained from the venom of the sea snake *Laticauda semifasciata* (Low *et al.*,1976; Low,1976, 1979). Interestingly, the chemical structure of this postsynaptic neurotoxin is very similar to that of DTX, in that it is composed of 59 amino acid residues with 3 disulfide bridges. Thus, the study of the 3-dimensional structure of the DTX rather than β-BuTx would be more feasible and productive as it comprises a single polypeptide chain of molecular weight of only 7000.

To elucidate the mechanism in which the binding sites of β-BuTx and DTX interact, further molecular characterization of the binding component(s) will be necessary. This thesis has demonstrated that the acceptor-proteins for these toxins are membrane-bound, and that they exhibit a relatively fast dissociation rate from the toxin-receptor complex. Thus, crosslinking the receptor-toxin complex *in vitro* would be required prior to solubilization with suitable detergents. Once solubilized, the molecular weights of the acceptor-proteins could be determined by gel-filtration or SDS gel electrophoresis. Solubilized receptors could also be used to investigate the interaction with either DTX or β-BuTx, and thus establish the nature of the antagonism observed in this study.

The ability of DTX and toxin I to antagonize the action and binding of β-BuTx should greatly facilitate the assay, purification and characterization of the β-BuTx -acceptor protein, by acting as independent agents for blocking β-BuTx binding. With the availability of techniques

for production of monoclonal antibodies, antibodies raised against solubilized β-BuTx receptor would be extremely useful for localizing the β-BuTx binding site and for cross-reactivity studies with DTX and toxin I. Similarly, the purification and characterization of the acceptor-protein for DTX could also be used in a complementary manner to that of β-BuTx in the localization and cross-reactivity studies of these toxins.

As it was not possible to identify the precise ultrastructural location of the binding sites of ^{3}H-β-BuTx and ^{125}I-DTX on synaptosomes, development of immunochemical methods which are of higher resolution will be required to pinpoint the exact areas of plasma membrane in which the sites reside, and also to establish the extent of any binding to non-neuronal material. Monoclonal antibodies against β-BuTx and DTX could facilitate greatly this study. At light-microscope level, the effect of β-BuTx on the labelling pattern of ^{125}I-DTX binding in hippocampal sections could clarify the similarities and differences observed in the labelling patterns of these toxins. Similarly, the effect of excess DTX on the localization of ^{3}H-β-BuTx binding sites in these sections could help elucidate the nature of their interaction, at least in this region of the brain. Specific lesioning studies using lesioning agents such as kainic acid should help to assess further the contribution of particular pathways and transmitters to the labelling patterns revealed by ^{3}H-β-BuTx and ^{125}I-DTX.

From the discussion above, it is clear that there is still a considerable way to go before the nature of interactions of β-BuTx and DTX with nerve terminals as well

as the mechanisms by which these toxins can cause their respective inhibitory or facilitatory actions, can be elucidated. However, research along these lines will definitely help the progress in the understanding of the molecular mechanisms of the process of neurotransmitter release.

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