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PURIFICATION OF β-BUNGAROTOXIN: ITS EFFECTS ON THE RELEASE OF NEUROTRANSMITTERS AND ITS BINDING TO BRAIN SYNAPTOSOMES

by

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A Thesis submitted for the degree of
Doctor of Philosophy in the University of London
and the Diploma of Imperial College

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1982

ABSTRACT

β-Bungarotoxin was purified from the venom of Bungarus multicinctus by ion exchange chromatography and isoelectric focusing. It was homogeneous on gel filtration (molecular weight 21000), polyacrylamide gel electrophoresis and isoelectric focusing (pI. 10.4). Electrophoresis in the presence of sodium dodecyl sulphate and urea gave a single protein band and, after reduction, two polypeptides of apparent molecular weights 9000 and 11000 were separated. The pure protein, which was highly toxic to mice after intraperitoneal injection, was even more potent when injected intraventricularly into rat brain; the toxin was lethal via these routes at 10 and 0.05 ng/g body weight, respectively. It caused an irreversible presynaptic blockade of neurotransmission at putative amino acid synapses in rat olfactory cortex.

β-Bungarotoxin induced the release, with similar concentration dependencies ($E.D._{50} = 5 \times 10^{-8}M$), of [^{3}H] acetylcholine, [^{3}H] choline and lactate dehydrogenase from rat brain synaptosomes, preloaded with [^{3}H] choline. Inhibition of the high affinity accumulation by synaptosomes of [^{3}H] choline may be a secondary effect of β-bungarotoxin-induced depolarisation of the nerve terminals, as was demonstrated for a scorpion toxin, tityustoxin. The Ca^{2+}-dependent phospholipase activity of β-bungarotoxin was inhibited by acylation of an essential histidine residue with p-bromophenacyl bromide or by replacement of Ca^{2+} with Sr^{2+}. Although these treatments greatly reduced the effects of the toxin on synaptosomes, an irreversible but somewhat attenuated blockade of neurotransmission in the olfactory cortex was still observed. Pure phospholipases A_2 were, generally, much less potent than β-bungarotoxin in mediating effects on transmitter release from synaptosomes and in olfactory cortex.

N-succinimidyl [2,3-^{3}H]propionate was used to produce an alkylated β-bungarotoxin derivative of high specific radioactivity; electrophoresis showed that the subunits were about equally labelled but a single peak of radioactivity was obtained on isoelectric focusing. [^{3}H] β-Bungarotoxin, which lacked phospholipase activity, blocked neurotransmission in the olfactory cortex and was lethal to rats when injected intraventricularly at 10 ng/g body weight. Binding of radiolabelled toxin to synaptosomes showed a saturable component (K_d 1.5 nM; 0.011p mol/mg of protein) together with sites of much lower affinity. The high affinity binding may be related to the effects of β-bungarotoxin on neurotransmitter release.

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ABBREVIATIONS

Acetylcholine	ACh
Adenosine triphosphate	ATP
γ-Aminobutyric acid	GABA
Batrachotoxin	BTX
Botulinum neurotoxin	BoNT
Botulinum toxin	BoTX
α-Bungarotoxin	α-BuTX
β-Bungarotoxin	β-BuTX
Central nervous system	CNS
Endplate potential	epp
Grayanotoxin	GTX
Isoelectric point	pI
Lateral olfactory tract	LOT
Miniature endplate potential	mepp
Postsynaptic potential	psp
Saxitoxin	STX
Sodium dodecylsulphate	SDS
Tetrodotoxin	TTX
Tityustoxin	TsTX

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ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor Dr. J.O. Dolly for his considerable help and patient encouragement. I thank Professor E.A. Barnard for allowing me the privilege of working with his department. Dr. C.K. Tse, Dr. J.V. Halliwell and Mr. I.B. Othman have been valued colleagues who have assisted with some experiments. I also thank Mr. D. Green and his staff for performing animal injections.

During the course of this work I was in receipt of a Medical Research Council postgraduate studentship for which I am very grateful.

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

Complete Purification of β-Bungarotoxin: Characterisation of its Action and that of Tityustoxin on Synaptosomal Accumulation and Release of Acetylcholine. Spokes, J.W. and Dolly, J.O. (1980). Biochimica et Biophysica Acta, 596, 81-93.

Interaction of β-Bungarotoxin with Synapses in the Mammalian Central Nervous System. Dolly, J.O., Halliwell, J.V. and Spokes, J.W. (1980). In 'Natural Toxins', edited by D. Eaker and T. Wadstrom, pp.549-559, Pergamon Press.

Effects of β-Bungarotoxin and Tityustoxin on Uptake and Release of Neurotransmitters. Dolly, J.O., Tse, C.K., Spokes, J.W. and Diniz, C.R. (1978). Biochemical Society Trans., 6, 652-654.

Pre- and Postsynaptic Effects of β-Bungarotoxin in the Mammalian Brain. Dolly, J.O., Halliwell, J.V., Schofield, C.N. and Spokes, J.W. (1980). J. Physiol. (London), 308, 70-71 P.

Biochemical and Electrophysiological Demonstrations of the Actions of β-Bungarotoxin on Synapses in Brain. Halliwell, J.V., Tse, C.K., Spokes, J.W., Othman, I. and Dolly, J.O. (1982). J. Neurochem., 39, 543-550.

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.

The experimental work described in this study was carried out between 1976 and 1979 as the initial part of a long term study to identify and characterise components of the mechanism of neurotransmitter release using specific neurotoxins as probes. Some of the experiments described are of an exploratory nature, particularly those involving the radiolabelling of β-bungarotoxin and its binding to synaptosomes (Chapter 5), and are discussed in relation to subsequent work in this laboratory using the techniques developed herein.

CHAPTER 1. GENERAL INTRODUCTION

1.1. The Events Involved in Release of Neurotransmitters

1.1.1. The neuromuscular junction

1.1.1.1. Electrophysiological observations

The mechanisms by which electrical impulses are transferred from one nerve cell to another or to an effector cell have been the subject of intense study for a considerable time. The process is best characterised at vertebrate motor endplates which are most amenable to electrophysiological studies and at the giant synapses in the stellate ganglia of squid where the mechanisms appear to be similar. Electrically excitable cells (principally nerve and muscle cells) maintain high internal concentrations of potassium ions (K^+) and low concentrations of sodium ions (Na^+), relative to the external medium, by means of energy dependent ion translocating systems (or ion pumps). Typically a nerve cell maintains, in this manner, a potential difference across its plasma membrane of -60 mV. The electrical depolarisation which occurs during the passage of an impulse is a result of Na^+ and K^+ moving down their concentration gradients via channels in the membrane. Since reduction of the membrane potential itself causes these channels to open, a wave of depolarisation (or action potential) is propagated along the membrane. The electrochemical gradients are subsequently restored by the ion pumps (Katz, 1966).

Arrival of an action potential at the unmyelinated nerve terminal triggers a series of events, including an influx of calcium ions (Ca^{2+}) into the nerve terminal down its electrochemical gradient (Stinnakre, 1977), which results in the release of a chemical transmitter. It is well established, at the vertebrate neuromuscular junction, that this release takes the form of large numbers of discrete packages (or quanta) of acetylcholine (ACh) (del Castillo and Katz, 1954). Inter-

action of the ACh with its receptors on the postsynaptic membrane, following its diffusion across the synaptic cleft, gives rise to a local depolarisation (end plate potential or e.p.p.) which, if it exceeds a certain threshold value triggers an action potential in the muscle membrane. Spontaneous release of transmitter also occurs in quanta which may be detected, by intracellular recording, as miniature end plate potentials (mepp's) (Katz, 1966). Nerve terminals may be depolarised experimentally, by increasing the external K^+ concentration or by electrical stimulation. It was observed that, when the membrane potentials of nerve terminals were electrically altered for prolonged periods, graded depolarisation of the nerve terminal resulted in a graded increase in spontaneous release, as measured by the frequency of m.e.p.p.'s (Katz and Miledi, 1967 a and b). ACh released by one action potential must be inactivated before the arrival of the next. This is carried out at cholinergic synapses by enzymatic hydrolysis; large amounts of acetylcholinesterase are located in the synaptic cleft; reviewed by Barnard et al. (1973). Cholinergic neurones reaccumulate the choline via a specific high afinity transport system (Pert and Snyder, 1974).

It is now well established that release of ACh is highly dependent on the concentration of Ca^{2+} inside the nerve terminal. Injection of Ca^{2+} into nerve terminals at squid giant synapses was shown to increase the rate of quantal transmitter release (Miledi, 1973). Intracellular recording at the giant synapse when Na^+ and K^+ channels were blocked by tetrodotoxin (TTX) and tetraethylammonium ions (TEA) respectively, allowed direct measurement of the inward Ca^{2+} flux and the concomitant postsynaptic response to released transmitter (Katz and Miledi, 1969). Aequorin, a protein which emits light on binding Ca^{2+} was injected into squid nerve terminals and light emissions measured which coincided with the arrival of action potentials (Llinas and Nicholson, 1975). Finally, the influx of radioactive $^{45}Ca^{2+}$ into

nerve terminals following depolarisation has been measured.

It is, therefore, accepted that this influx of Ca^{2+} occurs via specific, voltage-dependent channels in the nerve terminal membrane; although the early part of the Ca^{2+} current may occur via the Na^{+} channels (Baker et al., 1971). The time lag between the depolarisation of the nerve terminal and the onset of ACh release, about 200 μsec. at the squid giant synapse, can mostly be accounted for by the time required to activate Ca^{2+} entry (Llinas, 1977). Extracellular Mg^{2+}, although reported to increase mepp frequency at the neuromuscular junction at very low concentrations of Ca^{2+}(c.a. 10^{-7}M) (Hubbard et al., 1968a), generally inhibits release by competing with external Ca^{2+} (Hubbard et al, 1968 a and b). Sr^{2+} can substitute for Ca^{2+} in supporting transmitter release evoked by nerve impulses (Dodge et al., 1969), although on a molar basis it is much less effective. However, when release is evoked by prolonged depolarisation with increased extracellular K^{+} concentration, Ca^{2+} and Sr^{2+} are equally effective (Mellow, 1979).

The postsynaptic depolarisation, caused by released transmitter, persists for a few milliseconds after the presynaptic calcium current has ceased (Llinas 1977), indicating the period required for the removal of intraterminal Ca^{2+}. The increased frequency of mepp's observed during this period, termed "delayed release", has been associated with this persistence of an increased intracellular Ca^{2+} concentration (Rahamimoff 1976). A related phenomenum is that of "facilitation". If two presynaptic action potentials are separated by an appropriate time interval, (Katz, 1966) then the ACh release evoked by the second is increased, apparently as a result of the residual high intracellular Ca^{2+} concentration (Katz and Miledi 1968). Similar, but more pronounced, enhancement of ACh release is observed following tetanic nerve stimulation (Katz, 1966).

When nerve terminals are electrically depolarised with brief (1-10 ms) current pulses the amplitude of the epp's elicited show an

S-shaped dependence on the strength of the current used to depolarise the nerve terminal (Katz and Miledi, 1967 b). This relationship may be explained by the similar dependence of the rate of Ca^{2+} influx on the presynaptic depolarisation at the squid synapse (Llinas, 1977). The relationship between the amount of transmitter released in response to an action potential and the external Ca^{2+} concentration is non linear with a sigmioidal start (Rahamimoff, 1976), the release being proportional to the 4th power of the Ca^{2+} concentration. However, using voltage clamp techniques it was shown, at the squid synapse, that the amount of transmitter released was directly proportional to the rate of Ca^{2+} entry into the nerve terminals (Llinas, 1977). A possible explanation is that the rate of evoked transmitter release has a non-linear dependence on the intraterminal Ca^{2+} concentration. This seems probable since otherwise rates of release which are obtainable would require very high intracellular Ca^{2+} concentrations (Kelly et al., 1979a). Alternatively, the relationship between Ca^{2+} currents and internal Ca^{2+} concentration may be non-linear. Inside the nerve terminal, Ca^{2+} binding proteins, mitochondria and other organelles (Blaustein et al., 1977; Llinas and Heuser, 1977) are available for the sequestration of Ca^{2+}. The accumulation and release of Ca^{2+} by these stores may lead to a complex inter-relationship between intracellular and extracellular Ca^{2+} and the depolarisation of the nerve terminal. Furthermore, the concentration of Ca^{2+} may not be the same throughout the cytoplasm of the nerve terminal. The Ca^{2+} concentration around vesicle release sites on or near the plasma membrane may not, therefore, be the same as the total intracellular concentration.

Spontaneous release of transmitter can occur in the complete absence of external Ca^{2+} (Hubbard et al., 1968a). However, as described above, the frequency of spontaneous release is increased by treatments which are thought to raise the intraterminal Ca^{2+} concentration. Conversely, when the Ca^{2+} electrochemical gradient is reversed by a very low

extracellular Ca^{2+} concentration, arrival of an action potential causes a decrease in the rate of release of transmitter (Rotshenker et al., 1976). It is thought, therefore, that the Ca^{2+}- independent ACh release is due to the availability of Ca^{2+} from intracellular stores in accordance with findings for other secretory systems (Lowe et al., 1976).

1.1.1.2. Morphological observations

The most striking ultrastructural feature of nerve terminals, when examined in thin section under the electron microscope, is the large number of small membrane-bound vesicles which fill large proportions of the cytoplasmic space and are particularly dense near the presynaptic plasma membrane, for review see Jones (1975). The observation of such synaptic vesicles, concurrently with the discovery of the quantal nature of ACh release, led to the hypothesis that each vesicle contained one quantum of transmitter molecules which was released by fusion of the vesicle and plasma membranes in an exocytotic process (Katz, 1966). This was supported by the finding that synaptic vesicle fractions isolated from mammalian brain (Whittaker and Sheridan, 1965) and from electric organs of elasmobranchs (Whittaker et al., 1972) were enriched in ACh. The latter organs, which contain solely cholinergic nerve terminals have been used as a source of highly purified synaptic vesicles whose internal ACh concentrations (Wagner et al., 1978; Ohsawa et al., 1976) correlate well with electrophysiological observations of the number of molecules of ACh in one quantum at the vertebrate neuromuscular junction (Kuffler and Yoshikami, 1975). These vesicles appear to contain integral membrane proteins (Wagner et al., 1978) which may correspond to the intramembranous particles seen in synaptic vesicles in freeze-fracture studies of frog neuromuscular junctions (Heuser, 1976). They also contain large quantities of ATP (Dowdall et al., 1974).

The interaction of synaptic vesicles with the plasma membrane has been most studied, by thin section and freeze-fracture techniques,

at the frog neuromuscular junction (Heuser, 1976). The synaptic vesicles, as seen in thin sections, cluster around heavily staining regions of the presynaptic membrane which are opposed to the clefts in the highly enfolded postsynaptic membrane. The vesicles are closest to the plasma membrane in the areas immediately adjacent to these densities (Birks et al., 1960; Heuser, 1976). Freeze-fracture studies have revealed the presence of ridges in the presynaptic plasma membrane, raised towards the synaptic cleft, and running perpendicularly to the long axis of the nerve terminal branch (Heuser, 1976). These ridges, of which there are approximately 500 per endplate, are also marked by a double row of large intramembranous particles (Heuser, 1976). When frog muscles were indirectly stimulated at high frequency during fixation and subsequently sectioned, electron micrographs appeared to show vesicles fusing with the plasma membrane on either side of the presynaptic densities (Couteaux and Pecot-Dechavassine, 1970). These regions were termed "active-zones". Freeze-fracture studies on muscles fixed, with aldehydes during indirect stimulation, as viewed from the synaptic cleft revealed the presence of "dimples" in the plasma membrane. These indentations occurred on each side of the ridges described above and were interpreted as showing vesicles in the act of fusion with the presynaptic membrane (Heuser, 1976; Ceccarelli et al., 1979a). However, due to the slow nature of the aldehyde fixation, the numbers of such fusing vesicles were less than expected (Heuser, 1976). Rapid freezing of the muscles during stimulation, in conjunction with treatment with aminopyridine which greatly increases the number of quanta released by each impulse, allowed the latter to be correlated with the numbers of indentations seen at the active zones (Heuser et al., 1979). When black widow spider venom (1.3.1) was used, in the absence of Ca^{2+}, to cause massive ACh release a very large increase was seen in the number of dimples (Ceccarelli et al., 1979a). These presumed sites of vesicle fusion were again associated with the active

zones although the organisation of the latter was somewhat disrupted by the action of the venom. Following prolonged indirect stimulation (Heuser, 1976) or treatment with 20mMK^+ (Ceccarelli et al.,1979b) there was a very large increase in the number of dimples in the regions between the active zones. These indentations also increased when muscles were fixed during periods of recovery from repetitive stimulation (Ceccarelli, et al.,1979b and are thought to represent sites of endocytosis.

Vesicles fusing with the plasma membrane as part of an exocytotic process could not, on purely morphological grounds, be distinguished from the reverse process of endocytosis. Horse radish peroxidase, a protein of molecular weight 40000, was shown to be taken up into nerve terminals, presumably by endocytosis (Heuser and Reese, 1973). It appeared first in vesicles with filamentous "coats", then in the large vacuoles or cisternae which develop in nerve terminals during stimulation and finally in uncoated synaptic vesicles clustered near the plasma membrane. Coated vesicles have a diameter of about 700 Å and a high buoyant density and can be separated from uncoated vesicles (Pearse, 1976). Preparations of coated vesicles from pig brain contained 75% protein and 25% phospholipid by weight: 70 - 90% of the protein was clathrin which has a molecular weight of 180,000 daltons which forms polyhedral lattices of which the "coat" is made up (Crowther et al., 1976; Pearse, 1976). 10% of the protein in coated vesicles consists of two proteins which resemble the two major components of the Ca^{2+} - dependent ATPase from sarcoplasmic reticulum; this is significant in view of the ability of coated vesicles to accumulate Ca^{2+} (Blitz et al., 1977). A process of recycling of synaptic vesicles was suggested on the basis of the foregoing, morphological observations (Heuser, 1976).

The above provides convincing evidence of exocytosis at motor nerve terminals in conditions under which the release of ACh is stimulated. The final proof of the "vesicular" hypothesis, that the vesicles which fuse with the plasma membrane are actually those which

contain ACh, has not yet been forthcoming. This will be further discussed in the light of the biochemical evidence obtained from studies on synaptosomes and their subcellular fractions (1.1.3.).

1.1.2. Synapses in the central nervous system

Although there is a great deal of diversity in the types of synapses which have been studied, particularly in the molecules used as neurotransmitters, they possess many common features. Quantal release of transmitter has now been observed at synapses in vertebrate spinal cord (Kuno, 1964), autonomic ganglia (Blackman et al., 1963), and invertebrate neuromuscular junctions (Dudel and Kuffler, 1961). Transmitter release in response to action potentials at these synapses is dependent on Ca^{2+} and is inhibited by Mg^{2+}. The ultrastructure of nerve terminals in the central nervous system is very similar to that of the vertebrate neuromuscular junction; intraterminal mitochondria, synaptic vesicles and active zones have all been observed in the electron microscope by thin section and freeze-fracture technique (Akert et al., 1975).

1.1.3. Synaptosomes

It was discovered that when tissue from mammalian brain was homogenised under the appropriate conditions, nerve terminals were pinched off and became resealed. These nerve terminal vesicles, or "synaptosomes", could then be isolated by sedimentation through discontinuous density gradients (Whittaker, 1969; Cotman, 1974). When observed in the electron microscope, the intact synaptosomes (about 50% of the purified fraction) were seen to contain mitochondria and synaptic vesicles. Synaptosomes from mammalian brain also contained coated vesicles. When depolarised synaptosomes were incubated in the presence of horseradish peroxidase,

Ca^{2+} - dependent uptake of this marker enzyme into synaptic vesicles was observed (Blaustein et al., 1977). Lysed synaptosomes have been further fractionated by differential centrifugation. The soluble fraction contains, in addition to normal cytoplasmic markers such as lactate dehydrogenase, enzymes required for the synthesis of neurotransmitters such as choline acetyltransferase and glutamic acid decarboxylase (Whittaker, 1969). The microsomal fraction is enriched in acetylcholine and catecholamines and in small, membrane-bound, vesicles which are presumed to be synaptic vesicles (Whittaker and Sheridan, 1965; Nagy et al., 1977).

Synaptosome preparations, which also contain free mitochondria, typically maintain the metabolic and functional properties of nerve terminals for 3-4 hours (Bradford et al., 1975). They respire, metabolising a wide range of substrates including glucose, pyruvate and glutamate and producing ATP and phosphocreatine (Bradford, 1969; Bradford and Thomas, 1969); they also synthesise phospholipids (Abdel-Latif et al., 1968). Synaptosomes actively transport ions, maintaining membrane potentials which have been measured using $^{45}Ca^{2+}$ and fluorescent dyes (Blaustein and Weisman, 1970; Sen and Cooper, 1978) and are similar to those of intact nerve terminals. Synaptosome preparations contain a Na^{+} - dependent high-affinity system for accumulating choline (Yamamura and Snyder, 1973); evidence exists for similar systems for the uptake of catecholamines, putative amino acid transmitters and other metabolites (Levi and Raiteri, 1976).

Depolarisation of synaptosomes by electrical stimulation, increased external K^{+} concentration or by treatment with veratradine causes Ca^{2+} - dependent increases in the release (50 - 200%) of ACh (Wonnacott and Marchbanks, 1976), catecholamines (Mulder et al., 1975), GABA (Levy et al., 1974) and putative amino acids neurotransmitters (De Belleroche and Bradford, 1977). These increases in transmitter release are dependent

upon the presence of Ca^{2+} and are inhibited by Mg^{2+}. Uptake of $^{45}Ca^{2+}$ into synaptosomes is also stimulated by depolarisation with high K^+ concentrations, veratradine or scorpion venom and competitively inhibited by Mg^{2+} (Blaustein, 1975).

In vitro preparations of nerve terminals have proved extremely useful in biochemical studies on neurotransmitter release. Synaptosomes may be suspended at concentrations which greatly facilitate measurements of the binding of toxins and drugs, the transport or metabolism of ions and substrates as well as synthesis and release of neurotransmitters. In addition, ions, substrates or effectors may be added to the medium at any desired concentration without problems of tissue penetration associated with most electrophysiological preparations and brain slices. Since there is a limited number of preparations suitable for electrophysiological studies, much of the current information on neurotransmitter release in the central nervous system has been gained from experiments using synaptosomes. For example, both Ca^{2+} uptake and nonadrenaline release from synaptosomes from rat brain were shown to have a sigmoidal dependence on external K^+ concentration (Blaustein et al., 1977). These studies also indicated that extrusion of Ca^{2+} from nerve terminals occurs principally through a Na^+/Ca^{2+} exchange system, the low intracellular Na^+ concentration being maintained by Na^+/K^+ ATPase (Blaustein et al., 1977; Sanchez-Armass and Blaustein, 1982), An ATP-dependent accumulation of Ca^{2+} was observed in disrupted synaptosomes in the presence of uncouplers of oxidative phosphorylation and an inhibitor of mitochondrial Ca^{2+} uptake (Blaustein et al., 1977); it was suggested that this represented a system for sequestering intra-terminal Ca^{2+} into synaptic vesicles.

The availability of a concentrated synaptosome preparation also allows direct biochemical studies on moleculer components of nerve terminals. Ca^{2+} influx into nerve terminals whether induced by depolarisation or Ca^{2+} ionophores has been associated with phosphorylation

of specific proteins in the plasma membranes of intact synaptosomes (Kruegar et al., 1977) which may be involved in the process of transmitter release.

Purely cholinergic nerve terminals have also been isolated from the electric organs of elasmobranchs such as Torpedo marmorata (Israel et al., 1976; Dowdall and Zimmermann, 1976). Ca^{2+} - dependent ACh release from depolarised Torpedo synaptosomes has been measured (Michaelson and Sokolovsky, 1978). These preparations also contain a Na^{+} - dependent high affinity uptake system for choline (Dowdall et al.,1977).

Although synaptosomes are a very useful biochemical preparation, as indicated above,they have a number of limitations and the results obtained must be cautiously interpreted. Ca^{2+} flux into depolarised synaptosomes from rat brain (0.02-0.5μA/cm^2, Blaustein, 1975) is much less than that into intact squid nerve terminals (35μA/cm^2, Llinas, 1977). These studies are further complicated by the ability of contaminating mitochondria and glial cells to sequester Ca^{2+}. Glial cells in rat dorsal root ganglia were also shown to release acetylcholine in a Ca^{2+} - dependent manner (Minchin and Iversen 1974). Contaminating cells and organelles, as well as synaptosomes, maintain membrane potentials. Attempts to measure these potentials must therefore represent an average of all such components in the preparation. Since brain tissue contains neurones using a wide range of neurotransmitters, measurements using a single transmitter compound may involve only a small proportion of the synaptosome population. The transmitter released by depolarisation usually represents only a small proportion of the total amount present in the synaptosomes (Kelly et al., 1979a) and occurs against a relatively large background "leakage" of transmitter as well as other synaptosomal contents. Wonnacott and Marchbanks (1976) measured appreciable release, in control incubations of both [^{14}C]ACh and [^{14}C] choline from synaptosomes which had been preincubated with the latter. Wernicke et al., (1974)

measured the release of 3% of the tissue content of the cytoplasmic marker enzyme, lactate dehydrogenase. Due to the nature of the synaptosome preparation, complete integrity of their compartmentation and control systems would not be expected. It is also possible that such leakage occurs from intact nerve terminals in a non-quantal manner and is, therefore, not apparent in electrophysiological experiments (Kelly et al., 1979a).

As has been previously discussed, there is considerable evidence, from morphological observations, that exocytosis occurs in the nerve terminal during the process of neurotransmission. However, it has not been possible to prove directly that the vesicles seen fusing with the presynaptic membrane do contain neurotransmitter. The difficulty of homogenising skeletal muscle has precluded the isolation of synaptic vesicles from muscle and the analysis of their contents. Direct comparison of the number of ACh molecules per vesicle and the number per quantam has not therefore been possible. Synaptic vesicles have been isolated from mammalian brain, where ACh is only one of a number of transmitters, and from the purely cholinergic electroplaques of electric fish. Whilst all such studies have shown that neurotransmitter is present in synaptic vesicles there is a lack of agreement as to its distribution between the vesicles and the intraterminal cytoplasm. Probably the main cause of this is that some transmitter is liberated from vesicles which are damaged during the homogenisation and isolation procedures. When fractionating the electric organ of Torpedo marmorata, Marchbanks and Israel (1971) reported two fractions containing ACh, one bound or vesicular and one free and highly labile. In contrast, Whittaker and co-workers interpreted their results as showing two particulate fractions containing ACh; VP_1 which corresponds to the vesicular fraction of Marchbanks and Israel and a heavier VP_2 fraction (Suszkiw et al., 1978).

A number of attempts have been made to discover from which ACh

pool the transmitter is released in the physiological process. When electroplaque tissue was incubated with radiolabelled choline, the ACh which was subsequently released on stimulation was found to have the same specific radioactivity as that of the free fraction (Dunant et al., 1972) or that in the VP_2 fraction (Suszkiw et al., 1978). Whittaker and his colleagues also allowed electroplaque tissue to synthesis transmitter from a mixture of choline and a choline analogue, in this case, the ratio of true : false transmitter released by the tissue on stimulation was similar to the ratio observed in the VP_2 vesicle fraction and different to that in other fractions (Schwarzenfeld et al., 1979). Similar experiments identified a corresponding vesicle fraction (H) in guinea pig cortex which also preferentially accumulated newly synthesised transmitter and had the same ratio of true : false transmitter as was observed in the perfusion medium following high frequency stimulation (Schwarzenfeld et al., 1979).

However, radioactive tracer experiments have been performed using preparations from mammalian central nervous system, on neurotransmitters such as glutamate, aspartate and GABA; De Belleroche and Bradford (1977) have shown that newly accumulated or synthesised transmitter, which has the highest specific radioactivity, is present in the free "compartment" and is of the same specific radioactivity as that released following stimulation. These results have been interpreted by its opponents as evidence against the vesicular hypothesis. Those in favour argue that only a sub-population of vesicles is actually active in the release process and that this sub-population is either too small to be detected by biochemical methods or is labile and gives rise to the apparently cytoplasmic transmitter.

Israel and co-workers have developed a method for assaying, by chemiluminescence, the ACh released from a suspension of Torpedo synaptosomes (Israel and Lesbats, 1981). A single freezing and thawing of the same suspension liberated into the medium a fraction of ACh which was assumed to be cytosolic, allowing it to be measured directly. The

remaining ACh (60-70% of the total), which could only be released by treatment with detergent, was taken to represent vesicular ACh. The former ACh fraction was the one which was released from the synaptosomes in a Ca^{2+} - dependent manner, by increased K^{+} (Israel and Lesbats, 1981). This provided strong evidence that ACh was released from the cytoplasm of the nerve terminals rather than from vesicles; nevertheless, it is possible that the subpopulation of vesicles (VP_2) postulated by Whittaker and co-workers as the immediate source of released ACh may be labile to the freezing and thawing cycle. Subsequently this technique was used to assay ACh released from resealed synaptosomal sacs which were devoid of synaptic vesicles. The amount of ACh release by the calcium ionophore A23187 was found to be dependent on the concentration of the transmitter in the medium in which the sacs were resealed (Israel et al.,1981). These experiments demonstrate that ACh can be released directly from the cytosolic compartment of nerve terminals but how closely this release is related to the physiological process remains to be shown.

Similar arguments apply to experiments in which the number of vesicles in the nerve terminals is compared to the amount of quantal release. Motor nerve terminals can be depleted of vesicles by treatment with black widow spider venom which as previously discussed, causes a very large burst of transmitter release and increases the number of exocytotic events (Ceccarelli et al., 1979a). After vesicle depletion no quantal release was observed by these workers although some ACh remained in the tissue. Torpedo electric organs were depleted of vesicles by repetitive stimulation. When the tissue was allowed to recover only a few synaptic vesicles were present when neurotransmission was fully recovered (Zimmermann and Whittaker, 1974).

An attempt was made to selectively destroy cytoplasmic ACh by injecting acetylcholinesterase into cell bodies of cholinergic interneurones in the buccal ganglion of Aplysia (Tauc et al., 1974).

The enzyme was assumed not to enter the synaptic vesicles and, therefore, not to affect their content of ACh. Post-synaptic potentials (psp's) declined and were eventually abolished coincident with the transport of AChE to the nerve terminals, as followed by histochemical staining. The action potential in the interneurnones was unaffected and heat inactivated AChE had no effect on psp's. This seems to provide a clear evidence that cytoplasmic rather than vesicular ACh is involved in quantal release. However, hydrolysis of ACh releases H^+ and it has been estimated that if the cytoplasmic ACh has a concentration of 20 mM its hydrolysis would produce a pH shift in the nerve terminal from 7.4 to 6.6 (Van der Kloot, 1977).

Biochemical and electrophysiological experiments have not yet provided conclusive evidence for or against the vesicular hypothesis of neurotransmitter release. Whether or not the vesicular hypothesis proves to be correct, it is still very likely that specific components of the presynaptic membrane are involved in the release process, be they sites of vesicle attachment and fusion or gated channels which allow transmitter efflux from the cytoplasm. The premise of this study is that such components of the release mechanism may be amenable to investigation using toxins which specifically interact with them.

1.2 The Use of Neurotoxins in Biochemical Studies of Neurotransmission

The molecular components involved in the process of neurotransmission are present, in tissue, in extremely low amounts. Levels of nicotinic acetylcholine receptors are typically only a few picomoles per g wet weight of muscle (Dolly, 1979). Moreover, since each quantum is estimated to contain a maximum of 10^4 molecules of ACh (Kuffler and Yoskikami, 1975), components involved in release mechanisms may well be present at levels some orders of magnitude lower. Most, if not all,

of the macromolecules involved are likely to be integral membrane proteins; the physiological effects which they mediate may therefore not be measurable after their extraction from the membrane. Biochemical studies of such molecules in solution therefore require probes which bind them specifically and which can themselves be readily followed.

1.2.1. α-Neurotoxins

The α-neurotoxins, basic polypeptides from snake venoms have proved to be ideal probes for the nicotinic acetylcholine receptor which they specifically block; for reviews see Dolly (1979) and Conti-Tronconi and Raftery (1982). α-Bungarotoxin from the venom of Bungarus multicinctus has a molecular weight of 8000; it has been radioactively labelled with both ^{3}H and ^{125}I and show to retain its very high affinity for the receptor molecule ($K_D < 10^{-10}M$); its specific binding is inhibited by cholinergic ligands. Nicotinic acetylcholine receptors have been localated by the visualisation of α-bungarotoxin binding sites using a variety of scintillation, autoradiographic, fluorescent and histochemical techniques, quantitative autoradiography has been used to determine the density of receptors (Dolly, 1979). ^{3}H- and ^{125}I- α-Bungarotoxin derivatives, by making possible a simple and reproducible assay for the acetylcholine receptor have enabled the extraction and purification of the receptor and allowed a wide range of biochemical studies to be performed. Affinity chromatography using a second α-toxin of lower affinity is an important part of the purification of a receptor from detergent extracts of denervated skeletal muscle or elasmobranch electric organs. The physico-chemical properties and ligand binding characteristics of receptors from both sources have now been widely investigated (Dolly, 1979; Conti-Tronconi and Raftery, 1982). Partial sequencing of all four subunits of the electric organ acetylcholine receptor has been achieved (Raftery et al., 1980). Subsequently cloning of cDNA derived from mRNA isolated from

electric organ has allowed the complete sequence of the ACh-binding subunit to be determined (Sumikawa et al., 1982; Noda et al., 1982).

1.2.2. Toxins which affect ion channels

Several toxins have been isolated from widely different sources which affect the functioning of Na^+ channels in nerve and muscle membranes (Catterall, 1980). They fall into three distinct groups, Tetrodotoxin (TTX), from the ovaries of the puffer fish and saxitoxin (STX), from the Alaskan butterclam (Saxidomus giganteus) are water soluble heterocyclic compounds which specifically inhibit conduction through potential-dependent Na^+ channels (Narahashi et al., 1964; Hille, 1968). Radiolabelled derivatives of these toxins show reversible binding to the ion filters of Na^+ channels in non-myelinated nerves (Colquhoun et al., 1972; Henderson et al., 1974; Ritchie et al., 1976; Ulbricht, 1979) and, despite their different structures, compete for the same site. Radiolabelled derivatives of TTX and STX show saturable and specific binding to common sites on various excitable tissues with dissociation constants in the range of 1-5 nM (Catterall et al., 1982). The TTX/STX receptor has been solubilised and partially purified from eel electroplax (Agnew et al., 1978), mammalian brain (Catterall et al., 1982; Kreugar et al., 1979; Lazdunski et al., 1979) and skeletal muscle (Barchi et al., 1980). The TTX/STX receptors from brain and electroplax have components of molecular weight 250000-270000 daltons (Agnew et al., 1980; Catterall et al., 1982); the former has, in addition, subunits of molecular weight 32000 to 38000 daltons (Catterall et al., 1982) and the latter has minor components of 46000 and 59000 daltons (Agnew et al.,1980). The receptor isolated from skeletal muscle has subunits of 64000, 60000 and 53000 daltons (Barchi and Murphy, 1980).

The second group of toxins consists of lipid-soluble, non-proteinaceous compounds which depolarise excitable cells by increasing

Na^+ permeability (Catterall, 1980). They include Grayanotoxins (GTX), a series of closely related polycyclic compounds found in the leaves of plants of the Encaceae family (Narahashi, 1979), the alkaloids veratridine and aconitine (Catterall, 1980; Narahashi, 1979) and batrachotoxin, from the skin secretion of the Columbian frog Phyllobates aurotaenia (Alburquerque et al.,1973). These toxins all cause a shift in the voltage dependencies of the opening and closing of Na^+ channels. This results in an increase in the proportion of the channels which are open and a corresponding inhibition of the inactivation of the channels (Catterall, 1979; Narahashi, 1979). There is considerable variation between these toxins not only in their affinities for their putative binding sites on the molecule(s) which modulate(s) the Na^+ channel conductance through the ion filter but also in the proportion of the channels which they affect (Catterall, 1979). Ion flux studies have shown that these toxins bind to a common site (Catterall, 1980). Specific binding of ^3H-Batrochotoxin A 20-α-benzoate to rat brain synaptosomes (K_D 35nM) was shown to be inhibited by batrochotoxin,veratrine and aconitine (Catterall et al., 1981). Scorpion toxin (see below) which binds at a separate site enhanced the specific binding of the batrochotoxin derivative.

The third group of toxins affecting Na^+ channels are low molecular weight (4000-90000 daltons), single chain, basic polypeptides which have been isolated from a number of scorpion (Diniz, 1978; Linden and Raftery, 1976; Romey et al., 1976; Tazieff-Depierre, 1975; Rochat et al., 1979) and sea anemone (Rathmeyer, 1979) venoms. They cause an essentially irreversible, Na^+-dependent depolarisation of nerve and muscle membranes which is blocked by tetrodotoxin and potentiated by veratridine. These toxins inhibit the inactivation of Na^+ channels (Catterall, 1979). The specific binding to neuroblastoma cells and synaptosomes of radiolabelled derivatives of scorpion toxins (K_D's 0.5-15nM) was inhibited by depolarisation of the cells (Rochat et al., 1979; Catterall, 1977), was mutually competitive and also occurred at the same

site as the binding of a sea anemone toxin (Catterall, 1980). This site is separate from those which bind the first and second group of toxins which affect Na^+ channels although their potentiation of each others action suggests a strong interaction between the sites of binding of the second and third groups (Catterall, 1980). Since the voltage dependence of scorpion toxin binding resembles that of Na^+ channel activation, rather than inactivation, it has been suggested (Catterall, 1980) that these toxins may bind to that part of the Na^+ channel complex which responds to the depolarisation of the membrane.

These three groups of toxins have been very useful in studying the various components of the Na^+ channels involved in propagation of action potentials and are likely to prove increasingly so. Despite these advances very little is known of the molecular mechanisms by which neurotransmitters are released in response to depolarisation of nerve terminals. There are, however, a series of neurotoxins which specifically affect presynaptic processes and may prove to be useful probes for components of the transmitter release mechanism. A brief survey of these toxins is carried out in the following section.

1.3. Presynaptic Neurotoxins and Their Potential Usefulness as Probes

1.3.1. Toxins from black widow spider venom

An aqueous extract of the venom glands of the black widow spider (Lactrodectus mectans tredecimguttatus) causes a massive quantal release of neurotransmitter when applied to nerve-muscle preparations which use different neurotransmitters such as those from frog (cholinergic) (Longenecker et al., 1970), lobster (glutamatergic and gabaminergic) (Kawai et al., 1972) and locust (glutamatergic) (Cull-Candy et al., 1973). When applied to a frog nerve muscle preparation, the venom caused, after a lag of several minutes, about a 1000-fold increase in the frequency

of mepp's which then gradually declined. Amplitude of epp's was at first increased, concomitant with peak mepp frequency, but then fell to zero (Longenecker et al., 1970). The increase in transmitter release was paralleled by a virtually complete depletion of synaptic vesicles, slight swelling of the nerve terminals and infolding of the plasma membrane with no other observable effects on the ultrastructure of the endplate or on surrounding tissue (Clark et al., 1970; Clark et al., 1972; Cull-Candy et al., 1973).

The aqueous extract of the venom glands of the black widow spider was shown to contain at least four components each having similar effects to whole venom. These proteins had similar molecular weights (130000 daltons), isoelectric points (pH 5.2-5.5) and were immunologically indistinguishable (Frontali et al., 1976). These different forms of the toxin showed specificities for different species. α-Latrotoxin which is the most potent component of the venom towards vertebrates shows little specificity for synapse types (Tzeng and Siekevitz, 1979). It caused release of ACh, noradrenaline and GABA from cerebral cortex and depleted vesicles from this tissue (Tzeng and Siekevitz, 1979). It also increased release of catecholamines from a neurosecretory cell line PC 12 as well as a causing a large inward Ca^{2+} flux (Grasso et al., 1980). It was suggested by these authors that the toxin either activates physiologically voltage-dependent Ca^{2+} channels or induces new channels in the membrane similar to those which the venom forms in artificial lipid bilayers (Finkelstein et al., 1976). Specific binding of radiolabelled α-latrotoxin to synaptosomal membranes and PC 12 cells has been measured with dissociation constants in the range 0.5-2nM (Grasso et al., 1982).

1.3.2. Bacterial toxins

1.3.2.1. Botulinum toxin

The eight strains of Clostridium botulinum (A-G) so far isolated produce extremely potent neurotoxins with widely different species specificity (Smith, 1977), of which strain A is the most toxic to man and the most widely studied. The neurotoxic protein is synthesised as a progenitor toxin which is activated by a protease and which is stabilised in a complex with haemaglutinin; for reviews see Smith (1977) and Simpson (1979). Type A botulinum toxin (BoTX) was prepared in a crystalline form with a molecular weight of about 900,000 daltons (Putnam et al., 1948) which contained two haemaglutinin molecules and one neurotoxin (Wagman, 1954). The neurotoxin (BoNT), molecular weight 150000 daltons, can be isolated from the complex by ion-exchange (Dasgupta and Boroff, 1968) or affinity (Moberg et al.,1978; Tse et al., 1982) chromatography. BoNT contains two polypeptide chains of 53,000 and 97,000 daltons which are linked by disulphide bridges (Dasgupta and Sugiyama, 1972).

Botulinum toxin acts in vivo by blocking cholinergic transmission in the peripheral nervous system. The action of BoTX on neuromuscular transmission has been investigated in electrophysiological experiments using crystalline preparations of toxin (Boroff et al., 1974). The purified neurotoxin, however, shows similar effects at the mammalian neuromuscular junction (Tse et al., 1982). Nerve-muscle preparations from rats poisoned with BoTX showed a blockade of evoked transmitter release (Cull-Candy et al.,1976). Crystalline BoTX produced a progressive and irreversible decline of indirectly stimulated muscle contraction and e.p.p. amplitude in skeletal nerve-muscle preparations from rat, with no postsynaptic effects (Chang and Huang, 1974; Cull-Candy et al., 1976; Simpson, 1981). The decline in transmitter release was preceded by a latent period, even

at the highest toxin concentrations. Irreversible interaction of the toxin with the nerve terminal occurred within 20 minutes and was not dependent on temperature or neuronal activity. The rate of subsequent blockade was highly dependent on both temperature and the frequency of nerve stimulation and was inhibited by replacement of Ca^{2+} in the medium with Mg^{2+} (Cull-Candy et al., 1976; Simpson, 1980). The toxin was inactivated by antitoxin in the period immediately following its binding but subsequently became resistant suggesting that it is removed from contact with the external medium (Simpson, 1980).

Once developed, the neuromuscular blockade produced by BoTX can be partially and temporarily relieved by treatments which increase the intra-terminal Ca^{2+} concentration, i.e. tetanic stimulation, increased extracellular Ca^{2+} concentration, Ca^{2+} ionophores, black widow spider venom and 4-aminopyridine or tetraethylammonium ions which block K^{+} channels (Cull-Candy et al., 1976; Lundh et al., 1977; Simpson, 1978). Batrachotoxin, which activates Na^{+} channels, had no effect on BoTX poisoned synapses (Simpson, 1978). It was concluded that while BoTX does not block transmembrane flux of Ca^{2+} it does greatly reduce the efficacy of intraterminal Ca^{2+} in triggering transmitter release (Cull-Candy et al., 1976; Simpson, 1978).

BoTX abolished stimulation evoked fusion of synaptic vesicles with the presynaptic plasmalemma as shown by freeze-fracture electron microscopy (Pumplin and Reese, 1977). Vesicle fusion at active zones caused by black widow spider venom was also abolished by BoTX but no effect of the toxin was observed on the extrazonal vesicle fusion promoted by the spider venom in the absence of Ca^{2+} (Pumplin and Reese, 1977). Following treatment of BoTX-poisoned muscles with black widow spider venom, clumping of synaptic vesicles around release sites was observed in otherwise depleted nerve terminals (Kao et al., 1976). These

findings indicated that the toxin caused direct inhibition of the process of vesicle fusion with the nerve terminal membrane. Specific localisation of ^{125}I-labelled crystalline BoTX binding at neuromuscular junctions in mouse diaphragm was shown by autoradiography at the light microscope level (Hirokawa and Kitamura, 1975).

There has been only one report of an action of BoTX in the central nervous system *in vivo* when it affected the E.E.G. pattern in monkeys injected intravenously with toxin (Polley *et al.*, 1965). However some crystalline or more crude BoTX preparations have been shown to block depolarisation evoked release of radiolabelled ACh from synaptosomes (Wonnacott and Marchbanks, 1976) or slices (Gundersen and Howard, 1978) of mammalian brain tissue, preloaded with radiolabelled choline, at very high doses and with a considerable latency. Although Wonnacott and Marchbanks (1976) found no effect of their toxin on the accummulation of [^{14}C] choline and synthesis of [^{14}C] ACh, Gundersen and Howard (1978) reported that uptake of choline into mouse brain slices was inhibited following pretreatment with BoTX; this can probably be attributed to the effect of the toxin on ACh release. The Ca^{2+} ionophore A23187 increased release of both radiolabelled choline and ACh from normal and bolutinum poisoned synaptosomes (Wonnacott *et al.*, 1978). Purified botulinum neurotoxin (4.3nM) was found to decrease by 52% the release of [^{3}H] ACh from preloaded rat cortex synaptosomes which was induced by K^{+}(55mM) and by 57% the [^{3}H] ACh release from unstimulated synaptosomes (Dolly *et al.*, 1981a). [^{3}H] choline accumulation by the synaptosomes was decreased slightly, but Ca^{2+} influx was not inhibited (Dolly *et al.*, 1981a, 1982).

Some evidence was obtained of binding of ^{125}I-labelled crystalline BoTX (Haberman, 1974) and purified neurotoxin (Kitamura, 1976) to central nervous system preparations. However these studies did not conclusively demonstrate that this binding was either saturable

or to specific sites. However an indication of specificity of this interaction was the localisation, by an immunocytochemical method, of neurotoxin binding sites solely on the extracellular face of nerve terminal membranes (Hirokawa and Kitamura, 1979). Finally, saturable binding of a well characterised ^{125}I-BoNT to synaptosome membranes has been measured, with dissociation constant of 2 x 10^{-10}M and a maximum binding capacity of 150 f mol. mg of protein^{-1}, (Dolly et al., 1981a, 1982).

The extreme potency and irreversibility evidently requires very tight as well as very specific binding. For this reason BoTx, and more especially the purified neurotoxin component, seemed likely to prove an extremely valuable probe for the components of the release mechanism at both peripheral and central synapses. However, in view of the difficulties experienced in obtaining sufficient quantities of this toxin at the time this study was commenced it was not used in work described herein.

1.3.2.2. Tetanus toxin

A neurotoxic protein of molecular weight 150000 daltons consisting of covalently linked subunits of approximately 100000 and 50000 daltons has been isolated from cultures of Clostridium tetani. It produces muscle rigidity when injected into mammals, probably by blocking the release of neurotransmitters at inhibitory synapses (Kryzhanovsky, 1973). It inhibits release of GABA and putative amino acid transmitters from synaptosomes (Osborne and Bradford, 1973). ^{125}I-labelled tetanus toxin has been shown to ascend the spinal column by reverse axoplasmic transport (Haberman, 1973; Price et al., 1975) and to bind to neuronal cells in culture (Dimpfel et al., 1975; Mirsky et al., 1978). Its action at the neuromuscular junction has many similarities to that of BoTX; it produces a blockade of both evoked and spontaneous ACh release which can be partially reversed by repetitive nerve stimulation but not raised extracellular K^+ concentration.

The amplitude distribution of mepp's is skewed, with a disproportionately large number of low amplitude (Duchen and Tonge, 1973). ^{125}I-Tetanus toxin was localised at motor nerve terminals following intramuscular injection and at synaptic terminals in spinal chord following injection into the ventral horn (Price et al., 1977). Tetanus and botulinum toxins which are similar, both in their bacterial origins and their structures, may therefore inhibit release of neurotransmitters by similar molecular mechanisms despite having specificities for different types of synapses. Both toxins, when available in sufficient quantities in a homogeneous form may prove to be useful tools for investigating the molecular mechanism involved in neurotransmitter release.

1.3.3 Toxins from Snake Venoms

Presynaptically acting toxins have been found in the venoms of many elapid snakes including, principally Bungarus multicinctus (Chang et al., 1973), Bungarus caeruleus (Lee et al., 1976), Notechis scutatus scutatus (Harris et al., 1973; Karlsson et al., 1972) and Oxyurarus scutellatus (Fohlman et al., 1976; Kamenskaya and Thesleff, 1974) and in that of the crolatid snake Crolatus durissus terrificus (Vital Brazil and Excell, 1971).

These toxins, all of which produce an eventual blockade of ACh release at the vertebrate neuromuscular junction, have a number of striking similarities. They all contain, either in their single polypeptide chains or in one of their subunits a basic phospholipase A which has considerable sequence homology to other phospholipases A in snake venoms or mammalian pancreas (Karlsson, 1979; Kondo et al., 1978b). The effects of these toxins are only observed after a short latent period during which an irreversible interaction of toxin and nerve terminal occurs. Except for those from Notechis scutatus (Chang, 1979; Chang and Su, 1982) all of these toxins have a triphasic mode of action on

quantal ACh release at the neuromuscular junction, both spontaneous release and that evoked by nerve stimulation. An initial decrease in release is followed by a transient increase and subsequently, a decline to complete blockade; the first phase of this action is particularly apparent in media with low Ca^{2+} concentrations (Abe et al., 1977; Chang et al., 1977a and b; Chang 1979; Chang and Su, 1982). The rates at which they produce neuromuscular blockade are dependent on temperature and on the frequency of nerve stimulation and are decreased by high concentrations of Mg^{2+}. β-BuTX, crotoxin and taipoxin have been shown to mutually potentiate each others actions at neuromuscular junctions in mice, suggesting that, despite their similar effects, they act at different sites (Chang and Su, 1980).

1.3.3.1. Notexin

Three homologous proteins have been isolated from the venom of the Australian tiger snake (Notechis scutelatus scutelatus) by gel filtration and ion-exchange chromatography (Karlsson et al., 1972). Notexin and notechis II-5, single chain proteins (13500 daltons) contain 119 amino acids differing in seven amino acid positions (Halpert and Eaker, 1975, 1976). Notexin and notechis II-5 are toxic to mice (LD_{50} following i.v. injection 17 and 45 ng/g body weight, respectively) (Karlsson, 1979) and have weak Ca^{2+}-dependent phospholipase A activities which are activated by deoxycholate (Halpert et al., 1976); notechis II-5 is less toxic but has a greater phospholipase activity than notexin. Notechis II-2, the third protein, is non toxic and has no enzyme activity (Karlsson, 1979). Notexin loses its enzyme activity and lethality when a single histidine residue is modified with p-bromophenacyl bromide (Halpert et al., 1976). There is a 33% sequence homology between these proteins and phospholipases A_2 from porcine pancreas and other elapid venoms (Karlsson, 1979).

Notexin inhibited the release of neurotransmitters; it

reduced the frequency of mepps (Chang and Su, 1982) and inhibited choline and GABA accumulation by rat cortex synaptosomes (Harris and MacDonell, 1979; Sen et al., 1978) and increased ACh release (Sen et al., 1978). However, it also decreased the resting membrane potential in mouse muscle fibres (Harris et al., 1973) and, at a concentration of 7×10^{-8}M, it significantly decreased the postsynaptic response to ACh in chick muscle (Lee et al., 1976). Severe muscle necrosis was also observed following its subcutaneous injection (Harris et al., 1979). Since notexin consists of only one polypeptide chain which must be involved in both pre- and postsynaptic actions, this toxin is unlikely to prove a specific probe for the mechanism of neurotransmitter release.

1.3.3.2. Taipoxin

Gel filtration of the venom of the Australian taipan (Oxyuranus scutellatus scutellatus) followed by zone electrophoresis on a cellulose column at pH 7.5 separated an extremely potent neurotoxin. This protein, taipoxin, consists of three subunits in a complex which is non-covalently linked (Fohlman et al., 1976); it blocked release of ACh at the neuromuscular junction (Chang et al., 1977b; Kamenskaya and Thesleff, 1974). The α subunit is very basic (13,800 daltons, pI $\geq$ 10), has a weak phospholipase activity and is toxic to mice following intravenous injection of 300 ng/g body weight (Karlsson, 1979). The β and γ subunits (13,500 daltons, pI = 7 and 18,400 daltons, pI < 2.5, respectively) have no appreciable toxicity or phospholipase activity (Karlsson, 1979). The enzyme activity of the α subunit is almost abolished on formation of the ternary complex which, however, has a greatly enhanced toxicity (LD_{50} = 2.1 ng/g body weight following intravenous injection into mice) (Karlsson, 1979). It was reported by Kamenskaya and Thesleff (1979) that modification of two subunits of the toxin by p-bromophenacyl bromide

reduced but did not abolish its effects on neurotransmission; they also found no loss of neuromuscular blocking action when Ca^{2+} was replaced by Sr^{2+}. It was later reported (Fohlman et al., 1979) that alkylation of both the α and β subunits or of the α subunit alone decreased the lethality of taipoxin by 350-fold, but reduced by only 2 to 3-fold, its effect on the high affinity uptake of choline into T sacs from Torpedo marmorata. Modification of the β subunit alone decreased lethality five-fold but did not reduce the effect of the toxin on choline uptake.

Taipoxin affects the release of ACh in a similar manner to other presynaptic toxins (1.3.3) 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., 1979). The ternary taipoxin complex is dissociated by treatments such as low pH and by ion exchange chromatography (Karlsson,1979); radiolabelling of this toxin with the retention of biological activity may, therefore, be particularly difficult.

1.3.3.3 Crotoxin

The major neurotoxin in the venom of the South American rattlesnake (Crotalus durissus terrificus) was isolated by precipitation at its isoelectric point (pH 4.7 - 4.8) from a solution of the venom in dilute HCl (0.013 - 0.016M) which had been heated to 70°C for 10 min. This toxin (crotoxin) can be crystallised from pyridine acetate at pH 4.4 by slow cooling from 55°C; for a review see Karlsson (1979). Crotoxin is composed of two subunits, a very basic phospholipase A_2 (crotoxin B, 13,000 - 16,000 daltons) which has a low toxicity (440 ng/g body wt of mouse) (Marlas and Bon, 1982) and an acidic protein (m.w. 8,500 - 9,500) (crotoxin A or crotopotin) which consists of three polypeptide chains

linked by disulphides and which has no toxicity or enzyme activity (Horst et al., 1972; Breithaupt et al., 1974; Karlsson, 1979). The amino acid sequence of crotoxin B has considerable homology to other snake venom phospholipases (Fraenkel-Conrat et al., 1979) and acylation of an essential histidine residue with p-bromophenacyl bromide destroys the enzyme activity (Karlsson, 1979).

When crotoxin B is complexed with crotopotin its toxicity is greatly enhanced. The LD_{50} of crotoxin following intravenous injection is 25 ng/g body weight (Marlas and Bon, 1982). 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 (1.3.3; 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 endplates to ACh (Vital Brazil and Excell, 1971) and electroplaque (Marlas and Bon, 1982) to CCh. Crotoxin and component B inhibit the carbamyl choline-induced influx of $^{22}Na^{+}$ into microsacs from the electroplaque of Torpedo marmorata (Hanley, 1978; Marlas and Bon, 1982) but do not compete with α-bungarotoxin binding. Component A (crotopotin) has no blocking activity but potentiates the action of component B when complexed with it to form crotoxin (Hanley, 1978; Marlas and Bon, 1982). Radiolabelled crotoxin B shows non-saturable binding to receptor-rich Torpedo membranes whereas crotopotin shows very little binding. When added as a complex, the binding of crotoxin B is limited to saturable, high affinity sites and crotopotin is released into the medium (Bon and Marlas, 1979). The binding does not require Ca^{2+} and itself does not affect receptor function which is impaired by a second Ca^{2+}-dependent step (Bon and Marlas, 1979). Very similar results were obtained for the binding of [^{3}H] crotoxin to mouse diaphragm (Chang and Su, 1981).

Although the additional postsynaptic actions of crotoxin make it a poor candidate as a probe for transmitter release mechanism, the synergistic action between the basic phospholipase and the acidic crotopotin subunits may indicate mechanisms by which the other phospholipase neurotoxins exert their highly potent and specific effects.

1.3.3.4 β-Bungarotoxin

i) Source, structure and toxicity

Ion-exchange chromatography of the venom of the Formosan banded krait, Bungarus multicinctus, separated several very basic polypeptides which irreversibly blocked neurotransmission in an in vitro nerve-muscle preparation but did not affect the post-synaptic response to bath-applied ACh (Dryden et al., 1974; Lee et al., 1972). These toxins were termed β-toxins to distinguish them from the post-synaptically acting α-toxins. They all have, with the exception of the most acidic and the most basic (Hanley et al., 1977; Tobias et al., 1978), molecular weights of about 22,000 daltons and consist of two subunits linked by disulphide bridges (Hanley et al., 1977). The most abundant (by weight) of these toxins, which is also one of the most potent, has been further purified by a number of workers (2.1) and its mode of action extensively studied. This toxin will, henceforward, be referred to by the name β-bungarotoxin (β-BuTX), coined by Lee et al. (1972).

β-BuTX has an LD_{50} following intraperitoneal injection into mice of approximately 0.01 μg/g body weight (Lee et al., 1972; Strong et al., 1976). The whole animal toxicity and the potency of the toxin in producing blockade of a vertebrate, skeletal nerve-muscle preparation, together with the latencies which are observed in these effects vary considerably between different animal species (Chang and Huang, 1974). The effect of the toxin is irreversible after the

first 15-20 mins (Chang et al., 1973; Kelly and Brown, 1974). The rate of onset of neuromuscular blockade is to some extent dependent on the frequency of nerve stimulation and is antagonised by low Ca^{2+} or high Mg^{2+} concentrations (Chang et al., 1973).

β-BuTX shows a phospholipase A_2 activity which is dependent on the presence of Ca^{2+} and is greatly stimulated by the detergent deoxycholate (Chapter 4). The larger of the two subunits (13,000 daltons) has a considerable degree of sequency homology to pancreatic and other snake venom phospholipases A_2 whilst the sequence of the second subunit (7,000 daltons) is similar to those of certain protease inhibitors (Kondo et al., 1978b). The phospholipase activity can be diminished by chemical modification of the toxin with p-bromophenacyl bromide or by replacement of Ca^{2+} in the medium with Sr^{2+}. Although it is required for both whole animal toxicity and full expression of the neuromuscular blocking action of β-BuTX, (Abe et al., 1977; Chapter 3), the enzyme activity is not sufficient to account for the potency and specificity of action of the toxin (Strong et al., 1976). Recent experiments using enzymically inactive derivatives of β-BuTX as well as crotoxin and notexin produced residual, transient inhibition of ACh release at vertebrate neuromuscular junctions (Chang and Su, 1982). β.Bungarotoxin, therefore, interacts specifically with the nerve terminal to cause an inhibition of transmitter release and is potentially a very useful probe for the mechanisms involved in this process.

Abe et al. (1977), using a different procedure to that of Lee et al. (1972), separated four presynaptically acting toxins, with molecular weights of 22,000 from Bungarus multicinctus venom. These varied widely in toxicity but all showed Ca^{2+}-dependent phospholipase activity in the presence of deoxycholate, which was abolished by acylation with p-bromophenacyl bromide. These workers did not examine the most basic component of the venom, the last to be eluted on ion exchange chromtography. Due to the different numbers of toxins separated from Bungarus multicinctus venom by essentially the same

procedure, it is not certain that the finally eluted component represents the same toxin in every study in which its properties have been examined. Two groups reported that the most basic toxin produces a solely presynaptic blockade of neuromuscular transmission (fraction 8, Lee et al.,1972; fraction 13, Dryden et al.,1974). Hanley et al. (1977) showed that the most basic protein which was separated by their procedure consisted of a single polypeptide chain (13,800 daltons) with an amino acid composition similar to that of notexin and reported that it had myotoxic effects similar to those of the latter. A single chain toxin (11,500 daltons), with an amino acid composition similar to that reported by Hanley et al. (1977) was the last to be eluted on ion-exchange chromatography of Bungarus multicinctus venom by Tobias et al. (1978). This toxin, which possessed phospholipase activity in the presence of Ca^{2+} and deoxycholate, had a triphasic effect on the spontaneous and evoked release of ACh at the vertebrate neuromuscular junction, similar to that of β-BuTX (Livengood et al., 1978).

A toxin was isolated from the venom of Bungarus ceruleus which had a similar subunit composition to β-BuTX, showed phospholipase activity in the presence of Ca^{2+} and deoxycholate and, although much less potent, produced a triphasic effect on ACh release at the neuromuscular junction similar to that of β-BuTX (Abe et al., 1977).

ii) The actions of β-bungarotoxin at the vertebrate neuromuscular junction

β-Bungarotoxin has, in common with other presynpatic toxins from snake venoms, a triphasic effect on both spontaneous and evoked release of ACh and the neuromuscular junctions of frog and rat. Addition of β-BuTX (3 - 20 μg/ml) at 20°C to an indirectly stimulated

nerve-muscle preparation gives rise, within a few minutes, to a decline in mepp frequency (Abe et al., 1976; Abe et al., 1977) and in the force of isometric muscle contraction (Chapter 2; MacDermot et al., 1978a). During the first phase of β-BuTX action the frequency of mepps may be reduced to 20% or less of the control value (Abe et al., 1976); the decrease in evoked release is more pronounced and can be seen more clearly following treatment of the preparation with d-tubocurarine (2.5 µg/ml, in low Ca^{2+} medium or at low temperature (Abe et al., 1976; Abe et al., 1977; Caratsch et al., 1981).

Phase 2 of the neuromuscular blocking action of β-BuTX consists of a period of increased ACh release somewhat longer in duration than the initial inhibition. Spontaneous release of transmitter is increased, usually to levels considerably in excess of the control (Chang et al., 1973; Kelly and Brown, 1974; Abe et al., 1976; MacDermot et al., 1978a). Bursts of m.e.p.p.s appear with synchronous release of several ACh quanta and periods of high frequency spontaneous release are observed; spontaneous contractions of the muscle may also occur (Abe et al., 1976; Alderdice and Volle, 1978). It was reported that only at K^+ concentrations of 7.5 to 10 mM was an increase in m.e.p.p. frequency always elicited by β-BuTX (0.5 µg/ml) (Alderdice and Volle, 1978); other workers observed increases in spontaneous release by similar toxin concentrations at lower concentrations of K^+. Evoked released of ACh is also increased during this phase of β-BuTX action. The quantal content of epps is increased (Abe et al., 1977; Strong et al., 1977) and abnormally large as well as multiple, muscle contractions are observed on indirect stimulation (Kelly et al., 1979b). These increases are not observed in the absence of Ca^{2+} (Strong et al., 1977).

The period of increased release is followed by a gradual, steady decline of both spontaneous (Chang et al., 1973; Kelly and Brown, 1974; Abe et al., 1976; MacDermot et al., 1978a) and evoked (Chang et al., 1973; Kelly et al., 1975; Abe et al., 1977) ACh release resulting in complete blockade of neurotransmission. β-Bungarotoxin does not change the size of quanta of transmitter which are released (Kelly and Brown, 1974), a further indication that the toxin has no effect on the postsynaptic membrane or its acetylcholine receptors. Furthermore, the resting and action potentials in both muscle and nerve axons are unaffected by toxin treatment (Chang et al., 1973); a temporary increase in m.e.p.p. frequency at β-BuTX poisoned endplates can be elicited by increased K^+ concentration, indicating that action potentials continue to invade the nerve terminals (Chang et al., 1977a). The rate of onset of neuromuscular blockade produced by β-BuTX is dependent on temperature (Chang and Huang, 1974) and on the frequency of nerve stimulation (Chang et al., 1973). It is delayed by treatments which inhibit transmitter release such as low Ca^{2+} and high Mg^{2+} concentrations (Chang et al., 1973). It appears, from these observations, that β-BuTX blocks neurotransmission by specifically inhibiting the release of ACh and that this inhibition is not due to depletion of neurotransmitter from the nerve terminals.

The decline in isometric twitch tension produced by β-BuTX is paralleled by an increasingly rapid fall-off of muscle tension during tetanic nerve stimulation (Kelly and Brown, 1974). It was also reported that, during this third phase of β-BuTX action, most m.e.p.p.s were of low amplitude (MacDermot et al., 1978a). The delayed release of ACh, i.e. the amount of quantal transmitter release in the period of about 100 ms following the response to nerve stimulation was found to decline

with the same time course as e.p.p. amplitude following β-BuTX addition to rat diaphragm nerve muscle preparations (Strong et al., 1977). The toxin was also observed to temporarily restore neurotransmission which had been blocked by low Ca^{2+}.

The phospholipase activity of β-BuTX is required for the second and third phases of its action. Phase I, the initial decrease in transmitter release is however independent of the enzyme activity. This finding, and the similar observations made for β-ceruleotoxin and the 11,000 dalton, very basic "β-type" toxin from Bungarus multicinctus venom are discussed in detail in the introduction to Chapter 4 (4.1). An irreversible interaction of β-BuTX with the nerve terminals occurs after a short latent period; after this time, washing a β-BuTX poisoned nerve muscle preparation has no effect on the rate of the subsequent blockade (Chang et al., 1973; Kelly and Brown, 1974). It has been postulated that the initial Ca^{2+} and phospholipase-independent inhibition of transmitter release could be the result of β-BuTX binding to the nerve terminal membrane (Abe and Miledi, 1978), thereby conferring a specificity for the presynaptic membrane on the phospholipase activity of the toxin. β-BuTX has been shown to specifically disrupt nerve terminals with no observable ultrastructural effects on surrounding muscle or Schwann cells (Strong et al., 1977). It also causes denervation similar to that observed after transection of the motor nerve (Abe et al., 1976). These morphological effects are, however, only seen at concentrations (ca. 100 µg/ml) greatly in excess of those needed to produce electrophysiological blockade.

iii) The actions of β-bungarotoxin at other synapses

The release of ACh from the superior cervical ganglion of the cat, which was evoked by stimulation of the preganglionic nerve, was rapidly and irreversibly inhibited by β-BuTX (10 - 20 µg/ml). Spontaneous release of ACh was at first increased and subsequently returned to the

very low, control levels following toxin treatment (Kato et al., 1977). β-BuTX did not, however, block muscarinic cholinergic transmission in the isolated rat ileum (Chang and Lee, 1963; Kato et al., 1977) nor glutamtergic transmission at the locust neuromuscular junction (Tse et al., 1980). The toxin did not block the release of noradrenaline from the nerve terminals in the cat nictitating membrane preparation. It also had no effect on the sensitivity to noradrenaline of smooth and cardiac muscle (Kato et al.,1977).

A number of biochemical studies have been performed on the effects of this toxin on the accumulation and release of neurotransmitters by synaptosomes (see Chapter 3). No evidence has been obtained in these studies for neurotransmitter specificty of β-BuTX action, such as that found at non-central synapses. β-BuTX inhibits the accumulation by synaptosomes of choline (Wernicke et al., 1974; Sen et al., 1976; Spokes and Dolly, 1980) GABA (Wernicke et al., 1974; Tse, Dolly and Diniz, 1980), glutamate (Tse, Dolly and Diniz, 1980), noradrenaline and serotonin (Wernicke et al., 1974); it stimulates the release of previously accumulated ACh (Sen et al., 1976), glutamate (Tse et al., 1980); GABA (Wernicke et al., 1974; Tse et al., 1980) and noradrenaline (Wernicke et al., 1974).

1.4 Objectives of the Present Study

It is evident from the foregoing that β-bungarotoxin is a potentially useful probe for investigations of the mechanism of neurotransmitter release since it is likely to interact directly with one of the macromolecules involved in this system. Electrophysiological experiments carried out as part of this study on the effects of β-BuTX on neurotransmission in slices of rat olfactory cortex demonstrated, for the first time, inhibition of neurotransmitter release at intact

central synapses by this toxin (Chapter 4; Dolly et al., 1980a and b). β-BuTX is, therefore, an even more valuable toxin for use in investigations of neurotransmitter release in the central nervous system. The most straightforward approach to such an investigation was to attach a radioactive label to the toxin whilst retaining its ability to affect neurotransmitter release. Such a derivative might then be used as a marker for the components of the transmitter release mechanism. As a preliminary to radiolabelling β-BuTX it was necessary to obtain and characterise a completely homogeneous preparation of this toxin, hitherto unavailable (Chapter 2). The advantages of synaptosomes prepared from rat cerebral cortex for biochemical studies on neurotransmitter release in the central nervous system have already been discussed (1.1.3). The effects of pure β-BuTX on the accumulation and release of radiolabelled neurotransmitter compounds by this in vitro preparation of nerve terminals were measured (Chapter 3). They were compared with the toxin's action on intact synapses in the olfactory cortex of the rat, measured by extracellullar recording. The involvement of the phospholipase activity of the toxin in its action on these two systems was also investigated (Chapter 4). A radiolabelled derivative of β-BuTX was then prepared by the gentlest available method, characterised, and its binding to purified nerve terminals measured (Chapter 5).

CHAPTER 2. PURIFICATION AND CHARACTERISATION OF β-BUNGAROTOXIN

2.1 Introduction

The complexity of the neurotoxic effects of Bungarus multicinctus venom and the presence of several enzyme activities suggested that it contained a number of different protein species. The first attempt to resolve these components, by zone electrophoresis (Chang and Lee, 1963), gave four fractions, three of which were neurotoxic. The α fraction was shown to act by preventing the postsynaptic response to acetylcholine whereas the other two fractions, β and γ, acted pre-synaptically causing an eventual blockade of transmitter release. Subsequent ion-exchange chromatography of the venom (Lee et al., 1972) revealed a multiplicity of both pre- and post-synaptic toxins. Three postsynaptic or "α-type" neurotoxins were separated all of which were eluted from a CM-Sephadex column at lower salt concentrations than five presynaptic or "β-type" toxins. The most abundant, by weight, of the latter group which was also the most potent, was termed β-bungarotoxin by these workers.

The large number of very basic toxins in the venom and the variation in the conditions for the initial venom fractionation used by different groups of workers (Dryden et al., 1974; Eterovic et al., 1975; Kelly and Brown, 1974; Wernicke et al., 1974) led to some confusion, at the time this study was started, as to the identity and purity of the various preparations. The β-BuTX fractions obtained by Lee et al. (1972) initially contained a small amount of hyaluronidase activity which was removed by rechromatography on CM-cellulose; nevertheless, a contaminant remained which was detectable by electrophoresis in cellulose acetate. Strong et al. (1976) used a second

ion-exchange step to further purify the β-BuTX obtained from the initial fractionation of the venom (Kelly and Brown, 1974). This preparation was reported as giving a single protein band on sodium dodecyl sulphate polyacrylamide gel electrophoresis in non-reducing conditions and on isoelectric focusing over a broad pH range; the pI of the protein was not given. A β-BuTX preparation purified by gel filtration and a second ion-exchange step, following the initial venom fractionation, appeared to be pure when subjected to both native and sodium dodecyl sulphate polyacrylamide gel electrophoresis (Wernicke et al., 1974). However, it was shown to be heterogeneous on electrophoresis in cellulose acetate (Wernicke et al., 1975).

In view of this uncertainty, it was necessary to develop a method for preparing pure β-BuTX and to firmly establish its homogeneity by methods which depend on both molecular size and charge. This was an obvious prerequisite for the preparation and purification of a radiolabelled derivative of the toxin and the investigation of its interaction with nerve terminals. The criteria used for the purity of β-BuTX were given additional importance by the reported presence, in previous preparations, of a phospholipase A_2 activity (Strong et al., 1976; Wernicke et al., 1975). This enzyme activity was shown to be required for the lethality and neuromuscular blocking action of the toxin (Strong et al., 1976; Abe et al., 1977). Specific hydrolysis of phospholipids, unique to nerve terminals, was a possible mechanism of β-BuTX action. It was important, therefore, to determine whether the phospholipase activity resided in the homogeneous toxin or was a contaminant of the preparations discussed above. Since these preparations appeared to be pure on SDS polyacrylamide

gel electrophoresis it seemed likely that the contaminants could best be separated on the basis of their different charges.

2.2 Materials and Methods

2.2.1 Materials

Bungarus multicinctus venom was obtained from Miami Serpentarium Laboratories, U.S.A. Pharmalyte carrier ampholytes (pH 8 - 10.5) and Sephadex C-50,SP-50 and G-75 resins were all purchased from Pharmacia. Bee venom phospholipase A_2 was the gift of Dr R. Shipolini. α Chymotrypsinogen, cytochrome C, ovalbumin and myoglobin were supplied by Sigma. Ultrodex and Ampholine carrier ampholytes (pH 7 - 9 and 9 - 11) were from L.K.B. Biolyte carrier ampholytes (pH 8 - 10), carboxymethyl cellulose resin (CM-52) and egg yolk lecithin (grade 1) were obtained from Bio-Rad Laboratories, Whatman and Lipid Products, Surrey, U.K., respectively. All other chemicals used were of analytical grade.

2.2.2 Fractionation of Bungarus multicinctus venom

Ion-exchange chromatography of Bungarus multicinctus venom was performed by a modification of the procedure of Lee et al., (1972). Venom (1g) in 6 ml of ammonium acetate (0.05M, pH 7.0) was applied to a column of carboxymethyl-Sephadex C-50 resin (2.4 x 67 cm) which had been equilibrated with ammonium acetate (0.05M, pH 7.0). The sample was eluted from the column with a convex gradient produced from 400 ml of the same buffer in a stirred, airtight, flask connected to a reservoir of ammonium acetate (0.9M, pH 7.4). The initial flow rate was 20 ml/hr and 4 ml fractions were collected. All procedures were performed at 4°C. The protein concentration in the fractions was

measured by A_{280nm} and the ammonium acetate concentration was calculated from conductivity measurements. Peak V (0.87 to 0.95 dm^3) was pooled, desalted by gel filtration on a column of Sephadex G-25 and freeze dried.

2.2.3 Ion-exchange chromatography of partially purified β-bungarotoxin

The freeze dried material obtained from fractionation of the whole venom was re-chromatographed on the following series of ion-exchange columns using linear gradients of salt concentration or pH, as indicated. Columns i-iii were all carboxymethyl cellulose CM-52 (1.1 x 50 cm) and all procedures were carried out at 4°C.

i) The column was equilibrated with ammonium acetate (0.2M; pH 6.5) and eluted with a gradient of ammonium acetate (0.3M; pH 6.6 to 0.9M; pH 7.2 in 200 ml).

ii) The column was equilibrated with ammonium acetate (0.3M; pH 6.6) and eluted with a gradient of ammonium acetate (0.3M; pH 6.6 to 0.6M; pH 7.5 in 600 ml).

iii) The column was equilibrated with ammonium acetate (0.05M; pH 5.0) and eluted with a gradient of ammonium acetate (0.3M; pH 5.5 to 0.7M; pH 6.5 in 200 ml) (Strong et al., 1976).

iv) A sulphopropyl Sephadex C-50 column (1.5 x 15 cm) was equilibrated with ammonium acetate (0.05M; pH 5.0) and eluted with a gradient of sodium chloride (0.2M to 0.5M) in 500 ml of the same buffer. All procedures were carried out at 22°C (MacDermot et al., 1978).

2.2.4 Gel filtration of partially purified β-bungarotoxin

Further purification of β-BuTX, on the basis of size, was attempted by gel filtration on Sephadex G-75 (superfine) at 4°C. Partially purified β-BuTX from the initial venom fractionation (Fig. 2.1)

was dissolved in 1 ml of ammonium acetate (0.05M) and applied to a column (1 x 60 cm) of Sephadex G-75 equilibrated with the same buffer. The molecular weight of β-BuTX was measured by calibration of the column using ovalbumin, α-chymotrypsinogen and ribonuclease as markers.

2.2.5 Preparative isoelectric focusing

Final purification of β-bungarotoxin was carried out by isoelectric focusing in a flat bed of Sephadex G-75 using an L.K.B. 2117 multiphor apparatus (Winter et al., 1975). Two carrier ampholyte systems were used. Biolyte (pH 8-10) initially gave satisfactory separations which, however, could not be consistently reproduced with a later batch. Pharmalyte (pH 8 - 10.5) was then found to produce similar results with more reproducible pH gradients and a much reduced focusing time.

G-75 resin (1.6 g) was mixed with deionised water (38 ml) and 2 ml of carrier ampholyte solution, either Biolyte pH 8 - 10 or 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 (11.5 x 23 cm). 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 slurry was then air dried to a predetermined weight limit (Winter et al., 1975). The G-75 resin used (Ultrodex) had a factory determined evaporation limit of 65%, i.e. 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 cold tap water was circulated. The sample to be focused, 10 - 20 mg of protein dissolved in approximately 0.5 ml of water was applied in a narrow band towards the cathode end of the bed. Electrical contact

with the electrodes was via further wicks at each end. The solutions in which these wicks were soaked and the running conditions used with each ampholyte system are given below.

Biolyte: Cathode: NaOH (1M)

Anode: Phosphoric acid (1M)

Focused for 12 - 16 hours at 8 W constant power

Pharmalyte: Cathode: NaOH (1M)

Anode: Pharmalyte 8 - 10.5 (1%)

Prefocused for 45 minutes at 8 W constant power and focused, after sample addition, for 4½ hours at 10 W constant power. The focusing times were determined by trial runs using cytochrome C as a visual marker. When focusing was complete the position of the protein bands was determined by laying 2 - 3 mm wide filter paper strips along the bed; these were then fixed in trichloroacetic acid (10%), stained with Coomassie R 250 (0.2%) in methanol/water/acetic acid (5 : 5 : 1 by volume) and destained in the same solvent mixture. The portion of resin bed containing the major protein band was cut out. The Biolyte carrier ampholytes were removed by dialysis against ammonium acetate (0.05M) after the sample had been eluted from the resin in a small column with two volumes of the same buffer. The Pharmalyte carrier ampholytes were removed by placing the slurry taken from the bed (approximately 1 ml) on top of a Sephadex G-50 column and eluting with ammonium acetate (0.05M). The protein samples, in both cases, were then freeze dried.

2.2.6 Analytical isoelectric focusing

Focusing of small amounts (50 - 100 µg) of protein samples was carried out for 4 hours at 3 W constant power in a horizontal, water cooled slab of polyacrylamide gel (115 mm x 50 mm). Two carrier ampholyte systems were used for which the following gel

solutions were mixed:

	L.K.B. Ampholine system	Pharmalyte system
Acrylamide (15%)) Methylene bisacrylamide (0.42%))	4 ml	4 ml
N,N,N',N'Tetramethylethyl diamine (TEMED) (6%)	60 µl	60 µl
Ampholine pH 9 - 11 (20%)	0.6 ml	-
Ampholine pH 7 - 9 (40%)	0.3 ml	-
Pharmalyte pH 8 - 10.5 (20%)	-	0.5 ml
Sucrose (20%)	5.44 ml	5.0 ml
	Degas	
Ammonium persulphate (1%)	0.7 ml	0.7 ml

Electrical contact, between platinum wire electrodes and the gel surface, was made through filter paper wicks soaked in the following solutions:

Ampholine system:	Cathode	Ampholine pH 9 - 11 (1%)
	Anode	Ampholine pH 7 - 9 (1%)
Pharmalyte system:	Cathode	NaOH (1M)
	Anode	Pharmalyte pH 8 - 10.5 (1%)

The samples were applied to the surface of the polyacrylamide gel in volumes of 3 - 5 µl. The completion of focusing was indicated by the behaviour of coloured marker samples of cytochrome C. The position of the protein bands was determined by precipitation with trichloroacetic acid (40%) followed by washing in trichloroacetic acid (10%) to remove ampholytes.

2.2.7 Polyacrylamide gel electrophoresis

This was carried out, in non-denaturing conditions, at pH 4.5 in a β-alanine/acetate disc system as described by Riesfeld et al. (1962). The resolving gel contained 15% (w/v) acrylamide and 0.1% (w/v) methylene bisacrylamide. A current of 6 - 8 mA per gel was applied and bromocroesol green was used as a tracking dye. Protein bands were stained with Coomassie G-250 (0.04%; w/v) in perchloric acid (3.5%; w/v).

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis was carried out in a system for resolving small proteins described by Swank and Munkres (1971). The vertical slab gel contained 12.5% (w/v) acrylamide, 1.25% methylene bisacrylamide and 8M urea; samples were run under reducing (β-mercaptoethanol; 10% (v/v) added to the sample) and non-reducing conditions. After fixing in 40% trichloroacetic acid the gel was stained with Coomassie R-250 as described by Swank and Munkres. α-Chymotrypsinogen, myoglobin, cytochrome C and cyanogen bromide cleavage fragments of the latter were used as molecular weight markers.

2.2.8 Toxicity and phospholipase assays

The lethality of β-BuTX was determined following administration by two different routes:

a) Intraperitoneal injection of toxin, dissolved in NaCl (0.85%) containing bovine serum albumin (0.1 mg/ml), into 25 - 30 g mice.

b) Sprague-Dawley rats (150 g) were anaesthetised with and positioned in a stereotaxic apparatus. Toxin samples (10 µl) dissolved in 0.9% saline were injected into the cerebral intraventricular space at coordinates L-17, S-(-0.2) and D-4.0 mm according to the stereotaxic altas of Koenig and Klippel (1963).

The ability of the toxin to block neuromuscular transmission was also tested by measuring its effect on isometric contractions of a sartorius muscle isolated from frog in response to stimulation of the nerve. The force of the isometric contraction was measured using a Grass F.T. 03C transducer and Devices MX 212 recorder. Stimulation (1 - 2 v) was at a frequency of 0.1 Hz.

Phospholipase activity was assayed using a Radiometer autotitrator as a pH stat (Abe et al., 1977). Egg yolk lecithin, stored in solution in chloroform/methanol, was evaporated to dryness in a stream of nitrogen. The substrate was subsequently dispersed in the reaction mixture by sonication for three periods of 10 secs; it was cooled on ice for 1 - 2 mins between each period of sonication. The normal reaction mixture (volume 5.5 ml) contained lecithin (1.8 mM), NaCl (100 mM), $CaCl_2$ (10 mM) and sodium deoxycholate (0.18 mM). Where indicated the $CaCl_2$ was replaced by $SrCl_2$ (10 mM) or EGTA (5 mM); in some experiments the deoxycholate was omitted. Assays were carried out at 37°C and were initiated by addition of solutions of toxin or enzyme in water (10 - 25 µl). The H^+ produced was titrated with NaOH (0.01M) and the phospholipase activity, calculated from the initial slope, was expressed as µmol H^+ liberated. min^{-1}. mg of $protein^{-1}$.

2.3 Results

2.3.1 Ion-exchange chromatography of Bungarus multicinctus venom

The venom was eluted from the Sephadex C-50 column in this study with a gradient of ammonium acetate concentration (Fig. 2.1) which was convex and had a small increment in pH (7.0 to 7.4). Consequently, the latter part of the gradient, the region in which the presynaptic toxins are eluted, was much shallower than those previously

Figure 2.1 Ion-exchange chromatography of Bungarus multicinctus venom

Chromatography of venom (1g) was carried out as described in Section 2.2.2. Elution from a carboxymethyl Sephadex C.50 column was performed using a convex gradient of ammonium acetate concentration (●) and pH; 4 ml fractions were collected and the protein concentration measured by A_{280nm} (○).

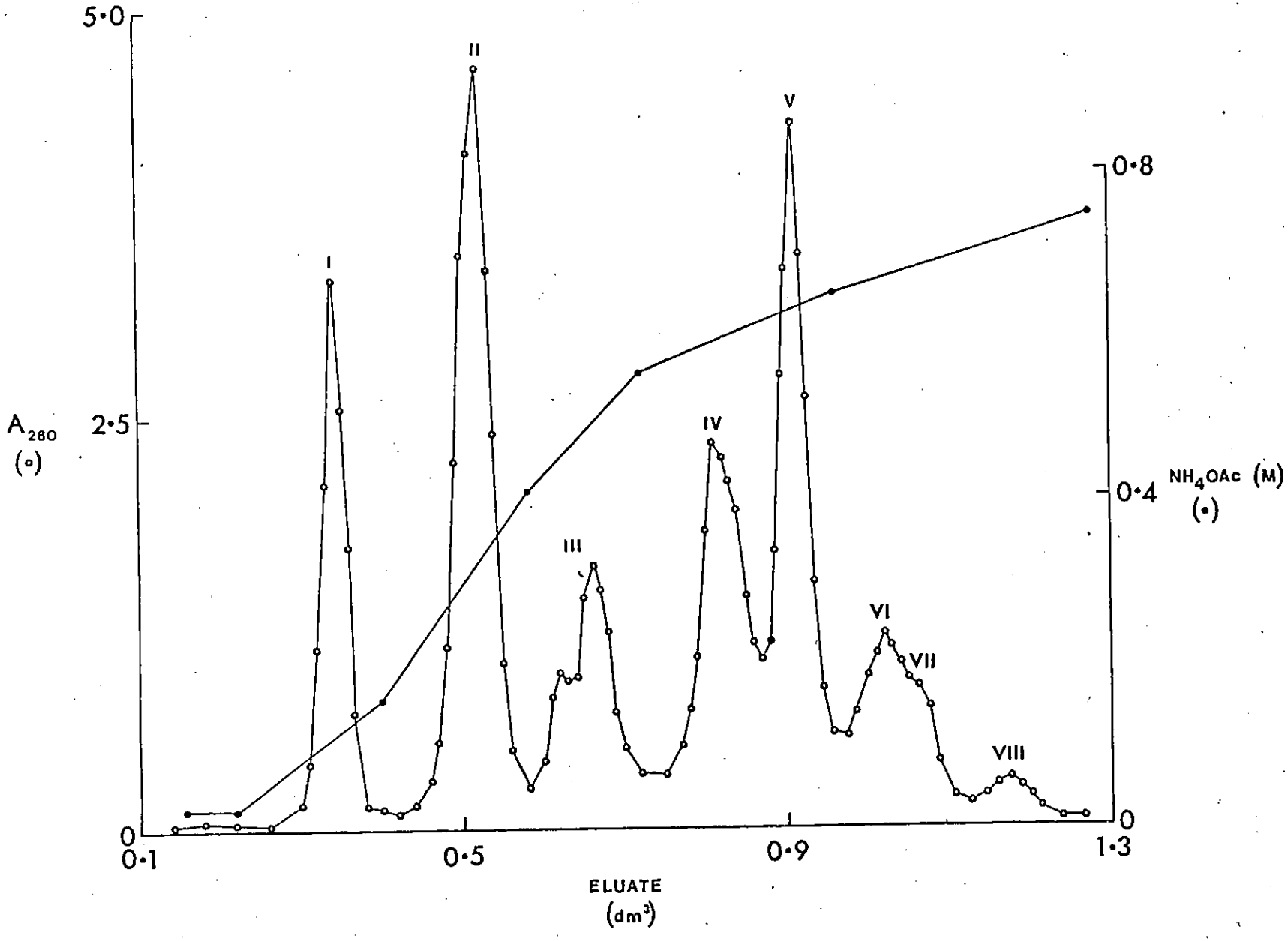

5·0
2·5
0
A_{280}
(o)
I
II
III
IV
V
VI
VII
VIII
0·8
0·4
0
NH_4OAc (M)
(•)
0·1
0·5
0·9
1·3
ELUATE
(dm^3)

Figure 2.2 Polyacrylamide gel electrophoresis of β-BuTX

Electrophoresis was carried out at pH 4.5 in a β alanine/ acetate buffer system as described in Section 2.2.7. Samples (25 μg) were applied at the anodic end of the gel; protein bands were stained with Coomassie G-250 (0.04%, w/v) in perchloric acid (3.5% w/v).

a) Peak V from CM-Sephadex column (Fig. 2.1)
b) Material from CM-cellulose CM52 chromatography (2.2.3.iii)
c) Material from SP-Sephadex chromatography (2.2.3.iv)
d) Pure β-BuTX obtained from preparative isoelectric focusing of peak V material (2.3.2.3).

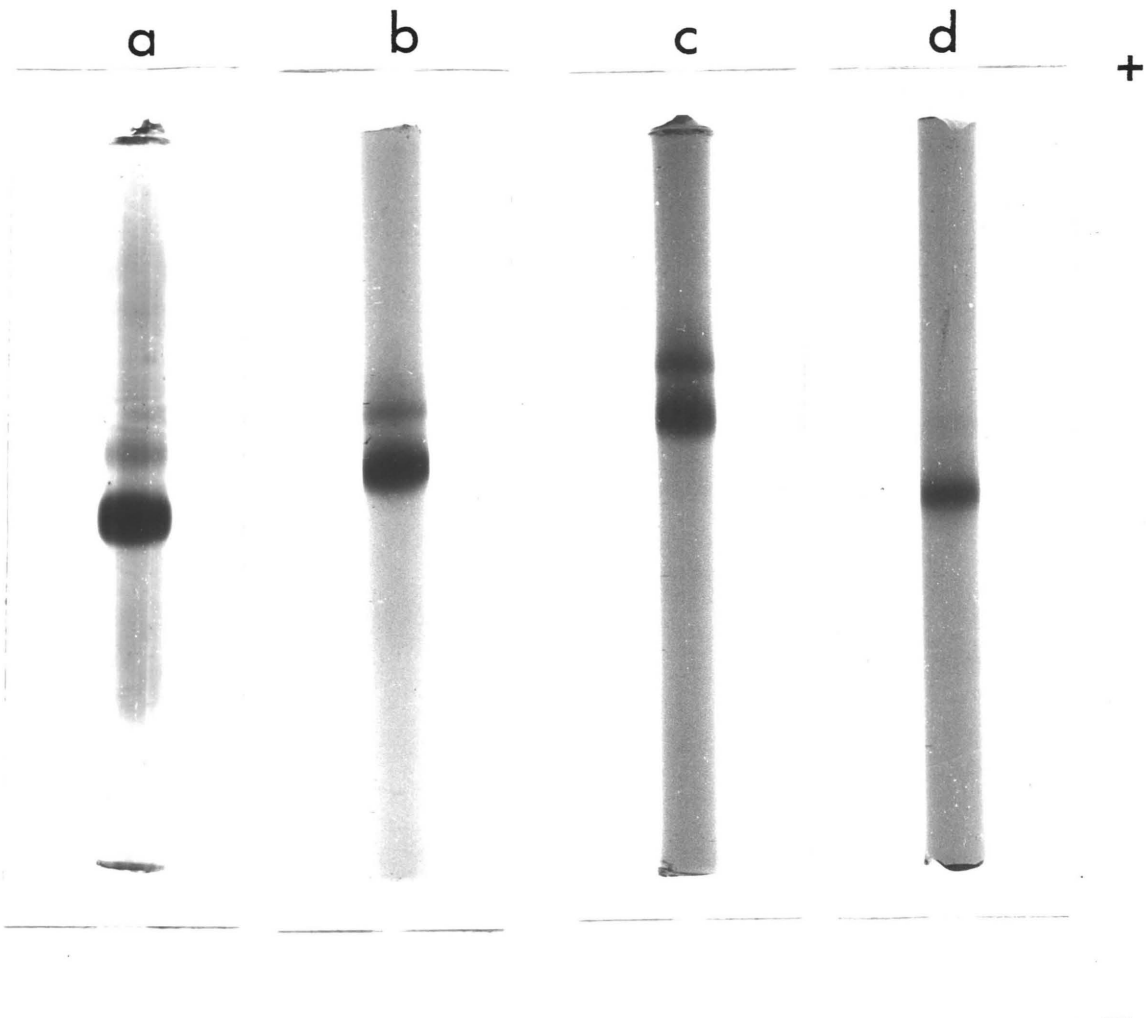


Figure 2.3 Analytical isoelectric focusing of β-BuTX

Focusing of material from peak V of initial venom fraction (60 µg) (a) and pure β-BuTX (60 µg) (b) was carried out in a polyacrylamide gel as described in Methods. After focusing, the pH gradient (●) was measured with a micro-electrode. The proteins in the gel were precipitated with 40% trichloroacetic acid, washed in 10% trichloroacetic acid to remove the ampholytes and scanned at 450 nm.

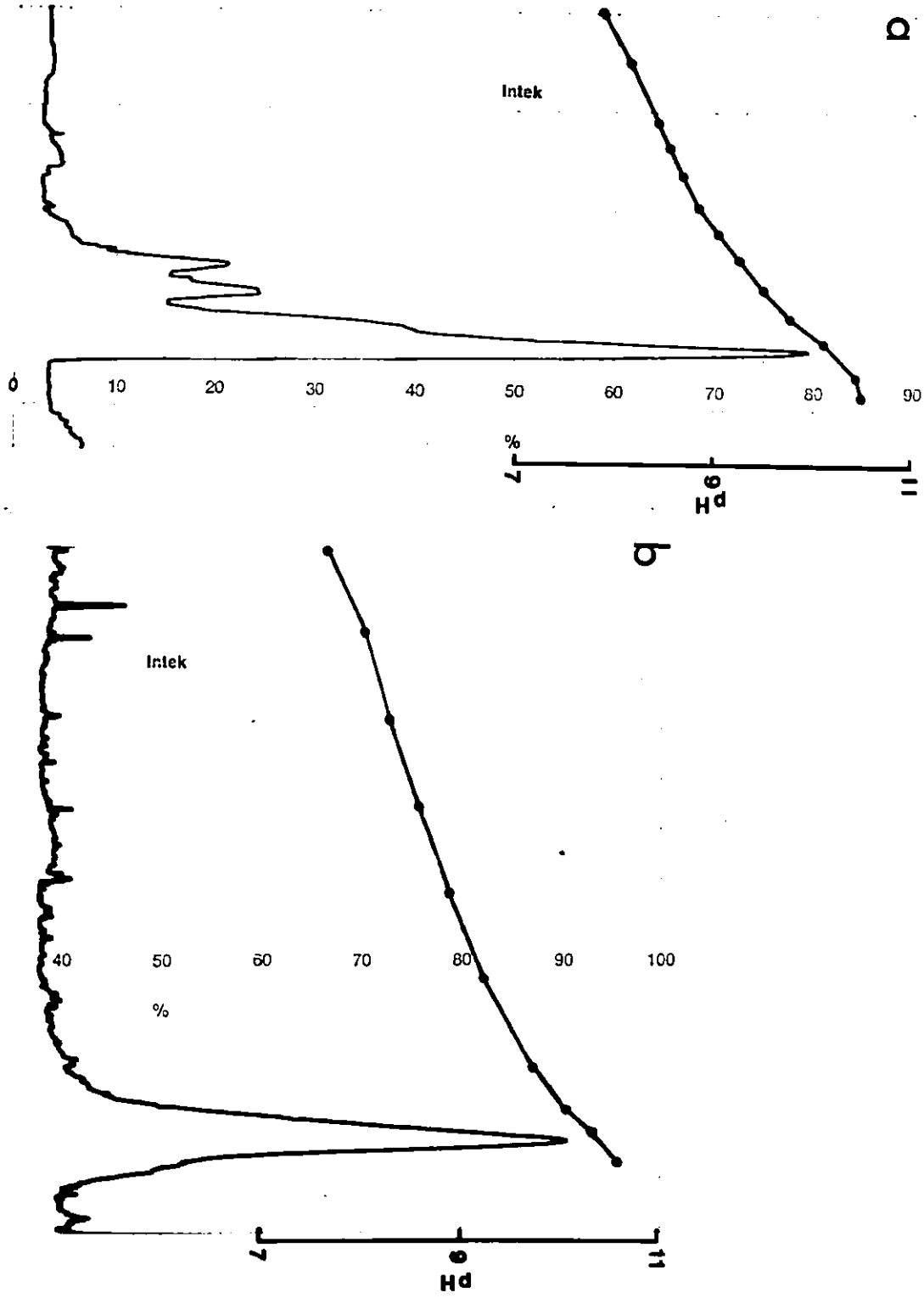

a
Intek
0
10
20
30
40
50
60
70
80
90
%
7
9
11
pH
b
Intek
40
50
60
70
80
90
100
%
7
9
11
pH

Figure 2.4 Sodium dodecylsulphate polyacrylamide gel electrophoresis of β-BuTX

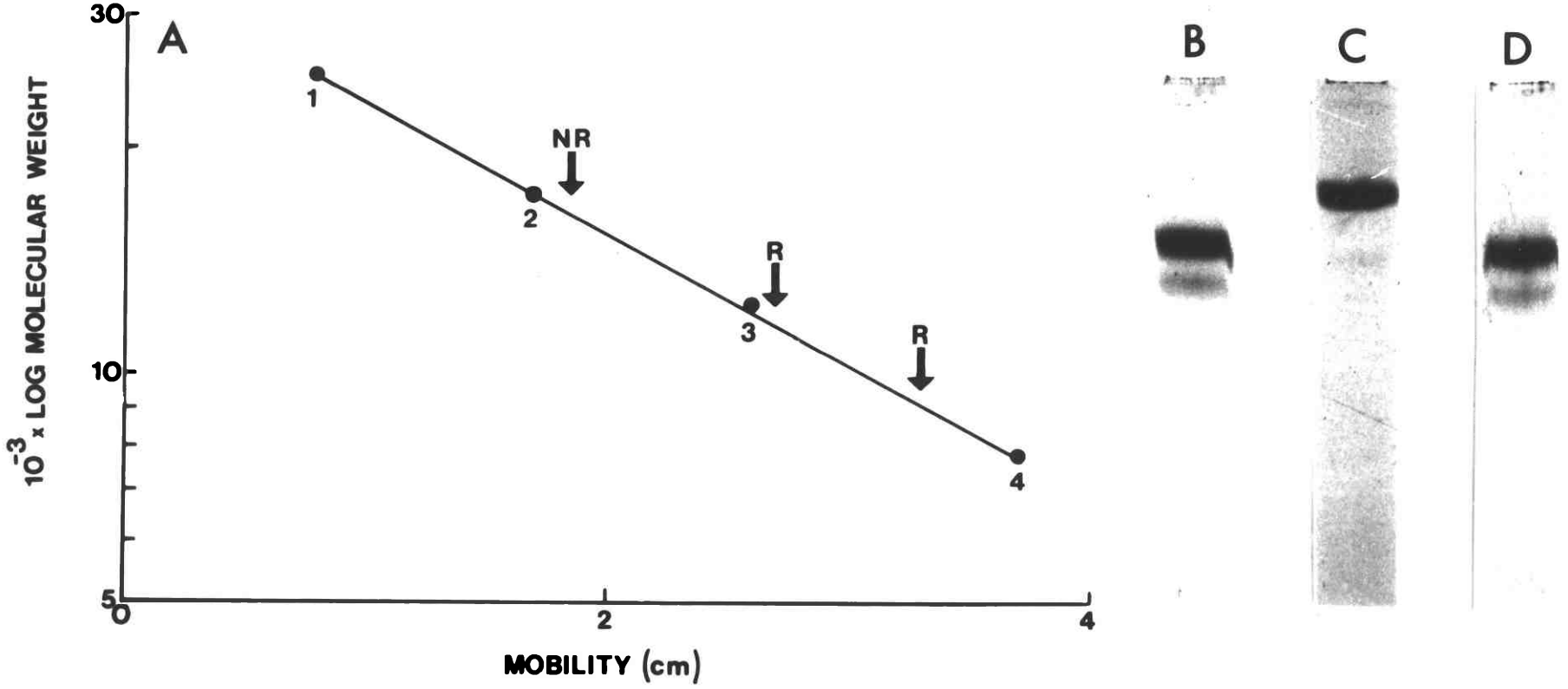
Electrophoresis was carried out in a gel containing 12.5% acrylamide, 1.25% bis-acrylamide and 8M urea as described in Section 2.2.7.

(A) Semilogarithmic plot of the relative mobilities and molecular weights of protein standards: α chymotrypsinogen (1), myoglobin (2), cytochrome c (3) and the largest fragment obtained from cleavage of (3) with cyanogen bromide (4). Arrows indicate the position of toxin bands.

(B) Pure β-BuTX (after preparative isoelectric focusing) under reducing conditions.

(C) Pure β-BuTX under non-reducing conditions.

(D) Impure β-BuTX (before preparative isoelectric focusing) under reducing conditions.


A
30
10
5
0
2
4
10^{-3} x LOG MOLECULAR WEIGHT
MOBILITY (cm)
1
2
3
4
NR
R
R
B
C
D

used. These fractionations of Bungarus multicinctus venom had all employed linear gradients of ammonium acetate concentration and pH (0.05M, pH 5.0 to 0.9 - 1.0M, pH 6.8 - 7.0) of varying slopes, to elute the proteins from CM Sephadex (Dryden et al., 1974; Eterovic et al., 1975; Lee et al., 1972) or CM cellulose (Wernicke et al., 1974) columns. Nevertheless, the elution profile obtained (Fig. 2.1) was similar to that of Lee et al. (1972) and the α and β-BuTX peaks, II and V respectively, were easily identified. Peak III of the profile shown by Lee et al. was partially resolved into two components whereas peaks VI and VII were less well separated. Peak V, representing 14% by weight of the freeze dried venom, was pooled, desalted by gel filtration on Sephadex G-25 and freeze dried.

This preparation was not homogeneous. Polyacrylamide gel electrophoresis at acid pH showed a toxin band together with one contaminant band (Fig. 2.2a) and analytical isoelectric focusing resolved three minor components from the major toxin band (Fig. 2.3a). These impurities were not revealed by SDS polyacrylamide gel electrophoresis which gave a single protein band in the absence of a reducing agent and two bands when the sample was treated with 10% β-mercaptoethanol (Fig. 2.4).

2.3.2 Further purification of β-bungarotoxin

2.3.2.1 Ion-exchange chromatography

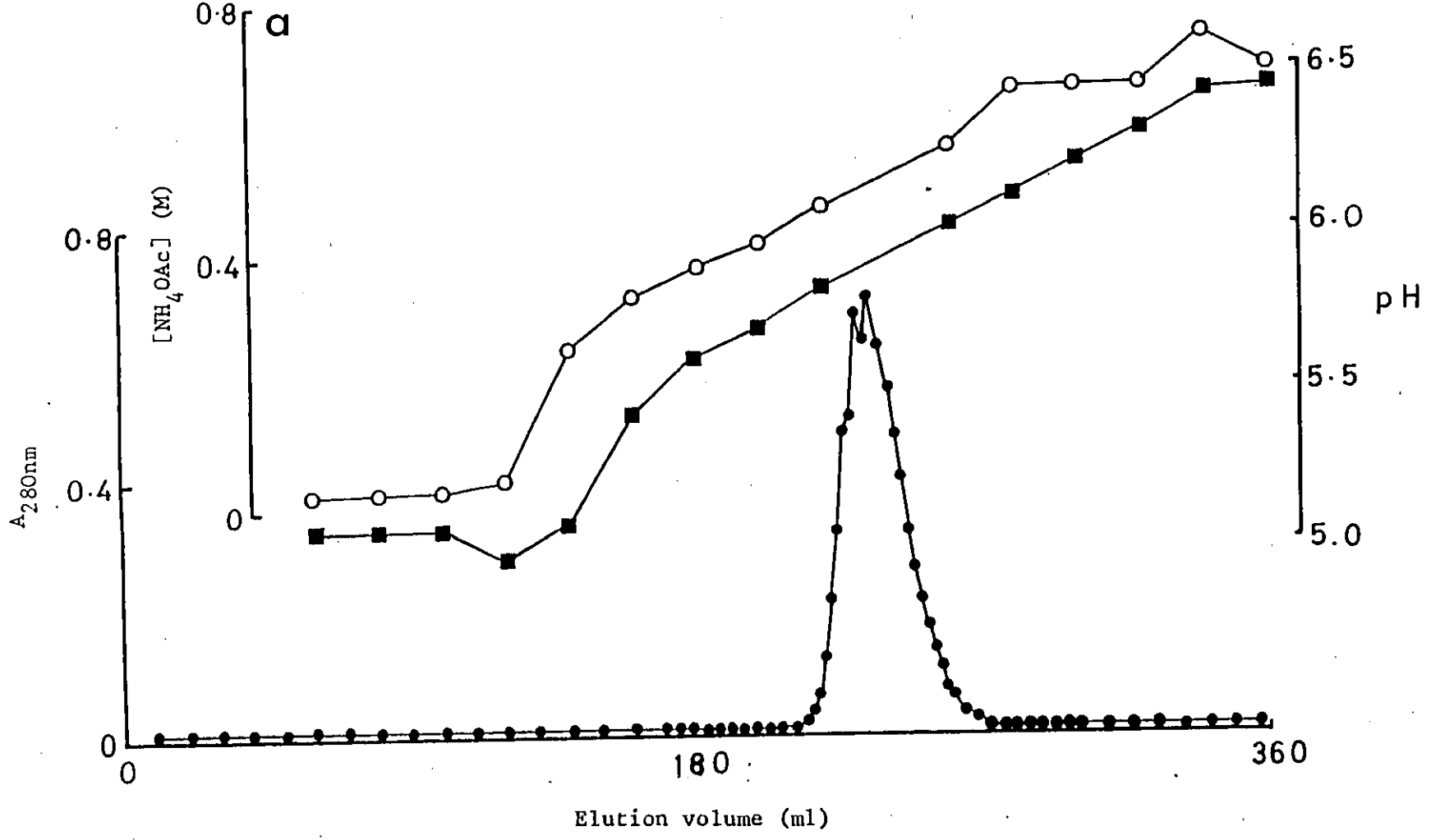
The material from peak V of the venom fractionation was subjected to chromatography on a series of CM cellulose columns (i - iii) as detailed in Section 2.2.3. A single protein peak was observed in each case. Fig. 2.5a illustrates a typical result, using the third of these gradients which is similar to that used by Strong et al. (1976).

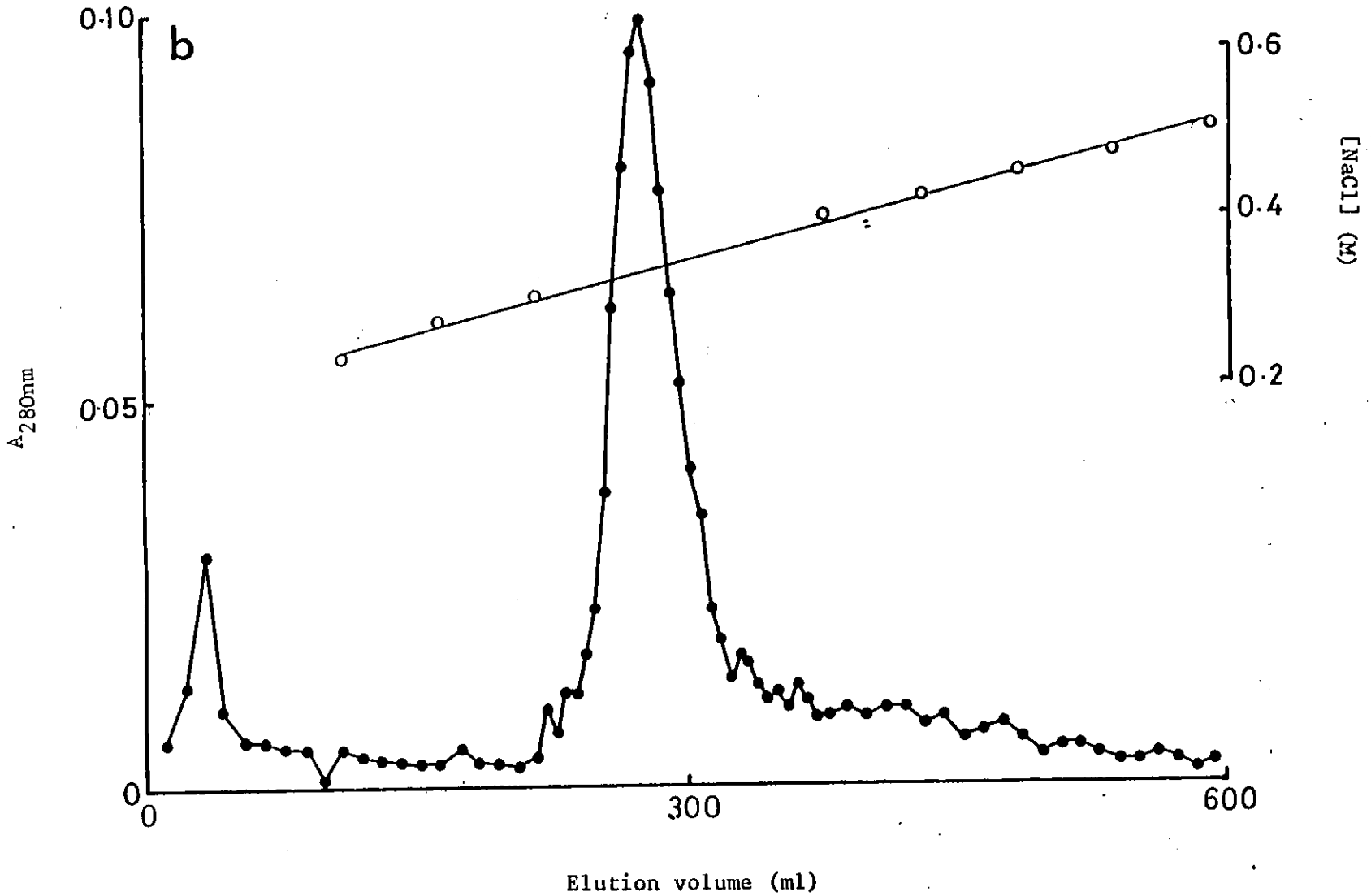
Figure 2.5 Ion-exchange chromatography of partially purified β-BuTX

(a) The protein sample (15 mg) was loaded onto a pre-equilibrated carboxymethyl cellulose column (1.15 x 50 cm) and eluted, as described in Section 2.2.3, with a linear gradient of ammonium acetate concentration (○) and pH (■); 2 ml fractions were collected and the protein concentration measured by A_{280nm} (●). (Strong et al., 1976)

(b) A sulphopropyl Sephadex C-50 column (1.5 x 15 cm) was equilibrated with ammonium acetate (0.05M; pH 5.0) as described in Section 2.2.3. Partially purified β-BuTX (4.7 mg) was loaded onto the column in this buffer and eluted with a linear gradient of NaCl concentration (0); 5.4 ml fractions were collected and the protein concentration measured by A_{280} (●). (MacDermot et al., 1978a)

Salt concentrations were calculated from conductivity measurements.


a
0·8
0·4
0
[NH4OAc] (M)
0·8
0·4
0
A280nm
6·5
6·0
5·5
5·0
pH
0
180
360
Elution volume (ml)


b
0·10
0·05
0
0
300
600
A_{280nm}
0·6
0·4
0·2
[NaCl] (M)
Elution volume (ml)

Although, in this case, there was a slight indication of a multiple peak, polyacrylamide gel electrophoresis of samples from fractions in different parts of the peak showed no significant decrease in the intensity of the contaminant band (Fig 2.2b). Towards the end of this study it was reported that β-BuTX obtained by CM Sephadex chromatography of the venom was further purified using an SP Sephadex column (MacDermot et al., 1978a). Such a column, run under similar conditions (Section 2.2.3.iv and Fig. 2.5b) failed to separate any additional components from the protein in peak V and polyacrylamide gel electrophoresis showed that the contaminant band was still present (Fig. 2.2c).

2.3.2.2 Gel filtration

A separation of the minor components was attempted on the basis of molecular size, rather than charge, by gel filtration of the peak V material on a column of Sephadex G-75 superfine (Fig. 2.6). A single, symmetrical peak of molecular weight 21,000 was obtained. Polyacrylamide gel electrophoresis of various fractions from this peak confirmed that the contaminant band was not removed.

2.3.2.3 Preparative isoelectric focusing of β-bungarotoxin

The β-BuTX obtained by ion-exchange chromatography of Bungarus multicinctus venom was resolved into a major and three minor components by analytical isoelectric focusing in a polyacrylamide gel using a narrow-range pH gradient (Fig. 2.3a). In view of the failure of column chromatographic techniques to separate these contaminants, a method for carrying out isoelectric focusing on a preparative scale was developed. Two carrier ampholyte systems were used to focus partially purified β-BuTX in a flat bed of Sephadex G-75 (2.2.5). The system which was initially used, employing Biolyte carrier

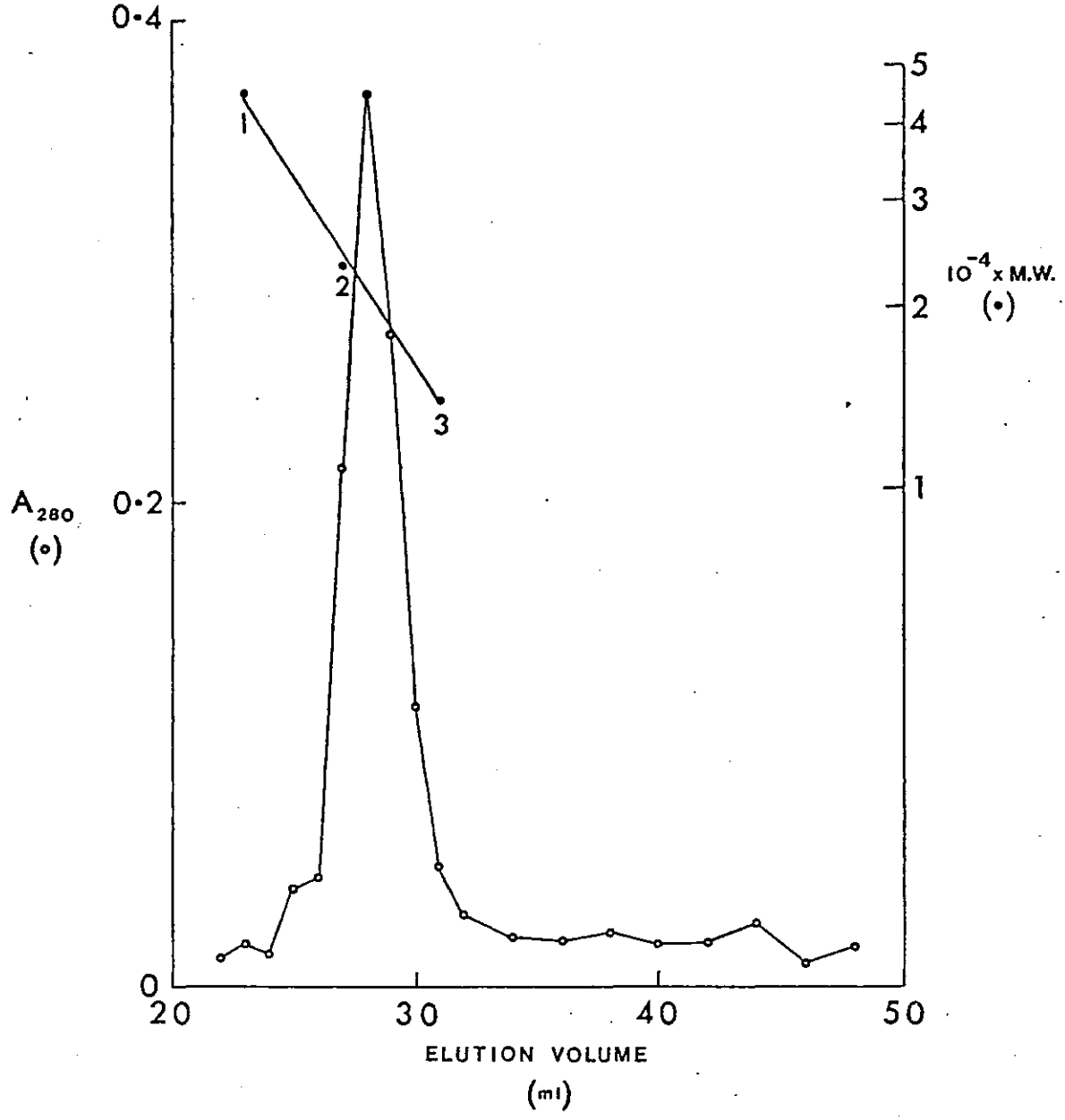


Figure 2.6 Gel filtration of partially purified β-BuTX

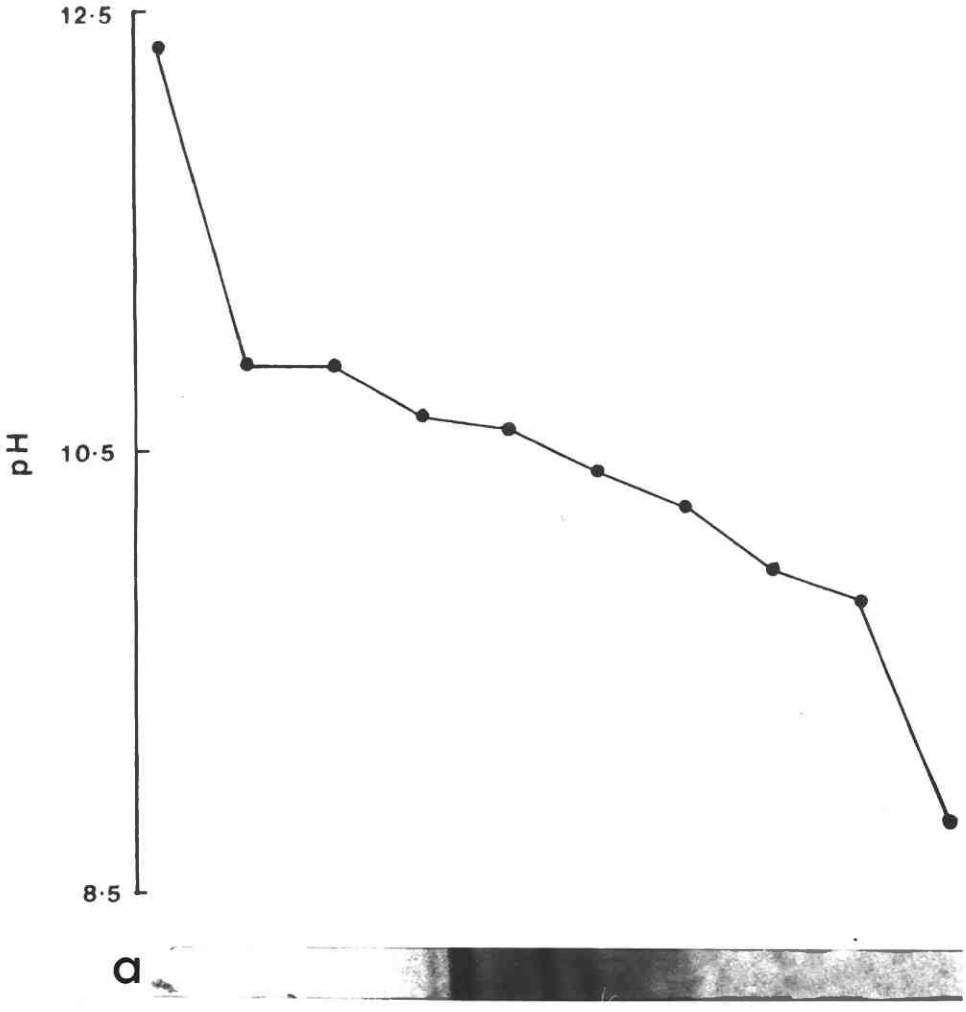
Chromatography was carried out at 4°C on a Sephadex G75 superfine column (1 x 60 cm) equilibrated with ammonium acetate (0.05M). The sample volume was 1 ml and 1 ml fractions were collected. Protein was measured by absorbance at 280 nm (O). The column had previously been calibrated using the following molecular weight markers: 1) ovalbumin, 2) α chymotrypsinogen, 3) ribonuclease.

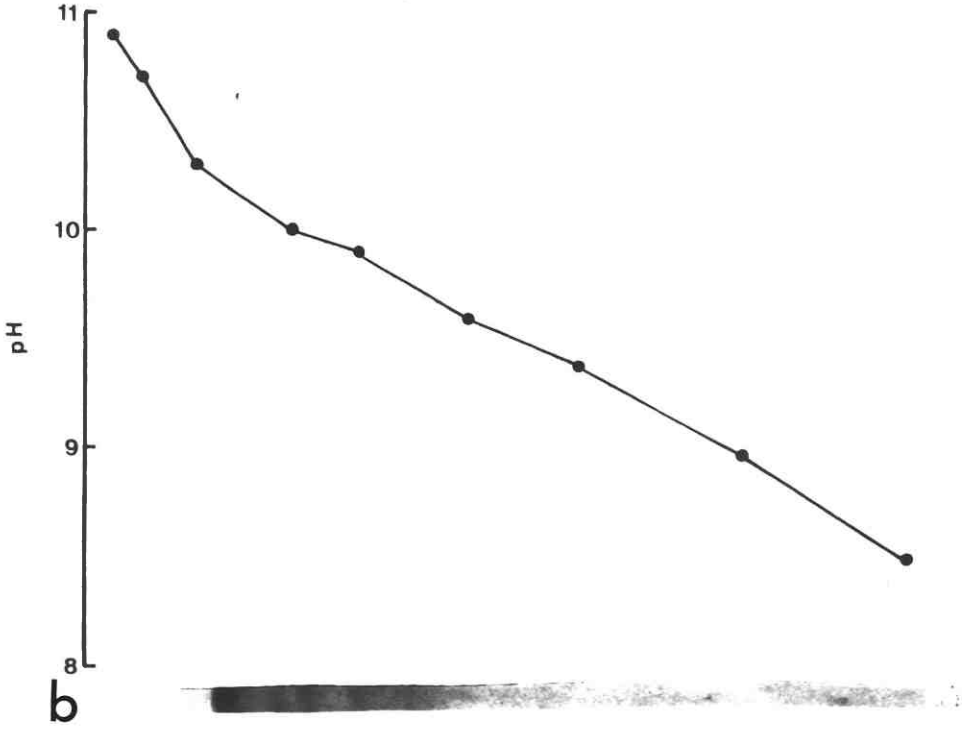
Figure 2.7 Preparative isoelectric focusing of β-BuTX

Material from peak V from the CM-Sephadex C50 (Fig. 1) column was focused in a flat bed of Sephadex G75 as described in Section 2.2.5. After focusing the pH gradient (●) was measured with a microelectrode and a paper print of the bed was fixed with trichloroacetic acid and stained with Coomassie R250 as described in Section 2.2.5.

a) Print of bed after focusing of Peak V material (20 mg) in a bed prepared from a slurry containing 1% (w/v) Biolyte pH 8 - 10 carrier ampholytes.

b) Print of bed following focusing of Peak V material (15 mg) using 2% (w/v) Pharmalyte pH 8 - 10.5


12·5
10·5
8·5
pH
a


11
10
9
8
pH
b

ampholytes, separated four contaminants more acidic than the major component (pI 10.6) together with one which was more basic; the latter and two of the former were stained at a rather low intensity (Fig. 2.7a). Focusing on a preparative scale using Pharmalyte carrier ampholytes also resolved four minor bands that were more acidic than the major band (Fig. 2.7b). The pI of the major band in this system was 10.2.

In view of the fact that the linear region of the pH gradient produced by the Biolyte system has a slope similar to that of the Pharmalyte system (Fig. 2.7), two factors may account for the slight differences observed in the patterns of protein bands and their pI's. The material which was purified using the Pharmalyte system was obtained from a different batch of venom to that previously used; on analytical isoelectric focusing, although these protein bands were again observed, their relative positions resembled those shown in Fig. 2.7b more closely than those in Fig. 2.3a. In addition the filter paper strip represented in Fig. 2.7b was much narrower than that shown in Fig. 2.7a ; the resultant edge effects caused some detail to be lost from the print.

The protein eluted from the major band, in both systems, was shown to be homogeneous on polyacrylamide gel electrophoresis (Figure 2.2d) and analytical isoelectric focusing (Fig. 2.3b) and will, henceforth, be referred to as β-bungarotoxin (β-BuTX). The toxin used in both the above mentioned figures was purified using the Biolyte system.

2.3.3 Criteria of purity of β-bungarotoxin

The isoelectric point of β-BuTX was measured as 10.4 $\pm$ 0.2 (n = 4) (Figs. 2.3 and 2.7). The pure toxin also gave a single band

on SDS polyacrylamide gel electrophoresis with an apparent molecular weight of 16,800 ± 800 (n = 3) and, after reduction, two bands corresponding to molecular weights of 9,000 ± 300 (n = 3) and 11,400 ± 250 (n = 3) (Fig. 2.4). This preparation appeared therefore, homogeneous with respect to size as well as charge.

The subunit molecular weights are in agreement with the molecular weight of 21,000 daltons, measured by gel filtration of the toxin (Fig. 2.6). The size of unreduced β-BuTX measured by SDS polyacrylamide gel electrophoresis was lower than expected. This may have been 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). Interestingly a molecular weight of 18,500 daltons was obtained by equilibrium sedimentation (Kelly and Brown, 1974).

2.3.4 Toxicity and phospholipase activity of β-bungarotoxin

The data shown in Table 2.1a show that the MLD of β-BuTX, i.e. the minimum lethal dosage after intraperitoneal injection into mice, could be estimated as 25ng/g before and 10ng/g after final purification. In accordance with previous observations (Chang and Lee, 1963), the toxin took at least one hour to kill the mice even at the highest doses used. The pure toxin was much more potent when injected intraventricularly (Table 2.1b), in agreement with previous reports (Hanley and Empson, 1979).
The blockade of an *in vitro* nerve muscle preparation by β-BuTX (Table 2.2.) was also similar to that reported for other

Table 2.1. Whole animal toxicity of β-bungarotoxin

See Section 2.2.8. for details of injections.

a) Intraperitoneal injection

i) Impure β-BuTX (major peak in Fig. 2.1.)

Dose (µg toxin/g body weight)	Number of animals injected	Number of animals killed
0.1	2	2
0.05	2	2
0.025	2	1
0.01	2	0

ii) Pure β-BuTX

Dose (µg toxin/g body weight)	Number of animals injected	Number of animals killed
0.10	2	2
0.075	3	3
0.050	6	6
0.01	6	4

b) Intraventricular injection of pure β-BuTX

Dose (ng toxin/g body weight)	Number of animals injected	Number of animals killed
10	2	2
5	2	2
1	1	1
0.5	2	2
0.05	2	2
0.025	2	0
0.005	2	0

Table 2.2 Neuromuscular blocking activity of pure β-Bungarotoxin

Times required for toxin to completely block neurotransmission in frog sartorius nerve-muscle preparation as detected by muscle twitch. (Section 2.2.8.).

Dose (μg/ml)	Time (min)
0.5	415
2.75	252
5.0	112

Table 2.3 Characteristics of phospholipase activity of β-BuTX

Assays were carried out at 37°C using the titration described in Section 2.2.8. The reaction mixture (vol. 5.5 ml) contained 10 μmol of dispersed egg yolk lecithin and NaCl (100 mM). Additions of $CaCl_2$ (10 mM), $SrCl_2$ (10 mM), EGTA (0.1M) and sodium deoxycholate (0.18 mM) were made as indicated.

	Phospholipase activity ($\mu mol.\ min^{-1}$ mg of $protein^{-1}$)	
	β-BuTX	Bee venom phospholipase
$CaCl_2$	0.6	327
$CaCl_2$ + deoxycholate	63	1345
EGTA + deoxycholate	0	0
$SrCl_2$ + deoxycholate	2.6	532

toxin preparations (Chang et al., 1973; Dryden et al., 1974; Kato et al., 1977). Following complete blockade of the muscle response with a very high dose (100 µg/ml) of the protein in the major β-toxin peak, prolonged contraction was elicited by 10 mM carbamyl choline. This indicated that this toxin acted presynaptically, as would be expected for β-BuTX.

It was important, in view of the number of contaminants separated by isoelectric focusing, to establish the level of phospholipase activity in the pure toxin (Table 2.3). Negligible activity was found in the absence of deoxycholate; in contrast, when the detergent was added, appreciable phospholipase activity was observed comparable to that previously reported (Abe et al., 1977; Strong et al., 1976). In the presence of deoxycholate, the phospholipase activity was greatly reduced when Sr^{2+} was substituted for Ca^{2+}; moreover, it was completely abolished in the absence of calcium and in the presence of 0.1 mM EGTA. Bee venom phospholipase A_2 had a considerably greater specific enzyme activity than the toxin. This activity was increased about four-fold in the presence of deoxycholate and was abolished when Ca^{2+} was completely removed in the presence of EGTA (Table 2.3). The activity of this enzyme in Sr^{2+} medium was greater than expected (Table 2.3); this may have resulted from residual Ca^{2+} in the assay medium.

2.4 Discussion

Peak V in the elution profile shown in Fig. 2.1 is equivalent to peak V in the very similar profile of Lee et al. (1972). It contains a very basic protein which is composed of two, covalently linked, polypeptide chains whose molecular weights (11,400 and 9000), as measured by sodium dodecylsulphate polyacrylamide gel electrophoresis (Fig. 2.4) were similar to those reported for other β-BuTX

preparations (Abe et al., 1977; Hanley et al., 1977; Kelly and Brown, 1974; Kondo et al., 1978a). These molecular weights also agree closely with that of the native toxin (21,000 daltons) as determined by gel filtration (Fig. 2.6; Kelly and Brown, 1974). However, they are somewhat different to those calculated from their amino acid sequences of 13 500 and 7000 (Kondo et al., 1978b). Possible reasons for discrepancies in the molecular weights of small proteins were discussed in Section 2.3.3. The isoelectric point was 10.4 as compared with a value of 9.5 measured in a system of much greater range which was linear only up to pH 10.0 (Kondo et al., 1978a). Stable pH gradients extending beyond pH 10.0 are not easily obtained; this is reflected in isoelectric point of 10.6 measured for α-bungarotoxin using systems similar to those described herein (Lo et al., 1981) as compared with a value of 9-10 obtained in more conventional systems (Karlsson, 1979). The lethality (Table 2.1), phospholipase activity (Table 2.3) and presynpatic mode of action of this toxin preparation were similar to those reported elsewhere (Lee et al., 1972; Kelly and Brown, 1974; Abe et al., 1977).

Following the example of Lee et al. (1972) most workers have carried out chromatography of Bungarus multicinctus venom on carboxymethyl ion-exchange columns using linear gradients of ammonium acetate concentration and pH (Dryden et al., 1974; Eterovic et al., 1975; Kelly and Brown, 1974; MacDermot et al., 1978a; Tobias et al., 1978; Wernicke et al., 1974). The variation in the slopes of the gradients used was probably the main reason for the differences in the elution profiles obtained, the number of protein peaks varying between six and thirteen. Miledi and co-workers (Abe et al., 1977) used a sodium chloride concentration gradient to elute the more basic components of venom from a CM-Sephadex column and obtained six fractions; the most abundant of these was taken to contain β-BuTX. Gel filtration of the venom on Sephadex G-50 (Kato et al., 1977) gave five protein peaks, the most abundant of which again contained β-toxin activity.

Minor contaminants were later removed from these initial β-BuTX fractions by ion-exchange chromatography (Hanley et al., 1977; Kato et al., 1977; MacDermot et al., 1978a; Strong et al., 1976; Wernicke et al., 1975), gel filtration (Kondo et al., 1978a) or a combination of these techniques (Abe et al., 1977). The β-BuTX peak from the venom fractionation shown here (Fig. 2.1) could not be further purified by gel filtration using columns of Sephadex G-75 (Fig. 2.6) or G-50 (C.K. Tse, unpublished observation). Ion-exchange chromatography, using SP-Sephadex (Fig. 2.5b) under the conditions used by MacDermot et al. (1978) or CM-cellulose (Fig. 2.5a) which included a column similar to that used by Strong et al. (1976), also failed to resolve the contaminants. The impurities separated on rechromatography of corresponding toxin preparations by these other workers appear to have been removed during the initial CM-Sephadex C-50 chromatography of the venom (Fig. 2.1) or were absent from the venom samples used in this study. It is interesting to note that, due to the elution conditions used here, the slope of the ammonium acetate gradient in the region of peak V is much shallower than that employed in other purification schemes.

The peak V material did, however, contain several minor contaminants which were resolved by narrow range isoelectric focusing (Figs. 2.3 and 2.7; Dolly et al., 1978; Spokes and Dolly, 1980). Some or all of these contaminants were detectable as a single band on polyacrylamide gel electrophoresis at pH 4.5 (Fig. 2.2). They were not shown by sodium dodecylsulphate polyacrylamide gel electrophoresis (Fig. 2.4) and are therefore of similar size and subunit composition to β-BuTX. It is clear that the electrophoresis at acidic pH and especially the narrow range isoelectric focusing are very important criteria of purity for this toxin (Spokes and Dolly, 1980). Only two β-BuTX preparations have, to date, been reported to give

single bands on electrophoresis at acid pH (Abe et al., 1977; Kondo et al., 1978a). One of these preparations was reported as giving a single band on narrow range isoelectric focusing (Kondo et al., 1978a) but using a pH gradient which was steeper than that shown herein. The second preparation was later shown to contain at least two components on two dimensional narrow range isoelectric focusing and SDS polyacrylamide gel electrophoresis (Abe et al., 1980). It should also be noted that the amino acid compositions of four purified β-BuTX preparations are similar (Abe et al., 1977; Hanley et al., 1977; Kondo et al., 1978a; Strong et al., 1976); together with their similar molecular weights this suggests that species separated by narrow range isoelectric focusing may represent "isotoxins" similar in composition to the major form (Kondo et al.,1982). These contaminants, although invariably present, appeared to vary in their relative abundance from one venom sample to another. The activities and specificities of action of these contaminants could be very different from those of β-BuTX. It is therefore necessary to completely remove them both for studies on the mode of action of toxin and before producing a radiolabelled derivative with which to investigate the binding of the toxin to nerve terminals. Additionally, it will be seen in Chapter 5 that the radiolabelling procedure chosen in this study produces a derivative which is less basic than the native toxin and has an isoelectric point in the same region as some of the contaminant bands.

The pure β-BuTX showed a phospholipase activity (Table 2.3) which was dependent on the presence of deoxycholate and Ca^{2+}, in agreement with reports on other toxin preparations (Abe et al., 1977; Chang et al., 1977b; Howard and Truog, 1977; Kondo et al., 1978a; Strong et al., 1976) and the high degree of sequence

homology between the larger A chain of the toxin and other phospholipases A_2 (Kondo et al., 1978b). The requirement for the presence of detergent may be explained by the observation of Strong and Kelly (1977) that the phospholipase activity of β-BuTX on phosphatidyl choline bilayers is maximal at the phase transition temperature of the lipid and is inhibited by the presence of cholesterol in the membrane. It has been suggested (Verheij et al., 1980) that pancreatic phospholipase A_2, the sequence of which is very similar to that of the β-BuTX A chain (Kondo et al., 1978b), contains a site for binding lipid/water interfaces which is separate from the active site. The presence of such a site in the β-BuTX molecule may explain why treatment of the lecithin substrate with detergent may increase the enzyme activity of the toxin.

CHAPTER 3. THE EFFECTS OF β-BUNGAROTOXIN ON SYNAPTOSOMES PURIFIED FROM RAT CEREBRAL CORTEX

3.1 Introduction

Pure β-BuTX was much more toxic when injected directly into the cerebral intraventricular spaces than when administered peripherally (2.3.4.), suggesting that it has direct toxic effects in the central nervous system. Mammalian brain, as has already been discussed (1.1.3.) is also the most convenient source from which to purify pinched off nerve terminals which retain many of their metabolic functions including the accumulation and release of neurotransmitters. Suspensions of these synaptosomes can be loaded with radiolabelled neurotransmitters which they subsequently release in a Ca^{2+}-dependent manner in response to electrical stimulation or depolarisation by increased external K^+ concentration (Wonnacott and Marchbanks, 1976; De Belleroche and Bradford, 1977). A simple assay system is, therefore, available in which the effects of neurotoxins on the release of a number of transmitters can be directly measured. Subsequently, the binding of radiolabelled toxin derivatives to this same preparation can be measured.

This chapter describes experiments which investigated the effects of homogeneous β-BuTX on the release of radiolabelled ACh from synaptosomes purified from rat cerebral cortex. Different preparations of β-BuTX have been shown to increase the release of previously accumulated noradrenaline, GABA, and the non-transmitter substance 2-deoxyglucose (Wernicke et al., 1974, Wernicke et al., 1975) as well as ACh (Sen et al., 1976). These toxin preparations were also reported to inhibit the net accumulation by brain synaptosomes of the putative neurotransmitters γ-aminobutyric acid (GABA) and noradrenaline (Wernicke et al., 1974; Wernicke et al., 1975),

the transmitter precursor, choline (Sen et al., 1976) and 2 – deoxyglucose (Wernicke et al., 1975); in addition β-BuTX decreased the uptake of choline into synaptosomes prepared from the electric organs of the fish Torpedo marmorata (Dowdall et al., 1977). A series of experiments was therefore carried out to measure the effects of homogeneous β-BuTX, prepared as described in Chapter 2 on the release of $[^3H]$ ACh from synaptosomes and to investigate whether it perturbed these two processes by a similar mechanism. A parallel study, by other workers in the same laboratory involved the putative neurotransmitters GABA and glutamate (Dolly et al., 1978; Tse et al., 1980) in order to test the specificity of pure β-BuTX for nerve terminals using different neurotransmitters.

The effects of β-BuTX were compared to those of tityustoxin (TsTX), which has been purified from the venom of the Brazilian yellow scorpion Tityus serrulatus (Diniz, 1978). This toxin binds with high affinity to potential dependent Na^+ channels and decreases the rate at which they are inactivated, thereby causing depolarisation of the membranes (1.2.2.).

3.2 Materials and Methods

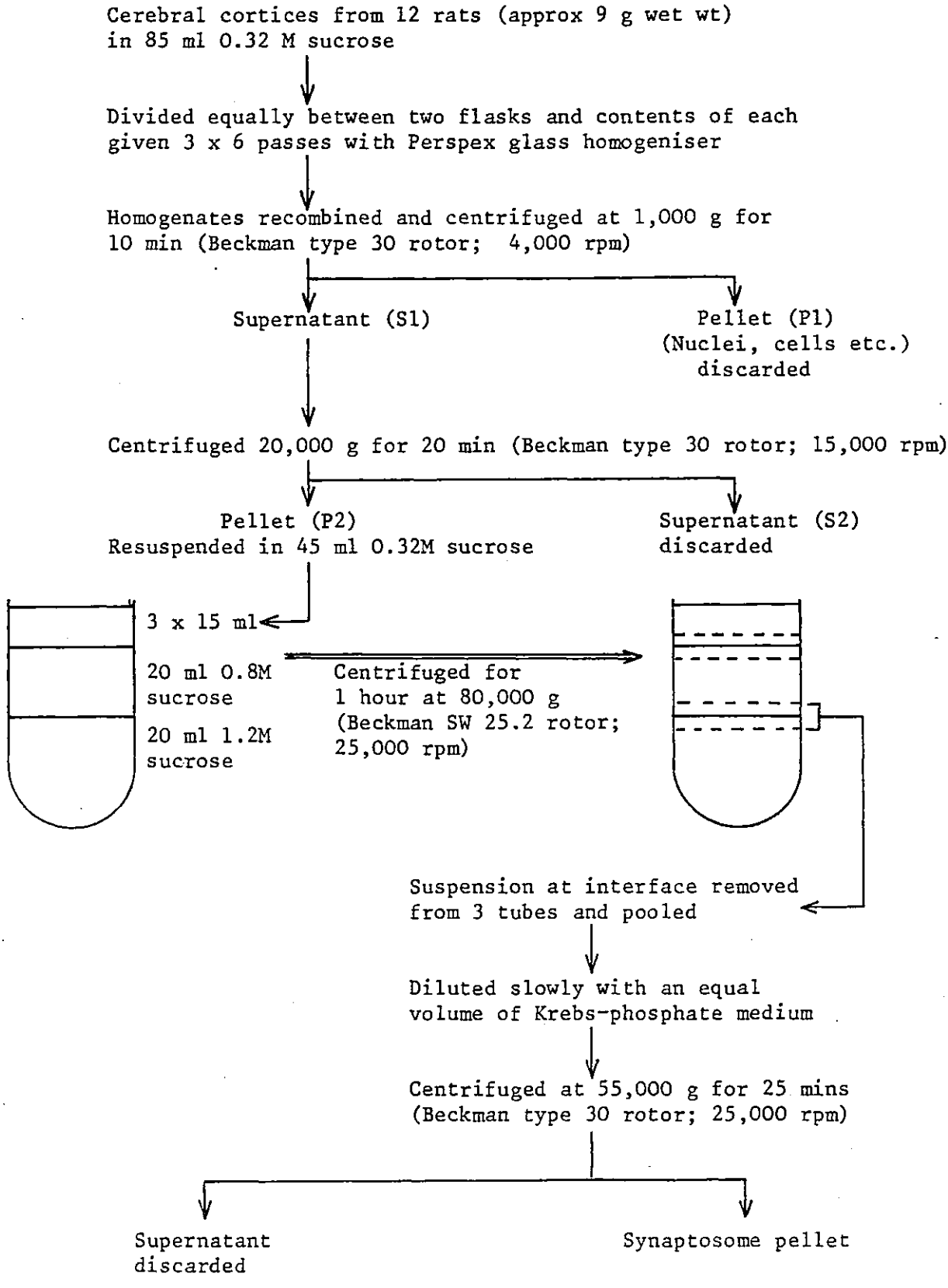
3.2.1. Materials

Tityustoxin, purified from the venom of the Brazilian yellow scorpion Tityus serrulatus and shown to give a single band in polyacrylamide gel electrophoresis at pH 4.3 and 8.2 (Diniz, 1978) was kindly provided by Dr. C. Diniz. Its LD_{50} measured in mice was 0.1 µg/g body weight. Tetrodotoxin (TTX), veratrine (a mixture of veratridine with other alkaloids including a muscarinic antagonist), eserine sulphate, choline chloride, acetylcholine chloride and EGTA were supplied by Sigma Chemical Co. [Methyl-^{3}H] choline chloride (specific radioactivity 13 Ci/m mol) was purchased from the Radio-Chemical Centre, Amersham, U.K. Soluene was supplied by Pharmacia.

3.2.2. Preparation of synaptosomes

The procedure used was slightly altered from that of Bradford (1969), itself a slight modification of the method of Gray and Whittaker (1962). All solutions were in deionised water and all operations were performed at 4°C. Adult female Sprague-Dawley rats (200-250g) were killed by dislocation of their necks and the cerebral cortices rapidly removed. A 10% (w/v) homogenate in 0.32M sucrose was formed by three series of six up and down strokes in a Perspex and glass tissue homogeniser of radial clearance 250 µm (Aldridge et al., 1960) with the Perspex pestle rotating and 1000 rev/min. Each series of strokes was performed alternately on half of the homogenate while the other half was cooled on ice. A synaptosome pellet was prepared from the final homogenate by a series of centrifugations as shown in Figure 3.1. The synaptosome suspension removed from the second interface of the discontinuous density gradient contained approximately equal amounts of 0.8M and 1.2M sucrose giving a final concentration of about 1.0M. This was diluted with an equal volume of cold Krebs-phosphate medium (20mM Na_2HPO_4, 1.25mM KH_2PO_4; 125mM NaCl, 5mM KCl, 0.74mM $CaCl_2$, 1.3mM $MgSO_4$

Figure 3.1 Protocol for preparation of synaptosomes from rat cerebral cortex



and 10mM glucose, saturated with 95%O_2/5% CO_2 and adjusted to pH 7.4; Wonnacott and Marchbanks, 1976) rather than with cold deionised water as in the method of Bradford (1969). The viability of the pelleted synaptosomes, resuspended by trituration in Krebs-phosphate medium was tested by measurement of the rate of lactate production, using a spectrophotometric assay (Bergmeyer, 1965) and the rate of O_2 uptake, using Warburg manometry.

3.2.3. Measurement of choline accumulation by synaptosomes

The uptake of [^{3}H] choline was measured using a filtration assay (Wernicke et al., 1974). Synaptosomal pellets were resuspended in Krebs-phosphate medium to a concentration of 4-5 mg of protein/ml. This suspension was divided according to the number of incubations to be performed. Each of thes smaller volumes (0.5 ml) of synaptosome suspension was then diluted with an equal volume of Krebs-phosphate medium containing [^{3}H]choline (1.0 µM; specific radioactivity 4 Ci/m mol (or 4.0 µM; specific radioactivity 1 Ci/m mol to initiate the measurement of uptake and incubated at 25°C. Small volumes (5 to 25 µl) of toxin solutions in water were added to the requisite incubation tubes at the appropriate times. At various times after the addition of [^{3}H] choline, the synaptosome suspensions were briefly mixed and aliquots (100 µl) removed from each suspension. These aliquots were then placed on to glass-fibre filters (Whatman GF/C) mounted on supports connected to a manifold which was under suction from a water pump. The filters were pre-wetted with Krebs-phosphate medium and the aliquots of synaptosome suspensions were applied under suction. The filters were then washed twice with 2.5 ml Krebs-phosphate medium, dried in air and counted in a toluene based scintillation cocktail containing 10% (v/v) Soluene. Blank samples, containing synaptosomes osmotically lysed in water, were incubated at 4°C and otherwise treated similarly.

3.2.4. Measurement of release of ACh and choline from synaptosomes

Suspensions of synaptosomes were preloaded with [^{3}H] choline (1.7µM;

specific radioactivity 1 Ci/m mol) for 20 min at 37°C in a modified Krebs-phosphate medium containing 1.8 mM KCl and 128.2mM NaCl. The suspension was washed twice by centrifugation at 27000 g for 2 min followed by resuspension in modified Krebs-phosphate medium; final resuspension was in the same medium containing eserine sulphate(0.1 mM), a competitive inhibitor of acetylcholinesterase. Experiments which examined the requirement for Ca^{2+} were carried out by preloading the synaptosomes using medium in which calcium salts were replaced iso-ostomically by sucrose; this medium was also used to dilute the synaptosome fraction from the density gradient (3.2.2.). Subsequent washing and final resuspension were in medium containing $CaCl_2$(0.74 mM) or sucrose (2.24 mM) and EGTA (5 mM). Equal volumes of the bulk, preloaded suspension (200µl) were then transferred to tubes containing the requisite amounts (5-20µl) of toxin solutions and/or KCl and/or equivalent volumes of water; KCl was added to a final concentration of 23mM. In experiments involving TTX, a solution of the toxin or the same volume (5µl) of water was added to the bulk synaptosome suspension and preincubated for 5 min at 25°C before the suspension was transferred to incubation tubes.

The incubations were carried out at 25°C; aliquots were taken at appropriate intervals and centrifuged at 10000 g for 5 min. The resultant supernatants were diluted with equal volumes of formic acid/acetone (1:3) and recentrifuged. Aliquots of the latter supernatants were subjected to electrophoresis at pH 2.1 and 3.8 kV on Whatman 3MM paper for 25 min (Sen et al., 1976) to separate choline and acetylcholine. Unlabelled ACh and choline (100-200 n moles) were added to each sample as carriers; the resultant spots were stained with iodine vapour, cut out and after the iodine had been allowed to evaporate their radioactivity determined by liquid scintillation counting in a xylene based cocktail containing 10% Triton X-100. Two control incubations, with no toxin or KCl added, were performed; one of these was terminated as the same time as the test samples while the second was terminated at 0 min. The values obtained

for this "zero time" sample were subtracted from those of all other samples to give the amounts of radioactive ACh and choline released during the incubation period.

3.2.5 Other determinations

Lactate dehydrogenase activity and lactate were assayed as described by Bergmeyer (1965). Acetylcholinesterase activity was measured spectrophotometrically,using acetylthiocholine as substrate, by the method of Ellman et al., (1961). Synaptosome pellets were digested in 0.31 M NaOH and protein was assayed by method of Lowry et al, (1951) using bovine serum albumin as a standard.

3.3 Results

3.3.1. Characterisation of synaptosomes purified from rat cerebral cortex

The rates of both O_2 uptake and lactate production by synaptosome suspensions at 37°C were very low in medium from which glucose was omitted; they increased from 0.5 to 21 and from 2.7 to 14.1 μ mol. hr^{-1} 100 mg of $protein^{-1}$, respectively on the addition of 10mM glucose (Table 3.1). This indicates that glycolytic and mitochondrial oxidation pathways were intact in this preparation. Furthermore, when the synaptosomes were depolarised by increasing the external K^+ concentration to 55mM the rates of lactate production and oxygen consumption increased 1.2 fold and 3.6 - 4.9 fold, repsectively. This shows that the preparations increased their rates of glucose metabolism in response to stimulation which also increases the rate of neurotransmitter release (Figs. 3.3 and 3.4), an important criterion of their viability. The rates of oxygen consumption shown here are in good agreement with reports by Bradford et al.,(1975) and Bradford and Thomas (1969) for rat cerebral cortex synaptosomes prepared in a very similar manner.

3.3.2 Characterisation of the synaptosomal uptake and release systems

The synaptosomes showed at 25°C, a rapid uptake of [^{3}H] choline (approx 2.3 pmol. min^{-1} mg of $protein^{-1}$) during the first 15 mins after it was added to a synaptosome suspension at a concentration of 0.5μM (Fig 3.2a).

Table 3.1 Lactate production and O_2 consumption by synaptosomes

Synaptosome suspensions (50 - 100 µg of protein/ml) were incubated at 37°C in Krebs-phosphate medium (10 mM glucose; 5 mM K^+) which was modified, where indicated, by the omission of glucose, the addition of KCl (50 mM) or the addition of β-BuTX (1 µM). The amounts of lactate produced were measured spectrophotometrically by a coupled enzyme reaction (3.2.5) and O_2 consumption was measured by Warburg manometry. The results shown are from four separate experiments; b and d were performed in collaboration with Dr C.K. Tse. The rates of both O_2 consumption and lactate production were constant over the 30 - 60 min duration of these experiments.

	Lactate production (µmol hr^{-1} 100 mg of $protein^{-1}$)		O_2 consumption (µmol hr^{-1} 100 mg of $protein^{-1}$)	
	a	b	c	d
No Glucose	2.7	-	0.5	-
10 mM Glucose	14.1	9.4	21	46
K^+ (55 mM)	-	11.2	103	164
β-BuTX (1 µM)	-	11.6	-	150

After 15 mins the rate of accumulation was much less (approx. 0.3p mol. min^{-1} mg of $protein^{-1}$). should be noted that, as the incubation proceeds, the radiolabelled choline in the medium would be diluted by any unlabelled endogenous choline released from the synpatosomes thus diminishing the apparent accumulation. After 40 mins only 9.4% of the added radioactivity had been taken up. The accumulation by a suspension of osmotically lysed synaptosomes at $4^{o}C$ was relatively low indicating that the bulk of the observed uptake was not due to low affinity binding of choline to membranes. It may be assumed that at the concentration of [^{3}H] choline used, it is taken up via the high affinity transport system which has a K_m of 1μM (Yamamura and Snyder, 1972) and that the contribution of the low affinity uptake system ($K_m = 9 \times 10^{-5}M$) is negligible (Yamamura and Snyder, 1972) their V_{max} values being comparable (Yamamura and Snyder,1973).

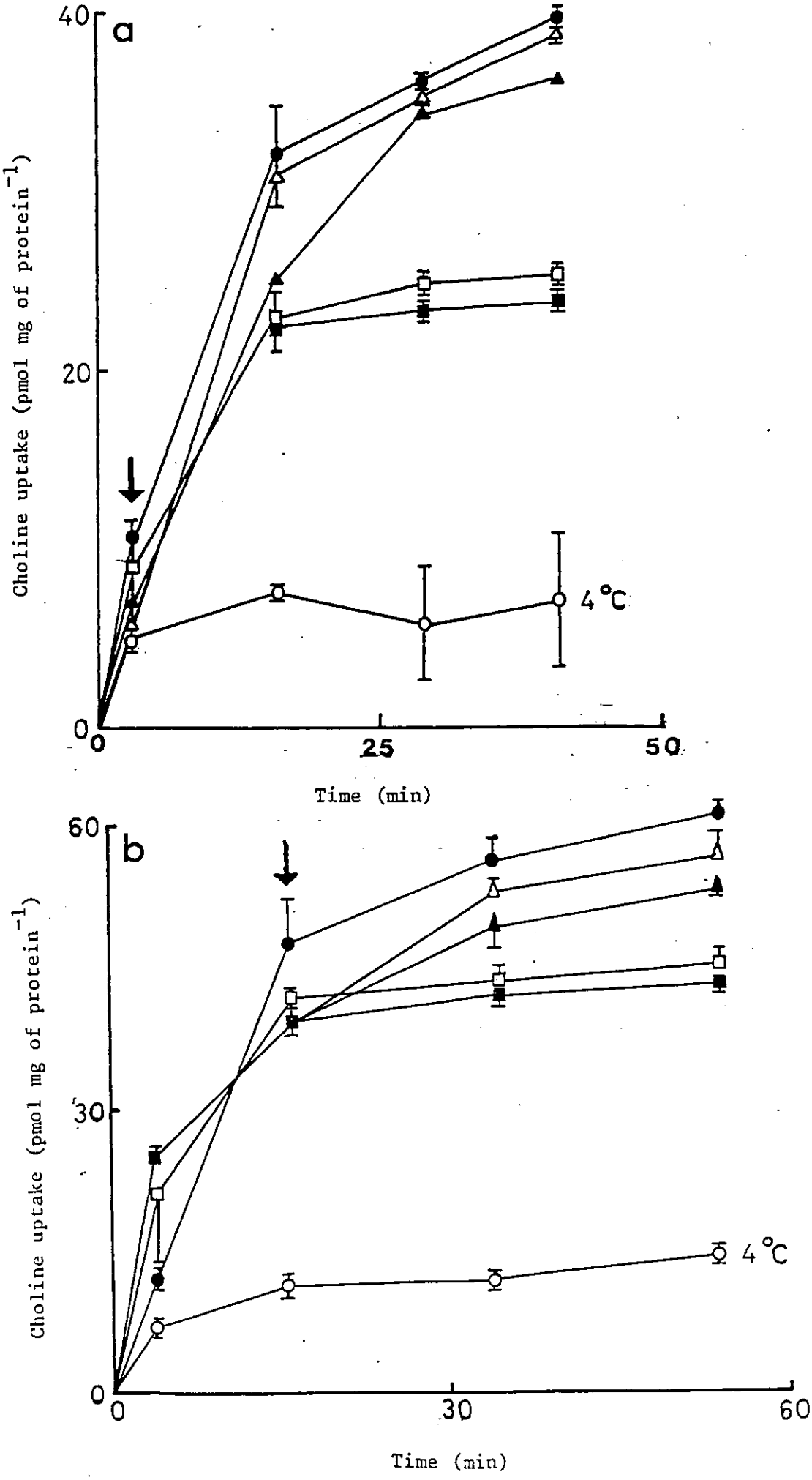
Synaptosomes which had been preloaded with [^{3}H] choline showed an increased release of [^{3}H] ACh when the K^{+} concentration was raised from 3 to 23 mM (Figs. 3.3 and 3.4); the evoked release was dependent on the presence of Ca^{2+} (Fig. 3.3) an important characteristic of the process of neurotransmitter release in vivo. The amount of choline released under the same conditions was smaller and was not Ca^{2+}-dependent (Fig. 3.3), this was possibly due to a small amount of leakage of [^{3}H] choline from the preloaded synaptosomes and to the displacement, by the increased salt concentration of some [^{3}H] choline which was non-specifically bound to the outside of the synaptosomes and other membrane fragments. Acetylcholinesterase activity was undetectable when aliquots of synaptosome suspensions were assayed in the presence of eserine sulphate; it seems unlikely, therefore, that this enzyme activity contributed significantly to the amount of [^{3}H] choline in the supernatant. Wonnacott and Marchbanks (1976) also observed increases in [^{3}H] choline release from guinea-pig synaptosomes although they were somewhat smaller.

Veratrine also stimulated ACh release; at a concentration of 25 μg/ml it gave a 6 to 7-fold increase (in 20 min,) over the amount released in the presence of 3mM K^{+} and its action was completely inhibited by TTX ($5 \times 10^{-6}M$) (Fig. 3.4). Veratrine also produced a "leakage" of

Figure 3.2 Effects of β-BuTX and TsTX on high affinity uptake of [^{3}H] choline by synaptosomes

The time course of accumulation of radioactivity by synaptosomes in Krebs phosphate medium containing [^{3}H] choline was measured by filtration assay as described in Section 3.2.3. Control incubations (●) contained no toxin additions and were incubated at 25°C. Other samples incubated at 25°C contained 2.3×10^{-7} M β-BuTX (□); 3×10^{-6} M TsTX (■); 1.5×10^{-5} M TTX (Δ); and 3×10^{-6} M TsTX and 1.5×10^{-5} M TTX (▲). TTX was added at zero time; β-BuTX and TsTX were added after 3 min (a) or after 16 min (b) as indicated by the arrows. Each point plotted is a mean of values from duplicate incubations. The error bars indicate the range of these values.

Blanks (O) were incubated at 4°C and contained synaptosomes which had been osmotically lysed in cold de-ionised water.


a
40
20
0
0
25
50
Choline uptake (pmol mg of protein^{-1})
Time (min)
4°C
b
60
30
0
0
30
60
Choline uptake (pmol mg of protein^{-1})
Time (min)
4°C

[^{3}H] choline similar to that observed with increased K^+. It is noteworthy that three methods of stimulating synaptosomes thought to act solely by their depolarising effects, i.e. K^+ (Fig. 4.5) veratrine (Tse et al., 1980) and TSTX (Fig. 3.5) failed to increase the release of the cytoplasmic marker enzyme, lactate dehydrogenase. These treatments seem, therefore, not to affect the integrity of the synaptosomes.

3.3.3. Effects of β-BuTX and TsTX on choline accumulation by synaptosomes

Both β-BuTX (2.3×10^{-7}M) and TsTX (3×10^{-6}M) caused almost complete inhibition of the uptake of [^{3}H] choline by synaptosome suspensions (Fig. 3.2). This action of TsTX was almost completely inhibited by TTX (1.5×10^{-5}M) which, on its own, had little effect on uptake (Fig 3.2). It is interesting to note that addition of β-BuTX and TsTX 2-3 min after resuspension of the synaptosomes in medium containing radioactive choline (0.5μM) produced only slight effects on accumulation earlier than 15 min (Fig 3.2a); thereafter no net accumulation occurred. A separate experiment measured the uptake by synaptosomes of [^{3}H] choline at a concentration of 2μM (Fig 3.2b) and a similar time course was observed to that shown in Fig 3.2a. The rate of uptake was approx. 3.0 pmol. min^{-1} mg of $protein^{-1}$ during the first 15 min and approx. 0.5 pmol. min^{-1} mg of protein thereafter. After 55 min, 7.7% of the [^{3}H] choline had been taken up into the synaptosomes. In this second experiment, addition of either β-BuTX or TsTX at 16 min immediately produced a nearly complete inhibition of choline accumulation. It is possible, therefore, that the apparent inhibition of [^{3}H] choline uptake by these toxins can be manifested only after an appreciable amount of the radioactivity has been accumulated.

Neither β-BuTX nor TsTX at the concentrations used in these experiments, caused a net decrease in the radioactive content of the synaptosomes which had been preloaded with [^{3}H] choline. In addition, incubation of synaptosomes for 20 min at 25°C with a range of concentrations of TsTX (10^{-10} to 10^{-4}M) caused negligible release of the cytoplasmic marker enzyme lactate dehydrogenase; β-BuTX at a concentration of 10^{-5}M

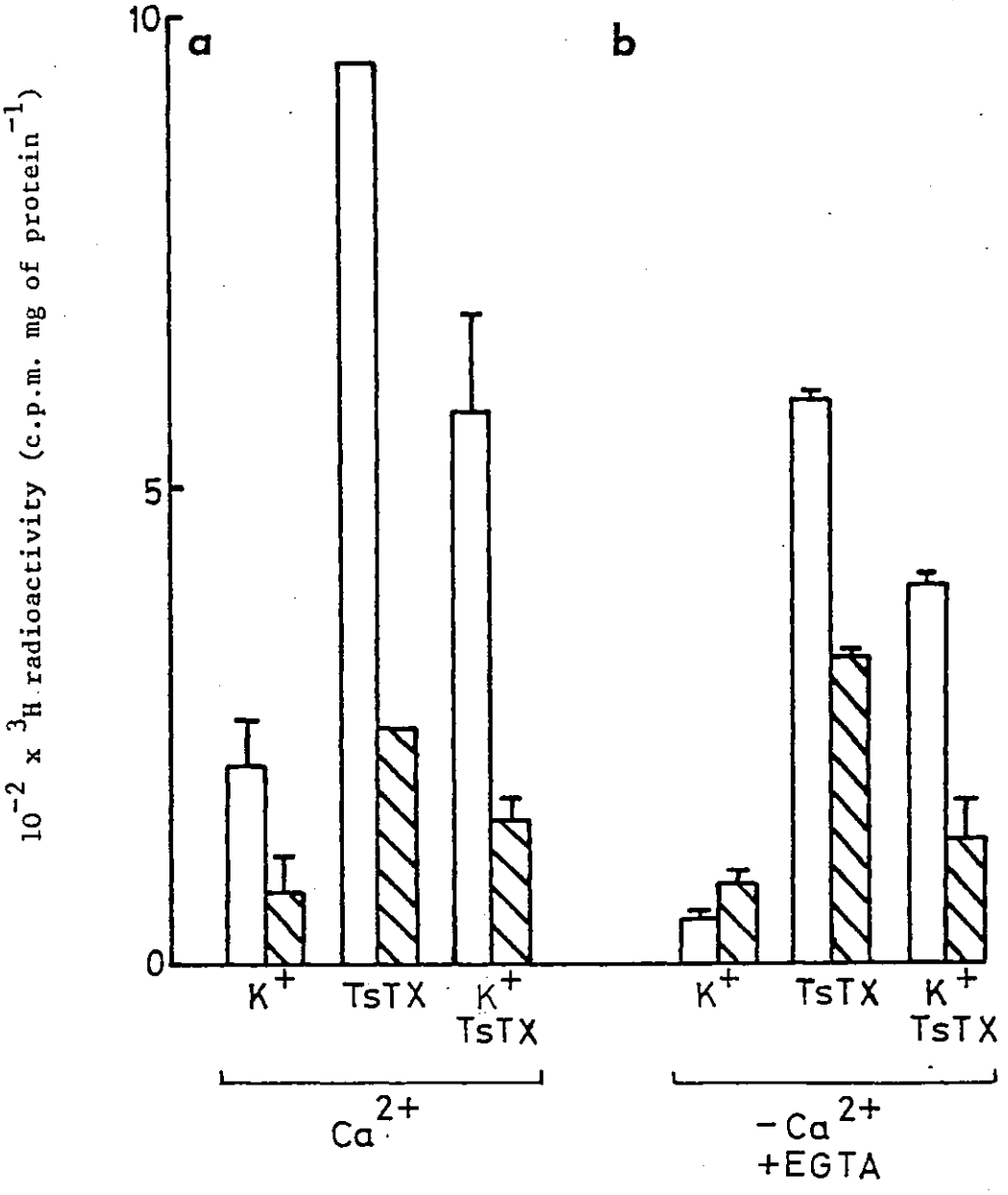


Figure 3.3 Ca^{2+} dependence of K^+- and TsTX-evoked release of ACh and choline from synaptosomes

The amounts of [^{3}H]ACh (open bars) and [^{3}H] choline (hatched bars) released into the medium, from synaptosomes which had been preloaded with the latter, during 20 min incubations at 25°C were determined as described in Section 3.2.4. The values shown are the means of those obtained from duplicate incubations; the error bars indicate the range. Control values (no additions) have been subtracted. Incubations were performed in (a) modified Krebs-phosphate medium (3.2.4) or (b) similar medium in which Ca^{2+} was iso-osmotically replaced by sucrose and with the inclusion of EGTA (5 mM). Where indicated, there was increased K^+ concentration (23 mM) and/or addition of TsTX (10^{-5} M).

released only 20% of the lactate dehydrogenase activity which was liberated by complete disruption of the synaptosomes with Triton X-100 (2%v/v) (Fig.3.5) It seems unlikely, in view of this, that either of these toxins causes gross lysis of synaptosomes.

3.3.4. Effects of β-BuTX and TsTX on the release of ACh and choline from synaptosomes

β-BuTX (5×10^{-6}M) produced a 1.7-fold increase in the rate of release of [^{3}H] ACh from synaptosomes which had been preloaded with [^{3}H] choline. The rate of release of radioactive choline was negligible in control samples but increased, in the presence of the toxin, to 75% of that measured for ACh. The rates of [^{3}H] ACh and [^{3}H] choline release were constant for 20 min following resuspension of the preloaded synaptosomes and addition of toxins at time zero (Fig. 3.6). Therefore, in all subsequent experiments, the effect of β-BuTX was measured by the increase in the amounts of radioactive ACh and choline released into the medium between 0 and 20 min. However, the rates of release varied between individual synaptosome preparations, the discrepancies were worsened by the low levels of radioactivity released. In the presence of 3mM K^+ and 0.74mM Ca^{2+} the amounts of [^{3}H] ACh and [^{3}H] choline in the medium after 20 mins. were, respectively, 200 and 10 cpm greater than their levels at 0 min in the experiment shown in Fig. 3.5., whereas 100 cpm [^{3}H] ACh and 35 cpm [^{3}H] choline were released in the same conditions over the same time in the example illustrated in Fig. 3.3. Such variation made it difficult to compare the effects on ACh and choline release of different conditions and toxin treatments except within each experiment.

The effects of β-BuTX on ACh and choline release showed similar concentration dependence (Fig. 3.5a) but the toxin released smaller amounts of choline than ACh. β-BuTX produced no increase at 10^{-8}M and a near maximal effect at 10^{-7}M; the EC_{50} was approximately 5×10^{-8}M. This suggests that there is a common mechanism by which β-BuTX affects release of the neurotransmitter, ACh and the non-transmitter, choline. Note that,

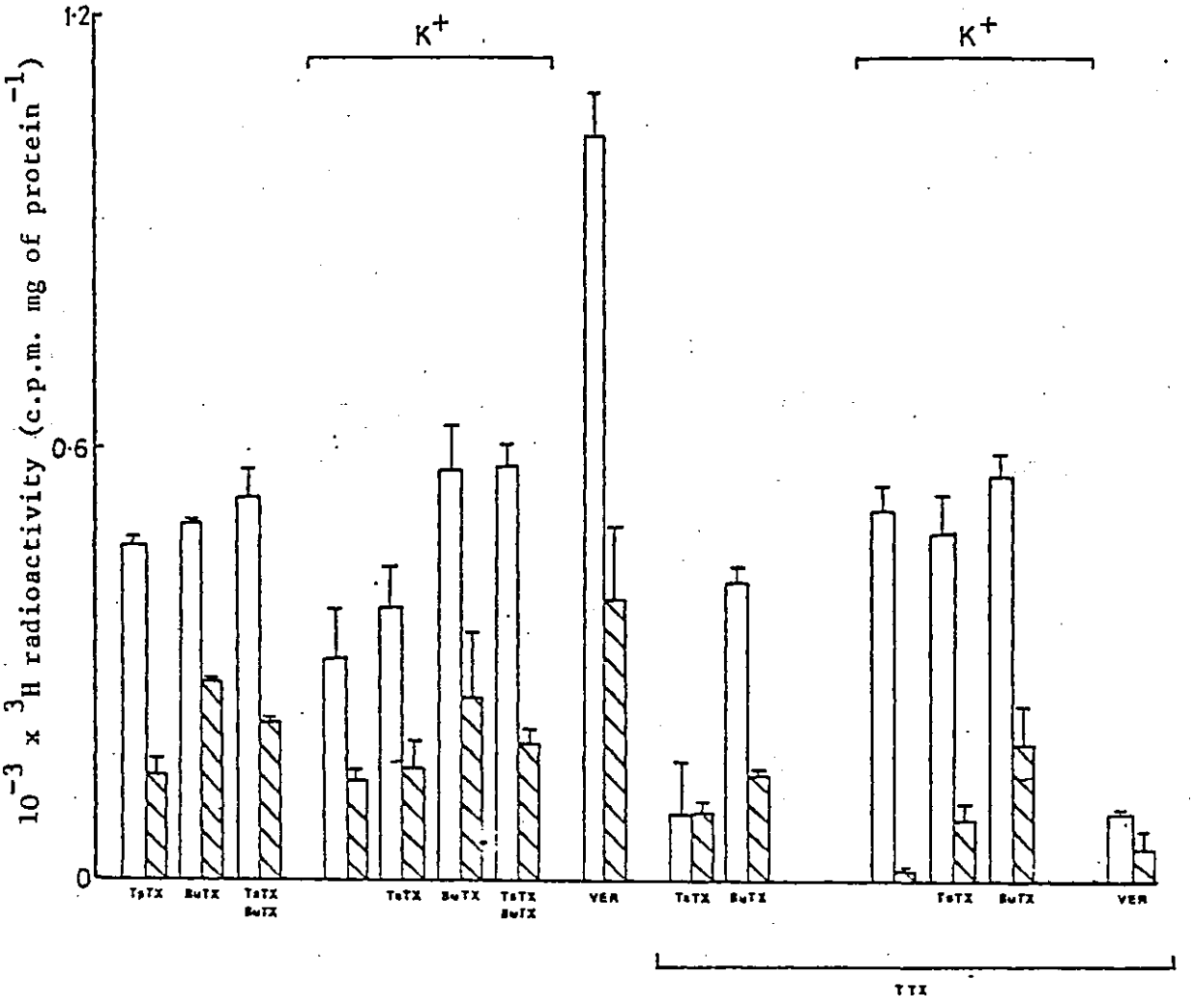


Figure 3.4 The effects of β-BuTX, TsTX and TTX on release of ACh and choline from synaptosomes

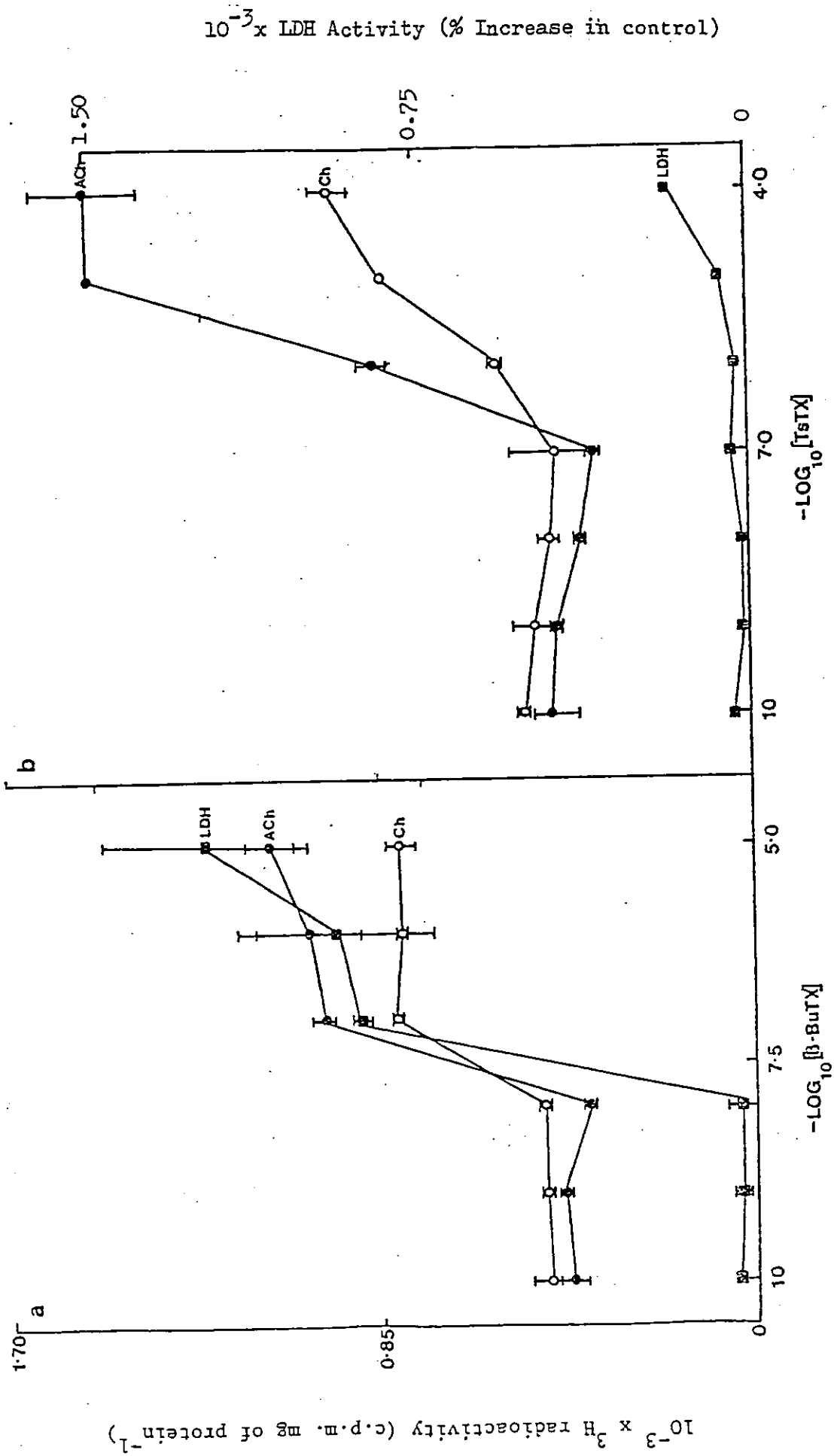
Released ACh (open bars) and choline (hatched bars) were determined as described in Section 3.2.4. Samples were pre-incubated for 5 min at 25°C, with TTX (5 x 10^{-6} M) where indicated. Additions were then made of β-BuTX (5 x 10^{-8} M), TsTX (10^{-6} M), veratrine (25 µg/ml) and increased K^+ concentration (23 mM) and the samples incubated for 20 min at 25°C. The values shown are the means from duplicate incubations with those obtained from control incubations subtracted. The error bars indicate the range.

in the same experiment, TsTX produced a large increase in ACh but not choline release (Fig. 3.5b); in view of this, and the lack of measurable acetylcholinesterase activity it would seem to be unlikely that the increased choline levels produced by β-BuTX were due to hydrolysis of released ACh. Also, since β-BuTX possesses a phospholipase activity (2.3.4.), the possibility that the toxin caused release of previously accumulated choline and ACh by lysing the synaptosome was investigated. β-BuTX was found to produce a large increase in the release, from synaptosomes, of the cytoplasmic marker enzyme lactate dehydrogenase (LDH); the concentration dependence of this effect was similar to those for its stimulation of both ACh and choline release. (Fig. 3.5.a). Although the LDH activity released by β-BuTX represents only 20% of that liberated by 2% Triton X-100, the release of such a large molecule (140000 daltons) must represent considerable disruption of the synaptosomal membranes. Depolarisation of the nerve terminals is an inevitable consequence of such perturbation of the membranes. It was, therefore, not surprising that when synaptosomes were depolarised with 23mM K^+, the addition of β-BuTX caused only a small additional release of ACh and choline compared to that produced in the presence of 3mM K^+ (Fig. 3.5a and b).

TsTX produced an increased rate of ACh release from synaptosomes (Figs. 3.3, 3.4, 3.5 and 3.6) which was linear for at least 40 mins. (Fig. 3.6); its EC_{50} was approximately 10^{-6}M (Fig. 3.5b) and it is, therefore, considerably less potent than β-BuTX (Fig. 3.5a). Unlike β-BuTX, TsTX had much less effect on choline than on ACh release (Figs. 3.4a and 3.5) and furthermore, the concentration dependence curve was shifted to higher toxin concentrations (Fig. 3.5b). In direct contrast to β-BuTX, it released negligible lactate dehydrogenase activity except at very high toxin concentrations (10^{-4}M) (Fig. 3.5b). TsTX, unlike β-BuTX, therefore, appears not to affect the integrity of synaptosomal plasma membranes. This is consistent with current understanding of the

Figure 3.5 Concentration dependence of β-BuTX and TsTX-induced release of ACh, choline and lactate dehydrogenase from synaptosomes

The amounts of [^{3}H] ACh (●), [^{3}H] choline (O) and lactate dehydrogenase (■) released after incubation of preloaded synaptosomes for 20 min at 25°C were determined as described in Sections 3.2.4 and 3.2.5. Released lactate dehydrogenase is expressed as a percentage increase in the amount present in the supernatant from control samples incubated without toxin addition. The values shown are the means from duplicate sets of incubations; the error bars indicate the range.


10^{-3} x LDH Activity (% Increase in control)
1.50
0.75
0
ACh
Ch
LDH
4·0
7·0
10
$-LOG_{10}$[TsTX]
b
LDH
ACh
Ch
5·0
7·5
10
$-LOG_{10}$[β-BuTX]
a
1·70
0·85
0
10^{-3} x ^{3}H radioactivity (c.p.m. mg of protein^{-1})

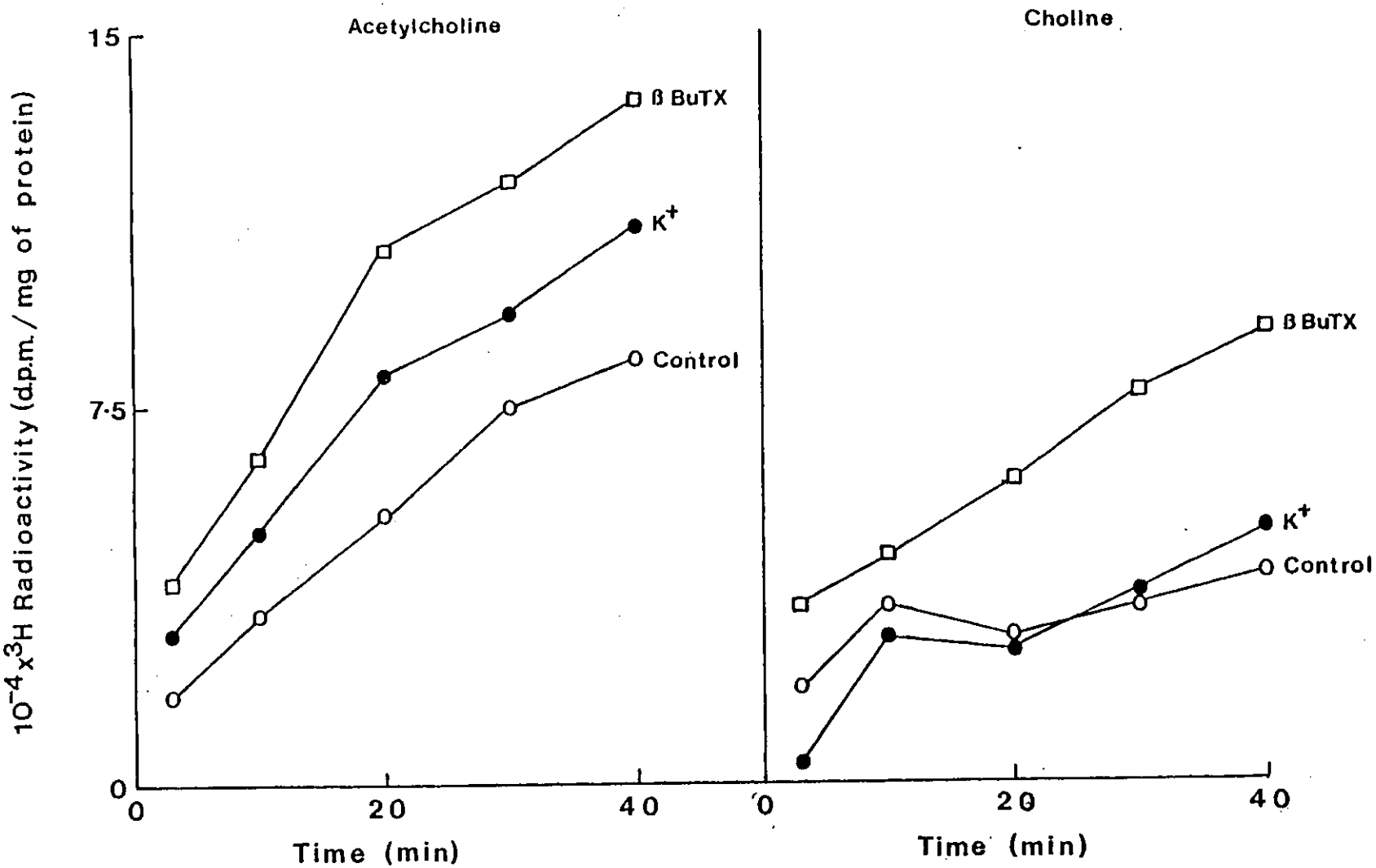
mechanism of action of TsTX, that it causes depolarisation of neuronal membranes by acting directly on the Na^+ channels and prolonging their inactivation (1.2.2.). Accordingly, removal of Ca^{2+} from the medium reduced the effect of TsTX on ACh, but not choline, release from synaptosomes (Fig. 3.3). Likewise TTX, markedly inhibited the effects of TsTX on ACh release but only slightly decreased its, much smaller, effects on choline release, (Fig. 3.4.). When synaptosomes were incubated with 23mM K^+, addition of TsTX caused a further increase in the release of ACh together with a smaller amount of choline (Fig. 3.3). Since both treatments cause Ca^{2+} dependent transmitter release by depolarising the synaptosomes, their separate effects were not expected to be additive (Fig. 3.3.). Indeed, the increased transmitter release given jointly by 23mM K^+ and TsTX was less than that due to the same concentration of the toxin alone. This was also as expected from the observation that the binding of a related scorpion toxin to neuroblastoma cells is dependent on the membrane potential (Catterall,1977).

When preloaded synaptosomes were incubated with either 3mM K^+ or 23mM K^+, the amounts of [^{3}H] ACh and [^{3}H] choline released when β-BuTX and TsTX were added together were only slightly greater than the amounts released in the presence of each toxin independently (Fig. 3.4.). This lack of additivity in the effects of these two toxins is consistent with the hypothesis that both cause increased release of transmitter by depolarising synaptosomes, although they may produce this depolarisation by different mechanisms. The non-additivity was not due to a maximal rate of release being reached since, in the same experiment, veratrine (at a concentration of 25μg/ml) released almost twice as much ACh over the same incubation period.

In the experiment illustrated in Fig. 3.4, β-BuTX (5×10^{-8}M) released 490 cpm [^{3}H] ACh mg of protein^{-1}, in 20 min., from synaptosomes which had been preloaded with [^{3}H] choline. In the presence of TTX

Figure 3.6 Time courses of the effects of β-BuTX and K^+ on the release of ACh and choline from synaptosomes

Synaptosomes which had been preloaded with [^{3}H] choline were incubated in modified Krebs-phosphate medium (3 mM K^+) at 25°C. The amounts of radioactive ACh (a) and choline (b) released into the medium were measured, after various time periods, as described in Section 3.2.4. The values plotted are the means of duplicate incubations; the error bars indicate the range. β-BuTX (5×10^{-6} M) (□) and K^+ (23 mM) (●) were added at zero time. Control incubations contained no toxin additions (○).


Acetylcholine
Choline
15
7·5
0
$10^{-4} \times {}^{3}H$ Radioactivity (d.p.m./mg of protein)
ß BuTX
K^+
Control
0
20
40
Time (min)

(5×10^{-6}M), a specific blocker of Na^+ channels, β-BuTX caused the release of 430 cpm [^{3}H]ACh. mg of protein^{-1} in the same period. If the action of β-BuTX involved depolarisation via Na^+ channels, as has recently been suggested (Smith et al, 1980), a much greater inhibition of its effects by TTX would be expected. Similarly, the effects of β-BuTX on ACh and choline release in the presence of either 23mM K^+ or TsTX (10^{-6}M) are not inhibited by TTX (Fig. 3.4); indeed, in these instances, the amounts of ACh released appear to be greater in the presence of TTX. These experiments were performed at room temperature 25°C to facilitate the manipulations; it has been assumed that the effects of toxins would be qualitatively similar at 37°C.

3.4 Discussion

3.4.1. Differentiation of toxin actions on synaptosomal uptake and release

Both pure β-BuTX and TsTX decreased the rate of choline uptake by nerve terminals purified from rat cerebral cortex (Fig. 3.2.). They reduced the accumulation, by synaptosomes, of glutamate and GABA (Dolly et al., 1978; Tse et al., 1980). Both toxins also caused an increase in the rate of release of previously accumulated ACh and choline (Fig. 3.4.) as well as the two putative amino acid neurotransmitters (Dolly et al., 1978; Tse et al., 1980). This confirmed previous reports, using other β-BuTX preparations, of similar effects on a number of neurotransmitter substances (3.1.). The similar lack of transmitter specificity in the case of TsTX was expected since the voltage dependent Na^+ channels that it affects are present in all neurones.

It was necessary to establish whether or not the apparent decreases in the overall rate of accumulation of radiolabelled transmitters or precursor, produced by β-BuTX and TsTX, represented direct inhibition of the Na^+-dependent high affinity transport systems. Alternatively, these may have been indirect effects resulting from the stimulation of release of already accumulated transmitter. Furthermore, both TsTX, by its well

characterised effects on Na^+ channels (3.1) and β-BuTX (Sen and Cooper, 1978) appear to cause depolarisation of synaptosomes. It has been reported that transport of GABA into brain synaptosomes was dependent on membrane potential (Blaustein and King, 1976; Martin, 1976). Toxin-induced depolarisation of synaptosomes may, therefore, decrease uptake in addition to stimulating the release of neurotransmitters. TTX had no effect on the net accumulation by synaptosomes of choline (Fig. 3.2), glutamate or GABA (Tse et al, 1980). It did not inhibit the binding, to the membranes of electrically excitable neuroblastoma cells, of a toxin from the venom of the scorpion Leiurus quinquestratus, whose action is similar to that of TsTX (Catterall, 1977). Nevertheless TTX, by directly blocking the ion filter, does inhibit the depolarisation of the membrane which results from TsTX binding at another site on the Na^+ channel complex (1.2.2.). Thus, if the effect of TsTX on transmitter uptake was a direct one due to its binding at a site other than the potential-dependent Na^+ channel complex, its decrease of transmitter accumulation should not be affected by TTX. In fact, TTX did inhibit the effect of TsTX on the accumulation of choline (Fig. 3.2), GABA and glutamate (Tse et al., 1980). It may reasonably be concluded, therefore, that the observed effects of TsTX were due solely to membrane depolarisation.

Unfortunately due to the lack of a suitable antagonist, it is not yet possible to separate the effects of β-BuTX on accumulation and release of transmitters. However, it is interesting that following early addition of either β-BuTX or TsTX to synaptosomes freshly suspended in medium containing radiolabelled choline a period of several minutes was required before more than a slight effect on the time course of accumulation was seen (Fig. 3.2a); later addition of toxin gave an immediate and almost complete inhibition. Thus the apparent blockade of [^{3}H] choline uptake produced by these toxins was only seen after a considerable amount of radioactivity had been accumulated within the nerve terminals.

This is difficult to reconcile with a direct effect of these toxins on the high affinity transport system. Another β-BuTX preparation (Sen et al., 1976) was reported to cause a large inhibition of high affinity accumulation of [^{3}H] choline (0.5μM) by a crude synaptosomal preparation, a P2 fraction of rat cerebral cortex. These workers reported that when added, "during the uptake process", β-BuTX (5×10^{-7} M) produced an inhibition of choline uptake within 3 min. This was presumably analogous to the effect shown in Fig. 3.2b. When synaptosomes were preincubated with this same toxin preparation (Sen et al., 1976) the rate of uptake of [^{3}H] choline was reduced from the time of its addition. In this case, preincubation with toxin may have disrupted the electrochemical gradients of the nerve terminals, thereby inhibiting the Na^+-dependent high affinity uptake system. A third β-BuTX preparation (4.5×10^{-8} M) caused an immediate inhibition of [^{3}H] choline accumulation by purely cholinergic nerve terminals (T sacs) isolated from the electric organ of Torpedo marmorata (Dowdall et al., 1977). However, in this instance the toxin was again added after 10 min. incubation with [^{3}H] choline.

It has been suggested that β-BuTX, by blocking uptake of choline, eventually depletes nerve terminals of transmitter (Dowdall et al., 1977; Sen et al., 1976). Indeed, since it also stimulates release of ACh a decrease in the transmitter content of nerve terminals would be expected. However, electrophysiological observations at synapses blocked by β-BuTX suggest that transmitter is not depleted (Kelly et al., 1975), and can be released from toxin-poisoned nerve terminals (Chang et al., 1977a). Ultrastructural studies showed that treatment with very high toxin concentrations (1-10μM) did not significantly decrease the number of synaptic vesicles in synaptosomes (Sen et al., 1976) or at the neuromuscular junction (Strong et al., 1977), except when the latter was also subjected to high frequency

stimulation (Chang et al., 1973). Thus, there are several indications that the decrease in choline accumulation which was produced by β-BuTX was not due to direct action of the toxin on the uptake system. Rather, it was probably mediated by the same mechanism by which the toxin increased ACh and choline release. This decrease in accumulation was similar to that produced by TsTX which, as described above, was shown to be a secondary effect of the depolarisation caused by TsTX binding to Na^+ channels. No similar antagonist of β-BuTX action is yet available. However, botulinum toxin shows some antagonism to the effects of β-BuTX, such as the release of ACh during the second phase of its action at the neuromuscular junction (Chang and Huang, 1974). If shown to affect specifically Ca^{2+}-dependent transmitter release in response to depolarisation, without affecting high affinity transport, this toxin may be used to show the extent to which increased transmitter release contributes to the apparent decrease of choline uptake caused by β-BuTX. This would not exclude the possibility that uptake is inhibited by β-BuTX evoked depolarisation of the nerve terminals rather than by a direct action of the toxin. Since β-BuTX is known to cause depolarisation of nerve terminals (Sen and Cooper, 1978) and most of its observed effects can be explained in terms of such depolarisation it may be unnecessary to invoke a separate blockade by the toxin, of transmitter accumulation. It should be noted, however, that chemically modified taipoxin which lacked phospholipase activity, was 300 times less lethal to mice than native toxin and did not inhibit neuromuscular transmission but was only three times less potent than the native toxin at inhibiting the uptake of $[^3H]$ choline into T sacs purified from the electric organ of Torpedo marmorata (Fohlman et al., 1979). This suggests that taipoxin, may have some phospholipase-independent action on the choline transport system. A recent study employing a gas chromatographic-mass spectrographic assay showed that β-BuTX (5×10^{-8} M) caused a large release of endogenous choline from P_2 fractions of rat brain

and suggested that the resultant dilution of labelled choline in the medium was the cause of apparent inhibition of choline uptake (Gundersen and Jenden, 1981). Surprisingly, however, in this study, β-BuTX failed to affect the accumulation of deuterated choline and caused only a small increase in ACh release from the synaptosome preparation.

3.4.2. The involvement of membrane perturbation and depolarisation in the action of β-BuTX

The effects of β-BuTX on the release of ACh and choline were not additive with those of TsTX or raised external K^+ concentration (Fig. 3.4). This observation is consistent with the involvement of membrane depolarisation in its mechanism of action. In the presence of Ca^{2+} such depolarisation would lead to increased transmitter release. Depolarisation of synaptosomes by the toxin has been measured using the potential-sensitive fluorescent dye 3,3'-dipentyl-2-2' oxycarbocyanine (Sen and Cooper, 1978). Previously, these workers had found, in agreement with observations described herein, that β-BuTX greatly increased the release of radiolabelled ACh from preloaded synaptosomes in the presence of 5.3mMK^+, but when the synaptosomes were depolarised with 53mMK^+ the toxin caused little further release. This was initially interpreted as an inhibition by the toxin of K^+-stimulation ACh release (Sen et al., 1976). It was subsequently shown, using the cyanine dye, that the addition of depolarising concentrations of K^+ to synaptosome suspensions which already contained β-BuTX caused no further depolarisation (Sen and Cooper, 1978). Furthermore, in the absence of Ca^{2+}, increased external K^+ concentrations continue to depolarise synaptosomes suspension whereas β-BuTX, which requires Ca^{2+} for its phospholipase activity, did not (Sen and Cooper, 1978).

As previously indicated the effects of β-BuTX on synaptosomes are not significantly inhibited by TTX as are those of TsTX (3.3.3; 3.3.4. Halliwell et al., 1982). The release of ACh from a P2 fraction of rat cerebral cortex by β-BuTX was also unaffected by TTX (Sen and Cooper, 1978). Similarly, TTX has little or no effect on the action of β-BuTX

on the accumulation or release by synaptosomes of other neurotransmitters, glutamate, GABA and noradrenaline (Tse et al., 1980; Wernicke et al., 1975). β-BuTX has also been shown to block neurotransmission at frog neuromuscular junctions in the presence of TTX (Alderdice and Volle, 1978). These observations strongly suggest that the depolarisation of nerve terminals caused by β-BuTX is not mediated via Na^+ channels. The release of glutamate and GABA from synaptosomes induced by β-BuTX was not affected by the presence of tetraethylammonium ions which block K^+ channels (Tse et al., 1980). This indicates that these channels, also are not involved in β-BuTX action on synaptosomes.

Unlike TsTX, β-BuTX caused the release from synaptosomes of choline and a cytoplasmic marker, lactate dehydrogenase, in addition to ACh; the concentration dependence for its effect on all three was very similar (Fig. 3.5). This strongly suggests that the mechanism by which β-BuTX increased release of the transmitter substance, its precursor and the cytoplasmic enzyme were related and, in view of the phospholipase activity of the toxin, that it involved some perturbation of the plasma membrane. However, the action of β-BuTX did not result in gross lysis of the nerve terminals as the maximum amount of lactate dehydrogenase activity released was approximately 20% of that liberated by 2% Triton X - 100, a treatment which was assumed to completely disrupt the synaptosomes. Also, in uptake experiments, treatment with the toxin did not lead to a net decrease in the radioactive content of synaptosomes. Furthermore, ultrastructural damage to nerve terminals has only been observed, in the electron microscope, at very high toxin concentrations (Sen and Cooper, 1976; Strong et al., 1977; Tse et al., 1980).

The involvement of the phospholipase activity of β-BuTX in its actions in the central nervous system are considered more fully in the following chapter. The effects of β-BuTX on neurotransmission, recorded

electrophysiologically at intact synapses in rat olfactory cortex will be described and discussed in relation to both the phospholipase activity of the toxin and its effects on synaptosomes.

CHAPTER 4. INVOLVEMENT OF THE PHOSPHOLIPASE ACTIVITY OF β-BUNGAROTOXIN IN ITS ACTION ON NEUROTRANSMITTER RELEASE AT SYNAPSES IN THE CENTRAL NERVOUS SYSTEM

4.1 Introduction

β-BuTX had been shown, in the previous chapter, to cause large increases in the rate of release of radiolabelled neurotransmitters from synaptosomes, probably as a result of depolarisation. It was necessary to examine two further questions: Firstly, to what extent are the observations using synaptosomes a good measure of the effect of β-BuTX on intact synapses in the central nervous system? Secondly, how is the phospholipase activity of the toxin involved in these actions?

To investigate the first of these points the effects of the toxin were measured, electrophysiologically, on intact synapses in a preparation from the central nervous system. Prior to this study the action of this toxin on such a preparation had not been observed. A series of experiments was therefore carried out, in collaboration with Dr J.V. Halliwell at the School of Pharmacy, University of London, to measure by extracellular recording the effect of β-BuTX on synaptic transmission in the rat olfactory cortex following stimulation of the lateral olfactory tract. This experimental system, as well as synaptosome preparations was then used to investigate the extent to which the phospholipase activity of β-BuTX contributes to its action at central synapses.

The phospholipase A_2 residing in the A chain of β-BuTX, as has previously been described, requires deoxycholate for maximal activity on pure phospholipid substrates (2.3.4; Abe et al., 1977; Howard

and Truog, 1977; Strong et al., 1976) and also varies with the chain length of the substrate (Howard and Truog, 1977). The enzymic activity of the toxin, on phosphatidylcholine liposomes as substrate,was greatly enhanced at their phase transition temperature and was inhibited when cholesterol was included in the vesicles (Strong and Kelly, 1977). Thus, the phospholipase A_2 activity of β-BuTX seems to be highly dependant on the fluidity and phase properties of the substrate. Very sensitive assays have been carried out using membranes and radiolabelled fatty acids. The toxin was reported to release labelled fatty acids from synaptosomal membranes in the presence of deoxycholate (Sen and Cooper,1978; Othman et al., 1982) and to hydrolyse bacterial membrane phospholipids in the absence of the detergent (Howard and Truog,1977; Wernicke et al., 1975). The toxin caused little hydrolysis of the membranes of intact erythrocytes (Lee et al., 1972; Strong et al., 1977; Wernicke et al., 1975) but produced complete lysis when entrapped in resealed red cell "ghosts" (Strong et al., 1977). In the experiments described in this chapter, the phospholipase activity of β-BuTX was inhibited either by changing the ionic composition of the medium or by chemical modification of the toxin.

Phospholipases A are, in general, highly dependent on the presence of Ca^{2+} (Pieterson et al., 1974b). The enzyme activity of β-BuTX is strongly inhibited if Ca^{2+} is replaced by Sr^{2+} and is completely abolished in Ca^{2+}-free medium containing the chelating agent EGTA (2.3.4). Since Sr^{2+} can substitute for Ca^{2+} in the process of neurotransmitter release (Dodge et al., 1969) this provides a method for studying the action of β-BuTX under conditions in which its phospholipase activity is minimised. When Ca^{2+} was replaced by Sr^{2+} the toxin did not produce either the second or third phases of its action on

spontaneous release of ACh (Strong et al., 1977) (but see also Alderdice and Volle, 1978). This ionic substitution also inhibited the effect of β-BuTX on release of ACh at vertebrate neuromuscular junctions in response to indirect stimulation, as measured by both intracellular recording (Abe and Miledi, 1978; Kelly et al., 1979b) and muscle contraction (Chang et al., 1977b; Kelly et al., 1979b). Nevertheless a decrease, corresponding to phase one, was observed in all these measures when Sr^{2+} substituted for Ca^{2+}, and in one instance (Abe and Miledi, 1978) this persisted for several hours. Recently, both time to the onset of phase one and the time required for 50% inhibition of epp amplitude during this phase were found to be both shorter and strongly temperature-dependent when Ca^{2+} was present rather than Sr^{2+} (Caratsch et al., 1981). This was interpreted as indicating a Ca^{2+}-dependent interaction of β-BuTX with nerve terminal membranes.

Chemical modification may also be used to remove the enzyme activity of the toxin. Porcine pancreatic phospholipase A_2, to which the A chain of β-BuTX has considerable sequence homology (Kondo et al., 1978b) may be specifically and irreversibly inactivated by acylation of a histidine residue in its active site, using p-bromophenacyl bromide (pBPB) (Volwerk et al., 1974). When the phospholipase activity of β-BuTX was abolished by a similar modification its toxicity was greatly reduced (Abe et al., 1977; Kelly et al., 1979b) and it produced only the initial decrease in frequency of spontaneous miniature end-plate potentials (Abe et al., 1977), evoked end-plate potential amplitude (Abe et al., 1977) or force of muscle contraction following nerve stimulation (Kelly et al., 1979b). It was also observed that the time required for β-BuTX to produce a neuromuscular blockade was

increased four-fold when the preparation was pretreated with a four-fold higher concentration of modified toxin (Abe et al., 1977). This partial protection was observed even if the preparation was washed between the two treatments (Kelly et al., 1979b).

As previously mentioned (1.3.3.4) the most basic component of the venom of Bungarus multicinctus is a single chain, presynaptic toxin with a triphasic effect similar to that of β-BuTX (Livengood et al., 1978). After inactivation of its enzyme activity, by boiling for 3 min this toxin also showed the first but not the second and third phases of its action.

The decreased ability of a β-BuTX preparation to cause the release of [^{14}C] GABA from rat cerebrocortical synaptosomes when Ca^{2+} was replaced by Sr^{2+} (Tse et al., 1980), provided evidence that phospholipase activity was also involved in the actions of the toxin in the central nervous system. This was supported by the finding that β-BuTX and another snake venom phospholipase A_2 caused similar depolarisation and ACh release from P_2 fractions of rat cerebral cortex (Sen and Cooper, 1978). As in the case of its blockade of neuromuscular transmission, it seemed unlikely that the effects of β-BuTX in the central nervous system could be due, solely, to its enzyme activity. Other phospholipases, when injected into the brain (Lee and Chen, 1977) are much less lethal than β-BuTX administered in this way (2.3.4). Also, when β-BuTX was reacted with ethoxyformic anhydride (EOFA), whilst its enzyme active site was protected with a pseudo-substrate, an enzymically active but non-neurotoxic derivative was formed. Its ability to depolarise synaptosomes and inhibit their accumulation of GABA was greatly impaired (Ng and Howard, 1978).

In this study the contribution of the enzyme activity of β-BuTX to its action at synapses in the central nervous system was examined by both biochemical and electrophysiological techniques. The phospholipase activity was inhibited by replacement of Ca^{2+} by Sr^{2+} or by chemical modification with p-bromophenacyl bromide and its effects examined on transmitter release from purified brain nerve terminals (see Chapter 3) and at synapses in slices of rat olfactory cortex. These effects were also compared to those of several pure phospholipases in an attempt to determine the specificity of the actions of β-BuTX in the central nervous system.

4.2 Methods

4.2.1 Materials

The following pure phospholipases were generously supplied by Dr R. Shipolini: Bee venom phospholipase A_2 (BVPL), Naja melanoleuca phospholipase A_2 (NMPL) and two enzymes from the venom of Vipera ammodites a heterodimer (VA_{5-7}) and a homodimer (VA_{6-8}). [Methyl-^{3}H] choline (specific radioactivity 13 Ci/mmol) and L-[U-^{14}C]-glutamate (290mCi/mmol) were purchased from the Radiochemical Centre, Amersham, U.K. p-Bromophenacyl bromide and egg yolk lecithin (Grade 1) were supplied by Fluka A.G. and Lipid Products, Surrey, U.K. respectively. Acetylcholine chloride and choline chloride were from Sigma. All other chemicals were of reagent grade. All solutions were made in deionised water.

4.2.2 Chemical modification of β-BuTX with p-bromophenacyl bromide

The activity of phospholipase A_2 enzymes can be abolished by chemical modification using the alkylating agent p-bromophenacyl bromide (Volwerk et al., 1974). A similar modification of β-BuTX was performed by incubating the toxin (7×10^{-5} M) at 30°C for 12 hours in the presence of p-bromophenacyl bromide (BPB); the reaction was carried out at pH 6.0 in sodium cacodylate buffer (0.1M). A tenfold molar excess of p-bromophenacyl bromide was added, dissolved in a small volume of acetone; however, its solubility in water is only 10^{-4}M and this was therefore the concentration which was maintained throughout the incubation as more of the reagent dissolved to replace that which had reacted with the toxin. Under similar conditions, a linear decrease with time in the phospholipase activity of β-BuTX was reported (Abe et al., 1977). A typical reaction mixture was as follows:

β-BuTX	(5 mg/ml)	0.620 ml
p-bromophenacyl bromide	(10 mg/ml in acetone)	0.039 ml
sodium cacodylate	(1M; pH 6.0)	0.200 ml
water		1.250 ml

The reaction was terminated after 12-16 hrs by gel filtration on a Sephadex G-75 (superfine) column, equilibrated with5mM Tris-HCl pH 7.4, 0.1M NaCl, which separated the modified toxin from unreacted BPB. The concentration of protein in the pooled toxin fractions was assayed by the method of Lowry et al. (1951) using unmodified β-BuTX as a standard. The absorbance of aqueous solutions of β-BuTX and BPB were measured at 271 nm and their molar extinction coefficients were calculated to be 19670 and 11950 cm^{-1} respectively. The number of moles

of BPB incorporated per mole of toxin was then calculated from the difference in absorbance at 271 nm between modified and native β-BuTX.

4.2.3 Measurement of the release of ACh, glutamate and GABA from synaptosomes

Nerve terminals were purified from rat cerebral cortex as previously described (3.2.2). In order to examine the effects of β-BuTX on ACh and choline release when its phospholipase activity is inhibited, some experiments were performed in which Ca^{2+} was replaced by Sr^{2+}. For these experiments, the synaptosomes were preloaded with [^{3}H] choline as before (3.2.4) except for the omission of Ca^{2+} from the medium used for both the loading incubation and the subsequent washing of the synaptosomes. Final resuspension of the loaded synaptosomes was in modified Krebs-phosphate medium (3.2.4) containing either $CaCl_2$ or $SrCl_2$ (0.74 mM). Equal volumes of these suspensions (200 µl) were added to tubes containing, where appropriate, aqueous solutions of β-BuTX and KCl or the corresponding small volumes (10 - 25 µl) of water. The incubations were carried out at 25°C and terminated after 20 minutes by centrifugation at 10,000 g for 5 minutes. Control incubations and the separation plus measurement of released ACh and choline were as previously described (3.2.4).

β-BuTX was found to cause a large increase in the release of [^{14}C] glutamate and [^{14}C] GABA from preloaded synaptosomes, similar to its effect on ACh release (Tse et al., 1980). Since measurement of glutamate and GABA release did not routinely involve high voltage paper electrophoresis it was more easily performed and was, therefore, used in experiments which compared the actions on transmitter release of native and chemically modified β-BuTX and phospholipases. For these experiments, the loading of synaptosomes was carried

out by incubation with [^{14}C] glutamate (0.65μM; specific radioactivity 0.29 Ci/mmol) or [^{14}C] GABA (0.65μM; specific radioactivity 6 Ci/mmol) for 20 minutes at 37°C in modified Krebs-phosphate medium (3.2.4). Washing, final resuspension and incubation of the synaptosomes for 20 minutes at 25°C were carried out as in the ACh release experiments; similar controls were performed. The incubations were again terminated by centrifugation at 10,000 g for 5 minutes and the radioactivity released into the supernatant was measured by liquid scintillation counting using a xylene based cocktail containing 10% Triton X-100. In similar experiments, high voltage paper electrophoresis, at pH 6.5, of aliquots of the supernatants followed by autoradiography showed that radiolabelled glutamate or GABA comprised 70% of the radioactivity released by K^+ stimulation or toxin (Tse et al., 1980).

4.2.4 Other determinations

Phospholipase activities towards egg yolk lecithin in the presence of Ca^{2+} and deoxycholate were measured as previously described (2.2.8). Lactate dehydrogenase activity was assayed spectrophotometrically (Bergmeyer, 1965). Following centrifugation of synaptosome suspensions at 10,000 g for 5 minutes, aliquots of the supernatants (30 μl) were added to a mixture consisting of potassium phosphate buffer (18 mM, pH 7.4), NADH (8×10^{-5} M) and sodium pyruvate (36 mM) in a quartz cuvette and the decrease in absorbance due to NADH at 340 nm was monitored. Enzyme activity, μmoles of pyruvate reduced per minute, was calculated from the initial rate of disappearance of this absorbance and expressed relative to the protein content of synaptosomes. In order to measure this, the pellets were broken up by agitation on a vortex mixer in 0.4 ml of water and solubilised in 0.9 ml of 0.45M NaOH; protein concentrations of the digests were determined by the

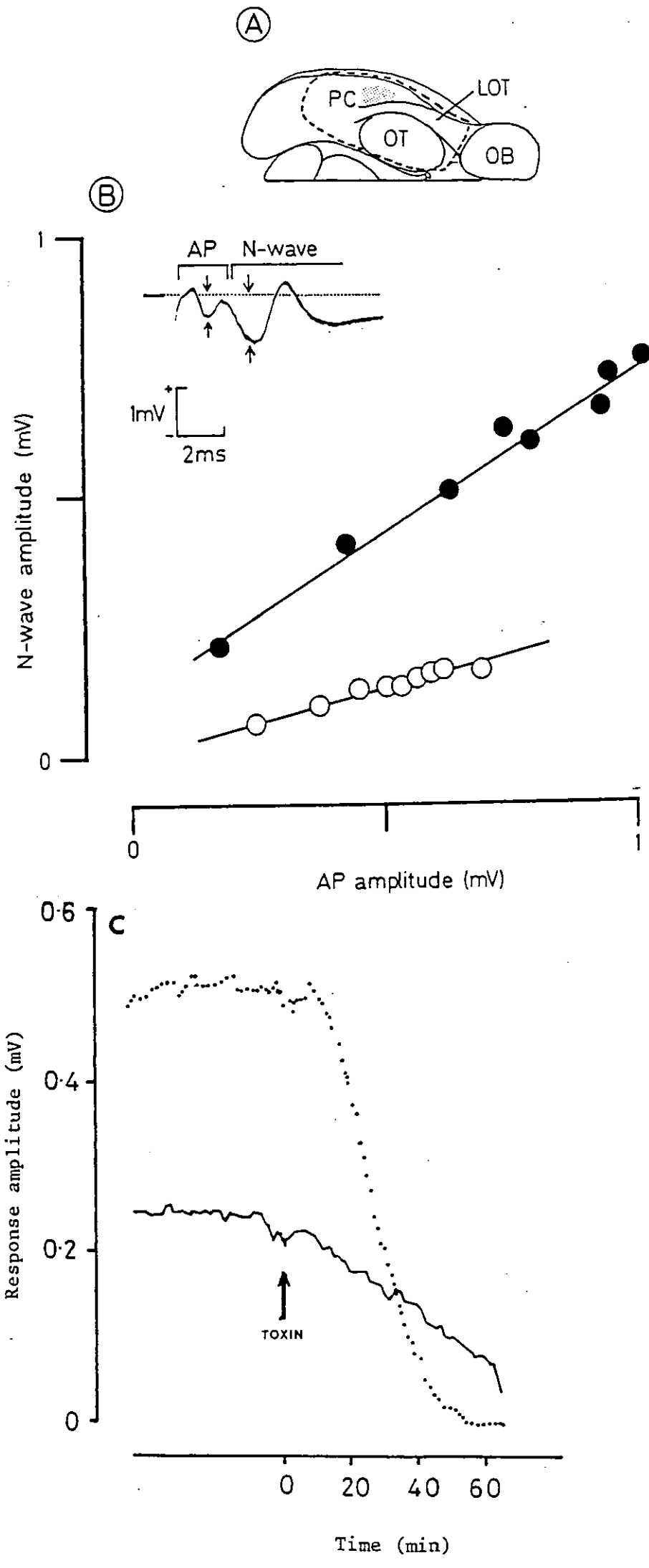
method of Lowry et al., (1951) using bovine serum albumin as a standard. Whole animal toxicities were determined by intraperitoneal injection into mice as previously described. (2.2.8).

4.2.5 Electrophysiological recordings on rat olfactory cortex slices

Extracellular recordings were made from surface slices (400μM thickness) of rat olfactory cortex (Dolly et al., 1980b). The lateral olfactory tract (LOT) runs across the surface of these slices (Fig. 4.1a) and fibres from this form synapses with the dendrites of cortical cells just below the surface. The cortical cell bodies lie deeper within the slices, forming one of the outer layers of the piriform cortex (Price et al., 1973). Single slices were incubated at 28°C in a chamber specially constructed for these measurements (Brown and Halliwell, 1980). The perfusion medium was a modified Krebs solution containing: Na^+, 118 mM; K^+, 6 mM; Ca^{2+}, 2.5 mM; Mg^{2+}, 1.2 mM; Cl^- 125 mM; HCO_3^-, 25 mM; $H_2PO_4^-$ 1.0 mM; D-glucose, 11 mM; pH 7.4 (when saturated with 95% O_2/5% CO_2). In some experiments Ca^{2+} was replaced by Sr^{2+} (5 mM). "Cation-free" medium was without Ca^{2+} and Mg^{2+} and contained EGTA (100μM); the change in osmolarity was < 4% and was not corrected for. The anterior end of the LOT was stimulated with regular pulses (50-100 μs) above a threshold amplitude from bipolar electrodes. Extracellular recordings from the surface of the piriform cortex, of the potentials evoked by this stimulation consisted of a triphasic complex (Fig. 4.1b). These phases represented the action potentials in the LOT fibres followed by a slower negative potential (N-wave) produced by the summation of the postsynaptic currents induced in the cortical cells (Fig. 4.1b). Superimposed on the N-wave was a positive deflection, the P spike, due to the firing of the cortical cells. Over a range of stimulus intensities, a linear relationship was observed between the amplitudes of positive wave, representing the summated LOT action

Figure 4.1 Extracellular recording of neurotransmission in slices of rat olfactory cortex

A diagram of the ventral view of the rat brain is given in A. The dotted line indicates the area removed when taking an olfactory cortex slice. The stippled region shows the area from which recordings are typically taken. The abbreviations used are LOT: lateral olfactory tract; OB: olfactory bulb; OT: olfactory tubercle; PC: piriform cortex. In B is a typical potential evoked by stimulation of the LOT showing the presynaptic volley (AP) and the postsynaptic negative wave (N-wave) and points of measurement. The positive deflection superimposed on the N-wave occurring at a latency of about 4 ms represents the synchronous discharge of many cortical cells to the excitatory input. The linear relationship between the presynaptic volley and postsynaptic response amplitudes determined in a typical experiment is shown in B. A range of stimuli from 100 - 500 μA, 100 μs pulse width were delivered to the LOT at 0.2 - 0.5 Hz; the amplitude of the AP is plotted (abscissa) against the height of the N-wave measured at a fixed latency before the N-wave peak (ordinate). Plots are given for responses in normal Krebs solution (filled circles) and in modified medium when Ca^{2+} (2.5 mM) was replaced by Sr^{2+} (5 mM) (open circles). (From Dolly et al., 1980b). C shows the effect of β-BuTX (230 nM) on presynaptic (—) and postsynaptic (•) response amplitudes.


A
PC
LOT
OT
OB
B
AP
N-wave
1mV
2ms
N-wave amplitude (mV)
1
0
0
1
AP amplitude (mV)
C
0·6
0·4
0·2
0
Response amplitude (mV)
TOXIN
0
20
40
60
Time (min)

potentials and the N-wave representing the postsynaptic response to released transmitter. When Ca^{2+} was replaced by Sr^{2+} this relationship of pre-: post-synaptic responses remained linear but with altered slope. The results of these measurements were expressed in terms of relative synaptic efficiency as given by the ratio of post- :pre-synaptic response amplitudes.

4.3 Results

4.3.1 Chemical modification of β-BuTX with p-bromophenacyl bromide

Following a 12 hour incubation of β-BuTX in the presence of an excess of BPB at 30°C (4.2.2), the alkylating reagent was separated from the toxin species by gel filtration of the reaction mixture (2.1 ml) on a column of Sephadex G-75 (Fig. 4.2). The absorbance of the fractions was measured at 280 nm, a wavelength at which both β-BuTX and BPB strongly absorb. The toxin peak fractions 19 - 26 (Fig. 4.2) was pooled and the toxin concentration, measured by the method of Lowry *et al.* (1951) using unmodified β-BuTX as a standard was 0.75 mg/ml. The molar extinction coefficients of β-BuTX and BPB at 271 nm had previously been measured at 19670 and 11950 cm^{-1} respectively. The absorbance, at 271 nm of the pooled toxin peak was 1.11 cm^{-1}; from these data it was calculated that 1.08 moles of the alkylating reagent were incorporated into each mole of toxin. Phospholipase activity was not detectable in the modified toxin, its specific activity being less than 1% of that of native β-BuTX, in the presence of Ca^{2+} and deoxycholate. This is consistent with the alkylation of residues essential for the enzyme activity. The modification was probably to a single histidine residue; the modification of such a residue in the amino acid analysis of β-BuTX on treatment with BPB was demonstrated by Abe *et al.* (1977) and an

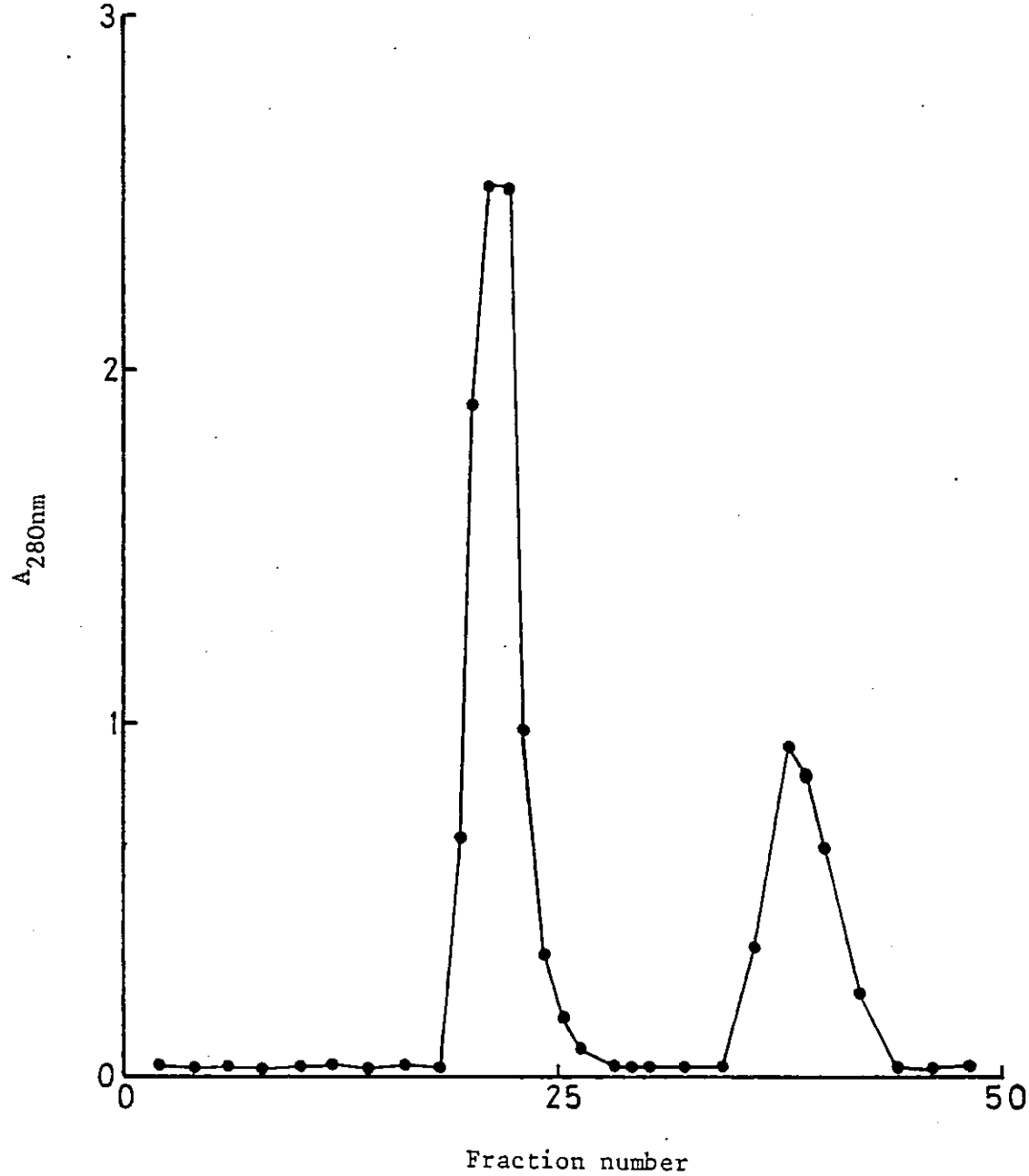


Figure 4.2 Separation of modified β-BuTX and unreacted p-bromophenacyl bromide by gel filtration

β-BuTX was modified by incubation with BPB as described in Methods (4.2.2). The reaction mixture (2.1 ml) was gel filtered on a Sephadex G-25 superfine column (1 x 47 cm) in 5 mM Tris HCl pH 7.4, 0.1 M NaCl. Fractions (0.5 ml) were collected and their absorbance at 280 nm measured. Fractions 19 - 26 were pooled and assayed for phospholipase activity.

essential histidine is known to occur in the active site of homologous phospholipases. Subsequently, a more sensitive assay using radio-labelled synaptosomal membranes showed that BPB-β-BuTX had 2.2% of the native toxin enzyme activity (Othman et al., 1982).

The lethality of β-BuTX to mice was, as expected, also greatly reduced when treated with BPB. It became non-toxic to whole animals when administered to mice by a peripheral route, intraperitoneal injection; the minimum lethal dose follwing its intraventricular injection into rat brain was increased from 0.05 to 12 ng/g body weight (Othman et al., 1982). These results again emphasise that the phospholipase activity is required for β-BuTX to show lethal effects. Nevertheless, as has been discussed before (1.3.3.4) its high neurotoxicity cannot be accounted for by its enzyme activity alone.

4.3.2 The actions of β-BuTX on synaptic transmission in slices of rat olfactory cortex

β-BuTX (230 nM) produced, after a latent period of 5 - 10 minutes, a monotonic and irreversible decline in the amplitude of the N-wave (Fig. 4.1c), although on three out of twelve occasions a clear potentiation preceded the depression. The toxin also caused a decline in the amplitude of the presynaptic action potential but with a slower time course (Fig. 4.1c; Halliwell and Dolly, 1982a). The blockade of neurotransmission was not reversed by washing for up to 18 hours. The rate of blockade was concentration dependent over the range 46-460 nM; the half times for blockade at the highest and lowest doses were 23 and 60 min respectively. Expose to toxin (230 nM) for 20 min was sufficient to abolish transmission within 90 min (Dolly et al., 1980b). The toxin caused an inhibition of transmitter release in

the preparation, as measured by the ratio of post : presynaptic measures. It was also demonstrated, using intracellular recording from cortical cells, that β-BuTX (250 nM) after 1 hour at 24°C abolished the epsp initiated by stimulating the LOT with little effect on membrane potential (Dolly et al., 1980a). The depolarisation induced by bath application of either of the putative neurotransmitters glutamate (Bradford and Richards, 1976) or aspartate (Collins, 1979) was not inhibited at this stage indicating that the toxin does not act postsynaptically to affect the sensitivity of cortical cells to neurotransmitters (Dolly et al., 1980a). In the early stages of its action on this preparation it appears that the major effect of β-BuTX is on the release of neurotransmitters. It is unlikely that this is due to the failure of action potentials to invade the nerve terminals since the toxin does not affect the conductance of unmyelinated vagal C-fibres (S.J. Marsh, unpublished observations). There is however a decrease in cell resistance, from 31.8 ± 1.9 MΩ to 8.5 ± 2.5 MΩ, and in the excitability of the postsynaptic cortical cells which casts doubt on the specificity of the action of β-BuTX on this preparation; in addition, the toxin eventually abolishes the extracellularly recorded LOT action potential (Fig. 4.1c). A possible explanation of the latter effect may be the presence, along the LOT, of terminal boutons and short branches which are potential sites of toxin action. Similarly, sites for toxin binding may exist on regions of the cortical cells removed from those innervated by the LOT; action of the toxin at such sites may be responsible for the observed decreases in cell resistance and

excitability. The possibility that these effects are due to a non-specific action of the phospholipase activity of β-BuTX will be examined elsewhere in this chapter. Very similar effects of this β-BuTX preparation were measured on neurotransmission in slices of rat hippocampus (Halliwell and Dolly, 1982b).

4.3.3 The inhibition of the phospholipase activity of β-BuTX and its action on preparations from the central nervous system

4.3.3.1 Replacement of Ca^{2+} by Sr^{2+}

The phospholipase activity of β-BuTX on phosphatidylcholine substrate, in the presence of deoxycholate was reduced from 63 to 2.6 μmol. min^{-1} mg of $protein^{-1}$ when Sr^{2+} was substituted for Ca^{2+} (Table 2.3). Sr^{2+} can, however, replace Ca^{2+} in supporting neurotransmitter release (Dodge et al., 1969). Indeed in the experiment illustrated in Figure 4.3, the release of [^{3}H] ACh and [^{3}H] choline from synaptosomes, induced by depolarisation with 23 mm K^+, was enhanced when $SrCl_2$ was substituted for $CaCl_2$. This is in apparent contradiction to the observation that Sr^{2+} is less effective than Ca^{2+}, on a molar basis, in supporting ACh release at the neuromuscular junction, as measured by e.p.p. amplitude (Dodge et al., 1969). However, when ACh release at endplates in frog muscle was stimulated by increased extracellular K^+ rather than via the nerve, it was found that there was no change in m.e.p.p. frequency when Ca^{2+} was replaced by the equivalent concentration of Sr^{2+} (Mellow, 1979). It should be noted in the experiment illustrated in Figure 4.3 the release of ACh in the control in the presence of Ca^{2+} was somewhat greater than normal. Nevertheless, this ionic substitution allows the effects of β-BuTX on the release of neurotransmitters to be measured under conditions in which its phospholipase activity is inhibited. When $SrCl_2$ was substituted for $CaCl_2$ the release of [^{3}H] ACh and [^{3}H] choline from synaptosomes induced by β-BuTX was markedly reduced, both in medium

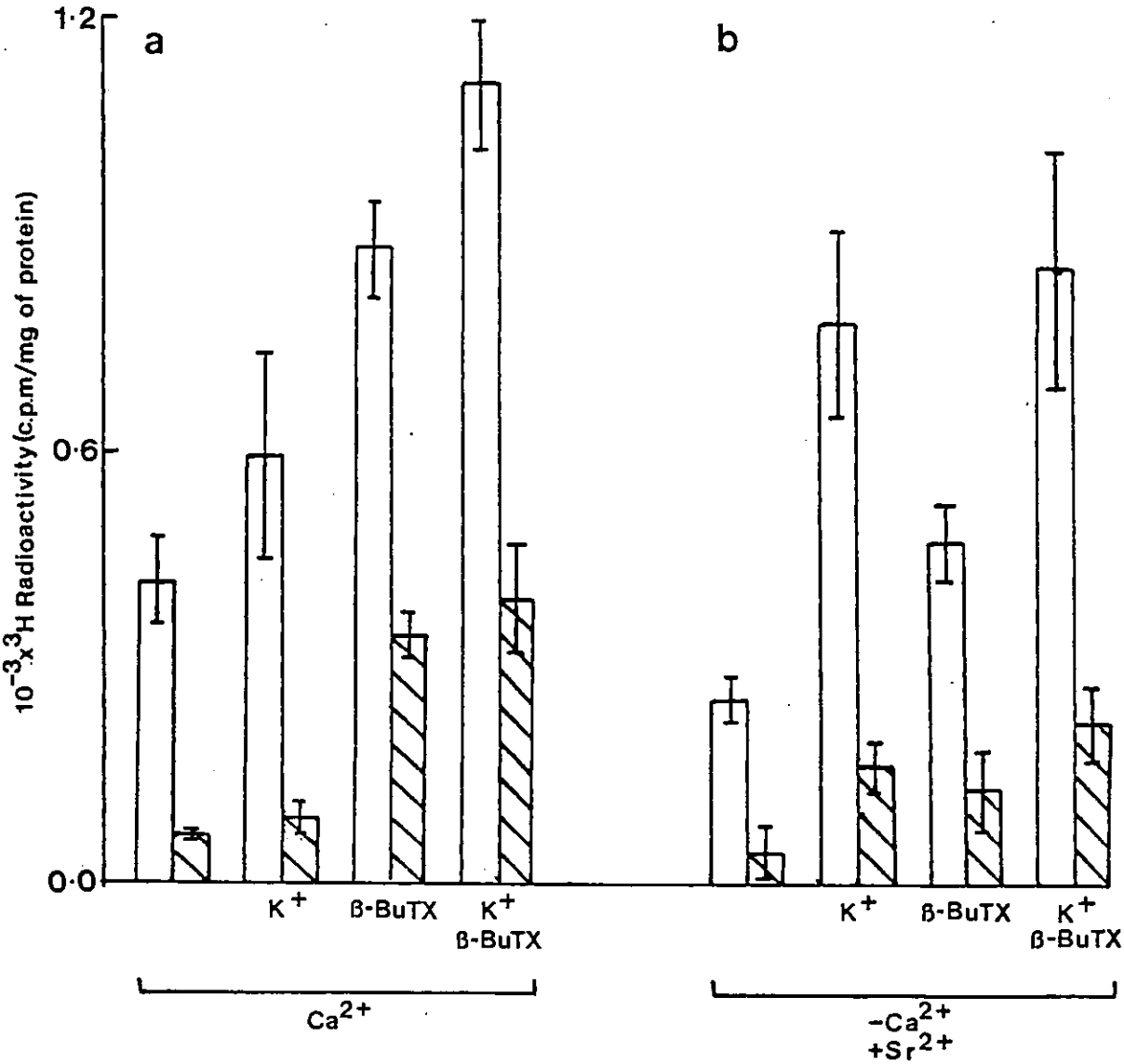


Figure 4.3 The effects of β-BuTX on the release of ACh and choline from rat cortex synaptosomes in the presence of Ca^{2+} or Sr^{2+}

Measurement of the amounts of [^{3}H] ACh (open bars) and [^{3}H] choline (hatched bars) released over 20 mins at 25°C from synaptosomes preloaded with the latter was as described in Methods. Control incubations were performed in (a) modified Krebs-phosphate medium and (b) the same solution except that Ca^{2+} was iso-osmotically replaced by Sr^{2+}. Test samples contained, in addition, 10^{-7} M β-BuTX and/or 23 mM K^+ as noted. The values shown are the means of duplicate incubations; the error bars indicate the range.

containing 3 mM K^+ and when the synaptosomes were depolarised with 23 mM K^+ (Fig. 4.3). This ion substitution also inhibited the toxin-induced release from synaptosomes of [^{14}C] GABA and lactate dehydrogenase (Tse et al., 1980). These observations suggest that the phospholipase activity of β-BuTX plays a large part in its effects on synaptosomes.

The action of β-BuTX at intact synapses in the olfactory cortex was investigated using a similar ionic substitution. When Ca^{2+} (2.5 mM) was removed from the bathing medium and EDTA (50 μM) added the N-wave was abolished (Fig. 4.4a and b) whilst the amplitude of the LOT action potential was decreased by 50%. Removal of the EDTA and addition of Sr^{2+} (5 mM) quickly restored the N-wave amplitude to 40% of its original level (Fig. 4.4c); both the pre- and post-synaptic responses continued to show a slower recovery while in Sr^{2+}-medium. Addition of β-BuTX (230 nM) caused a decline in the N-wave (Fig. 4.4d) but not the presynaptic volley. The toxin was, however, much less effective in Sr^{2+}-medium; the half-time for the depression of the N-wave was prolonged from 27 - 43 minutes (n = 4) to more than 60 minutes (range: 60 - 110 min; n = 4). Following 50 min. exposure to the toxin in Sr^{2+}-medium, a change to toxin-free medium containing Ca^{2+} (2.5 mM) produced a transient recovery of the postsynaptic response followed by its rapid decline (Fig. 4.4e) accompanied by a slower decline in the presynaptic action potential. When, following incubation with toxin, the tissue was washed for 20 min with toxin-free Sr^{2+}-medium a similar effect was still observed on restoration of Ca^{2+}. This indicates an irreversible interaction of toxin with the preparation in Sr^{2+}-medium. The effects of Sr^{2+}-medium alone were completely reversible.

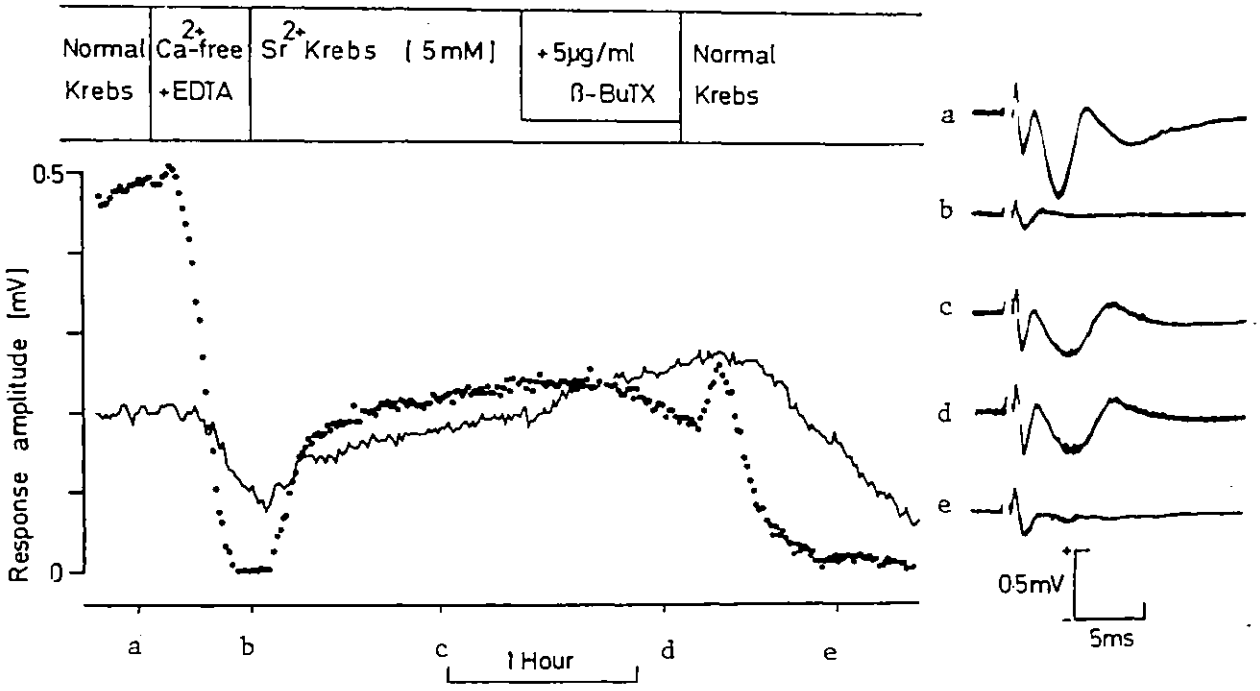


Figure 4.4 The effects of β-bungarotoxin on neurotransmission in olfactory cortex in the absence of Ca^{2+}

Presynaptic (–) and postsynaptic responses (●) were measured as described in Section 4.2.5, during the indicated solution changes. Ca^{2+} (2.5 mM) was removed from the tissue with EDTA (50 µM) before being replaced by Sr^{2+} (5 mM); β-BuTX (230 nM) was then administered. Responses a - e on the right were recorded at the times indicated on the abscissa. (From Dolly et al., 1980b).

The substitution of Sr^{2+} for Ca^{2+} did not totally inhibit the effects of β-BuTX on synaptosomes or on neurotransmission in the olfactory cortex; nevertheless, the observed effects in this medium appear to be greater than can be accounted for by the 4% of its phospholipase activity which the toxin retains under these conditions (Table 2.3). Residual Ca^{2+} associated with the synaptosomes or olfactory cortex slices was probably extremely low since the former were prepared loaded and washed in medium free from Ca^{2+} and the latter were preincubated for 30 min in Ca^{2+}-free medium containing EDTA (50 μM). There remains however, from these experiments, some doubt as to how much of the effect of the toxin is independent of its enzyme activity.

4.3.3.2 Chemically modified β-BuTX

β-BuTX which had been alkylated with p-bromophenacyl bromide and which totally lacked any measurable phospholipase activity was used to obtain clearer evidence as to the involvement of the enzyme activity in the actions of this toxin. BPB-β-BuTX was very much less effective than the native toxin in causing the release of [^{14}C] GABA (Fig. 4.5a) or [^{14}C] glutamate (Figs. 4.5b and c) from preloaded synaptosomes. Indeed, in three out of five such experiments performed, including that shown in Figure 4.5b, the modified toxin produced no increase in transmitter release over that in the control. Similarly, BPB-β-BuTX produced a negligible increase in the amount of the cytoplasmic marker, lactate dehydrogenase, released from synaptosomes (Figures 4.5a and b). This represents further evidence that the mechanism by which β-BuTX increases the release of transmitter and non-transmitter substances alike requires the phospholipase activity.

There was considerable variation in the ability of BPB-β-BuTX to antagonise the action of the native toxin on transmitter release

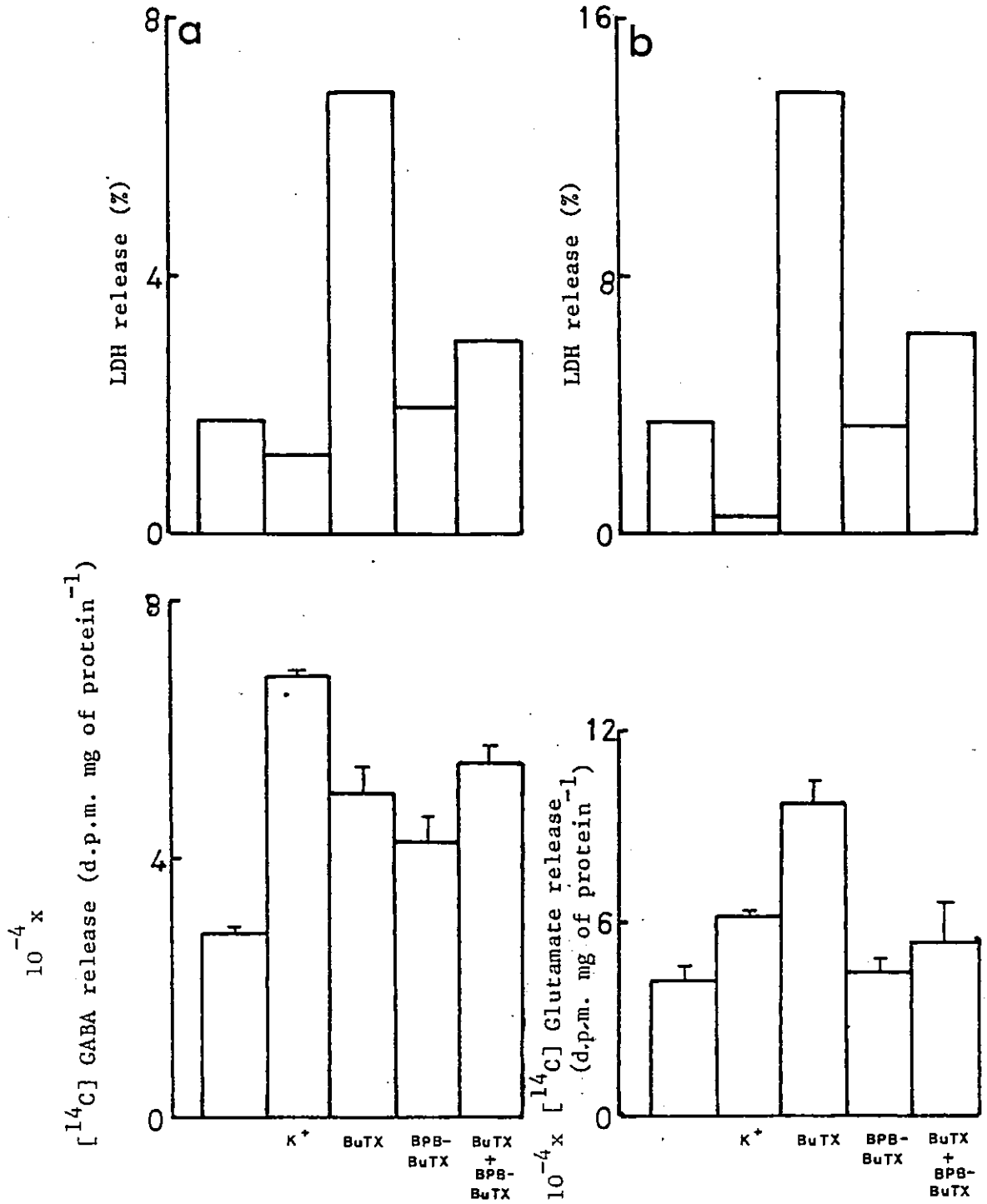
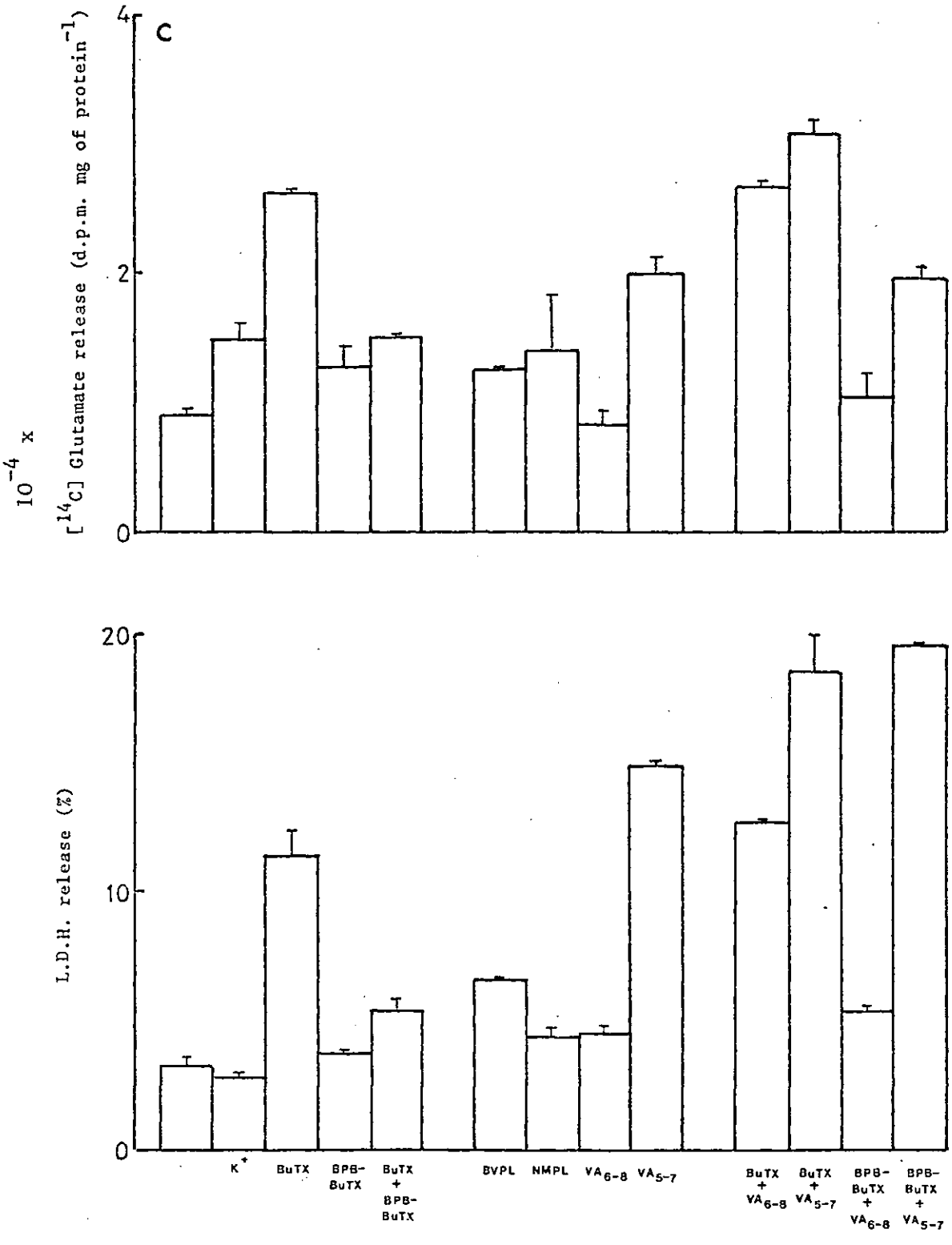


Figure 4.5 Effects of β-BuTX, BPB-β-BuTX and pure phospholipases on release, from synaptosomes of [^{14}C] glutamate, [^{14}C] GABA, and lactate dehydrogenase

Synaptosomes which had been preloaded with [^{14}C] glutamate (a and c) or [^{14}C] GABA (b) were incubated for 20 min at 25°C in modified Krebs-phosphate medium with, as noted, the following additions: KCl (23 mM), β-BuTX (2 x 10^{-8} M; 28 units of phospholipase activity l^{-1}), BPB-β-BuTX (8 x 10^{-7} M; 0.2 units l^{-1}), bee venom phospholipase A_2 (BVPL; 7 x 10^{-9} M; 430 units l^{-1}), Naja melanoleuca phospholipase A_2 (NMPL, 2.4 x 10^{-8} M; 590 units l^{-1}), Vipera ammodites phospholipase-heterodimer (VA_{5-7}; 3 x 10^{-8} M; 560 units l^{-1}) and Vipera ammodites phospholipase A_2-homodimer (VA_{6-8}; 2.4 x 10^{-8} M; 540 units l^{-1}). The amounts of [^{14}C] glutamate, [^{14}C] GABA and lactate dehydrogenase released were measured as described in Sections 4.2.3 and 4.2.4. The values shown are the means obtained from duplicate incubations; the error bars indicate the range. (^{1}unit = 1 μmol of H^+ released per min)


C
10^{-4} x [^{14}C] Glutamate release (d.p.m. mg of protein^{-1})
4
2
0
L.D.H. release (%)
20
10
0
K$^+$
BuTX
BPB-BuTX
BuTX + BPB-BuTX
BVPL
NMPL
VA$_{6-8}$
VA$_{5-7}$
BuTX + VA$_{6-8}$
BuTX + VA$_{5-7}$
BPB-BuTX + VA$_{6-8}$
BPB-BuTX + VA$_{5-7}$

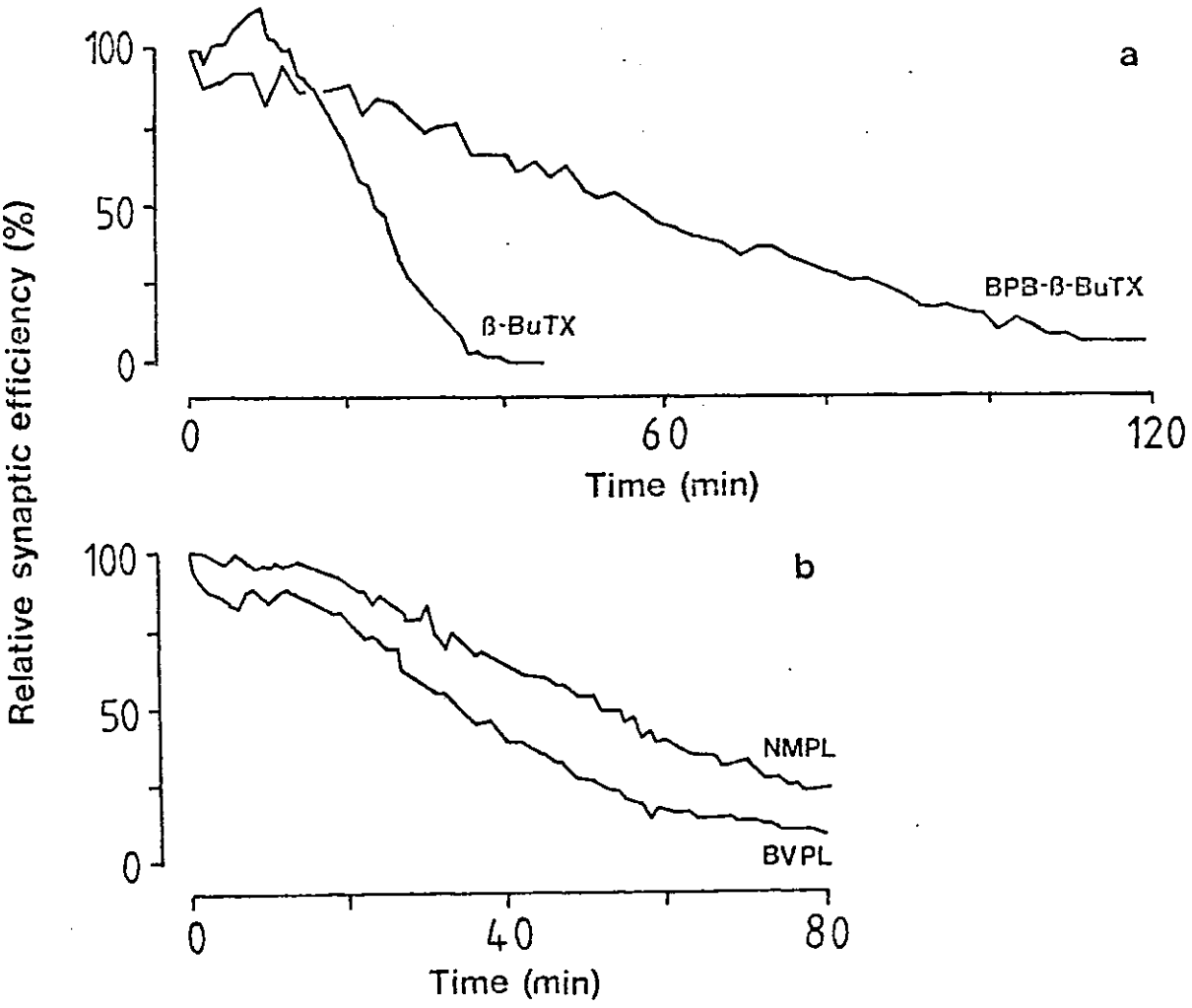


Figure 4.6 Comparison of the effects of β-BuTX, BPB-β-BuTX and pure phospholipases A_2 on neurotransmission in slices of olfactory cortex

Synaptic efficiency was determined as the ratio of post : presynaptic response amplitudes measured as described in Section 4.2.5. It is expressed here as the percentage of the average response ratio before addition of toxin or enzyme at zero time. The 95% confidence limits for the average are 97 - 103%.

a) β-BuTX (230 nM) was applied for 20 mins and BPB-β-BuTX (230 nM) for 60 min.

b) Bee venom phospholipase A_2 (BVPL: 500 nM) was applied for 50 min and Naja melanoleuca phospholipase A_2 (NMPL: 500 nM) for 70 min.

from synaptosomes as shown by Figure 4.5. In the three experiments illustrated a 40-fold molar excess of the modified toxin caused no inhibition of the GABA release induced by β-BuTX (Fig. 4.5a) and 76% and 78% reductions in the toxin-induced glutamate release (Figs 4.5b and c). It should be noted that in Fig. 4.5a the β-BuTX induced release of GABA is lower than expected. In a fourth experiment (not shown) the modified toxin failed to inhibit the effect of β-BuTX on glutamate release. In contrast to its variable effect on toxin-induced transmitter release, BPB-β-BuTX inhibited the release of lactate dehydrogenase by β-BuTX to the same extent in all experiments (76% 74% and 73% in Figs. 4.5a, b and c respectively).

Native β-BuTX decreased synaptic efficiency in the olfactory cortex slices (Fig. 4.6a);BPB-β-BuTX also inhibited synaptic transmission (Fig. 4.6a) but at a slower rate, similar to that observed in Sr^{2+}-medium. The half-time for the decrease in relative synaptic efficiency was increased from 27 - 43 min (n = 4) for native toxin (230nM) to 65 - 150 min (n = 3) in the case of BPB-β-BuTX (230 nM) (Halliwell and Dolly, 1982a) and to 60 - 110 min (n = 4) in the case of native toxin in Sr^{2+}-medium (Dolly et al., 1980b). The chemically modified toxin, like the native toxin in Sr^{2+}-medium, did not affect LOT action potentials (Halliwell and Dolly, 1982a). As with the native toxin, the effects of BPB-β-BuTX were not reversed by washing.

4.3.4 Comparison of the effects of β-BuTX and pure phospholipases on preparations from the central nervous system

In view of the involvement of phospholipase activity in the actions of β-BuTX, its effects on the release, from synaptosomes, of glutamate (Fig. 4.5c) and lactate dehydrogenase (Fig. 4.5c) were compared with those of a number of pure phospholipases A_2 which, although having much greater specific enzyme activities, were much less toxic than β-BuTX when injected into mice (Table 4.1).

Table 4.1 Toxicities and phospholipase activities of β-BuTX, BPB-β-BuTX and pure phospholipases

Toxicities were determined by intraperitoneal injection into mice of toxin solutions in 0.8% NaCl containing bovine serum albumin (0.5 mg/ml). Phospholipase activities were assayed using egg yolk lecithin substrate in the presence of Ca^{2+} and deoxycholate as described in Section 2.2.8.

	β-BuTX	BPB-β-BuTX	BVPL	NMPL	VA (6-8)	VA (5-7)
Molecular weight	21,000	21,000	40,000	27,000	29,000	31,000
Toxicity in mice (ip. inj.) (Approx MLD µg/g body wt)	< 0.01	> 10	> 5	> 5	> 5	0.5
Phospholipase activity (µmol H^+ released/min/nmol)	1.4	< 0.01	70	23	23	17

The enzymes tested were all much less potent than β-BuTX in causing the release of [^{14}C] glutamate (Fig. 4.5c) and lactate dehydrogenase (Fig. 4.5c) from synaptosomes. A phospholipase A_2 from the venom of Naja melanoleuca (NMPL) and a homodimeric enzyme from Vipera ammodites (VA_{6-8}) when added at the same concentration as β-BuTX (2×10^{-8} M) showed little or no effects (Fig. 4.5c, columns 7 and 8) despite having, respectively, 16.5 and 16.2-fold greater specific enzyme activity (Table 4.1). Pure phospholipase A_2 from the venom of the honey bee, Apis mellifera (BVPL) which possessed much the highest specific enzyme activity was added at 20 - 30% of the molarity of the other enzymes and β-BuTX. Although it was present at a much higher concentration in terms of units of enzyme activity it was less effective than β-BuTX (2×10^{-8} M) in causing release of glutamate and lactate dehydrogenase (Fig. 4.5c, columns 6 and 3).

A heterodimeric enzyme from the venom of Vipera ammodites (VA_{5-7}), added at a concentration of 3×10^{-8} M, produced a much greater release of glutamate and lactate dehydrogenase than did the other enzymes (Fig. 4.5c, column 9). Interestingly, the heterodimeric enzyme released more of the cytoplasmic marker than did β-BuTX indicating that it caused considerable disruption to the synaptosomal membranes. It was also the only one of the enzymes tested which was appreciably toxic to mice (Table 4.1), although it was approximately 50-fold less lethal on a molar basis than β-BuTX. This, once again, implies that the neurotoxicity of β-BuTX cannot be accounted for simply by the indiscriminate effects of its phospholipase activity and that its action shows a further degree of specificity.

The effects of the simultaneous addition to synaptosomes

of phospholipases from Vipera ammodites and either native or enzymically inactive toxin, were tested (Fig. 4.5). The homodimeric enzyme (VA_{6-8}), which itself caused little or no release of glutamate or lactate dehydrogenase, did not appreciably affect the actions of either β-BuTX or BPB-β-BuTX and its own action was not potentiated by either of these (Fig. 4.5c, columns 10 and 12). Thus the presence of β-BuTX, whether or not enzymatically active, is unable to potentiate the effects on membrane disruption or neurotransmitter release of a non-neurotoxic phospholipase. β-BuTX itself loses the capacity to cause such effects when its own phospholipase activity is inhibited. This leads to the suggestion that its potency depends on the presence of a phospholipase very closely associated with another part of the toxin molecule which confers specificity of the sites of action. The same was usually the case for BPB-β-BuTX and the heterodimeric enzyme VA_{5-7}, although in the experiment illustrated herein (Fig. 4.5c, column 13) there was an apparent potentiation of their individual effects on release of lactate dehydrogenase. When phospholipase VA_{5-7} and native β-BuTX were added together their combined effects, on both release of glutamate and lactate dehydrogenase, whilst greater than either of their individual effects were by no means fully additive (Fig. 4.5c, column 11). This was not unexpected since it has already been demonstrated that both these polypeptides cause considerable membrane disruption.

The actions of two pure phospholipases on the olfactory cortex preparation were examined. Bee venom phospholipase A_2, at a concentration (10 n M) that showed equivalent enzyme activity to 230 nM β-BuTX (Table 4.1) was ineffective and even at a concentration of 500 nM the time course of its decrease of synaptic

efficiency was longer than that of β-BuTX (230 nM) (Fig. 4.6b). Phospholipase A_2 from the venom of Naja melanoleuca, which was ineffective at 50 nM, depressed synaptic transmission at 500 nM but more slowly than the bee venom enzyme at the same concentration (Fig. 4.6b); its specific enzyme activity being 16-fold greater than that of β-BuTX (Table 4.1). The rate of blockade by both of these enzymes was considerably less than that by β-BuTX (230 nM). These two enzymes are therefore much less potent than β-BuTX in their effects on both synaptosomes (Fig. 4.5) and intact central synapses (Fig. 4.6). In addition these two enzymes, unlike β-BuTX, did not decrease the presynaptic action potentials (Halliwell and Dolly,1982a); in fact, bee venom phospholipase A_2 (500 nM) produced a sustained increase in this measure, similar to that observe by Abe and Miledi (1978) at the neuromuscular junction. As previously discussed (3.4.) this latter effect of β-BuTX is probably due to its action at terminal boutons along the lateral olfactory tract.

4.4 Discussion

4.4.1 Comparison of the actions of β-BuTX on synaptosomes and olfactory cortex slices

β-BuTX does not show a triphasic effect on neurotransmitter release in the olfactory cortex as is observed at the vertebrate neuromuscular junction. Rather, it causes a monotonic decline in synaptic efficiency (Figs.4.1 and 4.6a). It may be argued that since the extra cellular recordings represent an averaging of the responses at a great many synapses throughout each tissue slice, with a great variation in accessibility to the toxin, the different phases of the action of β-BuTX may be masked (Dolly et al., 1980b). If this is the case then not only

must this masking be complete but the first two phases of β-BuTX action in the CNS must be much more rapid than at the neuromuscular junction. Alternatively, in the central nervous system, phase one may be sufficient to completely inhibit neurotransmitter release. This is consistent with the blockades of synaptic transmission in the olfactory cortex produced by BPB-β-BuTX (Fig. 4.6a) and by native toxin in Sr^{2+}-medium (Fig. 4.4), although at a slower rate than that at which the native toxin acted in conditions under which its phospholipase activity was unimpaired. It has recently been demonstrated that at low temperature ($5^{o}C$) or at high toxin concentration (3 μM or higher) when Ca^{2+} was replaced by Sr^{2+}, phase 1 of the action of β-BuTX led to an almost complete blockade of neuromuscular transmission (Caratsch et al., 1981).

This second hypothesis is, however, very difficult to reconcile with the actions of β-BuTX on synaptosomes where an increase in transmitter release is seen (Chapter 3). It is therefore quite possible that the measurements using synaptosomes and olfactory cortex slices represent separate effects of β-BuTX in the olfactory cortex. Non-quantal release, or "leakage", is likely to account for a large proportion of the neurotransmitter released from synaptosomes. It has been estimated that, at the neuromuscular junction, such a process accounts for 99% of the transmitter released from resting nerve terminals (Katz and Miledi,1977). The contribution of non-quantal release could therefore be masking that of quantal release in the biochemical measurements made using synaptosomes but, since it would not give rise to post synaptic potentials, it would not be detected by electrophysiological techniques either in the olfactory cortex or at the neuromuscular junction. The action of the toxin on synaptosomes is clearly much more dependent

on its phospholipase action than is its effect on the olfactory cortex (4.3.3); a relatively unspecific effect of the phospholipase activity, increasing non-quantal release of neurotransmitters in addition to non-neurotransmitter substances and the marker enzyme lactate dehydrogenase, may explain this. Finally, the actions of β-BuTX measured in the olfactory cortex may be atypical of its action at central synapses in general and hence also synaptosomes prepared from whole cortex. In this regard it is interesting that when the phospholipase activity of the toxin was removed with p-bromophenacyl bromide it retained appreciable potency towards olfactory cortex slices (Fig. 4.6a) but did not induce transmitter release from synaptosomes (Fig. 4.5) and its lethality following intraventricular injection was greatly reduced (Table 6.1; Othman et al., 1982).

4.4.2 The involvement of phospholipase activity in the actions of β-BuTX

Regardless of whether the effects of β-BuTX measured using synaptosomes and olfactory cortex slices occur by similar or different mechanisms, it is clear that the phospholipase activity is necessary for its full potency. Nevertheless,it is also clear that an irreversible interaction occurs between the toxin and nerve terminals in the absence of the enzyme activity. In an experiment similar to that shown in Figure 4.4 prolonged (20 min) washing failed to reverse the blockade of cortical synapses produced by β-BuTX in medium in which Sr^{2+} replaced Ca^{2+} and a more rapid decline in synaptic activity was observed on the subsequent restoration of Ca^{2+} (Dolly et al., 1980b). Similarly, when β-BuTX was added for 20 - 30 mins and then washed out whilst the olfactory cortex preparation was bathed in medium containing no divalent cations, the synaptic transmission failed to recover

following the restoration of Ca^{2+} and Mg^{2+} (Halliwell et al., 1982). The same cortical slice had previously been shown to recover its original level of synaptic efficiency after similar treatment in the absence of toxin.

Phospholipases A_2 can, however, bind monomeric substrate independently of the presence of Ca^{2+} although the subsequent hydrolysis is inhibited (Pieterson et al., 1974a). This ability to bind the substrate is lost when a histidine residue in the active site is chemically modified with p-bromophenacyl bromide (Verheij et al., 1980). Since the action of BPB-β-BuTX on the olfactory cortex (Fig. 4.6a) was not reversed after washing for as long as 60 mins, it seems unlikely that the interaction of toxin with nerve terminals occurs via the binding of a specific phospholipid substrate. An interesting parallel may be drawn with taipoxin which retained ability to inhibit accumulation of choline by synaptosomes prepared from the electric organ of Torpedo marmorata following similar modification with p-bromophenacyl bromide (Dowdall et al., 1979). On the other hand, when β-BuTX was modified with BPB its toxicity when injected intraventricularly was reduced to a much greater extent (240-fold) than would be expected from the comparatively small decrease (c.a. 5-fold) in the rate of blockade of olfactory cortex synapses produced by the same modification. Also, although BPB-β-BuTX, which itself had little or no effect on neurotransmitter release from synaptosomes, did antagonise the effects of the native toxin, providing some evidence for a common, phospholipase independent binding site (Fig. 4.5); this antagonism was rather variable despite the modified toxin being present in a 40-fold molar excess. These results, and the similarly poor ability of BPB-β-BuTX to protect peripheral synapses from native toxin may be accounted for by the inefficiency of BPB-β-BuTX in antagonising ^{3}H-β-BuTX binding to rat cortex synaptosomes (Othman et al., 1982). These points will be discussed in Chapter 6.

Phospholipases A_2 possess, in addition to a site for binding monomeric substrate, a recognition site for lipid-water interfaces (Pieterson et al., 1974a). Two lines of evidence indicate that this site is not involved in the specific interaction of β-BuTX with nerve terminals. The first of these concerns a β-BuTX derivative produced using ethoxyformic acid, the second is a comparison of the effects of the toxin with those of non-neurotoxic phospholipases. β-BuTX has been treated with the ethoxyformic anhydride (EOFA) whilst protecting its phospholipase activity with dihexanoyl-lecithin (Howard and Truog, 1977). The resulting derivative retained full phospholipase activity, and was presumably unmodified at either active or lipid-water interface sites. It was not neurotoxic and had lost most of its ability to decrease synaptosomal uptake of deoxyglucose and GABA, to increase the release of the latter, to depolarise synaptosomes and reduce stores of ATP (Ng and Howard, 1978).

While β-BuTX appears to require phospholipase activity for its full potency it is much more toxic than other phospholipases when injected both peripherally (Table 4.1; Strong et al., 1976) and into the C.N.S. (Hanley and Empson, 1979). This, together with the much lower potency of the enzymes as compared to β-BuTX on synaptosomes (Fig. 4.5) and in the olfactory cortex (Fig. 4.6b) suggests that β-BuTX has a specificity of action which is not shared by non-neurotoxic phospholipases A_2 and it is very unlikely that this specificity resides in either the monomeric substrate binding site or lipid-water interface recognition site of the toxin.

CHAPTER 5. RADIOLABELLING OF β-BUNGAROTOXIN AND INVESTIGATION OF ITS BINDING TO NERVE TERMINALS IN THE CENTRAL NERVOUS SYSTEM

5.1 Introduction

Completely pure β-BuTX had now been shown to affect both neurotransmission at central synapses, by a presynaptic action, and release of neurotransmitters from cortical synaptosomes. It has also been established that whilst the phospholipase activity of the toxin contributes greatly to these effects some other factor confers a specificity of action and, hence, high neurotoxicity on this protein. According to the most straightforward hypothesis, this factor is the specific and irreversible binding of β-BuTX to nerve terminals. The final part of this study therefore involved the preparation of a radiolabelled derivative of β-BuTX which was active at synapses in the C.N.S. and an examination of its interaction with cortical synaptosomes, a preparation containing the highest achievable concentration of putative toxin binding sites. It seemed important that the gentlest possible method of labelling be employed. The procedure selected uses N-succinimidyl [2,3-^{3}H] propionate that introduces tritiated propionyl groups, preferentially into lysine residues. This reagent, which had already been successfully used to label α-bungarotoxin (Dolly et al., 1981b) has a high specific radioactivity (c.a. 50 Ci/mmol.).

There were two previous reports of preparations of radiolabelled β-BuTX. Oberg and Kelly (1976a) labelled their toxin with ^{125}I by the chloramine T method. The specific radioactivities obtained corresponded to an incorporation of 0.1 to 0.28 mol of ^{125}I per mol of toxin, both subunits being labelled. The enzyme activity of the toxin

was reduced to 85%, however the effect of this procedure on toxicity was not stated. This preparation showed, in a gel filtration assay, saturable binding to synaptosome membranes with a dissociation constant of 1.7 nM but the extent to which this was blocked by native toxin was not determined. MacDermot et al., (1978b) reported that they were unsuccessful in attempts to retain activity after incorporating ^{125}I into their β-BuTX preparation by a more gentle method, employing the enzyme lactoperoxidase. Instead, they were able to use pyridoxal 5'phosphate to form a Schiff base with an amino group on the protein, probably a lysine residue, which was then stabilised by reduction using $NaB[^3H]_4$ 6 (Ci/mmol). After removal of excess pyridoxal phosphate and borohydride using a column of Sephadex C-25, the specific radioactivity of the labelled toxin varied from 1.4 to 8.4 Ci/mmol. In this case the lethality of the toxin to mice was reduced 10-fold after the labelling. It also blocked a nerve-muscle preparation in vitro but the concentration required was not given and the time required for blockade was much greater than with native toxin. Binding of this toxin derivative to synaptosomes was measured by a centrifugation assay; saturable binding was observed with a rather high dissociation constant of 0.21 - 0.37 μM. Competitive binding experiments showed that this β-BuTX derivative had a drastically reduced affinity for its binding sites: the dissociation constant of native toxin was estimated to be 25 nM.

The aim of the work described in this chapter was to establish a method for producing a preparation of $[^3H]$ propionylated β-BuTX which was free of unlabelled toxin. This preparation was characterised in terms of (i) the number of $[^3H]$ propionyl moieties incorporated into the toxin molecules, (ii) the subunits which were labelled, (iii) the enzyme activity of the tritiated toxin, (iv)

the effect of the labelling procedure on lethality following both central and peripheral administration and (v) the action of [^{3}H]β-BuTX on neurotransmission in the rat olfactory cortex. Finally, some preliminary studies were carried out on the binding of [^{3}H]propionylated β-BuTX to synaptosomes from rat cerebral cortex.

5.2 Methods

5.2.1 Materials

Three batches of N-succinimidyl [2,3,^{3}H] propionate (specific radioactivities: 53 Ci mmol^{-1}, 37 Ci mmol^{-1} and 43 Ci mmol^{-1}) in toluene were supplied by the Radiochemical Centre, Amersham, U.K. Sephadex G-25 superfine and CM52 Cellulose were purchased from Pharmacia and Whatman, respectively.

5.2.2 Labelling of [^{3}H]β-bungarotoxin with N-succinimidyl [2,3-^{3}H] propionate

Three preparations of [^{3}H]β-BuTX will be described; one in which an excess of toxin over reagent was used during the labelling reaction (Preparation I) and two which used an excess of reagent (Preparations II and III). Aliquots (200 µl) of N-succinimidyl-[2,3-^{3}H]propionate in solution in toluene (2 mCi/ml) were added to a plastic Eppendorf tube, the solvent being evaporated under a stream of nitrogen gas between each addition until the requisite amount of the labelling reagent was present. A solution of pure β-BuTX in 20 mM sodium phosphate buffer (pH 7.5) was added, mixed vigorously for 5 min and incubated for 2 hr at 22°C with constant slow stirring. After removal of an aliquot of the mixture for scintillation counting, the

reaction was terminated by gel filtration on a Sephadex G-25 superfine column (1 x 29 cm) at 4°C.

Preparation I: β-BuTX (1 mg; 4.6×10^{-8} moles) in 100 µl of sodium phosphate buffer was added to an Eppendorf tube containing 4.6×10^{-9} moles of N-succinimidyl [2,3-^{3}H] propionate. When the incubation was completed the solution was diluted to 0.5 ml with sodium phosphate buffer and gel filtered on a column equilibrated with 30 mM ammonium acetate, pH 5.6.

Preparation II: β-BuTX (0.5 mg; 2.3×10^{-8} moles) in 200 µl sodium phosphate buffer was added to a 3-fold molar excess of labelling reagent. The incubation was terminated by gel filtration on a column similar to that used for Preparation I.

Preparation III: Sodium phosphate buffer (0.44 mls) containing β-BuTX (0.44 mg; 2.0×10^{-8} moles) was added to a tube containing a 5-fold molar excess of reagent. The increase in the volume of the reaction mixture and in the period of vortex mixing to 10 min was in an attempt to improve the recovery of reagent from the walls of the plastic tube (see 5.3.2). The Sephadex column used for gel filtration of this preparation was equilibrated with 30 mM ammonium acetate, pH 7.4.

5.2.3 Ion-exchange chromatography of [^{3}H] β-BuTX

The material from the pooled radiolabelled toxin peak following gel filtration of Preparation I (Fig. 5.1a) was applied to a CM52 cellulose ion exchange column (1.2 ml) equilibrated with 0.03 M ammonium acetate, pH 5.6. Labelled and unmodified toxin species were eluted with a 100 ml linear gradient of ammonium acetate concentration and pH from 0.03M, pH 5.6 to 0.60M, pH 6.5 at a flow rate of 5.5 ml per hr; 0.5 ml fractions were collected. All operations were carried out at 4°C.

5.2.4 Isoelectric focusing of [^{3}H] β-BuTX

5.2.4.1 Preparative

This was carried out in a flat bed of Sephadex G-75 using Biolyle carrier ampholytes (pH 8 - 10.5) as previously described (2.2.5). The bed was separated, longitudinally into two tracks. Freeze dried samples of radiolabelled (4×10^{-4} Ci) and native β-BuTX (2.5 mg) were dissolved in 0.5 ml of Biolyte solution (1%) and applied to the centre of separate tracks. When focusing was complete and the pH gradient had been measured, a paper print was taken of the track containing native β-BuTX which was fixed and stained for protein as previously described (2.2.5). The track to which radiolabelled toxin had been applied was cut into 3 mm slices; the slices were removed and mixed with 300 µl of ammonium acetate solution (0.05M). The radioactive content of each slice was determined by counting a 20 µl aliquot of the slurry in a toluene-based scintillation cocktail containing 10% (v/v) Soluene.

5.2.4.2 Analytical

Small aliquots of [^{3}H] β-BuTX were mixed with solutions of unlabelled toxin in water; 5 µl of these mixtures, containing 60 - 100 µg of toxin, were subjected to isoelectric focusing in slabs of polyacrylamide gel as previously described (2.2.6). After focusing was complete and pH gradients were measured, the proteins were precipitated with 40% trichloroacetic acid followed by washing in 10% trichloroacetic acid to remove ampholytes from the gel. The positions of protein bands were measured before each track was cut into 2 mm slices for scintillation counting in a Soluene/toluene cocktail.

5.2.5 SDS gel electrophoresis of [^{3}H] β-BuTX

Samples were denatured and reduced by boiling for 5 mins in sample buffer containing 10% β-mercaptoethanol. Electrophoresis in a polyacrylamide slab gel containing 12.5% acrylamide, 1.25% methylene bisacrylamide, 0.2% SDS and 8M Urea was as previously described (2.2.7). Tracks containing radioactive toxin were cut into 2 mm slices for scintillation counting in a Soluene/toluene cocktail; the remainder of the gel was fixed and stained for protein as before (2.2.7).

5.2.6 Measurement of toxicity and enzyme activity

Whole animal toxicity was determined both by injection into the cerebral intraventricular space of rats and by intraperitoneal injection into mice as previously described (2.2.8).

The effects of a [^{3}H] β-BuTX on neurotransmission in slices of rat olfactory cortex were measured by extracellular recording as previously described (4.2.5).

Phospholipase activities towards egg yolk lecithin substrate were measured titrimetrically in the presence of Ca^{2+} and deoxycholate as before (2.2.8).

5.2.7 Measurement of [^{3}H] β-BuTX binding to synaptosomes

Attempts to develop a filtration assay for [^{3}H] toxin binding were unsuccessful due to high non-specific absorption of toxin onto all types of filters tested; hence,a centrifugation assay was employed. Synaptosomes from rat cerebral cortex (3.2.2) were suspended, at a concentration of 10 - 20 mg of protein ml^{-1} in Krebs-phosphate medium containing bovine serum albumin (1mg/ml). Serial dilutions of [^{3}H] β-BuTX were made in the same buffer solution.The requisite small volumes of these solutions and/or unlabelled β-BuTX were added to 100 - 200 µl

aliquots of the synaptosome suspension in plastic microfuge tubes. Incubations were carried out at 37°C and were terminated by centrifugation at 10,000 g for 5 mins at 4°C and removal of the supernatant. The pellets were washed four times by resuspension and recentrifugation at 4°C, twice in Krebs-phosphate medium and twice in 5 mM Tris HCl (pH 7.6)/0.1 mM EGTA, the latter facilitatinglysis of the synaptosomes. The pellets were digested in Soluene (200 µl) before the lower part of the plastic tubes were cut off and placed in Soluene/toluene cocktail for liquid scintillation counting. Pellets from duplicate aliquots of the synaptosome suspension were digested in 400 µl of H_2O and 900 µl of 0.45M NaOH; aliquots of these digests were assayed for protein by the method of Lowry et al. (1951).

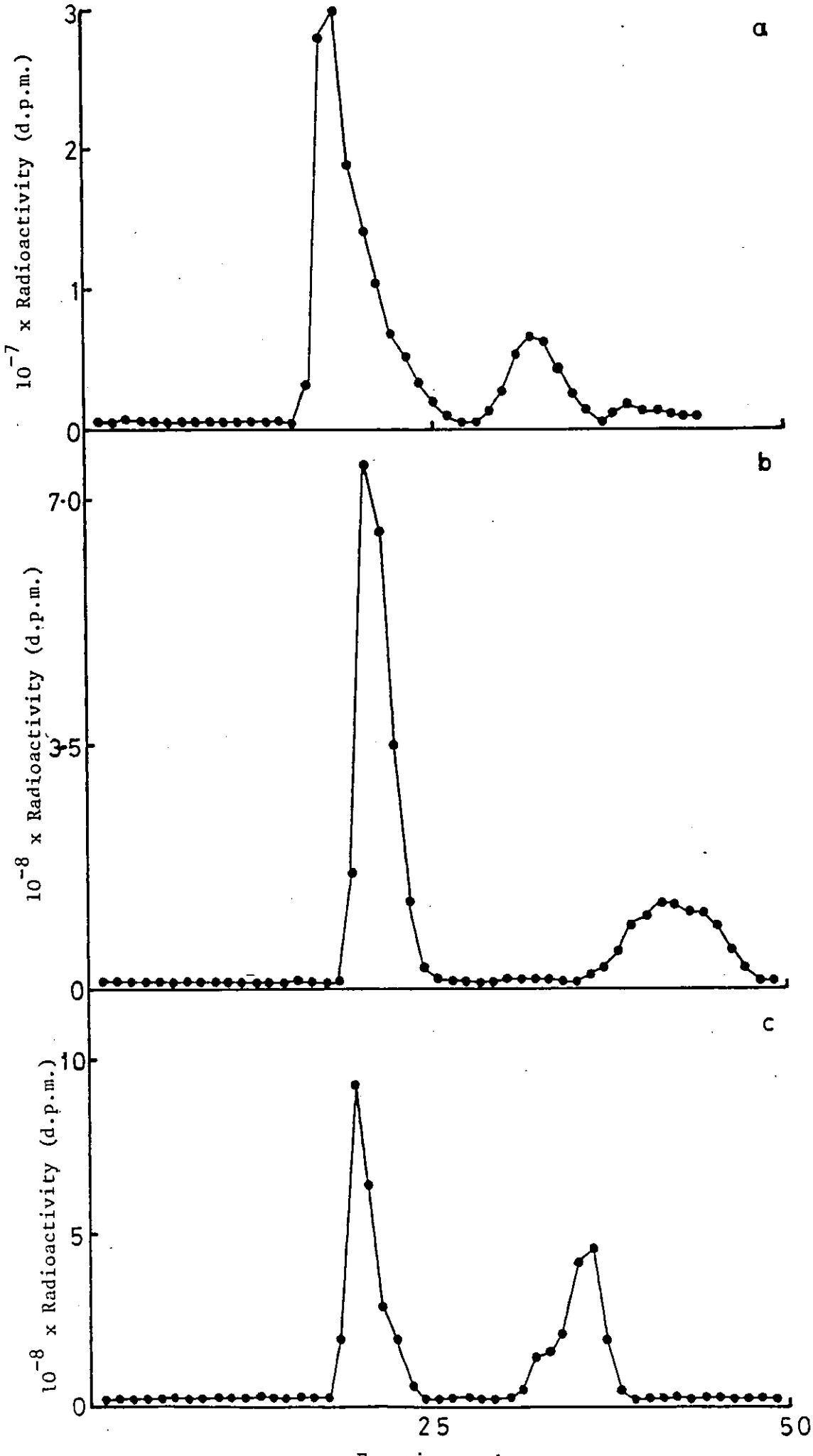
5.3 Results

5.3.1 [^{3}H] Propionylation of β-BuTX in the presence of an excess of toxin

The reaction between β-BuTX and N-succinimidyl [2,3-^{3}H] propionate was first carried out in the presence of a large excess of the toxin in order to ensure that only mono-propionylated derivatives of the toxin were formed. In the making of Preparation I (5.2.2) the amount of N-succinimidyl [2,3-^{3}H] propionate (53 Ci mmol^{-1}) dried onto the walls of the reaction tube was sufficient to give a 10-fold molar excess of the β-BuTX (1 mg) which was subsequently added in a very small volume (100 µl) to keep the reagents at high concentration in solution. After 2 hrs of constant agitation at 22°C, when the reaction mixture was diluted to 500 µl and a 10 µl aliquot counted, it was found that only 40% of the total radioactivity was present in solution. Thus, the actual ratio of toxin to labelling

Figure 5.1 Separation of toxin and N-succinimidyl [2,3-^{3}H] propionate by gel filtration

Similar Sephadex G-25 superfine columns (1 x 29 cm) were used to terminate the reaction in each of the three preparations of [^{3}H] propionylated β-BuTX described in the text: (a) Preparation I; (b) Preparation II; (c) Preparation III. The columns were equilibrated and eluted with 30 mM ammonium acetate pH 5.6; 0.5 ml fractions were collected and the radioactivity determined by counting 5 µl aliquots in a toluene-based scintillation cocktail containing 10% (v/v) soluene. In (c) the buffer used was 30 mM ammonium acetate pH 7.6.


a
b
c
10^{-7} x Radioactivity (d.p.m.)
10^{-8} x Radioactivity (d.p.m.)
10^{-8} x Radioactivity (d.p.m.)
3
2
1
0
7·0
3·5
0
10
5
0
25
50
Fraction number

reagent was 25 : 1. A lack of adequate mixing due to the very small volume of the solution was probably responsible for the poor recovery of N-succinimidyl [2,3-^{3}H] propionate from the walls of the vessel. The diluted reaction mixture was applied to a Sephadex G-25 column (Fig. 5.1a) from which the recovery of radioactivity was 79%; 80% of this was eluted in the void volume, corresponding to [^{3}H] propionylated β-BuTX and 20% was eluted in a second peak as the unreacted reagent.

The material in the toxin peak had a specific activity of 1.6Ci mmol^{-1} corresponding to an incorporation of 0.03 moles [^{3}H] propionate into each mole of toxin. This was calculated for the pooled toxin fractions from the radioactive content, determined by liquid scintillation counting and the concentration of β-BuTX, determined by measurement of absorbance at 280 nm (E = 24600 cm^{-1}). It was therefore, very likely that any toxin derivatives formed were mono-propionylated. This was supported by the observation that the radioactive material was eluted as a single peak from a CM-52 cellulose ion-exchange column by a gradient of ammonium acetate (Fig. 5.2) slightly before the peak of u.v. absorbance which corresponds to the unlabelled toxin. Unfortunately, the overlap of these two peaks was such that it was not possible to completely separate the propionylated from the native toxin. When subjected to preparative isoelectric focusing in a flat bed of Sephadex G-75 over a narrow pH range, the radioactive material again formed a single symmetrical peak (Fig. 5.3). Although in this particular bed the pH value at which the native toxin focused was rather high (pH 11.0) the [^{3}H] propionylated peak occurred at a less basic value (pH 10.5). SDS polyacrylamide gel electrophoresis showed that the toxin was labelled equally on its two subunits (Fig. 5.4a). Assuming that all the toxin derivatives were indeed mono-propionylated,

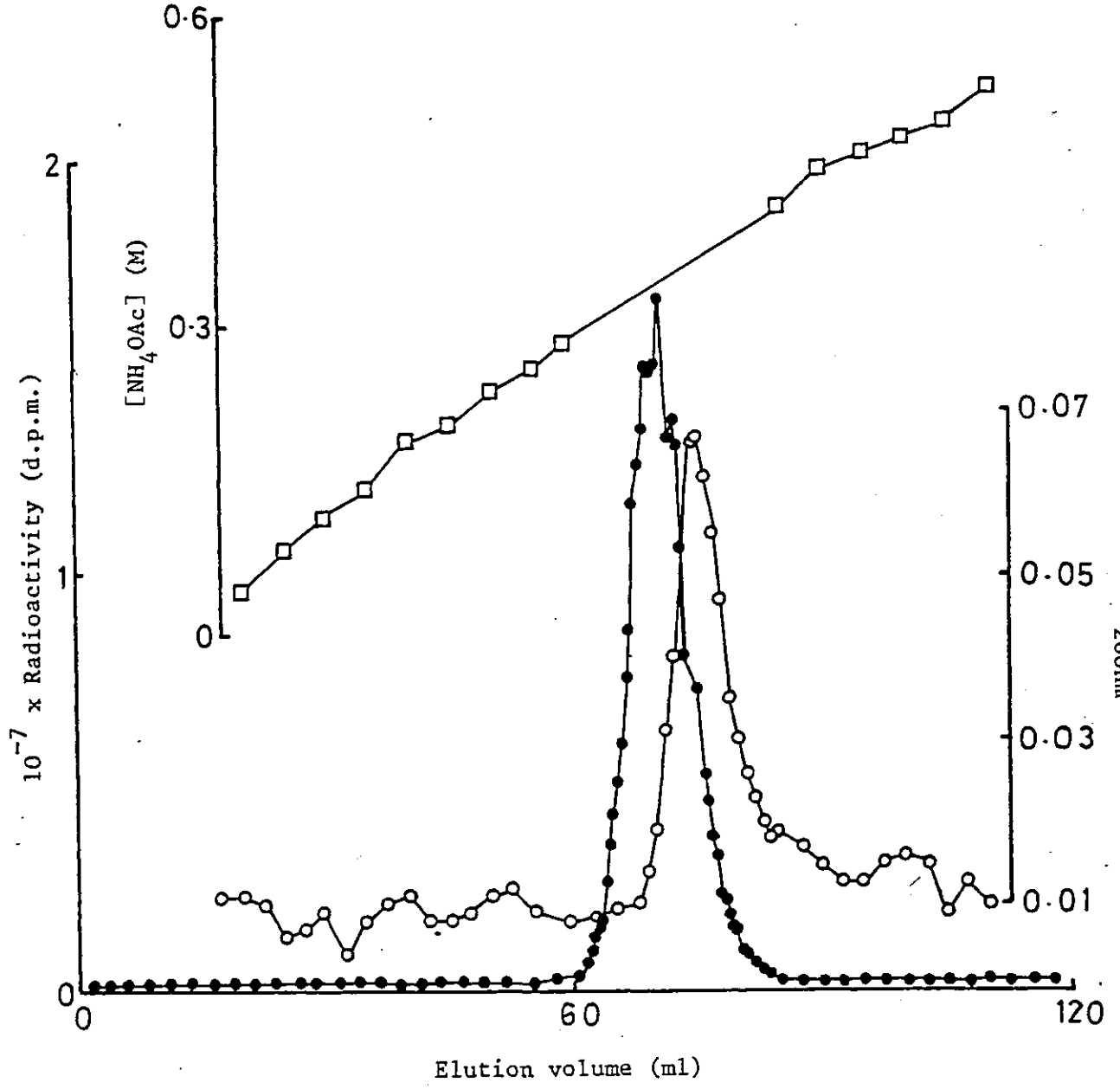
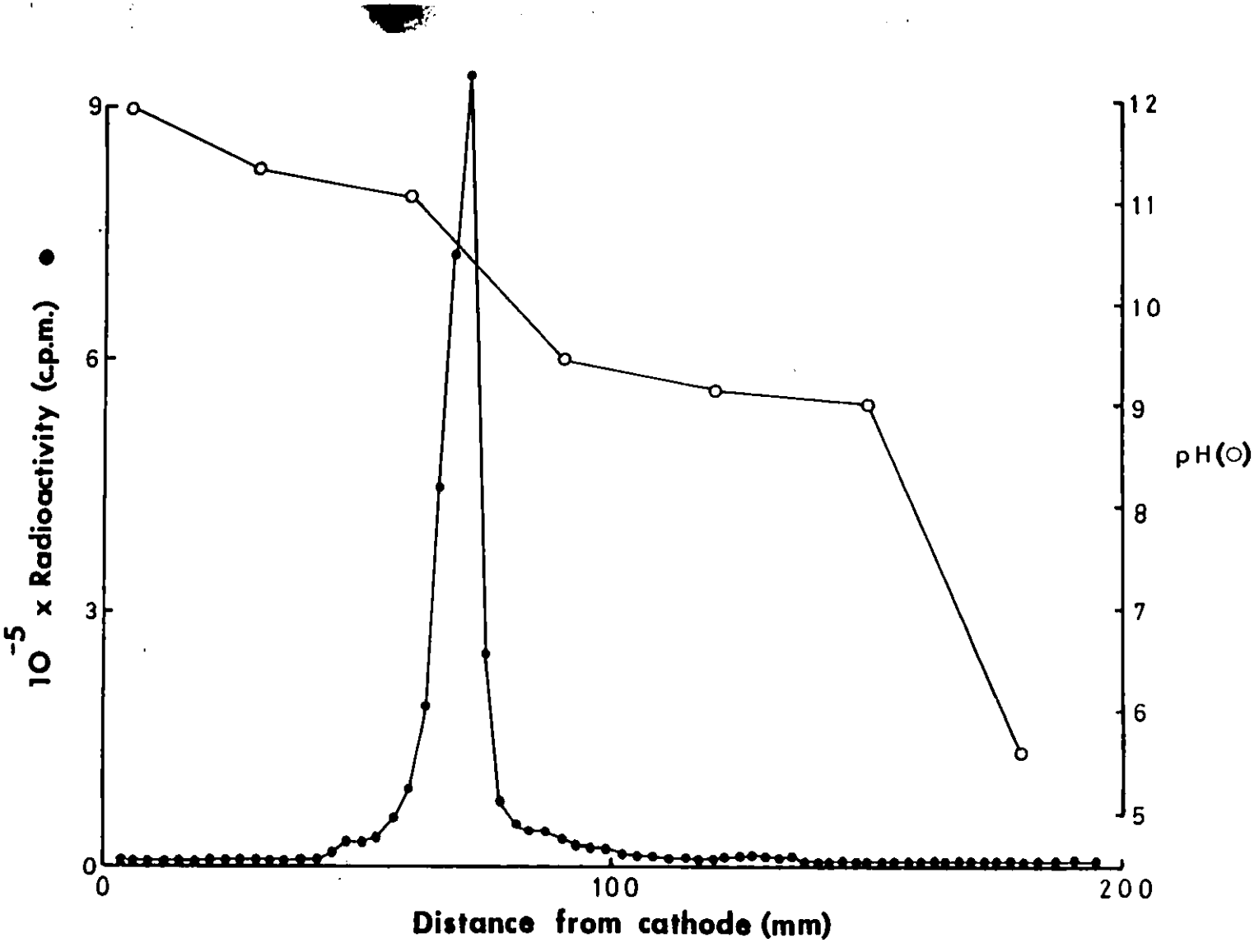


Figure 5.2 Ion-exchange chromatography of native and [^{3}H] propionylated β-BuTX

Radiolabelled toxin from Preparation I was applied to a CM52 cellulose column (0.4 x 9.6 cm) and eluted with a linear gradient of ammonium acetate concentration (□) as described in Methods (5.2.3). The concentrations of ammonium acetate were calculated from conductivity measurements; 0.5 ml fractions were collected. Radioactivity (●) was determined by counting 10 µl aliquots in a xylene based scintillation cocktail containing Triton X-100 (10% v/v). Protein (O) was detected by measuring absorbance of the fractions at 280 nm using water as a blank; 0.6 M ammonium acetate had an absorbance of zero at this wavelength.

Figure 5.3 Preparative isoelectric focusing of [^{3}H]propionylated β-BuTX

Focusing was carried out in a flat bed of Sephadex G-75 as described in Section 5.2.4. The upper part of this figure shows the paper print of the track containing 2.5 mg of native β-BuTX. The print was fixed in 10% TCA and stained for protein with Coomassie R250 as described in Section 2.2.5. The lower part of the figure shows the pH gradient (O) measured with a microelectrode when focusing was complete and the radioactivity (●) in the 3 mm slices into which the bed was subsequently cut.


9
6
3
0
10^{-5} x Radioactivity (c.p.m.) ●
12
11
10
9
8
7
6
5
pH(○)
0
100
200
Distance from cathode (mm)

the preparation must contain at least two such species. However, due to trailing of the unlabelled β-BuTX the resolution obtained by preparative isoelectric focusing still was not sufficient to completely remove free toxin from the labelled sample with an acceptable yield of radiolabelled toxin. It was therefore decided to carry out the reaction in an excess of N-succinimidyl [2,3-^{3}H] propionate in an effort to leave none of the toxin unlabelled.

5.3.2 [^{3}H] Propionylation of β-BuTX in the presence of an excess of N-succinimidyl [2,3-^{3}H] propionate

Preparation II involved the reaction of 0.5 mg of β-BuTX (2.3×10^{-8} moles) with, theoretically, a 3-fold molar excess of N-succinimidyl [2,3-^{3}H] propionate (37 Ci mmol^{-1}). However, despite the greater volume of the reaction mixture (0.2 ml), once again only a fraction (48%) of the labelling reagent dissolved in sodium phosphate buffer. The actual ratio of toxin : reagent in the solution was therefore 1 : 1.4. When the reaction was terminated by gel filtration on a Sephadex G-25 column (Fig. 5.1b) 80% of the radioactivity was recovered from the column; 68% of the radioactivity eluted in the void volume as [^{3}H] propionylated β-BuTX.

The [^{3}H] propionylated toxin had a specific radioactivity, determined as described for Preparation I, of 36.5 Ci mmol^{-1} corresponding very closely to an overall incorporation of 1 mole of [^{3}H] propionate into 1 mole of toxin. When subjected to isoelectric focusing on an analytical scale in a polyacrylamide gel and over a narrow pH range, the material from Preparation II showed a single peak of radioactivity, with some trailing, which focused at a different point in the pH gradient to native toxin (Fig. 5.5a). Preparation II had a phospholipase activity on egg yolk lecithin substrate in the

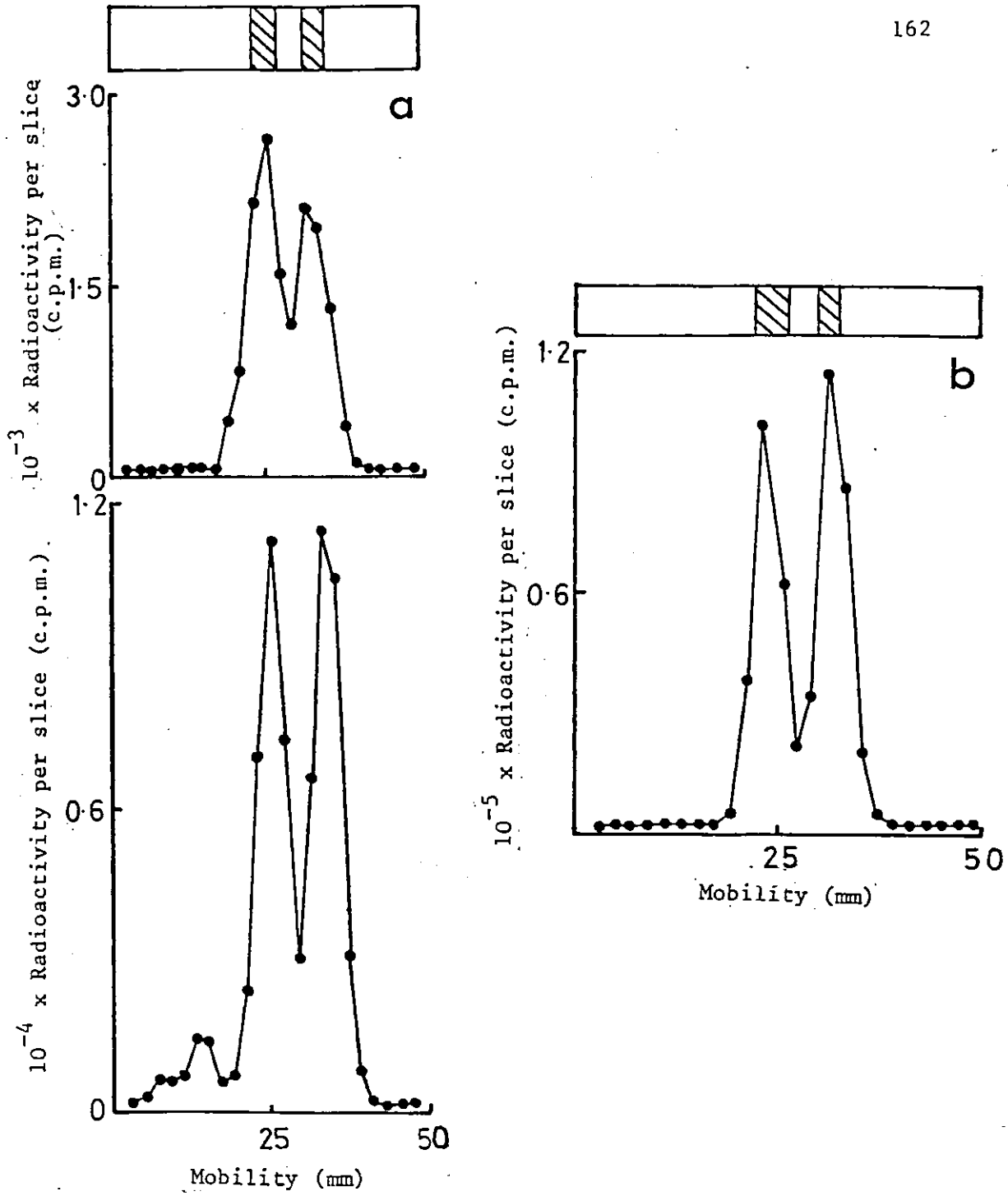


Figure 5.4 Sodium dodecyl-sulphate polyacrylamide gel electrophoresis of [^{3}H] propionyl β-bungarotoxin

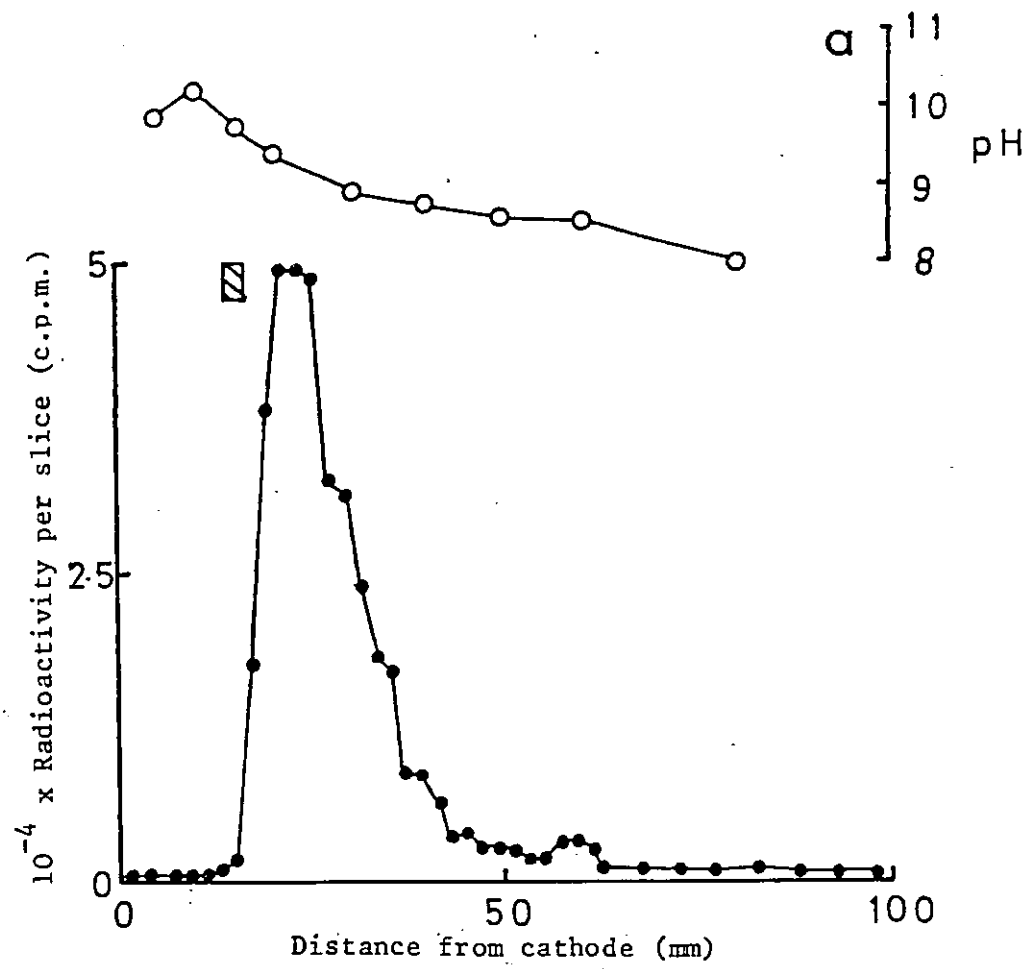
Electrophoresis was carried out under reducing conditions as described in Section 5.2.5. When electrophoresis was complete, those tracks with radiolabelled samples were cut into 2 mm slices. The radioactivity in each slice (●) was determined by liquid scintillation counting (5.2.5). Different tracks in the same gels contained pure β-BuTX (25 μg); the protein bands were stained with Coomassie R-250 as described in Section 2.2.7 and their positions are shown by the hatched bars.

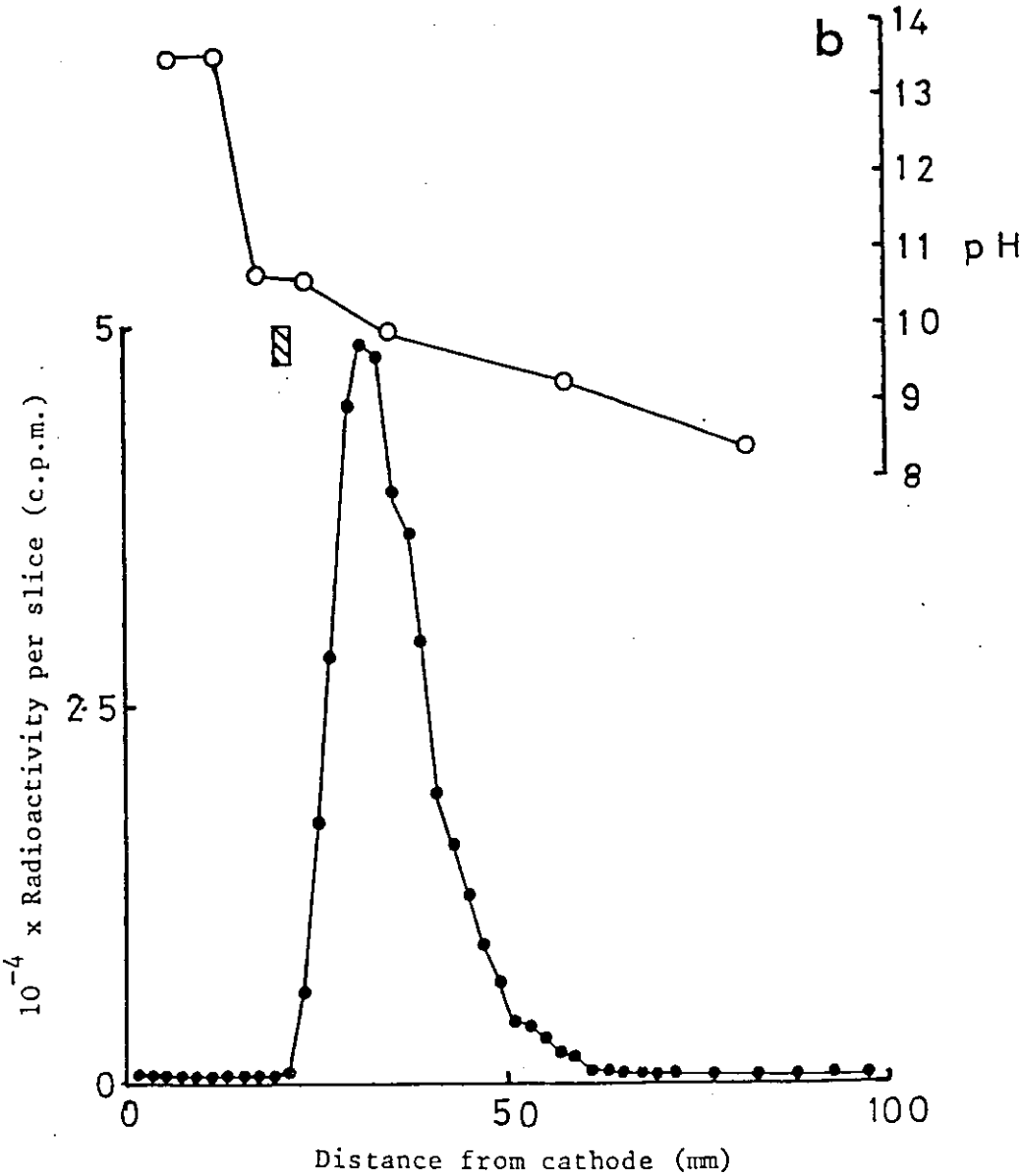
a) This gel contained samples of Preparation I (upper trace) and Preparation II (lower trace).

b) This gel contained a sample of Preparation III.

Figure 5.5 Isoelectric focusing of [^{3}H] propionyl β-bungarotoxin on an analytical scale

Focusing of [^{3}H] β-BuTX was carried out as described in Section 5.2.4.2. Samples of Preparation II (a) and Preparation III (b) were mixed with unlabelled toxin and applied to gels constituted as described in Section 2.2.5. When focusing was complete the pH (O) was measured at regular intervals along the gel. The protein bands were then fixed and visualised in trichloroacetic acid (5.2.4.2.). The position of these bands is indicated by the hatched bars. The gels were then sliced and the radioactivity in each slice (●) determined by liquid scintillation counting.


a
11
10
9
8
pH
5
2·5
0
10^{-4} x Radioactivity per slice (c.p.m.)
0
50
100
Distance from cathode (mm)


b
14
13
12
11
10
9
8
pH
5
2·5
0
10^{-4} x Radioactivity per slice (c.p.m.)
0
50
100
Distance from cathode (mm)

presence of deoxycholate and Ca^{2+} of 37 μmol min^{-1} mg of $protein^{-1}$ which was 60% of the activity of native β-BuTX measured in the same series of assays (Table 5.1). As with Preparation I, SDS polyacrylamide gel electrophoresis showed that almost equal labelling had occurred on the subunits of the toxin, 51% of the radioactivity migrating with the smaller polypeptide chain and 49% with the larger (Fig. 5.4a). The larger polypeptide chain has a residue, presumed to be a lysine, which can be modified with ethoxyformic anhydride and which is essential for the phospholipase activity (Howard and Truog, 1977). Labelling of this residue in Preparation II would account for its decreased enzyme activity. The presence of two or more mono-propionylated species, labelled on different parts of the toxin molecule may partly explain the relatively broad peak seen on isoelectric focusing.

Preparation III was carried out using sufficient N-succinimidyl [2,3-^{3}H] propionate (43 Ci $mmol^{-1}$) to give a 5-fold molar excess over the 0.44 mg (2×10^{-8} moles) of β-BuTX used. The volume of the reaction mixture was increased to 0.44 ml and the initial period of vigorous mixing increased to 10 mins; nevertheless, only 44% of the N-succinimidyl [2,3-^{3}H] propionate which was dried onto the walls of the reaction vessel was recovered in the sodium phosphate buffer. The ratio of toxin : reagent in solution was therefore 1 : 2.2. The incubation was terminated by gel filtration of the reaction mixture on a Sephadex G-25 column (Fig. 5.1c) with 99% recovery of the radioactivity; 55% of this was eluted as [^{3}H] propionylated toxin in the void volume.

When the toxin fractions were pooled the specific activity was determined as previously described (5.3.1) and found to be 82 Ci $mmol^{-1}$, a value very nearly twice that of the N-succinimidyl [2.3-^{3}H] propionate. This indicates an overall incorporation of 2 moles

Table 5.1 Chemical Properties of [^{3}H]-β-Bungarotoxin Preparations

	Preparation I	Preparation II	Preparation III
1. Molar ratio of toxin : label) Theoretical	10 : 1	1 : 3	1 : 5
) Actual	25 : 1	1 : 1.4	1 : 2.2
2. Specific radioactivity (Ci m mol^{-1})	1.6	36.5	82
3. Incorporation of label			
i) Overall (moles per mole of toxin)	0.03	1	2
ii) A chain %	52	49	47
iii) B chain %	48	51	53
4. Phospholipase activity[a] (μmol.min^{-1} mg.$^{-1}$)	-	37	0

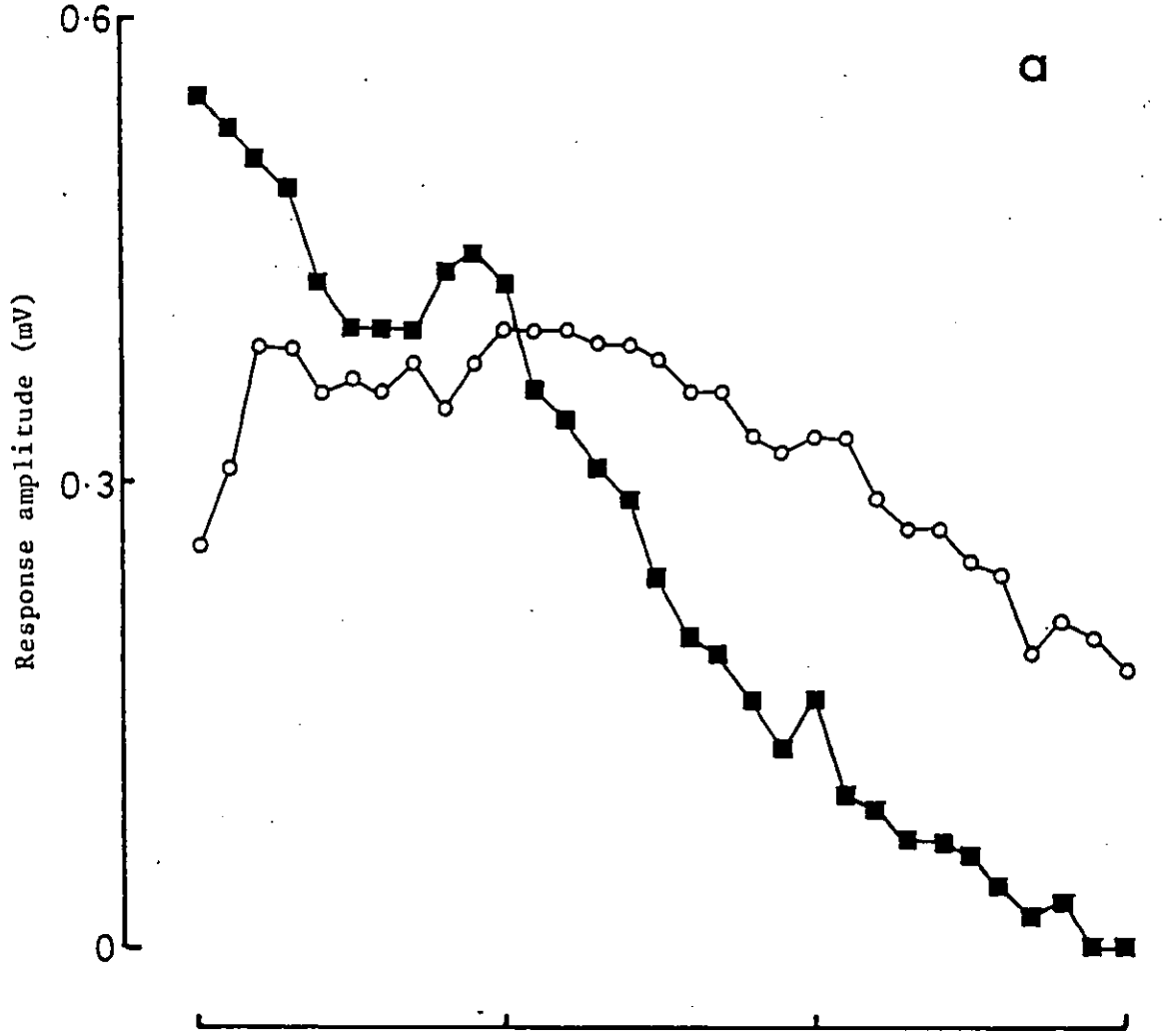
a. Measured on lecithin substrate in the presence of Ca^{2+} and deoxycholate (2.2.8)

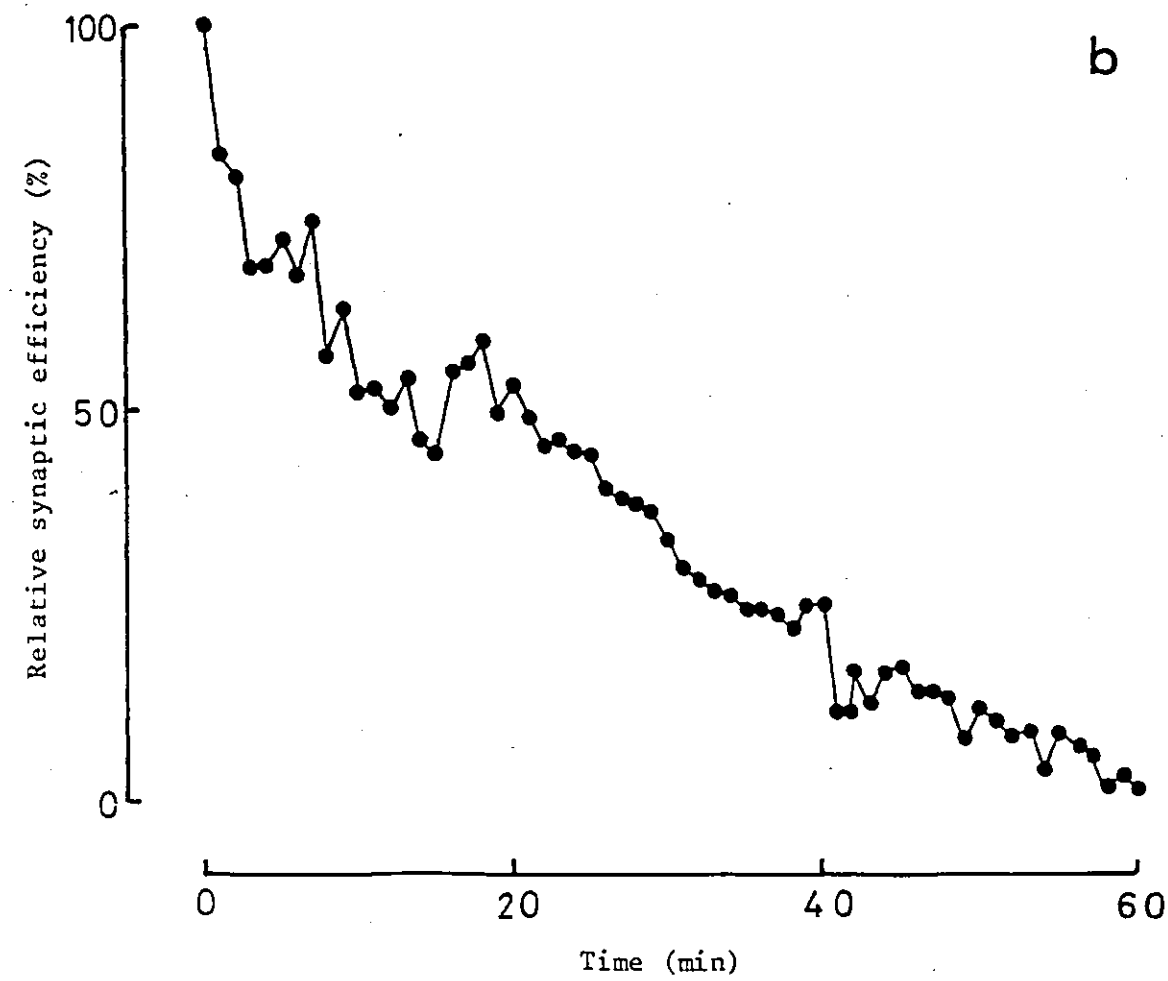
of [^{3}H] propionate into each mole of toxin. Analytical isoelectric focusing over a narrow pH range of this preparation gave a single peak of radioactivity with less trailing than was observed with Preparation II (Fig. 5.5b); once again the [^{3}H] β-BuTX peak was clearly focused in a different position to native toxin in the same track (Fig. 5.5b). As in the case of the two previous preparations of tritiated toxin, SDS polyacrylamide gel electrophoresis of Preparation III showed almost equal labelling of the two polypeptide chains; 53% of the radioactivity was associated with the smaller subunit and 47% with the larger (Fig. 5.4b). However, in contrast to Preparation II, this sample of [^{3}H] propionylated β-BuTX retained had no measurable phospholipase activity on dispersed egg yolk lecithin substrate in the presence of deoxycholate and Ca^{2+}. This lack of enzyme activity suggests that this preparation did not contain significant unmodified toxins and, by inference of its specific radioactivity, contains only di-propionylated species. Determinations of the lethalities or Preparation II and III were severely limited due to a shortage of material and were performed on a very small number of animals; the measurements made were sufficient only to establish that they possessed biological activity. Intraperitoneal injection of Preparation II killed two out of three mice at 0.5 µg/g body weight but surprisingly none at 0.1 µg/g body weight. Preparation III, administered by the same route, killed all three mice at 0.5 µg/g body weight but none at a ten-fold lower dose. Following intraventricular injection into rat brain, Preparation III was lethal at 10 ng/g body weight but not 1 ng/g body weight. These values for Preparation III are of the same order as the accurately determined lethalities of di-[^{3}H] propionylated β-BuTX subsequently prepared in the laboratories of Dr. Dolly by the same proceedure (Othman et al., 1982; 5.4.1)

Figure 5.6 The effect of [^{3}H] propionyl β-bungarotoxin on neurotransmission in rat olfactory cortex

a) Presynaptic (O) and postsynaptic (■) responses were measured as described in Section 4.2.5. Both measures were stable for 20 min prior to the addition of [^{3}H]β-BuTX (250 nM) at zero time.

b) Synaptic efficiency, the ratio of the amplitudes of postsynaptic to presynaptic responses, was calculated at each time point and expressed as a percentage of the value at zero time.


a
0·6
0·3
0
Response amplitude (mV)


b
100
50
0
Relative synaptic efficiency (%)
0
20
40
60
Time (min)

5.3.3 The actions of [^{3}H] propionylated β-BuTX in the central nervous system

Further evidence that this radiolabelled toxin was active at synapses in the central nervous system was provided by its effects on neurotransmission in slices of rat olfactory cortex (Fig. 5.6). [^{3}H] β-BuTX (Preparation III) at a concentration of 230 nM caused a decline in N-wave amplitude leading to a complete blockade (Fig. 5.6a). Immediately after its addition there was a 45% increase in the amplitude of the action potentials recorded extracellularly from the lateral olfactory tract (Fig. 5.6a). There followed a 10 min period during which there was some fluctuation in both the pre- and post-synaptic responses, after which the presynaptic action potential declined steadily but more slowly than the postsynaptic response, as was observed with native β-BuTX (Fig. 4.3). The ratio between these two measures, the relative synaptic efficiency, showed a mostly steady decline, after the addition of [^{3}H] β-BuTX, to produce a complete blockade of neurotransmission after 60 mins (Fig. 5.6b); 50% blockade was reached after 10 - 20 minutes. The unexpected effects seen immediately after addition of [^{3}H] β-BuTX were probably due to the presence of ammonium acetate in which the toxin sample was dissolved and which had a final concentration in the bath of 1.2 mM.

Similar experiments performed using a later preparation of di-[^{3}H] propionylated β-BuTX, from which the ammonium acetate had been removed by freeze drying, showed qualitatively similar results but without the period of fluctuation or increase in the presynaptic response.

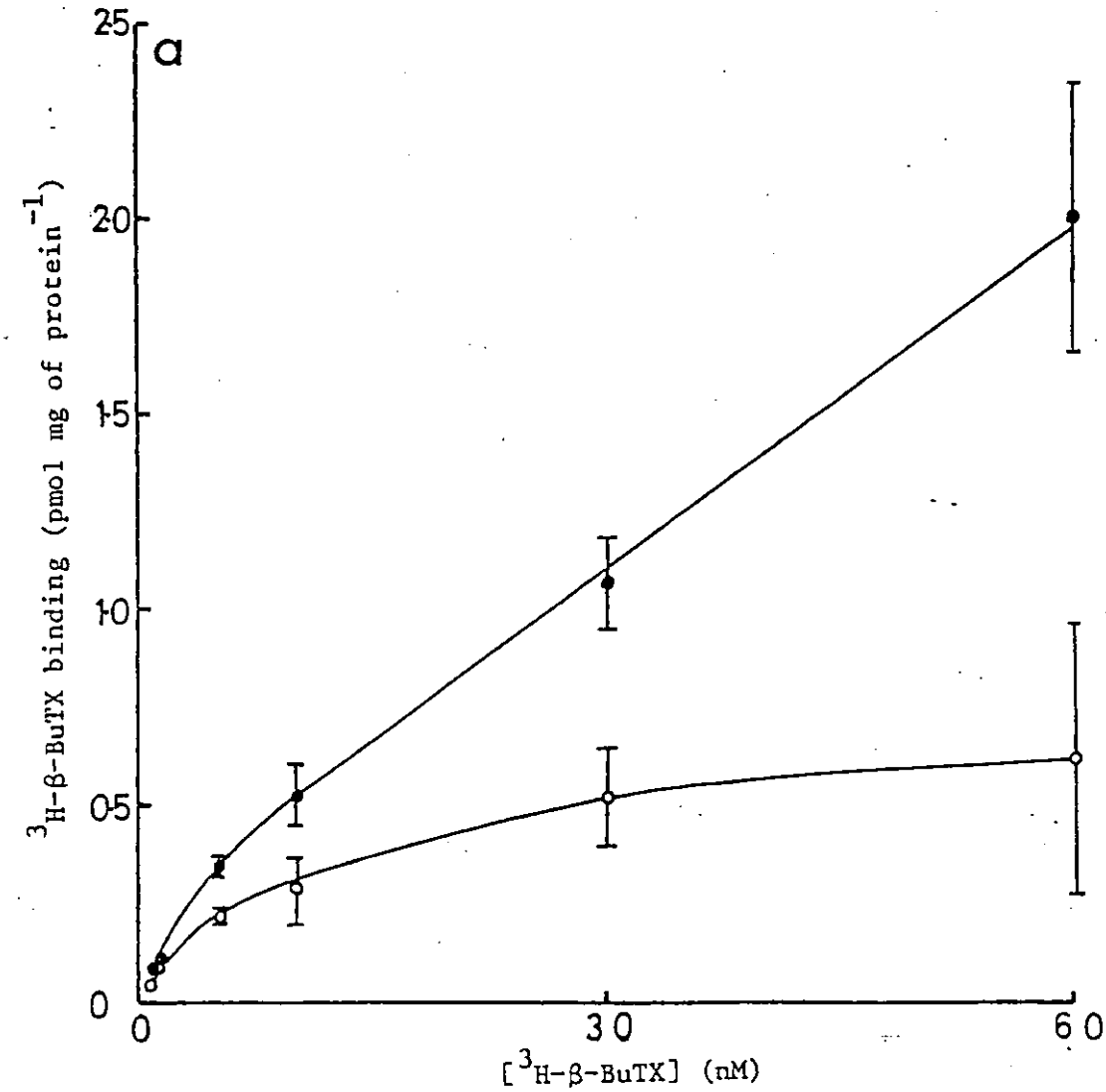
5.3.4 The binding of [^{3}H] propionylated β-BuTX to synaptosomes

Equilibrium binding of both preparations II (0.5-60 nM) and III (0.5-100 nM) of [^{3}H] β-BuTX to nerve terminals, purified from rat

Figure 5.7 Binding of [^{3}H] propionyl β-bungarotoxin (Preparation II) to synaptosomes

a) Suspensions of rat cerebrocortical synaptosomes were incubated at 37°C for 2 hours with various concentrations (0.5 - 60 nM) of [^{3}H] β-BuTX (Preparation II). The incubations were terminated by centrifugation and the amounts of radioactivity bound to the synaptosome pellets were determined as described in Section 5.2.7. The results were expressed in terms of pmoles of ^{3}H-toxin bound per mg of protein. The values obtained for non-specific binding in the presence of 6 μM unlabelled β-BuTX, were subtracted from those for total binding (●) to give the amount of specific binding (O) at each concentration of [^{3}H]-β-BuTX. The values shown are the means of those from duplicate samples; the error bars indicate the range.

b) Scatchard plot of the data in (a).


a
25
20
15
10
05
0
0
30
60
^{3}H-β-BuTX binding (pmol mg of protein^{-1})
[^{3}H-β-BuTX] (nM)

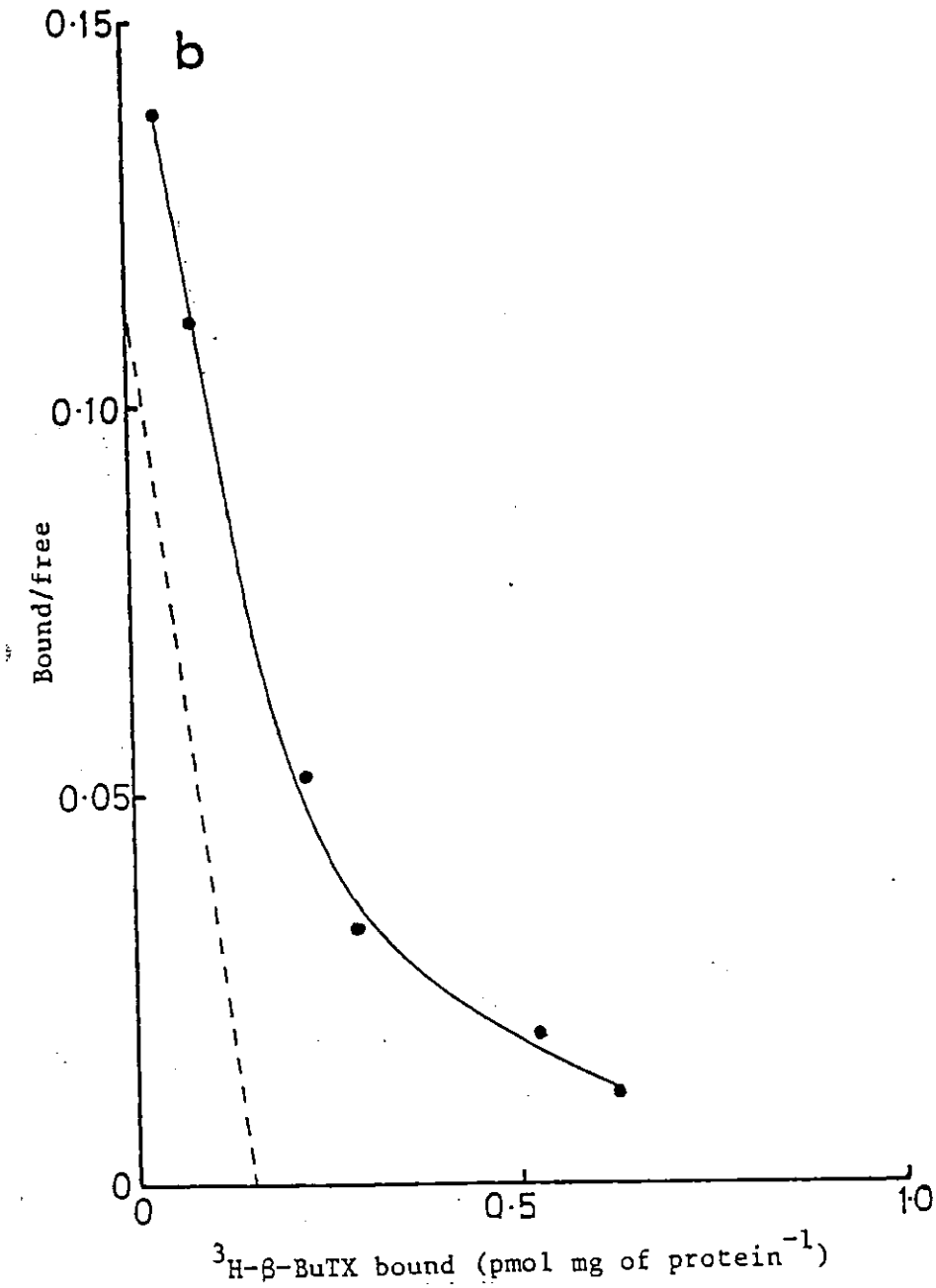
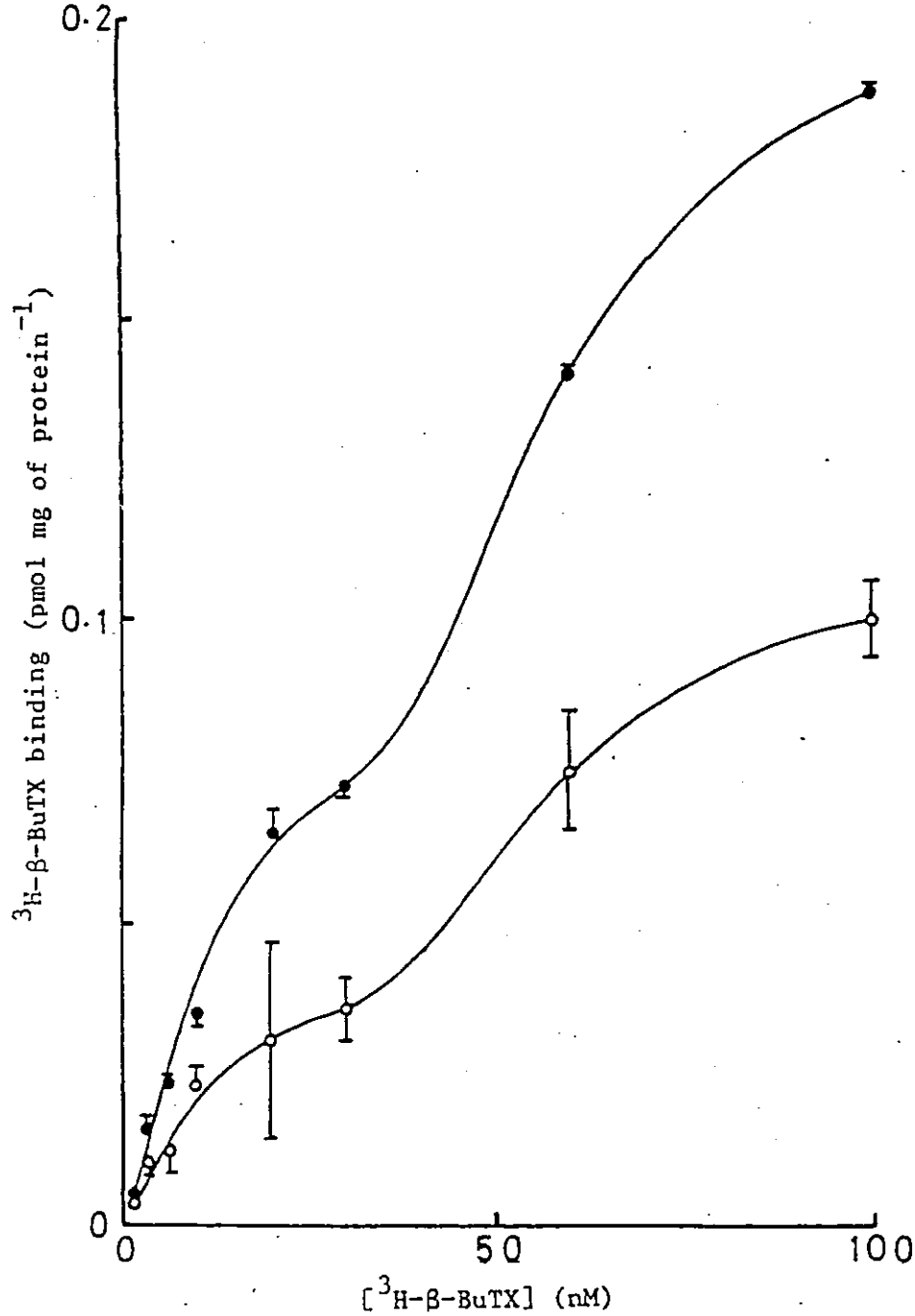
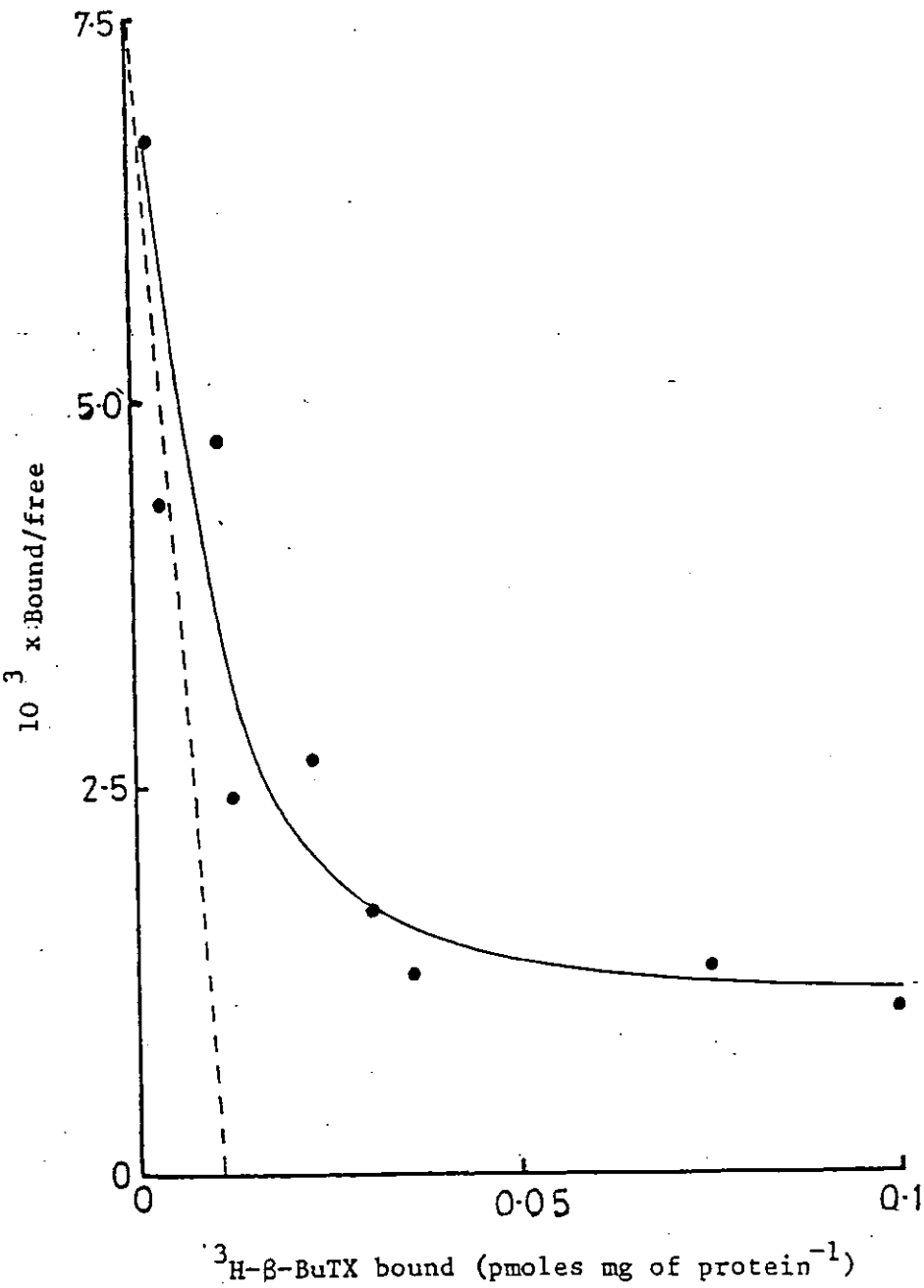

b
0·15
0·10
0·05
0
Bound/free
0
0·5
1·0
^{3}H-β-BuTX bound (pmol mg of protein^{-1})

Figure 5.8 Binding of [^{3}H] propionyl β-bungarotoxin (Preparation III) to synaptosomes

a) The binding of [^{3}H]-β-BuTX (Preparation III), at concentrations between 0.5 and 100 nM, was assayed as in Figure 5.7. Non-specific binding was measured in the presence of 10 μM unlabelled β-BuTX and was subtracted from the total binding (●) to obtain the amount of specifically bound [^{3}H]-β-BuTX (O) at each concentration. The binding is expressed as pmoles of toxin bound per mg of protein and the values shown are the means obtained from duplicate samples; the error bars indicate the range.

b) Scatchard plot of the data in (a).


0·2
0·1
0
0
50
100
^{3}H-β-BuTX binding (pmol mg of protein^{-1})
[^{3}H-β-BuTX] (nM)


7·5
5·0
2·5
0
0
0·05
0·1
10^3 x Bound/free
^{3}H-β-BuTX bound (pmoles mg of protein^{-1})

cerebral cortex, was measured at 37°C. When the amount of radioactivity which bound in the presence of a 100-fold molar excess of native β-BuTX was subtracted from the total binding measured in its absence, the binding curves for both preparations showed a marked tendency towards saturation. The degree of non-specific binding although large, was acceptable in both cases (Figs. 5.7a and 5.8a). The binding curve for preparation III suggested that two sets of binding sites were present; Scatchard analysis of binding curves for both preparations also indicated the existence of two classes of sites (Figs. 5.7b and 5.8b). In the case of Preparation II the Scatchard analysis (Fig. 5.7b) showed that the higher affinity sites had a maximum binding capacity of 0.16 pmoles/mg of protein^{-1} and a K_D of 1.4 x 10^{-9} M. Preparation III, which had no phospholipase activity (Table 5.1) and was shown to block neurotransmission in rat olfactory cortex (Fig. 5.6b) also appeared to have two groups of binding sites (Fig 5.8b). However, the values obtained from Scatchard analysis were somewhat different to those for Preparation II; the higher affinity sites had a K_D of 1.5 x 10^{-9} M and the maximum binding capacity (0.011 pmoles/mg of protein^{-1}) was 8-fold lower than the corresponding value for Preparation II.

5.4 Discussion

5.4.1 Radiolabelling of β-BuTX with N-succinimidyl 2,3-[^{3}H] propionate

In this study procedures were developed for successfully incorporating a tritiated label into β-BuTX at high specific radioactivity, for characterising the labelled toxin and for measuring its binding to purified nerve terminals. These studies have since been extended and refined in the laboratory of Dr. Dolly. The biggest single problem encountered in performing the labelling reaction was absorption of the reagent onto the walls of the microfuge tubes. The plastic material of

the tubes may have been attacked by the toluene in which the N-succinimidyl [2,3-^{3}H] propionate was dissolved thereby exacerbating this problem. If it was the case, the poor recovery of the labelling reagent in the reaction mixture might be improved by changing to a vessel of a different material. A subsequent [^{3}H] β-BuTX preparation in the same laboratory used a silicon coated glass vial and 78% of the radioactivity wasrecovered in the reaction mixture enabling a molar ratio of reagent : toxin of 3 : 1 to be attained.

Comparison of the specific radioactivities of Preparations II and III with the specific radioactivities of the respective batches of N-succinimidyl [2,3-^{3}H] propionate, quoted by the manufacturers, indicated that these preparations incorporated respectively 1 and 2 moles of the reagent per mole of toxin. However, it should be pointed out that some evidence was obtained, through studies on tritiated α-bungarotoxin, that the specific radioactivities of certain batches of N-succinimidyl [2,3-^{3}H] propionate were significantly different from those given by the manufacturers (J.O. Dolly, unpublished observations). A method was developed to measure the specific radioactivity of the labelling reagent which involved reaction of the ester with the chromophore, p-nitrophenylalanine in sodium borate buffer (0.1M, pH 8.0). The [^{3}H] propionylated reaction production (R_f 0.75) was separated by thin layer chromatography on silica-coated plates in a solvent system of chloroform : ethanol : acetic acid (8 : 1 : 1) and eluted from the plate in borate buffer. The specific radioactivity of the purified reaction product, and hence of the N-succinimidyl [2,3-^{3}H] propionate, was calculated from the radioactive content of the eluate and the concentration of the chromophore. This was determined by its absorbance at 275 nm (molar extinction coefficient $\varepsilon_{275} = 10.2 \times 10^3$ cm^{-1}). Unfortunately, of the

batches of N-succinimidyl [2,3-^{3}H] propionate used in this study, insufficient remained for this determination to be carried out. Nevertheless, this is a valuable method for checking batches of the labelling reagent used in future research.

It is clear from SDS gel electrophoresis (Fig. 5.4) that residues (probably lysine) that are almost equally reactive exist on both polypeptide chains. This is especially indicated by the SDS gel electrophoresis of Preparation I (Fig. 5.4a), which since it was labelled in large toxin excess was very unlikely to contain di-labelled or higher derivatives. In the case of Preparation II a 40% loss of phospholipase activity (Table 5.1) is reasonably consistent, within experimental error, with 49% of this toxin being modified on the larger chain (Fig. 5.4a) in which the enzyme activity resides. The lack of measurable enzyme activity strongly indicates that, all molecules of Preparation III are labelled on both subunits.

A subsequent preparation of [^{3}H] propionylated β-BuTX was made at the higher reagent : toxin ration of 3 : 1. This produced an incorporation of 2 moles of reagent per mole of toxin, equal labelling of the two subunits and abolition of the phospholipases on lecithin substrate (Othman et al., 1982). It showed only 1.3% of the phospholipase activity of native toxin in a more sensitive assay using radiolabelled cerebrocortical synaptosomes (Othman et al., 1982). This preparation of [^{3}H] β-BuTX produced a single, very sharp and symmetrical peak on isoelectric focusing; the shift in its isoelectric point from that of native toxin was 0.3 pH units. The lethality of this toxin, when administered by both peripheral and central routes, was decreased five-fold with respect to native toxin; the minimum lethal dose was increased from 10 to 50 ng/g body weight following intraperitoneal injection and from 0.05 to 0.24 ng/g body weight following intraventricular injection (Othman et al., 1982). The blockade of neurotransmission in the olfactory

cortex by enzymatically inactive [^{3}H] β-BuTX (5.3.3.) offers further evidence that the phospholipase activity is not necessary for the action of β-BuTX; since this derivative is more active than pure phospholipases tested in Chapter 4 (Fig. 4.6b) it supports the existence of specific sites of action on nerve terminals for which it may be used as a probe.

5.4.2 Binding of [^{3}H] β-BuTX to synaptosomes

Preparations II and III were similar in that they both showed two classes of binding sites on cortical synaptosomes with K_D values of the same order of magnitude. Those for the higher affinity sites were 1.4 nM and 1.5 nM for Preparations II and III, respectively, compared with a value of 1 - 2 nM for a preparation of ^{125}I-β-BuTX (Oberg and Kelly, 1976a).

The experiments described in this chapter formed a preliminary investigation of the binding of [^{3}H] β-BuTX to synaptosomes; their significance lies only partly in the actual values obtained. This work did, however, establish a method for radiolabelling β-BuTX and for characterising the species produced. It showed that such derivatives were active in the central nervous system and that the saturable binding of this toxin to purified nerve terminals could be measured. On the basis of this a preparation of [^{3}H] β-BuTX was subsequently made, as previously mentioned, which appeared to be a single di-propionylated species having negligible phospholipase activity but which retained 20% of its toxicity in the peripheral and central nervous systems (Othman et al., 1982). This toxin showed saturable binding to

synaptosomes of higher affinity than that measured for previous preparations; the single class of sites which were assayed had a K_D of 0.57 nM and the content of these sites was 135 - 160 f moles of protein^{-1}. The reasons for these discrepancies must be either with the [^{3}H] β-BuTX preparations themselves or with the way in which the binding studies were carried out. Preparation II is clearly different from the other two preparations, being only monolabelled, and equally clearly contains more than one labelled species. The possibility that it contains unlabelled toxin cannot be excluded. Preparation III is much less likely to contain native toxin; however, it too gave a much broader peak on isoelectric focusing (Fig. 5.5b) as compared with the later di-[^{3}H] propionylated preparation (Othman et al., 1982) and had less well characterised toxicity. Non-specific binding to synaptosomes of both Preparations II and III was a rather large proportion of the total. The protocol used for these measurements involved termination of the incubation of [^{3}H] β-BuTX with synaptosomes by centrifugation (5.2.7). The washing procedure was rather long due to the resuspension of pellets and re-centrigugation; this may have allowed bound ^{3}H-toxin to disaociate from its binding sites. The protocol used for the subsequent experiments (Othman et al., 1982) gave much more efficient termination of incubations, by dilution in medium at 4°C prior to centrifugation, and more rapid washing of the resulting pellets. The filtration assay which was also used in some of these later experiments is a yet further improvement (Othman et al., 1982). This binding was blocked by native β-BuTX with K_I of 0.29 nM indicating that, within experimental error, the labelling did not change the affinity of the toxin for its binding sites. The rate of association of the toxin with synaptosomes was very rapid (k_{+1} = 7.8 x 10^5 M^{-1} s^{-1} at 37°C) and is, therefore, unlikely to be the rate limiting step in its action. The dissociation rate (k_{-1} = 5.6 x 10^{-4} s^{-1} at 37°C) was somewhat faster than expected from the apparent

irreversability of the action of the toxin in the olfactory cortex (Othman et al., 1982). Enzymically inactive BPB-β-BuTX weakly inhibited [^{3}H] β-BuTX binding to synaptosomes (Othman et al., 1982). Removal of Ca^{2+} did not inhibit the binding of [^{3}H] toxin. This was also 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 = 5.9 nM) ten-fold lower than that of native toxin; it was not effective in Ca^{2+}-free medium. The foregoing provides further evidence that the binding of β-BuTX to nerve terminals is not due to direct enzyme-substrate interaction.

Saturable binding also occurred to lysed synaptosomes indicating that the interaction is not due to internalization of the toxin. Treatment of the synaptosomes with trypsin for 60 min at 37°C abolished the binding indicating that the membrane component involved is proteinaceous in nature (Othman et al., 1982).

Neither α-latrotoxin (Grasso et al., 1978) nor TsTX (3.3.4), which produce large increases in neurotransmitter release from synaptosomes, has any effect on the binding of di-[^{3}H] propionylated β-BuTX; this was interesting in view of the fact that the rate of blockade of neuromuscular transmission by β-BuTX is dependent on the frequency of nerve stimulation and the suggestion that β-BuTX might bind to sites exposed during the release process (Simpson, 1976). Botulinum neuro-toxin was also ineffective at inhibiting the binding of di-[^{3}H] propionylated β-BuTX to synaptosomes indicating that this toxin does not interact with the same sites on the nerve terminal as β-BuTX; taipoxin was weakly effective, similar to bee venom phospholipase. This later work did, however, show that toxin I from the venom of Dendroaspis polylepis, which increases release of ACh at the neuromuscular junction (Harvey, 1982), inhibited the binding of [^{3}H] β-BuTX to synaptosomes with a K_I of 0.07 nM (Othman et al., 1982).

Toxin I is the first specific inhibitor of β-BuTX binding to nerve terminals and will greatly facilitate investigation of the membrane component involved.

Saturable binding of an ^{125}I β-BuTX derivative to synaptic membranes from chick brain has recently been reported (Rehm and Betz, 1982) although this toxin was not fully characterised. A single class of high affinity binding sites was measured similar to that for [^{3}H]-propionylated β-BuTX on rat brain (Othman et al., 1982) (K_d = 0.47 nM, k_{+1} = 4.3 x 10^{6} M^{-1} s^{-1}, k_{-1} = 1.08 x 10^{-4} s^{-1}). The binding of ^{125}I-β-BuTX was Ca^{2+}-dependent; Co^{2+}, Sr^{2+} but not Mg^{2+} could substitute for Ca^{2+}. The level of binding sites for [^{3}H] propionylated β-BuTX on synaptosomes (135 - 160 fmol/mg of protein) was similar to that reported for a ^{125}I-labelled derivative of a single chain toxin from Bungarus multicinctus venom with a similar action to β-BuTX (Donlon et al., 1979), although in the latter case non-specific binding was not measured. The level of binding of the ^{125}I-β-BuTX preparation mentioned above to chick synaptic membranes was somewhat lower (25 fmol/mg of protein) (Rehm and Betz, 1982).

CHAPTER 6. GENERAL DISCUSSION

6.1. The Specificity of Action of β-BuTX

It has been known for several years that the killing action of β-BuTX, following its intravenous or intraperitoneal injection into mammals, is by a specifically presynaptic neuromuscular blockade (1.3.3.4). This is the eventual result of triphasic action of the toxin on both spontaneous and evoked release of ACh from the nerve terminals (Chapter 3) for which its phospholipase A_2 activity is required (Chapter 4). Furthermore, β-BuTX appears to exhibit, in the peripheral nervous systems, a specificity of action on nicotine cholinergic synapses (1.3.3.4.iii). This thesis describes the complete purification of β-BuTX (Chapter 2). Previous observations on the actions of this toxin at central synapses were confirmed and extended (Chapters 3 and 4). Finally, the nature of the interaction of β-BuTX with nerve terminals was investigated using a radiolabelled derivative of the toxin (Chapter 5).

When β-BuTX was administered to rats by injection into the lateral ventricle of the brain its lethality was increased by about one thousand-fold (Table 2.1; Hanley and Empson, 1979). This increase in potency of β-BuTX strongly suggest a specific action of the toxin on the central nervous system. The specificity shown by β-BuTX for nicotinic cholinergic synapses in the peripheral nervous system does not apply to its actions in the central nervous system. β-BuTX affects the release of several neurotransmitters including glutamate, GABA, noradrenaline and ACh from purified brain nerve terminals (Chapter 2); the toxin caused a blockade of neurotransmission in slices of olfactory cortex from rat, a system in which glutamate and aspartate are the putative transmitters (4.3.2), and hippocampal slices (Halliwell and Dolly, 1982b).

Extracellular recording in the olfactory cortex showed that β-BuTX affects primarily neurotransmitter release with secondary effects on conduction of action potentials along the lateral olfactory tract (4.3.2) which may, in part, account for the monophasic effect of the toxin in this system. Intracellular recording from the neurones of the olfactory cortex showed that β-BuTX caused a steady decline in the e.p.s.p.s induced by stimulation of the lateral olfactory tract with no effect on the membrane potential of the postsynaptic cells (Dolly et al., 1980b). The postsynaptic responses to bath applied glutamate or aspartate were unaffected in preparations which were completely blocked by β-BuTX indicating a presynaptic action (Dolly et al., 1980b). However, prolonged exposure to β-BuTX also reduced the excitability and attenuated the action potentials in the postsynaptic neurones (Dolly et al., 1980a). The postsynaptic neurones, in common with the cells of the LOT, possess nerve terminals; local disruption of the plasma membrane at these sites may reduce the excitability and affect action potentials in the postsynaptic cell. The action of Na^+/K^+ ATPase would probably be sufficient to maintain the membrane potential at the sites of postsynaptic intracellular recording, distal from the nerve terminal regions. β-BuTX has also been shown to cause a blockade of neurotransmission in terminal rich areas of rat hippocampus (Halliwell and Dolly, 1982b). Intracellular recordings from these regions showed that, following blockade by the toxin, the postsynaptic neurones maintained sensitivity to excitatory amino acids glutamate, aspartate and DL-homocysteic acid. In some experiments there was evidence of reduced excitability of these cells by depolarising currents (Halliwell and Dolly, 1982b) reminiscent of that seen in olfactory cortex. Extracellular recordings or pre- and post-synaptic potentials, and hence synaptic efficiency showed very similar effects of β-BuTX to those seen in olfactory cortex (Halliwell and Dolly, 1982b).

Autoradiography in the light microscope on cryostatic sections of rat brain has also shown that binding of di[^{3}H] propionylated β-BuTX, displacable by unlabelled toxin, is localised at terminal rich layers of the hippocampus and in the cerebellum. Specific localisation of toxin binding to the plasma membranes of synaptosomes has also been shown by autoradiography using the electron microscope (Othman, I. and Dolly, J.O. unpublished observations). Specific binding of β-BuTX, restricted to nerve terminals, has not yet been demonstrated at the neuromuscular junction. Nevertheless, it appears that peripheral nicotinic cholinergic nerve terminals share with a wide range of central nerve terminals a component or property which makes them susceptible to β-BuTX action. There appears, however, to be some variation in the mode of action and the potency of native β-BuTX and its enzymatically inactive derivatives in the different systems used to test them. Following modification with N-succinimidyl [2, 3 ^{3}H] propionate the toxin retained considerable (20%) lethality when injected by both peripheral and central routes. (Table 6.1). [^{3}H]β-BuTX also showed the same degree of activity as native toxin in blocking neurotransmission in rat olfactory cortex (5.3.3.). This is wholly consistent with the small decrease in the affinity of toxin for synaptosomal membranes produced by this modification (Othman et al, 1982). Modification of the toxin with p-bromophenacyl bromide evidently perturbed that part of the molecule involved in binding to nerve terminals in addition to removing the phospholipase activity since BPB-β-BuTX only weakly antagonised [^{3}H]β-BuTX binding to synaptosomes. It is not surprising that its lethality following both peripheral and central administration was very much less than that of native toxin (Table 6.1) and that it showed rather variable ability to antagonise the effects of native toxin on synaptosomes even

Table 6.1 Toxicities and Phospholipase Activities of β-Bungarotoxin and Derivatives

	Native	BPB-β-BuTX	^{3}H-β-BuTX	Sr^{2+} medium
Peripheral toxicity				
Intraperitoneal injection (ng/g body wt.)	10	> 10^{4}	50	-
Central toxicity				
Intraventricular injection (ng/g body wt.)	0.05	12	0.24	-
Rate of blockade in olfactory slices (Time for 50% blockade : min)	27-45	65-150	10-20	60-110
Phospholipase activity[a] µmol lecithin hydrolysed/min/mg	68.5	0	0	2.6

a) Measured in the presence of deoxycholate 37°C

when present in a large molar excess (4.3.3.2). BPB-β-BuTX did however retain considerable potency in blocking neurotransmission in rat olfactory cortex (4.3.3.2); it produced a blockade at approximately the same rate as did the native toxin in Sr^{2+}-medium. It may be that the olfactory cortex preparations contain sites at which BPB-β-BuTX can act but which are not typical of the cortex or CNS in general. Notably, BPB-β-BuTX showed antagonism of β-BuTX action at the neuromuscular junctions at odds with its much reduced affinity for synaptosomes (4.1).

6.2 The Nature of the Specific Interaction of β-Bungarotoxin with Nerve Terminals

6.2.1 Lack of involvement of phospholipase activity

The specificity of β-BuTX for sites of action in nerve terminals could be conferred either by high affinity binding or by a highly selective phospholipase activity or, indeed, by a combination of the two. Phospholipases are generally much less lethal than specific neurotoxins when administered either by intraperitoneal injection (4.3.4; Strong *et al.*, 1976) or directly into the central nervous system (Hanley and Empson, 1979; Othman *et al.*, 1982). Although there is considerable variation in their lethality, basic phospholipases A_2 tend to be more toxic than acidic enzymes (Rosenberg, 1979). The covalent bonding of the acidic phospholipase subunit of β-BuTX with a very basic polypeptide might therefore be expected to increase its toxicity. It is interesting, in this regard, that a heterodimeric phospholipase A_2 from the venom of *Vipera ammodites* (VA_{5-7}) was more effective than a less basic homodimeric enzyme from the same venom (VA_{6-8}) in causing the release from synaptosomes of glutamate and

lactate dehydrogenase (4.3.4). However, it should be noted that in these experiments high concentrations of enzymes were added directly to purified nerve terminals; effects seen here do not necessarily indicate that any of these enzymes have the specificity required in order to act solely at nerve terminals following their injection into whole animals.

It is possible that the catalytic activity of β-BuTX has an extra degree of specificity for a substrate found only in target membranes; however, no evidence has yet been found to indicate that the substrate specificity of β-BuTX is different to that of other phospholipases A_2 (Kelly et al., 1979a). Indeed, analysis of the products of toxin action on erythrocyte (Wernicke et al., 1975) synaptic (Sen and Cooper, 1978) and radiolabelled bacterial membranes (Wernicke et al., 1975) has shown that, in common with non-neurotoxic phospholipases A, β-BuTX converts phospholipids to their lyso-derivatives. It has been confirmed, using artificial lipid substrates that the hydrolysis of phospholipids catalysed by β-BuTX is not affected by the nature of their polar head groups (Kelly et al., 1979b). Their hydrophobic tails may be of significance since they affect the fluidity of the membrane and hence the accessibility of the toxin to the sites at which hydrolysis occurs. β-BuTX is similar to non-neurotoxic phospholipases such as the pancreatic enzyme in that its hydrolysis of artificial lipid substrates is stimulated by their dispersion with detergent (2.3.4). It is greatly increased when the fluidity of the substrate is increased either at the phase transition temperature (Strong and Kelly, 1977) or by including lipids of shorter chain length (Howard and Truog, 1977) and is inhibited by the presence of cholesterol which decreases

their fluidity (Strong and Kelly, 1977). The membranes of mitochondria, sarcoplasmic reticulum and bacteria which contain low proportions of cholesterol are all readily hydrolysed by β-BuTX whilst those of red blood cells, with a high cholestrol content, are a poor substrate.

Despite the unquestioned involvement of its phospholipase activity in its mechanism of action there is a large body of evidence to indicate that the specificity of β-BuTX is not conferred by its enzyme action, i.e. by the binding of substrate to the enzyme active site (Chapter 4). This argument is considerably strengthened by the finding that di-[^{3}H] propionylated β-BuTX, which has negligible enzyme activity and retains considerable biological activity in the central nervous system, binds specifically and with high affinity to cerebrocortical synaptosomes (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. The enzyme activity of BPB-β-BuTX is similarly inhibited and its albeit weak, antagonism of [^{3}H] β-BuTX binding is further evidence that it occurs other than through the phospholipase activity (Othman et al., 1982). Furthermore, [^{3}H] β-BuTX binding was also unaffected by a poorly neurotoxic phospholipase (NMPL). The antagonism of [^{3}H] β-BuTX binding to synaptosomes by bee venom phospholipase, together with its protective effect against β-BuTX at the neuromuscular junction (Abe and Miledi, 1978), was probably due to perturbation of the membrane by its phospholipolytic action; in Ca^{2+}-free medium it had no inhibitory effect on [^{3}H] β-BuTX binding (Othman et al., 1982). Recently, the binding of an ^{125}I-labelled β-BuTX derivative to synaptic membranes from chick brain was found to be Ca^{2+}-dependent, in apparent contradiction to the above (Rehm and Betz, 1982). However the binding affinity (K_d = 0.47 nM) is too high to be accounted for by an enzyme-substrate interaction; the interaction with synaptosomes of a phospholipase A_2 homologous to the A chain of β-BuTX

shows a K_d of 10 μM (Volwerk et al., 1974)

6.2.2. Nature of β-bungarotoxin binding sites on nerve terminals

Binding of [^{3}H] propionylated β-BuTX occurred equally well to lysed as to intact synaptosomes (Othman et al., 1982) indicating that the binding sites are membrane bound. The binding sites have recently been shown to be sensitive to both heat treatment and trypsinisation indicating that they are proteinaceous in nature (Othman et al., 1982). Binding of the toxin to a type of membrane protein unique to nerve terminals would confer the necessary specificity on its action. The phospholipase activity might also act to best effect on surrounding lipids at the phase transition between the fixed lipid anulus associated with an integral membrane protein and the fluid lipid layer. Recently, specific β-BuTX sites have been visualised on synaptosomes and in the central nervous system by autoradiography in the light and electron microscope (I.Othman and J.O. Dolly, unpublished observations).

The nature of the protein which might be involved in β-BuTX binding remains a matter for speculation. Na^+, K^+ and Ca^{2+} channels are all candidates; however, as previously discussed (3.4.2) there is strong evidence that the depolarisation of nerve terminals by β-BuTX is not mediated by Na^+ or K^+ channels (Halliwell et al., 1982). Likewise they seem unlikely to have a role as toxin binding sites; they are not confined to nerve terminals and TsTX failed to antagonise the binding of [^{3}H] β-BuTX to synaptosomes (Othman et al., 1982). No evidence is available as to whether β-BuTX binds to Ca^{2+} channels; although it does cause a large increase in the flux of $^{45}Ca^{2+}$ into synaptosomes (Tse, C.K.V. and Dolly, J.O., unpublished observations), this is probably a consequence of the depolarisation which it produces. The rate at which β-BuTX produces a blockade of neurotransmission at the neuromuscular junction shows a strong dependence on the frequency of stimulation of the nerve

(1.3.3.4.ii). In view of this it was suggested that the toxin might interact with sites exposed by the process of transmitter release (Simpson, 1976). The failure of α-latrotoxin to affect the binding of [^{3}H]β-BuTX to intact synaptosomes (Othman et al., 1982) suggests that this is not the case, at least not in the central nervous system.

Toxin I from the venom of Dendroaspis polylepis (Strydom, 1976) was the only one of the presynaptic neurotoxins tested to potently antagonise [^{3}H] β-BuTX binding to synaptosomes (Othman et al., 1982). Its K_i was 0.07 nM, as compared with 0.29 nM for native β-BuTX, indicating that the affinity of toxin I is four-fold greater than the latter for their common binding sites. Toxin I increases the rate of ACh release at the neuromuscular junction and a very similar toxin, Dentrotoxin, from the venom of Dendroaspis angusticeps antagonises the neuromuscular blocking action of β-BuTX (Harvey, 1982). Toxin I has a low toxicity following peripheral injection (Strydom, 1976) but reduces the lethality of β-BuTX when the two toxins are injected together (Othman et al., 1982). This antagonism may be due as much to the opposing effects of these toxins as to their common binding sites.

Toxin I is very potent following its intraventricular injection into rat brain (Othman et al., 1982). The discovery of Toxin I is a considerable step forward in the study of the action of β-BuTX on nerve terminals since it is, as yet, the only antagonist of saturable β-BuTX binding. Since it affects the release of neurotransmitters it provides further circumstantial evidence that β-BuTX binds to a component of the nerve terminal membrane which is involved in the process of transmitter release mechanism.

6.3 Possible Mechanisms of Synaptic Blockade by β-bungarotoxin

There has emerged, throughout this study, a strong argument that the specificity of action of β-BuTX on nerve terminals is by virtue of its binding to a membrane component and that this binding is independent of its phospholipase activity. Subsequent to this initial binding the enzyme activity is required for the full potency of the toxin, at both the vertebrate neuromuscular junction and in the central nervous system. It is believed that inhibition of ACh release during phase I of the action of β-BuTX at the neuromuscular junction is associated with the binding of the toxin to nerve terminals (Caratsch et al., 1981). Except under very special conditions of low Ca^{2+} concentration and low temperature (Caratsch et al., 1981) this inhibition is both incomplete and transient. In the absence of the phospholipase activity the subsequent two phases of the action of β-BuTX are not observed; eventual neuromuscular blockade is completely dependent on the enzyme activity as is the lethality of the toxin when injected by a peripheral route (Chapter 4). Thus, at the neuromuscular junction the phospholipase-independent and phospholipase-dependent effects do not appear to operate separately, rather, one follows as a consequence of the other.

In the central nervous system the situation appears to be more complicated. When the enzymatic activity of β-BuTX is destroyed by chemical modification with p-bromophenacyl bromide or [2,3-^{3}H] N-succinimidyl propionate the toxin can still cause complex blockade of neurotransmission in slices of rat olfactory cortex (Chapters 4 and 5), although in the former case its rate of action was reduced 2-3 fold (Table 6.1). Both derivatives killed rats following intraventricular injection although much higher doses were required than for native toxin; the lethalities of [^{3}H] β-BuTX and BPB-β-BuTX are respectively 5- and 240-fold less than native toxin (Fig. 6.1). Furthermore, when the enzyme activity was largely, but not completely, inhibited by replacing Ca^{2+} with Sr^{2+}, β-BuTX still blocked neurotransmission in the olfactory

cortex at a rate 2-3 fold less than when Ca^{2+} was present and caused substantial release of neurotransmitters from synaptosomes (Chapter 4). In the central nervous system 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 at least, that the sites are part of complexes which perform similar functions in the two systems but which differ somewhat in composition. This phospholipase-independent blockade is greatly enhanced by the presence of the enzyme activity which causes perturbation of the plasma membrane, presumably in the vicinity of toxin binding sites. That such perturbation occurs is made evident by the great increase in the leakage from synaptosomes of lactate dehydrogenase caused by β-BuTX and the fact that this is dependent on the phospholipase activity of the toxin (4.3.3). Prolonged treatment with high toxin concentrations leads to disintegration of the nerve terminal and eventual denervation (Strong et al., 1977).

Initially the depolarisation of nerve terminals arising from such membrane disruption would probably lead to an increase in transmitter release as seen in phase II of β-BuTX action at the neuromuscular junction and as observed using synaptosomes loaded with radiolabelled transmitter. Continued breakdown of the membranes would, however, lead to an inhibition of transmitter release in a number of ways. The rate of quantal transmitter release has been demonstrated to depend heavily on the intraterminal Ca^{2+} concentration (1.1.1.1). Disruption of the plasma membrane would inevitably lead to Ca^{2+} loss from the nerve terminal and hence an inhibition of transmitter release. Abolition of ion gradients, particularly that for Na^{+} would inhibit the active transport of metabolites such as glucose as well as amino acids and choline (1.1.3). Thus, not only transmitter but also energy stores would eventually be depleted.

In addition, it has been shown that oxidative phosphorylation by rat brain mitochondria is inhibited by factors, probably free fatty acids, released by the action of β-BuTX on synaptosomal membranes (Howard 1975). The action of the phospholipase would therefore lead to a decreased rate of ATP synthesis and also to an increase in the demand for ATP in the nerve terminals due to the increased phospholipid turnover and increased rate of energy-dependent ion translocation to re-establish ion gradients. Supplies of ATP might therefore be expected to run down quite rapidly before any major ultrastructural damage occurred. Such a reduction in ATP levels in β-BuTX-treated synaptosomes has been measured (Ng and Howard, 1978). Release of lysolecithin from the hydrolysis of phospholipids would accelerate the breakdown of internal membranes as well as the plasma membrane.

6.4 [2,3 ^{3}H] Propionyl-β-bungarotoxin: Usefulness as a Probe

Although its mode of action is complex, a problem greatly exacerbated by the presence of its phospholipase activity, it is clear that β-BuTX interacts specifically and with high affinity with a component of nerve terminal membranes (6.1, 6.2) which may well be involved in the process of neurotransmitter release. The [^{3}H] β-BuTX derivative produced by the methods developed in this study has a number of advantages for use as a probe for this nerve terminal component. It is labelled to high specific radioactivity and can, therefore, be measured at very low levels in any sample by liquid scintillation counting. Its position in gels used for electrophoretic separation, such as polyacrylamide, and in tissue slices can be detected by autoradiographic and fluorographic techniques. Although these latter techniques require considerably longer periods of time for tritium labelled probes than for those using isotopes which are γ-emitters, such as ^{125}I, they do not undergo inactivation by self-irradiation.

The stability of di-[^{3}H] propionylated β-BuTX (it can be stored at -20°C for several months) and the relatively long half-life of the isotope constitute an important practical advantage of this derivative, making repeated preparations unnecessary. Di-[^{3}H] propionylated β-BuTX has been shown to bind saturably and with high affinity to purified nerve terminals (Othman et al., 1982). The specificity of this binding is demonstrated by its inhibition with Toxin I and by its localised binding in the central nervous system. As previously discussed (6.2.2) this antagonism also provides a further indication that β-BuTX binds to a component of the neurotransmitter release mechanism.

6.5 Suggestions for Further Studies

Investigations to date suggest that the β-BuTX receptor is a membrane-bound protein. Further biochemical studies require that the receptor be available in solution and this can be achieved by extraction of synaptic membranes with suitable detergents. Unfortunately, the dissociation rate of the β-BuTX-receptor complex is probably too fast to allow it to be extracted intact. It would therefore be necessary to covalently crosslink the receptor-toxin complex in situ. Suitable conditions for the extraction of the crosslinked complex could then be investigated. Once solubilised the molecular weight of the oligomer could be determined by gel filtration or SDS gel electrophoresis.

Monoclonal antibodies against β-BuTX could prove exceptionally useful in this study. Such antibodies could provide highly specific antagonists of [^{3}H] β-BuTX binding to its receptor. Alternatively, an antibody might be isolated which bound the toxin molecule but did not inhibit the binding of the latter to its receptor. This might be used as the basis of an assay for solubilised receptor-toxin complex by means of immuno-precipitation. If the isoelectric points of

β-BuTX and the toxin-receptor complex are sufficiently different an assay similar to that used for the nicotinic acetylcholine receptor (Dolly 1979) could be developed. In this procedure the receptor is assayed by incubating it with a large excess of radiolabelled α-βUTX. The mixture is applied to ion exchange discs which are then washed, the pH being such that [^{3}H]toxin-receptor complex remains bound to the discs whilst the toxin is washed off. Liquid scintillation counting is then used to determine the amount of receptor bound to the filters. Such an assay, if the washing were sufficiently fast and efficient could be used to assay the levels of β-BuTX receptor in solution and, for example, to compare the amounts in detergent extracts of different tissues.

Such an assay would be necessary for purification of the receptor. A major step in this would be affinity chromatography using either toxin linked to the matrix of a column but able to bind to the receptor or a column to which is attached monoclonal antibody against the toxin and able to bind toxin-receptor complex.

The discovery of toxin I, an antagonist for the action and binding of β-BuTX, should greatly facilitate assay, purification and characterisation of the β-BuTX binding sites, in the ways mentioned above, by providing an independent agent for blocking β-BuTX binding. Indeed, radiolabelled toxin I could be used in a fashion complementary to radiolabelled β-BuTX and may make a better probe due to the higher affinity of its binding. It would be interesting, for instance, to see whether both toxins bind to the same solubilised membrane component and, if so, whether they bind to the same parts of the molecule. Immunochemical studies aimed at localising β-BuTX binding sites using antibodies raised against this toxin have not, so far, proved very successful. These small toxin molecules presumably have relatively few antigenic determinants and it is possible that those of β-BuTX are not accessible to the antibodies when the toxin is bound to its receptor site. Alternatively, binding may produce a conformational change in the

molecule such that it is no longer recognised by the antibodies. It may be that antibodies raised against toxin I can be more successfully employed in such studies. If toxin I and β-BuTX do indeed bind to the same component of the nerve terminal membrane then more detailed studies of the mode of action of toxin I should provide evidence as to the nature of this common site.

Finally, the development of techniques for producing monoclonal antibodies means that antibodies specific to a particular molecule can be produced from preparations of that molecule which are not homogeneous. Thus, partial purification of solubilised β-BuTX receptor might enable production of antibodies directed specifically against the receptor molecule. Such antibodies would be of great value in localising the β-BuTX binding site. By inhibiting the functioning of the β-BuTX receptor in the in vitro preparations described herein they would facilitate the investigation of its role. It may therefore be necessary to go a considerable way towards purifying the component of nerve terminals with which β-BuTX interacts before determining whether, as suspected, it is closely connected with the process of neurotransmitter release.

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