

IDENTIFICATION OF FUNCTIONAL
NEURONAL ACCEPTORS FOR
DENDROTOXIN

by

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To my family:

my parents BILLY and JOAN

and my sister LORNA,

for their lifelong friendship and support.

ABSTRACT

Dendrotoxin, a 7,000 M_r protease inhibitor homologue from the venom of Dendroaspis angusticeps, belongs to a group of excitatory neurotoxins which potentiate transmitter release at both central and peripheral synapses through blockade of voltage sensitive K^+ currents. It also has considerable sequence homology with the lighter B chain of β -bungarotoxin, a presynaptic inhibitory neurotoxin. Dendrotoxin antagonises biological actions of β -bungarotoxin as well as its binding to rat cerebrocortical synaptosomes ($K_I = 0.5nM$).

Radioiodination of dendrotoxin to high specific radioactivity ($1.1-1.5 \times 10^{16}$ Bq/mol) was achieved by an adaption of the chloramine-T method. The resultant derivative had the same biological activity as the native toxin and, consistent with the potent convulsive action, bound saturably to a set of kinetically homogeneous proteinaceous acceptor sites in rat cerebrocortical synaptic membranes ($K_D = 0.3nM$, $B_{max} = 1.5pmol/mg$ protein). As β -bungarotoxin only inhibited this binding with low affinity ($K_I \sim \mu M$), the interaction of these two toxins was unclear. This was elucidated by the use of chick synaptic membranes. Here, two classes of dendrotoxin acceptors were apparent from kinetic measurements ($K_D \sim 0.5$ and $15nM$, $B_{max} \sim 90$ and $400fmol/mg$ protein, respectively), the less abundant of which displayed high affinity for β -bungarotoxin ($K_I = 0.8nM$).

Cross-linking studies, using bis-imidoesters, revealed two polypeptide components ($M_r = 75,000$ and $69,000$) of the dendrotoxin acceptors from chick brain. β -bungarotoxin could only partially block the cross-linking to both of these; showing that the identified polypeptides are subunits of the two classes of acceptors. Likewise, studies with rat synaptic membranes also revealed two acceptor components of similar size to those from chick brain. Thus, both acceptor types from these two species appear to exhibit some structural similarities.

(iv)

An optimised procedure for detergent extraction of dendrotoxin acceptors from rat brain was developed. The binding properties and pharmacological specificity of the solubilised acceptors ($K_D = 0.5\text{nM}$; $B_{\text{max}} = 1.6\text{pmol/mg protein}$) were essentially unchanged from those seen in the membrane. A small fraction of ^{125}I -dendrotoxin binding could be inhibited by near equimolar concentrations of β -bungarotoxin, showing that the two classes of dendrotoxin acceptors exist in rat as well as in chick brain. The molecular weights of the solubilised acceptors ($M_r = 390,000\text{--}450,000$) were significantly larger than their size in the membrane-bound state ($M_r = 250,000$) derived from radiation inactivation data, suggesting that the DTX binding components are present in larger complexes.

On the basis of the good correlation of the affinities of dendrotoxin homologues with their central toxicities and abilities to inhibit K^+ conductances, it was concluded that the acceptors identified herein are directly, or indirectly, involved with voltage-sensitive K^+ channels.

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ABBREVIATIONS

A ₂₈₀	Absorbance at 280nm.
BPTI	Bovine pancreatic trypsin inhibitor.
β-BuTX	β-Bungarotoxin.
DMS	Dimethyl suberimidate
DTX	Dendrotoxin: Peak 9 from ion-exchange chromatography of <u>Dendroaspis angusticeps</u> venom Peak III.
D.V.14	Facilitatory neurotoxin from the venom of <u>Dendroaspis viridis</u> .
EDTA	Ethylenediaminetetra-acetic acid.
³ H-DTX	³ H-Propionylated DTX.
Hepes	N-2-Hydroxyethylpiperazine-N ¹ -2-ethanesulfonic acid.
HPLC	High performance liquid chromatography.
IC ₅₀	A concentration which results in 50% inhibition.
I-DTX	DTX preparation labelled to low specific activity with an isotopic mixture of ¹²⁵ I and ¹²⁷ I.
¹²⁵ I-DTX	Preparation of high specific radioactivity ¹²⁵ I-radioiodinated DTX.
MLD	Minimum lethal dose.
M _r	Relative molecular weight(s).
PAGE	Polyacrylamide gel electrophoresis.

PLA ₂	Phospholipase A ₂ .
PMSF	Phenylmethylsulphonyl fluoride.
SANPAH	N-Succinimidyl-6(4'-azido-2'-nitrophenyl-amino)-hexanoate.
SDS	Sodium dodecyl sulphate.
STI	Soy bean trypsin inhibitor.
TEMED	N,N,N ¹ ,N ¹ tetramethylethylene diamine.
TEA-HCl	Triethanolamine-HCl buffer.
Tris	Tris(hydroxymethyl) aminomethane.
v/v	Volume/volume.
w/v	Weight/volume.
w/w	Weight/weight.

CHAPTER 1

GENERAL INTRODUCTION

1.1 The Nervous System and the Mind.

When, in the 4th century B.C., Aristotle asked (in *De anima*) whether states of the soul were "all affections of the complex of body and soul", or whether any among them were "peculiar to the soul by itself", he reflected man's fundamental concern with the nature of his mind, its relationship with his body and the causes of mental disease. The Ancients associated at least some functions of the mind with the head (e.g. Plato in *Timaeus*) and by mediaeval times it was accepted that the mind resided in the brain, which communicated with the rest of the body through the nerves (e.g. Vesalius, 1543). At this stage, the mechanisms by which the nervous system functioned were unknown, being described in such terms as "animal spirits . . . distributed through the nerves as through little tubes". However, it is obvious that a detailed knowledge of these mechanisms is needed in order to understand the relationship of the nervous system with mental activity and disease.

1.2 Basic Components of Nervous Activity.

1.2.1 The neuron and the action potential.

By the end of the last century, work culminating in that of Ramon y Cajal had established the neuron as the fundamental, excitable unit of the nervous system (for an historical review, see van der Loos, 1967). Although the appearance of individual neurons differ, they conform to the same underlying structure: there is a central soma, or cell body, from which project dendrites and a single axon. In general, the dendrites and soma form the receptive field, collecting and integrating signals from other neurons. The axon is a specialised structure, which carries signals from the soma to the target of the neuron (this can be a muscle fibre, another neuron or the circulatory system). At its terminal, the axon forms a specialised structure with its target cell,

the synapse, (Sherrington, 1906) which is responsible for the transmission of signals from one cell to the other. (For a more detailed description of the various parts of the neuron, see Stevens, 1979 and Peters et al., 1976).

Concomitant with the identification of neurons, the nature of the signal which they carry was being elucidated. It was established that electrical currents were the basis of nervous impulses (Du Bois-Reymond, 1877) and the "membrane theory" was put forward to explain these (Bernstein, 1902). The nerve impulse, or action potential, is created by the differential distribution of ions across the neuronal plasma membrane, coupled with its selective and variable ion-permeability. Neurons are enriched in K^+ and Cl^- and depleted in Na^+ , Ca^{2+} and Mg^{2+} , relative to their external environment (Hodgkin, 1958). These ionic gradients, which form the immediate source of energy for nerve impulses, are maintained by a variety of mechanisms, e.g. the ubiquitous ATP driven Na^+-K^+ exchange pump (Skou, 1965), a Ca^{2+}/Na^+ exchange (Baker et al., 1969) and an ATP driven Ca^{2+} pump (Gill et al., 1981). At rest the neuronal membrane has a small but finite K^+ conductance (Hodgkin and Huxley, 1952). This, coupled with the uneven distribution of this ion, produces a resting membrane potential of $\sim -70mV$, which is close to the reversal potential for K^+ ($\sim -75mV$; Hodgkin, 1964). Depolarising the membrane beyond a threshold level ($\sim 55mV$) brings about a transient, voltage-dependent increase in Na^+ conductance (Hodgkin and Huxley, 1952). As the reversal potential for Na^+ is $\sim +55mV$ (Hodgkin, 1964), the associated entry of this ion brings the membrane potential up to $40-50mV$ (Curtis and Cole, 1942; Hodgkin and Huxley, 1939). Accompanying the rise in Na^+ conductance, there is a slower, voltage-dependent rise in K^+ permeability which, together with the transient nature of the Na^+ conductance, returns the membrane potential to its resting level. Movement of the action

potential down the axon is brought about by local currents flowing from positively charged membrane regions; these depolarise adjoining sections beyond the threshold level, initiating the above sequence of events therein.

As well as the two ion channels responsible for the above conductances, others are known to be involved with the action potential; for example, voltage-sensitive Ca^{2+} channels (Hagiware and Byerly, 1981) which are responsible for the entry of Ca^{2+} required for neurotransmitter release (Katz and Miledi, 1965; Llinas et al., 1981). Several other classes of K^+ channel, which can modulate neuronal excitability, have also been identified, e.g. Ca^{2+} -dependent K^+ channels, responsible for the after-hyperpolarization of the action potential (Meech, 1978); a transient voltage-sensitive K^+ channel, responsible for the A-current (Connor and Stephens, 1971; Gustaffson et al., 1982) and transmitter-sensitive K^+ channels such as the M and S currents modulated by muscarinic agonists and serotonin, respectively (Brown and Adams 1980; Klein et al., 1982).

As these channels regulate neuronal excitability, it is not surprising that their activities are under a variety of metabolic controls. For example, the degree of glycosylation of the voltage-sensitive Na^+ channel affects its rate of turnover (Waechter et al., 1983). Also, phosphorylation modulates the activity of this channel (Costa and Catterall, 1984), as well as that of others [e.g. Ca^{2+} -dependent K^+ channels (de Peyer et al., 1982; Ewald et al., 1985)]. An illustration of the physiological importance of such modulation can be seen with respect to memory; alteration of the A-current, brought about by phosphorylation of membrane proteins, seems to be involved with conditioned learning (Alkon, 1984).

1.2.2 Neurotransmission.

Conduction of nerve impulses is obviously only one part of neuronal activity, the other being the communication of those impulses to other neurons or tissues. This occurs by two basic mechanisms; chemical and electrical transmission. On a limited number of synapses where the gap between the cells, the synaptic cleft, is very narrow [$\sim 2\text{nm}$ (Pappas, 1975)], transmission is electrical (Furshpan and Potter 1959; Llinas et al., 1974) and the impulse is passed from one cell to the other by local currents, in essentially the same way as it is conducted down the axon. At chemical synapses, the arrival of the action potential causes the entry of Ca^{2+} into the neuron (through voltage-sensitive Ca^{2+} channels) which initiates the release of neurotransmitters in discrete packets or quanta (Katz and Miledi, 1965; del Castillo and Katz, 1954). The method of quantal release is uncertain and two explanations have been put forward. One proposes that transmitters are released directly from synaptic vesicles following their fusion with the plasma membrane, the other explains quantal release by the presence of channels which open for a fixed period of time. For reviews of the evidence for these two hypotheses and possible mechanisms underlying them, see Tauc (1979) and Ceccarelli and Hurlbut (1980).

Following their release, neurotransmitters diffuse across the synaptic cleft to the post synaptic membrane, where, by binding to specific receptors, they produce their effects; these can be either excitatory or inhibitory, depending on the particular transmitter and tissue involved. (Details of many of these can be found in Kuffler et al., 1984 and Bradford, 1985.) Specific mechanisms then inactivate the neurotransmitters either by enzyme degradation [e.g. acetylcholine esterase (Nachmansohn, 1954)] or uptake into neurons and glial cells (e.g. Iversen, 1971), thus limiting the signal passed from one cell to the other.

1.3 Dendrotoxin as a Probe for Components Involved in Neuronal Excitability.

1.3.1. The general strategy.

The preceding, albeit brief, description of neuronal communication demonstrates that it involves the complex interaction of many components. In order to understand the underlying mechanisms of this system, its components will have to be dissected and their precise molecular functions elucidated. As this requires the identification and purification of neuronal constituents involving the disruption of their physiological environment (e.g. solubilisation of membrane proteins), probes for these components are essential. Hence, it is fortunate that evolution has provided many natural toxins which interact with specific elements of neuronal activity. The usefulness of such neurotoxins can be demonstrated by reference to one example, α -neurotoxins, which block the nicotinic actions of acetylcholine (Lee 1972). Following their radiolabelling (e.g. Vogel et al., 1972; Dolly et al., 1981), α -neurotoxins have been invaluable in the identification, purification, characterisation (reviewed in Conti-Tronconi and Raftery, 1981) and, sequencing (e.g. Noda et al., 1983; Conti-Tronconi et al., 1985) of these receptors. Similarly, toxins which interact with the voltage-sensitive Na^+ channel have been used in its characterisation (Catterall, 1982) and have led to its cloning and sequencing (Noda et al., 1984). Likewise, other toxins are being used for similar studies, e.g. dihydropyridines, antagonists of voltage-sensitive Ca^{2+} channels (e.g. Venter et al., 1983) and apamin, which is thought to interact with one class of Ca^{2+} -dependent K^+ channel (Romey and Lazdunski, 1984; Schmid-Antomarchi et al., 1984).

In the above examples, the components which the toxin affects are known. However, this is not a prerequisite for their use. For example, although the mechanism by which botulinum neurotoxins inhibit

neurotransmitter release is unknown, their extreme potency ensures that present studies, which have already identified their neuronal acceptors (Williams et al., 1983) and shown that these mediate their internalisation (Black, 1984), will lead to valuable information concerning the processes underlying transmitter release.

1.3.2 Structure and actions of dendrotoxin and its homologues.

Dendrotoxin (DTX) is a 7 000 Mr, single chain polypeptide, containing 59 amino acid residues and 3 disulphide bridges, found in the venom of the Eastern green mamba (Dendroaspis angusticeps). (Harvey and Karlsson, 1980, 1982; Joubert and Taljaard, 1980). Despite its close homology to bovine pancreatic trypsin inhibitors (BPTI) (Table 1.1), DTX is not an antiprotease (Joubert and Taljaard, 1980) nor does it have any discernible enzyme activity (Harvey and Gage, 1981).

The first indication that DTX might prove to be a useful probe for neuronal components was the report that it was the major constituent responsible for the facilitatory action of Dendroaspis angusticeps venom at the neuromuscular junction (Harvey and Karlsson, 1980). DTX is a potent facilitator of neurotransmitter release, with concentrations as low as 70nM causing a two-fold augmentation in the response of chick biventer muscle to indirect stimulation (Harvey and Karlsson, 1980). This was due to an increase in the level of acetylcholine released (Harvey and Gage, 1981), rather than an effect on the sensitivity of the muscle (Harvey and Karlsson, 1980) or inactivation of transmitter (Rodriguez-Ithurralde et al., 1983).

Several congeners of DTX from Dendroaspis venoms, i.e. toxin I and K from Dendroaspis p. polylepis (Strydom, 1973) and a toxin designated D.V.14 from Dendroaspis viridis (R. Shipolini, personal communication), were also found to facilitate transmitter release (Harvey and Karlsson, 1982; A.L. Harvey, personal communication); this

Protein	Z	P	R	R	K	L	C	I	L	H	R	N	P	G	R	C	Y	D	K	I	P	A	F	Y	Y	N	Q	K	K	K	Q	C	E	R	F	D	W	S	G	C	G	G	N	S	N	R	F	K	T	I	E	E	C	R	R	T	C	I	G				
DTX	Z	P	L	R	K	L	C	I	L	H	R	N	P	G	R	C	Y	Q	K	I	P	A	F	Y	Y	N	Q	K	K	K	Q	C	E	G	F	T	W	S	G	C	G	G	N	S	N	R	F	K	T	I	E	E	C	R	R	T	C	I	R	K			
Toxin I	L	Q	H	R	T	F	C	K	L	P	A	E	P	G	P	C	K	A	S	I	P	A	F	Y	Y	N	W	A	A	K	K	C	Q	L	F	H	Y	G	G	C	K	G	N	A	N	R	F	S	T	I	E	K	C	R	H	A	C	V	G				
Toxin E			R	P	Y	A	C	E	L	I	V	A	A	G	P	C	M	F	F	I	S	A	F	Y	Y	S	K	G	A	N	K	C	Y	P	F	T	Y	S	G	C	R	G	N	A	N	R	F	K	T	I	E	E	C	R	R	T	C	V	V				
Toxin B			A	A	K	Y	C	K	L	P	L	R	I	G	P	C	K	R	K	I	P	S	F	Y	Y	K	W	K	A	K	Q	C	L	P	F	D	Y	S	G	C	G	G	N	A	N	R	F	K	T	I	E	E	C	R	R	T	C	V	G				
Toxin K			A	A	K	Y	C	K	L	P	V	R	Y	G	P	C	K	K	K	I	P	S	F	Y	Y	K	W	K	A	K	Q	C	L	Y	F	D	Y	S	G	C	G	G	N	A	N	R	F	K	T	I	E	E	C	R	R	T	C	V	G				
DV-14			R	Q	R	H	R	D	C	D	K	P	P	D	K	G	N	C	-	G	P	V	R	A	F	Y	Y	D	T	R	L	K	T	C	K	A	F	Q	Y	R	G	C	D	G	D	H	G	N	F	K	T	E	T	L	C	R	C	E	C	L	V	Y	P
β-BuTX			R	P	D	F	C	L	E	P	P	Y	T	G	P	C	K	A	R	I	I	R	Y	F	Y	N	A	K	A	G	L	C	Q	T	F	V	Y	G	G	C	R	A	K	R	N	N	F	K	S	A	E	D	C	M	R	T	C	G	G	A			
BPTI			R	P	D	F	C	L	E	P	P	Y	T	G	P	C	K	A	R	I	I	R	Y	F	Y	N	A	K	A	G	L	C	Q	T	F	V	Y	G	G	C	R	A	K	R	N	N	F	K	S	A	E	D	C	M	R	T	C	G	G	A			

$$\begin{array}{c} | \\ \infty \\ | \end{array}$$

puts DTX into a group of homologous excitatory neurotoxins (Table. 1.1). The actions of DTX and its homologues are not specific to a particular neurotransmitter as they facilitate the release of noradrenaline in vas deferens (Harvey et al., 1984a). Interestingly, the effect on vas deferens could be readily reversed by washing, whereas that at the neuromuscular junction could not, suggesting distinct modes of action on these two preparations.

In keeping with their peripheral facilitatory action, DTX and toxin I are potent convulsants in the central nervous system (Othman et al., 1982; Othman, 1983), where they increase both transmitter release and cell excitability (Docherty et al., 1983). Voltage-clamp studies in rodent hippocampus have shown that these toxins inhibit the A-current (Dolly et al., 1984; Halliwell et al., 1985), a transient voltage-sensitive K^+ conductance activated by membrane depolarisation, which is intimately involved in the control of cell excitability (Gustaffson et al., 1982). An effect of DTX on K^+ channels was also observed more recently at the frog node of Ranvier (Weller et al., 1985).

The potent and novel action of DTX makes it a promising probe for components involved with neuronal excitability. Its simple chemical structure ensures that studies will not be hindered by such complications as enzymic activity.

1.3.3. Interaction of DTX with β -bungarotoxin and other inhibitory presynaptic neurotoxins.

DTX shows considerable homology with the lighter B-chain of β -bungarotoxin (β -BuTX) (Kondo et al., 1982; Table 1.1). However, these two toxins differ in both structure and effect; β -BuTX contains 2 polypeptides, the heavier (A-chain) of which is thought to contain its

phospholipase A₂ (PLA₂) activity (Strong et al., 1976; Kondo et al., 1978). It belongs to a group of PLA₂ containing inhibitory pre-synaptic neurotoxins, which includes crotoxin, taipoxin and notexin (Lee and Ho, 1978). However, it is the only one of these to show no post-synaptic or myotoxic activity (Hanley, 1978; Harris et al., 1979). β -BuTX has a triphasic action at the neuromuscular junction (Abe et al., 1977). It initially causes a decrease, followed by an increase and finally a total blockade of neurotransmitter release; only the last and possibly the second of these is dependent on the enzymic action of the toxin (Howard and Gundersen, 1980). The other PLA₂ containing neurotoxins [except textilotoxin (Su et al., 1983)] also display a triphasic effect on transmitter release (Chang, 1985); thus, although some species differences are evident [e.g. the initial transient blockade by β -BuTX, but not notexin, is insignificant in rat diaphragm; (Chang et al., 1973; Su and Chang, 1981)], these toxins appear to act by very similar mechanisms. Despite this, their sites of action are obviously distinct, since β -BuTX can potentiate the action of crotoxin and taipoxin (Chang and Su, 1980).

β -BuTX also blocks transmitter release in the central nervous system, where it acts preferentially at the synapse but also effects cell excitability in hippocampal slices (Halliwell and Dolly, 1982). Consistent with this, components which bind β -BuTX with high affinity ($K_D = 0.5\text{nM}$, $B_{\text{max}} = 50\text{-}150\text{fmol/mg protein}$) have been identified in both rat (Othman et al., 1982) and chick (Rehm and Betz, 1982) brain membranes. These are large oligomeric proteins (Rehm and Betz 1983 and 1984) and, like their physiological equivalents, do not bind other presynaptic inhibitory neurotoxins (Othman et al., 1982; Rehm and Betz, 1982).

In light of the sequence homology and opposing pharmacological actions of β -BuTX and DTX, it is interesting that both DTX and toxin I are able to inhibit the peripheral toxicity of β -BuTX (Othman, 1983). This interaction has also been explored at the chick neuromuscular junction (Harvey and Karlsson, 1982), where pretreatment with DTX significantly increased the time for β -BuTX blockade of neurotransmitter release. Also, pretreatment of β -BuTX (in Sr^{2+} containing medium, to prevent its PLA_2 dependent action) blocked the facilitatory action of DTX. As might have been expected, such mutual antagonism was not seen with crotoxin, taipoxin or notexin which had no effect on the actions of DTX (in Sr^{2+} containing media). The ability of DTX to potentiate the actions of these toxins is probably mediated by its effect on cell excitability [increasing nerve stimulation has a similar effect (Chang et al., 1973; Cull-Candy et al., 1976)]. This suggests that the physiologically active sites for DTX are also sites for β -BuTX but not for other PLA_2 containing neurotoxins, at least on the chick motoneuron terminal.

Similar interactions of DTX and β -BuTX were seen in the central nervous system. Although DTX had little effect on rat cerebrocortical synaptosomes, it was able to block the depolarisation (Nicholls et al., 1985) and increase in neurotransmitter release (Karen Joseph, personal communication) caused by β -BuTX. Direct evidence for their interaction at common acceptor sites came from the reported ability of DTX and toxin I to block the binding of radiolabelled β -BuTX to rat cerebrocortical synaptosomes (Othman, 1983; Othman et al., 1982) with high affinity ($K_1 \sim 0.5\text{nM}$ and 0.06nM , respectively). This apparent antagonism between these toxins should prove of great value in the study of the neuronal component with which they are involved.

1.4 Conclusion

This introduction has shown that elucidation of the molecular mechanisms by which DTX facilitates neurotransmitter release, its involvement with a class of voltage-sensitive K^+ channels and its interactions with β -BuTX, will provide vital information about components of neuronal communication, which is essential for a better understanding of the relationship of the body with the mind and mental disease.

CHAPTER 2

RADIOLABELLING OF DTX AND IDENTIFICATION OF ITS NEURONAL ACCEPTORS

2.1 Introduction

2.1.1 Radiolabelling of DTX: a prerequisite for its use as a probe.

To exploit the potential of DTX as a probe for functional components involved with neuronal excitability, it was essential to produce a radiolabelled derivative that retained the original activity of the toxin. Radiolabelling, rather than other methods of visualisation (e.g. fluorescent labelling), was required, as it lends the greatest sensitivity to assay systems (many radioisotopes can be readily detected at sub-femtomolar levels) and gives derivatives which are suitable for the kinetic analyses described in Appendix A.2. Thus, radiolabelled toxins can be applied to the identification of specific neuronal components, both in the membrane and during their subsequent purification and characterisation (cf. α -neurotoxins). Two main isotopes have been used for such radiolabelling, namely tritium (^3H) and iodine-125 (^{125}I). Proteins can be labelled with ^3H by using alkylating agents, e.g. N-succiminidyl propionate (Dolly et al., 1981; Sugiyama and Yamashita, 1981), N-bromophenylacetyl bromide (MacDermot et al., 1978) or acetic anhydride (Barnard et al., 1971). Due to the low energy of the β -particles emitted by ^3H , together with its long half-life (12 years), tritiated proteins are generally stable. This, together with the relatively low cost of these reagents, enables large amounts of labelled toxin to be produced in a single batch, saving both time and money. However, the slow rate of decay of the isotope means that these derivatives have low specific activity (normally $<4 \times 10^{15}$ Bq/mol), so reducing their usefulness. Alkylation can also be used for iodination, e.g. using Bolton-Hunter reagent (Bolton and Hunter, 1973). However, unlike ^3H , iodine can be easily incorporated directly into proteins following its oxidation by e.g. lactoperoxidase (Sutter et al., 1979) or chloramine-T (Greenwood et al., 1963). ^{125}I emits higher energy

γ -particles, which tend to elicit autoradiolysis of labelled proteins. This, coupled to its short half-life (60 days), necessitates radioiodination at short intervals. This disadvantage is countered by the high specific activity of the isotope (8×10^{16} Bq/mol). All of these labelling methods incorporate groups which are small compared with the toxin and should have a negligible effect on its structure. However, if the site of labelling is in an area of the toxin responsible for its biological activity, the latter may be affected profoundly.

2.1.2 Methods used to radiolabel DTX.

The N-hydroxy succinimidyl ester N-succinimidyl (2, 3^3H) propionate (Sugiyama and Yamashita, 1981; Dolly et al., 1981) was used in an attempt to tritiate DTX. This was chosen for several reasons; (a) it has been used successfully to tritiate β -BuTX to 6×10^{15} Bq/mol (Othman et al., 1982) whereas other methods produced anomalous binding (MacDermot et al., 1978); (b) it alkylates proteins by the same reaction as Bolton-Hunter reagent [N-succinimidyl 3-(4-hydroxy phenyl) propionate (Bolton and Hunter 1973)]; thus, if labelling were successful, very similar methods could be used for both tritiation and iodination; (c) considerable experience had been gained by members of this laboratory in its use by labelling α -bungarotoxin and β -BuTX. With DTX, which has a blocked N-terminus (Harvey and Karlsson, 1980), an 3^3H -propionyl group must be incorporated on to the ϵ -amine of lysine, as the reagent reacts with free amino groups (Anderson et al., 1964). The removal of a positively charged group changes the electrochemical properties of proteins, often enabling labelled and native proteins to be separated (e.g. Dolly et al., 1981; Othman et al., 1982).

A mild chloramine-T method was chosen for iodination, as it was again regularly used in this laboratory to radiolabel botulinum neurotoxins (Williams et al., 1983). The reaction relies on the slow

release of hypochlorous acid from chloramine-T in aqueous solution (Hunter, 1978). This oxidises molecular iodine to I^+ which attacks tyrosines, labelling them in the meta positions. Below pH 7.5 tyrosine is usually the only group to be iodinated, although reversible substitution into histidine, tryptophan and cysteine can also occur (Hughes, 1957). Iodination increases the acidity of the phenolic hydroxyl of tyrosine, again often enabling separation of the labelled and non-labelled species (e.g. Vogel et al., 1972).

2.1.3 The choice of tissue for initial studies. As both toxin I (Othman et al., 1982) and DTX (see later) are potent central convulsants in rodents, where they increase synaptic transmission and cell excitability (Docherty et al., 1983; Dolly et al., 1984), rat brain was an obvious source of physiologically relevant acceptors for DTX, particularly in view of the ease with which neuronal preparations can be made from this tissue (Jones and Matus, 1974; de Belleruche and Bradford, 1977). Cerebrocortical synaptosomes were used in initial studies for two main reasons; (a) to enable comparison with the binding of ^3H - β -BuTX which had been studied in this preparation (Othman et al., 1982) and (b) perhaps more importantly, inhibition of ^3H - β -BuTX binding by toxin I and DTX (Othman et al., 1982; Othman, 1983) had revealed high affinity acceptors for these latter toxins in this tissue.

2.2 Methods

2.2.1 Purification of DTX.

This was performed by an adaption of the method described by Harvey and Karlsson (1980).

(a) Gel filtration of whole venom from Dendroaspis angusticeps. A solution of venom (1.5g) in 10ml of 0.1M ammonium

acetate, pH 4.7 was centrifuged at 1000 x g for 10 minutes to remove insoluble material. The supernatant was gel filtered at 4°C on a Sephadex G-50 superfine column (2.5 x 150cm), equilibrated with 0.1M ammonium acetate, pH 4.7, at a flow rate of 24ml/h. The protein content of the fractions collected (4ml) was determined by their absorbance at 280nm (A_{280}). Fractions comprising Peak III (Figure 2.1) were pooled and combined with the corresponding peak from an identical gel filtration of a further 1.5g of venom.

(b) Ion-exchange chromatography of Dendroaspis angusticeps venom Peak III. Peak III was lyophilised, resuspended in 15ml of deionised water and the pH of this solution (pH 5.5) was adjusted to pH 7.3 using 10mM ammonium acetate, pH 8.8. The molarity (as determined by its conductivity) was adjusted to 10mM with deionised water. The above solution (320ml) was then applied to a Bio-Rex 70 column (2 x 43cm) equilibrated with 0.1M ammonium acetate, pH 7.3. After washing with 130ml of the equilibration buffer, elution of the bound protein was carried out with a 2-step gradient, consisting of 800ml of 0.1-0.35M and 1200ml of 0.35-1.4M ammonium acetate, pH 7.3, with an 80ml wash using 0.35M buffer between the two steps. The flow rate of the column was 35ml/h; 4ml fractions were collected and their protein content (A_{280}) was assessed. Protein Peak 9 (Figure 2.2) was pooled, desalted on a Sephadex G-10 column (2.3 x 83cm, equilibrated with 10mM ammonium acetate, pH 7.3) and lyophilised repeatedly to remove the volatile salt. Samples were then stored at -20°C with desiccant until required.

2.2.2 High performance liquid chromatography of Peak 9 from ion-exchange chromatography.

Lyophilised Peak 9, dissolved in 0.1% trifluoroacetic acid, was subjected to high performance liquid chromatography (HPLC) on a molecular sizing column [two Waters I-125 (7.8 x 300mm) in series], equilibrated in the same buffer, at a flow rate of 0.8ml/min. Elution of protein was monitored by its A_{280} and peaks were collected manually.

2.2.3 Polyacrylamide gel electrophoresis (PAGE).

(a) Sodium dodecyl sulphate-PAGE (SDS-PAGE). This was carried out on 10% rod gels by the method of Weber and Osborn (1969). Samples run under reducing conditions were incubated for 10 minutes at 95°C in the presence of 5% β -mercaptoethanol. Samples (50-100 μ l) were loaded and the gels (10%) were run at 8mA per gel until the bromophenol blue marker approached the bottom of the gels. They were stained with Coomassie Brilliant Blue R-250 (0.5% w/v) in 50% (v/v) methanol and 10% (v/v) acetic acid, followed by destaining in 10% (v/v) methanol and 10% (v/v) acetic acid. Gels containing radioactive samples were sliced (2mm sections) and the distribution of radioactivity was determined by scintillation counting of the slices at 40% efficiency in a Soluene-based toluene scintillant.

(b) SDS-gradient-PAGE. This was an adaption of the method of Laemmli (1970). Stock solutions of 15% and 22% (w/v) acrylamide were prepared, containing 6.6% and 15% glycerol respectively in 0.375M Tris-HCl, pH 8.8., 0.1% (w/v) SDS, 0.008% (v/v) N, N, N', N' tetramethylethylenediamine (TEMED). The percentage of bis-acrylamide in the stock solution was 2.5% of the total acrylamide. These solutions were degassed, chilled on ice and 17.3ml of each was poured into the two chambers of a linear

gradient maker. Following the addition of ammonium persulphate to a final concentration of 0.01% (w/v), the solutions were stirred whilst being rapidly pumped into a 2 x 150 x 175mm mould, generating a 15-22% linear gradient of acrylamide in the gel. A 3% acrylamide stacking gel (in 0.125M Tris-HCl, pH 6.8, 0.1% SDS) was cast on top of this. Protein (10µg) samples in 10µl of sample buffer (10mM Tris-HCl, pH 6.8, 1% SDS, 1mM EDTA, 10% glycerol, plus or minus 5% β-mercaptoethanol) were loaded and the gels were electrophoresed at 200V for a period of two hours after the tracking dye (bromophenol blue) had left the bottom of the gels. Subsequent to this, gels were fixed with 10% formaldehyde, stained as described in (a), then soaked in a 50% (v/v) methanol, 5% (v/v) glycerol solution before being dried between dialysis tubing under vacuum. They were then subjected to autoradiography by exposure to Fuji X-ray film at -80°C. After 2-4 days autoradiograms were developed using a Kodak industrial X-omat processor.

(c) Native-PAGE was performed by the method of Reisfeld et al., (1962), except that 2mm "slab" gels were used. Samples (30-50µl) were applied to the gels and electrophoresed at 25mA until the methylene green marker approached the bottom of the gels. Fixation was in 15% trichloroacetic acid and the staining procedure used is described in (a). Gels containing ³H-DTX were sliced and subjected to scintillation counting to determine the distribution of radioactivity. Gels containing ¹²⁵I-DTX were soaked in 30% methanol, dried and subjected to autoradiography as described in (b).

2.2.4 Assessment of the biological activity of DTX preparations.

(a) Central toxicity. This was determined by injection of toxin samples into the third ventricle of rats (two animals being

used for each dose). The animals (~200g) were anaesthetized with Flouthane (1-2% and O_2) before being placed in a David Kopf small animal stereotaxic instrument. A hypodermic needle was inserted through a pre-drilled hole in the skull to the co-ordinates L-1.7, S-(-0.2) D-0.4mm (Koenig and Klippel, 1963) and 10 or 20 μ l samples were injected. After a pause to allow for pressure equilibration, the needle was removed and the bore hole was plugged with bone wax. The animals were then allowed to recover from anaesthetic, observed for 48h and their body weights were determined.

(b) Potentiation of synaptic transmission in guinea pig ileum preparations. Sections of ileum were removed from guinea pigs and submerged in Krebs-ringer solution which was aerated with 95% O_2 /5% CO_2 . One end of the tissue was immobilised; the other was tied to an isotonic transducer and tension was maintained at 1.5g. Field stimulation (10V) was applied for 0.2s every 20s and the induced contractions were recorded. After these had stabilised, toxins were added to a final concentration of 190nM for DTX and 10nM for atropine.

(c) Other criteria of activity. The DTX preparation was also tested for its effects on neuromuscular transmission (Harvey and Karlsson, 1980) and for its activity in rodent hippocampal slices (Dolly et al., 1984); these tests were kindly performed by Drs. A.L. Harvey and J.V. Halliwell, respectively. As a final check, its ability to inhibit the binding of ^3H - β -BuTX was assessed (as described in Othman et al., 1982).

2.2.5 ^3H -Propionylation of DTX.

This was carried out by an adaption of the method used by Dolly et al. (1981) to radiolabel α -bungarotoxin. N-succinimidy1

(2, 3³H)-propionate (with a specific radioactivity of 1.5×10^{15} Bq/mol) was mixed with unlabelled N-succinimidyl propionate to give a reagent with a specific radioactivity of 7.8×10^{13} Bq/mol. The solvent was then removed from 50nmol of this by evaporation under a stream of N₂ prior to the addition of DTX (175μl; 25nmol in 20mM sodium phosphate, pH 7.5). Following rapid mixing, the reaction was allowed to proceed, with gentle stirring, for 2h at 23°C. The reaction mixture was gel filtered on a Sephadex G-10 column (1 x 25cm) in 25mM ammonium acetate, pH 7.5. Fractions (0.3ml) were collected and their radioactive content was determined by scintillation counting; the peak corresponding to the tritiated toxin (³H-DTX) was pooled. The amount of protein in this pool, determined by the Folin-Lowry method (Lowry et al., 1951) with DTX as a standard, was used to calculate its specific radioactivity.

2.2.6 Iodination of DTX to high specific radioactivity.

The chloramine-T method (Greenwood et al., 1963) for iodinating DTX was derived from the conditions optimised for labelling botulinum neurotoxin (Williams et al., 1983). Na ¹²⁵I ($\sim 7.4 \times 10^{10}$ Bq; 20μl; 8×10^{16} Bq/mol) was added to DTX (20μg; 2.9nmol) in 10μl of 100mM sodium phosphate, pH 7.4, containing 0.02% Triton X-100. The reaction was started by addition of 5μl of chloramine-T (freshly dissolved in the above buffer) to a final concentration of 0.21mM (60μg/ml). After vortexing for 30s, the reaction was stopped by dilution to 1ml with 20mM sodium phosphate, pH 7.4. Duplicate aliquots were removed for determination of the specific radioactivity by acid precipitation (as detailed below). The remainder was applied to a CM-Sepharose C1-6B ion-exchange column (2ml) equilibrated with 20mM sodium phosphate, pH 7.4. The column was washed with 2ml of the above buffer, followed by

elution of the bound protein with a 10ml linear NaCl gradient (0-1M) in the equilibration buffer; 0.5ml fractions were collected and their radioactivity was determined by γ -counting. Those containing ^{125}I -DTX were stored at 4°C and diluted prior to use. The specific activity of the iodinated DTX preparation (^{125}I -DTX) was determined by precipitation of protein in the aliquots removed from the reaction mixture. These were added to 1% bovine serum albumin and trichloroacetic acid [to 10% (w/v)] was added to precipitate the protein. After 20 min on ice, the mixture was centrifuged to sediment precipitated protein. The pellet was then washed with 1ml of 10% (w/v) trichloroacetic acid and its radioactive content was determined by γ -counting. As the amount of protein in the aliquots was known, the specific radioactivity could be calculated. In order to assess the validity of this procedure, the specific radioactivity of ^{125}I -DTX obtained after chromatography was also assessed by the Folin-Lowry method as used for ^3H -DTX (Section 2.2.5).

2.2.7 Production of low specific radioactivity iodinated DTX with an isotopic mixture of ^{125}I and ^{127}I .

Trace amounts of Na ^{125}I were added to Na ^{127}I (0.26mM) in 100mM sodium phosphate, pH 7.4, containing 150mM NaCl, to give a final specific activity of 7×10^{13} Bq/mol. An aliquot (0.6ml; 160nmol) of this was added to 300 μg of DTX (43nmol in 60 μl) in the same buffer, followed by addition of 10 μl of a fresh solution of chloramine-T to a final concentration of 0.42mM (120 $\mu\text{g}/\text{ml}$). The reaction was allowed to continue, with mixing, for 1 min, before being quenched by addition of tyrosine (0.9mM). After a further 2 min, the incubation was gel-filtered on a Sephadex G-10 column (1 x 25cm equilibrated with 100mM sodium phosphate, pH 7.4, containing 150mM NaCl) in order to separate the DTX preparation labelled with low specific radioactivity iodine

(I-DTX) from other radioactive species and tyrosine. The radioactive content of collected fractions (0.5ml), was determined by γ -counting and the first fraction containing I-DTX was used to determine both its specific radioactivity (by the Folin-Lowry method) and its biological activity.

2.2.8 Preparation of rat cerebrocortical synaptosomes.

Whole cerebral cortices were removed in less than 30s from adult Sprague-Dawley rats (150-200g), previously stunned and killed by cervical dislocation. The tissue was frozen in liquid N_2 and stored at $-80^\circ C$ or placed into ice-cold 0.32M sucrose. Synaptosomes were prepared from these cortices by an adaption of the method of Gray and Whittaker (1962), as described by de Belleruche and Bradford (1977). All manipulations were carried out at $4^\circ C$. Cortices from four rats were homogenised in 25ml of 0.32M sucrose using a glass-perspex homogeniser with a clearance of 0.5mm (12 strokes at 900 rpm). The P_1 (crude nuclear fraction) was removed by centrifugation in a Beckman type 30 rotor at 4000 rpm ($1000 \times g$) for 10 min in a Beckman L-series centrifuge. The supernatant (S_1) was recentrifuged in a type 30 rotor at 15,000 rpm ($20,000 \times g$) for 20 minutes in order to pellet the crude mitochondrial fraction (P_2). This was resuspended in 15ml of 0.32M sucrose and carefully layered onto a discontinuous sucrose density gradient, consisting of 20ml of 0.8M sucrose on top of 20ml of 1.2M sucrose in a 60ml Beckman centrifuge tube. Following centrifugation in a Beckman SW 25.2 rotor at 24,000 rpm ($76,000 \times g$) for 1h, the synaptosomal fraction (P_2B) was collected from the interface of the 0.8M and 1.2M sucrose. The P_2B preparation was diluted to $\sim 0.45M$ by dropwise addition of ice-cold deionised water and the synaptosomes were sedimented in a type 30 rotor at 24,000 rpm ($54,000 \times g$) for 30 min. The pellet was resuspended in an appropriate volume of Krebs

medium (124mM NaCl, 5mM KCl, 20mM Na₂ HPO₄, 1.2mM KH₂ PO₄, 1.3mM MgSO₄, 0.75mM CaCl₂, 10mM D-glucose, pH 7.4). Synaptosomes were either used fresh or stored at -80°C until required.

As the data obtained was the same when using synaptosomes produced from fresh or frozen cortices and using synaptosomes which had or had not been frozen prior to use, for convenience frozen synaptosomes produced from frozen cortices more routinely used.

2.2.9 Characterisation of the binding of ¹²⁵I-DTX to rat cerebrocortical synaptosomes.

Binding was measured by a centrifugation washing technique as described by Othman et al. (1982). Synaptosomes (~100µg of protein) were incubated with ¹²⁵I-DTX in 250µl of Krebs phosphate medium. After 30 min at 23°C, 1ml of ice-cold Krebs phosphate medium was added and the membranes were rapidly pelleted in an Eppendorf microcentrifuge. The supernatant was removed and the membranes were washed with a further 1ml of ice-cold Krebs phosphate medium. Radioactivity in the pelleted membranes was then quantified by γ-counting at 50% efficiency. The level of non-displaceable binding was determined by inclusion of a 100-fold excess of native toxin (over ¹²⁵I-DTX) in the incubation medium. Competition experiments were carried out by labelling the membranes with 1.2nM ¹²⁵I-DTX in the presence of the stated levels of competing toxins. In order to determine the rate of dissociation of the acceptor/toxin complexes, synaptosomes were prelabelled with ¹²⁵I-DTX (1.2nM) for 30 min; at zero time DTX was added to 120nM and the level of toxin binding was determined at times thereafter. Binding at each competitor concentration or time point was determined in duplicate; on the rare occasions when the error in the duplicate values was >±7%, these were omitted from further calculations.

A slightly different procedure was used to determine the concentration dependence of toxin binding. Synaptosomes were incubated for 40 min with varying levels of ^{125}I -DTX, and they were pelleted without addition of ice-cold Krebs phosphate medium, the supernatant being removed for determination of the concentration of free ^{125}I -DTX. The membranes were washed subsequently with 2 x 1ml of Krebs phosphate medium before toxin binding was assessed.

The protein content of incubations was determined by the method of Bradford (1976) using bovine serum albumin as a standard and kinetic analyses were performed as detailed in Appendix A.2.

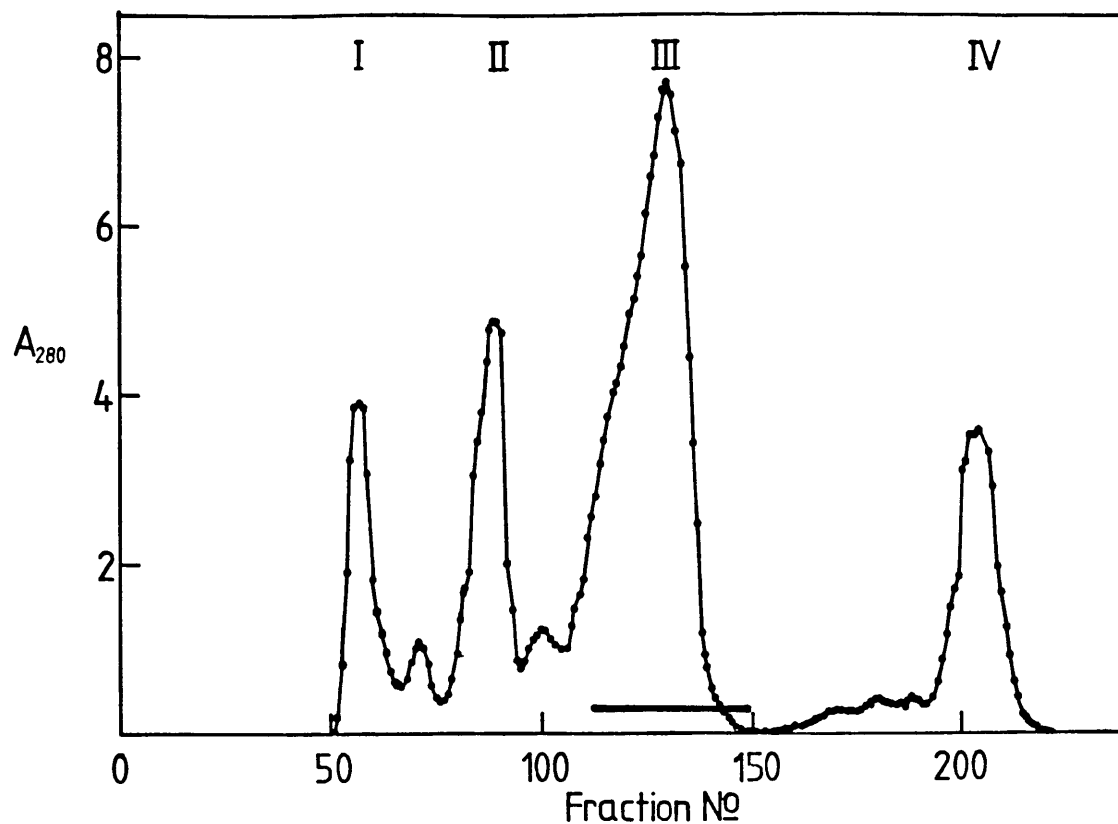
In order to investigate the proteinaceous nature of DTX acceptors, synaptosomes were subjected to the treatments detailed below, before their ability to bind ^{125}I -DTX (1.2nM) was determined. Heat treatment of synaptosomes was carried out at 95°C for 30 min. Control samples were incubated at 37°C for 15 min. Acidification of samples was in 1M HCl for 40 min at 37°C, followed by washing (2 x 1ml) with Krebs phosphate medium; HCl was omitted for controls. Proteolysis of synaptosomes (1-2mg in 150 μ l) was carried out by incubation at 37°C with 1mg/ml of trypsin or chymotrypsin. After 40 min, soy bean trypsin inhibitor (STI), to 5mg/ml (for trypsin), or phenylmethylsulphonyl fluoride (PMSF), to 5mM (for chymotrypsin), was added to stop the reaction. Control samples were prepared by addition of the appropriate inhibitor prior to the protease.

2.3 Results

2.3.1 Purification of DTX.

(a) Separation of DTX from other venom components. The eultion profile of Dendroaspis angusticeps venom from the Sephadex G-50 column (Figure 2.1) was essentially the same as that

Figure 2.1 Gel filtration of venom from *Dendroaspis angusticeps*.

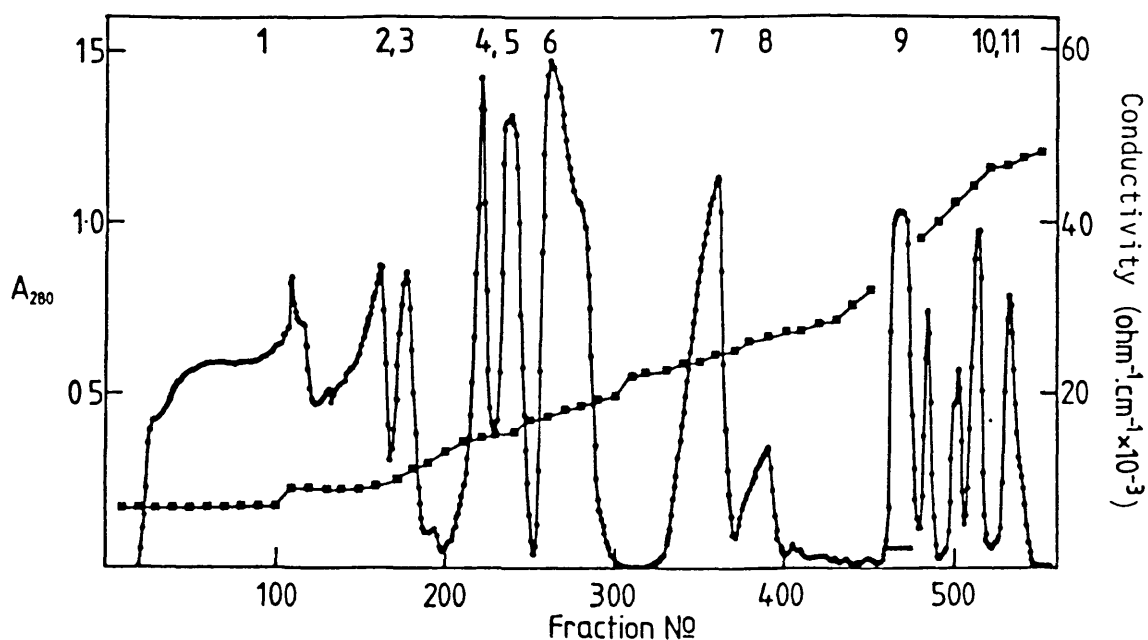


Venom (1.5g) in 10ml of 0.1M ammonium acetate buffer, pH 4.7, was loaded onto a Sephadex G-50 column (2.5 x 150cm), equilibrated with the same buffer, and was eluted at a flow rate of 24ml/h. Fractions (4ml) were collected and their absorbance at 280nm (A_{280}) was measured. Peaks are numbered according to Harvey and Karlsson (1980). The horizontal bar shows the pooled fractions of Peak III which were subsequently subjected to ion-exchange chromatography.

obtained by Harvey and Karlsson (1980), where Peak III contained DTX; this peak was pooled and subjected to ion-exchange chromatography. In order to improve resolution of the peaks close to DTX (Peak 9) on the Bio-Rex column (Harvey and Karlsson, 1980), a linear, rather than a concave, salt gradient was used. For this reason, and due to the larger volume loaded, the profile in Figure 2.2 differs from that of Harvey and Karlsson (1980). As hoped, a better resolution of protein Peaks 7-11 was achieved, with almost total separation of Peak 9 from its shoulder. Although the profile of the later peaks is readily comparable with that of Harvey and Karlsson (1980), the compression of the earlier peaks (1-6) makes their identification more difficult; hence, the assignments in this region are only tentative.

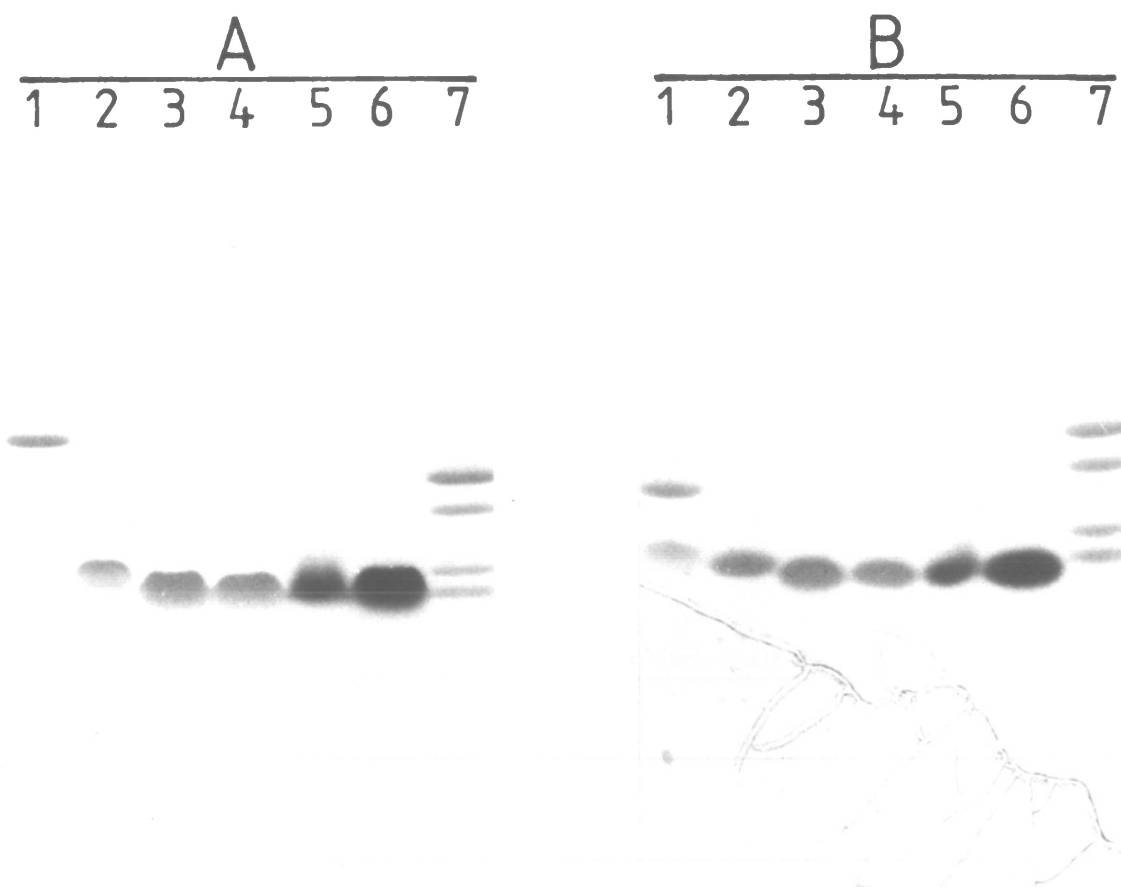
(b) Criteria of purity of Peak 9. Peak 9 appeared as a single band on SDS-gradient-PAGE (Figure 2.3) and native-PAGE (Figure 2.4), showing that it comprised only one major component which migrated slightly ahead of α -bungarotoxin ($M_r = 8000$) consistent with the size of DTX. Only when it was subjected to HPLC (Figure 2.5) was a minor amount of a larger species seen (<10%). This contaminant did not affect the results obtained. Peak 9 was indistinguishable from the major component obtained from HPLC (HPLC purified DTX) in binding studies (Black et al., 1985), toxicity studies (Mehraban et al., 1985), in its actions on the neuromuscular junction (A.L. Harvey, personal communication), hippocampal slices (J.V. Halliwell, personal communication) and guinea-pig ileum (see below). The purity of Peak 9 could not be assessed by isoelectric focussing as the protein migrated to the end of the gels; however, this showed that its pI value was >10.8, consistent with that of 10.5 previously reported for DTX (Othman, 1983).

Figure 2.2 Ion-exchange chromatography of *Dendroaspis angusticeps* venom
Peak III.



Peak III from gel filtration was lyophilised and resuspended to give a final volume of 320ml in 10mM ammonium acetate pH 7.4. This was loaded onto a Bio-Rex 70 column (2 x 43cm), washed with 0.1M ammonium acetate pH 7.4 and eluted with a two step gradient (0.1-0.35M and 0.35-1.4M ammonium acetate, pH 7.4) at a flow rate of 35ml/h. The absorbance at 280nm (A_{280}) and conductivity of the collected fractions (4ml) were measured and those corresponding to Peak 9 (Harvey and Karlsson, 1980) were pooled as indicated by the horizontal bar. Symbols represent conductivity (■) and A_{280} (•).

Figure 2.3 Analysis of DTX preparations and ^{125}I -DTX by SDS-gradient PAGE.



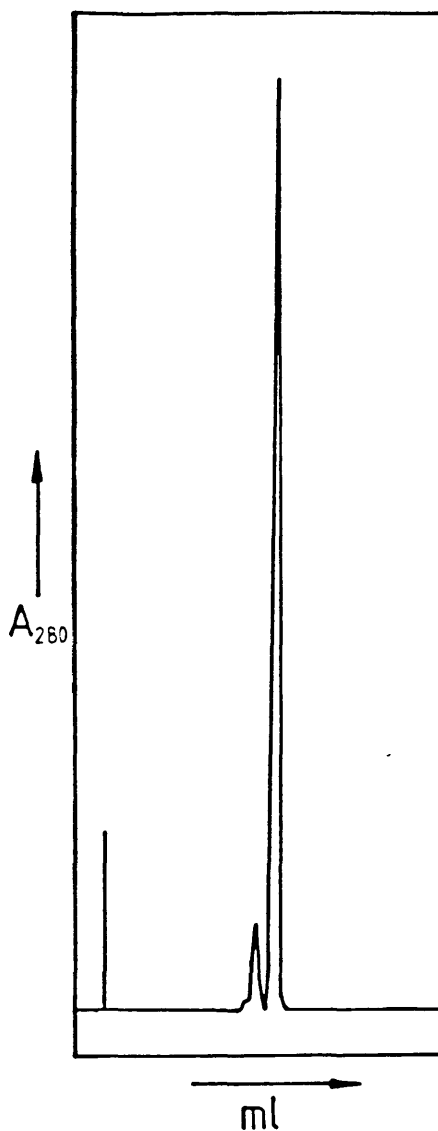
- (A) Electrophoresis under non-reducing conditions. Samples were subjected to electrophoresis in a 15-22% gradient polyacrylamide gel containing 0.1% SDS. The gel was stained with Coomassie Brilliant Blue R-250, dried and autoradiographed. Tracks contained: (1) β -BuTX; (2) α -bungarotoxin; (3) HPLC purified DTX; (4) Peak 9 from ion-exchange chromatography; (5) ^{125}I -DTX, visualised by autoradiography; (6) ^{125}I -DTX and Peak 9; (7) myoglobin and its CNBr cleavage products, which had M_r values of 17,200, 14,400, 8,200 and 6,400.
- (B) Electrophoresis under reducing conditions. As in (a) except that samples were reduced prior to electrophoresis by incubation for 15 min at 95°C with 5% β -mercaptoethanol, prior to electrophoresis.

Figure 2.4 Analysis of DTX preparations and ^{125}I -DTX by native-PAGE.



Protein samples were subjected to a discontinuous electrophoresis under non-denaturing conditions in gels of 15% polyacrylamide. The gels were stained, dried and subjected to autoradiography. Tracks are as follows: (1) β -BuTX; (2) HPLC purified DTX; (3) Peak 9 from ion-exchange chromatography; (4) ^{125}I -DTX and DTX (as carrier) visualised by autoradiography and staining, respectively.

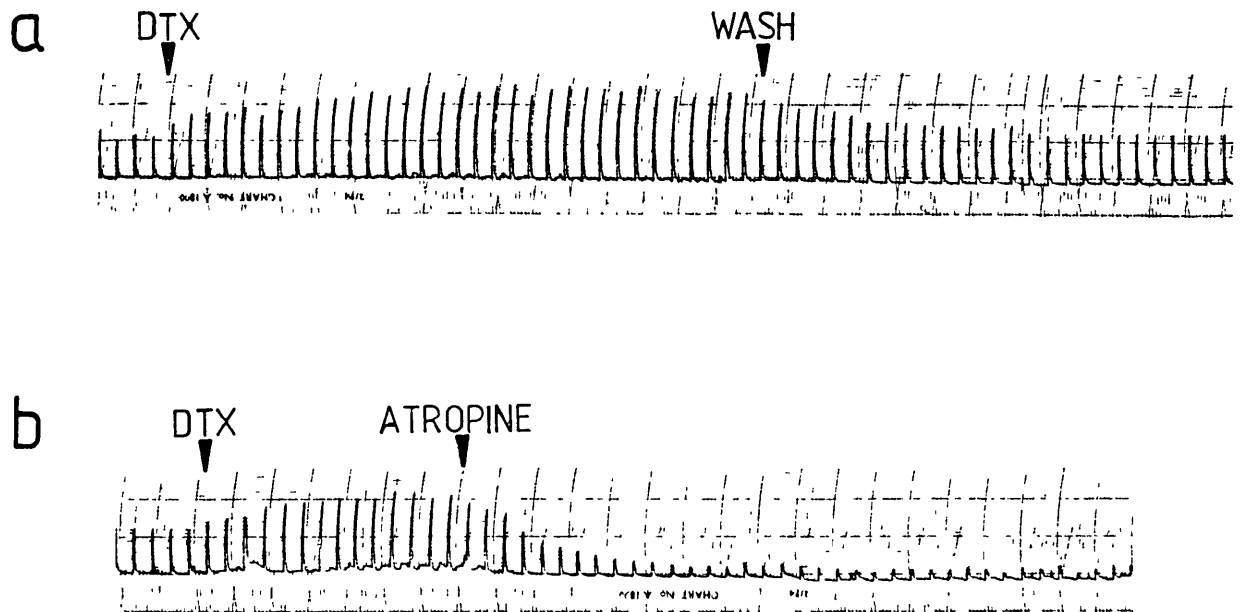
Figure 2.5 HPLC of Peak 9 from ion-exchange chromatography.



Peak 9, dissolved on 0.1% trifluoroacetic acid was applied to a sizing column [two Waters I-125 sizing columns (7.8 x 300mm) in series] equilibrated in the same buffer. The absorbance of the eluate (flow rate of 0.8ml/min) was monitored at 280nm (A_{280}).

(c) Biological activity of Peak 9. Following intraventricular injection, Peak 9 produced severe convulsions; the minimum lethal dose (MLD) was 2.5ng/g body weight in rats. Animals given doses below this amount developed convulsive symptoms but appeared to recover completely from intoxication over a period of 15h. The convulsive effects and toxicities were the same as those seen with DTX previously prepared by I.B. Othman and the symptoms observed were indential to those reported for toxin I (Othman et al., 1982; Othman, 1983). Peak 9 was also indistinguishable from DTX by its effects on peripheral and central neurons. In chick biventer muscle preparations both potentiated neurotransmitter release in an identical manner and were devoid of postjunctional activity (A.L. Harvey, personal communication); in rodent hippocampal slices they gave the same increases in transmitter release and cell excitability (J.V. Halliwell, personal communication). Also consistent with Peak 9 being DTX, it potentiated contractions of guinea pig iluem (Figure 2.6), as did HPLC purified DTX. These contractions were blocked by atropine, showing that Peak 9 and HPLC purified DTX were affecting synaptic activity (Figure 2.6a). The augmentation was almost totally reversed by washing (Figure 2.6b), as were the similar effects of DTX on other autonomic preparations (Harvey et al., 1984). As it was able to inhibit the binding of β -BuTX to rat cerebrocortical synaptosomes [150nM could block 97 + 2% of saturable ^3H - β -BuTX (1.5nM) binding], the results in (b) and (c) show collectively that Peak 9 behaved identically with DTX; thus, this preparation was used without further purification and will be referred to as DTX. When calculating concentrations of DTX (by weight), the minor amounts of contaminants in Peak 9 were ignored.

Figure 2.6 The effect of DTX on nerve-evoked twitch of guinea-pig ileum.



Sections of ileum (3-4cm), bathed in oxygenated Krebs-ringer solution at 37°C, were subjected to field stimulation (10V; 0.2s) every 20s and the resultant twitch was recorded with an isotonic transducer (1.5g tension).

- (a) As indicated, DTX (Peak 9 from ion-exchange chromatography) was added to the bath, to give a final concentration of 190nM, and was washed from it by continuous replacement of the ringer over a 5 min period.
- (b) DTX was added as in (a) and atropine was added to 10nM.

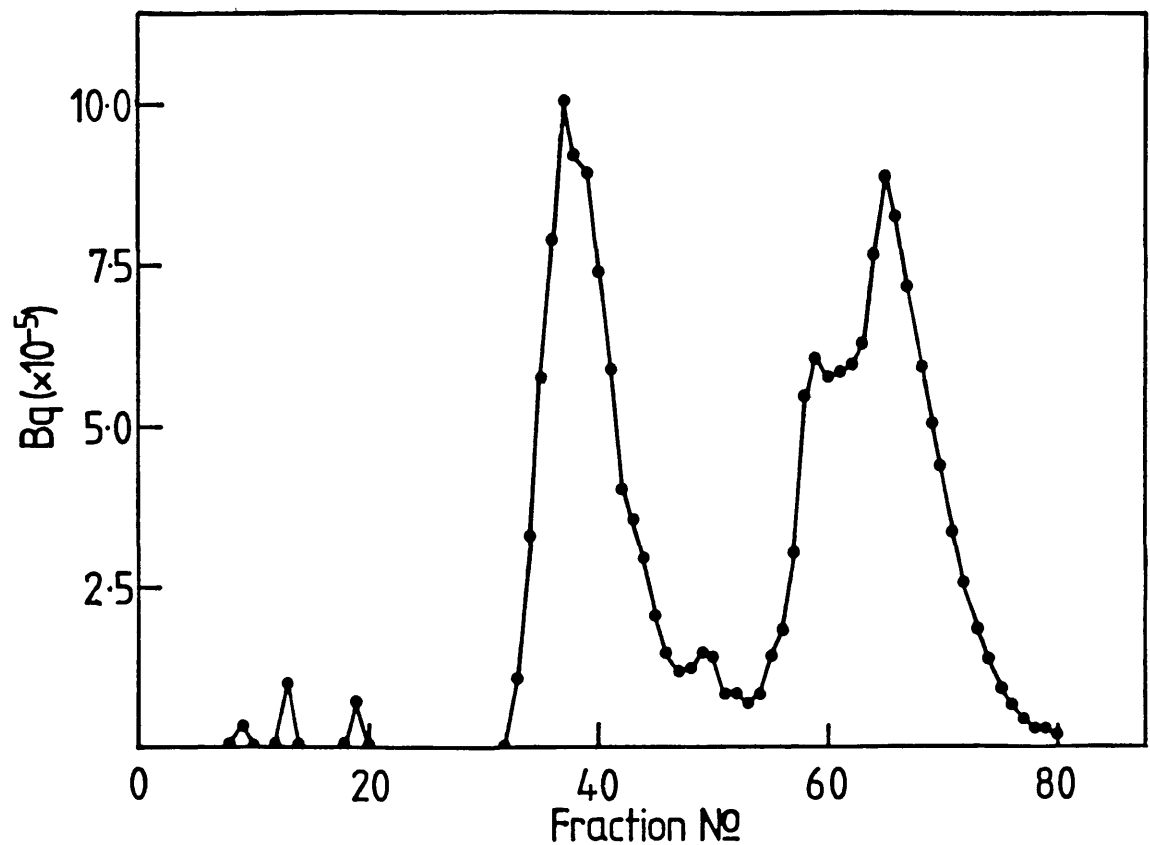
2.3.2 ³H-Propionylation of DTX.

To evaluate the utility of propionylation as a method for radiolabelling DTX, a pilot experiment was conducted with N-succinimidyl propionate of low specific radioactivity. The recovery of reagent into the reaction mixture was 37%, giving a final molar ratio of reagent to toxin of 0.7. Following its separation from the free reagent (Figure 2.7), the specific activity of the propionylated DTX preparation (³H-DTX) was found to be 7.8×10^{12} Bq/mol. This, together with the total radioactivity, showed that 10% of the toxin molecules had been labelled and that 13% of the reagent had been incorporated. Although ³H-DTX and DTX behaved identically on SDS-PAGE (Figure 2.8, A and B), they were clearly separated by native-PAGE (Figure 2.8c) reflecting the alteration of charge caused by propionylation.

2.3.3 Radioiodination of DTX.

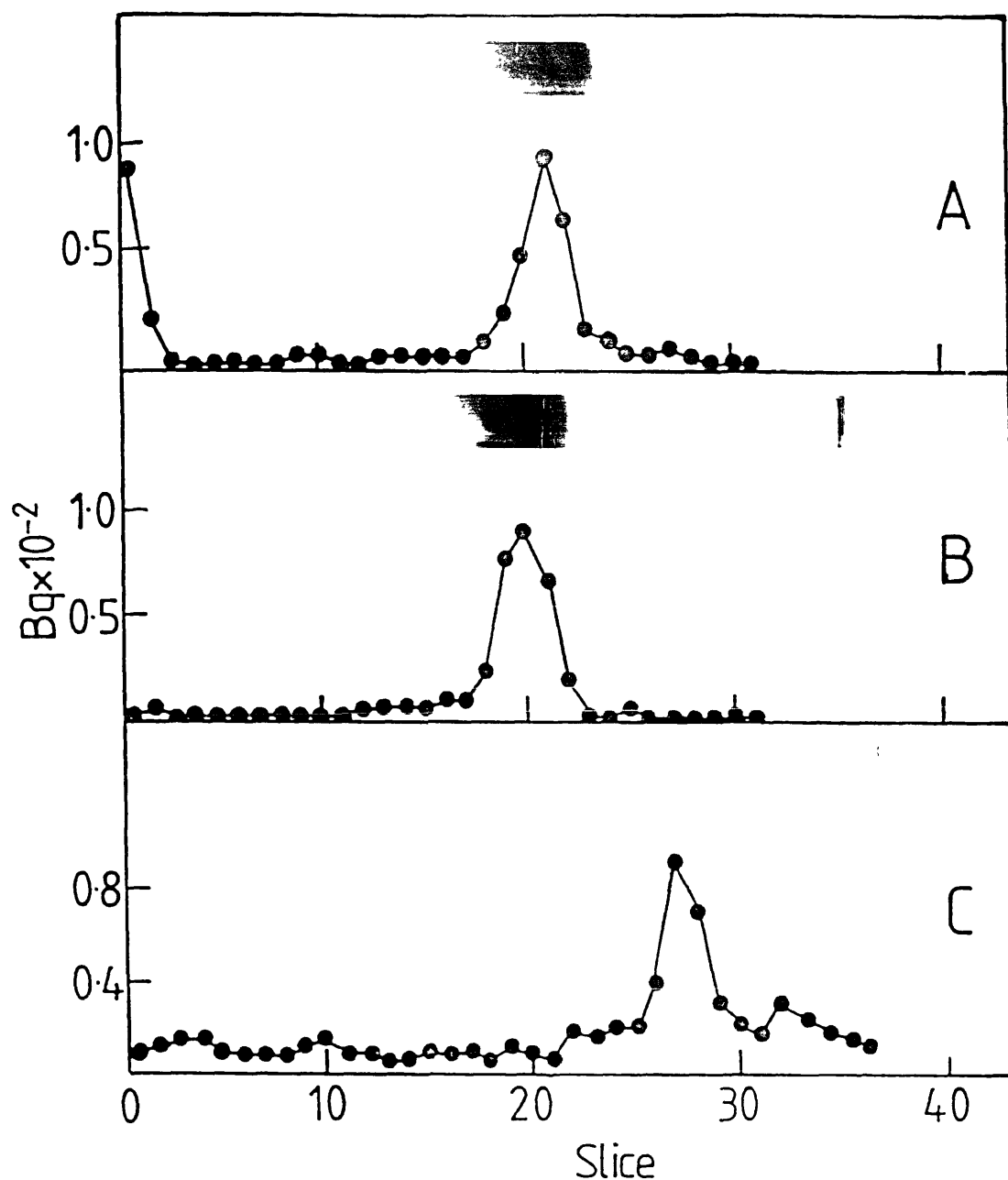
The mild chloramine-T method used for iodination of botulinum neurotoxin (Williams *et al.*, 1983) had to be modified as this toxin is 20-fold larger than DTX. After initial experiments, the optimised amounts for iodination were 10µg DTX per 10µl of Na ¹²⁵I ($\sim 3.5 \times 10^{15}$ Bq/ml) as these conditions consistently produced ¹²⁵I-DTX of high specific radioactivity. From the radioactivity eluted from the ion-exchange column, the recovery of both toxin and radioactivity could be estimated as >95%. Free iodine was eluted from the column in the initial wash (Figure 2.9) and was clearly separated from ¹²⁵I-DTX; >98% of the radioactivity corresponding to the latter could be precipitated by 10% trichloroacetic acid. The specific radioactivity of ¹²⁵I-DTX was between 1.1 and 1.5×10^{16} Bq/mol. In conjunction with the amount and radioactivity of ¹²⁵I used, this indicated an incorporation of $\sim 50\%$ of the iodine into $\sim 20\%$ of the toxin molecules. When the values of specific radioactivities determined for

Figure 2.7 Gel filtration of the reaction mixture for propionylation of DTX.



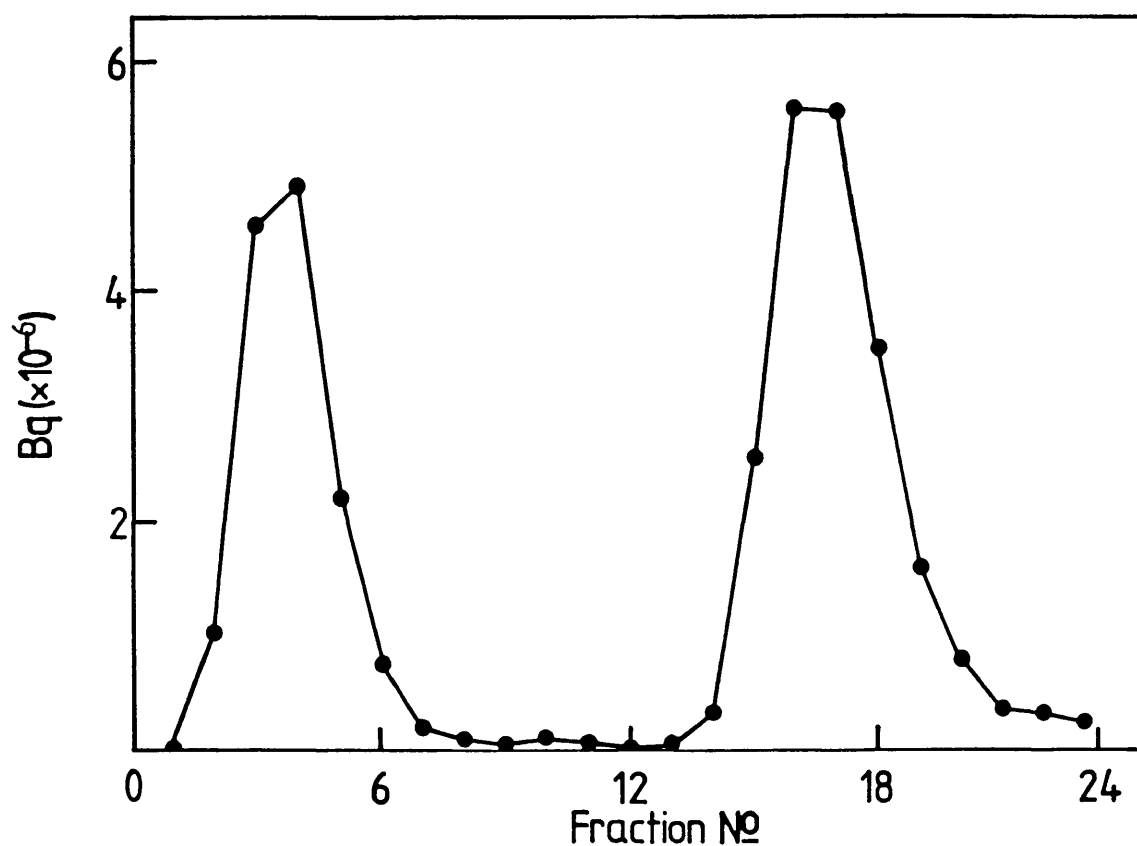
The reaction mixture was subjected to chromatography on a Sephadex G-10 column (1 x 25cm) equilibrated with 25mM ammonium acetate, pH 7.4, and the radioactive content of the collected fractions (0.3ml) was determined.

Figure 2.8 Electrophoretic analysis of ^3H -DTX



Samples were subjected to SDS-PAGE under reducing (A) or non-reducing (B) conditions or to cathodic native-PAGE (C). The radioactive content of tracks (2mm slices) containing ^3H -DTX is shown alongside tracks containing DTX which were electrophoresed simultaneously and stained.

Figure 2.9 Ion-exchange chromatography of the reaction mixture for iodination of DTX.



The reaction mixture was applied to a CM-Sepharose C1-6B column (2ml) equilibrated with 20mM sodium phosphate buffer, pH 7.4. The column was washed with 2ml of the equilibration buffer and bound protein was eluted with 10ml gradient of 0-1M NaCl. Fractions (0.5ml) were collected and the radioactive content of 5 μ l aliquots of these was determined.

the same preparation by the acid precipitation and measured protein content were compared, they were found to be practically the same (1.3 and 1.4×10^{16} Bq/mol, respectively). Thus, only the safer and more convenient acid-preparation method was used routinely.

SDS-gradient-PAGE under non-reducing and reducing conditions (Figure 2.3 A and B) could not separate ^{125}I -DTX and DTX, showing that the iodination procedure did not alter appreciably the structure of DTX. As expected, the charge of the toxin did change upon labelling; native-PAGE (Figure 2.4) revealed a major radioactive species together with a small amount of a less mobile component, presumably representing mono- and di-iodinated DTX; both of these were separated from DTX.

2.3.4 Biological activity of iodinated-DTX.

To establish whether iodination altered the biological activity of DTX, a preparation with high incorporation of iodine, but of low specific activity, was required to facilitate safe injection for toxicity testing. This was produced with an isotopic mixture of ^{125}I and ^{127}I . Ion-exchange chromatography was not used to separate I-DTX from free iodine (and tyrosine), as the final samples had to be in a buffer suitable for injections. Instead, gel filtration on a Sephadex G-10 column, equilibrated with 100mM sodium phosphate, pH 7.4, containing 150mM NaCl, was used. This gave a separation similar to that in Figure 2.7. The specific radioactivity of 1.3×10^{14} Bq/mol, obtained for I-DTX, corresponded to greater than equimolar labelling, with incorporation of 1.8 iodine molecules per toxin molecule. Presumably this preparation comprised mainly di-iodinated toxin with fairly small amounts of mono-iodinated and negligible levels of unlabelled DTX. When injected into rat brain, I-DTX produced convulsive symptoms with an MLD of 2.0ng/g body weight. This was practically the same as the toxicity of native DTX (MLD = 2.5ng/g body weight), showing

that iodination did not significantly alter the biological activity of the toxin. Thus, iodinated derivatives of DTX are valid probes for the neuronal components with which it interacts.

2.3.5 Identification of neuronal acceptors for DTX.

Incubation of synaptosomes with varying concentrations of ^{125}I -DTX revealed saturable binding (Figure 2.10a). The non-specific fraction of the observed binding was at an acceptably low level, between 20% and 30% at 1.2nM ^{125}I -DTX (the concentration used for further studies in this system). This rose only slightly over 3-5 weeks, demonstrating the stability of ^{125}I -DTX. Scatchard analysis of the saturable binding (Figure 2.10b) indicated the presence of non-interacting and kinetically homogeneous acceptors with respect to ^{125}I -DTX binding, displaying a K_D of 0.3nM and a B_{max} between 870 and $1050\text{ fmol/mg protein}$ (5 experiments). The sensitivity of these high affinity acceptors to heat, acid and protease treatments (Table 2.1) showed that they were proteins.

The dissociation of the acceptor/toxin complexes, revealed by addition of excess DTX, was a simple first-order reaction (Figure 2.11) with a $t_{1/2}$ of 11 min at 23°C ($k_{-1} = 1.1 \times 10^{-3}\text{s}^{-1}$), again indicative of a kinetically homogeneous population. As the association rate was too rapid to be measured by the present technique, the K_D could not be confirmed by kinetic data. However, from the k_{-1} and K_D , a k_{+1} of $4.4 \times 10^6\text{ M}^{-1}\text{s}^{-1}$ was calculated.

2.3.6 Inhibition of ^{125}I -DTX binding to rat brain synaptosomes by DTX and β -BuTX.

In competition studies (Figure 2.12) the ratio of the DTX concentrations required to inhibit 90% and 10% of the saturable binding of ^{125}I -DTX was $60\text{-}80$ (5 experiments). This is consistent with

Figure 2.10 Concentration dependence of ^{125}I -DTX binding to synaptosomes from rat cerebral cortex.

- (a) A suspension of synaptosomes (0.5mg/mg of protein) was incubated in Krebs phosphate medium at 23°C for 40 min with various concentrations of ^{125}I -DTX (final volume 250 μl). Bound and free toxin were separated by centrifugation; the supernatant was removed and the pellet was washed twice with ice-cold Krebs phosphate (2 x 1ml). The radioactivity associated with the washed synaptosomes and in the original supernatant was quantified by γ -counting. Non-specific binding was determined likewise in the presence of 100-fold molar excess of unlabelled DTX. Specific binding (●) was calculated as the difference between the total (▲) and non-specific (■) binding. Membrane protein was determined by the method of Bradford (1976).
- (b) A Scatchard plot of the specific binding data in (a).

Figure 2.10 Concentration dependence of ^{125}I -DTX binding to synaptosomes from rat cerebral cortex.

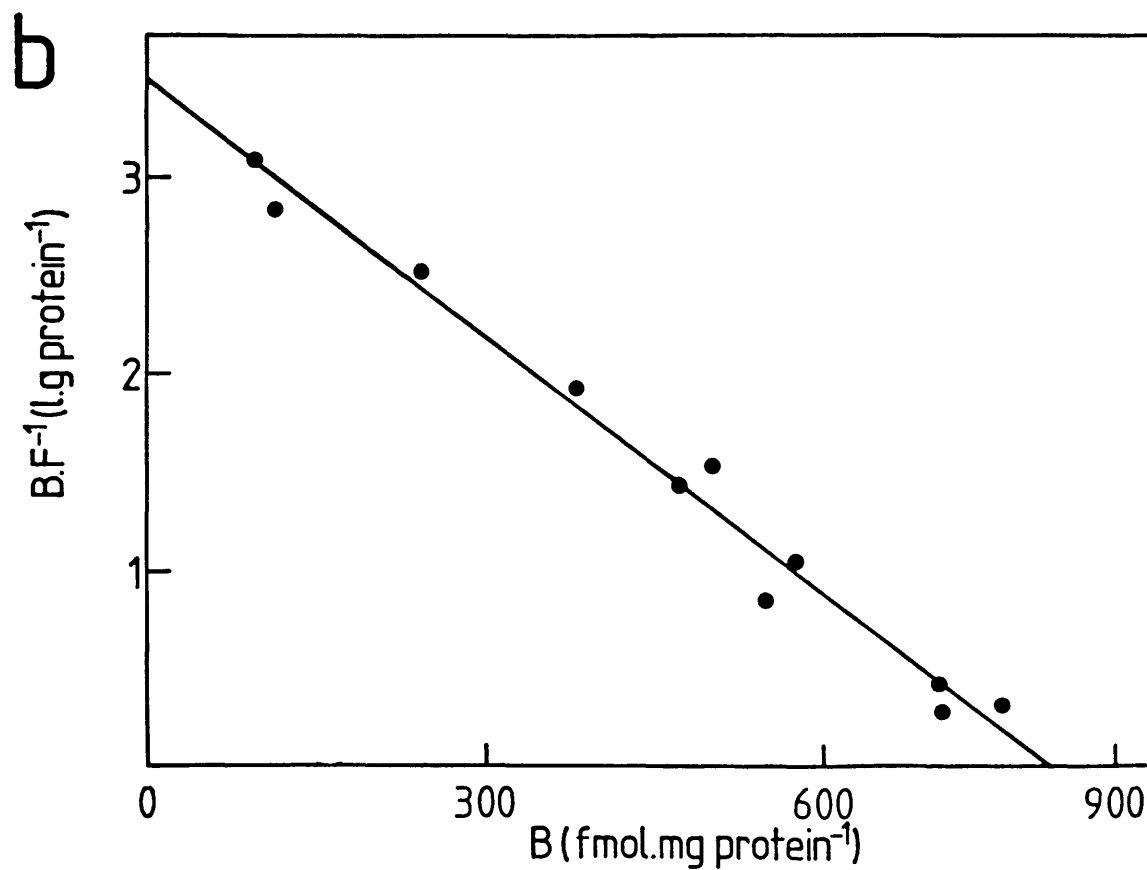
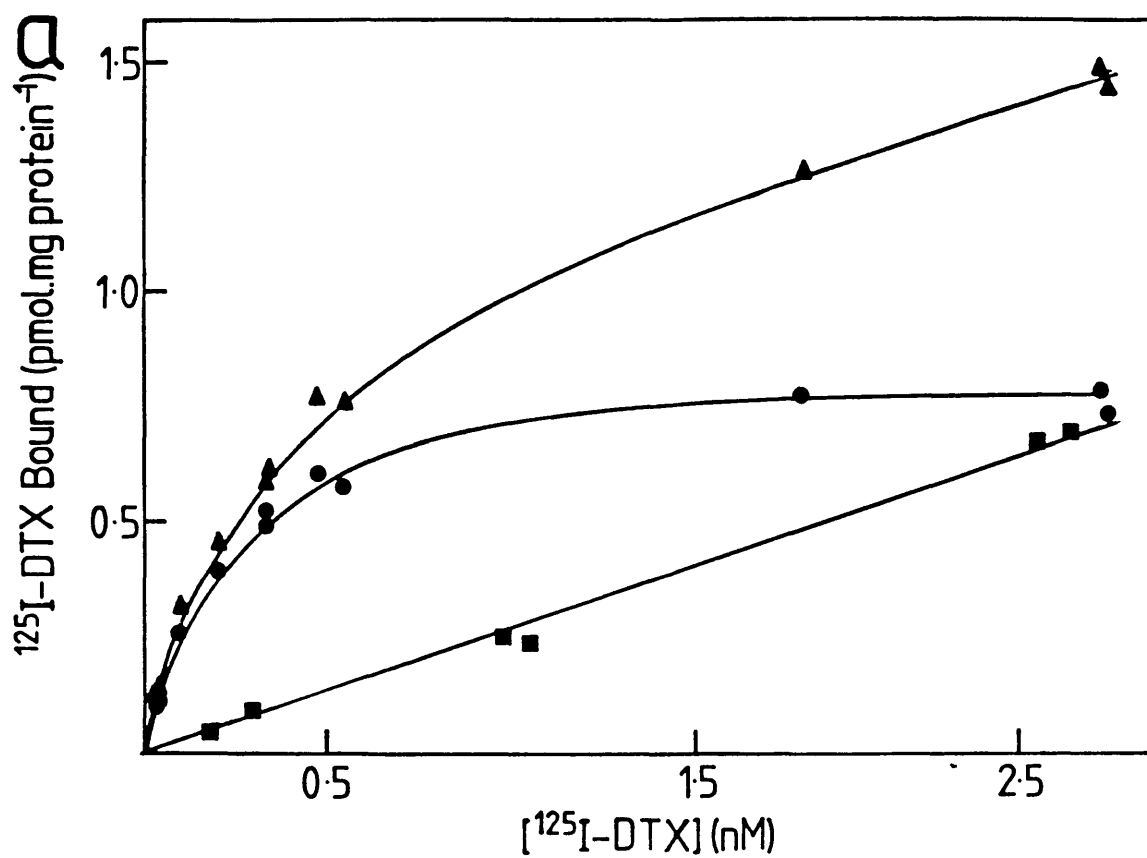
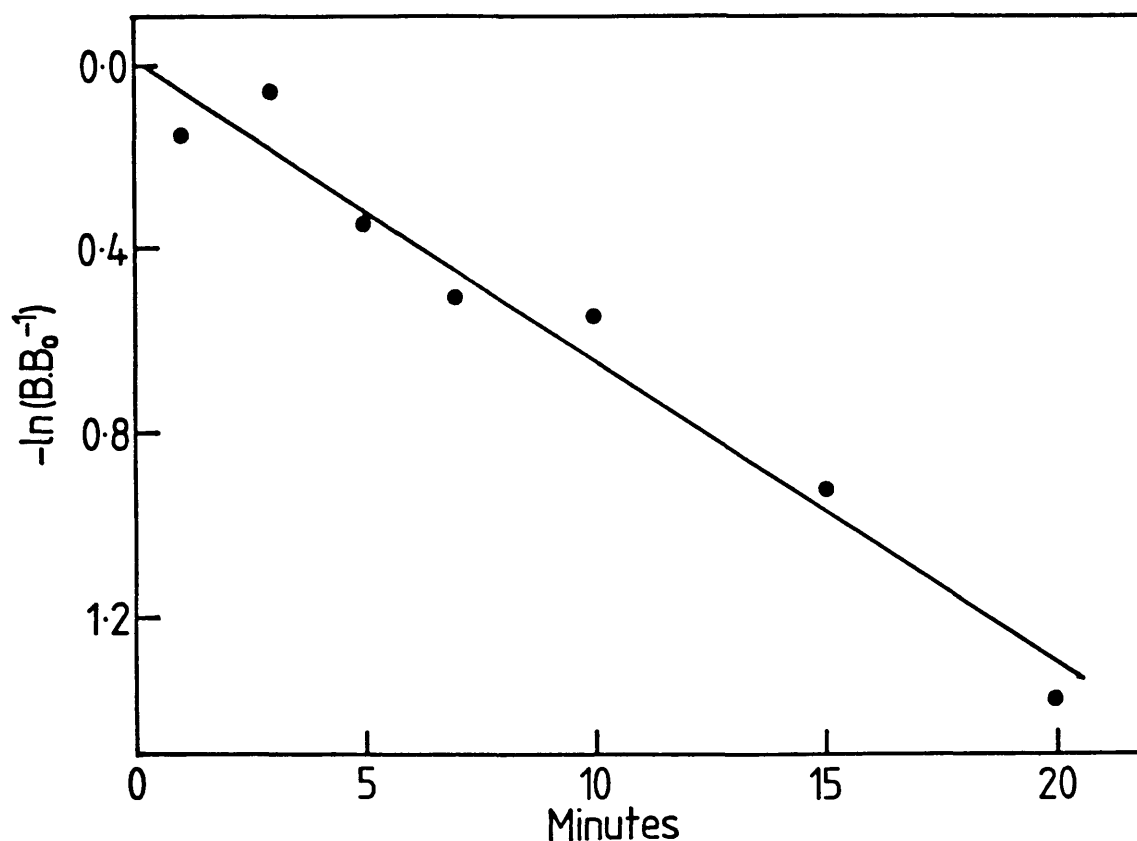


Table 2.1 The effect of various treatments on the DTX acceptors in synaptosomes from rat cerebral cortex.

<u>Treatment</u>	Relative specific binding of <u>^{125}I-DTX (%)</u>
Chymotrypsin (1mg/ml; 37°C for 40 min)	36 $\pm$ 16
Trypsin (1mg/ml; 37°C for 40 min)	34 $\pm$ 13
Heat (95°C for 30 min)	2 $\pm$ 2
Acidification (1M HCl; 37°C for 40 min)	3 $\pm$ 1

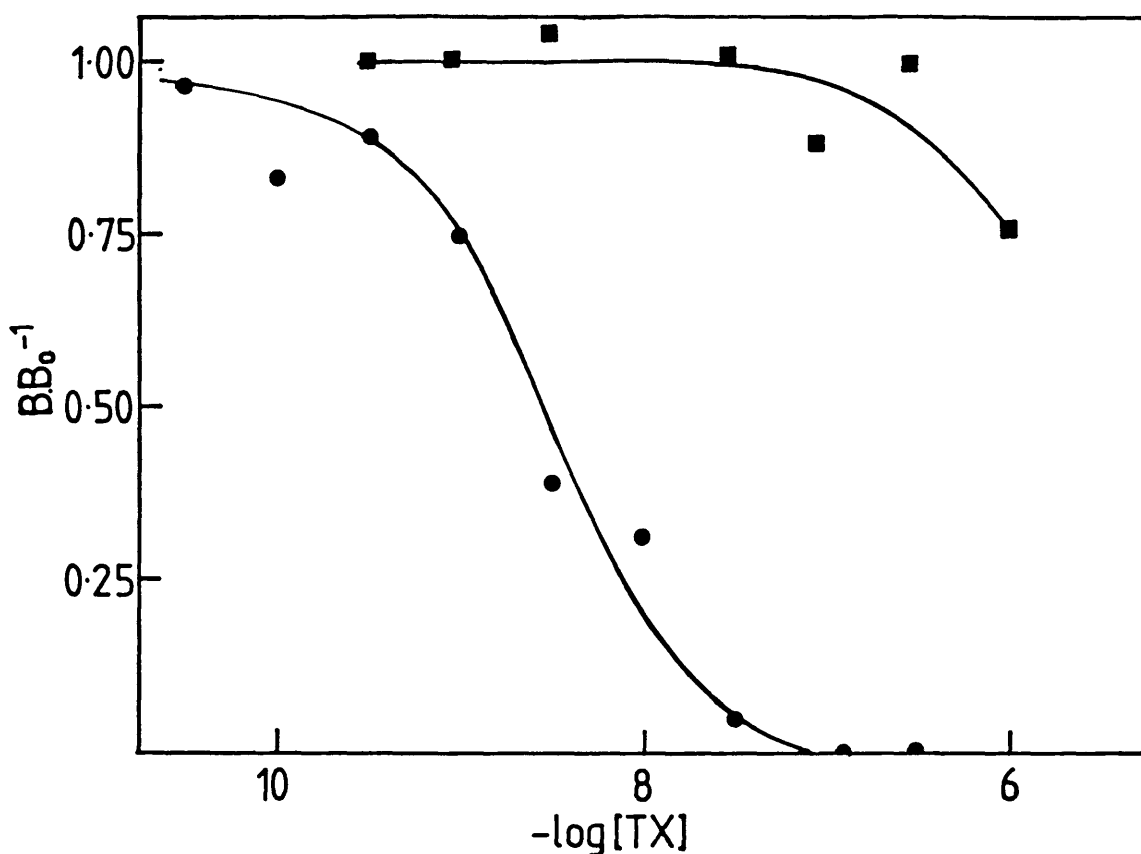
Synaptosomal suspensions in Krebs phosphate medium were subjected to the specified incubations. Proteolysis was stopped by addition of 5mg/ml STI (for trypsin) or 5mM PMSF (for chymotrypsin) and acidification was stopped by washing (2 x 1ml of Krebs phosphate) prior to assay for ^{125}I -DTX (1.2nM) binding as described in Section 2.2.9. Non-saturable binding, measured likewise in the presence of 120nM DTX, was subtracted from the total binding; the resultant specific binding is expressed relative to that obtained for respective control samples (650-750fmol/mg protein). These were incubated similarly, except that protease inhibitors were added prior to proteases or synaptosomes were incubated in Krebs phosphate medium at 37°C for 30 min (for heat treatment) or 40 min (for acidification). The data are averages from four separate experiments ($\pm$ S.E.).

Figure 2.11 Dissociation of ^{125}I -DTX/acceptor complexes in rat brain membranes.



^{125}I -DTX (1.2nM) was incubated at 23°C for 30 min with a synaptosome suspension (0.3mg protein/ml) in Krebs phosphate medium. Unlabelled DTX (120nM) was added and the amount of bound radioactivity was quantified as described in Section 2.2.9. Non-specific binding was measured by including 120nM DTX in the initial incubation mixture. The data is presented as a first-order plot of the dissociation of acceptor/toxin complexes where B_0 and B represent the specific binding at zero time and times thereafter, respectively. $B_0 = 700$ fmol/mg protein.

Figure 2.12 The effect of DTX and β -BuTX on specific ^{125}I -DTX binding to synaptosomes.



Synaptosomes (0.3mg protein/ml) were incubated at 23°C for 30 min in Krebs phosphate medium in the presence of 1.2nM ^{125}I -DTX and various concentrations of DTX (●) or β -BuTX (■). Bound ^{125}I -DTX was separated from free labelled toxin by centrifugation and washing as described in Section 2.2.9 and was quantified by γ -counting. Non-specific binding was determined in the presence of 120nM DTX. B_0 and B represent the amount of ^{125}I -DTX specifically bound in the absence and presence of competing ligand, respectively: $B_0 = 700\text{fmol/mg protein}$.

interaction at a set of homogeneous sites (theoretical ratio = 81). As with toxicity, competition studies showed that iodination did not significantly alter the properties of DTX because the affinities of DTX ($K_I = 0.4\text{nM}$) and ^{125}I -DTX ($K_D = 0.3\text{nM}$) were practically identical.

Surprisingly, β -BuTx could only inhibit ^{125}I -DTX binding with low efficacy (Figure 2.12) and, therefore, is able to interact only weakly with the majority of DTX acceptors.

2.4. Discussion

2.4.1 Radiolabelling of DTX.

Of the two radiolabelling techniques studied, only iodination proved successful; ^3H -propionylation led to very low incorporation of label into the toxin. Despite this, DTX could be propionylated to give a homogeneous derivative (Figure 2.8); if a more stable radiolabelled DTX derivative were required, further studies may be able to increase the efficiency of the reaction. However, the biological activity of the derivative would need to be determined, especially since it has been proposed that the biological activity of DTX is dependent on specific lysine residues (Harvey *et al.*, 1984b). The high specific radioactivity and stability of ^{125}I -DTX, together with its full retention of both convulsive and binding activity, shows that this preparation is both a valid and practical probe for its neuronal acceptors. The slightly higher affinity and toxicity recorded for labelled preparations can be ascribed to the greater accuracy with which their concentrations can be estimated.

Although iodinated DTX could be separated from the native toxin by virtue of its charge (Figure 2.4), this could not be done preparatively by either ion-exchange chromatography or isoelectric focussing (I.B.

Othman, personal communication). Fortunately, such separation is not necessary as both behaved identically in all other respects, showing that the presence of a mixture of DTX derivatives did not affect the results obtained.

2.4.2 The neuronal acceptors for DTX.

In keeping with its central activity and inhibition of β -BuTX binding, equilibrium measurements revealed saturable binding of DTX to rat cerebrocortical synaptosomes (Figure 2.10a). Scatchard analysis (Figure 2.10b) and dissociation kinetics (Figure 2.11) revealed the existence of high affinity ($K_D = 0.3\text{nM}$; $B_{\text{max}} = 870\text{ }1050\text{fmol/mg}$ protein), non-interacting acceptors which are homogenous with respect to toxin binding. These neuronal acceptors appear to be located on the plasma membrane as ^{125}I -DTX binding is enriched 1.6 fold in synaptic plasma membranes [$B_{\text{max}} = 1.4\text{-}1.7\text{pmol/mg}$ protein (Section 5.3.4)] relative to that seen in synaptosomes. The proteinaceous nature of the DTX acceptors was established by their sensitivity to protein denaturing agents and proteolytic enzymes (Table 2.1). Note that although incomplete inactivation of the acceptors in the membrane bound states was produced by trypsin or chymotrypsin, the same treatment abolished the DTX binding activity of the solubilised acceptors (Section 5.3.4); this is indicative of the acceptor proteins being protected in the membrane, as already shown for the nicotinic acetylcholine receptor (Conti-Tronconi and Raftery, 1982). Certain proteins exhibiting PLA_2 activity were found by I.B. Othman, to inhibit the binding of ^{125}I -DTX in the presence of Ca^{2+} (Black et al., 1985). Although this may indicate the occurrence of lipid moieties in the DTX binding component, it was also seen with the acceptors for botulinum neurotoxin (Williams et al., 1983) and probably reflects a conformational dependence of these binding components on their membrane environment.

Data obtained by others (I.B. Othman and A.L. Breeze of this laboratory) showed that apart from their interaction with β -BuTX, detailed below (Figure 2.12), the acceptors studied herein are highly specific to DTX and its neurotoxic homologues (see Chapter 7); no inhibition of ^{125}I -DTX binding was seen with PLA_2 containing presynaptic neurotoxins (in the absence of Ca^{2+}), other neurotoxins or various ion channel blockers (Black et al., 1985).

In the light of the inhibition of ^3H - β -BuTX binding by DTX (Othman, 1983), it is surprising that β -BuTX exhibits only low affinity for DTX acceptors [$K_I \sim \mu\text{M}$ (Figure 2.12) compared with the K_D of 0.5nM for ^3H - β -BuTX binding (Othman et al., 1982)] As this low affinity inhibition was also seen in the absence of Ca^{2+} (Black et al., 1985), it cannot be ascribed to PLA_2 activity. Therefore, β -BuTX would appear to bind the bulk of DTX acceptors with low affinity, perhaps due to the homology of its B chain to DTX (Kondo et al., 1982). However, the content of acceptors measured for ^{125}I -DTX is 7-10 fold higher than that seen for ^3H - β -BuTX in rat brain by Othman et al. (1982). Therefore, the expected level of high affinity inhibition by β -BuTX of DTX binding would be close to the resolution limit of the system used; moreover, it would tend to be masked by the inhibition seen with high concentrations of β -BuTX (Figure 2.12). Although the most obvious explanation for the data is that a small proportion of DTX acceptors also bind β -BuTX with high affinity, a non-competitive interaction could not be excluded and further studies were required to distinguish these possibilities.

2.4.3 Summary

A highly radioactive iodinated derivative of DTX has been produced using a mild chloramine-T method. This has enabled the characterisation of its binding to proteinaceous, membrane-bound acceptors which have

been shown by others (Black et al., 1985) to be selective for neurotoxic protease inhibitor homologues. Only low affinity inhibition of ^{125}I -DTX binding by β -BuTX could be detected: hence the relationship of the acceptors for these two toxins remained unclear. Despite this, the identification of these acceptors should lead the way to characterisation of components involved with neuronal excitability.

CHAPTER 3

TWO CLASSES OF DTX ACCEPTOR IN CHICK BRAIN
DIFFERENTIABLE BY β -BuTX

3.2 Introduction

3.1.1 The rationale for the study of DTX binding in chick brain.

Species differences are often useful for unravelling apparently complex systems. This is seen for the central α -bungarotoxin binding component. In mammals, some doubt exists as to the function of these acceptors, whereas in chick optic lobe they have been shown to be functional nicotinic acetylcholine receptors (Oswald and Freeman, 1981). Sequencing of the chick optic lobe receptor has shown that it is homologous to those seen in peripheral tissues (Conti-Tronconi et al., 1985); furthermore, antibodies raised against the chick receptor (Norman et al., 1982; Betz and Pfeiffer, 1984) can be applied to the mammalian system to help in discerning the function of the central α -bungarotoxin binding component therein.

The pharmacology and interactions of DTX homologues and β -BuTX show species differences. For example, both are much more potent in chick biventer muscle preparations than on mouse phrenic nerve (cf. Chang, 1985). Likewise, their mutual interaction at the chick neuromuscular junction was not seen in mouse preparations (cf. Chang, 1985). In this context, the demonstrated high affinity binding of ^{125}I -BuTX in chick brain membranes [$K_D = 0.5\text{nM}$, $B_{\text{max}} = 50\text{ fmol/mg protein}$ (Rehm and Betz, 1982)] was of interest, especially since it was very similar to the binding reported from this laboratory of ^3H - β -BuTX to rat cerebrocortical synaptosomes (Othman et al., 1982). When it was recently reported that, as in rat brain (Othman, 1983), β -BuTX binding to its chick brain acceptors was inhibitable by equimolar amounts of DTX (Rehm and Betz, 1984), it was decided to study ^{125}I -DTX binding to chick synaptic membranes. The strategy was to exploit possible species differences in order to decipher the basis of the interaction between these two toxins, as well as to provide further information about their acceptors.

3.2 Methods

3.2.1 Preparation of chick brain synaptic membranes.

This was accomplished by an adaption of the method of Rehm and Betz (1982). Cerebral cortices were removed within 30s after death from 1 day old leghorn chicks killed by decapitation. The tissue was frozen immediately in liquid N₂ and stored at -80°C until required. Prior to use, 6 cortices were thawed in ice cold 25mM sodium phosphate buffer, pH 7.4, 0.28M sucrose. All subsequent steps were performed at 4°C. The cortices were homogenised in the above buffer, using a glass/perspex homogeniser with a clearance of 0.5mm (2 x 6 strokes at 900 rpm). The homogenate was centrifuged at 4,000 rpm (1,000 x g) in a Beckman type 30 rotor. After discarding the pellet, the resultant supernatant was centrifuged at 11,000 rpm (10,000 x g) in the same rotor. The membrane pellet was resuspended in 20ml of 5mM Tris-HCl, pH 7.4, and the sucrose concentration was adjusted to 15% (w/v) using a 60% (w/v) sucrose solution. This suspension was layered on top of 25ml of 35% (w/v) sucrose in a 60ml centrifuge tube; 5ml of 5mM Tris-HCl, pH 7.4, was layered on top of this prior to centrifugation at 25,000 rpm (80,000 x g) for 50 min in a Beckman SW 25.2 rotor. Membranes were collected from the interface of the 15% and 35% sucrose layers and were pelleted at 27,000 rpm (60,000 x g) in a Beckman type 30 rotor for 40 min. The membranes were resuspended in deionised water and stored at -80°C until required. Prior to use they were thawed and homogenised (in a 15ml Jencons glass/PTFE homogeniser) in order to disperse any membrane aggregates.

3.2.2 Characterisation of the saturable binding of ¹²⁵I-DTX to chick synaptic membranes.

For equilibrium measurements, membranes (200-400µg protein) were incubated for 30 min at 23°C, with ¹²⁵I-DTX at the stated

concentrations in 250 μ l of 50mM Tris-HCl, pH 7.4, 100mM NaCl, 1mM MgCl₂ and either 2mM CaCl₂ or SrCl₂ (assay buffer). (For determination of the divalent cation dependence of binding, this buffer was changed as stated in Table. 3.3). Non-saturable binding was determined by inclusion of 200 or 400nM DTX in the incubation medium. Bound and free ¹²⁵I-DTX were separated using a rapid oil centrifugation method (Akerman and Nicholls, 1981; Black et al., 1985); 200 μ l of the incubation medium was layered on to 200 μ l of a mixture (~50%, see later) of dinonyl phthalate and Dow-Corning MS-550 silicone fluid in an Eppendorf centrifuge tube. The tubes were then centrifuged for 1 min in an Eppendorf microcentrifuge. For Scatchard analysis, aliquots of the aqueous supernatant were removed for determination of the free ¹²⁵I-DTX concentration. The aqueous layer was removed and the walls of the tube were washed with 1ml of 0.45M NaOH. ¹²⁵I-DTX levels in the pelleted membranes and in the aqueous phase were determined by γ -counting at 50% efficiency. The precise proportions of the dinonyl phthalate and silicone fluid used were determined empirically for each experiment; they were engineered so that membranes could pellet clearly through the organic phase (with a 10s centrifugation for kinetic measurement) whilst the incubation medium remained above it. In competition studies, various ligands were included in the incubation medium at the stated levels.

For measurement of the dissociation kinetics the same conditions were used, except that the incubation volumes were increased to 5ml. Membranes were prelabelled by incubation with 2nM ¹²⁵I-DTX in Sr²⁺ containing assay buffer in the presence or absence of 500nM β -BuTX. To prevent binding of ¹²⁵I-DTX, either 200nM DTX or 500nM β -BuTX was added. Duplicate aliquots (200 μ l) were removed for assay at times after these additions. Non-saturable binding was determined by inclusion of

200nM DTX and/or 500nM β -BuTX in the incubation medium, with addition of buffer at zero time.

Binding data were analysed as described in Appendix A.2. Protein was measured using bovine serum albumin as a standard (Bradford, 1976).

3.2.3 Treatment of chick synaptic membranes.

Proteolysis was performed by incubating membranes (1.5-3mg protein in 350 μ l) with either 1mg/ml trypsin or protease K at 37°C. After 40 min the reaction was stopped by the addition of STI to 5mg/ml (for trypsin) or PMSF to 5mM (for protease K). Controls were prepared by adding STI or PMSF prior to the proteases. Treatment of membranes (200-400 μ g protein in 50 μ l) with deoxyribonuclease I (40 units/mg protein of membranes), bee venom PLA₂ (1 μ M in the presence of 2mM CaCl₂) or neuraminidase (5 units/ml) was carried out for 40 min at 37°C. The enzymes were omitted from the control samples. One unit of deoxyribonuclease I (EC. 3.1.2.11.) will produce a ΔA_{280} of 0.001 per min per ml at pH 5.0 at 25°C using DNA (type I or III) as a substrate (Mg ion concentration = 4.2mM). One unit of neuraminidase (EC.3.2.1.18.) will liberate 1.0 μ mol of N-acetylneuraminic acid per min at pH 5.0 at 37°C using bovine submaxillary mucin as a substrate.

Heat treatment was carried out for 15 min at 95°C; control samples were incubated at 37°C for 15 min. Acid treatment was carried out in 1M HCl for 40 min at 37°C, followed by washing (2 x 1ml) with assay buffer; controls were prepared by omission of HCl.

3.2.4 Intraventricular injection of 1 day old chicks.

The co-ordinates of the ventricles of 1 day old leghorn chicks were determined using a method similar to that of van Tienhoven and Juhasz (1962) by D.G. Green in this department.

For injection, chicks were anaesthetized with Fluothane (1-2% + O₂) using a Boc King Boyles apparatus, before being placed in a David Kopf small animal stereotaxic instrument. The tip of the beak was placed on the incisor bar, which was set to 0°, and the ear bars and needle were zeroed for sagittal co-ordinates. Their scalps were plucked and the needle was inserted through the intact scalp and skull (using the Jenier screw adjustment) to the co-ordinates S-1mm (anterior), L-1.5mm and Dorsal-Ventral - 2.5mm. Samples (2μl) of various concentrations of DTX in phosphate buffered saline were injected into the ventricle. After a pause to allow for pressure equilibration, the needle was removed and the needle puncture (skull) was plugged with bone wax. The chicks were then removed from the apparatus and allowed to recover from the anaesthetic. They were kept warm under a 60W light bulb, observed for 48h for effects of the toxin and their body weights were determined.

3.3 Results

3.3.1 Identification of DTX acceptors on chick neuronal membranes.

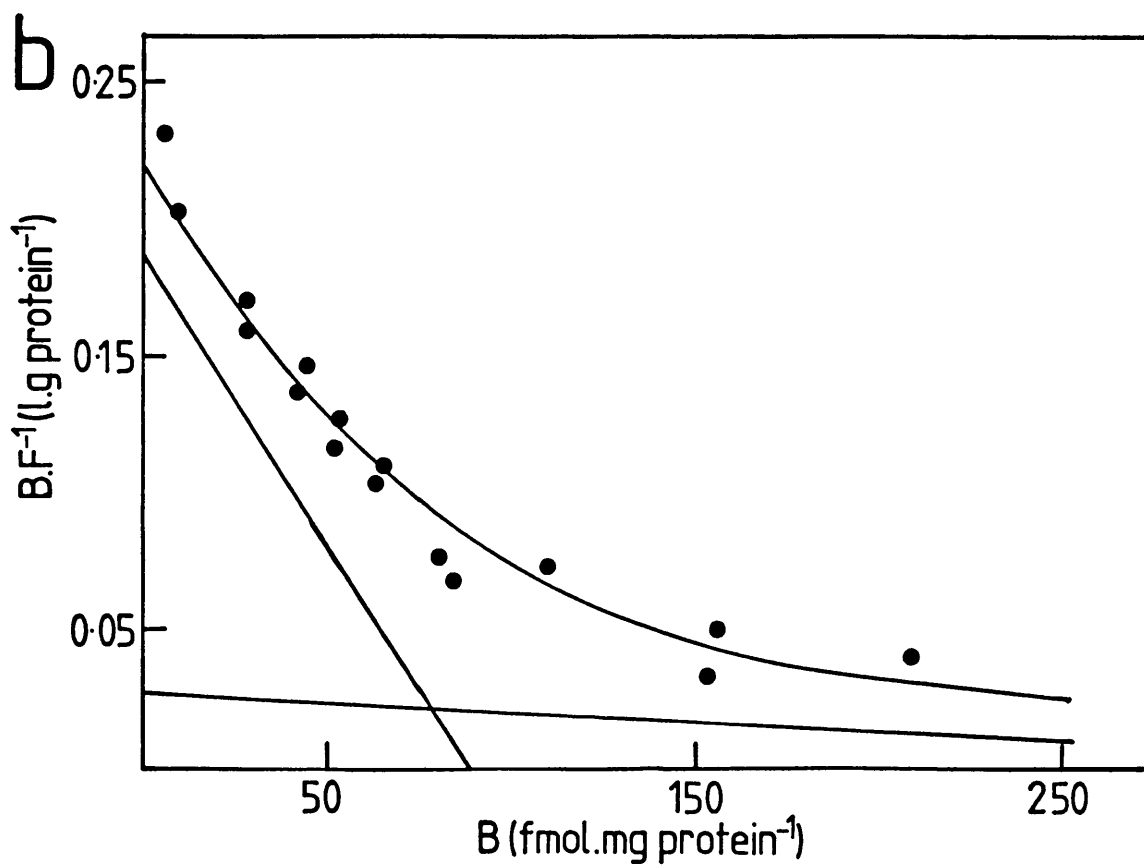
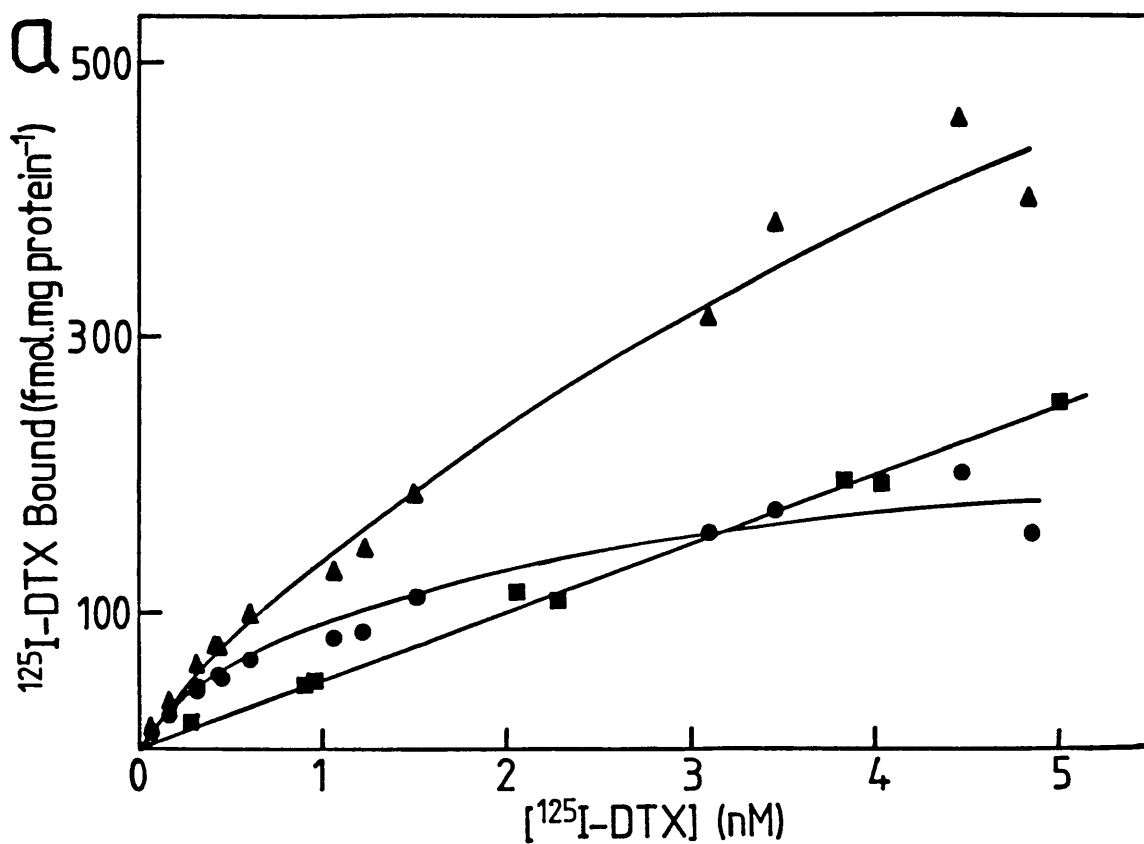
The relevance of studying DTX binding in chick brain was highlighted by the potent central action of the toxin in this species. Following intraventricular injection, doses as low as 1ng/g body weight produced severe convulsions in day-old chicks and its MLD was 19ng/g body weight. Chicks given doses below this appeared to recover completely from the pronounced effects of the toxin. Consistent with this central toxicity, binding of ¹²⁵I-DTX to chick synaptic membranes was observed (Figure 3.1a). The larger part of this was displaceable by excess DTX, with the non-saturable component constituting 30-40% of the total binding at 2nM ¹²⁵I-DTX (the concentration used in most further experiments). The saturable binding appeared extended suggesting that

Figure 3.1 Saturable binding of ^{125}I -DTX to chick synaptic membranes.

- (a) Chick synaptic membranes (200–400 μg) were incubated in the presence of various concentrations of ^{125}I -DTX in 250 μl of assay buffer containing 2mM CaCl_2 (see Section 3.2.2). After 30 min at 23°C, bound and free (F) ^{125}I -DTX were separated by rapid centrifugation through oil and quantified by γ -counting. Non-specific binding (■) was determined similarly in the presence of 400nM DTX and was subtracted from the total binding (▲) to give the specific binding (●). Membrane protein was determined by the method of Bradford (1976).
- (b) The specific binding data from (a) presented on a Scatchard plot: the lines shown are derived from graphical analysis based on the presence of two independent sites.

Figure 3.1 Saturable binding of ^{125}I -DTX to chick synaptic membranes.

d in the presence



multiple sites exist for ^{125}I -DTX in these membranes (Figure 3.1a). Heterogeneity of the acceptors was emphasised by Scatchard analysis of the specific binding, which produced an upward curve (Figure 3.1b). This could be graphically analysed as being due to 2 sites, one with high affinity ($K_D \sim 0.5\text{nM}$; $B_{\text{max}} \sim 90 \text{ fmol/mg protein}$) and another with lower affinity but more abundant ($K_D \sim 15\text{nM}$; $B_{\text{max}} \sim 400 \text{ fmol/mg protein}$). This analysis could be applied to the binding data from two further experiments, allowing for slight variations (<20%) in total binding. Note, however, that the low affinity site is poorly defined by the toxin concentrations used. The existence of multiple binding sites was confirmed by the competition and kinetic studies detailed below. The acceptors for ^{125}I -DTX were seen to be proteinaceous, as pretreatment of the membranes with heat, HCl, trypsin or protease K, considerably reduced their ability to bind ^{125}I -DTX (Table 3.1). Inhibition studies with β -BuTX on the remaining active acceptors (see later) indicated that the two sets of sites were similarly affected (Table 3.1, legend). Involvement of phospholipid or carbohydrate in these acceptors is unlikely, due to the lack of effect produced by PLA_2 , neuraminidase (Table. 3.1) or the lectins concanavalin A or wheat germ agglutinin (Table 3.2). Cytoskeletal components would not appear to be involved directly because deoxyribonuclease I had no effect on toxin binding (Table 3.1) excluding the involvement of actin (Mannhertz et al., 1975).

3.3.2 Selectivity of chick brain DTX acceptors.

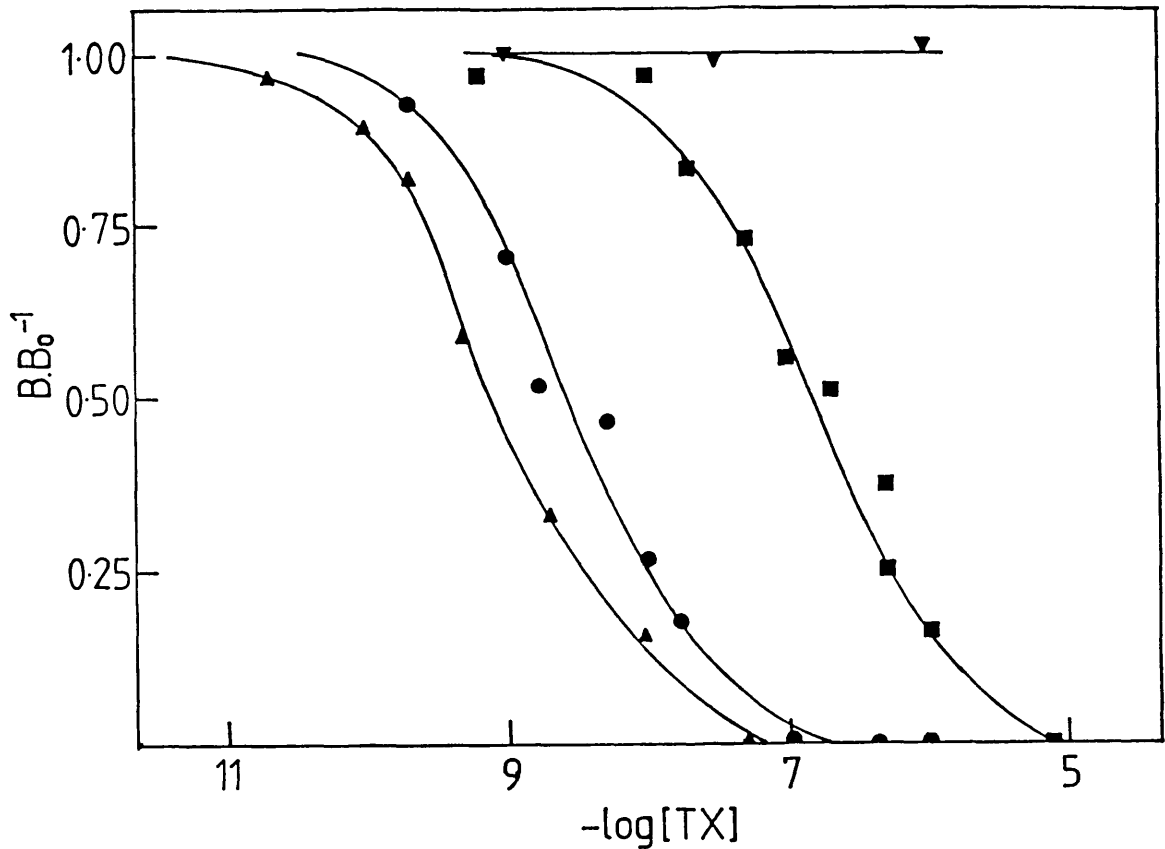
Equimolar amounts of DTX ($\text{IC}_{50} = 3.8 \pm 0.85\text{nM}$) were able to inhibit approximately half of the saturable binding of ^{125}I -DTX (Figure 3.2), confirming the observation (Chapter 2) that iodination has no significant effect on the affinity of the toxin. Toxin E ($\text{IC}_{50} = 129 \pm 29\text{nM}$) and toxin I ($\text{IC}_{50} = 0.4 \pm 0.2\text{nM}$) totally inhibited

Table 3.1 The effect of various treatments of chick synaptic membranes on ^{125}I -DTX acceptors.

<u>Treatment</u>	<u>Relative specific binding (%)</u>
Heat (95°C, 15 min)	18 ± 5
Acidification (1M HCl, 40 min)	15 ± 4
Protease K (1mg/ml, 40 min)	10 ± 2
Trypsin (1mg/ml, 40 min)	16 ± 4
Bee venom PLA ₂ (1μM, 40 min)	99 ± 10
Neuraminidase (0.5 units/ml)	90 ± 10
Deoxyribonuclease I (40 units/mg of membrane protein)	92 ± 4

The specific binding of ^{125}I -DTX (2nM) shown for the various samples was quantified in assay buffer containing 2mM CaCl_2 as detailed in Section 3.2.2 and expressed relative to each specified control. (Values for bee venom PLA₂, but not other treatments, are expressed as relative to pelletable membrane protein.) Average values (± error) of duplicate samples from at least 2 separate experiments are given. In all cases, inclusion of 500nM β -BuTX in the assay medium reduced the extent of specific binding of ^{125}I -DTX by 50-65%. The level of specific binding in control samples ranged from 110 to 125 fmol/mg protein.

Figure 3.2 Inhibition of ^{125}I -DTX binding to chick synaptic membranes by DTX and its homologues.



The membranes (200-400 μg protein) were incubated at 23°C for 30 min in the presence of 2nM ^{125}I -DTX and various concentrations of DTX (●), toxin I (▲), toxin E (■) or toxin B (▼) in 250 μl of assay buffer containing 2mM CaCl_2 ; binding was assayed as in Section 3.2.2. Non-specific binding, determined by including 200nM DTX in the incubation medium, was subtracted from the total binding to give the values shown. The data (B ; averages of duplicates, error $<\pm 7\%$) was plotted relative to the specific binding observed in the absence of competing ligand ($B_0 = 120\text{-}135$ fmol/mg protein).

the saturable binding, whereas no inhibition was seen with toxin B (Figure 3.2). The rank order of potency for these homologues in inhibiting saturable ^{125}I -DTX binding, i.e. toxin I < DTX < toxin E << toxin B, was the same as observed in rat and showed that, of the DTX homologues, only those which are neurotoxic could bind to these acceptors (Black *et al.*, 1985; Table 7.1). The ratio of the concentrations of these toxins required to give 10% and 90% of the inhibition of ^{125}I -DTX binding was 160 ± 27 ($n = 3$), 155 ± 11 ($n = 3$) and 165 ± 25 ($n = 2$) for DTX, toxin I and toxin E, respectively. These values are consistent with the existence of multiple sites for DTX because they are all significantly larger than the value of 81 expected for interaction with a single acceptor type (see Section A.2.4). As the ratios are essentially the same for all the toxins, the relative affinities of the DTX homologues would appear to be the same at all of the sites. The selectivity of the DTX acceptors was highlighted by the inability of the other neurotoxins, crotoxin (subunit A or B) and apamin, or the non-neurotoxic protease inhibitors from bovine pancreas and soy bean (Table 3.2) to inhibit the saturable binding of ^{125}I -DTX.

3.3.3 Two ^{125}I -DTX acceptor types distinguishable by β -BuTX.

In contrast to the facilitatory toxins, β -BuTX was only able to partially inhibit the saturable binding of ^{125}I -DTX (Figure 3.3a). The ratios of β -BuTX concentrations producing 90% and 10% inhibition were between 60 and 95, indicating that β -BuTX was preventing ^{125}I -DTX binding to a single class of acceptors. This antagonism was at a higher affinity set of DTX acceptors as the proportion of the binding inhibitable by β -BuTX decreased with increasing ^{125}I -DTX concentration. A Dixon plot together with a plot of L/B against [I] (Figure 3.3b and c) showed that the interaction was competitive and

Table 3.2 Pharmacological specificity of the saturable binding of ^{125}I -DTX to chick synaptic membranes.

<u>Conditions</u>	<u>Relative specific binding (%)</u>
Concanavalin A (1 μM)	108 $\pm$ 18
Wheat germ agglutinin (1 μM)	100 $\pm$ 3
Crotoxin subunit A (1 μM)	113 $\pm$ 5
Crotoxin subunit B (1 μM)	102 $\pm$ 13
Apamin (1 μM)	96 $\pm$ 2
BPTI (1 μM)	114 $\pm$ 6
STI (700 μM)	105 $\pm$ 5

The amount of ^{125}I -DTX (2mM) specifically bound was measured in assay buffer containing 2mM CaCl_2 as detailed in Section 3.2.2. Mean values ($\pm$ S.E) from at least 3 separate experiments are expressed relative to those observed for membranes in assay buffer without any additional ligands added. In all cases 500nM β -BuTx inhibited 65-72% of the saturable binding (110-130 fmol/mg protein).

Figure 3.3 Inhibition of the saturable binding of ^{125}I -DTX to chick synaptic membranes by β -BuTX.

- (a) Chick synaptic membranes (200-400 μg protein in 250 μl) were incubated with either 0.5nM (■), 2nM (●), 3.5nM (▲) ^{125}I -DTX in assay buffer containing CaCl_2 and various concentrations of β -BuTX. Open circles (○) show the binding of 2nM ^{125}I -DTX in assay buffer devoid of divalent cations and containing 4mM EDTA. The specific binding shown (averages of duplicates; error $<\pm 7\%$), was the difference between the binding seen in the presence and absence of a 100-fold excess of DTX; these were quantified as detailed in Section 3.2.2. Binding in the presence of β -BuTX (B) is expressed relative to the total values observed ($B_0 = 69, 110$ and 155 fmol/mg protein for 0.5, 2.0 and 3.5nM ^{125}I -DTX, respectively).
- (b) Dixon plot of the partial inhibition seen in (a), where the total binding was taken to be that inhibitable by $1\mu\text{M}$ β -BuTX (51, 71 and 82 fmol/mg protein using 0.5, 2.0 and 3.5nM ^{125}I -DTX, respectively).
- (c) The partial inhibition in (a) plotted as $[\text{L}]/\text{B}$ against $[\text{I}]$.

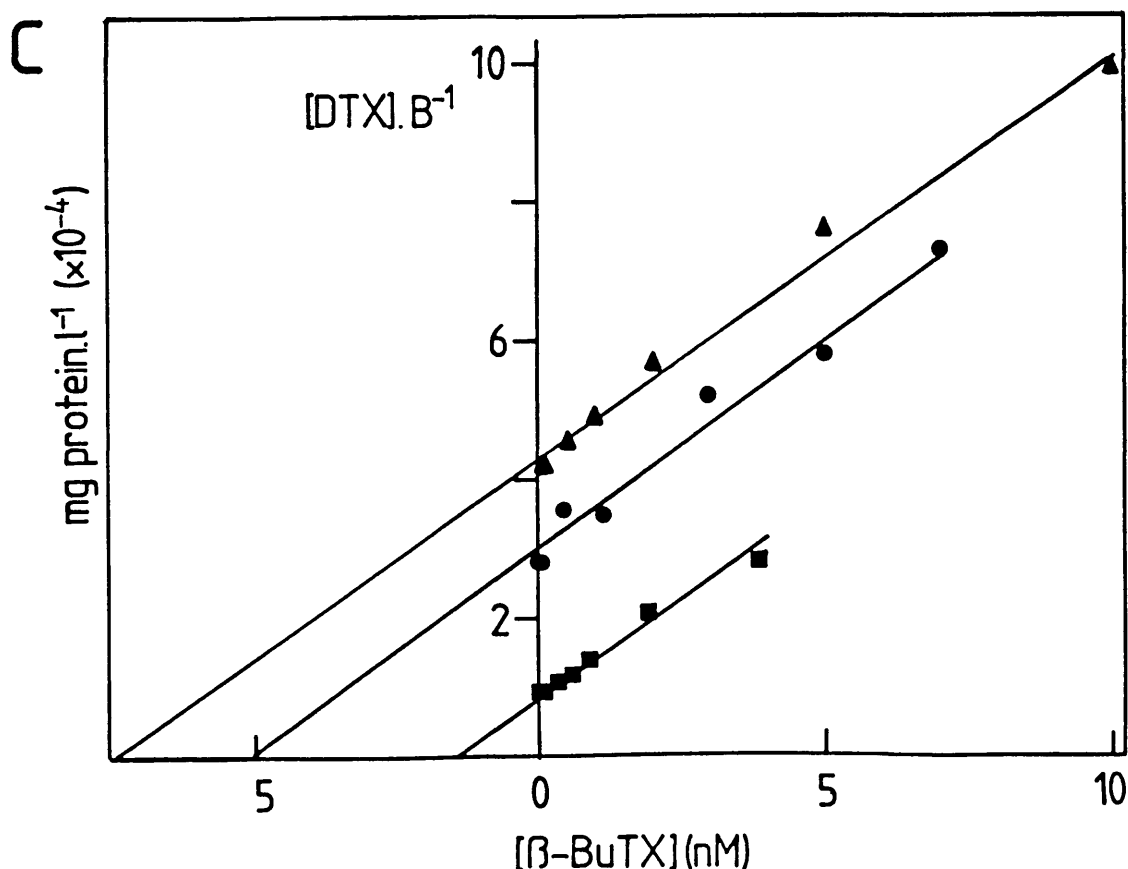
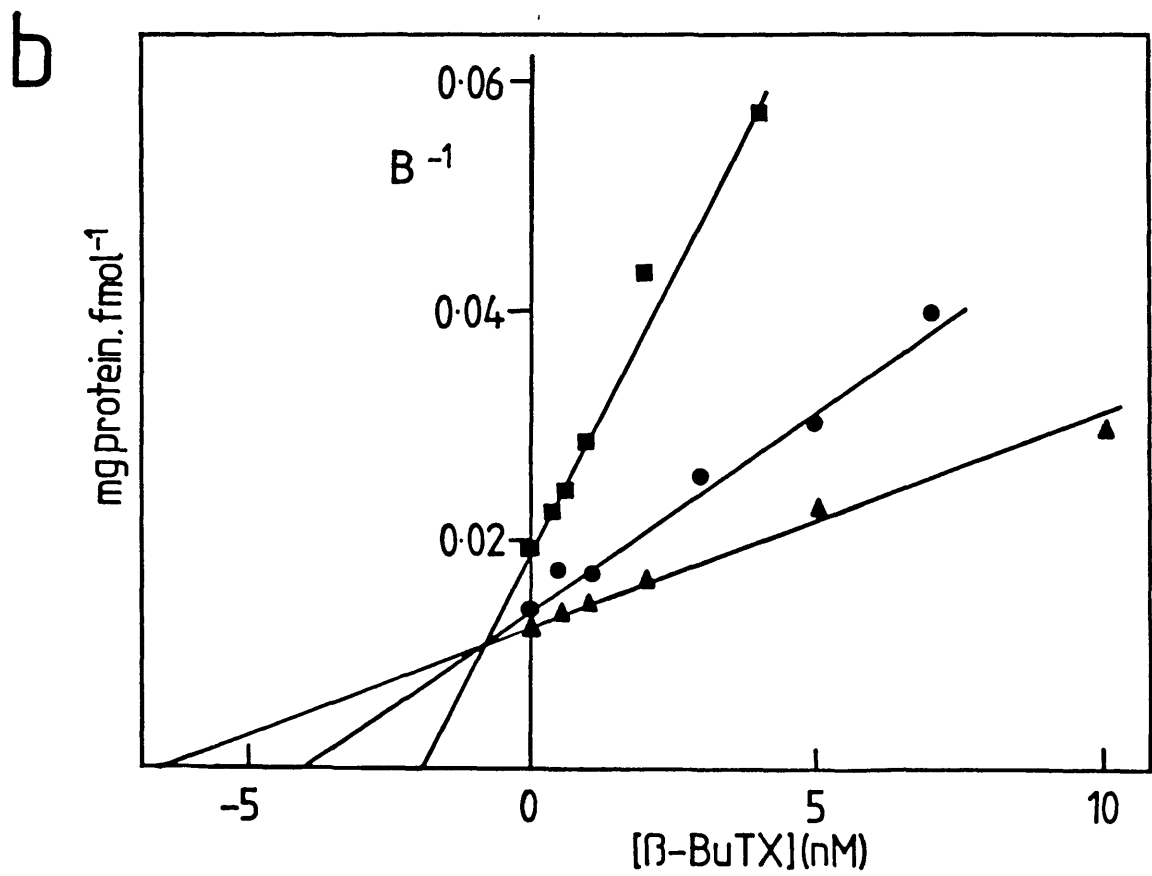
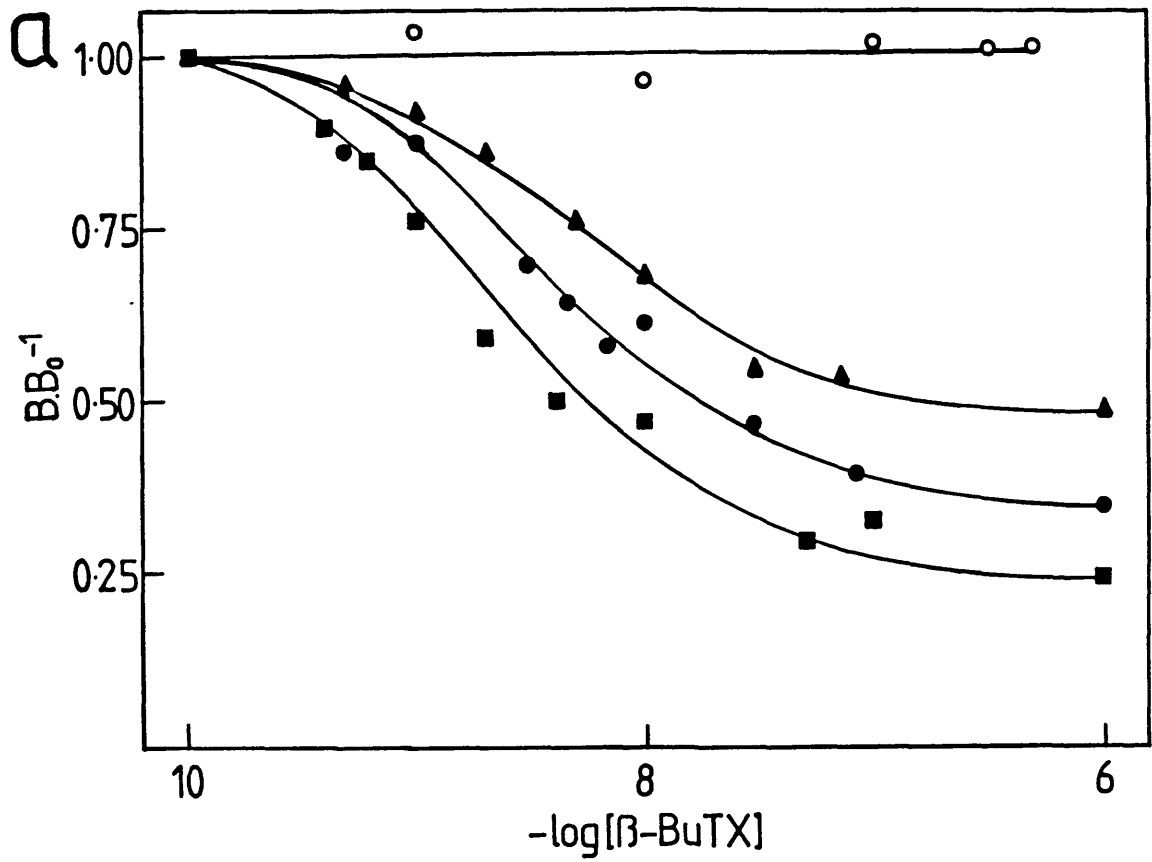


Figure 3.3 Inhibition of the saturable binding of ^{125}I -DTX to chick synaptic membranes by β -BuTX.

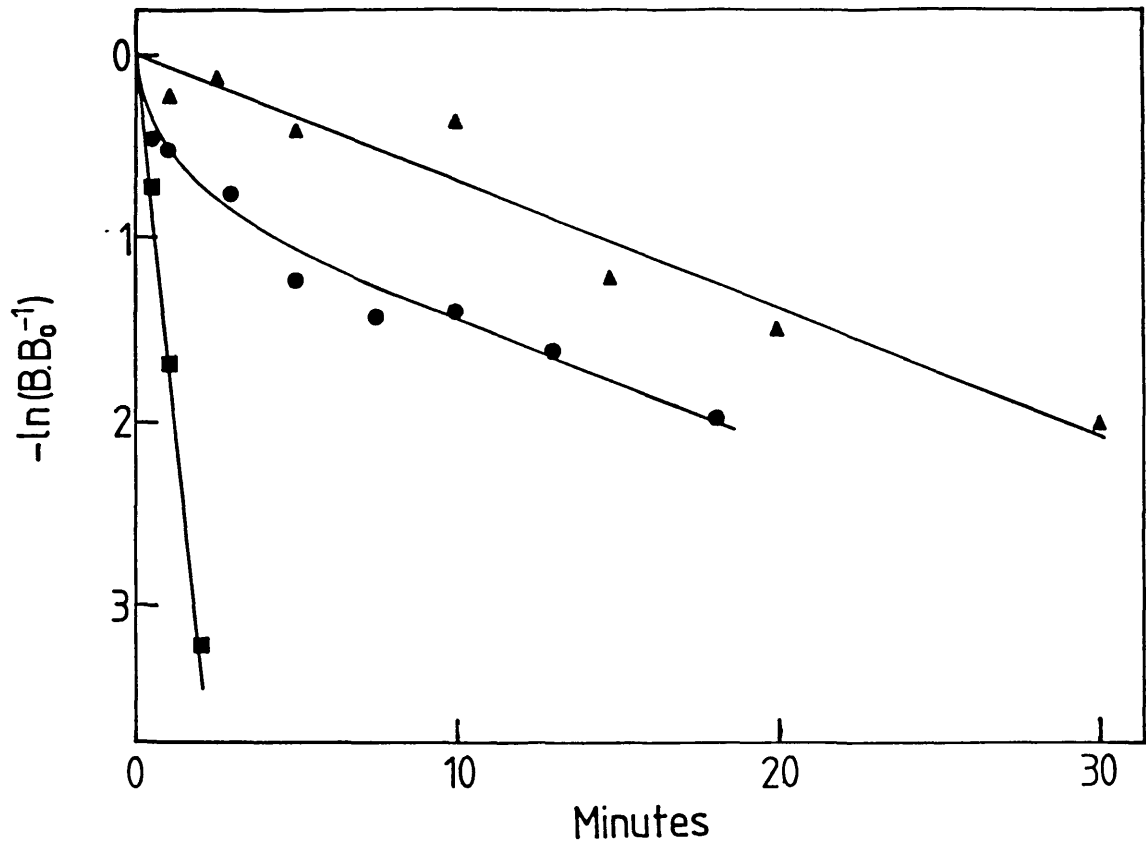


gave a K_I of 0.8nM for β -BuTX and a B_{max} of 90fmol/mg protein for the sites it shares with ^{125}I -DTX. The K_I value, together with the IC_{50} values for the partial inhibitions, gave a K_D of $0.46 \pm 0.5\text{nM}$ for ^{125}I -DTX. This shows that β -BuTX was binding to the higher affinity site for DTX as seen from Scatchard analysis. The absolute levels of ^{125}I -DTX binding observed (69, 110 and 155 fmol/mg protein at 0.5, 2.0 and 3.5nM ^{125}I -DTX) agree well with those calculated for the combined occupancy of the two classes of DTX acceptors, confirming the validity of the graphical analysis.

A semi-log plot of the dissociation rate of ^{125}I -DTX/acceptor complexes, monitored after the addition of a 100-fold excess of DTX, was non-linear (Figure 3.4). This supports the multiple-site model; the upward curve cannot be due to co-operativity as the occupancy of sites remained constant under the conditions used. Since β -BuTX could distinguish a population of binding sites for ^{125}I -DTX, it was used to separate the dissociation of ^{125}I -DTX from its acceptor types. Addition of an excess of β -BuTX to prelabelled membrane revealed a simple first-order dissociation (Figure 3.4) with a $t_{1/2}$ of 11 min ($k_{-1} = 1.2 \times 10^{-3}\text{s}^{-1}$) at 23°C for ^{125}I -DTX from its higher affinity acceptors. In order to investigate the dissociation from the other DTX sites, membranes were prelabelled with ^{125}I -DTX (2nM) in the presence of 500nM β -BuTX; the dissociation, visualised by addition of 200nM DTX, was again linear and gave a k_{-1} of $2.8 \times 10^{-2}\text{s}^{-1}$ ($t_{1/2} = 45\text{s}$). This identification of two simple first-order dissociations provides further convincing evidence for the two site model for DTX binding.

The association rate of ^{125}I -DTX binding to its acceptor types was not amenable to separation by β -BuTX and was too rapid for other methods of dissection ($t_{1/2} \sim 1$ min using 2nM ^{125}I -DTX). However, using the K_D values extrapolated from Scatchard analysis and the above

Figure 3.4 Dissociation of ^{125}I -DTX/acceptor complexes in chick brain membranes.



Membranes (1.2mg protein/ml) were incubated in Sr^{2+} containing assay buffer for 30 min at 23°C with 2nM ^{125}I -DTX in the presence (■) or absence (●, ▲) of 500nM β -BuTX. DTX (to a final concentration of 200nM; ●, ■) or β -BuTX (to a final concentration of 500nM; ▲) was added and aliquots (200 μ l) were removed at appropriate times. The amount of bound ^{125}I -DTX was quantified immediately (see Section 3.2.2).

Non-displaceable binding, determined by the inclusion of 500nM β -BuTX (▲), 200nM DTX (●) or both (■) in the labelling medium, was subtracted from the total binding. A semi-log plot of the data is shown (averages of duplicates, error $\leq \pm 7\%$). The levels of specific binding in incubates performed in the absence and presence of β -BuTX were 115 and 45 fmols/mg protein, respectively; B_0 and B represent the amount of ^{125}I -DTX/acceptor complex at time zero and time t .

dissociation rates, values of $k_{+1} \sim 2.4 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ and $k_{-1} \sim 1.9 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ could be calculated for the high and low affinity acceptors, respectively.

3.3.4 Divalent cation dependence of binding to DTX acceptors.

In view of the finding that DTX and β -BuTX share some common acceptors, it was perhaps surprising that their binding differed in its cation dependence. ^{125}I -DTX binding showed no such dependence; it was unaffected by the buffer used [except that non-saturable binding was slightly higher in phosphate, triethanolamine-HCl (TEA-HCl) or Hepes than in Tris-HCl], by replacement of Na^+ by K^+ or Ca^{2+} by Sr^{2+} in assay buffer, or by the omission of all divalent cations and the addition of EDTA to the assay medium (Table. 3.3). In contrast, no inhibition by β -BuTX of ^{125}I -DTX binding was detectable when using buffer devoid of divalent cations and containing EDTA (Figure 3.3a and Table 3.3, legend). β -BuTX inhibition of DTX binding was observed in TEA-HCl pH 8.5 and when EDTA was added to complete Sr^{2+} containing assay buffer (Table 3.3 and legend). This showed that very low levels of divalent cations fulfil this dependence of β -BuTX binding.

3.4 Discussion

3.4.1 Two classes of DTX acceptors, one of which is the binding component for β -BuTX.

The data presented here show saturable binding of ^{125}I -DTX to two acceptor sites ($K_D \sim 0.5$ and 15nM and $B_{\text{max}} \sim 90$ and 400fmol/mg protein, respectively), with the higher affinity type corresponding to the binding components described for ^{125}I - β -BuTX by Rehm and Betz (1982). This interpretation is strongly favoured for the following reasons: (a) the concentration dependence of saturable ^{125}I -DTX binding was complex (Figure 3.1a); (b) Scatchard analysis of this

Table 3.3 Specific binding of ^{125}I -DTX to chick synaptic membranes in various buffer systems and cation concentrations.

<u>Buffer</u>	<u>Added Cations</u>	<u>Specific binding (%)</u>
Tris-HCl, pH 7.4	Na^+ , Mg^{2+} , Ca^{2+}	100
Tris-HCl, pH 7.4	K^+ , Mg^{2+} , Ca^{2+}	99 $\pm$ 3
Tris-HCl, pH 7.4	Na^+ , Mg^{2+} , Sr^{2+}	102 $\pm$ 6
Hepes, pH 7.4	Na^+ , Mg^{2+} , Ca^{2+}	100 $\pm$ 6
TEA-HCl, pH 7.4	K^+ , Mg^{2+} , Sr^{2+}	98 $\pm$ 2
TEA-HCl, pH 8.5	None	96 $\pm$ 10
Hepes Tris HCl, pH 7.4	Na^+ , Mg^{2+} , Ca^{2+} + EDTA	94 $\pm$ 10
Tris-HCl, pH 7.4	Na^+ + EDTA	110 $\pm$ 5*

Binding was assayed using 2nM ^{125}I -DTX as indicated in Section 3.2.2, except that it was performed in the media indicated above. All buffer concentrations were 50mM except TEA-HCl, pH 8.5, which was 250mM. Cations were added as their chloride salt to the following concentrations Na^+ and K^+ , 100mM; Mg^{2+} , 1mM; Ca^{2+} and Sr^{2+} , 2mM. EDTA was added as its sodium salt to 4mM. Non-specific binding was determined by including 200nM DTX in the incubation medium. Data show saturable binding from two separate experiments ($\pm$ error) expressed as a percentage of that seen in Ca^{2+} containing assay buffer. In all cases, between 66% and 72% of the saturable binding (125-135 fmol/mg protein) could be inhibited by 500nM β -BuTX, except where divalent cations were omitted and EDTA was added (*); here no such inhibition was detected.

binding produced an upward curve, which could be graphically analysed as the two sites (Figure. 3.1b); (c) "chase" experiments using DTX gave a biphasic first-order dissociation for the ^{125}I -DTX/acceptor complexes, ruling out negative co-operativity as a cause of the non-linear Scatchard plot (Figure 3.4); (d) using β -BuTX, two monophasic first-order dissociation rate were revealed (Figure 3.4), showing that β -BuTX could distinguish two populations of DTX acceptors; (e) β -BuTX was only able to partially inhibit the saturable binding of ^{125}I -DTX; this inhibition was from a single class of DTX acceptors (Figure 3.4) and was competitive (Figure 3.3b); (f) the K_I for β -BuTX inhibition (Figure 3.3b) gave a K_D for ^{125}I -DTX at their shared sites of $\sim 0.5\text{nM}$, the same as that obtained for the high affinity site by a Scatchard analysis; (g) the B_{max} of β -BuTX inhibitable ^{125}I -DTX binding [90 fmol/mg protein; (Figure 3.3b)] was the same as that determined for the high affinity site; similarly, the amounts of ^{125}I -DTX binding accorded with the combined occupancy of the two sites, highlighting the accuracy of the graphical analysis; (h) both the K_I value (0.8nM) seen for β -BuTX and the abundance of the high affinity ^{125}I -DTX site agree closely with the K_D (0.5nM) and B_{max} (50 fmol/mg protein) seen for ^{125}I - β -BuTX binding to chick synaptic membrane (Rehm and Betz, 1982) as did the K_I (0.5nM) for DTX inhibition of ^{125}I - β -BuTX binding (from data in Rehm and Betz, 1984) and the K_D of the ^{125}I -DTX at its high affinity sites. Also, in keeping with the identity of the high affinity DTX site and that for β -BuTX, inhibition of ^{125}I -DTX binding by β -BuTX (Table 3.4, legend and Figure 3.3a) and ^{125}I -BuTX binding in chick brain (Rehm and Betz, 1982) were dependent on the presence of divalent cations. Whether the difference in divalent cation dependence of the binding of DTX and β -BuTX resides in association of ions with β -BuTX or the acceptors will require further study. Apart from the interaction of β -BuTX with the

high-affinity sites, both types of DTX acceptors are specific for DTX and neurotoxic homologues; non-neurotoxic congeners and protease inhibitors or other neurotoxins (Figure 3.2, Table 3.2) were unable to inhibit ^{125}I -DTX binding.

Sensitivity of the acceptors to protein denaturing agents and proteolytic enzymes, together with their resistance to PLA_2 , neuraminidase and lectins (Table 3.1 and 3.2), demonstrated that they are proteins, an observation confirmed by the cross-linking experiments described in Chapter 4. The incomplete inactivation produced by the protein denaturing treatments cannot be attributed to a selective action on one class of acceptor, as the fraction of DTX binding that was inhibitable by β -BuTX remained essentially unchanged (Table 3.1, legend). In the case of proteolytic digestion, the residual binding activity probably arises from the acceptors being partially inaccessible in the membrane (cf. Section 2.4). The inability of the other treatments to eradicate acceptor activity was also seen, at least to some extent, with ^{125}I - β -BuTX binding (Rehm and Betz, 1982). It may reflect some contribution of a non-proteinaceous component to the binding activity of the acceptor proteins or, equally, a stabilising influence of their membrane environments.

It is unclear why the effects of bee venom PLA_2 observed for the binding of ^{125}I - β -BuTX binding in chick (Rehm and Betz, 1982) and ^{125}I -DTX binding in rat (Black et al., 1985) were not observed here. This may reside in the enzymic action reducing the level of membranes retained in the assay system used; note that in the present study, PLA_2 treatment reduced both the amount of pelletable protein and toxin binding by 30%. Additionally, a difference in the phospholipids associated with the acceptors in rat and chick and/or in the enzyme preparations used (e.g. mellitin content or substrate specificity) may account for these discrepancies.

3.4.2 The relationship between the two classes of DTX acceptor in rat and chick brain.

Notable similarities are apparent in the properties of the acceptors in chick and rat synaptic membranes (Chapter 2), suggesting that they are related in the two species. The rank order of the affinities of DTX and its homologues was the same in both cases, as were their relative affinities [apart from toxin E whose relative affinity was 7-fold lower in chick brain (cf. Black et al., 1985 and Table 7.1)]. Although no high-affinity inhibition of ^{125}I -DTX binding by β -BuTX was seen in rat brain, the high affinity inhibition of ^3H - β -BuTX binding by both DTX and toxin I ($K_{\text{I}} = 0.5$ and 0.06nM , respectively; Othman, 1983; Othman et al., 1982) suggests that β -BuTX can also distinguish two classes of DTX acceptors therein. Failure to detect potent inhibition of DTX binding by β -BuTX probably results from the large discrepancy in the stoichiometry of ^{125}I -DTX binding to the two acceptor types in rat brain (Chapter 2; Othman et al., 1982). The only important species difference resides in the DTX affinities of these two classes of acceptor; in rat brain these are very similar (see later), whereas in chick they differ by a factor of 30. This has allowed chick synaptic membranes to be used in the first clear demonstration of competitive interaction between DTX and β -BuTX at a single acceptor type. Thus, it seems reasonable to divide DTX acceptors into two classes on the basis of differential interaction with β -BuTX, at least in those species studied to date. Nonetheless, the two acceptor types appear to be closely related to each other. As measured by the inhibition of ^{125}I -DTX binding, the relative affinities of facilitatory neurotoxins were apparently similar at the two sites in chick brain. An analogous situation was seen in rat brain, where the affinities of DTX and toxin I

were essentially the same at the two acceptor types. Their K_I values were practically unchanged when measured using ^{125}I -DTX [where inhibition is mainly at sites exhibiting high affinity for DTX homologues only (Othman 1983; Black et al., 1985)] or ^3H - β -BuTX [which binds avidly to the shared sites only; Othman 1983; Othman et al., (1982)], further highlighting the close relationship between the two classes of acceptors and the acceptors in the two species.

3.4.3 Summary

In keeping with its potent convulsive action in day-old chick brain, DTX bound to proteinaceous acceptors in chick synaptic membranes. Two types of acceptor, which bound the toxin with different affinities, were observed. β -BuTX and DTX interacted competitively only at the high affinity set of acceptors which appears to be the component reported to bind ^{125}I - β -BuTX. As similarities were apparent between the binding of these toxins in chick brain and that in rat, it is proposed that DTX acceptors can be divided into two classes by virtue of their differential binding of β -BuTX.

CHAPTER 4

IDENTIFICATION OF POLYPEPTIDE COMPONENTS OF DTX ACCEPTORS

4.1 Introduction

4.1.1 Identification of acceptor polypeptides by affinity labelling.

Having identified proteinaceous neuronal acceptors for DTX, the next stage in their characterisation was to obtain information about their structure. This can be readily achieved by affinity labelling. A radioactive ligand is allowed to bind to its acceptors in membranes or in solution and the bound ligand is then covalently coupled to acceptor polypeptides. As the ligand will be concentrated on its acceptors, appreciable cross-linking should only occur between the ligand and components of its acceptors. Subsequently, when the samples are subjected to analysis by SDS-PAGE, constituents of the acceptor which are coupled to the radio-ligand will appear as radioactive bands. For these to be ascribed to components of the acceptors, it must be shown that they result from the saturable binding of the radio-ligand; therefore, they should be absent from control samples, which are preincubated with an excess of the native ligand or acceptor antagonists (when available).

The covalent coupling can be achieved using bifunctional alkylating agents (e.g. Hucho et al., 1978) as these contain two reactive groups capable of cross-linking proteins. Alternatively, photoactivatable groups can be used, e.g. azido groups. These are non-reactive when kept in the dark; however, exposure to light produces highly reactive groups which react with proteins. Photoactivatable groups can be coupled to radio-ligands, to produce photoaffinity derivatives, prior to interaction with the acceptors (e.g. Sharkey et al., 1984). On the other hand, they can be linked to alkylating agents and used in a similar way to bifunctional alkylating agents (e.g. Betz et al., 1982).

The major limitation of this method is that a ligand, bound to its acceptor, can be cross-linked to any component within the range of the cross-linker used. However, for cross-linking to non-acceptor polypeptides to be detected, it must occur consistently, and is thus most likely to reflect a physiological interaction of the acceptors.

4.1.2 Studies which have used cross-linking of ^{125}I -DTX and ^{125}I - β -BuTX to identify components of their acceptors.

The cross-linking of ^{125}I -DTX and ^{125}I - β -BuTX to their acceptors from rat (Mehraban et al., 1984) and chick (Rehm and Betz, 1983) brain is of direct relevance to the present study. ^{125}I -DTX was cross-linked to rat synaptic membranes using dimethyl suberimidate (DMS) (Mehraban et al., 1984) a bis-imidoester which reacts with free amines to produce amidines (Hunter and Ludwig, 1962). Analysis of the cross-linked product by SDS-PAGE revealed a single band, which was absent from controls, corresponding to an acceptor polypeptide with an apparent M_r of 65,000. ^{125}I - β -BuTX was cross-linked to chick synaptic membranes using N-succinimidyl-6(4'-azido-2'-nitrophenyl-amino)-hexanoate (SANPAH) (Rehm and Betz, 1983). This asymmetric bifunctional reagent contains an N-hydroxysuccinyl ester, reactive with free amines as described in Chapter 2, and a photoactivatable azido group; illumination of the later produces an extremely reactive aryl nitrene (Cavalla and Neff, 1985). Here SDS-PAGE analysis also revealed a specifically cross-linked radioactive band, corresponding to a polypeptide in the chick brain β -BuTX acceptors with an apparent M_r of 95,000. To enable comparison with these two sets of data, cross-linking of ^{125}I -DTX to its acceptor in chick brain was performed, using both SANPAH and bis-imidoesters. Also, the cross-linking of ^{125}I -DTX to rat synaptic membranes with DMS was analysed in more detail and compared with the pattern obtained with chick membranes.

4.2 Methods

4.2.1 Preparation of rat synaptic membranes.

Synaptic plasma membranes were prepared essentially by the method of Jones and Matus (1974). Sprague-Dawley rats (~200g) were killed by cervical dislocation and their cerebral cortices were removed in less than 30s. The tissue was immediately frozen in liquid N₂ and stored at -80°C until required. All subsequent steps were performed at 4°C. Cortices from 4 rats were thawed in 25ml of 10% (w/w) sucrose containing 5mM Hepes, pH 7.4, 50μM CaCl₂ and homogenised with a glass/teflon homogeniser (0.5mm clearance; 12 strokes at 900 rpm). The resultant homogenate was centrifuged at 4,000 rpm (1000 x g) in a Beckman type 30 rotor and the supernatant was centrifuged at 11,000 rpm (10,000 x g) in the same rotor. The resultant pellet was resuspended in about 10ml of 5mM Tris-HCl, pH 8.1, and the suspension was left on ice for 45 min to lyse the synaptosomes. It was then homogenised gently and made up to three times the volume with 48% (w/w) sucrose in 5mM Hepes, pH 7.4, 50μM CaCl₂ to give a final sucrose concentration of 34% (w/w). This suspension was placed in a 60ml Beckman centrifuge tube and 25ml of 28.5% (w/w) sucrose in the same buffer was layered over it; 10ml of 10% (w/w) sucrose was layered on the top and the tubes was centrifuged at 25,000 rpm (80,000 x g) for 85 min. The synaptic plasma membrane fraction was collected from the 28.5%/34% sucrose interface and, following dilution, was pelleted at 24,000 rpm (54,000 x g) in a Beckman type 30 rotor for 20 min. Samples were resuspended in deionised water and stored at -80°C until required.

4.2.2 Cross-linking of ¹²⁵I-DTX to chick synaptic membranes using SANPAH.

This was done by a modification of the method used by Rehm and Betz (1983) to cross-link ¹²⁵I-β-BuTX. Membranes (1-2mg protein),

prepared as in Section 3.2.1, were incubated with 2nM ^{125}I -DTX in 500 μl of 15mM sodium phosphate, pH 7.4, 1mM CaCl_2 , 0.5mM MgCl_2 , 90mM NaCl at 23°C. Non-specific cross-linking was determined by including 200nM DTX in the incubation. After 30 min, SANPAH (10-50 μl dissolved in dimethyl sulphoxide) was added in the dark to give the stated concentration. After 10 min the tubes were illuminated for a period of 10 min with a Thorm EMI 60W bulb held at a distance of 12cm. The membranes were then washed (2 x 1ml) with 100mM Tris-HCl and analysed as described in Section 4.2.4.

4.2.3 Cross-linking of ^{125}I -DTX to synaptic membrane using bis-imidoesters.

Synaptic membranes from chick (1-2mg) or rat (1-1.5mg) brain were prelabelled with 2nM ^{125}I -DTX in 500 μl of Sr^{2+} containing assay buffer (described in Section 3.2.2) at 23°C. After 30 min, the membranes were pelleted and washed with 1ml of ice-cold TEA-HCl, pH 8.5, (to remove the Tris, a free amine, and the unbound toxins as well as optimising the pH) and were immediately resuspended in the same buffer (at 23°C), but containing various concentrations of the bis-imidoester indicated. After 30 min exposure to bis-imidoester, the membranes were pelleted and washed (2 x 1ml) with 100mM Tris-HCl, pH 7.4. Alternatively, prelabelling was carried out in TEA-HCl, pH 8.5, and the bis-imidoester was added (20 μl) as a concentrated solution to the incubation, without removal of unbound toxins. After 15 min the membranes were washed as above. In both cases, control samples were produced by prelabelling in the presence of 200nM DTX. The pharmacological specificity of the cross-linking was determined by including competing ligand at the stated level in the initial incubation medium.

4.2.4 Analysis of the cross-linked products.

Cross-linked samples were incubated for 10 min in a cocktail of protease inhibitors (100mM imidazole, pH 7.4, 2mM EDTA, 0.02% (w/v) bacitracin, 0.04% (w/v) STI, 2×10^{-4} M benzamidine, 8×10^{-4} M PMSF, 2mM dithiothreitol) to inactivate membrane bound proteases, prior to addition of 150µl of 1% SDS [sample buffer (Section 2.2.3)] and solubilisation at 95°C for 15 min. (Due to its short half life in aqueous solution, PMSF was dissolved in absolute alcohol immediately prior to its use, following addition to the buffer as a concentrated solution.) Unless otherwise stated, samples were reduced by using sample buffer containing 5% (v/v) β-mercaptoethanol. Samples were centrifuged for 15 min in an Eppendorf microcentrifuge to pellet remaining membranes and 75µl of the supernatant was subjected to electrophoresis at 200V until the tracking dye approached the bottom of the gel (linear gels) or until 2 h after it had left the gel (gradient gels). Linear 8% (w/v) acrylamide gels were prepared in the buffer systems described in Section 2.2.3(b) by the method of Laemmli (1970). Gradient gels were prepared as in Section 2.2.3(b) except that 5% and 20% (w/v) acrylamide solutions were used, which contained 0.06% (v/v) TEMED and 0.035% (w/v) ammonium persulphate.

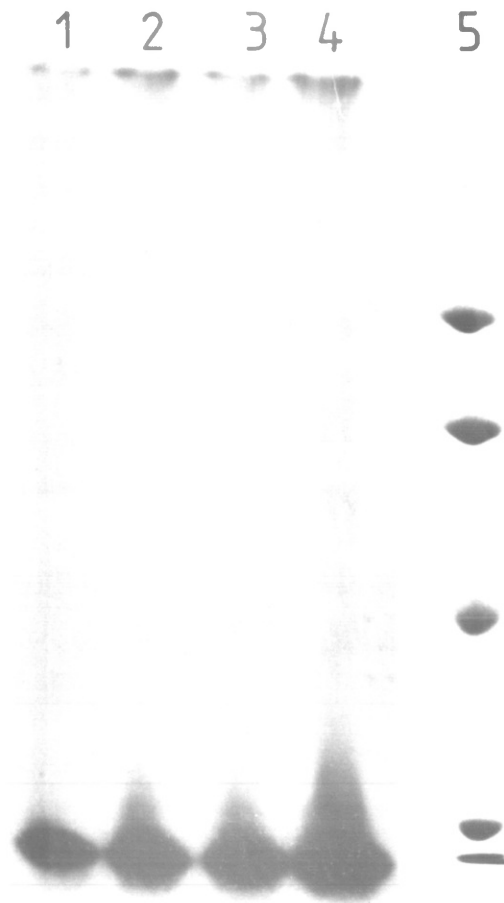
After electrophoresis, the gels were stained, dried and autoradiographed as described in Section 2.2.3(b) (except that linear gels were washed in 5% glycerol before drying).

4.3 Results

4.3.1 Components of the DTX acceptors from chick brain.

No specific cross-linking of ^{125}I -DTX to synaptic membrane proteins was seen using SANPAH (Figure 4.1), perhaps reflecting the weak reaction of N-hydroxy succinimidyl esters with DTX observed in Chapter 2. The radioactive material which failed to enter the gel can

Figure 4.1 Cross-linking of ^{125}I -DTX to chick synaptic membranes using SANPAH.

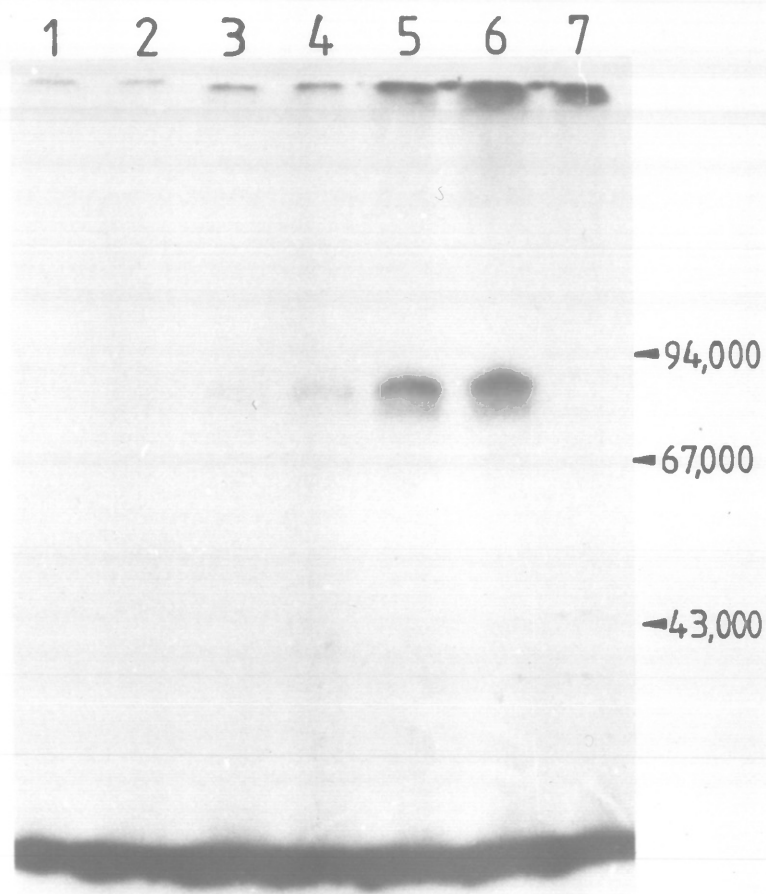


Membranes (1-1 5mg protein) were labelled with ^{125}I -DTX (2nM) in the absence (track 1, 2 and 4) or presence of 200nM DTX (track 3). After 30 min at 23°C, SANPAH was added to give final concentrations of 1mM (track 1), 3mM (track 2 and 3) or 5mM (track 4). After illumination for 10 min, samples were washed and cross-linking was analysed by SDS-PAGE and autoradiography. Track 5 shows protein standards visualised by protein staining. These had M_r values as follows: phosphorylase b (94,000); bovine serum albumin (67,000); ovalbumin (43,000); soy bean trypsin inhibitor (20,000).

be ascribed to ^{125}I -DTX cross-linked into large aggregates. As this material became greater with increasing SANPAH concentrations (Figure 4.1), it shows that, although unable to produce specific cross-linking, the reagent was active. The radioactive band at the dye front is presumably due to free toxin, although some cross-linking to low molecular weight material cannot be excluded.

In contrast to SANPAH, bis-imidoesters were able to specifically cross-link ^{125}I -DTX to membrane polypeptides. Figure 4.2 shows the pattern obtained when various concentrations of DMS were used to cross-link ^{125}I -DTX to chick synaptic membranes. In all cases, as described above, most of the radioactivity migrated with the dye front and some failed to enter the gel. With DMS concentrations above 0.05mg/ml, a radioactive band with an apparent M_r of 82,000 could be seen (Figures 4.2 and 4.3D); with increasing DMS concentrations (>0.1mg/ml), a second (apparent M_r = 76,000) was observed (Figures 4.2 and 4.3D). Neither of these bands was present when membranes had been labelled in the presence of excess DTX and, therefore, both result from saturable binding of ^{125}I -DTX. Minor amounts of non-specifically cross-linked material could often be seen below the two major bands. These presumably arose because, after the rapid washing protocol used, only ~30% of the ^{125}I -DTX present was bound to its acceptors. Similarly, faint bands due to specific cross-linking were often seen above the main bands, which are presumably due to intra-membrane cross-linking and/or to cross-linking of ^{125}I -DTX with further components of its acceptors. Intra-membrane cross-linking does not appear to contribute to the major cross-linked species as: (a) they both appeared at DMS concentrations at which no other specific cross-linking was seen (Figure 4.2); (b) no additional protein bands were produced when membranes were cross-linked with 0.5mg/ml DMS [except for some highly cross-linked material which failed to enter the gel;

Figure 4.2 Dependence of the cross-linking of ^{125}I -DTX to chick synaptic membranes on DMS concentration.



Membranes (1-1.5mg protein) were prelabelled with 2nM ^{125}I -DTX in 500 μl Sr^{2+} containing assay buffer. After 30 min at 23°C unbound ^{125}I -DTX was removed by washing with 1ml of ice-cold 0.25M TEA-HCl, pH 8.5 and the membranes were incubated at 23°C in 500 μl of the same buffer containing various concentrations of DMS. After 30 min, membranes were washed repeatedly with 100mM Tris-HCl buffer, pH 7.4, and analysed by SDS-PAGE (8%) and autoradiography. Non-specific cross-linking (track 7) was determined by inclusion of 200nM DTX in the initial incubation medium. DMS concentrations (mg/ml) used in the respective tracks were: (1) 0.005; (2) 0.01; (3) 0.05; (4) 0.1; (5) 0.5; (6) and (7) 1. Arrows show the positions and M_r values of Coomassie stained protein standards: phosphorylase b, bovine serum albumin and ovalbumin.

(Figure 4.3A)]. Cross-linking between toxin molecules cannot account for the specifically cross-linked bands. Apart from some highly cross-linked material concentrated at the top of the gel, only free toxin running at the ion front was seen when membranes were omitted during cross-linking (Figure 4.3B).

Although reduction of samples increased the resolution of the gel system, it did not affect the relative mobility of the cross-linked species (Figure 4.3C). This observation shows that the cross-linked membrane polypeptides are not linked to other membrane components through disulphide bridges.

Both of the major radioactive bands were also seen when bis-imidoesters with shorter carbon chains than DMS were used (Figure 4.4). However, dimethyl adipimate, the shortest, was only able to cross-link the lighter band relatively weakly (Figure 4.4, track 2), demonstrating that the reactive groups in the bound toxin were closer to those in the heavier polypeptide than to those in the lighter.

Cross-linking to these two bands was abolished by prelabelling in the presence of excess DTX, toxin I or E, whereas toxin B or BPTI was ineffective (Figure 4.5). This shows that the cross-linked products result from interaction of toxin with the sites identified in binding studies. (More detailed study of the efficiency of these homologues was precluded by the semi-quantitative nature of the autoradiography.) Interestingly, β -BuTX was only able to partially inhibit both of these bands when cross-linking was carried out with or without removing unbound toxins (Figure 4.5, tracks 6 and 7, Figure 4.6). Thus, binding of ^{125}I -DTX to both of its acceptor types contributes to the cross-linking of the two bands.

A contribution of proteolysis to the appearance of the two major polypeptides seems unlikely, due to the differential cross-linking by various bis-imidoesters and the use of protease inhibitors during

Figure 4.3 SDS-PAGE analysis of various samples treated with DMS.

- (A) The effect of DMS on the protein pattern of membranes. Following incubation of chick synaptic membranes in 0.25M TEA-HCl, pH 8.5, with (track 3) or without (track 2) 0.5mg/ml DMS for 30 min at 23°C, membranes (~0.25mg protein) were solubilised, analysed by SDS-PAGE and gels were Coomassie stained. Track 1 shows protein standards (with respective M_r values) phosphorylase b (94,000); bovine serum albumin (67,000); catalase (60,000); ovalbumin (43,000) and carbonic anhydrase (30,000).
- (B) Crosslinking of ^{125}I -DTX by DMS. ^{125}I -DTX (2nM) was incubated with 0.5mg/ml DMS as described for membranes in (A) and cross-linking was analysed by SDS-PAGE and autoradiography. Track 5 shows the Coomassie stained standards phosphorylase b, bovine serum albumin and ovalbumin.
- (C) The effect of reduction on the pattern of cross-linking of ^{125}I -DTX to chick synaptic membranes. ^{125}I -DTX (2nM) was incubated with chick synaptic membranes (1-1.5mg protein) in the presence (tracks 7 and 10) or absence (tracks 6 and 9) of 200nM DTX. Cross-linking was performed using 0.5mg/ml DMS following removal of unbound toxin and was analysed by SDS-PAGE under reducing (tracks 6,7 and 8) or non-reducing (tracks 9, 10 and 11) conditions followed by autoradiography. Tracks 8 and 11 show protein standards as in (A).
- (D) Semi-log plot of the electrophoresis of protein standards in (C). The positions of the two cross-linked species in track 6 are shown by the arrows.

Figure 4.3 SDS-PAGE analysis of various samples treated with DMS.

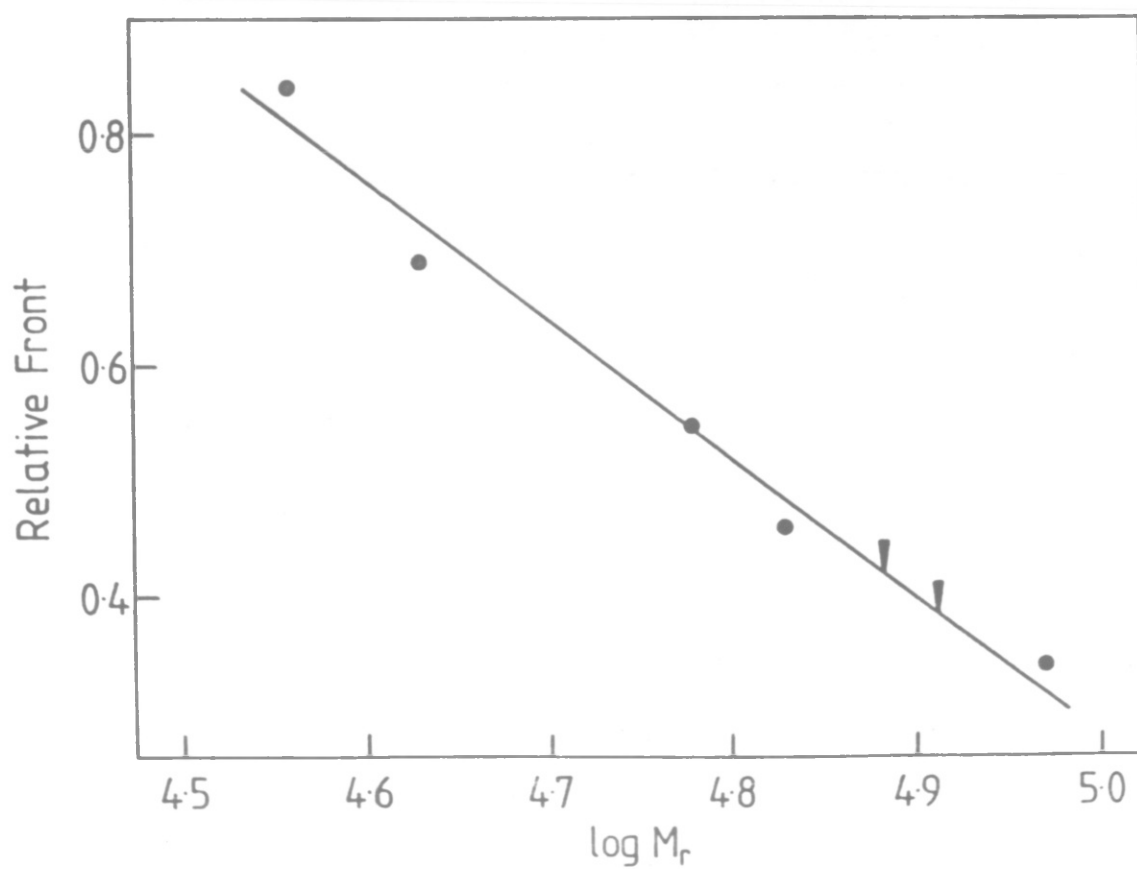
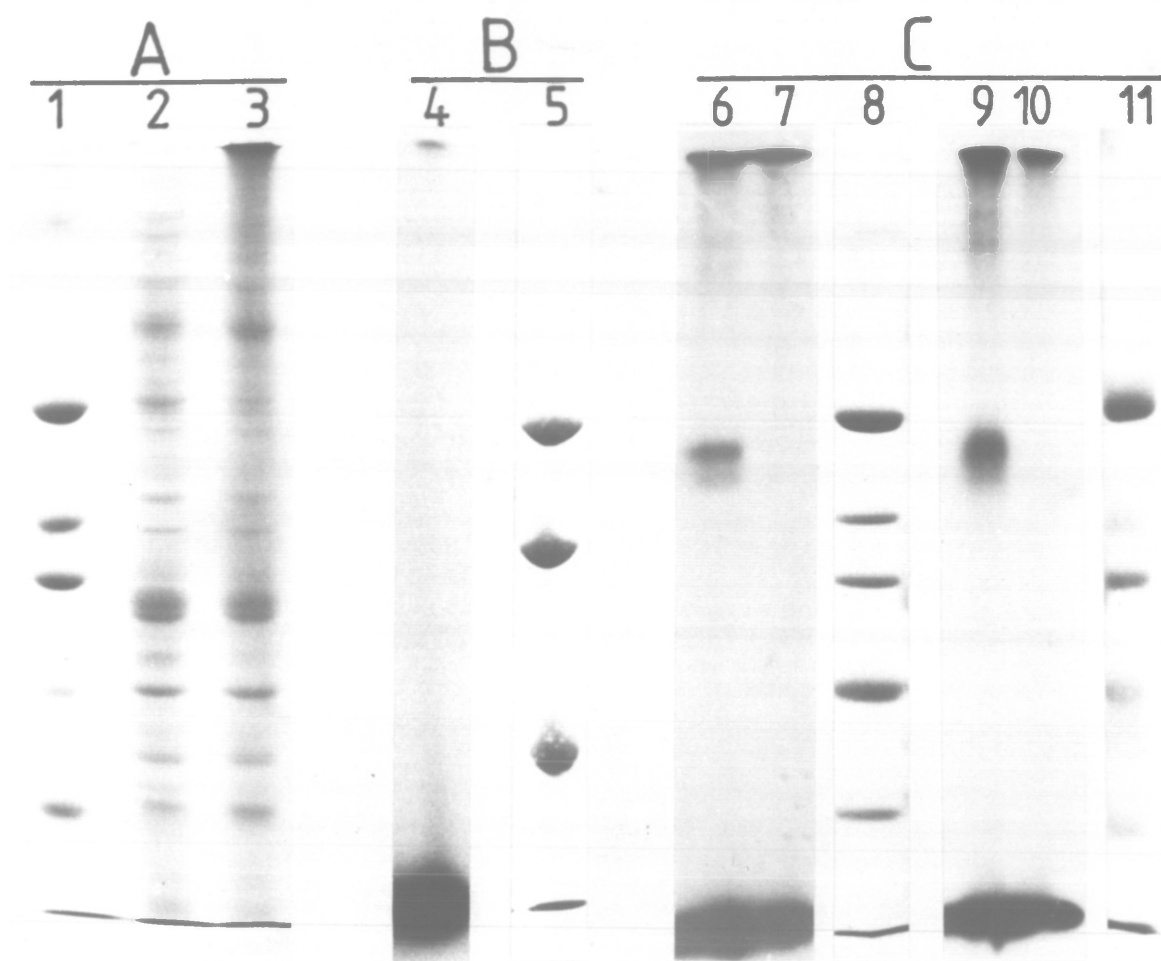


Figure 4.4 Cross-linking of ^{125}I -DTX to chick synaptic membranes using bis-imidoesters of various chain lengths.



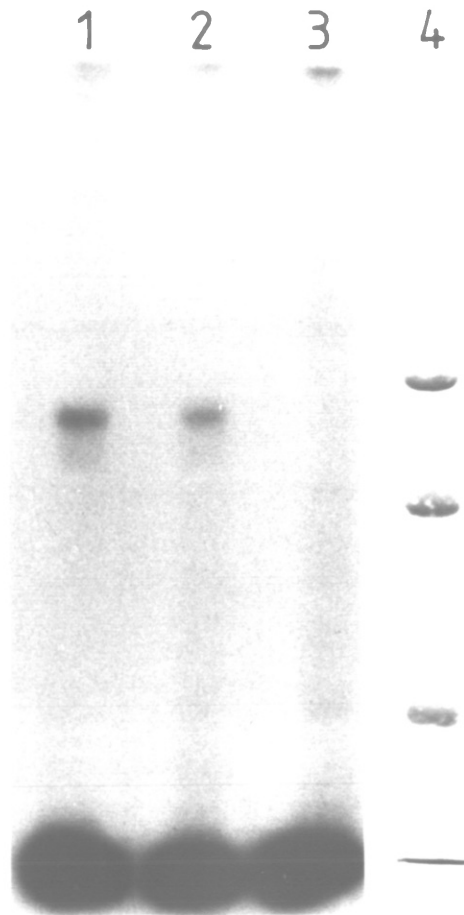
Membranes were prelabelled with 2nM ^{125}I -DTX as described in Section 4.2. After removal of free ^{125}I -DTX by washing, the membranes were exposed to bis-imidoesters (0.5mg/ml) in 500 μl of 0.25M TEA-HCl, pH 8.5, for 30 min and then washed repeatedly with 100mM Tris-HCl, pH 7.4. The products were analysed by SDS-PAGE (8%) and autoradiography. Non-specific cross-linking was determined by inclusion of 200nM DTX in the original incubation (1, 3 and 5). bis-Imidoesters used were dimethyladipimate (1 and 2), DMS (3 and 4) and dimethylpimilimide (5 and 6). Stained protein standards are shown in track 7. These are (with M_r value): phosphorylase b (94,000), bovine serum albumin (67,000), catalase (60,000), ovalbumin (43,000) and carbonic anhydrase (36,000).

Figure 4.5 Pharmacological specificity of the cross-linking of ^{125}I -DTX to chick synaptic membranes by DMS.



Membranes were labelled with 2nM ^{125}I -DTX in Sr^{2+} containing assay buffer in the presence of the various agents. After removal of free ^{125}I -DTX by washing, membranes were cross-linked for 30 min at 23°C using 0.5mg/ml DMS in 0.25M TEA-HCl, pH 8.5. After repeated washing, the membranes were subjected to SDS-PAGE analysis, followed by autoradiography. Agents included in the initial incubations were 200nM DTX (1), 200nM toxin I (2), 1 μM toxin E (3), 1 μM toxin B (4), 1 μM BPTI (5), none (6), and 1 μM β -BuTX (7). Arrows show the positions of the protein standards (visualised by staining); phosphorylase b (94,000), bovine serum albumin (67,000) and ovalbumin (43,000).

Figure 4.6 Cross-linking of ^{125}I -DTX to chick synaptic membranes in the presence of unbound toxins.



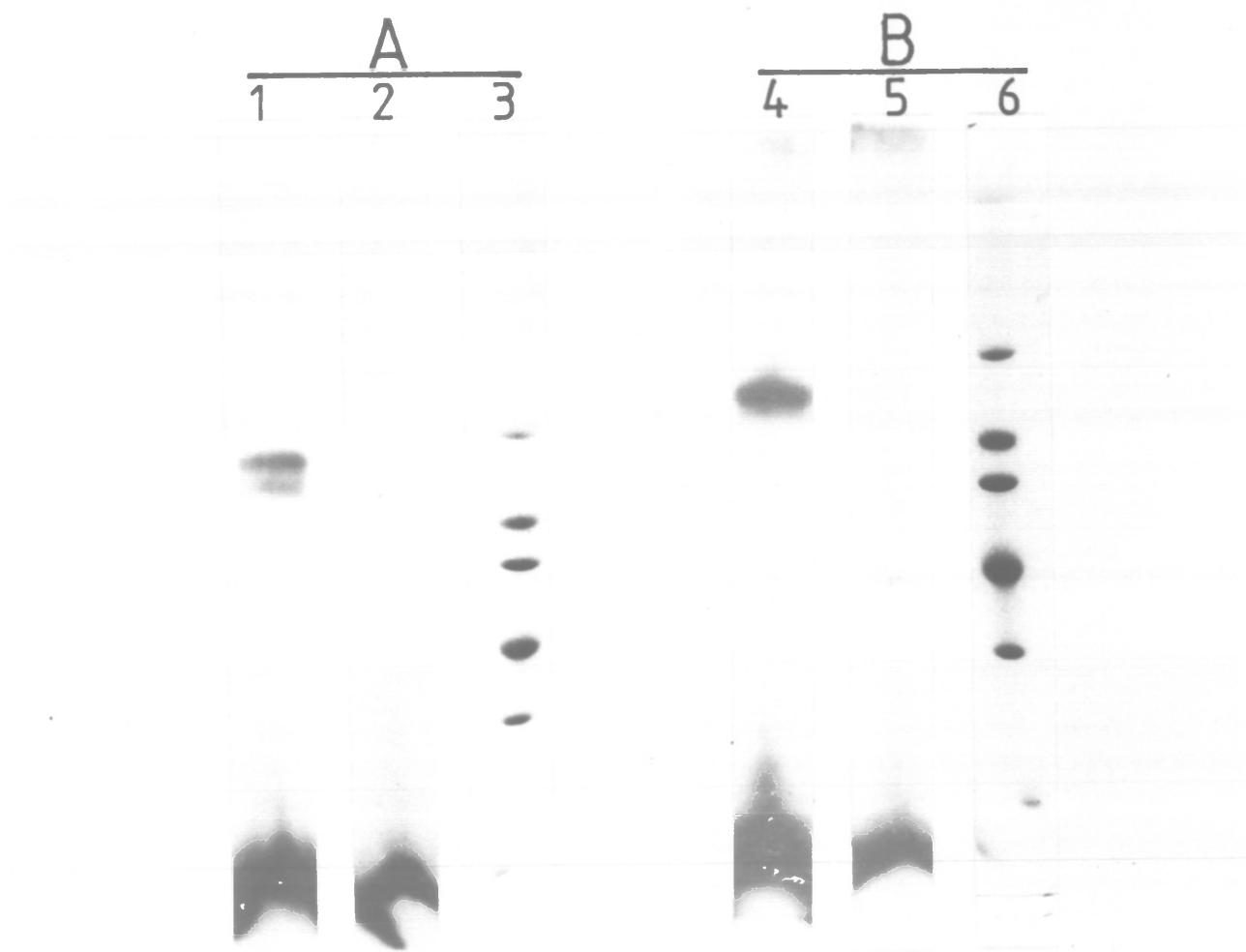
Membranes (1-1.5mg protein) were labelled with 2nM ^{125}I -DTX, in the absence (track 1) or presence of 50nM β -BuTX (track 2) or 200nM DTX (track 3) DTX in 0.25 TEA-HCl, pH 8.5. After 30 min at 23°C, 10 μ l of 25mg/ml DMS was added. Membranes were washed repeatedly after a further 15 min and were analysed by SDS-PAGE and autoradiography. Coomassie stained protein standards are shown in Track 4. These are: phosphorylase b (94,000), bovine serum albumin (67,000) and ovalbumin (43,000).

solubilisation (where proteolysis is most likely to occur). Thus, it seems reasonable to assume that the two bands arise from separate polypeptides of the DTX binding component. Assuming equimolar cross-linking of toxin (see Discussion), these two acceptor polypeptides would have apparent M_r of 75,000 and 69,000. The presence of acceptor polypeptides besides those identified above seems likely because of the small amounts of specifically cross-linked material seen in addition to the major cross-linked species. Further, the relatively low levels of toxin which were cross-linked (<5% of that present during the coupling reaction) indicates that only the more reactive species have been identified.

4.3.2 SDS-gradient-PAGE analysis of the products of ^{125}I -DTX cross-linking to rat and chick synaptic membranes.

When the cross-linking of ^{125}I -DTX to chick synaptic membranes using DMS was analysed on SDS-gradient-PAGE, two cross-linked radioactive bands, which were absent in the control, were again seen (Figure 4.7A). These had apparent M_r of 86,000 and 79,000 which agree with those seen on SDS-linear-PAGE. Analysis of the cross-linking to rat synaptic membranes by SDS-gradient-PAGE also revealed specific cross-linking to a major species together with a lesser amount of a smaller polypeptide (Figure 4.7B). However, these were only partially resolved, which explains why they appeared as a single band in linear gels (Mehraban *et al.*, 1984). The apparent M_r of these bands was 82,000 and 77,000 which is in reasonable agreement with that of 72,000 found from linear gels.

Figure 4.7 Analysis of ^{125}I -DTX cross-linking to synaptic membranes by SDS-gradient-PAGE.



Chick (A) and rat (B) membranes were labelled with ^{125}I -DTX (2nM) in the presence (tracks 2 and 5) or absence (tracks 1 and 4) of 200nM DTX and cross-linking was performed using 0.5mg/ml DMS as described in Section 4.2. The samples were then solubilised with SDS and electrophoresed on a 5-20% acrylamide gel which were subsequently dried and autoradiographed. Tracks 3 and 6 indicate the positions of standard proteins (Coomassie stained) with the stated M_r values: phosphorylase b (94,000), bovine serum albumin (67,000), catalase (60,000), ovalbumin (43,000), carbonic anhydrase (36,000) and ferritin (18,500).

1971) and often extract membrane proteins in an active form. On the other hand, detergents comprised of aliphatic chains with cationic or anionic head groups, e.g. SDS, are not generally applicable. These detergents bind to most proteins in a co-operative manner, resulting in the disruption of the tertiary and quaternary structures (Reynolds et al., 1967; Nelson, 1971; Nozaki et al., 1974).

5.1.2 Factors affecting the activity of detergent solubilised proteins.

Specific interactions with membrane lipids may be required to maintain the conformation of certain proteins; in these cases solubilisation will lead to loss of activity if the detergent used cannot act as a satisfactory substitute. When this occurs, the activity can sometimes be retained by changing the detergent; e.g. the interaction of γ -amino-butyric acid and [^{3} H]flunitrazepam at the benzodiazepine receptor was seen when CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulphonate) but not deoxycholate was used for extraction (Mernoff et al., 1983). In other cases no detergent can be found to replace the interaction of membrane lipids and specific lipids have to be added. This was necessary for the voltage-sensitive Na^+ channel, where phosphatidyl choline was added to stabilise its saxitoxin binding activity (Hartshorne and Catterall, 1984). As with the last example, the requirement for specific lipids may not lead initially to loss of activity. If the essential lipids are bound strongly to the protein, they will dissociate slowly (Tanford and Reynolds, 1976), resulting in instability of the active extracts.

Frequently, conformational changes are induced by solubilisation which lead to inactivation of the protein; this can often be prevented by the presence of particular ligands in the extraction. For example,

when solubilised with CHAPS and digitonin, voltage-sensitive Ca^{2+} channels required Ca^{2+} to retain the ability to bind [^3H]nimodipine (Glossmann and Ferry, 1983).

Certain membrane proteins are more readily extracted by particular detergents. This can also affect the activity of soluble extracts (Liljas *et al.*, 1974; Hjerten and Johansson, 1972). Selectivity of solubilisation can also be obtained by varying the ionic strength (Kyte, 1971). Thus, by altering the conditions, considerable enrichment of a particular membrane component in the extract can be obtained (e.g. Kuboyama *et al.*, 1972).

From the cited examples, it is obvious that the conditions of extraction need to be optimised to obtain maximal production of solubilised membranes proteins in an active form. Therefore, the effectiveness of various detergents and conditions should be evaluated.

5.1.3 Assay systems for binding of ligands to their solubilised acceptors.

One of the problems in the study of solubilised acceptors is the task of separating free ligands from ligand/acceptor complexes. Several separation methods have evolved which include differential precipitation by polyethylene glycol (Stephenson *et al.*, 1982) or ammonium sulphate (Gavish *et al.*, 1979), adsorption of acceptor/ligand complex onto ion-exchange discs [e.g. the DEAE disc assay for α -bungarotoxin binding to acetylcholine receptor (Schmidt and Raftery, 1973)] or gel filtration (Caron and Lefkowitz, 1976). The usefulness of these systems is limited by their ability to differentiate bound and free ligand. If >5-10% of the free ligand is not separated from the acceptor/ligand complexes, the sensitivity of the assay will be greatly reduced.

5.1.4 The choice of tissue for the present study.

Until the solubilisation conditions are optimised, it is best to use a tissue with the highest possible content of acceptors. For the present study, it was decided to use synaptic membranes purified from rat cerebral cortex, which have a higher level of DTX acceptors than rat synaptosomes (~1.5 times) or chick synaptic membranes (~3 times) (Chapters 2 and 3).

In the following sections of this chapter, the extraction of active DTX acceptors from these membranes, together with the identification and binding characteristics of these solubilised acceptors, are described.

5.2 Methods

5.2.1 Suitability of assay systems for measuring ^{125}I -DTX binding to its solubilised acceptors.

In order to assess the ability of various assay systems to separate bound from free toxin, these were applied to toxin (5-10nM) in 20mM Hepes, pH 7.4, 50mM KCl containing either 1.5% (w/v) Triton X-100 or deoxycholate, in the absence of solubilised extract. Polyethylene glycol and ammonium sulphate precipitation assays were carried out by adaptations of the methods of Stephenson et al. (1982) and Gavish et al., (1979), respectively. All procedures were carried out at 4°C. Bovine γ -globulin (15 μ l of 33mg/ml) was added to 240 μ l of ^{125}I -DTX followed by 255 μ l of 60% saturated ammonium sulphate or 80 μ l of 35% (w/v) polyethylene glycol. These mixtures were vortexed and the precipitate was pelleted at 9,000 x g for 5 min in an Eppendorf microcentrifuge. The supernatant was removed and the amount of ^{125}I -DTX in the pellets was quantified (by γ -counting) following superficial washing with 1ml of 100mM NaCl in 20mM Hepes, pH 7.4. The DEAE disc assay was adapted from

the method of Cremona et al (1981); ^{125}I -DTX (50 μl) was added to 2.3cm Whatman DE-81 discs. After 1 min, these were washed with 3 x 2ml of 50mM sodium phosphate, pH 7.4, 0.02% (w/v) Triton X-100 by filtration under vacuum. Discs were then dried and subjected to γ -counting at 50% efficiency.

The gel filtration assay was essentially that of Caron and Lefkowitz, (1976); ^{125}I -DTX (200 μl) was loaded onto Sephadex G-50 columns (0.5 x 10cm), equilibrated at 4°C with the incubation buffer, except that it contained only 0.02% (w/v) of the appropriate detergent. The samples were then eluted, under gravity; fractions (40 μl) were collected and γ -counted. The radioactivity seen in the initial peak (void volume) was taken as the amount of toxin which would have been detected along with that bound to acceptors. Rapid gel filtration was carried out by the adaption of the method of Lefkowitz et al. (1972) reported by Rehm and Betz (1984). This used a different buffer system. Biogel-P60 columns (2ml) were prepared in 2ml syringes and washed with 1ml of 1mg/ml bovine serum albumin followed by 2 x 1ml of 50mM imidazole-HCl buffer, pH 7.4, 100mM KCl containing 0.3% (w/v) Triton X-100. These were placed inside AS144 Sterilin tubes and centrifuged for 3 min at 2,400 rpm (1,000 x g) in an International Equipment Company portable centrifuge. The columns were then transferred to new Sterilin tubes and 200 μl of ^{125}I -DTX (5nM) in 50mM imidazole-HCl, pH 7.4, 100mM KCl containing 0.3% (w/v) Triton X-100 or deoxycholate, was loaded and the columns were centrifuged as described above. The eluted liquid (~200 μl), in which acceptor complexes would appear, was subjected to γ -counting at 50% efficiency.

5.2.2 Preparation of soluble extracts of rat synaptic membranes.

For detergent extracts, 50mM imidazole-HCl, pH 7.4, containing the stated levels of the various cations (chloride salts), detergents, and a

cocktail of protease inhibitors (1mM EDTA, 0.01% (w/v) bacitracin, 0.02% (w/v) STI, 10^{-4} M benzamidine, 5×10^{-4} M PMSF) was added to pelleted rat synaptic membranes (Section 4.2.1); 1ml was added per 15mg membrane protein. The mixture was vortexed and then shaken gently at 4°C. After 1h the incubation mixture was pelleted at 24,000 rpm (34,000 x g) for 30 min in a Beckman type 50 rotor, using 2ml adaptors. The supernatant was recentrifuged at 40,000 rpm (100,000 x g) for 1h in the same rotor and the final supernatant was collected.

Salt extract was prepared by omission of detergent and with the inclusion of 1M NaCl and 0.25M KCl in the extraction medium.

5.2.3 Binding of ^{125} I-DTX to its solubilised acceptors.

Except where otherwise stated, extract was produced routinely as in Table 5.1; 10 μ l was added to the binding medium in a final volume of 250 μ l and final concentrations of 50mM imidazole-HCl, pH 7.4, 10mM KCl, 20mM NaCl and ~0.16% Triton X-100. After 15 min at 23°C, 200 μ l of the incubation was subjected to rapid gel filtration as described in Section 5.2.1, except that columns were equilibrated with the incubation medium. ^{125} I-DTX and competing ligands were included in the incubations at the stated levels; non-saturable binding was determined in the presence of 120nM DTX (or 200nM DTX for Scatchard analysis). Free ^{125} I-DTX was taken as being the difference between the total toxin loaded onto the column and that present in the eluate. For measurement of the rate of dissociation of the acceptor ligand complexes, acceptors were labelled with ^{125} I-DTX (1.2nM) as above and 120nM DTX was added at the stated times before the incubation mixture was added to the columns. Non-displaceable binding was determined by including 120nM DTX in the initial incubation and adding buffer at appropriate times. Protein was measured by the method of Bradford (1976) using bovine serum albumin as a standard. Kinetic analyses were performed as described in Appendix A.2.

Table 5.1 Optimised conditions for the solubilisation of neuronal acceptors for DTX.

15mg protein of rat synaptic membranes were solubilised in 1ml of:

50mM imidazole-HCl, pH 7.4, containing:

2 x 10⁻⁴M benzamidine
0.02% (w/v) STI
0.01% (w/v) bacitracin
1mM EDTA
4 x 10⁻⁴M PMSF

4% (w/v) Triton X-100

500mM NaCl

250mM KCl;

for 1h at 4°C.

Membranes were removed by pelleting at 34,000g for 30 min, followed by pelleting at 100,000g for 1h.

5.2.4 Treatment of membranes and soluble extracts.

Rat synaptic membranes were treated at 95°C or with 1M HCl and controls were prepared as described for synaptosomes in Section 2.2.8. Following extraction (Table 5.1), binding was assayed as described in Section 5.2.3. For proteolysis, extracts were produced as described in Table 5.1 except that the NaCl and the protease inhibitors were omitted; the extract was incubated with 1mg/ml trypsin or chymotrypsin at 37°C. After 40 min, STI (to 5mg/ml for trypsin) or PMSF (to 5mM for chymotrypsin), was added. The incubation mixture was diluted with 50mM imidazole-HCl, pH 7.4, and assayed as described in Section 5.2.3. Controls were prepared by addition of STI or PMSF prior to the appropriate proteases.

5.3 Results

5.3.1 Evaluation of assay systems for the separation of bound and free ^{125}I -DTX.

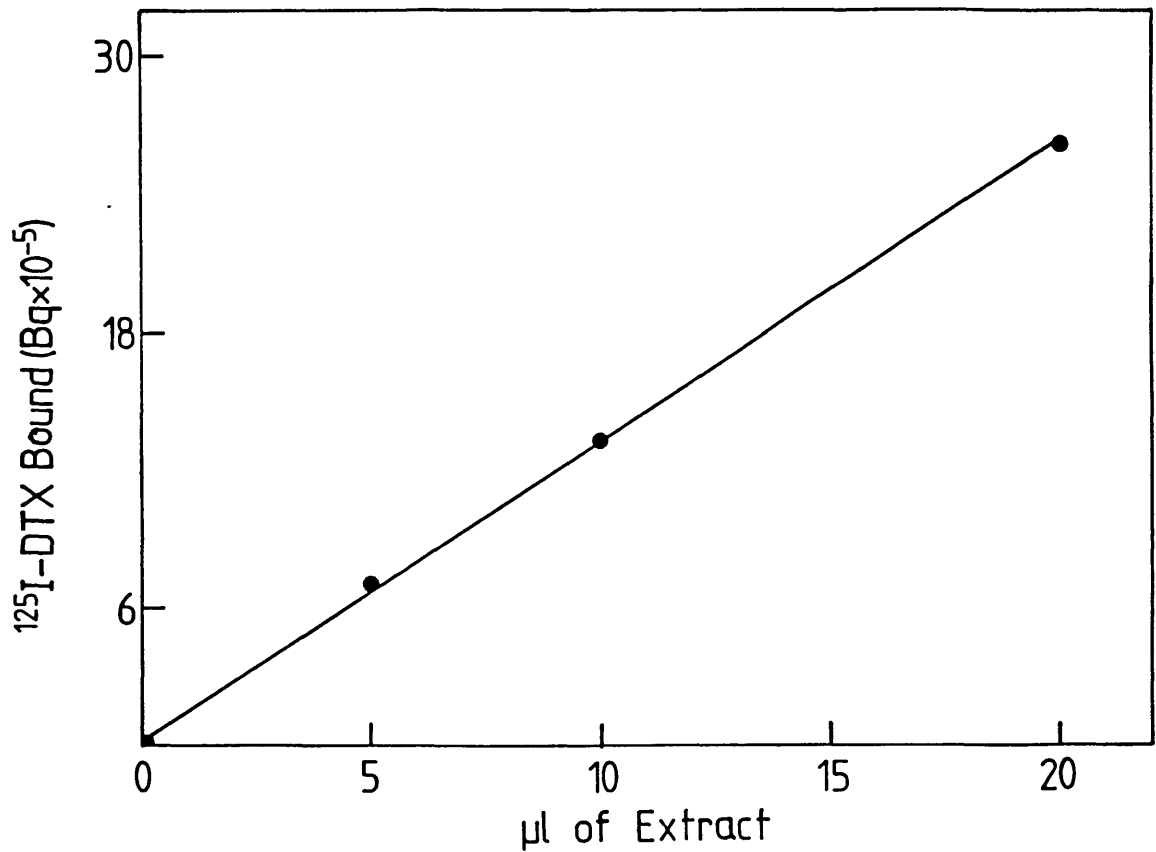
Table 5.2 shows the proportion of the "free" ^{125}I -DTX which would not be separated from the acceptor/toxin complex by the various assay systems. In the ammonium sulphate, polyethylene glycol precipitation and DEAE disc assay, this was unacceptably high (>10%). Only with the two gel filtration assays was the value reasonably low (~4%). Later experiments (Figure 5.1) showed that the saturable ^{125}I -DTX binding seen with the rapid gel filtration assay was proportional to the amount of extract present. Also, the amount of resin in the columns could be varied by $\pm 25\%$ without affecting the amounts of either "free" toxin or acceptor/toxin complex eluted, showing that the maximum recovery of the bound toxin and separation of free toxin from acceptor/toxin complexes was achieved. Thus, the rapid gel filtration assay is a valid and practicable system for studying the binding of ^{125}I -DTX to its solubilised acceptors.

Table 5.2 Assessment of assay systems for measuring ^{125}I -DTX binding to its solubilised acceptors.

<u>Assay Method</u>	<u>^{125}I-DTX detected (%)</u>	
	<u>Triton X-100</u>	<u>Deoxycholate</u>
DEAE disc	31 ± 3	24 ± 1
(NH ₄) ₂ SO ₄ (30%) precipitation	16 ± 2	-
Polyethylene glycol (8%) precipitation	15 ± 4	-
Gel filtration	4 ± 1	5 ± 1
Rapid gel filtration	3 ± 1	3 ± 2

^{125}I -DTX (5-10nM) in either 20mM Hepes, pH 7.4, 50mM KCl or 50mM imidazole-HCl, pH 7.4, 100mM KCl (rapid gel filtration only) containing 1.5% (w/v) detergent was subjected to the various assay systems as described in Section 5.2.1. The amount of ^{125}I -DTX detected by the assays was determined by γ -counting the discs (DEAE disc assay), precipitate [polyethylene glycol and (NH₄)₂ SO₄ precipitation assays] or the eluate (gel filtration assays). Values are average from at least 3 experiments ($\pm$ S.E.) expressed as a percentage of the total toxin present.

Figure 5.1 The relationship between the amount of soluble extract and saturable ^{125}I -DTX binding detected by the rapid gel filtration assay.



Binding of ^{125}I -DTX (1.2nM) in 5, 10 or 20 μl soluble rat brain extract (produced as in Table 5.1) was performed in 250 μl of 50mM imidazole-HCl, pH 7.4, containing ~0.32% (w/v) Triton X-100, 20mM KCl, 40mM NaCl and assayed by a rapid gel filtration assay. The data are averages of duplicates (error $\leq \pm 7\%$) and show saturable binding (that displaced by 120nM DTX).

5.3.2 Requirements for the extraction of active DTX acceptors from rat synaptic membranes.

Both deoxycholate and Triton X-100 were able to extract appreciable amounts of the acceptor activity. DTX acceptors appeared to be intrinsic membrane proteins because they could not be extracted by high salt (Table 5.3). Both of the detergents used extracted similar amounts of membrane protein; however, Triton X-100 extracted considerably more active acceptors (Table 5.3) and was, therefore, used in further studies.

The activity of solubilised DTX acceptors was dependent upon the presence of K^+ in the extraction medium; it could be replaced by Rb^+ but not Na^+ (Table 5.4). This was not due to a K^+ dependence of the binding alone as inclusion of K^+ in the assay medium, following extraction in K^+ free media, produced no specific binding (Table 5.4).

5.3.3 Optimisation of the conditions for extraction of active DTX acceptors.

The level of ^{125}I -DTX binding activity seen in Tables 5.3 and 5.4 (140fmol/mg protein) was 8 times lower than in the original membranes [$B_{max} = 1.4-1.7\text{pmol/mg protein}$ (see later)] and the yield of acceptor was only ~4% (Table 3.3), highlighting the need to optimise the conditions of extraction. Figure 5.2 shows the K^+ concentration dependence of solubilisation. Although K^+ had no effect on the level of protein in the extract, it did have a profound effect on solubilised acceptor activity. No specific ^{125}I -DTX binding activity was seen when <20mM K^+ was present in the extraction mixture. However, acceptor activity became greater and reached a plateau with increasing K^+ concentration. As 250mM was the concentration of K^+ at which the acceptor activity reached its plateau, this level was used in further studies because it allowed for the greatest exploration of the effect of

Table 5.3 Extraction of DTX acceptors from rat synaptic membrane by detergents and high salt.

<u>Extractant</u>	<u>Binding in extract</u>		<u>Protein in extract</u>	<u>Recovery of acceptors</u>
	<u>fmol/ml</u>	<u>fmol/mg protein</u>	<u>mg/ml</u>	<u>%</u>
Triton X-100	730	140	5.6	~4
Deoxycholate	430	60	7	~3
High Salt	10	12	0.9	~0.06

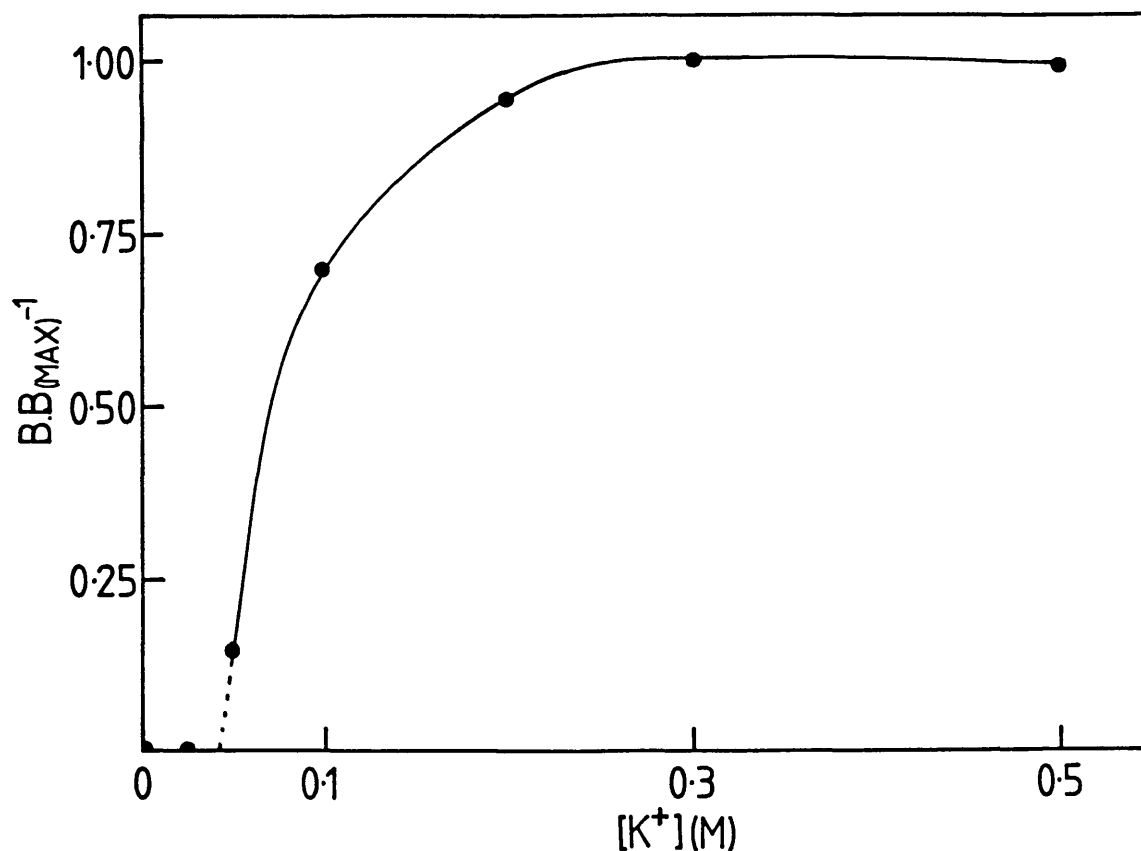
For detergent extracts, membranes (3.8mg protein) were extracted for 1h in 250μl of 50mM imidazole-HCl, pH 7.4, containing 100mM KCl and 1.5% (w/v) detergent. Salt extracts were produced in the same buffer but containing 1M NaCl and 0.25M KCl. Binding of 1.2nM ¹²⁵I-DTX to 50μl of extract was assayed by the rapid gel filtration assay following incubation in extraction buffer [except high salt extract (10μl) which was assayed in 50mM imidazole-HCl, pH 7.4, 10mM KCl and 40mM NaCl]] in the presence (for non-specific binding) or absence of 120nM DTX. Values [averages of duplicates (error <±7%)] are for specific binding and are representative of 3 experiments.

Table 5.4 Cation requirements for the extraction of DTX acceptor activity.

<u>Cation added</u>	<u>Specific ^{125}I-DTX binding (fmol/mg protein)</u>
K^+	140 $\pm$ 15
Rb^+	115 $\pm$ 4
Na^+	0.9 $\pm$ 0.1
Na^* (with K^+ in the assay)*	1.3 $\pm$ 0.3

Soluble extract of rat synaptic membranes (3.8 mg protein) was produced by incubation for 1 h at 4°C in 250 μl of 50mM imidazole, pH 7.4, containing 100mM of the stated cations (chloride salt) and 1.5% (w/v) Triton X-100 followed by centrifugation (as in Table 5.1). Binding of 1.2nM ^{125}I -DTX to the extract (50 μl) was assayed following incubation in 250 μl of the extraction medium [except (*) where 100mM KCl and 20mM NaCl were present], followed by rapid gel filtration. Data is specific binding (that which could be displaced by 120nM DTX) and is the average ($\pm\text{SE.}$) of 3 experiments.

Figure 5.2 K^+ dependence of extraction of DTX acceptors.



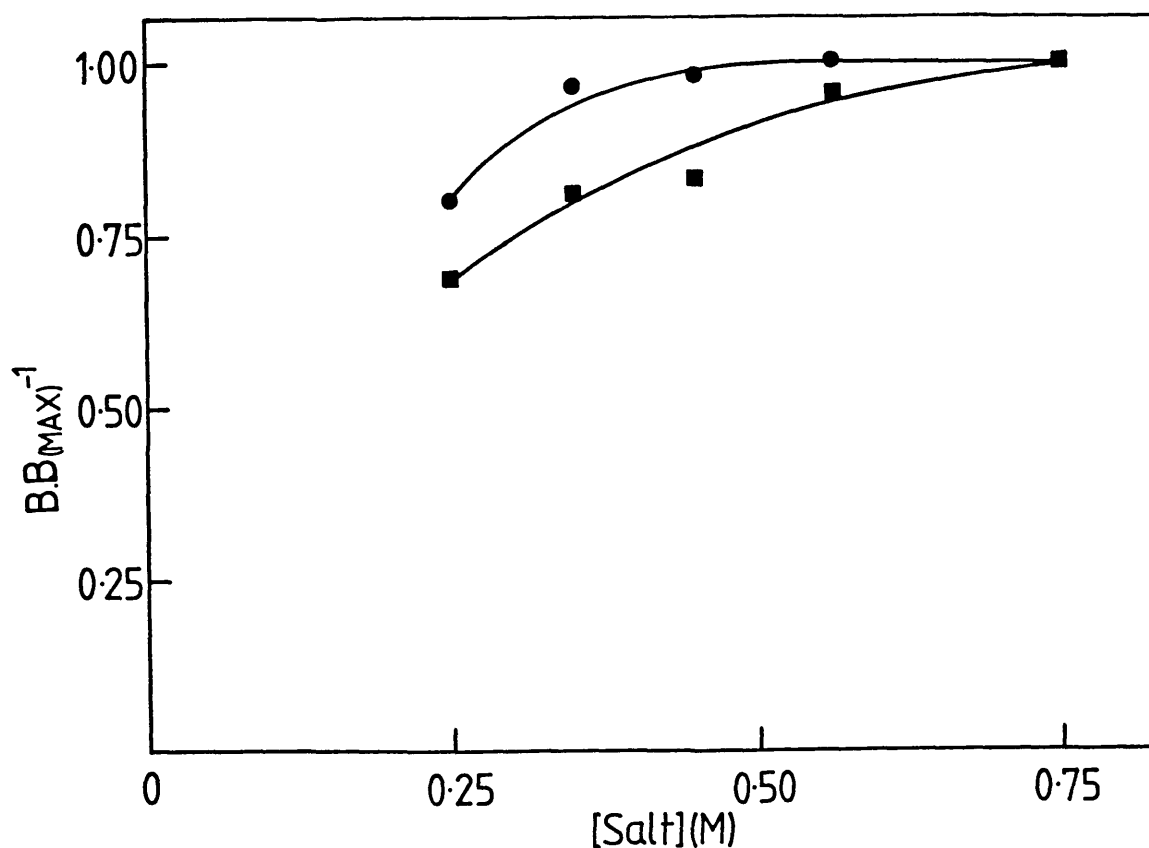
Extracts were produced as described in Table 5.1, except that membranes (3.8mg protein) were solubilised in 250 μ l of 50mM imidazole-HCl, pH 7.4, containing 4% (w/v) Triton X-100, the stated K^+ concentrations (chloride salt) in which the salt concentration was fixed at 750mM using NaCl. Binding of ^{125} I-DTX (1.2nM total) to 10 μ l of extract was performed in 250 μ l of 50mM imidazole-HCl, pH 7.4 containing ~0.16% (w/v) triton, 75mM KCl and 20mM NaCl and was quantified by a rapid gel filtration assay. Non-specific binding was determined by incubation with 120nM DTX. Data are averages of duplicates (error $\leq \pm 7\%$) and show the saturable binding expressed relative to that seen at 500mM K^+ (B_{max} = 700fmol/mg protein and 3.9 pmol/ml extract; yield ~25%).

also improved the ^{125}I -DTX binding activity of the solubilised extract (Figure 5.3). This had a greater effect on absolute binding of ^{125}I -DTX than on its binding relative to total protein. Thus, apart from increasing the efficiency of the detergent (Helenius and Simons, 1975), high ionic strength increased the relative ability with which DTX acceptors can be solubilised. The salt concentration used for optimal extraction was 750mM, since this produced the greatest extraction of acceptor activity both overall and with respect to total protein. Changing the concentrations of Triton X-100 also affected the final activity (Figure 5.4). Whilst the absolute activity continued to increase with the detergent levels, activity relative to total protein dropped at concentrations above 4% and, hence, this level was subsequently used.

In initial experiments, insoluble material was removed by a single centrifugation (100,000 x g for 1h); however, the effect of an initial low speed spin (34,000 x g for 30 min) was tested following the report that this was required for β -BuTX binding activity in soluble chick brain extracts (Rehm and Betz, 1984). Inclusion of the additional centrifugation step increased both ^{125}I -DTX binding activity and the level of protein in the final extract by 50%; thus, it was included in routine preparations. The final protocol chosen for optimised extraction of DTX acceptors is detailed in Table 5.1. This led to a 50% extraction of membrane protein and total acceptor activity (as determined by Scatchard analysis, see later), although, when the same concentrations were used but the volume was decreased, the extraction of protein was slightly lower, as was the relative extraction and yield of acceptors.

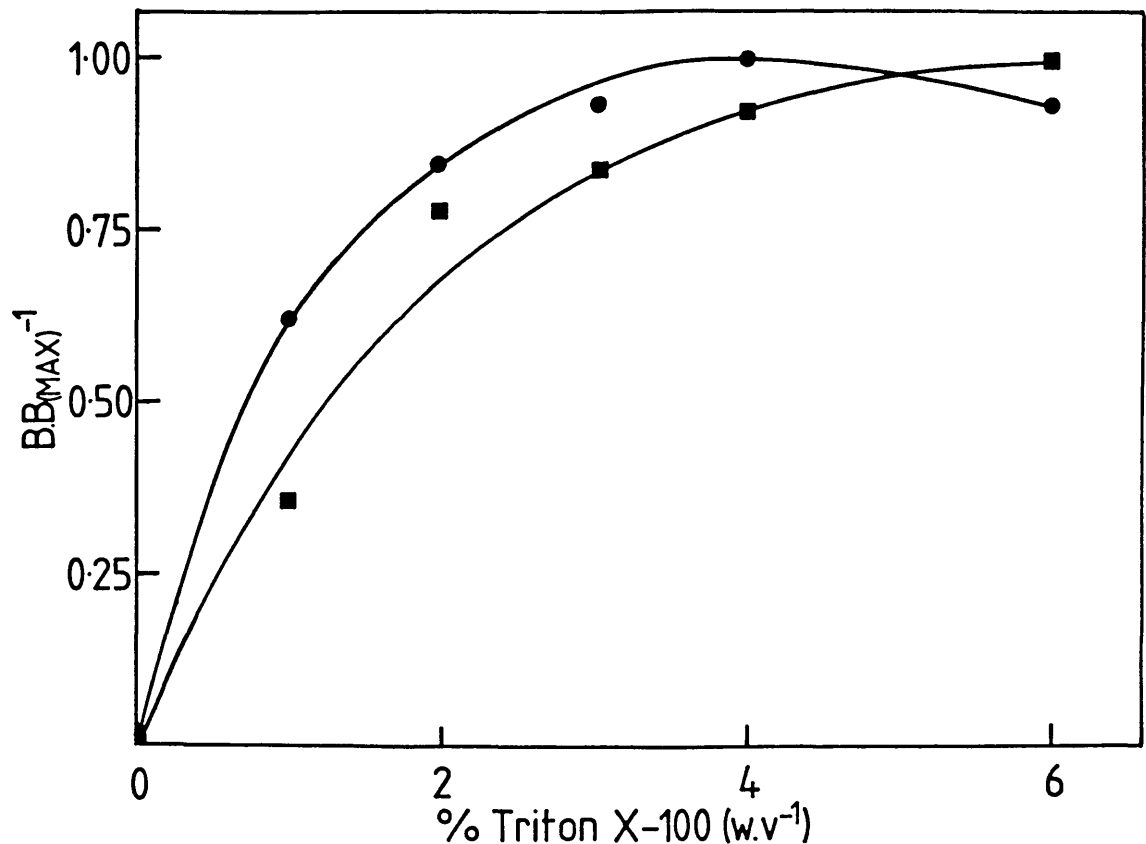
In order to optimise observed acceptor activity, the above parameters were also tested for their effects on ^{125}I -DTX binding in

Figure 5.3 Salt concentration dependence of extraction of DTX acceptors.



Extracts were produced and binding of ^{125}I -DTX (1.2nM) was assessed as described in Figure 5.2, except that solubilisation was in 50mM imidazole-HCl, pH 7.4, containing 4% (w/v) Triton X-100, 250mM KCl and various concentrations of NaCl. Data are averages of duplicates (error $<\pm 7\%$) and show saturable binding (that displaced by 120nM DTX) expressed relative to that seen at 750mM salt [$B_{max} = 750\text{fmo1/mg}$ (●); 4.2pmol/ml extract (■)]; yield $\sim 26\%$.

Figure 5.4 Triton X-100 concentration dependence of solubilisation of DTX acceptors.



Extracts were produced as described in Table 5.1 except that the stated levels of Triton X-100 were used to solubilise 3.8mg protein of membranes in 250 μ l of extraction buffer. Binding of ¹²⁵I-DTX (1.2nM) to 10 μ l of extract was performed in 250 μ l of 50mM imidazole-HCl, pH 7.4, containing ~0.24% (w/v) Triton X-100, 10mM KCl and 20mM NaCl and was assessed by a rapid gel filtration assay. Non-saturable binding was determined in the presence of 120nM DTX. Data are averages of duplicates (error $\leq \pm 7\%$) and show saturable binding (B) expressed relative to that seen using 4% (■; $B_{max} = 675\text{fmol/mg protein}$) or 6% Triton X-100 (●; $B_{max} = 4\text{pmol/ml extract}$; yield ~ 25%).

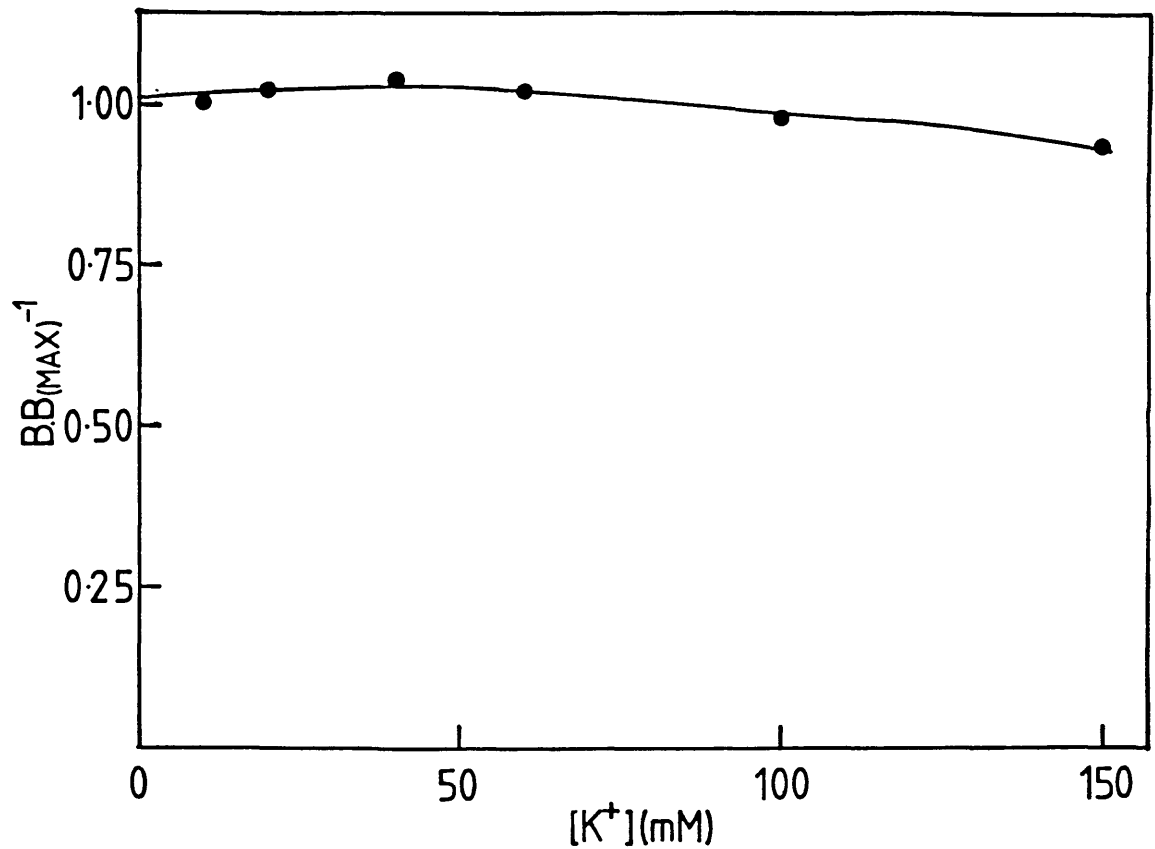
extracts. Figure 5.5. shows that K^+ (and total salt) concentrations between 10mM and 100mM had no significant effect on DTX binding to solubilised acceptors. However, Triton X-100 did have an inhibitory effect (Figure 5.6), reflecting an adverse interaction with the acceptors. Unfortunately, detergent cannot be excluded from the assay. In order to reduce its effect, extract was routinely diluted for assay to give a final concentration of $\sim 0.16\%$, where the inhibition should be $<10\%$. Where this was not possible, a higher, yet constant, detergent concentration was present during binding ($\sim 0.32\%$ and $\sim 0.24\%$ for (Figures 5.1 and 5.4, respectively).

5.3.4 Characterisation of the toxin binding of solubilised DTX acceptors.

A typical isotherm of ^{125}I -DTX binding to its acceptors in soluble extract is shown in Figure 5.7a. The non-specific component of this binding was between 15-20% at 1.2nM (the concentration used in further studies) and was mainly due to the "free" toxin which could not be separated from the acceptor/toxin complexes by the assay system. Scatchard analysis of the saturable component revealed a set of kinetically homogeneous, non-interacting sites ($K_D = 0.5\text{nM}$; $B_{\text{max}} = 1.4\text{-}1.8\text{pmol/mg protein}$) (Figure 5.7b). The insert in Figure 5.7b shows Scatchard analysis of ^{125}I -DTX binding to rat synaptic membranes assayed as described for synaptosomes in Section 2.2.9. Although the membranes have a marginally lower K_D (0.3nM), the B_{max} (1.4-1.7pmol/mg protein) was essentially the same as that following solubilisation, showing that extraction caused no significant inactivation or enrichment of the acceptors.

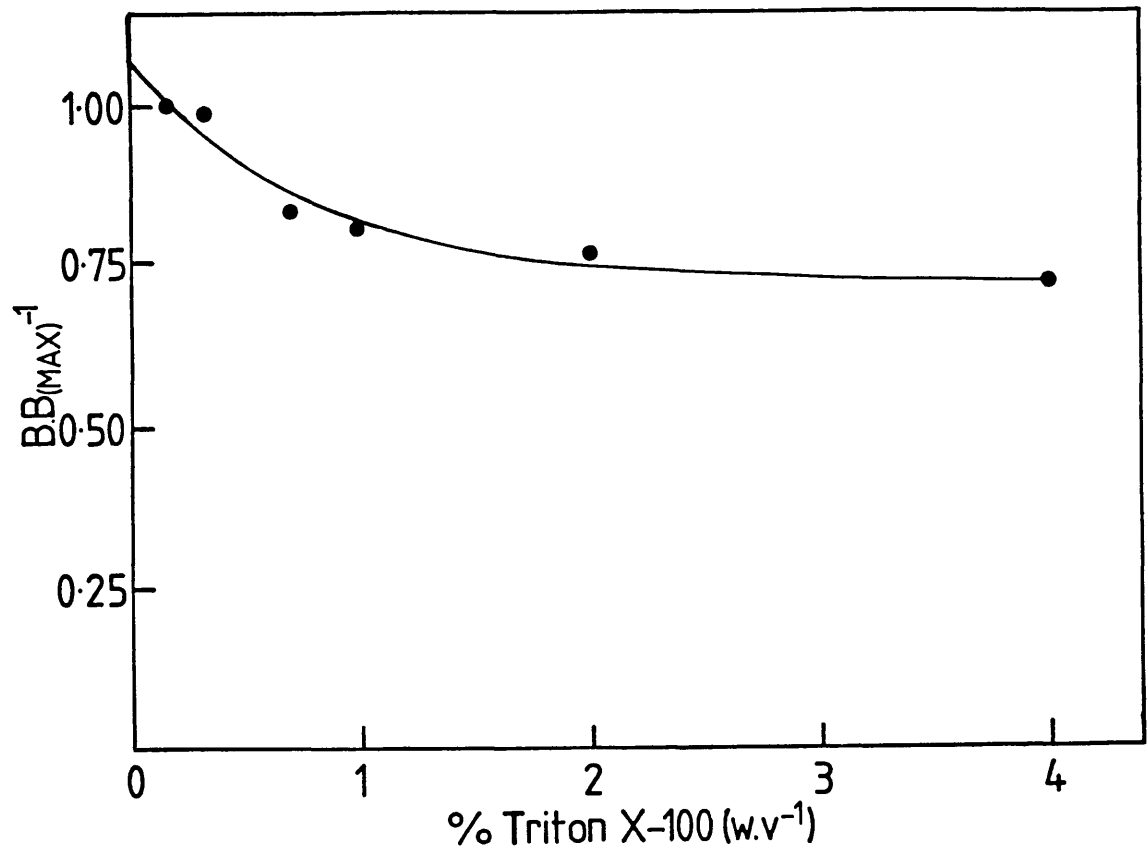
The dissociation of ^{125}I -DTX from its soluble acceptors also revealed a set of kinetically homogeneous sites; "chase" experiments (Figure 5.8) showed a linear first-order dissociation for the

Figure 5 5 K⁺ dependence of ¹²⁵I-DTX binding to solubilised DTX acceptors.



Binding of ¹²⁵I-DTX (1.2nM) in soluble rat brain extract was assessed by a rapid gel filtration assay, as described in Section 5.2.3, except that the K⁺ concentration in the incubations was varied. Data are averages of duplicates (error $\leq \pm 7\%$) and show saturable binding (that which could be displaced by 120nM DTX) expressed relative to that seen in 10mM KCl (950fmol/mg protein).

Figure 5.6 The effect of Triton X-100 on ^{125}I -DTX binding to its solubilised acceptors.



Binding of 1.2nM ^{125}I -DTX to solubilised extract was assessed as described in Figure 5.5, except that Triton X-100 rather than KCl concentration was varied. Data are averages of duplicates (error $\leq \pm 7\%$) and show saturable binding (that displaced by 120nM DTX), expressed relative to that seen in $\sim 0.16\%$ Triton X-100 (980fmol/mg protein). Triton X-100 concentrations were estimated assuming that the concentration in the extract was 4% .

Figure 5.7 Equilibrium binding of ^{125}I -DTX to its solubilised acceptors.

- (a) Binding of various concentrations of ^{125}I -DTX in soluble extract (produced as in Table 5.1) was assessed by a rapid gel filtration assay as described in Section 5.2.3. Free toxin concentrations were estimated by subtracting the bound toxin from the total amount present in the incubations. Non-saturable binding (■) was assessed by inclusion of 200nM DTX and was subtracted from the total binding (▲) to give the level of saturable binding (●).
- (b) Scatchard analysis of the saturable binding in (a). The insert shows a Scatchard plot of the saturable ^{125}I -DTX binding to the original membranes, assayed by the centrifugation washing method (Section 2.2.8).

Figure 5.7 Equilibrium binding of ^{125}I -DTX to its solubilised acceptors.

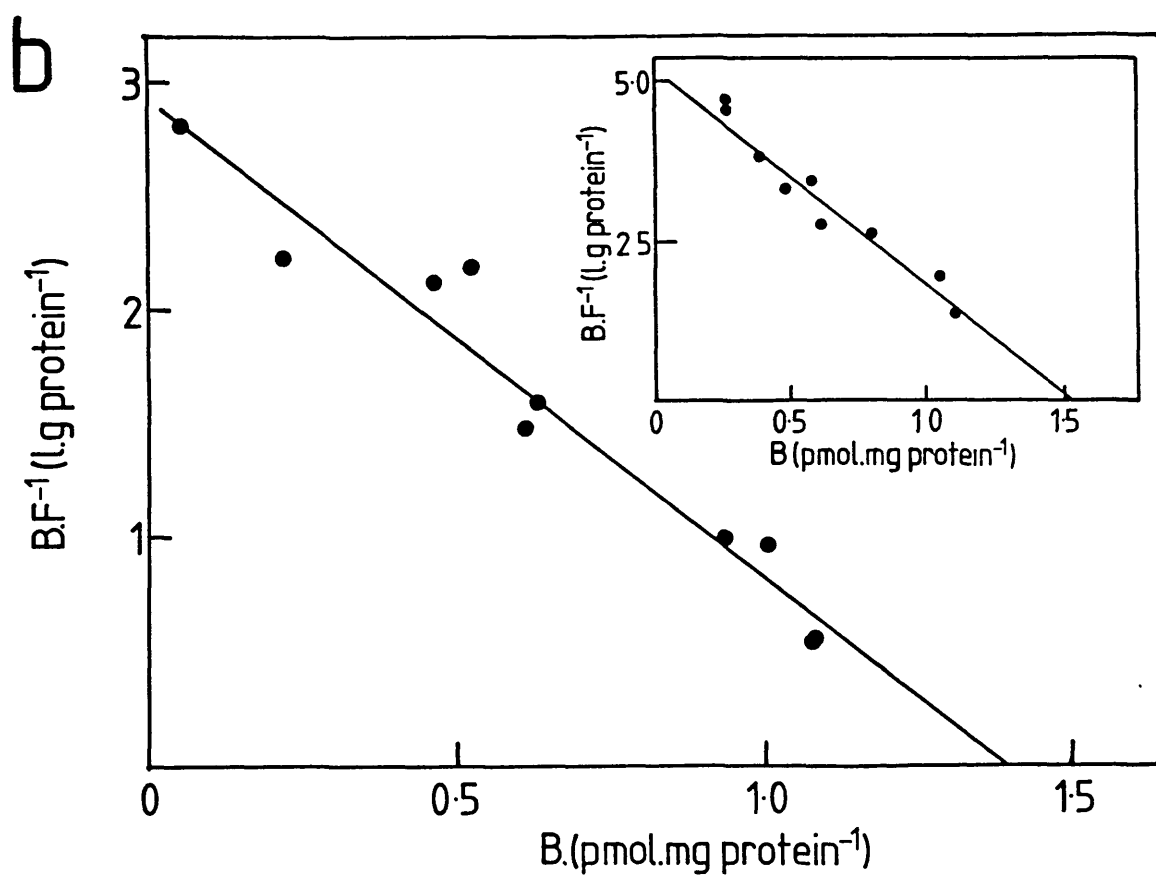
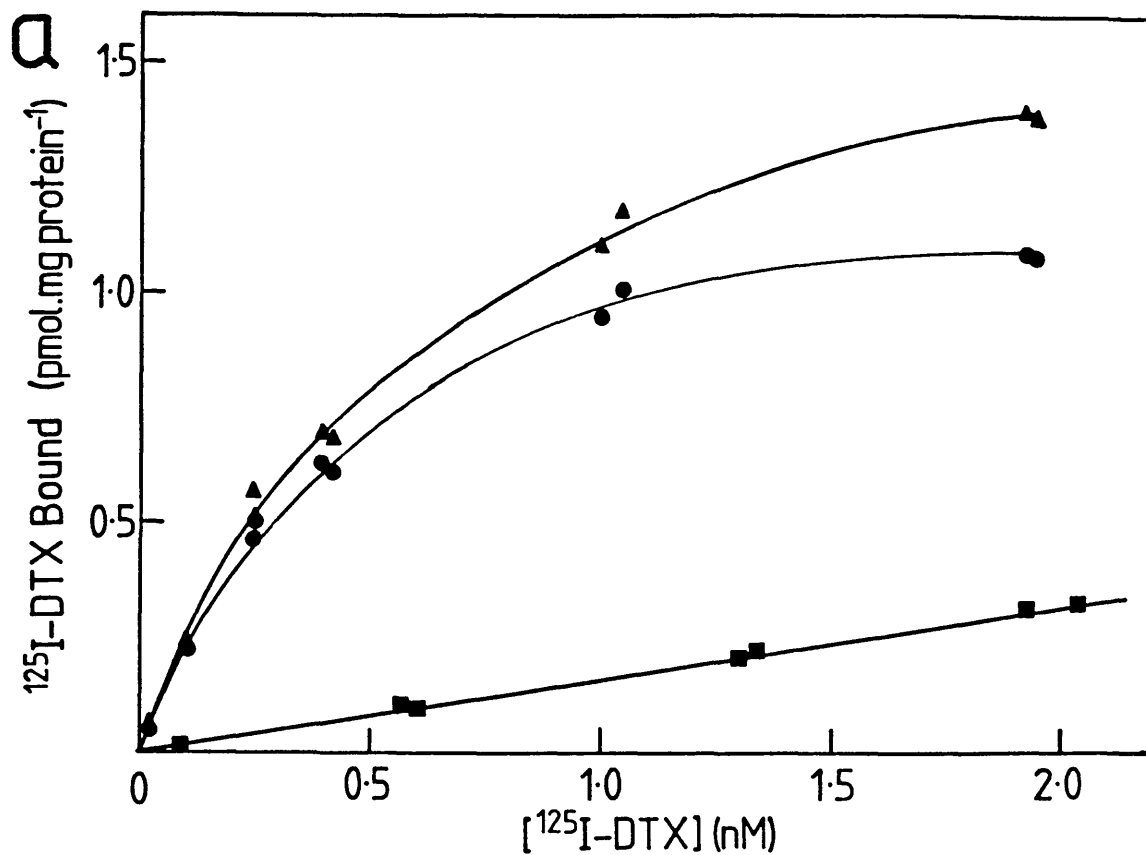
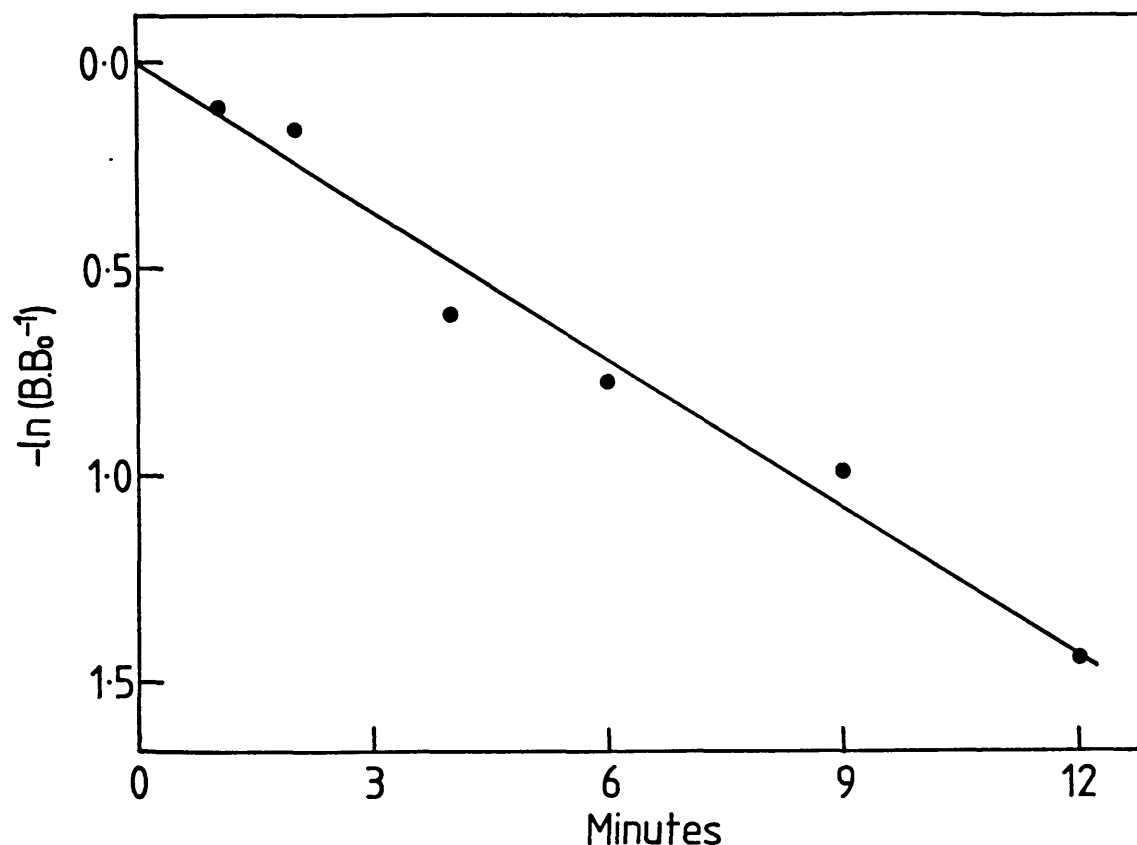


Figure 5.8 Dissociation of ^{125}I -DTX from its soluble acceptors.



After ^{125}I -DTX (1.2nM) had been preincubated (15 min) with solubilised extract (as in Section 5.2.3), 200nM DTX was added at various times prior to assay of binding by a rapid gel filtration method. For assessment of non-displaceable binding, 120nM DTX was present during preincubation and buffer was added prior to assay. Data show averages of duplicates (error $<\pm 7\%$) for the specific binding (B) which is expressed relative to the displaceable binding ($B_0 = 900\text{fmol/mg protein}$) seen in samples where 120nM was added immediately before the ^{125}I -DTX binding was assayed.

^{125}I -DTX/solubilised acceptor complexes (Figure 5.8) with a k_{-1} of $2 \times 10^{-3} \text{ s}^{-1}$ and $t_{1/2}$ of ~ 6 min.

No attempt was made to measure the association rate of the ^{125}I -DTX soluble acceptor complex, but a k_{+1} of $4 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ can be calculated from the k_{-1} and the K_D , showing that the association was rapid.

Pretreatment of the membranes at 95°C or with 1M HCl or pretreatment of the soluble extract with trypsin or chymotrypsin virtually abolished the binding of ^{125}I -DTX to its solubilised acceptors (Table 5.5), demonstrating their proteinaceous nature. DTX acceptors solubilised as described in Table 5.1 were unstable. Freezing the extract totally inactivated its ^{125}I -DTX binding activity and the extract lost 40%-60% of its activity in 14h at 4°C . Thus, where possible, all experiments were conducted using extract within 3h of its production (where $<10\%$ of activity was lost).

5.3.5 The effect of DTX, homologous toxins and β -BuTX on the binding of ^{125}I -DTX to its solubilised acceptors

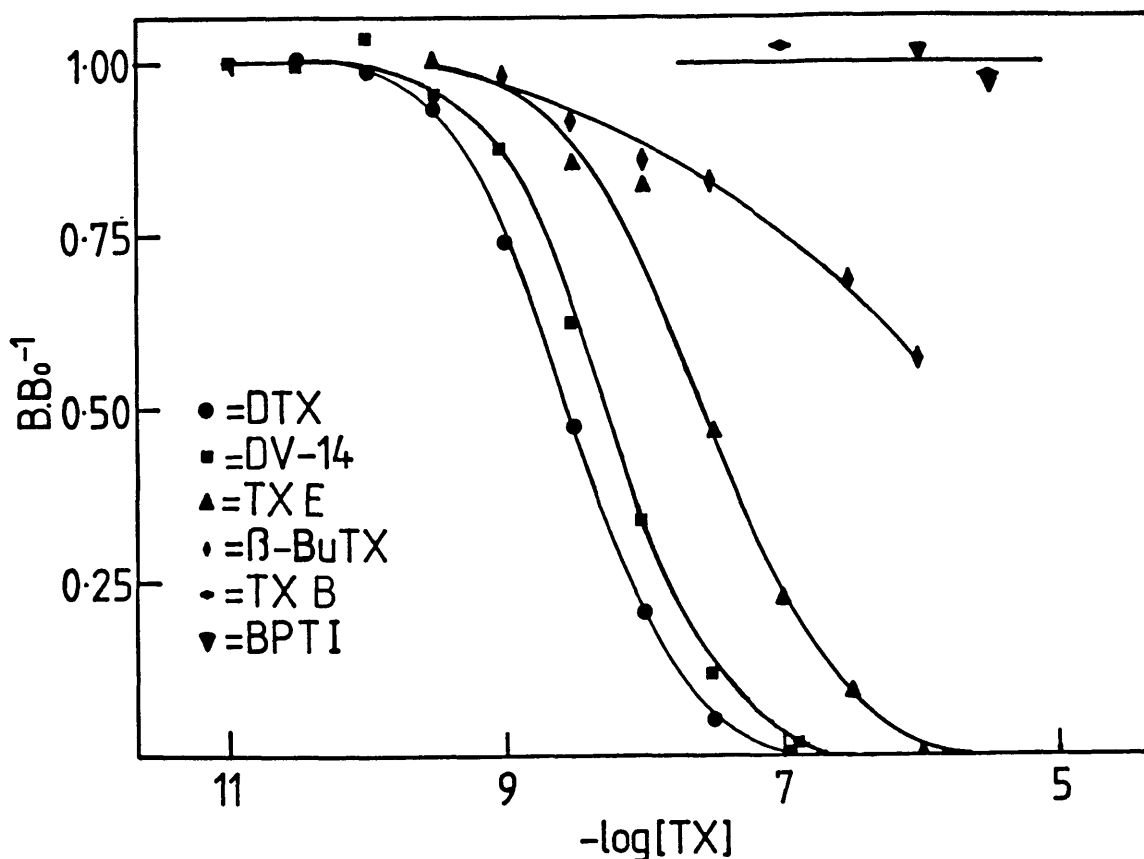
The specificity of solubilised DTX acceptors for protease inhibitor homologues was the same as that of the membrane bound acceptors (Black et al., 1985). DTX, D.V.14 and toxin E were all able to totally inhibit the specific binding of ^{125}I -DTX, with K_I values of 0.8nM , 2.5nM and 7.7nM , respectively (Figure 5.9). In contrast, toxin B and BPTI had no effect (Figure 5.9). When slight reduction in affinity produced by solubilisation is taken into account, the K_I values seen here agree closely with those determined simultaneously in rat synaptic membranes (DTX = 0.4nM ; D.V.14 = 1.1nM ; toxin E = 3.0nM) and reasonably with those previously obtained with rat synaptosomes (Black et al., 1985, Table 7). Collectively, these showed that the

Table 5.5 The effect of various treatments on the DTX acceptors
solubilised from rat synaptic membranes.

<u>Treatment</u>	<u>Relative specific binding of ^{125}I-DTX (%)</u>
Chymotrypsin (1mg/ml; 37°C for 40 min)	4 ± 5
Trypsin (1mg/ml; 37°C for 40 min)	6 ± 3
Heat (95°C for 30 min)	4 ± 1
Acidification (1M HCl; 37°C for 40 min)	3 ± 1

Soluble extract (prepared in 250 μ l of buffer as described in Section 5.2.4) was incubated with proteases as stated. After addition of PMSF to 5mM (for chymotrypsin) or STI to 5mg/ml (for trypsin), binding of 1.2nM ^{125}I -DTX was assayed as in Section 5.2.3. Protease inhibitors were added before the protease in control samples. Heat treatment and acidification (stopped by washing 2 x 1 ml in 50mM imidazole-HCl, pH 7.4) were carried out on the membranes prior to solubilisation in a volume of 250 μ l, as described in Section 5.2.4. Controls were prepared by incubating at 37°C for 30 min for heat treatment or 40 min in the absence of HCl for acidification. Values are for specific binding (700-800 fmol/mg), expressed relative to that seen in the respective controls, and are averages ($\pm$ S.E.) of 4 separate experiments.

Figure 5.9 Interaction of homologous toxins and β -BuTX with ^{125} I-DTX binding to its solubilised acceptors.



Binding of 1.2nM ^{125} I-DTX in soluble rat brain extract was assayed by a rapid gel filtration assay as described in Section 5.2.3. Competing ligands were included at the stated concentrations and non-saturable binding was determined in the presence of 120nM DTX. Data are averages of duplicates (error $\leq \pm 7$) and show saturable binding (B) relative to that seen in the absence of competing ligand ($B_0 = 900-1000\text{fmol/mg protein}$). Toxins are: ●, DTX; ■, D.V.14; ▲, toxin E; ◆, β -BuTX; ◄, toxin B; and ▼, BPTI.

binding characteristics of the DTX acceptors are not significantly changed by solubilisation.

Despite the marked similarities between the membrane-bound and solubilised acceptors, inhibition of ^{125}I -DTX binding by β -BuTX did change upon solubilisation. Although β -BuTX inhibited the majority of ^{125}I -DTX binding with only low efficacy, a small component of high affinity inhibition was obvious (Figure 5.9). Therefore, as proposed previously, rat brain contains two classes of DTX acceptors, which can be differentiated by β -BuTX (cf. Chapter 3).

5.4 Discussion

5.4.1 Methods for measurement of ^{125}I -DTX binding to solubilised acceptors.

The high levels of free ^{125}I -DTX detected in the polyethylene glycol and ammonium sulphate precipitation assays were not surprising as DTX is itself a polypeptide and some precipitation by these agents would be expected. Also, as the toxin is a highly basic molecule, it will bind to any negatively charged groups in the support of the DEAE discs, explaining the high level of binding to these. The toxin which would not have been separated from the acceptor/toxin complexes by the gel filtration assays was presumably due to incorporation of ^{125}I -DTX into detergent micelles as these would appear in the void volume of the columns. However, it only represented a small proportion of the total toxin present (~4%) and should not have an adverse effect on the assay.

Although some early experiments were conducted with the conventional gel filtration assay, the rapid method was used following its publication by Rehm and Betz (1984), because it was both more convenient and reproducible. Furthermore, the linear relationship between the amount of extract present and the binding detected by this

assay system, showed that it was well suited to the study of ^{125}I -DTX binding to its soluble acceptors.

5.4.2 Conditions for the solubilisation of DTX acceptors.

Although both Triton X-100 and deoxycholate, but not high salt, were able to solubilise DTX acceptors in an active form, lower levels of activity were seen with deoxycholate. This is presumably due to a more selective solubilisation of DTX acceptors by Triton X-100 or a greater stabilisation of the acceptors [either directly or through a less rapid exchange of lipids (Tanford and Reynolds, 1976)]. The requirement for K^+ (or Rb^+) during solubilisation was also seen for ^{125}I - β -BuTX binding in soluble chick brain extracts (Rehm and Betz, 1984) i.e. for solubilisation of the high affinity acceptor for DTX therein (cf. Chapter 3). This is interesting in view of the proposed involvement of DTX with voltage-sensitive K^+ conductances (Dolly *et al.*, 1984; Halliwell *et al.*, 1985; Weller *et al.*, 1985) as it reflects the selectivity of the channels responsible.

The extraction conditions have been optimised with respect to K^+ , salt and Triton X-100 concentration levels (Figures 5.2, 5.3 and 5.4). Those finally chosen, listed in Table 5.1, give the maximal extraction of binding activity, which allows the greatest acceptor concentrations with respect to total protein. Although extract produced under these conditions retained full ^{125}I -DTX binding activity, the solubilised DTX acceptors were unstable. This was not a major problem for the present study (this Chapter and Chapter 6); however, for the lengthy manipulations involved in purification of the acceptors, stabilisation of the extract will be required, perhaps by addition of certain lipids.

5.4.3 Binding characteristics of solubilised DTX acceptors.

Comparison of the binding of ^{125}I -DTX to membrane and in solubilised extract showed that solubilisation had little effect on the properties of its acceptors. In both cases they were susceptible to protein denaturing agents (Table 5.5; Chapter 2) and bound only neurotoxic homologues of DTX (Black *et al.*, 1985; Table 7.1; Figure 5.9). Scatchard analysis and kinetic measurements also revealed that solubilisation did not affect the level of acceptor activity (Figure 5.7) and only produces a minimal change in affinity (Figures 5.7, 5.8 and Chapter 2).

The only significant difference between binding in the membrane and soluble extract was in the pattern of inhibition by β -BuTX. This showed that a small proportion (15%-20%) of the solubilised DTX acceptors could bind β -BuTX with high affinity, confirming the proposal that two populations of DTX acceptor can be distinguished by β -BuTX in rat brain as well as in chick brain (cf. Chapter 3). The appearance of high affinity inhibition by β -BuTX upon solubilisation would appear to be due to a greater stability of the acceptor type which binds both DTX and β -BuTX. Following sucrose density centrifugation and gel filtration, where ~80% of the acceptor activity is lost, 100nM β -BuTX was able to inhibit ~50% of the binding of ^{125}I -DTX (Chapter 6). Additionally, interconversion or selective solubilisation of the acceptor types may contribute to this observation.

5.4.4 Summary

A procedure for optimised solubilisation of rat brain DTX acceptors has been described. Solubilisation had little adverse effect on the binding of ^{125}I -DTX to its proteinaceous acceptors, which displayed the same characteristics as those identified in the membrane. However, two classes of these acceptors could be distinguished by virtue of the ability of only one to bind β -BuTX with high affinity.

CHAPTER 6

PHYSICAL PROPERTIES OF DTX ACCEPTORS

6.1 Introduction

6.1.1 General perspective.

Following extraction from the membrane in an active form, the next stage in the study of DTX acceptors was to determine their molecular sizes in both the membrane-bound and solubilised states. In conjunction with cross-linking data, these give an insight into the structure of the acceptors and should help to assess any effects that solubilisation may have had on their properties. Knowledge of the size and hydrodynamic properties of the acceptors is a prerequisite for future investigations: for example, it can be used in monitoring the integrity of the acceptors at each stage of purification and in predicting their behaviour in various preparative procedures.

As DTX acceptors only represent a very small proportion of the protein present in neuronal membranes, only those techniques which enabled their detection by saturable ^{125}I -DTX binding could be used for their characterization. This requirement was fulfilled by two methods: a combination of sucrose density gradient centrifugation and gel filtration, which gives the hydrodynamic properties and size of solubilized acceptors (Siegel and Monty, 1966; Stephenson et al., 1982), and radiation inactivation, which can yield the size of both membrane-bound and solubilised acceptors (Lea, 1955; Lo et al., 1982).

6.1.2 Determination of hydrodynamic properties and molecular weight using sucrose density gradient centrifugation and gel filtration.

The theoretical basis for determination of the size of the particle from its hydrodynamic properties lies in the derivation of the Svedberg equation:

$$M_r = \frac{6\pi N n_{20,w} R_s S_{20,w}}{1 - \bar{v} \rho_{20,w}} \quad (\text{Formula 6.1})$$

where N is Avagadro's number, $\eta_{20,w}$ and $\rho_{20,w}$ are the viscosity and density of water at 20°C, $S_{20,w}$ and $\bar{v}$ are the sedimentation coefficient (in water at 20°C) and partial specific volume of a particle with a Stokes radius, R_s . Sucrose density gradient centrifugation can be used to determine two of the unknowns in Formula 6.1, as:-

$$r = k S_{20,w} (1 - \bar{v} \rho_{1/2}) \quad (\text{Formula 6.2})$$

where r is the distance travelled by a particle down a sucrose gradient, k is a constant for a solvent of a given density and $\rho_{1/2}$ is the density of the gradient at $r = 1/2$ (Meunier et al. 1972). A plot of the distance travelled by standard soluble proteins [which do not bind significant amounts of detergent (Clarke, 1975)] against values for $S_{20,w} (1 - \bar{v} \rho_{1/2})$ will give a straight line with a slope of k . If this is done for two solvents of different density (e.g. $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$), $S_{20,w}$ and $\bar{v}$ can be determined for an unknown by substituting its distance of travel in the two solvents and the respective k values into Formula 6.2, followed by the use of simultaneous equations.

The Stokes radius of a particle (the radius of a sphere with the same frictional coefficient) can be determined by gel filtration, using a variety of analyses (Porath, 1963; Laurent and Killander, 1964; Ackers, 1964), all of which have been shown to be applicable to a wide variety of proteins (Seigel and Monty, 1966). The method used here is that of Porath (1963) which relates the partition coefficient ($K_d^{1/3}$) of a particle to its elution volume (V_e) by the formula:-

$$K_d^{1/3} = \left(\frac{V_e - V_o}{V_i - V_o} \right)^{1/3} = \alpha - (\beta R_s) \quad (\text{Formula 5.3})$$

where V_o is the void volume of the column, (V_i) is the volume of

solvent in the column, α and β are undefined constants. Formula 6.3 predicts that a plot of $K_d^{1/3}$ against Stokes radius will be a straight line. V_i can be defined as the elution volume of a particle which is small enough to enter all available spaces within the gel matrix, e.g. ferricyanide; V_o can be defined as the elution volume of a large molecule, e.g. Dextran blue, which will be totally excluded from the gel matrix. Thus, $K_d^{1/3}$ can be calculated easily from the elution volume of any particle and its Stokes radius can be read directly from the straight line plot of $K_d^{1/3}$ against Stokes radius of standard proteins.

The values for Stokes radius together with the values obtained from sucrose density centrifugation, determined by specific toxin binding, can be substituted into Formula 6.1 to give the M_r of the acceptor/lipid/detergent complex. The weight fraction of the acceptor (X_a) in this complex can be calculated by the formula:

$$X_a = \frac{\bar{v}_c - \bar{v}_{d:l}}{\bar{v}_a - \bar{v}_{d:l}} \quad (\text{Formula 6.4})$$

where $\bar{v}_c$, $\bar{v}_a$ and $\bar{v}_{d:l}$ are the partial specific volumes of the complex, acceptor and detergent/lipid, respectively (or estimates thereof). Hence, the M_r of the acceptor protein can be estimated easily.

Another useful parameter which can be calculated from hydrodynamic properties is the frictional ratio ($f/f_{\min}$) of the acceptor/lipid/detergent complex. This parameter is defined as:

$$f/f_{\min} = Rs \left(\frac{4\pi N}{3M_r (\bar{v} + \delta/\rho)} \right)^{1/3} \quad (\text{Formula 6.5})$$

where δ is the degree of hydration (w/w) of the complex and ρ is the density of water, other parameters are as defined previously. $f/f_{\min}$ gives the ratio of the frictional coefficient of the complex to that of a sphere with the same mass, partial specific volume and degree of hydration. As asymmetry will increase the frictional coefficient, a value of >1 will indicate an elongated molecule, which can be equated to an equivalent ellipsoid to give an estimate of its axial ratio (Schachman, 1959).

Despite possible problems caused by dissociation (Hjelmeland et al., 1983), or association (Simons et al., 1973) of membrane proteins upon solubilisation, the anomalous behaviour of certain molecules on gel filtration (Nozaki et al., 1976), the need to estimate the partial specific volume in Formula 6.4, preferential hydration in sucrose solutions (Timasheff et al., 1976) or $^1\text{H}/^2\text{H}$ exchange, this method has given accurate estimates of the size of neurotoxin acceptors. The value of 340,000 obtained for the M_r of the Electrophorus nicotinic acetylcholine receptor (calculated from data in Meunier et al., 1972, using $\bar{v} = 0.91$ for Triton X-100) agrees well with that of 280,000 for the Torpedo receptor obtained from sequencing data (Noda et al., 1982, 1983; Claudio et al., 1983).

6.1.3 Target theory analysis.

Use of the target theory to relate the size of a protein to inactivation of its biological activity by ionising radiation relies on two basic assumptions (Kepner and Macey, 1968): (a) primary energy releases generated by the interaction of ionising radiation with matter occur as discrete localised clusters of ionisations; (b) when a single ionising event occurs within the "effective volume" of a particular function, the energy released must inactivate that function of the target molecule through disruption of its structure. A further

requirement for the application of target theory is that primary energy releases must occur at random with respect to volume; therefore, sparsely ionising radiation, such as γ -rays or high energy electrons, have to be used. Also, samples must be immobilised by freezing or by lyophilisation in order to remove secondary effects due to the diffusion of free radicals.

When these requirements are met, the probability of a particular functional volume being inactivated follows a Poisson distribution: remaining activity is related to radiation dose in an exponential manner, i.e. :

$$A/A_0 = e^{-VD} \quad \text{(Formula 6.6)}$$

where A_0 is the original activity of a target molecule with an "effective volume" of V and A is the remaining activity after a dose of ionising radiation, D . As the target for ionising radiation is matter, Formula 6.6 gives the mass of the target directly. Two approaches have been used to calculate molecular mass from inactivation data. The first put forward by Kepner and Masey (1968) uses the dose which reduces the activity to $1/e$ ($\sim 37\%$) of its original value, defined as D_{37} , which equals the reciprocal of the mass of the target (from Formula 6.6). From the empirically derived energy of a primary ionisation (66eV), the number of primary ionisations produced by 1rad was calculated to be 1.6×10^{-12} per dalton. Hence, molecular weight (M_{wt} , in daltons) is related to D_{37} (in rads) by the formula:

$$M_{wt} = \frac{6.3 \times 10^{11}}{D_{37}} \quad \text{(Formula 5.7)}$$

As inactivation by ionising radiation is temperature-dependent (Kempner and Schlegel, 1979), this formula is only valid for experiments conducted at 30°C and empirically derived correction factors have to be

applied when other temperatures are used e.g. Formula 6.7 must be multiplied by 2.59 for inactivations at -110°C (Nielsen et al., 1981).

Due to the empirical nature of the above approach and the difficulties in measuring the dose accurately, a second method using protein standards was developed (Lo et al., 1982). Formula 6.6 predicts that a plot of radiation dose against $\ln A/A_0$ will give a straight line, the slope of which is proportional to the M_r of the target. Thus, by plotting the relative rates of inactivation of standard enzymes against their M_r , the mass associated with any function can be determined.

It should be noted that the mass obtained from radiation inactivation is the "effective volume" of the function under study. Although this can be the complete oligomeric protein, it can also represent a subunit (Kempner et al., 1980) or a functional region of a polypeptide [as with the domains of immunoglobulin (Fewtrell et al., 1981)]. Thus, radiation inactivation may reveal valuable information about the structure of a protein rather than its actual size. Despite this and possible changes in structure produced by freezing or lyophilisation, target theory analysis has given valid estimates of the sizes of the nicotinic acetylcholine acceptor [300,000 (Lo et al., 1982)]. Similarly, the M_r value of 230,000 obtained from inactivation of the Electrophorus electroplax Na^+ channel (Levinson and Ellory, 1973) agrees with its actual size of 268,000 obtained from sequencing data (Noda et al., 1984).

6.1.4 Conclusion.

The two methods described above have given good estimates of the sizes of neurotoxin acceptors and were thus applied to the DTX acceptors with the hope of obtaining valuable information about their structure and hydrodynamic properties.

6.2 Methods

6.2.1 Sucrose density gradient centrifugation.

Solutions of 50mM potassium phosphate, pH 7.4, containing 0.2% (w/v) phosphatidylcholine, 0.8% (w/v) Triton X-100 and 5% or 20% (w/v) sucrose were prepared with either deionised water ($^1\text{H}_2\text{O}$) or deuterium oxide ($^2\text{H}_2\text{O}$). Using these, 11ml linear sucrose gradients (5%- 20%) in $^1\text{H}_2\text{O}$ or $^2\text{H}_2\text{O}$ were poured by a Buchler Auto-Densi-Flow II on top of a cushion of 60% sucrose (0.5ml) in 12ml Beckman centrifuge tubes. The gradients were cooled on ice. When rat synaptic membranes were solubilised as in Table 5.1, the resultant extract was denser than the top of the $^1\text{H}_2\text{O}$ gradients. However, the profiles obtained using this extract on $^2\text{H}_2\text{O}$ gradients did not differ from those obtained with extract produced as in Table 5.1 except with the omission of NaCl. Thus, the latter extraction procedure was used to obtain less dense extract for hydrodynamic studies. Extract (200 μ l) containing standard protein markers [10 units Escherichia coli β -galactosidase (16S), 30 units catalase (11.3S), 4 units horse liver alcohol dehydrogenase (4.8S) and 200 μ g cytochrome c (1.8S) (for references see Stephenson et al., 1982)] was layered on top of the $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ gradients. These were centrifuged simultaneously at 4°C and 39,000 rpm (190,000 x g) for 19h. Gradients were fractionated using the Densi-Flow II and identical fractions (0.45-0.5ml) were collected for all gradients in one experiment. These were then assayed for enzyme and ^{125}I -DTX binding activities.

6.2.2 Gel filtration.

Solubilised rat brain extract (1ml produced as in Section 6.2.1) containing standard protein Stokes radius markers [25 units β -galactosidase (6.4nm), 250 units catalase (5.2nm), 60 units yeast alcohol dehydrogenase (4.6nm) and 400 μ g cytochrome c (1.87nm), (Stokes

radii from Stephenson et al., 1982; Seigel and Monty, 1966)] was loaded onto a Sepharose Cl-6B column (1 x 54cm) equilibrated with 50mM potassium phosphate, pH 7.4, containing 0.2% (w/v) phosphatidylcholine and 0.8% (w/v) Triton X-100. The sample was washed into the column with 1ml of the equilibration buffer and was eluted at a flow rate of 12ml/h. Fractions (1ml) were collected and assayed for enzyme and ^{125}I -DTX binding activities. As thyroglobulin (8.5nm) has no enzymic activity, its elution position had to be determined by the protein content of the fractions collected; thus, it was chromatographed separately from the extract. Chromatography of 15mg of thyroglobulin was as before; in order to check that the elution characteristics of the column were unaltered, catalase (250 units) was included. The void volume and inclusion volumes of the column were determined similarly in the presence of catalase, using Dextran blue and ferricyanide, respectively.

6.2.3 Assessment of the various activities in fractions from density gradients and gel filtration.

- (a) ^{125}I -DTX binding activity: Aliquots (200 μl) of each fraction were mixed with 50 μl of ^{125}I -DTX (in the presence or absence of a 100-fold excess of DTX) to give a final concentration of 1.2nM; binding was assayed by the rapid gel filtration assay described in Section 5.2.3.
- (b) β -galactosidase activity; (Lederberg, 1950). Aliquots (1ml) of 100mM sodium phosphate buffer, pH 7.2, containing 2mM o-nitrophenyl- β -D-galactopyranoside were added to 10 μl of each fraction. After ~1h at 23°C the level of o-nitrophenol produced in each sample was measured by its absorbance at 416nm.

(c) Catalase activity. (Beers and Sizer, 1952). Following sucrose density centrifugation, catalase activity was assayed by mixing aliquots (10 μ l) of the fractions with 1ml of 50mM sodium phosphate buffer, pH 7.0, containing 0.05% (v/v) hydrogen peroxide. The rate of hydrogen peroxide reduction was monitored by the decrease in absorbance at 240nm. Following gel filtration, catalase was quantified by its absorbance at 403nm.

(d) Alcohol dehydrogenase activity. (Adapted from Vallee and Hoch, 1955.) To measure the amount of alcohol dehydrogenase in each fraction, an aliquot (10-20 μ l) was added to 1ml of 50mM Tris-HCl buffer, pH 8.8, containing 0.3M ethanol and 7.5mM nicotinamide dinucleotide (NAD). The production of NADH was monitored by the increase in absorbance at 340nm

(e) Detection of other markers. Cytochrome c levels in fractions showing a brown-red colour were measured by their absorbance at 410nm. The levels of Dextran blue and ferricyanide were detected similarly by their absorbance at 600nm and 450nm, respectively. The position of thyroglobulin was detected by the protein content of 100 μ l of the fraction measured by the method of Bradford (1976).

(f) Estimation of the $\rho_{1/2}$ values of sucrose density gradients. The densities of the stock sucrose solutions were determined by weighing 3 x 1ml of each. These values were then taken as the densities of the first (5%) and penultimate (20%) fractions obtained from the gradient and the $\rho_{1/2}$ values were extrapolated from this

6.2.4 Radiation inactivation.

The method used was essentially that of Lo et al., (1982).

Synaptosomes were prepared from rat cortices as described in Section

2.2.8 except that the final pellet was resuspended in deionised water and repelleted to remove any residual sucrose. The pellet was again resuspended in deionised water and 200 μ l of this suspension ($\sim$ 600 μ g protein) was placed into a series of 15ml pyrex glass tubes. These samples and tubes containing enzyme molecular weight markers [rabbit muscle pyruvate kinase ($M_r = 224,000$), rabbit muscle phosphocreatine kinase ($M_r = 170,000$) and horse liver alcohol dehydrogenase ($M_r = 84,000$)] were frozen to -80°C and the samples were lyophilised ($\sim$ 15h). The tubes were sealed under high vacuum (2×10^{-2} mm Hg) by melting the necks whilst taking care not to heat the dried samples. They were then maintained at -80°C . Irradiation was performed using the 8MeV linear electron accelerator formerly at Hammersmith Hospital, London. Samples were placed in an aluminium holder and suspended in a bath of acetone containing solid CO_2 . Irradiation was in a uniform beam of 8MeV electrons at a rate of 0.4Mrad/min. At $\sim$ 4 minute intervals the solid CO_2 was replenished. Triplicate samples were given various integrated doses (0-12 Mrad) by exposure to the beam for different lengths of time. Prior to assay, the tubes were equilibrated to 23°C and the tops of the sealed tubes were removed. Samples were resuspended in 200 μ l of 5mM Tris-HCl, pH 7.5, containing 1mg/ml bovine serum albumin, by trituration through a hypodermic needle. DTX binding activity was determined in the presence of $1.5\text{nM } ^{125}\text{I-DTX}$ as described in Section 2.2.9. The enzyme assays used (listed below) were adapted for use in an LKB 8600 reaction rate analyser from published methods (Liu *et al.*, 1980 for pyruvate kinase and phosphocreatine kinase; Vallee and Hoch, 1955 for alcohol dehydrogenase).

(a) Pyruvate kinase.

Solution A: 50mM Tris-HCl, pH 7.5, containing 0.1M KCl, 20mM MgSO_4 , 1mM EDTA, 0.25mM NADH, 7.5mM phosphoenol pyruvate and 25 units/ml D-lactic dehydrogenase.

Solution B: 0.1M ADP.

(b) Phosphocreatine kinase.

Solution A: 0.1M TEA-HCl, pH 7.0, containing 20mM glucose, 10mM Mg acetate, 1mM ADP, 10mM AMP, 0.6mM NADP, 1.25 units/ml hexokinase, 1.25 units/ml glucose-6-phosphate dehydrogenase and 9mM glutathione.

Solution B: 35mM creatine phosphate.

(c) Alcohol dehydrogenase.

Solution A: 100mM Tris-HCl, pH 8.8, containing 0.2M ethanol.

Solution B: 50mM NAD.

In each case, 25µl of solution B was automatically dispensed into, and mixed with, 0.5ml of solution A containing 10-25µl of the irradiated samples. After preincubation for 2 min the rate of change of absorbance at 340nm was recorded.

6.2.5 Evaluation of the effect of lyophilisation on DTX acceptors.

Synaptic membranes or synaptosomes, prepared from fresh rat cortices (Section 2.2.8 and 4.2.1) were lyophilised and resuspended (in deionised water) as described previously. ^{125}I -DTX binding activity was then assayed as described in Section 2.2.9; after solubilisation, sucrose density gradient centrifugation in $^1\text{H}_2\text{O}$, gradients was performed as described in Section 6.2.1.

6.2.6 Definition of enzyme units.

As defined by Sigma Chemical Co., enzyme units are as follows:

(a) Alcohol dehydrogenase (EC.1.1.1.1)

One unit will convert 1.0µmol of ethanol to acetaldehyde per minute at pH 8.8 at 25°C.

(b) Catalase (EC 1.11.1.6)

One unit will decompose 1.0 μ mol of H₂O₂ per minute at pH 7.0 at 25°C, while the H₂O₂ concentration falls from 10.3 to 9.2 μ mol per ml of reaction mixture.

(c) β -Galactosidase (EC 3.2.1.23)

One unit will hydrolyze 1.0 μ mol of o-nitrophenyl- β -D-galactoside to o-nitrophenol per minute at pH 7.3 at 37°C and using o-nitrophenol as standard.

(d) Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)

One unit will oxidise 1.0 μ mol of D-glucose-6-phosphate to D-glucose in the presence of NADP at pH 7.4 and 25°C.

(e) Hexokinase (EC 2.7.1.1)

One unit will phosphorylate 1.0 μ mol of glucose per minute at pH 8.5 at 25°C.

(f) D-Lactic dehydrogenase (EC 1.1.1.28)

One unit will reduce 1.0 μ mol of pyruvate to D-lactate per minute at pH 7.0 at 25°C.

(g) Phosphocreatine kinase (EC 2.7.3.2)

One unit will transfer 1.0 μ mol of phosphate from phosphocreatine to ADP per minute at pH 7.4 at 30°C.

(h) Pyruvate kinase (EC 2.7.1.40)

One unit will convert 1.0 μ mol of phospho(enol)pyruvate to pyruvate per minute at pH 7.6 at 37°C.

6.3 Results

6.3.1 Sucrose density gradient centrifugation of solubilised DTX acceptors from rat brain.

A single peak of saturable ¹²⁵I-DTX binding activity was seen

following centrifugation in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ linear sucrose gradients (Figure 6.1a). Saturable binding in the peak from $^2\text{H}_2\text{O}$ gradients was inhibited to 50% by 100nM β -BuTX (Figure 6.1a), showing that it contained both classes of DTX acceptors (see Discussion).

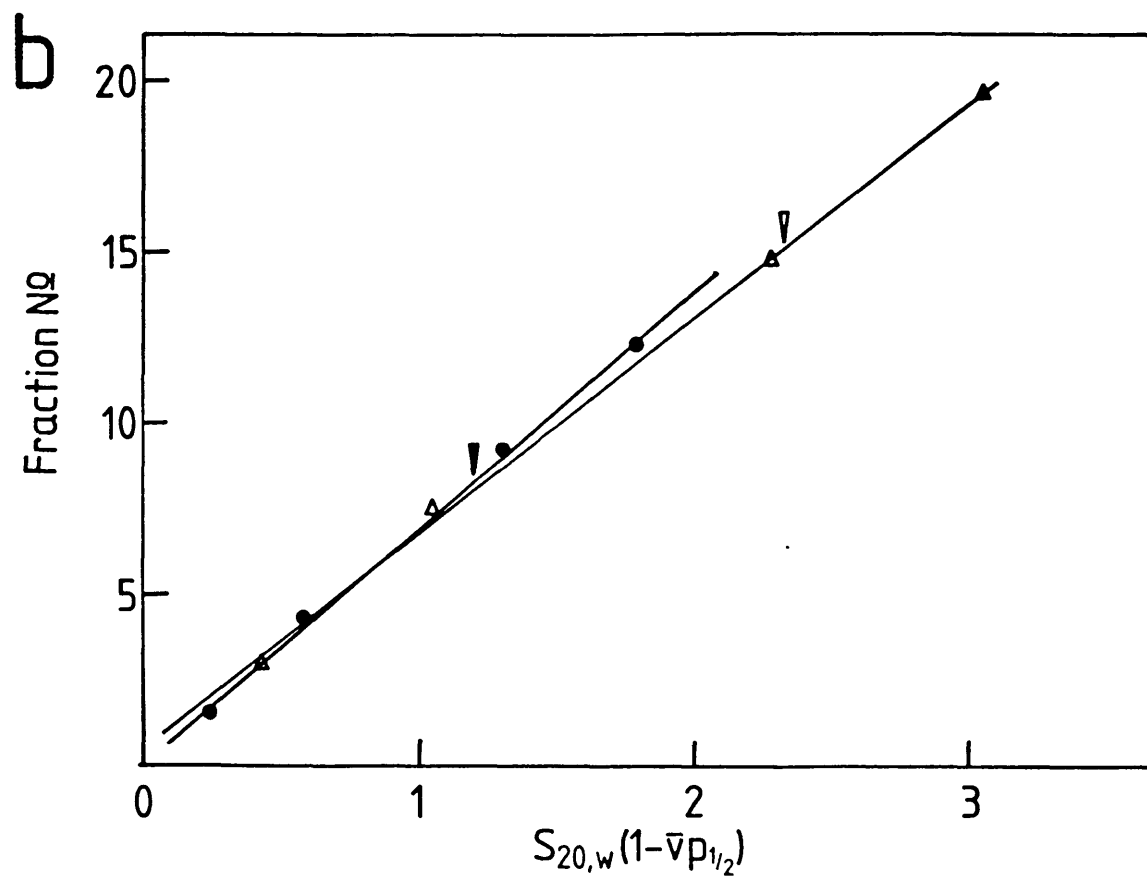
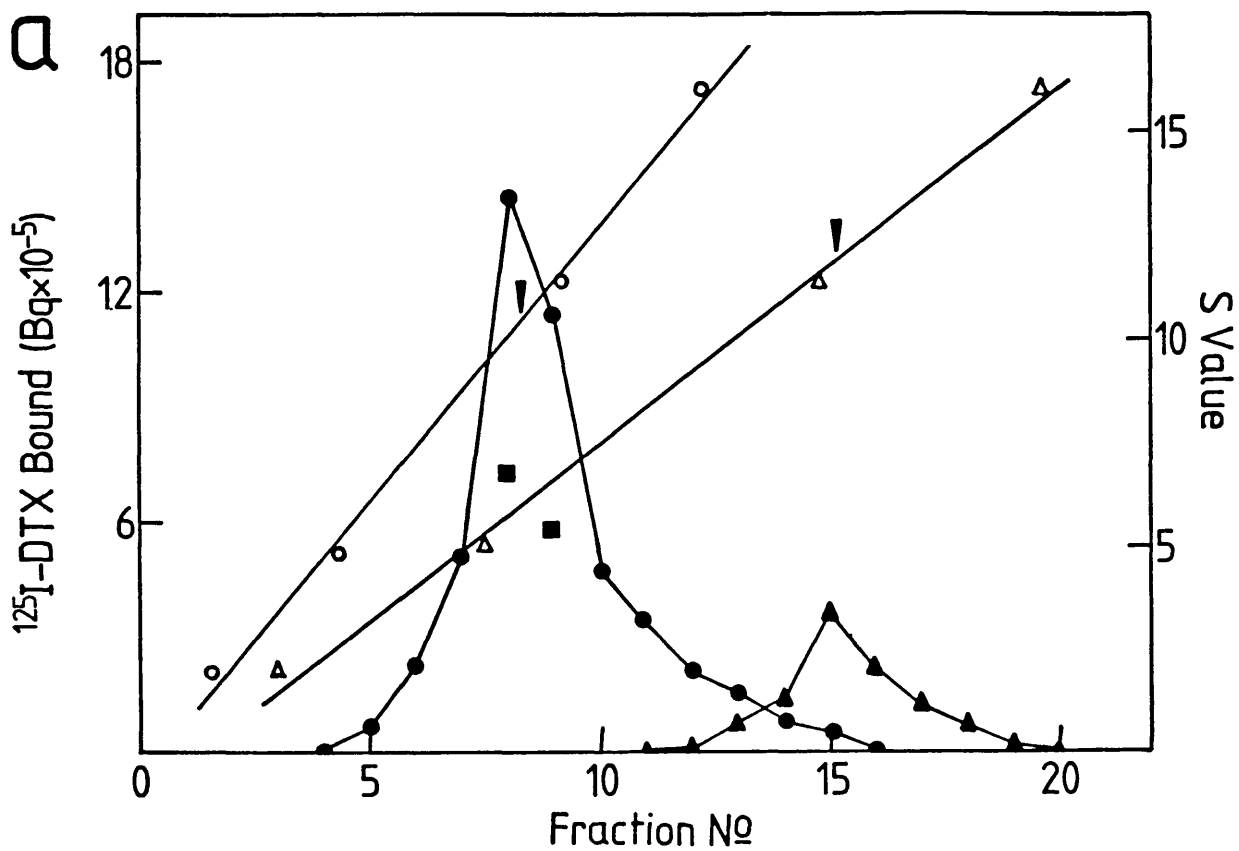
The recoveries of ^{125}I -DTX binding activity following centrifugation was ~5% and ~20% in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ gradients, respectively, reflecting the instability of the acceptors. The reason for the greater stability in $^2\text{H}_2\text{O}$ gradients is unknown. However, this has been seen for other membrane proteins (Cleanthi Mamalaki, personal communication [GABA receptor]; Rehm and Betz, 1984) and presumably reflects the different interaction of the two solvents with proteins (Henderson et al., 1970). By comparison with standard proteins (Figure 6.1a) the apparent sedimentation coefficients for the DTX acceptors were $11.7 \pm 0.2\text{S}$ ($n = 9$) and $10.6 \pm 0.1\text{S}$ ($n = 9$) in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$, respectively. Plotting these data as distance travelled against $S_{20,w} (1-\bar{v}\rho_{1/2})$ (Figure 6.1b) produced a straight line for each solvent. From this a partial specific volume of $0.747 \pm 0.003\text{ml/g}$ and an $S_{20,w}$ of $12.7 \pm 0.1\text{S}$ for the DTX acceptor/lipid/detergent complexes were calculated (Formula 6.2).

To calculate the weight fraction of protein in the acceptor/detergent/lipid complex, it was assumed that the partial specific volume of Triton X-100 was 0.910ml/g [as measured in micelles (Pollet et al., 1981)] and that of the lipid was 0.981 ml/g [for phosphatidylcholine vesicles (Huang and Charlton, 1971)]. It was also assumed that the weight ratio of detergent/lipid in the complex was the same as that in the gradients (4/1), which gives a partial specific volume of 0.924ml/g for the bound detergent/lipid. The partial specific volume of the acceptors was taken as being between that of the Na^+ channel (0.70ml/g) and the nicotinic acetylcholine receptor (0.73ml/g), as calculated by the method of Cohn and Edsall (1943)

Figure 6.1 Sucrose density gradient centrifugation analysis of DTX acceptors.

- (a) Soluble extract of rat brain and standard proteins [β -galactosidase (16S), catalase (11.4S), yeast alcohol dehydrogenase (4.8S) and cytochrome c (1.8S)] were centrifuged ($190,000 \times g$; 19h) simultaneously through 5-20% sucrose gradients made with either $^2\text{H}_2\text{O}$ ($\bullet, \blacksquare, \circ$) or $^1\text{H}_2\text{O}$ ($\blacktriangle, \triangle$). Points shown for standard proteins (open symbols) are their peak positions estimated from their levels in each fraction. Closed symbols show the saturable binding of ^{125}I -DTX (that displaceable by 120nM DTX) in fractions in the absence ($\bullet, \blacktriangle$) or presence ($\blacksquare$) of 100nM β -BuTX. Arrows show the estimated peak position of saturable binding.
- (b) Data in (a) re-arranged according to Meunier et al., 1972 (Formula 6.2). Open and closed symbols are for $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ gradients, respectively.

Figure 6.1 Sucrose density gradient centrifugation analysis of DTX acceptors.



(McMeekin and Marshall, 1952), from their amino acid compositions (Miller et al., 1983; Vandlen et al., 1979) and by assuming that the partial specific volume of their carbohydrate component was that of hexose [0.613ml/g (Barnard, 1984)]. These values gave a weight fraction of acceptor in the complex of between 0.79 and 0.91g/g (Formula 6.4).

6.3.2 Gel filtration of solubilised DTX acceptors from rat brain.

Following gel filtration of solubilised rat synaptic membranes, ~25% of the ^{125}I -DTX binding activity was recovered. As with density gradient centrifugation, this activity was seen in a single peak (Figure 6.2a) which appeared to contain both of the proposed classes of acceptor for DTX; a 60% reduction in saturable ^{125}I -DTX (1.2nM) binding was produced by 100nM β -BuTX (Figure 6.2a). The straight line plot of the Stokes radii of the standard proteins against their partition coefficient ($K_d^{1/3}$) (Figure 6.2b) gave a Stokes radius for the DTX acceptor of $8.6 \pm 0.3\text{nm}$ ($n = 3$). When this value and those derived from density centrifugation, summarised in Table 6.1, were substituted into Formula 6.1, an M_r of $490,000 \pm 30,000$ for the acceptor/lipid/detergent complex was obtained; hence, the M_r of the acceptor can be estimated as 390,000-450,000.

Taking the hydration of the acceptor protein as 0.3g/g and that of the detergent/lipid portion as 1.75g/g (Pollet et al., 1981), the frictional ratio of the acceptor/lipid/detergent was calculated as 1.4 (Formula 6.5). This corresponds to an axial ratio of 8:1 for an equivalent prolate ellipsoid (Schachman, 1959).

6.3.3 Radiation inactivation of lyophilised rat cerebrocortical synaptosomes.

Binding curves (Figure 6.3a) and Scatchard analysis (Figure 6.3b) showed that lyophilisation of synaptosomes had no significant effect on

Figure 6.2 Gel filtration of solubilised DTX acceptors.

- (a) Solubilised extract and standards (Dextran blue; thyroglobulin (8.5nm); β -galactosidase (6.4nm); catalase (5.2nm); yeast alcohol dehydrogenase (4.6nm); cytochrome c (1.87nm) and ferricyanide were chromatographed through a Sepharose C1-6B column (1 x 54cm) as described in Section 6.2.2. Arrows show the peak positions of standards estimated from their activity in individual fractions. Saturable binding of 1.2nM ^{125}I -DTX (that displaceable by 120nM DTX) in the presence (■) or absence (●) of 100nM β -BuTX was quantified by a rapid gel filtration assay.
- (b) Data in (a) replotted according to Porath (1963) (Formula 6.3). The arrow shows the peak position of DTX acceptors.

Figure 6.2 Gel filtration of solubilised DTX acceptors.

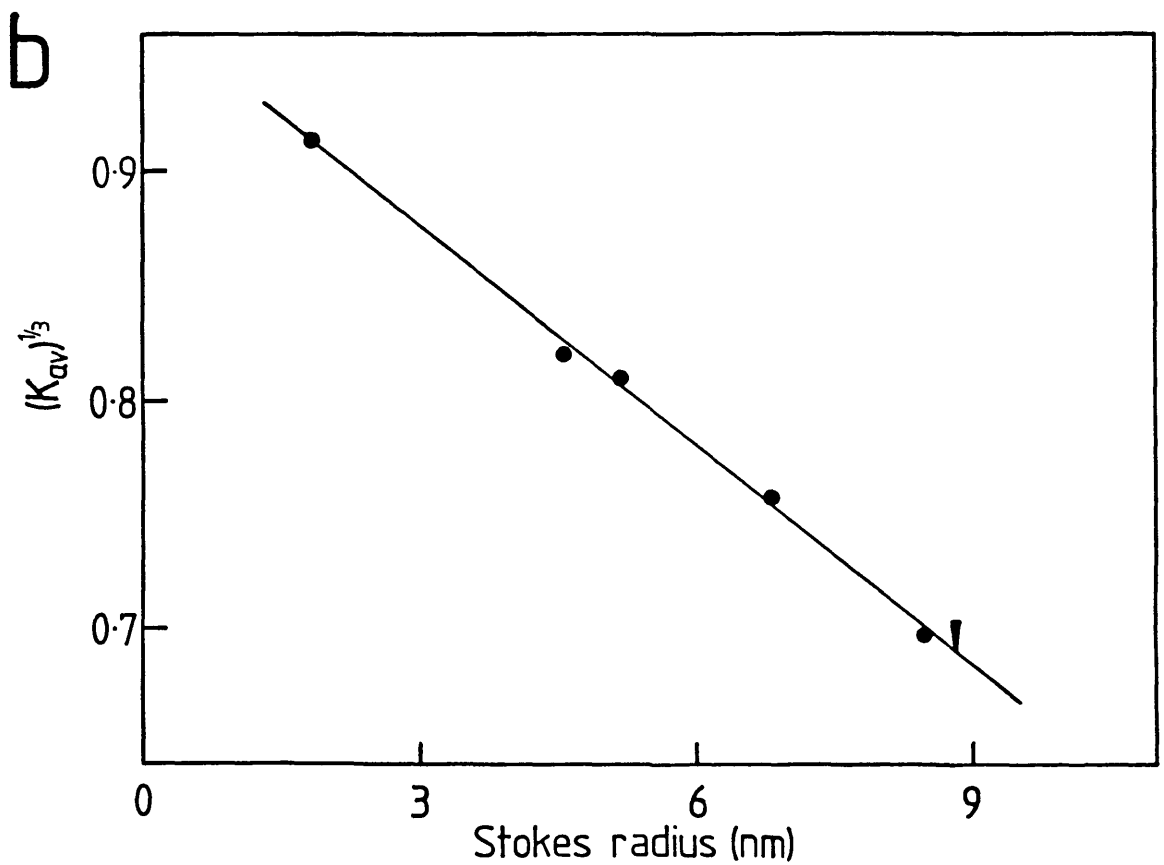
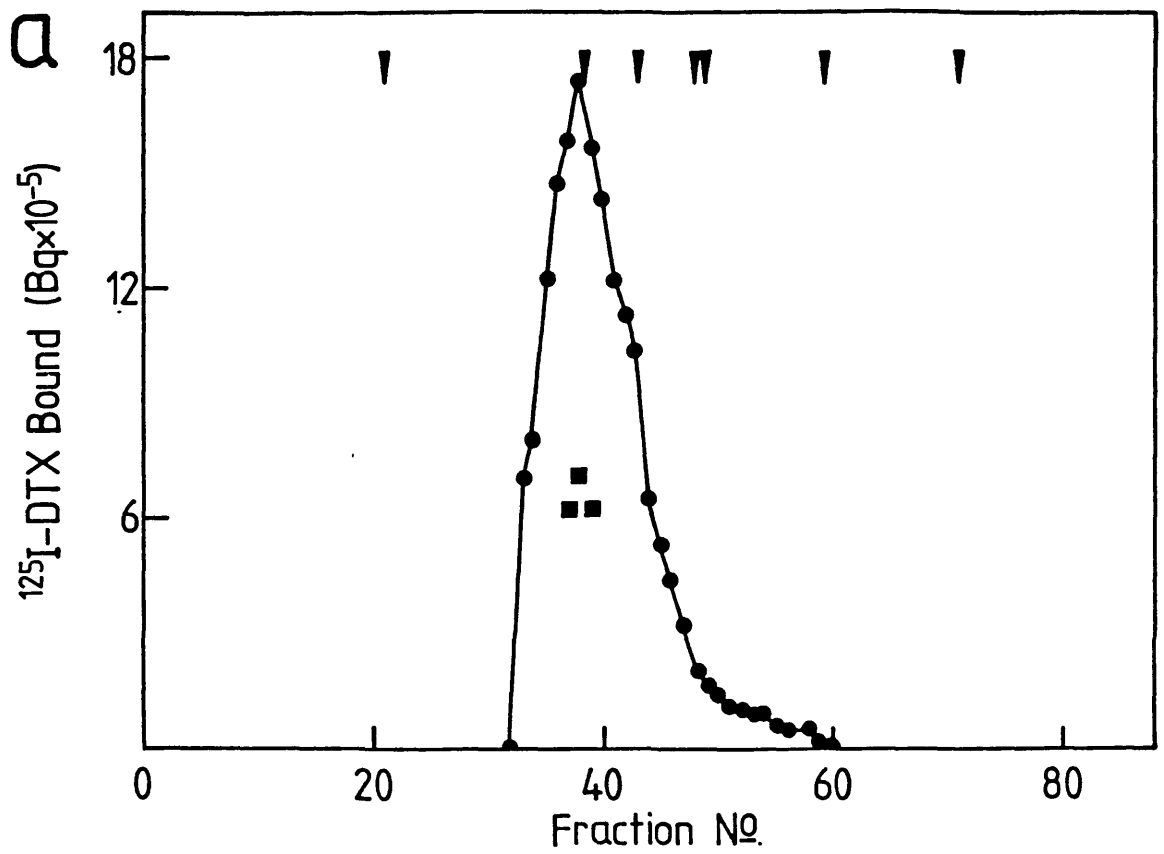


Table 6.1 Physical properties of DTX acceptors from rat brain.

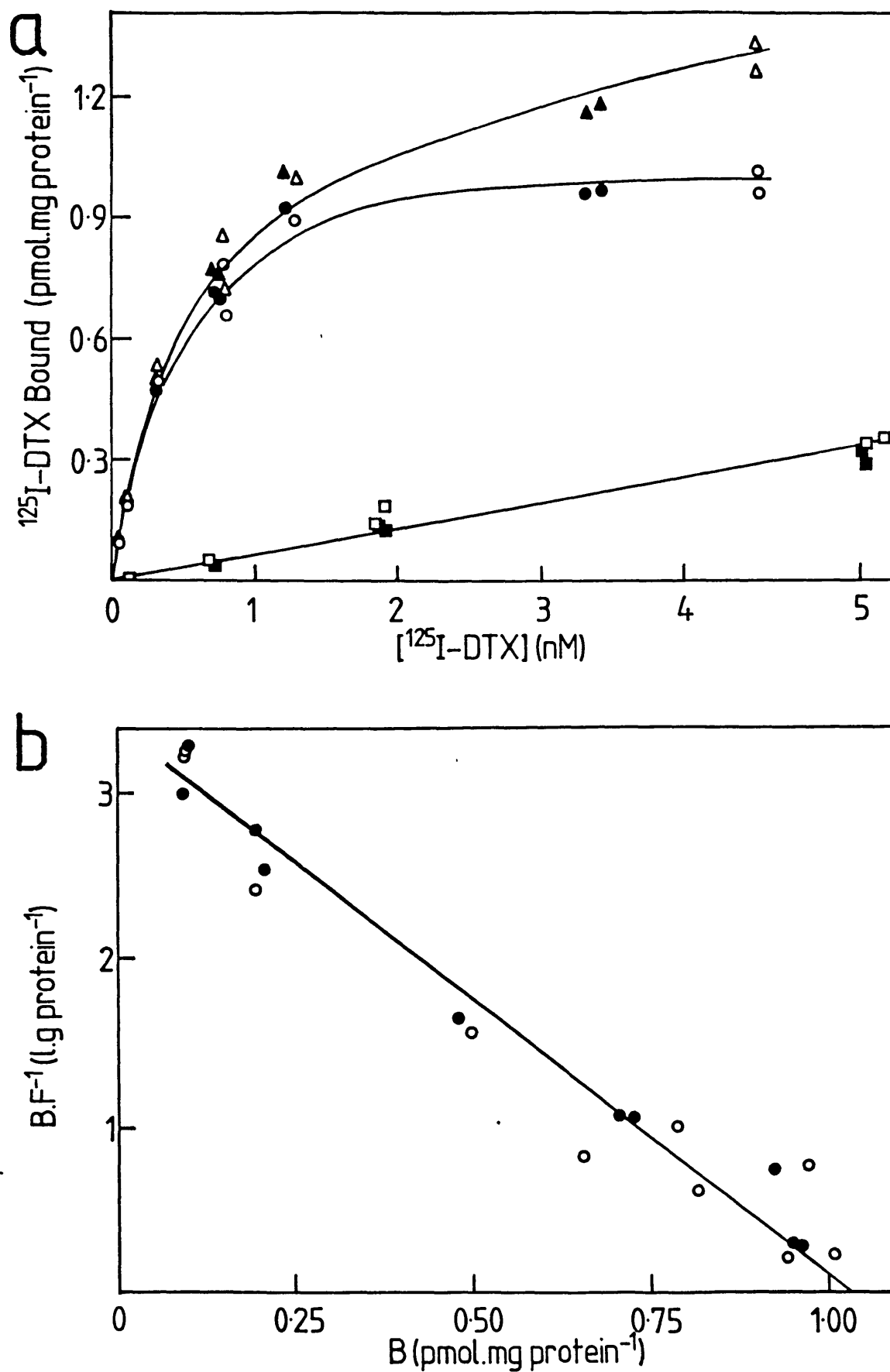
(a) Detergent/lipid/acceptor complex.		
S value (H ₂ O)		12
S value from lyophilised membranes (H ₂ O)*		12 α 8
Partial specific volume (ml/g)		0.747 ± 0.003
Weight fraction of protein (g/g)		0.79 - 0.91
Frictional ratio (f/f _{min})		1.4
Stokes radius (nm)		8.6 ± 0.3
Molecular weight (M _r)		492,000 ± 28,000
(b) Solubilised acceptor		
S _{20,w}		12.7 ± 0.1
Molecular weight (M _r)		390,000 - 450,000
(c) Membrane bound acceptor		
Molecular weight (M _r)		250,000 ± 14,000

Data in (a) and (b) are taken from sucrose density centrifugation and gel filtration of soluble extract produced from frozen rat synaptic membranes except for (*) where lyophilised membranes were used. Those in (c) are from radiation inactivation of lyophilised synaptosomes. Values are averages of 3 experiments (±S.D. where appropriate).

Figure 6.3 Binding of ^{125}I -DTX to lyophilised and non-lyophilised synaptosomes.

- (a) Binding in Krebs phosphate was assayed by a centrifugation washing technique as described in Section 2.2.9. Open and closed symbols represent data for lyophilised and non-lyophilised synaptosomes, respectively. Non-specific binding ($\square, \blacksquare$) was assessed by incubation with 500nM DTX and was subtracted from total binding ($\Delta, \blacktriangle$) to give specific binding ($\circ, \bullet$).
- (b) Scatchard analysis of the specific binding in (a).

Figure 6.3 Binding of ^{125}I -DTX to lyophilised and non-lyophilised synaptosomes.



either the affinity ($K_D = 0.3\text{nM}$) or extent of ^{125}I -DTX binding ($B_{\text{max}} = 1.05$ and 1.03pmol/mg protein before and after lyophilisation, respectively). Due to lack of material, full competition experiments could not be conducted using homologues of DTX; however, $1\mu\text{M}$ toxin E was able to totally inhibit saturable ^{125}I -DTX binding to lyophilised synaptosomes (saturable binding = -3% of control) whereas $1\mu\text{M}$ toxin B or BPTI had no effect (saturable binding = 97% and 93% of control, respectively). Thus, the selectivity of the acceptors for DTX homologues appeared unaltered by lyophilisation. Collectively, these data show that lyophilised synaptosomes are a valid system in which to conduct inactivation studies on the DTX acceptors.

The inactivation of saturable ^{125}I -DTX binding in lyophilised synaptosomes was related to the radiation dose in a simple exponential manner (Figure 6.4a) showing that, over the doses applied, the DTX acceptors presented a single target size to ionising radiation. Assuming a temperature correction factor of 1.95 for experiments conducted at -80°C (extrapolated from values in Angelides *et al.*, 1985 and Nielson *et al.*, 1981), the D_{37} value of this inactivation gave a target size for the DTX acceptors of $225,000 \pm 16,000$ ($n = 3$). The size was also obtained from the straight line produced when the inactivation rates of the standard enzymes (Figure 6.4a) were plotted against their molecular weight (Figure 6.4b). This gave a very similar value of $250,000 \pm 14,000$ ($n = 3$).

Because of the differences in the sizes obtained for the DTX acceptors from target analysis, and from their hydrodynamic properties, it was decided to ascertain whether the discrepancy was due to an effect of lyophilisation. Thus, lyophilised synaptosomes and synaptic membranes were solubilised and subjected to sucrose density gradient centrifugation. As can be seen in Figure 6.5a and b, this treatment did not effect the majority of observed DTX acceptors, which sedimented as a

Figure 6.4 Radiation inactivation of DTX acceptors.

- (a) Lyophilised rat cerebrocortical synaptosomes and standard proteins were irradiated simultaneously at -80°C in a uniform electron (8MeV) beam. Specific binding of 1.5nM ^{125}I -DTX and enzyme activities were quantified as described in Section 6.2.4. Symbols represent; $\blacktriangle$, horse liver alcohol dehydrogenase ($M_r = 84,000$); $\blacktriangledown$, phosphocreatine kinase ($M_r = 170,000$); $\blacksquare$, pyruvate kinase ($M_r = 224,000$); $\bullet$, saturable ^{125}I -DTX binding (that displaceable by 150nM DTX).
- (b) Gradients of curves in (a), relative to that seen with pyruvate kinase (I_{PK}), plotted against the molecular weight of the respective proteins. The arrow shows the gradient of the curve for ^{125}I -DTX binding.

Figure 6.4 Radiation inactivation of DTX acceptors.

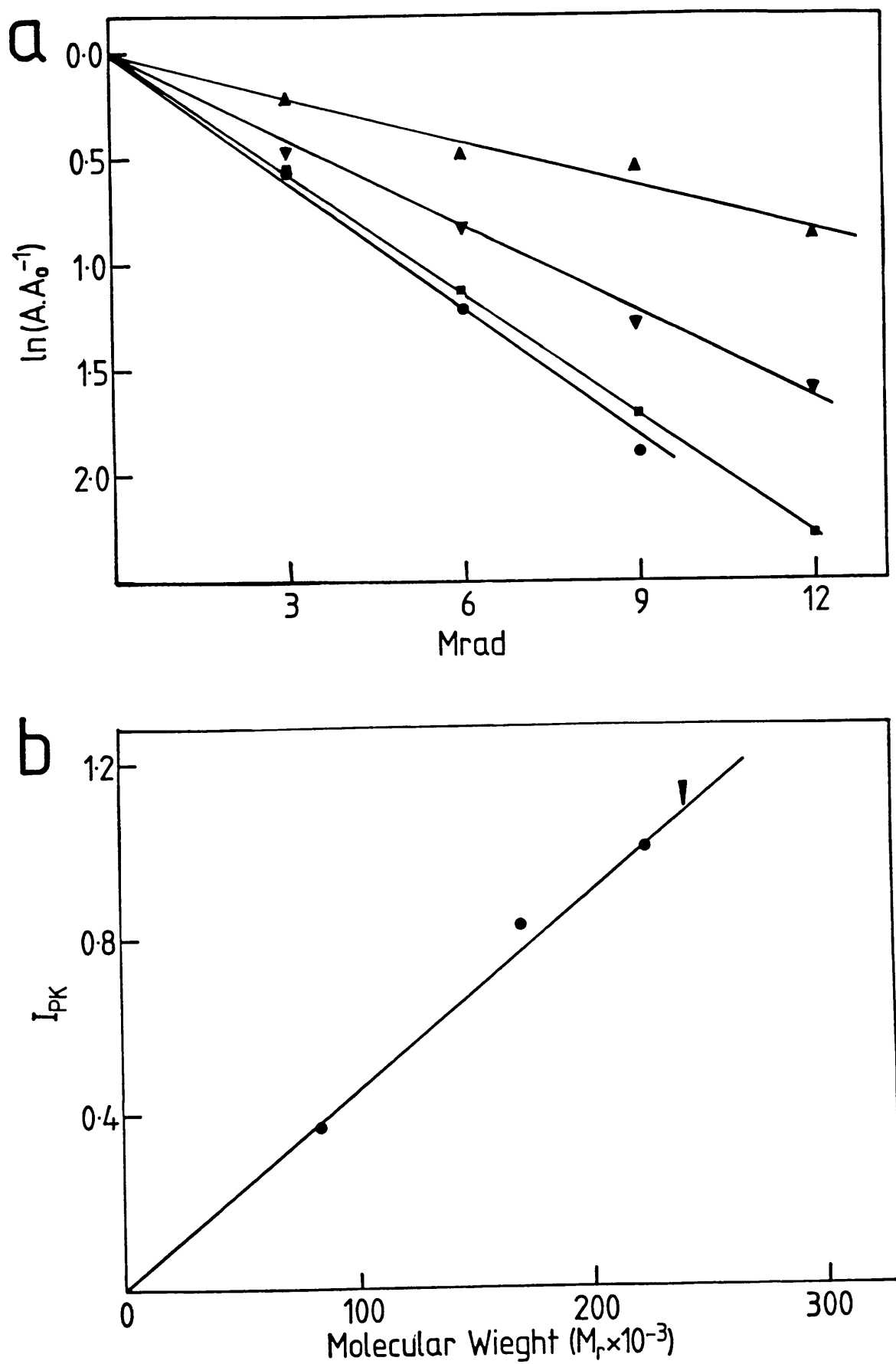
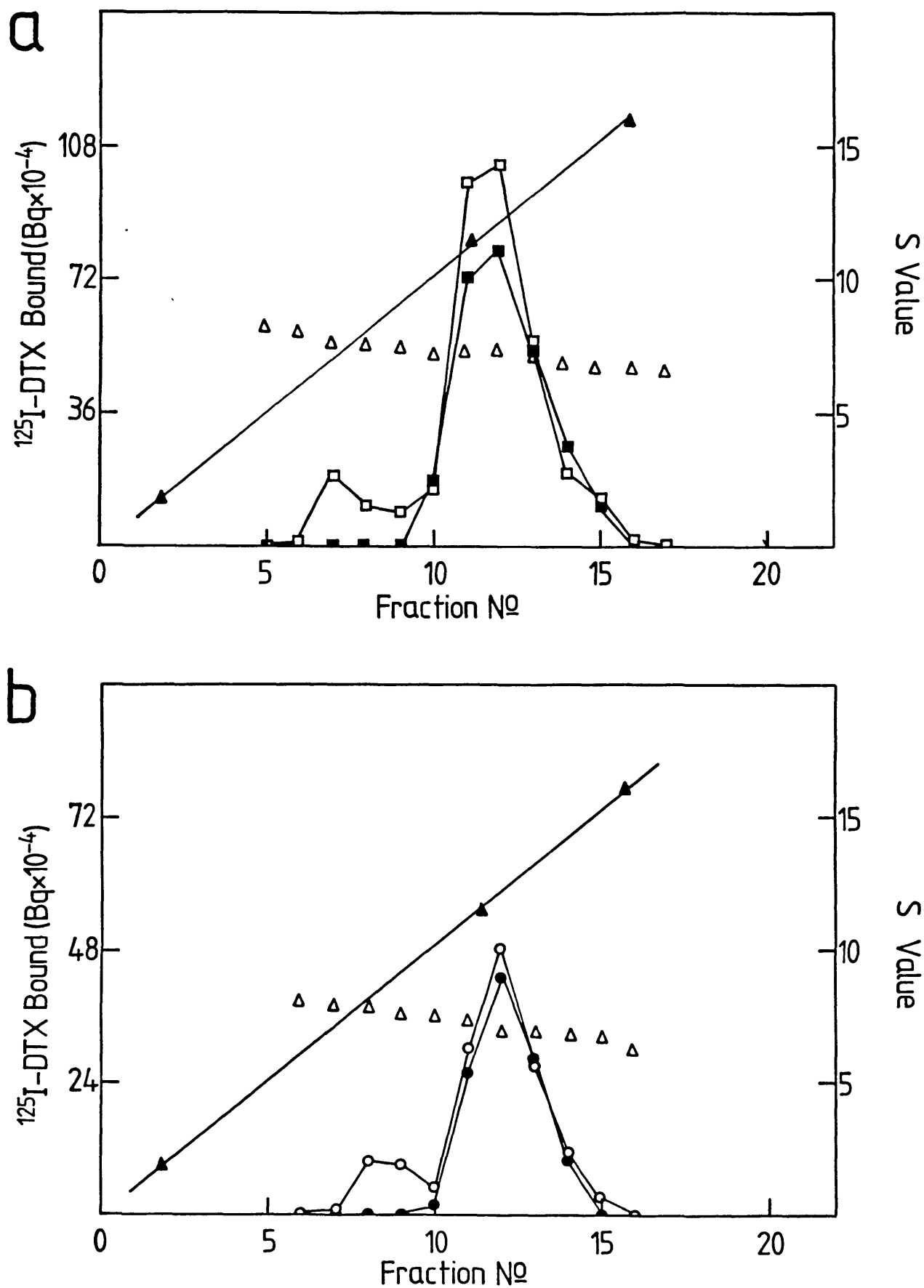


Figure 6.5 Sucrose density gradient centrifugation analysis of DTX acceptors solubilised from lyophilised and non-lyophilised synaptosomes or synaptic membranes.

Soluble extract of rat synaptosomes (a) or synaptic membranes (b), together with standard proteins [β -galactosidase (16S), catalase (11.4S) and cytochrome c (1.8S)], were centrifuged ($190,000 \times g$; 19h) simultaneously through 5-20% sucrose gradients. Levels of enzyme standards ($\blacktriangle$) and saturable binding of $1.2\text{nM } ^{125}\text{I-DTX}$ (circles and squares) were assayed as in Section 6.2.1. Open triangles ($\triangle$) represent the level of non-specific binding observed in the presence of 120nM DTX which was subtracted from the total binding to give the saturable binding data shown. Open ($\square, \circ$) and closed ($\blacksquare, \bullet$) symbols represent the specific binding of $1.2\text{nM } ^{125}\text{I-DTX}$ in gradients containing extracts from lyophilised and non-lyophilised samples, respectively.

Figure 6.5 Sucrose density gradient centrifugation analysis of DTX acceptors solubilised from lyophilised and non-lyophilised synaptosomes or synaptic membranes.



12S species. However, following lyophilisation, a small peak of saturable ^{125}I -DTX activity was obvious above the non-specific binding (being ~25% of this latter value); this represented an acceptor species of $7.9 \pm 0.4\text{S}$ ($n = 4$).

6.4 Discussion

6.4.1 Hydrodynamic properties of solubilised DTX acceptors.

From sucrose density centrifugation and gel filtration, it appears that the structures of the two classes of DTX acceptor from rat brain are very similar. In each case, there was a single peak of ^{125}I -DTX binding activity which was half inhibited by 100nM β -BuTX (Figures 6.1a and 6.2a). If only one class were present, either negligible or complete inhibition would be expected by this concentration of β -BuTX (cf. Figure 5.9). Thus, all of the hydrodynamic properties described here can be taken as applying to both of the DTX acceptor types. Despite the similarity of structure, the class of DTX acceptor which binds β -BuTX with high affinity appeared to be the more stable, the extent of inhibition by β -BuTX seen after analysis being somewhat greater than that seen before (Figures 6.1a, 6.2a and 5.9).

As expected from the difference in the sedimentation coefficients obtained using $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ gradients, DTX acceptors appear to bind significant amounts of detergent/lipid; the estimated weight fraction of acceptor protein in the acceptor/detergent/lipid complex was only 0.79-0.91g/g. It should be noted that the values of partial specific volume for the Na^+ channel (0.71ml/g) and the nicotinic acetylcholine receptor (0.73ml/g) used to obtain these figures vary significantly from each other and it would not be surprising if they deviated from those of the DTX acceptors. Thus, the weight fraction of

acceptor in the complex and the size of the acceptor derived from it should only be taken as estimates. Collectively, the data obtained from gel filtration and sucrose density gradient centrifugation (Table 6.1) gave an M_r of $490,000 \pm 30,000$ for the acceptor/lipid/detergent complexes and an M_r of 390,000–450,000 for the acceptors themselves. From the frictional ratio of 1.4, the DTX acceptors appear to be elongated molecules which is consistent with their proposed role as K^+ channels (see Chapter 7).

The hydrodynamic properties of the rat brain DTX acceptors bear a striking similarity to those seen for the solubilised β -BuTX/DTX acceptor from chick brain (Chapter 3; Rehm and Betz, 1984), where the $S_{20,w}$ (12.8S) of the complex and the derived molecular weight of the acceptor (431,000) agree closely with these seen here. The only significant difference arose from a greater amount of detergent bound to the chick brain acceptors ($X_a = 0.74\text{g/g}$). Thus, as seen from cross-linking data (Chapter 4), it would appear that although some differences exist, the structures of the DTX acceptors in these two species are similar.

6.4.2 The size of DTX acceptors in the membrane and their relationship with solubilised acceptors.

The M_r obtained by target theory analysis of irradiated rat brain synaptosomes can be ascribed solely to the class of DTX acceptors which bind only DTX homologues with high affinity; the system used may not detect the small proportion of the other class of acceptors (cf. Chapter 2). Even so, the M_r of 250,000 is significantly different from that obtained for the solubilised acceptors (Table 6.1). This discrepancy cannot be explained by the detection of a minor form of DTX acceptors following gel filtration or sucrose density centrifugation; these would represent at least 25% of the total DTX acceptors and would,

therefore, be detected in irradiation experiments. Following solubilisation of lyophilised membranes, an 8S species of DTX acceptors was observed, which would be about half the size of the major 12S species (Figure 6.5, Table 6.1). This shows that DTX binding activity can reside in a smaller structure than normally seen for the solubilised acceptors and presumably explains the lower M_r obtained from inactivation data. Further, it argues against non-specific association of membrane proteins upon solubilisation as lyophilisation would not be expected to affect this. Due to the low amounts of the smaller species recovered from density gradients, it was not possible to determine its relationship with the two acceptor types. In order to do this, analysis of density gradients and irradiated samples with labelled β -BuTX will be required.

There are several possible explanations for the discrepancy between the hydrodynamic and inactivation data. Due to the relative sizes obtained (Table 6.1), the DTX acceptor could exist as a dimer which inactivates as a monomer, like the dimeric nicotinic acetylcholine receptor (Lo *et al.*, 1982). Alternatively, there could be a regulatory protein associated with the DTX acceptors which is "uncoupled" under the conditions used for inactivation, similar to the situation seen for adenylate cyclase (Schlegel and Kempner, 1984). Equally, the size obtained from inactivation experiments could represent that of a functional unit within the DTX acceptors, or acceptor components could reversibly dissociate when lyophilised.

In order to investigate these possibilities further, it would be interesting to study the effect of possible regulatory ligands on the target size of DTX acceptors, as well as to perform inactivation on frozen membranes and soluble extract. These studies have not been carried out to-date as soluble extract loses activity on freezing and

because the linear accelerator used is now an exhibit in the Herriot Watt Museum, Edinburgh.

In conjunction with the cross-linking studies (Chapter 4) the results presented in this chapter indicate that DTX acceptors are oligomeric proteins as the acceptors (M_r at least 250,000) must contain multiple copies of the identified polypeptides ($M_r \sim 70,000$) or components which have yet to be identified.

6.4.3 Summary

Hydrodynamic properties and radiation inactivation were used to determine the molecular weight of solubilised and membrane-bound DTX acceptors, respectively. These showed that DTX binding activity can reside in a smaller structure than that normally seen upon solubilisation, suggesting that the acceptors are part of a larger complex.

CHAPTER 7

GENERAL DISCUSSION

7.1 Physiological Role of DTX Acceptors.

The production of ^{125}I -DTX has enabled the identification of acceptors for DTX in neuronal membranes. Interestingly, these could be separated into two classes by virtue of a differential interaction with β -BuTX, one class being a common high affinity acceptor for β -BuTX and DTX. When the structures of these acceptors were studied, both in the membranes by cross-linking and radiation inactivation and following their active solubilisation, they were found to be large oligomeric proteins. Further, the two classes of acceptors from both of the species studied appeared to be closely related. Thus, it seems that all DTX acceptors may have evolved from a common precursor, as has been proposed for α -bungarotoxin binding components (Conti-Tronconi et al., 1985).

7.1.1 DTX acts on voltage-sensitive K^+ currents.

The first step in determining the physiological relevance of the identified acceptors for DTX is obviously to determine the precise biological actions of the toxins with which they interact.

The PLA_2 activity of β -BuTX contributes to its potent neurotoxicity. In the presence of Sr^+ or following chemical modification, where this activity is virtually abolished, the potency of β -BuTX is greatly reduced in both central and peripheral tissue (Halliwell and Dolly, 1982; Abe et al., 1977; Chang et al., 1977). However, in the absence of its PLA_2 activity, β -BuTX still affects neurotransmission. At frog neuromuscular junction, the use of p-bromophenylacetyl bromide (Abe et al., 1977) and Sr^{2+} (Caratsh et al., 1981) has shown that only the initial depression of transmitter release is independent of PLA_2 activity.

In mouse, however, both the initial depression and later facilitation were unaffected by the use of Sr^{2+} containing media (Chang et al., 1977), suggesting that both of these actions of β -BuTX are independent of PLA_2 activity in this species. Thus, although the total blockade of neuromuscular transmission is a result of the enzymic activity of β -BuTX at least the first phase of its action is directly mediated by acceptor binding. In the central nervous system, β -BuTX is able to block irreversibly neurotransmission both in the presence of Sr^{2+} and following p-bromoacylphenyl bromide treatment, albeit with a much slower time course than the native toxin (Halliwell and Dolly, 1982). However, it is not clear if this blockade is due to residual PLA_2 activity of β -BuTX in Sr^{2+} media (Strong et al., 1976) or following modification (Chang, 1985), or solely to the binding of the toxin. In conclusion, it is evident that, at least in the peripheral tissue, the acceptors for β -BuTX are involved in cell excitability but, due to the PLA_2 activity of the toxin, the precise role of these acceptors is unclear.

With DTX, which displays no enzymic activity, it has been possible to gain a clear picture of the mechanisms underlying the effects of the toxin. Electrophysiological evidence suggests that DTX is acting exclusively through attenuation of rapid, transient, voltage-sensitive K^+ current. Voltage clamp studies on the cell bodies of CA_1 neurons have shown that DTX inhibits the A-current, a transient K^+ current, whilst not directly affecting other K^+ conductances or Ca^{2+} currents (Dolly et al., 1984; Halliwell et al., 1985). More recently, the effects of DTX have been measured at the frog node of Ranvier (Weller et al., 1985). The toxin did not directly affect Na^+ conductance but significantly reduced the observed voltage-sensitive K^+ current. DTX reduced the activation rate of the remaining K^+ conductance, which is consistent with blockade of the rapid transient

K⁺ currents in frog node (Dubois, 1981).

Although DTX has not been shown to affect this type of K⁺ current in synapses, the effects of the toxin on synaptosomes suggest that it does. DTX causes a slight depolarisation of synaptosomal membrane potential (Weller et al., 1985; Nicholls et al., 1985) which is thought to mediate its effects on neurotransmitter release (Weller et al., 1985). Evidence that these actions were mediated by attenuation of rapid transient K⁺ currents came when DTX was tested in conjunction with 4-aminopyridine, a selective inhibitor of this type of current (Weller et al., 1985). The effects of 4-aminopyridine on synaptosomes were very similar to DTX, causing a slight depolarisation and release of transmitter. Interestingly, when the toxin and 4-aminopyridine were added together, their effects were less than additive. In fact, at the highest concentrations, no significant addition of effects was seen indicating that these two agents act upon a common component. Although both DTX and aminopyridines facilitate transmitter release at peripheral synapses to about the same extent (Harvey and Karlsson, 1980, 1984), they acted over different times and the relationship between the components they effect has not been determined. However, the similar specificity for DTX homologues in the central nervous system and at motor nerve terminals (see later) suggests that the acceptors in these two locations are closely related. The effect of DTX on rapid transient K⁺ currents could account for all of the observed actions of the toxin. As these serve to limit cell excitability (Gustaffson et al., 1982), their attenuation by DTX should increase the excitability of neurons, thus leading to both the convulsive and facilitatory actions of the toxin. Further, through its action on the A-current, DTX also increases Ca²⁺ channel opening in cell bodies (Halliwell et al., 1985); were this to occur at nerve terminals, it would, by itself, increase transmitter release.

It is not clear whether DTX binds directly to K^+ channels or if its influence is indirect. Its rapid and reversible effects on axonal K^+ currents (Weller et al., 1985) and in the autonomic nervous system (Harvey et al., 1984b, Chapter 2) indicate a direct action. On the other hand, the delayed and irreversible effects at the neuromuscular junction suggests that DTX is acting indirectly. It is interesting to note that even where the toxin has a reversible effect, reversibility is incomplete (Chapter 2, Weller et al., 1985). Thus, the less direct mode of action seems to occur at all toxin sites. Patch clamp studies, in which DTX can be applied to the "patch" or to the surrounding membrane should help to clarify this situation. At present, all that can be concluded is that the DTX acceptors are in some way selectively involved with K^+ channels.

Definite proof that the acceptors identified in the present work are responsible for biological actions of DTX will only come after their purification and active reconstitution (which has been made possible by the solubilisation of active DTX acceptors as detailed in Chapter 5). However, strong evidence that both classes of these acceptors are relevant has been obtained from structure/activity studies using DTX homologues (Black et al., 1985) as well as from autoradiographic studies (Halliwell et al., 1985).

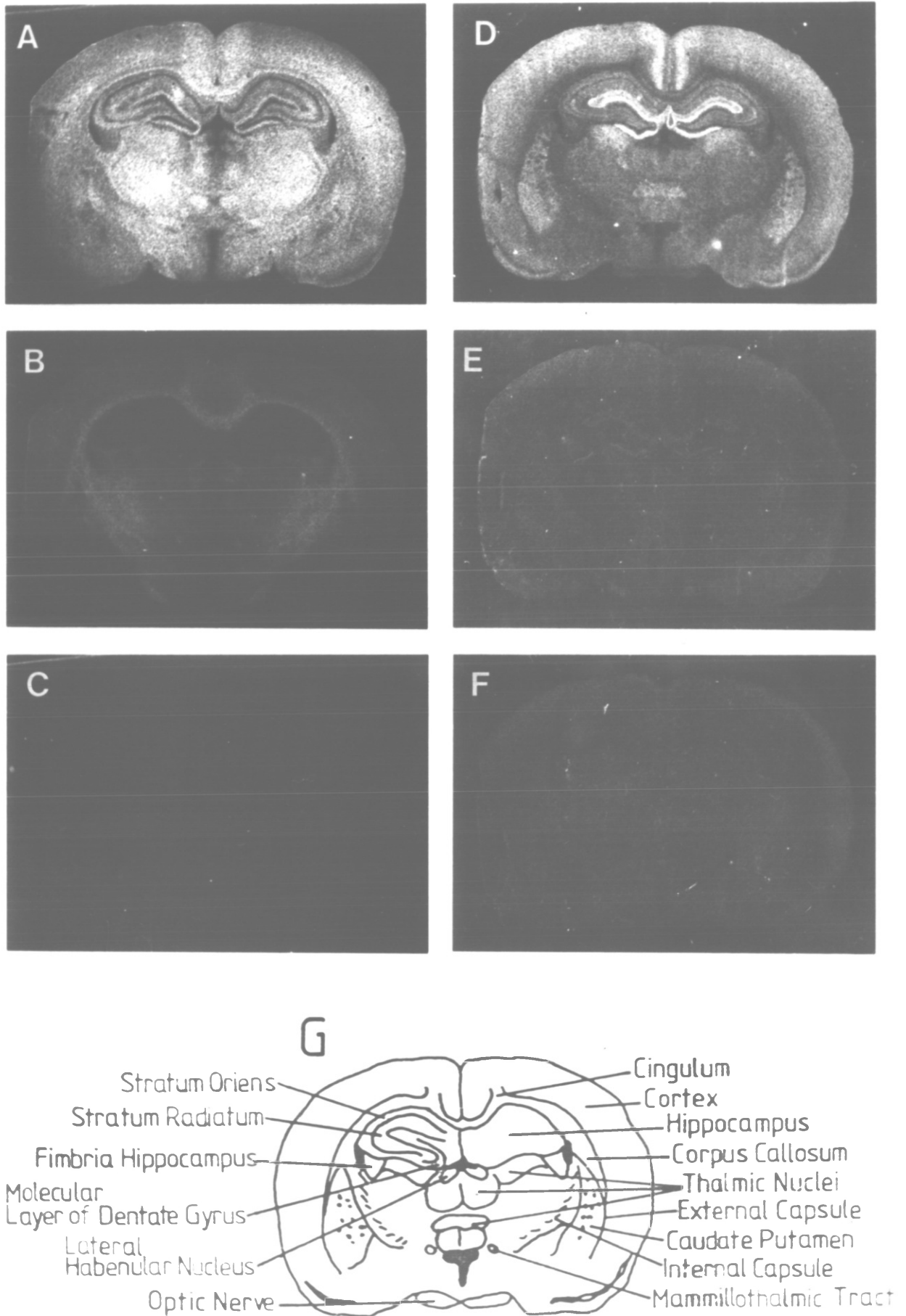
7.1.2 Localisation of DTX acceptors.

By using ^{125}I -DTX produced in Chapter 2, Annegret Pelchen-Matthews of this laboratory has been able to localise DTX acceptors in rat brain sections. Specific ^{125}I -DTX binding has a widespread distribution throughout the forebrain (Figure 7.1 A and C) except in the hippocampus, where it is present mainly in synaptic rich regions [strata oriens and radiatum, and the molecular layer of the dentate gyrus (Halliwell et al., 1985)]. Due to their different affinities for

Figure 7.1 Autoradiographic localisation of ^{125}I -DTX and ^{125}I - β -BuTX binding in rat brain.

Cryostat sections (10 μm) of lightly fixed rat brain were incubated in Krebs-ringer with 2nM ^{125}I -DTX (A, B and C) or 2nM ^{125}I - β -BuTX (D, E and F) in the absence (A and D) or presence of either 1 μM DTX (C and E) or 1 μM β -BuTX (B and F). After 45 min at 23°C sections were washed (3 x 5 min) at 4°C. Slides were dried and subjected to autoradiography by exposure to LKB [^3H]-sensitive Ultrafilm for 24h (A, B and C) or 48h (D, E and F). The photographic dark-field images (4.6 fold magnification) were produced directly from the film. The sections correspond to level A4 380 μ of Koenig and Klippel (1963). The diagram (G) indicates the positions of some of the areas labelled by ^{125}I -DTX and ^{125}I - β -BuTX. This figure was kindly provided by Annegret Pelchen-Matthews.

Figure 7.1 Autoradiographic localisation of ^{125}I -DTX and ^{125}I - β -BuTX binding in rat brain.



β -BuTX it was possible to localise the two classes of DTX acceptors individually. The acceptors which bind both DTX and β -BuTX with high affinity were localised as the binding of ^{125}I - β -BuTX which could be displaced by DTX. Unlike overall DTX binding, these acceptors were found to be enriched in specific areas, i.e. synaptic rich regions of the hippocampus, caudate putamen, thalamic nuclei and throughout the cerebral cortex (Figure 7.1, D-F). The distribution of the other class of acceptors which bind only DTX homologues with high affinity were localised by ^{125}I -DTX (2nM) binding which was insensitive to $1\mu\text{M}$ β -BuTX. This concentration of β -BuTX should totally block ^{125}I -DTX binding to the common sites whilst having only a slight effect on the remainder of its binding (cf. Figures 2.12, 3.3 and 5.9). Under these conditions, ^{125}I -DTX was concentrated in areas of white matter (the corpus collosum, cingulum, internal and external capsules, optic tracts, mammillothalamic tracts, and lateral habenular nuclei) where little ^{125}I - β -BuTX binding is seen.

It should be noted that these results are only qualitative, and that, in keeping with previous binding studies (Chapter 2), the amounts of ^{125}I -DTX binding, in the presence or absence of β -BuTX, were higher than those seen for ^{125}I - β -BuTX. Nevertheless, the data show that the two classes of DTX acceptors have distinct distributions in the brain, suggesting that each acceptor type is functional. Further, the different overall distribution of the two acceptor types indicates that their roles are not the same.

Recently, electron microscope autoradiography has provided additional evidence favouring the significance of the identified DTX acceptors. Specific ^{125}I -DTX binding has been located on cell bodies and myelinated axons, where the toxin affects K^+ currents, as well as on the synaptic bouton, where DTX facilitates transmitter release (Halliwell *et al.*, 1985; Annegret Pelchen-Matthews, personal communication).

7.1.3 The relationship between the identified DTX acceptors and biological activities of DTX homologues.

When I.B. Othman and A.L. Breeze, of this laboratory, compared the central toxicities of DTX homologues with their abilities to inhibit ^{125}I -DTX binding in rat brain, they found a good correlation between these two parameters (Black *et al.*, 1985; Table 7.1). If iodinated DTX, which has practically the same toxicity and binding affinity as the native toxin, is included in this correlation, it extends to 6 DTX congeners and provides strong evidence that the acceptors seen in rat brain membranes are those which mediate the convulsive actions of the toxins.

A similar study has not been carried out in day-old chicks due to the difficulties arising from the small size of their ventricles. However, the potency with which homologous toxins inhibit ^{125}I -DTX binding to chick synaptic membranes also agrees with their toxicity in rat brain (Table 7.1), suggesting that the acceptors identified in this species are also biologically active. Since toxin I was more potent than DTX in attenuating the A-current (Halliwell *et al.*, 1985) as well as inhibiting ^{125}I -DTX binding to synaptic membranes, the acceptors of DTX which have been identified at the nerve terminal would seem to be involved with the A-current, as proposed earlier. DTX homologues which can block ^{125}I -DTX binding are facilitatory at the chick neuromuscular junction; DTX, toxin I and D.V.14 were active in both cases, whereas toxin B and BPTI had no such effects (Black *et al.*, 1985; Chapters 3 and 5; Harvey and Karlsson, 1982; Harvey *et al.*, 1984a; A.L. Harvey, personal communication). This suggests that the peripheral acceptors are similar to those characterised in the CNS. The one exception to the relationship is toxin E, which showed no facilitatory action at the avian neuromuscular junction (Harvey *et al.*, 1984a), despite its convulsive action in rat brain and its ability to inhibit ^{125}I -DTX

Table 7.1 Correlation of the central neurotoxicities of DTX congeners with their relative affinities for the acceptor in rat and chick synaptic membranes.

	Central toxicity (MLD; <u>ng/g body wt)*</u>	Affinity in rat brain <u>K_I (nM)*</u>	Affinity in chick brain <u>IC₅₀ (nM)**</u>
Toxin I <u>(Dendroaspis polylepis)</u>	0.5	0.05	0.4
DTX <u>(Dendroaspis angusticeps)</u>	2.5	0.4	3.8
Dv-14 <u>(Dendroaspis viridis)</u>	2.5	0.4	-
Toxin E <u>(Dendroaspis polylepis)</u>	25	1.5	129
Toxin B <u>(Dendroaspis polylepis)</u>	150	2000	1000

* Values taken from Black et al., 1985 and refer to affinities and toxicities in rat.

** C₅₀ values taken from Chapter 3 are for inhibition of the specific binding of 2nM ¹²⁵I-DTX.

binding (Black et al., 1985; Table 7.1). This may reflect slight species or tissue differences between the acceptors involved. [N.B. toxin E has a ~8 fold lower affinity relative to DTX in chick brain compared to rat brain (cf. Table 7.1).] However, contamination of toxin E preparations seems a more likely cause of the difference. In this context, it is interesting that the toxin E used in studies on central toxicity and inhibition of ^{125}I -DTX binding (Black et al., 1985, Appendix A.1) were provided by Dr. A.L. Harvey, who performed the studies at the neuromuscular junction (Harvey et al., 1984a). Thus, contamination by facilitatory components of Dendroaspis. p. polylepis venom may explain the central actions of toxin E, but it is unlikely to account for the observed discrepancy. The most likely explanation for this is the reported 10-15% contamination of toxin E preparations by an α -neurotoxin, which accounts for its peripheral toxicity (Joubert and Strydom, 1978). Since α -neurotoxins are not toxic in rat brain (Schmidt et al., 1980) their presence would not affect the central convulsive actions of toxin E. On the other hand, by blocking acetylcholine receptor, this contaminant would mask facilitatory action of a DTX homologue at chick neuromuscular junction. This latter explanation seems to be correct, since toxin E has been reported to block neuromuscular transmission (Harvey et al., 1984a). Thus, the observed lack of facilitatory action of toxin E does not detract from the relationship between inhibition of ^{125}I -DTX binding by DTX homologues and their biological activity.

The correlation of toxicity and binding affinity holds for each of the two classes of DTX acceptors, as the affinities of DTX homologues at the two classes of acceptor in chick brain appeared very similar (Chapter 3). In rat brain, ^{125}I -DTX binding is predominantly to acceptors exhibiting very low affinity for β -BuTX; therefore the relevant data in Table 7.1 will only apply to this class of acceptors.

However, toxin I is more potent than DTX in inhibiting ^3H - β -BuTX binding in rat brain (Othman, 1983), showing that the relationship is the same for the acceptors common to DTX and β -BuTX, at least with respect to these two toxins.

In accord with the apparent relevance of both classes of DTX acceptors, a similar classification of DTX acceptor with respect to their interactions with β -BuTX has been seen in a physiological study. The mutual interactions of DTX homologues and β -BuTX at the neuromuscular junction (Harvey and Karlsson, 1982) and the ability of DTX to block the actions of β -BuTX on synaptosomes (Nicholls et al., 1985; Karen Joseph, personal communication) show that there are functional acceptors which are common to these two toxins. The physiological equivalent of the acceptors which bind only DTX homologues with high affinity can be seen in guinea pig ileum. Here, DTX causes a marked facilitation of nerve-evoked twitch (Chapter 2) whereas β -BuTX was without effect (Chang and Lee, 1963; Gundersen and Jenden, 1981). Although it remains to be demonstrated whether β -BuTX is able to bind to the acceptors in this tissue, this seems unlikely. As the irreversible blockade of transmitter release caused by β -BuTX is due to its PLA_2 activity, it should be expressed in any tissue to which the toxin binds. The only instance where physiological interactions of DTX and β -BuTX cannot be readily explained by the presence of only two classes of acceptor is in isolated mouse diaphragm/phrenic nerve preparations. Here, toxin I was unable to inhibit the action of β -BuTX (cf. Chang, 1985). However, both toxin I and DTX are able to increase the time to death caused by peripheral injections of β -BuTX in mouse (Othman et al., 1982; Othman, 1983). As β -BuTX kills through respiratory paralysis, it would seem that pharmacological interaction of these toxins does occur on the phrenic nerve of mouse. This may not be seen in isolated preparations due to the resistance of mice to the actions of β -BuTX. As toxin I only

delays the eventual blockade of transmitter release caused by β -BuTX at chick neuromuscular junction (Harvey and Karlsson, 1982), this action may not be obvious in mouse diaphragm preparations, where the action of β -BuTX is much slower (Chang and Huang, 1974).

The preceding arguments strongly support the physiological relevance of both classes of identified DTX acceptors. Thus, it is interesting that DTX has recently been shown to attenuate two subtypes of voltage-sensitive K^+ channels; those in hippocampal CA₁ cell bodies which inactivate rapidly and a variant in nodose ganglia which are more sustained (Halliwell et al., 1985). However, further work will be required to see whether this reflects the interaction of DTX with its two acceptor types.

The only possible objection to the physiological relevance of the acceptors identified with ¹²⁵I-DTX is the discrepancy between their K_D (0.3-15nM) and the concentrations required to produce many of the effects of the toxin [$\sim$ 50nM at the neuromuscular junction and on hippocampal slices (Harvey and Karlsson, 1980; Halliwell et al., 1984)]. However, at least in brain slices, where recordings were made at a depth of 200-300 μ m (Halliwell et al., 1985), this discrepancy seems to be due to diffusional barriers, as proposed for similar observations with tetrodotoxin (Richards, 1982). By using autoradiography, the penetration of DTX into brain slices was seen to be very poor (Halliwell et al., 1985); therefore, little of the toxin in the external media would be expected to reach the recording locus. It has also been argued that the level of acceptors in central tissue ($\sim$ 1pmol/mg protein) would be the equivalent of a μ M concentration in the extra cellular space, assuming 5-10% extracellular fluid (Halliwell et al., 1985). This would be expected to contribute to the delayed entry of the toxin into the slice. In accord with diffusion being the cause of the relatively large amounts of toxin required to produce biological effects, DTX is

extremely potent at dissected frog node of Ranvier, where access is unrestricted. Here, ~90% of the effects of the toxin are produced by a concentration of 10nM (Weller et al., 1985). This corresponds to a K_D of ~1nM (cf. Formula A.2), which is in excellent agreement with the affinity observed in the biochemical studies.

Altogether, the above arguments indicate that the two classes of DTX acceptors identified in Chapter 2 and 3 mediate the biological activity of the toxin and are in some way involved with voltage sensitive K^+ channels. Thus, the present study and its continuation are clearly justified.

7.2 Concluding Statement.

By identifying and partially characterising two classes of acceptors for DTX which appear to be involved with K^+ channels, much knowledge has been gained about important components associated with the regulation of neuronal excitability.

The present study has opened the way to the complete characterisation of these components; coupled with further physiological studies, this should lead to a full understanding of the molecular mechanisms by which binding of DTX to its acceptors affects nerve activity. In this way, it has made a positive contribution to the study of the brain and represents, therefore, a step towards greater knowledge of the mind/body relationship and the cause of mental disease.

APPENDIX

A.1 Materials

<u>Material</u>	<u>Source</u>
Sephadex G10 and G50	Pharmacia Fine Chemicals
Sepharose C1-6B	"
CM-Sepharose C1-6B	"
Bio-Rex 70	Biorad Laboratories
Biogel-P60	"
Radioisotopes	Amersham International PLC
<u>Dendroaspis angusticeps</u> venom	Sigma Chemical Company Ltd.
Proteases	"
Protease inhibitors	"
Deoxyribonuclease I (type 3)	"
Neuraminidase (from <u>Clostridium perfringens</u>)	"
Coomassie Brilliant Blue R-250	"
Chloramine-T	"
SDS	"
Deuterium oxide (99%)	"
Standard proteins	Sigma and Pharmacia
Acrylamide	BDH Chemicals Ltd.
Bisacrylamide	"
TEMED	"
Dow-Corning MS-550 silicone fluid	"
Dinonyl phthalate	"
β -BuTX	Prepared as described in Othman et al. 1982 by Annegret Petchen-Matthews.
Toxin I	Kindly provided by
Toxin E	Drs. A. Harvey
Toxin B	and E. Karlsson.
Apamin	Kind gift from
Bee venom PLA ₂	Dr. Rudolf Shipolini.
D.V.14	
Crotoxin subunits A and B	Kind gift from Dr. C. Bon.

All other reagents were of analytical grade.

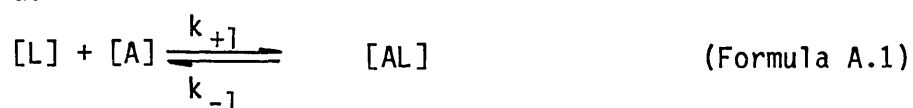
A.2 Analysis of Radioligand Binding.

A.2.1 The Langmuir isotherm and Scatchard analysis.

Radiolabelled ligands (such as ^{125}I -DTX) can be used to study the interactions of their acceptors. To do this the acceptors are incubated with the radioligand, followed by separation and quantification of the bound and free ligand. Several methods of separation have been used in this thesis and these are described in the relevant chapters.

The binding of any ligand to preparations containing its acceptors can be divided into two components; (a) specific binding to its acceptor and (b) non-specific binding, with low affinity, to multiple components in the preparation. As there are a limited number of acceptors, the specific binding will be saturable. Conversely, due to the large number of sites involved, the levels of ligands used will not saturate the non-specific binding. Thus, these two components of the binding can be easily differentiated by using an excess of unlabelled ligand (100-fold over radioligand and acceptor K_D). This will saturate the specific binding sites, thereby blocking the binding of radioligand to its acceptor, whilst having a negligible effect on non-specific binding. Hence, the level of saturable binding (i.e. the radioligand/acceptor complex) can be obtained from the difference between the binding seen in the absence and presence of excess unlabelled ligand. It is analysis of this specific binding which will be described below.

The reaction of ligand binding to its acceptor is described by the formula:



where [L] is the free ligand concentration, [A] and [AL] are the concentrations of free acceptor and of the acceptor/ligand complex, respectively. k_{+1} relates [A] [L] to the rate of formation of [AL] and k_{-1} relates [AL] to its dissociation. By applying the law of mass action, this can be rearranged into the Langmuir isotherm, which gives the proportion of acceptors occupied by ligand (r):

$$r = \frac{[AL]}{[AL] + [A]} = \frac{[L]}{K_D + [L]} \quad (\text{Formula A.2})$$

Here the term K_D , the equilibrium dissociation constant, is the ratio of k_{-1} over k_{+1} which at equilibrium is defined as:

$$K_D = \frac{k_{-1}}{k_{+1}} = \frac{[L][A]}{[AL]} \quad (\text{Formula A.3})$$

and is thus equal to the concentration of ligand which half saturates the acceptors (i.e. when $[A] = [AL]$).

Formula A.2 shows that a plot of [L] against [AL] (saturably bound ligand) gives a rectangular hyperbola asymptotic with lines $[AL] = \text{total acceptor } (B_{\max})$ and $[L] = -K_D$. Although [AL] and [A] are strictly concentrations, for comparative purposes they are normally expressed relative to the amount of tissue etc. in the incubation; here, per mg protein in the membranes or extract.

Although the Langmuir isotherm defines binding with respect to its affinity (K_D) and the number of acceptors ($B_{\max}$), it is difficult to determine these values accurately due to its curved nature; thus, several methods for its linearisation have been developed, e.g. 'Hill' plot (Hill, 1913) and the 'double-reciprocal' plot (Lineweaver and Burk, 1934). That used here is Scatchard analysis (Scatchard, 1949) which is based on the formula:

$$\frac{B}{F} = \frac{B_{\max} - B}{K_D} \quad (\text{Formula A.4})$$

where $B = [AL]$ and $F = [L]$.

A plot of B/F against B will give a straight line for the binding of a ligand to its acceptors with a slope of $-1/K_D$ and an abscissa intercept of $B_{\max}$. The linear relationship only holds if the acceptor sites are kinetically homogeneous and non-interacting. The presence of kinetically distinct populations of acceptors will cause the Scatchard plot to curve upwards, as the higher affinity sites will be occupied first and the average K_D will decrease with increased binding. Scatchard analysis is relatively poor at resolving multiple acceptor sites, e.g. if the ratio of 2 sites was 10:1, they would not be detected if the difference in their affinity was less than a factor of 10 (Burgisser, 1984). If negative co-operativity exists, i.e. the affinity of the acceptor sites decreases with their increasing occupancy, a Scatchard plot which curves upwards will be produced. This is the same situation as seen with heterogeneity and further analysis such as kinetic and competition studies (see later) are necessary to distinguish these two possibilities. With positive co-operativity, where K_D increases with occupancy, a downward curving Scatchard plot is obtained.

A.2.2 Graphical analysis of curved Scatchard plots.

If further analysis reveals that a curved Scatchard plot is due to multiple sites, an approximation of the binding parameters of these sites is obviously advantageous. This can be done by graphical analysis (Rosenthal, 1968). When more than one class of binding sites is present, the free ligand will be in equilibrium with the ligand bound to each of the sites. Thus, the levels of B and B/F are the sum of these

values for each of the sites at that ligand concentration. As the free ligand concentration is constant along a straight line drawn through the origin on a Scatchard plot (defined by B/B_F), the length of a line from the origin to any point on the experimentally derived curve will be equal to the sum of the distance along that line, from the origin to the Scatchard plots for occupancy of the different sites. Thus, by manipulating theoretical lines so that the above relationship with the experimental data holds for their entire length, an approximation of the K_D and B_{max} values of the individual sites can be derived.

A.2.3 Measurement of k_{+1} and k_{-1} .

From Equation 2.1, the rate of dissociation of the ligand/acceptor complex is $k_{-1}[AL]$. At equilibrium this is balanced by the equal rate of association ($k_{+1}[L][A]$) and, therefore, methods of differentiating these are required. The dissociation of a radioligand can be visualised by two methods; (a) by adding an excess (~100-fold) of the unlabelled ligand which will effectively block its association ("chase" experiments), and (b) by diluting the reaction mixture which, provided the radioligand concentration $\gg$ acceptor concentration, will reduce the association rate to a smaller but constant level. The decrease in binding [measured at various times after addition or dilution in (a) and (b)] will be a first order reaction; being related to k_{-1} by:

$$\ln B/B_0 = -k_{-1}t \quad (\text{Formula A.5})$$

In "chase" experiments, where excess ligand is added, B_0 and B are respectively the levels of specific binding at the time of the addition and at various times after it. Where dilution is employed, the meanings are the same except that B_0 and B are defined as the difference between the observed binding and that seen at equilibrium after the

dilution. A plot of $\ln B/B_0$ against time will give a straight line with a slope of k_{-1} .

As with Scatchard analysis, this linear relationship only holds for a kinetically homogeneous population of non-interacting sites. If the sites are heterogeneous with respect to dissociation, the plot will curve upwards, as the more rapid dissociation will occur first.

Co-operativity produces different effects for the two methods used.

Following dilution, negative and positive co-operativity will give a semi-log plot with an upward and downward curve, respectively. However, if addition of unlabelled ligand is used, the overall occupancy of the acceptor sites remains constant; thus a linear first-order dissociation will be obtained if co-operativity is present. This difference between the effects of heterogeneity and co-operativity on dissociation visualised by addition of cold ligand can be used to determine which of these is responsible for a non-linear Scatchard plot.

The rate of binding of a ligand to its acceptors is defined by the differential equation:-

$$d[AL]/dt = k_{+1} [A][L] - k_{-1} [AR] \quad (\text{Formula A.6})$$

Although this can be integrated to give k_{+1} with respect to binding and time, the solution is complex. However, when ligand concentration $\gg$ total acceptor, $[L]$ in Formula A.6 is essentially constant and $[A]$ tends to zero. Thus, the reaction becomes pseudo first-order, i.e.

$$[L] \xrightleftharpoons[k_{-1}]{k_{+1}} [AL] \quad (\text{Formula A.7})$$

which can be solved to give

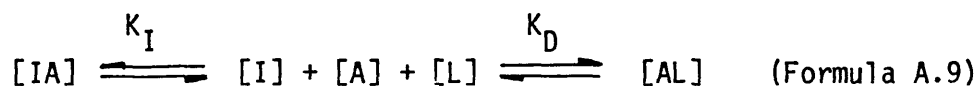
$$\ln \left(\frac{[B_{eq}]}{[B_{eq}] - [B]} \right) = ([L] k_{+1} + k_{-1})t \quad (\text{Formula A.8.})$$

where B_{eq} and B are the levels of ligand binding at equilibrium and at various times (t) after addition of the ligand $[L]$ to the reaction. Thus, k_{+1} , can easily be determined using the k_{-1} value obtained as described above.

Often, association rates are too rapid to be measured by the separation techniques used; here k_{+1} , can be estimated by placing k_{-1} and K_D into Formula A.3.

A.2.4 Binding of unlabelled ligands: competition studies.

For identifying acceptor sites it is important, not only to characterise their interaction with the radioligand, but also their interactions with unlabelled ligands. These interactions will detect any effects of radiolabelling, as well as helping to differentiate heterogeneous acceptors and in the identification of pharmacological relevance. If an unlabelled ligand competes for the radioligand binding sites, Formula A.1 becomes



where $[I]$ and $[IA]$ are the concentration of competing ligand and its complex with the acceptor $[A]$ respectively, K_I is the dissociation equilibrium constant for $[IA]$. Thus, the Langmuir isotherm becomes:

$$r = \frac{[AL]}{[A] + [AL] + [AI]} = \frac{[L]}{[L] + K_D + ([I]K_D/K_I)} \quad (\text{Formula A.10})$$

This predicts that a plot of $\log [I]$ against r will be sigmoidal and the ratio of the inhibitor concentrations required to inhibit 90% and 10% of the radioligand binding is 81.

From Formula A.9, the relationship between K_I and the concentration of competitor required to inhibit half of the binding of the radioligand (IC_{50}) can be derived (Cheng and Prusoff, 1973) i.e.

$$K_I = \frac{IC_{50}}{1 + [L]/K_D} \quad (\text{Formula A.11})$$

where $[L]$ is the free concentration of radioligand which has an equilibrium dissociation constant of K_D . Although $[L]$ is the concentration of free ligand, as long as the total ligand $\gg$ acceptor-bound ligand, $[L] \sim$ total ligand and need not be measured directly.

The mode of inhibition of binding by a unlabelled ligand, i.e. either competitive, non-competitive or mixed, can be determined by the use of a Dixon plot (Dixon, 1953) in conjunction with a plot of $[L]/B$ against $[I]$ (Cornish-Bowden, 1974). A Dixon plot is a plot of $1/B$ against inhibitor concentration $[I]$ for a series of radioligand concentrations. With competitive inhibition where:

$$1/B = 1/B_{\max} + \frac{(1 + [I]/K_I)K_D}{B_{\max} [L]} \quad (\text{Formula A.12})$$

the Dixon plot gives a series of straight lines which intersect in the fourth quadrant at a point $-K_I$ and $1/B_{\max}$. With mixed inhibition, where binding of ligand changes the affinity of inhibitor binding:

$$1/B = \frac{(1 + [I]/K_I)K_D}{B_{\max} [L]} + \frac{1 + [I]/K_I'}{B_{\max}} \quad (\text{Formula A.13})$$

Here K_I' is the dissociation equilibrium constant for the inhibitor/ligand/acceptor complex ([IAL]). This also predicts that a Dixon plot will have an intersect in the fourth quadrant for mixed inhibition. Fortunately these two types of inhibition can be readily distinguished on a plot of $[L]/B$ against $[I]$ for various ligand concentrations. Here competitive inhibition gives a series of parallel lines, whereas with mixed inhibition the lines intersect in the third or fourth quadrant at a value of $I = K_I'$. For non-competitive inhibition, where binding of inhibitor, and thus the proportion of inhibition, is independent of ligand concentration (i.e. $K_I = K_I'$), the intersect is on the abscissa in both cases. These analyses also have the advantages that they give the K_I and K_I' values (and the $B_{\max}$ for a competitive inhibitor) directly, without reference to the radioligand affinity.

Obviously, Formulas A.9 - A.13 apply to ligands binding to a homogeneous set of acceptor sites. If competition is at kinetically distinct populations of acceptors, additional terms will be introduced into Formula A.9 which will extend the range of the inhibition, i.e. the ratio of competitor required for 90% and 10% inhibition will be greater than 81. Under these circumstances, the IC_{50} value obtained will be an aggregate of that for the various sites and thus Formula A.10 cannot be used. On the other hand, if there are distinct populations of sites for the radioligand, binding to only one of which is inhibited by a particular ligand, the other sites are irrelevant to this competition and Formulas A.9 - A.13 as well as Dixon plots and plots of $[L]/B$ against $[I]$ can be used, provided that values are taken for the partial inhibition.

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