

DIC

TO MY PARENTS

for their continued support and encouragement

CHARACTERISATION AND NEURONAL SPECIFICITY
OF ACCEPTORS FOR BOTULINUM NEUROTOXINS

by

David Mark Evans
Department of Biochemistry
Imperial College of Science and Technology
University of London

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

October, 1987

Abbreviations.

ACh	Acetylcholine
AChE	Acetylcholine esterase
4-AP	4-aminopyridine
ATP	Adenosine tri-phosphate
bis	NN'-Methylenebisacrylamide
BWSV	Black Widow spider venom
BoNT	Botulinum neurotoxin
BoTx	Botulinum toxin complex
[Ca ²⁺] _i	Intracellular calcium concentration
ChAT	Choline acetyl transferase
Cl.	Clostridium
CNS	Central nervous system
CURL	Compartment of uncoupling of receptor and ligand
DEAE	Diethylaminoethyl
DTPA	diethylenetriaminepentaacetic acid
DTT	Dithiothreitol
DTX	Dendrotoxin
EDTA	Diaminoethanetetraacetic acid; disodium salt
epp	Endplate potential
FCCP	fluoro-carbonyl cyanide phenylhydrazone
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid
¹²⁵ I-BoNT	¹²⁵ I-labelled botulinum neurotoxin
K _d	Dissociation constant
mepp	Miniature endplate potential
MLD ₅₀	Mouse lethal dose ₅₀ (the amount of toxin required to kill 50% of injected mice within 4 days)
M _r	Relative molecular weight
NADH	Reduced form of NAD
NAG	N-acetylglucosamine
NANA	N-acetylneuraminic acid
NBM	nucleus basalis magnocellularis
NMJ	Neuromuscular junction
PAGE	Polyacrylamide gel electrophoresis
QAE	Diethyl- (2-hydroxypropyl) aminoethyl
Q ₁₀	Temperature coefficient
RME	Receptor-mediated endocytosis
SDS	Sodium dodecyl sulphate
SER	Smooth endoplasmic reticulum
TEMED	N,N,N',N'-Tetramethylethylene diamine
TLE	Trypsin-like enzyme
WGA	Wheat germ agglutinin

CONTENTS

	Page No.
Abbreviations	3
Table of contents	4
List of tables	9
List of figures	10
Acknowledgements	13
Abstract	14

Chapter One General Introduction.

1.1.	<u>Botulism: introduction.</u>	17
1.2.	<u>Botulinum toxin structure.</u>	18
1.2.1.	Production and activation of BoTx.	18
1.2.2.	Neurotoxin subunits.	22
1.3.	<u>Postulated mechanisms of neurotransmitter release.</u>	24
1.3.1.	The vesicular hypothesis; quantal release.	25
1.3.2.	Cytoplasmic release of neurotransmitter.	28
1.3.2.1.	The acetylcholine channel.	29
1.3.2.2.	The 'gate' hypothesis.	30
1.4.	<u>Mechanism of the inhibitory action of BoNT on transmitter release.</u>	34
1.4.1.	Toxin binding to neuronal membranes.	34
1.4.2.	Toxin internalisation.	36
1.4.3.	Pharmacological action of BoNT.	37
1.4.3.1.	Lack of an effect on propagation of the action potential.	37
1.4.3.2.	Lack of an effect on acetylcholine synthesis/storage.	37
1.4.3.3.	Blockade of ACh release.	39
1.4.4.	Role of Ca ²⁺ in intoxication.	40
1.5.	<u>Tetanus toxin: similarities and differences with BoNT.</u>	41

Chapter Two

Large Scale purification of BoNT: radioiodination and interaction with rat brain synaptosomal membranes.

2.1.	<u>Introduction.</u>	45
2.2.	<u>Materials and Methods.</u>	47
2.2.1.	Large scale purification of BoNT A and B.	47
2.2.2.	Analytical procedures.	48
2.2.3.	Preparation of rat cerebrocortical synaptosomes.	49
2.2.4.	Radiolabelling the BoNT.	50
2.2.5.	Characterisation of ^{125}I -BoNT (A and B).	51
2.2.6.	Measurement of ^{125}I -BoNT binding to synaptosomes.	52
2.2.7.	Biochemical treatment of synaptosomal membranes; effect on ^{125}I -BoNT binding.	52
2.3.	<u>Results.</u>	54
2.3.1.	BoNT purification and iodination.	54
2.3.2.	Demonstration of distinct populations of acceptors for ^{125}I -BoNT B on rat cerebrocortical synaptosomal membranes.	61
2.3.3.	The nature of synaptosomal acceptors for ^{125}I -BoNT B.	65
2.3.3.1.	Trypsin treatment of synaptosomal membranes.	67
2.3.3.2.	Heat treatment of synaptosomal membranes.	67
2.3.4.	Sialic acid moieties in the acceptors for ^{125}I -BoNT B.	71
2.3.5.	Scatchard analysis of ^{125}I -BoNT B binding under different incubation conditions.	76
2.4.	<u>Discussion.</u>	80
2.4.1.	Purification and radioiodination of BoNT B.	80
2.4.2.	BoNT acceptors.	82
2.4.2.1.	The nature of the acceptors for BoNT B.	83
2.4.3.	Scatchard analysis; critical appraisal.	85
2.4.4.	Gangliosides as toxin acceptors.	86
2.4.5.	BoNT binding; comparison with tetanus toxin.	87

Chapter Three

Neuronal specificity of BoNT A and B.

	Page No.
3.1. <u>Introduction.</u>	91
3.2. <u>Materials and Methods.</u>	93
3.2.1. Immuno-affinity purification of cholinergic nerve terminals from a crude population of rat cerebrocortical synaptosomes.	93
3.2.2. Enzyme assays.	94
3.2.3. Analysis of neurotransmitter content of the cholinergic-enriched synaptosomal fraction.	96
3.2.4. Binding of ^{125}I -BoNT A and B to crude and cholinergic-enriched nerve terminals from rat cortex.	97
3.2.5. Immunolysis of cholinergic nerve terminals using anti-(Chol-1) antibodies; protection by BoNT B.	98
3.2.6. Cholinergic lesioning of the rat brain and ^{125}I -BoNT binding.	99
3.3. <u>Results.</u>	101
3.3.1. Purity of cholinergic nerve terminals.	101
3.3.2. Comparison of the binding of ^{125}I -BoNT (A and B) to cholinergically-enriched and untreated terminals.	102
3.3.3. Identification of the acceptor moiety for BoNT B.	110
3.3.4. Binding of ^{125}I -BoNT to slices from cholinergically lesioned rat brain.	111
3.4. <u>Discussion.</u>	120
3.4.1. Isolation of cholinergic nerve terminals from the CNS.	120
3.4.2. Cholinergic specificity of BoNT in the CNS.	120
3.4.3. The acceptor for BoNT B in the CNS.	124
3.4.4. Possible function(s) of Chol-1.	127
3.5. <u>Conclusions.</u>	128

Chapter Four
Calcium homeostasis and neurotransmitter release in
synaptosomes: effect of BoNT A and B.

	Page No.
4.1. <u>Introduction.</u>	130
4.2. <u>Materials and Methods.</u>	131
4.2.1. Quin 2; measurement of $[Ca^{2+}]_i$.	131
4.2.2. BoNT intoxication of synaptosomes and quin 2 loading.	132
4.2.3. Fluorescent measurements.	132
4.2.4. Determination of synaptosomal respiration rates.	133
4.3. <u>Results.</u>	134
4.3.1. Quin 2; characterisation of fluorescent spectra.	134
4.3.2. Effect of pre-incubation on $[Ca^{2+}]_i$.	134
4.3.3. Effect of BoNT A and B on basal and K^+ -depolarised $[Ca^{2+}]_i$ levels.	140
4.3.4. Effect of BoNT on respiration rates in synaptosomes.	140
4.3.5. Effect of 4-aminopyridine (4-AP) on $[Ca^{2+}]_i$.	143
4.4. <u>Discussion.</u>	145
4.4.1. The role of intracellular Ca^{2+} in neurotransmitter release.	145
4.4.2. Quin 2; measurement of $[Ca^{2+}]_i$.	146
4.4.3. Effect of BoNT on $[Ca^{2+}]_i$.	148

Chapter 5
General Discussion.

	Page No.
5.1. <u>Introduction.</u>	152
5.2. <u>The nature of ganglioside molecules.</u>	152
5.3. <u>Possible functions of gangliosides in toxin action.</u>	154
5.3.1. Gangliosides as acceptors for bacterial toxins.	154
5.3.2. Receptor-mediated endocytosis (RME): possible role in BoNT internalisation.	156
5.3.3. Role of gangliosides in neurotransmitter release.	159
5.4. <u>Possible mechanism(s) of action of BoNT.</u>	162
5.5. <u>BoNT as a clinical tool.</u>	166
References	168

List of Tables.

Page No.

Table 2.1.	Purification of <i>Cl. botulinum</i> type B neurotoxin.	58
Table 3.1.	Amino acid contents of crude and cholinergic-enriched nerve terminals; comparison with the fold purification.	104
Table 3.2.	Effect of lesioning of the nucleus basalis magnocellularis (NBM) on specific binding of ^{125}I -BoNT to rat brain slices	118
Table 4.1.	Effects of BoNT (A and B) on $[\text{Ca}^{2+}]_i$ in synaptosomes.	142
Table 4.2.	Concentration dependence of 4-AP on $[\text{Ca}^{2+}]_i$ in synaptosomes.	144

List of Figures.

Page No.

Fig. 1.1.	Clostridial toxin structure.	20
Fig. 1.2.	Separation of the constituent fragments of type A BoNT by ion-exchange chromatography.	23
Fig. 1.3.	Hypothetical model of neurotransmitter release.	32
Fig. 2.1.	Purification of BoNT B by affinity and ion-exchange chromatography.	55
Fig. 2.2.	Electrophoretic analysis of BoNT samples.	57
Fig. 2.3.	Crossed immuno-electrophoresis of purified BoNT B.	59
Fig. 2.4.	Trypsin treatment of BoNT B.	60
Fig. 2.5.	Autoradiographic distribution of radiolabel in the subunits of BoNT B (Danish strain).	62
Fig. 2.6.	Equilibrium binding of ^{125}I -BoNT B to rat brain synaptosomal membranes.	63
Fig. 2.7.	Concentration dependence of trypsin on synaptosomal binding of ^{125}I -BoNT B.	66
Fig. 2.8.	Equilibrium binding of ^{125}I -BoNT B to trypsin-treated membranes.	68
Fig. 2.9.	Specific binding of ^{125}I -BoNT B to heat treated synaptosomal membranes.	70
Fig. 2.10.	Concentration dependence of neuraminidase on synaptosomal binding of ^{125}I -BoNT B.	72
Fig. 2.11.	Equilibrium binding of ^{125}I -BoNT B to neuraminidase-treated synaptosomal membranes.	73
Fig. 2.12.	Effect of wheat-germ agglutinin on ^{125}I -BoNT B binding to synaptosomal acceptors.	75
Fig. 2.13.	Equilibrium binding of ^{125}I -BoNT under conditions of low ionic strength and low pH buffer.	78

List of Figures (contd).

	Page No.
Fig. 3.1. Sagittal and coronal sectional diagrams of rat brain showing site of ibotenate injection and neuronal pathway together with regions subjected to densitometric analysis	103
Fig. 3.2. Equilibrium binding analysis of BoNT acceptors on cholinergic-enriched and crude populations of synaptosomes.	106
Fig. 3.3. Both high- and low- affinity binding components for ^{125}I -BoNT B exist on cholinergic nerve terminals.	107
Fig. 3.4. Co-purification of the BoNT acceptors with cholinergic nerve terminals.	108
Fig. 3.5. Inhibition by BoNT B of complement-mediated lysis of synaptosomes using anti-(Chol-1) antibodies.	112
Fig. 3.6. Inhibition by BoNT of complement-mediated immunolysis by sheep anti-(Chol-1) serum.	113
Fig. 3.7. ^{125}I -BoNT A binding to brain slices from lesioned animals.	114
Fig. 3.8. ^{125}I -BoNT B binding to brain slices from lesioned animals.	116

List of Figures (contd.)

Page No.

Fig. 4.1.	Structure and fluorescent emission spectra of quin 2 and its ester (quin 2-AM).	135
Fig. 4.2.	Typical trace from a single experiment showing method of calibration of $[Ca^{2+}]_i$ from the fluorescence signal.	137
Fig. 4.3.	Basal and K^+ -stimulated levels of $[Ca^{2+}]_i$ in synaptosomes in the presence and absence of BoNT.	139
Fig. 4.4.	BoNT: effect on respiration rates in synaptosomes.	141
Fig. 5.1.	Ganglioside structure.	153

Acknowledgements.

I would like to thank the following people for their assistance during my studies. I am grateful to my supervisors, Dr. J.O. Dolly and Prof. J. Melling for providing the means by which to pursue this interesting avenue of research and for their advice and encouragement; to the SERC for 3 years of financial support; Dr. Cliff Shone for helpful and interesting discussions on various aspects of the project, and provision of some toxin samples, together with Dr. Peter Hambleton, Nigel Bailey and Roger Rhind-Tutt for their help and technical support whilst at the PHLS, Porton Down; Ian and Mary Ingrey-Counter and family for their excellent hospitality during my frequent stays in Salisbury over the last 5 years. I would also like to thank Anne Maisey, Tony Ashton and my successor, John Wadsworth for their help and advice within the laboratory together with Alex Breeze and David Parcej who also provided dendrotoxin. The following people have also contributed to this work and, without whose help certain aspects of this research could not have been performed; Dr. Peter Richardson for provision of materials and demonstration of the cholinergic purification procedure; Dr. Alan Fine for the cholinergic lesions; Annegret Pelchen-Mathews for encouragement and cryostat sectioning; Dr. Maureen Docherty for amino acid analysis of synaptosomes; Dr. Dan Cutler for supplying the initial cultures of PC12 cells; Dr. Peter Tatum for assistance in analysis of the quin 2 data; Dr. David Nicholls and co-workers in Dundee for their hospitality and assistance during my studies there; Glyn Millhouse for excellent photography, often at short notice; and, finally, Dr. Roy Burns and Dr. Peter Hollenbeck for help in microtubule assembly and fast axonal transport.

ABSTRACT.

The botulinum neurotoxins (BoNT), types A and B, produced in association with a number of non-toxic moieties (including haemagglutinin), by the anaerobic bacterium, C. botulinum, were purified to homogeneity using a combination of ion-exchange and affinity chromatography. The yield of these presynaptically active toxins, exhibiting high levels of toxicity ($0.5-2 \times 10^8$ mouse LD₅₀/mg protein), represented $\approx 15\%$ of the initial toxicity. Radiolabelling of both toxin types (using ^{125}I -iodine) to high specific activities (500-3000Ci/mmol) was accomplished using a modification of the chloramine-T procedure. Such labelled toxins (^{125}I -BoNT) retained an appreciable proportion of their initial toxicities (65-80%). SDS polyacrylamide gel electrophoresis of ^{125}I -BoNT B (Danish strain), under reducing conditions, demonstrated preferential labelling of the heavy subunit ($M_r \approx 101000$) with little incorporation of ^{125}I into the light chain ($M_r \approx 53000$).

^{125}I -BoNT bound saturably to rat synaptosomal membranes and equilibrium binding studies revealed the presence of two distinct populations of acceptors; a low content ($B_{\text{max}} \approx 60\text{fmol/mg}$ protein) of acceptors exhibiting relatively high affinity ($K_d 0.3-0.5\text{nM}$) for the toxin with a much larger population ($B_{\text{max}} \approx 7\text{pmol/mg}$) of acceptors of lower affinity ($K_d 16-25\text{nM}$). Whilst treatment of synaptosomal membranes with neuraminidase decreased the specific binding of ^{125}I -BoNT B to both acceptor sites, only those acceptors exhibiting low affinity for the toxin were destroyed by heat (95°C) or trypsin. This would suggest that sialic acid moieties play an important functional role in both acceptor types, those of lower affinity also requiring the presence of a proteinaceous component(s) (possibly sialoglycopeptides) and implicating a ganglioside as the high affinity acceptor component.

Comparison of the binding of labelled BoNT (A and B) to cholinergic synaptosomes (immuno-affinity purified on cellulose beads bearing antibodies raised against the cholinergic-specific ganglioside marker, Chol-1) with that observed in the crude

population suggested that acceptors exhibiting high and low affinity for each toxin type are present almost exclusively on cholinergic nerve terminal membranes. Immunolysis of cholinergic nerve terminals using anti-(Chol-1) antibodies, could be effectively inhibited by low concentrations of BoNT B whilst being unaffected by high concentrations of BoNT A. The possibility that Chol-1 is the high-affinity acceptor moiety for BoNT B is discussed. Cholinergic specificity of BoNT acceptors in the CNS (as previously demonstrated in the PNS) was further analysed by quantifying the amount of ^{125}I -BoNT binding to brain sections from rats subjected to cholinergic lesioning. Lesions were made by injection of the excitotoxin, ibotenate, into the cholinergic cell bodies of the nucleus basalis magnocellularis. A 50% reduction in the cholinergic-specific enzyme markers, ChAT and AChE was observed in animals killed 6 weeks after lesioning; whilst there was a concomitant decrease in binding of ^{125}I -BoNT A in the lesioned relative to the control side of such brain sections, little or no difference in ^{125}I -BoNT B binding was observed. Similarly, no change in the immunostaining pattern using anti-(Chol-1) antiserum was seen, apparently confirming the stability of gangliosides in the membranes and further supporting the observations that the high-affinity acceptor for BoNT B may contain or be associated with Chol-1.

Studies were made on the effect of BoNT on Ca^{2+} homeostasis in synaptosomes using the fluorescent indicator dye, quin 2. Whilst BoNT A had little effect on resting levels of intracellular Ca^{2+} , BoNT B apparently produced a slight increase in the basal Ca^{2+} concentration. Both toxins caused a slight decrease in the maximal levels of $[\text{Ca}^{2+}]_i$ obtained upon depolarisation with K^+ , although this was more marked in the case of BoNT B. These results are discussed in terms of the possible mechanism of intoxication produced by BoNT in this model system.

CHAPTER 1

General Introduction.

General Introduction.

1.1 Botulism: Introduction.

Botulinum neurotoxins are amongst the most toxic naturally occurring products known to Man (with a lethal dose in mice of only $\approx 20\text{ng/Kg}$ body weight). Eight immunologically distinguishable forms of these proteins (labelled A, B, C₁, C₂, D, E, F, and G) are produced by different strains of *Clostridium botulinum* - a strictly anaerobic, spore-forming bacterium found commonly in the soil. These proteins are of similar molecular weight and, with the exception of type C₂ toxin (which has enterotoxin-like activity) they act primarily in the peripheral nervous system (PNS) inhibiting release of neurotransmitter from cholinergic nerve endings. Consequently, this leads to the classical medical condition of botulism, with widespread muscular paralysis and possibly death due to respiratory failure. The symptoms of botulism were first described many centuries ago and the initial discoveries of its site of action made in the early part of this century, yet it is only in the last 20 years that significant progress has been made on the path to elucidating the specific biochemical nature of the blockade. This advancement has only been made feasible by the large scale purification (from a mixture of other, non-toxic proteins) and characterisation of the neurotoxic moieties as well as by concurrent advances in the fields of pharmacology, neurochemistry and neurobiology. The action of these toxins is believed to proceed via a number of defined steps, namely; binding to a membrane acceptor, subsequent internalisation and eventual inactivation of a process that is crucial to excitation-secretion coupling. Researchers have focussed on each of these steps, together with studies on the structure-activity relationship of the neurotoxic proteins, in an attempt to elucidate the molecular mechanism by which minute quantities of the toxin can so effectively inhibit transmitter release. As improved methodologies for studying the exocytotic process become available, so the mechanism of the toxins' action appears

yet more elusive, further emphasizing their specificity within the neuron. Ultimately, the botulinum neurotoxins may prove to be invaluable tools and probes for dissecting the mechanism of neurotransmitter release but, at present they provide a constant challenge to the neurobiologist.

1.2. Botulinum toxin structure.

1.2.1 Production and activation of BoTx.

Crystallisation of botulinum type A (Abrams, 1946; and later Sugiyama and Moberg, 1977) produced a protein ($M_r \approx 900\text{Kd}$) containing both toxic and non-toxic moieties. The crystalline toxin was found to possess haemagglutinin activity and could be resolved into a number of components by anion-exchange chromatography (DasGupta et al., 1966, 1968). The initial peak (labelled α by the above authors) was a reasonably pure preparation of type A neurotoxin with a molecular weight (determined by gel filtration) of $\approx 150,000$. The second peak (β) was haemagglutinin ($M_r \approx 500,000$). Similar studies on other toxin types (B-F) have also shown a heterogeneous mixture of proteins in the toxin complexes. Electron microscopy studies by Boroff et al. (1972) on the toxic and haemagglutinin moieties of type A led to the suggestion that the neurotoxin molecule(s) form a central rod-shaped core (diameter $\approx 9\text{nm}$) surrounded by a coil of haemagglutinin (diameter $\approx 20\text{nm}$; Fig. 1.1a). This structure is not common to all toxin types, however, since type E and certain strains of type D (Miyazaki et al., 1977) lack this haemagglutinin moiety. The non-toxic components, including the haemagglutinin moieties, in the toxin complexes are believed to protect the neurotoxin from the environment of the gut prior to its absorption into the lymph and blood system (Sugii et al., 1977a,b). This is reflected in higher oral toxicities for these complexes over those observed in the neurotoxins (Ohishi, 1984). The progenitor neurotoxin molecules are synthesized as single polypeptide chains which subsequently undergo further post-translational processing (Fig. 1.1b; Habermann and Dreyer,

1986). The neurotoxins produced are similar in molecular size, ranging in molecular weight from 140,000 to 160,000, and share a common subunit structure (for review see Simpson, 1981). Beers and Reich (1969), using type B neurotoxin purified from a proteolytic culture, first demonstrated by polyacrylamide gel electrophoresis that, not only was the toxin composed of two chains (Mr. 104,000 and 59,000), but that these chains could be separated by disulphide reducing agents suggesting the existence of at least one interchain disulphide bond. With the exception of C₂ toxin in which the subunits are not covalently linked (Ohishi et al., 1984) and type G (uncharacterised), the presence of interchain disulphide bonds has since been demonstrated in all toxin types (for review see Simpson, 1981). However, whilst some strains of toxin types A, B and F are readily converted to the dichain form simply by reduction of these disulphide bond(s), type E toxin is insensitive to such treatment and is recovered as a single chain molecule suggesting that a further (proteolytic) cleavage between the heavy and light chains (see Fig. 1.1b) may be necessary for separation of the latter.

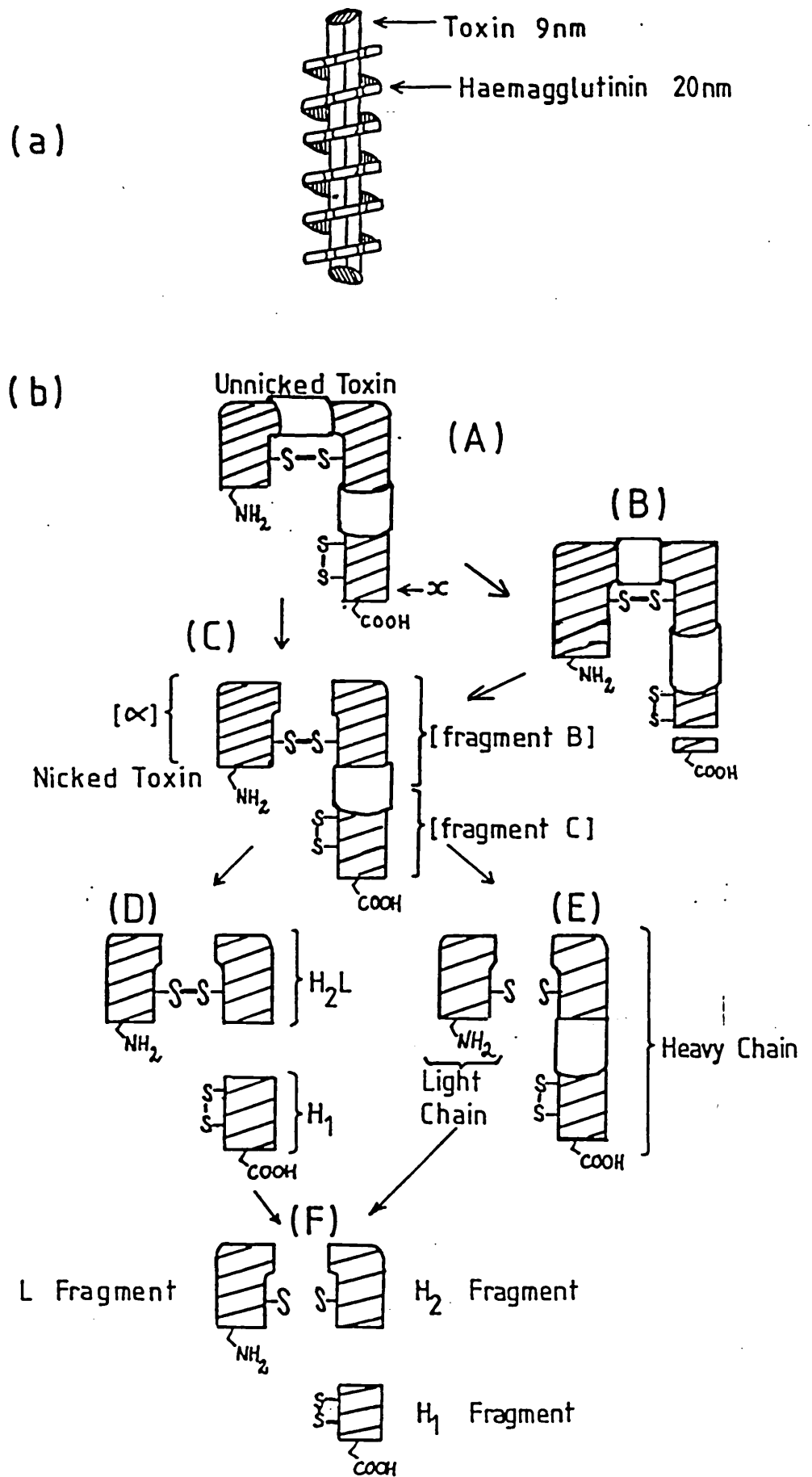
DasGupta and Sugiyama (1972a,b) noted that proteolytic enzyme activity in the bacterial culture is important in determining the form in which the neurotoxin will eventually be purified. A protease having trypsin-like specificity has been found in all tested proteolytic cultures of botulinum types A, B and F whilst absent from the non-proteolytic cultures producing type E. Consequently, BoNT E is found only as a single chain, although trypsinisation yields its dichain form (DasGupta and Sugiyama, 1972a,b). The latter authors thus suggested the presence of natural 'trypsin-like enzyme(s)' (TLE) in the bacterial cultures. These enzymes were proposed to differ from trypsin in their bond specificities; both enzymes acting on peptide bonds formed by carboxyl groups of arginyl and lysyl molecules but, in contrast to trypsin, TLE showing preference for arginyl residues (DasGupta and Sugiyama, 1980) resulting in structure (B) in Fig. 1.1b. This would serve to explain the observation that whilst proteolytic cleavage of the toxin

Fig. 1.1 Clostridial neurotoxin structure.

Fig. 1.1 a) Possible native arrangement of the molecules of the toxin and haemagglutinin of *Cl. botulinum* type A as determined by electron microscopy studies by Boroff et al. (1972). The neurotoxin molecule forms a central core surrounded by a coil of haemagglutinin.

Fig. 1.1 b) Structure, activation and nomenclature of Clostridial neurotoxins. These are synthesised intracellularly as single chain or unnicked molecules (A). Proteases may cause scission of an arginyl bond at the C terminus immediately subterminal (position 'x' in structure A), resulting in partially activated BoNT (B). In addition/alternatively endogenous or exogenous proteolytic enzymes (Trypsin-like enzymes) produce nicking of the toxin and removal of a fragment between the heavy and light chains to yield the dichain molecule (C). Structure (C) represents the fully activated toxin molecule in which both nicking and removal of the subterminal fragment has already occurred. Conversion of structure (B) to (C) can be achieved by proteolytic nicking using trypsin. Further cleavage of BoNT by trypsin (or tetanus by papain), results in the conversion of structure (C) to (D) producing the H₂L and H₁ fragments, whilst disulphide bond reduction of (C) results in the separation of the heavy and light chains of the neurotoxin (structure E). If structure (C) is subjected to both further proteolysis and disulphide bond reduction this results in the production of a number of fragments having similar molecular weights - the L, H₂ and H₁ fragments shown in structure (F). The above pathways of activation and nicking are very similar for botulinum and tetanus toxins - the labels in square brackets refer to the nomenclature used for tetanus toxin (from Bizzini, 1979), whilst all other labels also apply to the botulinum toxins (see Habermann and Dreyer, 1986). As can be seen from this figure, the multiple enzymic interactions possible mean that the activation and processing of the toxins need not occur via a linear pathway but several different pathways may result in the production of similar molecular structures.

Fig 1.1 Clostridial toxin structure



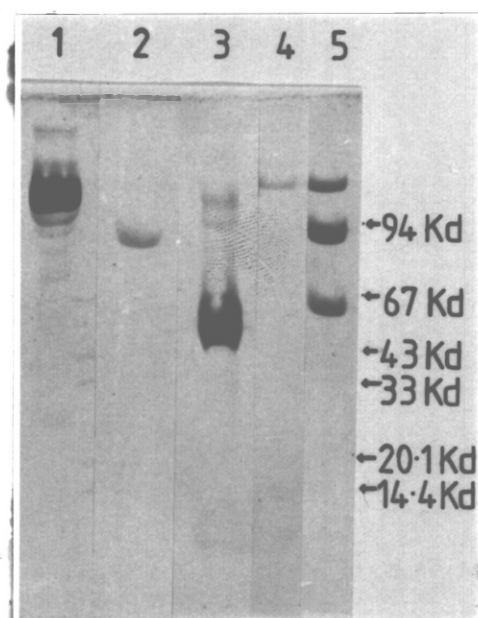
molecules and removal of a fragment between the heavy and light chains increased the specific toxicity of these proteins, complete activation of the neurotoxin required cleavage of both arginyl and lysyl bonds (structure (C); Fig. 1.1b), whilst cleavage by TLE occurs at arginyl bond(s) and results in only partial activation (structure (B); Fig. 1.1b). Complete activation would be observed in the presence of trypsin however due to cleavage at both arginyl and lysyl residues.

The process of removal of a fragment from the progenitor toxin resulting in the formation of the dichain molecule (a heavy (H) chain (molecular weight $\approx 100,000$) and a light (L) chain ($\approx 50,000$) linked by a disulphide bridge) is termed 'nicking' whilst an increase in toxicity over that observed in the progenitor toxin is termed 'activation'. As previously shown by Ohishi and Sakaguchi (1977) these events need not necessarily be linked and activation of type B toxin by trypsin at pH 4.5 was shown to precede nicking whilst at pH 6.0 both events occurred concurrently. The proposed model for activation and nicking of BoNT is summarised in Fig.1.1b.

1.2.2. Neurotoxin subunits.

Reduction of the disulphide bridge(s) linking the heavy and light subunits of BoNT generally leads to a loss of toxicity (Sugiyama et al., 1973). Concomitantly there is a reduction in solubility of the toxin and, at low ionic strength, a precipitate forms at concentrations $>1\text{mg/ml}$. In the case of type B toxin, the subunits can be maintained in solution by the inclusion of 1-2M urea in the reduction buffer and thus can be separated by gel filtration (Kozaki and Sakaguchi, 1975). Purification of the subunits from type A toxin by reduction of its disulphide bond(s) whilst bound to a QAE-Sephadex column has also been reported (Kozaki et al., 1981). Using this method the light chain is eluted leaving the heavy chain bound to the column. The heavy chain is subsequently recovered by elution in the reduction buffer containing 0.2M NaCl. As can be seen (Fig. 1.2) some cross-contamination of the subunits is usually observed in this

Fig. 1.2 Separation of the constituent fragments of type A BoNT by ion-exchange chromatography.



Purified neurotoxin type A was separated into its constituent subunits using ion-exchange chromatography as previously described by Kozaki et al. (1981). Briefly, type A neurotoxin (5mg; purified as described by Williams et al. (1983)), dialysed against sodium borate-phosphate buffer, pH 8.5, was adsorbed onto a column of QAE-Sephadex A-50 equilibrated in the same buffer, reduced with DTT (100mM) in the presence of 2M urea, and the two chains (heavy and light) separated from each other by step-wise elution using 2M urea and 10mM DTT followed by the same buffer containing 0.2M NaCl. All procedures were performed at 4°C. Samples from each of the eluted peaks were pooled and dialysed against sodium borate-phosphate buffer, pH 8.5, containing 2M urea and 10mM DTT. Aliquots were subjected to SDS-gel electrophoresis on 4-30% polyacrylamide gradient pore gels (Pharmacia PAA 4/30 gels) and visualised as detailed in section 2.2. Track number: 1) Intact native BoNT A 2) Separated heavy chain 3) light chain 4) Renatured heavy and light chain to produce the intact neurotoxin 5) Native toxin run under reducing conditions (in the presence of 5% β -mercaptoethanol) allowing the separation of the heavy and light chains of the nicked toxin molecules. The mobilities of protein markers of known molecular weights run on the same gel are shown.

protocol but modification of the latter has resulted in the purification of A, B and E neurotoxin subunits (Sathyamoorthy and DasGupta, 1985).

Individually, the toxin subunits exhibit low toxicities ($\leq 0.5\%$ of the intact toxin) and residual toxicity is believed to be due to minute contamination with native toxin. The subunits appear to maintain their structural integrity since intact neurotoxin may be recovered by mixing equimolar quantities of each and the slow removal of the sulphhydryl reducing agent by dialysis. Approximately 30% of the potential toxicity was recovered in this way in the samples shown (Fig. 1.2, track 4).

Using similar methodologies to those described above, purification and amino acid analysis of the subunits from neurotoxin types A, B, C, and E reveals a greater degree of homology between the light subunits of these neurotoxins than the heavy subunits (Sathyamoorthy and DasGupta, 1985; Syuto and Kubo, 1981). The light chains are richer in the α -helix-breaking amino acid proline but otherwise several of the amino acids in the heavy chains are present in concentrations close to twice those in the respective light chain. This has led to the suggestion that the 150,000d neurotoxin may have arisen by a process of duplication of an ancestral gene coding for a 50,000 protein (Sathyamoorthy and DasGupta, 1985; Simpson, 1986).

1.3. Postulated mechanisms of neurotransmitter release.

The process of neurotransmitter release has been the subject of much study in the last century but progress in understanding its mechanism was rapidly accelerated by the use, in the last 40-50 years, of electrophysiological techniques. The basic model for transmitter release is as follows; spike activity results in depolarisation of the nerve which allows the rapid influx of Ca^{2+} into the neuron. The Ca^{2+} concentration within the nerve ending rises from a resting level of $\approx 100\text{nM}$ to $\approx 1\mu\text{M}$. This, in turn, causes the release of transmitter (synthesized and stored in the presynaptic terminal) into the synaptic cleft,

although the exact mechanism linking Ca^{2+} entry and release is not yet known. The concentration of transmitter in the synaptic cleft between the pre- and post-synaptic membranes rises and it diffuses outwards from the point of release towards the post-synaptic membrane. Here, its action on post-synaptic receptors is to modify ionic movements across the membrane thus altering the excitability of the post-synaptic neuron. The transmitter's actions are only transient, however, since its levels in the synaptic cleft are rapidly decreased due to enzymatic degradation, reuptake into the presynaptic neuron or diffusion away from its site of action.

This conversion of an electrical impulse into a chemical signal requires energy and is not economical. However it has a number of advantages, namely; 1) Signal amplification is introduced: one or two transmitter molecules may open a single channel, allowing the passage of several thousand ions; 2) The selectivity of these channels opened by the transmitter may elicit either excitatory or inhibitory responses; and 3) the complexity of the chemical interactions, both at the presynaptic as well as the post-synaptic sites, provides a possible modulation of the efficacy of the transmission process, an essential requirement of central synapses.

1.3.1. The Vesicular hypothesis: Quantal release.

Quantal release is (probably) the major mechanism of transmission of impulses from nerve to muscle. Fatt and Katz (1952) first proposed the release of transmitter as discrete units (quanta) to explain the appearance of spontaneously occurring miniature endplate potentials (m.e.p.ps). Quantal release implies liberation of neurotransmitter by nerve terminals as multi-molecular packets of a constant size. These "packets" of transmitter are released into the synaptic cleft and interact with the post-synaptic membrane (the muscle membrane) resulting in local depolarisations having fixed amplitude and time-course. These m.e.p.ps, which can occur spontaneously in the resting synapse, are believed to sum in a stepwise manner to compose the

endplate potential (EPP) produced in the muscle fibre by the nerve impulse. The designated containers for each quantum were proposed to be the synaptic vesicles (DelCastillo and Katz, 1957) which were later shown to be filled with neurotransmitter (DeRobertis et al, 1962; Whittaker et al, 1963). It was postulated that these vesicles move randomly within the presynaptic terminal and occasionally touch the presynaptic membrane at critical contact zones, causing fusion with the cytoplasmic membrane and release of the vesicular contents into the synaptic cleft. This so-called "vesicular hypothesis" (reviewed by Ceccarelli and Hurlbut, 1980; Israel et al, 1979) was readily accepted since it not only provided an explanation of the occurrence of both m.e.p.ps and the quantal nature of the EPPs but also explained how these quanta are stored in the nerve terminal ready for rapid release. However, initial experiments tended to suggest that synaptic vesicles were an artifact of the preparation procedure for electron microscopy (Gray, 1976, 1977), but this was later dismissed (Gray et al., 1979). Since molecules such as dextran (Zimmermann and Denston, 1977), peroxidase (Heuser and Reese, 1973; Hurlbut and Ceccarelli, 1974) and acetylcholine esterase (AChE; Politoff et al., 1975) could be found in these vesicular structures it was believed that most synaptic vesicles are formed by recycling of presynaptic nerve terminal membrane in an endocytotic cycle after exocytosis has taken place (Heuser and Reese, 1973; Hurlbut and Ceccarelli, 1974; Zimmermann and Denston, 1977a).

If the vesicular hypothesis was correct it would be expected that the following criteria would apply to varying degrees :

- 1) It should be possible to show that one vesicle contains the amount of transmitter necessary for one m.e.p.p. i.e. one quantum.
- 2) There should be a large enough content of synaptic vesicles to allow a suitable postsynaptic response with a surplus of unused vesicles.
- 3) Fusion of a synaptic vesicle with the presynaptic membrane and release of neurotransmitter into the synaptic cleft should precede the appearance of the associated m.e.p.p.
- 4) Vesicular transmitter should be released

preferentially and directly without first entering another compartment. 5) Other compounds present in synaptic vesicles should also be released simultaneously, and in a stoichiometric ratio, with the neurotransmitter. Proof of the above criteria would support the vesicular hypothesis and has been much sought after, as shown by the literature (for reviews see Tauc, 1982; Dunant, 1986). In an attempt to elucidate the relationship between vesicular transmitter content and quantal transmitter content, Kuffler and Yoshikami (1975), by iontophoretic application of acetylcholine onto the neuromuscular junction, estimated the amount of ACh in one quantum to be less than 10,000 molecules. Heuser and Reese in a series of papers (1977, 1979a,b), showed that the number of membrane invaginations, believed to be formed as vesicles fuse with the presynaptic membrane during exocytosis, correlated well with the physiological estimates of the number of quanta released, and concluded that one vesicle discharges one quantum.

Evidence against the existence of vesicular release is provided by the observation of small-mode mepps (s-mepps) each representing $\approx 1/10$ th of a mepp (Bevan, 1976; Kriebel et al., 1974). The amplitude distribution of these s-mepps indicate several pools of transmitter, suggesting the presence of quantal subunits. This led to the proposal that the larger classical mepps were composed of synchronously released subunits - each subunit producing a single s-mepp (Kriebel et al., 1976; Matteson et al., 1979). Within the vesicular hypothesis, these s-mepps could be due to the release of partially filled vesicles or to the fact that each vesicle contains enough transmitter only for one s-mepp. In the case of the latter, this would require the synchronous release of 10 vesicles to produce a single mepp and, thus, the proposed direct correlation between the vesicles and observed quantal numbers (Heuser and Reese, 1979a,b) would be invalid. Vesicles would have to contain the amount of transmitter required for an s-mepp which would consequently mean that there are not enough vesicles in close proximity to the plasma membrane to account for the postsynaptic response upon depolarisation

(Katz and Miledi, 1979). Furthermore, the process of vesicle fusion with the presynaptic membrane and subsequent release of neurotransmitter would need to take place extremely rapidly since it has been shown (Llinas, R.R., 1977) that the time between Ca^{2+} entry and the start of the postsynaptic potential in stimulated squid terminals is approximately 200 μ seconds. It would be expected that, within this time span, fusion of vesicles with the presynaptic membrane would be observed. However, Heuser et al. (1973) using electron microscopic studies on rapidly frozen tissues (<1mS) demonstrated that, whilst most transmitter was released within 3mS after stimulation of the frog neuromuscular junction (NMJ) using 4-aminopyridine (4-AP), characteristic structures of exocytosis (coated pits etc.) were only observed 5mS after the stimulus; this suggested that transmitter was not released from vesicles. Finally, if transmitter were released from discrete vesicles then a stoichiometric co-release of non-transmitter substances would also be expected to be observed. Whilst these substances are found in perfusates they are present at lower concentrations than would be expected if stoichiometric release of all soluble components of a vesicle occurred during exocytosis (Smith, 1971, 1972). In order to explain some of the above observations a process involving direct release of transmitter from the cytoplasmic pool was proposed as described below.

1.3.2. Cytoplasmic release of transmitter.

Evidence for non-vesicular release of transmitter has been obtained from a number of sources. Normal synaptic transmission has been demonstrated in chick ciliary ganglia at a developmental stage where the low numbers of morphological synapses present contained few or no synaptic vesicles (Landmesser, L. and Pilar, G., 1972). The transmitters, dopamine, ACh and catecholamines have all been shown to be liberated from neurons lacking synaptic vesicles (Bjorklund and Lindvall, 1975; Neicoullon et al, 1977; Johnson and Pilar, 1979; Suetake et al, 1981). Furthermore, Israel and Lesbats (1981a) have proved that

upon stimulation it is the cytoplasmic ACh which is preferentially released over vesicular ACh (which remained stable even at a stage when the consumption of cytoplasmic transmitter was complete). These authors, using a chemiluminescent assay of ACh release, have also shown that synaptosomal "ghosts" (lacking internal organelles such as vesicles but containing a 'free-cytosolic-pool' of ACh) could release ACh in a Ca^{2+} -dependent manner, providing further support for the theory that transmitter is released from the cytoplasm and that vesicular release is not necessary for stimulated release (Israel et al., 1981b).

How then would neurotransmitter pass from the cytosol of the nerve terminal, across the presynaptic membrane and into the synaptic cleft in a manner reconcilable with the observations made above (section 1.3.1). Many proposals have been made for the mechanism of cytoplasmic release of transmitter and these are discussed below:

1.3.2.1. The Acetylcholine channel. It was proposed that there may exist a presynaptic channel which was sensitive to changes in intracellular Ca^{2+} concentration. The influx of Ca^{2+} upon depolarisation would cause this channel to open, allowing the passage of ACh in the proximity of the presynaptic plasma membrane through the channel following its electrochemical gradient. However, evidence against the existence of such a channel is quite marked e.g. changing the concentration of ACh within the presynaptic cytoplasm would be expected to alter the quantity of ACh corresponding to a quantum (released from a single channel). However, injection of AChE (Tauc, 1977; Tauc and Baux, 1982) or choline oxidase (Poulain et al., 1986) directly into presynaptic neurons depleted the cytosolic transmitter levels and decreased the number of quanta (quantal content) rather than the size of each quantum. Similarly, intracellular injection of ACh into presynaptic neurons led to an increase in the postsynaptic response by increasing the quantal content whilst the size of each quantum remained constant (Tauc and Baux, 1982). Whilst these observations provide further proof that

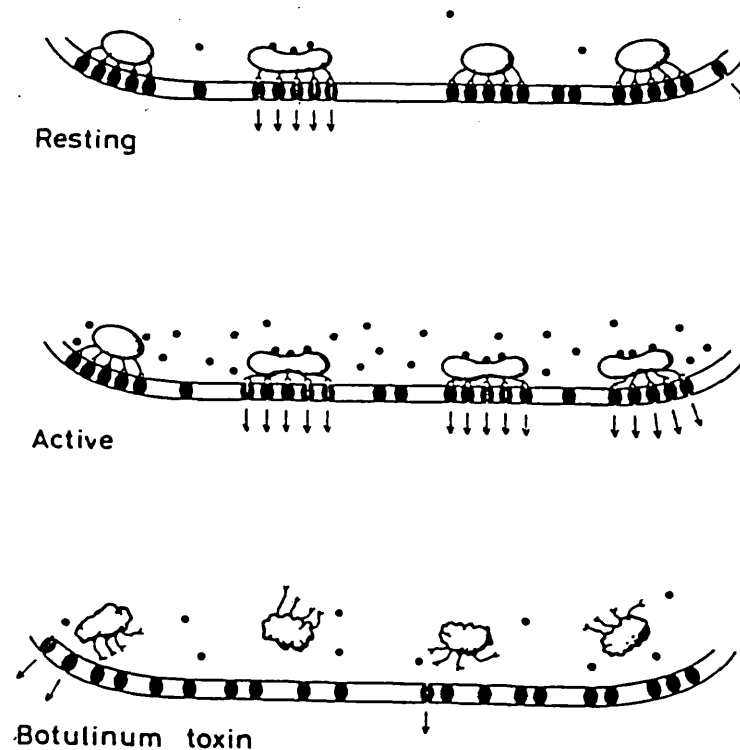
transmitter is released from a cytoplasmic rather than a vesicular pool (which remained unchanged by the above treatments), it tends to suggest that this release does not simply occur via the opening of a channel and therefore led to the modification of this mechanism detailed below.

1.3.2.2. The 'gate' hypothesis. In order to explain the quantal nature of cytoplasmic release and the dependence of quantal content on the amount of intracellular (non-vesicular) transmitter, a mechanism involving the binding of ACh to a saturable 'gate' was proposed in which Ca^{2+} influx triggers exocytotic release of cytoplasmic ACh from a triple tetrameric macromolecule that binds ACh to saturation (≈ 480 molecules-enough to explain a small mepp). Ten such molecules acting in synchrony (called an 'operator') could release a quantum (Israel et al., 1979). This hypothetical 'operator' responsible for Ca^{2+} -dependent cytoplasmic release of ACh from Torpedo electric organ synaptosomes (Israel et al., 1981b) has since been partially purified and demonstrated Ca^{2+} -dependent release of acetylcholine when incorporated into proteoliposomes (Israel et al., 1984; Birman et al., 1986). The molecule responsible for this release process was shown by the latter authors to be on the intracellular face of the presynaptic membrane and, after extraction under alkaline conditions, demonstrated preferential release of ACh over either choline or ATP. It was named a 'mediatophore' and exhibited a high apparent molecular weight on cholate solubilisation (200Kd-600Kd) which represented an aggregated form of the molecules. It was postulated that these mediatophores exist as pentameric structures in the membrane - the monomers are not covalently linked and consist of two chains - each with a molecular weight $\approx 17\text{Kd}$ (Israel et al., 1986). This mechanism would therefore serve to explain the observed quantal release of transmitter. However, cytoplasmic release resulting directly from the nerve terminal in a non-quantised manner has also been demonstrated and, like the quantal release above, is Ca^{2+} -dependent and also increases upon stimulation (Vizi and

Vyskocil, 1979; Mitchell and Silver, 1963). At rest quantal release accounts for only 1-2% of the total release of ACh (Vizi and Vyskocil, 1979; Mitchell and Silver, 1963). The large proportion of spontaneous transmitter release has been proposed to have a trophic role in maintenance of neuromuscular innervation and, in non-synaptic regions, it is postulated that this, non-quantal, release may play a role in neuromuscular transmission (Vizi and Vyskocil, 1979).

In an attempt to explain the different modes of transmitter release, Dunant (1986) proposed a unifying concept involving one common, basic mechanism. This is dependent on the hypothetical existence, in the presynaptic cholinergic membrane, of two types of moieties. The ACh-releasing elements (or 'mediatophores')- each one of which is capable of releasing approximately 1/10th of a quantum of ACh - are proposed to exist in groups of 10, and, unstimulated, give rise to sub-m.e.p.p.s as the elements open sporadically and independently of one another. The second hypothetical moiety has been named 'calsynchronin'. This molecule is proposed to respond to the increase in intracellular Ca^{2+} concentration during an action potential and, under these conditions, synchronise the release of transmitter from a group of mediatophore elements culminating in the release of one quantum of neurotransmitter per group. Only those mediatophore elements in a group are proposed to respond to calsynchronin and thus the individual (isolated) elements would be insensitive to Ca^{2+} influx. However, individual elements would still be capable of spontaneous release and responsible for the production of sub-miniature potentials (with a corresponding reduction in the number of mediatophores available for evoked quantal release). Dunant fits the work on botulinum toxins into this model by suggesting that these act by destroying or disconnecting calsynchronin, resulting in all of the mediatophore population being left in the isolated state -leading to a blockade of quantal release with the maintenance of subminiature potentials. A cartooned illustration of the mediatophore hypothesis is shown (Fig.1.3; Dunant, 1986).

Fig. 1.3. Hypothetical model of neurotransmitter release.



A cartooned illustration of the mediatophore hypothesis. In the pre-synaptic membrane a certain proportion of mediatophore units are grouped under the control of a molecule of calsynchronin. In the resting state, single mediatophore-calsynchronin complexes are occasionally activated and release the amount of transmitter corresponding to a miniature endplate potential. Isolated units may also "open", generating a subminiature potential. On the arrival of an action potential, calcium enters the synapse and synchronously activates a large number of calsynchronin-mediatophore complexes: an impulse is transmitted. The bottom figure represents the possible alteration of this system by botulinum toxin. The calsynchronin molecules are inactivated, or decoupled from the mediatophores. ACh can no longer be released in a calcium dependent quantal manner. The transmitter, however, is present in the synapse and some spontaneous release still takes place through the dispersed mediatophores. This generates sub-miniature potentials and, when "opening" is protracted, giant and slow events.

KEY: • = Ca^{2+}
 ○ = Calsynchronin
 || = Mediatophores

If these mediatophores prove to be the sole mechanism of ACh release and the neurotransmitter was shown to be released from a cytoplasmic pool as indicated (Dunant, 1986) rather than by vesicle fusion with the presynaptic membrane (Heuser and Reese, 1973) then it would be necessary to identify a possible role for these presynaptic vesicles. Tauc (1982) proposed that they act as depositories for unwanted products of synaptic transmission e.g. Ca^{2+} , and that they are part of the homeostatic mechanism within the neuron maintaining intracellular Ca^{2+} at a low level - a necessary function since Israel et al. (1986) have demonstrated that, in the presence of low levels of Ca^{2+} the mediatophore becomes desensitized in its ability to release ACh. Uptake of Ca^{2+} by the vesicles may be ATP-dependent and has a K_m in the micromolar range (Israel et al., 1980), rapid enough, in conjunction with uptake by the mitochondria and smooth endoplasmic reticulum (SER) (Blaustein et al., 1980), to buffer cytoplasmic Ca^{2+} at a low level.

Thus, in summary, it can be seen from this introduction that much controversy exists in the current literature with respect to the mechanism of neurotransmitter release from presynaptic nerve terminals. Groups of workers tend to take a stance on either the 'vesicular' hypothesis or the 'cytoplasmic' hypothesis. However, it is perfectly feasible that both types of transmitter release occur to varying degrees, each having a particular role to play in synaptic communication. It may very well be that a hybrid of the two hypotheses could be correct, in which, upon influx of Ca^{2+} due to nerve depolarisation, the 'mediatophores' (saturated with cytoplasmic ACh) release their transmitter. However, under heavy or repetitive stimulation they are replenished by transmitter released from the pool of metabolically active vesicles in the vicinity of the mediatophores hence maintaining a high local concentration of ACh in the region of the releasing sites. Due to their unique molecular action in inhibiting transmitter release, the botulinum neurotoxins are going to prove invaluable tools in verifying the

mechanism of neurotransmitter release as their mode, and site, of action become better understood.

1.4. Mechanism of the inhibitory action of BoNT on transmitter release.

1.4.1. Toxin binding to neuronal membranes.

The primary site of action of BoNT has long been established as the cholinergic nerve endings innervating the neuromuscular junction (Burgen et al., 1949; Brooks 1956). It was therefore pertinent to try and determine whether the neurotoxin bound to this tissue in a specific and saturable manner. The presence in these neurons of such high-affinity acceptors for the toxin would explain its unique potency. Initial studies to localise the binding of toxin at the NMJ used impure toxin-haemagglutinin complexes labelled with fluorescent or ferritin markers (Zacks et al., 1962, 1968). The validity of such localisation studies was questioned, however, since it was shown that tritiated toxin-haemagglutinin complex bound to non-neuronal as well as neuronal tissues (Dolly et al., 1981) and that the majority of this binding was abolished by galactose, an inhibitor of haemagglutinin. Attempts to use ^{125}I -labelled toxin were of limited use since these preparations were of low specific activity and unstable upon storage (Habermann, 1974; Hirokawa and Kitamura, 1975). Moreover, the techniques used were unable to differentiate between a pre- or a post- synaptic localisation of toxin binding. However, using a highly radiolabelled preparation of BoNT A, that exhibited a high retention of specific neurotoxicity, direct evidence for the saturable interaction of toxin solely with the motor nerve terminal (determined by electron microscope autoradiography) was presented for the first time (Dolly et al., 1984). Further studies have allowed the quantitation of these saturable binding sites for radioiodinated types A and B BoNT on the presynaptic nerve terminal (Black and Dolly, 1986a, 1986b). Acceptor densities of

150 acceptors/ μm^2 and $\approx 630/\mu\text{m}^2$ were obtained for BoNT A and B, respectively. From these studies, it was clear that ^{125}I -BoNT A bound specifically to motor nerve terminals with no binding seen on Schwann cells, blood vessels, myelin sheath, muscle fibre or collagen (Black and Dolly, 1986a). The toxin's specificity for nerve terminals was further emphasized by its demonstrated inability to bind to regions of motor neurons of newborn rats which had yet to be myelinated. Binding of toxin to such acceptors has been shown to be mediated by the heavy subunit both in the PNS ((Black and Dolly, 1986b) as well as in the CNS (Williams et al., 1983; Kozaki, 1979). Whilst an excess of BoNT A can partially inhibit ($\approx 24\%$) the binding of ^{125}I -BoNT B, BoNT B had no effect on the binding of ^{125}I -BoNT A (Black and Dolly, 1986a). This observation would tend to suggest that BoNT A interacts indirectly with at least some of the acceptors for BoNT B but otherwise emphasises that these toxin types have distinct membrane acceptor moieties. These acceptors for BoNT A, at least in the PNS, may be similar to those for tetanus toxin since high concentrations of the latter could partially inhibit (by $\approx 60\text{--}70\%$) the binding of ^{125}I -BoNT A to motor nerve terminals (Black and Dolly, 1986b).

Characterisation of the nature of such binding components was not possible at the neuromuscular junction due to the unsuitability of such a preparation for biochemical analysis. Thus, it was fortuitous that binding of ^{125}I -BoNT A to the synaptosome fraction of rat brain was demonstrated (Habermann, 1974; Kitamura, 1976) and this became the model system on which much of the current work on toxin-acceptor binding was performed. As is the case in the PNS, binding of type A neurotoxin in the CNS was shown to occur at the presynaptic membrane of nerve terminals (synaptosomes; Black and Dolly, 1987; Dolly et al., 1987). It is not known whether these acceptor moieties also play a physiological role during neuronal activity but, from the viewpoint of intoxication, their presence on the external surface of the plasma membrane may serve to selectively concentrate the toxin molecules at a site which allows a more rapid and efficient

uptake into the neuron than would otherwise occur during bulk fluid pinocytosis (Dautry-Varsat and Lodish, 1984). However, it must be noted that, in the CNS, little internalisation of toxin, specifically bound to its acceptors was observed (Black and Dolly, 1987), possibly due to the uptake mechanism being less efficient in this region than at the NMJ. Further discussion on the biochemical nature of these acceptors is provided in section 2.4.

1.4.2. Toxin internalisation.

Evidence that BoNT binding is proceeded by an internalisation step and subsequent retrograde intra-axonal transport (previously shown for tetanus toxin; Price et al., 1975) is provided by; (a) The observed latent period between irreversible binding of toxin and the onset of paralysis (Burgen et al., 1949); (b) the fact that antitoxin antibodies inactivated toxin bound to the nerve membrane but could not prevent intoxication beyond this step (Simpson, 1974) and (c) the demonstrated presence of labelled toxin within the nerve terminal (Black and Dolly, 1986a,b). The internalisation step appears to be energy-dependent since it is effectively prevented by incubation at reduced temperature (4°C) or in the presence of inhibitors of ATP production such as azide or dinitrophenol (Black and Dolly, 1986b). This would indicate that entry of the toxin into the neuron proceeds via acceptor-mediated endocytosis. Further evidence for such an entry mechanism has been provided by the observations that radiolabelled neurotoxin, within the neuron, was associated with vacuolar structures - possibly endosomes (Black and Dolly, 1986b). Lysosomotropic agents which prevent the acidification of these latter organelles (DeDuve et al., 1974) also seem to prevent the transfer of radiolabelled toxin into the cytoplasm (the believed site of the toxin's action) and thus prolong the time to intoxication (Black and Dolly, 1986b). Due to the raised intraendosomal pH, BoNT cannot undergo the conformational changes postulated to be required for entry into the cytoplasm (Shone et al., 1987; Hoch et al., 1985)

or the endosomes do not fuse with lysosomes to allow the toxin's breakdown. Consequently, the toxin is recycled to the plasma membrane (Black and Dolly, 1986b) and maintained at an antitoxin-sensitive site (Simpson, 1982, 1983). Thus, in the same way as nerve stimulation accelerates the rate of toxin internalisation (Burgen et al., 1949) and thereby results in a more rapid blockade of neurotransmission (Hughes and Whaler, 1962), so inhibition of the acceptor-mediated pathway can prevent the toxin's access to its ultimate site of action and the associated expression of toxicity.

1.4.3. The pharmacological action of BoNT.

The most valuable insight into the nature of the blockade in neurotransmission produced by BoNT has been afforded by electrophysiological recordings. In this respect, the mouse phrenic nerve hemidiaphragm preparation introduced by Burgen et al. (1949) has proved to be an extremely useful model system of the toxin's peripheral action. These authors demonstrated that the paralysis produced by BoTx was accompanied by a decrease in the release of the neurotransmitter, ACh. This decrease was hypothesised to be due to one of three possibilities; 1) blockade by the toxin of impulse flow along the fine, terminal arborisations of the nerve; 2) blockade of ACh synthesis or storage; 3) inhibition of transmitter release from the nerve terminals.

1.4.3.1. Lack of an effect on propagation of the action potential.

Work by Dickson and Shevsky (1923), Thesleff (1960) and Harris and Miledi (1971) has clearly demonstrated that the toxin does not affect the propagation of action potentials along the nerve terminals and hence dismisses the first hypothesis.

1.4.3.2. Lack of an effect on ACh synthesis or storage.

As shown in section 1.3 the release of ACh into the synaptic cleft and its subsequent interaction with postsynaptic

receptors on the muscle endplate results in the production of an endplate potential (epp) and subsequently muscle contraction. When the nerve is not being stimulated, mepps, due to random exocytosis of transmitter, can be recorded from the muscle endplates. In the initial stages of BoNT poisoning, mepps greatly decrease in frequency.

End-plates examined within 24hrs of BoTx treatment showed a skewed distribution of mepp amplitudes with a predominance of small mepps (c.f. Harris and Miledi, 1971). The mepps seen after BoTx poisoning were shown to be due to ACh quanta since addition to the perfusing medium of D-tubocurarine (Cull-Candy et al., 1976; Thesleff, 1984) or α -bungarotoxin (Thesleff, 1984) completely abolished all activity, whilst mepp amplitude is increased by blockers of cholinesterase (Thesleff, 1984). Presumably these mepps result from ACh release from the nerve (and not from Schwann cells) since they permanently disappear following surgical denervation of the muscle (Cull-Candy et al., 1976). The decreased mepp amplitude in intoxicated motor endplates may imply that the quantal amount of ACh released at these nerve endings is reduced. Based on observations made at the frog NMJ (Boroff et al., 1974) it was proposed that the toxin alters the properties of the vesicle membranes such that it impairs both vesicle loading and their subsequent fusion with the presynaptic membrane (hence producing a reduction in both quantal content and mepp frequency). However, such an effect of the toxin on vesicle fusion is at odds with the data provided by studies using either black widow - or brown widow- spider venom. These venoms overcome BoNT intoxication by evoking a large increase in mepp frequency in both test and control samples. Furthermore, the amplitudes of these induced mepps are similar, suggesting that the ACh content of the vesicles is unaltered by the toxin's action (Cull-Candy et al., 1976; Kao et al., 1976; Simpson, 1978). After prolonged intoxication, mepps exhibiting larger amplitudes appear (Cull-Candy et al., 1976; Gundersen, 1980; Colmeus et al., 1982), similarly, lowering the temperature (from 16°C to 6°C) results in an increased amplitude of the epps

produced by indirect stimulation of the nerve (Molgo and Thesleff, 1984) - inexplicable by a mechanism of impaired transmitter synthesis or vesicle loading.

1.4.3.3. Blockade of ACh release.

The rise-times of the mepps remaining after intoxication are slower than in unpoisoned muscles and their amplitude distribution is skewed rather than the normal bell-shaped curve (Harris and Miledi, 1971; Cull-Candy et al., 1976; Kriebel et al., 1976; Tse et al., 1982). Studies using mouse phrenic nerve hemidiaphragm preparations intoxicated with pure neurotoxin (BoNT A) *in vitro* further demonstrated the existence of two species of mepp in controls - only one of which was affected by the toxin (Dolly et al., 1987). The population unaffected by the toxin exhibited slow rise-times, a broad amplitude distribution and comprised just 1-2% of the total mepps in controls. The population affected by the toxin demonstrated fast rise-times with a gaussian amplitude distribution in the controls but were markedly reduced in frequency upon intoxication. The quantal content of the nerve-evoked epp is also greatly reduced whilst the rise-times of these epps is unaffected by the toxin (Cull-Candy et al., 1976; Dolly et al., 1987). The reduction in epp amplitude followed a similar time course to the observed decrease in fast rise-time mepp frequency, suggesting a common mechanism for the two processes (Dolly et al., 1987). Since the latter authors indicate that only the fast rise-time mepps form the quantal component of epps (produced by near-simultaneous release of packets of ACh with those producing mepps; DelCastillo and Katz, 1954) they propose that the similar action of the toxin on fast rise-time mepps and epps suggests that it blocks release from the active zones. The slow rise-time mepps (unaffected by the toxin) were proposed to be due to release from sites near to, but not at, the active zones (as proposed by Thesleff and Molgo, 1983)). The latter authors reported a similar population of slow rise-time mepps in the rat after chronic intoxication with BoNT which responded differently from normal mepps to procedures which

increased internal Ca^{2+} (e.g. high K^+ or ouabain; Thesleff and Molgo, 1983). Dolly et al. (1987) propose that this is due to a different release process for the slow rise-time mepps as compared with the fast rise-time population.

1.4.4. The role of Ca^{2+} in intoxication.

Studies on the nature of the blockade at the neuromuscular junction have revolved around trying to alleviate the inhibition of transmitter release by the elevation of intracellular levels of Ca^{2+} since this causes a shift toward a more normal amplitude distribution of mepps by increasing spontaneous transmitter release. Such procedures include the use of Black Widow spider venom (BWSV), Ca^{2+} ionophore A23187 (together with high Ca^{2+} concentrations), or high frequency stimulation of the nerve (see review by Sellin, 1981). Elevation of extracellular Ca^{2+} levels alone does not cause an increase in the rate of mepps in poisoned terminals (Harris and Miledi, 1971; Boroff et al., 1974; Cull-Candy et al., 1976) even though the toxin has no effect on presynaptic Ca^{2+} currents (Molgo and Thesleff, 1984; Gundersen et al., 1982). A23187 acts as a Ca^{2+} ionophore, allowing the passage of Ca^{2+} across biological membranes and, in the presence of high extracellular Ca^{2+} concentrations ($>4\text{mM}$), caused a massive release of transmitter from control endplates with a concomittant increase in mepp frequency ($>200/\text{s}$). In BoTx poisoned muscles, the ionophore also caused an increase in transmitter release to levels comparable to those observed in the controls (Cull-Candy et al., 1976). The fact that it is possible, in a poisoned muscle, to restore both spontaneous and impulse-evoked transmitter release to about normal levels by methods which raise intracellular Ca^{2+} concentrations indicates that the release mechanism in BoTx poisoned terminals is essentially intact, but shows lower sensitivity to raised intracellular levels of this cation. Thus larger intracellular levels of Ca^{2+} are required for 'normal' release to occur (Cull-Candy et al., 1976; Lundh et al., 1977; Molgo and Thesleff, 1984). Molgo and Thesleff (1984) proposed

that the toxin had an enzymic action, causing increased intracellular metabolic processes involved in the disposal of free Ca^{2+} from the cytoplasm. An enhanced disposal of Ca^{2+} could lower the concentration to a level where the amount of Ca^{2+} entering the terminal on depolarisation would not reach the threshold level necessary for transmitter release to occur (Katz, 1969). Evidence for such an enzymic action of the toxin comes from work showing that the neuromuscular blockade by BoTx has a very high temperature sensitivity (lower temperatures reducing the block and enhancing release - in contrast to the effect seen at unpoisoned junctions (Molgo and Thesleff, 1984)).

Studies on the central effects of BoNT, together with its effect on levels of intracellular Ca^{2+} , are discussed further in Chapter 4.

1.5 Tetanus toxin: similarities and differences with BoNT.

Tetanus neurotoxin (Tetx), another clostridial toxin, has also been extensively studied in an attempt to characterise its binding site(s), mode of internalisation and mode of action. Many similarities and differences exist between this toxin, and BoNT (recently compared in a number of reviews: Habermann and Dreyer, 1986; Simpson, 1986).

Tetx acts primarily at the spinal cord causing blockade of neurotransmitter (glycine) release from the Renshaw cells (Wellhoner, 1982; Bizzini, 1979); this leads to muscle rigidity, convulsions and, without treatment, death due to respiratory failure. Like BoNT, Tetx is produced by an anaerobic bacterium (*Clostridium tetani*) and its synthesis and proteolytic processing is virtually identical to that of BoNT (Habermann et al., 1986; see Fig. 1.1b).

Whilst the nomenclature differs slightly for the subunits of Tetx (Habermann, 1986), the generalised structure is similar to that seen for BoNT i.e. the toxin is composed of both a heavy chain ($M_r \approx 100,000$) and a light chain ($\approx 50,000$) linked by a disulphide bridge. As with BoNT, the carboxyterminus of the heavy

chain of Tetx (see Fig.1b) is proposed to be responsible for binding to the nerve terminal, whilst the amino terminus of the heavy chain is thought to mediate internalisation (Habermann, 1986). The light chain, as proposed for BoNT (Shone et al., 1985) is thought to be responsible for the toxicity to the nerve terminal, presumably by some enzymatic/ catalytic mechanism (Simpson, 1986). The apparent chemical similarities between Tetx and botulinum A, B and E toxins is highlighted by Habermann and Dreyer (1986) and is remarkable when considering that the former is encoded by a plasmid whilst the genes for the latter are present on the chromosome.

Based on the similarities in the amino acid composition between the heavy and light chains of Tetx, it has been proposed (Taylor et al., 1983) that the gene for Tetx arose through duplication events. This hypothesis has also been proposed for BoNT (Simpson, 1986) but has yet to be confirmed (by DNA sequencing) for either toxin type. Post-translational processing of these toxins appears to involve proteolytic activation (by extrinsic or intrinsic proteases) of all BoNT (except type E) whereas the data for Tetx are ambiguous. Like BoNT, nicking of Tetx can also take place but, additional proteolysis, mimicked by papain, can cause further cleavages resulting in a non-toxic moiety ($M_r \approx 45,000$), containing a disulphide loop, which retains its ability to bind to gangliosides (for review see Habermann and Dreyer, 1986). The N-terminal moiety has been sequenced and appears to occupy the C-terminal of the heavy chain (Matsuda and Yoneda, 1977), sometimes obtained as two fragments hydrolysed within the intrachain disulphide bond at the C-terminal (similar to observations made for the heavy chains of A and B BoNT (Shone et al., 1985; DasGupta, 1981)). The heavy chain of Tetx appears to mediate the binding of the toxin to the presynaptic membrane since fragment C of this chain (see Fig.1b) appears to share binding sites with whole toxin on brain preparations (Goldberg et al., 1981). Binding of Tetx has also been observed in kidney (Habermann and Albus, 1986) and on thyroid plasma membranes (where it appears to interact with the ganglioside moieties of

the receptor for thyrotropin (Ledley et al., 1977; Morris et al., 1980)).

As observed with BoNT (Williams et al., 1983; Shone et al., 1985), the toxicity of isolated heavy and light chains of Tetx is minimal and any residual toxicity is thought to be due to contamination with intact toxin (Helting and Zwisler, 1977). However, Penner et al. (1986) demonstrated, by intracellular injection into chromaffin cells, that, whilst no effect on transmitter release (as measured by changes in cell-capacitance under patch-clamp) was observed using fragment C of Tetx ($M_r \approx 50,000$), fragment B ($M_r \approx 100,000$, consisting of the light chain covalently linked to the part of the heavy chain remaining after cleavage by papain) was almost as effective as native toxin at inhibiting release. This would seem to confirm the hypothesis that fragment C is the binding moiety and that internalisation is necessary for expression of toxicity.

Although Tetx is known for its effects on inhibitory interneurons it has also been shown to weakly inhibit ACh release at the neuromuscular junction (Habermann et al., 1980) giving rise to botulinum-like symptoms (Matsuda et al., 1982). Like BoTx (Simpson, L.L., 1986), Tetx appears to also proceed through a sequence of steps, involving extracellular binding, membrane penetration and, finally, an intracellular poisoning step (Schmitt et al., 1981).

The action of tetanus and botulinum toxins on synaptic transmission in cultured mammalian spinal cord neurons (Dreyer et al., 1984) and at the mammalian neuromuscular junction (Dreyer and Schmitt, 1981) seems to have subtle differences (interpreted as being due to the toxins' having separate and distinct mechanisms of inhibition). Dreyer et al. (1984) suggested that, since Tetx preferentially blocked the release of so-called large-mode m.e.p.p.s (without affecting the small-mode ones) and BoNT A strongly inhibited both types, that Tetx may act intracellularly at a stage responsible for vesicle translocation to the active zones whilst BoNT A acts at the point of vesicle fusion with the presynaptic membrane.

CHAPTER 2

Large scale purification of botulinum
neurotoxins: radioiodination and
interaction with rat brain synaptosomal
membranes

2.1 Introduction

Cultures of *Cl. botulinum* are broadly classified either in terms of the type of neurotoxin they produce or are divided into 2 groups, proteolytic or non-proteolytic. It is now more common for the former classification to be used and eight immunologically distinguishable toxin types are recognised (A, B, C₁, C₂, D, E, F and G). However, in some reports, two distinct toxin types (A and F) have been shown to be produced from a single culture (Gimenez and Ciccarelli, 1970; Sugiyama et al., 1972). This has also been shown to be the case for certain strains of type C and D cultures since type C_α cultures produce types C₁, C₂ and D toxins, with type C₁ as the dominant species; type D cultures produce the same mixture with type D dominant; and C_β cultures produce only C₂ toxin (Jansen, B.C., 1971; Eklund and Poysky, 1972). More recent work showed that the toxins produced by strains of C and D cultures are encoded by bacteriophages and that a strain producing type C toxin could be converted to producing type D (and vice versa) by cross-infection of these culture types (Eklund and Poysky, 1974). Toxin-producing cultures can be divided into 4 separate groups if a combination of the two classification schemes outlined above is adopted (Smith, L. DS, 1977; Sugiyama, 1980) : Group I cultures are proteolytic strains producing toxin types A, B or F; Group II cultures are non-proteolytic strains producing toxin types B, E or F; Group III cultures are weakly proteolytic or non-proteolytic strains that produce toxin types C₁, C₂ or D; and Group IV contains the proteolytic but non-saccharolytic strain that produces type G toxin. Except in terms of the antigenic properties of their toxins (Sakaguchi et al., 1974; Shimizu and Kondo, 1973), a culture of type B from group I is very similar to a culture of type A but differs from a type B culture from group II (Sugiyama, 1980). Miyazaki et al. (1976) have shown that not only the toxic but also the non-toxic components produced by genetically and biologically distinct organisms (producing type B toxin) are immunologically and physicochemically similar.

Many of these toxins are produced and released into the culture fluid in the form of complexes in association with other, non-toxic, proteins. In attempts to elucidate the molecule(s) responsible for the neuromuscular condition of 'botulism' these toxic complexes were isolated and were initially thought to be the purified neurotoxin. Types A and B neurotoxins are produced in association with a molecule exhibiting haemagglutinin activity (Kozaki et al., 1974; DasGupta and Boroff, 1968; Sugiyama, 1980; Simpson, 1981; Fig.1.1a), thought to protect the neurotoxic moiety from denaturation following oral ingestion (Ohishi et al., 1977). Wagman (1954) demonstrated that the haemagglutinin moiety could be removed from the toxin molecule without loss of toxicity and with an increase in the specific toxicity. Using ion-exchange chromatography on DEAE-Sephadex A50 (DasGupta et al., 1966) or DEAE-cellulose (DasGupta and Boroff, 1967, 1968) it was possible to remove this haemagglutinin contaminant during large scale purification of the neurotoxic moiety. Moberg and Sugiyama (1978) significantly improved the purification procedure by the introduction of an affinity chromatography step utilising p-aminophenyl- β -D-thiogalactopyranoside (an analogue of galactose; a potent inhibitor of haemagglutinin (DasGupta and Sugiyama, 1977)) immobilised on CH-Sepharose 4B. Large scale purification of type A BoNT was thus achieved by a combination of affinity- and ion-exchange (using DEAE-Sephacel)- chromatography (Tse et al., 1982). A modification of this procedure (Tse et al., 1982) has allowed the large scale purification, to homogeneity, of the neurotoxic moiety from a culture of type B (Danish strain; Evans et al., 1986). Toxin purified from this strain typically yielded a much lower amount of 'nicked' toxic molecules compared with that observed for another strain (Okra) producing type B BoNT (Williams, 1984).

Using a modification of the radioiodination procedure described by Greenwood et al. (1963), previously shown to be successful for the iodination of type A (Williams et al., 1983), radiolabelling of BoNT B, using ^{125}I -iodine, to high

specific activities was also accomplished (Evans et al., 1986). This radiolabelled BoNT was used to quantify the toxin acceptors present on rat synaptosomal membranes. Biochemical treatments of such membrane preparations allowed the nature of these acceptor moieties to be determined. The observed similarities and differences between these acceptors for BoNT B and those previously reported for BoNT A (Williams et al., 1983) are discussed.

2.2

MATERIALS AND METHODS

2.2.1 Large scale purification of BoNT A and B.

Cl. botulinum cultures producing type B neurotoxin (Danish strain) were grown in 20 litre incubation chambers for 48h before precipitation ^{of the supernatant} at pH 3-3.5, using 3N H₂SO₄. Acid-precipitated crude type B toxin (100-200g) was resuspended in sodium-phosphate buffer (300ml; 0.2M, pH 6.0) using a Colworth Stomacher homogeniser (Seward laboratories) and the pH was adjusted to 6.0 by the addition of 1M NaOH. The suspension was stirred for 1h at room temperature and the pH maintained at 6.0 during this time. Solid matter was removed by centrifugation (30,000 x g, 30min, 4°C) and the sediment reextracted using the above procedure. Extracts containing the toxin solution were pooled and first treated with ribonuclease (100µg/ml, 60min, 34°C) prior to the slow addition of solid ammonium sulphate to the stirred suspension (final concentration, 60% (w/v)). The toxin solution was stirred for 20min prior to centrifugation (20,000 x g, 20min). The resulting precipitate was resuspended in 60ml of sodium phosphate buffer (0.1M, pH 5.8) and dialysed against 5 litres of the same buffer overnight at 4°C. The dialysed toxin solution was centrifuged (30,000 x g, 30min), loaded onto a column of DEAE-Sephacel (10 x 2.5cm) equilibrated in sodium phosphate (0.1M, pH 5.8) and eluted with 30ml of the same buffer. This latter step removed low molecular weight, brown-pigmented contaminants which interfered with the subsequent affinity

chromatography of the toxin solution. The eluate of the latter column was allowed to run directly onto an affinity column (7 x 1.0cm) of *p*-aminophenyl- β -D-thiogalactopyranoside covalently coupled to Sepharose 4B (Moberg and Sugiyama, 1978). After transfer of the toxin to the affinity column, the latter was disconnected from the DEAE-Sephacel column. Elution of unbound protein from the affinity column was accomplished using 60ml of sodium phosphate buffer (0.1M, pH 5.8) and neurotoxin bound to the column subsequently eluted with 0.15M Tris-HCl (pH 7.9) containing 1M sodium chloride (an elution profile from these columns is shown; Fig. 2.1a). Fractions (2ml) of this eluate, having an absorbance at 280nm greater than 0.5, were pooled and dialysed overnight at 4°C against 2 x 2 litres of Tris-HCl (0.1M, pH 7.9).

Neurotoxin, in Tris-HCl buffer (0.1M, pH 7.9), was further purified by chromatography on a DEAE-Sephacel column (10 x 0.6cm) equilibrated in - and subsequently washed with 20ml of - the same solution. Neurotoxin was eluted in a step-wise manner with 30ml of Tris-HCl (0.15M, pH 7.9) containing 10mM NaCl (Fig. 2.1b) and a second protein peak, comprising both neurotoxin and other contaminating proteins, could be eluted using Tris-HCl (0.15M, pH 7.9) containing 0.5M NaCl. Those fractions containing purified neurotoxin, with an absorbance at 280nm greater than 0.5, were pooled and dialysed overnight at 4°C against Tris-HCl (0.15M, pH 7.9).

The above purification procedure was found equally applicable to the purification of type A neurotoxin (from cultures of NCTC 2916) with the only modification being in the elution of the neurotoxin from the last DEAE-Sephacel column (Tse et al., 1982; Williams et al., 1983) ; elution of pure type A neurotoxin being by the stepwise addition of 0.14M Tris-HCl, pH 7.9, to the ion-exchange column pre-equilibrated in the same buffer.

2.2.2 Analytical procedures. Purity of neurotoxin was determined at various steps in the purification procedure by sodium-dodecyl-

1

sulphate (SDS) gel electrophoresis on gradient-pore slab gels (4-30%; PAA 4/30, Pharmacia). Gels were electrophoresed at 75V for 1h prior to sample application and subsequently at 125V (at 4°C) until migration of the tracking dye off the gel, after which the voltage was lowered to 100V for a further 2h. Samples were heated (60°C, 10min) in 10mM Tris-HCl, pH 8.0/ 1mM EDTA/ 2% SDS/ 10% glycerol in the presence (reducing conditions) or absence (non-reducing conditions) of β -mercaptoethanol (5% v/v). The electrode buffer consisted of 40mM Tris, 20mM sodium acetate, pH 7.4, containing 2mM EDTA and 0.2% SDS. Following electrophoresis, gels were fixed (25% isopropanol/ 10% acetic acid), stained (0.1% Coomassie blue R250/ 25% methanol/ 10% acetic acid) and destained (25% methanol/ 10% acetic acid followed by 10% methanol/ 10% acetic acid). Molecular weights of protein samples were estimated by comparison of their mobilities with those of protein standards (thyroglobulin, 669000 and 330000; ferritin, 440000, 220000; catalase, 23200 or 60000; lactate dehydrogenase, 140000 or 36000; albumin, 67000; phosphorylase b, 94000; ovalbumin, 43000; carbonic anhydrase, 30000; trypsin inhibitor, 20100; α -lactalbumin, 14400) as detailed in the figure legends. Crossed immunoelectrophoresis was performed as detailed elsewhere (Weeke, 1973); the procedure is summarised in the legend to Fig. 2.4.

Samples for determination of toxicity were serially diluted into 70mM sodium phosphate buffer, pH 6.5, containing 0.2% gelatin. Aliquots (0.5ml) were injected intraperitoneally into groups of 4 mice ($\approx$ 20g) and the amount which killed 50% of the mice after 4 days (mouse LD₅₀) determined.*

Protein concentrations were determined colourimetrically using the Folin-phenol reagent (Lowry et al., 1951) or a Coomassie blue G-250 protein dye assay (Bradford, 1976), using bovine serum albumin as a standard.

2.2.3 Preparation of rat cerebrocortical synaptosomes. These were routinely purified from brains of female Sprague-Dawley rats ($\approx$ 200g) using discontinuous sucrose density-gradient centrifugation either a) as described by deBellerocche and

- 49 -
* - FOOTNOTE: This mouse bioassay is subject to large errors and must be treated as a purely qualitative (rather than quantitative) measure of toxin potency.

Bradford (1977) or b) using a modification of this technique (Dodd et al, 1981). In the earlier method, after dissection of the brain and removal of the white matter, the cortex was homogenised in 0.32M sucrose and centrifuged (800x g, 10min). The crude nuclear pellet (P_1) was discarded and the low speed supernatant (S_1) centrifuged (20000x g, 20min) to sediment the crude synaptosomal fraction (P_2). The P_2 was homogenised in 0.32M sucrose and layered (15ml per gradient) onto a discontinuous sucrose gradient consisting of 1.2M sucrose (20ml) and 0.8M sucrose (20ml). The gradients were centrifuged in a swing-out rotor (SW 25.2) for 60min at 60000 x g. Purified synaptosomes were collected from the 0.8-1.2M sucrose interface and diluted slowly to ≈ 0.45 M sucrose using ice-cold deionised water, before collection by centrifugation (54000 x g, 20min) and resuspension in Krebs-phosphate buffer, pH 7.4 (containing the following concentrations of salts (mM), NaCl, 124; KCl, 5; Na_2HPO_4 , 20; KH_2PO_4 , 1.2; MgSO_4 , 1.3; CaCl_2 , 0.75 and glucose, 10. All procedures were performed at 4°C.

In the modification of the above method, described by Dodd et al (1981), the low speed supernatant (S_1 ; 40ml) was layered directly onto 30ml of 1.2M sucrose and centrifuged (150,000 x g, 25min, type 45Ti rotor). The interface was collected in ≈ 12 ml, diluted to 40ml with 0.32M sucrose and layered onto 0.8M sucrose (30ml). This gradient was centrifuged as for the previous step and the pellet (representing the synaptosomal fraction) resuspended in Krebs-phosphate buffer, pH 7.4, as described above. Lysed synaptosomal preparations were obtained either by freeze-thawing or treatment of synaptosomes with 50mM Tris-HCl buffer, pH 8.0, for 30min at 4°C followed by recentrifugation and suspension in assay buffer.

2.2.4 Radiolabelling the BoNT. Type A was routinely radiolabelled with ^{125}I -iodine to high specific radioactivities (as described by Williams et al., 1983) using a modification of the Chloramine-T method (Greenwood et al., 1963). The same methodology was found to be equally effective in the radiolabelling of type B toxin to

similar (if not higher) levels of specific radioactivity (Evans et al., 1986). BoNT (A or B) (100-200 μ g) in a maximum volume of 200 μ l was added to 5mCi (50 μ l) carrier-free Na¹²⁵I (Amersham International, Amersham) and the reaction initiated by the addition of Chloramine-T (10 μ l; final concentration, 0.22mM) in 100mM sodium phosphate buffer, pH 7.4). The reaction was quenched after 40s by the addition of an excess of L-tyrosine (25 μ l; 2mg/ml in 100mM sodium phosphate buffer, pH 7.4, containing 150mM NaCl). ¹²⁵I-labelled toxin types A or B [¹²⁵I-BoNT (A) or (B) respectively] were separated from tyrosine and free iodine by gel filtration on a column (5 x 1.5cm) of superfine Sephadex G-25, pre-equilibrated with 100mM sodium phosphate buffer, pH 7.4, containing 150mM NaCl. To maximise stability of the radiolabelled toxins in dilute solution, they were diluted with 2.5% (w/v) gelatin to a final concentration of 0.25% (w/v), after removal of aliquots for determination of protein and radioactivity. The amount of free radiolabel present in a labelled toxin sample (10 μ l) was determined by precipitation with trichloroacetic acid, 10% (v/v), using bovine serum albumin (100 μ g) as a carrier protein. Protein and toxicity determinations were performed as described previously (see Methods 2.2.2).

2.2.5 Characterisation of ¹²⁵I-BoNT (A and B). Electrophoretic mobility and distribution of radioactivity in the subunits of the toxin types was determined by discontinuous SDS-gel electrophoresis (Laemmli, 1970) on a 10% polyacrylamide gel run under non-reducing and reducing (5% 2-mercaptoethanol in the sample buffer) conditions (described in Methods 2.2.2). After fixing, staining and destaining, the gels were washed extensively in deionised water, followed by immersion in 10% glycerol/ 5% polyethylene glycol (PEG; 1500 av.Mr) for 12-18hr prior to being dried and submitted to autoradiography.

2.2.6 Measurement of ^{125}I -BoNT (A and B) binding to synaptosomes. Purified synaptosomes were diluted into Krebs-phosphate buffer, pH 7.4, containing 1mg/ml bovine serum albumin (BSA) [binding buffer]. Synaptosomal suspensions (0.5-1.2 mg/ml) were incubated with increasing concentrations of ^{125}I -labelled BoNT (A or B) for 60min at 25°C followed by centrifugation (9000 x g, 2min). Aliquots were taken from the supernatants for determination of free toxin concentrations and the synaptosomal pellet was washed twice (within 20min) by sedimentation and resuspension in ice-cold binding buffer prior to γ -radiation counting. Non-specific binding was determined at various concentrations of ^{125}I -BoNT (A or B) as the amount remaining in the presence of a 100-fold molar excess of unlabelled native toxin over the highest concentration of radiolabelled toxin used. Subtraction of the non-specific binding from the total yielded the amount of toxin specifically bound.

2.2.7 Biochemical treatment of synaptosomal membranes; effect on ^{125}I -BoNT binding. Synaptosomal membranes were assessed for their capacity to bind ^{125}I -BoNT (A or B) after a variety of treatments. Basically, two rationales were employed to determine a differential effect of these treatments on the two acceptor populations previously identified by Scatchard analysis (Fig. 2.6).

Membranes (2-6 mg/ml) were incubated with increasing concentrations of trypsin, neuraminidase or wheat-germ agglutinin in the presence or absence of their specific inhibitors or subjected to heat treatment (95°C) for various times (up to 20min).

Treatments were as follows:

a) Trypsin: Aliquots of synaptosomal membrane suspensions (4-6mg/ml assay buffer) were incubated (40min, 37°C) with a series of trypsin concentrations (0-1mg/ml; final concentration). Control samples were incubated with the enzyme in the presence of a 5-fold molar excess of soybean trypsin inhibitor. At the end of the incubation period proteolysis was terminated by the addition of a

5-fold molar excess of trypsin inhibitor to each sample. Membranes were then washed once in Krebs-phosphate, pH 7.4, containing 1mg/ml trypsin inhibitor and 1mg/ml BSA, before resuspension ($\approx$ 2mg synaptosomal protein/ml) in this assay buffer.

b) Neuraminidase: Aliquots of synaptosomal membrane suspensions (2-4mg/ml assay buffer) were incubated (40min, 37°C) with increasing concentrations (0-0.5 units/ml) of neuraminidase. Control incubations also contained 20mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (a specific inhibitor of neuraminidase). After treatment, membranes were washed once in binding buffer, centrifuged and resuspended in the same buffer containing the above inhibitor.

c) Wheat-germ agglutinin (WGA): Membrane suspensions (50-100 μ g protein) were preincubated (60min, 4°C) with increasing concentrations (0-600 μ g/ml; final concentration) of WGA in the presence or absence of a 100-fold excess of its specific inhibitor N-acetylglucosamine. All incubations were diluted, sedimented and resuspended in assay buffer prior to determination of BoNT binding.

d) Heat treatment: Membrane suspensions (4-6mg/ml assay buffer) were incubated for increasing times (0-20min) at 95°C. At the appropriate time, treated samples were immediately cooled on ice. Samples were washed twice in ice-cold binding buffer prior to determination of specific ^{125} I-BoNT binding as detailed above.

BoNT binding was quantified by incubation of such treated membranes with either 1nM or 10nM ^{125} I-BoNT (final concentration) for 60min at 25°C followed by washing using the centrifugation assay described in section 2.2.6.

Saturation studies of ^{125} I-BoNT B binding to such treated membranes were also performed as described in section 2.2.6. Using the optimal conditions from the concentration dependence of the above treatments the differential effects of

trypsin (1mg/ml) and neuraminidase (1unit/ml) on binding of ^{125}I -BoNT B to the two acceptor populations was further assessed by Scatchard analysis.

2.3.

RESULTS.

2.3.1. BoNT purification and iodination.

Using a combination of ion-exchange- and affinity-chromatography, pure neurotoxin, with a molecular weight ≈ 155000 (Fig. 2.2; track 5), could be separated from a mixture of non-toxic proteins (including haemagglutinin) in a protocol that resulted in a greater yield of neurotoxin ($\approx 15\%$ recovery; Table 2.1) and in a shorter time than previously reported (DasGupta and Sugiyama, 1976; Kozaki et al., 1977). The neurotoxin obtained in this way was essentially pure as determined by SDS-gel electrophoresis on polyacrylamide gradient gels (Fig. 2.2, track 5). Further analysis of neurotoxin purity was made using crossed immuno-electrophoresis (Fig. 2.3). A single peak was observed using this technique, indicating recognition by horse antiserum (raised against a crude preparation of type B toxin) of just a single proteinaceous component and further emphasizing the homogeneity of the neurotoxin. The neurotoxin existed mainly as the unnicked molecule (only $\approx 5\%$ of the toxin was nicked; see Figs. 2.2, track 8 and 2.4, track 2) as compared with BoNT A in which the extent of nicking was 60-80% (Williams et al., 1983). Upon treatment with trypsin the intact toxin molecules could be converted to the dichain form (Fig. 2.4, track 4) with a concomitant increase in the specific toxicity (from $\approx 2 \times 10^7$ $\text{MLD}_{50}/\text{mg}$ protein to $\approx 2 \times 10^8$ $\text{MLD}_{50}/\text{mg}$ protein; Table 2.1). However, the trypsinisation conditions used to produce a significant increase in the amount of the dichain form and result in maximum toxicity also caused further proteolytic breakdown of the individual toxin chains (Fig. 2.4). This could be minimised by reduction of the incubation temperature, for trypsinisation, from

Fig. 2.1. Purification of BoNT B by affinity and ion-exchange chromatography.

(A) Chromatography of type B neurotoxin-haemagglutinin complex on DEAE-Sephacel and p-aminophenyl- β -D-thiogalactopyranoside-Sepharose 4B. The sample was applied to the DEAE-Sephacel column in 0.1M sodium phosphate buffer, pH 5.8, eluted directly onto the affinity column after which (arrow A) the former column was disconnected. Unbound material was washed from the affinity column with the above buffer and the neurotoxic material was subsequently eluted (arrow B) with 0.15M Tris/HCl buffer, pH 7.9, containing 1M NaCl.

(B) Affinity-purified material was applied to the DEAE-Sephacel column in 0.1M Tris/HCl buffer, pH 7.9, and washed with the same buffer. The neurotoxin was then eluted with 0.15M Tris/HCl buffer, pH 7.9 containing 10mM NaCl. A second protein peak could be eluted by a stepwise addition of 0.15M Tris HCl, pH 7.9, containing 0.5M NaCl (arrow A). In both A) and B); 1.5ml fractions were collected.

Fig. 2.1.

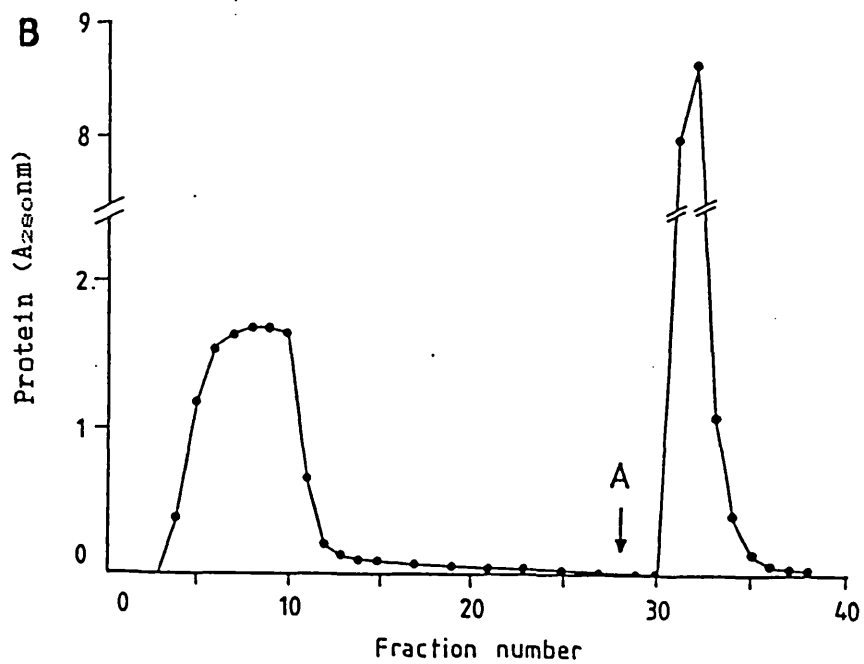
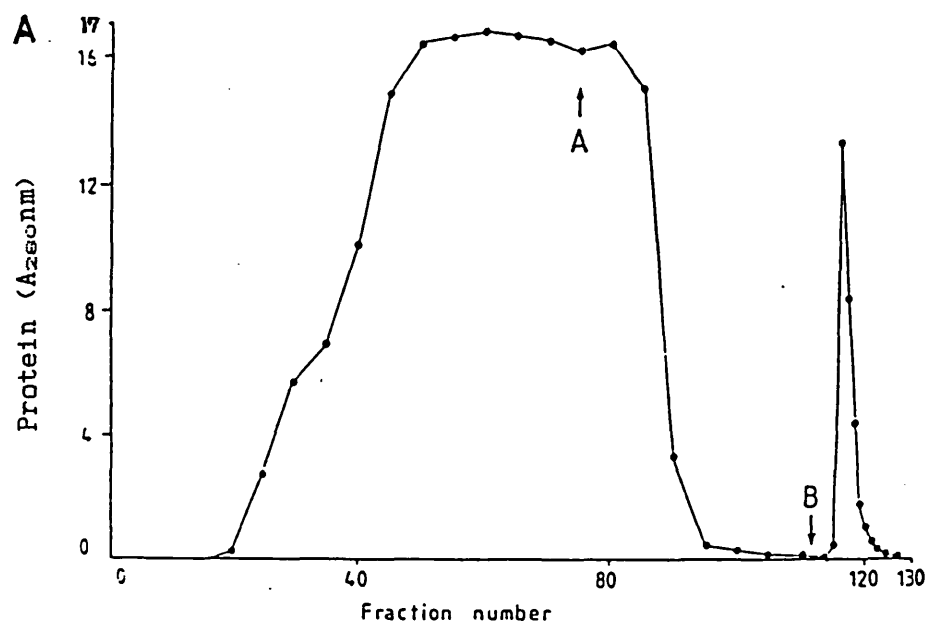
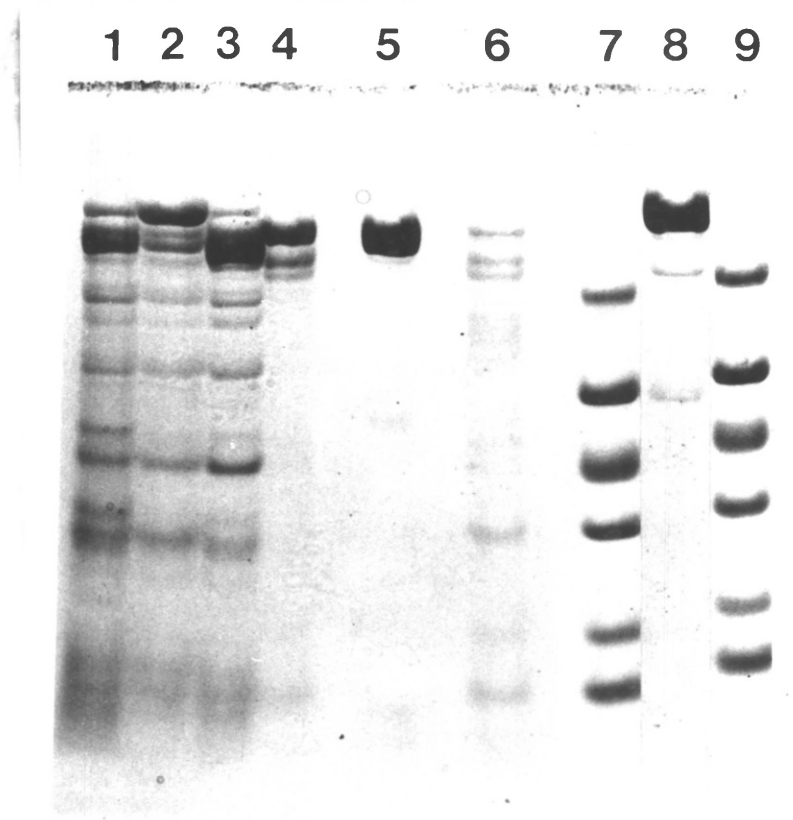


Fig. 2.2. Electrophoretic analysis of BoNT samples.



Gradient-pore gel electrophoresis was performed in 4-30% polyacrylamide gels containing SDS (as detailed in section 2.2.2). Samples were denatured by heating at 60°C for 15min in sample buffer, with and without the inclusion of reducing agent; the amount of protein applied from samples at various stages of BoNT purification ranged over 10-30µg. After electrophoresis, the gels were fixed and stained with Coomassie G-250. Samples run in the absence of reducing agent; tracks: 1, redissolved acid-precipitated material; 2, material that remained soluble in 20% $(\text{NH}_4)_2\text{SO}_4$; 3, protein precipitated with 60% $(\text{NH}_4)_2\text{SO}_4$; 4, protein eluted from the affinity column; 5, pure BoNT (first peak) eluted from the DEAE-Sephacel column ($M_r \approx 155000$); 6, material in the second peak from the DEAE-Sephacel column; 7, standard molecular weight marker proteins (Pharmacia): α -lactalbumin (M_r 14000), trypsin inhibitor (20100), carbonic anhydrase (30000), ovalbumin (43000), albumin (67000) and phosphorylase b (94000). 8, pure BoNT and 9, the above standards run under reducing conditions.

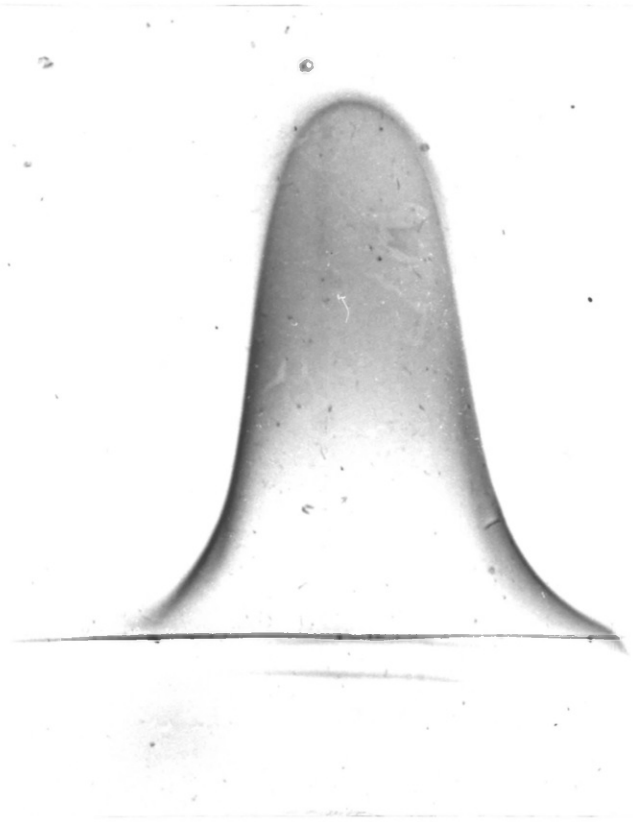
Table 2.1. Purification of *Clostridium botulinum* type B neurotoxin.

Stage	Volume (ml)	Toxicity* (mouse LD ₅₀ units/ml)	Protein (mg/ml)	Specific toxicity (LD ₅₀ units /mg protein)	Yield (%)
Acid precipitated suspension	580	4.5 x10 ⁷	n.d.	n.d.	100
Phosphate buffer (0.2M, pH6) extract	680	3.5 x10 ⁷	25.0	1.4 x10 ⁶	92
20% (NH ₄) ₂ SO ₄ supernatant	550	4.0 x10 ⁷	11.7	3.4 x10 ⁶	85
60% (NH ₄) ₂ SO ₄ precipitate	130	1.0 x10 ⁸	17.0	1.6 x10 ⁷	50
Affinity chromatography: Salt eluate	12	6.0 x10 ⁸	6.2	9.7 x10 ⁷	28
DEAE-Sephrose chromatography Peak I	13	3.0 x10 ⁸ (2.0 x10 ⁷)**	1.5	2.0 x10 ⁸	15
Peak II	6	6.0 x10 ⁷	5.0	1.2 x10 ⁷	n.d.

*Measured after trypsinisation (8µg/ml, 30min, 23°C), as described in section 2.2.2.

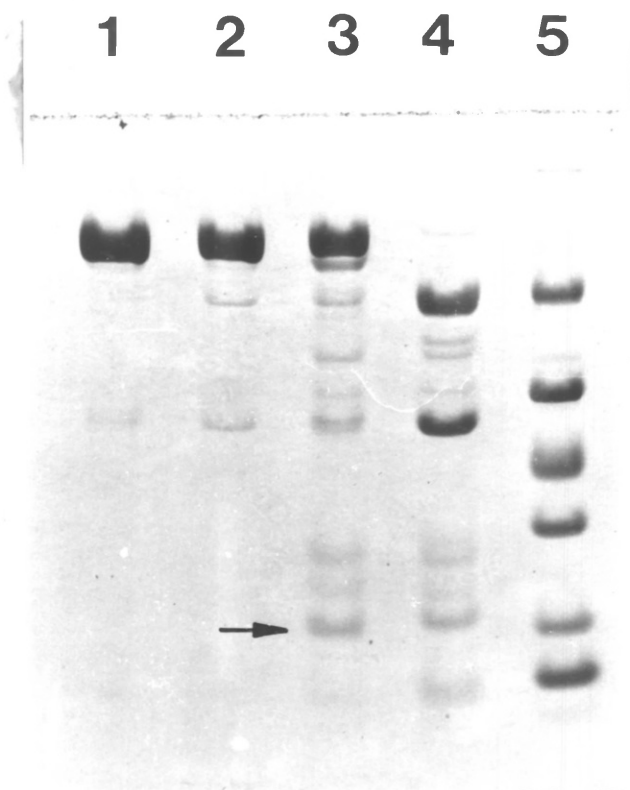
**Value observed prior to trypsinisation; although the equivalent values for three other preparations were in the range 3-5 x 10⁷ mouse LD₅₀ units/mg, the maximum level of specific toxicity after trypsinisation did not exceed 2 x 10⁸ LD₅₀ units/mg protein. n.d.= not determined.

Fig. 2.3. Crossed immunoelectrophoresis of purified BoNT B.



The sample (15 μ g) was run in the first dimension (right to left) in a 1% agarose gel at 10V/cm for 2h. The gel track was then superimposed on a 1% agarose gel containing horse antiserum (50 units/ml) raised against a crude preparation of type B toxin and electrophoresis performed at 1.5 V/cm for 16h at 10°C. After electrophoresis the gel was pressed, washed, dried, stained and destained as detailed elsewhere (Weeke, 1973).

Fig. 2.4. Trypsin treatment of BoNT B.



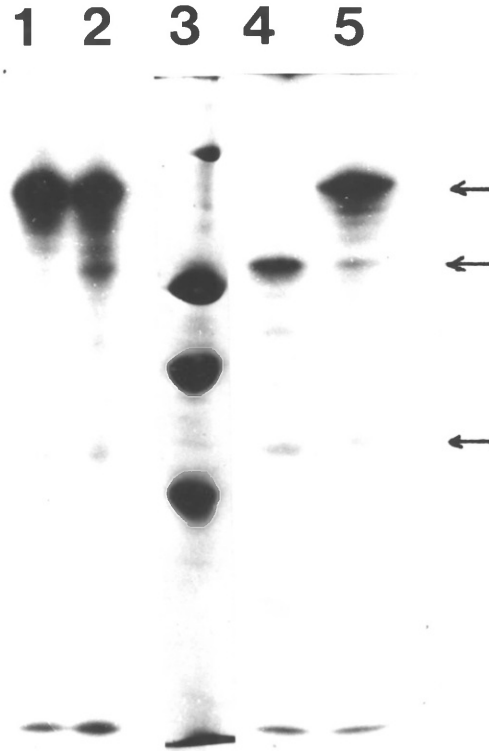
Purified BoNT B (30 μ g) was incubated (30min, 34°C) in the presence or absence of trypsin (final concentration, 8 μ g/ml). Proteolysis was inhibited by the addition of a 10-fold molar excess of soyabean trypsin inhibitor to the trypsinised samples. Aliquots ($\approx$ 10 μ g protein) were subjected to SDS gel electrophoresis on a 4-30% polyacrylamide gradient gel (Pharmacia PAA 4/30) in the presence (reducing conditions; tracks 2,4 and 5) or absence (non-reducing conditions; tracks 1 and 3) of β -mercaptoethanol (5% v/v). Bands were stained and destained as detailed in section 2.2.2. Tracks 1 and 2, BoNT B incubated in the absence of trypsin; 3 and 4, BoNT B incubated in the presence of trypsin; 5, low molecular weight protein markers (phosphorylase b, 94000; bovine serum albumin, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; soybean trypsin inhibitor, 20,100; α -lactalbumin, 14,400). The position of the trypsin inhibitor added to the treated samples is arrowed.

34°C to 23°C. Whilst the levels of toxicity expressed by BoNT B prior to trypsinisation typically varied between $\approx 1 \times 10^7$ and $\approx 1 \times 10^8$ MLD₅₀/mg protein, the maximal level observed after trypsin treatment was always around 3×10^8 MLD₅₀/mg protein.

A modification of the radioiodination procedure described by Greenwood et al. (1963), allowed labelling of BoNT A with ¹²⁵I-iodine to high specific activities (Williams et al., 1983). Application of the same protocol to purified BoNT B resulted in similar, if not higher, specific activities (500-3000 Ci/mmol) to those observed for BoNT A. Such highly labelled neurotoxin retained an appreciable proportion (>65%) of its initial toxicity demonstrating that the labelling procedure did not significantly affect its biological activity. The incorporation of the label into the subunits of type B neurotoxin was assessed by autoradiography after electrophoresis on an SDS-polyacrylamide gel. From Fig. 2.5 it can be seen that the radioiodinated toxin is homogeneous (track 1) and very few of the toxin molecules are nicked in the absence of trypsin (track 5). In order to separate the chains and determine the distribution of the label between the two subunits, the toxin was 'nicked' by incubation with trypsin (tracks 2 and 4) and the two chains separated by reduction of the interchain disulphide bond using β -mercaptoethanol (track 4). From the latter it can be seen that virtually all of the radiolabel is incorporated into the heavy chain with little/no incorporation into the light chain. Another interesting point to note is that the contaminant bands produced upon trypsinisation (c.f. Fig. 2.4, tracks 3 and 4) are not labelled.

2.3.2. Demonstration of distinct populations of acceptors for ¹²⁵I-BoNT B on rat cerebrocortical synaptosomal membranes. Binding of increasing concentrations of ¹²⁵I-BoNT B (0.05-30nM) to rat cerebrocortical synaptosomal membranes, at room temperature for 60min, showed that the extent of measurable specific binding reached a plateau at the highest concentrations used (Fig. 2.6, inset). Non-specific binding, determined as the binding remaining

Fig. 2.5. Autoradiographic distribution of radiolabel in the subunits of BoNT B (Danish strain).



Unlabelled BoNT B (30 μ g) together with aliquots of ^{125} I-labelled toxin of known specific activity were 'nicked' by incubation with trypsin (8 μ g/ml final concentration, 30min, 23°C) (Tracks 2 and 4). Proteolytic activation was inhibited by the addition of a 20-fold molar excess of soyabean trypsin inhibitor to each of the samples. Tracks 1 and 5; toxin samples treated with trypsin inactivated by preincubation with trypsin inhibitor. Samples were diluted in SDS sample buffer and electrophoresed on a polyacrylamide slab gel (10%; Laemmli, 1970) in the presence (tracks 4 and 5) or absence (tracks 1,2 and 3) of 5% (v/v) β -mercaptoethanol. Following electrophoresis, the gel was stained, with Coomassie R-250, destained, dried and exposed to autoradiographic film. Track 3, stained molecular weight protein markers: phosphorylase b (94000), albumin (67000) and ovalbumin (43000). Arrows indicate the positions of the native neurotoxin together with its constituent polypeptides.

Fig. 2.6. Equilibrium binding of ^{125}I -BoNT B to rat cerebrocortical synaptosomal membranes.

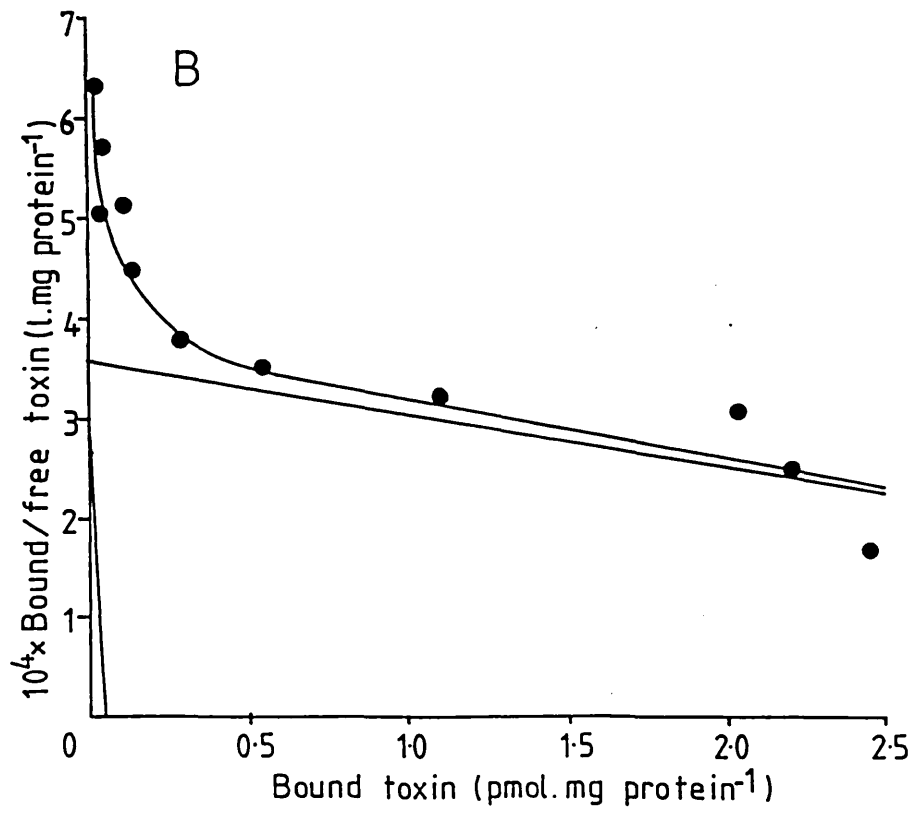
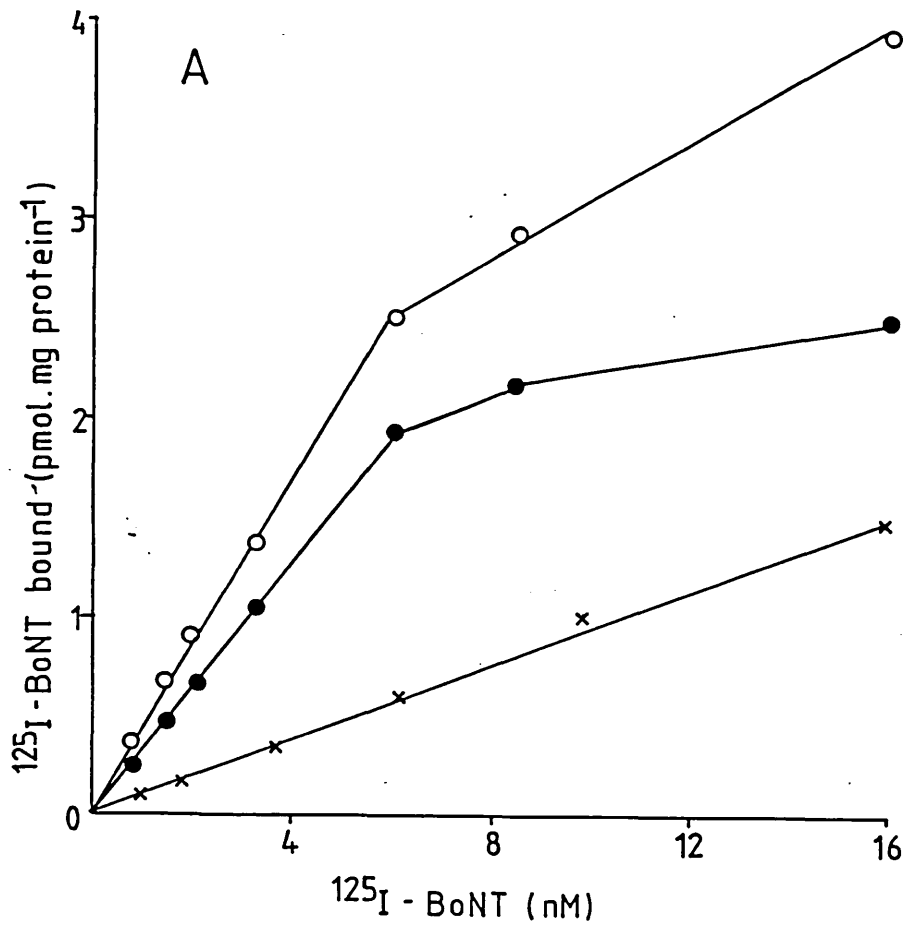
Aliquots of cerebrocortical membrane suspension (80-120 μg protein) in assay buffer (Krebs-phosphate buffer, pH 7.4, containing 1mg/ml BSA) were incubated at room temperature with increasing concentrations of ^{125}I -BoNT B (final volume 100 μl). Binding was terminated after 60min by dilution with ice-cold assay buffer, and subsequent centrifugation. The sedimented pellets were washed once at 4°C before γ -radiation counting. Non-specific binding was quantified, likewise, in control samples by inclusion of a 100-fold excess of native BoNT over the highest concentration of ^{125}I -BoNT B used. In each experiment measurements of total and non-specific binding were made in duplicate. Each of the points shown in the saturation curve (A) represent the averages of these determinations.

A) (Inset) The data is shown plotted as amount of toxin bound against the free toxin remaining in the supernatant at equilibrium. Specific binding ($\bullet$) was calculated by subtracting non-specific binding (x), from the total binding ($\circ$).

B) The data in A) above is represented in a Scatchard plot*. The plot of specific binding was non-linear and, on graphical analysis (Rosenthal, 1967), could be analysed as having high-affinity ($K_d=0.25\text{nM}$; $B_{\text{max}}=45\text{ fmol/mg}$) and low-affinity ($K_d=20\text{nM}$; $B_{\text{max}}=7\text{ pmol/mg}$) sites. Further Scatchard plots for untreated membranes are shown in Figs. 2.8 and 2.11.

* N.B. Binding of ^{125}I -BoNT B is reversible (Williams, 1984) a prerequisite for Scatchard analysis.

Fig. 2.6.



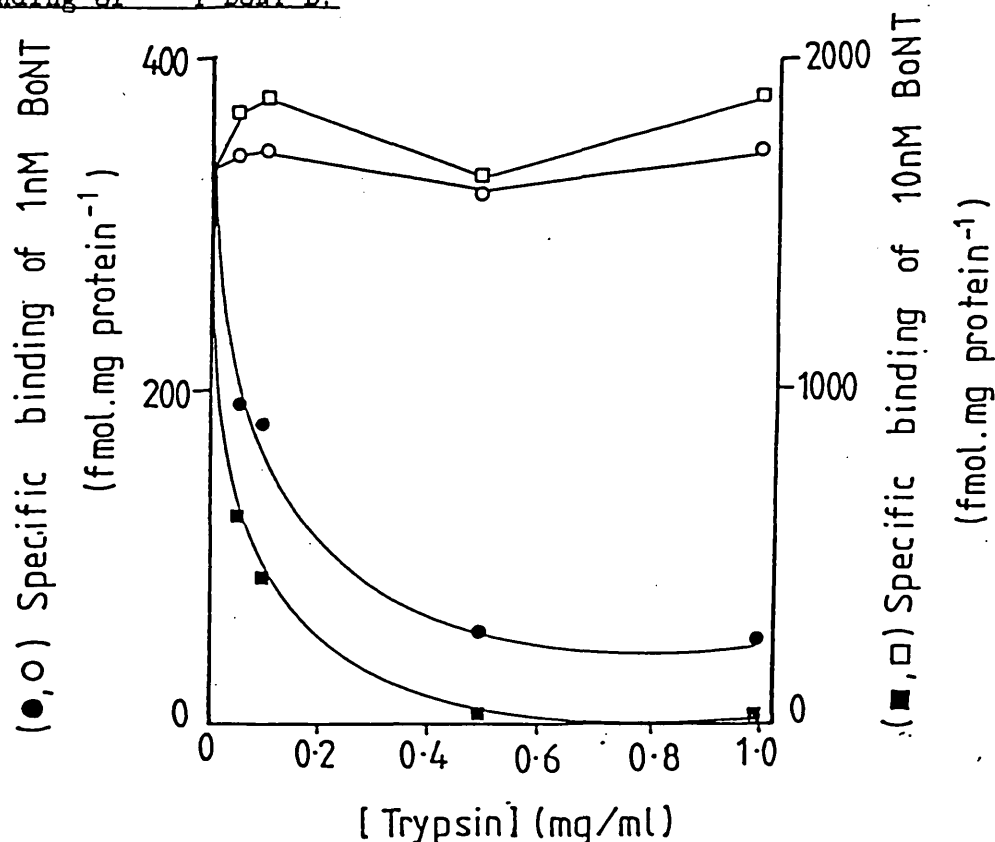
in the presence of a 100-fold excess of unlabelled BoNT over the highest concentrations of ^{125}I -BoNT used, represented an acceptably low proportion of the total, indicating the suitability of this labelled preparation for such binding studies. A Scatchard plot (Scatchard, 1949), of the specific binding curve was found to be non-linear (Fig. 2.6) and, using the method of graphical analysis (described by Rosenthal, 1967), two distinct binding components could be resolved. A low content of high-affinity acceptor sites ($K_d=0.3-0.5\text{nM}$; $B_{\text{max}}=30-60\text{ fmol/mg protein}$) could be distinguished from a much larger number of sites exhibiting lower affinity ($K_d>16-21\text{nM}$; $B_{\text{max}}\approx 7\text{ pmol/mg protein}$; in many experiments the B_{max} of the latter was sometimes as high as $10-12\text{ pmol/mg protein}$). Similar heterogeneous populations of acceptor sites were found when using either intact synaptosomes or purified synaptic plasma membranes (data not shown).

2.3.3 The nature of synaptosomal acceptors for ^{125}I -BoNT B.

Upon successful radioiodination of type B BoNT, and the demonstration of two acceptors having similar affinities to those for BoNT A (Williams et al., 1983), it was necessary to determine whether the acceptors for the two toxin types shared the same structural moieties. To characterise the differential effects of a variety of treatments (similar to those used for BoNT A; Williams et al., 1983) on the two acceptor sites, direct comparisons were made of the specific binding of ^{125}I -BoNT B to treated and untreated synaptosomal membranes using concentrations of BoNT (1nM and 10nM) which might distinguish the high- and low-affinity components.* In order to quantitate these changes to each of the two sites, saturation curves of ^{125}I -BoNT B binding were made using treated and untreated membranes in simultaneous, identical, binding assays.

* From the Scatchard plot for ^{125}I -BoNT B binding, at 1nM ^{125}I -BoNT B the occupancy of the high-affinity binding site represented $\approx 9\%$ of the total number of sites containing bound toxin at this total concentration.

Fig 2.7. Concentration dependence of trypsin on synaptosomal binding of ^{125}I -BoNT B.



Aliquots of synaptosomal membrane suspensions (4-6mg/ml assay buffer) were incubated (37°C, 40min) with increasing trypsin concentrations (●, ◻), or with trypsin in the presence of a 5-fold molar excess of soyabean trypsin inhibitor (○, ◻). At the end of this incubation period, proteolysis of the test samples was halted by the addition of a 5-fold molar excess of trypsin inhibitor to each sample. Treated membranes were then washed once in Krebs-phosphate, pH 7.4, containing 1mg/ml trypsin inhibitor, and 1mg/ml BSA before resuspension ($\approx 2\text{mg/ml}$) in this assay buffer. Membrane samples (50-100 μg) were then incubated (60min, 23°C) with either 1 or 10nM ^{125}I -BoNT B and specific binding determined by subtraction of the non-specific binding from the total as described in section 2.2.6. Specific binding of 1nM (circles) and 10nM (squares) ^{125}I -BoNT B to such treated membranes is shown. Points shown represent the average of duplicate determinations from a single experiment although similar results were obtained in at least two separate experiments.

The proteinaceous nature of the acceptors for ^{125}I -BoNT B.

2.3.3.1. Trypsin treatment of synaptosomal membranes.

Proteolysis of synaptosomal membranes using increasing concentrations of trypsin (0-1.0 mg/ml; Fig. 2.7) resulted in an extensive inactivation of the specific binding of ^{125}I -BoNT B. Whilst almost complete abolition of specific binding was observed using 10nM labelled toxin at the highest enzyme concentration, some specific binding (representing $\approx 50\text{fmol/mg}$ protein) could not be abolished when measured using 1nM ^{125}I -BoNT (even at the highest concentration of trypsin used). The specificity of the effect of trypsinisation is apparent from the ability of soybean trypsin inhibitor to prevent this inactivation (Fig. 2.7).

Using the concentration of trypsin (1mg/ml) which produced the differential reduction in ^{125}I -BoNT B binding indicated above, equilibrium binding of increasing concentrations of ^{125}I -BoNT B to treated and untreated membranes was performed in parallel and the data represented by Scatchard plots. This enabled the comparison between the affinity and contents of those acceptor sites remaining after proteolysis of the membranes to be compared to those sites in the absence of any treatment (Fig. 2.8). From such data it was found that, after enzymic digestion, two populations of acceptors for ^{125}I -BoNT B could still be distinguished [Fig. 2.8(A)]. However, whilst the content of those acceptors exhibiting low affinity for the toxin was reduced significantly ($\approx 80\%$) upon trypsin treatment ($B_{\text{max}} \approx 2\text{pmol/mg}$ protein c.f. $B_{\text{max}} \approx 10\text{pmol/mg}$ protein in the control [Fig. 2.8(B)] the content of high-affinity sites ($K_d \approx 0.3\text{nM}$) was apparently unaltered ($B_{\text{max}} \approx 60\text{fmol/mg}$ protein).

2.3.3.2. Heat treatment of synaptosomal membranes.

Membranes incubated at 95°C showed a rapid decrease with time in specific toxin binding assayed at both 1 and 10nM toxin concentrations (Fig. 2.9). Maximal inhibition of binding (at both concentrations of toxin used) occurred

Fig. 2.8. Equilibrium binding of ^{125}I -BoNT B to trypsin-treated synaptosomal membranes.

A suspension of synaptosomal membranes (4-6mg/ml Krebs assay buffer containing 1mg/ml BSA) was incubated (40min, 37°C) with trypsin (1mg/ml final concentration) prior to the addition of a 5-fold molar excess of trypsin inhibitor, centrifugation (9000 x g, 2min) and resuspension in assay buffer containing 1mg/ml trypsin inhibitor. Aliquots of treated membranes (50-100 μg) were incubated with increasing concentrations (0.02-30nM) of ^{125}I -BoNT B and specific binding determined as in Fig. 2.6a. A) Saturation curve (inset) and Scatchard plot (see Fig. 2.6) of specific binding to such treated membranes is shown. In each experiment, binding to untreated membranes was also determined in parallel (B) so that quantitative analysis of the changes in each of the acceptor types could be made.

	High affinity site		Low affinity site	
	B_{max}	Kd	B_{max}	Kd
Trypsin treated	65fmol/mg	0.26nM	2pmol/mg	6.3nM
Untreated	80fmol/mg	0.3nM	10pmol/mg	16nM

Fig. 2.8.

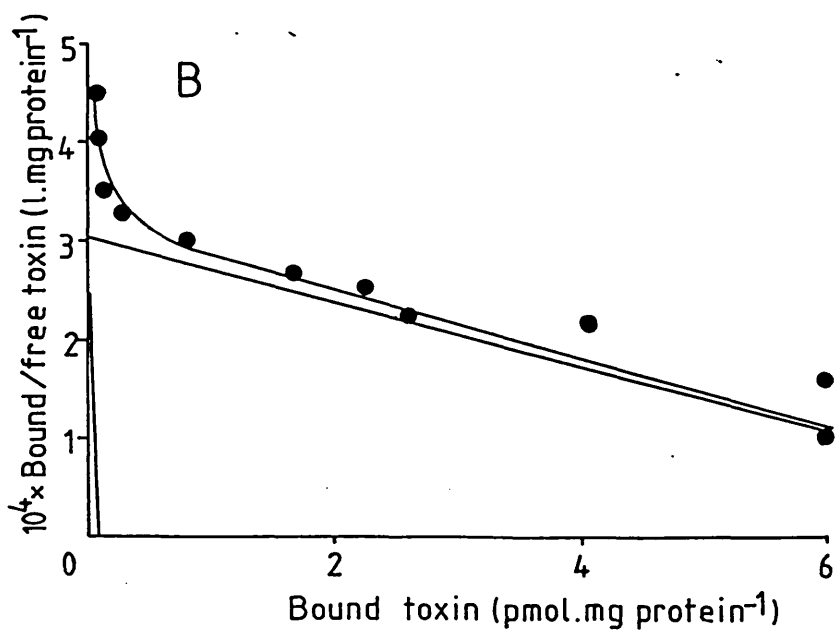
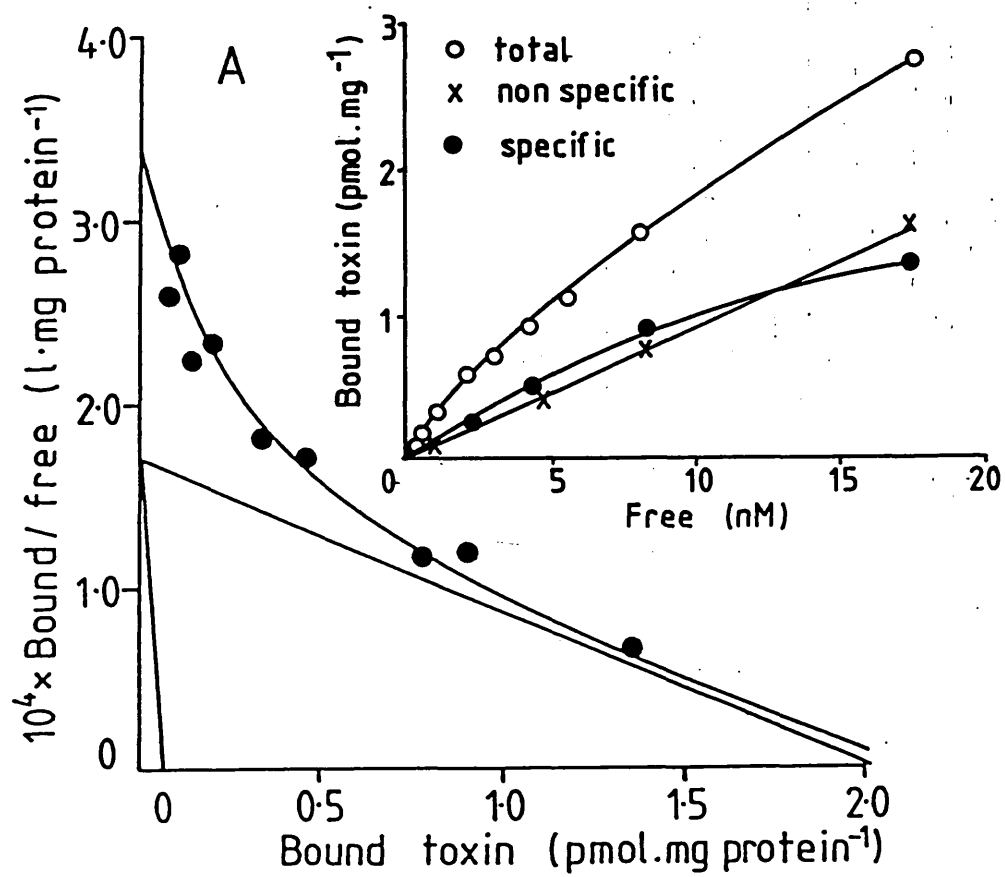
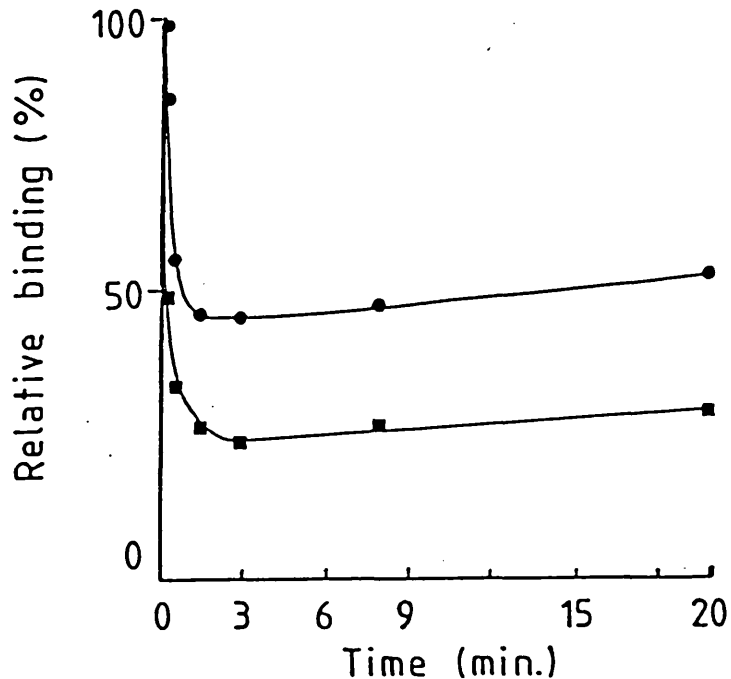


Fig. 2.9. Specific binding of ^{125}I -BoNT B to heat treated synaptosomal membranes.



Aliquots of synaptosomal membrane suspensions (4-6mg/ml assay buffer) were incubated for varying times at 95°C. Treated samples were cooled on ice, washed twice in ice-cold Krebs-phosphate binding buffer (pH 7.4) containing 1mg/ml BSA and resuspended (at $\approx 2\text{mg/ml}$) in the same buffer. Binding of ^{125}I -BoNT B to such treated aliquots was determined by incubation of membrane samples (50-100 μg) with 1 or 10nM labelled toxin (60min, 23°C - as described in section 2.2.6). Non-specific binding, defined as that remaining upon incubation with a 100-fold molar excess of unlabelled toxin over that of the labelled toxin, was subtracted from the total to give the specific binding capacities of such membranes at 1nM (●) and 10nM (■) ^{125}I -BoNT B is shown. Values are expressed as a percentage of the control (100%) value- determined as the specific binding to untreated membranes at each concentration of toxin studied. Points shown are the means of 6 determinations (SEM < 5%).

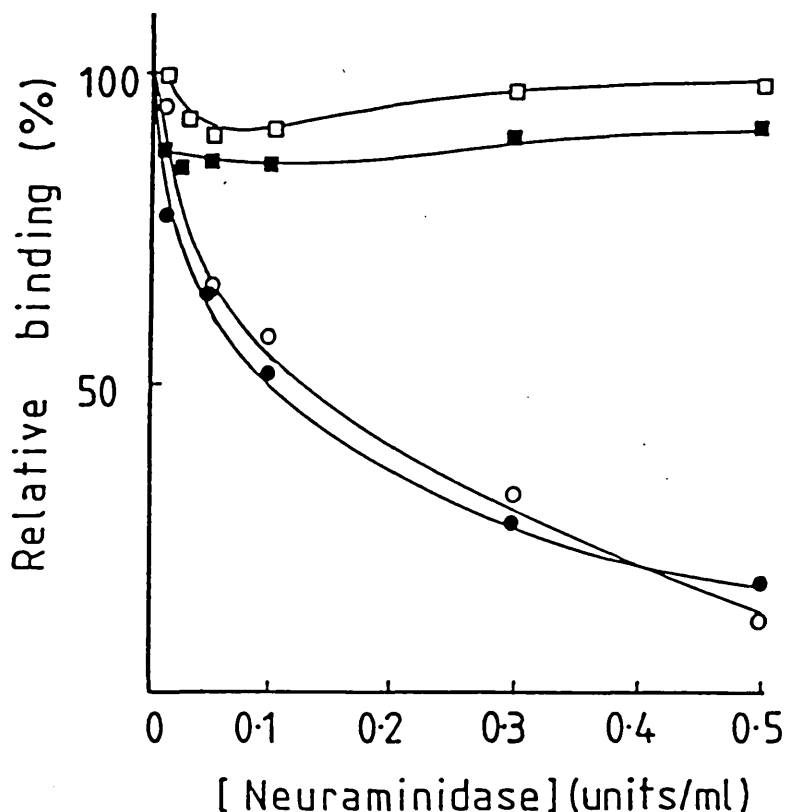
after incubation of the membranes for just 3min at 95°C. However, unlike the results obtained with trypsin treatment of synaptosomal membranes (Fig. 2.7) complete abolition of specific binding using 10nM ^{125}I -BoNT B was not found. In fact, in membranes heated for >3min, the level of specific binding apparently increased due to a decrease in the non-specific binding determined at each time point (Fig. 2.9).

2.3.4. Sialic acid moieties in the acceptors for ^{125}I -BoNT B.

Since the presence of N-acetylneuraminic acid residues on the acceptors for BoNT-A had previously been implicated (Williams et al., 1983), their involvement in the acceptor binding of ^{125}I -BoNT B was also assessed by preincubation of synaptosomal membranes with increasing concentrations of Neuraminidase (E.C.3.2.1.18) or WGA. Binding of ^{125}I -BoNT B to such treated membranes was carried out using concentrations of ^{125}I -BoNT which would distinguish the high- and low-affinity binding sites (see figure legends). Increasing neuraminidase concentrations caused a decrease in the observed specific binding of ^{125}I -BoNT B at both concentrations of toxin used and this reduction was maximal at 0.5 unit/ml neuraminidase (Fig. 2.10). This is a specific effect of the enzyme and not due to protease contamination since incubation of membranes with neuraminidase in the presence of its specific inhibitor (2,3 dehydro-2-deoxy-N-acetylneuraminic acid) negated this effect (Fig. 2.10).

In order to further assess the nature of the effect of the neuraminidase on the two acceptor populations for ^{125}I -BoNT B, synaptosomal membranes were incubated with the enzyme (1 unit/ml) for 40min at 37°C and the reaction terminated by addition of 20mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid, centrifugation and resuspension of the membranes in assay buffer containing this inhibitor. Total and non-specific binding of ^{125}I -BoNT were quantified as detailed earlier (see section 2.2.6) and a Scatchard plot of the specific binding was constructed [Fig. 2.11(A)]. Graphical analysis of the data yielded B_{max} values of 15 and 1850 fmol/mg of protein, respectively, for the two sets of

Fig. 2.10. Concentration dependence of neuraminidase on synaptosomal binding of ^{125}I -BoNT B.



Aliquots of synaptosomal membrane suspensions (2-4mg/ml assay buffer) were incubated (37°C, 40min) with increasing concentrations of neuraminidase (Sigma; E.C.3.2.1.18) in the presence (controls; squares) or absence (tests; circles) of 20mM 2,3 dehydro-2-deoxy-N-acetylneuraminic acid (a specific inhibitor of neuraminidase). Membranes were washed once, centrifuged (9000 x g, 2min) and resuspended ($\approx$ 2mg synaptosomal protein/ml) in assay buffer containing the above inhibitor. Aliquots of these treated membrane suspensions were then incubated (60min, 23°C) with 1 or 10nM ^{125}I -BoNT B and specific binding determined as detailed in section 2.2.6. using the standard centrifugation assay. The effects of neuraminidase on specific binding of 1nM (●, ■) and 10nM (○, □) ^{125}I -BoNT B are shown. Values are expressed as a percentage of the specific binding observed on untreated membranes. Points represent the means of duplicate determinations although similar results were obtained using 0.1, 1, 5 and 10nM ^{125}I -BoNT B.

Fig. 2.11. Equilibrium binding of ^{125}I -BoNT B to neuraminidase-treated synaptosomal membranes.

After incubation of synaptosomal membranes with neuraminidase (1 unit/ml) for 40min at 37°C, the reaction was stopped by addition of 20mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid, centrifugation and resuspension of the membranes in assay buffer containing the inhibitor. Total and non-specific binding of ^{125}I -BoNT B were quantified, as detailed in section 2.2.6. and a Scatchard plot of the specific binding to treated membranes is shown (A). Graphical analysis of the data yielded B_{max} values of 15 and 1850 fmol/mg protein, respectively, for the two sets of sites compared with 60 and 6900 fmol/mg protein for binding of ^{125}I -BoNT B to untreated membranes in this particular experiment (B). Apparently their K_d values were unaltered by the enzymic digestion.

Fig. 2.11.

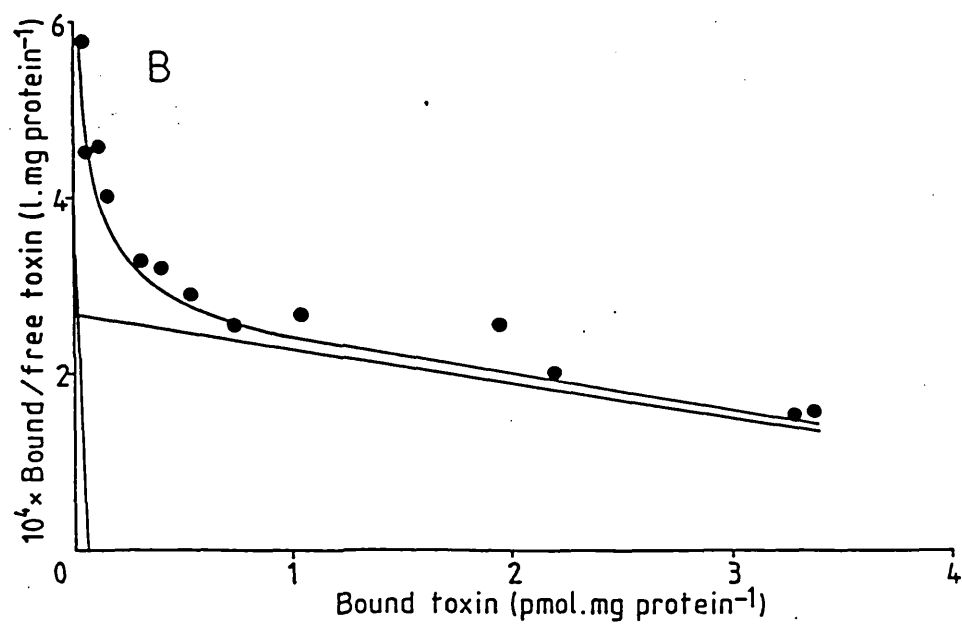
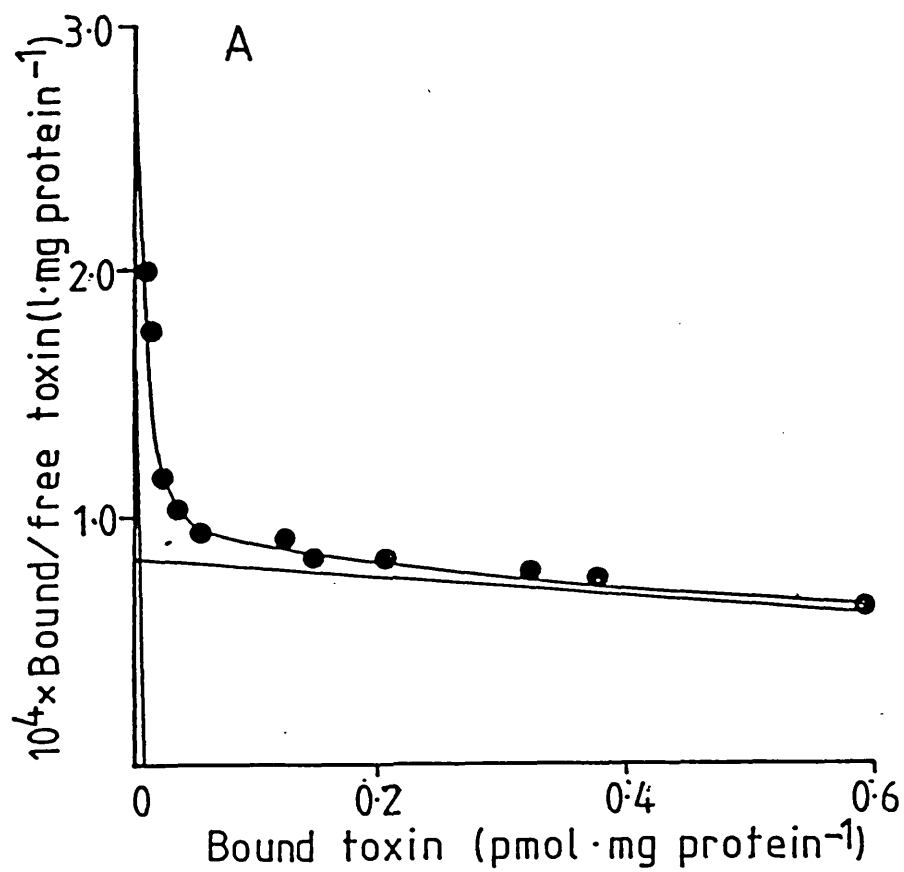
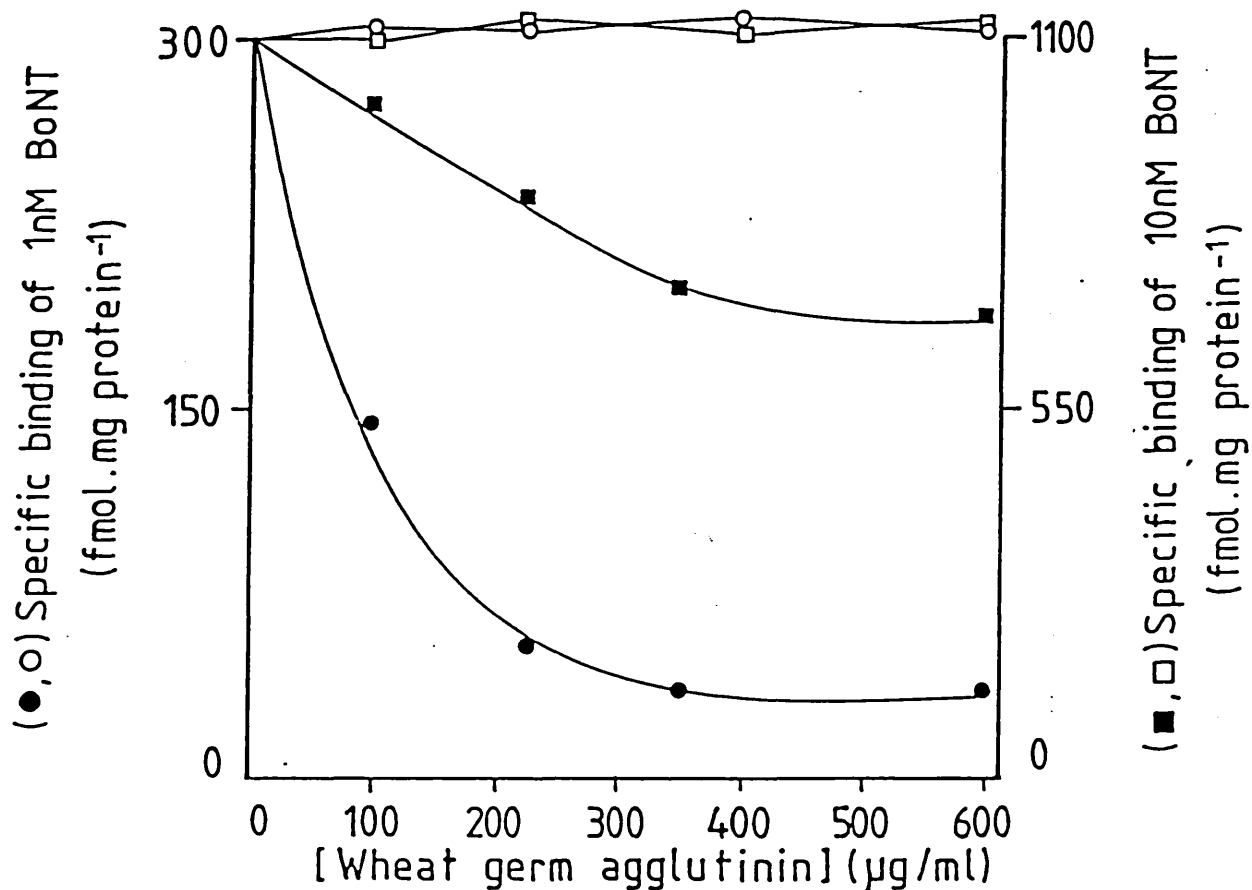


Fig. 2.12. Effect of wheat-germ agglutinin on ^{125}I -BoNT B binding to synaptosomal acceptors.



Membrane suspensions (50-100µg of protein/incubation) in assay buffer (see section 2.2.6) were preincubated for 60min with varying concentrations of wheat-germ agglutinin at 4°C in the presence (○, □) or absence (●, ■) of a 100-fold excess of its specific inhibitor N-acetylglucosamine. Control incubations were carried out in a similar manner but without any additions to the membranes. All the incubations were then diluted with assay buffer, sedimented and resuspended. Following addition of 1nM (○, ●) or 10nM (□, ■) ^{125}I -BoNT B and incubation at 37°C for 40min, the amount of specific toxin binding to treated and untreated samples were determined.

sites, compared with 60 and 6900 fmol/mg protein for the untreated samples in this particular experiment [Fig. 2.11(B)]. Apparently, no significant changes in the K_d values of the remaining sites, after enzymic digestion, were observed. This result would seem to indicate that both the high- and the low-affinity binding components for BoNT B are equally susceptible to neuraminidase treatment and implies a significant role in toxin binding for N-acetylneuraminic acid in both receptor types.

WGA was found to compete effectively for toxin binding and, interestingly, the extent of this inhibition was more pronounced with 1nM rather than 10nM ^{125}I -BoNT (Fig. 2.12). The specificity of the lectin interaction with the acceptors was ascertained from its inhibition by N-acetylglucosamine (Fig. 2.12). The observed inhibitory action of this lectin which binds a number of sugar molecules, including N-acetylglucosamine and N-acetylneuraminic acid (Monsigny et al., 1980), implicates such moieties in toxin binding by these acceptors. Other lectins tested (concanavalin A and soybean agglutinin) had no significant effect on toxin binding to membranes (Williams, R.S. Ph.D. thesis, London, 1984).

It would appear, from the above observations, that there exist two distinct populations of acceptors for type B BoNT; a high-affinity component which is resistant to proteolytic digestion and heat treatment and a site (or sites) of lower affinity which is sensitive to such treatments. Both types of site are sensitive to removal of sialic acid moieties or blocking of sugar residues.

2.3.5 Scatchard analysis of ^{125}I -BoNT binding under different incubation conditions. In order to study further the molecular nature of the BoNT acceptors and their interactions with the toxin, a variety of treatments were used in which the pH and/or the ionic strength of the incubation media were changed. A comparison in the B_{max} and K_d values of the remaining sites with those observed in Krebs-phosphate, pH 7.4 (c.f. Fig. 2.6) was made

by analysis of Scatchard plots of the data. The following incubation conditions using labelled toxin on rat brain synaptosomal membranes have been studied (all buffers contained 1mg/ml BSA);

(1) Low ionic strength Krebs buffer (in which the NaCl was omitted), pH 7.4

(2) Low pH buffer (25mM sodium acetate), pH 6.0, containing 150mM NaCl.

(3) Low ionic strength, low pH buffer (25mM sodium acetate, pH 6.0)

No differences in the profiles of either BoNT A or BoNT B binding (expressed graphically on Scatchard plots) were observed between (1) and (2) above and that seen in normal ionic strength Krebs-phosphate buffer, pH 7.4, containing 1mg/ml BSA (data not shown). This would suggest that the apparent affinities and contents of the high- and the low- affinity binding components for this toxin are not altered by such treatments. In contrast, however, a marked difference in the toxin-receptor binding was noted under the conditions of low pH and low ionic strength indicated in (3) above (c.f. that observed in Krebs-phosphate, pH 7.4).

Under these conditions, Scatchard analysis of the specific binding of increasing concentrations of ^{125}I -BoNT B indicated the presence of a large content ($B_{\text{max}} \approx 35$ pmol/mg protein) of a single population of acceptors exhibiting a moderately high affinity for the toxin [$K_d = 7\text{nM}$; Fig. 2.13(B)]. These sites are apparently in far greater abundance than those observed under 'physiological' binding conditions where a low number of high-affinity sites ($B_{\text{max}} \approx 50\text{fmol/mg}$ protein; $K_d = 0.3\text{nM}$) and a larger population ($B_{\text{max}} \approx 7\text{pmol/mg}$ protein) of low-affinity acceptor sites can be resolved (Fig. 2.6; Evans et al., 1986). Similarly, equilibrium binding studies using ^{125}I -BoNT A under these incubation conditions (low ionic strength, low pH) also revealed an apparent single population of acceptors ($K_d \approx 4\text{nM}$) present in large quantities ($B_{\text{max}} \approx 24\text{pmol/mg}$ protein) [Fig. 2.13(A)].

Fig. 2.13. Equilibrium binding of ^{125}I -BoNT under conditions of low ionic strength and low pH.

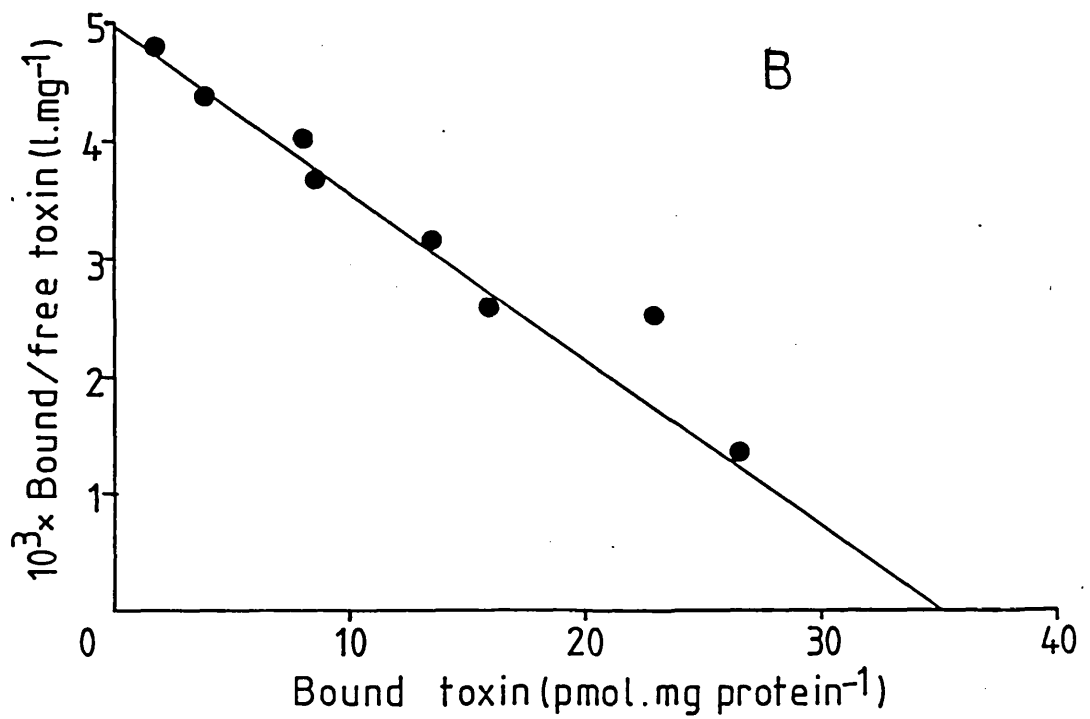
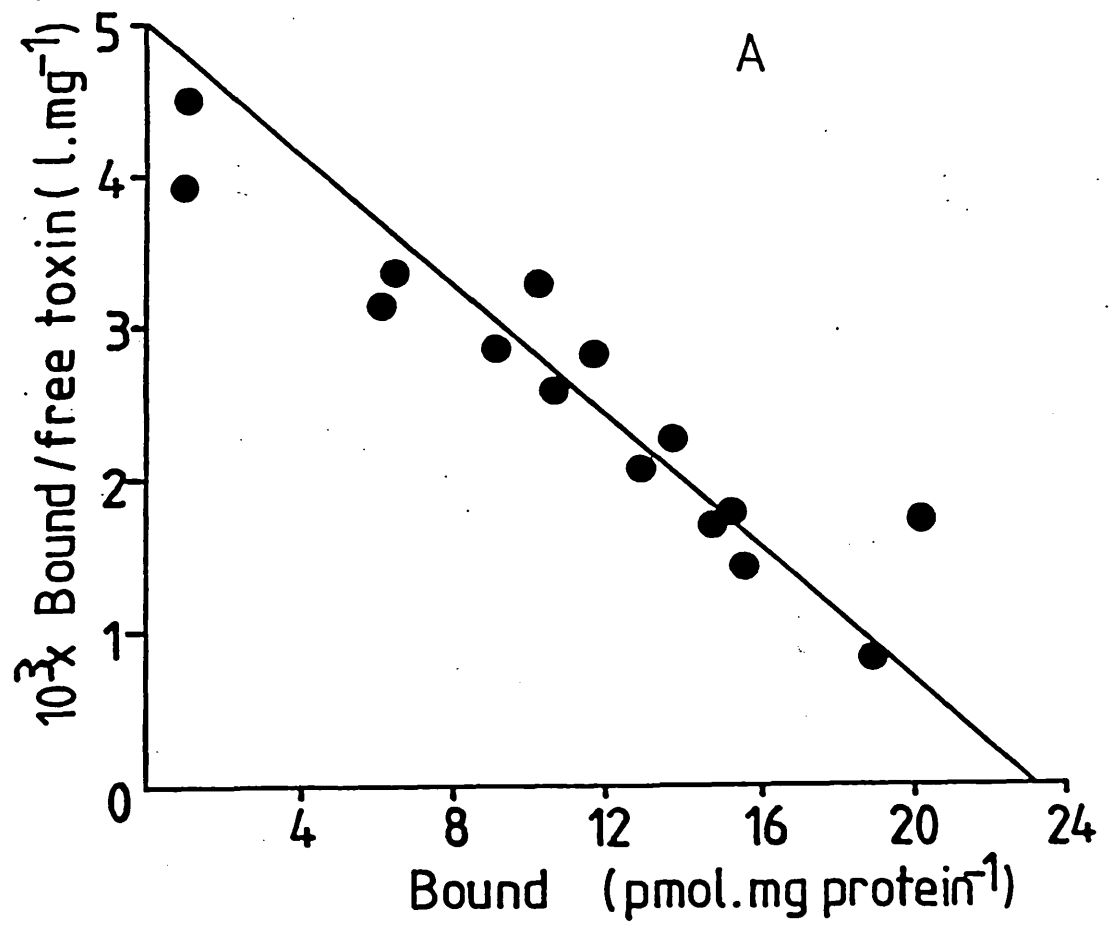
Aliquots ($\approx 100\mu\text{g}$ protein) of rat synaptosomal membranes were incubated (23°C , 60min) with increasing concentrations (0.02-30nM) of ^{125}I -BoNT (A or B) in 25mM sodium acetate, pH 6.0, containing 1mg/ml BSA (final volume 100 μl). Non-specific binding was determined in control samples by inclusion of a 100-fold excess of native BoNT over the highest concentration of labelled toxin used. After incubation, the membranes were pelleted, aliquots removed from the supernatant to determine the amount of unbound toxin and subsequently washed (2 x 1ml) in the same buffer at 4°C using the centrifugation assay described (section 2.2.6). Specific binding was quantified by γ -radiation counting as detailed in section 2.2.6.

The data is expressed graphically as a Scatchard plot.

A) Equilibrium binding of ^{125}I -BoNT A under the above conditions.
($B_{\text{max}} \approx 24\text{pmol/mg protein}$; $K_d = 4.4\text{nM}$)

B) Equilibrium binding of ^{125}I -BoNT B under the above conditions.
($B_{\text{max}} \approx 35\text{pmol/mg protein}$; $K_d \approx 7\text{nM}$)

Fig. 2.13.



2.4 Discussion

2.4.1. Purification and radioiodination of BoNT B.

A slight modification of a protocol, previously developed to purify type A BoNT to homogeneity (Tse et al., 1982), has proved to be equally applicable in the large scale purification of BoNT B from a culture of *Cl. botulinum* (Danish strain; Evans et al., 1986). The neurotoxin obtained in this way was shown to be homogeneous by a number of criteria (including SDS-gel electrophoresis and crossed immunoelectrophoresis) and could be obtained more rapidly (typically ≈ 72 h) and in greater yield ($\approx 15\%$) than previously reported (DasGupta and Sugiyama, 1976; Kozaki et al., 1977). The purified neurotoxin was of similar molecular weight (≈ 155000) to that published for other strains of type B (150000, Kozaki et al., 1974; 163000, Ohishi and Sakaguchi, 1977; 167000, Beers and Reich, 1969; 170000, Kozaki and Sakaguchi, 1975). Likewise, upon nicking (using trypsin) and reduction, the molecular weights of the toxin's two subunits (determined by polyacrylamide gel electrophoresis) were similar to those indicated by the latter authors. Since trypsin treatment of the native toxin produces the dichain form with a concomitant increase in specific neurotoxicity whilst also causing further proteolytic breakdown, it may be that, as seen with another strain of type B toxin (Okra; Ohishi and Sakaguchi, 1977), activation of type B from Danish strain may precede nicking of the molecule.

This species of neurotoxin could be radioiodinated to higher specific activities (up to 3000Ci/mmol) than previously reported for other toxins [e.g. type A, 1750 Ci/mmol; Williams et al., 1983; type B (Okra), 300Ci/mmol; Kozaki, 1979; types C and D, 600Ci/mmol; Murayama et al., 1984 or type E, ≈ 100 Ci/mmol; Kozaki and Sakaguchi, 1982]. The specific activity of the labelled toxin was accurately determined to allow quantitative analysis of toxin binding to neuronal membranes. Such highly labelled toxin retained an appreciable proportion (65-90%) of the specific neurotoxicity of the native toxin implicating that the residues which are

labelled are not essential for toxicity. The remaining toxicity of the labelled neurotoxin would not appear to be due to unlabelled toxin since appreciable toxicity of labelled BoNT was retained at specific activities »2000 Ci/mmol, representing an equimolar ratio of ^{125}I -iodine to protein. One might thus expect the majority of labelled toxin to be the mono-iodinated species with a much smaller number of molecules containing doubly or higher labelled species. Previous attempts to separate the various iodinated species of type A BoNT from the unlabelled toxin (using narrow range IEF, chromatofocussing and ion-exchange chromatography) proved unsuccessful (Williams R.S., Ph.D. thesis, London 1984). Some inherent microheterogeneity may exist in the mono-iodinated toxin (since it was shown that either of the subunits of BoNT A may be labelled) and this may affect the toxin binding characteristics to neuronal membranes. However, in the case of type B BoNT, the problems associated with microheterogeneity of the iodinated species were not significant since labelling was found primarily in the heavy chain of the toxin. This difference in the distribution of ^{125}I -iodine label between the subunits of types A and B BoNT may be due to the fact that the light chain from type B BoNT contains fewer residues which are accessible to the ^{125}I substitution i.e. tyrosine and histidine residues. In fact amino acid compositional analysis of the light chains of the two toxin types shows that they both contain similar proportions of tyrosine residues (25 residues/mol; 5.2% of total residues for BoNT A compared with 22 residues/mol; 4.9% of total residues for BoNT B light chain; Williams, R.S., 1984). Thus, the lack of iodination in the light chain of BoNT B may be due to a conformational difference in the toxin structures such that those residues which would be labelled in the light chain of BoNT B are 'protected' in some way whilst some of those in type A are exposed.

2.4.2. BoNT acceptors.

Identification of the nature of the presynaptic acceptor sites for BoNT may prove of interest since, it is not known whether these moieties play a functional role in neuronal communication (e.g. possibly in cell-cell recognition during synapse formation or in maintaining synaptic contact). Furthermore, due to the inaccessibility of the peripheral nervous system (the primary site of toxin action) to biochemical analysis, it is important to use other model systems e.g. synaptosomes, in an attempt to elucidate the mechanism of toxin action. Hopefully the acceptors demonstrated in the CNS will be similar/identical to those shown to be responsible for binding and energy-dependent internalisation of the toxin in the PNS (Black and Dolly, 1986a, b).

Saturation binding studies using BoNT A revealed biphasic Scatchard plots which could be resolved by graphical analysis (Rosenthal, 1967) to reveal the presence of at least two acceptor sites; a low content of acceptors exhibiting high affinity ($K_d=0.6\text{nM}$) and a much larger number of acceptors having lower affinity ($K_d\approx 25\text{nM}$) for the toxin (Williams et al., 1983). Studies, using radiolabelled BoNTs C₁ and D have also revealed heterogeneous binding components for these toxins [a small pool of high-affinity sites (K_d 0.08nM and 0.023nM respectively) together with a much larger pool of acceptors of lower affinity ($K_d\approx 35\text{nM}$; Agui et al., 1983, 1985; Murayama et al., 1984)]. However, it is questionable whether specific binding of the low concentrations of toxin required to distinguish such high affinity components could be measured or these sites resolved from the large content of low-affinity acceptors. In contrast, a study of ^{125}I -BoNT E binding to mouse brain synaptosomes indicated that the latter recognised only a single component of very low affinity ($K_d\approx 100\text{nM}$; Kozaki and Sakaguchi, 1982). However, the lowest concentration of labelled ligand used was $\approx 10\text{nM}$ and hence the latter authors may have failed to detect a site exhibiting higher affinity for this toxin type which may have been saturated at this relatively high concentration of toxin. Binding of BoNT A, B, C₁

and D, appears to be mediated by their respective heavy chains (Williams et al., 1983; Kozaki, 1979; Murayama et al., 1984) since large concentrations of the latter effectively inhibit binding of the intact toxin to synaptosomal membranes. The light chain of these preparations also caused a small reduction in toxin binding but this was thought to be due to contamination with native BoNT (Williams et al., 1983; Kozaki, 1979; Murayama et al., 1984).

2.4.2.1. The nature of the acceptors for BoNT B.

In this report and elsewhere (Evans et al., 1986) we have similarly demonstrated the existence of at least two populations of acceptor sites for another botulinum toxin - type B; a high-affinity component ($K_d \approx 0.3-0.5 \text{ nM}$) present in low numbers (B_{max} 60-100 fmol/mg protein) together with a much larger population ($B_{max} \approx 7 \text{ pmol/mg protein}$) of sites exhibiting lower affinity for the toxin ($K_d \approx 16-20 \text{ nM}$). In order to try and identify the chemical nature of these acceptor sites in synaptosomal membranes, their differential sensitivities were assessed by measuring the remaining toxin binding activity after a variety of treatments; two concentrations of toxin were employed which would primarily differentiate between the high- and the low-affinity components. Further studies were then made using the optimum incubation conditions for synaptosomal membranes to cause a significant reduction in the number of binding sites. Equilibrium binding studies were performed on such treated membranes using increasing concentrations of labelled BoNT and the results expressed as Scatchard plots (Scatchard, 1949). Graphical analysis of such data (Rosenthal, 1967) allowed the comparison between the affinity and content of the remaining sites with those in the control sample (measured during each experiment). Due to the inherent inaccuracies in such analysis (see section 2.4.3) these experiments were repeated two to three times in order to allow a more accurate comparison between the differential effects observed on the two sites.

The data obtained indicated a preferential sensitivity of the low affinity component to heat and trypsin, with such

treatment of synaptosomal membranes resulting in a decrease in the content of such sites (up to 80%) with no apparent decrease in the content or affinity of the remaining sites for ^{125}I -BoNT B. In contrast, both sites appeared equally sensitive to treatment with neuraminidase with a reduction of 50% in the numbers of both acceptor populations and no apparent changes in their respective K_d values. As in the case of trypsin; this was again due to the specific action of the neuraminidase and not contaminating protease since inclusion of 2,3-dehydro-2-deoxy-N-acetylneuraminic acid negated this effect. WGA is a protein with a molecular weight of 36000 composed of two polypeptide chains each possessing two binding sites for sugar residues (Nagata and Burger, 1974). This lectin shows a marked specificity for binding non-reducing terminal N-acetylneuraminic acid residues as well as internal N-acetylglucosamine residues (Bhavanandan and Katlic, 1979; Monsigny et al., 1980). WGA produced a marked reduction in ^{125}I -BoNT B binding when preincubated with synaptosomal membranes; N-acetylglucosamine or N-acetylneuraminic acid antagonised this effect. Thus, it would appear from this collective data that the the two acceptor sites for BoNT B contain sialic acid moieties and, whilst the low affinity acceptor appears to involve a proteinaceous component, the high-affinity site is resistant to proteolytic digestion or denaturation, suggesting that this component may be a ganglioside.

Binding of ^{125}I -BoNT B under physiological conditions was partially inhibited ($\approx 40\%$) by high concentrations ($\approx 1\mu\text{M}$) of BoNT A (Kozaki, 1979; Evans et al., 1986). Scatchard analysis of binding of ^{125}I -BoNT B in the presence of a large concentration of type A in each incubation revealed that this partial inhibition by BoNT A was not due to competition with the high-affinity acceptor for ^{125}I -BoNT B but rather with an acceptor component exhibiting extremely low affinity for the latter (data not shown). This result suggests that the low affinity 'acceptor' for BoNT B may comprise a large number of different components exhibiting a wide range of affinities for the toxin which cannot be resolved by the normal technique of Scatchard analysis.

2.4.3. Scatchard analysis; critical appraisal.

Some of the above implications are based on values obtained from graphical analysis of Scatchard plots and, as such, must be treated with some caution since there are many problems inherent with this method. The principle source of errors arises from incomplete separation of bound from free ligand (Klotz, 1982; Burgisser, 1984); failure to do so results in an underestimation of specific binding and gives rise to errors in the determination of the dissociation equilibrium constants (Bonafacino and Paladini, 1981). Such error was minimised since, using the centrifugation assay indicated, an aliquot could be removed to determine the concentration of free toxin whilst still at equilibrium and rapid washing at 4°C allowed the remaining free labelled ligand to be removed effectively with minimal displacement of any bound toxin. To determine accurately the concentrations of bound and free toxin and, hence, gain accurate values for the K_d and B_{max} of a binding site, the specific activity of the radiolabelled ligand must be accurately determined (Munson, 1984). This was in fact the case for each batch of iodinated toxin. Klotz (1982) and Burgisser (1984) point out that errors in obtaining parameters from Scatchard analysis arise from a) too low a ligand concentration range, or b) artifacts such as improper subtraction of the background radioactivity or the non-specific binding. A suitably large toxin concentration range (0.02-30nM) within the realms of practicality, was chosen and care was taken to ensure that the background radioactivity did not account for a significant proportion (>1%) of the total counts even at the lowest concentrations of toxin used. A 100-fold excess of unlabelled toxin over the highest concentration of iodinated ligand was used to determine the non-specific binding. Increased levels of unlabelled ligand were not used due to the possibility that these high concentrations of inhibitor may start competing for specific toxin binding to components exhibiting low affinity (Chamness and McGuire, 1975).

From semi-log plots ([bound] vs log[free]) it has been shown that, even at the highest concentrations of labelled toxin

used (30nM), complete saturation of all binding sites on untreated synaptosomal membranes was not achieved. However, estimates for the low affinity binding sites for BoNT B, from graphical analysis of Scatchard plots, gave K_d values of 16-25nM. In order to achieve 90% occupancy of receptor sites, a radioligand concentration 9-10 fold higher than the K_d value is required (Burgisser, 1984). This would require a concentration of ^{125}I -BoNT of at least 200nM. What is more, in order to determine the level of non-specific binding, Chamness and McGuire (1975) suggest a final concentration of unlabelled ligand of 50 times this amount i.e. 10 μM , a condition which is clearly impractical.

In order to minimize the aforementioned errors all saturation binding experiments were performed simultaneously on both test and control membrane samples.

2.4.4. Gangliosides as toxin acceptors.

The implication, from the above observations, that a ganglioside moiety is the high-affinity component for BoNT B, is in line with other experimental results indicating that gangliosides may be the binding components on the neuronal membrane for other clostridial neurotoxins. Simpson and Rapport (1971) first demonstrated that type A toxin complex lost toxicity when incubated with sialic-acid containing moieties whilst being unaffected by preincubation with sphingolipids, steroids or fatty acids. The ability of these gangliosides to inactivate the toxin was dependent on the number of sialic acid residues in the lipid; a larger number resulting in increased potency of the ganglioside molecule in inhibiting toxicity of the complex, whilst removal of sialic acid residues abolished any such effect. A similar study using purified type A neurotoxin also demonstrated that gangliosides could inhibit the toxin's action, possibly by preventing it binding to its neuronal acceptor (Kitamura et al., 1980). These results indicated that the location, as well as the number, of sialic acids in ganglioside molecules was important, with GT1b and GQ1b being extremely effective at detoxification of

BoNT A. Similar studies on the inactivation of a variety of botulinum toxins (A-F) by a ganglioside mixture and purified GT1b showed that whilst the toxicities of types A and B BoNTs were reduced by the mixture, type D was unaffected (Kozaki et al., 1984). As expected, purified GT1b was more effective than the crude mixture and the effect was only observed if the incubations were made at low ionic strength. Neuraminidase treatment of the GT1b removed its ability to detoxify BoNT B whilst having no effect on its potency for type A, concurring with the observations made here that BoNT B requires the specific presence of NANA in its binding moiety. Using immunostaining techniques (Takamizawa et al., 1986), the binding of type A BoNT to a variety of gangliosides run on TLC has been demonstrated to be both pH- and ionic strength- dependent (with maximal binding observed at pH 7.2 and low ionic strength to ganglioside GQ1b). Attempts to provide further evidence for the ganglioside nature of the high-affinity acceptor for ^{125}I -BoNT B by direct binding to rat brain gangliosides run on TLC did not prove successful using the binding conditions under which this component was detectable on synaptosomal membranes. This may have been due to the fact that the receptor is present in such low numbers or that, under the conditions of physiological pH and relatively high ionic strength (0.15M) the toxin no longer recognises these molecules, casting doubts on the ability of gangliosides *per se* to act as high affinity acceptors for BoNT.

2.4.5. BoNT binding: comparison with tetanus toxin.

Gangliosides (GT1 and GD1b) similar to those implicated in the binding of BoNT have also been suggested as the binding components for another clostridial toxin similar to BoNT - Tetx (see General Introduction 1.6 for comparison) (Rogers and Snyder, 1981; Goldberg et al., 1981; Morris et al., 1980). However, binding of labelled Tetx was performed under conditions of low ionic strength (25-50mM Tris-acetate and low pH, 6.0). These conditions were found to be optimal for Tetx binding to rat brain membranes and, on Scatchard analysis, revealed a single population

of acceptor sites having an apparent high affinity for the toxin ($K_d \approx 1-2.5 \text{ nM}$). These acceptors were present in large numbers ($B_{max} \approx 4 \text{ nmoles/mg protein}$) and which were insensitive to protease treatment whilst sensitive to neuraminidase (Rogers and Snyder, 1981). The apparent inability of other workers to observe this high-affinity binding component for Tetx was attributed to the use of higher tissue concentrations, or the radioiodination procedure used (Rogers and Snyder, 1981). However, it has recently been shown that the choice of binding buffer plays a vital role in the observed nature of these acceptors (Critchley et al., 1986; Pierce et al., 1986) and that this apparent binding of toxin to gangliosides may only take place under non-physiological conditions. Acceptors for Tetx, analysed using low salt, low pH buffer, appeared to comprise a single, high-affinity, ganglioside component (Critchley et al., 1986; Pierce et al., 1986), as previously indicated by Rogers and Snyder (1981). However, under more physiological conditions, Tetx binding was greatly reduced and appeared to be due to two populations of sites; a high affinity proteinaceous component ($K_d 0.42 \text{ nM}$; $B_{max} 0.90 \text{ pmol/mg}$) and a larger population of low-affinity sites ($K_d 146 \text{ nM}$; $B_{max} 179 \text{ pmol/mg}$), thought to be due to binding to gangliosides (Pierce et al., 1986). As pointed out by these latter authors, BoNT A (and now type B) binding to rat brain membranes in physiological buffers is remarkably similar to that observed for Tetx. It was therefore of interest to determine whether BoNT would yield only a single binding site under the conditions previously used for Tetx (low ionic strength, pH 6.0). Indeed, binding of ^{125}I -BoNT A in this buffer revealed only a single set of binding sites exhibiting relatively high-affinity ($K_d \approx 4 \text{ nM}$) present in much larger content ($B_{max} \approx 24 \text{ pmol/mg}$) than the two binding components observed in physiological buffer [$B_{max} \approx 100 \text{ fmol/mg}$ for the high-affinity site ($K_d \approx 0.6 \text{ nM}$) $\approx 2 \text{ pmol/mg}$ for the low ($K_d \approx 25 \text{ nM}$)]. However, if either the pH was raised to 7.4 or the ionic strength increased (by addition of 0.15 M NaCl) analysis of the binding data revealed the reappearance of two sites, each with similar K_d and B_{max} values to those observed under physiological

conditions. Similarly, BoNT B binding under these conditions (low ionic strength, low pH) also revealed an apparent single site (B_{max} 35pmol/mg; $K_d \approx 7nM$). However, it must be noted that, under the conditions used, the extremely large content of sites exhibiting low affinity may be masking the resolution of a small population of high affinity acceptors. Since under physiological conditions BoNT A recognizes components which are sensitive to both proteolytic degradation and neuraminidase treatment (possibly sialoglycopeptides) it might be proposed that, under the other binding buffer, a combination of both conformational change of the toxin (and/or possibly the acceptor component) due to the lowering in the pH, together with increased interaction with its acceptor due to the low ionic strength, allows the toxin to recognise a much larger range of sialic-acid containing moieties (probably including many gangliosides) with medium affinity. Much work could still be carried out in determining the nature, together with possible interactions of the BoNT and Tetx with these sites. In the meantime it can be seen that much care must be exercised in stating the absolute quantity and affinity of binding sites for these toxins since a change in the binding conditions can lead to such marked differences in the apparent nature/content of these acceptors.

CHAPTER 3

Neuronal specificity of BoNT A and B.

3.1. Introduction.

It has long been established that in the PNS BoNT acts primarily (if not exclusively) at cholinergic nerve terminals, producing inhibition of ACh release. Recent findings show that specificity of the neurotoxin for peripheral cholinergic nerves is due to the presence, exclusively on the latter, of acceptors which specifically bind BoNT. Using pure preparations of ^{125}I -BoNT (A and B), acceptors have been demonstrated on these nerve terminals (appearing to be absent from adrenergic or other cell types (Black and Dolly, 1986a,b)). The toxin, bound to these presynaptic acceptors, is taken up into the nerve terminal by a receptor-mediated process, possibly endocytosis (Dolly et al., 1984; Black and Dolly, 1986a,b). As indicated in Chapter 2, the use of highly radioactive toxin has enabled the nature of these acceptors in the CNS to be determined. Comparison of the localisation of ^{125}I -BoNT with immunocytochemical staining (using antibodies raised against ChAT; Houser et al., 1983) of cholinergic nerve terminals in similar sections of rat brain suggested that BoNT acceptors in the CNS are apparently localised in cholinergic-rich regions (Black and Dolly, 1987) like their counterparts in the PNS (Black and Dolly, 1986a, b). However, this autoradiographic localisation technique suffers from poor resolution and binding of BoNT to other terminal types (especially in the cerebellum) could not be excluded (Black and Dolly, 1987). Furthermore, these techniques did not lend themselves to biochemical analysis and therefore, in order to clarify the neuronal specificity of the toxins' acceptors a preparation would be required in which cholinergic nerve terminals could be isolated and studied independently of other transmitter-specific types. Whilst synaptosome preparations, homogeneous with respect to their transmitter content, have been made from non-mammalian tissues (e.g. the cholinergic electric organ of *Torpedo*) these have proved unsuitable for BoNT binding measurements due to the unacceptably high proportion of non-specific binding (Williams, 1984). Moreover, extrapolation of

data from these tissues to mammals is questionable and may only serve to confuse rather than clarify the issue. Recently, the preparation of an antiserum against the cholinergic synaptosomes of Torpedo has allowed the purification of intact cholinergic nerve terminals from a crude population of mammalian synaptosomes (Richardson and Luzio, 1983; Richardson, 1986). This antiserum was shown, upon complement-mediated lysis, to preferentially release ChAT from guinea pig brain synaptosomes and, using immunofluorescence, to label cholinergic nerve terminals in both guinea pig and rat (Richardson, 1981). It has subsequently been demonstrated that this antiserum recognizes a ganglioside (Chol-1) which is present on the cholinergic nerve terminals of many mammalian species as well as those of the Elasmobranch (Richardson et al., 1982; Richardson, 1983).

In order to isolate small particles such as subcellular fractions or nerve terminals it is best to use indirect immunoaffinity techniques where a second (anti-immunoglobulin) antibody immunoadsorbent is used to isolate terminals bearing a first (sensitizing) antibody. In this study, immunoaffinity purification of cholinergic nerve terminals and subsequent analysis of BoNT binding to the latter indicated that both the high- and the low-affinity acceptors for BoNT A and B appear to be present almost exclusively on these central cholinergic nerve terminals. Furthermore, since the Chol-1 ganglioside (Richardson et al., 1982; Ferreti and Borroni, 1984) is also present on both peripheral and central cholinergic nerves, the possible involvement of this ganglioside in the binding of BoNT was assessed.

3.2. Materials and methods.

3.2.1 Immunoaffinity purification of cholinergic nerve terminals from a crude population of rat cerebrocortical synaptosomes.

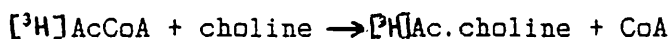
In this protocol the following buffers were used:

- 1/ 0.32M Sucrose : containing 4mM HEPES, 1mM EDTA, pH7.4
- 2/ EDTA solution: (mM) NaCl 145, KCl 3, EDTA 2, glucose 10, HEPES 10, pH7.4
- 3/ Percoll suspension: 45ml Percoll, 55ml above EDTA buffer, NaCl 70mM, pH7.4 (with 1M HEPES).

In all preparations described here, the cholinergic terminals were purified as previously described by Richardson (1984; 1986). Cortices, dissected from the brains of two female Sprague-Dawley rats (150-250g) were scraped to remove any residual myelin, and homogenised in 20ml ice-cold buffered sucrose solution. The resulting homogenate (10% w/v) was centrifuged (1000g, 10min, 4°C) in a Sorvall RC-5 centrifuge (SS-34 rotor) and the supernatant (representing the S₁ fraction of Gray and Whittaker, 1962) incubated (40min, 4°C) with a 1:80 dilution of sheep anti-(Chol-1) serum, prepared as previously described (Richardson et al., 1984). The pellet was washed 3 times, by centrifugation (16000g, 14min) and resuspension in 0.32M sucrose solution, before resuspension in 2.5ml 45% Percoll suspension. The terminals (representing the highly washed P₂ fraction of Gray and Whittaker (1962)) were then centrifuged (9000g, 2min) in an Eppendorf benchtop centrifuge and the intact synaptosomes (found layered on the surface of the Percoll suspension) were harvested and diluted 3 times into EDTA buffer prior to recentrifugation (9000g, 2min). The resulting pellets were resuspended in 5.5 ml EDTA solution and this crude synaptosomal suspension divided into two equal aliquots. Only one of the above aliquots was subjected to the following purification steps, the remaining aliquot (representing the total terminal population prior to purification) was kept at 4°C until required. Terminals, representing 5-20mg of ^{synaptosomal}Protein, were incubated with 1-4mg of a cellulose immunoabsorbent bearing a mouse monoclonal

anti-(sheep IgG) antibody (prepared as described by Richardson and Luzio (1983); kindly provided by Dr. Richardson). After 40min at 4°C, with occasional mixing, 2.5ml 45% Percoll (in EDTA buffer) was added to each centrifuge tube and the bound terminals separated from the free by centrifugation at 16000g for 12min. After a further two washes the immunoabsorbent-bound nerve terminals were washed once with Krebs-phosphate, pH7.4, (described in section 2.2.3.) and, immediately prior to the ¹²⁵I-BoNT binding assay, resuspended in 1.2ml of the same buffer containing 0.01% (v/v) final concentration Triton X-100 (binding buffer). Likewise, the crude synaptosomal preparation was pelleted, washed in Krebs-phosphate, pH7.4, and resuspended in 2ml of the above binding buffer containing 0.01%(v/v) Triton X-100.

3.2.2 Enzyme assays Choline acetyltransferase (ChAT, E.C.2.3.1.6) was assayed as a cholinergic marker by the method of Fonnum (1975) and lactate dehydrogenase (LDH, E.C.1.1.1.27) as a general cytoplasmic marker by the methods of Keiding et al. (1974). In the measurement of ChAT activity, a known volume (max.50μl) was incubated (37°C, 30min) with 50μl of ChAT cocktail containing the following (final concn; mM): NaCl 300, NaH₂PO₄ 50, pH7.4, choline chloride 10, eserine sulphate 0.1, acetyl-CoA 0.1, 0.2%(v/v) Triton X-100 together with 5.5μCi (≈22μl) [³H]-labelled acetyl-CoA (5.3Ci/mmol; Amersham, Bucks) per ml of cocktail. ChAT catalyses the conversion of acetyl-CoA and choline into ACh and CoA in the following reaction:



After incubation (in a microtube) the sample was dropped directly into a scintillation vial containing 5ml Krebs-phosphate, pH7.4. Tetraphenylboron (TPB; 2ml, 5g.l⁻¹) in acetonitrile was then added. The vial was shaken gently, followed by the addition of 10ml toluene scintillant (5g PPO, 0.2g POPOP/litre). The resultant mixture was shaken lightly for 1min allowing the positively charged ACh and choline to be extracted into the

organic scintillant with TPB, whilst any unconverted acetyl-CoA is left in the aqueous phase (Fonnum, 1975). The vial was placed in a scintillation counter and the two layers allowed to separate for 10min before counting. In each experiment a set of blanks, consisting of Krebs-phosphate, pH7.4, lacking the ChAT enzyme, were also assayed in the same manner to determine the amount of non-specific carry-over between the aqueous and organic phases of the unconverted [^{3} H]-acetyl-CoA. The values obtained for the blanks were averaged and subtracted from each of the experimental determinations and were typically found to be less than 5% of the total counts for the crude synaptosomal population.

Measurement of lactate dehydrogenase was by a spectrophotometric assay, following the decrease in $A_{340\text{nm}}$ with time. A known volume of the sample (50 μ l max) was incubated with 1ml assay cocktail (mM); Tris pH7.4, 20; EDTA, 1; NADH, 0.18M; Triton X-100 1%(v/v). The reaction mixture was centrifuged (9000g, 5min) to remove the immunoabsorbent cellulose beads (which interfere with the assay) and the supernatant transferred to a cuvette. The reaction was started by the addition of 100 μ l (50mM) pyruvic acid to the cuvette followed by rapid mixing and measurement of the decrease in $A_{340\text{nm}}$ with time: LDH activity was expressed as $\mu\text{mol}/\text{hour}/\text{mg}$ tissue.

3.2.3 Analysis of neurotransmitter content of the cholinergic-enriched synaptosomal fraction. Synaptosome samples, for measurement of catecholamine and indoleamine levels, were extracted and subjected to HPLC in the same buffer (0.1M KH_2PO_4 , 186 μM disodium EDTA, 387 μM sodium acetyl sulphonate, 14% methanol; pH 3.5: Docherty et al., 1986). Extraction by homogenisation in this buffer at 4°C, containing appropriate concentrations of 3,4-dihydroxybenzylamine and 5-hydroxyindole as internal standards, was followed by centrifugation (100,000 x g, 10min, 4°C). The resulting supernatants were filtered, using regenerated cellulose microfilters (0.2 μm , Anachem), prior to separation by reverse phase HPLC. Measurement of the transmitter levels was by electrochemical detection using an LCA 15 electrochemical detector (EDT Research). Determination of the amino acid transmitter content was by the method described by Young and Bradford (1986). Samples for determination of the amino acid content of the purified cholinergic terminals and comparison with the crude synaptosomal population, were sedimented by centrifugation, acidified by the addition of an equal volume of trichloroacetic acid [final concentration 5% (w/v)] and stored at 0-4°C pending analysis. After acidification, by the addition of a 10-fold excess of 0.025M HCl containing norleucine (500nM) as an internal standard, the sample was diluted two-fold, by the addition of methanol, mixed and centrifuged at 10,000 x g for 5min. The supernatant was removed, filtered (using regenerated cellulose microfilters, 0.2 μm , Anachem) and evaporated to dryness. After a further resuspension in methanol, the sample was again dried down and redissolved in water prior to autoanalysis of the amino acid contents by the method described by Norris et al. (1980). Peak areas were determined 'on-line' with a Digico Micro 16V computer using Rank-Hilger routines. 3 separate runs were made from each of 3 samples from 2 separate purification procedures exhibiting different extents of purification as determined by the increased ChAT/LDH ratio.

3.2.4 Binding of ^{125}I -BoNT A and B to crude and cholinergic-enriched nerve terminals from rat cortex. Specific binding of ^{125}I -BoNT A and B (0.05-30nM) was assessed using the standardised centrifugation assay previously described (section 2.2.6.). It was found necessary, however, to modify the Krebs-phosphate binding buffer by the addition of 0.01%(v/v) Triton X-100 (final concentration) and 0.1% (w/v) BSA in order to reduce non-specific adsorption of the labelled toxins to the cellulose beads of the immunoabsorbent. The incubations were started by the addition of aliquots of the crude synaptosomal suspensions or the cholinergic-enriched fraction (immunoaffinity purified and bound to cellulose beads). After incubation (60min, 23°C) the suspensions were centrifuged (9000g, 2min) and an aliquot taken to determine the free toxin concentration. The pellet was then washed twice in ice-cold binding buffer before being subjected to γ -radiation counting to determine the amount of bound toxin. As it was inappropriate to measure the amount of synaptosomal protein bound to the cellulose beads,* aliquots of the samples were taken for assay of LDH (and ChAT) activity. Values for the specific binding of radiolabelled toxin to crude terminals and those enriched for cholinergic nerve endings could then be rationalised as "amount of toxin bound (fmols) per unit LDH activity". Analyses of this data were then made using Scatchard plots and graphical analysis (see Chapter 2 for details of these procedures).

* FOOTNOTE — SINCE the sensitizing antibody is present in vast excess over the amount of synaptosomal protein recovered ($\approx 6-8 \mu\text{g}$).

3.2.5. Immunolysis of cholinergic nerve terminals using anti-(Chol-1) antibodies; protection by BoNT B. Using sheep anti-(Chol-1) polyclonal antisera together with human complement it has previously been shown that it is possible to selectively lyse cholinergic terminals by complement-mediated lysis (Richardson, 1981).

a) Cholinergic terminal lysis: antibody concentration dependence.

Interaction of BoNT B with the acceptor(s) for anti-(Chol-1) antiserum, was assessed by measuring its ability to inhibit complement-mediated lysis of cholinergic nerve terminals, using a range of antisera concentrations (1:1600-1:10; final dilutions). Inhibition of antibody binding by 1nM BoNT B was assessed by its ability to reduce the level of complement-mediated lysis of the cholinergic nerve terminals, as judged by the release of ChAT into the supernatant. Synaptosomes (fraction P₂B-Gray and Whittaker, 1962) prepared from 1gm of cerebral cortex were resuspended in ≈20-40ml ice-cold Krebs-phosphate buffer, pH7.4, containing 1mg/ml BSA. Aliquots (150μl) of this synaptosomal suspension were added to 100μl of the above buffer containing the appropriate antisera dilutions in the presence or absence of BoNT B and incubated for 15min at 4°C. The terminals were pelleted in an Eppendorf centrifuge and resuspended in 1ml of binding buffer. Aliquots were taken at various times (see (b) below) for determination of ChAT activity.

b) BoNT concentration dependent inhibition of complement-mediated lysis. Using the P₂B fraction of synaptosomes diluted as above, 150μl aliquots were added to 100μl of the binding buffer containing varying concentrations of BoNT (0-100nM; types A and B) and incubated for 30min at room temperature. Anti-(Chol-1) antibodies were added (1:100 final dilution) and incubation at 4°C continued for another 40min. Synaptosomes were centrifuged (9000g, 2min) in an Eppendorf benchtop centrifuge and each of the resultant pellets resuspended in 1ml assay buffer. Immediately, two aliquots (200μl each) were removed; one was used for measuring the total ChAT activity and the other centrifuged, as

above, and the supernatant assayed for any residual ChAT activity (to determine the initial proportion of terminals lysed). [Crude serum (as a source of human complement) was prepared from blood given by donors who had not previously been immunised against BoNT. Fresh blood was left at 4°C for ≈4hr before centrifugation at 1000g to remove the clotted blood products. The serum was removed and stored in 1ml aliquots at -70°C]. 70μl of this human serum was added to the remaining terminals and incubation, at 37°C, continued for 20-40min before another aliquot was removed, centrifuged and its supernatant assayed for ChAT activity to determine the amount of lysis occurring in the terminals due to the complement reaction. Control experiments (to determine the amount of non-specific lysis) included incubation with complement in the absence of toxin or antibody. The lysis of the cholinergic synaptosomes was evaluated as the percentage of ChAT activity released according to the formula:

$$100 \times \frac{\text{ChAT released in test} - \text{ChAT released in control}}{\text{Total amount of ChAT in the sample}}$$

ChAT activity was assayed by the method of Fonnum (1975).

3.2.6. Cholinergic lesioning of the rat brain and ¹²⁵I-BoNT binding.

Unilateral lesioning studies were performed by Dr. Alan Fine as detailed elsewhere (Fine et al., 1985a,b). Female Sprague-Dawley rats (≈200g) were anaesthetised using equithesin (0.3ml/100g) and large unilateral lesions of the right nucleus basalis magnocellularis (NBM) were produced by 2 stereotaxic injections of 5μg of ibotenic acid (Sigma) in 0.5μl 0.1M phosphate buffer, pH 7.4. The co-ordinates for the rostral injection were A (to bregma)=1.0mm, L (to midline)=2.6mm and V (below dura)=7.3mm with the incisor bar set 5.0mm above the interaural line, and for the caudal injection the co-ordinates were A=0.2, L=3.4 and V=7.0 (the approximate position of the NBM

is indicated in the sagittal section of the rat brain shown in Fig.3.1). Both injections were made in a single session - each made over 2min, with the needle left in place for a further 2min. Lesioned animals were killed after 1,6 or 8 weeks by transcardial perfusion under barbiturate anaesthesia. Animals intended for biochemical analysis (of ChAT levels) were perfused with ice-cold 0.1M phosphate buffer, pH 7.4, their brains removed rapidly and sliced as described (Fine et al., 1985a,b). ChAT activity was determined by the micro-method of Fonnum (1975). Animals intended only for histochemical analysis were perfused with heparinised saline followed by 0.08M phosphate buffer, pH 7.4, containing 40g/l paraformaldehyde, 0.02M L-lysine and 2.5mM sodium periodate. Brains were removed intact and immersed in the fixative for 4h at 4°C followed by 24h at 4°C in 0.1M phosphate buffer containing 30% sucrose, pH 7.4. The brains were frozen (-20°C) and sections cut using a Reichert-Jung Cryocut E cryostat. Starting at the rostral pole, 10µm vertical sections from at least 4 different brain levels were cut and placed onto gelatin coated microscope slides for analysis of BoNT binding. At each brain level, 25µm sections were also taken for histochemical analysis of AChE by the method of Schwarcz (1979) as detailed by Fine et al. (1985b). Brain slices from lesioned animals were stored dessicated at -20°C until required. Binding of ¹²⁵I-BoNT was determined by application to each of these slices of 1nM labelled toxin in Krebs-phosphate buffer, pH 7.4, containing 0.1% (w/v) BSA (final vol. 200µl). Incubation was allowed to continue for 60 min at room temperature prior to extensive washing (3x15 min; 300ml binding buffer per wash). To inactivate any residual toxin bound to these brain slices, they were washed briefly in deionised water prior to immersion in 3% paraformaldehyde for 15 min at room temperature. Finally, the slides were rinsed with water and allowed to air-dry before exposure to autoradiographic film. Non-specific binding was determined, on adjacent slices to those used to measure the total binding, by preincubation of each slice with 1µM unlabelled neurotoxin for 20 min prior to the addition of 1nM (final concn.) labelled BoNT and incubation for a

further 60 min at 23°C. At this stage the control samples were washed and processed for autoradiography as detailed above. The resultant autoradiograms were subjected to 2-dimensional laser densitometric scanning using a Bio-Med 2-D laser scanner and the data stored and processed on an Apple II microcomputer interfaced with the scanner. Densitometric readings were made in the cortical regions of both the lesioned and the non-lesioned sides of each brain slice. 9 readings were made in each of 4 distinct regions of the cortex as shown in Fig. 3.1b. The response of ³H Ultrofilm to autoradiography and quantitation of the densitometric readings was characterised using brain paste standards uniformly labelled with ¹²⁵I as described elsewhere (Pelchen-Matthews and Dolly, 1987). Accurate determination of the specific activity of the labelled toxin and exposure time for autoradiography allowed comparisons to be made with density readings of these standards. Hence data was expressed in terms of toxin bound. Non-specific binding was subtracted from the total to give values for the specifically bound toxin and comparisons made between the specific binding in the unlesioned and lesioned sides of the brain.

3.3. RESULTS.

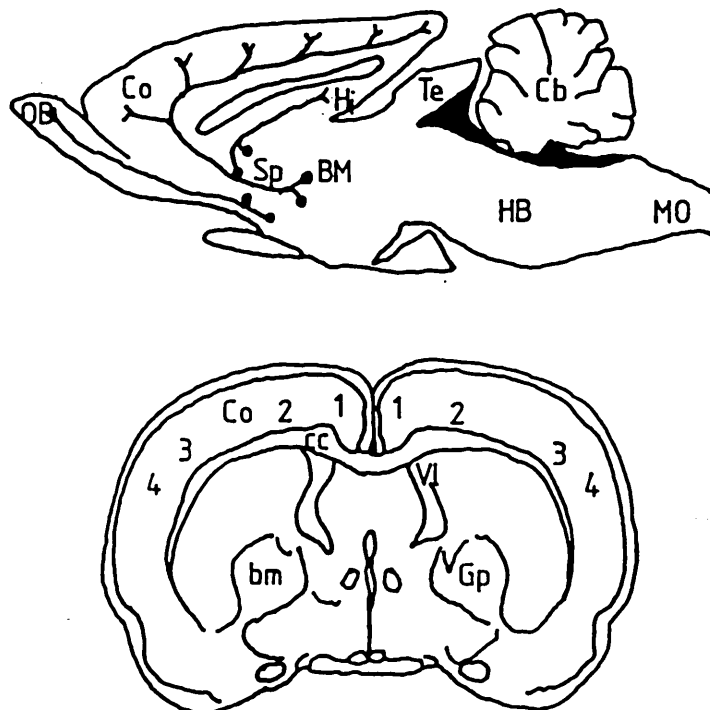
3.3.1 Purity of cholinergic nerve terminals.

The recovery and purification of these cholinergic nerve terminals was assessed by comparison of the ChAT to LDH ratios between the purified terminals and the initial, crude population. Typically, the increase in ChAT (a cholinergic-specific enzyme marker) compared with LDH (found in all terminals), was between 6- and 15- fold (Fig. 3.4). The recovery of such terminals was between 1 and 8% of the total (decreasing with an increase in the fold purification obtained. In order to assess whether any other neurotransmitter-specific terminals were co-purified with the cholinergic nerve endings, comparisons were made between the

amino-acid and catecholamine neurotransmitter profiles of the cholinergic nerve terminals and the initial population (as described in the Methods: 3.2.3). In the values shown (Table 3.1) for co-purification of the amino acids the increase in ChAT/LDH i.e. the purification of cholinergic terminals, was 15-fold, whilst for the catecholamines there was a 6.3-fold enrichment in cholinergic terminals over those in the starting material. From Table 3.1 it can be seen that ^{whilst} there is little or no concomitant increase in the proportions of the catecholamines in such purified cholinergic nerve terminals ^{some amino acids may show a slight} enrichment in these parameters.

3.3.2 Comparison of the binding of ^{125}I -BoNT (A and B) to cholinergically-enriched and untreated terminals. Purified cholinergic nerve terminals (still attached to the cellulose immunoadsorbent beads) were used in the standard centrifugation assay described in section 2.2.6. Using the binding conditions previously used for synaptosomal membranes a comparison of the nature of the binding sites between the cholinergically-enriched fraction (abbreviated to CT) and the crude terminal population (TT) could be made by determining the binding parameters for ^{125}I -BoNT (A and B) on these terminals. The level of non-specific binding to the cholinergic terminals on the beads was too large (60-80%) to allow accurate determinations of the specific binding to be made (presumably due to interaction of the labelled toxin with the cellulose beads). This high level of non-specific binding could be reduced to an acceptable level (<30%) by the addition, to the above binding buffer, of Triton X-100 (0.01% (v/v), final concentration). Under these conditions, binding of increasing concentrations of ^{125}I -BoNT A (Fig. 3.2) or B to these terminals was assessed and expressed in terms of the levels of LDH present in each of the populations (since, due to the presence of large concentrations of antibody on the cellulose

Fig. 3.1 Sagittal and coronal sectional diagrams of rat brain showing site of ibotenate injection and neuronal pathway together with regions subjected to densitometric analysis of ^{125}I -BoNT binding.



a) Sagittal section of rat brain. The excitotoxin ibotenate was injected into the nucleus basalis magnocellularis (BM) whose, primarily cholinergic, neurones extend to the cortex (Co). Injection of the cell bodies in the BM causes destruction of the cholinergic axons invading the cortex. OB, olfactory bulb; Sp, septum; Hi, hippocampus; Te, optic tectum; Cb, cerebellum; HB, hindbrain; MO, medulla oblongata.

b) Coronal section through mid-brain region showing approximate areas in which densitometric scans were made (marked 1-4). Densitometric readings in the areas of the cortex (Co) were made from 2-dimensional scans of autoradiograms (see Figs. 3.7 and 3.8) of ^{125}I -BoNT (A and B) binding to $10\mu\text{m}$ coronal sections from lesioned rat brains as detailed in the Methods (3.2.6). cc, corpus callosum; bm, nucleus basalis magnocellularis; Gp, globus pallidus; Vl, ventricle.

Table 3.1. Amino acid contents in crude and cholinergic-enriched nerve terminals: comparison with the fold purification.

Cholinergic nerve terminals were separated from a crude preparation of synaptosomes using the immunoaffinity technique described in section 3.2.1. The degree of enrichment was assessed by comparison of the ChAT/LDH ratios between the cholinergic terminals and the crude population of synaptosomes. Aliquots of each preparation were processed and analysed for their contents of a variety of amino acids, catecholamines and indoleamines by HPLC as detailed in section 3.2.3. Comparison of the peak areas obtained with known concentrations of standards, included in each HPLC run, allowed the quantitation of the neurotransmitter contents in both samples.

A) Amino acid contents of the crude, and cholinergically-enriched, synaptosomal populations. The apparent fold purification of these transmitters is shown. The degree of enrichment of cholinergic terminals was ≈ 15 fold.

B) The fold purification of the catecholamines between the purified cholinergic terminals and the crude synaptosomal preparation. The degree of purification in this experiment was ≈ 6.3 fold. (n.d. = not detectable).

Table 3.1 Amino acid contents in crude and cholinergic-enriched nerve terminals: comparison with the fold purification.

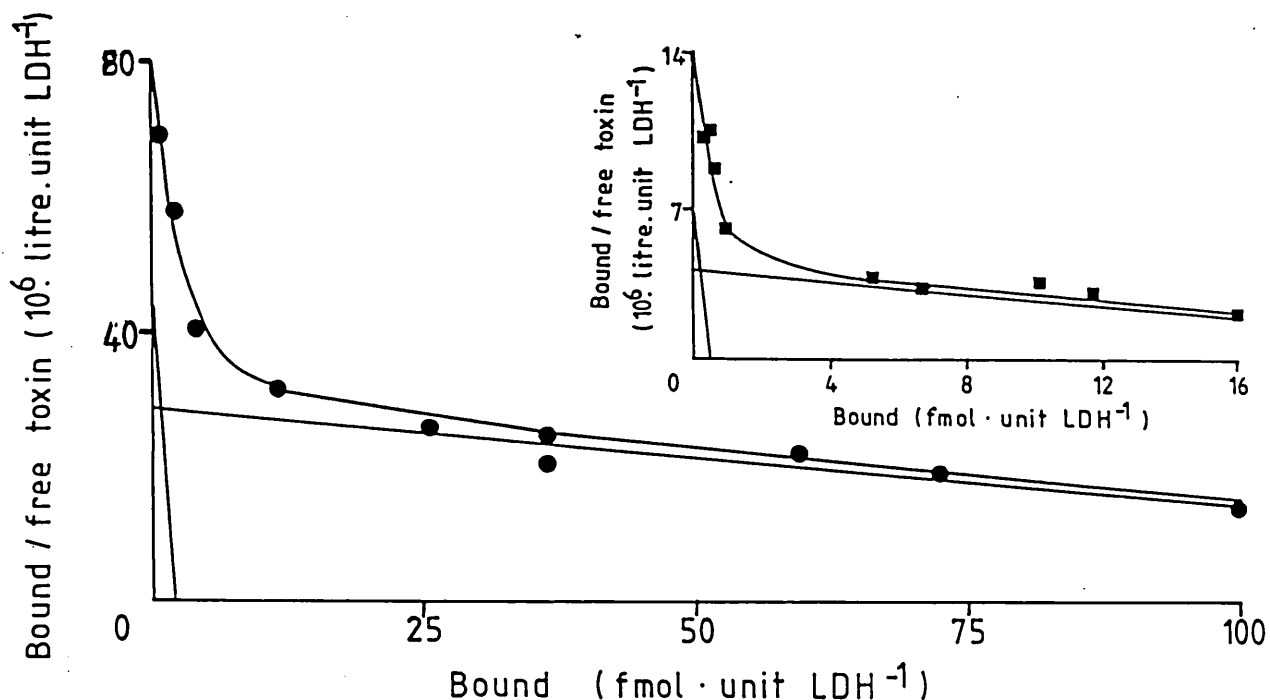
A)

AMINO ACID	Crude fraction (TT) pmol/unit LDH	cholinergic- enriched fraction (CT)	Fold Purification
			ChAT/LDH=15
*ASP	692±53	1777±201	2.57
*TYR	191±32	609±78	3.19
*GLU	1236±155	1810±219	1.46
*GLY	587±69	2549±243	4.34
*GLN	304±51	386±53	1.27
*GABA	239±40	298±32	1.24
*TAU	719±83	920±86	1.28
THR	255±31	798±82	3.13
SER	792±83	2751±304	3.47
ALA	466±52	1462±131	3.14
VAL	165±19	588±32	3.14
ILE	128±15	462±51	3.61
LEU	135±16	458±30	3.39
PHE	142±16	470±52	3.31
HIS	138±17	517±40	3.75
ARG	209±35	911±82	4.36
MET	n.d.	336±42	n.d.
TRP	n.d.	n.d.	n.d.

B)

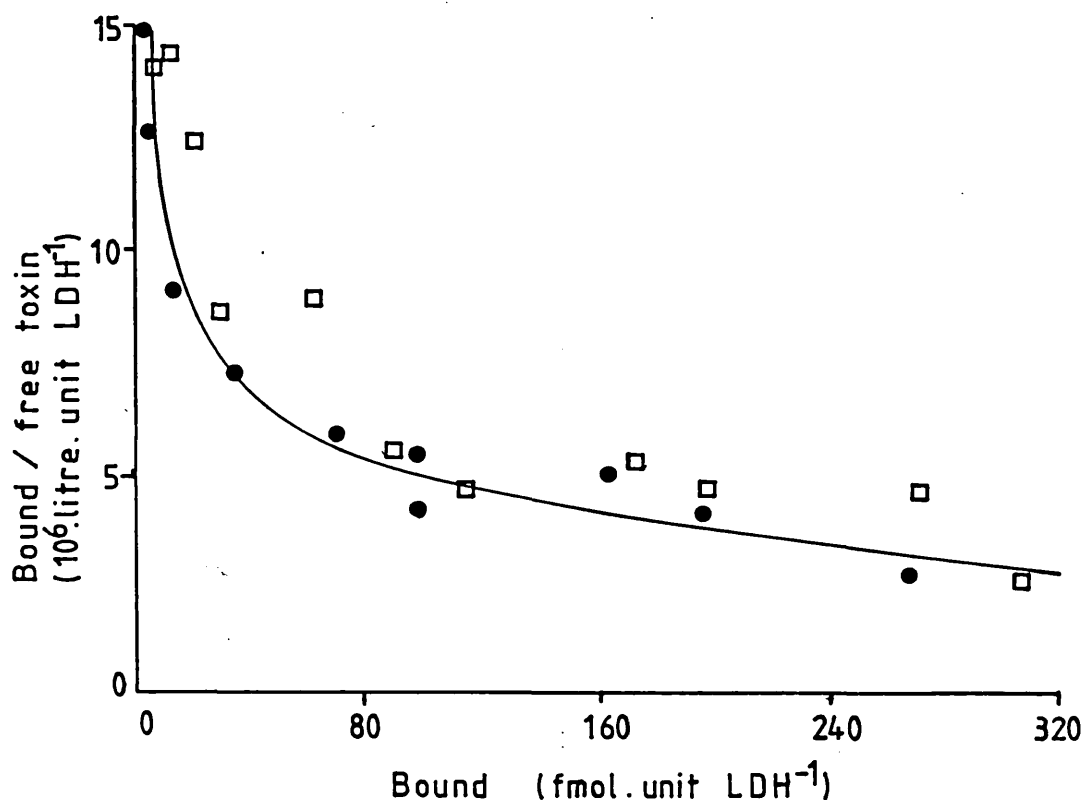
Catecholamine	(TT) pmol/mg	(CT) pmol/mg	Fold Purification
			ChAT/LDH=6.3
NA	440	37	0.08
DA	44	n.d.	n.d.
5-HT	7.7	n.d.	n.d.

Fig. 3.2. Equilibrium binding analysis of BoNT acceptors on cholinergic-enriched and crude populations of synaptosomes.



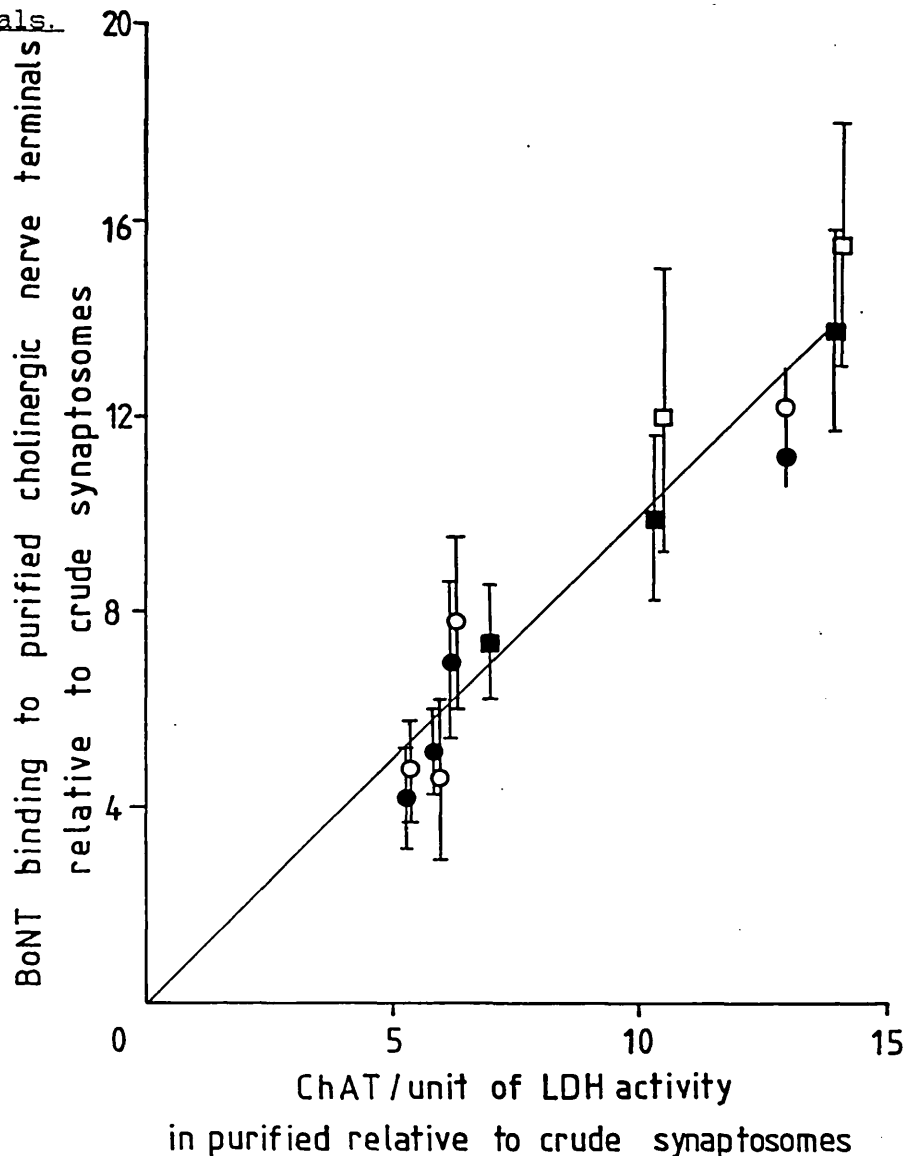
Rat cerebrocortical synaptosomes, isolated by density gradient centrifugation, were affinity purified as described in Methods (3.2.1). Specific binding of ^{125}I -BoNT A to the cholinergic nerve terminals bound to the cellulose beads was measured using the standard centrifugation assay (see section 2.2.6), except that the buffer used in the incubation and for washing the labelled membranes contained 0.01% (v/v) Triton X-100; this reduced the level of non-specific BoNT binding which was quantified in the presence of a 100-fold molar excess of the appropriate unlabelled toxin. Scatchard plots of ^{125}I -BoNT binding to [(a); insert] unfractionated synaptosomes and (b) affinity-purified cholinergic nerve terminals are shown. The straight lines represent the high- and low-affinity components, as determined by graphical analysis (Rosenthal, 1967). Note that BoNT binding is expressed relative to LDH activity since it is inappropriate to measure protein concentration. For the cholinergic-enriched terminals, B_{max} for the high-affinity component was $\approx 2 \text{ fmol} \cdot \text{unit LDH}^{-1}$ and the low $\approx 200 \text{ fmol} \cdot \text{unit LDH}^{-1}$. For the crude synaptosomal preparation the appropriate values were ≈ 0.3 and $30 \text{ fmol} \cdot \text{unit LDH}^{-1}$ respectively.

Fig. 3.3. Both high- and low- affinity binding components for ^{125}I -BoNT B exist on cholinergic nerve terminals.



Cholinergic nerve terminals were purified, and specific ^{125}I -BoNT B binding assessed, as detailed in the legend to Fig. 3.2. The data obtained were expressed in terms of the ratio of ChAT/LDH activities for each sample. In this experiment, the increase in the ChAT/LDH ratio in the cholinergic-enriched terminals over that in the crude preparation was 14.3 fold. Expression of the specific binding in terms of similar levels of LDH activity demonstrates that the acceptor components for BoNT are cholinergic-specific since the Scatchard plot of this data is directly superimposable on that obtained for the crude preparation. (□), specific binding to immunoaffinity purified cholinergic nerve terminals and (●), the crude synaptosomal preparation. Similar results were obtained for the specific binding of ^{125}I -BoNT (A and B) in experiments yielding a variety of purification ratios (6-15 fold; see Fig. 3.4)

Fig. 3.4 Co-purification of BoNT acceptors with cholinergic nerve terminals.



Rat cerebrocortical synaptosomes, isolated by density gradient centrifugation, were affinity purified as described (section 3.2.1). The purity of the isolated terminals was assessed by measuring the % recoveries of ChAT (as a cholinergic marker), and LDH (as a general cytoplasmic marker), and expressing them as a ratio. Specific binding of 0.2nM (○, □) and 0.5nM (●, ■) ^{125} I-BoNT A (circles) and B (squares) to impure and purified samples was determined from saturation binding studies similar to those used to plot the Scatchards shown in Figs. 3.2 and 3.3. The regression line shown for the composite values for BoNT A and B acceptors gives a correlation coefficient of 0.98.

beads, protein determination is not valid). Scatchard plots of the binding data for both the CT and the TT fractions are non-linear. Graphical analysis (Rosenthal, 1967) of the curves indicated the presence of a small population of acceptor sites having high-affinity for the toxin and a much larger population of acceptors demonstrating lower affinity (similar to that observed in rat cerebrocortical synaptosomal membranes: c.f. Fig.2.6). Rationalisation of the data for binding to cholinergic terminals (c.f. the crude population) in terms of the same levels of LDH activity, resulted in the Scatchard plots for both sets of data being directly superimposable (Fig. 3.3). Similar results were obtained with ^{125}I -BoNT A (data not shown) indicating further that both acceptor types (for both BoNT A and B) are present, almost exclusively, on cholinergic nerve terminals. Further evidence for the neuronal specificity of the high-affinity acceptors for BoNT A and B was obtained by analysis of the binding of labelled toxin to both CT and TT terminals from a variety of preparations having a broad range of recoveries and purification factors for the cholinergic nerve terminals. Comparison between the absolute amounts of binding activity in purified and crude terminals and the degree of purification of cholinergic terminals showed a linear relationship at 0.2 and 0.5nM BoNT (which ought to distinguish the high-affinity component). Upon linear-regression, the data was shown to fit well to a straight line, for both type A and type B BoNT binding, with a correlation coefficient of 0.98 (Fig. 3.4) suggesting that there is a direct relationship between the increase in toxin binding to its high-affinity acceptor and the degree of cholinergic enrichment, providing further evidence that this population of acceptors exist exclusively on cholinergic terminals.

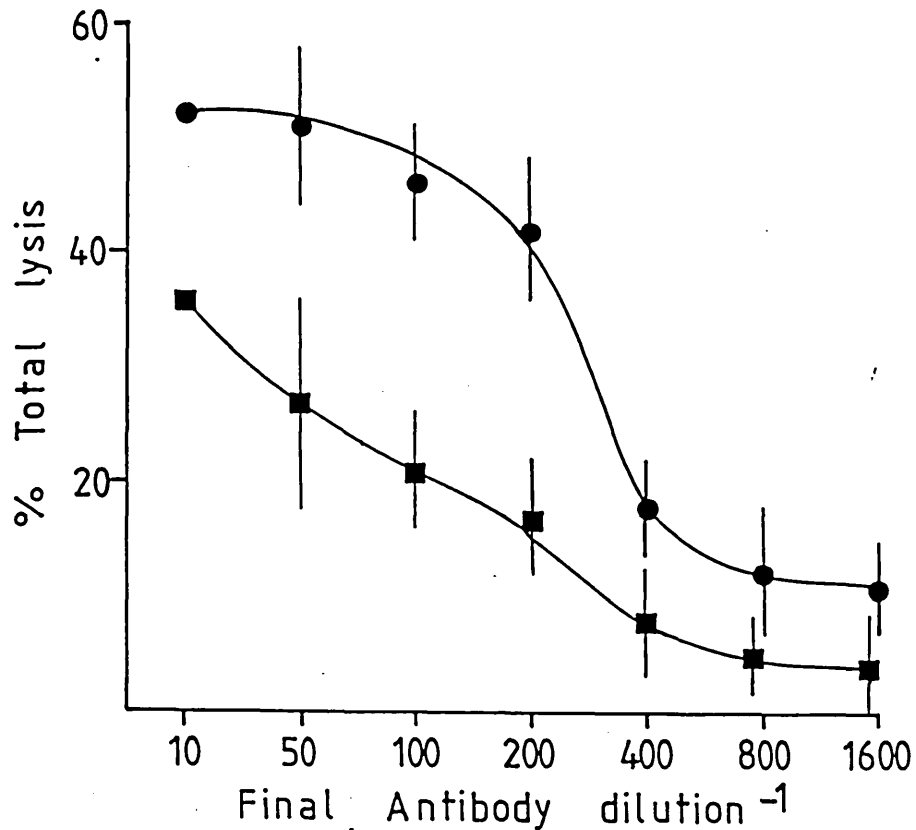
3.3.3 Identification of the acceptor moiety for BoNT B.

Since the indications, from biochemical analysis of ^{125}I -BoNT B binding to treated synaptosomal membranes were that the high affinity acceptor site contain sialic acid (see Chapter 2), and from the above evidence that these high-affinity acceptors for BoNT A and B are present almost exclusively on cholinergic nerve terminals, it was of interest to test the possibility that the cholinergic-specific ganglioside marker (Chol-1) could be associated with the high-affinity acceptor component(s) for either of these toxin types. The availability of the sheep polyclonal (Anti-(Chol-1); kindly provided by Dr. Peter Richardson) allowed this hypothesis to be tested. Initial studies, in which the ability of the antibody to inhibit the binding of low concentrations of ^{125}I -BoNT (1nM) to their acceptors was assessed, were of limited success. Relatively low levels of inhibition of toxin binding ($\approx 30\%$) was achieved only with very high concentrations of the antibody and, although the effect was more marked with ^{125}I -BoNT B, some inhibition of ^{125}I -BoNT A binding was also observed but the specificity of any such interaction could not be determined. This effect may be a result of the antibody having lower affinity for the acceptor than the toxin ($K_d \approx 3 \times 10^{-10}\text{M}$) although this would be inconsistent with ability of the serum to be used in the immunoaffinity purification procedure. However, the ability of the toxin types to compete for the antibody acceptor site (Chol-1) and thus prevent the immuno-specific complement-mediated lysis of the cholinergic terminals was investigated. Indeed, 1nM BoNT B was very effective at inhibiting the immunolysis at all antibody dilutions studied (Fig.3.5). Using the optimum antibody dilution (1:100), producing maximal lysis in the presence of complement, lysis of cholinergic terminals (by measuring released ChAT activity, as detailed in Methods: 3.2.2.) was determined in the presence of increasing toxin concentrations. Results from a number of separate experiments are shown (Fig.3.6) (in all experiments maximal lysis (100%) represented $\geq 50\%$ of the total releasable ChAT activity and was often as high as 80%). It can be

seen that BoNT B is very effective at inhibiting this antibody-specific immunolysis, resulting in almost complete inhibition of lysis at 100nM. BoNT A had no effect on the lytic reaction even at the highest concentration used, further indicating the specificity of the competition between BoNT B and the antibody, suggesting that they may possibly recognise the same component(s) - the ganglioside, Chol-1.

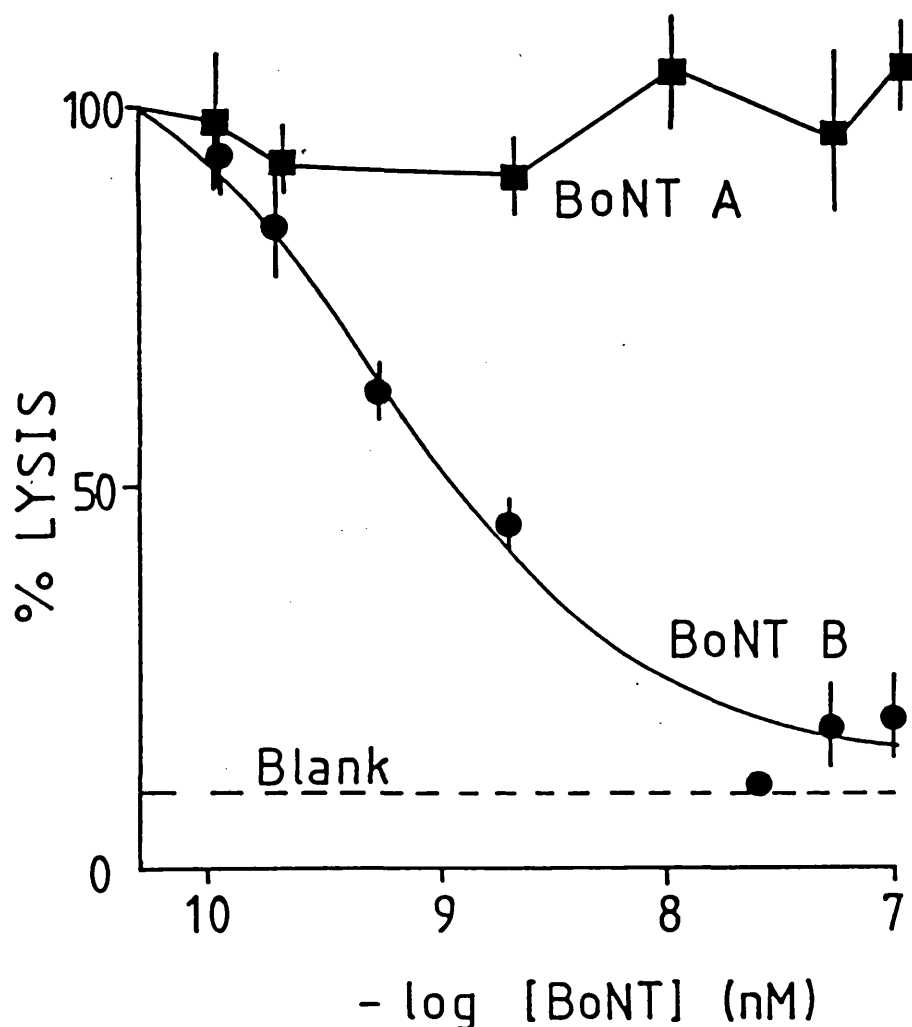
3.3.4. Binding of ^{125}I -BoNT to slices from cholinergically lesioned rat brain. Rats were subjected to lesioning of the central cholinergic tracts by unilateral injection of the excitotoxic amino acid - Ibotenate- into the cell bodies of the NBM as described in Methods (3.2.6.). This procedure results in the death of the cholinergic axons giving rise to extrinsic innervation of the neocortex and essentially causes denervation-like effects such as reduction in AChE levels and ChAT activity between the lesioned and non-lesioned halves of the brain. The extent of lesioning was dependent on the time after injection of the ibotenic acid, with a 40% decrease in AChE or ChAT content in lesioned c.f. intact contralateral levels 1 week after surgery (Fine et al., 1985a,b). The levels of these marker enzymes was further decreased (to $\approx 50\%$) in animals killed 6 weeks after surgery. However, little decrease in staining for the cholinergic marker, Chol-1, was observed at this time. Binding of 1nM ^{125}I -BoNT (A or B) to such brain slices was performed as described in Methods (3.2.6) and autoradiograms from such experiments were prepared and developed using the protocol described (section 3.2.6.). Although the labelling pattern was similar to that previously reported (Black and Dolly, 1987), no visual difference was obvious between the control and lesioned sides of the cortex (Fig. 3.7 and 3.8). However, quantitative analysis of the autoradiograms by laser densitometry (Table 3.2) showed that some reduction in toxin binding occurred in the lesioned relative to the control side. Although there was a significant reduction ($\approx 40\%$) in enzyme parameters measured in animals killed 1 week

Fig. 3.5 Inhibition by BoNT B of complement-mediated lysis of synaptosomes using anti-(Chol-1) antibodies.



Rat cerebrocortical synaptosomes (P_2 fraction; 50-150 μ g protein) were incubated in Krebs-phosphate, pH 7.4, containing 1mg/ml BSA with (■) and without (●) 1nM BoNT B for 30min at 20°C, and then with various dilutions of polyclonal anti-(Chol-1) serum for 15min at 4°C. After washing, the pellets were resuspended in a 1:10 dilution of human serum as a source of complement. Following incubation at 37°C for 15min, lysis was assessed by the release of ChAT activity, measured by the method of Fonnum (1975). Values obtained from samples treated with non-immune sheep serum in the presence of complement were subtracted from all determinations; extents of lysis were then expressed relative to the total ChAT activity detectable prior to the addition of complement. Error bars (S.D.) are shown for the mean of four experiments.

Fig. 3.6 Inhibition by BoNT of complement-mediated immunolysis by sheep anti-(Chol-1) serum.



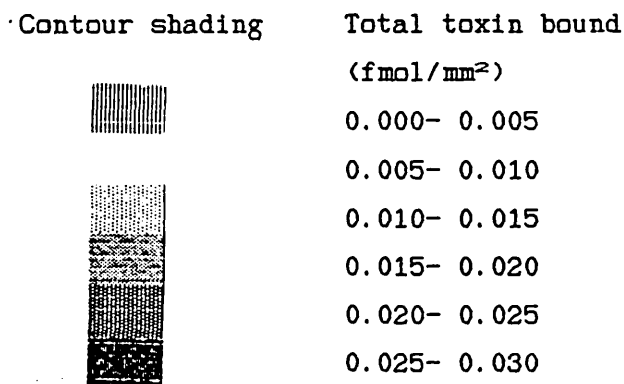
Rat cerebrocortical synaptosomes (P_2 fraction; 80-150 μ g protein) in Krebs-phosphate, pH 7.4, containing 1mg/ml BSA were incubated (30min, 23°C) with increasing concentrations (0-100nM) of botulinum neurotoxin. At this time the suspensions were cooled on ice, anti-(Chol-1) serum added (1:100, final dilution) and incubation continued for 40min. Synaptosomes were pelleted and resuspended in 1ml of the above buffer and aliquots (2 x 200 μ l) removed for determination of initial levels of ChAT activity. 70 μ l of human serum (as a source of complement) was added to the remaining suspension and incubation continued at 37°C for 10-20min prior to centrifugation (9000 x g, 1min) and removal of an aliquot of the supernatant for measurement of released ChAT activity. ChAT was assayed as detailed in the Methods. Values are expressed relative to the amount of lysis in the absence of toxin (typically representing 50-80% of the total releasable ChAT). The effect of increasing concentrations of BoNT A (■) or BoNT B (●) on the immunolysis of cholinergic terminals is shown. (---) represents the amount of complement-mediated lysis in the absence of either antibody or toxin. Error bars (S.D.) are shown for the means of four experiments.

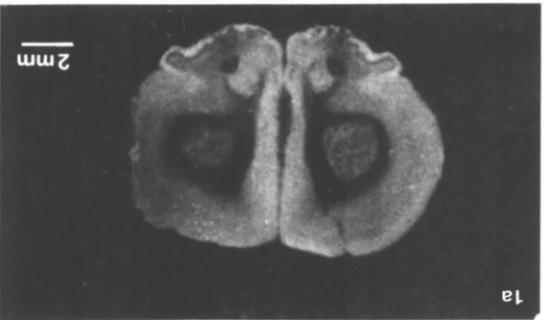
Fig. 3.7 ^{125}I -BoNT A binding to brain slices from lesioned animals.

Rats were lesioned (as detailed in section 3.2.6) by unilateral injection into the nucleus basalis magnocellularis (NBM) of the excitotoxin ibotenate. Six weeks after surgery the rats were killed, the brains removed intact, fixed (in 4% (w/v) paraformaldehyde) and frozen at -20°C . Coronal sections ($10\mu\text{m}$) were cut, using a Reichert-Jung cryostat, and placed onto gelatinised microscope slides. Total binding of ^{125}I -BoNT was determined by application to each of these slices of 1nM labelled toxin in Krebs-phosphate buffer, pH 7.4, containing 0.1% (w/v) BSA (final vol. $200\mu\text{l}$). Incubation (60 min, 23°C) was followed by extensive washing (3 x 15 min; 300ml per wash) in the above binding buffer. Slices were then rinsed briefly in deionised water prior to immersion in 3% paraformaldehyde (15 min, 23°C). Sections were then rinsed again in water, air-dried and exposed to autoradiographic film. Non-specific binding was determined, on adjacent slices to those used to measure the total binding, by preincubation with $1\mu\text{M}$ unlabelled BoNT for 20 min prior to incubation for a further 60 min at 23°C with 1nM (final conc) of labelled BoNT. The autoradiograms were developed and scanned using a 2-dimensional laser densitometer as detailed in Methods (3.2.6).

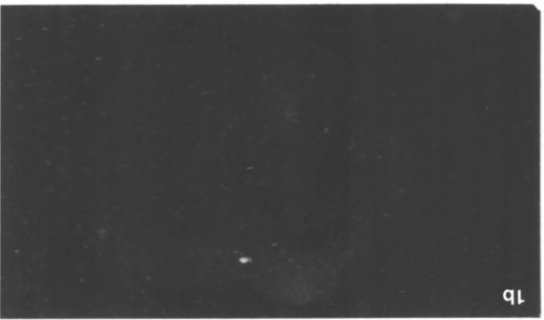
The left column of the figure shows the resultant autoradiographic distribution of toxin labelling in; (1) Frontal brain sections; (2) Mid-brain sections and (3) Rear brain sections of lesioned animals. The lesioned side is shown on the right hand side of these pictures. Pictures labelled (a) represent the total binding whilst those labelled (b) represent the non-specific binding to the corresponding brain slice. Scale bar in 1a represents 2mm on original brain slice.

The right hand column shows the corresponding densitometric scans of these autoradiograms and the amounts of toxin binding (calculated as detailed earlier) is shown by contour shading as indicated in the key below:

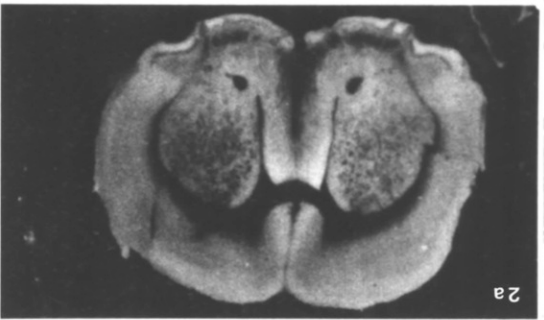




1a



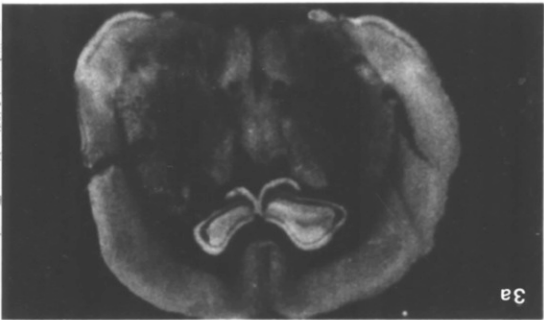
1b



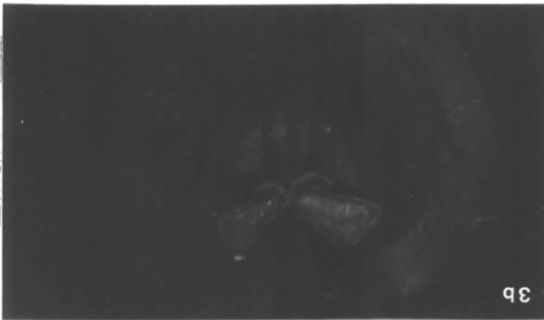
2a



2b



3a



3b

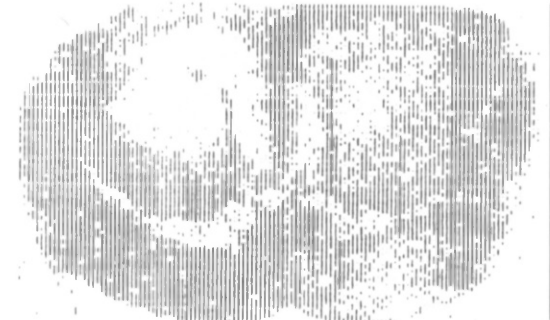


Fig. 3.8 ^{125}I -BoNT B binding to brain slices from lesioned animals.

Rats were lesioned and ^{125}I -BoNT binding to such treated brain slices was performed exactly as detailed in the legend to Fig. 3.7. Autoradiograms were developed and densitometric scans also made as in Fig. 3.7.

The left column of the figure shows the resultant autoradiographic distribution of toxin labelling in sections (1-3) from areas of the mid-brain in which a significant decrease in binding of ^{125}I -BoNT A was observed in the lesioned c.f. the non-lesioned sides. Lesioned side is on the left side of each of these pictures. Pictures labelled (a) show total ^{125}I -BoNT B binding whilst those labelled (b) represent the non-specific binding of toxin to adjacent brain slices. Scale bar in 1a represents 2mm on original slices.

The right column shows the corresponding densitometric scans for the specific binding of the toxin (calculated by subtraction of the non-specifically bound values from the totals). Contour shadings of these densitometric scans are indicated in the key below:

Contour shading	Specific toxin bound (fmol/mm ²)
	0.00- 0.02
	0.02- 0.04
	0.04- 0.06
	0.06- 0.08

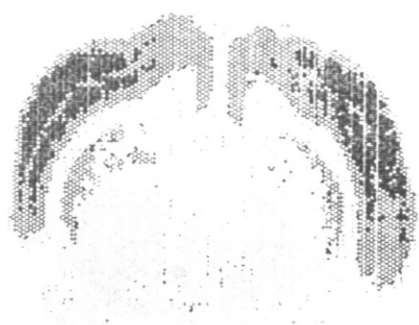
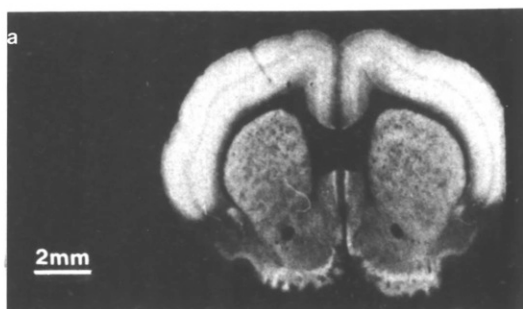


Fig.
3.8

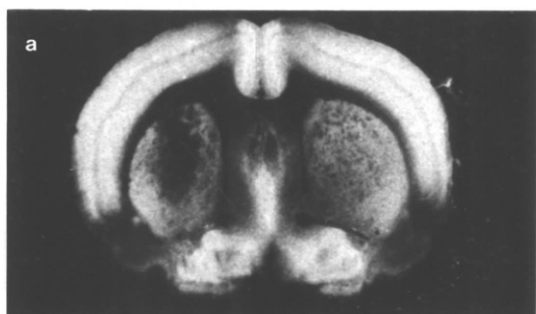
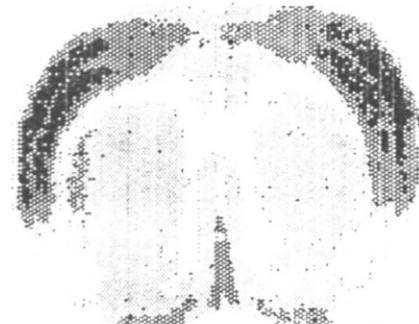
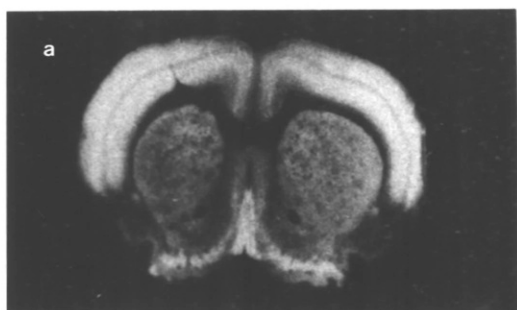
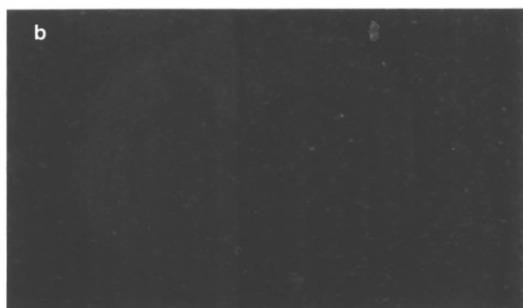


Table 3.2 Effect of lesioning of the nucleus basalis magnocellularis (NBM) on specific binding of ^{125}I -BoNT to brain slices.

Toxin type	Region	Specific binding (fmol/mm ²)		% of control	Average
		Control side	Lesioned side		
BoNT A	Rostral	0.029	0.029	100 ^a	98
		0.029	0.028	96 ^b	
	Medial	0.034	0.028	82 ^a	77
		0.020	0.014	70 ^b	
	Caudal	0.028	0.018	64 ^a	58
		0.027	0.014	52 ^b	
BoNT B	Rostral	0.061	0.055	90 ^a	95
		0.040	0.041	101 ^b	
	Medial	0.077	0.077	100 ^a	100
		0.063	0.064	101 ^b	
	Caudal	0.086	0.077	90 ^a	94
		0.069	0.067	98 ^b	

Rats were subjected to lesioning by unilateral injection of the excitotoxin ibotenate into the NBM. Animals were killed 6 weeks after surgery and the brains removed, fixed, frozen and sliced using a cryostat as detailed elsewhere (see legend to Fig. 3.7 and 3.8). Total and non-specific binding of 1nM (final concn.) ^{125}I -BoNT to 10 μm brain sections was determined by 2-dimensional laser densitometric scanning of resultant autoradiograms and the specific binding in the non-lesioned (control) and lesioned sides of the brain calculated. The table shows the data from the binding of both types A and B BoNT assayed on sections from at least two lesioned animals (^a, ^b; experiments with different lesioned animals and labelled toxin preparations). The average reduction in the cholinergic enzyme markers ChAT and AChE in the lesioned c.f. the intact contralateral sides in each of the above experiments was ~55%.

after surgery, no significant decrease in the specific binding of ^{125}I -BoNT (A or B) in the cortex of the lesioned/untreated sides was observed (data not shown). However, 6 weeks after lesioning, a significant reduction in both AChE and ChAT activity (average $\approx$ 50-60% of original; A. Fine, personal communication) was observed in the lesioned c.f. the non-lesioned sides. A similar decrease in the specific binding of ^{125}I -BoNT A between the two sides was also seen, but little or no effect however was observed on the specific binding of ^{125}I -BoNT B in the lesioned/unlesioned regions of adjacent sections from the same experimental animals (Table 3.2; values denoted *). Repetition of this experiment gave similar results (Table 3.2; values denoted $\square$).

3.4. Discussion.

3.4.1. Isolation of cholinergic nerve terminals from the CNS.

Using the immunoaffinity purification procedure described (Section 3.2.1; Richardson, 1986), relatively high levels of purification (≈ 15 -fold) of cholinergic nerve terminals from a crude synaptosomal population could be accomplished. Analysis of the transmitter contents of these purified terminals (Table 3.1) revealed that, whilst there was little or no co-purification in the catecholamines studied, a slight enrichment (≈ 4 -fold) in some of the amino acids was noted, however, this was not as high as the enrichment in ChAT/LDH (≈ 15 -fold). This would seem to confirm the cholinergic specificity of the antiserum reported by Richardson (1983) in which complement-mediated lysis of guinea pig synaptosomes using anti-(Chol-1) released ChAT, a small amount of LDH and enolase but had no effect on the release of serotonin, NA, DA, GABA, glutamate, aspartate or taurine.

3.4.2. Cholinergic specificity of BoNT in the CNS.

Using the radiolabelled toxin preparations previously described (section 2.2.4) it was possible to measure the specific toxin binding to both the crude and the cholinergic-enriched fraction of rat brain synaptosomes (still attached to the immunoadsorbent). Scatchard plots of the specific binding data revealed that the cholinergic nerve terminals possessed a heterogeneous population of acceptor sites (for both BoNT A and B) similar to that observed in the crude synaptosomal population i.e. a small number of sites exhibiting high-affinity for each of the toxin types and a much larger population of acceptors of lower affinity. The binding data is expressed in terms of ChAT/LDH activities since, due to the presence of the antisera, it is inappropriate to express these values in terms of protein content. Rationalisation of the binding data, for both the crude population and cholinergic fraction

of the synaptosomes, in terms of the same levels of LDH activity, resulted in the Scatchard plots for both sets of data being directly superimposable - regardless of the degree of cholinergic enrichment (Fig. 3.3). Comparisons of the amount of protein and LDH activities in the crude synaptosomal population allowed the approximation of the specific binding data in terms of protein content. This conversion resulted in comparable values for the affinities and contents of the two sites in the cholinergic-enriched fraction with those observed in the untreated membranes studied in Chapter 2 (see Fig. 2.6). This would tend to indicate that both the high- and the low- affinity acceptor sites demonstrable in a crude synaptosomal preparation are present, almost exclusively, on cholinergic nerve terminals. However, due to the inaccuracies in Scatchard analysis (see Discussion, Chapter 2), the absolute content of low-affinity acceptors on such terminals is difficult to assess accurately and, therefore, the presence of these sites on other, non-cholinergic terminals cannot be excluded. Further evidence for the cholinergic specificity of the high-affinity acceptors for BoNTs A and B was demonstrated by analysis of the binding of 0.2 and 0.5nM labelled toxin to both the crude population and the cholinergic-enriched terminals from a variety of preparations exhibiting a broad range of recoveries and purification factors for the cholinergic nerve terminals. Comparison between the absolute amounts of binding activity in purified and crude terminals and the degree of purification of cholinergic nerve terminals showed a linear relationship at this toxin concentration (which should preferentially label the high-affinity component) suggesting a direct relationship between the increase in toxin binding to its high-affinity acceptor and the degree of cholinergic enrichment. In an attempt to verify the cholinergic specificity of the acceptors for the BoNT in the CNS, specific binding of radiolabelled toxin to sections from rat brain subjected to unilateral lesioning of the NBM (see Methods 3.2.6) was assessed and comparisons made between the

lesioned and contralateral (control) sides (see Table 3.2). Evidence that the extrinsic cholinergic innervation of the mammalian neocortex is derived mainly from the ventral forebrain NBM was first provided by Shute and Lewis (1967). The cholinergic nature of the neurons projecting from the basal forebrain to virtually all areas of the cortex has since been studied using neurochemical measurements within the cortex following lesions (Johnston et al., 1979; Wenk et al., 1980) or AChE histochemistry combined with retrograde tracing (Wainer et al., 1984). However, with the advent of specific anti-ChAT antibodies, the identity of these cell groups in rats have now been confirmed (Houser et al., 1983). There appears to be good correlation between these areas and those in which specific ^{125}I -BoNT binding was observed (Black and Dolly, 1986a,b; 1987). The cell groups of the NBM form a continuum along the ventral portion of the telencephalon and are responsible for the major component (80-90%) of basal forebrain cholinergic projections to the mesocortical-neocortical mantle (Wainer et al., 1984; Rye et al., 1984). Lesions in the basal forebrain have been shown to produce a reduction in presynaptic cholinergic markers (e.g. AChE, ChAT, ACh and the high-affinity, Na^+ -dependent choline uptake) within the neocortex (Johnston et al., 1979; Lehman et al., 1980; Wenk et al., 1980). Whilst large bilateral lesions of the NBM in rat produce profound disruption of appetitive behaviour often with fatal consequences (Fine et al., 1985a), large unilateral NBM lesions leave one side intact to maintain normal eating and drinking, have no effect on spontaneous locomotor activity and provide an internal control for biochemical and histochemical analysis. Such lesions are performed by the stereotaxic injection into the NBM of the excitotoxin ibotenate. The site of this injection is shown (Fig. 3.1; Fine, 1986) and 1 week after lesioning a reduction in ChAT activity levels to $\approx 43\%$ in dorsolateral frontal and parietal cortex compared with intact contralateral levels has been reported (Fine et al., 1985a,b). The decrease in observed AChE activity due to the NBM

lesions persisted 6 months after surgery as did the levels of ChAT activity (Fine et al., 1985a). In animals killed 6 weeks after surgery there appeared to be a good correlation between the percentage decrease in these cholinergic-specific markers and the reduction observed in the specific binding of ^{125}I -BoNT A in the lesioned compared with the control (non-lesioned), sides (Table 3.2). Such lesions, as already indicated, do not completely abolish all cholinergic innervation in the cortex and a residual amount of ChAT activity in this region is always seen (Johnston et al., 1979; Lehman et al., 1980). This would tend to suggest that intrinsic cortical cholinergic neurons are present or that the basal forebrain lesions have not been extensive enough to completely abolish all of the projecting cholinergic cells (Fibiger, 1982). Cortical undercutting indicated that 20-50% of cortical ChAT is derived from these intrinsic cortical neurons (Emson and Lindvall, 1979) confirming that the NBM lesions reported by Fine et al. (1985a) are nearly complete. The remaining specific binding of ^{125}I -BoNT A in the lesioned side of the brain sections is therefore probably due to these remaining intrinsic cortical neurons. In adjacent sections, from animals killed 6 weeks after lesioning, no difference in the specific binding of ^{125}I -BoNT B was observed between the lesioned and contralateral sides (Table 3.2). This may reflect a difference in the nature of the acceptors for the two toxin types rather than one of transmitter specificity and will be discussed later. Gliosis was seen in some of the sections (Fig. 3.7 (2a), Fig. 3.8 (3a)) in the region of the injection site as previously observed in similar experiments where gliosis and neuronal degeneration associated with the NBM lesion were pronounced (Fine et al., 1985b). This gliosis was not responsible for the observed decrease in BoNT binding however, since it was restricted to areas of the ventral globus pallidus, substantia innominata and lateral hypothalamus and did not extend to the neocortex where density measurements of toxin binding were made. Furthermore, the decreased binding in the lesioned side

was not simply due to the surgical procedure used since animals subjected to lesioning surgery and killed after just one week showed no decrease in specific toxin binding whilst the levels of AChE had decreased $\approx 40\%$. This would tend to suggest that the acceptors for the botulinum toxins are more stable and more persistent (taking longer to be degraded) than these cholinergic enzyme markers.

3.4.3 The acceptor for BoNT B in the CNS.

Since the acceptors for BoNT A and B appeared to reside solely on cholinergic terminals in the rat brain and these acceptors appeared to involve sialic-acid-containing moieties (see Discussion Chapter 2), the possibility that the antigen Chol-1 could specifically bind these neurotoxin molecules was investigated. Inhibition of specific ^{125}I -BoNT binding to rat synaptosomal membranes by sheep polyclonal anti-(Chol-1) antiserum proved of limited success - resulting in inhibition of binding of 1nM ^{125}I -BoNT only at very high antibody concentrations. This suggested either a non-specific interaction or that the antibody-antigen interaction was of lower affinity than the toxin-acceptor binding ($K_d \approx 3 \times 10^{-10}\text{M}$). If the latter were the case then it would not be reconcilable with the use of the antiserum in the immunoaffinity procedure. However, low concentrations (1nM) of BoNT B produced a significant decrease in the ability of the antiserum to cause complement-mediated lysis of cholinergic nerve terminals (Fig. 3.5) even at relatively low dilutions of the antiserum ($\approx 50\%$ reduction at 1:50 final antibody dilution). Moreover, at a fixed antibody concentration (1:100), low concentrations of BoNT B caused a significant decrease in the lysis (Fig. 3.6) whilst BoNT A was without effect at the highest concentration used (100nM). The possibility that this inhibition was due to an effect on the steps in the complement pathway rather than a specific interaction with the antigen was dismissed since the ability of a general rabbit anti-(rat cerebrum) antiserum (DAKO Immunoglobulins, Denmark) to

produce specific complement-mediated release of ChAT from these terminals was unaffected even at the highest toxin concentrations studied (100nM; data not shown). However, the possibility that the toxin causes inhibition of antibody binding simply by steric hindrance cannot be ignored. If this were the case it would infer a similar (co-) localisation of acceptors for BoNT B with the Chol-1 antigen.

If Chol-1 were to play a role in binding BoNT then it would be expected that immunofluorescent localisation of this cholinergic marker would agree with the localisation of specific ^{125}I -BoNT binding. Immunofluorescent studies in *Torpedo marmorata* are not conclusive however, and, whilst Jones et al. (1981) indicate that Chol-1 is present only in the synaptic region of the neuron and absent from cholinergic cell bodies and axons, Ferreti and Borroni have observed immunochemical staining for the antigen on neuronal cell bodies (*personal communication*). Thus, the observations that BoNT binding in the CNS (Black and Dolly, 1986a,b; 1987) is localised in the presynaptic plasma membrane and is not found on cell bodies or myelinated tracts would seem at variance with the possibility that Chol-1 is the sole acceptor for BoNT. However, whilst lesioning of the NBM (see earlier in Discussion) results in a marked decrease ($\approx 50\%$) in the levels of AChE and ChAT activities in rat brains 6 weeks after lesioning surgery, little or no change in the immunohistochemical staining with the anti-(Chol-1) antiserum was observed. Similarly no decrease in specific binding of ^{125}I -BoNT B to such lesioned brain sections was detected suggesting that the acceptor for this toxin is extremely stable.

Estimates for the half-lives of gangliosides in the nervous system vary considerably and are subject to error depending on the protocol used for their detection. The half-lives of glycoconjugates are often determined by measuring the disappearance of saccharide moieties that have previously been labelled. Using such a technique, Ferwerda et al. (1981) estimated the turnover rate

of lipid-bound sialic acid to be approximately 25 days. However recycling of labelled sugars may prolong the apparent turnover time. Calculations by the same authors using another method based on radiolabel incorporation rate yielded shorter half-lives for the sialic acid residues of gangliosides of 6-8 days. Studying the decay curves for labelled gangliosides in optic lobe, Rosner and Merz (1982) demonstrated that the half-lives of gangliosides increased in order of increasing complexity - GD1a < GQ1b < GT1b < GD3 and averaged ≈ 36 days. Furthermore, neuraminidase (the principle degradative enzyme for gangliosides), although detectable in synaptosomes in assays carried out at pH 3.9 (Yohe and Rosenberg, 1977) under conditions of more physiological pH (6.8) appreciable endogenous neuraminidase activity was not detectable (Landa et al., 1981). It is therefore highly probable that the decrease in ganglioside markers from lesioned neurons would not be as marked 6 weeks post-lesioning as the disappearance of the cholinergic-specific enzyme markers, AChE and ChAT. What is more, such gangliosides are able to rapidly exchange between membranes and have been shown to be incorporated by whole cells and subcellular fractions (Callies et al., 1977; Fishman and Brady, 1976; Toffano et al., 1980) as well as by synthetic and synaptic plasma membranes (Masserini and Freire, 1987). This transfer of gangliosides between membranes appears to occur through the aqueous phase and not by micelle-membrane collision-mediated transfer (Masserini and Freire, 1987). If such a process were to take place in degenerating, lesioned, neurons then the cholinergic-specific ganglioside, Chol-1, may be inserted into the membranes which are still in the vicinity of the original cholinergic innervation; such a transfer would be undetectable by immunohistochemistry and light microscopy, thereby resulting in no change in the apparent density of ^{125}I -BoNT B binding in the cortex. If this were the case then Chol-1 would not be expected to remain tissue-specific. However, interaction of the large carbohydrate head groups of gangliosides with membrane

proteins, or other components, may regulate the rate of ganglioside exchange by promoting specific ganglioside interactions at the cell surface (Masserini and Freire, 1987), thus preventing such exchange of Chol-1 in healthy neurons but allowing this transfer to occur in degenerating neuronal membranes following lesioning. Due to the inability of such gangliosides to flip-flop in these membranes (Felgner et al., 1981), the hydrophilic carbohydrate binding moiety would remain exposed to the extracellular surface of the membrane resulting in normal interactions with antibody or toxin molecules.

3.4.4 Possible function(s) of Chol-1.

Whilst the role of gangliosides in the nervous system is discussed in more detail later (See General Discussion), it is of interest to note that these moieties have been implicated in a variety of cellular functions. Bacterial toxins (e.g. Cholera toxin), antigens and Ca^{2+} have all been proposed to bind to gangliosides and induce sequential activations of membrane-associated enzymic reactions and cellular metabolism (Fishman and Brady, 1976; Hakamori, 1981; Ando, 1983). No direct evidence has yet been presented for the function of Chol-1 in the nervous system but the degree of its conservation in many species suggests that it is of crucial importance in the function of cholinergic terminals. It has been postulated that it may play a role as a receptor (e.g. for the high-affinity choline uptake mechanism; Richardson et al., 1982), or possibly it is responsible for the specificity of cell-cell recognition during neuronal development and synapse formation or in maintenance of these synaptic contacts once formed. Work by Ferretti and Borroni (1986) would tend to support the latter of these proposals since, in the chicken brain, Chol-1 seems not to be present during development and appears only after birth suggesting a role in 'cementing' already formed neuronal contacts rather than directing their growth.

Ganglioside acceptors have been implicated in the regulation of specific enzymes or cellular processes e.g. gangliosides have been shown to activate adenylate cyclase (Partington and Daly, 1979), and may activate Mg^{2+} -ATPase, Ca^{2+} -ATPase or (Na^+, K^+) ATPase to varying degrees (possibly by modification of lipid environment; Caputto et al., 1977; Leon et al., 1981). However, whilst the acceptors for BoNT appear to be specific for cholinergic nerve terminals, the toxin inhibits many neurotransmitters with similar dose-response curves (Dolly et al., 1987). This would tend to indicate that the toxin affects a common component in the release process and that intoxication requires the internalisation of the toxin molecule (see General Discussion). It is therefore unlikely that BoNT has a direct effect on release simply by their interaction with such moieties.

3.5. Conclusions.

In conclusion it would appear that BoNT A and B preferentially bind to cholinergic nerve terminals in the CNS as has previously been demonstrated in the PNS (Black and Dolly, 1986a,b). This specificity is conferred by the presence, exclusively on these membranes, of both populations of acceptors for these toxin types. Evidence is presented herein which would indicate that the high-affinity acceptor for BoNT B may be associated with the ganglioside antigen Chol-1. If this is indeed the case then the question arises as to whether this same moiety is responsible for the high-affinity binding of the neurotoxins to the cholinergic nerve terminals observed in the periphery (Black and Dolly, 1986a,b). This would not be unexpected since this molecule appears to demonstrate a remarkable degree of conservation and is found, at least in the CNS, in many different species (Richardson et al., 1982).

CHAPTER 4

Calcium homeostasis and neurotransmitter release in
synaptosomes: effect of BoNT A and B

As mentioned in the previous chapter (section 3.4), the acceptors for BoNT A and B appear to reside almost exclusively on cholinergic nerve endings in the CNS, possibly conferring some specificity of the toxin for inhibition of ACh release from synaptosomes. However, recent work in this laboratory has indicated that, at very high concentrations (200nM), the toxin also inhibits the release of other neurotransmitters (e.g. dopamine, noradrenaline, GABA and glutamate) from these nerve terminal preparations* (Dolly et al., 1987; Sanchez-Prieto et al., 1987). Thus, it would appear to act on a mechanism which is common to the release of many neurotransmitter types. Since the toxins' action does not appear to be on the synthesis or storage of transmitter, attention has been focussed on the events which occur prior to transmitter release, i.e. the Ca^{2+} -mediated release process. The action potential evoked influx of Ca^{2+} into nerve endings is a key event in the stimulation of transmitter release (Rubin, R.P., 1970) and is common to all neurotransmitter types. BoNT could exert its action at any point at, or distal to, this primary influx of Ca^{2+} ions as follows:

(1) BoNT could inhibit the influx of Ca^{2+} across the presynaptic nerve terminal membrane during nerve stimulation, thereby depriving the release mechanism of this vital trigger.

(2) The toxins could act to stimulate the outflow of Ca^{2+} from the cytosol of the nerve terminal (either by increasing its uptake into intracellular organelles thought to be responsible for buffering its levels (Nicholls, 1978b) or by some process resulting in its rapid extrusion into the extracellular space). Either of these processes would result in a much reduced level of intracellular calcium ($[\text{Ca}^{2+}]_i$) such that it never reaches the required threshold level for transmitter release to occur.

(3) Lastly, BoNT may have a direct action on the release mechanism itself - possibly by lowering the affinity for Ca^{2+} of a process crucial to neurotransmission such that stimulated transmitter release cannot take place.

* FOOTNOTE: Synaptosomes apparently lack⁻¹³⁰⁻ the specific receptor-mediated uptake of BoNT observed at the PNS (Black and Dolly, 1987c) and toxin appears to enter these terminals by some form of non-adsorptive pinocytosis. This would explain the apparent lack of transmitter specificity even though the acceptors for BoNT appear to be localised almost exclusively on cholinergic nerve terminals (see Chapter 3).

This latter proposal is difficult to test biochemically since the substrate for the toxin's action has yet to be determined. However, with the advent of the fluorescent indicator dyes such as Quin-2 (Tsien, R.Y., 1981; Tsien et al., 1982) it has become possible to accurately measure the intracellular (cytosolic) levels of Ca^{2+} in a variety of tissue preparations (e.g. synaptosomes, Richards et al., 1984; lymphocytes, Tsien et al., 1982; cloned neuronal cells, Meldolesi et al., 1984; and muscle cells, Ambler et al., 1986). The use of such a technique could allow the first two of the above hypotheses to be tested. Rat cerebrocortical synaptosomes were chosen as a model system for such studies since stimulated release of many neurotransmitter types from these preparations has been well characterised and is effectively inhibited by BoNT (Dolly et al., 1987b; Sanchez-Prieto et al., 1987). In this chapter, the effect of BoNT A and B on Ca^{2+} influx upon depolarisation or Ca^{2+} disposal within rat brain synaptosomes were examined.

4.2 Materials and Methods.

4.2.1. Quin 2: Measurement of $[\text{Ca}^{2+}]$.

Stock solutions of the acetoxymethylester of quin 2 (quin 2-AM, Sigma; for structure see Fig. 4.1a) were made up as described (Tsien et al., 1982) by dissolving the solid in dry dimethylsulphoxide (DMSO; final concentration 6mM) and stored desiccated at -20°C until use.

Synaptosomes were prepared from rat cortex by the method of Dodd et al. (1981), modified as described in section 2.2.3. Aliquots of nerve terminals to be incubated immediately with quin 2-AM were loaded with the dye by a modification of the procedure described by Nachsen (1985). Synaptosomes were resuspended (at $\approx 4-6 \text{ mg/ml}$) in ice-cold Na^{+} solution, unless otherwise indicated containing (mM): NaCl , 145; KCl , 5; MgCl_2 , 1; CaCl_2 , 0.02; glucose, 10; Na pyruvate, 0.1; diethylenetriaminepentaacetic acid

(DTPA) a heavy metal chelator, 0.005; N-2-hydroxyethyl piperazine-N'-2-ethanesulphonic acid (HEPES), 10; pH 7.4 with NaOH. The diluted suspensions were centrifuged briefly in an Eppendorf benchtop centrifuge (9000 x g, 20s) and the resultant pellets (4-6mgs protein) resuspended in 1ml of the same buffer containing 1.2mM (final concentration) CaCl_2 (Ca^{2+} assay buffer). Quin 2-AM was added to a final concentration of $\approx 50\mu\text{M}$. Incubation was continued for a further 30min at 37°C under a 95% O_2 /5% CO_2 atmosphere. The synaptosome suspensions were then diluted 10-fold with Ca^{2+} assay buffer and centrifuged (1500g, 5min) in an MSE Centaur centrifuge. The resultant pellet was resuspended in 400 μl of the same buffer and subjected to fluorescence measurements as detailed below.

4.2.2. BoNT intoxication of synaptosomes and Quin 2 loading.

Synaptosomes were incubated (60min, 37°C) with 200nM BoNT (A or B) in Ca^{2+} assay buffer prior to the addition of quin 2-AM ($\approx 50\mu\text{M}$, final concentration). Incubation was continued at 37°C for a further 30min at which time the synaptosomes were diluted, pelleted and resuspended ($\approx 10\text{mg protein/ml}$) in Ca^{2+} assay buffer. All incubations were performed under an atmosphere of 95% O_2 /5% CO_2 . Control samples were treated as above in the absence of toxin but preincubated (60min, 37°C) with the same volume of the buffer in which the toxin is prepared (0.14M Tris HCl, pH 7.9; final concentration in these incubations of 3.6mM). Quin 2 loaded synaptosomes (50 μl , $\approx 10\text{mg protein/ml}$) were added to 2.5ml of Ca^{2+} assay buffer and fluorescent measurements made as described below (section 4.2.3).

4.2.3. Fluorescent measurements

These were made using a Perkin-Elmer MPF-44B spectrofluorimeter in ratio mode as described elsewhere (Tsien et al., 1982). Standard monochromator settings were 339-nm excitation with 4-nm slits; 490-500-nm emission, 10-nm slits. The resuspended synaptosomes were transferred to a 3ml quartz cuvette and the fluorimeter adjusted to give a baseline of $\approx 50\%$ on the chart recorder. Nerve terminals were prevented from

aggregation by occasional resuspension using a 1ml pippette. Once a steady baseline was achieved, the synaptosomes were depolarised by the addition of KCl (50mM) and the increase in fluorescence, due to increased Ca^{2+} uptake, monitored until the transient had declined significantly. Calibration of fluorescence, in terms of $[\text{Ca}^{2+}]_i$, was made as detailed in the legend to Fig. 4.2.

The effect of 4-AP on $[\text{Ca}^{2+}]_i$ was also studied (see figure legends for more detailed methodology).

4.2.4. Determination of synaptosomal respiration rates.

Synaptosomes were prepared from the cerebral cortices of Dunkin-Hartley strain guinea pigs by Ficoll gradient centrifugation as described previously (Nicholls, 1978a). The nerve terminals (2.2mg of protein/ml of incubation mixture) were suspended in Ca^{2+} -free incubation medium (containing (mM); NaCl, 122; KCl, 3.1; MgSO_4 , 1.2; NaHCO_3 , 5; Tes, 20; glucose, 10; pH 7.4, together with 16 μM -albumin) and divided equally between two identical oxygen-electrode chambers. 1.3mM Ca^{2+} was added to each sample together with 7mM Tris HCl, pH 7.9 (control) and 400nM BoNT (test). Incubation was continued for 90min at 37°C at which time the rate of oxygen consumption of both samples had reached a steady state. 100 μM -veratridine, 10 μM -ouabain and 1 μM FCCP (fluoro-carbonyl cyanide phenylhydrazone) were added sequentially to each of the samples and the changes in oxygen consumption monitored on a chart recorder.

4.3. RESULTS

4.3.1. Quin 2: characterisation of fluorescent spectra.

Addition of quin 2-AM to synaptosomes allows a very rapid uptake of the lipid permeable molecule into the cytoplasm of these preparations. Hydrolysis of the quin 2-AM to the impermeant parent compound, quin 2 (for structures see Fig. 4.1a), is accomplished by endogenous cytosolic esterases and produces the shift in the emission spectrum shown in Fig. 4.1b. From this spectrum (excitation at 339nm) it can be seen that there is a marked shift from an emission wavelength maximum of 432nm for quin 2-AM to 490nm for the free acid.

Using the monochromator settings indicated, traces similar to that shown in Fig. 4.2. could be obtained. Calibration of such traces in terms of the levels of $[Ca^{2+}]_i$ was performed as detailed by Komulainen and Bondy (1987) and is described in more detail in the legend to Fig. 4.2.

4.3.2. Effect of pre-incubation on $[Ca^{2+}]_i$.

From Fig. 4.3 it can be seen that both the basal level of $[Ca^{2+}]_i$, together with the increase upon K^+ -depolarisation, were dependent on the time of pre-incubation of these terminals. Synaptosomes, resuspended and incubated immediately with quin 2-AM (30min, 37°C), demonstrated lower basal levels of $[Ca^{2+}]_i$ (212 ± 10 nM; sem, n=13) than those preincubated (60min, 37°C) prior to further incubation with the dye ($[Ca^{2+}]_i = 266$ nM $\pm$ 22nM; sem, n=13). The differences in the maximal increase in $[Ca^{2+}]_i$ upon depolarisation with 50mM KCl between those synaptosomes incubated with quin 2-AM for just 30min/37°C (461 ± 16 nM) and those pre-incubated for 60min/37°C prior to quin 2 loading (492 ± 33 nM) were, however, less marked. It was therefore felt that the 60min preincubation of these terminals (necessary to produce maximal toxicity when incubated in the presence of BoNT A or B) prior to incubation with the ester would not significantly affect the levels of $[Ca^{2+}]_i$, 'per se' and would, thus, allow the use of such

Fig. 4.1. Structure and fluorescent emission spectra of quin 2 and its ester (quin 2-AM).

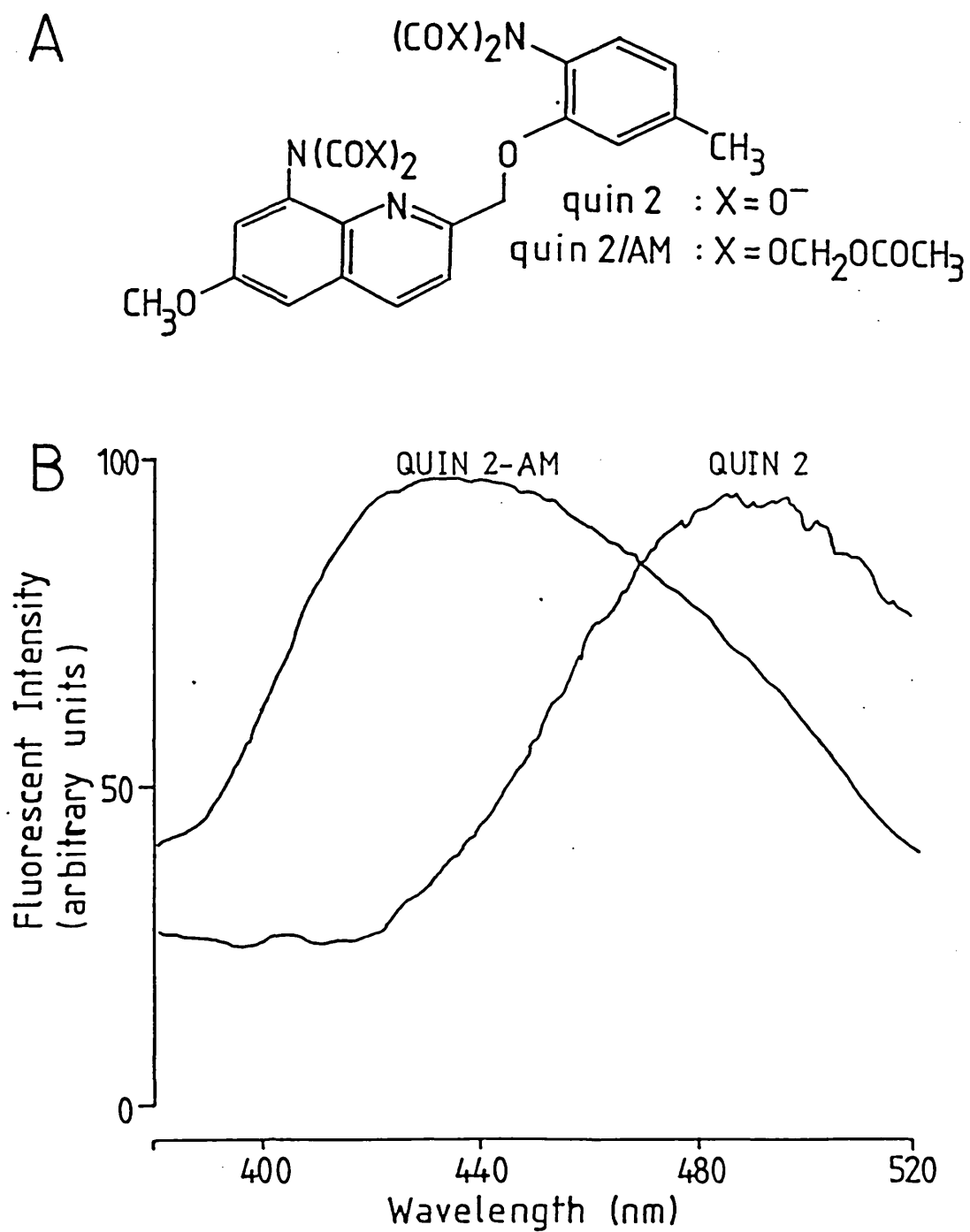
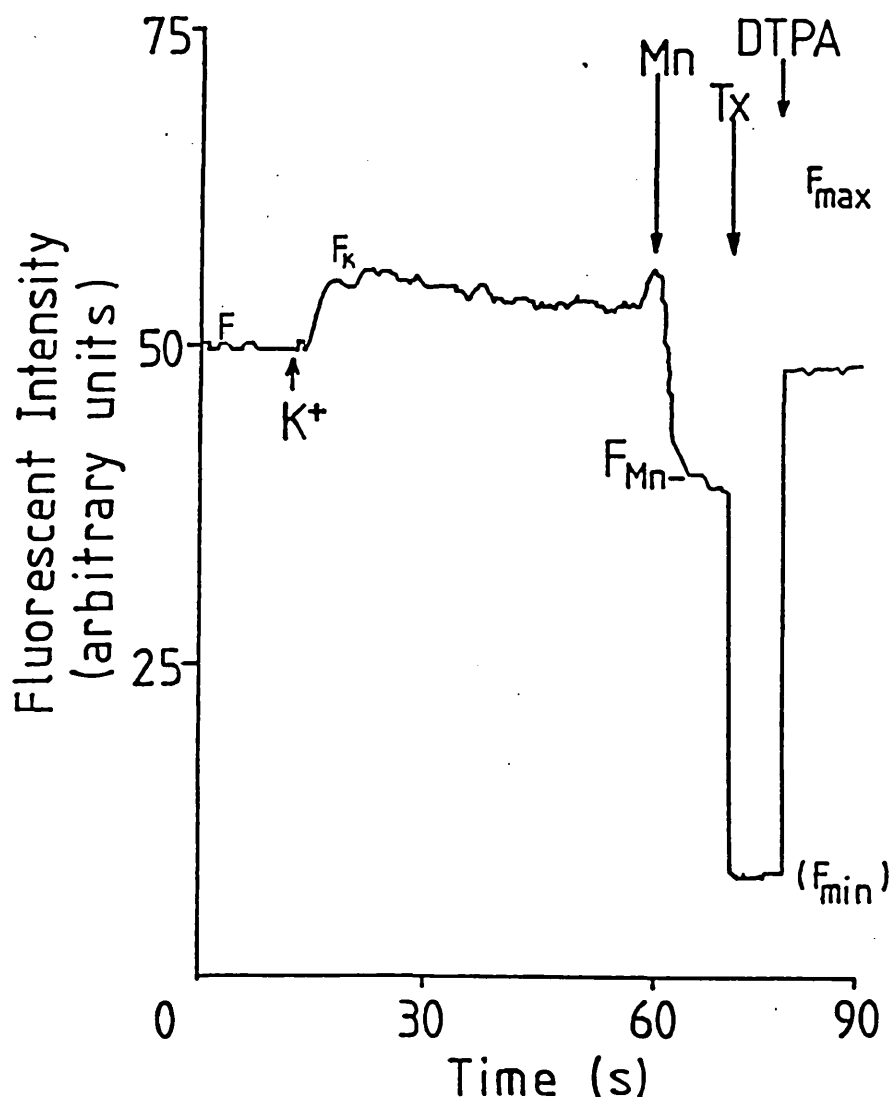


Fig. 4.1. Structure and fluorescent emission spectra of quin 2 and its ester (quin 2-AM).

A) Structure of the fluorescent Ca^{2+} -indicator dye, quin 2, and quin 2-AM. Hydrolysis of the ester yields the chelator tetra-anion, 4 molecules each of acetate and formaldehyde, and 8 protons (from Tsien et al., 1982; Tsien, 1981).

B) Fluorescent emission spectra of quin 2 and quin 2-AM. Samples were incubated with quin 2-AM ($50\mu\text{M}$) as detailed in section 4.2.1. Aliquots of synaptosomes for emission spectra ($\approx 0.5\text{mg}$ protein) were either taken immediately or after 30min at 37°C and resuspended in 2.5ml of Ca^{2+} assay buffer containing 1.2mM Ca^{2+} . Fluorescent measurements were made using a Perkin Elmer MPF 44B spectrofluorimeter in ratio mode as described elsewhere (Section 4.2.3; Tsien et al., 1982). The excitation wavelength used was 339nm (slit width 4nm) and the emitted fluorescence scanned between 380 and 520nm (slit width 10nm). From the resultant curves it can be seen that an aliquot taken immediately (consisting primarily of the lipophilic quin 2-AM) exhibits a single peak at an emission wavelength of 432nm whilst, after 30min at 37°C the hydrolysis of the ester (by endogenous esterases in the cells or synaptosomes) to produce the free acid, quin 2, has resulted in a shift in the emission peak to $\approx 490\text{nm}$.

Fig. 4.2. Typical trace from a single experiment showing method of calibration of $[Ca^{2+}]_i$ from fluorescence.

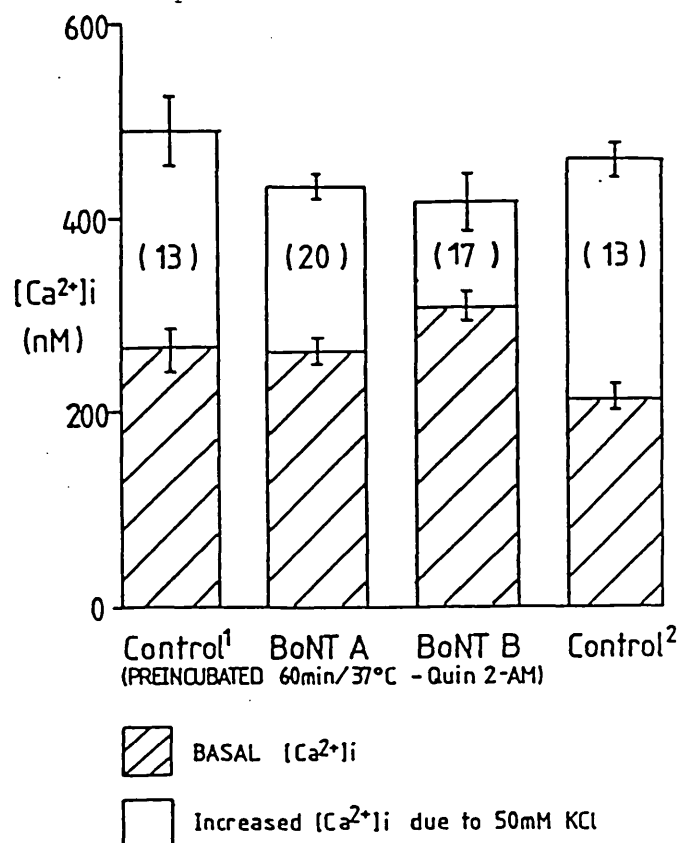


Synaptosomes, incubated with quin 2-AM as indicated in Materials and Methods, were resuspended in Ca^{2+} assay buffer containing 1.2mM Ca^{2+} and placed in a quartz cuvette. Samples were maintained at 30°C by pumping water from a thermostatted water bath around the cuvette holder. After adjusting the signal gain and sensitivity controls to give a 50% reading on the chart recorder, the sample was resuspended several times using a 1ml pipette and measurements continued until a steady signal (representing the basal level of $[Ca^{2+}]_i$; (F)) had been achieved. At this time the chart recorder was started; KCl (50 μ l; 50mM,

final concentration) was added at point 'K' resulting in a rapid increase in the fluorescent signal to a maximum (F_K) as the cells are depolarised and the level of $[Ca^{2+}]_i$ rises. After this transient had declined significantly, the fluorescence values were calibrated, in terms of $[Ca^{2+}]_i$, by the following additions: $MnCl_2$ (15 μ l; 0.5mM, final concentration) was added at the point shown (Mn) and rapidly caused a decrease in the signal to a point F_{Mn} as the Mn^{2+} displaced Ca^{2+} from any leached or unreacted extracellular quin 2. This decrease in the signal represents the contribution, to the overall fluorescence, of the extracellular dye and this value (F_{Mn}) was subtracted from the previous fluorescence values (F and F_K) prior to calculation of $[Ca^{2+}]_i$. (N.B. it is only the initial, rapid decrease in the fluorescent signal upon the addition of $MnCl_2$ which is used in the calibration procedure since after $\approx 20s$ the Mn^{2+} causes a slower, but pronounced decrease in the signal as it enters the cells or synaptosomes and quenches any intracellular quin 2). At this point Triton X-100 (10 μ l; 0.05% (v/v), final concentration) was added (Tx) to lyse the samples and allow access of the Mn^{2+} to the intracellular quin 2. This results in a marked decrease in fluorescence as the amount of quin 2- Ca^{2+} complex is reduced. This represents the minimum fluorescence (F_{min}) as the apparent $[Ca^{2+}]_i < 1nM$. Addition of the heavy metal chelator agent, DTPA (50 μ l; 1.1mM final concentration) to the cuvette produces the maximum fluorescent signal (F_{max}) as the Mn^{2+} is chelated and the quin 2 is saturated with Ca^{2+} . Similar traces were obtained from samples incubated in DMSO in the absence of quin 2-AM in order to determine the amount of autofluorescence of each sample. Autofluorescent values were subtracted from the above prior to calculation of the $[Ca^{2+}]_i$ using the following equation:

$$[Ca^{2+}]_i = K_d (F - F_{min}) / (F_{max} - F)$$

Fig. 4.3. Basal and K^+ -stimulated levels of $[Ca^{2+}]_i$ in synaptosomes in the presence and absence of BoNT.



Synaptosomes from rat cortex (see section 4.2.1) were either loaded immediately with quin 2-AM (Control²) or preincubated (60min, 37°C) in the presence of 3.6mM Tris HCl, pH 7.9 (Control¹) or 200nM toxin (BoNT A or BoNT B) prior to quin 2 loading. Fluorescence measurements were made and calibrated in terms of $[Ca^{2+}]_i$ as indicated in the legend to Fig. 4.2. Values for the basal $[Ca^{2+}]_i$, together with those upon depolarisation with 50mM KCl were determined for each of the above samples. Mean values are shown for the basal $[Ca^{2+}]_i$ for each sample (shaded bars), together with the maximal level reached after K^+ -depolarisation (unshaded bars). Error bars represent $\pm$ sem for the number of replicates shown in brackets (n).

a model system in assessing the effects of these toxins on resting and K^+ -depolarised $[Ca^{2+}]_i$.

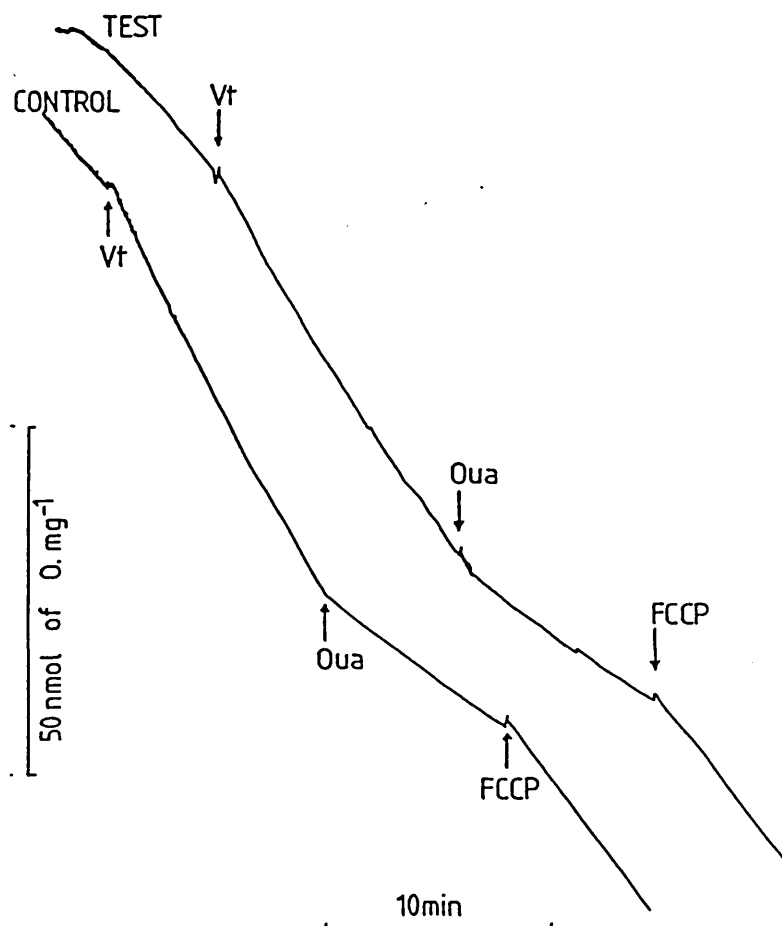
4.3.3. Effect of BoNT A and B on basal and K^+ -depolarised $[Ca^{2+}]_i$ levels.

The basal $[Ca^{2+}]_i$ in control synaptosomes, incubated in the absence of toxin, was found to be 266 ± 22 nM (sem, $n=13$; Fig. 4.3), similar to the values observed in samples intoxicated with BoNT A (262 ± 8 nM). Preincubation with BoNT B gave a slightly higher value for the mean level of basal $[Ca^{2+}]_i$ (306 ± 16 nM, representing a 15% increase over the mean value for the controls). Depolarisation with 50 mM KCl increased the $[Ca^{2+}]_i$ in controls to 492 ± 33 nM whilst in samples intoxicated with BoNT it was increased to ≈ 450 nM. Thus, BoNT A appears to have no effect on resting levels of $[Ca^{2+}]_i$ whilst causing a slight decrease in the level upon K^+ -depolarisation, BoNT B also produces a slight increase in the basal $[Ca^{2+}]_i$ with a subsequent decrease in the maximal level of $[Ca^{2+}]_i$ upon depolarisation. The effects of BoNT on these parameters are summarised in Table 4.1.

4.3.4. Effect of BoNT on respiration rates in synaptosomes.

Incubation (90 min, 37°C) with high concentrations of BoNT B (400 nM) had no significant effect on the respiration rates of synaptosomes treated with veratridine, ouabain and FCCP (Fig. 4.4). Similar results were obtained with synaptosomes intoxicated with 400 nM BoNT A (data not shown). Release of the amino acid, glutamate (measured as described elsewhere (Sanchez-Prieto et al., 1987)), was shown to be effectively inhibited in parallel samples incubated with these high concentrations of BoNT. The respiratory stimulation observed upon the addition of veratridine is presumably due to the enhanced demand for ATP to drive the cycling of Na^+ between the $(Na^+ + K^+)$ -dependent ATPase and the voltage-dependent Na^+ channel. Since BoNT had no effect on the veratridine-stimulated respiration rate, this suggests that the $(Na^+ + K^+)$ ATPase, the Na^+ channel, mitochondrial substrate supply, the respiratory chain, the ATP synthetase and the

Fig. 4.4. BoNT: effect on respiration rates in synaptosomes.



Synaptosomes were prepared and resuspended in Ca^{2+} -free incubation medium (final protein concentration 2.2.mg/ml) as described in section 4.2.6. Incubation (90min, 37°C) in the presence (test) or absence (control) of 400nM BoNT B was performed in identical oxygen electrode chambers whilst the rate of oxygen consumption was monitored. At the points indicated (arrows) 100 μM veratridine (Vt), 10 μM -ouabain (Oua) and 1 μM FCCP were added to each chamber and the changes in oxygen consumption monitored on a chart recorder. Parallel incubations under the same conditions as above were made prior to measurement of stimulated glutamate release as detailed elsewhere (Sanchez-Prieto et al., 1987). BoNT resulted in $\geq 80\%$ inhibition of K^{+} -dependent transmitter release observed in these samples.

Table 4.1. Effects of BoNT (A and B) on $[Ca^{2+}]_i$ in synaptosomes.

Synaptosomes					
	Control	BoNT A		BoNT B	
	$[Ca^{2+}]_i$	$[Ca^{2+}]_i$	% control	$[Ca^{2+}]_i$	% control
BASAL	266±22nM (n=13)	262±8nM (n=20)	98	306±16nM (n=7)	115
(50mM) K ⁺ depolarised	492±33nM (n=13)	456±10nM (n=20)	93	442±30nM (n=7)	89
% increase in $[Ca^{2+}]_i$ due to K ⁺	184	174		144	

Synaptosomes were pre-incubated (60min, 37°C) in the presence of (final concentrations) 3.6mM Tris HCl, pH 7.9 (Controls) or 200nM BoNT (A or B) prior to incubation for a further 30min at 37°C with quin 2-AM. Synaptosomes were washed and resuspended at ≈10mg/ml in Ca^{2+} assay buffer; fluorescent measurements and calibration of $[Ca^{2+}]_i$ were made as detailed in Fig. 4.2. Values for the basal and K⁺-stimulated $[Ca^{2+}]_i$ are expressed as a percentage of the mean control values. Similarly, the % increase of $[Ca^{2+}]_i$ upon K⁺-depolarisation is also shown for controls and in the presence of each of the toxin types. Values are shown in terms of $[Ca^{2+}]_i$ (nM) ± sem (the number of replicates for each set of experiments (n) is shown in brackets).

mitochondrial adenine-nucleotide translocator are all still active in intoxicated nerve terminals (Nicholls et al., 1985). After ouabain addition, the subsequent rate of respiration represents the basal respiration of the mitochondria, together with that required to maintain synaptosomal ATP requirements other than those due to the Na^+ pump. FCCP acts as a mitochondrial uncoupler by catalysing proton uniport across the mitochondrial membrane. The increased respiration rate observed in the presence of FCCP is due to removal of respiratory control and reversal of the ATP synthetase, resulting in increased ATP hydrolysis (Nicholls, 1982).

4.3.5. Effect of 4-aminopyridine (4-AP) on $[\text{Ca}^{2+}]_i$

Addition of 4-AP (10-1000 μM) to quin 2 loaded synaptosomes was followed by a protracted increase in the fluorescence signal; reaching a maximum after $\approx 30\text{s}$. The increase in $[\text{Ca}^{2+}]_i$ produced by 4-AP was dose-dependent but appeared to be reaching saturation at the highest concentration used (1000 μM ; Table 4.2). However, the percentage increase in $[\text{Ca}^{2+}]_i$ at this concentration of 4-AP was not as large as that produced upon depolarisation using 50mM KCl (Table 4.1).

Table 4.2.

Concentration dependence of 4-aminopyridine on $[Ca^{2+}]_i$.

4-Aminopyridine			
[4-AP] μM	BASAL $[Ca^{2+}]_i$	4-AP $[Ca^{2+}]_i$	% increase over basal
10	278 $\pm$ 16nM (n=6)	318 $\pm$ 16nM (n=6)	14
100	288 $\pm$ 4nM (n=7)	370 $\pm$ 9nM (n=7)	28
1000	300 $\pm$ 23nM (n=8)	400 $\pm$ 32nM (n=8)	33

Synaptosomes were prepared and loaded with quin 2 as indicated in section 4.2.1. After a steady fluorescent signal had been attained for the resting level of $[Ca^{2+}]_i$, 4-aminopyridine (4-AP) at the final concentrations shown (10,100,1000 μM) was added to the suspension and fluorescent measurements continued. The 4-AP produced a gradual increase in fluorescence, reaching a maximum after $\approx$ 30s. The signals were calibrated as indicated earlier (see legend to Fig. 4.2) and the mean values for both the resting $[Ca^{2+}]_i$ and the maximal level after 4-AP addition are shown together with the % increase over basal levels. Values are shown $\pm$ sem for the number of replicates indicated in brackets (n).

4.4.

Discussion.

4.4.1. The role of intracellular calcium in neurotransmitter release.

The regulation of cytosolic Ca^{2+} plays an important role in the modulation of many cellular processes. Depolarisation of the nerve membrane results in an increase in the cytosolic Ca^{2+} activity which appears to act as a trigger for the release of neurotransmitter. Due to the maintenance of a charge distribution across neuronal membranes, a membrane potential can be measured using ion distribution techniques (Akerman and Nicholls, 1981a). The main component of this potential is a K^+ equilibrium potential. Depolarisation-induced Ca^{2+} influx and subsequent increase in $[\text{Ca}^{2+}]_i$ occurs via specific voltage-dependent Ca^{2+} channels and can be induced artificially by increasing the external K^+ concentration, (thus reducing the membrane potential) and can be selectively blocked by some organic compounds such as verapamil, D-600 and nifedipine (Baker, 1972; Akerman and Nicholls, 1981b). Since the extracellular Ca^{2+} concentration is usually around 10^{-3}M , active transport of Ca^{2+} is required to maintain the gradient of this cation ($\text{Ca}^{2+}_{\text{out}}/\text{Ca}^{2+}_{\text{in}} = 10^4\text{--}10^5$) across the plasma membrane. Intraterminal organelles such as mitochondria and endoplasmic reticulum can accumulate substantial amounts of Ca^{2+} 'in vitro' (Blaustein et al., 1980), however, McGraw et al. (1982) showed that mitochondria were unable to sequester Ca^{2+} if the free concentration is $< 5 \times 10^{-7}\text{M}$. Thus, at the levels of $[\text{Ca}^{2+}]_i$ typically present in nerve terminals ($\approx 10^{-7}\text{M}$) mitochondria are unlikely to regulate the resting level of $[\text{Ca}^{2+}]_i$. However, upon depolarisation, the increased $[\text{Ca}^{2+}]_i$ ($> 10^{-6}\text{M}$) is probably buffered by these organelles (Akerman and Nicholls, 1981c) which may serve to return the $[\text{Ca}^{2+}]_i$ to its initial basal level. The main regulator of cytosolic Ca^{2+} in non-depolarised neurons is

thought to be the $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism (Nachsen, 1985; Blaustein, 1974) in which Ca^{2+} efflux occurs in exchange for 3-4 Na^+ ions. Ca^{2+} may also be extruded from the cytosol by an enzymic mechanism linked directly with ATP hydrolysis, catalysed by a $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATPase (Sobue et al., 1979; Gill et al., 1981).

4.4.2. Quin 2: measurement of $[\text{Ca}^{2+}]_i$

Fluorescent Ca^{2+} -indicator dyes have a number of useful properties. These include having a high affinity for Ca^{2+} , low affinity for Mg^{2+} and H^+ , large absorbance and fluorescence changes resulting from Ca^{2+} chelation (approximately 5-fold increase from Ca^{2+} -free to Ca^{2+} -saturated forms) and little or no binding to membranes (Tsien et al., 1982b). However, quin 2 has a number of drawbacks as an intracellular Ca^{2+} indicator, for example it is relatively insensitive to Ca^{2+} concentrations above micromolar levels, its extinction coefficient and quantum yield are not very high and its absorption spectrum overlaps substantially with that of endogenous fluorescent chromophores. Thus, in order to overcome the contribution due to cellular autofluorescence, it is necessary to load the cells with millimolar concentrations of the dye. This may result in the quin 2 acting as a Ca^{2+} buffer and may slow $[\text{Ca}^{2+}]_i$ transients upon depolarisation. For the types of experiment described herein any such effect is negligible since we are only interested in making a comparison in the levels of fluorescence between two sets of samples (in the presence and absence of toxin) loaded with the dye in exactly the same way. Thus any contribution by the dye to the buffering of $[\text{Ca}^{2+}]_i$ transients would be equal in both the tests (in the presence of toxin) and the controls. Larger intracellular quin 2 concentrations create a number of problems: 1) the products of quin 2-AM hydrolysis (formaldehyde) are toxic to synaptosomes and 2) since quin 2 is such a good buffer of Ca^{2+} , the larger the intracellular concentration of the dye, the greater the apparent $[\text{Ca}^{2+}]_i$. Another problem in the use of quin 2 in such measurements arises from the overlap in the emission spectra between the free acid and its precursor, quin 2-AM (Fig.

4.1b). Inefficient hydrolysis of the ester to the free acid can result in spurious signals as some contribution from the quin 2-AM to the overall fluorescence occurs. This has previously been indicated to be a problem in synaptosomes due to a lack of endogenous esterases (Meldolesi et al., 1984). However, extensive washing of synaptosomes effectively removed all unreacted ester and its hydrolysis to the free acid was routinely monitored as shown in Fig. 4.1b.

In order to allow for the contribution to the signal of any quin 2 which had leached from the samples, calibration was made by a modification of that described by Komulainen and Bondy (1987) and Hesketh et al. (1983; see Fig. 4.2) in which Mn^{2+} was used to compete with Ca^{2+} for binding by quin 2. The affinity of quin 2 for Mn^{2+} is $10^{2.7}$ greater than its affinity for Ca^{2+} and Mn^{2+} (0.5mM) quenches quin 2 fluorescence by >99% in the presence of 1mM Ca^{2+} (Hesketh et al., 1983). The initial decrease in fluorescence upon Mn^{2+} addition was rapid, presumably due to quenching of extracellular quin 2. Thereafter, the value of $[Ca^{2+}]_i$ decreased more slowly, perhaps due to Mn^{2+} entering the samples (Komulainen and Bondy, 1987) and thus all readings of extracellular dye were made within 20s of the addition of the Mn^{2+} .

It is important to note here that due to their nature, synaptosomes are very sensitive to the time of incubation since they are unable to maintain continued energy supplies and, hence, ion gradients. Thus, as the levels of ATP decline with increased time of incubation, the levels of $[Ca^{2+}]_i$ rise. This is particularly evident in Fig. 4.3 where it can be seen that those synaptosomes used immediately for fluorescence measurements yielded typically lower levels of basal $[Ca^{2+}]_i$ than those preincubated for 90min at 37°C (≈ 212 nM c.f. ≈ 266 nM respectively). These values agree well with the observations made by Nachsen (1985) in which he notes that the basal $[Ca^{2+}]_i$ in synaptosomes within 100min was ≈ 218 nM and, upon incubation at 30°C, gradually increased to ≈ 292 nM over the next 140min. Other authors have estimated the resting levels of intrasynaptosomal calcium to be

around 100nM (Meldolesi et al., 1984; Richards et al., 1984; Ashley et al., 1984), 220nM (Hansford and Castro, 1985; Nachsen, 1985) or even as high as 370nM (Komulainen and Bondy, 1987). The methods to determine these values appear to vary in the procedures employed for synaptosome preparation, the levels of quin 2 used in loading and the method of calibration of the fluorescence in terms of $[Ca^{2+}]_i$. One explanation for the discrepancies in these values may be that more rapid synaptosomal preparations have higher esterase activities (resulting in efficient cleavage of the quin 2-AM) and higher levels of ATP (enabling more efficient maintenance of ionic gradients). Finally, measurements of changes in $[Ca^{2+}]_i$ levels would be irrelevant if loading with high concentrations of quin 2 resulted in a marked reduction in the stimulated neurotransmitter release. However, although modulation of $[Ca^{2+}]_i$ transients at high $[quin\ 2]_i$ would be expected, this was shown to have no effect on stimulated neurotransmitter release from synaptosomes (Meldolesi et al., 1984; Richards et al., 1984).

4.4. . Effect of BoNT on $[Ca^{2+}]_i$

Studies using the cytosolic fluorescent indicator dye, quin 2, have allowed a number of the hypotheses of BoNT action on $[Ca^{2+}]_i$ (see General Introduction; 1.4.4) to be tested. The basal level of cytosolic Ca^{2+} , the maximal increase upon K^+ -depolarisation and the rate at which this transient declined could all be monitored directly and comparisons made between the levels of $[Ca^{2+}]_i$ in samples incubated in the presence and absence of BoNT.

From the results obtained, it is apparent that, whilst BoNT A has little effect on basal $[Ca^{2+}]_i$, it causes a small decrease in K^+ -stimulated levels of $[Ca^{2+}]_i$. BoNT B, under the same conditions, produces a slight increase in the basal $[Ca^{2+}]_i$, with a concomitant decrease in the respective value observed upon K^+ -depolarisation (Fig. 4.3; Table 4.1). The increase in $[Ca^{2+}]_i$ upon K^+ -depolarisation was due to influx of Ca^{2+} via the voltage-

dependent Ca^{2+} channels since this was effectively blocked in the presence of $10\mu\text{M}$ verapamil (data not shown). These observations would appear to confirm those made previously for BoNT A, i.e. that the toxin does not inhibit the depolarisation-induced influx of Ca^{2+} into the nerve terminal and, similarly, does not increase the enzymic disposal mechanisms for $[\text{Ca}^{2+}]_i$, since the basal level of $[\text{Ca}^{2+}]_i$ is similar to that in the control. Likewise, the rate of decline of the depolarisation-induced $[\text{Ca}^{2+}]_i$ maximum (due to $[\text{Ca}^{2+}]_i$ disposal) was also not significantly altered in either of the systems tested. Furthermore, BoNT was shown to have no effect on synaptosomal respiratory capacity, respiratory control (see Results), ATP synthesis, plasma membrane potential or $^{45}\text{Ca}^{2+}$ uptake upon depolarisation (Sanchez-Prieto et al., 1987). Based on the above and the further observation that intoxication was not overcome by increased extracellular Ca^{2+} but was reversed by the $\text{Ca}^{2+}/2\text{H}^{+}$ -exchange ionophore, ionomycin, it was proposed that exocytosis is triggered by the penetration of Ca^{2+} into an intracellular hydrophobic milieu and that this step is blocked by the toxin because ionomycin is able to bypass it and deliver Ca^{2+} to the exocytotic apparatus (Sanchez-Prieto et al., 1987). However, whilst 4-AP has previously been reported to overcome the blockade of transmitter release induced by BoNT in the PNS (Cull-Candy et al., 1976), presumably by raising $[\text{Ca}^{2+}]_i$, recent observations have suggested that 4-AP is unable to overcome the intoxication produced by BoNT in central preparations (Ashton and Dolly, 1988). This would seem to be confirmed by the small increment in $[\text{Ca}^{2+}]_i$ observed in similar preparations (Table 4.2) and would suggest a differential sensitivity of the two systems to this compound.

BoNT B caused a slight decrease in the maximal level of $[\text{Ca}^{2+}]_i$ upon K^{+} -depolarisation of synaptosomes, exaggerated by its slight increase in basal $[\text{Ca}^{2+}]_i$ (Table 4.1). This was not considered to be a significant change due to an increased rate of cytosolic Ca^{2+} disposal since, upon incubation with high concentrations of BoNT B (400nM) neither a change in the ATP/ADP ratio of such treated nerve terminals (Sanchez-Prieto et al.,

1987) nor an increase in energy metabolism was observed in toxin-treated c.f. control preparations (Fig. 4.4). This change in the level of K^+ -induced Ca^{2+} influx cannot be ignored, ^{however} since, in the release of DA from synaptosomes, ^{and Sanchez-Armass} Nachsen (1987) ~~have~~ shown, using Fura 2, that transmitter release increases steeply in a non-linear relationship with increased $[Ca^{2+}]_i$. This would imply that small changes in the inward Ca^{2+} current, or in the resting Ca^{2+} level in the cytosol of the nerve terminals, may have disproportionately large effects on transmitter release. One possibility is that BoNT may act by selectively blocking a small population of Ca^{2+} channels in the immediate vicinity of the active zones. This would cause a localised decrease in $[Ca^{2+}]_i$, which may result in the Ca^{2+} -dependent 'trigger' mechanism failing to produce transmitter release.

In summary, whilst BoNT A or B, under conditions shown to produce significant inhibition of neurotransmitter release from synaptosomes, may exert their effects by selectively blocking the entry of Ca^{2+} upon depolarisation, thus lowering the depolarisation-induced maximal $[Ca^{2+}]_i$, they do not appear to act by lowering the basal $[Ca^{2+}]_i$ by stimulating energy-dependent disposal systems. However, BoNT may have a direct action on the release mechanism itself - possibly by lowering the affinity for Ca^{2+} of an enzymic step crucial to the exocytotic process such that stimulated release cannot take place.

CHAPTER 5

GENERAL DISCUSSION

5.1. Introduction.

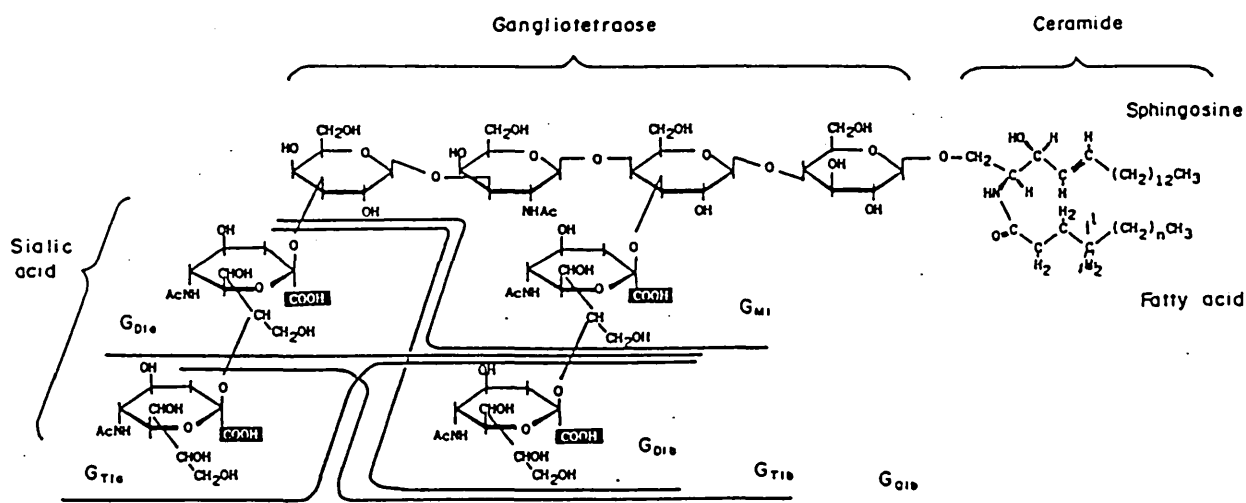
From the observations presented herein (see Chapters 2 and 3) it would appear that, as previously reported for BoNT A (Williams et al., 1983), the acceptor for BoNT B in the CNS may involve sialic acid containing moieties. Indeed, the evidence would appear to suggest a possible association of ganglioside moieties with the acceptor components exhibiting high affinity for the latter toxin type. In this chapter, the nature of these moieties, their possible functions and the ways in which these may be linked with the intoxication produced by BoNT are evaluated. Furthermore, putative mechanisms of the intracellular action of BoNT are discussed in relation to the experimental evidence.

5.2. The nature of ganglioside molecules.

Gangliosides, first identified in brain by Klenk (1935), comprise a family of acidic glycolipids that are characterised by the presence of negatively charged sialic acid moieties. They are amphipathic molecules i.e. contain both a hydrophilic carbohydrate group and a hydrophobic 'tail' region. The carbohydrate portion consists of sialic acid molecules, hexoses and N-acetylated hexosamines. The hydrophobic (ceramide) moiety is believed to act as an anchor in the plasma membrane and consists of a long-chain fatty acid linked to the C-2 carbon atom of the amino alcohol sphingosine by an amide bond. Oligosaccharides are linked via a glycosidic bond to C-1 of the sphingosine portion of ceramide. In the case of mammalian brain gangliosides, sialic acids are located at the non-reducing and/or internal galactose residues of neutral saccharide chains. Although also present on non-neuronal tissues as very minor constituents (Wiegandt, 1982; Ando, 1983), sialoglycolipid accounts for an estimated 10% of the total lipid content of the neuronal plasma membrane (Ledeen, 1978). The amount of lipid-bound sialic acid is approximately twice that observed for protein-bound sialic acid in the neuron, a situation reversed in other cell types. These structures show great diversity as a result of the combination of neutral oligosaccharides of various chain lengths and different numbers of sialic acid residues. The

length of the spacer neutral oligosaccharides, together with the degree of branching, as well as the number of sialic acid residues, may affect the density of negative charge at the membrane surface and regulate specific binding phenomena. Even the distribution of gangliosides on the cell surface may be of functional importance, since it has been observed that they do not appear evenly distributed, but rather exist in clusters surrounding membrane-bound proteins as functional aggregates (Sharom and Grant, 1978; Yamakawa and Nagai, 1978; Lee and Grant, 1980).

Gangliosides are normally classified according to their backbone structure of neutral oligosaccharides (Svennerholm, 1963). The structures of the major gangliosides are shown below (from Ando, 1983).



5.1. Structures of mammalian gangliosides with a gangliotetraose (GgOse₄) backbone.

The enzymes responsible for ganglioside biosynthesis are present on the cell surface membrane and may be associated as an enzyme complex (Roseman, 1970). Gangliosides in a precursor pool and newly formed molecules are less accessible to neuraminidase than older molecules arranged in membranes (Caputto et al., 1976). Soluble forms of gangliosides, even though at very low concentrations, have been demonstrated in rat brain synaptosomes

(Ledeen et al., 1976) as in bovine brain cortex (where cytosolic gangliosides form soluble complexes with protein in a definite ratio; Sonnino et al., 1979). Gangliosides are principally synthesized in neuronal perikarya and transported to nerve endings together with macromolecules (Ledeen et al., 1976; Landa et al., 1979) although local synthesis within the axon and nerve endings (Ledeen et al., 1976; Tettamanti et al., 1980) or at the plasma membrane (Prete et al., 1980) cannot be excluded.

5.3. Possible functions of gangliosides in toxin action.

5.3.1. Gangliosides as acceptors for bacterial toxins.

Since the observation by van Heyningen (1971) that cholera toxin may bind to cell membranes through gangliosides these moieties have been implicated in the specific binding of other bacterial toxins, including Tetx (van Heyningen, 1974) and BoNT (Kitamura et al., 1980; see also Chapters 2 and 3). The binding of cholera toxin (consisting of 1A and 5B subunits) to the monosialoganglioside G_{M1} has been well-characterised (van Heyningen et al., 1971; Cuatrecasas, 1973; Holmgren et al., 1973) and appears to be mediated by the five B subunits (Cuatrecasas, 1973; Holmgren et al., 1973; van Heyningen, 1974; DeWolf et al., 1981) whilst the A subunit, or part of it, crosses the membrane and is involved in the activation of adenylate cyclase (Gill et al., 1975; Bennett et al., 1975). The conformational change required for the insertion of the A subunit may be as a result of multivalent binding of one toxin molecule to several ganglioside acceptors and a redistribution of surface components by lateral movement of the toxin-ganglioside complex (patching and capping) in the fluid phase since this has been demonstrated in lymphocytes (Bennett et al., 1975; Revesz and Greaves, 1975).

Binding of Tetx to its ganglioside acceptor(s) appears to be followed (at 37°C) by the rapid internalisation of the toxin-acceptor complex as visualised by insensitivity to neuraminidase in cerebral neuron cultures (Yavin et al., 1983).

However, as detailed by these authors, this insensitivity to the enzyme could be due to a number of possibilities including; (1) temperature-dependent transfer of the toxin from its ganglioside acceptor to another receptor lacking sialic acid; (2) a chemical modification of the sialic acid moiety or steric hindrance by the toxin-receptor complex preventing access of the enzyme to its substrate or (3) the existence of two classes of acceptors for the toxin, one of which is insensitive to the enzyme. Similar work on primary cultures of mouse spinal cord neurons (Critchley et al., 1985) and on a neuroblastoma-retina hybrid cell line (N18-RE-105; Staub et al., 1986) using antiserum to Tetx and ¹²⁵I-labelled protein A chase experiments (together with indirect immunofluorescence) or pronase digestion of ¹²⁵I-toxin-acceptor complex has further indicated this rapid (<15min/37°C) internalisation of Tetx. This internalisation process appears to be energy-dependent since metabolic inhibitors prevented uptake of the toxin with no apparent effect on its binding (Staub et al., 1986). Thus, it would appear that a binding step is followed by a rapid, energy-dependent, internalisation process. However, in these as with other studies (Schwab and Thoenen, 1978; Montesano et al., 1982), uptake of toxin bound to its acceptor on the cell surface is not complete and may confirm the possible existence of 'effective and ineffective' receptor components as proposed by Zimmerman and Piffaretti (1977). Furthermore, the internalised toxin appears to be associated primarily with non-lysosomal structures (smooth vesicles) and not with coated pits (Critchley et al., 1985; Montesano et al., 1982) which may explain the toxins' exceptionally long half-life (≈6 days; Critchley et al., 1985).

There appears to be a remarkable degree of similarity between Tetx and BoNT in their structure, binding activity and possibly their mode of action. Some of these are detailed elsewhere (section 1.5; Habermann and Dreyer, 1986). Evidence, presented herein (Chapters 2 and 3) would tend to indicate that the acceptor for BoNT B may be associated with a ganglioside moiety. The nature of the acceptors for the other

botulinum toxin types, whilst also seeming to involve sialic acid moieties, possibly in association with a proteinaceous component(s) (at least for BoNT A; Williams et al., 1983) is less well defined but again implicates the possible functional importance of the gangliosides. In the case of BoNT these extracellular acceptors appear to confer specificity on the toxins' interaction with the presynaptic cholinergic nerve terminal in both the PNS (Dolly et al., 1986a, b) and CNS (see Chapter 3).

If the acceptor moieties on presynaptic neurons in the PNS, responsible for the translocation of BoNT from the extracellular membrane to an intracellular compartment (Dolly et al., 1986a, b), and ultimately, to their site(s) of action are similar/identical to those observed in the CNS then this would suggest a role for sialic acid-containing moieties, and possibly gangliosides, in receptor-mediated endocytosis. This raises a number of questions however: What is receptor-mediated endocytosis? Can gangliosides participate in this process? and does this play an important role in the pathology of intoxication?

5.3.2. Receptor-Mediated Endocytosis (RME): possible role in BoNT internalisation.

This is an energy-dependent mechanism whereby ligands bound to high-affinity acceptors on the extracellular membrane are internalised. Many of the studies of this process have been made on fibroblasts which possess receptors for insulin, epidermal growth factor (EGF), low-density lipoprotein (LDL), diphtheria, shigella and pseudomonas toxins. These receptor-ligand (R-L) complexes move to specialised regions of the membrane by random diffusion and form clusters in areas known as coated pits - first demonstrated by Roth and Porter (1964). These coated pits provide an entry route for a variety of ligands interacting with physiologically important receptors as above and calculations based on the mobilities of fluorescently labelled lipids or proteins have indicated that each receptor will encounter a coated

pit every 4-5 seconds (Schlessinger et al., 1978; Maxfield et al., 1981), fast enough to account for the rapid internalisation of some ligands. Phase-contrast microscopy revealed that the uncoated vesicles containing the ligands (receptosomes) are probably not lysosomes (which are 'phase-dense'; Pastan and Willingham, 1981b). These receptosomes (ranging in size from 2500-4000Å in diameter) are often seen near coated pits (1500Å in diameter) and are believed to be derived from these structures.

Materials which do not bind to specific receptors enter cells via non-specific endocytosis in another organelle termed a 'pinosome' which fuses with lysosomes and whose contents are then rapidly degraded (Willingham et al., 1979).

Galloway et al. (1983) used the fluorescein spectrum to demonstrate that isolated endocytotic vesicles became acidified in the presence of ATP. This would appear to confirm the existence in these organelles of the ATP-driven proton pump described by Schneider (1981). Many ligands dissociate from their receptors at low pH (including LDL, lysosomal enzymes and asialoglycoproteins) and would thus allow the unoccupied receptors to return to the cell surface. This effect of pH on ligand-receptor uncoupling and recycling is most clearly demonstrated in the studies made on transferrin (Dautry-Varsat et al., 1983).

Opportunistic ligands, including toxins, can also use the acid pH of the endosome to gain entry into the cytoplasm e.g. diphtheria, Tetx and BoNT are believed to bind to cell-surface receptors and enter the endosome where the acid pH induces a conformational change that allows an active portion of the toxin to cross the membrane and enter the cytoplasm (Donovan et al., 1981; Boquet and Duflot, 1982; Shone et al., 1987; Hoch et al., 1985). In the cases of BoNT and Tetx the acid pH (<5) in the endosome may allow a conformational change in the heavy chains that exposes a hydrophobic region which can insert into the lipid membrane and form pores. These pores are large enough to allow the passage of NAD^+ as well as K^+ ions. The process of pore formation appears to be mediated by the 50000 NH_2 terminal of the heavy chains of both toxins (the H_2 fragment of BoNT; Shone et al.,

1987) whilst the light chains are inactive at any pH. Hoch et al. (1985) proposed that these toxin fragments may act as 'tunnel proteins', allowing the transport of active peptide fragments of the toxins across the endosomal membrane of acidic vesicles. Thus, it would appear that the 3 distinct regions of BoNT (namely H₁, H₂ and light chain; see Fig. 1.1) are each responsible for one of the steps in the process of intoxication (Shone et al., 1987). Binding of the toxin to the presynaptic nerve surface is mediated by the H₁ fragment (Shone et al., 1985). The toxin, bound to its acceptor is endocytosed, apparently by acceptor-mediated endocytosis (Dolly et al., 1984) and the vesicle is acidified (Galloway et al., 1983) resulting in a conformational change in the H₂ fragment allowing its insertion into the membrane and the possible translocation into the cytosol of an 'active fragment' which is proposed to be either the light subunit or the whole toxin - the latter apparently being more likely since intracellular injection of intact BoNT A directly into the cholinergic cell bodies of *Aplysia* inhibited transmitter release (Dolly et al., 1987c). This proposed pathway of RME and subsequent processing of BoNT has apparently been demonstrated in the peripheral nervous system (Dolly et al., 1984; Black and Dolly, 1986a,b, 1987) and it would appear that, if the acceptors for these toxins are similar in the PNS to those observed in the CNS, this process is mediated by sialoglycopeptides (for BoNT A; Williams et al., 1983) or gangliosides (BoNT B; Evans et al., 1986).

In the cases of BoNT A and B, binding of toxin to a single acceptor moiety may exhibit low-affinity whilst cross-linking of groups in the membrane by a single toxin molecule (similar to the multivalent binding of cholera toxin to G_{M1} gangliosides (DeWolf et al., 1981)) may result in the apparent high-affinity sites seen under Scatchard analysis. This possible cross-linking reaction has been shown to be a prerequisite in the endocytosis of some molecules and may also be important in the rapid internalisation of the BoNT (although no evidence exists for this hypothesis). Furthermore, Gammon and Ledeen (1985) demonstrated the presence in brain of a cytoplasmic protein

capable of ganglioside transfer from membrane to membrane. If this transfer were to occur in the presence of bound toxin this protein may play a role in trans-synaptic transport of BoNT. The possible involvement of gangliosides per se in the binding and internalisation of both Tetx and BoNT is complicated by the fact that these charged moieties may interact with one-another, with surrounding membrane components or with the toxin.

Thus, in answer to the questions proposed earlier, it would appear that ganglioside moieties may well play a role in some form of acceptor-mediated endocytosis since they can be internalised through several pathways. * However, whether this mechanism is similar to the classical energy-dependent receptor-mediated endocytotic pathway is not clear from the literature. Furthermore, the importance of the internalisation process is questionable since injection of native toxin directly into the neurons of *Aplysia* also produces a blockade in release of neurotransmitter (Dolly et al., 1987c), suggesting that some form of toxin processing during RME is not essential for expression of toxicity.

5.3.3. The role of gangliosides in neurotransmitter release.

As indicated in the General Introduction (see section 1.3) there are several steps involved in neurotransmitter release. Depolarisation of the neuronal membrane results in Ca^{2+} influx into the presynaptic nerve terminal which causes evoked release of neurotransmitter, possibly via fusion of synaptic vesicles with the presynaptic membrane or cytosolic release via a direct gating system (for review see Dunant, 1986). Ganglioside moieties may play a direct/indirect role at any one of these steps and hence modulate the neurotransmitter release process. Reports that an antibody directed against the monosialoganglioside G_m1 can enhance the depolarisation-induced release of neurotransmitter GABA from rat brain slices have been published (Frieder and Rapport, 1981, 1987). Furthermore, this effect appears to implicate a role for this ganglioside species in ion channels, producing increased K^{+} -

or veratridine-stimulated GABA release whilst not affecting that *NB. Temperature-dependent uptake of cholera toxin, which binds specifically to the ganglioside GM_1 , has previously been demonstrated in cultured chicken sympathetic neurons and avian erythrocytes (Joseph et al., 1978, 1979). Endocytosis into the GERL occurred within 30 min and the cholera toxin was associated with these organelles for a further 24h (Joseph et al., 1979). Thus, BoNT may also be endocytosed in a similar manner in the nervous system.

observed in the presence of the Ca^{2+} -ionophore A23187 (Frieder and Rapport, 1987). Since elimination of the involvement of Na^+ channels did not affect the action of the antibodies, whereas elimination of the involvement of Ca^{2+} channels abolished their effect, it was suggested that the ganglioside, GM_1 , may participate in the function of Ca^{2+} channels involved in GABA release. Antibodies to these gangliosides localise on the cell surface rather than intracellularly (Laev et al., 1978) and these ganglioside moieties possess a high affinity for Ca^{2+} and Mg^{2+} ions (Sharom and Grant, 1978). Furthermore, since binding of these divalent cations is postulated to alter the excitability of membranes (Rahman, 1983), ganglioside moieties may play a role in Ca^{2+} binding to - and transport through - the membrane. The observation that these same antibodies were ineffective on other neurotransmitter systems (serotonin and noradrenaline; Frieder and Rapport, 1981) was explained by a topographical distribution of the ganglioside GM_1 with respect to the ion channels which varies in the different neurotransmitter-specific terminal membranes. Further evidence for an interaction between glycoconjugates and a Ca^{2+} channel has been obtained by studying the effect of the tetravalent lectin, Concanavalin A (Con A), on K^+ -dependent uptake of $^{45}\text{Ca}^{2+}$ in PC12 cells (Greenberg et al., 1987). Contrary to the effect observed in lymphocytes (Tsien et al., 1982) Con A did not produce membrane depolarisation in PC12 cells nor did it result in increased binding of $^{45}\text{Ca}^{2+}$ to the cell membrane but rather appeared to have a specific action on the voltage-dependent Ca^{2+} channel which, as assessed by ^3H -labelled antagonist binding, appears to be a glycoprotein (Curtis and Catterall, 1984; Borsotto et al., 1985). From these observations and those made in the discussion to Chapter 4, it is possible that the effect of BoNT on inhibition of neurotransmitter release may be as a direct consequence of binding to similar glycoproteins in the neuronal membrane which represent a subpopulation of Ca^{2+} channels in the vicinity of the active release zones (see later in discussion for more detail).

In contrast to the observations that gangliosides could mediate ionic events at the membrane surface which modulate transmitter release, the possibility of an intracellular role for such moieties has also been proposed (Simmoneau et al., 1980). Direct injection of either ruthenium red or neuraminidase into both cholinergic or non-cholinergic neurons of *Aplysia*, produced a blockade in synaptic transmission with no apparent effect on membrane conductance, spike conductance or Ca^{2+} permeability in the presynaptic terminal, and no effect on postsynaptic ACh receptors. Based on these observations Simmoneau et al. (1980) proposed that sialic acid fragments of gangliosides and/or sialoglycoproteins, capable of binding Ca^{2+} , were located in close proximity to the Ca^{2+} channels of the terminals and that, during the presynaptic depolarisation, Ca^{2+} enters, binds to the negatively charged sialic acids and causes conformational changes of the macromolecules involved in transmitter release. Removal of these intracellular sialated moieties abolishes these changes and hence prevents transmitter release. Since gangliosides are important in regulating the negative charge of the presynaptic membrane and the fluidity of membranes, all of the above observations could be dismissed as secondary effects or artifacts due to the decreased negative charge of the membrane by the aforementioned treatments. However, if the above observations were correct then the possibility would exist that the ganglioside/glycoprotein acceptor moieties for BoNT could also be functional components which, upon binding of BoNT prevented transmitter release. If the extracellular presynaptic sialated acceptors for BoNT were functional then toxicity would be observed upon toxin binding and this would be expected to be reversed by removal of the bound toxin using antibodies. This does not appear to be the case however, as Simpson (1981) proposed that there are at least 3 distinct phases of toxin action; 1) binding 2) internalisation and 3) expression of toxicity (with both of the first two steps being required before the expression of the latter since, using anti-toxin antibodies, bound toxin could be removed

from its acceptors and its effects on transmitter release negated (Simpson, 1981)).

5.4. Possible mechanism(s) of action of BoNT.

In attempting to elucidate the molecular mechanism(s) of the action of BoNT, a variety of tissue preparations have been studied including the neuromuscular junction, synaptosomes and neuronal hybrid cell lines. Much data has accumulated from each of these model systems and yet controversy in these findings has meant that the mechanism of toxin action is still not fully characterised. Problems arise in that gross extrapolation of data from one model system to another, differing intoxication conditions (e.g. incubation time and temperature) and utilisation of different toxin types (even different strains of the same toxin) has resulted in many hypotheses from one system subsequently being disproved in another. Furthermore, the possibility exists that each of the toxin types (A-G) may have a different site of action within the neuron.

Since, at the neuromuscular junction, nerve stimulation accelerates the rate of muscular paralysis (Hughes and Whaler, 1962) and the intoxication produced is dependent on the presence of Ca^{2+} ions in the incubation fluid (Simpson, 1971, 1973), it would appear that intracellular Ca^{2+} or initiation of transmitter release is necessary before the toxin can exert its paralytic effect. However, it is not known whether the process of nerve stimulation and/or the presence of Ca^{2+} play an important role in the final stages of inhibition or whether they are involved in the latter stages of internalisation into the nerve endings. From the observed ability of the toxin to inhibit the release of many transmitter types (see earlier) it would appear that intoxication was due to the inhibition of a process in the release mechanism which may be common to many neurotransmitter-specific terminals.

In order to try and elucidate the mechanism of BoNT action we studied the effect of BoNT A on the assembly and disassembly of dissociated chick microtubules 'in vitro' and on

fast axonal transport 'in vivo' using excised chick dorsal root ganglia in primary culture and phase-contrast microscopy. However, no effect on either the rate of microtubule assembly/disassembly, the maximal level of assembled microtubules at equilibrium, the rate of fast axonal transport or the numbers of vesicles and other organelles being transported was detected (data not shown). Thus it appears that the toxin does not prevent transmitter release by retarding/preventing the delivery of essential elements to the presynaptic membrane. This is consistent with the observed ability of certain pharmacological agents to overcome this blockade (see General Introduction). Habermann and Dreyer (1986) have also indicated that they find no effect of either Tetx or BoNT on interactions with the aggregation of microtubules or Ca^{2+} uptake, nor on protein phosphorylation, Na^+, K^+ -ATPase or on changes in adenylate or guanylate cyclase activity. In contrast to the latter observations, however, a report has been published which indicates that both BoNT and Tetx partially inhibit (60%) both the soluble and particulate guanylate cyclase activity in both permeabilised guinea pig synaptosomes and cultured nerve cells (NG108-15; Smith and Middlebrook, 1985); such an effect has yet to be fully characterised. Another study has suggested that botulinum toxin (types C₁ and D) ADP-ribosylate specific proteins ($\text{Mr} \approx 24\text{Kd}$) in membrane preparations of PC12 cells and in neuron-rich cultures of foetal rat brain cells ($\text{Mr} \approx 25-26\text{Kd}$) - suggesting that their targets may be novel GTP-binding proteins localised on cell membranes (Matsuoka et al., 1987). Similarly, a 21Kd membrane protein of bovine adrenal gland has also been shown to serve as the substrate for BoNT D-catalysed ADP-ribosylation (Ohashi and Narumiya, 1987). However, these results have been questioned and are apparently not mediated by the 150Kd neurotoxin molecule but by a contaminant protein ($\text{Mr} \approx 23\text{Kd}$) present at very low concentrations ($<0.1\%$ of the total protein) in preparations of types C₁ and D toxins. Thus, these apparently 'homogeneous' preparations of neurotoxin are contaminated with a minute quantity of a highly active molecule (called C₃) which is invisible on

Coomassie-stained polyacrylamide gels (M.Gill, unpublished observations).

Of all the hypotheses proposed to date, those implicating the role of Ca^{2+} , both in transmitter release and in the intoxication process, appear to be favoured. The specificity of BoNT A for the inhibition of Ca^{2+} -dependent release of glutamate from synaptosomes is extremely marked - having no effect on Ca^{2+} -independent release from the same terminal preparations (Sanchez-Prieto et al., 1987). As indicated earlier, BoNT may have a small effect on K^+ -depolarised Ca^{2+} uptake into these terminals via the voltage-dependent Ca^{2+} channels with little or no effect on basal $[\text{Ca}^{2+}]_i$ (see Chapter 4; Sanchez-Prieto et al., 1987). This would seem at variance with the hypothesis that BoNT lowers $[\text{Ca}^{2+}]_i$ levels such that Ca^{2+} influx upon depolarisation cannot reach the threshold level required to stimulate release. BoNT cannot be acting via such an energy-dependent mechanism since it is clearly shown (Fig. 4.4) that it has no effect on synaptosomal respiration rates. Furthermore, the ability of ionomycin (an electroneutral $\text{Ca}^{2+}/2\text{H}^+$ exchange ionophore) to overcome almost complete blockade by BoNT of KCl-induced release of glutamate cannot simply be due to its ability to overcome a blockade in Ca^{2+} entry (although see discussion Chapter 4). Thus the results observed with ionomycin are probably due to the toxins' action in decreasing the sensitivity of the exocytotic mechanism to cytosolic Ca^{2+} such that normal entry of this cation through Ca^{2+} channels is insufficient to activate exocytosis whilst the gross elevation of cytosolic Ca^{2+} by the ionophore is sufficient to overcome this insensitivity. Therefore, the possibility exists that the neurotoxin acts by decreasing the affinity for Ca^{2+} of a molecule which synchronises release of transmitter from a number of 'releasing sites'. This molecule could be similar to that of 'Calsynchronin' proposed by Dunant (1986) in which this molecule is responsible for synchronising the release of transmitter from a number of 'mediatophore' elements (see General Introduction for more detail). The existence of such a distinct entity in close proximity to the releasing sites has

not been demonstrated but the possibility exists that its function is mediated by components of the mediatophores. For example, the mediatophore elements are believed to be glycosylated - these carbohydrate chains possibly exhibiting low-affinity for Ca^{2+} as proposed for similar ganglioside moieties (Sharom and Grant, 1978). Upon depolarisation, Ca^{2+} rapidly enters the cytosol of the neuron and increases its intracellular concentration by as much as 10-fold. This large concentration of $[\text{Ca}^{2+}]_i$ may bind to the glycosylated moieties of the 'mediatophores' or similar releasing sites and cause a conformational change in the moieties allowing the release of their bound transmitter. Modification of the sialic groups on these elements may thus reduce their affinities for Ca^{2+} such that, even under depolarisation-induced Ca^{2+} influx, the levels of Ca^{2+} do not reach the threshold level required to promote transmitter release. Alternatively, as proposed in the discussion to Chapter 4, a sub-population of Ca^{2+} channels sensitive to BoNT blockade may exist in the immediate vicinity of these releasing sites. Blockade of a small number of Ca^{2+} channels by BoNT may thus prevent sufficient increase in $[\text{Ca}^{2+}]$ at the release site to allow a conformational change and hence transmitter release to occur. Indeed, in this context, Simmoneau et al. (1980) have demonstrated that injection of the enzyme neuraminidase directly into the presynaptic neuron of *Aplysia* causes inhibition of transmitter release from the nerve terminal, possibly by cleaving such sialic-acid-containing moieties responsible for binding Ca^{2+} and initiating the conformational changes required for release. Due to their unique potency of action it has long been postulated that BoNT act by modifying some enzymic process or even possess some inherent enzymic activity. If the above hypotheses are correct then this activity could involve modification of the mediatophore elements themselves or, as postulated by Dunant (1986) the molecules synchronising transmitter release from these elements. Thus, the possibility that the toxins may act by cleaving or modifying essential sialic-acid-containing groups on the mediatophores has been investigated to determine whether BoNT possess neuraminidase-

like activity. To this end synaptosomes were intoxicated by incubation with BoNT (A or B) under conditions known to produce maximal inhibition of transmitter release. The synaptosomes were then lysed and sialic-acid moieties labelled with ^3H by oxidation with periodate followed by reduction using tritium-labelled borohydride. The labelled glycoproteins were separated on a 5-20% SDS polyacrylamide gel and subjected to autofluorography. Comparisons were made between the labelling patterns observed in the presence and absence of BoNT in order to determine whether cleavage of the labelled groups had indeed taken place. No significant difference in the labelling patterns was observed. However, the fact that BoNT inhibits the release of many neurotransmitter types from synaptosomes with similar dose-response curves (Dolly et al., 1987b), would suggest that, if it were acting to modulate these mediatophore elements, similar mechanisms must exist in all transmitter-specific nerve terminals.

5.5. BoNT as a clinical tool.

To reiterate, BoNTs high-affinity binding to cholinergic-specific membrane acceptors, both in the periphery and in the central nervous system (at least in rats), is believed to be mediated by the heavy subunit whilst it is proposed that the light subunit possesses some enzymic action and is the 'active component' responsible for the inhibition of transmitter release. The two subunits are linked by a disulphide bridge and can be separated from one another and purified almost to homogeneity (see Fig. 1.2) and neither expresses toxicity on its own. The unique potency, together with their target specificity make BoNT a potentially useful pharmacological tool in the treatment of some disorders. The intact toxin is already undergoing clinical trials in the cure of both strabismus (ocular squint; Scott, 1980; Elston et al., 1985) and blepharospasm (which can cause almost permanent blindness due to the inability of the patient to control facial muscular spasms resulting in closure of the eyes for long periods). Injection of the toxin into discrete facial muscles prevents this spasm and is far more effective, less time-consuming

and less traumatic than the surgical methods used previously (Elston and Russell, 1985; Elston, 1986).

The observation that BoNT is toxic when injected intracellularly (Dolly et al., 1987c; Penner et al., 1986) suggested that processing of the toxin by some mechanism during receptor-mediated endocytosis does not appear to be essential for the expression of this toxicity. Therefore, upon separation of the two chains of BoNT, highly useful molecular probes would be generated. The purified heavy subunit could act as a highly selective probe for targetting drugs or antibodies to cholinergic nerve endings. Radiolabelled, this moiety may provide a useful cholinergic terminal marker for histochemistry. As is the case with the light subunit, the possession of at least one free sulphhydryl group would allow relatively straightforward coupling of the heavy chain to the molecule of interest. Such 'chimeric' molecules have already been manufactured by coupling the A-subunit of diphtheria toxin or ricin with lectins, other toxin subunits and antibodies (Cumber et al., 1985; Raso and Basala, 1984; Middlebrook and Kohn, 1981). The possible uses include prevention/recovery from toxin poisoning since heavy chain, coupled to either an antibody against the active light chain, or possibly an inhibitor/promoter of the enzyme modified by the toxin, may allow recovery of the nerve terminals from intoxication. Thus no quotation would appear to be more apt for the future applications of BoNT than that made by Dr. Haven Emson (1874-1957)

"The poisons are our principal medicines, which kill the disease, and save the life."

REFERENCES.

REFERENCES

- Abrams, A., Kegeless, G. and Hottle, G.A. (1946) J. Biol. Chem. 164: 63-79.
- Agui, T., Syuto, B., Oguma, K., Iida, H. and Kubo, S. (1983) J. Biochem. 94: 521-527
- Agui, T., Syuto, B., Oguma, K., Iida, H. and Kubo, S. (1985) J. Biochem. 97: 213-218.
- Akerman, K.E.O. and Nicholls, D.G. (1981a) Eur. J. Biochem. 115: 67-73
- Akerman, K.E.O. and Nicholls, D.G. (1981b) Eur. J. Biochem. 117: 491-497
- Akerman, K.E.O. and Nicholls, D.G. (1981c) Biochem. Biophys. Acta. 645: 41-48
- Akerman, K.E.O. and Nicholls, D.G. (1983) TIBS 8: 63-64
- Ambler, S.K. and Taylor, P. (1986) J. Biol. Chem. 261: 5366-5371
- Ando, S. (1983) Neurochem. Int. 5: 507-537
- Ashley, R.H., Brammer, M.J. and Marchbanks, R. (1984) Biochem. J. 219: 149-158.
- Ashton, A.C. and Dolly, J.O. (1988) in press.
- Baker, P.F. (1972) Prog. Biophys. Mol. Biol. 24: 177-223
- Beers, W.H. and Reich, E. (1969) J. Biol. Chem. 244: 4473-4479.
- Bennett, V., O'Keefe, E. and Cuatrecasas, P. (1975) Proc. Nat. Acad. Sci. 72: 33
- Bevan, S. (1976) J. Physiol. (Lond.) 258: 145-155.
- Bhavanandan, V.P. and Katlic, A.W. (1979) J. Biol. Chem. 254: 4000-4008.
- Birman, S., Israel, M., Lesbats, B. and Morel, N. (1986) J. Neurochem. 47: 433-444.
- Bizzini, B. (1979) Microbiol. Rev. 43: 224-240.
- Bjorklund, A. and Lindvall, O. (1975) Brain Res. 83: 531-537.
- Black, J.D. and Dolly, J.O. (1986a) J. Cell. Biol. 103: 521-534.

- Black, J.D. and Dolly, J.O. (1986b) J. Cell. Biol. 103: 535-544.
- Black, J.D. and Dolly, J.O. (1987) Neurosci. (in press)
- Blaustein, M.P. (1974) Rev. Physiol. Biochem. Pharmacol. 70; 34-82
- Blaustein, M.P., McGraw, C.F., Somlyo, A.V. and Schweitzer, E.S. (1980) J. Physiol. (Paris) 76: 459-470.
- Blaustein, M.P., Ratzlaff, R.W. and Schweitzer, E.S. (1980) Fed. Proc., Fed. Am. Soc. Exp. Biol. 39; 2790-2795
- Bonafacino, J.S. and Paladini, A.C. (1981) Anal. Biochem. 118: 213-220.
- Boquet, P. and Duflot, E. (1982) Proc. Nat. Acad. Sci. 79; 7614-7618
- Boroff, D.A., delCastillo, J., Evoy, W.H. and Steinhardt, R.A. (1974) J. Physiol. (Lond.) 150: 105-168.
- Boroff, D.A., Nyberg, S. and Hoglund, S. (1972) Infect. Immun. 6: 1003-1007.
- Borsotto, M., Barhanin, J., Fossett, M. and Lazdunski, M. (1985) J. Biol. Chem. 260; 14255-14263
- Bradford, M.M. (1976) Anal. Biochem. 72: 248-254
- Brooks, V.B. (1956) J. Physiol. (Lond.) 134: 264-277.
- Burgen, A.S., Dickens, V.F. and Zatman, L.J. (1949) J. Physiol. (Lond.) 109: 10-24.
- Burgisser, E. (1984) TIPS 5: 142-144.
- Callies, R., Schwarzmann, G., Radsak, K., Siegert, R. and Wiegandt, H. (1977) Eur. J. Biochem. 80: 425-432
- Caputto, R., Maccioni, H.J., Arce, A. and Cumar, F.A. (1976) In: 'Ganglioside Function: Biochemical and pharmacological implications' (Porcellati, G., Ceccarelli, B. and Tettamanti, G., eds) Adv. Exp. Med. Biol. 71; 72-144
- Caputto, R., Maccioni, A.H.R. and Caputto, B.I. (1977) Biochem. Biophys. Res. Commun. 74: 1046-1052
- Ceccarelli, B. and Hurlbut, W.P. (1980) Physiol. Rev. 60: 396-441
- Chamness, G.C. and McGuire, W.L. (1975) Steroids 26: 538-542.
- Colmeus, C., Gomez, S., Molgo, J. and Thesleff, S. (1982) Proc. R. Soc. Lond. Ser. B. 215: 63-74

- Critchley, D. R., Habig, W. H. and Fishman, P. H. (1986) J. Neurochem. **47**: 213-222.
- Critchley, D. R., Nelson, P. G., Habig, W. H. and Fishman, P. H. (1985) J. Cell Biol. **100**; 1499-1507
- Cuatrecasas, P. (1973) Biochemistry **12**; 3547-3581.
- Cuatrecasas, P., Parikh, I. and Hollenberg, M. D. (1973) Biochemistry **12**; 4253-4264.
- Cull-Candy, S. G., Lundh, H. and Thesleff, S. (1976) J. Physiol. **260**; 177-203.
- Cumber, A. J., Forrester, J. A., Foxwell, B. M. J., Ross, W. C. J. and Thorpe, P. E. (1985) Methods in Enzymology, Academic Press, N.Y. **112**; 207-225.
- Curtis, B. M. and Catterall, W. A. (1984) Biochem. **23**; 2113-2118
- DasGupta, B. R. and Boroff, D. A. (1967) Biochem. Biophys. Acta **147**: 603-605.
- DasGupta, B. R. and Boroff, D. A. (1968) J. Biol. Chem. **243**: 1065-1072.
- DasGupta, B. R., Boroff, D. A. and Rothstein, E. (1966) Biochem. Biophys. Res. Commun. **22**: 750-756.
- DasGupta, B. R. and Sugiyama, H. (1980) Biochem. Biophys. Res. Commun. **93**: 369-375.
- DasGupta, B. R. and Sugiyama, H. (1972a) Infect. Immun. **6**: 587-590.
- DasGupta, B. R. and Sugiyama, H. (1972b) Biochem. Biophys. Acta **268**: 718-729.
- DasGupta, B. R. and Sugiyama, H. (1976) Infect. Immun. **14**: 680-686.
- DasGupta, B. R. and Sugiyama, H. (1977b) Can. J. Microbiol. **23**: 1257-1266.
- Dautry-Varsat, A., Ciechanover, A. and Lodish, H. F. (1983) Proc. Nat. Acad. Sci. **80**; 2258-2262
- DeBelleoche, J. S. and Bradford, H. F. (1977) J. Neurochem. **29**; 335-343.
- DeDuve, C., DeBarsy, T., Poole, B., Trouet, A., Tulkens, P. and Van Hoof, F. (1974) Biochem. Pharmacol. **23**; 2495-2531.
- DelCastillo, J. and Katz, B. (1954) J. Physiol. (Lond.) **124**; 560-573.
- DelCastillo, J. and Katz, B. (1957) In: *Microphysiologie Comparee des Elements Excitables*. Paris: Centre Natl. Rech. Sci. Colloq. Int. **67**: 245-258

- DeRobertis, E.D.P., Pellegrino de Iraldi, A., DeLores Arnais, G.R. and Salganicoff, L. (1962) J. Neurochem. 9: 23-35
- DeWolf, M.J.S., Fridkin, M. and Kohn, L.D. (1981) J. Biol. Chem. 256: 5489-5496.
- Dickson, E.C. and Shevky, R. (1923a) J. Exp. Med. 38: 327-346.
- Dickson, E.C. and Shevky, R. (1923b) J. Exp. Med. 37: 711-731.
- Docherty, M., Bradford, H.F. and Joh, T.H. (1986) F.E.B.S. 202: 37-40
- Dodd, P.R., Hardy, J.A., Oakley, A.E., Edwardson, J.A., Perry, E.K. and Delaney, J.-P. (1981) Brain Res. 226: 107-118.
- Dolly, J.O., Black, J.D., Williams, R.S. and Melling, J. (1984) Nature 307: 456-460.
- Dolly, J.O., Lande, S. and Wray, D.W. (1987a) J. Physiol. (Lond.) 386: 475-484.
- Dolly, J.O., Ashton, A.C., Evans, D.M., Richardson, P.J., Black, J.D. and Melling, J. (1987b) in: "Cellular and Molecular Basis of Cholinergic Function." (eds., Dowdall, M.J. and Hawthorne, J.N.) Ellis Horwood, Chichester. p517-533.
- Dolly, J.O., Maisey, A., Poulain, B., Wadsworth, J. and Tauc, L. (1987c) In: Neurotoxins as tools in neurochemistry. (J.O. Dolly, ed.), Ellis Horwood, Chichester.
- Dolly, J.O., Tse, C.K., Black, J.D., Williams, R.S., Wray, D., Gwilt, M., Hambleton, P. and Melling, J. (1981) in Biomedical Aspects of Botulism (Lewis, G.E., ed.) pp 47-64. Academic Press, New York.
- Dolly, J.O., Williams, R.S., Black, J.D., Tse, C.K., Hambleton, P. and Melling, J. (1982) Toxicon. 20: 141-148.
- Donovan, J.J., Simon, M.I., Draper, R.K. and Montal, M. (1981) Proc. Nat. Acad. Sci. 78: 172-176
- Dreyer, F., Becker, C., Bigalke, H., Funk, J., Penner, R., Rosenberg, F. and Ziegler, M. (1984) J. Physiol. (Paris.) 79: 252-258.
- Dreyer, F., Mallart, A. and Brigant, J.L. (1983) Brain Res. 270: 373-375.
- Dreyer, F. and Schmitt, A. (1981) Neurosci. Letts. 26: 307-311.
- Dunant, Y. (1986) Progress in neurobiol. 26: 55-92.
- Eklund, M.W. and Poysky, F.T. (1972) Appl. Microbiol. 24: 108-113.
- Eklund, M.W. and Poysky, F.T. (1974) Appl. Microbiol. 27: 251-258.

- Elston, J.S. (1986) J. Neurol. Neurosurg. and Psychiatry. 49; 827-829.
- Elston, J.S. and Ross Russell, R.W. (1985) Br. Med. J. 290; 1857-1859.
- Emsen, P.C. and Lindvall, O. (1979) Neurosci. 4: 1-30
- Evans, D.M., Williams, R.S., Shone, C.C., Hambleton, P., Melling, J. and Dolly, J.O. Eur. J. Biochem. 154; 409-416
- Fatt, P. and Katz, B. (1952) J. Physiol. (Lond.) 117: 109-128.
- Feldman, R.A., Morris, J.G. and Pollard, R.A. (1981) in Biomedical Aspects of Botulism. (Lewis Jr., G.E., ed.) p.271-284, Academic Press Inc., New York.
- Felgner, P.L., Freire, E., Barenholz, Y. and Thompson, T.E. (1981) Biochemistry 20: 2168-2172
- Ferretti, P. and Borroni, E. (1984) J. Neurochem. 42; 1085-1093.
- Ferretti, P. and Borroni, E. (1986) J. Neurochem. 46: 1888-1894
- Ferwerda, W., Blok, C.M. and Heijlman, J. (1981) J. Neurochem. 36: 1492-1499
- Fibiger, H.C. (1982) Brain Res. Rev. 4: 327-388
- Fine, A. (1986) Scientific American 255: 42-50
- Fine, A., Dunnett, S.B., Bjorklund, A. and Iversen, S.D. (1985a) Proc. Nat. Acad. Sci. (USA). 82: 5227-5230
- Fine, A., Dunnett, S.B., Bjorklund, A., Clarke, D. and Iversen, S.D. (1985b) Neurosci. 16: 769-786
- Fishman, P.H. and Brady, R.O. (1976) Science (Washington D.C.) 194: 906-915
- Fonnum, F. (1975) J. Neurochem. 24; 407-409.
- Frieder, B. and Rapport, M.M. (1981) J. Neurochem. 37; 634-639
- Frieder, B. and Rapport, M.M. (1987) J. Neurochem. 48; 1048-1052
- Galloway, C.J., Dean, G.E., Marsh, M., Rudnick, G. and Mellman, I. (1983) Proc. Nat. Acad. Sci. 80; 3334-3338
- Gammon, C.M. and Ledeen, R.W. (1985) J. Neurochem. 44; 979-984

- Gill, D.C., Grollman, F. and Kohn, L.D. (1981) J. Biol. Chem. 256: 184-192
- Gill, D.M. and King, C.A. (1975) J. Biol. Chem. 250: 6424-6432.
- Gimenez, D.F. and Ciccarelli, A.S. (1970) Zentralbl. Bakteriол. Parasitenkd. Infektionskr. Hyg. Abt. Orig. 215: 221-224.
- Goldberg, R.L., Costa, T., Habig, W.H., Kohn, L.D. and Hardegree, M.C. (1981) Molecular Pharmacol. 20: 565-570.
- Gray, E.G. (1976) in Progress in Brain Research. Perspectives in Brain Research. (Corner, M.A. and Swaab, D.F., eds.) Elsevier, Amsterdam. Vol. 45: 207-234.
- Gray, E.G. (1977) in Synapses. (Cotrell, G.A. and Usherwood, P.N.R., eds.) Blackie, London. p6-18.
- Gray, E.G., Hughjones, D. and Barron, J. (1979) J. Neurocytol. 8: 675-685.
- Gray, E.G. and Whittaker, V.P. (1962) J. Anat. 96: 79-88
- Greenberg, C.A., Carpenter, C.L. and Messing, R.O. (1987) J. Neurochem. 48: 888-894
- Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963) Biochem. J. 89: 114-123.
- Gundersen, C.B. (1980) Prog. Neurobiol. (Oxford) 14: 99-119.
- Gundersen, C.B., Katz, B. and Miledi, R. (1982) Proc. R. Soc. B. 216: 369-376
- Habermann, E. (1974) Naunyn-Schmiedeberg's Arch. Pharmacol. 281: 47-56.
- Habermann, E. and Albus, U. (1986) J. Neurochem. 46: 1219-1226.
- Habermann, E., Bigalke, H., Dreyer, F. and Streitziq, P. (1980) in 'Natural Toxins' (eds. Eaker, D. and Wadstrom, T.), pp. 539-599 Pergamon Press, Oxford
- Habermann, E. and Dreyer, F. (1986) Current topics in Microbiol. and Immunol. 129: 93-179.
- Hakamori, S-I. (1981) A. Rev. Biochem. 50: 733-764
- Hansford, R.G. and Castro, F. (1985) Biochem. J. 227: 129-136.
- Harris, A.J. and Miledi, R. (1971) J. Physiol. (Lond.) 217: 497-515.
- Helting, T.B. and Zwisler, O. (1977) J. Biol. Chem. 252: 187-193.

- Hesketh, T.R., Smith, G.A., Moore, J.P., Taylor, M.V. and Metcalfe, J.C. (1983) J. Biol. Chem. **258**: 4876-4882
- Heuser, J.E. (1977) In : Society for Neuroscience symposia (ed. Cowan, A.M. and Ferrandelli, J.A.). Bethesda, MD : Soc. Neurosci. **2**: 215-239
- Heuser, J.E. and Reese, T.S. (1973) J. Cell. Biol. **57**: 315-344.
- Heuser, J.E. and Reese, T.S. (1979a) In: *The Neurosciences: Fourth study program* (ed. Schmitt, F.O. and Worden, F.G.). Cambridge, MA: MIT Press, p573-600
- Heuser, J.E., Reese, T.S., Dennis, M.J., Jan, Y., Jan, L. and Evans, L. (1979b) J. Cell Biol. **81**: 275-300
- Hirokawa, N. and Kitamura, M. (1975) Naunyn-Schmiedeberg's Arch. Pharmacol. **287**: 107-110.
- Hoch, D.H., Romero-Mira, M., Ehrlich, B.E., Finkelstein, A., DasGupta, B.R. and Simpson, L.L. (1985) Proc. Nat. Acad. Sci. **82**: 1692-1696
- Holmgren, J., Lonnroth, I. and Svennerholm, L. (1973) Infect. Immun. **8**: 208-214.
- Houser, C.R., Crawford, G.D., Barber, R.P., Salvaterra, P.M. and Vaughan, J.E. (1983) Brain Res. **266**: 97-119
- Hughes, R. and Whaler, B.C. (1962) J. Physiol. (Lond.) **160**: 221-233
- Hurlbut, W.P. and Ceccarelli, B. (1974) Adv. Cytopharmacol. **2**: 141-154.
- Israel, M., Dunant, Y. and Manaranche, R. (1979) Prog. Neurobiol. (Oxford). **13**: 237-275.
- Israel, M. and Lesbats, B. (1981a) J. Neurochem. **37**: 1475-1483.
- Israel, M., Lesbats, B. and Manaranche, R. (1981b) Nature (Lond.). **294**: 474-475.
- Israel, M., Lesbats, B., Morel, N., Manaranche, R., Krzywicki, G. and Dedieu, C. (1984) Proc. Nat. Acad. Sci. (USA). **81**: 277-281.
- Israel, M., Manaranche, R., Marsal, J., Meunier, F.M., Frachon, P. and Lesbats, B. (1980) J. Physiol. (Paris) **76**: 479-485.
- Israel, M. and Meunier, F.M. (1978) J. Physiol. (Paris) **74**: 485-490
- Israel, M., Morel, N., Lesbats, B., Birman, S. and Manaranche, R. (1986) Proc. Nat. Acad. Sci. (USA). **83**: 9226-9230.

- Jansen, B.C. (1971) Ondespoort. J. Vet. Res. 38: 93-98.
- Johnson, D.A. and Pilar, G. (1979) J. Physiol. (Lond.) 299: 605-619.
- Johnston, M.V., McKinney, M. and Coyle, J.T. (1979) Proc. Nat. Acad. Sci. (USA) 76: 5392-5396
- Jones, T., Walker, J.H., Richardson, P.J., Fox, G.Q. and Whittaker, V.P. (1981) Cell Tiss. Res. 218: 355-373
- JOSEPH, K.C., KIM, S.U., STIEBER, A. and GONATAS, N.K. (1978) Proc. Nat. Acad. Sci. 75; 2815-2819
- ↑ Kao, J., Drachman, D.B. and Price, D.L. (1976) Science 193: 1256-1258
- JOSEPH, K., STIEBER, A. and GONATAS, N.K. (1979) J. Cell. Biol. 81; 543-554.
- Katz, B. (1969) in The release of neural transmitter substances. Liverpool University Press, Liverpool.
- Katz, B. and Miledi, R. (1979) Proc. R. Soc. Lond. Ser. B. 205; 369-378.
- Keiding, R., Horder, M., Gerhardt, W., Pitkanen, E., Tenhunen, R., Stromme, J.H., Theordersen, L., Waldenstrom, J., Tryding, N. and Westlund, L. (1974) Scand. J. Clin. Lab. Invest. 33; 291-306.
- Kitamura, M. (1976) Naunyn-Schmiedeberg's Arch. Pharmacol. 295: 171-175.
- Kitamura, M., Iwamori, M. and Nagai, Y. (1980) Biochem. Biophys. Acta. 628: 328-335.
- Klenk, E. (1935) Z. Physiol. Chem. 235; 24.
- Klotz, I.M. (1982) Science. 217: 1247-1249.
- Klotz, I.M. (1983) TIPS. 4: 253-255.
- Komulainen, H. and Bondy, S.C. (1987) Neurochem. Int. 10; 55-64
- Kozaki, S. (1979) Naunyn-Schmiedeberg's Arch. Pharmacol. 308: 67-70.
- Kozaki, S., Miyazaki, S. and Sakaguchi, G. (1977) Infect. Immun. 18: 761-766
- Kozaki, S. and Sakaguchi, G. (1975) Infect. Immun. 11; 932-936.
- Kozaki, S. and Sakaguchi, G. (1982) Toxicon. 20; 841-846.
- Kozaki, S., Sakaguchi, G., Nishimura, M., Iwamori, M. and Nagai, Y. (1984) FEMS Microbiol. Letts. 21: 219-223.
- Kozaki, S., Sakaguchi, S. and Sakaguchi, G. (1974) Infect. Immun. 10: 750-756.
- Kozaki, S., Togashi, S. and Sakaguchi, G. (1981) Jap. J. Med. Sci. Biol. 34: 61-68
- Kriebel, M.E.F. and Gross, C.E. (1974) J. Gen. Physiol. 64; 85-103.

- Kriebel, M.E.F., Llados, F. and Matteson, D.R. (1976) J. Physiol. (Lond.) 262: 553-581
- Kuffler, S.W. and Yoshikami, D. (1975) J. Physiol. (Lond.) 251: 465-482
- Laemmli, U.K. (1970) Nature 227; 680-685.
- Laev, H., Rapport, M.M., Mahadik, S.P. and Silverman, A.J. (1978) Brain Res. 157; 136-141
- Landa, C.A., Maccioni, H.J.F. and Caputto, R. (1979) J. Neurochem. 33; 825-838.
- Landa, C.A., Defilpo, S.S., Maccioni, H.J.F. and Caputto, R. (1981) J. Neurochem. 37: 813-823
- Landmesser, L. and Pilar, G. (1972) J. Physiol. (Lond.) 222: 691-713.
- Ledeer, R.W. (1978) J. Supramol. Structure 8; 1-17
- Ledeer, R.W., Skrivane, J.A., Tirri, L.J., Margolis, R.K. and Margolis, R.U. (1976) 'Gangliosides of the neuron: localisation and origin.' In: Ganglioside function: Biochemical and pharmacological implications (Porcellati, G., Ceccarelli, B. and Tettamanti, G., eds). Adv. Exp. Med. Biol. 71; 83-103, Plenum Press, New York.
- Ledley, F.D., Lee, G., Kohn, L.D., Habig, W.H. and Hardegree, M.C. (1977) J. Biol. Chem. 252; 4049-4055
- Lee, P.M. and Grant, C.W.M. (1980) Biochem. Biophys. Res. Commun. 95; 1299-1305
- Lehman, J., Nagy, J.I., Atmadja, S. and Fibiger, H.C. (1980) Neurosci. 5: 1161-1174
- Leon, A., Facci, L., Toffano, G., Sonnino, S. and Tettamanti, G. (1981) J. Neurochem. 37: 350-357
- Llinas, R.R. (1977) Soc. Neurosci. 11: 139-160.
- Llinas, R.R. and Nicholson, C. (1975) Proc. Nat. Acad. Sci. 72; 187-190
- Longenecker, H.E., Hurlbut, W.P., Mauro, A. and Clark, A.W. (1970) Nature 225; 701-703.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193: 265-275
- Lundh, H., Leander, S. and Thesleff, S. (1977) J. Neurol. Sci. 32: 29-43.
- MacKenzie, I., Burnstock, G. and Dolly, J.O. (1982) Neurosci. 7: 997-1006

- Matsuda, M., Sugimoto, N., Ozutsumi, K. and Hirai, T. (1982) Biochem. Biophys. Res. Commun. 104: 799-805.
- Matsuda, M. and Yoneda, M. (1977) Biochem. Biophys. Res. Commun. 77: 268-274.
- Masserini, M. and Freire, E. (1987) Biochemistry 26: 237-242
- Matsuoka, I., Syuto, B., Kurihara, K. and Kubo, S. (1987) FEBS Letts. 216: 295-299
- Matteson, D.R., Kriebel, M.E. and Llados, F. (1979) Neurosci. Letts. 15: 147-152.
- Maxfield, F.R., Willingham, M.C., Pastan, I., Dragsten, P. and Cheng, S.-Y. (1981) Science 211: 63-64.
- McGraw, C.F., Nachsen, D.A. and Blaustein, M.P. (1982) in: 'Calcium and Cell Function' vol II, ed. Cheung, W.Y. pp 81-110. New York: Academic Press
- Meldolesi, J., Huttner, W.B., Tsien, R.Y. and Pozzan, T. (1984) Proc. Nat. Acad. Sci. (USA) 81: 620-624
- Middlebrook, J.L. and Kohn, L.D. (1981) in: "Receptor-mediated binding and internalisation of toxins and hormones." Academic Press, N.Y.
- Mitchell, J.F. and Silver, A. (1963) J. Physiol. (Lond.) 165: 117-129.
- Miyazaki, S., Iwamori, M. and Sakaguchi, G. (1977) Infect. Immun. 17: 395-401.
- Miyazaki, S., Kozaki, S., Sakaguchi, S. and Sakaguchi, G. (1976) Infect. Immun. 13: 987-989.
- Moberg, L.J. and Sugiyama, H. (1978) App. Environ. Microbiol. 35: 878-880.
- Molgo, J. and Thesleff, S. (1984) Proc. R. Soc. B. 214: 229-247.
- Molgo, J. and Thesleff, S. (1984) Brain Res. 297: 309-316.
- Monsigny, M., Roche, A.C., Sene, C., Maget-Dara, R. and Delmotte, F. (1980) Eur. J. Biochem. 104: 147-153.
- Montesano, R., Roth, J., Robert, A. and Orci, L. (1982) Nature. 296: 651-653.
- Morris, N.P., Consiglio, E.P., Kohn, L.D., Habig, W.H., Hardegree, M.C. and Helting, T.B. (1980) J. Biol. Chem. 255: 6071-6076.

- Munson, P.J. (1984) IN Principles and methods in receptor binding. Life Sciences 72: 1-12. (Series A) Plenum Press, New York (Cattabeni, F. and Nicosia, S., eds.)
- Murayama, S., Syuto, B., Oguma, K., Iida, H. and Kubo, S. (1984) Eur. J. Biochem. 142: 487-492
- Nachsen, D.A. (1984) Biophys. J. 45; 265a
- Nachsen, D.A. (1985) J. Physiol. 363: 87-101
- Nachsen, D.A. and Sanchez-Armass, S. (1987) J. Physiol. 387: 415-423.
- Nagata, Y. and Burger, M.M. (1974) J. Biol. Chem. 249: 3116-3122.
- Nicholls, D.G. (1978a) Biochem. J. 170: 511-522.
- Nicholls, D.G. (1978b) Biochem. J. 176: 463-474.
- Nicholls, D.G. (1982) In: 'Bioenergetics, an introduction to the chemiosmotic theory.' Academic Press, London.
- Nicholls, D.G., Rugolo, M., Scott, I.G. and Meldolesi, J. (1982) Proc. Nat. Acad. Sci. (USA) 79: 7924-7928
- Nicholls, D.G., Snelling, R. and Dolly, J.O. (1985) Biochem. J. 229: 653-662.
- Norris, P.J., Smith, C.C.T., DeBellerroche, J., Bradford, H.F., Mantle, P.G., Thomas, A.J. and Penny, R.H.C. (1980) J. Neurochem. 34: 33-42
- Ohashi, Y. and Narumiya, S. (1987) J. Biol. Chem. 262; 1430-1433.
- Ohishi, I. (1984) Infect. Immun. 43; 487-490.
- Ohishi, I. and Sakaguchi, G. (1977) Infect. Immun. 17; 402-407.
- Ohishi, I., Sugii, S. and Sakaguchi, G. (1977) Infect. Immun. 16: 107-109.
- Partington, C.R. and Daly, J.W. (1979) Molec. Pharmacol. 15: 484-491
- Pastan, I.H. and Willingham, M.C. (1981a) Ann. Rev. Physiol. 43; 239-250.
- Pastan, I.H. and Willingham, M.C. (1981b) Science 214; 504-509.
- Pelchen-Matthews, A. and Dolly, J.O. (1987) Brain Res. 'in press.'
- Penner, R., Neher, E. and Dreyer, F. (1986) Nature (Lond.) 324; 76-78
- Pierce, E.J., Davison, M.D., Parton, R.G., Habig, W.H. and Critchley, D.R. (1986) Biochem. J. 236: 845-852.

- Politoff, A., Blitz, A.L. and Rose, S. (1975) Nature. 256: 324-325.
- Poulain, B., Baux, G. and Tauc, L. (1986) Proc. Nat. Acad. Sci. 83: 170-173.
- Preti, A., Fiorilli, A., Lombardo, A., Caimi, L. and Tettamanti, G. (1980) J. Neurochem. 35: 281-296
- Price, D.L., Griffin, J., Young, A., Peck, K. and Stocks, A. (1975) Science 188: 945-947.
- Rahman, H. (1983) Neurochem. Int. 5: 539-547
- Raso, V. and Basala, M. (1984) J. Cell Biol. 259: 1143-1149.
- Revesz, T. and Greaves, M. (1975) Nature 257: 103-106.
- Richards, C.D., Metcalfe, J.C., Smith, G.A. and Hesketh, T.R. (1984) Biochem. Biophys. Acta. 803: 215-220.
- Richardson, P.J. (1981) J. Neurochem. 37: 258-260.
- Richardson, P.J. (1983) J. Neurochem. 41: 640-648.
- Richardson, P.J. (1984) Biochem. J. 210: 647-654.
- Richardson, P.J. (1986) J. Neurochem. 46: 1251-1255.
- Richardson, P.J. and Luzio, P. (1983) Biochem. Soc. Trans. 11: 273-275.
- Richardson, P.J., Walker, J.H., Jones, R.T. and Whittaker, V.P. (1982) J. Neurochem. 38: 1605-1614
- Rodbard, D., Munson, P.J. and Thakur, A.K. (1980) Cancer. 46: 2907-2918.
- Rogers, T.B. and Snyder, S.H. (1981) J. Biol. Chem. 256: 2402-2407.
- Roseman, S. (1970) Chem. Phys. Lipids 5: 270
- Rosenthal, H.E. (1967) Anal. Biochem. 20: 525-532.
- Rosner, H. and Merz, G. (1982) Brain Res. 236: 63-75
- Roth, T.F. and Porter, K.R. (1964) J. Cell Biol. 20: 313-332.
- Rubin, R.P. (1970) Pharmacol. Rev. 22: 389-428
- Rye, D.B., Wainer, B.H., Mesulem, M-M., Mufson, E.J. and Saper, C.B. Neurosci. 13: 627-643
- Sakaguchi, G., Sakaguchi, S., Kozaki, S., Sugii, S. and Ohishi, I. (1974) Jpn. J. Med. Sci. Biol. 27: 161-172.

- Sanchez-Prieto, J., Sihra, T.S., Evans, D.M., Ashton, A., Dolly, J.O. and Nicholls, D.G. (1987) Eur. J. Biochem. **165**; 675-681.
- Sathyamoorthy, V. and DasGupta, B.R. (1985) J. Biol. Chem. **260**; 10461-10466.
- Scatchard, G. (1949) Ann. N.Y. Acad. Sci. **51**; 660-672.
- Schlessinger, J., Schechter, Y., Cuatrecasas, P., Willingham, M. and Pastan, I. (1978) Proc. Nat. Acad. Sci. **75**; 5353-5357.
- Schmitt, A., Dreyer, F. and John, C. (1981) Naunyn-Schmiedeberg's Arch. Pharmacol. **317**; 326-330.
- Schneider, D.L. (1981) J. Biol. Chem. **256**; 3858-3864.
- Schwab, M.E. and Thoenen, H. (1978) J. Cell Biol. **77**; 1-13.
- Schwab, M.E., Suda, K. and Thoenen, H. (1979) J. Cell Biol. **82**; 798-810.
- Schwarcz, R., Hokfelt, T., Fuxe, K., Jansson, G., Goldstein, M. and Terenius, L. (1979) Exp. Brain Res. **37**; 199-216.
- Scott, A.B. (1980) J. Pediatr. Ophthalmol. Strabismus. **17**; 21-25.
- Sellin, L.C. (1981) Med. Biol. **59**; 11-20.
- Sharom, F.J. and Grant, C.W.M. (1978) Biochem. Biophys. Acta. **507**; 280-293
- Shimizu, T. and Kondo, H. (1973) Jpn. J. Med. Sci. Biol. **26**; 269-271.
- Shone, C.C., Hambleton, P. and Melling, J. (1985) Eur. J. Biochem. **151**; 75-82
- Shone, C.C., Hambleton, P. and Melling, J. (1987) Eur. J. Biochem. 'in press'
- Shute, C.C.D. and Lewis, P.R. (1967) Brain **90**; 497-522
- Simmoneau, M., Baux, G. and Tauc, L. (1980) J. Physiol. (Paris). **76**; 427-433
- Simpson, L.L. (1971) Neuropharmacol. **10**; 673-684.
- Simpson, L.L. (1973) Neuropharmacol. **12**; 165-176.
- Simpson, L.L. (1974) Neuropharmacol. **13**; 683-691.
- Simpson, L.L. (1978) J. Pharmacol. Exp. Ther. **206**; 661-669.
- Simpson, L.L. (1981) Pharmacol. Revs. **33**; 155-188.

- Simpson, L. L. (1982) J. Pharmacol. Exp. Ther. 222: 43-48.
- Simpson, L. L. (1983) J. Pharmacol. Exp. Ther. 225: 546-552.
- Simpson, L. L. (1986) Ann. Rev. Pharmacol. Toxicol. 26; 427-453.
- Simpson, L. L. and Rapport, M. M. (1971a) J. Neurochem. 18: 1341-1343.
- Simpson, L. L. and Rapport, M. M. (1971b) J. Neurochem. 18: 1751-1759.
- Smith, A. D. (1971) Philos. Trans. R. Soc. London Ser. B. 261; 363-370
- Smith, A. D. (1972) Biochem. Soc. Symp. 36; 103-131
- Smith, L. (1977) in Botulism: the organism, its toxins, the disease. Charles C. Thomas, Bannerstone House, Springfield III.
- Smith, L. A. and Middlebrook, J. L. (1985) Toxicon. 23; 611-613.
- Sobue, K., Ichida, S., Yoshida, H., Yamazaki, R. and Kakiuchi, S. (1979) FEBS Letts. 99; 199-202.
- Sonnino, S., Ghidoni, R., Marchesini, S. and Tettamanti, G. (1979) J. Neurochem. 33; 117-121
- Stadler, H. and Fuldner, H. H. (1980) Nature 286: 293-294.
- Staub, G. C., Walton, K. M., Schnaar, R. L., Nichols, T., Baichwal, R., Sandberg, K. and Rogers, T. B. (1986) J. Neurosci. 6; 1443-1451
- Suetake, K., Kojima, H., Inanaga, K. and Koketsu, K. (1981) Brain Res. 205: 436-440.
- Sugii, S., Ohishi, I. and Sakaguchi, G. (1977a) Infect. Immun. 16; 910-914.
- Sugii, S., Ohishi, I. and Sakaguchi, G. (1977b) Infect. Immun. 17; 491-496.
- Sugiyama, H. (1980) Microbiol. Revs. 44: 419-448.
- Sugiyama, H., DasGupta, B. R. and Yang, K. H. (1973) Proc. Soc. Exp. Med. Biol. 145; 1306-1309.
- Sugiyama, H., Mizutani, K. and Yang, K. H. (1972) Proc. Soc. Exp. Biol. Med. 141: 1063-1067.
- Sugiyama, H. and Moberg, L. T. (1977) Appl. Environ. Microbiol. 33: 963-966.
- Svennerholm, L. (1963) J. Neurochem. 10; 613-623
- Syuto, B. and Kubo, S. (1981) J. Biol. Chem. 256; 3712-3717.

- Takamizawa, K., Iwamori, M., Kozaki, S., Sakaguchi, G., Tanaka, R., Takayama, H. and Nagai, Y. (1986) FEBS 201: 229-232.
- Tauc, L. (1977) in: "Synapses" (Cottrell, G.A. and Usherwood, P.N.R., Eds.), Blackie, Glasgow. p64-78.
- Tauc, L. (1982) Physiol.Revs. 62: 857-893.
- Tauc, L. and Baux, G. (1982) J.Physiol.(Paris) 78: 366-372.
- Taylor, C.F., Britton, P. and van Heyningen, S. (1983) Biochem.J. 209: 897-899.
- Tettamanti, G., Preti, A., Cestaro, B., Venerando, B., Lombardo, A., Ghidoni, R. and Sonnino, S. (1980) Adv.Exp.Med.Biol. 125: 263-280
- Thesleff, S. (1960) J.Physiol.(Lond.) 151: 598-607.
- Thesleff, S. (1984) J.Physiol.(Paris) 79: 192-195.
- Thesleff, S. and Molgo, J. (1983) Neurosci. 9: 1-8.
- Toffano, G., Benvegnu, D., Bonetti, A.C., Facci, L., Leon, A., Orlando, P., Ghidoni, R. and Tettamanti, G. (1980) J. Neurochem. 35: 861-866
- Tse, C.K., Dolly, J.O., Hambleton, P., Wray, D. and Melling, J. (1982) Eur.J.Biochem. 122: 493-500.
- Tsien, R.Y. (1980) Biochemistry 19: 2396-2404.
- Tsien, R.Y. (1981) Nature 290: 527-528
- Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982a) Nature (Lond.) 295: 68-71
- Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982b) J. Cell Biol. 94: 325-334
- Van Heyningen, S. (1974) Science 183: 656-657.
- Van Heyningen, W.E. (1974) Nature (Lond.) 249: 415-417
- Van Heyningen, W.E., Carpenter, C.C.J., Pierce, N.F. and Greenough, W.B. (1971) J.Infect.DIS. 124: 415-420
- Vizi, E.S. and Vyskocil, F. (1979) J.Physiol.(Lond.) 286: 1-14.
- Wagman, J. (1954) Arch.Biochem.Biophys. 50: 104-112.
- Wainer, B.H., Levey, A.I., Mufson, E.J. and Marsel-Mesulem, M. (1984) Neurochem. Int. 6: 163-182

- Weeke, B. (1973) in A manual of quantitative immunoelectrophoresis: methods and applications. (Axelson, N.H., Kroll, J. and Weeke, B., eds.) Blackwell Scientific Publications, p47-56.
- Wellhoner, H.H. (1982) Rev. Physiol. Pharmacol. 93; 1-68.
- Wenk, H., Bigl, V. and Meyer, U. (1980) Brain Res. Rev. 2: 295-316
- Whittaker, V.P., Michaelson, I.A. and Kirkland, R.J.A. (1963) Biochem. Pharmacol. 12: 300-302
- Wiegandt, H. (1982) "The Gangliosides" in: "Advances in Neurochemistry" Plenum Press, N.Y. 4; p149-223 (Agranoff BW, Aprison, M.H., Eds.)
- Williams, R.S. (1984) Ph.D. thesis, University of London.
- Williams, R.S., Tse, C.K., Dolly, J.O., Hambleton, P. and Melling, J. (1983) Eur. J. Biochem. 131: 437-445.
- Willingham, M.C., Maxfield, F.R. and Pastan, I. (1979) J. Cell Biol. 82; 614-625.
- Yamakawa, T. and Nagai, Y. (1978) Trends Biochem. Sci. 3; 128-131
- Yavin, E., Yavin, Z. and Kohn, L.D. (1983) J. Neurochem. 40; 1212-1219
- Yohe, H.C. and Rosenberg, A. (1977) J. Biol. Chem. 252: 2412-2418
- Young, A.M.J. and Bradford, H.F. (1986) J. Neurochem. 47: 1399-1404
- Zacks, S., Metzger, J.F., Smith, C.W. and Blumberg, M. (1962) J. Neuropathol. Exp. Neurol. 21; 610-626.
- Zimmermann, H. and Denston, C.R. (1977a) Neurosci. 2: 695-714.
- Zimmerman, J.M. and Piffaretti, J.-Cl. (1977) Naunyn-Schmiedeberg's Arch. Pharmacol. 296; 271-277