THE INTERACTION OF TETANUS TOXIN WITH ADRENAL CHROMAFFIN CELLS

CAROLINE ANNE COLVILLE

Ph.D.

UNIVERSITY OF EDINBURGH
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To my Mum and Dad

PREFACE

The work reported in this thesis was carried out between 1st October 1987 and 30th September 1990 under the supervision of Dr Simon van Heyningen and Dr John Phillips at the Department of Biochemistry, University of Edinburgh, Scotland. All material presented in this thesis, unless otherwise stated, is the sole work of the author, as is the composition.

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ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine Serum Albumin
Ci	Curie
CNS	Central Nervous System
C. tetani	*Clostridium tetani*
DEAE-	Diethylaminoethyl-
DMEM	Dulbecco's Modified Eagle Medium
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
EF2	Elongation Factor 2
FCS	Foetal Calf Serum
FITC	Fluorescein isothiocyanate
G_s	Stimulatory regulatory subunit of adenylate cyclase
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
GM1	Galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosyl-glucosylceramide
GM3	[N-acetylneuraminyl]-galactosyl-glucosylceramide
GD1a	[N-acetylneuraminyl]-galactosyl-N-acetylgalactosaminyl-[N-acetyl-neuraminyl]-galactosylglucosylceramide
GD1b	Galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl-N-acetyl-neuraminyl]-galactosylglucosylceramide
GT1	[N-acetylneuraminyl]-galactosyl-N-acetylgalactosaminyl-[N-acetyl-neuraminyl-N-acetylneuraminyl]-galactosylglucosylceramide
HEPES	N-2-hydroxyethylpiperazine-N-2'-ethane sulphonic acid

IgG	Immunoglobulin G
IU	International Unit
K_d	Dissociation constant
K_i	Inhibition constant
kDa	Kilodaltons
LY	Lucifer Yellow
MLD	Minimum Lethal Dose
NANA	N-acetylneuraminic acid
PBS	Phosphate-Buffered Saline
pI	Isoelectric point
PMSF	Phenylmethylsulphonylfluoride
SDS	Sodium Dodecyl Sulphate
Tris	Tris[hydroxymethyl]aminomethane
TBS	Tris-Buffered Saline
v/v	volume/volume

ABSTRACT

Tetanus toxin exerts its primary biological effect by impairing the release of inhibitory neurotransmitters in the central nervous system; the mechanism of this blockade, however, remains unknown. Studies using adrenal medullary chromaffin cells, which are closely related to the toxin's target neurones but much more accessible to biochemical investigation, have the potential to provide information on various aspects of the intoxication process of tetanus. Nicotine-evoked (but not basal) secretion of catecholamines from intact chromaffin cells was inhibited by tetanus toxin in a dose-dependent fashion up to a maximum of 75%, half-maximal inhibition being achieved at 0.7 nM toxin (in single- or double-chain form). The time course of this inhibition was long, approximately 16 hours. Catecholamine release evoked by Ba^{2+} ions was not affected by the toxin, Pre-incubation of cells with ganglioside GT1, a specific ligand of the toxin, in the absence of toxin itself inhibited nicotine-evoked release of catecholamines (50% inhibition with 24 μM GT1), making it impossible to evaluate the role of GT1 in mediating the action of the toxin. Tetanus toxin (radiolabelled) was also shown to bind in a specific fashion to chromaffin cells. Toxin binding under less physiological conditions of pH and ionic strength was of a higher capacity (B_{max} 0.7-1.2 pmol/mg protein) than that found under more physiological conditions (B_{max} 0.2-0.3 pmol/mg protein); this is also characteristic of toxin binding to synaptic membranes. In both cases there appeared to be at least two components to toxin binding, a higher affinity component with an apparent Kd value of

approximately 1 nM (which did not account for more than 20% of total binding capacity), and a lower affinity binding with a K_d value of 10-25 nM. As expected, pre-incubation of chromaffin cells with ganglioside GT1 enhanced their toxin-binding capacity, but did not noticeably affect K_d values, perhaps indicating that gangliosides mediate binding of tetanus toxin to untreated chromaffin cells. This was further suggested by the finding that neuraminidase treatment of cells markedly reduced toxin-binding capacity (by 50% at pH 7.4, 90% at pH 6.0). Mild trypsinisation of chromaffin cells completely abolished toxin-binding under both sets of conditions, suggesting the involvement of a protein component in toxin binding also. Similar observations were made when toxin binding was visualised using immunocytochemical techniques. No binding of tetanus toxin to purified chromaffin granules was observed. In order to analyse the nature of the interaction between chromaffin cells and tetanus toxin further, the ganglioside composition of membranes from adrenal medullary microsomes was determined; a component with the same mobility on thin-layer chromatograms as GM3 was found to be the predominant species, while a small amount of a disialoganglioside, perhaps GD1b, was also evident. Overlay with radiolabelled tetanus toxin of these extracted gangliosides, separated on thin-layer chromatograms, indicated an interaction with both the mono- and disialylated species, as well as some toxin-binding to a GT1-like ganglioside. There was no detectable interaction between tetanus toxin and any protein components of the chromaffin cell membrane separated by SDS-polyacrylamide gel electrophoresis.

CHAPTER ONE.

INTRODUCTION

1.1 CLINICAL DEFINITION OF TETANUS

The disease tetanus, with its characteristic muscular spasms, must surely be one of the world's most feared afflictions; not only are its victims subjected to excruciating pain, but the risk of death is substantial as well. It is a disease of the central nervous system, caused by the presence in a wound of the bacillus *Clostridium tetani*. This micro-organism produces a neurotoxic protein, tetanus toxin, which is solely responsible for the symptoms of the disease.

Clinical tetanus has traditionally been divided into four types:

a) GENERALISED TETANUS: The most commonly recognised form of the disease is termed generalised tetanus, and is characterised by a spastic paralysis over the whole body, starting first in the jaw, and then moving towards the trunk and outer limbs; for this reason, this form of tetanus is sometimes referred to as the descending type. The victim is rigid, with arched back, clenched hands and feet and his jaw set in a permanent grimace - the "sardonic smile" of tetanus. His muscles are continually contracting against each other, so he exerts tremendous energy and is in constant pain. Death usually results from exhaustion, heart failure or the collapse of the lungs.

b) LOCAL TETANUS: This is the second form of the disease, consisting of paralysis of the muscles of a specifically infected limb; indeed, it may be induced by intramuscular

injection of the toxin. It is often mild, and may persist for some time without further development; in some cases however, it may precede the onset of generalised tetanus.

c) CEPHALIC TETANUS: Cephalic tetanus is an uncommon variant of local tetanus affecting the facial and lower cranial musculature. It usually follows head injuries, and the risk of death is high.

d) NEONATAL TETANUS: It is a matter for debate as to whether this type of tetanus deserves a separate classification, since it is identical to the generalised form of the disease, except that it is exclusive to the newborn. It is recognisable in the first two weeks after birth, and is seen primarily in areas where tradition dictates such practices as coating the freshly severed umbilical cord with cow dung or wrapping it in old rags. Neonatal tetanus carries an extremely high mortality.

For general references on the clinical features of tetanus, see reviews by van Heyningen (1968), Habermann (1978), van Heyningen (1980), Bleck (1986), and Cross & Sadoff (1988).

1.2 HISTORY OF TETANUS

Clear descriptions of tetanus occur in the literature throughout medical history. Hippocrates, twenty-four centuries ago, wrote of it as a disease whose victims "die within four days, or if they pass these, they recover".

Our understanding of the pathogenesis and prophylaxis of tetanus stems from the end of the nineteenth century. Prior to this time, a severe lack of both the knowledge and the means for effective treatment of the disease persisted, and the physician had little success in his attempts to arrest the disease; the first hint of a rational therapeutic approach came with the discovery of the muscle-paralysing properties of curare, and its subsequent use in 1859 in the treatment of a wounded soldier affected by tetanus after the battle of Magenta.

The infectious nature of tetanus was demonstrated in 1884 by Carle and Rattone, who took a pustule (believed to be the entry point of the illness) from a patient and injected it as a water homogenate into twelve healthy rabbits; all but one of the animals developed tetanus and later died. Further, they were able to show that a toxic agent could be transferred from the nervous tissue of these rabbits to fresh animals. In the same year, Nicolaier succeeded in producing experimental tetanus after injecting samples of soil into various animals. Following microscopic examination of material taken from the innoculation site, he described the micro-organisms responsible for the disease as elongated, thin bacilli. He proposed that the germ remained in the innoculation site, and attributed the development of tetanus to a strychnine-like substance produced and diffused in the blood. The tetanus bacilli were demonstrated in man by Rosenbach in 1886, although he was unable to isolate them. This was achieved three years later by Kitasato, who was able to show their identity with Nicolaier's bacilli; he also established the morphology of the germ, as well as some of its biological properties.

It is generally considered that Faber was the first to describe the tetanus toxin, in 1890. He demonstrated that the cell-free liquid obtained by filtering a culture of bacilli, when injected in minimal doses into an animal, resulted in a sickness identical in every respect to natural tetanus. The outstanding toxic power of the culture filtrate was immediately evident; less than one hundred thousandth of a millilitre was enough to kill an animal. It was subsequently discovered that a protein secreted by the micro-organisms was responsible for this remarkable toxicity.

The discovery of tetanus antitoxin by Behring and Kitasato in 1890 was a milestone in the history of tetanus (indeed, tetanus toxin proved to be an important tool which allowed the first steps towards immunohaematology). They carried out active immunisation of rabbits, and subsequently achieved passive immunisation in mice. Passive immunisation in man was first carried out by Behring and Knorr in 1896.

Around the turn of the century, the important pharmacokinetic and pathobiochemical properties of the toxin were also described, such as Wassermann and Takaki's finding in 1898 that tetanus toxin was fixed by brain matter, or Meyer and Ransom's discovery in 1903 of the intra-axonal ascent of the toxin to the central nervous system. Since this time, few further fundamental insights have been gained; the most important practical achievements of this century lie in the application of active immunisation in man.

1.3 BACTERIOLOGY

The genus *Clostridium*, to which the causative organism of tetanus belongs, contains those Gram-positive bacilli that grow only under strict anaerobic conditions and which are capable of forming endospores. Included in this group along with *Clostridium tetani* are a number of species pathogenic to man, such as *Clostridium botulinum*, responsible for botulism, and *Clostridium perfringens*, which causes gas-gangrene.

Clostridium tetani is a ubiquitous organism, commonly found in soil, air and even on clothing. However, soil rich in animal manure, in a sufficiently hot and wet climate, may be regarded as the main reservoir of tetanus bacilli; in this environment, the organism takes part in the anaerobic decomposition of organic matter, although the role of *Clostridium tetani* in the biocenosis of the soil is totally obscure. The organisms are motile rods approximately 0.4 x 6 μm in size; young bacteria contain a single cilium at one end, while older bacilli may have a tuft of cilia protruding from one pole. Later still, bacilli are surrounded by about a dozen cilia which are lost gradually with further aging.

After two to ten days in culture, the bacilli start to form spherical terminal spores, giving the organism a characteristic "drumstick" appearance; in the environment, low temperatures and lack of moisture also stimulate sporulation. These spores are resistant to oxygen, moisture and extremes of temperature, and so enable the organism to survive in soil indefinitely. When innoculated into a wound, the spores will germinate and if the

oxygen tension is sufficiently low, the organisms will proliferate and produce their exotoxins tetanospasmin, responsible for the clinical manifestations of tetanus, and tetanolysin, a protein of uncertain importance.

Clostridium tetani bacilli are generally cultured on synthetic medium supplemented with beef heart infusion (Mueller & Miller, 1954) and a complex array of nutrients required for normal growth, multiplication and toxin production (Latham *et al.*, 1962). Optimal growth occurs when pH is maintained between 7.0-7.6, and at temperatures of 35-38°C, and the oxygen-reduction potential (Eh) should be +0.01V or less, since the organism is a strict anaerobe. Under anaerobic conditions, motile cells can film the surface of solid media with either a continuous or broken transparent membrane; this is known as "swarming growth". The edges of such a translucent sheet are usually irregular and filamentous.

Like all strict anaerobes, *Clostridium tetani* lacks cytochromes, cytochrome oxidase, peroxidase and catalase, and does not seem to be very active in the majority of biochemical laboratory tests. Gas formation is low, and proteolysis is weak, although gelatinase activity is evident (it is not known whether gelatinase, or tetanolysin, is produced in vivo; if so, this would enable the organism to damage surrounding tissues, and to create better conditions for its growth). Production of tetanus toxin is a unique biochemical property, and is useful for identification.

For general references concerning the bacteriology of tetanus, see Habermann (1978), Bizzini (1979) and Adams *et al.* (1969).

1.4 EPIDEMIOLOGY

The World Health Organisation regards tetanus as a serious health problem, not only because of the vast numbers of people affected but also the lethality of the disease. Its mortality rate is between 50% and 80%, and it is estimated that well in excess of one million deaths occur each year due to tetanus infection (Bytchenko, 1975). Accurate figures are not available, as tetanus is not a notifiable disease in about half the countries of the world, and is commonest where arrangements for collecting medical data are most primitive.

Studies on the global distribution of tetanus show that typically it is a tropical disease, and that its incidence depends largely on socio-economic, demographic and environmental factors. It is more common in areas with a warm, humid climate, fertile highly cultivated soil, a substantial population of both man and animals, and a low standard of living. Neonatal tetanus is the most prevalent form of the disease; in many developing countries it still accounts for approximately 50% of neonatal deaths. It is estimated that worldwide about one million newborn children contract neonatal tetanus each year and that 80% of these die. This is due to multiple failures in the health systems of these countries; failure to protect expectant mothers with tetanus toxoid, coupled with unhygienic practices which bring tetanus spores in contact with the umbilical cord. The persistence of this disease is partly due to the ignorance of health authorities about the true scale of the problem. Recently, however, immunisation programmes in developing countries have been undergoing a period of

rapid acceleration. This is due to the implementation of the Expanded Programme on Immunisation, launched in 1974 by the World Health Organisation.

The magnitude of non-neonatal tetanus is still ill-defined. Available data are incomplete, but suggest mortality rates of 0.5-1.4/100,000 population in Latin America, 1.2-2.4 in South Asia and Africa, and less than 0.2 in Europe. These figures are probably lower than reality; it has been estimated that 400,000 deaths occur annually due to non-neonatal tetanus. It is interesting to note that in developing countries non-neonatal tetanus still affects mainly young people (half to two-thirds of patients are under the age of twenty) while in industrialised countries, the incidence increases relative to age, with 60-90% of patients being over sixty years of age. This pattern of "residual" tetanus can be explained as a result of incomplete immunisation programmes, having forgotten or neglected to immunise older adults.

For references to the above section, see Adams *et al.* (1969) and Habermann (1978).

1.5 TREATMENT OF TETANUS

Treatment of the tetanus patient, an expensive and complicated procedure, may be aimed at interrupting one or more of the three main stages of the disease process. The first means of intervention is to prevent the invasion of toxin from *Clostridium tetani*. This may be achieved by exhaustive cleansing of the wound, which will reduce the toxin load and improve the oxygen-reduction

potential of the damaged tissue, making conditions less favourable for bacterial growth. The administration of antibiotics may also be of use; *C. tetani* is sensitive to penicillins, cephalosporins, tetracyclines and erythromycin. However, apart from the fact that a wound may not be obvious, and that antibiotic treatment will not alter toxin already produced or inactivate spores, this form of treatment is virtually ineffective if tetanic symptoms are already in evidence, and so is really a preventative measure rather than a therapeutic one.

The second form of treatment involves the neutralisation of the toxin before it is able to reach the central nervous system and be adsorbed to its cell receptor (see section 1.8.2). This is primarily accomplished through the administration of tetanus anti-toxin, a form of treatment whose value is now beyond question. Intramuscularly injected human anti-toxin will neutralise both circulating tetanus toxin and that toxin in the wound not yet intraneuronal. However, as with the first approach, it is important that treatment is carried out as soon as possible after injury before any toxin can reach its target cells in the central nervous system, since as yet it is not known how to inactivate bound toxin.

The third type of treatment is to suppress the effect of toxin having reached the central nervous system; this mainly consists of limiting spasticity, and of preventing and counteracting the effects of spasms on vital functions, especially respiration. There are many sedative and muscle-relaxing drugs available, although the large doses often necessary carry their own risk of

side-effects. Hypnotics and sedatives such as barbiturates and paraldehyde lower the general state of excitability in the central nervous system, and reduce the effect of sensory input which may provoke spasms, while general anaesthetics also cause widespread suppression; however, these have now been largely replaced by centrally acting muscle relaxants such as benzodiazepines (e.g. valium) and phenothiazines (e.g. chlorpromazine) which depress reflex activity and reduce motor output from the central nervous system. These drugs remain the basis of tetanus therapy in many parts of the world.

In theory complete paralysis and artificial respiration for about two weeks should be the ideal treatment of severe tetanus. Respiratory management of the tetanus patient is vital; adequate ventilation and protection of the lung largely decide the outcome of the disease. Another point to note is that, since the amount of tetanus toxin produced in clinical tetanus is insufficient to be immunogenic, all patients must also receive active immunisation (see Section 1.6) to prevent further attacks.

For general references on the therapy of clinical tetanus, see Adams *et al.* (1969), Rey *et al.* (1981) and Bleck (1986).

1.6 PREVENTION OF TETANUS

In contrast to the treatment of tetanus, prevention of the disease is relatively simple and inexpensive. The attainment of more sanitary conditions in developing countries, where the disease is most prevalent, would go a long way to reducing its incidence, but

this is a lot easier said than done, and will not be possible until economic conditions are improved. However, immunisation can prevent tetanus regardless of insanitary environments. Direct active immunisation is the most effective form of prevention available, antibodies being elicited against tetanus toxin. The toxin itself cannot be used directly, since its immunogenic capacity is far exceeded by its toxicity; instead it is first "toxoided" by treatment with formaldehyde to yield an atoxic derivative which is still immunogenic. The usual course for immunisation is an intramuscular injection of toxoid followed by boosters after six weeks and six months. Further boosters may then be given at five or ten year intervals to maintain a high antibody titre.

Passive immunisation has some value in preventing tetanus, but the immunity afforded is shorter lived than with active immunisation, and there is a risk of allergic reaction, since it is not human but equine antiserum which is generally used. For this reason, the administration of tetanus antiserum has now been discontinued in many countries.

For references to the above section, see Adams *et al.* (1969), Habermann (1978), Veronesi (1981) and Bleck (1986).

1.7 THE TETANUS TOXIN MOLECULE

Ever since the realisation that it is a toxin secreted by *Clostridium tetani* which is responsible for the production of the tetanic state, much research has been undertaken with the aim of

elucidating the structural properties of this molecule. The last twenty-five to thirty years have been the most productive, with the development of methods which enable the toxin to be prepared to a high degree of purity. This has facilitated a concomitant increase in our knowledge of its physical characteristics.

Tetanus toxin is produced by *Clostridium tetani* at the end of the germination phase, when it can constitute more than five percent of the dry weight of the organism (Mellanby, 1968). Purification of the toxin may start either from an extract of the clostridia, or from the culture filtrate; most procedures start with cell extracts (where for instance the toxin is released from inside washed bacteria following ultrasonication or treatment with hypertonic solutions; Raynaud, 1947), proceed by either salt or organic solvent precipitation, then by filtration through a basic ionic exchanger such as DEAE-cellulose. It is sometimes necessary to include a gel permeation step as well, to produce a protein homogeneous on polyacrylamide gel electrophoresis.

The toxin molecule is a simple protein without lipid or carbohydrate, which may be purified in one of two forms; the so-called "intracellular" and "extracellular" forms. This is due to proteolytic processing; initially the toxin is synthesised as a single chain polypeptide, but upon secretion into the extracellular medium, or autolysis, it undergoes a proteolytic cleavage to yield the dichain form of the molecule. (However, nicking of the toxin <u>within</u> the bacterial cell cannot be excluded; Helting *et al.* (1979) have reported that extracted tetanus toxin may consist of up to 50% of the two-chain form). This cleavage has been shown by various

workers (Bergey *et al.*, 1989; Weller *et al.*, 1988) to increase the potency of the toxin dramatically; indeed, the weak activity of the single-chain toxin appears to be due to small amounts of contaminating two-chain toxin. It is assumed that under natural conditions, "intracellular" toxin is nicked by one or more tryptic clostridial enzymes to become "extracellular" toxin. Indeed, Helting *et al.* (1979) have succeeded in isolating an enzyme with a molecular weight of 27 kDa from culture filtrate, which has a very high cleaving activity against tetanus toxin.

Thus the "extracellular" toxin differs from the "intracellular" form in that one peptide bond in the single chain precursor is cleaved by a clostridial enzyme. Robinson & Hash (1982) have noted that the endogenous protease or proteases which act on the unnicked toxin produce multiple species of nicked toxin, and hence heterogeneity in the heavy and light chains. The resulting two chains of the extracellular toxin are still held together by one disulphide linkage (Craven & Dawson, 1973); in agreement with this, Matsuda & Yoneda (1974) showed that the "extracellular" toxin could be dissociated into two polypeptide chains by treatment with dithiothreitol and sodium dodecyl sulphate, while the "intracellular" form was undissociable by this treatment; however, the two chains could be obtained from "intracellular" toxin if prior to reduction the toxin was subjected to mild trypsinisation.

Prior to the advent of modern molecular biological techniques for obtaining molecular weight and sequence data for proteins, the structure of the toxin was investigated by various analytical methods. Ultracentrifugation (Mangalo *et al.*, 1968, Robinson

et al., 1975), gel filtration chromatography (Murphy *et al.*, 1968) and polyacrylamide gel electrophoresis (Matsuda & Yoneda, 1974) were all used to determine its molecular weight, and all yielded values between 140 kDa and 160 kDa. This has since been confirmed by nucleotide sequence data (see below). The molecule has also been shown to contain ten half-cystine residues, present as two disulphide bridges and six free sulphydryl groups (Murphy *et al.*, 1968). The alpha-helical content of the molecule has been estimated to be about 20%, and data from circular dichroism studies suggest that much of this is confined to the amino terminal of the heavy chain (Robinson *et al.*, 1982); according to these authors, this may be of some importance when assigning functions to specific parts of the toxin molecule. Beta structure is said to account for a further 20% of the molecule. A pI value of 5.1 has been reported (An der Lan *et al.*, 1980), which agrees well with amino acid composition data showing about 300 acidic but only 150 basic residues.

The generally accepted model for the structure of tetanus toxin is summarised in Figure 1.1; the two chains, termed "heavy" and "light", have molecular weights of 100 kDa and 50 kDa respectively. The individual toxin chains are non-toxic, but may be reunited to form a toxic molecule by removal of dissociating agents by dialysis under aerobic conditions (Matsuda & Yoneda, 1976). The two chains of the toxin must be held together strongly by non-covalent forces in the native molecule, since it is very difficult to separate them quantitatively, even under conditions where the covalent disulphide bonds are broken. This would suggest a complex three-dimensional structure, with the chains tightly interwoven; however, circular

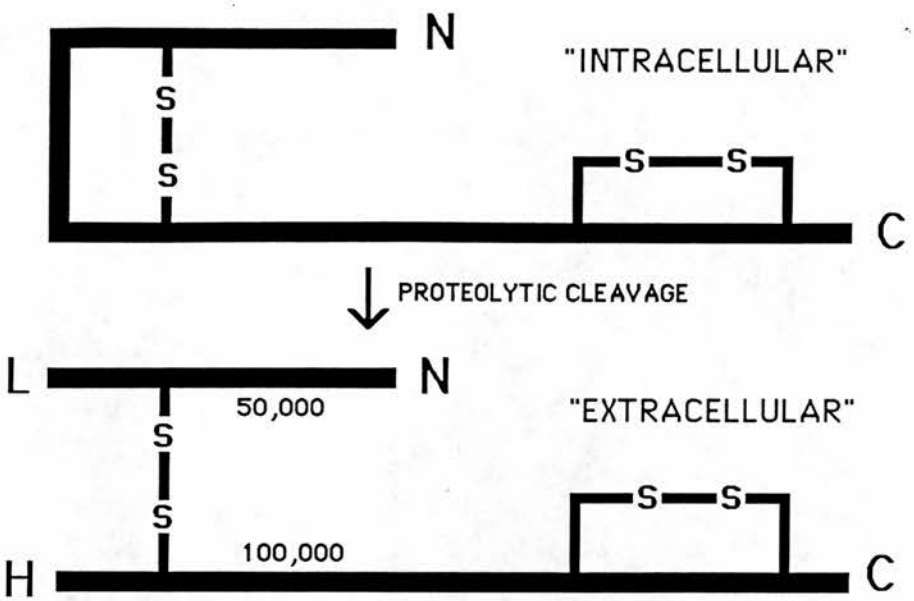


FIGURE 1.1 Structure of Tetanus Toxin

Proteolytic cleavage of the "intracellular" form of the toxin produces the "extracellular" form, composed of the light chain (L) with a molecular weight of 50 kDa, and the heavy chain (H) with a molecular weight of 100 kDa.

dichroism spectra show little difference between the three-dimensional conformation of the chains when they are isolated or combined, suggesting that they may make up relatively separate domains in the intact toxin (Robinson *et al.*, 1982). Recently, structural analysis of the toxin molecule by electron crystallography has shown that it has an asymmetric three-lobed structure (Robinson *et al.*, 1988).

Further attempts to probe the structure of the tetanus toxin molecule have been made by looking at various fragments produced from it, either by freezing and thawing or, more commonly, by enzymatic degradation; these isolated fragments differ in their structures and properties depending on the conditions used. For example, fragments produced by intrinsic proteases were isolated by Bizzini *et al.* (1977); a 46 kDa fragment, designated BIIb, was found to be atoxic, although still able to bind ganglioside with an affinity even greater than that of the toxin (see Section 1.8.1). It could also migrate by retrograde axonal transport towards the central nervous system. Similarly, using papain digestion, Helting & Zwisler (1977) produced two fragments with molecular weights of 95 kDa and 47 kDa, which they called B and C respectively. Analysis showed that the smaller fragment represented the carboxyl end of the toxin heavy chain, while the larger B fragment consisted of the light chain and the remainder of the heavy chain. Both fragments reacted with tetanus antitoxin although were non-toxic themselves. Later work by Bizzini *et al.* (1981) produced similar fragments, and again the smaller one was shown to bind gangliosides. Studies of this kind have been of much use when looking at structure-activity relationships of tetanus toxin, which

will be discussed in later sections of this chapter. The general structure of tetanus toxin after nicking, and the nomenclature of fragments (by different authors using different fragmentation techniques) is shown in Figure 1.2.

The structural gene (tox^+) for tetanus toxin has been found to be encoded by plasmid DNA in toxigenic strains of *Clostridium tetani* (Laird *et al.*, 1980; Finn *et al.*, 1984). The complete nucleotide sequence of the toxin has been published (Eisel *et al.*, 1986; Fairweather & Lyness, 1986); it contains one open reading frame encoding a protein of 1315 amino acids with a molecular weight of 150,491. Despite the suggestion of Taylor *et al.* (1983), that there are similarities in the sequences of the heavy and light chains of the toxin, based on their amino acid compositions, the DNA sequence shows no significant internal duplications. Interestingly, there is a marked homology of the light and heavy chain sequences with those of botulinum A,B and E neurotoxins (Eisel *et al.*, 1986; DasGupta & Foley, 1989), suggesting that the neurotoxins from *Clostridium tetani* and *Clostridium botulinum* are derived from a common ancestral gene. This sequence homology with botulinum toxins has also been detected using antipeptide antibodies (Halpern *et al.*, 1989). Further, there has been the suggestion of some similarity betweun tetanus toxin and the haemagglutinin of influenza virus (Montecucco *et al.*, 1987), based on the fact that both proteins are able to bind to sialic acid residues, which are components of gangliosides (see Section 1.8.1).

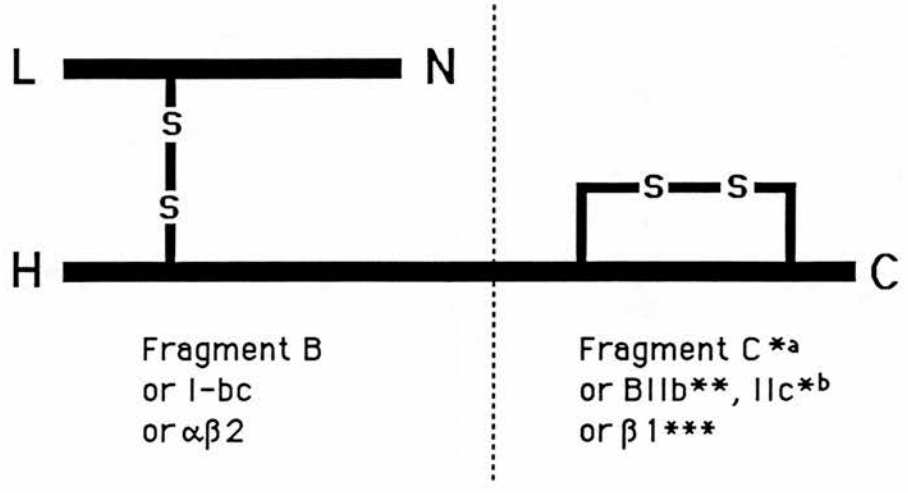


FIGURE 1.2 General Structure of tetanus toxin after nicking and nomenclature of fragments

* Produced from tetanus toxin by papain: a) Helting & Zwisler (1977)
b) Bizzini *et al.* (1981)

** Produced from tetanus toxin by intrinsic protease(s): Bizzini *et al.* (1977)

*** Produced from tetanus toxin by trypsin : Matsuda & Yoneda (1977)

Several different estimates of the toxicity of purified tetanus toxin have been published. There is much variation between laboratories, but a typical value seems to be in the region of $2x10^8$ mouse MLD (minimum lethal doses)/mg (Dawson & Mauritzen, 1975), making it one of the most poisonous substances known. However, no-one has yet been able to explain why *Clostridium tetani* synthesises this potent toxin, and there has been much speculation as to how it originated. One theory is that the toxin gene may have evolved from viral DNA, while another suggests a recombination event between viral and eukaryotic gene material. Regardless of its origin however, the toxin is of no obvious benefit to the bacterium; this serves to make it an extremely intriguing molecule.

1.8 FIXATION OF TETANUS TOXIN

1.8.1 Binding to Ganglioside

It is nearly one hundred years since it was first discovered that tetanus toxin is fixed preferentially by nerve tissue when, in 1898, Wassermann & Takaki observed that the toxicity of a toxin solution was reduced when mixed with an emulsion of brain. This reaction appears to be a specific one, since tetanus toxoid and other proteins are not fixed, and other tissues are relatively ineffective in toxin binding.

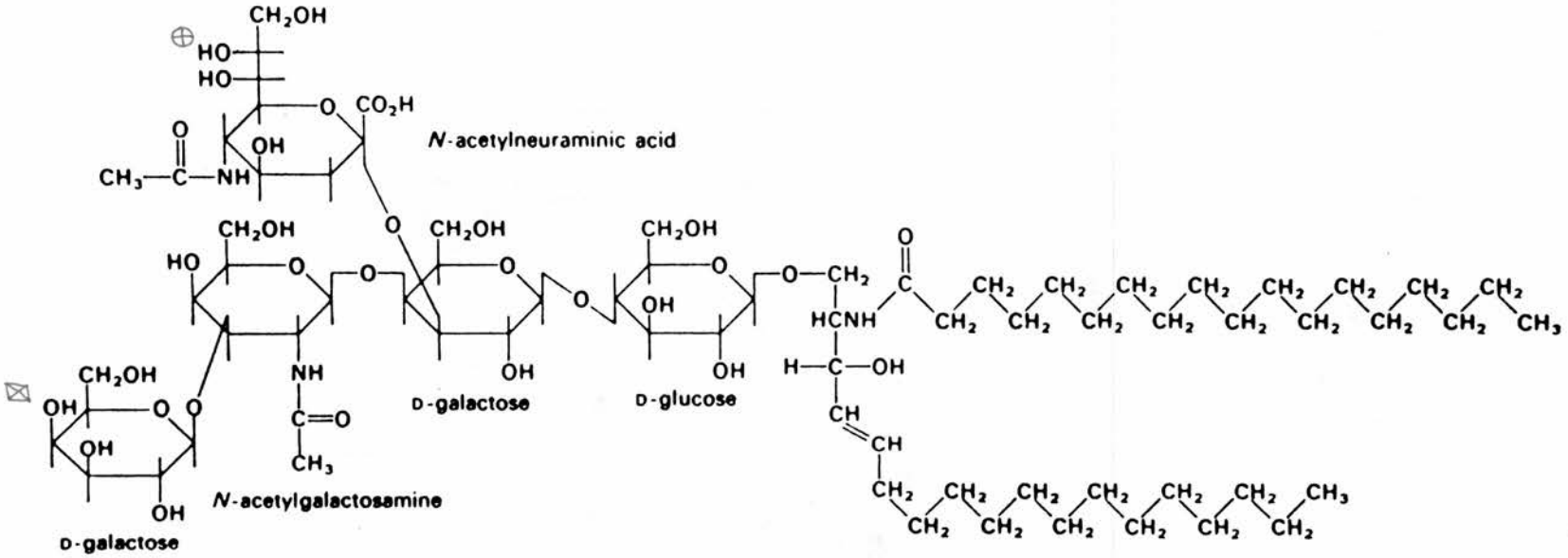
Much work went into trying to identify the receptor for the toxin, and in 1959 van Heyningen put forward the proposal that it was a ganglioside present in nervous tissue which was responsible for toxin binding (van Heyningen, 1959). Gangliosides are complex sialic acid-containing glycolipids found in almost all plasma

membranes, but with highest concentrations in neuronal tissue (for a typical structure see Fig. 1.3). Most of the different types of ganglioside seem to be tissue specific, with the tetanus toxin-binding gangliosides (see below) being predominant in nerve tissue. Gangliosides are amphiphilic compounds i.e. they contain both hydrophobic and hydrophilic domains; they also have a strong negative charge, due to the presence of one or more sialic acid residues. The hydrophobic portion of the molecule consists of two long hydrocarbon chains (one of which is a fatty acid) linked to sphingosine, while the hydrophilic region is made up of carbohydrates (glucose, galactose, N-acetylgalactosamine and N-acetylneuraminic acid (sialic acid), the proportions of which determine the type of ganglioside). This structure makes gangliosides very suitable candidates for cell surface receptors, since the hydrophobic portion confers lipid solubility to the molecule, enabling it to dissolve in the lipid bilayer, while the hydrophilic domain is dissolved in the aqueous environment outside the cell membrane, where it can complex ligands such as tetanus toxin.

The fact that gangliosides form micelles in aqueous solution (they have a very low critical micelle concentration, about 10^{-8} M; Formisano *et al.*, 1979) made quantitation of ganglioside-toxin binding difficult. The first experiments to demonstrate this binding used the analytical ultracentrifuge, where the separation of free toxin and toxin-ganglioside complex was shown in a sedimentation velocity run (van Heyningen & Miller, 1961). These authors also demonstrated that the N-acetylneuraminic acid residues of gangliosides were crucial for toxin fixation (removal of these

Figure 1.3 The Structure of Gangliosides

The structure of ganglioside GM1, with the complete tetrasaccharide backbone, and one N-acetylneuraminic acid residue, is shown. Ganglioside GD1b has an additional N-acetylneuraminic acid residue substituted at the position marked ⊕, while ganglioside GT1 has N-acetylneuraminic acid residues at positions ⊕ and ⊠.


N-acetylneuraminic acid
D-galactose
D-glucose
N-acetylgalactosamine
D-galactose
Hydrophilic portion
sugars
Hydrophobic portion
ceramide

residues abolished binding ability), and that complexing with the toxin did not alter ganglioside structure. Another method involved complexing ganglioside with cerebroside (an unreactive insoluble lipid) and incubating a suspension of this complex with tetanus toxin; binding of the toxin to the complex was then estimated by centrifuging the suspension and determining the toxicity of the supernatants (van Heyningen & Mellanby, 1968). By using this approach, the relative toxin-binding capacities of different ganglioside species could be assessed, and it was shown that GD1b and GT1 fixed tetanus toxin more than ten times better than other gangliosides. Here the nomenclature of gangliosides needs to be explained. It is based primarily on the number of sialic acid residues and on the carbohydrates forming the oligosaccharide portion of the molecule, such that GM indicates a monosialo -, GD a disialo- and GT a trisialoganglioside; the numbers refer to the oligosaccharide chain such that "1" indicates the major neutral tetrasaccharide, "2", the chain less the terminal galactose and "3" the chain lacking galactosyl-N-galactosamine. Subscript letters distinguish between species containing the same number of sialic acid residues but at different positions (Svennerholm, 1970). The important feature of tetanus toxin binding to gangliosides is that it shows an enzyme-like specificity for gangliosides containing two sialic acid residues linked in tandem to the galactose residue in the lactose moiety of the tetrasaccharide backbone, namely GD1b and GT1 (see Fig. 1.3). This moiety appears to be essential for good tetanus toxin-binding; the bond between the two sialic acid residues is neuraminidase-labile, and after hydrolysis the molecules no longer bind toxin with a high affinity.

Other methods have also been employed to study ganglioside-toxin interactions which have yielded more accurate and quantitative binding data. Helting *et al.* (1977) demonstrated binding between the two components using tetanus toxin adsorbed to a Sephadex matrix and radioactively labelled ganglioside. When the ganglioside was passed through the column, radioactivity eluting from it was decreased by the amount of ganglioside which had bound to the toxin. The amount of radiolabel adsorbed was shown to be proportional to the amount of toxin applied to the column, and it was calculated that in the nanomolar concentration range, tetanus toxin became half-saturated at about 5×10^{-8} M ganglioside. This suggests a very tight binding, especially as some of the ganglioside could have been in micelles and so perhaps not directly involved in the binding. A molar ratio of one to one, toxin to ganglioside, was demonstrated for GD1b by this method; surprisingly, however, it was claimed that GM1 ganglioside gave the same result, contrary to previous reports (van Heyningen, 1963). Holmgren *et al.* (1980) have used a ganglioside enzyme-linked immunosorbent assay to measure the binding of toxin to different gangliosides adsorbed on polystyrene. (Adsorption was through the ceramide moiety by strong hydrophobic bonding, leaving the oligosaccharide portion free to react with ligands). Again, a strong specific fixation of tetanus toxin to gangliosides of the G1b series in general, and to GD1b, GT1b and GQ1b (a tetrasialoganglioside) in particular, was observed. Gangliosides GM1 (which binds cholera toxin very strongly; van Heyningen, 1973) and GD1a also bound toxin but with much less affinity. This is more evidence that the disialic acid moiety is needed for toxin binding. Holmgren postulated that the natural binding structure

had the end sequence Galβ1→3GalNAcβ1→4Gal(2←8∝NANA2←3∝NANA), which occurs in the appropriate gangliosides, and possibly as oligosaccharide chains in certain cell membrane glycoproteins (see Section 1.8.2).

In contrast to the binding of cholera toxin to GM1 ganglioside, which has been investigated in great detail, little has been published on the nature of tetanus toxin binding to G1b gangliosides. Presumably the specificity is elicited by the carbohydrate moiety of the ganglioside molecule, since removal of the ceramide portion does not affect the binding (Helting *et al.*, 1977). Toxin fixation is reversible and non-covalent, and as mentioned already, there is no evidence to suggest that the ganglioside is changed in any way by reacting with toxin.

Gangliosides have been shown to bind a number of different ligands other than bacterial toxins. Strychnine, brucine and thebaine, all drugs with the same neurophysiological effects as tetanus toxin, bind to gangliosides (van Heyningen, 1963), as do interferon (Besancon *et al.*, 1976) and glycoprotein hormones such as thyrotropin (Fishman & Brady, 1976). This would appear to support a role for gangliosides as cell surface receptors, although in most cases their physiological functions remain unknown. Possible roles might include conferring structural rigidity on membranes, or as cell differentiation and growth markers.

As well as ascertaining which particular gangliosides have the highest affinity for tetanus toxin, it was obviously also of great importance to determine which part of the toxin molecule

facilitates binding. Investigation has shown that of the two chains of the toxin, only one actually binds ganglioside. This has been demonstrated in two different ways. In the first method (van Heyningen, 1976a), GT1 ganglioside was insolubilised by complexing with cerebroside as described above, then either whole toxin, heavy chain or light chain was incubated with it. The protein: ganglioside:cerebroside complex was then centrifuged, and the supernatant analysed by SDS-polyacrylamide gel electrophoresis to identify any molecular species of tetanus toxin left. Toxin bound to the complex could be dissociated with 8 M urea and identified in the same way. It was shown that both whole toxin and heavy chain were quantitatively adsorbed to the complex, while light chain did not adsorb at all. Helting *et al.* (1977) confirmed these results using their Sephadex binding assay. Heavy chain exhibited a binding activity of the same order of magnitude as that observed with the native toxin, while the light chain showed no interaction with ganglioside.

These experiments show that the binding site for ganglioside must lie on the heavy chain. Further, Helting's group showed that proteolytic fragment B (light chain and part of the heavy chain, see earlier) did not bind any significant amount, while fragment C showed some binding, suggesting that the binding site lies towards the carboxyl end of the heavy chain. The fact that the heavy chain still retains ganglioside binding ability when it is free from the light chain suggests that the conformation of the former does not change much on binding to the light chain; this is surprising in view of the fact that the two are so difficult to separate.

A point to note is that while cholera toxin complexed to purified GM1 is completely inactive *in vivo*, such is not the case with tetanus toxin and G1b gangliosides (van Heyningen & Mellanby, 1973). Only partial inactivation occurs; this could just be a question of the tightness of binding of ganglioside to tetanus toxin, but it does raise the question of whether gangliosides that fix tetanus toxin are genuine *in vivo* receptors, or possibly only part of a more complex receptor system; this will be discussed in detail later.

1.8.2 Binding of Toxin to Whole Cells and Subcellular Fractions

Since the original observation nearly a century ago that tetanus toxin is fixed by nervous tissue, it has become possible to be rather more specific. With the development of subcellular fractionation methods, Mellanby *et al.* (1965) were able to study the fixation of tetanus toxin by various fractions of the brain, and to show that the synaptosomal fraction (containing isolated nerve endings) had the greatest toxin-binding capacity. Further fractionation demonstrated that this was a property of the synaptic membrane, which had a ten-fold greater affinity for toxin than that of the synaptic vesicle fraction (Mellanby & Whittaker, 1968). Habermann (1973) also demonstrated binding of toxin to spinal cord synaptosomes using radiolabelled ligand. Further, Habermann (1976) was able to demonstrate that tetanus toxin could be affinity-purified by chromatography on a column of synaptosomes adsorbed to an insoluble support such as bromoacetyl cellulose; toxin was adsorbed to the column at low ionic strength and eluted at a higher ionic strength. Experiments using radiolabelled toxin fixed to the

synaptosomes showed that even large excesses of cold toxin could not elute all the label from the column, implying either a very large number of possible receptor sites on the synaptosomes, or perhaps uptake of toxin into the vesicles such that displacement of label was no longer possible.

Working with primary cultures from neuronal tissue, Dimpfel *et al.* (1975) showed by autoradiography that tetanus toxin fixed selectively to neuronal and not glial cells; this raised the suggestion that tetanus toxin could make a good marker of neuronal cells in culture. This work was carried further, in a thorough study of toxin binding by different kinds of cell (Dimpfel *et al.*, 1977). It was found that primary cell cultures derived from embryonic mouse central nervous system could bind ^{125}I-labelled tetanus toxin, but continuous cell lines failed to do so. This binding was subsequently shown to correlate with non-synthesis of gangliosides (see Section 1.8.1) in continuous cultures; toxin binding could be enhanced by *in vitro* addition and uptake of the relevant gangliosides into the cell membrane (this has been shown by other workers also; Yavin (1984), working with somatic neurohybrid cells, observed an eight-fold enhancement of binding after ganglioside supplementation, and Pierce *et al.* (1986) were able to produce a similar effect using rat brain membranes). Further, hybrid cells (neuroblastoma and glioma fusions) which contained relatively low levels of GD1b and GT1 gangliosides bound only small amounts of toxin. Experiments by Yavin & Habig (1984) gave similar results; they investigated the interaction of ^{125}I-labelled toxin with several somatic neural hybrid cell lines, and

found that the capacity of the hybrid cells to bind tetanus toxin correlated well with the complexity exhibited by their cellular gangliosides.

In another type of study, Mirsky *et al.* (1978) used immunofluorescence to show that tetanus toxin can distinguish neuronal from non-neuronal cells in a wide variety of dissociated cell cultures. They investigated cells from cortex, cerebellum, spinal cord and dorsal root ganglia from rats, and spinal cord, optic lobe, retina and dorsal root ganglia from chicks. Tetanus toxin was found to bind highly specifically to neuronal cells from all parts of the central nervous system in a ganglioside-dependent fashion. However, although the binding specificity of the toxin for neurones is marked, it is not absolute. Thus tetanus toxin does not bind to some large neurones present in late culture (Raju & Dahl, 1982), and, in the retina, binding to neurones is stronger than to other cells but not exclusive to them (Beale *et al.*, 1982). The toxin will also bind to pancreatic islet cells, which are known to have some cell surface differentiation markers in common with neurones (Eisenbarth *et al.*, 1982), and to thyroid plasma membranes (Ledley *et al.*, 1977). This suggests that the restricted binding of toxin to particular neurones *in vivo* is therefore due to limited accessibility rather than preferential binding.

Numerous other experimental arguments favour the assumption that specific gangliosides are involved in binding tetanus toxin in nervous tissue. Binding of toxin to rat brain membranes has been shown to be inhibited in a dose-dependent manner by gangliosides, with GT1b (K_i = 6nM) and GD1b (K_i = 10nM) being the most potent

(Rogers & Snyder, 1981); similar results were obtained by Critchley *et al.* (1986) under the same conditions. Further, Rogers & Snyder were able to show that GT1b and GD1b gangliosides could readily release toxin associated with mammalian brain membranes in a highly specific manner; they also observed that binding of the toxin was not affected by pretreatment of the neuronal membranes with trypsin or protein modification reagents, but was substantially reduced by neuraminidase. (Neuraminidase treatment has also been shown to reduce binding at 0°C and 37°C to neural cells in culture, while treatment by proteases before the addition of toxin has only a marginal effect in diminishing subsequent binding; Yavin, 1984).

Further evidence was to come from the finding that the non-toxic fragment C of tetanus toxin, which retains the capacity to interact with gangliosides, competes with the intact toxin for binding both *in vivo* (Simpson, 1984) and *in vitro* (Goldberg *et al.*, 1981); this gives more support to the idea of an *in vivo* ganglioside receptor. Also, it has been argued (Goldberg *et al.*, 1981) that the large number of toxin binding sites present in neuronal membranes is compatible with the reported concentration of GT1b and GD1b in rat brain (Ando *et al.*, 1978).

Recently, some confusion arose when Lazarovici *et al.* (1984) reported that radiolabelled tetanus toxin could be separated by ganglioside-affinity chromatography into two populations, one of which failed to bind to the solid-phase ganglioside column or to nerve cells, but which retained full toxicity. However, this claim not only contradicted the results obtained by affinity chromatography on synaptosome columns (Habermann, 1976), but also

could not be reproduced with the same type of solid-phase ganglioside (Habermann & Tayot, 1985). The importance and relevance of these findings is thus very much open to question.

It would seem then that there is clear evidence from experiments using synaptosomes, brain membranes and primary neuronal cell cultures to suggest a role for gangliosides as cell surface receptors for tetanus toxin. However, over the past several years, a significant number of reports have appeared which disagree markedly with this view. One of the first suggestions of a non-ganglioside receptor came when Zimmerman & Piffaretti (1977) distinguished between what they interpreted as two independent types of toxin binding. What they called "non-effective" binding, which was observed only with non-differentiating mouse neuroblastoma cells in culture, produced no biological effect and was shown by neuraminidase treatment to be ganglioside-dependent. "Effective" binding on the other hand was not associated with gangliosides (i.e. neuraminidase was ineffective), and led to shortening of cell processes and reduction in cell adherence to glass; this was observed only with differentiating cells. They postulated that non-effective binding was followed by pinocytosis and degradation by lysosomes, while effective binding led to internalisation of the toxin for subsequent retrograde axonal transport (see Section 1.9.3). However, since it is not clear what toxin concentrations were used in these experiments, it is difficult to assess their physiological relevance.

Several experimental observations suggest that the neurotoxic activity of tetanus toxin may be mediated by components other than gangliosides. For instance, Stoeckel *et al.* (1977) reported that exogenous gangliosides (either GT1 alone or a bovine ganglioside mixture) caused only a 50% blockade of the retrograde axonal transport of the toxin, while total blockade would be expected if gangliosides alone were responsible for toxin fixation (pre-incubation of ^{125}I-labelled cholera toxin with GM1 ganglioside, to which it binds specifically, completely blocked its retrograde axonal transport); however, this incomplete blockade may have been due to hydrolysis of GT1 to other ganglioside species, a phenomenon which is known to occur since GT1 is not very stable (indeed, rechromatography of purchased "purified" GT1 may reveal a mix of different hydrolysis products). The existence of an extra-axonal mode of transport for the toxin within the central nervous system has also been cited as evidence for a non-ganglioside toxin receptor (Erdmann *et al.*, 1975). Further, the observation of Habermann (1981) that neuraminidase treatment was unable to suppress the inhibitory effect of the toxin on the K^+-evoked release of [^{3}H]-noradrenaline from preloaded particulate rat forebrain cortex strongly suggests that the toxin effect is mediated by its binding to some cell membrane component other than a ganglioside. Similarly, Habermann *et al.* (1980) showed that the blocking effect of the toxin on the neuromuscular junction of the mouse phrenic nerve-hemidiaphragm could not be prevented by neuraminidase, while Bigalke *et al.* (1981) demonstrated the inability of neuraminidase to suppress toxin effects on K^+-evoked release of [^{3}H]-acetylcholine from brain slices. If toxin binding to ganglioside is important for its mechanism of action, it can be

predicted that neuraminidase treatment should confer at least partial protection from intoxication. Since this is not the case, it would appear that gangliosides are not physiological toxin receptors; the most obvious explanation for these observations is that the interaction of tetanus toxin with the cell surface may be more complex and require additional components.

Several other considerations suggest that the G1b gangliosides are not solely responsible for tetanus toxin binding to neuronal membranes. For instance, although there is no doubt that tetanus toxin binds to gangliosides and that there is some specificity with respect to the type of ganglioside, this specificity is not particularly marked. While cholera toxin has a binding preference for GM1 over other gangliosides by at least three orders of magnitude (as expressed in moles of toxin fixed per mole of ganglioside), tetanus toxin shows only a sevenfold preference for GD1b ganglioside over GM1 (van Heyningen, 1974). Considering the potency of tetanus toxin, one would expect a much greater specificity in a true toxin-receptor interaction (however, it may be that the specificity of binding is not a crucial factor, in other words it may not be important how the toxin binds to the cell as long as this binding is able to support entry of the toxin into the cell). Further, gangliosides are present on neuronal cells in enormous excess with respect to tetanus toxin at clinical doses (Mellanby & Green, 1981), whereas specific receptor systems (e.g. for hormones) are usually restricted in number. While some have suggested that an extreme excess of binding sites may contribute to the potency of the toxin (e.g. Habermann & Dreyer, 1986), it may be that this large number of gangliosides represents a sink of non-

productive toxin binding, as Zimmermann & Piffaretti (1977) have suggested; however, since there is likely to be a vast excess of toxin present anyway, this may not be an important consideration. No-one has shown definitive data to correlate binding of toxin to gangliosides and cellular responses to toxins; even experiments where a decrease in toxicity is observed after pre-binding toxin with gangliosides should not necessarily be interpreted as evidence of structural similarities between the protective ganglioside and the toxin receptor.

It is very important to note that most of the early binding studies done with tetanus toxin (e.g. those cited previously, Rogers & Snyder, 1981; Lee *et al.*, 1979; Morris *et al.*, 1980) which suggested a receptor role for gangliosides, were carried out under conditions previously shown to be optimum for toxin binding *in vitro* i.e. in a low salt, pH 6.0 buffer (Ledley *et al.*, 1977); therefore it is not impossible that binding of tetanus toxin under these conditions is to components, maybe gangliosides, that are not necessarily involved in binding under physiological conditions. Also, it would seem that no specific measures were taken to limit proteolysis during membrane isolation procedures, so that candidate proteinaceous toxin receptors might have been destroyed. More recent binding studies have been conducted under more physiological conditions of ionic strength and pH; this reduces toxin binding, with a lowering of both affinity and number of sites (Weller *et al.*, 1986). An investigation of toxin binding to cerebral neurones in monolayer culture by Yavin & Nathan (1986) showed that under physiological conditions, binding could be reduced by approximately 40% if the cells were treated with trypsin prior to toxin

incubation; trypsin-insensitive binding could be eliminated by neuraminidase treatment or cell extraction with methanol. Experiments looking at the effect of cross-linking agents such as formaldehyde on binding suggested that a substantial portion of the binding sites contained a sialo cross-linkable protein component, but that there was also a sialo component unaffected by cross-linkers. On the basis of these observations, the authors proposed that cerebral neurones possess two classes of ganglioside receptor, one of which may be modulated by cell surface proteins.

Critchley *et al.* (1986) did similar experiments using rat brain membranes. Again it was demonstrated that binding at pH 7.4 (in Tris-saline) was of lower affinity and capacity than at pH 6.0 (in Tris-acetate). Binding under both conditions was reduced by neuraminidase treatment, but protease treatment only decreased the physiological binding. The authors conclude that the toxin interaction with rat brain membrane gangliosides is much reduced under physiological conditions; this is further supported by the finding that the concentration of gangliosides needed to inhibit toxin-binding was 100-fold higher at pH 7.4 than at pH 6.0. In an attempt to analyse the nature of the toxin receptors in rat brain membranes further, membrane components were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose which was then overlaid with radioactive tetanus toxin; the toxin bound only to material that migrated at the dye front and that was extracted by lipid solvents (presumably gangliosides). When binding of toxin to nitrocellulose was determined in the Tris-saline buffer (pH 7.4), the toxin bound to the same components but the extent of binding was markedly reduced

compared with the low salt and low pH conditions. The authors were unable to detect any toxin binding to membrane proteins, but this may have been because the secondary or tertiary structure of the protein was altered such that the toxin could no longer interact with it. Critchley concludes that membrane components other than gangliosides, such as sialoglycoproteins, may act as toxin receptors under physiological conditions. Pierce *et al.* (1986) arrived at similar conclusions; in physiological buffers, tetanus toxin binds with high affinity to a protein receptor, while gangliosides represent only a low affinity site. Parton *et al.* (1988) obtained similar results working with spinal cord cultures, as did Lazarovici & Yavin (1986) using guinea pig brain synaptic membranes. Interestingly, the latter authors suggest that the toxin interaction with neuronal membranes may also require an appropriate lipid environment; they observed that binding was facilitated, at least in part, by phospholipase-sensitive components.

Another theoretical suggestion, which can explain many of the findings reported above, has been put forward by Montecucco (1986); he proposes that binding to both a ganglioside and a protein acceptor is important to toxin action. The high concentrations of gangliosides present in neuronal membranes are envisaged to bind toxin through low affinity interactions trapping it at the cell surface. Rapid diffusion of this complex in the membrane would then lead to collision with low abundance high affinity protein acceptors involved in toxin internalisation.

In any case, further progress would seem to depend on purification of the protease-sensitive toxin acceptor. Traditional methods for purification of membrane protein receptors generally depend on a convenient assay that can be used with solubilised material. Such an assay system may be difficult to develop in the case of the toxin receptor, given the large size of the toxin itself; therefore it may be necessary to follow the more arduous route of screening for monoclonal antibodies to neuronal membranes which block toxin binding. Such antibodies could then be used to characterise the receptor via Western blotting and to affinity purify the component for reconstitution studies.

Thus our knowledge about the true physiological receptor for tetanus toxin is incomplete. While there is no doubt that polysialogangliosides will bind tetanus toxin, many experiments are incompatible with the view that gangliosides are the only receptors for the toxin; much evidence points to the existence of another membrane component, perhaps a protein, which facilitates toxin binding. One point which has not been considered during this discussion is, what do we actually mean when we speak of a toxin receptor? It is really a matter of convenience that the cell surface binding sites for tetanus toxin should be termed "receptors" at all; "receptor" would imply that the main, if not only, function of the cell surface molecule (whether it be ganglioside or otherwise) is to bind tetanus toxin and facilitate its subsequent internalisation. This is certainly not the case; it is obvious that a cell will not express a receptor on its surface specific for a toxic molecule. Thus it would seem that the toxin acts in an opportunistic fashion, utilising a moiety present on the

cell surface for some other purpose as a means of gaining access to its intracellular target. It is perhaps more correct, then, to refer to toxin binding molecules as "acceptors" rather than "receptors".

It must be realised too, that there are no published data to show that for instance the tetanus toxin receptor in brain is the same as that in peripheral nerve; it is possible that receptors in different cell types may be distinct molecular species. With this in mind, it is interesting to note that in recent years, several groups have used various model cell systems to study the binding and subsequent physiological effect of the toxin. Thus, Staub *et al.* (1986) investigated toxin-binding to a neuroblastoma retina hybrid cell line N18-RE-105, and found it to be very similar to binding to rat synaptic membranes; further, it was demonstrated that these cells contained polysialogangliosides, although the effect of neuraminidase or proteolytic enzymes on toxin binding was not examined. Similarly, Wellhoner & Neville (1987) showed that tetanus toxin bound with high affinity to neuroblastoma x glioma hybrid cells NG108-15; this could be prevented by pretreatment with neuraminidase, as could toxin inhibition of acetylcholine release from these cells. These authors suggested that neuraminidase-sensitive receptors serve as productive receptors for tetanus intoxication in differentiated NG108-15 cells; these could be either gangliosides or sialoglycoproteins. Finally, Walton *et al.* (1988) and Fujita *et al.* (1990) have used phaeochromocytoma PC12 cells to study tetanus toxin binding. Walton's group reported that the major ganglioside species present in these cells was GT1b and that radiolabelled toxin bound with high affinity, while Fujita

et al. observed very low levels of binding to untreated cells, which was enhanced ten-fold after nerve-growth factor-induced differentiation; neuraminidase treatment reduced binding by about 40%, but the nature of the neuraminidase-resistant sites was unclear. How relevant the findings of experiments in these model systems are to the true *in vivo* case, however, remains to be seen.

1.9 INTERNALISATION OF BOUND TOXIN

1.9.1 Introduction

It is generally believed that tetanus toxin shares the tripartite mechanism of action employed by other bacterial toxins with intracellular targets, namely binding of the toxin to the cell membrane, as discussed above, followed by its internalisation, and finally modification of an intracellular target (e.g. Simpson, 1986). There are two distinct binding sites for tetanus toxin in the body; firstly, after the release of toxin at the site of infection, it is seen to bind to nerve endings at the neuromuscular junction, while a second binding site is at its site of action, the pre-synaptic membranes of certain inhibitory synapses (see Section 1.10). In both instances, binding is followed by an internalisation step; in the case of the neuromuscular junction, this leads to subsequent axonal transport of the toxin to the central nervous system (see Section 1.9.3), while at the inhibitory synapse, internalisation is followed by intracellular intoxication. There is no reason to believe that there is any difference between the mechanisms involved in each case (if indeed a specific internalisation mechanism does operate, see below).

1.9.2 Possible Mechanisms of Membrane Translocation

Since toxin-binding occurs on the neuronal cell surface, and intoxication is presumed to occur intracellularly (see Section 1.10), it would appear that the toxin must translocate across the membrane to the cell interior. In recent years considerable progress has been made in elucidating the morphological basis of tetanus toxin internalisation, although it can perhaps be argued that a specific mechanism is not necessarily essential for internalisation to occur. There are several possible routes into the cell, including receptor-mediated endocytosis, pinocytosis or even direct passage across the plasma membrane; whether one of these phenomena occurs in preference to the others is still a somewhat open question.

Schwab & Thoenen (1978) carried out some of the early experiments in this area. They demonstrated that when tetanus toxin adsorbed to colloidal gold was injected into the anterior eye chamber of rats, it was found 1-2 hours later in smooth, membranous elements within axons; there was very little incorporation into lysosomes. Similar studies with peroxidase-conjugated toxin also indicated that transport occurred via smooth vesicles (Schwab *et al.*, 1979). Much of the toxin was ultimately incorporated into lysosomes, but a small amount was specifically accumulated in presynaptic nerve terminals where it was contained within small vesicles of 50-100 nm diameter. Unfortunately, however, it could not be concluded from these studies whether internalisation at the neuromuscular junction (or trans-synaptic movement of the toxin) was mediated by coated pits. Later, Montesano *et al.* (1982) studied the internalisation of tetanus toxin-gold particles by cultured cells, and also found

these to be localised in smooth vesicles. These authors found no evidence for the involvement of coated pits; however, since internalisation via this mechanism is a rapid process, the experimental procedure adopted was such that it may not have been detected even if it was in fact occurring. It should be noted also that the neurotoxicity of the tetanus toxin-gold was not established, and that it was used at very high concentrations (1 mg/ml). Moreover, the cells used were not of neuronal origin, so the relevance of these findings is to be questioned.

More recent studies on tetanus toxin internalisation have been conducted on neuronal cell cultures. Critchley *et al.* (1985) bound tetanus toxin to primary mouse spinal cord cell cultures at 4°C, and followed internalisation (after warming cells to 37°C) by measuring the accessibility of toxin to anti-toxin. They found that some surface-bound toxin was rapidly internalised (within 5 minutes). Immunofluorescence experiments confirmed this, and indicated that internalised toxin was distributed in a punctate pattern, reminiscent of ligands internalised via coated pits. Parton *et al.* (1987), using the same cells, adsorbed tetanus toxin to colloidal gold, and used electron microscopy to define the mode of internalisation more precisely. Quantitation of the distribution of toxin-gold particles bound to cell bodies at 4°C showed that they were concentrated in coated pits; few particles were found within cells under these conditions. Five minutes after warming the cells to 37°C however, toxin-gold was found within coated vesicles, endosomes and tubules. After 15 minutes at 37°C internalised toxin was found mainly in endosomes, and after 30 minutes largely in multivesicular bodies. Tetanus toxin-gold was

also shown to enter nerve terminals and axons via coated pits, accumulating in synaptic vesicles and intra-axonal uncoated vesicles respectively (given that the toxin inhibits the release of neurotransmitters, toxin accumulated here may be more relevant to intoxication than that internalised by the cell body). Interestingly, much of the toxin-gold remained bound at the cell surface but was not rapidly internalised. Parton suggests that perhaps toxin internalised via coated pits is that bound to the protease-sensitive site which has been detected in mouse spinal cord cells and which may be equivalent to the small number of protease-sensitive high affinity sites in rat brain membranes (see Section 1.8.2). Toxin not rapidly internalised may represent binding to the lower affinity sites, possibly gangliosides, which may not be able to support internalisation via coated pits. In agreement with this idea, it was noted that cholera toxin, specific for ganglioside GM1, was partially excluded from coated pits; also, Montesano *et al.* (1982) had previously shown that cholera toxin entered non-neuronal cells via smooth membrane invaginations.

In an attempt to determine whether tetanus toxin bound to gangliosides can enter cells via coated pits, Parton *et al.* (1988) incorporated ganglioside GT1 into the plasma membrane of mouse fibroblast Balb/c3T3 cells which lack endogenous toxin-binding activity. Using toxin-gold, it was demonstrated that at 4°C, toxin was concentrated in non-coated cell surface membrane invaginations; on warming to 37°C, toxin was internalised via non-coated vesicles and accumulated within multivesicular bodies and lysosomes. In contrast, there was negligible labelling of coated pits. These results, the authors conclude, show that toxin bound to ganglioside

receptors does not enter cells via coated pits, and thus lend support to the view that toxin internalised by neuronal cells in this fashion must be that bound to a protein receptor. While it is highly likely that some of the toxin bound to neuronal cells is associated with the abundant low affinity ganglioside receptors, toxin-gold particles were not observed to enter neuronal cells via non-coated invaginations. However, it is not impossible that this phenomenon occurs; cholera toxin can undergo retrograde axonal transport (see Section 1.9.3), suggesting that ligands bound to gangliosides can indeed be internalised into a vesicular compartment in nerve terminals. If this is so, then the question must be asked: does tetanus toxin internalised via both coated pits and non-coated invaginations contribute to intoxication? The fact that cholera toxin, unlike tetanus toxin, does not undergo trans-synaptic transfer (see Section 1.9.3) suggests that perhaps ligands entering neurones via a ganglioside receptor may be processed differently from those internalised via a protein receptor. Tetanus toxin entering cells via coated pits passes into the acidic endosomal compartment where it remains bound to the limiting endosomal membrane; this may account for the ability of a significant portion of the toxin to escape degradation and hence exert its neurotoxic effects. On the other hand, toxin bound to gangliosides and internalised via non-coated invaginations (at least in fibroblasts) would seem to be directly routed to lysosomes; assuming that this can be extrapolated to neuronal cells, it appears that toxin internalised by this route is largely degraded and therefore probably not neurotoxic. The view that an acidic compartment is important to toxin action is supported by the observation that lysosomotropic agents which raise the pH inside

endosomes (e.g. ammonium chloride, methylamine hydrochloride) cause a significant delay in the onset of toxin-induced spastic paralysis (Simpson, 1983); however, it would be interesting to know whether an *in vitro* effect of these agents could also be observed.

Once within the inhibitory neurone, trapped inside the endosome, the toxin molecule must presumably reach the cell cytoplasm to exert its toxic effect. It has been proposed that the low pH which is achieved in the lumen of the endosome somehow causes a conformational change in the toxin, such that its hydrophobic surfaces are exposed, facilitating its penetration and translocation of the endosome membrane (Boquet & Duflot, 1982). These workers demonstrated that tetanus toxin fragment B (light chain plus the amino-terminal half of the heavy chain) could release K^+ ions from single-walled asolectin vesicles at low pH; since light chain alone was unable to provoke K^+ release, it was concluded that the amino-terminal of the heavy chain could facilitate the formation of a channel that might be involved in the transport of an active toxin fragment (presumably the light chain) into the cytosol.

Hoch *et al.* (1985) also found that the heavy chain formed channels at low pH in planar lipid bilayer membranes. Selectivity experiments with different cations and anions showed that the channel was potentially large enough to serve as a "tunnel protein" for translocation of the light chain. In agreement with this, Roa & Boquet (1985) have demonstrated that two polypeptides which localise to the amino-terminal of the heavy chain are protected against proteolysis when a mixture consisting of asolectin vesicles

and ^{125}I-labelled tetanus toxin is subjected to a pH drop from 7.2 to 3.0. Further, Johnstone *et al.* (1990) have demonstrated that the heavy chain of tetanus toxin can facilitate the entry of gelonin (a cytotoxic cell-impermeant analogue of the ricin A chain) into intact cultured HT29 cells (a human colonic carcinoma cell line). Although this experiment provides no information about the actual mechanism of toxin internalisation, it does lend further support to the notion that the heavy chain may mediate the entry of the light chain into cells.

However, Montecucco *et al.* (1986) have shown, using hydrophobic labelling with radioactive photoactivatable phospholipids, that both toxin chains interact with the fatty acid portion of the lipid bilayer at low pH. (This is supported by the finding that, at low pH, many binding sites for Triton X-100 appear on both toxin chains; Boquet *et al.*, 1984). On the basis of these findings, Montecucco proposes a model in which both chains of tetanus toxin penetrate the bilayer, with their hydrophobic surfaces exposed to the membrane lipids and their hydrophilic residues facing each other. He further suggests that this penetration is not necessarily followed by translocation of the toxin across the membrane, as proposed by Hoch *et al.* (1985), and that it is not impossible that the toxin may carry out its enzymatic function while embedded in the endosome membrane. Indeed, it should be pointed out that although there is mounting evidence to suggest that endocytosis is a prerequisite for intoxication (Simpson, 1986), it has not been conclusively proven that tetanus toxin does not reach its cytoplasmic substrate from the plasma membrane, as is the case with cholera and pertussis toxins.

(One point to bear in mind when looking at many of these experiments is that they were conducted in very artificial systems. Therefore their true similarity to the *in vivo* situation, and thus their biological relevance, is perhaps open to question.)

1.9.3 Retrograde Axonal Transport and Trans-synaptic Transfer

(This is a very large field, which will only be discussed in brief, as it is of little relevance to the work presented in this thesis).

The fact that the site of tetanus infection and the focus of the disease are not the same is obvious, implying that there must be some transport of the toxin to its site of action. The mechanism of this transport has been studied in great detail and is one of the few problems concerning the action of tetanus toxin which can perhaps be said to have been solved at least in outline. For many years there were two basic theories, one proposing that the toxin reached the spinal cord via the blood (e.g. Abel *et al.*, 1935) while another idea, first introduced by Meyer & Ransom (1903), suggested that it reached the spinal cord via a neural route of some sort. The latter has since been shown to be the means by which tetanus toxin reaches its site of action. Transport of the toxin has been observed to be intra-axonal (Erdmann *et al.*, 1975) and in a retrograde direction (Price *et al.*, 1975). Further, Stoeckel *et al.* (1975) have demonstrated that toxin is transported in all peripheral neurones, especially in motor, sensory and adrenergic types, although it seems likely that it is that which ascends in motor neurones which constitutes the bulk of the toxin reaching the CNS. Transport in motor neurones is mainly confined

to α-neurones (Green *et al.*, 1977), with little or no transport via γ-neurones. Toxin has been shown to be transported within vesicles and branched cisternae of the smooth endoplasmic reticulum (Griffin *et al.*, 1977).

Once the toxin has reached the motor neuronal cell body by axonal transport, it may either be transported up the next motor neurone, or interact with the pre-synaptic membrane of an inhibitory neurone synapsing with the motor neurone. Either way, the toxin is required to cross a synaptic cleft. This trans-synaptic migration of the toxin has been demonstrated by Schwab & Thoenen (1976), and appears to be specific to the toxin; other macromolecules such as cholera toxin, wheat germ agglutinin and ricin, which may be transported in the same motor neurones as tetanus toxin, are not transferred (Schwab *et al.*, 1979). Finally, evidence suggests that it is the intact whole toxin which is transported (Dumas *et al.*, 1979).

For further discussion on the transport of tetanus toxin, see Bizzini, 1979; van Heyningen, 1980; Mellanby & Green, 1981; Habermann & Dreyer, 1986.

1.10 THE ACTION OF TETANUS TOXIN ON THE NERVOUS SYSTEM

Once within the inhibitory neurone, the toxin is able to express its biological effects. It has been well established that tetanus toxin blocks the action of inhibitory neurones, much as strychnine does, although the molecular mechanism involved in producing this effect remains unclear. It is known, however, that the toxin acts

pre-synaptically (in contrast to strychnine), and it is the release of inhibitory neurotransmitter (both spontaneous and evoked) rather than its synthesis which is impaired (Curtis & DeGroat, 1968; Fedinec & Shank, 1971; Osborne & Bradford, 1973).

The toxin has been shown to block neurotransmission at a number of different sites in the central nervous system, including pre- and post-synaptic inhibitory synapses where δ-aminobutyric acid (GABA) is the transmitter (Curtis *et al.*, 1973; Davies & Tongroach, 1979), as well as post-synaptic inhibitory synapses utilising glycine (Curtis & DeGroat, 1968). This indicates that tetanus toxin action is not specific to a particular transmitter or to a particular type of synapse. Indeed, although central inhibitory synapses are the main target of the toxin, clinical observations and experiments with *in vivo* administration of tetanus toxin show that the toxin also affects peripheral neurotransmission, albeit with a lower potency. Duchen & Tonge (1973) have shown the toxin to block transmission at excitatory neuromuscular junctions in the mouse, while Diamond & Mellanby (1971) have demonstrated the toxin-sensitivity of the neuromuscular junction of the skeletal muscle in the goldfish fin. A peripheral toxin effect has also been demonstrated in the rabbit iris, where cholinergic parasympathetic synapses are affected (Ambache *et al.*, 1948). The relevance of this peripheral blockade remains unclear, although studies on these relatively simple peripheral systems might help us to understand the complex central effects of the toxin. There is also some experimental evidence for a direct action of tetanus toxin on the autonomic nervous system (Paar & Wellhoner, 1973).

The effects of the toxin are very long-lasting and indeed it seems that, at least in the case of the neuromuscular junction, recovery requires nerve-terminal sprouting and the formation of new synapses (Duchen & Tonge, 1973). It is unclear whether this longevity of toxicity is due to the continued presence of the toxin at the site of action, or to an immediate action of the toxin with prolonged sequelae.

In recent years, there have been several reports of tetanus toxin action *in vitro*, e.g. on particulate preparations, synaptosomes and cultured cells. Some experiments have shown a direct paralytic effect of the toxin *in vitro* (e.g. Habermann *et al.*, 1980), while others have concentrated on demonstrating the inhibitory effect of tetanus toxin on the uptake and release of neurotransmitters (e.g. Bigalke *et al.*, 1978; Habermann *et al.*, 1981; Bigalke *et al.*, 1981; Collingridge *et al.*, 1981; Collingridge & Davies, 1982; Pearce *et al.*, 1983; Wendon & Gill, 1982). These kinds of neurophysiological experiments serve to demonstrate that the toxin can exert its effects in a wide variety of *in vitro* systems, but do not yield any information on the mechanism by which inhibition of secretion is achieved. It is also important to keep in mind that it is very difficult to assess the relevance of this kind of experiment to the *in vivo* situation; there may be substantial differences between results obtained using *in vitro* systems and what occurs *in vivo*.

Recent experiments have been able to provide more useful information about tetanus toxin action. Firstly, evidence has been provided which strongly suggests that the toxin does in fact act intracellularly to block secretion (up until recently this was only

an assumption). This has come from the work of Penner *et al.* (1986), who have demonstrated that intracellularly injected tetanus toxin is able to strongly inhibit exocytosis in bovine adrenal chromaffin cells. Further, experiments have been carried out which indicate that it is the light chain of the toxin molecule which is responsible for intracellular poisoning. Ahnert-Hilger *et al.* (1989) have shown that when chromaffin cells permeabilised with streptolysin O are incubated with solutions of the light chain, and subsequently challenged with secretagogues, an inhibition of exocytosis is observed. Also, in a series of very elegant experiments, Mochida *et al.* (1990) have succeeded in expressing exogenous mRNA encoding tetanus toxin light chain in *Aplysia* neurones and demonstrating a subsequent inhibition of neurotransmitter release.

Thus the predominant, although not the only, effect of tetanus toxin *in vivo* is a preferential blockade of inhibitory neurotransmitter release, resulting in a loss of activity of inhibitory pathways acting on both α and γ motor neurone systems. This causes hyperactivity of spinal cord motor neurones, which in turn results in increased muscle tone and the dramatic spasms of tetanus. It would appear that it is the light chain of the tetanus toxin molecule which facilitates intracellular poisoning, but the molecular mechanism responsible for this is not known. Various proposals have been put forward, and these will be discussed elsewhere in this thesis.

For more detailed reviews on the action of tetanus toxin, see Mellanby & Green (1981); Habermann & Dreyer (1986).

1.11 COMPARISON OF TETANUS TOXIN WITH OTHER BACTERIAL TOXINS

Tetanus toxin may be compared with other bacterial protein toxins on the basis of similarities in either toxin structure or the nature of the toxin receptor; since the molecular basis of tetanus intoxication remains unclear, comparisons with regard to the mechanism of toxin action are not possible.

Of all the other bacterial toxins studied, it is not surprising that those produced by *Clostridium botulinum*, a micro-organism closely related to the causative agent of tetanus, show the greatest similarity to tetanus toxin. The various strains of *C. botulinum* produce at least eight different toxins, seven of which (A, B, C1, D, E, F and G) are neurotoxins (botulinum C2 toxin is not neurotoxic but cytotoxic, acting to ADP-ribosylate actin (Aktories *et al.*, 1986) and so decreasing the ability of the microfilament protein to polymerise). Like tetanus toxin, these are produced as exotoxins formed in the bacterium mainly during the stationary phase and are released upon cell lysis. They are synthesised as single-chain polypeptides with a molecular weight of 150 kDa, and like tetanus toxin undergo a proteolytic cleavage to yield a two-chain molecule consisting of a heavy chain (100 kDa) and a light chain (50 kDa) held together by a disulphide bond. Further, there is a marked similarity between the gene sequences of the heavy and light chains of tetanus toxin and botulinum toxins A, B and E (Eisel *et al.*, 1986; DasGupta & Foley, 1989), suggesting a common ancestral gene.

However, it is not only at the structural level that tetanus and botulinum toxins show common features. These molecules act in a similar fashion as well, by blocking the evoked and spontaneous release of neurotransmitters; the main difference lies in their sites of action within the body. While the principal effect of tetanus toxin is the blockade of central inhibitory mechanisms, botulinum toxin has been shown to impair neuromuscular transmission, so that tetanus characteristically involves spasticity with convulsions, while botulism causes a generalised flaccid paralysis. It seems possible that the mode of action of both toxins at the molecular level might be similar, and that the differences in their clinical effects are due to some as yet undefined properties of the toxin molecules that determine which synapses they act upon. (Originally, when it was found that tetanus toxin bound fairly specifically to nervous tissue and that this was apparently due to gangliosides, it seemed that it was this that directed tetanus toxin towards the CNS; however, it has been shown that botulinum toxin can also bind to nervous tissue (Habermann & Heller, 1975), albeit less avidly than tetanus toxin, so it is no longer thought that the synaptic specificity exhibited by the two toxins is due to their different affinities for gangliosides. Furthermore, botulinum toxin can also be transported by retrograde axonal transport up motor neurones (Wiegandt & Wellhoner, 1974), so this is not the explanation either.)

Tetanus toxin may further be compared to several other bacterial toxins on the basis of similar structures or receptors. For instance, the receptor for cholera toxin is also a ganglioside, in this case the monosialoganglioside GM1. The structure of cholera

toxin however, (which incidentally is similar to tetanus toxin in that it also affects secretion across a cell membrane, causing electrolyte and water losses from epithelial cells of the small intestine) is quite different to that of tetanus toxin; it is composed of two different types of subunit (five identical B subunits and one A subunit). However, both toxins contain specific parts (the heavy chain in tetanus toxin, the B subunit in cholera toxin) responsible for binding to receptors; also, the A subunit of cholera toxin is the active moiety, and similarly it would appear that the light chain of tetanus toxin is the active part of the molecule (see Section 1.10). These comparisons may also be made between tetanus toxin and the heat-labile toxin of *E. coli*, since the latter has the same subunit structure (Clements *et al.*, 1980), and indeed the same biological effects, as cholera toxin.

Structurally, the diphtheria toxin molecule, synthesised and secreted by *Corynebacterium diphtheriae*, is very similar to tetanus toxin in that it is also composed of two chains linked by a disulphide bridge. It has an A chain, which again is the active part of the molecule, and a B chain, required for toxicity but atoxic by itself, which presumably facilitates binding of the toxin to the cell surface. Diphtheria toxin, like tetanus toxin, is synthesised as a single polypeptide and must be nicked by a protease to form the active molecule (van Heyningen, 1976b). This structural similarity is also shared with exotoxin A of *Pseudomonas aeruginosa* (Vasil *et al.*, 1977).

A summary of the comparative features of bacterial toxins is presented in Table 1.1.

Table 1.1 The Component Structure of Various Toxins (adapted from van Heyningen, 1984)

TOXIN	MOL.WT. OF TOXIN	ACTIVE COMPONENT			BINDING COMPONENT		
		STRUCTURE	MOL.WT.	TARGET	STRUCTURE	MOL.WT.	TARGET
NEUROTOXINS							
Tetanus	150,000	L chain	50,000	Unknown	H chain	100,000	Gangliosides GT1b, GD1b
Botulinum	150,000	L chain	50,000	Unknown	H chain	100,000	Unknown, may be a glycolipid
AFFECTING ADENYLATE CYCLASE							
Cholera	82,000	Subunit A (A1 peptide +A2 peptide)	27,000 22,000 5,000	G_s protein of adenylate cyclase	Five B subunits	11,600 each	Ganglioside GM1
E. coli heat labile	91,000	Subunit A (A1 peptide +A2 peptide)	30,000 25,000 5,000	G_s protein of adenylate cyclase	Five B subunits	11,800 each	Ganglioside GM1 and glycoproteins
Pertussis	117,000	S-1	28,000	G_i protein of adenylate cyclase	S-2 S-3 Two S-4 S-5	23,000 22,000 11,700 9,300	Unknown, may be a glycoprotein
AFFECTING PROTEIN SYNTHESIS							
Diphtheria	62,000	A chain	24,000	EF2 (diphthamide)	B chain	38,000	Glycoprotein
Ps. aeruginosa exotoxin A	71,000	A fragment	27,000	EF2 (diphthamide)	B fragment	45,000	Has no clear function

1.12 THE ADRENAL CHROMAFFIN CELL

Mammalian adrenal glands, situated in the body just above the kidneys, consist of two morphologically distinct tissues, the medulla and the cortex, both of which may be observed following a lateral section of a gland. The cells of the adrenal medulla function to manufacture, store and secrete a mixture of hormones, the most important of which is adrenaline; in the case of the bovine adrenal medulla, approximately 75% of the cells produce adrenaline, while only about 25% synthesise noradrenaline (these numbers vary to some extent between species). These specialised cells were termed chromaffin cells by Alfred Kohn at the beginning of this century, owing to the chemical reaction of adrenaline with chromium salts, which produces a yellowish-brown colour.

Chromaffin cells are closely related to sympathetic neurones, sharing the same embryological origin in the neural crest. When allowed to differentiate *in vitro*, they develop long axon-like processes or "paraneurones". Like the neurones of the sympathetic system, the adrenal medulla is controlled by nerves originating in the spinal cord (see below), and its primary hormone, adrenaline, is closely related to noradrenaline, the characteristic neurotransmitter of the sympathetic nerves. Moreover, the adrenal medulla itself secretes some noradrenaline, and it also releases neurologically active neuropeptides (see below). The implications of these similarities will be discussed further in Section 1.13.

The catecholamine hormones of chromaffin cells are not stored as free molecules in the cytosol, but are contained within subcellular organelles known as chromaffin granules, small vesicles approximately 0.3 μm in diameter, which may number up to 30,000 in a single chromaffin cell. In addition to the catecholamines, these granules contain several proteins, at a total concentration of approximately 200 mg/ml; chromogranins A and B (which are as yet of unknown function) are the major protein components in bovine cells (Winkler *et al.*, 1986), together with the enzyme dopamine-β-hydroxylase (which is involved in noradrenaline synthesis), and a variety of derivatives of proenkephalin. Adrenaline and noradrenaline (about 0.5 M), ATP (0.1 M) and ascorbate (which serves as a co-factor for dopamine-β-hydroxylase) are the major small molecules. ATP, a very important granule constituent, serves to maintain osmotic stability within the granule.

Chromaffin cells not only store all these compounds, but release them as a mixture, by the process of exocytosis. These cells are particularly suitable for the study of exocytosis, since they are readily available in a relatively pure form, and so their secretory mechanism has been extensively researched. However, detailed knowledge still remains sparse.

1.12.1 An Overview of Secretion

In exocytosis a vesicle moves towards the surface of the cell, the vesicle membrane fuses with the plasma membrane, and the vesicle is opened to the exterior of the cell. The entire content of the vesicle is thereby emptied into the extracellular space. This is a

very efficient mechanism of release; it delivers compounds to the cell exterior at a much higher concentration than would be possible if they were secreted from the cytosol.

Exocytosis from chromaffin cells *in vivo* is triggered by stimulation of the splanchnic nerve, which provides cholinergic innervation to the adrenal medulla from the sympathetic nervous system. This stimulation evokes the release of acetylcholine from nerve terminals synapsing on the chromaffin cells, which in turn stimulates secretion from the chromaffin cells themselves. It is believed that acetylcholine sets up a chain of events in which its binding to a nicotinic acetylcholine receptor leads to the opening of a receptor-linked Na^+ channel, generating action potentials and the opening of voltage-dependent Ca^{2+} channels. The resulting increase in the intracellular concentration of Ca^{2+} is thought to be the signal for secretion to begin; this is supported by the work of Baker & Knight (1978) showing that, in a "leaky cell" preparation, secretion can be triggered without a cholinergic stimulus simply by raising intracellular Ca^{2+} concentrations to micromolar levels. Just how this comes about, though, is not clear.

In view of the fact that exocytosis is a Ca^{2+}-dependent event, much effort has gone into looking for Ca^{2+}-dependent interactions of chromaffin granules (or resealed granule membranes) with cytoskeletal or cytosolic components, in an attempt to identify some of the molecular events involved in secretion. For instance, with respect to cytoskeletal components, it has been shown that chromaffin granule membranes can bind F-actin (Fowler & Pollard,

1982), and that at least part of this binding appears to result from the presence of the actin-binding protein α-actinin on the cytoplasmic surface of the granule membrane (Aunis *et al.*, 1980). The fact that non-muscle α-actinins are calcium-sensitive with respect to their ability to cross-link actin filaments (this being inhibited by free Ca^{2+} levels of approximately 1 μM; Burridge & Feramisco, 1981) suggests that the interaction of granule membranes with actin via α-actinin could be Ca^{2+}-sensitive. This is consistent with a model in which Ca^{2+}-influx following stimulation of intact chromaffin cells results in the release of chromaffin granules from their association with an actin network; this release may be necessary to allow granule movement to the site of exocytosis. However, it must be remembered that these are experiments conducted *in vitro*, and the question remains as to whether or not chromaffin granules interact with actin *in vivo*.

Various cytosolic components have also been shown to interact with chromaffin granules *in vitro*. Thus, Geisow & Burgoyne (1983) have demonstrated that there are calmodulin binding sites (both Ca^{2+}-dependent and Ca^{2+}-independent) present on the chromaffin granule membrane. Binding of calmodulin to granule membranes results in the stimulation of a calmodulin-dependent protein kinase and the binding of other cytosolic proteins (see below). The high affinity of these calmodulin binding sites and the level of Ca^{2+} required for binding (Burgoyne & Geisow, 1981) are consistent with an increase in the binding of calmodulin to the Ca^{2+}-dependent sites following Ca^{2+} influx; this suggests that granule-bound calmodulin may play a role at some stage of the secretory process, perhaps at the level of fusion of granule and plasma membranes. Further

evidence comes from the finding of Trifaro & Kenigsberg (1983) that exocytosis is inhibited when antibodies against calmodulin are microinjected into chromaffin cells.

As well as calmodulin, several minor cytosolic proteins also show reversible Ca^{2+}-dependent binding to the cytoplasmic surface of granule membranes (Geisow & Burgoyne; 1982,1983), suggesting that they too become associated with the chromaffin granule membrane *in vivo* following stimulation. The binding of several of these proteins to the granule membrane appears to depend on the presence of calmodulin; however, the functions of these proteins are as yet unknown.

The requirement of Mg-ATP for secretion suggests the possibility that protein phosphorylation may also be involved in exocytosis; however, it is not clear whether or not this is the case. Examination of protein phosphorylation in chromaffin granule membrane fractions (Burgoyne & Geisow, 1981) has shown that the granule membrane contains an endogenous calmodulin-dependent protein kinase (activated half-maximally at around 5 μM Ca^{2+}) which phosphorylates various granule membrane polypeptides; whether the phosphorylation of any of these proteins is regulated during secretion, however, is not yet known. Similarly, experiments examining the effects of a phorbol ester on secretion from leaky chromaffin cells have suggested the possible involvement of protein kinase C in exocytosis (Knight & Baker, 1983a), since the phorbol ester was seen to increase the sensitivity of exocytosis to Ca^{2+}; however, the identity of the substrate for protein kinase C, involved in the regulation of exocytosis, is unknown.

On the basis of these findings, then, it is possible to construct a hypothetical scheme of some of the events involved in secretion from chromaffin cells. The scheme suggests that the translation of a rise in intracellular Ca^{2+} levels into an exocytotic event involves, at least in part, the dissociation of actin filaments from granules, the binding of calmodulin and other cytosolic granule-binding proteins to granules, and phosphorylation of some granule membrane proteins. It remains to be determined if any or all of these events are indeed essential aspects of the secretory process.

1.13 AIMS OF THE PROJECT

From the preceding discussion, it is clear that adrenal medullary chromaffin cells provide an ideal system in which to study exocytotic mechanisms. Further, since both chromaffin cells and sympathetic neurones synthesise and store catecholamines, and release them in response to cholinergic stimulation, it is not unreasonable to regard the chromaffin cell as a model of the neurone. Indeed, much has been learnt about the production and secretion of neurotransmitters through studies of the chromaffin cell. Also, as already mentioned, chromaffin cells cultured *in vitro* develop axon-like "paraneurones", so in this respect they are perhaps a better model of neuronal cells than many preparations of more directly neuronal origin that lose the ability to behave in this way when cultured *in vitro*.

It is also apparent that tetanus toxin interferes with neurotransmitter release at certain inhibitory synapses in the central nervous system. There is no reason to believe that the mechanism of this release in one type of nerve cell is any different to that operating in any other type of neurone, or, indeed, in other secretory cells. It seems probable that tetanus toxin could inhibit secretion in a variety of cell types, provided that the toxin could gain access to them.

This cell system has previously been used to investigate the inhibition of secretion by various neurotoxins. One of the first reports of an effect of toxins on secretion from chromaffin cells came in 1985, when it was shown that both basal and evoked secretion were inhibited by 50% or more after pre-incubation for up to several days with botulinum toxins A, B and D (Knight *et al.*, 1985; Knight, 1986). At about the same time, Figliomeni & Grasso (1985) reported that tetanus toxin inhibited catecholamine release from PC12 cells (phaeochromocytoma cells, a permanent cell line derived from a rat adrenal tumour), while Penner *et al.* (1986) demonstrated depressed exocytosis from chromaffin cells following intracellular injection of tetanus toxin. More recently neosurugatoxin, from the Japanese ivory mollusc, has been shown to inhibit catecholamine release (Bourke *et al.*, 1988), while it has also been reported that pertussis toxin facilitates this process (Tanaka *et al.*, 1987).

Thus the main aim of this project was to investigate the interaction between tetanus toxin and the intact adrenal chromaffin cell. It was thought that the chromaffin cell would provide an

excellent model system for studying the effect of tetanus toxin on its target cell; they are available in a relatively pure form and are more accessible to biochemical investigation than sympathetic neurones, which are widely distributed throughout the body. Further, expertise in primary culture of chromaffin cells was already available in the department. Some preliminary work in the laboratory had suggested that there might be some effect of tetanus toxin on secretion from intact chromaffin cells, so the project was aimed firstly at confirming this, and then characterising this effect. Once it had been established that tetanus toxin was indeed capable of inhibiting exocytosis from these cells, the binding of the toxin to the cells was to be investigated and characterised as far as possible, and its similarity to binding to neurones assessed. Binding was to be studied by various techniques, including direct binding studies with radiolabelled tetanus toxin, and immunocytochemical methods. The project also aimed to analyse toxin-binding to chromaffin cells at a more biochemical level, by extracting various components from cell membranes and investigating the interaction, if any, of toxin with these components. It was also anticipated that internalisation of the toxin by chromaffin cells could be demonstrated, using either biochemical or immunocytochemical methods. Finally, we hoped that the results obtained from this work would enable us to decide one way or the other whether the chromaffin cell is indeed a suitable neuronal model for this kind of study.

CHAPTER TWO.

MATERIALS AND METHODS

2.1 MATERIALS

Tetanus toxin was the kind gift of Dr N.F. Fairweather, Wellcome Biotechnology Ltd, Beckenham, Kent, and also of Dr E. Habermann, Buchheim Institut fur Pharmakologie der Justus-Liebig-Universitat, Giessen, West Germany. It was prepared using the method of Ozutsumi *et al.* (1985). Briefly, cells from 10 l of a 3-day culture of *C. tetani* were collected by centrifugation and lysed in 1 M NaCl, 0.1 M sodium citrate; after removal of cell debris, the toxin was precipitated with 40% ammonium sulphate, resuspended in 0.1 M sodium/potassium phosphate buffer, pH 7.5 and freeze-dried. The toxin from Wellcome was almost entirely in the single-chain form as judged by polyacrylamide gel electrophoresis in SDS (see Fig. 3.2), while the Giessen toxin was predominantly in the nicked form. Because of its high toxicity, great care was taken in handling the toxin; all glassware and surfaces that became contaminated were washed immediately in dilute acid, which destroys the toxin very rapidly.

Radiolabelled iodine ($Na^{125}I$, from Amersham International, Little Chalfont) was generously supplied by the Radioimmunoassay Section, Department of Clinical Chemistry, University of Edinburgh. Iodogen was from Pierce & Warriner (UK) Ltd., Chester.

Standard gangliosides, collagenase, protease (type XIV), neuraminidase, adrenaline bitartrate and noradrenaline bitartrate were obtained from Sigma Chemical Co., Poole, Dorset.

Thin-layer chromatography plates were obtained from BDH Ltd, Poole, Dorset, or Camlab (Cambridge).

For tissue culture, Dulbecco's Modified Eagle Medium, foetal calf serum, gentamycin, penicillin/streptomycin and fungizone were purchased from Gibco Ltd., Paisley, Scotland; cytosine arabinoside was obtained from Aldrich Chemical Co., Gillingham, Dorset; deoxyribonuclease and fluorodeoxyuridine were purchased from Sigma Chemical Co., Poole, Dorset; Percoll was obtained from Pharmacia Ltd., Milton Keynes.

Multiwell tissue culture plates (24- and 96-well) were from Gibco Ltd., Paisley, Scotland; petri dishes and culture flasks were obtained from Becton Dickinson Ltd., Oxford.

All antibodies were purchased from Sigma Chemical Co., Poole, Dorset, with the exception of the human anti-tetanus antibody, which was a gift from the Scottish National Blood Transfusion Service, Edinburgh.

Phosphate-buffered saline routinely used had the following composition: 154 mM NaCl, 1.9 mM NaH_2PO_4, 8 mM Na_2HPO_4, 3 mM KCl, pH 7.4.

All other reagents were of analytical grade.

2.2 METHODS

2.2.1 Isolation and Culture of Adrenal Chromaffin Cells

Bovine adrenal medullary chromaffin cells were isolated essentially by the method of Knight & Baker (1983b), with minor modifications. All solutions used in the isolation procedure were either obtained sterile or were prepared in deionised water and sterilised by filtration, and all glassware was autoclaved prior to use. Usually four glands were processed at one time.

Fresh, intact adrenal glands were obtained from the local slaughter house and transported at ambient temperature to the laboratory in calcium- and magnesium-free Krebs-Ringer buffer (145 mM NaCl, 5 mM KCl, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM HEPES, pH 7.4) containing 50 units/ml penicillin and 50 µg/ml streptomycin. After removal of adhering fat, each gland was perfused with 20 ml of buffer to flush out red blood cells and tissue debris; this was achieved by applying gentle pressure to a buffer-filled syringe introduced into the adrenal vein. Proteolytic digestion was then carried out using a 0.2% solution of protease (Sigma Type XIV) in Krebs-Ringer buffer; 5 ml was injected into each gland through the adrenal vein, and the glands incubated in a 37°C water bath for 15 minutes. The whole digestion procedure was carried out a second time, and the glands were then sliced laterally and the medullae dissected out and minced finely in a petri dish with a small amount of buffer. The material was then transferred to a 75 cm^2 tissue culture flask, gassed thoroughly with a mixture of 95% O_2/5% CO_2, and incubated, with shaking, in 50 ml of Krebs-Ringer buffer containing 0.1% collagenase and 15 µg deoxyribonuclease (Type I) per ml for 20

minutes. After filtration through 250 μm nylon mesh (or alternatively, muslin cloth), dissociated cells were collected by centrifugation at 1000 r.p.m. for 10 minutes in a bench centrifuge, then resuspended in Krebs-Ringer buffer, filtered through 85 μm nylon mesh and centrifuged again at 1000 r.p.m. for 10 minutes. This was carried out twice, after which time the cells were resuspended in 28 ml of Dulbecco's Modified Eagle Medium (DMEM), and mixed with 25 ml of balanced salt-Percoll (prepared by mixing 9 volumes of Percoll with 1 volume of 10x Krebs-Ringer buffer, and adjusting to pH 7.4 with 2.0 M HCl). The cell suspension was placed in sterile polycarbonate centrifuge tubes with caps and centrifuged at 21,000 g (15,000 r.p.m., 50.2 Ti rotor) for 20 minutes (Wilson & Viveros, 1981). The chromaffin cell fraction was then collected by aspiration, washed twice by centrifugation in DMEM and finally resuspended in plating medium (DMEM containing 20 mM HEPES, 2% foetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamycin, 0.25 μg/ml fungizone, 25 μg/ml fluorodeoxyuridine, 50 μg/ml ascorbic acid (pH 7.4) and 3 μg/ml cytosine arabinoside). Cells were counted in a haemocytometer, their viability being estimated using trypan blue; this was usually greater than 90%. The cells were then diluted in plating medium to yield 0.5×10^6 cells/ml (for transfer to 24- or 96-well tissue culture plates) or 0.3×10^6 cells/ml (for transfer to petri dishes containing cover slips). After plating, cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2, and were generally used for experimental purposes within three to six days of preparation. The yield from this preparation procedure was typically 50×10^6 cells per gland.

2.2.2 Preparation of Membranes from Adrenal Medullary Microsomes

The method of Gavine *et al.* (1984) was used in the preparation of microsomal membranes from adrenal medullae. Bovine adrenal glands (usually 15-20) obtained fresh from the local slaughterhouse were kept on ice, then laterally dissected and the medulla scraped away from the cortex with a scalpel and placed in ice-cold 0.3 M sucrose, 20 mM HEPES-Tris pH 7.0. The medullae were then passed through a steel mincer with holes of 2 mm diameter, and homogenised in 8 volumes of ice-cold buffered sucrose (in a motor-driven homogeniser with a loose-fitting teflon pestle) until no large lumps of tissue remained. All subsequent procedures were performed at 0-4°C. The homogenate was centrifuged at 800 g (3300 r.p.m., JA20 rotor) for 10 minutes, and the supernatant thus obtained recentrifuged one more time under the same conditions; the supernatant obtained here was then centrifuged at 20,000 g (16,000 r.p.m., JA20 rotor) for 20 minutes. This supernatant was then centrifuged over a cushion of 1.4 M sucrose for 60 minutes at 160,000 g (45,000 r.p.m., 45Ti rotor). Membranes from the 0.3 M/1.4 M sucrose interface were collected using a Pasteur pipette, diluted in 20 mM HEPES, pH 7.0 and centrifuged for 90 minutes at 160,000 g (45,000 r.p.m., 45Ti rotor). The pelleted membranes were then taken up in approximately 2 ml of buffered sucrose (0.3 M), and stored at -20°C until required, either for protein estimation or ganglioside extraction (see Section 2.2.13).

In order to achieve some degree of fractionation of microsomal membranes, an additional step was sometimes included in the protocol, after collection of membranes from the 0.3 M/1.4 M sucrose interface. The membranes were made 1.4 M in sucrose (using

2 M sucrose stock, and determining sucrose molarity using a refractometer), and 5 ml aliquots were then overlaid with 6.7 ml of 1.15 M sucrose, 6.7 ml of 0.85 M sucrose and 5 ml of 0.5 M sucrose in 50.2Ti centrifuge tubes. These discontinuous sucrose gradients were then centrifuged at 180,000 g (45,000 r.p.m., 50.2 Ti rotor) for 60 minutes, after which time material at each interface was recovered using capillary tubing attached to a peristaltic pump. Each of the three membrane fractions thus obtained was then diluted to a volume of 70 ml with 20 mM HEPES, pH 7.0 and centrifuged at 160,000 g (45,000 r.p.m., 45Ti rotor) for 60 minutes. Membranes were finally resuspended in approximately 2 ml of buffered sucrose (0.3 M), and stored at -20°C until required.

2.2.3 Preparation of Chromaffin Granules

A crude preparation of chromaffin granules was produced by the method of Phillips (1974). An homogenate of adrenal medullary material was made in the same way as outlined in Section 2.2.2, diluted in 0.3 M sucrose, 20 mM HEPES-Tris pH 7.0 and centrifuged at 1700 g (4,000 r.p.m., JA14 rotor) for 5 minutes to remove cell debris. The supernatant thus obtained was then centrifuged at 18,500 g (14,000 r.p.m., JA14 rotor) for 30 minutes. The pellet after this centrifugation consisted of a lower layer of chromaffin granules with overlaying mitochondria; the latter were removed by gently swirling buffer over the surface of the pellet. The granules were then resuspended in buffered sucrose, homogenised again and centrifuged at 18,000 g (15,000 r.p.m., JA20 rotor) for 20 minutes. Washing, resuspension and centrifugation was generally

carried out a second time to give a visibly clean pellet of granules, which was finally collected in approximately 5 ml of buffered sucrose.

This "crude granule" fraction was then purified by centrifugation on discontinuous Percoll gradients, composed of 4 ml 60% (bottom), 3 ml 45% (middle) and 4 ml 20% (top) Percoll (in 0.35 M sucrose, 20 mM HEPES-Tris pH 7.0) in 15 ml Corex tubes. 0.5 ml aliquots of granules (10-20 mg protein/ml) were layered on top of the gradients, which were then centrifuged at 11,000 g (12,000 r.p.m., JA20 rotor) for 20 minutes at 4°C. Purified chromaffin granules were collected from the 60% Percoll region and the 60%/45% Percoll interface, diluted in 0.35 M sucrose 20 mM HEPES-Tris, pH 7.0, and recentrifuged at 18,000 g (15,000 r.p.m., JA20 rotor) for 20 minutes to remove the Percoll. The granule pellet was then resuspended in a small volume of 0.35 M sucrose, 20 mM HEPES-Tris, pH 7.0, kept on ice and used within a few hours.

2.2.4 Stimulation of Exocytosis From Adrenal Chromaffin Cells

Chromaffin cells were routinely tested after two days in culture for their ability to release their granule contents by exocytosis, using secretagogues such as nicotine. Medium was removed from cells in 24-well plates by aspiration, and 1 ml of Locke's solution, composed of 154 mM NaCl, 5.6 mM KCl, 5.6 mM glucose, 5 mM HEPES, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, (Kilpatrick *et al.*, 1980) containing 10 μM nicotine was added to each well; cells were then incubated for 10 minutes at room temperature, after which time the supernatant was carefully removed, and stored at 4°C until assayed for catecholamines. Cells were then lysed using 1 ml of Locke's

solution containing 1% (w/v) Triton X-100, and collected for determination of unreleased catecholamines. Basal release of catecholamines was also measured, using cells incubated with Locke's solution alone.

2.2.5 Fluorimetric Assay of Catecholamines

Catecholamines were assayed fluorimetrically using the trihydroxyindole method of von Euler & Lishajko (1961). 0.5 ml aliquots of supernatant or 0.2 ml aliquots of cell suspension were adjusted to pH 6.2 with 0.1 ml of 0.1 M potassium phosphate buffer (pH 6.2), and diluted to a final volume of 1 ml with distilled water. Oxidation of catecholamines was carried out for three minutes by the addition of 0.1 ml of 0.25% (w/v) potassium ferricyanide (freshly prepared), then 2 ml of alkaline ascorbate solution (4 M NaOH/ethylene diamine/2% ascorbic acid 9:0.2:1) was added to each tube and mixed in thoroughly. Addition of the alkali causes formation of strongly fluorescent trihydroxyindoles, which are stabilised by including ascorbic acid and ethylene diamine in the mixture. Blanks were prepared in the same way using Locke's solution (both with and without 1% w/v Triton X-100). The fluorescence of samples was then read after 15 minutes (but within two hours) using a Perkin Elmer 300 fluorescence spectrophotometer at two sets of wavelengths; (a) excitation 395 nm, emission 490 nm, and (b) excitation 436 nm, emission 540 nm. Calibration was carried out by using comparison with known adrenaline and noradrenaline standards, which fluoresce with different intensities at the two sets of wavelengths.

Catecholamine content was calculated as follows:

Aa = fluorescence of 1 nmol of adrenaline at wavelengths a)

Ab = fluorescence of 1 nmol of adrenaline at wavelengths b)

Na = fluorescence of 1 nmol of noradrenaline at wavelengths a)

Nb = fluorescence of 1 nmol of noradrenaline at wavelengths b)

$$\text{Noradrenaline (nmol)} : y = \frac{(M.Ab/Aa) - N}{(Na.Ab/Aa) - Nb}$$

$$\text{Adrenaline (nmol)} : x = \frac{N - y.Nb}{Ab}$$

where M = sample fluorescence at 395/490 nm

N = sample fluorescence at 436/540 nm.

(The derivation of these equations is given in Appendix 1).

Results were corrected to give catecholamine content (nmol/ml) and secreted catecholamines were expressed as a percentage of the total catecholamine content of the cells. All release experiments were carried out on triplicate wells.

This method allowed a differential estimation of noradrenaline and adrenaline to be made by measuring the fluorescence of a single sample at two different sets of wavelengths. Fluorescence readings were found to be stable for at least two hours (after an initial increase in the first 15 minutes), allowing many samples to be processed simultaneously. Standards were always assayed in triplicate, dilutions being made fresh each time from a stock

solution (the concentration of the stock was adjusted by measuring the absorbance at 280 nm of a 1/100 dilution, and using a molar extinction coefficient (ϵ) of 27 l mol^{-1} cm^{-1}; the stock (approximately 10 mM) was then diluted accordingly to give a working concentration of 1 mM).

2.2.6 Inhibition of Exocytosis by Tetanus Toxin

Plating medium was removed from cells in 24-well plates after approximately three days in culture, and the cells were incubated (in triplicate) at 37°C in DMEM containing different concentrations of toxin (7 pM - 70 nM, equivalent to 1 ng/ml - 10 µg/ml) for various times as indicated. After toxin incubation, the medium was removed, the cells washed once with Locke's solution and then stimulated with 10 uM nicotine, as outlined at Section 2.2.4.

2.2.7 Preparation of Fluorescent GT1

Fluorescent ganglioside was prepared by the method of Spiegel (1985). Ganglioside GT1 (3 mg) was dissolved in 3 ml of 100 mM sodium acetate buffer (pH 5.5) containing 150 mM NaCl and 2 mM $NaIO_4$, and oxidised for 30 minutes at 0°C. The reaction was stopped by the addition of 0.3 ml of 50% glycerol, and the solution was dialysed extensively against water, then lyophilised. The oxidised GT1 was then dissolved in 3 ml of PBS and, after addition of Lucifer Yellow CH to 5 mM, the solution was incubated overnight at 0°C and dialysed against PBS. The material was reduced with 10 mM $NaCNBH_3$ (15 minutes at 23°C), dialysed against distilled water, and lyophilised. The modified GT1 was analysed by thin-

layer chromatography (see Section 2.2.14), and visualised with resorcinol reagent (see Section 2.2.15); it was also easily detected on chromatograms under UV illumination.

2.2.8 Incorporation of Fluorescent GT1 into the Chromaffin Cell Membrane

The method of Spiegel *et al.* (1984) was used for studying the incorporation of fluorescent ganglioside into chromaffin cell membranes. Cells cultured on coverslips were washed extensively with Locke's solution (see Section 2.2.4) to remove any serum-containing medium which would interfere with ganglioside uptake by the cells, then incubated in serum-free medium containing GT1-Lucifer Yellow (0.25 μg/ml) for various times at 37°C in a humidified incubator. The coverslips were then thoroughly washed in Locke's solution, and the cells fixed in 10 mM PBS containing 3.7% formaldehyde and 5% sucrose for 60 minutes. After further washing in Locke's solution, coverslips were mounted in 50% glycerol and the cells examined for fluorescence using a Leitz Ortholux 2 microscope. Fluorescence of single cells was measured using the photometer of a Leitz Vario Orthomat 2 automatic microscope camera, and compared with that of control cells incubated with medium containing no fluorescent ganglioside.

2.2.9 Preparation of ^{125}I-Labelled Tetanus Toxin

Radiolabelled tetanus toxin was prepared essentially by the method of Salacinski *et al.* (1981), using the reagent 1,3,4,6-tetrachloro-3α,6α-diphenyl glycouril (Iodogen). Usually 100 μg of toxin was iodinated each time, using 0.5 mCi of Na^{125}I.

Iodogen (1 mg) was dissolved in 2 ml of chloroform, and 200 µl of this solution dispersed in the bottom of a small glass vial. The solution was then evaporated to dryness at room temperature under nitrogen; this removed the chloroform to produce a film of Iodogen, ensuring that the Iodogen did not form a suspension, which could give variable iodinations. These dried vials could be stored for up to six months at -20°C.

Prior to iodination, the coated vial was rinsed with buffer (usually PBS, pH 7.2), then the toxin* was added (100 µg in 0.1 ml of 0.15 M potassium phosphate buffer, pH 6.5), followed by $Na^{125}I$ (0.5 mCi, 11 µM, 5 µl). The iodination was allowed to proceed for 15 minutes at room temperature, then terminated by decanting the reaction mixture. To separate iodinated protein from free iodide, the mixture was centrifuged through a Biogel P6-DG column (packed in an Eppendorf tube) at 1400 r.p.m. in a bench centrifuge for one minute; ^{125}I-labelled tetanus toxin was eluted, while free $^{125}I^-$ remained on the column. The radiolabelled toxin was stored at 4°C, and 0.25% bovine serum albumin was added to increase its stability. Protease inhibitors were also added, to the following final concentrations: benzamidine (5 mM), 6-aminohexanoic acid (10 mM), EDTA (10 mM), N-ethyl maleimide (1 mM) and freshly prepared phenylmethylsulphonylfluoride (1 mM).

* Tetanus toxin was generally supplied to our laboratory as a 10 mg/ml solution in 1.5 M potassium phosphate buffer, pH 6.5. For iodination, 10 µl of this solution was diluted to 0.1 ml with double-distilled water.

2.2.10 Binding of ^{125}I-Labelled Tetanus Toxin to Chromaffin Cells

Measurement of binding of radiolabelled tetanus toxin to chromaffin cells was performed essentially by the method of Staub *et al.* (1986). Growth medium was removed from cells cultured for 5-7 days in 96-well tissue culture plates, and the cells in each well washed twice with 200 µl of ice-cold rinse buffer (either 0.25 M sucrose, 20 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 6.0 (low ionic strength buffer), or 0.25 M sucrose, 20 mM Tris, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 30 mM NaCl, pH 7.4 (high ionic strength buffer), as indicated in the text). Cells were then incubated for three hours (unless otherwise indicated) at 0°C in 100 µl of binding buffer (rinse buffer supplemented with 2.5 mg/ml BSA) containing 0-20 nM ^{125}I-labelled tetanus toxin; incubations were carried out in triplicate. After toxin incubation, the radioactive medium was removed, and cells were washed three times in ice-cold rinse buffer, before being lysed in 200 µl of 0.5 M NaOH. The resulting cell lysates were then carefully transferred to test-tubes and their radioactivity determined using a γ-radiation counter (with a counting efficiency of 83%). Non-specific toxin binding was determined by incubating cells with radiolabelled toxin in the presence of a 100-fold excess of unlabelled toxin.

For investigating the effect of exogenously added gangliosides on the toxin-binding capacity of chromaffin cells, the cells were incubated in serum-free medium containing 25 µg/ml of the specified ganglioside for two hours at 37°C, then washed extensively with ice-cold rinse buffer, prior to toxin addition. Similarly, when evaluating the effect of neuraminidase or trypsin treatment on

toxin binding, cells were pre-incubated with the enzymes prior to addition of toxin (0.1 IU/ml neuraminidase for one hour at 37°C, or 25 μg/ml trypsin for 10 minutes at 37°C).

2.2.11 Binding of ^{125}I-Labelled Tetanus Toxin to Chromaffin Granules

The method of Lazarovici *et al.* (1989) was used to measure tetanus toxin-binding to chromaffin granules. Fresh granules (prepared as outlined in Section 2.2.3) were assayed for protein, then diluted in 0.32 M sucrose, 25 mM Tris-HCl pH 7.4 to achieve a final protein concentration of 3.6 mg/ml. Aliquots of 50 μl were then pippetted into Eppendorf tubes (pre-coated with bovine serum albumin to reduce binding of toxin to the plastic), and 50 μl of radiolabelled toxin (0-40 nM in 0.32 M sucrose, 25 mM Tris-HCl pH 7.4) was added to each tube to give final toxin concentrations in the range 0-20 nM. The granules were then incubated in suspension with the tetanus toxin for 2 hours at 4°C; incubations were performed in triplicate. After 2 hours, the suspensions were centrifuged for 5 minutes in a microfuge at maximum speed (13,000 r.p.m.) and the supernatants removed by aspiration. The pellets were washed with incubation buffer and recentrifuged; this was carried out a second time to ensure adequate washing. After aspiration of the supernatant, the radioactivity bound to the pellets was measured in a δ-counter.

2.2.12 Internalisation of Tetanus Toxin by Chromaffin Cells

The protease assay of Staub *et al.* (1986) was used to investigate possible internalisation of ^{125}I-tetanus toxin by chromaffin cells. Cells grown in 24-well tissue culture plates were washed with 2 ml

of rinse buffer (pH 7.4; see Section 2.2.10), and then incubated in triplicate with 2 nM ^{125}I-labelled tetanus toxin in 1 ml of binding buffer (pH 7.4) for three hours at 0°C or 37°C. The incubation medium was then removed by aspiration, and the cells washed three times with 2 ml of rinse buffer. Cells were then incubated at 37°C for 10 minutes in 1 ml of rinse buffer containing either 0, 5, 20 or 100 µg/ml of pronase; proteolytic activity was stopped by the addition of 100 µl of an inhibitor cocktail containing 1 mM PMSF, 1 mM benzamidine and 5 mM ε-aminocaproic acid. After gentle washing three times with 2 ml of rinse buffer, cells were lysed in 1 ml of 0.5 M NaOH, and their radioactivity counted using a γ-radiation counter.

2.2.13 Isolation of Gangliosides from Membrane Fractions

Preparation of gangliosides from chromaffin cell membrane fractions was carried out using the procedure of Svennerholm & Fredman (1980). Membranes, in 0.3 M sucrose, 20 mM HEPES pH 7.0, were homogenised for two minutes with a Potter-Elvehjem homogeniser, and the homogenate poured into 10.8 ml of methanol at room temperature under constant stirring. 5.4 ml of chloroform was then added to the mixture, which was left to stir for 30 minutes at room temperature. Following this, the mixture was centrifuged at 2000 g (5000 r.p.m., JA20 rotor) for 30 minutes at 4°C, and the supernatant removed and saved. The pellet was re-extracted once by homogenisation in 2 ml of distilled water, then poured into 8 ml of chloroform/methanol (1:2) and left to stir for a further 30 minutes at room temperature; centrifugation was then performed as before. The two supernatants were then combined in a separating funnel, and distilled water added to achieve a final chloroform/methanol/water

(+tissue) ratio of 1:2:1.4. The solvents were carefully mixed by turning the funnel up and down several times (shaking was omitted to prevent emulsification), and the phases left to separate overnight. After separation, the lower phase was removed and the upper phase set aside; 0.5 volumes of methanol was then added to the lower phase, followed by 0.22 volumes of 0.01 M KCl (in water). After thorough mixing, phases were separated by centrifugation at 2000 r.p.m. in a bench centrifuge for 10 minutes and the upper phase removed. The two upper phases were then combined and evaporated to dryness, after addition of 10 ml of isobutanol to prevent foaming. The dried residue was taken up in 2 ml of distilled water, sonicated for 5 minutes, and then dialysed extensively against distilled water over a 3-4 day period (with several changes of water). The dialysed material was then lyophilised, redissolved in 500 ul of chloroform/methanol (2:1) and stored at -20°C until required.

2.2.14 Thin-Layer Chromatography of Gangliosides

Chromatographic separation of ganglioside species was carried out using a modification of the method of Dreyfus *et al.* (1975). Gangliosides, dissolved in chloroform:methanol (1:1 v/v), were applied as thin streaks to precoated silica gel thin-layer plates (0.25 mm thickness), and the plates developed in chloroform: methanol:0.02% $CaCl_2$ (60:40:10 v/v) in a saturated chamber. After development, plates were air-dried and then subjected to one of three treatments:

i) overlaying with radioactive tetanus toxin (see Section 2.2.16);

ii) spraying with resorcinol reagent, a specific stain for sialic acid residues (see Section 2.2.15); or

iii) exposure to iodine vapour, a non-specific stain for double bonds, which will therefore stain all lipids.

2.2.15 Sialic Acid Assay

The yield of gangliosides from each isolation was measured with the assay of lipid-bound sialic acid using the resorcinol method of Svennerholm (1957). Aliquots of the lipid extract, containing 10-30 nmol of sialic acid were added to pyrex tubes and made up to a volume of 2 ml with distilled water; standards, in the range 0-30 nmol sialic acid, were prepared in the same way. To each tube was added 2 ml of resorcinol reagent (10 ml of 2% aqueous resorcinol, added to 80 ml of concentrated HCl containing 0.25 ml of 0.1 M $CuSO_4$, and made up to 100 ml with distilled water); tubes were then capped securely and heated for 15 minutes in a boiling water bath. The tubes were then cooled quickly in an icebath, the chromogen extracted with 4 ml of butyl acetate/butanol (85:15 v/v) (Miettinen & Takki-Luukkainen, 1959), and the solvent mixture centrifuged at 500 r.p.m. in a bench centrifuge for 5 minutes. The absorbance of the upper organic phase was then read at 580 nm.

The same resorcinol reagent was used in the detection of gangliosides separated on TLC plates. Developed plates were air-dried, sprayed with the reagent and then sandwiched between two glass plates before being incubated at 110°C for 15 minutes, or until ganglioside spots appeared.

2.2.16 Ganglioside Overlay with ^{125}I-Labelled Tetanus Toxin

Binding of ^{125}I-labelled tetanus toxin to gangliosides on TLC plates was performed using the method of Magnani *et al.* (1980), with minor modifications. Chromatograms developed in the usual manner (see Section 2.2.14) were air-dried, and then soaked in 40 ml of pre-chilled phosphate-buffered saline (0.15 M NaCl, 10 mM sodium phosphate, pH 7.2) containing either 1% polyvinylpyrrolidone and 0.2% BSA, or 1% BSA and 0.2% Tween (as indicated), to decrease non-specific binding of tetanus toxin. The plates were shaken in this solution for approximately one hour, then placed in fresh buffer containing 10^6 cpm/ml of ^{125}I-labelled tetanus toxin and incubated for a further 60 minutes. The radioactive medium was then carefully pipetted off, and the chromatograms given several quick rinses with PBS. Further washing was carried out over the next 4-5 hours (with the rinse buffer being renewed approximately every 30 minutes); chromatograms were then air-dried, wrapped in clingfilm and exposed to X-ray film for 1-4 days as outlined in Section 2.2.20.

2.2.17 Immunocytochemistry

Chromaffin cells cultured on glass coverslips were used for immunocytochemical experiments. The typical procedure is outlined here; variations and additional steps are as described in the text.

After 5-7 days in culture, cells on coverslips were washed twice with PBS and then incubated with tetanus toxin (10 µg/ml in the specified buffer) for at least three hours at 4°C. After thorough washing with PBS (3 x 5 minute washes), cells were fixed in 3.7% formaldehyde in PBS for 10 minutes, followed by extensive washing

with Tris-buffered saline (TBS; 150 mM NaCl, 10 mM Tris-HCl pH 7.4) to wash out excess fixative. Cells were then incubated with a 1/100 dilution of human anti-tetanus antibody (IgG) in TBS/5% FCS for 1 hour at room temperature. Coverslips were again washed extensively with TBS, and then incubated for 3-4 hours at room temperature with a 1/400 dilution of secondary antibody (either alkaline phosphatase-conjugated goat anti-human IgG, or biotinylated goat anti-human IgG) in TBS/5% FCS. After further washing with TBS, cells treated with alkaline phosphatase-conjugated antibody were ready for incubation with chromogen, while those treated with biotinylated antibody were first incubated for one hour with a 1/300 dilution of streptavidin-horseradish peroxidase (in TBS supplemented with 1 mg/ml BSA), prior to incubation with chromogen. It should be noted that in each experiment, controls omitting toxin, anti-tetanus antibody or secondary antibody were routinely included.

2.2.17.1 Alkaline Phosphatase staining (Ormerod & Imrie, 1989) 5 mg of naphthol AS BI phosphate (sodium salt) were dissolved in a few drops of dimethylformamide in a glass tube, and added to 5 mg of Fast Red TR salt dissolved in 10 ml of 20 mM Tris, pH 9.2. Levamisole, at 1 mg/ml, was also added to block endogenous enzyme. The solution was filtered through glass fibre paper, and coverslips were then incubated at 37°C for one hour or until cells were sufficiently stained relative to controls. Coverslips were then washed thoroughly in water, mounted in Uvinert mountant (BDH) on glass microscope slides and examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

2.2.17.2 Horseradish Peroxidase staining (Graham & Karnovsky, 1966)

10 mg of 3,3',4,4'-diaminobenzidine was dissolved in 10 ml of 0.1 M Tris-HCl, pH 7.4. To this was added 0.3 ml of 1% $NiCl_2$ (to enhance staining) and 10 µl of 30% hydrogen peroxide. Cells were incubated for 30 minutes in this solution, washed thoroughly in TBS, then mounted and examined as above.

2.2.18 Determination of Protein

Protein was determined by one of two methods. The protein content of membrane fractions and granule preparations was estimated by a modification of the method of Bradford (1976). A stock solution was first prepared by dissolving 100 mg of Coomassie Brilliant Blue G in 50 ml of 95% ethanol, and adding 100 ml of 85% orthophosphoric acid. 18 ml of this stock solution was then diluted to 100 ml with distilled water, and filtered through Whatman No. 1 paper. For protein estimation, 0.5 ml of sample (containing 10-50 µg of protein) was added to 2.5 ml of the diluted Bradford reagent, and the absorbance at 595 nm measured after 30 minutes. A standard curve was constructed using bovine serum albumin.

Cell lysates were assayed for protein using a modified version of the method of Markwell *et al.* (1978). 0.1 ml of lysate (containing 10-100 µg of protein) was made up to 0.5 ml with 0.5 M NaOH, and to this was added 2 ml of reagent C, an alkaline copper reagent (reagent C was made by mixing 100 parts of reagent A [2% Na_2CO_3, 0.4% NaOH, 0.16% sodium tartrate, 1% SDS] with 1 part of reagent B [4% $CuSO_4.5H_2O$]). Samples were incubated at room temperature for 30 minutes, and then mixed vigorously with 0.2 ml of Folin-

Ciocalteu phenol reagent, diluted 1:1 with water. The absorbance of samples was read at 660 nm after 45 minutes. A standard curve was constructed using bovine serum albumin dissolved in 0.5 M NaOH.

2.2.19 SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was routinely carried out under denaturing conditions by the addition of 0.1% (w/v) SDS in both the separating and stacking gels. The gel and buffer system used was based upon that of Laemmli (1970), with the addition of 2 mM EDTA to chelate any metal ions which may interfere with the polymerisation of acrylamide and cause aggregation of proteins (Douglas & Butow, 1976). Protein samples were applied to the gel and electrophoresed for one hour at 100 volts while the proteins moved into the stacking gel, and then at 50 volts overnight. Gels were fixed in 20% (v/v) methanol, 10% (v/v) acetic acid for 15 minutes, stained in 0.25% Coomassie Brilliant Blue R-250, 45% (v/v) methanol, 9% (v/v) acetic acid for 15 minutes at 60°C, then destained in 5% (v/v) methanol, 7.5% (v/v) acetic acid at 60°C until the gel background was sufficiently clear. The addition of pieces of polystyrene foam quickened the destaining process by absorbing the dye.

For determination of molecular masses, gels were calibrated with the following standard proteins: bovine erythrocyte carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine serum albumin (66 kDa), rabbit muscle phosphorylase b subunit (97.4 kDa), *E. coli* β-galactosidase subunit (116 kDa) and rabbit muscle myosin subunit (205 kDa). These proteins were supplied as a high molecular weight standard mixture from Sigma Chemical Co.

2.2.20 Autoradiography

Polyacrylamide gels containing ^{125}I-labelled tetanus toxin, after destaining, were dried using a Bio-Rad model 443 slab gel dryer onto Whatman 3 mm filter paper under vacuum for 1 hour at 80°C. Before drying, gels were covered with Saran Wrap, a non-porous plastic film. Once dried, the gels were exposed to Agfa-Gevaert X-ray film in a Dupont Cronex cassette containing "lightning plus" intensifying screens, and left at -70°C for 1-4 days, depending on the amount of radiolabel present; overlaid thin-layer chromatograms were exposed under the same conditions. Films were developed automatically using a Fuji RG 11 X-ray film processor.

2.2.21 Blotting of Proteins onto Nitrocellulose

Proteins separated by polyacrylamide gel electrophoresis were transferred to nitrocellulose sheets essentially by the method of Towbin *et al.* (1979). A sheet of nitrocellulose (0.45 µm pore size) was wetted with water and laid on a Scotch-Brite pad supported on a plastic grid. The gel was placed on the nitrocellulose, care being taken to remove any air bubbles trapped between the two layers. Another pad and plastic grid were added and the "sandwich" was firmly clipped together so that the gel was firmly and evenly pressed against the nitrocellulose. The assembly was put into an electrophoretic blotting tank containing electrode buffer (25 mM Tris, 192 mM glycine, pH 8.3), with the nitrocellulose facing the anode. A voltage gradient of 6V/cm was then applied for one hour.

CHAPTER THREE.

INHIBITION BY TETANUS TOXIN OF EXOCYTOSIS FROM CULTURED ADRENAL CHROMAFFIN CELLS

3.1 INTRODUCTION

As discussed in Section 1.10, it has become well established that the primary action of tetanus toxin *in vivo* is an inhibition of neurotransmitter secretion from certain synapses; the mechanism of this inhibition, however, remains unknown. This situation has largely arisen because of the limitations imposed by the gross anatomy of the toxin's target neurones; they are widely distributed throughout the body, making them relatively difficult to isolate and culture *in vitro*.

However, it should not be assumed that this is the only site where the toxin can exert its effects. Indeed, as discussed earlier, it does not seem unreasonable to suppose that tetanus toxin could inhibit secretion from a wide variety of cell types, provided that the toxin could gain access to them. In support of this idea, Ho & Klempner (1985) have pointed out the similarities between neuronal neurotransmitter release and stimulation-secretion in phagocytes, and have demonstrated a dose-dependent inhibition of secretion of lysosomal contents from human macrophages by tetanus toxin. While this clearly indicates the potential of tetanus toxin to act as an inhibitor of secretion in a wide variety of cell types, not necessarily neuronal, the question must always be borne in mind: "how relevant is the action of tetanus toxin on these cells to neurotoxicity?" Clearly, a cell-culture model of neuronal origin would be more appropriate for studying the mechanism of toxin action. According to Wellhoner & Neville (1987) a model system should "consist of only one type of cell of neuronal origin, the quality of action of tetanus toxin should be in the inhibition of

the stimulated transmitter release, and cells should be sensitive to toxin concentrations comparable to those found in animals suffering from tetanus".

The virtues of chromaffin cells for this kind of study have already been discussed; indeed, experiments with chromaffin cells have already provided some useful information on the action of tetanus toxin (see Section 1.10). However, these studies have not been carried out using "intact" chromaffin cells as such; either cells have been permeabilised with digitonin or streptolysin O, or the toxin has been introduced into the cell by microinjection, thereby artificially bypassing the chromaffin cell membrane. To date there have been no convincing reports of an effect of tetanus toxin on exocytosis from intact chromaffin cells; Marxen & Bigalke (1989) have reported a very slight inhibitory effect of tetanus toxin (less than 10%) if offered in low ionic strength solution to ganglioside-untreated chromaffin cells, but this is hardly a significant result. On the other hand, there have been several reports stating emphatically that tetanus toxin does not affect exocytosis from intact chromaffin cells (e.g. Knight *et al.*, 1985; Bittner & Holz, 1988; Marxen & Bigalke, 1989).

However, some work carried out previously in our laboratory indicated that there might in fact be some effect of tetanus toxin on these cells. Since it is generally believed that the mechanisms of botulinum toxin and tetanus toxin are virtually the same at the molecular level, it was thought that the results obtained by Knight and his coworkers (i.e. botulinum toxin effective at inhibiting secretion from chromaffin cells, while tetanus toxin was

ineffective) might be caused by the inability of tetanus toxin to bind to and enter these cells. If this were the case, pre-incubating chromaffin cells with gangliosides should introduce binding sites; when ganglioside micelles are incubated with cells *in vitro*, they are incorporated into the membrane and behave in all known respects like endogenously synthesised lipid (see Section 4.3.2). This sort of experiment has been done often with cholera toxin and GM1 (van Heyningen, 1983). Experiments showed that, as expected, tetanus toxin inhibited nicotine-evoked secretion from ganglioside-treated cells, but that there was also some reproducible inhibition using cells <u>not</u> pre-treated with ganglioside. The effect was more marked, however, when the cells had been pre-incubated with ganglioside; in this case, an inhibition of approximately 30% was achieved. Other workers have similarly reported an enhancement of toxin effect following incubation of cells with toxin-binding gangliosides (e.g. Marxen *et al.*, 1989; Marxen & Bigalke, 1989).

Thus this cell system seemed valuable for future work. It appeared relatively easy to work with, and displayed one of the most marked inhibitory effects of tetanus toxin so far reported. The aim of the work presented in this chapter, then, was to confirm these preliminary findings of an effect of tetanus toxin on intact chromaffin cells, and to characterise this effect as much as possible, with respect to time course, dose-dependency and so on. It was also of interest to investigate whether single- and double-chain forms of the toxin were equally active in producing this effect, and to determine whether the toxin could inhibit secretion evoked by different secretagogues. It was hoped that the results

obtained would provide evidence that the chromaffin cell is indeed a valid model system for studying the intoxication process of tetanus.

Note: Much of the work presented in this chapter was carried out in collaboration with Dr M.K. Bansal, Department of Biochemistry, University of Edinburgh.

3.2 METHODS

3.2.1 Stimulation of Exocytosis from Chromaffin Cells

Release of granule contents from chromaffin cells by exocytosis was evoked using secretagogues such as nicotine and Ba^{2+} ions, as outlined in Section 2.2.4. Cells were routinely tested for their ability to respond to these secretagogues prior to experiments investigating the effect of tetanus toxin on this process.

3.2.2 Fluorimetric Assay of Catecholamines

Exocytosis from chromaffin cells was quantitated by measuring the levels of catecholamines both released into the supernatant or remaining intracellular, using the fluorimetric assay of von Euler & Lishajko (1961), as described in Section 2.2.5.

3.2.3 Inhibition of Exocytosis by Tetanus Toxin

The effect of tetanus toxin on evoked secretion from chromaffin cells was determined basically as outlined in Section 2.2.6, or with modifications as described in the text. The toxin used was almost entirely in the single-chain form, as judged by polyacrylamide gel electrophoresis in SDS (Fig 3.1).

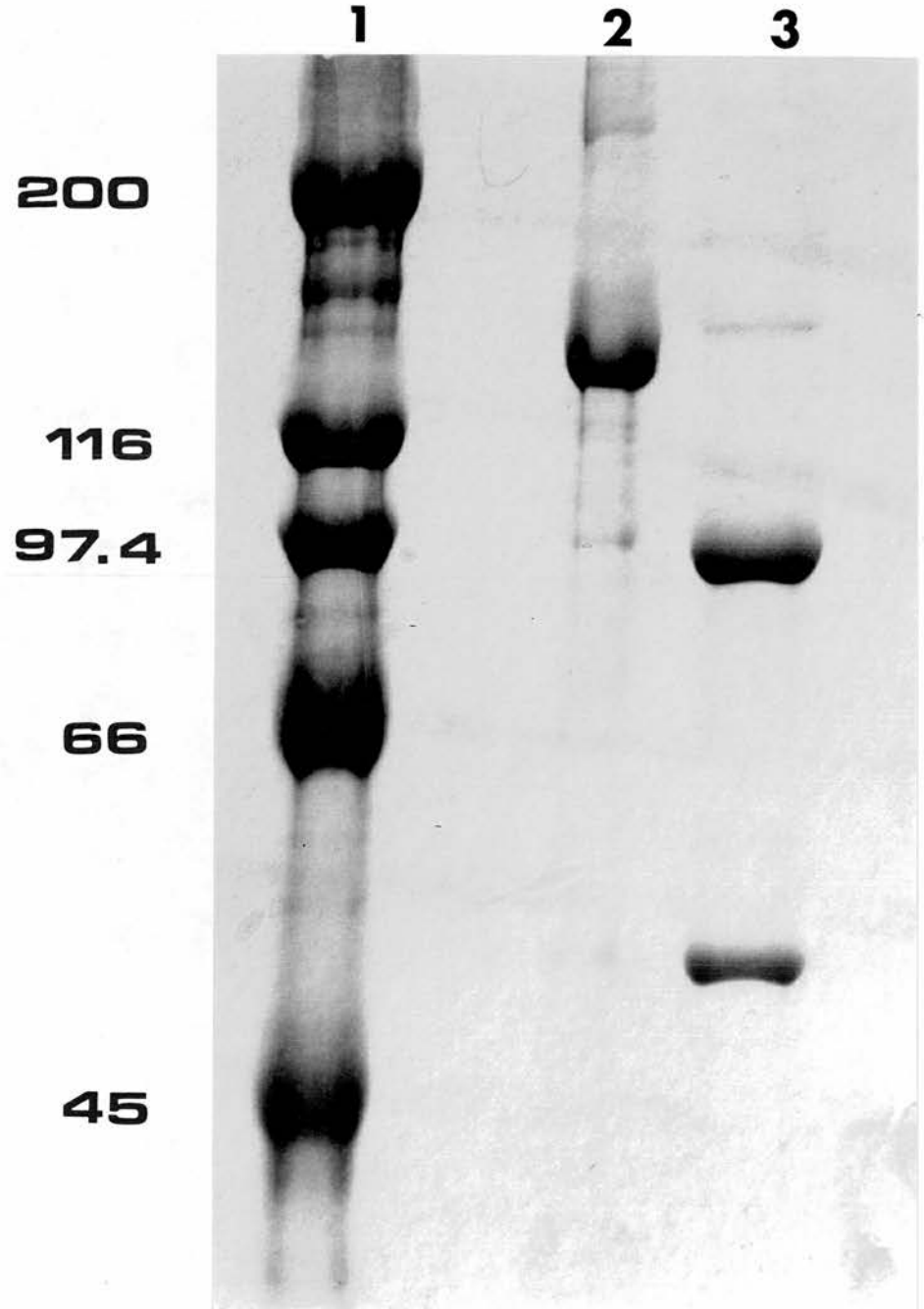


Figure 3.1 **SDS-Polyacrylamide Gel Electrophoresis of Tetanus Toxin**
Samples of toxin (10 μg) reduced with β-mercaptoethanol were subjected to electrophoresis as outlined in Section 2.2.19; bands were visualised by staining with Coomassie Blue. Lane 1: molecular weight markers (kDa); Lane 2: native tetanus toxin; Lane 3: toxin treated with trypsin (see text for details).

3.3 RESULTS AND DISCUSSION

3.3.1 Optimisation of Conditions for Stimulation of Secretion

Since the main objective of this work was to demonstrate an inhibitory effect of tetanus toxin on exocytosis from chromaffin cells, it was important first to establish conditions that promoted optimal levels of secretion; obviously changes in the extent of exocytosis occurring will be more evident if "control" levels are high. The secretagogue of choice was nicotine (even though others may evoke higher levels of secretion; see Table 3.1), the main reason for this being that nicotine had been used in the preliminary studies reported in Section 3.1.

Nicotine evokes exocytosis by binding to nicotinic acetylcholine receptors in the chromaffin cell membrane, thus setting in motion the chain of events described in Section 1.12.1. (Bovine chromaffin cells have muscarinic cholinergic receptors also, but only the nicotinic receptors are involved in acetylcholine-induced secretion of catecholamines). High concentrations of K^+ ions (e.g. 56 mM) cause direct depolarisation of the cell membrane, thus facilitating movement of Na^+ and Ca^{2+} ions, while veratridine is an alkaloid which activates Na^+ channels. The mechanism by which Ba^{2+} ions stimulate secretion is not certain, but it is thought that they may be able to permeate the cell membrane more readily than Ca^{2+} (i.e. without the need for depolarisation), so that they can gain access to the cell interior where they may be able to activate secretion directly.

Table 3.1 Exocytosis from Chromaffin Cells Evoked by Different Secretagogues

Chromaffin cells were stimulated by incubation for 10 minutes in Locke's solution containing secretagogue. Released catecholamines were then measured as described in Section 2.2.5.

SECRETAGOGUE	EVOKED RELEASE (% of cell content)
Nicotine (10 μM)	7.8 ± 0.7
Ba^{2+} ions (5 mM)	24.4 ± 1.2
K^{+} ions (55 mM)	7.5 ± 0.4
Acetylcholine (100 μM)	16.1 ± 1.0
Veratridine (100 μM)	3.0 ± 0.4

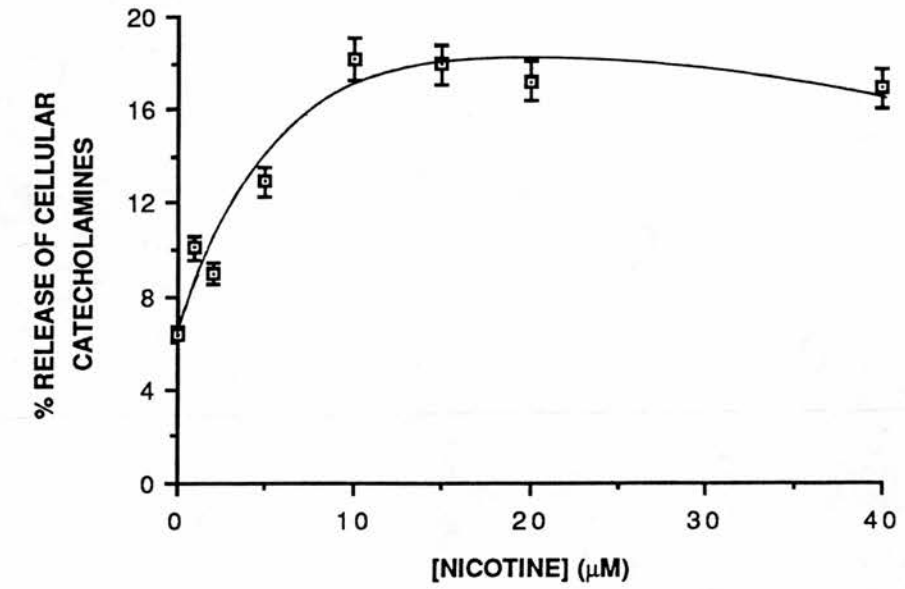
The values shown are typical of 3 independent experiments. Variability between data obtained in different experiments was less than 10%.
Students t-test: $p < 0.01$ for stimulatory effect of nicotine, Ba2+ ions, K+ ions and acetylcholine on catecholamine release; $p < 0.05$ for effect of veratridine.

N.B. Evoked release = total release - basal release

10 μM nicotine was found to be sufficient to evoke maximal levels of secretion, as measured by release during a 10 minute incubation (Fig. 3.2a); concentrations higher than this (up to 40 μM) did not cause any further enhancement of the response. In similar experiments, Kilpatrick *et al.* (1980) have found that nicotine concentrations higher than 30 μM may produce a sub-maximal response as a result of receptor desensitisation. On investigating the effect of different incubation times on evoked secretion, it was found that approximately 65% of the maximal response was achieved within five minutes, with a slow increase to maximal levels of exocytosis after 10-15 minutes (Fig. 3.2b); presumably receptor desensitisation is significant.

The effect of replacing the maintenance medium on levels of basal and evoked secretion was also examined. Perhaps not surprisingly, it was found that cells which had not recently been subjected to medium replacement (i.e. within the 24 hours immediately prior to experimentation) showed significantly lower levels of basal secretion and higher levels of evoked secretion than cells which had (Table 3.2). In accordance with this observation, Wilson & Viveros (1981) have commented that chromaffin cells seem to thrive better if the medium is not replaced too frequently; presumably some kind of conditioning factor(s) is produced by the cells that facilitates cell survival and maintenance of cell function.

To check for non-exocytotic release of catecholamines occurring during experiments i.e. that due to "leakiness" of cells, the release during stimulation of the cytosolic enzyme lactate



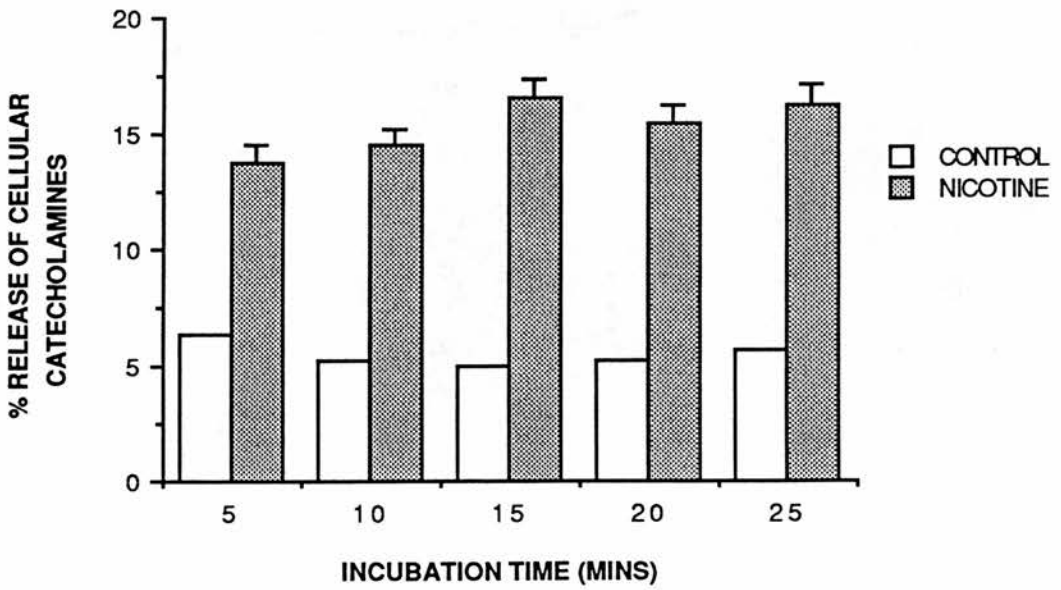


Figure 3.2 Optimisation of Catecholamine Secretion from Chromaffin Cells

(a) Chromaffin cells were incubated for 10 minutes with various concentrations of nicotine between 0-40 uM.

(b) Chromaffin cells were stimulated with 10 uM nicotine for various times up to 25 minutes.

Released catecholamines were then measured as outlined in Section 2.2.5; each result is the mean of 3 different wells ± standard deviation. Results shown in each case are typical of 2 separate experiments; variability between experiments in each case was less than 10%.

Table 3.2 Effect of Medium Replacement on Levels of Exocytosis from Chromaffin Cells

Catecholamine release was stimulated and measured as outlined in Sections 2.2.4 and 2.2.5. Figures represent the mean of 3 different wells ± standard deviation.

CELL TREATMENT	BASAL RELEASE (% of cell content)	EVOKED RELEASE (% of cell content)
Cells not subjected to medium replacement	2.9 (± 0.6)	16.9 (± 0.9)
Medium on cells replaced 24 hours prior to stimulation with nicotine	5.5 (± 0.9)	12.2 (± 0.5)

N.B. Evoked release = total release - basal release
The values shown are typical of two independent experiments; variability between data obtained in separate experiments was not more than 5%
Students t-test: $p < 0.01$ for effect of medium replacement on basal catecholamine release and evoked catecholamine release.

dehydrogenase into the medium was measured (these assays were carried out by Dr M.K. Bansal); negligible quantities of this protein were found in the medium, confirming that the cells were intact and that catecholamines present in the medium had been secreted by exocytosis.

(In all experiments, Ca^{2+} was maintained at 2.2 mM, the optimal concentration, as suggested by Fenwick *et al.* (1978).

3.3.2 Effect of Tetanus Toxin on Secretion from Intact Chromaffin Cells

Table 3.3 shows the results of a preliminary experiment in which the nicotine-evoked release of catecholamines from cells subjected to various treatments was determined. The effect of tetanus toxin 10 μg/ml, 67 nM) on secretion from native chromaffin cells was investigated, and the influence of ganglioside GT1 (50 μg/ml, 33 μM) on this effect was also examined. (It should be noted here that cells incubated with tetanus toxin, when viewed under the microscope, were indistinguishable from untreated cells, plated down normally and excluded trypan blue, suggesting that cell integrity was not affected by the toxin). The results indicate that, as suggested by the earlier experiments, tetanus toxin does have a significant inhibitory effect on catecholamine release from intact, native chromaffin cells; treatment of these cells with toxin for 24 hours at 37°C led to a 69% inhibition of evoked exocytosis (there was little or no effect on basal secretion). This inhibition was more marked when the cells had been pre-incubated with ganglioside; under these conditions, evoked exocytosis was reduced by 80%.

Table 3.3 Evoked Release of Catecholamines from Adrenal Chromaffin Cells

Cells were incubated in maintenance medium with or without ganglioside GT1 (50 μg/ml) for 1 hour, and then with 10 μg/ml toxin (also pre-incubated with or without the same concentration of GT1 for 1 hour) for 24 hours. The release of catecholamines evoked by 10 μM nicotine was measured using the assay procedure described in Section 2.2.5. Figures represent the mean of three wells ± standard deviation.

CELLS AND TOXIN	BASAL RELEASE (% of cell content)	TOTAL RELEASE (% of cell content)	TOTAL -BASAL	% INHIBITION OF RELEASE
Native cells, no toxin	7.4 (± 0.60)	17.2 (± 0.82)	9.8	-
Native cells, treated with toxin (24 hours, 37°C)	7.1 (± 0.36)	10.1 (± 0.32)	3.0	69 *
Cells pre-incubated with GT1 (1 hr, 37°C), no toxin (24 hrs)	4.9 (± 0.68)	13.6 (± 0.49)	8.7	11
Cells pre-incubated with GT1 (1 hr), then toxin (24 hrs)	7.4 (± 0.22)	9.4 (± 0.45)	2.0	80 *
Toxin pre-incubated with GT1 (1 hr), cells untreated (24 hrs)	6.9 (± 0.31)	6.8 (± 0.27)	-0.1	100 *
Toxin and cells both pre-incubated with GT1	6.5 (± 0.43)	8.1 (± 0.35)	1.6	84 *

* $p < 0.01$ for effect of treatment on evoked catecholamine release

However, if the toxin was pre-incubated with ganglioside GT1 and the toxin-ganglioside complex then added to the cells for 24 hours, exocytosis was completely abolished, suggesting that this preformed complex was being taken up by the cells very efficiently. This result was of interest since there had been no previous demonstration of an *in vitro* effect of the toxin which could be significantly enhanced by the introduction of gangliosides into the cell membrane, and was clearly worth further investigation (see Section 3.3.8).

3.3.3 Time Course of Inhibition of Secretion

Having observed an inhibition of exocytosis by tetanus toxin, we next characterised the time course of this inhibition; the results presented in Table 3.3 were obtained following a 24 hour incubation of chromaffin cells with tetanus toxin, and it seemed reasonable to assume that a shorter incubation time should be sufficient to produce the same effect. Figure 3.3 shows that there was no significant inhibition of secretion of catecholamines when chromaffin cells were incubated with toxin for various times up to 6 hours; maximal inhibition, of 70%, was observed after 16 hours incubation, and this was maintained during further prolonged incubation. This incubation time is longer than has been observed by some workers using PC12 cells (e.g. Sandberg *et al.*, 1989a), where maximal levels of inhibition were reached within 4 hours, but shorter than the three to six day incubation found by Marxen & Bigalke (1989) for maximal inhibition by tetanus toxin of [^{3}H]-noradrenaline secretion from chromaffin cells pre-incubated with ganglioside.

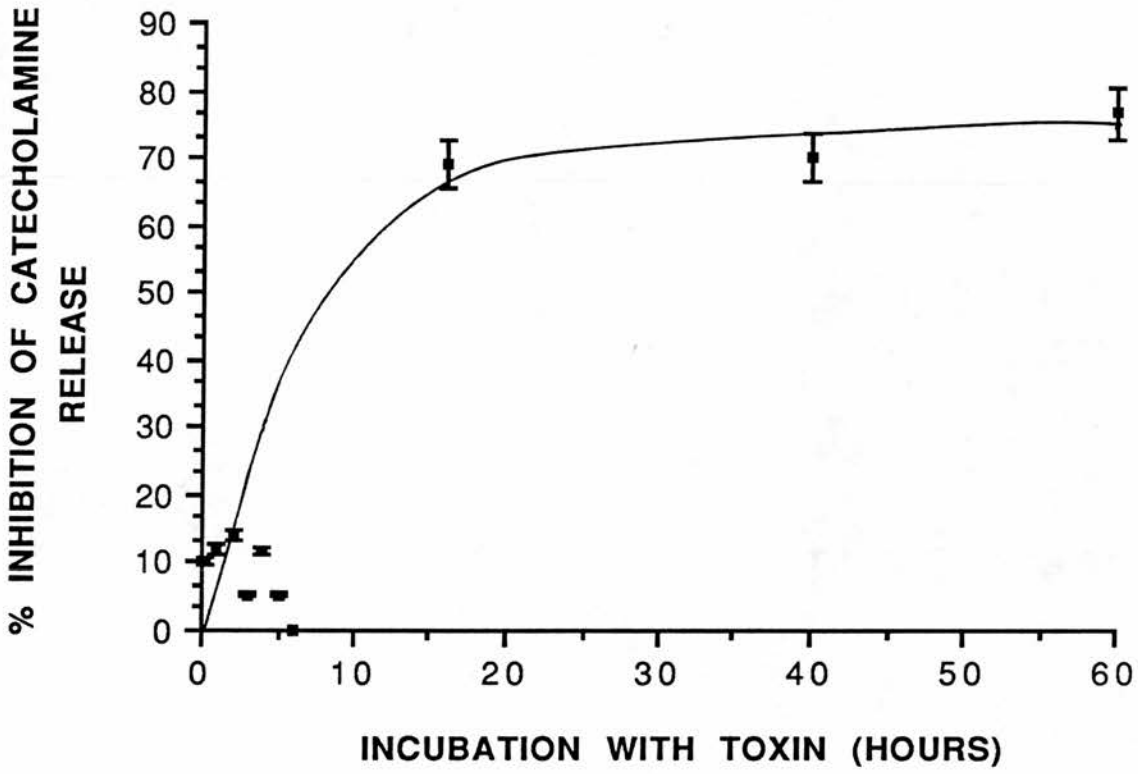


Figure 3.3 Time Course of Inhibition of Secretion from Intact Chromaffin Cells by Tetanus Toxin

Chromaffin cells were incubated for various times up to 60 hours with tetanus toxin (70 nM) in DMEM. Medium was then removed, the cells stimulated with 10 μM nicotine and released catecholamines measured as outlined in Sections 2.2.4 and 2.2.5. Each result is the mean of 3 different wells ± standard deviation.

Variability between data obtained in 3 independent, replicate experiments was less than 10%.

It is not possible from this type of experiment to determine which stage of the intoxication process is responsible for this long time course. Indeed, this is still an unanswered question; work with PC12 cells has indicated that binding and internalisation are relatively rapid events, and that this lag phase probably results from steps that occur in the intracellular compartment after internalisation (Sandberg *et al.*, 1989a), while experiments with permeabilised cells suggest that the intracellular action of the toxin is rapid also (e.g. Bittner & Holz, 1988; Bittner *et al.*, 1989a).

3.3.4 Dose-Dependency of Toxin Inhibition of Secretion

Experiments were next carried out to determine the toxin concentration dependence of inhibition. The dose-response curve thus obtained (typical of several different experiments) is shown in Fig. 3.4. When chromaffin cells were incubated for 16 hours in the presence of increasing concentrations of native tetanus toxin, catecholamine release was inhibited by up to 75% (the maximum observed); the half-maximal effect was found at 0.7 nM toxin. It is unclear why the remaining 25-30% of evoked release could not be inhibited, but it is possible that this relates to the exocytosis of those secretory granules already positioned close to the plasma membrane, such that if, for instance, the toxin acts to inhibit the movement of the granules to the cell surface, those already there will not be affected. This incomplete inhibition has been noted by other workers; Marxen & Bigalke (1989) were not able to achieve higher than 80% inhibition using chromaffin cells pre-incubated with ganglioside GT1, while Bittner *et al.* (1989b) and Stecher *et al.* (1989) have reported similar findings with botulinum toxin A.

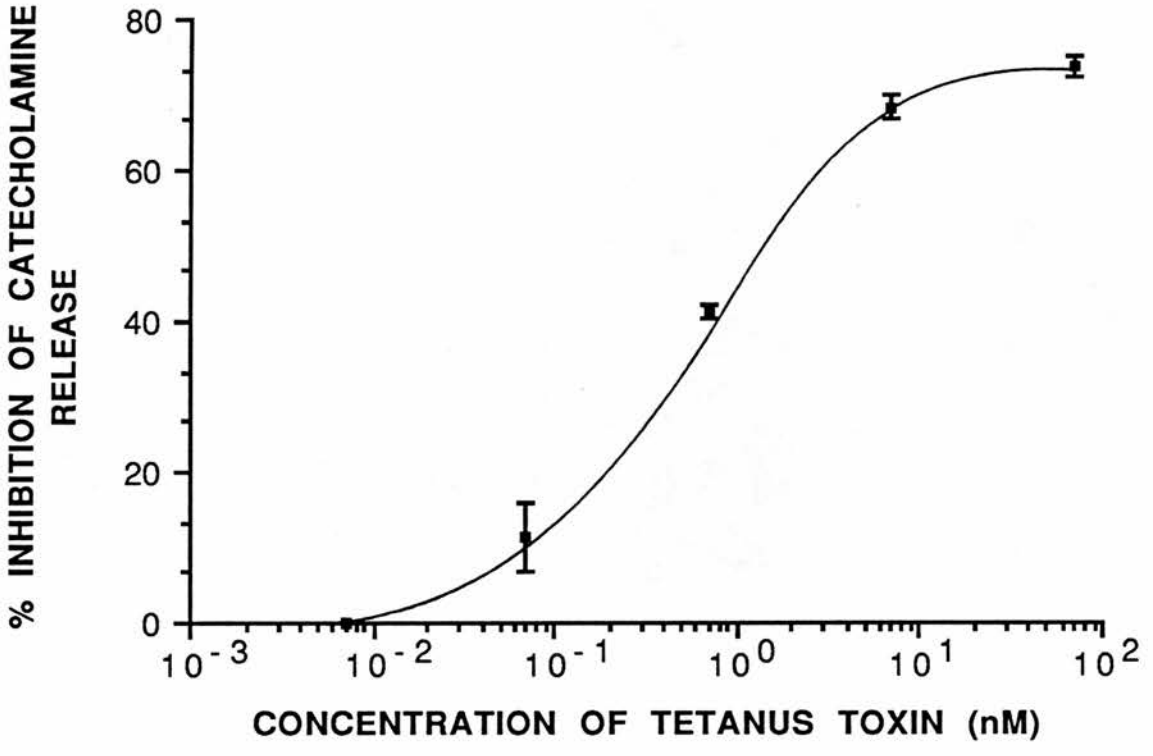


Figure 3.4 Effect of Tetanus Toxin on Nicotine-evoked Catecholamine Release from Intact Chromaffin Cells
Cells were incubated for 16 hours in the presence of concentrations of tetanus toxin between 7 pM and 70 nM in DMEM, then treated as described in Section 2.2.6. Each result is the mean of three different wells ± standard deviation.

The values shown are typical of 3 independent experiments; variability between data obtained in separate experiments was not more than 10%.

The concentrations of tetanus toxin that were found to inhibit catecholamine release by intact chromaffin cells are comparable to those found to be effective in PC12 cells (Sandberg *et al.*, 1989a), and are considerably lower than the concentrations found to be required when the cells are permeabilised (Penner *et al.*, 1986; Bittner & Holz, 1988). The reason why a higher concentration is needed when the cells are permeabilised is not clear, but receptor-mediated endocytosis may be an important feature in the mechanism of toxin action.

3.3.5 Reproducibility of Inhibition

Although this effect of toxin was seen in many of our experiments, an inhibition of secretion was not always observed; only about half of our cell preparations (of which there were about thirty) that responded to secretagogue also showed sensitivity to toxin. It should be stressed, however, that the inhibitory effect of the toxin was observed on numerous occasions, and is undoubtedly a genuine phenomenon. The data presented in the figures are representative of the cells that responded, and depict the largest responses seen, since the effectiveness of the toxin on different cell preparations was all-or-nothing and not graded i.e. the cells responded similarly to the results shown in the figures or not at all.

The reasons for this inconsistency are not clear, but it may be that some completely undiscovered property of the chromaffin cells affects their response to toxin. It is perhaps worth pointing out that Sandberg *et al.* (1989a) have reported that cultures of PC12 cells will only bind tetanus toxin with high affinity and therefore

become sensitive to the toxin after treatment with nerve growth factor; apparently the differentiation state of these cells is a critical factor in determining their sensitivity to toxin. It is not impossible that a similar explanation applies in the case of chromaffin cells. Another reason for the observed lack of consistency could be that the toxin-binding sites on the surface of chromaffin cells are damaged by the enzymes used during the isolation of the cells, and are not restored over the culture period. As mentioned in Section 1.8.2, there have been several reports of a trypsin-sensitive component in the toxin receptor on nerve cells (e.g. Yavin & Nathan, 1986; Pierce *et al.*, 1986), and it is also well known that the responsiveness of chromaffin cells to secretagogues such as nicotine can be impaired as a result of collagenase digestion (Almazon *et al.*, 1984).

As indicated above, other groups have not observed inhibition of release from intact cells. It is likely that this is related to the inconsistency of the cell preparations that we observe. It may be relevant that, in our experiments, we measured release of endogenous catecholamines directly using a fluorescent assay, whereas other groups have measured the release of radioactive catecholamines with which the cells have been preloaded. It is well known that such loading with labelled catecholamine does not label chromaffin granules uniformly; rather, the radioactive amine is more readily released than endogenous amine.

It appears that the toxin has little or no inhibitory effect on basal levels of secretion from chromaffin cells, which is somewhat surprising since it is well established that tetanus toxin not only

blocks evoked release of transmitter but is also able to block most (if not all) of the spontaneous neurotransmitter release from the synapses on which it acts. However, the precise mechanism of the basal release that is always observed is unclear.

3.3.6 Effect of Trypsinisation of Toxin on Inhibition of Secretion

The experiments reported thus far had been carried out using tetanus toxin in its single-chain form (as judged by SDS-polyacrylamide gel electrophoresis; see Fig. 3.1). To determine whether the two-chain form was equally active at inhibiting exocytosis, nicked toxin was prepared by proteolysis wih trypsin (trypsin:toxin ratio 1:50) for 30 minutes at room temperature, followed by addition of soybean trypsin inhibitor (2.5 μg/ml); formation of the heavy and light chains was confirmed by gel electrophoresis (see Fig. 3.1). Single-chain toxin and toxin nicked with trypsin (both 70 nM) were subsequently found to be equally potent, each causing approximately 65% inhibition of nicotine-evoked release (Table 3.4), and so it would seem that if there is any, the gain in toxicity by nicking is small. However, it is possible that the originally intact toxin had been nicked during the incubation by slight residual proteolytic activity in the toxin preparation or in the cell culture; other workers (e.g. Ahnert-Hilger *et al.* (1989), studying inhibition of Ca^{2+}-stimulated catecholamine release from permeabilised chromaffin cells) have reported that the single-chain form of the toxin is ineffective, and that chain separation, as well as reduction of the interchain disulphide bridge, must precede inhibitory action on exocytosis (in disagreement with the latter condition, however, is the report by

Table 3.4 Effect of Trypsinisation of Tetanus Toxin on Inhibition of Secretion

Chromaffin cells were incubated with or without 70 nM toxin (native or trypsinised) in DMEM for 16 hours, and then treated as described in Sections 2.2.4 and 2.2.5. The toxin was nicked as outlined in the text. Each result is the mean of 3 different wells ± standard deviation.

CELL TREATMENT	SECRETION (% of cell content)	% INHIBITION
None (i.e. basal secretion)	4.5 (± 0.3)	
Nicotine stimulation	17.0 (± 0.9)	
Native toxin, then nicotine	8.9 (± 0.5)	65%
Trypsinised toxin, then nicotine	9.1 (± 0.7)	63%
Trypsin*, then nicotine	18.9 (± 0.8)	0%

* Cells were exposed to trypsin itself (in the presence of excess soybean trypsin inhibitor) to check for any effect of this treatment on levels of secretion.

Results shown are typical of 2 separate experiments; variability between data obtained in separate experiments was less than 10% Students t-test: $p < 0.01$ for effect of toxin on nicotine-evoked secretion

Schiavo *et al.* (1990) that an intact interchain disulphide bond is required for the neurotoxicity of tetanus toxin). Similarly, Bittner *et al.* (1989b) report increased potency of botulinum toxins B and E in inhibiting exocytosis from permeabilised chromaffin cells following mild trypsinisation.

3.3.7 Effect of Tetanus Toxin on Exocytosis Evoked by Different Secretagogues

As discussed in Section 3.3.1, catecholamine release from chromaffin cells can be stimulated by a number of different secretagogues. Having established that tetanus toxin inhibits nicotine-evoked release, it was of interest to determine whether exocytosis evoked by other means was also affected. Previously, Sandberg *et al.* (1989a), using NGF-treated PC12 cells, have reported that tetanus toxin inhibits secretion of [^{3}H]acetylcholine evoked by Ba^{2+} ions, veratridine and carbachol (which induces secretion via muscarinic receptor activation), suggesting that a crucial step common to secretion evoked by distinctly different secretagogues is inhibited by the toxin. Similarly, Knight *et al.* (1985) observed an inhibitory effect of botulinum toxin type D on the response of intact chromaffin cells to acetylcholine, veratridine, high [K^+] and Ba^{2+}.

Table 3.5 clearly demonstrates that tetanus toxin had no inhibitory effect on catecholamine release from chromaffin cells evoked by Ba^{2+} ions. This is in sharp contrast to the findings of Sandberg and her coworkers, who reported that tetanus toxin inhibited barium-evoked release by as much as 82%. However, it is perhaps dangerous to assume that exocytotic mechanisms operating in PC12

Table 3.5 Effect of Tetanus Toxin on Exocytosis Evoked by Ba^{2+} ions

Chromaffin cells were incubated for 16 hours with or without tetanus toxin (70 nM) in DMEM, and then stimulated with either nicotine (10 μM) or Ba^{2+} ions (5mM). Released catecholamines were measured as described in Section 2.2.5. Each result is the mean of 3 wells ± standard deviation.

CELL TREATMENT	% RELEASE OF CATECHOLAMINES	% INHIBITION
None i.e. basal release	5.8 (± 0.7)	
Nicotine	16.2 (± 1.1)	
Toxin, then nicotine	8.4 (± 0.9)	75%
Ba^{2+} ions	37.1 (± 1.7)	
Toxin, then Ba^{2+} ions	38.3 (± 2.1)	0%

The results shown are typical of 2 independent experiments; variability between data obtained in separate experiments was less than 8%.
Students t-test: $p < 0.01$ for effect of toxin on catecholamine secretion evoked by nicotine.

cells (which have really been studied in very little detail) are identical to those found in chromaffin cells. Further, it is maybe not surprising that tetanus toxin does not affect Ba^{2+}-evoked release; while Ba^{2+} can substitute for Ca^{2+} in entering nerve terminals and stimulating neurosecretion (Nachshen & Blaustein, 1982), the mechanism by which Ba^{2+} ions promote exocytosis is not well defined. Evidence is accumulating, though, to suggest that this may involve processes that are not entirely identical to those utilised by Ca^{2+}. For instance, Robinson & Dunkley (1985) have reported that while Ca^{2+} ions are able to modulate depolarisation-dependent protein phosphorylation and dephosphorylation (presumably through activation of protein kinases), Ba^{2+} ions cannot do this; presumably, then, Ba^{2+} ions stimulate secretion via a different route. Further, the observation of Armstrong & Taylor (1980) that Ba^{2+} ions can interact with K^+ channels and competitively inhibit K^+ fluxes suggests that Ba^{2+} ions, in addition to utilising Ca^{2+} transport mechanisms, may also be able to obtain access to intracellular release sites via K^+ channels.

In further experiments conducted in the laboratory, it was found that K^+-evoked secretion from intact chromaffin cells was inhibited much less than that evoked by nicotine: about 30% after 3 days incubation with 70 nM toxin (data not shown; experiments carried out by Dr M.K. Bansal). This difference between the effectiveness of the toxin on nicotine- and K^+-evoked release suggests that it might be acting on some component of the cytoskeleton. It has been reported by Cheek & Burgoyne (1986) that nicotinic stimulation of chromaffin cells results in cortical actin disassembly, whereas depolarisation with high K^+ does not, suggesting that with K^+ there

is mainly exocytosis of those granules already close to the plasma membrane, while with nicotine there is mobilisation of more distant granules. Since some inhibition of K^+-evoked release by toxin was observed, it is possible that the toxin may also have a target at the plasma membrane.

3.3.8 Effect of Gangliosides on Toxin Inhibition of Secretion

In Section 3.3.2, it was described how pre-incubation of tetanus toxin with ganglioside GT1, prior to its introduction to chromaffin cells, resulted in an enhancement of the toxin's inhibitory action. This observation prompted us to carry out more experiments using this preformed toxin-ganglioside complex, with a view to evaluating the role of gangliosides in mediating the action of the toxin. In an initial experiment to determine the dose-dependency of the toxin effect in the presence of ganglioside, different concentrations of tetanus toxin from 0.01-33 μg/ml (67 pM-220 nM) were pre-incubated with 50 μg/ml GT1 for one hour prior to addition to cells. However it was soon apparent that even at the lowest tetanus toxin concentration (0.01 μg/ml), maximal inhibition of catecholamine secretion was still occurring. This was a somewhat puzzling result until, on running a ganglioside control (i.e. incubating cells with ganglioside only, then evoking secretion), it was discovered that all the inhibition observed could be attributed to a ganglioside-mediated effect. Further evidence that this was not a toxin effect came from the finding that the time course of inhibition with the toxin-ganglioside complex was complete within one hour (see Fig. 3.5); tetanus toxin alone requires much longer than this to exert its inhibitory effects (Section 3.3.3). On investigating the dose-

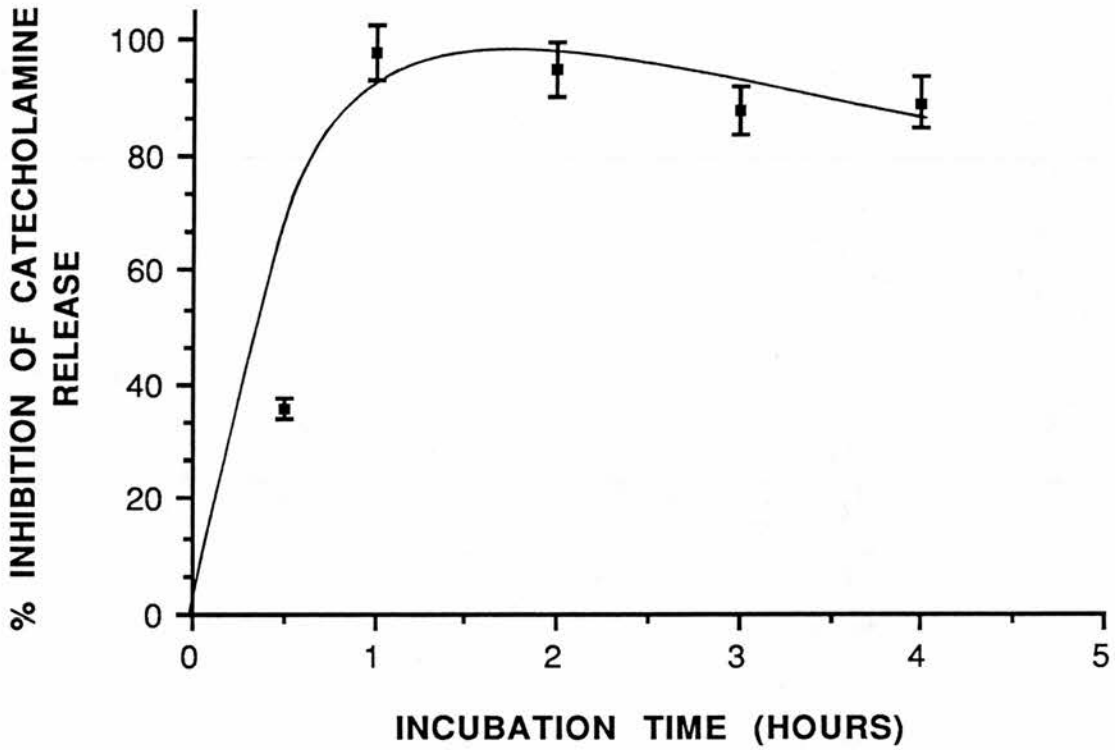


Figure 3.5 Time Course of Inhibition of Catecholamine Secretion from Intact Chromaffin Cells by Tetanus Toxin Pre-Incubated with Ganglioside

Cells were incubated for various times up to 4 hours with tetanus toxin (70 nM) which had been pre-incubated for one hour with ganglioside GT1 (50 μg/ml) in DMEM. The medium was removed, the cells stimulated with 10 μM nicotine, and released catecholamines measured as outlined in Sections 2.2.4 and 2.2.5. Each result is the mean of three different wells ± standard deviation.

This result is typical of 2 independent experiments; variability between data obtained in replicate experiments was less than 7%.

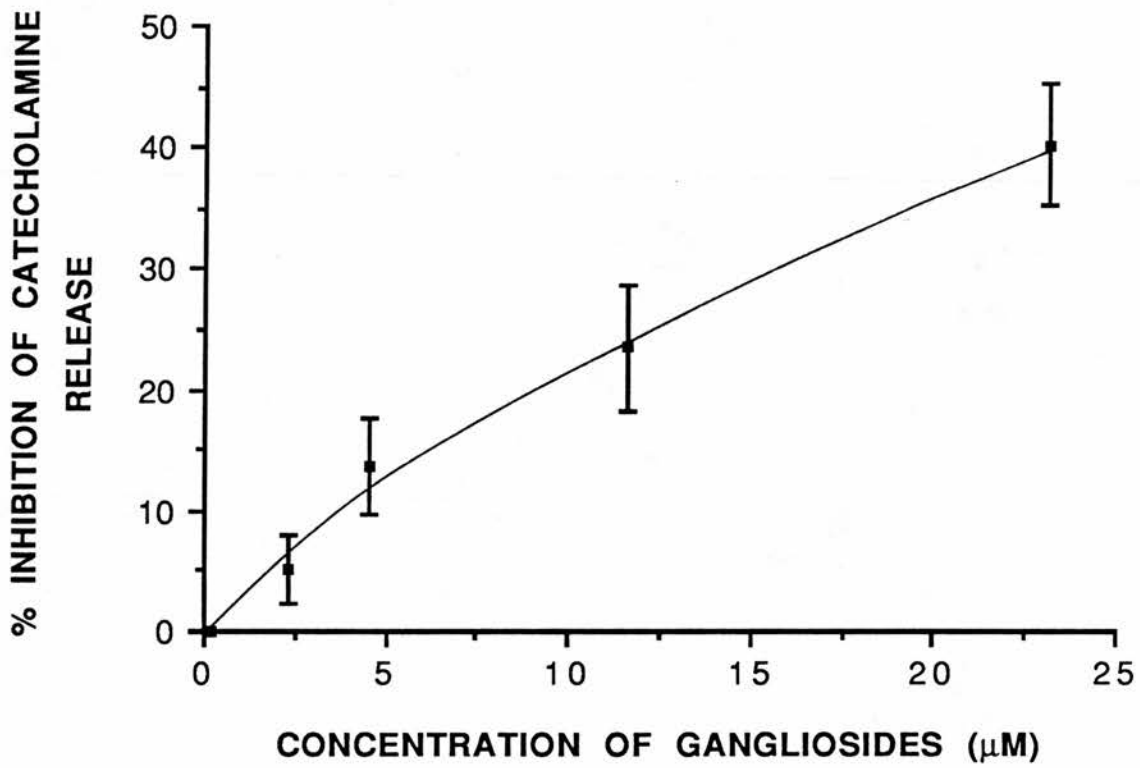


Figure 3.6 Effect of Ganglioside GT1 on Release of Catecholamines from Intact Chromaffin Cells

Cells were incubated for 16 hours in the presence of various concentrations of ganglioside GT1 in DMEM. The medium was then removed, the cells stimulated with 10 μM nicotine, and released catecholamines measured as outlined in Sections 2.2.4 and 2.2.5. Each result is the mean of three different wells ± standard deviation.

This result is typical of 2 independent experiments; variability between data obtained in replicate experiments was less than 10%.

dependency of this ganglioside-mediated effect, it was discovered that as little as 5 μM ganglioside GT1 inhibited catecholamine release by 12% (Fig. 3.6).

Thus incubation of chromaffin cells with ganglioside alone is inhibitory to nicotine-evoked secretion. This makes it very difficult, if not impossible, to determine whether pre-incubation of the cells with ganglioside, which should increase the binding of tetanus toxin, would also increase its inhibition of exocytosis. (An alternative approach would perhaps have been to investigate the effect of neuraminidase treatment of chromaffin cells on the ability of tetanus toxin to inhibit their catecholamine release). It is unclear how the ganglioside is interfering with the secretory process; one can only speculate that it is perhaps acting at the level of the cell membrane to interact with nicotinic receptors in some way. One point to note is that in the experiment reported in Section 3.3.2, cells pre-incubated with ganglioside GT1 (50 μg/ml, 33 μM) alone did not show a significant level of inhibition of evoked catecholamine release; this may have been due to the fact that in this case, the cells were replaced in maintenance medium for 24 hours after incubation with GT1 and prior to nicotinic stimulation; perhaps this allowed the cells time to "recover" from the effects of incubation with ganglioside.

3.4 CONCLUDING REMARKS

The work presented in this chapter clearly demonstrates that tetanus toxin is able to inhibit catecholamine release from intact chromaffin cells, lending considerable support to the notion that

the toxin may have the potential to act as a general inhibitor of secretion from a wide variety of cell types, not necessarily neuronal, as long as it can gain access to these cells; the specificity of the toxin's action on the nervous system *in vivo*, then, may well be a result of limited accessibility rather than preferential binding of the toxin to particular neurones.

Essentially nothing is known about how the toxin inhibits exocytosis once it has entered the cell; exhaustive experiments in many laboratories including our own have failed to find any ADP-ribosylation catalysed by tetanus or botulinum neurotoxins (see Adam-Vizi *et al.*, 1988), an enzymatic activity found in other toxins such as cholera, diphtheria and pertussis, while Wendon & Gill (1982) were unable to find any toxin-catalysed protein phosphorylation. Mellanby (1988) has suggested that the toxin might be a specific phospholipase, acting on a membrane component such as phosphatidylserine which is required to increase the sensitivity to Ca^{2+} of protein kinase activation; this would reduce the sensitivity to Ca^{2+} of the exocytotic mechanism. Recently an involvement of tetanus toxin in the mobilisation of protein kinase C in NG-108 cells has been reported (Considine *et al.*, 1990), and Sandberg *et al.* (1989b) have suggested that its action in PC12 cells is connected with alterations in the metabolism of cyclic GMP. More recently, Marxen & Bigalke (1991) have proposed that tetanus and botulinum A toxins inhibit exocytosis by interfering with stimulus-evoked F-actin rearrangement. It remains to be seen, however, whether any of these possible actions of the toxin do in fact occur *in vivo*.

Other work in our laboratory (carried out by Dr M.K. Bansal) has concentrated on characterising the effect of tetanus toxin on catecholamine release from permeabilised chromaffin cells; similar results to those recently published have been obtained. The effect of tetanus toxin on pertussis toxin-stimulated exocytosis has also been examined. (Pertussis toxin catalyses the ADP-ribosylation of a 40 kDa GTP-binding protein in chromaffin cells; this stimulates secretion by increasing the affinity of exocytosis for Ca^{2+}, suggesting that G proteins are involved in the direct control of exocytosis). Tetanus toxin has been found to inhibit catecholamine release stimulated by pertussis toxin, showing that the two toxins probably act at different sites i.e. tetanus toxin probably does not inhibit exocytosis by affecting G proteins. This finding is in accord with the inability of workers to find evidence of any ADP-ribosylation.

In summary, then, it can be said that intact chromaffin cells are indeed sensitive to externally applied tetanus toxin, given the appropriate conditions. Unfortunately this effect, although quantitatively reproducible, is not very consistent, for reasons that are by no means obvious. Chromaffin cells may therefore have the potential to provide useful information on the mechanism of action of this toxin.

CHAPTER FOUR.

BINDING OF ^{125}I-LABELLED TETANUS TOXIN TO CULTURED ADRENAL CHROMAFFIN CELLS

4.1 INTRODUCTION

In Chapter 3, evidence was presented to show that tetanus toxin is a potential inhibitor of exocytosis from intact chromaffin cells. Since it is generally thought that tetanus toxin shares the same mechanism of action as that displayed by other bacterial toxins (e.g. cholera, diphtheria), which involves binding of the toxin to the cell membrane, internalisation of the toxin and finally intracellular poisoning, it would seem that a binding event must be occurring prior to chromaffin cell intoxication and inhibition of secretion. For the purposes of this project, it seemed a logical step to go on from demonstrating an inhibition of secretion to attempt to show that this binding did in fact take place, and to characterise it as fully as possible.

To date there have been no published reports of tetanus toxin-binding by adrenal chromaffin cells; in fact, there have been several which state that tetanus toxin <u>does not</u> bind to these cells. Most of these claims, however, have come from workers who have been unable to demonstrate an inhibitory effect of tetanus toxin on exocytosis from intact chromaffin cells, and who have concluded that this must be a reflection of the inability of these cells to bind and internalise the toxin (Knight *et al.*, 1985; Bittner & Holz, 1988; Penner *et al.*, 1986; Marxen *et al.*, 1989). It seems that little effort has been made to demonstrate tetanus toxin binding to chromaffin cells <u>directly</u>. (Lazarovici *et al.* (1989) have reported that they were unable to observe any binding

of ^{125}I-labelled tetanus toxin to intact chromaffin cells; however, they provide no details of the binding assay or of the toxin used, so it is difficult to assess the validity of this report.)

It would obviously be advantageous, then, if toxin-binding could in fact be demonstrated in our system. Further, since one of the aims of this project was to assess the suitability of the chromaffin cell as a model for studying the intoxication process of tetanus, it was of interest to compare the characteristics of toxin-binding by chromaffin cells to binding by true neuronal tissue. For instance, does binding of tetanus toxin to chromaffin cells exhibit the same dependence on pH and ionic strength as binding to rat brain membranes? Does pre-treatment of chromaffin cells with neuraminidase or trypsin have any effect on their subsequent toxin-binding capacity? Further, what effect does pre-incubation of cells with ganglioside GT1 have on toxin-binding, and is the binding seen in these cells of the same nature as that seen with native cells? Also, if it is possible to demonstrate the specific fixation of tetanus toxin to chromaffin cells, can any subsequent internalisation of the toxin be detected? By answering these questions, we may be better placed to draw conclusions as to how similar the binding of tetanus toxin by chromaffin cells is to binding occurring to toxin receptors *in vivo*.

4.1.1 Parameters of Ligand Binding That Define a Receptor

There are several biophysical characteristics of ligand binding that define a true receptor (although it must be borne in mind that the cell-surface moieties which bind tetanus toxin *in vivo* can not strictly be regarded as "receptors", for reasons stated earlier).

The first of these is saturation; this is a direct consequence of the limited number of receptors that should be present on a target cell. Associated with this is the concept of high affinity; not only should there be a finite number of receptors but in addition, their affinity for the ligand should be high. The dissociation constant, K_d, is a measure of the tightness of ligand binding and can be defined as $K_d = [R][L]/[RL]$, where the brackets signify the concentrations at equilibrium, of the respective components R (receptor), L (ligand) and RL (ligand-receptor complex). Further, according to simple dissociation theory, K_d will also be equivalent to the concentration of ligand that produces half-saturation of the available binding sites; the smaller the value of K_d, the higher the binding affinity. There is no absolute value for K_d that defines high affinity; however, a value greater than 1-10 μM is relatively weak and represents the lower-affinity end of the range for ligand-receptor binding constants, whereas K_d values less than 100 pM are rare and define the upper limits.

The third criterion defining a true receptor is reversibility; after forming, a ligand-receptor complex will dissociate with time, according to the dissociation rate constant, k_{off}. A consequence of this is that, if k_{off} is small, then toxin molecules may spend a long time on their receptors, allowing plenty of time for extensive cellular processing to occur. The final biophysical criterion to satisfy is specificity; that is, the binding of a ligand to its specific receptor should be affected by other ligands that interact with the same receptor. In all instances, the addition of an excess of the same unlabelled ligand should compete for the binding measured.

An important point to keep in mind when carrying out these kinds of studies is that there may exist ligand-binding sites with all the biophysical features characteristic of a true receptor, yet those sites have nothing to do with mediating the biological response to the ligand. Therefore, as well as meeting biophysical criteria, a receptor must possess a number of biological correlates i.e. a definite correlation between ligand-binding and the biological response must be observed. It is interesting to note that as yet such a correlation has not been clearly demonstrated for tetanus toxin binding to gangliosides (or any other cell surface molecules).

4.2 METHODS

4.2.1 Incorporation of Fluorescent GT1-LY into Chromaffin Cell Membranes

Fluorescent ganglioside GT1 was prepared as outlined in Section 2.2.7, and analysed by thin-layer chromatography (Section 2.2.14). This ganglioside derivative was then used to determine the time course of incorporation of exogenous gangliosides into the plasma membranes of intact chromaffin cells, as described in Section 2.2.8.

4.2.2 Binding of ^{125}I-labelled Tetanus Toxin to Chromaffin Cells

The binding of radiolabelled tetanus toxin to cultured chromaffin cells was measured using the procedure described in Section 2.2.10. Experiments were first carried out to determine the time course of toxin-binding, the linearity of toxin-binding with respect to the amount of cell protein, and the effect of pre-incubation of

chromaffin cells with different concentrations of GT1 on subsequent toxin-binding ability. These experiments established i) the optimal length of incubation of chromaffin cells with tetanus toxin, ii) the optimal amount of cell protein to have present in an assay well, and iii) the optimal concentration of GT1 to use to achieve maximal (or near-maximal) enhancement of toxin-binding ability.

4.2.3 Binding of ^{125}I-labelled Tetanus Toxin to Chromaffin Granules

The binding of radiolabelled tetanus toxin to chromaffin granules was measured as described in Section 2.2.11. In some cases, granules were pretreated with 50 mU/ml neuraminidase or 1 mg/ml trypsin (in incubation buffer) for 30 minutes at 37°C prior to incubation with toxin. Non-specific binding of toxin was measured by incubating granules with radiolabelled toxin in the presence of a 100-fold excess of unlabelled toxin.

4.2.4 Internalisation of Tetanus Toxin by Chromaffin Cells

The procedure outlined in Section 2.2.12 was used to investigate toxin internalisation by chromaffin cells. This method makes use of the fact that surface-bound toxin is susceptible to pronase digestion, while internalised tetanus toxin is pronase-resistant. All incubations were performed in triplicate. Optimal conditions for the degradation of ^{125}I-labelled tetanus toxin were determined by assessing toxin degradation on an SDS-polyacrylamide gel after the toxin had been exposed to different concentrations of enzyme (as described below).

4.3 RESULTS AND DISCUSSION

4.3.1 Analysis of Lucifer Yellow CH-Labelled Ganglioside

Oxidation of the sialic acid residues of ganglioside GT1 with sodium periodate, and subsequent reaction of the resulting aldehydes with the hydrazide moiety of the naphthylamide dye Lucifer Yellow CH led to the formation of fluorescent derivatives of GT1 ganglioside. The oxidation conditions used (2 mM sodium periodate for 30 minutes at 0°C) ensure the specific oxidation of the sialic acid residues, and not the periodate-sensitive galactose residue of GT1 (Veh *et al.*, 1977).

Three fluorescent ganglioside derivatives were produced, which could be readily separated by thin-layer chromatography, as shown in Fig. 4.1. All of these derivatives reacted with resorcinol reagent, indicating the presence of sialic acid residues and suggesting that they were indeed gangliosides. A small amount of underivatised GT1 was also evident; this may have been due to incomplete oxidation of the ganglioside prior to the addition of the Lucifer Yellow, which was present in considerable excess. Two of the derivatives had a lower mobility on thin-layer chromatograms than native GT1; this is not unexpected, since Lucifer Yellow is both polar and negatively charged. The third fluorescent compound, however, was more mobile than native GT1; it seems unlikely that this is a derivative of GT1, but rather of some minor contaminating ganglioside species present in the GT1, perhaps a disialo-ganglioside. It is worth noting that the fluorophore itself is immobile in the solvent system used here, so any fluorescence due to free Lucifer Yellow CH would have remained at the origin.

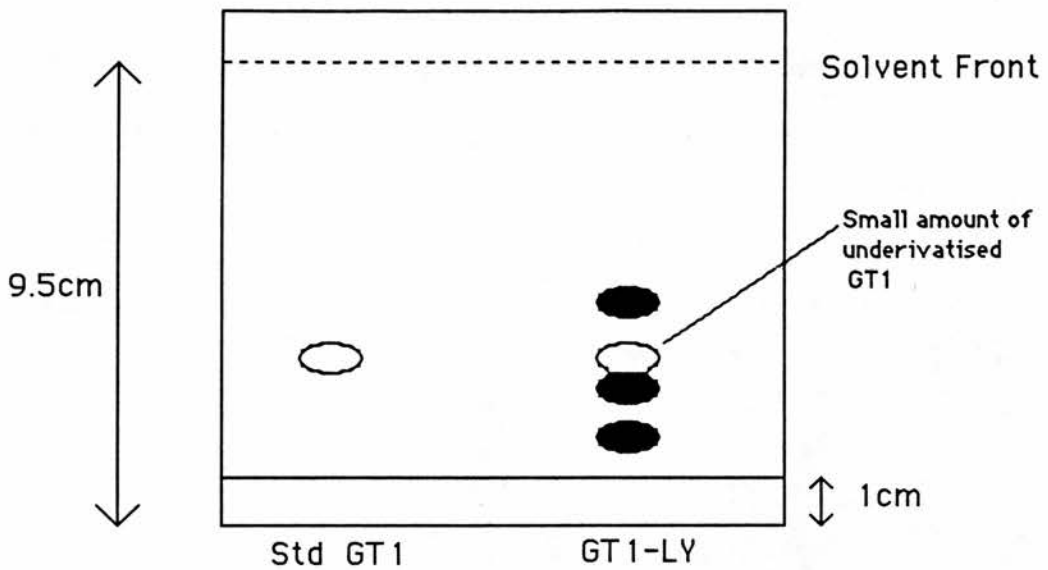


Figure 4.1 Representation of Thin-Layer Chromatogram of Fluorescent Ganglioside GT1

Standard and fluorescently-labelled GT1 were subjected to thin-layer chromatography as described in Section 2.2.14. Gangliosides were visualised by resorcinol staining; fluorescent derivatives were also visible under ultraviolet illumination.

N.B. Thin-layer chromatography of GT1-LY was carried out three times, with identical results.

There are several possible explanations to account for the formation of more than one derivative of GT1. Since this ganglioside species contains three sialic acid residues, it is possible that different GT1 molecules were conjugated with Lucifer Yellow CH on different residues; indeed, it could even be that some ganglioside molecules had more than one sialic acid residue modified. Another possibility is that residues on different molecules could be derivatised on different carbon atoms; oxidation of gangliosides with sodium periodate can form aldehydes at Carbon-7 or Carbon-8 of sialic acid residues (Veh *et al.*, 1977). Further, Lucifer Yellow has a free amino group in addition to hydrazide (the usual reactive part of the molecule), which could react with aldehyde groups. It is not easy, however, to establish which of these explanations is the correct one.

Further purification of the individual GT1 derivatives could have been carried out by performing preparative thin-layer chromatography, then scraping the bands from the chromatograms and eluting the silica gel with solvent. However, this was deemed unnecessary since it seemed evident that all the fluorescent material was indeed ganglioside (owing to its reactivity with resorcinol and its mobility relative to standard ganglioside on thin-layer chromatograms). The fact that there was more than one ganglioside derivative was not considered to be a problem for our purposes, since all gangliosides are likely to incorporate into membranes at essentially the same rate and to the same extent, regardless of their hydrophilic moiety.

4.3.2 Incorporation of GT1-Lucifer Yellow into Chromaffin Cell Membranes

The reason for preparing a fluorescent derivative of ganglioside GT1 was that this provided a convenient means of monitoring the incorporation of exogenous gangliosides into chromaffin cell membranes. Since one of the questions to be addressed later in this study was whether or not exogenous ganglioside could enhance the ability of adrenal chromaffin cells to bind tetanus toxin, it was important i) to establish that these cells did in fact incorporate exogenous ganglioside into their plasma membranes and ii) to determine the time course of this process. This sort of experiment has been done often with cholera toxin and its receptor ganglioside GM1; Cuatrecasas (1973) showed that the response of fat cells to cholera toxin was increased ten-fold when the cells were pre-incubated with ganglioside, while Gill & King (1975) reported similar findings with pigeon erythrocytes. In these experiments, incorporation of tritiated ganglioside into the cell membrane was shown directly and correlated with toxin binding. Similarly, Hollenberg *et al.* (1974) demonstrated that transformed mouse fibroblasts which contained no GM1 (but which did have an adenylate cyclase that responded to cholera toxin after lysis of these cells) and were therefore normally unresponsive to toxin when intact, could take up as much as 10^5 molecules of [^{3}H]GM1 per cell and then respond to toxin. All these experiments provide good evidence that when ganglioside molecules in solution are incubated with cells, their hydrophobic portions insert into the lipid membrane and they presumably become indistinguishable from endogenous ganglioside.

When cultures of chromaffin cells were incubated with the ganglioside derivatives at 37°C they became highly fluorescent, in a time-dependent manner. The fluorescence was evenly spread over the whole of the cells, suggestive of surface labelling. Fig. 4.2 shows the results of a typical "incorporation experiment"; values plotted are an average of 20 measurements taken at each time point, and are typical of three such experiments. The graph indicates that after a two hour incubation period, there is very little further increase in the level of incorporated ganglioside. This time course agrees well with that found by other workers; Facci *et al.* (1984) observed that incorporation of [^{3}H]GM1 by neuroblastoma cells reached 95% of its maximum after approximately two hours, while Leskawa *et al.* (1989) reported very similar findings using the same cells.

It was concluded, therefore, that a two hour incubation of chromaffin cells with ganglioside was sufficient to achieve maximum incorporation into the plasma membrane. Although it is likely that some of the cell fluorescence was due to internalised ganglioside, the fact that the whole of the cells appeared to be evenly labelled, without any obvious signs of compartmentalisation, was a good indication that much of the ganglioside was present at the level of the cell membrane. Further, the finding by Spiegel (1985) that fluorescent GM1 could act as a receptor for cholera toxin in GM1-deficient rat glioma C6 cells after a three hour incubation under the same conditions as used here strongly suggests stable incorporation into the cell membrane, as it seems unlikely that different cell types will vary much in their ability to incorporate exogenous gangliosides. Another point to note is that although it

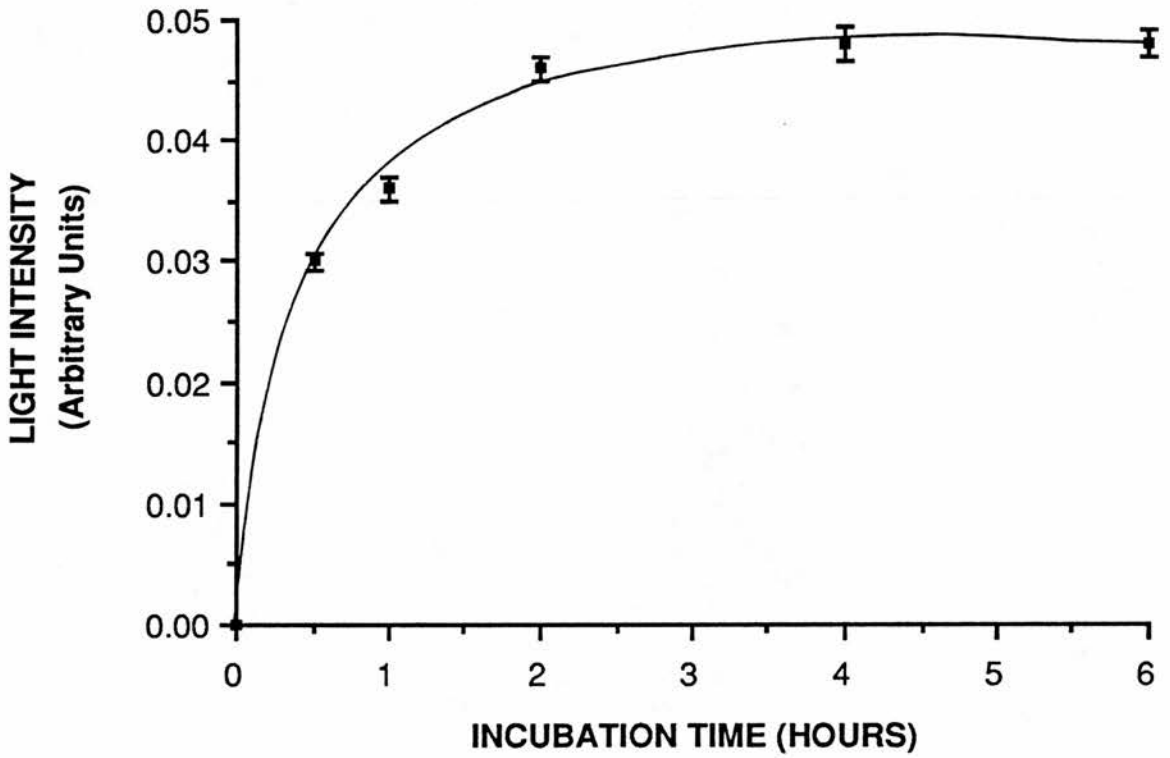


Figure 4.2 Incorporation of Fluorescent GT1 into Chromaffin Cell Membranes

Chromaffin cells were incubated with GT1-LY as outlined in Section 2.2.8. then the fluorescence of single cells was measured using the photometer of a Leitz Vario Orthomat 2 automatic microscope camera. Each result represents the mean of 20 measurements ± standard deviation.

This experiment was conducted three times; variability between data obtained in separate experiments was less than 10%.

can really only be assumed that the gangliosides are inserted into the plasma membrane in the correct orientation, one would expect on energetic grounds that both hydrocarbon chains would be inserted into the bilayer, leaving the carbohydrate portion exposed at the cell surface (indeed, Spiegel's results suggest this to be the case). Presumably, also, only the monomeric and not the micellar form of ganglioside is stably inserted. Extensive work in other systems has never shown any difference between endogenous and exogenous ganglioside.

4.3.3 Iodination of Tetanus Toxin

Radiolabelled tetanus toxin was routinely prepared to a specific radioactivity of 0.5-1.5 μCi/μg protein. Its purity was determined by SDS-polyacrylamide gel electrophoresis and subsequent analysis of the distribution of radioactivity on the gel by autoradiography. A typical result is shown in Fig. 4.3. Three bands of radioactivity comigrating with holotoxin (150 kDa), heavy chain (100 kDa) and light chain (50 kDa) were detected; very little contaminating radioactivity was present. Most of the radiolabel appeared to be incorporated into the heavy chain of the toxin; this is perhaps due to the presence of more tyrosine residues in the heavy chain, or maybe reflects easier accessibility of the heavy chain to the iodinating reagents. Each batch of iodinated toxin was kept for a maximum of two months, and was checked routinely by gel electrophoresis and autoradiography for signs of degradation.

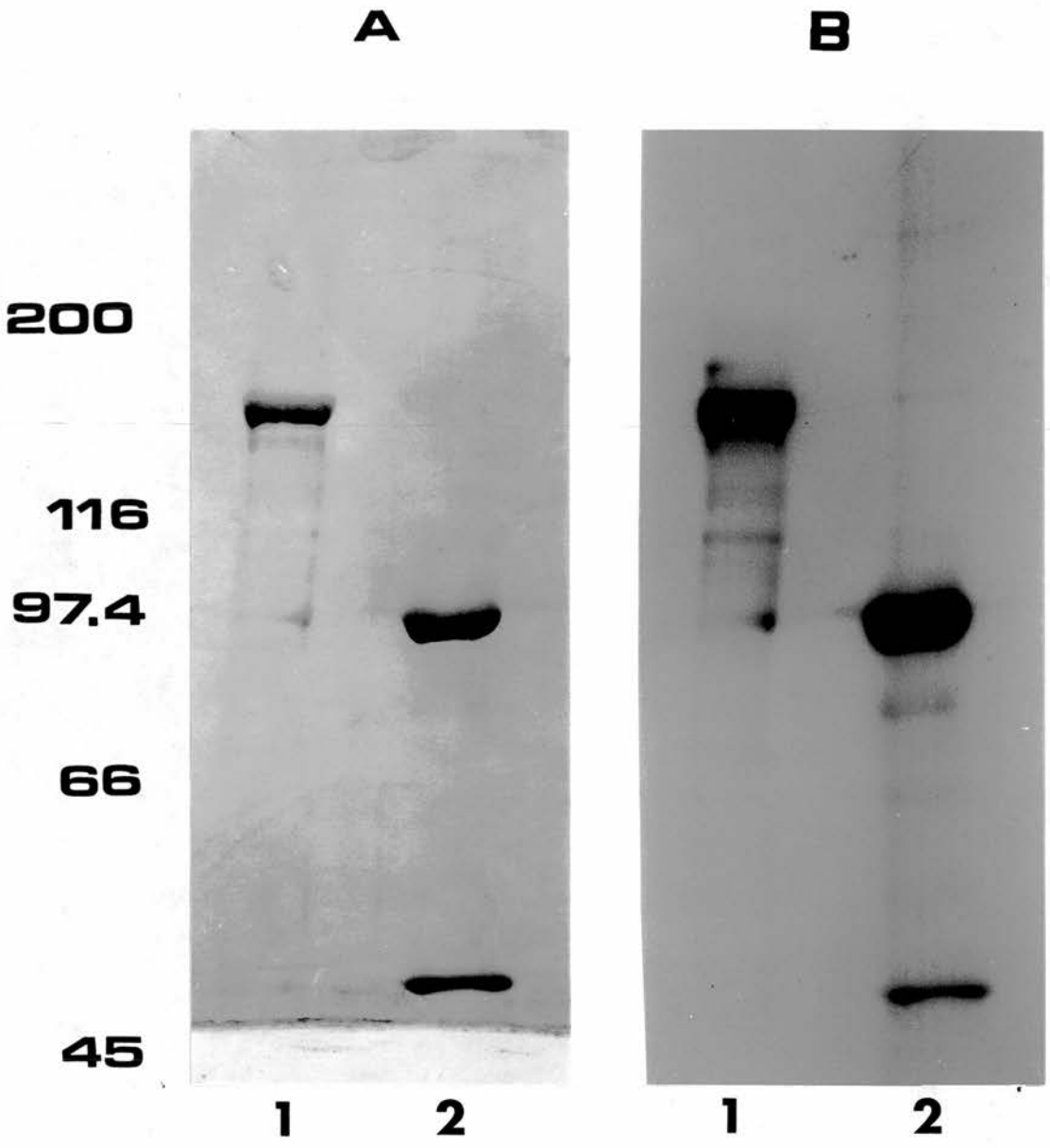


Figure 4.3 **SDS-Polyacrylamide gel (a) and corresponding autoradiograph (b) of ^{125}I-Labelled Tetanus Toxin**

Gel electrophoresis and autoradiography were carried out as outlined in Sections 2.2.19 and 2.2.20. Track 1 contains non-reduced tetanus toxin; track 2 contains reduced tetanus toxin. Molecular weight markers ran at the positions indicated.

4.3.4 Binding of ^{125}I-Labelled Tetanus Toxin to Native Chromaffin Cells

The objective of this section of work was to produce some quantitative information about the tetanus toxin-binding capacity of chromaffin cells, and to characterise this binding as much as possible. Since at temperatures higher than about 15°C some uptake or internalisation of the toxin could possibly occur (which would complicate interpretation of results), the decision was made to conduct all binding experiments (both at pH 6.0 and 7.4) at 0-4°C, at which temperature no significant sequestration of radiolabelled toxin by the cells should occur.

4.3.4.1 Time Course of Toxin Binding

To determine the optimum length of time for incubating cells with toxin, a time course experiment was conducted in which cells were incubated with a fixed concentration of radiolabelled toxin (2 nM) for various times; the amount of cell-associated toxin was then determined in each case. The experiment was carried out using native cells, cells pre-incubated with ganglioside GD1a or cells pre-incubated with ganglioside GT1 (see below). The results for the experiment carried out in pH 7.4 incubation buffer are shown in Fig. 4.4; a virtually identical result was obtained when cells were incubated at pH 6.0. Three conclusions can be drawn from this graph. Firstly, the fixation of tetanus toxin to chromaffin cells is complete after a three hour incubation period; this agrees well with the incubation times used by workers investigating tetanus toxin binding by other cell types e.g. Wellhoner & Neville (1987) working with neuroblastoma x glioma NG108-15 hybrid cells, and Walton *et al.* (1988), using PC12 phaeochromocytoma cells.

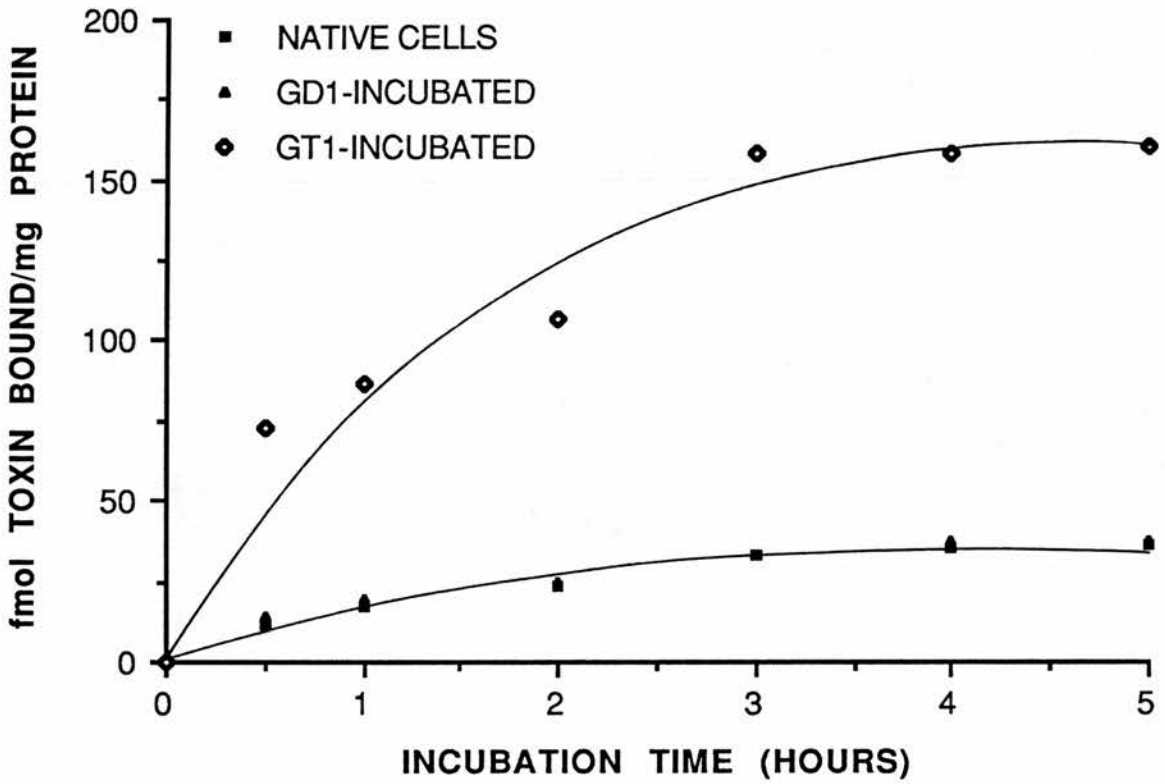


Figure 4.4 Time Course of Binding of ^{125}I-Labelled Tetanus Toxin to Chromaffin Cells

Chromaffin cells were pre-incubated for 2 hours with either DMEM alone, or medium containing gangliosides GD1a or GT1 (50 µg/ml). Cells were then incubated with tetanus toxin (2 nM) for various times in pH 7.4 binding buffer, and bound toxin measured as described in Section 2.2.10.

This result is typical of 3 independent experiments; variability between data obtained in replicate experiments was less than 10%.
Students t-test: $p < 0.01$ for effect of ganglioside GT1 in enhancing toxin-binding (at 3-5 hours incubation time).

Secondly, pre-incubation of chromaffin cells with ganglioside GT1 causes, as expected, a significant enhancement of the toxin-binding capacity of these cells, without altering the time course over which maximal binding occurs. Thirdly, this enhancement is not a non-specific effect, since pre-incubation of cells with GD1a ganglioside, which is known not to bind tetanus toxin to any great extent, has virtually no effect on the level of toxin binding.

4.3.4.2 Linearity of Toxin-Binding with Varying Cell Protein

In order to detect variations in ligand binding to cells, it is essential that experiments are carried out under conditions in which binding is linear with respect to the amount of cell protein present. Fig. 4.5 shows that, under both sets of conditions used, binding of toxin to chromaffin cells is linear up to approximately 40 μg of cell protein. Future experiments were therefore conducted using cells plated at a density of 1×10^5 cells/assay well; this corresponds to approximately 25 μg protein, which is within the range of linear toxin-binding.

4.3.4.3 Effect of Ganglioside Concentration on Toxin Binding

Since it was intended that binding of tetanus toxin (at both pH values) would be studied both in native chromaffin cells and cells treated with ganglioside GT1, the question of what concentration of ganglioside to pre-incubate cells with, prior to incubation with tetanus toxin, had to be addressed. So as to avoid using unnecessarily high concentrations of GT1, it was important to determine the minimum concentration of ganglioside that could be used which would give maximal, or near-maximal, enhancement of toxin binding. Table 4.1 shows the results of such an experiment.

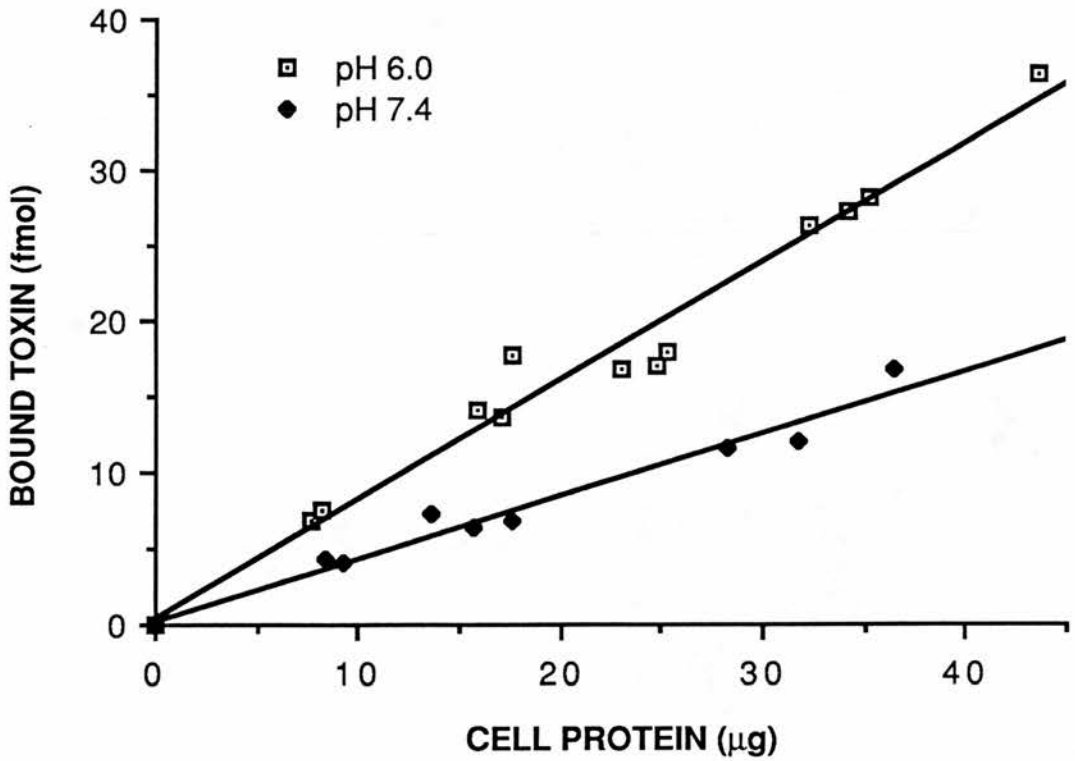


Figure 4.5 Linearity of Toxin-Binding with Varying Cell Protein

Chromaffin cells plated at various densities between 5 x 10^4 and 2 x 10^5 cells/assay well were incubated for 3 hours at 0°C with 20 nM ^{125}I-labelled tetanus toxin in either pH 6.0, low ionic strength, or pH 7.4, high ionic strength buffer; bound toxin was then measured as described in Section 2.2.10, and cell protein determined as outlined in Section 2.2.18.

Similar results were obtained in 3 independent experiments; variability between data from separate experiments was less than 10%.

Table 4.1 Effect of GT1 Concentration on Toxin-Binding by Chromaffin Cells

Cells were pre-incubated for 2 hours with different concentrations of GT1 as indicated, then toxin-binding was measured as described in Section 2.2.10. Figures represent the mean of three different wells ± standard deviation.

[GT1] (μg/ml)	TOXIN BOUND (pmol/mg protein)
0	0.027 (± 0.002)
10	0.043 (± 0.003)
25	0.062 (± 0.001)
50	0.069 (± 0.003)

This result is typical of 2 independent experiments; variability between data from separate experiments was not more than 10%.
Students t-test: $p < 0.01$ for effect of ganglioside GT1 in enhancing toxin-binding (at all concentrations tested).

It is clear that a significant increase in toxin binding occurs when the concentration of GT1 is raised from 10 μg/ml to 25 μg/ml, but that by doubling the GT1 concentration to 50 μg/ml, only a negligible increase in binding is achieved. Thus subsequent pre-incubations of chromaffin cells were carried out using a ganglioside concentration of 25 μg/ml.

4.3.4.4 Characteristics of Toxin-Binding to Chromaffin Cells

It was now possible to begin to investigate the binding of tetanus toxin to native chromaffin cells. Binding was to be studied under two different conditions; i) in physiological, high ionic strength buffer, pH 7.4, and ii) in non-physiological, low ionic strength buffer, pH 6.0. Previous binding studies with tetanus toxin and rat brain membranes (e.g. Critchley *et al.*, 1986; Pierce *et al.*, 1986) had shown that while binding could be observed under physiological conditions of pH and ionic strength, this appeared to be non-saturable, and was of a lower capacity and affinity than that seen under low-salt and -pH conditions; further, binding under the latter conditions was shown to be saturable. In this study, we wished, first, to demonstrate whether chromaffin cells are capable of binding tetanus toxin in a specific fashion and, second, to evaluate whether any binding found is similar to that observed with true neuronal tissue, such as rat synaptic membranes.

Binding curves obtained upon incubation of native chromaffin cells with various concentrations of ^{125}I-labelled tetanus toxin (in the range 0-20 nM), under both sets of conditions, are shown in Fig. 4.6 and Fig. 4.7; in each case, non-specific binding (which represented less than 1% of added radioactivity and was most likely

A

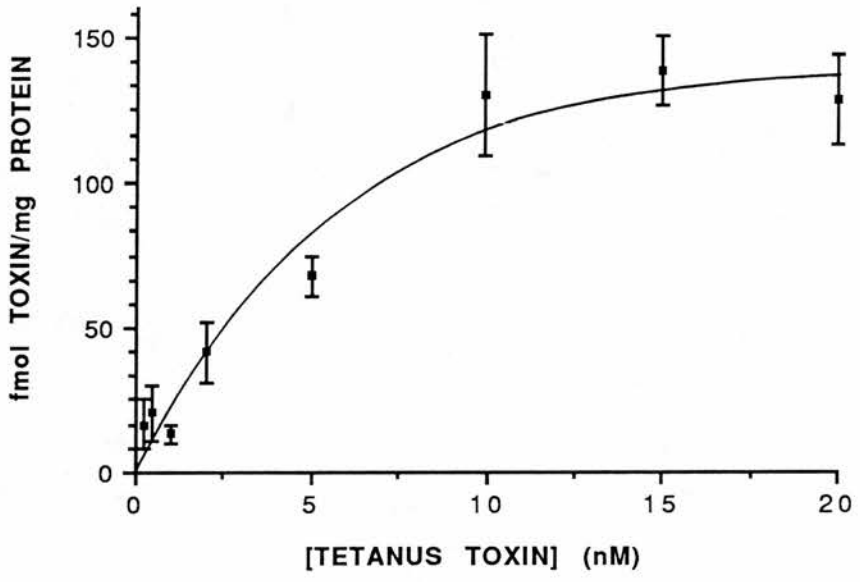
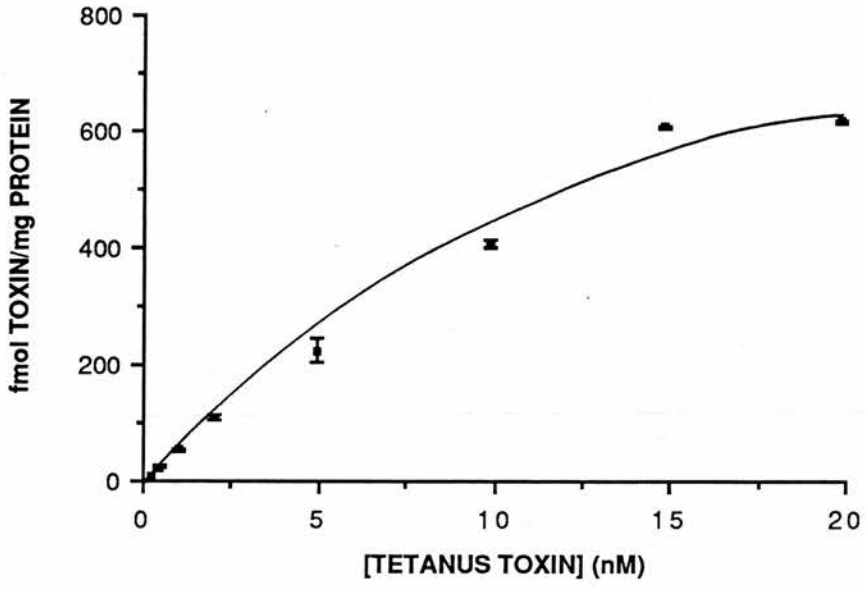


Figure 4.6 Binding of ^{125}I-Labelled Tetanus Toxin to Native Chromaffin Cells in pH 6.0, Low Ionic Strength Buffer

Chromaffin cells plated at 1 x 10^5 cells/assay well were incubated for 3 hours at 0°C with various concentrations of ^{125}I-labelled toxin (in the range 0-20 nM) in 100 µl of binding buffer. Bound toxin was then measured as described in Section 2.2.10. Each point is the mean of 3 wells ± standard deviation; separate graphs represent the results of binding experiments conducted on different preparations of cells.

B


800
600
400
200
0
fmol TOXIN/mg PROTEIN
0
5
10
15
20
[TETANUS TOXIN] (nM)

C

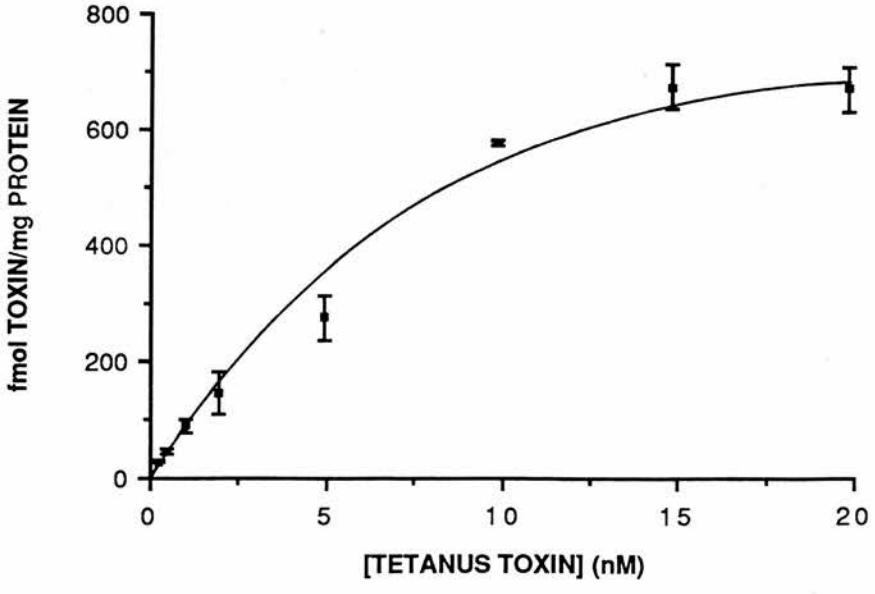
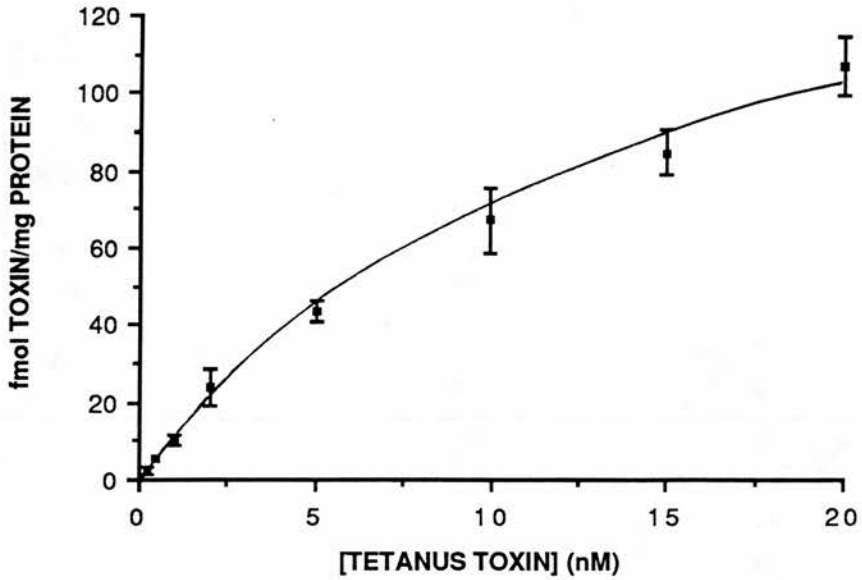

800
600
400
200
0
fmol TOXIN/mg PROTEIN
0
5
10
15
20
[TETANUS TOXIN] (nM)

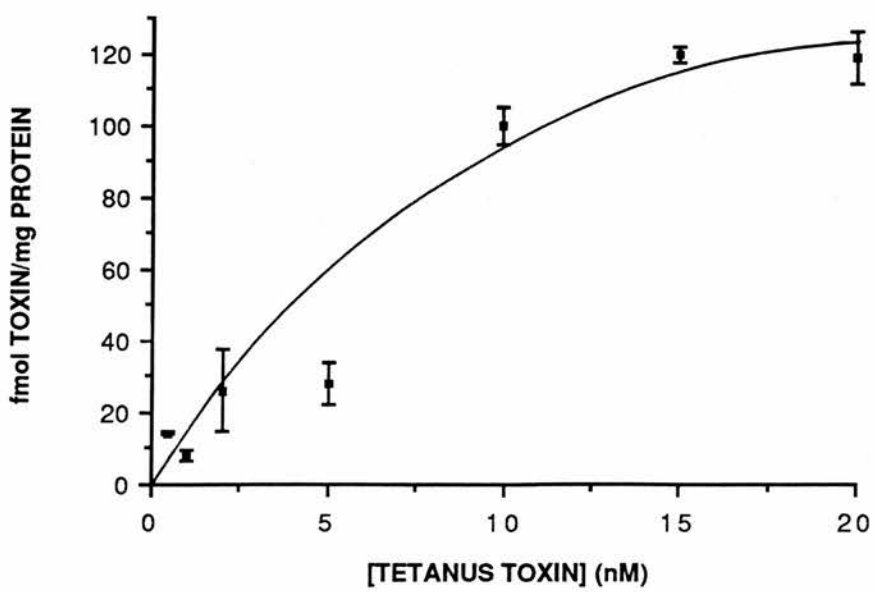
Figure 4.7 Binding of ^{125}I-Labelled Tetanus Toxin to Native Chromaffin Cells in pH 7.4, High Ionic Strength Buffer

Chromaffin cells plated at 1 x 10^5 cells/assay well were incubated for 3 hours at 0°C with various concentrations of ^{125}I-labelled toxin (in the range 0-20 nM) in 100 µl of binding buffer. Bound toxin was then measured as described in Section 2.2.10. Each point is the mean of 3 wells ± standard deviation; separate graphs represent the results of binding experiments conducted on different preparations of cells.

A


fmol TOXIN/mg PROTEIN
120
100
80
60
40
20
0
0
5
10
15
20
[TETANUS TOXIN] (nM)

B


fmol TOXIN/mg PROTEIN
120
100
80
60
40
20
0
0
5
10
15
20
[TETANUS TOXIN] (nM)

due to adsorption of the toxin to the plastic surface of the cell culture wells) has been corrected for, by incubating cells with radiolabelled toxin in the presence of a 100-fold excess of unlabelled toxin. The first point to note is that, as with synaptic membranes, binding at pH 6.0 is of a markedly higher capacity than that observed at pH 7.4; typically, under the experimental conditions used, maximal binding at pH 6.0 appeared to be approximately five times greater than that occurring at pH 7.4. Second, it would appear from looking at these graphs (prior to any thorough analysis of the data) that binding at pH 6.0 is saturating within the range of toxin concentrations used, while, perhaps unexpectedly, pH 7.4 binding also appears to be beginning to saturate, an observation not previously made in other binding studies with tetanus toxin. This is also suggested when the data are plotted on a semi-logarithmic scale (as in Fig. 4.8), where saturation is indicated by the curve flattening out to a plateau. (This suggestion of saturation, however, really only rests on data obtained using one toxin concentration, albeit from several independent experiments; ideally there should be more points on these graphs at higher toxin concentrations to show definite plateaux. However, the amount of toxin that could be used in any one experiment was limited by the availability of pure toxin, and it was impracticable to increase the scale of these experiments). It is worth pointing out that these curves do not follow the shape of a "classical" isotherm of saturable binding; they tend to be linear over a substantial part of the graph i.e. the increase in bound toxin (at lower concentrations of free toxin) is more gradual than would be expected for single-site binding.

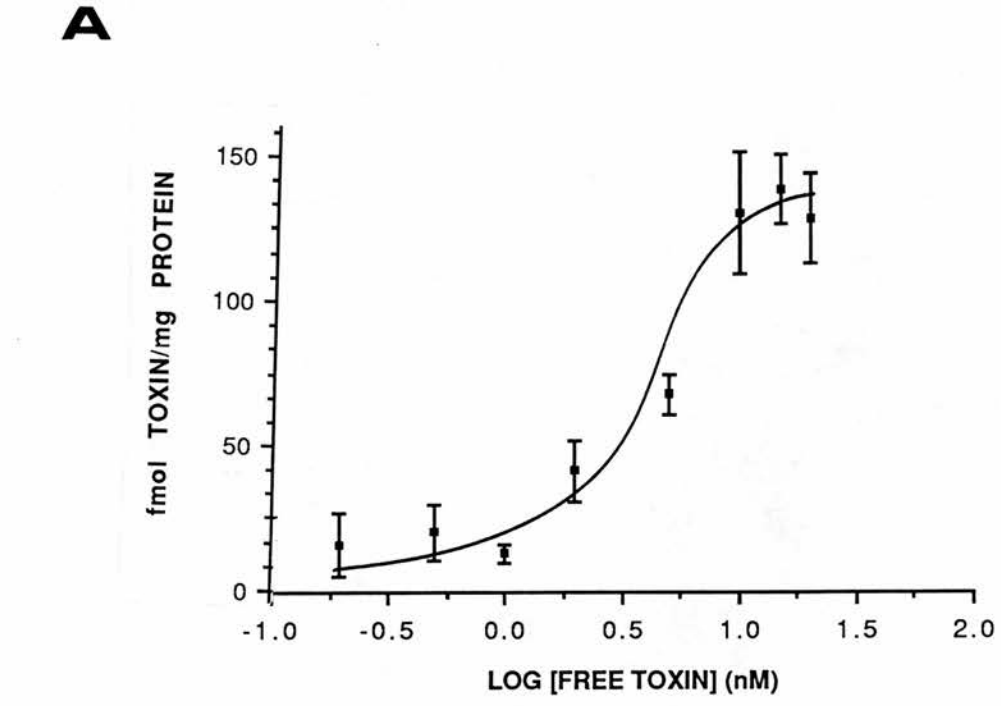
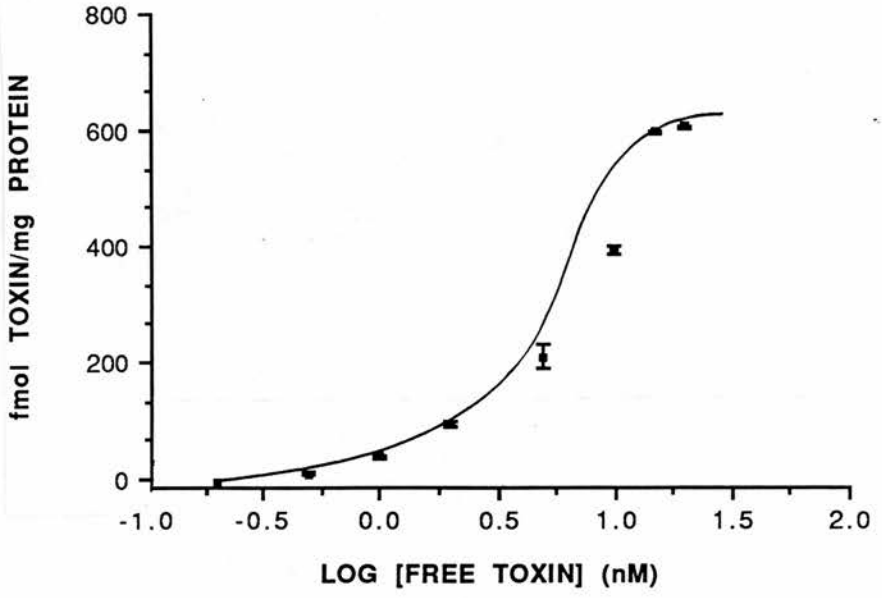


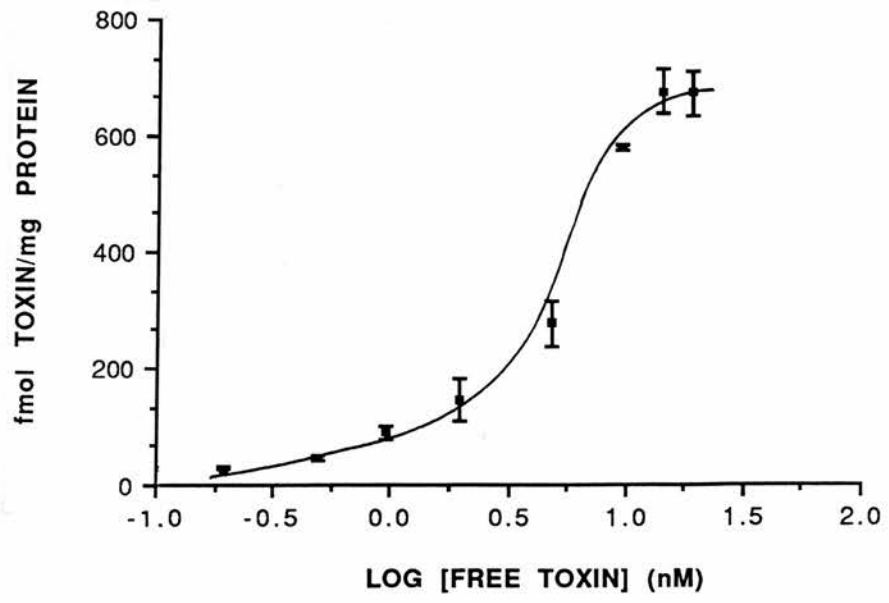
Figure 4.8 Semi-Logarithmic Representation of the Data from Figures 4.6 and 4.7

Graphs (A), (B) and (C) represent the data from binding experiments conducted in pH 6.0, low ionic strength buffer, while graphs (D) and (E) represent the data from experiments conducted in pH 7.4, high ionic strength buffer.

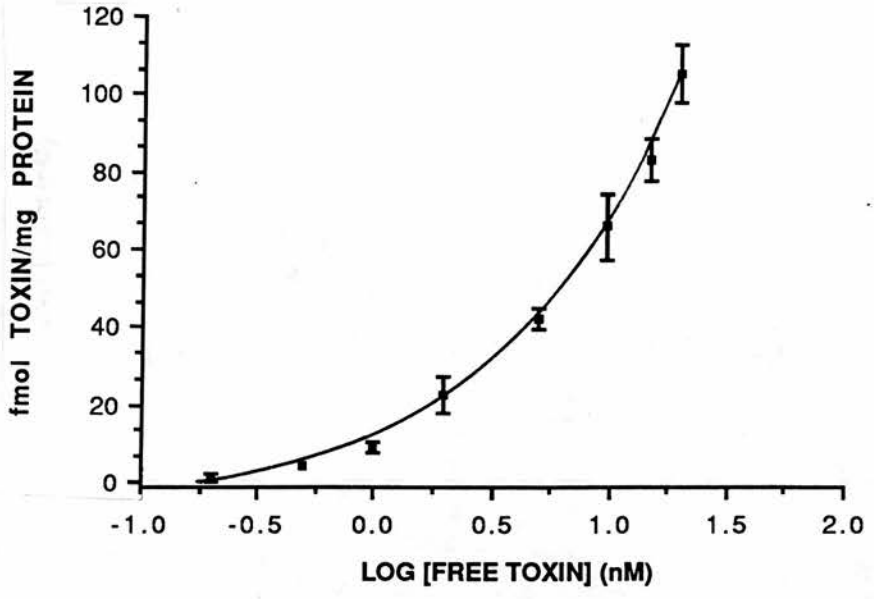
B


800
600
400
200
0
fmol TOXIN/mg PROTEIN
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
LOG [FREE TOXIN] (nM)

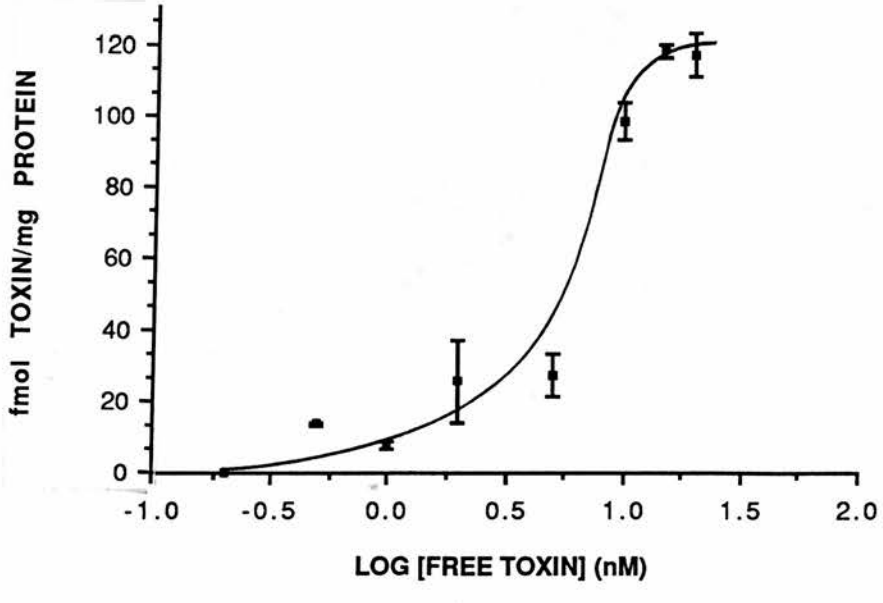
C


800
600
400
200
0
fmol TOXIN/mg PROTEIN
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
LOG [FREE TOXIN] (nM)

D


fmol TOXIN/mg PROTEIN
120
100
80
60
40
20
0
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
LOG [FREE TOXIN] (nM)

E


fmol TOXIN/mg PROTEIN
120
100
80
60
40
20
0
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
LOG [FREE TOXIN] (nM)

The semi-logarithmic plots shown in Fig. 4.8 suggest that there are at least two components to the binding of tetanus toxin to these cells. The curves are not symmetrical; in both cases (pH 6.0 and pH 7.4) there is a definite tailing of the graph at lower toxin concentrations. The simplest interpretation of this finding is that, in addition to the high capacity binding indicated by these graphs, there is also a higher affinity, low capacity component.

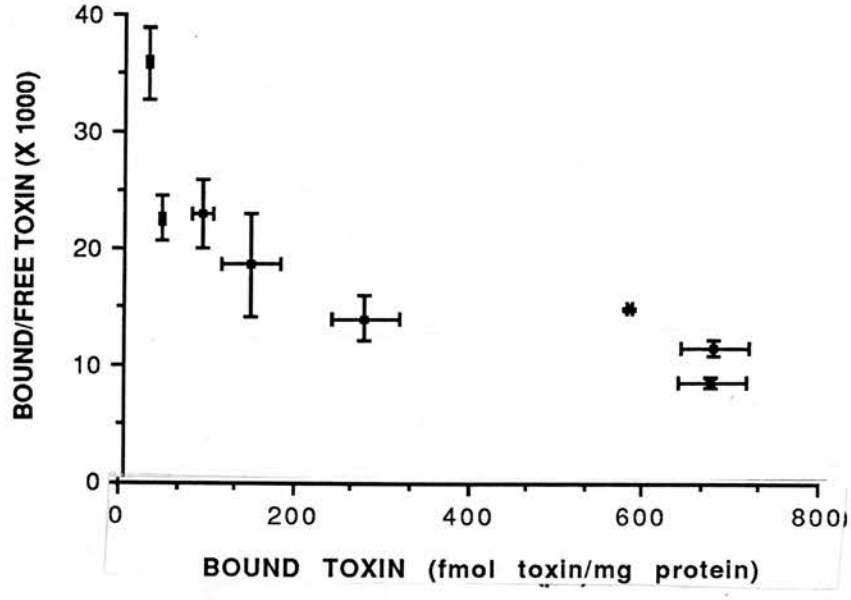
After having presented the binding data in this most direct form, the usual procedure would be to transform the data and carry out Scatchard analysis in order to obtain values for K_d (a measure of the binding affinity) and B_{max} (the total number of binding sites). In Scatchard analysis, the data obtained from a binding isotherm are used to calculate the bound and free ligand concentrations at each experimental point, and the ratio of bound to free ligand is plotted against the bound ligand. A straight line is taken as evidence of a single class of binding sites, with a dissociation constant defined by the slope of the line and B_{max} determined by the x-intercept.

However, when the data from these isotherms were transformed for Scatchard analysis, it was immediately apparent that it would not be at all easy, or indeed possible, to obtain any accurate quantitative information using this approach. When Scatchard data are plotted, there is always a strong, sometimes irresistible temptation to fit them to a straight line, either by eye or by least squares methods; however, the plots obtained are clearly non-linear (see Fig. 4.9), with several possible interpretations. It could be that there are two or more classes of binding site, with

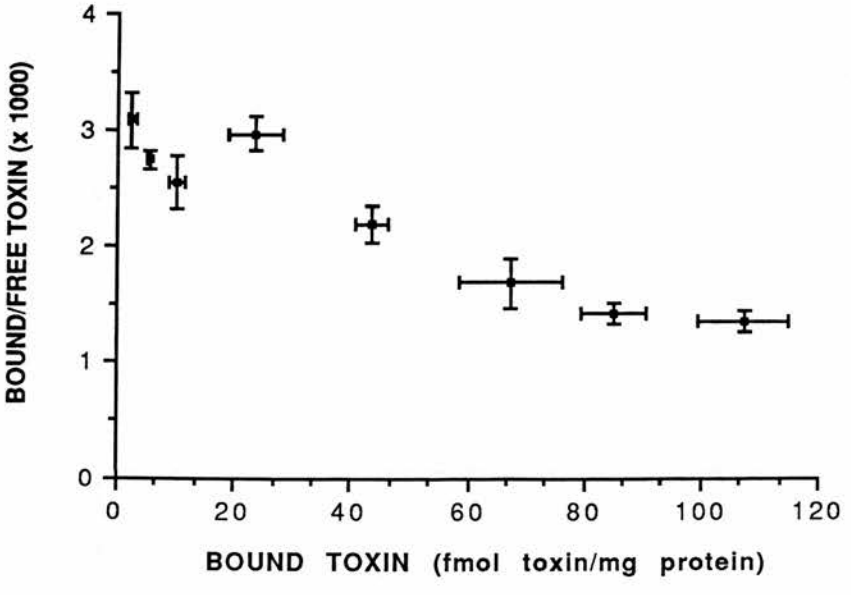
Figure 4.9 Scatchard transformations of the Data presented in Figures 4.6 (c) and 4.7 (a)

Graph (A) is a Scatchard transformation of data obtained from a binding experiment conducted in pH 6.0, low ionic strength buffer; graph (B) represents the same transformation of data obtained from an experiment conducted in pH 7.4, high ionic strength buffer.

A


BOUND/FREE TOXIN (X 1000)
40
30
20
10
0
0
200
400
600
800
BOUND TOXIN (fmol toxin/mg protein)

B


BOUND/FREE TOXIN (x 1000)
4
3
2
1
0
0
20
40
60
80
100
120
BOUND TOXIN (fmol toxin/mg protein)

different but fixed affinities that do not change with extent of occupancy by the toxin (as suggested above), or alternatively there could be a single class of identical binding sites whose affinity decreases with increasing occupancy by the toxin (negative co-operativity); there is no way of telling from binding data alone which of these factors is responsible for the observed curvature in these plots. It was felt that an alternative means of analysing the data should be used, since even when the curvature of a Scatchard plot is not overlooked, the graph can easily entice one into drawing incorrect conclusions. A common over-simplification is to draw two straight lines through the data, and to ascribe one of the lines, that of higher slope, to "high affinity" sites, and the other to low affinity sites. Even if one knows for certain that there are two classes of binding sites, the slopes and intercepts of such lines are complex functions, and are generally unsuited for evaluation of binding constants.

In order to gain some quantitative information from the binding data obtained, we decided to assume that there are two major components to the binding of tetanus toxin to chromaffin cells, the simplest interpretation of the previous plots. The "high affinity, low capacity" component is small, and thus difficult to quantitate, but from careful inspection of the semi-logarithmic plots (Fig. 4.8), it seems that at both pH values it has an apparent Kd value of approximately 1 nM, and that it does not account for more than 20% of the toxin-binding capacity of the cells.* We could, however, be somewhat more quantitative with respect to the lower affinity binding component. Data were analysed using a non-linear regression method, as carried out by Dr. G. Atkins, Department of

Biochemistry, University of Edinburgh; a regression of the concentration of bound toxin versus the concentration of free ligand was performed. The model used was

$$b = Nf/(K_d + f)$$

where b = concentration of bound ligand, N = concentration of binding sites, K_d = the dissociation constant of binding sites for the ligand, and f = concentration of free ligand. All data were weighted equally.

The values calculated for apparent Kd and Bmax for each set of data (for binding of tetanus toxin to native chromaffin cells) are shown in Table 4.2. The data appeared to fit satisfactorily to this model; residuals of positive and negative value were evenly distributed, and in all cases the number of iterations required to reach a minimum value for the sum of squares of residuals was acceptable.

It is clear that there is a significant degree of variation between results obtained when the same binding experiment is carried out using different cell preparations; this is perhaps not very surprising considering the biological nature of the system we are using. But, in addition to this variation, there were preparations of cells for which no binding of tetanus toxin was observed. There is no obvious explanation for this, but it seems more likely to be a feature of the cells than of the toxin, as the same preparation of radiolabelled toxin was used for several experiments, some of which did result in detectable binding, while others did not. Just what this feature of the cells is, however, remains unknown.

Table 4.2 Values of Apparent Kd and Bmax Calculated for High Capacity Binding of ^{125}I-Labelled Tetanus Toxin to Native Chromaffin Cells

	Apparent Kd (nM)	B_{max} (pmol/mg protein)	B_{max} (molecules/cell)
Binding in pH 6.0 low ionic strength buffer			
(a)	7.1 ± 2.7	0.19 ± 0.03	29,000 ± 4,000
(b)	24.4 ± 8.2	0.73 ± 0.15	109,000 ± 23,000
(c)	12.4 ± 3.9	1.17 ± 0.18	175,000 ± 27,000
Binding in pH 7.4 high ionic strength buffer			
(d)	18.6 ± 3.6	0.20 ± 0.02	30,000 ± 3,000
(e)	22.2 ± 17.3	0.27 ± 0.12	41,000 ± 19,000

Letters (a) to (e) refer to experiments with various preparations of cells. Experiments (b) and (d) were conducted on the same cell preparation, as were experiments (c) and (e). Experiment (a) has no counterpart at pH 7.4; no binding was observed at this pH with this batch of cells.

Since this problem of inconsistency was also encountered in the inhibition of secretion studies reported in Chapter 3, the obvious question to ask is whether or not it is possible to correlate the lack of binding of tetanus toxin to a particular cell preparation to the inability of the toxin to inhibit exocytosis from those cells. Unfortunately, however, these two separate aspects of the project were not carried out concurrently, so we do not have the relevant data to address this question.

Because of the inconsistency of the cell preparations and the not insignificant errors associated with the calculated $\hat{K}_d^{apparent}$ and B_{max} values, it is not possible to draw firm conclusions from the data. However, one or two points seem clear. Firstly, it would appear that in fact tetanus toxin binds with similar affinity to chromaffin cells at pH 6.0 and at pH 7.4; this is in contrast to binding to neurones, which displays markedly higher affinity under the less physiological condition of pH 6.0. (There is perhaps a slight suggestion of lower $\hat{K}_d^{apparent}$ values at pH 6.0, at least for the lower affinity binding component, but the variation in the data prevents us from putting much weight on this). The $\hat{K}_d^{apparent}$ values reported here, especially for the higher affinity binding component, are nonetheless comparable to those found with neuronal tissue. Most other published K_d values for tetanus toxin binding are in the range 0.2-4.0 nM e.g. Rogers & Snyder (1981), Goldberg *et al.* (1981), Staub *et al.* (1986), Critchley *et al.* (1986), Parton *et al.* (1989). One conclusion from the higher $\hat{K}_d^{apparent}$ values found with the lower affinity toxin binding to chromaffin cells is that the binding experiments should ideally have been conducted over a wider range of toxin concentrations, so that experimental points could

have been collected for free toxin concentrations significantly greater than the apparent Kd value; however, as discussed earlier, this was not possible in practice.

The second point to note from Table 4.2 is that the toxin-binding capacity of chromaffin cells seems consistently to be greater at pH 6.0 than at pH 7.4; this, too, is in agreement with previous toxin-binding studies. It is difficult to compare the results reported here with those published for neuronal material, since in most cases, toxin-binding capacity is given in the literature in terms of weight of membrane protein; obviously one cannot make direct comparisons between the relative toxin-binding capacities of membrane preparations and whole cells. Fujita *et al.* (1990) have reported a B_{max} value of 70 pmol toxin/mg protein for binding to PC12 cells, a value substantially higher than the figures reported here; however, they do not quote a K_d value for this binding, which may be of relatively low affinity and therefore somewhat non-specific. When the B_{max} values obtained for chromaffin cells are expressed as toxin molecules bound per cell, however, the numbers obtained are not at all unreasonable for specific, high capacity binding.

Thus it would seem that specific binding of tetanus toxin to adrenal chromaffin cells can and does occur, given the appropriate conditions. This is similar to the binding of toxin to cells of a more direct neuronal origin, with a substantial capacity and a similar dependence on ionic strength and pH. However, the nature

of the cell surface acceptor that mediates the binding of the toxin is not clear, nor whether the same acceptor is involved under both sets of conditions investigated.

Binding of ^{125}I-labelled tetanus toxin to chromaffin cells pre-incubated with ganglioside GT1 was investigated at the same time as the experiments with native cells, to see whether incorporation of exogenous ganglioside GT1 into the chromaffin cell membrane did in fact cause an enhancement of toxin-binding capacity, or result in a different binding affinity. Typical results are shown in Fig. 4.10; the data were analysed by non-linear regression as described above. The toxin-binding capacity of the cells was increased to 3.7 $\pm$ 0.7 pmol/mg protein at pH 6.0 and 2.4 $\pm$ 0.2 pmol/mg protein at pH 7.4. These values correspond to the incorporation of an additional 450,000 toxin-binding sites per cell at pH 6.0, and an extra 300,000 sites at pH 7.4, numbers easily achievable with the concentration of GT1 used in the pre-incubations. Perhaps more interesting is the observation that the $\hat{K}_d^{apparent}$ values calculated for GT1-treated cells (16.7 $\pm$ 5.9 nM at pH 6.0, 22.5 $\pm$ 2.7 nM at pH 7.4) are much the same as those values obtained for toxin binding to native chromaffin cells. On the basis of this observation, it is tempting to suggest that perhaps gangliosides are the receptors involved in mediating the binding of tetanus toxin to native chromaffin cells. However, this is mere speculation, and obviously further experiments are needed.

4.3.5 Effect of Enzymic Treatments on Toxin-Binding

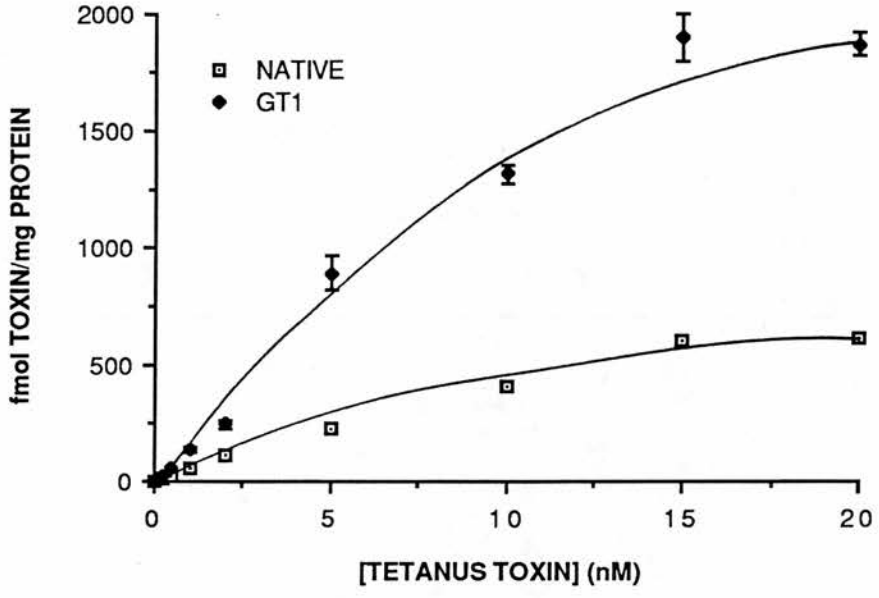
The approach taken to investigate the nature of the toxin-binding moiety on the surface of the adrenal chromaffin cell was to

Figure 4.10 Effect of Pre-Incubation of Chromaffin Cells with Ganglioside GT1 on Subsequent Toxin-Binding Capacity

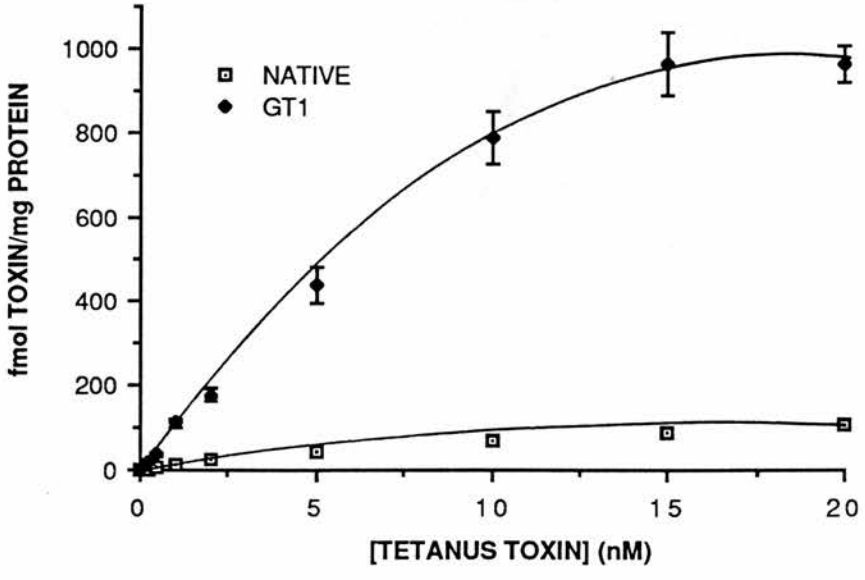
Chromaffin cells were pre-incubated with either DMEM alone, or DMEM containing GT1 (25 μg/ml), for 2 hours at 37°C, then toxin-binding was measured as described in Section 2.2.10. Graph (A) shows the result of the experiment when conducted in pH 6.0, low ionic strength buffer; graph (B) shows the result of the experiment conducted in pH 7.4, high ionic strength buffer.

The results shown in each case are typical of 3 separate experiments; variability between data obtained in different experiments was not more than 10%. Students t-test: $p < 0.01$ for effect of GT1 on toxin-binding capacity under both buffer conditions (at 15-20 nM tetanus toxin).

A


2000
1500
1000
500
0
NATIVE
GT1
fmol TOXIN/mg PROTEIN
0
5
10
15
20
[TETANUS TOXIN] (nM)

B


1000
800
600
400
200
0
NATIVE
GT1
fmol TOXIN/mg PROTEIN
0
5
10
15
20
[TETANUS TOXIN] (nM)

determine the effect of various enzymic treatments on toxin-binding. This method has been used previously by other workers (see Section 1.8.2), and has helped to shape current opinion on the nature of tetanus toxin receptors in neuronal tissue. Briefly, the consensus seems to be that, at least with brain and spinal cord tissue, gangliosides play a relatively minor role in toxin fixation under physiological conditions (as indicated by experiments using neuraminidase), while experiments with trypsin and other proteolytic treatments suggest the involvement of a protein (perhaps a sialoglycoprotein) in physiological toxin-binding. Toxin-binding under less physiological conditions of pH and ionic strength is very sensitive to neuraminidase, so therefore gangliosides would appear to bind tetanus toxin with high affinity under these conditions.

The effect of neuraminidase treatment on toxin-binding by chromaffin cells was investigated by pre-incubating cells with 0.1 IU/ml of the enzyme in pH 6.0 rinse buffer (see Section 2.2.10) for 1 hour at 37°C, and then comparing the binding of ^{125}I-labelled tetanus toxin to these cells (under both sets of conditions) with that to cells pre-incubated with buffer alone. The reason for using this buffer is that neuraminidase has a pH optimum of approximately 5.5, and so a relatively low pH buffer was needed to ensure adequate enzyme action on any chromaffin cell membrane gangliosides. It should be noted that protease inhibitors were also present in the pre-incubation buffer, since some preparations of neuraminidase are known to contain small quantities of such enzymes.

The results of these experiments are shown in Fig. 4.11. It is immediately apparent that the neuraminidase treatment has a much more marked effect at pH 6.0, as is the case with neuronal tissue; this is consistent with the notion that gangliosides are the major toxin-binding components in these cells under these conditions of pH and ionic strength. Compared with native chromaffin cells, the tetanus toxin-binding capacity of neuraminidase-treated cells is reduced by approximately 90% (B_{max} 1.17 $\pm$ 0.18 pmol/mg protein for native cells, B_{max} 0.128 $\pm$ 0.03 pmol/mg protein for treated cells). Neuraminidase also had a significant effect on toxin binding under physiological conditions of salt and pH, although the reduction in this case was less pronounced, around 50% (B_{max} 0.27 $\pm$ 0.12 pmol/mg protein for native cells, B_{max} 0.136 $\pm$ 0.09 pmol/mg protein for treated cells). On the basis of these results, then, it would appear that binding of tetanus toxin to chromaffin cells in both cases is mediated at least in part by gangliosides, or perhaps some other sialic acid-containing molecule.

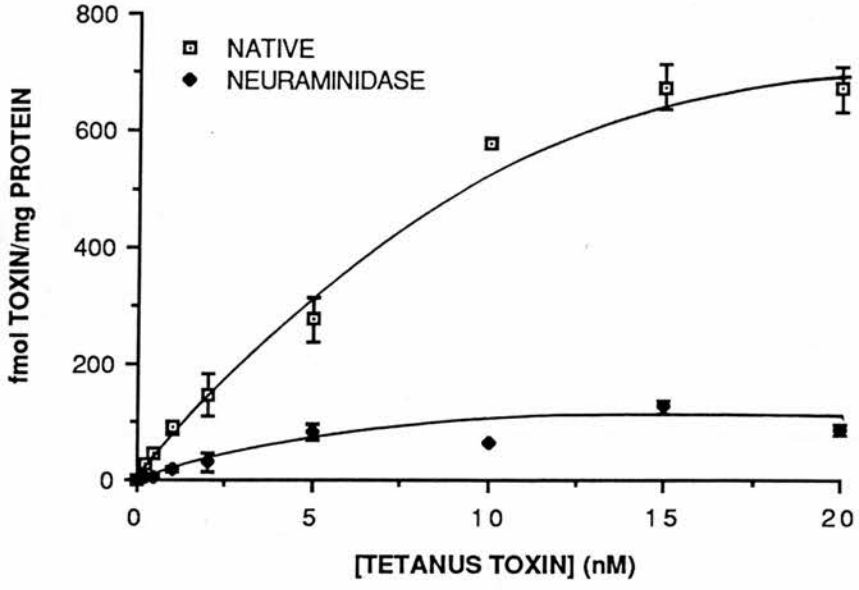
Since there have been reports suggesting that tetanus toxin-binding to neuronal tissue may be mediated by some kind of protein component *in vivo* (e.g. Critchley *et al.*, 1986; Pierce *et al.*, 1986; Lazarovici & Yavin, 1986), the effect of trypsinisation of the cells on their subsequent toxin-binding ability was investigated. (Limited proteolysis is a widely used approach to study surface macromolecule interactions with ligands and the subsequent biological signals that follow). The cells were subjected to mild proteolysis, using 25 µg/ml trypsin (from bovine pancreas) in pH 7.4 rinse buffer (see Section 2.2.10) for 10 minutes at 37°C; the enzyme activity was then inhibited by dilution

Figure 4.11 Effect of Neuraminidase Treatment of Chromaffin Cells on Subsequent Toxin-Binding Capacity

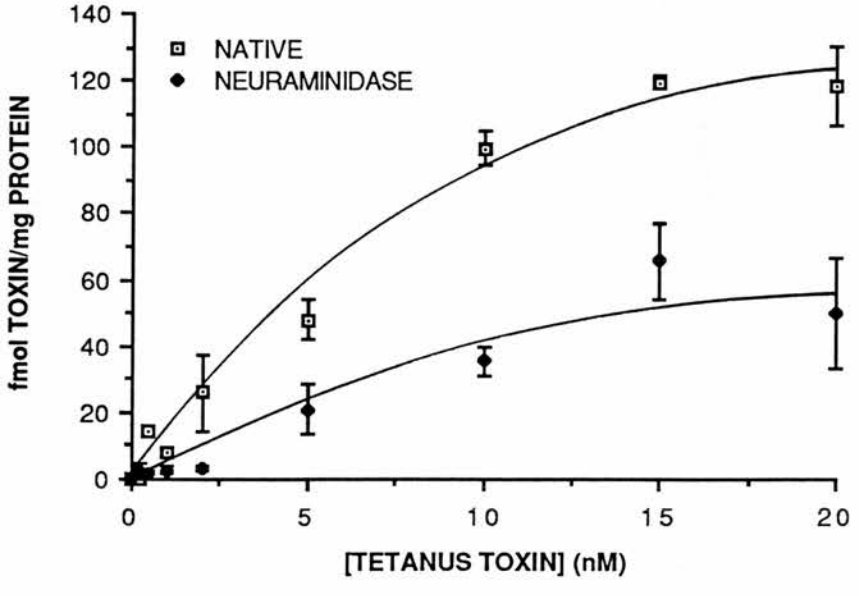
Chromaffin cells were pre-incubated with either pH 6.0 rinse buffer alone, or buffer containing neuraminidase (0.1 IU/ml), for 1 hour at 37°C, then toxin-binding was measured as described in Section 2.2.10. Graph (A) shows the result of the experiment when toxin-binding was conducted in pH 6.0, low ionic strength buffer; graph (B) shows the result of the experiment conducted in pH 7.4, high ionic strength buffer.

The results shown in each case are typical of 3 separate experiments; variability between data obtained in different experiments was not more than 10%.
Students t-test: $p < 0.01$ for effect of neuraminidase on toxin-binding capacity under both buffer conditions (at 15-20 nM tetanus toxin).

A


800
600
400
200
0
NATIVE
NEURAMINIDASE
fmol TOXIN/mg PROTEIN
0
5
10
15
20
[TETANUS TOXIN] (nM)

B


140
120
100
80
60
40
20
0
NATIVE
NEURAMINIDASE
fmol TOXIN/mg PROTEIN
0
5
10
15
20
[TETANUS TOXIN] (nM)

with ice-cold buffer containing excess soybean trypsin inhibitor (0.5 mg/ml). The binding of tetanus toxin to these cells was then compared to that to untreated control cells. (It is important to note here that the cells did not appear to be affected morphologically by the trypsinisation; they still remained attached to the tissue culture plates, and retained their integrity as judged by their ability to exclude trypan blue).

Using this method, Yavin & Nathan (1986) have shown that after treatment of cerebral neurons with trypsin, a reduction in tetanus toxin binding of approximately 40% is observed. Similarly, Fujita *et al.* (1990) have demonstrated that trypsinisation of guinea pig brain synaptosomes leads to a 90% reduction in toxin-binding under physiological conditions. In our experiments, however, trypsinisation of chromaffin cells led to a complete abolition of toxin-binding under both sets of conditions, even when lower concentrations of trypsin were used. It is not easy to reconcile this observation with those made in other studies. The fact that trypsin is totally effective at abolishing toxin binding at pH 7.4, while neuraminidase is less so, is similar to previous findings, and perhaps indicates the importance of a membrane protein component under these conditions, with gangliosides taking a lesser role, or maybe a sialoglycoprotein that is more sensitive to proteolysis than to desialylation. The results at pH 6.0, however, are less straightforward; trypsin has not previously been shown to be effective at reducing toxin-binding under these less physiological conditions. It is perhaps somewhat unwise to read too much into the results of the trypsinisation experiments; it is not impossible that the trypsin is somehow exerting some kind of

indirect inhibitory effect on toxin binding. However, assuming that the results obtained are valid, how can we interpret them? Since binding at pH 6.0 appears to be totally abolished by both neuraminidase and trypsin, this suggests the crucial involvement of both gangliosides and a membrane protein, or of a sialoglycoprotein. Perhaps, in line with the suggestion of Montecucco (1986), tetanus toxin binds first, rather loosely, to a ganglioside and this complex subsequently forms a more stable association with a protein; in the absence of gangliosides (i.e. after neuraminidase treatment), little or no toxin fixation can occur, while in the absence of the appropriate protein, the toxin dissociates from the ganglioside and is observed experimentally as an abolition of toxin binding. Under more physiological conditions, however, the ganglioside assumes a lesser role, with the toxin perhaps able to form an association with the protein independently.

It is not possible to conclude from these experiments the exact nature of the interaction between adrenal chromaffin cells and tetanus toxin. The results obtained suggest the involvement of neuraminidase-sensitive, and perhaps also protease-sensitive, components in toxin-binding, but beyond this it is difficult to be specific. For this purpose experiments of a more biochemical nature, as will be described later in this thesis, are necessary.

4.3.6 Binding of ^{125}I-labelled Tetanus Toxin to Chromaffin Granules

Since we were able to demonstrate the specific binding of tetanus toxin to adrenal chromaffin cells, and since an inhibition by the toxin of exocytosis from intact chromaffin cells was also observed (as reported in Chapter 3), it seemed logical to assume that the

toxin must be acting on an intracellular target of some kind. Obviously there are many possible ways in which the toxin could be interfering with the secretory mechanism, a thorough investigation of which would be a major undertaking beyond the scope of this thesis. However, it was considered that it might be worthwhile, since we were conducting toxin binding experiments anyway, to determine whether or not any binding of the toxin to isolated chromaffin granules could be detected; one possibility is that the toxin could perhaps be exerting its effect by interacting with these secretory vesicles in some way such that their movement or their fusion with the plasma membrane is prevented. Previously, Lazarovici *et al.* (1989) have reported that tetanus toxin binds to intact chromaffin granules and isolated granule membranes in a neuraminidase-sensitive fashion; they assayed this biochemically and also claimed to visualise binding to the cytoplasmic face of the chromaffin granule membrane using electron microscopic techniques (interestingly though, they were unable to demonstrate any toxin-binding to intact chromaffin cells, either in suspension or as monolayers; however, as mentioned earlier, few experimental details are provided, so it is difficult to assess the validity of this report). On carrying out this same experiment twice, we were unable to detect any binding to chromaffin granules, even using concentrations of ^{125}I-labelled tetanus toxin as high as 20 nM. This is perhaps a more acceptable observation than that of Lazarovici *et al.*; they admit themselves that theirs is an unexpected result, since gangliosides are generally believed to reside solely on the inner face of the granule membrane (Westhead & Winkler, 1982). This seems logical enough, considering that on exocytosis it is the inner surface of the granule membrane that

becomes exposed to the cell exterior. Thus it would appear that the intracellular action of tetanus toxin, at least in adrenal chromaffin cells, does not involve an association of the toxin with the secretory vesicles but with some other as yet undetermined component of the exocytotic machinery.

4.3.7 Internalisation of Tetanus Toxin by Chromaffin Cells

Specific binding of tetanus toxin to chromaffin cells, if it is to lead to an intracellular poisoning event as documented in Chapter 3, is presumably followed by some kind of internalisation step, and so it was of interest to see if it was possible to demonstrate this. The rationale behind the method used is that toxin bound to cells at 0°C should remain at the cell surface and therefore be susceptible to proteolysis, while if cells are incubated with toxin at 37°C, a significant proportion of the toxin should be internalised and hence become protease-resistant.

Before conducting the experiment, it was necessary to establish conditions under which tetanus toxin was completely degraded by protease. Staub and his coworkers found that treatment of ^{125}I-labelled toxin with 5 μg/ml pronase at 37°C for 5 minutes could completely degrade free toxin, while 20 μg/ml pronase was required for toxin bound to microsomes. Similarly, it was found that in our hands 5 μg/ml of pronase was sufficient for degradation of free toxin (as analysed by gel electrophoresis of treated toxin samples and subsequent autoradiography), and so it was assumed that 20 μg/ml would be adequate for chromaffin cell-associated toxin. However, when two internalisation experiments were carried out, there was no difference observed between the proportions of pronase-

resistant radiolabel present at 0°C or 37°C; in both cases, approximately 75-80% of cell-associated toxin was removed. This unexpected result did not appear to be due to any morphological effect on the cells by the pronase; the cells remained attached to the tissue culture dishes prior to collection with sodium hydroxide, and it was also established that cells treated with pronase were still able to exclude trypan blue.

It may be that chromaffin cells, although capable of specifically binding tetanus toxin, do not possess a specific means of internalising it; a very small amount may reach the interior of the cell in some non-specific fashion, such that this event is undetectable using this experimental approach. After all, the tremendous potency of tetanus toxin means that very little of it is required to bring about cellular intoxication, and the specific activity of the radio-labelled toxin used may not have been sufficiently high for such a low level of internalisation to be detected. In any case, it was decided that this assay was perhaps not the best method for visualising toxin internalisation and that an immunocytochemical approach might be more successful (see Chapter 5).

4.4 SUMMARY AND CONCLUDING REMARKS

The work documented in this chapter set out to demonstrate the specific binding of ^{125}I-labelled tetanus toxin to cultured adrenal chromaffin cells, a phenomenon not previously observed. This binding was also to be characterised as much as possible, and its similarity to toxin-binding to neuronal tissue assessed.

Using cells grown as monolayers and radiolabelled tetanus toxin, we were able to demonstrate a specific binding event. This binding, like that observed with neuronal tissue, was found to be dependent on ionic strength and pH; binding measured in physiological buffer was of a lower capacity that than seen under conditions of lower pH and ionic strength, although in both cases the affinity of binding appeared to be similar. The calculated $K_d^{apparent}$ values for toxin binding to chromaffin cells were reasonably comparable to corresponding values obtained with cerebral neurons or rat brain membranes, and taken together with the calculated B_{max} values would seem to indicate that chromaffin cells express high affinity binding sites for tetanus toxin, in significant number, on their cell surfaces. It was not possible, however, to define the nature of these toxin acceptors accurately; experiments with neuraminidase- and trypsin-treated cells suggested that both sialic acid-containing and protein-containing membrane components are important for toxin-binding, but whether this is indicative of a sialoglycoprotein receptor, or of the involvement of both a ganglioside and a protein, is not clear.

CHAPTER FIVE.

IMMUNOCYTOCHEMICAL VISUALISATION OF TETANUS TOXIN BINDING TO ADRENAL CHROMAFFIN CELLS

5.1 INTRODUCTION

The results presented in Chapter 4 served to demonstrate that tetanus toxin is capable of binding to chromaffin cells in a specific fashion, and that the toxin-binding capacity of these cells may be increased or reduced by various treatments. The aim of the work detailed in the current chapter was to visualise toxin-binding using immunocytochemistry, and to investigate whether similar observations could be made using this approach; in other words a qualitative back-up to the ^{125}I-labelled toxin-binding experiments. Further, this technique has the potential to show the distribution of toxin acceptors on the cell surface.

Immunocytochemistry is a powerful technique for localising specific biochemical components on the surface of, or within, particular cells. Early procedures involved the use of antibodies (directed against cell components) which were conjugated to fluorescent molecules, but more recently antibodies conjugated to specific enzymes have become common. These enzymes, such as horse-radish peroxidase or alkaline phosphatase, convert their substrates into densely-coloured reaction products; this facilitates the localisation of the antibody-enzyme conjugate using bright-field microscopy.

Other workers have previously used immunocytochemical techniques to demonstrate the binding of tetanus toxin to various types of cells. For instance, Zimmerman & Piffaretti (1977) have shown toxin-binding to cultured mouse neuroblastoma cells using indirect immunofluorescence (human antitoxin followed by fluorescently

labelled anti-human immunoglobulin), while Mirsky *et al.* (1978) used the same approach to demonstrate that tetanus toxin-binding is a general property of all neurones, and can therefore be used to distinguish them from non-neuronal cells. Similarly, Raju & Dahl (1982) have stained cultured neurones from rat embryo with tetanus toxin, while Eisenbarth *et al.* (1982) used immunofluorescence to show that tetanus toxin binds specifically to the plasma membrane of pancreatic islet cells.

5.2 METHODS

5.2.1 Dopamine-β-Hydroxylase Staining of Chromaffin Cells

Chromaffin cells cultured on coverslips were washed twice with PBS, fixed in 3.7% formaldehyde in PBS for 10 minutes, then rinsed thoroughly with TBS to wash out excess fixative. Coverslips were then incubated with 1% Triton X-100 in TBS for 5 minutes (to solubilise cell membranes), washed in TBS and incubated for 3 hours at 37°C with a mouse monoclonal antibody against dopamine-β-hydroxylase (provided by Bulent Tugal, Department of Biochemistry, University of Edinburgh). This was followed by further washing in TBS, then incubation with alkaline phosphatase-labelled sheep anti-mouse IgG (1/400 dilution in TBS/5% FCS) for 3 hours at 37°C. Cells were then incubated with chromogen, and examined as described in Section 2.2.17.1.

5.2.2 Tetanus Toxin-Binding to Chromaffin Cells

Binding of tetanus toxin to cells cultured on coverslips was visualised by immunocytochemistry as outlined in Section 2.2.17. Toxin-binding to native chromaffin cells was investigated under

both buffer conditions used in Chapter 4; the effects of pre-treatment of cells with ganglioside GT1 (25 μg/ml in DMEM for 2 hours at 37°C), neuraminidase (0.1 IU/ml in pH 6.0 rinse buffer [see Section 2.2.10] for 1 hour at 37°C), or trypsin (25 μg/ml in pH 7.4 rinse buffer [see Section 2.2.10] for 10 minutes at 37°C), prior to incubation with toxin, were also examined.

To confirm that treatment of chromaffin cells with neuraminidase was in fact removing sialic acid residues from gangliosides (and other glycolipids), the ability of peanut agglutinin to bind to these cells before and after exposure to the enzyme was assessed (neuraminidase treatment should expose galactose residues previously masked by sialic acid residues, and therefore enable cells to bind the agglutinin). Cells were fixed for 10 minutes with 10% formaldehyde in PBS, washed thoroughly with TBS and then incubated at 37°C for 1 hour with buffer containing 0.1 IU/ml neuraminidase. After further washing, cells were incubated with biotinylated peanut agglutinin (1/100 dilution in TBS) for 3 hours, washed, then incubated with streptavidin-horseradish peroxidase (1/300 dilution in TBS) for a further 60 minutes. Coverslips were then washed, incubated with chromogen and examined as described in Section 2.2.17.2. Control coverslips were incubated either with buffer containing no neuraminidase, or with peanut agglutinin which had been pre-incubated for 15 minutes with 200 mM galactose.

5.2.3 Internalisation of Tetanus Toxin by Chromaffin Cells

Two different approaches were taken in attempting to visualise the internalisation of tetanus toxin by chromaffin cells. In the first method, cells were incubated with tetanus toxin at 4°C for 3 hours

as previously described, washed quickly with ice-cold rinse buffer to remove unbound toxin, and then either fixed immediately, or warmed to 37°C (in binding buffer containing no toxin) for 3 hours prior to fixation and immunocytochemical processing (as outlined in Section 2.2.17). Coverslips were then examined by microscopy to determine whether incubation at 37°C led to a reduction in the level of surface-binding, indicating possible internalisation of the toxin. The second method was very similar, except that after incubation with tetanus toxin, cells were fixed and then treated with 1% Triton X-100 in TBS (as described in Section 5.2.1) prior to antibody incubations. It was envisaged that, since treatment with Triton X-100 is presumed to solubilise the plasma membrane (at least partially), any toxin located within the cells could be visualised directly using this approach.

5.3 RESULTS AND DISCUSSION

5.3.1 Dopamine-β-Hydroxylase Staining of Chromaffin Cells

The enzyme dopamine-β-hydroxylase, which is involved in catecholamine biosynthesis, catalyses the conversion of dopamine to noradrenaline and is found in both soluble and membrane-bound forms within the chromaffin granule. Since this enzyme is unique to chromaffin granules, it may be used as a marker to identify positively chromaffin cells in culture. Such an experiment was conducted on the cell preparations to be used in this immunocytochemical study, the results of which are shown in Fig. 5.1. It is clear that the majority of the cells were stained by this procedure and can therefore be identified as chromaffin cells; observation of cell cultures under phase contrast illumination did not reveal

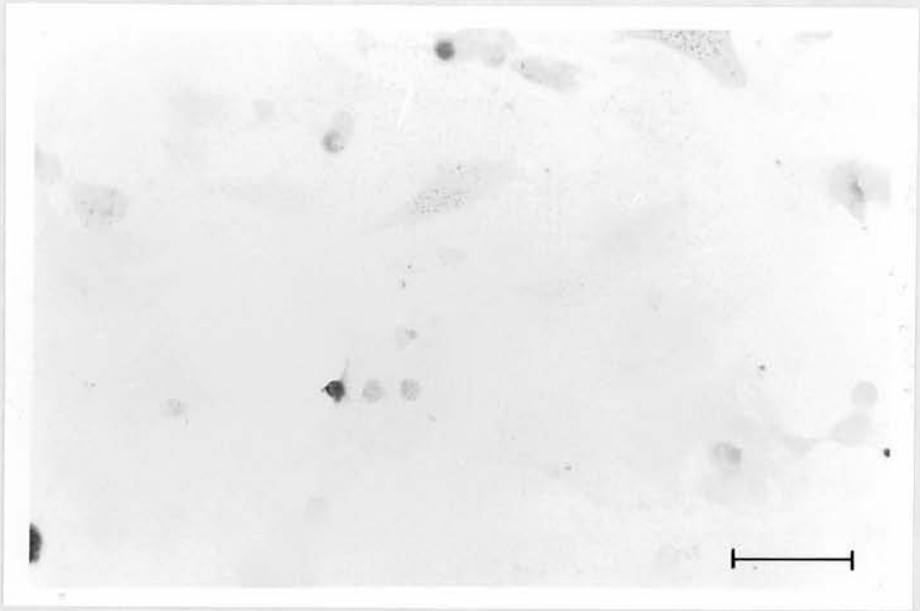
Figure 5.1 Dopamine-β-Hydroxylase Staining of Chromaffin Cells

Chromaffin cells cultured on coverslips were fixed, treated with Triton X-100, then sequentially incubated with mouse anti-dopamine-β-hydroxylase IgG and alkaline phosphatase-labelled sheep anti-mouse IgG before incubation with chromogen, as described in Sections 5.2.1 and 2.2.17.1. Cells were examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

(a) Control cells - not incubated with mouse anti-dopamine-β-hydroxylase antibody
(b) Bright-field micrograph of dopamine-β-hydroxylase staining of chromaffin cells
(c) Phase contrast micrograph corresponding to (b)

Scale bar represents 5 um
The results shown are typical of 3 separate experiments.

A



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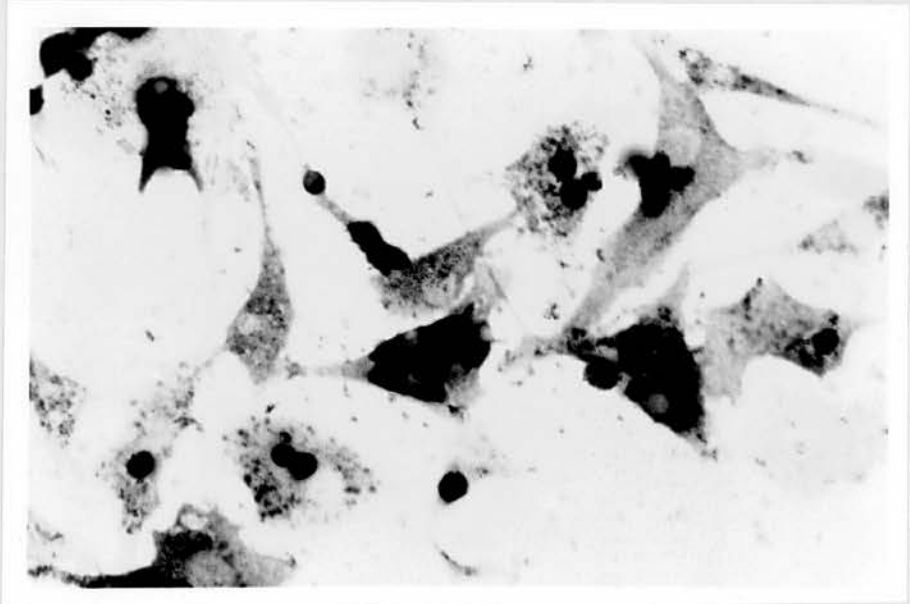

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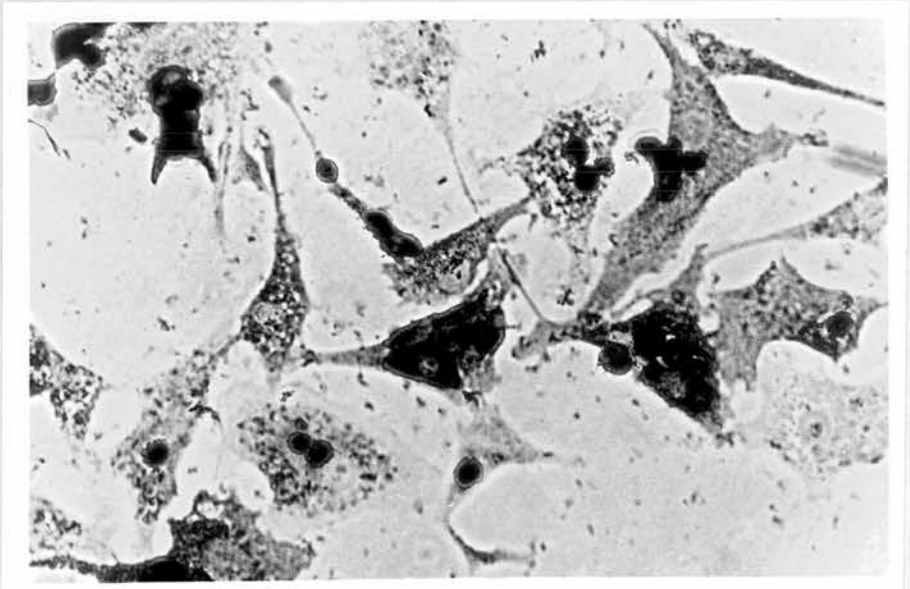


significant numbers of unstained cells. Further, the punctate distribution of the label within the cells is typical of an intracellular marker such as dopamine-β-hydroxylase. Another point to note is the variable extent to which individual cells are stained by this procedure; the reason for this is unclear, although it could be related to the length of time of the cells in culture.

5.3.2 Tetanus Toxin-Binding to Native Chromaffin Cells

As mentioned above, previous studies have employed indirect immunofluorescence to visualise toxin-binding to cells. However, this technique was not particularly successful in the present study. The levels of non-specific background binding were relatively high, and although enhanced antibody-binding to toxin-treated chromaffin cells could be seen by eye through the microscope, the extent to which the cells were fluorescently labelled was insufficient to allow satisfactory photographs to be produced. It was decided, therefore, to use alkaline phosphatase-labelled second antibodies in these experiments.

Native chromaffin cells were shown by this method to bind tetanus toxin under both non-physiological and physiological conditions of pH and ionic strength (the same conditions as those used in the binding studies in Chapter 4), as shown in Figs. 5.3 and 5.4 respectively; Fig. 5.2 indicates that levels of toxin-binding achieved when one of either toxin, human anti-toxin, or enzyme-conjugated anti-human IgG was omitted were negligible. It is clear from Figs. 5.3 and 5.4 that the staining of the cells by the toxin is typical of toxin-binding to the plasma membranes of the cells. Well-spread cells are uniformly stained, with dark edges of cells

Figure 5.2 Controls for Immunocytochemical Detection of Tetanus Toxin-Binding to Chromaffin Cells

Chromaffin cells cultured on coverslips were processed for immunocytochemical detection of toxin-binding in **pH 6.0, low ionic strength buffer**, as described in Section 2.2.17, except that in:

(a) cells were incubated with pH 6.0 buffer containing no tetanus toxin;
(b) cells were incubated with TBS/5% FCS containing no human anti-tetanus toxin IgG;
(c) cells were incubated with TBS/5% FCS containing no alkaline phosphatase-labelled goat anti-human IgG.

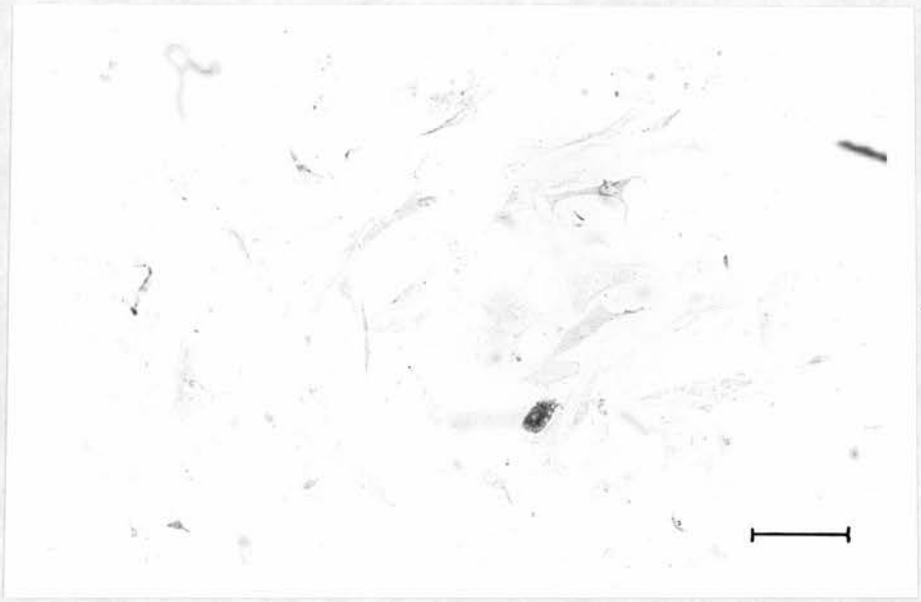
Chromaffin cells cultured on coverslips were processed for immunocytochemical detection of toxin-binding in **pH 7.4, high ionic strength buffer**, as described in Section 2.2.17, except that in:

(d) cells were incubated with pH 7.4 buffer containing no tetanus toxin;
(e) cells were incubated with TBS/5% FCS containing no human anti-tetanus toxin IgG;
(f) cells were incubated with TBS/5% FCS containing no alkaline phosphatase-labelled goat anti-human IgG.

Cells were then examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

Scale bar represents 10 um
Results shown are typical of at least 12 separate experiments.

A



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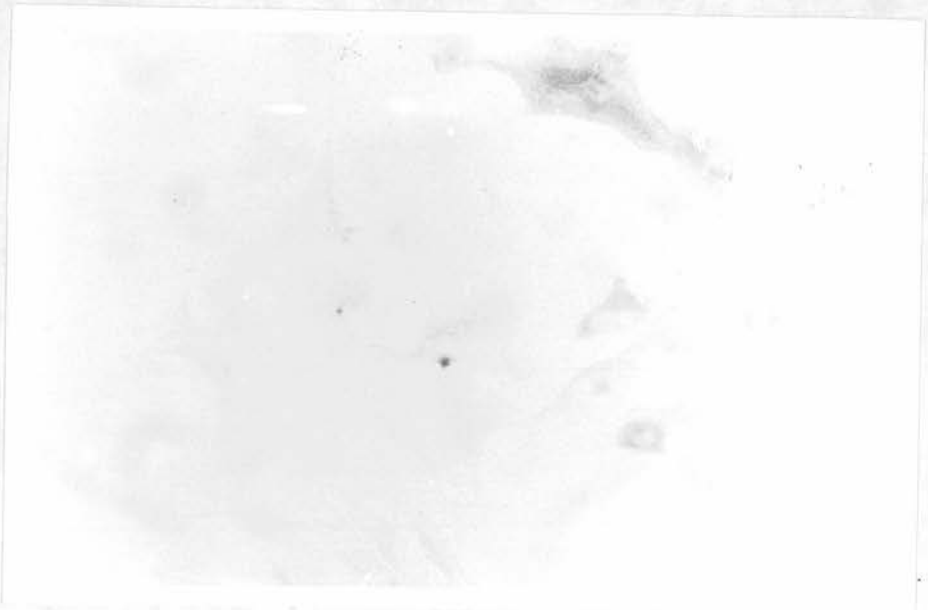

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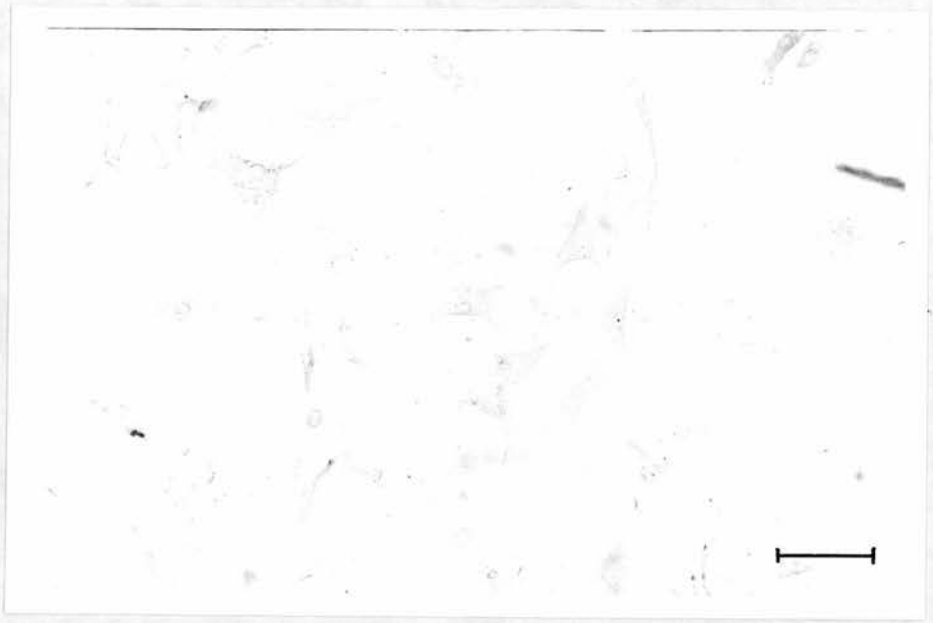

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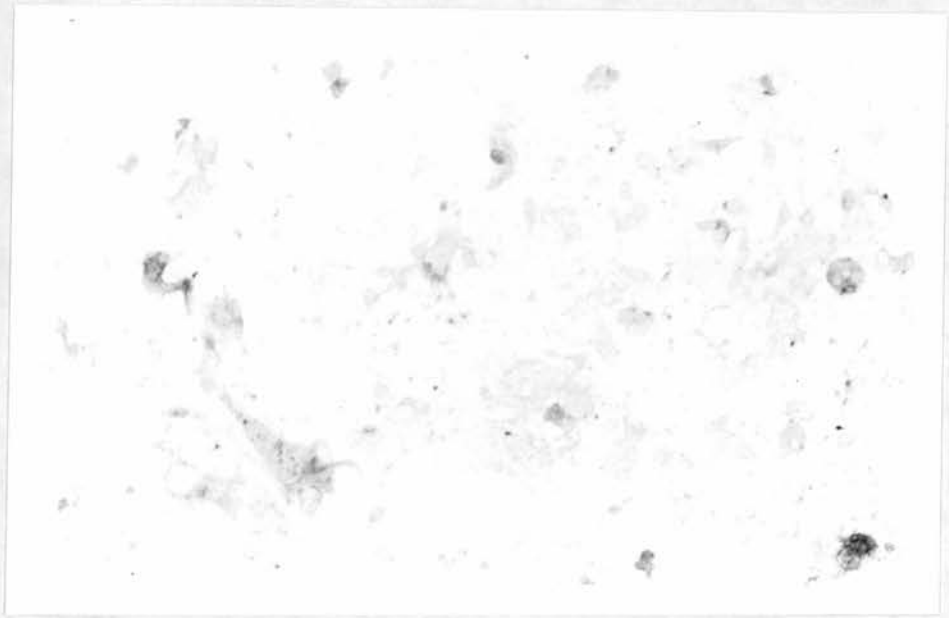

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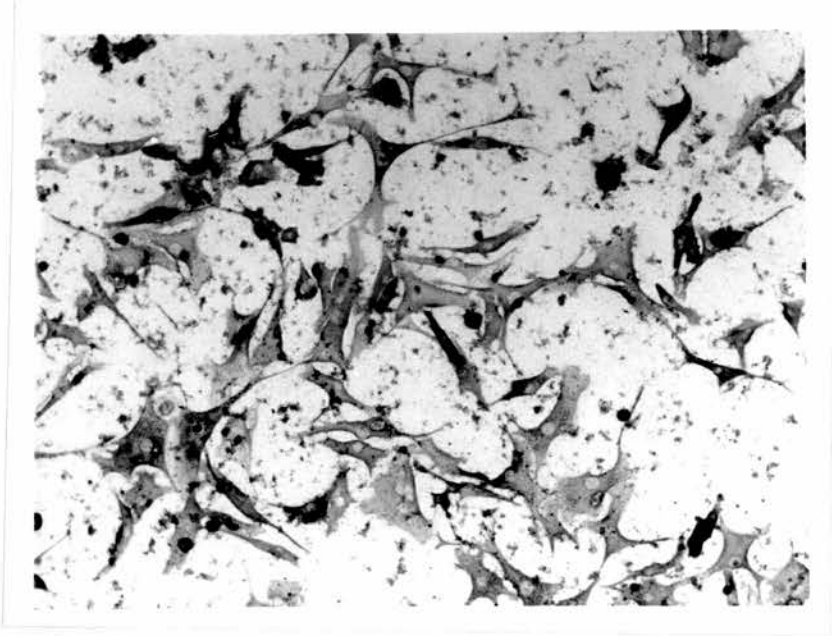
Figure 5.3 Immunocytochemical Detection of Tetanus Toxin-Binding to Native Chromaffin Cells in pH 6.0, Low Ionic Strength Buffer

Chromaffin cells cultured on coverslips were incubated for 3 hours at 0-4°C with tetanus toxin (10 µg/ml) in pH 6.0, low ionic strength buffer (Section 2.2.10), then processed for immunocytochemistry as described in Section 2.2.17. Cells were examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

Scale bar represents 10 um
Results shown are typical of at least 5 separate experiments.



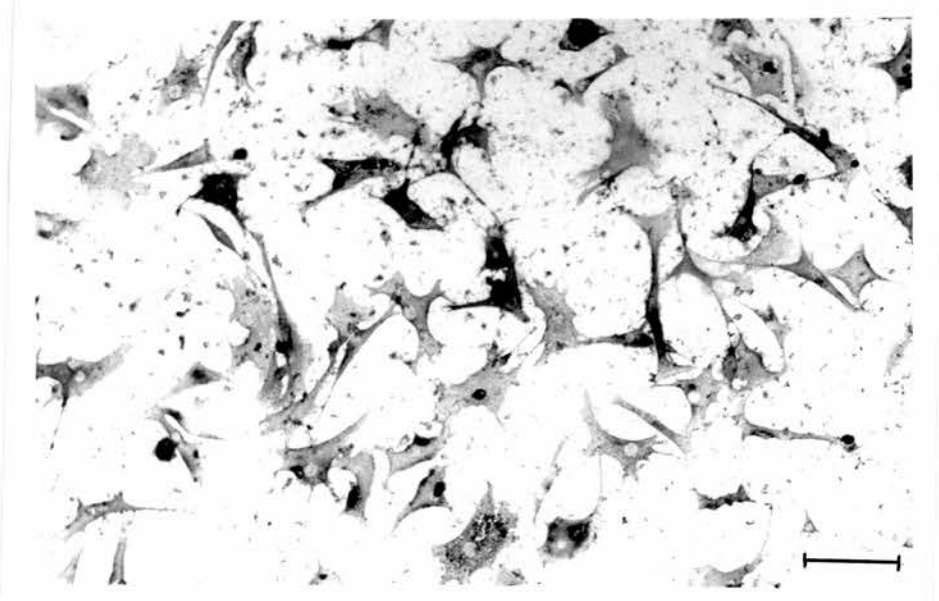


Figure 5.4 Immunocytochemical Detection of Tetanus Toxin-Binding to Native Chromaffin Cells in pH 7.4, High Ionic Strength Buffer

Chromaffin cells cultured on coverslips were incubated for 3 hours at 0-4°C with tetanus toxin (10 µg/ml) in pH 7.4, high ionic strength buffer (Section 2.2.10), then processed for immunocytochemistry as described in Section 2.2.17. Cells were examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

Scale bar represents 10 um
Results shown are typical of at least 5 separate experiments.

often being observed; this is in contrast to the staining of dopamine-β-hydroxylase (Fig. 5.1), where the intense (and, at high magnification, punctate) staining is typical of an intracellular antigen. The dark specks on the photographs are suggestive of some precipitation of toxin on the coverslips, despite filtration of all the solutions.

Interestingly, while it was observed in Chapter 4 that the toxin-binding capacity of these cells was several-fold greater under less physiological buffer conditions, this was not reflected in immunocytochemical experiments; in general, the intensity of staining of cells under both sets of conditions appeared to be similar. This is presumably related to the rather non-quantitative nature of this technique, and the difficulty of photographing different cell preparations under identical conditions. There also seemed to be some variation in the extent of staining of individual cells subjected to the same treatment; the most obvious explanation for this is that cells have varying levels of the receptor in their plasma membranes. Whether this is also the case *in vivo*, or is due to some aspect of the cell isolation procedure, for instance uneven exposure of cells to protease and/or collagenase, is not known.

From these photographs, it appears that the tetanus toxin acceptors in chromaffin cell membranes are evenly distributed over the whole cell surface; both the cell bodies and the long axon-like processes are stained with the toxin. This is in agreement with the findings of Mirsky *et al.* (1978), who reported that in their experiments showing the binding of tetanus toxin to a wide variety of neurones, both the neuronal soma and the processes were stained. In

contrast. Zimmerman & Piffaretti (1977) found that surface-bound toxin appeared as bright fluorescent patches; they interpreted these as aggregates of toxin due to migration of the receptor-toxin complex within the membrane. These different observations may be due to the fact that Mirsky's group conducted their toxin-binding experiments at room temperature, while Zimmerman & Piffaretti incubated their cells with tetanus toxin at 37°C; this increased temperature would certainly facilitate the movement and subsequent patching of the toxin-receptor complex within the cell membrane.

5.3.3 Effect of Exogenous Ganglioside on Toxin-Binding

In Chapter 4 it was reported that the incorporation of exogenous GT1 ganglioside into chromaffin cell membranes led to an increase in the capacity of the cells to bind ^{125}I-labelled tetanus toxin, both under physiological and non-physiological conditions of pH and ionic strength. Similar observations were made when toxin-binding was visualised by immunocytochemical techniques. Fig. 5.5 indicates that cells pre-treated with GT1 prior to incubation with tetanus toxin were frequently significantly more stained than untreated chromaffin cells, suggestive of higher levels of toxin-binding. Further, the intensity of staining of the treated cells is relatively uniform over the whole cell surface, indicating an even incorporation of the exogenous ganglioside into the cell membrane.

There appears, however, to be considerable variation in the intensity of staining of individual GT1-treated cells. This would suggest varying levels of ganglioside incorporation, although there is no obvious reason why this should be the case, and indeed no

Figure 5.5 Binding of Tetanus Toxin to Chromaffin Cells Pre-Incubated with Ganglioside GT1

Chromaffin cells cultured on coverslips were incubated for 2 hours at 37°C with GT1 (25 μg/ml in DMEM), prior to incubation with tetanus toxin (10 μg/ml) in either (a) pH 6.0, low ionic strength buffer, or (b) pH 7.4, high ionic strength buffer (Section 2.2.10) for 3 hours at 0-4°C. Cells were then processed for immunocytochemistry as described in Section 2.2.17, and examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

Scale bar represents 10 um
Results shown are typical of 3 separate experiments.

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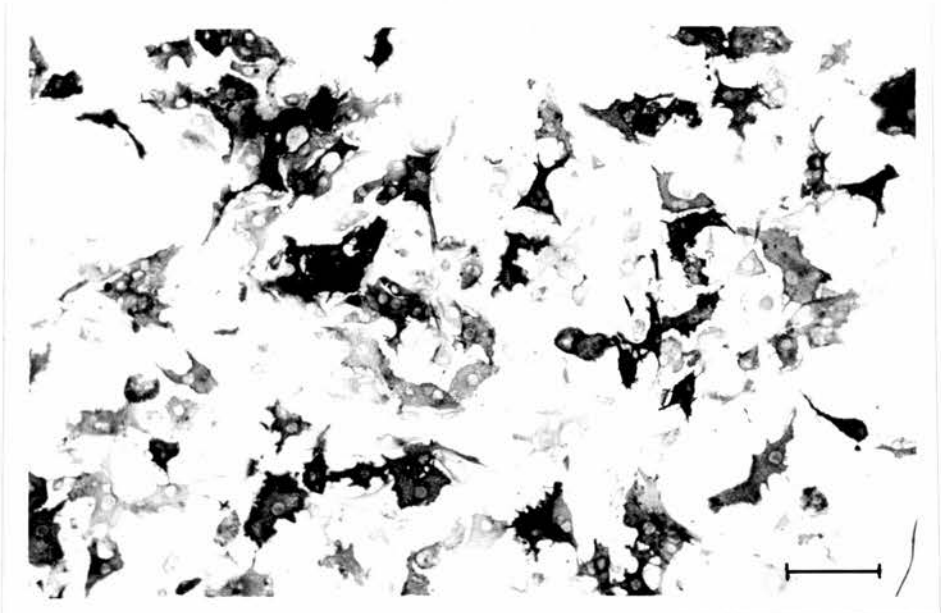

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evidence for this; in the experiments reported in Section 4.3.2, investigating the incorporation of fluorescent GT1 into chromaffin cell membranes, there was no large standard deviation in the measurements obtained, suggesting that all cells incorporated similar amounts of ganglioside. Further, this variability is not likely to be due to toxin internalisation by some cells during incubation, since although pre-incubation with ganglioside was carried out at 37°C, incubation with toxin was always performed at 4°C.

5.3.4 Effect of Enzymic Treatments on Toxin-Binding

Both neuraminidase treatment and trypsinisation have previously been shown to reduce the binding of ^{125}I-labelled tetanus toxin to chromaffin cells (see Section 4.3.5). It was of interest in this study to determine whether similar observations could be made using an immunocytochemical approach.

After confirmation that incubation of the cells with neuraminidase was in fact removing sialic acid residues from cell membrane gangliosides and other glycolipids, (see Fig. 5.6), the effect of this treatment on subsequent toxin-binding ability was evaluated. Fig. 5.7 indicates that, in agreement with the observations made in Chapter 4, the capacity of chromaffin cells to bind tetanus toxin is noticeably diminished, but not abolished, by neuraminidase treatment. Treated cells are less intensely stained, although the remaining toxin-binding appears to be distributed similarly to that occurring with native cells. Somewhat surprisingly, there does not appear to be any significant difference between the effects of the enzyme on toxin-binding observed under the two different buffer

Figure 5.6 Action of Neuraminidase on Chromaffin Cells

(a) Chromaffin cells cultured on coverslips were fixed with 10% formaldehyde in PBS, then incubated sequentially with neuraminidase (0.1 IU/ml in pH 6.0 rinse buffer), biotinylated peanut agglutinin, streptavidin-horseradish peroxidase and chromogen as described in Section 5.2.2. Cells were then examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

(b) Control cells - incubated with pH 6.0 rinse buffer containing no neuraminidase.

(c) Control cells - incubated with peanut agglutinin which was pre-incubated for 15 minutes with 200 mM galactose.

Scale bar represents 10 um
Results shown are typical of 3 separate experiments.

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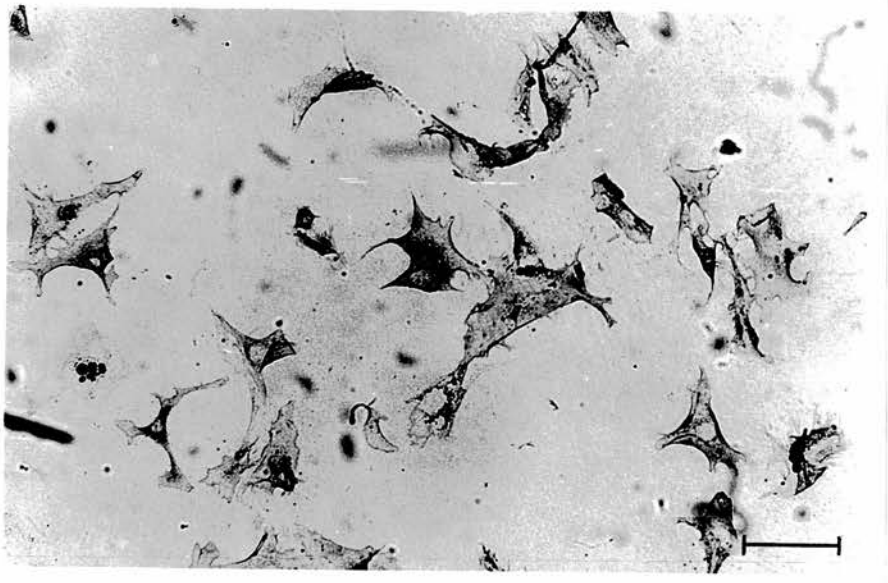

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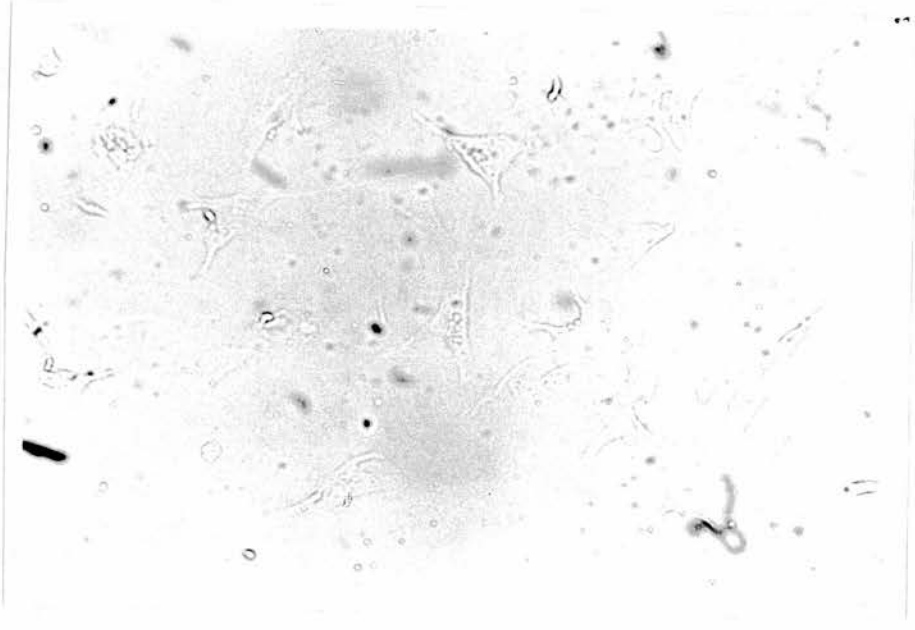

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Figure 5.7 Binding of Tetanus Toxin to Chromaffin Cells Pre-Treated with Neuraminidase

Chromaffin cells cultured on coverslips were incubated for 1 hour at 37°C with neuraminidase (0.1 IU/ml in pH 6.0 rinse buffer), prior to incubation with tetanus toxin (10 ug/ml) in either (a) pH 6.0, low ionic strength buffer, or (b) pH 7.4, high ionic strength buffer (Section 2.2.10) for 3 hours at 0-4°C. Cells were then processed for immunocytochemistry as described in Section 2.2.17, and examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

Scale bar represents 10 um
Results shown are typical of 3 separate experiments.

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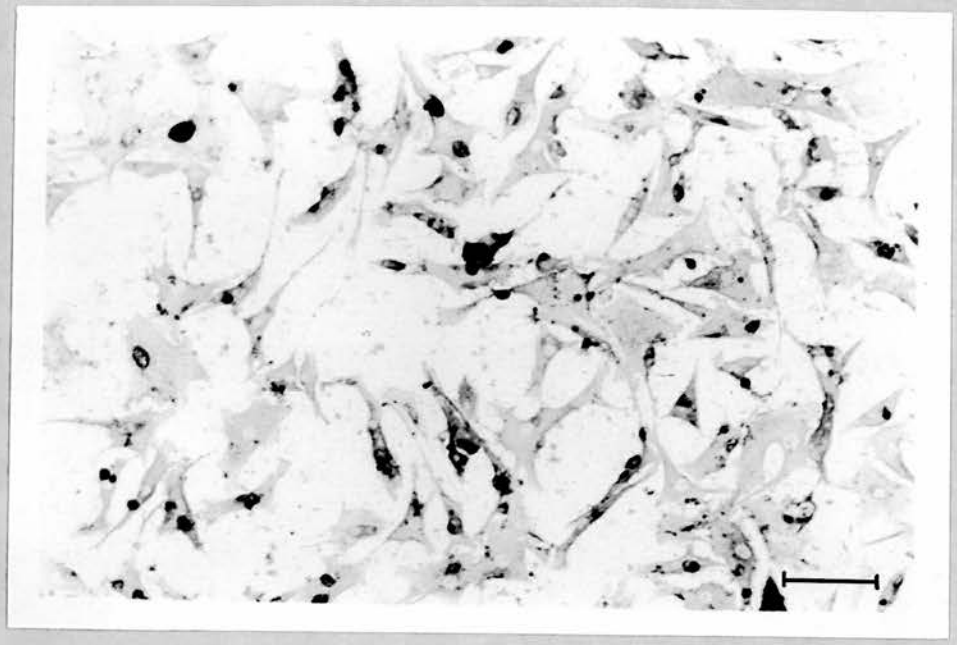

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conditions, as was noted in the ^{125}I-labelled toxin binding experiments; presumably this is again related to the rather non-quantitative nature of this approach.

Immunocytochemical visualisation of toxin-binding following mild trypsinisation indicated that this treatment drastically reduces the binding capacity of these cells under both physiological and non-physiological conditions; Fig. 5.8 shows that staining of these cells was not enhanced compared with that found with control cells. This is in agreement with the results reported in Section 4.3.5, and further suggests that toxin-binding to chromaffin cells is mediated, at least in part, by a membrane protein.

5.3.5 Internalisation of Tetanus Toxin by Chromaffin Cells

Since previous attempts to demonstrate internalisation of tetanus toxin by chromaffin cells had met with failure (see Section 4.3.7), it was thought that an immunocytochemical approach might be more successful. Figure 5.9 shows the results of such an experiment. Comparison of the intensity of staining of cells in photograph (a), where cells were incubated with toxin at 0°C then fixed, with those in photograph (b), where cells were first heated to 37°C prior to fixation, shows that the latter are somewhat less stained, suggestive of lower levels of surface-bound toxin. (Interestingly, there does not appear to be any evidence of patching of the toxin on the cell surface). This could be due to internalisation of some of the toxin, or alternatively to its detachment from the cell surface during the rather long incubation time. However, examination of photographs (c) and (d), where cells were treated with Triton X-100 (so intracellular as well as surface-bound toxin

Figure 5.8 Binding of Tetanus Toxin to Chromaffin Cells Pre-Treated with Trypsin

Chromaffin cells cultured on coverslips were incubated at 37°C for 10 minutes with trypsin (25 ug/ml in pH 7.4 rinse buffer), prior to incubation with tetanus toxin (10 ug/ml) in either (a) pH 6.0, low ionic strength buffer, or (b) pH 7.4, high ionic strength buffer (Section 2.2.10) for 3 hours at 0-4°C. Cells were then processed for immunocytochemistry as described in Section 2.2.17, and examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

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Results shown are typical of 2 separate experiments.

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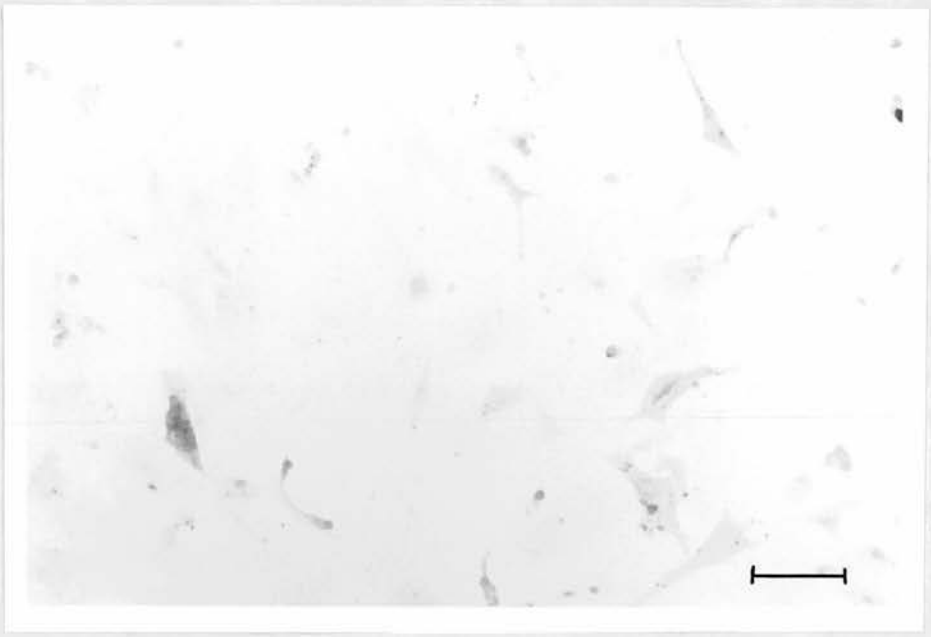

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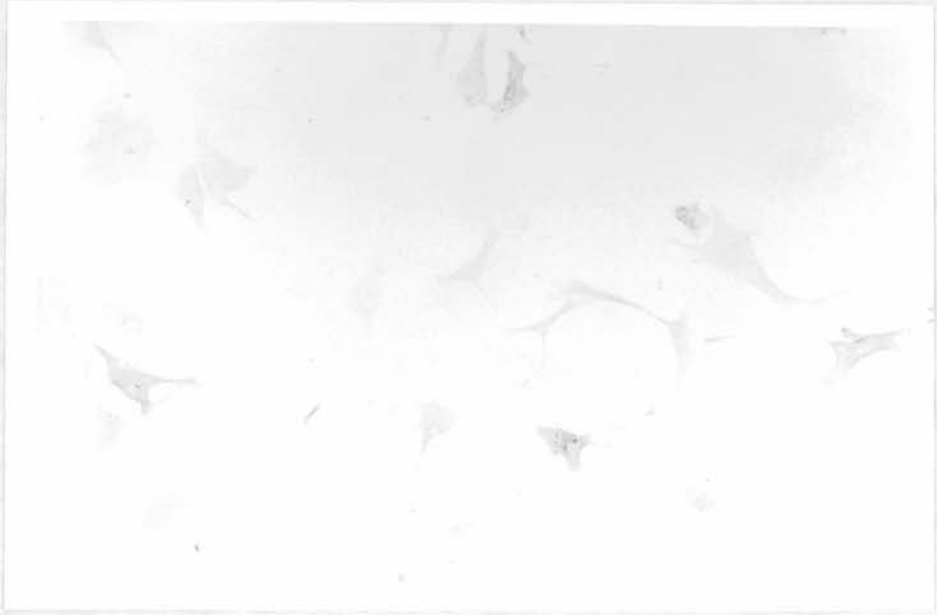

Figure 5.9 Internalisation of Tetanus Toxin by Chromaffin Cells

(a) Chromaffin cells cultured on coverslips were incubated with tetanus toxin (10 ug/ml in pH 7.4, high ionic strength buffer) for 3 hours at 4°C, washed with ice-cold rinse buffer, fixed and processed for immunocytochemistry as described in Section 2.2.17.

(b) Cells were incubated with tetanus toxin at 4°C for 3 hours and washed with ice-cold rinse buffer as above, then incubated in pH 7.4 binding buffer at 37°C for a further 3 hours, prior to fixation and immunocytochemical processing.

(c) Cells were incubated with tetanus toxin at 4°C for 3 hours, washed, fixed and treated with Triton X-100 prior to immunocytochemical processing.

(d) Cells were incubated with tetanus toxin at 4°C for 3 hours, washed, then incubated in pH 7.4 binding buffer at 37°C for a further 3 hours. This was followed by fixation and treatment with Triton X-100, prior to processing for immunocytochemistry.

Cells were then examined using a Leitz Ortholux 2 microscope and a Leitz Vario Orthomat 2 automatic microscope camera.

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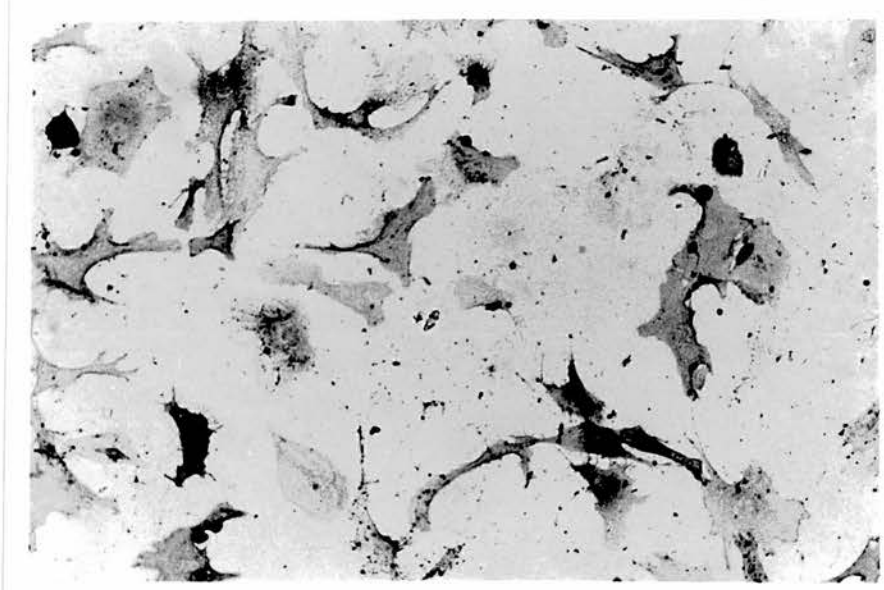

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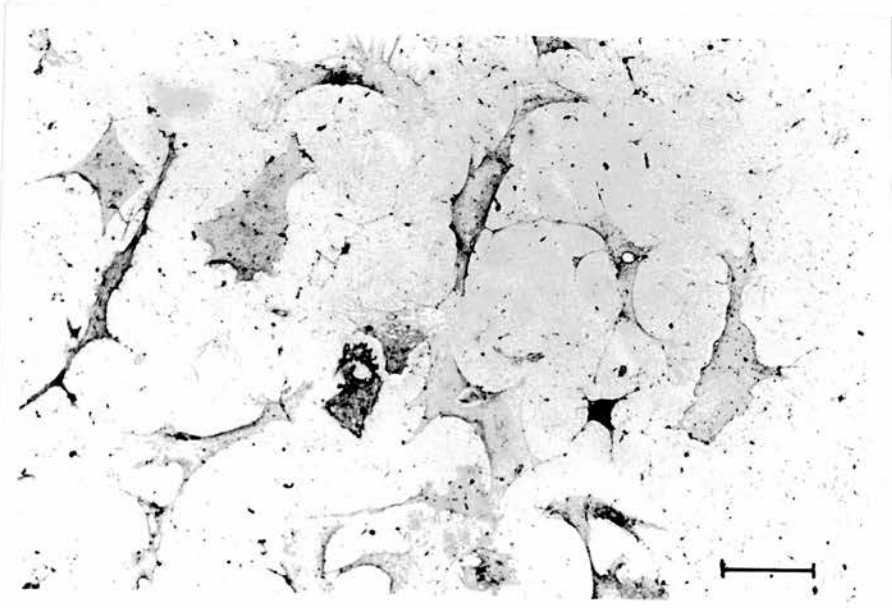

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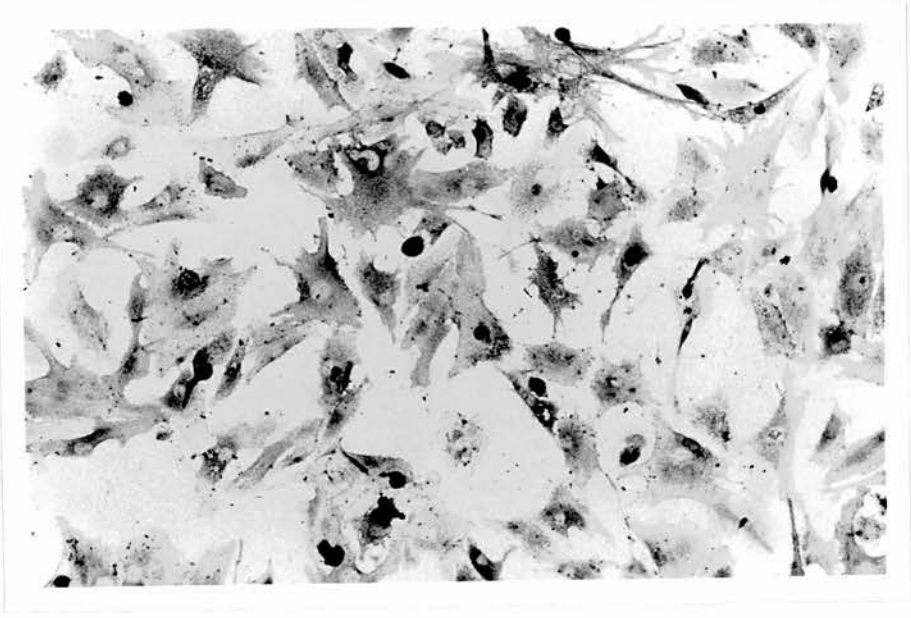

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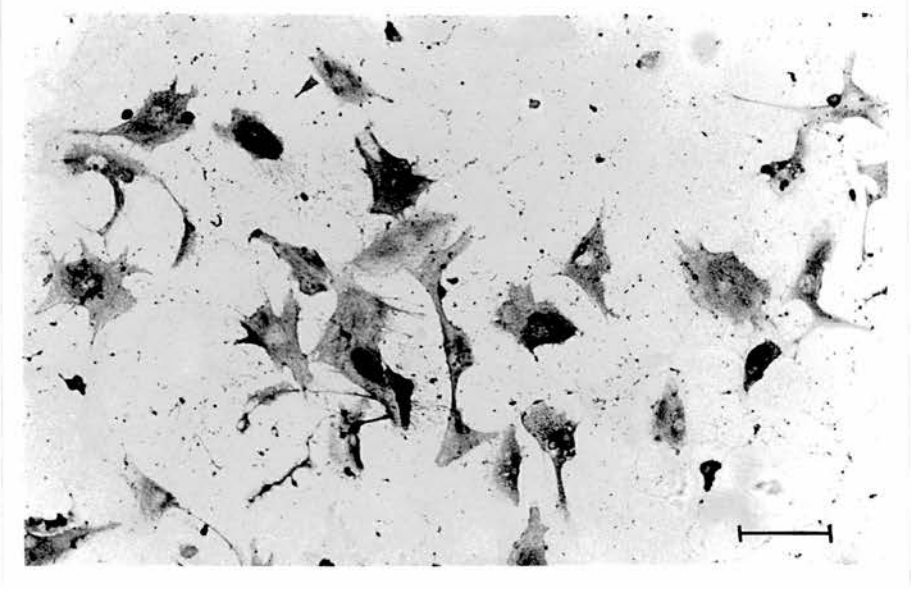

could be detected), suggests that some toxin internalisation is indeed occurring. The intensity of cell staining is similar in both cases, whereas it would be less in (d) if toxin had been lost into the medium and no internalisation had occurred. It is not possible, however, to state with any degree of confidence that the staining pattern of the cells warmed to 37°C is significantly different to those incubated with toxin solely at 0°C (i.e. that there is any evidence for intracellular compartmentalisation of the toxin), and so no firm conclusions can be drawn from this experiment. Presumably, though, some internalisation must be occurring in order for the toxin to inhibit exocytosis from these cells; it may be, therefore, that the amount of toxin reaching the cell interior is so little that immunocytochemical methods are not sensitive enough to detect this event.

5.4 CONCLUDING REMARKS

Using a different experimental approach, it has been possible to show again that tetanus toxin is able to bind to chromaffin cells, in a manner that is sensitive to both neuraminidase and trypsin. These observations serve as a qualitative back-up to the results obtained in Chapter 4, and lend further support to the notion that gangliosides (or at least some sialic acid-containing cell surface moiety) and perhaps also a protein are required for toxin-binding to chromaffin cells. Further, some evidence for internalisation of tetanus toxin by chromaffin cells has been presented. In this regard, then, chromaffin cells would appear to be similar to cells of a more direct neuronal origin.

CHAPTER SIX.

BIOCHEMICAL CHARACTERISATION OF TOXIN-BINDING SITES ON CHROMAFFIN CELL MEMBRANES

6.1 INTRODUCTION

Previously in this thesis, we have considered a wealth of experimental evidence which suggests that tetanus toxin displays a high affinity for gangliosides of the G1b series, and that it will bind readily to nerve cells containing significant levels of these compounds, or to other cells artificially supplemented with them. It has also been noted that numerous other observations suggest that the neurotoxic activity of tetanus toxin may be mediated by membrane components other than gangliosides. Since the main aim of this work is to assess the suitability of the chromaffin cell as a neuronal model for this kind of study, it is of obvious interest to define as specifically as possible the nature of the toxin-binding moieties present in chromaffin cell membranes.

The results presented in Chapters 4 and 5 indicate that adrenal chromaffin cells are able to bind tetanus toxin in a specific manner, with considerable affinity and capacity. Further, in line with the points made above, experiments with trypsin-treated and neuraminidase-treated chromaffin cells suggest the possible involvement of gangliosides (or some other sialic acid-containing compound) and maybe proteins in mediating this toxin-binding. The work presented in this chapter set out to study this binding on a more analytical level, in an attempt to identify the component(s) within the chromaffin cell membrane responsible for conferring toxin-binding ability, and to ascertain their similarity to those present in neuronal membranes.

The ganglioside composition of the chromaffin cell plasma membrane has not previously been investigated in detail, but chromatographic analysis of extracted gangliosides should indicate whether or not there are any likely toxin-binding species present. Any interaction between these gangliosides and tetanus toxin can then be detected by overlaying the separated gangliosides with radiolabelled toxin. This technique has been used successfully to demonstrate binding of cholera toxin to its receptor, ganglioside GM1 (Magnani *et al.*, 1980). Further, since any sialoglycoproteins present will be insoluble in the organic solvents used and will therefore not migrate on the chromatograms, it should be possible to assess the relative roles of gangliosides and glycoproteins as toxin receptors under varying conditions of pH and ionic strength.

Identifying a proteinaceous toxin receptor is not a straightforward task. Although there is good evidence for the involvement of a protein in toxin-binding to neuronal cells, such a protein has not been isolated; no group has yet reported detectable binding of ^{125}I-labelled toxin to rat brain membrane components separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. This may be due to alteration of the secondary or tertiary structure of the protein such that the toxin can no longer recognise it; care must therefore be taken to minimise denaturation of any potential protein toxin receptors.

6.2 METHODS

6.2.1 Extraction of Gangliosides from Membranes of Adrenal Medullary Microsomes

Membranes were prepared from adrenal medullary microsomes using the procedure outlined in Section 2.2.2; isolation of gangliosides from these membranes was then performed as described in Section 2.2.13. The yield of extracted gangliosides was quantitated by measuring lipid-bound sialic acid, as outlined in Section 2.2.15.

6.2.2 Analysis of Extracted Gangliosides

Gangliosides isolated from membrane fractions were analysed by chromatography on thin-layer plates, followed by exposure to iodine vapour or treatment with resorcinol reagent, as outlined in Section 2.2.14. Tentative identification of isolated ganglioside species was made by comparing their mobilities on thin-layer plates with those of standard gangliosides.

6.2.3 Ganglioside Overlay with ^{125}I-Labelled Tetanus Toxin

Chromaffin cell gangliosides separated on TLC plates were examined for their ability to bind tetanus toxin by using the overlay procedure described in Section 2.2.16. Preliminary experiments were conducted to confirm that, under the conditions used, tetanus toxin-binding to ganglioside GT1b was stronger than that to other ganglioside species.

6.2.4 Binding of ^{125}I-Labelled Tetanus Toxin to Membrane Proteins of Adrenal Medullary Microsomes

Microsomal membrane components were separated by SDS-polyacrylamide gel electrophoresis as described in Section 2.2.19, and were then blotted onto nitrocellulose as outlined in Section 2.2.21. The nitrocellulose sheets were then soaked for at least one hour at 4°C in the specified buffer containing 3% bovine serum albumin, before being examined for the presence of toxin-binding components by the same procedure as that used for TLC plates.

6.3 RESULTS AND DISCUSSION

6.3.1 Extraction and Analysis of Gangliosides from Adrenal Medullary Microsomes

In order to determine whether the ganglioside content of chromaffin cells could account for their sensitivity to tetanus toxin, we first analysed the distribution of these glycolipids in membranes prepared from both adrenal medullary microsomes and chromaffin granules. Previously, Dreyfus *et al.* (1977) have reported that 95% of the total gangliosides present in chromaffin granule membranes are monosialylated GM3 species. These workers detected three distinct GM3 bands on thin-layer chromatograms, which were shown to differ in their fatty acid composition. Other gangliosides co-migrating with GD3 and GD1a standards were also detected, but these accounted for less than 5% of the total ganglioside content of these membranes. More recently, Marxen *et al.* (1989) have analysed the ganglioside content of whole chromaffin cells grown in monolayer culture, and have reported that they contain small amounts of GD1a, but no detectable amounts of any other

gangliosides, including GT1b, GD1b or GM3; however, their chromatogram did contain unidentified lipids which migrated above the GM1 standard; they do not comment on these, but it is possible that one or more of these bands may be other monosialogangliosides, perhaps GM3. Interestingly, Walton *et al.* (1988) have reported that seven ganglioside species can be detected in extracts from PC12 cells, with tri- and tetrasialogangliosides making up over 30% of the total; they claim that GT1b is the major ganglioside component in these cells.

Figure 6.1(a) shows a typical resorcinol-stained thin-layer chromatogram of gangliosides extracted from adrenal medullary microsomes; the same result was obtained using ganglioside extracts from chromaffin granule membranes. Comparison with reference gangliosides indicates that the most prevalent species in these extracts is GM3, or at least a compound with the same mobility as GM3; ideally, isolation of gangliosides and determination of their structures should be carried out together with chromatographic analysis to ensure positive identification. There is also a detectable amount of a disialoganglioside present; this agrees with the findings of Dreyfus and his co-workers, suggesting that gangliosides found in adrenal medullary microsomes and chromaffin granules are indeed similar. Quantitation of the proportion of each species present in the extract by densitometric scanning is not easy, since resorcinol staining increases proportionally with the number of sialic acid residues present in the ganglioside, but it would seem reasonable to state that the monosialylated component represents at least 80% of the total extracted ganglioside. Obviously, then, the ganglioside composition of chromaffin cell

Figure 6.1 Thin-Layer Chromatography of Gangliosides Extracted from Adrenal Medullary Microsomes

Gangliosides were extracted from membranes of adrenal medullary microsomes as detailed in Section 2.2.13; yield was quantitated by determination of lipid-bound sialic acid (Section 2.2.15). A volume of extract containing approximately 5 μg of extracted ganglioside-sialic acid was applied to a thin-layer chromatographic plate, together with 10 μg each of the standard gangliosides GM1, GM3, GD1a and GT1. Chromatography was performed as described in Section 2.2.14, and plates were then either (a) sprayed with resorcinol reagent to visualise gangliosides, or (b) exposed to iodine vapour to reveal other lipids.

The results shown are typical of at least 3 separate adrenal medullary microsome ganglioside preparations.

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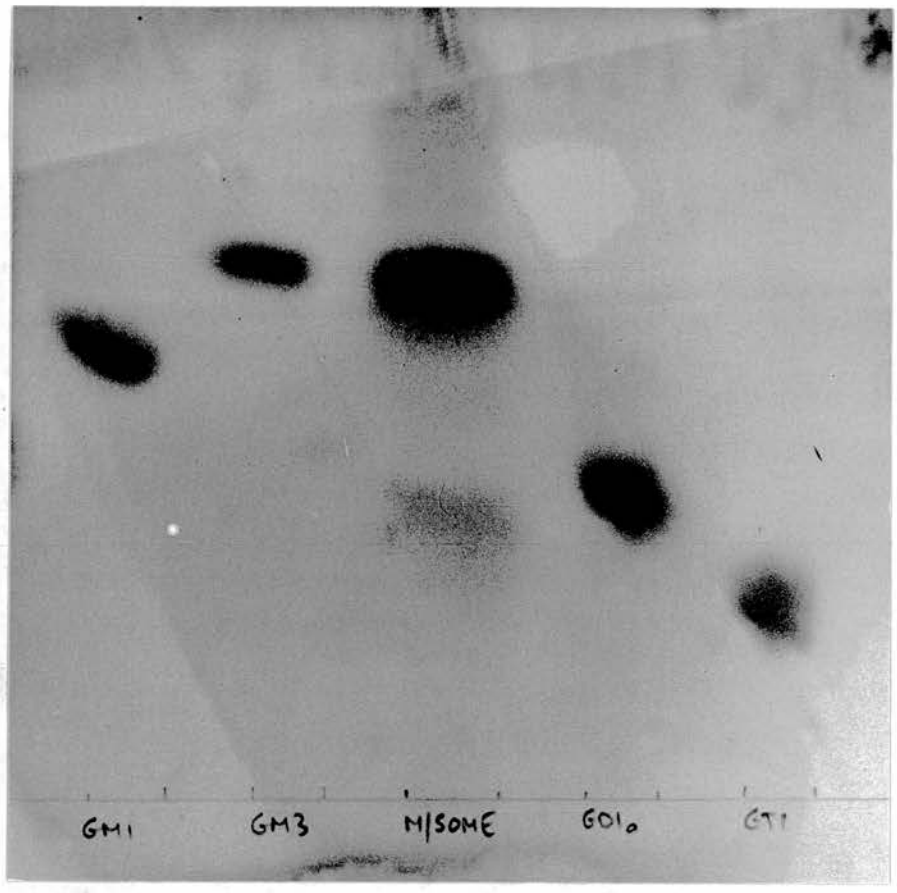

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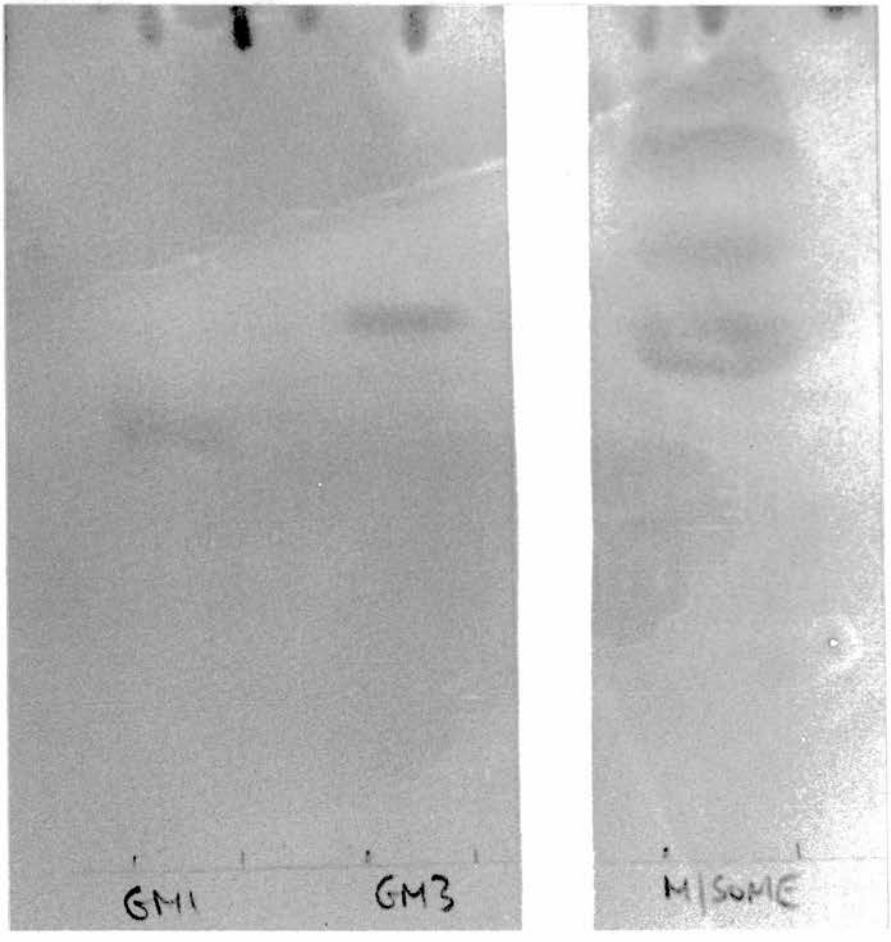

membranes (from both microsomes and granules) is quite unlike that characteristic of the central nervous system, where polysialo-gangliosides are the major constituents and monosialylated species represent only a small proportion of the ganglioside population.

The yield of gangliosides extracted from both microsomal and granule membranes in three separate extractions was typically within the range 2-4 μg ganglioside-sialic acid/mg protein; this is similar to the figure reported by Dreyfus for chromaffin granule membrane gangliosides (3.8 ± 0.9 μg ganglioside-sialic acid/mg protein). Figure 6.1(b) indicates that the extraction procedure does not completely separate gangliosides from other lipids; there are several non-sialylated species evident on exposure of the chromatogram to iodine vapour. The presence of these lipids, however, was not thought likely to be a problem in future experiments using these ganglioside extracts.

6.3.2 Detection of Toxin-Binding Gangliosides in Membranes of Adrenal Medullary Microsomes

The technique of overlaying gangliosides separated on thin-layer chromatograms with radiolabelled tetanus toxin, in order to identify those species with toxin-binding ability, has been used previously by several groups. Critchley *et al.* (1985), using gangliosides from primary cultures of mouse spinal cord, have demonstrated an interaction of toxin mainly with GD1b and GT1b, but also some binding to GD1a and GM1. Later, they obtained similar results using rat brain membrane gangliosides; binding of the labelled toxin was shown to be blocked following its pre-incubation with gangliosides, or by the addition of an excess of unlabelled

toxin to the overlay buffer. Further, they showed that the pattern of toxin-binding to these gangliosides was identical whether the overlay buffer was Tris-acetate (pH 6.0) or Tris-saline (pH 7.4), although binding was greatly reduced in the latter case, in agreement with the notion of a lower affinity of tetanus toxin for gangliosides under more physiological conditions of salt and pH (Critchley *et al.*, 1986). Habermann & Albus (1986) have similarly shown an interaction of radiolabelled tetanus toxin with gangliosides isolated from rabbit kidney membranes; these unidentified gangliosides had a mobility greater than that of GD1b. Finally, Nathan & Yavin (1989) have shown an interaction of tetanus toxin with GT1b isolated from PC12 cells using this overlay technique.

It was necessary to confirm that under the overlay conditions used, binding of radiolabel to ganglioside GT1b was greater than that to the other standard gangliosides available; GM1, GM3 and GD1a. This was carried out by overlaying a chromatogram to which equimolar amounts of the standard gangliosides (5 nmoles) had been applied. The result is shown in Fig. 6.2. Clearly there is very little interaction with gangliosides other than GT1; the arrows indicate the positions of very faint bands which may correspond to toxin binding to GD1a and GM1. (The dark spots on the autoradiograph probably result from aggregated toxin binding non-specifically to the chromatogram.)

Overlay experiments were next carried out, using gangliosides extracted from microsomal and granule membranes. Figure 6.3 shows the result of one such experiment, where the toxin was overlaid in

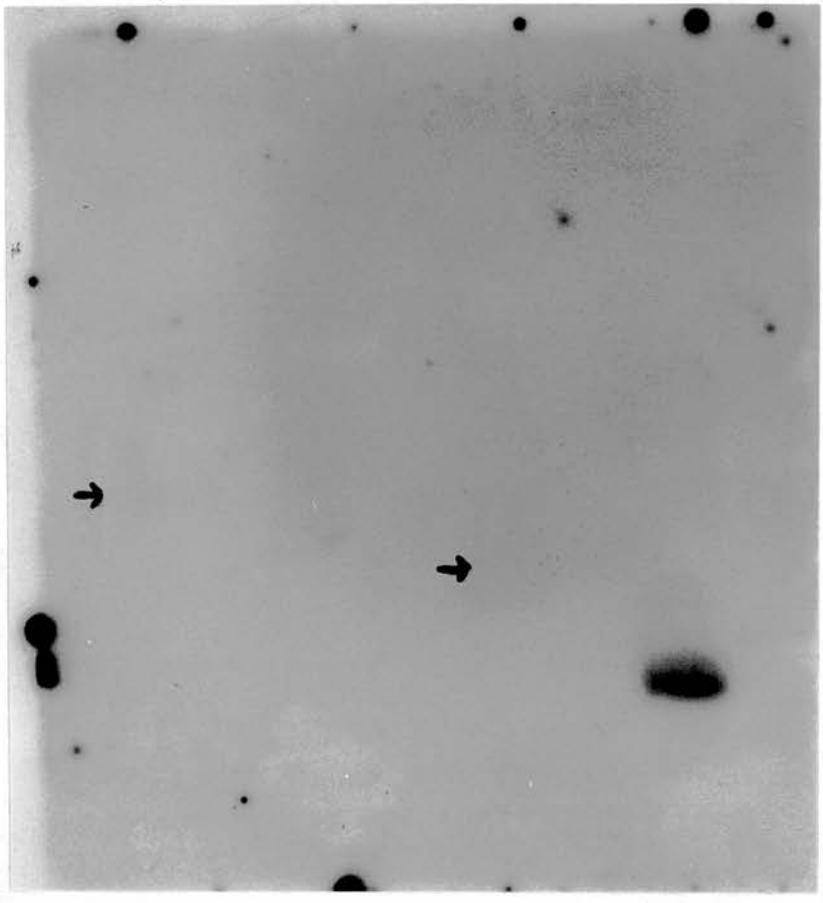

Figure 6.2 Binding of ^{125}I-Labelled Tetanus Toxin to Standard Gangliosides on Thin-Layer Chromatograms

5 nmoles of each of the standard gangliosides GM1, GM3, GD1a and GT1 were applied to a thin-layer plate; chromatography was then performed as outlined in Section 2.2.14, followed by overlay with ^{125}I-labelled tetanus toxin as described in Section 2.2.16. Toxin was used at 1 x 10^6 cpm/ml (equivalent to 12 nM). Binding of radiolabelled toxin was visualised by autoradiography (Section 2.2.20).

This result was obtained in 2 independent experiments.

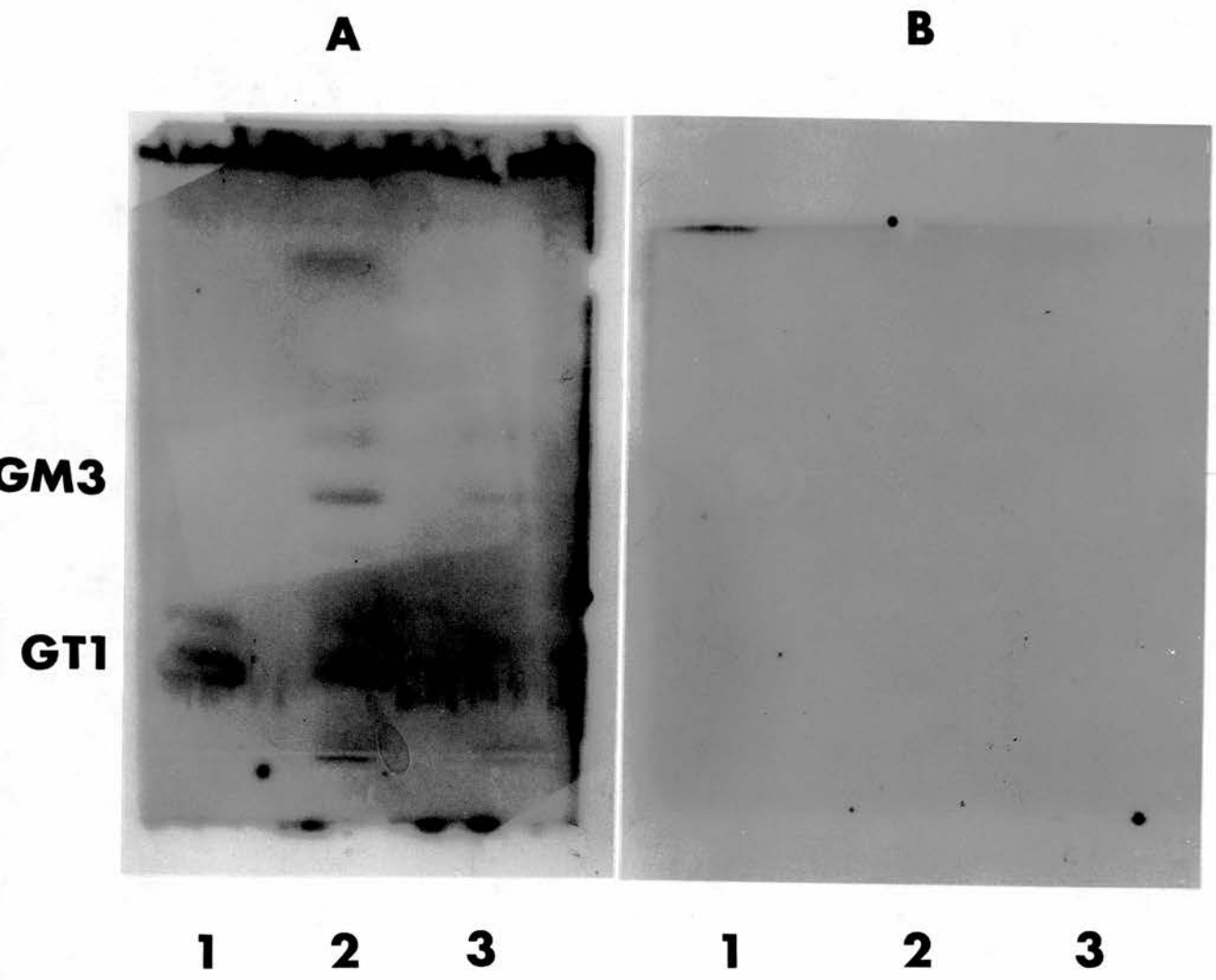

Figure 6.3
Overlay with ^{125}I-Labelled Tetanus Toxin of Gangliosides from Adrenal Medullary Microsomes and Chromaffin Granule Membranes

(a) Gangliosides extracted from membranes of adrenal medullary microsomes (approximately 5 μg of ganglioside-sialic acid), chromaffin granule membranes (approximately 1 μg of ganglioside-sialic acid) and standard GT1 (10 μg) were applied to a thin-layer plate. Chromatography was then performed as described in Section 2.2.14, followed by overlay with ^{125}I-labelled tetanus toxin as outlined in Section 2.2.16. Toxin was used at 1 x 10^6 cpm/ml (equivalent to 12 nM). Binding of radiolabelled toxin was visualised by autoradiography (Section 2.2.20).

(b) Chromatography and overlay with radiolabelled tetanus toxin was carried out as in (a), except that an excess of unlabelled toxin (25 μg/ml, 170 nM) was included in the overlay buffer.

1 = standard GT1; 2 = microsomal membrane gangliosides;
3 = chromaffin granule membrane gangliosides

low ionic strength, pH 6.0 buffer; there is clearly some interaction with the ganglioside material extracted from microsomal membranes. Surprisingly, the strongest band corresponds in mobility to GT1, a species which was not detected by resorcinol staining of microsomal gangliosides; the most plausible explanation for this is that these membranes do indeed contain very small amounts of GT1, which are not detectable by normal staining procedures, but which are nevertheless able to bind substantial amounts of tetanus toxin. There is also a significant amount of toxin bound to the monosialylated species tentatively identified earlier as GM3, as well as some interaction with a lower band which corresponds in mobility to the disialoganglioside detected in Fig. 6.1. Further, there is some evidence of toxin interacting with material of a higher mobility than GM3, albeit to a much lesser extent. A virtually identical pattern of toxin-binding was seen with the gangliosides extracted from chromaffin granule membranes, although these bands are not so strong, because less material was applied to the chromatogram. The second autoradiograph indicates that inclusion of an excess of unlabelled tetanus toxin in the overlay buffer (25 μg/ml, 170 nM) prevented binding of the labelled toxin.

It perhaps seems puzzling that binding of toxin to chromaffin granule membrane gangliosides was detected in this experiment, while no binding to purified granules was noted in Section 4.3.6. This can be explained, however, by the observation that the oligosaccharide portions of gangliosides are located on the inner face of the granule membrane (Winkler & Westhead, 1982).

Fractionation of microsomal membranes was next carried out, in an attempt to obtain a more pure plasma membrane fraction for use in future experiments; the procedure used (Section 2.2.2) is presumed to separate plasma (top interface), Golgi (middle interface) and endoplasmic reticulum (bottom interface) membranes (Gavine *et al.*, 1984). However, the ganglioside profiles of the three fractions were found to be identical (in two fractionations), and since the recovery of membranes at each interface was relatively poor, it was decided to pursue further experiments using gangliosides extracted from unfractionated microsomal membranes.

It was envisaged that this overlay technique could be used to ascertain whether toxin-binding to gangliosides from adrenal medullary microsomes was reduced under more physiological conditions of salt and pH. However, difficulties were encountered upon carrying out further overlay experiments. In some cases, no toxin-binding could be detected, and concentration of the ganglioside extracts (in order to increase the amount of material that could be applied to the chromatogram) was unsuccessful, since the ganglioside material was prone to precipitation. Similarly, increasing the concentration of radiolabelled toxin in the overlay buffer to any extent resulted in unacceptable levels of non-specific background binding, and sometimes even "negative staining" of the ganglioside bands. In conducting at least twelve overlay experiments, it seemed impossible to achieve the correct balance of ganglioside material and labelled toxin, given the low affinity of the binding interaction, to obtain satisfactory results routinely.

Alternative means of visualising an interaction between gangliosides from adrenal medullary microsomes and tetanus toxin were thus sought. One approach taken was a modification of the conventional procedure for processing Western blots; gangliosides separated on a chromatogram were overlaid with unlabelled tetanus toxin (30 μg/ml, 200 nM), then sequentially incubated with human anti-tetanus antibody and horseradish peroxidase-labelled anti-human IgG prior to development with chloronaphthol reagent. However, this procedure also failed to indicate any toxin-binding; presumably this method was insufficiently sensitive to detect any low affinity interaction. Similarly, a modified "enhanced chemi-luminescence" technique did not produce any detectable binding.

So, although successful in demonstrating an interaction between tetanus toxin and gangliosides from adrenal medullary microsomes, we were unable to analyse this binding to any extent, or to acquire any quantitative information. It would have been interesting, for instance, to estimate the amount of toxin bound per microgram of sialic acid on the chromatogram, and to relate this to the results obtained from the direct binding experiments in Chapter 4. It is possible that gangliosides, when organised in membranes, are more accessible for toxin-binding than when they are adsorbed onto a thin-layer chromatogram; in other words, it may be that whether or not a binding interaction is detected, especially one of low affinity, may depend on how the gangliosides are presented. For instance, while Holmgren *et al.* (1980) detected binding of toxin to GM1 and GD1a in a solid phase assay using gangliosides adsorbed to polystyrene, Habermann & Albus (1986) did not, using gangliosides on silica gel plates as we have done. It may be that the blocking

step, necessary to prevent non-specific binding of the radio-labelled toxin, may abolish some low affinity toxin-ganglioside interaction. (It should be noted here that the affinity of GM1 for cholera toxin does not seem to alter significantly when studied using gangliosides inserted in membranes, adsorbed to plastic, or free in solution; Critchley *et al.*, 1989).

Another point to consider is the question of whether iodination of the toxin may change its specificity for gangliosides. However, this is not generally the case, since Critchley *et al.* (1986) obtained identical results whether they used radiolabelled toxin, or unlabelled toxin followed by anti-toxin and ^{125}I-labelled protein A.

6.3.3 Detection of Toxin-Binding Proteins in Membranes of Adrenal Medullary Microsomes

Experiments were next carried out in an attempt to identify any membrane proteins that could also interact with the toxin. Overlay of microsomal membrane proteins (separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose) with ^{125}I-labelled toxin did not, however, result in any detectable binding in two independent experiments of the radiolabel to proteinaceous material, regardless of the buffer conditions used in the incubation. Even when precautions were taken to minimise possible irreversible denaturation of any potential protein receptors (i.e. running membrane samples dissolved in SDS without heating, with or without reduction with β-mercaptoethanol) there was no indication of any toxin binding. Therefore it would seem that if there is a sialoglycoprotein present in chromaffin cell membranes that is involved in toxin-

binding, it is unlikely that the toxin is simply binding to the carbohydrate portion of the molecule, since this should not be inactivated by treatment with SDS. Instead, this result implies that the secondary or tertiary structure of such a protein is also critical for toxin-binding. This will make the identification of the protein a difficult task.

6.4 CONCLUDING REMARKS

The work presented in this chapter indicates that there are some similarities between the binding of tetanus toxin to its target neurones and to chromaffin cells. It appears that gangliosides present in the chromaffin cell membrane are able to bind tetanus toxin, and may be able to mediate its action. Apart from the possible involvement of a GT1-like species in this process, both a disialoganglioside and a monosialoganglioside also seem to be capable of toxin-binding, with the latter being a major binding component; even if binding to this ganglioside is weak, its importance presumably relates to its abundance in the chromaffin cell membrane relative to other species. This is not a particularly surprising result; previously, as mentioned above, Holmgren *et al.* (1980) have reported some binding of tetanus toxin to GM1, while Helting *et al.* (1977), using tetanus toxin adsorbed to Sephadex and radiolabelled ganglioside, have claimed that toxin-binding to GM1 is as strong as that to GD1b.

These findings are in agreement with the results of the binding experiments conducted on neuraminidase-treated cells reported in Chapter 4. Such treatment will convert the disialoganglioside (as

well as any GT1 present) to GM1, while neuraminidase will also remove the sialic acid residue from GM3 (Miller-Podraza *et al.*, 1982); presumably, the absence in GM3 of the terminal galactosyl-N-galactosamine moiety of the tetrasaccharide backbone, which is present in GM1, renders the sialic acid residue susceptible to enzyme attack.

Attempts to identify any protein(s) of the chromaffin cell membrane able to mediate toxin-binding were not successful. This problem has been encountered by workers searching for toxin-binding proteins in neuronal membranes also; obviously, alternative means of showing such interactions will be required.

CHAPTER SEVEN.

CONCLUDING SUMMARY AND FUTURE WORK

Although it is well known that tetanus toxin acts *in vivo* to inhibit the exocytotic release of neurotransmitters from certain synapses, the means by which this blockade of secretion is achieved is not clear. One of the main reasons for this is the fact that the toxin's target neurones are difficult to isolate and culture *in vitro*, and are therefore not very accessible to scientific study. A suitable neuronal cell model would therefore be useful for investigation of its action. The main purpose of this study was thus to investigate the interaction between tetanus toxin and adrenal medullary chromaffin cells, with a view to assessing the suitability of these cells as neuronal models.

Other workers (e.g. Penner *et al.*, 1986; Ahnert-Hilger *et al.*, 1989) have previously shown an inhibitory effect of tetanus toxin on exocytosis from chromaffin cells. However, in these experiments the toxin was introduced directly into the cytoplasm of the cells, either by microinjection or permeabilisation of the plasma membrane; further, it was the release of radiolabelled catecholamines with which the cells had been preloaded that was measured. Release of radiolabelled catecholamine frequently does not parallel that of endogenous catecholamine, and extrapolation of the results to the *in vivo* situation has been difficult in consequence.

The present study was to be conducted using <u>intact</u> chromaffin cells, and initial experiments were aimed at demonstrating an inhibitory effect of tetanus toxin on nicotine-evoked secretion from these cells; the secretion of <u>endogenous</u> catecholamines was measured, thus keeping conditions as close as possible to those occurring *in vivo*. Tetanus toxin was shown to be capable of

inhibiting exocytosis from intact chromaffin cells in a dose-dependent fashion, with half-maximal inhibition being achieved at 0.7 nM toxin. Since it is not inconceivable that the toxin could reach such concentrations in a tetanus victim, this would appear to be a physiologically relevant effect. However, it would seem that some as yet undiscovered property of these cells determines their sensitivity to toxin, since about 50% of our cell preparations were not affected by incubation with the toxin, and were able to secrete their granule contents as efficiently as untreated cells. The reason for this observation is not clear, but it may be that, for instance, the differentiation state of the cells is an important factor in determining their sensitivity to toxin; this has been found to be the case with PC12 cells (Sandberg *et al.*, 1989a), and is perhaps worthy of future investigation. Alternatively, as discussed in Section 3.3.5, it could be some feature of the cell isolation procedure which is responsible for this lack of consistency.

Catecholamine release evoked by Ba^{2+} ions was shown to be unaffected by the toxin, while its effect on K^+-evoked secretion was never greater than 30% (compared with a maximal 75% inhibition of nicotine-stimulated secretion). This finding, that the extent to which evoked secretion from chromaffin cells is affected by the toxin differs depending on the secretagogue used, may prove to be very useful in future in the search for the toxin's intracellular substrate. It is known, for instance, that nicotinic stimulation of chromaffin cells results in disassembly of actin, while

depolarisation by K^+ ions does not; this would suggest that the toxin is acting at the level of the cytoskeleton, and deserves further investigation.

For the past 30 years, ganglioside GT1 has been considered a likely candidate for the *in vivo* cell surface acceptor for tetanus toxin. However, to date there has been no convincing demonstration of a correlation between binding of the toxin to cell membrane gangliosides and a subsequent cellular response (i.e. decreased levels of exocytosis). We thought that it might be possible to show such a correlation by incorporating exogenous gangliosides into chromaffin cell plasma membranes and demonstrating a subsequent enhancement of the toxin's inhibitory effect, due to the introduction of new toxin-binding sites. Unfortunately, we discovered that the ganglioside itself could inhibit nicotine-evoked exocytosis, at least at the concentrations of GT1 being used (5-24 μM), making it impossible to evaluate the role of GT1 in mediating the action of the toxin. Time constraints prevented us from conducting further experiments in this area, but it may be worth pursuing this type of approach in the future, using lower concentrations of GT1; there is no reason to believe that an enhancement of toxin-binding, and hence inhibition of exocytosis, could not be brought about by pre-incubation of chromaffin cells with much lower levels of ganglioside. [It should be noted here that the work of Marxen and her colleagues (Marxen & Bigalke, 1989; Marxen *et al.*, 1989), describing how exogenous gangliosides could mediate the inhibitory effect of tetanus toxin on chromaffin cells, was published after our experiments in this area were undertaken. Furthermore, these workers used very high concentrations of

gangliosides (1 mg/ml), and did not appear to check for any inhibition of exocytosis due to the gangliosides themselves.] Finally, in line with findings made later in this study (see below), it would be interesting to investigate the effect of neuraminidase treatment or mild trypsinisation of native chromaffin cells on their subsequent sensitivity to tetanus toxin. These experiments may provide further clues as to what type of cell surface acceptor is actually mediating the cellular response to the toxin.

With the knowledge that tetanus toxin is indeed able to inhibit exocytosis from intact chromaffin cells, we next wished to demonstrate the specific binding of the toxin to these cells. Binding of ^{125}I-labelled toxin to native chromaffin cells was observed under both non-physiological and physiological buffer conditions; this binding showed characteristics similar to toxin-binding to cells of a more direct neuronal origin, with a substantial capacity and a similar dependence on pH and ionic strength. However, like the "inhibition-of-secretion" experiments discussed above, results were not consistent, in that some preparations of chromaffin cells were unable to bind the toxin; the reason for this observation is not clear, and this should be investigated in the future. It would also be very useful to be able to correlate this lack of binding to a lack of susceptibility to the toxin; this would be a good indication that the observed binding was indeed mediating the toxin-induced inhibition of exocytosis. Ideally, future binding studies should be carried out using a wider range of toxin concentrations, in order to obtain

more accurate values for $^{apparent}K_d$ and B_{max}; unfortunately this was not possible in the present study owing to the limited availability of tetanus toxin.

Binding of tetanus toxin to chromaffin cells under both conditions studied was found to be a complex phenomenon, with at least two components to binding in evidence, while experiments with neuraminidase-treated and trypsinised cells suggested the possible involvement of both sialic acid-containing and proteinaceous moieties in mediating toxin-binding. Future work could perhaps be directed towards identifying the cell membrane components that are responsible for mediating these two apparently distinct types of binding. This could perhaps be achieved by conducting more detailed experiments with neuraminidase-treated cells; careful analysis of the toxin-binding remaining after neuraminidase treatment and calculation of $^{apparent}K_d$ may be useful in this regard. Similarly, a more thorough investigation of the effect of trypsinisation on toxin-binding might be worthwhile, using much reduced concentrations of trypsin.

Toxin-binding by chromaffin cells was also visualised using immunocytochemical techniques. The difference in the toxin-binding capacity of native chromaffin cells under the two different buffer conditions was not so evident here as in the direct binding experiments, although an effect of pre-incubation of cells with GT1 in enhancing toxin-binding was apparent. Similarly, neuraminidase-treated and trypsinised cells were less intensely stained compared to native cells. These findings serve to confirm the observations made in the direct binding experiments.

Further work was aimed at defining as specifically as possible the nature of the toxin-binding components of chromaffin cell plasma membranes. Analysis by thin-layer chromatography of gangliosides extracted from adrenal medullary microsomes indicated that the major species present was GM3; a small proportion of a disialylated species was also evident. The identity of these gangliosides should ideally be confirmed using specific antibodies. (Anti-ganglioside antibodies could also perhaps be used to evaluate the role of gangliosides in mediating the effect of the toxin on native chromaffin cells, although they have been found to display rather low affinity for their antigens; S van Heyningen, personal communication). Overlay of adrenal medullary gangliosides with radiolabelled toxin indicated that they were in fact able to interact with the toxin (and that there might in fact be very low levels of GT1 present which can bind substantial amounts of toxin), adding further support to the idea that toxin-binding to native chromaffin cells may be mediated, at least in part, by gangliosides. Unfortunately, we were unable to characterise this interaction as much as we would have liked; this is an aspect of the study that future work may concentrate on. It would be useful to be able to obtain some quantitative information on the binding of tetanus toxin to these ganglioside species, and to compare this binding under the two different sets of conditions used in the direct binding experiments.

Attempts to identify a proteinaceous toxin acceptor in chromaffin cell membranes were unsuccessful. This was presumably due to the conditions used (separation of proteins by polyacrylamide gel electrophoresis in SDS, followed by Western blotting and overlay

with radioactive toxin) disrupting the secondary or tertiary structure of the putative protein such that the toxin could no longer recognise it. Perhaps an alternative approach to identifying such a molecule, that could be taken in the future, would be to screen for monoclonal antibodies that block exocytosis from chromaffin cells, and to use these to characterise the acceptor by Western blotting techniques.

Attempts to visualise the internalisation of tetanus toxin by chromaffin cells met with limited success. Although there was some suggestion that warming of the cells resulted in a reduction in the levels of surface-bound toxin, there was no clear evidence of any intracellular compartmentalisation of the toxin, and so these experiments were somewhat inconclusive. It seems likely that the methods used in this study were insufficiently sensitive to allow internalisation of the toxin to be detected, and that future efforts in this area should therefore perhaps use the approach taken by other workers (e.g. Parton *et al.*, 1987, 1988) in using toxin-colloidal gold complexes and electron microscopy. In this way, the mechanism of toxin internalisation by chromaffin cells may also be elucidated; however, it could be that these cells do not possess a specific means of toxin internalisation, and that it is toxin that reaches the cell interior in some non-specific fashion that is responsible for the observed inhibition of exocytosis. It does not necessarily follow that all, or indeed any, of the toxin bound specifically to the cell membrane will eventually reach the cell cytoplasm.

The work presented in this thesis has gone some way towards characterising the interaction between adrenal chromaffin cells and tetanus toxin. It would appear that binding of the toxin to chromaffin cells shows similar characteristics to toxin-binding to its target neurones, and that it is mediated by similar components. Further, the toxin is able to inhibit exocytosis from these cells as it does from neuronal cells; indeed, the levels of inhibition achieved in this study (up to 75%) are among the highest so far reported. The general conclusion from these findings, then, would seem to be that the chromaffin cell is a suitable neuronal cell model, and that future experimentation with these cells has the potential to provide useful information on the mechanism of action of tetanus toxin.

APPENDIX 1

Derivation of Equations for Calculating Catecholamine Content of Samples

Noradrenaline (nmol): $$y = \frac{(M.Ab/Aa) - N}{(Na.Ab/Aa) - Nb}$$

Adrenaline (nmol): $$x = \frac{N - y.Nb}{Ab}$$

where Aa = fluorescence of 1 nmol adrenaline at 395/490 nm
Ab = fluorescence of 1 nmol adrenaline at 436/540 nm
Na = fluorescence of 1 nmol noradrenaline at 395/490 nm
Nb = fluorescence of 1 nmol noradrenaline at 436/540 nm

M = sample fluorescence at 395/490 nm
N = sample fluorescence at 436/540 nm

It follows that: $M = x.Aa + y.Na$
$N = x.Ab + y.Nb$

Therefore: $x = \frac{N - y.Nb}{Ab}$; equally $x = \frac{M - y.Na}{Aa}$

$$\frac{N - y.Nb}{Ab} = \frac{M - y.Na}{Aa}$$

so $$N - y.Nb = \frac{M - y.Na}{Aa}.Ab$$

so $$N - y.Nb = M.Ab/Aa - y.Na.Ab/Aa$$

so $$N = M.Ab/Aa - y.Na.Ab/Aa + y.Nb$$

$$= M.Ab/Aa - y[(Na.Ab/Aa) - Nb]$$

$$y[(Na.Ab/Aa) - Nb] = M.Ab/Aa - N$$

Therefore: $$y = \frac{(M.Ab/Aa) - N}{(Na.Ab/Aa) - Nb}$$

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