THE STRUCTURE AND ACTION OF TETANUS TOXIN.

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for my mother

ABSTRACT

The neurotoxin from Clostridium tetani is a protein of 150,000 M_r that has one chain of 50,000 M_r (the light chain) joined by disulphide bonds to a 100,000 M_r chain (the heavy chain). Separation of the reduced and carboxymethylated chains was achieved by electroelution of the proteins from SDS/polyacrylamide gels or by ion exchange on cellulose CM52 at pH 5.1 in urea. Separation of reduced chains was achieved by electroelution of the reduced proteins from SDS/polyacrylamide gels but isolation of the reduced chains by ion exchange was only achieved by modifying the thiols with Ellman's reagent (5,5 -dithiobis(2-nitrobenzoate)) before ion exchange on CM52 at pH 5.1 in urea. Pure light chain was obtained in a high yield from ion exchange but the heavy chain, although free of light chain was obtained in a poor yield.

The chains have different functions; the heavy chain interacts with gangliosides in the cell membrane but no function has yet been determined for the light chain. However, quantitative comparison of the amino acid compositions of the heavy and light chains by the method of Cornish-Bowden ((1983) Methods, Enzymol. 91, 60-75) suggests that there is sequence homology between the heavy and light chains. Examination of peptides produced from the chains by proteolytic cleavage supports this idea. Either V8 proteinase from S. aureus or cyanogen bromide was used to cleave the isolated chains and the digests were resolved by either SDS/polyacrylamide gel electrophoresis or reverse-phase HPLC. On SDS/polyacrylamide gels there were several peptides derived from the light chain that had the same mobility as peptides derived from the heavy chain (Taylor et al., (1983) Biochem. J. 209, 897-899). There is also a thiol containing peptide of 25,000 Mr in the V8 digest that is common to both; this was detected by autoradiography of digests of chains modified at the thiols with iodo [2 - ^{14}C] acetic acid. Monoclonal antibodies which had previously bound one of whole H or whole L were shown by immunoblotting to bind particular peptides in the digests derived from both the chains. Further evidence was obtained from HPLC of the

cyanogen bromide fragments of both chains; there were several peptides of the same retention time and one of these had the same tetrapeptide sequence at the N terminal.

The molecular action of the toxin was investigated by determining the effect of thetoxin on the calcium-calmodulin dependant phosphorylation of synaptosomal proteins. Using [γ- ^{32}P] ATP and lysed synaptosomes, tetanus toxin was shown to significantly decrease the calcium-calmodulin dependant phosphorylation without affecting the phosphorylation that occurs in the absence of calcium. The decrease in the intensity of the calcium-calmodulin dependant phosphorylations was also seen when the synaptosomal ATP was radiolabelled by incubation of the synaptosomes with [^{32}P] orthophosphate, but in this case changes in the intensity of some phosphoproteins were also seen in the absence of calcium.

Declaration

This thesis is my own composition. The experimental work is also my own except those parts where I collaborated with Dr. M. Richardson (Durham, U.K.) or Dr. K. Goretzki (Giessen, F.R.G.) and their contributions are noted in the text. Parts of the thesis have been published; C.F. Taylor, P. Britton & S. van Heyningen (1983) Biochem. J. 209, 897-899 and C.F. Taylor & S. van Heyningen FEMS Microbiol. Lett. (in press). A copy of the former is bound in the thesis.

Clare F. Taylor

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CHAPTER 1 : INTRODUCTION

1.1. The toxin and the disease

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1.1. The toxin and the disease

The disease, tetanus, is regarded as a serious health problem by the World Health Organisation, owing in part to the large number of people affected but also to the lethality of the infection. Tetanus is not a debilitating disease, it is a killer; the mortality rate is around 50-80% and the worldwide death rate has been estimated at 0.2 million per annum. (For a general introduction to the toxin and the disease see van Heyningen, 1968, and for recent reviews of the structure and function of the toxin see Mellanby & Green, 1981, and van Heyningen, 1980).

The classic symptom of tetanus is a spastic paralysis brought about by a suppression of inhibitory neurones in the spinal cord. The disease is a consequence of the infection of a wound by *Clostridium tetani*. This non-invasive bacillus can secrete a protein which is the sole cause of clinical tetanus. Tetanus toxin is one of the most toxic substances known; the estimated lethal dose for man is 60 ng (400 fmols). The high toxicity of tetanus toxin is only biological specificity writ large; the toxin is bound only to the target cells and unfortunately the target cells themselves are the control system in the body: the central nervous system.

1.2 The transport of tetanus toxin in the body and the specific binding of tetanus toxin to neuronal cells

The disparity between the site of infection and the focus of the disease is obvious and indeed was the original inspiration for the suggestion by Nicolair in 1885 (as quoted by van Heyningen, 1968) that a toxin was responsible for the disease. The means by which the toxin travels from the site of infection to the spinal cord is not relevant here and is discussed further in Bizzini (1979), van Heyningen (1980) and Mellanby & Green (1981). The toxin binds to neuronal membranes and in particular to gangliosides GD1b and GT1 (van Heyningen, 1963). The binding to

gangliosides is thought to be the first step in a 3 stage process of binding, sequestration and internalisation of the toxin (Yavin et al., 1981, 1982, 1983). The binding and internalisation of toxin is associated with non-coated membrane invaginations (Montesano et al., 1982).

1.3 The action of the toxin on the central nervous system

1.3.1 Inhibition of evoked release of neurotransmitters by tetanus toxin

The mode of action of tetanus toxin is not known. Early work established that the toxin blocked the action of inhibitory neurones, much like strychnine, but that the loci for the bacterial toxin were presynaptic rather than postsynaptic, in contrast to strychnine (van Heyningen,1968). The specificity of the toxin for inhibitory synapses seen *in vivo* is not reflected *in vitro*, and it is thought likely that the characteristic symptoms of the disease are a consequence of pharmacokinetics(Mellanby & Green, 1981, Bigalke et al.,1981 b). The toxin specificity *in vitro* is not limited to the class of neurotransmitters released. The evoked release of γ-aminobutyric acid and glycine from slices (Collingridge & Davies, 1982, Collingridge et al.,1980), synaptosomes (Bigalke et al.,1981 b) and cultured neurones (Pearce et al.,1983) is diminished by tetanus toxin, but so also are noradrenaline and acetylcholine (Bigalke et al., 1978, Bigalke et al.,1981 a,b, Habermann,1981). The latter group of transmitters are usually classed as excitatory neurotransmitters. Inhibition of release is not confined to amino acid and monoamine neurotransmitters as shown recently by Janicki & Habermann(1983) where the toxin completely abolished the evoked release of Met-enkephalin in particulate fraction of rat brain. The toxin sensitive release mechanism in cerebellar neurones in culture takes some days to develop and it parallels the appearance of action potential Na^+channels (Pearce et al., 1983).

There are several properties characteristic of tetanus toxin poisoning of the evoked release mechanism in the above processes. The toxin significantly decreases the extent of the evoked release alone; it does not alter the basal release of neurotransmitters. There is a lag of at least 60 minutes between addition of the toxin and any noted effect on release. During this

time the inhibition is sensitive to antisera but once the inhibition is manifest then the antitoxin sera has no protective ability. The degree of inhibition of the evoked release is dose and time dependant (i.e. beyond the lag phase).

1.3.2. Possible control points in the synthesis and release of neurotransmitters

The evoked release of neurotransmitters could be inhibited by blocking any one of the following processes;

(i) the uptake and synthesis of neurotransmitters or precursors.

(ii) the movement of Ca^{++} from the extracellular medium

(iii) the activity of macromolecules involved in the secretion mechanism.

Each of these processes will be discussed in more detail in the following sections.

1.3.2.1 Tetanus toxin and the metabolism of neurotransmitters

The most obvious target for a specific blockage of the release of neurotransmitters is the uptake or synthesis of neurotransmitters but this is not the case with tetanus toxin. The uptake of neurotransmitters or precursors is not altered by the toxin *in vivo* or *in vitro*. Collingridge et al., (1980) injected toxin into the nigrostriatal region of brain and some days later, when physiological changes had occurred, excised the tissue and compared both content of endogenous neurotransmitter and also the uptake with tissue from untreated animals. They found no difference in these properties but a marked decrease in the evoked release of neurotransmitter. The same result has also been seen in neuronal cells in culture (Pearce et al., 1983), and also in brain homogenate (Bigalke et al., 1981 a), and synaptosomes (Bigalke et al., 1981 b).

The conclusion of the kinetic studies was supported by direct measurement of the activity of some of the enzymes involved in neurotransmitter synthesis. Choline acetyltransferase (E.C. 2.3.1.6.) and glutamate decarboxylase (E.C. 4.1.1.15) from tetanus-toxin-poisoned preparations had activities similar to

untreated controls (Bigalke et al., 1978 and Kryzhanovskii et al., 1983 respectively). It would seem that tetanus toxin exerts its inhibitory effect on neurotransmitter release by some mechanism other than neurotransmitter metabolism.

1.3.2.2 Tetanus toxin and Ca^{++} mobilisation

An attractive alternative target, bearing in mind the central role of Ca^{++} in evoked release, is the influx of Ca^{++} or the release of Ca^{++} from intracellular stores. Although the inhibition produced by tetanus toxin is transiently alleviated by mechanisms designed to increase the intracellular Ca^{++} concentration, the movement of Ca^{++} seems not to be affected by tetanus toxin. Bigalke et al. (1981 a) measured the uptake of $^{45}Ca^{++}$ by toxin treated forebrain slices in response to high K^{+} or sea-anemone tox II. (ATX II poisons the Na^{+} pump and so causes uncontrolled entry of Ca^{++} to the neurone.) The uptake of $^{45}Ca^{++}$ induced by these agents was not altered by tetanus toxin and furthermore the presence of these agents did not prevent the inhibition of transmitter release by the toxin. The authors suggested that the toxin impeded the Ca^{++} dependant mobilisation of neurotransmitter at some stage subsequent to Ca^{++} entry.

The failure of high external Ca^{++}, A23187 or 4-aminopyridine to influence tetanus toxin induced paralysis at neuromuscular junctions also implies that tetanus toxin modifies the secretion mechanism after Ca^{++} entry (Habermann et al., 1980, Wendon, 1979). The results with A23187, (the Ca^{++} ionophore), and 4-aminopyridine (which is thought to mobilise intracellular Ca^{++} stores) suggests that not only is Ca^{++} influx not the target for the toxin but that neither are any intracellular Ca^{++} stores. Further evidence for the post Ca^{++}-entry site of action for tetanus toxin comes from Wendon (1979) who suggested this on the basis of a rather novel observation that the toxin inhibited the response of the nerve-muscle preparation to hyperosmolarity. Apparently if the fluid perfusing an isolated nerve-muscle preparation is hypersmotic then a Ca^{++} independant release of neurotransmitter occurs.

1.3.2.3. Tetanus toxin and synaptosomal macromolecules

There has been little research on identifying macromolecules in the nerve terminal that could be possible targets for the toxin. Kryzhanovskii and his colleagues found that the toxin had no effect on the sodium-potassium ion ATP-ase (Kryzhanovski et al., 1977) but later demonstrated a toxin specific inhibition of the interaction of the actomyosinlike protein in synaptic membranes and the myosinlike protein in synaptic vesicles (Kryzhanovskii et al., 1980). This latter report is interesting but it is difficult to assess the degree of credibility in these results mainly because of the minimal descriptions of method and results given. Habermann recently reported that the toxin had no effect on the adenylate or guanylate cyclase nor the calmodulin activation of phosphodiesterase but rather surprisingly these experiments were not published and the results only appeared as a closing statement in the discussion of a paper covering some other aspect of tetanus toxin function (Janicki & Habermann, 1983).

1.3.2.4. Summary and re-evaluation of possible macromolecular targets for the toxin

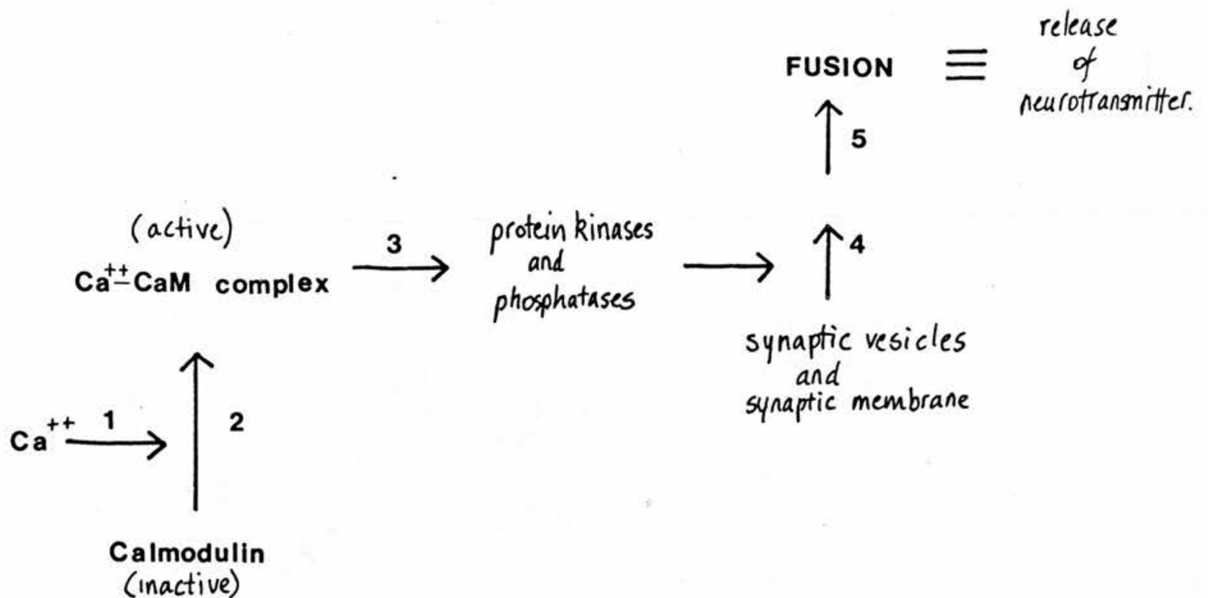
Evidence has been presented in the above section to show that tetanus toxin blocks neuronal activity at inhibitory synapses by inhibiting the release of neurotransmitter. There is evidence that it is the Ca^{++} dependant process itself that is inhibited by the toxin and not some aspect of neurotransmitter metabolism. Further the Ca^{++} mobilisation seems not to be affected by the toxin and so it would seem that the toxin interferes in the activity of the neurone at some point in the secretion process subsequent to Ca^{++} entry but before release of neurotransmitter. Perhaps a knowledge of the molecular aspects of Ca^{++} dependant secretion may clarify matters and help to identify possible targets.

1.3.3 Ca^{++}- Calmodulin and the control of neurosecretion

Stimulation of a nerve causes a wave of depolarisation to pass along the axon to the terminal where it causes a local influx of Ca^{++}. Neurotransmitter release is a consequence of the transient increase in Ca^{++} concentration (Baker et al, 1971). The molecular basis underlying the Ca^{++} dependant secretion of

Diagram 1.1.

Ca^{++}-Calmodulin Dependant Secretion and Possible Sites for Inhibition of The Whole Process.



(1) The movement of Ca^{++} (from either extracellular medium or intracellular stores).

(2) The binding of Ca^{++} by calmodulin and the resultant formation of the active complex.

(3) The interaction of the active complex with the dependant kinases or phosphatases.

(4) The phosphorylation or dephosphorylation of a particular substrate protein.

(5) The interaction of vesicles and membranes "primed" by phosphorylation changes.

neurotransmitters is not fully understood. The lack of knowledge is due to the complexity of the process itself as well as our limited understanding of the tissue in which it is observed.

The main calcium binding protein in eukaryotic cells is calmodulin, and it is now widely accepted that this protein is the major means by which calcium acts as a second messenger. Calmodulin binds Ca^{++} and the now activated complex changes the activity of a number of proteins whose biochemical activity is thought to be related to the physiological activities of Ca^{++} (Klee et al., 1980). In the brain the Ca^{++}- calmodulin complex activates a number of Ca^{++}– CaM dependant kinases and phosphatases and the resultant change in phosphorylation state of the substrate proteins following the entry of Ca^{++} is thought to be the causal event in neurotransmitter release (for review of Ca^{++} and brain phosphorylation see Nestler & Greengard, 1983, Schulman et al., 1980 and DeLorenzo, 1980.) There has been a suggestion that the change in phosphorylation state of the proteins in the synaptic membrane and the synaptic vesicle allows the structures to fuse together and so release neurotransmitter by exocytosis of vesicle contents, into the synaptic cleft (see De Lorenzo, 1980).
However the activity of many of the substrate proteins is not known and some are implicated in the release process only by the change in their phosphorylation level e.g. Protein 1 (Nestler & Greengard, 1983.) The significance of some of the other proteins to neurosecretion is known e.g. tyrosine hydroxylase, an enzyme in neurotransmitter synthesis. (The Ca^{++} – calmodulin dependant phosphorylation of tyrosine hydroxylase increases the activity of the enzyme which is useful as neurotransmitter stores are concomitantly being decreased by secretion.)

1.3.4. Control of the Ca^{++}- calmodulin system

Ca^{++}- calmodulin dependant stimulus secretion occurs in several stages and so the whole process is therefore labile at any one of the steps outlined in diagram 1.1.

The mobilisation of Ca^{++} and the metabolism of neurotransmitters are unaffected by tetanus toxin (as discussed in sections 1.3.2.1 and 1.3.2.2.). However, there is a report that

tetanus toxin does have an inhibitory action on the interaction of synaptic vesicles and synaptic membranes but it is not clear if this is the actual event in tetanus intoxication or just a consequence (Kryzhanovskii et al., 1980 and section 1.3.2.3.)

As the site of action of the toxin is apparently at some stage subsequent to Ca^{++} entry then a working hypothesis would be that it affects the Ca^{++}- calmodulin system. The toxin could inhibit K^{+} evoked release of neurotransmitter by altering the activity of proteins at any one of three main stages in the secretion process. The toxin could inhibit the stimulus-secretion process at the beginning by altering either calmodulin or a hypothetical calmodulin regulatory protein. Neurosecretion could also be inhibited by altering the activity of the Ca^{++}- CaM dependant kinases or phosphatases. A final possible target for the toxin could be the modification of a particular protein such that a certain phosphorylation is either prevented or that the effect of that phosphorylation is negated.

The Ca^{++}- CaM dependant phosphorylation changes are an ideal model to investigate further the molecular basis of the toxin's action as changes in the pattern could answer questions as to the functional integrity of the various components of the secretion process. Surprisingly, there have been no reports in the literature on the effect of tetanus toxin on the phosphorylation state of synaptosomal proteins in the "resting state" and after depolarisation. The effect of tetanus toxin on this aspect of neurosecretion is described in Chapter 5.

1.4 The structure of the toxin

The reasons for studying the structure of a protein are twofold. Firstly, the structure per se may be interesting. The structure at the sequence or higher levels may reveal information on the evolutionary history of the gene e.g. that coding for the mammalian serine proteases, or even on the structure of the gene itself e.g. the mechanism of antibody specificity or the discovery of splicing in the eukaryotic virus SV40. Secondly and more frequently, information on the structure is necessary for elucidating the molecular basis of the protein's activity. At a

mundane level, without considering such an ethereal example as a complete synthesis of the data from primary sequence, X-ray crystallography and affinity labelling of the active site, such humble characteristics as determining the N terminal residue, the amino acid composition or homogeneity on polyacrylamide gel electrophoresis can determine if the biological activity under test is a consequence of a pure protein.

Such data as the amino acid composition, the N-terminal amino acids and the thiol content have all been reported for tetanus toxin. Unfortunately, apart from the composition and the molecular weight, the results from various laboratories disagree.

1.4.1. The molecular weight of tetanus toxin

The molecular weight (M_r) of 150,000 has been determined by polyacrylamide gel electrophoresis in SDS, gel filtration and ultracentrifugation (Holmes & Ryan, 1971, Bizzini et al., 1973a, Craven & Dawson, 1973, Matsuda & Yoneda, 1974, Robinson et al., 1975, Britton, 1981.) There are two forms of the toxin; the "intracellular" which is obtained by lysis of a young culture (i.e. 2 days of growth), and the "extracellular" which is obtained from the filtrate of an old i.e. 6 day culture. There is no apparent difference in molecular weight.

1.4.2. The chain composition of tetanus toxin

Craven and Dawson (1973) showed that treatment of the extracellular toxin by reduction in urea or by oxidative sulphitolysis yielded two polypeptide fragments. The chains were the heavy chain (H) of 100,000 M_r and the light chain (L) of 50,000 M_r. The intracellular toxin could not be cleaved by disulphide scission but was otherwise identical to the extracellular form. (The criteria used were amino acid composition, thiol and disulphide content, antigenicity and molecular weight on acrylamide gels in SDS.) Matsuda and Yoneda (1974) confirmed the chain structure of the extracellular toxin. They also reported the conversion of the intracellular form to the extracellular form by digestion with trypsin. The trypsin cleavage products were thought to be identical with the heavy and light chains by mobility on acrylamide gels in SDS alone. However the intracellular toxin cleaved by trypsin was

not identical to the extracellular form, as they claimed, since two-thirds of the former could be resolved into chains of 100,000 M_r and 50,000 M_r in the absence of dithiothreitol i.e. there were two populations of tetanus toxin molecules in the trypsin cleavage products; both being two chain molecules but in one case the chains were held together by a disulphide bond. This implies that there were at least two proteolytic sites for trypsin in the intracellular form; at least one on either side of a disulphide bond connecting fragments of approximately 100,000 M_r and 50,000 M_r. Neubauer and Helting (1979) also generated fragments of 100,000 M_r and 50,000 M_r using trypsin under unspecified conditions. They found that the N terminal of the 100,000 M_r fragment was heterogenous and did not contain the leucine terminal of the heavy chain. Also either their toxin or proteolytic conditions differed from Matsuda and Yoneda (1974) as Neubauer and Helting (1979) did not get fragments released on polyacrylamide electrophoresis in the presence of SDS of their trypsin treated toxin in the absence of reducing agent.

Helting et al., (1979) isolated a protease capable of converting the intracellular toxin to the extracellular form. (The cleaved form of the toxin is sometimes called "nicked toxin" and the uncleaved similarly is called "unnicked toxin".) They were unable to determine whether the enzyme was free in the medium or attached to the cell membrane. They therefore could not say if the conversion occurred before release into the medium or in the medium itself. That Matsuda and Yoneda (1974) generally found about 5% of the extracellular toxin to be unnicked may suggest that the nicking occurred in the medium or at the very least was not mandatory for exit of the toxin from the bacillus. Another role suggested for the nicking is the activation of the toxin. Matsuda and Yoneda (1974) suggested that the intracellular toxin was a less active progenitor of the extracellular. They based this on a threefold increase in toxicity of the intracellular toxin after trypsinization (of which they gave no evidence), and by analogy with diphtheria toxin. There have been no other reports of differential toxicity of the intra- and extracellular toxins; but as it is possible that the former could be cleaved _in vivo_, then a reliable

test of this "relationship" will need the development of a suitable *in vitro* assay.

1.4.3. The amino acid composition of the toxin and of the heavy and light chains

The amino acid composition of tetanus toxin determined by different laboratories is fairly similar (Dawson & Mauritzen, 1967, Bizzini et al., 1970, Holmes & Ryan, 1971, Craven & Dawson, 1973, Robinson et al., 1975, Britton, 1981.) There are no unusual amino acids or irregularities in the number of particular amino acids to suggest possible function for the protein e.g. the high glycine and proline content of collagen. The amino acid compositions of the extracellular and intracellular forms were found to be similar and Bizzini et al. (1970) assumed that this was so. This conclusion was put on a sounder basis by Craven and Dawson (1973) who extended the comparison by also comparing thiol and disulphide content, immunological properties and molecular weight studies.

Amino acid composition data was of use in showing that the two forms of the toxin were the same but a more interesting finding emerged from the amino acid composition of the chains. The amino acid composition of the heavy and light chains were determined independantly by Britton (1981) and DiMari et al., (1982a). However both groups failed to notice the remarkable similarity between the compositions of the chains. This similarity was marked enough to suggest sequence homology between the two chains. This will be discussed in fuller detail later (Chapter 4).

1.4.4. The N terminal amino acid and the N terminal sequence of tetanus toxin and the constituent chains

As well as giving information on the purity of a protein preparation, or the processing of a protein e.g. cleavage of the signal sequence, amino terminal analysis can further peptide mapping of homologous proteins by amino-typing the peptides produced (see Chang (1983) for the application of this technique.)

Until fairly recently very little information on tetanus toxin could be gleaned from published data on the amino terminal. The identity or even the presence of the N terminal amino acid was in dispute, with various authors reporting different

Table 1.1. The N terminal amino acids of the two forms of tetanus toxin and the constituent chains.

Toxin[1]	Intracellular toxin	Extracellular toxin	Heavy chain	Light chain	Reference
asp					Soru et al.,(1958)
		blocked[2]			Craven & Dawson (1973)
	blocked[3]				Murphy et al., (1968)
gly					Holmes & Ryan (1971)
leu					Bizzini et al.,(1970)
	pro-ile	pro leu	leu	pro-ile	Neubauer & Helting (1979)
	pro-ile		blocked	pro-ile	Britton (1981)
	pro	pro(major) asn ser	ser asn	pro	Di Mari et al.,(1982b)

Notes (1) not specified in reference whether toxin was intra-or extracellular.
(2) assumed to be extracellular form as paper mentions two chains.
(3) assumed to be intracellular form as paper mentions one chain.

or blocked N terminals (Table 1.1). However Neubauer and Helting (1979) have clarified the situation and also produced a model for the linear arrangement of the heavy and light chains within the intracellular form of the toxin. They found that the intracellular form of the toxin had only one amino terminal viz pro-ile, but that the extracellular form (as expected for a two-chain molecule) had two different N terminals; pro-ile and leu. The chains were separated by polyacrylamide electrophoresis in SDS and the amino terminal of the light chain was found to be pro-ile and that of the heavy chain to be leu. Neubauer and Helting therefore deduced that the light chain was the N terminal fragment of the intracellular toxin.

Two groups have attempted to sequence the N terminal of the heavy and light chains. The sequences determined for the N terminal of the L chain are:-

Pro-Ile-Thr-Ile-Leu-Asp-Phe .... Britton (1981)
Pro-Ile-Lys-Leu-Asn-Asn-Phe-Arg-Tyr .... Robinson & Hash (1982)

These are obviously preliminary results and so nothing can be said of the differences. The dipeptide pro-ile found by Neubauer and Helting (1979) is confirmed in each case.

Both groups met difficulties in determining the N terminal sequence of the heavy chain. Di Mari et al., (1982b) found serine or asparagine followed by microheterogeneity and they also had difficulties with the solubility of their heavy chain preparation. Britton (1981) also found heterogeneity but discerned a pattern giving
Asp-Leu-Lys-Asp-Asn-Gly-Glu-Tyr-Met-Asp

The microheterogeneity at the N terminal of the heavy chain may be due to low specificity of the processing enzyme (section 1.4.2) or an artefact occurring during the purification of the toxin. Di Mari et al. (1982b) report the appearance of new amino terminals with time on storage of the toxin at 4°C which suggests that their preparation is contaminated with proteases. (This is the same group as Robinson & Hash, 1982.)

TABLE 1.2. The thiol content of tetanus toxin

Reference	Protein species	Total thiol	Free thiol	Disulphide	Reagent
Craven & Dawson (1973)	intracellular	9	6	1.5	)2-aldrithiol $\pm$ reductant)sulphitolysis
	extracellular	9			)2-aldrithiol $\pm$ reductant)sulphitolysis
	heavy chain	4	0.1 3 (+urea)	0.5	)^{14}C-IAA)$\pm$ dithiothreitol
	light chain	4	0.1 3 (+urea)	0.5	))
Murphy et al., (1968)	intracellular (?)	10	0 4 (+urea) 2 (+urea) (+HQS)	2	"by alkylation": no other details given
Bizzini et al.,(1970)	"toxin"	8 (+urea)	0 4 (+urea)	2	) IAA
		10(+6MGdHCl)	3(+2MGdHCl) 4(+4MGdHCl) 6(+6MGdHCl)	2	) IAA

(contd.)

TABLE 1.2. The thiol content of tetanus toxin (contd.)

Reference	Protein Species	Total thiol	Free thiol	Disulphide	Reagent
Bizzini et al., (1970) contd.	"toxin"	9(+urea)	2.7(-urea)		ethylene imine
		10	6		
			7.6(+urea)		PCMB
Britton (1981)	total toxin	10 ± 2			^{14}C-IAA/DTT/GdHCl)
	unnicked toxin	6			) Reduced and carboxymethylated
	heavy chain	2			) after elution from gel
	light chain	3			) ^{14}C-IAA/urea/DTT)
	total toxin	10	0	5	NEM/IAA/urea/DTT
	total toxin	10	1(+GdHCl)	4	^{3}H IAA/^{14}C-IAA
	total toxin	13			Performic Acid

1.4.5. The cysteine and cystine content of the toxin and chains

Cysteine residues can play either functional or structural roles in proteins, acting as strong nucleophiles e.g. the active site thiol in papain, or by forming disulphide bonds e.g. those in the immunoglobulins. To have a useful model of the structure of tetanus toxin, and possibly of its mechanism of action, certain information on the nature of the thiols is required. For example, the number of disulphide bonds that are intermolecular, and those that are intramolecular and also if the disulphides are easily reduced or if a denaturant is required. We also need to know the number of free thiols on each chain and if they are hidden or labile. A very labile thiol suggests that that particular cysteine is involved in the active site. Attempts have been made to obtain this information.

There seems to be general agreement on the number of total cysteines; a value of 10 is most often found. However the results on the number of disulphide bridges differ (Table 1.2.) Murphy et al., (1968) state, with no experimental detail, that a single chain form of the toxin had two disulphide bonds and six free thiols. They did not find the thiols unless the toxin was alkylated after denaturation in 8 M urea and 8 hydroxyquinoline-5-sulphonic acid.

Similarly Bizzini et al., (1970) found two disulphides and six free thiols using iodoacetic acid and 6M guanidine hydrochloride. They also found the free thiols to be innaccessible to iodoacetic acid in the absence of a denaturant. The extent of modification by iodoacetic acid was also dependant on the nature and concentration of the denaturant. The reactivity of the thiols was also dependant on the reagent used eg performic acid oxidation yielded 10 cysteic acid residues in comparison to 8 carboxymethyl-cysteines on alkylation after 2-mercaptoethanol reduction or even use of iodoacetic acid in the absence of urea gave no modified thiols whereas the use of ethylene imine revealed 3.

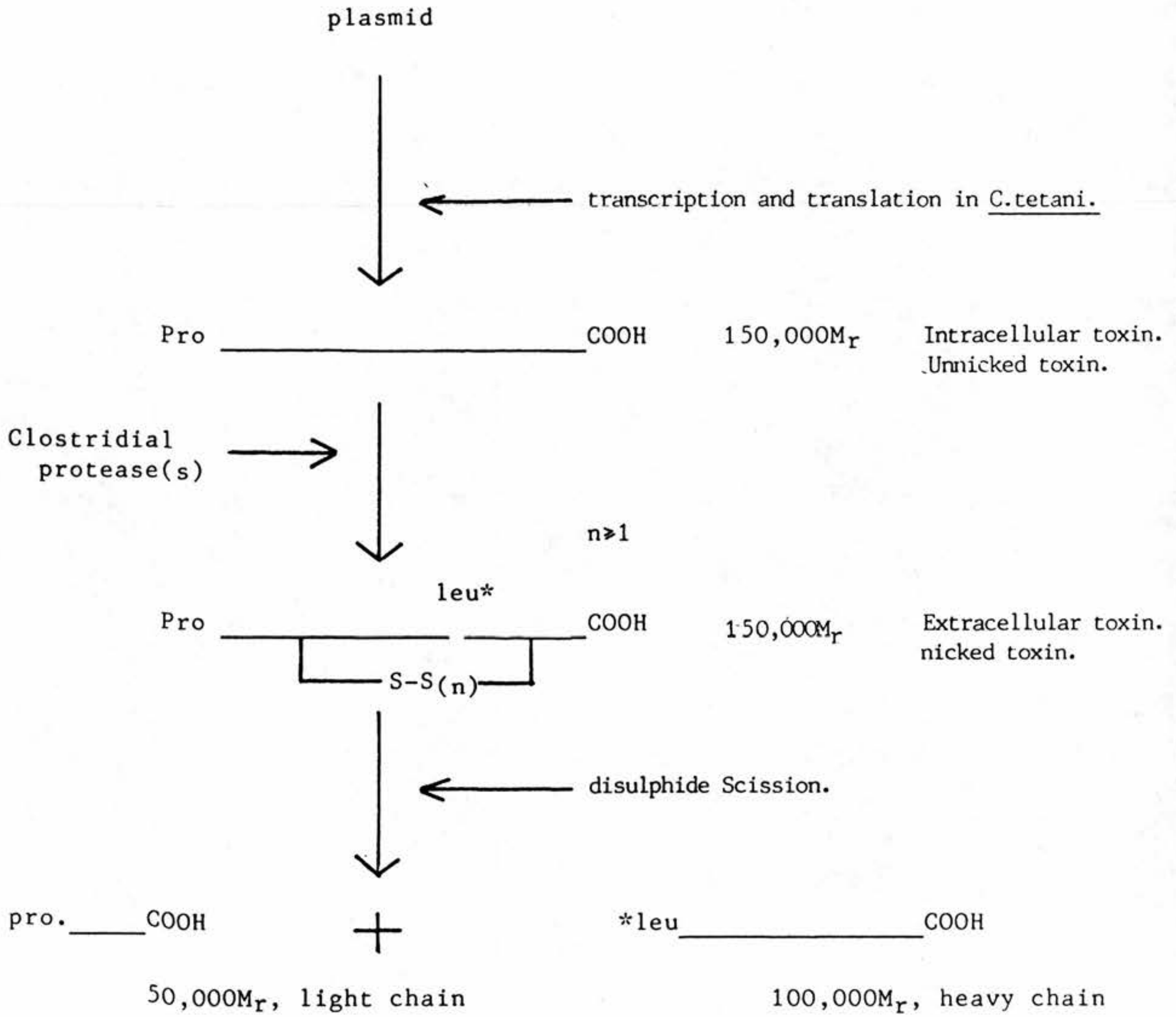
Craven and Dawson (1973) determined a total of 9 cysteines by using $NaHSO_3$ or reaction with aldithriol after reduction (without denaturant.) They also found 6 free thiols but only one

disulphide in extracellular toxin using iodo [2-^{14}C] acetic acid after reduction in 8M urea. The disulphide was intermolecular and the cysteines were equally distributed among the heavy and light chains. The free thiols were "hidden" in the sense that urea was required before any reaction was seen with the iodoacetic acid.

In common with Bizzini et al., (1970) and Murphy et al. (1968), Britton (1981) found a total of 10 cysteines using iodo [2-^{14}C] acetic acid after reduction of denatured toxin, but he obtained 13 cysteic acid residues on performic acid oxidation. A more marked difference is that Britton apparently had a much more oxidised form of toxin. By pretreating in urea or guanidine hydrochloride with N-ethylmaleimide and then alkylating with iodo [2-^{14}C] acetic acid after full reduction he found that all 10 cysteines were involved in disulphide bridges. However, on repeating with ^{3}H - labelled iodoacetic acid instead of N-ethylmaleimide he found one free thiol which implied only four disulphides and at least one free thiol (table 1.2). He found that the cysteine content was approximately the same in each chain but the low value he obtained for the unnicked toxin (6 in comparison to 10) suggests that the efficiency of recovery of his modified chains was not equal in all cases. There is the possibility that the unnicked toxin cannot be used as an internal standard in this technique as it may not be equivalent to whole toxin, however this possibility can be discounted as Craven and Dawson (1973) found that extra- and intracellular toxin had the same content of both disulphide and thiol.

The disagreement on the thiol and disulphide content of the toxin (and chains) is not too surprising because of the different means used to estimate the protein content. Three of the groups used a calculated extinction coefficient, but none of these values agreed with the others (Murphy et al., 1968, Bizzini et al., 1970, Craven & Dawson, 1973.) Britton (1981) determined the amount of protein present by amino acid analysis but when determining the thiol content of isolated chains made no correction for loss of material in recovering chains from polyacrylamide gels.

Diagram 1.2. The Structure of Tetanus Toxin.



* possibly heterogenous.

1.4.6. A structural model of tetanus toxin

Despite several inconsistencies and gaps, such as the "raggedness" or otherwise at the N terminal of the heavy chain, or the number of inter and intramolecular disulphides, a model of tetanus toxin has been built up. It is a protein of 150,000 Mr which is found in two forms: the intracellular and the extracellular. The former is processed by proteolysis to give the extracellular form which has two chains. The chains are of 50,000 Mr (the light chain) and 100,000 Mr (the heavy chain) and are linked by disulphide bonds (diagram 1.2).

1.5 The structure of the toxin in relation to its function

1.5.1 Modification of protein side chains

Formaldehyde treatment of tetanus toxin makes the protein atoxic and immunogenic. Widespread immunisation with the toxoid made this way is the major reason for the disappearance of the disease from developed countries (see van Heyningen, 1968). The protein chemistry of the conversion of toxin to toxoid is intriguing as it occurs in such a way that most of the tertiary structure (as seen by antigenic properties) is not changed yet the toxicity is entirely lost. Investigations on side chain reactivity in tetanus toxin have centred on this conversion as implicating certain residues at the "toxic site" of the protein. (The concept that the toxin has a "toxic site" is possibly rather dubious.)

Bizzini et al., (1970) found lysine and tyrosine to be the only modified residues in amino acid hydrosylates of tetanus toxoid. Covalent modification of lysine is not thought to be important in the loss of toxicity as reductive alkylation (i.e. formaldehyde treatment of toxin in presence of sodium borohydride) of the toxin does not affect toxicity (Stein & Biel, 1973), or only slightly (Robinson et al., 1975) . Circular dichroism spectra of the toxin and lysine-modified toxin were similar implying that there was no great change in the conformation of the toxin (Robinson et al., 1975). Stein and Biel (1973) also suggested that tyrosine, histidine and probably tryptophan were involved in the "toxic site" as chemical modification of these residues led to a decrease in

toxicity but apparently did not affect the flocculation value. (The flocculation value is a measure of the interaction of the modified toxin with anti-toxoid sera). The involvement of tyrosine was also investigated by Bizzini et al., (1973b) but in a more quantitative manner. They found that 3 of the 81 tyrosines were rapidly modified by tetranitromethane (TNM) in under 5 minutes and that nitration of 4-5 tyrosine residues led to an 80% decrease in toxicity and that a further 5 tyrosines modified meant a 99% loss of toxicity. The antigenicity of the protein was only altered when greater than 33 tyrosines were modified. The symptoms produced by a tri-nitrated tetanus toxin derivative were different from normal. Illness was delayed and the mice had dyspnoea before dying from flaccid rather than spastic paralysis. The tri-nitrated toxin derivative was also not fixed by nerve cells, which suggests that some, if not all, of the three modified tyrosines were involved in the binding site.

One of the difficulties with the above studies has been that the functional assay for the toxin is the toxicity in vivo, but this is not a clearly defined system as it involves the interaction of the protein with the immune system, the binding to neuronal cells and the subsequent inhibition of neurotransmitter release. A decrease in toxicity could reflect changes in any one or all of these interactions. Another difficulty in further interpreting the results is that there is neither a topological nor structural map of the toxin e.g. accessible tyrosines may be in the active site or the surface of the toxin or the nitration of 9 tyrosines may reflect summation of a heterogenous population of modified residues (see Glazer et al. 1975 for a discussion of this phenomenon).

A common theme running through the literature was the acceptance of the concept that the toxoiding by formaldehyde had the observed effect by modifying residues important in either the actual active site or causing small changes in the conformation of the active site. The toxoid was known, from the antisera reaction and the circular dichroism spectra, not to have a different conformation. They did not consider that the formation of inter- and intrachain methylene bridges could lead to the formation of a crosslinked molecule that would have the structure of the native toxin but

would be constrained and unable to undergo induced conformational change or separation of chains, both of which may be important in the "active" form of the protein. It may be relevant here that the tetranitromethane reaction with toxoid (Bizzini et al., 1973b) could only achieve 50% of the toxin modification, this may be that the tyrosines are already modified but it could also be that the molecule (i.e. the toxoid) cannot breathe.

1.5.2. The activity of isolated chains

As with all multichain proteins the active site(s) of tetanus toxin may lie between the chains, like the antigen binding site formed by the heavy and light chains of the imunoglobulins, or another possibility is that the active sites are located on particular chains. Many plant and bacterial protein toxins have a common scheme of action in which an enzymatically active chain is linked by disulphide bonds to a cell binding polypeptide (van Heyningen, 1982).

Tetanus toxin does have two chains joined by disulphide bonds. The action of the toxin on the nervous system is a consequence of the whole protein as isolated chains are atoxic (Matsuda & Yoneda, 1975). There is evidence that the ganglioside binding site is in the heavy chain (van Heyningen, 1976, Helting et al., 1977). However there is no evidence that the intracellular action of the toxin is either enzymic or a function of the light chain alone.

1.5.3. The activity of proteolytic fragments

Partial proteolysis of tetanus toxin has been used to define further the structure-function relationship in the heavy and light chains. Helting and Zwisler (1974, 1977) isolated two major fragments on treating the toxin, whether intra- or extra-cellular, with papain. The B fragment of 100,000 Mr, contained two chains of 48,000 Mr and 46,000 Mr linked by disulphide bonds, and the C fragment was a single chain molecule of 50,000 Mr. By analysis on polyacrylamide gel electrophoresis in SDS and of immunological cross reactivity with the heavy and light chains, fragment C was found to be part of the heavy chain and fragment B to be the light

chain plus the complementary fragment of the heavy chain. On the basis of N terminal determinations alone, the heavy chain derived fragment in B was identified as the amino-terminal of the parent chain (Neubauer & Helting, 1981, and see section 1.4.4.). They placed the C fragment at the carboxyl terminal of the heavy chain as the N terminal determined for the C fragment was not that found for the heavy chain. A small criticism is that the C fragment was not homogeneous as they claimed. There were 3 bands on a native gel and 2 bands on polyacrylamide gel electrophoresis in SDS, one of which was lost on reduction of the samples (Helting & Zwisler, 1977). Amino terminal analysis of fragment C gave several amino acids, one of them being leucine (the N terminal amino acid of the heavy chain) and it was only by eluting one of the bands from the SDS polyacrylamide gels that they obtained a single amino terminal. They suggested that the heterogeneity on gels was due to different levels of amidation in the same protein. Deamidation of glutamine and asparagine does occur in aqueous solution but probably not to the extent as is claimed in the literature as an excuse for heterogeneity in supposedly pure proteins.

Despite these small structural anomalies the function of fragment C is known. Radio-labelled fragment C has the same specificity and kinetics as radio-labelled toxin in binding to either brain membranes or gangliosides (Morris et al., 1980, Goldberg et al., 1981). These findings contradict earlier work which showed fragment C to have only 0.2% of the ganglioside binding capacity of whole toxin (Helting et al., 1977). As determined earlier with the heavy chain, fragment C was also found to be non-toxic despite the binding ability. This absence of neurotoxicity further emphasises that in tetanus toxin poisoning of a neurone the membrane binding event is not sufficient in the same way that say adrenaline binding to its receptor on the cell membrane alters the metabolism of that cell.

Fragment B had no binding abilities and was also thought to be nontoxic but later work showed that high doses of

fragment B were lethal (Helting & Zwisler, 1977 and Helting et al., 1978, respectively). However, the symptoms were not those of classical tetanus toxin poisoning ie.the characteristic spastic paralysis, but rather death was due to several factors including general exhaustion and failure to take fluids or food. Although injection of 200 μg of fragment B into a mouse would only have to contain a 4.5 x 10^{-6}% w/w impurity of toxin in order to provide the toxin lethal dose, the activity is apparently fragment B specific because of the effect of antisera and also the atypical symptoms. Anti-C fragment sera protected animals against tetanus toxin poisoning but had no effect on the B fragment toxicity. The authors suggest that the observed differences in symptoms and toxicity are due to an impaired capacity of B to bind to toxin receptors in the nervous system, yet they still propose disturbance of the nervous system by fragment B as the cause of death. They did not consider some other non-neuronal effect of tetanus toxin, knowing that the toxin affected the nervous system they assumed that any fragment having activity would follow suit. It is conceivable that removal of the nervous system specific binding agent i.e.fragment C, and the concomitant loss of the ability to be specifically taken up by the nervous system might leave the altered protein, i.e. fragment B, free to react with other cells and so have the observed effect. As an example, the adrenal of all poisoned animals was grossly distended and it was the organ with the most marked difference to non-poisoned animals. The adrenal is an extremely important part of the hormone system and it is possible that altering it might lead to death.

Other workers have tried to reproduce the papain cleavage of Helting and Zwisler (1974, 1977) but with varying success. In general other people get much more proteolysis and the fragments obtained differ from those of Helting and Zwisler in molecular weight, immunological properties and thiol lability.

Robinson et al. (1978) obtained a fragment of 56,000 Mr containing two chains of 32,000 Mr and 23,000 Mr linked by disulphide(s). Optical rotatory dispersion and circular dichroism of the fragment showed it to have a stable tertiary

structure and the secondary structure to be mainly β sheet. They suggested that this fragment was equivalent to Helting's fragment C. They had no evidence for this and their fragment differed in molecular weight and was a two chain molecule and not a one chain molecule. Similarly Matsuda and Yoneda (1977) only obtained one fragment when using the Helting & Zwisler (1974) papain procedure but they obtained two immunologically distinct fragments by using milder digestion conditions. These fragments were similar to those of Helting & Zwisler (1974, 1977) in that one was part of the heavy chain, and the second fragment contained the light chain and some part of the heavy chain. They differed in that both of Matsuda and Yoneda's fragments were thiol sensitive and became low molecular weight molecules lacking any immunological characteristics common to the fragments or chains. It is regrettable that the characterisation of the Matsuda & Yoneda fragments was by immunoelectrophoretic properties alone and that there was no further analysis e.g. by gel electrophoresis in SDS.

Sato et al.(1979) digested toxin with subtilisin and obtained an immunogenic atoxic fragment, which they designated S. Fragment S had 3 bands on native gels and was partially identical with whole toxin in double immunodiffusion experiments. There was no further chemical characterisation of this fragment.

Bizzini and his colleagues obtained equivalent fragments by either freeze-thawing the toxin or using papain. They found fragments of 30,000 Mr to be toxic, but at the toxicity levels seen this is more likely to be toxin impurities in the fragment preparations. They also found fragments of 100,000 Mr and 50,000 Mr that were said to be equivalent to the B and C fragments of Helting (Bizzini, 1978, Bizzini et al.1977, 1980, 1981).

Boquet & Duflot (1982) claimed that the heavy chain and the B fragment (both supplied by Bizzini) formed channels in single walled asolectin phospholipid vesicles at pH3 and that this channel forming was relevant to the mode of action of the toxin. In their system it is not possible to differentiate between the exposure of hydrophobic residues by conformational changes brought about by 'activation' of the toxin or fragment (i.e. physiologically

meaningful) or by acid denaturation of the toxin (which is more likely).

It is unfortunate that the chemical typing, with respect to peptide mapping or thiol location, of the chains and fragments was not at a more advanced stage as perhaps then the differences between the fragments produced by the various laboratories could be resolved and a fuller picture of the molecule obtained. As yet all that can be said is that the protein appears to have structural domains and that one of these domains, Helting's fragment C, has a function i.e. binding to the toxin's membrane receptor and that the "enzymc" activity of the toxin may reside in the light chain or the N terminal domain of the heavy chain.

1.6. The scope of the present work

The aim of the experimental work in the present study was threefold; the development of a method for the separation of the chains of tetanus toxin, the determination of the homology between the chains and finally an investigation of the involvement of tetanus toxin with the Ca^{++}-calmodulin dependant phosphorylations in rat brain.

Chapter 2 : Methods,

2.1 Purification of Tetanus Toxin.

2.2 Ouchterlony Technique.

2.3 Polyacrylamide Gel Electrophoresis.
2.3.1 Buffer Systems.
2.3.1.1 Weber and Osborne.
2.3.1.2 Laemmli.
2.3.2 Detection of Proteins in Gels.
2.3.2.1 Coomassie Brilliant Blue.
2.3.2.2 Silver Staining of Proteins in Polyacrylamide Gels.
2.3.2.3 Detection of Radioactive Proteins in Gels.
2.3.2.3.1 Autoradiography of Gels.
2.3.2.3.2 Liquid Scintillation Counting of Gel Pieces.
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2.3.3 Elution of Proteins from Polyacrylamide Gels.

2.4 Covalent Modification of Protein Thiol Groups.
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2.5 Proteolytic Cleavage.
2.5.1 Chemical Cleavage of Proteins.
2.5.1.1 Cyanogen Bromide.
2.5.1.2 Ō-Iodosobenzoic Acid.
2.5.2 Limited Proteolysis in Sodium Dodecyl Sulphate (The Cleveland Technique).

2.6 Analysis of Proteolytic Digests.
2.6.1 Polyacrylamide Gels in Sodium Dodecyl Sulphate.
2.6.2 High Performance Liquid Chromatography, (HPLC).
2.6.3 N Terminal Sequence Analysis.

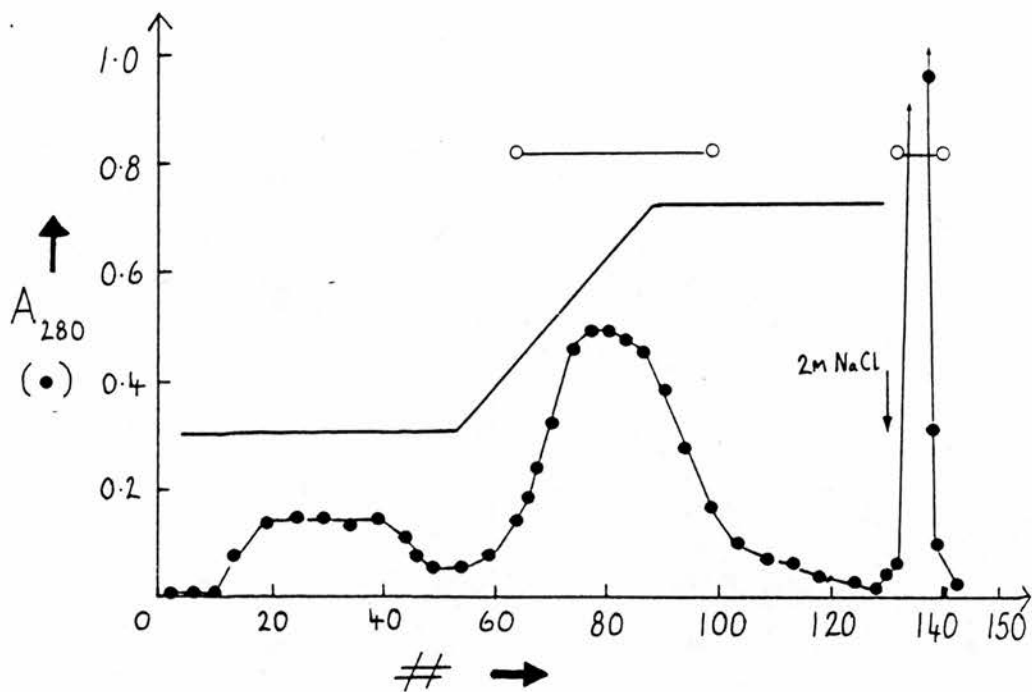
The methods for separating the chains of tetanus toxin are described in chapter 3 and all methods for isolation of synaptosomes or phosphorylation of synaptosomal proteins are described in chapter 5.

2.1. Purification of Tetanus Toxin.

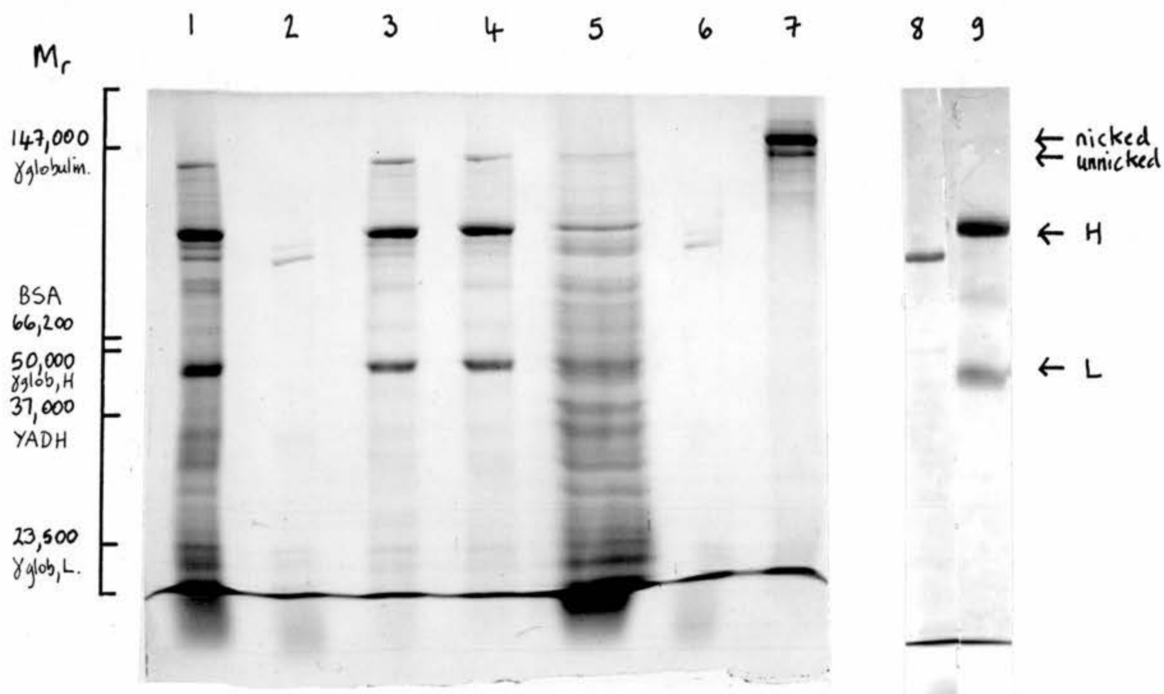
Tetanus toxin was purified from a crude culture filtrate obtained from Dr R.O. Thomson of the Wellcome Research Laboratories, Beckenham, Kent. All the usual precautions were taken in handling this toxic material viz all workers were immunized against the toxin, protective clothing was worn when handling the toxin and all glassware in contact with the toxin was acid washed after use. The toxin was purified on a linear phosphate gradient on DEAE Cellulose at pH7.2 (van Heyningen, 1976)

300mg of crude toxin (weight including salt) were dissolved in 10ml of 10mM sodium phosphate pH7.2 containing 1mM benzamidine, 0.1mM triethylenetetramine. The crude protein was preincubated at 4°C for one hour in the presence of 0.1mM phenylmethyl sulphonyl fluoride (PMSF) before dialysis against the 10mM phosphate buffer. The non-diffusable material was diluted ten fold in the 10mM phosphate buffer and run onto a Whatman DE52 DEAE - Cellulose ion exchange chromatographic column (2cm x 6cm) previously equilibrated with the same buffer. The flow rate was 2.5ml min^{-1}. The column was washed with 10mM phosphate buffer containing 0.1mM PMSF to remove any unabsorbed material. The toxin was eluted using a linear gradient of 10-100mM sodium phosphate. Protein was detected by measuring the absorbance of the fractions at 280nm. The tetanus toxin - specific peaks were detected by using equine antitetanus toxin in the double diffusion technique of Ouchterlony (section 2.2) and the purity of these fractions were assayed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE, section 2.3). Two of the peaks contained material reacting with antitoxin in an Ouchterlony immunodiffusion, they were eluted at 100mM NaCl and at 1M NaCl. The latter samples were shown by SDS-PAGE to contain mostly impurities and the former was pure toxin. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate of the purified toxin showed one band in non reducing conditions and three bands in the

(a)



(b)



presence of reducing agents. The two main bands were the heavy chain (H) of 100,000 M_r and the light chain (L) of 50,000 M_r. Whether or not there was any unnicked toxin (U) of 150,000 M_r was dependant on the batch preparation eg Wellcome TD822D always contained unnicked toxin whereas Wellcome batch TD881C did not. (see figure 2.1). The yield of toxin was generally 70%.

2.2. Ouchterlony Double Diffusion.

Double diffusion plates were prepared by the method described by Ouchterlony (1958). Six microlitres of horse anti-tetanus toxin serum (Wellcome) was put in the centre well within a ring of six other wells. Aliquots (6 μl) of the samples under test were put in the exterior wells (at a distance of 2mm from the centre well). Precipitin bands were visible after diffusion overnight at 4°C or in 2 hours if at room temperature.

2.3. Polyacrylamide Gel Electrophoresis.

2.3.1. Buffer System.

Two types of sodium dodecyl sulphate systems were used; the sodium phosphate method of Weber and Osborne (1969) and the Tris chloride discontinuous method of Laemmli (1970).

2.3.1.1. Weber and Osborne.

Gels referred to as "tube gels" were 5% acrylamide gels (of 7cm in length and 0.5cm diameter) that were prepared and electrophoresed as described by Weber and Osborne (1969).

2.3.1.2. Laemmli.

Slab gels (15cm x 15cm x 0.13cm) were electrophoresed in vertical tanks as described by Studier (1973). The gel and buffer systems were those of Laemmli (1970) with the modifications introduced by Douglas and Butow (1976). The modifications were the inclusion of 2mM EDTA in the gels and the electrophoresis buffer, and the addition of polyacrylamide ($M_r > 5x10^6$) to a final concentration of 0.5% (w/v) in the separating gel to increase the tensile strength.

2.3.2. Detection of Proteins and Peptides in Gels.

2.3.2.1. Coomassie Brilliant blue.

All fixing, staining and non-electrophoretic destaining was at 55°C and with continuous agitation. Slab gels were fixed in 20% (v/v) MeOH/10% (v/v) HAc for 15 minutes and stained in 2.5% (w/v) coomassie brilliant blue R150 in 45% (v/v)

MeOH/10% (v/v) HAc for 15 minutes. The gels were destained in 5% (v/v) MeOH/7.5% (v/v) HAc in under two hours if small pieces of polyurethane foam were added to absorb the dye. Gels were dried onto Whatman 3mm paper under vacuum and with heating using a Hoeffer slab gel dryer.

If the proteins were to be eluted from the gel then they were stained for 10 minutes without prior fixing and rapidly destained in 50% (v/v) MeOH/7% (v/v) HAc (about 10-15 minutes) before excision of the bands from the gel (see section 2.3.3 for details on the elution of proteins).

Tube gels were stained for one hour without prior fixing and destained in 7.5% (v/v) HAc/5% (v/v) MeOH by transverse electrophoresis.

2.3.2.2. Silver Staining of Proteins in Polyacrylamide Gels.

The recently developed technique of silver staining of proteins in polyacrylamide gels is 10-100 times more sensitive than coomassie brilliant blue. The method of Wray et al. (1981) was found to be the most reliable and the least time consuming.

The slab gels are fixed in the normal manner and then soaked in three changes of 50% (v/v) MeOH to remove glycine or acetic acid. The stain was prepared by adding solution A (0.8g $AgNO_3$ in 4ml of distilled water) dropwise to solution B (a mixture of 21ml of 36% (w/v) NaOH and 1.4ml of 14.8 M NH_4 OH) with constant stirring. The stain was made to 100ml with distilled water and used within 5 minutes of preparation. The stain had to be clear and if at any time during the preparation a cloudy brown precipitate formed then the stain was discarded. The gel was stained for 15 minutes at room temperature with constant agitation. The stain was then removed and the gel washed in distilled water for 5 minutes. (The ammonicial silver solution in the used stain is explosive and so was disposed of by adding to a saturated solution of sodium chloride). The developer was prepared freshly by mixing 2.5ml of 1% (w/v) citric acid and 250 μl of 38% (w/v) formaldehyde and making to 500ml with distilled water. The gel was immersed in the developer for 8-15 minutes with shaking at room temperature. Developing beyond 15 minutes was to no avail as the gel would develop within this time or not at all. The development was stopped by immersing the gel in 50% (v/v) MeOH/10% (v/v) HAc.

If the gel was overstained then the staining was reversed in 1-2 minutes by using a destain made by mixing equal volutions of solution I and solution II (personal communication, John Coggins, University of Glasgow.) Solution I was made by dissolving 3.7g sodium chloride and 3.7g cupric sulphate in 85ml of distilled water and adding ammonia until a deep blue colour was obtained, then the solution was made to 100ml with distilled water. Solution II was 43.6g sodium thiosulphate per 100ml of distilled water.

2.3.2.3. Detection of Radioactive proteins in gels.

Generally autoradiography was used to detect radioactive bands and densitometric traces of the autoradiograph, or liquid scintillation counting of excised gel pieces was used to quantify the radioactivity.

2.3.2.3.1. Autoradiography of Gels.

Radioactive proteins or peptides were detected either directly or by fluorography depending on the activity of the samples. In direct autoradiography the dried gels were placed in contact with AGFA GEVAERT curix RP1 X-ray film in the dark at room temperature for the required time.

The detection of ^{14}C labelled peptides in gels was enhanced by soaking the gel in 1M sodium salicylate for 30 minutes and exposing the dried film to preflashed film at -70°C (Chamberlain, 1979).

The detection of high energy nuclides such as ^{32}P or ^{125}I was enhanced by using Dupont Cronex Lightening Plus intensifying screens and preflashed film (Laskey & Mills, 1977, Laskey, 1980).

The films were developed by 5 minute immersions in developer, followed by stop solution and finally in fixer. The developer was 17% (v/v) Agfa G150, the stop was 5% (v/v) acetic acid and the fix was 19.5% (v/v) Agfa G334/2.4% (v/v) Agfa aditan hardener.

2.3.2.3.2. Preparation of Gel pieces for Liquid Scintillation Counting.

Dried gels were cut in 1mm slices. The slices were reswollen by soaking overnight in 0.5ml of distilled water. The swollen slices plus the soak were counted in a Searle Mark III scintillation counter, after the addition of 10ml of scintillation

fluid to the samples. The scintillation fluid was 700ml toluene containing 0.15g 1,4-di-[2-(4-methyl-5-phenyloxazolyl)]-benzene, 5.5g of 2,5 diphenyloxazole made up to 1 litre with triton X-100.

2.3.2.4. The Immune Replica Technique (Towbin et al, 1979).

A replica of the pattern of proteins or peptides separated on a polyacrylamide gel in sodium dodecyl sulphate can be transferred to nitrocellulose by electroblotting. The nitrocellulose electroblot is then decorated with antibodies specific for some component of the protein mixture. The presence and position of the antigen is revealed because the antibody binding to the antigen can be detected by isotopic or chromogenic techniques (Towbin et al, 1979). The isotopic method originally used was autoradiography of nitrocellulose membranes after incubation with ^{125}I labelled protein A (Protein A from Staph Aureus binds to Fc region of antibodies, see Surolia et al., 1982 and Surolia, 1982.) However equally sensitive results were obtained but more rapidly by the chromogenic assay introduced to our laboratory by Dr K. Goretzki (Giessen, F.R.G.) and so this method was used preferentially.

The proteins were transferred to the nitrocellulose by electroelution in a Shandon Electroblot Apparatus at 10 V for 2-3 hours. The transfer solution was 20mm $Na_2 HPO_4$ /0.2% (w/v) SDS/20% (v/v) MeOH (D.K.Apps, Edinburgh). The subsequent incubations of the blot were at room temperature with shaking. Non specific binding to the nitrocellulose was saturated by 30-45 minutes incubation with 50mM Tris-chloride pH7.4 containing 150mM NaCl/10% sheep serum /1% (w/v) bovine serum albumin /0.1% Tween 20 (saturation solution). The monoclonal antibody was added to the saturation solution at a dilution of 10^{-2} to 10^{-4} of culture supernatant fluid and the incubation was continued for a further 2 hours. After three washings of 15minute duration with 50mM Tris-chloride pH7.4, the anti-mouse γ globulin (sheep) linked to peroxidase, at 10^{-2} to 10^{-4} dilution in saturation buffer was added and the nitrocellulose filter incubated for 2 hours. The washing with 50mM Tris-chloride was then repeated. For the colour reaction the blots were soaked in a solution of 0.06% (w/v) 4-chloro-1-naphthol/0.01% H_2O_2 /150mM NaCl/50mM Tris chloride pH7.4. This was prepared freshly from stock solutions of 3mgml $^{-1}$ 4 chloro-1-naphthol in methanol and 30% H_2O_2. The reaction was continued for 10-30 minutes and terminated

by washing with water. The antigen antibody complexes stained as blue bands against a white background.

2.3.3. Elution of Proteins From Gels.

Proteins were recovered from SDS-polyacrylamide gels using the method of Walker et al. (1982). The proteins were detected by rapid staining and destaining in unfixed gels with coomassie (section 2.3.2.1). The bands were excised and cut into small pieces using a scalpel. Walker et al. (1982) used conventional tube gel apparatus as the elution chamber and had disposable plastic pasteur pipettes as supports for the gel pieces. The plastic pipettes were unsuitable and instead small glass tubes (7cm x 0.5cm internal diameter, widened at one end to 1.5cm internal diameter) were used.

A small plug of acid washed glass wool was inserted inside the tube and the tube inserted into the top compartment (cathode) of the tube gel apparatus. An 11cm length of 8/32 dialysis tubing was pushed onto the narrow end of the tube and knotted so that the knot was just above the platinum electrode when the end of the tube was put into the bottom reservoir (anode). The gel pieces were put inside the glass tube and a second glass wool plug was inserted on top to keep the gel pieces in place. Both compartments of the tube gel apparatus were filled with elution buffer (the Laemmli electrophoresis buffer, section 2.3.1.2) and any air bubbles in the tubes were removed using a hypodermic syringe. The protein was eluted from the gel pieces by electrophoresis at 3mA/tube for 16 hours. The sodium dodecyl sulphate and coomassie were removed by dialysis against 10% (v/v) EtOH for 24-36 hours, freeze drying and washing the protein in acidified acetone (0.15% (w/v) HCl in acetone). Using this method the protein was routinely isolated in a high yield (80%) and in a pure form eg diagram 3.1.

2.4. Covalent Modification of Protein Thiol Groups.

2.4.1. Reduction of Disulphide Bonds.

Pure toxin was dissolved in 0.1M Tris chloride pH8.2 containing 8M urea/1mM benzamidine/ 2mM EDTA. Phenyl methyl sulphonyl fluoride (PMSF) $10mgml^{-1}$ in propan 2-ol, was added to a final concentration of 0.1mM. Dithiothreitol at $15.4mgml^{-1}$ (aq) or in the above buffer was added to a tenfold molar excess to protein

disulphide and the sample left for 1 hour under an atmosphere of nitrogen. The reaction volume was generally 100-200μl or sometimes 5ml for a large scale preparation of reduced toxin.

2.4.2. Carboxymethylation of thiol groups Using Iodoacetic Acid.

Iodoacetic acid (IAA) was recrystallised from n-heptane. A 10g sample of IAA was dissolved in 20ml of n-heptane by heating in a steam bath. The solution was filtered through a hot filter funnel under vacuum. The IAA was then recrystallised by cooling the solution on ice. The white crystals of IAA were then filtered off and recrystallised once more. The IAA was dried in an evacuated desiccator in the dark. The IAA was kept in dark bottles in a desiccator at 4°C. It was dissolved shortly before use in the Tris urea buffer (section 2.4.1) and the pH readjusted to pH8.2 with concentrated HCl.

To avoid unwanted side reactions the pH of the modification was 8.2 and the reaction and subsequent dialysis were in the dark to prevent formation of the iodine radical.

IAA in Tris urea buffer was added, in a tenfold molar excess to total thiol, to a protein sample reduced as described in section 2.4.1 and the sample left for a further hour in the dark at room temperature. The reaction was terminated by the addition of dithiothreitol to a slight excess over the alkylating agent. The sample was dialysed extensively against 50mM NH_3 in the dark and the protein recovered by lyophilization.

The toxin was radiolabelled at the thiol groups following the same procedure as above by treatment of the reduced toxin with Iodo [2-^{14}C] acetic acid (25μCi of 6.25mCi/mmol).

2.4.3. Reversible Covalent Modification of Protein Thiols.

Ellman's reagent, 5,5'-dithiobis (2-nitrobenzoic acid) dissolved in 0.1M Tris chloride pH8.0 containing 8M urea/2mM benzamidine/2mM EDTA was added to toxin reduced as above (section 2.4.1), in a 20 fold molar excess to the total thiol content. The pH was rapidly adjusted to 7.5 with additions of 0.1M NaOH. The sample was left under nitrogen for 2-4 hours. The modified protein was dialysed against several changes of the loading buffer for the ion exchange (see section 3.2.2.3). The method for reversing this modification is described in section 3.2.2.4.

2.5. Proteolytic Cleavage.

Except where noted, all proteins were reduced and carboxymethylated (section 2.4.1 and 2.4.2) before cleavage to avoid any microheterogeneity arising from oxidation of thiols or disulphide rearrangement and to minimise side reactions eg ō - Iodosobenzoic acid oxidises cysteine to either sulphenic acid or to unwanted disulphide species (Mahoney & Hermodson, 1979).

2.5.1. Chemical Cleavage of Proteins.

2.5.1.1. Cyanogen Bromide.

The proteins were dissolved in 100 μl of 70% (v/v) formic acid containing an equal weight of cyanogen bromide and the samples left for 24 hours in a stoppered vial at room temperature. The reaction was terminated by diluting the sample tenfold with distilled water and freezedrying. The digest was resuspended in distilled water and freezedried again, this was repeated.

2.5.1.2. ō - Iodosobenzoic Acid, (ō-IBA).

The ō-IBA was preincubated in 80% (v/v) HAc containing 4M guanidine hydrochloride and a 0.1 M equivalent of p-cresol for 2 hours in the dark at room temperature. The preincubation with p-cresol was to limit the selective cleavage to tryptophan. The proteins were dissolved in 100 μl of this solution at a 1:2 w/w ratio of protein : ō-IBA and left for a further 24 hours in the dark at room temperature. The reaction was terminated by the addition of thiol (DTT) to a slight molar excess. The peptides were recovered by diluting the digest tenfold with distilled water and freezedrying. (Fontana et al., 1981).

2.5.2. Limited Proteolysis in SDS (The Cleveland Technique).

Peptide mapping after limited proteolysis was performed according to Cleveland et al. (1977). The toxin was first reduced and carboxymethylated as described in sections 2.4.1 and 2.4.2., and the modified protein was subjected to electrophoresis in 8% polyacrylamide gels (Laemmli, 1970, and also see section 2.3.1.2.). The gel was stained with coomassie and destained briefly as described in section 2.3.2.1., and bands containing the heavy chain, the light chain or the unnicked form of the toxin were excised with a scalpel blade and then soaked for 30 minutes at room temperature with occasional swirling in 10ml of 125mM Tris-chloride pH6.8 containing 0.1% (w/v) SDS and 1mM EDTA. (At this stage the gel pieces were sometimes stored at -20°C). The gel pieces were placed in the sample wells of a second gel and overlayed with 10-20 μl of the soaking buffer containing 20% (v/v) glycerol and 0.01% (w/v) bromophenol blue. Each gel slice was then overlaid with 10μl of soaking buffer containing 10% (v/v) glycerol and *Staph. aureus* V8 proteinase at 16μgml^{-1}. Electrophoresis was performed as usual except that the voltage was turned off for 30 minutes when the

bromophenol blue reached the bottom of the stacking gel.

2.6. Analysis of Proteolytic Digests.

2.6.1. Polyacrylamide Gels in Sodium Dodecyl Sulphate.

Slab gels of the Laemmli type were prepared and run as described in section 2.3.1.2. The total peptides were detected by silver staining of the gels and the [2-^{14}C]- carboxymethyl peptides were detected by fluorographic enhancement as described in sections 2.3.2.2. and 2.3.2.3.1. respectively.

2.6.2. High Performance Liquid Chromatography, (HPLC).

The isolated reduced and carboxymethylated chains were treated with cyanogen bromide as described earlier, and the resulting fragments were fractionated by reverse phase HPLC. The method used was that of Campos & Richardson (1983) but for further details on seperation of peptides by this technique see Hermodson & Mahoney (1983).

The fragments from each chain were dissolved (3mgml^{-1}) in 6M guanidine in 0.1% trifluoroacetic acid. Both solutions were blue presumably because the chains had been isolated by the SDS-PAGE method (section 3.2.1.) and contained traces of coomassie brilliant blue. The samples were viscous and so were centrifuged to remove insoluble material. (This additional step did not alter the peptide profile obtained after HPLC but it did relieve pressure problems met in the initial runs).

The peptides were fractionated by reverse phase HPLC on a Varian model 5000 HPLC fitted with a μ Bondapack C-18 column (0.5cm x 2cm) and using a linear gradient of 0-70% acetonitrile in 0.1% trifluoroacetic acid. The peptides were detected by absorbance at 214nm and all peaks were collected and lyophilized.

2.6.3. N Terminal Sequences Analysis.

The reduced and carboxymethylated toxin and cyanogen bromide fragments from the heavy and light chains were subjected to micro-sequence analysis using the 4-N,N- dimethylaminoazobenzene -4'-isothiocyanate/phenyl - isothiocyanate (DABITC/PITC) double coupling technique (Chang et al., 1978) with a few minor modifications.

The freeze dried cyanogen bromide peptides and the reduced and carboxymethylated toxin (approx 10nmol of the latter) were dissolved in 100μl of aq. 50% (v/v) pyridine and 80μl was transferred to an acid washed tube (0.6 - 0.8cm diameter x 5cm) fitted with a

Quickfit glass stopper. The tube was flushed with nitrogen while 40 μl DABITC solution (10nmol/μl pyridine freshly prepared) was added. The tube was stoppered and left in an oven at 52°C for 50 minutes. After the first coupling, 10 μl PITC was added, the tube was flushed with nitrogen, sealed and vortexed and left in the oven at 52°C for a further 20 minutes. After the reaction, the excess reagents and by-products were removed by mixing the reaction mixture with three portions of 250μl of heptane/ethyl acetate (2:1,v/v) on a vortex mixer and centrifuging. The organic phase was removed carefully and discarded. The mixture was evaporated in a high vacuum. The dried residue was dissolved in 40 μl 50% aq trifluoroacetic acid, flushed with nitrogen, sealed with a glass stopper and left in the oven at 52°C for 5-10 minutes. The sample was evaporated in a high vacuum and dissolved in 50 μl of water. The cleaved DABTZ-amino acids (and PTZ-amino acids) were extracted by mixing with 150 μl of butyl acetate on a vortex, centrifuging and removing the organic phase. The peptide in the water phase was freeze dried and subjected to the next degradation cycle. The organic phase was evaporated and the thiazolinone amino acids were converted to thiohydantoins by the addition of 50 μl of 50% trifluoracetic acid and heating at 80°C in an oven for 10 minutes. The sample was dried and redissolved in 5μl ethanol and 2 μl of ethanol extract was used for TLC identification. The samples were run on polyamide sheets (2.5cm x 2.5cm) in acetic acid/water (1:2,v/v) as the first dimension and toluene/n-hexane/acetic acid (2:1:1, by volume) as the solvent for separation in the second dimension. The plates were dried and exposed to HCl vapour.

Chapter 3 : The Separation of The Heavy and Light Chains of Tetanus Toxin.

3.1 Introduction.

3.2 Methods.

3.2.1 Separation of The Chains by Polyacrylamide Gel Electrophoresis in SDS.

3.2.2 Separation of The Chains by Ion Exchange.

3.2.2.1 Development of System.

3.2.2.2 Preparative Ion Exchange of Reduced and Carboxymethylated Toxin.

3.2.2.3 Preparative Ion Exchange of DTNB - Modified Toxin.

3.2.2.4 Removal of the 2 Nitro - 5 - Thiobenzoate Group and Re-Oxidation of Protein Thiols to Disulphides.

3.2.3 Separation of Chains by HPLC.

3.3 Results.

3.3.1 The Separation of The Chains of Tetanus Toxin by Electrophoresis in SDS.

3.3.2 Separation of Reduced and Carboxymethylated Chains on Cellulose CM 52.

3.3.3 Separation of reduced Chains and of DTNB modified Chains on Cellulose CM52.

3.3.4 Isolation of The Chains by HPLC.

3.4. Discussion.

3.1. Introduction.

Tetanus toxin is a multifunctional protein. There are parts of the protein responsible for receptor binding, membrane translocation, and an unknown activity that inhibits release of neurotransmitter. All of these functions are on a two - chain molecule of 150,000M_r. A dissection of the protein into smaller units would be useful in simplifying studies in structure and function. Possible means of splitting the molecule would be interdomain cleavage (section 1.5.2) or separation of the chains, and the latter will be discussed here.

There have been reports published on the protein chemistry of the separated chains eg the N terminal amino acids, the number of cystine and cysteine residues per chain and even the N terminal sequences (sections 1.4.4. & 1.4.5.). However the results do not agree. This inconsistency could be explained by the existence of variants of the toxin but may also be explained by the use of impure chain preparations. Hence there is a real need for a reliable method of separation.

The need for isolated chains is obvious in structural studies of multichain proteins but rather less so in functional studies. As the toxin is composed of two chains then either the active site(s) may lie in interchain pockets like those of the antigen binding sites in immunoglobulins or the activities may be located on particular chains as they are in diphtheria toxin where the cell binding component is the B chain and the A chain is an ADP - ribosyl transferase (Uchida, 1982). The resolution of the "whole toxin activity" and "chain activity" dichotomy would need a highly efficient means of chain separation because of the toxin's extremely high activity.

A further reason for isolating the chains is the generation of non toxic derivatives of tetanus toxin for use in the production of antitoxin antibodies, both for clinical use or for research on the antigenicity of the toxin.

There have been several attempts to separate the heavy and light chains by column chromatography, but none have been particularly successful. The most widely used method is that of Matsuda & Yoneda (1975), which is gel filtration of the previously reduced toxin on Ultragel AcA44 in buffers containing urea and dithiothreitol. Several workers using this method have claimed that it is an

improvement on other gel filtration methods eg Biogel and Sepharose 6B (van Heyningen, 1976) and Sephadex G150 (Helting & Zwisler, 1977).

The chains isolated in this way are unsuitable for chemical or biological studies as there is contamination of one chain by the other eg Matsuda & Yoneda (1975) reported that there was a 2% impurity of light chain in the heavy chain fraction and in a later paper they removed the toxicity of this heavy chain preparation by treatment with anti - light - chain antibody (Matsuda & Yoneda 1976). A second problem is the insolubility of the chains on the removal of urea.

To avoid the solubility problem, Helting & Zwisler (1977) used the Matsuda & Yoneda procedure to separate the chains of a toxin preparation that had previously been partially modified with formaldehyde. They then fully modified the isolated chains with formaldehyde to keep them in a soluble form. This procedure allows for the production of light and heavy chain derivatives suitable for raising chain specific antibodies but obviously is of no use for either structural or functional studies.

Because of the drawbacks of separation by gel filtration the recent work on the structure of the pure chains has been achieved by using polyacrylamide gel electrophoresis to isolate the chains.

DiMari et al. (1982a) criticised the results from column chromatography and developed a separation method based on preparative gel electrophoresis in a non - SDS buffer system using a Buchler polyprep 200 apparatus. They noted the instability (with respect to solubility) of both chains in the reducing and denaturing medium and circumvented this by bringing the toxin, the reductant and urea together only in the stacking gel of a discontinuous Davis - style gel. This method cannot be considered as an improvement on the gel filtration for several reasons viz the purity, the yield and the insolubility of the chains. Each peak was impure and contained proteins other than the heavy or light chains. Continuous elution is a method that is well known to have poor resolution (Spiker & Isenberg, 1983). The yield of the light and heavy chains was calculated to be approximately 60%, which is poor for a gel method. Finally the isolated heavy and light chains were unstable in that both chains aggregated in the elution buffer and underwent proteolytic digestion on storage.

Better resolution is obtained on polyacrylamide gels if the electrophoresis is run in the presence of SDS (Spiker & Isenberg, 1983), and this technique has been used by Neubauer & Helting (1979) and Britton (1981) to isolate the heavy and light chains. In order to determine the arrangement of the heavy and light chains within the extracellular form of the toxin, Neubauer & Helting (1979) determined the N terminal amino acids of the intracellular toxin, the extracellular toxin and the heavy and light chains. They obtained the heavy and light chains by running tetanus toxin under reducing conditions on polyacrylamide tube gels in SDS. (They had previously used Matsuda & Yoneda's procedure to obtain the heavy and light chains and it is not clear if their decision to use a new method was because of an appreciation of the limitations of the gel filtration method.) The position of each of the chains was established by staining one of the gels. The chains were eluted from the gel pieces by diffusion into a detergent (0.3% w/v SDS). This attempt by Neubauer and Helting (although fine in principle and sufficient for the purpose,) is limited as a method for separation of the chains ; mainly because of the almost arbitrary assignment of the chain position and the inefficient means of protein recovery.

Britton (1981) also used polyacrylamide gel electrophoresis in SDS to separate the reduced chains but he recovered the protein from the gel pieces by electroelution. There are two criticisms of his method. He determined the position of the chains by precipitating the protein - SDS complex by immersion of the gels in 4M sodium acetate. This procedure is extremely insensitive and is also not precise enough, as an extra 40% of area of gel around the protein band is unavoidably cut out. There was also no attempt made to remove the SDS.

There is therefore a need for a reliable method of separation of the heavy and light chains. The requirements are that the chains are pure and that the method gives proteins which are soluble.

3.2. Methods for Separation of Chains.

3.2.1. Separations of the Heavy and Light Chains by Polyacrylamide Gel Electrophoresis in SDS.

Samples of tetanus toxin and reduced and carboxymethylated tetanus toxin were dissolved in dissociation buffer (62.5mM Tris -

chloride/2%(w/v) SDS/2mM EDTA/10% glycerol) at a protein concentration sufficient to give 5-15 μg of the light chain per slot. The unmodified tetanus toxin samples were reduced by adding β mercaptoethanol and boiling for 2 minutes. The samples were run on 8% polyacrylamide gels in SDS (section 2.3.1.2). The chains were isolated by electrophoretic elution from the gel as described in section 2.3.3.

3.2.2. Ion Exchange.

3.2.2.1. Development of System.

Small ion exchange columns (1.5cm x 0.5cm) were made by pouring 1ml of preequilibrated CM52 cellulose suspension into pasteur pipettes plugged with glasswool. The ion exchanger was assayed for discriminatory binding of the chains at different pH's and ionic strength. The analysis of the eluant was by polyacrylamide gel electrophoresis in SDS as there are no functional assays for the isolated chains.

The reduced toxin sample was prepared by dissolving 0.5 - 1.0mg of pure toxin in 0.5ml of the starting buffer containing 2mM dithiothreitol (DTT) and 8M urea. The reduced and carboxymethylated toxin was likewise dissolved in the same buffer without dithiothreitol, as was the DTNB modified toxin. The protein sample was loaded and the column washed with 3 x 0.5ml of starting buffer containing 10mM NaCl followed by 1.0ml of the same buffer containing 100mM NaCl.

3.2.2.2. Preparative Ion Exchange of Reduced and Carboxymethylated Toxin.

Reduced and carboxymethylated toxin (12ml) at a concentration of 0.85mgml^{-1} in 17.5mM - sodium acetate/7.5mM - acetic acid, pH 5.1 containing 8M urea / 1mM benzamidine /2mM citrate (starting buffer) was loaded onto a (2cm x 5cm)CM 52 cellulose at a flow rate of 2.5ml min^{-1}. The column was washed with 40ml of the starting buffer, followed by 30ml of the same buffer but containing 100mM NaCl. The eluant was assayed for protein by measurement of absorbance at 280nm and the purity of the protein peaks were determined by polyacrylamide gel electrophoresis in SDS. The isolated chains were dialysed against 50mM NH_3 and lyophilized.

3.2.2.3. Preparative Ion Exchange of DTNB Modified Toxin.

The DTNB modified toxin (45ml) at a protein concentration of 0.33mg ml^{-1} (Bradford dye binding assay) in 17.5mM - sodium acetate

/7.5mM acetic acid, pH5.1 containing 8M urea / 1mM benzamidine / 2mM citrate /10mM NaCl (starting buffer) was loaded onto a (2.5 x 1.0 internal diam. cm) CM52 cellulose column at a flow rate of 2.2ml min^{-1}. The column was washed with 100ml of the starting buffer, followed by 50ml of the same buffer but containing 100mM NaCl. The eluant was assayed for modified protein by absorbance at 327nm and for protein at 280nm and less often by the Bradford dye binding assay (Bradford, 1976). The UV-VIS spectrum of the DTNB - modified protein has a λmax of 327nm and a significant overlap at the λmax of the aromatic amino acids (ie 280nm) (see diagram 3.4). This means that the A_{280} of a DTNB modified protein is not a reliable indication of the amount of protein present.

3.2.2.4. Removal of The 2 Nitro - 5 - Thiobenzoate Group from Protein Thiols and Reformation of The Protein Disulphide Bonds.

The DTNB modification of thiols is reversible (equation 3.1) and the 2 nitro - 5 - thiobenzoate group can be removed by treatment with dithiothreitol.

$$R{-}S^- + O_2N{-}C_6H_3(COO^-){-}S{-}S{-}C_6H_3(COO^-){-}NO_2 \rightleftharpoons R{-}S{-}S{-}C_6H_3(COO^-){-}NO_2 + {}^-S{-}C_6H_3(COO^-){-}NO_2 \quad (+DTT \rightarrow R{-}S^-)$$

Equation 3.1

Pooled fractions from the ion exchange column corresponding to light and heavy chains were concentrated (by freeze - drying or ultrafiltration against Sephadex as both chains bound irreversibly to Amicon membranes) and dialysed against 0.1M Tris chloride pH 8.2 containing 8M urea / 2mM EDTA / 1mM benzamidine. (Dialysis was the preferred method in desalting samples of tetanus toxin as the toxin interacted with the gel matrix and was retarded to a noticeable extent in gel filtration). The chains were reduced after dialysis by the addition of dithiothreitol at a tenfold molar excess over the protein thiol and the sample left for 2 hours at room temperature under nitrogen. The protein was reoxidised by dialysing against 3 changes of 0.1M Tris chloride pH 8.2 at room temperature (the reoxidation buffer was made with tap water and not distilled water). UV -VIS scans were taken of the DTNB modified proteins before and after the reduction steps to ensure that the 2 - nitro - 5 - thiobenzoate group had been removed.

3.2.3. Separation of The Heavy and Light Chains by HPLC.

1.2mg of reduced and carboxymethylated toxin was dissolved in 200 μl of 50mM sodium phosphate pH7.5 containing 2.5M urea and 0.1M sodium chloride. The buffers for the ion exchange or gel filtration were not the optimal for the separation of the chains but were those in use at a Waters HPLC demonstration. The toxin solution was diluted twofold in either 20mM Tris chloride pH8.5 (the loading buffer for the ion exchange on a Waters PROTEIN - PAK DEAE 5PW) or 50mM - dipotassium hydrogen phosphate / 50mM - potassium dihydrogen phosphate pH 7.0. The latter was the mobile phase for the gel filtration on a Waters PROTEIN - PAK 300 SW. The gradient for the ion exchange was 0 - 100% B, where eluent B was 20mM Tris chloride pH 8.5 containing 0.5M - sodium chloride. In each case the volume injected was 25 μl. The peaks were detected by absorbance at 280nm and the peaks from the gel filtration were collected. The peaks were analysed by polyacrylamide gel electrophoresis in SDS after dialysis against 50mM NH_3 to remove any potassium (which forms an insoluble complex with SDS).

3.3. Results.

3.3.1. The Separation of The Chains of Tetanus Toxin by Polyacrylamide Gel Electrophoresis in SDS.

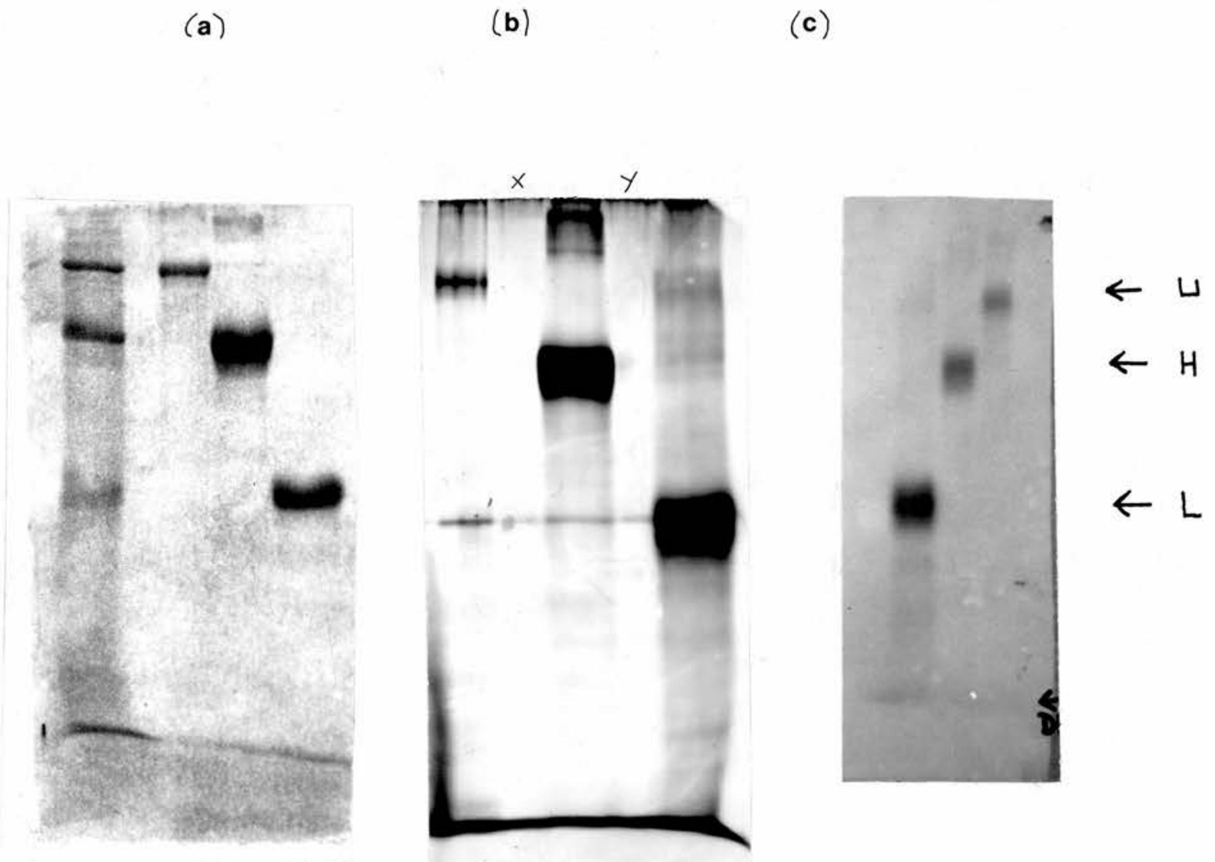
Either reduced or reduced-and-carboxymethylated chains could be separated by this procedure. Each of the heavy chain, the light chain or the unnicked form of the toxin, whether reduced or reduced-and-carboxymethylated were obtained in a pure form with no cross contamination at a yield of at least 80% (diagram 3.1). The chains were stored at -20°C in Tris buffers containing SDS as either gel slices (in which case the buffer was 125mM Tris chloride pH 6.8 containing 2mM EDTA and 0.1% SDS) or in solution in 25mM Tris/0.2M Glycine/2mM EDTA/0.1% SDS pH8.3 after electroelution from the gel. The alkylated chains were soluble in 50mM NH_3, and in 10% (v/v) EtOH but not in other aqueous solutions such as phosphate, without urea. They remained in solution in the organic solvents used for chemical proteolysis (ie 70% (v/v) formic acid) or N terminal sequence analysis (ie aq 50% (v/v) pyridine) or in the presence of SDS (ie suitable for digestion by Staph.aureus V8 proteinase).

3.3.2. Separation of Reduced and carboxymethylated Chains on CM52.

The conditions for separation of the reduced -and

Diagram 3.1. Isolation of The Heavy Chain, Light Chain and Unnicked Form of The Toxin by Polyacrylamide Gel Electrophoresis in SDS.

The heavy, light and unnicked chains of tetanus toxin isolated by elution from polyacrylamide gels as shown by protein staining ((a) coomassie dye (b) silver staining) or by autoradiography of ^{14}C - carboxymethyl chains (c). In (b) the line at 50,000M_r is an artefact of the stain (it is present in tracks x and y which have no sample). In each case the percentage of polyacrylamide in the separating gel was 8%.



carboxymethylated heavy and light chains of tetanus toxin on CM52 Cellulose were found by assaying the binding of the chains to a series of mini ion exchange columns. The pH of the columns was varied at 0.5pH units over a range from pH 5.0 to pH 7.5. Complete separation of the heavy and light chains was obtained at pH 5.1 (in urea). The reduced-and-carboxymethylated light chain did not bind to the column whereas the heavy chain did, but could be eluted along with the unnicked chain (when present) at higher salt concentrations (Table 3.1).
The process was scaled up on a 2cm x 5cm column (diag 3.2). The yield of the reduced-and-carboxymethylated light chain was approximately 90% and was routinely free of heavy chain. The heavy chain was eluted with buffer containing 100mM NaCl at a maximum yield of 50% and if the pH was raised a second heavy chain fraction was obtained. The heavy chain peak also contained the unnicked fraction (when unnicked toxin was present in the batch) and was therefore unsuitable for structural studies without further purification. The isolated reduced-and-carboxymethylated chains were soluble in 50mM NH_3 and were dialysed against this to remove the urea and generally stored as a freeze dried powder.

3.3.3. Separation of Reduced Chains, and DTNB Modified Chains on Ion Exchange.

Unfortunately the conditions found for successful separation of the reduced-and-carboxymethylated chains were not suitable for the separation of the reduced toxin (Table 3.1). Although pH5.1 was again found to be the optimum pH for separation of the chains there was always cross contamination of one chain by the other in the reduced toxin samples. As the chain separation occurred in the case where the thiols were irreversibly modified and as this was the only difference between the two experiments then it seemed that the two chains would reassociate (in 2mM DTT), even in 8M Urea, unless the thiols were modified. The aim of the separation of the reduced chains was to have a preparation of isolated light and heavy chains where each has the native structure (apart from the interactions between the heavy and light chains and in particular the intermolecular disulphide(s)) but as the reduced sample did not produce isolated chains then the approach taken was the reversible modification of the thiols produced on reduction of the toxin. After

Table 3.1. Determination of Conditions for Separation of Heavy and Light Chains on Cellulose CM52.

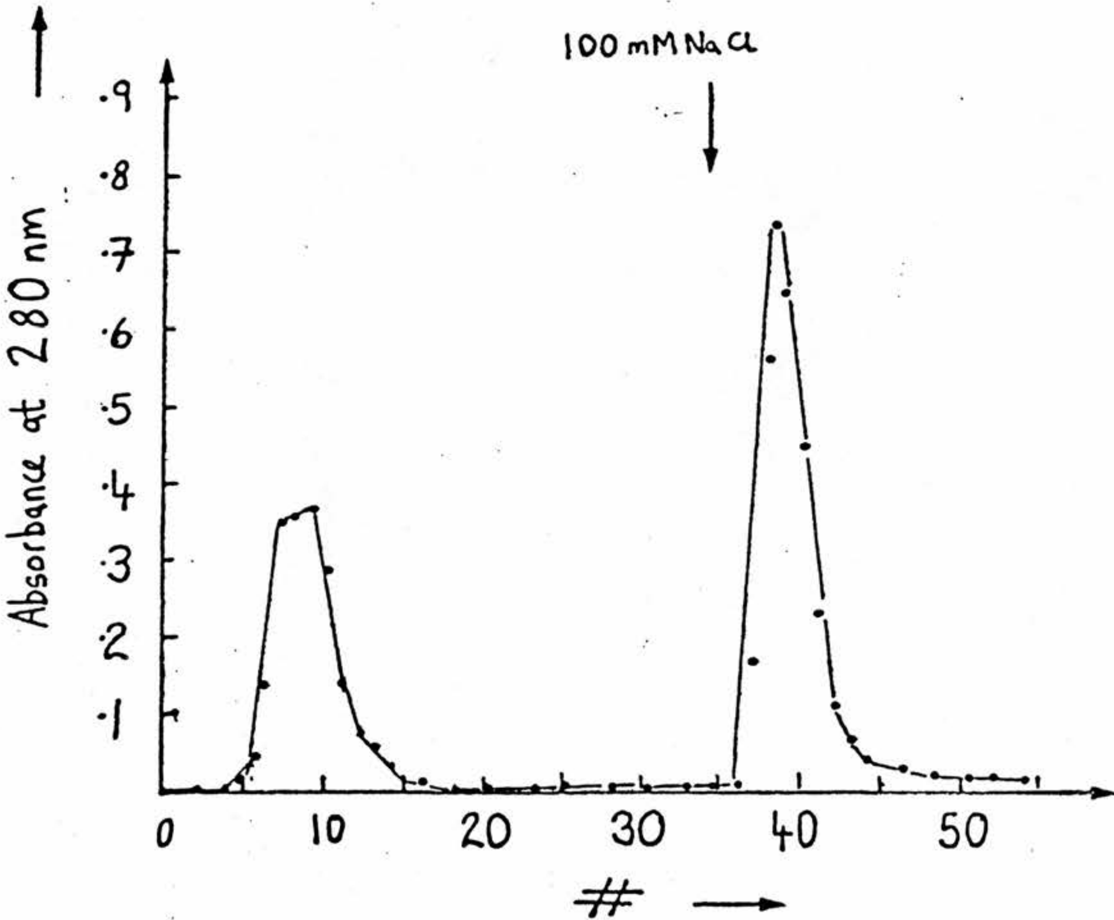
1. no binding to column 2. partial binding at 10mM NaCl and elution at 100mM NaCl 3. binding to column at 10mM NaCl and elution at 100mM NaCl	pH				
	7.5	6.5	5.7	5.5	5.1
reduced and carboxymethylated toxin					
light chain	1	1	2	2	1
heavy chain	1	1	2	3	3
unnicked toxin (A)	1	1	2	3	3
reduced toxin					
light chain	1	1	2	2	2
heavy chain	1	1	2	2	2
unnicked toxin (A)	1	1	2	2	2
DTNB modified toxin					
light chain	N.D	N.D	N.D	N.D	1
heavy chain	N.D	N.D	N.D	N.D	3
unnicked toxin (A)	N.D	N.D	N.D	N.B	3

The chains (0.5ml) were loaded onto the minicolumns in buffers of the appropriate pH containing 8M urea, 10mM NaCl, 1mM benzamidine and 2mM citrate. The columns were washed with 2 x 0.5ml of loading buffer followed by 3 x 0.5ml of the same buffer except that the NaCl concentration was 100mM. The eluants were assayed by polyacrylamide gel electrophoresis in SDS. (N.D.) means not determined, (A) when present.

Diagram 3.2. The Separation of The Constituent Chains of Reduced and Carboxymethlated Toxin on Cellulose CM52.

(a) Elution profile of the separation of the two constituent chains of reduced and carboxymethylated tetanus toxin by ion exchange on cellulose CM52.

(b) Polyacrylamide gel electrophoresis in SDS of carboxymethylated light and heavy chain isolated by ion exchange (Tracks 1 and 2,3 respectively in diagram 3.4.b). The slight contamination of the light chain with heavy chain could be removed by a second passage on the column. The heavy chain fraction contained unnicked toxin when this was present in the toxin batch.



(a)

chain separation the modification could be reversed and the proteins oxidised to the native form. The reagent chosen was 5,5-dithiobis-(2-nitrobenzoic acid)(Ellman's reagent).

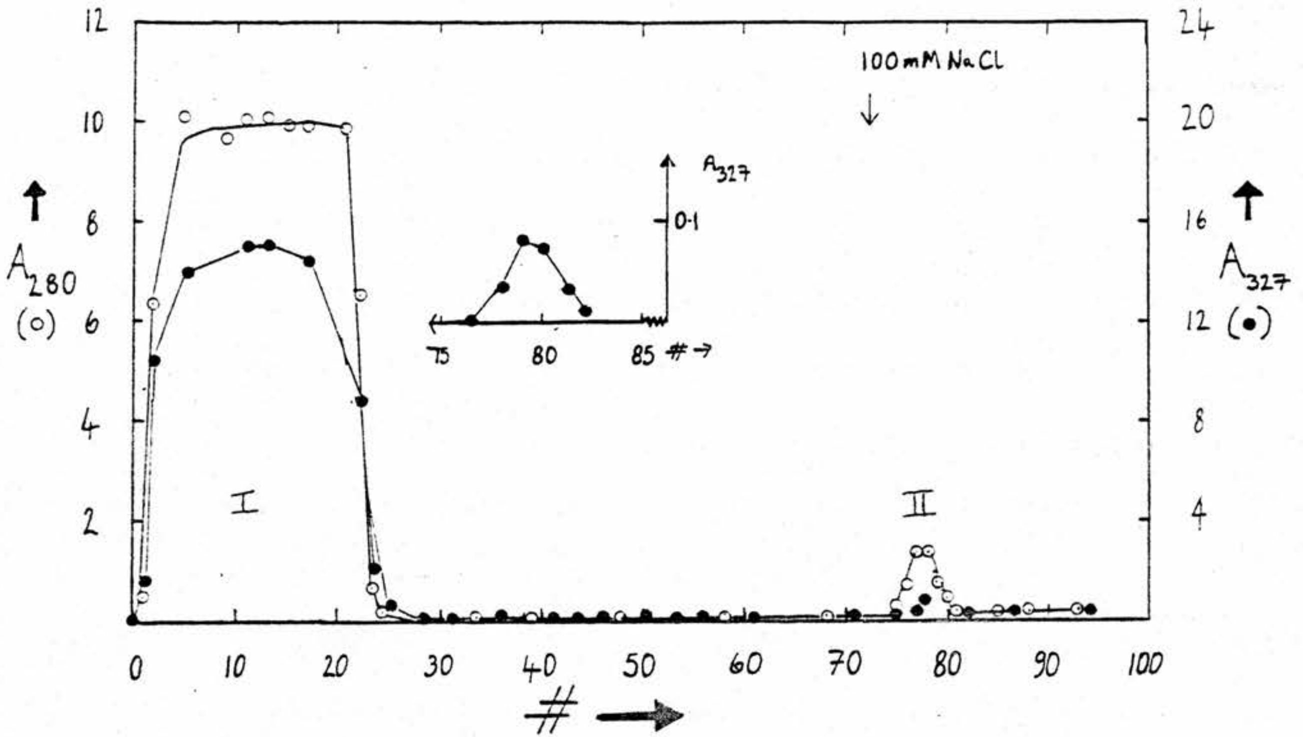
The DTNB modified toxin had the same behaviour as the carboxymethylated toxin on cellulose CM52 at pH 5.1 in urea, ie the modified light chain did not bind to the column whereas the heavy chain and the unnicked form of the toxin did bind (diagram 3.3). The difference lay in the yield of the proteins. The light chain (peak I) was obtained at 27 - 30% of total protein onto the column ie at approximately 90% of the calculated light chain, but the heavy chain yield was only 13% of the calculated (as estimated by Bradford). Increasing the ionic strength and / or varying the pH had no effect on the yield of the heavy chain and unnicked fraction. Even such drastic washes as 3M KI had no effect. Washing the column with buffer containing dithiothreitol turned the column yellow due to release of 2 nitro-5-thiobenzoate anion but only achieved at maximum a further 10% of the heavy chain and unnicked form.

The extent of DTNB modifications in peak II material ie the heavy and unnicked is extremely low (diagram 3.3), the A_{327} is almost negligible in comparison to that of the peak I material. Comparison of the A_{327} and the protein concentration of the light fraction with that of the whole toxin before the ion exchange shows that the absorbancy of the whole toxin is due mainly to the light chain (diagram 3.4). The heavy chain seems to be modified little, if at all.

Polyacrylamide gel electrophoresis in SDS of the light chain after removal of the 2 nitro-5-thiobenzoate by dithiothreitol and reoxidation of the protein, showed a single protein of $50,000M_r$ in the absence and presence of β-mercaptoethanol (diagram 3.5). (It was always cautionary to run a light chain sample in the presence of β-mercaptoethanol as well, as sometimes there was a faint trace of heavy chain which only entered the 5% polyacrylamide gel in reducing conditions. If the heavy chain was present then the light chain was recycled). The light chain was obtained in a high yield after the removal of the 2,nitro-5-thiobenzoate and the reoxidation. The light chain was stable (in that it was still present) in the oxidation buffer at -20°C for 18 months.

Light chain prepared in this way was immunologically

Diagram 3.3. The Separation of The Two Chains of DTNB Modified Tetanus Toxin on Cellulose CM52.



(a)

(a) Elution profile of the separation of the two chains by ion exchange on cellulose CM52.

(b) Polyacrylamide gel electrophoresis of chain isolated in this manner see diagram 3.4 (b) track 4 (light chain), and tracks 6,7 showing light chain and "heavy chain" after removal of 2,nitro-5-thiobenzoate group.

Diagram 3.4. Reaction of DTNB with Reduced Tetanus Toxin Results in Modification of The Light Chain Alone.

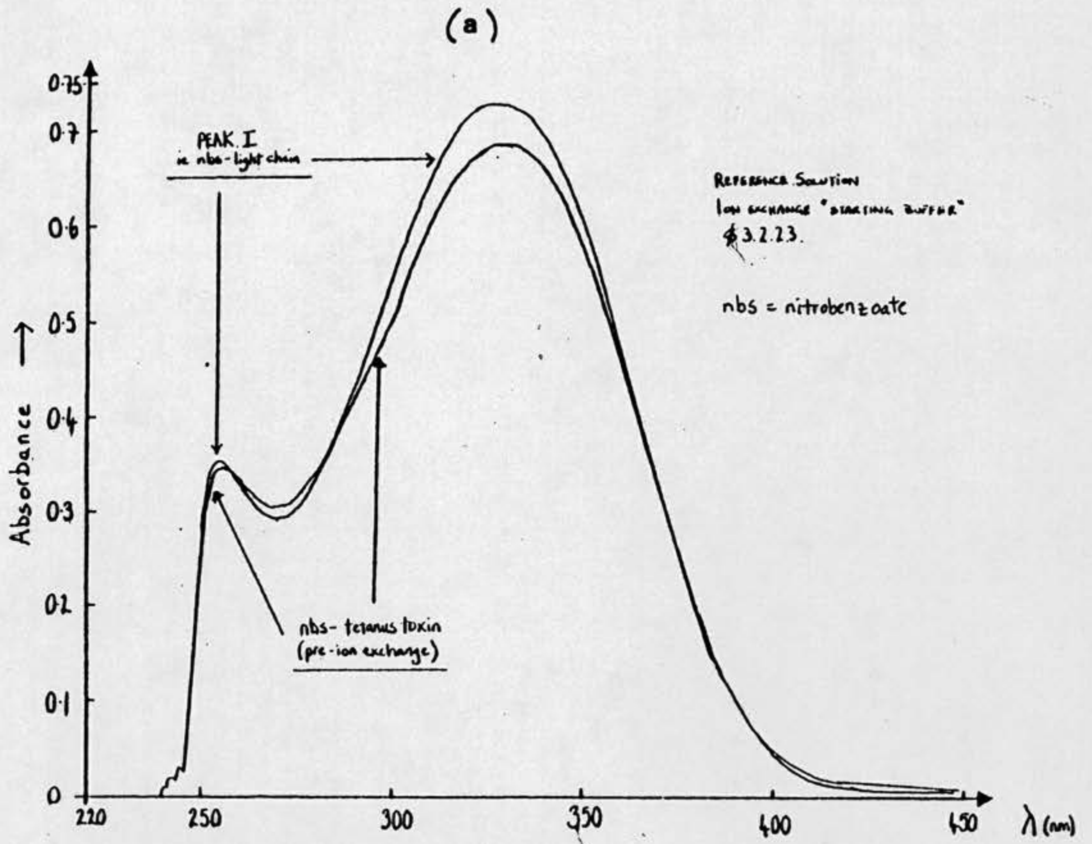
(a) UV-VIS scan of (i) the DTNB-modified toxin before ion exchange and (ii) the DTNB-modified light chain after ion exchange (the peak I material of diagram 3.3). The protein concentration (Bradford,1976) of the light chain was $0{\cdot}096mgml^{-1}$ and that of the total toxin was $0.4mgml^{-1}$. The A_{327} of a tenfold dilution of the L chain was 0.73, and that of the total toxin was 0.68 difference of 0.05, ie the A_{327} values are nearly equivalent despite a fourfold difference in protein concentration. As the L chain is one-third of the total toxin then these values imply that it is mainly L chain that is modified on treatment of reduced toxin with DTNB.

(b) 5% polyacrylamide gels in SDS of;

1 reduced and carboxymethylated L isolated by ion exchange.
2 reduced and carboxymethylated H(+U) isolated by ion exchange.
3 reduced and carboxymethylated H isolated by ion exchange
4 DTNB-modified L post cellulose CM52 (trace of H present).
5 DTNB-modified toxin pre cellulose CM52.
6 re-oxidised light chain.
7 re-oxidised heavy chain.

The gels of interest here are tracks 4 and 5, and these samples each contained an equivalent amount of material with respect to the absorbance at 327nm, but track 4, the L chain, contained only 14μg of protein whereas track 5, total toxin, contained 60μg of protein.

(a)



(b)

equivalent to that prepared by Habig (U.S.A.) but was totally dissimilar to that prepared by Bizzini (France). (Analysis by Dr. K. Goretzki, Giessen, F.R.G.)

Analysis of the "heavy chain" fraction on polyacrylamide gel electrophoresis in SDS showed both the heavy chain and the unnicked form of the toxin to be present if the sample had been treated with β-mercaptoethanol but the gel was blank if the sample had not been reduced (diagram 3.5). In other words the heavy chain and the unnicked form of the toxin formed a large molecular weight aggregate (either hetero- or homologous) that was unable to enter a 5% polyacrylamide gel in the presence of SDS and which was presumably disulphide linked. The "heavy chain" fraction was insoluble in the absence of 8M urea and was not stable over a long time, even in this denaturant.

3.3.4 The Isolation of Reduced and Carboxymethylated Chains by HPLC.

Gel filtration on a Waters PROTEIN - PAK 300 SW resolved the reduced and carboxymethylated chains into three components (diagram 3.6). The peaks were analysed by polyacrylamide gel electrophoresis in SDS. The first peak consisted of heavy and light chain that still associated throughout the gel filtration (although not in SDS), the second peak was mainly heavy chain and the third peak was apparently pure light chain. The ion exchange on the Waters PROTEIN - PAK DEAE 5PW showed 2 doublet peaks (diagram 3.6) but the composition of these peaks is not known as the samples were not collected.

3.4. Discussion.

The separation of the chains by polyacrylamide gel electrophoresis in SDS is an ideal method of obtaining pure chains when the amount of toxin available is small. The method described here is rapid, precise, efficient and reproducible. The disadvantage of this proceedure is an integral part of its success : the use of SDS. The SDS gives the system high resolution and avoids the solubility problems associated with the isolated chains, but may cause denaturation of the protein. Therefore, because of this risk, the method was used mainly for the isolation of reduced and carboxymethylated chains for structural studies, and not usually for the isolation of reduced chains. The reduced chains are unsuitable for peptide mapping etc. and unless it is possible to recover tetanus

Diagram 3.5. The Re-Oxidised Forms of The Heavy and Light Chains of Tetanus Toxin.

(a) Spectrophotometric traces of 5% polyacrylamide gels run in SDS, and stained with Coomassie Brilliant Blue R.

1. Light chain.
2. Light chain plus 2-mercaptoethanol.
3. Heavy chain.
4. Heavy chain plus 2-mercaptoethanol.
5. "Recycled" light chain plus 2-mercaptoethanol.

(b) Tracks 6 and 7 in diagram 3.4. b show the re-oxidised light chain and the re-oxidised heavy chain respectively.

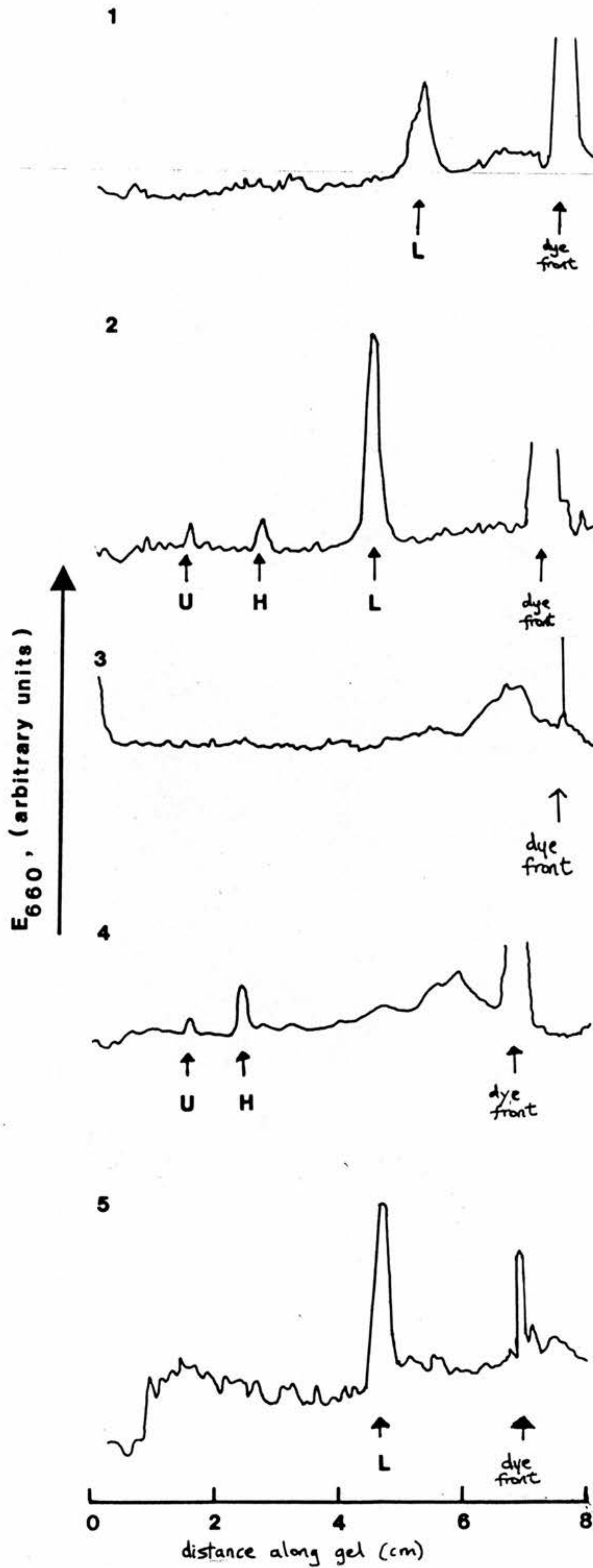
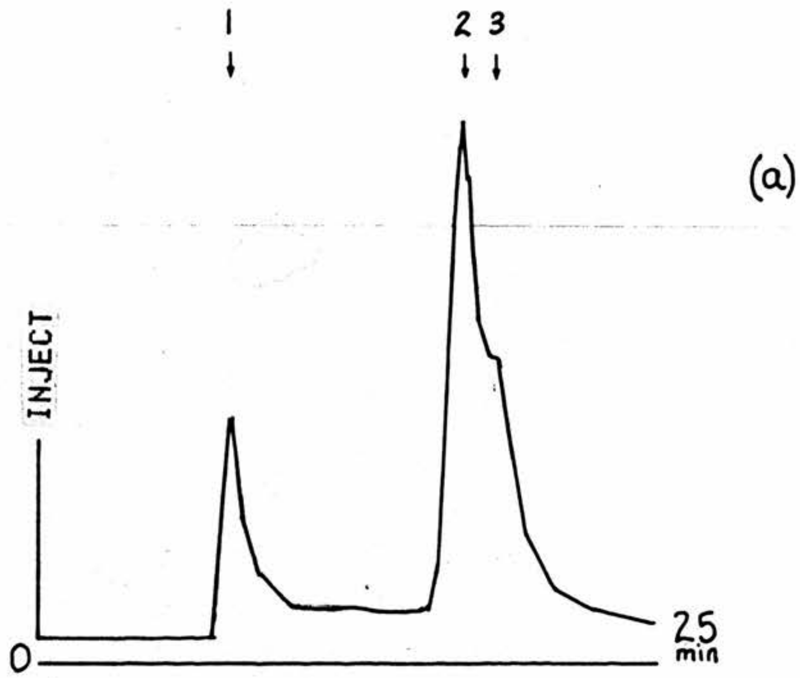

1
L
dye front
2
U
H
L
dye front
3
dye front
4
U
H
dye front
5
L
dye front
E_{660}, (arbitrary units)
0
2
4
6
8
distance along gel (cm)

Diagram 3.6. The Separation of The Reduced and Carboxymethylated Heavy and Light Chains by HPLC.

(a) Rapid separation of heavy and light chains from reduced and carboxymethylated tetanus toxin (25μg) by gel filtration on Waters PROTEIN-PAK 300SW. Eluent : 0.1M phosphate buffer pH 7.0. Peak 1 was heavy and light chain, peak 2 was mainly heavy chain and peak 3 was light chain.

(b) Resolution of reduced and carboxymethylated tetanus toxin (25μg) into two main peaks by ion exchange on Waters PROTEIN-PAK DEAE 5PW. Gradient conditions ; Eluent A : 0.02M Tris HCl pH 8.5, Eluent B: 0.02M Tris HCl, pH 8.5 plus 0.5M NaCl. 0-100% B, 25 minutes.



A_{280}, (arbitrary units)

(b)

INJECT

0

25 min

toxin or the chains in an active form from SDS gels (as has been found for other proteins, Spanos & Hübscher, 1983) then this method is not the one of choice for the production of heavy and light chains for functional studies. (Reduced chains are unsuitable for structural studies as they contain reactive thiols which are generally disadvantageous, a point that seems to have escaped the attention of other workers, including DiMari and Robinson whose apparent aim is the sequence of the protein.) It is possible, however, to use chains obtained in this way for the raising of antibodies specific to either reduced chains or reduced and carboxymethylated chains despite the presence of SDS (Stumpf et al., 1974).

However there is a need for a method suitable for processing larger amounts of toxin for structural studies and possibly for functional purposes. The singular difficulty encountered in separating the chains by the most obvious method for such dissimilar - sized proteins viz gel filtration, suggests that the separation is more complicated than previously thought. The problems are that the chains remain associated in reducing conditions in urea or guanidine hydrochloride (although not in SDS) and that the isolated chains are insoluble even in urea. This instability may be a reflection of the disulphides and / or because the heavy chain may need the light chain to stabilize its secondary and tertiary structure (as the C terminals of proteins are generally less well folded than the N terminal). The problem then simplified to one of how to keep the thiols stable ie chemically inert, and the answer was obviously to modify them after reduction. It was necessary to have two kinds of modification : one irreversible for structural work and one reversible for functional studies. A further change was the use of ion exchange rather than gel filtration.

The ion exchange chromatography of the reduced and carboxymethylated toxinwas a success in that reduced and carboxymethylated heavy chain and reduced and carboxymethylated light chain were obtained in a pure form at a reasonable yield and were soluble in solvents used in the structural studies. The ion exchange of DTNB modified material was a limited success : it was wholely successful for thelight chain but was of no use for the isolation of the heavy chain as it bound irreversibly to the column. The purification of light chain in this way is useful as the product is pure and stable (insofar as solubility is concerned) and it can be used in functional studies or to generate anti-light chain antibodies. The irreversible binding of the heavy chain

chain is probably due to denaturation although as it is the cell binding polypeptide then the possibility remains that this is a specific interaction (but unlikely as ion exchange of the reduced toxin in presence of a range of sugars or washing of column by 3M KI was to no avail). A further point is that the heavy chain was not modified by DTNB and presumably the differential reactivity of the heavy chain thiols to DTNB and iodoacetic acid was due to the different size and charge of the reagents; DTNB is large and hydrophobic whereas iodoacetic acid is small and electronegative.

In the introduction to this chapter the problems blocking the separation of the chains were said to be the tight association of the chains and the insolubility of the heavy chain once removed from the light chain. Ion exchange coupled with modification of the thiols solves the first problem but the second remains. The answer could be further chemical modification of the chains or the introduction of an entirely new method. In the case of both irreversibly - modified and reversibly - modified toxin, citraconylation of the proteins may help in solubulising the chains. The reversible modification of thiol groups (before ion exchange) should not be abandoned as a possible means of obtaining "native" heavy and light chains but perhaps other reversible thiol agents such as cysteine or glutathione (which are both small and electronegative) should be tried. The new method refered to is HPLC, either by gel filtration or ion exchange. Under less than ideal conditions (ie no denaturant present) a sample of reduced and carboxymethylated toxin was split into heavy and light chains by gel filtration on a Waters PROTEIN - PAK 300SW. This result is encouraging and probably the best approach for separation of the heavy and light chains or either structural or functional studies will be HPLC. The main difference between this technique and the classical column methods is the extreme rapidity and this is probably the main reason for the success in separating the heavy and light chains.

Chapter 4. Homology Between The Heavy and Light Chains of Tetanus Toxin.

4.1 Similarities in The Amino Acid Compositions.

4.2 Statistical Test of Sequence Homology from Composition Data.

4.2.1 $S\Delta n$ as an Indicator of Sequence Relatedness.

4.2.2 Calculation of Interdomain Homology in Proteins of Known Sequence.

4.2.3 Calculation of Relatedness Between The Chains of Tetanus Toxin.

4.3 Proteolytic Fragments of The Heavy and Light Chains of Tetanus Toxin.

4.3.1 Preparation of Chains for Digestion.

4.3.2 Proteolytic Cleavage, and Separation and Analysis of Peptides.

4.3.3 Analysis of Peptides Derived from Heavy or Light Chains by Silver Staining of Peptides in Polyacrylamide Gels.

4.3.4 Epitope mapping of V8 Proteinase Digests of The Heavy and Light Chains.

4.3.5 Thiol Peptides.

4.3.6 Analysis of Cyanogen Bromide Fragments by Reverse Phase HPLC and N Terminal Sequence Analysis.

4.4 Discussion.

4.1 Similarities in The Amino Acid Compositions.

The synthesis and structure of the toxin are discussed in detail in Chapter 1. The following statement is a brief summary to place the contents of this chapter in context.

Tetanus toxin is first synthesized as a single chain of 150,000 M_r that is converted by proteolysis to an extracellular form having two polypeptide chains joined by one or more disulphide bonds. The two chains are the heavy chain (H) of 100,000M_r and the light chain (L) of 50,000 M_r. Experiments on the isolated chains have apparently shown them to be functionally different and to possess different antigenic determinants.

The experiments on the function and immunological properties of the isolated chains reflect higher levels of protein structure and may mask similarities in the amino acid sequence. Despite the functional and possible conformational differences between the two chains there is evidence from the amino acid composition data that the chains have regions of homology.

The amino acid composition has been determined for the heavy and light chains isolated by polyacrylamide gel electrophoresis in an SDS-system (Britton,1981) and a non SDS system (DiMari et al., 1982a). In each case the compositions of the two chains are remarkably similar for two supposedly distinct proteins. The heavy chain (880 residues) is twice the size of the light chain (437 residues) and for each residue the total number for a particular amino acid in the heavy chain is almost twice that of the light chain (Table 4.1). The compositions are similar but some method is needed to decide whether this similarity is significant.

4.2 Statistical Test of Sequence Homology for Composition Data.

4.2.1 SΔn as an Indicator of Sequence Relatedness.

Ath 1 Cornish - Bowden has developed the theoretical basis for an index that estimates the amount of relatedness between two proteins using the amino acid compositions alone. He defines SΔn as follows (Equation 4.1),

$$S\Delta n = 0.5 \sum (n_{iA}-n_{iB})^2 - 0.035 (N_A - N_B)^2 + 0.535 |N_A - N_B| \quad \text{(Equation 4.1)}$$

Where A and B are two proteins and N_A and N_B signify the total number of residues in that protein and of which n_{iA} in A and n_{iB} in B are the number of residues of the ith type of amino acid and where the summation is over the range of amino acids determined in the analysis.

Table 4.1 The amino acid composition of the chains of tetanus toxin

	moles of residue per mole protein			
	H chain	half-H chain	L chain	$(n_{\underline{i}H} - n_{\underline{i}L})^2$
Cys	6	3	4	1
Asx	129	64.5	68	12.25
Thr	53	26.5	24	6.25
Ser	56	28	26	4
Glx	84	42	41	1
Pro	40	20	25	25
Gly	59	29.5	32	6.25
Ala	61	30.5	25	30.25
Val	54	27	27	0
Met	23	11.5	13	2.25
Ile	59	29.5	27	6.25
Leu	86	43	40	9
Tyr	45	22.5	22	0.25
Phe	27	13.5	16	6.25
His	13	6.5	7	0.25
Lys	63	31.5	29	6.25
Arg	22	11	11	0
Total	880	440	437	116.5

The amino acid compositions are taken from Britton (1981). The reduced and carboxymethylated heavy and light chains were eluted from SDS-polyacrylamide gels. The heavy chain is an average of three analyses and the light chain of two analyses.

$S\Delta n$ predicts the number of differences between the sequences of A and B and it has a coefficient of variation of 38% ie

$$S\Delta n = M\ (1 \pm 0.38) \qquad \text{(Equation 4.2)}$$

where M is the actual number of sequence differences.

The theoretical background for $S\Delta n$ is given in Cornish - Bowden (1979, 1983). The usefulness of $S\Delta n$ can be found by comparing it with 0.42N and 0.93N (where N is the absolute number of residues in the smaller protein). These values are not arbitary. Cornish - Bowden (1979) has shown that 95% of comparisons between pairs of unrelated proteins give $S\Delta n$ values greater than 0.42N, therefore any value less than 0.42N would imply that the proteins were related. This was referred to as the "strong test" as it almost always gives a negative result when applied to unrelated proteins, however it may fail to detect genuine relatedness. Unrelated proteins are typically identical at about 7% of the loci by chance alone, therefore any $S\Delta n$ that is greater than 0.93N means that the proteins are probably unrelated. The 'weak test' falls between these two limits ie a value for $S\Delta n$ that is greater than 0.42N but less than 0.93N passes the weak test and implies that the proteins are related. The weak test is nearly certain to give a positive result when applied to proteins with an appreciable amount of homology but it may give a wrong answer in about 10% of comparisons between unrelated proteins. As an example the weak composition test erronously indicated similarity in only 16 comparisons out of a possible 140 whereas it correctly assigned 22 of the 23 related pairs (Cornish-Bowden, 1980).

4.2.2. Calculation of Interdomain Homology in Proteins of Known Sequence.

Calmodulin and Immunoglobulin EU consist of several structural domains that are homologous in sequence. As such they are ideal examples for testing the Cornish-Bowden method (which until now has only been used to determine homology between proteins rather than within proteins). In both cases the $S\Delta n$ for each domain was calculated here from the amino acid composition and used to determine if there was any homology, and, if so; the extent of this homology. The calculated homology was then compared with the actual homology from the calmodulin sequence (Klee et al., 1980) or the immunoglobulin sequence (Edelman, 1973).

There are four Ca^{++} binding domains in calmodulin and therefore 6 possible comparisons. From the sequence data the extent of homology in these 6 comparisons ranges from 23-57%.

The calculated values of $S\Delta n$ and the comparison with 0.42N, 0.93N and M are detailed in tables 4.2 and 4.3. The calculated $S\Delta n$ for all comparisons suggests, with the exception of comparison I vs IV, that the domains are related and indeed for the II vs III comparison there is a strong indication that this is so (Table 4.2). The agreement of the calculated number of differences, $S\Delta n$, with the actual number of diferences (M) is good for all comparisons except (I vs IV) and (II vs III). In the former case the actual homology is not seen and in the latter case the homology is overestimated (Table 4.3).

The constant regions in the heavy and light chains of Immunoglobulin EU have about 30% homology as shown by amino acid sequence. The values of $S\Delta n$ calculated from the amino acid composition, and the comparison with 0.42N, 0.93N and M are given in tables 4.4 and 4.5. All comparisons between the constant regions, except that between C_H1 and C_H2, pass the weak test in that the $S\Delta n$ is between 0.42N and 0.93N ie there is a weak indication that the domains are related. The calculated number of differences, $S\Delta n$, was equal to the actual number of differences (M) or within the theoretical coefficient of variation for all comparisons with the exception of that between C_H1 and C_H2 (Table 4.5). The discrepancy between $S\Delta n$ and the known homology between C_H1 and C_H2 probably arises from the noticeable difference in the serine content in the compositions of the two domains.

The two model proteins show that the Cornish - Bowden compositional index, $S\Delta n$ is a reliable index of the existence of homology between domains but that the relationship between $S\Delta n$ and M (Equation 4.2) has too great a coefficient of variation ie the value of $\pm$ 0.38M is so large as to make the value of $S\Delta n$ of little use in predicting the percentage homology.

4.2.3 Calculation of Relatedness Between Chains of Tetanus Toxin.

The heavy chain of tetanus toxin is twice the size of the light chain and the arithmetic difference of 443 in number of residues is obviously too large a number for the molecular weight correction factor in equation 4.1. The comparison of the amino acid compositions of chains of such unequal length by the method of Cornish - Bowden is useless, unless the assumption is made that the heavy chain is composed of two similar halves. The comparison is now between the composition of the light chain and the halved composition

Table 4.2. Prediction of Homology Between The Domains of Calmodulin.

comparison	$S\Delta n$(1)	N(2)	0.42N	0.93N	comment	actual homology (%)
i vs ii	14	30	13	28	passes weak	40
i vs iii	14				passes weak	57
i vs iv	35				fails both	23
ii vs iii	10				passes strong	30
ii vs iv	15				passes weak	40
iii vs iv	14				passes weak	33

Notes (1) $S\Delta n = \frac{1}{2} \sum (\Delta n)^2$ as the molecular weights of the domains are identical.

(2) N chosen as 30, domain i,ii,iii,iv were residues 10-39, 46-75, 83-112, and 119-148 respectively.

(3) calculated from sequence in Klee et al., (1980).

Table 4.3 Comparison of Calculated Number of Differences (SΔn) with the actual Number of Differences (M), and Showing Upper and Lower Limits of SΔn, for Calmodulin.

comparison	calculated difference (SΔn)	actual difference (M)	limits[4] upper	limits[4] lower
i vs ii	14	18	25	11
i vs iii	14	13	18	8
i vs iv	35	23	32	14
ii vs iii	10	21	29	13
ii vs iv	15	18	25	11
iii vs iv	14	20	28	12

Notes (4) The limits for SΔn are $\pm$ 0.38M.

Table 4.4 Prediction of Homology Between The Constant Domains of Immunoglobulin EU .

comparison[1]	$S\Delta n$[2]	N[3]	0.42N	0.93N	comment
C_L vs C_H1	72	103	43	96	passes weak
C_L vs C_H2	78	106	45	99	passes weak
C_L vs C_H3	59	106	45	99	passes weak
C_H1 vs C_H2	115	103	43	96	fails both
C_H1 vs C_H3	85	103	43	96	passes weak
C_H2 vs C_H3	70	107	45	100	passes weak

Notes (1) The domains are those designated by Edelman, (1973).

(2) $S\Delta n$ calculated using equation 4.1.

(3) N is the number of residues in the domain with the lowest M_r; C_L has 106 residues, C_H1 has 103, C_H2 has 108 and C_H3 has 107.

(4) Calculations from sequence given in Edelman (1973).

Table 4.5. Comparison of Calculated Number of Differences ($S\Delta n$) with The Actual Number of Differences (M), and Showing Upper and Lower Limits of $S\Delta n$, for Immunoglobulin EU.

comparison	calculated difference ($S\Delta n$)	actual difference (M)	limits[5]	
			upper	lower
C_L vs C_H1	72	71	98	44
C_L vs C_H2	78	72	99	47
C_L vs C_H3	59	74	102	46
C_H1 vs C_H2	115	77	106	48
C_H1 vs C_H3	85	76	105	47
C_H2 vs C_H3	70	79	101	41

Notes (5) The limits for $S\Delta n$ are $\pm 0.38M$.

of the heavy chain (Table 4.1).

The values calculated for $S\Delta n$ from the data of Britton (1981) and DiMari et al. (1982a) are given in table 4.6, which also has the values for 0.42N and 0.93N. The compositional index for the reduced - and - carboxymethylated chains (Britton 1981) is 60. The value from the compositions of the reduced chains (DiMari et al., 1982a) is 162. These values for $S\Delta n$ which were calculated from the data of two different groups agree qualitatively as they are both less than 0.42N. Thus the result satisfies the most stringent test for sequence similarity which " is a strong indication, amounting almost to certainty, that the proteins are related". (Cornish - Bowden, 1983).

Whether the chains have some homology can only be answered by a knowledge of the complete sequence of the protein or the gene coding for the toxin. A more practical approach is to look at the products of chemical and enzymic cleavage of the two chains.

4.3 Proteolytic Fragments of The Heavy and Light Chains of Tetanus Toxin.

Proteolytic digests would give structural information in two ways ie the presence of "common" peptides and also the total number of peptides produced after specific cleavage. However both approaches are open to criticism. The first is only strictly correct when "common" refers to the composition or sequence of such peptides rather than more arbitary characteristics such as polarity or hydrophobicity. The absolute number of peptides found after total cleavage is of use if there is a very large discrepancy between the expected number of peptides and those actually found because this could be due to internal repeats. This approach was used to show that the two polypeptides of the arom multienzyme complex of N.crassa were identical (Lumsden & Coggins, 1978). The disadvantage of this approach is that it relies on the specific cleavage of the protein going to completion and that the method chosen to analyse the digest is capable of resolving all the peptides.

4.3.1 Preparation of Chains for Digestion.

Pure tetanus toxin was reduced and carboxymethylated according to the method described in section 2.4.2. The reduced and carboxymethylated chains were separated by polyacrylamide gel electrophoresis in SDS as described in section 3.2.1 and isolated by

Table 4.6. The Compositional Index for The Heavy and Light Chains of Tetanus Toxin.

N (1)	437
0.42N	184
0.93N	406
$S\Delta n^{(2)}$	60 (Britton, 1981) 162[3](DiMari et al.,1982a)

Notes (1) N is taken as the number of residues in the smaller of the proteins in the comparison. As H/2 is 440 and the value for L is 437, then N=437.

(2) $S\Delta n = 0.5 \sum (n_{iH}-n_{iL})^2 - 0.035(N_H-N_L)^2 + 0.535(N_H-N_L)$ for "Britton"

$S\Delta n = 0.5 \sum (n_{iH}-n_{iL})^2$ for "DiMari"

(3) In this case N was assumed to be 437 as no value was given in DiMari et al.,(1982a)

elution of the protein from gel slices (section 2.3.3). There was no crosscontamination of U, H or L using this procedure (see diagram 3.1).

4.3.2 Proteolytic Cleavage, and Separation and Analysis of Peptides.

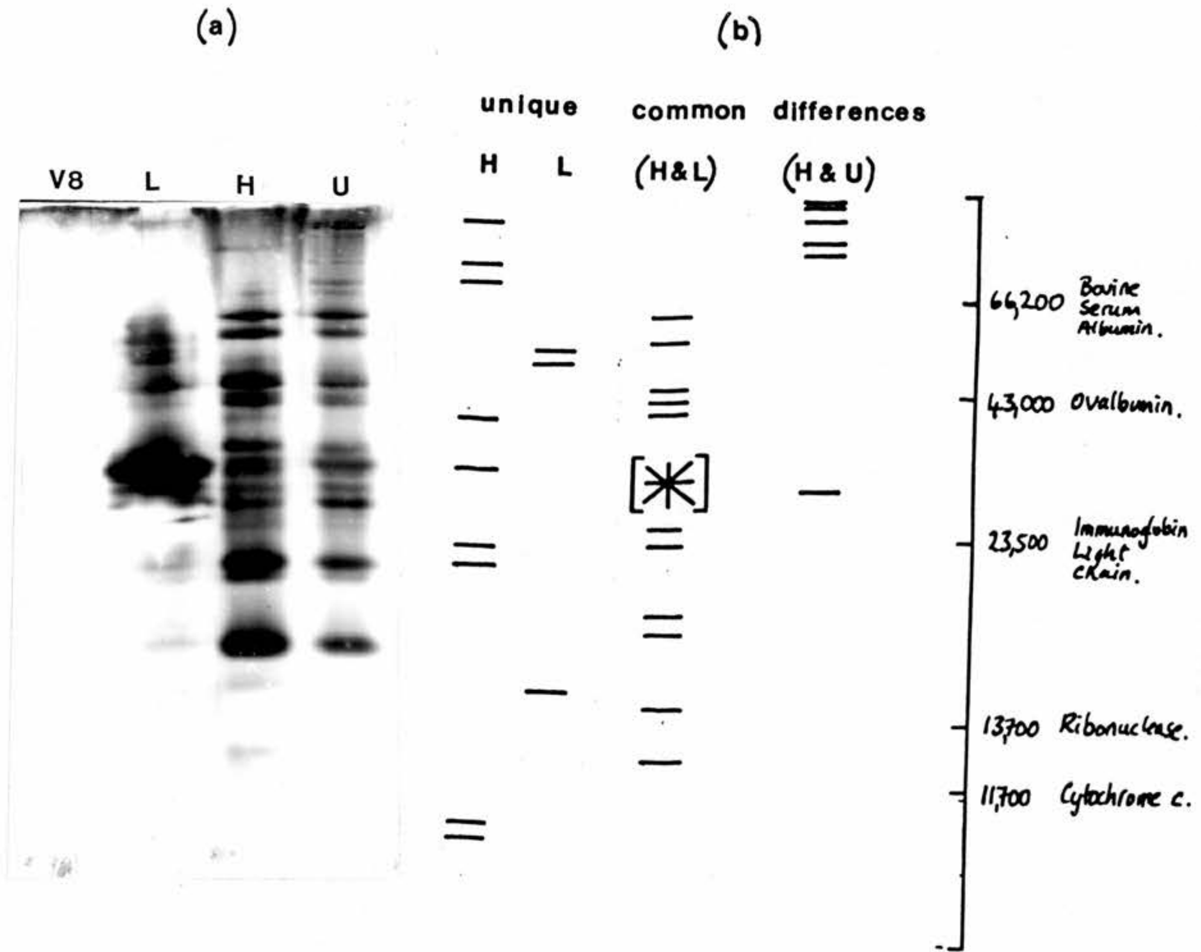
The reduced and carboxymethylated chains were cleaved using cyanogen bromide (section 2.5.1.1), ō-Iodosobenzoic acid (section 2.5.1.2) or the V8 proteinase from *Staph. aureus* (section 2.4.2). The digests were fractionated by polyacrylamide gel electrophoresis in SDS (section 2.3.1.2) or by HPLC (section 2.6.2). The peptides resolved by polyacrylamide gel electrophoresis in SDS were analysed by staining of all peptides (using the silver stain of Wray et al. (1981) as in section 2.3.2.2), determining the location of thiol containing peptides (by autoradiography of peptides derived from [2-^{14}C] carboxymethyl chains,see section 2.4.2 and 2.3.2.3.1) and by epitope analysis using monoclonal antibodies supplied by Professor E. Habermann, Giessen. F.R.G. (section 2.3.2.4). The peptides purified by HPLC were further analysed by N Terminal sequence analysis (Chang et al., (1978) and section 2.6.3).

4.3.3 Analysis of Peptides Derived from Heavy or Light Chains by Silver Staining of Peptides in Polyacrylamide Gels.

The amounts of whole toxin and isolated chains were limited and so peptides were detected by silver staining of polyacrylamide gels rather than the less sensitive coomassie brilliant blue dye.

Diagram 4.1 shows the cleavage pattern from obtained from reduced - and - carboxymethylated heavy and light chains and unnicked toxin after limited cleavage by the V8 proteinase from *Staph. aureus.* There are about 24 peptides resolved in the track for the unnicked toxin and about 20 peptides in that for the heavy chain. The light chain is much less digested than either the heavy chain or the unnicked toxin, there are about 14 resolved peptides and 1 group of unresolved peptides. There are 11 peptides in the reduced - and - carboxymethylated light chain pattern that have the same mobility as peptides in the heavy chain. Also of note is the marked similarity between the digests of the heavy chain and the unnicked toxin. One third of the whole toxin is light chain and by proportionality one would expect to see a greater difference between the heavy and the unnicked chain if there were no similar regions in the light and

Diagram 4.1. SDS/Polyacrylamide - Gel Electrophoresis of Peptides from The Chains of Tetanus Toxin after Limited Digestion with V8 Proteinase.



(a) An SDS/12% polyacrylamide gel showing the products of limited cleavage with V8 proteinase. The gel is silver - stained and there was no visible staining in a parallel track containing the same amount of V8 proteinase only. Peptides common to the heavy and light chains are marked.

(b) A diagram, taken from the actual gel, showing the unique and common peptides in the heavy and light chains. Also shown are differences in peptides pattern between the heavy chain and the unnicked form of the toxin. [*] difficult to asses due to high amount of material in L track.

heavy chains. The common peptides were not from the enzyme (V8 proteinase) as there was no staining in an "enzyme only" track even on loading twice the amount used in the digestion. The differences between the heavy chain and the unnicked toxin are also noted: they consist of some peptides in the 25,000M_r region and some larger molecular weight peptides, any one of these could contain the link region between the light and heavy chains. There are no peptides in the light chain digest that are not present in the unnicked toxin digests also.

Diagram 4.2 shows the products of cyanogen bromide cleavage of the heavy and light chains. There is always a bad background in silver stained gels of cyanogen bromide digests despite extensive removal of the cyanogen bromide and the solvent used in the cleavage. There are 15 groups of peptides in the digest derived from the heavy chain and there are 11 in that from the light chain, of which 5 have the same mobility as peptides from the heavy chain digest.

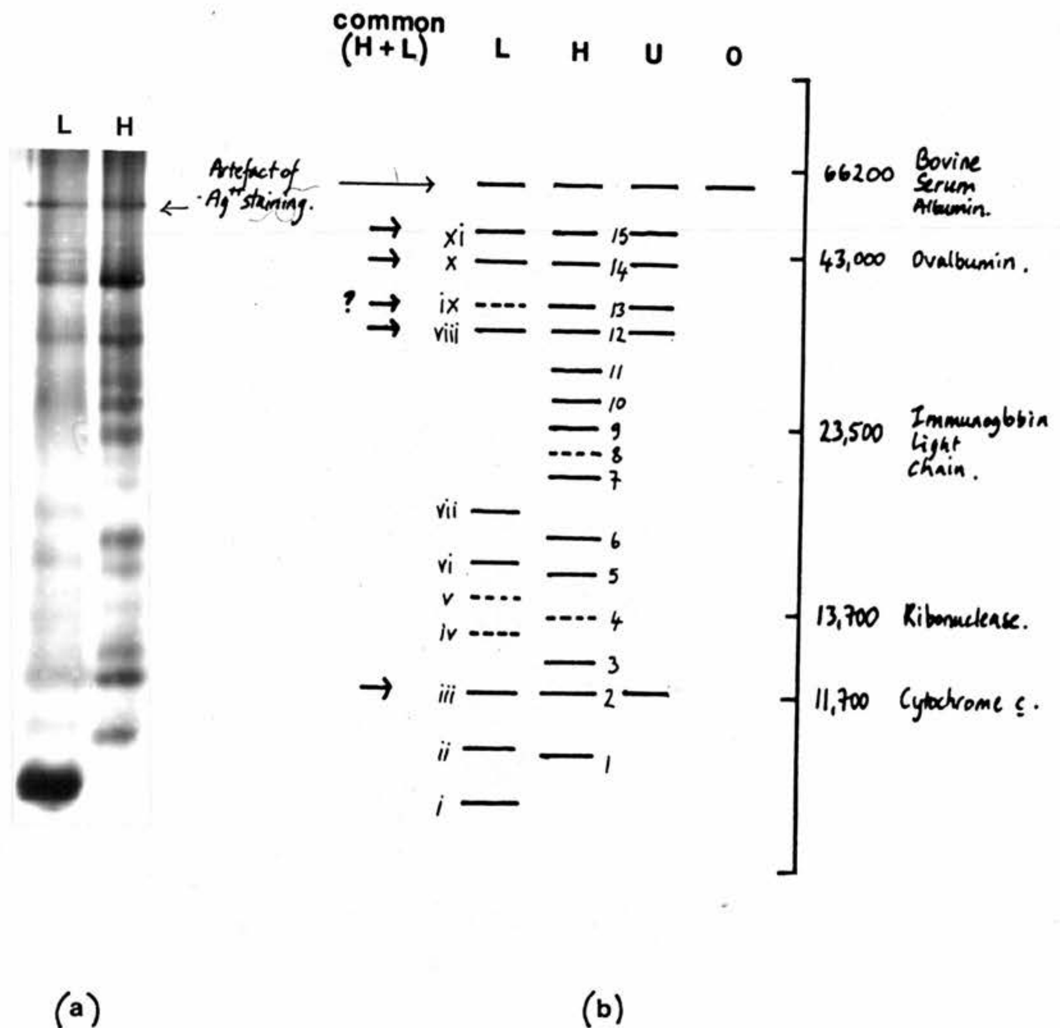
Cleavage at tryptophan residues using ō-Iodosobenzoic acid (section 2.5.1.2) was attempted but was not successful. The experimental conditions for cleavage of proteins using this reagent caused irreversible precipitation of the reduced-and-carboxymethylated heavy chain, light chain or unnicked toxin.

4.3.4 Epitope Mapping of V8 Proteinase Digests of The Heavy and Light Chains.

This work was a collaboration with Professor E. Habermann and Dr K. Goretzki of the Institüt fur Pharmakologie der JLU, Giessen, F.R.G.. Professor Habermann's group has prepared a number of monoclonal antibodies against the toxin or toxoid (Ahnert-Hilger et al., 1983). Gels of digests of the separated chains using Staph. aureus V8 proteinase were made by the author and Dr Goretzki used the immunoblotting technique (section 2.3.2.4) to test the peptides produced in this way for reactivity against the monoclonal antibodies

The antibody samples were;a polyclonal serum that was active against toxin and two monoclonal antibody preparations that had previously been shown to react with one or other of the heavy or light chains of tetanus toxin (by immunodotting or the ELISA technique). The monoclonal antibody (P883D5II2A), which had previously bound only whole light chain now bound particular peptides in the digests derived from both of the chains. The monoclonal

Diagram 4.2. SDS/Polyacrylamide - Gel Electrophoresis of Peptides from The Chains of Tetanus Toxin after Cleavage with Cyanogen Bromide.



(a) A 13% gel showing the products of cleavage with cyanogen bromide. Peptides common to the heavy and light chains are marked. The gel is silver stained.

(b) A diagram, taken from the actual gel, showing the common and unique peptides more clearly. Arabic numerals correspond to peptides in the heavy chain digest, and roman numerals to those in the light chain digest. Peptides in the unnicked digest are named as for the heavy chain fragments.

antibody (P104A1B268) which had previously bound whole heavy chain was an antibody of low avidity and it was only just possible to see the binding of this monoclonal antibody with peptides in the heavy chain digest. In this case there was no discernible binding to peptides in the light chain digest. The polyclonal sera (AK III) which was included as a control did bind peptides in the digest derived from each of the chains (diagram 4.3).

4.3.5 Thiol Peptides.

Diagram 4.4 shows an autoradiograph of a Cleveland gel of [2-^{14}C] carboxymethyl - heavy and light chains. There is a peptide of 25,000M_r that is present in both the heavy and light chains.

4.3.6 Analysis of Cyanogen Bromide Fragments by HPLC and N Terminal Sequence Analysis.

This work was done at the Department of Botany of the University of Durham with the help of Dr M. Richardson, who did the N terminal sequence for peptides L2CN23, L2CN24 and H2CN20 that are reported here.

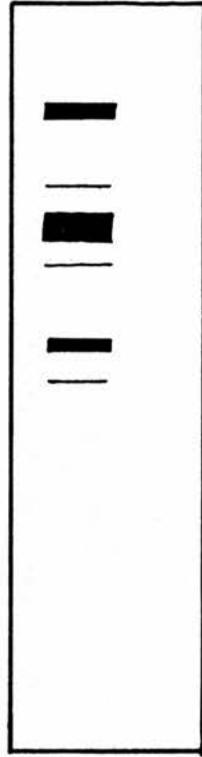
There were 30 peaks in the traces from the cyanogen bromide cleavage of the reduced and carboxymethylated light chain, and 29 for that of the heavy chain (diagrams 4.5, & 4.6). There were peptides from each digest that had the same retention time. The N terminal amino acids (and in a few cases the second and third amino acid) for some of these fragments are given in table 4.7. Fragments 4 & 5 are thought to be artefacts of some kind. Two of the peptides in the light chain digest were coloured : L2CN24 had a blue green colour in solution and L2CN28 had a blue tinge, (presumably some component of the coomassie dye binding to a particular peptide). Peptides with these same properties were also present at the same retention time in the digest of the heavy chain ie H2CN20 and H2CN24 respectively. The N terminal amino acids for both of the 'green' peptides were determined and were similar; the terminal dipeptide was Tyr-(Ile/Leu or Ala), (and see (ii) on table 4.7). This result is extremely encouraging as it means that in both the heavy and lights chains there is a sequence (see overleaf),

Diagram 4.3 Epitope Mapping of The Light and Heavy Chains of Tetanus Toxin.

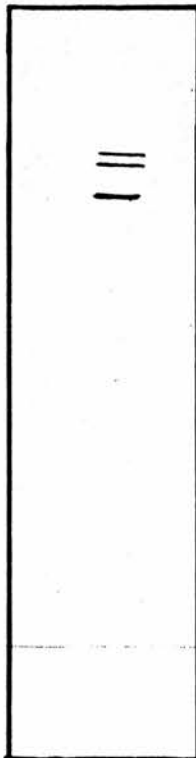
1 and 2 refer to V8 proteinase digests of the light and heavy chain respectively, whereas a,b and c refer to the antibody used.

a. AK 111, a mouse polyclonal sera.
b. PI04A1B268, a mouse monoclonal antibody specific to heavy chain.
c. P883D5112A, a mouse monoclonal antibody specific to light chain.

1. a b c



2. a b c



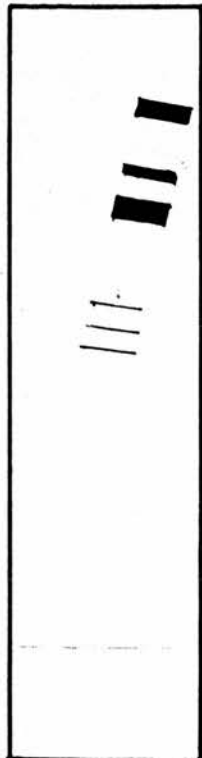
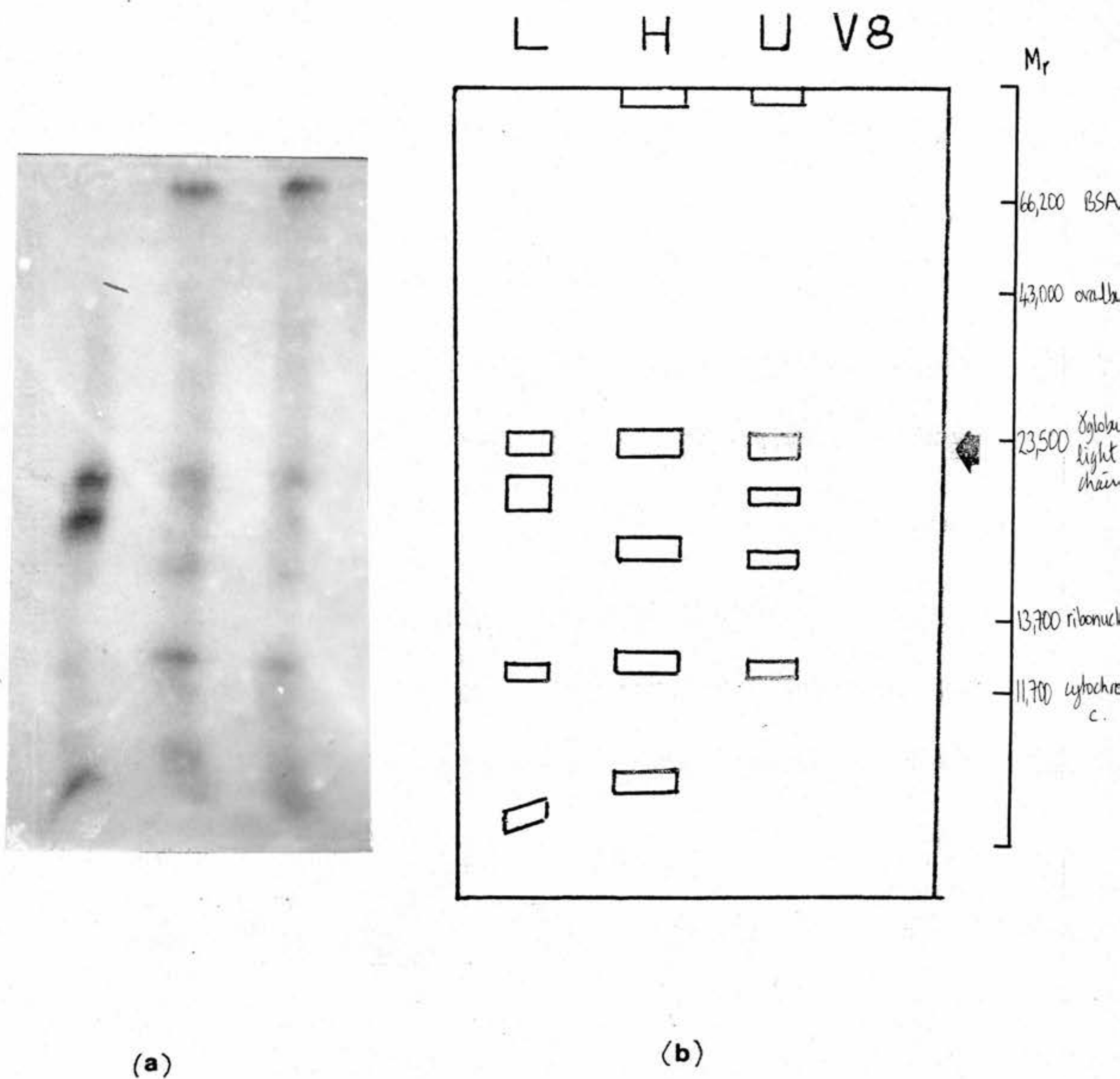
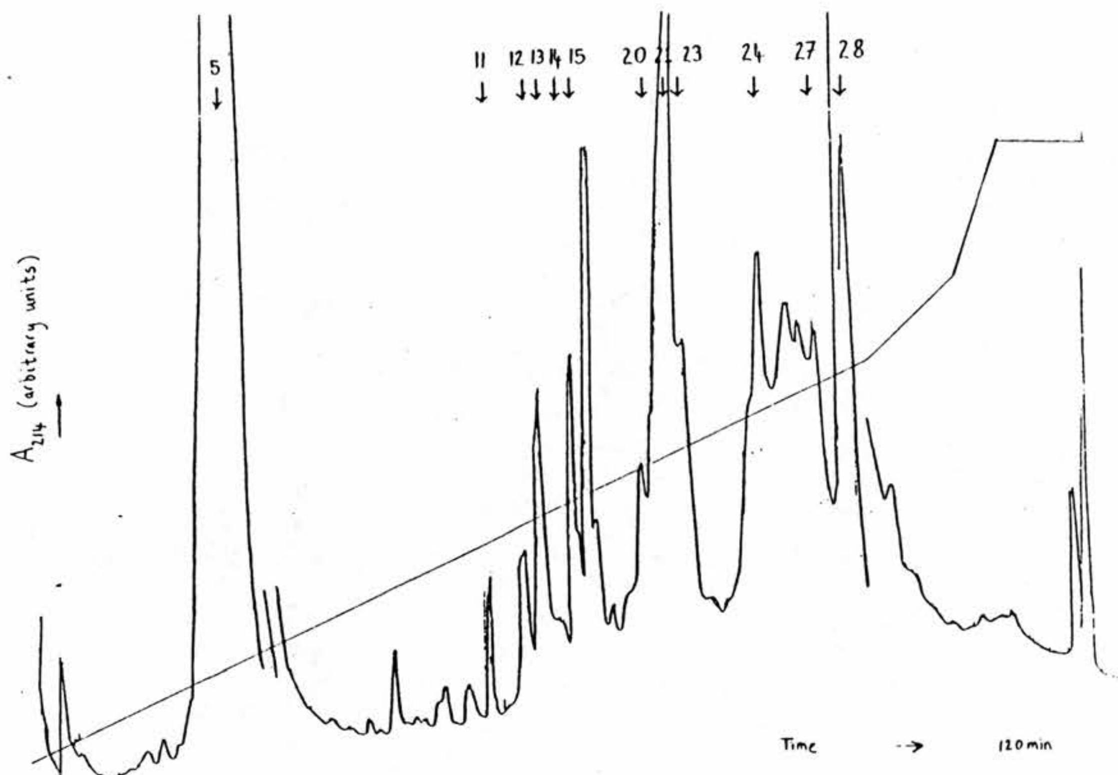


Diagram 4.4. SDS/Polyacrylamide - Gel Electrophoresis of Thiol Peptides from The Chains of Tetanus Toxin after Limited Digestion with V8 Proteinase.



Autoradiography of a 12% gel showing the ^{14}C - carboxymethyl-peptides obtained after limited cleavage with V8 proteinase. (a) is a photograph and (b) a tracing of the autoradiograph.

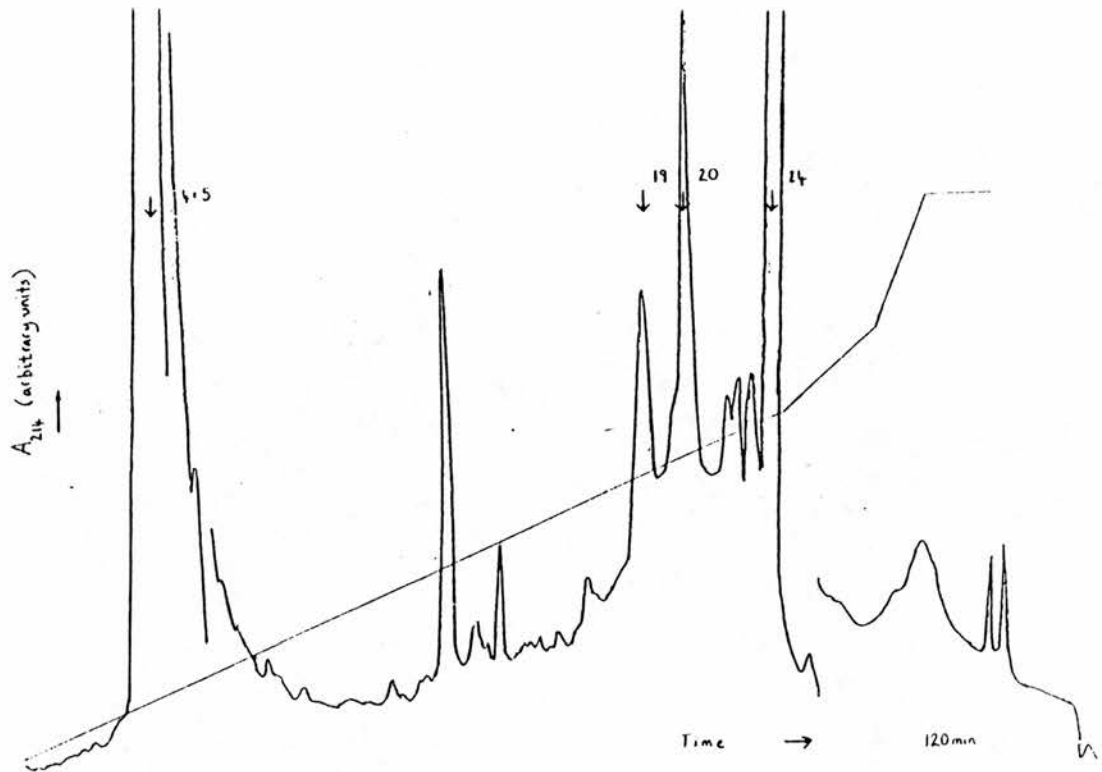
Diagram 4.5. Peptide Map (by HPLC) of the Light Chain of Tetanus Toxin after Cyanogen Bromide Cleavage.



200 µl cyanogen bromide digest of reduced and carboxymethylated light chain, $3mgml^{-1}$ in 6M guanidine / 0.1% Tri-fluoroacetic acid. The column was µ Bondapack C_{18} (5mm x 25cm). The flow rate was 1ml min^{-1} and detection of peptides was by absorbance at 214nm. The gradient conditions were : eluant A, 0.1% Tri-fluoroacetic acid, eluant B, acetonitrile in 0.1% Tri-fluoroacetic acid, 0-50% B, 50-70% B and 70-100% B. (120 minutes)

A discontinuity in the line is a scale change in the absorbance : generally $0 \rightarrow 0.5$ but was changed to $0.5 \rightarrow 1.0$ and $1.0 \rightarrow 2.0$ at high peaks.

Diagram 4.6. Peptide Map, by HPLC, of The Heavy Chain of Tetanus Toxin after Cyanogen Bromide Cleavage.



180 μl cyanogen bromide digest of reduced and carboxymethylated heavy chain, 3mgml^{-1} in 6M guanidine /0.1% Trifluoroacetic acid. The column was μ Bondapack C_{18} (5mm x 25cm). The flow rate was 1ml min^{-1} and detection of peptides was by absorbance at 214nm. The gradient conditions were those noted in diagram 4.5. A discontinuity in the line signifies a change of scale in the A_{214}, generally 0→0.5 but changed to 0.5→1.0 and 1.0→2.0.

Table 4.7. N Terminal Amino Acids of Cyanogen Bromide Fragments from The Heavy and Light Chains of Tetanus Toxin.

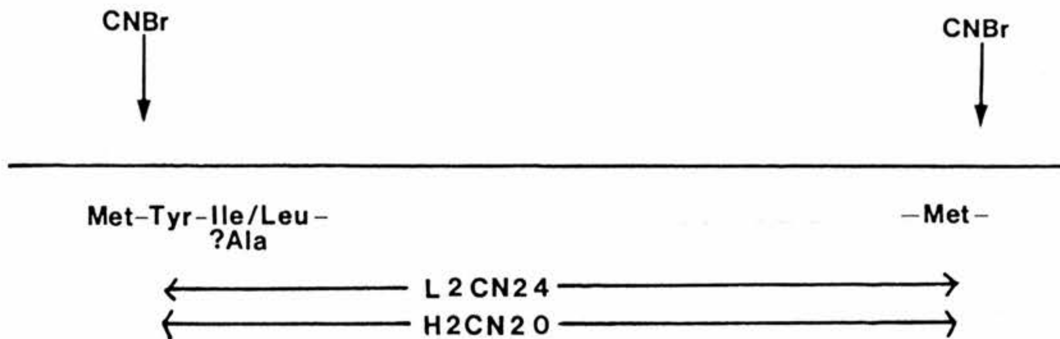
Fragment (1)	Retention Time (min)	Nterminal amino acid	Penultimate amino acid	antepenultimate amino acid	comment
L2CN 11	52	Arg?			
L2CN 12	55.9	Pro	Ile/Leu		
L2CN 13	57.4	Pro	Ile/Leu		
L2CN 14	57.9	Pro			
L2CN 15	61.2	Tyr	Ile/Leu		
L2CN 20	68.8	Tyr	Ile/Leu		
		Pro	Pro		
		Asp			
L2CN 21	71.4	Ile/Leu	Thr	many	
		Glu	Ile/Leu		
		Arg	Val		
			Pro		
L2CN 23	73.2	Pro	Ile/Leu	Thr	Nterminal of light chain ?
H2CN 19	76.1	Phe			
		Tyr			
L2CN 24	81.3	Tyr	Ile/Leu or Ala(ii)		green tinge to solution(iii)
H2CN 20	81.0	Tyr	Ile/Leu or Ala(ii)		green tinge to solution(iii)
L2CN 27	87.2	Val	Ile/Leu		
		Ala			
L2CN 28	90.1	Val	Ile/Leu		blue solution(ii
		Ala			
H2CN 24	90	?	?		blue solution(iii

notes overleaf.

Notes (i) L2CN refers to cyanogen bromide fragments of reduced and carboxymethylated light chain; similarly H2CN refers to those of the heavy chain.

(ii) The HPLC run was duplicated: in the first of the ' L digest ' this sequence was Tyr-Ala and on the second Tyr-Ile/Leu, in the first run of the H digest this sequence was Tyr-Ile/Leu and on the second Tyr-Ala.

(iii) Colours refer to peptide in the acetonitrile post elution.



and the peptide must have similar amino acids as the retention times for H2CN20 and L2CN24 are the same.

Peptide L2CN23 in the light chain digest may be the N terminal peptide of the light chain as the first two amino acids are the same as those in the sequences reported by Britton (1981) and Robinson & Hash (1982) (and see section 1.4.4). This peptide is not present in the cyanogen bromide cleavage pattern of the heavy chain.

4.4 Discussion.

The similarity in amino acid composition of the two chains of tetanus toxin is so unusual as to suggest that there are internal repeats within this protein ie that the heavy chain has two similar halves, each of which are homologous with the light chain.

The objections to the proposed homology rest on several properties of the two chains where they do not crossreact. These objections and the arguements that refute them are set out later in this section.

The constant domains in the immunoglobulins viz C_L,C_H1,C_H2 and C_H3 have different functions although the amino acid sequences are 40% homologous and the domains have the same secondary and tertiary folding pattern "the immunoglobin fold" (Stryer, 1981). At the other end of the spectrum even small changes in the amino acid sequence of closely related proteins can produce changes in function. The haemagglutinin glycoproteins of influenza virus membrane bind the virus to cells by interacting with receptors on the cell membrane. The specificity of binding varies for different influenza viruses and this differential recognition of cellular receptors rests on a single amino acid substitution. The residue at position 226 of the haemagglutinin glycoproteins can be leucine, glutamate or methionine as a result of base changes in triplet nucleotides 754-756, and the specific sugar that is recognised by the haemagglutinin is dependant on whichever of these three amino acids is present. (Rogers et al.,1983)

A second example of small changes in protein sequence causing a change in the activity of a protein is the recent report of Wilkinson et al.(1984) who engineered two point mutations in a tyrosyl tRNA synthetase. They obtained a large improvement in the activity of the enzyme by changing one particular threonine to either an alanine or a proline by oligodeoxynucleotide - directed mutagenesis.
Therefore the hypothesis that there are regions in the heavy chain that are homologous to regions in the light chain does not conflict with the observation that the various parts of the toxin have different functions. The proteins can be different in structure and function yet have similarity at the level of the primary sequence. The two chains are sufficiently different in composition generally (table 4.1) to have different functions. There is a particular difference in composition that is interesting; the apparent inverse relationship between the proline and alanine content of the two chains. The light chain has five more proline residues and five less alanine residues than the heavy chain per molecule of 50,000M_r. If this difference in amino acid composition reflects a true difference in the sequence then it would be due to a single base change (CCN for proline to GCN for alanine); that, because of the special properties of proline, would produce a marked change in structure and hence in function.

The results from the epitope mapping experiments would suggest that the chains do have different conformations but that there are regions of sequence homology. Although the experiments are preliminary, the results illustrate that the reaction with the monoclonal antibodies is complex. The implication from the immunoblotting of the digests is that the monoclonal antibody P883D5 II2A is specific for an amino acid sequence common to both chains and available for interaction with the antibody in the whole light chain yet masked in the conformation of the whole heavy chain.

The significance of the observed similarity between the amino acid compositions of the chains to any sequence homology was estimated by the method of Cornish - Bowden (1983). In the present work this method was used to determine intraprotein homology rather than interprotein homology and therefore the method was first tested by calculating the homology between domains in proteins that have internal repeats and comparing with the actual homology from the

amino acid sequence. The proteins chosen were calmodulin, which is an example of a protein where all the domains (4) have the same function (ie binding Ca^{++}), and the other immunoglobin EU as an example of a protein where the domains have different functions, but have evolved from a common ancestor. In both cases the calculated number of differences, $S\Delta n$, and the actual number of differences, M, were in agreement (section 4.2.2). However as $S\Delta n$ is equal to $M \times (1 \pm 0.38)$, then it may be prudent to use $S\Delta n$ qualitatively as a predictor of homology rather than quantitatively as an estimate of actual homology.

The value of $S\Delta n$ calculated from the light chain and the halved composition of the heavy chain of tetanus toxin is less than 0.42N. As this value was found for both the data of Britton (1981) and that of DiMari et al (1982a) it means that the compositional similarity was not due to some particularities in the Edinburgh amino acid analysis. These values of $S\Delta n$ imply that there is a strong probability that sequence homology will be found between the heavy and light chains and that the heavy chain consist of two similair halves.

Further evidence for the hypothesis was obtained from the products of proteolytic cleavage. The initial chosen method for separating the peptides was polyacrylamide gel electrophoresis in SDS rather than more discriminatory techniques such as chromatography or electrophoresis. A one amino acid difference in a peptide could have a noticeable effect on the polarity or hydrophobicity of the peptide but the length of the peptide (which is the parameter measured in SDS -PAGE) would not be altered unless the mutation involved an amino acid at a cleavage site.

The peptides pattern for H,L after limited proteolysis by the V8 proteinase from Staph. aureus are distinct but similar. Each pattern contains some peptides of the same mobility as peptides present in the other. Despite the limitations of the Cleveland method (eg. limited proteolysis) this degree of similarity is important as this technique tends to emphasize differences between proteins. The evidence for the latter statement can be taken from Cleveland's own paper. The Cleveland analysis of α and β tubulin shows them to be distinct proteins (Cleveland et al.,1977) despite similar amino acid compositions and N terminal amino acids (Luduena &

Woodward, 1973). The SΔn calculated from the data in Luduena & Woodward (1973) for this comparison passes the strong test yet the protein digests show distinct patterns with some common peptides in Cleveland's gels (Cleveland et al., 1977).

The SDS - Polyacrylamide gels of the digests of the heavy and light chains also showed "common" peptides when the isolated chains were cleaved with cyanogen bromide. However the main question with chemical cleavage remained unanswered viz whether the absolute number of peptides in the heavy chain digest was (n + 1), ($^n/_2$ + 1) or some integer between the two, where n is the number of methionine residues in the heavy chain. It was impossible to say with confidence that the number of peptides in the digest (15 instead of 24) was due to repeating sequence or limitations of the system in sensitivity and resolution.

Another approach was taken to fractionate the cyanogen bromide fragments by using HPLC. There were some peptides present in the digests of both chains and the most interesting of these was one that had the same tripeptide at the N terminal. As mentioned in section 4.3.6 this peptide is strong evidence for the presence of homologous regions in the chains. Unfortunately this technique may have been too discriminating to study the number of total fragments eg two peptides that differ only in the product of the cyanogen bromide reaction either of homoserine or homoserine lactone at the "new" C terminal can have different retention times in reversed phase HPLC. This point and the possibility of incomplete cleavage are some explanations for the light chain digest showing 30 peptides when the whole light chain contains only 13 methionine residues (table 4.1). There are 29 peaks from the heavy chain digests and as there are 23 methionine residues in the heavy chain then it would seem likely that there are no internal repeats in this chain, but this statement cannot be made with certainty or at all, because of the greatly increased number of light chain fragments.

Robinson (Vanderbilt University, Tennessee, U.S.A., personal communication) has evidence for a repeating structure in tetanus toxin from gel filtration of cyanogen bromide digests of the toxin and chains, but no details have been published.

An alternative approach to examining the total digest pattern is to look at the peptide environment of certain amino acids.

Cystine residues are generally well preserved in the evolution of a protein as disulphide bridges play an important role in tertiary and quaternary structure. The autoradiograph of a Cleveland gel (V8 proteinase) of heavy and light chains labelled with ^{14}C at the thiol group showed a radioactive peptide of 25,000M_r that was present in the digests of both chains. The presence of this common peptide in itself is not sufficient proof for the homology but it is a start to obtaining information on the composition and sequence of the "common" peptides.

There is evidence in the literature which on first inspection seems to be contrary to there being any homology but which on a second look can either be accommodated or dismissed. , The heavy and light chains do not interconvert in function. This is not suprising, many proteins are similar in either one or both of primary or secondary structure but do not have the same function. Previous work on the antigenicity of the isolated chains has shown that the chains are immunologically distinct. The difference between the experiments reported here and these other experiments are that the latter were investigations of the reaction of polyclonal sera with the whole chains, generally on agar plates whereas here the emphasis was more on the reactivity of antibodies to particular peptides (which may reflect primary structure more than the whole chains). A third point is that Britton (1981) found the pI and the N terminal sequences of the chains to be different, these findings do not argue against the homology as the hypothesis is that there are regions of homology not that the chains have complete identity.

A more serious objection is that there is a complementary set of amino acid composition data ie that obtained by Helting and Zwisler (1977) for the fragments of tetanus toxin produced by papain cleavage. As discussed in section 1.5.3. Fragment B of 100,000M_r was found to be the light chain joined by disulphide bonds to the N terminal of the heavy chain, and fragment C of 50,000M_r is the remainder of the heavy chain. If the internal homology idea is correct, then these two fragments should show the same compositional relationship as the heavy and light chains. This is not the case. If $S\Delta n$ is calculated from the compositions of B and C given by Helting & Zwisler then the value is found to be greater than N. Such a result means that either the fragment compositions are incorrect or

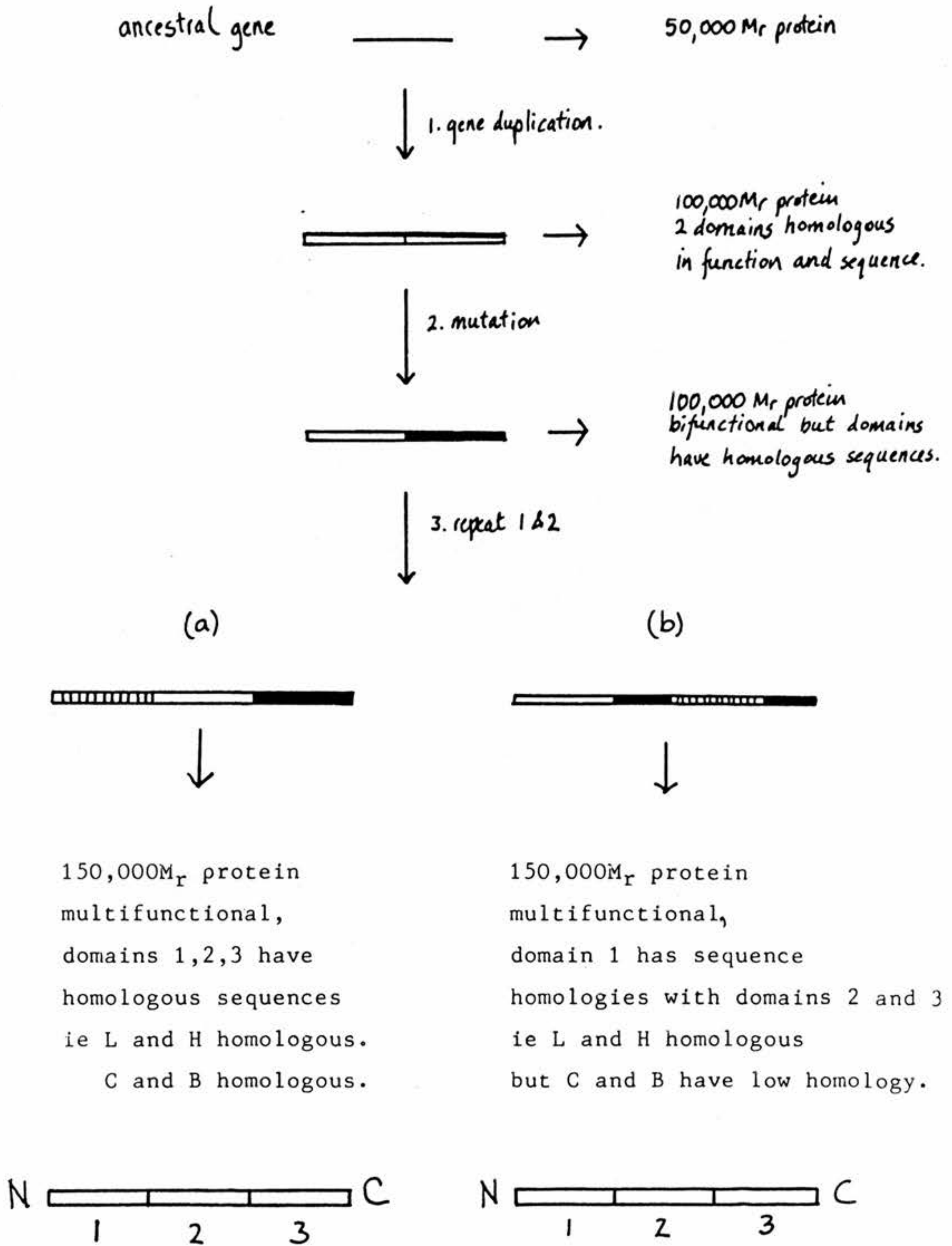
that the homology idea must be modified or discarded. It is unlikely that the amino acid compositions were technically wrong but they could be incorrect in the sense that the fragments are obtained from proteolysis of the toxin by an exogeneous enzyme and the cleavage, in this case, may remove larger peptides than the clostridial cleavage.

If the fragments can be regarded as conceptually equivalent to the heavy and light chains then the homology idea must be modified. The original suggestion was that the heavy chain has two similar halves, each of which are homologous with the light chain. There is another arrangement which would still maintain the homology idea but which can be best introduced after a discussion on the evolution of the toxin. The observed sequence homologies between the light and heavy chains could have arisen by gene duplication and rearrangements in the evolution of the toxin. If the "repeating unit" in the toxin is a protein region of 50,000M_r then one could speculate about an ancestral gene coding for a 50,000M_r protein that by gene duplication and fusion now codes for a protein that consists of three different but related domains, one in the light chain and two in the heavy. This arrangement for the development of the heavy and light chains of the toxin is illustrated in diagram 4.7. Also in the diagram is an alternative scheme of gene fusion and rearrangement where the gene product ie the protein would have statistical properties such that the light chain amino acid composition would be half of the heavy chain but that fragments B and C would have unrelated amino acid compositions.

It is relevant to note here that the toxin is thought to be coded on a plasmid (Laird et al., 1980) as duplication and rearrangements of such genes are thought to occur at a faster rate than genes coded on the chromosome. Plasmids are seen as vehicles for rapid evolution of new protein activities because of the potential the gene has to move around the plasmid and between the plasmid and the chromosome.

A final point is that the suggestion for the sequence homology between the light and heavy chains can only be proven one way or the other when the sequence of the protein or the gene coding for the protein is known.

Diagram 4.7. Possible Mechanisms for The Evolution of The Light and Heavy Chain.



Chapter 5. Tetanus Toxin and Ca^{++}-Calmodulin Dependant Phosphorylation Changes in Synaptosomes.

5.1 Introduction.

5.2 Methods.

5.2.1 Isolation of Synaptosomes.

5.2.2 Phosphorylation of Proteins in Lysed Synaptosomes.

5.2.2.1 Binding of Toxin To Whole Synaptosomes.

5.2.2.2 Protein Phosphorylation in Lysed Synaptosomes.

5.2.3 Phosphorylation of Proteins in Thawed Synaptosomes.

5.2.4 Phosphorylation of Proteins in Intact Synaptosomes.

5.2.4.1 Preparation of Tetanus Toxin.

5.2.4.2 Binding of Toxin To Whole Synaptosomes.

5.2.4.3 Protein Phosphorylation in Intact Synaptosomes.

5.2.4.4 Isolation of Crude Protein 1.

5.3 Results.

5.3.1 The Effect of Tetanus Toxin on Phosphorylation in Lysed Synaptosomes.

5.3.2 The Effect of Tetanus Toxin on Phosphorylation in Thawed Synaptosomes.

5.3.3 The Effect of Tetanus Toxin on Phosphorylation in Whole Synaptosomes.

5.4 Discussion.

5.1 Introduction.

The aim of the present study was to determine the effect of tetanus toxin on the Ca^{++}-calmodulin - dependant phosphorylations in synaptosomes. As previously mentioned in section 1.3 the effect of the toxin on the phosphorylation changes is a logical development in the determination of the molecular activity of the toxin. Changes in the phospho-protein pattern could answer questions as to the functional integrity of the various components of the stimulus - secretion process viz calmodulin, the calmodulin - dependant kinases and phosphatases or the substrate proteins.

5.2 Methods.

The effect of toxin on the Ca^{++} - calmodulin dependant phosphorylation was studied using adenosine 5 '-[γ-^{32}P] triphosphate ([γ-^{32}P] ATP) or inorganic [^{32}P] phosphate. In both sets of experiments, the tetanus toxin was incubated with whole synaptosomes but when adenosine 5'-[γ-^{32}P] triphosphate was used the synaptosomes were lysed after the toxin incubation but before the addition of the [γ-^{32}P] ATP. These synaptosomes will be referred to as "intact" and "lysed" respectively. In another set of experiments using [γ-^{32}P] ATP the toxin was added to lysed synaptosomes sometime before the addition of [γ-^{32}P] ATP. These preparations are called "thawed synaptosomes" as they were not fresh but had been stored at -20°C.

5.2.1 Isolation of Synaptosomes.

Synaptosome suspensions were prepared by the method of Dodd et al., (1981). Sprague Dawley or Wistar rats were used. Adult female rats weighing 120-150g were killed by cervical fracture and decapitation. The brains were removed rapidly, chopped and placed in 9ml of ice-cold unbuffered 0.32M sucrose and 100μl of PMSF (10mgml^{-1} propan -2-ol) was added. The tissue was dispersed using a homogenizer by eight up and down strokes of the pestle. The centrifugation steps were carried out at 4°C in a Beckman L8 centrifuge fitted with a 70Ti rotor. The initial homogenate was centrifuged at 5000rpm for 10 minutes. The low speed supernatant fraction (ca 6ml) was layered onto 4ml of ice cold 1.2M sucrose and centrifuged at 50,000rpm for 15 minutes. The synaptosomes, myelin and some microsomes were retained at the gradient interface. This material was collected (ca 1-2ml) and diluted 2:5 with ice-cold 0.32M sucrose. The diluted suspension (ca 6ml) was layered onto 4ml of

0.8M sucrose and centrifuged at 50,000rpm for 15 minutes. This final centrifugation yielded a synaptosomal pellet ready for suspension in medium.

5.2.2 Phosphorylation of proteins in Lysed Synaptosomes.

5.2.2.1 Binding of Toxin to Whole Synaptosomes.

The pellet was resuspended at approximately 3-5$mgml^{-1}$ in oxygenated calcium - free Krebs Ringer (40mM Tris. Chloride pH 7.6 containing 130mM NaCl / 4mM KCl / 1mM EGTA and 20mM glucose). 27μg of tetanus toxin (1.35$mgml^{-1}$ in 5mM-sodium phosphate pH 7.2 containing bovine serum albumin at 2$mgml^{-1}$) was added to 200μl aliquots of the synaptosome suspension and the volume made to 250μl with Ca^{++}-free Krebs Ringer. The control for the toxin was an equal volume of Ca^{++}-free Krebs Ringer. The intact synaptosomes were incubated with the toxin for 90 minutes at 37°C. Any unbound toxin was removed by adding 1ml of calcium free Krebs-Ringer to each sample and centrifuging for 5 minutes in a Beckmann microfuge. The supernatant was removed and the synaptosomal pellet resuspended in 200μl of the calcium-free Krebs Ringer. Toxin-free samples were also washed by this proceedure.

5.2.2.2 Protein Phosphorylation in Lysed Synaptosomes.

100μl aliquots of suspension were added to Krebs Ringer buffer with or without the addition of 20μl of 10mM $CaCl_2$, to a final volume of 210μl. The suspensions were sonicated for 5 seconds and the phosphorylation reaction begun by the addition of 40μl of 4mM $MgCl_2$ and adenosine 5'-[γ-^{32}P] triphosphate (concentration of [γ-^{32}P]-ATP was 33μM, 3000Ci/mol). The samples were sonicated for a further 5 seconds and incubated at 37°C for 5 minutes. The samples were divided in two and the reaction terminated by either the addition of an SDS stop solution or by acid precipitation of the proteins.

The SDS stop soln was 30mM Tris chloride pH 7.4 containing 5% (w/v) SDS/5% (w/v) sucrose/3mM EDTA/2mM DTT/0.01% (w/v) bromophenol blue and 50μl of this was added to 125μl of the synaptosomal suspension. The samples were run on polyacrylamide gels in SDS (section 2.3.1.2). The gels were fixed, stained, destained and dried and autoradiographed as described previously (section 2.3.2.1 and 2.3.2.3.1). Densitometric traces were made of the autoradiographs. The traces gave the same profile as that obtained by liquid scintillilation counting of 1mm slices of the dried gels (section

2.3.2.3.2) and so for convenience autoradiography was the method routinely used to quantify incorporation of ^{32}P. An alternative approach to quantifying the ^{32}P incorporation was acid precipitation of proteins followed by liquid scintillation counting of the precipitate, but the results were disregarded as reproducibility was poor using this technique.

5.2.3 Phosphorylation of Proteins in Thawed Synaptosomes.

The toxin preparation and all buffers etc. were the same as those used in section 5.2.2, the only difference being the protocol. Aliquots (50μl) of the synaptosomal suspension were stored at -20°C for 72 hours. The thawed synaptosomes were sonicated and incubated at room temperature for 15 minutes with or without the addition of 27μg of tetanus toxin and the final volume adjusted to 160 μl with Ca^{++} -free Krebs Ringer. The phosphorylation reaction was begun by adding 20 μl of 10mM $CaCl_2$ and 20μl of 4mM $MgCl_2$ /33μM [γ-^{32}P] ATP (3000Ci/mmol) and sonicating the samples once more. The incubation was continued for 5 minutes at 37°C and terminated by addition of the SDS stop soln. The samples were analysed by autoradiography of the dried gels The ^{32}P incorporation was determined by densitometric traces of the autoradiographs and also by slicing the dried gels in particular regions and counting the slices (section 2.3.2.3.2).

5.2.4 Phosphorylation of Proteins in Intact Synaptosomes.

5.2.4.1 Preparation of Tetanus Toxin.

Even after extensive dialysis the toxin samples contained sufficient inorganic phosphate (40fM to 20pM) to alter the specific activity of the added inorganic [^{32}P] phosphate (diagram 5.3). Therefore the controls were (i) a non toxin control (an equal volume of the last diffusate) and (ii) a boiled toxin control (an equal volume of supernatant from tetanus toxin that had been boiled for 5 minutes and then centrifuged.

5.2.4.2 Binding of The toxin to Whole Synaptosomes.

The pellet was resuspended at 5 $mgml^{-1}$ in oxygenated Krebs Ringer buffer ("KR1":-40mM-Trischloride pH 7.6 containing 130mM NaCl / 4mM KCl / 1.2mM $MgSO_4$ /1mM EGTA and 20mM glucose). The synaptosome suspension was divided in three, and either tetanus toxin (0.5ml of 4$mgml^{-1}$ in KR-1) or 0.5ml of one of the nontoxin controls (see 5.2.4.1) was added and the intact synaptosomes (final volume 2ml) were incubated at 37°C under O_2/CO_2 for 60 minutes to allow binding

of the toxin. Inorganic [^{32}P]phosphate (20μl of 10mCi/ml per 2ml of sample) was added and the synaptosomes were incubated for a further 30 minutes at 37°C under O_2/CO_2 in order to label the intrasynaptosomal ATP.

5.2.4.3 Protein Phosphorylation in Intact Synaptosomes.

Membrane depolarisation in the synaptosomes was induced by the addition of a Krebs Ringer buffer (KR2) that contained K^+ in a high concentration, and also Ca^{++}. The isotonicity was maintained by decreasing the NaCl concentration in an inverse relationship to the KCl concentration. KR2 was 40mM Tris.Chloride pH 7.6 containing 70mM NaCl/60mM KCl/1.2mM $MgSO_4$/2.2mM $CaCl_2$ and 20mM glucose. Aliquots (200 μl) of the synaptosomal suspension was added to 200 μl of either KR1 or KR2 and the incubation continued for 2 minutes at 37°C. The reaction was terminated by the addition of 100 μl of a sodium dodecyl sulphate stop solution (0.5M Tris chloride pH 6.8 containing 8% (w/v) SDS/40% (v/v) glycerol / 10mM EDTA / 10mM Benzamidine / 30mM Na F / 0.01% (w/v) bromophenol blue and 0.02% (v/v) 2-mercaptoethanol (added freshly).) to the sample followed by immediate boiling (2 minutes). The samples (125 μl aliquot) were run on 6%, 8% or 12% polyacrylamide gels in SDS. The gels were fixed, stained, destained and dried and autoradiographed as described in sections 2.3.2.1 and 2.3.2.3.1.

5.2.4.4 Isolation of Crude Protein 1.

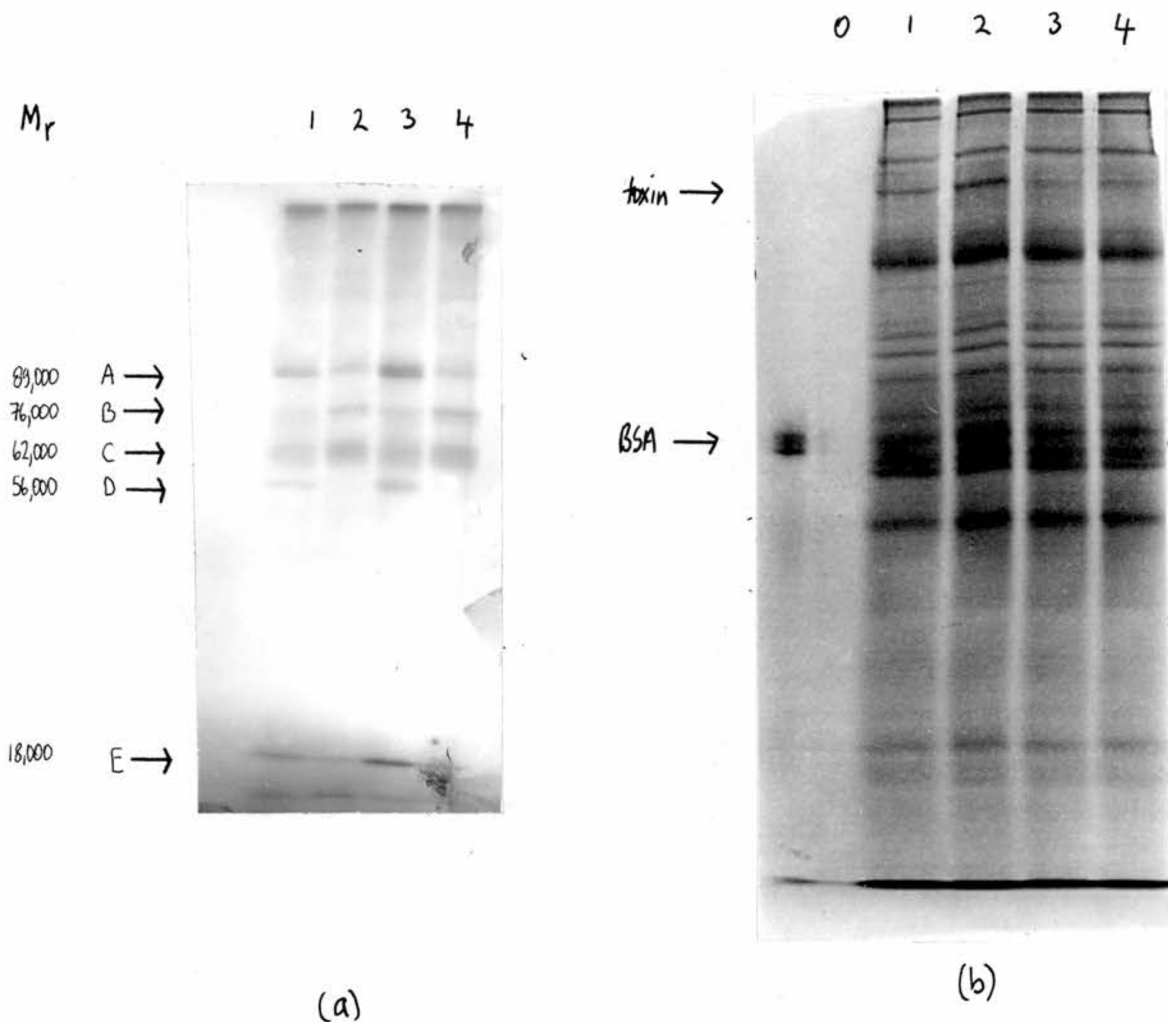
The radioactive bands containing protein 1 were cut from a dried 6% polyacrylamide gel using the autoradiograph as a guide. (The cut gel was re-exposed to check that the band had been correctly removed.) The gel pieces were reswollen for one hour in 125mM Tris-chloride pH 6.8 containing 0.1% (w/v) SDS and the protein was then peptide mapped by the method of Cleveland et al., (1977). The separating gel was 15% acrylamide and the mass of Staph. aureus V8 proteinase per slot was 0.16 μg. After electrophoresis the gel was dried and autoradiographed in the normal manner.

5.3 Results.

5.3.1 The Effect of Tetanus Toxin on Phosphorylation in Lysed Synaptosomes.

The pattern of protein phosphorylation in lysed synaptosomes is shown in diagram 5.1. The addition of Ca^{++} resulted in increased phosphorylation of three proteins, A (89,000 M_r), D (56,000M_r), E (18,000M_r) and decreased phosphorylation of protein B

diagram 5.1. The effect of tetanus toxin on the Ca^{++}- calmodulin dependant phosphorylations in lysed synaptosomes

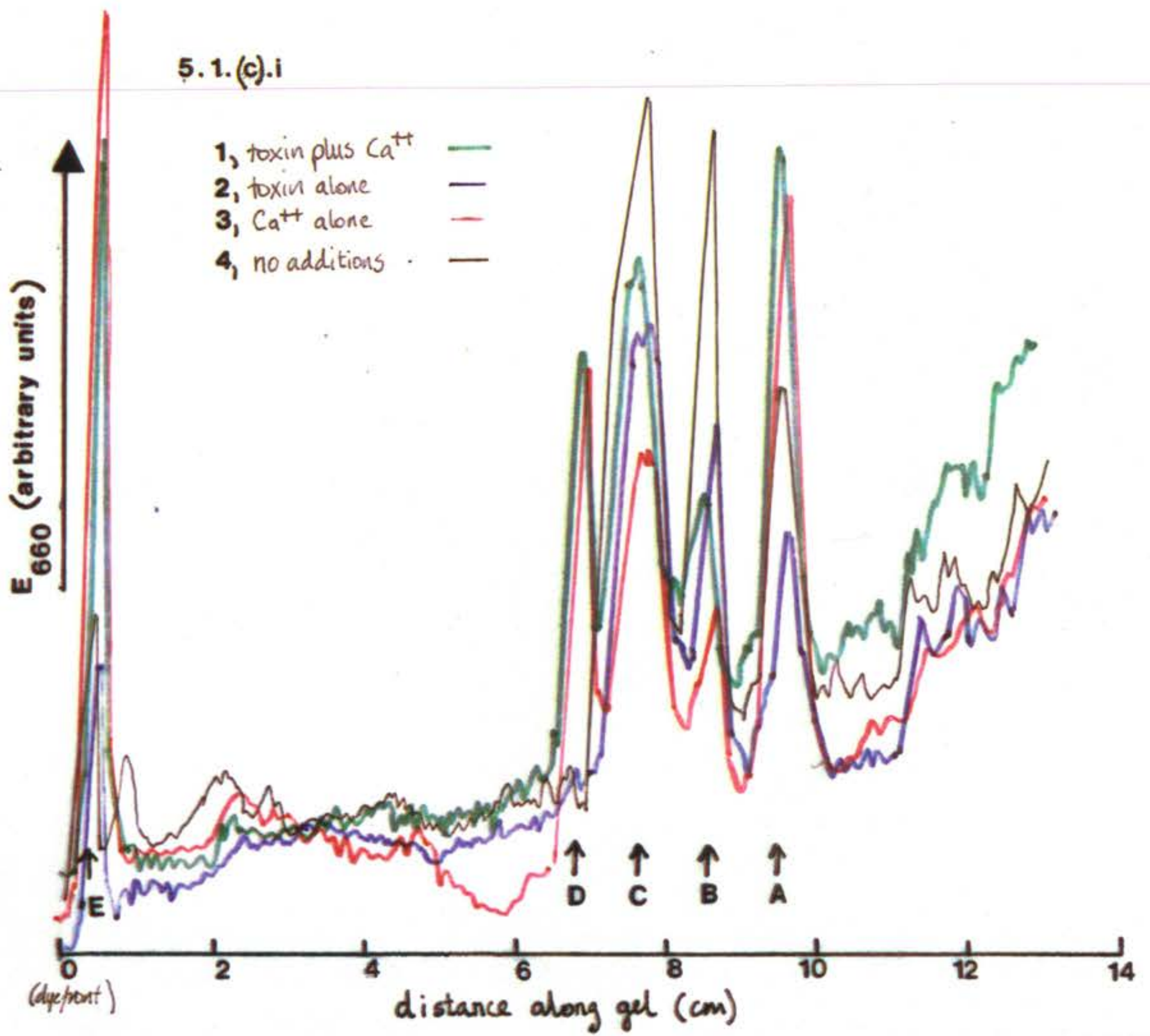


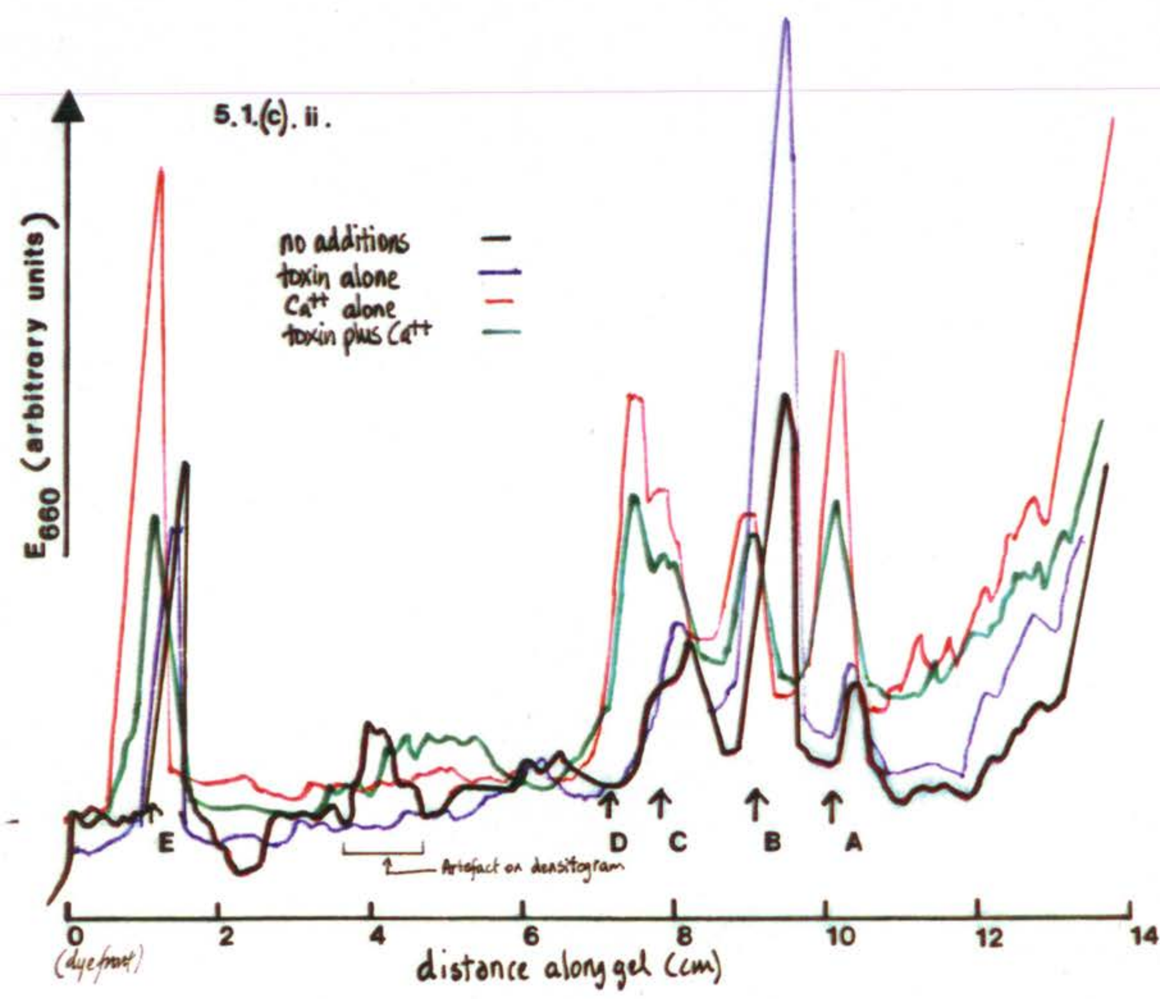
Tracks 1 and 3 are in the presence of Ca^{++}. Tracks 1 and 2 are proteins from synaptosomes incubated with tetanus toxin.

(a) An autoradiograph of the SDS/8% polyacrylamide gel.

(b) The same gel stained by coomassie brilliant blue. Tetanus toxin (150,000 Mr) is marked. The absence of bovine serum albumin in the washed synaptosomes implies that the association of the tetanus toxin with the synaptosomes is specific (BSA is the 66,000 Mr protein in track 0 of photograph (b)).

(c) Densitometric traces of the autoradiographs are shown on the following pages.


5. 1. (c). i
1, toxin plus Ca++
2, toxin alone
3, Ca++ alone
4, no additions
E660 (arbitrary units)
D
C
B
A
E
0
2
4
6
8
10
12
14
(dye front)
distance along gel (cm)


5.1.(c). ii.
E_{660} (arbitrary units)
no additions
toxin alone
Ca++ alone
toxin plus Ca++
E
D
C
B
A
Artefact on densitogram
0
2
4
6
8
10
12
14
(dye front)
distance along gel (cm)

($76,000M_r$) and to a lesser extent, if at all, C ($62,000M_r$).

Preincubation of synaptosomes with tetanus toxin before lysing, resulted in a change in the Ca^{++}-dependant phosphorylation pattern. The effect of the toxin was to diminish the Ca^{++}-dependant changes (see scan 5.1, and diagram 5.1). The only exception is the Ca^{++}-dependant dephosphorylation of protein B. Whereas the other Ca^{++}-dependant changes were partially inhibited in toxin treated synaptosomes, the dephosphorylation of protein B still occurred. Tetanus toxin had no effect on the Ca^{++}-independant phosphorylations eg compare the phosphorylation level of proteins A,C,D,and E in toxin treated and untreated synaptosomes in the absence of Ca^{++} (diagram 5.1). However there is again an exception in the case of protein B and this change is inconsistent. In one instance the incubation with toxin caused a dephosphorylation of protein B and yet in another experiment there was an apparent increase in phosphorylation (traces C(i) and C(ii) in diagram 5.1). There was no obvious difference in the protocol of these experiments to explain this small difference in the results.

The toxin binds specifically to synaptosomes as Coomassie staining of the gels showed the tetanus toxin in synaptosomal samples whereas the BSA was removed by the washing step (diagram 5.1).

5.3.2. The Effect of The Toxin on Phosphorylation in Thawed Synaptosomes.

The phosphorylation pattern in thawed synaptosomes was similar to that in freshly lysed synaptosomes except that protein B was absent. Preincubation of the thawed and lysed synaptosomes with toxin had no effect on the phosphorylation pattern seen in the absence of Ca^{++} (diagram5.2). Addition of Ca^{++} to synaptosomes previously treated with toxin resulted in a much diminished Ca^{++} effect (diagram 5.2). This experiment may show that the binding of toxin to neuronal cell membrane is not the actual biological event in tetanus intoxication and that the lag period of 1 hour generally observed in inhibition of transmitter release is due to binding and translocation of the toxin. However a firm statement on these two points would require time course studies, which unfortunatly was not possible in the present work.

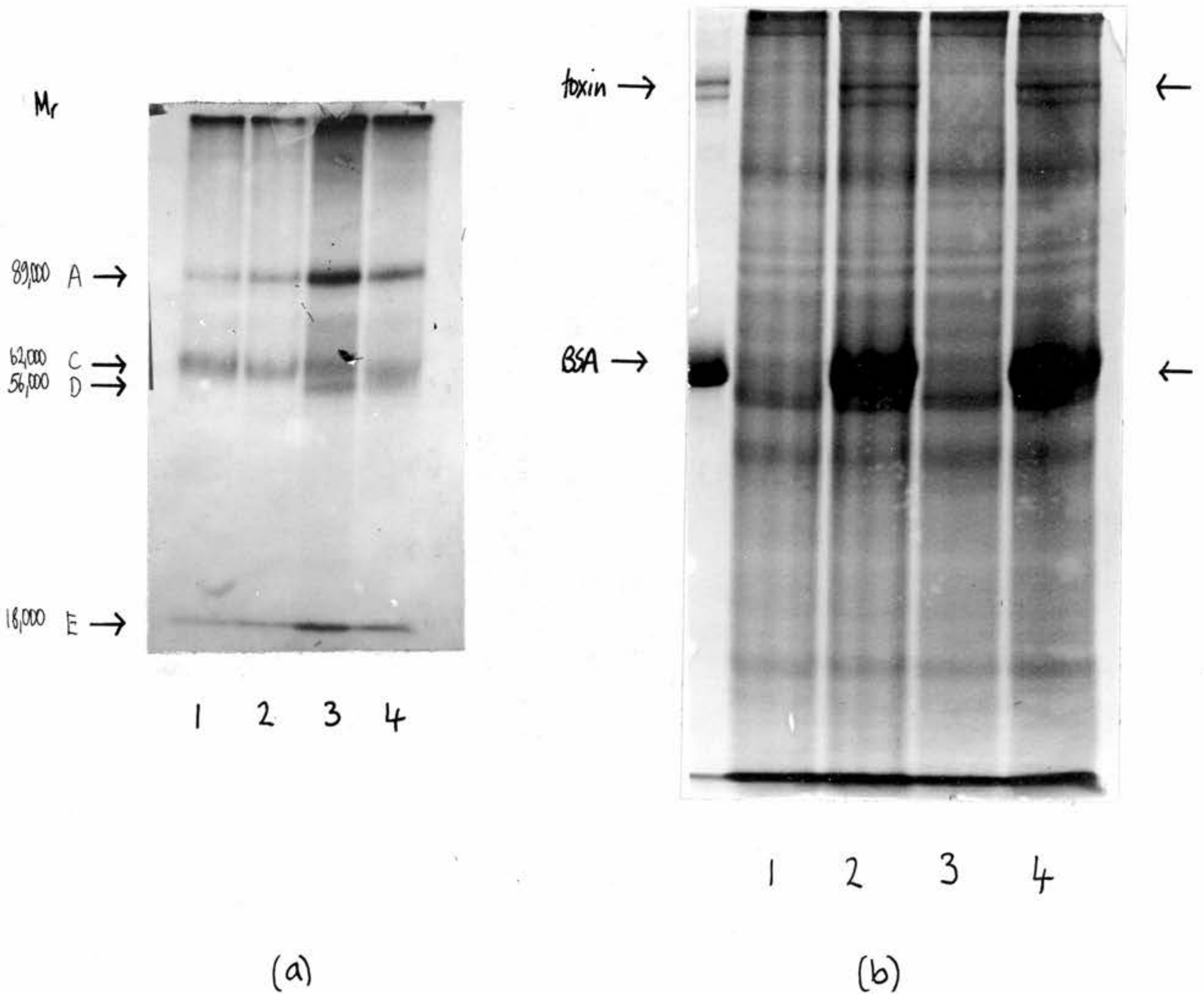
diagram 5.2. The effect of tetanus toxin on the Ca^{++}- calmodulin dependant phosphorylation in thawed synaptosomes

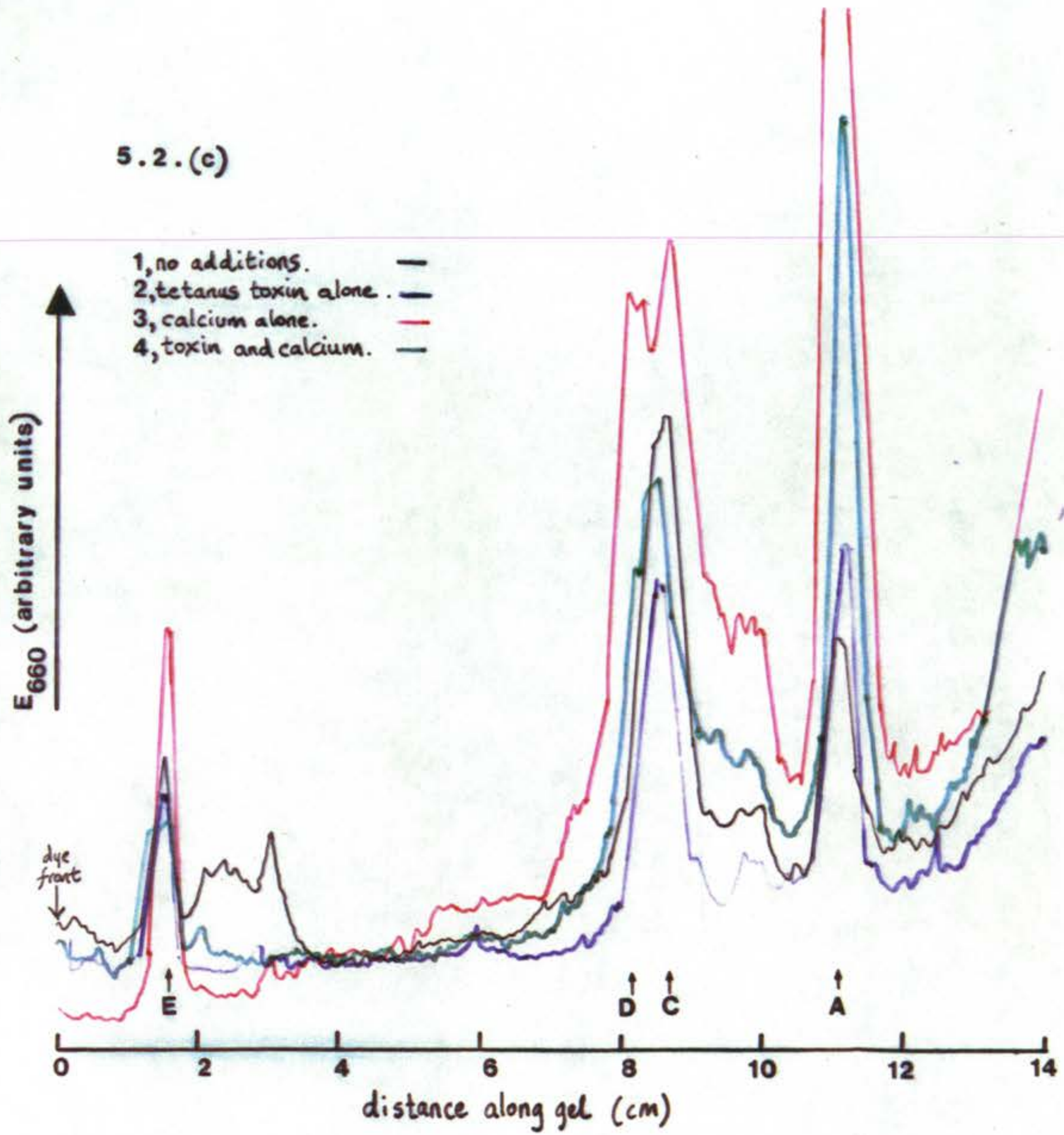
Tracks 3 and 4 are in the presence of Ca^{++}. Tracks 2 and 4 are proteins from synaptosomes incubated with tetanus toxin.

(a) An autoradiograph of the SDS/8% polyacrylamide gel.

(b) The same gel stained by coomassie brilliant blue; tetanus toxin (150,000 Mr, 2 bands : the nicked and the unnicked) and bovine serum albumin (66,000 Mr) are marked.

(c) Densitometric traces of autoradiograph (a) are shown on the following pages.




5.2.(c)
1, no additions.
2, tetanus toxin alone.
3, calcium alone.
4, toxin and calcium.
E660 (arbitrary units)
dye front
E
D
C
A
0
2
4
6
8
10
12
14
distance along gel (cm)

5.3.3. The Effect of The Toxin on Phosphorylation in Whole Synaptosomes.

The pattern of protein phosphorylation in intact synaptosomes preincubated with inorganic [^{32}P] phosphate is shown in diagram 5.3. Addition of a depolarising agent like 60mM K^+ in the presence of Ca^{++} causes a change in the pattern (contrast tracks 5,6 and 7,8, diagram 5.3) Ca^{++} promotes the dephosphorylation of a 120,000M_r band (x) and the phosphorylation of a protein of 90,000M_r (y).

Diagram 5.3 also illustrates the need for the two nontoxin controls described in section 5.2.4.1. The toxin is purified on a phosphate gradient on Cellulose DE52 and despite extensive dialysis to a calculated 'safe' concentration of inorganic phosphate, toxin samples still contained sufficient unlabelled inorganic phosphate to decrease the specific activity of the synaptosomal ATP significantly (tracks 1-4 & 9-12)

The effect of tetanus toxin on the phosphorylation of proteins in intact synaptosomes is shown in diagram 5.4. In some instances the effect of tetanus toxin was to diminish only the Ca^{++}-dependant phosphorylation changes. These results were in agreement with the results of the experiments using [γ-^{32}P] ATP and lysed synaptosomes. However in other experiments, although the Ca^{++}-dependant phosphorylation of the 90,000M_r band was blocked, the Ca^{++} dependant dephosphorylation of the 120,000M_r band was apparently unaffected. The surprise was that both of these effects were also seen in the absence of Ca^{++} (diagram 5.4). This observation is in marked contrast to the results of the other experiments where the toxin had no effect on the Ca^{++}-independant phosphorylation of protein 1. (Protein 1 is the 90,000M_r band)

An easier approach to the measurement of the effect of the toxin on the Ca^{++}-dependant phosphorylations is to examine the individual sites in protein 1 that are phosphorylated by depolarisation induced Ca^{++} influx. This approach was first taken by Huttner & Greengard (1979) to demonstrate that the phosphorylation of sites in protein 1 were differentially regulated by CAMP and Ca^{++}.

Crude protein 1 was purified from both toxin-treated and untreated synaptosomes by excising a radioactive band of 90,000M_r from an SDS-polyacrylamide gel. The autoradiograph of this gel (diagram 5.5) showed a difference between the toxin treated and untreated synaptosomes. the quality of the autoradiograph is poor but it would

Diagram 5.3. Phosphoprotein Pattern Changes in Intact Synaptosomes After Depolarisation.

Autoradiograph of an SDS/8% polyacrylamide gel. Tracks 3,4,7,8,11,12, are in the presence of Ca^{++}. This experiment was not adequately controlled for the presence of inorganic phosphate in the toxin preparation: tracks 1-4 and tracks 9-12 are the toxin treated samples (9-12 had 80 μl of $10 mgml^{-1}$ toxin in KR1 added to the usual quantity of synaptosomes, section 5.2.4.2, and 1-4 had a tenfold dilution of toxin)

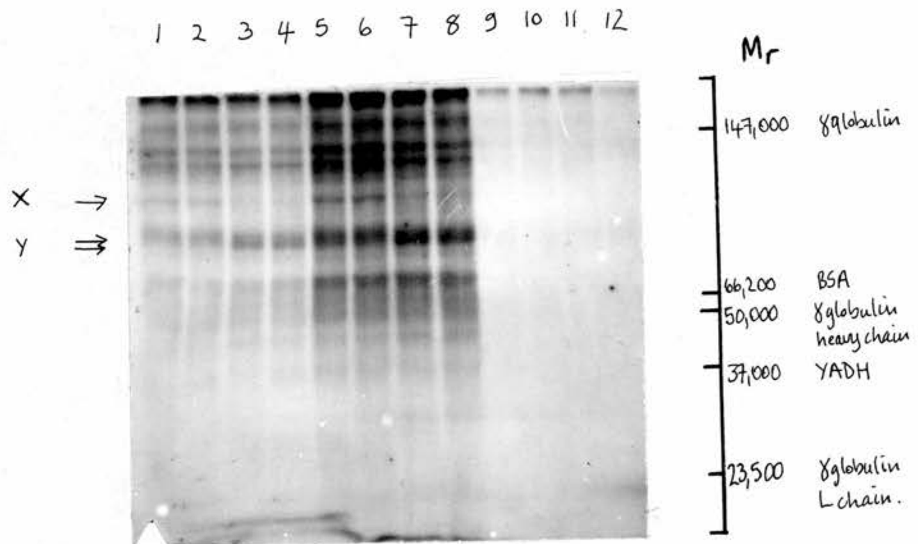


Diagram 5.4. The Effect of Tetanus Toxin on Phosphorylation intact Synaptosomes, in The Absence and Presence of Ca^{++}.

Autoradiograph of an SDS/8% polyacrylamide gel that shows tetanus toxin altering the phosphorylation of proteins of 90,000M_r and 120,000M_r. Tracks 1-4 and 9-11 are the nontoxin and boiled toxin samples. Tracks 5-8 are the tetanus toxin treated samples. Tracks 3,4,7,8,11 are in the presence of Ca^{++}. The duplicates are duplicate experiments, not duplicate electrophoresis samples.

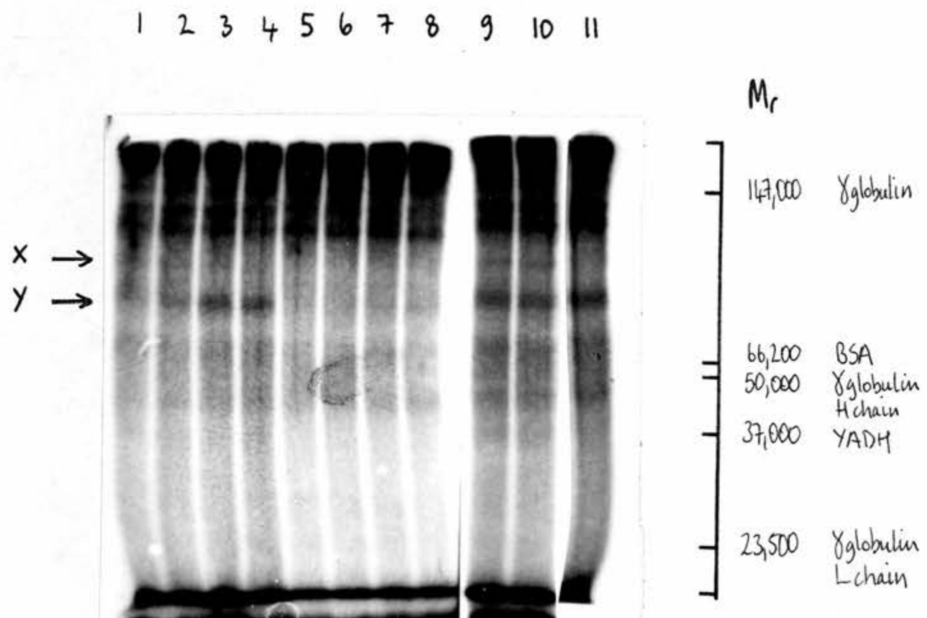
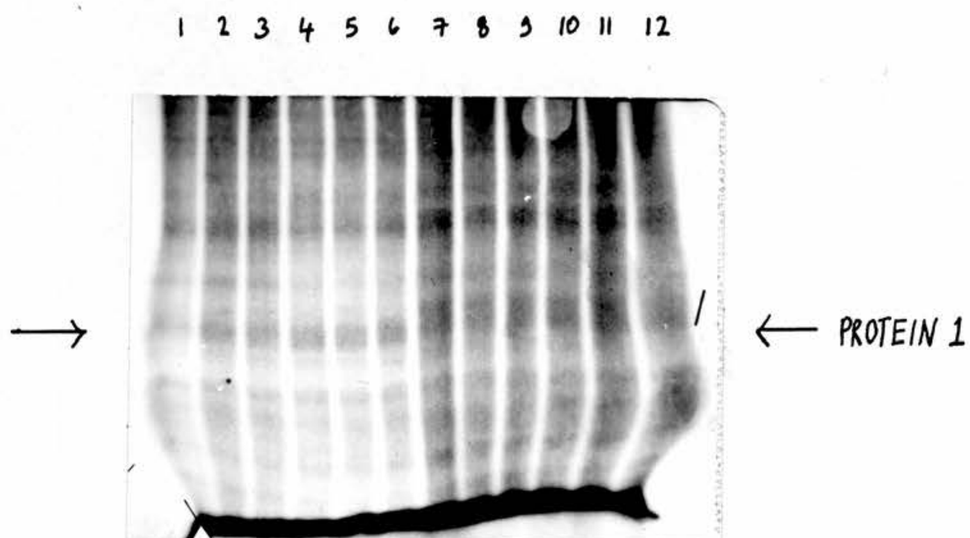


Diagram 5.5 Autoradiograph of SDS/6% Polyacrylamide Gel from which Protein 1 was Excised.

Tracks 4-6 and 10-12 are in the presence of Ca^{++}. Tracks 7-12 are the tetanus toxin treated samples. Protein 1 is marked.



seem that the band equivalent to protein 1 is more diffuse in the tetanus treated synaptosomes than in the untreated and this is so whether Ca^{++} is present or not.

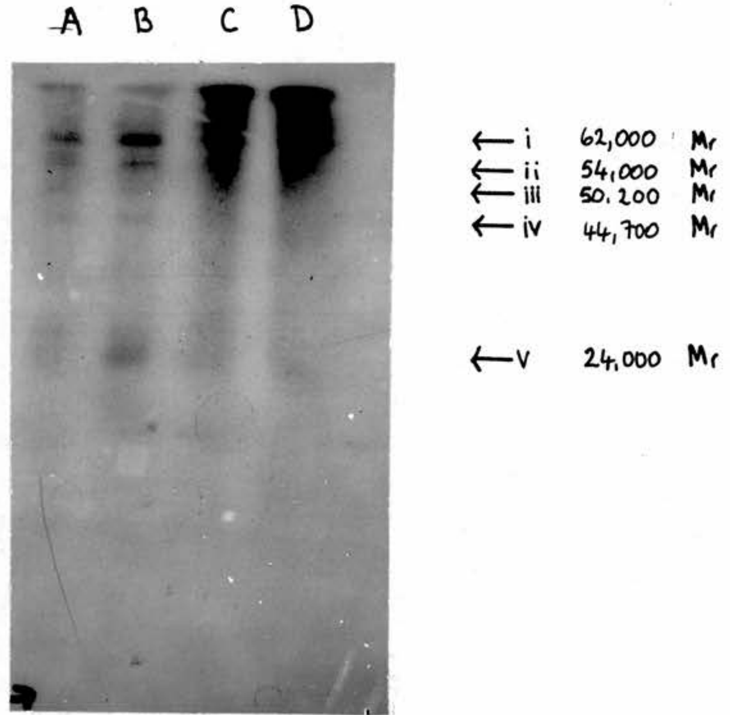
The phosphopeptide pattern of protein 1 obtained after digestion with the V8 proteinase of Staph. aureus is shown in diagram 5.6. Depolarisation induced Ca^{++} influx stimulated the phosphorylation of peptides $\bar{i}$ and $\bar{v}$. The incubation of intact synaptosomes with tetanus toxin altered the phosphopeptide maps in the absence and the presence of Ca^{++}. There was more undigested protein 1 at the top of the gel in the toxin treated synaptosomes. Phosphopeptides ii - iv were absent as was phosphopeptide $\bar{v}$, the Ca^{++} dependant site. Either all the phosporylation sites have been altered or incubation of the synaptosomes with the toxin somehow changed the susceptibility of protein 1 to digestion with the V8 proteinase.

5.4. Discussion.

The Ca^{++}-dependant phosphorylation patterns observed here in lysed and intact synaptosomes are similar to those reported initially by Krueger et al. (1977) and De Lorenzo (1977) and later shown by Greengard and his colleagues to be calmodulin dependant (Schulman & Greengard, 1978a, Schulman & Greengard, 1978b).

Little is known of many of the phosphoproteins other than that the level of ^{32}P incorporation changes on the addition of Ca^{++}. The phosphoprotein of approximately $90,000M_r$ designated here as A in lysed synaptosomes and as Y in whole synaptosome experiments, is known as Protein 1 (and more recently as synapsin see Nestler & Greengard, 1983). It is found only in neurones and it is thought to be involved in the secretion of neurotransmitters. It may be a structural protein similar to collagen as it is rapidly degraded by highly purified collagenase (Huttner & Greengard, 1979). Phosphoprotein D ($56,000M_r$) may be the DPH-M phosphoprotein described by De Lorenzo (1977), the molecular weight is different but the overall pattern of phosphorylation is similar to the pattern seen here in lysed synaptsomes. De Lorenzo (1977) found that addition of calcium caused an increase in the incorporation of ^{32}P phosphate from $[\gamma-^{32}P]$ ATP to several proteins but that the presence of diphenylhydantoin significantly inhibited the Ca^{++}-dependant increase in phosphorylation of two proteins, designated DPH-L and DPH-M. Diphenylhydantoin is an anticonvulsant drug which has antagonistic effects to that of calcium on the release of

Diagram 5.6 The Effect of Tetanus Toxin on The Phosphorylation of Protein 1.



The phosphopeptide map of protein 1 obtained after digestion with V8 proteinase, B and D are from 'depolarised' synaptosomes (ie in presence of Ca^{++}). Tracks C and D are from synaptosomes incubated with tetanus toxin.

neurotransmitters. The phosphoprotein of 18,000M_r seen in experiments using [γ- ^{32}P] ATP may be myosin light chain since myosin light chain kinase is activated by the Ca^{++}-calmodulin complex (Hathaway et al., 1981). Myosin light chain is thought to be involved in stimulus-secretion (Scholey et al., 1980).

The experiments described here show that the addition of tetanus toxin to synaptosomes alters the level of ^{32}P incorporation to phosphoproteins. A change in the intensity of a band on an autoradiograph could also reflect differences in specific activity rather than an altered rate of incorporation. There were several controls in each experiment to offset this possibility. The total amount of radioactivity in each sample was always determined by liquid scintillation counting and there was no significant difference between samples. Each gel (with the exception of the Cleveland digest of protein 1) was stained with coomassie blue before drying for autoradiography and the addition of the toxin preparation to the synaptosomes did not alter the synaptosomal proteins. Also the toxin would seem not to have had any effect on the synaptosomal pool of ATP as there was no difference in the intensity of phosphoproteins in the absence of calcium (the exception being protein B).

Judging by some experiments, tetanus toxin has an inhibitory effect on the Ca^{++}-calmodulin dependant phosphorylations. The observed decrease in the responce to Ca^{++} by synaptosomes incubated with toxin implies that the target for the toxin is at some stage in the process at, or near to, the interaction of the Ca^{++}-calmodulin complex with the dependant kinases and phosphatases.

However there are some experiments where the results do not fully agree with the above conclusion and therefore may be open to other interpretations. There are two experiments where the results could imply that the presence of the toxin had caused a modification of the kinase,or of protein 1 such that it is unable to accept a phosphoryl group. These results are shown in diagram 5.4 where the toxin appears to block the basal phosphorylation of protein 1 that occurs in the absence of calcium, and in diagram 5.6 where the phosphopeptide maps of protein 1 in the absence and presence of tetanus toxin are compared. A second inconsistency that does not agree with the proposed inhibition of the Ca^{++}-calmodulin complex is the behaviour of protein X (120,000M_r) in the intact synaptosomes and protein B in the lysed synaptosomes. The addition of calcium to intact or lysed synaptosomes respectively

causes these proteins to dephosphorylate and so inhibition of the Ca^{++} -calmodulin system should mean that these proteins should remain in the phosphorylated state. This does not happen. And further, the intensity of protein B changes when toxin is present and calcium is absent ; a change that is consistent within experiments but not between experiments (eg contrast traces (i) and (ii) in diagram 5.1). A pertinent point is that at present there is no clear idea of the means by which the formation of the Ca^{++}-calmodulin complex causes stimulus secretion and therefore these results which do not agree with the Ca^{++}-calmodulin hypothesis, may so behave simply because of the present state of ignorance with respect to the process.

The analysis of the phosphorylation state of synaptosomal proteins in the presence and absence of calcium has been shown here to be a useful system for the determination of the molecular activity of tetanus toxin. However despite these promising results this line of research was abandoned at this stage : the major difficulty was consistently reproducing the toxin effect. For no apparent reason consecutive experiments with no noticeable change in any parameters would have different results ; either the toxin would inhibit the Ca^{++} -calmodulin phosphorylation or the toxin would have no effect. (The results were at least consistent in one respect in that there were no cases where the intensity of the Ca^{++}-calmodulin dependant phosphorylations increased in the presence of the toxin ie the observation was not a statistical variation, the toxin either inhibited the phosphorylation or had no effect). Synaptosomes are a notoriously difficult preparation and this may be the reason for the non-reproducibility. An obvious failure in the protocol was that the release of neurotransmitter was not measured concomitantly with the analysis of the phosphorylation.

By analogy with other bacterial protein toxins it seems likely that the activity of tetanus toxin is probably enzymic covalent modification. A possible covalent mechanism could be methylation. Methylation has already been shown to have an important role in control of cellular activity in prokaryotes (Springer et al.,1979), and in eukaryotes, methylation of some proteins in vitro has beenshown to alter their activity eg calmodulin (Gagnon et al., 1981). Although the experiments described here were designed to show any effect of the toxin on the Ca^{++}-calmodulin phosphorylation, they also show that tetanus toxin is probably not a kinase or a phosphatase. Other

bacterial toxins eg diphtheria or cholera toxins achieve their effect on cellular metabolism by transferring an ADP-ribose group onto the target protein (van Heyningen, 1982). During the course of this work Wendon & Gill (1982) reported that tetanus toxin did not ribosylate or phosphorylate proteins in neuroblastoma cells in culture. They found that incubation of the cells with toxin under conditions that had previously been shown to abolish K^+-invoked neurotransmitter release, had no effect on ^{32}P incorporation from [^{32}P-both phosphates] NAD or [α-^{32}P] ATP (sic). Assuming that they mean [γ-^{32}P] ATP, the phosphoprotein patterns shown for the cells do not look like those obtained here or reported in the literature. They do say that the toxin sample was very impure and that significant proteolysis occurred in neuronal proteins in the presence of toxin. The proteolysis may explain the so-called tetanus toxin inhibition of neurotransmitter release etc. that Wendon and Gill observed.

Chapter 6: FUTURE WORK

In comparison to what is not known of tetanus toxin the known data is sparse indeed for a toxin that has been studied for the past 100 years. It is no exaggeration to say that it is perhaps only recently that both the methodology and knowledge have appeared that can be exploited in order to answer several outstanding questions on the toxin.

The primary structure of the toxin

The hypothesis presented in chapter 4 is that tetanus toxin has a significant degree of internal homology. Further evidence either for or against the proposed homology would come from an extension of the approaches begun here, viz. the peptide mapping of the chain digests by HPLC or immunoblotting. The most obvious test of the hypothesis and possibly the most rapid is the determination of the sequence of the gene coding for the toxin . Unfortunately there are difficulties at present with regard to these kinds of experiments as, in the USA a moratorium has been declared on genetic manipulation of any part of the genome of *C. tetani*.

The production of a synthetic vaccine for tetanus toxin

The vaccine in use today is tetanus toxoid, an attenuated form of toxin prepared by modifying the toxin with formaldehyde. The toxoid is an excellent vaccine and its widespread use is a major reason for the marked decrease in the incidence of the disease in the West (van Heyningen, 1968). Even so, a synthetic vaccine would be advantageous. Synthetic vaccines are a recent trend in the prevention of disease. Immuno-reactive peptides are determined in the sequence of proteins such as toxins or external proteins of bacteria or viruses, and are synthesized by solid-phase methods or by genetic manipulation. The newly synthesized peptide is then used to raise antibodies against the "parent"

protein. The usefulness of the peptides is that the antigen is now in a stable and safe form for the large-scale production of antibodies. The epitope mapping experiments described in chapter 4 could be extended with the aim of finding a particular reactive peptide in either of the chains.

The function of the toxin

The involvement of the toxin with the Ca^{++}- Calmodulin dependant phosphorylation of proteins in synaptosomes discussed in Chapter 5 must be verified by concomitant studies of neurotransmitter release and phosphorylation in toxin treated and untreated preparations. If the Ca^{++}- calmodulin dependant phosphorylations are diminished in toxin treated samples (as found here) then this development would be of great significance in determining the molecular activity of the toxin. The activity of the toxin is important as far as the disease and treatment of the disease is concerned but also if the activity were known then tetanus toxin would be an important tool in elucidating the molecular basis of neurosecretion and the control thereof.

Structure-function relationships in tetanus toxin and pharmacological significance

At present there is no "magic bullet" in the treatment of tetanus; such a drug can only be developed when more is known about the molecular activity of the toxin (excepting serendipity, of course). However, the possibility that the various activities of tetanus toxin may be a function of individual domains may either be tested or used in the emerging technology of hybrid toxins.

If the activities of a protein toxin can be physically separated then it is possible to exploit either function (i.e. the binding or intracellular activity). This is the principle behind the synthesis of hybrid toxins. The approach is to take the enzyme component of one toxin and alter the cell specificity by binding it covalently to another carrier molecule. Carrier molecules used have been antibodies and their fragments (both serum and

monoclonal), lectins or less often the binding chains of polypeptide hormones or other toxins (for a review see Olnes, 1981 or Moller, 1982).

The emphasis in these hybrids can be on either the membrane event i.e. binding and subsequent translocation, or the intracellular event. In the first case hybrids can be used to select mutant cells in culture with defective routes of internalisation or to delineate the minimum structural requirements for the translocation of the A chain (or total toxin) into the cytosolic compartment. An example of the former is the internalisation of diphtheria toxin A chain by a diphtheria-toxin-transport mutant via the ricin transport pathway (Youle & Neville, 1979).

There has been more progress in the other aspect of hybrid toxins i.e. the specific modification or destruction of a cell by a hybrid toxin without harm to other cells in a mixed population. This has possibilities as a new type of chemotherapy in cancer studies. Many conjugates containing the active chain of diphtheria or ricin have been shown tobe selectively cytotoxic *in vitro* (Gilliland et al., 1978, Oeltmann & Heath, 1979a, 1979b, Ross et al., 1980) and Blythman et al (1981) have described the first *in vivo* evaluation of hybrid cytotoxicity. Initially the hope was that hybrid toxins could be used against all tumours but although this seems not to be so, there does seem to be a role for artificial hybrids between tumour specific antibodies and the A chain of ricin (Krolick et al., 1982) or of diphtheria (Thorpe et al., 1982) in the removal of malignant cells during autologous bone marrow transplants in the treatment of leukemia.

So far most of the emphasis in the pharmacology of hybrid toxins has been on creating specific cytotoxic agents but the cytospecific modulation of cellular metabolism is potentially more useful clinically than cytotoxicity. Toxins that modulate a cellular activity that does not lead to cell death are cholera toxin and *E. coli* heat labile toxin (the activation of adenylate cyclase) and tetanus and botulinum toxins (the inhibition of neurotransmitter release). There has been one report of a hybrid

toxin capable of modulating the metabolism of cells *in vitro* (van Heyningen, 1983).

Tetanus toxin inhibits the release of neurotransmitters and if stimulus-secretion in cells other than neurones, follows the same general mechanism then hybrid toxins containing whole tetanus toxin or the active moiety of the toxin (which is often assumed to be the light chain by analogy with other bacterial toxins) and some carrier molecule could be a means by which the molecular action of the toxin could be found and also could be the prototypes for the synthesis of hybrid toxins that would be pharmacologically useful blockers of stimulus-secretion. Non-neurone cells to be considered would be blood cells like platelets, mast cells or macrophages.

ABBREVIATIONS

ADP:	adenosine 5' - pyrophosphate
ATP:	adenosine 5' - triphosphate
ATXII:	Sea-anemone tox II
CaM:	calmodulin
CM-cellulose:	carboxymethylcellulose
DABITC:	dimethylaminoazobenzene isothiocyanate
DABTC:	dimethylaminoazobenzene thiocarbamoyl
DABTH:	dimethylaminoazobenzene thiohydantoin
DABTZ:	dimethylaminoazobenzene thiazolinone
DEAE-cellulose:	diethylaminoethylcellulose
DTT:	dithiothreitol
DTNB:	5,5' - dithiobis (2-nitrobenzoate)
EDTA:	ethylenediaminetetra-acetate
EGTA:	ethyleneglycolbis (amino-ethylether) tetra-acetate
H:	the heavy chain (100,000 M_r) of tetanus toxin
HPLC:	high performance liquid chromatography
HQS:	8 hydroxyquinoline 5-sulphonic acid
IAA:	iodoacetic acid
L:	the light chain (50,000 M_r) of tetanus toxin
M:	the actual number of differences in a comparison of the sequence of two proteins
M_r:	relative molecular mass
NEM:	N-ethylmaleimide
ō-IBA:	ō-iodosobenzoic acid
page:	polyacrylamide gel electrophoresis
PCMB:	p-chloromercuribenzoate
PITC:	phenyl isothiocyanate
PMSF:	phenyl methyl sulphonyl fluoride
PTH:	phenylthiohydantoin
S∆N:	the Cornish-Bowden compositional index, the value of which approximates to the actual number of differences in a comparison of the sequences of two proteins
SDS:	sodium dodecyl sulphate
TNM:	tetranitromethane
Tris:	2-amino-2-hydroxy-methylpropane-1,3-diol

ABBREVIATIONS

(Continued)

U:	the unnicked form (150,000 M_r) of tetanus toxin
U.V-vis:	ultraviolet-visible

MATERIALS

Common laboratory reagents were obtained from either BDH Chemicals Ltd. or Sigma Chemical Co. Ltd., specific details are only recorded here for unusual material or in cases where the quality differs from supplier to supplier.

DEAE and CM cellulose ion exchangers: Whatmann, Maidstone, Kent.

Sodium dodecyl sulphate, "specially pure": BDH Chemicals Ltd. Poole, Dorset.

Acrylamide, "electrophoresis grade": Fisons Scientific Apparatus, Loughborough.

Coomassie Brilliant Blue R150 and 4-chloro-1-naphthol: Sigma (London) Chemical Company Ltd., Kingston-upon-Thames.

Nitrocellulose membrane filters; Schleicher & Schüll, supplied in UK by Anderman, East Molesey KT8 0QZ.

The animal sera and the mouse monoclonals were a gift from Professor E. Habermann, Rudolf-Buchheim-Institut für Pharmakologie der Justus Liebig-Universität, Giessen, F.R.G.

Anti-Mouse-γ-globulin (sheep) linked to peroxidase; Scottish Antibody Production Unit, Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke.

Tetanus toxin, tetanus toxoid and equine anti-toxoid were a gift from Dr. R. O. Thomson of the Wellcome Research Laboratories, Beckenham, Kent.

ō - Iodosobenzoic acid: Pierce & Warriner (UK) Ltd., Chester CH1 4EF.

V8 proteinase from <u>Staph</u>. <u>Aureus</u>,; Miles Laboratories Ltd., P.O. Box 37, Slough, Berks.

The suppliers and the grade of the reagents used in the HPLC of the cyanogen bromide fragments and the N terminal sequence analysis are given in Campos & Richardson, (1983).

All radiochemicals were supplied by Amersham International plc., Amersham, Bucks., (albeit inadvertently in the case of the $[\gamma-{}^{32}P]$ ATP).

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