STRUCTURAL ASPECTS OF

TETANUS TOXIN

by

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To my Mother and Father

PREFACE

The experimental work for this thesis was carried out in the Department of Biochemistry, and I thank the Head of Department, Professor G.S. Boyd, for making these facilities available to me.

I am also grateful to Dr. S. van Heyningen in his capacity as my supervisor, for the use of his laboratory facilities, advice, patience and friendship throughout my stay. I am indebted to many other members of the Department, past and present, for their readiness to advise and assist me. I am particularly grateful to Dr. G. Pettigrew (Department of Biochemistry, University of Edinburgh Veterinary School) for his instruction on protein chemical techniques and for the use of his laboratory facilities. I am also grateful to Dr. P. Cohen (Department of Biochemistry, University of Dundee) for the use of his protein sequenator and analytical ultracentrifuge; Dr. J. Fothergill (Department of Biochemistry, Marischal College, University of Aberdeen) for the use of his solid phase sequenator; Dr. R. Ambler (Department of Molecular Biology, University of Edinburgh) for the use of his high voltage paper electrophoresis apparatus for the separation of dansyl amino acid derivatives; Dr. J. Mellanby (Department of Experimental Psychology, University of Oxford) for carrying out an in vivo bio-assay on a sample of purified tetanus toxin. I would also like to thank Dr. R.O. Thomson (Wellcome Research Laboratories, Kent) for his generous gifts of tetanus toxin, tetanus toxoid and Equine anti-tetanus toxin. I am very grateful to Mrs. Glenda Sharpe for the typing of my thesis and to Mrs. Kathleen Brown for her proof reading.

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I certify that this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration, nor has any of the work been submitted for any other degree. Various sections of this work were presented on a poster at the 13th FEBS meeting in Jerusalem (Britton and van Heyningen, 1980), a copy of the poster abstract is included in the back of the thesis. Some of the work has been published elsewhere; Ward, Britton and van Heyningen (in press).

Paul Britton
August 1981

ABBREVIATIONS

Some of the abbreviations listed below are as defined in Biochem. J. (1981) 193, 1-27, the other abbreviations are included for clarity of definition because of their use in the text.

AEAPG	3-aminoethyl-(3-aminoethyl) porous glass
ANS	8-anilino-1-naphthalene sulphonate
APS	Ammonium persulphate
ATPase	Adenosine triphosphatase
bis-acrylamide	N,N'-methylene bisacrylamide
BPB	Bromophenol blue
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate (adenosine 3':5'-phosphate)
CBB	Coomassie brilliant blue
cGMP	Cyclic guanosine monophosphate (guanosine 3':5'-phosphate)
Cl. botulinum	Clostridium botulinum
Cl. septicum	Clostridium septicum
Cl. sordelli	Clostridium sordelli
Cl. tetani	Clostridium tetani
Cl. welchii	Clostridium welchii
CNS	Central nervous system
c.p.m.	Counts per minute
c.p.s.	Counts per second
CTAB	Cetyltrimethylammonium bromide
DATD	N,N'-diallyltartardiamide
DEAE-	Diethylaminoethyl-
dimethyl POPOP	1,4 bis-2-(4-methyl-5-phenyl oxazolyl)
DITC	p-phenyl diisothiocyanate
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
DNP-	2,4-dinitrophenyl
DNS-	Dansyl
DNS-Cl	Dansyl chloride (N,N-dimethylaminonaphthalene-5-sulphonyl chloride)
DNS-OH	N,N-dimethylaminonaphthalene-5-sulphonic acid
DPCC	Diphenyl carbamyl chloride
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)

DTT	Dithiothreitol
E. coli	Escherichia coli
EDTA	Ethylenediamine tetra-acetate (used as disodium salt)
FDNB	2,4-dinitrofluorobenzene (fluorodinitrobenzene)
GABA	γ-amino butyric acid
GuHCl	Guanidine hydrochloride or guanidinium chloride
HDL	High density lipoprotein
HPLC	High pressure liquid chromatography
IAA	Iodoacetic acid
IgG	Immunoglobulin G
LDL	Low density lipoprotein
M. butyricum	Mycobacterium butyricum
2-ME	2-mercaptoethanol or β-mercaptoethanol
MeOH	Methanol
MLD	Minimum lethal dose
Mol.Wt.	Molecular weight
NaN_3	Sodium azide
NEM	N-ethylmaleimide
NGF	Nerve growth factor
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric point
PITC	Phenylisothiocyanate
PMSF	Phenyl methylsulphonyl fluoride
PPO	2,5-diphenyl oxazole
PTH	Phenylthiohydantoin
SDS	Sodium dodecyl sulphate (sodium lauryl sulphate)
SER	Smooth endoplasmic reticulum
STI	Soya bean trypsin inhibitor
TCA	Trichloro acetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TET	Triethylenetetramine
TFA	Trifluoro acetic acid
TRIS	Tris(hydroxymethyl)methylamine [2-amino-2-hydroxymethyl-propane-1,3-diol]
TSH	Thyrotropin (thyroid stimulating hormone)
UV	Ultra violet
V. cholerae	Vibrio cholerae
WGA	Wheat germ agglutinin

AMINO ACID ABBREVIATIONS

Most of the amino acids abbreviations are as defined in Biochem. J. (1972) 126, 773-780, others are included for clarity of definition because of their appearance in the text.

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
CMcys	Carboxymethyl cysteine
Cys	Cysteine
CysA	Cysteic acid
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Met(O_2)	Methionine sulphone
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Prefixes for units:-

mmol	-milli-	10^{-3}mol
µmol	-micro-	10^{-6}mol
nmol	-nano-	10^{-9}mol
pmol	-pico-	10^{-12}mol
fmol	-femto-	10^{-15}mol
amol	-atto-	10^{-18}mol

CONTENTS

INDEX TO TABLES

INDEX TO DIAGRAMS

INDEX TO PHOTOGRAPHS

STRUCTURAL ASPECTS OF TETANUS TOXIN

by P. BRITTON.

Abstract

Tetanus toxin is a neurotoxic protein produced by Cl. tetani. It is produced as a single polypeptide (Mol.Wt. approximately 150 000 daltons), which is subsequently 'nicked' by a protease into two polypeptides, H- and L-chains (Mol.Wts. approximately 100 000 and 50 000 daltons), joined by two or more disulphide bridges. The toxin was purified by ion exchange chromatography on DEAE cellulose in the presence of two protease inhibitors, phenyl methylsulphonyl fluoride and benzamidine. The purified toxin was shown to be toxic and gave a single band in a non-denaturing polyacrylamide gel and two bands in a reducing SDS polyacrylamide gel. 'Unnicked' toxin was also observed in some reducing SDS gels. Initially the separation of the two chains was done either on Ultrogel AcA44 or Sephacryl S-200, usually using 2M urea in the presence of 2mM DTT, after a reductive incubation in 8M urea and 100mM DTT. The L-chain was purified to give a single band on a reducing SDS gel, but the H-chain was always contaminated with L-chain. After formaldehyde treatment, antibodies were raised against L-chain in a rabbit and purified by precipitation in 18% (w/v) Na_2SO_4; the anti-L-chain antibodies partly cross-reacted with whole toxin. The gel filtration method was tried under a number of different conditions without successful purification of H-chain, indicating that the two chains are tightly bound together. The two chains have been successfully purified by preparative SDS-PAGE. The intact chain and L-chain have N-terminal proline but the H-chain appears to be heterogeneous at the N-terminus, suggesting a "ragged" cleavage by a protease. Charge shift electrophoresis indicated that the toxin molecule is hydrophilic though upon separation L-chain appears hydrophilic but H-chain was shown to be amphiphilic. H-chain is known to bind to specific gangliosides but L-chain does not. It appears that upon fixation to ganglioside SGGnSSLC (G_{T1}) that the toxin molecule may undergo some conformational change.

SECTION 1.

INTRODUCTION

1.1. Tetanus the Disease

1.1.1. Definition of Clinical Tetanus

Tetanus is a disease of the central nervous system (CNS), brought about by the presence, in a wound, of a gram positive anerobic bacterium, Clostridium tetani, which produces a neurotoxic protein, tetanus toxin, causing hyperexcitation. The disease may be found as three different types, as follows.

1.1.1.1. Spastic Paralysis

This is the form of the disease that is most recognisable as tetanus. The wound in which Cl. tetani may grow can vary from a tiny pin prick to a deep cut, or even a broken bone. The usual symptoms are an arched back with clenched hands and feet and the jaw is set in a permanent grimace, the risus sardonicus (sardonic smile), often referred to as lockjaw. A victim suffering from this form of tetanus is in a state of spastic paralysis, paralysed because all the muscles are contracting and opposing each other. The victims are conscious but unable to control their movements. Convulsions usually occur in response to the slightest external stimuli, such as a light being switched on, or the movement of bed clothes. Such stimuli, cause the whole body to be contorted into a major spasm. The victims are fully conscious and sweat profusely, they are usually exhausted from continuous muscular activity and in pain. Death usually occurs as a result of exhaustion, heart failure, or the collapse of the lungs. It has been estimated (Weinstein, 1966) that the overall mortality from this form of tetanus is 45% to 55%.

There is no tissue damage; in fact there are no post-mortem signs of the disease. The wound containing Cl. tetani may be too slight to be noticed or even have healed over. The average incubation period following infection is 3 to 21 days; the average time for the development of the initial symptoms is 14 days. Injection of large amounts of purified toxin into experimental animals can induce severe symptoms within a few hours, indicating that the limiting factor for the onset of the symptoms is the growth of the infective organism and the production of the toxin.

The disease is never epidemic, but is endemic because of the near universal presence of spores produced by Cl. tetani.

Hörtnagl et al. (1979) produced evidence that certain symptoms such as hypertension and sweating which occur in severe tetanus may be due to the over-activity of the sympathetic nervous system. The symptoms are similar to those seen in patients suffering from phaeochromocytoma (a tumour developed in the adrenal glands that gives rise to phaeochromocytes, microbodies that stain brown with chromic salts), and the elevated catecholamine levels associated with the disease have been observed in the plasma and urine of patients suffering from severe tetanus. Death due to heart failure in victims suffering from tetanus may thus be due to the over-activity of the sympathetic nervous system, as well as exhaustion due to the hyperactivity of the motor system.

1.1.1.2. Tetanus Neonatorum

In this form of the disease, the victim is in a state of spastic paralysis as described in section 1.1.1.1., but the cause of the disease is more specific. This form of the disease exclusively affects the newborn, and is recognisable in the first two weeks after birth. Tetanus neonatorum is usually caused by insanitary conditions, and as a result of the severing of the umbilicus. The disease is prevalent mainly in third world countries owing to the use of unhygenic instruments, and in some cases the practice of coating the freshly severed umbilicus with cow dung. Cl. tetani is transferred to the severed umbilicus and is able to produce tetanus toxin, giving rise to the spastic form of tetanus. Tetanus neonatorum accounts for 30-80% of deaths from tetanus in tropical countries. The incubation period for this form of the disease may be as short as 4-6 days. The first symptoms observed are crying and refusal to suckle.

1.1.1.3. Local Tetanus

In man, the general form of tetanus, the spastic paralysis, is the usual type observed. The general form is also known as the descending type, owing to the fact that tetanus in humans initially

manifests itself in the muscles whose neurones originate from the medulla oblongata and the upper part of the spinal cord, and later reaches the muscles of the trunk and the extremities. Another form of tetanus may be observed in man and is easily reproducible in experimental animals by the injection of tetanus toxin. In this form of tetanus a total spastic paralysis is not initially observed, but a local paralysis is. Symptoms are observed immediately in local tetanus and appear in the muscles surrounding the toxin pool. The cause is believed to be due to the inhibition of the neurotransmitter along a particular nerve (see section 1.2.5.1.). Symptoms of local tetanus spread along the neuraxis, and this often classifies local tetanus as ascending tetanus.

Local tetanus can be induced by the injection of small quantities of tetanus toxin directly into a muscle (usually the hind limb) of experimental animals, or by the injection of large doses of tetanus toxoid or tetanus antitoxin before the injection of toxin. The question whether local tetanus exists as a natural phenomenon or as an artefact brought about by localised injections has yet to be answered. A local tetanus may arise from specifically localised sites of Cl. tetani, due to the introduction of a splinter or a nail into a muscle. Millard (1954) estimated that local tetanus has a mortality of about 1%.

For references to the above sections, see general reviews by: van Heyningen (1968), Adams et al. (1969), Zacks and Sheff (1970), Adams (1971), Curtis (1971), van Heyningen and Mellanby (1971), Mellanby et al. (1973), Habermann and Wellhorner (1974), Fedinec (1975), Bizzini (1976), Arbuthnott (1978), Habermann (1978), Bizzini (1979) and van Heyningen (1980).

1.1.2. The History and Discovery of Tetanus Toxin

Tetanus has been well documented from very early times. Hippocrates twenty-four centuries ago described a disease that consisted of a triad of symptoms: a wounding, a lockjaw and finally death. The disease described is without doubt the disease we now know as tetanus. Credit for the discovery of the tetanus bacillus, Cl. tetani, must go to A. Nicolaier in 1884. He was a twenty-two year old medical student at Göttingen working on his doctoral thesis.

He was studying the production of tetanus-like symptoms in experimental animals following the injection of soil samples. He observed that the causative, organism presumed to cause tetanus (which at the time had not been classified), was not distributed throughout the body. Nicolaier also observed that there was little change in the animals (rabbits) at post-mortem, except for a small amount of pus at the site of inoculation. The pus was shown to contain a variety of bacilli, but overwhelmingly some fine slender bacilli a little larger than the bacilli of Koch's mouse septicaemia (recently discovered at the time). Heated soil samples produced no effect but clear sieved extracts of soil produced tetanus symptoms. Inoculation of pus from animals that had died following an injection of soil also produced tetanus, with a reduced incubation time. Nicolaier also noted that the symptoms of tetanus were similar to those produced by the alkaloid poison, strychnine, and went on to postulate in 1885 that the causative organism acted by producing a strychnine-like poison.

In 1884, Carle and Rattone demonstrated that an infective agent from a human pustule could be transferred to rabbits, and that a toxic agent could be further transferred from the nervous tissue of the rabbits to fresh animals. Rosenbach in 1886 was the first person to demonstrate the presence of the tetanus-causing bacillus in man, but was unable to isolate the bacillus. In 1889 a Japanese bacteriologist, S. Kitasato, working with Emil von Bohering in Koch's laboratory in Berlin isolated Cl. tetani from a human victim. The organism Kitasato isolated was shown to be identical to the one isolated by Nicolaier, and to reproduce tetanus by inoculation with uniform cultures.

Tizzani and Cattani in 1890 were the first to show that the tetanus bacillus did produce a toxin. A month after Tizzani's and Cattani's report a Danish investigator, K. Faber (often credited with the first demonstration of tetanus toxin), independently reported the same result as Tizzini and Cattani, all three confirming Nicolaier's original idea. Both groups of workers produced tetanus in experimental animals by the injection of filtrates from Cl. tetani containing no bacilli. Nicolaier has been reported never to have tried the effect of bacteria-free culture filtrates, a test which would have confirmed his hypothesis. In 1889 the first bacterial exo-toxin, diphtheria toxin, was identified by E. Roux and A. Yersin at the

Institut Pasteur in Paris. Tetanus toxin was the second such bacterial toxin to be identified. In 1896 E. van Ermengem identified the toxin responsible for a form of food poisoning produced by Cl. botulinum, botulinus toxin.

The existence of diphtheria, tetanus and botulinus toxins was postulated about the same time as that of another toxin, namely cholera toxin, produced by Vibrio cholerea responsible for another of the major diseases at the time. Unlike the first three toxins which were all identified at about the same time cholera toxin was not identified until much later by S.N. De in 1959. See Table 1. For references to the above section see, van Heyningen (1968) and Habermann (1978).

1.1.3. Clostridium tetani

1.1.3.1. Morphology

Cl. tetani is a rod shaped bacterium of about 5-8μm by 0.4μm, capable of becoming filamentous, up to 30μm in length, it is a gram positive anaerobic endospore former. Unlike the less strict anaerobes such as Cl. welchii (perfringens) which can tolerate some oxygen, Cl. tetani is a strict anaerobe and cannot grow in the presence of oxygen owing to the lack of an enzyme (peroxidase) to destroy H_2O_2. Most subtypes of Cl. tetani are actively motile and can swarm on agar plates. The bacteria contain a single cilium at one end; later a tuft of cilia protrude from one pole; later still other cilia may form along the bacillus but disappear on ageing. Two to ten day old cultures of Cl. tetani start to form spores. Spores may appear at one or both poles of the bacillus in the form of round bodies, giving the bacterium its shape from which the terms 'PLECTRIDIUM' (from the Greek πλῆκτρον = plectrum or drumstick) and 'CLOSTRIDIUM' (from the Latin colus = distaff or the Greek equivalent, closter = spindle), have been derived.

Cl. tetani, when compared to other strains of clostridia, shows slightly different biochemical patterns that are used in identification. Gas formation is very low, and proteolysis is fairly weak, as can be shown by a low gelatinolytic activity. Though there are contrasting views concerning the proteolytic activity of Cl. tetani, Williams (1971). The production of tetanus toxin is

TABLE 1.

EVENTS IN THE DISCOVERY OF TETANUS TOXIN AND THE DISCOVERY OF OTHER BACTERIAL TOXINS

YEAR	AUTHOR	EVENT
5th century BC	Hippocrates	Description of local and general tetanus as a sequelae of symptoms.
1884	Carle and Rattone	Transfer of an infective agent from a human pustula to rabbits; Transfer of a toxic agent from the nerve tissue of the rabbits to a fresh animal.
1884	Nicolaier	Experimental tetanus by inoculation with soil; Culture of rod like bacteria and reproduction of the disease by injection of the passaged bacteria. Proposal of a strychnine like poison.
1886	Rosebach	Demonstration of tetanus bacilli in man. Near isolation of the bacteria.
1889	Kitasato	Isolation of tetanus bacilli from a human victim; Identification as Nicolaier's bacilli; Reproduction of the disease by inoculation with uniform cultures.
1889	Roux and Yersin	Isolation of first bacterial exo-toxin -Diphtheria Toxin.
1890	Tizzoni and Cattani)	Independently showed proof for the
	)	existence of a thermolabile toxin in
	)	tetanus; Reproduction of the disease
1890	Faber)	by sterile culture filtrates. The isolation of the second bacterial exo-toxin -Tetanus Toxin.
1886	Von Ermengem	Isolation of Botulinus Toxin.
1952	De	Isolation of Cholera Toxin.

a unique biochemical property and is useful for identification, so it is difficult to identify non-toxigenic strains of Cl. tetani. Cl. tetani also produces and releases another toxin, tetanolysin (see section 1.2.1.), which causes the haemolysis of red blood cells under reducing conditions; in this respect it is like the θ-toxin from Cl. welchii.

As Cl. tetani is a strict anaerobe, the spores require an oxidation-reduction potential of + 0.01V or less at pH 7.0 for germination with an optimal growth temperature of 37°C. Vegetative bacilli are killed by heat and antiseptics; the spores are destroyed by heating to 100°C for 1 hr or autoclaving at 121°C for 10 min.

Clostridia are principally anaerobic so should not be able to infect higher organisms to any great extent. Cl. tetani and other clostridia, eg. Cl. welchii, Cl. septicum, Cl. sordelli, and Cl. botulinum are exceptional amongst bacteria, as snakes are amongst vertebrates, in that they can produce many diverse toxic substances. Toxigenesis appears to be for most Clostridia a valuable method of producing anaerobiosis. Cl. welchii is able to produce toxins (enzymes in this context) such as lecithinase (α-toxin), collagenase (κ-toxin), proteinases (ɪ-toxin) and cytolysins which are all able to damage living tissue and thus create an anaerobic environment. In this context the products resemble viperid venoms, which also contain enzymes as well as toxins. Cl. tetani and Cl. botulinum do not fit this concept as they produce neurotoxins which can be paralleled with the neurotoxins of elpoid or crotoloid snakes eg. Cobra, although the paralysis caused by a snake's venom is apparently advantageous to the snake, the benefit for the micro-organism of the flaccid paralysis caused by botulinus toxin or the spastic paralysis of tetanus toxin is less understood.

For general references to the above section, see Habermann (1978), Bizzini (1979), Adams et al. (1969) and Levy (1973).

1.1.3.2. Growth

As described in section 1.1.3.1., for the successful growth of Cl. tetani either in culture, its natural environment, or a wound, a strict anaerobic environment is required. The growth conditions of the Harvard strain of Cl. tetani are described by van Heyningen and

Mellanby (1971), who list the two main types of media required, as originally described by Mueller and Miller (1954) and Lantham et al. (1962). The Harvard strain of Cl. tetani is the strain most commonly used for experimental purposes. To grow spores in culture a complicated medium is required consisting of different amino acids and salts, see Shoesmith and Holland (1967, 1972).

Growth in a wound requires the obligatory anaerobic environment, a condition that does not exist in a clean, healthy and healing wound. Thus for the growth of Cl. tetani in a wound either a dirty non-healing wound is required or a wound infected with organisms like Cl. welchii which are capable of forming an anaerobic environment as described in section 1.1.3.1. For the germination of spores in a wound, a redox potential of less than half that of normal human tissue is required. Thus the growth of spores in normal tissue is highly improbable, though viable spores in normal tissue may persist for up to 14 years. In experimental work a lower redox potential may be obtained by the injection of tissue damaging agents such as 1% $CaCl_2$.

For general references to the above section, see: Habermann (1978), Bizzini (1979), Shoesmith and Holland (1967), Shoesmith and Holland (1972), Murphy and Miller (1967), Mueller and Miller (1954), Lantham et al. (1962), Mellanby (1968) and van Heyningen (1980).

1.1.3.3. Habitat

The spores of Cl. tetani are ubiquitous, they are mostly present in soil, the air and even on clothing. Owing to the anaerobic nature of Cl. tetani, its natural habitat is soil. Cl. tetani can also be classified as an enterobacterium because of its presence in the intestine of some animals (especially herbivores, possibly in man and other omnivores, and in carnivores to a lesser extent). In fact the best habitat is hot damp soil rich in faeces. One of the causes of tetanus neonatorum (section 1.1.1.2.) is the covering of the freshly severed umbilicus with cow dung, evidence of an enteric environment for Cl. tetani.

The disease, tetanus, is brought about by the infection of a wound with either the bacillus or its spores under anaerobic conditions. A wound may range from being serious such as a deep cut or the breaking of a bone to a simple pin prick. The incidence of

tetanus increased during the late 1960's in America because of the use of unhygienic needles by drug addicts. The presence of spores from Cl. tetani in the air can be a specific danger, especially in hospital operating theatres. Récently two cases of tetanus, one ending fatally, occurred at Falkirk hospital in Scotland, possibly due to the presence of spores in the hospital's casualty room.

For general references to the above section, see: Habermann (1978), Bizzini (1979) and van Heyningen (1980).

1.1.4. The Course of Tetanus

After infection, an incubation period follows for either the germination of spores or growth of the bacilli. The bacilli then have to produce and secrete tetanus toxin in sufficient amounts to cause tetanus; approximately 400 fmols in man will cause death. As mentioned earlier the incubation time for tetanus varies a great deal; in fact dormant cultures may exist for years (up to 14 years for spores) and then be activated by a fall in the redox potential of the tissue. Artificial injection of large amounts of purified toxin gives rise to tetanus within a few hours. If the dose of toxin is sublethal recovery may take several weeks because of morphological changes in affected muscles, brought about by the prolonged contractions or damage to joints involved in tetanic spasms.

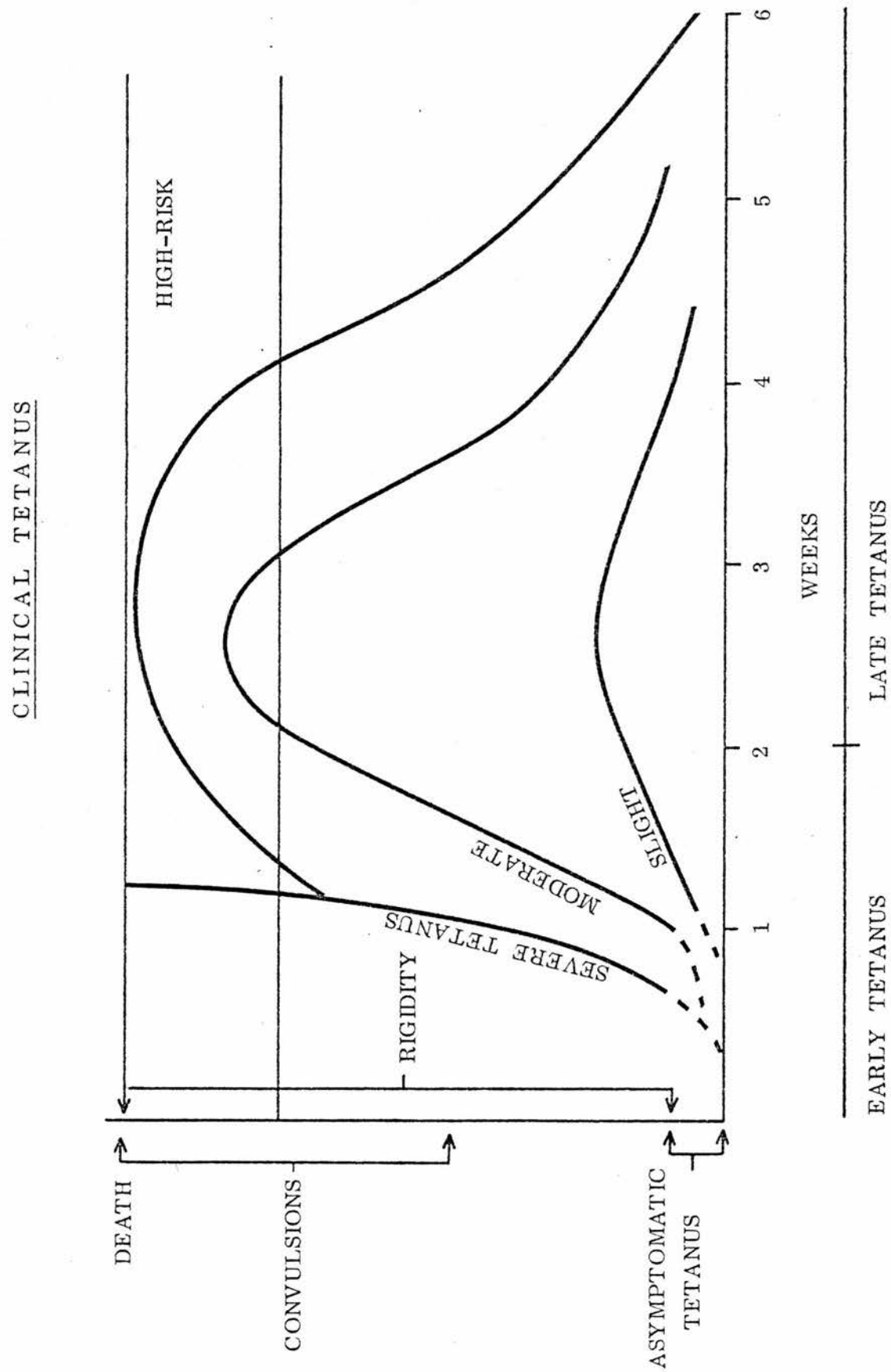
Depending on the amount of toxin produced a victim may go into either mild, intermediate or severe tetanus. By classification the worst has the shortest onset period. In mild tetanus, there is a long incubation period and spinal convulsions are usually absent, whereas in intermediate tetanus a few spinal convulsions may occur. See Diagram 1 for the course of clinical tetanus.

The incidence of fatalities appears to be dependant to some extent on age. In developing countries, tetanus is mainly a disease of neonates and children, whereas in developed countries it is a disease of the aged. The high death rate associated with neonates stems not only from a lower resistance but the fact that tetanus reduces the ability to suckle, and hence reduces the energy intake. For this reason neonates can die within 48 h of onset. In developed countries the aged are mainly affected, because of their lower immune status. In Britain many tetanus cases are due to

DIAGRAM 1. COURSE OF CLINICAL TETANUS

Reproduced from Haberman (1978), for an explanation see Section 1.1.4.

DIAGRAM 1.


CLINICAL TETANUS
HIGH-RISK
SEVERE TETANUS
MODERATE
SLIGHT
RIGIDITY
DEATH
CONVULSIONS
ASYMPTOMATIC TETANUS
1
2
3
4
5
6
WEEKS
EARLY TETANUS
LATE TETANUS

gardening accidents involving retired people.

The onset of tetanus is observed by the convulsions associated with the spastic paralysis and also the lockjaw, as described in section 1.1.1.1. An increase in the concentration of creatine phosphokinase in the blood stream is sometimes used as a marker; values higher than 3 Ucm^{-3} are indicative of tetanus.

Tetanus can be divided into an early and a late stage (Diagram 1). In early tetanus, spinal convulsions are the imminent threat to the victim as they lead to the impairment of respiration. The convex curvature of the spine during convulsions (kyphosis) may lead to fractures of the vertebrae. Up to two-thirds of tetanus victims who recover suffer from this type of fracture. Cardiovascular shock may be induced by hypoxia (oxygen-starvation) if the victim suffers from difficulties in breathing. Deaths from tetanus result mainly from respiratory or cardiovascular disturbances. Tetanus toxin may possibly affect the sympathetic nervous system, therefore affecting the heart directly (see section 1.1.1.1.). Since the muscle spasms can be controlled in intensive care units, death from early tetanus should be very rare in developed countries.

Death in late tetanus results from multifunctional effects; in the case of severe tetanus initial damage from hypoxia, brain damage, or the spastic convulsions leading to damage of the spinal cord may have resulted from the early tetanus. Difficulties in oral feeding decrease the victim's energy intake during late tetanus leading to disturbances in the victim's homeostasis. The most noticeable changes are in acid-base balance and fluid balance; this often leads to pneumonia.

As tetanus develops from its early to late stage the cause of death results more from metabolic disturbances than from the actual tetanus. The chances of heart failure or respiratory failure greatly increase. Exhaustion is another problem, caused by the continuous muscular contractions and low levels of food intake, both of which delay recovery.

A victim who survives the disease has no immunity to further attacks, as the dose of toxin needed to cause death is lower than the amount needed to activate the immunological system. The lethal dose required to kill a mouse is 20 pg ($\sim$100 amols); the dose required to kill a man is believed to be about 50 ng ($\sim$ 400 fmols).

For general references to the above section, see: Habermann (1978) and van Heyningen (1977).

1.2. The Role of Tetanus Toxin in Tetanus

The action of the toxin is assumed to be the same whether produced by Cl. tetani at the site of a wound or by the injection into experimental animals.

1.2.1. Production of Toxin by Clostridium Tetani

Cl. tetani is able to produce two toxins; one, tetanolysin, is believed to be produced during the growth or log phase of the organisms growth cycle and tetanospasmin (tetanus neurotoxin) produced mainly in the stationary or non-growth phase (no increase in the overall population, cell death rate equals cell division rate). Tetanolysin is not very toxic; it is only active under reducing conditions as it is oxygen labile, and it causes the haemolysis of red blood cells. Tetanospasmin or more commonly tetanus toxin is the neurotoxic protein, the subject of this thesis.

Tetanus toxin is synthesised within the bacterium. The amount produced is related to the particular strain of Cl. tetani; some non-toxigenic strains exist without any detriment to the organism's growth in culture. The type of growth medium also plays a role in the amount of toxin produced. In culture the synthesis of toxin is influenced by the presence of certain amino acids. How tetanus toxin is stored in the cells is not known, but it has been suggested (Habermann, 1978) that a progenitor form of the toxin may exist; a complex between toxin and some non-toxic components, similar to those for botulinus toxins. How tetanus toxin is released is also not known, though evidence suggests that it may follow from the autolysis of the cells at the stationary phase of the cell cycle. Though recent evidence has shown that two bacterial toxins, diphtheria toxin (Smith et al., 1980) and cholera toxin (Nichols et al., 1980), are synthesised as precursor forms; on membrane-bound polysomes for diphtheria toxin and on free polysomes for cholera toxin. Diphtheria toxin has been shown to be secreted cotranslationally and that the toxins immunological activity is only found external to the cytoplasmic

membrane. This is very similar to the method or protein secretion in eucaryotes as described by Blobel and Dobberstein (1975).

Some exotoxin-producing bacteria have been shown to carry information for their toxin on some extrachromosomal genome. One of the best examples is diphtheria toxin. Only strains of Corynebacterium diphtheria that are lysogenic for a bacteriophage containing a tox^+ gene are able to produce that toxin. The structural information for toxin biosynthesis is entirely on phage DNA, but expression of the tox^+ gene is a function of the bacterial genome. Cl. botulinum type C has also been shown to carry the toxin producing gene on phage DNA. The toxin produced by certain strains of E. coli is also expressed by extrachromosomal DNA, in this case by a plasmid, unlike V. cholerae which appears to carry the genetic information for cholera toxin on its own genome, see van Heyningen (1977).

Recent work (Hara et al., 1977) has shown that the treatment of different cultures of Cl. tetani with various mutagens (acridine orange, N-Methyl-N' - Nitro-N-Nitrosoguanidine, rifampicin or ultra violet light) produced non-toxigenic derivatives at a higher frequency than would be expected for a mutation in a bacterial chromosome. The non-toxigenic derivatives were stable, and no toxigenic revertants were recovered upon repeated subculture. The non-toxigenic strains which produced no toxin or fragments of toxin grew at the same rate as the parent strains which produced as much as 5-10% of their weight as toxin. It was proposed at one time that a phage associated with Cl. tetani might govern production of toxin, but a phage both inducible and morphologically identical to the one found in the parent strains was found in the non-toxigenic derivatives. Thus it was concluded that the presence of the phage was not directly responsible for toxin production. This was in contrast to the result observed for Cl. botulinum where toxigenic cultures could be "cured" of their prophage and toxigenicity simultaneously. Hence it was concluded from the loss of toxigenicity associated with the stability to revertants and the restriction to the loss of only one component, toxin, that the genetic factor controlling the production of toxin must be extrachromosomal, a plasmid, in Cl. tetani. The fact that toxin does not appear to be essential (in culture anyway) for the

growth of the bacteria, also suggests that toxin is not encoded on the bacterial genome. The agents used by Hara et al. (1977) to induce mutations in Cl. tetani are often used to remove extra-chromosomal elements in bacteria. The plasmid believed to encode tetanus toxin appears not to be associated to the phage carried by Cl. tetani, though the plasmid may be transduced by the phage. Before tetanus toxin can be conclusively identified as a plasmid product and the role of the phage determined, a strain of Cl. tetani which has been "cured" of its prophage but still retains toxigenicity must be isolated. Also direct transmission of toxigenicity from toxigenic to non-toxigenic derivatives of Cl. tetani by mixed culture must be shown. Work of this type is still being carried out by Hara. The possibility that tetanus toxin may be encoded by a plasmid has been confirmed by Laird et al. (1980).

1.2.2. The release of Tetanus Toxin

Tetanus toxin is a relatively simple protein, as it contains no carbohydrate or lipid moieties. The molecular weight of the toxin has been determined by several workers (Holmes and Ryan, 1971; Bizzini et al., 1973; Craven and Dawson, 1973; Matsuda and Yoneda, 1974; Robinson et al., 1975) using either gel-permeation chromatography, gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) or in the ultracentrifuge; most workers reporting a value at around 150 000 daltons. Work presented in this thesis, see section 3, shows values of 154 000 daltons for the molecular weight of tetanus toxin when determined by gel electrophoresis in the presence of SDS or 142 000 daltons using the ultracentrifuge.

The accepted structure for tetanus toxin (see diagram 2) is of a protein composed of two polypeptide chains linked by disulphide bonds: the H- or α-chain, molecular weight of about 100 000 daltons, and the L- or β-chain, molecular weight of about 50 000 daltons (Craven and Dawson, 1973; Matsuda and Yoneda, 1974; van Heyningen, 1976a; Helting and Zwisler, 1977; Britton and van Heyningen, 1980). There is now evidence (Murphy et al., 1968; Bizzini et al., 1973; Matsuda and Yoneda, 1974; Habermann, 1978; van Heyningen, 1977; Britton and van Heyningen, 1980; as reviewed in; Bizzini 1976, 1979; Habermann, 1978; van Heyningen

DIAGRAM 2.

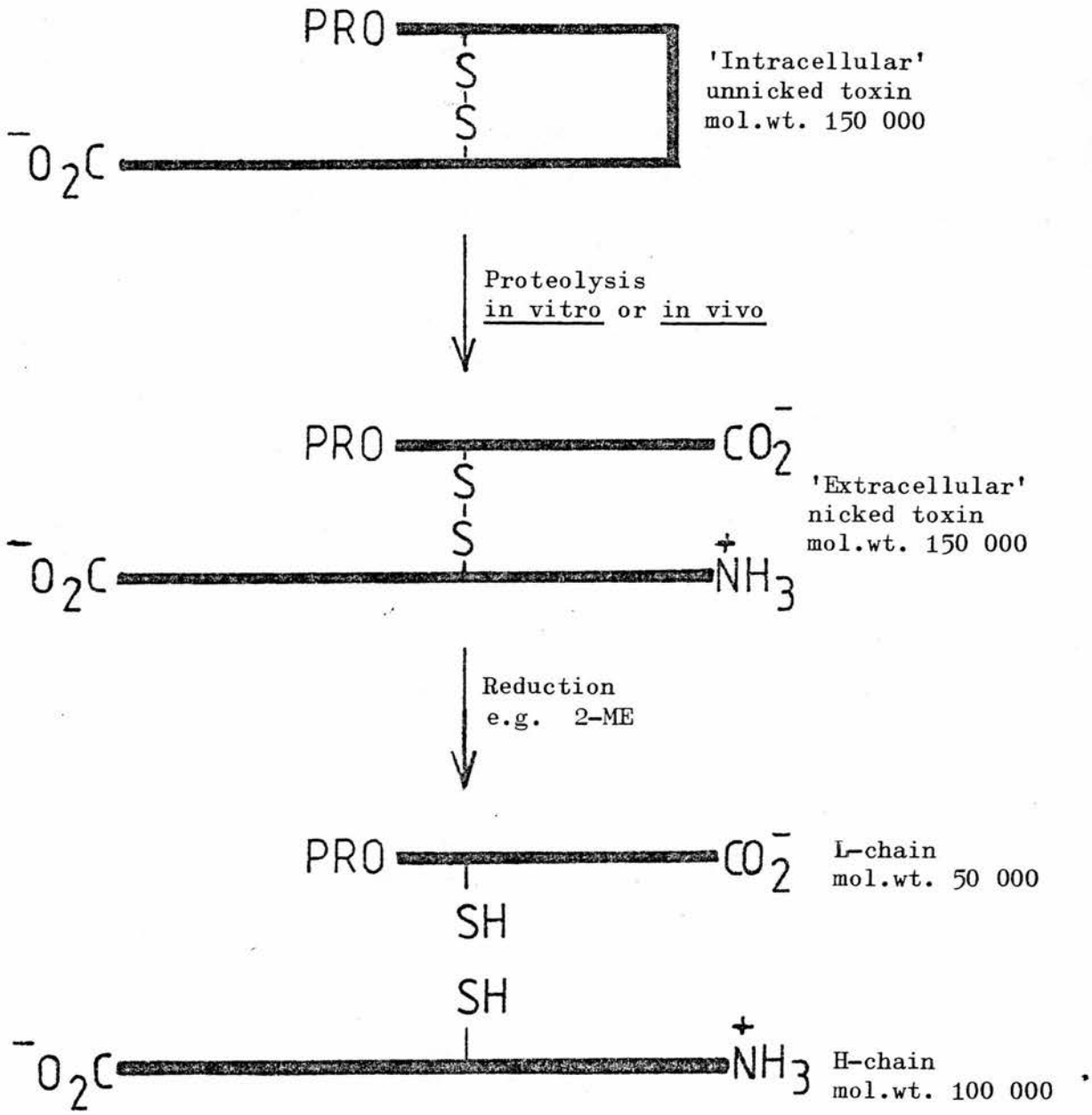


DIAGRAM 2. A SCHEMATIC MODEL FOR THE STRUCTURE OF TETANUS TOXIN

1977, 1980; Helting and Zwisler, 1977; Neubauer and Helting, 1979; Helting et al., 1979) that there are two forms of tetanus toxin. One form is a single polypeptide (molecular weight about 150 000 daltons) isolated from bacterial extracts (see below), which Matsuda and Yoneda (1974) termed 'intracellular' or 'unnicked' toxin. This form of the toxin gave only a single band up on investigation using SDS-polyacrylamide gel electrophoresis (PAGE) in the presence of reducing agents. They showed that it could be cleaved by limited trypsinolysis to give the H-chain and L-chain. They postulated that the 'intracellular' form of the toxin was still composed of the two chains but that the chains were also joined by a peptide bond that could be cleaved by trypsin or some endogenous proteases. The other form of tetanus toxin was termed 'extracellular' or 'nicked' toxin. By using SDS-PAGE in the presence of reducing agents this form of the toxin was shown to have the two chains linked by disulphide bonds only (see Diagram 2).

Most workers use the method of Raynaud (1947) to extract toxin from Cl. tetani in order to obtain the 'intracellular' form of the toxin. The method initially consists of inoculating a 100 cm^3 of a 2% glucose solution with 5 cm^3 of a 12h (later altered to 1-3 days) culture and incubating the inoculum at 37°C for 5h. The newly grown cells are then centrifuged, washed and re-suspended in a solution of 1M NaCl and 0.1M sodium citrate at 0°C for 12h (later altered to 5 days). The extracted cells are then centrifuged and the supernatant filtered. If the NaCl and sodium citrate are replaced by water during the extraction step the filtrate is non-toxigenic. The toxin extracted consists mainly of the 'intracellular' form, whereas toxin from a culture filtrate of autolysed cells is of the 'extracellular' form.

Although the exact mechanism of release from the cell is unknown, some evidence (as described above) suggests there may be a difference between the structures of 'intracellular' and 'extracellular' toxin. It appears that, upon release, 'intracellular' toxin is 'nicked' from a single polypeptide chain to the 'extracellular' form of the toxin. The reason why toxin is 'nicked' has yet to be answered, nor is it known whether 'nicking' is a pre-requisite for toxicity. Diphtheria toxin and the A-subunit of cholera toxin are activated by 'nicking' and remain non-toxic if unnicked. Some forms

of botulinus toxin are 'nicked' as indicated by reduction and separation on SDS-PAGE, but whether the unnicked forms are 'nicked' at a later stage is not known.

Helting et al. (1979) believe that they have isolated an enzyme from the culture filtrate of Cl. tetani that is responsible for the 'nicking' of the intracellular toxin. Limited trypsinolysis (Matsuda and Yoneda, 1974) has been used to try and convert intracellular toxin to the extracellular form, though whether this is the same as "native" extracellular form is not known.

In our laboratory, we found that the toxin extract we received consisted of two types. One was received as a re-suspension of a potassium phosphate precipitate and contained no 'unnicked' toxin; the second type was a freeze dried preparation and contained some 'unnicked' toxin. We also used two protease inhibitors namely phenylmethylsulphonyl fluoride (PMSF) and benzamidine to protect the toxin at all times from proteolysis by serine proteases. It appeared that the re-suspended toxin was 'nicked' by proteinases before the addition of the above inhibitors (see section 3.1.). This was not a problem with the freeze dried material as it was protected as soon as it was redissolved; any 'nicking' probably occurred before it was received. Helting et al. (1979) have shown that their 'nicking' enzyme is inhibited to a greater extent by benzamidine than PMSF.

It appears that toxin is released as a single polypeptide and 'nicked' in culture rather than 'nicked' on release. The question of whether 'unnicked' toxin is toxic or not is difficult to answer at the present as any 'unnicked' toxin is without doubt 'nicked' in the victim's blood stream. A way to try and answer the question would be to use cross-linked toxin, but unfortunately there is no in vitro assay for tetanus toxin. Merely injecting cross-linked toxin into animals could give unreliable negative results, as one would not know if one had destroyed toxicity or caused the toxin to be destroyed in vivo. Although a positive result would be of great interest.

Presumably in a wound the toxin is released by autolysis of the bacteria, followed by transportation, in the blood stream or the lymphatic system. Toxin released into lymph will eventually

be released into the jugular vein via the thoracic duct or released directly into the blood stream by diffusion from lymph vessels. Thus the main destination after release is the blood stream, where most toxin is destroyed by the various defence systems.

1.2.3.1. Fixation of Tetanus Toxin

It has been known for nearly 100 years that tetanus toxin is fixed preferentially by nerve tissue. Wassermann and Takaki (1898) showed that the toxicity of tetanus toxin, isolated from the culture filtrate of Cl. tetani, was reduced when it was mixed with an emulsion of brain. Marie (1898) showed that grey matter from brain had a greater affinity for toxin than had the white matter. Mellanby et al. (1965) studied the fixation of tetanus toxin by various subcellular fractions of brain. They showed that synaptosomes (isolated nerve endings) had the greatest affinity for toxin fixation. Mellanby and Whittaker (1967) went on to show that the synaptic membrane obtained from synaptosomes had a factor of ten times greater affinity for toxin than any other synaptosome fraction.

The receptor for tetanus toxin was tentatively identified as a ganglioside by van Heyningen (1959) and later confirmed to be a ganglioside, present mainly in nervous tissue, by van Heyningen and Miller (1961). The binding of tetanus toxin to a ganglioside was demonstrated by the use of the analytical ultracentrifuge, where the separation of free toxin and the toxin-ganglioside complex was shown in a sedimentation velocity run. Van Heyningen and Miller (1961) also showed that the N-acetyl neuraminic (sialic) acid residues of the ganglioside are essential for toxin fixation, and that the complexing with toxin does not alter the ganglioside structure.

An initial problem that caused the delay in finding the appropriate receptor for tetanus toxin lies in the fact there are two main types of lipids present in nervous tissue in greater quantities than in other tissues, both of which could have been suitable receptors. One type of lipid is the cerebrosides, which are hydrophobic and are now known not to fix tetanus toxin. The other type of lipid is the gangliosides; these are amphiphilic and are able to bind tetanus toxin. Gangliosides cannot be extracted directly from membranes with water as they are complexed

to hydrophobic components of the membrane such as the cerebrosides. Ganglioside-cerebroside complexes, termed 'protagon', can be extracted and are able to bind tetanus toxin because of the ganglioside moiety. Early workers extracted 'protagon' from nervous tissue but at the time they did not know what the components consisted of, though they were able to show that 'protagon' complexed tetanus toxin. After the discovery of cerebrosides it was believed these were the components in 'protagon' that were capable of complexing toxin, though doubt was introduced when it was discovered that the grey matter of brain contained less cerebrosides than white matter, contrary to Marie's observation. When gangliosides were discovered and found to bind tetanus toxin the discrepancy was cleared up as grey matter was shown to contain gangliosides predominantly, confirming Marie's observation.

Van Heyningen and Mellanby (1968) showed that a complex of 25% ganglioside with 75% cerebroside (in a mole:mole ratio) were the best proportions for complexing tetanus toxin, as opposed to say 5% or 50% ganglioside. Thus by changing the type of ganglioside in the above complex, they were able to show that the gangliosides G_{D1b} and G_{T1} (Svennerholm's nomenclature, Svennerholm (1963)) or GGnSSLC and SGGnSSLC (McCluer's nomenclature, McCluer (1970)) fixed tetanus toxin by a factor of twelve times that of other gangliosides such as G_{M1} (GGnSLC) and G_{D1a} (SGGnSLC). (See Table 2 for classification of gangliosides). Throughout the rest of this thesis McCluer's nomenclature will be used preferentially, as it gives one more information on the structure of a ganglioside. Svennerholm's nomenclature will be included in brackets as at the moment this appears to be more widely used. Binding to the complex was determined by incubating toxin of known toxicity with the various ganglioside-cerebroside complexes, centrifuging the complexes and determining the toxicity of the supernatants.

Gangliosides are complex amphiphilic lipids found in almost all plasma membranes but with the highest concentrations in neural tissue. Most of the different types of gangliosides appear to be tissue specific; the tetanus toxin binding gangliosides are predominantly found in nervous tissue. The ganglioside molecule is composed of two parts. There is a hydrophobic portion consisting of a long chain fatty acid (such as stearic acid) linked to sphingosine,

TABLE 2.

CLASSIFICATION OF GANGLIOSIDES

SVENNERHOLM (1963).	KHUN AND WIEGANDT (1963).	McCLUER (1970).
G_{M1}	G_{I}	GGnSLC
G_{D1a}	G_{II}	SGGnSLC
G_{D1b}	G_{III}	GGnSSLC
G_{T1}	G_{IV}	SGGnSSLC

G = GALACTOSE.
Gn = N-ACETYL GALACTOSAMINE.
S = N-ACETYL NEURAMINIC ACID = SIALIC ACID.
L = LACTOSE = GALACTOSE-GLUCOSE.
C = CERAMIDE = SPHINGOSINE-STEARIC ACID.

these parts confer lipid solubility on the molecules which enables them to dissolve into lipid membranes. The hydrophilic portion consists of the carbohydrates; glucose, galactose, N-acetyl glucosamine and N-acetyl neuraminic acid (sialic acid), the proportions of which determine the type of ganglioside. The hydrophilic portion is on the outside of the cell membrane and is dissolved in the aqueous environment where it is capable of complexing with ligands, such as tetanus toxin. See Diagram 3 for the general structure of a ganglioside. The two tetanus-toxin-binding gangliosides GGnSSLC (G_{D1b}) and SGGnSSLC (G_{T1}) both have a neuraminidase-labile bond between two N-acetyl neuraminic acid residues, after hydrolysis the molecules no longer bind toxin with a high affinity. Ganglioside SGGnSSLC (G_{T1}) has another neuraminidase-labile bond: hydrolysis converts it to GGnSSLC (G_{D1b}) with no noticeable decrease in the affinity for tetanus toxin. See Diagram 3 for positions of neuraminidase-labile bonds, and Diagrams 4a and 4b for the structure of ganglioside SGGnSSLC (G_{T1}).

Gangliosides have been found to bind a number of different ligands. Van Heyningen (1963) showed that strychnine, brucine and thebaine, drugs having the same neurophysiological effect as tetanus toxin, were bound to gangliosides. Cholera toxin has been shown to be preferentially bound to ganglioside GGnSLC (G_{M1}), (van Heyningen, 1974). Interferon (Besancon et al., 1976) and glycoprotein hormones such as thyrotropin (TSH) (Fishman and Brady, 1976), have also been shown to be complexed by gangliosides. Thus it appears that gangliosides are good candidates for the role of cell-surface receptors, although in most cases their function is unknown.

Van Heyningen and Mellanby (1973) and van Heyningen (1974) have shown that cholera toxin complexed to purified ganglioside GGnSLC (G_{M1}) is completely inactive in vivo. In contrast, tetanus toxin was shown not to be completely inactivated in vivo, when complexed to purified gangliosides GGnSSLC (G_{D1b}) and SGGnSSLC (G_{T1}). This observation poses the question of whether the gangliosides that fix tetanus toxin are the actual receptors or possibly only part of the receptor, which would explain the incomplete inactivation by purified gangliosides. The possibility that there may be more than one receptor for tetanus toxin will be discussed later. Another possible explanation for the incomplete inactivation of the

DIAGRAM 3. A GENERALISED STRUCTURE FOR GANGLIOSIDES

For a complete description of gangliosides see Section 1.2.3.1.

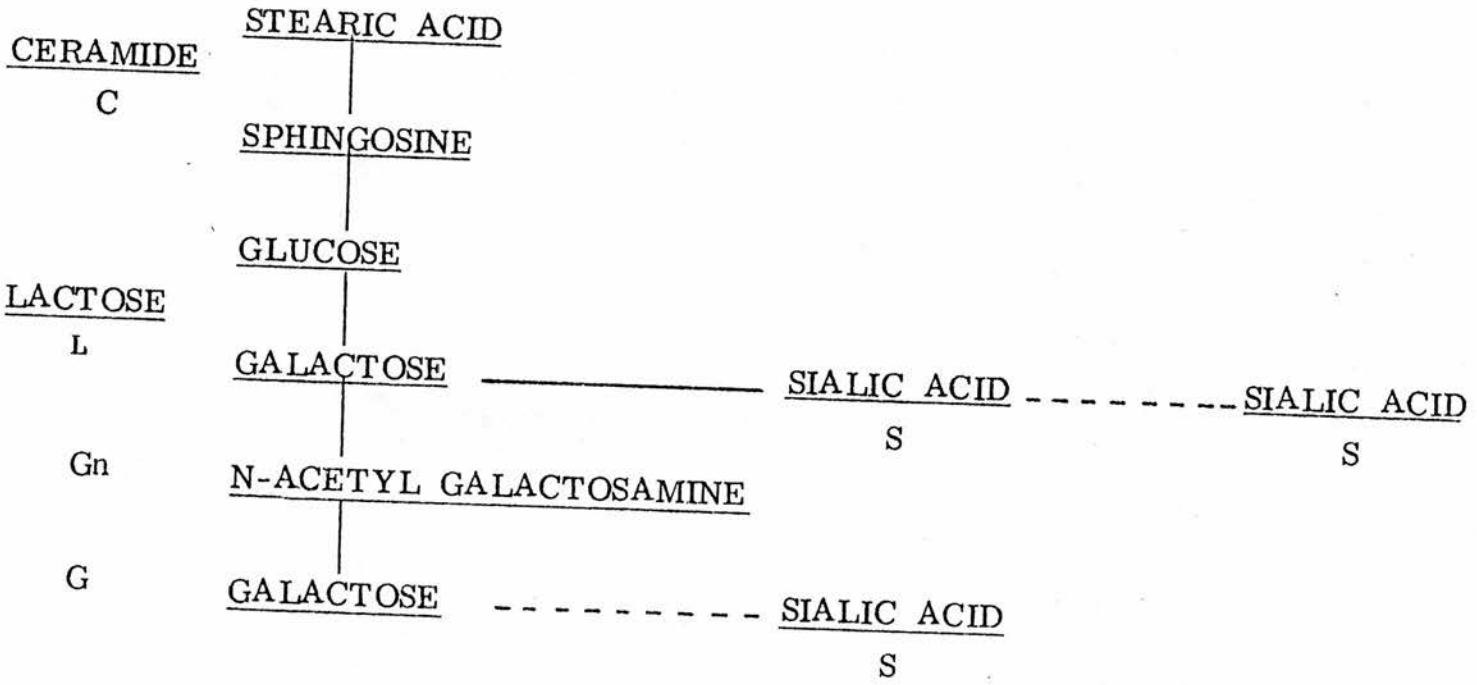


DIAGRAM 3.

S = SIALIC ACID ≡ N-ACETYL ACID.

BASIC STRUCTURE OF A GANGLIOSIDE NAMELY SGnGSSLC (G_{T1})

_____ REPRESENTS THE NEURAMINIDASE LABILE BONDS.

DIAGRAM 4a. THE STRUCTURE OF GANGLIOSIDE SGGnSSLC (G_{T1})

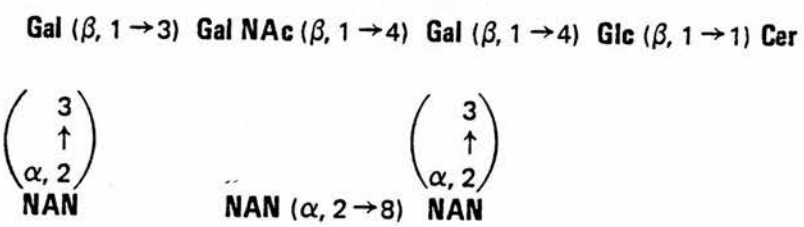
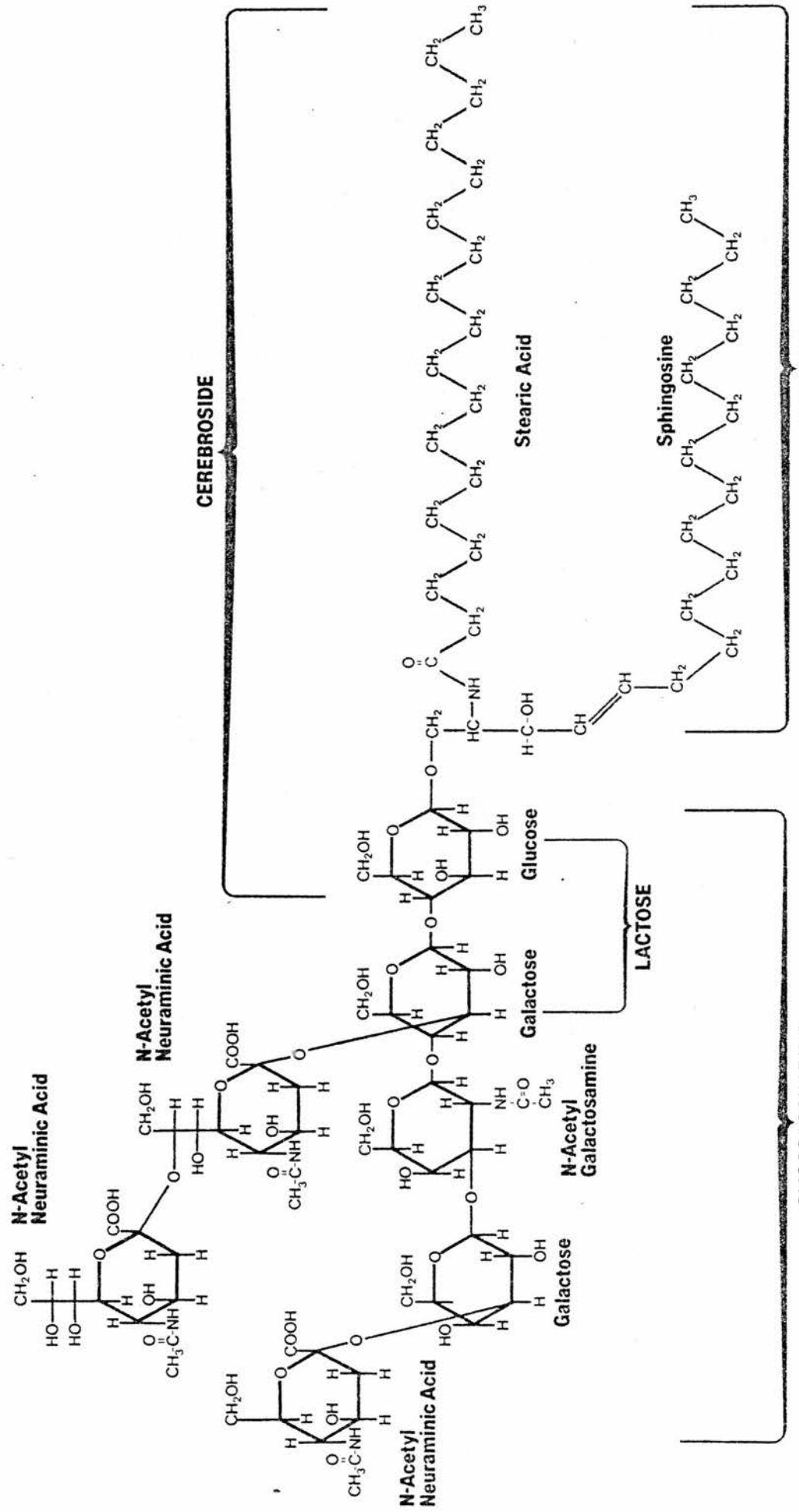


DIAGRAM 4b. A SIMPLIFIED STRUCTURE OF GANGLIOSIDE SGGnSSLC (G_{T1}) showing the bonding between the sugar residues

DIAGRAM 4a.


CEREBROSIDE
CERAMIDE
OLIGOSACCHARIDE
LACTOSE
Stearic Acid
Sphingosine
Glucose
Galactose
N-Acetyl Galactosamine
Galactose
N-Acetyl Neuraminic Acid
N-Acetyl Neuraminic Acid
N-Acetyl Neuraminic Acid

toxin:ganglioside complex in vivo, is the break down of the complex after injection to yield active toxin.

The di-N-acetyl neuraminic acid moiety joined to the galactose residue in the lactose moiety (see Diagram 3) in tetanus toxin binding gangliosides, appears to be essential for toxin binding. Serotonin (5-OH tryptamine) is known to bind to a ganglioside, SSLC, (van Heyningen, 1974) so tetanus toxin may bind to its ganglioside receptors via a tryptophan residue.

A new method capable of giving more accurate results and possibly quantitative binding data is that of Helting et al. (1977), using tritiated gangliosides, prepared by reduction with KB^3H_4, of gangliosides previously oxidised using galactose oxidase. Two different binding assays were used. In the first a small column of Sephadex G-100 was equilibrated with a buffer containing ^{3}H-ganglioside and a sample of tetanus toxin then applied to the column. For reasons not entirely clear, the toxin remained bound to the column, and the amount of radioactive ganglioside eluted decreased by the amount that complexed to the toxin. In the second method, toxin was applied to the column in the absence of ganglioside, and [^{3}H]-ganglioside was then run through the column. Any ganglioside not eluting must have bound to the toxin. Bound radioactive complexes in both cases were eluted using 0.1% NaOH. Helting's group used the gangliosides GGnSSLC (G_{D1b}) and GGnSLC (G_{M1}), the tetanus toxin binding di-sialo ganglioside and the cholera toxin binding mono-sialo ganglioside. They were able to show for ganglioside GGnSSLC (G_{D1b}) that there was a one to one molar binding ratio of toxin to ganglioside. They also claimed that GGnSLC (G_{M1}) also gave the same result, contrary to previous reports, (van Heyningen, 1963).

Van Heyningen (1976a) showed that ganglioside SGGnSSLC (G_{T1}) is able to bind the H-chain of tetanus toxin but not the L-chain. The ganglioside was made insoluble by complexing with cerebroside, then either whole toxin, H-chain in 1M urea (to keep it solubilised), or L-chain were incubated with the insolubilised ganglioside. The protein:ganglioside:cerebroside complex was then centrifuged down and the supernatant analysed by SDS polyacrylamide gel electrophoresis to identify any molecular species of tetanus toxin left. The insoluble complex was dissociated in 8M urea to release

the bound protein for identification. Thus H-chain was found, after incubation with the ganglioside:cerebroside complex, to be absent from the supernatant but to be released from the complex by urea. L-chain gave no evidence of adsorption to the ganglioside:cerebroside complex. Helting et al. (1977) were able to confirm van Heyningen's results; they found that the ganglioside binds only the H-chain of tetanus toxin or to their proteolytic fragment C of H-chain (prepared by the proteolytic digestion of H-chain by papain). (See section 3).

Mellanby and Pope (1975) showed that increasing the Ca^{2+} concentration, interferes with the binding of toxin to either synaptic membranes or ganglioside:cerebroside complexes. They also found that raising the temperature released some bound toxin from synaptic membranes, an action enhanced at higher Ca^{2+} concentrations.

Habermann (1976) was able to show that tetanus toxin can be purified by affinity chromatography using a column of synaptosomes adsorbed to either bromoacetyl cellulose or kieselguhr. Toxin was adsorbed to the synaptosomes at low ionic strength and eluted at a higher ionic strength, indicating that electrostatic forces are responsible for the fixation rather than hydrophobic interactions. Experiments using [^{125}I]-tetanus toxin fixed to the synaptosomes, showed that even large excess of unlabelled toxin could not elute much of the labelled toxin, implying a large number of possible receptor sites on the synaptosomes.

Holmgren et al. (1980) used another method, in which the ceramide moiety of a ganglioside was adsorbed spontaneously (by strong hydrophobic bonding) to polystyrene leaving the oligosaccharide portion of the ganglioside free to react with specific ligands. They were able to show that GGnSSLC (G_{D1b}) and SGGnSSLC (G_{T1}) but not GGnSLC (G_{M1}) (in contrast to Helting et al. (1977)), were able to bind tetanus toxin. They went on to show that a tetra-sialo ganglioside, namely SSGGnSSLC (G_{Q1b}) was also able to bind tetanus toxin to the same extent. This observation shows more evidence that the di-N-acetyl neuraminic acid portion is necessary for the binding of tetanus toxin. They postulated that the natural binding structure has the end sequence Galβ1 → 3Galβ1 → 4Gal(2← 8αNeuAc2← 3αNeuAc) which occurs in the appropriate gangliosides and possibly as oligosaccharide chains in certain cell membrane glycoproteins.

1.2.3.2. The Fixation of Tetanus Toxin to Whole Cells

As described above, a possible receptor for tetanus toxin has been identified, namely specific gangliosides, but as yet no evidence has been presented for the binding of toxin to whole cells. Dimpfel *et al.* (1977) showed that primary cell cultures derived from embryonic CNS were able to bind [^{125}I]-tetanus toxin, but continuous cell lines did not. The continuous cell lines did not contain any long chain gangliosides such as GGnSSLC (G_{D1b}). The use of [^{14}C]-glucosamine (a sugar that is specifically incorporated into ganglioside type lipids) for the *de novo* synthesis of gangliosides, showed that only the primary cell cultures produced gangliosides. Hybrid cells (neuroblastoma and glioma fusions) were shown to bind only small quantities of tetanus toxin, because of the lack of relevant gangliosides. Binding was increased by the *in vitro* addition and uptake of the relevant purified gangliosides into the cell membranes. The use of neuraminidase resulted in near complete prevention of [^{125}I]-tetanus toxin binding. These experiments also provided evidence that tetanus toxin may be used as a neuronal marker in primary tissue culture cells. Using this method, Dimpfel *et al.* (1975) were able to show that neuronal cells but not glial cells in culture were able to bind tetanus toxin.

Mirsky *et al.* (1978) have shown (by using immunofluorescence) that tetanus toxin can distinguish neuronal cells from non-neuronal cells in a wide variety of dissociated cell cultures. They used rabbit anti-tetanus toxin immunoglobulins and goat anti-rabbit gamma globulins conjugated with a fluorescent label. The cell types they investigated were neuronal cells from the cortex, cerebellum, spinal cord and dorsal root ganglia obtained from rats and spinal cord, optic lobe, retina and dorsal root obtained from chicks. All were shown to bind tetanus toxin. The use of tetanus toxin as a neuronal marker has increased so much recently that it is now used as a tool in neurobiology for the identification of neuronal cells. For example Raff *et al.* (1979) and McGeer and McGeer (1979) have used the toxin as a marker. Thus from the evidence using gangliosides, ganglioside/cerebroside complexes, synaptosomes and primary neuronal cell cultures it appears the gangliosides may be the receptors for tetanus toxin.

Zimmerman and Pifaretti (1977) disagree with the evidence for a ganglioside binding site and suggest two types of binding sites. One is a 'non-effective' binding site that is ganglioside dependant (shown by neuraminidase experiments), observed only with non-differentiating mouse neuroblastoma cells in culture and produces no biological effect on the growth of the cells. The second type an 'effective' binding, not associated with gangliosides (not effected by pre-treatment with neuraminidase), is observed only with differentiating cells and leads to a shortening of cell processes, a decrease in population and a decrease in the cells adherence to glass. They postulate binding to a 'non-effective' site leads to uptake by pinocytosis and degradation by lysosomes, whereas binding to an 'effective' site leads to internalisation for transport (see section 1.2.4.).

1.2.3.3. The Possible Binding of Tetanus Toxin to Other Receptors

Recent work carried out on the thyrotrophin (TSH) receptor in the thyroid gland has introduced some very interesting information about the possible receptor for tetanus toxin. It is known that the TSH receptor in the thyroid gland is composed of two components; a glycoprotein and a ganglioside, both of which are required for message transmission by TSH. Ledley *et al.* (1977) have shown that thyroid plasma membranes are capable of adsorbing [^{125}I]-tetanus toxin and that the binding characteristics such as time, pH and ionic strength were similar to those of [^{125}I]-TSH. The [^{125}I]-tetanus toxin binding was shown to be blocked or chased by the addition of either unlabelled tetanus toxin or TSH, but not by glucagon, insulin, diphtheria toxin, prolactin or human chorionic gonadotropin, though cholera toxin did affect both [^{125}I]-tetanus toxin and [^{125}I]-TSH binding. Certain gangliosides especially GGnSSLC (G_{D1b}) and SGGnSSLC (G_{T1}) inhibited the binding of [^{125}I]-TSH, indicating that the gangliosides play some role in the receptor. Recent work by Morris *et al.* (1980) has shown that Fragment C (see section 3), a proteolytic fragment of H-chain, retains most of the determinants of the toxin molecule for interaction with neural or thyroid membranes.

Another approach was adopted by the same group (Habig *et al.*, 1978); they studied the binding of tetanus toxin and TSH to normal

rat thyroid membranes and to membranes from rats suffering from a thyroid tumour. The tumour cells had a TSH receptor defect which greatly reduced the binding of TSH. The same cells were also shown not to adsorb tetanus toxin, indicating some similarity between the TSH receptor and the receptor for tetanus toxin.

The glycoprotein component of the TSH receptor was purified, following solubilisation from the membranes with lithium di-iodosalicylate, (Lee et al., 1978). The binding characteristics of TSH and tetanus toxin were then determined on the purified glycoprotein either in solution or after incorporation into liposomes. The same characteristics as previously described were observed as regards binding inhibition or non-inhibition. The work was continued, (Lee et al., 1979), by studying the interactions of tetanus toxin and TSH on preparations of brain cell membranes obtained from rats. It was observed that the brain cell membranes bound [^{125}I]-tetanus toxin 20-40 times better than [^{125}I]-TSH and that thyroid membranes bound [^{125}I]-TSH four times better than [^{125}I]-tetanus toxin. A strange result was observed; [^{125}I]-TSH binding to brain cell membranes was inhibited by unlabelled tetanus toxin but [^{125}I]-tetanus toxin binding was enhanced by unlabelled TSH. It was suggested that [^{125}I]-TSH might occupy the 'non-effective' sites postulated by Zimmerman and Piffaretti allowing [^{125}I]-tetanus toxin to bind more readily to the 'effective' sites. The observation appears to be a property of a tetanus toxin to ganglioside interaction since analogous results were observed using liposomes containing mixed brain gangliosides, but not with liposomes containing the glycoprotein component of the thyroid TSH receptor.

1.2.3.4. The Binding Sites for Tetanus Toxin

After the release of tetanus toxin into the blood stream by autolysis of Cl. tetani, the toxin is observed to bind to nerve endings at the neuromuscular junction. Direct binding to the neuromuscular junction was shown by autoradiography using [^{125}I]-toxin, (Wernig et al., 1977). The binding of toxin to nerve terminals at the neuromuscular junction is now known to be a prerequisite for axonal transport (see section 1.2.4.). Another binding site for tetanus toxin is at its site of action, the pre-synaptic membranes of certain inhibitory synapses (see section 1.2.5.).

As discussed in section 1.2.3., the nature of the receptor for tetanus toxin is uncertain. It appears from recent evidence that as well as gangliosides containing the di-N-acetyl neuraminic acid group there is the possibility of the involvement of a glycoprotein being involved in the receptor. The glycoprotein probably contains a similar oligosaccharide structure to the gangliosides involved in the fixation of tetanus toxin. For a review on toxin interactions with glycoconjugates see Wiegandt (1979).

1.2.4.1. Neuronal Transport of Tetanus Toxin

The main problem to be answered once tetanus toxin has bound to its receptor at the neuromuscular junction concerns the question of what the ultimate destination of the toxin is and how it arrives there.

Two hypotheses have been suggested; the first was that toxin is transported via the blood stream to its site of action. This is now believed not to be true because toxin cannot cross the blood brain barrier and is fixed at the neuromuscular junction. The second theory was first introduced by Meyer and Ransom (1903), and involves the neuronal ascent of toxin to the spinal cord. Such ascent of the toxin could be intra-axonal or extra-axonal, (see Zacks and Sheff, 1970). Many experiments were carried out by different research groups over many years in order to try and answer the question. This thesis will review only the recent work as earlier workers tended to use impure toxin and ultimately produced conflicting data. Also the introduction of labelled toxin only occurred in 1970, and this was essential for determining the route of transport for tetanus toxin.

Recent work has shown that tetanus toxin is transported intra-axonally. Erdman *et al.* (1975) used histo-autoradiography on particular neurones after the intramuscular injection of [^{125}I]-tetanus toxin and showed the toxin was transported intra-axonally. Transport was found to be confined to the ventral roots and the spinal cord, and the amount of toxin transported could be reduced by the use of colchicine and vinblastine (alkaloids used to disrupt microtubules which are known to be required for intracellular transport in axons). This confirmed the work of Dimpfel

and Habermann (1973), who used [^{125}I]-tetanus toxin to show that toxin was localised in the spinal cord in general tetanus with the possibility of intra-cellular accumulation of toxin. Price et al. (1975) were able to show direct evidence for intra-axonal transport in a retrograde direction, opposite to the usual antegrade direction from the cell body to the nerve ending. They injected rats or mice in a leg muscle with [^{125}I]-tetanus toxin and the nerves supplying the muscle were crushed, labelled toxin was found to accumulate at the distal side of the crush but not at the proximal side. Thus they were able to show that toxin is only taken up at the nerve terminal and not along the axon.

Stockel et al. (1975) showed that tetanus toxin is retrogradely transported intra-axonally in all peripheral neurones, especially in motor, sensory and adrenergic types. They also showed that nerve growth factor (NGF) is only transported retrogradely in adrenergic and sensory neurones. The mechanism for the transport for both tetanus toxin and NGF was shown to be colchicine-sensitive and the rates of transport were the same, 7.5 mmh.$^{-1}$ Stockel et al. (1977) went on to show that cholera toxin and wheat germ agglutinin (WGA) as well as tetanus toxin are transported in all peripheral neurones. This led to the idea that the uptake and the retrograde axonal transport of these macromolecules may depend upon some common property of the neurones. It was shown when cholera toxin was bound to its receptor ganglioside GGnSLC (G_{M1}) that no retrograde transport occurred. If tetanus toxin was bound to SGGnSSLC (G_{T1}) the amount transported was reduced by 50%, indicating that gangliosides may be a general receptor for the uptake of proteins at nerve endings. Stockel et al. (1977) also showed that tetanus toxin was transported at a rate of 3 mm h^{-1} in adrenergic neurones, 7.5 mm h^{-1} in motor neurones and 13 mm h^{-1} in sensory neurones. The same rates were also observed for cholera toxin and NGF. They postulated that the 50% reduction in uptake of tetanus toxin when complexed to ganglioside SGGnSSLC (G_{T1}) or mixed gangliosides may be due to a second site available for the binding of tetanus toxin which may not be a ganglioside, possibly confirming the work of Zimmerman and Piffaretti (1977, see above).

Price and Griffin (1977) showed that systematically (intraperitoneally) injected toxin was taken up by nerve endings.

They injected mice intraperitoneally with [^{125}I]-tetanus toxin then ligated one sciatic nerve. After 24 h they killed the mice. Tetanus toxin was shown to have accumulated within the axons distal to the ligature, as shown previously for intra-muscularly injected [^{125}I]-tetanus toxin. Thus they had answered the age-old problem of the fate of blood-borne tetanus toxin. This observation was also confirmed by Erdman and Habermann (1977).

Green et al. (1977) showed that the retrograde transport of toxin in motor neurones is mainly confined to the α-neurones. These are the main neurones conveying signals to the muscles from the CNS. They showed there was little or no transport via the γ-neurones, the slower acting motor neurones.

Schwab and Thoenen (1978) used gold-labelled toxin to show selective binding (in contrast to gold-labelled albumin) to the nerve terminals of the rat iris. They were also able to show that the gold-labelled toxin was absorbed into the terminals and retrogradely transported. The position of the toxin was detected by the use of an electron microscope because of the electron dense colloidal gold marker. They observed that the toxin complexed to the neuro-plasma membrane of the nerve terminal prior to uptake, confirming the results of Price et al. (1975). Uptake was followed by the complexing of the labelled toxin to smooth-endoplasmic-reticulum-(SER)-like membrane components. The toxin-gold complex was then observed to be transported retrogradely within the axon, and to reach the adrenergic ganglion cell bodies of the superior cervical ganglia after 14 h. It had been shown earlier (Erdman et al, 1975) that the retrograde transport is colchicine sensitive but nothing is known about the possible interaction of the SER-like components with microtubules and the localisation of the force generating mechanisms. This also confirmed the work of Griffin et al. (1977) who showed that [^{125}I]-tetanus toxin was transported in vesicles and branched tubules of SER.

A problem involved in using either [^{125}I]-tetanus toxin or gold-labelled toxin is the fact that one does not know if it is the protein, the complex or even a degradation product of labelling, that is being transported. This problem was resolved by Carroll et al. (1978), by the use of unlabelled tetanus toxin. They ligated the sciatic nerve and detected toxin by the use of immunoperoxide and immunofluorescent techniques. Rats were taken and two ligatures were placed 5 mm apart

on both sciatic nerves, and tetanus toxin was injected into the right hind limb. After 24 h, the rats were killed and the sciatic nerves removed. Sections of the nerve were overlayed with goat anti-toxin followed by either rabbit anti-goat antibodies conjugated to horse-radish peroxidase or conjugated to fluorescein isothiocyanate. The horse-radish peroxidase samples were then incubated in diaminobenzidine-hydrogen peroxide and counter stained with toluidine blue. Both methods showed either stain or fluorescence at the distal side of the distal ligature; no evidence of toxin proximal to the distal ligature or proximal to the proximal ligature was observed. The movement of unlabelled toxin to the CNS was confirmed by Chaudhury et al. (1978) using fluorescent labelled anti-tetanus toxin. Thus it appears tetanus toxin is finally adsorbed to nerve-endings, and internalised for internal retrograde transport.

1.2.4.2. The Absorbtion of Tetanus Toxin Prior to Transport

It is known that certain proteins and peptides can enter cells by receptor-mediated endocytosis. The process consists of the extracellular protein or peptide binding to a specific receptor on the cell surface followed by rapid internalisation into the cell. Internalisation appears to follow the clustering of receptors in specialised regions of the cell surface, called coated pits, that can invaginate to form intracellular coated vesicles (Goldstein et al., 1979). Most proteins are transported to the lysosomes for degradation, but some proteins may not be, for example NGF appears not to be degraded but transported retrogradely up the axon inside its vesicle (Goldstein et al., 1979). It has been shown that as well as NGF, tetanus toxin is transported retrogradely inside vesicles and cisternae of SER, (Schwab and Thoenen, 1976b, 1976c, 1978). Thus it is possible that tetanus toxin may be internalised by receptor-mediated endocytosis, due to its fixation to a specific receptor and intracellular transport without degradation.

1.2.4.3. The Trans-Synaptic Migration of Tetanus Toxin

Another problem associated with intra-axonal transport is the fate of the toxin once it has reached the cell body of the neurone. It is known that toxin can either be transported up the next neurone

or interact with the pre-synaptic membrane of certain inhibitory neurones (see section 1.2.5.1.), both processes requiring toxin to cross a synaptic cleft. Schwab and Theonen (1976a) observed that rats injected with [^{125}I]-tetanus toxin showed signs of rigidity 12-13 h after injection and death occurred 1-2 h later. Electron microscopic autoradiography showed that a large proportion of radioactive label was located in motor neurones 7 and 14 h after injection. A great majority of the label was found over synaptic termini afferent to the motorneurones; glial cells were not labelled to any extent. Thus the observation indicated evidence of trans-synaptic migration by the toxin. They later elaborated on this work, (Schwab et al., 1979), by showing that cholera toxin and some lectins such as WGA and ricin were transported retrogradely in the same motor neurones as tetanus toxin, but unlike tetanus toxin were incorporated into the lysosomes in the cell body. Tetanus toxin was found to be present in pre-synaptic terminals afferent to the cell body, probably due to the selective release of the toxin from the post-synaptic dendrites followed by the uptake into the pre-synaptic terminals. Dumas et al. (1979) showed that labelled toxin after trans-synaptic transfer was still intact and could undergo further retrograde transport. See Dimpfel (1980) for a short review on the transport of tetanus toxin in neurones.

It was suggested by Erdman and Habermann (1977) that 'general' tetanus is a multiple 'local' tetanus. This was postulated from the evidence that one still finds the neural transport of toxin in local tetanus and that the peripheral spread of multiple sites of local tetanus to one site could manifest itself as general tetanus. In the last few years the evidence tends to agree with this theory especially from experiments showing that blood-borne toxin is eventually transported intra-axonally.

1.2.5.1. The Mechanism of Action of Tetanus Toxin

Tetanus toxin does not destroy any isolated cell nor does it produce any pathological lesions. Changes have been shown to occur, but never consistently, and have not been reported after the injection of purified toxin. Thus any lesions attributed to injected crude toxin is probably due to contaminating enzymes (proteinases) and other

substances accompanying the toxin. Without the production of lesions during intoxication there are no clues to the mode of action of the toxin at the histological level. The greatest evidence for the mode of action has been derived from physiological evidence due to its action on the nervous system (van Heyningen and Mellanby, 1971).

In 1884 Sir James Simpson commented upon the similarity between the symptoms of tetanus and strychnine intoxication. It was due to this similarity that the mode of action of tetanus toxin was initially unravelled. Curtis (1959) was able to show that the action of strychnine was to abolish inhibition in the CNS, which allows the uncontrolled spread of nerve impulses initiated anywhere in the CNS.

Curtis and DeGroat (1968) showed, using Renshaw cells, that the action of tetanus toxin was also to block the inhibitory pathways of the CNS. They showed that both tetanus toxin and strychnine block the synaptic process at inhibitory synapses, resulting in the dis-inhibition of spinal inhibition. The major difference observed between the action of the two toxins was that strychnine acted post-synaptically but tetanus toxin acted by some other mechanism. They concluded that tetanus toxin did not act post-synaptically because electrophoretically administered glycine (the postulated inhibitory neurotransmitter) when added with tetanus toxin reversed the effect of the toxin. When glycine was added with strychnine there was no reversal of the effect caused by strychnine. They postulated that tetanus toxin interfered with the release of glycine rather than interfering with its synthesis, indicating a pre-synaptic mechanism for the action of tetanus toxin. The work was confirmed by Fedinec and Shank (1971), who showed that tetanus toxin during tetanus induced no significant changes in the levels of glycine, γ-aminobutyric acid (GABA), glutamate or aspartate in the spinal cord of rats. Thus they were able to show that the amino acid pool in the spinal cord did not change and that if glycine was the spinal cord inhibitory neurotransmitter, tetanus toxin appeared to interfere with the release rather than deplete the transmitter's stores.

Earlier work (Ambache et al., 1948a, 1948b) showed that tetanus toxin can interfere with the release of acetyl choline from the nerve endings of the rabbit's iris. Treatment of tetanus toxin with the cerebroside-ganglioside complex inhibited the toxin's ability to paralyse the cholinergic neurones in the sphincter pupillae of the

rabbit's iris (Mellanby et al., 1968).

Osborne and Bradford (1973) postulated that as well as the release of glycine being inhibited by tetanus toxin, the release of another inhibitory neurotransmitter, GABA, may also be inhibited. This confirmed the work of Curtis et al. (1973) who showed that tetanus toxin abolished GABA mediated inhibition in Purkinje cells of cats. This has been confirmed more recently, (James and Collingridge, 1979), by showing that GABA plays a role in the substantia nigra in rats by inhibiting its release with tetanus toxin. Hillbig et al. (1979) showed by the use of in vivo experiments that glycine release was impaired by tetanus toxin in rat spinal cord and by the use of [^{14}C]-glycine that there was no uptake of glycine. Davies and Tongroach (1979), showed that tetanus toxin only affected GABA mediated synaptic inhibition in the rat substantia nigra and not mono-amine mediated inhibition; they suggested a pre-synaptic action. Collingridge et al. (1980) concluded that tetanus toxin inhibits the release of GABA directly and not by interfering with the metabolism, in the substantia nigra and striatum of rats.

Ambache et al. (1948a, 1948b) were able to show a peripheral action of tetanus toxin using the iris muscle (sphincter pupillae) in a rabbit's eye. Results showed that the injection of tetanus toxin into the anterior chamber of the eye abolished the constriction of the pupil in response to light. Injection of acetyl choline into the anterior chamber, of toxin treated rabbits caused the sphincter pupillae to contract very quickly. They concluded that the toxin blocked the cholinergic neuromuscular transmission to the sphincter pupillae. Diamond and Mellanby (1971) produced further evidence for the action of tetanus toxin, using neuromuscular transmission in goldfish. Tetanus toxin was shown to kill goldfish by producing a flaccid paralysis rather than a spastic paralysis, due to the blocking of the action of the neuromuscular transmitter acetyl choline. This led to the postulation by Mellanby et al. (1973) of the similarity between the actions of tetanus and botulinus toxins. Botulinus toxin was known to inhibit the release of acetyl choline at nerve endings of the neuromuscular junction. Habermann et al. (1980) showed that tetanus toxin blocked the nerve action at the neuromuscular junction in the mouse diaphragm in a similar mechanism to that of botulinus toxin, supporting the hypothesis of Mellanby et al. (1973). Previously Bigalke et al. (1978) showed that both

tetanus toxin and botulinus toxin suppressed the release of acetyl choline upon potassium ion depolarisation in primary nerve cultures derived from embryonic rat CNS.

Thus it appears the action of tetanus toxin is to inhibit, pre-synaptically, the release of neurotransmitters, namely glycine and GABA in the CNS, responsible for the inhibition of motor responses and possibly of acetyl choline at the neuromuscular junction. The possible actions of tetanus toxin are summarised in Diagram 5.

1.2.5.2. Possible Mechanisms by which Tetanus Toxin may Inhibit the Release of Neurotransmitters

The mechanism by which tetanus toxin inhibits the release of neurotransmitters is not really understood but a few mechanisms have been suggested. Kryzhanovsky (1973) showed that tetanus toxin disrupted the neuromuscular transmission in the rat diaphragm; electron microscopic investigation revealed no ultrastructural changes in the synapse apart from the accumulation of synaptic vesicles.

Duchen and Tonge (1973), working on the action of tetanus toxin at the neuromuscular junction in the hind legs of mice showed that raising the external calcium ion concentration did not restore neuromuscular transmission. They also showed that raising the external concentration of potassium ions did not restore neuromuscular transmission. They suggested that tetanus toxin causes an impairment between terminal depolarisation and transmitter release.

Kryzhanovsky (1975, and Kryzhanovsky *et al.*, 1975) postulated that tetanus toxin inhibited the contractile ability and ATPase activity of a brain or spinal cord actinomyosin-like protein. It had been suggested that the actinomyosin-like protein played a role in mediator release processes.

Fedinec *et al.* (1976) showed that glycine and theophylline (a drug that inhibits the breakdown of cyclic mucleotides by inhibiting phosphodiesterases) reversed tetanus-toxin-induced paralysis of the sphincter pupillae of the rabbit's iris. Thus they showed tetanus toxin might affect the levels of cyclic nucleotides, which are known to play a role in the release of neurotransmitters. King *et al.* (1978) went on to show that the sphincter pupillae paralysed by tetanus toxin could be made to contract by the use of cGMP but not cAMP.

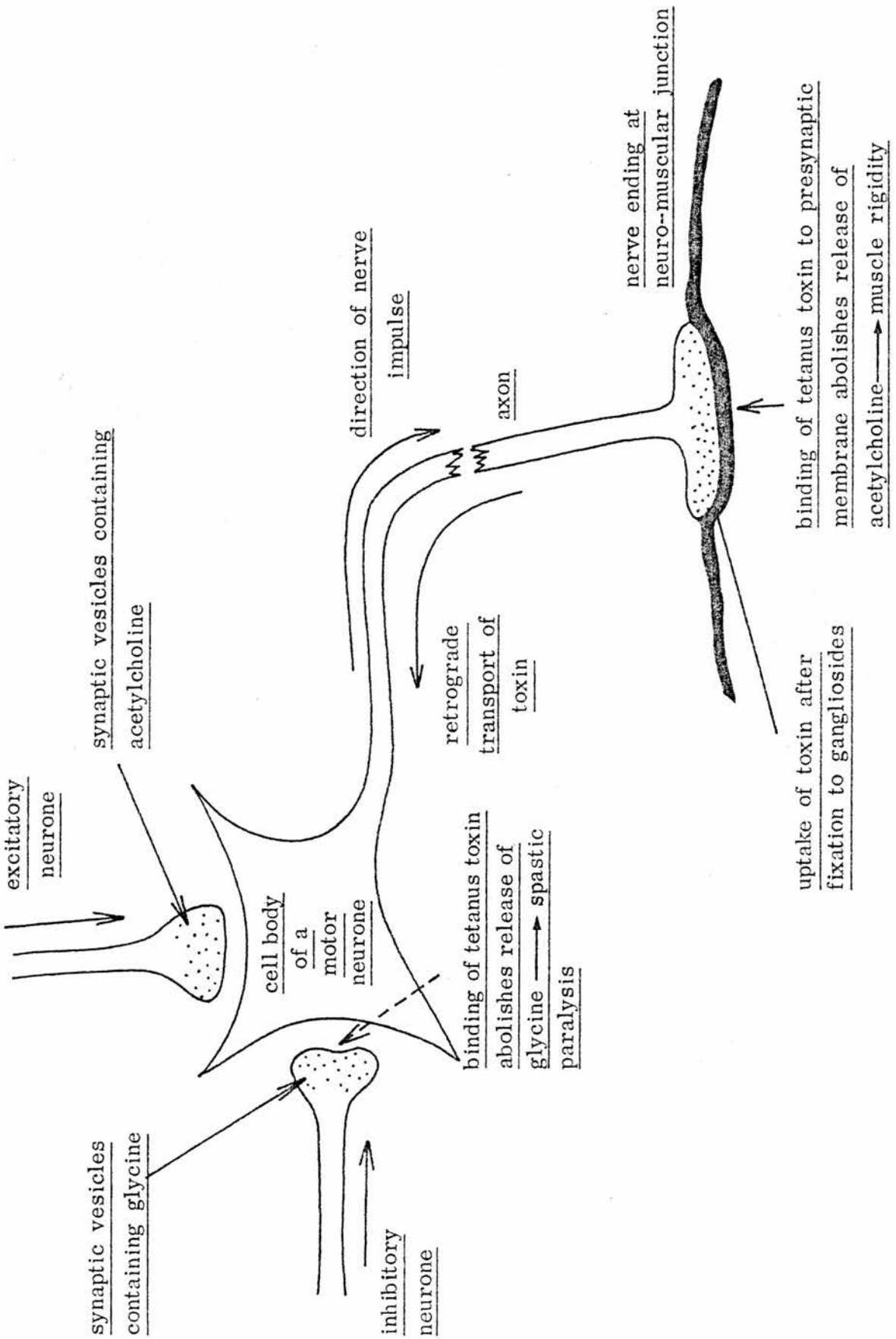
DIAGRAM 5. POSTULATED ACTIONS OF TETANUS TOXIN

In a normal muscular movement the brain sends nerve impulses to the muscles via the motor neurones to cause contraction. The nerve impulse travels due to the depolarisation of the nerve cell along the axon. Sodium ions are allowed to pass into the cell making it more positive on the inside as compared to the outside. Normally Na^+ are kept out by an active process involving an Na^+/K^+ ATPase. When the impulse reaches a nerve ending, the pulse crosses the synaptic cleft via a chemical messenger, the release of an excitatory neurotransmitter such as acetyl choline. The neurotransmitter is normally stored in synaptic vesicles, upon depolarisation an uptake of Ca^{2+} occurs, which possibly binds to calmodulin, and with an increase in the concentration of cAMP causes the activation of a protein kinase which in turn phosphorylates two proteins causing the release of the neurotransmitter, Marx (1980). The neurotransmitter crosses the synaptic cleft and binds to a receptor on the post-synaptic membrane causing a depolarisation effect in the axon, possibly via a cAMP mediated phosphorylation process. The depolarisation effect passes down the axon to the nerve ending as a nerve impulse.

In inhibitory neurones the depolarisation causes a release of a neurotransmitter at a nerve ending; but this time an inhibitory one such as glycine or GABA. Inhibitory neurotransmitters are normally released due to messages from the brain to stop unnecessary motor impulses. Binding of an inhibitory neurotransmitter to a motor neurone causes a hyperpolarisation, there is an increase in the permeability of the nerve cell to Cl^- and not Na^+, thus the interior of the cell becomes more negative. The hyperpolarisation stops any depolarisation of the motor neurone that may be occurring via excitatory neurones. Thus the inhibitory neurones are able to control the excitatory messages to muscles via the motor neurones, abolishing unnecessary and involuntary contractions.

From the diagram one can see that tetanus toxin works by abolishing the action of inhibitory neurones allowing uncontrolled impulses from the excitatory neurones. In some cases tetanus toxin may work by blocking the release of the neuromuscular transmitter that conveys the message from the nervous system to the muscle.

DIAGRAM 5.


excitatory neurone
synaptic vesicles containing acetylcholine
direction of nerve impulse
axon
nerve ending at neuro-muscular junction
cell body of a motor neurone
retrograde transport of toxin
binding of tetanus toxin abolishes release of glycine ⟶ spastic paralysis
binding of tetanus toxin to presynaptic membrane abolishes release of acetylcholine ⟶ muscle rigidity
uptake of toxin after fixation to gangliosides
synaptic vesicles containing glycine
inhibitory neurone

Ramos et al. (1979) prepared synaptosomes isolated from guinea pigs' brains and showed by using the 'lipophilic ion' tetraphenylphosphonium (TPP^+) in the presence and absence of tetanus toxin that the toxin caused an enhanced uptake of TPP^+ into the synaptosomes. They concluded that tetanus toxin caused hyperpolarisation of the synaptosomes (an increase in Cl^- uptake, to increase the negative potential inside the synaptosomes) opposing the depolarisation effect required for the release of neurotransmitters.

Wendon (1979) showed that tetanus toxin had no effect on the entry of Ca^{2+} into cells, concluding that tetanus toxin may act on transmitter release after entry of extracellular calcium ions into the nerve terminal. This was shown to also occur for the inhibition of acetyl choline release by botulinus toxin, but here it was postulated that the botulinus toxin probably interfered with intracellular Ca^{2+}, (Collingridge et al., 1980).

Thus the mechanisms of how tetanus toxin inhibits the release of neurotransmitters is not yet known though evidence is slowly pointing towards some sort of membrane potential effect or the interference of Ca^{2+} and cAMP mediated reactions.

1.2.6. Comparison of Tetanus Toxin to Other Bacterial Toxins

Tetanus toxin can be compared to several other bacterial toxins on the basis of similarities between structure and the nature of the receptors. Van Heyningen (1973) compared tetanus toxin to cholera toxin because both toxins affect secretory processes across cell membranes. Tetanus toxin abolishing neurotransmitter release in nerve terminals and cholera toxin causing electrolyte and water losses from the epithelial cells of the small intestine. The structure of the two toxins is very different, namely two chains for tetanus toxin termed H and L, and two different subunits for cholera toxin; five identical B-subunits (choleragenoid) and one A-subunit, the A-subunit is composed of two polypeptide chains A_1 and A_2 (van Heyningen, 1977; Lai, 1980). Both toxins contain specific parts namely H-chain in tetanus toxin and B-subunit in cholera toxin, responsible for binding to their receptors, specific gangliosides. The A-subunit of cholera toxin is the active moiety and the L-chain has been suggested to be the active part for tetanus toxin though this has yet to be proven.

Mellanby et al. (1973) compared tetanus toxin with botulinus toxin because of the similarities in the actions of both toxins, namely the ability to inhibit pre-synaptically the release of neurotransmitters. There is also some structural analogy between the two toxins (see Table 3) as described by Sugiyama (1980).

Tetanus toxin can also be compared to the glycoprotein hormones, especially TSH, because of their bi-partite structure and the fact that glycoprotein hormones may work by some membrane effect, (van Heyningen, 1977 and Ledley et al., 1977).

Tetanus toxin can be compared to diphtheria toxin on a structural basis. Diphtheria toxin is composed of two subunits linked by a disulphide bridge, an A-subunit which is the active moiety and a B-subunit required for toxicity but atoxic by itself when assayed in cell lysates. The B-subunit probably interacts with the cell membrane; but not with gangliosides. Diphtheria toxin like tetanus toxin is synthesised as a single polypeptide and must be nicked by a protease to form an active molecule, (van Heyningen, 1976b). See Tables 3 and 4 for the comparison of tetanus toxin with other toxins.

1.2.7. Comparison of Tetanus Toxin with Other Neurotoxins

Other toxins not of bacterial origin have been shown to have similar effects as tetanus toxin. Taipoxin and notoxin, polypeptides from the venom of two Australian elopidae are potent blockers of neuromuscular transmission, by the pre-synaptic inhibition of acetyl choline. It appears that taipoxin and notoxin cause an interference in the recycling of synaptic vesicles, possibly inhibiting the reformation of vesicles from the cell membrane by endocytosis, (Thesleff, 1977).

Other neurotoxins such as Scorpion toxin, tetrodotoxin, saxitoxin and Sea anemone toxin II affect sodium channels. It was found that Sea anemone toxin II caused a 35-fold increase in the levels of cGMP when used to activate the sodium channel, in primary nerve cell cultures obtained from mice. Tetanus toxin was shown to have no effect on the cyclic nucleotide levels in the same system, Ahnert et al. (1979), though it was shown earlier that cGMP can be used to reverse the action of tetanus toxin, see section 1.2.5.2.

TOXIN	SUB-UNITS	BINDING PROPERTIES	ACTIVE MOIETY
CHOLERA	A = 27K 5 x B = 56K	B ⟶ Gangliosides	A
DIPHTHERIA	A = 21K B = 41K	B ⟶ Phospholipids ? (not a ganglioside)	A
TETANUS	H = 100K L = 50K	H ⟶ Ganglioside	L?
BOTULINUS A, B, F	100K 50K	Does not bind to gangliosides	?
D	110K 60K		
E	150K		
ABRIN	A = 30K B = 35K	B ⟶ Ganglioside/ Glycoprotein	A
RICIN	A = 32K B = 34K	B ⟶ Ganglioside/ Glycoprotein	A
GLYCOPROTEIN HORMONES	α β	β ⟶ Ganglioside? Glycoprotein	α

TABLE 3. A STRUCTURAL COMPARISON OF TETANUS TOXIN TO OTHER BACTERIAL TOXINS, TWO PLANT TOXINS AND GLYCOPROTEIN HORMONES

TOXIN	SITE OF ACTION	MECHANISM OF ACTION
CHOLERA	Intestinal lumen	ADP-Ribosylation of GTPase in adenyl cyclase system → ↑cAMP → loss of water.
DIPHTHERIA	Any cell death due to susceptability of heart cells	ADP-Ribosylation of EF2 Inhibiting protein biosynthesis.
TETANUS	CNS/neuromuscular junction	Inhibition of the release of the inhibitory neuro transmitter Glycine ⟶ spastic paralysis.
BOTULINUS	Peripheral nervous system at the neuromuscular junction	Prevents the release of acetylcholine stopping neuromuscular transmission ⟶ flaccid paralysis.
ABRIN and RICIN	Any cell	Modifies the 60S ribosome interfering with the GTPase site blocking the binding of EF2 ⟶ inhibition of protein biosynthesis.
GLYCOPROTEIN HORMONES	Target tissue	Activation of adenylate cyclase.

TABLE 4. A COMPARISON OF THE MECHANISMS OF ACTION OF TETANUS TOXIN, OTHER BACTERIAL TOXINS, TWO PLANT TOXINS AND GLYCOPROTEIN HORMONES

1.3. The Treatment of Tetanus

1.3.1. Treatment by the Use of Drugs

The treatment of tetanus can be divided into three main types:-

a) to interrupt the invasion of the toxin via Cl. tetani.

b) to neutralise the toxin on its way from the bacterium to the CNS.

c) to suppress the effect of the toxin having reached the CNS.

Initially the treatment required is to clean the wound; as mentioned Cl. tetani cannot grow in a clean healing wound. Antibiotics such as penicillin are administered to prevent the growth and multiplication of the bacilli.

In the second type of treatment the main course of action is the administration of anti-toxin. Originally horse anti-toxin was used but it has now been replaced by human antitoxin. The horse anti-toxin often caused serum sickness and anaphylactic shock because of the sensitivity of the patient to the presence of horse protein. Many people died due to the administration of horse anti-toxin used to prevent tetanus rather than from the actual tetanus. Once the tetanus toxin has left the bloodstream either by being adsorbed to its receptor or absorbed into nerve cells it can no longer be neutralised by anti-toxin.

In the third type of treatment a number of drugs can be employed to counteract the symptoms of tetanus allowing the toxin to be eventually destroyed by protein turnover processes. The convulsions and spasticity result from exaggerated reflex responses to afferent simuli as a result of the dis-inhibition of central inhibitory processes, these may be controlled by drugs acting at different sites along the reflex pathways:-

i) Hypnotics and sedatives reduce the effect of sensory input and general state of excitability in the CNS, eg. barbiturates or paraldehyde.

ii) General anaesthetics - produce widespread suppression of the CNS.

iii) Centrally acting muscle relaxants which depress reflex activity and reduce motor output from the CNS. Drugs available here are the phenothiazines, eg. chlorpromazine and the benzodiazerpines such as diazepam (valium).

The benzodiazerpines, mainly diazepam, are used in many early cases of tetanus as they do not induce unconsciousness in a patient and probably exert their effect by reducing impulses in both the α and γ motor neurones.

iv) Peripherally acting muscle relaxants, the neuromuscular blocking agents such as tubocurazine and gallamine.

For the case of a patient suffering from tetanus all three groups of treatment are usually employed. The drugs used depend upon the state of the tetanus. In severe tetanus the only course of treatment may be the use of muscle relaxants, where the patient has to be maintained on life support systems because of the relaxation of the muscles involved in respiration. Recovery in developed countries with well-equipped hospitals is very good. Unfortunately in less developed countries the survival rate depends on the speed of treatment and the facilities available. See Adams et al. (1969), Rothstein and Baker (1978) and Habermann (1978) for the different methods of treatment available.

1.3.2. Prevention of Tetanus by Immunization

The most effective way of preventing tetanus is by active immunization, where antibodies are elicited against tetanus toxin. Tetanus toxin cannot be used directly due to its toxicity being much greater than its immunogenicity. The toxic activity can be destroyed by the 'toxoiding' of the toxin with formaldehyde, which produces an atoxic derivative of the toxin which still retains its immunogenicity. The course for immunization usually takes the form of an intramuscular injection of tetanus toxoid followed by two boosters after six weeks and six months. Boosters should then be given every five to ten years to maintain a high titre of antibodies. People who have been immunized against tetanus are usually protected against any normal levels of tetanus toxin, (Rothstein and Baker, 1978).

1.4. Purpose of the Work

There have been many investigations into the structure of tetanus toxin over the last twenty-years, leading to many conflicting results about various aspects of the structure of tetanus toxin. It appears that the earlier workers may have used toxin containing other proteins, that at the time of their investigations would not have been detectable with the techniques available. One of the aims of this project was to re-investigate certain chemical aspects of the toxin using toxin purified to certain criteria, as described in Section 4. One of the main techniques used to investigate the purity of the toxin was SDS-PAGE, which is able to detect very low concentrations of contaminating proteins. The various chemical aspects investigated were the amino acid composition of tetanus toxin, the number and identification of N-terminal amino acid residues and the number of cysteine and cystine residues.

The physiological action of tetanus toxin is partially understood, as described earlier in Section 1., but the molecular action of the toxin is still unknown. To be able to understand the molecular action of the toxin one needs to have some information about the structure of the toxin at the chemical level and about how various parts of the molecule are associated with each other. At the start of the work for this thesis two different models for the structure of the toxin molecule had been suggested, as described in Section 4, and it was one of the aims of the project to try and elucidate which of the two models was consistent with the results of the various structural investigations. It was also hoped to be able to compare the possible structure of tetanus toxin with the structures of other bacterial exotoxins and ascertain whether there were any analogies between the various toxins with the possibility of trying to find possible molecular actions of tetanus toxin.

Experiments were carried out to try and purify the constituent chains of tetanus toxin and to see how they related to the overall structure of the toxin molecule and how they fitted the proposed models of the structure of tetanus toxin. Other experiments were carried out to try and see how the two chains are related to each other and to investigate whether they are hydrophilic or amphiphilic. Experiments were carried out to see how the two chains

behaved in the "native" structure of tetanus toxin after the fixation of the toxin molecule to one of the proposed membrane receptors, ganglioside SGGnSSLC (G_{T1}). It was also hoped to be able to try to relate some of the structural information to some of the roles in proposed molecular actions of the toxin molecule.

SECTION 2.

MATERIALS AND METHODS

2. MATERIALS AND METHODS

The sources of the chemicals varied, and are listed in the Appendix. The Tris used throughout the thesis was Trizma base obtained from Sigma. The methods listed below are general; the more specialised methods are described in more detail in their specific Results chapter.

2.1. Preparation of Buffers

All buffers contained chemicals of analar grade unless otherwise stated. In some cases (e.g. guanidine hydrochloride) chemicals were purified by recrystallisation and the purity checked by determining the melting point. Buffers containing urea (BDH) usually had Tris present to help remove any cyanate ions (Hirs, 1967b). The buffers containing urea of a concentration equal to or greater than 4M were filtered through glass fibre filter papers to remove any insoluble material, a step found necessary when they were to be used for column chromatography. The urea used was purchased from BDH and was found to contain a high proportion of insoluble material. Another source of urea (Koch Light) was tried and found to be worse: it required de-colourisation by activated charcoal.

The pH of buffers was adjusted at room temperature. In most cases they were made 0.01% (w/v) in NaN_3 to prevent the growth of bacteria. Most of the column buffers were stored at $4^{\circ}C$. Buffers used at room temperature were usually made fresh or in the case of electrophoresis buffers, made at high concentration, and diluted before use. Phosphate buffers were made up by mixing the necessary quantities of 0.5M sodium dihydrogen phosphate and 0.5M disodium hydrogen phosphate solutions to give the required pH.

2.2. Preparation of Dialysis Tubing

All dialysis tubing was boiled in either 7% acetic acid or 50mM EDTA for 5 min to remove any plasticisers. After this treatment, the tubing was thoroughly washed in distilled water and washed in the dialysis buffer for a few minutes.

2.3. Preparation of Columns for Column Chromatography

2.3.1. The Types of Gel Matrices Used

Three main types of gels were used; the first type was the polyacrylamide - agarose based gels produced by LKB or the similar dextran- N,N' -methylene bisacrylamide cross-linked gels produced by Pharmacia, namely the Ultrogels and Sephacryls respectively. The second type of gel used was the cross-linked dextrans produced by Pharmacia, namely the Sephadex gels. The third type of gel used was a DEAE ion exchange resin produced by Whatman, namely DE-52.

The Ultrogel or Sephacryl and Sephadex type gels were used for gel permeation or exclusion chromatography, either for the separation and purification of proteins, using Ultrogel or Sephacryl, or for the separation of macromolecules from small molecules (desalting) using the Sephadex type gel. Two types of Ultrogel were used, namely AcA 44 which has an exclusion limit of 200 000 daltons and AcA 34 which has an exclusion limit of 750 000 daltons, for proteins. Both were received pre-swollen in a solution containing an antibacterial agent, and were stored at $4^{o}C$. The Sephacryl gel used was S-200 superfine, which has an exclusion limit of 250 000 daltons for proteins. It was also supplied pre-swollen and stored at $4^{o}C$. The Sephadex gel used was G-25 fine, which had to be swollen and de-fined for use. Other types of Sephadex gels were not used because the acrylamide-based gels were preferred. The higher chemical and physical stabilities of the acrylamide-based gels enabled one to use faster equilibration times with constant flow rates. The ion-exchange resin used was an anionic type, supplied as a pre-swollen powder requiring de-fining.

The Ultrogel and Sephacryl gels required little preparation. The gels were washed once and then diluted to twice the column volume, and degassed. Whatman DE-52 was made into a slurry using the appropriate buffer and de-fined by repeated decanting. Once the fines were removed the gel was degassed and immediately poured into the column. The Sephadex G-25 was swollen by adding $6cm^{3}$ of buffer per 1g of gel and either boiled on a water bath for one hour or allowed to swell at room temperature for three hours. After the swelling process, the gel was de-fined by repeated decantation, and degassed for use. Any excess gel was stored at $4^{o}C$.

2.3.2. Pouring of Columns

In all cases the manufacturer's recommended flow rates were used in order to prevent overpacking of the columns which causes reduced or inconsistent flow rates. Sephadex G-25 is assumed to consist of rigid spheres capable of withstanding high flow rates, therefore was immediately poured into the column and allowed to pack down to the desired height with continuous topping up to prevent layering. If the flow rate dropped, it was increased by merely increasing the head height of the buffer reservoir without any detriment to the gel matrix. Gel filtration columns using either Ultrogel or Sephacryl required more carefully controlled packing, though in contrast to Sephadex gels greater than G-50, the gels could be packed at greater flow rates than those required for high resolution during chromatographic runs. To pack columns with Ultrogel or Sephacryl; a gel reservoir, consisting of a filter funnel sealed to the top of the column with parafilm and supported by a retort stand and clamp, was used. All the degassed gel slurry was poured down the side of the column and the excess into the funnel. The slurry was allowed to settle for 10 mins before the bottom of the column was opened to give the required flow rate and allow the gel to pack. In all gel exclusion chromatographic runs the buffer reservoir was a Mariotte flask. The head height was determined by the distance between the bottom of the column and the bottom of the inlet tube of the Mariotte flask, which gave a constant flow rate even though the level of the buffer dropped. All columns run in this manner had safety loops, where the tubing from the Mariotte flask to the top of the column hung below the bottom of the column, so that the column could not run dry even if the buffer supply was exhausted. The buffers used in ion exchange chromatography were pumped onto the column at a constant flow rate, using a peristaltic pump.

2.3.3. Storage of Columns

All columns except the Sephadex G-25 columns were run and stored at $4^{o}C$ using buffers containing 0.01% NaN_3 (w/v) as an antibacterial agent. The Sephadex G-25 columns were usually run at room temperature and if not in use for any length of time were also

stored at 4°C in the presence of 0.01% w/v NaN_3. All the columns were washed with 2M NaCl after use to remove any adsorbed protein, and then washed with appropriate buffer for storage.

2.4. Recrystallisation of Chemicals

2.4.1. Guanidine Hydrochloride

Commercial guanidine hydrochloride (GuHCl) even at analar grade contains a high proportion of impurities and the better aristar grade is extremely expensive. A recrystallisation method described by Nozaki (1972) was used to purify commercial technical grade GuHCl. 100g of GuHCl was dissolved in 130 cm^3 of re-distilled or analar methanol (MeOH) by heating on a steam bath. The dissolved GuHCl gave a yellow-coloured solution which was decolourised by adding freshly activated Norit GSX charcoal. The charcoal was filtered from the GuHCl solution with glass fibre filter paper. The GuHCl was recrystallised by cooling the MeOH/GuHCl solution in an acetone dry ice mix for six hours. The white crystals were then filtered from the methanol, and washed with 50 cm^3 of MeOH previously cooled in a mixture or dry ice and acetone. The GuHCl was then dried in a desiccator under vacuum. The final yield was about 50%.

2.4.2. Iodoacetic Acid

Iodoacetic acid (IAA) tends to absorb water from the atmosphere and break down releasing iodine. Thus samples of commercial IAA tend to contain a low proportion of IAA with a high concentration of iodine present. The technical grade of IAA obtained from BDH was found to be dark brown in colour and was recrystallised using n-heptane. A 10g sample of IAA was dissolved in 20 cm^3 of n-heptane by heating on a steam bath. The solution was then filtered through a hot filter funnel under vacuum, to remove any insoluble material. The IAA was then recrystallised by cooling the solution on ice, leaving a Mother Liquor which was usually purple because of the dissolved iodine. The white crystals of IAA were then filtered off and recrystallised once again. At the end of the second recrystallisation, the IAA was washed with

ice cold n-heptane and dried in an evacuated desiccator placed in the dark. The IAA was then stored in dark bottles in a desiccator at 4°C. Yields of about 40% were usually obtained. The melting point was 81-82°C. (Literature value: 82-83°C, Merck Index, 1976).

2.4.3. Magnesium 8-anilino-1-naphthalenesulphonate (Mg^{2+}-ANS)

The magnesium salt of 8-anilino-1-naphthalenesulphonic acid (ANS) was required for the fluorescent staining of proteins in polyacrylamide gels and was prepared from the ammonium salt according to the method of Hartman and Udenfriend (1969). About 10g of NH_4^+- ANS was dissolved in boiling water containing some Norit GSK activated charcoal, and then filtered to remove the charcoal. Excess saturated $MgCl_2$ was slowly added to the ANS solution kept constantly boiling. The solution was then cooled in ice, which caused the crystallisation of thin green needle-like crystals of Mg^{2+}- ANS. The Mg^{2+}- ANS was then filtered off and recrystallised twice from an aqueous solution. After the final recrystallisation, the crystals were washed with ice-cold water and dried in an evacuated desiccator.

2.4.4. Cholic Acid

The cholic acid was dissolved in a hot solution of 30% ethanol in water (v/v), which produced a yellow-coloured solution. The solution was decolourised by adding freshly activated Norit GSX charcoal and a little celite. The solution was then filtered through a hot Buchner filter funnel; the celite stopped particles of carbon blocking the filter paper. The cholic acid was recrystallised by cooling the solution in ice, washed with an ice-cold 30% ethanol solution, and dried in an evacuated desiccator. If sodium cholate was used it was first acidified with HCl, as the sodium salt is very soluble in water.

2.5. Polyacrylamide Gel Electrophoresis

In all cases the solutions required were made as stock solutions and stored at room temperature unless otherwise stated. The exception was ammonium persulphate (APS, BDH) which was prepared

fresh. The acrylamide and N,N'-methylene bisacrylamide (bis-acrylamide) were purchased from BDH of the grade "especially purified for gel electrophoresis" and were not purified further. Stock solutions of acrylamide contained the appropriate amounts of acrylamide and bis-acrylamide were filtered through Whatman No.1 filter paper to remove any insoluble material. The acrylamide stocks were stored in dark-brown bottles at 4°C. See Tanaka (1981) for polymerisation reactions.

2.5.1. SDS Polyacrylamide Gel Electrophoresis Systems

Two main types of SDS systems were used; the sodium phosphate method by Weber and Osborn (1969) and the Tris-glycine discontinuous system of Laemmli (1970).

2.5.1.1. Weber and Osborn System

Stock Solutions

Stock acrylamide solutions contained 22.2% (w/v) acrylamide and 0.6% (w/v) bis-acrylamide in distilled water. The gel buffer consisted of 0.14M Na_2HPO_4, 0.056M NaH_2PO_4 and 0.2% w/v SDS at pH 7.0. The electrode buffer was a 1:1 dilution of the gel buffer.

Production of 10% Cross-Linked Polyacrylamide Gels

In most cases 10% acrylamide gels were used for this system; gels containing less acrylamide were made by simply adjusting the quantities of the appropriate stock solutions. The gels were prepared by mixing stock solutions as follows:-

		Final Concentrations
Acrylamide stock	$6.75cm^3$	10% : 0.27% (w/v)
Gel buffer	$7.5\ cm^3$	0.1M phosphate, 0.1% (w/v) SDS
APS $14mgcm^{-3}$	$0.75cm^3$	0.07% (w/v)
TEMED	$0.025cm^3$	0.17% (v/v)

All the above components were mixed except for the TEMED (BDH) and then degassed; TEMED was then added to start the polymerisation of the gels. Chromic acid washed tubes of 8cm by 0.5cm

were sealed at the bottom with suba-seals (small rubber bungs with a hole small enough to seal the glass tubes), filled with the polymerising gel solution to about 1cm from the top and overlayed with a saturated solution of butan-2-ol to produce a flat gel surface (Neville, 1971). The gels were allowed to polymerise, for about 20 mins, when a phase difference between the gel and the butanol appeared. The top of the gel was thoroughly washed and overlayed with electrode buffer until it was to be used.

Sample Preparation and Running

Protein samples of up to $0.1cm^3$ (30 µg) were used and made 1% (w/v) with SDS (BDH, specially purified for biochemical work), 1% (v/v) with 2-mercaptoethanol (2-ME, BDH) and 0.001% with bromophenol blue (BPB, BDH). The samples were then put in a boiling water bath for 3 mins to ensure that the proteins were fully denatured and reduce any proteolysis (Pringle, 1970) and, a drop of glycerol (BDH) was added to make the solutions more viscous so that they did not diffuse into the buffer. After the samples had cooled they were layered on top of their appropriate gel, already placed in the electrophoresis apparatus; with the bottoms immersed in electode buffer connected to the anode (+), and the tops below the surface of the upper electrode buffer connected to the cathode (-). The samples were electrophoresed at 8mA per gel until the BPB dye front reached the bottom of the gels, which usually took five hours at room temperature. The gels were removed from the tubes by inserting a hypodermic syringe needle between the gel and the glass tube and lubricating the gel with water. The loosened gel could then be gently eased out by the use of a pipette teat. The position of the BPB was marked by injecting Indian ink at the front of the dye band.

Staining and Destaining

The gels were stained in a 0.25% (w/v) solution of Coomassie brilliant blue R-250 (CBB, Sigma) in 45% (v/v) methanol, 10% (v/v) acetic acid and 45% water for 1h at $55^{o}C$. The CBB solution was prepared by dissolving the CBB in the methanol with continuous stirring followed by the acetic acid and made up to

volume with distilled water. The solution required filtering through Whatman's No.1 filter paper before use because of undissolved CBB. The gels were electrophoretically destained in 7.5% (v/v) acetic acid, 5.0% (v/v) methanol and 87.5% distilled water for 1h, by transverse electrophoresis.

2.5.1.2. Laemmli System

Stock Solutions

Stock acrylamide solutions contained 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide in distilled water. This system is discontinuous and therefore requires three different types of buffer systems. The stock separating gel buffer consisted of 1M Tris-HCl and 5.35mM EDTA (disodium salt, BDH) at pH 8.8. The stock stacking gel buffer consisted of 1M Tris-HCl and 16mM EDTA at pH 6.8. The electrode buffer was made up as a ten times concentrated solution to help preserve it against the growth of bacteria and for ease of handling. It consisted of 0.248M Tris, 1.92M glycine, 1.0% (w/v) SDS and 20mM EDTA at pH 8.3, and was diluted 1:9 just before use. The EDTA was used to remove any contaminating heavy metal ions which may interfere with polymerisation and cause aggregation of proteins, 2mM was used throughout a gel system as described by Studier (1973) and Douglas & Butow (1976).

Production of Gels

Three types of gel were produced: a 20% spacer gel, a separation gel (of different strengths), and a 3.7% stacking gel. The 20% spacer gel consisted of:-

		Final Concentrations
Stock acrylamide solution	$8.35cm^3$	20% : 0.53% (w/v)
1M Tris-HCl, 5.35mM EDTA pH 8.8	$2.32cm^3$	0.184M Tris-HCl 1mM EDTA
10% (w/v) SDS	$0.17cm^3$	0.13% (w/v)
APS $30mgcm^{-3}$	$0.75cm^3$	0.18% (w/v)
Water	$1.00cm^3$	
TEMED	$0.01cm^3$	0.08% (v/v)

The separating gel consisted of various percentages of acrylamide:-

% Acrylamide Stock soln. (cm^3)	4%	5%	6%	8%	10%	12%	Final Conc.
Acrylamide	6.67	8.33	10.0	13.35	16.7	20.2	
1M Tris-HCl,)	18.75	18.75	18.75	18.75	18.75	18.75	0.375M
5.35mM EDTA pH 8.8)							2mM
10% (w/v) SDS	0.50	0.50	0.50	0.50	0.50	0.50	0.1%
3% (w/v)polyacrylamide	8.4	8.4	8.4	8.4	8.4	8.4	0.5%
Water	14.63	12.97	11.3	8.0	4.6	1.5	
APS 30mgcm^{-3}	1.0	1.0	1.0	1.0	1.0	1.0	0.06%

The gels were polymerised, by the addition of 0.03cm^3 TEMED (0.06% (v/v)), for 30 mins. The polyacrylamide was obtained from BDH and consisted of polymerised acrylamide of molecular weight greater than 5,000,000 daltons without any cross-linking. It was used to strengthen the gels and stop any cracking during the drying down process as described by Douglas and Butow (1976). The stock solution of polyacrylamide also contained 0.1% (w/v) NaN_3 to stop any bacterial growth. The stacking gel contained 3.7% acrylamide (final concentration) and consisted of:-

		Final Concentrations
Stock acrylamide	2.6cm^3	3.7% : 0.01% (w/v)
1M Tris-HCl 16mM)	2.66cm^3	0.125M Tris-HCl
EDTA pH 6.8)		2mM EDTA
10% (w/v) SDS	0.22cm^3	0.1% (w/v)
Water	15.28cm^3	
APS 30mgcm^{-3}	0.5cm^3	0.075% (w/v)
TEMED	0.014cm^3	0.066% (v/v)

The gels were prepared by a method modified from the one described by Laemmli (1970). A cassette for the gel was prepared by covering the outer edges of two Perspex strips to a depth of 0.25cm with vacuum grease (Ames, 1974). The two Perspex strips were then sandwiched between two glass plates, one measuring 20cm by 15cm and the other 18cm by 15 cm, and held together with four clips. The bottom of the cassette was then placed in an aluminium foil boat into which the 20% acrylamide solution was poured. The acrylamide solution

was taken up into the cassette to depth of 1cm by capillary action and set after 5 mins, sealing the bottom of the cassette. The separation gel was then poured into the cassette to a depth of 14cm, overlayed with butan-2-ol (Neville, 1971), and allowed to set for 30 mins. When the gel had set, the butan-2-ol was washed off with copious amounts of water. If the gel was to be run the following day, it was overlayed with buffer of the same concentration and pH as the separation gel. Normally gels were run the same day, and after the removal of the butan-2-ol, the stacking gel was poured to the top of the smaller plate, i.e. a depth of 3cm. As soon as the gel solution had been placed into the cassette the sample comb was inserted into the solution, giving either a continuous slot or 9, 10, 13, 16 sample wells. In cases where the gel was left overnight, the buffer was poured away and the top of the gel layered with a weak solution of sodium dithionite to remove any excess oxygen, which can give rise to poor adhesion between the separation gel and the stacking gel, this could make the two gels tear apart when the sample comb is removed. The dithionite was then washed away and the gel treated as above. The sample comb was carefully removed and the sample wells washed with water to remove any unpolymerised acrylamide. The gels produced for analytical purposes were 2mm thick and those for preparative work were 4mm thick.

The cassette was then clamped to the electrophoresis apparatus described by Studier (1973) with the smaller plate facing the apparatus. The buffer reservoirs filled and the samples loaded into their specific well. The samples were then electrophoresed from the cathode (-) to the anode (+) at a constant voltage of 50V overnight, at room temperature. The current was initially at 70mA and usually dropped to 20mA at the end of a run. Running at constant current caused a rise in voltage altering the rate of electrophoresis so was not used.

Preparation of Samples

The dissociation buffer was made as a four times concentrated solution consisting of 0.25M Tris-HCl, 8% (w/v) SDS, 8mM EDTA and 40% (v/v) glycerol at pH 6.8. Samples containing 20-30 µg of protein were diluted to 0.05cm^3 with water. 0.025cm^3

of dissociation buffer, 0.012cm^3 of 2-ME and 0.013cm^3 0.02% (w/v) BPB wereadded to the samples, and they were boiled for 3 min.

Fixing, Staining and Destaining

After electrophoresis the gels (except for the 20% spacer gel) were removed from the cassette, and placed in fixing solution (20% (v/v) methanol and 10% (v/v) acetic acid in water) for 20 mins at 55°C. The fixing solution precipitated the polypeptides in the gel stopping any diffusion, but more importantly, removed excess SDS which can precipitate the CBB stain in the gel. After fixing the gels were stained in the same staining solution as used for Weber and Osborn system, for 15 mins at 55°C. To destain, the gels were initially immersed in the same destaining solution as described above but at 55°C for 20 mins. The destain was then discarded and fresh destain added with either a few pieces of sponge or white wool (to adsorb the stain) and further destained at 55°C. The gels were usually destained within 60 mins in the second destain solution with continuous shaking. The gels were then photographed using polaroid film, and then dried down on Whatman 3MM paper using a Bio-Rad gel drier.

2.5.2. Non-SDS Gel Electrophoresis System

The non-SDS system used was the discontinuous system described by Davis (1964), initially using tube gels but converted to a slab gel system later. Stock solutions were prepared that were either used for tube gels or slab gels.

Stock solution A : 3M Tris-HCl and 0.25% (v/v) TEMED pH 8.9.
Stock solution B : 0.5M Tris-HCl and 0.5% (v/v) TEMED pH 6.7.
Stock solution C : 28% (w/v) acrylamide and 0.74% (w/v) bis-acrylamide.
Stock solution D : 10% (w/v) acrylamide and 2.5% (w/v) bis-acrylamide.
Stock solution E : 0.004% (w/v) riboflavin.
Stock solution F : 40% (v/v) glycerol.
Stock solution G : 1.4% (w/v) APS prepared just before use.

The electrode buffer for tube gels consisted of 0.05M Tris and 0.4M glycine at pH 8.3 (pH usually without adjustment), diluted ten times

before use. The electrode buffer for the slab gel system was 0.248M Tris, 1.92M glycine and 20mM EDTA at pH 8.3, diluted ten times before use.

2.5.2.1. Tube Gel Production

The separation gel was 7% acrylamide and made up as follows:-

		Final Concentrations
Solution A	$1.5cm^3$	0.375M Tris-HCl, 0.03% (v/v) TEMED.
Solution C	$3.0cm^3$	7% (w/v) acrylamide, 0.19% (w/v)bis-acrylamide.
Solution G	$6.0cm^3$	0.7% (w/v) APS.
Water	$1.5cm^3$	

The stacking gel consisted of 2.5% acrylamide and made up as follows:-

		Final Concentrations
Solution B	$1.0cm^3$	0.0625M Tris-HCl, 0.0625% (v/v) TEMED.
Solution D	$2.0cm^3$	2.5% (w/v) acrylamide, 0.625% (w/v)bis-acrylamide.
Solution E	$1.0cm^3$	0.0005% (w/v) riboflavin.
Solution F	$4.0cm^3$	20% (v/v) glycerol.

Chromic acid washed tubes (8cm by 0.5cm) were sealed at the bottom with suba-seals and filled to 6cm with the separation gel solution overlayed with butan-2-ol and allowed to polymerise for 20 mins. After the gels had polymerised the butan-2-ol was washed away, and the stacking gel solution poured into each gel tube to a depth of 1cm, layered with butan-2-ol, and polymerised under UV light for 20 mins.

Protein samples of up to $0.1cm^3$ were diluted with an equal volume of solution F to which $0.01cm^3$ of a 0.02% (w/v) BPB solution was added. The samples were electrophoresed from the cathode (-) to the anode (+) at 1mA per gel until the sample had stacked and then electrophoresed at 2mA per gel until the dye reached the bottom of the gel. The gels then underwent the same process as the Weber and Osborn tube gels.

2.5.2.2. Slab Gel Production

The slab gels consisted of three types of gel, a 20%

acrylamide spacer gel, a separation gel of variable acrylamide content and a 3.6% acrylamide stacking gel. All the solutions contained EDTA of a final concentration of 2mM throughout the gel. Otherwise the stock solutions were the same as for the tube gels except for solution E, which was not used.

The 20% spacer gel was prepared as follows:-

		Final Concentrations
Solution C	$8cm^3$	20% (w/v) acrylamide, 0.53% (w/v) bis-acrylamide.
Solution A	$2.4cm^3$	0.643M Tris-HCl, 2mM EDTA, 0.05% (v/v) TEMED.
Solution G	$0.8cm^3$	0.1% (w/v) APS.

The separation gel was variable in its acrylamide content and prepared from one of the following solutions:-

% Acrylamide	6%	7%	8%	10%	12%	Final Conc.
Solution C (cm^3)	10.2	12.0	13.7	17.1	20.9	
Solution A (cm^3)	6.0	6.0	6.0	6.0	6.0	0.375M Tris-HCl,0.025%TEMED
3% polyacrylamide(cm^3)	8.0	8.0	8.0	8.0	8.0	0.5% (w/v).
Water (cm^3)	21.4	19.6	17.9	14.5	10.7	
Solution G (cm^3)	2.4	2.4	2.4	2.4	2.4	0.075% (w/v).

The 3.6% stacking gel was prepared as follows:-

		Final Concentrations
Solution D	$8.7cm^3$	3.6% (w/v) acrylamide, 0.9%(w/v)bis-acrylamide.
Solution B	$3.0cm^3$	0.0625M Tris-HCl, 2mM EDTA, 0.0625% (v/v) TEMED.
Solution F	$12.0cm^3$	20% (v/v) glycerol.
Solution G	$0.3cm^3$	0.02% (w/v) APS.

The gels were prepared, samples electrophoresed and gels stained as described for the Laemmli slab gels. The samples were prepared in the same way as for the tube gels.

2.5.3. Two-Dimensional Electrophoresis

The method used was that of O'Farrell (1975).

2.5.3.1. First Dimension - The Electrofocusing Gel

The first dimension gels were used to electrofocus the

proteins in the samples by relying on the fact that proteins at their isoelectric point carry no net charge, so no longer move in an electric current. The principle of the system relies in setting up a pH gradient throughout the gel through which the proteins are electrophoresed until they move to the pH of their isoelectric point and no longer move in the gel. The constituents required for three gels were:-

		Final Concentrations
Aristar urea (BDH)	1.19g	10M urea
Stock soln.of 45% (w/v)acrylamide and 0.6% (w/v) bis-acrylamide	$0.21cm^3$	4.7% : 0.06%
Nonidet P-40 non-ionic detergent 10%(w/v)	$0.4cm^3$	2% (w/v) NP-40
LKB Ampholines pH 3.5-10 40%(w/v) and pH 9-11 20%(w/v) in ratio 90:10	$0.1cm^3$	2%(w/v) ampholines
Water	$0.58cm^3$	

The urea was fully dissolved and the solution then degassed. The gel solution was polymerised by adding $0.01cm^3$ of a 10% (w/v) APS solution. (No TEMED was required as the ampholines acted as initiators of polymerisation). Glass tubes 14cm long by 0.25cm internal diameter were used and filled up to an 11cm mark with the above gel solution. The bottom of the tubes were sealed with parafilm rather than suba-seals as the suba-seals tended to exert too much suction on the gels, and introduce air bubbles when removed. The gel solution was injected into the narrow tube with a long hypodermic needle. The gel solution was then overlayed with butan-2-ol, and allowed to polymerise for 30 mins.

A sample buffer was prepared and stored at $-20^{\circ}C$. The constituents were:-

		Final Concentrations
Aristar urea	0.57g	9.5M urea.
10%(w/v) P-40 (non-ionic detergent)	$0.2cm^3$	2% (w/v) NP-40.
Ampholines ($0.045cm^3$ of a 40% (w/v) soln. pH 3.5-10 and $0.01cm^3$ of a 20% (w/v) soln. pH 9-11)	$0.055cm^3$	2%(w/v) ampholines.
2-mercaptoethanol	$0.05cm^3$	5%(v/v) 2-ME
Water	$0.3cm^3$	
BPB $1mgcm^{-3}$	$0.001cm^3$	0.1%(w/v) BPB.

An overlay solution was made up that contained 5M aristar urea and 0.4% (w/v) ampholines. There were two different electrode solutions: an acidic one comprising of 0.2% (v/v) phosphoric acid used at the anode (+), and a basic solution comprising of 0.4% (v/v) ethanolamine used at the cathode (-).

Samples to be run in the first dimension were diluted in two volumes of sample buffer up to a maximum volume of $0.1cm^3$. Normally the samples contained 30µg of protein. The butan-2-ol was washed from the tops of the polymerised gels with sample buffer and the gels mounted in the electrophoresis tank. The samples were layered on top of their appropriate gel, followed by $0.01cm^3$ of overlay solution. The cathode solution was placed in the top reservoir (causing a slight loss in the high pH end of the gradient). The samples were electrophoresed at 100V for 10 mins, then at 200V for 15 mins, then at 300V for 30 mins and finally at 400V for 12 h, all at room temperature. The current was always below 0.2mA per tube and fell during electrophoresis. After the electrophoresis, the gels were removed and either used for electrophoresis in the second dimension, or stored at $-20^{\circ}C$.

2.5.3.2. Second Dimension - SDS Polyacrylamide Gel Electrophoresis

The Laemmli discontinuous slab gel system was used, with a stacking gel 1.5cm deep reaching the top of the smaller plate. The separation gel used was 8% acrylamide. The first dimension gel was soaked for 1 h in the following buffer solution:-

		Final Concentrations
10% (w/v) SDS	$30cm^3$	3% (w/v) SDS.
2-Mercaptoethanol	$3cm^3$	3% (v/v) 2-ME
0.5M Tris-HCl pH 6.8	$10cm^3$	0.05M Tris-HCl.
Glycerol	$10cm^3$	10% (v/v) glycerol.
BPB $1mgcm^{-3}$	$0.01cm^3$	0.00001% (w/v) BPB.
Water	to $100cm^3$	

For each gel run, $2cm^3$ of the above buffer had agarose added to a concentration of 1% (w/v) (ME agarose Miles Laboratories Ltd., Lot No. 61999). The pre-soaked first dimension gel was placed on the ledge of the slab gel apparatus next to the stacking gel. The stacking

gel was then overlayed with the freshly prepared agarose solution; into which the first dimensional gel was then pushed. The tube gel and stacking gel was then covered with the rest of the agarose solution to seal the first gel in place. Once the agarose had set, the electrode buffer was added, and the sample electrophoresed as described previously.

Staining

A problem associated with the staining of the second dimension gels is the presence of the ampholines, which are also stained by CBB. For this reason the normal procedure for staining slab gels cannot be used. The ampholines were partially washed out of the gel by agitation in a solution of 25% (v/v) propan-2-ol, 10% (v/v) acetic acid and 65% (v/v) water at 55°C for 20 mins. The gels were then stained in a solution of 0.25% (w/v) CBB R-250, 0.1% (w/v) Crocein Scarlet, 0.5% (w/v) $CuSO_4$, 27% (v/v) propan-2-ol, 10% (v/v) acetic acid and 63% water at 55°C for 30 mins with continuous agitation. The $CuSO_4$ was dissolved in the above solution before the addition of the propan-2-ol and was used to prevent the staining of the ampholines (Righette and Drysdale, 1974). The gels were then destained in 0.5% (w/v) $CuSO_4$, 12% (v/v) propan-2-ol, 7% (v/v) acetic acid 81% water at 55°C with continuous agitation until they were destained.

2.5.4. Preparative SDS Polyacrylamide Gel Electrophoresis

Preparative gels were prepared according to the method of Laemmli as described earlier except that they were 4mm thick as compared to the 2mm thick analytical gels. A continuous slot was moulded into the stacking gel rather than the multi-sample type used in analytical work. The acrylamide concentration was always 6% as this gave very good separation between the polypeptides under investigation. The setting times were lengthened by decreasing the amount of APS by half, this allowed the gels to set more evenly; gels allowed to set too rapidly developed distortion lines. This was because of areas of the gel setting at different times. The use of a continuous slot allowed the sample volume to be as high as $4cm^3$ containing 5mg of protein.

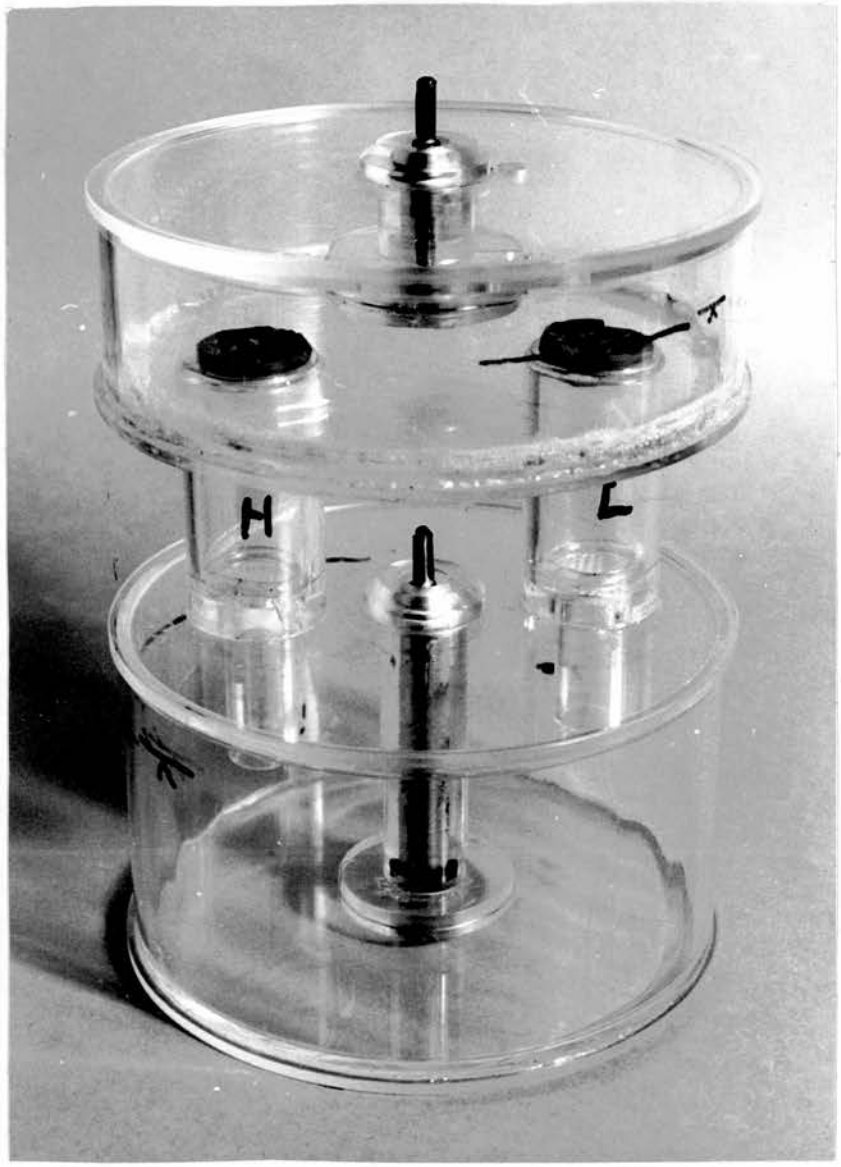
Detection of Polypeptides in Preparative Gels

Polypeptides in gels can be detected very rapidly by the immersion of the whole gel into 4M sodium acetate at room temperature, as described by Higgins and Dahmus (1979). However in these experiments it was observed that the SDS/polypeptide complex precipitated out and appeared as a white band against a clear background within 15 sec whereas Higgins and Dahmus (1979) observed clear bands containing the SDS/polypeptide complex against a white background of precipitated SDS. This could not have been because the gel was extra thick since it was also observed for 1mm thick gels. However proteins such as bovine serum albumin (BSA) gave the same result that Higgins and Dahmus (1979) observed. Thus it was concluded that the effect was associated with the nature of tetanus toxin and its chains. The precipitated SDS/polypeptide complexes were usually allowed to develop for five minutes but were observable within 15 secs. The bands were best viewed on a dark background with a light shone on them at a 45^{o} angle. The gels shrank to about a third of their size because of the loss of water into the high concentration of sodium acetate. The shrinking could be reversed by placing the gel in water.

2.5.5. Electrophoretic Elution

Preparative electrophoresis was carried out as described in section 2.5.4., and the polypeptides were identified by 4M sodium acetate precipitation. The polypeptide complexes were then electrophoretically eluted by a similar method to the one described by Stephens (1975). The polypeptides were not dansylated as suggested by Stephens (1975) as dansylated toxin was found either to aggregate or to be too hydrophobic to enter the gel.

A piece of apparatus was specially designed for eluting two samples at once (see photograph 1). The bands of polypeptide detected in the preparative gel were cut into 1cm by 1cm pieces and loaded into the appropriate sample chamber of the electrophoresis elution apparatus. The bottom of each chamber had a fresh dialysis bag attached with elastic bands and filled with Laemmli electrode buffer (Section 2.5.1.2.). The bottom of the dialysis bag was then



PHOTOGRAPH 1. ELECTROPHORETIC ELUTION APPARATUS

For a full description of its use see Section 2.5.5.

dipped into the bottom reservoir, connected to the anode of the power pack. The gel chambers were filled with electrode buffer, all air bubbles were removed, and the chambers were then sealed with rubber bungs with holes drilled through. Tightly fitting bungs were found to be necessary as the shrunken gels on swelling pushed glass wool plugs out. The top reservoir was then connected to the cathode and the proteins electrophoretically eluted from the gel at 40V for 15h. The current was observed to rise initially but then to fall. The elution of the polypeptides was observed to be completed by a change in the refractive index of the solution in the dialysis bags. The polypeptides were at the bottom of the dialysis bag.

The electrode buffer was carefully removed from above the polypeptide samples, which were in turn removed by using a long Pasteur pipette. The samples were then dialysed against 2M urea to remove as much SDS as possible without precipitating the proteins as suggested by Weber and Kuter (1971). The further purification step they suggested using Dowex AG 1-X2 was tried but without any success. Samples were then exhaustively dialysed against water before further use.

2.5.6. Limited Proteolysis in SDS Polyacrylamide Gels

Peptide mapping by limited proteolysis in SDS was carried out by a modified form of the method described by Cleveland *et al.* (1977). The modified form of the method was described by Apps and Schatz (1979).

An 8% Laemmli gel 1mm thick was initially used for the preparation of toxin and its chains. No individual sample wells were used, but a single slot in order to produce bands of material right across the gel. After electrophoresis the polypeptide bands were detected by using 4M sodium acetate (Section 2.5.4.), and not by staining the gel in CBB as suggested by Apps and Schatz (1979), to remove the chance of any acid hydrolysis. When radio-iodinated samples were used, the gel had to be stained by CBB as the 4M sodium acetate method was not sensitive enough. In fact, even the CBB method was not really sensitive enough in all cases, so the gels after fixation were stored in water, and samples from the gels autoradiographed overnight to confirm the position of the polypeptide bands.

After the polypeptide bands were detected and sliced out of the gel, the samples of interest were soaked in an eight-fold diluted solution of Laemmli's pH 6.8 stacking gel buffer (final concentration was 0.125M Tris-HCl, 0.1% (w/v) SDS and 2mM EDTA). The samples were stored at -20°C in this buffer. A Laemmli gel containing 10% acrylamide in the separation gel and 2mm thick was then prepared. Each sample well before use was filled with a four-fold diluted sample of Laemmli's stacking gel buffer, i.e. twice the normal strength as described above. A piece of preparative gel containing the appropriate polypeptide was put into each well and then pushed to the bottom taking care not to trap any air bubbles. The cassette was then clamped to the gel apparatus as described earlier, and the buffer reservoirs filled with electrode buffer. The gel slices were then overlayed with 50µl of a four-fold diluted sample of Laemmli's pH 6.8 dissociation buffer containing 20% (v/v) glycerol and 0.0001% (w/v) BPB.

The proteases used were either chymotrypsin or trypsin and were prepared as $1mgcm^{-3}$ stock solutions in 1mM HCl (pH 3). The stock solutions were stored at -20°C and diluted to give 10µl samples containing 0.01µg, 0.1µg, 1.0µg or 10µg (20µl) enzyme protein in 10% (v/v) glycerol. The 10µl protease samples were then overlayed on the 20% (v/v) glycerol layer. The samples were electrophoresed at 10mA until the dye was well into the separation gel. After electrophoresis non-radiolabelled gels were stained as described previously whereas radiolabelled gels were immediately dried down and autoradiographed.

2.5.7. Charge Shift Electrophoresis

Charge shift electrophoresis was carried out according to the method of Helenius and Simons (1977). Electrophoresis was carried out using 1% agarose gels on 7.5cm by 2cm microscope slides in a flat bed apparatus. The gel buffer consisted of 0.05M glycine-NaOH, 0.1M NaCl at pH 9.0 containing either 0.5% (v/v) Triton X100, 0.5% (v/v) Triton X100 plus 0.25% (w/v) sodium cholate (recrystallised) or 0.5% (v/v) Triton X100 plus 0.05% (w/v) cetyltrimethylammonium bromide (CTAB). The electrode buffer was the same as the particular gel buffer except when Triton X100

alone was used in the gel buffer, in which case no detergent was used in the electrode buffer. The gels had either sample wells cut in the middle or a continuous slit. (A well held about 10µl whereas a slit could hold up to 50µl). Gels were pre-electrophoresed for 15 mins at 4.5 Vcm^{-1}, and after addition of sample and 1µl of 0.02% (w/v) BPB for 5h at 4.5 Vcm^{-1}.

After electrophoresis the gels were placed in the fixer solution (described in Section 2.5. earlier) to fix the proteins, and remove excess detergent (which was found to interfere with staining). The gels were then dried down and stained in a 2% (w/v) solution of CBB R250 in methanol : acetic acid : water in the ratio 5 : 1 : 5 for 5 mins, and destained in the same solution less the CBB for 5 mins. Gels with radio-iodinated samples were immediately dried down after electrophoresis and autoradiographed.

2.6. Immunological Techniques

2.6.1. Ouchterlony Double Diffusion Plates

Double diffusion plates were prepared by the method described by Ouchterlony (1949). A 3% (w/v) solution of noble agar (Difco) was prepared in boiling water containing 0.02% (w/v) NaN_3, and then divided into $5cm^3$ samples and allowed to set. When needed the agar solution was melted in a boiling water bath, and diluted with an equal volume of 0.1M sodium phosphate, 1.8% (w/v) NaCl and 0.01% (w/v) NaN_3 pH 7.0. Five 7.5cm by 2cm microscope slides (chromic acid washed) were taken, onto which $2cm^3$ of the 1.5% (w/v) agar solution was pipetted, and allowed to set at room temperature. Once the agar had set (approximately 4 mins) the plates were placed in a sealed humid box and stored at 4^oC. Sample wells were cut with a special cutter, which produced a set of six equally spaced wells of 6µl capacity surrounding at an equal distance a seventh well also of 6µl capacity. The centre well usually contained the antibody solution, and the six wells around it had the samples containing the suspected antigen solutions. The distance between the wells was 2mm and the solutions were allowed to diffuse, interact and possibly produce antigen:antibody precipitin bands overnight at 4^oC. Precipitin bands were visible

without staining but for storage they were stained after several washes in a solution of 0.9% (w/v) NaCl and 0.02% (w/v) NaN_3 over 48 h to remove any unreacted protein. The salt solution was then washed out of the gel by soaking in water and the gel dried down. The gel was covered by a piece of Whatman's No.50 filter paper and placed in a hot oven for 1 h, for drying down. The filter paper was wetted for removal and the gel placed in a solution of CBB (prepared as described for polyacrylamide gel electrophoresis, (Section 2.5.1.1.), for 20 mins. The gel was then destained in a methanol : acetic acid : water solution in the ratio 5 : 5 : 1.

2.6.2. Two-Dimensional Immunoelectrophoresis

Two-dimensional immunoelectrophoresis was originally described by Laurell (1965, 1966) and modified by Clarke and Freeman (1968). The method described here was a further modification and improvement by Bradwell and Burnett (1975) of the method described by Clarke and Freeman (1968).

The electrode buffer contained 0.06M sodium barbitone and 0.1% (w/v) NaN_3 pH 8.6. The buffer was either made up using diethyl-barbituric acid (barbitone) which needed a few pellets of sodium hydroxide added, to produce the sodium salt, because of the insolubility of barbitone in water in the acidic form. Alternatively the buffer was made using diethyl-barbituric acid and sodium barbitone. (Sodium barbitone buffer is often referred to as veronal).

The gels for the system were made using agarose from Miles Laboratories Limited (Lot No. 61999). A 1% (w/v) agarose solution was prepared using equal volumes of water and electorde buffer plus 3% (w/v) polyethylene glycol 6000 (BDH). The agarose solution was boiled, divided into $10cm^3$ aliquots, and stored at 4^oC.

2.6.2.1. First Dimension

Molten agar solution ($2cm^3$) was poured onto chromic acid washed 7.5cm by 2cm microscope slides. After the agar had set, the slides were placed at 4^oC for 5 mins to harden the agar, each had a 2mm diameter well cut 2mm from the edge and side of the slide.

The slides were then placed in a flat bed electrophoresis tank, and connected to the electrode buffer reservoirs by paper wicks cut from Whatman's 3MM paper. A 6µl sample containing the antigen was placed into the well along with 1µl of 0.02% BPB. The samples were then electrophoresed from the cathode (-) to the anode (+) at $10Vcm^{-1}$ until the dye reached the end of the gel.

2.6.2.2. Second Dimension

The first dimension gel was removed from the microscope slide and transferred to a glass plate, 8cm by 10.5cm, with the edge containing the sample to the inside of the new plate. A $10cm^3$ aliquot of the 1% (w/v) agar was melted at 100°C and cooled to 56°C, and an appropriate amount of antibody added to it just before use. For the work carried out in this thesis a 10µl sample of a 1:10 diluted solution of tetanus antitoxin No. RX 6720/24/23 (from Dr. R.O. Thomson, Wellcome Laboratories) was found to be sufficient. Once the antibody solution had been added to the liquid agar it was mixed by inversion and the agar was poured onto the plate above the first dimensional gel taking care to make sure there was good contact between the two gels. The gel was then connected to the electrode buffer as described for the first dimensional gel, and electrophoresed from the cathode to the anode at $3Vcm^{-1}$ for 20 h.

Staining

The gel was initially soaked in 0.9% (w/v) NaCl and 0.01% (w/v) NaN_3 to remove any excess antibody and washed to remove the salt by several soakings in water. At this stage, a faint precipitin band in the shape of a rocket (characteristic of this technique) was usually visible. The gel was then dried down under a piece of Whatman's No.1 paper at room temperature overnight. The paper was removed by wetting, and the plate with the dried-down gel on, placed in a 2% (w/v) CBB solution containing methanol : acetic acid : water in the ratio 5 : 1 : 5 for 5 mins. Excess stain was washed off using water and the background of the gel destained using the above solution minus the CBB.

2.6.3. Raising of Antibodies

2.6.3.1. Toxoiding of Proteins

Although the chains obtained from tetanus toxin are supposed to be devoid of biological activity (Matsuda and Yoneda, 1975) it was decided to toxoid any fragments obtained from the toxin; for the raising of antibodies because of the toxin's very high biological activity. Toxoiding was carried out in a similar manner to that described by Helting and Zwisler (1977). The proteins were initially dialysed twice against 1M urea, 0.1M sodium phosphate and 0.01% (w/v) NaN_3 pH 7.2. The second change of buffer was then made 0.19% (v/v) with formaldehyde (37%-40% (w/v), BDH) and the samples dialysed for 72 h at room temperature. The excess formaldehyde was removed by dialysis with the initial buffer system and the samples divided into aliquots containing $1mgcm^{-3}$ of antigen. The samples were stored at $-20^{o}C$. $0.5cm^3$ samples were used for antibody production.

2.6.3.2. Injection of Rabbits

The rabbits used were two female New Zealand whites that weighed 4.8Kg and 6.4Kg. The heavier rabbit was used for raising antibodies against H-chain and the other for antibodies to L-chain. Both rabbits were intially checked for reaction against tetanus toxoid to rule out the possibility of them having any naturally cross-reacting material.

The rabbits were injected intra-muscularly with $0.5cm^3$ of material into each hind limb. The samples injected consisted of $0.5cm^3$ of toxoided protein solution and $0.5cm^3$ of adjuvant. The first injection contained Freund's complete adjuvant (Difco) which contains dried Mycobacterium butyricum cells as well as two oils. M. butyricum probably stimulates the immune system by stimulating the reticuloendothelial system possibly causing T lymphocyte proliferation and the production of B-cell stimulating factors. The presence of the oils causes an emulsion to be formed on mixing which causes the antigen to be slowly released from the small droplets, Levy et al. (1973). A second injection followed a week later, this time containing Freund's incomplete adjuvant which does not contain M. butyricum. A third injection was given

three weeks after the second injection, again containing Freund's incomplete adjuvant. Injections following the third injection always contained Freund's incomplete adjuvant and were given whenever the antibody titre seemed to fall.

2.6.3.3. Collection of Blood

The first collection of blood for the detection of antibodies followed one week after the third injection. The rabbits were placed in a constraining box, and a section of the outer part of the ear shaved for easy access to the ear vein. The ears were gently warmed by rubbing, or by shining a table lamp onto the ear. Once the ear vein had swollen owing to an increase in blood flow, it was pierced using a sterile hypodermic needle. The blood that flowed out was collected in a conical MSE bench centrifuge tube. Blood flow was continually stimulated by rubbing the ear. Samples of $1cm^3$ were collected for testing and later $20cm^3$ were collected for antibody purification.

2.6.3.4. Isolation and Purification of Immunoglobulin G (IgG)

Tetanus immunity is determined by immunoglobulin type G antibodies (Yount et al., 1968), so a general IgG purification was carried out according to the method of Kekwick (1940). The collected blood was allowed to clot for several hours at room temperature or overnight at $4^{o}C$. The clot was removed as much as possible and the rest was then centrifuged at 4,000 rev. min^{-1} for 10 mins in a MSE bench centrifuge. Small plastic beads of a density slightly lower than the density of red blood cells were added during the centrifugation step so that any non-aggregated red blood cells were pelleted below the beads, this makes it easier to remove the serum supernatant. The serum was then tested against the appropriate antigen using the double diffusion technique of Ouchterlony (1949). All sera were protected from the growth of micro-organisms by the addition of NaN_3 (0.01% (w/v)).

The IgG fraction of the serum was then partially purified by dialysing the serum initially against 18% (w/v) sodium sulphate. A second dialysis against 15% (w/v) sodium sulphate was used, both of three hours duration at room temperature. The IgG fraction

precipitated out by the above concentrations of sodium sulphate. The precipitated IgG's were then centrifuged down in an MSE bench centrifuge at 4,000 rev. min^{-1} for 15 mins, resuspended in 15% (w/v) sodium sulphate and centrifuged as above, the supernatants in each case were discarded. After the second centrifugation step the precipitated IgG's were re-dissolved in a minimal amount of water with a little NaN_3.

2.7. Amino Acid Analysis

2.7.1. Total Acid Hydrolysis

Before hydrolysis samples of tetanus toxin (3nmol) or its chains were initially dialysed against water in order to remove any salts. The dialysed samples were then placed in persulphuric acid washed hydrolysis tubes and freeze dried. The persulphuric acid was produced by adding ammonium persulphate to concentrated sulphuric acid, a method preferred to the usual chromic-acid washing process, as the glass is not etched by the persulphuric acid, and there is no deposition of chromic salts. The freeze-dried protein samples were then re-dissolved in $1cm^3$ 6M aristar HCl, made up immediately before use. The tops of the tubes were then heated in an oxygen and gas flame until the glass was pliable and the tubes gently pulled in order to form a narrow constriction at the top of the tube. The tubes were allowed to cool, and then placed in propan-2-ol at $-70^{\circ}C$, (to cool the 6M HCl and stop the sample from bumping under reduced pressure). The cooled tubes were evacuated using an oil pump to about 15mmHg, and sealed under vacuum by heating the constricted neck of the tubes in a flame.

The protein samples were then hydrolysed at $106^{\circ}C$ for either 5 h, 24 h, 48 h or 72 h; 72 h was the usual time, especially for analytical purposes. Later hydrolyses were carried out in the presence of 0.05% (v/v) thioglycolic acid in order to stop the oxidation of carboxymethyl cysteine and methionine.

After hydrolysis, the tubes were opened, and the samples dried down in an evacuated desiccator in the presence of NaOH pellets. The amino acids were dissolved in an appropriate amount of pH 2.2 citrate buffer (see below, Section 2.7.5.1.). For a review of the method see Perham (1978).

2.7.2. Performic Acid Oxidation

Performic acid oxidation was used to oxidise cysteine and cystine in the proteins to cysteic acid in order to determine the total cysteine content. Samples oxidised with performic acid gave no methionine on analysis owing to its oxidation to methionine sulphone and methionine sulphoxide. Tyrosine was partially oxidised, so its composition could not be determined correctly. Histidine could not be determined, as the oxidised products of tyrosine co-eluted with it.

Performic acid was produced by adding $0.5cm^3$ of 30% hydrogen peroxide (BDH, stored at 4^oC), to $4.5cm^3$ of 100% formic acid (BDH) and leaving at room temperature for 1 h. The performic acid was then cooled to 0^oC and 100-200µl was added to a dialysed and freeze-dried protein sample (5nmol), which was then incubated at 0^oC for 1 h. The sample was made up to $1cm^3$ with water, and frozen in liquid nitrogen for freeze drying. It was observed during the freeze drying process that the samples thawed out, because the excess formic acid lowered the freezing point of the solutions.

After freeze-drying, the samples were treated as a sample for total acid hydrolysis (Section 2.7.1.). The cysteic acid was eluted at the very start of the elution from the automatic amino acid analyser. The colour constant for apartic acid was used to determine the amount of cysteic acid present. The method was based on Hirs (1956, 1967a).

2.7.3. Determination of Tryptophan

Tryptophan cannot be determined by acid hydrolysis because of its destruction under acid conditions. To determine tryptophan, 3M mercaptoethane sulphonic acid was used according to the method of Penke *et al.* (1974). A 5.5 nmol salt-free, freeze-dried sample of tetanus toxin had $0.3cm^3$ of 3M mercaptoethane sulphonic acid (Pierce) added. The samples were hydrolysed in evacuated tubes at 106^oC for 96 h. After hydrolysis $0.6cm^3$ 1M NaOH was added, and 0.3 nmol of toxin applied directly to the amino acid analyser. The colour constant for lysine was used to determine the amount of tryptophan.

2.7.4. Detection of Carboxymethyl Cysteine

Carboxymethyl cysteine is easily detected by the use of an automatic amino acid analyser, (Spackman et al., 1958; Gurd, 1967, 1972; and Hirs, 1967b). In the normal buffer system used in the analyser at Edinburgh (see Section 2.7.5.) one could not detect carboxymethyl cysteine as it co-eluted with aspartic acid. In order to detect carboxymethyl cysteine, the pH of the first buffer was lowered from pH 3.27 to pH 3.05, and the elution time increased to 50 min. At the new pH, carboxymethyl cysteine was detected as a separate peak immediately before aspartic acid. Unfortunately the lowering of the pH caused valine and cysteine to co-elute in the standard runs and as a consequence the colour constant of methionine was used to calculate the amount of valine. Because of the susceptibility of carboxymethyl cysteine to oxidation, the hydrolysis samples were made 0.05% (v/v) in thioglycolic acid.

2.7.5. Automatic Amino Acid Analysis

A Locarte floor-standing amino acid analyser was used, with a 20cm by 1cm sulphonated polystyrene resin ion exchange column. The method used for the analysis of amino acids was based on that of Spackman et al. (1958).

2.7.5.1. Sample Buffer

All the dried-down hydrolysates were re-dissolved in an appropriate amount of sodium citrate buffer pH 2.2. The amount of sample loaded depended on the amount of individual residues in the sample. The optimal loading used in standard runs was 25nmol of each amino acid. In the case of tetanus toxin about 0.3nmol was found to give the best ratios for the residues. The loaded samples were usually of 100µl in 0.1M sodium citrate (Sigma) and 0.5% (v/v) thiodiglycol (BDH), pH 2.2. The citrate used was the monohydric acid, the solution was altered to the correct pH with 0.21M sodium hydroxide, added in the pellet form, and 0.51M aristar HCl.

For sample loading the old buffer was removed from the appropriate loading chamber, and the sample loop washed with $2cm^3$

of the above buffer. The amino acid mixture was then pushed into the loop behind the above buffer, using nitrogen and sandwiched behind with a further $1cm^3$ sample of the citrate buffer. The loading chamber was then filled with 20% (v/v) methanol pH 3.25 citrate buffer.

2.7.5.2. First Buffer

The first buffer was used to elute the acidic amino acids from the ion exchange column. The buffer was essentially citrate at pH 3.25 but as mentioned (Section 2.7.4.) the pH of this buffer had to be reduced to pH 3.05 in order to elute carboxymethyl cysteine. The buffer was run for 40 min at a temperature of $50^{\circ}C$ in order to elute the acidic amino acids. All were separated to base line (peaks with base line between) except threonine and serine which seemed to separate better in the lower pH buffer. The buffer consisted of 0.1M citrate, 0.12M NaCl and 0.5% (v/v) thiodiglycol pH 3.25, containing 6.3g Brij 35 and 30 drops of pentachlorophenol per $9dm^3$. Brij 35 was a detergent used to improve the flow rate of the column and the pentachlorophenol was a bacteriocide. The sodium chloride was produced by adding NaOH pellets (0.21M) and concentrated HCl (0.12M). The citrate was added as the monohydric acid.

2.7.5.3. Second Buffer

The second buffer eluted the neutral amino acids and was run for 44 min at a temperature of $50^{\circ}C$. The buffer consisted of 0.1M citrate, 0.055M NaCl, 0.5% (v/v) thiodiglycol pH 4.25 containing the same amount of Brij 35 as buffer 1. Again, the sodium chloride was produced by adding sodium hydroxide pellets (0.21M) and concentrated HCl (0.055M). In this buffer the citrate was added as the monohydric acid.

2.7.5.4. Third Buffer

This buffer was used to elute the basic amino acids lysine and arginine, and ammonia which was eluted between the two amino acids. Tryptophan was also eluted by this buffer but could only be detected as described above (Section 2.7.3.). The buffer was run

for 100 min at 63°C. The buffer consisted of 0.1M citrate, 0.033M borate and 0.5% (v/v) dithioglycol pH 9.35 containing Brij 35 as described above. The buffer was altered to the correct pH by adding sodium hydroxide to give 0.068M; the citrate used was the trisodium citrate dihydrate (Sigma).

The ion exchange column was then washed with 0.2M NaOH for 20 min and re-equilibrated with the first buffer for 60 min.

2.7.5.5. Detection of the Amino Acids

The amino acids eluted from the column were reacted with ninhydrin reagent at 100°C to form a blue-coloured product, diketohydrindylidene-diketohydrindamine. The amount of product was measured by its absorbance at 570nm in a photocell. Proline is not a primary amine amino acid, so reacts differently with ninhydrin. It produces a yellow-coloured product that was monitored at 440nm. All the amino acids were monitored at both wavelengths but only the respective traces at the mentioned wavelengths were used for quantitative analysis. The results of the photometric analysis were plotted directly onto logarithmic chart paper as dots at intervals of 12 seconds on each trace. Every fourth dot on each trace was black to help determine the number of dots printed. The ninhydrin reagent consisted of 2% (w/v) ninhydrin, 0.04% (w/v) stannous chloride in 75% (v/v) methyl cellosolve 25% (v/v) 4M sodium acetate pH 5.5. The ninhydrin reagent was prepared by initially dissolving 1Kg of sodium acetate trihydrate (Koch Light) in $1dm^3$ of de-ionised water at 90°C plus $184cm^3$ glacial acetic acid and then making up to $1840cm^3$ with de-ionised water, giving a 4M sodium acetate solution at pH 5.5. The above solution was then added to $4020cm^3$ methyl cellosolve (Richard Smith's methyl oxytol) previously checked for peroxide ions. The solution was continually stirred and flushed with nitrogen. After 15 min, 107.2g of ninhydrin (Cambrian Chemicals N362) was added to the above mixture, followed by 2.1g stannous chloride (BDH aristar). The ninhydrin solution was stored under nitrogen. After the ninhydrin solution was connected to the analyser, it was again flushed with nitrogen before use, and used within one month, otherwise the ninhydrin tended to precipitate out and block the lines.

2.7.5.6. Calculation of the Amounts of the Amino Acids

The amino acids were eluted from the analyser column in the following order: cysteic acid (CysA), carboxymethyl cysteine (CMcys), aspartic acid (Asp), threonine (Thr), serine (Ser), glutamic acid (Glu), proline (Pro), glycine (Gly), alanine (Ala), valine (Val), cysteine (Cys), methionine (Met), isoleucine (Ile), leucine (Leu), tyrosine (Tyr), phenylalanine (Phe), histidine (His), tryptophan (Trp), lysine (Lys), ammonia (NH_3) and arginine (Arg). The standard amino acid mixture was obtained from Sigma and contained all the above amino acids except CysA, CMcys and Trp, in 25nmol per $0.1cm^3$ quantities. Standard runs were carried out before most unknown runs and for new batches of ninhydrin. An integrator was used to determine the areas of the peaks printed onto the chart paper except for CysA, CMcys, Pro and Trp (due to no standards). Each amino acid produced a slightly different colour with ninhydrin giving rise to different absorbance values at 570nm and so to different peak areas even with the standards. From the peak area and the known quantity of standard amino acid, the integrator was able to determine different colour constants for each standard amino acid. During a sample run, the integrator was able to determine the amounts of the amino acids from the new peak areas and the colour constants obtained from the standard run.

If the integrator was not used, the peak areas for both standard and sample runs were determined by hand. The peak area was determined as a product of the peak height and its width at half height. The peak widths were calculated from the number of spaces between the dots printed between the two half-height values. The dots were printed at regular time intervals. The greater the concentration of amino acid the longer the distance between the dots, which gave a high narrow peak. Lower concentrations of amino acid gave a short wide peak. The colour constants for the standards were determined from the ratio between the peak area and the amount of standard amino acid, known to be 25nmol, as above. For the unknown samples, the amounts of amino acids were determined from the ratio between peak height and the colour constant of the particular amino acid. Proline was always calculated by hand as the integrator did not work using the 440nm information. Cysteic acid and tryptophan were also calculated by hand using the colour constants of aspartic acid and lysine respectively.

2.7.5.7. Calculation of the Number of Residues in a Protein

The method of calculating the number of residues in a protein at a given molecular weight is fairly tedious and time consuming for several runs. Therefore a computer program was devised by Dr A.P. Ryle from the Department of Biochemistry, Edinburgh University, in order to obtain more accurate results and be less time consuming. The program was devised from a program published by Bryce (1979), using a method published by Black and Hogness (1969). The program calculated from the determined amino acid quantities and a given molecular weight of the protein, the number of residues of each amino acid in the protein. The program essentially calculated the molecular weight of the protein from the sum of the products of the frequencies of the amino acids and their residue weights (defined as the molecular weight of the amino acid less the molecular weight of water). The difference between the obtained and actual molecular weight was then calculated, and the amounts of the amino acids altered to give the actual molecular weight of the protein. These new quantities being the number of residues per molecule of protein.

2.8. High Voltage Paper Electrophoresis

The apparatus used and the methods followed were as described by Ryle *et al.* (1955) and Perham (1978). Paper electrophoresis was carried out at pH 2.0 for amino acids, and at pH 2.0 and pH 3.5 for peptides. The separations of amino acids were carried out on protein samples hydrolysed as described previously, except that in this case the amino acids were re-dissolved in 50mM ammonia. The separation of peptides was carried out directly from the sample in buffer.

2.8.1. The Separation of Amino Acids by High Voltage Paper Electrophoresis

The same amount of protein hydrolysate was used for paper electrophoresis as for the separation of amino acids by the analyser. Samples were applied to an origin 10cm from the bottom of Whatman

No.1 paper and at least 4cm from either edge. Usually several samples were applied to the same paper in 1cm strips 2cm apart. The paper was then wetted with electrophoresis buffer, (formic acid : acetic acid : water in the ratio 1 : 3.5 : 35.5, pH 2.0), on either side of the origin in such a manner that the buffer fronts joined along the origin concentrating the samples on the origin. The excess buffer was then blotted off the paper, and the paper was loaded into the electrophoresis tank with the bottom edge in the electrophoresis buffer attached to the anode. The coolant system consisted of white spirit which was in turn cooled by water running through a heat exchanger. The samples were electrophoresed at 5KV for 25 min from the anode to the cathode.

Two types of markers were used for each run: R, (CysA, Met, Met-sulphone, Tyr, Phe, Pro, Leu, Val, Arg and Lys) and, T, (Asp, Glu, Thr, Ile, Ser, Ala, Gly and His). The standards were $1mgcm^{-3}$ solutions in 1mM HCl and 5µl was applied. A $1mgcm^{-3}$ solution of carboxymethyl cysteine was also used as a standard on its own. At pH 2.0 all the ionisable groups of the amino acids are fully protonated except for the α-carboxyl groups which are only partially protonated (pK_a approximately 2). Thus most of the amino acids separated very well though Arg and His ran very close together as did Leu, Ile, Val and Ser. The papers were dried immediately after the electrophoresis run.

Staining

The amino acids were stained using a solution of 0.2% (w/v) ninhydrin in acetone to which a few drops of 2,4,6 collidine (2,4,6 trimethyl pyridine) technical grade had been added (see Levy and Chung, 1953; and Perham, 1978). The papers were then heated to 100°C for 3 min to allow the colours to develop. Collidine reacts with the ninhydrin amino acid complexes to give a variety of coloured complexes depending on the amino acid. The mechanism of this reaction for collidine is unknown, but it is useful for a better identification of an amino acid. The colours observed were:-

Lys	Blue	Leu/Ile	Blue	Phe	Brown
His	Brown	Thr	Grey/Blue	Cys	Purple
Gly	Green/Brown	Ser	Green	Tyr	Brown
Ala	Blue	Pro	Yellow	Asp	Turquoise
Val	Blue	Asn	Orange/Yellow	CysA	Blue
Glu	Blue	Gln	Grey	CM-Cys	Grey/Green

The amino acids were identified as soon as the paper was dry since the colours faded after a few days and in some cases after a few hours.

2.8.2. The Separation of Polypeptides by High Voltage Paper Electrophoresis

The polypeptide solution used was a trypsin digest of a $16mgcm^{-3}$ solution of Insulin B-chain. Samples of 20µl (320ng) were applied on an origin 10cm above the bottom of Whatman 3MM paper, treated as above and ran at pH 2.0 as described above. Samples were also ran at pH 3.5 on Whatman 3MM paper. Here the samples were loaded 15cm above the bottom of the paper and electrophoresed in pyridine : acetic acid : water in the ratios 1 : 10 : 89. The pH 2.0 electrophoresis was carried out at 2KV for 90 min ($180KV\ min^{-1}$) and the pH 3.5 electrophoresis carried out at 2KV for 115 min ($230KV\ min^{-1}$). The wetting solution for the pH 3.5 run was half the strength of the electrophoresis buffer. In both cases the electrophoresis tanks were cooled with white spirit.

The standard solution used was "Wondermix", containing, Arg, Cys, His, Gly-gly, Gly, Leu, Asp, Glu, CysA, Taurine (Tau), Lys-lys, ε-DNP-lys (ε(2,4-dinitro-phenyl)lysine) and xylene cyanol FF(XCFF). The separation of the peptides at the different pH's were different because the pK_a of the α-carboxyl group is 3.5 (for peptides) and the carboxyl groups of the side chains were only partially protonated at pH 3.5, but fully protonated at pH 2.0 (the basic groups were fully protonated in each case). The papers were dried immediately after the electrophoresis runs.

Staining

The peptides were stained by spraying the papers with a 0.25% (w/v) solution of ninhydrin in ethanol, and heating at 100°C for 20 min. The peptides and amino acids were visualised as purple spots. The ninhydrin spots faded after a few days, but were made permanent by spraying with a copper nitrate solution. This solution was made by adding $2cm^3$ of a saturated copper nitrate solution to $0.4cm^3$ of a 10% (v/v) solution of nitric acid and making up to $100cm^3$ with 95% ethanol. The purple ninhydrin spots sprayed with the copper nitrate solution immediately turned to a pinky/orange colour.

2.8.3. Elution of Amino Acids from Electrophoresis Papers

Carboxymethyl cysteine (CMcys) was eluted from the electrophoresis papers according to the method of Ingram (1963) using the apparatus described in that paper. A total protein hydrolysate containing CMcys was run as a 2cm strip. Standard samples were run on either side of the protein sample, and were cut away after the electrophoresis step and stained. The stained strips were then aligned against the preparative strip and the CMcys band identified. The piece of paper containing the amino acid was then cut out of the main strip and trimmed to 3cm x 2cm. One end of the strip was then cut to a point and the other end folded about 0.5cm from the end, sandwiched between two microscope slides, and placed in the elution apparatus. The slides were placed with the opposite end to the paper in a trough containing 0.1M ammonia. The pointed end of the paper was then placed in the top of a small acid washed test tube positioned below the trough. The ammonia solution worked its way between the microscope slides by capillary action and moved through the paper eluting the amino acid, which was collected in the test tube. The elution process took three hours at room temperature. The eluted sample was then dried down and taken for further analysis.

2.9. Autoradiography

Autoradiography was carried out on dried-down gels, either polyacrylamide or agarose, and electrophoretically separated carboxymethyl cysteine on Whatman No.1 paper. The radioisotopes used were either ^{14}C or ^{125}I. The samples were placed against Agfa-Gevaert Curix RP1 X-ray film (13cm by 18cm) in light-proof folders at room temperature. The length of the exposure depended upon the isotope used and the specific activity of the sample. Gels containing bands of [^{125}I]-radiolabelled proteins or polypeptides were exposed from 24 h up to two weeks depending on the activity of the samples. Samples containing [^{14}C]-carboxymethyl cysteine either in polyacrylamide gels or on paper were exposed for about one month.

After exposure the X-ray films were developed in Agfa-Gevaert G150 developer, (prepared as stated in the manufacturer's instructions), for 5 min. The developed film was then placed in a 5% (v/v) acetic acid stop bath for two minutes in order to destroy

any excess developer. After the stop bath, the film was washed in water for one minute and placed in fixer for two minutes. The fixer consisted of Agfa-Gevaert G334 X-ray film fixer plus 0.25% (v/v) Agfa-Gevaert Aditan. After the films had been fixed they were thoroughly washed in running water for one hour.

2.10. Radio-Iodination of Proteins

Two types of radio-iodination techniques were carried out, one in the presence of SDS and the other in the absence of SDS. In both cases the radio-iodinations were carried out using 1mCi of ^{125}I as sodium iodide dissolved in dilute sodium hydroxide pH 7-11. The radioisotope was supplied by The Radiochemicals Centre, Amersham, and was part of batch No.24B. All the reactions used for the labelling of tetanus toxin were carried out in a specifically designated room under the constant monitoring of a Geiger-Muller tube. The experiments were carried out under the supervision of Dr D.K. Apps, Department of Biochemistry, University of Edinburgh, using a method of his design.

2.10.1. Radio-Iodination in the Presence of SDS

A $1cm^3$, $1mgcm^{-3}$ sample of tetanus toxin in 40mM sodium phosphate and 1mM benzamidine pH 7.4 was denatured by the addition of 10% (w/v) solution of SDS, resulting in a 0.5% (w/v) SDS concentration in the toxin solution. The denatured toxin was radio-iodinated with the $Na^{125}I$ by initially adding the toxin solution to the opened vial containing the radioisotope. The solution was then carefully transferred to a small test tube, to which was added 100µl of a $10mgcm^{-3}$ solution of chloramine-T (BDH). The sample was incubated at room temperature for 50s, allowing the chloramine-T to oxidise the iodide to iodine, which in turn reacted with the tyrosine residues in the protein. After the oxidation step the sample was then reduced with 100µl of a $20mgcm^{-3}$ solution of sodium meta-bisulphite ($Na_2S_2O_5$) (BDH), which reduced any unreacted free iodine to the relatively safer iodide.

The radio-iodinated toxin was then separated from any unreacted iodide and salts by gel filtration on a Sephadex G-25 column (20cm by 1cm), previously equilibrated with the phosphate buffer.

The radioactive protein peak was monitored using a Geiger-Muller tube as it progressed down the column. After a few minutes of column chromatography, two radioactive peaks were observed; a leading fraction consisting of the radio-labelled protein and a trailing peak consisting of the unreacted iodide. When the radio-labelled protein peak reached the bottom of the column it was collected as six drop fractions in small plastic tubes. After the protein peak had eluted, the bottom of the column was sealed off, and the unreacted iodide was allowed to decay in a lead-lined cupboard before disposal. The extent of radio-labelling was monitored by taking 2µl samples of each fraction diluting to 50µl with water and measuring the counts per second (cps) over 30s in a Wilj gamma counter.

2.10.2. Radio-Iodination in Absence of SDS

The procedure used was as described in section 2.10.1. except that after the addition of chloramine-T the sample was incubated at $0^{o}C$, for 10 min and then reduced with $Na_2S_2O_4$.

2.11. Preparation of Insolubilized Ganglioside

The method used was as described by van Heyningen (1976a). Insolubilized ganglioside was prepared by dissolving 1mg of ganglioside SGGnSSLC (G_{T1}) and 9mg of brain cerebrosides in $1cm^3$ of chloroform and $1cm^3$ methanol, and then evaporating a suitable volume to dryness in vacuo. The ganglioside : cerebroside complex was then resuspended in 0.1M Tris-HCl pH 7.0.

2.12. Determination of Inorganic Phosphate

The samples for analysis were diluted to $1cm^3$ with de-ionised water before use. A $3cm^3$ solution of 10% (v/v) $HClO_4$ and $0.5cm^3$ of concentrated $HClO_4$ was then added to the samples immediately followed by $1cm^3$ of a 2.5% (w/v) ammonium molybdate solution. The samples were then heated at $60^{o}C$ for 5 min. After the incubation, $1cm^3$ of a freshly prepared 0.1M ascorbic acid solution was added, followed by a further incubation at $60^{o}C$ for 15 min, during which

a blue colour developed. After the second incubation step, the samples were allowed to cool, and the absorbance was read at 650nm in a Cecil spectrophotometer. A standard curve was produced using inorganic phosphate between 0.0 - 1.0μmol.

2.13. Preparation of 5,5'-Dithiobis-(2-Nitrobenzoic acid)

A solution of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) or Ellman's reagent was used to detect the presence of thiol group containing compounds. For references on the mechanism of the reaction and determination of the amount of thiol present (Ellman, 1959; Sedlak and Lindsay, 1968; Habeeb, 1972).

A stock solution of 1mM DTNB (Sigma) in 0.5M sodium phosphate pH 7.0 was made, and stored in dark brown bottles wrapped in aluminium foil. The stock solution was colourless when freshly made and developed a slight yellow colour upon storage. When an appropriate amount of the stock DTNB solution was added to a solution with thiol-group-containing compounds present, an intense yellow colour was produced owing to the production of 5-mercapto-2-nitrobenzoate. The DTNB had to be used in excess over the thiol reagent to make sure all the thiol groups of the test compound had reacted. The reaction relies upon the thiol anion reacting with the DTNB producing a modified and blocked thiol group and 5-mercapto-2-nitrobenzoate. The amount of thiol was equivalent to the amount of 5-mercapto-2-nitrobenzoate, (since the reaction is one to one) and was measured in a spectrophotometer at 412nm. The 5-mercapto-2-nitrobenzoate has a molar extinction coefficient of 13600 $M^{-1}cm^{-1}$ at 412nm (Ellman, 1959).

2.14. Scintillation Counting

A Searle liquid scintillation counter Mark III was used, using a variable quench programme. Each sample was initially counted twice for forty seconds in order for the counter to measure the quenching in the sample. The sample was then counted for the set period, usually ten minutes. The counter was able to convert the cpm to dpm directly using the quenching constant. The scintillation fluid used was a Triton X-100 (Koch Light scintillation

grade) toluene (Koch Light scintillation grade) based mixture. Triton X-100 was used in the scintillant for emulsifying aqueous samples which are normally insoluble in scintillation cocktails. The scintillant contained 0.55% (w/v) 2,5 diphenyl oxazole (PPO) and 0.015% (w/v) 1,4 bis-2-(4-methyl-5-phenyl oxazolyl) benzene (dimethyl POPOP) (both of scintillation grade from Koch Light) 70% (v/v) toluene and 30% (v/v) Triton X-100. The PPO was the primary solute in the scintillation fluid with the dimethyl POPOP as the secondary solute. The PPO has an average fluorescence of 370nm and the dimethyl POPOP at 427nm acting as a sensitiser for any weaker disintegrations. The scintillation fluid was able to dissolve $2cm^3$ of aqueous solutions in $10cm^3$.

2.15. Protein Concentration Determination

For general purpose methods, the protein concentration of a sample was determined by measuring the absorbance of the solution at 280nm, using an arbitrary extinction index of $E^{1\%}_{280} = 10$. The protein concentration for more accurate work was determined by amino acid analysis of a sample using the automatic analyser. Amino acid analyses in such cases were carried out in triplicate and the average value determined.

In a few cases the protein concentration was assayed using the modified version by Spector (1978) of the CBB method of Bradford (1976). The assay solution contained 0.01% (w/v) CBB G-250, 4.7% (v/v) ethanol and 8.5% (v/v) phosphoric acid in water. The solution was filtered through Whatman No.1 filter paper to remove any undissolved CBB. Samples of $0.1cm^3$ were used either directly or made up to $0.1cm^3$ with the particular buffer. The higher the amount of assay solution the greater the amount of protein detectable, so either of the following ranges were used:-

ASSAY SOLN. (cm^3).	RANGE FOR LINEAR RESPONSE (µg).
0.5	0.5 - 5.0
1.0	1.0 - 10.0
2.0	2.0 - 20.0
5.0	5.0 - 50.0

The sample and assay solutions were mixed by immediately inverting the assay tube several times after the addition of the

two solutions. The colour developed after two minutes and the absorbance was measured at 595nm within twenty minutes. A standard 1mgcm^{-3} BSA solution containing the appropriate buffer was used for producing a standard curve. The concentration of the standard BSA solution was determined by measuring the absorbance at 280nm and using the extinction index of $E^{1\%}_{280} = 6.7$. Both standard and unknown samples were carried out in triplicate and the average value determined. Many of the buffers used throughout this thesis were found to interfere with the protein:CBB complex, so this method of protein concentration determination was of limited value.

2.16. Analytical Ultracentrifuge

A Spinco Model E analytical ultracentrifuge was used both for a sedimentation velocity run and for a sedimentation equilibrium run using the meniscus depletion method. In both cases a AN-D rotor was used using double sector cells. A sample of 4mgcm^{-3} tetanus toxin in 0.1M sodium phosphate buffer pH 7.0 containing 1mM benzamidine and 0.01% (w/v) NaN_3 was used. The toxin was exhaustively dialysed against the above buffer, and the final dialysis solution was retained for diluting the toxin solution and for running in one sector of the double sector cells. The cell centre, of the double sector cells was composed of charcoal-filled epon and was used with sapphire windows. Both in the sedimentation velocity run and the sedimentation equilibrium run photographs were taken on Ilford glass plates. The distance moved by the peak in the sedimentation velocity run and the increase in height of the interference fringes in the sedimentation equilibrium run were measured using a Nikon model 6CT microcomparator. The methods followed were those of Cohen (1973) and Nimmo et al. (1976) using a general method according to Bowen (1970).

2.16.1. Sedimentation Velocity

The left-hand channel of the double sector cell was loaded with 0.4cm^3 of the dialysis buffer and the right-hand channel with 0.4cm^3 of the 4mgcm^{-3} toxin solution. The sample was centrifuged at 56,000 rev.min^{-1} at 18°C and photographs were taken through a

Schlieren optical system at eight-minute intervals after ten minutes using an exposure of twelve seconds. The partial specific volume ($\bar{v}$) was determined from the amino acid analysis of the toxin using the partial specific volumes of the amino acids as listed by Lee and Timasheff (1974).

2.16.2. Sedimentation Equilibrium

The above sample of tetanus toxin was diluted with the dialysis buffer to give a 0.4 $mgcm^{-3}$ solution of which 0.11cm^3 was loaded into the right-hand channel of a double sector cell. The left-hand channel was loaded with 0.125cm^3 of the dialysis buffer. The sample was then centrifuged at 16,000 $rev.min^{-1}$ at 25°C for twenty hours. Once the sample had reached equilibrium (20 h) pictures were taken, through a Rayleigh interference system, with an exposure of fifteen seconds. On the Beckman instrument the Rayleigh system is converted from the Schlieren system by a few simple changes to the optical system.

SECTION 3.

RESULTS.

Chapter 1

3.1. Purification of Tetanus Toxin

3.1.1. Introduction

For progress to be made in the understanding of the chemistry of a protein, a homogeneous sample is essential. Various properties have been published for tetanus toxin (i.e. molecular weight and subunit structure); they will be discussed in this section and in Section 4. The variety of differing properties proposed for tetanus toxin probably arose from several different factors.

One factor is that the different strains of Cl. tetani may produce slightly different toxins, though (as mentioned in Section 1) the Harvard strain of Cl. tetani is the usual source of toxin for research purposes. It was also conceivable that a single strain upon sub-culture may mutate to produce structurally differing toxins. But the most probably reason for the discrepancies in the published properties of tetanus toxin is the presence of contaminating proteins. With the advancement of techniques in protein chemistry over the last decade, the purity of proteins has increased; which has in turn led to a greater understanding of their structure and function.

Pillemer et al. (1948) developed a method for the purification of tetanus toxin using methanol precipitation. By this technique they were able to crystallise tetanus toxin either from a very dilute toxin solution, between pH 4.5 and pH 5.4, or from a 20% (v/v) methanol solution at pH 6.0 at $-5^{\circ}C$. The colour of the crystals they obtained was yellow, indicating that the toxin they classified as pure may have contained contaminants. They also showed that the toxin contained no carbohydrate and that it had an isoelectric point of pI 5.1, determined from minimum solubility studies. From analytical ultracentrifugation studies they showed that the toxin contained two components, observable as two schlieren peaks during a sedimentation velocity run. The two components had sedimentation values of 4.5S and 7.0S. The 7.0S component was shown to be atoxic, and they postulated that it was a dimer of the 4.5S

component. The 4.5S component was shown to be toxic, and they classified it as tetanus toxin.

Dawson and Mauritzen (1967) improved on the purification method of Pillemer et al. (1948) by the introduction of ion-exchange chromatography. The methanol-precipitated toxin was further purified on a DEAE-cellulose column at pH 7.2 using a linear gradient of sodium phosphate at a concentration from 0.01M to 0.05M. They also carried out analytical ultracentrifugation studies on their purified toxin and identified a single component of 7.0S, which was shown to be toxic. Murphy and Miller (1967) included gel-permeation chromatography using Sephadex G-100, after ion-exchange chromatography on DEAE-cellulose. They showed that their purified toxin consisted of a single component by using immuno-electrophoresis and polyacrylamide gel electrophoresis. Analytical ultracentrifugation studies also indicated a single component of 6.4S.

Several authors have used the above systems for the purification of tetanus toxin, using either ion-exchange chromatography alone or followed by gel-permeation chromatography and in some cases using gel-permeation chromatography alone. The different authors produced various results as regards amino acid composition, number of sulphur groups (present as cysteine and cystine) and differing N-terminal amino acids (see Section 3.3), which led to a variety of models for the structure of tetanus toxin (see Section 4).

A modification of the method developed by Dawson and Mauritzen (1967) was used for the work presented in this thesis. The introduction of a linear gradient of sodium phosphate at a concentration betwen 0.01M and 0.04M with the addition of 0.1mM triethylenetetramine (TET) instead of EDTA was described by van Heyningen (1976a). Tetanus toxin was shown to be eluted from the DEAE-cellulose column at a sodium phosphate concentration between 0.03M and 0.04M (see Photograph 3). Thus the shallower gradient was found to be sufficient to elute the toxin without the need to increase the phosphate concentration and possibly elute other proteins; Ognyanova (1974) used an upper concentration of 0.1M sodium phosphate and Bizzini (1976) recommended using 0.01M EDTA at pH 7.6 for both toxin solvent and column eluent, tetanus toxin was observed to run straight through the column but

contaminants were observed to be adsorbed to the column. The use of 0.1mM TET as opposed to 0.1mM EDTA was preferred as it allows a quicker equilibration time for the preparation of columns and improves reproducibility. EDTA is adsorbed to ion-exchange columns because it has a high affinity for the DEAE (diethylaminoethyl) exchanger group. Because of the low concentration of EDTA a larger quantity of buffer would be required to completely saturate the exchanger resin, otherwise a mixture of EDTA and non-EDTA resin would occur and the reproducibility of the chromatographic step would depend on obtaining the same mixture each time. In contrast, TET does not have a high affinity for the exchanger so it does not need long equilibration times, but still acts as a heavy-metal chelator.

A further modification was the addition of two protease inhibitors, 0.1mM phenylmethylsulphonyl fluoride (PMSF, Sigma, Fahrney and Gold (1963)) and 1mM benzamidine (Sigma, Mares-Guia and Shaw (1965)). PMSF is a potent and highly specific irreversible protease inhibitor. It is specific for serine type proteases, i.e. proteases having a serine residue at the active site. A problem associated with PMSF is insolubility and stability in aqueous buffers. PMSF has to be initially dissolved in either 96% ethanol or propan-2-ol before addition to aqueous buffers and is only soluble up to a concentration of 1mM. Because of the insolubility of PMSF "salting-out" occurs if the ionic strength of the buffer is increased. PMSF is also hydrolysed in aqueous buffers and has a half-life of about 100 min at pH 7.0 at $4^{o}C$, which is decreased at higher pH values (Gold, 1967; Pringle, 1975; Lumsden and Coggins, 1977). Because of the problems associated with PMSF the other protease inhibitor, benzamidine, was also included in the purification of tetanus toxin. Benzamidine is also a serine protease inhibitor but it is a reversible inhibitor. Benzamidine in contrast to PMSF is soluble in aqueous buffers and is not hydrolysed. Benzamidine was primarily included in the ion-exchange purification procedure to protect the toxin from any proteases not inactivated by PMSF, because of the fall in concentration of PMSF brought about by "salting-out" and hydrolysis. PMSF is probably "salted-out" as a result of the increase in phosphate ion concentration and also lowered in concentration as a result of

hydrolysis during the several hours needed for the chromatographic purification of tetanus toxin. The benzamidine was used at a fairly high concentration to ensure that any active serine proteases were saturated with the reversible inhibitor. The use of protease inhibitors throughout a purification procedure is essential, their use is now becoming more widespread, especially with proteins obtained from micro-organisms because of the fairly high concentrations of proteases present. It is becoming increasingly clear that the purification methods used to purify proteins can also lead to the co-purification and activation of proteases necessitating the need for protease inhibitors throughout a purification procedure rather than at the beginning and end of the method (a process tended to be used previously).

3.1.2. Purification Method for Tetanus Toxin

As mentioned in the Introduction (Section 1.2.2.) the toxin used for the work described in this thesis was obtained from Dr. R.O. Thomson of the Wellcome Research Laboratories, Beckenham, Kent, and consisted of two types. Initially the toxin was sent as a partially purified sample (see below) obtained from a resuspension of potassium phosphate precipitated material. Because of possible spillage in transit the toxin was later sent as a freeze-dried sample of the above toxin.

In both cases the toxin was derived from the Harvard strain of Cl. tetani, by taking the extracellular filtrate and intracellular material after auto-lysis of the cells. The toxin was partially purified by precipitation with 1.4M potassium phosphate at pH 7.0, the precipitate was then re-dissolved in water containing 0.01M thiomersalate as an antibacterial agent. The various batches of toxin used were TD 771B, TD 772B, TD 801B and TD 803C, which were all re-suspended potassium phosphate precipitations, and batch TD 812B which was a freeze-dried sample.

For further purification either $6cm^3$ (120mg protein determined by absorbance at 280nm, $E^{1\%}_{280}=10$, see Section 2.15) of the toxin solutions were used or 0.6g (400mg protein determined by absorbance at 280nm) of the freeze-dried sample was used. The toxin solutions yielded about 68mg of purified toxin and the freeze-dried

sample about 74mg of toxin, both determined by absorbance at 280nm. The initial procedures slightly differed for the two types of toxin.

Samples from batches TD 771B, TD 772B, TD 801B and TD 803C were centrifuged at 5°C in a Ti50 rotor using a Beckman L2 65B preparative ultracentrifuge at 50 000 rev.min^{-1} (105 000g) for 1 h to remove any insoluble material. The supernatant was retained (a straw-coloured solution) and the pellet was washed twice by re-suspending in 0.01M sodium phosphate buffer at pH 7.2 (for full constituents see below) and then centrifuging as described above; the washings were pooled with the original supernatant. The pellet, after the final wash, was observed to contain very little toxin in comparison to the supernatants (see Photograph 5). The pooled supernatants were exhaustively dialysed against 0.01M sodium phosphate buffer at pH 7.2 at 4°C, and then treated as described later.

The freeze-dried toxin was carefully re-dissolved in 10cm^3 of a buffer containing 0.01M sodium phosphate, 0.1mM TET, 0.01% (w/v) NaN_3, 1mM benzamidine and 0.1mM PMSF pH 7.2 (throughout the thesis this buffer will be referred to as 0.01M sodium phosphate buffer pH 7.2 for convenience), at 4°C for 3 h. After the toxin had been re-dissolved there was no evidence of any insoluble material, so no centrifugation step was required. The straw-coloured toxin sample was exhaustively dialysed against 0.01M sodium phosphate buffer pH 7.2 at 4°C.

After the dialysis steps, the two different toxin samples were treated in the same way. The dialysed samples were run onto a Whatman DE-52 DEAE-cellulose ion-exchange chromatographic column (2cm by 6cm) that had been prepared as described in Section 2.3. The column was equilibrated, before the addition of toxin, by passing 0.01M sodium phosphate buffer pH 7.2 through the column overnight at 4°C; usually 600cm^3 of the buffer was sufficient. The column was observed to be equilibrated when the pH of the effluent was the same pH as the eluent. The dialysed toxin samples were diluted to 100cm^3 with sodium phosphate buffer pH 7.2, containing fresh PMSF, before running onto the column to make sure the ionic strength of the toxin containing buffer was the same as the ionic strength of the column buffer. This was initially checked with a conductivity meter. A Gilford peristaltic pump was used to run the sample onto the column and for all the following steps in order

to ensure a constant flow rate; $3cm^3$ fractions were collected. Once all the sample had been run onto the column the adsorbed toxin was washed with $50cm^3$ of 0.01M sodium phosphate buffer pH 7.2, containing fresh PMSF, to remove any unadsorbed material. The toxin was then eluted from the column using a linear gradient of $75cm^3$ of 0.01M sodium phosphate buffer pH 7.2 and $75cm^3$ of 0.04M sodium phosphate buffer pH 7.2 (same constituents as 0.01M sodium phosphate buffer pH 7.2) containing fresh PMSF. Any remaining toxin was eluted using $70cm^3$ of 0.04M sodium phosphate buffer pH 7.2 and the column was finally washed, to remove any adsorbed protein, using $70cm^3$ of 2M NaCl. A straw-coloured protein solution was removed from the column during this final step. See Photograph 2 for the general steps used in toxin purification.

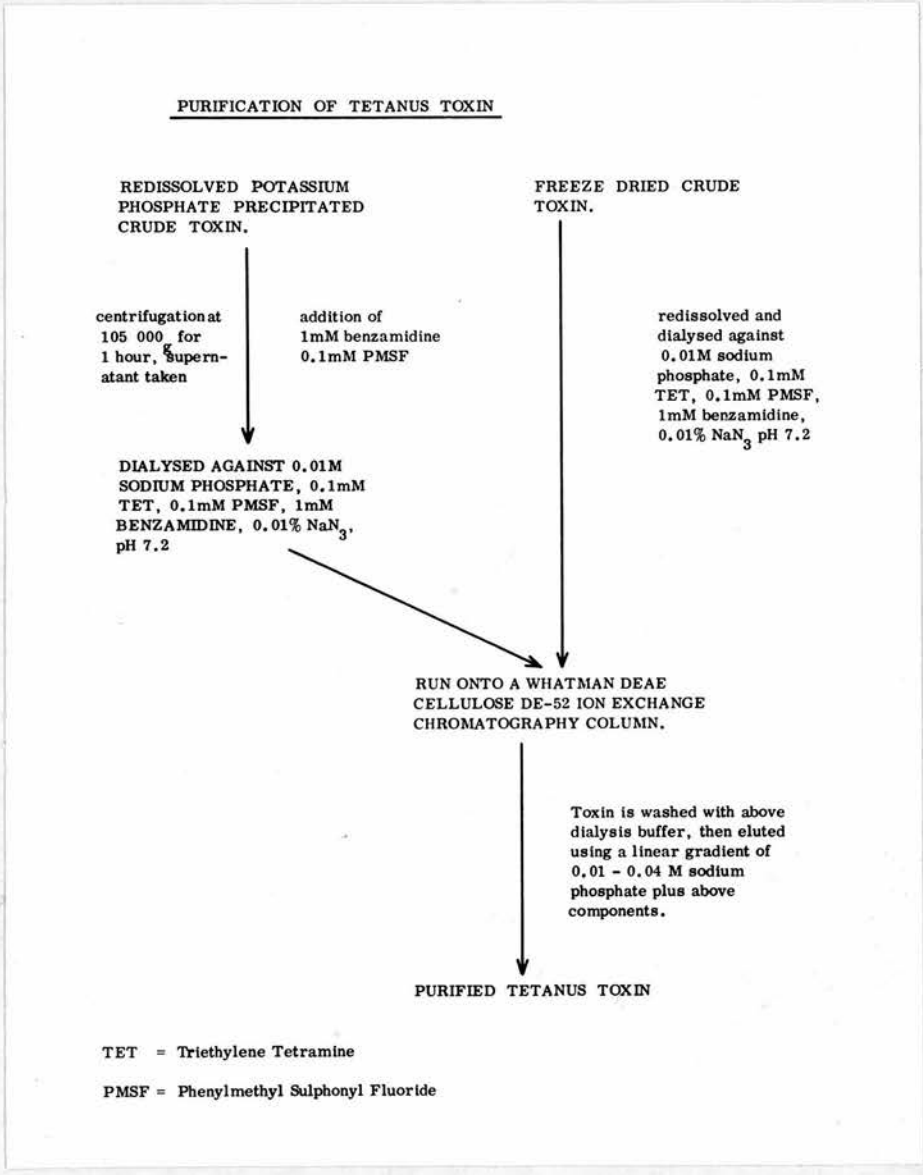
3.1.3. Detection of Tetanus Toxin

The amount of protein eluted into each fraction was determined by absorbance at 280nm. The elution profile (Photograph 3) showed that tetanus toxin was eluted between sodium phosphate concentrations of 0.035M and 0.04M. The toxin-containing peak was identified by using the double diffusion technique developed by Ouchterlony (1949), as described in Section 2.6.1. Samples (6µl) were taken from each fraction eluted from the DEAE-cellulose column and were tested against 6µl of equine anti-toxin (supplied by Dr. R.O. Thomson of the Wellcome Research Laboratories). A white precipitin band was observed when tetanus-toxin-containing fractions cross-reacted with the anti-toxin, Photograph 4. The presence of toxin was confirmed using SDS-PAGE, a method which also checked the purity of the toxin-containing fractions, see Section 3.1.4.

3.1.4. The Purity of Tetanus Toxin

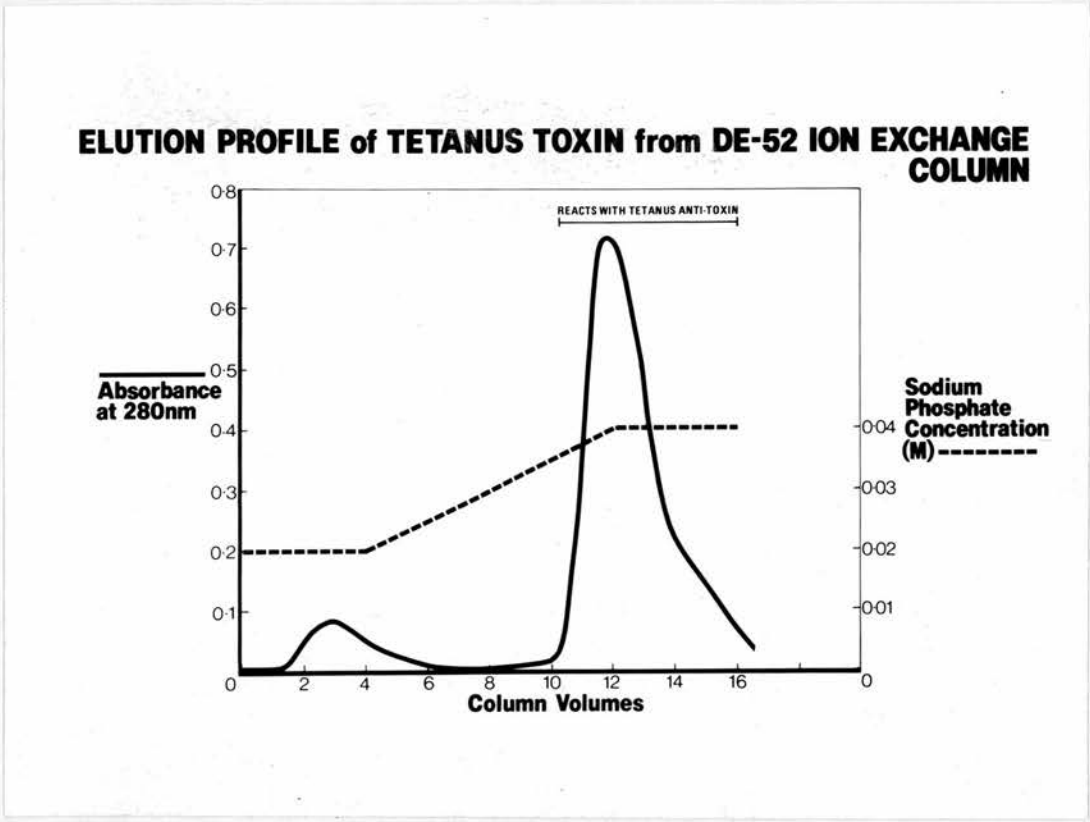
3.1.4.1. SDS-Polyacrylamide Gel Electrophoresis

The purity of the toxin eluted from the DEAE-cellulose ion-exchange column was initially checked by SDS-PAGE. The system initially used was that described by Weber and Osborn (1969), as



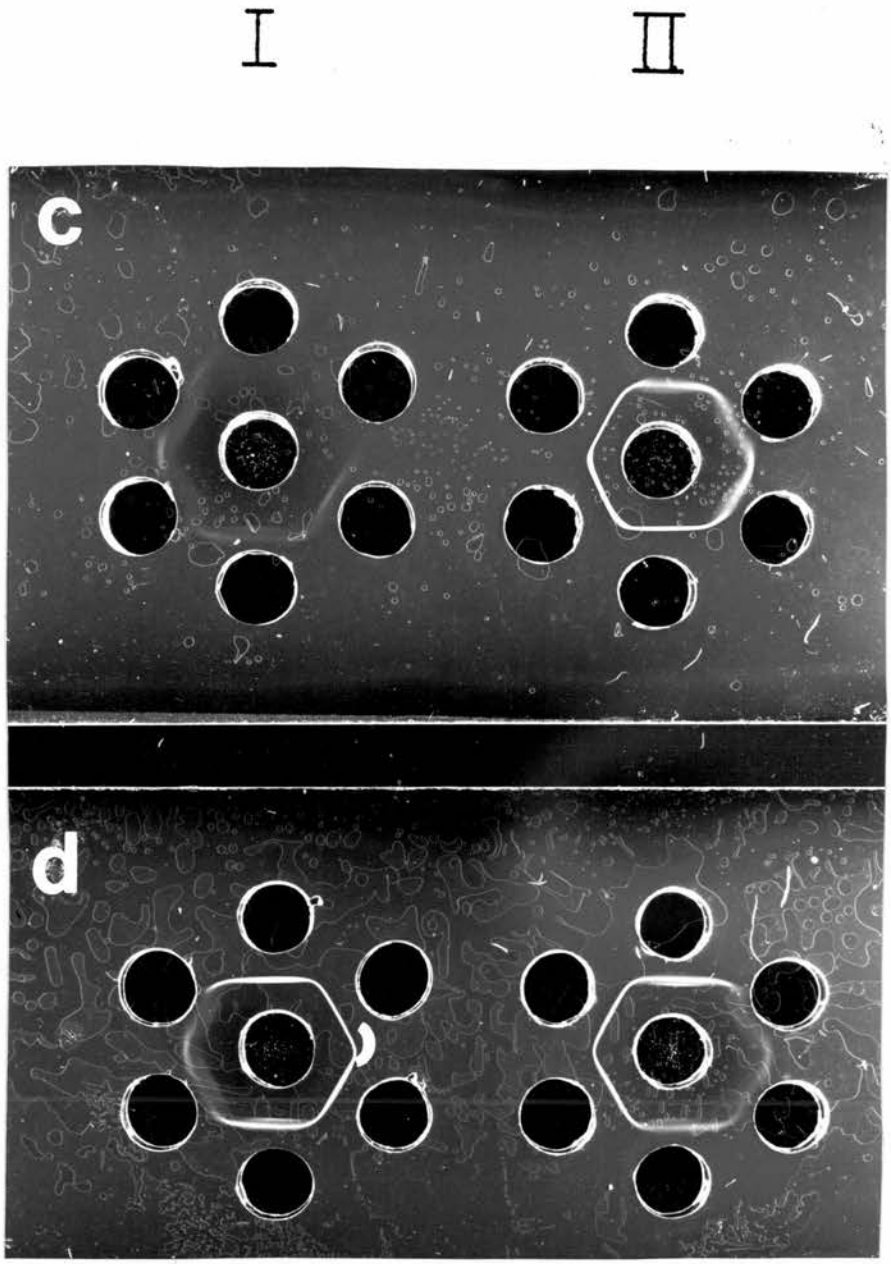
PHOTOGRAPH 2. **SUMMARY OF THE PURIFICATION PROCEDURES FOR TETANUS TOXIN**

See Section 3.1.2. for details.



PHOTOGRAPH 3. THE ELUTION PROFILE OF TETANUS TOXIN FROM A DE-52 ION-EXCHANGE CHROMATOGRAPHIC COLUMN

See Section 3.1.2. for details of the procedure.
The continuous black line shows the protein profile measured by absorbance at 280 nm.
The broken line shows the increase in phosphate ion concentration, determined as described in Section 2.12.



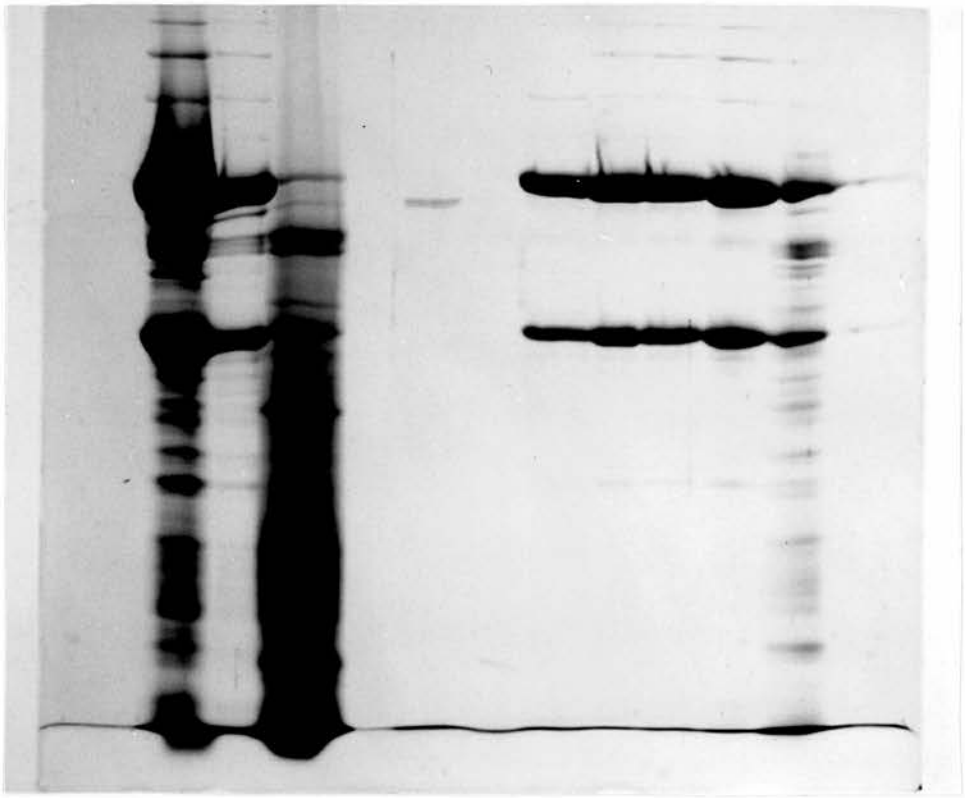
PHOTOGRAPH 4. IDENTIFICATION OF TETANUS TOXIN CONTAINING FRACTIONS BY THE DOUBLE IMMUNO-DIFFUSION TECHNIQUE OF OUCHTERLONY (1949)

See Section 3.1.3. for details. Each outside well contained a different sample of purified toxin obtained from the various fractions eluted from the DE-52 column. The inner wells contained tetanus anti-toxin. cI contained samples at the start of the toxin peak. cII and dI contained samples from the peak. dII contained samples eluted at the end of the peak and from the 2M NaCl wash.

described in Section 2.5.1.1., using gels containing 10% acrylamide. The discontinuous slab gel system of Laemmli (1970) (Section 2.5.1.2.) was used later and was preferred because of the better resolution obtained and the ease of running large numbers of samples. In order to obtain better resolution of the two chains (the criterion used to identify tetanus toxin) 8% acrylamide was used in the analytical slab gels.

The method of SDS-PAGE was used to show the difference between the two toxin preparations (see Photograph 5 and Photograph 6). The freeze-dried samples of tetanus toxin were observed to contain a protein of molecular weight 150 000 daltons (from SDS-PAGE data) which was classified and later identified (by the N-terminus amino acid, Section 3.3.2.1.) as 'unnicked' or 'intracellular' toxin. There was never any evidence of 'unnicked' toxin present in the samples of toxin sent as re-suspensions. It was suspected that the re-suspended samples of toxin contained no 'unnicked' toxin because of the presence of active endogenous proteases; and that the freeze-dried samples contained 'unnicked' toxin because of the immediate use of the protease inhibitors PMSF and benzamidine.

The purity of the toxin (re-suspended sample only) was also checked using non-denaturing gels, according to the method of Davis (1964), as described in Section 2.5.2. As can be seen from Photograph 7, material is removed by the purification method described in Section 3.1.2. There is very little resolution between tetanus toxin and the contaminants, perhaps because of similarities in charge or size or from both factors. The protein present in a purified sample of tetanus toxin was identified by staining with Mg^{2+}-ANS (0.01% (w/v) in 0.1M sodium phosphate pH 7.0 for 10 min at room temperature), prepared as described in Section 2.4.3., in the Davis gels for further analysis. The Mg^{2+}-ANS was preferred to the normal CBB method in order to avoid any acid hydrolysis of the protein during the staining procedure. The protein after complexing with the ANS was identified under ultra-violet light and sliced out of the gel. The protein was then eluted out of the gel into $0.3cm^3$ of 0.1% (w/v) SDS, 1mM benzamidine and 0.1mM PMSF at $25^{\circ}C$ overnight. The eluate was removed and stored at $4^{\circ}C$ whilst the rest of the slice was re-eluted with a further $0.1cm^3$ of the above solution at $25^{\circ}C$ for 5 h.



PHOTOGRAPH 5. SDS-POLYACRYLAMIDE GEL OF THE PURIFICATION OF RE-SUSPENDED TOXIN

See Section 3.1.4.1. for details.

Track 1. Crude toxin.

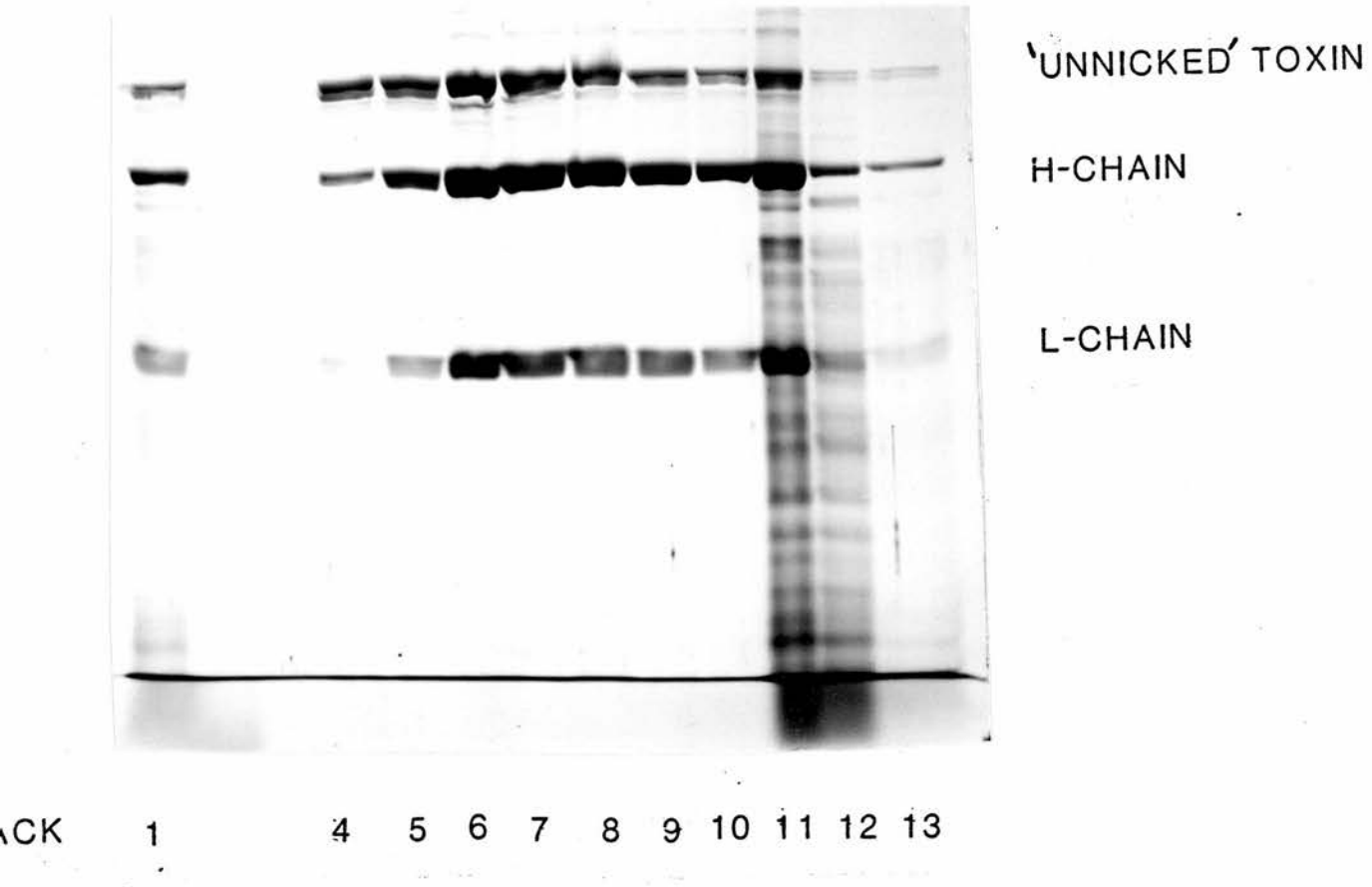
Track 2. Centrifuged and dialysed toxin.

Track 3. Pellet obtained from crude toxin.

Track 5. Protein eluted at the first peak of the DE-52 column. (PHOTOGRAPH 3).

Tracks 7-10. Samples from various fractions of purified toxin (second peak) eluted from the DE-52 column.

Tracks 11 and 12. Samples from the 2M NaCl wash of the DE-52 column.



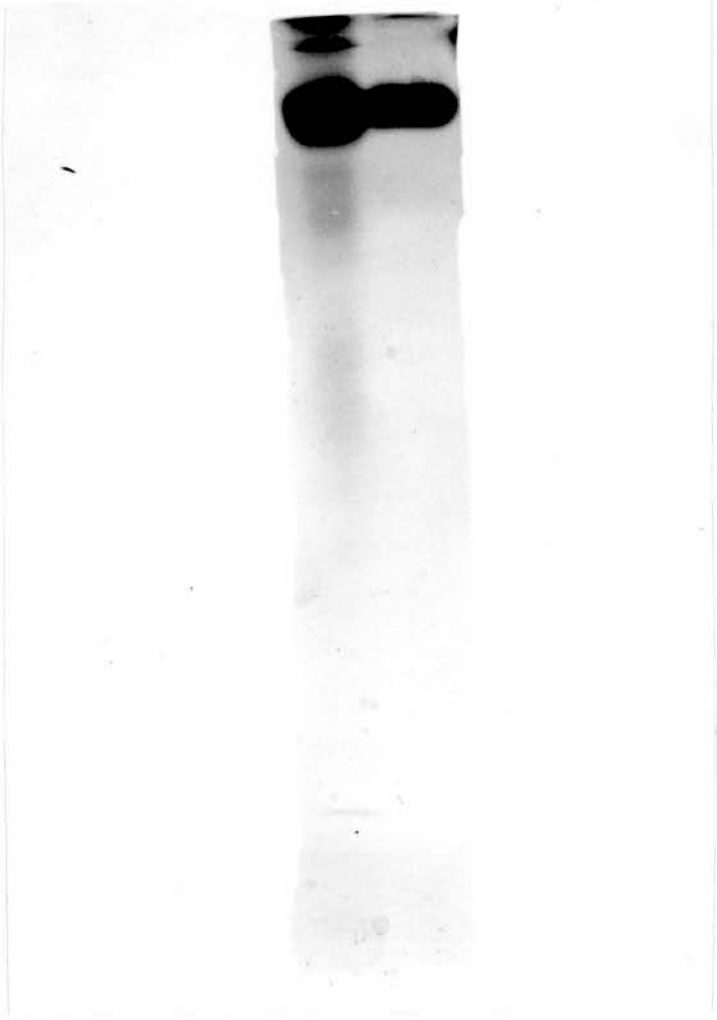
PHOTOGRAPH 6. SDS-POLYACRYLAMIDE GEL OF THE PURIFICATION OF FREEZE-DRIED TOXIN

See Section 3.1.4.1. for details.

Track 1. Crude toxin.

Tracks 4-10. Samples from various fractions of purified toxin eluted from the DE-52 column.

Tracks 11-13. Samples from the 2M NaCl wash of the DE-52 column.



A B

PHOTOGRAPH 7. SEPARATION OF CRUDE AND PURIFIED TOXIN ON A NON-DENATURING DAVIS GEL

See Section 3.1.4.1. for details.

A Sample of crude toxin.

B Sample of purified toxin.

The two eluates were then pooled, freeze-dried and re-dissolved in $0.1cm^3$ of water. The samples were then treated for electrophoresis into 10% acrylamide Weber and Osborn gels as described in Section 2.5.1.1. Two bands were detectable in the gels and corresponded to H- and L-chain.

3.1.4.2. Two-Dimensional Polyacrylamide Gel Electrophoresis

Another method used to check the purity of the toxin (freeze-dried sample only) was two-dimensional-PAGE, according to the method of O'Farrell as described in Section 2.5.3. The first dimension was isoelectric focusing and the second dimension SDS-PAGE.

In this method, proteins are separated in one dimension under denaturing conditions (9.5M urea and 2% (w/v) nonidet P-40 a non-ionic detergent) as a result of the difference in their net charge. In order to separate the proteins only by net charge, a pH gradient is required. The pH gradient is set up throughout the length of a gel by using ampholines. In the work described in this thesis the range of ampholines used was pH 3.5-10.0. The ampholines are mixed in with the rest of the gel components and form a pH gradient in the gel as soon as an electric current is passed down the gel. For experiments described in this thesis the ampholines were arranged with the alkaline end at the top of the gel and the acidic end at the bottom of the gel (see Section 2.5.3.1.). Proteins electrophoresed into such a gel move because of their net charge, which varies according to the pH of their environment. The variability of the net charge of the protein depends on the number and nature of charged amino acid residues in the protein. When a protein reaches the pH equivalent to its isoelectric point (pI) it no longer has a net charge so does not move from that point and is said to be focused. In theory the protein should no longer move in the gel but in practise the pH gradient set up by the ampholines moves slowly down the gel taking the focused proteins with it.

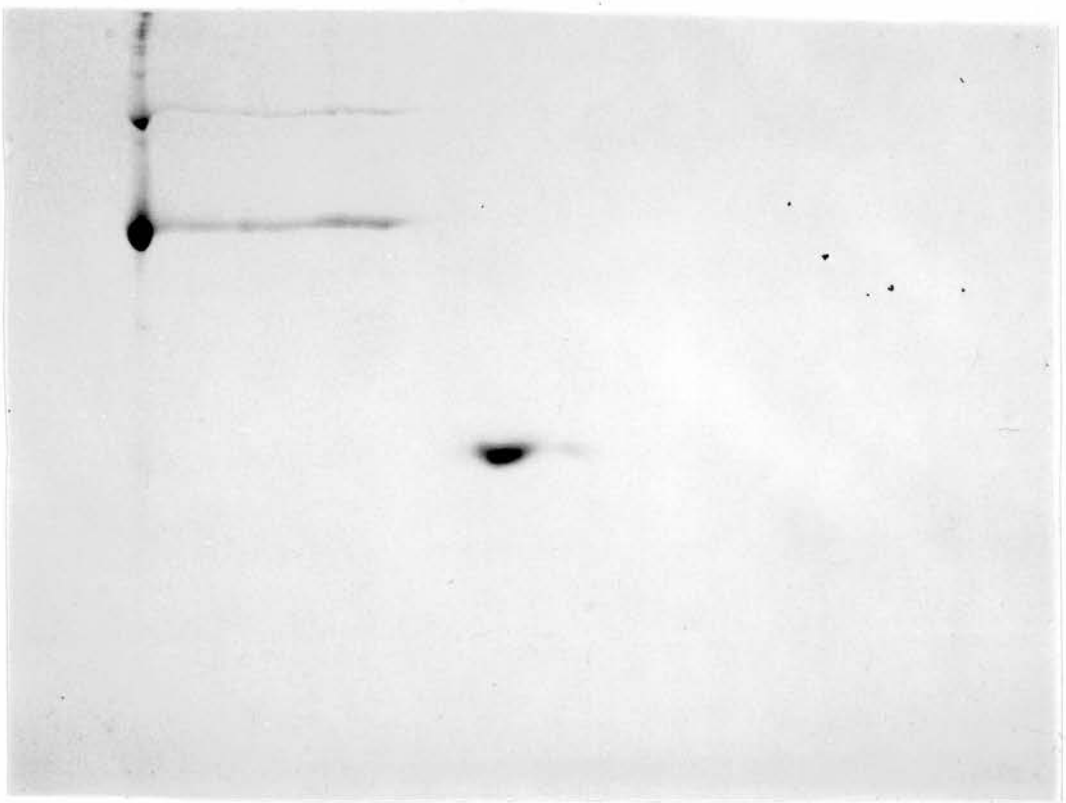
After the proteins had been focused in the first dimensional gel they were re-electrophoresed at right angles to the first gel into an SDS slab gel (see Section 2.5.3.2.). The proteins in the second dimensional gel are separated solely by a size difference

from their focused positions in the first dimensional gel (the SDS gives each protein an equivalent negative charge to mass ratio). From the position of the protein in the second dimensional gel the distance into the first dimensional gel can be calculated. By determining the pH gradient in the first dimensional gel (see below) an approximate isoelectric point for each protein can be determined. According to O'Farrell (1975) 1100 protein components have been resolved by this method, from E. coli, differing by as little as a single charge, indicating the great potential of the method.

Purified samples (40µg) of tetanus toxin containing both 'unnicked' and 'nicked' toxin (determined by the use of SDS-PAGE) were subjected to two-dimensional electrophoresis, using ampholines of the range pH 3.5-10.0 and an acrylamide concentration of 8% in the second dimensional gel. Two-dimensional electrophoresis was used to determine whether any other proteins co-migrated with either 'unnicked' toxin or the two chains of 'nicked' toxin in SDS-PAGE. It would be highly unlikely that any proteins of the same molecular weight of either tetanus toxin or its chains would also carry the same charge. Evidence from the Davis gels (Section 3.1.4.1.) indicated that no other proteins of similar size and charge co-migrated with tetanus toxin. But two-dimensional electrophoresis is a more refined method separating proteins initially by charge only and with high resolution.

Gels containing no protein were also run with the protein samples in order to determine the pH gradient set up by the ampholines during the electrophoretic run. After the samples were electrophoresed, as described in Section 2.5.3., the pH gradient of the ampholines was determined by slicing the gel, without any protein, into 1mm slices and eluting the ampholines into 0.5cm^3 of water overnight. The pH of each slice eluate was measured, using a micro-pH electrode; the value obtained was an average value for the slice as each slice consisted of a micro-pH gradient.

After two-dimensional electrophoresis only three proteins or polypeptides were observed corresponding to 'unnicked' toxin and the two chains from 'nicked' toxin (Photograph 8). From Photograph 8 it appears that 'unnicked' toxin and H-chain have only just entered the first-dimensional gel. If no urea was used in the first-dimensional gel all three proteins appeared in a



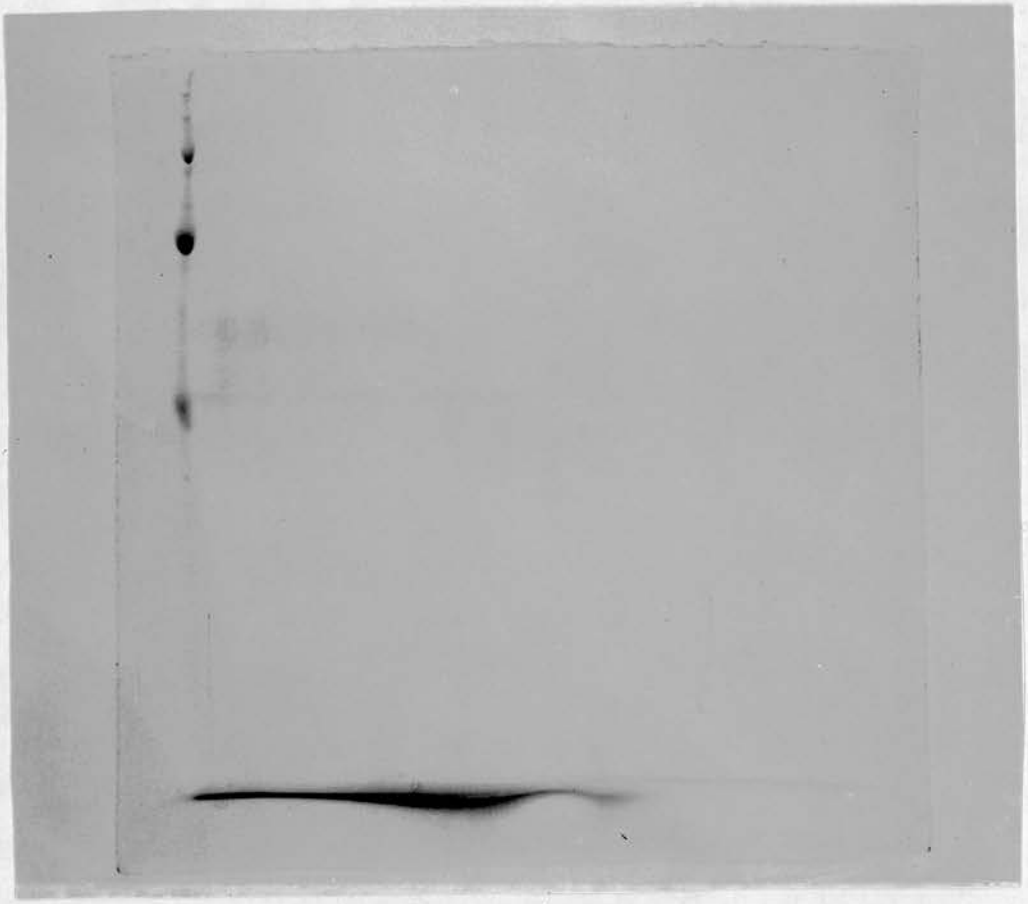
PHOTOGRAPH 8. 2-DIMENSIONAL ELECTROPHORETIC SEPARATION OF TETANUS TOXIN

See Section 3.1.4.2. for details. The first dimensional gel (electrophoresed from left to right, cathode to anode) consisted of 7% acrylamide, 9.5M urea and 2% nonidet P-40. The second dimensional gel (electrophoresed top to bottom, cathode to anode), consisted of an 8% Laemmli SDS gel.

straight line in the second-dimensional gel (Photograph 9). Quite clearly the L-chain had remained associated with the H-chain in the first-dimensional gel even after reduction in the first-dimensional gel. This indicated that more denaturation was required than obtained with nonidet P-40 in order to separate the two chains after reduction. If urea was used in the first-dimensional gel but no 2-mercaptoethanol some L-chain was observed to be associated with H-chain in the first-dimensional gel and some to be separated (see Photograph 10). This indicated that upon denaturation the two chains might have slightly separated, possibly by slight reduction either by the ampholines or the low oxygen-tension of the gel.

The observation that both 'unnicked' and 'nicked' toxin run together in the first-dimensional gel in the absence of urea suggests that the proteins are very similar in structure because of the similarity in the net charges of the proteins even though the 'unnicked' toxin has an extra constraint on its ability to undergo conformational changes because of the extra peptide linkage. The nonidet P-40 is a non-ionic detergent so should only bind to the available hydrophobic portions of the proteins forming micelle-like clusters, allowing the proteins to retain their "native" conformations (Helenius and Simmons, 1977).

A problem to be answered is why H-chain carries a similar charge to whole toxin whether 'nicked' or 'unnicked'. A possible answer is aggregation in urea, which is also a possible reason for the streaking observed in Photograph 8 and Photograph 10, (though the streaking problem appears to be a common phenomenon associated with two-dimensional electrophoresis). From the movement of whole toxin into the gel it can be assumed that it carries a slightly net negative charge as also observed with the Davis gel. But after the addition of urea to allow the separation of the L-chain one would expect H-chain to have altered its position because of the loss of the net negative charge carried by the L-chain. An explanation for the retention of the same net charge for H-chain compared to whole toxin is that upon removal of L-chain, the H-chain is able to undergo a conformational change which increases the net negative charge of the molecule in order to replace the charge value lost by the



PHOTOGRAPH 9. 2-DIMENSIONAL ELECTROPHORETIC SEPARATION OF PURIFIED TETANUS TOXIN IN THE ABSENCE OF UREA FROM THE FIRST DIMENSIONAL GEL

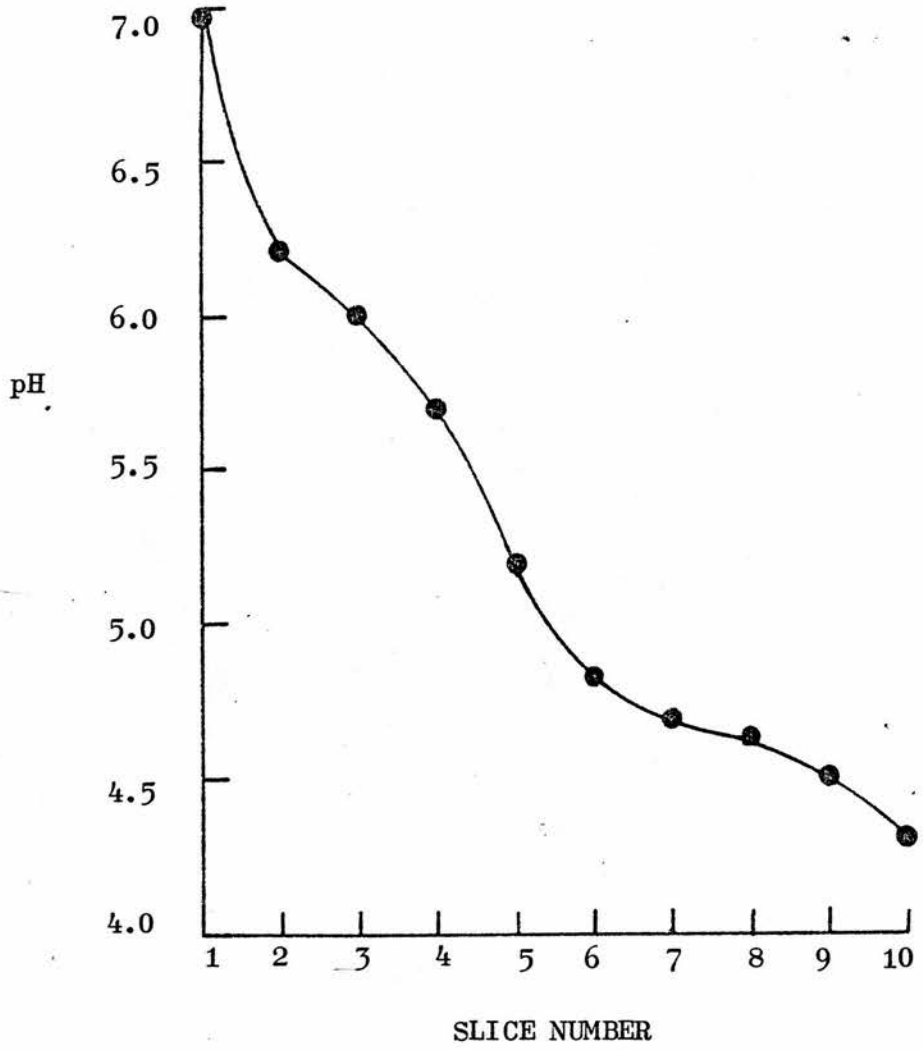
See Section 3.1.4.2. for details. The conditions are as described for PHOTOGRAPH 8, except no urea was included.

PHOTOGRAPH 10. 2-DIMENSIONAL ELECTROPHORETIC SEPARATION OF PURIFIED TETANUS TOXIN IN THE ABSENCE OF 2-MERCAPTOETHANOL

See Section 3.1.4.2. for details. The conditions are as described for PHOTOGRAPH 8, except no 2-mercaptoethanol was included.

removal of L-chain. The fact that after reduction there is no separation of the two chains in non-denaturing conditions suggests a tight association between the chains which is abolished by denaturation. If H-chain undergoes a conformational change, to increase the number of negative charges the possibility of a hydrophobic structure for H-chain must be considered where the charged amino acid residues are internal in the "native" structure. The fact that 'unnicked' toxin has the same charge under non-denaturing and denaturing conditions suggests that the presence of the L-chain, even after reduction, influences the conformational structure of H-chain. The same phenomenon is observed from Photograph 10 where 'nicked' toxin is denatured but not reduced and that the undissociated L-chain not held to H-chain by a peptide bond is able to influence the conformational state of H-chain, i.e. if it did not the overall net charge of denatured H-chain plus undissociated denatured L-chain would be different to either whole toxin or H-chain and be identifiable as another spot. An observation in support of the hydrophobic nature of H-chain is its insolubility in non-denaturing conditions (van Heyningen (1976a) observed that it could only be kept soluble in 1M urea or in SDS). If L-chain does not influence H-chain a difficulty arises in how to explain the non-movement of 'unnicked' toxin under denaturing and non-denaturing conditions. It could be explained by the newly exposed charges opposing one another, negative for L-chain as observed from 'nicked' toxin and by inference more positive for H-chain. But this cannot be so, as H-chain in 'nicked' toxin would be observed to be more positively charged.

From the pH gradient (see Diagram 6) of the ampholines along the first dimensional gel one can determine the approximate isoelectric points of tetanus toxin and its two chains. The isoelectric point of L-chain was determined to be pI 4.8 and for 'unnicked' toxin, 'nicked' toxin and H-chain to be pI 6.2, which is in contrast to Pillemer et al. (1948) who determined it to be pI 5.1 though there is doubt about the purity of their toxin as they determined its s value to be 4.5S whereas it now appears to be about 7.0S (Murphy and Miller, 1967; Robinson et al., 1975).



GRAM 6. pH GRADIENT OF THE AMPHOLINES ALONG THE FIRST DIMENSIONAL GEL

See Section 3.1.4.2. for details.

An isoelectric point of pI 6.2 for tetanus toxin would be in agreement with its purification on a weak anion exchanger. The fact that tetanus toxin is bound to DEAE-cellulose at pH 7.2 suggests that it carries a net negative charge; a net negative charge would only occur above the isoelectric point. During the purification of tetanus toxin the initial buffer concentration was 0.01M sodium phosphate pH 7.2 (ionic strength of 0.03) and the toxin was eluted with 0.03M sodium phosphate pH 7.2 (ionic strength of 0.09), indicating that the toxin was bound weakly to the column. The evidence from the ion-exchange chromatographic step suggests an isoelectric point of about one pH unit below the pH of the chromatography buffer.

3.1.4.3. Two-Dimensional Immunoelectrophoresis

Another method used to check the purity of the purified tetanus toxin was two-dimensional immunoelectrophoresis. The purified toxin was compared to the proteins eluted by the salt wash from the DEAE-cellulose ion-exchange column.

In this method the proteins were initially separated in a 1% agarose gel at pH 8.6 as a result of their net charge at that pH, according to the method described in Section 2.6.2.1. The proteins were then electrophoresed at right-angles to the initial dimension into a 1% agarose gel containing anti-tetanus-toxin (Section 2.6.2.2.). The antiserum was obtained from toxin (not purified as described above), that had been toxoided; Antiserum production was carried out by Dr. R.O. Thomson at The Wellcome Laboratories in Kent. The antiserum contained a variety of antibodies because of the different antigens present in the toxoided material. The electrophoresis system was carried out at pH 8.6 so that the antibodies carried no net charge (pI of gamma-immunoglobulins is 8.6) and were not electrophoresed in the second-dimensional gel.

Separate antigen:antibody precipitin arcs are formed as each antigen combines with its specific antibody. Precipitin arcs are formed in contrast to the normal precipitin bands observed in double immunodiffusion because the antigen is initially in excess

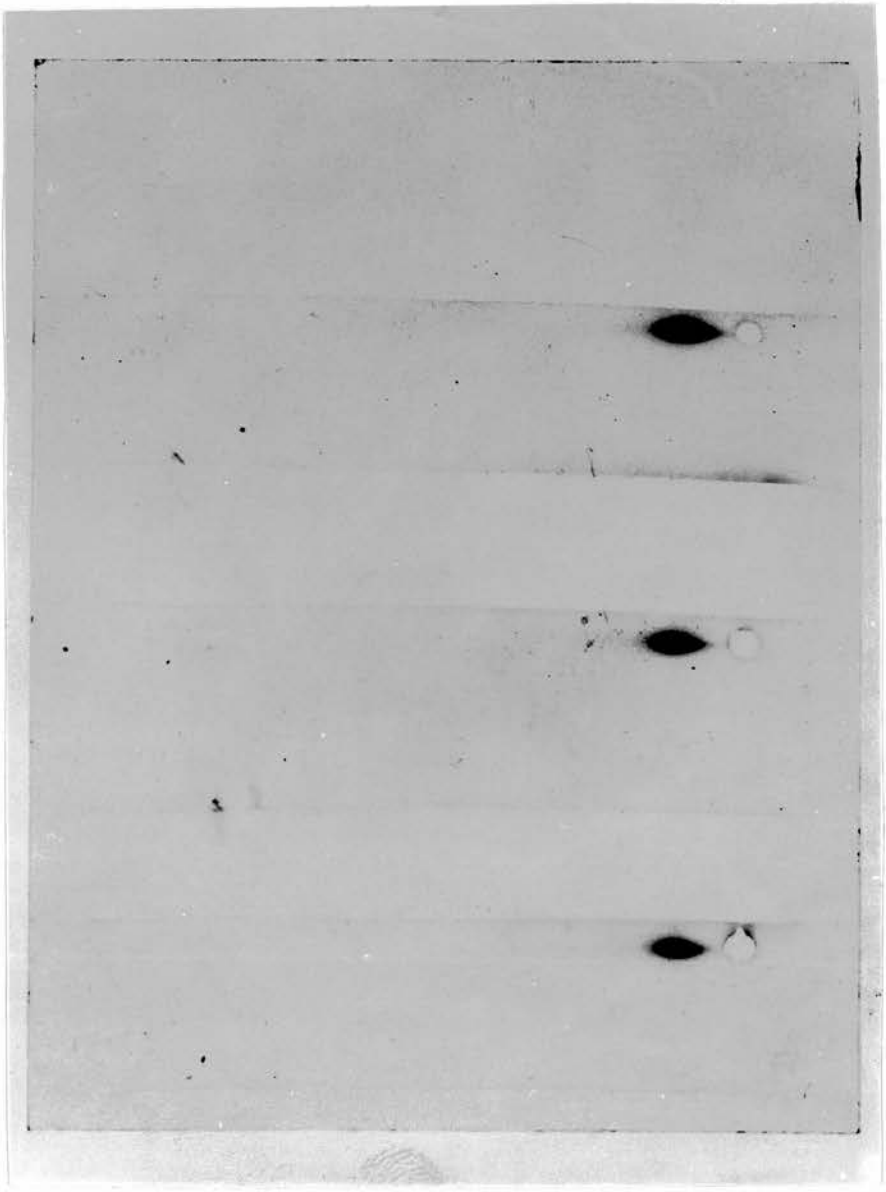
and so redissolves the antigen:antibody complexes except at the outer edges. The precipitin band of the arc forms as each antigen reacts with an equivalent amount of specific antibody. As more antigen is precipitated at the edges the amount of antigen decreases until it is precipitated at the leading edge or top of the precipitin arc. If there are a variety of antigens and antibodies present the specific precipitin arcs may be superimposed on each other and/or form overlapping arcs.

Photograph 11 shows that the purified toxin moved as a single spot in the first-dimensional gel. Photographs 12 and 13 show that the purified toxin formed a single rocket peak (arc) from the single spot in the first dimension. Photograph 12 shows the precipitin arc stained with CBB, which is able to stain any superimposed precipitin arcs and Photograph 13 is of a precipitin arc complexed to Mg^{2+}-ANS (Section 2.4.3.) which appears to enhance the outer edges of the precipitin arcs; and so would show any overlapping arcs. The single precipitin arc for purified toxin is in contrast to the multiple arcs formed from a sample taken from the 2M NaCl wash of the DEAE-cellulose ion-exchange column, Photograph 14. At least six different precipitin arcs are visible and are composed of superimposed and overlapping arcs. It can be concluded that the antitoxin used contained several different types of antibodies including antibodies to tetanus toxin and that after the purification of tetanus toxin the contaminating antigens are removed.

3.1.4.4. Analytical Ultracentrifugation

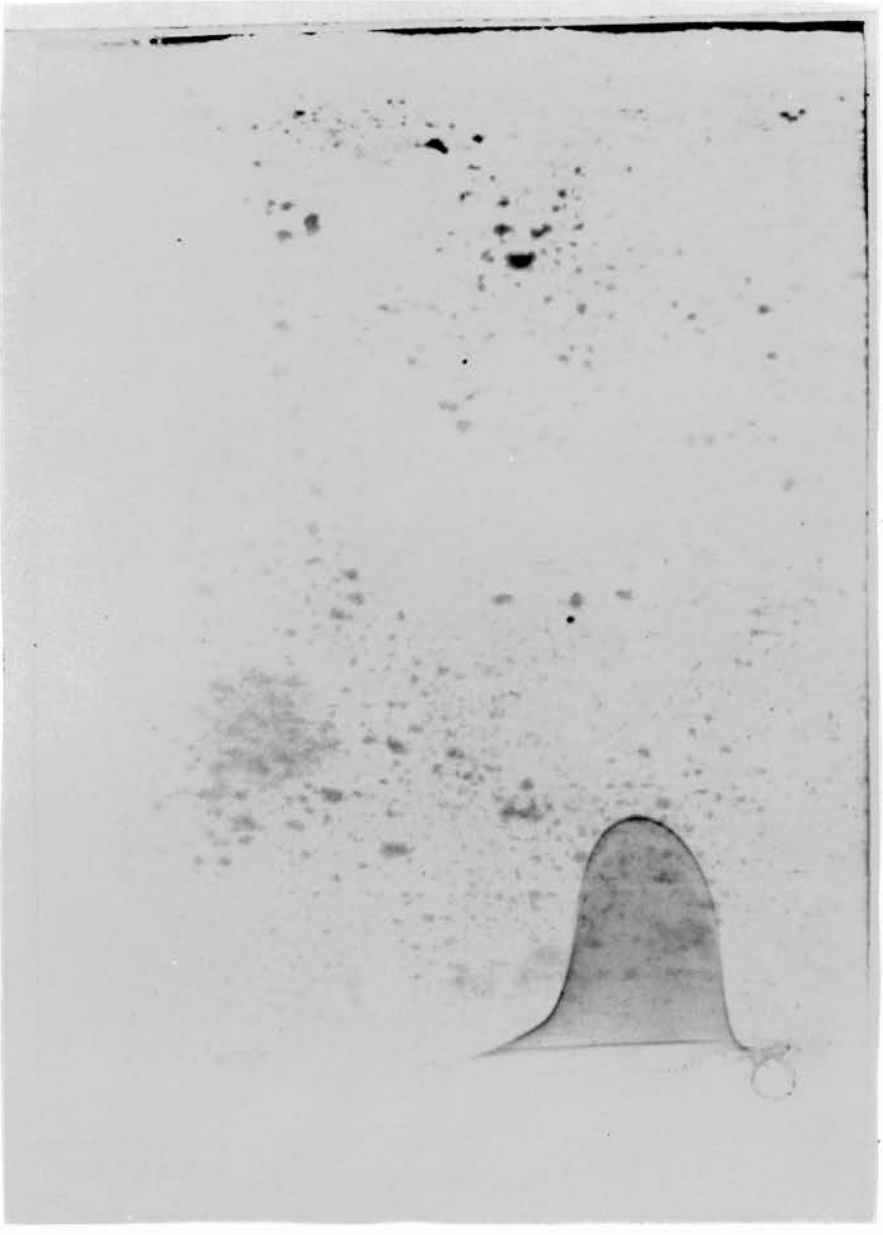
A final method to check the purified tetanus toxin was using the analytical ultracentrifuge, initially by a sedimentation velocity run and later by a sedimentation equilibrium run (as described in Section 2.16.). The sedimentation velocity run gave a sedimentation constant $s_{20,w}$ equal to 7.26S for tetanus toxin, as well as information about the purity of the sample. The purified sample of tetanus toxin used was from batch No. TD 812B and was centrifuged in a Spinco analytical ultracentrifuge (Model E) as described in Section 2.16.1.

ORIGIN



PHOTOGRAPH 11. 1ST-DIMENSIONAL ELECTROPHORESIS OF TETANUS TOXIN FOR 2-DIMENSIONAL IMMUNOELECTROPHORESIS

See Section 3.1.4.3. for details.
The electrophoretic separation was carried out at pH 8.6 in 1% agarose.



PHOTOGRAPH 12. 2-DIMENSIONAL ELECTROPHORESIS OF PURIFIED TOXIN FOR 2-DIMENSIONAL IMMUNOELECTROPHORESIS USING CBB AS STAINING REAGENT

See Section 3.1.4.3. for details. The photograph shows the characteristic rocket precipitin arcs produced by the interaction of tetanus toxin with tetanus anti-toxin in the second gel. The gel was stained using CBB which appeared to enhance any superimposed ones.

PHOTOGRAPH 13. 2ND-DIMENSIONAL ELECTROPHORESIS OF PURIFIED TOXIN FOR 2-DIMENSIONAL IMMUNOELECTROPHORESIS USING Mg^{2+}-ANS AS STAINING REAGENT

See Section 3.1.4.3. for details. As PHOTOGRAPH 12 except Mg^{2+}-ANS was used to stain the arc which appeared to enhance any overlapping arcs. The arc was visualised by photographing the gel in U.V. light.
ANS bound to protein fluoresces green in the presence of U.V. light.

PHOTOGRAPH 14. 2-DIMENSIONAL IMMUNOELECTROPHORETIC PATTERN OF A PROTEIN SAMPLE ELUTED FROM THE DE-52 COLUMN BY 2M NaCl

See Section 3.1.4.3. for details. The gel was stained by CBB and as can be observed there are at least six precipitin arcs visible. The smaller arc appears to correspond to tetanus toxin.

A single Schlieren peak was observed without any sign of heterogeneity which would have been observable from an asymmetric peak, see Photograph 15. A plot of dlogx versus dt gave a straight line (Diagram 7) again indicating a homogeneous sample; any deviation from a straight line would indicate the presence of more than one protein. The $s_{observed}$ was calculated from the equation :

$$s_{observed} = \frac{2.303}{\omega^2 60} \times \left(\frac{dlogx}{dt}\right)$$

where :- ω $\frac{2\pi}{60} \times N^o$ rev min^{-1}

t length of time of the run

x distance moved from the centre of the rotor axis to the centre of the Schlieren peak

The $s_{observed}$ was found to be 6.47S by using the above equation and the slope of the graph $dlog_{10}x$ versus dt. From the $s_{observed}$ value, the $s_{20,w}$ was determined by using the equation :

$$s_{20,w} = s_{observed}\left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta_{sol}}{\eta_w}\right)\left(\frac{(1-\bar{v}\rho)_{20,w}}{(1-\bar{v}\rho)_{20,w}}\right)$$

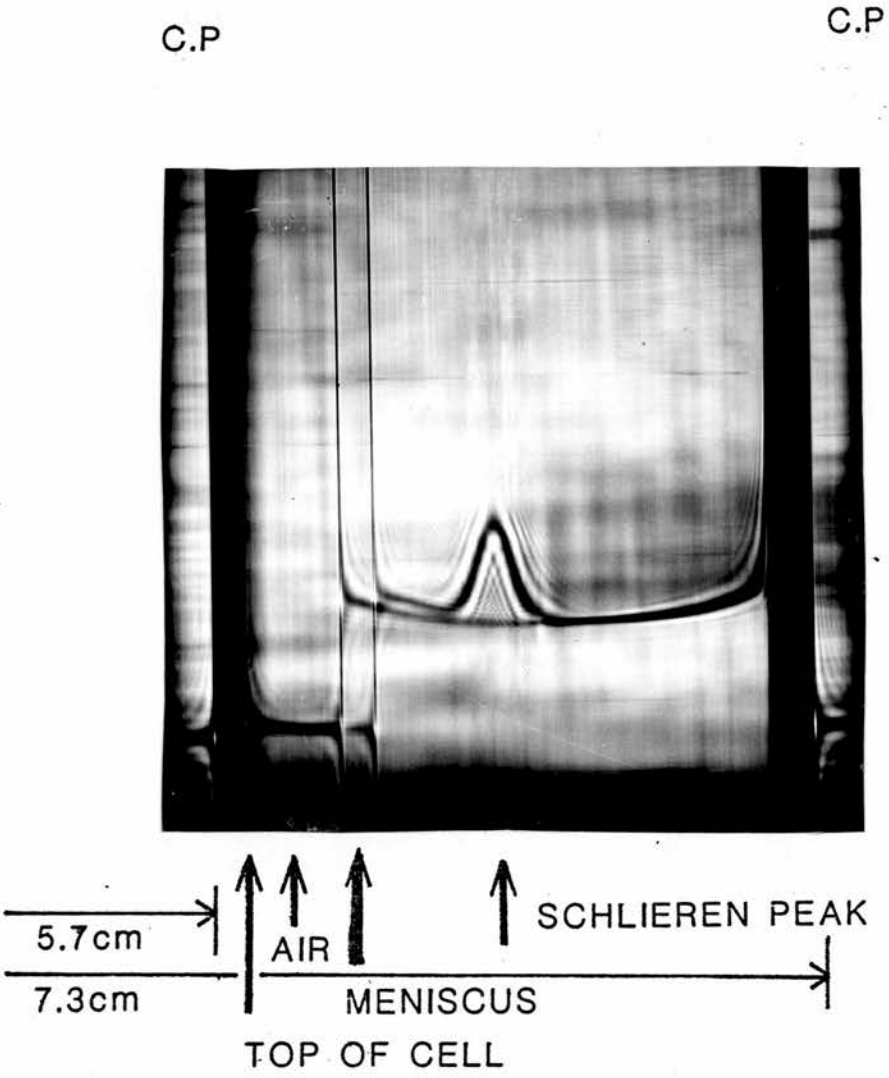
where:- $\bar{v}$ Partial specific volume of tetanus toxin 0.732 from the amino acid analysis as described in Section 3.3.3.4.

η_t Viscosity of H_2O at the run temperature (18^oC)

η_{20} Viscosity of H_2O at 20^oC

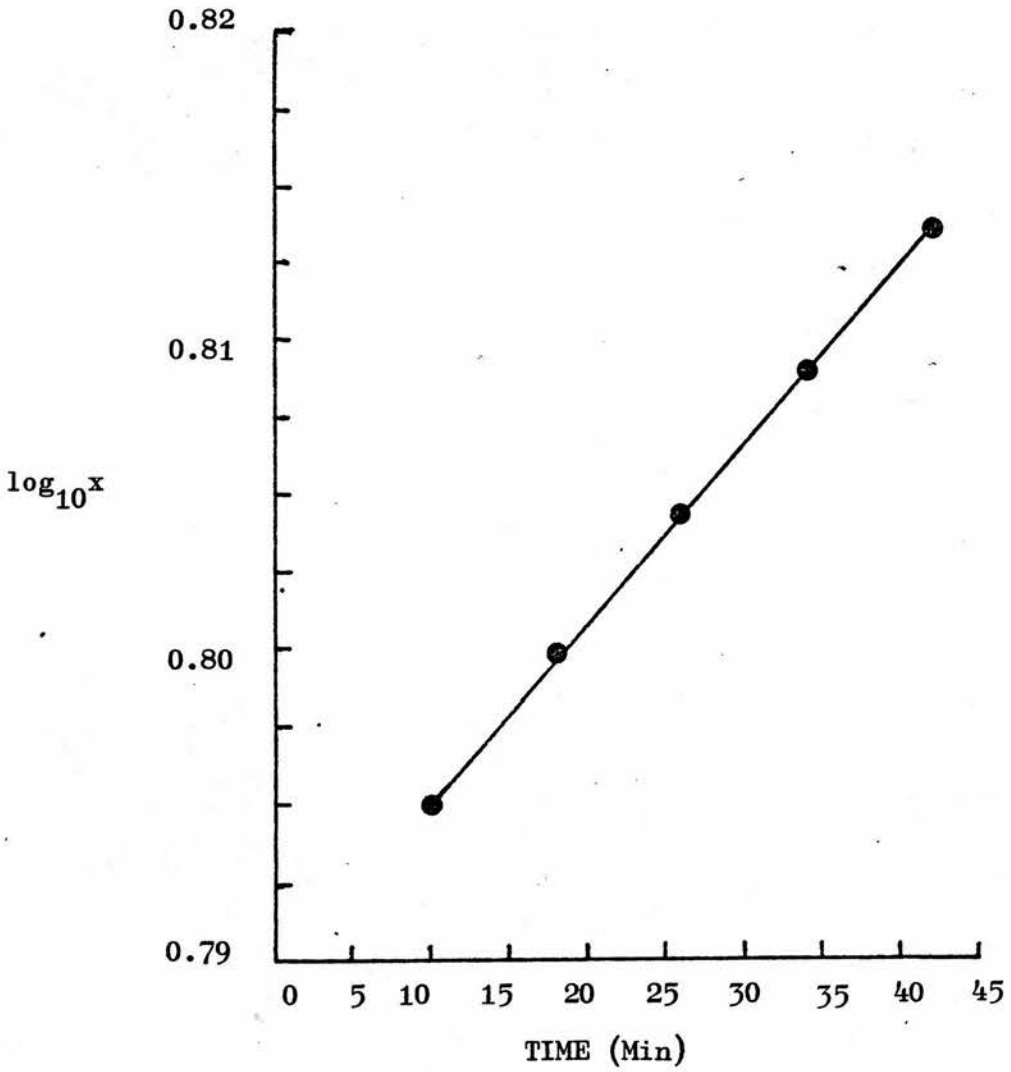
η_{sol} Viscosity of solute

η_w Viscosity of H_2O



PHOTOGRAPH 15. SCHLIEREN PEAK OF PURIFIED TETANUS TOXIN

For details see Section 3.1.4.4. The data refer to the Beckman machine. The distance x is measured from the centre of the rotor axis to the centre of the peak.
C.P. refers to counterpoise reference holes in the centrifugation cell.



GRAM 7. PLOT OF logx VERSUS t FOR A SEDIMENTATION VELOCITY CENTRIFUGATION OF TETANUS TOXIN

See Section 3.1.4.4. for details.

$\rho_{20,w}$ Density of H_2O at 20°C

$\rho_{20,\ sol}$ Density of solute at 20°C

and:-

$$\left(\frac{\eta_t}{\eta_{20}}\right) = 1.052$$

$$\left(\frac{\eta_{sol}}{\eta_w}\right) = 1.032$$

$$\rho_{20,w} = 0.9982$$

$$\rho_{20,sol} = 1.01$$

By using the above data and equations, the $s_{20,w}$ for tetanus toxin was found to be 7.26S. Previous values quoted in the literature were 4.5S (Pillemer *et al.*, 1948), 7.0S (Dawson and Mauritzen, 1967), 6.4S (Murphy and Miller, 1967) and 7.5S (Robinson *et al.*, 1975).

3.1.5. Toxicity of the Purified Tetanus Toxin

A sample of purified tetanus toxin was sent to Dr. J. Mellanby of the Department of Experimental Psychology, University of Oxford who carried out a bio-assay on the toxin to check for any toxicity. There is no *in vitro* assay for tetanus toxin, for determination of toxicity, the only method for determining toxicity is to carry out experiments on whole animals, the *in vivo* bio-assay. The *in vivo* bio-assay used for tetanus toxin was as described by van Heyningen and Mellanby (1971) and consisted of injecting various dilutions of the purified toxin into experimental animals (mice). The toxicity of a sample is determined by measuring the minimum lethal dose (MLD) of the sample. The MLD is in turn determined from the LD_{50} i.e. where 50% of the mice are killed by the toxin at the particular dose used within 4 - 7 days after the injection. The bio-assay has to be carried out by an experienced

person with a specially obtained license to practise on experimental animals. In practice the animals are not allowed to die of tetanus because of the distressing symptoms of the disease as described in Section 1.1. A mouse is usually injected intramuscularly in the hind limb with $0.5cm^3$ of the diluted toxin sample, characteristic and clearly definable symptoms of increasing severity occur in a given time by increasing doses of toxin. Mellanby et al. (1968a) have assigned numerical values to the type and time of the symptoms of tetanus observed. The amount of toxin which produces symptom 3 (a limp in the injected limb with no impediment in walking) by day 3 is interpreted as equivalent to one LD_{50} dose, death would follow by day 6 so the animal is usually killed to prevent further distress (van Heyningen and Mellanby, 1971).

The sample of purified tetanus toxin sent to Dr. Mellanby was found to have a higher MLD than an equivalent amount of unpurified toxin, i.e. from equivalent amounts of protein. Unfortunately not all the fractions obtained from the DEAE-cellulose column could be tested for toxicity because of the cost of the bio-assay. But from the information obtained it was quite obvious that the protein suspected of being tetanus toxin was capable of giving rise to the disease tetanus.

3.1.6. The Use of PMSF and Benzamidine

Two experiments were carried out to try and show the presence of a protease in unpurified toxin samples that was capable of cleaving 'unnicked' toxin into 'nicked' toxin and that the protease was sensitive to PMSF and benzamidine.

In the first experiment, unpurified freeze-dried tetanus toxin (batch No. TD 812B) was dissolved in 0.04M sodium phosphate buffer at pH 7.2, with and without 0.1mM PMSF and 1mM benzamidine. The samples were incubated at 37^oC for 3h and $0.1cm^3$ samples were taken at intervals in order to determine the amount of digestion. The $0.1cm^3$ samples were immediately made 5% (w/v) with trichloroacetic acid (TCA) and the proteins were allowed to precipitate at 0^oC for 20 min. The precipitated proteins were centrifuged down and washed with water before being solubilised in undiluted SDS-dissociation buffer (Section 2.5.1.2.), in the presence of 2-ME by

boiling for 3 min. The solubilised proteins were then electrophoresed into 8% polyacrylamide gels according to the method of Laemmli as described in Section 2.5.1.2.

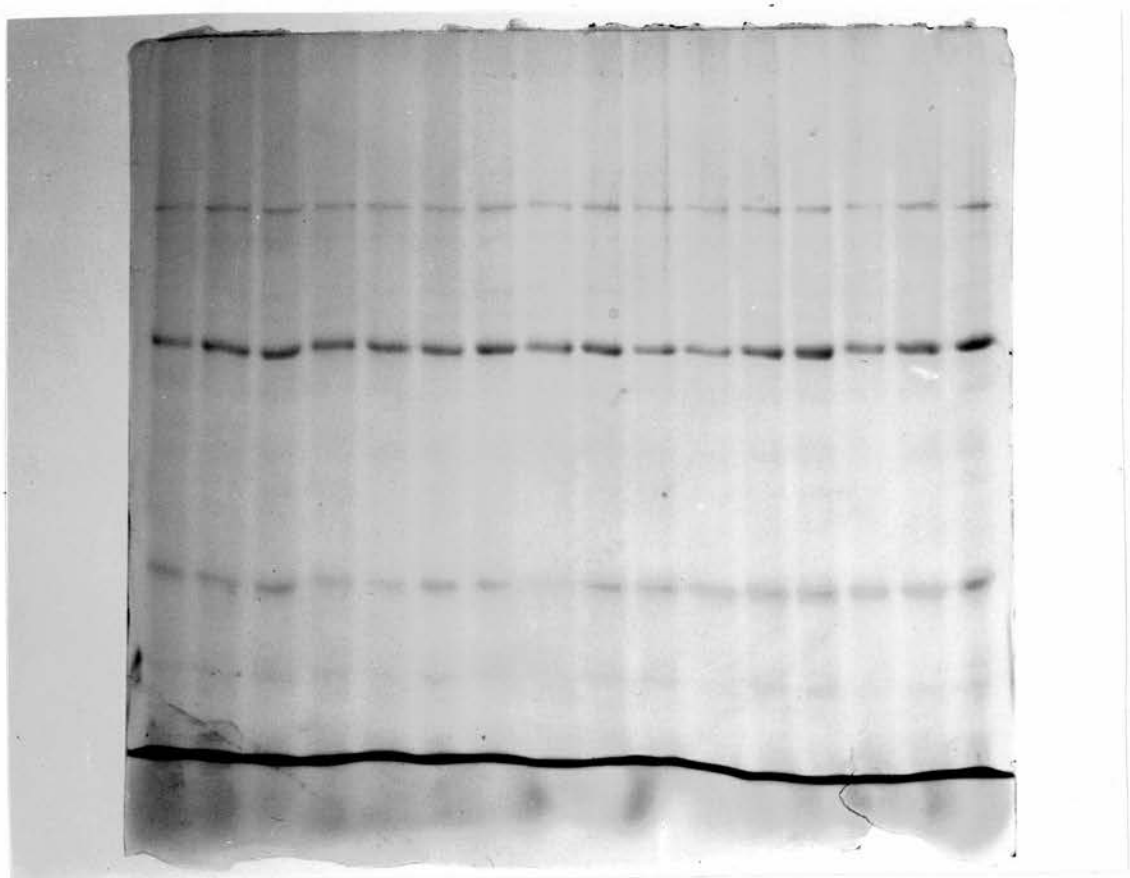
From Photograph 16, where PMSF and benzamidine were used, there is no observable digestion of the 'unnicked' toxin. In contrast, Photograph 17, shows that there was some digestion of 'unnicked' toxin after an incubation of 150 min in the absence of PMSF and benzamidine.

In the second experiment tetanus toxin was again dissolved in 0.04M sodium phosphate buffer at pH 7.2 in the presence and absence of PMSF and benzamidine. But this experiment was carried out at 30°C over 5 days. 0.1 cm^3 samples were once again taken at various times but this time immediately frozen and stored at -20°C. The samples were once again analysed by SDS-PAGE as described above in order to check the samples for any proteolysis.

Photograph 18 shows that there was digestion of 'unnicked' tetanus toxin and there is also the appearance of another band below H-chain, possibly due to some further digestion of either whole toxin or H-chain (this band was sometimes observable in some fractions of purified toxin obtained from earlier batches of toxin). There is some decrease in 'unnicked' toxin in the protease treated samples, probably because of the hydrolysis and inactivation of PMSF, but the samples having a low amount of 'unnicked' toxin also have correspondingly lower amounts of H-chain and L-chain indicating a variability in the amount of sample electrophoresed into the gel. Photograph 19 shows the untreated samples alone and there is a distinct decrease in the amount of 'unnicked' toxin present.

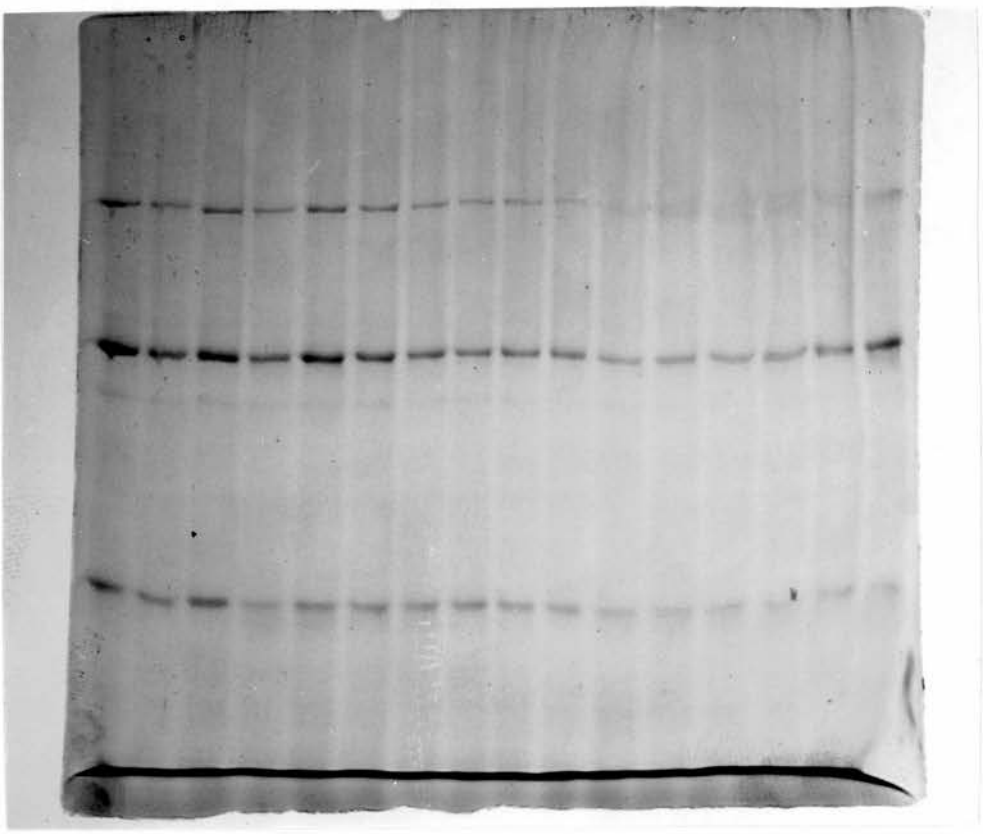
3.1.7. Further Purification of Tetanus Toxin

Tetanus toxin can be further purified using the method of van Heyningen (1976a) by gel-permeation chromatography. This purification step followed from the ion-exchange chromatography procedure. The purified fractions of tetanus toxin from the ion-exchange column were pooled and then concentrated using an Amicon ultrafiltrator with a PM30 membrane (retains macromolecules with molecular weight greater than 30 000 daltons). Tetanus toxin was found to be denatured or irreversibly aggregated when concentrated using ultra-filtration so its use was avoided if possible. After the toxin was concentrated it was then dialysed against 0.1M Tris-HCl,



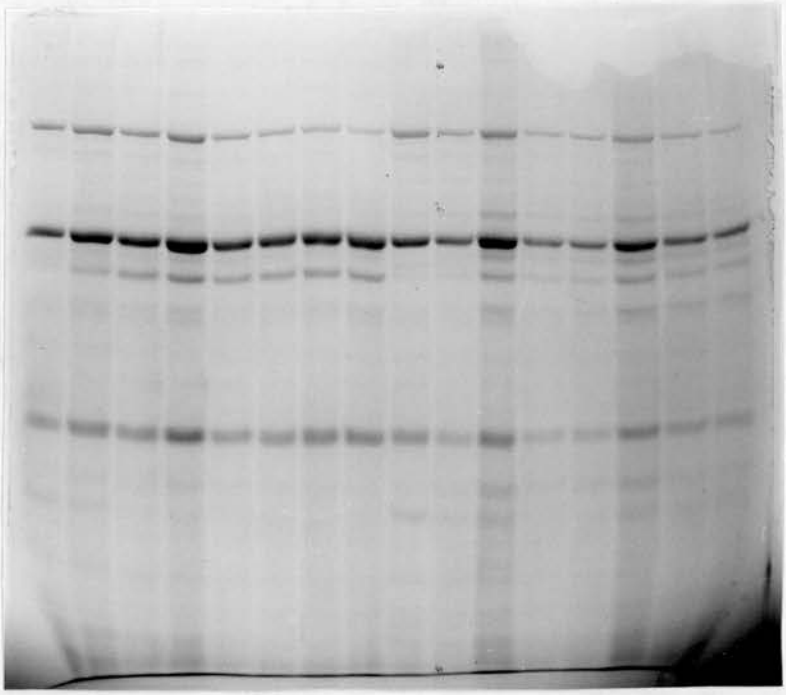
PHOTOGRAPH 16. TCA PRECIPITATED TOXIN AFTER INCUBATION IN THE PRESENCE OF PMSF AND BENZAMIDINE

For details see Section 3.1.6.
As can be observed there is no decrease in the amount of 'unnicked' toxin.



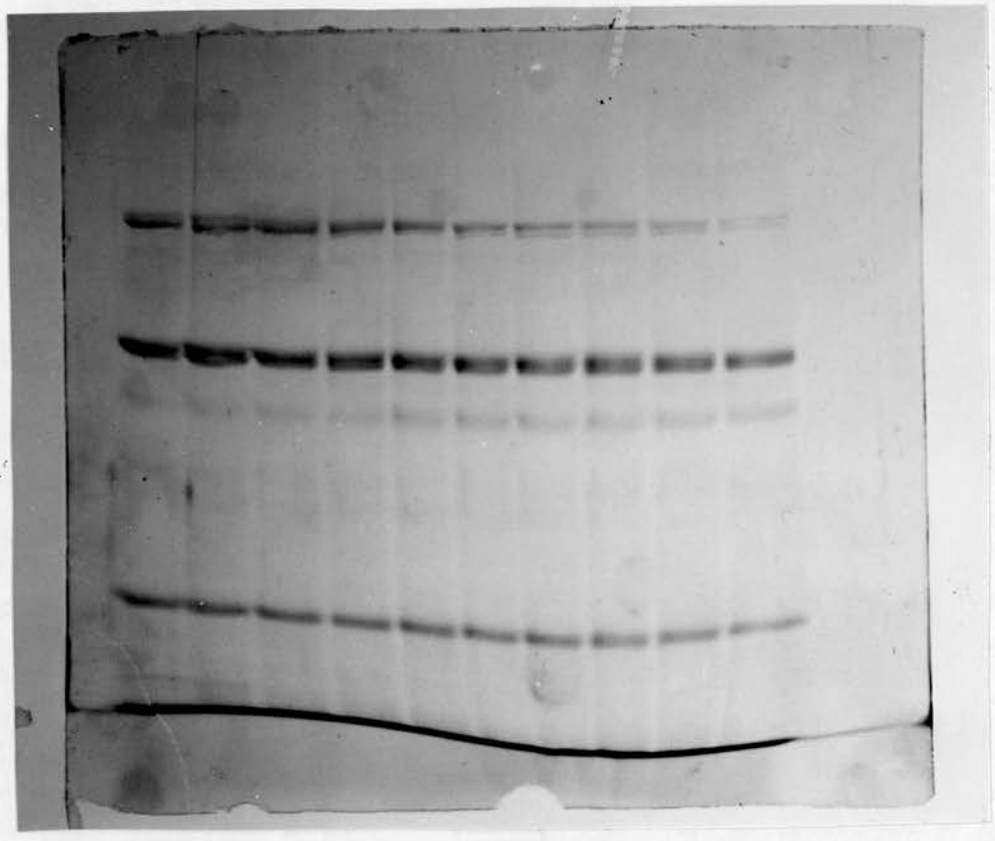
PHOTOGRAPH 17. TCA PRECIPITATED TOXIN AFTER INCUBATION IN THE ABSENCE OF PMSF AND BENZAMIDINE

For details see Section 3.1.6.
As can be observed the amount of 'unnicked' toxin slowly decreases.



PHOTOGRAPH 18. DIGESTION OF 'UNNICKED' TOXIN OVER A FIVE-DAY PERIOD IN THE PRESENCE AND ABSENCE OF PMSF AND BENZAMIDINE

See Section 3.1.6. for details.
Tracks 1-8 represent samples without PMSF and benzamidine.
Tracks 9-16 represent samples containing PMSF and benzamidine.
As can be observed there is a decrease in the amount of 'unnicked' toxin in the untreated samples with progressive increase in digestion product D. There is little digestion of 'unnicked' toxin in treated samples.



PHOTOGRAPH 19. DIGESTION OF 'UNNICKED' TOXIN OVER A FIVE-DAY PERIOD IN THE ABSENCE OF PMSF OR BENZAMIDINE

See Section 3.1.6. for details.
The gel shows there was progressive digestion of 'unnicked' toxin with the increase of a digestion product D. There appeared to be no decrease in the amounts of H- and L-chain. The tracks refer to the different sample times 0, 2, 4, 7, 20.5, 25, 29, 45, 71.5 and 119.5 h.

0.2M NaCl and 1mM EDTA pH 7.5 (later 0.1mM PMSF and 1mM benzamidine were also used). The toxin was then further purified by gel-permeation chromatography on an Ultrogel AcA34 column (2.6cm by 100cm) using the above buffer system.

This method was found to be unnecessary after the introduction of both PMSF and benzamidine in the DEAE-cellulose ion-exchange chromatography procedure. In fact this method also caused lower yields of purified toxin due to the ultrafiltration step.

3.2. Separation and Purification of the Two Chains Derived from 'Nicked' Tetanus Toxin

3.2.1. Introduction

Craven and Dawson (1973) were able to partially separate H- and L-chain derived from 'extracellular' (later known as 'nicked') tetanus toxin. They used the oxidative sulphitolysis method of Chang (1969) to break the disulphide bridges, known to hold the two chains together in the "native" structure of tetanus toxin. Craven and Dawson (1973) showed that oxidative sulphitolysis in the presence of 8M urea caused the scission of the two chains present in 'extracellular' toxin allowing their separation by PAGE but in contrast the two chains in 'intracellular' (later known as 'unnicked') toxin could not be separated using PAGE. Craven and Dawson (1973) also attempted to purify the two chains after oxidative sulphitolysis using gel-permeation chromatography on Bio-Gel A-15 in the presence of 7M urea. By using 15mg of tetanus toxin they were able to obtain two poorly resolved protein containing fractions, with molecular weights of 95 000 daltons and 55 000 daltons after analysis with SDS-PAGE. They also observed that the H-chain aggregated in the presence of urea.

Dawson (1975) carried out oxidative sulphitolysis in the presence of Cu^{2+} and was able to show considerable scission of both 'unnicked' and 'nicked' toxin. The cleavage products of 'unnicked' toxin led Dawson to suggest that sulphitolysis in the presence of Cu^{2+} was likely to involve cleavage at at least three different sites in addition to the scission of the disulphide bridges. Dawson (1975) carried out the experiments for ten days at 6°C in the presence of 30mM Na_2SO_3, 50µM $CuSO_4$ and 70mM sodium phosphate at pH 7.5 or in 10mM EDTA, 50mM glycine and 70mM sodium phosphate at pH 7.5 in the presence or absence of 30mM Na_2SO_3. He was able to show that the original MLD values of the toxin were maintained in the sodium phosphate buffers containing EDTA and glycine. But, in contrast, there was a hundred-fold decrease in toxicity of the toxin after sulphitolysis in the presence of

Cu^{2+}. It was concluded that the toxin was protected from sulphitolysis because of the removal of trace heavy metal ions by the EDTA. Analysis by SDS-PAGE showed that oxidative sulphitolysis in the presence of Cu^{2+} caused considerable cleavage in both 'unnicked' and 'nicked' toxins and in contrast there was very little cleavage in the presence of EDTA. Dawson (1975) also showed that there was very little change in the antigenicity of the toxin even though there was a large decrease in the toxicity, and several cleavage products were formed after oxidative sulphitolysis in the presence of Cu^{2+}. In all the experiments carried out by Dawson (1975) no protease inhibitors were used. Thus the results of the oxidative sulphitolysis experiments could be due to proteolysis, especially when considering the cleavage of 'unnicked' toxin, as Dawson (1975) suggested that peptide bonds may be susceptible to cleavage by sulphite. There appears to be no scission products in the presence of EDTA, possibly because of inactivation of contaminating proteases by the EDTA.

Matsuda and Yoneda (1975) dissociated the two chains by using 100mM dithiothreitol (DTT) in the presence of 4M urea at pH 8.5. Van Heyningen (1976a) modified the method by using 8M urea rather than 4M urea because of his observation of incomplete dissociation of the two chains in 4M urea. Matsuda and Yoneda (1975) were also able to separate the two dissociated chains by gel-permeation chromatography using Ultrogel AcA44 in the presence of 1mM DTT and 2M urea at pH 8.5. Matsuda and Yoneda (1976) went onto show that tetanus toxin could be reconstituted from the purified chains by mixing them and dialysing away the DTT and urea. The reconstituted toxin was shown to be toxic, suggesting that the reconstituted toxin was very similar in structure to "native" toxin even if it did not have the same conformational structure as "native" toxin.

Helting and Zwisler (1977) used the method of Matsuda and Yoneda (1975) to purify the two constituent chains of tetanus toxin, but they observed an extra protein peak eluted after H-chain. The new peak was identified as undissociated tetanus toxin after analysis by SDS-PAGE. Helting and Zwisler (1977) suggested from their observations that the method of chain dissociation described by Matsuda and Yoneda (1975) did not always go to completion and

could consequently lead to the isolation of some undissociated toxin. None of the chromatographic methods are really satisfactory; all end up with considerable cross-contamination of the two chains.

Neubauer and Helting (1979) published another method of purifying the two constituent chains of tetanus toxin, which was independently confirmed at the time by work being carried out for this thesis. Neubauer and Helting (1979) and Britton and van Heyningen (1980) purified the two constituent chains by preparative SDS-PAGE. Neubauer and Helting (1979) dissociated tetanus toxin into its two chains in 1% (w/v) SDS and 5% (v/v) 2-ME and then separated them by PAGE according to the method of Weber and Osborn (1969). The positions of the H- and L-chains were identified by staining a few gels and the relevant regions of unstained gels were removed and homogenised in 0.03% (w/v) SDS and the proteins eluted from the gels overnight. This was in contrast to the earlier work of Helting and Zwisler (1977), where the gel-permeation chromatography method of Matsuda and Yoneda (1975) was preferred.

3.2.2. Methods and Results for the Separation and Purification of H-Chain and L-Chain Derived from 'Nicked' Tetanus Toxin

3.2.2.1. Gel-Permeation Chromatography

In order to separate and purify the two constitutive chains of tetanus toxin (H- and L-chains) to homogeneity, the gel-permeation chromatographic method initially described by Matsuda and Yoneda (1975) but modified by van Heyningen (1976a) was used.

Tetanus toxin ($10cm^3$), purified by DEAE-cellulose ion-exchange chromatography, was concentrated by using an Amicon ultrafiltrator with a UM10 membrane (which has a cut off at molecular weight 10 000 daltons) to about $2cm^3$ (1.5mg). Unfortunately, as mentioned earlier, concentrating tetanus toxin by this procedure caused losses either by denaturation on the membrane surface or by irreversible aggregation of the toxin at higher protein concentrations. The concentrated toxin (1.5mg of protein, measured by absorbance at 280nm) was made 8M with urea and incubated at $37^{\circ}C$ for 30 min in order to unfold the protein structure

of the toxin, PMSF and later benzamidine with PMSF were added to the solution in order to prevent proteolytic digestion of the toxin, observed by van Heyningen (1976a). An explanation for this observation could be that any contaminating proteases that were initially inactive would be activated by the denaturing conditions and the susceptibility of the toxin to proteases could be increased by the increase in the number of labile bonds as a result of denaturation in 8M urea. After the denaturation step the toxin was made 100mM with DTT and incubated at 37°C for 1.5h in order to reduce any disulphide bridges made available by denaturation in 8M urea; the high concentration of reductant inhibited the reformation of any disulphide bridges. The toxin was then directly chromatographed on an Ultrogel AcA44 gel-permeation column (1cm by 100cm) and eluted with 0.05M Tris-HCl, 0.6M glycine, 1mM EDTA, 2M urea, 0.3M NaCl, 0.01% (w/v) NaN_3, 1mM benzamidine, 0.1mM PMSF and 2mM DTT at pH 8.5. The column was previously equilibrated overnight with fresh DTT and the eluent checked for the presence of free thiol groups, (present as un-oxidised DTT) by using DTNB (Ellman's reagent) as described in Section 2.13., before the toxin sample was chromatographed. Initially 0.3M NaCl was included in the elution buffer, as described above, because polyacrylamide containing gel matrices tend to adsorb proteins. In order to prevent the non-specific adsorption of proteins the manufacturer advise the use of elution buffers with a high ionic strength. It was found later that the use of 0.3M NaCl prevented the separation of the two constituent chains of tetanus toxin and so it was left out of the above buffer system. However 0.3M NaCl was included in the above buffer for calibration of Ultrogel AcA44 columns as some of the standard proteins, e.g. cytochrome **c**, were found to be adsorbed to the gel matrix and required 2M NaCl to wash them from columns run in the absence of 0.3M NaCl.

A number of experiments were carried out as described above to try and separate and purify the constituent chains of tetanus toxin but without any success. Analysis of the reduced and denatured toxin samples used in the chromatographic step, by SDS-PAGE in the absence of 2-ME, indicated that the two chains had been completely dissociated although they had not separated. The eluent from the column did not drop below 1mM DTT throughout the chromatographic procedure, from analysis with DTNB.

The pre-incubation step was modified. The toxin was reduced first in the presence of 100mM DTT for 1 h at 37°C and then incubated in 8M urea for 3 h at 37°C. The sample was then chromatographed as described above but again there was no separation of the two chains by the chromatographic procedure.

The modified pre-incubation was tried again but using 200mM N-acetyl-L-cysteine (BDH) instead of 100mM DTT as the reducing agent. The elution buffer was as described above except 4mM N-acetyl-L-cysteine was used instead of 2mM DTT. The N-acetyl-L-cysteine was used instead of DTT in order to try and determine whether the DTT was somehow oxidised and rendered inactive as a reducing agent in either the pre-incubation step or the chromatographic step (though this was unlikely because of the positive DTNB reactions). Results using N-acetyl-L-cysteine indicated that there was a slight improvement in the purification of the two chains. Analysis of the fractions by SDS-PAGE indicated that H-chain was being eluted at a higher concentration and slightly before than L-chain though L-chain still contaminated H-chain.

A new approach was tried, using iodo-acetic acid (IAA) in order to S-carboxymethylate the reduced thiol groups thereby preventing any re-oxidation and subsequent re-formation of disulphide bridges, possibly leading to the linking of the two chains or the linking of two similar chains. The S-carboxymethylation was carried out essentially according to the method of Hirs (1967b). Purified tetanus toxin (6mg) was initially denatured in 8M urea in the presence of 0.5M Tris-HCl (to keep the pH of the reaction constant and allowing only S-carboxymethylation and not carboxymethylation of histidine residues which occurs at pH 6), 0.2% (w/v) EDTA (to prevent oxidation of thiols to disulphides by contaminating heavy metal ions) and 1mM benzamidine at pH 8.5. The solution was de-gassed and flushed with N_2 to slightly above atmospheric pressure and then incubated at 37°C for 1 h to allow unfolding of the protein structure with the increase in the availability of disulphide bridges. After the denaturation step the solution was made 0.6mM with DTT, flushed with N_2, and incubated at 37°C for 4 h for complete denaturation and reduction of the disulphide bridges. After the reduction step, 1.3mM recrystallised IAA in $0.5cm^3$ 1M NaOH was added to the solution (the pH was observed to fall from pH 8.5 to pH 4.5 so was immediately altered to pH 8.5

with 4M NaOH) in the dark and then incubated at 37°C for 1 h. The sample was then chromatographed on an Ultrogel AcA44 column and eluted with the buffer system previously described but without any reducing agent present. Again there was no real separation of the two chains (see Diagram 8) though the later fractions eluted from the column contained a higher proportion of L-chain when analysed by SDS-PAGE.

The experiment was repeated but the S-carboxymethylated toxin was de-salted on a Sephadex G-25 column (2cm by 20cm) using the buffer system described for the Ultrogel AcA44 chromatographic step. The fractions obtained from the Sephadex G-25 column were analysed for absorbance at 280nm and the toxin-containing fractions were pooled and freeze-dried. The freeze-dried sample of toxin was redissolved in the Ultrogel AcA44 elution buffer; urea was found to be necessary as the S-carboxymethylated toxin was less soluble than "native" toxin, and then chromatographed on an Ultrogel AcA44 column as described above. Again there was no observable purification of the two chains, though analysis of S-carboxymethylated toxin by SDS-PAGE in the absence of 2-ME indicated that the two chains had been dissociated.

The sulphitolysis method of Cole (1967) and Chan (1968) as described by Dawson (1975) was tried but using the Ultrogel AcA44 column as described above, but again without any success (see Diagram 9).

A sample of purified toxin (1.8mg) was treated using the mild trypsinolysis method of Matsuda and Yoneda (1974). The trypsinolysis experiment was carried out to make sure that the toxin being used was not a single polypeptide. The toxin sample was exhaustively dialysed against 0.05M Tris-HCl and 1mM EDTA pH 8.2 and then treated with 1.8µg of DPCC-trypsin (diphenyl carbonyl chloride-trypsin, Servac Laboratories), a form of trypsin specially treated in order to inactivate any contaminating chymotrypsin, at 25°C for 1 h. The trypsin activity was stopped by the addition of 2.7µg of PMSF. The sample was then treated in 100mM DTT and 8M urea as described above before being chromatographed on an Ultrogel AcA44 column as described above. There appeared to be no separation of the two chains (see Diagram 10) ruling out the possibility of the toxin being a single polypeptide.

DIAGRAM 8. ELUTION PROFILE OF S-CARBOXYMETHYLATED TETANUS TOXIN ON ULTROGEL AcA44

See Section 3.2.2.1. for details.

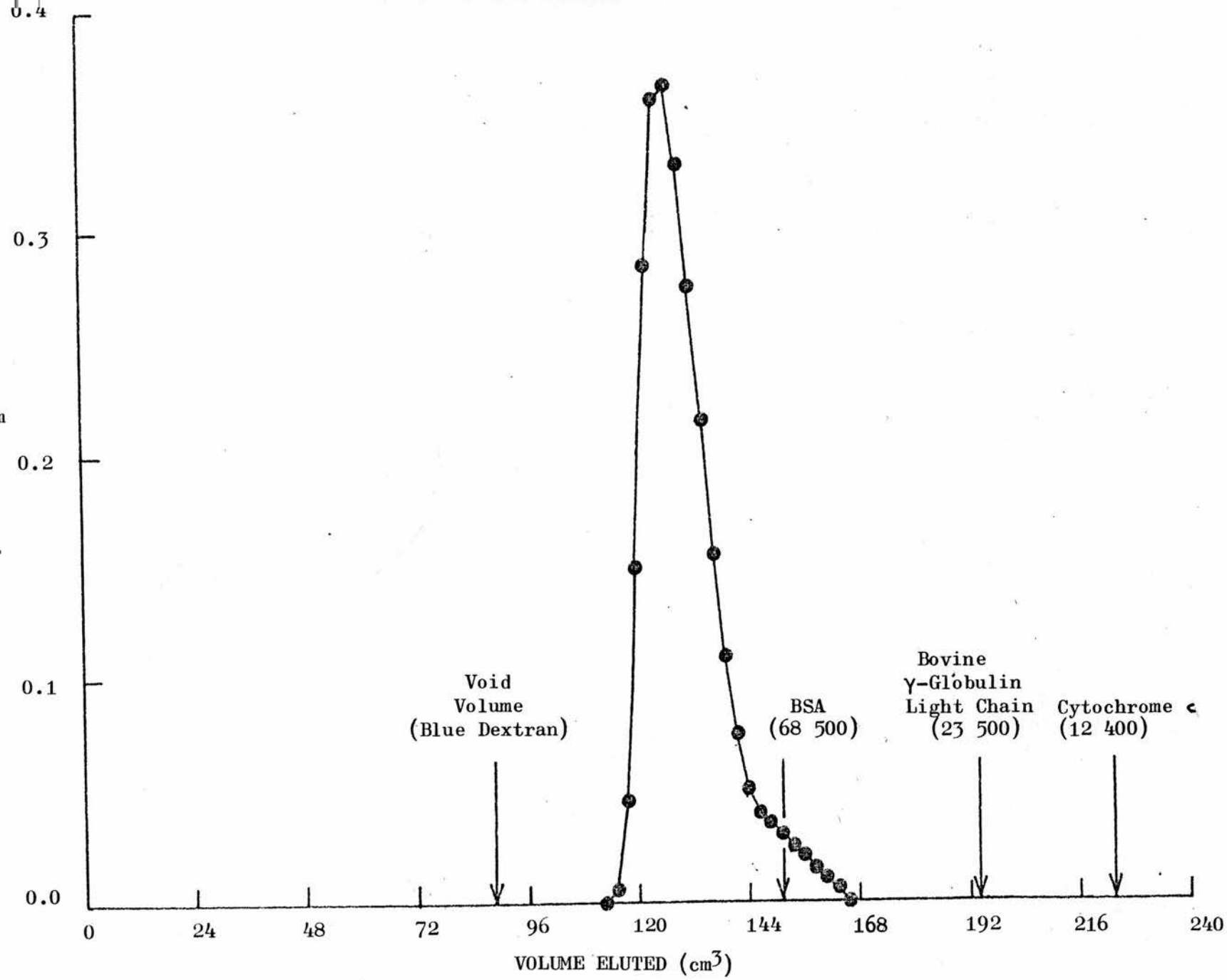
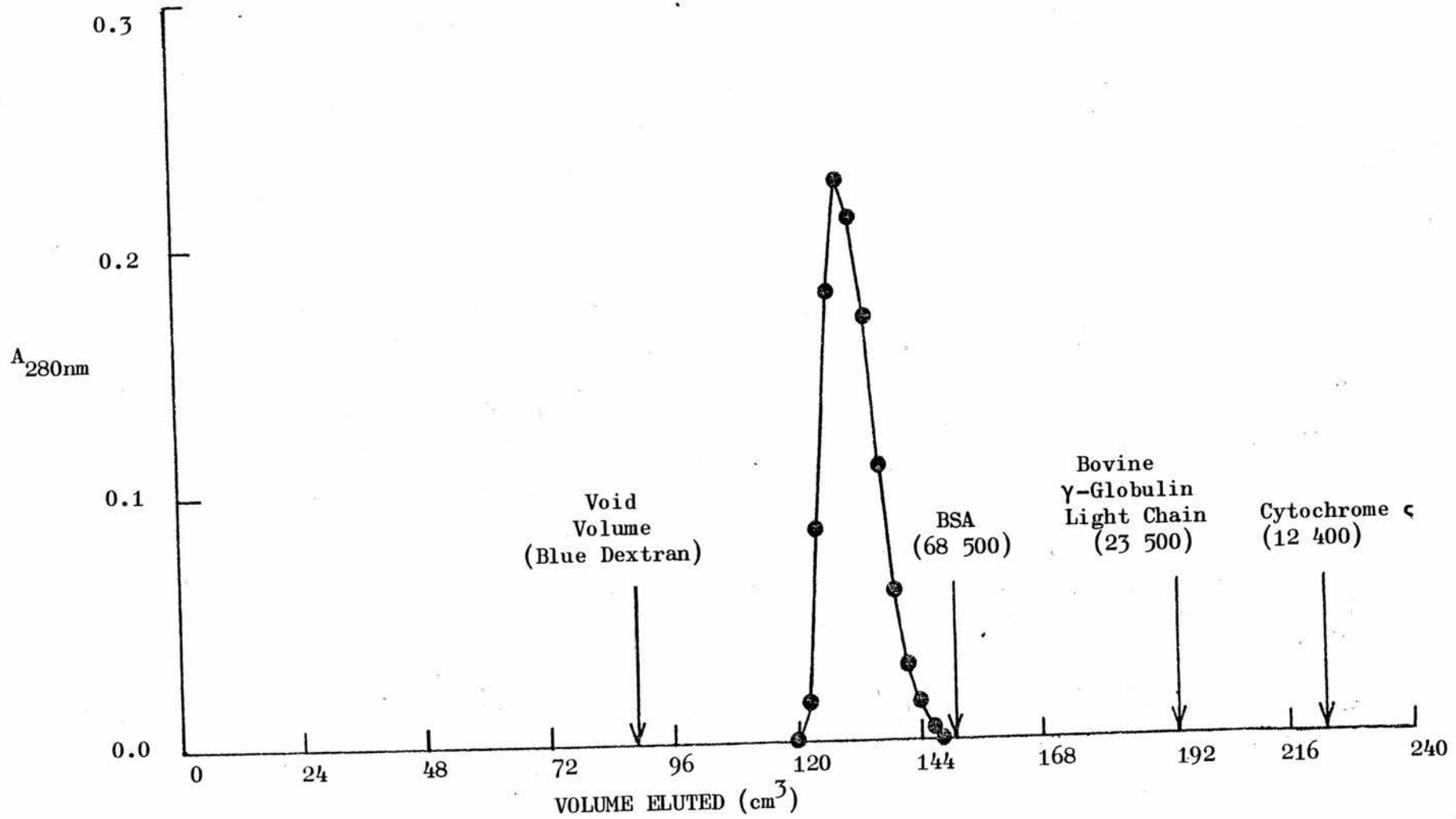
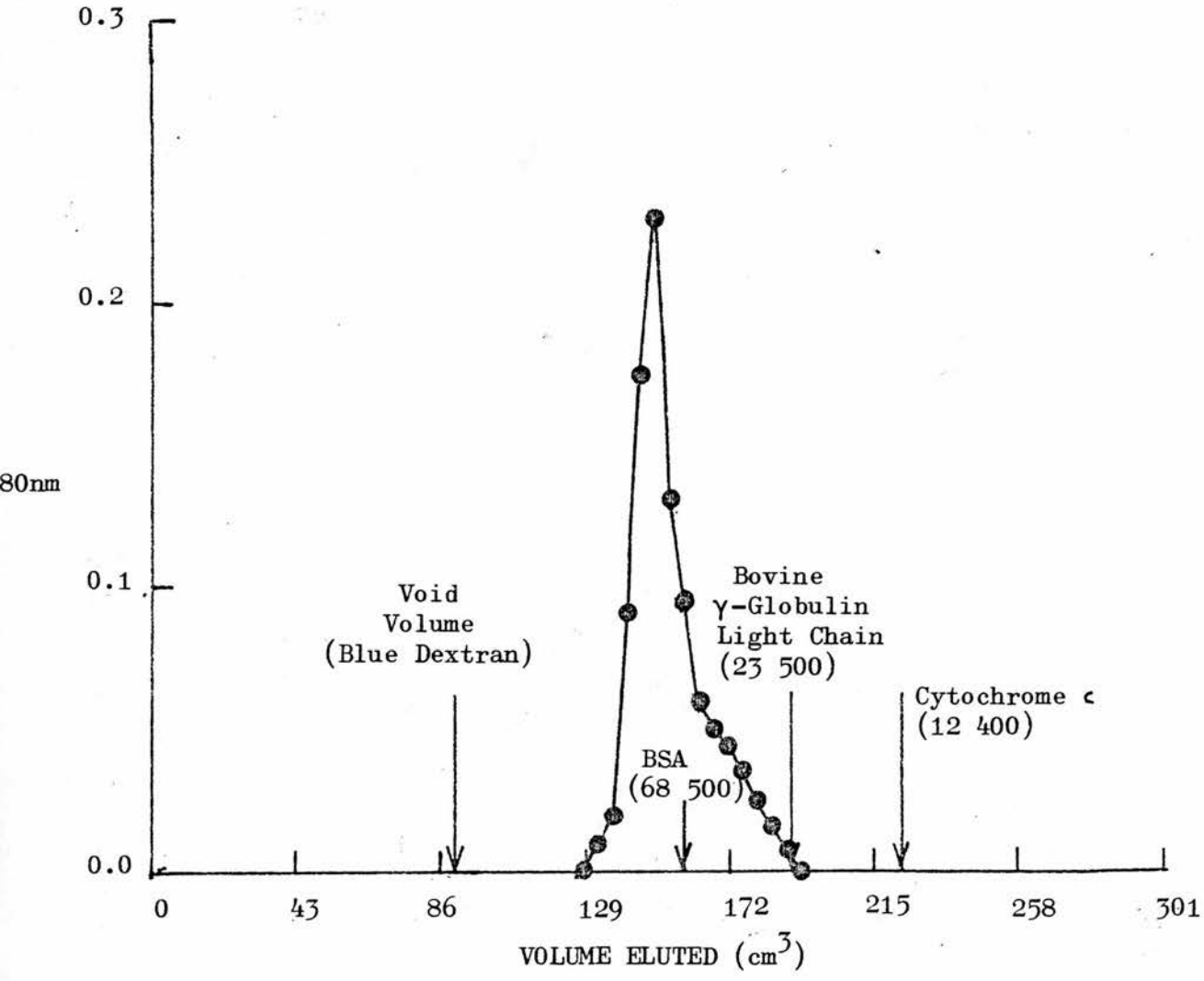

0.4
0.3
0.2
0.1
0.0
A_{280nm}
Void
Volume
(Blue Dextran)
BSA
(68 500)
Bovine
γ-Globulin
Light Chain
(23 500)
Cytochrome c
(12 400)
0
24
48
72
96
120
144
168
192
216
240
VOLUME ELUTED (cm^3)

DIAGRAM 9. ELUTION PROFILE OF TETANUS TOXIN, AFTER OXIDATIVE SULPHITOLYSIS, ON ULTROGEL AcA44

See Section 3.2.2.1. for details.


0.3
0.2
0.1
0.0
A_{280nm}
Void
Volume
(Blue Dextran)
BSA
(68 500)
Bovine
γ-Globulin
Light Chain
(23 500)
Cytochrome c
(12 400)
0
24
48
72
96
120
144
168
192
216
240
VOLUME ELUTED (cm^3)



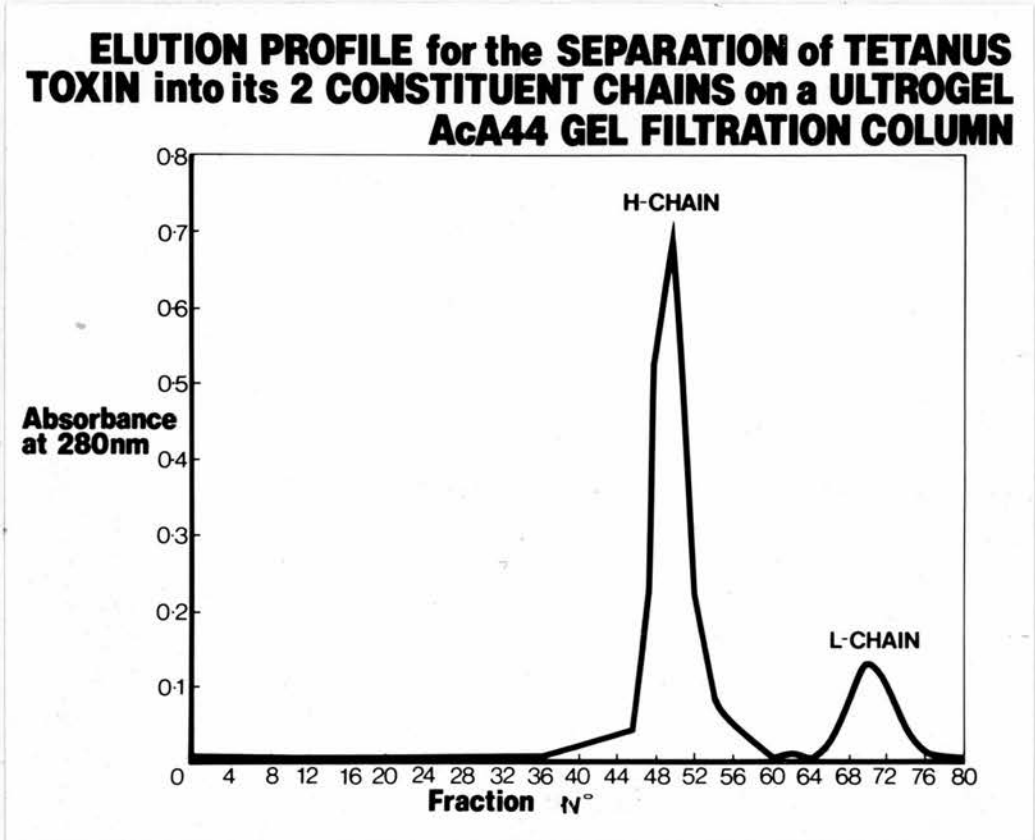
GRAM 10. ELUTION PROFILE OF TETANUS TOXIN, AFTER LIMITED TRYPSINOLYSIS, ON ULTROGEL AcA44

See Section 3.2.2.1. for details.

The initial experiments were repeated but using a Sephacryl S-200 superfine (Pharmacia) gel-permeation column instead of the Ultrogel AcA44 gel-permeation column. Sephacryl S-200 superfine allows the use of 8M urea in the elution buffer in contrast to Ultrogel AcA44 where the use of high concentrations of urea are not recommended. Again there was no separation of the two chains even at the higher urea concentration. Thus it appeared that simply using stronger denaturating conditions did not improve the separation of the two chains nor did the use of a different type of gel-permeation column, recommended to have a greater capacity for the separation of proteins, i.e. 5 000 - 250 000 daltons for Sephacryl S-200 superfine and an exclusion limit of 200 000 daltons for Ultrogel AcA44.

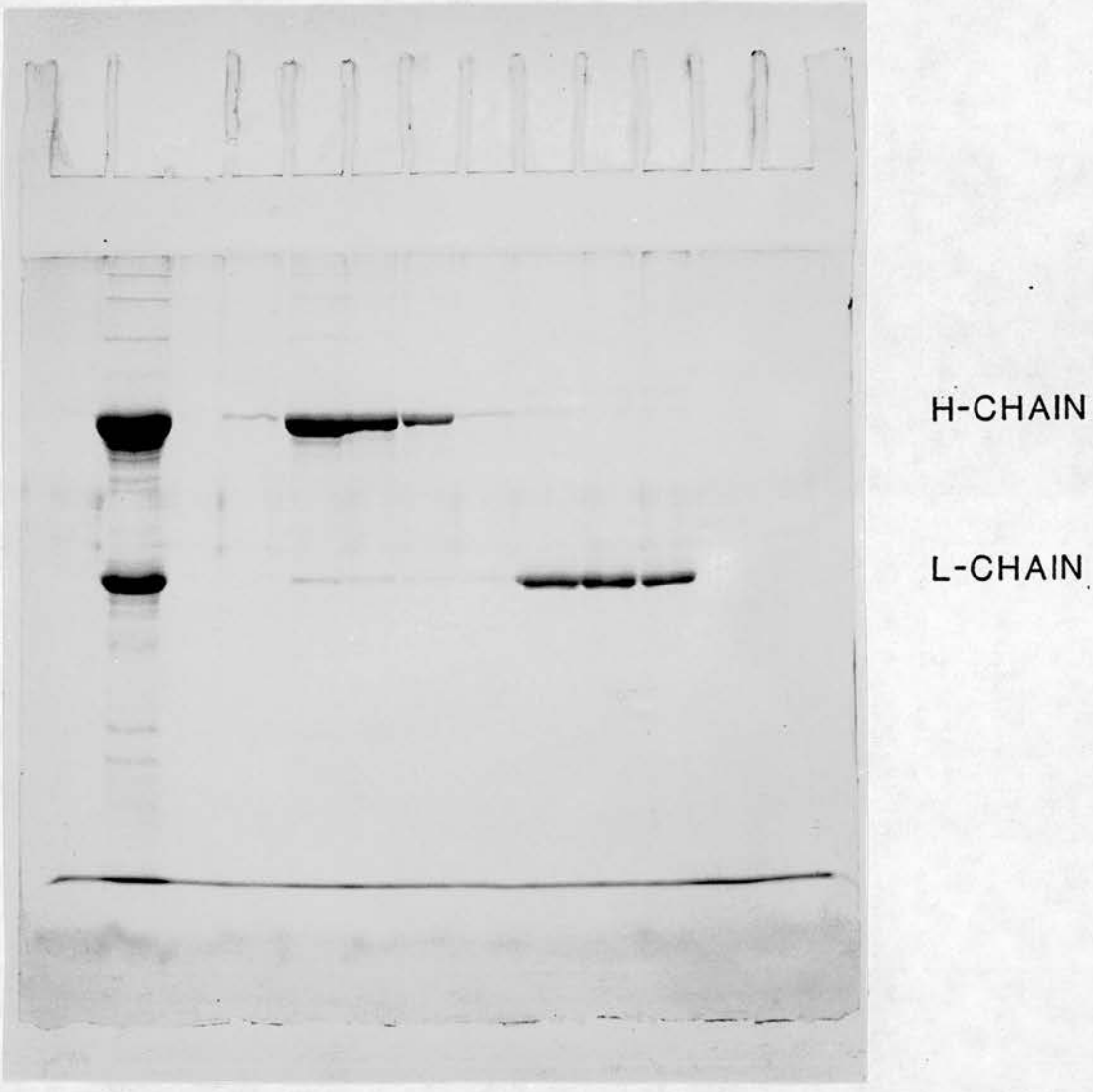
Experiments were then carried out using a further modification of the pre-incubation step, used to dissociate the two chains. Concentrated purified toxin, usually $1cm^3$ containing 4mg, was initially reduced in 100mM DTT at $37^{\circ}C$ for 1 h and then denatured, by the addition of solid urea (Aristar grade) to 8M, at $37^{\circ}C$ for 3 h. The sample was then dialysed against the 2M urea Ultrogel AcA44 elution buffer in the presence of 20mM DTT but without the 0.3M NaCl at $4^{\circ}C$ overnight. After the dialysis step the sample was again concentrated to $1cm^3$ using an Amicon ultrafiltrator with a YM10 membrane (cut off limit 10 000 daltons, a YM10 membrane was used in preference to the UM10 membrane as it is capable of withstanding high concentrations of denaturants, e.g. 8M urea). The sample was then chromatographed on an Ultrogel AcA44 gel-permeation column using the previously described buffer system but without 0.3M NaCl. This time there was some purification of the two chains, though the H-chain was always observed to be contaminated with L-chain, by analysis with SDS-PAGE. See Photograph 20 for the elution profile and Photograph 21 for the SDS-PAGE pattern for the various column fractions. The L-chain was observed to be sufficiently homogeneous for the raising of antibodies, which was carried out as described in Section 3.2.3.

The H-chain fractions, which were always observed to contain L-chain, were pooled from several preparations, concentrated by Amicon ultrafiltration and re-chromatographed on an Ultrogel AcA44 column (as described above), but without any improvement on the purification of H-chain. This possibly indicates that the H-chain



PHOTOGRAPH 20. ELUTION PROFILE OF THE SEPARATION OF TETANUS TOXIN INTO ITS TWO CONSTITUENT CHAINS BY GEL FILTRATION ON ULTROGEL AcA44

See Section 3.2.2.1. for details.



PHOTOGRAPH 21. SDS-POLYACRYLAMIDE GEL PATTERN OF VARIOUS FRACTIONS OF TETANUS TOXIN AFTER SEPARATION INTO ITS TWO CONSTITUENT CHAINS ON AcA44.

See Section 3.2.2.1. for details.

A a sample tetanus toxin.

B H-chain containing fractions with L-chain contaminating.

C L-chain containing fractions.

binds L-chain very tightly, which would also explain the poor success for the other chromatographic procedures.

3.2.2.2. The Purification of H-Chain and L-Chain by Preparative Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

The basis of this purification method was; separation by preparative SDS-PAGE followed by electrophoretic elution. Two pilot experiments were carried out to check the usefulness of this method. Cytochrome c (Sigma) was used initially as it was easily traceable throughout the experiment, because of the haem-group. Bovine γ-globulin (Sigma) was the second protein to be tested, as it is composed of two chains held together by disulphide bridges which can be dissociated into two subunits by reduction, resembling tetanus toxin in this manner. The two pilot proteins tested were analysed, after purification, in 10% Weber and Osborn poly-acrylamide gels (see Section 2.5.1.1.). Both cytochrome c and the two subunits of bovine γ-globulin were purified to homogeneity and so it was decided to use this method for the purification of the two composite chains derived from tetanus toxin.

Purified tetanus toxin (3-4mg) was electrophoresed into 6% double thickness acrylamide gels of the Laemmli type, prepared as described in Section 2.5.4. The gels were 4mm thick, in contrast to the 2mm thick gels used for analytical purposes, so that a higher concentration of protein could be electrophoresed into the gels. The lower percentage of acrylamide was preferred as it gave better resolution of the H- and L-chains and in later gels for 'unnicked' tetanus toxin as well. The gels, as described in Section 2.5.4., had to be poured more carefully than analytical gels (usually with the aid of a peristaltic pump) and required longer setting times, in order to prevent areas of heterogeneous gel concentrations resulting from uneven cross-linking caused from the polymerisation of the gel at various initiation sites.

An initial problem associated with the method was the detection of the protein bands within the gel. Coomassie brilliant blue R-form (BDH) was initially used, as described for protein detection in analytical gels in Section 2.5.1.2. In order to

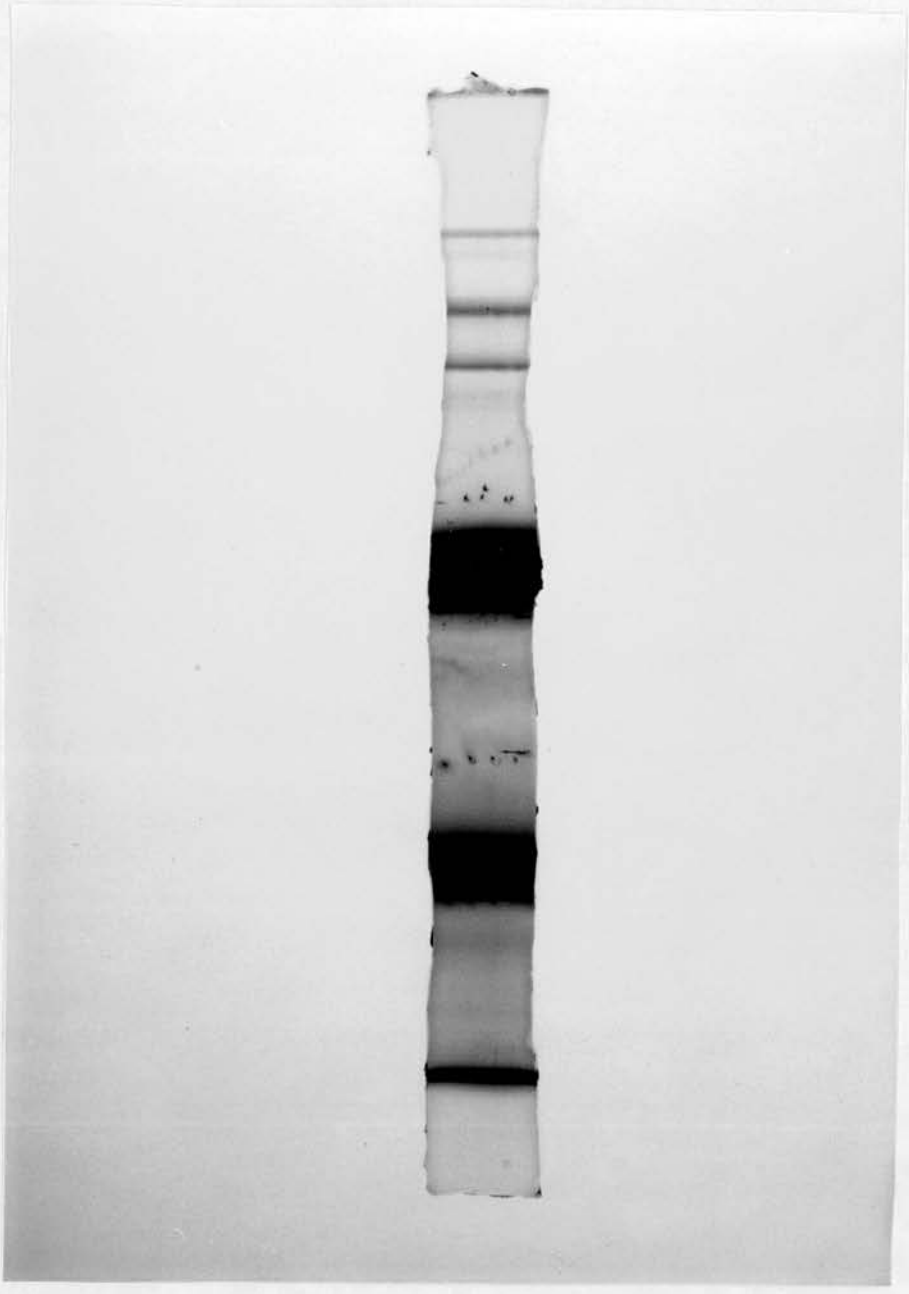
detect the position of the protein bands in the preparative gel two strips of gel, one either side of the gel, were sliced from the rest of the gel and fixed, stained and destained as described in Section 2.5.1.2. The stained strips were then re-aligned with the main gel, or as near as possible as the staining and destaining caused the strips to swell, and the positions of the proteins were thus identified. This detection method took at least 2 h and resulted in the loss of some material. A second detection method was tried, in order to speed up the process; the Mg^{2+}-ANS method of Hartman and Udenfriend (1969) as described by Nerenberg et al. (1971). Again two strips were removed from the preparative gel, fixed as for CBB staining (to remove excess SDS), and then soaked in a 0.01% (w/v) solution of Mg^{2+}-ANS in 0.1M sodium phosphate at pH 7.0. The slices were then aligned with the rest of the gel and the positions of the proteins identified by the fluorescence of the ANS:protein complexes under U.V. light. But again the method resulted in the loss of some material though the process was much quicker, about 25min. A third method was tried where a small amount of tetanus toxin was dansylated, using dansyl chloride, according to the method of Talbot and Yphantis (1971) as described by Stephens (1975). Unfortunately the dansylated tetanus toxin did not enter the preparative gel either because of aggregation or because it was too hydrophobic as a result of the dansyl groups. The final method tried was the SDS-precipitation method of Higgins and Dahmus (1979) (as described in Section 2.5.4.) using 4M sodium acetate. This method allowed a very rapid visualisation of the proteins in the preparative gel, they were visible within 15 - 30s after immersion in the 4M sodium acetate (though in most cases the gel was immersed for 3 - 5min), and there was no loss of sample. The proteins were identified as white bands against a clear background (as seen in Photograph 22). This was in contrast to Higgins and Dahmus (1979) who observed clear protein bands against a white background. Standard proteins (e.g. BSA) were also detected this way and were observed as according to Higgins and Dahmus (1979), so it was concluded that the precipitation of either tetanus toxin or its chains was a phenomenon of the toxin. To make sure the actual SDS:protein complex was being precipitated and not an SDS front

PHOTOGRAPH 22. PRECIPITATION OF SDS-PROTEIN COMPLEX BY 4M SODIUM ACETATE IN A 6% POLYACRYLAMIDE GEL

See Section 3.2.2.2. for details.

running before the complex a slice of gel was initially immersed in 4M sodium acetate and the positions of the precipitate front and back were marked by the injection of Indian ink into the gel. The strip was then fixed, stained in CBB and destained as described for analytical gels (see Section 2.5.1.2.), the Indian ink was not removed by the process. As can be observed from Photograph 23 the precipitated bands coincided with the CBB staining material. Thus this method was used for the visualisation of the proteins after electrophoresis in the preparative gels.

After the proteins had been identified in the preparative gels the appropriate area of gel was sliced out and cut into $1cm^2$ pieces. The gel pieces were then placed into the appropriate chamber of the electrophoretic-elution apparatus (as described in Section 2.5.5.) on top of a coarse plastic mesh secured by rubber rings. The bottom of each chamber had a freshly prepared dialysis bag attached (prepared by double-knotting one end of a piece of dialysis tubing) with rubber bands. The chambers of the electrophoretic elution apparatus and the dialysis bags were filled with the electrophoresis buffer described in Section 2.5.1.2. The tops of the chambers were sealed with rubber bungs that had small holes drilled through, and any trapped air was removed by gently squeezing the dialysis bags. The proteins were electrophoresed out of the gel pieces into the dialysis bags overnight at a constant voltage of 50V. The current was always observed to rise initially from about 80mA to 100mA but then fall gradually to about 20mA. The eluted proteins were observed to be electrophoresed to the tips of the dialysis bags, judging by a change in the refractive index of the electrode buffer. Stephens (1975) and Tijssen and Kurstak (1979) observed electrophoretically eluted proteins as a dense fluorescent solution at the tips of the dialysis bags as a result of running dansylated proteins. The rubber bungs were carefully removed and the pieces of gel were removed with forceps and discarded (samples were stained with CBB and shown to contain no protein). The plastic mesh was removed from each chamber by carefully removing the rubber rings with forceps and lifting the mesh out with the forceps. The protein samples were then removed from the bottom of each dialysis bag by using a long-tipped pasteur pipette and stored at $4^{o}C$. The protein samples



PHOTOGRAPH 23. PHOTOGRAPH TO SHOW THAT THE 4M SODIUM ACETATE PRECIPITATION BANDS RUN CONCURRENTLY WITH CBB STAINING MATERIAL

See Section 3.2.2.2. for details.
The gel was initially placed in 4M NaAc the bottom and top edges of the bands marked with Indian ink. The gel was then stained in CBB.

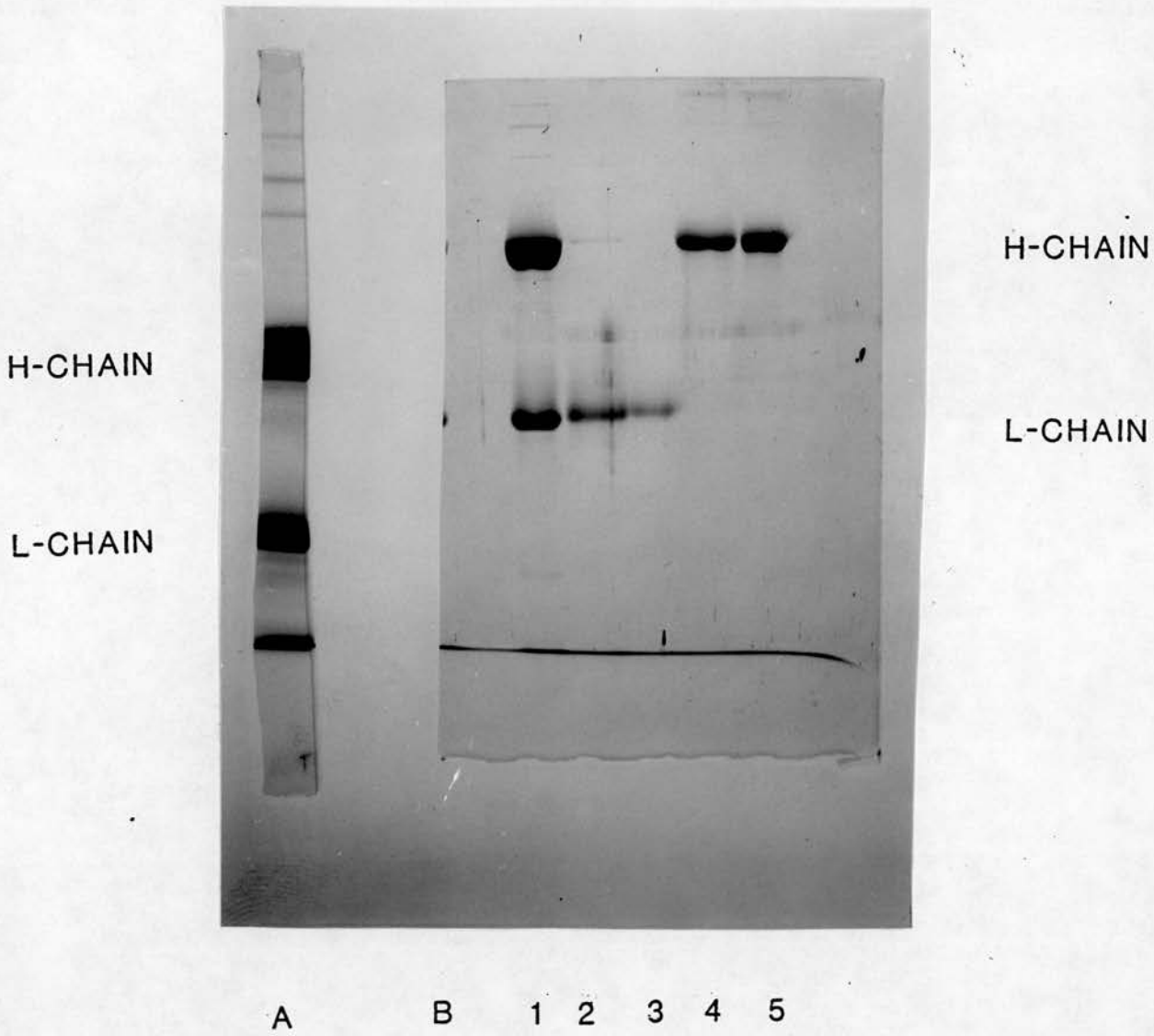
were usually dialysed against 8M urea in 0.1M sodium phosphate buffer at pH 7.0 containing 1mM benzamidine to try and remove as much SDS as possible, and then exhaustively dialysed against water to remove the urea before freeze-drying. The proteins were then redissolved in the appropriate buffer of the experiment they were used in.

Photograph 24 shows that the two chains were purified to single bands after analysis on 8% acrylamide gels. Photograph 25 shows that 'unnicked' tetanus toxin was also purified to a single component and was thus shown not to dissociate upon reduction.

3.2.3. The Raising of Antibodies to H-Chain and L-Chain

The use of proteins and polypeptides as antigens and the basic principles of antigen-antibody reactions are described by Maurer and Callahan (1980) and Kabat (1980). The methods used for the raising of antibodies to H- and L-chains in rabbits are described in Section 2.6.3. Initially the L-chain purified by gel-permeation chromatography on Ultrogel AcA44 was used and later H- and L-chains purified by preparative SDS-PAGE were used for the production of their respective antibodies. The two chains were toxoided, as described in Section 2.6.3.1., in order to destroy any possible toxigenicity of an individual chain but without any detriment to the immunogenicity of either chain.

The antibodies raised against toxoided L-chain (purified by gel-permeation chromatography) were found to react against toxoided L-chain, tetanus toxin and tetanus toxoid (Batch No. TD755, supplied by Dr. R.O. Thomson of The Wellcome Research Laboratories, Beckenham, Kent). The toxoided L-chain was never observed to react against Equine anti-toxoid. The anti-L-chain reacted as a single precipitin band (indicating a single component), see Diagram 11.



PHOTOGRAPH 24. SDS-POLYACRYLAMIDE GEL PATTERN OF THE PURIFICATION OF THE TWO CONSTITUENTS OF TETANUS TOXIN BY PREPARATIVE SDS-PAGE

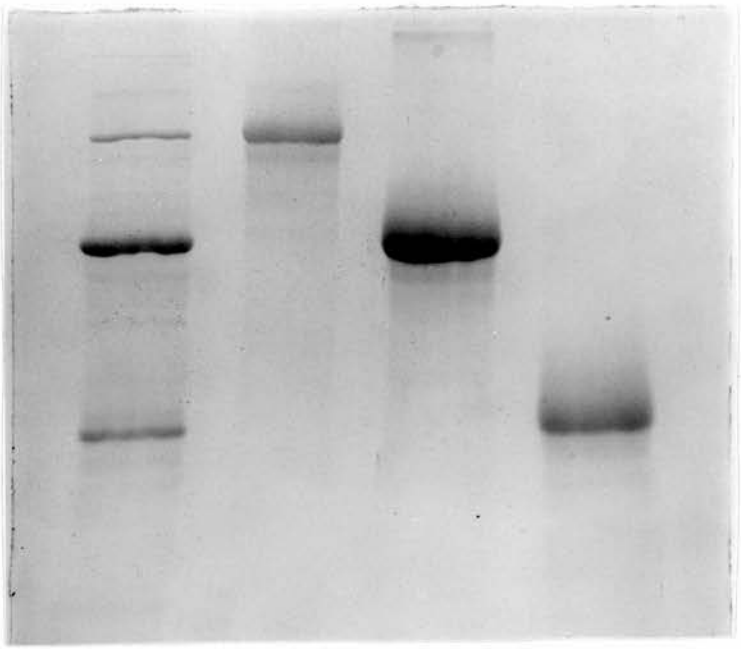
See Section 3.2.2.2. for details.

A a slice from a 6% Preparative gel.

B1 a sample of purified toxin used for the Preparative gel.

B2 and B3. Purified L-chain.

B4 and B5. Purified H-chain.



PHOTOGRAPH 25. SDS-POLYACRYLAMIDE GEL PATTERN OF THE PURIFICATION OF 'UNNICKED' TETANUS TOXIN, H- AND L-CHAINS BY PREPARATIVE SDS-PAGE

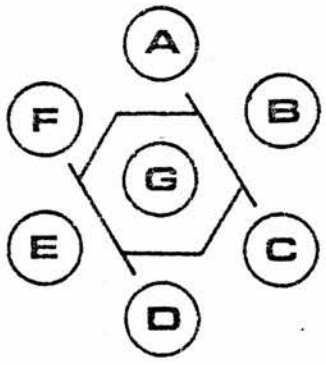
See Section 3.2.2.2. for details.

1 sample of purified toxin containing 'unnicked' toxin used for the preparative gel.

2 Purified 'unnicked' toxin.

3 Purified H-chain.

4 Purified L-chain.



A,C,D,F TETANUS TOXIN OR TOXOID

B,E TOXOIDED L-CHAIN

G ANTI-L-CHAIN

AGRAM 11. DOUBLE-IMMUNODIFFUSION PATTERN FOR THE CROSS-REACTION OF ANTI-L-CHAIN AGAINST TETANUS TOXIN OR TOXOID AND TOXOIDED L-CHAIN

See Section 3.2.3. for details.

Thus it appeared that the antibodies raised from the toxoided L-chain were able to cross-react with either tetanus toxin or tetanus toxoid, but with partial identity between either tetanus toxin or tetanus toxoid and toxoided L-chain. When tetanus toxin or tetanus toxoid cross-react with anti-tetanus toxoid no 'spurs' are observed, a confluence reaction, because both antigens are immunologically identical. The 'spurring' observed with either tetanus toxin or tetanus toxoid with toxoided L-chain results from only partial immunological identity between the two antigens. Since toxoided L-chain was not observed to react with the Equine anti-toxoid, it seems that the toxoided free L-chain is in some structural conformation that is different from what it is in whole toxoid. The fact that anti-L-chain toxoid is able to recognise some structural information on either tetanus toxin or tetanus toxoid suggests that the L-chain in the "native" conformation of tetanus toxin is immunologically available, i.e. present at least in in some part on the outside of the toxin molecule.

The antibodies raised against H- and L-chain purified by preparative SDS-PAGE were found only to react against either tetanus toxin or tetanus toxoid denatured by SDS as well as their respective SDS-denatured isolated chains. The antibodies raised against H-chain did not react with L-chain and vice versa. The fact that the L-chain antibodies, prepared from L-chain purified by preparative SDS-PAGE, reacted only against SDS denatured tetanus toxin or tetanus toxoid suggests that these L-chain antibodies are different to the L-chain antibodies prepared from L-chain purified by gel-permeation chromatography, as one would expect. Because of the SDS the two chains will be in rod-like structures with the result that more antigenic sites are made available, while some previous antigenic sites are lost as a result of the change in structure. Thus the different antibody preparations have different populations of antibodies capable of reacting with different antigenic sites. Because the differences between denatured and "native" L-chain are so large, and hence a difference in antigenic sites, one set of antibodies will not react with L-chain in a different conformation.

3.2.4. Molecular Weight Determination

Molecular weight determination was carried out using two methods; one was SDS-PAGE and the other by sedimentation equilibrium in the analytical ultracentrifuge.

3.2.4.1. Molecular Weight Determination by SDS-PAGE

In this method tetanus toxin and proteins of known molecular weight were complexed with SDS and reduced with 2-ME. The reduction of the disulphide bridges is important because the proteins must have complete conformational freedom for reaction with SDS. SDS-binding studies on a variety of proteins indicate that above an SDS monomer concentration of 8×10^{-4}M (0.02% SDS), 1.4g of SDS are bound to 1g of protein (Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970a,b). Thus, the number of SDS molecules bound to a protein or polypeptide chain is approximately half the number of amino acid residues in the protein or polypeptide chain. This level of binding, and the constant binding ratio, will in general "swamp out" the "native" charge contribution of most proteins, and an approximate constant negative net charge per unit mass will be obtained (Weber *et al.*, 1972). Hydrodynamic and optical studies indicate that the complexes behave like rod-like particles in which the particle length varies uniquely with the molecular weight of the protein or polypeptide chain (Reynolds and Tanford, 1970a,b; Fish *et al.*, 1970). The use of 2-ME is necessary not only for the reduction of inter-disulphide bridges but intra-disulphide bridges in order to give complete conformational freedom. Bovine serum albumin (BSA) has been found to bind only 0.9g of SDS per gram of protein, in the absence of 2-ME, instead of the generally found value of 1.4g, the latter value is achieved by extensive reduction (Weber *et al.*, 1972). PAGE is ideal for the separation of the SDS-complexed proteins which according to the above criteria will separate solely on a size basis or difference in molecular weight (Shapiro *et al.*, 1967; Weber and Osborn, 1969; Weber *et al.*, 1972).

The method used to calculate the molecular weight of H- and L-chain derived from 'nicked' tetanus toxin was initially that

of Weber and Osborn (1969) (see Section 2.5.1.1. for gel preparation). The reproducibility of the Weber and Osborn system is not very good as there are very often small differences in the mobilities of proteins between individual gels, resulting from small differences in gel composition; Weber et al. (1972) indicated that the reproducibility between gels may vary between 5% - 10%. Weber et al. (1972) suggested that a better sensitivity is obtained if the bands are made sharperin the gel, this results from an effective sample "stacking". Thus the discontinuous SDS system of Laemmli (1970) was preferred to the Weber and Osborn system. The slab gel system of Laemmli and Favre (1973) was preferred to the original tube gel system of Laemmli (1970). The SDS slab gel system produced a greater reproducibility than the Weber and Osborn system and the Laemmli tube gel system because each sample was run under the same conditions. A higher sensitivity was achieved as a result of the discontinuous system (a series of buffers at different pH values); which caused the proteins to be "stacked" in the stacking gel before being electrophoresed into the separating gel. The higher sensitivity or increase in resolution is the result of sharp protein bands in the gel. The advantages of molecular weight determination of protein - dodecyl sulphate complexes by gel electrophoresis in a discontinuous buffer system has been described by Neville (1971).

The protein samples containing SDS and 2-ME were heated to 100°C for 3min to minimize any possible proteolytic digestion a phenomenon known to occur in SDS solutions at room temperature (Pringle, 1970; Weber et al., 1972). The samples were complexed in 2% (w/v) SDS (as described in Section 2.5.1.2.) in order to make sure that the proteins were fully saturated with SDS, in the ratios described above. The importance of obtaining a high ratio of SDS to protein has also been described by Stoklosa and Latz (1975).

The amount of monomeric acrylamide used in the production of the gels and the amount of cross-linker (N,N'-methylenebis acrylamide) is also important for the determination of the molecular weight of the SDS-protein complexes (Neville, 1971). Ferguson (1964) showed that a graph of relative mobility against logarithm of molecular weight showed areas of linearity and non-linearity, in

agarose gels. Hedrick and Smith (1968) showed that this was also true for polyacrylamide gels. Neville (1971) was able to show that by altering the amount of acrylamide used in the production of the gels that the areas of linearity, in the graph of relative mobility versus log molecular weight, altered according to the molecular weights of the proteins used. Thus the higher the molecular weight of the protein the lower was the percentage of acrylamide needed in the composition of the gel and vice versa. A gel of one composition was only suitable for proteins of a certain molecular weight range (a linear plot on the graph of relative mobility versus log molecular weight). But by altering the composition of the gel one could either extend the molecular weight range at the higher end by lowering the acrylamide content of the gel, with a loss in molecular weight range at the lower end, or extend the molecular weight range at the lower end by increasing the acrylamide content with a loss in molecular weight range at the higher end. An area of non-linearity could lead to an error in the determination of the molecular weight of a protein of unknown molecular weight if its molecular weight fell into that range, unless a sufficient number of standards were used in that particular range. A linear plot allows one to use fewer standards, in cases where the number of suitable standards is limiting.

For the above reason three types of polyacrylamide gels were run: an 8% gel was used to determine the molecular weights of H-chain and L-chain and a 4% and 5% gel were run to determine the molecular weights of H-chain and 'unnicked' tetanus toxin. The 8% gel was very useful for determining the molecular weight of L-chain, as it was midway along the standard line (log molecular weight versus relative mobility), whereas H-chain was just within the limits of the linear line and tended to be away from the main number of standards. By using 4% and 5% gels the molecular weight for H-chain was determined more accurately as it was now central to several standard proteins. The 4% and 5% gels also gave reasonable results for 'unnicked' tetanus toxin.

As described above in order to determine the molecular weight of a protein one needs a set of standard proteins of known molecular weight whose molecular weights ideally lie to either side of the molecular weight of the protein under investigation.

10µg of the standard proteins used were treated in a similar way as the tetanus toxin samples.

The standard proteins used for the 8% gel were:-

PROTEIN	SOURCE	SUBUNIT MOL.WT.	REFERENCE
Muscle Glycogen Phosphorylase a	Sigma	97 400	Titani et al. (1977)
Bovine Serum Albumin (BSA)	Sigma	68 500	Tanford et al. (1967)
Muscle Pyruvate Kinase	Boehringer	57 000	Weber and Osborn (1969)
Bovine γ-Globulin	Sigma		
H-chain		50 000	Weber and Osborn (1969)
L-chain		23 500	Weber and Osborn (1969)
Ovalbumin	Sigma	43 000	Weber and Osborn (1969)
Muscle Aldolase	Boehringer	40 000	Weber and Osborn (1969)

The standard proteins used for the 4% and 5% gels were:-

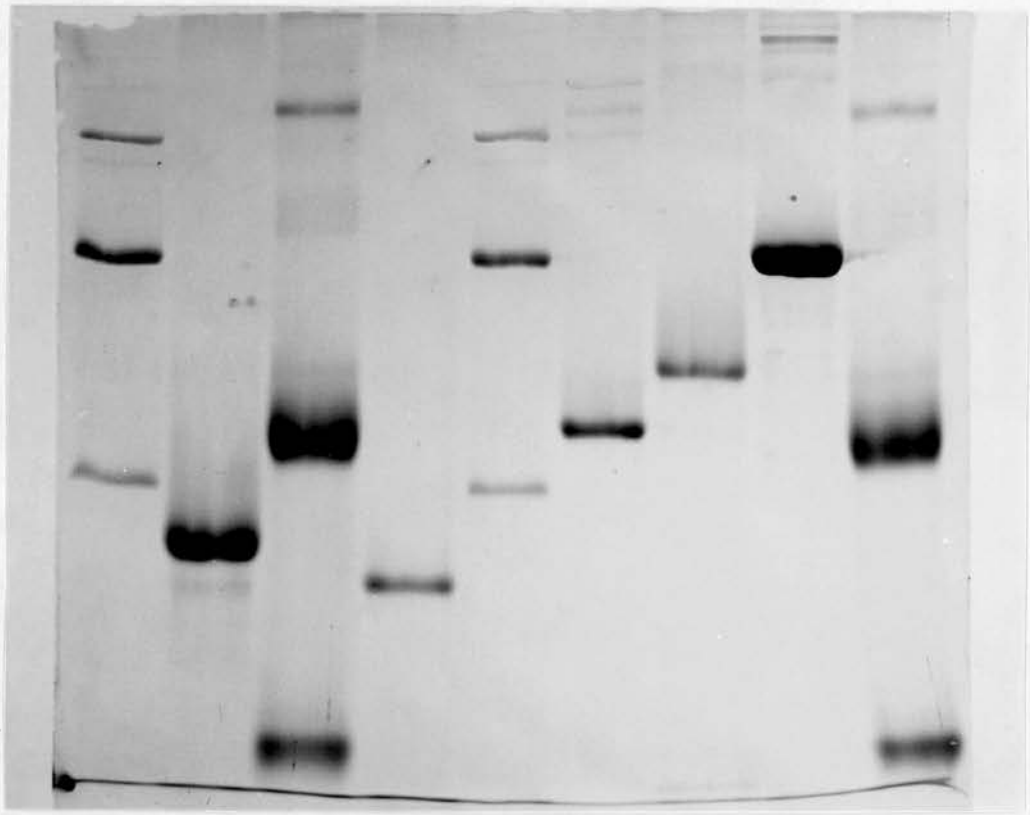
PROTEIN	SOURCE	SUBUNIT MOL.WT.	REFERENCE
Fatty Acid Synthetase	(1)	252 000	Hardie and Cohen (1978)
Myosin H-chain	(2)	210 000	Harrington and Karr (1965)
Muscle Phosphorylase Kinase	(3)		
α-Subunit		145 000	Cohen (1973)
β-Subunit		128 000	Cohen (1973)
Muscle Glycogen Phosphorylase b	(4)	100 000	Cohen et al. (1971)
Muscle Glycogen Phosphorylase a	Sigma	97 400	Titani et al. (1977)
Bovine Serum Albumin (BSA)	Sigma	68 500	Tanford et al. (1967)

Samples marked (1), (2), (3) and (4) were all a gift from Dr. P. Cohen (Department of Biochemistry, University of Dundee) and were supplied as $1 mg cm^{-3}$ samples containing 0.1% (w/v) SDS and 0.1% (v/v) 2-ME in sodium phosphate buffer at pH 7.0. Sample (1) was obtained from rabbit lactating mammary glands. Sample (3) also contained γ-subunit (mol.wt. of 45 000) and δ-subunit (mol.wt. of 17 000) but were not detectable on either the 4% or 5% gel as a result of their small size.

The relative mobilities of the standard proteins and either 'unnicked' tetanus toxin or its two chains were calculated, as described by Weber et al. (1972), by measuring the distance moved by the protein and the distance moved by BPB, the relative mobilities were then determined from the ratio of distance moved by the protein against distance moved by BPB (in both cases the distances were measured from the top of the gel to the front of the bands). The relative mobility of each standard was then plotted against the log of the molecular weight of the particular standard protein, a straight line was obtained in each case, as the standard proteins were chosen to give a linear graph from each of the gels used. See Photograph 26 for the positions of the standard proteins and H- and L-chains in the 8% gel. From Diagram 12, the graph of log mol.wt. versus relative mobility for the standard proteins in the 8% gel, the molecular weights of H-chain and L-chain were determined to be 97 000 daltons and 51 000 daltons.

In order to calculate the molecular weight of H-chain more accurately and calculate the molecular weight of 'unnicked' toxin the 4% and 5% gels described above were used using the standard proteins as described above. From the graphs of the log mol.wt. versus relative mobility for the standard proteins (Diagram 13) the molecular weights of H-chain and 'unnicked' tetanus toxin were found to be 99 000 $\pm$ 1 900 daltons and 150 000 $\pm$ 3 000 daltons from the 4% gel and 95 000 $\pm$ 2 000 daltons and 158 000 $\pm$ 2 000 daltons from the 5% gel.

A computer program was designed by Dr. G.L. Atkins (Department of Biochemistry, University of Edinburgh) in which the molecular weights of the standard proteins were converted to exponential logarithmic values and then compared to the relative mobilities. The computer calculated the best straight line obtainable for the values using the non-parametric method of Nimmo and Atkins (1976). The relative mobility values of the unknown proteins (H- and L-chains and 'unnicked' tetanus toxin) were then fed into the computer which calculated the appropriate molecular weights from the corrected values of the standard proteins. From the statistically "best" values of molecular weights, the values for H-chain and 'unnicked' tetanus toxin were found to be 98 000 $\pm$ 2 000 daltons and 150 000 $\pm$ 2 600 daltons from the 4% gel and 99 000 $\pm$ 2 200 daltons and 164 000 $\pm$ 2 000 daltons from the 5% gel.



PHOTOGRAPH 26. 8% SDS-POLYACRYLAMIDE GEL PATTERN OF TETANUS TOXIN AND VARIOUS MARKER PROTEINS FOR DETERMINATION OF THE MOLECULAR WEIGHTS OF H- AND L-CHAINS

See Section 3.2.4.1. for details.

Tracks 1 and 5	Tetanus toxin containing 'unnicked' toxin.
Track 2	Ovalbumin (43 000)
Track 3 and 9	Bovine γ-globulin (50 000 and 23 500)
Track 4	Muscle Aldolase (40 000)
Track 6	Pyruvate Kinase (57 000)
Track 7	BSA (68 500)
Track 8	Phosphorylase a (97 400)

DIAGRAM 12. PLOT OF LOG$_{10}$ MOL. WT. VERSUS RELATIVE MOBILITY FOR STANDARD PROTEINS ELECTROPHORESED ON AN 8% SDS-POLYACRYLAMIDE GEL

See Section 3.2.4.1. for details.

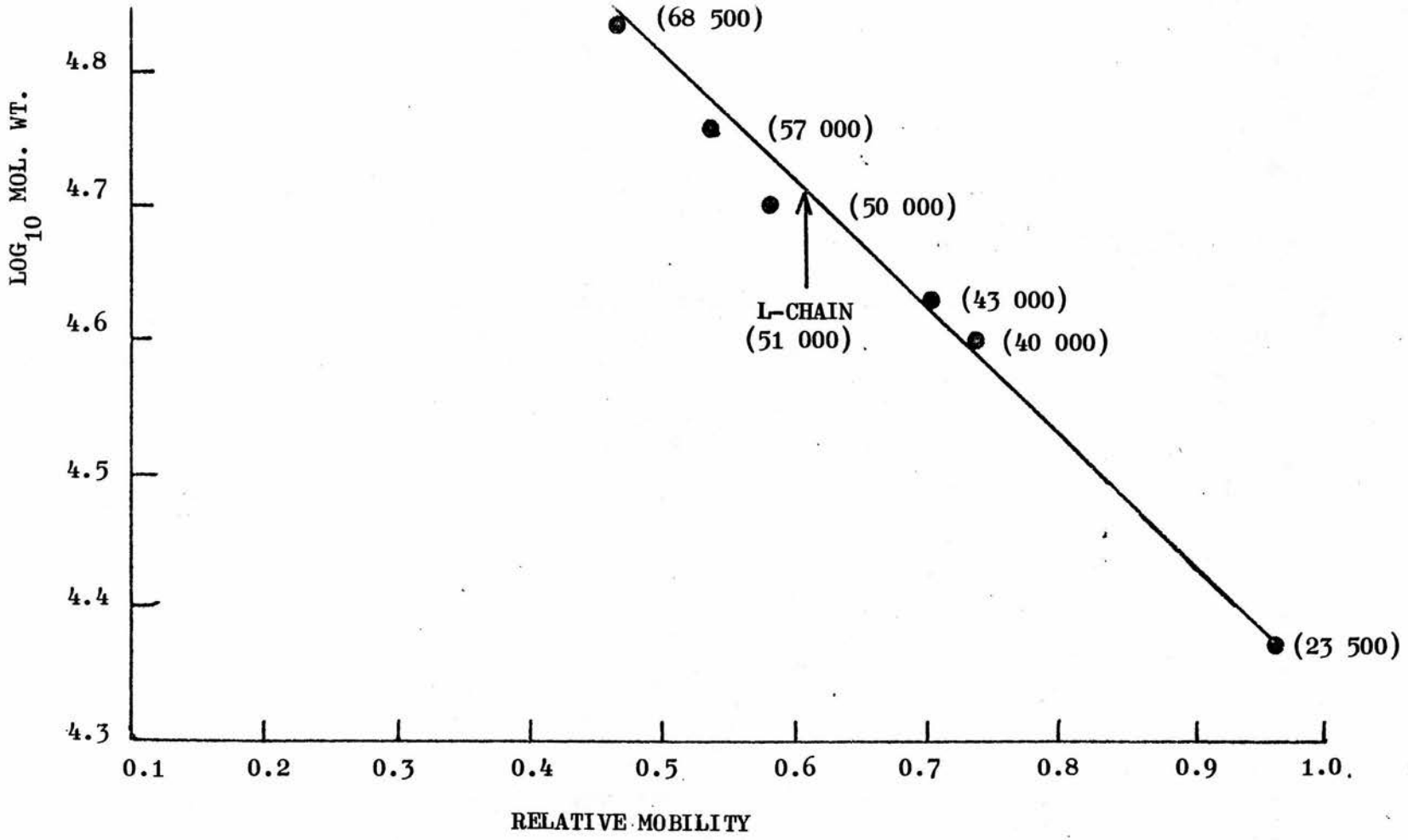
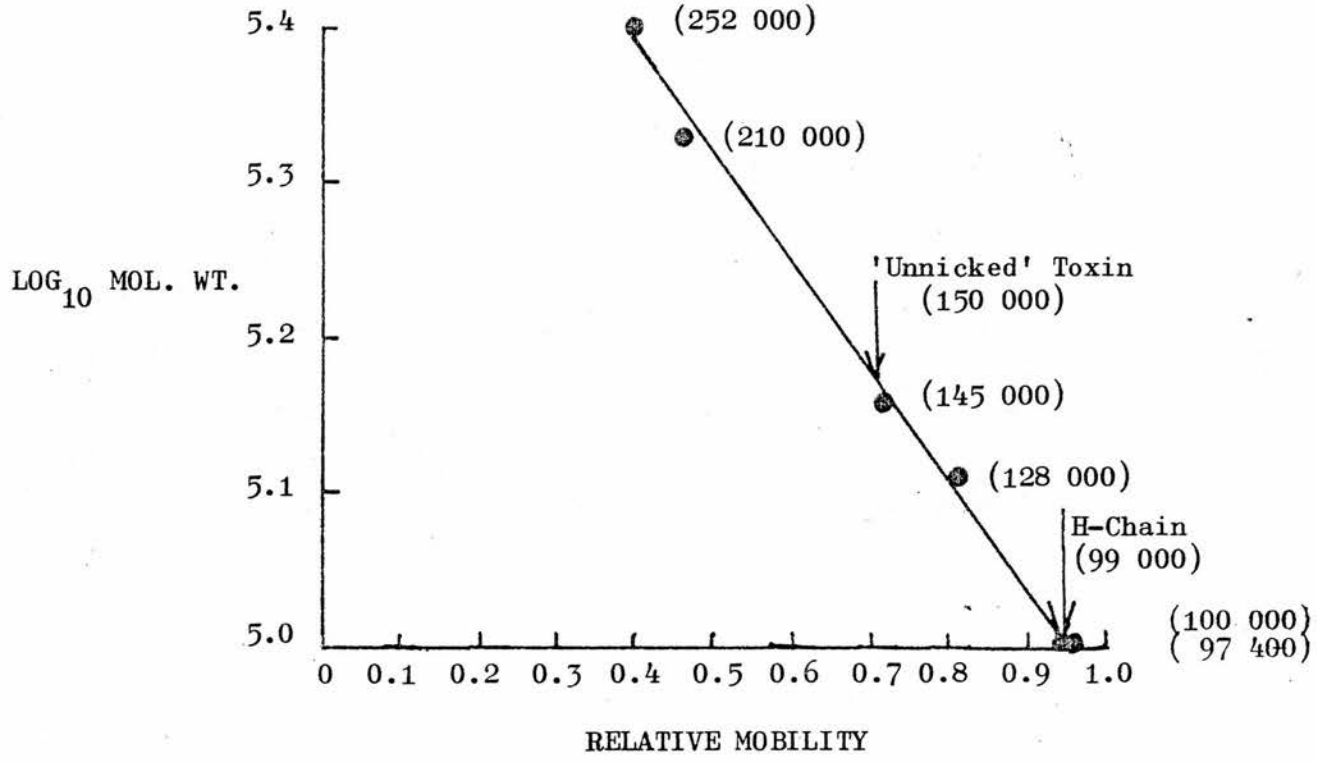

(68 500)
(57 000)
(50 000)
L-CHAIN
(51 000)
(43 000)
(40 000)
(23 500)
4.8
4.7
4.6
4.5
4.4
4.3
LOG10 MOL. WT.
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
RELATIVE MOBILITY

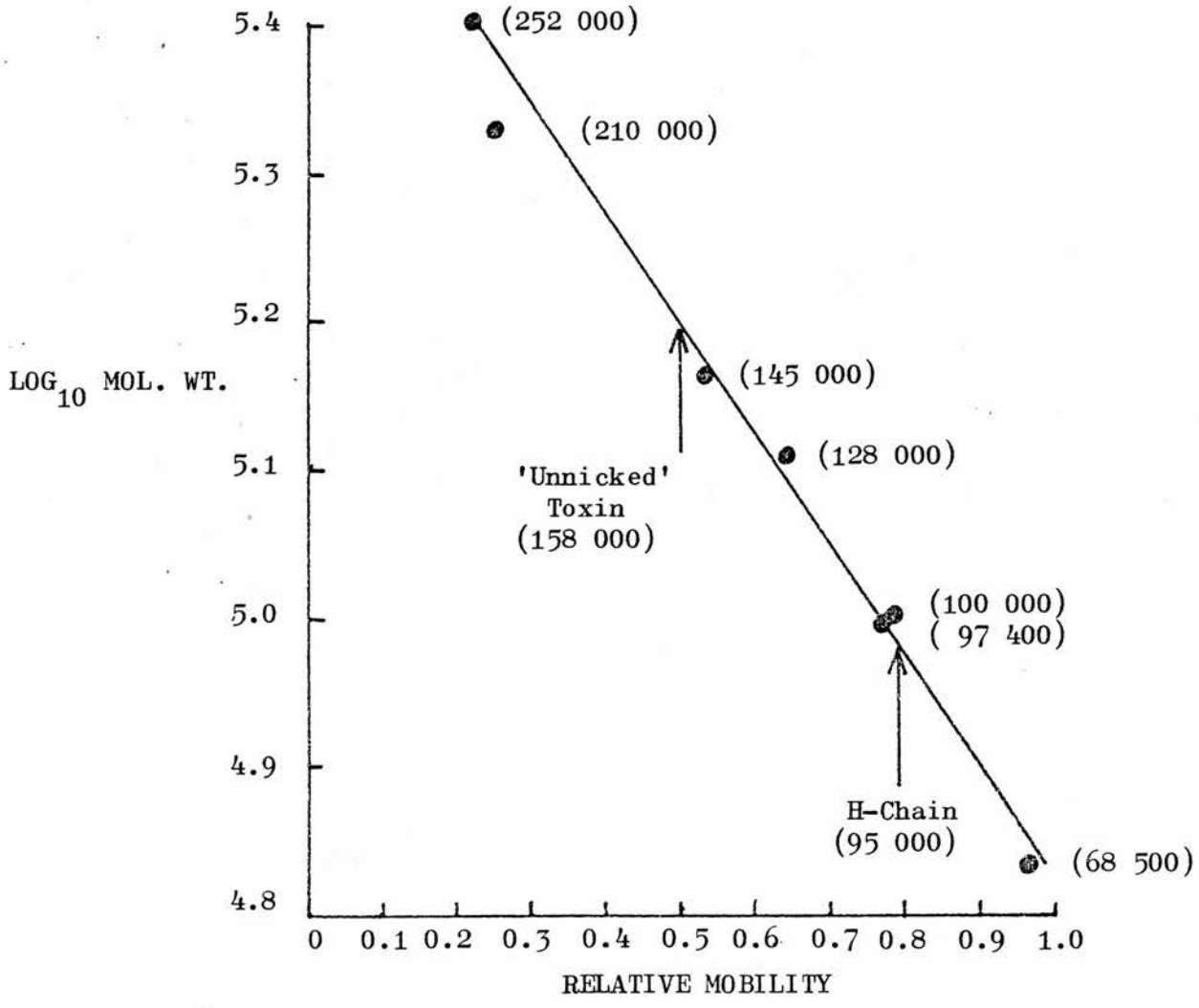
DIAGRAM 13. PLOT OF LOG_{10} MOL. WT. VERSUS RELATIVE MOBILITY FOR STANDARD PROTEINS ELECTROPHORESED ON 4% AND 5% SDS-POLYACRYLAMIDE GELS

See Section 3.2.4.1. for details.

4% GEL


5.4
5.3
5.2
5.1
5.0
LOG10 MOL. WT.
(252 000)
(210 000)
'Unnicked' Toxin
(150 000)
(145 000)
(128 000)
H-Chain
(99 000)
(100 000)
(97 400)
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
RELATIVE MOBILITY

5% GEL


5.4
5.3
5.2
5.1
5.0
4.9
4.8
LOG10 MOL. WT.
(252 000)
(210 000)
(145 000)
(128 000)
'Unnicked'
Toxin
(158 000)
(100 000)
(97 400)
H-Chain
(95 000)
(68 500)
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
RELATIVE MOBILITY

3.2.4.2. Molecular Weight Determination by Analytical Ultracentrifugation

The molecular weight of a mixture of purified 'nicked' and 'unnicked' tetanus toxin was determined by sedimentation equilibrium, using the meniscus depletion method of Yphantis (1964). The experimental procedures used were as described by Cohen (1973), Nimmo et al. (1976) and Hardie and Cohen (1978) and are summarised in Section 2.16.2.

The toxin sample was centrifuged for 20 h at a low speed until a steady state was reached between the sedimentation and diffusion of the toxin sample in the centrifuge cell. From the fringe pattern obtained (as described in Section 2.16.2.) the degree of purity of the sample and the molecular weight of the sample were deduced. A very straight base line was observed in the fringe pattern indicating a homogeneous protein sample. The evenness of the fringe pattern (spacing of the fringes) indicated that the tetanus toxin was globular and that there was no aggregation of the sample. In order to obtain the molecular weight of the toxin the following equation was used:-

$$M_w = \frac{2RT}{(1-\bar{v}\rho)\omega^2} \times 2.303 \frac{d(\log C)}{d(r^2)}$$

Where :-

R gas constant, 8.314×10^7 erg/mol/K

T Absolute temperature of the centrifugation run, 298K

$\bar{v}$ Partial specific volume of the toxin, 0.732 $cm^{-3}g^{-1}$ (see Section 3.3.3.4.)

ρ Density of the buffer, $1.01 gcm^{-3}$

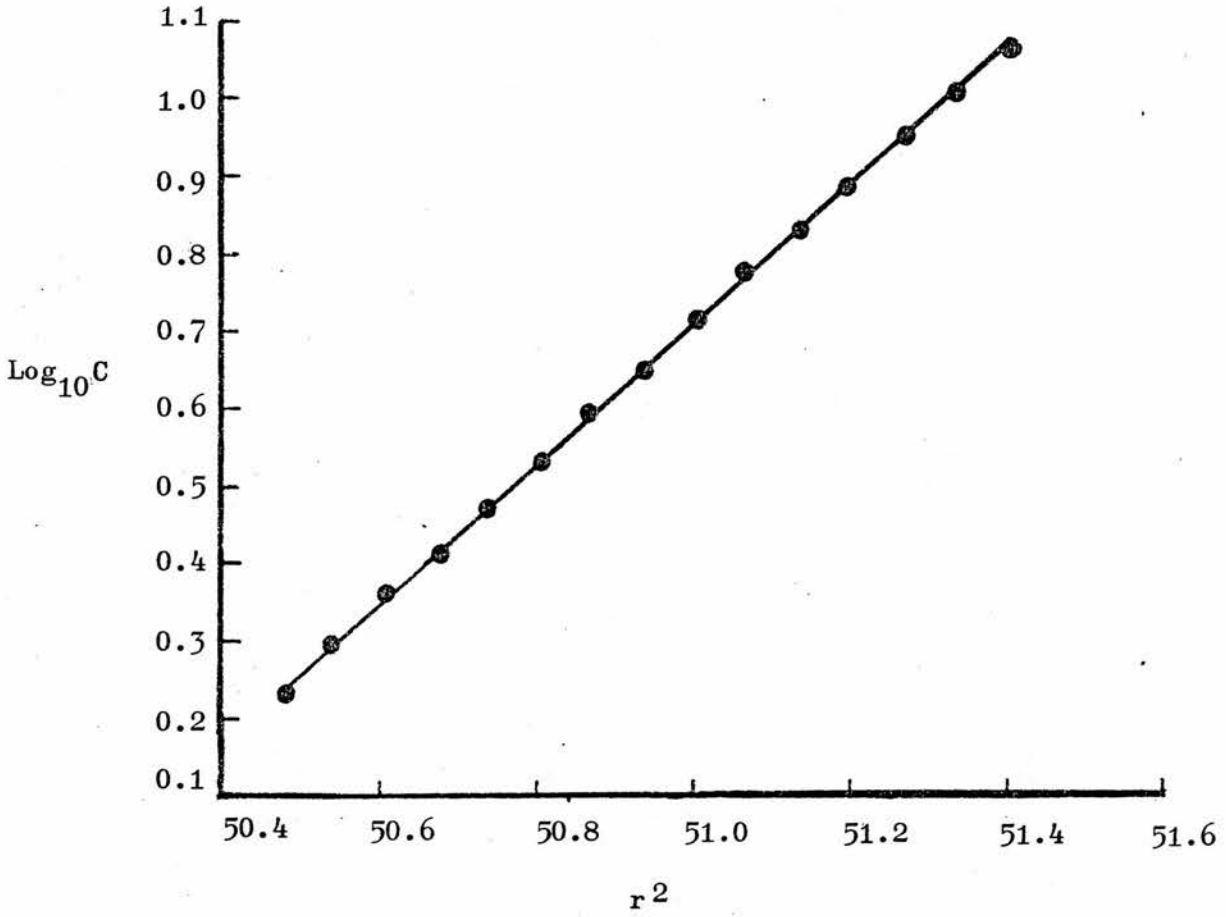
ω^2 Angular velocity of the rotor at equilibrium, 2.807×10^6 $rads^{-1}$

C Concentration of solute, Tetanus toxin concentration

r Distance from the centre of rotation to the protein band in the centrifuge cell.

From the fringe pattern one was able to plot log C against r^2 and from the slope determine $\frac{d(\log C)}{d(r^2)}$, see Diagram 14 for the graph of logC versus r^2.

The distance along the fringe was measured at various points and reduced by the magnification factor of the photosystem (known to be



IAGRAM 14. PLOT OF $LOG_{10}C$ VERSUS r^2 FROM SEDIMENTATION EQUILIBRIUM CENTRIFUGATION OF TETANUS TOXIN

See Section 3.2.4.2. for details.

2.165) to which was added the value 5.65cm (which represented the distance from the centre of the rotor axis to reference line of the centrifuge cell which was printed onto the photograph on the glass plate). The final values obtained were equal to r. The height of the fringe above the base line was determined, at the various points measured for r, the values of C were determined in terms of these heights. The vertical displacement of the fringes is proportional to the refractive index between the solute and solvent. The refractive index is in turn dependant upon the concentration of the solute. Thus the concentration of the solute can be measured as a change in refractive index. The values of r and C were averaged from their measurement on three different fringes. The slope of the graph of logC versus r^2 was observed to be 0.91. The molecular weight of both types of toxin, using the above values, was calculated to be 141 905 daltons, from the above equation.

3.3. Structural Investigation of Tetanus Toxin

This chapter describes experiments carried out to investigate some structural aspects of tetanus toxin. The experiments were designed to determine the N-terminal amino acids, the number of half-cystine residues, the number of disulphide bridges, and the amino acid composition of tetanus toxin and its constituent chains.

3.3.1. Introduction

Bizzini et al. (1970) were the first group to characterise tetanus toxin. They purified methanol-precipitated toxin by ion-exchange chromatography (DEAE-cellulose) followed by gel-permeation chromatography on Sephadex G-200. The amino acid composition of the purified toxin was determined and found to be similar to the analyses previously reported by Dawson and Mauritzen (1967) and Murphy et al. (1968). Bizzini et al. (1970) went on to show that the amino acid composition of tetanus toxin purified from a bacterial extract and toxin purified from culture filtrate were the same. They reported a single N-terminal amino acid, leucine. Experiments were carried out to determine the cysteine and cystine content of tetanus toxin, using a number of different methods, which gave a variety of results. Performic acid oxidation of tetanus toxin revealed ten half-cystine residues. They carried out experiments, using iodoacetic acid (IAA), on "native" toxin, which revealed the absence of any free thiol groups, indicating that there are either no free cysteine residues or none available for reaction with IAA. Denaturation of the toxin with either 8M urea or 6M GuHCl yielded four and five cysteines respectively available for reaction with IAA. Reduction of tetanus toxin yielded eight cysteine residues in the presence or 8M urea or ten cysteine residues in the presence of 6M GuHCl. Thus both experiments indicated that there were at least two disulphide bridges in the "native" structure of the toxin. Bizzini et al. (1970) also used ethylene-imine, according to the method of Raftery and Cole (1966), to try and determine the number of half-cysteine residues in tetanus toxin. "Native" toxin was found to contain two free cysteine

residues but after denaturation using 8M urea and reduction using 2-ME nine cysteine residues were identified. The treatment of "native" toxin with ethylene-imine caused a 50% decrease in toxicity.

Two different groups, Murphy et al. (1968) and Craven and Dawson (1973), failed to identify any N-terminal amino acids. Aspartic acid was identified as an N-terminal amino acid by Soru et al. (1958). Glycine was identified as an N-terminal amino acid by Holmes and Ryan (1971). Neubauer and Helting (1979) identified proline as the overall N-terminal amino acid for 'unnicked' toxin and proline as the N-terminal amino acid for L-chain with leucine as the N-terminal amino acid for H-chain. Proline was independently identified as the N-terminal amino acid for both 'unnicked' toxin and L-chain from work carried out in this thesis (van Heyningen, 1980; Britton and van Heyningen, 1980).

Murphy et al. (1968) showed that tetanus toxin contained ten cysteine residues of which four were involved in disulphide bridge formation. Craven and Dawson (1973) investigated the cysteinyl content of 'unnicked' toxin, 'nicked' toxin, H-chain and L-chain using 2-aldrithiol according to the method of Grassetti and Murray (1967). The samples were reduced with either sodium borohydride according to the method of Cavallini et al. (1966) or dithiothreitol (DTT) according to the method of Morino and Snell (1967). Craven and Dawson (1973) also investigated the cysteinyl content of 'nicked' toxin using [^{14}C]-IAA according to the method of Crestfield et al. (1963) in either the presence or absence of 8M urea and DTT. The distribution of the cysteinyl residues in the toxin molecule was also investigated using [^{35}S]-sodium sulphite and [^{14}C]-IAA by Craven and Dawson (1973). 'Unnicked' toxin was found to contain 6.5 free cysteine residues and either a total of 8.9 cysteine residues, after borohydride reduction, or 8.2 cysteine residues, after reduction with DTT, using 2-aldrithiol, and 8.9 cysteine residues by oxidative sulphitolysis. 'Nicked' toxin was found to contain 6.0 free cysteine residues and either a total of 9.4 cysteine residues, after borohydride reduction, or 9.0 cysteine residues, after reduction with DTT, using 2-aldrithiol, and 9.4 cysteine residues by oxidative sulphitolysis. S-carboxymethylation of H-chain indicated 0.1 free cysteine residues which was increased to 3.0 cysteine residues after denaturation in 8M urea. A total of

3.7 cysteine residues were detected in H-chain, after denaturation and reduction, using [^{14}C]-IAA. This was also confirmed by oxidative sulphitolysis in the presence of 8M urea. S-carboxymethylation of L-chain indicated 0.1 free cysteine residues which was increased to 2.7 cysteine residues after denaturation in 8M urea and further increased to 3.6 residues after denaturation and reduction. Oxidative sulphitolysis of L-chain in the presence of 8M urea indicated 3.9 cysteine residues. Thus Craven and Dawson (1973) showed that the cysteine residues present in tetanus toxin were equally distributed between the two chains.

The amount of toxin in all the experiments described above was determined by absorbance at 280nm using absorbance indices ($E^{1\%}_{280}$) of either 14.1 (Bizzini et al., 1970), 7.8 (Murphy et al., 1968) or 11.3 for 'unnicked' toxin and 12.4 for 'nicked' toxin (Craven and Dawson, 1973). Craven and Dawson (1973) determined an extinction index of 7.1 for L-chain using purified L-chain, obtained by oxidative sulphitolysis of tetanus toxin. H-chain was found to be very insoluble after preparation by oxidative sulphitolysis, so an absorbance index could not be directly determined. However, Craven and Dawson (1973) calculated an extinction index for H-chain of 13.7 by the difference between the absorbance indices of 'nicked' toxin and L-chain. Thus in each of the above experiments carried out to determine the sulphydryl content of tetanus toxin the determination of the amount of tetanus toxin is questionable. A suitable method for the determination of the extinction index of a protein would be the use of the analytical ultracentrifuge as described by Hardie and Cohen (1978). Though this method was not used in the experiments described above.

3.3.2. Methods

3.3.2.1. Determination of N-Terminal Amino Acids

3.3.2.1.1. Identification By Dansylation

Experiments to determine the N-terminal amino acids of tetanus toxin were carried out using 'unnicked' toxin, 'nicked' toxin,

H-chain and L-chain. The proteins were purified as described in Chapters 1 and 2.

The N-terminal amino acids were determined by the dansylation method first described by Gray and Hartley (1963a,b). The method uses N,N-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride, DNS-Cl), which was originally used by Weber (1952) and then by Hartley and Massey (1956) as a protein labelling compound for the elucidation of specific groups at the active sites of enzymes. It was during these experiments that DNS-Cl was discovered as an N-terminal reagent, a hundred times more sensitive than Sanger's 2,4-dinitrofluorobenzene (see Section 3.3.2.1.2.), though its potential as a N-terminal reagent was not utilised until the early 1960's (Hartley, 1970). The use of DNS-Cl has been very widespread over the last two decades not only as an N-terminal reagent but also for the identification of the new N-terminal amino acids produced after Edman degradation of proteins (Edman, 1956) during the elucidation of protein sequences (Gray, 1972b). The methods and the mechanism of the reactions of DNS-Cl with proteins have been published many times and may be found in Gray and Hartley (1963a,b); Brown and Hartley (1966); Gray (1967, 1972a); Hartley (1970); Seiler (1970); Thomas (1974) and Perham (1978).

The method used for work described in this thesis was based on the method of Gray (1967) and Perham (1978). DNS-Cl is susceptible to hydrolysis by water and hydroxyl ions. It reacts only with amino groups present as free bases ($R\text{-}NH_2$), and not with amino groups present as their conjugate acids ($R\text{-}\overset{+}{N}H_3$), so labelling was carried out at alkaline pH. The DNS-Cl was dissolved in acetone, because of its greater solubility in acetone, without any hydrolysis. The dissolved DNS-Cl was then added to the appropriate protein previously dissolved in 0.5M $NaHCO_3$, to give a final acetone concentration of 50% (v/v). The acetone also suppresses the ionisation of the amino groups of the proteins (Gray and Hartley, 1963a).

Four different purified protein samples were analysed with DNS-Cl. In each case 20nmol (3mg of either 'unnicked' or 'nicked' toxin, 2mg of H-chain and 1mg of L-chain) of freeze-dried protein, previously dialysed against de-ionised water, was dissolved in $0.5cm^3$ of freshly prepared 0.5M $NaHCO_3$ and 8M urea (aristar grade, BDH)

in an acid washed test tube. The urea was included to denature the proteins and increase the availability of the N-terminal amino acids for reaction with the DNS-Cl. Dansylation was carried out by adding 0.5cm^3 of 20$mgcm^{-3}$ DNS-Cl (BDH) in acetone (analar) to the protein solutions and incubating the samples for 1 h at 37°C. The test tubes containing the dansyl solutions were wrapped in aluminium foil and sealed with parafilm. The dansylation reaction was observed to be completed when the deep yellow colour of DNS-Cl was lost either as a result of reacting with the amino groups of the protein or as a result of hydrolysis. A precipitate was usually observed, probably as a result of the precipitation of the protein by the acetone; it usually redissolved after vigorous shaking. After the labelling step, the dansylated proteins were transferred to dialysis bags, diluted three-fold with distilled water, and dialysed with several changes against distilled water to remove the DNS-OH and urea. After about three hours a white precipitate formed in the dialysis bag. The precipitate was the dansylated protein, which was insoluble in aqueous buffers as a result of the covalently attached hydrophobic dansyl groups. The contents of the dialysis bags were transferred to large acid-washed hydrolysis tubes and freeze-dried. The samples were then hydrolysed in 6M HCl at 106°C for either 5 h or 15 h.

Dansyl-proline (DNS-Pro, in future all dansylated amino acids will be described with the abbreviation, DNS, before the abbreviation of the amino acid's full name) is known to be destroyed by acid hydrolysis and for a reasonable identification requires a short hydrolysis time, 5 h, Hartley (1970); Gray (1972a) and Perham (1978). The shorter hydrolysis time can also give rise to dansylated dipeptides resulting from the second amino acid in the protein sequence being still attached to the dansylated N-terminal amino acid.

The hydrolysed samples were dried down in an evacuated desiccator over NaOH pellets and then redissolved in 50µl of absolute ethanol or occasionally 50% (v/v) pyridine. Absolute ethanol was preferred, as pyridine tended to dissolve by-products of the dansylation reaction and lead to dirtier chromatograms. Absolute ethanol results in lower yields of DNS-Glu and DNS-Asp for the chromatograms, so samples were also dissolved in pyridine to check for these two dansylated amino acids.

There are two ways of identifying dansyl amino acids; either by high voltage electrophoresis (Gray, 1967, 1972a; Ambler, 1967) or by two-dimensional chromatography on polyamide sheets (Woods and Wang, 1967; Gray, 1972a; Perham, 1978). The high voltage electrophoresis method requires a very high capital outlay and is only useful in laboratories producing many routine samples. A high voltage electrophoretic run was carried out on a sample of dansylated H-chain with the help of Dr. R. Ambler (Department of Molecular Biology, University of Edinburgh). The method most frequently used and favoured by most laboratories is the two-dimensional chromatographic procedure carried out on polyamide sheets (Hartley, 1970; Ramshaw et al., 1970; Gray, 1972a; Perham, 1978). The polyamide sheets were obtained through British Drug Houses (BDH), Poole, Dorset, England, from the Cheng Chin Trading Company Taiwan, as 15cm by 15cm sheets which were cut into four 7.5cm by 7.5cm sheets for use.

The appropriate amount of dansyl amino acid mixture (usually 10µl, determined by trial and error) was spotted 1cm from the bottom and the right-hand side of one side of the polyamide sheet, and an equivalent amount was spotted directly over the sample but on the reverse side of the sheet. A 5µl sample containing a mixture of dansylated amino acids was spotted on top of one of the samples already spotted onto the plate. The standard mixture contained $0.1 mgcm^{-3}$ of the following dansylated amino acids in absolute ethanol; DNS-Ala, DNS-Lys, DNS-Leu, DNS-Phe, DNS-Pro, DNS-Val, DNS-Ser, DNS-Asp, DNS-Tyr and DNS-Arg. All were a gift from Dr. G. Pettigrew (Department of Biochemistry, University of Edinburgh Veterinary School); all standards were N^{ϵ}-dansylated.

The samples were developed in Solvent 1 (3% (v/v) formic acid), by ascending chromatography in a chromatographic beaker previously equilibrated with the solvent, until the solvent front reached the top of the polyamide sheet, which usually took 10min. The polyamide sheet was dried in a stream of warm air turned through 90^{o} and developed in Solvent 2 (toluene:glacial acetic acid, 9:1), by ascending chromatography as described for Solvent 1, which usually took 15 min. The polyamide sheet was then dried in a stream of warm air and the dansylated amino acids were observed as a result of their fluorescence in UV light (protective goggles

were worn) of long wave emission (366nm). The dansylated amino acids were observed as yellow fluorescent spots. DNS-NH_2 produced from the reaction of DNS-Cl with ammonia was identified as a greenish-blue spot and DNS-OH produced from the hydrolysis of DNS-Cl by water was observed as an intense-blue spot. Other dansylated derivatives of various amino acids were also observed; O-DNS-Tyr and N^6-DNS-Lys produced from the reaction of DNS-Cl with the -OH group of tyrosine and the amino group of the side chain of lysine. The derivatives fluoresced with a greater intensity than dansylated N-terminal amino acid residues because of their higher mol ratio. The presence of these fluorescent derivatives was useful as an indication that the dansylation reaction had taken place. The dansylated amino acids present in the toxin samples were identified from co-migration of the standard amino acids on the reverse side of the polyamide sheet. DNS-Leu and DNS-Ile run very close together, so it is very difficult to make a positive identification. However by including the sample under investigation with the standards one can distinguish between the two amino acids by including one of them in the standards. If two spots are observed in the vicinity of DNS-Leu and DNS-Ile on the standard side of the polyamide sheet one knows which of the amino acids are present from the known amino acid in the standards mix. If one spot occurs on the sample side and one spot on the standard plus sample side one deduces that the amino acid is the same as the one included in the standards.

Most amino acids are resolved in the two-dimensional system, though DNS-Asp/DNS-Glu and DNS-Thr/DNS-Ser can produce ambiguities. DNS-Asp and DNS-Glu are sometimes observed to be overlapped by DNS-OH and DNS-Thr and DNS-Ser may be observed to run as one spot. In order to resolve DNS-Asp/DNS-Glu and DNS-Thr/DNS-Ser a third solvent system was used. Solvent 3 consisted of butyl acetate:methanol:glacial acetic acid, 20:1:1. The chromatogram was developed in Solvent 3 in the same direction as for Solvent 2. This step usually took 15 min.

After use the polyamide sheets were washed in acetone:1M ammonia (1:1) for twelve hours. The washing process was carried out within one day of the polyamide sheets being used to avoid any irreversible binding of the dansylated amino acids to the polyamide.

The washed sheets were carefully checked under UV light for the presence of any fluorescent material before re-use.

Dansylation of Single Amino Acids

In one experiment DNS-Met was required in the standard mixture, in order to check its position on the chromatogram. A process suitable for dansylating single amino acids was used. A $1mgcm^{-3}$ solution of methionine (chromatographically pure, BDH) in water was prepared. A 7µl sample (50nmol, 6.85µg) was placed in an acid washed Durham tube, and dried down to remove any ammonia. The sample was re-dissolved in 10µl of 1% (w/v) $NaHCO_3$, dried down once again and finally redissolved in 10µl of deionised water. The methionine was dansylated by adding 10µl of $2.5mgcm^{-3}$ DNS-Cl in acetone and incubating the sample at 37°C until the solution went colourless (about 30 min). The sample was dried down and any unreacted DNS-Cl destroyed by adding 10µl 6M HCl. The solution was dried down and redissolved in 50µl of absolute ethanol.

3.3.2.1.2. Identification by Dinitrophenylation

2,4-dinitrofluorobenzene (FDNB, Sanger's reagent) was the first reagent to be successfully used for the identification and estimation of the amount of N-terminal amino acids in peptides and proteins. The method was used in this work to try and determine quantitatively the amount of N-terminal amino acids and to rule out the possibility that both chains in tetanus toxin had the same N-terminal amino acid. The method used was as described by Fraenkel-Conrat et al. (1955), and carried out on purified 'nicked' toxin.

A 110nmol (16.5mg) sample of purified 'nicked' toxin was dialysed several times against distilled water and then freeze-dried. The sample was redissolved in $6cm^3$ of freshly prepared 0.2M N-ethylmorpholine and 8M urea (aristar grade) at pH 8.5. 40µl of FDNB was carefully added to the toxin solution which was then flushed with N_2. The sample tube was wrapped in aluminium foil and sealed with a glass stopper, and the sample incubated at room temperature with continuous shaking for 24 h. Excess FDNB reagent was extracted once into $6cm^3$ of ether (top layer) and for a second time into $10cm^3$

of ether. The ether extracts were pooled and any toxin present back extracted with $1cm^3$ of water, which was then pooled with the main aqueous sample. The dinitrophenylated (DNP) protein sample was dialysed against water, freeze-dried and hydrolysed with $0.5cm^3$ of 6M HCl at 105^oC for 19 h. The hydrolysate was dried down and extracted three times using $0.5cm^3$ of ether; the extracts were pooled and re-extracted using $1cm^3$ 1M HCl. The ether extracts were dried down in a stream of N_2 and the DNP-amino acids dissolved in 30µl of absolute ethanol.

A set of standard DNP-amino acids were obtained from Dr. A.P. Ryle (Department of Biochemistry, University of Edinburgh) for the identification of DNP-amino acids obtained from the toxin sample. The standard DNP-amino acids were $2mgcm^{-3}$ in ethanol and consisted of DNP-NH_2, di-DNP-Tyr, di-DNP-Lys, DNP-Trp, DNP-Phe, DNP-Leu, DNP-Ile, DNP-Val, DNP-Met, DNP-OH, di-DNP-His, DNP-Ala, DNP-Pro, DNP-Met(SO_2) (methionine sulphone), DNP-Thr, DNP-Gly, DNP-Ser, DNP-Glu and DNP-Asp. A 100µl (200µg) sample of each standard DNP-amino acid was taken and pooled to make a standard mix. The mixture was dried down in a stream of N_2, and redissolved in $0.3cm^3$ of absolute ethanol. Two chromatograms were run using either 20µl or 40µl of the standard mix to ascertain the correct loading. The DNP-amino acids either from the standard mix or the ether extract were spotted onto the bottom right-hand corner of a sheet of Whatman No.1 paper (25cm by 25cm) 3cm from either edge. The chromatograms were mounted onto a metal frame for the development of several chromatograms. The chromatograms were developed in Solvent A, 2-methylbutan-2-ol:2M ammonia (4:1), by ascending chromatography for 16 h in a tank previously equilibrated with the solvent system. The chromatograms were dried down turned through 90^o, and developed by ascending chromatography in 1.5M sodium phosphate pH 6.0 for 8 h.

The DNP-amino acids are observed as yellow spots and identified by comparing their positions with the chromatograms of the standard DNP-amino acids. The identification of some DNP-amino acids, DNP-Trp, di-DNP-Tyr and DNP-Pro, was improved by observation under UV light (366nm) as they appeared as much darker spots. DNP-OH was also further identified by its disappearance upon exposure to 6M HCl fumes.

3.3.2.2. Determination of the Total Half-Cystine Residues

Disulphide bridges play an important role in stabilising the three-dimensional structure of proteins and bring different regions of the primary structure of a protein into close proximity with each other, which may be important for both structural reasons and for biological activity. To determine the number of disulphide bridges in a protein molecule one first needs to know the total number of half-cystine residues and the number of free cysteine residues and from the difference one can calculate the number of cysteine residues involved in disulphide bridges.

There are numerous reagents that are able to react with cysteine residues, e.g. DTNB, IAA, N-ethylmaleimide, iodoacetamide, and there are a number of reagents capable of reducing disulphide bridges, e.g. 2-ME, $NaBH_4$ and DTT. For the variety and mechanisms of reaction of these reagents see: Fontana and Scaffone (1969); Freedman (1971); Means and Feeney (1971); Jocelyn (1972); Friedman (1973); Anderson and Wetlaufer (1975); Kenyon and Bruice (1977); and Brocklehurst (1979). The reagents used to identify the cysteine residues in 'nicked' toxin, 'unnicked' toxin, H- and L-chains were either DTNB, IAA or N-ethylmaleimide.

3.3.2.2.1. Investigation Using DTNB

The method for determining the total half-cystine content of a protein using DTNB was first described by Cavallini *et al.* (1966) and later by Habeeb (1973). The disulphide bridges were reduced using sodium borohydride ($NaBH_4$) as initially described by Brown (1960) and later by Cavallini *et al.* (1966) and Habeeb (1973). The number of cysteine residues produced were detected using DTNB (Ellman, 1959). The method of Cavallini *et al.* (1966) was tested using BSA both in the presence and absence of either 8M urea, 6M GuHCl or 1% (w/v) SDS. 8M urea was found to be the most suitable denaturant as 6M GuHCl produced inconsistent results with BSA and SDS foamed too much during some of the experimental procedures.

The amount of BSA was determined by absorbance at 280nm using the extinction index $E_{280}^{1\%}$ of 6.7. Several graduated test tubes were used, and the following reagents were added to each tube

1.44g urea (aristar) 0.1cm^3 0.1M EDTA, 0.5-1.0cm^3 of protein solution, 1cm^3 2.5% (w/v) $NaBH_4$ (prepared immediately before use) and water to the 3cm^3 level of each tube. A drop of octanol was added to each sample (to act as an antifoaming agent). The samples were incubated at 37°C for 30 min with continuous shaking. 0.5cm^3 of 1M KH_2PO_4 containing 0.2M HCl was then added to each tube to destroy any excess $NaBH_4$. After a 5 min incubation period, at 37°C, any remaining $NaBH_4$ was destroyed by the addition of 2cm^3 acetone (Brown et al., 1955). The samples were flushed with N_2 for 5 min. The nitrogen was passed through a solution of pyrogallol and KOH to remove any O_2 and CO_2. The pyrogallol solution was prepared from 50g pyrogallic acid dissolved in 600cm^3 of water to which was then added to 250g KOH dissolved in 600cm^3 of water. After the solutions had been flushed with N_2, 0.5cm^3 0.01M DTNB in 0.5M sodium phosphate pH 7.2 was added to each sample, which were then flushed with N_2 and the tubes stoppered. The samples were incubated at room temperature for 15 min and the absorbance of each solution was measured at 412nm in a Cecil spectrophotometer. Control experiments were included where no protein was added, to determine any background absorbance. All reactions were carried out in duplicate and the final absorbances were determined from the two averages less any background absorbance. The number of cysteine residues (N) were determined using the equation:-

$$N = \frac{MW.\ A.V}{13600.M}$$

Where:-

MW	molecular weight of the protein in daltons.
A	absorbance of the sample at 412nm.
V	total volume of the sample.
M	weight in mg of the protein used.

The method was used to try and determine the total number of half-cystine residues and the number of free cysteine residues in 'nicked' tetanus toxin.

3.3.2.2.2. Investigation Using IAA

S-carboxymethylation using IAA (recrystallised as described

in Section 2.4.2.) was carried out according to the method of Hirs (1967b) and Gurd (1967, 1972) except DTT was used instead of 2-ME because of its greater reducing capacity (Konigsberg, 1972). The number of cysteine residues were determined by including iodo-[^{14}C]-acetic acid ([^{14}C]-IAA) in the 'cold' IAA. From the specific activity of the [^{14}C]-IAA one can determine the number of cysteine residues (van Heyningen, 1972; Perham, 1978). BSA was once again used in order to check the effectiveness of the system. 'Unnicked' toxin, 'nicked' toxin, H- and L-chain were then analysed using the method.

A known amount of protein was taken. The amount of BSA was determined as described above, and the amount of protein in the toxin samples was determined by amino acid analysis, carried out in triplicate. The protein samples were dialysed against distilled water, freeze-dried and redissolved in 6M GuHCl (recrystallised or aristar grade), 0.05M Tris-HCl, 0.2% (w/v) EDTA at pH 8.6 and then added to graduated test tubes. 8M urea was originally used as the denaturant but experience using BSA showed that 6M GuHCl was the better denaturant for this system; urea as the denaturant tended to give lower numbers of cysteine residues than published or as observed by using 6M GuHCl. The disulphide bridges were reduced by adding a known amount of DTT (from a freshly prepared stock solution), usually in the range of 50μmols, and incubating the samples at 37°C for 5 h.

The thiol groups were then S-carboxymethylated by adding a solution of [^{14}C]-IAA of known specific activity (usually about 70nCi μmol^{-1}, the amount of 'cold' IAA was determined by weighing). The amount of IAA added was always slightly greater than twice the amount of DTT because of the two thiol groups carried by each DTT molecule. The samples were incubated at 37°C for 15 min in the presence of IAA and the tubes were wrapped in aluminium foil. The reaction was carried out in the dark to prevent the photo-oxidation of the liberated iodide ions (produced as a result of the S-carboxymethylation reaction) to iodine which in turn is able to react with tyrosine. Excess IAA was destroyed by adding a few drops of 2-ME to each solution. Initially the samples were then dialysed to remove the iodide ions and other reagents, especially S-carboxymethylated DTT and 2-ME, but this was found to give inconsistent

results; not all the excess radiolabel was being removed. Samples were then desalted on Sephadex G-25 columns (fine grade, 2cm by 17cm) wrapped in aluminium foil. The samples were eluted from the columns using 4M urea, 0.5M Tris-HCl, 0.2% (w/v) EDTA at pH 8.0 and collected in $2cm^3$ fractions. The protein containing fractions were identified by taking $0.1cm^3$ samples for scintillation counting and measuring the absorbance, at 280nm, of the fractions, see Diagram 15.
The protein containing fractions were pooled and various volumes (usually $0.2cm^3$ to $1.0cm^3$) taken in duplicate for scintillation counting. It was at this stage a known volume, in triplicate, was taken for protein concentration determination. The samples for scintillation counting were made up to $2cm^3$ with the elution buffer and dissolved in $10cm^3$ Triton/toluene scintillant, as described in Section 2.14.

The total number of half-cystine residues were determined in 'unnicked' toxin, H- and L-chain purified by preparative SDS-PAGE. The samples were treated as described in Section 3.3.2.2.2., except that 8M urea was used instead of 6M GuHCl as the presence of SDS with GuHCl caused gelling of the GuHCl presumably from the formation of guanidine dodecyl sulphate. The protein concentrations of the samples were determined by amino acid analysis and found to be 6.86nmol for 'unnicked' toxin, 12.6nmol for H-chain and 13.5nmol for L-chain. The samples were reduced with 0.11mmol DTT at $37^{o}C$ overnight and labelled with [^{14}C]-IAA of specific activity $139nCi\mu mol^{-1}$ at $37^{o}C$ for 15 min. The samples were then treated as described earlier and analysed.

A number of experiments were carried out; to check that the incubation time was long enough, and that the samples were being S-carboxymethylated. The IAA incubation time was checked by taking a known amount of toxin (32nmol) and labelling with $38.64nCi\mu mol^{-1}$ [^{14}C]-IAA. Duplicate samples were taken from the reaction at times ranging from 0.5 min to 30 min. The samples were immediately treated with 13µmol DTT to destroy any IAA and the toxin precipitated by adding 10% (w/v) TCA and incubating at $0^{o}C$ for 20 min. The precipitated toxin was filtered onto Whatman GF/C (2.5cm) glass fibre filters previously soaked in 5% (w/v) TCA. The precipitates were washed thrice with $2.0cm^3$ 5% (w/v) TCA, $2.0cm^3$ redistilled ethanol and ether (1:1) and finally with $1cm^3$ ether. The

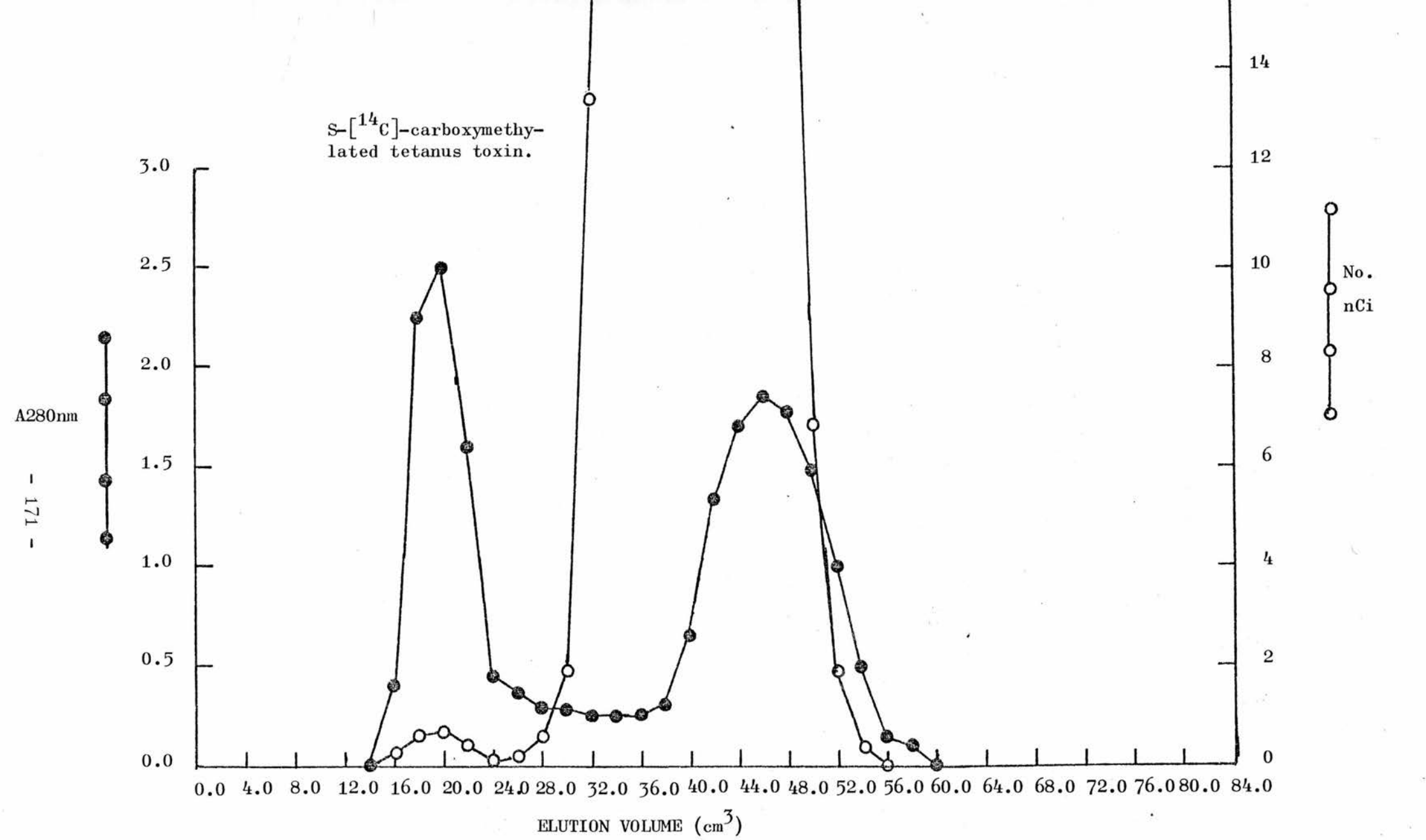


DIAGRAM 15. ELUTION PROFILE FOR THE IDENTIFICATION OF S-[^{14}C]-CARBOXYMETHYLATED TETANUS TOXIN FROM A SEPHADEX G-25 COLUMN.

filters were then dried and placed in scintillation vials. The precipitated proteins were dissolved out of the filters by incubating the filters in $0.7cm^3$ NCS solution (NCS:water, 9:1) at $60^{\circ}C$ for 3 h. The vials were allowed to cool and the NCS solution dissolved in $10cm^3$ Triton/toluene scintillant. The vials were stored at $4^{\circ}C$ overnight before counting to allow any photons produced by the NCS in the scintillant to dissipate.

To determine if the toxin was being S-carboxymethylated two types of experiments were carried out; analysis by SDS-PAGE and analysis of hydrolysed S-carboxymethylated toxin. In the first type of experiment S-carboxymethylated toxin was electrophoresed into 8% polyacrylamide gels and the channel containing the toxin sliced into 1mm slices. The slices were then incubated in $0.5cm^3$ NCS:water (9:1) at $50^{\circ}C$ for 4 h in order to swell the gel slices to allow any proteins to diffuse out of the slices. Because of the high pH of the NCS and the temperature of the incubation, the proteins originally present in the slice would be hydrolysed into peptides. The peptides were then dissolved in $10cm^3$ Triton/toluene scintillant. Unfortunately the number of counts detected were not significantly greater than the background counts. NCS is known to cause the production of photons which can interfere with scintillation counting if samples of low specific activity are analysed. A second approach was tried using the method of McKee et al. (1970) where the gel slices were hydrolysed in $1cm^3$ 6M HCl at $106^{\circ}C$ for 24 h. The acrylic acid, produced from the hydrolysis of the gel matrix, was precipitated by cooling the samples to $-50^{\circ}C$. The precipitates were centrifuged down, and the supernatants removed, dried down and redissolved in $1cm^3$ of water. The samples were dissolved in $10cm^3$ Triton/toluene scintillant and analysed. Again there was no significant difference between the radioactive slices and background pieces of gel, probably because of quenching caused by the presence of any unprecipitated acrylic acid and the low specific activity of the samples.

To rule out the possibility of interference from the solubilising reagents a series of gels were polymerised with N,N'-diallyltartardiamide (DATD, Eastman Kodak) instead of bis-acrylamide, used in a mol to mol replacement, Anker (1970). Gels polymerised with DATD were observed to have a larger pore size,

requiring a final acrylamide concentration of 12% to be equivalent of the normal 8% gels. A problem associated with DATD gels was the increase in water retention, after the staining and destaining procedures, resulting in much longer gels. 2mm slices were cut from the DATD gels and dissolved in $0.5cm^3$ 2% (w/v) periodic acid (Sigma) at $37^{\circ}C$ for 30 min. Any excess periodic acid was destroyed with $0.5cm^3$ 1M glucose. The samples were then dissolved in $10cm^3$ Triton/ toluene scintillant and analysed, no quenching is caused by the periodic acid. There was very little difference in the number of counts between the slices, so it was concluded that the specific activity of the S-[^{14}C]-carboxymethylated toxin was too low for analysis from gel slices. A normal 8% polyacrylamide gel greatly overloaded with a sample of S-[^{14}C]-carboxymethylated toxin was dried down and autoradiographed for two months, as described in Section 2.9. Faint radioactive bands were observed on the autoradiograph which corresponded to H- and L-chains.

In the second type of experiment S-[^{14}C]-carboxymethylated toxin or S-carboxymethylated BSA was hydrolysed in 6M HCl at $106^{\circ}C$ for 24 h, dried down and redissolved in 0.1M NH_3 and analysed by paper electrophoresis at pH 2.0. A sample of S-carboxymethylated cysteine (CMcys, Sigma) was added to the standard amino acids described in Section 2.8.1. An amino acid spot present in either hydrolysed toxin or hydrolysed BSA co-electrophoresed with the CMcys in the standard mix. Staining with collidine (Section 2.8.1.) showed that the amino acids stained the same colour, grey-green, evidence that they were equivalent. A sample of the [^{14}C]-CMcys from S-[^{14}C]-carboxymethylated toxin was eluted from the paper (as described in Section 2.8.3.) and analysed. The number of counts obtained from the suspected [^{14}C]-CMcys spot were found to be much higher than background counts. Autoradiography of the appropriate area of paper also showed the spot to be radioactive. The method described above was only used once as it was found to be easier to analyse the hydrolysate using the automatic analyser, after a new buffer system had been devised for detecting CMcys (Section 2.7.4.), see Diagram 16 for the separation of CMcys from Asp.

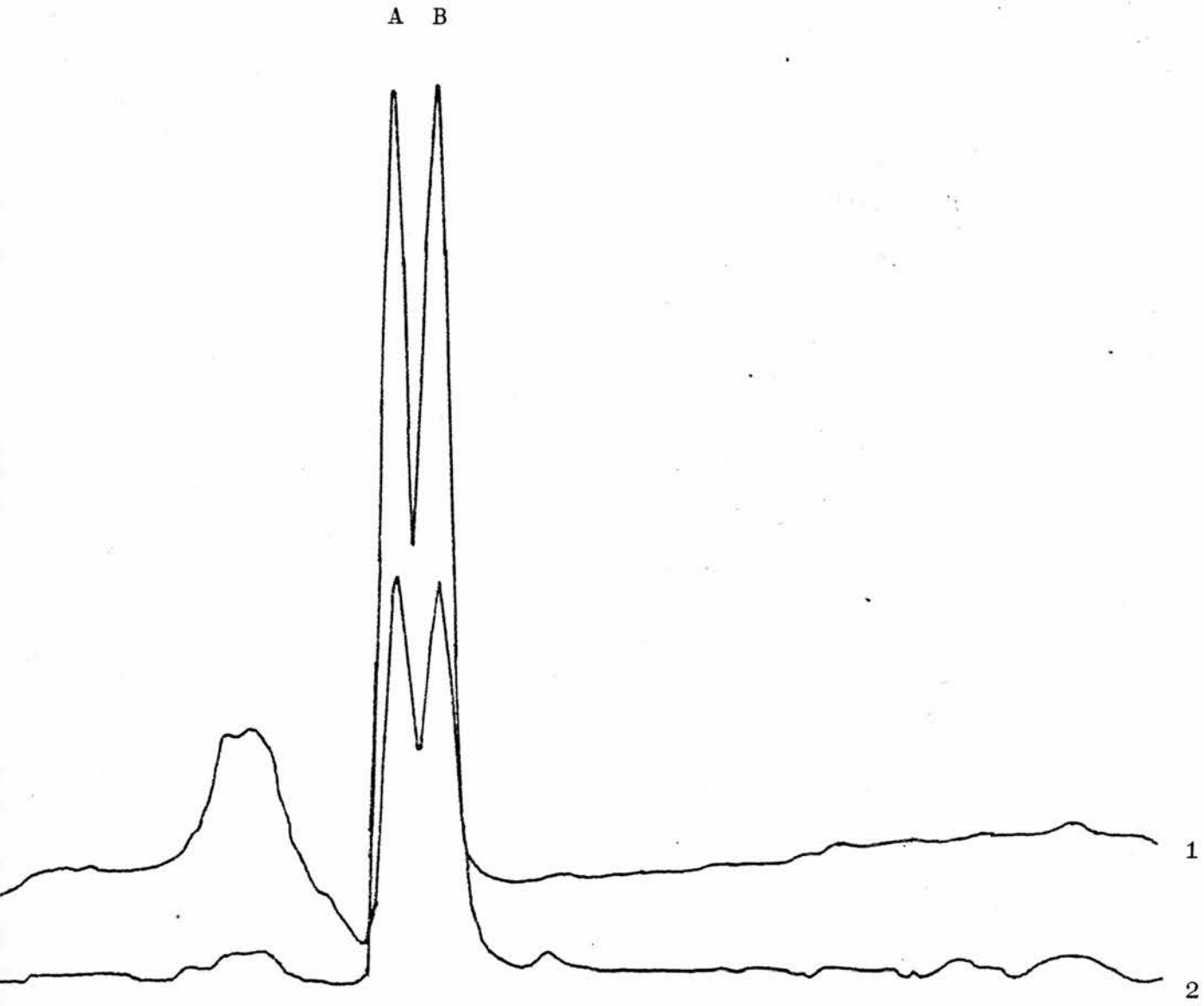


DIAGRAM 16. SEPARATION OF S-CARBOXYMETHYLATED CYSTEINE FROM ASPARTIC ACID AT pH 3.05 ON AN AUTOMATIC AMINO ACID ANALYSER

See Section 3.3.2.2.2. for details.

A S-carboxymethylated cysteine.

B Aspartic acid.

1 Absorbance at 440nm.

2 Absorbance at 570nm.

3.3.2.3. Determination of the Number of Free Cysteine Residues and the Number of Disulphide Bridges

Two different methods were used to try and determine the number of disulphide bridges involved in the "native" structure of 'nicked' toxin. In the first experiment 8.75nmol of purified toxin in 0.04M sodium phosphate buffer pH 7.2 (Section 3.1.2.) was reacted with 20mg (0.16mmol) N-ethylmaleimide (NEM) at 37°C for 1 h to block any free cysteine residues in the "native" structure of the toxin. The toxin was then denatured by adding solid urea to a concentration of 8M. The toxin was then incubated at 37°C for 2 h for the NEM to react with any cysteine residues made available for reaction after denaturation. The sample was dialysed several times against water and then freeze-dried. The freeze-dried toxin was then redissolved in $5cm^3$ 6M GuHCl, 0.5M Tris-HCl, 0.2% (w/v) EDTA and 48.7µmol (9.7mM) DTT at pH 8.5 and incubated at 37°C for 5 h. The reduced cystine residues were then reacted with [^{14}C]-IAA of specific activity $81.91nCi\mu mol^{-1}$ (prepared by dissolving 132µmol IAA in $0.3cm^3$ of 0.5M NaOH and then adding 350µl of $50\mu Cicm^{-3}$ [^{14}C]-IAA batch No.71, The Radiochemicals Centre, Amersham, the actual specific activity was determined by analysing the amount of radioactivity present in a known amount of IAA (determined by weighing))at 37°C for 15 min. The sample was then treated as described in Section 3.3.2.2.2., and the radiolabelled toxin analysed, except that duplicate samples ranging from $0.2cm^3$ to $3.5cm^3$ were made up to $4cm^3$ with the elution buffer and dissolved in $14cm^3$ of scintillant. Cysteine residues react with NEM to form S-succinyl cysteine (Scys) which was also detectable just before CMcys by automatic amino acid analysis.

The second type of experiment to try and determine the number of disulphide bridges was double labelling using [^{3}H]-IAA and [^{14}C]-IAA. The protocol of the experiments is shown in Diagram 17. The general outline of the experiment was to label initially all the free cysteine residues (initially in the "native" conformation and then after denaturation) with [^{3}H]-IAA, to reduce the toxin and label the cysteines derived from cystine residues with [^{14}C]-IAA. The Searle scintillation counter allowed analysis of double labelled samples by making the cross-channel corrections automatically.

DIAGRAM 17. **EXPERIMENTAL PROCEDURE FOR DETERMINING THE DISULPHIDE BRIDGE CONTENT OF TETANUS TOXIN BY DOUBLE LABELLING**

See Section 3.3.2.3. for details.

DIAGRAM 17

0.25μmol Tetanus Toxin in 0.04M sodium phosphate buffer pH 7.2

Dialysed against H_2O.

Sample for performic acid oxidation.

Cysteic acid content.

sample dialysed against 0.5M Tris-HCl, 0.2% EDTA pH 8.0. Concentrated using Amicon UM10 membrane.

Reacted with [^{3}H]-IAA

x2 samples dialysed against H_2O, freeze-dried and redissolved in 6M GuHCl, 0.5M Tris-HCl, 0.2% EDTA 1mM DTT at pH 8.0. Incubated at 37°C for 5 h and then reacted with either [^{14}C]-IAA or [^{3}H]-IAA.

+ 2-ME

Sample desalted and protein conc. determined.

Total number of free cysteine residues in "native" toxin from [^{3}H]-IAA.

Rest desalted on a Sephadex G-25 column. Eluted with 0.5M Tris-HCl 0.2% EDTA pH 8.0. Radio-labelled samples pooled and conc. using Amicon UM10 membrane and then made to 6M GuHCl and incubated at 37°C for 1 h. Reacted with [^{3}H]-IAA.

+ 2-ME

Sample desalted and protein conc. determined.

Total number of free cysteine residues in tetanus toxin from [^{3}H]-IAA labelling.

Desalted as above, pooled, fractions conc. using YM10 membrane. Denatured in 6M GuHCl as above and then reduced with 1mM DTT and reacted with [^{14}C]-IAA.

+ 2-ME

Desalted as described above except elution buffer also contained 0.1% 2-ME. Protein conc. determined.

Number disulphide bridges from [^{14}C]-IAA labelling.

+ 2-ME

Desalted on Sephadex G-25 column. Eluted with 4M urea, 0.5M Tris-HCl, 0.2% EDTA 0.1% 2-ME pH 8.0. Radiolabelled samples pooled and samples taken for protein conc. determination and determination of number cysteine residues.

Gave total number of cysteine residues from either [^{14}C]-IAA or [^{3}H]-IAA labelling.

The $[^3H]$-IAA used was from a $1mCicm^{-3}$ sample, The Radiochemicals Centre, Amersham, and the $[^{14}C]$-IAA as described earlier, the specific activities of the samples used were 1 184 000 $cpm\mu mol^{-1}$ for $[^3H]$-IAA and $75.2nCi\mu mol^{-1}$ for $[^{14}C]$-IAA. In both cases the actual specific activities were determined by analysis. In some of the experiments concentrating took place in the absence of a denaturant allowing a UM10 membrane to be used. Ultrafiltration in the presence of denaturant was carried out using a YM10 membrane.

3.3.2.4. Amino Acid Analysis of 'Nicked' Tetanus Toxin, H-Chain and L-Chain

The procedures used for the preparation of samples (Section 2.7.1.) and for their analysis were as described in Section 2.7.5. Initially toxin samples were hydrolysed in 6M HCl. Different hydrolysis times were used in order to determine the amounts of serine and threonine by extrapolation to zero time and to make sure there were no dipeptides still present after the shorter incubation times, i.e. Leu-Leu, Ile-Ile or Leu-Ile, which require longer hydrolysis times to hydrolyse the peptide bonds.

In later analyses, the number of cysteine residues were determined, after oxidation to cysteic acid, using performic acid as described in Section 2.7.2. Tryptophan is destroyed by acid hydrolysis so was determined after hydrolysis using 2-mercaptoethane sulphonic acid (Pierce, a gift from Dr. R. Ambler, Department of Molecular Biology, University of Edinburgh), as described in Section 2.7.3. Unfortunately there was only sufficient reagent to determine the tryptophan content of 'nicked' toxin.

72 h hydrolyses were carried out in triplicate for the determination of protein concentration. The amount of protein, in nmol, was determined from the reciprocal of the conversion factor needed to convert the number of detected amino acid to the actual number of residues per mol of protein. The conversion factor was determined from the average value of the three analyses.

3.3.3. Results

3.3.3.1. Determination of N-Terminal Amino Acids

Initially purified 'nicked' toxin was investigated using the method described in Section 3.3.2.1.1. After a 15 h hydrolysis of the dansylated toxin no N-terminal amino acid was observed. After a 5 h hydrolysis DNS-Pro was detected (see Diagram 18) as was also observed by Neubauer and Helting (1979). A dansyl spot was observed very close to DNS-Pro which was not identifiable as a DNS-amino acid; this spot was also observed by Neubauer and Helting (1979) who found it to be DNS-Pro-Ile. The presence of a DNS-dipeptide after a 5 h hydrolysis is not uncommon. Very faint spots corresponding to DNS-Val, DNS-Ile and DNS-Leu were also observed, in contrast to Neubauer and Helting (1979) who observed a strong DNS-Leu spot with the DNS-Pro.

The possibility of the N-termini of both chains being proline was investigated using the FDNB method (Section 3.3.2.1.2.) which allows quantitative analysis. Unfortunately DNP-Pro is very unstable to acid hydrolysis so was not detected. No other DNP-amino acid was observed.

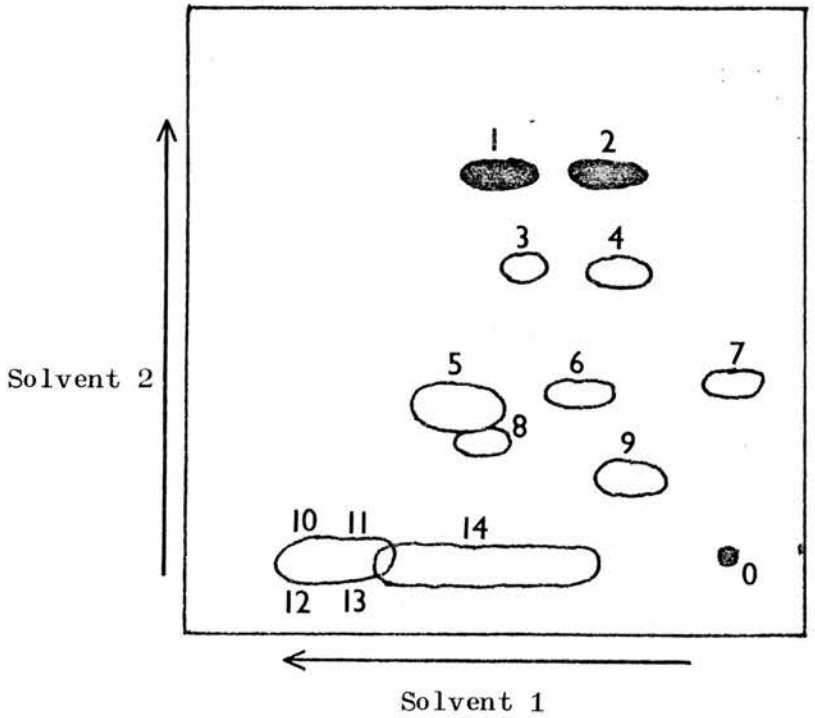
As a result of the incomplete data obtained from the dansylation experiments with 'nicked' toxin, H- and L-chain purified by SDS-PAGE were also dansylated. Both 5 h and 15 h hydrolyses were carried out on the dansylated chains. The 5 h hydrolysis of L-chain gave two dansyl spots corresponding to DNS-Pro and the DNS-Pro-Ile. A 15 h hydrolysis gave only a faint DNS-Pro spot. No detectable N-terminal amino acid was found for H-chain after either a 5 h or a 15 h hydrolysis, though O-DNS-Tyr and N^6-DNS-Lys were observed. Development of the L-chain and H-chain chromatograms in Solvent 3 gave no further information, apart from improving the separation of N^6-DNS-Lys. The dansylation experiments were carried out on H-chain several times without any further result. No new dansylated amino acids were observed if the dansylated samples were dissolved in 50% (v/v) pyridine. A sample of dansylated H-chain was analysed by high voltage electrophoresis. The sample was spotted onto Whatman 3MM paper and electrophoresed at 6 000V, 150mA for 2 h in a buffer system composed of 30cm^3 pyridine,

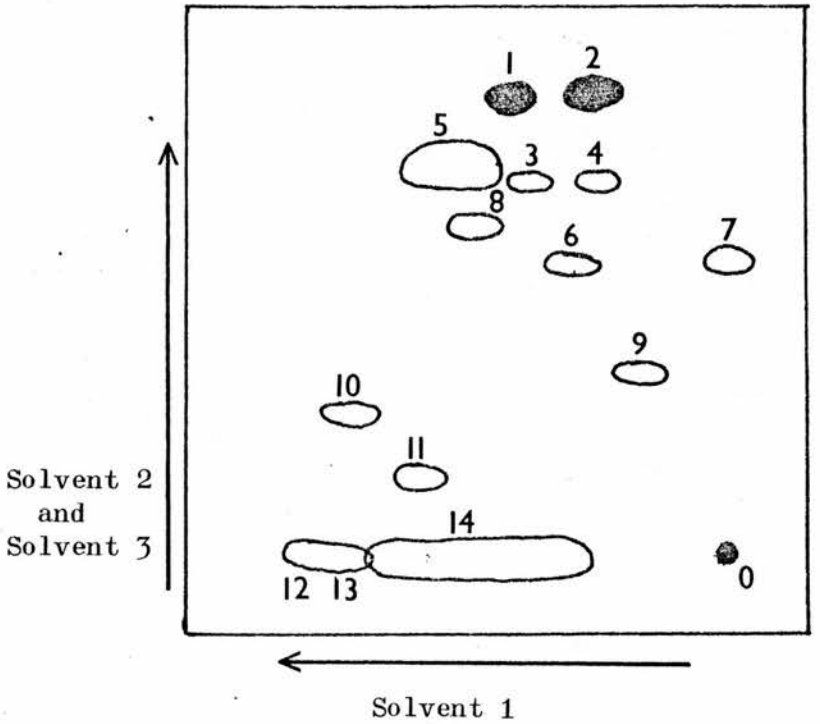
DIAGRAM 18. IDENTIFICATION OF DANSYL-PROLINE AND THE DANSYLATED-DIPEPTIDE DERIVED FROM 'UNNICKED' AND 'NICKED' TETANUS TOXIN OR L-CHAIN BY CHROMATOGRAPHY ON POLYAMIDE SHEETS

See Section 3.3.3.1. for details.

0 Origin
1 DNS-Pro
2 DNS-Dipeptide (DNS-Pro-Ile)
3 DNS-Val
4 DNS-Leu
5 DNS-NH_2
6 DNS-Phe
7 BIS-DNS-Tyr
8 DNS-Ala
9 BIS-DNS-Lys
10 DNS-Ser
11 DNS-Asp
12 DNS-Arg
13 N^6-DNS-Lys
14 DNS-OH

The black spots represent the positions of DNS-proline and the dansylated dipeptide.


Solvent 2
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
Solvent 1


Solvent 2
and
Solvent 3
1
2
3
4
5
6
7
8
9
10
11
12
13
14
0
Solvent 1

60cm^3 glacial acetic acid and 3.91cm^3 water at pH 4.35. A faint DNS-Asp spot was observed, though there was very high amounts of O-DNS-Tyr and N^6-DNS-Lys present.

To try and rule out the possibility of a blocked N-terminus on H-chain it was decided to try and obtain a partial N-terminal sequence. Purified samples of 'nicked' toxin H- and L-chains were analysed with Dr. P. Cohen (Department of Biochemistry, University of Dundee) using a Beckman 890C spinning cup automatic sequencer. Polybrene (1,5-dimethyl-1,5-diazoundecamethylene polymethobromide) was used to hold the protein samples in the spinning cup (Croft, 1980). A program devised by Dr. P. Cohen was run, using 0.2M Quadrol (N,N,N',N'-tetrakis-2-hydroxypropylethylene diamine) as the buffer. For the reagents used, the mechanisms of reaction and their general order in the process of automatic sequencing see Croft (1980). The thiozolinones, derived from the N-terminal amino acids at the cleavage step, were dried down and hydrolysed to the stable phenylthiohydantoins (PTH) of the amino acids using six drops of 20% (v/v) trifluoroacetic acid (TFA) at 80°C for 7 min. The PTH-amino acids were extracted into ethyl acetate, dried down and redissolved in 100µl of methanol and stored at -70°C. The PTH-amino acids were identified by high pressure liquid chromatography (HPLC) using a Sorbox ODS (octadecasilicate) reverse phase column (Dupont), a Water Associates Model 660 solvent programmer, a WISP 710 injector and a Water Associates Model 440 detector. The PTH-amino acids were eluted from the column using program gradient 11, elution took 14 min. The two solvent systems were A, 10% (v/v) CH_3CN in 0.01M acetate pH 4.75 and B, 45% (v/v) CH_3CN in 0.01M acetate pH 4.75. The PTH-amino acids were detected by absorbance in UV light at 260nm and identified from a sample of mixed standard PTH-amino acids.

Unfortunately the H- and L-chain samples could not be analysed in this system as the SDS present in the samples reacted with the quadrol producing derivatives that co-eluted with the PTH-amino acids. Twelve cycles of the sequencer were carried out for the 'nicked' toxin, each cycle gave rise to about twelve amino acids. The large number of amino acids could have arisen from contaminating proteins, but analysis of purified toxin was contrary to this. Certain sequence patterns were identified for the cycles carried out. A particular amino acid was observed to increase in

concentration throughout two cycles and then drop completely in concentration in the next cycle. The highest concentration was always observed during the second cycle of the pattern. The pattern was observed to occur throughout the twelve cycles with different amino acids rising and falling in concentration. Methionine was observed to be present in cycle 9 to increase in cycle 10 and then to be absent in cycle 11. Because methionine is a rare amino acid the liklihood of two methionine residues occurring consecutively is highly improbable, the methionine observation supports the sequence pattern. A possible explanation for this sequence pattern is a sequence in one of the chains been out of phase possibly a result of a "ragged" end. Proline was observed in a high concentration in the first cycle and isoleucine in a high concentration in the second cycle without any proline. This observation suggested that the L-chain (as proline is known to be the N-terminal amino acid from dansyl experiments) is in phase and as a consequence it must be concluded that H-chain is out of phase. Other amino acids were observed in smaller quantities in the cycles that could also be in some pattern but not as easily identifiable. Some of the amino acids were part of the sequence of L-chain and observed to be in greater amounts than the rest but without any observable pattern. The out of phase sequence and presence of smaller amounts of amino acids for H-chain suggested a 'ragged' N-terminus for H-chain possibly resulting from either a non-specific cleavage of 'unnicked' toxin or a trimming by a non-specific amino-peptidase.

From the sequence data two tentative sequences were derived:

L-chain
Pro-Ile-Thr-Ile-Leu-Asp-Phe-
H-chain
Asp-Leu-Lys-Asp-Asn-Gly-Glu-Tyr-Met-Asp-

Leucine was not detected to any great extent in the first cycle though it was in the second cycle.

To try and determine the N-terminal sequence (less the actual N-terminal amino acids) of purified H- and L-chain a solid phase sequencer was used. The samples were analysed with

Dr. J. Fothergill (Department of Biochemistry, Marischal College, University of Aberdeen). It was known that insulin in the presence of SDS could be analysed by solid phase sequencing but whether larger proteins could be analysed in the presence of SDS was not known. The method used was as described by Laursen et al. (1972), using a home-made solid phase sequencer. For a complete description of the general order and mechanisms of reaction see Croft (1980). The purified chains were labelled with [^{14}C]-IAA for identification purposes. The method initially consisted of coupling p-phenyl diisothiocyanate (DITC) to 3-aminoethyl-(3-aminoethyl) glass (AEAPG, constant pore glass beads of pore diameter of 7.5nm) in the presence of dimethyl formamide (DMF). A 100nmol of each chain was then coupled to the free isothiocyanate group of the AEAPG-DITC in the presence of 50% (v/v) N-methylmorpholine pH 9.0 at 45°C for 30 min. Proteins are coupled to the isothiocyanate groups via the N-terminal amino acid residue and the N^6-amino group of lysine residues. TFA was used in the first step of the sequencer to cleave the first peptide bond of the protein, creating a new N-terminal amino acid, corresponding to the second residue of the protein, capable of reacting with phenylisothiocyanate (PITC) in the first cycle of the sequence process (Croft, 1980). Any unreacted isothiocyanate groups were destroyed by adding ethanolamine to the sample after the coupling process. The glass beads were then washed with methanol and dried under vacuum. Samples of the washings and the beads were analysed by scintillation counting to determine if the proteins had coupled to the beads. Unfortunately the SDS prevented any efficient coupling to the glass beads as the protein was found to be present in the washings. The coupling process was observed to be working as a sample of S-[^{14}C]-carboxymethylated insulin in the presence of SDS, coupled concurrently with the two chains, was observed to be efficiently coupled to the glass beads. Thus no further information was gained regarding the possible 'ragged' N-terminus of H-chain.

A sample of the freeze-dried toxin was separated into its three components and analysed by dansylation, after both 5 h and 20 h hydrolyses. 'Unnicked' toxin after a 5 h hydrolysis gave DNS-Pro with a small amount of DNS-Ile and DNS-Val but no observable DNS-dipeptide. A 5 h hydrolysis of L-chain gave a DNS-Pro and a

DNS-dipeptide spot, after 20 h hydrolysis the DNS-Pro spot was very faint and the DNS-dipeptide spot had disappeared. Another spot was identified in the L-chain sample that could have been DNS-Met. A sample of DNS-Met was synthesised (see Section 3.3.2.1.1.) and included in the standard DNS-amino acids. The new spot present in the L-chain sample did not correspond to DNS-Met nor any of the other dansylated amino acid so was concluded to be a by-product of the dansylation. Analysis of H-chain gave no dansylated amino acids apart from O-DNS-Tyr and N^6-DNS-Lys. Development of all three types of chromatograms in Solvent 3 gave no further information.

Thus it was concluded that the N-terminus amino acid of L-chain and 'unnicked' toxin were the same, proline, indicating that the L-chain is at the N-terminal end of tetanus toxin and that after cleavage H-chain is produced. The N-terminus of H-chain is less clear: there is the possibility of either an out of phase sequence, a 'ragged' end, or possibly a combination of both. Heterogeneity in the N-terminus of H-chain is possible as it appears H-chain is the product of the cleavage of 'unnicked' toxin.

3.3.3.2. Determination of Sulphydryl Content of Tetanus Toxin

Experiments using DTNB on untreated and denatured BSA either in the presence or absence of DTT, as described in Section 3.3.2.2.1., gave results in agreement with literature values, i.e. a total of 35 half-cystine residues, 1 free cysteine residue and 17 disulphide bridges. Experiments using DTNB with tetanus toxin were inconclusive as the amount of toxin needed for the spectroscopic analysis of the cysteine residues was too high to be of practical use. Smaller amounts of toxin gave spurious results because of the errors of the procedure being too high. However it was concluded that the number of total half-cystine residues must be quite low.

Thus the $[^{14}C]$-IAA method described in Section 3.3.2.2.2., was used. A number of experiments were carried out to check that the labelling system worked. It was shown that tetanus toxin was fully labelled within 30 sec after the addition of $[^{14}C]$-IAA, indicating that the usual length of labelling, 15 min, was sufficient. Tetanus toxin was shown to be S-carboxymethylated either by incorporation of radioactivity by autoradiography and elution of S-$[^{14}C]$-

carboxymethyl cysteine, from the electrophoretogram, after the electrophoretic separation of acid hydrolysed labelled toxin or by the observation of CMcys in the automatic analyser after analysis of acid hydrolysed labelled toxin.

The total number of half-cystine residues in 'nicked' toxin was shown to be 10 $\pm$ 2, from the average of five experiments. By using NEM to label any free cysteine residues the number of cysteines involved in disulphide bridge formation were found to be 10, indicating 5 disulphide bridges. From the double labelling experiments where [^{3}H]-IAA was used to label free cysteines and [^{14}C]-IAA to label reduced cystines, the number of free cysteines in the "native" conformation were 0.5 and after denaturation 0.7, indicating 1 free cysteine residue. The number of cystéines involved in disulphide bridge formation were 8 indicating the possibility of 4 disulphide bridges. A sample of reduced and denatured toxin was labelled with [^{3}H]-IAA and shown to contain 10 cysteine residues. Performic acid oxidation of labelled toxin gave no cysteic acid. No alanine was detectable in control experiments where CMcys was treated with performic acid, ruling out the possibility of the oxidation of CMcys to alanine in the toxin sample. Performic acid oxidation of tetanus toxin yielded 13 cysteic acid residues (see Section 3.3.3.3.).

S-[^{14}C]-carboxymethylation of 'unnicked' toxin, H- and L-chain gave either 6 cysteines for 'unnicked toxin, 2 cysteines for H-chain and 3 cysteines for L-chain. The lower values for the number of cysteine residues probably resulted from the use of 8M urea as the denaturant instead of 6M GuHCl.

3.3.3.3. Amino Acid Analysis of Tetanus Toxin

Three types of analysis were carried out on 'nicked' toxin, acid hydrolysis, performic acid oxidation followed by acid hydrolysis and hydrolysis using 2-mercaptoethane sulphonic acid. Acid hydrolyses and performic acid oxidation was carried out on H- and L-chain. Table 5 lists the number of residues of each amino acid per mol of toxin 'nicked' or 'unnicked' and H- and L-chain. Table 6 shows the comparison of the number of residues of the amino acids in tetanus toxin from work carried out in this thesis and as published by other workers.

TABLE 5.

AMINO ACID ANALYSIS OF TETANUS TOXIN

Amino Acid	'Nicked'	'Unnicked'	H-Chain	L-Chain
Cys	13 ± 1	N.D.	6 ± 1	4 ± 1
Asp	204 ± 13	191 ± 2	129 ± 1	68 ± 2
Thr	77 ± 2	78 ± 2	53 ± 1	24 ± 1
Ser	107 ± 3	104 ± 1	56 ± 1	26 ± 1
Glu	131 ± 2	117 ± 1	84 ± 1	41 ± 2
Pro	74 ± 4	72 ± 4	40 ± 1	25 ± 2
Gly	105 ± 6	74 ± 1	59 ± 1	32 ± 2
Ala	77 ± 5	77 ± 1	61 ± 1	25 ± 1
Val	75 ± 2	79 ± 2	54 ± 2	27 ± 1
Met	28 ± 2	29 ± 2	23 ± 1	13 ± 1
Ile	103 ± 3	97 ± 2	59 ± 1	27 ± 2
Leu	115 ± 3	125 ± 2	86 ± 1	40 ± 2
Tyr	67 ± 4	71 ± 2	45 ± 1	22 ± 1
Phe	54 ± 2	55 ± 2	27 ± 2	16 ± 2
His	16 ± 2	16 ± 2	13 ± 2	7 ± 2
Lys	97 ± 3	95 ± 2	63 ± 1	29 ± 2
Arg	34 ± 2	38 ± 2	22 ± 1	11 ± 2
Trp	10 ± 1	N.D.	N.D.	N.D.

N.D. not determined.

'Nicked' toxin determined from 34 analyses except for cysteine determination which was from 2 analyses and tryptophan from 1 analysis.

'Unnicked' toxin determined from 3 analyses.

H-Chain determined from 3 analyses except for cysteine determination which was from 1 analysis.

L-Chain determined from 2 analyses except for cysteine determination which was from 1 analysis.

TABLE 6.

COMPARISON OF SEVERAL AMINO ACID ANALYSES OF TETANUS TOXIN

AMINO ACID	Dawson and Mauritzen (1967)	Murphy et. al. (1968)	Bizzini et. al. (1969)	Holmes and Ryan (1971)	Robinson et. al. (1975)	Britton
Asp	192	200	200	126	191	204 ± 13
Thr	71	72	67	62	61	77 ± 2
Ser	85	98	94	138	86	107 ± 3
Glu	108	111	107	93	104	131 ± 2
Pro	52	55	55	43	48	74 ± 4
Gly	55	63	62	185	59	105 ± 6
Ala	46	53	51	79	48	77 ± 5
Val	49	61	60	51	48	75 ± 2
Met	21	23	23	24	19	28 ± 2
Ile	106	126	129	74	96	103 ± 3
Leu	102	111	115	72	103	115 ± 3
Tyr	62	76	81	32	66	67 ± 4
Phe	48	56	58	28	48	54 ± 2
His	13	14	14	9	11	16 ± 2
Lys	98	104	106	63	96	97 ± 3
Arg	33	37	35	18	32	34 ± 2
Cys	9	10	10	14	10	13 ± 1*
Trp	n.d.	13	12	0	7	10 ± 1
TOTAL	1150	1283	1279	1111	1133	1387

n.d. = No data.

* = Determined as cysteic acid after performic acid oxidation.

From the number of residues of each amino acid per mol of toxin an approximate molecular weight can be calculated. The molecular weight of tetanus toxin was determined to be 178 700 ± 8 000 daltons, H-chain to be 112 300 ± 3 000 daltons and L-chain to be 58 200 ± 4 000 daltons. The values are slightly higher than those observed by physical methods, as described in Section 3.2.4.

3.3.3.4. Calculation of the Partial Specific Volume of Tetanus Toxin

From the amino acid analysis of tetanus toxin (Table 5) the partial specific volume ($\bar{V}$) for tetanus toxin was determined. The $\bar{V}$ of each amino acid was taken from Lee and Timasheff (1974), see Table 7. The partial specific volume was calculated using the equation (from Lee and Timasheff, 1974):-

$$\bar{V} = \frac{\Sigma N_i (W_i \bar{V}_i)}{\Sigma N_i W_i}$$

Where:-

N_i number of residues of amino acid (i) in one mol of protein

W_i residue weight of amino acid (i) (mol.wt. of i - 18)

$\bar{V}_i$ partial specific volume of amino acid (i) at 20°C.

The partial specific volume of tetanus was calculated to be $0.732\ cm^{-3}g^{-1}$

TABLE 7.

PARTIAL SPECIFIC VOLUME DATA FOR TETANUS TOXIN

Amino Acid	N_i	W_i	$\bar{V}_i$	$N_i W_i$	$W_i \bar{V}_i$
Asp	204 ± 13	115.1	0.60	23 480	60.060
Thr	77 ± 2	101.1	0.70	7 785	70.770
Ser	107 ± 3	87.1	0.63	9 320	54.873
Glu	131 ± 2	129.1	0.66	16 912	85.206
Pro	74 ± 4	97.1	0.76	7 185	73.796
Gly	105 ± 6	57.0	0.64	5 985	36.480
Ala	77 ± 5	71.1	0.74	5 475	52.614
Val	75 ± 2	99.1	0.86	7 433	85.226
Met	28 ± 2	131.2	0.75	3 674	98.400
Ile	103 ± 3	113.2	0.90	11 660	101.880
Leu	115 ± 3	113.2	0.90	13 018	101.880
Tyr	67 ± 4	163.2	0.71	10 934	115.872
Phe	54 ± 2	147.2	0.77	7 949	113.344
His	16 ± 2	137.2	0.67	2 195	91.924
Lys	97 ± 3	128.2	0.82	12 435	105.124
Arg	34 ± 2	156.2	0.70	5 311	109.340
Cys	13 ± 1	103.2	0.61	1 342	62.952
Trp	10 ± 1	186.2	0.74	1 862	137.788

N_i Number of residues of amino acid (i) in 1mol of tetanus toxin.

W_i Residue weight of amino acid (i) calculated from Mol.Wt. (i) -18

$\bar{V}_i$ Partial specific volume of amino acid (i), from Lee and Timasheff (1975), at 20°C.

Chapter 4

3.4. A Topographic Investigation of Tetanus Toxin

This chapter is an account of three types of experiments carried out to investigate some topographic aspects of the "native" structure of tetanus toxin. The first type of experiment was charge shift electrophoresis, which was used to try and determine whether tetanus toxin and its two constituent chains were hydrophilic or amphiphilic. The second type of experiment was the limited digestion of [^{125}I]-tetanus toxin, with different proteases, to investigate whether there was any constraint on the conformation of SDS-denatured 'unnicked' toxin. The third type of experiment was the investigation of whether there was any conformational change associated with the binding of tetanus toxin to ganglioside SGGnSSLC (G_{T1}).

3.4.1. Introduction

As described in Section 1.2.3.1., tetanus toxin is known to associate with gangliosides in cell membranes before exerting its biological activity. Several bacterial toxins (e.g. diphtheria and cholera toxins) are also known to associate with membrane components via a specific binding subunit, and as a result of this association another specific subunit is able to penetrate the cell membrane and bring about the biological activity of the toxin, usually by some enzymic activity, see Section 1.2.6. and Stephen and Pietrowski (1981). Less is known about the action of tetanus toxin, though it appears that some association to cell membranes is a prerequisite for the action of the toxin. By analogy with other bacterial toxins it is possible (though not yet proven) that L-chain may cross the cell membrane and exert its affect on some membrane or internal cell component, possibly by an enzymic activity (judging from the high biological activity of the toxin).

It was decided to investigate the possibility that tetanus toxin may be an amphiphilic protein using the charge shift electrophoretic method of Helenius and Simons (1977). It has been shown that ordinary soluble proteins and peripheral membrane proteins

bind little or no Triton X-100 (p-t-octylphenylpolyoxyethylene); in contrast amphiphilic membrane proteins bind large amounts usually in the range of 80-100 mol Triton X-100 per mol of protein, when solubilised from membranes (Helenius and Simons, 1972; Makino et al., 1973; Simons et al., 1973; Clarke, 1975; Fries, 1976; Helenius and Simons, 1975; Tanford and Reynolds, 1976). The bound detergent forms micelle-like clusters around the hydrophobic domains of amphiphilic proteins, usually without loss of the "native" conformation of the protein (Helenius and Simons, 1977). The extent of the binding of Triton X-100 is determined by the hydrophobicity of the protein. Helenius and Simons (1977) used a mixture of Triton X-100 and a charged detergent, sodium deoxycholate (carries a negative charge) or cetyltrimethylammonium bromide (CTAB, which carries a positive charge), and found that amphiphilic proteins formed detergent-protein complexes containing both neutral and charged detergent molecules (Helenius and Simons, 1975, 1977). The net charges of the complexes are thus dependant upon the charge of the detergent used, resulting in opposite electrophoretic mobilities for amphiphilic proteins when electrophoresed in the presence of cationic and anionic detergent mixtures. The electrophoretic mobilities of hydrophilic proteins, which do not form complexes with the detergent mixtures, remain unaffected by a change in the charge of the detergent used.

Boquet (1979) used the charge shift electrophoretic method of Helenius and Simons (1977) to investigate diphtheria toxin (Section 1.2.6.). Boquet (1979) showed that fragment B (binding component) of diphtheria toxin is an amphiphilic protein, implying the presence of a hydrophobic domain. In contrast the fragment A (active component) was shown to be a hydrophilic protein possessing no hydrophobic domain. Experiments were carried out for work in this thesis to try and determine whether tetanus toxin is a hydrophilic or amphiphilic protein and, if amphiphilic, to try and determine whether the hydrophobic domain is carried by the H- or L-chain or carried by both chains.

The second type of experiment carried out was to try and investigate whether there is any constraint on the conformation of denatured 'unnicked' toxin (constraint is defined as some structural force exerted on the whole molecule that is not present in the

'nicked' form of the toxin). The two-dimensional electrophoretic method described in Section 3.1.4.2. showed that there may be some constraint on the denatured conformation of 'unnicked' toxin. It is possible that any constraint present is caused by the extra peptide bond present in 'unnicked' toxin, which after being hydrolysed gives 'nicked' toxin. To investigate this phenomenon the peptide mapping method, using limited proteolysis in the presence of SDS, initially described by Cleveland et al. (1977) but as modified by Apps and Schatz (1979) was used.

Several groups have investigated the structure of tetanus toxin by looking at proteolytic fragments of the toxin. Helting and Zwisler (1974, 1977) digested tetanus toxin with papain and obtained two fragments, B and C, both of which reacted with anti-toxin but were shown to be atoxic. Analysis showed that fragment C (Mol.Wt. 47 000 daltons) was a portion of H-chain, and was later shown to be derived from the C-terminal end of 'unnicked' toxin. Fragment B (Mol.Wt. 95 000 daltons) was shown to form the rest of the toxin molecule, i.e. L-chain, and a portion of H-chain of Mol.Wt. 45 000 daltons. Fragment B was split into two chains in the presence of 2-ME. Antibodies raised against fragment C were shown to neutralise toxin in vivo. Purified fragment B, although not toxic at levels sufficient to promote antibody synthesis, was toxic at high concentrations (20µg per mouse, Helting et al., 1978). The symptoms observed were not the convulsions or paralysis associated with tetanus (Section 1.1.) but less specific symptoms involving weight loss and flaccidity. Antibodies raised against fragment C were also shown to interfere with ganglioside binding, possibly indicating that the binding site for adsorption to gangliosides present on H-chain is in or near the region comprised of fragment C.

Bizzini et al. (1977) isolated another fragment, fragment B-IIb, which appeared to be spontaneously produced, presumably by endogenous proteases (Bizzini stated that the fragment is produced by freezing and thawing tetanus toxin but gives no explanation of how this occurs so specifically); it gave a single band of Mol.Wt. 46 000 daltons when analysed by SDS-PAGE, so appears to be similar to the fragment C of Helting and Zwisler (1977). Fragment B-IIb was shown to be atoxic though it retained some of the properties of

"native" toxin in that it bound ganglioside and was capable of migrating by retrograde axonal transport towards the CNS. Bizzini et al. (1980) also digested tetanus toxin with papain, but under slightly different conditions from those described by Helting and Zwisler (1977), they also obtained two fragments, the fragment similar to fragment B was designated fragment Ibc. Fragment Ibc was not bound by gangliosides or isolated synaptic membranes nor was it retrogradely transported in axons. By conjugating fragment B-IIb to fragment Ibc, Bizzini et al. (1980) demonstrated that fragment Ibc was then retrogradely transported in axons.

Matsuda and Yoneda (1977) also reported similar results to Helting and Zwisler (1977). Their fragment β-1 seemed similar to fragment C and had similar antigenic properties.

Robinson et al. (1978) also digested tetanus toxin with papain and obtained a fragment whose molecular weight was 59 000 daltons which appeared (in that it had two chains) to be part of fragment B; they found no fragment equivalent to fragment C. They isolated a second fragment which was slightly smaller than H-chain. There is no explanation from the different authors for these discrepancies.

The third type of experiment was either the DPCC-trypsin (diphenyl carbamyl chloride) or α-chymotrypsin digestion of [^{125}I]-tetanus toxin either before or after fixation to ganglioside SGGnSSLC (G_{T1}). The basis of the experiment was to investigate the possibility of a conformational change in the toxin as a result of binding to the ganglioside. A ganglioside-cerebroside complex (insolubilised ganglioside) was used as the source of the ganglioside. If any conformational change was induced in the molecule by the fixation process one might expect to observe a different proteolytic digestion pattern between bound and unbound toxin. The different patterns would be the result of either production of new proteolytic labile sites or the loss of proteolytic labile sites or a mixture of the two possibilities.

3.4.2. Methods and Results

3.4.2.1. Charge Shift Electrophoresis

For the gel composition, production and electrophoretic conditions see Section 2.5.7. To investigate whether tetanus

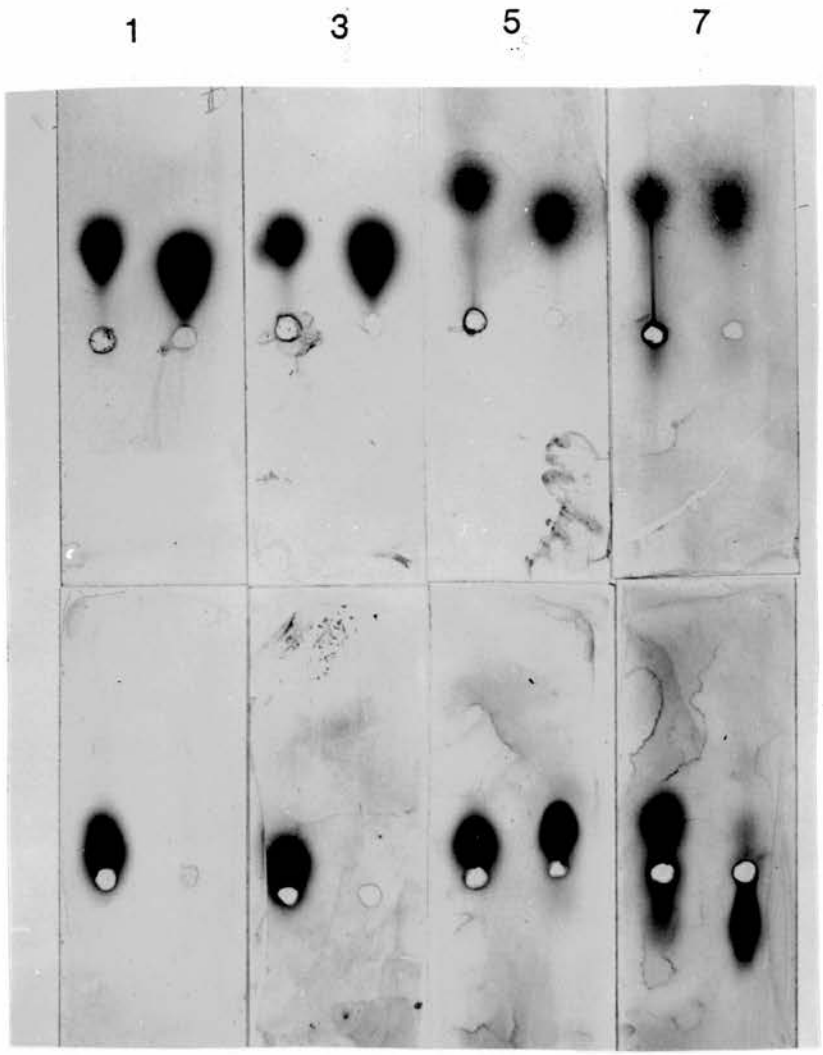
toxin was amphiphilic or hydrophilic a set of protein standards of known hydrophobicity was used; BSA (Sigma), ovalbumin (Sigma), bovine γ-globulin (Sigma) and diphtheria toxin (a gift of Dr. R.O. Thomson, Wellcome Research Laboratories, Kent) were used as hydrophilic proteins, human apoprotein A-1 (the major High Density Lipoprotein, HDL, Mol.Wt. 28 000 daltons) and human apoprotein B (a major Low Density Lipoprotein, LDL), both a gift of Dr. I. Craig (Department of Biochemistry, University of Edinburgh) were used as hydrophobic proteins. The system was also checked using Cytochrome b_{561}, an intrinsic membrane protein present in chromafin granule membranes, which was recently isolated and purified by Dr. D.K. Apps (Department of Biochemistry, University of Edinburgh) and suspected to be an amphiphilic protein.

Initially a modified Helenius and Simons (1977) system was used; it consisted of 4% polyacrylamide gels being run vertically. Because of complications associated with the acrylamide system the agarose system of Helenius and Simons (1977), was preferred. In the acrylamide system, Davis gels (Sections 2.5.2. and 2.5.2.2.) were prepared with and without the detergents (in the detergent buffer system) as described in Section 2.5.7., according to Helenius and Simons (1977). The proteins were then added to the appropriate detergent solution loaded onto the gel and electrophoresed into the gel. Tetanus toxin, ovalbumin, BSA and diphtheria toxin were all observed to run in similar patterns in gels with and without Triton X-100 or Triton X-100 plus sodium cholate (preferred to deoxycholate as it can be recrystallised as described in Section 2.4.4.). Cytochrome b_{561} was observed to have a faster mobility in the Triton X-100 plus sodium cholate mixture when compared to a gel without any detergent. It was observed to aggregate in a Triton X-100 gel. The use of CTAB in the acrylamide system would require a reversal of the electrodes with a resulting loss of hydrophilic proteins. It was because of this that the agarose system was chosen where the samples are loaded in the centre of the gel allowing migration in two directions.

A number of samples were used in the agarose system, including the two hydrophobic proteins described previously. Unfortunately Cytochrome b_{561} was not used because there was not enough left, though from the pilot experiment it appeared to be an

amphiphilic protein. Four different buffer systems were used in the agarose gels; buffer without any detergent, buffer with Triton X-100, buffer with Triton X-100 plus sodium cholate and buffer with Triton X-100 plus CTAB.

From Photographs 27, 28, 29 and 30 one can observe that BSA and ovalbumin behaved as hydrophilic proteins. This was confirmed in the CTAB system where the net migration to the anode was unaffected. γ-globulin did not migrate in any of the four systems but can be still classified as a hydrophilic protein because there was no alteration in migration in the presence of the two charged detergent systems. The non-movement of γ-globulin is a result of a net zero charge on the protein molecule at the pH of the charge shift experiments. Diphtheria toxin also appeared to behave as a hydrophilic protein, as observed by Boquet (1979). There was a component in the sample that was drawn towards the cathode in the presence of CTAB, but this was probably a contaminant because the sample of diphtheria toxin used had been stored for a long time. Quite clearly LDL behaved as a amphiphilic protein because the sample migrated towards the anode in the presence of cholate and towards the cathode in the presence of CTAB. The HDL protein appeared to be missing from samples containing detergent possibly as a result of elution from the gel during the fixing, staining and destaining procedures. Some highly hydrophobic proteins (especially membrane proteins) are known to be solubilised from gel matrices when complexed to detergents. Unfortunately the fixing procedure using 4% (w/v) 5-sulphosalicylic acid and 12% (w/v) TCA was not applicable in this agarose gel system as the 5-sulphosalicylic acid fixes proteins via their lysine residues to the acrylamide matrix of polyacrylamide gels. From Photograph 27, tetanus toxin initially appeared as a hydrophilic protein except that a component migrated towards the cathode in the presence of CTAB, whereas the bulk of the sample migrated towards the anode. An explanation of this phenomenon could be the diffusion of 2-ME from the LDL sample. This possibility was investigated by the electrophoresis of toxin and denatured toxin (4M urea) in the presence of 2-ME in the Triton X-100 plus cholate mixture and the Triton X-100 plus CTAB mixture. From Photograph 28, one can observe that the toxin did split into two components in the presence of CTAB, one migrated towards the

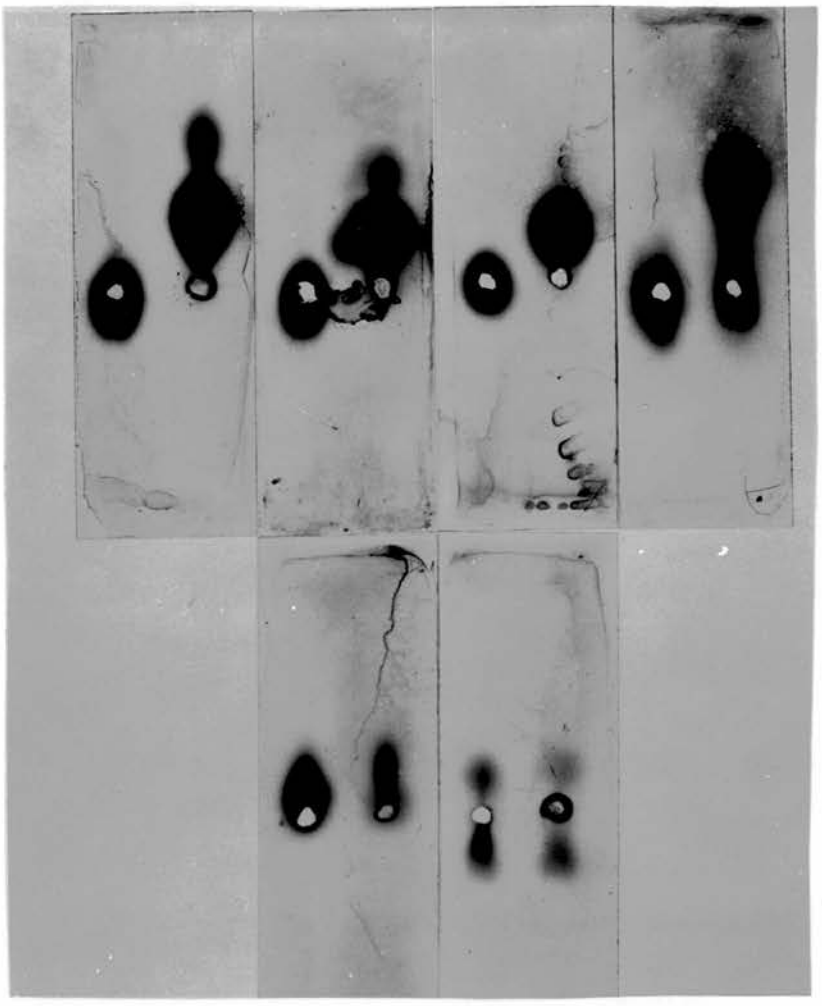


PHOTOGRAPH 27. CHARGE SHIFT ELECTROPHORESIS I

See Section 3.4.2.1. for details.

Gel	Detergent	Left Sample Well	Right Sample Well
1	None.	BSA.	Ovalbumin.
2	None.	Tetanus toxin.	HDL.
3	Triton.	BSA.	Ovalbumin.
4	Triton.	Tetanus toxin.	HDL.
5	Triton + cholate.	BSA.	Ovalbumin.
6	Triton + cholate	Tetanus toxin.	LDL.
7	Triton + CTAB.	BSA.	Ovalbumin.
8	Triton + CTAB.	Tetanus toxin.	LDL.

9 10 11 13 ANODE



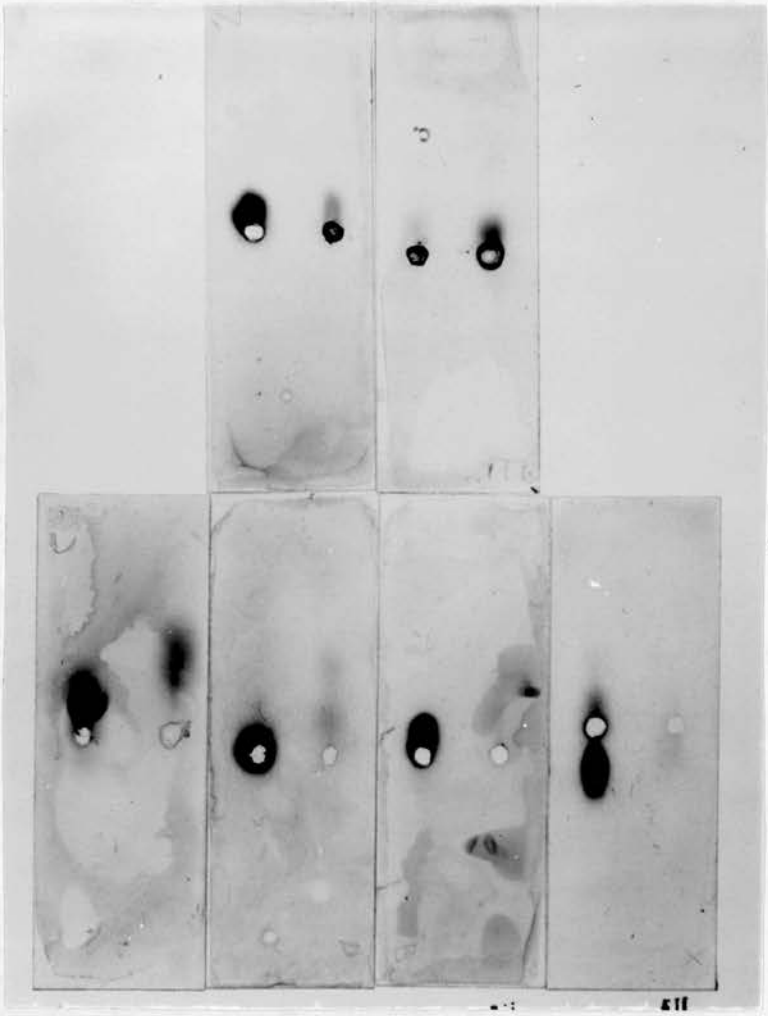
12 14 CATHODE

PHOTOGRAPH 28. CHARGE SHIFT ELECTROPHORESIS II

See Section 3.4.2.1. for details.

Gel	Detergent	Left Sample Well	Right Sample Well
9	None.	Bovine γ-globulin.	Diphtheria toxin.
10	Triton.	Bovine γ-globulin.	Diphtheria toxin.
11	Triton + cholate.	Bovine γ-globulin.	Diphtheria toxin.
12	Triton + cholate.	Tetanus toxin + 4M urea.	Tetanus toxin + 4M urea + 2-ME.
13	Triton + CTAB.	Bovine γ-globulin.	Diphtheria toxin.
14	Triton + CTAB.	Tetanus toxin + 4M urea.	Tetanus toxin + 4M urea + 2-ME.

15 16 ANODE



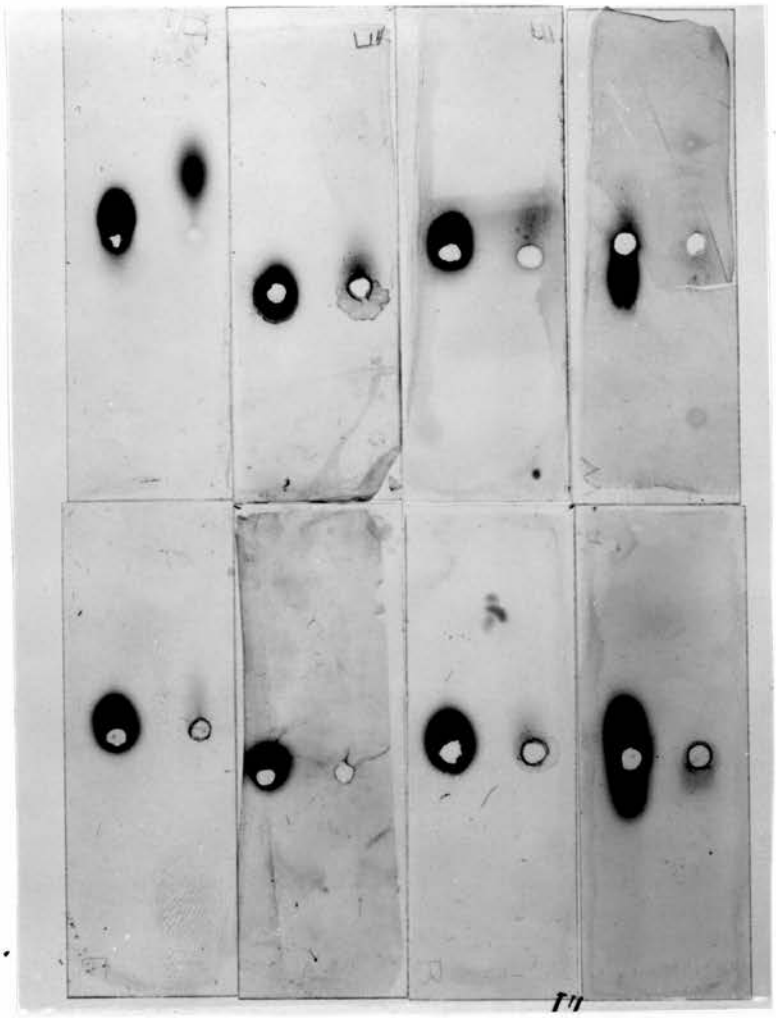
17 18 19 20 CATHODE

PHOTOGRAPH 29. CHARGE SHIFT ELECTROPHORESIS III

See Section 3.4.2.1. for details.

Gel	Detergent	Left Sample Well	Right Sample Well
15	Triton + cholate.	Tetanus toxin + 4M urea.	Tetanus toxin + 4M urea + 2-ME.
16	Triton + CTAB.	Tetanus toxin + 4M urea.	Tetanus toxin + 4M urea + 2-ME.
17	None.	LDL.	HDL.
18	Triton.	LDL.	HDL.
19	Triton + cholate.	LDL.	HDL.
20	Triton + CTAB.	LDL.	HDL.

21 22 23 24 ANODE



25 26 27 28 CATHODE

PHOTOGRAPH 30. CHARGE SHIFT ELECTROPHORESIS IV

See Section 3.4.2.1. for details.

Gel	Detergent	Left Sample Well	Right Sample Well
21	None.	LDL.	HDL.
22	Triton.	LDL.	HDL.
23	Triton + cholate.	LDL.	HDL.
24	Triton + CTAB.	LDL.	HDL.
25	None.	Tetanus toxin + 4M urea + 2-ME.	H-chain.
26	Triton	Tetanus toxin + 4M urea + 2-ME.	H-chain.
27	Triton + cholate.	Tetanus toxin + 4M urea + 2-ME.	H-chain.
28	Triton + CTAB.	Tetanus toxin + 4M urea + 2-ME.	H-chain.

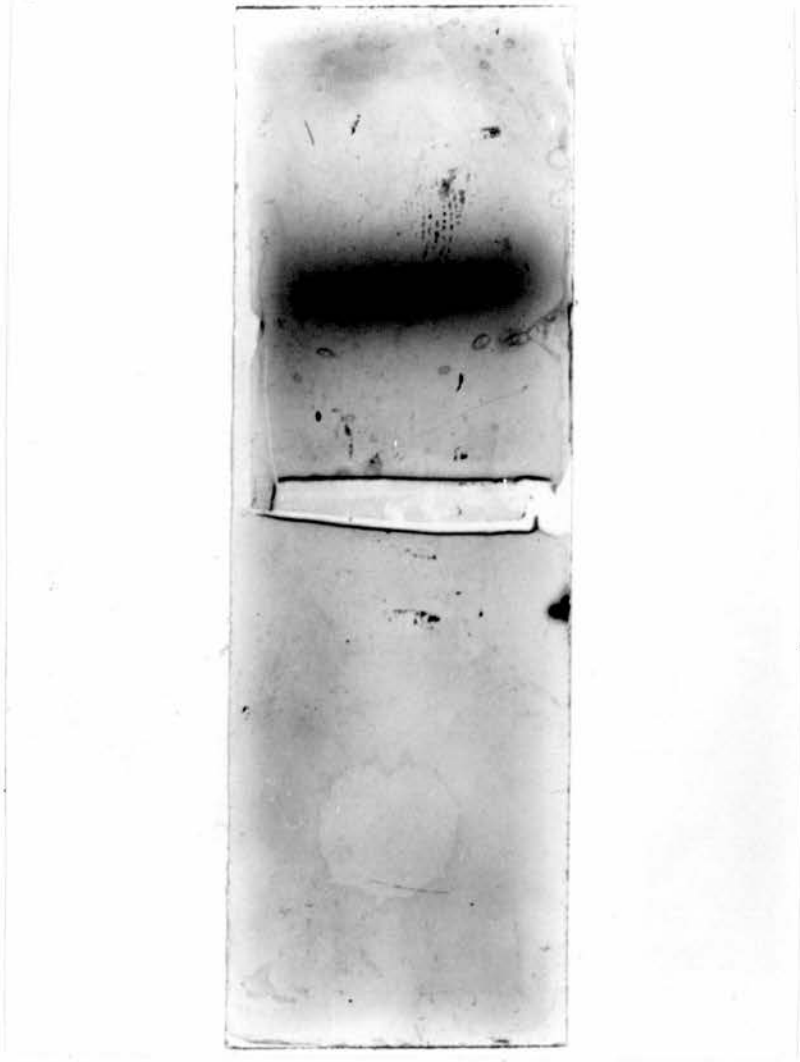
anode and the other towards the cathode. This indicated the presence of a hydrophilic and an amphiphilic component. This experiment was repeated several times with the same result. Tetanus toxin in the absence of 2-ME migrated towards the anode in the presence of CTAB (Photograph 31). A sample of reduced toxin was electrophoresed against a sample of partially purified H-chain (obtained by the gel-permeation method described in Section 3.2.2.1.). Unfortunately the amount of H-chain was very low but a faint spot was observed to have migrated towards the cathode in the presence of CTAB, indicating that the amphiphilic component might be H-chain (Photograph 30).

To try and identify the amphiphilic component a series of experiments using [^{125}I]-tetanus toxin were carried out. The toxin was labelled in the absence of SDS as described in Section 3.4.2.2. The labelled toxin was then denatured with 8M urea, reduced with 2-ME and electrophoresed into a CTAB gel. Gels were run in duplicate; one gel was dried down and autoradiographed, the other gel had pieces of agarose, containing proteins electrophoresed towards the anode and cathode, on either side of the origin, cut out. The pieces of agarose were then placed in the sample wells of an 8% Laemmli SDS gel and any protein electrophoresed out of the agarose into the polyacrylamide gel, which was then dried down and autoradiographed. The dried-down gel was covered in aluminium foil to decrease the amount of background radiation. From Photograph 32 the component observed to be electrophoresed towards the cathode appears to be H-chain, though the majority of toxin appeared to be electrophoresed towards the anode in the agarose gel, possibly because of insufficient solubilisation with CTAB.

3.4.2.2. Peptide Mapping by Limited Proteolysis

In initial experiments, tetanus toxin was digested with three different proteases; Papain (Sigma), α-Chymotrypsin (Sigma) and Staphylococcus aureus V8 protease (Sigma). The digestions were carried out as described by Cleveland et al. (1977). Approximately 0.9mg of toxin (resuspended sample) in 0.125M Tris-HCl, 0.5% (w/v) SDS and 10% (w/w) glycerol at pH 6.8 was boiled at

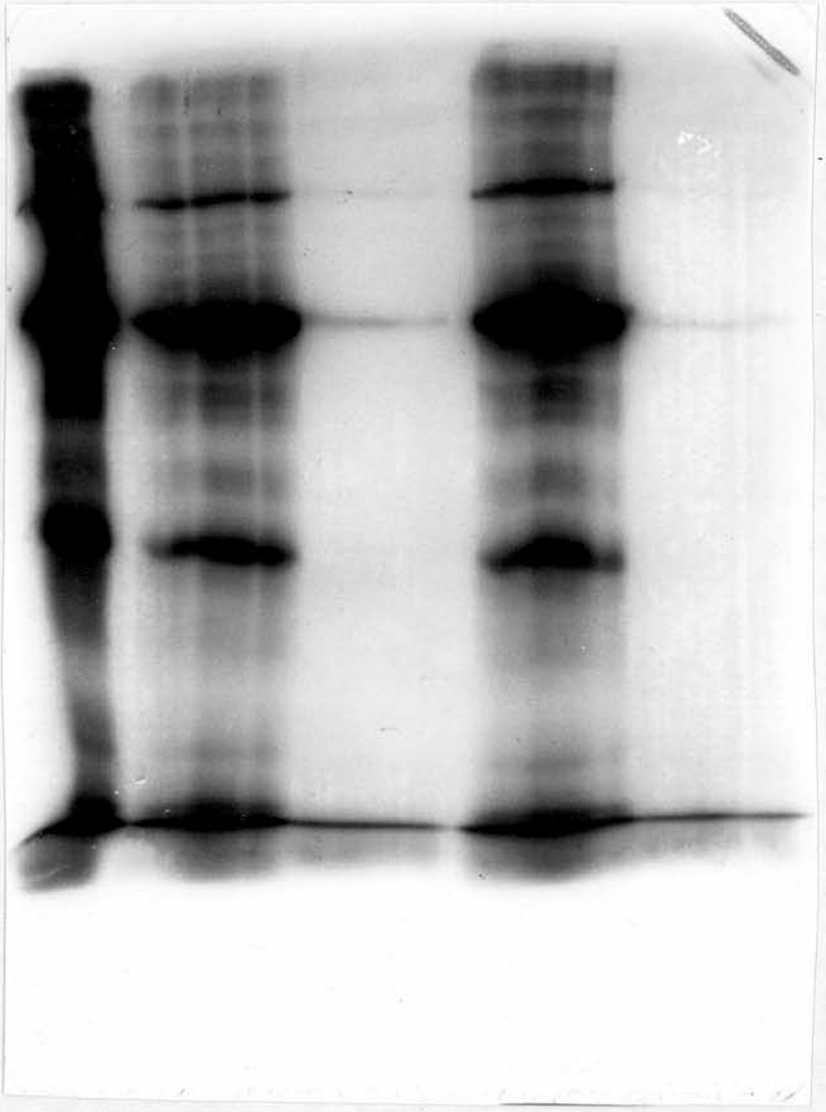
ANODE



CATHODE

PHOTOGRAPH 31. CHARGE SHIFT ELECTROPHORESIS OF TETANUS TOXIN IN THE ABSENCE OF 2-MERCAPTOETHANOL IN A MIXTURE OF TRITON X-100 AND CTAB

See Section 3.4.2.1. for details.



PHOTOGRAPH 32. AUTORADIOGRAPH OF [^{125}I]-TETANUS TOXIN ELECTROPHORESED IN AN 8% POLYACRYLAMIDE GEL AFTER SEPARATION BY CHANGE SHIFT ELECTROPHORESIS IN THE PRESENCE OF TRITON X-100 AND CTAB AND TREATMENT WITH 8M UREA AND 2-MERCAPTOETHANOL

See Section 3.4.2.1. for details.

A	Sample of [^{125}I]-tetanus toxin.
B and D	Proteins electrophoresed towards the anode of the charge shift gel.
C and E	Proteins electrophoresed towards the cathode of charge shift gel, mainly H-chain and some 'unnicked' toxin.

100°C for 2 min, cooled to 37°C, divided into 36µg lots and digested with 2.5µg of protease for various lengths of time. Digestion was stopped by making the samples 10% (w/v) with SDS and boiling for 2 min. The samples were split into two lots, one lot was reduced using 2-ME, and both sets of samples were electrophoresed into 12% polyacrylamide gels.

The digestion patterns observed for α-chymotrypsin and S. aureus V8 protease were complicated (see Diagram 19). There was little difference between a 2 min and 40 min digestion time with α-chymotrypsin; at least six major fragments of Mol.Wts. between 70 000 and 150 000 daltons were detected, all of which disappeared upon reduction of the sample. Digestion with S. aureus V8 protease gave a similar result, though a 40 min digestion period was required for complete digestion, see Diagram 19.

The pattern observed after digestion with papain (Photograph 33) had no fragment C, as described by Helting and Zwisler (1977), present. If fragment C had been present one would have observed one band in an SDS gel resulting from fragment C and the reduction products of fragment B (L-chain and half of H-chain) all of which have similar molecular weights. But under reducing conditions, as observed from Photograph 33, at least two main bands are visible, one with a molecular weight similar to H-chain and the other with a molecular weight slightly smaller than L-chain. It appeared that a small piece of L-chain is removed after papain digestion; this may be the small peptide visible at the bottom of the gel (Photograph 33). The observations from the various protease digestions imply that several disulphide bonds are present in the toxin molecule; which are intra-chain and which are inter-chain is not known.

Another approach using limited proteolysis was tried where purified 'unnicked' toxin, H- and L-chains were digested to try and investigate whether there was any similarities in the digestion patterns, between the chains and 'unnicked' toxin, and if there was any constraint on the denatured structure of 'unnicked' toxin. If there was no constraint one would expect the digestion pattern of 'unnicked' toxin to be composed of the digestion patterns of H- and L-chains. If there was a constraint on the

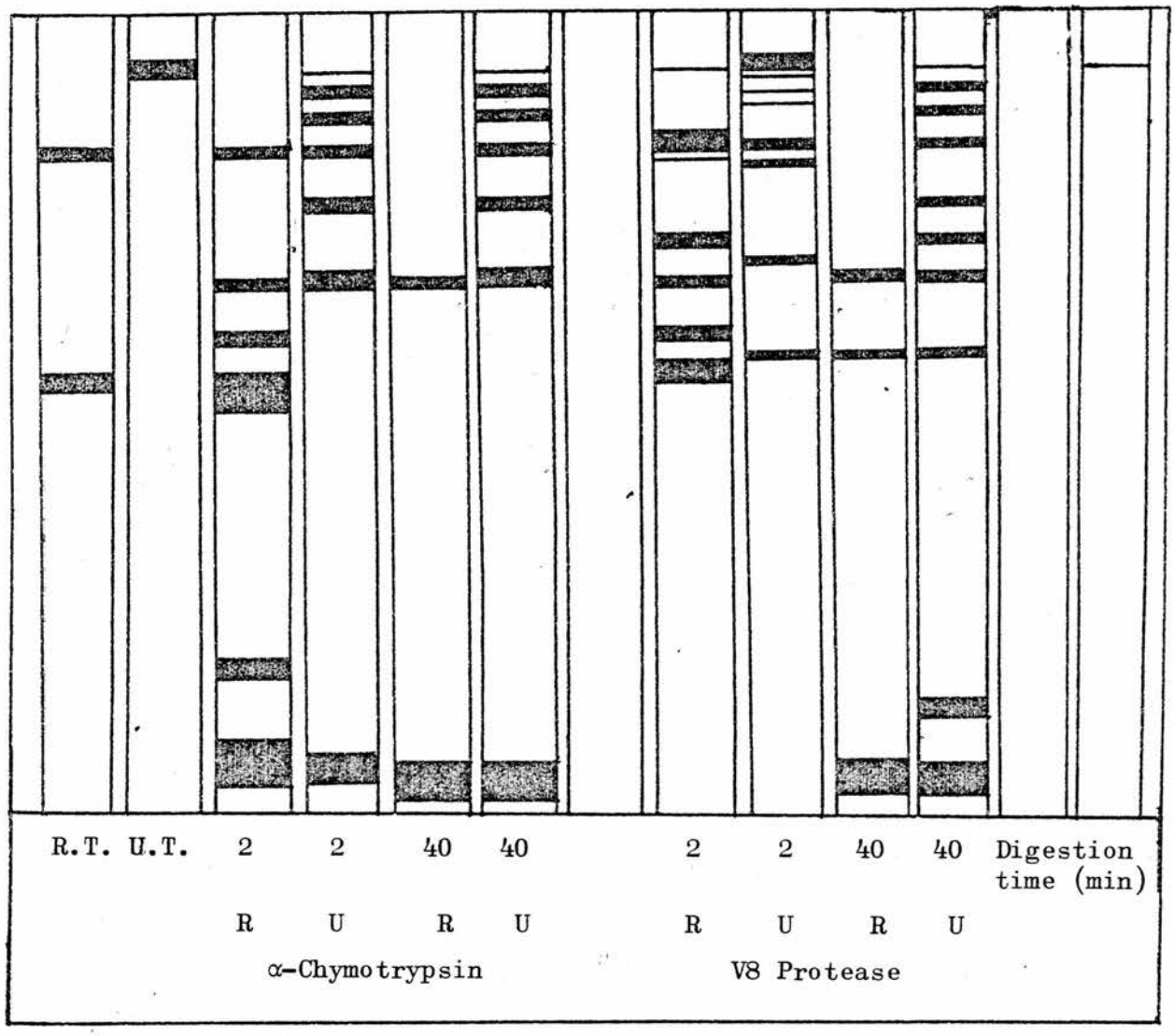
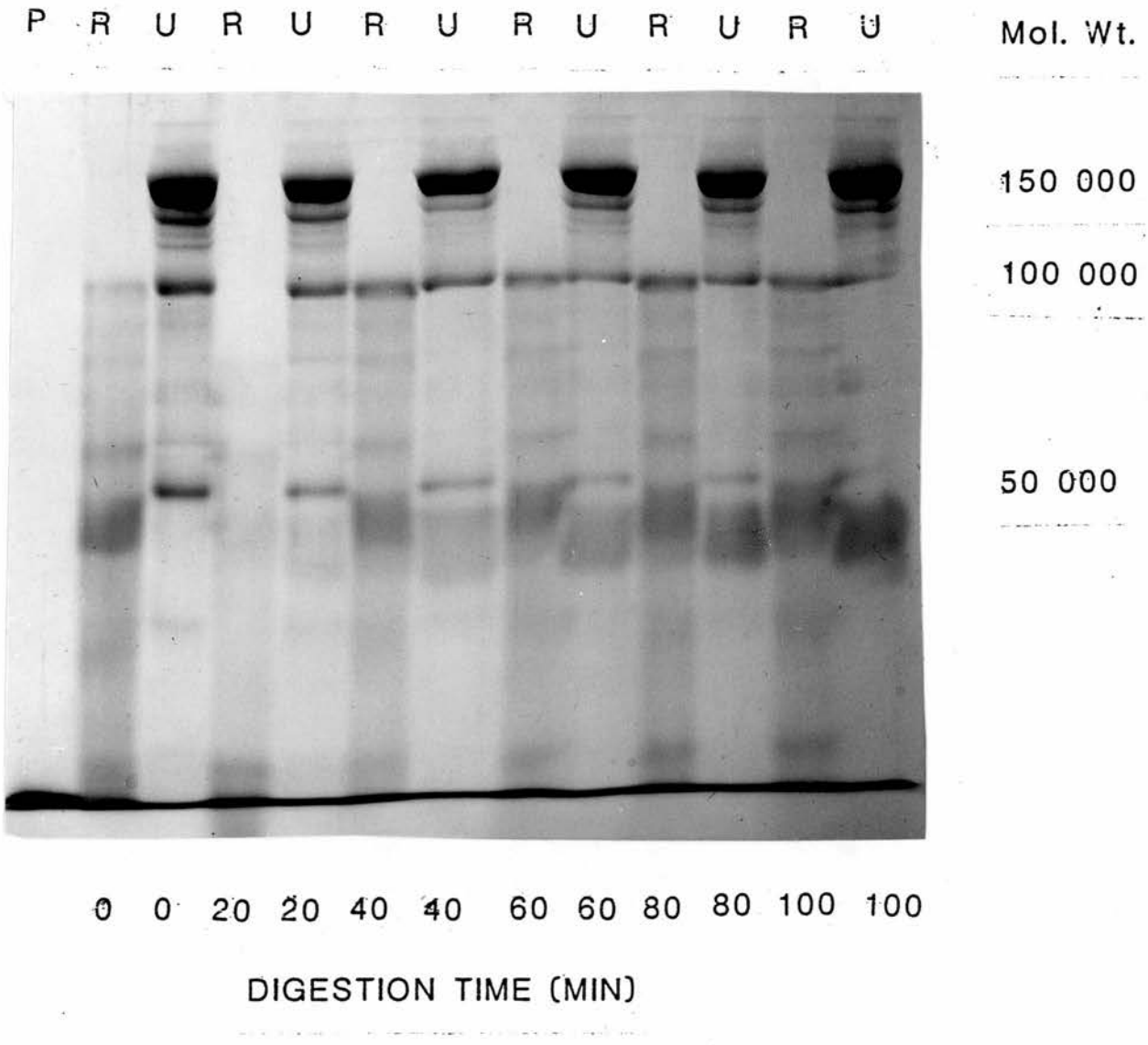


DIAGRAM 19. DIGESTION OF 'NICKED' TETANUS TOXIN WITH α-CHYMOTRYPSIN AND V8 PROTEASE

See Section 3.4.2.2. for details.

R.T.	Reduced toxin	(+ 2-ME)
U.T.	Unreduced toxin	
R	Reduced sample	(+ 2-ME)
U	Unreduced sample	
UN	UNNICKED TOXIN	
H	H-CHAIN	
L	L-CHAIN	



PHOTOGRAPH 33. DIGESTION PATTERN OF TETANUS TOXIN AFTER TREATMENT WITH PAPAIN

See Section 3.4.2.2. for details.

P PAPAIN ALONE
R SAMPLES REDUCED WITH 2-ME
U SAMPLES UNREDUCED

Note: Zero time control still digested possibly as a result of either incomplete activation or reactivation of papain after treatment with SDS and boiling.

denatured structure of 'unnicked' toxin one might expect to see a difference in the digestion patterns resulting from the loss of a protease-labile site. Near complete and accessible digestion is assumed as the proteins would be in rod-like particles as a result of the SDS. The digestion may not be complete as areas of protein may bind more SDS with a fall in the efficiency of digestion, but the digestion pattern can be assumed to be different to that of a protein in its "native" structure. The proteins were digested with papain, α-chymotrypsin (Sigma) and DPCC-trypsin (Seravac). The activity of DPCC-trypsin was checked by the digestion of insulin B-chain as described in Section 3.4.2.3.

In order to use smaller quantities of protein the modified method of Cleveland et al. (1977) as described by Apps and Schatz (1979) was used. Tetanus toxin was radio-labelled with iodine-125 in the presence of SDS as described in Section 2.10.1. A $1mgcm^{-3}$ sample of toxin had a final specific activity of $0.58mCimg^{-1}$. The digestions were carried out as described in Section 2.5.6. [^{125}I]-tetanus toxin corresponding to 750nCi was run into the first gel to give the material for digestion.

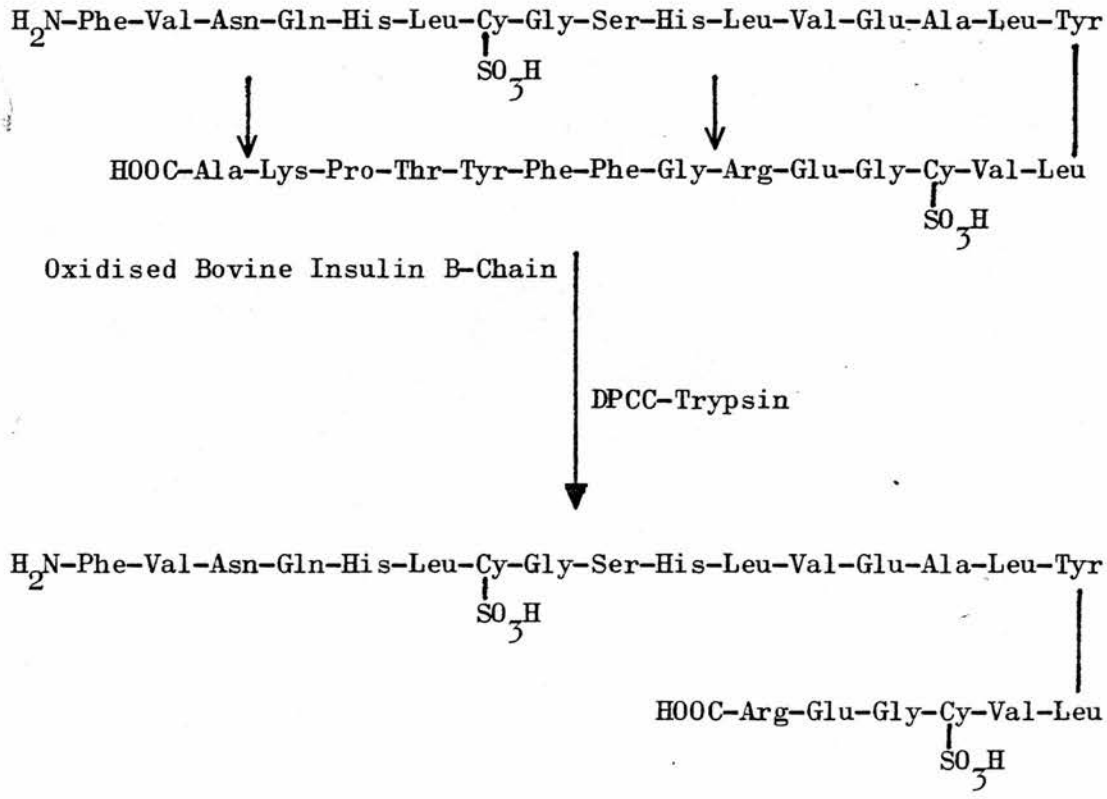
Digestion with papain resulted in complete digestion of all three proteins. Digestion with α-chymotrypsin resulted in the complete digestion of 'unnicked' toxin though H- and L-chains were not completely digested. Digestion with DPCC-trypsin resulted in near-complete digestion of 'unnicked' toxin, L-chain was digested into two main fragments of which only one was present in the digestion pattern obtained for 'unnicked' toxin. It appeared that 'unnicked' toxin was very susceptible to proteolytic digestion so its digestion patterns could not be compared to those for H- and L-chains. The observation that 'unnicked' toxin is so susceptible to proteolytic digestion, in contrast to H- and L-chains, could suggest that the conformation of denatured 'unnicked' toxin exposes more proteolytic labile sites than are present on the isolated chains.

3.4.2.3. Tryptic and Chymotryptic Digestion of Tetanus Toxin and Tetanus Toxin Complexed to Insolubilised Ganglioside SGGnSSLC (G_{T1})

DPCC-trypsin was once again used in preference to normal trypsin, as it contains no chymotryptic or pseudo-tryptic activity*. The activity of the DPCC-trypsin was checked by the digestion of insulin B-chain, and the products of the digestion were analysed by paper electrophoresis as described in Section 2.8. Trypsin is known to hydrolyse proteins at the carboxylic side of two amino acids, arginine and lysine. Bovine insulin B-chain contains only one arginine and one lysine residue (see Diagram 20) and as a result of tryptic digestion is split into two peptides and an alanine residue. If there is any chymotrypsin or pseudo-trypsin activity in the trypsin sample other peptides would be produced with the ones described. From Photographs 34 and 35, one can see that as a result of DPCC-trypsin digest insulin B-chain was split into only two peptides and a single amino acid, that was shown to co-migrate with a standard sample of alanine, after analysis by high voltage electrophoresis at pH 2.0 and pH 3.5. The Bovine insulin B-chain was a gift of Dr. A.P. Ryle (Department of Biochemistry, University of Edinburgh) and was used as described in Section 2.8.2. The sample of α-chymotrypsin used in the following experiments was from Sigma, obtained from bovine pancrease and was type II α-chymotrypsin.

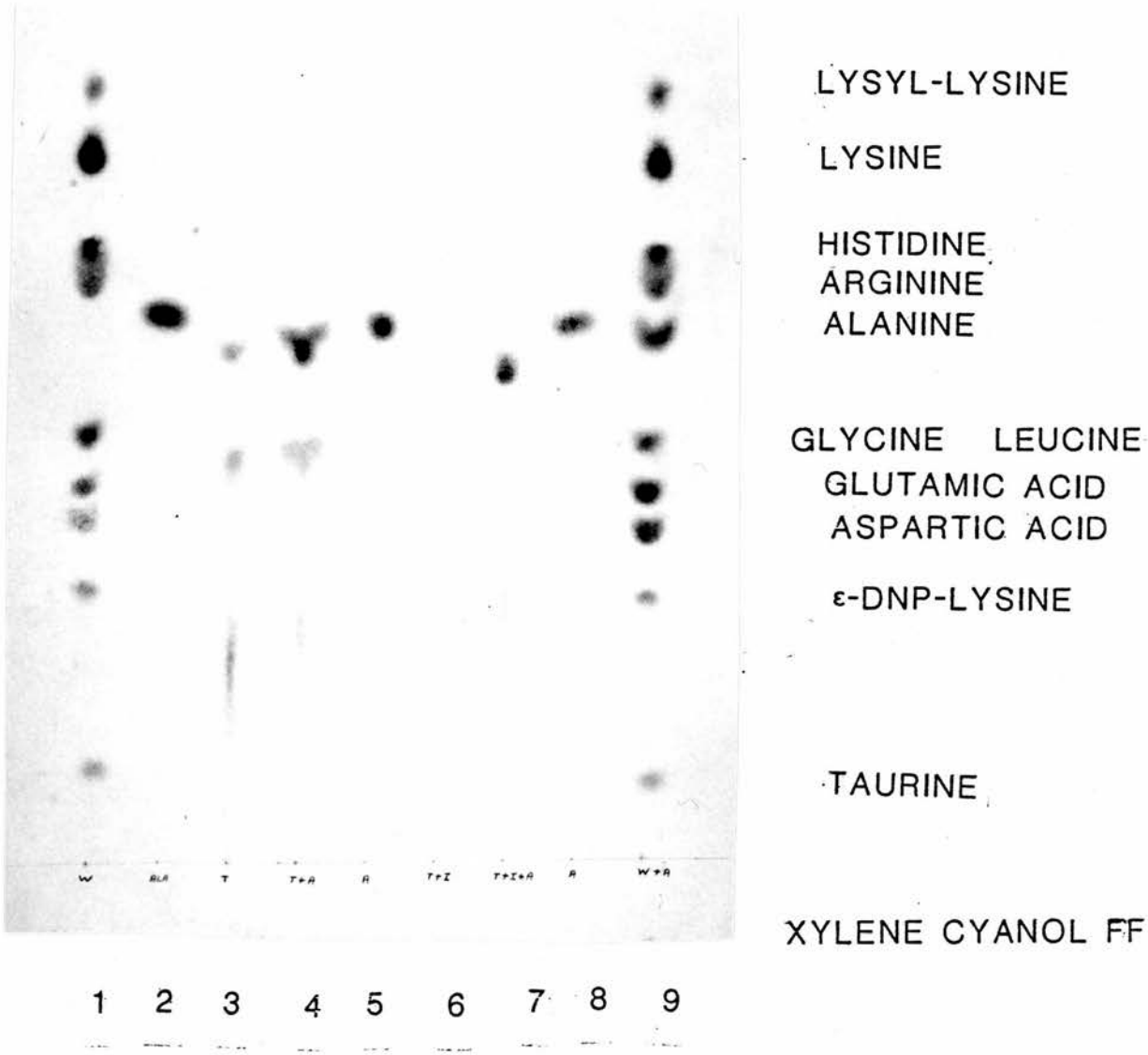
In order to be able to use small quantities of tetanus toxin, the toxin was radio-labelled with $Na^{125}I$, in the absence of SDS as described in Section 2.10.2. The $1mgcm^{-3}$ toxin sample had a final specific activity of $0.14mCimg^{-1}$, as opposed to the specific activity of $0.58mCimg^{-1}$ obtained for toxin labelled in the presence of SDS, indicating that most of the tyrosine residues

* see Smith, R.L. and Shaw, E. (1969) J.Biol.Chem. 244, 4704-4712.



↓ indicates the trypsin labile peptide bonds.

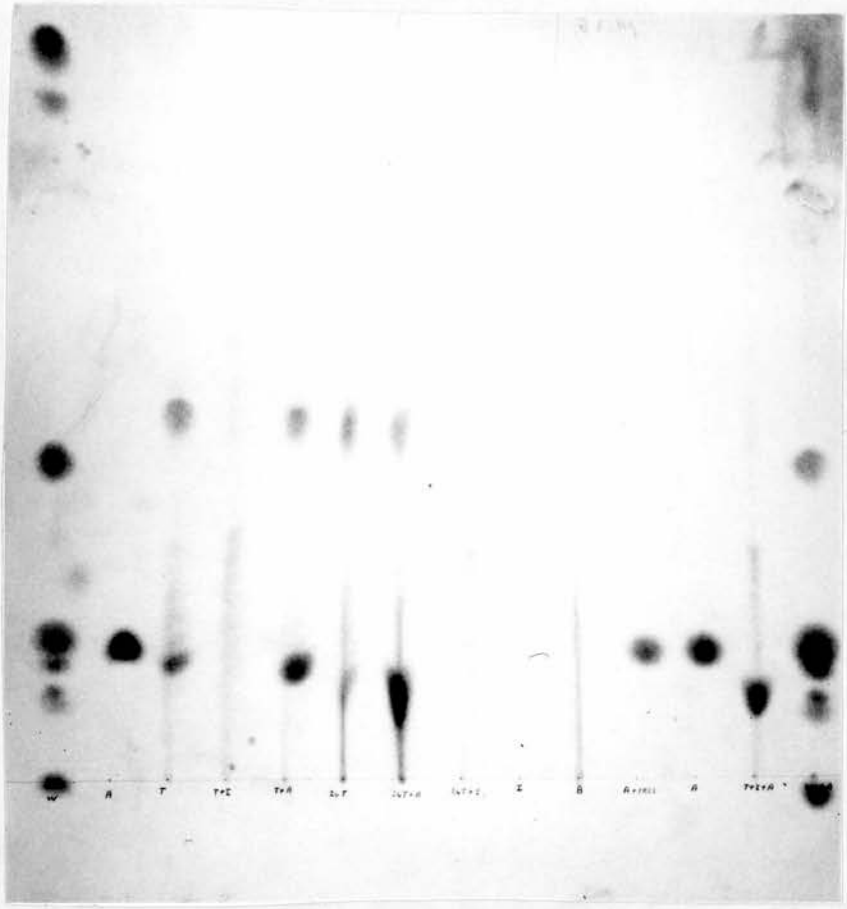
DIAGRAM 20. POSITIONS OF THE PEPTIDE BONDS HYDROLYSED BY TRYPSIN IN BOVINE INSULIN B-CHAIN



PHOTOGRAPH 34. HIGH VOLTAGE PAPER ELECTROPHORESIS, AT pH 2.0, OF BOVINE INSULIN B-CHAIN DIGESTED WITH DPCC-TRYPSIN

See Section 3.4.2.3. for details.

Track	Sample
1	"Wondermix".
2	Alanine.
3	Bovine insulin B-chain digested with DPCC-trypsin.
4	As 3 but with alanine added, to show co-migration of alanine from insulin B-chain with a sample of alanine.
5	Alanine.
6	As 3 but in presence of soya bean trypsin inhibitor.
7	As 4 but in presence of soya bean trypsin inhibitor.
8	Alanine.
9	"Wondermix" + alanine.



PHOTOGRAPH 35. HIGH VOLTAGE PAPER ELECTROPHORESIS, AT pH 3.5, OF BOVINE INSULIN B-CHAIN DIGESTED WITH DPCC-TRYPSIN

See Section 3.4.2.3. for details.

Track	Sample
1	"Wondermix".
2	Alanine.
3	DPCC-trypsin digest of insulin B-chain.
4	As 3 but in presence of soya bean trypsin inhibitor.
5	As 3 but plus alanine.
6	24 h digest of 3.
7	As 6 but plus alanine.
8	As 6 but in presence of soya bean trypsin inhibitor.
9	Soya bean trypsin inhibitor.
10	Insulin B chain.
11	Alanine plus Tris.
12	Alanine.
13	As 4 but plus alanine.
14	"Wondermix" plus alanine.

are unavailable for reaction in the "native" conformation of the toxin. The fractions, from the Sephadex G-25 column, containing the highest counts were used and were always re-analysed using the Wilj gamma counter before use in order to determine the amount of toxin used. Insolubilised ganglioside was prepared as described in Section 2.11.

Initial experiments were carried out using α-chymotrypsin to determine the best conditions for the various reactions and to check whether [^{125}I]-tetanus toxin was being adsorbed to the insolubilised ganglioside complex. 50µl of the ganglioside-cerebroside mixture, in chloroform and methanol, which corresponded to 0.025mg SGGnSSLC (G_{T1}) and 0.225mg cerebroside, was dried down with N_2 and re-dissolved in 50µl 0.1M Tris-HCl pH 7.0 for use where necessary. 10µl of [^{125}I]-tetanus toxin (2.6µg) which corresponded to about 3 x 10^5dpm was used as necessary. The initial experiments carried out were as follows:-

1) Incubation of [^{125}I]-tetanus toxin with insolubilised ganglioside for 2 h at 37°C. The toxin:ganglioside complex was centrifuged down in an Eppendorf 5412 microfuge for 10 min. The pellet was washed twice with 250µl of 0.1M Tris-HCl pH 7.0. The washings were discarded but the pellet and original supernatant were kept for further analysis.

2) Repeat of (1) but in the presence of 10µl 2-ME.

3) Repeat of (1) but in the presence of 4M urea.

4) Repeat of (1) but in the presence of 4M urea and 10µl of 2-ME.

5) Repeat of (1) but in the presence of 20µl 1mgcm^{-3} BSA; to check for the non-specific adsorption of proteins to ganglioside. By using a vast excess of BSA over tetanus toxin any non-specific binding of protein would reduce the binding of tetanus toxin unless the toxin was bound specifically.

6) Incubation of [^{125}I]-tetanus toxin with 1µg of α-chymotrypsin at 37°C for 5 min. The digestion was stopped by the addition of excess benzamidine.

7) Repeat of (6) but the digestion time was 15 min.

8) Repeat of (6) but after the digestion step the sample was incubated with insolubilised ganglioside for 2 h at 37°C. The supernatant and pellet were collected as described in (1).

9) Repeat of (8) but a digestion time of 15 min.

10) Repeat of (8) but 20µl of 1mgcm^{-3} BSA was added with the insolubilised ganglioside.

11) Repeat of (9) but using BSA as described in (10).

12) Repeat of (1) but the adsorbed toxin was digested as in (6). Supernatants and pellets were collected as described in (1).

13) Repeat of (12) but digestion time of 15 min.

The insolubilised ganglioside:toxin pellet was re-suspended in a known volume (usually 50µl) of 0.1M Tris-HCl pH 7.0. 10µl samples from each experiment, and where applicable of re-suspended pellets and supernatants, were counted in the Wilj gamma counter. From the total volumes of each sample the total number of counts in the samples were determined. 10µl samples were also electrophoresed, after treatment with dissociation buffer, into 8% polyacrylamide gels which were dried down without staining and autoradiographed as described in Section 2.9. As can be seen from Table 8 the toxin was adsorbed to the insolubilised ganglioside complex in a specific manner, observed from no decrease in the adsorption of the toxin to the ganglioside in the presence of excess BSA. The addition of 2-ME caused some decrease in the amount of toxin adsorbed which appeared, from Photographs 36 and 37, to be a result of the release of

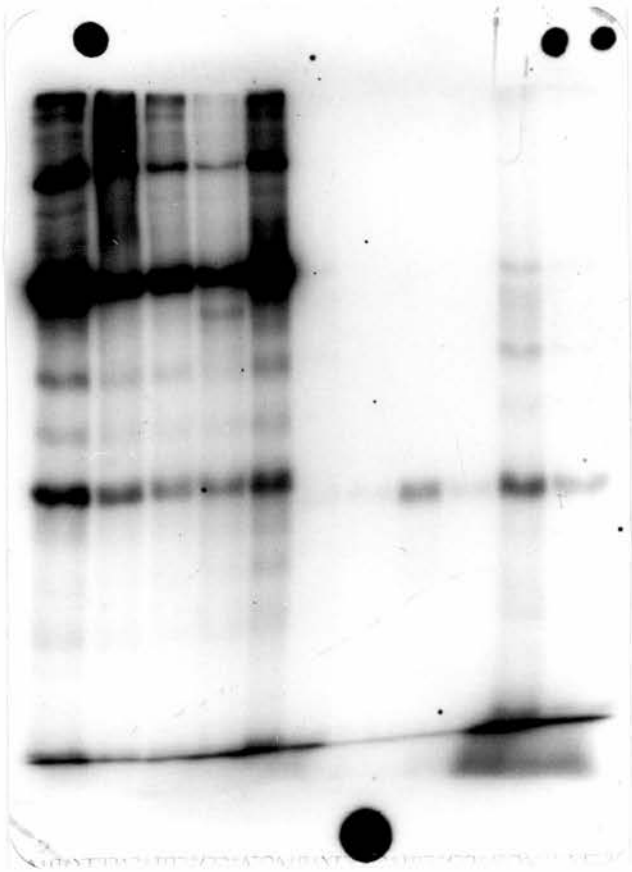
EXPERIMENT	TOTAL NUMBER OF CPM		EXPERIMENTAL CONDITIONS
	SUPERNATANTS	PELLETS	
1	53 000	216 000	Insolubilised ganglioside.
2	88 000	108 000	As (1) in presence of 2-ME.
3	88 000	71 000	As (1) in presence of 4M urea.
4	164 000	57 000	As (1) in presence of 4M urea and 2-ME.
5	55 000	196 000	As (1) in presence of BSA.
8	237 000	18 000	Toxin digested then adsorbed to insolubilised ganglioside.
9	259 000	8 000	As (8) but longer digestion time.
10	220 000	11 000	As (8) but in presence of BSA.
11	215 000	10 000	As (9) but in presence of BSA.
12	164 000	87 000	Digestion of adsorbed toxin.
13	206 000	42 000	As (12) but longer digestion time.

Samples from experiments (6) and (7) were not included as no insolubilised ganglioside was used. α-chymotrypsin was used for the digestions.

TABLE 8. DISTRIBUTION OF $[^{125}I]$-TETANUS TOXIN AFTER VARIOUS EXPERIMENTS USING INSOLUBILISED GANGLIOSIDE SGGnSSLC (G_{T1})

whole toxin from the ganglioside and not just L-chain as might have been expected. The addition of urea caused an increase in the amount of toxin unadsorbed to the ganglioside complex, possibly as a result of the disruption of the binding site on H-chain. As expected the addition of both 4M urea and 2-ME caused even less adsorption; H-chain appeared (from Photograph 36) to be bound in the largest quantity, indicating that, even in the presence of denaturant and reductant, H-chain still retains some binding capacity. Though this phenomenon may arise from the fact H-chain contains twice the amount of tyrosine, so giving rise to a stronger radioactive band. The digestion conditions used led to the complete digestion of the toxin, as observed from tracks 12 and 13 of Photograph 37. Though as seen from Photograph 36, tracks 6, 7 (very faint), 8, 9, 10 and 11, there is a new fragment of molecular weight less than L-chain which was observed to be adsorbed by insolubilised ganglioside, possibly from H-chain, and if so, possibly similar to the fragment C of Helting and Zwisler (1977). From track 10 (Photograph 36) one can observe a low amount of H-chain and a fragment migrating between H- and L-chains, which after a longer digestion time is further digested, as observed from track 11 - Photograph 36. The band migrating faster than L-chain was also observed to be adsorbed by insolubilised ganglioside after digestion of toxin. This adsorption was enhanced in the presence of BSA. By comparing tracks 8 and 10 of Photograph 36, one can observe that after adsorption of the toxin to the insolubilised ganglioside a conformational change may occur in the toxin which protects the H-chain from digestion because H-chain not adsorbed to ganglioside is completely digested.

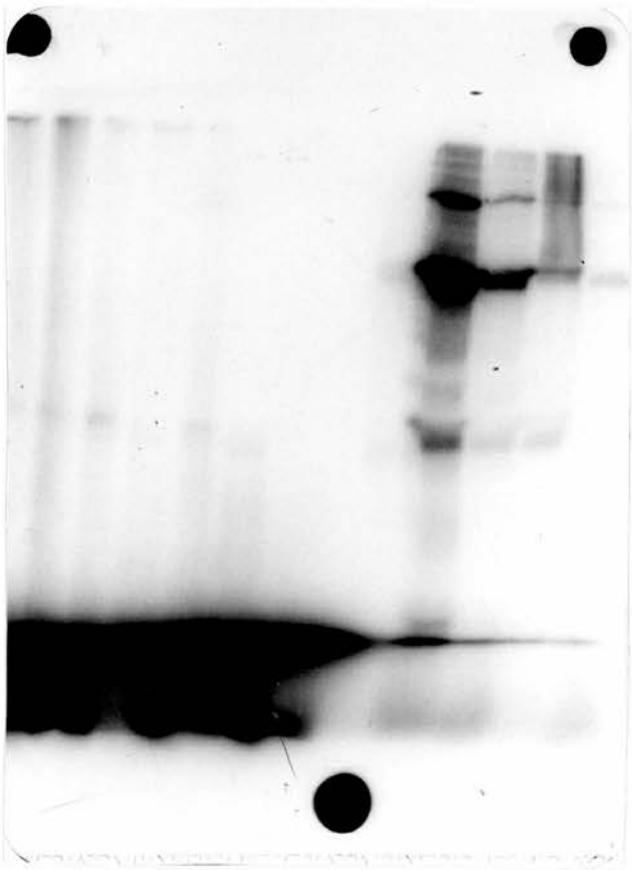
Most of the experiments were repeated but this time using DPCC-trypsin, known only to have tryptic activity, as determined from the digestion of Bovine insulin B-chain. The amount of trypsin used was lower than the amount of α-chymotrypsin and the digestion time was reduced, in order to try and identify any intermediate digestion products. The experiments carried out used the same amount of insolubilised ganglioside as the chymotrypsin experiments. Soya Bean Trypsin Inhibitor (STI, Sigma) was used to stop the digestion reaction, as this is known to work instantaneously and with high efficiency as observed



PHOTOGRAPH 36. AUTORADIOGRAPH OF BOUND [^{125}I]-TETANUS TOXIN AFTER DIGESTION BY α-CHYMOTRYPSIN IN THE PRESENCE AND ABSENCE OF INSOLUBILISED GANGLIOSIDE SGGnSSLC (G_{T1})

See Section 3.4.2.3. for details.

1 Pellet from experiment 1.
2 Pellet from experiment 2.
3 Pellet from experiment 3.
4 Pellet from experiment 4.
5 Pellet from experiment 5.
6 Pellet from experiment 8.
7 Pellet from experiment 9.
8 Pellet from experiment 10.
9 Pellet from experiment 11.
10 Pellet from experiment 12.
11 Pellet from experiment 13.



131211109 8 7 6 5 4 3 2 1

PHOTOGRAPH 37. AUTORADIOGRAPH OF UNBOUND [^{125}I]-TETANUS TOXIN AFTER DIGESTION BY α-CHYMOTRYPSIN IN THE PRESENCE AND ABSENCE OF INSOLUBILISED GANGLIOSIDE SGGnSSLC (G_{T1})

See Section 3.4.2.3. for details.

1 Supernatant from experiment 1.
2 Supernatant from experiment 2.
3 Supernatant from experiment 3.
4 Supernatant from experiment 4.
5 Supernatant from experiment 5.
6 Supernatant from experiment 8.
7 Supernatant from experiment 9.
8 Supernatant from experiment 10.
9 Supernatant from experiment 11.
10 Supernatant from experiment 12.
11 Supernatant from experiment 13.
12 Digestion experiment 6.
13 Digestion experiment 7.

from Photographs 34 and 35. The experiments carried out were:-

1) Digestion of [^{125}I]-tetanus toxin using 0.1µg of DPCC-trypsin at 37°C for 2 min. The reaction was stopped by the addition of 10µl 1mgcm^{-3} STI.

2) As (1) but a digestion time of 10 min.

3) Digestion of [^{125}I]-tetanus toxin with 0.1µg of DPCC-trypsin at 37°C for 2 min in the presence of insolubilised ganglioside. The reaction was stopped by the addition of 10µl 1mgcm^{-3} STI.

4) As (3) but a digestion time of 10 min.

5) Digestion of [^{125}I]-tetanus toxin with 0.1µg of DPCC-trypsin as (1) but after the addition of STI the sample was incubated with insolubilised ganglioside for 2 h at 37°C.

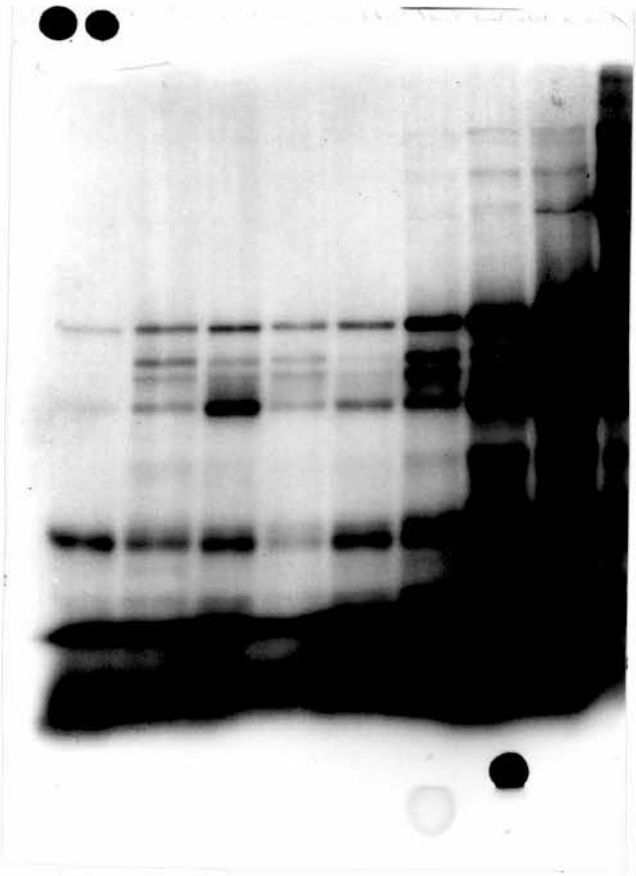
6) As (5) but a digestion time of 10 min.

7) [^{125}I]-tetanus toxin was incubated with insolubilised ganglioside at 37°C for 2 h and then digested with 0.1µg of DPCC-trypsin at 37°C for 2 min. The reaction was stopped by the addition of 10µl of 1mgcm^{-3} STI.

8) As (7) but a digestion time of 10 min.

In all experiments except the first two, the insolubilised ganglioside was centrifuged down as described earlier, washed twice with 250µl of 0.1M Tris-HCl pH 7.0, the washings were discarded and the pellets re-suspended in 50µl of 0.1M Tris-HCl pH 7.0, the original supernatant was kept. 20µl samples were taken from each experiment, and where applicable from both supernatant and re-suspended pellet, treated with SDS dissociation buffer and electrophoresed into 8% polyacrylamide gels which were dried down without staining and autoradiographed.

As can be seen from Photograph 38 (tracks 2 and 3) tetanus toxin was not completely digested, though several new fragments were produced, including a fragment that migrated slightly faster than L-chain. 'Unnicked' toxin was completely digested after a 10 min digestion period (track 3, Photograph 38). From Photograph 38 (tracks 4 and 5) and Photograph 39 (tracks 2 and 3) one can observe that the digestion of [^{125}I]-tetanus toxin by DPCC-trypsin,



PHOTOGRAPH 38. AUTORADIOGRAPH OF UNBOUND [^{125}I]-TETANUS TOXIN AFTER DIGESTION BY DPCC-TRYPSIN IN THE PRESENCE AND ABSENCE OF INSOLUBILISED GANGLIOSIDE SGGnSSLC (G_{T1})

See Section 3.4.2.3. for details.

1 Tetanus toxin.

2 Tetanus toxin digested with 0.1µg DPCC-trypsin for 2 min (Experiment 1).

3 Tetanus toxin digested with 0.1µg DPCC-trypsin for 10 min (Experiment 2).

4 Supernatant from experiment 3.

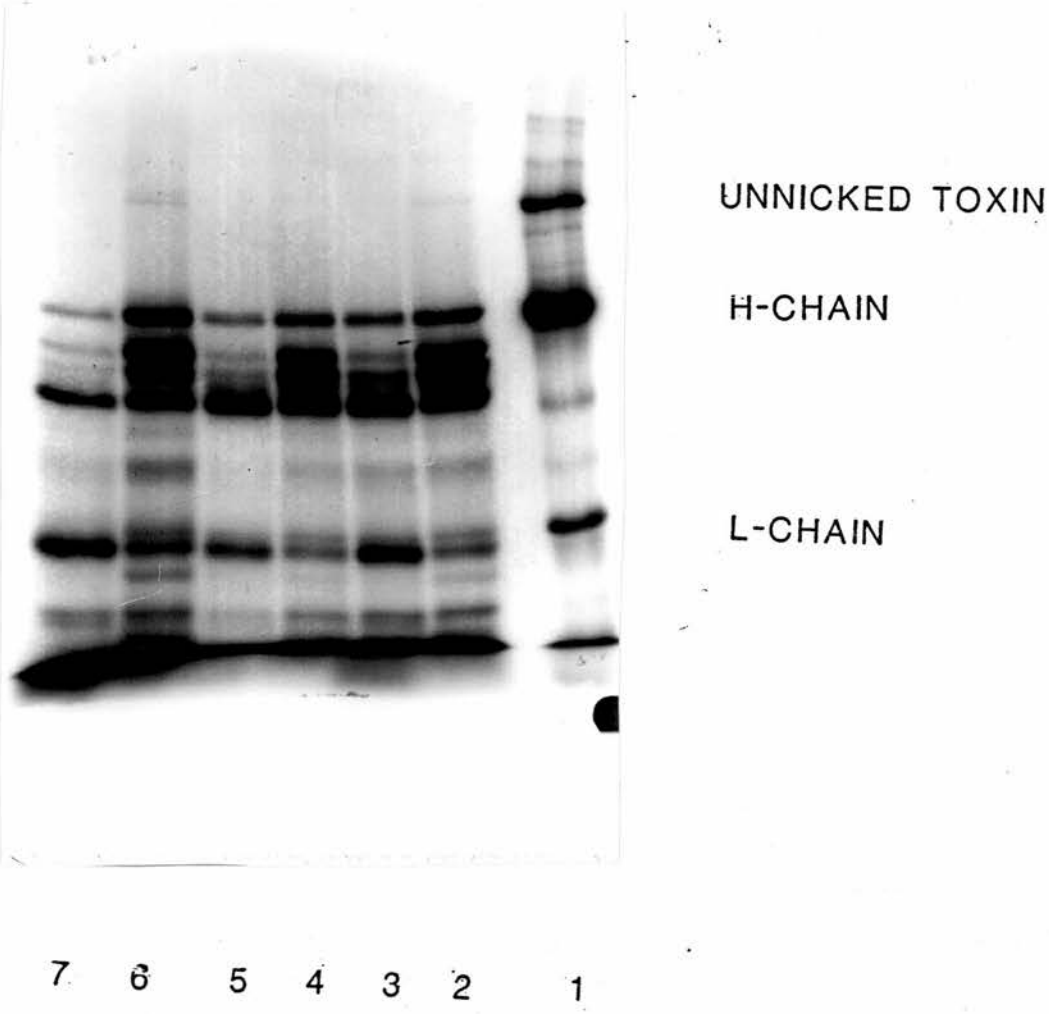
5 Supernatant from experiment 4.

6 Supernatant from experiment 5.

7 Supernatant from experiment 6.

8 Supernatant from experiment 7.

9 Supernatant from experiment 8.



PHOTOGRAPH 39. AUTORADIOGRAPH OF BOUND [^{125}I]-TETANUS TOXIN AFTER DIGESTION BY DPCC-TRYPSIN IN THE PRESENCE AND ABSENCE OF INSOLUBILISED GANGLIOSIDE SGGnSSLC (G_{T1})

See Section 3.4.2.3. for details.

1 Tetanus toxin.
2 Pellet from experiment 3.
3 Pellet from experiment 4.
4 Pellet from experiment 5.
5 Pellet from experiment 6.
6 Pellet from experiment 7.
7 Pellet from experiment 8.

in the presence of insolubilised ganglioside, produced similar patterns between pellet and supernatant samples. This indicated either that some toxin was adsorbed to the ganglioside and was digested in a similar manner to un-adsorbed toxin or that the digestion products of un-adsorbed toxin are able to be adsorbed. The electrophoretic pattern obtained from the pellets indicated that the fragments were still attached to digested 'nicked' toxin and were probably held by disulphide bridges. A way of testing this would have been carrying out the digestion process in the presence of 2-ME, but as shown in the chymotrypsin experiments 2-ME disrupts the binding of toxin to ganglioside.

The addition of insolubilised ganglioside to digested toxin produced a similar pattern between the pellets and supernatants. This again suggested that the toxin is in some structure whereby all the fragments are held as one molecule but the molecule is 'nicked' at a number of sites, and that the fragmented toxin is not adsorbed as strongly as undigested toxin. The digestion of adsorbed toxin produced a different pattern between the supernatants and pellets. By comparing track 9 of Photograph 38, to track 7 of Photograph 39, a major fragment (as marked x) is completely absent from the supernatant sample of the 10 min digested sample of toxin. Another fragment observed near the bottom of the gel (as marked y) is also missing. These observations may arise from a conformational change associated with the binding of toxin to ganglioside and that un-adsorbed toxin is more easily digested, i.e. more labile, than adsorbed toxin unless there was some protection by the ganglioside. The major fragment, mentioned above, appears to be bound in a higher concentration by the ganglioside, suggesting its origin is from H-chain. By comparing track 3 of Photograph 38 to track 9 of Photograph 38, once can observe that a fragment (as marked z) is absent from track 9, indicating the possibility of some protection of the adsorbed molecule. Thus it appears that there may be conformational changes associated with the binding of toxin to ganglioside SGGnSSLC (G_{T1}).

SECTION 4.

DISCUSSION.

Section 4

4.1. Discussion

It can be concluded, from Section 3.1., that the purification of tetanus toxin was sufficient for the further analysis. The criteria of purity can be summarised as:-

a) a single component in a non-denaturing polyacrylamide gel.
b) either two or three bands (depending on the type of toxin sample used) on an SDS-denaturing polyacrylamide gel; corresponding to 'unnicked' toxin, H- and L-chains.
c) three spots observed after the two-dimensional electrophoretic analysis of the freeze-dried toxin sample: again 'unnicked' toxin, H- and L-chains.
d) a single precipitin arc observed after two-dimensional immuno-electrophoretic analysis of the toxin.
e) a single component observed after sedimentation velocity centrifugation; the component showed no signs of heterogeneity.
f) the protein found to be homogeneous by criteria a - e, suspected of being tetanus toxin did produce the symptoms of tetanus (as described in Section 1.) in a mouse.

Most workers have judged that their samples of toxin were pure usually on the basis of either two or three components in an SDS-denaturing polyacrylamide gel (in the presence of a reducing agent). Work in this thesis has shown that the components observed in an SDS-denaturing polyacrylamide gel, and assumed to be single components, are in fact single polypeptides derived from a single protein. The importance of using purified toxin for physiological experiments, a situation that has not occurred in the past, has recently been discussed by Mellanby and Green (1981).

The molecular weight of 'unnicked' toxin was determined to be 150 000 $\pm$ 3 000 daltons using SDS-PAGE with a 4% polyacrylamide gel and to be 158 000 $\pm$ 2 000 daltons using a 5% polyacrylamide gel. The molecular weight of 'unnicked' toxin plus 'nicked' toxin was determined to be 142 000 daltons by sedimentation equilibrium in an analytical ultracentrifuge. The values given for the molecular

weight of whole tetanus toxin by other workers are 160 000 ± 5 000 daltons (Matsuda and Yoneda, 1974) and 150 000 daltons (Robinson et al., 1975), which are in agreement with the values described above. The molecular weight of H-chain was determined to be 97 000 daltons using SDS-PAGE with a 8% polyacrylamide gel, 99 000 ± 1 900 daltons with a 4% polyacrylamide gel and 95 000 ± 2 000 daltons with a 5% polyacrylamide gel. The average value was 97 000 daltons. The molecular weight of H-chain has been determined, by other workers, to be 95 000 daltons (Craven and Dawson, 1973), 107 000 ± 4 000 daltons (Matsuda and Yoneda, 1974) and 100 000 daltons (van Heyningen, 1976a) which are in agreement with the values described above. The molecular weight of L-chain was determined to be 51 000 daltons by SDS-PAGE with a 8% polyacrylamide gel. The molecular weight of L-chain has been determined, by other workers, to be 55 000 daltons (Craven and Dawson, 1973), 53 000 ± 3 000 daltons (Matsuda and Yoneda, 1974), between 46 000 - 48 000 daltons (Helting and Zwisler, 1977) and 50 000 daltons (van Heyningen, 1976a), which are in agreement with the values described above. Thus the evidence obtained from work carried out for this thesis and by other workers indicates that whole tetanus toxin can exist in two forms, a single polypeptide chain of molecular weight about 150 000 daltons, or a polypeptide of similar molecular weight that can dissociate, under denaturing and reducing conditions, into two polypeptide chains of molecular weights about 97 000 daltons and 51 000 daltons. Sedimentation equilibrium indicated that the "native" structure of the toxin molecule was a globular protein.

The toxin molecule was further analysed by various protein chemical techniques, as described in Section 3.3., in order to elucidate some of the chemistry of the molecule. A single N-terminal amino acid residue, proline, was found for 'unnicked' toxin and this was also shown to be the N-terminal amino acid residue for L-chain, indicating that L-chain is at the N-terminal end of the toxin molecule. No other N-terminal amino acid residue was found for either 'nicked' toxin, or for purified H-chain. This suggested that either H-chain had a "ragged" N-terminus or the N-terminus of H-chain was blocked, possibly arising from the cyclisation of a glutamine residue (the methylation or acetylation

of a newly produced N-terminus resulting from a proteolytic cleavage is unlikely). A "ragged" N-terminus could result from either the non-specific proteolytic cleavage of 'unnicked' toxin (but within a specific short sequence), or from the action of amino peptidases. Preliminary sequence work ruled out the possibility of a blocked N-terminus, because of the presence of several reactive N-terminal amino acids associated with purified 'nicked' toxin. The sequence work implied that the N-terminus of H-chain is "ragged". Several different groups have tried to identify any N-terminal amino acid residues for tetanus toxin; Murphy et al. (1968) and Craven and Dawson (1973) failed to identify any N-terminal amino acid residues, Bizzini et al. (1970) reported a single N-terminal amino acid residue which was leucine, Holmes and Ryan (1971) also reported a single N-terminal amino acid residue, but this time glycine. Neubauer and Helting (1979), independently to work carried out for this thesis, identified proline as the N-terminal amino acid residue for both 'unnicked' toxin and L-chain, but also identified a second N-terminal amino acid residue, leucine, present in 'nicked' toxin and H-chain. Leucine was observed as a possible second residue, aspartic acid being the first, in a tentative sequence for H-chain as described in Section 3.3.3.1.

It was shown in Section 3.1., that the freeze-dried toxin sample containing 'unnicked' toxin also contained a protease capable of converting 'unnicked' toxin to 'nicked' toxin, and the protease was also shown to be sensitive to PMSF and benzamidine. It is possible that the protease may be specific under certain conditions and less specific under different conditions. Neubauer and Helting (1979) were able to obtain tetanus toxin from their own cultures of Cl. tetani and purify the toxin immediately, possibly removing the toxin from the protease before it became less specific with "ageing" and thus obtaining a sample of toxin from which it was possible to identify the N-terminal amino acid residue of H-chain. The toxin samples used in this thesis were obtained from very large batch cultures, where the toxin was isolated for toxoiding, and it is possible that the longer delay between isolation and purification could have led to the protease becoming less specific and thus producing a "ragged" N-terminus for H-chain.

The sulphydryl content of tetanus toxin was investigated as described in Sections 3.3.2.2. and 3.3.2.3. The total number of half-cystine residues in whole tetanus toxin were determined to be 10 $\pm$ 2 using IAA, or 13 $\pm$ 2 by oxidising cysteine residues to cysteic acid which was then determined quantitatively in an automatic amino acid analyser. There was one free cysteine residue available for reaction with IAA, in the toxin in the presence of 6M GuHCl, but none were found to be available for reaction with IAA in the "native" conformation of the toxin molecule. At least eight cysteine residues were therefore found to be involved in disulphide bridge formation, but it is not known how many of the four disulphide bridges are inter-chain or intra-chain. The results indicated that the number of half-cystine residues are the same in both chains with the possibility (judging from experiments with IAA) that the free cysteine residue may be in the L-chain, though H-chain appeared to have more half-cystine residues than L-chain when the cysteine content is analysed as cysteic acid. This could imply that there are at least two cysteine residues in H-chain that are unavailable for reaction with IAA even in purified H-chain. The total half-cystine content of tetanus toxin has also been found to be about ten by Murphy *et al.* (1968), Bizzini *et al.* (1970) and Craven and Dawson (1973). Bizzini *et al.* (1970) found no free cysteine residues in the "native" structure of the toxin using IAA although they did identify two free cysteine residues using ethylene-imine. They also observed five cysteine residues available for reaction with IAA after denaturation of the toxin with 6M GuHCl. Craven and Dawson (1973) identified six free cysteine residues in the "native" conformation of tetanus toxin using 2-aldrithiol. Bizzini *et al.* (1970) and Murphy *et al.* (1968) observed four cysteine residues to be involved in disulphide bridge formation. Thus it appears that the various workers agree on the total half-cystine content of tetanus toxin but discrepancies occur as to whether the cysteine residues are available for reaction or are involved in disulphide bridge formation. It is possible that under denaturing conditions the free cysteine residues may be oxidised to form disulphide bridges in the presence of contaminating heavy metal ions though this is unlikely because of the 0.2% EDTA in the denaturation buffer system. It appears that some cysteine residues may be free to react with some sulphydryl reagents but

unavailable to react with IAA even in the presence of chaotropic reagents such as 6M GuHCl, though they may react with different sulphydryl reagents.

The amino acid analysis of purified toxin revealed little information. The analysis presented in this thesis is in agreement with analyses obtained by other workers as shown in Table 6 in Section 3.3.3.3. The exception is the analysis obtained by Holmes and Ryan (1971) which differs quite considerably from all the other analyses. Analysis of the individual purified chains showed that the amino acids were evenly distributed between the two chains as was also observed by Craven and Dawson (1973).

Purification of the two constituent chains was tried as described in Section 3.2.2. From the results of the gel-permeation chromatographic method, Section 3.2.2.1., it appears that the two chains are tightly associated, a phenomenon also observed and reported by other workers. The phenomenon is also supported by the results obtained from the two-dimensional electrophoretic analysis of the toxin, where the presence of L-chain appears to influence the net charge of H-chain. Because of this strong association between the two chains the gel-permeation method for the purification of the two chains was not very useful. The purification of the two chains, to single components as observed by SDS-PAGE, was possible by preparative SDS-PAGE, as described in Section 3.2.2.2. This method of purification allowed the DNS-Cl analysis of the two chains for the identification of any N-terminal amino acid residues, the amino acid analysis of each chain and the sulphydryl content of each chain. A disadvantage of this purification method was the presence of the SDS on the polypeptide chains which interfered with some experiments, e.g. N-terminal sequence studies.

Antibodies were raised against L-chain purified by gel-permeation chromatography, and against H- and L-chains purified by preparative SDS-PAGE. The antibodies raised against L-chain purified by gel-permeation chromatography were able to react with tetanus toxin and purified L-chain but not with purified H-chain. This observation suggested that the L-chain must be on the outside of the toxin molecule in the "native" conformation of the molecule. The antibodies raised against the chains purified by preparative SDS-PAGE cross-reacted only with SDS-denatured toxin, or with the

particular chain in the presence of SDS, indicating that all the structural information carried by the "native" conformation of the toxin molecule had been lost.

Analysis of whole tetanus toxin by charge shift electrophoresis, Section 3.4.2.1., indicated that the toxin is hydrophilic. Analysis of the toxin in the presence of urea and 2-ME with CTAB indicated that the toxin was split into two components one of which behaved as a hydrophilic protein but the other as an amphiphilic protein, indicating that it contained hydrophobic domains. Investigation using [^{125}I]-tetanus toxin revealed that the component behaving as a amphiphilic protein was H-chain, though some H-chain was still associated with L-chain indicating that the toxin was not fully dissociated. This result implied that H-chain contains some hydrophobic domains. The result that one chain is hydrophilic and that the binding component is amphiphilic, is similar to the result observed for diphtheria toxin (Boquet, 1979). In the case of diphtheria the hydrophilic subunit is also known to be the active moiety of the toxin. In contrast it has been shown that for cholera toxin both types of subunits are hydrophilic (Ward, Britton and van Heyningen, 1981, in press).

Limited proteolysis of tetanus toxin in the presence of SDS was used to try and investigate the association between the two chains. But inconclusive results were obtained arising from the fact whole toxin appeared to be very susceptible to proteolysis, and was usually completely digested under the conditions used whereas the two chains after purification were less susceptible to proteolysis. It is possible that the bound SDS is somehow able to protect the two chains from proteolytic digestion but the presence of the two chains together give rise to a structure labile to proteases. This possibly supports the hypothesis described above, where the presence of both chains is able to influence each other to a marked degree possibly a result of a tight association between the two chains.

Tryptic and chymotryptic digestion of toxin, and toxin complexed to insolubilised ganglioside, SGGnSSLC (G_{T1}) was carried out to investigate the possibility of any conformational change associated with the fixation of the toxin to ganglioside. It appeared from the results, Section 3.4.2.3., that there is the

possibility of a conformational change associated with the fixation of tetanus toxin to gangliosides. The conformational change may be needed to disrupt the tight association of the two chains for the initiation of the toxin activity. This evidence could support the theory that L-chain is the active moiety of tetanus toxin and that H-chain is only required for the adsorption of the toxin to specific cell membrane components in specific cells. It is not known at the present time whether one or both chains undergo a possible conformational change.

4.2. Conclusions

From work carried out in this thesis it can be concluded that:-

1) Tetanus toxin can exist in two forms, an 'unnicked' and a 'nicked' form both of molecular weight 150 000 daltons.

2) The toxin molecule gives a single component in sedimentation velocity centrifugation which has a sedimentation coefficient(s) of 7.26S.

3) The toxin molecule behaves as a globular protein as observed from sedimentation equilibrium centrifugation.

4) 'Nicked' toxin is composed of two chains of molecular weight 97 000 and 51 000 daltons.

5) The two chains are tightly associated.

6) The total half-cystine content of tetanus toxin is ten; one is available for reaction with IAA after denaturation of the toxin with the chaotropic reagent GuHCl. That at least eight of the cysteine residues present in tetanus toxin are involved in disulphide bridge formation.

7) The N-terminal amino acid residue of 'unnicked' toxin is proline.

8) The N-terminal amino acid of L-chain is proline indicating that L-chain is at the N-terminal end of tetanus toxin.

9) The N-terminus of H-chain consists of a "ragged" end.

10) There is a protease present in the unpurified toxin preparation capable of converting 'unnicked' toxin to 'nicked' toxin.

11) Tetanus toxin is a hydrophilic protein, though H-chain alone behaves as a amphiphilic protein indicating the presence of hydrophobic domains.

12) There is possibly a conformational change associated with the fixation of tetanus toxin to the ganglioside SGGnSSLC (G_{T1}).

13) Proteolysis experiments provided further evidence for the binding of H-chain to ganglioside SGGnSSLC (G_{T1}).

The structure of the toxin molecule as determined from work carried out for this thesis supports the model described by other workers but initially proposed by Craven and Dawson (1973), of a single protein composed of two chains. Another model for the structure of tetanus toxin was proposed by Bizzini et al. (1973). They postulated that tetanus toxin was composed of two identical subunits of molecular weight 75 000 daltons, and that each subunit was in turn composed of two non-identical chains of molecular weights 50 000 and 25 000 daltons which were held together by disulphide bridges. They deduced the structure from information obtained by SDS-PAGE and analytical ultracentrifugation. Matsuda and Yoneda (1975) agreed with the model proposed by Craven and Dawson (1973) but went on to show that tetanus toxin can also exist as a single polypeptide chain that cannot be dissociated into two chains. They postulated that the two-component form of tetanus toxin was the proteolytic cleavage product of the single-component toxin.

From the work presented in this thesis and by other workers it appears that the model proposed by Craven and Dawson (1973) and as improved by Matsuda and Yoneda (1975) is the correct structure for tetanus toxin which is now agreed upon by Bizzini and his co-workers. Work from this thesis, and independently shown by Neubauer and Helting (1979), expands the information of the model in that the N-terminal amino acid for 'unnicked' toxin is known and that L-chain is now known to comprise the N-terminal end of tetanus toxin. The overall model now suggested for tetanus toxin is as shown in Diagram 2 in Section 1.2.2. .

4.3. Future Work

There are still many questions to be answered regarding the structure of the toxin molecule:-

a) Investigation of the denaturation of tetanus toxin.

b) The use of chimeric forms of tetanus toxin to try and investigate the molecular action of the toxin.

c) The sequence of tetanus toxin to try and predict the quaternary structure of the molecule.

d) Investigation of possible conformational changes of the toxin molecule after fixation to its receptor.

e) Development of an *in vitro* assay for tetanus toxin.

f) An investigation of the protease responsible for the conversion of 'unnicked' toxin to 'nicked' toxin.

Experiments similar to those described by Creighton (1979) using polyacrylamide gel electrophoresis in the presence of an increasing gradient of urea would be useful to show how the toxin molecule unfolds in an increasing concentration of denaturant. If, as suspected, the two chains are tightly associated one might expect to see a very slow unfolding of the toxin molecule with complete or near complete unfolding of the chains at a high urea concentration.

Experiments using chimeric (a hybrid molecule composed or subunits obtained from different molecules) forms of tetanus toxin, similar to those described for diphtheria toxin by Gilliland *et al.* (1978), would be of great value for the understanding of the action of the toxin. As described in Section 1, the most likely action of the toxin is by some enzymic mechanism, because of the very high biological activity associated with a very low concentration. This mechanism appears to be a general feature of many bacterial exotoxins for example it has been described that a single molecule of diphtheria toxin is sufficient to kill a single cell. As described it appears that several toxins, e.g. cholera and diphtheria toxins, work by the ADP-ribosylation of a protein resulting in the inactivation of the protein. The combining of L-chain, from tetanus toxin, to Concanavalin A or to the binding subunit of either

the bacterial toxins, cholera or diphtheria toxins, or the plant toxins, Abrin and Ricin, could be a useful way of elucidating the possible enzymic reaction of tetanus toxin. Unfortunately with this type of experiment only a positive result would be of any use as a negative result could arise for several reasons, e.g. one would not know from a negative result whether the hybrid protein was inactive merely due to the incompatibility of the two different polypeptides. Tetanus toxin cannot be used to investigate its action at the molecular level because the cells sensitive to tetanus toxin cannot be purified to a level sufficient for this type of experiment. There has been a preliminary investigation (Wendon, 1980) into the possibility that tetanus toxin may work by some ADP-ribosylation mechanism, using neuroblastoma cells, though it appeared no ADP-ribosylation took place. A chimeric form of the toxin could be used on a more well-defined and relatively easily purified cell system, such as the rat liver cells, which have already been used to study the mechanism of the action of cholera toxin (Tait, 1980). The use of a chimeric form of the toxin could possibly answer the question of whether L-chain is the active moiety of the toxin molecule. A problem associated with the formation of a chimeric molecule is the purification of L-chain, as quite clearly the preparative SDS-PAGE system could not be used. Purification of L-chain by ion-exchange chromatography in the presence of urea is a possibility though the use of urea and a non-ionic detergent may be better. A problem with the use of urea would be the re-folding of L-chain into its active conformation. Though L-chain purified by gel permeation chromatography in the presence of urea still retains some of the immunogenicity associated with the L-chain in its "native" conformation.

The sequence of tetanus toxin is still required for the determination of the overall structure of the molecule and for determining the quaternary structure of the toxin. It has been reported that Robinson is at present attempting to sequence whole toxin and the two chains by amino acid sequencing. But the evidence that the toxin is expressed by plasmid DNA must give rise to the possibility of obtaining the amino acid sequence from the much easier and faster determined DNA sequence, though the

necessity of producing clones, for obtaining large quantities of DNA, may limit this investigation.

Further experiments to investigate a possible conformational change associated with the fixation of tetanus toxin to gangliosides are required. The attachment of a fluorescent label, in a low concentration, and looking for any change in a fluorescence pattern upon fixation of the toxin is a possibility. However, like the attachment of iodine-125 to the toxin, one does not know what effect these labels may exert on the molecule. Another possibility would be to observe an internally fluorescent molecule such as a tryptophan residue but this may not be sensitive enough as a result of the low levels of tryptophan present in the toxin. Another approach would be to observe the possible appearance or loss of an active molecule, such as cysteine, but again the low levels or lack of availability of cysteine residues may inhibit this type of investigation. Possibly the most likely method is circular dichroism.

The necessity of an *in vitro* assay for tetanus toxin still remains one of the greatest problems associated with research using tetanus toxin. Many experiments require the assaying of toxin activity but one cannot for both moral and economic reasons use bio-assays merely for routine screening of biological activity for many experiments. The requirement for a simple assay for tetanus toxin is required to answer questions such as whether both 'unnicked' and 'nicked' toxins are active or inactive and also whether L-chain is the active moiety of the toxin molecule.

An investigation of the protease responsible for the conversion of 'unnicked' toxin to 'nicked' toxin is also required. This has been partially investigated by Helting *et al.* (1979) who have shown that there are three proteases present in culture filtrates of *Cl. tetani* one of which converts 'unnicked' toxin to 'nicked' toxin. Evidence is still required regarding the specificity of this enzyme to determine whether or not it is able to become less specific under certain conditions. The protease itself seems to be unusual in that it appears to be very specific and possibly able to hydrolyse a particular peptide bond in the toxin molecule.

APPENDIX

CHEMICAL SOURCES

All of the common laboratory reagents were obtained from BDH and were of analar grade. Chemicals not obtained from BDH or of a less common use have their source indicated in brackets after their first appearance. Chemicals not of analar grade are also indicated in the text. The sources of the chemicals are as follows:-

Agfa-Gevaert Ltd., Dear Park Road, Wimbledon, London, SW19

Amicon Ltd., Amicon House, 2 Kingsway, Woking, Surrey, GU21 1UR

BDH Chemicals Ltd., Poole, Dorset, BH12 4NN

The Boehringer Corporation (London) Ltd., Bilton House, 54/58 Uxbridge Road, Ealing, London W5 2TZ

Cambrian Chemicals Ltd., Beddington Farm Road, Croydon, CR0 4XB

Difco Laboratories Ltd., P.O. Box 14, Central Avenue, East Molesly, Surrey

Eastman Kodak Ltd., Kirby, Liverpool, L33 7UF

Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, SL3 0BZ

LKB Instruments Ltd., 232 Addington Road, Selsdon, South Croydon, Surrey, CR2 8YD

Pierce and Warriner (UK) Ltd., 44 Upper Northgate Street, Chester, CH1 4EF

Pharmacia (Great Britain) Ltd., Prince Regent Road, Hounslow, Middlesex, TW3 1NE

The Radio Chemicals Centre, Amersham, Buckinghamshire, HP7 9LL

Servac Laboratories = Miles Laboratories Ltd., P.O. Box 37, Stoke Court, Stoke Poges, Slough, SL2 4LY.

Sigma Chemicals Company Ltd., Worbiton Station Yard, Kingston-upon-Thames, Surrey, KT2 7BH.

Whatman, Springfield Mill, Maidstone, Kent.

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C1-P73 The refolding of denatured creatine kinase

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Creatine kinase from rabbit muscle which has been denatured in 3M guanidine hydrochloride can be refolded with regain of full activity when the denaturant concentration is lowered to ≤ 0.1M. The refolded product has the same helix content and kinetic parameters as the native (dimeric) enzyme. The mechanism of the folding process has been studied by following the disappearance of reactive thiol groups during refolding and by monitoring the regain of quaternary structure using the cross-linking agent dimethylsuberimidate. These studies show that there is a rapid process, complete within 15 min, which yields a dimeric product containing two reactive thiols per subunit and which has 70% of the activity of the native enzyme; this species then undergoes a slow isomerisation to yield the final product (one reactive thiol group per subunit). These processes have also been monitored by spectroscopic methods to yield more information about the structural changes involved. The importance of the reactive thiol group in the refolding process has been demonstrated by studies of the refolding of modified creatine kinase.

C1-P74 DENATURED PROTEINS AS IMMUNOGENS AND ANTIGENS

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Detection of an immunological cross-reaction between two denatured proteins could be a rapid method to detect sequence homologies. In order to test the possibilities of such a methodology, using proteins of known structure, glyceraldehyde 3-phosphate dehydrogenases (GPDH) from different sources were compared. The antibodies raised against the denatured enzyme of E.coli K12 can recognize the GPDH from man, ostrich, chicken, sturgeon, halibut, lobster and yeast. In contrast, the antibodies directed against the native enzyme can only recognize the GPDH from B.stearothermophilus and to a lesser extent that from halibut.

This immunochemical approach was applied to the aspartokinases-homoserine dehydrogenases (AK-HDH) I and II and to the aspartokinase III (AK III) from E.coli K12. A cross-reaction between the denatured species but not between the native ones, was detected for the AK I-HDH I and AK II-HDH II enzymes and for the AK I-HDH I and AK III proteins. These results suggest that these proteins derive from a common ancestor.

The methodology was also applied in the search of some common structural features between the α and β_2 subunits of tryptophan synthetase and tryptophanase, proteins involved in the metabolism of tryptophan in E.coli K12. The lack of cross-reactivity observed between these proteins, even when in their denatured state, suggests that, despite their functional relationships, they probably do not derive from a common ancestor.

In conclusion, the present results suggest that the immunochemical detection of homologies among proteins using unfolded proteins has a wider application than that using native proteins, and that this method should be a useful tool in studying biochemical evolution.

C1-P75 REFOLDING OF A BIFUNCTIONAL ENZYME AND OF ITS TWO MONOFUNCTIONAL FRAGMENTS

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Aspartokinase I-homoserine dehydrogenase I (AK I-HDH I), a bifunctional and allosteric enzyme, has been renatured from its unfolded and separated polypeptide chains. Folding was measured by the reappearance of each of the two enzymatic activities, kinase and dehydrogenase, and of their allosteric inhibition by the same effector, threonine. The various observed properties yield different kinetics of folding, which shows the presence of intermediates having only part of the functional features of the native enzyme. Apparently, three successive steps can be detected during the folding of AK I-HDH I; first, a monomolecular step leads to a monomeric species with the kinase activity; then, an association step leads to a dimeric species with the kinase and dehydrogenase activities, and a threonine sensitive dehydrogenase; finally, a second association step leads to a tetrameric species with the two activities, both sensitive to threonine. The folding of this large protein appears as a sequential process during which the functional properties are regained successively, as the protein structure becomes more complex.

The two regions responsible for the kinase and dehydrogenase activities and located respectively in the N- and C-terminal part of the polypeptide chain can be obtained as monofunctional fragments. These two fragments have also been renatured from their unfolded and separated chains. The mechanism of folding of each of these fragments is the same as that of the corresponding region in the whole protein. During the folding process of AK I-HDH I, the two regions respectively responsible for the kinase and dehydrogenase activities seem to acquire their native conformation rather independently of each other.

C1-P76 "Fast" myosins in avian and mammalian skeletal muscle. A biochemical and immunological study.

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The biochemical analysis of myosin from avian fast muscle (Pectoralis-PECT), mammalian fast red (Masseter-MASS) and white (Tensor Fasciae Latae-TFL) muscles, using one- and double-dimensional SDS-gel electrophoresis, showed structural differences in light and heavy subunits. In particular, the avian LC1F light chain differed in its isoelectric point from LC1F of mammalian MASS and TFL. Tryptic fragments from heavy chains were markedly different in the three myosins. Antibodies raised against these myosins displayed a specific reactivity with the heavy chains of the respective immunogens, as revealed by GEDELISA (Gel Electrophoresis Derived Enzyme Linked Immuno Sorbent Assay) test. The three anti-myosin antisera stained selectively, in indirect immunofluorescence assay, fast fibers but showed limited interspecies reactivity. Segregation of different fast myosin isoenzymes in mammalian fast fibers was demonstrated in cross-absorption experiments. Anti-MASS myosin absorbed with insolubilized TFL myosin reacted selectively with fast red fibers; anti-TFL myosin absorbed with insolubilized MASS myosin stained only fast white fibers.

C1-P77 *Partial primary structure of Lobster muscle Arginine-kinase (EC.2.7.3.3...) C-1b*

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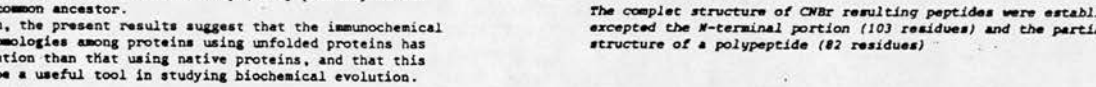
The N-terminal sequence of APK is N-acetyl-Ala-AsX-Ala-Ala-Thr.. and the C-terminal sequence is Lys-Glu-Methionine (COOH). The carboxyl end by the sequence Lys-Glu-Met (OH), this particular 3 residues sequence is repeated in three CNBr fragments. One of them a peptide of 14 amino acids residues was identified in the course of this study and its amino acid sequence determined its location at the COOH. Terminal end of the enzyme was recognized on the basis of investigations carried out with Des-Glu-Met (OH) arginine kinase a specific proteolytic derivatives.
The alignement of the eight CNBr-fragments which constitute the arginine-kinase molecule was established according to the sequential and compositional properties of seven unique tryptic methionyl peptides isolated from whole protein. The alignement was confirmed by using BNPS-Skatole fragments of the enzyme as another protein source.
The partial structure of CNBr fragments (Overlapping) was below



The complet structure of CNBr resulting peptides were established excepted the N-terminal portion (103 residues) and the partial structure of a polypeptide (82 residues)

C1-P78 The Protein Chemistry of Tetanus Toxin

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Tetanus toxin is a neurotoxic protein produced by Cl. tetani. It is produced as a single polypeptide m.wt 150 000, which is subsequently "nicked" by a protease into two polypeptides, m.wt 100 000 (H-chain) and 50 000 (L-chain), joined by one or more disulphide bridges. The toxin was purified by ion exchange chromatography on DEAE cellulose in the presence of protease inhibitors, producing a toxic and antigenic protein, giving a single band on a non-denaturing polyacrylamide gel and two bands on a reducing SDS gel. Initially the separation of the chains was done either on Ultrogel AcA44 or Sephacryl S-200, usually using 2M urea in the presence of 2mM dithiothreitol (DTT), after a reductive incubation in 8M urea and 100 mM DTT. The L-chain was purified to give a single band on a reducing SDS gel, but H-chain was always contaminated with L-chain. After formaldehyde treatment, antibodies were raised against L-chain in a rabbit and purified by precipitation in 18% Na_2SO_4; the anti-L-chain antibodies partly cross-reacted with whole toxin. The gel filtration method was tried under a number of different conditions without a successful purification of H-chain, indicating that the two chains are tightly bound together. The two chains have been successfully separated by preparative gel electrophoresis, identified in the gel by 4M sodium acetate precipitation of the SDS/polypeptide complex and eluted from the gel by electrophoresis. The intact chain and the L-chain have N-terminal proline but the H-chain appears to be heterogeneous at the N-terminus, suggesting a ragged cleavage by a protease. H-chain binds a specific ganglioside, G_{T1}, but the L-chain does not.