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Structure and Function of Pertussis toxin

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To my Mum and Dad

Preface

Work presented in this thesis was carried out solely by the author unless stated otherwise, between October 1st 1989 and September 30th 1992, supervised by Dr. Simon van Heyningen, University of Edinburgh Medical School, or under the supervision of Dr. L. Irons, PHLS Centre for Applied Microbiology and Research, Porton Down, Wiltshire.

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Contents

Abbreviations

ACT	adenylate cyclase toxin
ADP	adenosine diphosphate
AGG	agglutinogen
AMP	adenosine monophosphate
cAMP	cyclic adenosine monophosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAMR	Centre for Applied Microbiology and Research
CT	cholera toxin
CHO	Chinese Hamster Ovary
DMSO	dimethyl sulphoxide
DT	diphtheria toxin
DSP	dithiobis-(succinimidyl propionate)
DST	disuccinimidyl tartarate
DTE	dithioerythritol
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
Et_2O	diethyl ether

ΔF	change in fluorescence
FAB MS	fast atom bombardment mass spectrometry
FHA	filamentous haemaglutinin
fmoc	N-9-fluoroenylmethoxycarbonyl
G_i	inhibitory regulator of adenylate cyclase
G_s	stmulatory regulator of adenylate cyclase
GTP	guanosine triphosphate
HLT	heat labile toxin
HOBT	1-hydroxybenzotriazole
I.D.	internal diameter
K_a	association constant
K_d	dissociation constant
KLH	keyhole limpet haemocyanin
K_m	Michaelis constant
LPS	lipopolysaccharide toxin
$[M+H]^+$	protonated molecular ion
MAB	monoclonal antibody
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide ester
2-ME	2-mercaptoethanol

M_r	relative molecular mass
NAD^+	nicotinamide adenine dinucleotide
NCS	newborn calf serum
ODhbt	3-hydroxy-2,3-dihydro-4-oxo-benzotriazole
PBS	phosphate buffered saline (0.9%(m/v) NaCl, 50 mM sodium phosphate, pH 7.2)
Pfp	pentafluorophenyl
PHLS	Public Health Laboratories Service
R_i	inhibitory regulatory receptor of adenylate cyclase system
R_s	stimulatory regulatory receptor of adenylate cyclase system
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMPB	succinimidyl 4-(p-maleimidophenyl) butyrate
tboc	N-α-tert-butoxycarbonyl
TCT	tracheal cytotoxin
TEA.HCl	Triethanolamine hydrochloride
TFA	trifluoroacetic acid

Abstract

Pertussis toxin is a bacterial protein toxin consisting of five subunits, S1, S2, S3, S4 and S5, in a ratio of 1:1:1:2:1. The whole toxin has a molecular mass of 104 950, calculated from the nucleotide sequence of the toxin subunits.

Receptor-recognition of pertussis toxin is mediated by the B-oligomer, which consists of 1xS2, 1xS3, 2xS4 and 1xS5 and delivers the enzymatically active S1 subunit to target cells, where S1 catalyses the transfer of ADP-ribose from NAD^+ to G_i, a protein involved in regulation of adenylate cyclase activity. ADP-ribosylation of G_i prevents inhibition of adenylate cyclase activity and leads to a build-up of intracellular cAMP.

Overlapping decameric peptides derived from the entire amino acid sequence of the S1, S3 and S4 subunits of pertussis toxin were synthesised on polyethylene pins, to study the antigenicity of the S1, S3 and S4 pin decapeptides and fetuin binding to the S3 pin decapeptides. No major continuous antigenic sites were identified using the S1 and S3 pin decapeptides. One pin decapeptide derived from the amino acid sequence of the S4 subunit (residues 86-95, sequence QLTFEGKPAL) did react strongly with one rabbit anti-(pertussis toxin) antisera and the reaction was inhibited by pre-incubation of the anti-toxin sera with whole pertussis toxin. However, binding of the rabbit anti-toxin sera to whole toxin was not inhibited by antipeptide sera raised against a free peptide S4b that contained amino acids 86-95, possibly because anti-S4b did not bind strongly to whole toxin. No other polyclonal antisera or monoclonal antibodies bound to the pin decapeptide that contained residues 86-95, so this peptide does not form a major continuous antigenic site.

Fetuin bound to an S3-derived pin decapeptide (amino acids 46-55, sequence RQITPGWSIY). (E)S3c, a free peptide containing residues 44-58 also bound α-1-acid glycoprotein, mixed bovine brain gangliosides and fetuin. The ability of peptide (E)S3c to bind glycoprotein and glycolipid suggest that amino acids within the peptide are involved in toxin binding to target cells, so rabbit antisera raised against peptide (E)S3c were included in a pertussis toxin-mediated Chinese Hamser Ovary cell clumping assay. Rabbit anti-(E)S3c did not inhibit pertussis toxin-mediated clumping of Chinese Hamster Ovary cells, so (E)S3c is not likely to induce protective antibodies and be a candidate for replacement of detoxified pertussis toxin as a pertussis vaccine component.

Binding of NAD^+ to the S1 subunit of whole pertussis toxin, isolated S1 subunit and recombinant S1 analogues was investigated using intrinsic tryptophan fluorescence quenching with NAD^+. Dissociation constants (K_d values) were obtained at several temperatures for the whole toxin and isolated S1 subunit. K_d

values were similar for the whole toxin and isolated S1 subunit, suggesting that the B-oligomer is not essential to provide a suitable conformation for binding of NAD^+ to the S1 subunit.

Dissociation constants for binding of NAD^+ to a truncated S1 analogue, rS1d, lacking the C-terminal 47 amino acids and dissociation constants for binding of NAD^+ to rS1d containing a substitution of a glutamate residue at position 129 for aspartate were not dramatically different. This suggests that the C-terminal 47 amino acids and glutamate 129 are not essential for binding of NAD^+ to the S1 subunit.

The spatial arrangement of the toxin subunits was studied using reversible and non-reversible cross-linking reagents, one- and two-dimensional SDS PAGE and Western blotting. Results suggest that S4 and S3, S4 and S5 lie close together in the whole toxin, with S1 'on top' of the B-oligomer subunits. No cross-linked species that contained S2 were obtained, possibly because S2 was shielded from the cross-linkers by the other toxin subunits.

Chapter 1

Introduction

1.1 Pertussis : A brief overview

Pertussis (whooping cough) is an endemic disease with periodic epidemics affecting infants and young children. The disease was first recognised in Europe in the 16th century, and until the first recommendations for pertussis vaccination in 1957, whooping cough was a major cause of childhood mortality (Fine and Clarkson, 1987). A killed whole cell vaccine is in routine use in most countries (Robinson *et al.*, 1985). Uptake of the vaccine increased throughout the nineteen fifties and sixties, leading to a corresponding decline in the occurrence and severity of whooping cough (Miller *et al.*, 1982; Robinson *et al.*, 1985; Moxon and Rappuoli, 1990).

Reports of adverse reactions and variation in efficacy of this vaccine lead to decreased vaccine uptake in the mid 1970's (Ad hoc group for study of pertussis vaccines, 1988; Cherry *et al.*, 1988), and the argument over whether the vaccine is responsible for seizures and brain damage continues (Cherry, 1990; Griffin *et al.*, 1990), so the search for a better characterised, safer vaccine for pertussis has been given considerable momentum (Robinson *et al.*, 1985; Moxon and Rappuoli, 1990; Hewlett, 1992).

1.1.1 The cause of pertussis

Pertussis is caused by the gram negative bacterium *Bordetella pertussis*, which was first isolated by Bordet and Gengou (1906) and specifically colonises human ciliated respiratory epithelium (Mallory and Horner, 1912; Tuomanen and Weiss, 1985). The bacteria are transmitted by aerosol from infected individuals and the disease is common in crowded conditions (Muller *et al.*, 1986).

Many virulence factors are secreted by *B. pertussis*. Pertussis toxin may be the major one (Pittman, 1984; Monack *et al.*, 1989; Loosemore *et al.*, 1991; Miller *et al.*, 1991), and the importance of other virulence factors in vaccine development is still being studied.

1.1.2 The disease

The only natural host for *Bordetella pertussis* is man. The bacteria specifically attach to and colonise host ciliated respiratory epithelium. An incubation period of seven to ten days ensues. A catarrhal phase follows, with cold-like symptoms lasting up to two

weeks.

Ciliostasis and respiratory cell damage result in the paroxysmal (or spasmodic) coughing phase, which lasts approximately four weeks. As the patient attempts to clear the lungs of obstructing bacteria and mucus the characteristic 'whoop' of the disease, resulting from rapid inhalation after severe bouts of coughing may be heard in a small number of cases (Hewlett, 1992). Cyanosis, tongue protrusion, hernia, vomiting and seizures may be also be observed (Cherry *et al.*, 1988). Coughing gradually abates and a convalescent stage is reached, lasting about three weeks.

1.1.3 Prevention and Treatment

Antibiotics can be used to combat *Bordetella pertussis* infection, but by the time the disease is diagnosed, cell damage has been done and toxins have been released. Erythromycin is the most used antibiotic, but is only effective if given before the paroxysmal phase begins (Bass, 1985).

The killed whole cell vaccine (1.1) is administered with diphtheria and tetanus toxoids adsorbed onto aluminium salts (Robinson *et al.*, 1985). The UK has an established programme of three preschool immunisations at two, three and four months, with a booster at four years (Booy *et al.*, 1992). Vaccination of infants in the UK has recently risen to the highest level recorded (White *et al.*, 1991), but decreased public acceptance in the nineteen seventies and eighties, decreased efficacy and possible inherent toxicity of the whole cell vaccine, coupled with evidence that the whole cell vaccine may not give lifetime protection against infection (Linneman and Nasenby, 1977; Addiss *et al.*, 1991; Halperin and Marrie, 1991), have created the requirement for a better characterised, acellular vaccine.

Japan has an acellular vaccine which has been widely used since 1981 (Sato *et al.*, 1984) and was included in Swedish trials in the late nineteen eighties (ad hoc group for study of pertussis vaccines, 1988). It was given to children over two years old in the Japanese trials, and differences in efficacy were seen between the Swedish and Japanese trials. Consequently, the Japanese vaccine was not advised for routine vaccination of infants in the Western world.

Research into vaccine components continues; at least thirteen potential, acellular vaccines are at various stages of development at the moment (this number recently entered phase II clinical trials in the USA), containing a range of *B. pertussis* antigens (Podda *et al.*, 1992; Miller *et al.*, 1991; Pizza *et al.*, 1989; Ad hoc group for study of pertussis vaccines, 1988).

The next advances in acellular vaccine development will involve identification of subunits and peptides from *B. pertussis* antigens which contain epitopes, and are immunogenic and protective against the disease, without the toxicity associated with

the whole cell vaccine.

1.2 Virulence factors of *Bordetella pertussis*

There are several virulence factors produced by *B. pertussis*, including the agglutinogens, pertactin, adenylate cyclase toxin, endotoxin, tracheal cytotoxin, heat-labile toxin and pertussis toxin. They will be discussed in this section with the exception of pertussis toxin, which is covered in section 1.3.

1.2.1 Agglutinogens

1.2.1.1 A brief summary

Agglutinogens (Aggs) are generally described as serotype-specific proteins (M_r 10 000 to 23 000), which generate antibodies, which agglutinate bacteria. Agg 1 is thought to be a non-fimbrial protein common to all strains of *B. pertussis* , but its nature has not been determined; Fim 2 and Fim 3 (Robinson *et al.*, 1989), otherwise known as Agg 2 and 3 are fimbrial proteins, found in various combinations on different strains of *B. pertussis*, and are major serotyping antigens of *B. pertussis*. Aggs 4, 5, 6 and 7 are minor antigens : Agg 6 is thought to be identical to Fim 3 (Agg 3) and Agg 7 is common to all *Bordetella* species (Robinson *et al.*, 1989).

1.2.1.2 *B. pertussis* fimbriae

The major types of fimbriae of *B. pertussis* are fim 2 and fim 3 (M_r 22 500 and 22 000 respectively, as determined by SDS PAGE). Their amino acid sequences show a high degree of homology and they are antigenically distinct (Mooi *et al.*, 1990). *B. pertussis* has three sequenced fimbrial subunit genes : *fim2, fim3* and *fimX* (Livey *et al* ., 1987; Pedroni *et al* ., 1988; Mooi *et al* ., 1990) and a fourth (*fimY*) has been suggested. *FimX* and Y may be silent genes (Mooi *et al.*, 1992), or expressed at low levels (Riboli *et al.*, 1991).

Fim 2 and 3 monomers are assembled into fimbriae of about 100 nm length (Steven *et al* ., 1986), possibly involved in adhesion to the respiratory tract of mice and possibly humans (Robinson *et al.*, 1990). Fim 2 and 3 also protect against infection by *B. pertussis*, leading to their consideration for inclusion in an acellular vaccine (Miller *et al.*, 1991).

1.2.2 Filamentous Haemagglutinin

B. pertussis secretes two haemagglutinins, filamentous haemagglutinin (FHA) and pertussis toxin (see section 1.3). FHA is a non-fimbrial protein (apparent M_r 220 000) and has been sequenced (Relman *et al.*, 1989; Delisse-Gayothe *et al.*, 1990). It is required for adhesion of *B. pertussis* to ciliated epithelium, as well as to integrins on macrophages (Relman *et al.*, 1990; Shahin *et al.*, 1992;) and contains the amino acid triplet RGD (ArgGlyAsp) which acts as a cell binding site for some eukaryotic proteins (Ruoslahti and Pierschbacher, 1986). FHA also contains a lectin-like binding site; FHA binding to epithelial cells can be inhibited by galactose (Tuomanen *et al.*, 1988). FHA also binds sulphated saccharides (e.g. heparin) (Menozzi *et al.*, 1991), which may be the mechanism of binding of *B. pertussis* to sulphated glycolipids, present in large amounts in the human trachea and lung (Brennan *et al.*, 1991).

FHA's importance in adhesion has meant that it has been included in potential acellular vaccines (Ad hoc group for study of pertussis vaccines, 1988; Podda *et al.*, 1992).

1.2.3 Pertactin

Pertactin (P69) is a non - fimbrial agglutinogen (M_r 69 000) which has been sequenced (Brennan *et al.*, 1988; Charles *et al.*, 1989). Its role in pathogenesis is unclear, but it has been associated with the adenylate cyclase toxin (1.2.4) and contains the RGD sequence involved in adhesion to some eukaryotic proteins (Leininger *et al.*, 1991). It has also been linked to active uptake of *B. pertussis*, by interaction with eukaryotic cell receptors (Leininger *et al.*, 1992). There is a strong antibody response to pertactin (Anwar, 1991) and it protects against pertussis aerosol challenge (Shahin *et al.*, 1990) adding to the reasoning that it should be included in acellular pertussis vaccines (Podda *et al.*, 1992).

1.2.4 Adenylate cyclase toxin

B. pertussis secretes an adenylate cyclase toxin (ACT, cya A; Hewlett and Wolf, 1976) whose production is governed by the *cya* operon (containing five genes : *cyaA, B, C, D* and *E*) which codes for a protein of M_r about 200 000 (Glaser *et al.*, 1988). ACT is toxic to neutrophils, causes lysis of red blood cells and is partly responsible for the incidence of secondary bacterial infection due to impaired host immune responses.

ACT is activated by calmodulin binding (Wolf *et al.*, 1980), enters eukaryotic

cells, possibly by receptor-mediated endocytosis (Donovan and Storm, 1990) and transiently increases levels of intracellular cAMP (Confer and Eaton, 1982; Hanski and Farfel, 1985). This differs from toxins acting by ADP-ribosylation, which cause a more protracted increase in cAMP levels.

The N-terminal, catalytic region which binds calmodulin has recently been better defined (Ladant *et al.*, 1992); two insertions between amino acids 247 and 248, and 335 and 336, affect calmodulin activation of ACT. The C-terminal region of the protein is thought to be involved in invasion and haemolysis (Rogel *et al.*, 1991; Gross *et al.*, 1992).

In clinical trials acellular vaccines which were mostly based on pertussis toxin and FHA decreased the severity of the disease, but gave incomplete protection. (Storsaeter, 1990). The importance of ACT in virulence (Goodwin and Weiss, 1990; Khlef *et al.*, 1992) means it is a possible candidate for inclusion in an acellular vaccine.

1.2.5 Endotoxin (lipopolysaccharide)

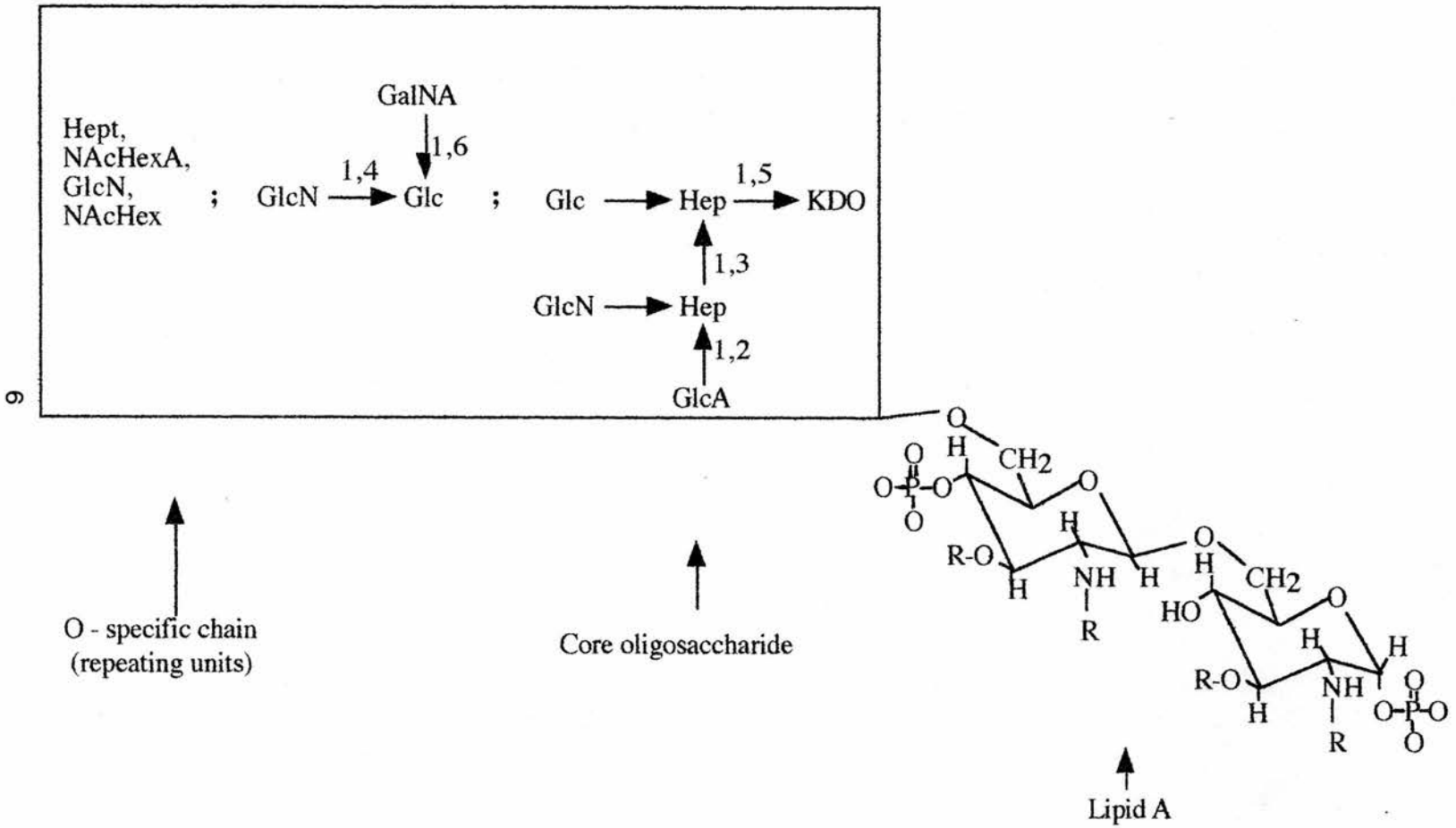
Endotoxins are lipooligo- or -polysaccharides (LPS) found in the outer membrane of gram negative bacteria (e.g. *Bordetella pertussis, Escherichia coli and Salmonella typhimurium*). They are variations of one molecule (fig. 1.1) which consists of lipid A linked by 3-deoxy-D-manno-2-octolusonic acid (kdo) to a core oligosaccharide, which is connected to a longer, more variable O-specific chain (consisting of repeating units of three to eight sugars). The O-chain is the most variable region and triggers a specific immune response (i.e. antibody production). The lipid A domain is mainly responsible for the non-specific immune response by macrophages, complement molecules and granulocytes.

B. pertussis produces two endotoxins, LPS-I which contains non - phosphorylated kdo, and LPS-II which contains phosphorylated kdo (LeDur *et al.*, 1980; Peppler, 1984). Endotoxins stimulate macrophages to produce mediators (proteins such as cytokines : tumour necrosis factor and interleukins 1, 6, and 8; lipids, some of which cause fever and are transformed into oxygen free radicals, which aid microbial killing). So if moderate amounts of endotoxin are released, macrophage stimulation leads to eradication of disease by a controlled localised immune response. If a more severe infection is encountered, larger amounts of endotoxin are released (Morrison and Ryan, 1987) which can result in hypotension, high fever and shock (leading to cell malfunction and cell death).

There are several possible mechanisms of endotoxin action. The endotoxin may bind a carrier, then possibly to CD14 receptors on macrophages, which then stimulate mediator release. Another view is that endotoxin directly binds receptors on

Fig. 1.1 Schematic diagram of *Bordetella* endotoxin

Six saturated fatty acid chains are linked to two glucosamine residues (GlcN) in lipid A The core oligosaccharide region of the lipooligosaccharide contains 3-deoxy-D-manno-2-octolusonic acid (kdo), glucose (Glc), heptose (hept), glucosamine (GlcN) and glucuronic acid (GlcA). The O-chain consists of repeating oligosaccharide units including heptose, N-acyl hexosamine (NAcHex), mono/di-N-acylaminohexuronic acid (NAchexA) and glucosamine.


Hept,
NAcHexA,
GlcN,
NAcHex
;
GalNA
1,6
1,4
GlcN
Glc
;
Glc
Hep
1,5
KDO
1,3
GlcN
Hep
1,2
GlcA
O - specific chain
(repeating units)
Core oligosaccharide
Lipid A

the macrophage surface without using a carrier, again stimulating mediator release (Raetz *et al* ., 1991).

Endotoxin is therefore thought to be responsible for the milder side effects of the whole cell vaccine (fever; inflammation around the injection site) and is not generally considered for inclusion in an acellular vaccine.

1.2.6 Tracheal cytotoxin

Tracheal cytotoxin (TCT) causes lung ciliostasis and cilial cell damage (Goldman *et al.*, 1982). TCT is produced by all strains of *Bordetella* and is a small glycopeptide, N-acetyl glucosaminyl-1,6-anhydro-N-acetylmuramyl-alanyl-glutamyldiaminopimelyl-alanine (M_r 921 Da), derived from cell wall peptidoglycans (Cookson *et al.*, 1989) and it inhibits DNA synthesis *in vitro*, so slowing replacement of static, damaged cilia (Goldman *et al.*, 1982).

The mechanism of action of TCT is not fully understood; it has no observed enzyme activity, so it may act via receptors on host cells.

1.2.7 Heat labile toxin

B. pertussis expresses a heat-labile toxin (inactivated by heating to 56°C; Munoz, 1971) also called dermonecrotic toxin because it causes lesions when injected into the skin. Its activity was first discovered by Bordet and Gengou (1909) and is a single polypeptide (M_r 140 000) (Zhang and Sekura, 1991) produced by all strains of *Bordetella*..

HLT may be involved in vasoconstriction (Endoh *et al.*, 1986),which could result in inflammation of highly vascularised tissues (e.g. lung, spleen), possibly enhanced by endotoxin, in the initial stages of pertussis infection.

HLT has low antigenicity; it interferes with the adjuvant effect of pertussis toxin, and relatively little is known about its biochemical action. It is not usually considered for use in acellular vaccines.

1.3 Pertussis Toxin

1.3.1 General Introduction

Many of the effects of *B. pertussis* infection stem from the action of one antigen called pertussis toxin (PT) (Pittman, 1979, 1984), which was known in the past under names such as lymphocytosis promoting factor (LPF), histamine sensitising factor (HSF) and islet activating protein (IAP).

Pertussis toxin is believed to be involved in bacterial adhesion to glycoproteins of cilia of the respiratory tract (3.3.8); it acts as an adjuvant, activates adenylate cyclase, causes lymphocytosis, histamine sensitisation, T-cell mitogenicity, haemagglutination, and increased insulin production leading to hypoglycaemia. Several of the effects of pertussis are produced by the toxin alone (Ui, 1988), so pertussis toxin is regarded as one of the major virulence factors of *B. pertussis* (Weiss and Hewlett, 1986; also see section 1.1.2).

The toxin is also involved in bacterial uptake by macrophages. It binds to carbohydrates on their surface, activating an integrin called CR3 which can then bind filamentous haemagglutinin, resulting in the bacteria being taken up into the macrophage (Hoepelman and Tuomanen, 1992; van't Wout *et al.*, 1992).

1.3.2 Structure

The estimated molecular mass of pertussis toxin differs slightly depending on whether it was measured by experimental methods, or was calculated from the nucleotide sequence of the toxin subunits. Tamura *et al.*, (1982) estimated the molecular mass at 117 000, using equilibrium ultracentrifugation. They also found that when the purified toxin was boiled with 1%(w/v) SDS, it dissociated into five subunits which were resolved by SDS-PAGE (Laemmli, 1970), in gradient gels of 10%(w/v) (top) to 30%(w/v) (bottom) acrylamide and the subunits were named S1, S2, S3, S4 and S5 according to decreasing molecular weight (S1 28 000, S2 23 000, S3 22 000, S4 11 700 and S5 9 300). These molecular masses add up to a total for the whole toxin of 105 700. The difference between this value and the 117 000 value could possibly be explained by the partial specific volume required for analytical ultracentrifugation calculations being calculated from amino acid composition, instead of being determined experimentally. Comparison of coomassie blue staining of the subunits by scanning densitometry of SDS-PAGE gels suggested that S1-S5 exist in a molar ratio of 1:1:1:2:1 in the holotoxin.

Sekura *et al.* (1983) purified pertussis toxin by affinity and gel filtration chromatography. They analysed their toxin preparation by SDS-PAGE in 15%(w/v) acrylamide gels and resolved only four subunits; the S4 and S5 subunits identified by Tamura *et al.*(1982) ran as one band in the Sekura *et al.*(1983) system, so Sekura *et al.* (1983) proposed that the stoichiometry of the toxin, based on scanning densitometry of SDS-PAGE gels was the toxin was a hexamer, S1, S2, S3 and S4 being in a 1:1:1:3 ratio. Molecular masses of each subunit also differed from those obtained by Tamura *et al.* (1982), S1 being 26 100, S2 25 300, S3 22 000, S4/S5 was a single band of 13 200, giving a total mass for the whole toxin of 113 000.

Other groups have estimated slightly different molecular masses for the whole

toxin and separated subunits by SDS-PAGE (Morse and Morse, 1976; Irons and MacLennan, 1979; Peppler *et al.*, 1985; Perera *et al.*, 1985), but the estimates are hard to compare as they were made using different protein standards, and/or different gel systems. All the experimentally-determined molecular masses are similar to those calculated by Locht and Keith (1986) and Nicosia *et al.*, (1986) (see section 1.3.3) with one exception. Cowell *et al.* (1982) estimated the molecular mass of the whole toxin to be 170 000 under non-dissociating conditions using PAGE at acid pH, but this is a notoriously difficult technique to interpret, so this value can be discounted.

The subunit stoichiometries obtained by Tamura *et al.*, (1982) and Sekura *et al.*, (1983) probably differ due to the acrylamide concentration used in SDS-PAGE; the gradient system used by Tamura *et al.*, (1982) having the better resolving power. Both groups based their stoichiometry results on the assumption that all the subunits have the same affinity for the Coomassie stain. This is not a wise assumption; since dye binding in dissociating agents is a poor method of determining the concentrations of different proteins; in particular, S5 is known to stain poorly on SDS-PAGE (Nicosia *et al.*, 1986).

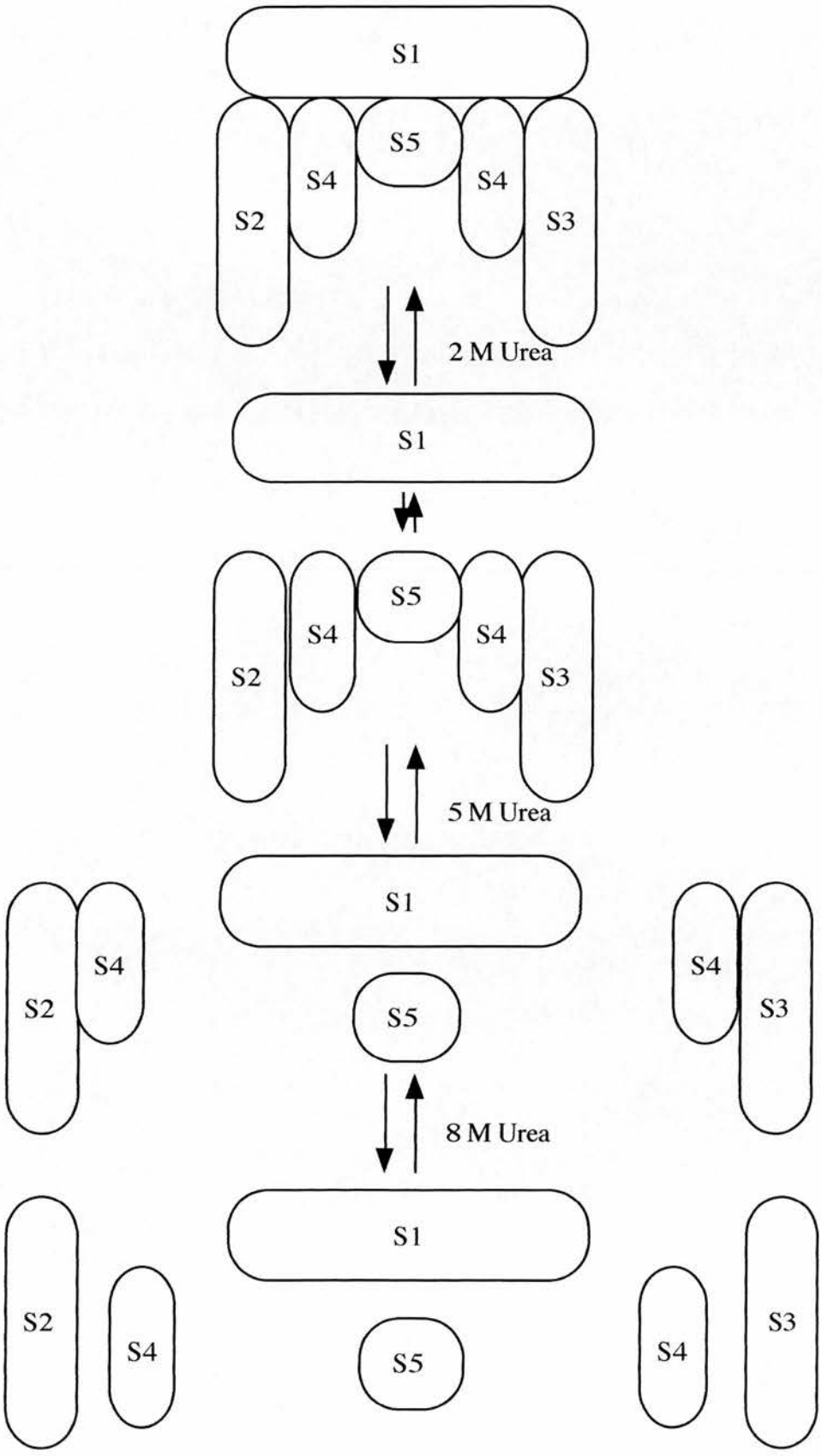
At present the spatial position of the subunits in the toxin can only be inferred, from toxin dissociation and association studies in urea (Tamura *et al.*, 1982) and exact subunit positioning awaits crystallisation of the protein (further investigation of subunit organisation using chemical cross-linking reagents is discussed in chapter 4). Tamura *et al.* (1982) dissociated the toxin into two dimers, 1 and 2, and two monomers, S1 and S5, after treatment with 5 M urea (pH 6.0) at 4°C for four days and separation by CM-sepharose cation exchange chromatography. Further treatment with 8 M urea (pH 8.4) at 4°C for 16 hours and separation by DEAE-sepharose anion exchange chromatography resolved dimer 1 into S2 and S4, while dimer 2 was resolved into S3 and S4 (Tamura *et al.*, 1982). This two-step purification was required for quantitative analysis because S5 was denatured by exposure to 8 M urea.

Toxin assembly in the periplasm may occur in three steps: S2 and S3 each combine with an S4 subunit; these dimers are then connected by one S5, making the B-oligomer. The S1 subunit binds only when the B-oligomer is formed (Tamura *et al.*, 1982; fig.1.2).

If S3 is missing or defective the subunits accumulate in the periplasm (Nicosia and Rappuoli, 1987), so S3 is needed for B-oligomer assembly. S1 is also needed for efficient release of the whole toxin into the extracellular medium, as strains with mutations within S1 still assemble the B-oligomer, which is released into the medium in low amounts in the absence of mammalian cells (pertussis toxin is also thought to be a bacterial adhesin) and S1 is not incorporated into the toxin (Pizza *et al.*, 1990).

Pertussis toxin is an A-B type toxin, like several other bacterial toxins, such as cholera toxin, *E.coli* heat labile toxin, diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A (Gill, 1976; Pappenheimer, 1977; Tamura *et al.*, 1982; Pastan and

Fig. 1.2. Schematic model of pertussis toxin dissociation in urea (from Tamura *et al.*, 1982)


S1
S5
S4
S4
S2
S3
2 M Urea
S1
S5
S4
S4
S2
S3
5 M Urea
S1
S4
S2
S4
S3
S5
8 M Urea
S1
S2
S4
S4
S3
S5

Fitzgerald, 1989). The A protomer is the S1 subunit of pertussis toxin, acting as an ADP-ribosyltransferase (section 1.3.6) and the B-oligomer is S2 to S5, which binds eukaryotic cells (section 1.3.5.and chapter 3). Pertussis toxin is also the only known AB type bacterial toxin that is involved in attachment of bacteria to mammalian cells (Tuomanen and Hendley, 1983)

Labelling of the holotoxin subunits has given another insight into subunit organisation (Montecucco *et al.*, 1986). To determine which subunits interact with membrane lipids, labelling of pertussis toxin with two phospholipid micelles was observed and the results agreed with the accepted model of pertussis toxin (Figures 1.2 and 1.3). It is thought that S1 is quite weakly attached to the B-oligomer (Tamura *et al.*, 1982), but precisely how the subunits of the B-oligomer are arranged, where they bind S1, and where the S3-S4 and S2-S4 dimers are held together is not known.

1.3.3 Cloning and sequencing of pertussis toxin

The DNA sequence of the pertussis toxin operon has been obtained (Locht and Keith, 1986; Nicosia *et al.*, 1986). The genes are organised in the order S1, S2, S4, S5, S3 (Fig 1.4) with the open reading frame for S4 overlapping the S2 and S5 genes. There is a promoter upstream of S1 and a site for a trans activating regulatory protein (produced by the bvg regulon, see 1.3.4), which switches expression of the toxin subunits on and off at the level of transcription (Nicosia and Rappuoli, 1987).

Each subunit has an N-terminal signal sequence of variable length. The subunits are secreted into the periplasm separately and their signal sequences are cleaved, to give the mature subunits with calculated molecular masses of :

S1	26 024 (234 amino acids (aa))	S4	12 058 (110 aa)
S2	21 924 (199 aa)	S5	11 013 (100 aa)
S3	21 873 (199 aa)		

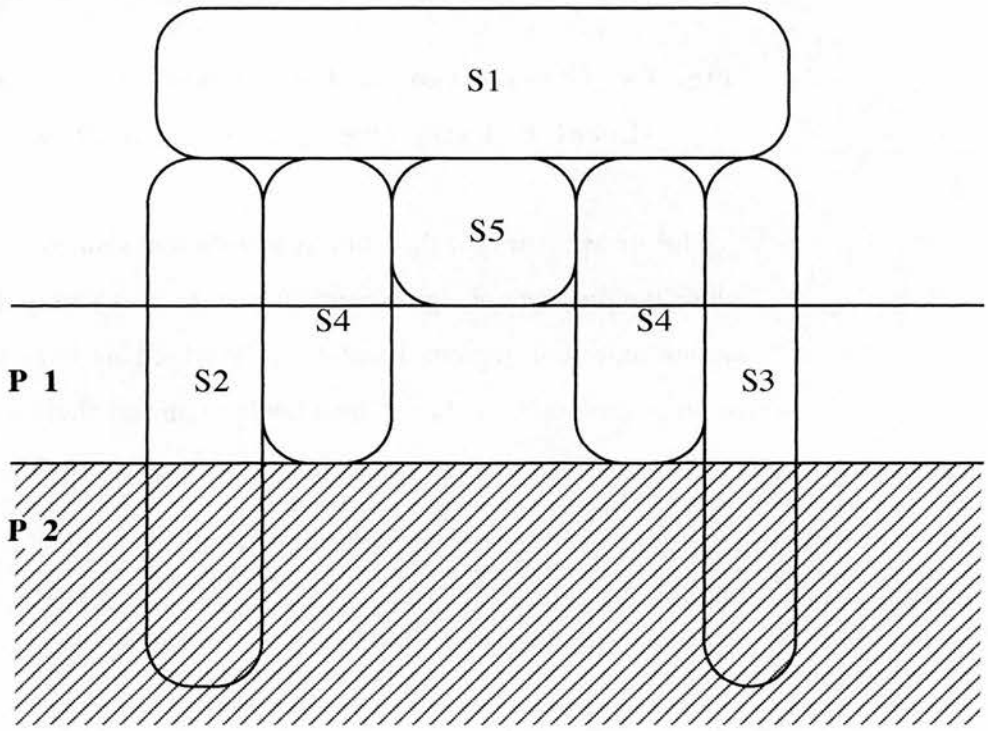
(Locht and Keith, 1986). S4 has a long signal sequence of 42 residues (Nicosia *et al.*, 1986) and a high positive charge at its N-terminal and this may mean that secretion of S4 is very efficient (Nicosia *et al.*, 1986). That there are two copies of S4 in the holotoxin produced from a single copy gene may be due to different promoter strengths controlling transcription. S1 has no lysine residues in its deduced amino acid sequence and contains two regions of homology with the A protomers of *Escherichia coli* heat labile toxin (HLT) and cholera toxin :

Fig. 1.3. Model of pertussis toxin interaction with phospholipid micelles

(Montecucco *et al.*, 1986)

Phospholipid 1 was labelled at the head group and interacted with parts of the toxin which did not penetrate deep into the micelle.

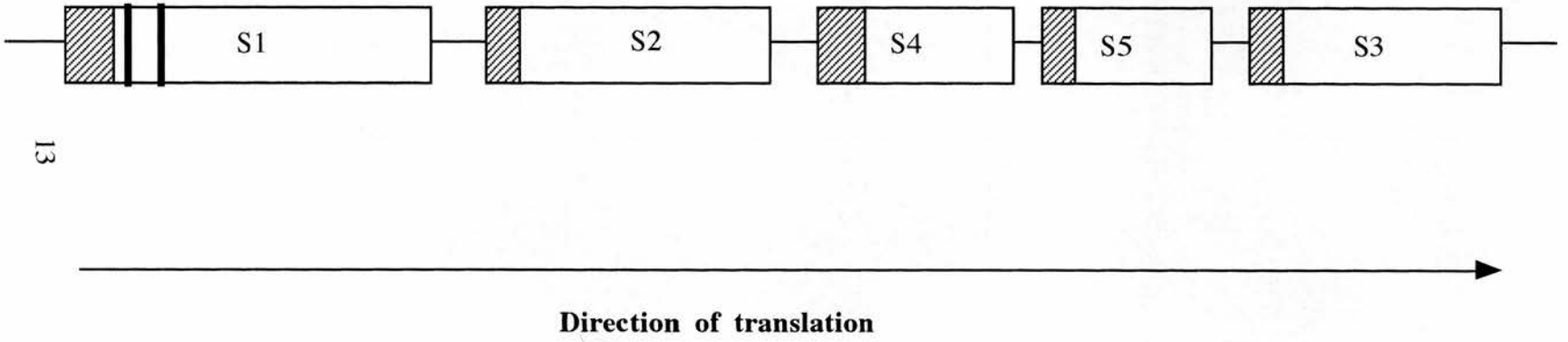
Phospholipid 2 was labelled at the fatty acid methyl terminus and interacted with parts of the toxin which did penetrate deep into the micelle.



P 1, P 2 = Phospholipids 1 and 2

Fig. 1.4. Organisation of the pertussis toxin operon (Locht & Keith 1986; Nicosia *et al* 1986)

The arrow shows the direction of translation; shaded boxes show the presence of signal peptides and the bars within the S1 subunit represent regions 1 and 2 (1.3.3) which are homologous to the A protomers of *E. coli* heat labile toxin and cholera toxin.


S1
S2
S4
S5
S3
Direction of translation

Region 1

Pertussis toxin S1 subunit	(8)	Y R Y D S R P P	(15)
Cholera toxin A subunit	(6)	Y R A D S R P P	(13)
E. coli HLT A subunit	(6)	Y R A D S R P P	(13)

Region 2

Pertussis toxin S1 subunit	(51)	V S T S S S R R	(58)
Cholera toxin A subunit	(60)	V S T S I S L R	(67)
E. coli HLT A subunit	(60)	V S T S L S L R	(67)

(Numbers in parentheses denote amino acid sequence number).

Mutations within region 1 affect the enzymic activity of the S1 subunit of pertussis toxin and the other two toxins (Cieplak *et al.*, 1988; Burnette *et al.*, 1991; Lobet *et al.*, 1991).

1.3.4 Regulation of expression of virulence factors

The expression of bacterial proteins is often regulated by environmental conditions. Many bacteria have a temperature-regulated method of expressing proteins needed for colonisation of a host (Miller *et al.*, 1989) and chemical agents can also control transcription of the 'virulence genes' giving rise to these proteins. *Bordetella pertussis* virulence genes are induced by both temperature and chemicals (Arico *et al.*, 1989; Roy *et al.*, 1989; Scarlato *et al.*, 1990, 1991).

Expression of the virulence factors of *B.pertussis* is under the control of the *bvg* locus, or regulon (*Bordetella virulence gene*), which contains several operons and unlinked genes, two of these coding for regulatory proteins BvgA and BvgS (originally called BvgABC), which have calculated molecular masses of 23 000 and 135 000. These are members of a family of signal transduction proteins, judged by sequence homology (Gross *et al.*,1989; Scarlato *et al.*, 1990).

A model suggested by Arico *et al.* (1989) and Stibitz and Yang (1990, 1991) is that BvgS is a monomeric trans-membrane protein; its N-terminal end senses external signals which trigger dimerisation of BvgS and transmits the signals to a cytoplasmic domain, which phosphorylates BvgA, enabling it to dimerise, bind DNA and activate

transcription.

All except one of the promoters controlling the *bvg* locus (called P_1, P_3,P_4; the exception being P_2) and the promoters of the *Bordetella* virulence genes are regulated by environmental factors. A temperature shift from 25 to 37°C immediately induces P_1 and P_4 of the bvg locus, P_{FHA} which produces filamentous haemagglutinin (FHA)and the fimbrial gene promoters (Willems *et al.*, 1990; Scarlato *et al.*, 1991). Other promoters are induced after a time lag of about two hours (eg. P_3 of the *bvg* locus; P_{TOX} and P_{AD} which control pertussis toxin and adenylate cyclase toxin transcription), which may be due to large amounts of BvgA and S being required for activation of these promoters (Scarlato *et al.*, 1991). In summary, genes involved in bacterial adhesion (eg. FHA, fimbriae) are switched on before those involved in systemic effects of the disease.

1.3.5. Interaction of pertussis toxin with cell membranes and entry into the cell

1.3.5.1 Binding to cell membrane receptors

Pertussis toxin interacts with cells via its B-oligomer, which can be said to have three functions; to bind receptors containing glycoproteins and glycolipids on the host cell surface, bind whole bacteria to cells (Tuomanen and Weiss, 1985) and to deliver S1 to its target cell. Purified B-oligomer causes erythrocytes to agglutinate, blocks entry of whole toxin to adipocytes and is also responsible for the mitogenic action of the toxin on T-cells.

Binding of the B-oligomer to eukaryotic cell membrane receptors has been well documented (Tuomanen *et al.*, 1988; Clark and Armstrong, 1990; Relman *et al.*, 1990; Saukkonen *et al.*, 1992). The B-oligomer binds a 165kDa glycoprotein on Chinese Hamster Ovary (CHO) cells (Brennan *et al.*, 1988), a 70kDa and a 43kDa membrane protein on T-lymphocytes (Clark and Armstrong, 1990; Rogers *et al.*, 1990) and a 115kDa protein in goose erythrocyte extracts (Witvliet *et al.*, 1989).

Certain carbohydrate residues are important in toxin binding to eukaryotic cells, but little is known about the mechanism of binding and entry of the toxin to the cell. Sialyllactosamine (NeuAc→ Galβ4) needs to be present for binding; a CHO cell line lacking terminal sialyllactosamine residues on N-linked oligosaccharides did not react to toxin treatment. When a normal CHO cell line is treated with toxin, morphological changes occur and the cells cluster (Hewlett *et al.*, 1983; Gillenius et *al.*, 1985; Witvliet *et al.*, 1989).

B-oligomer also binds to serum glycoproteins, eg. fetuin and haptoglobin

(Irons and MacLennan, 1979; Sekura *et al.*, 1983; Capiau *et al.*, 1986) and mucosal proteins such as α–1 acid glycoprotein (Armstrong *et al.*, 1988). Fetuin contains both N- and O-linked oligosaccharides. Removal of the O-linked chains has no effect on fetuin's ability to bind toxin (Sekura *et al.*, 1985), so binding must involve N-linked oligosaccharides. Further investigation of this observed binding was carried out by Armstrong *et al.*, (1988), who suggested that sialic acid (neuraminic acid), galactose and N-acetyl glucosamine are important in toxin binding, as are these residues' orientation within the carbohydrate chains. Monosaccharides were sequentially removed from the non-reducing termini of the carbohydrate chains of fetuin and then replaced one sugar at a time. Removal of sialic (neuraminic acid) creating asialofetuin, reduced toxin binding to fetuin considerably. Sialic acid replacement in the α 2,6 orientation, found only in N-linked oligosaccharides restored fetuin's pertussis toxin binding; replacement in the α 2,3 orientation found in both N- and O-linked oligosaccharides had little effect. Removal of galactose, linked β1,4 in the N-linked chains was also found to decrease binding of the toxin, as was removal of GlcNAc, only found in N-linked oligosaccharides.

B-oligomer receptor binding is mainly mediated by the S2 and S3 subunits (Capiau, *et al.*, 1986; Van't Wout *et al.*, 1991; 1992). S2 and S3 are each linked to one S4 subunit, these dimers are linked by an S5 subunit. Antipeptide antibodies to S2 or S3 peptides inhibited toxin-mediated erythrocyte agglutination (Schmidt *et al.*, 1989; 1991).

Recent data suggest that regions of S2 bind galactose-containing glycoconjugates and recognise respiratory cilia and T-cells, but do not bind galactose-containing glycoconjugates on macrophages, nor sialylated glycoproteins; regions of S3 recognise and bind sialylated glycoconjugates, for example on macrophages and monocytes (Rogers *et al.*, 1990; Saukkonen *et al.*, 1992; Van't Wout *et al.*, 1992); but do not bind respiratory cilia.

A study of peptides of pertussis toxin subunits involved in carbohydrate recognition, and attachment to eukaryotic cells, is discussed in more detail in chapter 3.

1.3.5.2 Pertussis toxin entry into the cell

Pertussis toxin S1 subunit needs to pass through the cell membrane to reach its substrates located on the inner face of the plasma membrane, and catalyse transfer of ADP-ribose from NAD^+ to intracellular proteins (1.3.6, 1.3.7).

The whole toxin's B-oligomer binds its cellular receptor, may penetrate the plasma membrane and bind ATP which is thought to weaken interactions between S1

is thought to weaken interactions between S1 and the B-oligomer and releases S1 (Burns and Manclark, 1986; Hausman *et al.*, 1990). How much of the B-oligomer needs to penetrate the plasma membrane to facilitate S1 entry is unknown.

The mechanism for S1 entry is unknown, but is thought to involve one of two mechanisms, which are endocytosis, like diphtheria toxin and possibly cholera toxin (Saelinger, 1990; Janicot *et al.*, 1991) or direct entry through the plasma membrane (cholera toxin is believed to enter some cells by this mechanism; see section 1.5.1 and Ribi *et al.*, 1988)

Diphtheria toxin requires an acidic environment to enter eukaryotic cells, an environment which is found in endosomes. Pertussis toxin appears not require an acidic environment for entry to cells (Hausman and Burns, 1992) as pertussis toxin intoxication of CHO cells was not reduced by abolition of membrane pH gradients by NH_4Cl.

Parts of the toxin interact with detergents (Moss *et al.*, 1986) and phospholipids (Montecucco *et al.*, 1986; Hausman and Burns, 1992). The latter workers suggest that as the isolated S1 subunit binds phospholipid-containing vesicles in the presence of reducing agents, which are required for enzymic activity (Kaslow and Lesikar, 1987), then S1 entry to cells may involve whole toxin binding to a cell receptor via its B-oligomer, ATP binding to the B-oligomer facilitating the release of S1, and direct interaction of S1 with the membrane.

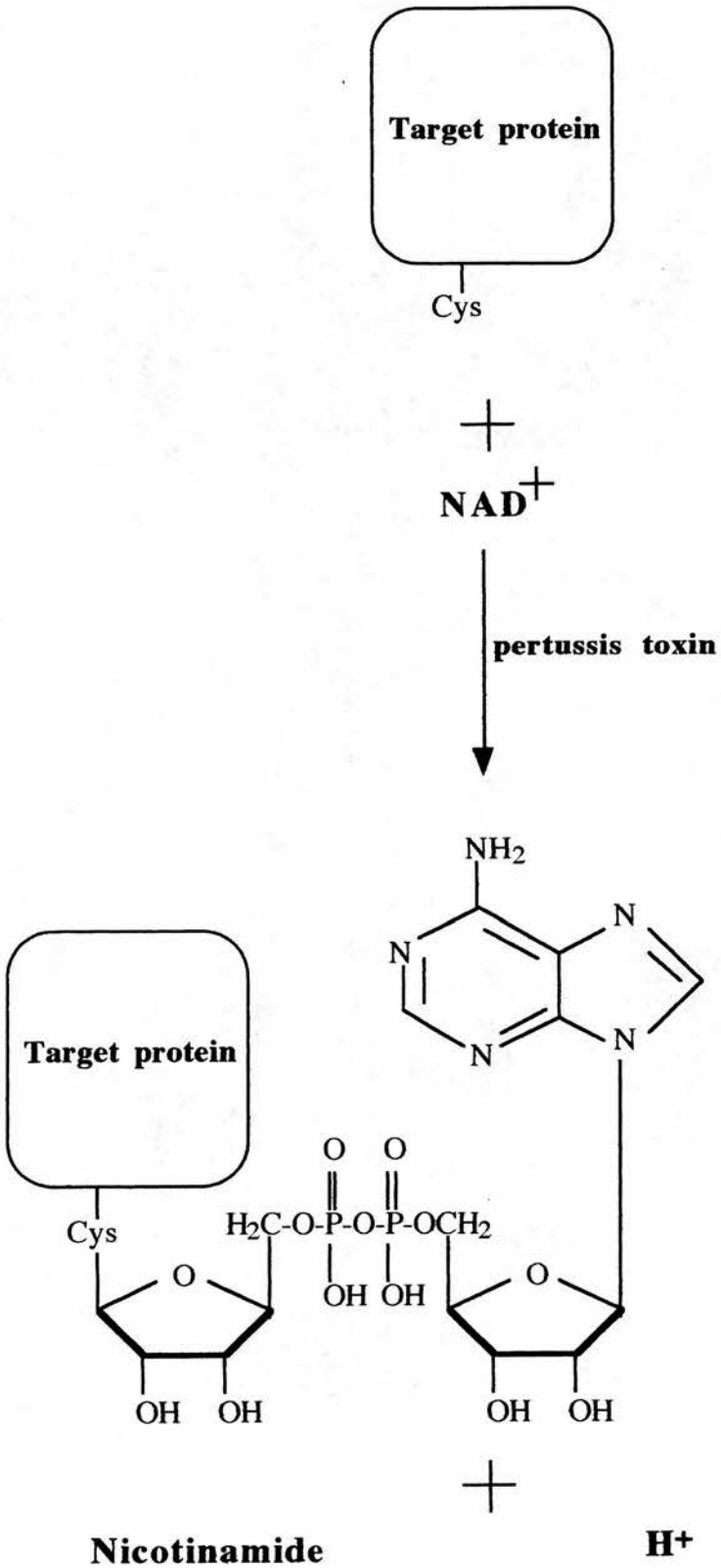
The degree of the B-oligomer's involvement in facilitating S1 entry and mechanism are still to be fully clarified. It is not known if the B-oligomer penetrates the plasma membrane, if so how far, and which subunits are involved in the penetration.

1.3.6 Effects of pertussis toxin on adenylate cyclase

Pertussis toxin prevents inhibition of eukaryotic membrane adenylate cyclase by ADP-ribosylation of a 41kDa membrane protein (Katada and Ui, 1982). The toxin does this by enzymically cleaving NAD and binding the ADP-ribose released to its eukaryotic protein substrate, which is Gi in the adenylate cyclase system, at a cysteine residue four residues from the C-terminus (Hurley *et al.*, 1984; West *et al.*, 1985).

The protein substrates ADP-ribosylated by pertussis toxin are a family of guanine nucleotide binding proteins (G proteins) involved in regulation of adenylate cyclase activity. The major G protein modified is Gi (inhibitory GTP binding protein, subunit M_r 41kDa) which normally mediates the effects of inhibitory hormones on adenylate cyclase (Smith and Limbird, 1982; Murayama and Ui, 1983; Gilman, 1987). The mechanism for ADP-ribosylation of Gi and other pertussis toxin sensitive G proteins is shown in fig. 1.5.

Fig. 1.5. ADP-ribosylation of pertussis toxin sensitive G proteins


Target protein
Cys
NAD+
pertussis toxin
NH2
N
N
N
N
Target protein
Cys
O
O
H2C-O-P-O-P-OCH2
OH
OH
O
O
OH
OH
OH
OH
Nicotinamide
H+

Two or more G proteins are involved in adenylate cyclase regulation; Gi and Gs (stimulatory GTP binding protein), Gs being the protein which normally mediates the effects of stimulatory hormones on adenylate cyclase and the substrate for cholera toxin. ADP-ribosylation of Gs leads directly to cAMP production; ADP-ribosylation of Gi removes inhibition of cAMP production induced by stimulatory hormone-receptor signals.

Gs and Gi are heterotrimeric proteins (M_r about 90kDa) which have highly conserved amino acid sequences, and are associated with the inner face of the plasma membrane. They contain one α (αi M_r 41kDa; Katada and Ui, 1982; Burns *et al.*, 1987; αs M_r 42kDa), one β (M_r about 35kDa) and one γ subunit (M_r about 5kDa). In their 'resting' state (Milligan, 1988), G proteins have GDP bound to their α subunit. When a ligand binds to its receptor, the ligand-receptor complex binds the G protein, enabling it to exchange GDP for GTP (Brandt and Ross, 1986) and dissociate to active α.GTP and a $\beta\gamma$ dimer (Hanski and Gilman, 1982; Bokoch *et al.*, 1983). The presence of $\beta\gamma$ increases dissociation of the G protein (Higashijima *et al.*, 1987). In the presence of Mg^{2+} ions, α.GTP can then bind its effector and either stimulate or inhibit its activity.

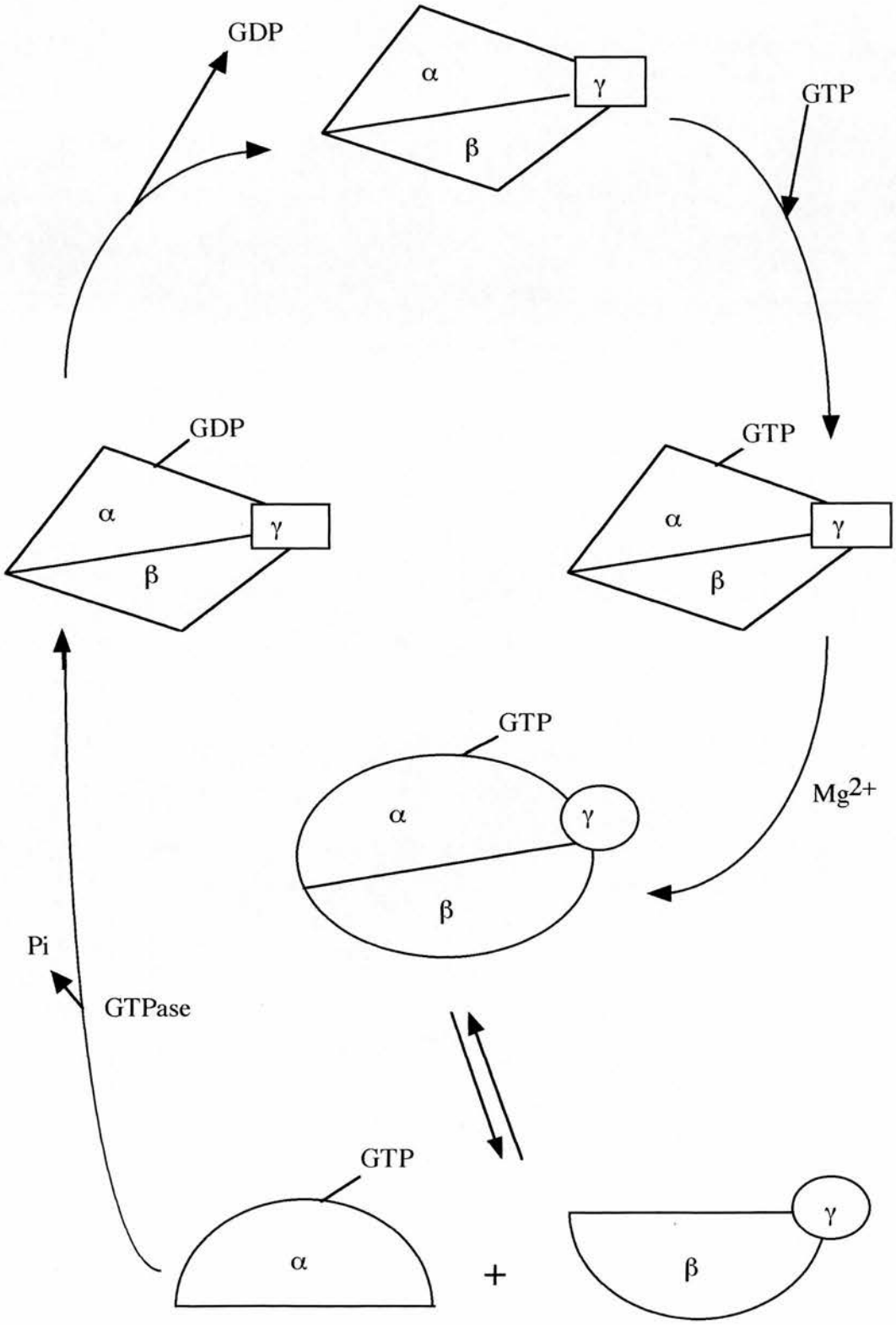
Normally, Gi α.GTP binds its effector, adenylate cyclase, and inhibits production of cAMP, which acts as a second messenger and activates protein kinase leading to phosphorylation of a range of proteins, altering their function. The α subunit acts as a GTPase, and the GTP bound to Giα.GTP is hydrolysed to GDP. The α subunit dissociates from its effector, reassociates with $\beta\gamma$ and returns to its resting state (fig. 1.6).

The C-termini of Gα subunits which are sensitive to pertussis are highly conserved (Price *et al.*, 1990) and are thought to interact with the receptor-ligand complex, promoting dissociation and activation of the G protein. The direct ADP-ribosylation of these G proteins by pertussis toxin occurs at a cysteine four residues from the C-terminal, blocking their interaction with the receptor-ligand complex and blocking binding of GTP to the α subunit. If the G protein is Gi, when it is ADP-ribosylated by pertussis toxin at its C-terminal, it can no longer interact with the inhibitory receptor-ligand complex, or bind GTP, and so can no longer inhibit adenylate cyclase, leading to unregulated production of cAMP (Gs has a tyrosine at this position and so is not a substrate for pertussis toxin; Fong *et al.*, 1988). One form of α i.GTP also activates potassium channels in heart and is involved in regulation of

Fig. 1.6. Activation/deactivation cycle of a G protein.

GTP binding results in dissociation of the G protein α subunit from the $\beta\gamma$ dimer (activation). GTP hydrolysis results in reassociation of the G protein subunits (deactivation).

Activation is dependent on GTP and magnesium ions.


GDP
α
γ
β
GTP
GDP
α
γ
β
GTP
α
γ
β
GTP
α
γ
β
Mg2+
Pi
GTPase
GTP
α
+
γ
β

Gsα.GTP binds adenylate cyclase and stimulates production of cAMP via stimulatory hormones. Gsα is ADP-ribosylated at an arginine residue and locked in its active state, with GTP bound (i.e.. the GTPase activity is inhibited), leading to continuous stimulation of adenylate cyclase and unregulated production of cAMP. The structure-function relationships of the various types of β and γ subunits are still to be fully defined. The two subunits exist as a dimer, and may anchor the G protein to the plasma membrane (Toro *et al.*, 1987; Birnbaumer, 1987, 1990; Boquet and Gill, 1991). β subunits are highly homologous even in G proteins with different functions (Tamir *et al.*, 1991), and recent data suggest that β subunits may regulate steps in the GTP hydrolytic cycle, as βγ has been found to interact directly with some of the specific effector systems linked to the GTP hydrolysis cycle (Okabe *et al.*, 1990; Stryer, 1991).

γ subunits are very divergent among species (Tamir *et al.*, 1991), possibly helping the βγ dimer to recognise and deactivate the α subunit from which they dissociated on activation of the G protein.

1.3.7 Other pertussis toxin-sensitive proteins

Several other G proteins are ADP-ribosylated at a cysteine four residues from their C-terminals by pertussis toxin, and these are also involved in signal transduction.

Gt (transducin) is a retinal rod protein which regulates cyclic GMP-phosphodiesterase (cGMP-PDE; Tsuda *et al.*, 1986). The α subunit (now called αt1) has an M_r of 39 000, β has an M_r of 36 000 and γ has an M_r of about 7 000. The initial 'ligand-receptor' interaction is the activation of rhodopsin by light (Gilman, 1987; Stryer, 1991). Normally, when light activates rhodopsin, it interacts with GDP-bound Gαt which exchanges GDP for GTP and dissociates from βγ as described in section 1.3.6. αt.GTP then stimulates cGMP-PDE, which leads to reduction of cGMP levels in the cytoplasm, closes cGMP-regulated ion channels and causes hyperpolarisation of rod outer segment plasma membranes (Stryer, 1991).

ADP-ribosylation of Gt caused by pertussis toxin in the dark locks it in its inactive form with GDP bound, so visual signals are blocked. Cortina *et al.* (1991) have shown that residues between 180 and 219 of the S1 subunit of pertussis toxin are needed for efficient ADP-ribosylation of Gt.

Another form of αt subunit (called αt 2) has been discovered in retinal cones and it is similar to that in rods.

An interesting development is the discovery of a protein in retinal rods, which has an M_r of 12 000 and appears to stimulate GTP binding to rod Gt α. The protein, called δ, occurs in 3-10% of the amount of γ. βδ stimulates GTP binding to α more than βγ, so may be involved in GTP binding to Gt and activation of cGMP-PDE (Umbarger *et al.*, 1992). This work needs clarification, as δ could have originated from retinal cones contaminating the rod preparation.

Another pertussis-sensitive G protein is Go; two forms of Go have been discovered called αo1 and αo2, which are both sensitive to pertussis toxin (Spicher *et al.*, 1992). They are widely distributed throughout the central nervous system (Hsu *et al.*, 1990) and as many as four Go-like proteins may exist, which have been resolved by ion exchange chromatography (Ianobe *et al.*, 1990). The two forms have molecular masses of 39 000 and may be involved in potassium channel regulation, inactivate calcium channels and activate phospholipase C (Schultz *et al.*, 1990; Brown *et al.*, 1991). The biochemical function of these G proteins remains to be fully determined. Several G proteins involved in activation of membrane phospholipases have still to be fully studied. They are called Gp; the class being subdivided into those which activate phospholipase C or A2 (PLC or PLA_2). Responses to vasopressin and angiotensin II are due to PLC stimulation by a pertussis-sensitive G protein. Receptor-mediated arachidonic acid release (eg. from macrophages) is due to PLA_2 stimulation.

ADP-ribosylation of G proteins by pertussis toxin can have a number of effects, for example, α-adrenergic receptors involved in regulation of insulin secretion can be uncoupled from adenylate cyclase by ADP-ribosylation of Gi, resulting in over-secretion of insulin leading to hypoglycaemia; this effect was noted by Katada and Ui (1979) as the islet-activating activity of pertussis toxin. Inhibition of histamine secretion could also be due to ADP-ribosylation of Gi, leading to over-secretion of insulin, increased vascular permeability, resulting in histamine sensitisation. Arciniega *et al.*, (1991) recombined a mutant form of S1 having no enzymic activity with toxin B-oligomer, resulting in a mutant holotoxin which had decreased histamine sensitising activity compared to native whole toxin. This suggests the activity belongs to the S1 subunit.

The effect of pertussis toxin on Chinese Hamster Ovary (CHO) cells may be due to ADP-ribosylation (Burns *et al.*, 1987; Nencioni *et al.*, 1991); CHO cells cluster and their shape alters after toxin treatment (see section 3.3.8.2.6.6). Nencioni *et al.*,

(1991) suggested that the effect is caused by the S1 subunit as isolated B-oligomer did not cause morphological changes in CHO cells. Askelof *et al.* (1990) suggest that Chinese Hamster ovary (CHO) cell clustering is due to a whole toxin-binding event involving part of the S1 subunit, as rabbit antiserum to an S1 subunit synthetic peptide conjugated to diphtheria toxin cross-reactive mutant protein (CRM) inhibited ADP-ribosylating activity of the toxin in CHO cells, but had no effect on CHO cell clustering. Antiserum to a different peptide conjugate whose sequence was derived from the S1 subunit had the converse effect.

Whether CHO cell clustering is a direct effect of ADP-ribosylation and exactly how toxin binding to the CHO cells is involved is yet to be fully defined.

It must be remembered that not all effects of pertussis toxin are due to ADP-ribosylation; toxin B-oligomer binding alone produces some of them (see section 1.3.5). A summary diagram of some of the pathways affected by pertussis toxin are shown in fig. 1.7.a and b.

1.4 Towards partially/wholly synthetic pertussis vaccines

1.4.1 Immune response to a foreign molecule

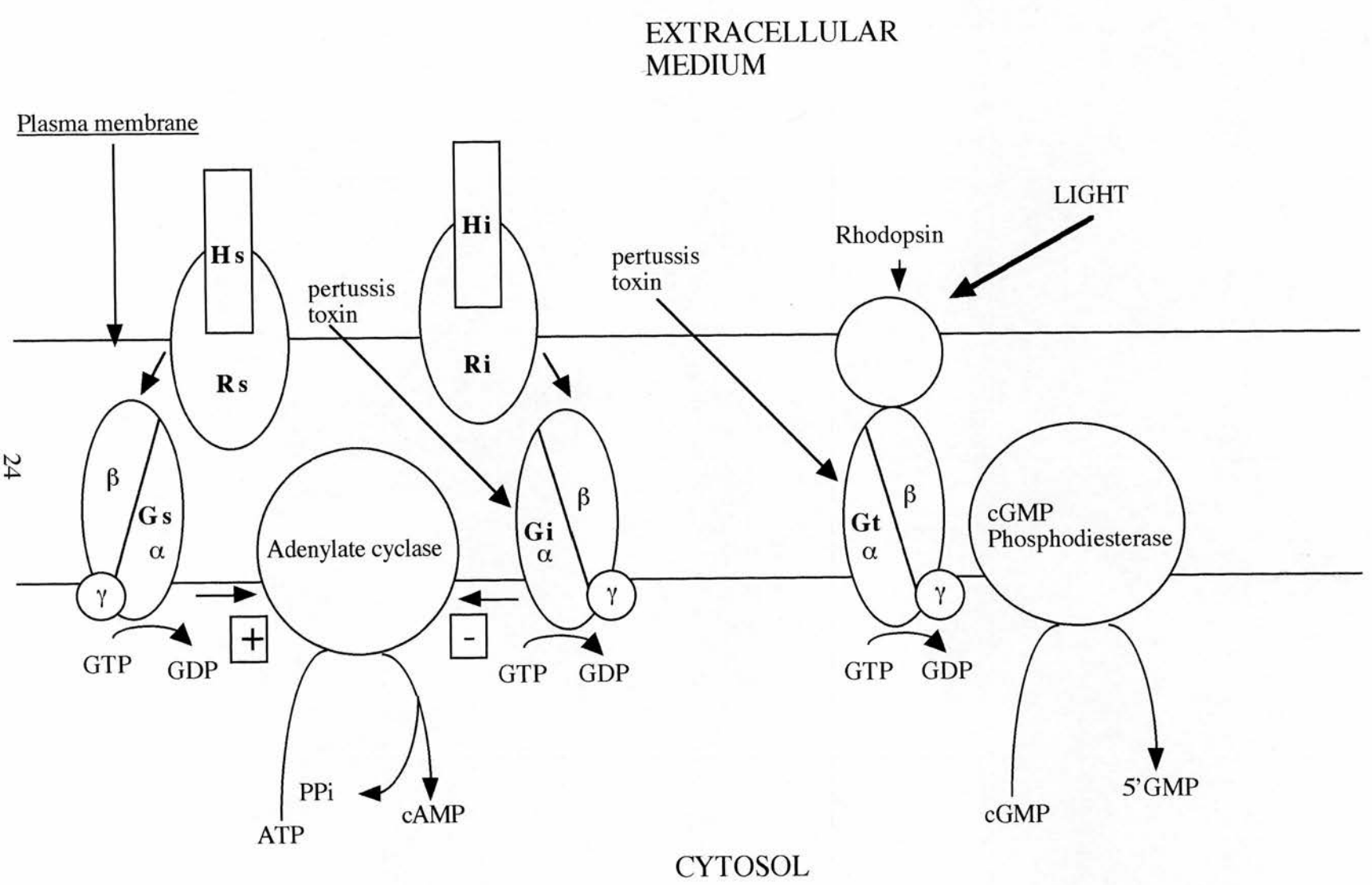
The immune response consists of several nonspecific components, including mononuclear phagocytes, polymorphonuclear leukocytes and the complement system. The specific recognition molecules of the immune system are the B- and T-lymphocytes. Usually both humoral (B lymphocytes, antibody) and T cell systems interact in response to an antigen.

Antibodies are secreted from B cells and bind antigen molecules or cells, via epitopes on the antigen. This binding is recognised by a responsive B cell and internalisation of the antigen-antibody complex is triggered. The native antigen is cleaved in the cytoplasm into peptides ten to twenty amino acids in length, that are presented on the B cell surface by type II major histocompatability complexes (MHC). T helper cells carrying a glycoprotein CD4 on their surface recognise the antigen-MHC II complex and B cell functions are altered, e.g. proliferation and isotype switching occur.

Another class of MHC molecule called type I are found on the plasma membrane of all cells with nuclei. Peptides of eight to ten residues are presented by MHC I and recognised by a subset of cytotoxic T cells important in lysis of virally infected cells, for example. These cytotoxic T cells recognise the MHC I-antigen complex via CD8 glycoprotein on their surface and lyse the infected cell.

There are several types of T cell, classified according to their various cell surface markers and functions. Primitive T cells develop T cell specific surface

Fig. 1.7a, b Other proteins sensitive to pertussis toxin (see section 1.3.7 for details)


EXTRACELLULAR
MEDIUM
Plasma membrane
Hs
Rs
Hi
Ri
pertussis
toxin
pertussis
toxin
LIGHT
Rhodopsin
β
Gs
α
γ
Adenylate cyclase
+
-
Gi
α
β
γ
Gt
α
β
γ
cGMP
Phosphodiesterase
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GDP
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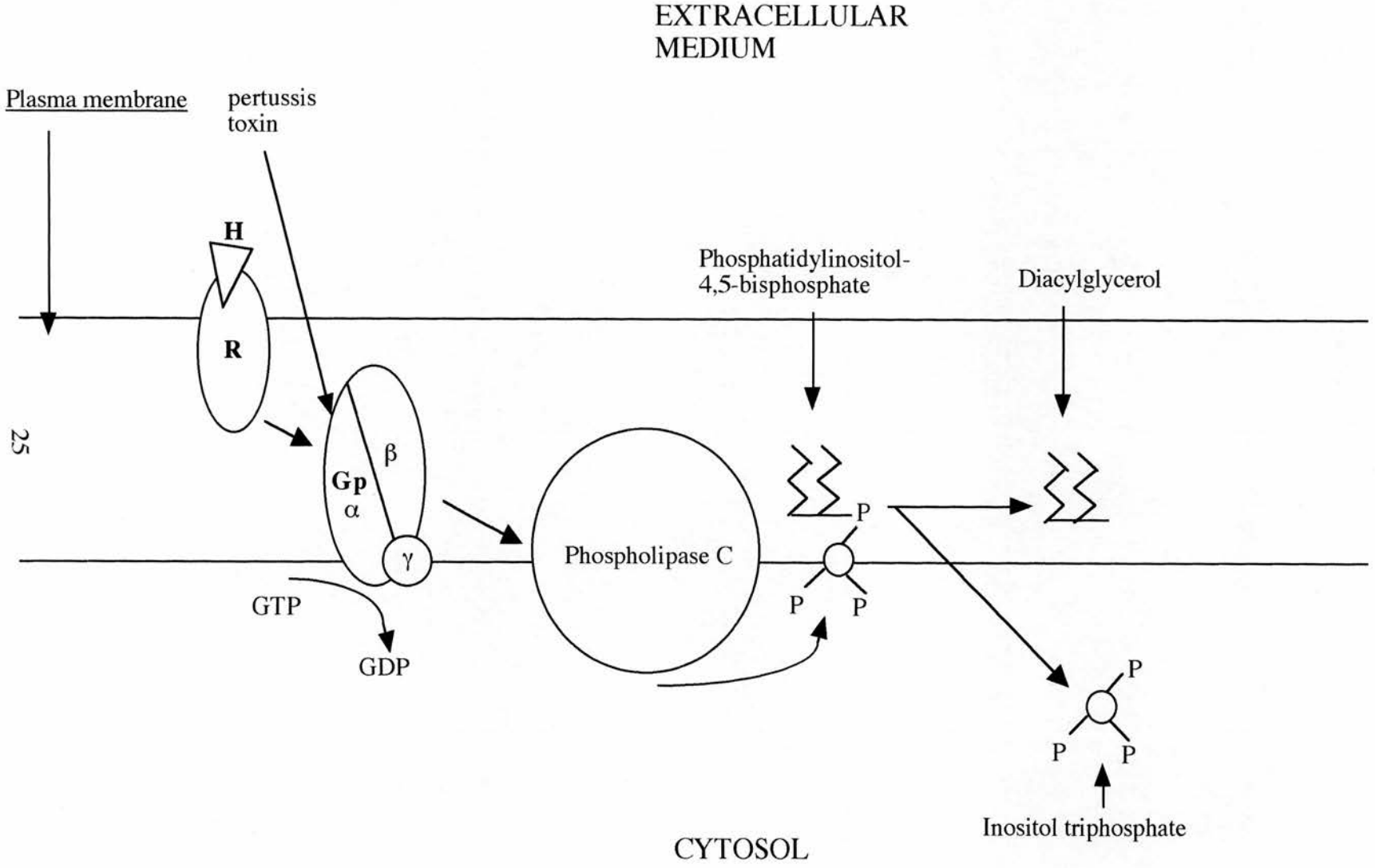
GDP
GTP
GDP
PPi
ATP
cAMP
cGMP
5'GMP
CYTOSOL


EXTRACELLULAR
MEDIUM
Plasma membrane
pertussis
toxin
H
R
Phosphatidylinositol-
4,5-bisphosphate
Diacylglycerol
Gp
α
β
γ
GTP
GDP
Phospholipase C
P
P
P
P
P
P
Inositol triphosphate
CYTOSOL

antigens in the thymus and when they mature they leave the thymus expressing CD4 or CD8 glycoproteins, for example.
B cells are specifically activated by antigen binding to an Ig isotype on the cell surface and nonspecific activation can be achieved by B cell mitogens.

Protein antigens can activate antibody response in two ways:

T-dependent antigen activation

When an MHC II-antigen complex is presented by a B cell to a T helper cell, it is activated and releases lymphokines. The antigen and lymphokines switch the B cell on to antibody secretion. The primary antibody response to antigen involves IgM secretion and this is switched by T helper cells to IgG, IgA and sometimes IgE in the secondary response. This type of activation can lead to a large pool of T and B memory cells.

T-independent antigen activation

Some antigens (e.g. polysaccharides) trigger B cell functions directly, needing no T cell help, but they induce little memory and produce mainly IgM responses.

There are two subclasses of T-independent antigen: Type I (e.g. LPS) that result in a large direct response but with little memory and Type II, a subclass including some linear antigens containing repeat sequences, that are not easily handled by the body and induce largely a direct IgM response.

In summary of the T cell dependent immune response, antigen binds Ig expressed on the surface of B cells and is internalised and degraded (priming). The peptides created are presented at the cell surface complexed with MHC II molecules and trigger T helper cells to secrete lymphokines, that switch B cells to antibody making B memory cells. This antigen priming makes for a faster secondary immune response to antigen.

1.4.2 Recognition of antigenic sites

Antigenicity is the ability of a molecule to be recognised by B cell (antibodies) and T cell receptor molecules. Structures recognised and bound by B or T cells are known as antigenic determinants, or epitopes. B cell molecules (antibodies) are thought to recognise mainly conformational epitopes formed by discontinuous sequences on the native protein surface (Blundell *et al.*,1987). Several 'linear' (continuous) sequences that would be surface-exposed on the native antigen are believed to form domains of these discontinuous epitopes and be recognised by antibody (Kazemi and Finkelstein, 1991). The minimum number of amino acids in

required to induce a B cell response has been put at six (Geysen *et al.*, 1988) when presented correctly to the B cells.

T cells recognise peptide fragments about 10 amino acids long (Horsfall *et al.*, 1991) presented by a major histocompatability complex (MHC). These T cell epitopes may originate from regions not exposed on the surface of the native antigen, so they have also been termed cryptotopes (Laver *et al.*, 1990).

1.4.3 Identification of antigenic epitopes

Several methods are available to aid in identification of protein epitopes. X-ray crystallography has identified discontinuous epitopes of lysozyme and influenza virus neuraminidase, for instance (Davies *et al.*, 1990; Tulip *et al.*,1990), each consisting of 15 -22 residues in contact with the antibody over an area of 65-90nm on the protein. This is a method that relies on crystallisation of the protein, and as relatively few crystal structures are known at the present time, other methods have to be employed.

Site-directed mutagenesis can be utilised to detoxify a protein without destroying antigenic sites, as used when a single mutation in the S1 subunit of pertussis toxin (R9 to K) was introduced to create a detoxified toxin in *Bordetella* (Burnette *et al.*, 1988; Pizza *et al.*,1989). Although whole recombinant toxin was assembled, it was at low levels and with the possibility of contamination with other *Bordetella* toxins (Weiss and Hewlett, 1986). Even so the recombinant toxoid was included in human vaccine trials in 1990 (Podda *et al.*, 1990).

Epitope libraries using phage lambda, with each virion expressing one peptide sequence on its surface, could be screened for antibody binding. A random library of peptides is created in this way and epitopes, or 'mimotopes' (sequences mimicking native protein epitopes) can be identified (Scott and Smith, 1990; Devlin et *al.*, 1990).

Computer prediction of antigenic regions, from primary amino acid sequence has been attempted by predicting β turn potential and surface exposure (Krchnak *et al.*, 1987). Molecular modelling of antibody-antigen interactions have been tried to identify antigenic sites (Blundell *et al.*, 1987).

Both free and immobilised peptides are used to identify antigenic sites (and receptor binding sites, for instance). Both were used in the study of antigenicity of pertussis toxin subunits, which is discussed in more detail in chapter 3.

1.4.4 Peptide synthesis

Development of automated long chain tBoc peptide synthesis by Merrifield (1963) opened the way for the study of structure and function of discrete regions of proteins. The tBoc (tertiary butyloxycarbonyl) group can be used to protect the amino

function of amino acids used in solid phase protein synthesis. Peptide synthesis has been refined and changed since then, for several reasons, some of which are listed below. Solid phase supports were developed that were more polar than the ones used originally, to increase peptide solvation. The tBoc group used in the Merrifield method was removed from the N-terminal amino acid by acidolysis using TFA before each amino acid coupling step. Acidolysis derivatises some peptides and side reactions side reactions can occur, for example t-butyl cations created in N-terminal deprotection can react with nucleophilic amino acid side chains (e.g. of histidine, tryptophan and tyrosine). As a result a protecting group for the amino function was sought that could be removed under mild alkaline conditions. The search resulted in the development of the fmoc group (9-fluorenylmethoxycarbonyl; Chang and Meienhofer, 1978; Atherton *et al.*, 1978) which is removed by treatment with a mild base. Other changes of the side chain protecting groups used, and the methods used for their removal, as well as improvement of conditions for peptide solvation have also occurred (for a review of fmoc solid phase peptide synthesis see Fields and Noble, 1990).

Solid phase peptide synthesis on derivatised 'pins' was developed by Geysen *et al.*, (1984; 1987). Peptides, usually six to ten amino acids long are covalently linked one at a time, to derivatised polypropylene pins arranged in blocks of 96 and screened for antibody binding using a modified ELISA system, with the aim of identifying antigenic sites (in this case linear peptides which may or may not be part of conformational epitopes on a protein). The technique has been used to map B cell epitopes of many proteins, for example from viruses, e.g. foot and mouth disease (Geysen *et al.*, 1984; Meloen and Barteling, 1986) and from bacteria, e.g. *E.coli* colonization factor antigen I (Cassels *et al.*, 1992). Cholera toxin B cell epitopes have been studied using this technique (Kazemi and Finkelstein, 1991) as have B cell epitopes of eucaryote proteins, for example bovine growth hormone (Beattie et *al.*, 1992) and dihydrofolate reductase (Tan *et al.*, 1990).

1.4.5 Peptide antigenicity

Synthetic peptides must be able to induce production of antipeptide antibodies that can bind specifically to the native antigen and some synthetic peptides can cross-react with antibodies to the native protein, suggesting that the peptides exist in the same (or a similar) conformation as in the native protein. Therefore, they may induce antibodies that neutralise the biological activity of the native protein when included in a vaccine.

A number of factors determine which sequences are antigenic, assuming that antibodies bind residues on the protein surface:

Regions of hydrophilicity may be antigenic, and charged residues are likely to be at the protein surface (Hopp and Woods, 1981).
Local mobility of proteins and mobility of peptides seems important in determining antigenicity (Tainer *et al* ., 1984; Westhof *et al.*, 1984). The tendency to form ß turns is also used (Hopp and Woods, 1981).
A linear peptide which binds antibody is termed a continuous epitope (Atassi and Smith, 1978) and most continuous epitopes occur in surface regions of a protein (Barlow *et al.*, 1986), have a convex shape, a high local mobility (Getzoff *et al.*, 1987) and are either chain termini or ß turns (i.e. on the protein surface). A continuous epitope may be part of a more complex, discontinuous epitope (Getzoff *et al.*, 1987; Rini *et al.*, 1992).

A problem of short peptides is that they may not be recognised by antibody as they may not have a recognisable structure and they may not be immunogenic. Peptides are assumed to exist in a number of random conformations and a small number of these correspond to the conformation in the native protein (Furie *et al.*, 1975; Dyson *et al.*, 1988a, b). About 90% of continuous epitopes are six amino acids or less; this is thought to be the minimum number to induce a B cell response (Geysen *et al* , 1988).

1.4.6 Requirements for a pertussis vaccine containing synthetic peptides

An ideal vaccine should be safe, immunogenic and produce a large pool of B and T memory cells to protect against a disease for an indefinite period. This should be backed by vaccine stability and the ability to produce it economically on a large scale. This has lead to attempts to develop more characterised vaccines containing immunogenic peptides with multiple T and B cell epitopes, to ensure that most host immune systems recognise some of the epitopes and produce a protective response. An antigen may contain one immunodominant B-epitope, which simplifies the choice of peptides for inclusion in a vaccine (e.g. the hypervariable loop of the gp120 protein of HIV). If there is no immunodominance, e.g. in the case of influenza virus haemagglutinin (Wang *et al.*, 1986) the range of peptides which may be suitable for inclusion in a vaccine expands.

1.4.7 Presentation of peptide

Peptides shorter than 20 amino acids may not be immunogenic, so they are linked to larger carriers, such as bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH), which are recognised by B cells. KLH is highly immunogenic,

so antigenic competition can occur, inducing a response to the carrier rather than the peptide. A carrier can alter the peptide's conformation and recognition may differ depending on whether the peptide is linked via its N- or C-terminal, so more than one carrier and method of conjugation is usually considered (Dryrberg *et al.*, 1986; Chong *et al.*, 1991; 1992).

If several administrations of a vaccine containing carrier-conjugated peptides are required, sensitization to the carrier can be induced. This may be reduced by use of an adjuvant, other than those used in rabbits and mice (e.g. Complete Freunds adjuvant) which causes inflammation and granuloma and so can not be used in humans. Another carrier system being investigated is the linking of a peptide to a detoxified toxin known to be immunogenic, such as tetanus toxoid, cholera and diphtheria toxoids and meningococcal outer membrane proteins (Ballou *et al.*, 1987; Ballou, 1991). A repeating unit of *Plasmodium falciparum* circumsporozoite protein has been linked to *Pseudomonas aeruginosa* exotoxin A and tested for immunogenicity in humans (Fries *et al.*, 1992).

1.4.8 Antigenicity of pertussis toxin

Pertussis toxin is a major protective antigen against pertussis, as shown by clinical trials of several vaccines (Ad hoc group for the study of pertussis vaccines, 1988; Miller *et al.*, 1989; Olin *et al.*, 1989) but is also a major virulence factor in pertussis, as discussed in section 1.3, complicating vaccine development.

Some vaccines currently in use employ chemically detoxified pertussis toxin. This detoxification method may alter antigenic regions of the protein and so affect immunogenicity of the toxoid. Also, rigorous purification procedures may not remove contamination by other *Bordetella pertussis* toxins (Weiss and Hewlett, 1986). Haptoglobin- or fetuin-affinity chromatography are most widely used for pertussis toxin purification and eluants used with these methods include high concentrations of magnesium chloride, which may alter the toxin structure (Chong and Klein, 1989), or thiocyanate, which is expensive to dispose of and highly toxic. These elution conditions also have the potential of leaching of the fetuin or haptoglobin into toxin preparations. Haptoglobin is purified from human plasma and transmission of blood-carried disease is possible, as well as sensitization to human and bovine (the source of fetuin) blood constituents can be induced.

So antigenic epitopes of pertussis toxin are being located (mapped) in the hope that some are immunogenic and can be used in the induction of protective immune responses to either detoxified whole toxin, subunits, or synthetic peptides in acellular vaccines. Multiple B and T cell epitopes of pertussis toxin subunits are being discovered, and B cell epitopes were the subject of studies by the author. B cell

antigenicity of particular subunits is discussed in chapter 3.

1.5 Other Toxins

There are a number of toxins with similarities to pertussis toxin, including their A-B type structures, the way they bind cells and their mechanism of action. Cholera toxin, diphtheria toxin, *E. coli* heat labile toxin and *Pseudomonas aeruginosa* exotoxin A are among the other examples of ADP-riboyslating toxins. These toxins, as well as tetanus and botulinum neuro- and cytotoxins will be briefly discussed in this section.

1.5.1 Cholera toxin and *Escherichia coli* heat labile toxin

Cholera is caused by the non-invasive organism *Vibrio cholerae* which infects the gastrointestinal tract. The resulting severe loss of fluid, diarrhoea and dehydration can kill, although fluid replacement is an effective treatment.

Some strains of *E. coli* secrete a toxin called heat labile toxin (HLT), which has about 80% amino acid identity with cholera toxin. HLT has been crystallised (Sixma *et al.*, 1991; 1992) and its crystal structure deduced from X ray diffraction is assumed to be almost identical to the crystal structure of cholera toxin. The crystal structure of cholera toxin is being investigated and structural similarities to HLT have been identified (Westbrook, personal communication). Both are A-B type toxins, having one A subunit of M_r about 27 000, and five B subunits of M_r about 11 000, coded for by the genes *ctxA and ctxB* (Mekalanos *et al.*, 1983) in *Vibrio cholerae* .

Both A and B are synthesised with signal peptides which are cleaved on secretion into the periplasm where the mature toxins are assembled. The A subunit of cholera toxin (CTX A) is modified further by proteolysis to give an N-terminal A_1 fragment (Mr about 22 000) and C-terminal A_2 fragment (Mr about 5 000) (Mekalanos *et al.*, 1979; van Heyningen, 1982) linked by a disulphide bond; cleavage of this bond is thought to be necessary for activation of the ADP-ribosyltransferase (Gill, 1976).

Like cholera toxin A subunit, HLT A is proteolytically nicked after binding its cell receptor, to give A_1 and A_2 fragments. A_1 is then internalised possibly by endocystosis (Janicot *et al.*, 1991).

The B subunits of both HLT and cholera toxin A bind GM_1 on the cell surface (van Heyningen, 1977; Moss and Vaughan, 1988) and crystallisation of HLT with lactose (Sixma *et al.*, 1992) has revealed the toxin binding site for GM_1 and shown that A_1 apparently faces away from the cell binding site, which is assumed to be the case in cholera toxin. The C-terminal of A_2 may interact with GM_1 ; A_1 appears flexible, a feature which may be important in membrane translocation.

The mechanism of entry of the A subunit to the cell is not understood. It could enter some cells by directly penetrating the membrane (van Heyningen, 1982; Ribi et *al.*, 1988), although endocytosis may be involved in entry to other cells (Janicot *et al.*, 1991).

Cholera toxin and HLT catalyse ADP-ribosylation of an arginine residue of Gsα (Moss and Vaughan, 1977), locking it in its GTP-bound, active form, leading to unregulated production of cAMP and disruption of the normal ion flow across the intestinal epithelium (fig. 1.7). Both cholera toxin A (CTX A) subunit and HLT have regions of almost identical sequence with pertussis toxin S1 subunit (see section 1.3.3; Locht and Keith, 1986; Nicosia *et al.*, 1986). The N-terminal of S1 is known to be part of a major protective epitope (Bartoloni *et al.*, 1988) and mutation of the arginine mentioned has been used to produce detoxified pertussis toxin (also mutated at a glutamate at position 129) in *Bordetella* for use in an acellular vaccine. The levels of toxin synthesised were not very high (Pizza *et al.*,1989), but the toxoid was included in human trials (Podda *et al.*, 1992).

1.5.2 Diphtheria toxin

Diphtheria toxin (DT) is an A-B type toxin secreted by pathogenic strains of *Corynebacterium diphtheriae*. It is synthesised as one polypeptide chain which is proteolytically cleaved, either before or after cell binding, to give the active form consisting of an ADP-ribosylating A fragment (M_r about 21kDa) and a B fragment (M_r about 37kDa), and its crystal structure was analysed by X ray diffraction and published by Choe *et al.*, 1992. The fragments are linked by a disulphide bond, which is probably reduced inside the target cell to activate the A fragment (Greenfield *et al.*, 1983). The B subunit binds the cell surface, probably to glycoprotein targets, and enters the cell by endocytosis (Saelinger, 1990).

When inside an endosome, the acidic pH causes a conformational change in the A fragment which is thought to enable it to cross the endosome membrane and find its target in the cytoplasm (Dumont and Richards, 1988; Olnes and Sandvig, 1985). DTA catalyses ADP-ribosylation of elongation factor 2 (EF-2). This blocks the elongation of polypeptides on ribosomes. This inhibition of protein synthesis causes cell death (Honjo *et al.* , 1968). The modified residue is diphthamide, which is a derivative of histidine and is unique to EF-2 (Robinson *et al.* , 1974; Van Ness *et al.* , 1980).

1.5.3 Pseudomonas aeruginosa exotoxin A

This toxin (ETA) also catalyses ADP-ribosylation of the diphthamide residue of EF-2, inhibits protein synthesis and causes cell death. ETA is synthesised as a single polypeptide proenzyme which appears to undergo covalent changes to activate it *in vivo* (Vasil *et al.*, 1977; Leppla *et al.*, 1978). The proenzyme has an N-terminal signal sequence which is cleaved on secretion (the calculated M_r of the proenzyme after signal sequence removed is 66 583). ETA and DT have no nucleotide sequence similarity, but their active sites contain corresponding amino acids (Carroll and Collier, 1988). ETA has been crystallised and its structure determined by X ray diffraction (Allured *et al.*, 1986) and is proposed to contain three domains. Domain I is the N-terminal half of the molecule and binds the cell receptor (binding domain). Domain II splits domain I in two, and seems to be involved in translocation of the toxin across the target cell membrane (Chaudhary *et al.*, 1988) but the precise mechanism of entry is unknown. Domain III is contained in the C-terminal part of ETA (Gray *et al.*, 1984; Hwang *et al.*, 1987) and is the enzymatic part of the toxin, so this is another example of an A-B type toxin.

1.5.4 Clostridial cytotoxins

Various clostridial toxins have ADP-ribosylating activity, their substrate being monomeric actin. They include Botulinum C2 toxin, *Clostridium perfringens* iota toxin, *Clostridium sporiforme* toxin and a toxin produced by *Clostridium difficile*. These toxins block polymerisation of monomeric actin (Aktories *et al.*, 1986).

Some strains of *Clostridium botulinum* types C and D produce a toxin C3, which modifies small GTP binding proteins (molecular mass 21-24kDa) (Braun *et al.*, 1989).

Botulinum C2 toxin is well studied, and has two components, one responsible for binding (I) and one which has enzymic activity (II). C2 is not a usual A-B toxin, as components I and II act as separate proteins in intoxication (Ohishi *et al.*, 1980; Simpson, 1982).

1.5.5 Clostridium botulinum neurotoxins

At least nine toxins are produced by *Clostridium botulinum*, including the cytotoxins C2 and C3 discussed above, and the neurotoxins A,B,C1,D,E,F and G. The neurotoxins are absorbed from gastrointestinal tract, reach susceptible neurons, bind presynaptic terminals and block exocytosis of acetylcholine resulting in

respiratory paralysis, inability to swallow and speech difficulties.

The neurotoxins have a similar overall structure with a light and a heavy chain linked by a disulphide bond.

1.5.6 Tetanus toxin

Clostridium tetani secretes a neurotoxin called tetanus toxin, which causes spastic paralysis due to block of neurotransmitters (especially gamma-amino butyric acid, GABA) from inhibitory neurons (Simpson, 1989), which leads to uninhibited neuronal firing.

The toxin is synthesised as a single chain precursor, cleaved by proteases to form a heavy chain, H(M_r 100 000) and a light chain, L(M_r 50 000). The H chain is involved in cell binding and the L chain is responsible for inactivation of a component in neuroexocytosis (Niemann, 1991). The two chains are linked by a disulphide bond.

Recently Schiavo *et al.*, (1992a) demonstrated that tetanus toxin L contains a zinc binding motif characteristic of metalloproteinases, and has a proteolytic effect on an integral membrane protein called VAMP(vesicle-associated membrane protein) found in synaptic vesicles (Schiavo *et al.*, 1992b).

1.6 Aims of the Project

The aims of the project were to investigate the spatial arrangement of pertussis toxin subunits within the whole toxin, using chemical cross-linking reagents. Reversible and non-reversible cross-linking, one- and two-dimensional SDS-PAGE, Western blotting techniques were to be used. A range of cross-linked species would generated and the cross-linked species separated from non-cross-linked material using SDS PAGE. Inter-subunit cross-links could then be broken and the component subunits of a particular species separated, again using SDS PAGE and the component subunits identified by their size. Cross-linked species with bridges that were difficult to break, could be separated on one-dimensional SDS PAGE and their composition wholly or partly identified using Western blotting using subunit specific antibodies.

Antigenicity (antibody binding) and glycoprotein-binding peptides derived from the whole amino acid sequence of pertussis toxin subunits S1, S3 and S4 were to be investigated, using the epitope mapping technique of Geysen *et al.* (1984) and free peptides. Glycoprotein-binding was used as an identifier of peptides that could be involved in receptor-recognition in the native toxin.

The Geysen *et al.*, (1984) technique involves manual synthesis of peptides which are covalently attached via their C-terminus, to derivatised polypropylene pins.

Anti-(pertussis toxin) antibody and glycoprotein-binding to the pin peptides was assayed using an ELISA technique. Only peptides whose entire sequence or part of that sequence was exposed on the surface of the whole toxin should be antigenic or bind glycoprotein, unless a peptide mimicked a binding site on a protein subunit for an antibody or glycoprotein. Antigenic and/or glycoprotein-binding peptides were to be studied for their ability to induce antibodies that recognised 'native' pertussis toxin and neutralised some of the toxin's biological activities.

Finally, the binding of NAD^+ to whole pertussis toxin S1 subunit is the initial event in both the ADP-ribosyltransferase and NAD^+-glycohydrolase activities of pertussis toxin, so that binding event was to be studied using intrinsic tryptophan fluorescence quenching. Whether NAD^+-binding to the S1 subunit was altered by S1 being associated with the B-oligomer of the toxin was to be assessed, using S1 subunit isolated from a preparation of whole pertussis toxin. Binding of NAD^+ to recombinant S1 subunit analogues was also to be looked at, to try to identify whether a specific region or residue of the S1 subunit was involved in binding of NAD^+ and hence whether regions or residues of S1 are likely to be surface-accessible.

Chapter 2

Materials and Methods

2.1 Materials

Dithiobis-(succinimidylpropionate) (DSP), Disuccinimidyl tartarate (DST) and succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB) were purchased from Pierce and Warriner Ltd., Chester, Cheshire, UK.

Solid phase peptide synthesis kits including polypropylene pins and polypropylene microtitre plates, activated amino acid esters and 1-hydroxybenzotriazole (HOBT) were from Cambridge Research Biochemicals, Northwich, Cheshire, U.K.

Phenol, piperidine, acetic anhydride, hydroxylapatite, silver nitrate, citric acid and triethanolamine hydrochloride were from BDH Chemicals, Hampshire, U.K.

Ethanedithiol, diisopropylethylamine, 3,3',5,5' - tetramethylbenzidine (TMB) and Triton X - 100 were purchased from Aldrich Chemical Co., Dorset, U.K.

Mixed bovine brain gangliosides (approx. 21% GM_1, 40% $GD1_a$, 16% $GD1_b$, 19% $GT1_b$, in 150 mM NaCl, 10 mM $NaHPO_4$, pH 7.2) were from Calbiochem, Nottingham, U.K.

Nicotinamide adenine dinucleotide (NAD^+) (grade 1, 100%) was from Boehringer Mannheim, East Sussex, UK.

Eagles Minimal Essential Medium (MEM) was obtained from Gibco Laboratories, Surrey, U.K.

Dithiothreitol was obtained from Bio-Rad, Hertfordshire, UK.

Fetuin, asialofetuin, alpha-1-acid glycoprotein, haptoglobin, Fast blue RR salt, Napthol AS-MX alkaline phosphate solution, anti-human and anti-bovine IgG's, horse radish peroxidase- and alkaline phosphatase-conjugated antibodies, glutaraldehyde (grade 1, 25% aqueous solution), Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid, DTNB) and glycerol were from Sigma Chemical Company, Poole, Dorset, UK.

Cyanogen bromide-activated sepharose 4B, DEAE-sepharose CL4B, Mono-Q anion

exchange FPLC columns and PD10-G25M gel filtration columns (pre-packed, 1 x 10 cm) were purchased from Pharmacia LKB, Buckinghamshire, U.K.

TMS-250 reverse phase FPLC column was from Anachem, Bedfordshire, U.K.

All other reagents were of the highest grade possible.

2.2 Methods

2.2.1 Pertussis toxin purification

Pertussis toxin was purified following the method below (Imaizumi *et al* ., 1983, Irons and MacLennan 1979):

1. 0.5 ml freeze-dried *Bordetella pertussis* (Wellcome strain 28) in culture medium (see below) was resuspended in water and grown at 35°C for 48 hours, on charcoal agar (oxoid CM 119) supplemented with 10%(v/v) defibrinated horse blood (Difco Lab., Surrey, U.K.).

2. Bacteria grown on this medium were used to inoculate three seed culture flasks containing 100 ml culture medium. These were incubated at 35°C for 24 hours on an orbital shaker.

To prepare 10 litres of culture medium, the following constituents were added to 7 litres of water:

Sodium glutamate	106.0 g
Tris (pH 7-9)	61.0 g
NaCl	25.0 g
Casamino acids	100.0 g
KH_2PO_4	5.0 g
$MgCl_2.6H_2O$	1.0 g
KCl	2.0 g
L-proline	2.4 g
Glutathione	1.5 g
Ascorbic acid	4.0 g
$CaCl_2$	0.4 g
O-methyl-β-cyclodextrin	10.0 g

Three stock solutions were prepared separately:

i) 0.5 g $FeSO_4.7H_2O$ in 200 ml water
ii) 0.2 g nicotinic acid in 200 ml water
iii) 2.5 g L-cysteine in 200 ml 2.5 M HCl

40 ml of each stock was added to the medium and the pH adjusted to 7.6 with concentrated HCl and the volume made up to 10 litres with water.

3. 30 x 2.5 l Thompson flasks containing 250-300 ml culture medium were inoculated with 9 ml of seed culture and incubated on a lateral shaker, on their sides at 35°C for 48 hours.

4. Bacterial cultures were centrifuged at 6000*g* for one hour at 6°C; remaining bacteria were removed by passing the supernatant through a capsule filter (Sartorius, Surrey, UK., 0.22 μm).

5. Spherical hydroxyl apatite (BDH Ltd., U.K.) was resuspended in 500 ml 0.1 M NaOH, allowed to settle and the procedure repeated. The hydroxyl apatite matrix was the washed several times with water until the pH of the decanted liquid was 8.0. The matrix was then resuspended in 10 mM potassium phosphate buffer, pH 8.0 and packed into a column (5 cm I.D. x 17 cm L) and equilibrated with the phosphate buffer. The filtered supernatant was passed through the column at 500 ml/hour, 4°C, to remove filamentous haemagglutinin (FHA), leaving pertussis toxin in the supernatant.

6. Column eluate was precipitated by addition of ammonium sulphate crystals, to 74% saturation and left over 18-48 hours, at 4°C.

7. Precipitated protein was centrifuged at 6000*g* for 20 minutes at 4°C. The pellet was retained washed three times with 100 ml 50 mM potassium phosphate, 0.5 M NaCl pH 7.5 and centrifuged at 12000*g* for 30 minutes, at 6°C. Supernatant was discarded, the pellet resuspended in 100-150 ml of the above buffer and dialysed against the above buffer for 48 hours at 4°C.

8. During the dialysis step, 15 g CNBr-activated sepharose 4B powder (Pharmacia) was suspended in 100 ml 1 mM HCl and filtered through a sintered glass funnel using about 150 ml 1 mM HCl/g powder. 200 mg of fetuin was dissolved in coupling buffer and added to the swelled matrix and the mixture was rotated end-over-end for 3.5 hours at room temperature. Excess, uncoupled fetuin was washed away by washing the matrix with coupling buffer and the filtrate tested for protein content using a Bradford assay with bovine serum albumin as standard. Successful coupling was indicated by a low protein content of the filtrate (<50 μg/ml). The remaining active groups were blocked using blocking buffer for two hours at room temperature. The coupled fetuin-sepharose 4B was then washed with three cycles of high and low pH, each cycle consisting of one wash with acetate buffer, pH 3.8 followed by one

wash with Tris buffer, pH 8.0. Fetuin-sepharose 4B was then packed into a column (2 cm I.D. x 10 cm L) and equilibrated with 10 column volumes of 50 mM potassium phosphate, 0.5 M NaCl pH 7.5.

Dialysis residue was applied at 1 ml/min to the fetuin-sepharose 4B affinity column and recirculated through the column three times.

9. After washing the column with 5-10 column volumes of 50 mM potassium phosphate, 0.5 M NaCl pH 7.5, pertussis toxin specifically bound to the column was eluted with 50 mM Tris-Cl, 130 mM NaCl, 2.7 M $MgCl_2$ pH 6.5 at 0.5 ml/min. 50 x 3 ml fractions were collected, their protein content estimated from their A_{280} and fractions estimated to contain most protein were run on 17.5%(w/v) SDS-PAGE to confirm their composition.

10. Fractions containing pertussis toxin (identified by the five resolved bands seen after silver or coomassie staining of the gels, see figure 4.2) were dialysed for 36 hours at 4°C against 50 mM Tris-Cl, 1M NaCl pH 7.5 to remove traces of $MgCl_2$.

11. The pertussis toxin was then dialysed against 10 mM phosphate, 0.5 M NaCl pH 7.5 overnight.

12. Toxin was precipitated to 1.5-3 mg/ml with ammonium sulphate (74% saturation) and stored in this form at 4°C, until used.

2.2.2. Pin peptide synthesis

Peptide synthesis was carried out using a kit (Cambridge Research Biochemicals, Cheshire, UK), according to the method of Geysen *et al* (1987). Polypropylene pins derivatised with an fmoc N-(9-fluoroenylmethoxycarbonyl) protected linker were provided in blocks of 96 to fit polypropylene microtitre plates. The linker allowed covalent bonding of the C-terminal of each peptide to to the pins. Consecutive cycles of washing, deprotection and amino acid coupling resulted in peptides of the required length, which were then N-terminal and side chain deprotected and acetylated, ready for use in subsequent binding assays.

2.2.2.1 Washing and N-terminal deprotection

The derivatised pins were washed before deprotection at room temperature with four cycles of:

DMF	100 ml	2 min
Methanol	100 ml	2 min
DMF	100 ml	2 min

Pins were N-terminal deprotected for 20 minutes at room temperature in 100 ml of 20%(v/v) piperidine in DMF. (Note that DMF was supplied standing over molecular sieve to remove amines).

Pins were then washed:

DMF	100 ml	5 min
Methanol	100 ml	2 min (repeated four times)
Air dry		≥10 min
DMF	100 ml	5 min (immediately before amino acid coupling).

2.2.2.2 Amino acid coupling

Pentafluorophenyl (Pfp) activated esters of L-amino acids were used in solid phase synthesis. 3-hydroxy-2,3-dihydro-4-oxo-benzotriazine (ODhbt) replaced Pfp for serine and threonine.

All coupling reactions were performed in 30 mM 1-hydroxybenzotriazole (HOBT) in DMF.

All amino acid N-terminals were fmoc protected. Side chains were protected where necessary by t-butyl derivatives, with the exception of arginine, which was protected by 4-methoxy-2,3,6-trimethylbenzene sulphonyl (Mtr).

100 μl aliquots of 30 mM amino acid ester and 30 mM HOBT in DMF were dispensed into the appropriate wells of a polypropylene microtitre plate. Deprotected pins were correctly oriented and immersed as blocks of 96 into the wells. Coupling was allowed to proceed for 18 hours at room temperature, with gentle shaking. Washing and N-terminal deprotection was repeated, ready for the next coupling reaction.

The cycle of washing, deprotection and coupling continued until peptides of the required length were reached.

2.2.2.3 Acetylation

The N-terminal amino acid (and side chain amino group of lysines) of each complete peptide was deprotected as in 2.2.2.1 and the N-terminus acetylated to remove the positive charge by immersing the pins in microtitre plate wells containing 100 μl 10%(v/v) acetic anhydride, 2%(v/v) diisopropylethylamine in DMF

for 90 minutes at room temperature, while shaking gently.
This was followed by a washing step:

DMF	100 ml	2 min
Methanol	100 ml	2 min (repeated four times)
Air dry		≥10 min

2.2.2.4 Side chain deprotection

Pin blocks were reacted with 100 ml 95%(v/v) TFA, 2.5%(w/v) phenol and 2.5%(v/v) ethanedithiol in sealed polypropylene boxes for four hours, followed by a final washing and neutralisation procedure:

Washing:

DCM	100 ml	2 min (repeated twice)

Neutralisation:

5%(v/v) diisopropylethylamine in DCM

	100 ml	5 min (repeated twice)
DCM	100 ml	5 min
Air dry		≥10 min
dH_2O	100 ml	2 min
Methanol	100 ml	18 hours

Pin peptides were then dried over silica in a desiccator at room temperature for 18 hours and stored in boxes containing silica gel at 4°C until use.

2.2.2.5 Disruption of peptides

Newly synthesised pin peptides were disrupted by sonication before their first enzyme linked immunoassay (ELISA) screen, to remove residual solvents, and after each subsequent assay, to remove bound antibody complexes from the pins. This involved sonication of the pins in pin peptide disruption buffer (100 mM NaH_2PO_4, pH 7.2, containing 0.1%(v/v) 2-ME and 1%(w/v)SDS), at 60°C for 30 minutes, followed by two washes in 100ml water for two minutes, at 60°C. The pins were then immersed in a bath of boiling methanol for two minutes, removed and dried in air for not less than 10 minutes, before either storage or the next screening.

2.2.3 Pin peptide screening by enzyme linked immunoassay (ELISA)

2.2.3.1 Antibody binding to pin decapeptides

Nonspecific binding to peptides was reduced by immersion of the pins in microtitre plate wells containing 200 μl blocking buffer (10% (v/v) newborn calf serum (NCS), 0.4%(v/v) Tween 20 in PBS, pH 7.2) for 90 minutes. Pin peptides were then incubated with 175 μl/well of an appropriate dilution of antibody, in blocking buffer overnight.

Pins were then washed four times for 2 minutes per wash in 0.1%(v/v) Tween 20 in PBS, pH7.2 and immersed in 175 μl/well 1:500 dilution of anti-IgG, species specific (whole molecule) horse radish peroxidase conjugate, in 0.1%(v/v) Tween 20 in PBS, for 90 minutes. The pins were then washed as above.

Colour was developed for 30 minutes by incubation with 150 μl/well substrate at room temperature:

0.05%(w/v) 2,2'-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid, 80 mM citric acid, 100 mM Na_2HPO_4, 0.01%(v/v) H_2O_2, pH 4.0.

The reaction was stopped by removal of pins from the substrate and colour development was measured at 405 nm on a Titertek Multiskan™. Pins were then disrupted (2.2.2.5). Residual binding to the pins after sonication was tested by the same ELISA: pins were incubated without test antibody (or glycoprotein) and the rest of the assay carried out as described above.

2.2.3.2 Binding of glycoprotein and glycolipid to pin decapeptides

Nonspecific binding to the pin peptides was reduced as described in the previous section. This was followed by incubation with the glycoprotein in blocking buffer (175 μl/well) for seven hours at room temperature. Three 5-minute washes in 0.1%(v/v) Tween 20 in PBS, pH 7.2 followed. The pin decapeptides were then incubated overnight in 1:250 dilution of affinity-purified rabbit anti-fetuin serum (Provided by Dr. L.A.E. Ashworth, PHLS CAMR Porton Down, U.K.) in blocking buffer, four 2-minute washes in 0.1%(v/v) Tween 20 in PBS and a 90 minute incubation with goat anti-(rabbit IgG) serum conjugated to horse radish peroxidase and washed four times as above.

Colour was developed as described in the previous section and was measured on a Titertek multiscan™ at 405 nm. Pins were then sonicated in disruption buffer, ready for the next assay.

2.2.4. Monoclonal antibody (MAB) purification

2.2.4.1 FPLC anion exchange chromatography

Monoclonal antibodies, as ascites fluid were a gift from Dr. L.A.E. Ashworth, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire and their preparation is described in Gorringe *et al.* (1985). The ascites fluid, containing monoclonal antibody (1 or 2 ml) were diluted to 10 ml using buffer A (5 mM Tris-Cl pH 8.0), filtered to remove insoluble matter and applied via a 10 ml superloop to a Mono-Q anion exchange column (Pharmacia, 1 cm I.D.x 5 cm L) equilibrated with buffer A.
The column was eluted with buffer B (5 mM Tris-Cl, 200 mM NaCl, pH 8.0) at a flow rate of 1 ml/min. Protein concentrations were estimated by A_{280} and fractions judged to contain protein were analysed for pertussis toxin binding activity, by ELISA (section 2.2.8.1)

2.2.4.2 Anion exchange chromatography

Ammonium sulphate was added to ascites fluid containing monoclonal antibody (1-3 ml) to a concentration of 50-60% saturation and agitated gently for 15 minutes. The precipitated protein was centrifuged at 10 000*g* for 30 minutes. The pellet was washed once with ammonium sulphate solution and the pellet resuspended in buffer A (10 mM Tris-Cl pH 8.0) and dialysed for 18 hours at 4°C. The dialysate was loaded onto a DEAE Sepharose CL-6B column (2 cm I.D x 20 cm L) equilibrated with buffer A and eluted with a gradient of 0% B to 100% B (10 mM Tris-Cl, 500 mM NaCl, pH 8.0) at a flow rate of 1 ml/min. Protein containing fractions (estimated by A_{280}) were assayed for pertussis binding by ELISA (section 2.2.8.1).

2.2.4.3 Protein A-sepharose affinity chromatography

For a column of dimensions 1 cm I.D. x 5 cm L of protein A-sepharose CL4B (Pharmacia LKB), 3 ml ascites fluid containing monoclonal antibody was dialysed against 140 mM sodium phosphate buffer, pH 8.0 overnight (buffer A). The dialysate was then applied to the column, which was equilibrated with buffer A and the fluid recirculated through the column for at least three hours at a flow rate of 5 ml/hour. The column was then washed with three column volumes of buffer A at 5 ml/hour, followed by three column volumes of 100 mM citrate/citric acid, pH 6.0, then three column volumes of 100 mM citrate/citric acid, pH 3.0 and 1 ml fractions collected throughout. Fractions containing protein estimated from A_{280} were pooled and

immediately dialysed against buffer A. Anti-(pertussis toxin)-IgG-containing fractions were identified by solid-phase ELISA using pertussis toxin as coating antigen (2.2.8.1).

2.2.5 Synthesis and purification of free peptides

2.2.5.1 Overview

Antigenic pin peptides were synthesised with a C-terminal amide from 4-[2',4'-dimethoxyphenyl-N-(9-fluorenylmethoxycarbonyl)-aminomethyl]phenoxy resin (Novabiocem, U.K.), using the Applied Biosystems Fastmoc synthesis system, by Dr. C. Shone (PHLS Centre for Applied Microbiology and Research, Porton Down, UK) using an Applied Biosystems A431A amino acid synthesiser. Isolation of the free peptide from the resin and deprotection were carried out by the author.

Peptide (CGE)S3c, which was to be conjugated to keyhole limpet haemocyanin (KLH) had a glycine residue used as a spacer and a cysteine (C) residue at its N-terminal. Peptide S4b had the same two residues at its C-terminal. Both peptides were coupled to KLH using a chemical cross-linker, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), which reacts with cysteine residues between pH 7 and 7.5. Peptide S4b was also coupled to bovine serum albumin (BSA) using glutaraldehyde as the cross-linking reagent. Peptide (E)S3c was to be conjugated to BSA by carbodiimide and had a glutamate added at its N-terminal (see table 3.2 for amino acid sequences of the free peptides), but conjugation of (E)S3c to BSA using glutaraldehyde generated an anti-(pertussis toxin) response judged to be adequate for the studies to be carried out.

2.2.5.2 Deprotection and removal of peptide from resin

For peptides (E)S3c and (CGE)S3:

0.75 g	phenol
0.25 ml	1,2 ethanedithiol
0.25 ml	deionised water
10 ml	TFA

in a round bottomed flask. The mixture was stirred on ice for two hours. Deprotected peptide was then isolated from the resin (2.2.5.3)

For peptide S4b(GC)

0.25 ml	1,2 ethanedithiol
0.25 ml	deionised water
9.5 ml	TFA

in a round bottomed flask. The mixture was stirred on ice for two hours and deprotected peptide was isolated from the resin (2.2.5.3).

2.2.5.3 Isolation and concentration of crude free peptide from resin

The mixture of deprotected free peptide and resin was vacuum filtered through a size 3 glass filter into a 250 ml round bottomed flask and the filter rinsed with 10-15 ml DCM. The yellow peptide solution was concentrated on a rotary evaporator to about 3 ml. The temperature of the water bath was kept between 30 and 37°C to retain the peptide in an undamaged form.

2.2.5.4 Precipitation of free peptide

About 50 ml cold diethyl ether (Et_2O) was added to precipitate the peptide (leaving a cloudy yellow suspension). The precipitate was filtered through a size 3 filter and washed with 10-15 ml Et_2O.

2.2.5.5 Drying and reprecipitation of free peptide

The precipitate from 2.2.5.4 was dried in a desiccator for two hours under vacuum, then redissolved in 10 ml 90%(v/v)TFA in water and 2.2.5.4 repeated. Purified peptide precipitate was again dried in a desiccator and assayed for purity by reverse phase HPLC and mass spectrometry (2.2.5.6 and 2.2.5.7). Remaining peptide was stored as solid at -20°C, until use.

2.2.5.6 Reverse phase chromatography of free peptide

3-5 mg of peptide was dissolved in 50 μl 0.1%(v/v) TFA in water (buffer A). The resulting solution was filtered through a 0.2 μm filter and loaded onto a C8 reverse phase HPLC column (Beckman, 1 cm I.D. x 20 cm L) equilibrated with buffer A. A 40 minute gradient from 100% A to 0.1% TFA, 60%(v/v) acetonitrile in water was run at a flow rate of 1 ml/min, peptide being detected by A_{220}.

2.2.5.7 Mass spectrometry of free peptide

Mass spectrometry was carried out using a Kratos MS80 RFA mass spectrometer fitted with an Iontech FAB 11 NF saddle field gun, using xenon as bombarding gas (thanks to Dr. R. Wait, PHLS Centre for Applied Microbiology and Research, Porton Down, UK) and calibrated with caesium ion clusters.

Peptides were dissolved in 30%(v/v) acetic acid and 1 μl containing about 5 μg of peptide was applied to the same volume of matrix on a stainless-steel probe tip. Spectra were recorded with a 5 KeV accelerating voltage and a scan rate of 30 s per decade.

Between 10 and 20 scans were obtained and averaged using the raw data facilities of the DS90 data system.

Matrix :	DTT : DTE (5:1, w/w)	(if peptide Mr > 1500)
	Glycerol	(if peptide Mr < 1500)

2.2.5.8 Solubilisation of free peptide

All peptides were dissolved to the required concentration in water, after preliminary observations of solubility in water plus or minus a drop of glacial acetic acid, or ammonium carbonate.

2.2.6 Peptide conjugation

2.2.6.1 Activation of keyhole limpet haemocyanin (KLH)

The method of Green *et al.* (1982) was used, with modifications.
4 mg/ml KLH was dissolved in 50 mM sodium phosphate buffer, pH 7.4 and stored at -20°C until use. 50 μl of 14 mg/ml m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) in DMF was added to 250 μl KLH solution and mixed at room temperature for 30 min.

2.2.6.2 Separation of activated KLH from unreacted KLH and MBS

The sample from 2.2.6.1 was loaded onto a PD10-G25 M column (1 cm I.D. x 10 cm L) equilibrated with 50 mM sodium phosphate buffer, pH 6.0. 10 x 1 ml fractions were eluted using the pH 6.0 buffer, protein content estimated by A_{280} and the first protein peak used in section 2.2.6.4.

2.2.6.3 Determination of peptide free thiol

This was done to estimate free thiol groups in the activated KLH that were available for coupling to KLH. The method used was that of Ellman (1959) with slight modifications. 100 μl of 4 mg/ml 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellmans reagent) in 100 mM sodium phosphate buffer, pH 7.4, containing 0.5 mg/ml EDTA was added to 5 μl of 5 mg/ml peptide in water. This was made up to 1 ml with the pH 7.4 buffer. After a standing time of five minutes absorbance of the sample at 412 nm was read. Moles of free thiol were estimated from a calibration curve of 0-100 nmoles L-methyl cysteine against absorbance at 412 nm

2.2.6.4 Coupling of peptide to activated Keyhole Limpet Haemocyanin (KLH)

The pH of the activated KLH (2.2.6.2) was raised to about 7 with 1M NaOH. A 40-100 molar excess of peptide (calculated from the free thiol content of each peptide in the previous section) was added and the sample mixed, for three hours at room temperature. The peptide-KLH conjugate was dialysed against PBS pH 7.2 at 4°C. Aliquots were tested for protein using an automated Lowry assay (Lowry *et al* ., 1951), with bovine serum albumin as standard, performed at the PHLS CAMR, Porton Down, U.K. The remainder was stored in small aliquots, at -20°C.

2.2.6.5 Peptide conjugation to bovine serum albumin (BSA)

About 2 mg peptide was added as solid to 250 μl (1 mg) of 4 mg/ml BSA (96-98% pure fraction V, Sigma Chem. Co., Dorset, UK) in PBS pH 7.2, on ice. 10 μl glutaraldehyde was added in 2.5 μl aliquots and the sample shaken vigorously. The sample was left on ice for 60 minutes and the reaction inhibited by addition of 0.1 mg sodium borohydride. Sample volume was made up to 1 ml with PBS pH7.2 and dialysed against PBS overnight. Protein estimation was carried out using an automated Lowry assay (2.2.6.4).

2.2.7 Peptide conjugate immunisation

2.2.7.1 Mouse polyclonal serum preparation

Mice were immunised by Dr. A. Robinson, (PHLS CAMR, Porton Down, UK) with 10 μg antigen adsorbed onto alhydrogel adjuvant and boosted with the same dose after three weeks. Sera were obtained six weeks after the initial dose.

2.2.7.2 Rabbit polyclonal serum preparation

New Zealand White rabbits were immunised at multiple intramuscular sites by Dr. L. A.E. Ashworth, (PHLS CAMR, Porton Down, UK) with 150 μg peptide (E)S3c-BSA-conjugate in 0.75 ml Freund's incomplete adjuvant and boosted with the same dose after three weeks and six weeks. Serum was obtained eight weeks after the initial dose.

150 μg peptide S4b-KLH-conjugate was administered in 0.75 ml incomplete Freund's adjuvant and the dose repeated after three weeks. Serum was harvested six weeks from the first dose.

2.2.8. Microtitre plate enzyme-linked immunoassay (ELISA) techniques

2.2.8.1 Antibody binding to antigen-coated plates

1. 2 μg /ml pertussis toxin, peptide or peptide-conjugate in 50 mM carbonate buffer, or free peptide in water, were coated onto microtitre plates (Nunc Immunoplates 1) overnight at room temperature (toxin and conjugates), or at 37°C (free peptide).
Peptide coated plates were then fixed with 50%(v/v) methanol in water for 15 minutes. All plates were washed three times with 300 μl/well PBS pH 7.2 containing 0.1%(v/v) Tween 20.
Nonspecific binding was blocked with 100 μl/well blocking buffer (PBS, pH 7.2 containing 10%(v/v) newborn calf serum (NCS) and 0.1%(v/v) Tween 20) for 90 minutes at room temperature. Each step was followed by washing (above).

2. Test antibodies were serially diluted in blocking buffer on a separate plate. 100 μl/well were transferred to the blocked, antigen-coated plate and incubated for 90 minutes. Plates were then washed.

3. 100 μl/well of 1: 625 dilution, species specific, anti-IgG (whole molecule) antibody, conjugated to horse radish peroxidase in blocking buffer was added, the plates incubated for a further 90 minutes and washed

4. Developer : 1 mg of 3,3',5,5'-tetramethylbenzidine (TMB), dissolved in 100 μl DMSO was added to 10 ml 50 mM acetate buffer pH 6.0, giving a final

concentration of 0.01 mg/ml TMB. H_2O_2 was added to 0.012%(v/v) and each well was incubated with 100 μl of the developer for 20 minutes at room temperature. Development was stopped by addition of 35 μl/well H_2SO_4. The resulting absorbance was read at 450 nm on a Titertek Multiskan.

2.2.8.2 Recognition of pertussis toxin captured by fetuin or gangliosides by antipeptide antibody

Experimental procedure was adapted from 2.2.8.1:

1. 2 μg/ml of glycoprotein or mixed gangliosides were coated onto microtitre plate wells as described in section 2.2.8.1.

2.(i) 50 μg/ml pertussis toxin in blocking buffer was added to the blocked antigen-coated plate and incubated for 90 minutes and the plate washed.
Antipeptide antibody was serially diluted in a microtitre plate in blocking buffer and 100 μl/well was transferred to the plate containing captured pertussis toxin, incubated for 90 minutes and the plate washed.
(ii) 100 μl/well diluted antipeptide antisera was added, incubated for 90 minutes, the plate washed and developed as described in section 2.2.8.1.

2.2.8.3. Binding of pertussis toxin or peptide to glycoprotein- or glycolipid-coated plates

2 μg/ml glycoprotein or mixed bovine brain gangliosides were coated onto microtitre plates and blocked as in 2.2. . Blocked plates were washed as before and incubated for 90 minutes with 100 μl /well toxin or peptide serially diluted in blocking buffer. The plates were washed and incubated for a further 90 minutes with anti-toxin antibody or anti-peptide antisera diluted in blocking buffer and washed again. Procedure then followed steps 3 and 4 of section 2.2.8.1.

2.2.8.4 Inhibition of binding to antigen

Procedure followed that of 2.2.8.1, with this addition:

2.(i) Test antiserum (giving an A_{450} of 1.2-1.5 on binding to antigen-coated plates) was pre-incubated with serially diluted inhibitor in blocking buffer for 90 minutes in a separate plate, while the coated plate was being blocked. 100 μl/well was transferred to the coated plate and incubated for a further 90 minutes.

2.2.9 SDS Polyacrylamide gel electrophoresis (SDS PAGE)

2.2.9.1 Molecular weight markers used for SDS PAGE

SDS PAGE was performed using the method of Laemmli (1970). Samples were prepared for electrophoresis in 1%(w/v) SDS, by boiling for three minutes. Glycerol was added to a final concentration of 5%(v/v) and bromophenol blue was added as a marker of protein migration. The prepared samples were then loaded onto SDS polyacrylamide gels and run to completion.
Gels were calibrated using one of two molecular weight marker kits:

1. Dalton mark VII-L (Sigma Chemical Co., Dorset, UK) which contained bovine serum albumin (Mr 66000), hen egg ovalbumin (Mr 45000), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (subunit, Mr 36000), carbonic anhydrase from bovine erythrocytes (Mr 29000), bovine pancreas trypsinogen (Mr 24000), soybean trypsin inhibitor (Mr 20100) and bovine milk α-lactalbumin (Mr 14200).

2. Bio-Rad (Hertfordshire, U.K.) low range SDS-PAGE markers, containing phosphorylase b (Mr 97000), bovine serum albumin, hen egg ovalbumin, bovine carbonic anhydrase, soybean trypsin inhibitor and hen egg white lysozyme (Mr 14400).

2.2.9.2 Coomassie staining

Gels were fixed in 20%(v/v) methanol, 10%(v/v) acetic acid in water for 1 hour. They were then transferred to a staining solution containing 0.25%(w/v) coomassie blue R-250, 45%(v/v) methanol, 9%(v/v) acetic acid in water at 60°C for 15 minutes. Protein bands were visualised by destaining the background with 10%(v/v) methanol, 7%(v/v) acetic acid in water.

2.2.9.3 Electroelution

Areas of gel containing the protein of interest were excised, cut into small pieces and placed in the larger cup of a concentrating apparatus (ISCO), resting in 25 mM Tris-Cl, 0.5 M glycine, pH 8.3, containing 1%(w/v) SDS. A constant current of 40 mA was applied for four hours. 50 μl concentrated, eluted protein solution was collected in the smaller cup.

2.2.9.4 Silver staining

The method followed was that of Wray (1981). Gels were soaked for one hour at 60°C in 50%(v/v) methanol in water, then for five minutes in water. 0.8 g silver nitrate was dissolved in 4 ml water in glass and added dropwise to 22.4 ml 0.36%(w/v) NaOH containing 2.2%(v/v) ammonia solution. This was made up to 100 ml with water and the gel soaked in it for 15 minutes at room temperature. Three five minute washes with water followed. The stain was developed by soaking the gel in 0.5%(w/v) citric acid, 0.02%(v/v) formaldehyde in water. Development was stopped by placing the gel in 5%(v/v) methanol, 10%(v/v) acetic acid in water.

2.2.9.5 Western blotting

Proteins separated by SDS-PAGE were transferred by Western blotting (Towbin *et al* 1979) onto nitrocellulose paper.
Blotting was performed in an LKB™ tank blotter at 1 A for two hours at room temperature using 20%(v/v) methanol, 0.02%(w/v) SDS, 20 mM Na_2HPO_4 as protein transfer buffer. The nitrocellulose was then cut into strips and nonspecific antibody binding was blocked for 90 minutes with 1%(w/v) BSA, 0.3% Tween 20 in PBS pH 7.2. The strips were then incubated in an appropriate dilution of test antibody in blocking buffer, for 90 minutes, then washed three times for 10 minutes with 0.1%(v/v) Tween 20 in PBS pH 7.2. This was followed by a 90 minute incubation with an appropriate dilution of anti-IgG (whole molecule), species specific antibody, conjugated to alkaline phosphatase, followed by three 10 minute washes with water. Blots were developed for 10 minutes using 2.5 ml developer: 12 mg fast blue RR salt dissolved in 50 ml water containing 0.01%(v/v) naphthol AS-MX alkaline phosphate solution (Sigma Chem. Co., U.K.). Development was stopped by rinsing the nitrocellulose with water.

2.2.10 Cross-linking of pertussis toxin subunits

2.2.10.1 Cross-linking of pertussis toxin and separation by SDS PAGE

A 2-3 mg/ml ammonium sulphate suspension of pertussis toxin in 10 mM sodium phosphate, 0.5 M NaCl pH 7.5 was centrifuged at 10 000 *g* for 20 minutes. The pellet was resuspended in cross-linking buffer (50 mM triethanolamine hydrochloride, 150 mM NaCl pH 8.3 containing 1%(v/v) Triton X-100, 2%(v/v) glycerol) to a final concentration of 0.35, 0.7, or 1.4 mg/ml and dialysed against

cross-linking buffer at 4°C overnight.

Cross-linking reagents were dissolved to 100 mM in DMSO and stored at -20°C until use.

Toxin was incubated with 0.1-10 mM cross-linker for 1-60 minutes at 30°C. The reaction was stopped by addition of 4 M Tris-Cl pH 7.8. Cross-linked protein was prepared for loading onto a 10-20%(w/v) gradient or 17.5%(w/v) SDS polyacrylamide gel.

2.2.10.2 Two-dimensional SDS-PAGE

Cross-linked pertussis toxin samples were run on SDS PAGE as described in section 2.2.9 . A strip was cut from this first dimension gel and coomassie or silver stained to determine the cross-linking pattern.
Another strip from the same gel was soaked in cross-link cleavage buffer:

1. Dithiobis-(succinimidyl propionate) (DSP): 100 mM Tris-Cl pH 8.9 containing 3%(v/v) 2-mercaptoethanol and 1%(w/v) SDS for 45 minutes at 60°C. The strip was washed twice for 5 minutes in equilibration buffer (50 mM Tris-Cl, 1 mM EDTA pH 6.7 containing 1%(w/v) SDS). It was then fixed to the stacking gel of a 17.5%(w/v) SDS polyacrylamide gel with 1%(w/v) agarose and run at 30 mA to completion.

2. Disuccinimidyl tartarate (DST): 30 mM sodium periodate in 100 mM Tris-Cl pH 6.0, containing 1%(w/v)SDS, for one to four hours, at 60°C. The strip was then washed in equilibration buffer, fixed to the stacking gel of a 17.5%(w/v) SDS polyacrylamide separating gel and run as above.
Cleaved cross-links were detected by coomassie or silver staining, or Western blotting using subunit specific antibodies.

Several conditions were tried for cross-link cleavage, leading to the methods described above :

i. A strip was cut from the first dimension gel and soaked in 100 mM Tris-Cl pH 8.9, 1%(w/v) SDS, 15 mM DTT (cleavage buffer) at room temperature for 60 minutes. The strip was then soaked in 50 mM Tris-Cl, 1%(w/v) SDS, (pH 6.7). The strip was then fixed to the stacking gel of the second dimension gel using 1% agarose.

ii. Next the concentration of DTT was increased to 30 and then 45 mM, the other conditions being the same as in the previous section.

iii. The method described in part i, above was used but 1%(v/v) 2-ME was substituted for DTT.

iv. The method described in part iii was used and the cleavage-reaction temperature was increased to 40, 50 and then to 60°C, for 20, 30, 40, 45 and 50 minutes. 45 minutes was judged to produce the highest yield of cleaved cross-linked species.

v. Finally the concentration of 2-ME used in part iii was increased to 2%(v/v) and then 3%(v/v). 3%(v/v) 2-ME was most successful.

2.2.10.3 HPLC-separation of cross-linked pertussis toxin subunits

Pertussis toxin was prepared in cross-linking buffer, as described in section 2.2.10.1 and buffer B (0.1%(v/v) TFA in acetonitrile) added to a final concentration of 15%(v/v) acetonitrile. The sample was centrifuged to remove debris and applied to a TMS-250 reverse phase column (0.46 cm I.D. x 7.5 cm L, Anachem, Bedfordshire, U.K.), equilibrated with 15% B and 85% buffer A (0.1% TFA in water).
The column was eluted with a gradient of 15-55% B over 120 minutes at a flow rate of 0.4 ml/min. Fractions containing protein estimated by A_{220} were evaporated to dryness in a Gyrovap vacuum centrifugal evaporator (Howe instruments, UK), resuspended in cross-linking buffer and run on SDS-PAGE (2.2.9).

2.2.11 Fluorescence measurements

Fluorescence measurements were performed in a Perkin Elmer 3000 spectrofluorimeter with a 1 cm pathlength and a thermostatically controlled cuvette holder. Samples were protected from incident light when readings were not being taken. Fluorescence was measured at 333 nm (excitation 285 nm), slit widths both being 10 nm.

All fluorescence data were corrected for dilution and inner filter effects (Honore and Pedersen 1989). Protein concentration was calculated using the Lambert-Beer law from A_{280} using the molecular mass of the whole toxin or S1 subunit, deduced from the DNA-sequence of the subunits (Locht and Keith, 1986). A molar extinction coefficient (ε) was calculated for pertussis toxin, isolated S1 subunit and the S1 subunit analogues, from the subunit amino acids that absorb light at 280 nm (tryptophan, tyrosine and cystines- assuming that all cysteines are disulphide-bonded) (Gill and von Hippel, 1989).

2.2.11.1 NAD^+ binding to pertussis toxin and isolated S1 subunit

Isolated S1 subunit was a kind gift of Dr. C. Capiau (Smith Kline Beecham, Rixensart, Belgium). 2 ml of 50 μg/ml pertussis toxin or isolated S1 subunit protein sample equilibrated in 5 mM potassium phosphate, 100 mM NaCl, pH 7.2) was titrated with 2-6 μl aliquots of 1-20 mM NAD at 14, 20, 23, 30 and 40°C, to a final concentration of between 160 and 200 μM, depending on the experiment.

2.2.11.2 NAD^+-binding to pertussis toxin and S1 subunit analogues

S1 subunit analogues rS1d and rS1dE129D were a kind gift from Dr. C. Locht (Institut Pasteur, Lille, France). 2 ml of 50 μg/ml subunit was equilibrated in 5 mM potassium phosphate, 100 mM NaCl, pH 7.2 and titrations were carried out as described in section 2.2.11.1, at 37°C.

2.2.12 Chinese Hamster Ovary (CHO) cell clumping

The method of Gillenius *et al.* (1985) was adapted to study pertussis toxin-mediated clumping of CHO cells. A CHO cell line (CB169, European Collection of Animal cell culture, PHLS Porton Down, U.K.) was maintained in Eagle's Minimal Essential Medium (MEM) supplemented with 10 %(v/v) foetal calf serum (Gibco Laboratories, Surrey, U.K.), 0.11 %(w/v) sodium bicarbonate, 15 mM Hepes buffer and 0.06% L-glutamine, 25 U/ml gentamycin (growth medium).

1. 25 μl growth medium was added to each well of a microtitre plate, then 25 μl of serum sample was added to row H of the test wells.

2. 25μl of the diluted sample was removed from row H and serially diluted one in two from row H to row A, leaving 25 μl of diluted sample in each well.

3. 25 μl of 20 ng/ml pertussis toxin (final concentration was the lowest found to clump the cells, to detect weak inhibition of clumping) was added to all the wells containing diluted sample and incubated for one hour.

4. The growth medium from a confluent monolayer of CHO cells was discarded and the cells washed with sterile PBS, pH 7.2, which was then discarded.

5. A single cell suspension was obtained by washing the cells with 2 ml of 0.1 %(w/v) trypsin and 0.02 %(w/v) EDTA in PBS, pH 7.2 and observed under a microscope until they were detached from the culture flask and were not attached to

any other cells.

6. The wall of the culture flask was then washed with 5 ml growth medium to remove any remaining single cells and the cell suspension was then centrifuged at 1000*g* and the supernatant discarded.

7. The cell pellet was resuspended in 10 ml growth medium and a viable cell count carried out in a Neubauer Haemocytometer. The cell suspension was then adjusted to 5×10^4 cells/ml and 200 μl added to each well of a microtitre plate.

8. The microtitre plate was sealed, placed in a humidified container with a sheet of damp paper touching the base of the microtitre plate, to prevent uneven distribution of the cells due to static electricity and incubated for 48 hours at 37°C in a CO_2 incubator.

9. The wells of the plate were scored as completely clumped (score 2), partially clumped (score 1) or not clumped (score 0). For direct comparison with the test rows, each plate contained a row with a serial toxin dilution used as a positive control (score 2) and a row containing neither toxin nor serum, used as a negative control (score 0). A row containing diluted serum sample with no toxin added was also included, to assess the effect of the sample on the CHO cells.

Chapter 3

Epitope mapping and glycoprotein binding to pertussis toxin subunits

3.1 Introduction

3.1.1 Binding to peptides attached to polyethylene pins

The aims of this section of work were to identify antigenic peptides (i.e. antibody-binding) and glycoprotein-binding peptides derived from the whole amino acid sequence of pertussis toxin. Those antigenic and glycoprotein-binding peptides were to be studied for their ability to induce antibodies that recognised 'native' pertussis toxin and neutralised some of the toxin's biological activities. In order to study the binding of antibody to subunits S1, S3 and S4 of pertussis toxin, and the glycoprotein binding properties of the S3 subunit, the solid phase epitope mapping procedure of Geysen *et al.* (1984) was used. This was employed as a primary screening technique and used a pin peptide synthesis kit supplied by Cambridge Research Biochemicals (U.K.). Polypropylene pins derivatised with an fmoc N-(9-fluoroenylmethoxycarbonyl) protected linker were provided in blocks of 96 to fit polypropylene microtitre plates. The linker allowed covalent bonding of the C-terminal of each peptide to to the pins. Consecutive cycles of washing, deprotection and amino acid coupling resulted in decapeptides, which were then N-terminal and side chain deprotected and acetylated, ready for use in subsequent binding assays.

This technique has been used to map 'linear' B-cell epitopes of many proteins (see section 1.4.4), for example respiratory syncytial virus fusion protein (Scopes *et al.*, 1990), *Clostridium perfringens* Alpha toxin (Logan *et al.*, 1991) and herpes-simplex-virus type 1 thymidine kinase (Zimmerman *et al.*, 1991).

Binding of murine monoclonal antibodies (either as ascites fluid and as affinity purified antibody), rabbit polyclonal and human polyclonal antibodies to pin peptides derived from the amino acid sequences of the S1, S3 and S4 subunits of pertussis toxin was studied. Glycoprotein and glycolipid binding of the S3 subunit, and to a lesser extent, of the S1 and S4 subunits, were also studied.

3.1.2 Binding to peptides in solution

Some of the peptides that produced interesting (see later in this section) results in the pin peptide form were chosen for synthesis as free peptides. There were several reasons for this secondary technique being employed. Firstly, the pin peptides were covalently linked to the pins at their C-termini. This linkage could have produced a

conformation not present in the native protein. Secondly, a spurious result could be produced by binding to the pin itself, which could account for the high background sometimes seen in the ELISA assay used to detect binding to the pin peptides. Thirdly, pin peptides cannot be used for immunisation, whereas free peptides can be prepared as peptide-carrier-conjugates and used to raise antipeptide antisera. Previous pin peptide mapping results have indicated that some antipeptide antibodies cross-react with peptide sequences that are found in the amino acid sequence of the carrier and not the peptide immunogen (Trifilieff *et al.*, 1991). Non-specific reaction of antipeptide antibodies with sequences unrelated to the peptide immunogen has also been reported (Savoca *et al.*, 1991) and may be due to peptide flexibility (Jemmerson *et al.*, 1985). For these reasons it was necessary to confirm interesting pin peptide results by carrying out studies using free peptides.

Several criteria had to be fulfilled before results were judged interesting:

1. The free peptide should inhibit binding of antibody or glycoprotein of interest to the pin-bound peptide, showing that free and bound peptide bind the same sites on the antibody or glycoconjugate.
2. Whole pertussis toxin should inhibit binding of antibody or other glycoconjugate of interest to the pin-bound peptide, showing that the peptide and native protein bind the same site, or adjacent sites on the antibody or glycoconjugate. That is, if an antibody bound both peptide and whole pertussis toxin, it would suggest that all or part of the peptide sequence is exposed on the surface of the native protein.
3. When linked to an immunogenic carrier, the free peptide should elicit antibodies that cross-react with the native protein, for example in a solid phase ELISA using pertussis toxin as the coating antigen as described in section 2.2.8. This cross-reaction should be inhibited by the free peptide in solution, which would again suggest that the peptide sequence is in a conformation which is at least similar to the native protein. Conversely, reaction of an antipeptide antibody with the peptide immunogen captured onto an ELISA plate should be inhibited by native protein in solution.

Is an antipeptide antibody truly capable of recognising the native protein from which its sequence was derived? To answer this question, it is necessary to ensure that assays used to determine cross-reactivity between peptides and proteins present antigen to antibody in a conformation as near to 'native' as possible.

Cross-reaction of antibodies between proteins and peptides may be due, at least in part to the presence of denatured protein in the immunogen, or in the antigen used to test the reaction of the antipeptide antibodies. This would mean that antibodies raised against a protein may contain populations of antibodies raised against the denatured portion of the protein. These anti-(denatured protein) antibodies could then recognise denatured antigen present in an assay supposed to test whether the antibody recognised

native protein!

ELISA assays in which the antigen is adsorbed to a plastic surface can be responsible for partial denaturing of the antigen used to test the anti-native protein reactivity of antipeptide antibodies (Darst *et al.*, 1988). Capture of the native antigen, for example by a monoclonal antibody, could present the antigen in a more native form for assay of cross-reaction of the captured antigen with an antipeptide antibody. This presumption was made for antipeptide antibody binding to pertussis toxin. As well as assessment binding to pertussis toxin directly captured onto a microtitre plate, binding of antipeptide antibody to the toxin captured by another antibody or other glycoconjugate was assessed and is described later in this chapter.

3.2 Methods

3.2.1 Pin decapeptides

The method of pin decapeptide synthesis is described in section 2.2.2, but briefly, short peptides of ten amino acids were synthesised spanning the entire amino acid sequence of subunits S1, S3 and S4. The peptides each had a sequence overlap with the next peptide synthesised, as seen in the example below:

Peptide 1 VAPGIVIPPK-------- Pin

Peptide 2 VIPPKALFTQ-------- Pin

Subunits S1, S3 and S4 were synthesised as ten amino acid peptides with a five amino acid overlap, in duplicate. The S3 decapeptide that contained the C-terminal residues of the subunit (peptide 39) overlapped with the previous peptide by six residues. S1 was also synthesised in duplicate as ten amino acid peptides with an eight amino acid overlap and decapeptide that incorporated the C-terminal end of the subunit amino acid sequence (peptide 115) overlapped the previous peptide by nine residues. The sequences of the pin decapeptides are given in appendix 1.

Activated amino acid esters were coupled one at a time to the developing peptide, which was covalently coupled to the polyethylene pin at its C-terminus by a linker consisting of acrylic acid and hexamethylene diamine coupled to β–alanine (fig. 3.1). The amino group of the β-alanine was protected by a base-labile group, called 9-fluorenylmethoxycarbonyl (fmoc), that was removed before the coupling reaction with the first amino acid ester.

Synthesis involved successive cycles of washing, N-terminal deprotection and amino acid coupling. Incorrect coupling and side reactions were avoided by using

Fig. 3.1. Pin decapeptide linker

The linker covalently attached the C-terminal of synthetic peptides to a polyethylene pin (see text for details).

Polyethylene pin
acrylic acid
hexamethylene diamine
Fmoc-β-alanine
CH2-CH2-C-NH-(CH2)6-NH-C-CH2-CH2-NH-Fmoc
O
O

amino acid esters that had acid-labile side-chain protecting groups.

On completion of the synthesis, the peptides were N-terminally deprotected and acetylated to remove their positive charge. Side-chain protecting groups were then removed and the pin peptides washed and sonicated in disruption buffer (see section 2.2.2), to remove remaining scavengers and cleaved protecting groups that could derivatise the peptides. Antibody and glycoconjugate binding to the pin peptides was estimated from the results of a modified ELISA (see section 2.2.3).

The pin decapeptide reactions were repeated at least once. A reaction which gave a repeatable pattern and reasonable agreement between duplicate peptides was considered positive.

A decapeptide was considered strongly reactive and worthy of further study when the absorbance at 405 nm was twice the mean value of the background non-specific binding and the actual absorbance was greater than 0.7. Weakly reactive peptides were considered to be those that produced an absorbance at 405 nm between 20% and 100% above the mean value of the background. The background binding was calculated by averaging the absorbances of the lowest 80% of the duplicate peptides.

It was assumed that pmol quantities of peptide were required for antibody detection (Geysen *et al.*, 1984). If synthesis was inefficient, e.g. with an overall yield of 1%, 1 mmol of activated amino acid ester should generate more than the pmol amounts necessary for a detectable reaction.

3.2.2 Free peptides

Peptides chosen for further work from the results of the pin peptide studies were synthesised with a C-terminal amide as described in section 2.2.5.

Three pin peptides (each ten amino acids) were chosen as the core sequences for free peptides. Two free peptides derived from the sequence of S3 were synthesised, from the pin peptide sequence 46-55. The first free peptide, (E)S3c, contained amino acids 44-58 of the S3 sequence, with an N-terminal glutamic acid, to facilitate coupling to BSA using carbodiimide (this was not carried out because (E)S3c coupled to BSA using glutaraldehyde raised antisera that cross-reacted with pertussis toxin). The second free peptide, (CGE)S3c, contained the same amino acids, with the addition of an N-terminal glycine and cysteine, to facilitate coupling to keyhole limpet haemocyanin (KLH). One free peptide was synthesised from the sequence of S4, and the pin peptide that it was based on contained amino acids 86-95, with a C-terminal glycine and cysteine, to facilitate KLH-conjugation. Their sequences are shown in table 3.2 and the methods of conjugation of the peptides to the carriers are described in section 2.2.6.

Each synthesised free peptide was cleaved from the resin, its purity confirmed by

Table 3.2 Anti-(pertussis toxin) titres and immunising peptides for rabbit antipeptide antisera

Antipeptide Antibody	Toxin subunit	Amino acid sequence position	Immunising peptide sequence	Anti-pertussis toxin titre *
S1a	S1	35-52	DDPPATVYRYDSRPPEDV(GC)	8152
S1c	S1	237-255	(Y)RQAESSEMAAWSERAGEA(GC)	3701
S3a	S3	94-115	AYGGIKDAPPGAGFIYRETFC	6950
S3b	S3	153-171	(KG)RDGQSVIGACASPYEGRYR	59
(E)S3c	S3	44-58	(E)YLRQITPGWSIYGLY	see table 3.4
(CGE)S3c	S3	44-58	(CGE)YLRQITPGWSIYGLY	not done
S4b	S4	86-95	QLTFEGKPAL(GC)	see table 3.4

* Results are shown as ELISA titres, i.e. reciprocal of end-point dilution that gave half-maximal colour (an absorbance of 1.0).

reverse-phase HPLC as described in section 2.2.5.6 and its composition confirmed by fast-atom bombardment mass spectrometry (FAB MS) as described in section 2.2.5.7.

After purification, (E)S3c and S4b were coupled to BSA using glutaraldehyde as the coupling reagent and (CGE)S3 and S4b were coupled to KLH using MBS as the coupling reagent.

Three mice were immunised with 10 μg each of peptide conjugate and given a booster immunisation of the same dose after three weeks. Antisera were harvested and tested using a solid phase ELISA for reaction with peptide, pertussis toxin, and both KLH- and BSA-conjugates coated directly onto the microtitre plate (see section 2.2.8.1). Reaction with a peptide from *B. pertussis* fim 3 was used as a negative control. The amino acid sequence of the fim 3 peptide is given in appendix 1.

If mouse antipeptide antisera cross-reacted most strongly with pertussis toxin, peptide and peptide conjugate captured onto ELISA plates,the peptide-conjugate used to immunise those mice were also used to immunise rabbits, to generate larger volumes of sera, needed for further studies. The rabbit antipeptide antisera were tested for reaction with pertussis toxin, peptide (sequence as in the immunogen) and peptide-conjugate as well as with a peptide derived from fim 3 of *Bordetella pertussis* in a solid phase ELISA assay. The rabbit antipeptide antisera were also tested for reaction with pertussis toxin captured onto ELISA plates by monoclonal antibody as described in section 2.2.8.1 and for reaction with pertussis toxin subunits separated by SDS PAGE. Other methods are described in each relevant section of the text.

3.2.3 Monoclonal antibodies

Monoclonal antibodies were prepared as described by Gorringe *et al.* (1985). Monoclonal antibody was purified from ascites fluid as described in section 2.2.4. The anti-pertussis toxin titre of each antibody in ascites fluid was measured using a solid phase ELISA as before and results are shown in table 3.1. Briefly, 1-2 ml was dialysed against column equilibration buffer and applied to a DEAE sepharose CL6B column after centrifugation to remove debris, or the ascites fluid was diluted to 10 ml using equilibration buffer and applied to a Mono Q HR 5/5 column through a superloop. Each column was eluted as described in section 2.2.4 and fractions containing protein determined by absorbance at 280 nm were tested for anti-pertussis toxin activity using a solid phase ELISA. Those fractions containing most anti-pertussis toxin antibody judged by the ELISA test were pooled (see table 3.1 for anti-pertussis toxin titres of the purified antibodies) and reacted with the pin decapeptides at decreasing dilution, the highest dilution being 1:200, decreasing to 1:100, 1:10 and 1:2 (the least dilution).

Purification of the antibodies gave similar chromatographic profiles,

Fig. 3.2 Typical anion exchange profile

Ascites fluid containing monoclonal antibody was diluted to 10 ml using 5 mM Tris-Cl, pH 8.0 and applied to a Mono-Q column through a superloop. The column was then eluted with 5 mM Tris-Cl, 200 mM NaCl, pH 8.0 and fractions containing assayed for anti-(pertussis toxin) activity using a solid phase ELISA using pertussis toxin as coating antigen.

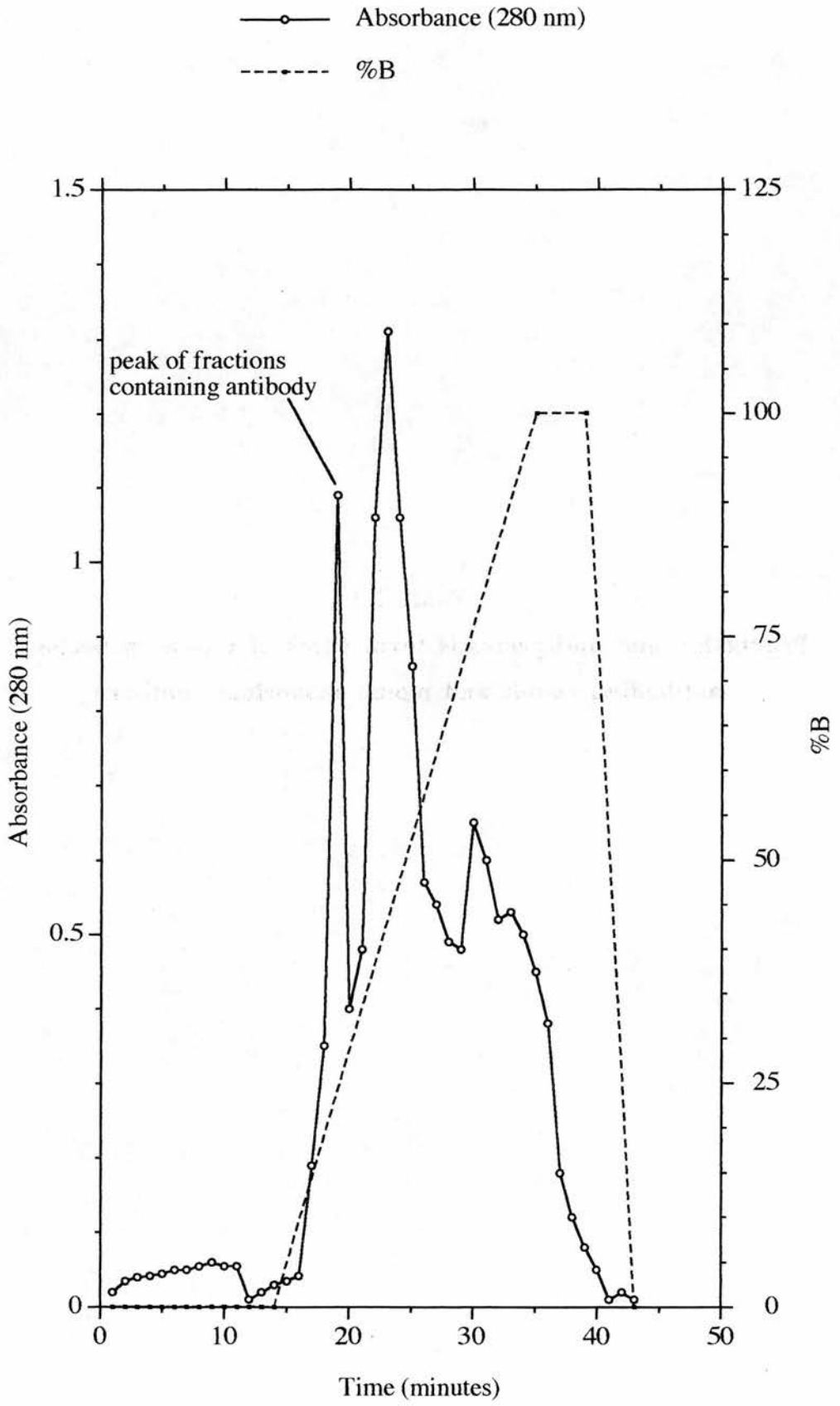

Absorbance (280 nm)
%B
peak of fractions
containing antibody
Absorbance (280 nm)
%B
Time (minutes)
1.5
1
0.5
0
125
100
75
50
25
0
0
10
20
30
40
50

Table 3.1

Production and anti-pertussis toxin titres of mouse monoclonal antibodies, rabbit and mouse monoclonal antisera

Antibody	IgG isotype	Immunising antigen		Anti-pertussis toxin titre (a)		
		primary	secondary	ascites fluid	purified antibody	serum
Mouse MAB						
L3	2a	dPT	PT	43721	/	/
L4	1	as above		18232	/	/
L5	1	"	"	15070	/	/
L6	/	"	"	199710	74595	/
L7	2a	"	"	118610	84585	/
L9	2a	"	"	153977	97634	/
L10	2a	"	"	559548	367616	/
L11	2a	"	"	4375	/	/
L12	1	"	"	298754	145478	/
L13	1	"	"	414086	120020	/
Mouse PAB						
4/292	/	"	"	/	/	86400
289/9	/	"	"	/	/	65214
Rabbit PAB						
12A20A	/	PT	PT	/	/	166600
985/989	/	PT	None	/	/	323487
978/983	/	PT	None	/	/	28676

(a). Results are expressed as ELISA titres (reciprocal of end-point dilution that gave an absorbance of 1.0 at 450 nm).

PT: pertussis toxin.
dPT: toxoided pertussis toxin.

independent of the two methods mentioned above and a typical anion exchange profile is shown in figure 3.2. The first peak, which can be seen at 19 minutes was found to contain the highest anti-pertussis toxin activity, shown in table 3.1, judged from a solid phase ELISA using pertussis toxin as coating antigen.

The monoclonal antibody L10 was the only antibody that was not purified efficiently using anion exchange, so 1 ml of L10 was purified from ascites fluid using protein A sepharose CL4B as described in section 2.2.4.3. Fractions containing protein (judged from A_{280} were tested for anti-pertussis toxin activity in a solid phase ELISA and those containing highest anti-toxin activity were pooled.

3.2.4 Efficiency of pin peptide synthesis

To assess the efficiency of the peptide synthesis indirectly, the reaction of a reference MAB provided by the suppliers of the epitope mapping kit, with two control tetrapeptides was assessed using the ELISA method described in section 2.2.3.1. One set of two tetrapeptides was synthesised simultaneously with the decapeptides and the other set was provided by the suppliers of the kit. One of the tetrapeptides, PLAQ, reacts strongly with the reference serum, but the other tetrapeptide, GLAQ, does not react. Comparison of the reaction of the reference MAB with the suppliers' control peptides and with the control peptides synthesised at the same time as the test decapeptides gives an indication of the success of the peptide synthesis.

A more direct way of assessing the efficiency of the synthesis was also used. Antisera raised against synthetic peptides (anti-S1a, -S1c, S3a, and S4b)whose sequences were derived from pertussis toxin subunits, were reacted at 1:300 dilution with the completed pin peptides, to determine whether the antisera recognised decapeptides located in the sequence of the immunising peptide.

3.3 Results

3.3.1 Western blotting of antibodies used in reaction with pin peptides

Figure 3.3 shows the subunit specificity of the antibodies reacted with the pin peptides, assessed by Western blotting of pertussis toxin subunits separated by SDS PAGE. Antibodies were reacted at 1:1000 dilution unless stated otherwise and all monoclonal antibodies were tested as ascites fluid.

As was expected, each antipeptide antibody reacted with the subunit from which the peptide sequence was raised at 1:1000 dilution.

Antibodies S1a and S1c (lanes one and two) reacted with subunit S1 and

weakly with subunits S2 and S3.

Antibodies anti-S3a, -S3b and -(E)S3c reacted with subunit S3 and weakly with S1 (lanes three to five). Cross-reaction of anti-(E)S3c was reduced but not eliminated by inclusion of 250 mM NaCl in the blot incubation buffer.

Reaction of subunit S4 with anti-S4b diluted 1:4000 in blocking buffer containing 250 mM NaCl is shown in lane six. At 1:1000 dilution anti-S4b reacted with the subunit from which the peptide was derived, and cross-reacted with subunit S3 (not shown). Several dilutions of anti-S4b were made in blot blocking buffer that contained increasing concentrations of NaCl. These dilutions were then incubated with the separated pertussis toxin subunits to assess nonspecific binding of the antiserum to immunoblots.

Monoclonal antibodies L3, L9 and L10 all reacted with subunit S1 and weakly with S3 (lanes 7, 12 and 13) and L12 only reacted with S1 (lane 15). Monoclonal L4 (lane 8) reacted very weakly with subunit S3 and L6 and L11 (lanes 10 and 14) did not show a detectable reaction with any of the subunits. Monoclonals L5, L7 and L13 (lanes 9, 11 and 16) reacted with subunit S3 and weakly with S1. When the toxin sample was prepared in a buffer containing 2-ME and blotted with L5, L7 and L13, L5 and L13 recognised subunit S3, but L7 did not.

Mouse polyclonal anti-toxin antiserum 4/292 bound all the separated subunits (lane 17). Anti-toxoid antibody 289/9 also bound the subunits, but more weakly (lane 18).

Rabbit polyclonal antiserum 12A20A bound to all the separated subunits (lane 19). 985/989 and 978/983 also bound the toxin subunits (lanes 20 and 21), but more weakly than 12A20A. The extent of binding of these antisera to the toxin subunits were not identical, possibly because the antisera contained antibodies of different clonal origin: Variations in the apparent position of the subunits in these lanes would be explained by this and possibly by slight variations in protein loading, resulting in wide bands on staining of the blots. Finally, lanes 22 and 23 show negative control immunoblots treated with anti-mouse IgG conjugated to horse radish peroxidase and anti-rabbit IgG conjugated to alkaline phosphatase, respectively.

3.3.2 Antipeptide antibody binding to pin decapeptides

3.3.2.1 Efficiency of pin peptide synthesis

Both the supplier's and the author's positive control tetrapeptides (PLAQ) gave an ELISA absorbance at 405 nm over 1.2 and the negative control tetrapeptides (GLAQ) had an absorbance less than 0.2, indicating successful synthesis of the control peptides and suggesting that the first four residues of the test decapeptides had been synthesised successfully.

Fig. 3.3

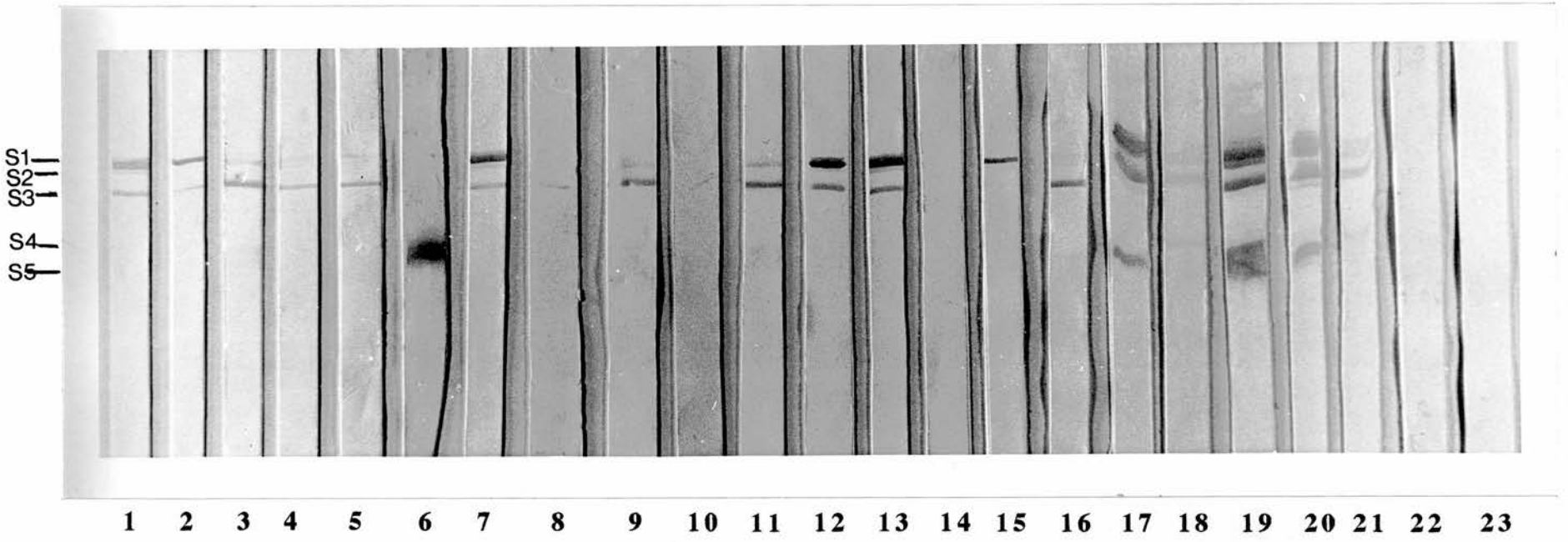
Western blotting of pertussis toxin subunits

Lane	Antibody	Subunit(s) bound
1	anti-S1a	S1 (also S2,S3)
2	anti-S1c	S1 (S2, S3)
3	anti-S3a	S3 (S1)
4	anti-S3b	S3 (S1)
5	anti(E)S3c	S3 (S1)
6	anti-S4b	S4
7	L3	S1 (S3)
8	L4	S3
9	L5	S3 (S1)
10	L6	None
11	L7	S3 (S1)
12	L9	S1 (S3)
13	L10	S1 (S3)
14	L11	None
15	L12	S1
16	L13	S3 (S1)
17	4/292	all (S1-S5)
18	289/9	all (S1-S5)
19	12A20A	all (S1-S5)
20	985/989	all (S1-S5)
21	978/983	all (S1-S5)
22	anti-mouse IgG conjugate (a)	None
23	anti-rabbit IgG conjugate (b)	None

(a), (b). anti-mouse and anti-rabbit IgG conjugated to alkaline phosphatase

(c). All antipeptide antisera reacted with blots in PBS pH 7.2 (final concentration of NaCl 250 mM)

Subunits in parentheses indicate weak cross-reaction with antibody


S1
S2
S3
S4
S5
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

Synthesis of the subunit decapeptides was confirmed indirectly using rabbit antipeptide antibodies. The amino acid sequences of the peptides used to raise these sera and anti-pertussis toxin titres of the sera in a solid phase ELISA (see section 2.2.3.1 for the ELISA method) are given in table 3.2. The antipeptide sera were reacted with the pin decapeptides from subunits S1, S3 and S4 and these were used as controls. For example, anti-S1a was reacted with S3 and S4 decapeptides, which contained decapeptides not in the immunising peptide.

Two rabbit-antipeptide antisera, anti-S1a and anti-S1c (kindly provided by Dr. R.N. Seabrook, PHLS, Porton, U.K.) were used to confirm the synthesis of the S1 subunit decapeptides. These antisera were raised against two synthetic peptides from the S1 amino acid sequence, each conjugated to a carrier. Their production is described elsewhere (Seabrook *et al.*, 1990).

Figure 3.4 shows the reaction of anti-S1a with S1 decapeptides with a five amino acid overlap. As was expected, the antibody recognised decapeptides that were in the sequence of the immunising peptide, confirming the peptide synthesis, but the strength of reaction differed between reacting peptides. Decapeptide 1 had the strongest reaction, followed by decapeptide 3, then 2, so residues within decapeptides 1 and 3 seem important for recognition by anti-S1a.

Figure 3.6 shows the reaction of anti-S1a with S1 decapeptides with an eight amino acid overlap. Anti-S1a bound decapeptides containing residues from the N- and C-terminal regions of the immunising peptide, confirming the peptide synthesis.

Again the strongest reaction was with decapeptides that were in the sequence of the immunising peptide and again the strength of reaction differed between reacting peptides. Decapeptide 1 from the N-terminal sequence of S1, (the same sequence as decapeptide 1 above) again had the expected strong reaction with anti-S1a, but peptide 2 did not react at all. This suggests that residues 1 and/or 2 of S1 are important in the binding of anti-S1a because only these residues differ between peptides 1 and 2. However, internal peptide residues may also be involved in the interaction.

Three more decapeptides, 5, 6 and 7, also found in the sequence of the immunogen, reacted strongly with anti-S1a, but peptide 4, which has only two amino acids different from peptide 5, did not bind anti-S1a. This implicates residues 17 and/or 18 in decapeptide 5, in the epitope bound by anti-S1a. Strength of reaction with anti-S1a decreased between decapeptides 6 and 7, implying that residues 19 and 20 (decapeptide 6) may also be involved in binding of this antibody. Weaker reaction was seen with decapeptide 7, so residues in this peptide could be involved in anti-S1a binding, as the binding to decapeptide 7 is lost in decapeptide 8.

Figure 3.5 shows anti-S1c binding to S1 decapeptides with a five amino acid overlap. Anti-S1c only recognised decapeptide 42 that contained residues in the sequence of the immunising peptide and so confirmed the synthesis. A strong reaction was seen with this peptide, suggesting that most, if not all residues necessary for

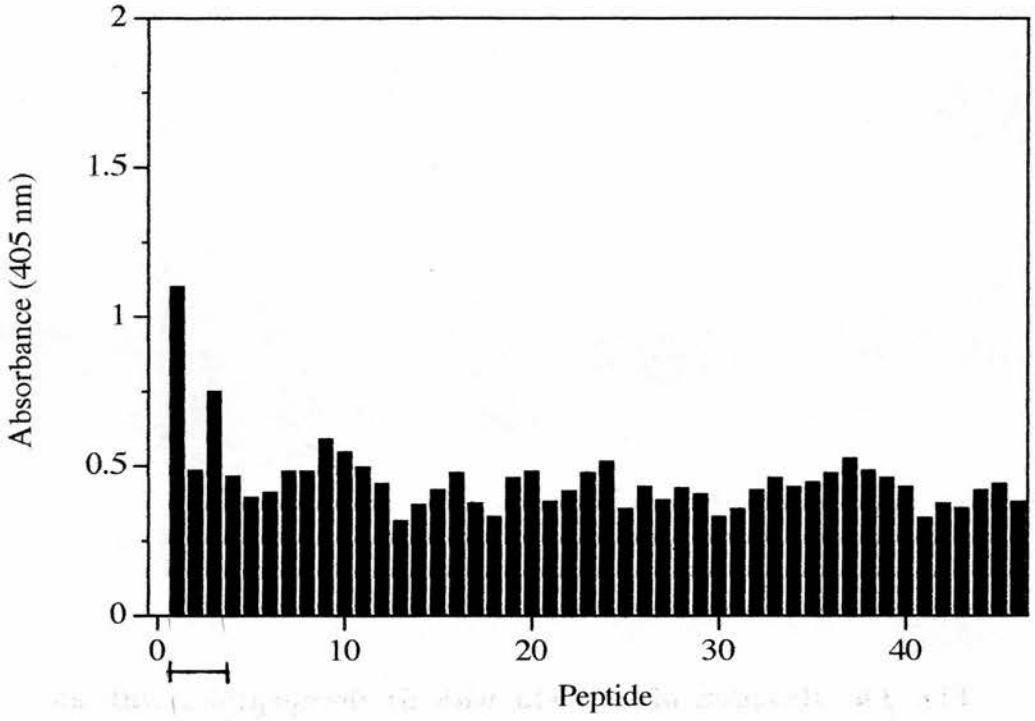
Fig. 3.4 Reaction of anti-S1a with S1 decapeptides with a five amino acid overlap

Decapeptides were incubated with blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v)Tween 20) for 90 minutes and then with rabbit anti-S1a at a dilution of 1:300, overnight. Reaction with the decapeptides was detected by ELISA (see section 2.2.3.1 for details).

The bar (⊢——⊣) shows pin decapeptides contained in the peptide immunogen.

Fig. 3.5 Reaction of anti-S1c with S1 decapeptides with a five amino acid overlap

Anti-S1c was reacted with the decapeptides at a dilution of 1:300, other experimental conditions being as above.


Absorbance (405 nm)
2
1.5
1
0.5
0
0
10
20
30
40
Peptide

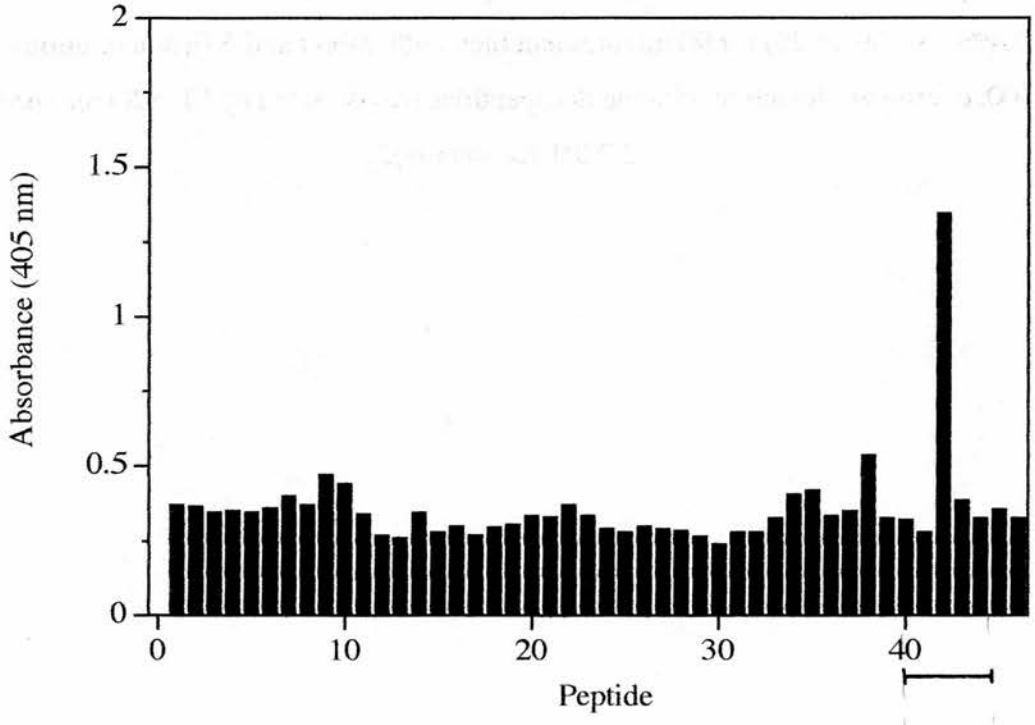

Absorbance (405 nm)
2
1.5
1
0.5
0
0
10
20
30
40
Peptide

Fig. 3.6 Reaction of anti-S1a with S1 decapeptides with an eight amino acid overlap

Decapeptides were incubated with blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v)Tween 20) for 90 minutes and then with rabbit anti-S1a at a dilution of 1:300, overnight. Reaction with the decapeptides was detected by ELISA (see section 2.2.3.1 for details).

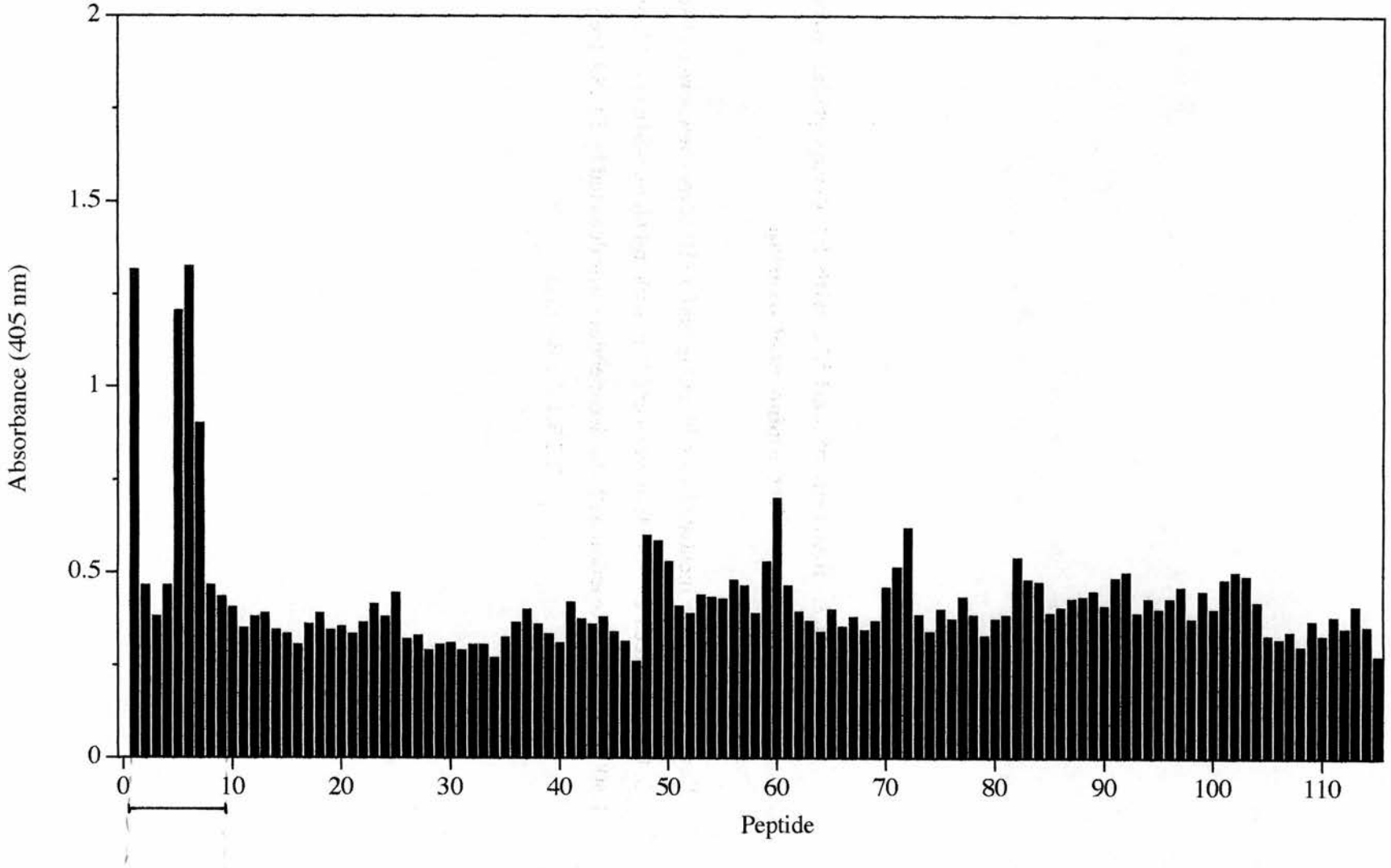
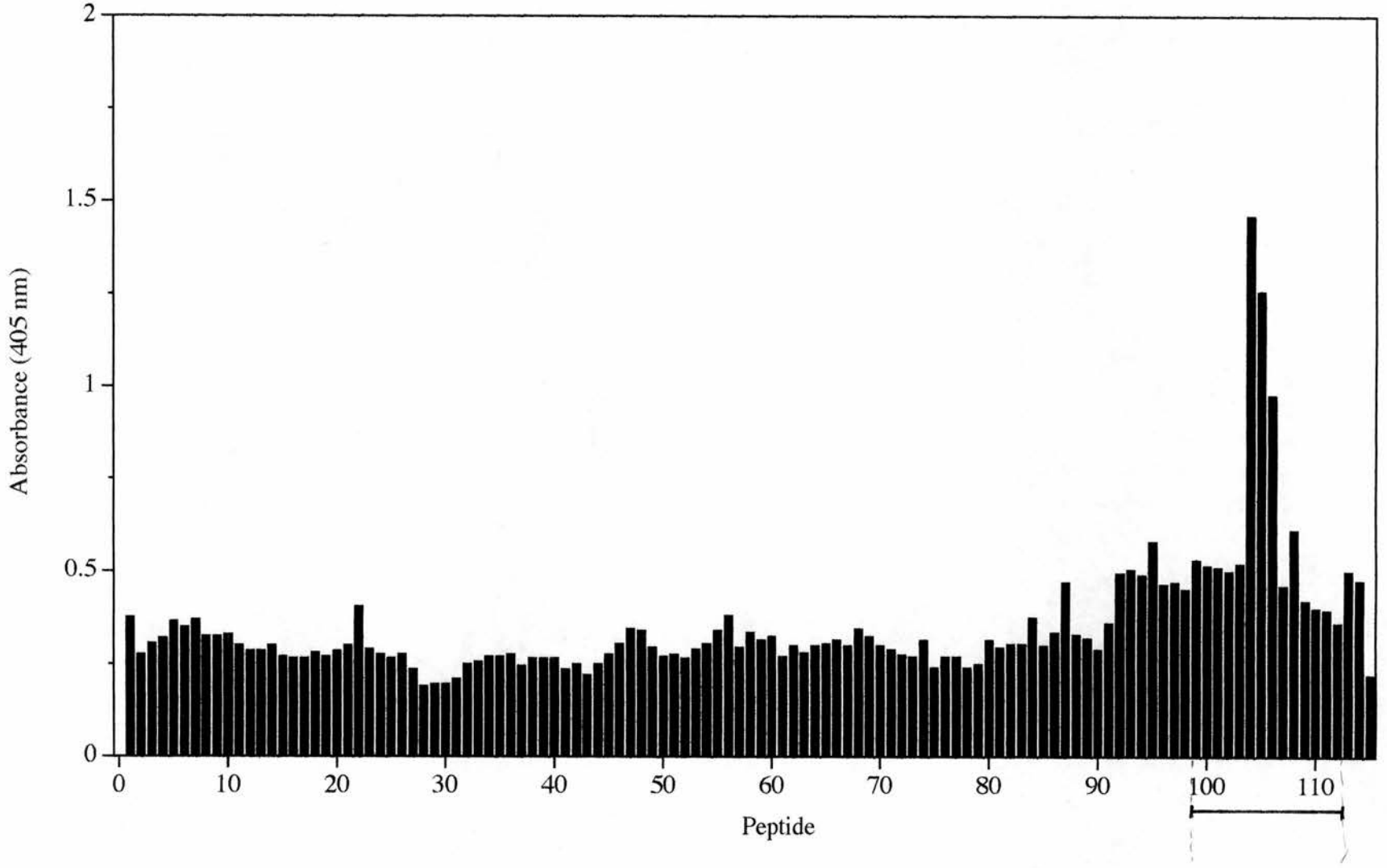

Absorbance (405 nm)
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
90
100
110
Peptide

Fig. 3.7 Reaction of anti-S1c with S1 decapeptides with a five amino acid overlap

Decapeptides were incubated with blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v)Tween 20) for 90 minutes and then with rabbit anti-S1c at a dilution of 1:300, overnight. Reaction with the decapeptides was detected by ELISA (see section 2.2.3.1 for details).


Absorbance (405 nm)
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
90
100
110
Peptide

antibody binding were contained in this decapeptide.

Anti-S1c was also tested against S1 decapeptides with an eight amino acid overlap (fig. 3.7) and was found to react strongly with peptides 104, 105 and 106 as expected because all three peptides have sequence in common with the reactive peptide (42) of S1 decapeptides with a five amino acid overlap. This suggests that the N-terminal residues of the immunising peptide (237-247) interacted with anti-S1c.

One rabbit-antipeptide antibody, anti-S3a, was used to confirm the synthesis of the S3 subunit decapeptides. It was kindly provided by Dr. R.N. Seabrook (PHLS, Porton, U.K.). Its production is described elsewhere (Seabrook *et al.*, 1990).

Figure 3.8 shows binding of anti-S3a to S3 decapeptides. Strong reactions were observed with decapeptides 14, 15 and 16 and all three peptides were contained within the sequence of the immunising peptide, so confirming the decapeptide synthesis.

The strongest binding was seen with decapeptide 15, compared with slightly weaker binding seen with decapeptides 16 and 14. This suggests that N-terminal residues in the immunising peptide bound anti-S3a more strongly than C-terminal residues in the immunising peptide, but that both regions contributed to the overall binding of anti-S3a.

The synthesis of the S4 decapeptides was also confirmed by reaction with an antipeptide antibody. At the time of synthesis, an anti-S4 antipeptide antibody that bound the pin peptides was not available, so a decapeptide that reacted strongly with a polyclonal antibody (12A20A) was synthesised and rabbit antisera raised against a KLH-conjugate of that peptide (section 3.3.6.5.1). The sequence of the peptide and the anti-pertussis toxin titre of the antibody are given in table 3.2. Anti-S4b was found to react with two S4 decapeptides (17 and 18, figure 3.9) that contained residues in the sequence of the immunising peptide and so confirmed the S4 peptide synthesis. The strongest reaction was with decapeptide 18, which consisted of the whole amino acid sequence of the immunising peptide. A slightly weaker reaction was seen with peptide 17, which only contained five from the ten amino acids used as immunogen. This indicates that amino acids in the N- and C-terminal of the immunogen were involved in binding of this antibody.

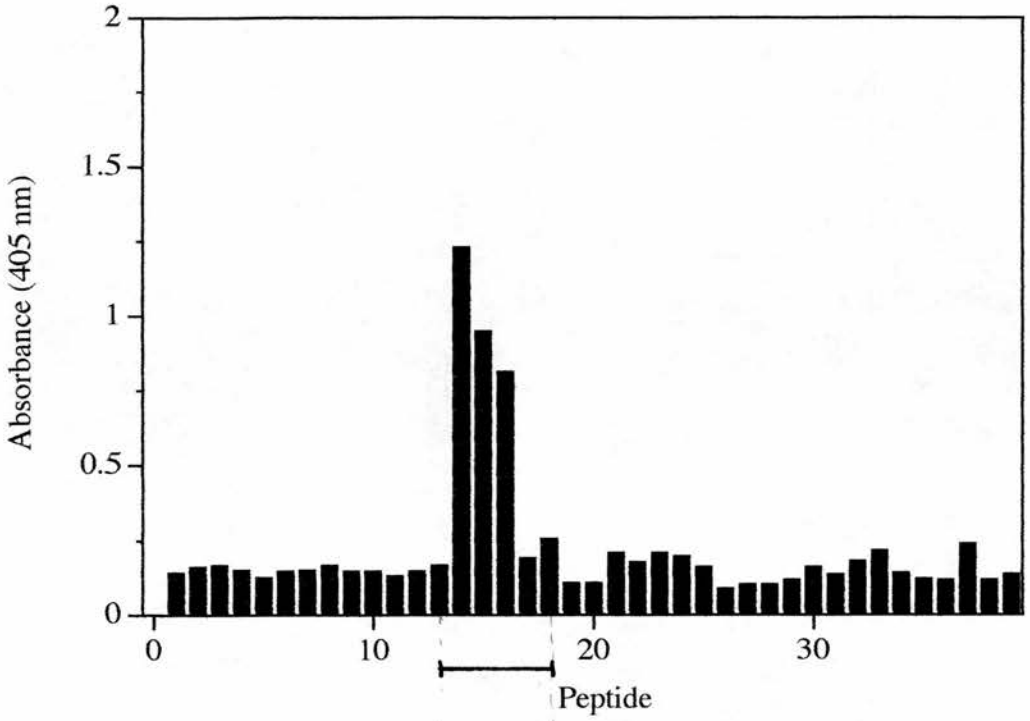
In summary, reactions of rabbit antisera raised against synthetic peptides with the decapeptides confirmed as far as possible that the syntheses consisted of the expected amino acid sequences. Each antibody reacted with pertussis toxin used as coating antigen in a solid phase ELISA, shown in table 3.2 and with pertussis toxin subunits separated by SDS PAGE and immunoblotted (fig. 3.3).

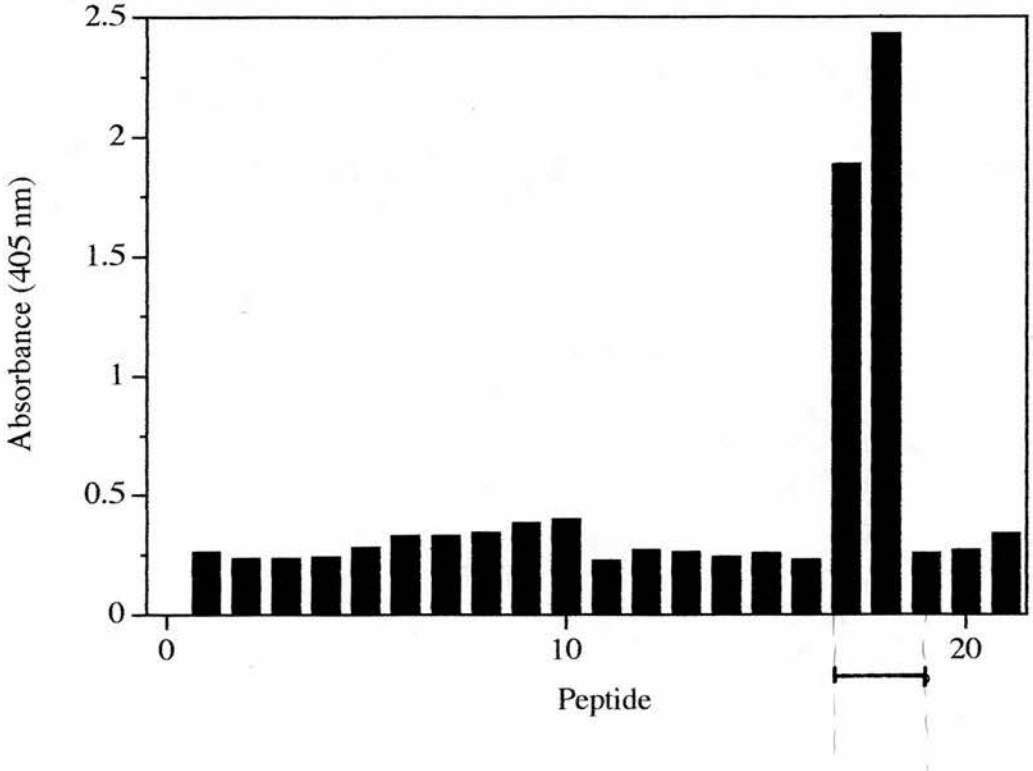
Fig. 3.8 Reaction of anti-S3a with S3 decapeptides with a five amino acid overlap

Decapeptides were incubated with blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v)Tween 20) for 90 minutes and then with rabbit anti-S3a at a dilution of 1:300, overnight. Reaction with the decapeptides was detected by ELISA (see section 2.2.3.1 for details). As previously, the bar below the graph shows decapeptides contained in the amino acid sequence of the peptide immunogen

Fig. 3.9 Reaction of anti-S4b with S4 decapeptides with a five amino acid overlap

Experimental conditions were as described above, the dilution of anti-S4b being 1:300.


Absorbance (405 nm)
2
1.5
1
0.5
0
0
10
20
30
Peptide


Absorbance (405 nm)
2.5
2
1.5
1
0.5
0
0
10
20
Peptide

3.3.2.2 Reactions of antipeptide antisera with all the pin peptides

Cross-reaction of antibodies with peptides that contain similar partial sequences have been reported (Jacob *et al.*, 1983; Kazemi and Finkelstein, 1991; Trifilieff *et al.*, 1991).

It was decided to investigate cross-reaction of a panel of rabbit antipeptide antibodies raised against pertussis toxin subunit sequences, at 1:300 dilution, with the pin peptides from the sequences of the other subunits that were synthesised in this study. Antipeptide antibodies to the S2 and S5 subunits were not tested as they were not available at the time of study.

Some of the antipeptide antisera in the study bound on Western blots to subunits other than the ones from which their peptide sequences were derived (figure 3.3), it was interesting to see if these antisera cross-reacted with pin peptides derived from the same subunit(s) as the ones they cross-reacted with on Western blots.

The panel of antibodies tested included anti-S1a, -S1c, -S3a, and -S3b antipeptide antibodies, which were raised in rabbits as mentioned above (Seabrook *et al.*, 1990). Their anti-pertussis toxin titres and sequences of the peptide immunogens are given in table 3.2 . Two other rabbit-antipeptide antibodies that were raised during this study were also tested, anti-S4b and -(E)S3c (see section 2.2.5 for details of their production and table 3.2 for the peptide sequences that they were raised against).

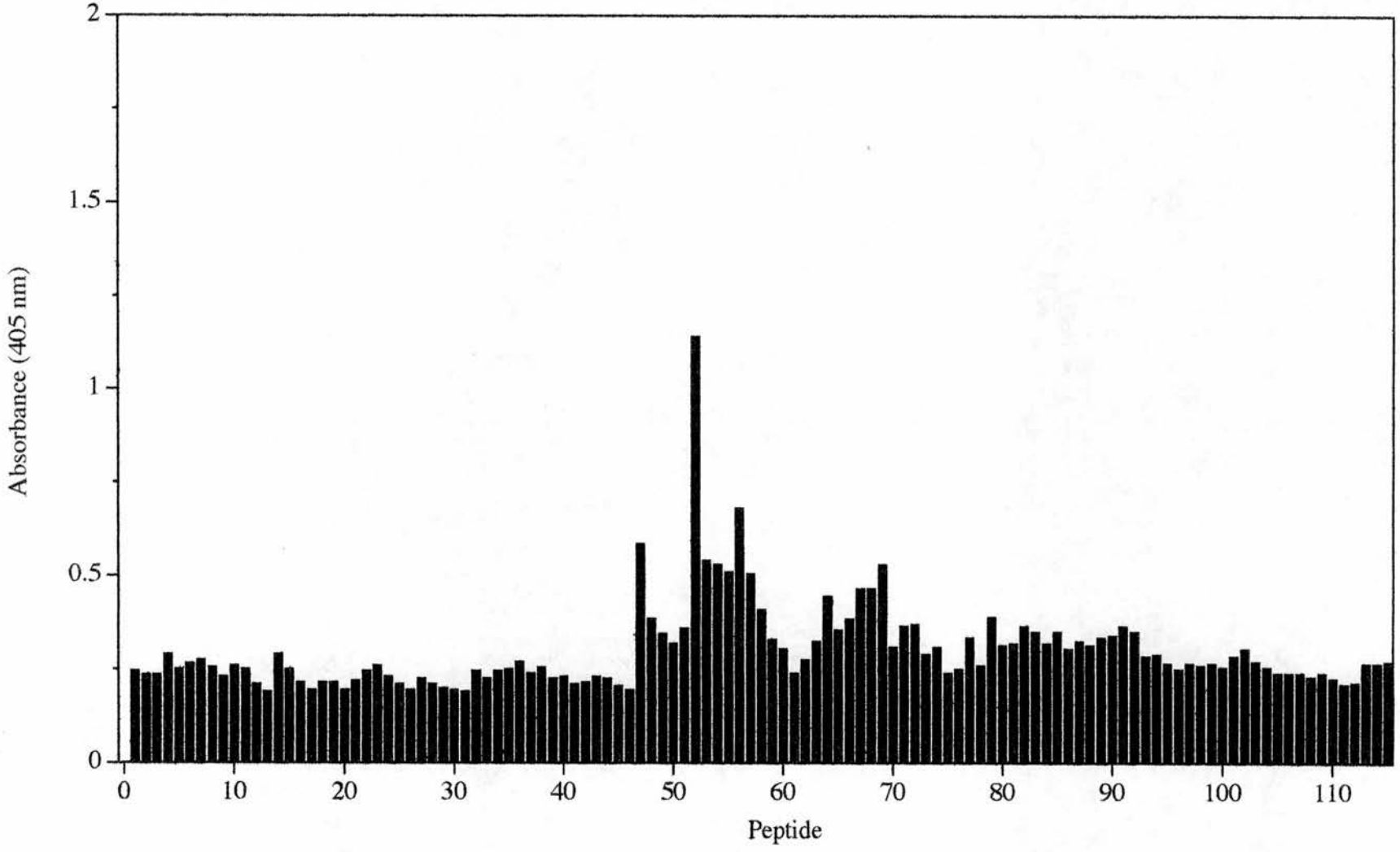
Anti-S1a did not cross-react with S3 or S4 decapeptides. Anti-S1c cross-reacted weakly (about 30% above the background absorbance at 405 nm of 0.32) with two S3 decapeptides (6 and 23), both of which have only two amino acids homologous with the peptide used to raise anti-S1c. This binding could be due to the partial sequence identity between the decapeptides and the peptide immunogen. Two amino acids identity in an S4 decapeptide (21) with the peptide immunogen used to raise anti-S1c could be responsible for the weak cross-reaction (25% above background absorbance at 405 nm of 0.27) of S4 decapeptides with anti-S1c.

Anti-S3a reacted strongly with one peptide (52) of S1 decapeptides with an eight amino acid overlap (figure 3.10). This decapeptide was part of a region of reactive decapeptides (52-57) and had three non-adjacent amino acids identity with the anti-S3a peptide immunogen. Anti-S3a also reacted with S1 decapeptide 47, which had only one amino acid in common with the peptide used to raise anti-S3a.

Anti-S3a did not cross-react with S1 decapeptides with a five amino acid overlap. Decapeptide 56 (8 residue overlap) had the same amino acid sequence as decapeptide 23 (5 residue overlap), but the latter decapeptide (23) did not react with anti-S3a, possibly because those pin peptides had been screened a considerable number of times when this reaction was performed.

Fig. 3.10 Reaction of anti-S3a with S1 decapeptides with an eight amino acid overlap

Decapeptides were incubated with blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v)Tween 20) for 90 minutes and then with rabbit anti-S3a at a dilution of 1:300, overnight. Reaction with the decapeptides was detected by ELISA (see section 2.2.3.1 for details).


Absorbance (405 nm)
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
80
90
100
110
Peptide

The lack of reaction of anti-S3a with S1 decapeptides with a five amino acid overlap and the strong reaction with S1 decapeptides with an 8 residue overlap highlights the problem that whatever arbitrary pin peptide overlap of amino acids (five, for instance) is decided upon, there is no guarantee that an antibody binding site of greater that five residues will be within one peptide.

Anti-S3a also cross-reacted with S1 when pertussis toxin subunits were separated by SDS PAGE. Binding of this antibody was not reduced to background by inclusion of 250 mM NaCl to either the pin or immunoblot antibody incubation buffers. (higher concentrations of NaCl were tried but binding to S1 was not totally inhibited).

Anti-S3a weakly cross-reacted with S4 decapeptide 17, which has only two amino acids identical with the peptide immunogen and could account for the weak reaction.

Anti-S3b did not cross-react with any S1 decapeptides with a five amino acid overlap or eight residue overlap or with S3 or S4 decapeptides. This antibody had a low anti-pertussis toxin titre (pertussis toxin coating antigen) as seen in table 3.2. A possibility for the lack of reaction with the pin peptides is that the peptide is that not all residues required for anti-S3b binding were contained in any S3 pin decapeptide.

Anti-(E)S3c reacted with weakly with S3 decapeptides 11 and 14, that were contained in the immunising peptide and with decapeptides 16, 25 and 27, all of which contained partial sequences in the immunising peptide sequence. None of the peaks were over 25% above background binding (absorbance average 0.32).

Anti-(E)S3c cross-reacted weakly with S1 decapeptides with an eight amino acid overlap: peptides 10-13 cross-reacted with anti-(E)S3c as did peptide 18. These peptides did not possess more than two amino acids identity with the immunising peptide and the cross-reaction was reduced to background levels by incubation of the antibody with the pins in a buffer containing 250 mM salt. The cross-reaction was also seen with Western blots using anti-(E)S3c against pertussis toxin subunits separated by SDS PAGE and was reduced by incubating the antibody with the immunoblots in a buffer containing 250 mM NaCl (see chapter 4). Anti-(E)S3c was also be expected to react with the S2 subunit, since peptide (E)S3c has sequence homology with the same region of S2, but cross-reaction with the S2 subunit was not detected on immunoblots.

Anti-S4b, raised against a pin decapeptide that was found to bind a polyclonal antibody, (the sequence of the peptide immunogen is given in table 3.2) cross-reacted with decapeptides 17 and 18 that were contained in the immunising peptide, the strongest reaction being with the decapeptide containing the whole sequence of the immunising peptide (fig. 3.9). Anti-S4b did not cross-react with the other pin decapeptides.

Table 3.3 Reaction of rabbit antipeptide antisera with pin decapeptides

(Subunit-specificity of rabbit antipeptide antisera)

a, b, c, d. Amino acid overlap of pertussis toxin subunit pin decapeptides

Antipeptide sera		Subunit	Decapeptides	
	S1 5 residue (a)	S1 8 residue (b)	S3 5 residue (c)	S4 5 residue (d)
anti-S1a	++	++	-	-
anti-S1c	++	++	+	+
anti-S3a	-	++	++	+
anti-S3b	-	-	-	-
anti-(E)S3c	+	+	+	-
anti-S4b	-	-	-	++

-: no cross-reaction
+: weak cross-reaction
++: strong cross-reaction

In short, some antipeptide antisera cross-reacted weakly with pin decapeptides with as few as two amino acids identity with the immunising peptide. The only antipeptide antibody that strongly cross-reacted with pin peptides was anti-S3a, which reacted with S1 decapeptides with an eight amino acid overlap (Table 3.3).
This means that reaction of antipeptide antibodies, especially weak reaction with the pin decapeptides, must be viewed with scepticism in the first instance, and even strong reactions with antipeptide antisera (e.g. anti-S3a with S1 decapeptides) could be due to reasons other than sequence identity, for example peptide flexibility, as mentioned earlier. Antiprotein antibodies are less subject to cross-reaction with the pin decapeptides than antipeptide antisera, as is shown in sections 3.3.3, 3.3.4.

3.3.3 Reaction of pin decapeptides with mouse polyclonal antibodies

Reactions of a mouse anti-(pertussis toxin) antibody (4/292), mouse anti-pertussis toxoid(289/9) antibody and convalescent mouse serum with the pin peptides were assessed and compared with the reactions of rabbit and human antisera. The convalescent mouse serum sample was kindly provided by Dr. K. Redhead (NIBSC, Potters Bar, U.K.) and the mouse anti-(pertussis toxin) antisera were generously supplied by Dr. A. Robinson, PHLS CAMR.

Table 3.1 shows the anti-pertussis toxin titre of antibodies 4/292 and 289/9 in a solid phase ELISA using pertussis toxin as the coating antigen. Just enough convalescent mouse serum was available to react with the pin decapeptides at 1:250 dilution in pin blocking buffer (2.2.3.1), so an anti-pertussis toxin titre was not obtained for this sample.

4/292 and 289/9 were reacted with the pins at both 1:300 and 1:200 dilution in pin blocking buffer. None of the three sera reacted against the pin decapeptides.

3.3.4 Reaction of monoclonal antibodies with pin peptides

The pin peptides were screened for reaction with a panel of mouse monoclonal antibodies (MABs), to identify linear antigenic determinants, i.e. peptides that bound antibody. Mouse monoclonal antibodies were used as ascites fluid or were purified by DEAE-sepharose or by Mono-Q anion exchange chromatography and then reacted with the pin peptides. As stated earlier, a large proportion of B cell epitopes are thought to be conformational, and the aim of these experiments was to identify peptides that either form subsites of discontinuous epitopes or at least mimic part of the antigenic epitope with which the antibody paratope interacts.

Monoclonal antibody (MAB) that was raised against another protein (fim 2 from pertussis toxin fimbriae) was used as a negative reaction control. This

monoclonal antibody did not react with the pin decapeptides, as ascites fluid or purified antibody and gave an average background of 0.2. Anti-pertussis toxin MABs that did not react with the pin peptides were also used as negative controls.

The panel of antibodies, their IgG isotype and anti-pertussis toxin titre is given in table 3.1. Each set of pin decapeptides from subunits S1 (both five and eight amino acid overlap), S3 and S4 (both five amino acid overlap) were reacted with each MAB as ascites fluid at 1:100 dilution and as purified antibody diluted as little as possible (maximum dilution 1:4), which was prepared at the same time as the pin peptides were screened with the ascites fluid.

There were three exceptions to this scheme, being L3, L5 and L11, which were reacted as ascites fluid only with the pin decapeptides. As they did not react at all with the decapeptides as ascites (generating a background level of absorbance of 0.15 or less), they were not purified as there was little chance of the purified antibody reacting with the pin peptides.

Most of the MABs did not cross-react with the pin decapeptides, either as ascites fluid or as purified antibody. An exception was L10, which reacted weakly with the S1 pin decapeptides (5 overlapping residues) 11, 23 and 24 (44, 37 and 46% above the average background of 0.4 at 405 nm as ascites fluid). It was hoped that purification of L10 using protein A-sepharose would reduce the background binding and reveal specific binding peaks. This cannot have been the case, because no reaction with the S1 decapeptides was seen, even when the purified L10 was reacted with the pins at 1:4 dilution (background level of absorbance was 0.25 at 405 nm).

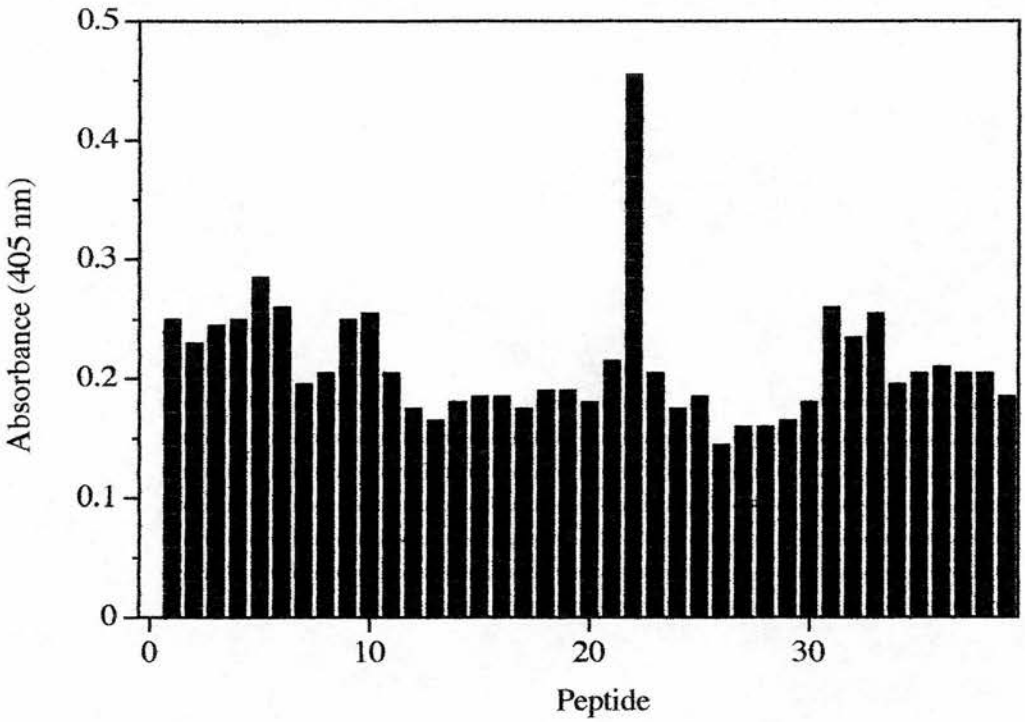
L10 reacted weakly with S3 decapeptides as ascites fluid. One S3 peptide (peptide 1, A_{405} 0.38) bound L10 to produce an absorbance almost double the background level (A_{405} 0.145). However, after purification L10 did not bind any of the S3 decapeptides above background level.

Monoclonal antibodies L7 and L9 both reacted weakly as ascites fluid with S3 decapeptides. Neither reacted with single peptides but they did react with regions of peptides. L7 as ascites fluid reacted weakly with decapeptides 6-8, 10, 21-23, 32 and 34 (background A_{405} 0.6, maximum peak absorbance 1.175). When reacted with purified L7, S3 decapeptides 22 and 39 generated an absorbance distinctly above the background level.

L9 as ascites fluid reacted weakly with S3 decapeptides 11 and 22 and the level of background binding was high at 0.45. Purified L9 bound to one decapeptide (22) to give an absorbance at 405 nm double that of the background (fig. 3.11).

Fig. 3.11 Reaction of purified monoclonal antibody L9 with S3 decapeptides with a five amino acid overlap

Decapeptides were incubated with blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v)Tween 20) for 90 minutes and then with L9 overnight. Reaction with the decapeptides was detected by ELISA (see section 2.2.3.1 for details).


0.5
0.4
0.3
0.2
0.1
0
Absorbance (405 nm)
0
10
20
30
Peptide

3.3.5 Binding of rabbit antisera by pin decapeptides

3.3.5.1 Results of binding of rabbit antisera

Three rabbit polyclonal antisera (12A20A, 985/989 and 978/983) raised against pertussis toxin were kindly provided by Dr. L. Irons (PHLS, Porton, U.K.). Sample 985/989 was raised with a primary dose of 50 μg pertussis toxin in incomplete Freund's adjuvant with no booster and harvested 16 weeks after the first dose.

Sample 12A20A was raised with a primary dose of 10 μg pertussis toxin in incomplete Freund's adjuvant , boosted at three weeks with 50 μg pertussis toxin in the same adjuvant and the serum harvested 20 weeks after the initial dose.

Sample 978/983 was raised with a primary dose of 50 μg pertussis toxin in alhydrogel adjuvant. No booster was administered and the serum was harvested after 16 weeks.

Anti-(pertussis toxin) titres in a solid phase ELISA using pertussis toxin as coating antigen are given in table 3.1.

Sample 978/983 did not cross-react with any pin decapeptides. This antibody was reacted with the pin decapeptides at dilutions ranging between 1:400 and 1:50 and generated no binding peaks in the pin ELISA test.

The rabbit anti-(pertussis toxin) antibody 12A20A bound pertussis toxin adsorbed onto ELISA plates, as did 985/989 (table 3.1). Despite the fact that 12A20A had a lower anti-pertussis toxin titre than 985/989, 12A 20A had the strongest reaction of all three rabbit antibodies with the pin decapeptides. 12A20A was reacted with the pin decapeptides at 1:500 to 1:100, but 1:100 dilution gave a high background level of binding to the decapeptides, so 1:400 was chosen for inclusion in figure 3.12a, because the background binding level was low.

Sample 12A20A (fig. 3.12a) bound strongly to peptides 3 and 41 of S1 (5 residue overlap), when diluted 1:400 and also cross-reacted with S1 decapeptides (8 residue overlap) as antibody 985/989 did when diluted 1:100. S1 decapeptides (8 residue overlap) 5 and 7, 102-104 were strongly bound and decapeptides 15,16 and 65 were bound more weakly (fig 3.13). Decapeptide 6 reacted weakly with 12A20A, possibly because this decapeptide was more difficult to synthesise than decapeptides 5 or 7.

The S1 decapeptides (8 overlap) bound were similar for 985/989 and 12A 20A but the level of reactivity with the peptides did differ. 12A 20A reacted most strongly with decapeptides 5 and 103 as can be seen in figure 3.13, whereas 985/989 reacted most strongly with decapeptide 6 and more weakly with decapeptide 102.

Sample 12A20A did not cross-react with S3 decapeptides at dilutions between 1:400 and 1:100. Lower dilutions were not tested because decreasing the antibody dilution only succeeded in raising the level of background binding.

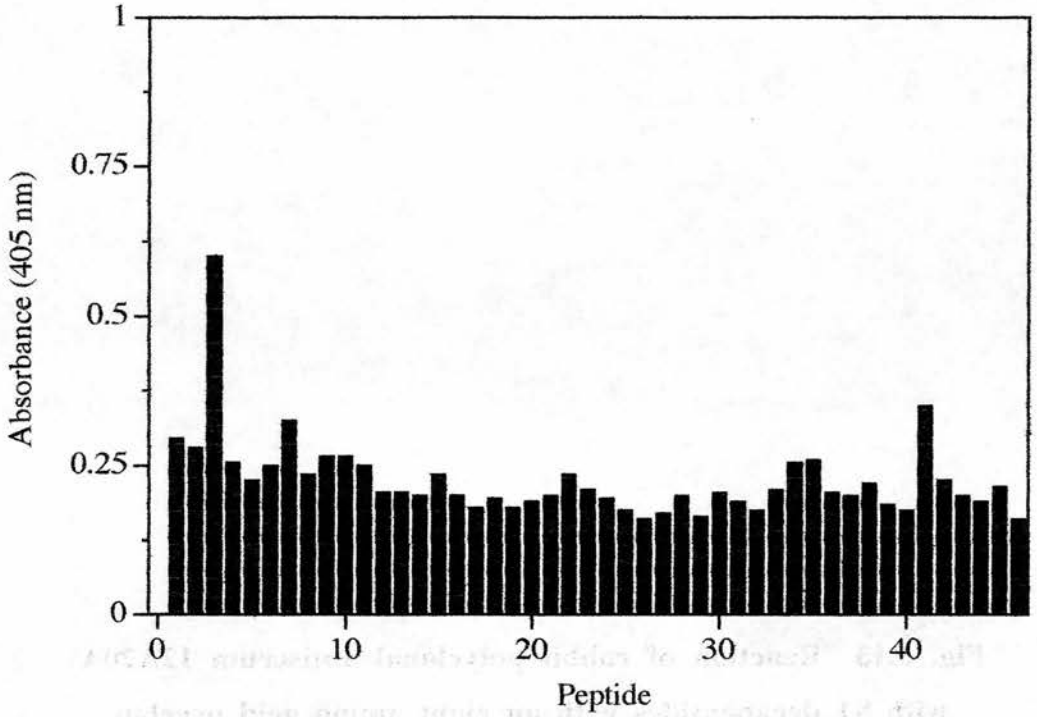
Fig. 3.12a Reaction of polyclonal antiserum 12A20A with S1 decapeptides with a five amino acid overlap

Decapeptides were incubated with blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v)Tween 20) for 90 minutes and then with 12A20A at a dilution of 1:400 overnight. Reaction with the decapeptides was detected by ELISA (see section 2.2.3.1 for details).

Fig. 3.12b Reaction of polyclonal antiserum 12A20A pre-incubated with 20 μg/ml pertussis toxin, with S1 decapeptides with a five amino acid overlap

Experimental conditions were as above, with 20 μg pertussis toxin added to the antibody incubation step.

a


Absorbance (405 nm)
1
0.75
0.5
0.25
0
0
10
20
30
40
Peptide

b.

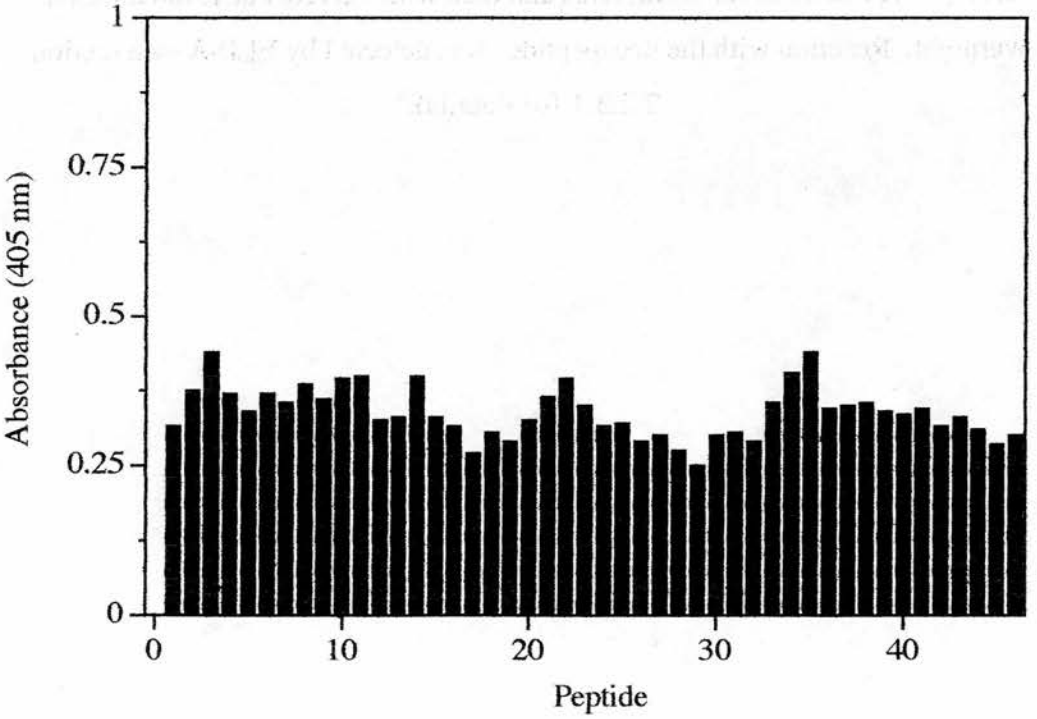
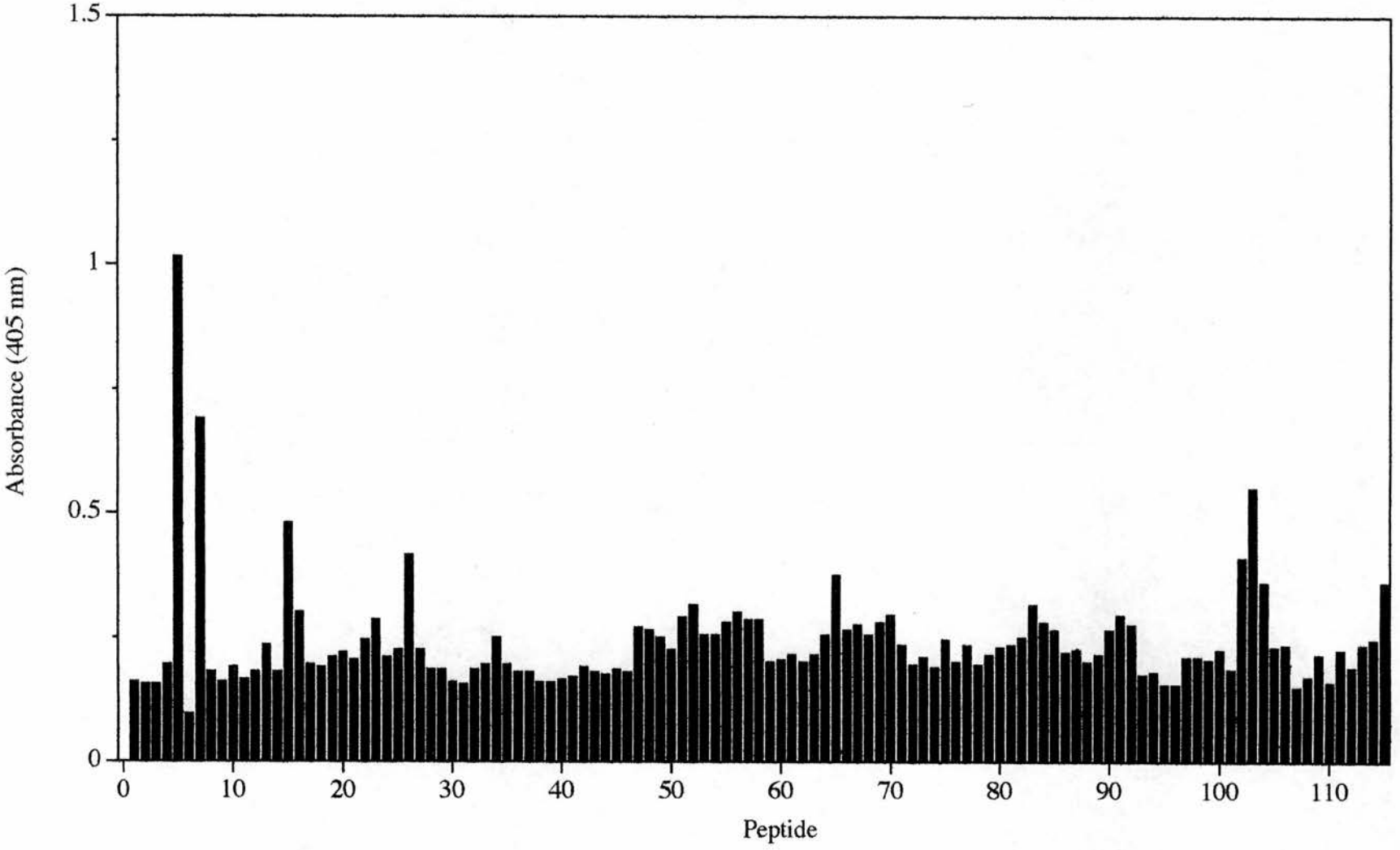

Absorbance (405 nm)
1
0.75
0.5
0.25
0
0
10
20
30
40
Peptide

Fig. 3.13 Reaction of rabbit polyclonal antiserum 12A20A with S1 decapeptides with an eight amino acid overlap

Decapeptides were incubated with blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v)Tween 20) for 90 minutes and then with 12A20A at 1:400 dilution overnight. Reaction with the decapeptides was detected by ELISA (see section 2.2.3.1 for details).


1.5
1
0.5
0
Absorbance (405 nm)
0
10
20
30
40
50
60
70
80
90
100
110
Peptide

Serum sample 12A20A (1:400) cross-reacted with the same S4 decapeptide (18, fig. 3.14a) as 985/989. Even though 12A20A was four times more dilute than 985/989 when reacted with the pin decapeptides, 12A20A reacted more strongly than 985/989 with both S1 and S4 decapeptides.

Sample 985/989 diluted 1:100 in pin blocking buffer reacted weakly with the same regions of S1 decapeptides (8 residue overlap) as sample 12A20A, but did not react at all with S1 decapeptides (5 residue overlap), possibly because the 5-residue overlapping decapeptides were synthesised less successfully than the 8-overlapping ones. Sample 985/989 did not react at all with S3 decapeptides, but did react with the same S4 decapeptide as sample 12A20A. Sample 985/989 did not cross-react with any S3 decapeptides, but did react with S4 decapeptide 18 (A_{405} nm 0.65), a strong reaction when compared to the average background binding (A_{405} nm 0.3).

In summary, samples 12A20A and 985/989 both reacted with the same regions of S1 decapeptides (8 residue overlap) and the same S4 pin decapeptide. None of the sera reacted with the S3 decapeptides.

3.3.5.2 Inhibition of rabbit polyclonal antibody binding to S1 and S4 pin decapeptides by pertussis toxin

Sample 12A20A bound strongly to S1 and S4 decapeptides, so the antibody (1:400) was preincubated with pertussis toxin to determine whether this inhibited antibody binding to the pin decapeptides. A 1:400 dilution was used because this was the highest dilution to react strongly with the pin decapeptides and produce a low background level of binding.

Several concentrations of pertussis toxin were tried for the preincubation (0.2, 2, and 20 μg/ml), diluted in the pin blocking buffer. 0.2 and 2 μg/ml did not inhibit 12A20A binding to S1 (5 residue overlap) or S4 decapeptides, but 20 μg/ml did

In fig. 3.12a , which shows 12A20A binding to S1 decapeptides (5 residue overlap), two peaks can be seen at decapeptides 3 (A_{405} 0.6) and 41 (A_{405} 0.35). After preincubation of 12A 20A with 20 μg/ml pertussis toxin, the two peaks at peptides 3 and 41 were reduced to background level (fig. 3.12b).

Figure 3.14a shows 12A20A binding to S4 decapeptide 18 (A_{405} 1.04). After preincubation with 20 μg/ml pertussis toxin (fig. 3.14b), the peak at decapeptide 18 had been reduced to background level.

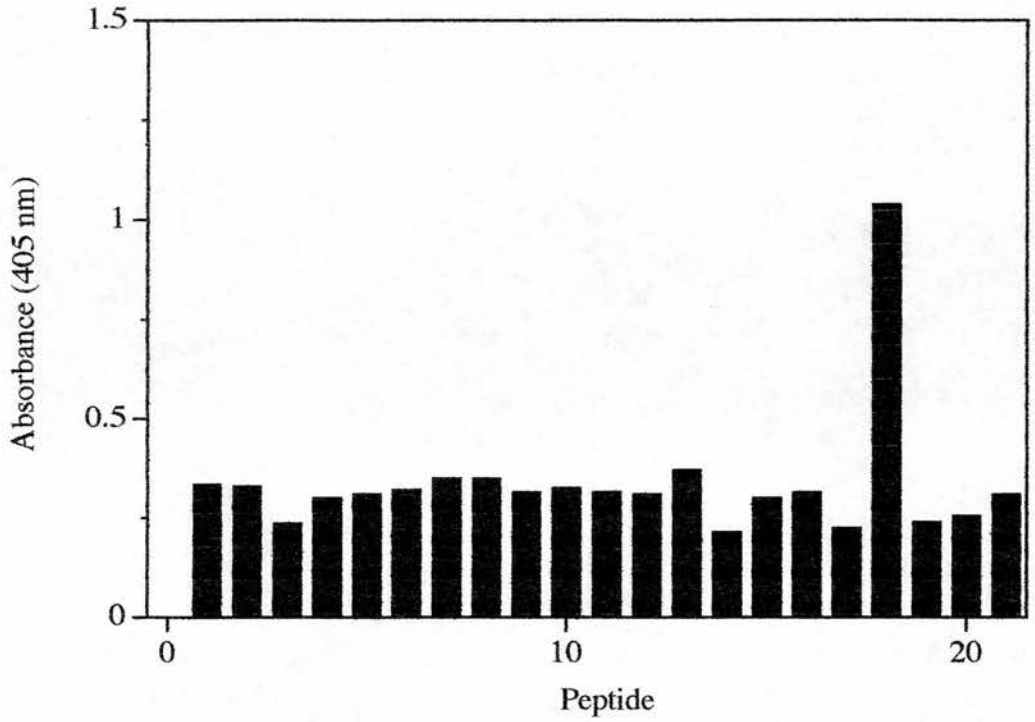
Fig. 3.14a Reaction of 12A20A with S4 decapeptides with a five amino acid overlap

Experimental conditions were as described for figure 3.12a and 3.12b.

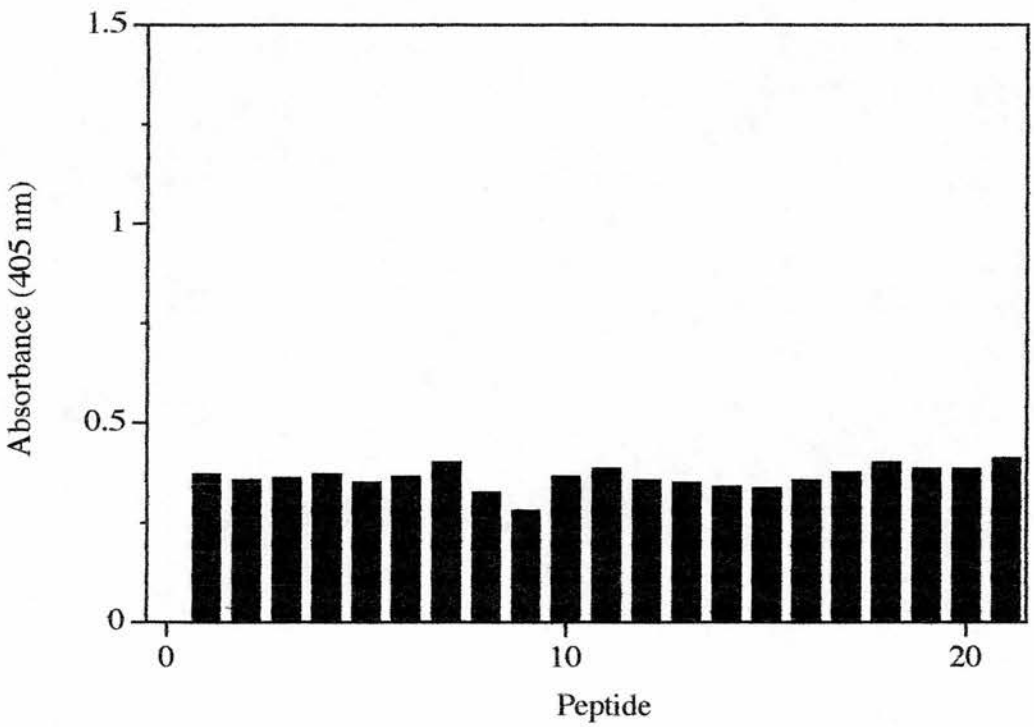
Fig.3.14b Reaction of 12A20A pre-incubated with 20 μg/ml pertussis toxin, with S4 decapeptides with a five amino acid overlap

Experimental conditions were as described for figure 3.12a and 3.12b.

a.


1.5
1
0.5
0
Absorbance (405 nm)
0
10
20
Peptide

b.


1.5
1
0.5
0
Absorbance (405 nm)
0
10
20
Peptide

3.3.6 Studies on peptide S4b

3.3.6.1 Synthesis of free peptide S4b

As both 12A20A and 985/989 bound to one S4 decapeptide, decapeptide 18 (amino acids 86-95, sequence QLTFEGKPAL) was synthesised as a C-terminal amide with a C-terminal glycine spacer followed by a cysteine residue and was named peptide S4b (table 3.2).

The glycine and cysteine residues were incorporated to enable coupling of S4b to KLH, using the thiol-reactive cross-linking reagent, MBS, in preparation for immunisation of rabbits.

The peptide was purified by reverse phase HPLC as described in section 2.2.5.6 S4b eluted as a single peak at 19.27 minutes (fig. 3.15).

3.3.6.2 Mass spectrometry of peptide S4b

The purification of S4b by HPLC only showed that the peptide was homogeneous. It did not indicate whether the peptide residues had been properly deprotected, had been derivatised in the deprotection process, or was of the expected mass, so an additional check on the peptide was made. Peptide S4b was subjected to fast atom bombardment mass spectrometry (FAB MS) as described in section 2.2.5.7. This technique was used to determine whether S4b had a molecular mass that would be expected for the residues which it contained, that is to determine if S4b had been derivatised during deprotection, or if a truncated form of S4b had been generated. Peptide S4b produced the expected measured mass of molecular ion ($[M + H]^+$) of 1262.8 Da, and the fragmentation pattern for the peptide fittedthe sequence of peptide S4b, shown in figure 3.16. This indicated that the peptide had not been truncated in synthesis and had not been derivatised during deprotection. For instance, if the preparation contained a full-length and a truncated peptide, two molecular ions would be generated, one having the calculated mass of the full-length peptide and one with a lower mass that would indicate an incomplete synthesis. Subtraction of the lower from the higher mass of molecular ion would enable calculation of the mass of the missing residues.

3.3.6.3 Inhibition of 12A20A binding to S4 pin peptides by peptide S4b

Antibody 12A20A (1:400) was preincubated with three concentrations of peptide S4b (1, 10 and 100 μg/ml) in pin blocking buffer and incubated with the S4 pin decapeptides. Peptide S4b was found to inhibit 12A20A binding to the same

Fig. 3.15 Reverse phase HPLC-purification of peptide S4b

See sections 2.2.5.6 for details.

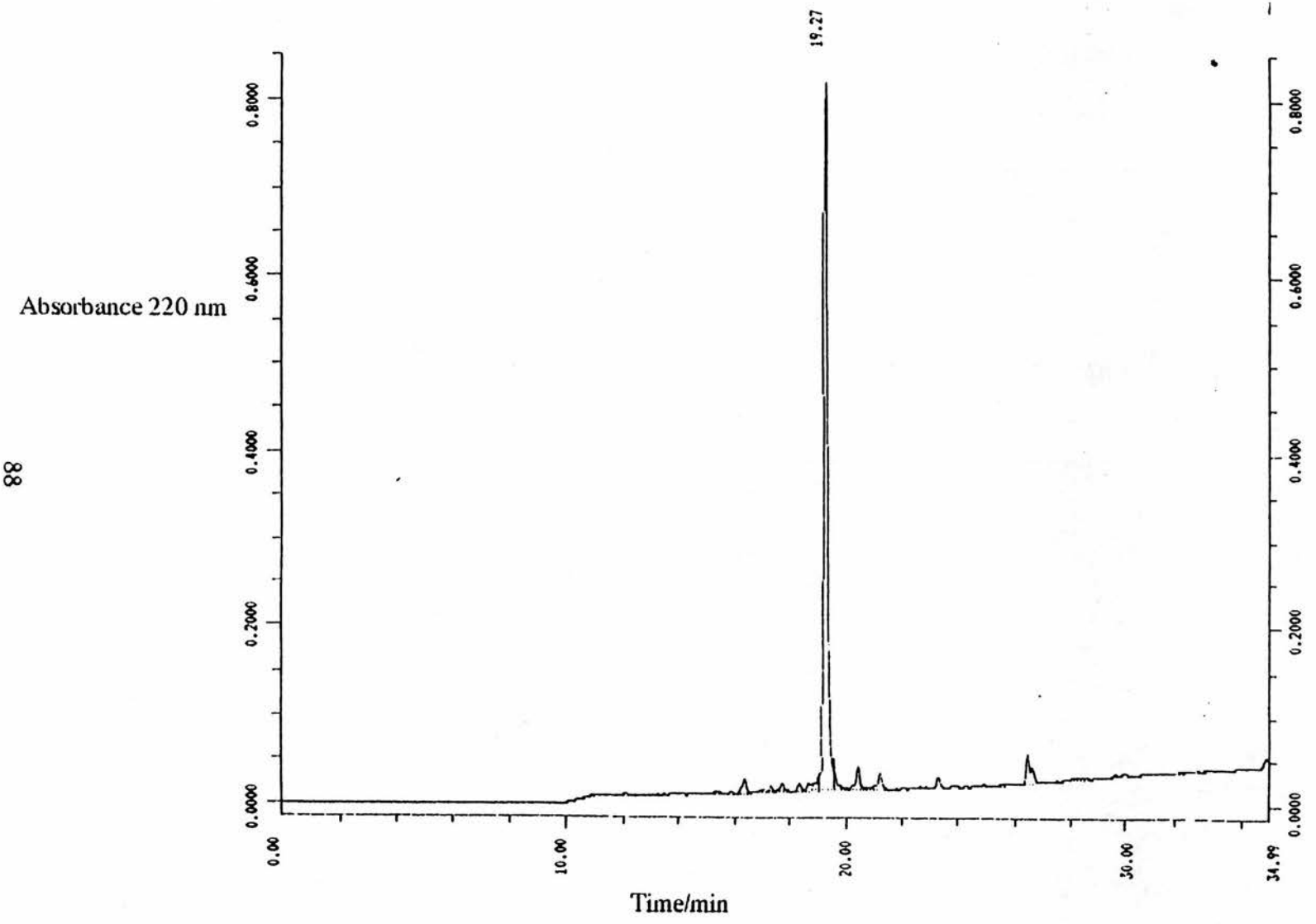
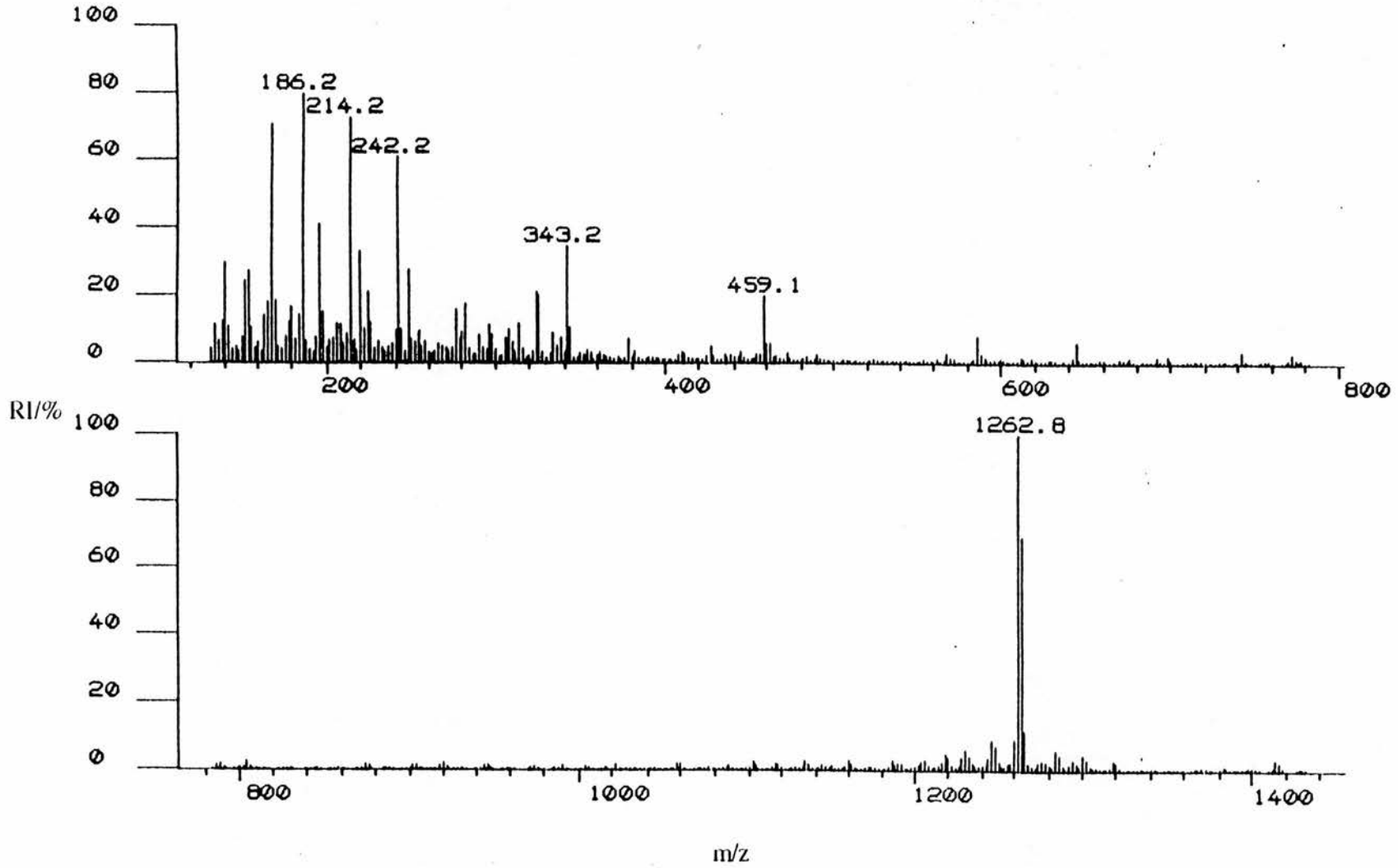

Absorbance 220 nm
19.27
0.8000
0.6000
0.4000
0.2000
0.0000
0.00
10.00
20.00
30.00
34.99
Time/min

Fig. 3.16 FAB mass spectrum of peptide S4b

See section 2.2.5.7 for details.


100
80
60
40
20
0
186.2
214.2
242.2
343.2
459.1
200
400
600
800
RI/%
100
80
60
40
20
0
1262.8
800
1000
1200
1400
m/z

extent as pertussis toxin, that is, to background levels (as in fig. 3.14b).

Binding of 12A20A to S4 pin decapeptides was inhibited by peptide S4b and pertussis toxin, suggesting that the 12A20A anti-pin peptide reaction was specific, i.e. directed against similar sites on the pin and free peptide and on pertussis toxin.

3.3.6.4 Mouse anti-S4b

Peptide S4b was conjugated to bovine serum albumin (BSA) using glutaraldehyde or conjugated to keyhole limpet haemocyanin (KLH) using m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) .

Anti-S4b sera were raised in mice against BSA-S4b and KLH-S4b using the method described in section 2.2.7.1. Nonspecific binding was controlled for using a peptide (3f) derived from *B. pertussis* fim 3 , whose sequence is given in appendix 1.

Antibodies to BSA-S4b and KLH-S4b recognised the homologous peptide, peptide conjugate and pertussis toxin, in a solid phase ELISA in which the antigen was coated directly onto the microtitre plate (table 3.4).

Anti-KLHS4b recognised homologous peptide and peptide conjugate with higher titres than anti-BSA-S4b. Anti-KLH-S4b also recognised BSA-S4b coated directly onto the microtitre plate, more strongly than anti-BSA-S4b recognised KLH-S4b coated onto the microtitre plate (table 3.4). Despite anti-KLH-S4b having a lower anti-pertussis toxin titre than anti-BSA-S4b, the larger amounts of antipeptide serum needed for further work was raised against KLH-S4b in rabbits. (NB. anti-carrier reactions were controlled for by measuring binding to carrier-coated plates).

3.3.6.5 Rabbit anti-S4b

3.3.6.5.1 Binding of rabbit anti-S4b to peptide S4b and pertussis toxin

The method of generation of rabbit antipeptide antibodies is described in section 2.2.7.2.

Rabbit anti-S4b was assayed for antipeptide and antiprotein reactivity by solid phase ELISA, in which the antigen was coated directly onto the plate, as with the mouse antipeptide sera above. A KLH-peptide conjugate (KLH-3f) whose synthetic peptide sequence was derived from *B. pertussis* fim 3 was used as a negative control. The sequence of the latter control peptide is given in appendix 1.

Anti-KLH-S4b bound to pertussis toxin adsorbed onto the microtitre plate, and more strongly to peptide S4b, KLH-S4b and BSA-S4b adsorbed directly onto the microtitre plate (table 3.4).

As 'native' antigen coated onto a microtitre plate could contain some denatured

Table 3.4 Solid phase ELISA titres for rabbit- and mouse-antipeptide antisera

Antipeptide Antibody	Solid phase ELISA coating antigen (a)							
	pertussis toxin	Homologous peptide	KLH-S4b	BSA-S4b	KLH-(CGE)S3c	BSA-(E)S3c	3f	KLH-3f
Murine								
anti-KLHS4b	131	24 832	58 296	21 017	-	-	<18	<18
anti-BSAS4b	262	3631	22	80 310	-	-	<18	<18
anti-BSA(E)S3c	470	4981	-	-	300	29 964	<18	<18
anti-KLH(CGE)S3c	61	205	-	-	71	506	<18	<18
Rabbit								
anti-KLHS4b	4283	202 050	106 557	80 328	-	-	<18	<18
anti-BSA(E)S3c	539	12 769	-	-	3609	10 464	<18	<18

(a). Results are shown as ELISA titres (reciprocal of end-point dilution that produced an absorbance at 450 nm of 1.0).

protein and coating the antigen directly onto the microtitre plate could also denature the antigen (Jemmerson and Blankenfeld, 1989), recognition of pertussis toxin captured by a monoclonal antibody (MAB L10) was assessed.
Anti-S4b recognised 'native' pertussis toxin captured by MAB L10 with a titre of 186, compared with 270 against pertussis toxin coated directly onto the ELISA plate, so although recognition of pertussis toxin was weak, a small part of the response appeared to be due to recognition of denatured antigen by the antipeptide antibody.

The subunit specificity of anti-S4b was assessed by Western blotting of pertussis toxin subunits separated by SDS PAGE (see fig. 3.3).

3.3.6.5.2 Anti-S4b binding to S4 pin decapeptides

Anti-S4b reacted with S4 pin decapeptides 17 and 18, both of which were contained in the sequence of the peptide immunogen (fig. 3.9).
This indicated that the pin peptide synthesis of S4 decapeptides was successful.

3.3.6.5.3 Inhibition of 12A20A binding to pertussis toxin

Anti-S4b and 12A20A cross-reacted with S4 decapeptide 18, so inhibition of 12A20A binding to pertussis toxin by peptide S4b was assessed, using a solid phase ELISA .

Serum 12A20A was diluted to give A_{450} 1.2-1.5 and preincubated with decreasing concentrations of peptide S4b. After 90 minutes this was incubated with microtitre plate wells coated with pertussis toxin for another 90 minutes. Binding of 12A20A was detected using anti-rabbit (IgG) conjugated to horse radish peroxidase. The reaction was expected to show an increase in A_{450} to 1.2-1.5 as peptide S4b was diluted, if 12A20A bound homologous sites on both S4b and whole pertussis toxin. However, no inhibition of 12A20A binding to pertussis toxin by peptide S4b was seen, possibly due to a small proportion of 12A20A antibodies that bind peptide S4b.

3.3.6.5.4 Inhibition of antibody binding to peptide S4b and pertussis toxin

Anti-S4b and 12A20A bound S4 decapeptide 18, as mentioned earlier, and the experiment described in the previous section did not show inhibition, so inhibition of anti-S4b and 12A20A binding to peptide S4b and whole pertussis toxin was attempted to show that the two antibodies recognised the peptide and toxin.

Mouse anti-S4b was diluted in a fixed concentration of 12A20A (A_{450} 1.2-1.5) and incubated for 90 minutes, then incubated with peptide S4b-coated plates or pertussis toxin-coated plates. Binding to the peptide or toxin was detected

with species-specific anti-rabbit IgG conjugated to horse radish peroxidase. The experiment was repeated with 12A20A diluted in anti-S4b and peptide binding detected with species-specific anti-mouse IgG conjugated to horse radish peroxidase. If anti-S4b and 12A20A bind the same site, then the absorbance at 450 nm was expected to increase to 1.2-1.5-fold in these two experiments. Anti-S4b and 12A20A diluted in ELISA blocking buffer were reacted with the peptide-coated wells as controls for peptide-binding.

This assay did not show that anti-S4b binding could be inhibited by 12A20A, nor vice versa. The above assay was repeated with pertussis toxin as the coating antigen, with the same results.

3.3.7 Reaction of Human antisera with pin decapeptides

To assess whether human sera reacted with continuous sequences of amino acids ('continuous epitopes'), the pin decapeptides of S1, S3 and S4 were reacted with a small panel of human sera, from subjects convalescent from pertussis. The sera were kindly provided by Dr. K. Redhead (NIBSC, Potters Bar, U.K.) and their characteristics and anti-pertussis toxin titres, determined by coating the antigen directly onto the microtitre plate in a solid phase ELISA are shown in table 3.5. It should be noted that due to limited sample availability, these sera were only reacted with the pin decapeptides once.

The panel included sera from subjects of varied age. Eight had the full course of three DTP vaccinations, one had one vaccination and four had none. One subject had mild disease, three had no disease and the rest had severe disease and all subjects but one were culture-positive.

One sample tested was the freeze-dried *Bordetella pertussis* reference serum (89/530), pooled by Dr. L.A.E. Ashworth, PHLS CAMR and supplied by the National Institute for Biological Standards and Control, Potter's Bar, U.K. The reference serum 89/530 was a pool that included convalescent sera from two adult pertussis patients and two adult sera from disease-free immunused individuals. No child sera could be obtained at the time that were of a high enough titre to be included in the pool. 89/530 was provided as a freeze-dried preparation which was reconstituted in 0.5 ml PBS pH 7.2 and diluted as the other convalescent sera were.

As a limited supply of each convalescent serum sample was available, the sera were reacted with the pin decapeptides at the highest concentration practicable and inhibition of their binding to the pin peptides by pertussis toxin in solution was not assessed

The human sera were reacted with the pin decapeptides at the lowest dilution possible for the sample size. The dilution used was 1:200 for all the samples except 169(1)2, which was reacted with the decapeptides at 1:300 dilution. Background

Table 3.5 Solid phase ELISA titres, age, DTP vaccination status and disease status for antisera from human convalescent and disease-free individuals

Sample code	DTP vaccinations (a)	Age/years	Severity of disease	Sampled/days (b)	Culture (c)	Anti-PT titre (d)
135	3	<1	no disease	4-6 months after 1st dose	N/A	21161
145	3	<1	no disease		N/A	11406
412	3	<1	no disease		N/A	18210
18(1)1	0	1.58	severe high lymphocytosis	26	+	3654
149(1)2	0	3.25	severe	53	+	10491
24(1)2	3	19.5	severe	24	-	11037
27(1)4	3	21	mild	48	+	14915
36(1)1	0	2	severe	26	+	1489
140(1)1	1	3	severe	25	+	20372
166(1)2	0	3	severe	39	+	17598
169(1)2	3	7	severe	31	+	66643
B. pertussis reference serum (89/530)	N/A (e)	N/A (e)	N/A (e)	N/A (e)	N/A (e)	82613

(a). A full vaccination course for pertussis (and diphtheria and tetanus) is 3 DTP vaccinations.
(b). Number of days after onset of cough that sample was taken.
(c). *B. pertussis* was found in cultured swab samples (+), or not (-).
(d). Results are expressed as anti-PT (pertussis toxin) ELISA titres (reciprocal of end-point dilution giving an absorbance of 1.0).
(e). 89/530 was obtained from the National Institute for Biological Standards and Control, U.K.

binding to the pin decapeptides was judged to be equal to the level of binding seen with any human serum sample that did not react with the pin decapeptides.

Sera 135, 145 and 412 had anti-pertussis toxin titres over 10 000 but below 22 000, about a quarter of the anti-pertussis toxin titre of the reference serum (table 3.5). All three samples came from individuals who had three DTP vaccinations and were disease free, so they were used to give an idea of the immune response (IgG) in vaccinated, disease-free individuals.

Sample 135 reacted strongly with decapeptide 114 (A_{405} 0.84, average background A_{405} 0.39) of S1 decapeptides (8 residue overlap). No other peaks were above background level and sample 135 did not react with either S3 or S4 decapeptides.

Sample 145 reacted weakly with S1 decapeptides (8 residue overlap) 23, 35, 47 and 59 and sample 412 did not react with any of the pin decapeptides.

Sample 27(1)4 was from the oldest individual in the panel and the most likely to have acquired immunity to pertussis through repeated exposure during the individual's lifetime. The individual had undergone a full course of three DTP vaccinations and the sample had an anti-pertussis toxin titre of 14 915 (table 3.5), when the antigen was coated directly onto the microtitre plate in a solid phase ELISA.

27(1)4 did not react with S1 decapeptides with a 5 residue overlap (average background A_{405} 0.17), but did react with S1 decapeptides with an 8 residue overlap. However, only one S1 decapeptide (8 residue overlap) reacted to give an absorbance at 405 nm that was equal to 0.5 (peptide 114), the rest giving absorbances below this value. The sequences of these weakly reactive decapeptides did not match those of S1 (5 residue overlap), which would explain the lack of reaction with S1 decapeptides (5 residue overlap). 27(1)4 reacted weakly with S3 decapeptides, but again the absorbances at 405 nm did not exceed 0.5. No peaks above the average background were observed when 27(1)4 was reacted with S4 decapeptides.

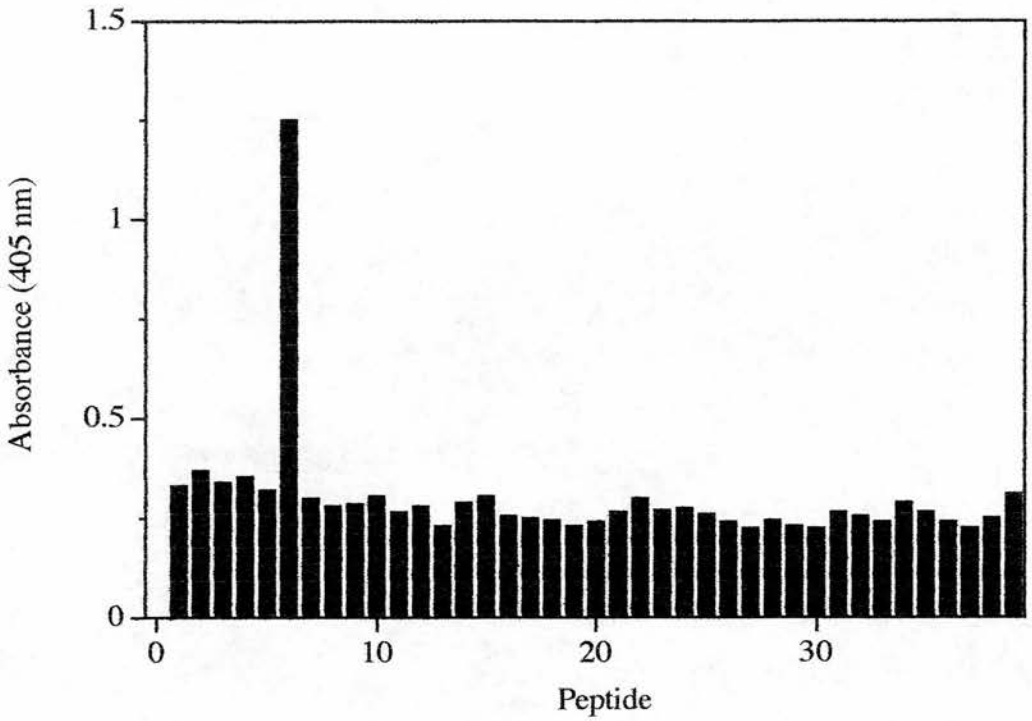
169(1)2 was taken from another individual who had three DTP vaccinations and was convalescent from pertussis. This sample had the highest anti-pertussis toxin titre in an ELISA in which the antigen was coated directly onto the ELISA plate (table 3.5), with the exception of the reference serum. Weak cross-reaction was seen with S1 decapeptides with a five residue overlap, but no reaction produced an absorbance greater than 0.5. When 169(1)2 was reacted with S1 decapeptides (8 overlap), no reaction giving an absorbance at 405 nm greater than 0.5 was seen with the peptides that had the same sequence as the weakly reactive S1 (5 residue overlap) decapeptides.

24(1)2 was taken from the second oldest individual and was the only culture-negative sample in the panel. The individual had been vaccinated three times with the DTP vaccine and the sample's anti-pertussis toxin titre is shown in table 3.5.

No peaks distinctly above background were observed when 24(1)2 was

Fig. 3.17 Reaction of human polyclonal convalescent serum 18(1)1 with S3 decapeptides with a five amino acid overlap

Decapeptides were incubated with blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v)Tween 20) for 90 minutes and then with 18(1)1 at a dilution of 1:200, overnight. Reaction with the decapeptides was detected by ELISA (see section 2.2.3.1 for details).


1.5
1
0.5
0
Absorbance (405 nm)
0
10
20
30
Peptide

reacted against S1, or S4 decapeptides. The sample reacted weakly with S3 decapeptide regions 1-12 and 21-24, but again no reaction produced an absorbance at 405 nm greater than 0.5.

18(1)1 was taken from an unvaccinated convalescent individual and had a low anti-pertussis toxin titre compared to the reference serum as shown in table 3.5. The sample produced the strongest reaction of the human sera and bound to one S3 decapeptide. This was decapeptide 6 (A_{405} 1.25) (fig. 3.17) and the background binding absorbance was 0.25. No peaks distinctly above background were observed with S1 or S4 decapeptides.

Sample 36(1)1 was also taken from an individual who had no DTP vaccinations and the sample gave a low anti-pertussis toxin titre compared to the reference serum (table 3.5). This serum did not react with the pin decapeptides with the exception of S1 decapeptide (8 residue overlap) 56. However, the sample did not react with S1 decapeptide (5 residue overlap) 23, which had the same amino acid sequence as decapeptide 56 mentioned above, so the reaction of 36(1)1 with decapeptide 56 of S1 decapeptides (8 residue overlap) was discounted as being nonspecific.

Sample 166(1)2 was also from an unvaccinated individual. This serum gave a higher anti-pertussis toxin titre than either 36(1)1 or 18(1)1, which were also taken from unvaccinated individuals. 166(1)2 was from a subject one year older than the other two and so may have been exposed more to pertussis antigens,which could explain the higher anti-pertussis toxin titre. Also, samples 36(1)1 and 18(1)1 were taken 26 days after onset of cough. 166(1)2 was taken 39 days after onset of cough, so increasing time for the immune system to generate IgG detectable by the pin peptide ELISA.

Although 166(1)2 had a higher anti-pertussis toxin titre than 36(1)1 or 18(1)1, it did not react distinctly with any of the pin decapeptides.

Finally, samples 140(1)1 and 149(1)2 did not react with the pin peptides.

That the human sera had quite high anti-pertussis toxin titres, and did not react well with the pin decapeptides, probably because of low amounts of correctly synthesised peptides on the pins.

3.3.8 Glycoconjugate binding to pin and free synthetic peptides

3.3.8.1 Introduction

The B-oligomer of pertussis toxin is involved in receptor recognition (see section 1.3.5) and is responsible for the toxin's ability to agglutinate erythrocytes (Nencioni *et al.*, 1991).

The B-oligomer is also an adhesin, enabling attachment of *B. pertussis* to cilia

of the respiratory tract (Saukkonen *et al.*, 1992).

Molecules acting as receptors for pertussis toxin have not yet been identified but studies have suggested that membrane proteins of different sizes are involved, size depending on the cell type. Serum glycoproteins such as haptoglobin and fetuin have also been used as model receptors (chapter 1).

Nogimori *et al.*, (1986) chemically modified the lysine residues of S3-S4 and this abolished the mitogenic, adjuvant and lymphocytosis activities of the whole toxin, which implicates S3 in receptor recognition.

Both S2 and S3 subunits have been shown to be involved in recognition of eukaryotic glycoproteins (Capiau *et al.*, 1986; Schmidt *et al.*, 1989; Schmidt *et al.*, 1991) via their carbohydrate recognition domains (Saukkonen *et al.*, 1992). Both subunits S2 and S3 are apparently involved in binding of pertussis toxin to sialylated glycoconjugate of macrophages (van't Wout *et al.*, 1992) and human T cells (Witvliet *et al.*, 1992).

Additional evidence for the involvement of S2 and S3 in binding to sialylated glycoconjugates (also see chapter 1) was provided by Witvliet *et al.* (1992), who found that sialidase treatment of a line of human T cells decreased binding of FITC-labelled pertussis toxin (FITC = Fluorescein isothiocyanate) to them and that binding apparently involved both S2-S4 and S3-S4 dimers, suggesting that divalent binding of pertussis toxin activates T cells.

CHO cell lines deficient in sialic acid moieties on cell surface glycoproteins were found not to be sensitive to ADP-ribosylation of G proteins by pertussis toxin, (Witvliet *et al.*, 1989) implying sialic acid on cell surface glycoconjugates is required for the binding and activity of pertussis toxin and only cells displaying sialylated glycoconjugates are sensitive to intoxication by pertussis toxin (Brennan *et al.*, 1988). Sialidase (neuraminidase) treatment of CHO cells decreases pertussis toxin binding to them (Brennan *et al.*, 1988) and sialic acid blocks S3-mediated binding of pertussis toxin to human macrophages (van't Wout *et al.*, 1992), implying that sialic acid moieties are important in toxin subunit-carbohydrate interactions.

Subunits S2 and S3 share about 80% amino acid sequence homology (Locht and Keith, 1986; Nicosia *et al.*, 1986) so it is possible that the recognition sites have some similarities. However, differences in binding (Francotte *et al.*, 1989; Witvliet *et al.*, 1989; Schmidt *et al.*, 1989; 1991; Saukkonen *et al.*, 1992) suggest that differences in structure and/or amino acid sequence may indicate regions that determine carbohydrate binding specificity.

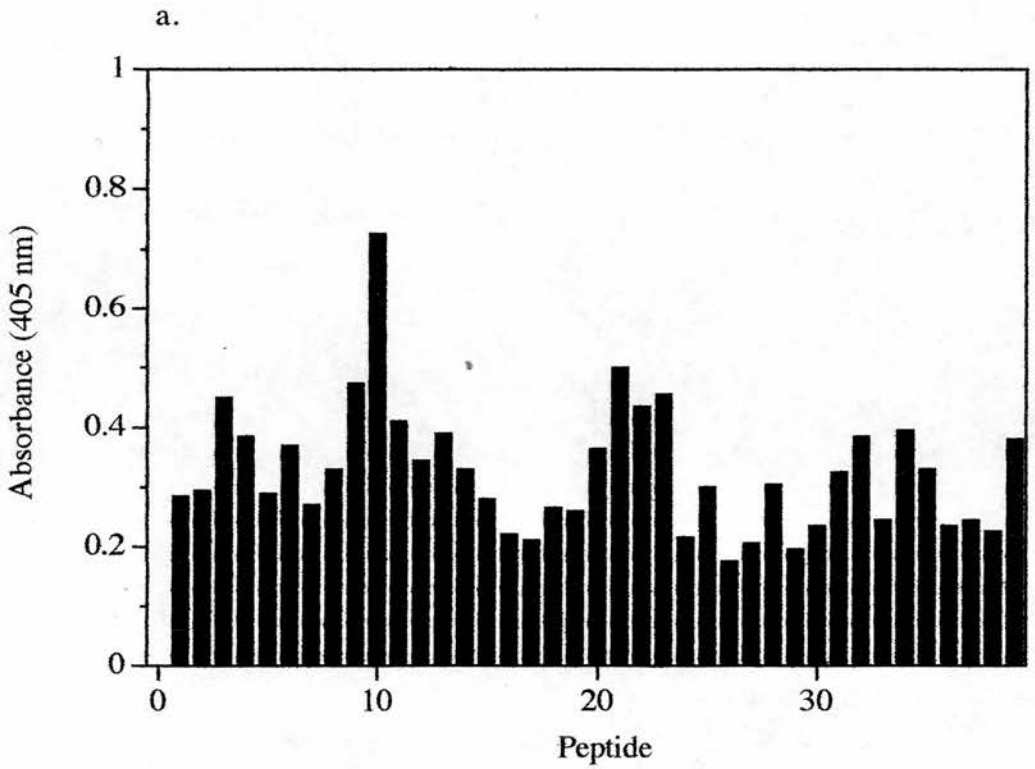
Linear peptide analogues have been used to mimic carbohydrate recognition domains of fibronectin (Pierschbacher and Ruoslahti, 1984; Yamada and Kennedy, 1984), so in this study, interaction of synthetic peptides from the S3 subunit of pertussis toxin with glycoconjugates, using pin-bound peptides (Geysen *et al.*, 1984; 1987) and free peptides were studied in order to identify glycoconjugate binding

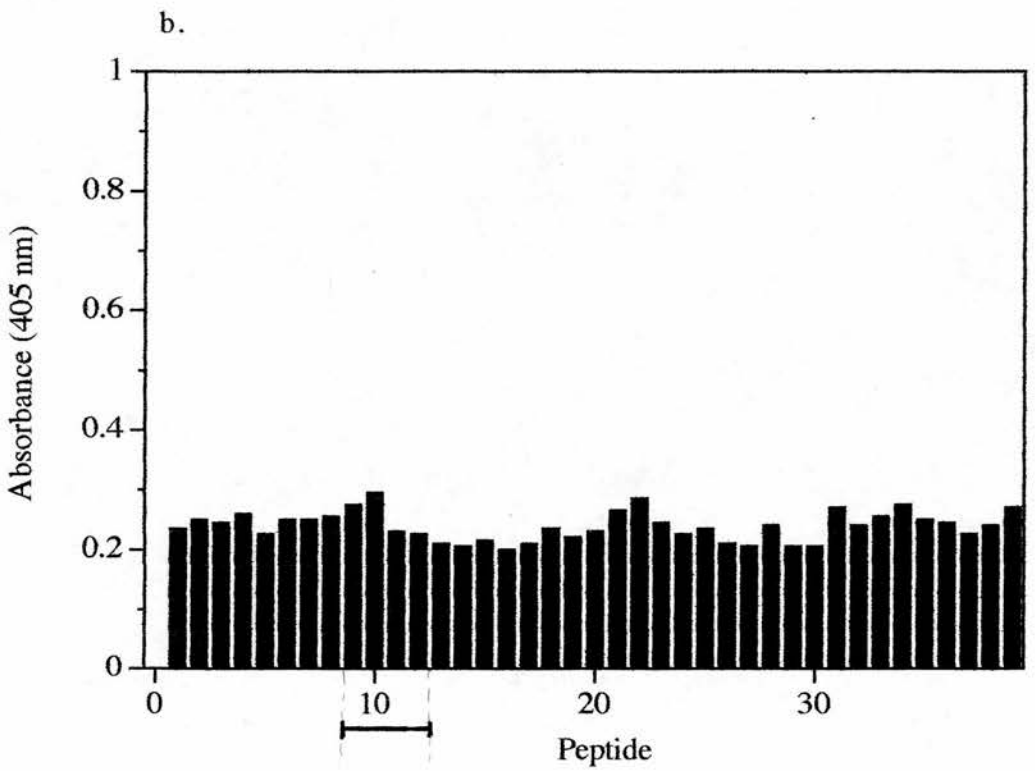
Fig. 3.18a Reaction of S3 decapeptides with a five amino acid overlap with 20 μg/ml fetuin in pin blocking buffer (10%(v/v) newborn calf serum, 0.4%(v/v) Tween 20 in PBS, pH7.2)

See section 2.2.3.2 for details.

Fig. 3.18b Reaction of S3 decapeptides with a five amino acid overlap with 20 μg/ml fetuin plus 100μg/ml peptide (E)S3c in pin blocking buffer

See section 2.2.3.2 for details.


a.
Absorbance (405 nm)
1
0.8
0.6
0.4
0.2
0
0
10
20
30
Peptide


b.
Absorbance (405 nm)
1
0.8
0.6
0.4
0.2
0
0
10
20
30
Peptide

domains.

3.3.8.2 Results of glycoconjugate binding to S3 peptides

3.3.8.2.1 Binding of fetuin to S3 pin decapeptides

S3 pin decapeptides were incubated with 20 μg/ml fetuin as described in section, 2.2.3.2 (fig.3.18a).

Fetuin recognised S3 decapeptide 10 (residues 46-55, amino acid sequence RQITPGWSIY). The absorbance was over double that of the average background (peptide 10 A_{405} 0.725, average background 0.32).

3.3.8.2.2 Synthesis of free peptide (E)S3c and (CGE)S3c

The glycoprotein fetuin bound to S3 decapeptide 10 (residues 46-55), so two free peptides, (E)S3c and (CGE)S3c consisting of S3 amino acids 44-58 were synthesised. Their sequences are shown in table 3.2.

The N-terminal glutamic acid was originally included in the synthesis of (E)S3c to enable efficient coupling to a protein carrier using carbodiimide as the coupling agent and raising of antipeptide antisera. However, this coupling was not done because antipeptide antisera raised against (E)S3c conjugated to BSA using glutaraldehyde reacted with pertussis toxin and the peptide immunogen. The glycine and cysteine residues were incorporated into (CGE)S3c to enable coupling to KLH, using the thiol-reactive cross-linking reagent, MBS, in preparation for immunisation of mice.

The peptides were purified by reverse phase HPLC and (E)S3c eluted as a single major peak at 23.29 minutes (fig. 3.19a). (CGE)S3c eluted as one major peak at 24.16 minutes (fig. 3.19b).

3.3.8.2.3 Mass spectrometry of peptides (E)S3c and (CGE)S3c

The purification of (E)S3c and (CGE)S3c by HPLC indicated that the peptide preparations contained no major contaminants, because they both eluted as one major peak on the chromatograms. It did not indicate whether peptide residues had been properly deprotected, or had been derivatised in the deprotection process, so an additional check on the peptide was made. Peptides (E)S3c and (CGE)S3c were subjected to fast atom bombardment mass spectrometry (FAB MS) as described in section 2.2.5.7. This technique was used to determine whether the peptides had a molecular mass that would be expected for the residues which it contained, that is to determine if amino acids had been derivatised during deprotection, or if a truncated

Fig. 3.19a Purification of peptide (E)S3c by reverse phase HPLC

See section 2.2.5.7 for details.

0.0000
0.5000
1.0000
1.5000
0.00
10.00
20.00
30.00
34.99
22.74
24.31
25.55
25.92
22.93; 23.08; 23.29
23.82
Time/min
Absorbance 220 nm

Fig. 3.19b Purification of peptide (CGE)S3c by reverse phase HPLC

See section 2.2.5.7 for details.

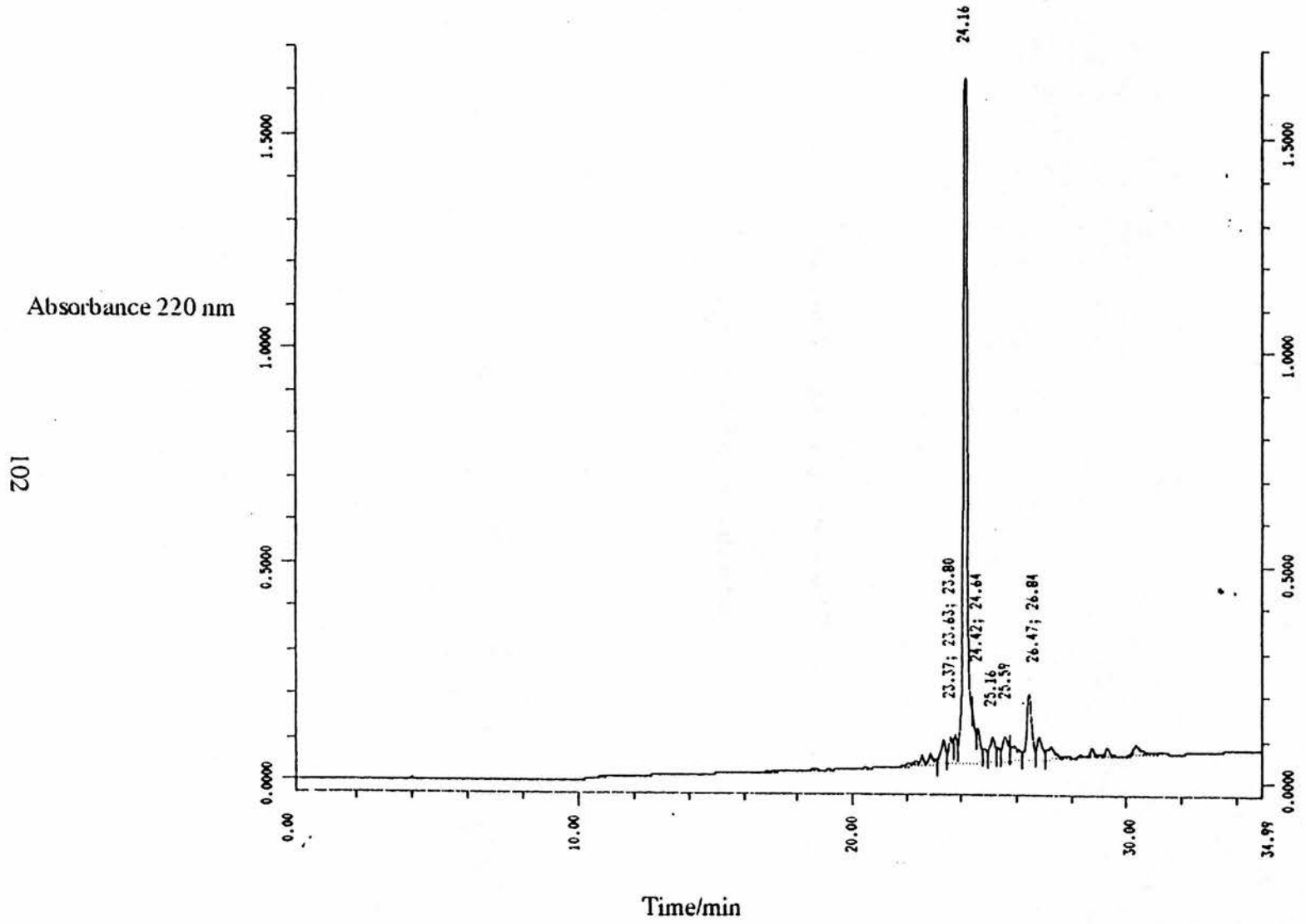

Absorbance 220 nm
1.5000
1.0000
0.5000
0.0000
24.16
23.37; 23.63; 23.80
24.42; 24.64
25.16
25.59
26.47; 26.84
0.00
10.00
20.00
30.00
34.99
Time/min

Fig. 3.20a FAB mass spectrum of peptide (E)S3c

See section 2.2.5.6 for details.

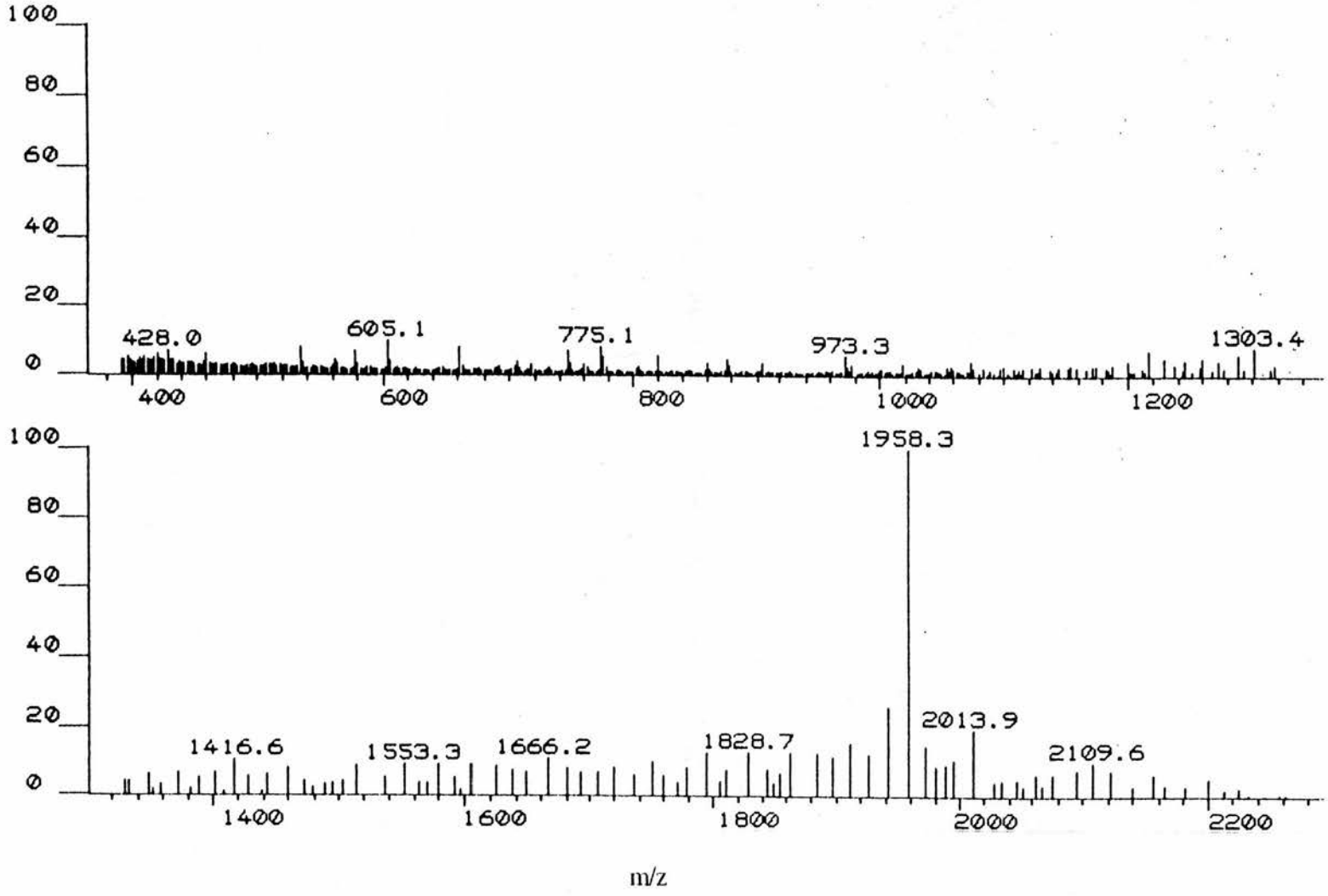
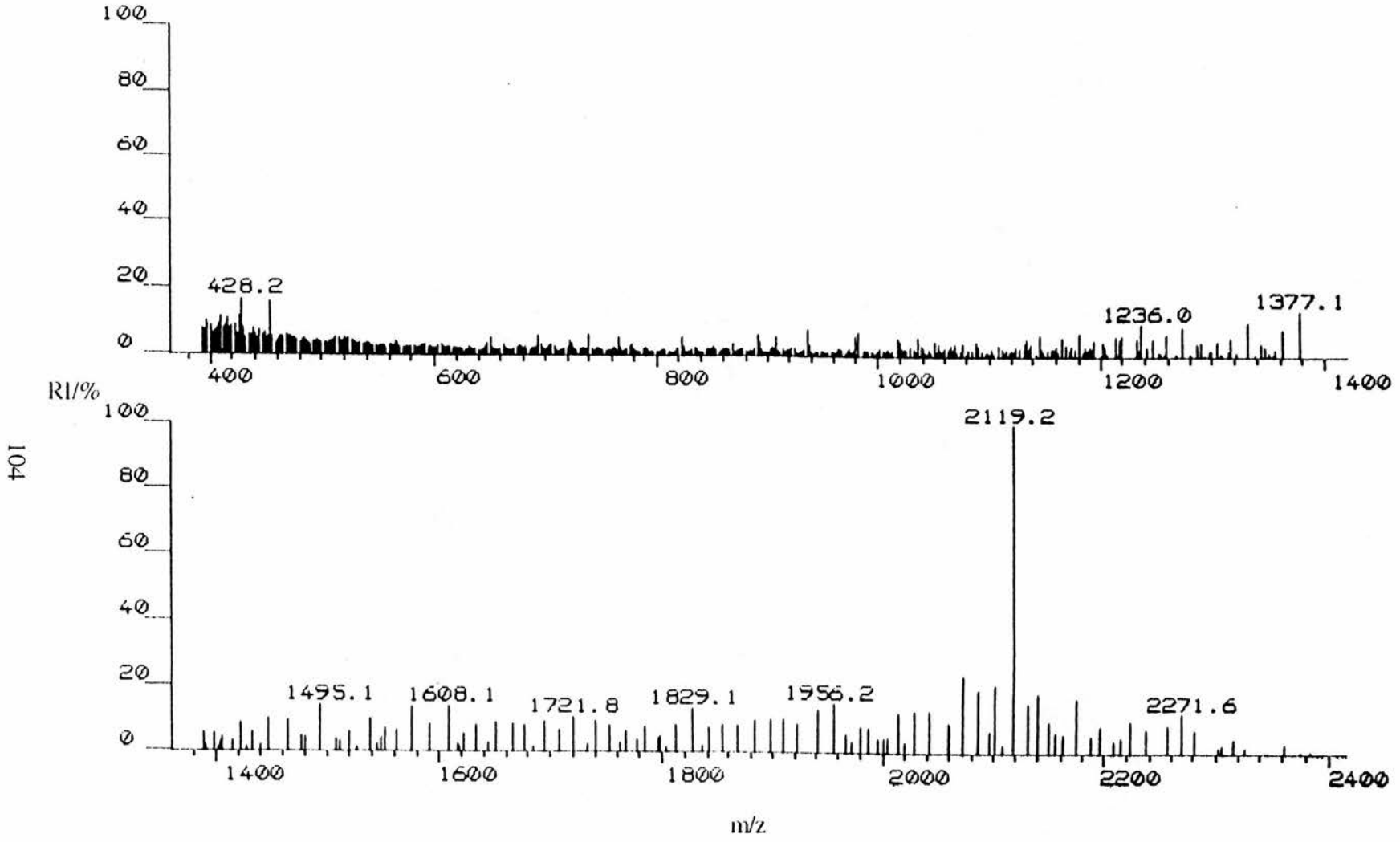

100
80
60
40
20
0
428.0
605.1
775.1
973.3
1303.4
400
600
800
1000
1200
RI/%
100
80
60
40
20
0
1958.3
2013.9
1416.6
1553.3
1666.2
1828.7
2109.6
1400
1600
1800
2000
2200
m/z

Fig. 3.20b FAB mass spectrum of peptide (CGE)S3c

See section 2.2.5.6 for details.


100
80
60
40
20
0
428.2
1236.0
1377.1
400
600
800
1000
1200
1400
RI/%
104
100
80
60
40
20
0
2119.2
1495.1
1608.1
1721.8
1829.1
1956.2
2271.6
1400
1600
1800
2000
2200
2400
m/z

form the peptide had been generated.

Peptide (E)S3c produced the expected measured average mass of molecular ion ($[M + H]^+$) of 1958.3 Da, and the fragmentation pattern for the peptide is shown in figure 3.20a. Peptide (CGE)S3c produced the expected measured average $[M + H^+]$ of 2118.4 Da and the fragmentation pattern for the peptide is shown in figure 3.20b. That one molecular ion of the expected mass was found, indicated that the peptides had not been truncated in synthesis and had not been derivatised during deprotection (subtraction of the masses of individual deprotected amino acids from the molecular ion indicated that no fragments had been derivatised).

3.3.8.2.4 Inhibition of fetuin binding to S3 pin decapeptides

To determine whether preincubation of peptide (E)S3c inhibited fetuin binding to the pin decapeptides, 20 μg/ml fetuin was incubated with peptide 20 μg/ml (E)S3c for 90 minutes at room temperature, at the same time as the S3 pin decapeptides were incubated with pin blocking buffer, to reduce nonspecific binding. The preincubated solution containing (E)S3c and fetuin was then incubated with the S3 pin decapeptides for 7 hours (section 2.2.3.2). Detection of fetuin binding to the S3 decapeptides was detected as described in section 2.2.3.1. 20 μg/ ml peptide did not inhibit fetuin binding to the S3 decapeptides, so the peptide concentration was increased to 100 μg/ml. The result of the latter experiment can be seen in figure 3.18b, which shows that preincubation with 100 μg/ml peptide totally inhibited fetuin binding to the decapeptides.

Incubation of the decapeptides with fetuin alone (fig. 3.18a) was used as a positive control (i.e. no inhibition of fetuin binding).

3.3.8.2.5 Mouse anti-(E)S3c and anti-(CGE)S3c antibodies

Peptide (E)S3c was conjugated to bovine serum albumin (BSA) using glutaraldehyde and peptide (CGE)S3c was conjugated to keyhole limpet haemocyanin (KLH) using the cross-linker MBS.

Anti-(E)S3c was raised in mice against peptide conjugated to BSA and anti-(CGE)S3c serum was raised in mice against peptide conjugated to KLH, using the method described in section 2.2.7.1.

Antibodies raised against (E)S3c recognised peptide, peptide conjugate and pertussis toxin, in a solid phase ELISA in which the antigen was coated directly onto the microtitre plate (table 3.4). Nonspecific binding to the peptide was controlled for using a peptide (3f) derived from *B. pertussis* fim 3 as coating antigen and the sequence of that peptide is given in appendix 1. Nonspecific binding to pertussis toxin was controlled for using *B. pertussis* fim 2 as coating antigen.

Anti-(CGE)S3c recognised pertussis toxin, peptide and homologous peptide conjugate with much lower titres than anti-(E)S3c in an ELISA assay in which the antigen was coated directly onto the microtitre plate.

Anti-(E)S3c also recognised KLH-(CGE)S3c peptide conjugate coated directly onto the microtitre plate, albeit rather weakly (table 3.4).

For these reasons, the larger amounts of antipeptide serum needed for further studies was raised in rabbits against (E)S3c (peptide conjugated to BSA).

3.3.8.2.6 Rabbit anti-(E)S3c

3.3.8.2.6.1 Binding of rabbit anti-(E)S3c to peptide S3c and pertussis toxin

The method of generation of rabbit antipeptide antibodies is described in section 2.2.7.2.

Rabbit anti-(E)S3c was assayed for antipeptide and antiprotein reactivity by solid phase ELISA, in which the antigen was coated directly onto the plate, as with the mouse antipeptide sera above. A peptide (3f) whose synthetic peptide sequence was derived from *B. pertussis* fim 3 was used as a negative control.

Anti-(E)S3c recognised pertussis toxin, (E)S3c, (E)S3c conjugated to BSA and (CGE)S3c conjugated to KLH in an ELISA assay in which the antigen was adsorbed onto the microtitre plate (tables 3.2 and 3.4).

As 'native' antigen coated onto a microtitre plate could contain some denatured protein and coating the antigen directly onto the microtitre plate could also denature the antigen, (Jemmerson and Blankenfeld, 1989) recognition of pertussis toxin captured by a monoclonal antibody (MAB L10) was assessed by solid phase ELISA. Anti-(E)S3c recognised pertussis toxin captured by MAB L10 as ascites fluid with a titre of 300, so only part of the anti-pertussis toxin response appeared to be due to recognition of denatured antigen by the antipeptide antibody.

The subunit specificity of rabbit anti-(E)S3c was assessed by Western blotting of pertussis toxin subunits separated by SDS PAGE. That is described in section 3.3.1. Results of binding of anti-(E)S3c to S3 pin decapeptides are described in section 3.3.2.2.

3.3.8.2.6.2 Binding of (E)S3c and pertussis toxin to glycoconjugates

The binding of (E)S3c and pertussis toxin to glycoconjugates other than fetuin was studied in a solid phase binding ELISA assay. Fetuin, asialofetuin, alpha-1-acid glycoprotein and mixed bovine brain gangliosides were coated onto the microtitre plate, incubated with serial dilutions of (E)S3c or pertussis toxin, then incubated with

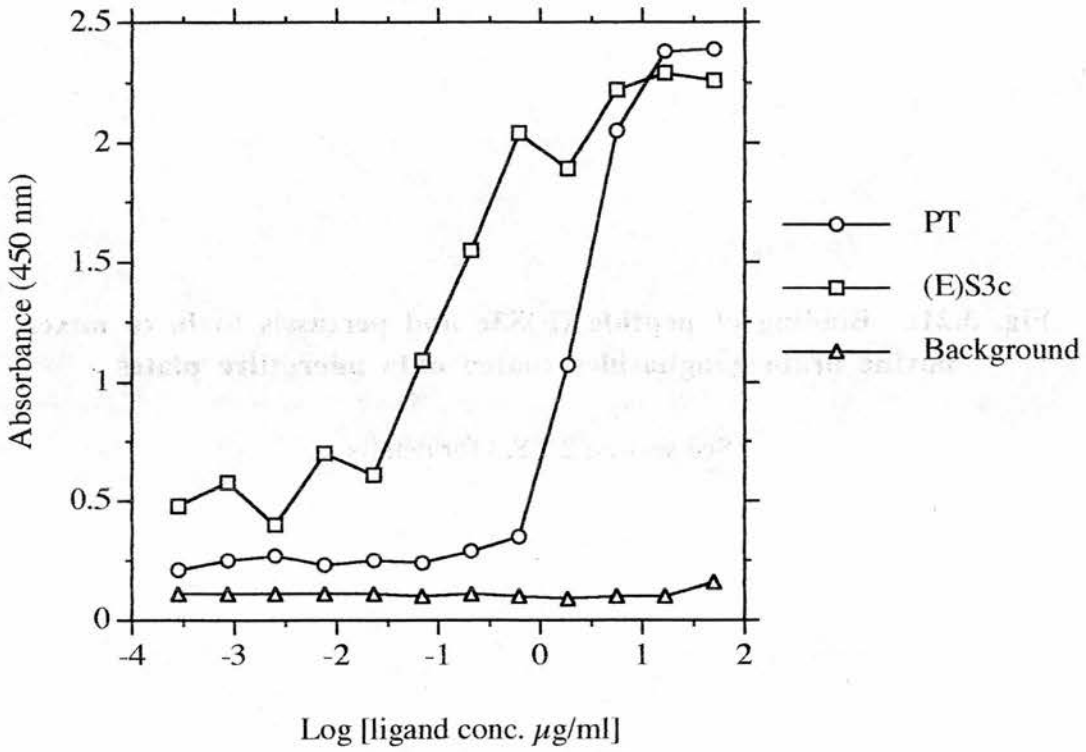
Fig. 3.21a Binding of peptide (E)S3c and pertussis toxin to fetuin coated onto microtitre plates

See section 2.2.8.3 for details.

Fig. 3.21b Binding of peptide (E)S3c and pertussis toxin to alpha-1-acidglycoprotein coated onto microtitre plates

See section 2.2.8.3 for details.

a.


Absorbance (450 nm)
2.5
2
1.5
1
0.5
0
-4
-3
-2
-1
0
1
2
Log [ligand conc. μg/ml]
PT
(E)S3c
Background

b.

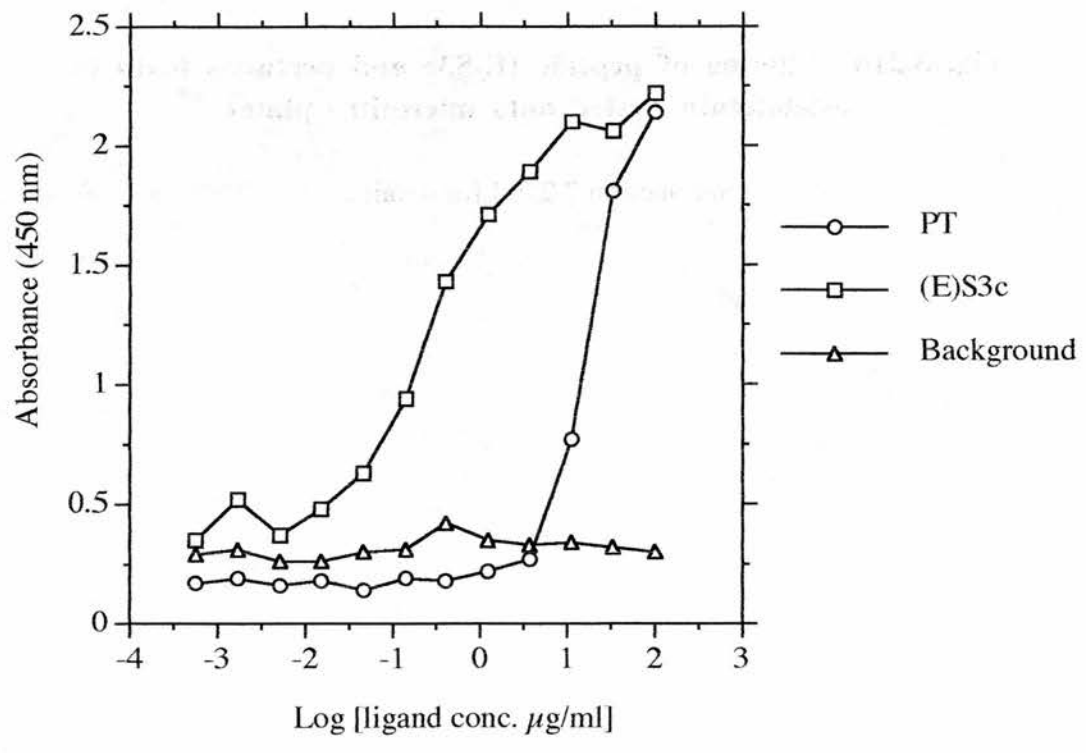

Absorbance (450 nm)
2.5
2
1.5
1
0.5
0
-4
-3
-2
-1
0
1
2
3
Log [ligand conc. μg/ml]
PT
(E)S3c
Background

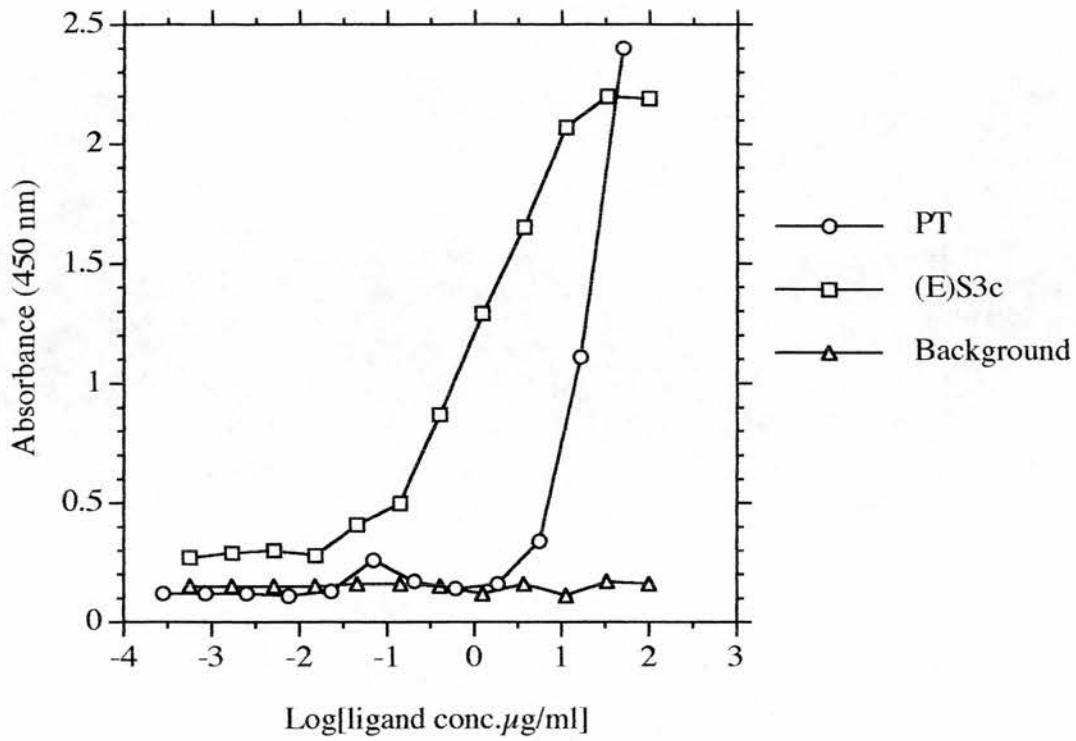
Fig. 3.21c Binding of peptide (E)S3c and pertussis toxin to mixed bovine brain gangliosides coated onto microtitre plates

See section 2.2.8.3 for details.

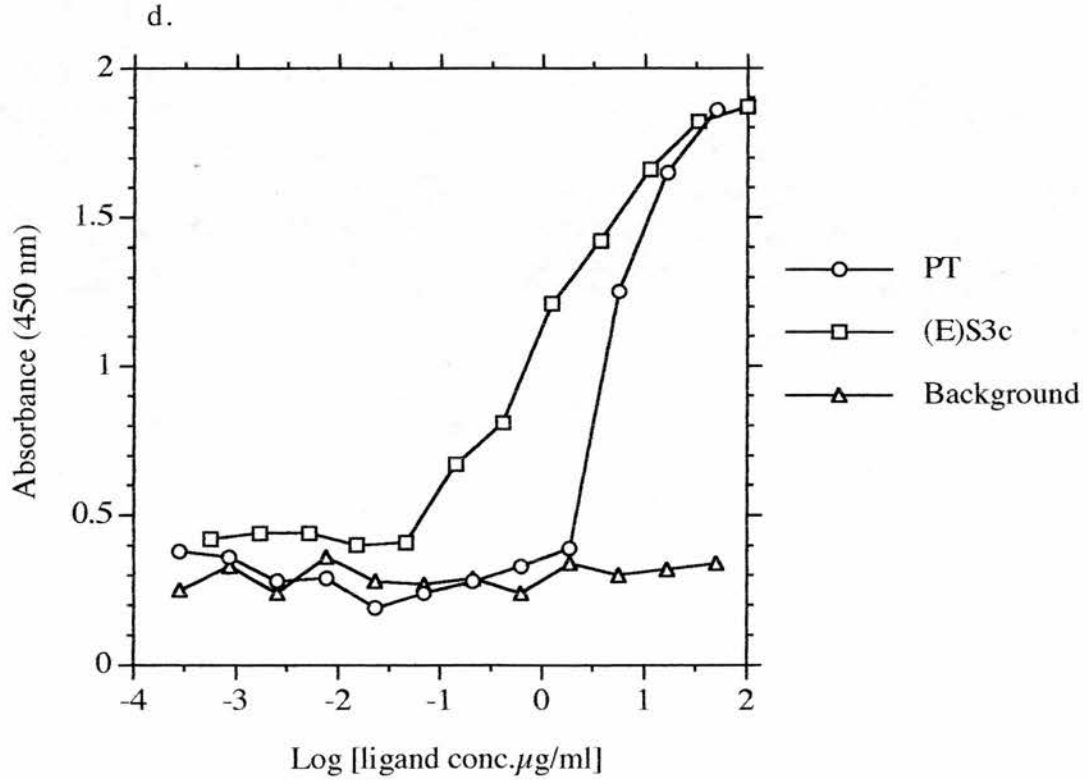
Fig. 3.21d Binding of peptide (E)S3c and pertussis toxin to asialofetuin coated onto microtitre plates

See section 2.2.8.3 for details.

c.


Absorbance (450 nm)
2.5
2
1.5
1
0.5
0
-4
-3
-2
-1
0
1
2
3
Log[ligand conc.μg/ml]
PT
(E)S3c
Background

d.


Absorbance (450 nm)
2
1.5
1
0.5
0
-4
-3
-2
-1
0
1
2
Log [ligand conc.μg/ml]
PT
(E)S3c
Background

rabbit anti-(E)S3c or mouse MAB L10 and then incubated with the species-specific anti-IgG antibody conjugated to horse radish peroxidase.

Background binding was anti-(E)S3c binding to the relevant glycoconjugate (the background was very similar to that seen with MAB L10).

The results of these experiments are shown in figures 3.21a to 3.21d. Apparent binding was taken to be a function of the absorbance (at 450 nm) generated in the ELISA assay. The higher the absorbance, the greater the apparent binding of the ligand to the glycoconjugate.

Pertussis toxin and (E)S3c apparently reacted most strongly with fetuin (fig.3.21a). Maximum binding with fetuin was seen at about 16 μg/ml pertussis toxin and about the same level with (E)S3c (log ligand concentration 1.2 in figure 3.21a).

Weaker binding was seen with alpha-1-acid glycoprotein (fig. 3.21b). Maximum binding of pertussis toxin and (E)S3c was not reached at 100 μg/ml of either the peptide or pertussis toxin (log ligand concentration 2 in figure 3.21b).

Weaker binding of (E)S3c and pertussis toxin to mixed bovine brain gangliosides was observed (fig. 3.21c), when results were compared with both fetuin and alpha-1-acid glycoprotein. Maximum binding was seen with 100 μg/ml peptide or 50 μg/ml pertussis toxin.

The weakest apparent binding activity was seen with asialofetuin (fig. 3.21d). Maximum absorbance (and binding activity) was lower than with fetuin, alpha-1-acid glycoprotein and mixed bovine brain gangliosides. Nonspecific binding,which is shown by the background in the figure, was higher than with the other glycoconjugates, so the interaction with asialofetuin was the weakest of the glycoconjugates tested.

3.3.8.2.6.3 Recognition of pertussis toxin by rabbit anti-(E)S3c

2 μg/ml pertussis toxin or (E)S3c were coated onto microtitre plate wells and reacted with rabbit anti-(E)S3c in a solid phase ELISA. Background binding was anti-(E)S3c binding to uncoated wells. The results of the experiment are shown in figure 3.22a and the absorbance at 450 nm was used as an estimate of antibody binding activity as in the previous section.

It can be seen from the figure that a high dilution of anti-(E)S3c was needed to titrate out the binding activity of the antibody to (E)S3c to background level. The absorbance (at 450 nm) did not fall below 2.5 until the antibody was diluted almost 1500-fold and background binding absorbance was seen with antibody diluted about 10^5-fold.

Binding of anti-(E)S3c to pertussis toxin was weaker. The absorbance was below 2.5 even at 1:54 dilution of antibody and background binding was reached at about 10^4-fold dilution of antibody, a lower dilution than seen with (E)S3c, above.

3.3.8.2.6.4 Recognition of pertussis toxin captured onto microtitre plates by glycoconjugates

Fetuin or mixed bovine brain gangliosides were captured onto microtitre plates, incubated with pertussis toxin and the pertussis toxin detected with anti-(E)S3c as described in section 2.2.8.2. If pertussis toxin bound each of the glycoconjugates to its S3 subunit, it might be expected that anti-(E)S3c would be sterically excluded from binding pertussis toxin. That is, the antibody (E)S3c was raised against an apparent fetuin-binding peptide. One could reasonably expect that occupation of the apparent binding site on S3 by fetuin would stop anti-(E)S3c from binding and because (E)S3c bound to mixed bovine brain gangliosides (see previous section), binding of the gangliosides to captured pertussis toxin should also exclude anti-(E)S3c from binding pertussis toxin.

Figure 3.22b shows the results of the experiment and that both pertussis toxin captured by fetuin and by mixed bovine brain gangliosides were recognised by anti-(E)S3c.

3.3.8.2.6.5 Inhibition of anti-(E)S3c binding to peptide (E)S3c

Fetuin and anti-(E)S3c bind to peptide (E)S3c, as described above, so the ability of fetuin to inhibit binding of anti-(E)S3c to peptide (E)S3c was assessed using a solid phase ELISA with the peptide as coating antigen (see section 2.2.8).

Anti-(E)S3c was diluted in a constant concentration of fetuin (concentration gives an absorbance at 450 nm of 1.2-1.5 in the absence of peptide) and incubated for 90 minutes, then incubated with the peptide. Binding of fetuin to the peptide was detected using an anti-fetuin antibody conjugated to horse radish peroxidase.

The assay was repeated with fetuin diluted in a fixed concentration of anti-(E)S3c. Anti-(E)S3c and fetuin diluted in ELISA blocking buffer were used as controls.

Both the controls were expected to give a decreasing absorbance at 450 nm as the ligand was diluted and that was the observed result.

As anti-(E)S3c was diluted in fetuin, the absorbance at 450 nm would be expected to increase to 1.2-1.5 because the amount of anti(E)S3c would decrease leaving more peptide free to bind to fetuin and be detected by the anti-fetuin antibody conjugated to horse radish peroxidase. A similar result was expected with fetuin diluted in a constant concentration of anti-(E)S3c. However, neither fetuin nor anti-(E)S3c inhibited the other's binding to peptide (E)S3c.

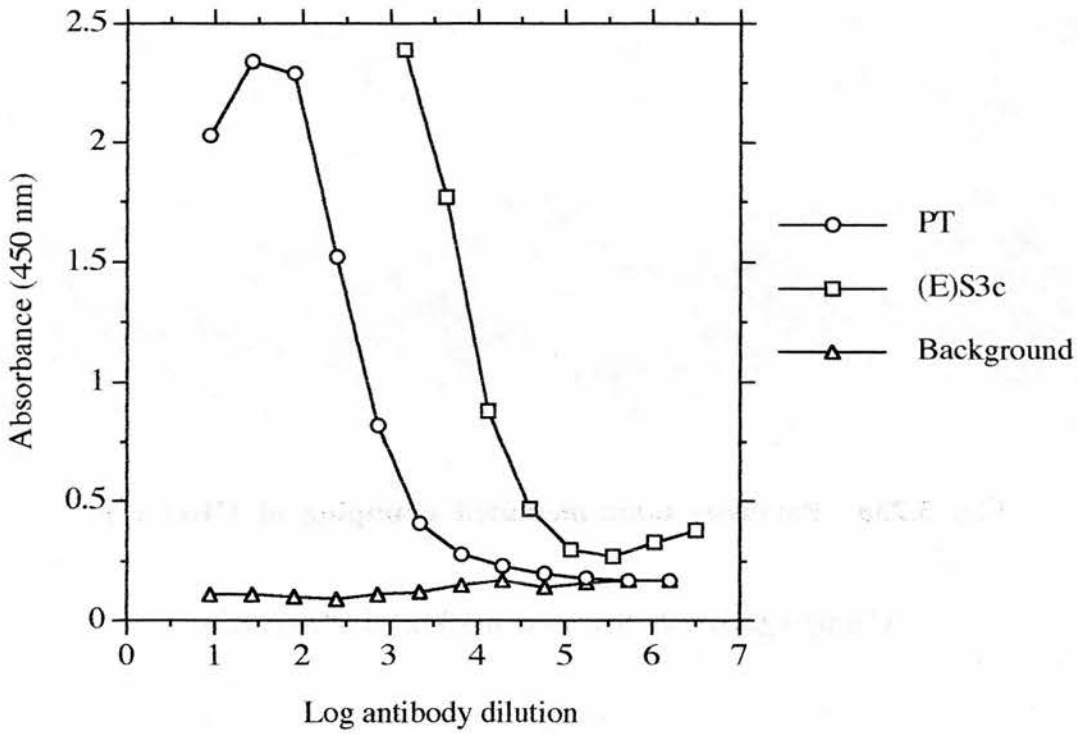
Fig. 3.22a Recognition of peptide (E)S3c and pertussis toxin coated onto microtitre plates, by rabbit anti-(E)S3c

See section 2.2.8 for experimental details.

Fig. 3.22b Recognition of pertussis toxin captured by mixed bovine brain gangliosides or fetuin, by rabbit anti-(E)S3c

See section 2.2.8.2 for details.

a.


Absorbance (450 nm)
Log antibody dilution
PT
(E)S3c
Background

b.

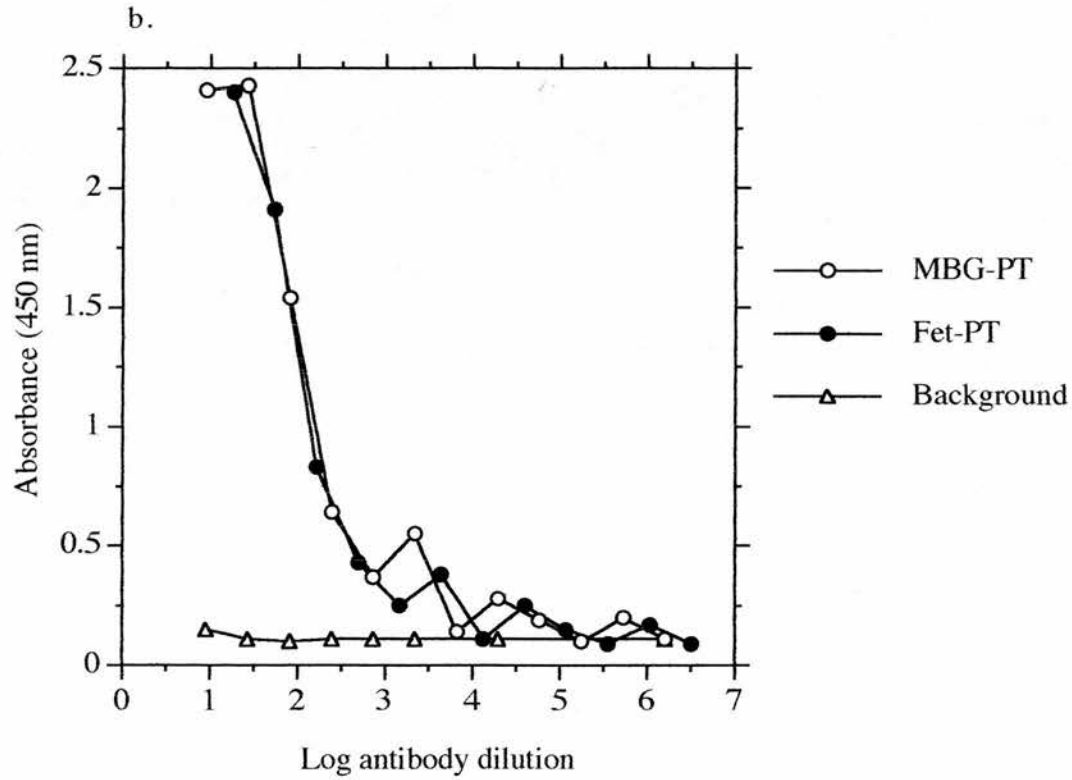

Absorbance (450 nm)
Log antibody dilution
MBG-PT
Fet-PT
Background

Fig. 3.23a Pertussis toxin-mediated clumping of CHO cells

(Clumping score 2. See section 3.3.8.2.6.6 for details)

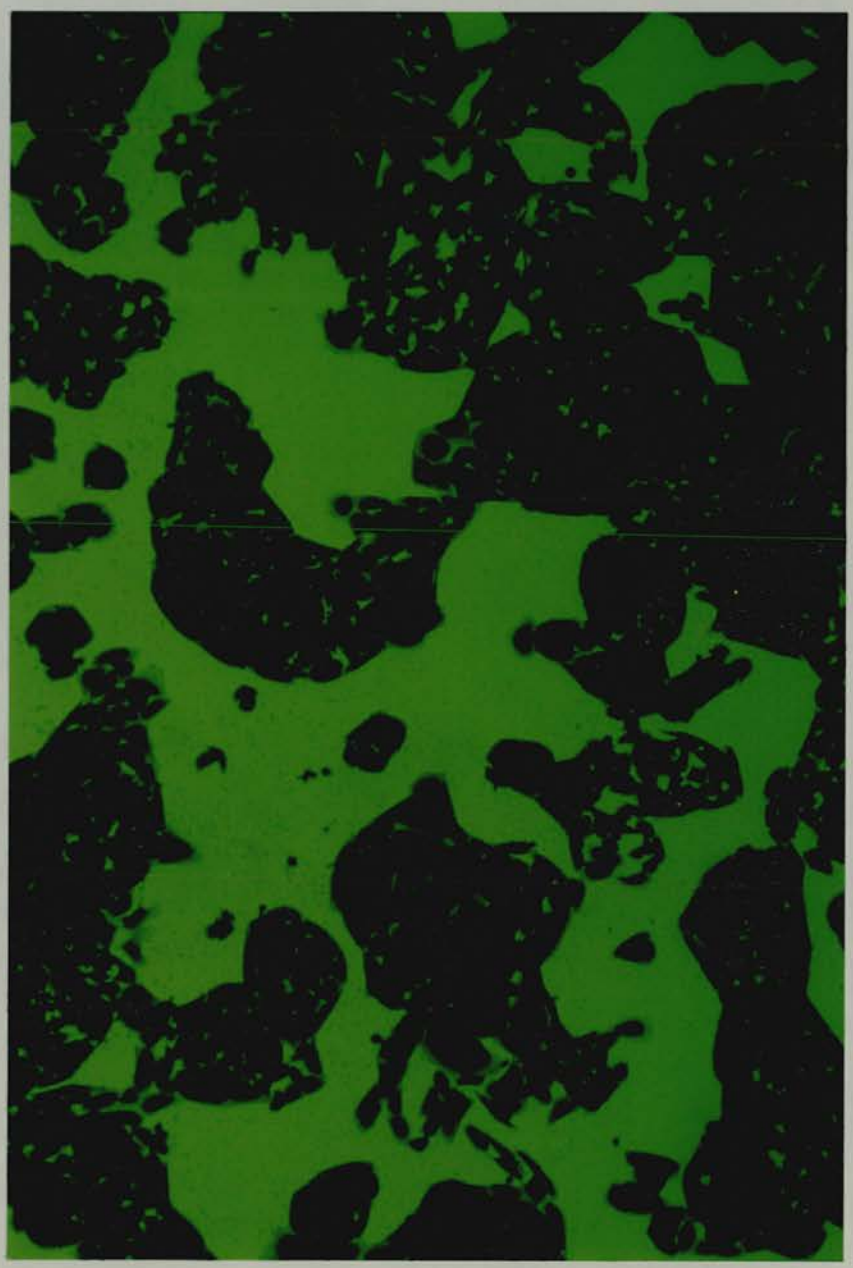


Fig. 3.23b Pertussis toxin-mediated clumping of CHO cells

(Clumping score 0. See section 3.3.8.2.6.6 for details)

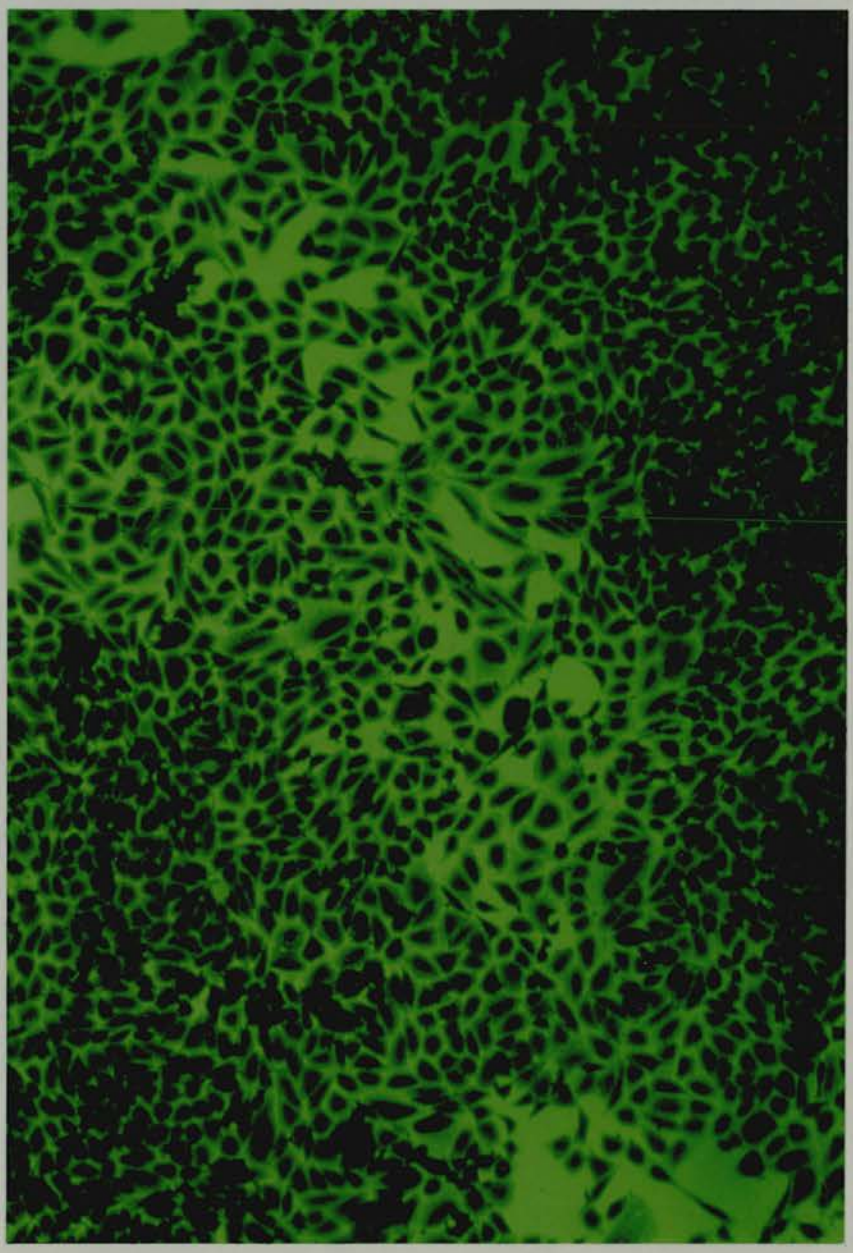
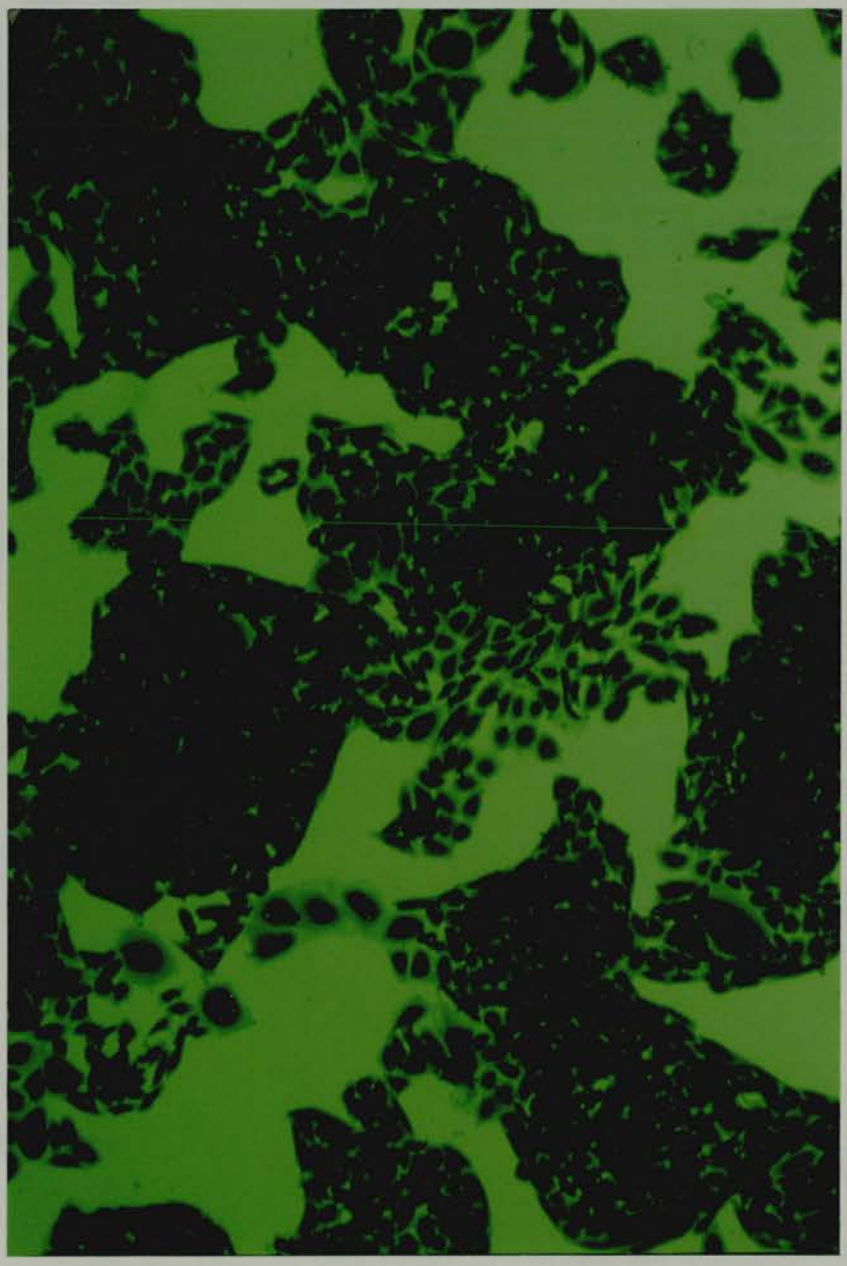


Fig. 3.23c Pertussis toxin-mediated clumping of CHO cells

(Clumping score 1. See section 3.3.8.2.6.6 for details)



3.3.8.2.6.6 Chinese hamster-ovary cell clumping

The ability of (E)S3c to bind fetuin led to assessment of the ability of anti-(E)S3c to inhibit pertussis toxin-mediated Chinese hamster ovary cell (CHO) clumping. The method of assay is described in section 2.2.12 and the effect of incubating anti-(E)S3c and pertussis toxin with CHO cells was compared to the effect of incubating CHO cells with and without pertussis toxin.

CHO cells in the presence of 2 ng/ml pertussis toxin (the lowest concentration found to clump the cells) were found to be be totally clumped and test microtitre plate wells containing totally clumped cells were given a score of 2 (fig. 3.23a). This concentration was the minimum found to clump all cells in a well. CHO cells not in the presence of pertussis toxin did not clump and test wells containing non-clumped cells were given a score of 0 (fig. 3.23b). Any state of clumping which was not total was given a score of 1 in the test plate wells (fig. 3.23c).

Neither (E)S3c or anti-(E)S3C inhibited pertussis toxin clumping of CHO cells at all (wells containing either were given a score of 2).

3.4 Discussion

In this chapter, studies on the antigenicity and glycoconjugate binding of synthetic peptides derived from the amino acid sequence of pertussis toxin are described.

The results obtained were to be used in an attempt to identify S3 subunit amino acids that were involved in B-oligomer binding to carbohydrate, and S1, S3 and S4 amino acids that may constitute 'linear' B cell epitopes, which in turn could be part of discontinuous B cell epitopes.

As stated in chapter 1, most protein epitopes are thought to be conformational. However, if overlapping pin decapeptides bind anti-protein antibodies, those decapeptides can be used as a template for the synthesis of free peptides. Those free peptides which raise antibodies that cross-react with native protein can be used to study the structure and function of a protein indirectly.

That few strong positive reactions were seen using the pin peptide technique suggests that low amounts of peptide were synthesised successfully, a possibility supported by the reaction of some of the antibodies and antisera with SDS-denatured toxin subunits on Western blots. Some of the test samples had quite high anti-pertussis toxin titres, so it was expected that they should react with both the pin decapeptides and the denatured subunits on Western blot.

3.4.1 Antibody binding to pertussis toxin subunit pin decapeptides

A panel of antibodies were tested for reaction with the pin decapeptides, with pertussis toxin coated onto microtitre plates in a solid phase ELISA and with pertussis toxin subunits separated by SDS PAGE and immunoblotted onto nitrocellulose.

All but three antibodies tested were reactive with the toxin subunits on immunoblots, suggesting that antibody binding domains may contain continuous ('linear') regions of amino acids and may bind to the pin decapeptides. MAB L4 weakly recognised subunit S3 and L6 and L11 did not recognise any pertussis toxin subunits on immunoblots. All the antibodies tested reacted with pertussis toxin in a solid phase ELISA, the lowest titre being with MAB L11.

3.4.1.1 Antipeptide antibodies

Antipeptide antisera raised against synthetic peptides derived from the amino acid sequence of pertussis toxin subunits were reacted with the pin decapeptides. Some were found to react weakly with decapeptides that had some amino acid identity with the peptide immunogen. The amount of identity for an antipeptide antibody against one pertussis toxin subunit to weakly bind a pin decapeptide derived from another subunit appeared to be two residues.

Only one antipeptide antibody (anti-S3a) bound strongly to decapeptides derived from the sequence of another toxin subunit (S1 decapeptides with an eight residue overlap). This antipeptide antibody did not react with S1 decapeptides with a five residue overlap, probably because all but one of these S1 decapeptides had different amino acid sequences to the decapeptides with an eight residue overlap.

This supported the findings of Trifilieff *et al.*, (1991), whose group found that antipeptide antibodies cross-reacted with peptides that contained similar partial sequences. Anti-S3a also bound to S3 and S1 subunits on immunoblots of pertussis toxin subunits separated by SDS PAGE, supporting the result that showed binding of anti-S3a to S1 decapeptides.

The results of pin decapeptide binding of antipeptide antibodies meant that cross-reaction of an antibody with the pin peptides was only viewed as suggestive of peptide antigenicity and not as concrete evidence.

3.4.1.2 Mouse antibodies

The three polyclonal antisera and most of the mouse-monoclonal antibodies did not react with the pin decapeptides. Two possible reasons for this could be that the ELISA assay used to detect binding to the pin peptides was biased to detection of relatively

high affinity binding, or that the antibody paratopes recognise conformational epitopes on the toxin subunits.

MAB L10 has been shown to neutralise the action of the toxin *in vitro* and to protect mice from intracerebral challenge with *B. pertussis* (Bartoloni *et al.*, 1988) and it has been suggested that L10 binds a discontinuous epitope contained in the N- and C-terminal regions of the S1 subunit of pertussis toxin. L10 did not bind to any of the S1 pin decapeptides above background levels, suggesting that L10 binds a conformational epitope, or that the antibody bound to the pin decapeptides with too low an affinity to be detected by the pin ELISA assay.

Cieplak *et al.*, (1988) and Burnette et *al.*, (1988a) had identified epitopes within S1 and found that immunisation with a recombinant S1 subunit did not induce protective immunity in mice, indicating that at least part of the B-oligomer is needed for protection against infection.

Only L7 and L9 reacted with the pin decapeptides when the antibodies were reacted as purified antibody. Both reacted weakly with S3 decapeptide 22. MAB L7 has been shown to neutralise pertussis toxin-mediated CHO cell clumping (Anwar *et al.*, 1987) and recognised mostly subunit S3 on immunoblots (fig. 3.3). L7 no longer recognised subunit S3, when 2-ME was included in the sample buffer, in preparation for electrophoresis and before immunoblotting with L7. This suggested that antibody L7 bound a conformational epitope on subunit S3. Decapeptide 22 of S3 may represent part of that conformational epitope, which could explain the weak binding of L7 to the peptide (A_{405} nm 0.365), but results of other studies have not suggested that the residues of decapeptide 22 are involved in a B cell epitope of pertussis toxin (Chong *et al.*, 1992; Lobet *et al.*, 1993), so it is unlikely that S3 decapeptide 22 is part of a B cell epitope.

3.4.1.3 Rabbit antibodies and binding to free peptide S4b

Two rabbit polyclonal antibodies reacted strongly with the same S1 and S4 pin decapeptides. The antibodies reacted mainly with two S1 decapeptides with a five residue overlap, one near from the N-terminal of the subunit sequence and one from near the C- terminal. Several B cell epitopes have been attributed to the S1 subunit of pertussis toxin and major B cell epitopes found using monoclonal and antipeptide antibodies are thought to be within residues 1-18 (Bartoloni *et al.*, 1988; De Magistris *et al.*, 1989; Chong et *al.*, 1991), and residues 201-235 (De Magistris *et al.*, 1989; Chong *et al.*, 1991).

The rabbit antibody (12A20A) which reacted most strongly was preincubated with pertussis toxin, before reaction with the pin decapeptides. That preincubation was found to inhibit antibody binding to both S1 and S4 decapeptides, suggesting that the antibody bound to homologous sites on the pin decapeptide and the whole toxin.

Preincubation of 12A20A with free peptide S4b (residues 86-94), which had the same amino acid sequence (plus two amino acids synthesised to couple S4b to an immunogenic carrier protein) as the decapeptide bound by 12A20A, also reduced 12A20A binding to the S4 pin decapeptides to background levels, suggesting that 12A20A bound homologous sites on both the pin-bound and free peptides, so antipeptide antisera were raised against peptide S4b conjugated to immunogenic protein carriers. Antisera to S4b conjugated to KLH weakly recognised whole pertussis toxin captured directly and indirectly to microtitre plate wells, suggesting that the sequence of S4b may be exposed on the surface of the whole toxin. Weak recognition of whole pertussis toxin by anti-S4b may be due to part-exposure of the sequence in the whole toxin, or a conformation of the whole toxin that was not favourable to strong binding of the antipeptide antibody.

Peptide S4b did not inhibit 12A20A binding to toxin coated onto microtitre plates in a solid phase ELISA, probably because 12A20A only weakly recognised the peptide. Anti-S4b and 12A20A did not inhibit each other's binding to peptide S4b or to pertussis toxin again probably because anti-S4b did not recognise pertussis toxin well and 12A20A did not recognise peptide S4b well.

Only two studies to date have looked at B cell epitopes of the S4 subunit and one did not identify any on that subunit (Seabrook *et al.*, 1990). The other study (Ibsen *et al.*, 1993) used free peptides spanning the whole amino acid sequence of the S4 subunit and screened them for reaction with a panel of murine monoclonal antibodies and various polyclonal antisera, by ELISA using the peptide as coating antigen. Ibsen *et al.* (1993) identified six antigenic regions (they bound polyclonal antisera in this case), one of which included residues 91-106 of S4 (the reactive S4 pin decapeptide 18, contained residues 86-95. Monoclonal anti-(pertussis toxin) antibodies did not recognise region 91-106 or any other S4 peptides, suggesting the existence of mostly conformational epitopes on the S4 subunit. Antipeptide antisera raised from the S4 peptides conjugated to diphtheria toxoid recognised pertussis toxin in an ELISA, but did not neutralise pertussis toxin-mediated Chinese Hamster ovary cell clumping, which could be partly due to the S4 subunit consisting mostly of discontinuous B cell epitopes. This could explain the low anti-(pertussis toxin) reaction of antisera S4b found by the author in ELISA and the lack of reaction of the author's panel of monoclonal antibodies with the S4 pin decapeptides.

T cell epitopes of S4 have recently been studied by Petersen *et al.* (1992; 1993). Two major and one minor human T cell epitope (residues 1-15, 21-45 and 41-65 respectively) and one corresponding epitope in mice which was the C-terminal portion of residues 1-15, as well as mouse T cell epitopes in residues 21-55, 72-88 and 93-106 were discovered. Peptide S4b has residues in common with two of the mouse T cell epitopes identified in the studies by the Petersen group, but the significance of that remains to be seen.

The results showed that peptide S4b is not likely to be a suitable candidate for inclusion in a vaccine containing antigens that stimulate B cell immunity.

3.4.1.4 Human antibodies

Samples 135 and 27(1)4 reacted mainly with one S1 decapeptide (8 residue overlap), number 114 (residues 225-234) derived from the C-terminal of the S1 amino acid sequence. Both individuals had three DTP vaccinations, but their ages were <1 and 21 respectively. Individual 135 was disease-free when the sample was taken whereas individual 27(1)4 had mild disease.

Sample 18(1)1 reacted with one decapeptide from the S3 subunit (decapeptide 6) that contained residues 26-35. This sample was from an individual 1.58 years of age who had no DTP vaccinations.

The panel of human sera was small, so no conclusions can be made apart from general ones and extensions of the work would require the testing of many more sera.

Reaction of a sample with the pin decapeptides could not be predicted from the characteristics of the individual that the sample was taken from. For example, samples 135, 145 and 412 were taken from a similar group, but only 135 reacted strongly with the pin decapeptides.

What was seen was that all three reactive sera were taken from individuals less than two years of age . It would be interesting to see if this becomes a trend if more samples were tested with the pin peptides. The two oldest subjects had three DTP vaccinations but that did not prevent their contracting the disease and neither reacted strongly with the pin decapeptides. Whether this means that the protection of the DTP vaccine decreases with age is obviously impossible to say at this early stage of investigation.

The number of DTP vaccinations and the effects on samples from a group of very similar age taken at similar times after onset of cough could also be studied in further work. Sample 140(1)1 was from a subject who had only one DTP vaccination and the sample had a high anti-pertussis titre (table 3.5).

3.4.1.5 Comparison of reaction of mouse and rabbit anti-pertussis toxin and human antibodies with the pin decapeptides

The results showed that there were different responses to the pin decapeptides both between and within species.

The two reactive rabbit antisera bound to decapeptides derived from near the N- and C-terminals of the S1 amino acid sequence and to a decapeptide derived from near to the C-terminal of the S4 amino acid sequence. One of the reactive human sera also bound to decapeptides derived from near the N- and C-terminal regions of S1

decapeptides (8 residue overlap), but the reactive regions were slightly different compared to the reactive rabbit antisera. The other two reactive human sera bound to S3 decapeptides, one binding to a peptide near the N-terminal and the other to an S3 decapeptide derived from the sequence near the C-terminal of the S3 subunit.
The reactive mouse antisera bound to one S3 decapeptide that did not correspond to the S3 decapeptides reactive with the human sera and the mouse sera reacted with the pin decapeptides with low affinity. That is, even though the most reactive decapeptides produced an absorbance at 405 nm that was double the background, the actual absorbance (taken to indicate antibody-pin peptide affinity) of the reactive decapeptides was below 0.5 at 405 nm.

The differences in response of different species has been reported previously using this pin peptide mapping method (Beattie *et al.*, 1992). The differences in specificity of response (B cell) to a protein antigen was to be expected in different animals, because of the effects of antigen processing and T cell help, for example.

3.4.2 Glycoconjugate binding to peptides derived from the amino acid sequence of the S3 subunit of pertussis toxin

A pin decapeptide derived from the amino acid sequence of the S3 subunit of pertussis toxin was found to bind the glycoprotein fetuin using the pin peptide mapping method of Geysen *et al.* (1984; 1987). The technique was used in an attempt to localise region(s) of the S3 subunit amino acid sequence that bound to the carbohydrate portion of some glycoproteins.

The binding of fetuin to the pin decapeptide identified (residues 46-55, sequence RQITPGWSIY) with pin peptide mapping was inhibited by a free peptide (E)S3c that had the same sequence as the pin decapeptide (with a C-terminal glutamic acid), suggesting that the pin and free peptide had comparable conformations.

An antipeptide antibody raised to peptide (E)S3c recognised pertussis toxin and free peptide in a direct and in an indirect capture ELISA assay, in which the toxin or peptide was either coated directly onto the microtitre plate, or was captured by a monoclonal antibody or glycoconjugate. This result showed that although reaction was weak, the antipeptide antibody was capable of recognising the intact toxin and that the (E)S3c peptide sequence is probably exposed at the toxin surface.

The antipeptide antibody anti-(E)S3c was used to detect binding of peptide (E)S3c to glycoconjugates. Binding of the peptide to fetuin, alpha-1-acid glycoprotein and mixed bovine brain gangliosides was detected. The highest activity appeared to be with fetuin, slightly weaker reaction was seen with alpha-1-acid glycoprotein, weaker still with mixed bovine brain gangliosides and the weakest apparent activity was seen with asialofetuin. An anti-pertussis toxin antibody that bound to S1 in immunoblots (L10) was used to detect pertussis toxin binding to the four glycoconjugates mentioned

above and showed that the toxin bound to all four, again with decreasing affinity.

The results showed that peptide (E)S3c acted like the whole toxin in binding to glycoconjugates (Armstrong *et al.*, 1988).

Anti-(E)S3c also recognised pertussis toxin bound to fetuin and mixed bovine brain gangliosides. Anti-(E)S3c may be expected not to bind to the toxin if it were bound to the glycoconjugate via the S3 subunit. However, the S2 subunit has an amino acid sequence about 70% similar to S3, so it is possible that the antibody cross-reacted with similar amino acids of S2. Another possibility is that pertussis toxin was bound to the glycoconjugate via its S2 subunit and that anti-(E)S3c bound to the S3 subunit. The latter possibility is supported by subunits S2 and S3 having some receptors (e.g. fetuin) in common (Witvliet *et al.*, 1989; Chong *et al.*, 1992). No inhibition of anti-(E)S3c binding to peptide (E)S3c by fetuin was seen, which could also be explained by the possible cross-reaction of anti(E)S3c with similar residues of S2 or that the toxin was bound to the S2 subunit, leaving the S3 subunit free to bind anti-(E)S3c. (The glycoconjugate-binding abilities of peptide (E)S3c were published in Tallett *et al.*, 1993).

The ability of (E)S3c to bind glycoproteins and glycolipids suggested that this region of S3 in the whole toxin may be involved in binding of the toxin to target cell receptors. The ability of anti-(E)S3c to inhibit pertussis toxin-mediated Chinese hamster ovary cell clumping tested this idea and the antibody did not inhibit the clumping. This means that peptide (E)S3c is not likely to induce protective antibodies and so is not likely to be included in a potential vaccine against whooping cough.

The region of the S3 subunit found to bind glycoconjugates in this study was found to be in the same region of the subunit as a domain similar to eukaryotic C-type carbohydrate recognition domains (Drickamer 1989) contained in residues 37-52 of S3 (Saukkonen *et al.*, 1992). Region 37-52 of S3 was found to bind leukocytic gangliosides and binding was inhibited by sialic acid (neuraminic acid). The same region of S2 was found to bind lactosylceramide (cilia) and mutation in that region of S2 or S3 resulted in interchanged carbohydrate specificity, indicating the region's possible role in receptor binding. Saukkonen's and the author's work could be expanded to identify the possible carbohydrate structure specificity and to determine whether differences in membrane structure or membrane components alter specificity.

Gangliosides were also found to bind peptide (E)S3c (Tallett *et al.*, 1993) using intrinsic fluorescence, but whether the interaction is with the carbohydrate portion or the lipid portion of the ganglioside remains to be seen, because (E)S3c was also found to bind phospholipid (L-α–lysolethicin) and N-acetylneuramin-lactose. (N-acetylneuramin-lactose is an analogue of the oligosaccharide part of fetuin and gangliosides).

Brennan *et al.*, (1988) found no binding of pertussis toxin to glycolipids using a TLC system, in contradiction to Saukkonen et *al.*(1992) and Tallett *et al.*, (1993),

described above.

However, Hausman and Burns (1993) detected binding of whole pertussis toxin and B-oligomer to lipid vesicles containing ganglioside Gd1a. Gd1a as a functional receptor was not addressed in this study, but the importance of sialic acid moieties in toxin binding was. They (Hausman and Burns) claimed that the reason that Brennan *et al.*, (1988) did not detect any glycolipid binding to pertussis toxin was because the vesicle-binding system that Hausman and Burns used was more sensitive than the TLC system used by Brennan's group. This reason could also apply to the detection of glycolipid binding using fluorescence.

Other regions may be involved in receptor recognition by the S3 subunit that were not detected by the pin peptide mapping procedure. For example residues 28-45 of S3 inhibited bacterial cells' binding to human macrophages (van't Wout *et al.*, 1992) and Loosemore *et al* . (1993) found that residues 149-176 of the S3 amino acid sequence blocked pertussis toxin-mediated clumping of Chinese Hamster Ovary cells, suggesting that this region is involved in binding of mammalian target cells.

The sequence of (E)S3c has little sequence similarity with other peptides involved in carbohydrate binding. Many of these other peptides (for example fibronectin) contain the peptide sequence RGD (Arg-Gly-Asp) at their recognition site (Ruoslahti and Pierschbacher, 1986). Peptide (E)S3c does contain a tripeptide PGW, that is found in the sequence of an integrin-binding peptide of type IV collagen (Chelberg *et al.*, 1990) and type IV collagen and pertussis toxin have been found to bind to laminin, an extracellular matrix component (Witvliet *et al.*, 1989; Mayo *et al.*, 1991), so it is possible that this recognition does not use the RGD sequence in cell adhesion.

In summary, a glycoconjugate-binding peptide (E)S3c was identified using pin peptide mapping and free peptide studies. The peptide did not inhibit pertussis toxin-mediated Chinese hamster ovary cell clumping, so is not likely to induce protective antibodies.

Chapter 4

Chemical cross-linking of pertussis toxin

4.1 Introduction to chemical cross-linking

In this chapter, chemical cross-linking is discussed as another approach for investigation of the spatial arrangement of toxin subunits and to reaffirm the 1:1:1:2:1 stoichiometry. Reversible and non-reversible cross-linking, one- and two-dimensional SDS-PAGE, Western blotting and FPLC were used.

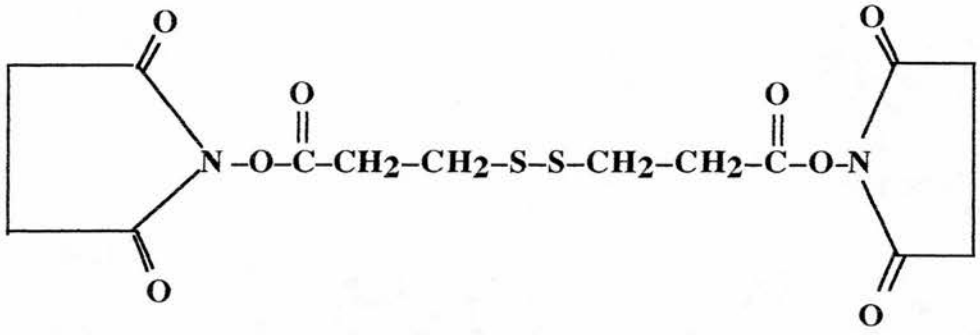
Chemical cross-linkers are bifunctional reagents which link neighbouring polypeptides and can be used to determine the tertiary or quaternary structure of a protein. One protein subunit may be linked to one or more neighbours, forming dimers, trimers and so on. Separation of cross-linked species and identification of their components can aid in mapping the location of subunits in an oligomer. However, formation of cross-linked species containing more than two subunits can complicate the development of a map.

Cross-linkers are grouped according to several factors including length, amino acids linked (or bridged), whether the amino acids linked are the same (homobifunctional) or different (heterobifunctional), whether they contain an internal cleavable site and whether the cross-linking reaction is triggered chemically or by light.

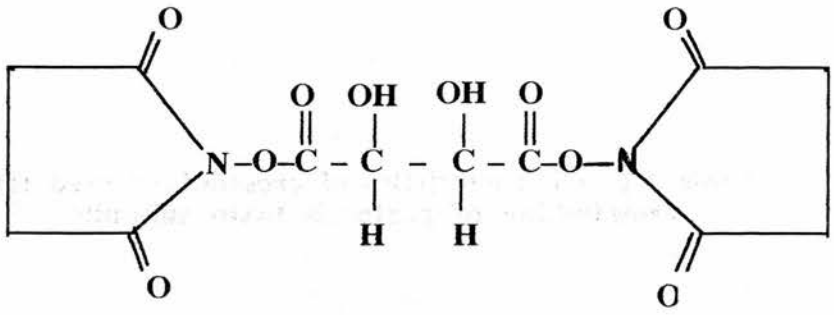
Chemical cross-linkers generally react with lysine and/or cysteine residues (Smith *et al.*, 1978; Geisler *et al.*, 1992). Three cross-linkers were used to study pertussis toxin subunits in this work. Two homobifunctional, cleavable reagents were used, dithiobis (succinimidyl propionate), DSP, and disuccinimidyl tartarate, DST. Their structures are shown in figure 4.1. They acylate amines of lysine residues or at the N-terminal. DSP has an internal disulphide bond cleavable by reducing agents (Lomant and Fairbanks, 1976) and DST contains a glycol bond, cleavable by treatment with periodate (Smith *et al.*, 1978). Cleavable cross-linkers were used because their cleavage should regenerate the monomers involved in creation of the cross-linked aggregates.

One non-cleavable, heterobifunctional cross-linker was used, succinimidyl 4-(p-maleimidophenyl) butyrate, SMPB. One functional group acylates primary amino groups (e.g.. lysine, N-terminal amine) and the other is a maleimide group which has a double bond that reacts with the thiol group of cysteine, to form a thioether bond. SMPB is longer than either DSP or DST and a comparison of their characteristics is given in table 4.1. SMPB, being the longest linker used, should be less subject to steric hindrance than DSP or DST, and was used to compare patterns of cross-linked aggregates with those obtained with DSP or DST.

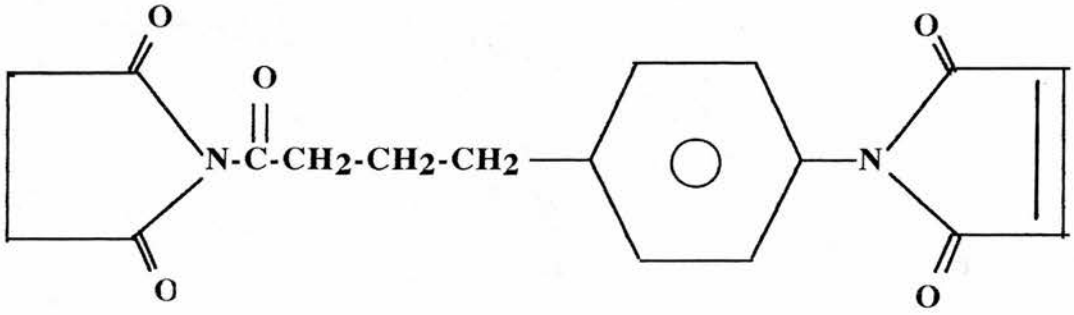
Fig. 4.1 Structures of DSP, DST and SMPB



DSP
Dithiobis(succinimidyl propionate)
M_r 404.4



DST
Disuccinimidyl tartarate
M_r 344.2



SMPB
Succinimidyl 4-(p-maleimidophenyl)
butyrate
M_r 356.3

Table 4.1 Characteristics of crosslinkers used for crosslinking of pertussis toxin subunits

Chemical Name	Abbrev.	Type	Length/nm	Cleavable?
Dithiobis(succinimidyl propionate)	DSP	homobifunctional	1.2	Yes (thiol)
Disuccinimidyl tartarate	DST	homobifunctional	0.64	Yes (periodate)
Succinimidyl 4-(p-maleimidophenyl) butyrate	SMPB	Heterobifunctional	1.45	No

The principles on which this work were based were that cross-linked aggregates could be separated by size on first dimension SDS-PAGE and cross-link bridges connecting the subunits within the aggregates could be cleaved and the regenerated monomers separated by size on a second dimension of SDS-PAGE . Separation of the cross-linked aggregates using FPLC was also tried. Uncleaved aggregates were also run on one dimensional SDS-PAGE and cross-linked aggregates were detected by using subunit-specific antibodies and Western blotting.

4.2 Methods

4.2.1 Time courses of cross-linking

The method of cross-linking pertussis toxin subunits is shown in section 2.2.10.1. In order to determine an appropriate time of cross-linking and cross-linker concentration to maximise cross-linked species for a known toxin concentration, time course experiments were carried out. Experiments were performed at pH 8.3, where primary amines reactive with the linker are less protonated, since this favours the cross-linking reaction. The pH used for cross-linking experiments using SMPB was also pH 8.3. DSP, DST and SMPB were dissolved in dimethyl sulphoxide (DMSO) to a final stock concentration of 100 mM. An aliquot of stock cross-linker was added to a known concentration of toxin in cross-linking buffer (pH 8.3), to give final cross-linker concentrations of 0.1, 0.5, 1.0, 5.0 or 10.0 mM and protein concentration of 0.35, 0.7, or 1.4 mg/ml. Cross-linking at lower protein concentrations, protein-precipitation and re-solubilisation was tried before SDS-PAGE, but the re-solubilisation step was not effective enough to be able to detect any cross-linked species by silver-staining.

The reaction mixture was agitated and incubated for 5, 10, 15, 20, 25, or 30 minutes at 30°C. The linking reaction was effectively stopped by addition of excess Tris-Cl pH 7.8 (decreased pH probably stopped the reaction) and samples were prepared for electrophoresis (section 2.2.9). Cross-linked species were separated using 17.5%(w/v) acrylamide SDS PAGE. A control was also run on SDS-PAGE, containing toxin and the appropriate amount of DMSO, but without cross-linker.

4.2.2 Two-dimensional electrophoresis

Cross-linked pertussis toxin was separated in a first dimension of 17.5%(w/v) acrylamide gel using no reducing agent, as described in section 2.2.9. A strip was cut from this first dimension and incubated in cleavage buffer for 45 minutes at 60°C. The strip was then washed and fixed to the stacking gel of a second dimension 17.5%(w/v) acrylamide gel. Pertussis toxin was used as a standard after either being boiled with

0.1%(w/v) SDS, 2%(v/v) 2-ME or with 0.1%(w/v)SDS alone, the latter being used to check the reduction of S1 after cross-link cleavage.

Several cross-link cleavage methods were tried for DSP, before a suitable method was found. Initially, results indicated either partial cleavage, or at worst, no detectable cross-link cleavage at all. Possible reasons for this are that the conformation of the cross-linked aggregates formed shielded the cross-link bridge from the reducing agent, or that the high concentration of the acrylamide in the gel increased the time taken for the reducing agent to gain access to the aggregates. In the first method, a strip was cut from the first dimension and soaked in cleavage buffer, 100 mM Tris-Cl, 1%(w/v) SDS, 15 mM DTT (pH 8.9) at room temperature for one hour. The strip was then soaked in 50 mM Tris-Cl, 1%(w/v) SDS, (pH 6.7) for five minutes, a step found to remove artifactual bands on the second dimension gel. The strip was then fixed to the stacking gel of the second dimension gel. No cross-link cleavage was detected by silver staining of the second dimension after electrophoresis.

Next the concentration of DTT was increased to 30 and then 45 mM, the other conditions being the same as used previously, but this had no effect on the cross-linked material.

Reduction of the cross-links using 1%(v/v) 2-ME was tried but to no avail, so the reaction temperature was increased to 40, 50 and then to 60°C. The last temperature was successful, but the cleaved monomers were created in such small amounts that the reaction time was reduced and a time of 45 minutes was decided upon (30 and 40 minutes produced smaller amounts of cleaved cross-linked subunits). Finally the concentration of 2-ME was increased to 2% and then 3%(v/v) in an attempt to decrease the reaction time and temperature. Although 3%(v/v) 2-ME was most successful, reduction of reaction time and temperature(from 60°C) appreciably decreased the amount of cleaved cross-linked material.

The final conditions used for cross-link cleavage were soaking of a strip of first dimension gel in cleavage buffer (100 mM Tris-Cl, 1%(w/v) SDS, 3%(v/v) 2-ME pH 8.9) at 60°C for 45 minutes followed by two washes in 50 mM Tris-Cl, 1%(w/v) SDS (pH 6.7). The strip was then attached to the stacking gel of a second dimension gel (2.2.10.2).

4.2.3 Western blotting

Western blotting of cross-linked aggregates is covered in section 2.2.9.5. Cross-linked species were separated in 17.5%(w/v) acrylamide gels using SDS PAGE (section 2.2.9). The gel lane containing separated cross-linked species was Western blotted onto nitrocellulose (Towbin *et al.*, 1979). Subunit specific antitoxin and antipeptide antibodies, the latter being polyclonal sera raised against synthetic peptides of S1, S3 and S4 (Seabrook *et al.*, 1990), were incubated with the immunoblotted

pertussis toxin subunits at an appropriate dilution. The antibodies and antisera were diluted in PBS buffer, pH 7.2, containing added NaCl, which reduced non-specific binding of antibody to the toxin subunits (NaCl concentrations used are quoted in the figure legend).

Nitrocellulose was then washed and incubated with second antibody (anti-mouse or anti-rabbit lgG conjugated to alkaline phosphatase). Bands were visualised using Fast blue RR salt (Sigma Chem. Co.) dissolved in Naphthol ASMX™ solution (Sigma Chem. Co.). The results of Western blotting are shown in figure 4.9

4.2.4 Reverse phase FPLC

Reverse phase FPLC was carried out on a Gilson FPLC system. Pertussis toxin was prepared as in section 2.2.10.1. 0.1%(v/v) TFA in acetonitrile (buffer B) was added to a final concentration of 15%(v/v) acetonitrile. Debris was removed by centrifugation and the sample applied to a TMS-250 column equilibrated with 15%(v/v) buffer B. The column was eluted, the fractions containing protein as detected by A_{220} were evaporated to dryness and redissolved in cross-linking buffer before SDS PAGE.

4.3 Results

4.3.1 Time courses

Figure 4.2 shows resolution of pertussis toxin into five subunits by SDS PAGE in 17.5%(w/v) acrylamide.

Figures 4.3, 4.4 and 4.5 show results of three time courses using the linkers DSP, DST and SMPB respectively. All three cross-linkers produced reproducible patterns of cross-linked aggregates in quantities detected by silver staining.

Figure 4.3 shows 0.7 mg/ml toxin cross-linked by 1 mM DSP. Several cross-linked species were generated and bands indicating S2, S3, S4 and S5 can be seen to decrease in stain intensity during the experiment, although a corresponding proportional increase in stain intensity of cross-linked species bands was not seen. Variation of linker and toxin concentrations all produced the same range of cross-linked aggregates, making first and second dimension gels difficult to interpret.

Figure 4.4 shows 0.7 mg/ml toxin cross-linked by DST. Three cross-linked species were created that were detected by silver staining, but in small amounts, as shown by the faint staining.

Figure 4.5 shows 1.4 mg/ml toxin cross-linked by 5 mM SMPB. Three cross-linked bands were detected by silver stain. In both SMPB and DST experiments,

Fig. 4.2 Resolution of pertussis toxin into five subunits by SDS PAGE in 17.5%(w/v) acrylamide

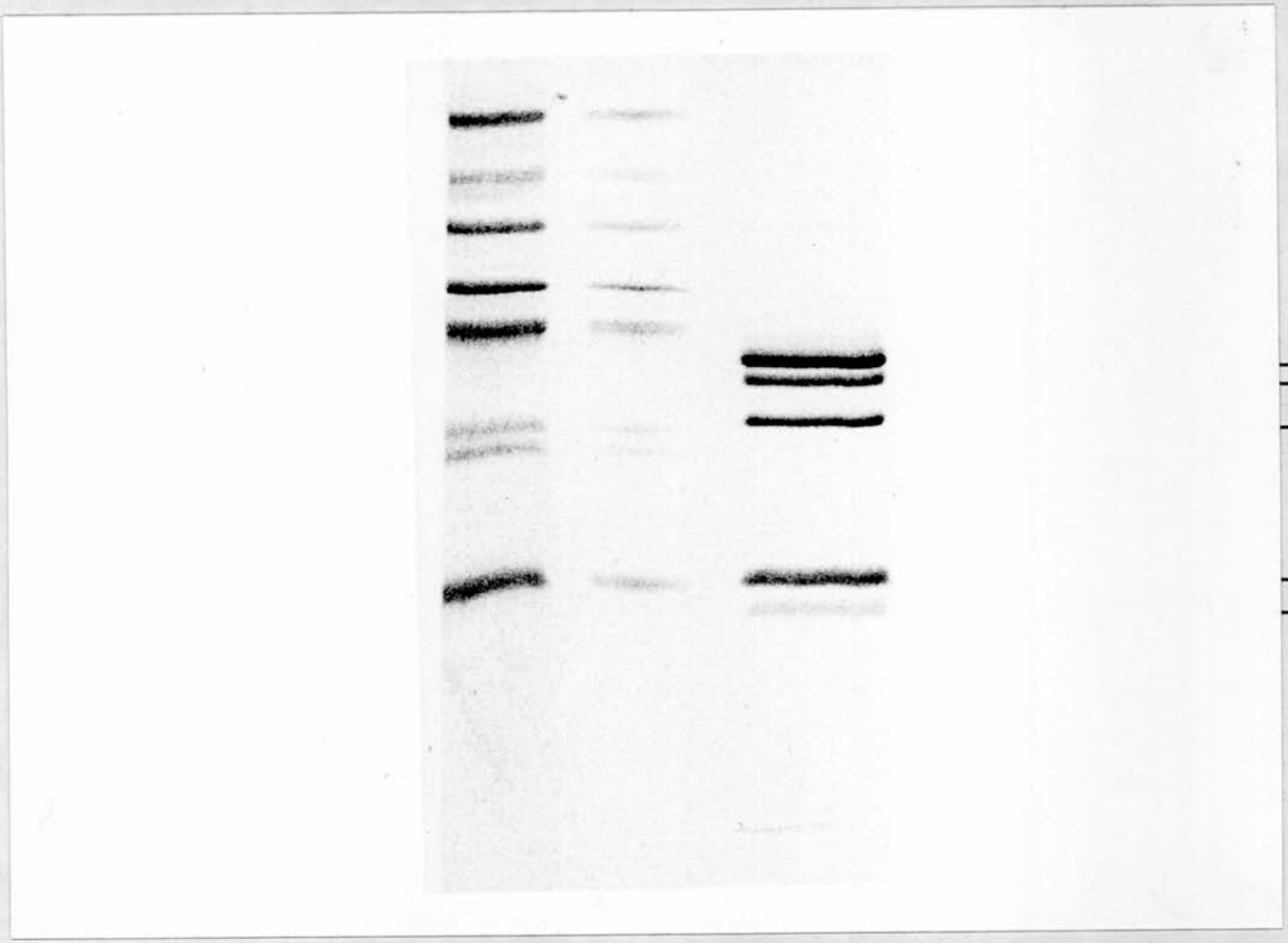

S1
S2
S3
S4
S5
M_r standards
pertussis toxin

Fig. 4.3 Separation of DSP-cross-linked pertussis toxin subunits by SDS PAGE in 17.5%(w/v) acrylamide

(Sigma molecular weight markers)

A: 66kDa, B: 45kDa, C: 36kDa, D: 29kDa, E: 24kDa, F: 20.1kDa

Lane 1 M_r standards
2 M_r standards
3 pertussis toxin (PT)
4 PT +DSP, 5 min
5 PT +DSP, 10 min
6 PT +DSP, 15 min
7 PT +DSP, 20 min
8 PT +DSP, 25 min
9 PT +DSP, 30 min

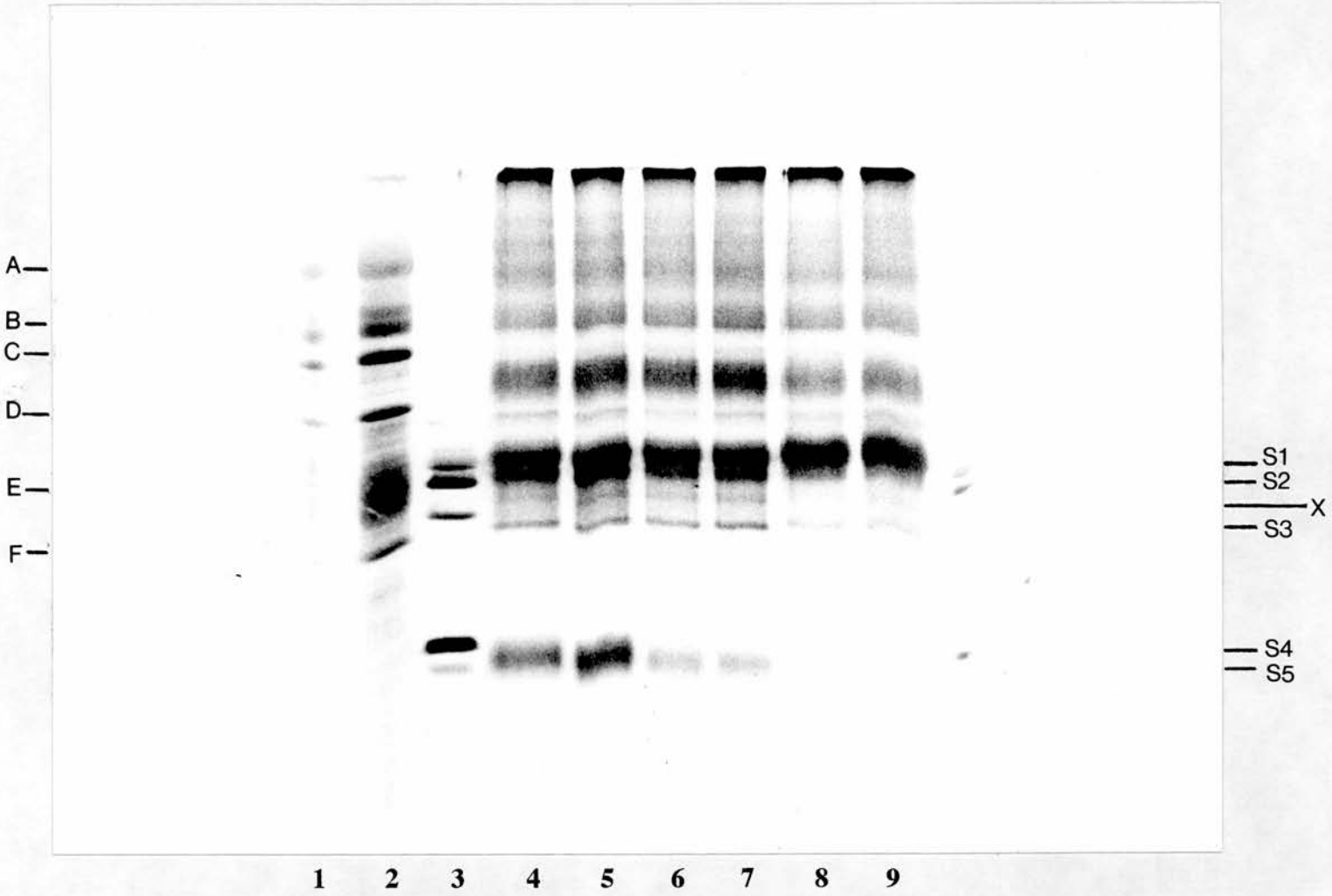

A
B
C
D
E
F
S1
S2
X
S3
S4
S5
1
2
3
4
5
6
7
8
9

Fig. 4.4 Separation of DST-cross-linked pertussis toxin subunits by SDS PAGE in 17.5%(w/v) acrylamide

(Bio Rad molecular weight markers)

A: 97kDa, B: 66kDa, C: 45kDa, D: 29kDa, E: 20.1kDa, F: 14.4kDa

Lane 1 M_r standards
2 M_r standards
3 pertussis toxin (PT)
4 PT +DST, 5 min
5 PT +DST, 10 min
6 PT +DST, 15 min
7 PT +DST, 20 min
8 PT +DST, 25 min
9 PT +DST, 30 min

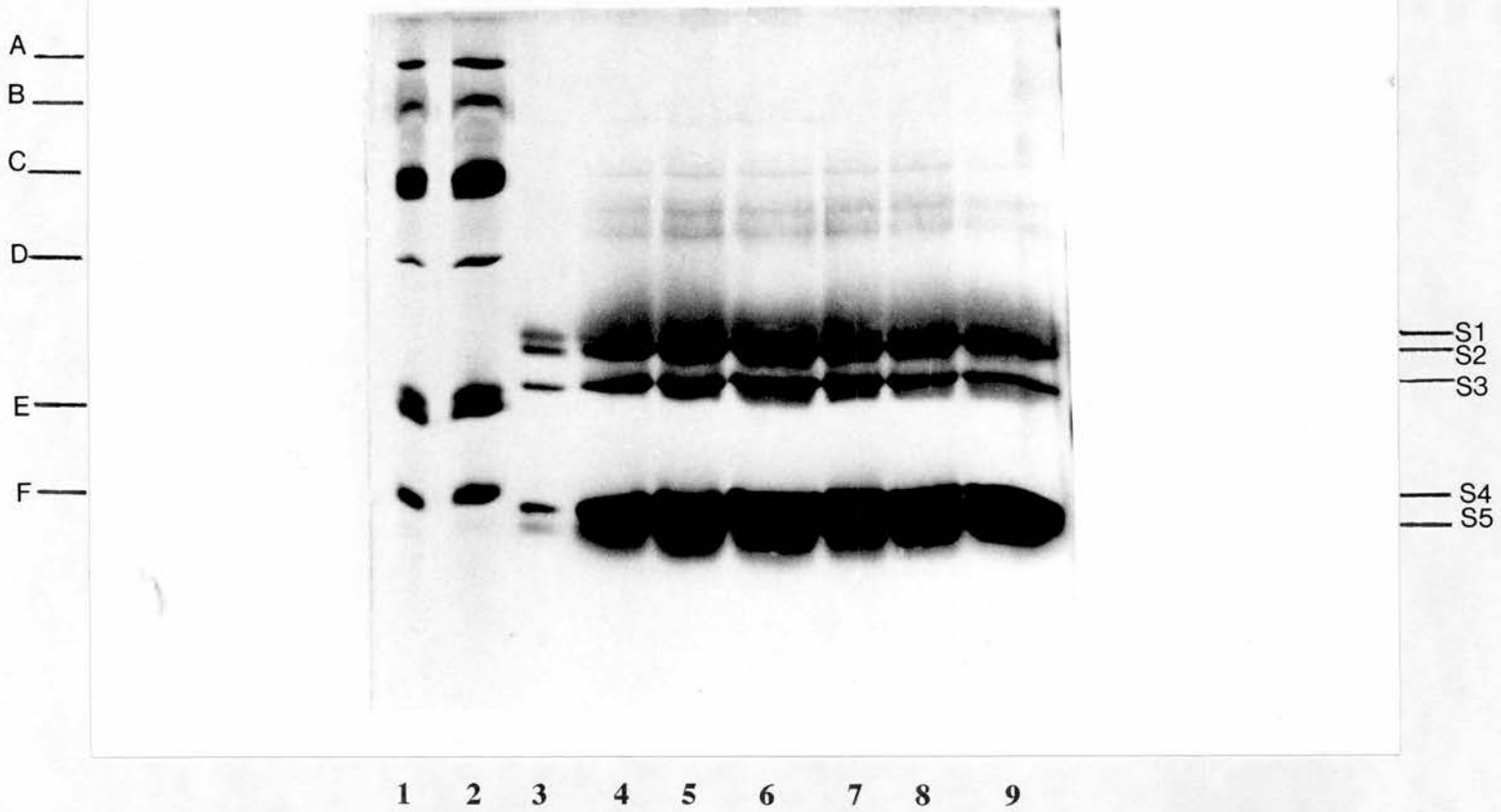
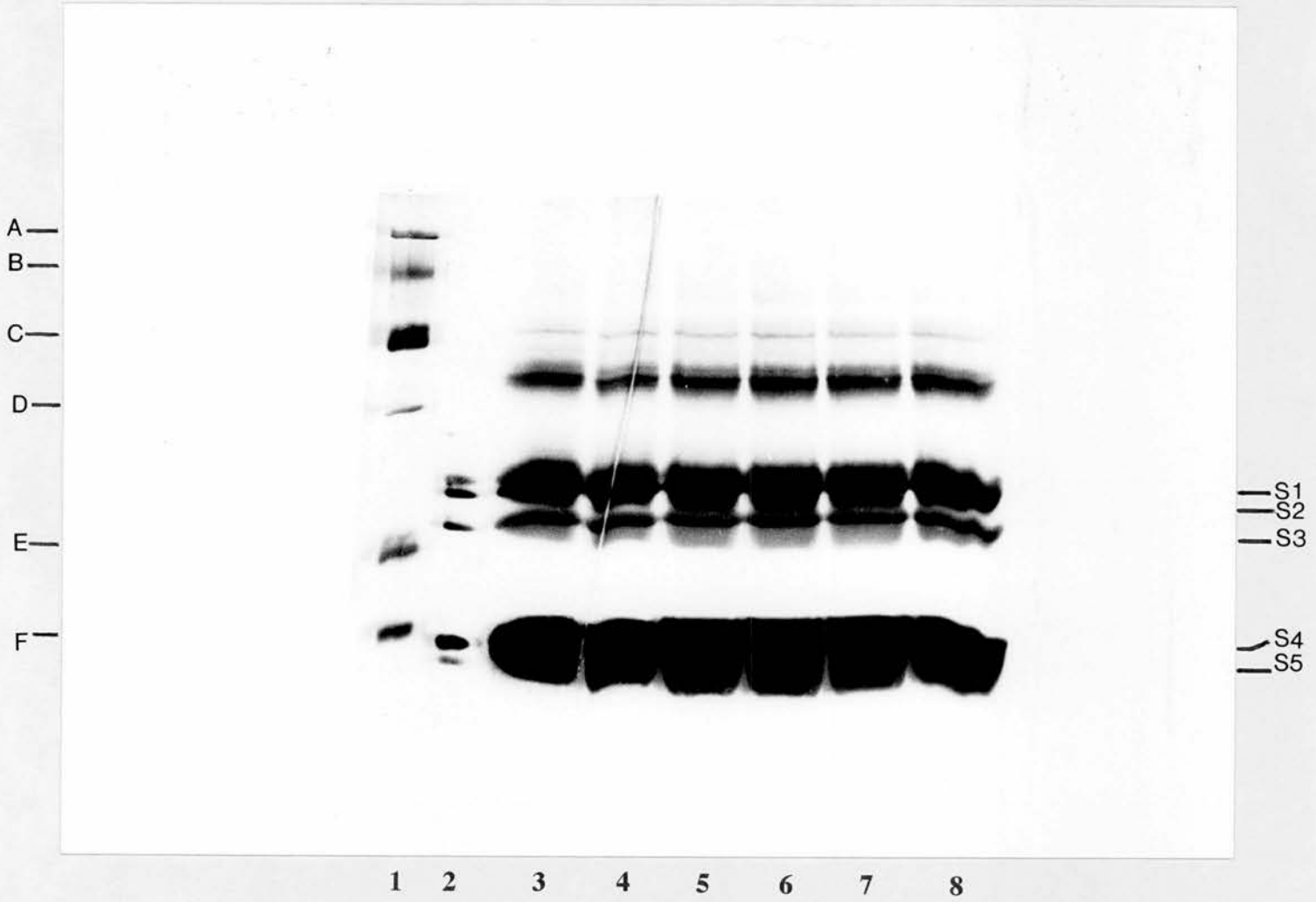

A
B
C
D
E
F
S1
S2
S3
S4
S5
1 2 3 4 5 6 7 8 9

Fig. 4.5 Separation of SMPB-cross-linked pertussis toxin subunits by SDS PAGE in 17.5%(w/v) acrylamide

(Bio Rad molecular weight markers)

A: 97kDa, B: 66kDa, C: 45kDa, D: 29kDa, E: 20.1kDa, F: 14.4kDa

Lane 1 M_r standards
2 pertussis toxin (PT)
3 PT + SMPB, 5 min
4 PT + SMPB, 10 min
5 PT + SMPB, 15 min
6 PT + SPMB, 20 min
7 PT + SMPB, 25 min
8 PT + SMPB, 30 min


A
B
C
D
E
F
S1
S2
S3
S4
S5
1
2
3
4
5
6
7
8

increasing protein concentration from 0.35, to 0.7 and to 1.4 mg/ml and/or increasing the cross-linker concentration from 0.1, to 0.5, to 1.0, to 5.0 and to 10.0 mM had little effect on the range of cross-links produced.

S1 was not reduced (by 2-ME) before SDS PAGE and the subunit migrated with an apparent molecular weight of 24 KDa. Subunits S2 and S3 have similar apparent molecular masses in SDS PAGE as do S4 and S5 so a cross-link band separated on an SDS PAGE gel could be formed from a number of subunit combinations. As a simple example, a band with a mobility that suggested an apparent M_r of 46.5 KDa may be a dimer of subunits S1 and S2, S1 and S3, or a trimer of subunits S1 S4 and S5, etc. To identify subunits in the cross-linked species, cleavage of the cross-links in a one-dimensional SDS PAGE gel was followed by a second dimension gel of regenerated subunits.

4.3.2 Two dimensional SDS PAGE

4.3.2.1 'Diagonal' two dimensional SDS PAGE

The principle of this two dimensional system is that a first dimension SDS polyacrylamide gel is used to separate cross-linked aggregates from non-cross-linked monomers by size. A strip of the first dimension gel is then soaked in a buffer that cleaves the cross-link bridges. The strip containing the cleaved species and monomers is then fixed to a second dimension gel.

Subunits that were part of a cross-linked aggregate in the first dimension migrate as monomers in the second dimension. Non-cross-linked subunits retain the same relative mobility in the first and second dimensions, and fall on a diagonal line. Protein subunits cleaved from a cross-linked aggregate migrate to a position below that diagonal because of their increased mobility relative to the aggregate from which they were derived. Subunits that were members of a cross-linked species can be identified by comparison with the mobility of monomeric subunits (see fig. 4.6-4.8). Cross-link cleavage and two dimensional SDS PAGE have been used in a number of systems and with a number of cross-linkers to identify protein-protein interactions (Wang and Richards, 1974; Smith *et al.*, 1978; Bragg and Hou, 1980).

From the time-course experiments, toxin and linker concentrations were chosen to produce maximum cross-linking without losing resolution of the cross-linked species, so that the likelihood of detection of cleaved monomers in the second dimension was improved.

DSP generated cross-linked aggregates in large amounts detected by silver staining of first dimension gels, as compared to DST. As DST generated such a low yield of cross-linked species, cleavage of cross-links within the first dimension

resulted in such losses of protein into the cleavage buffer that detection of cleaved monomer in the second dimension was not possible. As a result it was decided to concentrate efforts on identification of subunits cross-linked by DSP, and cross-linked species were identified.

Monomers S3 and S4 were cleaved from a cross-linked species that had a mobility suggestive of a possible dimer in the first dimension, and monomers from a cleaved trimer of S1, S3 and S4 were identifiable by silver staining. A cross-linked dimer which had an apparent mobility of about 46 KDa had been cleaved in the first dimension gel. Its components ran as monomers S1 and S3 in the second dimension and were detected by silver staining.

One band identified by silver staining of a first dimension gel had a mobility between that of the S2 and S3 subunits (see fig. 4.3, band x), suggesting that that band was a cross-linked species. Cleavage of that species in a first dimension gel produced a spot on the second dimension, which was 'off-diagonal', indicating that cross-link cleavage had occurred. The spot detected on the second dimension also had a mobility very like that of the monomers S4 and S5. This suggests that a cross-linked dimer of S4 and S5 had been separated and then cleaved in the first dimension and the regenerated monomers had been separated in the second dimension.

Figure 4.6 shows cleaved cross-links of pertussis toxin subunits in a second dimension gel. 0.7 mg/ml toxin was cross-linked using 1 mM DSP for 10 minutes at 30°C. Reduced toxin was used as a standard. 'Off-diagonal' spots are visible, indicating successful cleavage of cross-links.

Resolution is not good because of the overloading of the first dimension gel to reduce protein losses during cross-link cleavage, but it is possible to identify tentatively subunits involved in a cleaved dimer of S3 and S4, a trimer of S1, S3 and S4, and two larger cleaved aggregates whose composition is more complicated. The toxin standard on the left of the gel suggests that the largest cleaved aggregate contained all the toxin subunits.

An interesting cleaved species is visible in a position suggesting that S4 and S5 could have been linked by DSP to form a dimer (mentioned earlier). A combined molecular mass of S4 plus S5 is about 21 kDa, and a species of that molecular mass would be expected to run at a position between S2 and S3 on first dimension SDS PAGE. A species that could be this aggregate can be seen in such a position, between S2 and S3, on figure 4.3.

Figure 4.7 also shows cleaved cross-links. 0.7 mg/ml toxin was cross-linked using 10 mM DSP for 10 minutes at 30°C. This gel shows a range of cleaved cross-links, and their composition difficult to ascertain. However, there is a spot at a position indicating the possible cleavage of S4 and S5. Toxin was used as a standard but was left unreduced to check on the cleavage method, as some gels showed that not

Fig. 4.6 Second dimension gel of cleaved cross-linked aggregates of pertussis toxin

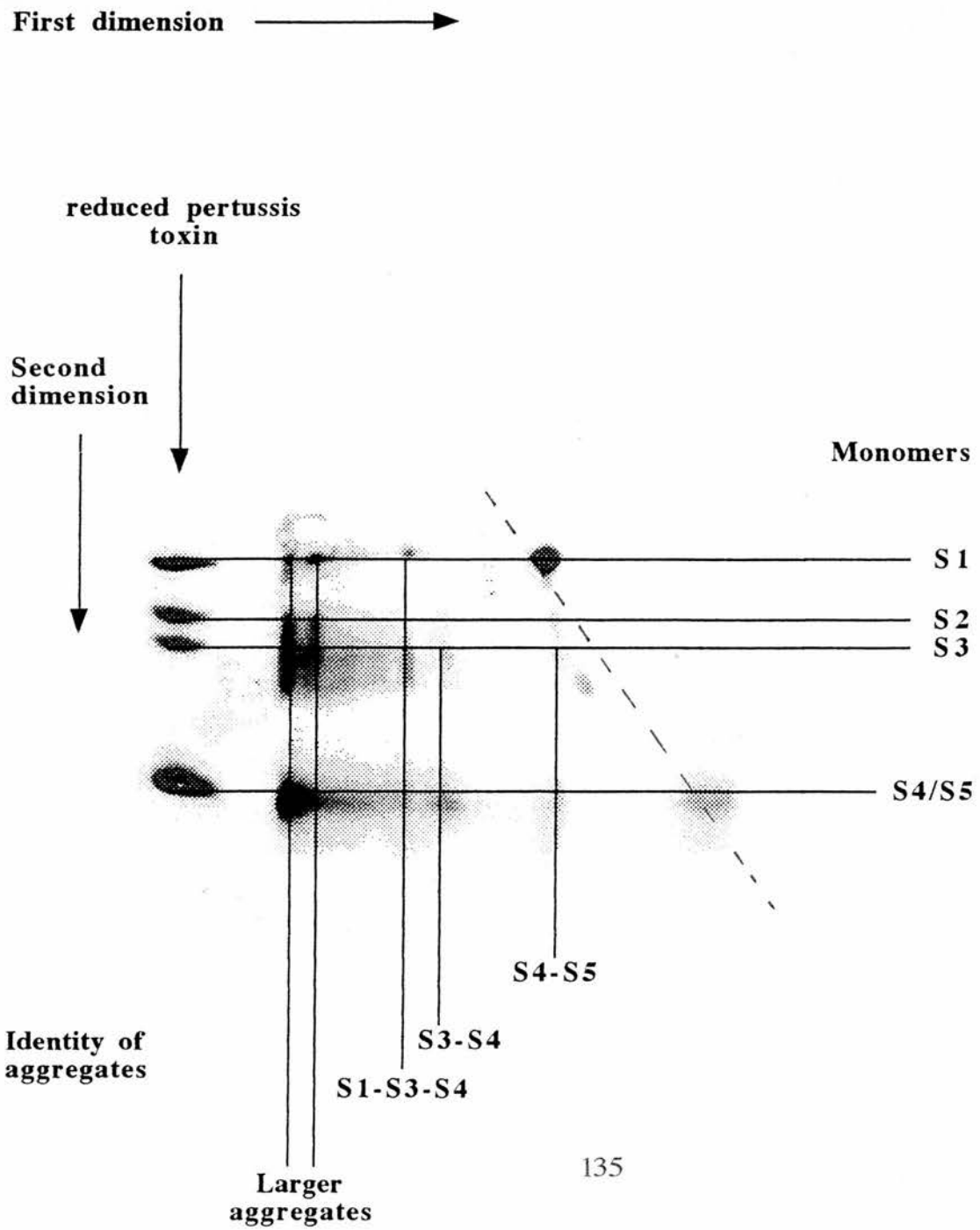
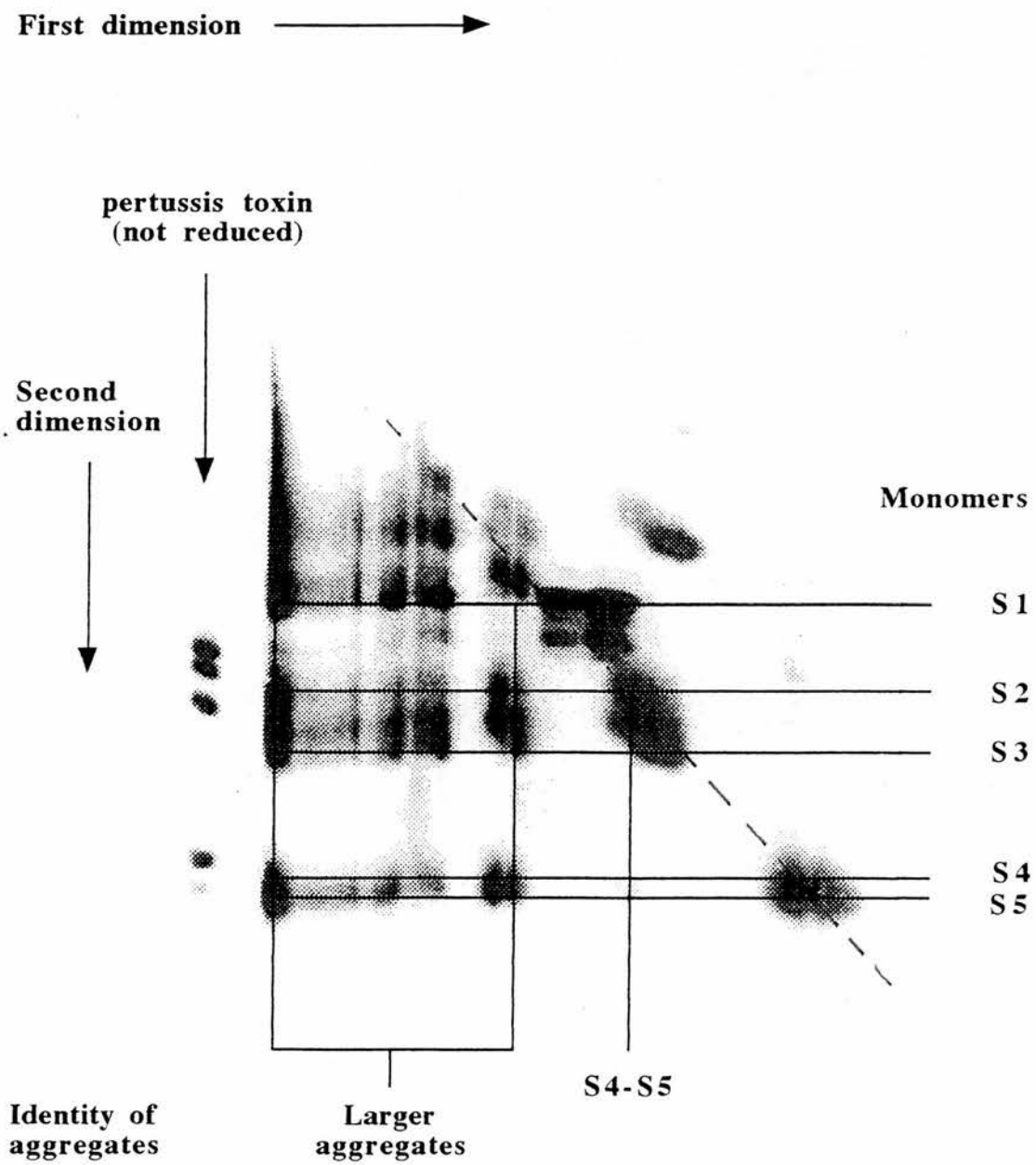


Fig. 4.7 Second dimension gel of cleaved cross-linked aggregates of pertussis toxin



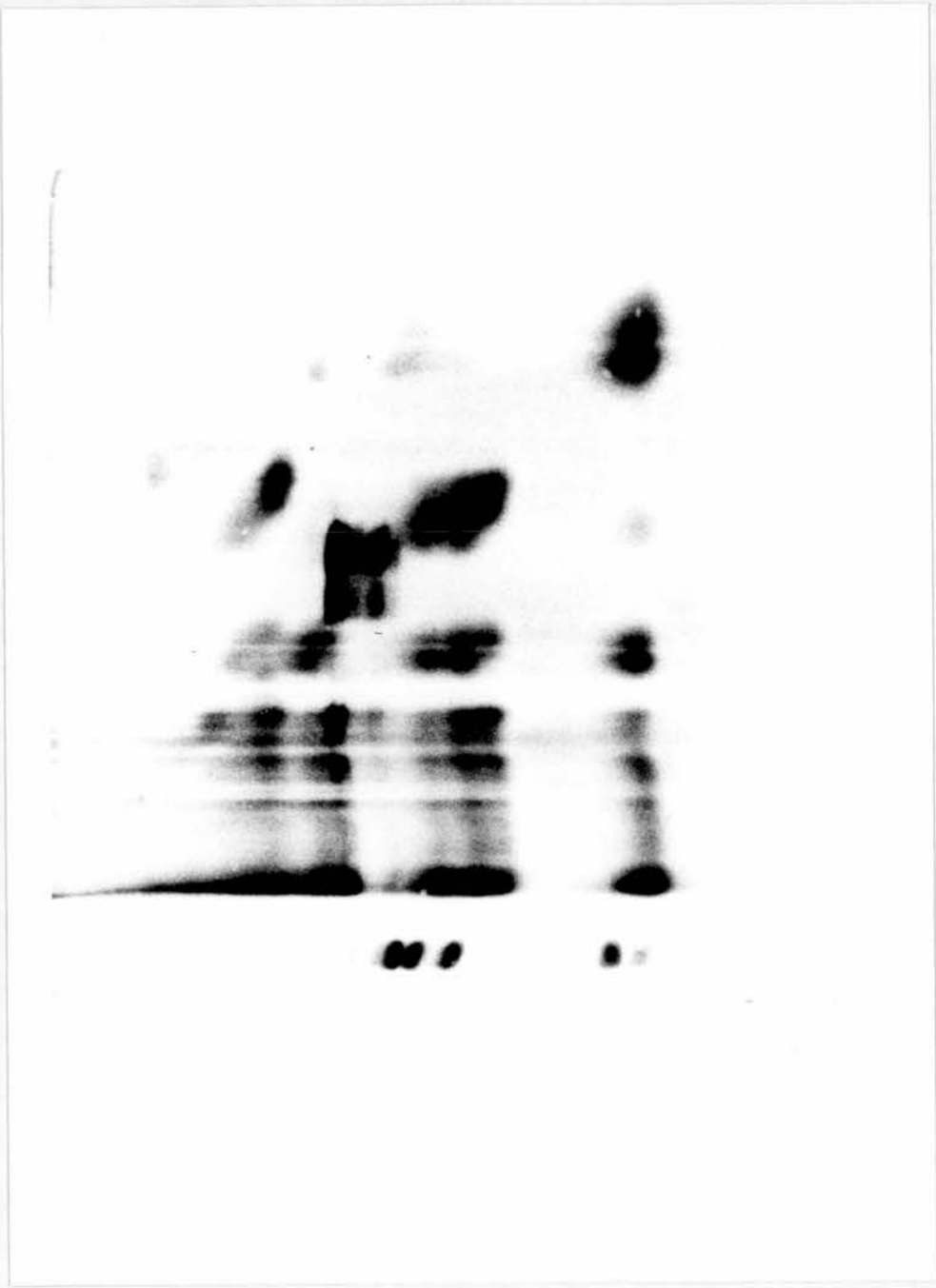
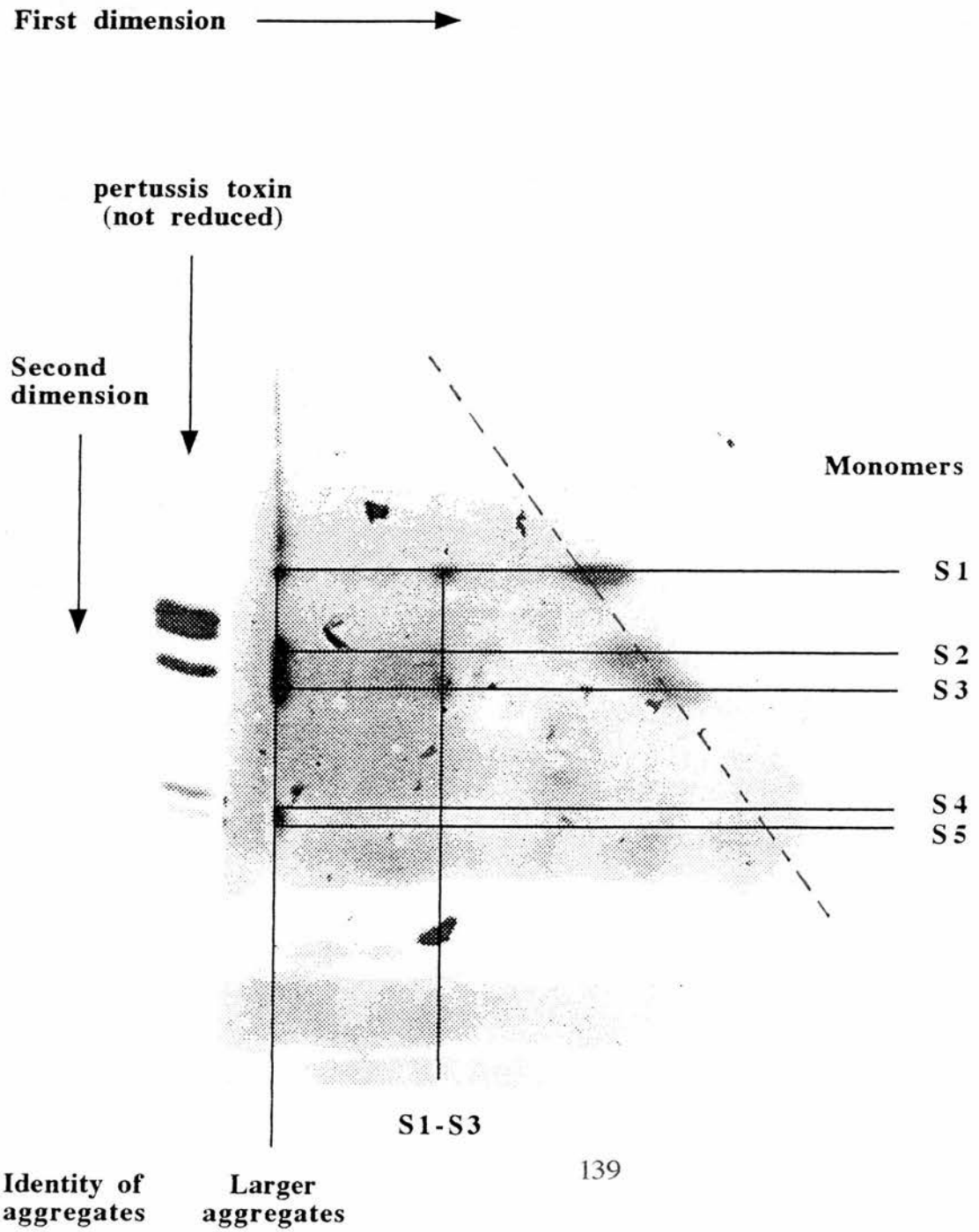
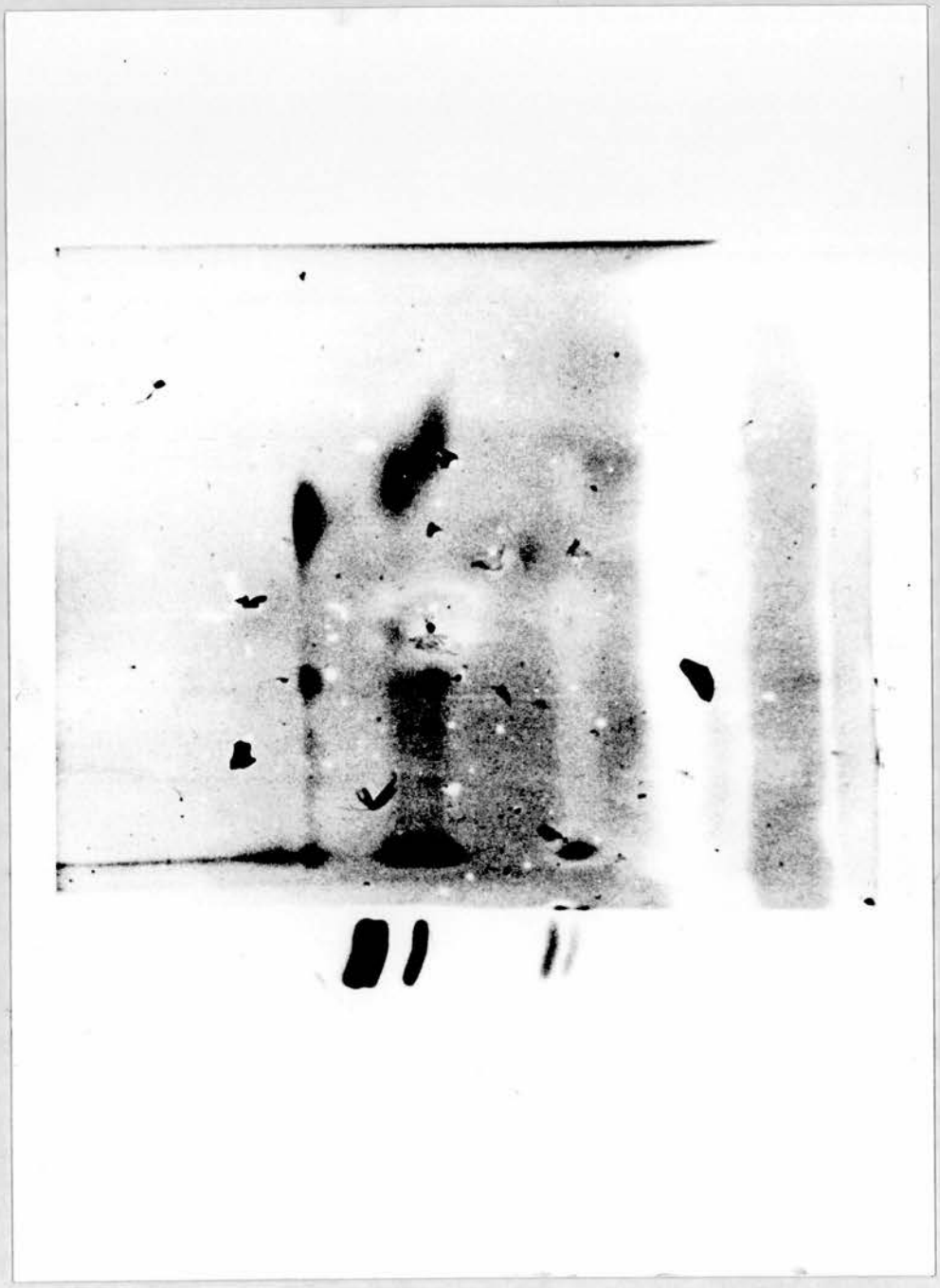


Fig. 4.8 Second dimension gel of cleaved cross-linked aggregates of pertussis toxin





all S1 was reduced by the cleavage treatment and the extra spots due to this complicated the second dimension gel.

Figure 4.8 shows a third two dimensional gel. 0.7 mg/ml toxin was cross-linked with 10 mM DSP for 30 minutes at 30°C. Two cross-linked species were cleaved, as indicated on the accompanying diagram. One contains S1 and S3. S4 and S5 did not stain well in this experiment, so this 'dimer' may contain S4. The other species cleaved appears to contain all the toxin subunits.

4.3.2.2 Electroelution

The two dimensional gel system did not identify subunits in cross-linked aggregates with total certainty, so electroelution of first dimension cross-links, cleavage in solution and electrophoresis on a second gel was tried. A strip from the first dimension DSP-cross-linked gel was silver stained and areas of unstained gel corresponding to cross-linked species were cut out. The protein in these areas was electroeluted as described in section 2.2.9.3. Electroeluted material was then treated with 3%(v/v) 2-ME, 1%(w/v) SDS in a boiling water bath for five minutes (for DSP-cross-linked toxin), or treated with 15, 30 or 60 mM sodium periodate for up to three hours at room temperature and the pH adjusted to neutral (for DST-cross-linked toxin). The material was then applied to another gel to resolve the subunits in the cleaved aggregates. This method was not successful, because periodate-treated proteins produced poorly resolved bands on the second gel. Periodate treatment can alter the electrophoretic mobilities of some proteins, which could explain this result (Peters and Richards, 1977). The same problem was not encountered with 2-ME treatment, but a large number of cross-links were generated and it was not possible to electroelute them one at a time (even when the cross-links were separated on a gradient gel of 10-20, or 5-20%(w/v) acrylamide).

4.3.3 Western blotting

Four antibodies, murine anti-S1 and -S3 antibodies and two rabbit antibodies, one raised against a peptide synthesised from the amino acid sequence of the S3 subunit ((E)S3c) and one against a peptide derived from the sequence of S4 (S4b) were used. The peptide amino acid sequences against which the rabbit antipeptide sera were raised are to be found in chapter 3.

Three antibodies were subunit-specific when reacted with immunoblots of non-cross-linked pertussis toxin subunits separated by SDS PAGE. The rabbit anti-S3 antipeptide antibody was found to cross-react with the S1 subunit monomer on immunoblots of separated cross-linked subunits and very weakly with S1 monomer on

blots of non-cross-linked pertussis toxin subunits. The cross-reaction was reduced by incubation of antibody with the immunoblots in a buffer containing 250 mM NaCl (higher concentrations were also tried). Non-specific binding of the other antibodies was also reduced by inclusion of up to 250 mM NaCl in the buffer in which the first antibody was diluted in (if NaCl was added, the concentration is quoted in the figure legend). Subunit-specific antibodies to subunits S2 and S5 were not available to the author at the time of the study.

Cross-linked bands were identified as containing S3-S4 and S1-S3-S4. These subunit combinations were compatible with the apparent M_r of the species. Other bands were visualised, but their subunit composition could not be identified using this method due to the lack of a range of subunit-specific antibodies to all five subunits of the toxin.

Only DSP formed cross-linked species detectable by Western blotting. DST and SMPB generated cross-links detected by silver stain but the first antibody concentration needed in the blotting method to detect cross-links was so low that non-specific binding of the antibodies to all the toxin subunits occurred. The non-specific binding could not be reduced so identification of component subunits in a DST- or SMPB-cross-linked species was not possible.

Lane one of figure 4.9 shows murine anti-(pertussis toxin) antibody binding to non-cross-linked pertussis toxin subunits and in lane two, the same antibody binding to cross-linked pertussis toxin species. Several cross-linked species were detected.

Lanes three, four, five and six show uncross-linked pertussis toxin blotted with murine monoclonal antibodies to S1 and S3 and rabbit polyclonal antipeptide antisera to S3 and S4 respectively.

Lanes seven to ten show the antibodies mentioned in the above paragraph binding to DSP-cross-linked toxin (0.7 mg/ml toxin cross-linked with 5 mM DSP for 10 minutes at 30°C). Lanes seven and eight show recognition of cross-linked toxin species by the murine monoclonal anti-S1 and anti-S3 antibodies respectively. The anti-S1 antibody recognised non-cross-linked S1 and one cross-linked species.

The rabbit anti-S3 antipeptide antibody (lane nine) and the murine anti-S3 antibody (lane eight) both recognised the same bands, but some cross-reaction of the anti-S3 antipeptide antibody with S1 can be seen in lane nine. Reduction of this cross-reaction was attempted by including NaCl in both the buffer used for diluting the first and second antibody in, and by reducing the temperature of the antibody binding reactions to 4°C. Neither of these methods succeeded.

The rabbit anti-S4 antipeptide antibody (lane ten) was used at very high dilution to reduce cross-reaction with subunit S3 seen at lower dilutions, and this reduced the amount of antibody available to bind S4. This meant that the bands detected by the anti-S4 antibody were faint.

One cross-linked band recognised by the anti-S3 antibodies was also bound by

Fig. 4.9 Western blots of cross-linked and non-cross-linked pertussis toxin subunits separated by SDS PAGE in 17.5%(w/v) acrylamide

Lane	Toxin status(a)	Antibody	Subunit(s) bound
1	-	anti-PT	S1-S5
2	+	anti-PT	S1-S5
3	-	anti-S1(b)	S1
4	-	anti-S3(b)	S3
5	-	anti-S3(c)	S3(S1)
6	-	anti-S4(c)	S4
7	+	anti-S1(b)	S1 +cross-links
8	+	anti-S3(b)	S3 +cross-links
9	+	anti-S3(c)	S3(S1) +cross-links
10	+	anti-S4(c)	S4 +cross-links
11,12	species-specific murine- and rabbit-IgG conjugated to alkaline phosphatase		

(a) Toxin cross-linked (+) by 5 mM DSP, or not cross-linked (-).

(b) Murine monoclonal antibody.

(c) Rabbit polyclonal antibody (note: both rabbit antipeptide antibodies were incubated with immunoblots in PBS, pH 7.2 with added NaCl (final concentration 250 mM).

1 2 3 4 5 6 7 8 9 10 11 12
S1
S2
S3
S4
S5

the anti-S4 antibody, indicating that the cross-linked species contained S3 and S4. The generation of a cross-linked dimer of S3 and S4 by DSP had been suggested by cleavage of the link and two dimensional SDS PAGE. Another band on the immunoblot indicates a species having the mobility of a cross-linked trimer. The species contained S1, S3 and S4, also suggested by cleavage of this cross-linked species followed by separation of its component subunits with the two dimensional gel system.

Other bands were detected by this method, but their identification was not possible, as subunit-specific anti-S2 and anti-S5 antibodies were not available, as mentioned earlier.

4.6.4 Reverse phase chromatography

This system (TMS-250 C_1 column) separated non-cross-linked toxin subunits, but a large portion of the sample did not interact with the column matrix and eluted in the void volume. This meant that when DSP-cross-linked material was applied to the column, almost all of the sample eluted in the void volume and cross-linked material was not detectable by A_{220}. Conditions of chromatography were varied in order to increase the amount of material interacting with the column matrix and the experiment attempted with a different (C_8) column . However, this method was abandoned because indication of components in cross-linked species was provided by other techniques.

This method was not attempted with DST or SMPB which generated less cross-linked material than DSP.

4.7 Discussion

Chemical cross-linking was used to study the arrangement of pertussis toxin subunits in space. Reversible and non-reversible cross-linking reagents were used with one and two dimensional electrophoresis, Western blotting and FPLC were used.

4.7.1 Time courses and two dimensional electrophoresis

Three cross-linking reagents, dithiobis(succinimidyl propionate),DSP, disuccinimidyl tartarate, DST and succinimidyl 4-(p-maleimidophenyl) butyrate, SMPB, were used. Reproducible patterns of cross-linking were created and resolved using SDS PAGE and these patterns gave an idea of the range and amount of cross-linked species for a given toxin and cross-linker concentration and reaction time. One dimensional SDS PAGE did not identify which subunits were involved in a cross-

linked aggregate as S2 and S3, S4 and S5 have similar molecular masses, so a cross-linked species resolved by a one dimensional gel may contain several combinations of those subunits.

To clarify which toxin subunits were involved in cross-linked aggregates, cleavage of DSP and DST-cross-linked aggregates and resolution by two dimensional electrophoresis was attempted. The cleavage method used, involving soaking of first dimension gel strips in a cleavage buffer, meant that too much DST-cross-linked protein was lost from the gel for any cleaved aggregates to be detected using silver staining.

DSP-cross-linked species proved difficult to reduce, possibly because the cross-linked subunits protect the disulphide bond of the cross-linker from the reducing agent and also difficult to separate using second dimension SDS PAGE. A temperature of 60°C and a concentration of 2-mercaptoethanol (2-ME) of 3%(v/v) were finally chosen and reduced cross-links, but spots indicating subunits cleaved from aggregates were not well resolved in all cases, as seen in figure 4.7, for example. This could be because the first dimension gel was overloaded, leading to streaking in the second dimension, or simply because too many cross-linked species were generated in the first dimension to be properly resolved in the second dimension (again, see fig. 4.7).

The results of diagonal two dimensional cross-linking with DSP add to the evidence of identification of an S3-S4 dimer (Tamura *et al.*, 1982; Sekura *et al.*, 1983; 1985). Interestingly S1, which contains no lysine residues in its amino acid sequence (Locht and Keith, 1986; Nicosia *et al.*, 1986), was found to be part of an aggregate that contained S3 and S4, so S1 may have been cross-linked to either S3 or S4 via its N-terminal amine. Linking of cysteine or arginine residues of S1 with S3 or S4 is also a possibility. An aggregate apparently consisting of S1 and S3 was also identified, as was an aggregate containing S4 and S5. The latter result could be clarified by Western blotting of cross-linked toxin separated by SDS PAGE with an antibody specific for S5.

4.7.2 Western blotting

Cross-linking a toxin which has several different subunits of similar molecular mass, results in an array of cross-linked species that could have been formed from various combinations of subunits. The two dimensional SDS PAGE system used during this study did not positively identify as many cross-linked species as hoped, so Western blotting was used to clarify the results of the diagonal two dimensional electrophoresis.

This method was unsuccessful for both DST and SMPB. This could be due to the small amount of cross-linked material generated, and non-specific binding to the

blot may have occurred, caused by the requirement for a low dilution of first antibody. Also, cross-linking may have obscured the antigenic sites for the antibodies.

A number of antibodies, both murine monoclonal and polyclonal and rabbit polyclonal, were tested to identify which, if any, were specific for one toxin subunit (section 3.3.1). The majority recognised S1 and/or S3, one recognised S4 alone and none specifically recognised S2 or S5.

Western blotting confirmed the cross-linked S3-S4 dimer and the aggregate containing S1, S3 and S4. The latter aggregate is unlikely to contain S5, as an aggregate containing S5 would be expected to have a lower mobility (about 4 mm higher on the gel) in the first dimension, than was observed.

The S4-S5 dimer suggested by two dimensional electrophoresis was not detected by the anti-S4 antibody, possibly because the yield of cross-linked material was too low to be detected. A cross-linked S4-S5 species was expected to have a mobility between that of S2 and S3 on a first dimensional gel, and a band detected by silver staining of DSP-cross-linked toxin (fig. 4.3) does in fact show such a band between S2 and S3. The positive identification of the S4-S5 dimer would require an increased yield of this species and blotting with both anti-S4 and -S5 antibodies. Increasing the yield of this aggregate could be a difficult task, if S5 is shielded from by the other toxin subunits, as proposed by Tamura *et al.* (1982) and Sekura et *al.* (1983). Such shielding may protect the subunit from the action of cross-linkers by 'hiding' surface-exposed primary amines.

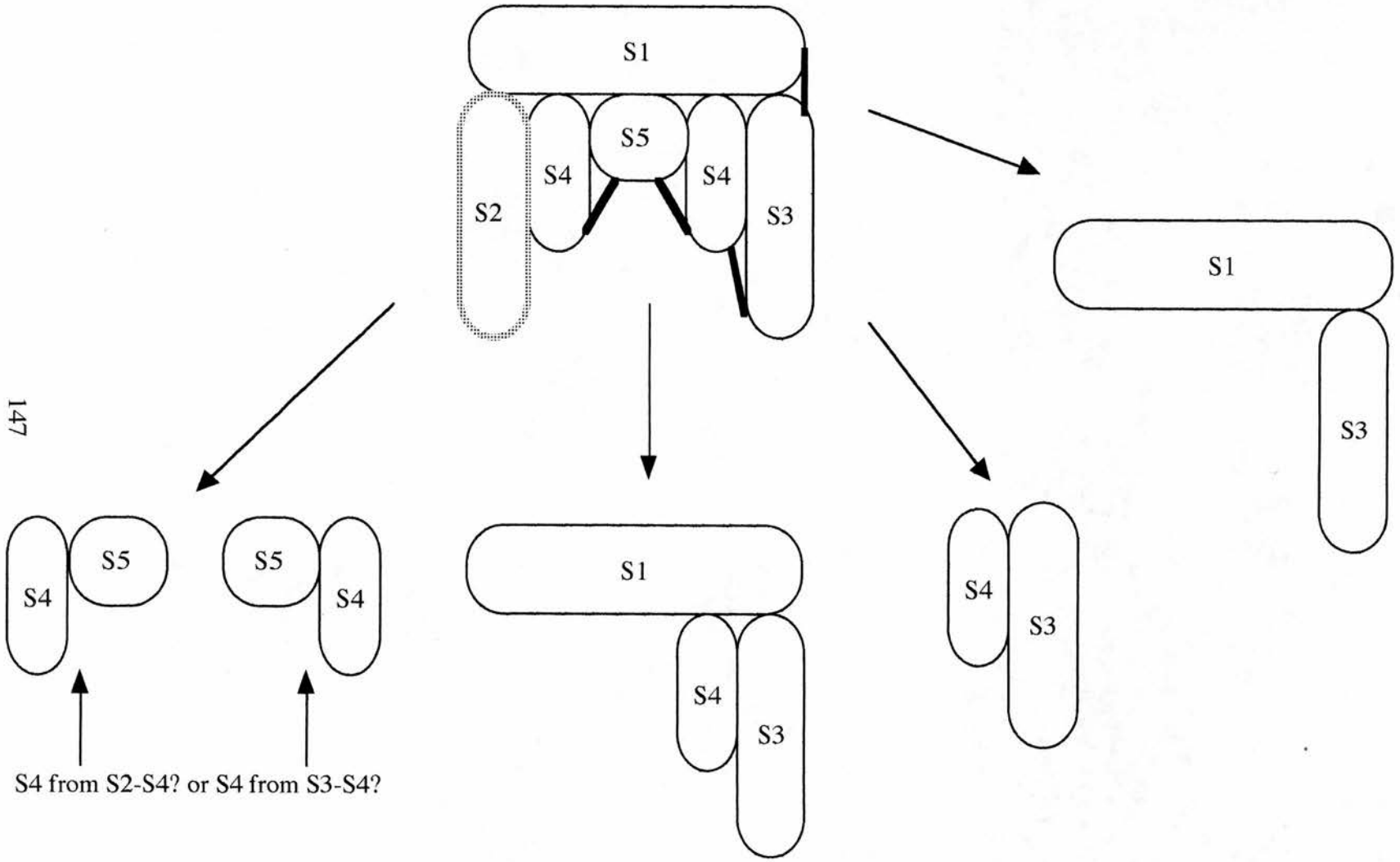
Immunoblotting did not indicate the presence of a cross-linked S1-S3 dimer seen using the two dimensional electrophoresis system in figure 4.8, possibly because the dimer formation by cross-linking obscured the antigenic sites of the subunit-specific antibodies. It is also possible that silver staining of the gel seen in figure 4.8 did not detect S4, explaining the apparent S1-S3 dimer observed.

It appears from the results using the 1.2 nm cross-linker DSP, that S1 may sit 'on top' of the S3 and S4 subunits with S5 'underneath' and shielded by the other toxin subunits (Fig. 4.10). No aggregates containing S2 were identified using DSP and the two dimensional electrophoresis system, but may be identifiable using other cross-linkers and by immunoblotting.

The currently accepted model of the toxin is supported by this study, but the results are incomplete and further work with other cross-linkers is required. A linker with a longer cross-link bridge than DSP but with the same amino acid specificity could be used and the cross-link map compared to one generated with a cleavable heterobifunctional linker, with one functional group having specificity for lysine residues and the other being specific for cysteine residues. This would help to generate a map of the toxin subunits that would be confirmed by the eventual determination of the toxin's crystal structure.

Fig. 4.10 Proposed structural model of pertussis toxin deduced by crosslinking with DSP (1.2 nm)

Figure shows subunits cross-linked by DSP and cleavage of cross-links which identified components of subunit aggregates


S1
S2
S4
S5
S4
S3
S1
S3
S5
S4
S5
S4
S1
S4
S3
S4
S3
S4 from S2-S4? or S4 from S3-S4?

Chapter 5

Binding of NAD^+ to pertussis toxin and its S1 subunit

5.1 Introduction

The S1 subunit of pertussis toxin catalyses ADP-ribosyl transfer from NAD^+ to a C-terminal cysteine residue of some G proteins (Ui, 1990). This results in uncoupling of some effectors (e.g. adenylate cyclase) from their respective cell surface receptors and generates some of the toxic effects of pertussis toxin, including histamine sensitisation and lymphocytosis (chapter 1).

When no protein substrate is available, water acts as the ADP-ribose acceptor and NAD^+ is hydrolysed to ADP-ribose and nicotinamide and binding of NAD^+ to the S1 subunit is the initial event in both the ADP-ribosyltransferase and NAD^+-glycohydrolase activities of pertussis toxin.

In the work described in this chapter, fluorescence quenching was used to study the interaction of NAD^+ with whole pertussis toxin and isolated S1 subunit purified from whole pertussis toxin, at various temperatures and interaction of NAD^+ with recombinant S1 subunit analogues.

Intrinsic tryptophan fluorescence quenching has been used to study the interaction of NAD^+ with diphtheria toxin fragment A (DTA, Kandel *et al.*, 1974), *Pseudomonas aeruginosa* exotoxin A (ETA, Chung and Collier, 1977). The quenching of DTA and ETA fluorescence seen on NAD^+-binding may be due to interaction with a tryptophan residue in each toxin and a tryptophan in ETA (W466) is thought to be involved in interaction with NAD^+ (Brandhuber *et al.*, 1988).

Although ETA and DTA have sequence similarity, they have little similarity with pertussis toxin, cholera toxin and *Pseudomonas aeruginosa* exotoxin A and ETA and DT act on a different target. However, experiments using site-directed mutagenesis have indicated that tryptophan 26 (W26) of the S1 subunit of pertussis toxin is important in the toxin's enzymic activity, that is, either the toxin's NAD^+-binding or catalytic activity (Cortina and Barbieri, 1989; Locht *et al.*, 1990; Loosemore *et al.*, 1990). It was shown in this study that the intrinsic tryptophan fluorescence of pertussis toxin, its isolated S1 subunit and S1 subunit analogues were all quenched on the addition of NAD^+, which suggests that a tryptophan may be involved in NAD^+-binding.

Other residues may be involved in NAD^+-binding. Glutamate 129 (E129) of

pertussis toxin S1 subunit is involved in expression of enzymic activity and alteration or replacement of this residue has been found to reduce enzymic activity (Pizza *et al.*, 1988; Loosemore *et al.*, 1990; Locht *et al.*, 1990). NAD^+ has been shown to interact with glutamate 129 of the S1 subunit using photoaffinity labelling (Cockle, 1989) and that photolabelling was undetectable when this residue was replaced by either an aspartate or glycine residue.

Glutamate 148 of DTA and glutamate 553 of ETA have both been shown to interact with NAD^+ (Carroll and Collier, 1987; Douglas and Collier, 1990) and mapping of the NAD^+-binding site of DTA and ETA using X-ray diffraction identified glutamate 148 and glutamate 553 as being at or near the NAD^+ active site, so glutamate 129 of the S1 subunit of pertussis toxin may have an analogous role in enzymic activity to glutamate 148 and glutamate 553 of DTA and ETA.

It has been established that tryptophan 26 (W26) and glutamate 129 (E129) of the S1 subunit of pertussis toxin are essential for the toxin's enzyme activity (Pizza *et al.*, 1988; Cortina and Barbieri, 1989; Locht *et al.*, 1989), but to what extent they are involved NAD^+-binding and/or catalytic activity is still being established.

NAD^+-binding to the S1 subunit of whole pertussis toxin and isolated S1 subunit was studied by the author to determine if binding was affected by S1 being attached to the B-oligomer of the toxin. NAD^+-binding to a truncated analogue of S1 (rS1d) and rS1d with the glutamate residue at position 129 altered by site-directed mutagenesis to an aspartate (rS1dE129D) was also studied to determine the importance of glutamate 129 in NAD^+-binding.

Tryptophan 50 (W50) and glutamate 148 (E148) of DTA, and tryptophan 466 (W466) and glutamate 553 (E553) in ETA are thought to be involved in the catalytic activity of both these toxins (Brandhuber *et al.*, 1988). Both fluorescence studies on DTA and ETA gave dissociation constants (Kd) for NAD^+ in the micromolar range and similar Km values were obtained for the proteins' NAD^+-glycohydrolase activities. Equilibrium dialysis, used by Lobban *et al.* (1991) also yielded dissociation constants for whole pertussis toxin that were of the same order as the K_m values obtained from NAD^+-glycohydrolase assays.

The similarities between DTA and ETA binding of NAD^+ using intrinsic fluorescence quenching, and the involvement of analogous tryptophan and glutamate residues in the enzymic activities of DTA, ETA and pertussis toxin suggested that intrinsic fluorescence quenching could be used to study NAD^+-binding to whole pertussis toxin, isolated S1 subunit and S1 subunit analogues.

Fluorescence quenching by NAD^+ was studied by the author using a recombinant S1 subunit lacking C-terminal amino acids (rS1d, consisting of amino acids 2-187) and rS1d that contained aspartate (D) instead of the wild-type glutamate (E) residue found at position 129 (rS1dE129D). The two recombinant S1 subunit analogues were produced in *E. coli* and were kind gifts of Dr. C. Locht, Institut Pasteur, France. Their production is described elsewhere (Locht *et al.*, 1987).

The emission spectrum of pertussis toxin's tyrosine and tryptophan residues was measured by excitation at 285 nm. The emission spectrum showed two wide peaks at 300-315 nm and 320-350 nm. The second peak was due to tryptophan fluorescence, giving similar maximum and peak-width as other tryptophan fluorophores (Burstein *et al.*, 1973) and the first peak was due to tyrosine fluorescence.

Quenching of fluorescence on addition of NAD^+ was observed at an excitation wavelength 285 nm and emission spectra recorded at a wavelength of 333 nm. Experimental conditions are described in chapter 2.

5.2 Results

5.2.1 Corrections and calculation of the apparent dissociation constant, K_d

Fluorescence quenching data were corrected for dilution of the solution on addition of NAD^+. A correction for the absorbance of the solution at the excitation and emission wavelengths was applied (Lakowicz, 1983):

$$F_{corr} = F_o \,.\, \text{Antilog}\,[(A_{ex}+A_{em})/2]$$

where Fcorr is the corrected fluorescence intensity, Fo is the observed fluorescence intensity and A_{ex} and A_{em} are the absorbances of the solution at the excitation and emission wavelengths respectively.

In the simple reaction below for protein P and ligand, L:

$$P + L \rightleftharpoons PL$$

the dissociation constant K_d is given by:

$$K_d = \frac{[P][L]}{[PL]} \qquad (1)$$

A binding site may or may not be saturated with L so the fractional saturation, r can be calculated:

$$r = \frac{[PL]}{[P] + [PL]} \qquad (2)$$

Substituting for [PL] from equation 1:

$$r = \frac{[P][L]}{K_d([P] + [P][L]/K_d)} \qquad (3)$$

Equation 3 simplified:

$$r = \frac{[L]}{K_d + [L]} \qquad (4)$$

Equation 4 was adapted for graphical calculation of the dissociation constant:

$$\frac{r}{[L]} = \frac{1}{K_d} - \frac{r}{K_d} \qquad (5)$$

A plot of r against r/[L] gives a slope of $-1/K_d$ and this graphical representation is shown in figures 5.1 to 5.13.

The number of NAD^+-binding sites was assumed to be one (n =1) on the S1 subunit (Lobban *et al*, 1991).

The maximum fluorescence quench (Δf_{max}), when the protein is saturated with ligand was measured. Δf_{max} was then used to calculate r, the fractional saturation of the protein with ligand:

$$r = \Delta f_o / \Delta f_{max} \qquad (6)$$

where Δf_o is the change in relative fluorescence at a particular concentration of ligand, L. The concentration of the protein was known, so the concentration of free ligand

could be calculated using the fractional saturation, r, and the manipulated fluorescence data are presented as plots of r/[free NAD^+] (μM) against r (Scatchard, 1949) as stated above and analysed using least squares best fit calculations. The gradient of each plot yielded a value for the apparent association constant, K_a and the reciprocal of this value is the apparent dissociation constant, K_d (μM).

5.2.2 Effect of temperature on the NAD^+-dissociation constant, K_d

Fluorescence titrations were done as described in section 2.2.11.1 at 14, 20, 23, 30, and 40°C. Results of the titrations of whole pertussis toxin are shown in figures 5.1 to 5.5
and results of titrations of isolated S1 subunit are shown in figures 5.6 to 5.10. K_d values were calculated from the reciprocal of the gradients and the K_d values are shown in table 5.1. The dissociation constants obtained for whole pertussis toxin were similar to those for isolated S1 subunit (table 5.1). The enthalpy of binding at constant pressure, ΔH (kJ mol^{-1}), was calculated from the relationship below:

$$\frac{d \ln K_a}{d T} = \frac{\Delta H}{RT^2} \qquad (7)$$

Where Ka is the association constant, T is the absolute temperature (i.e. in Kelvin) and R is the gas constant (8.314 J K^{-1} mol^{-1}). Integration of equation 7 gives:

$$\ln K_a = -\frac{\Delta H}{RT} + \text{constant}$$

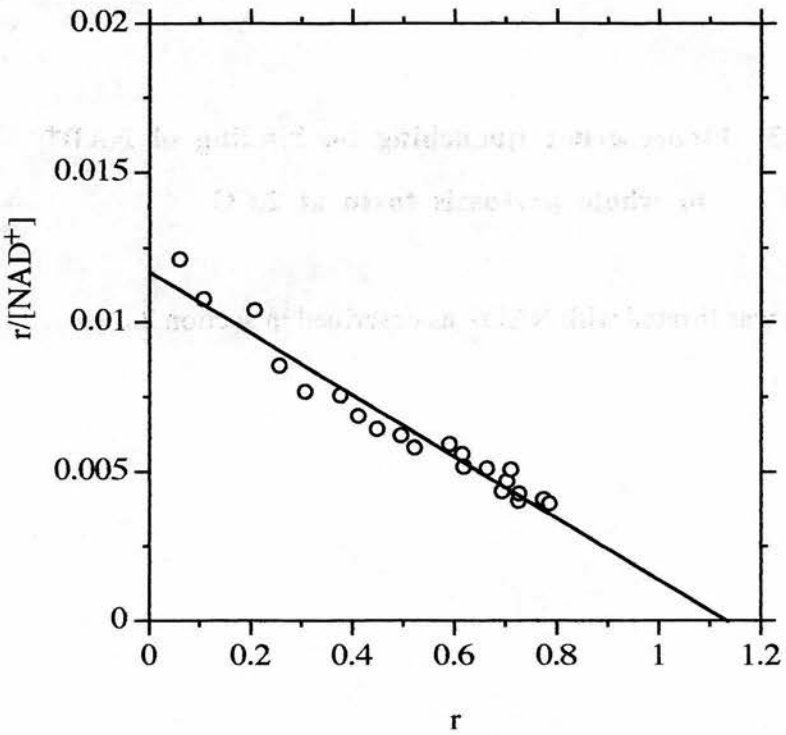
If log K_a is plotted against 1/T, a straight line will be obtained that will have a slope of $-\Delta H/2.303R$ and ΔH can be calculated.
The enthalpy of binding (ΔH) for whole pertussis toxin and isolated S1 subunit were similar (fig. 5.11), suggesting that the presence of the B-oligomer does not significantly affect NAD^+-binding.

Fig. 5.1 Fluorescence quenching on binding of NAD^+ to whole pertussis toxin at 14°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.

Fig. 5.2 Fluorescence quenching on binding of NAD^+ to whole pertussis toxin at 20°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.


0.02
0.015
0.01
0.005
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
1.2
r

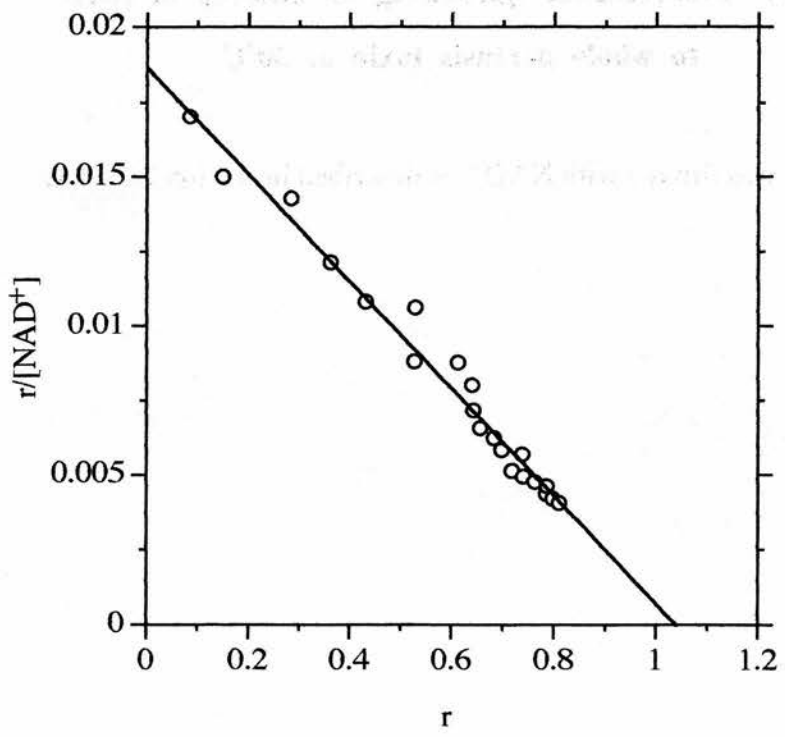
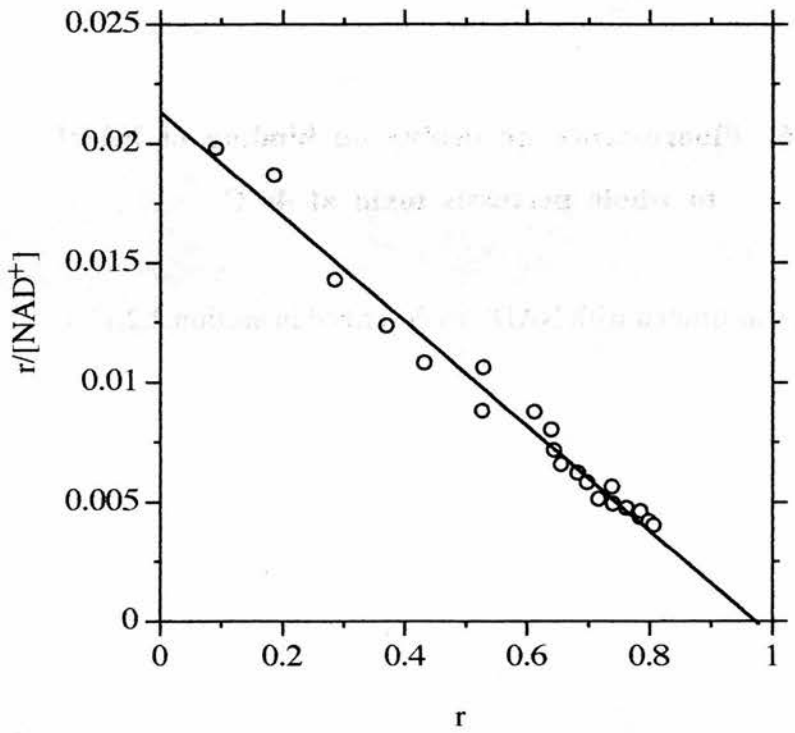

0.02
0.015
0.01
0.005
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
1.2
r

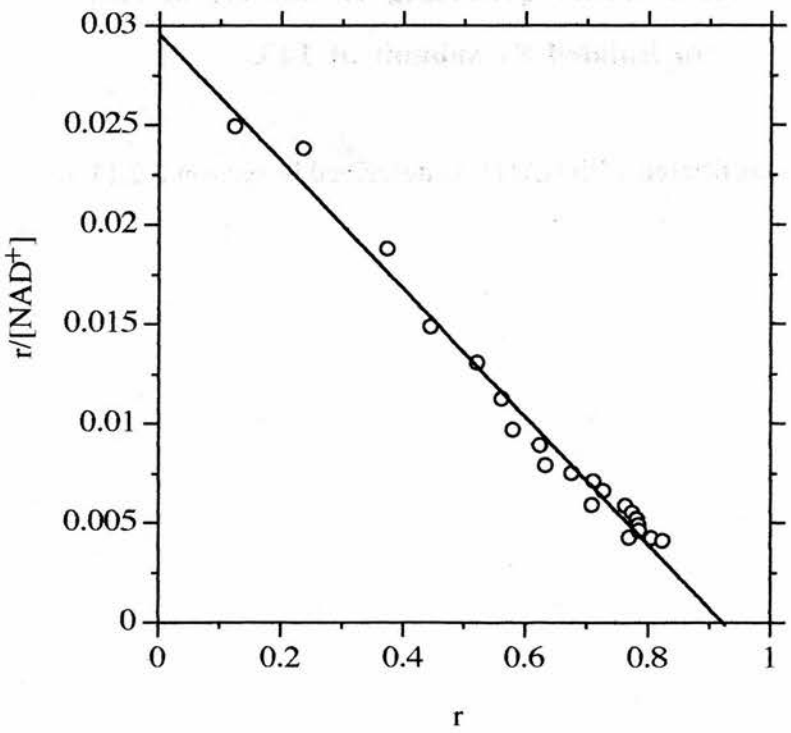
Fig. 5.3 Fluorescence quenching on binding of NAD^+ to whole pertussis toxin at 23°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.

Fig. 5.4 Fluorescence quenching on binding of NAD^+ to whole pertusis toxin at 30°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.


0.025
0.02
0.015
0.01
0.005
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
r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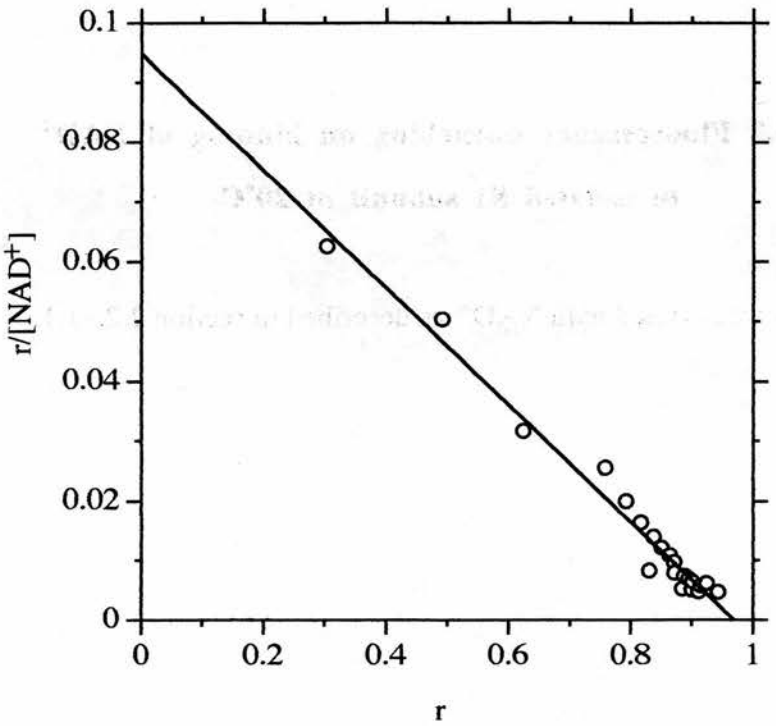
0.025
0.02
0.015
0.01
0.005
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
r

Fig. 5.5 Fluorescence quenching on binding of NAD^+ to whole pertussis toxin at 40°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.

Fig. 5.6 Fluorescence quenching on binding of NAD^+ to isolated S1 subunit at 14°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.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0.02
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
r

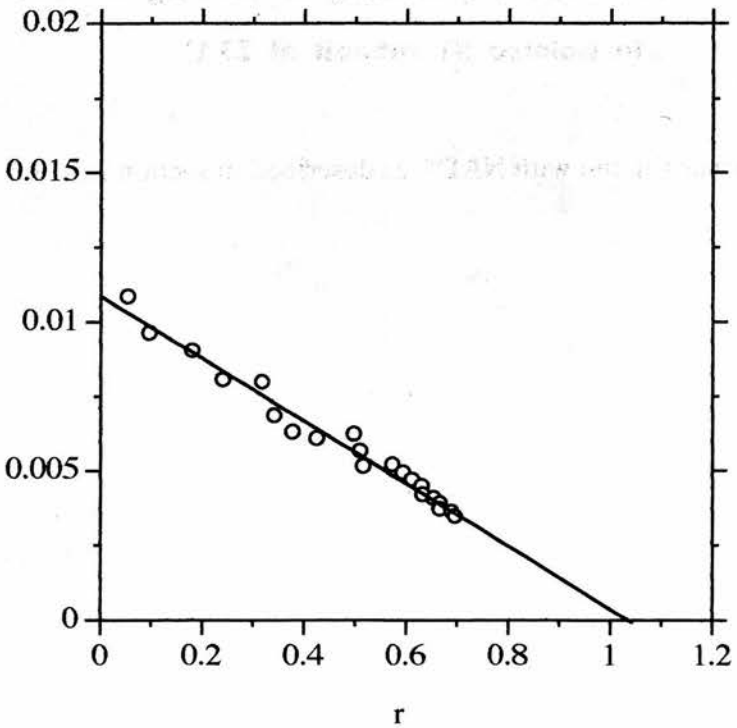
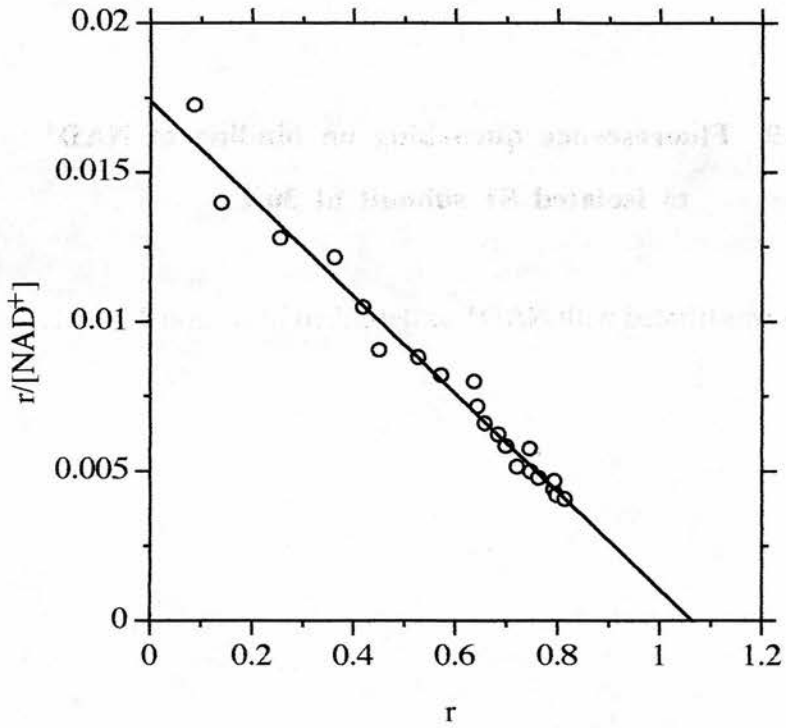

0.02
0.015
0.01
0.005
0
0
0.2
0.4
0.6
0.8
1
1.2
r

Fig. 5.7 Fluorescence quenching on binding of NAD^+ to isolated S1 subunit at 20°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.

Fig. 5.8 Fluorescence quenching on binding of NAD^+ to isolated S1 subunit at 23°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.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0.01
0.005
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
1.2
r

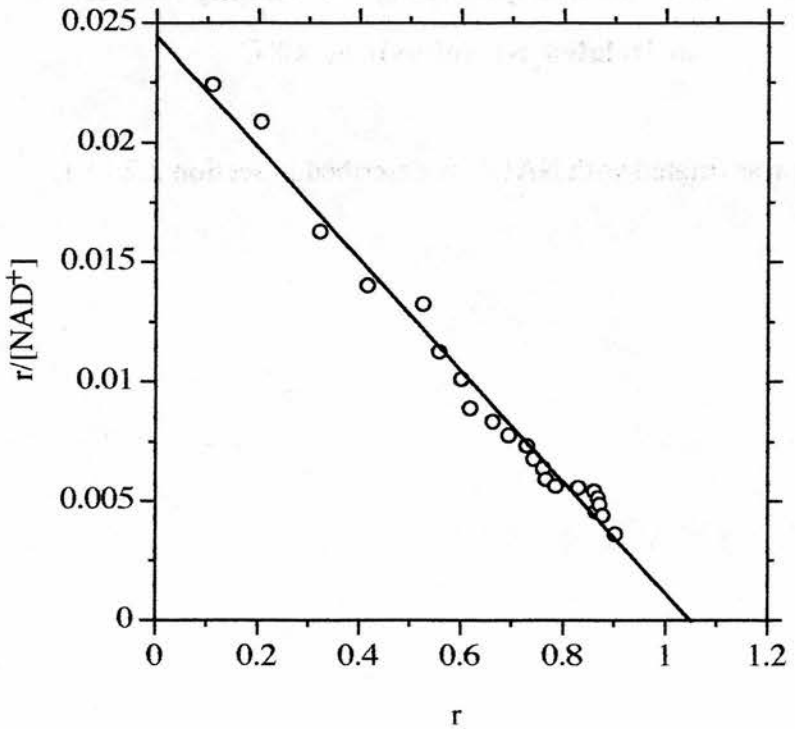
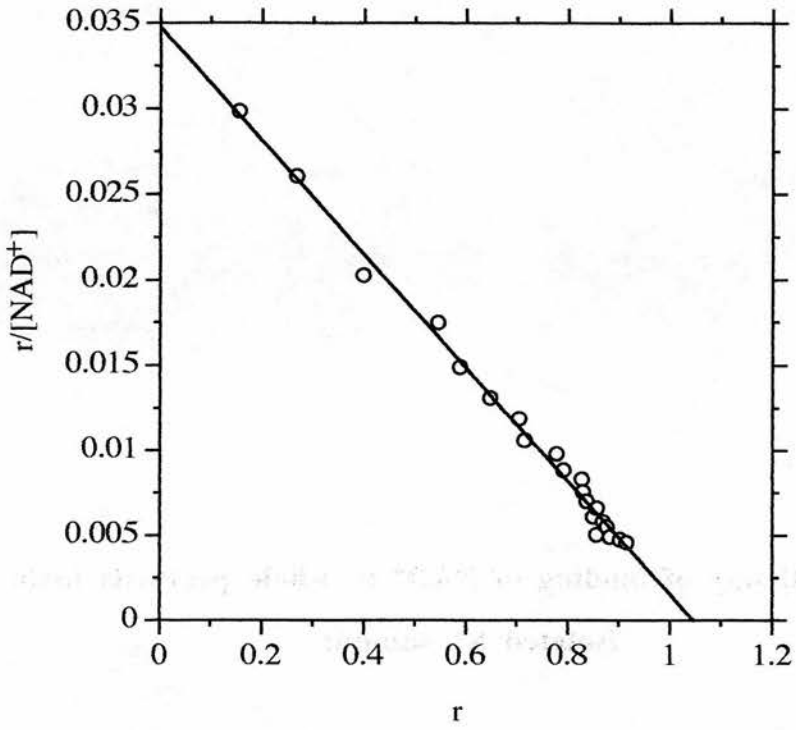

0.025
0.02
0.015
0.01
0.005
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
1.2
r

Fig. 5.9 Fluorescence quenching on binding of NAD^+ to isolated S1 subunit at 30°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.

Fig. 5.10 Fluorescence quenching on binding of NAD^+ to isolated S1 subunit at 40°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.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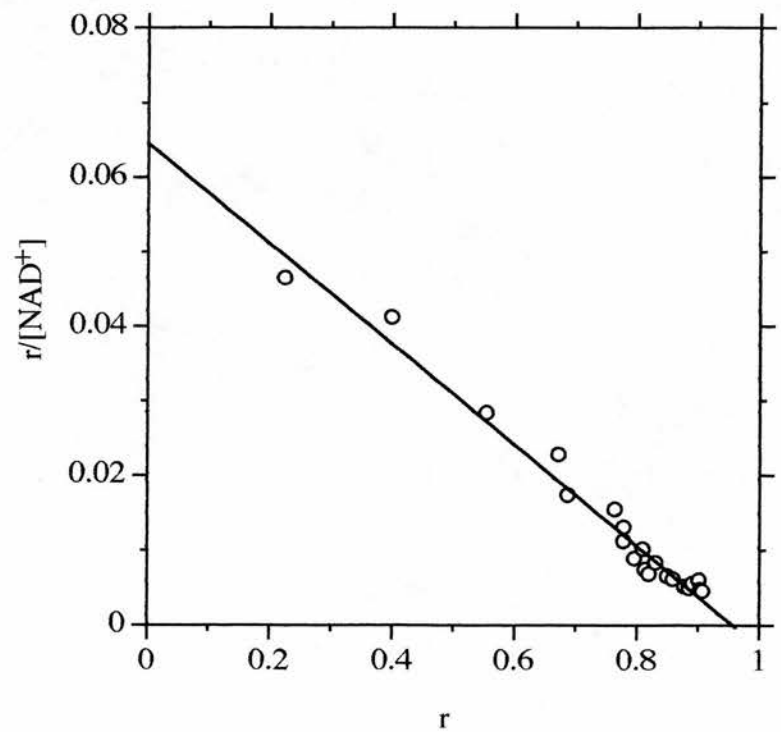
0.03
0.025
0.02
0.015
0.01
0.005
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
1.2
r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0.06
0.04
0.02
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
r

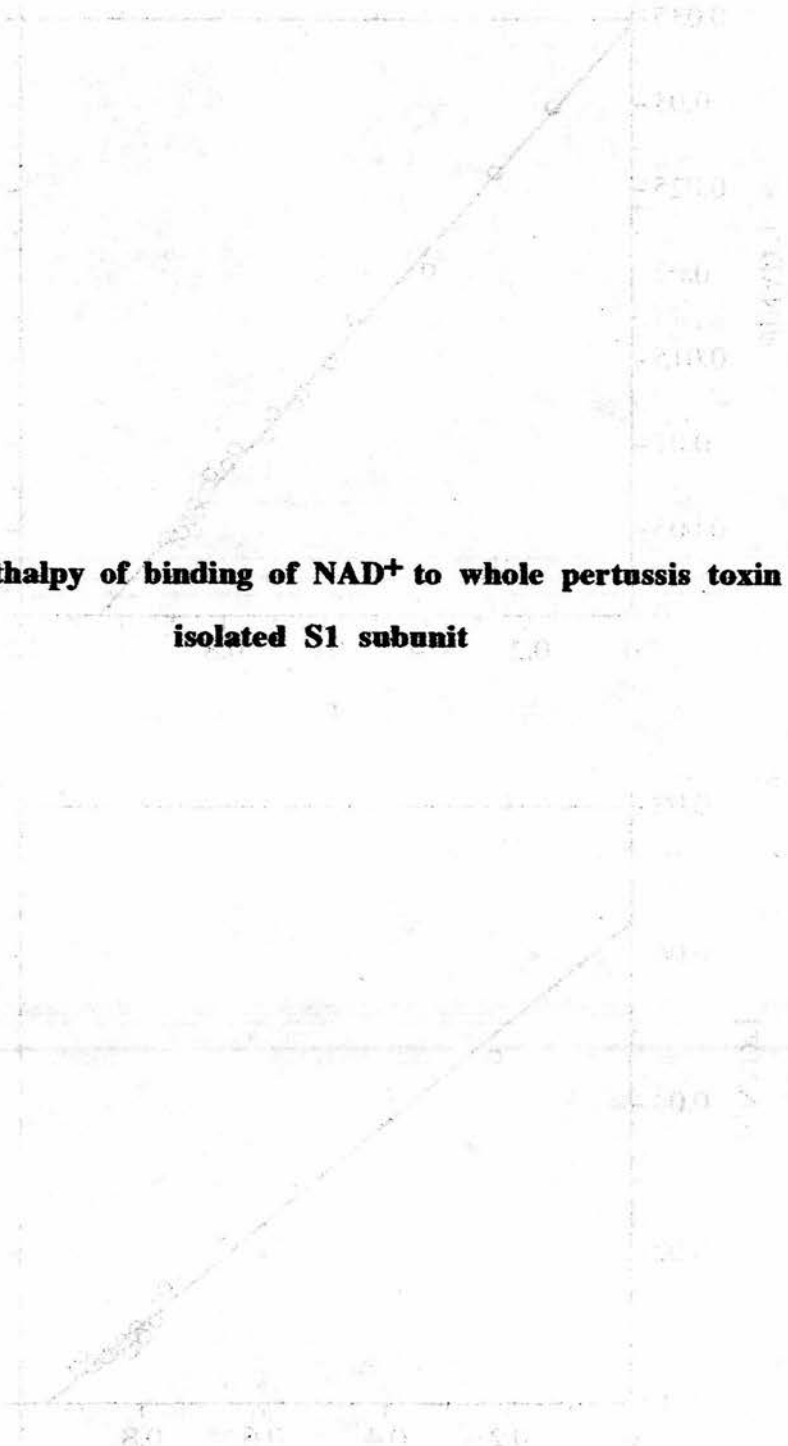
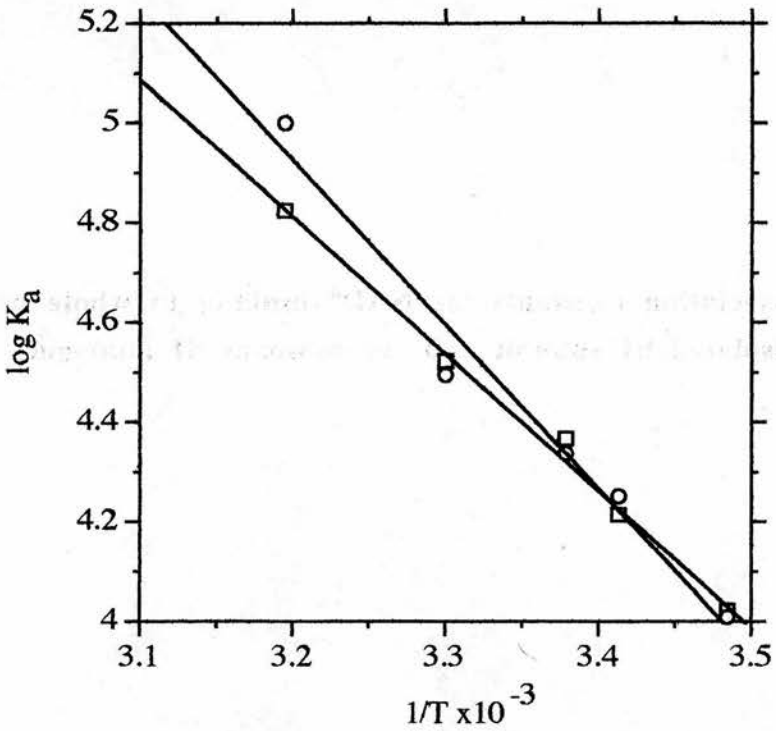


Fig. 5.11 Enthalpy of binding of NAD^+ to whole pertussis toxin and isolated S1 subunit



○ PT $\Delta H = -60 kJ\ mol^{-1}$

□ S1 $\Delta H = -53\ kJ\ mol^{-1}$

Table 5.1 Dissociation constants for NAD^+-binidng to whole pertussis toxin, isolated S1 subunit and recombinant S1 analogues

Temperature /°C	Dissociation constant / μM			
	whole pertussis toxin	Isolated S1	rS1d	rs1dE129D
14	98 +/-6	95	ND	ND
20	56 +/-4	62	ND	ND
23	46 +/-3	43	ND	ND
30	32 +/-2	30	ND	ND
40	10 +/-2	15	ND	ND
37	12 +/-2	ND	38	54

ND: Not done. (quenching experiments were not done using isolated S1 subunit at 37°C because of the low amounts of available material

Note: dissociation constants for pertussis toxin are given as means of three experiments carried out in duplicate in μM +/- standard deviation

5.2.3 NAD$^+$-binding to recombinant S1 subunit analogues

Fluorescence titrations were carried out as described in section 2.2.11.2 at 37°C using whole pertussis toxin, rS1d, and rS1dE129D. Results of the titrations are shown in figures 5.12, 5.13 and 5.14 respectively and the dissociation constants calculated from these plots are shown in table 5.1. The apparent dissociation constant for rS1d was 38 μM, which was about twice the apparent K_d obtained for whole pertussis toxin at the same temperature. The K_d obtained for rS1dE129D was 54 μM, slightly higher than the K_d for rS1d. (NB. limited amounts of isolated S1 subunit were available, so measurements of fluorescence quenching at 37°C with NAD$^+$ were not possible).

5.3 Discussion

5.3.1.1 Effect of interaction of the S1 subunit with the B- oligomer on NAD$^+$-binding

Binding of NAD$^+$ to whole pertussis toxin and isolated S1 subunit showed that there was little difference in dissociation constants across a range of temperatures and little difference in enthalpy of binding. The results suggest that the isolated S1 subunit and S1 attached to the B-oligomer do not have detectably different conformations and that the NAD$^+$-binding site is accessible on the surface of S1, independent of whether the S1 subunit is attached to the B-oligomer.

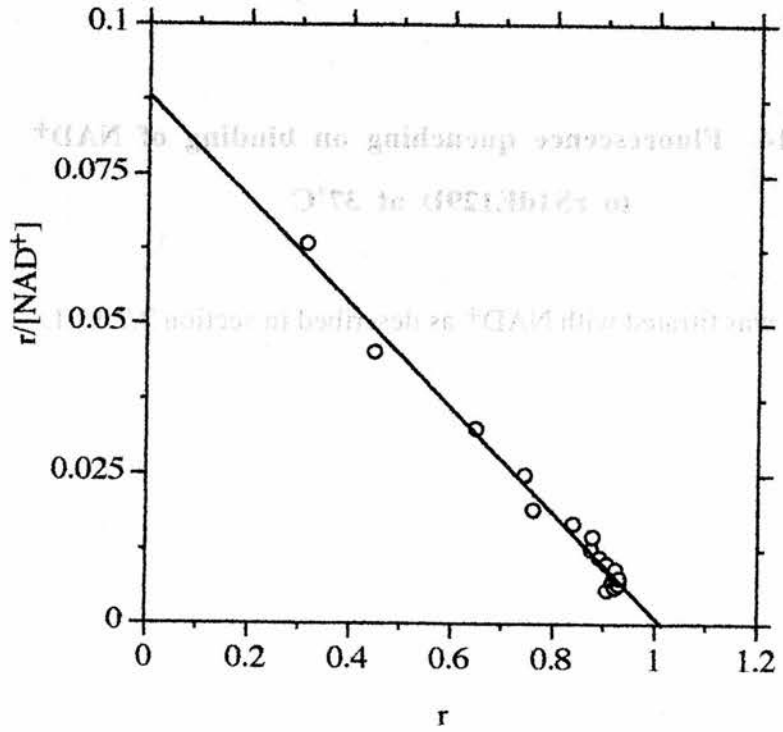
5.3.1.2 Binding of NAD$^+$ to S1 subunit analogues

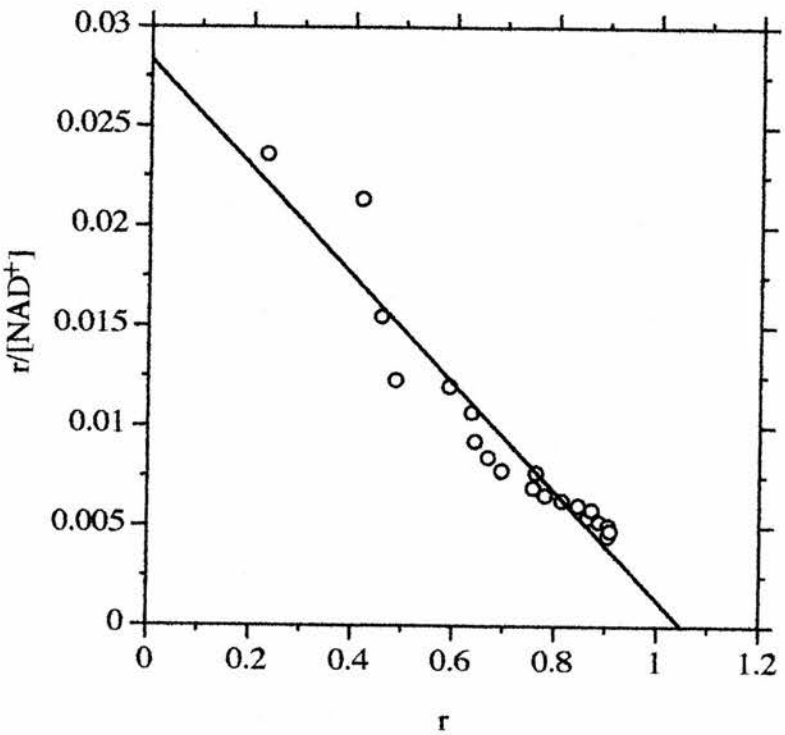
Quenching of intrinsic tryptophan fluorescence showed some difference in K_d between pertussis toxin (K_d 12 +/- 2 μM), rS1d (K_d 38 μM) and rS1dE129D (K_d 54 μM). The dissociation constant for rS1d is about two-fold higher than that for whole pertussis toxin and the dissociation constant for rS1dE129D was about five-fold higher than that for whole pertussis toxin. These results suggest that NAD$^+$-binding to the truncated S1 analogue (rS1d) and the mutated S1 analogue (rS1dE129D) is slightly disturbed, possibly due to slight changes in conformation of the analogues as compared to the wild-type whole toxin, and that glutamate 129 is not essential for NAD$^+$-binding, because the K_d values for the S1 analogues were not drastically different to the K_d for NAD$^+$ of the whole toxin.

Fig. 5.12 Fluorescence quenching on binding of NAD^+ to whole pertussis toxin at 37°C

Fig. 5.13 Fluorescence quenching on binding of NAD^+ to rS1d at 37°C

(Protein was titrated as described in section 2.2.11.1)


0.1
0.075
0.05
0.025
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
1.2
r


0.03
0.025
0.02
0.015
0.01
0.005
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
1.2
r

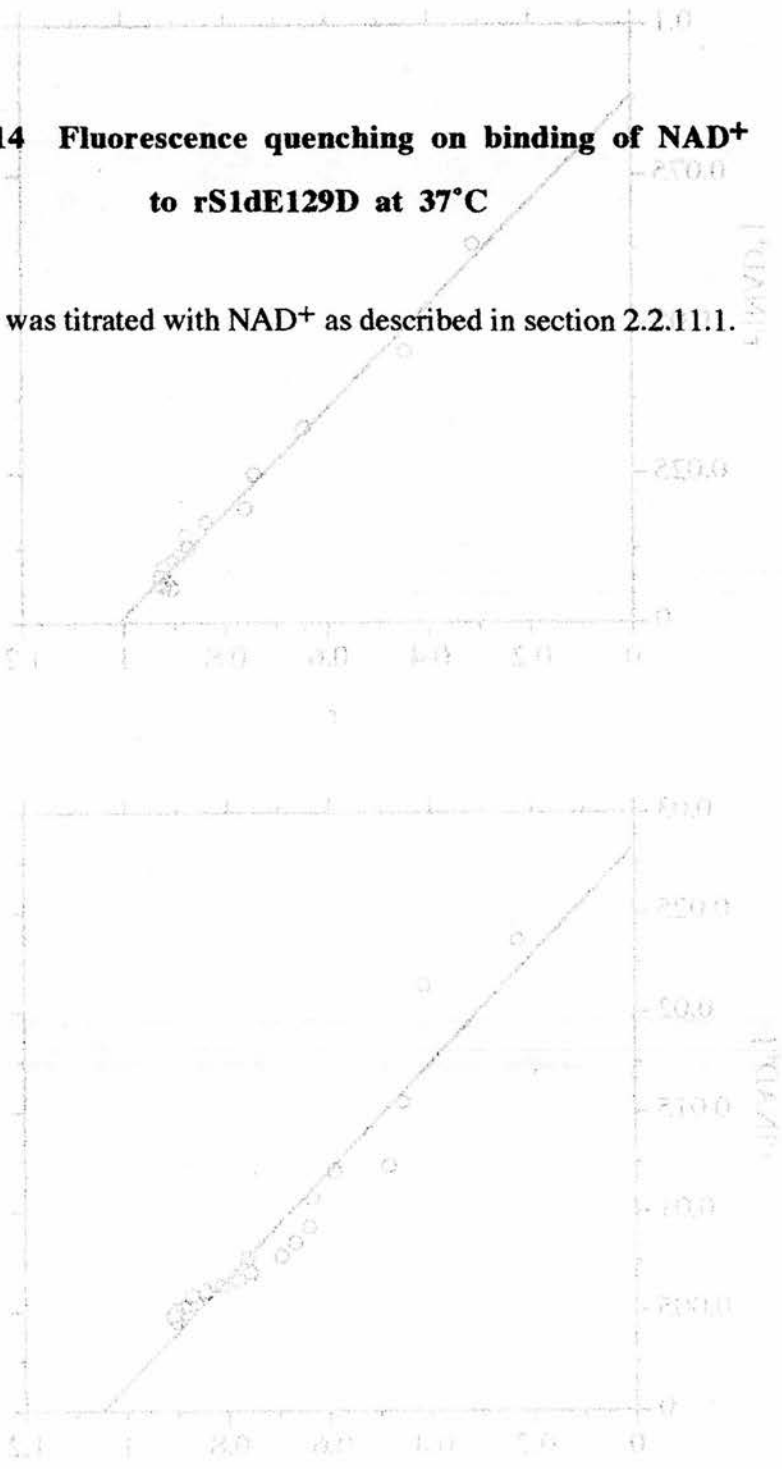
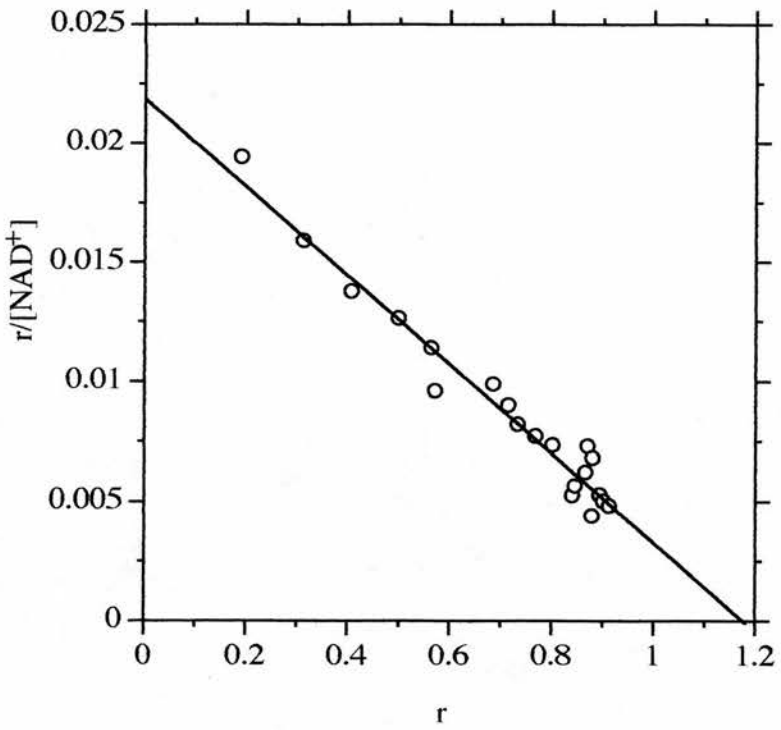


Fig. 5.14 Fluorescence quenching on binding of NAD^+ to rS1dE129D at 37°C

Protein was titrated with NAD^+ as described in section 2.2.11.1.


0.025
0.02
0.015
0.01
0.005
0
r/[NAD+]
0
0.2
0.4
0.6
0.8
1
1.2
r

That the conformation of rS1d has not been dramatically altered when compared to the conformation of native S1 subunit is supported by studies carried out by Locht *et al.* (1989). The group identified conformational epitopes on the S1 subunit of whole pertussis toxin that were recognised by anti-(pertussis toxin) antibodies and those antibodies were also found to react with the S1-analogue rS1d, indicating that the conformation of rS1d was not dramatically different to that of wild type S1 in the whole toxin.

Quenching of rS1d and rS1dE129D fluorescence studies carried out by the author and enzymatic analysis of the S1 subunit analogues were investigated in Antoine et *al* . (in preparation). The NAD^+-glycohydrolase assay method is described in Locht *et al.* (1987) and the assays were performed at 37°C. Truncation of S1 to produce rS1d, or modification of glutamate 129 by site-directed mutagenesis to an aspartate did not significantly affect the Michaelis constant for the NAD^+-glycohydrolase activity and the dissociation constants for NAD^+-binding to rS1d or rS1dE129D found by fluorescence quenching were not dramatically different. However, the catalytic efficiency of the mutated S1 analogue rS1dE129D was significantly affected when compared to rS1d in an NAD^+-glycohydrolase assay. The K_{cat} for rS1d in the NAD^+-glycohydrolase assay was 1.24 min^{-1} and the K_{cat} for rS1dE129D was more than 100-fold less than that of rS1d.

Anti-S1 antibodies known to recognise three-dimensional epitopes of S1 and rS1d (Locht *et al.*, 1989, mentioned above) also recognised rS1dE129D, indicating that the three-dimensional structure of rS1dE129D was not very different to S1 subunit of the whole toxin.

That the dissociation constants and Michaelis constants of rS1d and rS1dE129D for NAD^+ were not very different and the catalytic turnover of rS1dE129D was more than two orders of magnitude lower than rS1d suggested that glutamate 129 is involved mainly in catalysis, rather than the binding of NAD^+ and that the mutation of glutamate 129 to aspartate did not significantly affect the conformation of the molecule.

5.3.2 Other residues involved in the enzymic activity of pertussis toxin

Several other amino acid residues of S1 have been identified as important for the enzymic activity of pertussis toxin and others have been proposed as important and await further work.

An N-terminal region containing residues 8-15 of S1 is necessary for expression of enzymic activity of pertussis toxin, either in catalysis or NAD^+-binding

and aspartate 11 (D11), and arginine 13 (R13) are necessary for the enzymic activity of pertussis toxin (Pizza et *al.*, 1988; Burnette *et al.*, 1988; Locht *et al.*, 1989; Cieplak *et al.*, 1990). Site-directed mutagenesis has identified arginine 9 as being important for enzyme activity because substitution of this residue for lysine reduced the ADP-ribosyltransferase activity to below detectable levels (Burnette *et al.*,1988).

A histidine residue (H21) of diphtheria toxin fragment A has been shown to be involved in NAD^+-binding (Papini *et al.*, 1989), the imidazole of the histidine possibly interacting with the sugar moiety of the adenosine of NAD^+. A possible analogous histidine of pertussis toxin S1 subunit is histidine 35 (H35), which may contribute to enzymic activity (Domenighini *et al.*, 1991).

Chemical modification of the S1 subunit's two cysteines with sulphydryl-reactive compounds suggested that either one or both is involved in enzymic activity. The truncated recombinant S1 analogue rS1d has only one cysteine (C41) and lacks the other, which is located in the C-terminal region of rS1d. Analogue rS1d has similar NAD^+-glycohydrolase and ADP-ribosyltransferase activity to wild type S1 subunit (Locht *et al.*, 1987; 1990), suggesting that cysteine 200 is not required for enzymic activity. Site-directed mutagenesis has identified cysteine 41 as being involved in enzymic activity (Locht *et al.*, 1990), and a replacement of cysteine 41 with glycine altered the K_m for NAD^+, but not the k_{cat}, suggesting that cysteine 41 is involved in NAD^+-binding. Site-directed mutagenesis carried out by Cortina and Barbieri, (1989) also suggested that cysteine 41 could be involved in NAD^+-binding.

In summary, quenching of tryptophan fluorescence of S1 of whole pertussis toxin, isolated S1 subunit and S1 analogues suggests that a tryptophan (possibly tryptophan 26) may be involved in NAD^+ binding. However, binding of NAD^+ to another residue in the N-terminal region of S1 may result in the fluorescence quenching seen. The pertussis toxin B-oligomer is not essential for efficient binding of NAD^+ because dissociation constants for S1 subunit of whole pertussis toxin and isolated S1 subunit were very similar over a range of temperatures. Glutamate 129 of the S1 subunit does not seem essential for NAD^+-binding, as a mutated S1 analogue (rS1dE129D) bound NAD^+ in fluorescence quenching experiments with an affinity that was not greater than one order of magnitude different to the affinity of NAD^+ for whole pertussis toxin.

The involvement of residues of S1 in NAD^+-binding and catalysis can be implied from fluorescence quenching and kinetic studies of mutant proteins, but a full

picture of the residues involved awaits the resolution of the crystal structure of pertussis toxin by X-ray diffraction.

Chapter 6

Summary and Future Work

The subunit arrangement of pertussis toxin and the function of some of the subunits in terms of NAD^+-binding, antigenicity and glycoprotein-binding have been investigated by the author in this thesis.

The spatial arrangement of the subunits of pertussis toxin were investigated using chemical cross-linking reagents. Using the cross-linker DSP, it was shown that subunits S3 and S4 are close together in the whole toxin and S1 is close to subunits S3 and S4. S1 contains no lysine residues in its amino acid sequence, indicating that S1 was linked via its N-terminal to either S3 or S4. These two results were confirmed by Western blotting using subunit-specific monoclonal antibodies to subunits S1 and S3 and polyclonal antisera to subunits S4 , adding to the evidence of an S3-S4 association (Tamura *et al* ., 1982; Sekura *et al* ., 1983) in the whole toxin. One cleaved cross-linked species, that only contained subunits S4 and S5 was identified using two-dimensional SDS PAGE, but the yield of cross-linked material was too low to confirm that S4 was present and an anti-S5 antibody was not available to the author at the time of study. A cleaved cross-link species consisting of subunits S1 and S3 was also identified using two-dimensional SDS PAGE, but this result was not confirmed by Western blotting. No cross-linked species containing S2 were identified in the study, either because there were no accessible residues in the amino acid sequence of S2, or the yield of cross-links contaniing S2 was too low. No dimers were detected that contained S1 and S5 or S1 and S4, indicating that the subunits were not accessible to the cross-linker and suggesting that S1 may sit 'on top' of the S4 and S5 subunits, in agreement with Tamura *et al.* (1982).

The results obtained so far are in agreement with the proposed structure of the toxin to date and are supported by recent structural studies using X-ray crystallography (R. Reid, personal communication).

Further work to investigate the arrangement of the toxin subunits using cross-linkers would include obtaining of S5-specific antibodies to confirm the composition of the cross-linked species thought to contain S4 and S5 and S2-specific antibodies to detemine the compostion of other cross-linked species by Western blotting. Yields of cross-linked material may be increased from those obtained in the study by the use of water-soluble cross-linking reagents. The cross-linking reaction would then occur throughout the cross-linking solution, rather than at the aqueous-organic solvent interface. When using the linkers DSP, DST and SMPB, the linker was dissolved in DMSO and an aliquot added to the cross-linking buffer containing protein, so the cross-linking reaction only occurs at the interface of the two solvents.

Heterobifunctional cross-linkers would generate different cross-linked species to those generated using homobifunctional linkers and would help to clarify the subunit structure of the toxin further.

Antigenicity of pertussis toxin subunits was studied using decapeptides covalently bound to polypropylene pins. The amino acid sequences of the peptides were derived from the entire amino acid sequences of subunits S1, S3 and S4.

Although peptides that bound antibody were identified, using mouse monoclonal antibodies, rabbit polyclonal and human polyclonal antisera, pin peptide-binding patterns differed within and between species and no major continuous antigenic epitopes were identified. As synthesis of each pin decapeptide was carried out under the same conditions, the success of the syntheses probably varied and generated different amounts of each pin peptide. This may have been the case, for example the reaction of anti-S1a with S1 decapeptides with an eight amino acid overlap, where decapeptides 5 and 7 reacted with anti-S1a but decapeptide 6 did not (decapeptide 6 could have been a relatively unsuccessful synthesis). That quite low amounts of decapeptide were synthesised on the pins could account for the few reactive peptides seen with antibodies and antisera having quite high anti-(pertussis toxin) titres. The pin synthesis technique was useful for primary screening in this study and it should be emphasised that conclusions made from this work alone should at least be backed up by studies of free peptides, containing the reactive pin peptide sequence.

One pin decapeptide (sequence QLTFEGKPAL) bound a rabbit polyclonal anti-(pertussis toxin) antisera and that binding was inhibited by whole toxin, or a free peptide containing the same amino acids (peptide S4b, sequence QLTFEGKPALGC). However, rabbit anti-S4b antisera did not react strongly with whole pertussis toxin, suggesting that the sequence of the peptide in subunit S4 of the whole toxin may exist in a different conformation, or that only part of the peptide is exposed on the surface of the S4 subunit in the whole toxin, or that peptide S4b was not particularly immunogenic. These results suggested that peptide S4b was unlikely to induce protective antibodies and is unlikely to be a candidate for a new acellular pertussis vaccine.

Glycoprotein- and glycolipid-binding to the S3-derived pin decapeptides was investigated in an attempt to identify peptides which may be involved in cell-recognition. One pin decapeptide derived from the amino acid sequence of the S3 subunit (residues 46-55, sequence RQITPGWSIY) was found to bind the glycoprotein fetuin. Fetuin-binding of the pin decapeptide was inhibited by a free peptide containing the same amino acids (peptide (E)S3c, sequence EYLRQITPGWSIYGLY) and an antipeptide antibody raised against peptide (E)S3c weakly recognised whole pertussis toxin in an antigen-capture ELISA, indicating that all or part of the sequence of peptide (E)S3c is likely to be at the surface of subunit S3 in the whole toxin.

Pin peptide (residues 46-55) weakly bound only one other antisera, so the peptide does not constitute a major continuous antigenic epitope.

The author found that peptide (E)S3c also bound alpha-1-acid glycoprotein, mixed bovine brain gangliosides and asialofetuin each with decreasing affinity. Armstrong *et al.* (1988) had previously shown that whole toxin bound differentially to glycoproteins, so peptide (E)S3c acted like whole toxin in binding to glycoproteins. A region of S3 thought similar to eukaryotic C-type carbohydrate recognition domains (Saukkonen *et al* , 1992) contains residues contained in peptide (E)S3c, more evidence suggesting that at least some of the residues of peptide (E)S3c could be involved in cellular recognition.

Antisera raised against peptide (E)S3c did not inhibit pertussis toxin-mediated clumping of Chinese Hamster Ovary cells, which is thought to involve the S3 subunit, so peptide (E)S3c is unlikely to induce protective antibodies.

It would be interesting to investigate glycoprotein- and glycolipid- binding to pin decapeptides derived from the amino acid sequence of the S2 subunit of pertussis toxin. This would identify differences in glycoprotein-binding between peptides of subunits S3 and S2 and may provide information on the attachment of pertussis toxin to eukaryotic cells. Subunits S2 and S3 have 70% sequence similarity, so both subunits are very structurally similar and both have been implicated in the cell clumping phenomenon (Lang *et al* ., 1989), so the effects of a combination of antibodies raised against glycoprotein-binding regions of S2 and S3, on pertussis toxin-mediated clumping of Chinese Hamster ovary cells could be investigated.

Lastly, the binding of NAD^+ to whole pertussis toxin, isolated S1 subunit and recombinant S1 analogues was studied using intrinsic tryptophan fluorescence quenching. Dissociation constants were obtained at a number of temperatures for whole pertussis toxin and isolated S1 subunit and the K_d values were used to calculate the enthalpy change on binding of NAD^+ for both whole toxin and S1 subunit. The K_d values determined for whole toxin and S1 subunit using fluorescence quenching were similar. The K_d values for whole toxin were similar to those obtained by Lobban *et al.* (1991) using equilibrium dialysis.

The results suggest that the B-oligomer is not necessary for binding of NAD^+ to the S1 subunit.

Dissociation constants were determined for NAD^+-binding to rS1d, a C-terminally truncated recombinant analogue of the S1 subunit with better solubility than isolated S1 subunit and an S1 mutant analogue which contained one altered amino acid at position 129, called rS1dE129D. The K_d value for rS1dE129D (K_d 54 μM) was less than one order of magnitude greater than the K_d value for rS1d (38 μM) and whole pertussis toxin (12 μM). The results suggest that glutamate 129 is not at the

binding site for NAD^+ and that removal of the C-terminal region of the S1 subunit did not affect binding of NAD^+. Antibodies raised against whole pertussis toxin that are specific for conformational epitopes of S1 also recognise rS1d and rS1dE129D (Locht *et al* ., in preparation), so rS1d and rS1dE129D probably have three-dimensional structures comparable to the S1 subunit of whole pertussis toxin.

Future work would include investigation of the effects of other mutations within the amino acid sequence of the S1 subunit on binding of NAD^+. These would include mutations at position histidine 35 (H35), as this residue has been implicated in enzymic activity of the toxin (Kaslow *et al.*, 1989), cysteine 41 (C41; Locht *et al* ., 1989), arginine 9 (R9) and other mutations within region 8-15 of the S1 subunit, that are known to affect enzymic activity (Cieplak *et al.*, 1989). Effects of of alteration of the length of amino acid side chain, size and charge of the residues would be investigated along with binding of NAD^+-analogues to rS1d and mutated forms of rS1d.

Addiss, D.G., Davis, J.P. and Meade, B.D., Burstyn, D.G., Meissner, M., Zastrow, J.A., Berg, J.L., Drinka, P. and Phillips, R. (1991) J. Infect. Dis. **164**, 704-710

Ad hoc group for study of pertussis vaccines (1988) Lancet (**i**), 955-960

Aktories, K., Barmann, M., Ohishi, I., Tsuyama, S., Jacobs, K.H. and Habermann, E. (1986) Trends Pharmacol. Sci. **8**, 158-160

Allured, V.S., Collier, R.J., Carroll, S.F. and McKay, D.B. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 1320-1324

Anwar, H. (1991) Can. J. Microbiol. **37**, 590-593

Arciniega, J.L., Shahin, R.D., Burnette, W.N., Bartley, T.D., Whiteley, D.W., Mar, V.L. and Burns, D.L. (1991) Infect. Immun. **59**, 3407-3410

Arico, B., Miller, J.F., Roy, C., Stibitz, S., Monack, D., falkow, S., Gross, R. and Rappuoli, R. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6671-6675

Armstrong, G.D., Howard, L.A. and Peppler, M.S. (1988) J. Biol. Chem. **263**, 8677-7684

Askelof, P., Rodmalm, K., Wrangsell, G., Larsson, U., Svenson, S.B., Cowell, J.L., Unden, A. and Bartfai, T. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 1347-1351

Atassi, M.Z. and Smith, J.A. (1978) Immunochemistry **15**, 609-610

Atherton, E., Fox, H., Harkiss, D., Logan, C.J., Sheppard, R.C. and Williams, B.J. (1978) J. Chem. Soc. Chem. Commun., 539-540

Ballou, W.R., Hoffman, S.L., Sherwood, J.A., Hollingdale, M., Neva, F., Hockmeyer, W.T., Gordon, D.M., Wirtz, R.A., Young, J.F., Reeve, R., Diggs, C.L. and Chulay, J.D. (1987) Lancet, (**i**), 1277-1281

Ballou, W.R. (1991) In Cryz, S.J. (ed.) Vaccines and Immunotherapy pp 373-380, Pergamon Press, New York.

Barlow, D.J., Edwards, M.S. and Thornton, J.M. (1986) Nature **322**, 747-748

Bartoloni, A., Pizza, M., Bigio, M., Nucci, D., Ashworth, L.A., Irons, L.I., Robinson, A., Burns,D., Manclark, C.R., Sato, H. and Rappuoli, R. (1988) Biotechnology **6**, 709-712

Bass, J.W. (1985) Lancet (**ii**), 147

Beattie, J., Fawcett, H.A.C. and Flint, D.J. (1992) Eur. J. Biochem. **210**, 59-66

Birnbaumer, L. (1987) Trends Pharmacol. Sci. **8**, 209-211

Birnbaumer, L. (1990) Ann. Rev. Pharmacol. Toxicol. **30**, 675-705

Blundell, T.L., Sibanda, B.L., Sternberg, M.J.E. and Thornton, J.M. (1987) Nature **326**, 347-352

Bokoch, G.M., Katada, T., Northup, J., Hewlett, E.L. and Gilman, A.G. (1983) J. Biol. Chem. **258**, 2072-2079

Booy, R., Aitken, S.J.M., Taylor, S., Tudor - Williams, G., Macfarlane, J.A., Moxon, R., Ashworth, L.A.E., Mayton - White, R.T., Griffiths, S.H. and Chapel, H.M. (1992) Lancet **339**, 507-510

Boquet, P. and Gill, D.M. (1991) In Sourcebook of Bacterial and Protein Toxins. Alouf, J.E. and Freer, J.H. (eds.) pp23-44, Academic Press, London

Bordet, J. and Gengou, O. (1906) Ann. Inst. Pasteur **20**, 731-741

Bragg, P.D. and Hou, C. (1980) Eur. J. Biochem. **106**, 495-503

Brandhuber, B.J. and Mackay, D.B. (1988) Proteins **3**, 146-154

Brandt, D.R. and Ross, E.M. (1986) J. Biol. Chem. **261**, 1656-1664

Braun, U., Habermann, B., Just, I., Aktories, K. and Vandekerckhove, J. (1989) FEBS Lett. **243**, 70-76

Brennan, M.J., Li, Z.M., Cowell, J.L., Bisher, M.E., Steven, A.C. and Novotny, P. (1988) Infect. Immun. **56**, 3189-3195

Brennan, M. J., Hannah, J.H. and Leininger, E. (1991) J. Biol. Chem. **266**, 18827-18831

Brown, A.M., Yatani, A., Kirsch, G., Okabe, K., van Dongen, A.M.J. and Birnbaumer, L. (1991) J. Bioenerg. **23**, 499-507

Burnette, W.N., Cieplak, W., Mar, V.L., Kaljot, T., Sato, H. and Keith, J.M. (1988) Science **242**, 72-74

Burnette, W.N., Mar, V.L., Platler, B.W., Schlotterbeck, D., McGinley, M.D., Stoney, K.S., Rohde, M.F. & Kaslow, S. (1991) Infect. Immun. **59**, 4266-4270

Burns, D.L. and Manclark, C. R. 91986) J. Biol. Chem. **261**, 4324-4327

Burns, D.L., Kenimer,J.G. and Manclark, C.R. (1987) Infect. Immun. **55**, 24-28

Burstein, E.A., Vedenkina, N.S. and Ivkova, N.N. (1973) Photochem. Photobiol. **18**, 263-279

Capiau, C., Petre, J., van Damme, J., Puype, M. and Vaderkerkhove, J. (1986) FEBS Lett. **204**, 336-340

Carroll, S.F. and Collier, R.J. (1988) Mol. Microbiol. **2**, 293-296

Carroll, S.F. and Collier, R.J. (1987) J. Biol. Chem. **262**, 8708-8711

Cassels, F.J., Deal, C.D., Reid, R.H., Jarboe, D.L., Nauss, J.L., Carter, J.M., Boedeker, E.C. (1992) Infect. Immun. **60**, 2174-2181

Chang, C.-D. and Meienhofer, J. (1978) Int. J. Peptide Protein Res. **11**, 246-249

Charles, I.G., Dougan, D., Pickard, S., Chatfield, M., Smith, P., Novotny, P., Morrisey, P. and Fairweather, N.F. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3554-3558

Chaundhary, V.K., Xu, Y.-H., Fitzgerald, D., Adhaya, S. and Pastan, I. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2939-2943

Chelberg, M.K., McCarthy, J.B., Skubitz, A.P.N., Furcht, L.T. and Tsilbary, E.C. (1990) J. Cell Biol. **111**, 261-270

Cherry, J.D. (1990) J.A.M.A. **263**, 179-180

Cherry, J.D., Brunel, P.A., Golden, G.S. and Karzan, D.T. (1988) Pediatrics **81**, 939-984

Choe, S., Bennett, M.J., Fujii, G., Curmi, P.M.G., Kantardjieff, K.A., collier, R.J. and Eisenberg, D. (1992) Nature **357**, 216-222

Chong, P. and Klein, M. (1989) Biochem. Cell. Biol. **67**, 387-391

Chong, P., Sydor, M., Wu, E., Zobrist, G., Boux, H. and Klein, M. (1991) Mol. Immunol. **28**, 239-245

Chong, P., Zobrist, G., Sia, C., Loosemore, S. and Klein, M. (1992) Infect. Immun. **60**, 4640-4647

Chung, D.W. and Collier, R.J. (1977) Infect. Immun. **16**, 832-841

Cieplak, W., Burnette, W.N., Mar, V.L., Kaljot, K.T., Morris, C.F., Chen, K.K., Sato, H. & Keith, J.M. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 4667-4671

Cieplak, W., Locht, C., Mar, V.L., Burnette, W.N. and Keith, J.M. (1990) Biochem. J. **268**, 547-551

Clark, C.G. and Armstrong, G.D. (1990) Infect. Immun. 58, 3840-3846

Cockle, S.A. (1989) FEBS Lett. **249**, 329-332

Confer, D.L. and Eaton, J.W. (1982) Science **217**, 948-950

Cookson, B.T., Cho, H.-L., Herwaldt, L.A. and Goldman, W.E. (1989) Infect. Immun. **57**, 2223-2229

Cortina, G. and Barbieri, J.T. (1989) J. Biol. Chem. **264**, 17322-17328

Cortina, G. and Barbieri, J.T. (1991) J. Biol. Chem. **266**, 3022-3030

Cortina, G., Krueger,K.M. and Barbieri, J.T. (1991) J. Biol. Chem. **266**, 23810-23814

Cowell, J.L., Sato, Y., Sato, H., An Der Lan, B. and Manclark, C.R. (1982) Semin. Infect. Dis. **4**, 371-379

Darst, S.A., Robertson, C.R. and Berzofsky, J.A. (1988) Biophys. J. **53**, 533-539

Davies, D.R., Padlan, E.A. and Sheriff, S. (1990) Annu. Rev. Biochem. **59**,439-493

De Magistris, M. T., Romano, M., Bartioloni, A., Rappuoli, R. and Tagliabue, A. (1989) J. Exp. Med. **169**, 1519-1532

Delisse-Gayothe, A., Locht, C., Jacob, F., Raaschou-Nielsen, M., Heron, I., Ruelle, J., De Wilde, M. and Cabezan, T. (1990) Infect. Immun. **58**, 2895-2905

Devlin, J.J., Panganiban, L.C. and Devlin, P.E. (1990) Science **249**, 404-406

Domenighini, M., Montecucco, C., Ripka, W.C. and Rappuoli, R. (1991) Mol. Microbiol. **5**, 23-31

Donovan, M.G., and Storm, D.R. (1990) J. Cell Physiol. **145**, 444-449

Douglas, C.M. and Collier, R.J. (1990) Biochemistry **29**, 5043-5049

Drickamer, K. (1989) In Carbohydrate Recognition in Cellular Function. Bock, G. and Harnett, S. (eds.) Wiley, Chichester, U.K. pp 45-61

Dryrberg, T. and Oldstone, M.B.A. (1986) J. Exp. Med. **164**, 1344-1349

Dumont, M.E. and Richards, F.M. (1988) J. Biol. Chem. **263**, 2087-2097

Dyson, H.J., Rance, M., Houghten, R.A., Wright, P.E. and Lerner, R.A. (1988) J. Mol. Biol. **201**, 201-207

Ellman, G.L. (1959) Arch. Biochem. Biophys. **82**, 70-77

Endoh, M., Nagai, M. and Nakase, Y. (1986) Microbiol. Immunol. **30**, 1239-1246

Fields, G.B. and Noble, R.L. (1990) Int. J. Peptide Protein Res. **35**, 161-214

Fine, P.E.M. and Clarkson, J.A. (1987) Rev. Infect. Dis. **9**, 866-883

Fong, H.K.W., Yoshimoto, K.K., Eversole-Cire, P and Simon, M.I. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 3066-3070

Francotte, M., Locht, C., Feron, C., Capiau, C. and De Wilde, M. (1989) Vaccines (Cold spring Harbor) **89**, 243

Fries, L.F., Gordon, D.M., Schneider, I., Beier, J.C., Long, G.W., Gross, M., Que, J.D., Cryz, S.J. and Sadoff, J.C. (1992) Infect. Immun. **60**, 1834-1839
Fulgintini, V.A. (1992) Pediatrics **89**, 334-336

Furie, B., Schechter, A.N., Sachs, D.H. and Anfinsen, C.B. (1975) J. Mol. Biol. **92**, 497-507

Giesler, N., Schunemann, J. and Weber, K. (1992) Eur. J. Biochem. **206**, 841-852

Getzoff, E.D., Geysen, H.M., Rodda, S.J., Alexander, H., Tainer, J.A. and Lerner, R.A. (1987) Science **235**, 1191-1196

Geysen, H.M., Meloen, R.H. and Barteling, S.J. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 3998-4002

Geysen, H.M., Mason, T.J., Tribbick, G. and Schoofs, P.G. (1987) J. Immunol. Meth. **102**, 259-274

Geysen, H.M., Mason, T.J. and Rodda, S.J. (1988) J. Mol. Recognit. **1**, 32

Gill, D.M. (1976) Biochemistry **15**, 1242-1248

Gill, S.C. and von Hippel, P.H. (1989) Anal. Biochem. **182**, 319-326

Gilman, A.G. (1987) Ann. Rev. Biochem. 56, 615-649

Gillenius, P., Jaatmaa, E., Askelof, P., Granstrom, M. and Tiru, M. (1985) J. Biol. Stand. **13**, 61-66

Glaser, P., Ladant, D., Sezer, O., Pichot, F., Ullman, A. and Danchin, A. (1988) Mol. Microbiol. **2**, 19-30

Goldman, W.E., Klapper, D.G. and Baseman, J.B. (1982) Infect. Immun. **36**, 782-794

Goodwin, M.S. and Weiss, A.A. (1990) Infect. Immun. **58**, 3445-3447

Gorringe, A.R., Ashworth, L.A.E., Irons, L.I. and Robinson, A. (1985) FEMS Microbiol. Lett. **24**, 5-9

Gray, G.L., Smith, D.H., Baldridge, J.S., Harkins, R.N., Vasil, M.L., Chen. E.Y. and Heyneker, H.L. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 2645-2649

Green, N., Alexander, H., Olson, A., Alexander, S., Schinnick, T.M., Sutcliffe, J.G. and Lerner, R.A. (1982) Cell **28**, 477-487

Greenfield, L., Bjorn, M.J., Horn, D., Fong, D., Buck, G.A., Collier, R.J. and Kaplan, D.A. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6853-6857

Griffin, M.R., Ray, W.A., Mortimer, E.A., Fenichel, G.M. and Schaffner, W. (1990) J.A.M.A. **263**, 1641-1645

Gross, R., Arico, B. and Rappuoli, R. (1989) Mol. Microbiol. **3**, 16661-1667

Gross, M.K., Alu, D.C. Smith, A.L. and Storm, D.R. (1992) Proc. Natl. Acad. Sci. USA **89**, 4898-4902

Halperin, S.A. and Marrie, T.J. (1991) Rev. Infect. Dis. **13**, 1043-1047

Hanski, E. and Gilman, A.G. (1982) J. Cyclic Nucleitide Res. **8**, 323-336

Hanski, E. and Farfel, Z. (1985) J. Biol. Chem. **260**, 5526-5532

Hausman, S.Z., Manclark, C.R. and Burns, D.L. (1990) Biochemistry **29**, 6128-6131

Hausman, S.Z. and Burns, D.L (1992) J. Biol. Chem. **267**, 13735-13739

Hausman, S.Z. and Burns, D.L. (1993) Infect. Immun. **61**, 335-337

Hewlett, E. and Wolf, J. (1976) J. Bacteriol. **127**, 890-898

Hewlett, E., Sauer, K.T., Myers, G.A., Cowell, J.L. and Guerrant, R.L. (1983) Infect. Immun. **40**, 1198-1203

Hewlett, E.L. (1992) J. Med. Microbiol. **36**, 141-142

Higashijima, T., Ferguson, K.M., Sternweiss, P.C., Smigel, N.D. and Gilman, A.G. (1987) J. Biol. Chem. **262**, 762-766

Hoepelman, A.I.M. and Tuomanen, E.I. (1992) Infect. Immun. **60**, 1729-1733

Honjo, T., Nishizuka, Y. and Hayashi, O. (1968) J. Biol. Chem. **243**, 3553-3555

Honore, B. and Pedersen, A.O. (1989) Biochem. J. **258**, 199-204

Hopp, T.P. and Woods, K.R. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 3824-3828

Horsfall, A.C., Hay, F.C., Soltys, A.J. and Jones, M.G. (1991) Immunol. Today **12**, 211-213

Hsu, W.H., Rudolph, U., Stanford, J., Bertrand, P., Olate, J., Nelson, L.C., Moss, L.G., Boyd, A.E III., Codina, J. and Birnbaumer, L. (1990) J. Biol. Chem. **265**, 11220-11226

Hurley, J.B., Simon, M.I., Teplow, D.W., Robishaw, J.D. and Gilman, A.G. (1984) Science **226**, 860-862

Hwang, J., Fitzgerald, D.J., Adhaya, S. and Pastan, I. (1987) Cell **48**, 129-136

Ianobe, A., Shibasaki, H., Takahishi, K., Kobayashi, I., Tomita, U., Ui, M. and Katada, T. (1990) FEBS Lett. **263**, 369-372

Ibsen, P.H., Holm, A., Petersen, J.W., Olsen, C.E. and Heron, I. (1993) infect. Immun. **61**, 2408-2418

Imaizumi, A., Suzuki, Y., Ono, S., Sato, H. and Sato, Y. (1983) Infect. Immun. **41**, 1138-1143

Irons, L.I. and MacLennan, A.P. (1979) Biochim. Biophys. Acta **580**, 175-185

Jacob, C.O., Sela, M. and Arnon, R. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 7611-7615

Janicot, M., Fouque, F. and Desbuquois, B. (1991) J. Biol. Chem. **266**, 12858-12865

Jemmerson, R., Paterson, Y., Van Regenmortel, M.H.V., Altschuh, D., Tainer, J.A., Getzoff, E.D., Alexander, H., Houghton, R.A., Olson, A.J. and Lerner, R.A. (1985) Nature, **317**, 89-90

Jemmerson, R. and Blankenfeld, R. (1989) Mol. Immunol. **26**, 301-307

Kandel, J., Collier, R.J. and Chung, D.W. (1974) J. Biol. Chem. **249**, 2088-2097

Kaslow, H. and Lesikar, D.D. (1987) Biochemistry **26**, 4397-4402

Katada, T. & Ui, M. (1979) J. Biol.Chem. **260**, 469-479

Katada, T. & Ui, M. (1982) J. Biol. Chem. **257**, 7210-7216

Kazemi, M. and Finkelstein, R.A. (1991) Mol. Immunol. **28**, 865-876

Khlef, N., Sakamoto, H. and Guiso, N. (1992) Microbial Pathogen. (in press)

Krchnak, V., Mach, O. and Maly, A. (1987) Anal. Biochem. **165**, 200-207

Ladant, D., Glaser, P. and Ullman, A. (1992) J. Biol. Chem. **267**, 2244-2250

Laemmli, U.K. (1970) Nature **227**, 680-685

Lakowicz, J.R. (1983) Principles of Fluorescence spectroscopy, Plenum Press

Lang, A.B., Ganss, M.T. and Cryz, S.J. (1989) Infect. Immun. **57**, 2660-2665

Laver, W.G., Air, G.M., Webster, R.G. and Smith-Gill, S.J. (1990) Cell **61**, 553-556

LeDur, A., Chaby, R. and Szabo, L. (1980) J.Bacteriol. **143**, 78-88

Leininger, E., Roberts, M., Kenimer, J.G., Charles, I.G., Fairweather, N., Novotny, P. and Brennan, M.J. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 345-349

Leininger, E., Ewanowich, C.A., Bhargava, A., Peppler, M.S., Kenimer, J.G. and Brennan, M.J. (1992) Infect. Immun. **60**, 2380-2385

Leppla, S.H., Martin, O.C. and Muehl, L.A. (1976) Biochem. Biophys. Res. Commun. **81**, 532-538

Linneman, C.C. Jr. and Nasenbeny, J. (1977) Annu. Rev. Med. **28**, 179-185

Livey, I., Duggleby, C.J. and Robinson, A. (1987) Mol. Microbiol. **1**, 203-209

Lobban, M.D., Irons, L.I. and van Heyningen, S. (1991) Biochim. Biophys. Acta **1078**, 155-160

Lobet, Y., Cluff, C.W. & Cieplak, W.J. (1991) Infect. Immun. **59**, 2870-2879

Lobet, Y., Feron, C., Desquesne, G., Simoen, E., Hauser, P. and Locht, C. (1993) J.Exp. Med. **177**, 79-87

Locht ,C. and Keith, J.M. (1986) Science **232**, 1258-1264

Locht, C., Cieplak, W., Marchitto, K.S., Sato, H. and Keith, J.M. (1987) Infect. Immun. **55**, 2546-2553

Locht, C., Capiau, C. and Feron, C. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3075-3079

Locht, C., Lobet, Y., Feron, C., Cieplak, W. and Keith, J.M. (1990) J. Biol. Chem. **265**, 4552-4559

Logan, A.J., Williamson, E.D., Titball, R.W., Percival, D.A., Shuttleworth, A.D., Conlan, J.W. and Kelly, D.C. (1991) Infect. Immun. **59**, 4338-4342

Lomant, A.J. and Fairbanks, G. (1976) J.Mol. Biol. **104**, 243-261

Loosemore, S., Zealey, G., Boux, H., Cockle, S.A., Radika, K., Fahim, R.E.F., Zobrist, G.J., Yacoob, R.K., Chong, P.C.-S., Yao, F.-L. andKlein, M.H. (1990) Infect. Immun. **58**, 3653-3662

Loosemore, S., Cockle, S., Zealey, G., Boux, H., Phillips, K., Fahim, R. and Klein, M. (1991) Infect. Immun. **61**, 2316-2324

Lowry, O.H., Roseburgh, N.J., Farr, A.L. and Randall, R.J (1951) J. Biol.Chem. **193**, 265-275

Mallory, F.B. and Horner, A.A. (1912) J. Med. Res. **27**, 115-123

Mayo, K.H., Parra-Diaz, D., McCarthy, J.B. and Chelberg, M. (1991) Biochemistry **111**, 8251-8267

Mekalanos, J.J., Collier, R.J., and Romig, W.R. (1979) J. Biol. Chem. J. Biol. Chem. **254**, 5855-586

Mekalanos, J.J., Swartz, D.J., Pearson, G.D.N., Harford, N., Groyne, F. and deWilde, M. (1983) Nature, **306**, 551-557

Meloen, R.H. and Barteling, S.J. (1986) Virology **149**, 55-63

Menozzi, F.D., Gantiez, C. and Locht, C. (1991) FEMS Microbiol. Lett. **78**, 59-74

Merrifield, R.B. (1963) J. Am. Chem. Soc. **85**, 2149-2154

Miller, D.L., Alderslade, R. and Ross, E.M. (1982) Epidemiol. Rev. **4**, 1-24

Miller, E., Ashworth, L.A.E., Robinson, A., Waight, P.A. and Irons, L.I. (1991) Lancet **337**, 70-73

Miller, J.F., Mekalanos, J.J. and Falkow, S. (1989) Sceince **243**, 916-922

Milligan, G. (1988) Nature **255**, 1-13

Monack, D., Munoz, J.J., Peacock, M.J., Black, W.J. and Falkow, S. (1989) J. Infect. Dis. **159**, 205-210

Montecucco, C., Tomasi, M., Schiavo, G. and Rappuoli, R.(1986) FEBS Lett. **194**, 301-304

Mooi, F.R., Ter Avest, A. and van der Heide H.J.G. (1990) FEMS Microbiol. Lett. **66**, 327-332

Mooi, F.R., Jansen, W.H., Brunings, H., Gielen, H., van der Heide, H.J.G., Walvoort, H.C. and Guinee, P.A.M. (1992) Microb. Pathogen. **12**, 127-135

Morrison, D.C. and Ryan, J.L. (1987) Annu. Rev. Med. **38**, 417-432

Morse, S.I. and Morse, J.H. (1976) J. Exp. Med. **143**, 1483-1502

Moss, J. and Vaughan, M. (1977) J. Biol. Chem. **252**, 2455-2457

Moss, J., Stanley, S.J., Watkins, P.A., Burns, D.L., Manclark, C.R., Kaslow, C.R. anad Hewlett, E. (1986) Biochem. J. **25**, 2720-2725

Moss, J. and Vaughan, M. (1988) Adv. Enzym. **61**, 303-379

Moxon, E.R. and Rappuoli, R. (1990) Lancet **335**, 1324-1329

Muller, A.S., Leeuwenberg, J. and Pratt, D.S. (1986) Bull. WHO **64**, 321-331

Munoz, J. (1971) Protein toxins from *Bordetella pertussis*, in Kadis, S. and Montie, T.C. (ed.) Microbial toxins vol. II A., Academic press Inc., New York

Murayama, T and Ui, M. (1983) J. Biol. Chem. **258**, 3319-3326

Neer, E.J. and Clapham, D.E. (1988) Nature **333**, 129-134

Nencioni, L., Pizza, M., Volpini, G., De Magistris, M.T., Giovannoni, F. and Rappuoli, R. (1991) Infect. Immun. **59**, 4732-4734

Nicosia, A., Perugini, M., Franzini, C., Casagli, M.C., Borri, M.G., Antoni, G., Almoni, M., Neri, P., Ratti, G. and Rappuoli (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4631-4635

Nicosia, A. and Rappuoli, R. (1987) J. Bacteriol. **169**, 2843-2846

Niemann, H. (1991) In Alouf, J.E. and Freer J.H. (eds.), A Source book of Bacterial Protein Toxins pp303-348, Academic Press, London.

Nogimori, K., Tamura, M., Yajima, K., Ito, T., Nakamura, N., Kajikawa, Y., Marugama, Y. and Ui, M. (1984) Biochim. BIophys Acta **801**, 232-243

Ohishi, I., Iwasakl, M. and Sakaguchi, G. (1980) Infect. Immun. **30**, 668-673

Okabe, K., Yatani, A., Evans, T., Ho, Y.-K., Codina, J., Birnbaumer, L., and Brown, A.M. (1990) J. Biol. Chem. **265**, 12854-12858

Olin, P., Storsaeter, J. and Romanus, V. (1989) J. Am. Med. Assoc. **261**, 560

Olnes, S. and Sandvig, K. (1985) In Endocytosis (Pastan, I. and Willingham, M.C., eds.) pp196-394, Plenum Press, New York

Papini, E., Schiavo, G., Sandona, D., Rappuoli, R. and Montecucco, C. (1989) J. Biol. Chem. **264**, 12385-12388

Pappenheimer, A.M. Jr. (1977) Ann. Rev. Biochem. **46**, 69-94

Pastan, I. and Fitzgerald, D. (1989) J. Biol. Chem. **264**, 15157-15160

Pedroni, P., Riboli, B., De Ferra, F., Grandi, G., Toma, S., Arico, B. and Rappuoli, R. (1988) Mol. Microbiol. **2**, 539-543

Peppler, M.S. (1984) Infect. Immun. **43**, 224-232

Peppler, M.S., Judd, R.C. and Munoz, J.J. (1985) Dev. Biol. Stand. **61**, 75-87
Perera, Y.V., Rogers, H. and Freer, J.H. (1985) J. Gen. Microbiol. **131**, 1897-1901

Peters, K., and Richards, F.M. (1977) Annu. Rev. Biochem. **46**, 523-551

Petersen, J.W., Holm, A., Ibsen, P.H., Haslov, K., Capiau, C. and Heron, I. (1992) Infect. Immun. **60**, 3962-3970

Petersen, J.W., Holm, A., Ibsen, P.H., Haslov, K. and Heron, I. (1993) Infect. Immun. **61**, 56-63

Pierschbacher, M. and Ruoslahti, E. (1984) Nature **309**, 30-33

Pittman, M. (1979) Rev. Infect. Dis. **1**, 401-412

Pittman, M. (1984) Pediatr. Infect. Dis. **3**, 467-486

Pizza, M.G., Bartoloni, A., Prugnola, A., Silvestri, S. and Rappuoli, R. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7521-7525

Pizza, M.G., Covacci, A., Bartoloni, A. Perugini, M., Nencioni, L., De Magistris, M.T., Villa, L., Nucci, D., Manetti, R., Bugnoli, M., Giovannoni, F., Olivieri, R., Barbieri, J.T., Sato, H. and Rappuoli, R. (1989) Science **246**, 497-500

Pizza, M.G., Bugnoli, M., Manetti, R., Covacci, A. and Rappuoli, R. (1990) J. Biol. Chem. **265**, 17759-17763

Podda, A., De Luca, E.C., Titone, L., Casadei, A.M., Cascio, A., Pepploni, S., Volpini, G., Marsili, I., Nencioni, L. and Rappuoli, R. (1992) J. Pediatr. **120**, 680-685.

Price, S.R., Barber, A. and Moss, J. (1990) In ADP-Ribosylating toxins and G proteins. Insights into signal transduction. Moss, J. and Vaughan, M. (eds.) American Society for Microbiology, Washington D.C.

Raetz, C.R., Ulevitch, R.J., Wright, S.D., Sibley, C.H., Ding, A. and Nathan, C.F. (1991) FASEB. J. **5**, 2652-2660

Relman, D.A., Domenighini, M., Tuomanen, E., Rappuoli, R. and Falkow, S. (1989) Proc. Natl. Acad. Sci. USA. **86**, 2637-2641

Relman, D.A., Tuomanen, E., Falkow, S., Golenbock, D.T., Wright, S.D. and Kinzel, V. (1990) Cell **61**, 1375-1382

Ribi, H.O., Ludwig, D.S., Mercer, K.L., Schoolnik, G.K. and Konberg, R.D. (1988) Science **239**, 1272-1276

Riboli, B., Pedroni, P., Cuzzoni, A., Grandi, G. and De Ferra, F. (1991) Microb. Pathogen. **10**, 393-403

Rini, J.M., Schulze-Gahmen, U. and Wilson, I.A. (1992) Science **255**, 959-965

Robinson, A., Irons, L.I. and Ashworth, L.A.E. (1985) Vaccine **3**, 11-20

Robinson, A., Gorringe, A.R., Funnell, S.G.P. and Fernandez, M. (1989) Vaccine **7**, 321-324

Robinson, A., Irons, L.I., Seabrook, R.N., Pearce, A., Matheson, M. and Funnell, S.G.P. (1990) In Manclark (ed.) Proceedings of the Sixth International Symposium on Pertussis, DHHS Publication No. (FDA) 90-1164 pp 126-135, Department of Health and Human Services, Bethesda, Maryland

Robinson, E.A., Henriksen, O. and Maxwell, E.S. (1974) J. Biol. Chem. **249**, 5088-5093

Rogel, A., Meller, R. and Hanski, E. (1991) J. Biol. Chem. **266**, 3154-3161

Rogers, T.S., Crey, S.J. and Rosoff, P.M. (1990) J. Immunol. **145**, 678-683

Roy, C.R., Miller, J.F. and Falkow, S. (1989) J. Bacteriol. **171**, 6345-6448

Ruoslahti, E. and Piershbacher, M.D. (1986) Cell **44**, 517-518

Saelinger, C.B. (1990) Trafficking of Bacterial Protein Toxins (Saelinger, C.B., ed.) pp 26-48, CRC press, Inc., Boca Raton.

Sato, Y., Kimura, M. and Fukimi, H. (1984) Lancet (**i**), 122-126

Saukkonen, K., Burnette, W.N., Mar, V.L., Masure, H.R. and Tuomanen, E.I. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 118-122

Savoca, R., Schwab, C. and Bosshard, H.R. (1991) J.Immunol. Meth. **141**, 242-245

Scarlato, V., Prugnola, A., Arico, B. and Rappuoli, R. (1990) Proc. Natl. Acad.Sci. USA **87**, 6753-6757

Scarlato, V., Arico, B., Prugnola, A. and Rappuoli, R. (1991) EMBO J. **10**, 3971-3975

Scatchard, G. (1949) Ann. N. Y. Acad. Sci. **51**, 660

Schiavo, G., Poulain, B., Rossetto, O., Benfenati, F., Tauc, L. and Montecucco, C. (1992a) EMBO J. **11**, 3577-3583

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Lauretto, P., DasGupta, B.R. and Montecucco, C. (1992b) Nature **359**, 832-835

Schmidt, M.A. and Schmidt, W. (1989) Infect. Immun. **57**, 3828-3833

Schmidt, M.A. Raupach, B., Szulczynski, M. and Marzillier, J. (1991) Infect. Immun. **59**, 1402-1408

Schmitt, H.-J. and Wagner, S. (1993) Eur. J. Pediatr.

Schultz, G., Rosenthal, W. and Hescheler, J. (1990) Ann. Rev. Physiol. **52**, 275-292

Scopes, G.E., Watt, P.J. and Lambden, P.R. (1990) J.Gen. Virol. **71**, 53-59

Scott, J.K. and Smith, G.P. (1990) Science **249**, 386-390

Seabrook, R.N., Robinson, A., Sharma, R.P., Irons, L.I., Ashworth, L.A.E., Price, C.P. and At.kinson, T. (1990) Mol. Immunol. **27**, 777-785

Sekura, R.D., Fish, F., Manclark, C.R., Meade, B. and Zhang, Y. (1983) J. Biol. Chem. **258**, 14647-14651

Sekura, R.D., Zhang, Y. and Quentin-Millet, M.J. (1985) In Pertussis toxin (Sekura, R.D., Moss, J. and Vaughan, M., eds.) pp 45-64, Academic press, New York

Shahin, R.D., Amsbaugh, D.F. and Leef, M.F. (1992) Infect. Immun. **60**, 1482-1488

Shahin, R.D., Brennan, M.J., Li, Z., Meade, B.D. and Manclark, C.R. (1990) J. Exp. Med. **171**, 63-73

Simpson, L.L. (1982) J. Pharmocol. Exp. Ther. **223**, 695-701

Simpson, L.L. (ed.) (1989) Botulinum Neurotoxin and Tetanus toxin. Academic Press, San Diego

Sixma, T., Pronk, S.E., Kalk, K.H., Wartna, E.S., van Zanten, B.A.M., Witholt, B. and Hol, W.G.J. (1991) Nature **351**, 371-377

Sixma, T.K., Pronk, S.E., Kalk, K.H., van Zanten, B.A.M., Berghuis, A.M. and Hol, W.G. (1992) Nature **355**, 561-564

Smith, R.J., Capaldi, R.A., Muchmore, D. and Dahlquist, F. (1978) Biochemistry **17**, 3719-3723

Smith, S.K. and Limbird, L.E. (1982) J. Biol. Chem. **257**, 10471-10478

Spicher, K., Klinz, F.J., Rudolph, U., Codina, J., Birnbaumer, L., Schultz, G. and Rosenthal, W. (1992) Biochem. Biophys. Res. Commun. **175**, 473-479

Steven, A.C., Bisher, M.E., Trus, B.L., Thomas, D., Zhang, J.M. and Cowell, J.L. (1986) J. Bacteriol. **167**, 968-974

Stibitz, S. and & Yang. M.-S. (1990) In Manclark (ed.) Proceedings of the Sixth International Symposium on Pertussis, DHHS Publication No. (FDA) 90-1164, pp 126-135, Department of Health and Human Services, Bethesda, Maryland, U.S.A.

Stibitz, S. and Y & Yang. M.-S. (1991) J. Bacteriol. **173**, 4288-4296

Storsaeter, J., Hallander, H., Farrington, C.P., Olin, P., Mollby, R. and Miller, E. (1990) Vaccine **8**, 457-461

Stryer, L. (1991) J. Biol. Chem. **266**, 10711-10714

Tainer, J.A., Getzoff, E.D., Alexander, H., Houghten, R.A., Olsen, A.J., Lerner, R.A. and Hendrickson, W.A. (1984) Nature **312**, 127-134

Tallett, A., Seabrook, R.N., Irons, L.I., Robinson, A., van Heyningen, S. (1993) Eur. J. Biochem. **211**, 743-748

Tamir, H., Fawzi, A.B., Tamir, A., Evans, T. and Northup, J.K. (1991) Biochemistry **30**, 3929-3936

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M. and Ishii, S. (1982) Biochemistry **21**, 5516-5522

Tan, X.H., Ratnam, M., Huang, S.M., Smith, P.L. and Freisham, J.H. (1990) Eur. J. Biochem. **210**, 59-66

Toro, M.J., Montoya, E. and Birnbaumer, L. (1987) Mol. Endocrinol. **1**, 669-676

Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350-4354

Triffilieff, E., Dubs, M.C. and Van Regenmortel, M.H.V. (1991) Mol. Immunol. **28**, 889-896

Tsuda, M., Tsuda, T., Terayama, Y., Fukuda, Y., Akino, T., Yamakama, G., Stryer, L., Katada, T., Ui, M., and Ebvey, T., (1986) FEBS Lett **198**, 5-10

Tulip, W.R., Varghese, J.M., Webster, R.G., Air, G.M., Laver, W.G. and Colman, P.M. (1990) Quant. Biol. **54**, 257-63

Tuomanen, E.I. and Hendley, J.O. (1983) J. Infect. Dis. **148**, 125-130

Tuomanen, E., Towbin, H., Rosenfelder, G., Braun, D., Larson, G. and Hill, R. (1988) J. Exp. Med. **168**, 267-277

Tuomanen, E. and Weiss, A.A. (1985) J. Infect. Dis. **152**, 118-125

Ui, M. (1988) In Pathogenesis and Immunity in Pertussis (Wardlaw, A. and Parton, R., eds.), pp121-146, Wiley, New York

Ui, M. (1990) In ADP-ribosylating Toxins and G proteins. (Moss, J., and Vaughan, M., eds.) American Society for Microbiology, Washington D.C., U.S.A. pp45-61

Umbarger, K.O., Yamakazi, M., Hutson, L.D., Hayashi, F. and Yamakazi, M. (1992) J. Biol. Chem. **267**, 19494-19502

van Heyningen, S. (1977) Biol. Rev. **52**, 509-549

van Heyningen, S. (1982) Rev. Biosc. Rep. **2**, 135-146

Van Ness B.G., Howard, J.B. and Bodley, J.W. (1980) J. Biol. Chem. **255**, 10717-10720

Van't Wout, J., Burnette, W.N., Mar, V.L., Rozdinski, E., Wright, S.D. and Tuomanen, E.I. (1992) Infect. Immun. **60**, 3303-3308

Vasil, M.L., Kabat, D. and Iglewski, B.H. (1977) Infect. Immun. **16**, 353-361

Wang, K. and Richards, F.M. (1974) J.Biol. Chem. **249**, 8005-8018

Wang, M.-L., Skehel, J.J. and Wiley, D.C. (1986) J. Virol. **57**, 124-128

Weiss, A.A. and Hewlett, E.L. (1986) Ann. Rev. Microbiol. **40**, 661-686

West, R.E.Jr., Moss, J., Vaughan, M., Liu, T. and Liu, T.-Y. (1985) J. Biol. Chem. **260**, 14428-14430

Westhof, E., Altschuh, D., Moras, D., Bloomer, A.C., Mondragon, A., Klug, A. and Van Regenmortel, M.H.V. (1984) Nature **311**, 123-126

White, V.M., Hobday, S. and Begg, N.T. (1991) 'COVER' (cover of vaccination evaluated rapidly) Comm. Dis. Rep. 1, R140

Willems, R.P.A., van der Heide, H.J.G., ter Avest, A.R. & Mooi, F.R. (1990) EMBO. J. **9**, 2803-2809

Witvliet, M.H., Burns, D.L., Brennan, M.J., Poolman, J.T. and Manclark, C.R. (1989) Infect. Immun. **57**,3324-3330

Witvliet, M.H., Vogel, M.L., Wiertz, E.J. and Poolman, J. (1992) Infect. Immun. **60**, 5085-5090

Wolf, J., Cook, G.H., Goldhammer, A.R. and Berkowitz, S.A. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 3841-3844

Wray, W., Boulikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. **118**, 197-203

Yajima, M., Hosoda, K., Kanbayashi, Y., Nakamura, T., Nogimori, K., Nakase, Y. and Ui, M. (1978) J. Biochem. **83**, 295-303

Yamada, E.G. and Kennedy, D.W. (1984) J.Cell Biol. **99**, 29-36

Zhang, Y.L. and Sekura, R.D. (1991) Infect. Immun. **59**, 3754-3759

Zimmerman, N., Beck-Sickinger, A.G., Folkers, G., Krickl, S., Muller, I. and Jung, G. (1991) Eur. J. Biochem. **200**, 519-528

Appendix 1

Amino Acid Sequences of Pin Decapeptides and peptide 3f

S1 subunit (5 amino acid overlap)

peptide n°.	sequence	peptide n°.	sequence
1	DDPPATVYRY	**27**	LAHRRIPPEN
2	TVYRYDSRPP	**28**	IPPENIRRVT
3	DSRPPEDVFQ	**29**	IRRVTRVYHN
4	EDVFQNGFTA	**30**	RVYHNGITGE
5	NGFTAWGNND	**31**	GITGETTTTE
6	WGNNDNVLDH	**32**	TTTTEYSNAR
7	NVLDHLTGRS	**33**	YSNARYVSQQ
8	LTGRSCQVGS	**34**	YVSQQTRANP
9	CQVGSSNSAF	**35**	TRANPNPYTS
10	SNSAFVSTSS	**36**	NPYTSRRSVA
11	VSTSSSRRYT	**37**	RRSVASIVGT
12	SRRYTEVYLE	**38**	SIVGTLVRMA
13	EVYLEHRMQE	**39**	LVRMAPVIGA
14	HRMQEAVEAE	**40**	PVIGACMARQ
15	AVEAERAGRG	**41**	CMARQAESSE
16	RAGRGTGHFI	**42**	AESSEAMAAW
17	TGHFIGYIYE	**43**	AMAAWSERAG
18	GYIYEVRADN	**44**	SERAGEAMVL
19	VRADNNFYGA	**45**	EAMVLVYYES
20	NFYGAASSYF	**46**	VYYESIAYSF
21	ASSYFEYVDT		
22	EYVDTYGDNA		
23	YGDNAGRILA		
24	GRILAGALAT		
25	GALATYQSEY		
26	YQSEYLAHRR		

S3 subunit (5 amino acid overlap)

peptide n°.	sequence	peptide n°.	sequence
1	VAPGIVIPPK	**38**	LTGISLCNPA
2	VIPPKALFTQ	**39**	SLCNPAASIC
3	ALFTQQGGAY		
4	QGGAYGRCPN		
5	GRCPNGTRAL		
6	GTRALTVAEL		
7	TVAELRGNAE		
8	RGNAELQTYL		
9	LQTYLRQITP		
10	RQITPGWSIY		
11	GWSIYGLYDG		
12	GLYDGTYLGQ		
13	TYLGQAYGGI		
14	AYGGIIKDAP		
15	IKDAPPGAGF		
16	PGAGFIYRET		
17	IYRETFCITT		
18	FCITTIYKTG		
19	IYKTGQPAAD		
20	QPAADHYYSK		
21	HYYSKVTATR		
22	VTATRLLAST		
23	LLASTNSRLC		
24	NSRLCAVFVR		
25	AVFVRDGQSV		
26	DGQSVIGACA		
27	IGACASPYEG		
28	SPYEGRYRDM		
29	RYRDMYDALR		
30	YDALRRLLYM		
31	RLLYMIYMSG		
32	IYMSGLAVRV		
33	LAVRVHVSKE		
34	HVSKEEQYYD		
35	EQYYDYEDAT		
36	YEDATFQTYA		
37	FQTYALTGIS		

S4 subunit (5 amino acid overlap)

peptide n$^{o.}$	sequence	peptide n$^{o.}$	sequence
1	DVPYVLVKTN		
2	LVKTNMVVTS		
3	MVVTSVAMKP		
4	VAMKPYEVTP		
5	YEVTPTRMLV		
6	TRMLVCGIAA		
7	CGIAAKLGAA		
8	KLGAAASSPD		
9	ASSPDAHVPF		
10	AHVPFCFGKD		
11	CFGKDLKRPG		
12	LKRPGSSPME		
13	SSPMEVMLRA		
14	VMLRAVFMQQ		
15	VFMQQRPLRM		
16	RPLRMFLGPK		
17	FLGPKQLTFE		
18	QLTFEGKPAL		
19	GKPALELIRM		
20	ELIRMVECSG		
21	VECSGKQDCP		

S1 subunit (8 amino acid overlap)

peptide n$^{o.}$	sequence	peptide n$^{o.}$	sequence
		36	AVEAERAGRG
1	DDPPATVYRY	37	EAERAGRGTG
2	PPATVYRYDS	38	ERAGRGTGHF
3	ATVYRYDSRP	39	AGRGTGHFIG
4	VYRYDSRPPE	40	RGTGHFIGYI
5	RYDSRPPEDV	41	TGHFIGYIYE
6	DSRPPEDVFQ	42	HFIGYIYEVR
7	RPPEDVFQNG	43	IGYIYEVRAD
8	PEDVFQNGFT	44	YIYEVRADNN
9	DVFQNGFTAW	45	YEVRADNNFY
10	FQNGFTAWGN	46	VRADNNFYGA
11	NGFTAWGNND	47	ADNNFYGAAS
12	FTAWGNNDNV	48	NNFYGAASSY
13	AWGNNDNVLD	49	FYGAASSYFE
14	GNNDNVLDHL	50	GAASSYFEYV
15	NDNVLDHLTG	51	ASSYFEYVDT
16	NVLDHLTGRS	52	SYFEYVDTYG
17	LDHLTGRSCQ	53	FEYVDTYGDN
18	HLTGRSCQVG	54	YVDTYGDNAG
19	TGRSCQVGSS	55	DTYGDNAGRI
20	RSCQVGSSNS	56	YGDNAGRILA
21	CQVGSSNSAF	57	DNAGRILAGA
22	VGSSNSAFVS	58	AGRILAGALA
23	SSNSAFVSTS	59	RILAGALATY
24	NSAFVSTSSS	60	LAGALATYQS
25	AFVSTSSSRR	61	GALATYQSEY
26	VSTSSSRRYT	62	LATYQSEYLA
27	TSSSRRYTEV	63	LATYQSEYLA
28	SSRRYTEVEL	64	TYQSEYLAHR
29	RRYTEVELEH	65	QSEYLAHRRI
30	YTEVELEHRM	66	EYLAHRRIPP
31	EVELEHRMQE	67	LAHRRIPPEN
32	ELEHRMQEAV	68	HRRIPPENIR
33	EHRMQEAVEA	69	RIPPENIRRV
34	RMQEAVEAER	70	PPENIRRVTR
35	QEAVEAERAG	71	ENIRRVTRVY

72	IRRVTRVYHN
73	RVTRVYHNGI
74	TRVYHNGITG
75	VYHNGITGET
76	HNGITGETTT
77	GITGETTTTE
78	TGETTTTEYS
79	ETTTTEYSNA
80	TTTEYSNARY
81	TEYSNARYVS
82	YSNARYVSQQ
83	NARYVSQQTR
84	RYVSQQTRAN
85	VSQQTRANPN
86	QQTRANPNPY
87	TRANPNPYTS
88	ANPNPYTSRR
89	PNPYTSRRSV
90	PYTSRRSVAS
91	TSRRSVASIV
92	RRSVASIVGT
93	SVASIVGTLV
94	ASIVGTLVRM
95	IVGTLVRMAP
96	GTLVRMAPVI
97	LVRMAPVIGA
98	RMAPVIGACM
99	APVIGACMAR
100	VIGACMARQA
101	GACMARQAES
102	CMARQAESSE
103	ARQAESSEAM
104	QAESSEAMAA
105	ESSEAMAAWS
106	SEAMAAWSER
107	AMAAWSERAG
108	AAWSERAGEA
109	WSERAGEAMV
110	ERAGEAMVLV
111	AGEAMVLVYY
112	EAMVLVYYES
113	MVLVYYESIA
114	LVYYESIAYS
115	VYYESIAYSF

Amino acid sequence of peptide 3f:

KVTNGSKSYTLRYLASYVK(GC)

Appendix 2

Circular dichroism of peptide (E)S3c

Far u.v. circular dichroism spectra of peptide (E)S3c (kindly performed by Dr. L. Irons, PHLS, Porton Down, U.K.) in water showed a minimum at 194 nm with an peak height of about -16 000 deg.cm^2.dmol^{-1} (fig. A1). There were no other peaks in the spectrum, suggesting that (E)S3c had little secondary structure in water. When trifluoroethanol was added to (E)S3c in water the molar ellipticity at 194 nm moved to a positive intensity of about 7 000 deg.cm^2.dmol^{-1}

Short linear peptides are thought to exist in many rapidly changing conformations. Trifluoroethanol is thought to stabilise transient structures and is known to stabilise alpha-helix in peptides (Dyson *et al.*,1988), so its interaction with peptide (E)S3c was observed. The alpha helix content of the peptide in water appeared to be less than 5% estimated from the mean residue ellipticity at 222 nm, although it is not known what contribution the aromatic amino acids tyrosine and tryptophan make to the far u.v. C.D. spectra. Circular dichroism spectra of peptide (E)S3c will have to be supported by NMR studies to determine to what extent secondary structure exists in peptide (E)S3c.

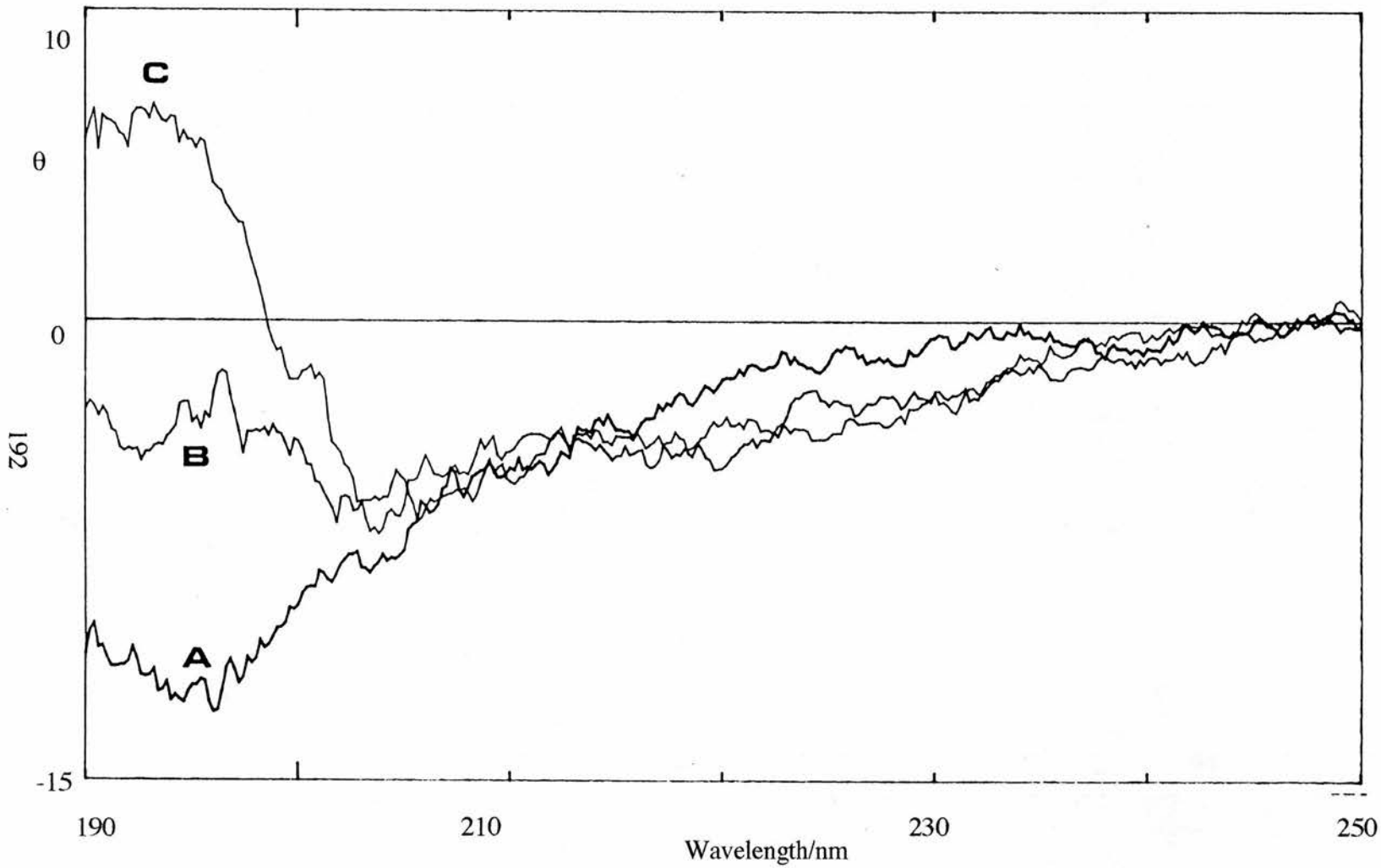
Fig. A1 Circular dichroism of peptide (E)S3c

A 0% Trifluoroethanol

B 50% Trifluoroethanol

C 90% Trifluoroethanol

θ mean residue ellipticity ($deg.cm^2.dmol^{-1}$)


10
θ
0
-15
192
C
B
A
190
210
230
250
Wavelength/nm

Appendix 3

Published Work

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Localisation of a receptor-recognition domain on the S3 subunit of pertussis toxin by peptide mapping

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Overlapping 10-amino-acid peptides, which consecutively span the amino acid sequence of the S3 subunit of pertussis toxin, were synthesised on polyethylene pins and screened for their ability to bind the glycoprotein fetuin. Fetuin binding was localised to a single peptide comprising amino acids 46–55. A free peptide, (E)S3c, of longer sequence (S3 amino acids 44–58) was also found to bind α-1-acid glycoprotein, mixed brain gangliosides and fetuin. (E)S3c also recognised asialofetuin but with a lower apparent affinity relative to fetuin. The single tryptophan residue of the peptide yielded a fluorescence-emission maximum of 355 nm. In the presence of either ganglioside or the phospholipid L-α-lysolecithin, but not *N*-acetylneuramin-lactose or lactosylceramide, the emission intensity of (E)S3c was enhanced and the emission maximum blue-shifted to 340 nm by ganglioside, or to 345 nm by L-α-lysolecithin. Monosialogangliosides, disialogangliosides, and trisialogangliosides, when fluorescence-titrated, were each found to bind the peptide with a similar dissociation constant of 4.4 ± 2.8 μM. These findings demonstrate that region 44–58 of the pertussis-toxin S3 subunit is likely to be involved in the recognition of both glycosylated and phospholipid constituents of target-cell membranes.

Pertussis toxin (PT) is an ADP-ribosylating toxin secreted by the causative agent of whooping cough, *Bordetella pertussis* (Weiss and Hewlett, 1986), and is currently considered an important virulence determinant involved in the disease process. PT can function as one of the *B. pertussis* adhesins, which facilitate the first stage of infection by enabling attachment of the bacteria to the cilia of the respiratory tract (Relman et al., 1990; Saukkonen et al., 1992). In addition, several of the systemic disease effects which may result from infection, such as lymphocytosis, enhanced vascular permeability and stimulation of insulin secretion, can be reproduced by PT in experimental models (Ui, 1988). Consequently in order to induce PT-neutralising antibodies, acellular vaccines designed to protect children from whooping cough contain a detoxified form of the protein (Robinson et al., 1985).

The PT molecule comprises of subunits S1/S2/S3/S4/S5 in the ratio 1:1:1:2:1 (Tamura et al., 1982). The ADP-ribosyltransferase activity and NAD-glycohydrolase activity are contained within the S1 subunit (A-protomer), while the remaining subunits form a pentameric B-oligomer which is involved in eukaryotic cell recognition (Ui, 1988). The A-B functional organisation of PT is similar to that of other bacterial protein toxins such as cholera toxin, *Escherichia coli* heat-labile toxin and diphtheria toxin. The interaction of the B-oligomer with eukaryotic membrane glycoproteins and glycolipids is primarily mediated by subunits S2 and S3 (Capiau et al., 1986; Schmidt et al., 1991), which are both closely associated in the B-oligomer with subunit S4 (Tamura et al., 1982). S2 and S3 differ in their range of receptor-binding specificities, but also possess some receptors in common (Witvliet et al., 1989; Saukkonen et al., 1992). Attachment of the holotoxin to a target cell is considered to be followed by penetration of the target cell membrane by the catalytic S1 subunit (Ui, 1988). However, there are some biological activites of PT that occur independently of the S1 subunit, including T-cell mitogenicity and erythrocyte agglutination, both of which have been directly attributed to B-oligomer cell attachment (Nencioni et al., 1991).

In addition to membrane glycoreceptors, the B-oligomer also mediates PT binding to sialylated serum proteins, such as fetuin and haptoglobin (Irons and Maclennan, 1979), and to mucosal proteins, such as α-1-acid glycoprotein (Armstrong et al., 1988). Optimal binding of PT to glycoprotein depends upon the presence of *N*-acetylneuraminic acid and lactosamine constituents in the oligosaccharide (Armstrong et al., 1988; Witliviet et al., 1989). Furthermore, the existence of carbohydrate recognising domains in both the S2 and S3 subunits, has recently been confirmed using site-directed mutants of PT (Saukkonen et al., 1992). Since certain carbohydrate-recognising domains of other proteins, such as

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Abbreviations. $AcTrpNH_2$, *N*-acetyl-L-tryptophanamide; Fmoc, *N*-(9-fluorenylmethoxycarbonyl); MBG, mixed brain ganglioside; PT, pertussis toxin.

Enzyme. NAD glycohydrolase (EC 3.2.2.5).

fibronectin (Pierschbacher and Ruoslahti, 1984), have been mimicked by linear-peptide analogues, we have chosen to assess whether a peptide from the amino acid sequence of the S3 subunit of PT could also bind either glycoprotein or glycolipid. To prepare consecutive and overlapping peptides spanning the S3 amino acid sequence, we employed the pin-peptide-mapping technique (Geysen et al., 1987) which has been successfully used to identify antibody-binding peptides from protein amino acid sequences (e.g. Kazemi and Finkelstein, 1991).

EXPERIMENTAL PROCEDURES

Materials

The following were purchased from Sigma Chemicals: *N*-acetylneuramin-lactose, *N*-acetyl-L-tryptophanamide (Ac-TrpNH$_2$), α-1-acid glycoprotein, antibody-enzyme conjugates, asialofetuin, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), bovine serum albumin, Chaps, fetuin, glutaraldehyde, lactosylceramide, L-α-lysolecithin, and Tween 20. Mixed brain ganglioside (MBG) and gangliosides G_{M1}, G_{D1a} and G_{T1b} were from Calbiochem. Organic solvents were of the highest grade available; diisopropylethylamine, ethanedithiol and phenol were obtained from Aldrich Chemicals; dichloromethane, dimethylformamide, methanol and trifluoroacetic acid were from Romil Chemicals; acetic anhydride and piperidine were both from BDH Chemicals. Derivatised polypropylene pins for overlapping peptide synthesis, *N*-(9-fluorenylmethoxycarbonyl) (Fmoc) amino acid pentafluorophenyl esters, 1-hydroxybenzotriazole were each purchased as a kit from Cambridge Research Biochemicals (ICI, Northwhich, UK). Reagents for the synthesis of the free peptide were from Applied Biosystems, and both newborn calf serum and Nunc Immuno-1 microtitre plates were from Gibco-BRL. PT was purified from culture supernatants by the method of Irons and Gorringe (1988).

Peptide synthesis

Pin peptides were simultaneously synthesised on a block of 96 derivatised pins (Geysen et al., 1987) according to the suppliers procedures. Essentially this involved consecutive cycles: (1) Fmoc deprotection of the block of pins using 20% (by vol.) piperidine in dimethylformamide for 20 min; (2) repeated pin washes with both dimethylformamide and methanol; (3) immersion of the block of pin tips into the corresponding wells of a polypropylene microtitre plate, which contained 100 µl/well of the appropriate 30 mM Fmoc-Xaa (mixed with 30 mM 1-hydroxybenzotriazole in dimethylformamide) and incubated for 18 h; (4) washing of the pin tips with dimethylformamide, methanol (4×) and again dimethylformamide; (5) repeat the cycle with step 1 for the next amino acid in the sequence. After 10 coupling cycles, the α-amino group of each pin peptide was deprotected (step 1) and acetylated [100 µl/well 10% (by vol.) acetic anhydride, 2% (by vol.) diisopropylethylamine in dimethylformamide for 90 min] then washed as described in step 4. The block of pin peptides was then transferred to a vessel containing 95% (by vol.) trifluoroacetic acid, 2.5% (mass/vol.) phenol and 2.5% (by vol.) ethanedithiol for 4 h to deprotect the amino acid side chains. The block was then washed with dichloromethane, neutralised with 5% (by vol.) diisopropylethylamine in dichloromethane, washed again with dichloromethane and dried before a final wash with both H_2O then methanol for 18 h. The block of pin peptides was dried over silica and stored dessicated.

Free peptide (E)S3c was prepared with a C-terminal amide from a 4-[2′,4′-dimethoxyphenyl-*N*-(9-fluorenylmethoxycarbonyl)-aminomethyl]phenoxy resin, using the Applied Biosystems FastMoc system and a 431A automatic synthesiser. Peptide purity was confirmed by reverse-phase HPLC using a Vydac C_8 column and composition was confirmed by mass spectrometry.

Pin-peptide binding assays

The pin-peptide tips were suspended from the block into the corresponding wells of a microtitre plate and incubated for 90 min in 200 µl diluent [10% (by vol.) newborn calf serum, 0.4% (by vol.) Tween 20, in 50 mM sodium phosphate, 0.9% (mass/vol.) sodium chloride, pH 7.2 (NaCl/P_i)] to reduce non-specific binding. This was followed by incubation for 7 h with 20 µg/ml fetuin (175 µl/well) in diluent; three 5-min washes with 0.1% (by vol.) Tween 20 in NaCl/P_i, overnight incubation with 175 µl/well of a 1:250 dilution of affinity-purified rabbit anti-fetuin serum (provided by Dr L. A. E. Ashworth) in diluent, four 2-min washes with 0.1% (by vol.) Tween 20 in NaCl/P_i, a 90-min incubation with a 1:500 dilution of goat anti-(rabbit IgG) serum conjugated to peroxidase (175 µl/well) and washed as previously described. Colour was developed by incubating the pin peptides with 150 µl/well 0.05% (mass/vol.) 2,2′-azinobis(3-ethylbenzthiazolone-6-sulphonic acid), 80 mM citric acid, 100 mM disodium hydrogen orthophosphate, 0.01% (by vol.) H_2O_2, (pH 4.0) for 30 min and was measured at 405 nm on a Titertek Multiscan.

To rejuvenate the pin peptides for subsequent assays, the block of pins was sonicated for 30 min at 60°C in disruption buffer [0.1% (by vol.) 2-mercaptoethanol, 1.0% (mass/vol.) SDS, 100 mM sodium dihydrogen orthophosphate, pH 7.2], followed by two H_2O washes at 60°C, immersed in boiling methanol and air dried for storage at 4°C.

Anti-peptide serum

Peptide conjugation to bovine serum albumin using glutaraldehyde has been described before (Seabrook et al., 1990). New Zealand white rabbits were each immunised with 150 µg of the peptide−bovine-serum-albumin conjugate dispersed in Freund's incomplete adjuvant. At 3 weeks and 6 weeks, the dose was repeated and serum was harvested at 8 weeks from primary immunisation.

ELISA procedures

ELISA have been described previously (Seabrook et al., 1990). PT and peptide (E)S3c recognition of receptor-coated ELISA plates was detected by a subsequent incubation with either mAb L10 (1:500 dilution; Irons and Gorringe, 1988) for PT, or by rabbit anti-(E)S3c serum (1:350 dilution) for peptide (E)S3c This was followed by an incubation with goat anti-(rabbit IgG) serum conjugated to peroxidase (1:625 dilution).

Fluorescence

Spectra and ligand-titration measurements were made at 30°C with a Perkin Elmer LS 5 spectrofluorimeter as previously described (Seabrook et al., 1991). The stock ligand

solutions were 1 mg/ml in NaCl/P_i for Chaps, *N*-acetylneuramin-lactose, gangliosides and l-α-lysolecithin. Lactosylceramide was 1 mg/ml in dimethylformamide. Inner filter corrections for ultraviolet absorbance were made using $F_{corr} = F \cdot \text{antilog}\,[(A_{ex} + A_{em})/2]$, where F is the observed fluorescence emission intensity and A_{ex} and A_{em} the absorbances of the solution at the emission and excitation wavelengths, respectively. Dissociation constants were computed using Enzfitter software (Elsevier Biosoft) from plots of the increase in fluorescence (ΔF) against ligand concentration.

RESULTS

Localisation of an S3 binding domain for glycoprotein

Consecutive overlapping 10-amino-acid peptides, spanning the complete DNA-derived amino acid sequence (Locht and Keith, 1986) of the S3 subunit, were synthesised in duplicate with a five-residue overlap (except for the final peptide, 39, which overlapped the previous peptide by six residues). To confirm the fidelity of the synthesis, an antibody previously raised to an S3 peptide sequence (Seabrook et al., 1990) was shown to bind its expected antigenic pin peptide (Fig. 1a). Subsequently, the pin peptides were assayed with the glycoprotein receptor fetuin, which was found to recognise pin-peptide 10 (amino acids 46–55; sequence RQITPGWSIY) with an absorbance almost double that of the average obtained for the remaining S3 peptides (Fig. 1b).

To explore the peptide/receptor interaction in greater detail, a free peptide (E)S3c, of sequence EYLRQITPGWSIY-GLY-$CONH_2$, was synthesised. This peptide consisted of S3 residues 44–58, preceded by an N-terminal glutamic acid. (The latter was incorporated to improve carbodiimide coupling effeciency to the carrier protein. However, we subsequently observed that immunisation with a glutaraldehyde-prepared conjugate yielded a higher antibody titre). The purified peptide produced the expected $[M+H]^+ = 1958.3$ Da by mass spectrometry. Peptide (E)S3c, when incubated beforehand with fetuin, was found to inhibit the subsequent binding of fetuin to the pin peptide (Fig. 1c), confirming the specificity of the peptide/glycoprotein interaction.

Several monoclonal and polyclonal antibodies were screened with the block of pin peptides, but none of the antibodies were found to recognise pin-peptide 10. This suggests that the region of the S3 subunit represented by the fetuin-binding peptide does not form part of an intrinsic contiguous S3 epitope.

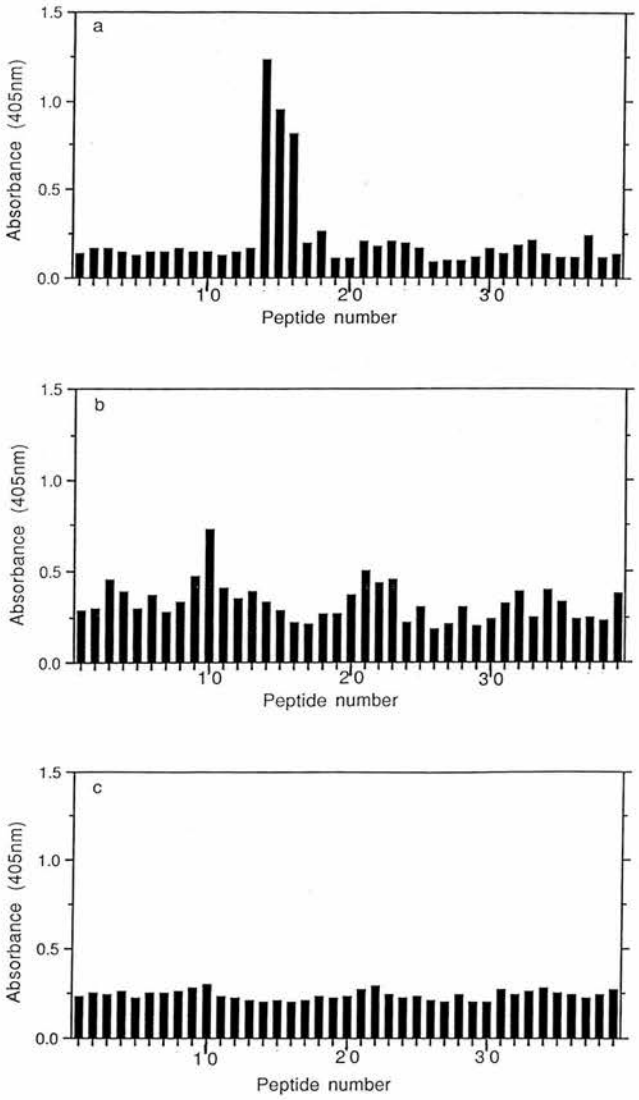

Fig. 1. Peptide mapping of (a) rabbit anti-(peptide S3a) serum (1:300 dilution), (b) fetuin (20 μg/ml) and (c) fetuin incubated with peptide (E)S3c (100 μg/ml). Fetuin recognition was detected with rabbit anti-fetuin serum (1:250) and anti-(rabbit IgG) serum conjugated to peroxidase (1:500) was used for colour production in each case.

Peptide (E)S3c recognition of glycoprotein and glycolipid

The ability of (E)S3c and PT to bind fetuin, α-1-acid glycoprotein, asialofetuin and MBG was compared in a solid-phase binding assay (Fig. 2). Both PT and the peptide demonstrated their highest apparent binding activity with fetuin as the receptor (Fig. 2a), slightly weaker binding with α-1-acid glycoprotein and ganglioside receptors (Fig. 2b and c) and their lowest apparent activity with asialofetuin (Fig. 2d). These results show that peptide (E)S3c was able to mimic some of the receptor binding characteristics of the holotoxin (Armstrong et al., 1988).

Anti-(E)S3c antibodies

Antibodies raised to an (E)S3c–bovine-serum-albumin conjugate were able to recognise both peptide and PT, when either antigen was coated directly onto the microtitre plate (Fig. 3a). This anti-peptide serum recognition of PT confirms the putative surface exposure of the receptor-recognition domain within the holotoxin (Westhof et al., 1984). The anti-peptide serum was also able to recognise fetuin-bound or ganglioside-bound PT (Fig. 3b). This result is interesting, since as found for cholera-toxin/G_{M1} interaction (Ghoshe and Karush, 1988), it might be expected that the receptor occupied domain would sterically exclude the anti-peptide serum from binding PT. However, the result may be explained by two different possibilities. Firstly, there is likely to be antigenic cross-reactivity with similar amino acids in the S2 subunit (Locht and Keith, 1986). Secondly, the S2 subunit of PT has been shown to have some glycoprotein receptors, such as fetuin, in common with S3 (Witvliet et al., 1989), therefore it is likely that some of the PT molecules will be bound to fetuin via S2, leaving S3 available for antibody binding.

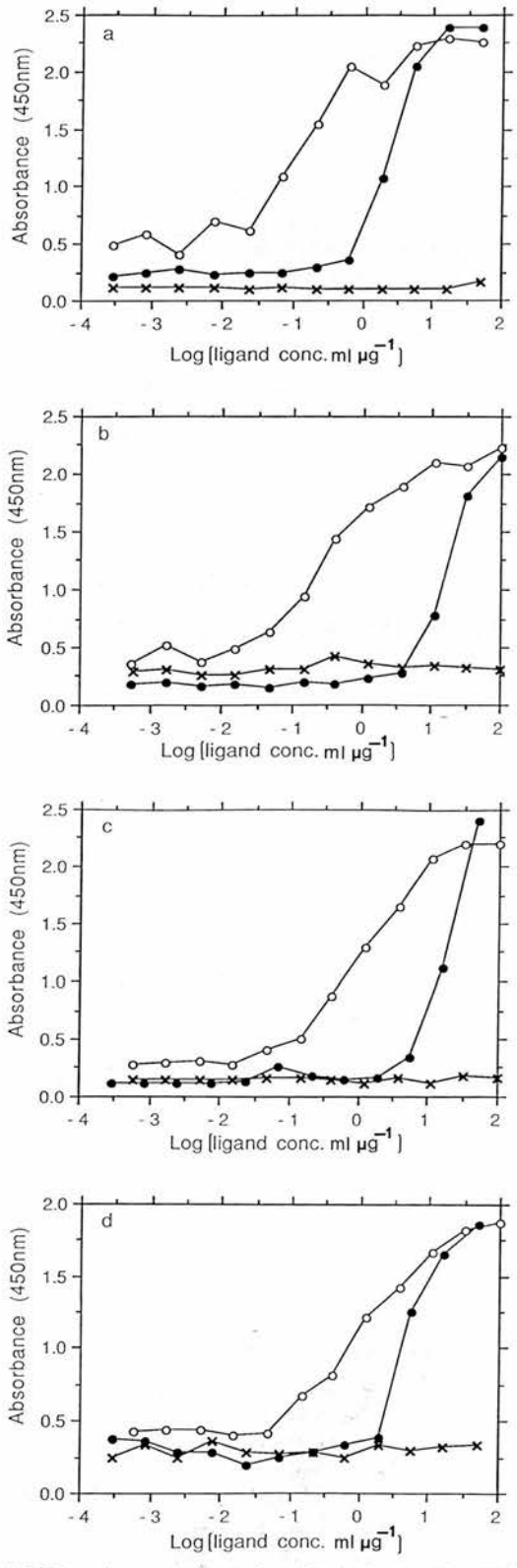

Fig. 2. ELISA of pertussis toxin (●) and peptide (E)S3c (○) recognition of (a) fetuin-coated, (b) α-1-acid glycoprotein-coated, (c) MBG-coated and (d) asialofetuin-coated wells (2 μg/ml). Various concentrations of pertussis toxin or peptide were incubated with the receptor-coated wells (2 μg/ml), followed by incubation with either anti-(pertussis toxin) mAb L10 (1:500) or anti-(E)S3c serum (1:350) and by a subsequent incubation with the corresponding species specific anti-IgG-serum conjugated to peroxidase (1:625). Background (X) was anti-(E)S3c serum recognition of receptor-coated wells (a similar background was obtained for mAb L10).

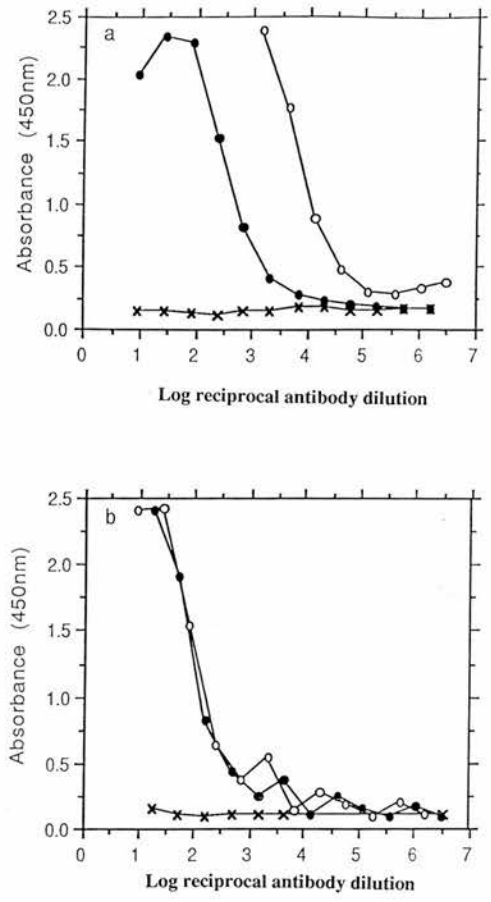

Fig. 3. ELISA of rabbit anti-(E)S3c serum recognition of (a) (●) pertussis toxin-coated and peptide-(E)S3c-coated (○) wells (2 μg/ml) and (b) of pertussis toxin (50 μg/ml) captured onto the ELISA plates by either (●) fetuin-coated or (○) MBG-coated wells (2 μg/ml). Background (X) is anti-(E)S3c serum recognition of fetuin-coated wells.

The anti-peptide serum did not neutralise the Chinese-hamster-ovary-cell-clumping activity of PT (Gellenius et al., 1985).

(E)S3c fluorescence

When excited at 280 nm, the peptide produced a fluorescence spectrum with a tryptophan emission maximum at 355 nm and a typically weak tyrosine emission at 306 nm (Lakowicz, 1983; Fig. 4a). On addition of MBG, a fluorescence-emission enhancement and a blue-shift in the tryptophan emission maximum was observed (Fig. 4a). At a saturating concentration of MBG, the tryptophan emission maximum had shifted by 15 nm to 340 nm. Both the ganglioside-induced fluorescence enhancement and the blue-shift in the emission maximum are consistent with a decrease in the solvent exposure of the peptide tryptophan (Burstein et al., 1973). Fluorescence changes were not observed for the model fluorophore $AcTrpNH_2$ or PT when either were mixed with MBG (data not shown). The enhancement of (E)S3c fluorescence intensity was titrated with gangliosides G_{M1} (one Neu residue), G_{D1a} (two Neu residues) and G_{T1b} (three Neu residues). The minimum amount of ganglioside required to achieve saturation of fluorescence enhancement was found to vary for the different gangliosides (Fig. 4b); however, assuming a 1:1 peptide/ligand complex (the precise stoichi-

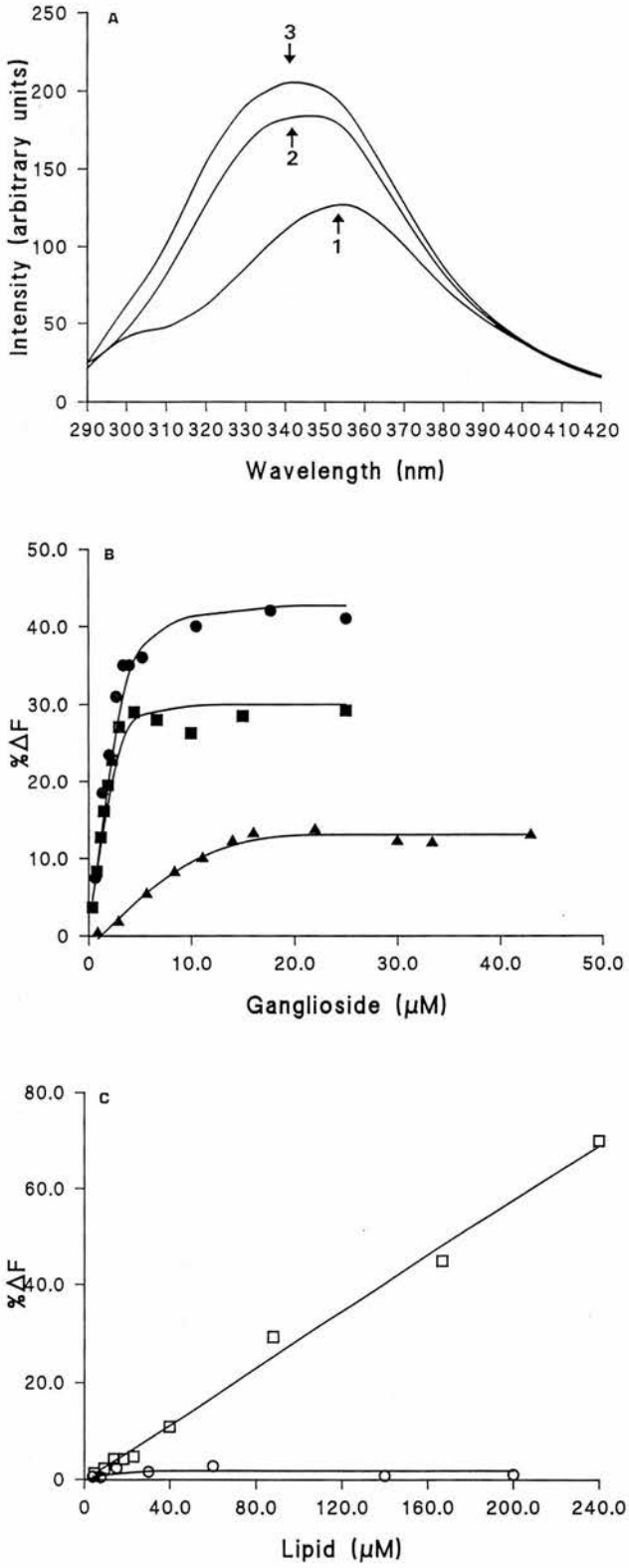

Fig. 4. Fluorescence characteristics of peptide (E)S3c. (A) Emission spectrum of 4 μM peptide (1), peptide mixed with 242 μM L-α-lysolecithin (2) and of peptide mixed with 30 μg/ml MBG (3). The excitation wavelength was 280 nm. (B) Increase in (E)S3c fluorescence intensity following the addition of either G_{M1} (▲), G_{D1a} (●) or G_{T1b} (■). (C) (E)S3c increase in fluorescence intensity resulting from the addition of L-α-lysolecithin (□) or Chaps (○). Excitation and emission slit widths were 2.5 nm and 5.0 nm, respectively. The excitation wavelength was 280 nm and the emission wavelength 355 nm for the titrations shown in (B) and (C).

ometry of binding is currently under investigation), dissociation constants of 7.2, 2.1 and 1.6 μM were obtained for G_{M1}, G_{D1a} and G_{T1b}, respectively. Both *N*-acetylneuramin-lactose (1−80 μM) and the cerebroside lactosylceramide (1−30 μM), which lacks the Neu and galactosyl(β1−3)*N*-acetylgalactosamine moieties of the ganglioside, were found not to perturb (E)S3c fluorescence when mixed with the peptide (data not shown). The findings with lactosylceramide are similar to those of Saukkonen et al. (1992), who found by TLC that this glycolipid was only recognised by the S2 subunit and not the S3 subunit of PT.

The phospholipid L-α-lysolecithin was also found to shift the (E)S3c emission maximum (Fig. 4a) and to enhance the fluorescence intensity, which had not reached saturation after the addition of 242 μM phospholipid (Fig. 4c). L-α-Lysolecithin and detergents such as Chaps have been shown to enhance the enzyme activity of PT (Moss et al., 1986). Chaps, however, did not enhance the (E)S3c emission intensity (Fig. 4c), which demonstrates that the fluorescence changes which were induced by either glycophospholipids or phospholipids are not the result of dielectric changes to the solvent.

The addition of 1.75 μM fetuin to a 4 μM solution of (E)S3c produced an 8.5% decrease in the initial (E)S3c fluorescence emission intensity (after correction for fetuin absorbance and fluorescence). Lower concentrations of fetuin also demonstrated (E)S3c fluorescence quenching (data not shown), but the results were not sufficiently reproducible for titration-curve construction.

DISCUSSION

A region of the S3 subunit of PT (amino acids 46−55, RQITPGWSIY) which binds the glycoprotein fetuin, was localised by the pin-peptide mapping procedure (Geysen et al., 1987). Previously, this technique has primarily been applied to the identification of peptides which represent subsites of anti-protein serum epitopes (Getzoff et al., 1987). The localised glycoprotein-binding region of S3 is closely analogous to the recently defined region II of a carbohydrate-recognition domain proposed for the S3 subunit (Saukkonen et al., 1992). The possibility of multiple regions of S3 contributing to receptor binding cannot be excluded on the basis of our peptide-mapping results. Indeed, during the course of our work, it has been shown that a peptide corresponding to S3 amino acids 28−45 inhibits bacterial cells from binding to macrophages (van't Wout et al., 1992).

In addition to sialylated glycoproteins, it has recently been demonstrated that sialylated glycolipids (gangliosides) can recognise the S3 subunit of PT (Saukkonen et al., 1992). In this paper, a free peptide, (E)S3c, containing S3 amino acids 44−58 (YLRQITPGWSIYGLY, preceded by an N-terminal glutamic acid and succeeded by an amide group), was able to bind MBG in solid-phase ELISA. Gangliosides were also found to enhance the intrinsic fluorescence emission intensity of peptide (E)S3c and blue-shifted the emission maximum. These changes are consistent with a decrease in the solvent exposure of the tryptophan residue. This decrease in solvent exposure may be due to the tryptophan interacting with the oligosaccharide moiety of the ganglioside, as proposed for G_{M1}-induced fluorescence changes in cholera toxin (Fishman et al., 1978). However, considering that phospholipids such as L-α-lysolecithin can induce (E)S3c fluorescence changes similar to those induced by ganglioside, and that *N*-acetylneuramin-lactose (an analogue of the oligosaccharide

moiety of both fetuin and ganglioside) does not perturb (E)S3c fluorescence, an alternative proposal is that the tryptophan is interacting with the lipid component of the ganglioside. This has been shown to be the case for the binding of glucagon to phospholipid vesicles, which also results in a blue-shift of the glucagon fluorescence emission maximum and an enhancement of the emission intensity (Schneider and Edelhoch, 1972; Kimura et al., 1992). Indeed, it has recently been proposed that tryptophan side chains can bury into the hydrophobic interior of a lipid bilayer, whilst the same amino acid maintains a hydrogen bond with the lipid polar head group (Schiffer et al., 1992).

The S3 amino acid sequence represented by the free peptide does not have any strong sequence similarity with other carbohydrate-binding peptides (Ruoslahti and Pierschbacher, 1986), except for the Pro-Gly-Trp sequence, which occurs in an integrin-binding peptide derived from type-IV collagen (Chelberg et al., 1990). Interestingly, both PT and type-IV collagen have been found to recognise a common glycoprotein, laminin (Mayo et al., 1991; Witvliet et al., 1989). The conformation in solution for the collagen-derived peptide, and for peptides containing the fibronectin recognition sequence Arg-Gly-Asp, appears to consist of consecutive or nested β-turn structures (Mayo et al., 1991; Reed et al., 1988). For peptide (E)S3c, the ability of antibodies raised against the peptide to also recognise the holotoxin, is consistent with a putative β-turn conformation (Krchnak et al., 1989; Westhof et al., 1984). However, clearly the elucidation of the peptide's secondary structure requires a detailed study by NMR which is currently in progress.

The ability of the S3-derived peptide, (E)S3c, to bind glycoprotein, glycolipid and phospholipid, suggests that the analogous region of the S3 subunit maybe involved in both holotoxin binding to target-cell receptors and also the subsequent penetration, by the toxin, of the cell-membrane phospholipid bilayer. Consequently, it was anticipated that anti-(E)S3c serum would inhibit the interaction of PT with target cells, such as Chinese-hamster-ovary cells. However, there was no attenuation by these antibodies of PT-induced Chinese-hamster-ovary-cell clumping. This finding is similar to results previously obtained with cholera toxin, where antibodies raised to a peptide (CTP-6), which incorporates the B-subunit tryptophan involved in G_{M1} binding, failed to inhibit cholera-toxin activity (Jacob et al., 1984). Thus, it is unlikely that a peptide analogue of the S3-subunit receptor-binding domain, will be able to induce protective antibodies and replace detoxified PT as a whooping-cough-vaccine component.

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REFERENCES

Armstrong, G. D., Howard, L. A. & Peppler, M. S. (1988) *J. Biol. Chem. 263*, 8677–8684.

Burstein, E. A., Vedenkina, N. S. & Ivkova, N. N. (1973) *Photochem. Photobiol. 18*, 263–279.

Capiau, C., Petre, J., van Damme, J., Pupe, M. & Vandekerkove, J. (1986) *FEBS Lett. 204*, 336–340.

Chelberg, M. K., McCarthy, J. B., Skubitz, A. P. N., Furcht, L. T. & Tsilibary, E. C. (1990) *J. Cell Biol. 111*, 261–270.

Fishman, P. H., Moss, J. & Osborne, J. C. (1978) *Biochemistry 17*, 711–716.

Gellenius, P., Jaatmaa, E., Askeloff, P., Granstrom, M. & Tiru, M. (1985) *J. Biol. Stand. 13*, 61–66.

Getzoff, G. D., Geysen, H. M., Rodda, S. J., Alexander, H., Tainer, J. A. & Lerner, R. A. (1981) *Science 235*, 1191–1196.

Geysen, H. M., Rodda, S. J., Mason, T. J., Tribbich, G. & Schoofs, P. G. (1987) *J. Immunol. 102*, 259–274.

Ghoshe, A. C. & Karush, F. (1988) *Mol. Immunol. 28*, 865–876.

Irons, L. I. & Gorringe, A. R. (1988) in *Pathogenesis and immunity in pertussis* (Wardlaw, A. C. & Parton, R., eds) pp. 95–120, Wiley, New York.

Irons, L. I. & MacLennan, A. P. (1979) *Biochim. Biophys. Acta 580*, 175–185.

Jacob, C. O., Sela, M., Pines, M., Hurwitz, S. & Arnon, R. (1984) *Proc. Natl Acad. Sci. USA 81*, 7893–7896.

Kazemi, M. & Finkelstein, R. A. (1991) *Mol. Immunol. 28*, 865–876.

Kimura, S., Erne, D. & Schwyzer, R. (1992) *Int. J. Pept. Protein Res. 39*, 431–442.

Krchnak, V., Mach, O. & Maly, A. (1989) *Methods Enzymol. 178*, 586–811.

Lakowicz, J. R. (1983) *Principles of fluorescence spectroscopy*, Plenum Press.

Locht, C. & Keith, J. M. (1986) *Science 232*, 1258–1264.

Mayo, K. H., Parra-diaz, D, McCarthy, J. B. & Chelberg, M. (1991) *Biochemistry 30*, 8251–8267.

Moss, J., Stanley, S. J., Watkins, P. A. Burns, D. L., Manclark, C. R., Kaslow, H. R. & Hewlett, E. L. (1986) *Biochemistry 25*, 2720–2725.

Nencioni, L., Pizza, M., Volpini, G., DeMagistris, M. T., Giovannoni, F. & Rappuoli, R. (1991) *Infect. Immun. 59*, 4732–4734.

Pierschbacher, M. D. & Ruoslahti, E. (1984) *Nature 309*, 30–33.

Reed, J., Hull, W. E., von der Leith, C.-W., Kubler, D., Suhai, S. & Kinzel, V. (1988) *Eur. J. Biochem. 178*, 141–154.

Relman, D., Tuomanen, E., Falkow, S., Golenbock, D. T., Saukkonen, K., Wright, S. D. & Kinzel, V. (1990) *Cell 61*, 1375–1382.

Robinson, A., Irons, L. I. & Ashworth, L. E. A. (1985) *Vaccine 3*, 11–22.

Ruoslahti, E. & Pierschbacher, M. D. (1986) *Cell 44*, 517–518.

Saukkonen, K., Burnette, W. N., Mar, V. L., Masure, H. R. & Tuomanen, E. I. (1992) *Proc. Natl Acad. Sci. USA 89*, 118–122.

Schiffer, M., Chang, H.-C. & Stevens, F. J. (1992) *Protein Eng. 5*, 213–214.

Schmidt, M. A., Raupach, B., Szulczynski, M. & Marziller, J. (1991) *Infect. Immun. 59*, 1402–1408.

Schneider, A. B. & Edelhoch, H. (1972) *J. Biol. Chem. 247*, 4986–4991.

Seabrook, R. N., Robinson, A., Sharma, R. P., Irons, L. I., Ashworth, L. E. A. Price, C. P. & Atkinson, T. (1990) *Mol. Immunol. 27*, 777–785.

Seabrook, R. N., Atkinson, T. & Irons, L. I. (1991) *Eur. J. Biochem. 198*, 741–747.

Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K. Ui, M. & Ishii, S. (1982) *Biochemistry 21*, 5516–5522.

Ui, M. (1988) in *Pathogenesis and immunity in pertussis* (Wardlaw, A. C. & Parton, R., eds) pp. 121–146, Wiley, New York.

Van't Wout, J., Burnette, W. N., Mar, V. L., Rozdinski, E., Wright, S. D. & Tuomanen, E. I. (1992) *Infect. Immun. 60*, 3303–3308.

Weiss, A. & Hewlett, E. L. (1986) *Annu. Rev. Microbiol. 40*, 661–686.

Westhof, E., Altschul, D., Moras, D., Bloomer, A. C., Mondragon, A., Klug, A. & van Reggenmortel, M. H. V. (1984) *Nature 311*, 123–126.

Witvliet, M. H., Burns, D. L. Brennan, M. J., Poolman, J. T. & Manclark, C. R. (1989) *Infect. Immun. 57*, 3324–3330.