Interaction of *Bordetella pertussis* adenylate cyclase toxin with target cells

by

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PUBLICATIONS AND PRESENTATIONS

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ABBREVIATIONS

AC	Adenylate cyclase toxin
ACT	Adenylate cyclase toxin
ADP	Adenosine diphosphate
Ag	Antigen
AGG	Agglutinogen
Akt A	*A.actinomycetemcomitans* leukotoxin
App A	*A.pleu*ropneumoniae haemolysin
ATP	Adenosine triphosphate
BG	Bordet Gengou medium
BHK	Baby hamster kidney cell line
BL3	Bovine lymphoma cell line
BP	*Bordetella pertussis*
Brk A	*Bordetella* resistance to killing factor
C-	Carboxy terminal
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CD	Cyclodextrin liquid medium
CHO	Chinese hamster ovary
cm	Centimetre(s)
cpm	Counts per minute
Cya A	Adenylate cyclase toxin
DAB	Diaminobenzidine
DCP	Dicetyl phosphate
DMSO	Dimethyl sulphoxide
DMPC	Dimyristoyl phosphatidyl choline
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
DSPC	Distearoyl phosphatidyl choline
EDTA	Ethylenediamine tetra-acetic acid
EGTA	Ethyleneglycol-bis N, N-tetra acetic acid
ELISA	Enzyme linked immunosorbant asssay
FHA	Filamentous haemagglutinin
g	Gram(s)
g	Gravity

GD_{1a}	Disialoganglioside-1a
GD_{1b}	Disialoganglioside-1b
GM_1	Monosialoganglioside
GT_{1b}	Trisialoganglioside-1b
GTP	Guanosine triphosphate
h	Hour(s)
HCl	Hydrochloric acid
Hepes	N-2-hydroxyethylpiperazine-N-2-ethanosulphonic acid
HH	Hanks HEPES
HLY	Haemolysin
Hly A	*E.coli* haemolysin
HRP	Horse radish peroxidase
HS	Hanks saline
IBMX	3-isobutyl-1-methylxanthine
IgG	Immunoglobulin G
IPTG	Isopropyl-β-thialogalactopyranoside
J774.2	Murine macrophage cell line
kDa	Kilodalton(s)
L	Litre(s)
Lkt A	*P.haemolytica* leukotoxin
LOS	Lipo-oligosaccharide
LPS	Lipopolysaccharide
M	Molar
mA	Milliamp(s)
MeCD	2, 6-O-Dimethyl β-cyclodextrin
mg	Milligram(s)
min	Minute(s)
ml	Millilitre(s)
mM	Millimolar
mm	Millimetre(s)
mV	Millivolt(s)
N-	Amino terminal
NAD	Nicotinamide adenine dinucleotide
Nana	N-acetyl neuraminic acid
nm	Nanometre(s)
°C	Degrees Celsius
OD	Optical density
OMP	Outer membrane protein(s)

P.69	Pertactin
PA	Protective antigen
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulphonyl fluoride
PRN	Pertactin
PT	Pertussis toxin
RBC	Red blood cell (s)
RGD	Arginine-glycine-aspartic acid
rpm	Revolutions per minute
RTX	Repeats in toxin
SA	Stearylamine
SAPU	Scottish antibody production unit
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide electrophoresis
sec	Second(s)
sRBC	Sheep red blood cell(s)
TBS	Tris buffered saline
TBSC	Tris buffered saline containing calcium chloride
TCF	Tracheal colonisation factor
TCT	Tracheal cytotoxin
TEMED	N, N, N, N- tetramethyl-ethylenediamine
Tn	Transposon
Tris-HCl	Tris hydrochloride
TSB	Transformation and storage buffer
V	Volt(s)
v/v	Volume/volume ratio
vol	Volume
w/v	Weight/volume ratio
YT	Yeast tryptone
"	Inch
%	Percentage
^{32}P	Phosphorus-32
69K	68 kilodalton antigen
^{125}I	Iodine-125
µCi	Microcuries
µg	Microgram(s)
µl	Microlitre(s)
µmol	Micromolar

SUMMARY

Adenylate cyclase toxin (CyaA) is one of the many virulence factors produced by *B.pertussis*, the causative organism of whooping cough. The mechanisms governing the interaction of CyaA with target cells and the role toxin-activation plays in these are unclear. This study investigated these mechanisms.

For the purposes of this study CyaA was purified from *B.pertussis* 348 (pRMB1) by a two step-process involving urea extraction of whole cells followed by calmodulin-agarose affinity chromatography. The purification of CyaA was confirmed by SDS-PAGE and Western blotting. The functional significance of toxin activation was studied by preparing activated and non-activated forms of CyaA from recombinant *E.coli* strains. Urea extraction from the recombinant strains yielded a 200kDa protein which was relatively free from contaminating host cell protein as determined by Coomassie blue staining. Enzymic assays showed that catalytically active forms of CyaA had been purified from the recombinant *E.coli* strains and from *B.pertussis* 348 (pRMB1). The activated form of CyaA possessed toxic activity which was absent from the non-activated form.

Binding studies were employed to investigate the interaction of CyaA with various cell types. It was found that activated CyaA could bind to / associate with sheep erythrocytes in a time and dose-dependent manner. Studies revealed that binding increased even at very high doses of toxin, suggesting that the binding process is non-saturatable and therefore non-specific.

Regarding the specificity of interaction of CyaA with cells it was found that pre-trypsinisation of sheep erythrocytes increased the susceptibility of the cells to the haemolytic activity of the toxin and this activity was directly dependent on the concentration of trypsin used to treat the erythrocytes. Trypsinisation of cells may have made membrane recognition sights more accessible for CyaA insertion and pore formation. The binding of CyaA to trypsinised cells was low compared to control cells

suggesting that the processes of binding and pore-formation are separable events. In this study it was found that none of the sugars tested; D-galactose, D-glucose, D-mannose, D-maltose, D-fucose or N-acetyl neuraminic acid (NANA), inhibited the binding of CyaA to sheep erythrocytes suggesting that these sugars may not play essential roles in toxin-cell interaction.

Interestingly, it was found that activated and non-activated derivatives of CyaA could both bind to / associate with sheep erythrocytes. However, binding of non-activated CyaA to cells from the murine macrophage cell line (J774.2) was much less when compared to the activated form. These results are the first evidence of differences in cell-affinity between activated and non-activated CyaA. No binding of activated or non-activated CyaA was detected with BHK or BL3 cells. It is possible that the solubilisation conditions of hypotonic lysis of these cell types may have resulted in the dissociation of bound toxin or that the assay was not sensitive enough to detect low levels of cell-bound toxin.

Sheep erythrocyte membrane proteins were investigated as potential receptors for CyaA. No binding was detected between CyaA and the membrane proteins isolated from sheep erythrocytes. To detect if CyaA interacts directly with glycolipid a thin-layer chromatogram overlay assay was developed. CyaA bound to a range of gangliosides (GM_1, GD_{1a}, GD_{1b}, and GT_{1b}) and to some but not all of the components in a sheep erythrocyte lipid extract. These results suggest that glycolipids may be possible membrane recognition sites for CyaA.

Activated CyaA extracts were found to be lytic towards sheep erythrocytes and liposomes in a time and dose dependent manner. Unlike haemolysis the lytic effect of CyaA on liposomes proceeded without a lag period. This may be because liposomes do not have the same constraints on membrane integrity that erythrocytes have and can leak contents without lysis. Toxin activation was required for haemolytic and liposome permeabilising activities. This is further evidence of the essential role activation plays in the productive interaction of CyaA with cell membranes.

The activity of CyaA was much greater towards liposomes with a net negative charge than to liposomes with no net charge or a net positive charge. Gangliosides were not essential and other negatively charged lipids could fulfil the same function. These results suggest that the interaction of CyaA with membranes may be at the level of a charge-charge interaction whereby negatively charged moieties on the membrane surface act as the initial recognition sites.

SECTION 1
INTRODUCTION

1.1 CHARACTERISTICS OF *BORDETELLA PERTUSSIS*

1.1.1 Classification of *Bordetella pertussis*

Bordetella pertussis is a member of the genus *Bordetella*. Originally 3 species were assigned to this genus; *Bordetella pertussis*, the agent responsible for human pertussis; *Bordetella parapertussis*, which causes a mild, pertussis-like disease in humans and *Bordetella bronchiseptica* , which is primarily an animal pathogen but may infect humans (Moreno-Lopez, 1952).

Recently, *Bordetella avium*, associated with respiratory illness in birds (Kersters *et al*, 1984), *Bordetella holmesii* cultured from the blood of human patients with septicaemia (Weyant *et al* , 1995) and *Bordetella hinzii* , isolated from poultry and humans (Vandamme *et al*, 1995) have also been identified as *Bordetella* spp. The Bordetellae are small Gram-negative, coccobacilli which are strictly aerobic and non-sporing. With the exception of *B.holmesii*, the Bordetellae are obligate parasites and pathogens of the upper respiratory tract. The different species can be differentiated by phenotypic characteristics such as motility, growth requirements and pathogenic tropism (reviewed by Parton, 1997). However, DNA homology studies have demonstrated that *B.pertussis* is so closely related to *B.parapertussis* and *B.bronchiseptica* that, rather than be considered as separate species, the organisms should be considered as subtypes of the same species (Kloos *et al*, 1981; Musser *et al*, 1986). The other 3 members of the genus, *B.avium*, *B.holmesii*, and *B.hinzii*, are distinct from these subspecies, and from each other, and can be considered as distinct species (Vandamme *et al* , 1995; Weyant *et al* , 1995).

1.1.2 Growth characteristics of *Bordetella pertussis*

B.pertussis was first described by Bordet and Gengou (1906) who isolated the organism on a glycerol-potato-blood medium. The organism is a non-motile, oxidase

positive, Gram-negative coccobacillus which grows optimally at 35-37°C. Originally it was thought that *B.pertussis* had complex growth requirements, requiring supplements such as blood, but it is now known that it can be grown on media containing just a few amino acids, growth factors and salts (Stainer and Scholte, 1971; reviewed by Stainer, 1988). However the organism is sensitive to growth inhibition by peptone, sulphur, peroxide, manganese, and fatty acids (Pollock, 1947 and 1949; Proom, 1955; Rowatt, 1957). A novel medium containing the cyclodextrin MeCD (2,6-O-dimethyl β-cyclodextrin) has now been developed which stimulates growth, even from small inocula of *B.pertussis* by neutralising growth inhibitors. This medium is of particular use because it promotes production of virulence factors which is of advantage in vaccine production and studies into the mechanisms of *B.pertussis* pathogenicity (Imaizumi *et al*, 1983 and 1984).

Importantly, *B.pertussis* grows in any of 4 serological phases (Phase I-IV) when cultured *in vitro*. Upon successive subculture, fresh isolates of *B.pertussis* are converted from phase I to phase IV, characterised by loss of haemolytic activity and an alteration in antigenic profile and colony morphology (Leslie and Gardner, 1931). The virulence potential of *B.pertussis* is environmentally regulated (Lacey *et al*, 1960). It is believed that the different phases displayed *in vitro* are a visual demonstration of the phase variation, antigenic modulation and serotypic variation which may occur in the clinical manifestation of pertussis (see section 1.3.3).

1.2 CLINICAL ASPECTS OF PERTUSSIS

1.2.1 Pathology of pertussis

Pertussis (whooping cough) was first described in the sixteenth century (cited by Holmes, 1940). Nowadays, pertussis is recognised as an endemic, and sometimes epidemic, upper respiratory tract disease of humans (Cherry, 1988). Even in this modern era, pertussis is a global disease which is responsible for 60 million cases and

half a million deaths annually worldwide (Muller *et al,* 1986). Mortality is highest in unimmunised infants, particularly in developing countries. The importance of pertussis as a disease has stimulated studies into its pathogenicity. *Bordetella pertussis* is the causative organism of whooping cough, although *B.parapertussis* and even *B.bronchiseptica*, albeit very rarely, may also cause the disease (Mertsola, 1985; Woolfrey and Moody, 1991).

The disease is transmitted by close contact with infected individuals, via respiratory secretions. The most infectious period is during the first week of the illness when the organism is most easily isolated (Hodder and Mortimer, 1992). There have been reports of transmission from people with a sub-clinical infection (Long *et al,* 1990) although the significance of this remains open to debate (Krantz *et al* , 1986).

Classical pertussis, the most common form of infection, generally lasts 4 to 8 weeks and presents itself as a disease of three stages, (catarrhal stage, paroxysmal stage and convalescent stage) although these stages do overlap. The most infectious stage is the catarrhal stage which lasts from 7-10 days. Patients present with a mild, non-specific cough, rarely accompanied by fever, which increases in severity with time. The disease then progresses into the paroxysmal stage, punctuated by bouts of paroxysmal coughing which are terminated by the distinctive 'whoop' resulting from a vigorous inspiration of air (Cherry, 1988). This paroxysmal stage usually lasts for several weeks, but may last for a number of months with the paroxysms becoming less frequent and less severe, as patients convalesce (Cherry, 1988; Walker, 1988).

Morbidity and mortality are caused by complications, usually arising during the paroxysmal stage of pertussis. Respiratory, cerebral and haemorrhagic *sequelae* are the major causes of long-term illness and loss of life (Hodder and Mortimer, 1992). Secondary infections may also occur as a result of damage to the airways (Swansea Research Unit, 1985). Complications are manifested most commonly, and most severely in young infants and in particular babies < 1 year old (Farizo *et al,* 1992).

1.2.2 Diagnosis and treatment of pertussis.

Pertussis is usually diagnosed on the clinical basis of a persistent cough for 14 days (Patriarca *et al* , 1988), accompanied by signs of lymphocyte proliferation, and isolation of *B.pertussis* from nasophyrangeal swabs (Onorato and Wassilak, 1987). However positive culture is only achieved in about 80% of cases, which diminishes if the disease has progressed, patients have received antibiotics or had previous immunisation (Onorato and Wassilak, 1987).Other diagnostic tests such as fluorescent-labelled antibodies, DNA probes, and ELISA techniques have been investigated but so-far these give variable results (Viljanen *et al*, 1982; Onorato and Wassilak, 1987).

Erythromycin is the drug of choice for treatment of pertussis, particularly if given early in infection (Bergquist *et al*, 1985). Ampicillin, chloramphenicol, and tetracycline, have also been investigated as antibacterials, but they are not as effective as erythromycin (reviewed by Hodder, 1992). However, antibiotic treatment is usually given too late and after patients are already paroxysmal. Treatment will therefore not prevent the harmful *sequelae* which sometimes occur at this stage although it is of importance in preventing secondary infection. Also by this stage the toxins and factors eliciting damage to the immune system will have already been released by *B.pertussis* (see section 1.3). Mortality and morbidity could be alleviated if earlier diagnosis was achieved thereby facilitating the use of antibiotics when they were most effective. However, the best solution is prevention by vaccination (see below).

1.2.3 Vaccination against pertussis

1.2.3.1 Conventional whole-cell vaccine

The discovery of *Bordetella pertussis* as the causitive organism of pertussis (Bordet and Gengou, 1906), encouraged the developement of whole cell vaccines against the disease (reviewed by Cherry, 1996). Mass vaccination was introduced in the United States in the 1950's and since then, vaccination programmes have been

introduced world wide. Vaccination in the United States has reduced the incidence of pertussis considerably (**Figure 1.1**). The efficacy of whole-cell vaccine preparations vary from country to country but they are all produced empirically by killing and partially detoxifying concentrated *B.pertussis* cultures by heat, chemical means or both (Cherry, 1988).

Despite the obvious efficacy of vaccination in controlling pertussis uptake rates of the vaccine have fallen in recent years and since 1980 there has been a resurgence in both incidence and mortality due to the disease (**Figure 1.1**). The drop in vaccination levels have been fueled by concern over the safety of the currently available whole cell vaccines (Cherry, 1996). Safety fears have been raised because of local transient reactions and reports of long-term neurological damage and even death linked to vaccination (reviewed by Ross, 1988). Short term reactogenicity takes the form of local inflammation, fever and and unexplained crying which, although distressing, is not life threatening. However, reports of vaccine-related seizure and encephalopathy are of concern (Alderslade *et al* , 1981; Fenichel, 1982). There is controversy as to whether the neurological side-effects are real or imaginary. Recent studies have concluded that 'pertussis vaccine encephalopathy is a myth' (Shields *et al*, 1986; Griffin *et al*, 1990; Gale *et al* , 1994).

1.2.3.2 Acellular pertussis vaccines

New pertussis vaccines have now been developed which do not have the short or long term complications associated with the whole cell vaccine. This should lead to improvements in the vaccine uptake rate within the population, with a corresponding reduction in the incidence of disease. Research has centered on acellular, component vaccines (Rappuoli, 1996).

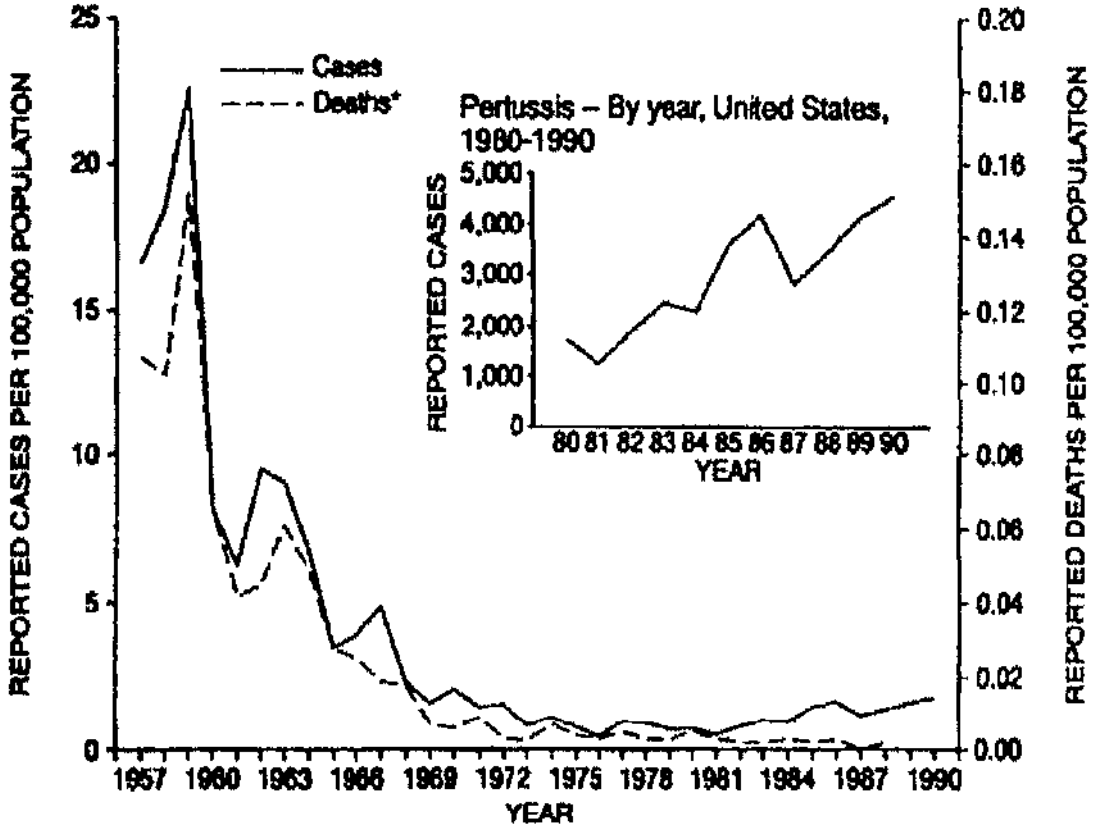


Figure 1.1 *Incidence of whooping cough in the United States, by year, 1957-1990*

Source, Hodder and Mortimer (1992)

The most studied candidate antigens for acellular vaccines are pertussis toxin (PT) and filamentous haemagglutinin (FHA), thought to be involved in tissue intoxication and bacterial adhesion respectively (see below). The first acellular vaccine was developed in Japan (Sato *et al*, 1984) and a number of vaccines, using either chemically of genetically detoxified components, are now at different stages of development (**Table 1.1**). The search for an acellular vaccine has added impetus to the study of the virulence factors of *B.pertussis* and the role of these factors in the pathogenesis of whooping cough.

1.3 PATHOGENICITY OF *BORDETELLA PERTUSSIS*

Bordetella pertussis produces an array of virulence factors which are thought to have a variety of roles in the pathogenesis of whooping cough (Weiss and Hewlett, 1986). Different factors are thought to be involved in adherence of the bacteria to the respiratory epithelium, avoidance of the host immune system, and tissue damage.

1.3.1 Adhesins of *Bordetella pertussis*

1.3.1.1 Filamentous haemagglutinin

Filamentous haemagglutinin (FHA) is a 220kDa protein loosely associated with the cell surface. FHA is the major adhesin of *B.pertussis,* and mediates attachment of the organism to epithelial cells *in vitro* and in animal models and also to macrophages (reviewed by Mooi, 1994). Unusually, FHA has at least three separate binding activities which have specificity for CR3 integrins of macrophages, ciliated cells and epithelial cells or the extracellular matrix (Locht *et al*, 1993). The binding specificities of FHA may have different roles at the different stages of pertussis.

Studies with mutants deficient in FHA demonstrated that FHA is required for optimal adherence of *B.pertussis* to ciliated respiratory cells in culture. Furthermore

Table 1.1 ***Development of acellular pertussis vaccines*** **(Source; Rappuoli, 1996)**

Vaccine components	Detoxification Method	Clinical Trials	Efficacy
PT	Hydrogen peroxide	Phase I, II, III	71%
	Tetranitromethane	Phase I, II	ND
	Formaldehyde	Phase I, II, III	54%
	Genetic	Phase I, II	ND
PT + FHA	Formaldehyde	Phase I, II, III	69%
	Formaldehyde	Phase I, II, III	58%
	Glutaraldehyde	Phase I, II, III	NA
PT + FHA + 69K	Formaldehyde	Phase I, II, III	63.9%
	Genetic	Phase I, II, III	84.2%
PT + FHA + 69K+ AGG	Formaldehyde	Phase I, II, III	85%

PT, pertussis toxin; FHA, filamentous haemagglutinin; 69K, 69 kDa antigen; AGG, agglutinogens
ND, not determined; NA, not available

when FHA was added exogenously to the FHA$^-$ mutant strains, adherence was restored (Tuomanen and Weiss, 1985). Strains carrying mutations in FHA are able to colonise the lungs but cannot persist in the upper respiratory tract of mice (Kimura *et al*, 1990; Mooi *et al*, 1992). These results suggest that FHA may be important in the initial stages of adherence but may be of less importance in colonisation of the lungs.

1.3.1.2 Fimbriae

Bordetella pertussis produces 2 types of proteinaceous fimbriae; agglutinogen 2 and 3. *B.pertussis* is unusual in that a particular strain may produce either type of fimbriae, both types or none (reviewed by Tuomanen, 1988). Fimbriae are of importance in mediating attachment of *B.pertussis* to various cell types *in vitro*, but their role in pathogenesis remains unclear (Mooi, 1994). Mutants lacking fimbriae retain their ability to colonise the nasopharynx and lung but are less able to colonise the trachea when compared to wild-type *B.pertussis* in the mouse model of infection (Mooi, 1992). However, non-fimbriated *B.pertussis* can adhere to a number of cell types including ciliated cells, suggesting fimbriae may not be essential for adherence. There is also some evidence that fimbriae are involved in promoting adhesion to and subsequent invasion of macrophages by *B.pertussis* (Mooi, 1994).

1.3.1.3 Processed outer-membrane proteins

Bordetella pertussis possesses at least three outer membrane proteins synthesised as large precursor polypeptides which are proteolytically cleaved at both the C- and N-termini before insertion into the outer cell membrane. The mature proteins travel anomalously on denaturing gels and all contain RGD motifs which are thought to facilitate binding to target cells (Charles *et al*, 1994; Fernandez and Weiss, 1994; Finn and Stevens, 1995).

Pertactin (P.69) is the protype of the family and has an apparent molecular weight of 69kDa as determined by SDS-PAGE (Montarez *et al*, 1985). Pertactin is important to *B.pertussis* for its attachment to and invasion of human cells (Leininger *et al*, 1991; Roberts *et al*, 1991). For this reason pertactin is being considered for inclusion in acellular pertussis vaccines (Rappuoli, 1996).

Recently, tracheal colonisation factor (TCF) and BrkA have been identified in *B.pertussis* (Fernandez and Weiss, 1994; Finn and Stevens, 1995). TCF is synthesized as a 68kDa protein and after proteolytic cleavage is retained in the membrane. A mutant of *B.pertussis* lacking TCF had significantly reduced ability to colonise the trachea when compared to the wild type strain but was still able to survive deeper in the lungs (Finn and Stevens, 1995).

Also produced by *B.pertussis* is BrkA. This protein shares sequence homology with pertactin and TCF (Fernandez and Weiss, 1994). *B.pertussis* mutants deficient in BrkA were less virulent, less adherent and more sensitive to killing by human serum (Fernandez and Weiss, 1994). These results suggest that BrkA may be an important virulence factor of *B.pertussis* with roles in both colonisation and resistance to host immunity.

1.3.2 Toxins produced by *Bordetella pertussis*

1.3.2.1 Tracheal cytotoxin

It is believed that the characteristic pathology of whooping cough: destruction of the ciliated respiratory epithelium, with corresponding effects on muco-cilliary function may lead to the generation of the paroxysmal cough associated with the disease (Olson, 1975). Studies on hamster tracheal rings demonstrated that a fraction isolated from the supernatant of *B.pertussis* cultures can mimic this pathology (Goldman *et al*, 1982). The so-called tracheal cytotoxin (TCT) has been subsequently purified and consists of a disaccharide tetra-peptide subunit of peptidoglycan (Cookson *et al*, 1989). TCT causes

ciliated cell-specific respiratory tract pathology *in vitro* that is indistinguishable from the pathology seen in infection. The toxin also inhibits DNA synthesis of respiratory epithelial cells (Cookson *et al*, 1989). TCT may contribute directly to the pathology of pertussis by damaging the respiratory epithelium thereby allowing *B.pertussis* to multiply without mucociliary clearance.

1.3.2.2 Dermonecrotic toxin

Dermonecrotic toxin (DNT) is a heat-labile 140 kDa polypeptide localised in the cytoplasm of *B.pertussis* and only released by disruption of cells (Cowell *et al*, 1979; Zhang and Sekura, 1991). The protein is characterised by its ability to cause dermonecrotic lesions when injected into various animals (Livey and Wardlaw, 1984; Parton, 1985). The role of DNT in the pathogenesis of whooping cough is uncertain since mutants lacking DNT retain their virulence in the infant mouse model of infection (Weiss and Goodwin, 1989). However, a related dermonecrotic toxin from *Bordetella parapertussis* has been reported to cause vasoconstriction in cell lines and organ strips and it has been suggested that DNT may play a role in the early stages of whooping cough by vasoconstriction of peripheral arterioles leading to localised inflammation and general respiratory degeneration (Endoh *et al* , 1988; Nakase and Endoh, 1988).

1.3.2.3 Lipo-oligosaccharide

Two types of lipo-oligosaccharide (LOS) have been described in *B.pertussis*, each consisting of a lipid A and oligosaccharide core, but no extensive O-antigen chain (reviewed by Chaby and Caroff, 1988). As well as possessing typical endotoxic activities such as general toxicity, pyrogenicity and adjuvanticity, the LOS of *B.pertussis* can also induce B-cell mitogenicity and polyclonal B-cell activation (Girard *et al* , 1981; Haeffner-Cavaillon *et al* , 1982) and also possesses anti-viral and anti-bacterial activities (Ayme, 1980).

1.3.2.4 Pertussis toxin

Pertussis toxin (PT) is the most extensively studied virulence factor of *B.pertussis*. PT is a 105 kDa hexameric protein composed an enzymically active A-subunit (PTA), and a B-oligomer (PTB), made up of 5 subunits (S2, S3, S4 and S5 in a 1:1:2:1 ratio), which is responsible for binding of the toxin to mammalian cells (Tamura, 1982). PT binds to carbohydrate moieties on target-cell surface glycosphingolipids or glycoproteins via the B-oligomer. This facilitates entry of the enzymic subunit into the cell (Hausman and Burns, 1992; Tyrell *et al*, 1989). Once inside the cell the toxin exerts its effects by catalysing the transfer of an ADP-ribose moiety of NAD to a family of GTP-binding regulatory proteins. The altered G-proteins are unable to function normally in signal transduction. It is generally believed that the numerous biological activities described for pertussis toxin result from its effects on cellular signal transduction (Krueger and Barbieri, 1995; Kaslow and Burns, 1992).

Despite the multitude of effects PT has on mammalian cells the role of PT in pertussis remains unclear. PT may exert its effect by inhibiting the cells of the immune sytstem. PT may also be an important adhesin for *B.pertussis*. Mutants lacking PT do not adhere to human-ciliated cells. Exogenous addition of PT to such mutants restores adherence (Tuomanen, 1988). Despite the debate about the specific role of PT in pertussis , its role as a virulence factor is not in doubt (Weiss and Hewlett, 1986).

1.3.2.5 Adenylate cyclase toxin

The toxin can penetrate mammalian cells where it is stimulated by host cell calmodulin to induce supraphysiological levels of intracellular cyclic adenosine monophosphate (cAMP) (Confer *et al*, 1984). The toxin can also lyse erythrocytes by colloid osmotic lysis (Ehrmann *et al*, 1991). Adenylate cyclase toxin (CyaA) is post-translationally activated and secreted by novel mechanisms and is a member of RTX

family of toxins (Welch *et al*, 1991) produced by other Gram-negative bacteria (see section 1.4.).

Demonstration of CyaA as an important virulence factor has been facilitated by the use of transposon (Tn5) mutants of *B. pertussis*. These mutants lacking adenylate cyclase as well as haemolytic activity are avirulent in an infant mouse model (Weiss *et al* , 1983). In addition to implicating CyaA in pathogenesis, this was the first evidence that the adenylate cyclase and haemolysin were products of the same locus, later confirmed by Glaser and co-workers (Glaser *et al* , 1988b). Recently it has been shown that CyaA is required for lethal infection of mice and *cya* A mutants are rapidly cleared from the lungs of intra-nasally challenged neonatal mice (Weiss and Goodwin, 1989; Goodwin and Weiss, 1990). Furthermore, both invasive and adenylate cyclase enzymic activities are required for colonisation of the mouse respiratory tract (Khelef *et al*, 1992). These results stress the importance of adenylate cyclase toxin as a colonisation factor. The toxic activity of CyaA impairs the functions of a number of immune effector cells, and it is believed that CyaA may be involved in immune evasion during pertussis infection (Confer and Eaton, 1982).

1.3.3 Regulation of virulence by *Bordetella pertussis*

The expression of the virulence determinants of *B.pertussis* is subject to phase variation and antigenic modulation (Robinson *et al*, 1986). At a frequency of 10^{-3}, *B.pertussis* can spontaneously and permanently change its phenotype between at least 4 phases (Leslie and Gardner, 1931; Weiss and Falkow, 1984)). The phase change is seemingly sequential and is characterised by the loss of virulence and virulence determinants such CyaA, PT, FHA and outer membrane proteins (Weiss and Falkow, 1984).

B.pertussis is also subject to antigenic modulation in response to environmental conditions (Lacey *et al*, 1960). Changes in certain environmental stimuli such as

temperature and the concentrations of various salts and acids can, reversibly, convert organisms from virulent to avirulent modes with a concomitant loss of virulence factors (Coote and Brownlie, 1988).

Phase variation and antigenic modulation in *B.pertussis* are underpinned by regulatory control mechanisms. The isolation of mutants of *B.pertussis* which were deficient in production of all virulence factors suggested that expression of these factors was under the control of a single genetic locus (Weiss *et al*, 1983). Subsequently, it has been found that virulence gene expression of *B.pertussis* is controlled by the products of the *bvg* A and *bvg* S loci. In this two-component regulatory system Bvg S acts as a sensor to detect changes in the external environment such as an increase in temperature and phosphorylates Bvg A. Phosphorylated Bvg A promotes expression of virulence activated genes (*vag*) such as CyaA, PT and FHA, and represses the expression of virulence repressed genes (*vrg*) as reviewed by Stibitz and Miller (1994).

Regulation allows *B.pertussis* to detect changes in the external environment and adapt by changing its phenotype. Differential production of virulence factors such as adhesins may be important in facilitating adherence of *B.pertussis* to different cell types during the different stages of pertussis.

1.3.4 Mechanisms of pathogenicity in pertussis

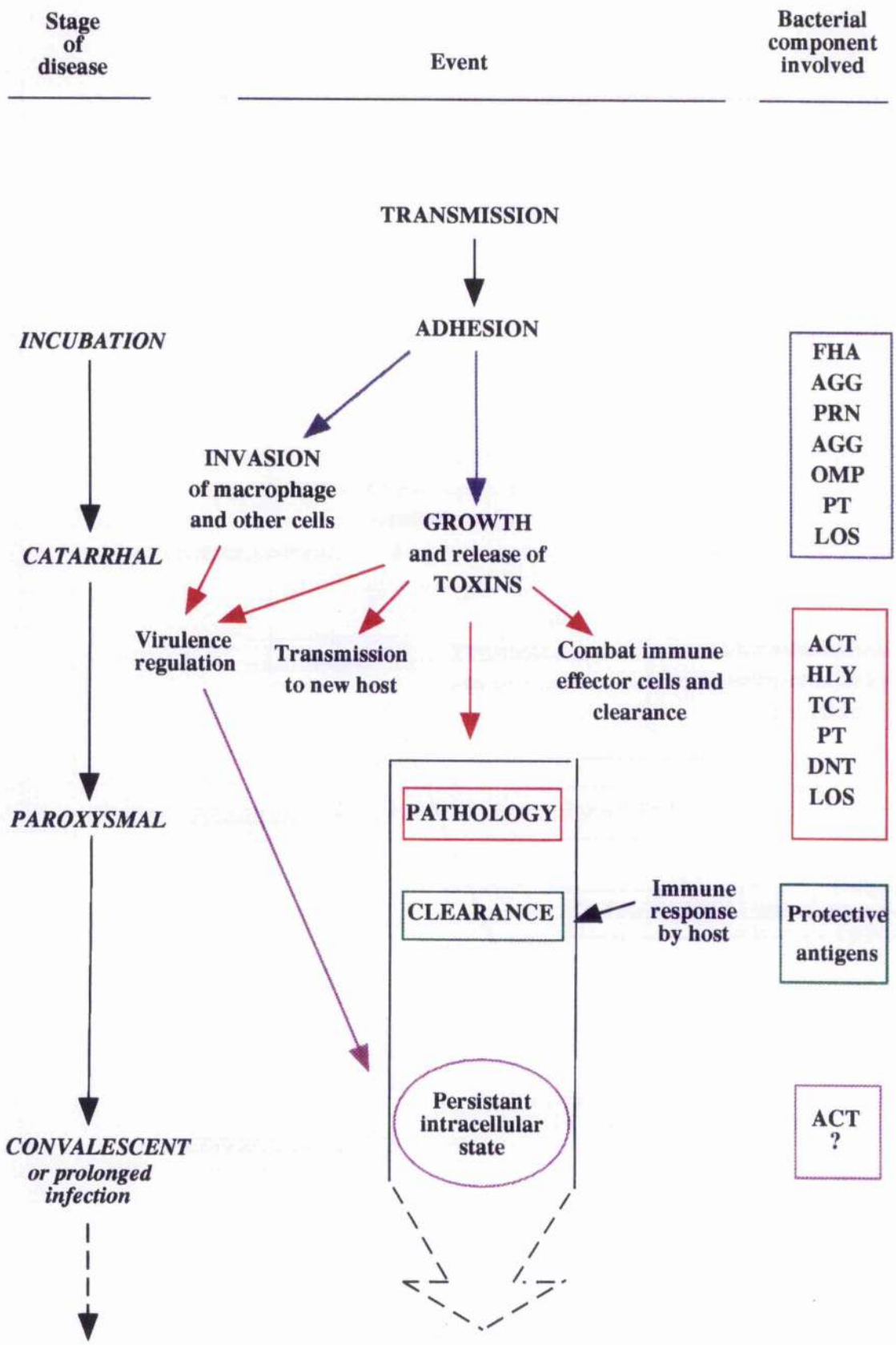
Considerable research has been carried out into the individual virulence factors of *B.pertussis* with respect to their chemical nature, genetics, immunogenicity and in particular their biological activities on cells and in animal models (reviewed by Wardlaw and Parton, 1988). However, the relevance of these activities *in vivo* and the mechanisms by which virulence determinants co-operate with each other are unknown.

From the evidence available a model (**Figure 1.2**) has been proposed for the involvement of the virulence factors in the different stages of pertussis (Wardlaw and Parton, 1988). When the bacteria infect their host they attach to the ciliated respiratory

Figure 1.2 ***Model for the sequence of events in pertussis*** [a]

The bacterial components involved are FHA, filamentous haemagglutinin; Agg, Fimbrial agglutinogens; PRN, pertactin; OMP, processed outer membrane proteins; PT, pertussis toxin; LOS, lipooligosaccharide; ACT, invasive adenylate cycalse activity of adenylate cyclase toxin; HLY, haemolytic activity of adenylate cyclase toxin; TCT, tracheal cytotoxin; DNT, dermonecrotic toxin.

[a] (Adapted from Parton, 1997)


Stage of disease
Event
Bacterial component involved
TRANSMISSION
ADHESION
INCUBATION
FHA
AGG
PRN
AGG
OMP
PT
LOS
INVASION of macrophage and other cells
GROWTH and release of TOXINS
CATARRHAL
Virulence regulation
Transmission to new host
Combat immune effector cells and clearance
ACT
HLY
TCT
PT
DNT
LOS
PAROXYSMAL
PATHOLOGY
CLEARANCE
Immune response by host
Protective antigens
Persistant intracellular state
ACT ?
CONVALESCENT or prolonged infection

epithelium of the airways and lungs via a co-operative mechanism between their adhesins (FHA, fimbriae, PT and outer membrane proteins). It is thought that the adherent bacteria liberate their toxic factors (ACT, TCT, PT, DNT, LOS) to facilitate persistence. The toxins probably act together to cause localised tissue damage to the ciliated epithelium and to inhibit immune effector cells therebye preventing clearance by the action of the mucocilliary escalator and by cell-mediated immunity respectively (**Figure 1.2**). *B.pertussis* may also establish an internal reservoir for infection as shown by its ability to survive intracellularly in various cell types (Ewanowich *et al,* 1989; Lee *et al,* 1990). Recently it has been found that other *Bordetella* spp can survive and grow outside their hosts in low nutrient environments (Porter and Wardlaw, 1993). This may reflect mechanisms by which bordetellae in general maintain an external reservoir for infection.

1.4 ADENYLATE CYCLASE TOXIN OF *BORDETELLA PERTUSSIS*

1.4.1 History

Fishel and co-workers were the first group to discover that *Bordetella pertussis* produced a factor which had adenylate cyclase activity (Fishel *et al* , 1970). In order to study the role of the adenylate cyclase in pathogenesis and its mode of action a number of investigators have subsequently attempted to purify it. Initial attempts to purify the toxin revealed a variety of forms ranging from 45 -700 kDa (Hewlett and Wolff, 1976; Shattuck *et al* , 1985; Kessin and Franke, 1986).

More recently, significant purification has been achieved and from this it is apparent that the adenylate cyclase exists in two forms: a 43-50 kDa form which is found in culture supernates, and has only catalytic activity, and a 200-220 kDa form, termed invasive adenylate cyclase which posseses enzymic activity, and can enter mammalian cells where, stimulated by host-cell calmodulin, it elevates intracellular cyclic AMP levels (Rogel *et al* , 1989; Bellalou *et al* , 1990; Leusch *et al* , 1990).

Rogel and co-workers demonstrated that the two forms were immunologically related suggesting the smaller form was derived from the degradation of the larger form (Rogel *et al* , 1988). This was confirmed when it was demonstrated that the smaller form corresponded to the amino terminal portion of the larger precursor (Ladant *et al* , 1989).

Cloning and sequencing of the adenylate cyclase gene, *cya* A, has indicated that the toxin is coded for by an open reading frame of 1706 codons, the product of which is a bifunctional protein (Cya A) with both invasive adenylate cyclase and haemolytic activities. (Glaser *et al* , 1988). Cya A displays low specificity, is toxic to a wide range of nucleated cells (Confer and Eaton, 1982) and haemolytic to erythrocytes (Rogel *et al,* 1991) from a number of species (see section 1.5).

1.4.2 Homology with the RTX-family of toxins

Cloning and sequencing of adenylate cyclase toxin genes from *B.pertussis* has also revealed that the toxin shows genetic similarity to the RTX family of toxins which are produced by other Gram-negative pathogens (reviewed by Welch, 1991 and Coote, 1992). The RTX toxins are important virulence factors to their producer organisms (Coote, 1992). The toxins are typified by *Escherichia coli* haemolysin (HlyA), *Pasteurella haemolytica* leukotoxin (LktA), *Actinobacillus actinomycetemcomitans* leukotoxin (AktA) and *Actinobacillus pleuropneumoniae* haemolysin (AppA) (**Table 1.2**). The group is expanding and recently related toxins have also been described in *Actinobacillus suis*, *Proteus vulgaris*, *Morganella morganii,* and in enteroaggregative and enterohaemorrhagic strains of *E.coli* (Koronakis *et al,* 1987; Lori *et al,* 1991; Baldwin *et al,* 1992; Schmidt *et al,* 1994).

The genes for synthesis, activation and secretion of the RTX toxins are organised in an operon structure (see below). These toxins are all synthesised as inactive precursors and activated post-translationally prior to secretion by a novel

Table 1.2 ***Properties of the representative RTX toxins*** [a]

Bacterium	Toxin	Amino acids	Mr (kDa)	No. of repeats
Bordetella pertussis	Adenylate cyclase/ haemolysin (CyaA)	1706	177	41
Escherichia coli	Haemolysin (HlyA)	1023	110	16
Pasturella haemolytica	Leukotoxin (LktA)	953	102	6
Actinobacillus actinomycetemcomitans	Leukotoxin (AktA)	1055	116	12
Actinobacillus pleuropneumoniae	Haemolysin (AppA)	956	103	9

[a] source, Coote (1992)

secretion process (see sections 1.4.2.2 and 1.4.2.3). *B.pertussis* CyaA also shares a number of functional similarities with the RTX toxins (see sections 1.4.3 and 1.4.4)

1.4.2.1 Genetic structure and organisation

Synthesis, activation and secretion of CyaA requires the interaction of at least five gene products arranged in an operon structure. The operon structure for production of CyaA is homologous to that of the RTX toxins so far described (**Figure 1.3**) and includes the structural gene encoding an inactive polypeptide (*cya* A), the gene involved in toxin activation (*cya* C) and *cya* B, *cya* D *and cya* E which are involved in toxin secretion (Glaser *et al,* 1988b; Masure *et al,* 1990; Barry *et al* , 1991). The mechanisms of toxin activation and secretion are discussed in sections 1.4.2.2 and 1.4.2.3 respectively. Cloning and sequencing of the genes controlling production of the other RTX toxins has revealed similar gene clusters containing contiguous C, A, B, and D genes with homologous products and functions (reviewed by Welch, 1991 and Coote, 1992).

1.4.2.2 Toxin activation

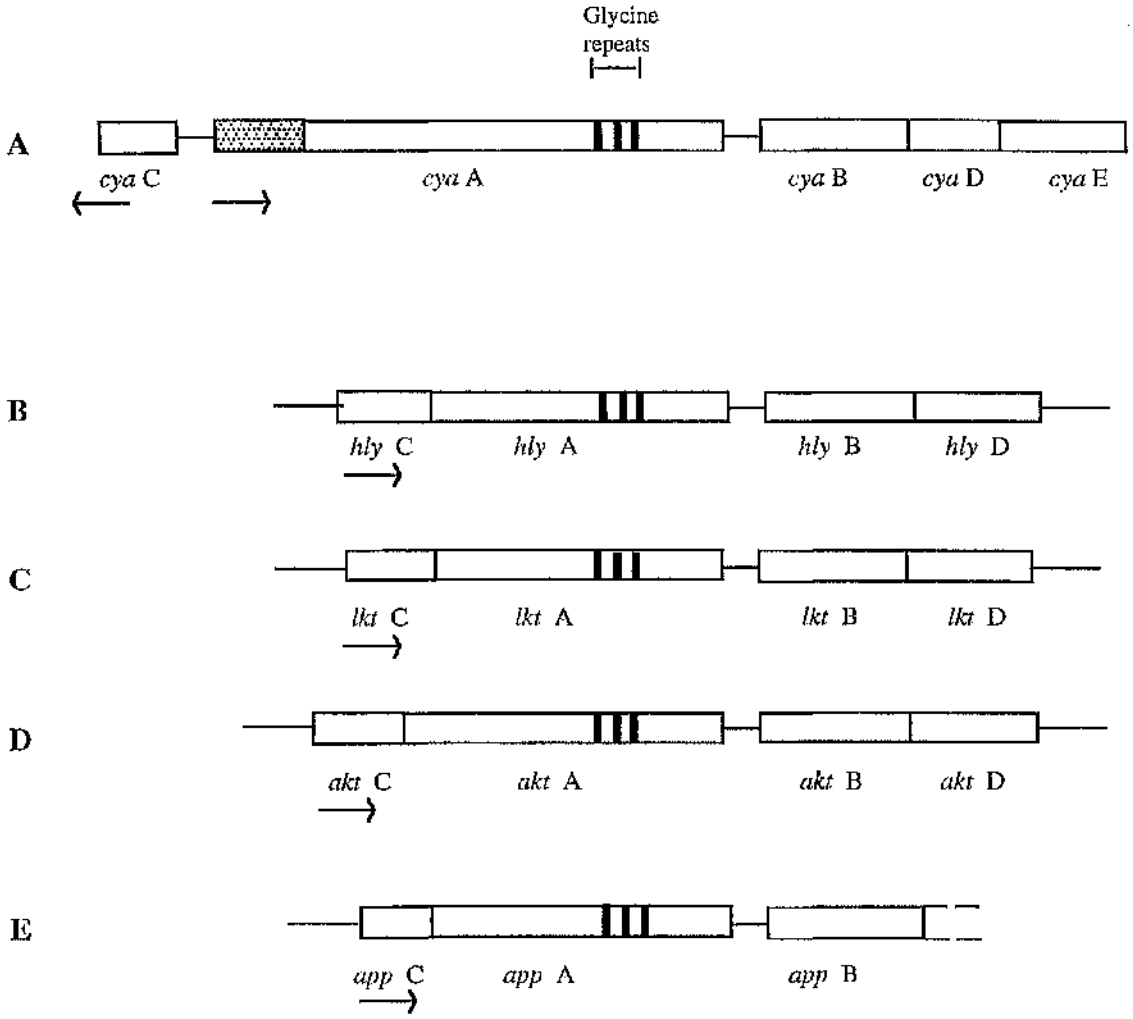
Bordetella pertussis CyaA is synthesised as an inactive protoxin and requires the collaboration of the product of the *cya* C gene for activation (Barry *et al,* 1991). The *cya* C mediated activation is necessary for the invasive and haemolytic activities of CyaA (Rogel *et al* , 1989; Betsou *et al* , 1993). The other members of the RTX family of toxins are also synthesised as inactive precursors and require prior activation for full biological activity (Nicaud *et al* , 1985; Wagner *et al,* 1988; Forestier and Welch 1990). This suggests that toxin activation is a prerequisite for a successful interaction of adenylate cyclase toxin and the other RTX toxins with target cells.

The molecular mechanism of activation of *E.coli* Hly A has been studied as a model for the activation the RTX toxins. Initial studies revealed that activation of HlyA

Figure 1.3 ***Genetic organisation of RTX toxins determinants*** [a]

The A gene corresponds to the structural (toxin) gene.The C gene products is required for toxin activation and the B and D gene products are required for secretion. **A** corresponds to operon of CyaA (Glaser *et al*, 1988a, 1988b; Barry *et al* , 1991). **B, C, D** and **E** are the operons controlling production of HlyA (Mackmann *at al*, 1988; Welch and Pellet, 1988), LktA (Lo *et al*, 1987; Strathdee and Lo, 1987), AktA (Kolodrubetz *et al* , 1989; Lally *et al* , 1989; Kraig *et al* , 1990) and AppA (Chang *et al*, 1989) respectively (see text). Also shown are the catalytic (stippled region) of CyaA, and the glycine rich repeat units of all the toxins. Arrows indicate the direction of transcription.

[a] source Coote (1992)


Glycine
repeats
A
cya C
cya A
cya B
cya D
cya E
B
hly C
hly A
hly B
hly D
C
lkt C
lkt A
lkt B
lkt D
D
akt C
akt A
akt B
akt D
E
app C
app A
app B

did not involve proteolytic C-terminal cleavage, glycosylation or phosphorylation (Nicaud *et al,* 1985; Felmlee *et al,* 1985; Koronakis *et al,* 1989). However, it was observed that activated Hly A had an altered migration on moderately denaturing SDS-PAGE gels, compared to unactivated HlyA (Nicaud *et al,* 1985; Wagner *et al,* 1988). It was also observed that activation and the altered electrophoretic mobility, were sensitive to phospholipase treatment, suggesting that phospholipids may have a role in the activation proess (Wagner *et al,* 1988). It has subsequently been shown that activation of HlyA by HlyC requires a cytsolic activating factor (Hardie *et al,* 1991) identified as acyl-carrier protein (Iaaartel *et al,* 1991). It is believed that HlyA is activated by the transfer of a fatty-acyl group from acyl carrier protein to HlyA. The activation site of HlyA has been localised by Pellet and co-workers who isolated a monoclonal antibody which only recognised the activated form of Hly A (Pellet *et al,* 1990). The epitope of this antibody was localised within the ß-turn domain of HlyA. Recently it has been found that activation of HlyA occurs by fatty acylation of two internal lysine residues (Stanley *et al,* 1994). It is thought that activation of CyaA may occur by a similar mechanism.

The C gene sequences are highly conserved among the RTX toxins and it is assumed that all toxins are modified by a similar mechanism (reviewed by Braun and Focareta, 1991). The structural similarity between the RTX toxins is exemplified by the finding that the C gene products from one toxin can activate the A gene products of others e.g. HlyC can activate LktA and AppA (Forestier and Welch, 1990; Gygi *et al,* 1990). However, CyaA cannot be activated by HlyC or LktC, suggesting that although the mechanism of activation of the RTX toxins may be similar, they are not identical (Rogel *et al,* 1989; Westrop *et al,* 1997). These differences may be important in determining the specificity or lack of specificity of CyaA and the other RTX toxins (see section 1.6)

1.4.2.3 Toxin secretion

Export of proteins across the Gram-negative membrane is normally a two step process involving initial targeting to the inner membrane by a hydrophobic N-terminal signal sequence followed by transfer across the outer membrane by a specific transfer mechanism and is exemplified by cholera toxin (Hirst and Welch, 1986; Hirst and Holmgren, 1987). The predicted N-terminal sequences of CyaA and HlyA do not display the typical features of hydrophobic signal sequences and it has been shown that they are secreted by a novel mechanism (Felmlee *et al*, 1985; Glaser *et al*, 1988b; Holland *et al*, 1990).

The secretion of HlyA has been studied in detail as a model for secretion of CyaA and the other RTX toxins. The secretion of HlyA is dependent upon the products of *hly* B, *hly* D and *tol* C , and an uncleaved C-terminal signal sequence (Koronakis *et al* , 1989; Holland *et al* , 1990; Wandersmann and Delepelaire, 1990)/ HlyB, HlyD, and TolC are transmembrane proteins (Mackmann *et al* , 1985; Blight and Holland, 1990). It is believed that they interact with the C-terminal signal sequence of HlyA to facilitate its transfer across the cytoplasmic and outer membranes (reviewed by Coote, 1992).

It has been shown that CyaA, can be efficiently secreted by *E. coli* , suggesting that the secretion machinery has a broad recognition profile (Masure, 1990). The B and D gene products of the *Bordetella pertussis* adenylate cyclase operon and the other RTX members show extensive homology (summarised by Welch, 1991) suggesting that these toxins are secreted in a similar manner to HlyA.

1.4.3 Functional domains of adenylate cyclase toxin

The adenylate cyclase toxin of *B.pertussis* is synthesised as a 1706 amino acid polypeptide and possesses invasive adenylate cyclase and haemolytic activities (Glaser *et al* , 1988b). Cloning and site directed mutagenesis have shown that the toxin is

composed of an N-terminal catalytic domain and a C-terminal channel-forming domain which is homologous to the RTX toxins and is required for haemolysis and delivery of the catalytic domain to target cells (**Figure 1.4**).

1.4.3.1 N-terminal catalytic domain

Studies have revealed that N-terminal 400 amino acids of adenylate cyclase corresponds to the calmodulin dependent catalytic portion of the toxin (Glaser *et al*, 1988a; Ladant *et al*, 1989). Further analyses have shown that an N-terminal fragment (residues 1-235) of the enzymic domain contains the active site for catalysis , and that a C-terminal fragment (residues 236-399) contains the calmodulin binding domain (Ladant *et al*, 1989; Glaser *et al* 1989). Lysine 58 and Lysine 65, which are located in a putative ATP binding site, have been implicated in catalysis and Tryptophan-242 has an important role in calmodulin binding (Moller and Amons, 1985; Glaser *et al*, 1989 and 1990)

1.4.3.2 C-terminal channel-forming domain

The C-terminal portion of CyaA (residues 400-1706) can independently act as a haemolysin (Sakamoto *et al*, 1992). This domain shows seqeunce homology with the RTX toxins (reviewed by Coote, 1992) and contains three highly conserved regions: a hydrophobic region, a region rich in ß-turns and a region which contains a variable number of glycine-rich tandem repeats (**Figure 1.4**).

Hydrophobicity plots of the amino acid sequence of CyaA have identified a 200 residue hydrophobic region containing four putative membrane-spanning domains starting at amino acid 530 (Eisenberg *et al*, 1984). Homologous hydrophobic regions have been found in the other RTX toxins (Coote, 1992). Mutations altering the hydrophobicity of CyaA abolish its pore-forming ability and toxicity (Glaser *et al* , 1988b; Bellalou *et al* , 1990). Studies with other RTX toxins such as HlyA (Ludwig

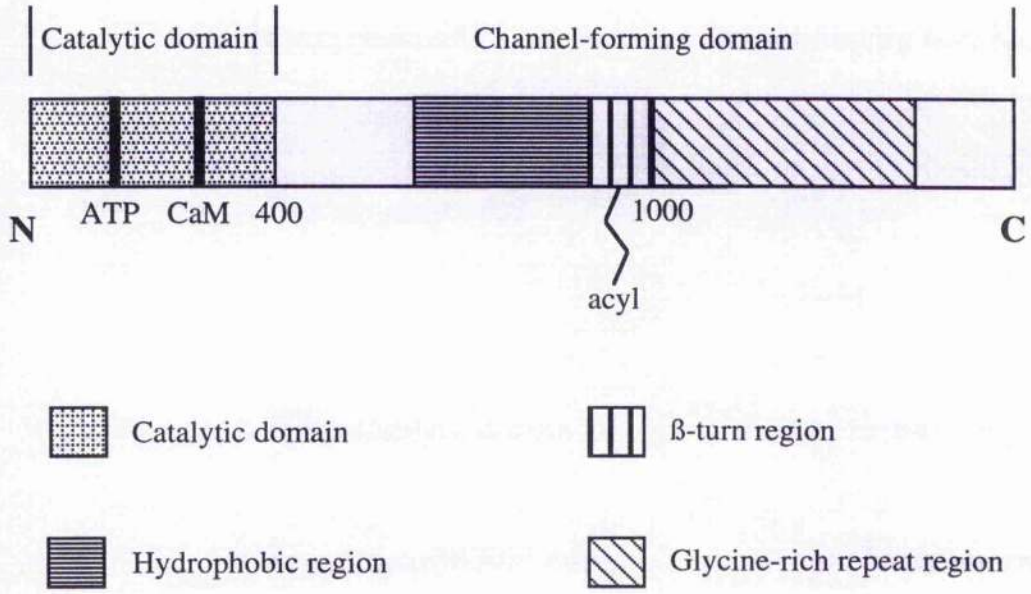


Figure 1.4 ***Diagrammatic representation of the domain structure of B. pertussis adenylate cyclase toxin*** [a]. The first 400 N-terminal amino acids correspond to the enzymic domain of CyaA. Within this domain, ATP and calmodulin (CaM) binding sites are shown (see section 1.4.3.1). The C-terminal amino acids show homology with the RTX cytolysins and are involved in channel formation in target cells. Also shown is the acyl-modification site involved in toxin activation (see section 1.4.2.2). N and C represent the N- and C- termini of the protein.

[a] based on a model by Hanski (1989)

et al, 1987 and 1988) and Lkt A (Cruz *et al* , 1990) have yielded similar results. Also, peptides prepared from the hydrophobic region of Hly A, retain pore-forming ability (Oropeza-Wekerle *et al* , 1992). These results have implicated the hydrophobic domains in pore formation (see section 1.4.4).

The hydrophobic region is followed by a region rich in β-turns which contains the putative acylation site required for activation (see section 1.4.2.2) of CyaA and HlyA (Stanley *et al* , 1994).

At the C-terminus of the CyaA and the other RTX toxins there is a region containing tandemly arranged glycine-rich nonapeptide repeat sequences from which their name was derived (Welch, 1991). The RTX toxins contain variable numbers of these repeats (**Table 1.2**). Rose *et al* (1995) have recently shown that the repeat region of CyaA can bind calcium and that binding to this region can induce a conformational change. Deletions within the repeat region of HlyA reduce or abolish haemolysis (Ludwig *et al,* 1988). It has also been demonstrated, with HlyA and LktA, that the repeats are actually involved in calcium binding and binding of the toxins to target cells (Ludwig *et al,* 1988; Boehm *et al* 1990a and 1990b). Analyses of the three dimensional structure of a similar repeat motif found in the alkaline protease of *Pseudomonas aeruginosa* have found that it constitutes a new type of calcium binding structure (Baumann *et al* , 1993). From these studies with the other RTX toxins the repeat region appears to be involved in the calcium-dependent interaction of CyaA and the other RTX toxins with target cells (see section 1.4.4.4)

1.4.4 Interaction of adenylate cyclase toxin with mammalian cells

1.4.4.1 Pore formation by adenylate cyclase toxin

The RTX toxins such as *E.coli* HlyA, *P.haemolytica* LktA, *A.pleuropneumoniae* AppA, and *A. actinomycetemcomitans* AktA lyse target cells by formation of transmembrane pores (Bhakdi *et al,* 1980; Lalonde *et al,* 1988; Clinkenbeard *et al* , 1989; Iwase *et al* , 1990).

Similarly, *B.pertussis* CyaA destroys erythrocytes by colloid osmotic lysis (Ehrmann *et al* , 1991). Studies with sheep erythrocytes have demonstrated haemolysis occurs after a lag period of 45-60 minutes (Ehrmann *et al* , 1991; Rogel *et al* , 1991). Osmotic protection studies have revealed that haemolysis by CyaA is brought about by the formation of pores with a diameter of approximately 0.6 nm (Ehrmann *et al* , 1991). The toxin can also disrupt liposome membranes (Gordon *et al* , 1989).

There is still debate as to whether the pores formed in cell membranes by CyaA are generated by toxin monomers or oligomers. As the prototype of the RTX-toxin family *E.coli* HlyA has been used as a model to describe pore formation by CyaA and other RTX toxins. HlyA forms voltage-dependent, cation-selective and ion permeable transmembrane pores in erythrocyte membranes (Bhakdi *et al*, 1987; Menestrina *et al* , 1987; Menestrina, 1988; Eberspacher *et al* , 1989)

The pores formed display similar properties to other membrane-seeking bacterial toxins including the colicins (Schein *et al* , 1978), diphtheria toxin (Shiver and Donovan, 1987), botulinum and tetanus toxin (Shone *et al*, 1987; Hoch *et al*, 1985). In contrast to the oligomeric pores formed by many cytolysins from Gram-positive organisms (Bhakdi and Tranum-Jensen, 1988) as well as complement (Bhakdi and Tranum-Jensen, 1987) and lymphocytolysins (Henkart, 1985), the pores formed by HlyA appear to be formed by toxin monomers (Jorgensen *et al* , 1980; Menestrina, 1988). However in one study it has been reported that toxin molecules act co-operatively to form oligomeric pores (Benz *et al* , 1988). In this study Benz *et al* were unable to reconcile their findings with the observations of other researchers and it may be that the pores are generated from pre-formed oligomers.

1.4.4.2 Invasive activity of adenylate cyclase toxin

In contrast to the other RTX toxins CyaA has toxic as well as lytic effects on mammalian cells. Confer and Eaton first demonstrated that CyaA could elevate cyclic AMP levels in macrophages by conversion of endogenous ATP to cAMP (Confer and Eaton, 1982). Since then a number of studies have demonstrated that CyaA is toxic to a wide range of cells, and in particular immune effector cells, leading to impairment of function (reviewed by Hewlett and Gordon, 1988). Further investigation has shown that intoxication is due to the activity of CyaA itself, rather than by activation of an intrinsic membrane-bound adenylate cyclase (Hanski and Farfel, 1985; Friedman *et al*, 1987). In contrast to haemolysis, which occurs after a lag period, intoxication by CyaA occurs immediately (Farfel *et al*, 1987; Friedman *et al*, 1987; Rogel *et al*, 1991). In these studies intracellular cAMP accumulated, inside CyaA treated cells, even when trypsin was present in the external medium (Farfel *et al*, 1987; Friedman *et al*, 1987). This was the first evidence that CyaA or at least the enzymic domain (see section 1.4.3.1) penetrates target cells.

The enzymic activity of CyaA is activated by the eukaryotic regulatory protein, calmodulin (Wolff *et al*, 1980; Greenlea *et al*, 1982; Kilhoffer *et al*, 1983). Exogenous addition of calmodulin to CyaA blocks intoxication of target cells (Shattuck and Storm, 1985). It has been suggested that the binding of calmodulin to CyaA outside cells may induce a conformational change, thus preventing the toxin from binding to or penetrating the cell membrane (Hanski, 1989). However, it has been found that exogenous addition of calmodulin actually potentiates haemolysis of sheep erythrocytes by CyaA (Rogel *et al*, 1991). The differences in the toxic and haemolytic properties of CyaA, such as calmodulin and calcium requirement (see below) and kinetics (discussed above) suggest that the toxic and lytic activities of CyaA are separable and may be mediated by different domains of the toxin.

1.4.4.3 Role of calcium in activities of adenylate cyclase toxin

In common with the other RTX toxins, CyaA is dependent on calcium for some of its biological activities. Hewlett and co-workers first demonstrated that CyaA was a calcium binding protein, and that binding of calcium altered the conformation of the toxin molecule (Hewlett *et al* , 1991). Recently it has been found that the repeat motifs of CyaA can bind calcium inducing a conformational change (Rose *et al*, 1995). However, there is debate as to the exact role of calcium in the different stages of cellular attack by CyaA. The catalytic acticity of CyaA is calcium-independent (Greenlea *et al*, 1982; Kilhoffer *et al* , 1983), whereas the invasive adenylate cyclase activity of CyaA requires calcium (Rogel *et al* , 1991). The involvement of calcium in the lytic activity of CyaA is still a matter of controversy. The majority of studies have shown that the lytic activity of CyaA requires calcium (Gordon *et al*, 1989; Ehrmann *et al*, 1991). However, Rogel and co-workers demonstrated that the binding of CyaA to sheep erythrocytes and subsequent haemolysis did not require calcium, and even occurred in the presence of the calcium-chelating agent, EDTA (Rogel *et al* , 1991). It is probable that CyaA encounters and binds calcium during growth of *B.pertussis* and purification of the toxin, and that results which suggests calcium-independence may be an artefact of this.

1.4.4.4 Molecular mechanisms for interaction of adenylate cyclase toxin with target cells

The process by which CyaA can form pores in cell membranes and also enter target cells, has still not been satisfactorily explained at the molecular level. Evidence so far has suggested that penetration occurs in consecutive steps involving initial cell binding, insertion and pore formation, and finally delivery of the catalytic domain leading to intoxication and/or cell lysis (Rogel and Hanski, 1992; Hewlett *et al* , 1993).

The first step in the interaction of CyaA with the target cell is initial association with the cell surface. Binding to target cells does not reqiuire calcium or prior activation of the toxin (Rogel *et al* , 1991; Hewlett *et al* , 1993). This is in contrast to *E.coli* HlyA, which requires calcium and an intact repeat region (see section 1.4.3.1) to bind to target cells. It may be that the initial association of CyaA is a non-specific one (see section 1.4.5).

The next stage is insertion into the cell membrane and pore formation. The hydrophobic membrane spanning region (see section 1.4.3.1) of CyaA is believed to be involved in the formation of pores in the cell membrane (Bellalou *et al*, 1990). Toxin activation and high calcium concentrations are required for this stage (Rogel and Hanski, 1992; Hewlett *et al* , 1993). Activation occurs by acylation of CyaA (see section 1.4.2.2), and this may reflect a mechanism of anchoring CyaA to cell membranes, analogous to the acylation of certain eukaryotic proteins which is thought to be involved in protein-protein and protein-lipid interactions (Towler *et al*, 1988; O'Dowd *et al*, 1989; Wedegaertner *et al*, 1993). Acylation may anchor CyaA in a position to allow calcium to induce a conformational change allowing the toxin to insert into the membrane (Hewlett *et al*, 1991 and 1993).

The next steps in the cellular attack by CyaA, intoxication due to invasive adenylate cyclase activity, and cell lysis, are separable events. Lysis requires relatively high concentrations of CyaA and occurs after a lag period, whereas intoxication is immediate and occurs in the presence of relatively low concentrations of CyaA (Rogel *et al*, 1991). It has been hypothesised that the invasive adenylate cyclase and haemolytic activities of CyaA are mediated by different domains of the toxin molecule (Rogel *et al*, 1991). Meanwhile studies using agents which interfere with endocytosis, or prevent acidification of endosomes, have revealed that the mechanism of entry of the toxin does not involve receptor-mediated endocytosis (Gordon *et al*, 1988 ; Donovan *et al*, 1991). Rogel and Hanski have demonstrated that the catalytic domain is cleaved and released inside target cells (Rogel and Hanski, 1992). From this it has been

hypothesised that the RTX toxin homologous channel-forming portion of CyaA forms a pore in target cell membranes (in an analogous manner to the other RTX toxins) whereupon the amino terminal catalytic domain is inserted and exerts its toxic effects (Rogel and Hanski, 1992). Lysis probably follows later when a sufficient number of pores have been formed. A hypothetical model for the interaction of CyaA with target cells, based on the evidence accumulated so far is shown in **Figure 1.5**.

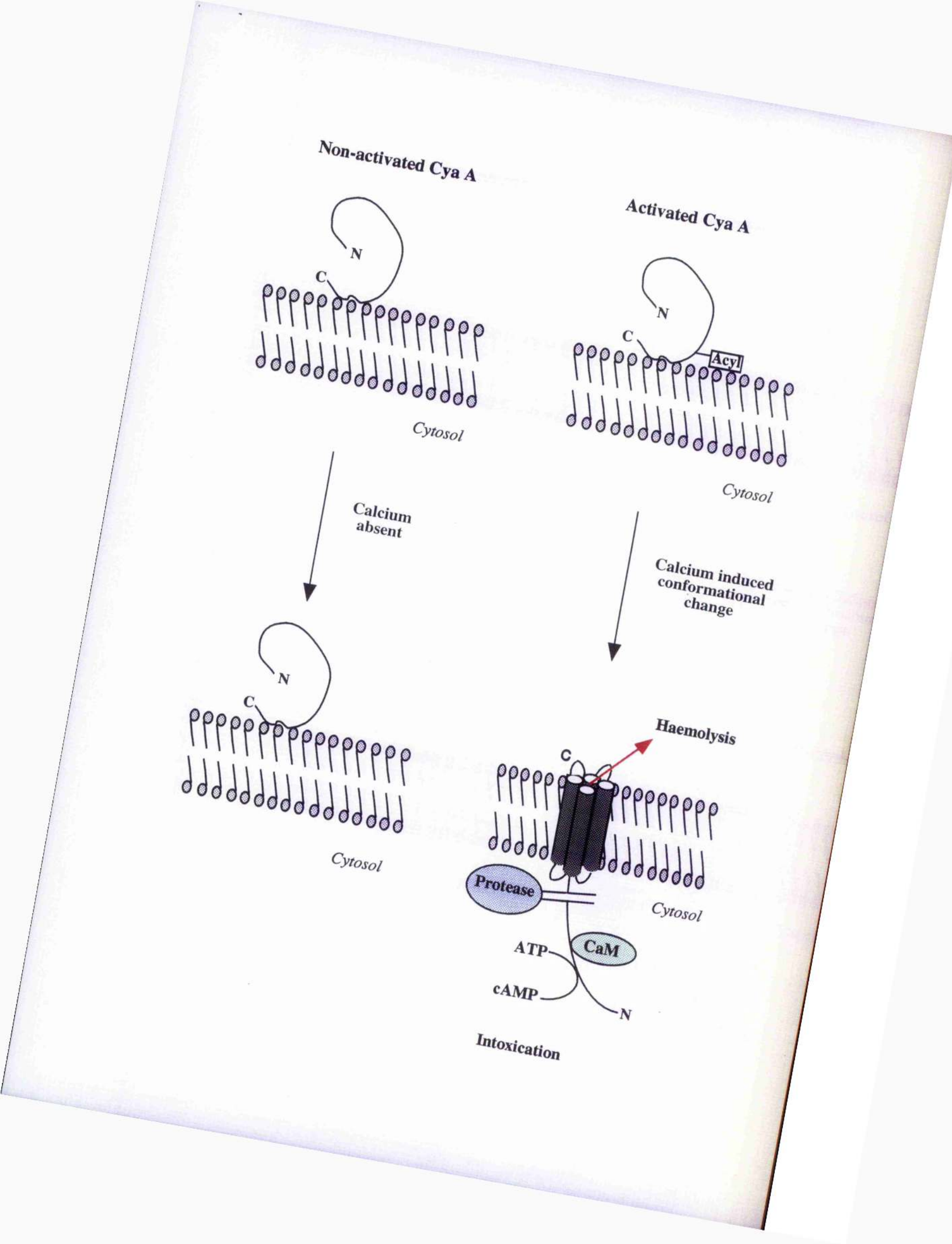
1.5 SPECIFICITY OF RTX TOXINS

Despite exhibiting structural and functional similarity, the RTX toxins display striking differences in their specificity. *B.pertussis* CyaA displays little specificity in terms of its lytic and toxic activities. CyaA is haemolytic to a broad range of cell types including erythrocytes from humans, sheep, rabbits and mice (Ehrmann *et al*, 1991; Rogel *et al* , 1991) and also toxic to a wide range of nucleated cells such as human macrophages, monocytes and natural-killer cells and also rat pituitary cells and murine lymphoma cells (Confer and Eaton, 1982; Hewlett *et al*, 1983; Hanski and Farfel, 1985; Weiss *et al* , 1986). Similarly other RTX toxins such as *E.coli* HlyA and *A.pleuropneumoniae* AppA are non-specific. HlyA lyses erythrocytes and nucleated cells from a wide range of species (Cavalier *et al* , 1984; Gadeberg and Orsklov, 1984; Bhakdi *et al* , 1989 ; Mobley *et al* , 1989) and AppA is lytic towards erythrocytes from porcine, equine , bovine and human origin (Maudsey and Kada, 1981; Rosendal *et al*, 1988) and toxic towards rabbit and porcine neutrophils and porcine macrophages (Bendixin *et al* , 1981; Rosendal *et al* , 1988).

However, other RTX toxins such as *P.haemolytica* LktA and *A.actinomycetemcomitans* Akt A, are highly specific. The activity of LktA is limited to ruminant leukocytes and platelets (Shewen and Wilkie, 1982; Clinkenbeard and Lipton, 1991), whereas Akt A is specific for leukocytes from certain primate species (Taichman *et al* , 1987).

Figure 1.5 ***Hypothetical model for the interaction of adenylate cyclase toxin with target cells*** [a]. Adenylate cyclase toxin (CyaA) is activated post-translationally by acylation of a region in the C-terminal half of the protein. Activated (acylated) and non-activated forms of CyaA can both bind non-specificially to target cells. Non-activated CyaA is unable to penetrate the the cell membrane and therefore cannot mediate cell intoxication or lysis. The activated form of CyaA may anchor the toxin to a receptor wherebye calcium induces a conformational change facilitating penetration of the toxin into the cell via a trans-membrane pore formed by the hydrophobic region of the protein. The catalytic domain of the protein is delivered to the target cell by a cellular protease. Inside the cell the catalytic domain binds to calmodulin and is activated to convert ATP to cAMP, leading to cell intoxication. Formation of pores in the cell membrane may also lead to cell lysis.

[a] based on models by Rogel and Hanski (1992); Hewlett *et al* (1993).


Non-activated Cya A
N
C
Cytosol
Activated Cya A
N
C
Acyl
Cytosol
Calcium absent
Calcium induced conformational change
N
C
Cytosol
Haemolysis
C
Protease
Cytosol
ATP
CaM
cAMP
N
Intoxication

According to these differences in specificity the RTX toxins can be divided into two distinct classes (see below).

Haemolysins - typified by HlyA, CyaA and AppA. The haemolysins have broad specificity and will lyse erythrocytes and nucleated cell types from a variety of species.

Leukotoxins - typified by Lkt A and Akt A. The leukotoxins are highly specific and their lethality is host and cell specific.

The molecular basis of the differences in specificity is unknown. Kraig and co-workers compared the predicted primary amino acid sequences derived from cloned toxin genes from a number of RTX toxins (Kraig *et al* , 1990). This study revealed no regions of homology within the haemolysin and leukotoxin classes of RTX toxin that could account for the differences in target cell specificity. Indeed the non-specific haemolysins AppA and HlyA are most closely related to the highly specific leukotoxins LktA and the AktA respectively (reviewed by Welch, 1991). Thus, the primary amino acid sequences do not reflect differences in specificity. Instead, it is likely that the tertiary / domain structure of the toxins will determine the specificity of interaction of these toxins with target cells.

Recently, gene hybrids have been constructed between different RTX toxin genes to determine whether or not certain domains are involved in determining target cell specificity (Forestier and Welch, 1991; McWhinney *et al*, 1992). These studies found that the domains which determine erythrocyte and leukocyte lysis are indeed separate. However, these reports conflict on the precise location of the domains involved. Forestier and Welch generated a number of different hybrids between the *hly* A and *lkt* A genes and reported that a significant portion of the ruminant cell specificity of Lkt A resides in the amino terminal one fifth of the protein, while a region within the repeat region of Hly A is critical for cytolysis of nucleated cells but not haemolysis (Forestier and Welch, 1991) . McWhinney and co-workers generated hybrids between

app A and *lkt* A and reported that the haemolytic and leukotoxic activities of App A were determined by different, perharps overlapping regions of the protein (McWhinney *et al*, 1992). However, in contrast to Forestier and Welch, McWhinney and co-workers reported that the leukocytic potential of AppA maps to the carboxy-terminal half of the protein. Further studies are required to determine if different domains are indeed involved in determining the degree of specificity of the various RTX toxins, and if so, which domains.

It has also been reported that specificity was dependent upon the RTX C-gene used for activation (Forestier and Welch, 1990). It is apparent that the specificity of the RTX toxins may be determined by differences in domain structure although as yet it is not clear which domains are ultimately involved. Specificity may or may not be dependent upon the process of activation. The generation of further gene hybrids and studies involving heterologous activation of RTX toxins should elucidate the domains involved in target cell recognition and the role activation plays in determining specificity.

1.6 CELLULAR RECEPTORS FOR BACTERIAL TOXINS

Biological receptors are defined as the binding-components on the cell surface which ultimately lead to the productive expression of toxicity of a particular toxin (Eidels *et al* , 1983) and biochemically defined as possessing specificity, high affinity, and saturatability (Cautrecasas, 1974). The majority of receptors for bacterial toxins are thought to involve carbohydrates (Eidels *et al*, 1983; Karlsson, 1989; Karlsson *et al*, 1991) present as glycoconjugates on glycolipids e.g. receptors for cholera toxin, botulinum toxin and tetanus toxin, or on glycoproteins e.g. receptors for diphtheria toxin and anthrax protective antigen (**Table 1.3**).

Carbohydrates, either linked to proteins or lipids, make up the major part of cell surfaces and are therefore primary collision partners for bacterial toxins. Cell surface

Table 1.3 ***Reported membrane receptors for selected bacterial toxins***

Bacterium	Toxin	Target tissue	Receptor	Reference
V.cholerae	Cholera toxin	Small intestine	Glycolipid	Merritt *et al*, 1994a
E.coli	Heat-labile toxin	Intestine	Glycolipid	Sixma *et al*, 1992
C. botulinum	Botulinum toxins	Nerve membranes	Glycolipid	Takimazawa *et al*, 1986
C. tetani	Tetanus toxin	Nerve membranes	Glycolipid	Schiavo *et al*, 1991
S.dysenteriae	Shiga toxin	Large intestine	Glycolipid	Lindberg *et al*, 1986
B. pertussis	Pertussis toxin	Respiratory tract	Sugars [a]	Hausman & Burns, 1993
C. diphtheriae	Diphtheria toxin	Respiratory tract	Glycoprotein	Mekada *et al*, 1991
B.anthracis	PA [b]	?	Glycoprotein	Escuyer & Collier, 1991
E.coli	Heat-stable toxin	Intestine	Glycoprotein	Dreyfus & Robertson, 1984

[a] Reported to bind to sugars groups on glycolipids and glycoproteins

[b] Protective Antigen (PA)

glycolipids and glycoproteins vary between cells and from one host to another, and this is thought to explain the host- and cell-specificity of many pathogens and their particular toxins (Karlsson *et al* , 1989).

Few receptors have been formally identified at the molecular level. Recently, the three dimensional stucture of a number of toxin-receptor complexes has been achieved (Sixma *et al* , 1992; Merritt *et al*, 1994a and 1994b; Stein *et al*, 1994). Of the receptors identified so far the majority of binding sites are internal carbohydrate residues of glycolipids or glycoproteins. Internal residues may be more susceptible to mutational shifts in receptor specificity, which is not possible with the functionally important terminal residues (Karlsson, 1989). The relevence of the binding affinities ascribed to toxins *in vitro* has still not been proven (Karlson, 1989). However, identification of further receptors and the domains of toxins involved in binding to these, will hopefully yield important information on the structure-function relationships and the mode of entry of bacterial toxins into eukaryotic cells. It has been hypothesised that toxins such as diphtheria toxin interacting with glycoproteins may enter cells by receptor-mediated endocytosis (Eidels *et al* , 1983). Glycolipid receptors, which have a close proximity to the lipid bilayer itself, may facilitate direct penetration into the membrane (Karlsson, 1991).

1.6.1 Receptors for RTX toxins

Research into the interaction of the RTX toxins with target cells so far has been centered on the influence of the toxins themselves on specificity (see section 1.5). Research into the influence of the target cell and its membrane recognition sites, which will ultimately define whether or not there will be a productive expression of toxicity, has been limited. It is likely that the broad specificity haemolysins such as *E.coli* HlyA, *B.pertussis* CyaA and *A.pleuropnuemoniae* AppA will interact with receptors common to a wide range of cell types, whereas the highly specific leukotoxins of

P.haemolytica and *A.actinomycetemcomitans*, will recognise receptors limited to particular cell types.

HlyA and AppA are active against artificial lipid vesicles, suggesting that these toxins can also interact directly with the lipid bilayer (Lalonde *et al* , 1988; Menestrina, 1988; Menestrina *et al*, 1987). Benz *et al* (1989) reported that permeabilisation of liposomes by HlyA was dependent upon the lipid composition. In this study, pore formation by HlyA was much higher in liposomes composed of asolectin (a crude lipid mixture from soyabean) than pure phospholipids. Hence, asolectin may contain a receptor for HlyA. However, Menestrina (1988) has reported that HlyA can permeabilize liposomes composed of pure phospholipids.

With respect to CyaA, it has been observed that intoxication of Chinese Hamster Ovary (CHO) cells by CyaA is unaffected by pre-treatment of the cells with trypsin or cyclohexamide suggesting that the toxin does not interact with cell-surface proteins (Gordon *et al*, 1989). Also, CyaA can permeabilise artificial lipid vesicles suggesting it can interact directly with the lipid bilayer (Gordon *et al*, 1989). The limited research carried out so far on CyaA has implicated gangliosides as putative membrane receptors. Gable and co-workers demonstrated that toxicity of CyaA towards polymorphonuclear leukocytes could be inhibited by pre-treating the cells with neuraminidase or by incubation of the toxin with bovine brain gangliosides (Gable *et al*, 1985). Gordon *et al* (1989) subsequently carried out a more extensive study of the target cell recognition sites for CyaA and showed that pre-incubation of CyaA with different gangliosides inhibited subsequent intoxication of CHO cells by the toxin.

Charge-charge interactions may be important in the interaction of Cya A with target cells. Rapitis *et al* (1989) reported that cationic molecules inhibited the interaction of CyaA with cells by blocking anionic sites on the membrane. It is possible that these anionic sites correspond to membrane gangliosides which are negatively charged. In accordance with the glycolipid type of receptor (Eidels *et al*, 1983) CyaA does not enter cells by receptor mediated endocytosis (Gordon *et al* , 1988; Donovan and Storm,

1991). Nevertheless, the interaction of CyaA and other RTX toxins with target cells is an area which requires further research.

OBJECTIVES OF RESEARCH

Bordetella pertussis produces an array of virulence factors which have been implicated in the pathogenesis of whooping cough. One such factor is adenylate cyclase toxin (CyaA), a unique bifunctional protein which possesses invasive adenylate cyclase and haemolytic activities. The mechanisms by which CyaA interacts with target cells are not clearly understood. This study aimed to investigate the binding/association of CyaA with eukaryotic cells, to understand the initial steps in the interaction of the toxin with cell membranes. CyaA exhibits broad cell specificity in terms of its toxic (invasive adenylate cyclase) and lytic activities. The study also aimed to investigate the specificity of CyaA by indentifying putative cell-surface receptor molecules.

CyaA is a member of the RTX family of genetically, functionally and immunologically related toxins. In common with other RTX toxins, CyaA is synthesised as an inactive precursor and is activated post-translationally. This study also aimed to investigate the role that activation plays in the binding of CyaA to target cells and its contribution towards subsequent pore formation in cells and model membranes.

SECTION 2
MATERIALS AND METHODS

2.1 ORGANISMS

2.1.1 *B. pertussis*

The *Bordetella pertussis* strains used in this study were obtained from the culture collection of the Laboratory of Microbiology, University of Glasgow (**Table 2.1**). *B.pertussis* 348 (pRMB1) was derived from *B.pertussis* 348, which carried a Tn5 insertion in the adenylate cyclase (*cya* A) gene (see section 1.4.2.1) and expressed no detectable enzymic or toxic activity (Glaser *et al.*, 1988). In *B.pertussis* 348 (pRMB1) the insertion mutation of *B.pertussis* 348 was complemented by pRMB1, a recombinant plasmid which contained the entire *cya* locus (Brownlie *et al*, 1988). *B.pertussis* 348 (pRMB1) had elevated expression of enzymic and toxic activities and was therefore useful in this study.

B.pertussis DE386 contained an oligonucleotide insertion which inactivated the *cyaC* gene product, but did not affect expression of the structural gene, *cyaA*. This strain produced full length protoxin which has adenylate cyclase enzymic activity, but was non-invasive for S49-lymphoma cells (Barry *et al* , 1991).

2.1.2 *E.coli*

Adenylate cyclase toxin (CyaA) was synthesized in the host strain *E.coli* DH5αF'IQ which had been transformed with DNA from the recombinant plasmids pCACT-3, pDLACT-1 and pICNOH (**Figure 2.1**). This plasmid expression system had been described previously (Betsou *et al*, 1993). Plasmid pCACT-3 encoded *cya* A, the structural gene for adenylate cyclase toxin., and also *cya* C, the gene involved in toxin activation (see section 1.4.2.2). Therefore, *E.coli* DH5αF'IQ harbouring this plasmid produced activated CyaA. When *E.coli* DH5αF'IQ was transformed with pDLACT-1, which encoded *cya* A alone, the non-activated form of CyaA was synthesized (**Table 2.2**). The host strain was also transformed with pICNOH, the vector for the *cya* inserts, as a control. The host *E.coli* strain and the plasmid DNA

Table 2.1 ***B.pertussis strains used in this study***

Strain	Relevant genotype
B.pertussis 348 (pRMB1)	*cyaA* + *cyaC*
B.pertussis DE 348	*cyaA*

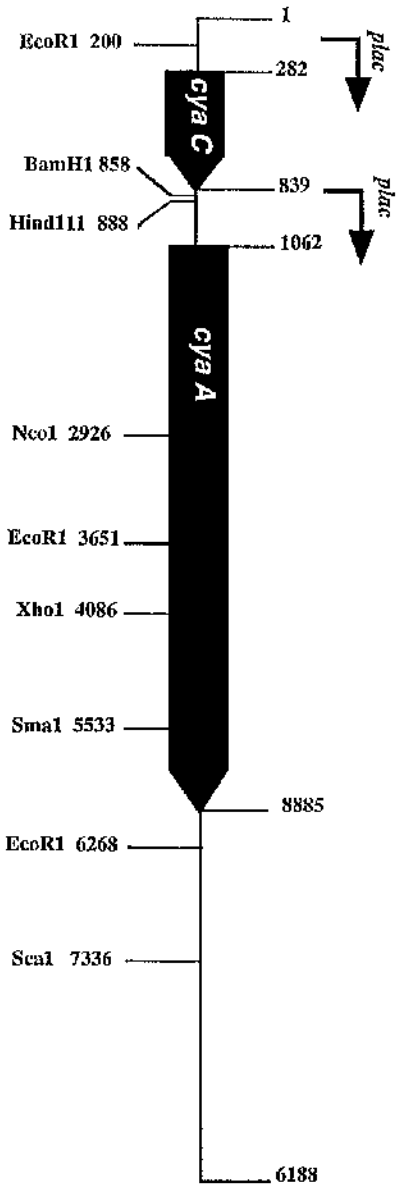
Table 2.2 ***Recombinant E. coli strains used in this study***

Strain	Relevant genotype
E.coli DH5aF'IQ	
pDLACT1	*cyaA*
pCACT3	*cya A* + *cya C*
pIC10H	-

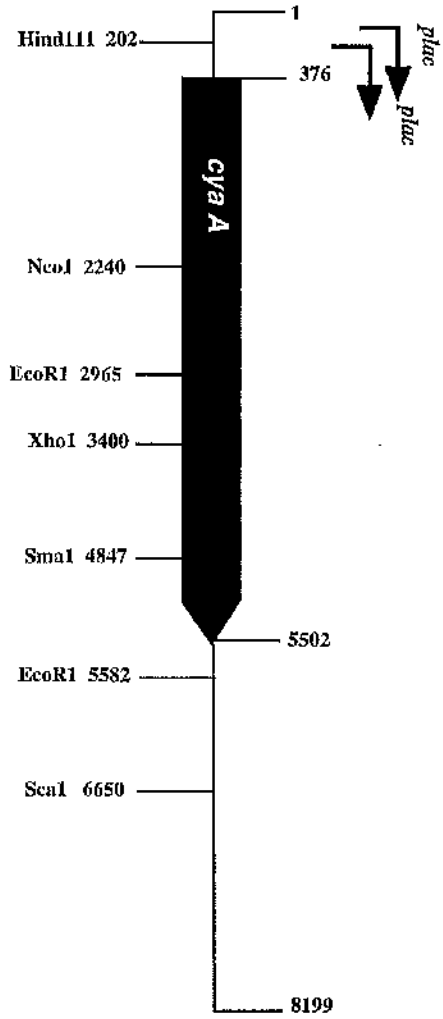
Figure 2.1 ***Representation of the plasmid system for expression of cya A and cya C genes in E.coli***

Adapted from Betsou *et al* (1993)

pCACT3



pDLACT-1



were kindly supplied by Dr Gareth Westrop (Division of Infection and Immunity, University of Glasgow).

2.2 PURIFICATION OF ADENYLATE CYCLASE TOXIN

2.2.1 Purification of adenylate cyclase toxin from *B.pertussis*

2.2.1.1 Growth conditions

B.pertussis was grown on Bordet Gengou (BG) medium (Gibco-BRL) containing 20% defibrinated horse blood, for 72h at 37°C in a humid atmosphere. Bacteria harvested from 2 lawn plates were inoculated into 1L of cyclodextrin (CD) medium (Appendix 1), in 2L dimpled flasks. *B.pertussis* 348 (pRMB1) was grown in the presence of tetracycline (10 μg/ml) and kanamycin (20 μg/ml). The flasks were incubated on an orbital shaker at 150 r.p.m. for 48h at 37°C. Cells were harvested by centrifugation at 9,000 x *g* for 30 min at 4°C (Sorval RC5B) and stored at -20°C.

2.2.1.2 Production of urea extracts

Cell pellets (4 grams wet weight) were resuspended in 82 ml of 10mM Tricine HCl, 0.5mM EDTA, 0.5mM EGTA, pH 8.0 (Buffer A) containing 4M urea and 1mM phenylmethylsulphonyl fluoride (PMSF). Suspensions were stirred for 1h at 4°C before centrifugation at 15,000 x *g* for 30 min at 4°C. The supernate was cleared by ultracentrifugation at 160,000 x *g* for 1h at 4°C and the resulting crude urea extracts stored at -20°C.

2.2.1.3 Calmodulin-agarose affinity chromatography

The urea extracts were diluted with an equal volume of Buffer A containing 5mM $CaCl_2$ to give a final urea concentration of 2M. The extracts were then mixed with 10ml of packed calmodulin agarose (Sigma) as described by Westrop *et al*

(1994). This slurry was mixed gently for 2h at 4°C. After loading on a column (Econocolumn, 10 x 2.5 cm), the agarose was washed with 3 bed volumes (30ml) of 10mM Tricine HCl, 1mM $CaCl_2$, 0.5M NaCl, pH 8.0 (Buffer B) followed by 30ml of Buffer A. Adenylate cyclase was then eluted with 30ml of Buffer A containing 8M urea. Purified urea extracts were stored at -70°C . The extracts were analysed by SDS-PAGE and Western Blotting (sections 2.6 and 2.7, respectively).

2.2.2 Purification of recombinant adenylate cyclase from *E.coli*

2.2.2.1 Transformation of competent cells with plasmid DNA

2.2.2.1.1 Production of competent cells

Host strain DH5α F'IQ was grown on 2 x Yeast Tryptone (2YT) agar (Appendix 2) containing kanamycin (50 µg/ml) for 16h at 37°C. An isolated colony was used to inoculate 10 ml of 2YT broth containing kanamycin (50 µg/ml). The flasks were incubated on an orbital shaker (150 rpm), overnight at 37°C. This overnight culture (0.5 ml) was used to inoculate 50 ml of 2YT broth containing kanamycin (50 µg/ml). The bacteria were grown on an orbital shaker (150 r.p.m) at 37°C, to exponential phase (OD_{650nm} = 0.5-0.6). Competent cells were prepared by the method of Chung and Miller (1988). The cells were pelleted by centrifugation at 1,000 x *g* for 10 min at 4°C (Sorval RC5B) and resuspended in 5 ml of Transformation storage buffer (TSB), (2YT broth containing 10% polyethylene glycol (Molecular weight 3,350), 10mM $MgCl_2$, 10mM $MgSO_4$ and 5% DMSO) and kept on ice for 10 min. The competent cells were stored at - 70°C until required.

2.2.2.1.2 Transformation of competent cells

Competent cells (100µl) and 2 µl of plasmid DNA (pDLACT-1, pCACT-3 or pIC20H) were mixed in 1.5 ml microfuge tubes and incubated on ice for 5 min. To these mixtures 900 µl of TSB, containing 2mM glucose, was added. The

transformation mixtures were then transferred to 20 ml universals and incubated for 60 min at 37°C. Transformation mixtures (100 µl) were spread on 2YT agar containing kanamycin (50 µg/ml) and ampicillin (100 µg/ml). These plates were incubated overnight at 37°C to give isolated colonies which were stable for 2-3 days.

2.2.2.2 Production of *E.coli* extracts from transformed cells

2.2.2.2.1 Growth conditions

Single colonies of the transformants were inoculated onto 2YT agar containing kanamycin (50 µg/ml) and ampicillin (100 µg/ml) and incubated overnight at 37°C to generate lawn plates. The lawn cultures were resuspended in 1ml of 2YT broth. The suspensions were then inoculated into 250 ml of 2YT broth containing kanamycin (50 µg/ml) and ampicillin (100µg/ml), at a starting OD_{650nm} of 0.01-0.02 . The cultures were incubated on an orbital shaker (130rpm) at 37°C. Samples were taken hourly and the OD_{650nm} determined. At an OD_{650nm} of 0.3-0.4 the cells were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1mM, and grown for a further 4h. To analyse expression of recombinant proteins 1ml samples were taken every hour and the cells harvested by centrifugation at 12,000 x *g* for 1 min at 4°C in a microfuge (Heraeus). The pellets were analysed by SDS-PAGE.

The cells were harvested by centrifugation at 9,000 x *g* for 10 min, at 4°C (Sorval RC5B) and pellets stored at -20°C.

2.2.2.2.2 Preparation of urea extracts

The harvested cell pellets were resuspended in 9 ml of 50mM Tris-HCl, 10mM EGTA and 20% sucrose (Buffer C). The cell suspension was then frozen in liquid nitrogen and thawed at 37°C. The cells were incubated in the presence of 1 ml lysozyme (10mg/ml) for 15 min at 4°C and then lysed by 2 cycles of freezing in liquid nitrogen and thawing at 37°C. To the lysed cells, $MgCl_2$ was added to a final

concentration of 20mM, and DNase 1 (prepared in 150mM NaCl) was added to a final concentration of 20 kunitz units /ml. The lysates were incubated at 37°C until they were no longer viscous, whereupon 20 ml of Buffer C was added. Insoluble protein was harvested by centrifugation at 17,000 x *g* for 10 min at 4°C and the supernate (soluble protein fraction) was stored at -20°C .The pellet was resuspended in 3 ml of 50mM Tris-HCl, 0.5mM EGTA, 0.5mM EDTA containing 8M urea and the proteins were extracted by rotation at 4°C for 2h. The extract was transferred to 1.5 ml microfuge tubes and cleared by centrifugation at 15,000 x *g* for 20 min at 4°C. The supernate (urea extract) and the pellet (urea insoluble fraction) were stored at -20°C. Samples of the soluble protein fraction, urea extract and the urea insoluble fraction were examined by SDS-PAGE (secton 2.6).

2.2.2.3 DIALYSIS OF EXTRACTS

Immediately before assays were carried out, urea was removed from extracts of adenylate cyclase by dialysis against the appropriate assay buffer. The extracts were dialysed in a dialysis membrane (size 1-8/32") against 500 volumes of the appropriate assay buffer for 2 x 1h at 4°C with continuous stirring.

2.3 PROTEIN ESTIMATION OF SAMPLES

Protein concentrations were determined by the Bicinchinconic acid method of Smith *et al* (1985). Bovine serum albumin was used to generate a standard curve, from which the concentration of unknown samples was estimated.

2.4 ADENYLATE CYCLASE ENZYMIC ASSAY

The enzymic activities of the adenylate cyclase (Cya A) extracts were determined by the method of Salomon *et al* (1974). The assay measured the conversion of (α^{32}P) ATP to (^{32}P) cAMP. Enzymic activity was determined at 30°C

in 100µl of 25mM Tris-HCl, 10mM $MgCl_2$, pH 7.5 containing 1µM Bovine-brain Calmodulin (Sigma), and 1mM ATP with 10^5 cpm [α-^{32}P] ATP (Amersham). Reactions were terminated after incubation for 10 min by the addition of 100 µl of a solution containing 1% SDS, 20mM ATP and 6.25mM cAMP and immediately transferred to an iced-water bath.

The substrate [(α^{32}P] ATP] and product of the reaction [(^{32}P) cAMP)] were then separated by a double chromatography method using a Dowex 50 x 4 (200-400 mesh) column and a Neutral alumina column. The Dowex column bound cAMP non-specifically and the majority of the labelled components were washed through. The bound cAMP and any remaining unreacted ATP were then washed onto the second column with distilled water. Bound cAMP was recovered from the column by washing with 0.3mM imidazole, pH 7.4 and quantified by liquid scintillation counting.

2.5 ADENYLATE CYCLASE SHAPE-CHANGE ASSAY

The shape-change assay is a toxicity assay based on the principle that agents which increase intracellular cAMP levels inside BHK cells cause a change in the shape of the cells, called stellation. Stellated cells have a decreased ratio of area to perimeter when compared to normal cells, and this ratio can be used as a measure of the toxicity of particular agents.

2.5.1 Tissue culture and preparation of cells

The cells spreading assay was performed according to the method of Westrop *et al* (1994). BHK 21 cells were grown to 60% confluence in Glasgow-modified MEM medium (Macpherson and Stoker, 1962; House, 1964) buffered with 22mM HEPES, pH 7.4. and supplemented with 2% calf serum. Assays were performed in Hank's HEPES (HH) buffer pH 7.4, containing 140mM NaCl, 5.4mM HCl, 1.27mM

$MgCl_2$, 1.3mM $CaCl_2$, 5.6mM D-glucose and 10mM HEPES pH 7.4. Monolayers were washed in Hanks Saline (Hanks HEPES without divalent cations) and cells removed from culture surfaces by the low trypsin-EDTA method (Edwards *et al* , 1975). Cells were aspirated in growth medium and washed twice by centrifugation and resuspension in Hanks saline. This procedure produced a planktonic suspension without affecting the rate of attachment and spreading on fibronectin-coated surfaces.

2.5.2 Shape-change assay

Bovine plasma fibronectin which had been purified from calf serum was kindly donated by Dr. Gareth Westrop (Division of Infection and Immunity, University of Glasgow). Cell spreading assays were performed in 24-well plastic tissue-culture plates. Wells were coated with 0.5 ml of fibronectin (25μg/ml) in HH for 15 min. This coating solution was replaced with 0.5 ml of HH containing 0.5mg/ml haemoglobin, (to block free adsorption sites) and the plates were incubated for a further 15min, before rinsing with HH. Cells (10^4) in trypsinised suspension were mixed with dialysed CyaA extracts in 0.5 ml (final volume) of HH, and added directly to coated wells. Negative and positive controls were set up by mixing cells with HH and HH containing 1mM dibutyryl cAMP, 0.1M 3-isobutyl-1-methylxanthine (IBMX), respectively. After incubation for 90 min at 37°C, cells were fixed for 15 min by addition of 0.25 ml of 4% formaldehyde in phosphate-buffered saline, pH 7.2 . Cells were stained with Kenacid blue (0.1% in water : methanol : acetic acid , 50:50:7 by volume), rinsed twice in water and mounted in Gurr's Clearmount.

2.5.3 Image analysis

Images were obtained with the 40X objective on a Leitz Diavert microscope equipped with a Hamamatsu Vidicon C1000 camera and were digitized to 512 x256

pixels and 64 grey shades using an Archimedes digitizer. Analysis was carried out with a programme written on Acorn Risc Machine Language for an Acorn Archimedes 310 microcomputer (Edwards *et al*, 1993). The programme measured cell perimeter and area and calculated the parameter dispersion as described by Dunn and Brown (1986). Dispersion was a measure of how much the cell shape differed from a circle. Stellation was accompanied by a decrease in the ratio of area to perimeter. The percentage of stellation caused by particular extracts was calculated by the following equation.

$$\text{Stellation (\%).} = \frac{\text{area/perimeter(HH)}^{a} - \text{area/perimeter (extract)}}{\text{area/perimeter (HH)} - \text{area/perimeter (positive control)}^{b}} \times 100$$

[a] buffer control

[b] HH containing 1mM dibutyryl cAMP, 0.1M IBMX

2.6 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970) on 1.5 mm thick SDS-polyacrylamide gels using a vertical-slab gel tank. The stock solutions and recipes for gel and buffer preparation are shown in Appendix 2. The stacking gels and resolving gels contained 4.5% (w/v) and 7.5% (w/v) acrylamide, respectively. Samples were solubilised in an equal volume of solubilising buffer and boiled in a water bath at 100°C for 3 min. To estimate the molecular weight of proteins in samples, SDS-6H molecular weight standards (Sigma) were run on all gels. Samples were electrophoresed through the stacking gel and separating gels at 20mA and 25mA respectively, until the tracking dye reached the bottom of the gel. Gels were stained either by the Coomassie blue staining method of Weber and Osborne (1969), or by the Silver staining method of Oakley *et al* (1980).

2.7 WESTERN BLOTTING.

Protein transfer from SDS-PAGE gels onto nitrocellulose membranes (Hybond C, Amersham) was carried out according to the method of Towbin *et al* (1979). Protein transfer was carried out in a BioRad trans blot cell. The transfer buffer consisted of 20mM Tris, 150mM glycine (pH 8.0) and 20% methanol . Proteins were transferred at 130mA / 50V for 1hr, 80mA / 30V overnight and then 130mA / 50V for 30min.

After transfer the proteins were temporarily stained with Ponceau S solution (0.5% Ponseau S, in 1% acetic acid in distilled water) for 3 min, followed by washing in distilled water for 2 min. The positions of molecular weight markers were marked on the membranes before completely destaining in PBS. The membranes were immersed in blocking buffer (3% powdered milk, 0.2% Tween-20 in PBS), and gently shaken for 1h at room temperature and then washed for 3 x 10 min in PBS. Blots were then probed with monospecific anti-CyaA (1/1000 dilution in blocking buffer) on a rocking platform for 1h at room temperature, followed by washing as described above. The membranes were then probed with HRP-labelled anti-rabbit IgG (SAPU, 1/1000 dilution in blocking buffer, with shaking for 1h at room temperature. The nitrocellulose membrane was washed, then developed with DAB developer [containing 0.05% w/v 3-3 diaminobenzidine tetrahydrochloride (DAB), 98ml PBS, 2ml $CoCl_2$ (1%), and 100µl H_2O_2 (30%)]. When the desired staining was achieved the reaction was stopped with distilled water. The membranes were then dried and stored in the dark.

2.8 PREPARATION OF ANTISERUM AGAINST ADENYLATE CYCLASE TOXIN

Unconcentrated monospecific anti-adenylate cyclase (anti-CyaA) serum was kindly supplied by Dr Yasmeen Kazi (Division of Infection and Immunity, University

of Glasgow). The antiserum was raised against calmodulin-agarose chromatography purified CyaA from *B.pertussis* 348 (pRMB1). The antiserum had been prepared by inoculating a New Zealand Albino rabbit with sonicated nitrocellulose containing CyaA (Dr Yasmeen Kazi, PhD Thesis, University of Glasgow, 1992).

2.8.1 Concentration of antiserum by salting-out

Unconcentrated anti-CyaA serum (50 ml) was mixed with 35 ml, 100% saturated ammonium sulphate, stirred for 1h at room temperature and then centrifuged at 6,000 x *g* for 10 min at 4°C (Sorval RC5B). The pellet was resuspended in 35 ml of 100% ammonium sulphate and stirred for 30 min at room temperature. This suspension was centrifuged again at 6,000 x *g* for 10 min at 4°C (Sorval RC5B) and the pellet resuspended in 10ml of 0.15 M NaCl. To remove the ammonium sulphate the suspension was dialysed for 2 x 2h, then overnight , at 4°C against 500 volumes of 0.15 M NaCl. The concentrated immunoglobulin was stored at -20°C until future use.

2.9 PREPARATION OF CELLS FOR ASSAYS

2.9.1 Isolation of sheep red blood cells

Defibrinated Sheep blood (5ml) was gently layered onto 10 ml Ficoll-Paque (Pharmacia) in 15ml conical centrifuge tube and centrifuged at 2,000 x *g* for 10 min (Medifuge). The supernate was discarded and the pellet resuspended in 15 ml of Tris buffered saline containing 2mM $CaCl_2$ (TBSC). The suspension was centrifuged again at 2,000 x *g* for 10 min, and the pellet washed a further 2-3 times until the supernate was clear. Finally the red blood cells (RBCs) were resuspended in 5ml TBSC. The cells were counted in an Improved Neubauer counting chamber and diluted to the appropriate concentration in the appropriate assay buffer.

2.9.2 Preparation of Baby Hamster Kidney (BHK) cells

Cells were grown and isolated by trypsinisation of monolayers as described in section 2.5.1. An aliquot (50µl) of the trypsinised cells was removed, stained with Trypan blue (50 µl), and the live cells were counted in a counting chamber. Cells were centrifuged at 5,000 x *g* for 10 min (Medifuge) and resuspended in HH to 10^6 cells/ml for use in cell binding assays.

2.9.3 Preparation of Bovine Lymphoma (BL3) cells

Cells were maintained in RPMI 1640 Medium (Gibco) containing 20% foetal calf serum and 2.5mM L-glutamine at between 1.0 x 10^5 and 1.0 x10^6 cells/ml in 25 cm^2 tissue culture flasks. Cells were gently resuspended and a sample removed and stained with Trypan blue before live cells were counted as above. Cells were centrifuged at 5,000 x *g* for 10 min (Medifuge) and resuspended in HH to 10^6 cells/ml for use in cell binding assays.

2.9.4 Preparation of murine macrophage cell line (J774.2)

Cells were maintained in RPMI 1640 Medium(Gibco) containing 10%.foetal calf serum, 2.5mM L-glutamine and amphoterocin B (2.5 µg/ml) at between 1.0 x 10^5 and 1.0 x10^6 cells/ml in 25 cm^2 tissue culture flasks. The cells were stained and counted as above. Cells were centrifuged at 5,000 x *g* for 10 min (Medifuge) and resuspended in HH to 10^6 cells/ml for use in cell binding assays.

2.10 CELL BINDING ASSAY

The cell binding assay was based on the method of Rogel and Hanksi (1991). Binding of CyaA to sheep RBCs, BHK cells, BL3 cells and J774.2 cells was investigated. The cells were isolated as described above and resuspended in HH

containing 2mM $CaCl_2$ (unless otherwise stated). The RBCs (2x 10^8 cells/assay) or BHK, BL3 and J774.2 cells (5 x 10^5 cells/assay) were incubated with dialysed extracts in 1ml (final volume) for 60 min at 37°C, unless otherwise stated. The mixture was then centrifuged at 12,000 x *g* for 5 min. The cell pellet was resuspended in 10mM Tris-HCl, 2mM $MgCl_2$, 1mm EDTA, pH 7.4 (TME buffer.) and cells lysed by 2 cycles of freezing in liquid nitrogen and thawing to 37°C. The membrane pellets were washed a further three times in TME and finally resuspended in 100 µl of TME. Membrane proteins (50µl) were then separated by SDS-PAGE and transferred to nitrocellulose by Western blotting, before probing with monospecific anti-CyaA serum for the presence of CyaA in the membrane fraction (section 2.7).

2.10.1 Binding assay using trypsinised erythrocytes

Sheep RBCs were isolated as above, resuspended to 2 x 10^8 cells/ml and treated with trypsin (25-100µg/ml), prepared in HH, for 60 min at 37°C. Cells were then centrifuged at 12,000 x *g* for 5 min and resuspended in 1ml of HH. To remove the trypsin the cells were then washed in HH a further 4 times. The binding assay was then carried out as above.

2.11 NITROCELLULOSE OVERLAY ASSAY

This procedure was developed to isolate protein receptors for CyaA and was a variation of a method previously described by Knowles and Ellar (1986). Membrane proteins from sheep erythrocytes were separated by SDS-PAGE, transferred to nitrocellulose and overlaid with CyaA. Binding of CyaA to the proteins was detected by use of labelled anti-CyaA antibodies.

2.11.1 Preparation of nitrocellulose for overlay

Sheep erythrocytes membranes were prepared as described in section 2.9.1.1. The membranes containing 5-10 µg protein, were separated by SDS-PAGE and either stained by the Silver staining method of Oakley *et al* (1980), or transferred to nitrocellulose by Western blotting (Towbin *et al,* 1979). The nitrocellulose was immersed in blocking buffer (3% powdered milk, 0.2% Tween-20 in TBS containing 2mM $CaCl_2$ (TBSC)) and gently shaken for 1h at room temperature.

2.11.2 Overlay of nitrocellulose with adenylate cyclase toxin

The blocked nitrocellulose was overlaid with 50 µg/ml CyaA purified from BP348 (pRMB1) in blocking buffer, overnight at 4°C unless otherwise stated. The membrane was washed for 3 x 10 min in blocking buffer before probing with monospecific anti-CyaA serum (1/1000 dilution in Blocking buffer) with shaking for 1h at room temperature, followed by washing as described above. Two procedures were then tested to detect the binding of CyaA to the sheep RBC membrane proteins and are described below in sections 2.11.3 and.2.11.4 and in **Figure 2.2.**

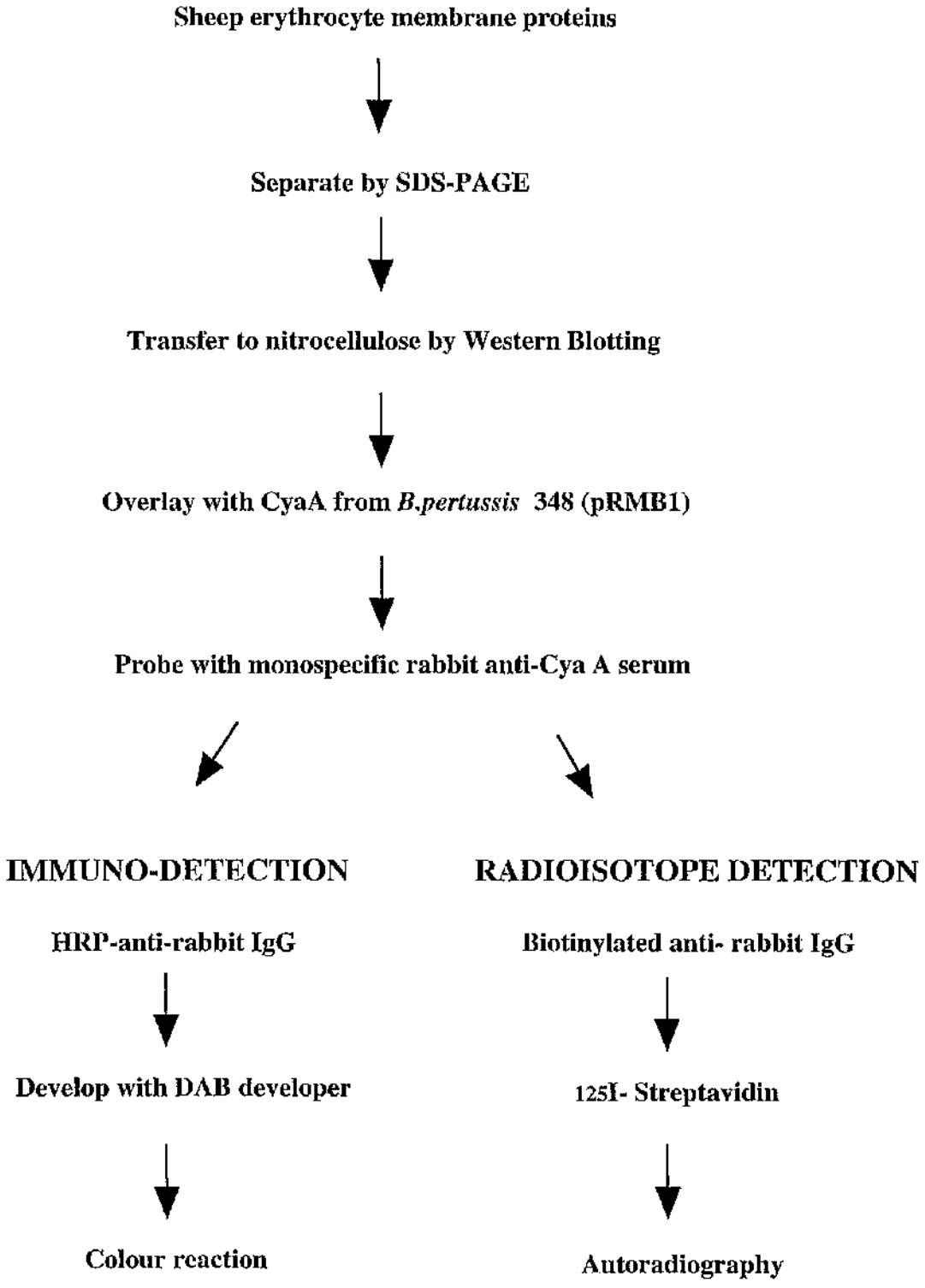
2.11.3 Immunodetection

In this procedure the nitrocellulose was further overlaid with HRP-labelled anti-rabbit IgG (SAPU, 1/1000 dilution in blocking buffer), and shaken for 1h at room temperature, washed and then developed with DAB developer solution as described in section 2.7.

2.11.4 Radioisotope-detection

In this procedure the nitrocellulose was further overlaid with biotinylated anti-rabbit IgG (Amersham), (1/400 dilution in blocking buffer) for 1h at room

Figure 2.2 ***Overlay assays to detect binding of CyaA to membrane proteins***


Sheep erythrocyte membrane proteins
Separate by SDS-PAGE
Transfer to nitrocellulose by Western Blotting
Overlay with CyaA from *B.pertussis* 348 (pRMB1)
Probe with monospecific rabbit anti-Cya A serum
IMMUNO-DETECTION
HRP-anti-rabbit IgG
Develop with DAB developer
Colour reaction
RADIOISOTOPE DETECTION
Biotinylated anti- rabbit IgG
125I- Streptavidin
Autoradiography

temperature. The nitrocellulose was then washed in blocking buffer and overlaid with 5μCi ^{125}I-streptavidin (Amersham) for 1h at room temperature. Unbound isotope was removed by washing (6 x 10min) in blocking buffer and the nitrocellulose was then exposed for 3-5 days to blue sensitive X-ray film (Genetic Research Instrumentation Ltd) with intensifying screens at 70°C.

2.12 THIN-LAYER CHROMATOGRAM ASSAY

2.12.1 Preparation of glycolipids

Sheep RBCs were isolated from fresh whole blood as described in Section 2.9.1.1. From 60 ml of defibrinated sheep blood, 30 ml of 50% volume RBCs were prepared. Glycolipids were isolated from RBCs by a method adapted from that of Saito and Hakamori (1971). Bovine brain gangliosides were obtained from Calbiochem, and consisted of a mixture of the gangliosides GM_1, GD_{1a}, GD_{1b}, and GT_{1b}.

RBCs were homogenised with 20 volumes (600ml) of chloroform: methanol (2:1) with a mortar and pestle. The homogenate was filtered through a Buchner funnel using 7.5 cm charcoal filter paper. The filter paper and retentate were re-extracted with 10 volumes (100ml) of chloroform: methanol (1: 2) + 3% H_20 and then filtered through a Buchner funnel. The combined filtrates were refiltered, shell dried in a rotary evaporator, and weighed. The yield of this procedure ranged from 200-500 mg. The lipid was resuspended in chloroform : methanol (2:1), filtered and stored at -20°C.

2.12.2 Chromatogram overlay assays

Glycolipids were chromatographed on aluminium-backed silica gel thin-layer plates (Merck LTD), which had been prebaked for 30 min at 100°C. The chromatograms were developed with chloroform:methanol : KCl (0.1%) [5: 4: 1, by

vol.] unless otherwise stated. To visualise the lipids the plates were stained with resorcinol reagent (Appendix 4). Unstained chromatograms were run in parallel for use in overlay assays.

Binding of CyaA to glycolipids separated on these chromatograms was investigated by a method adapted from that of Magnani *et al* (1980). Chromatograms were dipped into a solution of poly-isobutylmethacrylate (0.1%) in hexane, air dried and incubated in blocking buffer (3% powdered milk in TBS containing 2mM $CaCl_2$). The blocked chromatograms were then overlaid with 50µg/ml CyaA purified from BP348 (pRMB1), incubated overnight at room temperature (unless otherwise stated), and then washed (3x 10 min) with blocking buffer. The chromatograms were probed with monospecific anti-CyaA serum (1/1000 dilution in blocking buffer) with shaking for 1h at room temperature, followed by washing as described above. Two procedures were then tested to detect the binding of CyaA to the separated lipids as described below in sections 2.12.2.1 and 2.12.2.2 and in **Figure 2.3**.

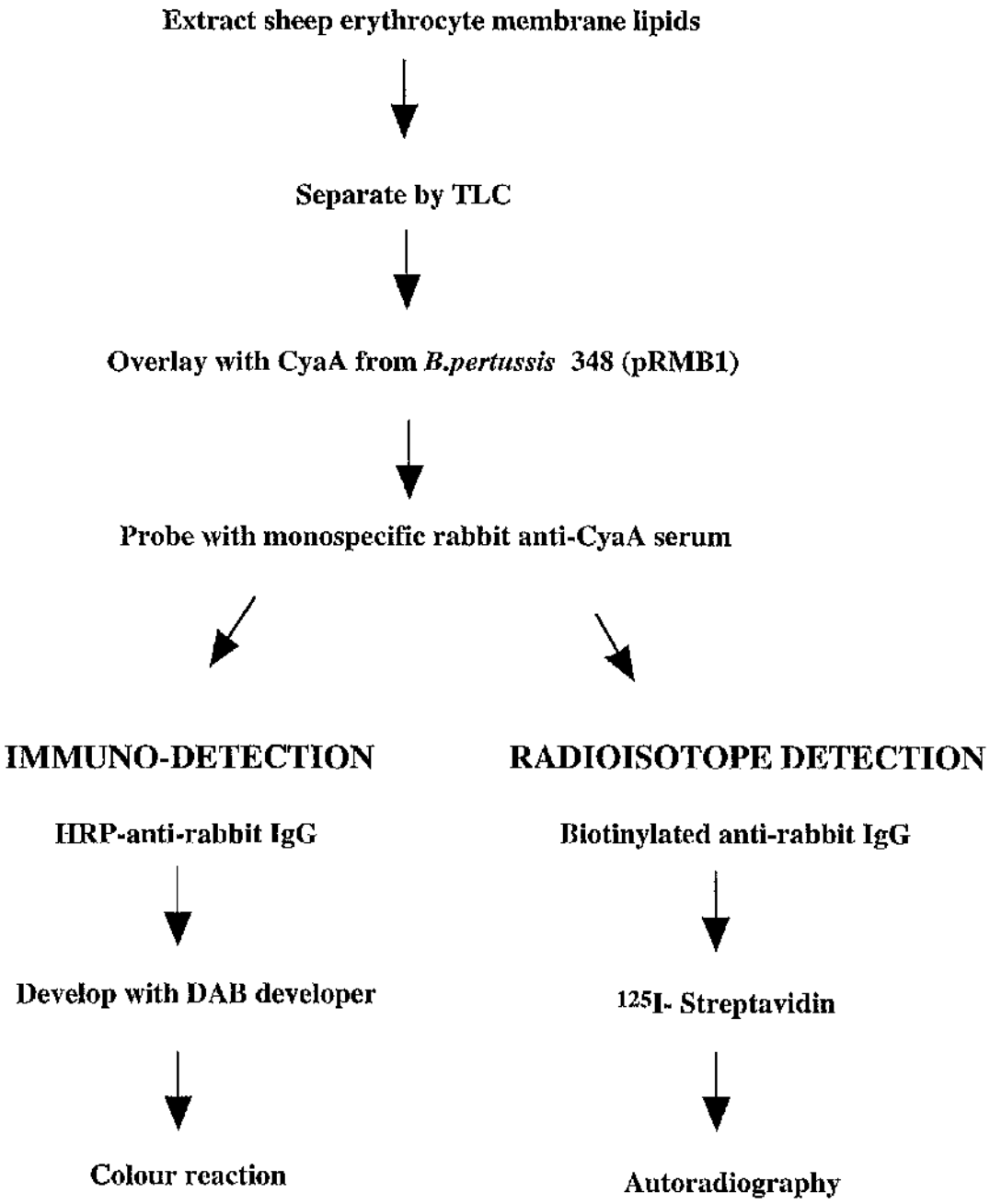
2.12.2.1 Immunodetection

Chromatograms were overlaid with HRP-labelled anti-rabbit IgG (SAPU, 1/1000 dilution in blocking buffer), shaken for 1h at oom temperature, washed and then developed with a DAB developer solution (section 2.7).

2.12.2.2 Radioisotope- detection

Chromatograms were overlaid with biotinylated anti-rabbit IgG (Amersham) (1/400 dilution in blocking buffer) for 1h at room temperature, washed in blocking buffer and overlaid with 5µCi of ^{125}I-streptavidin (Amersham) for 1h at room temperature. Unbound isotope was removed by washing (6x 10min) with distilled water and the chromatogram was exposed for 3-5 days to blue sensitive X-ray film (Genetic Research Instrumentation Ltd) with intensifying screens at 70°C.

Figure 2.3 ***Overlay assays to detect binding of CyaA to membrane lipids***


Extract sheep erythrocyte membrane lipids
Separate by TLC
Overlay with CyaA from *B.pertussis* 348 (pRMB1)
Probe with monospecific rabbit anti-CyaA serum
IMMUNO-DETECTION
HRP-anti-rabbit IgG
Develop with DAB developer
Colour reaction
RADIOISOTOPE DETECTION
Biotinylated anti-rabbit IgG
^{125}I- Streptavidin
Autoradiography

2.13 HAEMOLYSIS ASSAY

The haemolytic activity of native and recombinant Cya A was determined by a microtitre assay adapted from Hewlett *et al*, (1993). Sheep erythrocytes (sRBCs) were isolated from whole blood as described earlier (section 2.9.1.1), and diluted in haemolysis assay buffer (PBS cintaining 2mM $CaCl_2$). Haemolysis was measured after mixing sRBCs (10^8 cells) with dialysed extracts in 100 µl (final volume) and incubation for 3h at 37°C, unless otherwise stated. Reactions were also set up to measure any haemolysis induced by the appropriate buffers. Reactions were stopped by the addition of 150 µl of ice cold PBS to each well. From each well, 200 µl samples were removed and centrifuged at 2,000 x *g* for 10 min at 4°C (Biofuge). The supernatants (100µl) were then transferred to a fresh microtitre plate and OD $_{540nm}$ was determined in a spectrophotometer (Anthos Labtech instruments).

The background and 100% haemolysis values were determined by incubating sRBCs (10^8 cells) with haemolysis assay buffer and 1% saponin (final concentration), respectively, in an identical assay system. The haemolytic activity of extracts was determined by the following equation:

$$\text{Haemolysis (\%)} = \frac{\text{OD } 540\text{nm (extract)} - \text{OD } 540\text{nm (background)}}{\text{OD } 540\text{nm (saponin)}} \times 100$$

2.14 LIPOSOME ASSAY

2.14.1 Preparation of liposomes by cross-flow dialysis

Liposomes were prepared by cross flow dialysis of mixed micelles in an adaptation of methods described by Milsmann *et al* (1978) and Brunner *et al* (1976). The lipids used in the preparation of liposomes by this method,

distearoylphosphatidylcholine (DSPC) and dicetyl phosphate (DCP), were purchased from Sigma.

The lipids DSPC :DCP (molar ratio 9:1) were dissolved in chloroform and dried *in vacuo* at 40°C using a rotary evaporator (Buchi). A mixed micelle solution was then prepared by combining the dried lipid with the detergent, sodium cholate (Sigma) in a molar ratio 0.62 : 1 (lipid: detergent). The fluorescent calcein buffer (6 ml of 80mM calcein (Sigma), 150mM NaCl, 2mM $CaCl_2$, 0.5mM $MgCl_2$, pH 8.0) was then added. Liposomes encapsulating the fluorescent marker were prepared by removal of the detergent from the lipid by cross-flow dialysis through dialysis membranes (molecular weight cut off, 10,000 kDa) against 150mM NaCl, 2mM $CaCl_2$, 0.5mM $MgCl_2$, pH 8.0 using a Liposomat liposome maker (Dianorm). Flow rate was set at between 1 ml and 2 ml/min.

2.14.2 Preparation of liposomes by freeze-thawing and chromatography

The lipids used in the preparation of liposomes were dimyristoyl phosphatidyl choline (DMPC), cholesterol (C), dicetyl phosphate (DCP), stearylamine (SA), and trisialoganglioside (GT_{1b}) and were purchased from Sigma. Different combinations of these lipids were used to alter the charge and composition of liposomes. Liposomes were prepared from DMPC : C : x (molar ration of 1.0 : 0.75 : 0.03) where x was either DCP, SA or GT_{1b}, by adaptation of the method of Pick *et al* (1981).

Lipids were dissolved in chloroform, mixed in appropriate combinations (12.9µmol lipid in total) and dried *in vacuo* at 40°C using a rotary evaporator (Buchi). The dried lipid was emulsified in 3ml of calcein buffer (80mM calcein, Sigma, 150mM NaCl, 2mM $CaCl_2$, 0.5mM $MgCl_2$, pH 8.0) by sonication in a bath sonicator (Ultrasonics Ltd) for 2-3 min. The emulsion was then incubated for 90min at 37°C to allow the liposomes to swell. The multilamellar vesicles were observed

under phase contrast microscopy and then broken down to small unilamellar vesicles by sonication for 4 x 1min with a probe sonicator (Soniprep-150) at constant temperature.

Large unilamellar vesicles were prepared by 2 cycles of freezing multi-lamellar vesicles in liquid nitrogen and thawing at 37°C. The vesicles were equilibrated by incubation at 37°C for 90 min. To remove unsequestered calcein, the liposomes were passed twice through a Sephadex G-50 column (10 x 1.5 cm), and eluted with 50mM NaCl, 2mM $CaCl_2$, 0.5mM $MgCl_2$, pH 8.0) (Liposome assay buffer).

2.14.3 Pore formation assay

Release of the fluorescent marker (calcein) from liposomes was measured at room temperature using a fluorescence spectrophotometer (Cytofluor 2300). Excitation and emission were set at 485 nm and 530 nm respectively. Assays were carried out by mixing liposomes (50 µl) with the appropriate dialysed extracts in 200µl (final volume) or dialysed extract buffer as a control. These assays were carried out in flat bottomed 96 well plates (Nunc 96) and fluorescence was measured for 300 min. Spontaneous release (background) was determined by measuring marker release from the liposomes in the Liposome assay buffer (200 µl final volume). Full marker release (100%) was determined by treating liposomes with 2% Triton-X 100 (final concentration).

The activity of extracts on liposomes was determined by the following equation:

$$\text{Activity (\%)} = \frac{\text{Fluorescence (extract or buffer) - Fluorescence (background)}}{\text{Fluorescence (Triton X-100)}} \times 100$$

SECTION 3
RESULTS

3.1 PURIFICATION AND CHARACTERISATION OF ADENYLATE CYCLASE TOXIN

3.1.1 Purification of adenylate cyclase toxin from *B.pertussis* 348 (pRMB1)

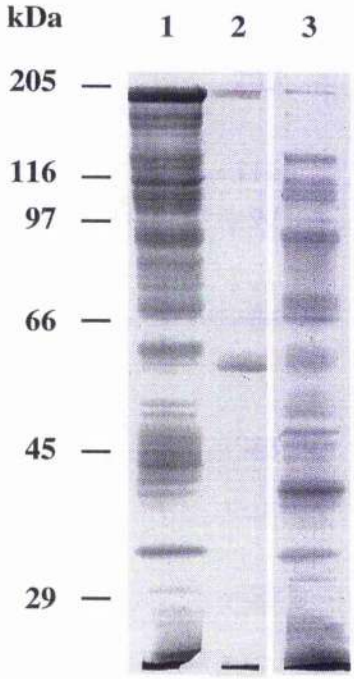
Adenylate cyclase toxin (CyaA) was prepared from *B.pertussis* 348 (pRMB1) by a two stage process involving an initial treatment of whole cells with urea, followed by purification of this urea extract by calmodulin-agarose (CaM)-affinity chromatography as described in section 2.2. Treatment of whole cells, from BP348 (pRMB1), with urea resulted in the extraction of a number of polypeptides, including high levels of a protein with a molecular weight of approximately 200 kDa (**Lane 1, Figure 3.1a**). When the urea extract was subjected to CaM-affinity chromatography, the 200 kDa protein, which was believed to be CyaA, was purified (**Lane 2, Figure 3.1a**). However, the chromatography step also resulted in the purification of a 60 kDa polypeptide (**Lane 2, Figure 3.1a**). This protein band was probably a degradation product of the full length adenylate cyclase toxin and corresponded to the catalytic domain of CyaA (see section 1.4.1.1). CyaA interacts with calmodulin, and this property was used in the purification process. The other components of the urea extract did not interact with the calmodulin and were collected in the flow through from the chromatography column (**Lane 3, Figure 3.1a**). Probing of an immunoblot with monospecific anti-CyaA serum confirmed that the 200 kDa protein purified by CaM-agarose chromatography, was CyaA (**Lane 2, Figure 3.1b**).

On a number of occasions attempts were made to purify CyaA from *B.pertussis* DE386, but the urea extract did not penetrate the calmodulin-agarose column.

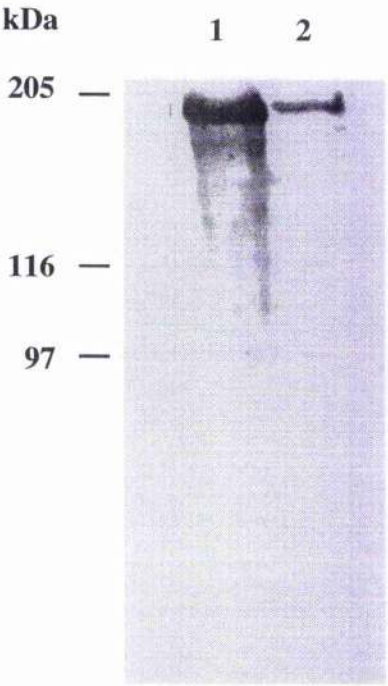
Figure 3.1a ***Purification of CyaA from B.pertussis 348 (pRMB1) - SDS-PAGE analysis.*** Crude urea extracts from *B.pertussis* 348 (pRMB1) were purified by CaM-agarose affinity chromatography. Samples (50μg) were separated by SDS-PAGE and the gels were stained with Coomassie blue. Lane 1, crude urea extract of CyaA from *B.pertussis* 348 (pRMB1); Lane 2, affinity column purified CyaA from *B.pertussis* 348 (pRMB1); Lane 3, flow through from affinity column.

Figure 3.1b ***Purification of CyaA from B.pertussis 348 (pRMB1) - Western blot analysis.*** Crude urea and CaM-agarose affinity column purified extracts from *B.pertussis* 348 (pRMB1) which were run on SDS-PAGE, transferred to nitrocellulose and probed with monospecific anti-CyaA serum as described in section 2.7. Lane 1, crude urea extract of CyaA from *B.pertussis* 348 (pRMB1); Lane 2, affinity column purified CyaA from *B.pertussis* 348 (pRMB1).

3.1a



3.1b



3.1.2 Purification of recombinant adenylate cyclase toxin from *E.coli*

Competent cells of *E.coli* DH5α F'IQ were transformed with DNA from the plasmids pCACT-3, pDLACT-1 and pIC20H (**Figure 2.1**). The recombinant plasmid genes were under control of the *lac* promoter and thus inducible with IPTG (Betsou *et al*, 1993). Growth of these strains was followed spectrophotometrically until mid-exponential phase, when they were induced with IPTG (**Figure 3.2**). After induction, *E.coli* DH5α F'IQ (pCACT-3) and *E.coli* DH5α F'IQ (pDLACT-1) demonstrated a considerable increase in synthesis of a 200 kDa protein with time (**Figure 3.3**). The control strain [*E.coli* DH5αF'IQ (pICNOH)], harbouring the vector plasmid which did not contain *cya* inserts, did not synthesize this 200 kDa protein. Treatment of the cells from *E.coli* DH5α F'IQ (pCACT-3) and *E.coli* DH5α F'IQ (pDLACT-1) with urea resulted in the partial purification of the 200 kDa protein (**Lane 4, Figure 3.4a**). This protein was not purified from the control strain (**Figure 3.4a**). When the urea extracts from *E.coli* DH5α F'IQ (pCACT-3) (**Lane 1**) and *E.coli* DH5α F'IQ (pDLACT-1) (**Lane 2**) were analysed by Western Blotting, using monospecific anti-CyaA serum, the 200 kDa protein was identified as CyaA (**Figure 3.4b**).

3.1.3 Enzymic activity of adenylate cyclase toxin

To further characterise the extracts purified from *B.pertussis* and *E.coli* an adenylate cyclase enzymic assay was carried out as described in the section 2.4 (Salomon *et al* , 1974). The assay measured the conversion of (α^{32}P) ATP to (^{32}P) cAMP. The specific activities for the different extracts are shown in **Table 3.1**. These results demonstrated that catalytically active forms of CyaA had been purified from the recombinant *E.coli* strains and from *B.pertussis* 348 (pRMB1). The extracts tested had similar enzymic activities which were within a factor of two. These results revealed that both activated and non-activated forms of CyaA, were catalytically active. Thus, enzymic activity was not dependent upon toxin activation. The specific activities for the

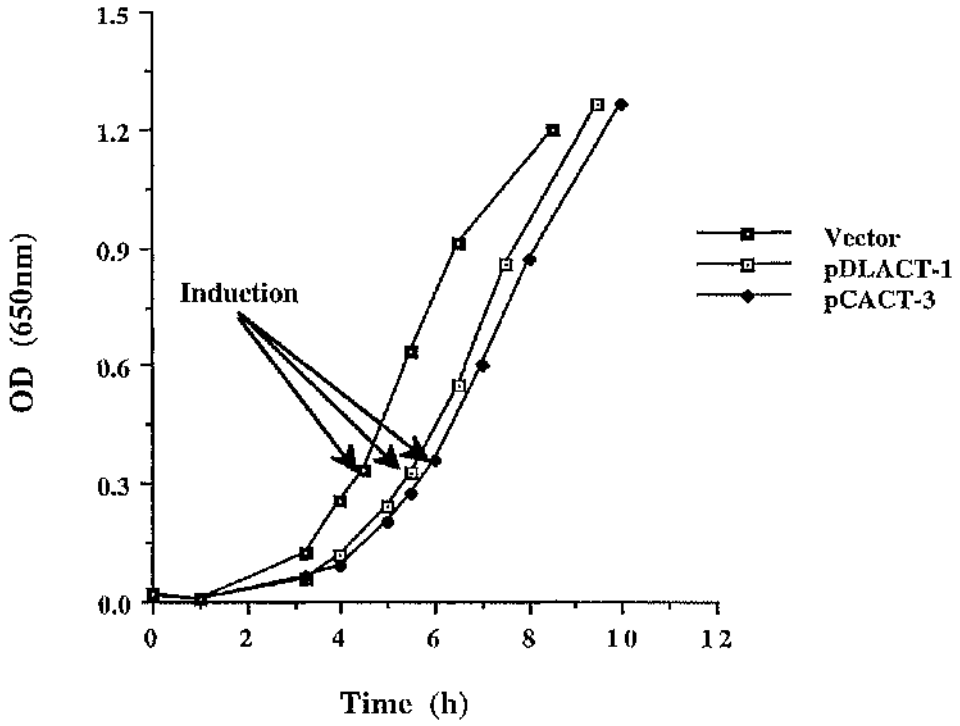


Figure 3.2 ***Purification of recombinant CyaA : Growth and induction of recombinant E.coli strains.*** The *E.coli* strains were grown at 37°C until OD_{650nm} reached 0.3-0.4, whereupon the cells were induced with 1mM IPTG and grown for a further 4h to express the recombinant proteins (see section 2.2.2). The strains grown were Vector = *E.coli* DH5αF'IQ (pICN0H), pDLACT-1 =*E.coli* DH5αF'IQ (pDLACT-1) and pCACT-3 = *E.coli* DH5αF'IQ (pCACT-3).

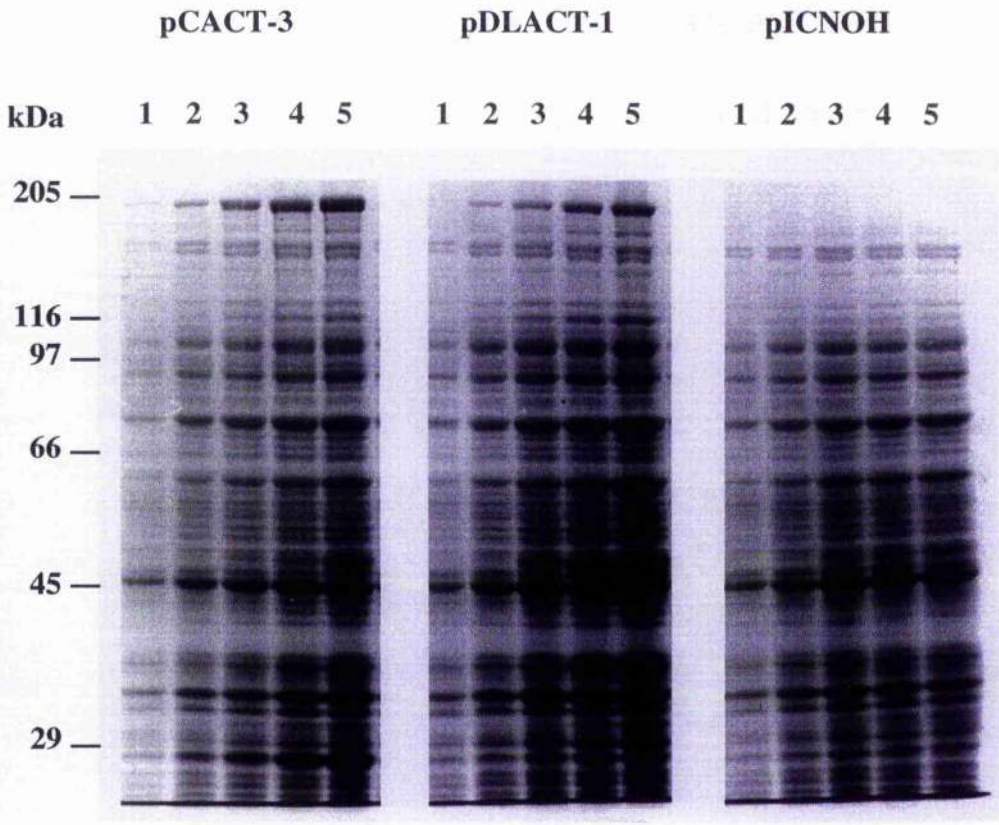
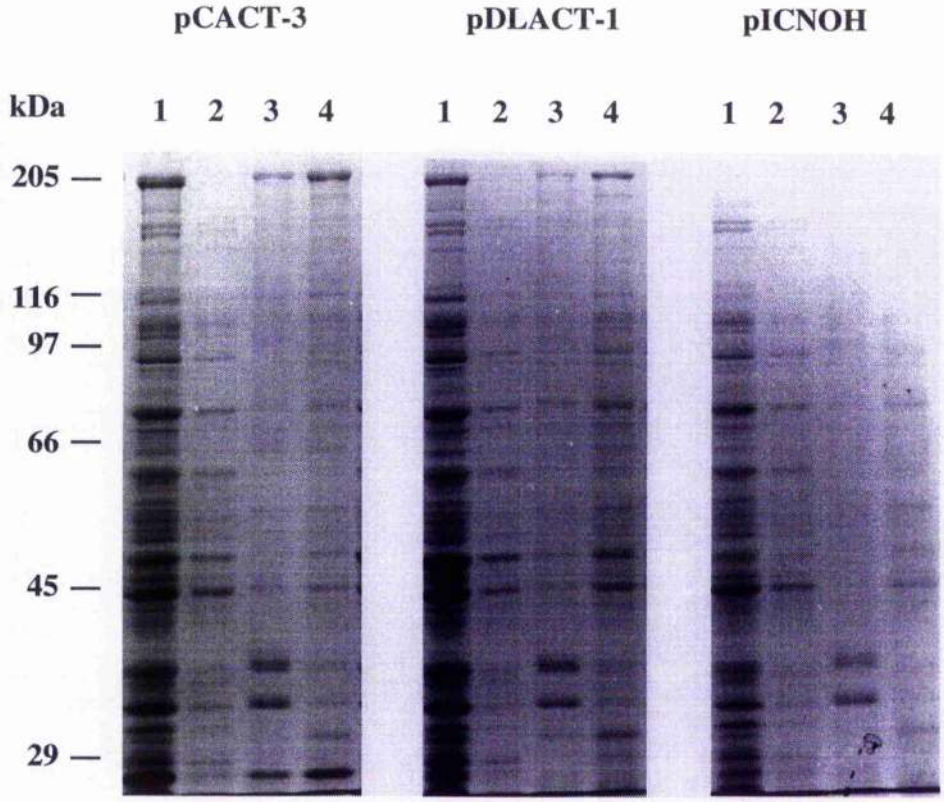


Figure 3.3 ***Purification of recombinant CyaA: Expression of recombinant CyaA in E.coli .*** Host strain *E.coli* DH5aF'IQ harbouring either pCACT-3, pDLACT-1 or pIC2OH was induced with IPTG. Samples were taken at intervals from the three strains and 50 µl of these were separated by SDS-PAGE. Gels were stained with Coomassie blue. Lane 1, non induced cells; Lane 2, 1h after induction; Lane 3, 2h after induction; Lane 4, 3h after induction; Lane 5, 4h after induction.

Figure 3.4a ***Purification of recombinant CyaA : Urea extraction of recombinant E.coli strains.*** Recombinant proteins were expressed in *E.coli* DH5αF'IQ harbouring either pDLACT-1, pCACT-3 or pICNOH and extracted with urea as described in section 2.2.2. The proteins (50μg) were then separated by SDS-PAGE and the gels were stained with Coomassie blue. Lane 1, total cell protein; Lane 2, soluble protein fraction; Lane 3, insoluble protein fraction; Lane 4, urea extract.

Figure 3.4b ***Purification of recombinant CyaA : Western Blot analysis.*** Urea extracts of recombinant proteins were separated by SDS-PAGE, transferred to nitrocellulose by Western Blotting and probed with monospecific anti-Cya A serum as described in section 2.7. Lane 1, CyaA from *E.coli* DH5αF'IQ (pCACT-1); Lane 2, CyaA from *E.coli* DH5αF'IQ (pDLACT-1); Lane 3, extract from *E.coli* DH5αF'IQ (pICNOH).

3.4a


pCACT-3
pDLACT-1
pICNOH
kDa
1 2 3 4
1 2 3 4
1 2 3 4
205
116
97
66
45
29

3.4 b

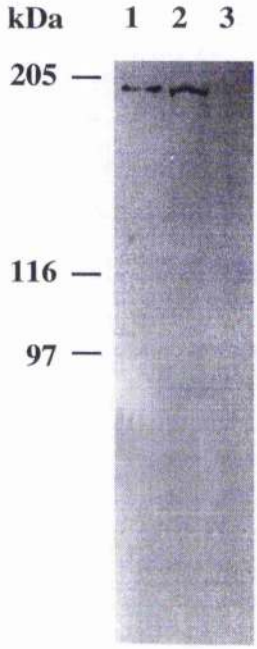

kDa
1 2 3
205
116
97

Table 3.1 ***Determination of adenylate cyclase enzymic activity of CyaA extracts by the Salomon assay***

Strain	Activation	Specific Activity [a] (µmol cAMP/min/mg protein)
B. pertussis 348 (pRMB1)[b]	activated	32
E.coli. DH5αF'IQ (pCACT-3)[c]	activated	42
E.coli DH5αF'IQ (pDLACT1)[c]	non-activated	34
E.coli (pGW22)[d]	non activated	26

[a]Mean of 3 determinations

[b]CaM-agarose affinity chromatography purified extract

[c]Urea extract

[d]Donated by Dr Gareth Westrop, University of Glasgow

recombinant CyaA proteins were slightly lower, but still similar to those achieved by other investigators (Westrop *et al*, 1986). It was decided to use the protein concentration of the extracts, as determined by the method of Smith *et al* (1985), to standardise them for future assays.

3.1.4 Toxic activity of adenylate cyclase toxin

To characterise the toxicity of extracts a Baby Hamster Kidney (BHK) cell shape change assay was carried out. The assay proved to be a rapid and sensitive assay for the invasive adenylate cyclase toxin (Westrop *et al*, 1994). The assay was based on the principal that increases of cAMP levels in BHK cells, induced by agents such as CyaA, resulted in altered cell morphology, called stellation. The degree of stellation was used as a measure of the toxicity of extracts (described in section 2.5). The shape change assay demonstrated that activated forms of CyaA purified from BP348 (pRMB1) and *E.Coli* DH5αF'IQ (pCACT-3) were toxic to BHK 21 cells. This toxicity was dose-dependent (**Figure 3.5**). Determination of the concentration of CyaA giving half-maximal stellation gave an indication of the relative toxicity of the extracts. For CyaA, purified from BP348 (pRMB1) and *E.coli* DH5αF'IQ (pCACT-3), half maximal stellation occurred at protein concentrations of 0.3 μg/ml, and 0.52 μg/ml, respectively. The half maximal values for the extracts were similar to those found by Westrop *et al* (1994).

The non-activated form of CyaA purified from *E.coli* DH5αF'IQ (pDLACT-1) was non-toxic (stellation < 5%) to BHK cells in the shape-change assay (**Figure 3.5**). These results indicated that activation of CyaA was important for toxicity and agreed with toxicity studies carried out on other cell types (Betsou *et al*, 1993; Hewlett *et al*, 1993).

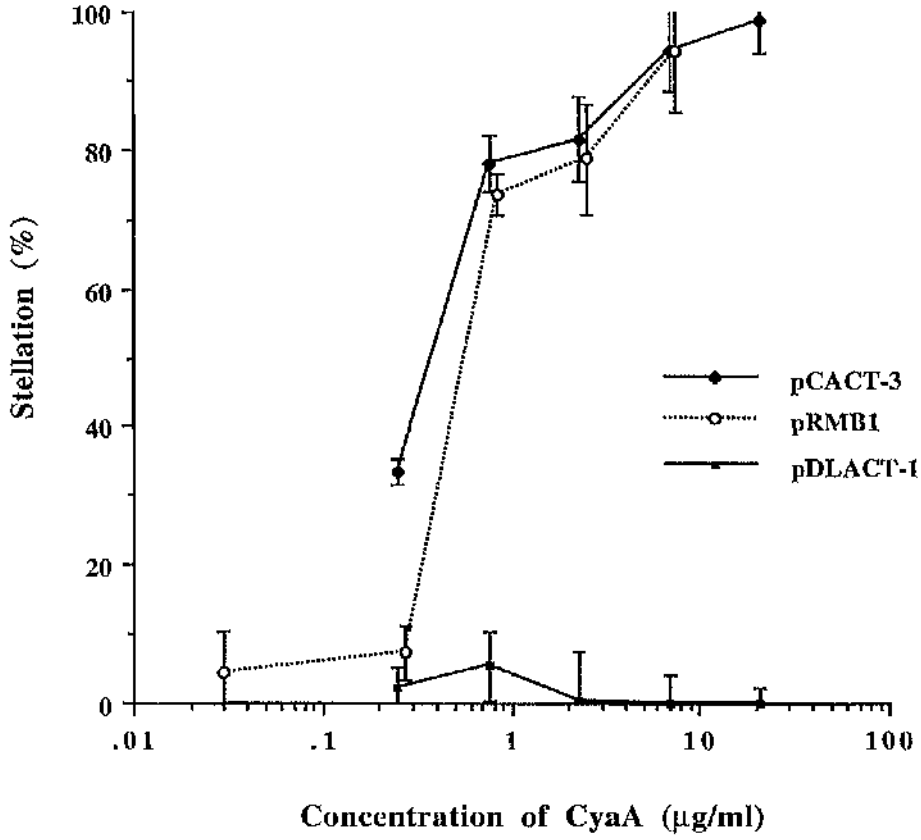


Figure 3.5 ***Stellation of BHK 21 cells by CyaA***

Toxicity of dialysed extracts was measured by the BHK 21 shape change assay. Mean cell area and perimeter were determined by analysis of 25 cells for each treatment. For the purposes of experiments, 100% stellation was defined as stellation caused by HH containing 1mM dibutyryl cAMP, 0.1 M IBMX, and test samples were compared to this value (Section 2.5). Bars represent the mean ± the S.E.M of 4 determinations from 2 independent experiments.

pRMB1 = CyaA purified from BP348 (pRMB1) by affinity chromatography

pCACT-3 = Urea extract of activated CyaA from *E.coli.* DH5αF'IQ (pCACT-3)

pDLACT-1 = Urea extract of non-activated CyaA from *E.coli.* DH5αF'IQ (pDLACT-1)

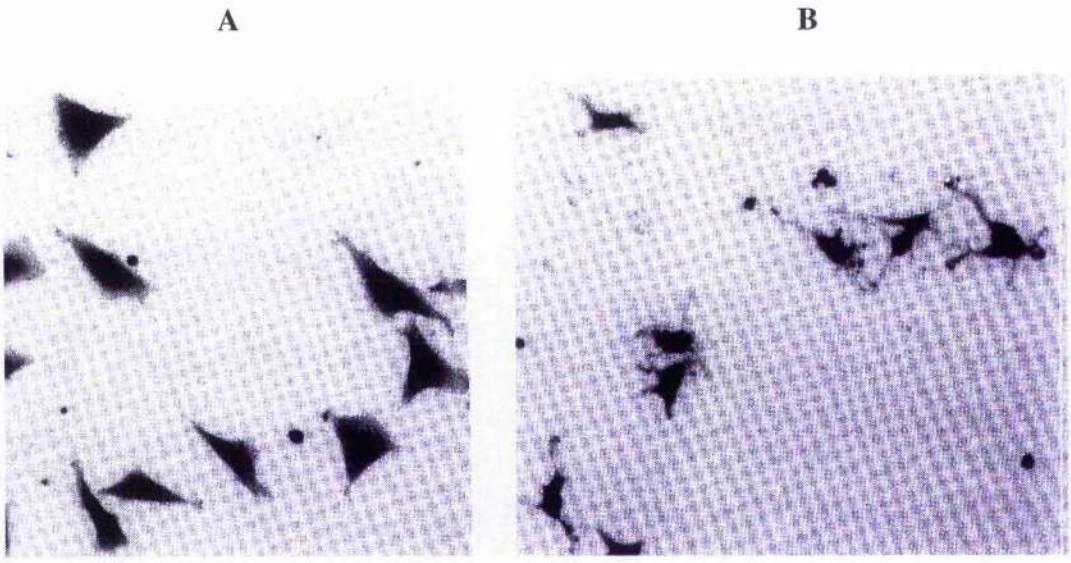


Figure 3.5a ***Stellation of BHK21 cells*** (Source: Westrop *et al*, 1994).
BHK shape change assay was performed by the same method as described in this thesis (section 2.5). Plate **A** shows unstellated control BHK cells. Plate **B** shows cells after treatment with 1mM disbutyryl cAMP and 0.1M IBMX. Similar results were obtained in this study when cells were treated with activated extracts of CyaA as described in **Figure 3.5**, but photographs were not available.

3.2 BINDING OF ADENYLATE CYCLASE TOXIN TO CELLS

3.2.1 Binding to sheep erythrocytes

To investigate the interaction of CyaA with target cells a whole-cell binding assay was employed. The binding assay was based on the fact that when CyaA bound to cell membranes, and the cells were lysed, the CyaA-membrane complex could be separated from unbound toxin and target cell components by centrifugation. When the membrane pellet was washed, resolved by SDS-PAGE, and transferred to nitrocellulose by Western Blotting, CyaA could be detected by probing with monospecific anti-CyaA serum (Section 2.10).

Before looking at the binding process in more detail it was necessary to demonstrate that CyaA would indeed bind to sheep erythrocytes, a convenient target cell system. As the incubation time of CyaA with sheep erythrocytes was increased there was an increase in the intensity of the 200 kDa CyaA protein band detected on an immunoblot from CyaA treated cells. The majority of CyaA was bound by 30 min (**Lane 3**) and increased only slightly after 60 min (**Lane 4**) and 120 min (**Lane 5, Figure 3.6**) as detected by the binding assay employed. Untreated erythrocytes did not show the presence of the 200 kDa protein band (**Lane 2, Figure 3.6**). When sheep erythrocytes were incubated with increasing concentrations of CyaA, there was a concomitant increase in the amount of toxin which was bound (**Lanes 3-8, Figure 3.7**). Binding could be detected when erythrocytes were incubated with as little as 5 μg/ml of CyaA (**Lane 7, Figure 3.7**). These results demonstrated that CyaA bound to sheep erythrocytes in a time-dependent (**Figure 3.6**) and dose-dependent (**Figure 3.7**) manner.

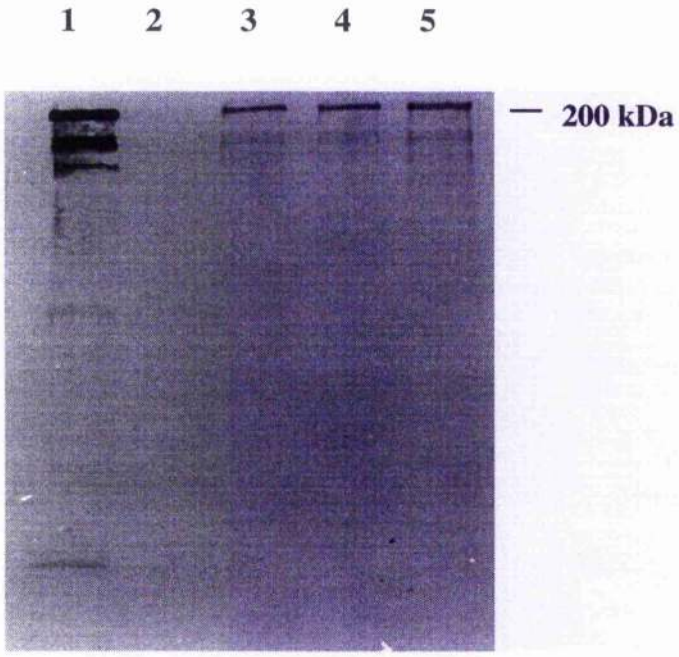


Figure 3.6 ***Effect of incubation time on binding of CyaA to sheep erythrocytes***
Sheep RBCs were incubated with dialysed CyaA (100 μg/ml), purified from BP348 (pRMB1). RBC membrane ghosts were prepared and resolved by SDS-PAGE. Bound toxin was detected by transferring membrane proteins to nitrocellulose and probing with anti-CyaA serum, as described in section 2.10. Lane 1, CyaA control (10 μg); Lane 2, untreated RBC control (10 μg); Lane 3, 4 and 5, RBCs incubated with CyaA for 30, 60, and 120 min, respectively.

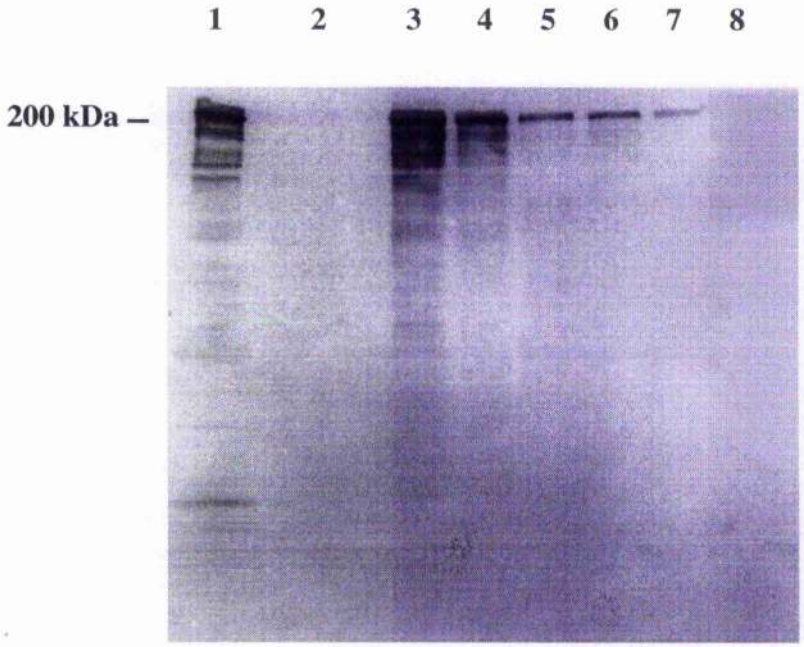


Figure 3.7 ***Effect of concentration of CyaA on its binding to sheep erythrocytes***
Sheep RBCs were incubated with dialysed CyaA, purified from BP348 (pRMB1), for 1h at 37°C. RBC membrane ghosts were prepared and resolved by SDS-PAGE. Bound toxin was detected by transferring membrane proteins to nitrocellulose and probing with anti-CyaA serum, as described in section 2.10.. Lane 1, CyaA control (10 µg); Lane 2, untreated RBC control (10 µg); Lanes 3-8, RBCs incubated with 100, 50, 20, 10, 5 and 1 µg/ml CyaA, respectively.

3.2.2 Binding of adenylate cyclase to trypsinised sheep erythrocytes

There has been limited research into the specificity of the interaction of CyaA with target cells. Therefore, a study was carried out to determine if proteolytic treatment of sheep erythrocytes affected the subsequent binding of CyaA. Erythrocytes were treated with a range of concentrations of trypsin as described in section 2.10.1. Treatment of sheep erythroctes with trypsin altered the SDS-PAGE profile of the erythrocyte membrane proteins (**Figure 3.8**). As the concentration of trypsin was increased, there was a subsequent increase in cleavage of the membrane proteins demonstrated by loss of a number of the protein bands (**Lanes 2-5, Figure 3.8**). This indicated that trypsinisation of the erythrocytes altered their surface composition.

When CyaA was incubated with trypsinised erythrocytes the toxin was not detected in the membrane fraction of the cells. (**Figure 3.9a**). This occurred when cells were treated with as low as 25μg/ml trypsin (**Lane 3, Figure 3.9a**). However, CyaA was detected in the membranes of the control cells (**Lane 2**) which were not trypsinised (**Figure 3.9a**). These results suggested that CyaA did not bind to trypsinised erythrocytes. In contrast, trypsinised erythrocytes were found to be more sensitive to haemolysis by CyaA. The haemolytic activity of CyaA was dependent upon the concentration of trypsin used to treat the erythrocytes.(**Figure 3.9b**).

Proteolytic degradation of CyaA, due to low levels of residual trypsin, may have resulted in the loss of binding of CyaA to trypsinised cells. To test this a binding assay was carried out in which a cocktail of protease inhibitors had been incorporated. Cells were treated with trypsin, washed as before, and then incubated with CyaA in the presence of PMSF (200μM), leupeptin, (1μM) and pepstatin (1μM). The cocktail provided a broad range of protease protection (Boehringer-Mannhein, Biochemical Information Leaflet, May 1987). However, CyaA did not bind to trypsinised erythrocytes, when co-incubated with the protease inhibitors (**Lanes 4-7, Figure 3.10**). This suggested that it was not degradation of CyaA by trypsin that prevented

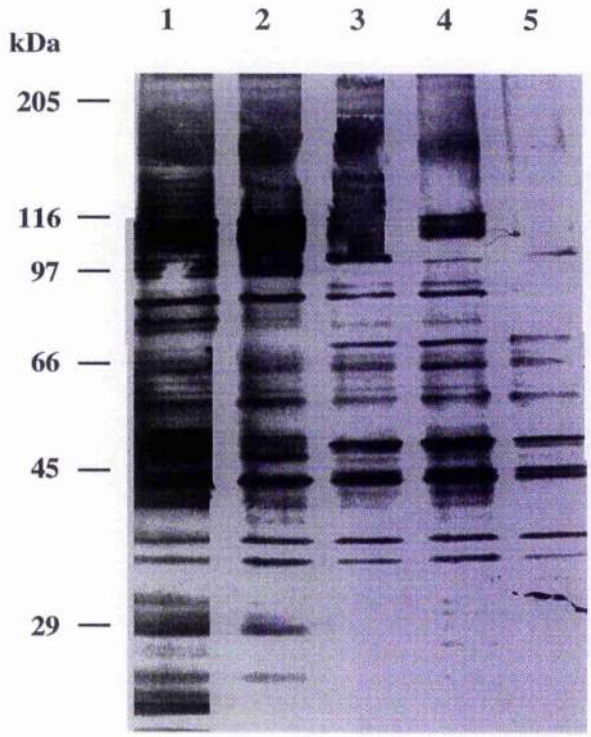


Figure 3.8 ***SDS-PAGE profile of trypsinised erythrocytes membrane proteins.***

Sheep RBCs were pretreated with trypsin (25-100 µg/ml) for 60 min at 37°C. After washing the RBC's were lysed and the membranes proteins were separated by SDS-PAGE and stained with Silver stain as described in section 2.6. Lane1, untreated RBC control (10 µg); Lanes 2-5 RB's treated with 25, 50, 75 and 100 µg/ml trypsin, respectively

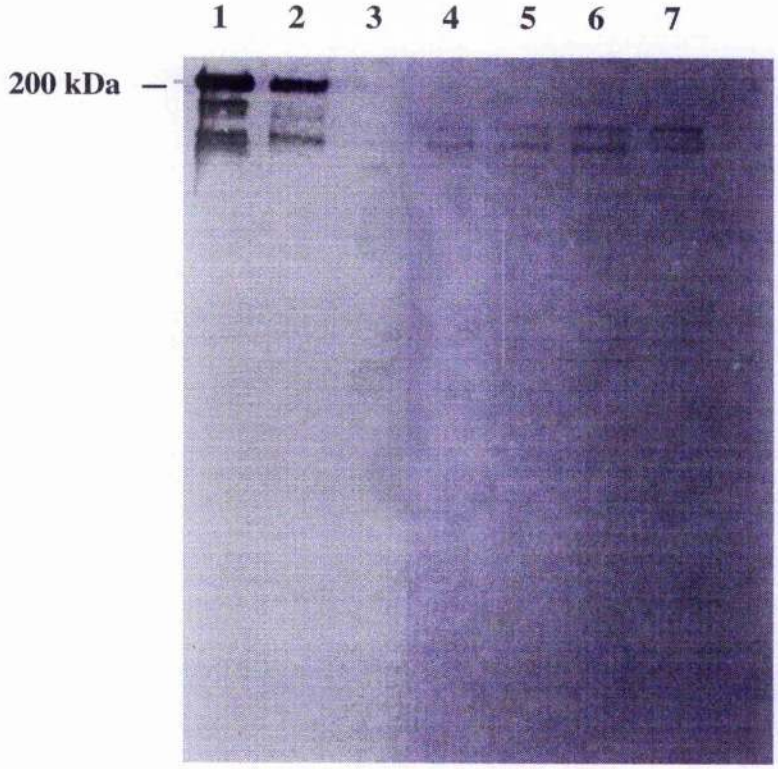
Figure 3.9a ***Binding of CyaA to trypsinised erythrocytes***

Trypsinised erythrocytes were incubated with 100 μg/ml of dialysed CyaA, purified from BP348 (pRMB1) , for 60 min at 37°C. RBC membrane ghosts were prepared and resolved by SDS-PAGE. Bound toxin was detected by transferring membrane proteins to nitrocellulose and probing with anti-CyaA serum, as described in section 2.10. Lane 1, CyaA control (10 μg) ; Lane 2, CyaA incubated with untreated RBCs; Lanes 3-6, CyaA incubated with RBCs pretrypsinised with 25, 50, 75 and 100 μg/ml trypsin respectively ; Lane 7 untreated RBCs.

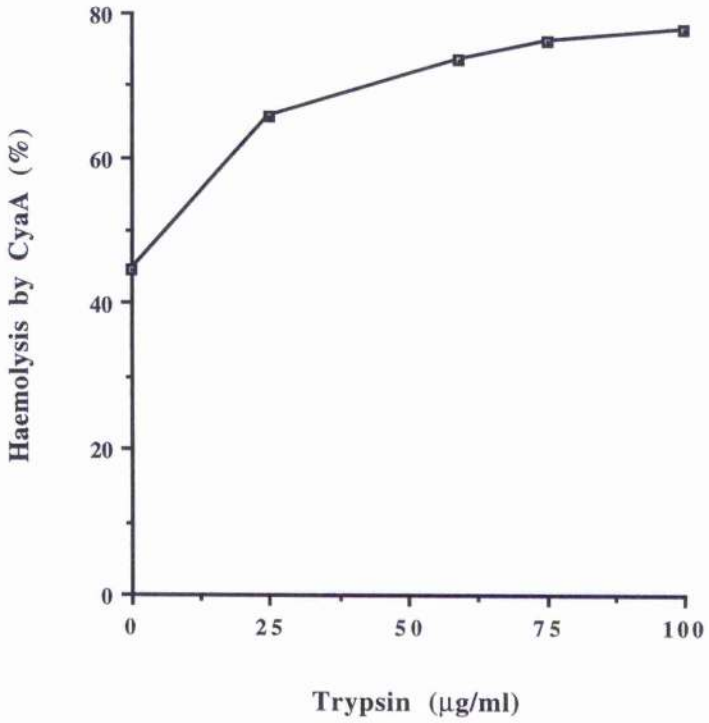
Figure 3.9b ***Haemolysis of trypsinised erythrocytes by CyaA***

Sheep RBCs were treated with a range of concentrations of trypsin. RBCs were washed and incubated with 100 μg/ml of dialysed CyaA, purified from BP348 (pRMB1), for 60 min at 37°C. Haemolysis of RBCs was measured as described in section 2.13.

3.9 a


1 2 3 4 5 6 7
200 kDa

3.9 b


Haemolysis by CyaA (%)
80
60
40
20
0
0
25
50
75
100
Trypsin (µg/ml)

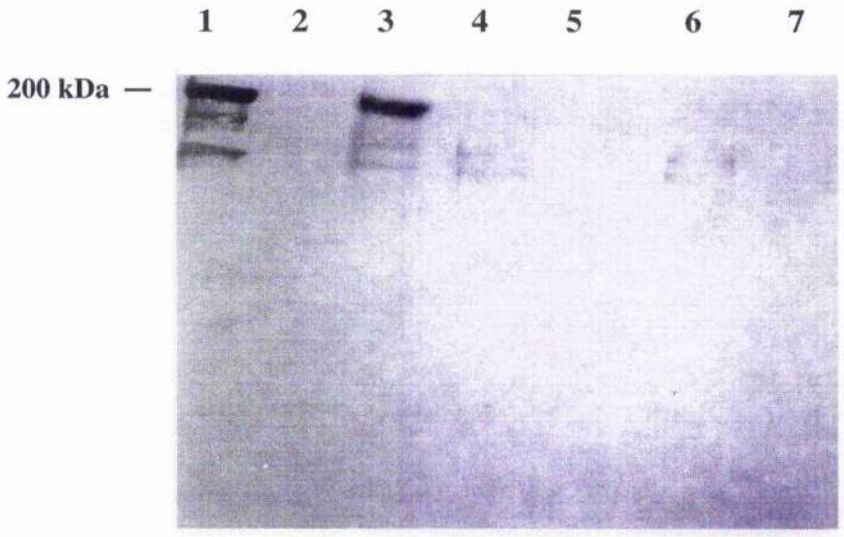


Figure 3.10 ***Binding of CyaA to trypsinised sheep erythrocytes in the presence of protease inhibitors.*** Trypsinised RBCs were incubated with 100 µg/ml of dialysed CyaA, purified from BP348 (pRMB1), along with a cocktail of protease inhibitors (see text) for 30min at 37°C. RBC membrane ghosts were prepared and resolved by SDS-PAGE. Bound toxin was detected by transferring membrane proteins to nitrocellulose and probing with anti-CyaA serum, as described in section 2.10. Lane 1, CyaA control (10µg); Lane 2, RBC control; Lane 3, CyaA/ inhibitors incubated with untrypsinised RBC's; Lanes 4-7, CyaA/inhibitors incubated with RBCs pre-trypsinised with 25, 50, 75 and 100 µg/ml trypsin, respectively.

the 'binding' of the toxin The loss of binding was probably due to the alteration in surface architecture of membrane receptors brought about by the protease activity.

3.2.3 Role of carbohydrates in cell-binding of adenylate cyclase toxin

The majority of receptors for bacterial toxins are carbohydrates and many of these contain contain sialic acid (see section 1.6). It has been suggested that the mammalian cell receptor for CyaA may also be a carbohydrate and in particular a ganglioside (sialic acid containing glycolipid), (Gable *et al*, 1985; Gordon *et al* , 1989). Therefore, the cell-binding assay was adapted to investigate if there was a carbohydrate involvement in the binding of CyaA to sheep erythrocytes. To test this a number of simple carbohydrates [D-galactose, D-glucose, D-mannose, D-maltose, D-fucose or N-acetyl neuraminic acid (NANA)] were examined to see if they blocked binding of CyaA to the cells by competition with a receptor. When CyaA was incubated with sheep erythrocytes, in the presence of these carbohydrates separately, it was found that none of the sugars tested prevented the binding of CyaA (**Lanes 4-9, Figure 3.11**), when compared to the binding control (**Lane 2, Figure 3.11**). This result suggests that either the carbohydrate moieties tested may not play an essential role in the binding of CyaA to erythrocytes, or that the receptor for CyaA may be an oligosaccharide of specific structure, not matched by these simple sugar residues.

3.2.4 Role of activation of adenylate cyclase toxin in cell-binding

B.pertussis CyaA is the product of the structural gene *cya* A. A number of the properties of CyaA are dependent upon prior activation, which is mediated by the product of the *cya* C gene (see section 1.4.2.2). To investigate the role activation played in the binding of CyaA to sheep erythrocytes, activated and non-activated forms of CyaA were prepared. Erythrocytes were incubated with the same concentration of activated and non-activated forms of CyaA and the binding analysed by the binding

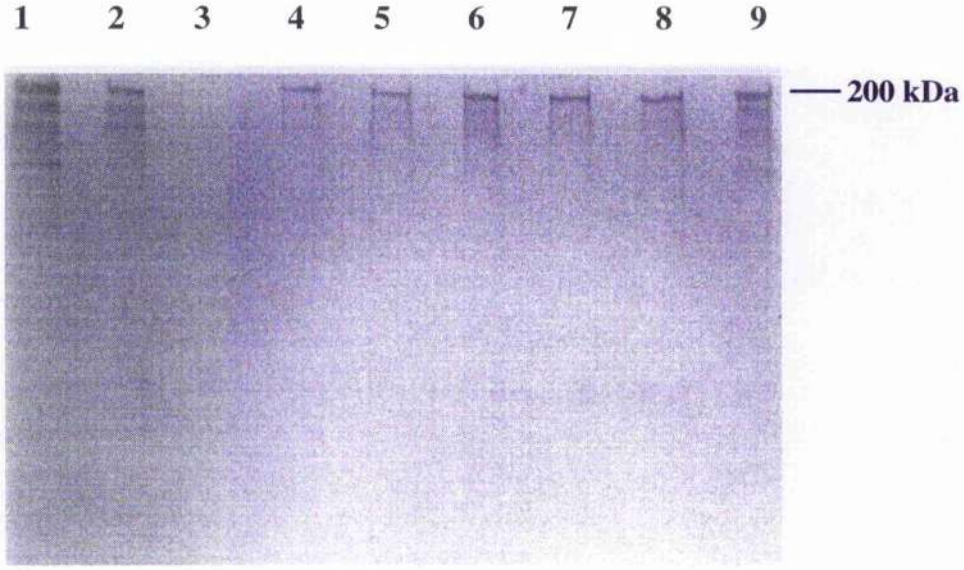


Figure 3.11 ***Binding of CyaA to sheep erythrocytes in the presence of different sugars***. Sheep RBCs were exposed to 100µg/ml of dialysed CyaA, purified from BP348 (pRMB1) and either 50 mM galactose, glucose, mannose, maltose, fucose or N-acetyl neuraminic acid (NANA). RBC membrane ghosts were prepared and resolved by SDS-PAGE. Bound toxin was detected by transferring membrane proteins to nitrocellulose and probing with anti-CyaA serum, as described in section 2.10. Lane 1, CyaA control (10 µg); Lane 2, RBC's incubated with CyaA ; Lane 3, RBC control; Lane 4-9, RB's incubated with CyaA and, Lane 4, galactose; Lane 5, glucose; Lane 6; mannose; Lane 7, maltose; Lane 8, fucose; Lane 9, NANA.

assay. **Figure 3.12** shows that both activated (**Lanes 6** and **8**) and non-activated forms (**Lanes 7** and **9**) of CyaA bound to sheep erythrocytes. However, visual analysis of the intensity of the 200 kDa toxin bands present indicated that slightly more activated CyaA (**Lanes 6** and **8**) had become bound to the erythrocytes in comparison to the non-activated extracts (**Lanes 7** and **9**) tested. This suggested that either more activated CyaA was bound, or that bound activated CyaA was bound more stongly, in comparison to the non-activated form. There was also some degradation of the CyaA protein demonstrated by the laddering pattern of the 200 kDa protein, particularly in **Lanes 1, 2, 6** and **7** of **Figure 3.12.**

The role of activation on binding specificity was further investigated by determining the binding of CyaA to various cell lines, namely Baby Hamster Kidney (BHK) cells , a murine macrophage cell line (J774.2) and a bovine lymphoma cell line (BL3). These cell lines were routinely cultivated in our department, and had been used by other groups investigating the toxicity of CyaA so were both convenient and of interest. The activated and non-activated forms of CyaA were also incubated with sheep erythrocytes as a control.

Figure 3.13 shows that activated CyaA purified from BP348 (pRMB1) (Lane 1) and *E.coli* DH5αF'IQ (pCACT-3) (**Lane 3**) bound to J774.2 cells. Non-activated CyaA, (supplied by Dr Gareth Westrop, Division of Infection and Immunity, University of Glasgow) did not bind to this cell type (**Lane 2, Figure 3.13**). Only a small amount of non-activated CyaA purified from *E.coli* DH5αF'IQ (pDLACT-1) bound to these cells (**Lane 4, Figure 3.13**). These results suggest that activation of CyaA is important in potentiating binding of the toxin to certain cell types. However, we did not detect binding of activated or non-activated forms of CyaA to either BHK or BL3 cells in this assay (**Figure 3.13**). The assay was repeated three times with BHK and BL3 cells, but no binding could be detected. BHK cells have been used routinely in adenylate cyclase toxicity assays by other investigators (Westrop *et al* ,1994; Westrop *et al* , 1996), strongly suggesting that the toxin does indeed bind to them. The

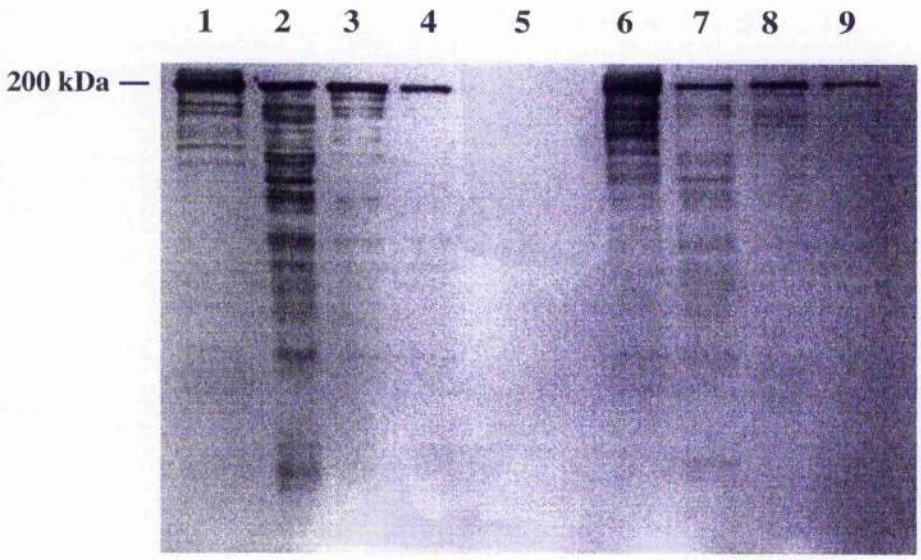


Figure 3.12 ***Role of activation in binding of CyaA to sheep erythrocytes.***

Sheep RBCs (2 x10^8 cells/assay) were incubated with 100 µg/ml of dialysed extracts of CyaA purified from *B.pertussis* 348 (pRMB1) and recombinant strains of *E.coli* for 60 min at 37°C. Bound toxin was detected by as described in section 2.10. In Lanes 1-4, 5 µg of toxin extracts were run as controls and in Lanes 5-9, 50µl samples were run. Lane 1, CyaA from BP348 (pRMB1); Lane 2, non-activated recombinant CyaA (pGW22)[a]; Lane 3, activated recombinant CyaA[b]; Lane 4, non-activated recombinant CyaA[c]; Lane 5, 10 µg of untreated RBC's; Lane 6-9 , RBC's exposed to: Lane 6, CyaA from BP348 (pRMB1); Lane 7, non-activated recombinant CyaA (pGW22); Lane 8, activated recombinant CyaA; Lane 9, non-activated recombinant CyaA.

[a] gift from Dr Gareth Westrop, University of Glasgow

[b] urea extract from *E.coli* DH5α F'IQ (pCACT-3)

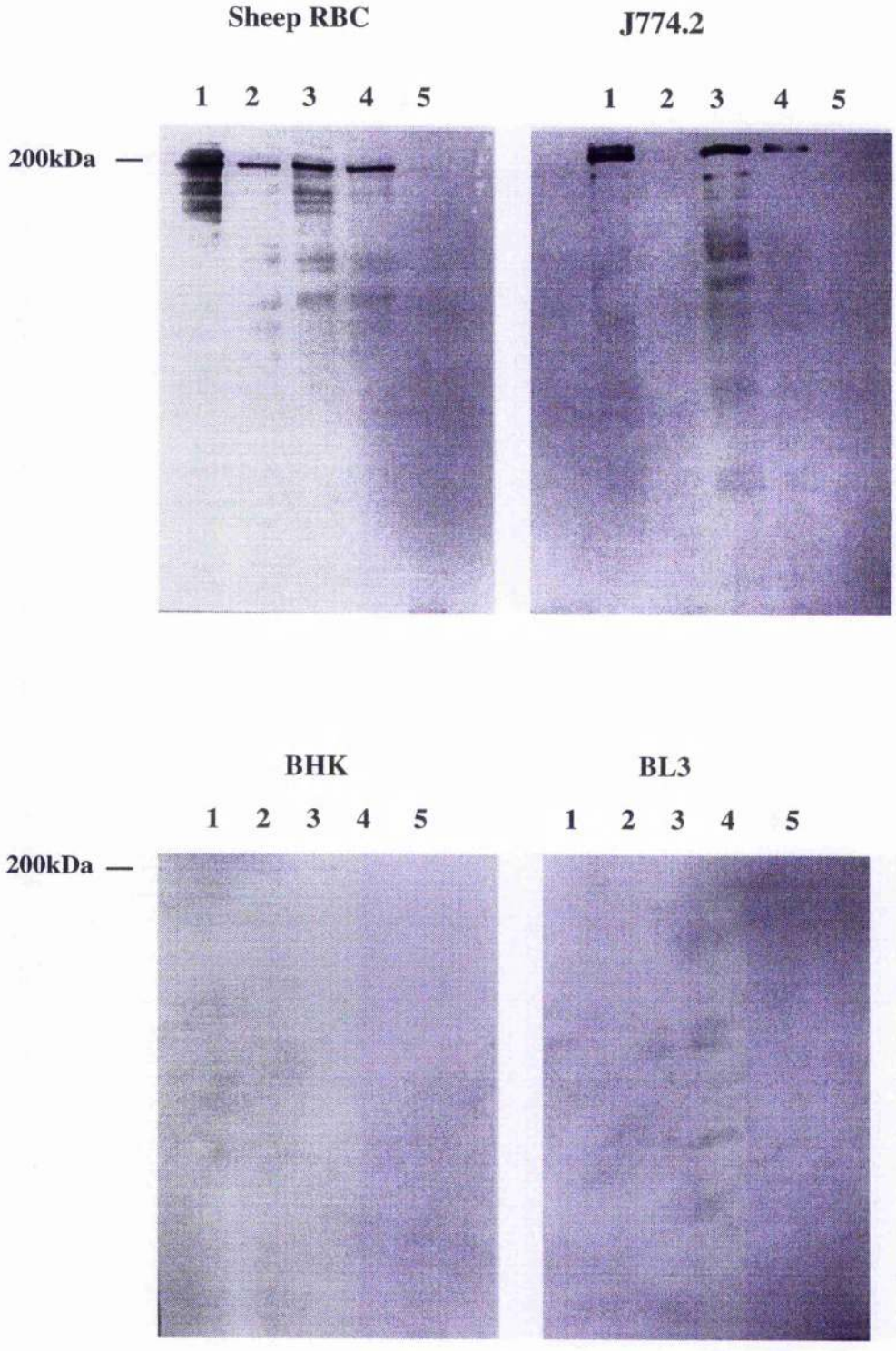
[c] urea extract from *E.coli* DH5α F'IQ (pDLACT-1)

Figure 3.13 ***Role of activation in binding of CyaA to different cell types***
Cells assayed were sheep erythrocytes (2 x 10^8/assay), and the J774.2, BHK and BL3 cell lines (5 x 10^5 cells/assay). Cells were incubated with 100 µg/ml of dialysed CyaA extracts for 60 min at 37°C. Bound toxin was detected as described earlier. Cells were incubated with: Lane 1, CyaA from BP348 (pRMB1); Lane 2, inactive recombinant CyaA (pGW22)[a]; Lane 3, active recombinant CyaA[b]; Lane 4, inactive recombinant CyaA[c]; Lane 5, 10µg of untreated cells.

[a] gift from Dr Gareth Westrop (University of Glasgow)

[b] urea extract from *E.coli* DH5αF'IQ (pCACT-3)

[c] urea extract from *E.coli* DH5αF'IQ (pDLACT-1)


Sheep RBC
J774.2
1 2 3 4 5
1 2 3 4 5
200kDa —
BHK
BL3
1 2 3 4 5
1 2 3 4 5
200kDa —

negative binding results obtained for this cell type may be due to an inherent insensitivity of the binding assay, resulting from the low number of BHK cells ($5x10^5$) used in the assay in comparison to erythrocytes ($2x10^8$). This may also explain the result obtained with the BL3 cell line. However,we also used relatively few ($5x10^5$) J774.2 cells in this assay, and detected binding of the activated form of CyaA. Therefore, it is possible that CyaA may have bound to BHK and BL3 cells in a manner which could not be detected by this assay system. Nevertheless, the results obtained with the sheep erythrocytes and J774.2 cells do suggest that activation may play a role in facilitating cell binding and / or determining cell specificity.

3.3 ANALYSIS OF RECEPTORS FOR ADENYLATE CYCLASE TOXIN

3.3.1 Investigation of protein receptors for adenylate cyclase toxin

A number of protein and in particular glycoprotein receptors for bacterial toxins, including those for diphtheria toxin (Makeda *et al* , 1991) and the ∂-endotoxin of *Bacillus thuringiensis* (Knowles and Ellar, 1986), have been described. With this in mind, analyses were carried out to determine if the receptor for CyaA on sheep erythrocytes was proteinaceous in nature. Silver staining of an SDS-PAGE gel of sheep RBC membrane proteins revealed a variety of different molecular weight proteins (**Lanes 1** and **2, Figure 3.14**). These proteins were potential receptors for CyaA. The binding of CyaA to these membrane proteins, was analysed by a nitrocellulose overlay assay as described in Materials and Methods. In this assay membrane proteins were separated by SDS-PAGE, blotted onto nitrocellulose and overlaid with adenylate cyclase. To detect binding of CyaA to these proteins a system of immunodetection was initially used **(Figure 2.2)** involving sequential overlays of antibodies culminating in a colourometric reaction.

When these sheep RBC membrane proteins were blotted and probed with CyaA at 37.5 µg/ml (**Blot A**) and 75 µg/ml (**Blot B**), no binding was detected at either

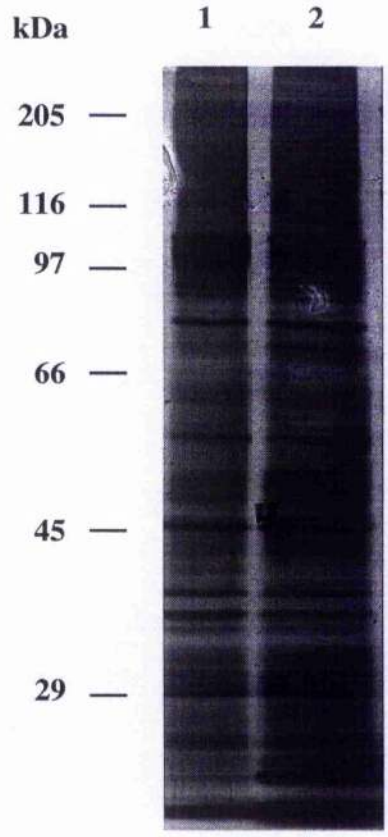


Figure 3.14 ***Silver stain of membrane proteins isolated fron sheep erythrocytes***
Membrane proteins were isolated from sheep erythrocytes (sRBCs), were separated by SDS-PAGE and visualised by silver staining as described in Materials and Methods. Lane 1, sRBC membrane proteins (5µg); Lane 2, sRBC membrane proteins (10µg).

concentration to any of them (**Figure 3.15**). A control blot (**Blot C**) was also performed, in which CyaA was run on the SDS-PAGE gel adjacent to the membrane proteins and blotted. This blot was overlaid with buffer as a control, instead of CyaA and then processed in the same manner as before. On this blot the 200 kDa CyaA protein was detected (**Lane 3, Blot C, Figure 3.15**) This demonstrated that the assay system could detect CyaA, which had initially been run on a gel and blotted. However, the assay may not have been sensitive enough to detect small amounts of CyaA bound to the membrane proteins on the overlay blots.

In an attempt to amplify any binding by CyaA to the blotted proteins a system of radioisotope detection was employed (**Figure 2.2**). When CyaA was incubated for 2 hours with blots of sRBC membrane proteins no binding was detected to any of the proteins using this system (**Lanes 1** and **2, Blot A, Figure 3.16a**). A similar result was obtained when CyaA was incubated overnight with an identical blot (**Lanes 1** and **2, Blot A, Figure 3.16b**). **Lane 3** of the control blot (**B**) in **Figure 16a** identified the 200 kDa CyaA toxin band which had been run on a gel adjacent to the membrane proteins.The control blot (**B**) in **Figure 3.16b** also showed the presence of 200 kDa CyaA in **Lane 3**, but there are also a number of protein bands showing up, and were probably degradation products of CyaA, or have resulted from over-exposue of the autoradiograph. Nevertheless, these results that CyaA run on a gel could be detected by this system and that the assay was working.

These results with the nitrocellulose overlay systems indicated that *B. pertussis* CyaA did not bind to proteins isolated from sheep erythrocytes membranes and suggested that such proteins would not act as receptors.

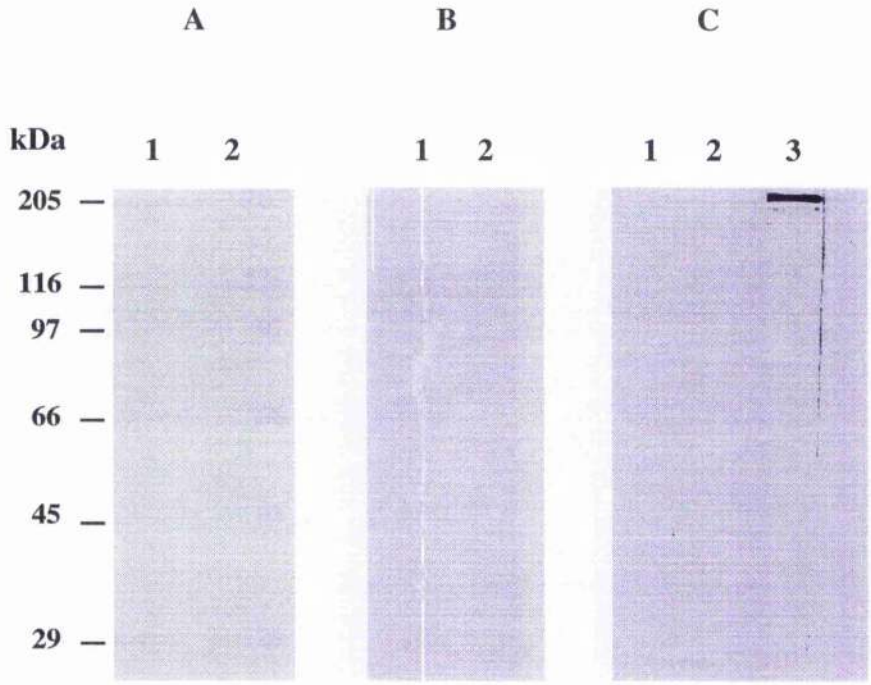
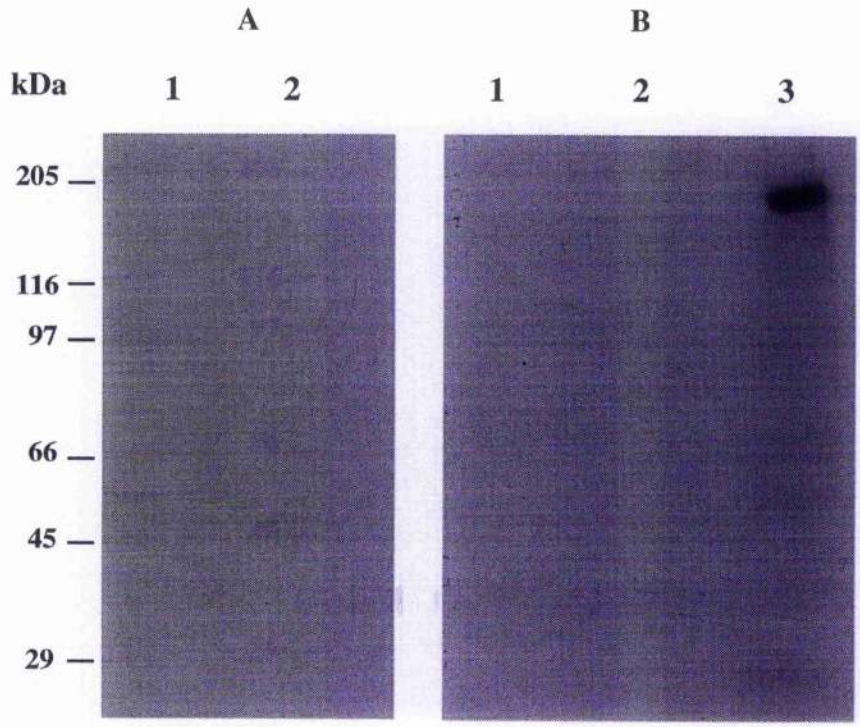


Figure 3.15 ***Binding of CyaA to membrane proteins separated by SDS-PAGE.*** Membrane proteins, isolated from sheep erythrocytes (sRBC's), were separated by SDS-PAGE and transferred to nitrocellulose membranes by Western blotting. The blots were overlaid overnight with dialysed CyaA from BP348 (pRMB1) at 37.5 (A) or 75 µg/ml (B) or with buffer (C) as a control.The overlaid blots were incubated with monospecific anti-CyaA serum prepared in rabbits. Binding was detected by means of peroxidase-labelled donkey, anti-rabbit antibodies as described in Materials and Methods. Lane 1, sRBC membrane proteins (5µg); Lane 2, sRBC membrane proteins (10µg); Lane 3, CyaA control (10µg).

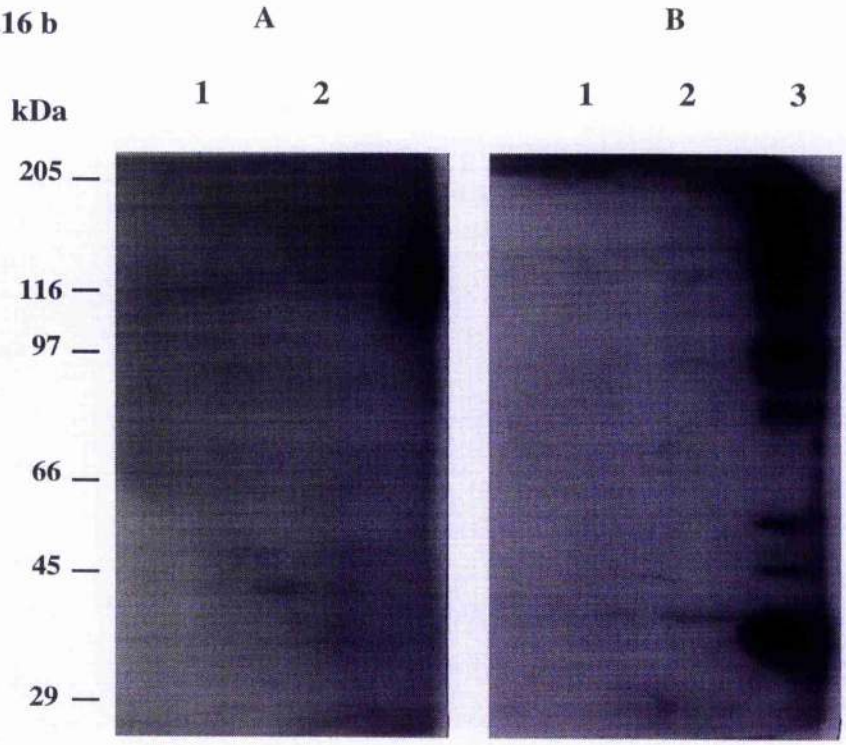
Figure 3.16a ***Binding of CyaA to membrane proteins separated by SDS-PAGE after a 2 hour incubation.*** Sheep RBC membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose by western blotting. The blots were overlaid with either 75 μg/ml dialysed CyaA, purified from BP348 (pRMB1) (A) or buffer (B) as a control for 2h. The overlaid blots were then incubated with monospecific anti-CyaA prepared in rabbits. Binding was detected by subsequent overlays of biotinylated anti-rabbit antibodies and ^{125}I-Streptavidin followed by autoradiography as described in Materials and Methods. Lane 1, sRBC (5 μg); Lane 2, sRBC (10 μg); Lane 3, CyaA (10 μg).

Figure 3.16b ***Binding of CyaA to membrane proteins separated by SDS-PAGE after an overnight incubation.*** Sheep RBC membrane proteins were separated by SDS-PAGE and transferred to nitrocellulose by western blotting. The blots were overlaid overnight with either 75 μg/ml of dialysed CyaA, purified from BP348 (pRMB1) (A) or buffer (B) as a control. The overlaid blots were then incubated with monospecific anti-CyaA prepared in rabbits. Binding was detected by subsequent overlays of biotinylated anti-rabbit antibodies and ^{125}I-Streptavidin followed by autoradiography as described in Materials and Methods. Lane 1, sRBC (5 μg); Lane 2, sRBC (10 μg); Lane 3, CyaA (10 μg).

3.16 a



3.16 b



3.3.2 Investigation of glycolipid receptors for adenylate cyclase toxin

To investigate glycolipid receptors for adenylate cyclase toxin the glycolipid fraction was obtained from sheep erythrocytes by extraction with chloroform : methanol as described in Materials and Methods (section 2.12.1). A chromatogram overlay procedure was employed to investigate the possible glycolipid receptors.

Before overlay analysis could be carried out, it was necessary to find a suitable solvent to separate and resolve the extracted lipids. Comparison of a range of solvents demonstrated that a solvent system of chloroform : methanol : KCl (0.1%) [5: 4: 1, by vol] resulted in the best separation of a control mixture of gangliosides and the sheep erythrocyte lipid extract (**Lanes 1** and **2, chromatogram A, Figure 3.17**). The TLC profile of the erythrocyte extract identified a number of components which were possible receptors for CyaA (**Lane 2, chromatogram A, Figure 3.17**). The other solvents systems tested, chloroform : methanol : water (65 : 25: 4, by vol) and chlorofrom : methanol : NH_3 (2.5M) (60 : 40 : 9, by vol) gave much poorer separation of the lipid components (**chromatograms B** and **C, Figure 3.17**). Therefore, the chloroform : methanol : KCl solvent system was subsequently used to separate lipid components before overlays were carried out.

Binding of CyaA to lipids separated by TLC was initially tested by an overlay immunodetection-system similar to that developed to investigate protein receptors (see **Figure 2.3**, Materials and Methods section). When separated lipids were overlaid with CyaA for 2h, the toxin appeared to bind to a number of gangliosides (**Lane 1, chromatogram B, Figure 3.18**). However, some gangliosides components showed up positive in the control overlay, in which CyaA had been replaced with buffer in the overlay sequence (**chromatogram C, Figure 3.18**). CyaA bound to the ganglioside, GD_{1b} (**Lane 1, chromatogram B, Figure 3.18**), which showed up much less strongly in the control blot (**Lane 1, chromatogram C, Figure 3.18**).

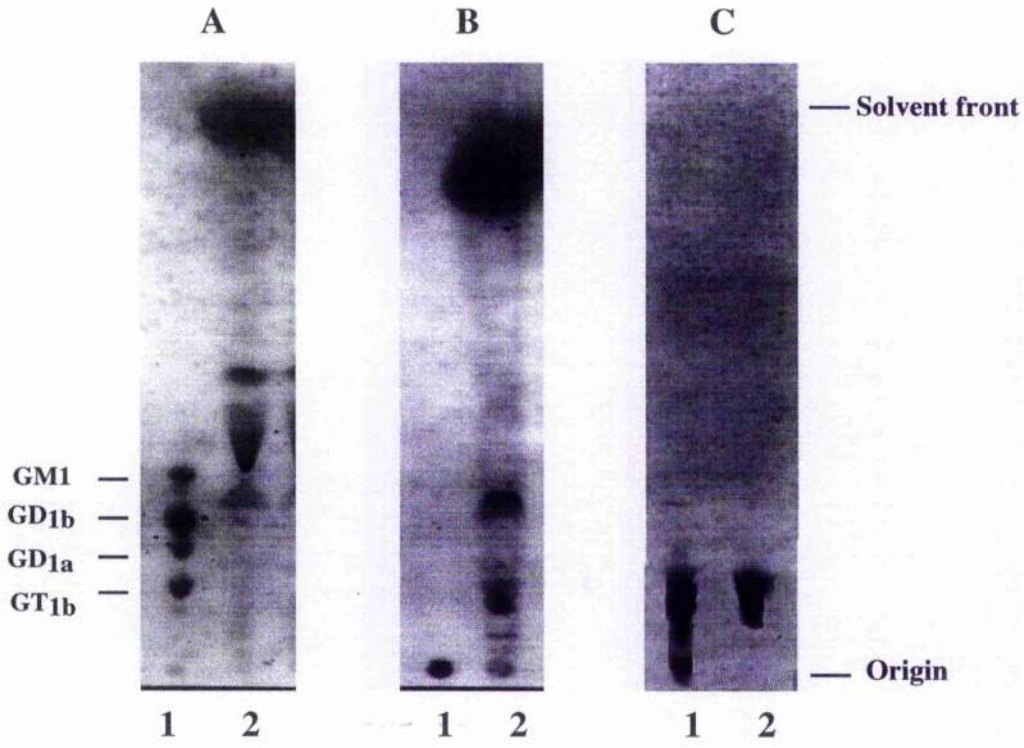


Figure 3.17 ***Comparison of solvents for lipid separation***

Sheep RBC lipids and gangliosides mixture (see text) were separated by TLC using three solvent systems: A, chloroform : methanol : KCl (0.1%) [5: 4: 1, by vol]; B, chloroform : methanol : water [65 : 25: 4, by vol] or C, chloroform : methanol : NH_3 (2.5M) [60 : 40 : 9 , by vol]. The TLC's were air dried and stained with resorcinol. Lane 1, Ganglioside mixture (150 μg); Lane 2 , sRBC total lipid (500 μg).

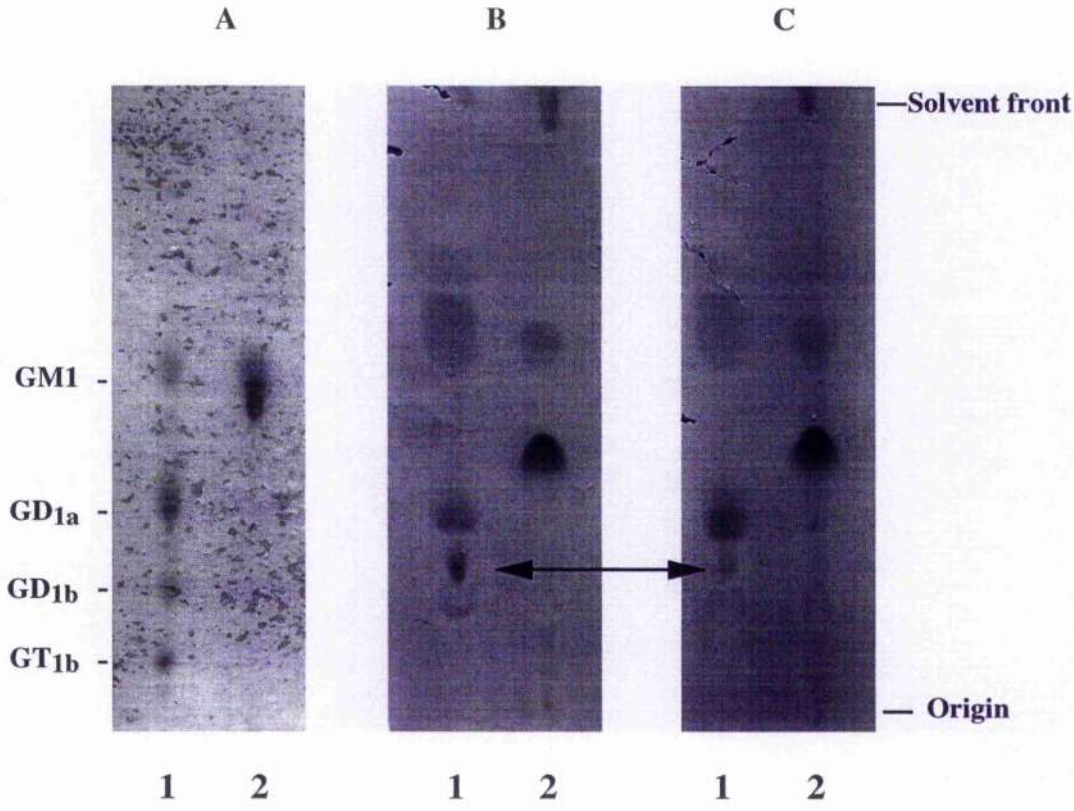


Figure 3.18 ***Binding of CyaA to lipids separated on thin-layer chromatograms - Immunodetection Run 1.*** TLC plates were prepared and run as described in Materials and Methods and either stained with resorcinol (A) or overlaid for 2h with 50 µg/ml of dialysed CyaA (B) or buffer (C) as a control. The overlaid TLC's were incubated with monospecific anti-AC prepared in rabbits. Binding was detected by means of peroxidase-labelled donkey anti-rabbit antibodies. Lane 1, Ganglioside mixture (150µg); Lane 2, sRBC total lipid (500µg). Arrow shows possible binding of CyaA to GD_{1b} (see text)

When CyaA was incubated with TLC plates overnight the control overlay showed a similar pattern to the CyaA overlay (**chromatograms C** and **B, Figure 3.19**). It was not possible to detect if CyaA bound strongly to the G_{D1b} as described above because, the background staining of the overlaid chromatograms was so dark that it was not possible to determine differences between the control overlay (**chromatogram C, Figure 3.19**) and the overlays with CyaA (**chromatogram B, Figure 3.19**) itself. It was possible that the DAB developer, used to visualise binding may have non-specifically stained the chromatograms. Therefore the overlay procedure was adapted with the purpose of eliminating this background staining of the TLC plates. In the new system overlay with CyaA was followed by sequential overlays of rabbit anti-CyaA serum, biotinylated anti-rabbit serum, and finally ^{125}I-Streptavidin. Autoradiography was used to visualise the binding of CyaA to lipids run on chromatograms (Materials and Methods, section 2.12.2). When TLC plates were run and incubated with CyaA or buffer (control) for 2 hours, a number of glycolipids showed up positive in the assay on both the CyaA overlay plate and control plate (**autorad's B** and **C, Figure 3.20**). It was possible that there were components in the anti-CyaA serum which were reacting with the chromatographed lipids and giving false positives in the controls. With the aim of reducing this, the incubation of CyaA with the TLC plate was increased from 2 hours to overnight and the subsequent incubation of anti-CyaA serum with the TLC plate was reduced from 1h to 30 min (**Figure 3.21**). However, when these changes were made lipids still showed up on the background (control) plate in which CyaA had been omitted (**autorad C, Figure 3.21**).

Anti-CyaA serum [1/1000 dilution in blocking buffer (see Materials and Methods) was incubated overnight with a TLC plate which had been run as above to absorb out any components of the serum which were reacting with the lipids. The overlay was repeated with preadsorbed anti-CyaA serum replacing normal anti-CyaA serum. CyaA bound to components in the sRBC lipid extract and gangliosides mixture

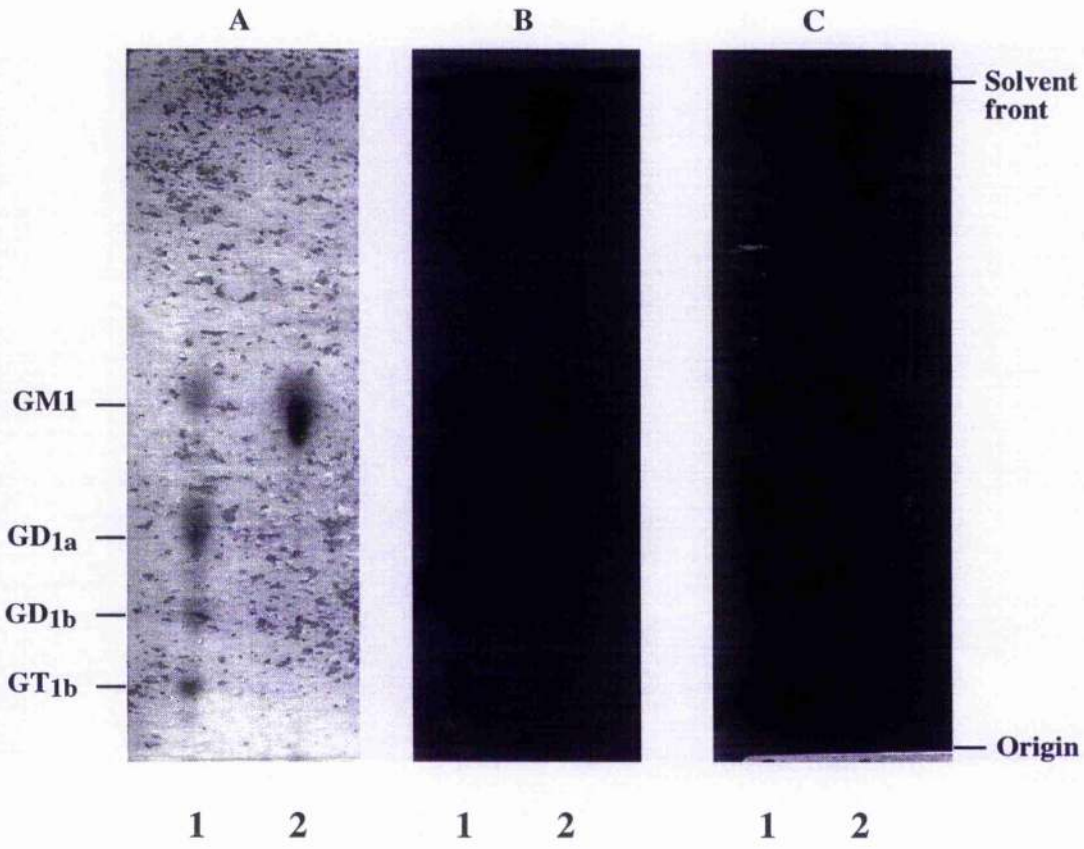


Figure 3.19 ***Binding of CyaA to lipids separated on thin-layer chromatograms - Immunodetection Run 2***. TLC plates were prepared as described in Materials and Methods. and either stained with resorcinol (A) or overlaid overnight with 50 µg/ml of dialysed CyaA (B) or buffer (C) as a control. The overlaid TLC's were incubated with monospecific anti-CyaA prepared in rabbits. Binding was detected by means of peroxidase-labelled donkey anti-rabbit antibodies. Lane 1, Ganglioside mixture (150 µg); Lane 2, sRBC total lipid (500 µg)..

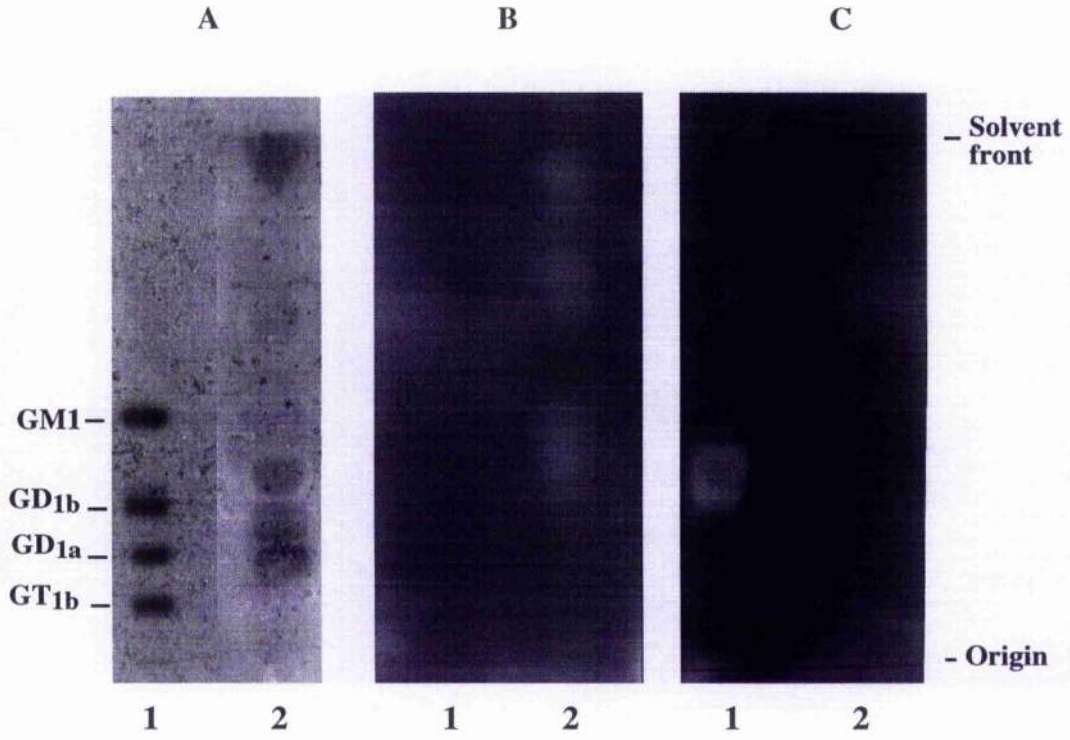


Figure 3.20 ***Binding of CyaA to lipids separated on thin-layer chromatograms - Radio-isotope detection Run 1.*** TLC plates were prepared and run as described in Materials and Methods, and either stained with resorcinol (A) or overlaid for 2h with 50 µg/ml of dialysed CyaA purified from BP348 (pRMB1) (B) or buffer (C) as a control. The overlaid TLC's were then incubated with monospecific anti-CyaA prepared in rabbits. Binding was detected by subsequent overlays of biotinylated anti-rabbit antibodies and ^{125}I-Streptavidin followed by autoradiography as described in Materials and Methods. Lane 1, Ganglioside mixture (150 µg); Lane 2, sRBC total lipid (500 µg).

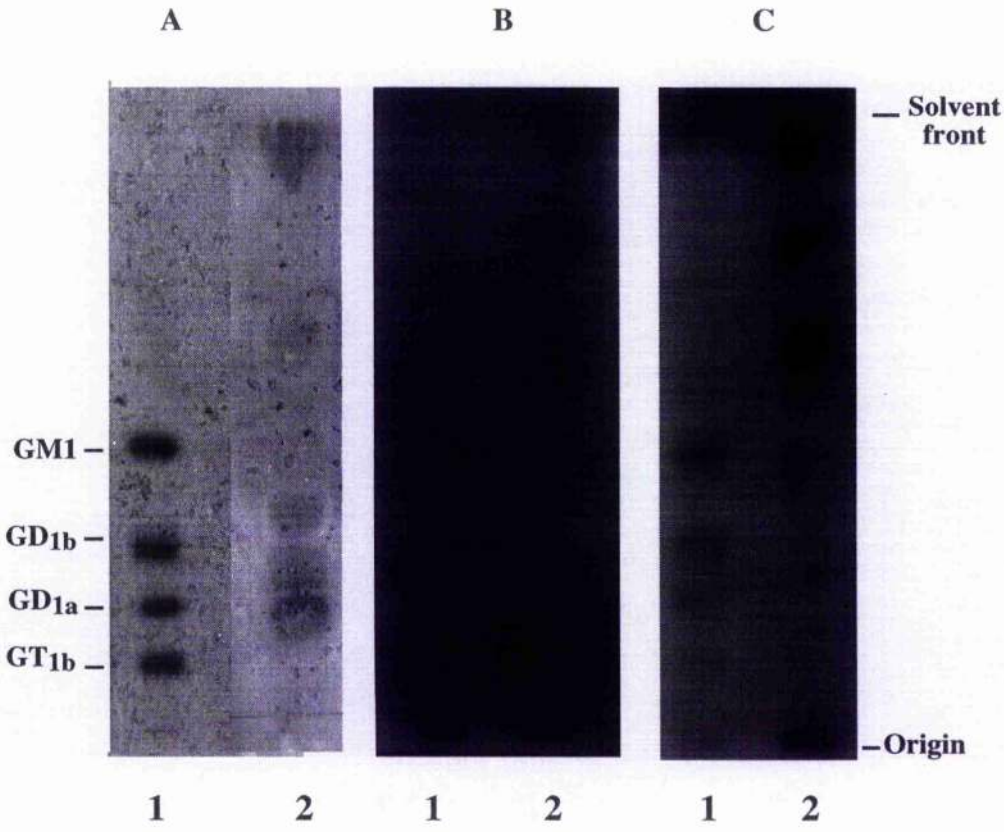


Figure 3.21 ***Binding of CyaA to lipids separated on thin-layer chromatograms - Radio-isotope detection Run 2.*** TLC plates were prepared and run as described in Materials and Methods. and either stained with resorcinol (A) or overlaid overnight with 50 µg/ml of dialysed CyaA (B) purified from BP348 (pRMB1) or buffer (C) as a control. The overlaid TLC's were then incubated with monospecific anti-CyaA prepared in rabbits. Binding was detected by subsequent overlays of, biotinylated anti-rabbit antibodies and ^{125}I-Streptavidin followed by autoradiography as described in Materials and Methods. Lane 1, Ganglioside mixture (150µg); Lane 2 , sRBC total lipid (500µg).

(**Lanes 1** and **2**, respectively, **autorad B, Figure 3.22**). Comparing the autoradiograph (**B**) with the stained plate (**A**) showed that CyaA bound to all the gangliosides in the mixture, namely GM_1, GD_{1a}, GD_{1b}, and GT_{1b} with similar intensity observed for each ganglioside (**Lane 1, autorad B, Figure 3.22**). There was also strong binding observed between CyaA and some of the components of the sRBC lipid extract, although binding was not detected to all these components (**Lane 2, autorad B, Figure 3.22**). Preadsorbing the anti-CyaA serum removed all the non-specific reactions with the gangliosides and most of the reaction with sRBC lipid components on the control plate (**autoradiograph C, Figure 3.22**). These results indicate that CyaA is able to bind to purified gangliosides and membrane glycolipids from sheep erythrocytes. However the observation that binding of CyaA was not detected to all the components of the sheep erythrocyte membrane extract suggests that there is a degree of specificity in the binding of CyaA

3.4 ANALYSIS OF PORE FORMATION BY ADENYLATE CYCLASE TOXIN

3.4.1 Pore formation by adenylate cyclase toxin in erythrocytes membranes

One of the *sequela* of the interaction of adenylate cyclase toxin with eukaryotic target cells is colloid osmotic lysis, due to pore formation. To study pore formation by CyaA in sheep erythrocyte membranes, haemolysis assays were carried out. Adenylate cyclase toxin purified from *B.pertussis* 348 (pRMB1) was haemolytic to sheep erythrocytes (**Figure 3.23a** and **b**). When CyaA was incubated with the erythrocytes there was an initial lag phase of approximately 60 min before haemolysis became evident. This was followed by a linear phase in which haemolysis increased with time and reached a maximum after 300 min (**Figure 3.23a**). CyaA also exhibited a linear

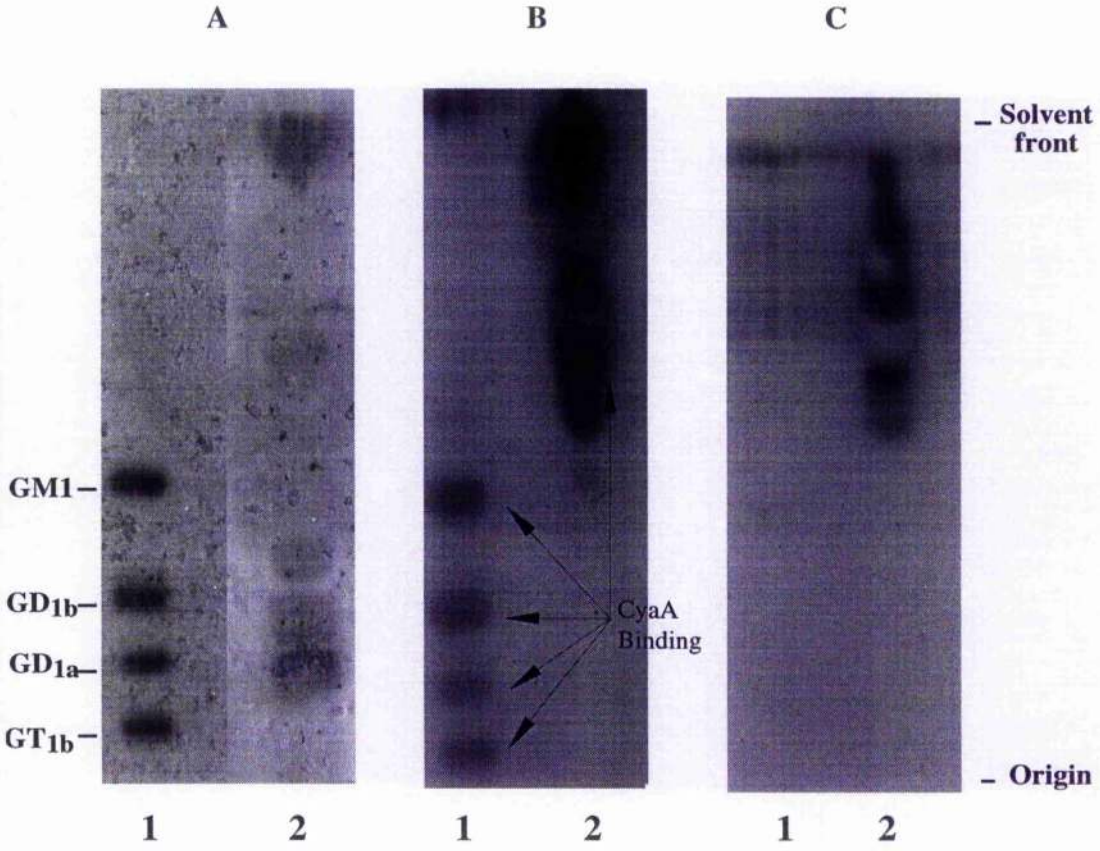
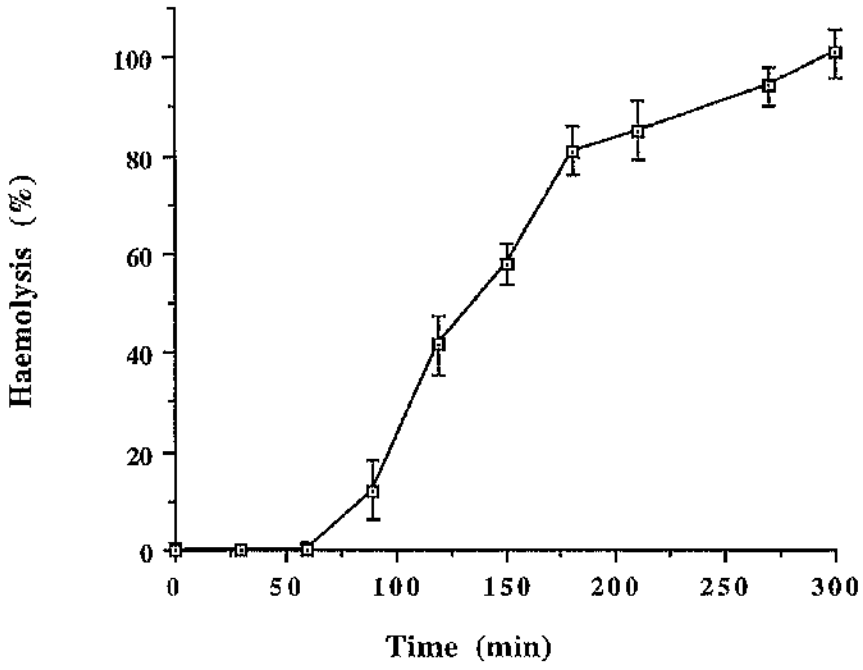


Figure 3.22 ***Binding of CyaA to lipids separated on thin-layer chromatograms - Radio-isotope detection Run 3*** . TLC plates were prepared as described in Materials and Methods, and either stained with resorcinol (A) or overlaid for overnight with 50 µg/ml of dialysed CyaA purified from BP348 (pRMB1) (B) or buffer as a control (C). The overlaid TLC's (B and C) were then incubated with anti-CyaA serum prepared in rabbits which had been preadsorbed to a TLC plate run as above. Binding of CyaA was detected by subsequent overlays of biotinylated anti-rabbit antibodies and ^{125}I-Streptavidin followed by autoradiography as described in Materials and Methods. Lane 1, Ganglioside mixture (150µg); Lane 2, sRBC total lipid (500µg). Arrows shows components in ganglioside mixture and RBC lipid extract which CyaA bound to.

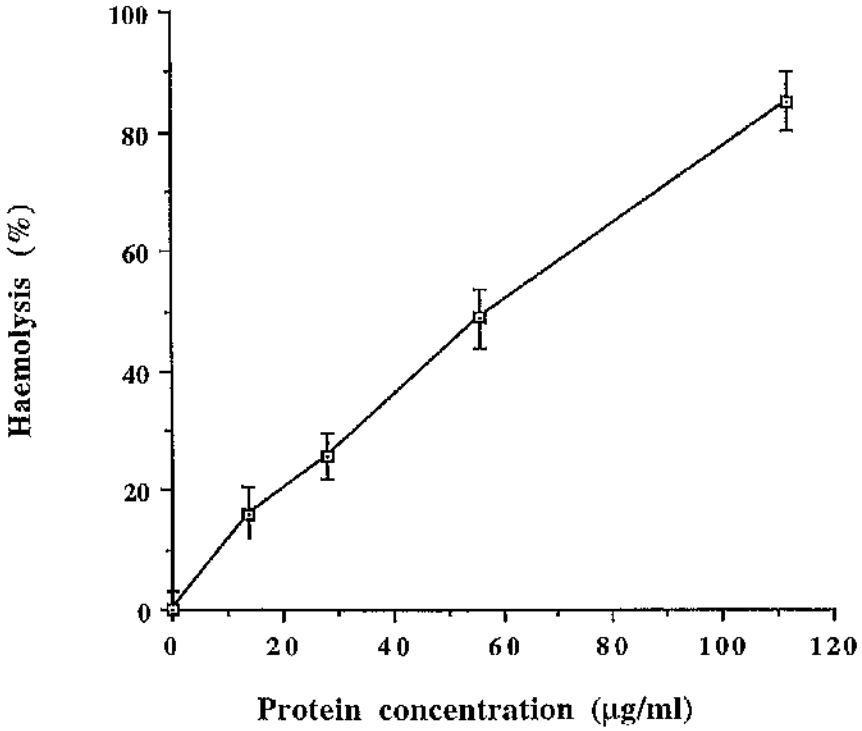
Figure 3.23a ***Haemolysis of sheep erythrocytes by CyaA - Time course.*** Sheep RBCs were incubated with 100 μg/ml of dialysed CyaA purified from BP348 (pRMB1) and haemolysis measured at OD_{540nm}. Percentage haemolysis was calculated as described in Materials and methods. Bars represemt the mean ± SEM from 3 determinations. Haemolysis by dialysed toxin buffer was < 1%.

Figure 3.23b ***Haemolysis of sheep erythrocytes by CyaA- Dose response.*** Sheep RBCs were incubated with dialysed CyaA purified from BP348 (pRMB1) for 300 min and haemolysis measured at OD_{540nm}. Percentage haemolysis was calculated as described in Materials and Methods. Bars represent the mean ± SEM from 3 determinations. Haemolysis by dialysed toxin buffer was < 1%.

3.23 a


Haemolysis (%)
100
80
60
40
20
0
0
50
100
150
200
250
300
Time (min)

3.23 b


Haemolysis (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
Protein concentration (μg/ml)

relationship between protein concentration and haemolysis. (**Figure 3.23b**). Therefore, haemolysis by CyaA was both time and dose-dependent.

The haemolytic activities of the recombinant and native forms of CyaA were compared (**Figure 3.24**). The recombinant form of CyaA exhibited similar kinetics of haemolysis to the native form, consisting of a lag phase followed by linear phase reaching a maximum after 300 min. Since the recombinant form of CyaA exhibited similar properties to the native form, and was therefore comparable, recombinant forms of activated and non-activated CyaA were subsequently used to examine the role activation played in pore formation. Non-activated CyaA did not exhibit haemolytic activity towards sheep erythrocytes after incubation for 300 min (**Figure 3.25**). Activation was therefore important in potentiating the haemolytic activity of CyaA.

3.4.2 Pore formation by adenylate cyclase toxin in liposomes

3.4.2.1 Preparation of liposomes

Liposomes were used as a model system to examine the specificity of the interaction of CyaA with membranes which leads to pore formation. Two methods of liposome preparation were tested. Aiming for reproduciblity and consistency of size, liposomes were initially prepared using a *Liposomat* (liposome maker). Liposomes were formed from mixed lipid:detergent micelles prepared in a buffer containing a fluorescent marker (calcein) by removal of the detergent by cross-flow dialysis (Section 2.14.1). The second method used freezing and thawing of lipid mixtures in the presence of calcein to fuse small liposomes into larger ones. The excess calcein was removed by column chromatography (Section 2.14.2).

The amount of calcein which had been trapped inside the liposomes prepared by the two methods was compared. It was evident that there was considerably more marker trapped by the freeze-thawing method than by cross-flow dialysis (**Figure 3.26**). Indeed cross-flow dialysis resulted in little marker being trapped. Pore

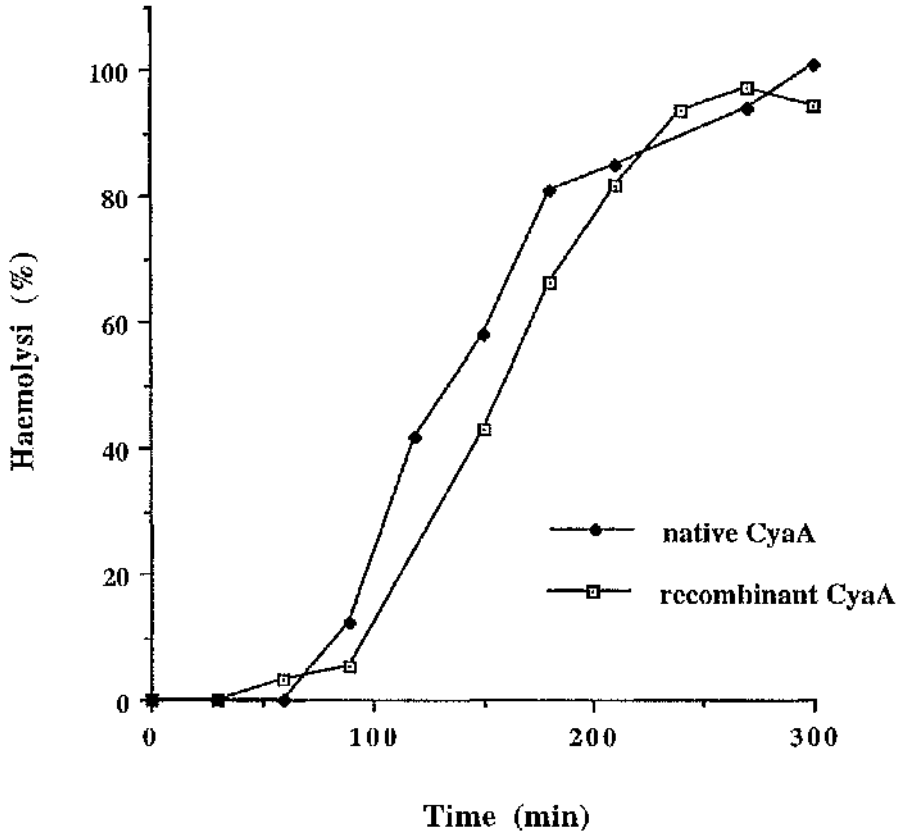


Figure 3.24 ***Effect of native and recombinant CyaA on sheep erythrocytes*** . Recombinant CyaA[1] and native CyaA[2] (100 µg/ml) were dialysed and incubated with sheep erythrocytes and haemolysis measured at OD_{540nm}. Percentage haemolysis was calculated as described earlier. Haemolyis by dialysed toxin buffers and by dialysed urea extract from *E.coli* DH5'α (pICNOH) was < 1%.

[1]Urea extract from *E.coli* DH5'α (pCACT-3).

[2]Extract from *B.pertussis* 348 (pRMB1) purified by calmodulin agarose chromatography.

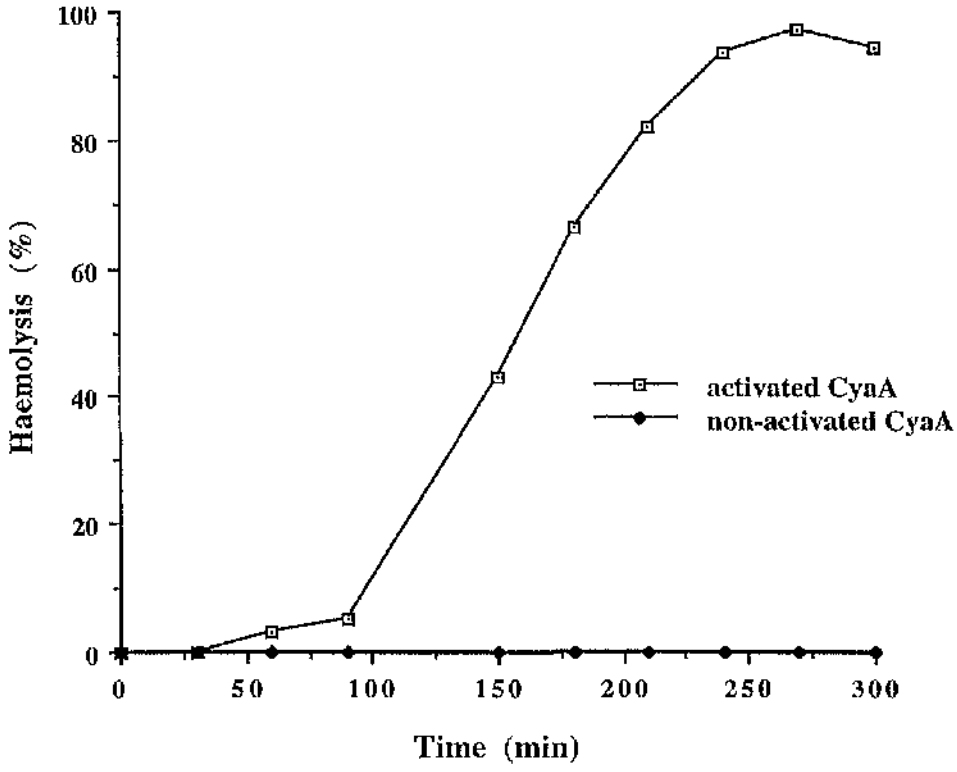


Figure 3.25 ***Effect of activation on haemolytic activity of CyaA***

Activated and non-activated CyaA were prepared as dialysed urea-extracts from *E.coli* DH5'α (pCACT-3) and *E.coli* DH5'α (pDLACT-1) respectively, as described in Materials and Methods. Sheep RBC's were incubated with 100 μg/ml of the extracts and haemolysis was measured at OD_{540nm} as described earlier. Haemolysis by dialysed toxin buffer and urea extract from *E.coli* DH5'α (pICNOH)was < 1%.

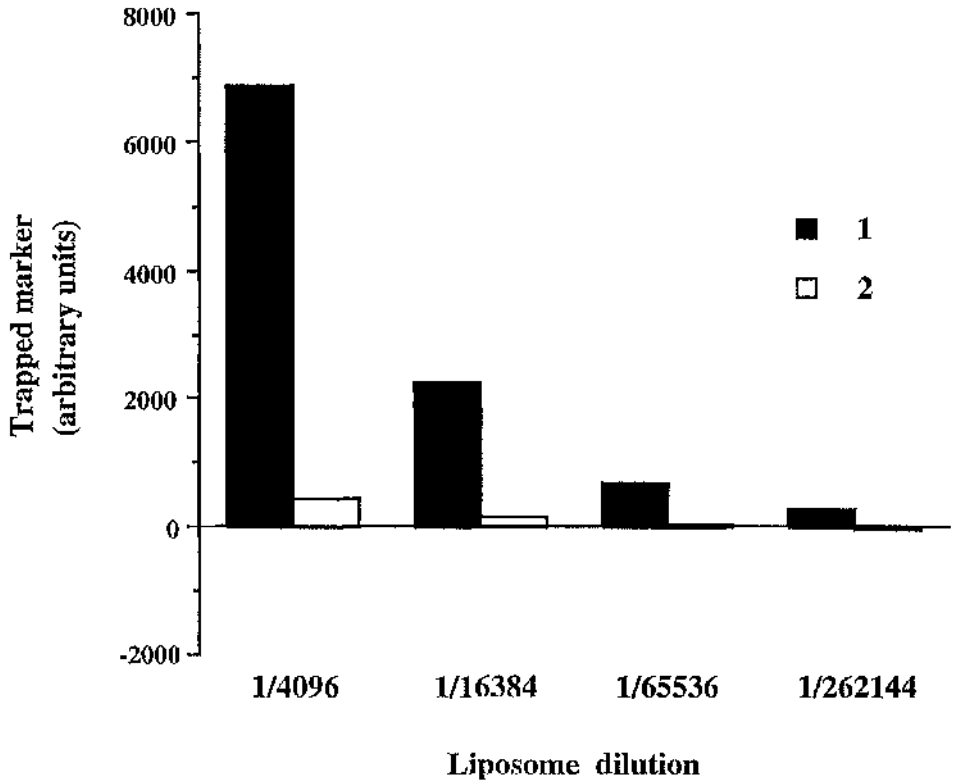


Figure 3.26 ***Trapping of fluorescent marker by two methods of liposome production.*** Liposomes were prepared by freezing and thawing followed by chromatography **(1)** or cross flow dialysis **(2)** as described in sections 2.14.1 and 2.14.2. After preparation liposomes were diluted in liposomes assay buffer (serial one in foue dilutions). The trapped fluorescent marker (calcein) was released by treating the liposomes with Triton X-100, and fluorescence (arbitrary units) measured after 30 min in a fluorescence spectrophotometer (Cytofluor 2300)

formation studies required liposomes which had a relatively large amount of fluorescent marker trapped inside in order to assay the release of this marker by lytic agents. Therefore, the freeze-thawing method of liposome production was employed in future studies.

3.4.2.2 Pore formation in liposomes

Native CyaA elicited marker release from liposomes (**Figure 3.27**). When the liposomes were treated with 75 μg/ml of CyaA there was no detectable lag period in the release of the marker. Marker release increased with time and reached a maximum of 40% after 300 min.The other concentrations of CyaA tested had similar effects on the liposomes but the rate of marker release was lower. These studies demonstrated that liposomolysis (lysis of liposomes) by native CyaA was both time- and dose-dependent.

The recombinant form of CyaA showed a similar pattern of marker release from liposomes when compared to the native form of CyaA (**Figure 3.28**) However, when the same concentration of native and recombinant CyaA were tested the native form showed slightly more marker release after 300 min than the recombinant form. We had a higher yield of the recombinant form and so used this in subsequent studies. The activated and non-activated forms of recombinant CyaA were examined to determine the role activation played in pore formation in liposomes (**Figure 3.29**). Non-activated CyaA did not elicit marker release from liposomes. This observation indicated that activation was important in potentiating pore formation by CyaA in liposomes.

The role of calcium, in the lysis of liposomes by activated CyaA, was investigated (**Figure 3.30**). Lysis of the liposomes by CyaA was absolutely dependent on the presence of calcium. The threshold level of calcium required for lysis

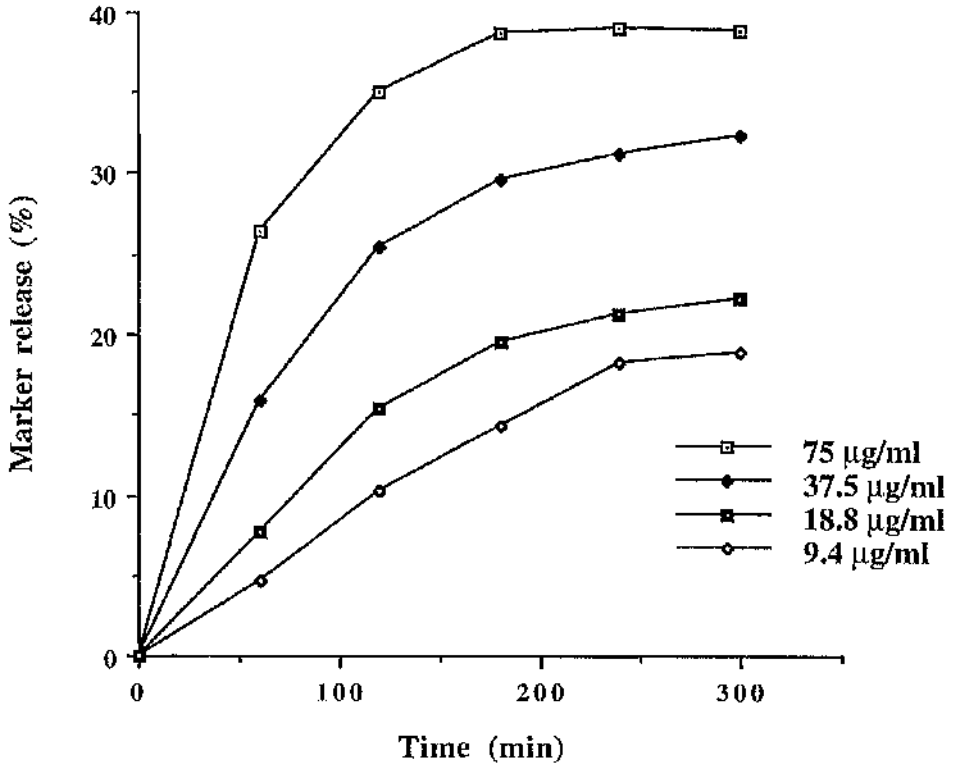


Figure 3.27 ***Pore formation by CyaA in liposomes***

Liposomes composed of DMPC : C : DCP (see section 2.14.2) were treated with various concentrations of dialysed CyaA purified from BP348 (pRMB1). Percentage marker release was calculated by comparison with release elicited by Triton-X 100. Marker release by dialysed toxin buffer was < 1%.

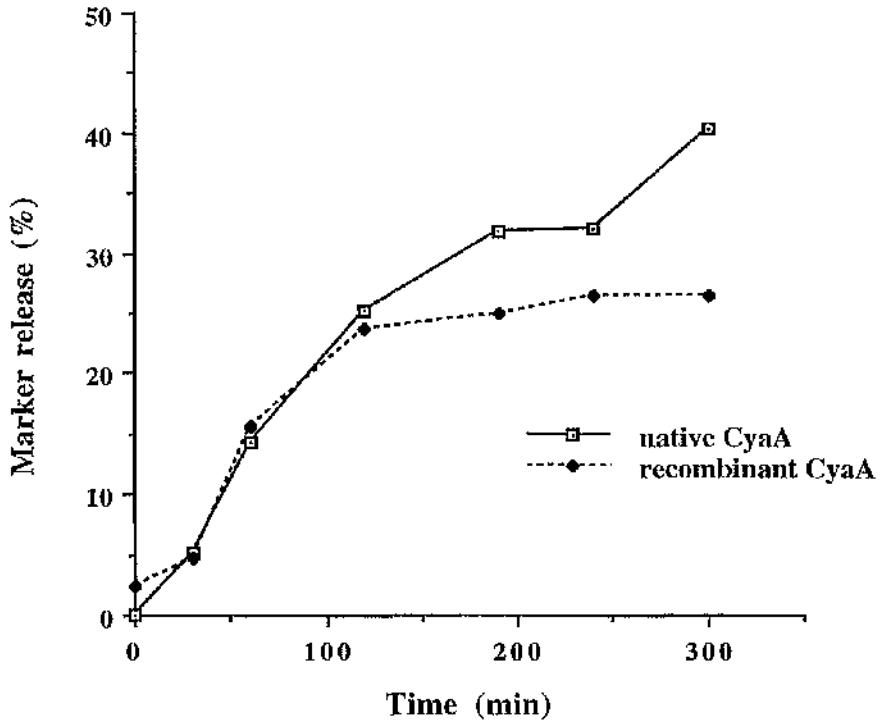


Figure 3.28 ***Effect of native and recombinant CyaA on liposomes***

Native and recombinant CyaA were prepared from BP348 (pRMB1) and *E. coli* DH5'α (pCACT-3), respectively (see Materials and Methods) and dialysed. Liposomes composed of DMPC : C :DCP (see section 2.14.2) were treated with the extracts (100 μg/ml). Percentage of marker release was calculated by comparison with release elicited by Triton-X 100. Marker release by dialysed toxin buffer and urea extract from *E.coli* DH5'α (pICNOH) was < 1%.

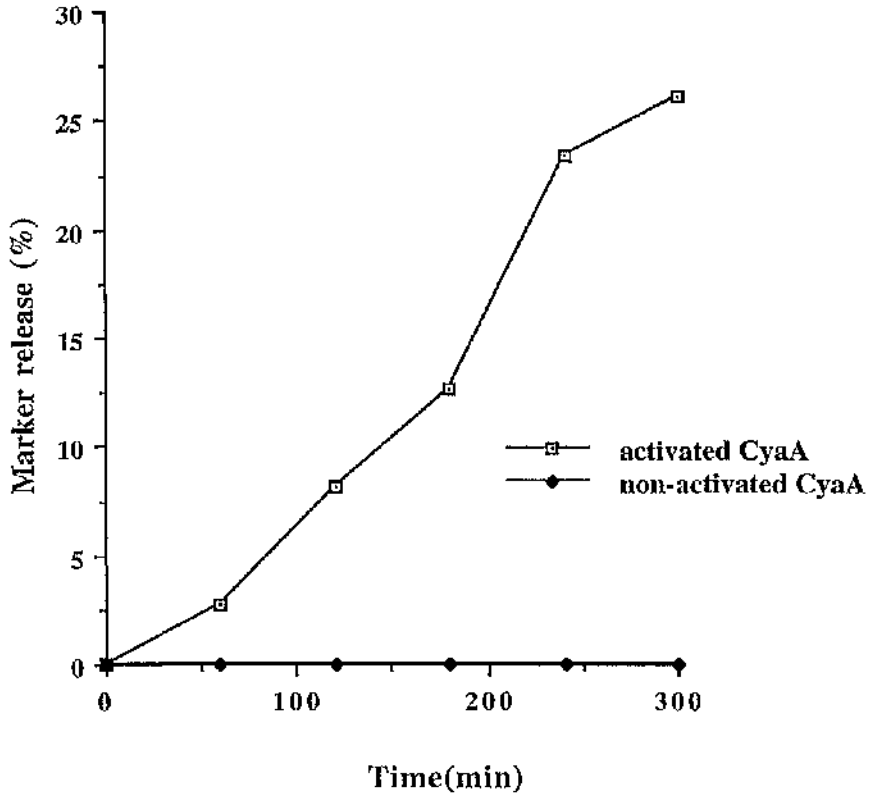


Figure 3.29 ***Effect of activation of CyaA on pore formation in liposomes.*** Activated and non-activated CyaA were prepared as dialysed urea-extracts from *E.coli* DH5'α (pCACT-3) and *E.coli* DH5'α (pDLACT-1) respectively, as described in Materials and Methods. Liposomes composed of DMPC : C :DCP (see section 2.14.2) were treated with the extracts (100 μg/ml). Percentage of marker release was calculated by comparison with release elicited by Triton-X 100. Marker release by dialysed toxin buffer and urea extract from *E.coli* DH5'α (pICNOH)was < 1%.

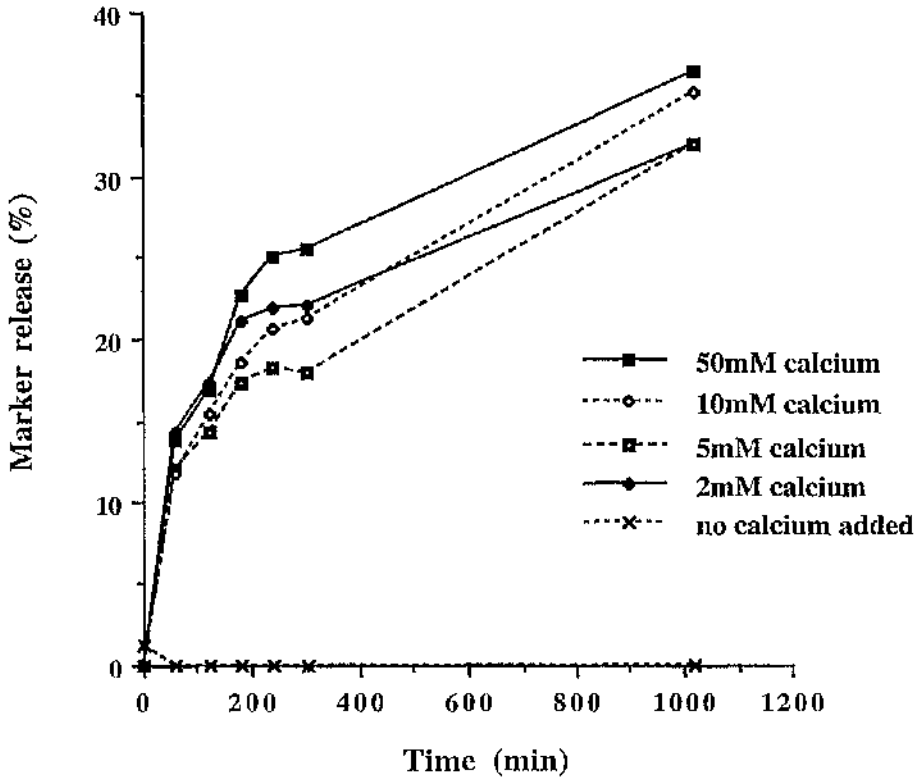


Figure 3.30 ***Effect of calcium on pore formation by CyaA in liposomes.*** Recombinant CyaA was dialysed against liposome assay buffer without calcium. Liposomes composed of DMPC :C:DCP were prepared as described in Materials and Methods in the absence of calcium. Liposomes were treated with the CyaA extract (100 µg/ml) in the presence of 0-50mM calcium. Percentage of marker release was calculated by comparison with release elicited by Triton-X 100. Marker release by dialysed toxin buffer and urea extract from*E.coli* DH5'α (pICNOH)was < 1%.

< 1%.

was between 0 and 2mM calcium. When the calcium concentration was increased above 2mM there was no significant increase in lysis mediated by CyaA.

3.4.2.3 Specificity of interaction of adenylate cyclase toxin with liposomes of different composition

With the aim of looking at the specificity of the interaction of CyaA, liposomes were prepared from different lipid mixtures. Varying the lipids used to prepare the liposomes altered the composition of the lipid membrane bilayer upon which the toxin acted. The importance of surface charge of target liposomes was examined (**Figure 3.31**). Adenylate cyclase mediated marker release without a lag period from liposomes with a negative surface charge. There was a lag period of 100-150min in marker release from liposomes with a positive or no net surface charge. Release from positive or uncharged liposomes reached much lower levels (5%), compared to negatively charge liposomes (25%), after 300min. Therefore the surface charge of liposomes had a considerable affect on subsequent lysis by adenylate cyclase toxin.

Liposomes were synthesized with a ganglioside [Trisialoganglioside (GT_{1b})] incorporated into their membranes with an equivalent surface charge density to the negatively charged liposomes prepared normally. Incorporation of the ganglioside had no effect on lysis of liposomes by CyaA when compared to negatively charged liposomes prepared normally (**Figure 3.32**). Lysis of liposomes with or without the ganglioside incorporated reached 25% after 300 min. These results suggested that membrane gangliosides, or GT_{1b} in particular, were not essential for initiation of pore formation by CyaA in liposmes. It is possible that a negative charge alone may be required for the successful interaction between CyaA and the liposomes.

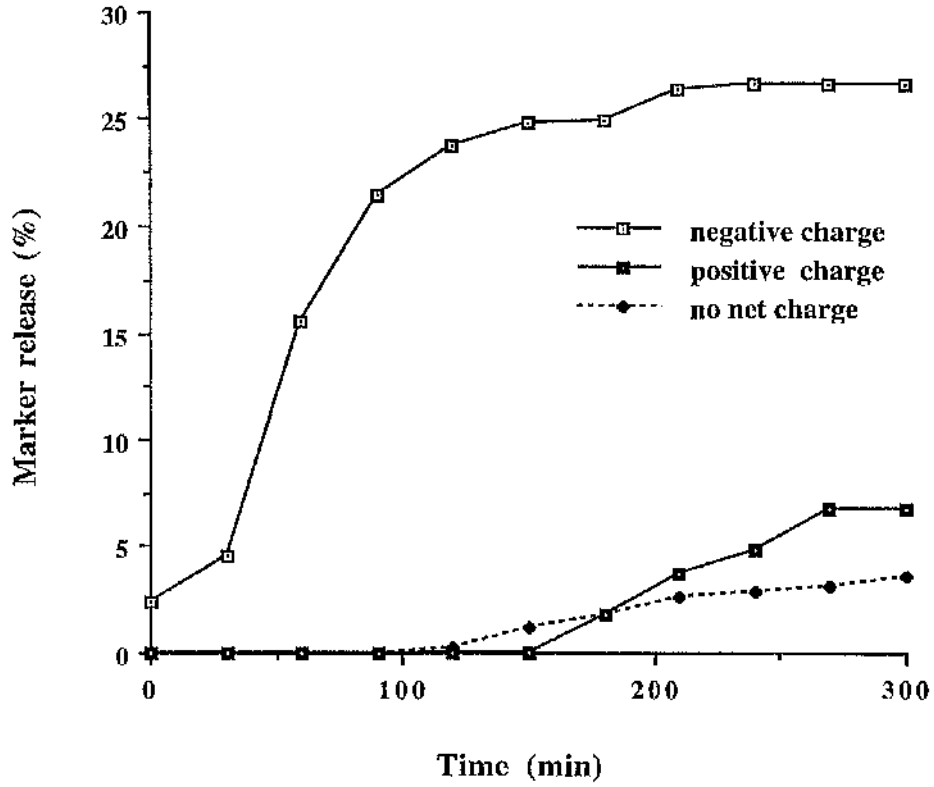


Figure 3.31 ***Effect of surface charge of liposomes on pore formation by CyaA.*** Liposomes were prepared with different surface charges by altering their composition as described in Materials and Methods. Negative, positive or liposomes with no net charge were prepared from lipid mixtures consisting of DMPC : C: DCP, DMPC: C: SA or DMPC : C, respectively. Liposomes were treated with dialysed recombinant CyaA (100 μg/ml). Percentage of marker release was calculated by comparison with release elicited by Triton-X 100. Marker release by dialysed toxin buffer and urea extract from*E.coli* DH5'α (pICNOH) was < 1%.

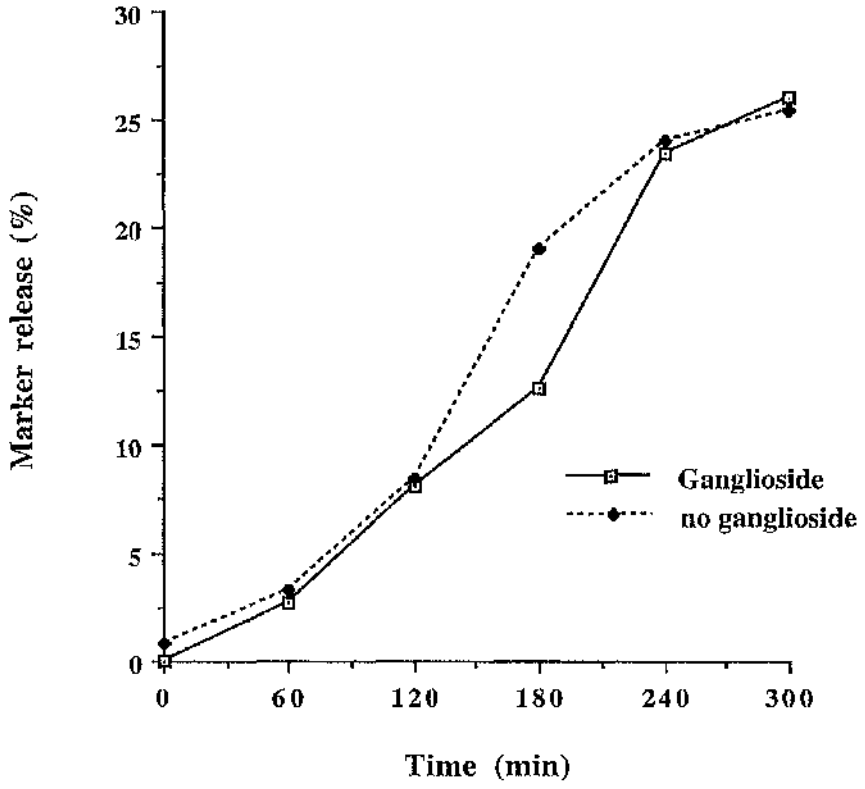


Figure 3.32 ***Role of gangliosides in pore formation by CyaA in liposomes.*** Liposomes were composed of DMPC : C G_{T1b} **(ganglioside)** or DMPC : C : DCP **(no ganglioside)** Liposomes were treated with dialysed recombinant CyaA (100 µg/ml). Percentage of marker release was calculated by comparison with release elicited by Triton-X 100. Marker release by dialysed toxin buffer and urea extract from *E.coli* DH5'α (pICNOH)was < 1%.

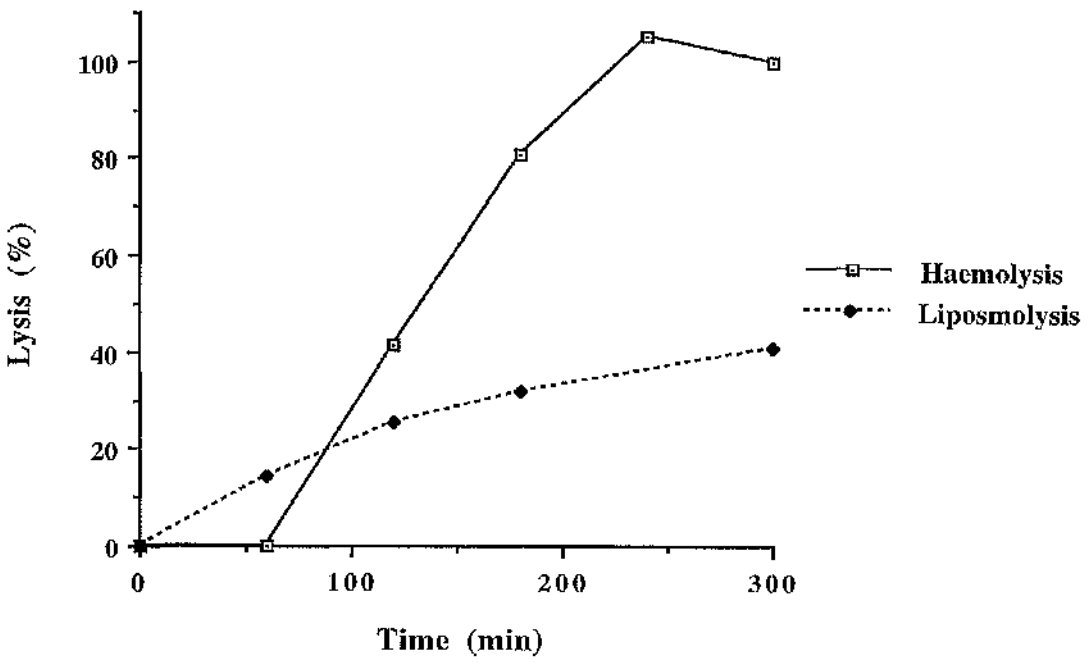
3.4.3 Pore formation by adenylate cyclase toxin in erythrocytes and liposomes

Liposomes were used as a model membrane system. In order to apply the results obtained above to eukaryotic cell membranes it was necessary to compare the lytic effects of CyaA on liposomes and erythrocytes (**Figures 3.33a and b**). Lysis of erythrocytes showed a lag period of approximately 60 min whereas lysis of liposomes occurred without a lag (**Figure 33b**). When the same amount of CyaA protein was applied to erythrocytes and liposomes, haemolysis reached completion (100%) whereas lysis of liposomes only reached (40%), after 300 min. Therefore, the toxin was either only lysing 40% of the liposomes or causing 40% marker release from all of them or both. Increasing the concentration used to treat the liposomes increased lysis, but the amount of liposome lysis after 300 min was still considerably lower than haemolysis (**Figure 3.33b**). Therefore, the toxin was either only able to lyse some of the liposomes or cause some marker release from all of them or a combination of both. It was therefore necessary to be careful when making comparisons between the model membrane system and the eukaryotic cells themselves.

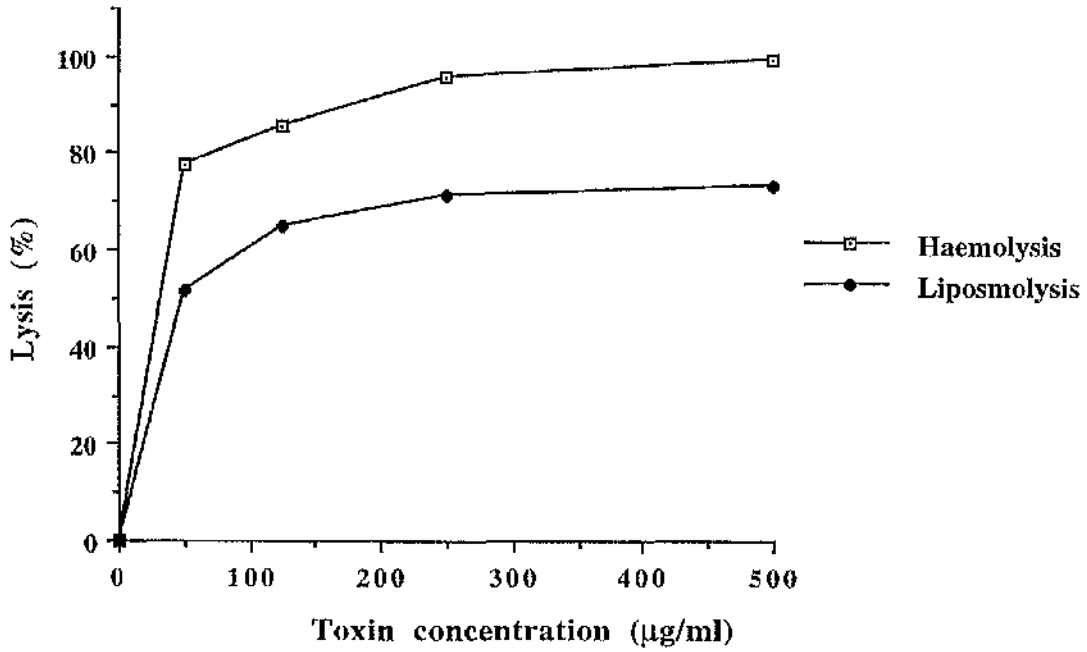
3.33a ***Lytic effect of CyaA on sheep erythrocytes and liposomes - Time course.*** Liposomes composed of DMPC : C : DCP and sheep erythrocytes were prepared as described in the Materials and Methods. The liposomes and erythrocytes were treated with dialysed recombinant CyaA (100 μg/ml) and lysis calculated as described earlier.

3.33b ***Lytic effect of CyaA on sheep erythrocytes and liposomes - Dose response.*** Liposomes composed of DMPC : C : DCP and sheep erythrocytes were prepared as described in the Materials and Methods. The liposomes and erythrocytes were treated with different amounts of dialysed recombinant CyaA for 300 min and lysis calculated as described earlier.

3.33a


100
80
60
40
20
0
Lysis (%)
0
100
200
300
Time (min)
Haemolysis
Liposmolysis

3.33b


100
80
60
40
20
0
Lysis (%)
0
100
200
300
400
500
Toxin concentration (µg/ml)
Haemolysis
Liposmolysis

SECTION 4
DISCUSSION

4.1 PURIFICATION OF ADENYLATE CYCLASE TOXIN

In this study *B.pertussis* 348 containing the plasmid pRMB1 was chosen for production of CyaA because this strain had been previously shown to produce CyaA with high enzymic and toxic (invasive adenylate cyclase) activities (Brownlie *et al* 1988; Westrop *et al*, 1994). Indeed, with enzymic and toxic activities reported to be as much as 3-5 times higher than the wild type *B.pertussis* Tohama strain (Brownlie *et al*, 1988), *B.pertussis* (pRMB1) was suitable for parts of this study which required high levels of CyaA. Since the discovery of CyaA (Fishel *et al*, 1970) a number of investigators have made attempts to purify and characterise it (see section 1.4.1.1). Complex methods for purification have been described (Hewlett *et al*, 1989; Gentile *et al*, 1989). These methods include hydrophobic interaction chromatography and sucrose gradient centrifugation as well as a calmodulin-affinity chromatography step.

. Urea extraction was the method chosen for the initial In this present study a two-step process was employed for purification of CyaA from *B.pertussis* 348 (pRMB1) extraction of CyaA from whole cells and this was first described by Confer and Eaton (1982). Treatment of whole cells with urea yields high levels of CyaA and was of particular use here because CyaA could be maintained in a stable form in urea solutions for several months (Confer and Eaton, 1982; Pearson *et al*, 1987; Kazi, 1992). Calmodulin-agarose (CaM) affinity chromatography was the next stage used in the purification of CyaA. This method of purification has been adopted by other investigators (Bellalou *et al*, 1990; Kazi, 1992; Westrop *et al*, 1994) and constituted a relatively simple and rapid method for purifying CyaA from *B.pertussis*.

By the two-step purification process full length (200 kDa) CyaA, which reacted with monospecific anti-CyaA serum, was purified (**Figures 3.1a** and **b**). In the purification process a 50-60 kDa polypeptide was also found in the CaM-agarose purified samples of CyaA (**Figure 3.1a**). A number of other studies have also reported the co-purification of a low molecular weight polypeptide during the

purification of CyaA (Hewlett and Wolff, 1976; Wolff *et al*, 1984; Shattuck *et al*, 1985; Kessin and Franke, 1986; Ladant *et al*, 1986; Friedman, 1987). This polypeptide is a degradation product corresponding to the N-terminal catalytic domain of CyaA (Rogel *et al*, 1988; Ladant *et al*, 1989; Bellalou *et al*, 1990). In the present study the N-terminal degradation product was probably co-purified with the holotoxin because, as has been reported previously, this fragment contains the CyaA calmodulin binding site (Glaser *et al*, 1988a; Ladant *et al*, 1989). Results obtained in the present study in subsequent toxicity and pore-formation assays were not attributed to this fragment because, as has been reported in other studies, it is non-toxic and non-lytic to target cells (Hewlett *et al*, 1989; Rogel *et al*, 1989; Rogel *et al* , 1991).

The functional significance of CyaA activation was studied by preparing activated and non-activated forms of CyaA from two recombinant *E.coli* strains which co-expressed *cya* A with *cya* C or expressed*cya* A alone in an expression system (**Figure 2.1**) described previously by Betsou *et al* (1993). Urea extraction from these recombinant strains yielded a 200kDa protein which was relatively free from contaminating host cell protein as determined by Coomassie blue staining of SDS-PAGE gels (**Figure 3.4a**). Any contribution of contaminating protein from the host strain (*E.coli* DH5αF'IQ) was controlled for in assays by the incorporation of a control consisting of a urea extract from the host strain which had not been transformed with the recombinant plasmids harbouring the *cya* inserts.

4.2 CHARACTERISATION OF ADENYLATE CYCLASE TOXIN

CyaA is an invasive adenylate cyclase toxin (Hanski, 1989). The toxin is characterised by its ability to enter mammalian cells whereby it is activated by host cell calmodulin to convert ATP to cAMP leading to impairment of cell function (Confer et al, 1984).

In the present study the extracts of CyaA were characterised for their enzymic activity and also their toxicity towards BHK cells. The *B.pertussis* and *E.coli* extracts of CyaA had relatively high enzymic activities of 26-42 µmol cAMP/min/mg protein. The specific enzymic activity of the calmodulin-agarose purified extract from *B.pertussis* 348 (pRMB1) was 20-fold higher than the levels reported for CyaA purified by a similar method from the same strain by Kazi (1992). Rogel *et al* (1989) also reported purification of CyaA with an enzymic activity of 387.5 µmol cAMP/min/mg protein from *B.pertussis* 348 (pRMB1). However, in the study by Rogel *et al* (1989) the urea extract from *B.pertussis* 348 (pRMB1) was concentrated by ultra-filtration and partially purified by gel-filtration prior to CaM-agarose chromatography purification of CyaA. The current study was not a study on methods of purification of CyaA rather it was a study on the mode of action. The extracts were relatively free from contaminating material as determined by Coomassie staining of SDS-PAGE gels and although the enzymic activity was lower than the activity described by Rogel *et al* (1989) further purification was not considered necessary for the purposes of the study .

The extracts from *B.pertussis* and *E.coli* were assayed for their toxic activity by a novel toxicity assay which has been developed by Westrop *et al* (1994). Invasive adenylate cyclase activity (toxicity) of CyaA is usually determined by measurement of intracellular cAMP levels. The procedure is lengthy and time consuming and after incubation with CyaA, cells require further extraction with HCl or sodium acetate followed by a number of centrifugation steps (Hanski and Farfel, 1985; Hewlett *et al,* 1989; Ladant *et al,* 1989; Leusch *et al,* 1990) with measurement by a radioimmuno protein-binding assay (Gillman, 1970). The shape change assay which was developed in this department was relatively quick, free of isotopes and required only 90 minutes of incubation between CyaA and BHK cells before cells were fixed, stained and toxicity measured by computer aided image-analysis. The assay has been previously shown to be >11-fold more sensitive than traditional methods of intracellular and cAMP

measurement and >150-fold more sensitive than haemolysis assays (Westrop *et al,* 1994). This novel toxicity assay demonstrated that the activated forms of CyaA purified from *B.pertussis* and *E.coli* possessed toxic activity which was absent from the non-activated form of CyaA, and was in agreement with results obtained using traditional toxicity assays (Barry *et al,* 1991; Betsou *et al,* 1993 Hewlett *et al,* 1993). Hewlett *et al* (1993) found that the absence of toxic activity of non-activated CyaA could be alleviated by artificially injecting CyaA into Jurkat cells.

4.3 CELL-BINDING OF ADENYLATE CYCLASE TOXIN

The full sequence of events during the interaction of CyaA with target cells has not yet been fully elucidated. However, the first stage in the successful interaction of CyaA and the other RTX toxins with target cells is cell-binding or cell-association. To examine the binding of CyaA to cells a whole-cell binding assay was employed which utilised the fact that a mono-specific anti-CyaA antibody was available which could detect the presence of CyaA in immunoblots of toxin-treated membranes (see section 2.10). The assay was relatively simple and gave some interesting qualitative data about the specificity of binding of CyaA to erythrocytes and nucleated cell types and the role that activation played in this association.

In this present study it was demonstrated that CyaA could bind to / associate with sheep erythrocytes in a time and dose-dependent manner (section 3.2.1). However, the majority of CyaA was bound within 30 minutes, and binding increased only slightly after 60 and 120 minutes, as detected by immunoblotting of toxin-treated membranes (**Figure 3.6**). In general, these results were in accordance with the findings of a study by Rogel *et al* (1991). In that study, which utilised a similar binding assay, binding of CyaA to sheep erythrocytes could be detected within 5 minutes. However, in contrast with the results presented here, Rogel *et al* detected a considerable increase in CyaA binding with time. These differences may be attributed to

the fact that in this study sheep erythrocytes were incubated with a 100µg dose of CyaA while Rogel *et al* used only 2.5µg. In the current study by using forty times more toxin than Rogel *et al* , it is possible that the erythrocytes were swamped with such high levels of toxin that increases in binding with time could not be detected or that the excess of toxin caused cell lysis leading to loss of bound material. However, a related study on *E.coli* HlyA, another RTX toxin, also found that the majority (85-95%) of toxin became bound within 60 minutes (Eberspacher *et al,* 1988).

Whereas time-dependent binding of CyaA to erythrocytes was not observed with the time-scale used in the present study, for the reasons described above, it was found that as the concentration of CyaA was increased from 5-100 µg/ml there was a visible and linear increase in cell-bound toxin and that there was no apparent end-point (plateau) of binding reached (**Figure 3.7**). This result concurs with an earlier study on *E.coli* HlyA which quantitatively demonstrated by ELISA that the amount of cell-bound HlyA increased as a linear function of the toxin dose applied such that HlyA did not display saturatability in its binding to erythrocytes (Eberspacher *et al,* 1988). The results presented here give further credence to the hypothesis that the binding of CyaA, and probably other RTX haemolysins is non-saturatable and therefore non-specific.

There has been limited research into the specificity of the initial interaction of CyaA with target cells. CyaA has broad range specificity so it unlikely to interact with a specific receptor and indeed may interact with molecules that are common to many if not all eukaryotic cell surfaces. A study by Gordon *et al* (1989) found that pre-treatment of CHO cells with trypsin (5-2000µg/ml) for 30 minutes did not affect subsequent intoxication by CyaA, as measured by intracellular cyclic AMP levels. The present study has extended and corroborated these findings by demonstrating that pre-trypsinisation not only did not inhibit haemolysis of sheep erythrocytes by CyaA but actually increased the susceptibility of the cells to the toxin (**Figure 3.9b**). Indeed, the haemolytic activity of CyaA was directly dependent on the concentration of trypsin used to treat the erythrocytes. However, in contrast, the binding of CyaA to trypsinised

cells, as detected by immunoblotting of toxin treated membranes, decreased considerably (**Figure 3.9a**). This was not due to degradation by residual trypsin or a cell surface protease (**Figure 3.12**). Trypsinisation of the erythrocytes may allow CyaA greater accessibility to the hydrophobic membrane and hence allow increased penetration by stripping-off hydrophilic cell surface structures such as glycoproteins.The increased lysis of the cells by the high levels of CyaA encounteriung the membranes may have resulted in release or solubilisation of cell-associated toxin such that it could not be detected by the binding assay.

The finding from this study that CyaA can productively associate with cells treated with high concentrations of trypsin and cause haemolysis along with the findings of Gordon *et al* (1989) are strong indications that CyaA does not interact with a protein receptor. Alternatively, CyaA may interact with a trypsin-resistant protein receptor. This is unlikely because it was also found that another protease (proteinase K) could also potentiate the haemolytic activity of CyaA (data not shown). These results were confirmed and extended by the overlay and liposomes assays (see sections 3.3 and 3.4)

To determine if CyaA interacted with specific sugar molecules, which are present on eukaryotic cell-surfaces on either glycolipids or glycoproteins, the inhibitory effects of a variety of sugars on the binding of CyaA to sheep erythrocytes was investigated. In this study it was found that none of the sugars tested; D-galactose, D-glucose, D-mannose, D-maltose, D-fucose or N-acetyl neuraminic acid (NANA), inhibited the binding of CyaA to sheep erythrocytes. It has been reported that treatment of human phagocytes with neuraminidase inhibits the toxicity of CyaA and suggested that neuraminic acid moieties on gangliosides may be receptors for CyaA (Gable *et al,* 1985). However in the present study NANA did not block binding of CyaA to erythrocytes. It is possible that the concentration of the sugars was too low to block binding of CyaA, but at a concentration of 50mM this is unlikely. A study by Escuyer and Collier (1991) into the receptor for*Bacillus anthracis* protective antigen (PA) used

13 monosaccharides, gangliosides and glycoproteins at the same concentration as in this study, to determine if they inhibited the interaction of PA with CHO cells, and binding-inhibition was detected.

It is probable that the initial interaction of CyaA with receptor-like molecules may be a weak non-specific one, facilitating penetration of the molecule into the membrane. Alternatively, in bulk solution the CyaA toxin molecule may be folded differently than when it is in association with cell membranes, possibly as a response to differing dielectric constant in the immediate vicinity of the membrane surface, and that an initial non-specific association of CyaA with the target cell membrane, may result in partial unfolding of the toxin molecule allowing it to anchor itself to the membrane (via negatively charged molecules such as gangliosides). This may be followed by pore formation and delivery of the catalytic unit to the cell interior.

4.4 ROLE OF ACTIVATION IN CELL-BINDING OF ADENYLATE CYCLASE TOXIN

CyaA is a member of the RTX toxins family and in common with the other RTX toxins it is now well established that it is activated by means of a C-gene-mediated modification (Barry *et al,* 1991; Coote, 1992). It has been suggested that activation of RTX toxins may be important in determining cell-specificity (Forestier and Welch, 1990; Westrop *et al,* 1997). To study this, the binding of activated and non-activated CyaA to different cell types was investigated (section 3.2.4). In this study it was found that activated and non-activated derivative of CyaA could both bind to / associate with sheep erythrocytes, although on visual examination of Western Blots (**Figure 3.12**) of toxin-treated membranes, slightly more activated CyaA had become bound. Using Jurkat cells as a model for the interaction of CyaA with cells, Hewlett *et al* (1993) have also demonstrated that activation is not required for the initial interaction of CyaA with cells. However, in addition they demonstrated a quantifiable difference between the

interaction of the different forms of CyaA with cells, in that 99% of the cell-associated non-activated toxin was sensitive to trypsinisation whereas 20% of cell-associated activated toxin was trypsin resistant. It can be concluded from their study that CyaA produced from cells lacking CyaC is defective in the insertion and delivery of the catalytic unit to cells, and therefore more sensitive to proteolysis.

Bauer and Welch (1996) have recently demonstrated that acylated and non-acylated HlyA bind equally well to sheep erythrocytes, and that acylated HlyA can displace unacylated HlyA from the cell surface. They suggested that activated HlyA has a slightly stronger affinity for the cells surface than the non-activated form which is in agreement with the results presented here for CyaA (**Figure 3.12**).

Whereas, there was only a minimal difference in the binding of the activated and non-activated forms of CyaA to sheep erythrocytes, this was not the case when the binding was examined using a murine macrophage cell line (J774.2). It was found that binding of non-activated CyaA was significantly lower and almost absent compared to the activated form (**Figure 3.13**). These results are the first evidence of differences in affinity between activated and non-activated CyaA. It is possible that the results with this cell line reflect the true significance of activation of CyaA, which may be to selectively target activated CyaA to cells of the immune system such as macrophage. The haemolytic activity of CyaA occurs after a 30-60 minute lag period and requires relatively high concentrations of toxin, whereas intoxication is without a discernable lag and occurs in the presence of relatively low concentrations of CyaA (Rogel *et al*, 1991). During the *B.pertussis* infection of the upper respiratory tract the organism and toxins liberated such as CyaA will rarely encounter erythrocytes unless there is damage to the respiratory epithelium. Therefore erythrocytes, although useful as models of pore-formation by CyaA, nucleated cell types may have more relevance to the *in vivo* situation which CyaA will encounter during pertussis.

Interestingly, no binding of activated or non-activated CyaA was detected with BHK or BL3 cells (**Figure 3.13**). This was particularly surprising since CyaA was

shown to be toxic to BHK cells in the Shape-Change assay (**Figure 3.5**). It is possible that the solubilisation conditions of hypotonic lysis of these cell types may have resulted in the dissociation of bound toxin such that in the subsequent washing steps CyaA was not recoverable or that the assay was not sensitive enough to detect the small amount of cell-bound toxin.

Recently the mechanism of activation of CyaA has been investigated, and it has been found that activation occurs by a similar mechanism to that described for HlyA by acylation of the protoxin (Hackett *et al*, 1994; Heveker *et al*, 1994). Heveker *et al* demonstrated that random transfer of fatty-acyl groups to inactive CyaA conferred haemolytic and toxic activities to the molecule (Heveker *et al*, 1994). Recently it has been shown that wild-type activated CyaA is palmitoylated at lysine-983 whereas inactive CyaA is not (Hackett *et al*, 1994).

A very recent and extensive study on *E.coli* haemolysin has produced some interesting findings which confirm the results presented here concerning the role of activation with regard to target cell specificity (Pellet and Welch, 1996). In the study the cytotoxic phenotype of *E.coli* haemolysin mutants bearing substitutions in or around the known *in-vitro* activation sites of HlyA was tested for cytotoxicity towards three different cell types: sheep erythrocytes, BL-3 cell line and Raji cell line (human B-cell lymphoma). The study demonstrated that the lytic activity of HlyA towards sheep erythrocytes and Raji cells depended on the presence of two acylated lysines in the putative toxin-activation site, whereas the cytotoxic activity of HlyA towards BL-3 cells did not require this modification. The results presented here along with the results described by Pellet and Welch (1996) suggest that *in vivo*, activation of CyaA and HlyA may be required for targeting these toxins towards different cell-types.

A recent study by Iwaki *et al* (1996) examined the toxic activities of a set of deletion derivatives of CyaA and found that there was no dedicated cell-binding domain of CyaA, rather that structural integrity of CyaA and co-operation of all the domains of CyaA and fatty acylation was required for cell-association and subsequent toxicity. This

is in contrast to other more specific toxins such as *Pseudomonas* exotoxin A which has a discrete cell-recognition domain essential for toxin-receptor interaction (Wick *et al,* 1990). Activation by acylation may be a means for directing and anchoring CyaA and other RTX toxins in target cell membranes. A recent study be Westrop *et al* (1997) has shown that a region spanning the proposed actylation site of the highly specific RTX leukotoxin LktA, produced by *Pasturella haemolytica* , and the adjacent glycine rich repeat region plays a major role in determining specificity.

It should noted that although traditionally *B.pertussis* has been considered an extra-cellular pathogen which multiplied on the surface of the respiratory epithelium without internal dissemination, it has also been found in alveolar macrophage early in infection (Gray and Cheers, 1967). In addition, *in vitro* can enter and survive intracellularly in various cell types, including macrophages, polymorphonuclear leukocytes, epithelial cells, Hela cells, HEp2 cells and CHO cells (Evanowich *et al,* 1989; Lee *et al,* 1990; Mouallem, 1990; Saukkonen *et al,* 1991; Steed *et al,* 1991; Roberts *et al,* 1991; Friedman *et al,* 1992; Masure, 1992). Recently it has been demonstrated that CyaA itself can be delivered by entry of whole bacteria into target cells, and that indeed in these circumstances the toxic activity of CyaA actually contributes to the survival of *B.pertussis* inside cells (Mouallem *et al* , 1990; Masure, 1993). Moreover, using a macrophage cell line Khelef *et al* (1993), showed that *B.pertussis* promotes macrophage apoptosis (programmed cell-death) and that CyaA was essential in this process whereas pertussis toxin was not required. This mode of entry may have considerable significance to the pathology of whooping cough whereby it may be a means by which the organism establishes an intracellular reservoir from which it can infect neighbouring cells and tissue surfaces and also other susceptible individuals. Repression of virulence factors may be importance if as part of its life cycle *B.pertussis* survives in its host in a quiescent intracellular state

4.5 CELLULAR RECEPTORS FOR ADENYLATE CYCLASE TOXIN

Glycoprotein receptors have been previously described for a number of bacterial protein toxins including *Bacillus anthracis* protective antigen (Escuyer and Collier, 1991), *E.coli* heat-stable toxin (Dreyfus and Robertson, 1984), diphtheria toxin (Mekada, 1991) and *Bacillus thuringiensis* ∂-endotoxin (Knowles and Ellar, 1984). A number of techniques have been applied to identify proteinaceous receptors. Loss of susceptibility to toxin after enzymic pre-treatment has been the first step in the identification of protein receptors for a number of bacterial toxins. Escuyer and Collier demonstrated that by pre-treating CHO cells with trypsin or pronase, the receptor for *B.anthracis* PA was proteinaceous in nature (Escuyer and Collier, 1991). Similar studies have been the starting point for identification of protein receptors for diphtheria toxin (Moehring and Crispell, 1974) and *E.coli* heat-stable enterotoxin (Frantz and Robertson, 1983). In this present study it was shown that pre-trypsinisation did not inhibit subsequent haemolysis of sheep erythrocytes by CyaA, rather it potentiated it (section 4.3). CyaA exhibits little or no specificity in its cellular attack and will lyse erythrocytes and is toxic to a wide range of nucleated cell types (see section 1.5). It is therefore unlikely that CyaA interacts with a specific receptor.

However, to investigate this further a nitro-cellulose overlay assay was developed. Nitro-cellulose overlay assays have been used previously by other investigators to isolate glycoproteins receptors for bacterial toxins such as *B.thuringiensis* ∂-endotoxin and *Pseudomonas aeruginosa* exotoxin A (Knowles and Ellar, 1986; Forristal *et al,* 1991; Thompson *et al,* 1991) and for other bacteria such as *Mycoplasma pneumoniae* and oral streptococci (Geary *et al,* 1990; Murray *et al,* 1992). The assay involved the transfer of sheep erythrocyte membrane proteins, previously separated by SDS-PAGE, to nitro-cellulose by Western blotting. The immobilised proteins were then probed with CyaA and binding analysed by means of antibodies. In

the present study no binding of CyaA was detected to the membrane proteins isolated from sheep erythrocytes.

Gordon *et al* (1988) found that agents such as cytochalasin D, ammonium chloride and chloroquinoline, which interfere with receptor-mediated uptake mechanisms, had no effect on the uptake of CyaA, prepared as a urea extract from whole cells of *B.pertussis* strain 338, into CHO cells (Gordon *et al,* 1988). A related study, which measured the entry of CyaA, partially purified from the culture supernatant of *B.pertussis* (Tohama phase 1 strain) into a murine neuroblastoma cell line (N1E-115), confirmed these earlier findings (Donovan and Storm, 1991). In general bacterial toxins like diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A that enter target-cells by receptor-mediated endocytosis interact with specific glycoproteins on cell surfaces (Morris, 1990; Mekada *et al,* 1991) whereas toxins such as cholera toxin and *E.coli* LT that penetrate cell membranes directly (Eidels *et al,* 1983) interact with glycolipid-type receptors. The evidence presented here suggests, in common with other bacterial toxins which do not enter cells by receptor-mediated endocytosis, that CyaA does not interact with specific glycoproteins

To further investigate the hypothesis that CyaA may interact directly with glycolipid-type receptor(s) a thin-layer chromatogram overlay assay was developed. The thin-layer chromatography overlay assay was first described by Magnani *et al* (1981) for detection of cholera toxin binding to gangliosides. Since then the technique has been applied successfully to identify the glycolipid-type receptors for a number of bacterial toxins including pertussis toxin (Brennan *et al,* 1991; Hausmann and Burns, 1993) shiga-like toxin (Samuel *et al,* 1990) and microbial adhesins (Hansson *et al,* 1985; Payne *et al,* 1993; Willemsen and Graaf, 1993; Cameron and Douglas, 1996). The technique uses the principal that lipids separated and immobilised by thin-layer chromatography can be overlaid with ligands such as bacterial toxins and adhesins and binding can be detected by enzyme- or isotope -labelled antibody systems.

In the current study a number of technical difficulties were encountered in the development of the TLC overlay technique. Initially using an immuno-detection method (see **Figure 2.3**) there was background staining of the chromatograms such that it was difficult to differentiate between the background and specific binding of CyaA. The system used to detect the binding of CyaA, i.e. initial incubation with CyaA followed by sequential overlay of TLC's with antibodies prior to visualisation of binding by means of diaminobenzidine (DAB) developer, was adopted from Western Blotting and was found to be unsuitable for TLC. This was probably due to the fact that once the TLC's had been run they were coated with a solution of poly-isobutylmethacrylate to present and orientate the lipids prior to the overlay stages. It is possible that there was a reaction occurring between the developer mixture and the coated surface of the chromatograms.

Another problem encountered with this technique was cross-reactivity between the anti-CyaA serum with components of the sheep erythrocyte extract. The serum was mono-specific and was raised by Dr Yasmeen Kazi (University of Glasgow) in a rabbit against sonicated nitro-cellulose containing CyaA. It is possible that the rabbit used to prepare the serum had previously encountered ovine antigens and elicited an immune response. This may have yielded some ovine-cross reactivity in the anti-CyaA serum. However, it is of note that in the cell-binding assays and nitro-cellulose overlay assays no cross-reactivity was detected between anti-CyaA and the sheep erythrocyte membrane proteins. Therefore, it is possible that the binding of the anti-CyaA serum to the lipid components of the membrane extract and gangliosides was a non-specific adsorption. Nevertheless, the cross-reactivity with the components was successfully removed from the anti-CyaA serum by pre-adsorbing the serum to pre-run components on a TLC plate.

The TLC overlay assay demonstrated that CyaA could bind to a range of gangliosides and that the toxin did not display a specificity for any of those tested (GM_1, GD_{1a}, GD_{1b}, and GT_{1b}). Binding of CyaA was detected to some but not all of

the components of the sheep erythrocyte lipid extract. This is the first evidence that CyaA can bind directly to lipid components extracted from sheep erythrocyte membranes and also purified gangliosides. Along with the finding that CyaA does not bind to membrane proteins from sheep erythrocytes these results suggest that CyaA does not interact with a specific receptor but may interact with a range of components on the cell surface which may facilitate insertion into the cell membrane. It would be interesting to determine the degree of binding of non-activated CyaA to the same components, but unfortunately when these experiments were carried out an extract of non-activated CyaA was not available.

4.6 PORE-FORMATION BY ADENYLATE CYCLASE TOXIN

It had been shown previously that CyaA can lyse erythrocytes by colloid osmotic lysis by formation of pores with an approximate diameter of 0.6 nm (Ehrmann *et al*, 1991; Rogel *et al*, 1991). The haemolytic activity of CyaA is weak compared to its toxic (invasive adenylate cyclase) activity and haemolysis is reported to occur after a lag phase of 30-60 minutes (Ehrmann et al, 1991).

The results presented here demonstrate the haemolytic activity of CyaA purified from *B.pertussis* 348 (pRMB1) and from a recombinant *E.coli* strain towards sheep erythrocytes. The haemolytic activities and kinetics of haemolysis exhibited by the CyaA extracts purified from *B.pertussis* and *E.coli* were similar. A study by Sebo *et al* (1991) found recombinant CyaA expressed an *E.coli* strain to have lower haemolytic activity then CyaA purified from *B.pertussis* 18323. In that study *cyaA* and *cyaC* were expressed on separate plasmids in the same *E.coli* host. The results presented here demonstrate that when the cyaA and cyaC genes are expressed in the same high-copy number plasmid then there is little difference in the haemolytic activities compared to *B.pertussis* CyaA. These results are in agreement with a study by Betsou *et al* (1993) which compared the haemolytic activities of CyaA produced by co-expression

of *cyaA* and *cyaC* on the same plasmid with a two plasmid system and found that co-expressing of the two genes to be more efficient for production of activated CyaA.

Haemolysis mediated by these extracts increased with the dose applied and the time of incubation. These results are in agreement with studies carried out by other investigators (Ehrmann *et al,* 1991; Rogel *et al,* 1991; Bellalou *et al,* 1993). There was a lag period of between 30-60 minutes before haemolysis became evident. Other studies have reported a lag-phase before haemolysis became apparent (Ehrmann *et al,* 1991; Rogel *et al,* 1991).The most recent studies into the nature of the pore formed by CyaA have indicated that the toxin forms small channels in membranes which are polarity and voltage dependent and suggest that the pore formed is an oligomer and produced by the interaction of at least three toxin monomers (Benz *et al,* 1994; Szabo *et al,* 1994; Otero *et al,* 1995).

Interestingly, considering that pertussis is a human disease, it has been reported that human erythrocytes are relatively resistant to haemolysis by CyaA but still sensitive to the toxicity of CyaA, albeit at lower levels compared to erythrocytes from other mammalian species (Rogel et al, 1991). This effect can abrogated by pre-treatment of cells with a protease inhibitor (N-ethyl malemide). It is likely that human erythrocytes, unlike erythrocytes from other species which will not have had the selective pressure since *B.pertussis* is a human pathogen, may have evolved to become resistant to haemolysis by CyaA.

In the study presented here liposomes were prepared to investigate the effect of membrane composition on the lytic activity of CyaA. These experiments demonstrated that CyaA could permeabilise liposomes in a time and dose-dependent manner, an observation which had been previously reported by Gordon *et al* (1989). Unlike haemolysis the lytic effect of CyaA on liposomes proceeded without a lag period. This is presumably because liposomes do not have the same constraints on membrane integrity that erythrocytes have and can leak contents without lysis. The lipsomes assay measured the release of a fluorescent marker calcein, whereas haemolytic assays

measure that release of haemolglobin a cellular protein. Membrane disruption in erythrocytes requires osmotic swelling before release of haemoglobin. Erythrocyte membrane permeablisation will by definition be much greater than that associated with permeability changes involving release of a smaller chemical marker from liposomes: hence the lag period.

Despite the absence of a lag period the final marker release from liposomes was low (30-40%) compared to the haemoglobin release in haemolysis (100%) for the comparable time period. In a study by Gordon *et al,* (1989) the marker release by CyaA from liposomes also plateaued at a similar low level (approximately 40%). The liposome permeability assays were carried out at room temperature without temperature control because no thermostatic control was available on the microplate reader in which the experiment was carried out. The haemolysis assays were carried out at 37ºC. This difference in marker release with time can probably be attributed to these temperature differences.

It has been previously reported that gangliosides may act as putative receptors for CyaA (Gable *et al,* 1985; Gordon *et al,* 1989). To investigate this a comparison was made of CyaA mediated marker release from liposomes with or without gangliosides incorporated. It was found that a negative charge on the liposomes was enough to make them susceptible to CyaA and that there was not an absolute requirement for the presence of gangliosides. Furthermore, assays in which the charge of the liposomes was altered showed that negatively charged liposomes were much more susceptible to CyaA than positively charged or neutral liposomes. These results are interesting in the light of a study performed by Rapitis *et al* (1989) who found that cations such as poly-L-lysine incubated with CHO cells could block the invasive activity of CyaA and concluded that the cations were blocking anionic sites on the membrane. In addition they found that treatment of CHO cells with neuraminidase would not block the CHO intoxication. The results presented here are further evidence to suggest that the interaction of CyaA with membranes may be at the level of a charge-charge interaction

with negatively charged moeties on the membrane surface acting as the initial recognition sites. It is possible that these sights may non-specifically interact with the positively charged glycine-rich repeats on the toxin molecule

The results presented here for haemolysis and marker release for liposomes demonstrate that CyaA activation is an absolute requirement for its haemolytic activity. Recently, other investigators have described a similar phenomenon with haemolysis (Betsou *et al,* 1993; Hewlett, *et al* 1993). It is possible that the initial non-specific charge association between CyaA and the target cell membrane is followed by a stronger anchoring in the membrane allowing penetration and expression of toxicity and eventual development of haemolysis.

In conclusion the interaction of CyaA with target cells is complex but it is gradually becoming understood.This study has revealed further aspects of the non-specific interaction of CyaA with cells and their lipid components. The importance of membrane surface charge has also been demonstrated. Further domain dissection studies will hopefully reveal more about the regions of the protein which interact with these negatively charged recognition sites. The role of activation of CyaA is still an area which requires intensive research but results from the present study indicate that activation may be a means of specifically targeting CyaA to cells of the immune system. Ultimately the interaction of CyaA with immune effector cells will be of most interest because the evidence suggests that it is a weak haemolysin which is ineffective on human erythrocytes. Nevertheless erythrocytes and model membrane systems such as liposomes, will continue to be useful tools for studying the interaction of CyaA with cell membranes.

SECTION 5
REFERENCES

ALDERSLADE, R., BELLMAN, M.H., RAWSON, N.S.B., ROSS, E.M. & MILLER, D.L. (1981). The National Childhood Encephalopathy study. In *Whooping Cough*, HMSO, London. 79-169.

AYME, G., CAROFF, M., CHABY, R., HAEFFNER-CAVAILLON, N., LEDUR, A., MOREAU, M., MUSET, M., MYNARD, M-C, ROUMIANTZEFF, M., SCHULZ, D. & SZABO, L.(1980). Biological activities of fragments derived from *Bordetella pertussis* endotoxin: Isolation of non-toxic, Shwartzman-negative lipid A possessing high adjuvant properties. *Infection and Immunity* , **27**: 739-745.

BALDWIN, T.J., KNUTTON, S., SELLERS, L., HERNANDEZ, H.A.M., AITKEN, A. & WILLIAMS, P.H. (1992). Enteroaggregative *Escherichia coli* strains secrete a heat-labile toxin antigenically related to *Escherichia coli* hemolysin. *Infection and Immunity*, **60**: 2092-2095.

BARRY, E.M., WEISS, A.A., EHRMANN, I.E., GRAY, M.C., HEWLETT, E.L. & GOODWIN, M.M. (1991). *Bordetella pertussis* adenylate cyclase toxin and hemolytic activities require a second gene, *cyaC*, for activation. *Journal of Bacteriology*, **173**: 720-726.

BAUER, M.E. & WELCH, R.A. (1996). Association of RTX toxins with erythrocytes. *Infection and Immunity*, **64**: 4665-4672.

BAUMANN, U., WU, S. FLAHERTY, K.M. & MCKAY, D.B. (1993). 3-Dimensional structure of the alkaline protease of *Pseudomonas aeruginosa* - a 2-domain protein with a calcium-binding parallel-beta roll motif. *EMBO Journal* , **12**: 3357-3364.

BELLALOU, J., LADANT, D. & SAKAMOTO, H. (1990). Synthesis and secretion of *Bordetella pertussis* adenylate cyclase as a 200-Kilodalton protein. *Infection and Immunity*, **58**: 1195-1200.

BELLALOU, J., SAKAMOTO, H., LADANT, D., GEOFFROY, C. & ULLMANN, A. (1990). Deletions affecting hemolytic and toxic activities of *Bordetella pertussis* adenylate cyclase. *Infection and Immunity*, **58**: 3242-3247.

BENDIXEN, D.H., SHEWEN. P.E., ROSENDALL. S. & WILKIE , B. (1981) Toxicity of *Haemophilus pleuropneumoniae* for porcine macrophage, peripheral blood monocytes and testicular cells. *Infection and Immunity*, **33**: 673-676.

BENZ, R., MAIER, E., LADANT, D., ULLMANN, A. & SEBO, P. (1994). Adenylate cyclase toxin (CyaA) of *Bordetella-pertussis* - evidence for the formation of small ion-permeable channels and comparison with HlyA of *Escherichia coli*.. *Journal of Biological Chemistry*, **269:** 27231-27239.

BENZ, R., SCHMID, A., WAGNER, W. & GOEBEL, W. (1989). Pore formation by the *Escherichia coli* hemolysin: Evidence for an association-dissociation equilibrium of the pore forming aggregates. *Infection and Immunity*, **57**: 887-889.

BERGQUIST, S.O., BERNANDER, S., DAHNAJO, H. & SUNDELOF, B. (1987). Erythromycin in the treatment of pertussis: A study of bacteriological and clinical effects. *Pediatric Infectious Disease Journal* , **6**: 458-461

BETSOU, F., SEBO, P. & GUISO, N. (1993). CyaC mediated activation is important not only for toxic but also for protective activities of *Bordetella pertussis* adenylate cyclase -haemolysin. *Infection and Immunity*, **61**: 3583-3589.

BHAKDI, S. & TRANUM-JENSEN (1987). Damage to mammalian cells by proteins that form pores. *Reviews in Physiology Biocheistry and Pharmacology*, **107**: 147-223.

BHAKDI, S. & TRANUM-JENSEN (1988). Damage to cell membranes by pore forming cytolysins. *Progress in Allergy*, **40**: 1-43.

BHAKDI, S., GREULICH, S., MUHLY, M., EBERSPACHER, B., BECKER, H., THEILE, A. & HGO, F. (1989). Potent leukocidal action of *Escherichia coli* hemolysin mediated by permeabilisation of target cell membranes. *Journal of Experimental Medecine*, **169**: 737-754

BHAKDI, S., MACKMAN, N., NICAUD, J.-M. & HOLLAND, I.B. (1986). *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. *Infection and Immunity*, **52**: 63-69.

BLIGHT, M.A. & HOLLAND, I.B. (1990). Structure and function of haemolysin B, P-glycoprotein and other members of a novel family of membrane translocators. *Molecular Microbiology*, **4**: 873-880.

BOEHM, D.F., WELCH, R.A. & SNYDER, I.S. (1990a). Calcium is required for binding of *Escherichia coli* haemolysin (hly A) to erythrocyte membranes. *Infection and Immunity*, **58**: 1951-1958.

BOEHM, D.F., WELCH, R.A. & SNYDER, I.S. (1990b). Domains of *Escherichia coli* hemolysin (Hly A) involved in binding of calcium and erythrocytes. *Infection and Immunity,* **58**: 1959-1964.

BORDET, J. & GENGOU, O.(1906). Le Microbe de la coqueluche. *Annales de L'institut Pasteur,* **20**: 731-741.

BRAUN, V., FOCARETA, T. (1991). Pore-forming bacterial protein hemolysins (cytolysins). *Critical Reviews in Microbiology* , **18** :115-158.

BRENNAN, M.J. HANNAH, J. & LEININGER, E. (1991). Adhesion of Bordetella pertussis to sulphatides and to the GalNAcβ4Gal containing lipids. *Journal of Biological Chemistry,* **266**: 18827-18831.

BROWNLIE, R.M., COOTE, J.G., PARTON, R., SCHULTZ, J.E., ROGEL, A. & HANSKI, E. (1988). Cloning of the adenylate cyclase genetic determinant of *Bordetella pertussis* and its expression in *Escherichia coli* and *Bordetella pertussis. Microbial Pathogenesis,* **4**: 35-344.

BRUNNER, J., SKRABAL, P. & HAUSER, H. (1976). Single bilayer vesicles prepared without sonication. Physico-chemical properties. *Biochimica et Biophysica Acta,* **455**: 322-311.

CAMERON, B.J. & DOUGLAS, L.J. (1996). Blood group glycolipids as epithelial cell receptors for *Candida albicans. Infection and Immunity,* **64**: 891-896.

CAUTRECASAS, P. (1974). Insulin receptor, cell membranes and hormone action. *Biochemistry and Pharmacology,* **23**: 2353-2361.

CAVALIER, S.J., BOHACH, G.A. & SNYDER, I.S. (1984). *Escherichia coli* α-haemolysin: characteristics and probable role in pathogenicity. *Microbiological Reviews,* **48**: 320-343.

CHABY, R. & CAROFF, M. (1988). Lipopolysaccharides of *Bordetella pertussis* endotoxin. In: *Pathogenesis and Immunity in Pertusssis* , eds Wardlaw, A,C., & Parton, R. John Wiley & Sons, Chichester. 247-271.

CHANG, Y., YOUNG, R. & STRUCK, D.K. (1989). Cloning and characterisation of a hemolysin gene from *Actinobacillus (Haemophilus) pleuropneumoniae. DNA,* **8**: 635-647.

CHARLES, I., FAIRWEATHER, N., PICKARD, D., BEESLEY, J., ANDERSON, R., DOUGAN, G. & ROBERTS, M. (1994). Expression of the *Bordetella pertussis* P.69 pertactin adhesin in *Escherichia coli* : fate of the carboxy-terminal domain. *Microbiology* , **140**: 3301-3308.

CHERRY, J.D. (1996). Historical review of pertussis and the classical vaccine.*The Journal of Infectious Diseases* , **174** (suppl 3): 259-263.

CHERRY, J.D., BRUNEL, P.A., GOLDEN, DARZOT, D.T. (1988). Report of the task force on pertussis and pertussis immunisation-1988. *Paediatrics*, **81** (suppl): 939-984.

CHUNG, C.T & MILLER, R.H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Research*, **16**: 3580.

CLINKENBEARD, K.D. & UPTON, M.L. (1991). Lysis of bovine platelets by *Pasteurella haemolytica* leukotoxin. *American Journal of Veterinary Research*, **52**: 453-457.

CLINKENBEARD, K.D., MOSIER, D.A. & CONFER, A.W. (1989). Transmembrane pore size and role of cell swelling in the cytotoxicity of *Pasteurella haemolytica* leukotoxin. *Infection and Immunity*, **57**: 420-425.

CONFER, D.L. & EATON, J.W. (1982). Phagocyte impotence caused by the invasive bacterial adenylate cyclase. *Science*, **217**: 948-950.

CONFER, D.L., SLUNGAARD, A., GRAF, E., PANTER, S.S. & EATON, J.W. (1984). *Bordetella* adenylate cyclase toxin: Entry of bacterial adenylate cyclase into mammalian cells. *Advances in Cyclic Nucleotide Research*, **17**: 183-187.

COOKSON, B.T., CHO, H-L., HERWALDT, L.A. & GOLDMAN, W.E. (1989). Biological activities and chemical composition of purified tracheal cytotoxin of *Bordetella pertussis*. *Infection and Immunity* , **57**: 2223-2229.

COOTE, J.G. & BROWNLIE, R.M. (1988). Genetics of virulence of *Bordetella pertussis* . In: *Pathogenesis and Immunity in Pertusssis* , eds Wardlaw, A.C. & Parton, R. John Wiley & Sons, Chichester: 39-74.

COOTE, J.G.C. (1992) Structural and functional relationships among the RTX toxin determinants of Gram-negative bacteria. *FEMS Microbiology Reviews*, **88**: 127-167.

COWELL, J.L., HEWLETT, E. & MANCLARK, C.R. (1979). Intracellular localisation of the dermonecrotic toxin of *Bordetella pertussis*. *Infection and Immunity*, **25**: 896-901.

CRUZ, W.T., YOUNG, R., CHANG, Y.F. & STRUCK, D.K. (1990). Deletion anelysis resolves cell binding and lytic domains of the *Pasteurella* leukotoxin, *Molecular Microbiology*, **4:** 1933-1939.

DONOVAN, M.G. & STORM, D.R. (1991). Evidence that the adenylate cyclase secreted from *Bordetrella pertussis* does not enter animal cells by receptor mediated endocytosis. *Journal of Cellular Physiology*, **145:** 444-449.

DREYFUS, L.A., ROBERTSON, D.C. (1984). Solubilization and partial characterization of the intestinal receptor for *Escherichia coli* heat-stable entero-toxin. *Infection and Immunity*, **46**: 537-543.

DUNN, G.A. & BROWN, F. (1985). Alingment of fibroblasts on grooved surfaces described by a simple geometric transformation. *Journal of Cell Science*, **88**: 363-368.

EBERSPACHER, B., HUGO, F. & BHAKDI, S. (1989). Quantitative study of the binding and hemolytic efficiency of *Escherichia coli* hemolysin. *Infection and Immunity*, **57**: 9830988.

EDWARDS, J.G., CAMPBELL, G, CARR, M. & EDWARDS, C.C.(1993). Shapes of cells spread on fibronectin: measurement of the stellation of BHK21 cells induced by using cAMP, and its reversal by serum and lysophosphatidic acid. *Journal of Cell Science*, **104**: 399-407.

EDWARDS, J.G., CAMPBELL, J.A., ROBSON, R.T., & VICKER, M.S. (1975). Trypsinised BHK21 cells aggregate in the presence of metabolic inhibitors and in the absence of divalent cations. *Journal of Cell Science*, **19**: 656-667.

EHRMANN, F.E., GRAY, M.C., GORDON, V.M., GRAY, L.S. & HEWLETT, E.K. (1991). Haemolytic activity of adenylate cyclase toxin from *Bordetella pertussis*. *FEBS Letters*, **278**: 79-83.

EIDELS, L., PROIA, R.L. & HART, D.A. (1983). Membrane receptors for bacterial toxins. *Microbiological Reviews* , **42**: 596-620.

EISENBERG, D., SCHWARZ, E., KOMAROMY, M. &WALL, R. (1984). Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *Journal Of Molecular Biology* , **199**: 125-142.

ENDOH, M., NAGAI, M. & NAKASE, Y. (1988). Contractile action of heat-labile toxin of *Bordetalla parapertsussis* on aortic smooth muscles of pigs. *Microbiology and Immunology* , **32**: 755-767.

ESCUYER, V. & COLLIER, R.J. (1991). Anthrax protective antigen interacts with a specific receptor on the surface of CHO-K1 cells. *Infection and Immunity*, **59**: 3381-3386.

EWANOWICH, C.A., MELTON, A.R., WEISS, A.A., SHERBURNE, R.K. & PEPPLER, M.S. (1989). Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. *Infection and Immunity* , **57**: 2698-2704.

FARFEL, Z., FRIEDMAN, E. &, HANSKI, E. (1987). The invasive adenylate-cyclase of *Bordetella pertussis* - intracellular-localization and kinetics of penetration into various cells.*Biochemistry Journal* , **243**: 153-158.

FARIZO, K.M., COCHI, S.L., ZELL, E.R., BRINK, E.W., WASSILAK, S.G.& PATRIARCA, P.A. (1992). Epidemiological features of pertussis in the United States, 1980-1989.*Clinical Infectious Diseases*, **14**: 708-719.

FELMLEE, T., PELLET, S., LEE, E.Y. & WELCH, R.A.(1985). The *Escherichia coli* hemolysin is released extracellularly without cleavage of a signal peptide. *Journal of Bacteriology* , **163**: 89-93.

FENICHEL, G.M. (1982). Neurological complications of immunisation. *Annals of Neurology* , **12**: 119-128.

FERNANDEZ, R.C. & WEISS, A.A. (1994). Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. *Infection and Immunity* , **62**: 4727-4738.

FINN, T.M. & STEVENS, L.A. (1995). Tracheal colonisation factor: *Bordetella pertussis* secreted virulence determinant. *Molecular Microbiology* , **16**: 625-643.

FISHEL, C.W., O'BRYAN, B.S., SMITH, D.L. & JEWEL, J.W. (1970). Conversion of ATP by mouse lung preparations and by an extract of *Bordetella pertussis*. *International Archives of Allergy*, **38**: 457-462.

FORESTIER, C. & WELCH, R.A. (1991). Identification of RTX toxin target cell specificity domains by use of hybrid genes. *Infection and Immunity,* **59**: 4212-4220.

FORESTIER, C. & WELCH, R.A.(1990). Non-reciprocal complementation of the hly C and Lkt Cgenes of the *Escherichia coli* haemolysin and the *Pasteurella haemolytica* leukotoxin determinants. *Infection and Immunity,* **58**: 838-632.

FORRISTAL, J.J., THOMPSON, M.R., MORRIS, K.E. & SAELINGER, C.B. (1991). Mouse liver contains a *Pseudomonas aeruginosa* exotoxin A-binding protein. *Infection and Immunity,* **59**: 2880-2884.

FRANTZ, J.C. & ROBERTSON, D.C. (1983). High affinity receptor for heat-stable enterotoxins (ST1) on rat intestinal epithelial cells. In: *Advances in research on cholera and related diarrheas,* eds Kuwahara, S. and Pierce, N.F. Martimus Nijhoff Publishers, Boston: 247-257

FRIEDMAN, E., FARFEL, Z. & HANSKI, E. (1987). The invasive adenylate cyclase of *Bordetella pertussis* : Properties and penetration kenetics. *Biochemistry Journal,* **243**: 145-151.

FRIEDMAN, R.L. (1987). *Bordetella pertussis* adenylate cyclase: isolation and purification by calmodulin sepharose 4B chromatography. *Infection and Immunity,* **55**: 129-134.

FRIEDMAN, R.L. NORDENSSON, K., WILSON, E., AKPORIAYE, E.T. & YOCUM, D.R. (1992). Uptake and intracellular survival of *Bordetella pertussis* in human macrophages. *Infection and Immunity,* **60**: 4578-4585.

GABLE, P., EATON, J.W. & CONFER, D.L. (1985). Intoxication of human phagocytes by *Bordetella perussis* adenylate cyclase toxin: implication of a ganglioside receptor. *Clinical Research,* **33**: 844A.

GADEBERG, D.V. & ORSKOV, I. (1984). *In vitro* cytotoxic effect of alpha-hemolytic *Escherichia coli* on blood granulocytes. *Infection and Immunity,* **45**: 755-760.

GALE, J.L., THAPA, P.B., WASSILAK, S.G., BOBO, J.K., MANDELMANN, P.M. & FOY, H.M. (1994). Risk of serious acute neurological illness after immunisation withdiphtheria-tetanus-pertussis vaccine. A population-based case-control study.*Journal of the American Medical Association* , **271**: 37-41.

GEARY, S.J., GABRIDGE, M.G. INTRES, R., DRAPER, D.L. & GLADD, M.F. (1990). Identification of mycoplasma binding proteins utilising a 100 Kilodalton lung fibroblast receptor. *Journal of Receptor Research,* **9**: 465-478.

GEBER, A., GRAY, M.C., PEARSON, R.D, HEWLETT, E.L.(1988) *Bordetella pertussis* (BP) adenylate cyclase toxin is lethal for cells of the J774.1A macrophage-like cell-line. *Clinical Research,* **36**: A456.

GENTILE, F. KNIPLING, L.G., SACKETT, D.L. & WOLFF, J. (1990). Invasive adenylyl cyclase of *Bordetella pertussis* . Physical, catalytic and toxic properties. *Journal of Biological Chemistry* , **265**: 10686-10692.

GILLMAN, A.G.A. (1990). A protein binding assay for 3',5'-cyclic adenosine monophosphate. *Proceedings of the National Academy of Sciences,* **67**: 305-312.

GIRARD, R., CHABY, R. & BORDENAVE, G. (1981). Mitogenic response of C3H/HeJ mouse lymphocytes to polyanionic polysaccharides obtained from *Bordetella pertussis. Infection and Immunity* , **31**: 122-128.

GLASER, P. EMAOGLOU-LAZARIDOU, A., KRIN, E., LADANT, D., BARZU, O. & DANCHIN. (1989). Identification of residues essential for catalysis and binding of calmodulin in *Bordetella pertussis* by site directed mutagenesis. *EMBO Journal,* **8**: 967-962.

GLASER, P., DANCHIN, A., BARZU, O. & ULLMANN, A. (1990). *Bordetella pertussis* adenylate cyclase. In: Bacterial Protein Toxins. Eds: Rappuoli, R., Alouf, J.E., Falmange, P., Fehrenbach, F.J., Freer, J.H., Gross, R., Jajaszewicz,J., Montecucco. C., Tomasi, M., Wadstrom, T. & Wiltholt, B.. Gustav Fisher, Stuttgart and New York: 375-382.

GLASER,P., LADANT, D., SEZER, O., PICHOT, F., ULLMAN, A. & DANCHIN, A. (1988a). The calmodulin sensitive adenylate cyclase of *Bordetella pertussis* - cloning and expression in *Escherichia coli* . *Molecular Microbiology* , **2:** 19-30.

GLASER, P., SAKAMOTO, H., BELLALOU, J., ULLMANN, A. & DANCHIN, A. (1988b). Secretion of cyclolysin, the calmodulin sensitive acenylate cyclase-haemolysin, bifunctional protein of *Bordetella pertussis. EMBO Journal,* **7**: 3997-4004.

GOLDMAN, W.E., KLAPPER, D.G. & BASEMAN, J.B. (1982). Detection, isolation , and analysis of a released *Bordetella pertussis* product toxic to cultured tracheal cells. *Infection and Immunity*, **36**: 782-794.

GOODWIN, M.S. & WEISS, A.A. (1990) Adenylate cyclase toxin is critical for colonisation and pertussis toxin is critical for lethal infection by *Bordetella pertussis* in infant mice. *Infection and Immunity,* **58**: 3445-3447.

GORDON, V.M., LEPPLA, S.H. & HEWLETT. E. L (1988). Inhibitors of receptor -mediated endocytosis block the entry of *Bacillus anthracis* adenylate cyclase toxin, but not that of *Bordetella pertussis* adenylate cyclase toxin. *Infection and Immunity,* **56**: 1066-1069.

GORDON, V.M., YUONG, W.W., LECHLER, S.M., GRAY, M.C., LEPPLA, S.H. & HEWLETT, E.L. (1989). Adenylate cyclase toxins from *Bacillus anthracis* and *Bordetella pertussis.* Different processes for interaction with and entry into target cells. *Journal of Biological Chemistry,* **264**: 14792-14796.

GRAY, D.F. & CHEERS, C. (1967). The steady state in cellular immunity. II. Immunological complaisance in murine pertussis. *Australian Journal of Experimental Biology and Medical Science,* **45**: 417-426.

GREENLEA, D.V., ANDREASEN, T.J. & STORM, D.R. (1982). Calcium-independent stimulation of *Bordetella pertussis* adenylate cyclase by calmodulin. *Biochemistry* , **21**: 2758-2764,

GRIFFIN, M.R., RAY, W.S., MORTIMER, E.A., FENICHEL, G.M. & SCHAFFNER, W. (1990). Risk of seizures and encephalopathy after immunisation with the diphtheria-tetanus-pertussis vaccine. *Journal of the American Medical Association* , **263**: 1641-1645.

GYGI, D., NICOLET, J., FREY, J., CROSS, M., KORONAKIS, V. & HUGHES, C. (1990). Isolation of the *Actinobacillus pleuropneumoniae* haemolysin gene and the activation and secrretion of the prohaemolysin by the Hly C, Hly B, Hly D proteins of *Escherichia coli. Molecular Microbiology,* **4**: 123-128.

HACKETT, M., GUO, L., SHABANOWITZ, J., HUNT, D.F. & HEWLETT, E.L. (1994). Internal lysine palmitoylation in adenylate cyclase toxin from *Bordetella pertussis. Science,* **266**: 433-435.

HAEFFNER-CAVAILLON, N., CAVAILLON, J-M. & SZABO, L. (1982). Macrophage- dependant polyclonal activation of splenocytes by *Bordetella pertussis* endotoxin and its isolated polysaccharide and lipid A regions. *Cellular Immunology* , **74**: 1-13.

HANSKI, E. & FARFEL (1985). *Bordetella pertussis* adenylate cyclase: partial resolution and properties of cellular penetration. *Journal of Biological Chemistry* , **260**: 5526-5532.

HANSKI, E. (1989). Invasive adenylate cyclase toxin of *Bordetella pertussis.Trends in Biochemical Sciences,* **14**: 459-463.

HANSSON, G.C., KARLSSON, K.-A., LARSON, N., STROMBERG, N. &THURIN, J. (1985). Carbohydrate-specific adhesion of bacteria to thin-layer chromatograms: a rationalised approach to the study of host cell glycolipid receptors. *Analytical Biochemistry,* **146**: 156-163.

HARDIE, K.R., ISAARTEL, J.-P., KORONAKIS, E., HUGHES, C. & KORONAKIS, V. (1991). In vitro activation of *Escherichia coli* prohaemolysin to the mature membrane-targetted toxin requires Hly C and a low molecular-weight cytosolic polypeptide. *Molecular Microbiology* , **5:** 1669-1679

HAUSMAN, S.Z. & BURNS, D.L. (1992). Interaction of pertussis toxin with cells and model membranes. *Journal of Biological Chemistry* , **267**: 13735-13739.

HENKART, P. (1985). Mechanism of lymphocyte cytotoxicity. *Annual Review of Immunology* , **3**: 31-58.

HEVEKER, N., BONAFFE, D. & ULLMANN, A. (1994). Chemical fatty acid acylation confers hemolytic and toxic activities to adenylate cyclase protoxin of *Bordetella pertussis* . *Journal of Biological Chemistry* , **269**: 32844-32847.

HEWLETT, E. & WOLFF, J. (1976). Soluble adenylate cyclase from the culture medium of *Bordetella pertussis* : Purification and characterisation. *Journal of Bacteriology,* **127**: 890-898.

HEWLETT, E.L. &GORDON, V.M. (1988). Adenylate cyclase toxin of *Bordetella pertussis.* In : *Pathogenesis and Immunity in Pertusssis* , eds Wardlaw, A.C. & Parton, R. John Wiley & Sons, Chichester: 193-209.

HEWLETT, E.L., GORDON, V.M., MCCAFFERY, J.D., SUTHERLAND, W.M. & GRAY, M.C. (1989). Adenylate-cyclase toxin from *Bordetella pertussis* - identification and purification of the holotoxin molecule.*Journal of Biological Chemistry*, **264**: 9379-19384.

HEWLETT, E.L., GRAY, L.,ALLIETTA, M., EHRMANN, I.E., GORDON, V.M. & GRAY, M.C.(1991). Adenylate cyclase of *Bordetella pertussis* : conformational change associated with toxin activity.*Journal of Biological Chemistry*, **228**: 17503-17508.

HEWLETT, E.L., GRAY, M.C., EHRMANN, I.E., MALONEY, N.J., OTERO, A.S., GRAY, L., ALLIETA, M., SZABO, G., WEISS,A.A. & BARRY, E.M. (1993) . Characterisation of adenylate cyclase toxin from a mutant of *B.pertussis* defective in the activator gene, *cya* C. *Journal of Biological Chemistry*, **268**: 7842-7848.

HEWLETT, E.L., SMITH, D.L., MYERS, G.A., PEARSON, R.A. & KAY, H.D. (1983). *In vivo* inhibition of human natural killer (NK) cells cytotoxicity by *Bordetella* cyclase and pertussis toxins. *Clinical Research* , **31**: 365A.

HIRST, T.R. & WELCH, R.A. (1986). Mechanisms of secretion of extracellular proteins by Gram-negative bacteria. *Trends In Biochemical Sciences*, **13**: 265-269.

HIRST, T.R., HOLMGREN, I. (1987).Transient entry of enterotoxin subunits into periplasm occurs during their secretion from *Vibrio cholerae*. *Journal of Bacteriology*, **169**: 1037-1045.

HOCH, D.H., ROMERA-MIRA, M., EHRLICH, B.E., FINKELSTEIN, A. , DAS-GUPTA, B.R. & SIMPSON,L.L. (1985). Channel formation by botulinum, tetanus and diphtheria toxin in planar lipid bilayers: relevance to translocation of proteins across membranes. *Proceedings of the National Academy of Sciences*, **87**: 1692-1696

HODDER, S.L. & MORTIMER, E.A. (1992). Epidemiology of pertussis and reactions to pertussis vaccine.*Epidemiological `Reviews* , **14**: 242-268.

HOLLAND, I.B., BLIGHT, M.A & KENNY, B. (1990). The mechanism of secretion of hemolysin and other polypeptides from Gram-negative bacteria. *Journal of Bioenergetics and Biomembranes*, **23**: 473-491.

HOLMES,W.H. (1940). Whooping cough or pertussis. In: *Bacillary and Ricketsial infections: acute and chronic., black death to white plague*. Ed Holmes, W.H. Macmillan, New York. 395-414.

HOUSE, W. (1964). Medical Research Council, Institute of Virology, University of Glasgow, Scotland, November, 1964.

IMAIZUMI, A. SUZUKI, Y., ONO, S., SATO, H. & SATO, Y. (1983). Heptakis (2,5-0-dimethyl cyclodextrin is a novel growth stimulant for *Bordetella pertussis* phase 1. *Journal of Clinical Microbiology* , **17:** 781-786.

IMAIZUMI, A. SUZUKI, Y., GINNAGA, A., SAKHUMA, S. & SATO, Y. (1984). A new culturing method for production of filamentous haemagglutinin of *Bordetella pertussis* . *Journal of Microbiological Methods*, **3**: 339-347.

ISAARTEL, J.-P., KORONAKIS, V. & HUGHES, C. (1991). Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty acylation. *Nature,* **351**: 759-761.

IWAKI, M., ULLMANN, A. & SEBO, P. (1995). Identification by *in vitro* complementation of regions required for cell-invasive activity of *Bordetella pertussis* adenylate cyclase toxin. *Molecular Microbiology,* **17**: 1015-1024.

IWASE, M., LALLY, E.T., BERTHOLD, P., KORCHAK, H.M. & TAICHMAN, N.S., (1990). Effects of cations and osmotic protectants on cytolytic activity of *Actinobacillus actinomycetemcomitans* leukotoxin. *Infection and Imununity,* **58**: 1782-1788.

JORGENSEN, S.E., HAMMER, R.F. & WU, G.K. (1980). Effects of a single hit from the α-hemolysin produced by *Escherichia coli* on the morphology of sheep erythrocytes. *Infection and Immunity* , **27**: 988-994

KARLSSON, K.-A. (1989) Animal glycosphingolipids as membrane attachment sites for bacteria . *Annual Review of Biochemistry,* **58**: 309-350.

KARLSSON, K.-A., ANGSTROM, J. & TENEBERG, S. (1991). On the characteristics of carbohydrate receptors for Bacterial toxins: Aspects of the analysis of binding epitopes. In *Sourcebook of Bacterial Protein Toxins* , Ed Freer, J.H., Academic Press Ltd (London): 431-439.

KASLOW, H.R. & BURNS, D.L. (1992). Pertussis toxin and target eukaryotic cells: binding entry and actibvation. *FASEB Journal* , **6**: 2684-2690.

KATES, M. (1972). Separation of lipid mixtures. In: *Techniques of Lipidology : Isolation , analysis, and identification of lipids*. Ed. Kates, M. North Holland Publishing Company, Amsterdam: 438-439.

KAZI, Y. (1992). Isolation and characterisation of different forms of *Bordetella pertussis* adenylate cyclase. PhD Thesis, University of Glasgow.

KERSTERS, K., HINZ, K.H., HERTLE, A., SEGERS, P., LIEVENS, A., SIEGMANN, O., DELEY, J.(1984). *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. *International Journal of Systematic Bacteriology*, **54**: 56-70.

KESSIN, R.H. & FRANKE, J. (1986). Secreted adenylate cyclase from *Bordetella pertussis* : Calmodulin requirements and parial purification of two forms. *Journal of Bacteriology*, **166**: 290-296.

KHELEF, N., SAKAMOTO, H. & GUISO, N. (1990). Both adenylate cyclase and hemolytic activities are required by *Bordetella pertussis* to initiate infection. *Microbial Pathogenesis*, **12**: 486-490.

KHELEF, N., ZYCHLINSKY, A. & GUISO, N. (1993). *Bordetella pertussis* induces macrophage apoptosis: Role of adenylate cyclase-hemolysin. *Infection and Immunity*, **61**: 4064-4071.

KILHOFFER, M.C., COOK, G.H. & WOLFF, J. (1983). Calcium-indepcndant activation of adenylate cyclase by calmodulin. *European Journal of Biochemistry*, **133**: 11-15

KIMURA, A., MOUNTZOUROS, K.T., RELMAN, D.D., FALKOW, S. & COWELL, J.L. (1990). *Bordetella pertussis* filamentous haemagglutinin: evaluation as a protective antigen and colonisation factor in a mouse respiratory infection model. *Infection and Immunity*, **58**: 7-16.

KLOOS, W.E., MOHAPTRA, N., DOBROGOSZ, W.J., EZZEL, J.W., & MANCLARK, C.R. (1981). Deoxyribonucleotide sequence relationships among *Bordetella* species. *International Journal of Systematic Bacteriology*, **31**: 173-176.

KNOWLES, B.H. & ELLAR, D.J.(1986). Characterisation and partial purification of a plasma membrane receptor for *Bacillus thuringiensis* var. *kurstatis* lepidopteran-specific δ– endotoxin. *Journal of Cell science*, **83**: 89-101.

KOLODRUBETZ, D., DAILEY, T., EBERSOLE, J. & KRAIG, E. (1989). Cloning and expression of the leukotoxin gene from *Actinobacillus actinomycetemcomitans* . *Infection and Immunity,* **57**: 1465-1469.

KORONAKIS, V., CROSS, M., SENIOR, B., KORONAKIS, E. & HUGHES, C. (1987). The secreted hemolysins of *Proteus mirabilis*; *Proteus vulgaris,* and *Morganella morganii* are genatically related to each other and to the α-hemolysin of *Escherichia coli* . *Journal of Bacteriology,* **169:** 1509-1515.

KORONAKIS, V., KORONAKIS, E., HUGHES, C. (1989). Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* hemolysin protein across both bacterial membranes. *EMBO Journal,* **8**: 595-605.

KRAIG, E., DAILEY, T. & KOLODRUBETZ, D. (1990). Nucleotide sequence of the leukotoxin gene from *Actinobacillus actinomycetemcomitans* : homology to the alpha hemolysin / leukotoxin gene family. *Infection and Immunity,* **58**: 920-929.

KRANTZ, I., ALESTIG, K., TROLLFORS, B. & ZACHRISSON, G. (1986). The carrier state in Pertussis. *Scandinavian Joiurnal of Infectious Diseases*, **18**: 121-123.

KREUGER, K.M. & BARBIERI, J.T. (1995). The family of bacterial ADP-ribosylating exotoxins. *Clinical Microbiology Reviews* , **8**: 34-47

LACEY, B.W. (1960). Antigenic modulation of *Bordetella pertussis*. *Journal of Hygeine,* **58**: 57-03.

LADANT, D., BREZIN, C., ALONSO, J-M, GRENON, J. & GUISO, N. (1986). *Bordetella pertussis* adenylate cyclase.*Journal of Biological Chemistry*, **261**: 16264-16269.

LADANT, D., MICHELSON, S., SARFATI, R., GILLES, A.-M, PREDELEANU, R. & BARZU, O. (1989). Characterisation of the calmodulin binding and catalytic domains of *Bordetella pertussis* adenylate cyclase. *Journal of Biological Chemistry,* **264** : 4015-4120.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, London, **227**: 680-685.

LALLY, E.T., GOLAB, E.E., KIEBA, I.R., TAICHMAN, N.S., ROSENBLOOM, J., ROSENBLOOM, J.C., GIBSON, C.W. & DEMUTH , D.R. (1989). Analysis of the *Actinobacillus actinomycetemcomitans* leukotoxin gene: delineation of unique features and comparison to homologous toxins. *Journal of Biological Chemistry,* **264**: 15451-15456.

LALONDE, G., McDONALD, T.V., GARDNER, P. & O'HANLEY, P.D. (1988). Identification of a hemolysin from *Actinobacillus pleuropneumoniae* and characterisation of its channsl properties in planar phospholipid bilayers. *Journal of Biological Chemistry,* **264**: 13559-13564.

LEE, C.K., ROBERTS, A.L., FINN, T.M. & MEKELANOS, J.J.(1990). Invasion of HeLa 229, Chinese Hamster Ovary, and U937 cells by *Bordetella pertussis.*. In: *Proceedings of the Sixth International Symposium on Pertussis.* Ed Manclark, C.R. Department of Health and Human services, United States Public Health Service, Bathesda, Maryland: 115-125.

LEININGER, E., ROBERTS, M., KENIMER, J.G., CHARLES, I.G., FAIRWEATHER, N.F., NOVOTNY, P. & BRENNAN, M.J. (1991). Pertactin, an Arg-Gly-Asp containing *Bordetella pertussis* surface protein that promotes adherence to mammalian cells. *Proceedings of the National Academy for Science* , USA, **88**: 345-349.

LESLIE, P.H. & GARDNER, A.D. (1931). The phase of *Haemophilus pertussis. Journal of Hygeine*, **31**: 423-434.

LEUSCH, M.S., PAULAITIS, S. & FRIEDMAN, R.L. (1990). Adenylate cyclase toxin of *Bordetella pertussis* : production, purification, and partial characterisation. *Infection and Immunity*, **58**: 3622-3626.

LINDBERG, A.A., SCHULTZ, J.E., WESTLING, M., BROWN, J.R., ROTHMAN, S.W., KARLSSON, K.-A. & STROMBERG, N. (1986). Identification of the receptor glycolipid for Shiga toxin produced by *Shigella dysenteriae* type 1. In: *Protein-carbohydrate interactions in biological systems* . Ed. Lark, D., Academic Press, Inc (London), Ltd., London: 439-436.

LIVEY, L. & WARDLAW, A.C. (1984). Production and properties of *Bordetella pertussis* heat labile toxin. *Journal of Medical Microbiology* , **17**: 91-103.

LO, R.Y.C., STRATHDEE, C.A. & SHEWEN, P.E. (1987) . Nucleotide sequence of the leukotoxin genes of *Pasteurella haemolytica* A1. *Infection and Immunity,* **55**: 1987-1996.

LOCHT, C., BERTIN, P., MENOZZI, F.D. &RENAULD, G. (1993). The filamentous haemagglutinin, multifacteted adhesin produced by virulent *Bordetella* spp. *Molecular Microbiology* , **9**: 653-660.

LONG, S.S., WELKON, C.J, & CLARK, J.L. (1990). Widespread silent transmission of pertussis in families: antibody correlation of infection and immunity.*Journal of Infectious Disease*, **16**: 480-486.

LORI, L., MERNIER, a. & LO, R.Y.C (1991). Characterisation of an *Actinobacillus suis* hemolysin. *Abstracts for the American Society for Microbiology* (1991), p32, B-43.

LUDWIG, A.T., JARCHAE, T., BENZ, R. & GOEBEL, W. (1988). The repeat domain of *Escherichia coli* hemolysin (Hly A) is responsible for its Ca^{2+} dependent binding to erythrocytes. *Molecular and General Genetics,* **214**: 553-561.

LUDWIG,A., VOGEL, M. & GOEBEL, W. (1987). Mutations affecting activity and transport of hemolysin in *Escherichia coli. Molecular and General Genetics,* **206**: 238=245.

MACKMAN, N., NICAUD, J.M., GRAY, L. & HOLLAND,I.B. (1988). Genetical and functional organisation of the *Escherichia coli* haemolysin determinant 2001. *Molecular and General Genetics,* **201**: 282-288.

MACPHERSON, I. & STOKER, M. (1962). Polyoma transformation of Hamster cell clones - an investigation of genetic factors affecting cell competence.*Virology* , **16**: 147-151.

MAGNANI, J.L., SMITII, D.F. & GINSBURG, V. (1980). Detection of gangliosides that bind cholera toxin: Direct binding of ^{125}I-labelled toxin to thin-layer chromatograms. *Analytical Biochemistry*, **109**: 399-402.

MASURE, H.R. (1992). Modulation of adenylate cyclase toxin production as *Bordetella pertussis* toxin enters human macrophages. *Proceedings of the National Academy of Sciences*, USA, **89**: 6521-6525.

MASURE, H.R. (1993). The adenylate cyclase toxin contributes to the survival of *Bordetella pertussis* within human macrophages. *Microbial Pathogenesis*, **14**: 253-260.

MASURE, H.R., AU, D.C., GROSS, M.K., DONOVAN, M.G. & STORM, D.A. (1990). Secretion of *Bordetella pertussis* adenylate cyclase from *Escherichia coli* containing the hemolysin operon. *Biochemistry* , **29**: 140-145.

MAUDSEY, J.R,, KADA, S. (1981). Growth and hemolysin production by *Haemophilus pleuropneumoniae* cultivated in a chemically defined medium *Canadian Journal of Micobiology,* **37**: 801-805.

McWHINNEY, D.R., CHANG, Y.-F., YOUNG, R. & STRUCK , D.K. (1992). Seperable domains define target cell specificities of an RTX hemolysin from *Actinobacillus pleuropneumoniae. Journal of Bacteriology,* **174**: 291-297.

MEKADA, E., SENOH, H., IWAMOTO, R., OKADA, Y., UCHIDA, T.(1991). Purification of diphtheria-toxin receptor from Vero cells.*Journal of Biological Chemistry*, **266**: 20457-20462.

MENESTRINA, G. (1988). *Escherichia coli* permeabilises small unilamellar vesicles loaded with calcein by a single hit mechanism. *FEBS Letters*, **232**: 217-220.

MENESTRINA, G., MACKMAN, N., HOLLAND, I.B. & BHAKDI, S. (1987). *Escherichia coli* hemolysin forms voltage dependent channels in lipid membranes. *Biochimica et Biophysia Acta,* **905**: 109-117.

MERRITT, E.A., SARFATY, S., VANDENAKKER, F., LHOIR, C., MARTIAL, J.A. & HOL, W.G.J. (1994a). Crystal-structure of cholera-toxin B-pentamer bound to receptor G(M1) pentasaccharide. Protein Science, **3**: 166-175.

MERRITT, E.A., SIXMA, T.K., KALK, K.H., VANZANTEN, B.A.M. & HOL, W.G.J. (1994b). Galactose-binding site in *Escherichia coli* heat-labile enterotoxin (LT) and cholera-toxin (CT). *Molecular Microbiology*, **13**: 745-753.

MERTSOLA, J. (1985). Mixed outbreak of *B.pertussis* and *B.parapertussis* in Finland. *European Journal of Clinical Microbiology,* **4**: 123-128.

MILSMANN, M.H., SCHWENDENER, R.A., & WEDER, H.G. (1978).The preparation of large single bilayer liposomes by a fast and controlled dialysis.*Biochimica Biophysica Acta*, **512**: 147-155.

MOBLEY, H.L.T., GREEN, D.M., TRIFILLIS, A.L., JOHNSON, D.E., CHIPPENDALE, G.R., LOCHALETTE, C.V., JONES, B.D. & WARREN, J.W. (1989). Pyelonephritogenic *Escherichia coli* killing of cultured human rnal proximal tubular epithelial cells: role of hemolysin in some strains. *Infection and Immunity,* **58**: 1281-1289.

MOEHRING, T.J. & CRISPELL, J.P. (1974). Enzyme treatment of KB cells: the altered effect of diphtheria toxin. *Biochemical and Biphysical research Communications*, 60: 1446-1452.

MOLLER, W. &AMONS, R. (1985). Phosphate binding sequences in nucleotide binding proteins. *FEBS Letters,* **18**: 1-7

MONTAREZ, J.A., NOVOTNY, P. & IVANYL, J. (1985). Identification of a 68-kilodalton protective protein antigen from *Bordetella bronchiseptica. Infection and Immunity*, **47**: 744-751.

MOOI, F.R. (1994). Genes for the filamentous haemagglutinin and fimbriae of *Bordetella pertussis* : Colocation, coregulation and cooperation?. In: *Molecular Genetics of Bacterial Pathogenesis* , Eds. MILLER, V.L., RAPER, J.B. *et al* . American Society for Microbiology, Wahshington DC: 144--155.

MOOI, F.R., JANSEN, W.H., BRANINGS, H., GIELEN, H., VAN DER HEILDE, H.G.J., WALVOORT, H.C. & GUINEE, P.A.M. (1992). Construction and analysis of *Bordetella pertussis* mutants defective in the production of fimbriae. *Microbial Pathogenesis*, **12**: 127-135.

MORENO-LOPEZ, M. (1952). El genero *Bordetella. Microbiology Espagnol*, **5**: 117-181.

MORRIS, R.E (1990). Interacation between pseudomonas exotoxin A and mouse LM fibroblast cells. In *Trafficking of bacterial toxins.* Ed. Saelinger C.B. CRC Press Inc., Boca Raton, Florida: 49-70.

MOUALLEM, M., FARFEL, Z. & HANSKI, H. (1990). *Bordetella pertussis* ademylate cyclase : intoxication of host cells by bacterial invasion. *Infection and Immunity,* **58**: 3759-3764.

MULLER, A.S., LEEUWENBURG, J. & PRATT, D.S (1986). Pertussis: epidemiology and control.*Bull. WHO*, **64**: 321-331.

MURRAY, P.A., PRAKOBPHOL, A., LEE, T., HOOVER, C.I. & FISHER, S. (1992). Adherence of oral streptococci to salivary glycoproteins. *Infection and Immunity*, **60**: 31-38.

MUSSER, J.M., HEWLETT, E.L., PEPPLER, M.S. & KASS, E.H. (1986). Genetic diversity and relationships in populations of *Bordetella* spp.*Journal of Bacteriology*, **166**: 230-237.

NAKASE, Y. & ENDOH, M. (1988). Heat-labile toxin of *Bordetella pertussis* . In: *Pathogenesis and Immunity in Pertusssis* , eds Wardlaw, A,C., & Parton, R. John Wiley & Sons, Chichester: 211-229.

NICAUD, J.M., MACKMAN, N., GRAY, L. & HOLLAND, I.B. (1985). Characterisation of Hly C and mechanism of activation and secretion of hemolysin from *Escherichis coli* 2001. *FEBS Letters*, **187**: 339-344.

OAKLEY, B.R., KIRCH, D.R. & MORRIS, N.R. (1980). a simple ultra sensitive silver stain for detection of proteins in polyacrylamide gels. *Analytical Biochemistry*, **105**: 361-363.

ODOWD, B.F., HNATOWICH, M., CARON, M.G., LEFKOWITZ, P.J., & BOUVIER, R.J. (1989). Palmitoylation of the human beta-2-adrenergic receptor - mutation of cys-341 in the carboxyl tail leads to an uncoupled nonpalymitoylated form of the receptor.*Journal Of Biological Chemistry*, **264**: 7564-7569

OLSON, L.C. (1975). Pertussis. *Medicine (Baltimore)*, **54**: 427-469.

ONORATO, I.M. & WASSILAK, S.G.F. (1987). Laboratory diagnosis of pertussis: the state of the art. *Paediatric Infectios Diseases*, **6**: 145-151.

OROPEZA-WEKERLE, R.-L., MULLER, S., BRIAND, J.-P, BENZ, R., SHMID, A. & GOEBEL, W. (1992). Haemolysin-derived synthetic peptides with pore forming and haemolytic activity. *Molecular Microbiology*, **6**: 115-121.

OTERO, A.S, YI, X.B., GRAY, M.C., SZABO, G., HEWLETT, E.L. (1995). Membrane depolarization prevents cell invasion by *Bordetella pertussis* adenylate cyclase toxin. *Journal of Biological Chemistry*, **270**: 9695-9697.

PARTON, R. (1985). Effect of prednisolone on the toxicity of *Bordetella pertussis* for mice.*Journal of Medical Microbiology*, **19**: 391-400.

PARTON, R. (1997). *Bordetella*. In: *Topley and Wilson's Microbiology & Microbial Infections*, Vol 2. Ed Collier, L. Edward Arnold, London.

PATRIARCA, P.A., BIELLIK, R.J., SANDEN, G., BURSTYN, D.G., MITCHELL, P.D., SILVERMAN, P.R., DAVIS, J.P. & MANCLARK, C.R.(1988). Sensitivity and specificity of clinical case definitions for pertussis. *American Journal of Public Health* , **78**: 833-836.

PAYNE, D. O'REILLY, M. & WILLIAMSON, D. (1993). The K88 fimbrial adhesin of enterotoxigenic *Escherichia coli* binds to β1-linked galactosyl residues on glycosphingolipids. *Infection and Immunity*, **61**: 3673-3677.

PEARSON, R.D., SYMES, P., CONBOY, M., WEISS, A.A. & HEWLETT, E.L. (1987). Inhibition of monocyte oxidative responses by *Bordetella pertussis* adenylate cyclase toxin. *Journal of Immunology*, **139**: 2749-2754.

PELLET, S & WELCH, R. (1996). *Escherichia coli* hemolysin mutants with altered target cell specificity. *Infection and Immunity*, **64**: 3081-3087.

PELLET, S., BOEHM, D.F., IRVIN, S., SNYDER, G.R. & WELCH, R.A. (1990). Characterisation of monoclonal antibodies against the *Escherichia coli* hemolysin. *Infection and Immunity*, **58**: 882-827.

PICK, U. (1981). Liposomes with a large trapping capacity prepared by freezing and thawing of sonicated phospholipid mixtures. *Archives of Biochemistry and Biophysics*, **212**: 186-194.

POLLOCK, M.R. (1947). The growth of *H.pertussis* on media without blood.*British Journal of Experimental Pathology*, **28**: 295-307.

POLLOCK, M.R. (1949). The effects of long-chain fatty acids on the growth of *Haemophilus pertussis* and other organisms. *Symposium of the Society of Experimental Biologists*, **3**: 193-216.

PORTER, J.F. & WARDLAW. A.C. (1993). Long-term survival of *Bordetella bronchiseptica* in lakewater and buffered saline without added nutrients. *FEMS Microbiology Letters*, **110**: 33-36.

PROOM, H. (1955). The minimal nutritional requirements of the organisms of the genus *Bordetella Lopez* . *Journal of General Microbiology*, **12**: 63-75.

RAPPUOLI, R. (1996). Acellular pertussis vaccines- a turning point in infant and adolescent vaccination. *Infectious Agents and Disease-Reviews Issues and Commentary* . **5**: 21-28.

RAPTIS, A., KNIPLING, L.G., GENTILE, F. & WOLFF, J. (1989). Modulation of invasiveness and catalytic activity of *Bordetella pertussis* adenylate cyclase by polycations. *Infection and Immunity,* **57**: 1066-1071.

RIBI, H.O., LUDWIG, D.S., MERCER, K.L., SCHOOLNIK, G.K. & KORNBERG, R.D. (1988). Three dimensional structure of cholera toxin penetrating a lipid membrane. *Science,* **239**: 1272-1276.

ROBERTS, M., FAIRWEATHER, N.F., LEININGER-ZEPATA, E., BRENNAN, M.J., PICKARD, D., HEWLETT, E.L., ROBINSON, A., HAYWARD, C., DOUGAN, G. & CHARLES, I.G. (1991). Construction and characterisation of *Bordetella pertussis* mutants lacking in the*vir* - regulated P.69 outer membrane protein. *Molecular Microbiology* , **5**: 1393-1404.

ROBINSON, A., DUGGLEBY, C.J., GORRINGE, A.R. & LIVEY, I. (1986). Antigenic variation in *Bordetella pertussis.* In: *Antigenic variation in Infectious Diseases* . Eds. Birkbeck,T.H. & Penn, P. IRL Press, Oxford and Washington DC.: 147-161.

ROGEL, A. & HANSKI, E. (1992). Distinct steps in the penetration of adenylate cyclase toxin of *Bordetella pertussis* into sheep erythrocytes. Translocation of the toxin across the membranc. *The Journal of Biological Chemistry*, **267**: 22588-23605.

ROGEL, A. & HANSKI, E. (1992). Translocation of adenylate cyclase toxin across target cell plasma membrane. In*Bacterial Protein Toxins* (WILTHOLT *et al* , eds) Gustav Fischer, Stuttgart, Jena, New York.

ROGEL, A., FARFEL, Z., GOLDSCHMIDT, S. , SHILOAH, J. & HANSKI, E. (1988). *Bordetella pertussis* adenylate cyclase toxin. Identification of multiple forms of the enzyme by antibodies. *Journal of Biological Chemistry*, **263**: 13310-13316.

ROGEL, A., MELLER, R. & HANSKI, E. (1991). Adenylate cyclase toxin from *Bordetella pertussis* : The relationship between induction of cAMP and hemolysis. *Journal of Biological Chemistry,* **266**: 3154-3161.

ROGEL, A., SCHULTZ, J.E., BROWNLIE, R.M., COOTE, J.G., PARTON, R. & HANSKI, E. (1989). *Bordetella pertussis* adenylate cyclase: Purification and characterisation of the toxic form of the enzyme. *EMBO Journal,* **8**: 2755-2760.

ROSE, T., SEBO, P., BELLALOU, J. & LADANT, D. (1995). Interaction of calcium with *Bordetella pertussis* adenylate cyclase toxin. *Journal of Biological Chemistry,* **270**: 26370-26376.

ROSENDAL, S., DEVENISH, J., MACINNES, J.I., LUMSDEN. J.H., WATSON, S. & XUN, H. (1988). Evaluation of heat-sensitive neutrophil-toxic and hemolytic avtivity of *Haemophilus (Actinobacillus) pleuropneumoniae. American Journal of Veterinary Research,* **33**: 673-676.

ROSS, E.M. (1988). Reactions to Whole-Cell Pertussis Vaccine. In: *Pathogenesis and Immunity in Pertusssis* , eds Wardlaw, A,C. & Parton, R. John Wiley & Sons, Chichester: 375-398

ROWATT, E.(1957). Some growth factors affecting growth of *Bordetella pertussis* . *Journal of General Microbiology* , **17**: 279-296.

SAITO, T. & HAKAMORI, S-I. (1971). Quantitative isolation of total glycosphingolipids from animal cells.*Journal of Lipid Research,* **12**: 257-259

SAKAMOTO, H., JACQUES, J., SEBO, P. & LADANT, D. (1992). *Bordetella pertussis* adenylate cyclase toxin. Structural and functional independence of the catalytic and hemolytic activities. *Journal of Biological Chemistry,* **267**: 13598-13602

SALOMON,Y., LONDOS, C. & RODBELL, M.(1974). A highly sensitive adenylate cyclase assay. *Analytical Biochenistry* , **56**: 341-548.

SAMBROOK, J. (1989). Bacterial Media, Antibiotics, and Bacterial Strains. In: *Molecular Cloning: A Laboratory Manual Book 3* . Eds, Sambrook, J., Fritsch, E.F. & Maniatis, T. Cold Spring Laboratory Press (USA). Appendix 3.

SAMUEL, J.E., PERERA, L.P., WARD, S., O'BRIEN, A.D., GINSBURG, V. & KRIVAN, H.C. (1990). Comparison of glycolipid receptor specificities of shiga-like toxin type II and shiga-like toxin type II variants. *Infection and Immunity,* **58**: 611-618.

SATO, Y., KIMURA, M. & FUKUMI, H. (1984). Development of a pertussis component vaccine in Japan. *Lancet* , **1**: 422-428.

SAUKKONEN, K., CABELLOS, C., BURROUGHS, M., PRASAD, S. & TUOMANEN. (1991). Integrin-mediated localisation of *Bordetella pertussis* within macrophages: role in pulmonary colonisation. *Journal of Experimental Medicine*. **173**: 1145-1149.

SCHEIN, S.J., KAYAN, B.L. & FINKELSTEIN, A. (1978). Colicin K acts by forming voltage dependent channels in phospholipid bilayer membranes. *Nature* (London), **266**: 159-161.

SCHIAVO, G., DEMEL, R. & MONTECUCCO, C. (1991). On the role of polysialoglycosphingolipids as tetanus toxin receptors- a study with lipid monolayers. *European Journal of Biochemistry*, **199**: 705-711.

SCHMIDT, H., KARCH, H. & BEUTIN, L. (1994). The large-sized plasmids of enterohemorrhagic *Escherichia coli* 0157 strains encode hemolysins which are presumably members of the *E.coli* α-hemolysin family. *FEMS Microbiolohy Letters*, **117**: 189-196.

SEBO, P., GLASER, P., SAKAMOTO, H. & ULLMANN, A. (1991). High-level synthesis of active adenylate cyclase toxin of *Bordetella pertussis* in a reconstructed *Escherichia coli* system.*Gene*, **104**: 19-24.

SHATTUCK, R.L. & STORM, D.R. (1985). Calmodulin inhibits entry of *Bordetella pertussis* adenylate cyclase toxin into animal cells. *Biochemistry* , **24**: 6356-6326.

SHATTUCK, R.L., OLDENBURG, D.J. & STORM, D.R.(1985). Purification and characterisation of a calmodulin-sensitive adenylate cyclase from *Bordetella pertussis. Biochemistry*, **24**: 6356-6362.

SHEWEN, P.E. & WILKIE, B.N. (1982). Cytotoxin of *Pasteurella haemolytica* acting on bovine leukocytes. *Infection and Immunity*, **35**: 91-94.

SHIELDS, W.D., NIELSON, C., BUCH, D , JACOBSEN, V., CHRISTENSON, P., ZACHAUCHRISTIANSEN, B. & CHERRY, J.D. (1988). Relationship of pertussis immunisation to the onset of neurological disorders: a retrospective epidemiology. *Journal of Paediatrics* , **113**: 801-805.

SHIVER, J.W. & DONOVAN, J.J. (1987).Interactions of diphtheria toxin with lipid vesicles- Determinants of ion channel formation *Biochimica et Biophysia Acta*, **903**: 48-55.

SHONE, C.C., HAMBLETON, P. & MELLY, J.(1987). A 50 kDa fragment from the NH2 terminus of the heavy subunit of *Clostridium botulinum* Type A neutotoxin forms channels in lipid vesicles. *European Journal of Biochemistry,* **167**: 175-180.

SIXMA, T.K., PRONK, S.E., KALK, K.H., VANZANTEN, B.A.M, BERGHUIS, A.M. & HOL, W.G.J. (1992). Lactose binding to Heat-labile enterotoxin revealed by X-ray crystallography. *Nature,* **355**: 561-564.

SMITH, P.K, KROHN, R.I, HERMANSON, G.T, MALLIA, A.K., GARTNER, F.H., PROVENZANO, M.D., FUJIMOTO, E.K., GOEKE, N.M., OLSON, B.J., & KLENK,D.C (1985) Measurement of protein using bicinchoninic acid. *Analytical Biochemistry,* **150**: 76-85.

STAINER, D.W. & SCHOLTE, M.J.(1971). A simple chemically defined medium for the production of phase 1 *Bordetella pertussis.Journal of General Microbiology,* **63**: 211-220.

STAINER, D.W. (1988). Growth of *Bordetella pertussis.* In: *Pathogenesis and Immunity in Pertusssis* , eds Wardlaw, A,C. & Parton, R. John Wiley & Sons, Chichester: 19-37.

STANLEY, P., PACKMAN, L., KORONAKIS, V. & HUGHES, C. (1994). Fatty acylation of two internal lysine residues required for the toxic activity of *Escherichia coli* hemolysin. *Science* , **286**: 1992-1966

STEED, L., MOREY, S., & FRIEDMAN, R.L. (1991). Intracellular survival of vurulent *Bordetella pertussis* in human polymorphonuclear leukocytes. *Journal of Leukocyte Biology,* **50**: 321-330.

STEIN, P.E., BOODHOO, A., ARMSTRONG, G.D., HEERZE, L.D., COCKLE, S.A., KLEIN, M.H. & READ, R.J. (1994). Structure of a Pertussis toxin sugar complex as a model for receptor-binding. *Nature Structural Biology,* **1**: 591-596.

STIBITZ, E. & MILLER, J.F. (1994). Co-ordinate regulation of virulence in *Bordetella pertussis* mediated by the *vir* (*bvg*). In : *Molecular Genetics of Bacterial Pathogenesis* , Eds. MILLER, V.L., RAPER, J.B. *et al* . American Society for Microbiology, Wahshington DC: 407-422.

STRATHDEE, C.A. & LO, R.Y.C. (1987). Extensive homology between the leukotoxin of *Pasteurella haemolytica* A1 and the alpha hemolysin of *Escherichia coli. Infection and Immunity,* **55**: 3233-3236.

SWANSEA RESEARCH UNIT OF ROYAL COLLEGE OF GENERAL PRACTITIONERS. (1985). Respiratory sequelae of whooping cough. *British Medical Journal* , **290**: 1937-1940.

SZABO, G., GRAY, M.C. & HEWLETT, E.L. (1994). Adenylate cyclase toxin from *Bordetella pertussis* produces ion conductance across artificial lipid bilayers in a calcium-dependent and polarity-dependent manner. *Journal of Biological* Chemistry, **269**: 22496-22499.

TAICHMAN, N.S., SIMPSON, D.L., SAKURADA, S., CRANFIELD, M., DIRENZO, J. & SLOTS, J. (1987). Comparitive studies of the biology of *Actinobacillus actinomycetemcomitans* leukotoxin in primates. *Oral Microbiology and Immunology,* **2**: 97-104.

TAKAMIZAWA, K., IWAMORI, M., KOZAKI, S.. SAKAGUSHI. G., TANAKA, R., TAKAYAMA, H. & NAGAI, Y. (1986). TLC immunostaining characterisation of*Clostridium botulinum* Type A neurotoxin binding to gangliosides and fatty acids. *FEBS Letters*, **201**: 229-232.

TAMURA, M., NOGIMURI, K., MURAI, S., YAJIMA, M., ITSO, K., KATADA, T., UI, M. % ISHUII, S. (1982). Subunit structure of the islet activating factor, pertussis toxin, in conformity with the A-B model. *Biochemistry* , **21**: 5516-5522.

THOMPSON, M.R., FORRISTAL, J., KAUFFMANN, P., MADDEN, T., KOZAK, R., MORRIS, R. & SAELINGER, C.R. (1991). Isolation and characterisation of *Pseudomonas aeruginosa* exotoxin A binding protein from mouse LM cells. *Journal of Biological Chemistry,* **266**: 2390-2396.

TOWBIN, H., STAEHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proceedings of the national Academy of Science,* USA, **76**: 4350-4354.

TOWLER, D.A., GORDON, J.I., ADAMS, S.P. & GLASER, L. (1988). The biology and enzymology of eukarytoic protein acylation. *Annual Review of Biochemistry,* **57**: 66-99.

TUOMANEN, E. & WEISS, A. (1985). Characterisation of two adhesins of *Bordetella pertussis* for human respiratory epithelial cells. *Journal of Infectious Diseases*, **152**: 118-125.

TUOMANEN, E. (1988). *Bordetella* adhesins. In: *Pathogenesis and Immunity in Pertusssis* , eds Wardlaw, A.C., & Parton, R. John Wiley & Sons, Chichester: 75-94.

TYRELL, G.J., PEPPLER, M.S., BONNAH, R.A., CLARK, C.G., CHONG, P. & ARMSTRONG, G.D. (1989). Lectinlike properties of pertussis toxin. *Infection and Immunity* , **57**: 1854-1857.

VANDAMME, P., HOMMEZ, J., VANCANNEYZ, M.MONSIEURS, M., HOSTE, B., COOKSON, B., VONKONIG, C.H.W., KERSTERS, K., BLACKALL, P.J. (1995).*Bordetella hinzii* Sp.nov. isolated from poultry and humans. *International Journal of Systematic Bacteriology*, **45**: 37-45.

VILJANEN, M.K., RUUSKANEN, O., GRANBERG, C. & SALMI, T.T. (1982). Serological diagnosis of pertussis- IgM, IgA AND IgG antibodies against *Bordetella-pertussis* measured by enzyme-linked immunosorbent assay (ELISA) *Scandinavian Journal of Infectious Diseases*, **14**: 117-122

WAGNER, W., KUHN, M. & GOEBEL, W. (1988). Active and inactive forms of hemolysin (Hly A) from *Escherichia coli. Biological Chemistry Hoppe-Seyler*, **36**: 39-46.

WALKER, E. (1988). Clinical aspects of pertussis. In: *Pathogenesis and Immunity in Pertusssis* , eds Wardlaw, A.C., & Parton, R. John Wiley & Sons, Chichester: 273-282.

WANDERSMAN, C. & DELEPELAIRE, B.P. (1990). Tol C, an *Escherichia coli* outer membrane protein required for hemolysin secretion. *Proceedings of the National Acacemy of Sciences* (USA), **87**: 4776-4781.

WARDLAW, A.C. & PARTON, R.(1988a). *Pathogenesis and Immunity in Pertusssis*, eds Wardlaw, A.C. & Parton, R. John Wiley & Sons, Chichester.

WARDLAW, A.C. & PARTON, R.(1988b). The host parasite relationship in Pertussis. In: *Pathogenesis and Immunity in Pertusssis*, eds Wardlaw, A.C. & Parton, R. John Wiley & Sons, Chichester: 327-347

WEBER, R. & OSBORNE, M. (1969) The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide electrophoresis.*Chemistry*, **244**: 4406-4412.

WEDEGAERTNER, P.B, CHU, D.H., WILSON, P.T., LEVIS, M.J., & BOURNE, H.R. (1993). Palmitoylation is required for signaling functions and membrane attachment of G(q) alpha andG(s)alpha *Journal of Biological Chemistry*, **268**: 25001-25008

WEISS, A., HEWLETT, E.L. &CRONIN, M.J. (1986). Bacterial adenylate cyclase increases cyclic AMP and hormone release in pituatary tumor cells. *Biochemical and Biophysical Research Communications* . **136**: 463-469

WEISS, A.A. & FALKOW, S. (1984). Genetic analysis of phase change in *Bordetella pertussis* . *Infection and Immunity* . **43**: 293-269.

WEISS, A.A. & GOODWIN, M.S.M. (1989). Lethal infection of *Bordetella pertussis* mutants in the infant mouse model. *Infection and Immunity* , **57**: 3757-3764.

WEISS, A.A. & HEWLETT, E.L. (1986). Virulence factors of *Bordetella pertussis* . *Annual Review of Microbiology* , **40**: 661-686.

WEISS, A.A., HEWLETT, E.L., MYERS, G.A. & FALKOW, S. (1983). Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. *Infection and Immunity* , **42**: 33-41.

WELCH, R.A. & PELLET, S. (1988). Transcriptional organisation of the *Escherichia coli* hemolysin genes. *Journal of Bacteriology*, **170**: 1622-1630.

WELCH, R.A. (1991). Pore forming cytolysins of Gram-negative bacteria. *Molecular Microbiology*, **5**: 521-528.

WESTROP, G.D., CAMPBELL, G., KAZI,Y., BILLCLIFFE, B., COOTE, J.G., PARTON, R., FREER, J.H.& EDWARDS, J.G. (1994). A new assay for the invasive adenylate-cyclase toxin of *Bordetella-pertussis* based on its morphological effects on the fibronectin-stimulated spreading of BHK-21-cells. *Microbiology*, UK, **140**: 245-253.

WESTROP, G.D., HORMOZI, E.K., DA COSTA, N.A., PARTON, R. & COOTE, J.G. (1996). *Bordetella pertussis* adenylate cyclase toxin proCyaA and CyaC proteins synthesised separately in *Escherichia coli* produce active toxin *in vitro*. *Gene*, **180**: 91-99.

WESTROP, G.D., HORMOZI, E.K., DA COSTA, N.A., PARTON, R. & COOTE, J.G. (1997) . Structure-function studies of the adenylate cyclase toxin of *Bordetella pertussis* and the leukotoxin of *Pasteurella haemolytica* by heterologous C protein activation and construction of hybrid proteins. *Journal of Bacteriology*, **179**: 871-879.

WEYANT, R.S., HOLLIS, D.G., WEAVER, R.E., AMIN, M.F.M., STEIGERWALT, A.G., O'CONNOR, S.P., WHITNEY, A.M., DANESHVAR, M.I., MOSS, C.W., BRENNER, D.J.(1995). *Bordetella holmseii*, sp. nov., a new gram-negative species associated with septicaemia. *Journal of Clinical Microbiology* , **33**: 1-7.

WICK, M.J., HAMOOD, A.N. & IGLEWSKI, H. (1990). Analysis of structure-function of *Pseudomonas aeruginosa* exotoxin A. *Molecular Microbiology*, **4**: 527-535.

WILLEMSEN, P.T.J. & GRAAF, F.K. (1993). Multivalent binding of K99 fimbriae to the *N* -Glycolyl-GM_3 ganglioside receptor. *Infection and Immunity*, **61**: 4518-4522.

WOLFF, J., COOK, G.H., GOLDHAMMER, A.R. & BERKOWITZ, S.A. (1980). Calmodulin activates prokaryotic adenylate cyclase. *Proceedings of the National Academy of Science*, **71**: 3841-3844.

WOLFF, J., COOK, G.H., GOLDHAMMER, A.R., LONDOS, C. & HEWLETT, E.L. (1984). *Bordetella pertsussis* : multiple attacks on host cell cyclic AMP regulation. *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, **17**: 161-172.

WOOLFREY, B.F. & MOODY, J.A. (1991). Human infections associated with *Bordetella bronchiseptica* . *Cinical Microbiology Reviews*, **4**: 243-255.

APPENDIX 1

CYCLODEXTRIN LIQUID MEDIUM (CD)

(Imaizumi *et al* , 1983 & 1984)

Cyclodextrin liquid medium (CD)

L-glutamate (monosodium salt)	10.72 g
L-proline	0.24 g
Sodium chloride (NaCl)	2.5 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	0.5 g
Potssium chloride (KCl)	0.2 g
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	0.1 g
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	0.02 g
Tris	6.075 g
Casamino acids [a]	10 g
Heptakis [2,6-O-Dimethyl β-cyclodextrin (MeβCD)]	1.0 g

[a] Casein acid hydrolysate (Peptone No.5, Gibco)

Dissolve CD medium in 900 ml distilled water and adjust pH to 7.4 with conc. HCl and make up to 990 ml with distilled water. Autoclave and store at 4°C. Before use add 10 ml of sterile vitamin supplements (see below).

Vitamin supplement

L-cysteine	0.4 g
Ferrous sulphate ($FeSO_4 . 7H_2O$)	0.1 g
Ascorbic acid	0.2 g
Nicotinic acid	0.04 g
Glutathionine	1.5 g

Dissolve in 100 ml distilled water, and filter sterilise through a Millipore filter (0.45μm) before use..

APPENDIX 2

2 X YEAST TRYPTONE MEDIUM (2 X YT)

(Sambrook, 1989)

2 x Yeast Tryptone Broth

Tryptone	16 g
Yeast Extract	10 g
NaCl	5 g
Distilled water	900 ml

Adjust pH to 7.0 with 5N NaOH, make volume up to 1000 ml with distiled water, and autoclave.

2 x Yeast Tryptone Agar

As above but containing 1.2% Technical Agar (Oxoid)

APPENDIX 3

SDS-PAGE BUFFERS AND STOCK SOLUTIONS

(Laemmli, 1970)

STOCK SOLUTIONS

Acrylamide / Bis

Acrylamide	30 g
N,N-Methylene-bis-acrylamide	0.8 g

Dissolve in 100ml (final volume) distilled water, and filter before use.

Lower buffer

Tris	18.1 g
SDS	0.4 g
Distilled water	70 ml

Adjust pH to 8.9 with conc. HCl and make up to 100 ml with distilled water.

Upper buffer

Tris	6.06 g
SDS	0.4 g
Distilled water	70 ml

Adjust pH to 6.8 with conc. HCl and make up to 100 ml with distilled water.

Solubilising buffer

Glycerol	10 ml
2-mercaptoethanol	5 ml
SDS	3.O g
Bromophenol blue	0.01 g

Make to 100ml with Upper buffer (1 in 8 dilution).

Running Buffer

Tris	6.06 g
Glycine	28.8 g
SDS	2.0 g
Distilled water	1800 ml

Adjust pH to 8.3 with conc. HCl and make volume up to 2 Litres with distilled water.

Coomassie-Blue staining solution (x10)

Coomassie-Blue R250 (BDH)	1.25 g
50 % (v/v) methanol	454 ml
Glacial acetic acid	46 ml

Filter and dilute 1 in 10 in distilled water before use.

Destaining solution

Methanol	50 ml
Glacial acetic acid	75 ml
Distilled water	875 ml

GEL PREPARATION

Lower separating gel - 7.5 %

Lower buffer	10 ml
Distilled water	20 ml
Acrylamide / Bis	10 ml
Ammonium persulphate (10%)[a]	200 µl
TEMED	20 µl

[a] freshly prepared

Upper stacking gel - 4.5 %

Upper buffer	5 ml
Distilled water	12 ml
Acrylamide / Bis	3 ml
Ammonium persulphate (10%)[a]	60 µl
TEMED	40 µl

[a] freshly prepared

APPENDIX 4

RESORCINOL REAGENT

(Katcs, 1972)

Preparation of Reagent

Dissolve 2 grams of resorcinol in 100ml of water (stable solution). At least 4 h before use add 10ml of this solution to 80ml of conc. HCl (Analar reagent grade). containing 0.5ml of 0,1M copper sulphate (2.5g $CuSO_4 \cdot H_2O$ in 100ml of water), and dilute mixture to 100ml with water.

Procedure

Spray the dried plate with the resorcinol reagent: cover the sprayed TLC plate carefully with a glass plate and heat in an oven at 120°C. Gangliosides or sialic acid derivatives appear as a blue-violet spots,