CHARACTERISATION OF *CAMPYLOBACTER JEJUNI* TOXINS AND THEIR EFFECTS ON CULTURED MAMMALIAN CELLS.

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Table Of Contents

List Of Figures

List of Tables

List of Abbreviations

ABC	Antigen binding capacity
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
APS	Ammonium persulphate
Arg	Arginine
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BHI-	Brain heart infusion without yeast extracts
BHI+	Brain heart infusion with yeast extracts
BSA	Bovine serum albumin
C6	Human glioma
C(L)DT	Cytolethal distending toxin
C(L)RT	Cytolethal rounding toxin
CHO	Chinese hamster ovary cells
CNF	Cytotoxic necrotizing factor
Cr	Chromium
CT	Cholera toxin
DMEM	Dulbecco's modified eagle's medium
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNT	Dermonecrotic toxin
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence

ECV	Human umbilical cord endothelial
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
ETA	Exotoxin A
ETCC	European tissue culture collection
FITC	Fluorescein isothiocyanate isomer 1
GBS	Guillian-Barre syndrome
Gln	Glutamine
Glu	Glutamate
GTP	Guanosine triphosphate
HeLa	Human cervical carcinoma tissue culture
HPLC	High performance liquid chromatography
Ig	Immunoglobulin
LPS	Lipopolysaccharide
LT	Lethal toxin
MAP	Mitogen activated protein
MAPK	Mitogen-activated protein kinase
MES	Microtubule extraction suspension
MFS	Miller Fisher syndrome
MTT	Methyl blue tetrazolium
N2a	mouse neuroblastoma
NBT	Nitro blue tetrazolium
NFH	Neurofilament heavy chain
P125fak	Focal adhesion kinase
PBS	Phosphate buffered saline

PEG	Polyethylene glycol
PMSF	Phenyl methyl sulphonyl fluoride
PT	Pertussis toxin
PVDF	Polyvinyl diisorpropyl fluoride
RAPD	Random amplified polymorphic DNA
RPMT	Recombinant *Pasteurella multocida* toxin
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
spp	Plural of species
TBS	Tris buffered saline
TEMED	N, N, N, N-tetramethyl-ethylenediamine
Thr	Threonine
Tris	Tris(hydroxymethyl)aminomethane
UDP	Uridine diphosphate

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DECLARATION

This Work has not been accepted for any other degree and is not being currently submitted for any other degree.

This is to certify that the candidate carried out the work being submitted and that due acknowledgement has been made of any help received.

Signed.....

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(Director of Studies)

Abstract

Campylobacter jejuni and other related strains are important pathogens in human enterocolitis, the most common bacterial cause of diarrhoea in the industrialised countries.
In order to achieve a better level of understanding towards the role of toxins in *Campylobacter* infection the aims of this thesis were; to determine the optimal toxin producing *Campylobacter jejuni* strain from the three strains made available (namely; 11351, 11322, 11168), to optimise bacterial culture conditions for toxin production; to initially characterise the sub lethal effects of culture extracts with regards to their effects on cell morphology and cytoskeletal networks; to purify a novel *Campylobacter jejuni* toxin for N-terminal sequencing and characterisation at the molecular level.
Studies of mammalian cytotoxicity suggested that the bacterial strain *Campylobacter jejuni* 11351 was the most consistently toxic compared to other strains. BHI broth culture supernatants and the blood agar cell sonicates produced the most consistent cytotoxic effects on cultured mammalian cell lines (N2a and ECV). Results of cytotoxicity assays indicated that two types of toxins were being produced in the two bacterial culture conditions. Firstly, there was a secreted heat/trypsin labile exotoxin produced by bacteria grown in BHI broth cultures, and secondly, a heat/trypsin stable cell-attached endotoxin produced by bacteria grown on blood agar plates.
Sub-cytotoxic effects levels of the bacterial broth culture supernatant caused elongation of cell bodies in CHO cells, suggesting the presence of a cytolethal distending toxin (CDT). When introduced to ECV cells the toxin induced cell body rounding, suggesting the presence of a cytolethal rounding toxin (CRT). Neurotoxic effects, as demonstrated by reduction in the outgrowth of axon like neurites, were observed in N2a cells treated with both BHI broth culture supernatant and blood agar cell sonicate. These effects were heat sensitive for the former but heat resistant for the latter, suggesting a secreted proteinaceous neurotoxic exotoxin and an endotoxin, respectively. Probing of western blots of cell extracts treated with sub-cytotoxic levels of toxins revealed changes in the levels of microtubule, intermediate filament and microfilament proteins. In the case of BHI extracts, toxicity was also associated with disruption of microtubule and microfilament networks, as determined by confocal fluorescence microscopy.
N-terminal sequencing a 50 kDa polypeptide purified from blood agar cell sonicates by a combination of ion exchange chromatography and electro elution revealed a sequence which, when compared with known protein sequences using BLAST and LALIGN databanks, showed a most likely match for the major outer membrane protein reported as a cytotoxic porin.
In order to fully determine the identity and mechanism of action of the toxin, further work is required in the purification process, along with further characterisation of the toxin activity at the molecular level.

CHAPTER 1

1. Introduction

1.1 Importance of Campylobacter spp as a pathogen responsible for human enterocolitis

Campylobacter jejuni and other related strains are important pathogens in human enterocolitis, the most common bacterial cause of diarrhoea in the industrialised countries. At the moment the biology and mechanisms of action of the bacteria are still poorly understood. The species presently included in the *Campylobacter* genus include *C. jejuni, C. coli, C. upsaliensis, C. hyointestinalis* and *C. lari*. The first two types are responsible for 80-90% of enteric *Campylobacter* infections (Walker *et al* 1986; Ketley 1997; Wassenaar 1997).

Campylobacter are 1.5-6.0μm long and 0.2-0.5μm wide gram negative rods, usually possessing a flagellum at each pole (Ketley 1997). The 2 main species are microaerophilic and require oxygen concentrations of 3-15% and a CO_2 concentration of 3-5%. As a thermophilic organism, *C. jejuni* has an optimal growth temperature of 42°C, which may reflect an adaptation to habitat temperatures found in the gut of warm blooded hosts (Ketley 1997). The *Campylobacter* genome size is small, containing approximately 1.6-1.7 Mb of AT-rich DNA (Owen and Leaper 1981, Chang and Taylor 1990), which may explain the requirement for complex media for growth and their inability to ferment carbohydrates and degrade complex substances (Griffiths and Parkes 1996).

Infection with *Campylobacter spp.* occurs most often through ingestion of contaminated liquid or food, with an infective dose of as few as 500-800 organisms (Wallis 1994; Walker *et al* 1986). The clinical manifestations of the disease range from severe inflammatory diarrhoea to generally mild, non-inflammatory, watery diarrhoea. The first case is accompanied by acute abdominal pain, often with fever and general malaise. This progresses to profuse diarrhoea with an incubation time of 1 to 7 days until the symptoms appear (Wassenaar 1997; Ketley 1997; Walker *et al* 1986).

Relapse symptoms may occur several weeks after initial infection, but these are less severe than those observed initially. The illness can last for more than a week, but is normally self-limiting with complications uncommon (Ketley 1997), although complications associated with *Campylobacter* infection include Reiter's syndrome (Johnsen *et al* 1983), reactive arthritis (Ebright *et al* 1984) and Guillain-Barre syndrome (Allos 1998; van Belkum *et al* 2001; Ang *et al* 2002).

From similarities to other enteropathogens and the typical motility of *Campylobacter*, four virulence properties have been recognised: motility, adherence, invasion and toxin production (Wassenaar 1997, Walker *et al* 1986). Motility is required by the bacteria to reach attachment sites and for penetration of intestinal cells. Adherence to the epithelial surface is important for colonisation and may increase local concentration of secreted bacterial products. Although invasion (penetration) has been identified *in vivo* and *in vitro* (Blaser *et al* 1981; Konkel *et al* 1989), invasion levels detected *in vitro* are normally low (less than 1% of applied bacteria invade a monolayer in cell

culture) (Blaser *et al* 1981). Therefore, toxin production plays an important role in *Campylobacter* enteritis pathogenicity. A better understanding

of *Campylobacter* toxins may be achieved by a brief general introduction to bacterial toxins.

1.2 Introduction to bacterial toxins

Bacterial toxins were first discovered in 1888, when Roux and Yersin isolated toxins from *diphtheria bacillus*, which they tested on guinea pigs with fatal results. Within the following decade two more toxins were isolated, namely tetanus and botulinum. The discovery of bacterial toxins started a new field of study where injection of bacterial cells and their products into animals was used to investigate effects and find anti-serum therapies.

Toxins are the principal pathogenic factors of microorganisms causing infectious diseases. The term itself describes products of microbial metabolism that are lethal to cells or affect their functions negatively at very low concentrations (Balfanz *et al* 1996).

The three above mentioned toxins discovered in the 19th century are exotoxins, which are mainly proteins secreted into the surrounding medium (Boquet *et al* 1998), whereas endotoxins are lipopolysaccharides making up part of the cell wall of gram-negative bacteria (Balfanz *et al* 1996). The fundamental differences between the 2 types of toxins are not related to whether they are found inside or outside the bacterial cell, but to their chemical structures (Arbuthnott 1978). Exotoxins are antigenic proteins

probably without any non-protein residues, whereas endotoxins consist of a lipid moiety, lipid A. This region is covalently linked to a conserved inner-core oligosaccharide, which is followed by a highly variable O-specific polysaccharide chain (Lagrange *et al* 1995; Lerouge *et al* 2002; Caroff *et al* 2002). The lipid A region is responsible for the endotoxicity of the molecule (Poxton 1995). The non-repeating inner-core oligosaccharide region, which, because it is reasonably well conserved, provides the possibility of an epitope capable of inducing an antibody of broad enough specificity to cross-react antigenically with the LPS of many different gram negative strains (Erridge *et al* 2002; Raetz and Whitfield 2002).

Finally, the outer-core, or distal polysaccharide (Raetz and Whitfield 2002) of the O-polysaccharide region, which is the outer most part of the endotoxin and is therefore the major antigen targeted by host antibody responses (Erridge *et al* 2002).

Endotoxins are derived from the cell wall, playing an important part in cell survival and division. Toxicity is minimal during normal bacterial growth and significant release into the surrounding medium requires cell lysis after phagocytosis, complement-mediated attack or by antibiotic therapy (Williams and Clarke 1998). However, exotoxins can be secreted and appear in the culture medium during the logarithmic or declining phase of growth. Heyningen (1970) suggested that exotoxins are less heat-stable than the endotoxins (due to chemical structure), which are particularly less toxic than the exotoxins.

The principal toxigenic pathogenic bacteria and their exotoxins can be seen in table 1.1.

Table 1.1. Summary of pathogenic bacteria and their exotoxins.

Bacterium species	Toxin	Main biological effect	Reference
Corynebacterium diphtheriae	Diphtheria toxin	lethal, dermonecrotising	Arbuthnott 1978
Staphylococcus aureus	α-toxin, β-toxin, γ-toxin	lethal, dermonecrotising, haemolytic	Balfanz *et al* 1996
Streptococcus pyogenes	streptoylsin O, streptolysin S	cardio-toxic, haemolytic	Mitchell 1998
Bacillus anthracis	complex, oedema-producing toxin	adenylate cyclase	Balfanz *et al* 1996
Vibrio cholerae	enterotoxin	diarrhoeagenic	Ganguly and Kaur 1996
Shigella dysenteriae	haemorrhagin	neurotoxin, enterotoxin	Buchanan and Pearce 1979
E.coli	Heat labile/Stable enterotoxins, etc	diarrhoeagenic, cytotoxic	Chart 1998
Salmonella typhimurium	Salmonella toxin	diarrhoeagenic, haemolytic	Ganguly and Kaur 1996
Campylobacter jejuni	Campylobacter toxin, etc	diarrhoeagenic, ADP-ribosylate	Wassenaar 1997

The biological activities of toxins can be specific and diverse - many toxins can target a number of cell types, but some restrict attacks to a single cell type. Cytotoxic toxins, such as diphtheria α-toxin produced necrosis when injected into the skin (Arbuthnott 1978). Other toxins are not necrotic but produce oedemas and/or haemorrhage after injection into the skin *e.g.* anthrax toxin and cholera toxin (Brossier and Mock 2001). The dysentery toxin is neurotoxic and has no effect on the skin (Haijo *et al* 1968; Balfanz *et al* 1996). Toxins that haemolyse red blood cells are called oxygen-labile haemolysins, which include streptolysin O (Mitchell 1998). Other toxins are known to act on the human gut, for example the staphylococcal enterotoxin causes emesis and cholera toxins cause diarrhoea. This can be fatal, and successful treatment requires effective rehydration and antibiotic administration to decrease disease duration (Zhang *et al* 1997; Balaban and Rasooly 2000).

Potent neurotoxins such as those from *Clostridium botulinum* and *C. tetani* have characteristic paralytic actions, implying action on nervous tissue in a non-degradative manner (Heyningen 1970; Johnson and Bradshaw 2001).

The classification of bacterial toxins is a complex process due to the physiological events relevant to their synthesis and release. The toxins can be generally classified into four groups according to their cellular location:

1. *Intracytoplasmic protein toxins of gram-negative bacteria*, such as *Cholera* endotoxins and *Shigella* neurotoxins. These toxins are strongly linked to the bacterial cell wall and are never released into the culture medium during the active phases of growth (Buchanan and Pearce 1979). Appearance of the

endotoxins in the medium after cell lysis suggests location in the cytoplasm as opposed to the cell wall (Arbuthnott 1978).

2. *Complex lipopolysaccharide/protein toxins of the cell walls of gram-negative bacteria.* These toxins are produced by *Enterobacteriaceae.* They are constituent parts of the bacterial cell wall and thus are located at the surface of the organism. The endotoxins are strictly cell-bound, particularly during the logarithmic phase of the growth cycle (Koller *et al* 1994; Lagrange *et al* 1995).

3. *True protein exotoxins.* The toxins have characteristics such as synthesis and release into the medium by actively growing cells, no increase in toxin levels in the stationary phase, and negligible intracellular concentrations of toxin. The toxins can be released immediately after growth or after a lag period (Balfanz *et al* 1996; Arbuthnott 1978).

4. *Protein toxins with both intracellular and extracellular location during logarithmic growth phase.* These toxins are found in the gram-positive *Clostridium* bacteria. In this case, large amounts of the toxins accumulate intracellularly during the log phase. At the same time, significant quantities of these toxins may be found in the medium. Cells grown beyond the log phase release large amounts of the toxin into the medium reducing intracellular levels of the toxin (Arbuthnott 1978).

1.2.1. Clinical effects of enteric pathogen infection.

There have been a number of clinical studies on the effects of bacterial toxins and the symptoms they induce. In an Australian study of 3 cases of haemolytic uraemic syndrome, *E.coli* strains were isolated from one patient (Miles *et al* 1996). In an attempt to identify the pathogens in patient food sources, *Shigella*-like toxins were found in local butcher meats, but no match was made with patient stool samples. Environmental factors were also thought to be a consideration in a *Campylobacter* infection. The incidence of the disease is thought to be dependent on the frequency of meat testing for *Shiga*-like toxins I and II and additional educational and research programmes (Miles *et al* 1996).

In a study investigating the prevalence of clinical manifestations present in Austrian children, 280 patients were analysed for the presence of free faecal *Shiga* toxins (Stx) 1 and 2 using an enzyme immunoassay (EIA) and also by culture on sorbitol MacConkey agar (Allerberg *et al* 1996). Further testing of EIA positive specimens included a cytotoxicity assay, PCR analysis and a colony hybridization test. The agar culture revealed 3 *E.coli* strains in stool samples. A further six samples were found positive by EIA, and analysis of stools for a variety of enteric pathogens revealed that *E.coli* produced *Shiga*-like toxins and was the third most common bacterial pathogen. The average age of children suffering from Shiga toxin-producing *E.coli* (STEC) -induced diarrhoea was 27.6 months. *Salmonella* and *Campylobacter* infections were associated with younger children in general, whereas rotavirus infections were associated with older children (Allerberg *et al* 1996; Terajima *et al* 1999).

An Italian team studied 618 children with infectious diarrhoea and 135 controls for the presence of viral, bacterial and parasitic enteric pathogens (Caprioli *et al.*, 1996). Using gene probes specific to different virulence factors, they identified diarrhoeagenic *E.coli.* Free bacterial toxins were also identified in 59% of children with diarrhoea and 10.4% of asymptomatic controls, using cell culture cytotoxicity assays on stool filtrates. The main agents associated with the disease were rotaviruses (23.6%), *Salmonella* (19.2%) and *Campylobacter* (7.9%), the latter being highly associated with outpatients. *Camplyobacter* infections were found to be predictable through the presence of blood and/or leukocytes in stool samples. The presence of diarrhoeagenic *E.coli, Shigella flexneri, Yersinia enterocolitica, Crystosporidium* and *Giardia* was also observed in a small number of paediatric patients (Caprioli *et al* 1996). The results of such clinical studies can be used for planning strategies to prevent and control diarrhoeal diseases in the future.

These and other new discoveries are highly important considering the fact that microbial diseases continue to be a leading cause of morbidity and mortality worldwide. Epidemiological studies predict that their incidence will increase as the world population grows.

Several new fields of research have been developed to increase our knowledge of microbial pathogenesis (Lee 2000), such as studies on the molecular basis of diseases through the genetic manipulation of pathogens, for example *Shigella* and *Salmonella*.

For example, new techniques have been developed to study the bacteria at the cellular and molecular levels, such as detection of virulence factors *in vivo* (using methods such as sensitive imaging systems following fluorescent

protein expression). Molecular techniques have been used to identify genes induced in *in vivo* bacterial infection but not in *in vitro* situations. The continuing development of *in vitro* systems along with techniques such as fluorescence, confocal, video enhanced and electron microscopy is proving very useful, together with new methods such as the ability to generate and express transdominant negative forms of numerous cytoskeleton rearrangements and signalling pathways (Finlay and Cossart 1997).

For example, such approaches aided the discovery that bacteria such as *Salmonella* and *Shigella* infect mammalian cells by mimicking the action of epidermal growth factor (EGF), thus inducing membrane ruffling and actin polymerisation. The membrane ruffling is involved in the internalisation of the bacteria (Cossart *et al* 1996; Tran Van Nhieu and Sansonetti 1999; Bourdet-Sicard and Van Nhieu 1999).

Another system of infection, through the disruption of the cytoskeleton and cellular signal transduction is carried out by *Yersinia*. The bacteria secrete Yop proteins as part of a type III secretion system. Once in contact with the host cells the Yop proteins are injected into the cell and halt phagocytosis. The Yop protein's specific target site is unknown, but they do disrupt the actin cytoskeleton of infected cells. The proteins show homology with exoenzyme S from *Pseudomonas aeruginosa,* which modifies small G proteins involved in the regulation of the actin network (Barbieri 2000). Through Yop interference with phagocytosis the bacteria can remain extracellular, allowing survival and multiplication (Finlay and Cossart, 1997).

1.2.2. Clinical effects of *Campylobacter spp* infection.

A study amongst the U.S. military based in Thailand, where *Campylobacter* is the leading cause of traveller's diarrhoea, compared microbiologic findings in 169 personnel with acute diarrhoea with 77 asymptomatic subjects. *Campylobacter* was found to be strongly associated with disease (1 in 37), with a more severe clinical presentation, and 96% of isolates showing ciproflaxin resistance *in vitro* (Sanders *et al* 2002). The paper highlighted the importance of *Campylobacter* infection as a major cause of severe traveller's diarrhoea, and also illustrated the gaining problem of drug-resistant strains and associated treatment problems.

This worrying trend was also highlighted in a paper by Aarestrup and Engberg (2001), who suggested that anti-microbial resistance has emerged among *Campylobacter* as a consequence of antimicrobial agents in food animal production. Elaborating, that resistance to fluoroquinolones and macrolides is mediated by chromosomal mutations and resistance to other antimicrobial agents is mediated by acquired resistance genes (originating from both gram positive and negative bacterial species).

In a case report involving a patient who had acquired immunodeficiency syndrome, a multi-drug resistant *Campylobacter fetus* infection proved fatal, resulting in complicated symptoms, such as hypersplenism, splenic infarction and histiocytic hyperplasia with haemophagocytosis. Effective therapy was delayed due to multi-drug resistance and the prolonged infection/antigenic stimulation promoted splenomegaly and hypersplenism (Anstead *et al* 2001).

Similar results were found in a study comparing HIV-infected patients and non-HIV-infected subjects. Where *Campylobacter* infection of HIV-infected

patients was found to be often severe, debilitating febrile illness, requiring multiple and prolonged antibiotic therapy. Where-as in non-HIV-infected patients, the illness was an acute onset of fever associated with self-limiting enteritis, with fever resolving rapidly with antibiotic treatment (Tee and Mijch 1998).

1.3 Molecular basis of toxin action

Toxins can be classified into three main divisions, according to their specific sites of action within the host cell. The first group of toxins act on the plasma membrane, where they interfere with transmembrane signalling pathways. Toxins included in this group are the heat-stable enterotoxin ST (from *E.coli*) which acts on the transmembrane enzyme guanylate cyclase in intestinal cells (Chart 1998). The second group of toxins alter membrane permeability, for example pore-forming toxins of the streptolysin O family (Bhakdi *et al* 1998). The third group of toxins act inside the cell by targeting specific cytosolic components and modifying them enzymatically (Thelestam and Florin 1994). These toxins can be further divided into categories according to their enzymatic activity. The toxins are most potent through their catalytic actions and can often result in changes in the cytoskeleton or cell signalling pathways (Finlay and Cossart, 1997).

1.3.1 ADP-ribosylation

The majority of ADP-ribosylating toxins act on GTP-binding proteins (Krueger and Barbieri 1995). As mentioned earlier (section 1.2), *Vibrio cholerae* produces the cholera toxin (CT) which was first demonstrated in cell-free culture filtrates (De 1959) and was eventually purified (Finkelstein and Lospalluto 1969). The toxin belongs to the bacterial family of ADP-ribosylating toxins, which catalyse the transfer of the ADP-ribose portion of NAD to a nucleophilic acceptor. CT specifically acts on Arg-201 in the guanine nucleotide binding subunit of the stimulatory regulator of adenyl cyclase (Gsα) (Moss and Vaughan 1988). Through the use of CT and other ADP-ribosylating toxins, a number of proteins (ADP-ribosylation factors [ARFs]) have been identified that show critical roles in intracellular vesicular transport. A number of bacterial toxins are structurally related to CT, such as the heat-labile *E.coli* enterotoxins, which consist of a single catalytic A subunit and five B subunits (Ganguly and Kaur 1996). Another example of an oligomeric toxin is the *pertussis* toxin (PT) secreted by *Bordetella pertussis*. PT has the most complicated structure of the various bacterial toxins known to date (Locht and Antoine 1997). It is a member of the A-B family of toxins, but is made up of 5 different sub-units (S1-S5), where S1 is the A (enzyme) moiety and S2-S5 are the B (receptor-binding) domain, connected by intermolecular disulphide bonds (Antoine and Locht 1990). Monomeric protein ADP-ribosylating toxins include the diphtheria toxin from *Corynebacterium diphtheriae* and exotoxin A (ETA) from *Pseudomonas aeruginosa.* These toxins ADP-ribosylate elongation factor 2, leading to inhibition of protein synthesis and cell death

(Zdanovskaia et al 2000; Pastan and FitzGerald 1989; Yates and Merrill 2001).

CT is an 84 kDa protein containing an A subunit and five B subunits (Lencer 2001). The B subunits forms a pentameric ring to which the A subunit is anchored. One face of this ring contains five binding sites for ganglioside GM1, which is the mammalian cell surface binding site for CT. Through an unknown mechanism, binding of the pentamer results in endocytosis of the toxin. This could be partially due to two effects observed after CT binding. The first is a change in membrane lipid packing and the second is an increase in calcium influx (Lencer *et al* 1999). CT is endocytosed into Golgi-endoplasmic reticulum lysosomes, where it is transferred to a compartment in which reduction occurs; this produces enzymatically active toxin and the ADP-ribosylation of Gsα, resulting in the activation of adenylate cyclase (Zhang *et al.*, 1997).

The *Pertussis* toxin (PT) is a 105 kDa hexameric enzyme. It ADP-ribosylates Gα isoforms of the Gi class of G proteins, catalysing the hydrolysis of NAD+ and resulting in an ADP+ moiety which is transferred to a cysteine residue near the C-terminal of the α subunit (Locht and Antoine 1997). This results in the prevention of transmembrane receptor interaction with the G proteins, which are anchored to the cytoplasmic surface. The result is the formation of a functional receptor-G-protein uncoupling. Again, PT comprises A and B subunits. The B subunit is involved in binding to the surface of eukaryotic cells and possibly toxin translocation across the plasma membrane, whereas the reduction of a disulphide bond to separate the A subunit is required for toxin activation (Antoine and Locht 1990). Depending on the target cell, the

physiological effects of PT vary greatly, but the effects are responsible for most of the symptoms associated with whooping cough (Pittman 1984). PT receptors can be found on the surface of most cells, although they have yet to be characterised; they are thought to be sialoglycoproteins (Locht and Antoine, 1997).

Another group of exoenzymes has been found to ADP-ribosylate small GTPases that are involved in the control of the actin cytoskeleton and its role in the control of cell migration and cell shape. For example, C3 is one of the exoenzymes produced by *Clostridium botulinum* type C and is distinct from C1 neurotoxin and the actin ADP-ribosylating C2 toxin (Saito and Narumiya 1997). C3 ADP-ribosylates members of the Rho GTPase family, specifically at Arg-41, resulting in protein inactivation. The resultant inactivation of the Rho GTPase has revealed a number of functional properties of Rho. For example, studies using the C3 exoenzyme have shown that Rho is involved in the formation of stress fibres (Paterson *et al* 1990), contractile rings during cytokinesis (Mabuchi *et al* 1993) and amongst other things, cell adhesion, cell motility and cell proliferation (Saito and Narumiya 1997).

As mentioned, there are a number of clostridial toxins that ADP-ribosylate the ATP-binding protein actin. Actin depolymerization is thus induced by *C. botulinum* C2 toxin, *C. perfingens iota* toxin, *C. spiroforme* toxin and certain *C. difficile* toxins that contain transferase activity (Kiefer *et al 1996; Gulke* et al 2001; Sklyarova *et al* 1995; Chowdhury *et al* 1999; Nagahama *et al* 2000). Again, the toxins are characterised by an A-B (enzyme-binding) component structure, but unlike the cholera or pertussis toxins, they are separate proteins which are not bound either covalently or non-covalently (Aktories *et al* 1997).

The cell surface receptor for the C2 toxin is unknown but internalization is thought to be due to receptor-mediated endocytosis followed by translocation of the enzyme component into the cytosol. The subsequent ADP-ribosylation of actin results in dramatic redistribution of F-actin. The redistribution of the polymerised F-actin is an indirect effect of the ADP-ribosylation of the monomeric G-actin by C2. This inhibits actin polymerisation, most probably by steric hindrance, because the Arg-177 site of ribosylation is near an actin-actin contact site (Aktories *et* al 1986). The ADP-ribosylated actin interacts with unmodified actin and binds like a capping protein to the barbed (fast-polymerising) end of F-actin, this interaction inhibits further association with monomeric actin at this end (Wegner and Aktories 1988). Polymerisation at the pointed end of the actin filaments is not affected by ADP-ribosylated actin, which results in an imbalance in the equilibrium of actin polymerisation, thus producing a redistribution of the microfilament network (Saito and Narumiya 1997). The modification of actin cross-linking complexes may also play a role in the cytotoxic or cytopathic effects of the toxin (Aktories *et al* 1997).

The above biological effects of ADP-ribosylation were supported by a recent paper investigating the effects of microinjecting ADP-ribosylated G-actin into cultured cells in the absence of toxins in order to study the effects on the actin cytoskeleton (Kieffer *et al* 1996). This was carried out because C2 and *iota* toxins alter monomeric G-actin at Arg-177, but do not affect polymeric F-actin directly; this results in the ADP-ribosylated G-actin having the properties of a (+) end capping protein (Wegner and Aktories 1988). This prevents further actin assembly at the (+) end of microfilaments, while net disassembly may occur at the (-) ends. Microinjection of the altered G-actin resulted in the

retraction of the cell body, together with redistribution and depolymerization of the actin cytoskeleton in a concentration and time dependent manner. This may explain the cytopathic effects of ADP-ribosylating toxins on microfilaments, even though F-actin is not directly affected (Kieffer *et al* 1996). A new *Clostridium difficile* isolate has been discovered which produces an actin-specific ADP-ribosyltransferase with homology to the *iota* and *C. spiroforme* toxins (Just *et al* 2001; Perelle 1997a). The locus has been cloned and sequenced. The site contains 2 genes (cdtA and cdtB) which produce the enzymatic and binding components of an enzyme. These have 81 and 84% identity, respectively, to the components present in the *iota* toxin. These genes have been identified in 3 of 24 clinical isolates, suggesting that some *C. difficile* strains synthesize a binary toxin which could be a new virulence factor (Perelle *et al* 1997b).

1.3.2 Monoglycosylation

Another 2 major bacterial toxins are produced by *Clostridium difficile*; known as toxins A and B, these molecules also affect cytoskeletal organization, but they lack ADP-ribosyltransferase activities (Dillon *et al* 1995; Borriello 1998; Limaye 2000). The toxins have an actinomorphic, cytopathogenic effect on cells. These toxins were the first bacterial toxins found to affect the actin cytoskeleton while other major cytoskeleton structures were left intact. The toxins act intracellularly after uptake by endocytosis into the cytosol, where intracellular processing of the toxins is required to produce their cellular

effects. Both proteins have been shown to affect Rho, Ras and Cdc42 proteins of the GTP-binding family (Thelestam *et al* 1997).

Similarly the α-toxin produced by *Clostridium noyvi,* which is a lethal and oedema-inducing toxin, causes cell rounding of cultured cells by redistribution of the actin cytoskeleton and has been reported to catalyse modification of Rho (Busch *et al* 2000; Selzer *et al* 1996). Like toxins A and B, α-toxin also acts on Cdc42 and Rac proteins through the incorporation of GlnNAc at Thr-35 (equivalent to Thr-37 in Rho) via the activated UDP precursor. This results in the inactivation of the GTPase and redistribution of the actin cytoskeleton (Selzer *et al* 1996).

Toxin B has also been shown to act on the low molecular weight GTPase proteins of the Rho family, by using the selective co-substrate UDP-glucose to transfer the glucose moiety to Rho, Ras and Cdc42 proteins, acting specifically at Thr-37 in Rho and Thr-35 of Ras and Cdc42. These glucose acceptors reside in the effector domains of the proteins and their glycosylation results in the inactivation of the GTPases. The toxin is co-expressed along with toxin A, with which it shows 63% amino acid homology. Both toxins A and B are single-chained exotoxins with molecular masses of 270 and 380 Da, respectively (Just *et al* 1997).

Toxin B is characterized by three structural domains (Hofmann *et al* 1997). The C-domain, which contains a repeated oligopeptide sequence, is thought to be involved in binding to target eukaryotic cells via glycoconjugates. The middle-domain, which contains a small hydrophobic region with putative membrane spanning regions is assumed to be involved in membrane translocation. Finally the N-terminal domain is thought to contain a nucleotide

binding site and to harbour the cytotoxic activity of the toxin (Chaves-Olarte *et al* 1997). The nucleotide binding region present in the N-terminal (amino acids 651-683) was thought to be essential for cytotoxicity (Barroso *et al* 1990). However, deletion studies using toxin fragments (amino acids 1-546) showed glycosylation levels comparable to those produced by the intact toxin (Hofmann *et al* 1997). The N-terminal also contains several cysteines that are highly conserved in *C.difficile* toxins and were thought to have an important role in the holotoxin cytotoxicity. However, the same deletion studies showed little effect on GTPase glycosylation in the absence of the residues, although the cysteines may have a role in endocytosis or translocation of the toxin into the cytosol. The site of the cytotoxic harbouring region in the N-terminal was localized in these deletion studies, in which deletion of amino acids 517-546 resulted in 1,000 fold reduction in toxicity, therefore suggesting that amino acids 517-546 are essential for enzyme activity and/or toxin B structure (Hofmann *et al.*, 1997).

Because of the known action of toxin B, it has been used in a number of studies investigating the roles of low molecular weight GTPases in signal transduction. For example in a study by Just *et al* (1997), toxin B-catalysed monoglycosylation of the Rho proteins resulted in the inhibition of an antigen-induced signal transduction (IgE-stimulated serotonin release). The substrate Rho proteins were therefore thought to be involved in the FcεRI-stimulated signal pathway (this is the activation of rat basophilic leukemia (RBL) cells by antigens and relies on IgE-receptor complexes and a resultant cascade). The pathway stimulates tyrosine kinases and phospholipase Cγ, which results in mobilization of calcium and protein kinase C (Just *et al* 1997).

Further evidence for the alteration of the Rho proteins by toxin B involved the inhibition of 90% Rho protein through ADP-ribosylation by treatment with *C. botulinum* transferase C3 (Prepens *et al* 1996). C3 induced ADP-ribosylation of Rho proteins did not result in inhibition of the RBL cells, providing evidence that the toxin B induced gylcosylation of Cdc42 proteins was responsible for the antigen-induced secretion from RBL cells (Prepens *et al* 1996).

Another *Clostridium* toxin which has 90% homology with toxin B and 73% homology with toxin A is the cytotoxic, oedema-causing lethal toxin (LT), produced by *C sordellii* and is associated with diarrhoea and enterotoxemia (Just *et al* 1996). The toxin belongs to the family of large clostridial cytotoxins, characterized by a single-chain structure and has a molecular mass of 250-300 kDa. LT affects cell monolayers in culture by redistributing the actin cytoskeleton, producing pronounced rounding of cells, which become grouped in small clusters (Popoff *et al* 1996). Like toxins A and B, LT contains C-terminal located repetitive peptide sequences, probably involved in cell receptor binding. In a recent review, LT was shown to have glucosyltransferase activity, specifically acting on low molecular weight GTP-binding proteins Rac and Ras (Aktories 1997). Like toxins A and B, LT also selectively uses UDP-glucose as a cosubstrate to modify Rac proteins (which are involved in membrane ruffling) and, unlike the *C. difficile* toxins, alters Ras proteins (which are, along with Rho proteins, involved in the activation of transcription factors).

Therefore LT can work in 2 separate ways: firstly, through the inactivation of regulatory proteins of the actin cytoskeleton; secondly, they may interfere with gene expression by inhibiting signal cascades which lead to the activation of

transcription factors. Again, like toxins A and B, LT activity is dependent on the cofactor Mn^{2+} as most glucosyltransferases require the presence of divalent cations for activity (Ciesla and Bobak 1998). Sequential glycosylation and deglycosylation techniques have shown that the target amino acid in Rac proteins is the same for LT and toxin B. Ras inactivation by LT results in the blocking of the MAP kinase pathway and glycosylation of Rac proteins results in the depolymerization of actin filaments (Just *et al* 1997).

A novel isoform of the Ras-modifying clostridial cytotoxins was reported to modify Ral proteins, along with glycosylation of Ras and Rap proteins. The LT toxin produced by *C.sordellii* strain 6018 acts preferentially on the GDP-bound form of Ral and the acceptor amino acid for glucose is Thr-46 (equivalent to Thr-35 in H-Ras), which is located in the effector region (Hoffmann *et al* 1996).

1.3.3 Deamidation.

A new toxin-mediated reaction discovered recently is the deamidation of GTP-binding proteins belonging to the Rho family (Boquet 2000; Lerm *et al* 2000; Aktories 1997). The toxin involved is the cytotoxic necrotizing factor (CNF) produced by the uropathogenic (CNF1) or enteropathogenic (CNF2) *E. coli* strains. Exposure of cells to CNF toxins produces a decrease in migration of the Rho GTP-binding proteins on SDS-PAGE and may constitutively activate the proteins (Flatau *et al* 1997). This change also correlates with an increase in actin stress fibre formation and focal adhesion contacts in cultured cells. The specific target site of deamidation is a Gln residue at position 63. CNF1

catalyses the deamidation and changes the Gln to Glu; this inhibits intrinsic GTP-hydrolysis, which is stimulated by its GTPase activating protein (GAP). Therefore the alteration induced by CNF1 results in the constitutive activation of the Rho protein, which induces reorganization of actin stress fibres. These observations have also been seen in *in vitro* assays, in which recombinant RhoA shows similar electrophoretic mobility shifts after incubation with purified CNF1 (Boquet 2001; Horiguchi 2001).

Another example of a toxin with deamidase activity is the dermonecrotic toxin (DNT) secreted by *Bordetella bronchiseptica*, which shows homology to CNF1. DNT also promotes actin reorganization and a mobility shift in the Rho protein, suggesting a deamidation of Gln63 (Flatau *et al* 1997; Horiguchi 2001).

1.3.4 Tyrosine Phosphorylation

CNF1 and DNT induce other modifications to cytoskeletal proteins and a non-receptor protein. Both toxins induce p21rho-stimulated tyrosine phosphorylation of paxillin and focal adhesion kinase (p125fak). The biochemical process itself has been identified as an early event in the action of diverse signalling molecules that mediate cell growth and differentiation.

Paxillin is a multidomain protein which may function as an adaptor, capable of complex formation with p125fak and other proteins of the focal adhesion complex (Turner 1998). p125fak has been shown to have a critical role in embryonic development and cell locomotion. The result of increased tyrosine phosphorylation of both proteins is a profound alteration in actin organisation

and in the assembly of focal adhesion complexes. The small G protein p21rho, a member of the Ras family of GTP-binding proteins, has been implicated in the tyrosine phosphorylation of the 2 proteins and has also been associated with the mitogen-stimulated formation of focal adhesions and actin stress fibres (Lacerda *et al* 1997).

As mentioned earlier, CNF1 and DNT induce actin reorganization and multinucleation in several cell types. The toxin induced tyrosine phosphorylation of the 2 proteins results in a concomitant increase in the number of focal adhesion plaques in cultured fibroblast cells. Along with these morphological changes, the activation of p21rho proteins is thought to lie in a novel signal transduction pathway that regulates cell motility and cell proliferation (Lacerda *et al* 1997).

The fact that treatment with cytochalasin D, an actin cytoskeleton and focal adhesion plaque-disrupting agent, resulted in abolition of tyrosine phosphorylation, suggests that an intact cytoskeleton is essential for activity (Lacerda *et al* 1997; Garcia *et al* 1997). The toxins also induce DNA synthesis in the absence of detectable activation of p42MAPK and p44MAPK (protein members of the MAPK phosphorylation cascade pathway, as mentioned earlier), providing additional evidence for a novel p21rho-dependent signalling pathway leading to cell entry into the S phase (Lacerda *et al* 1997).

Another potent mitogen for 3T3 fibroblast cells is the recombinant *Pasteurella multocida* toxin (rPMT), which promotes anchorage-independent growth of Rat-1 cells. The toxin has been cloned and sequenced and has partial sequence homology to CNF1 and CNF2 (Lacerda *et al* 1996). rPMT is

thought to enter host cells and act intracellularly in the initiation and sustainment of DNA synthesis. Its entry occurs via endosomal/lysosomal compartments, followed by initiation of signal transduction cascades, including p125fak and paxillin tyrosine phosphorylation pathways (Pullinger *et al* 2001). In similar studies, carried out by Lacerda *et al*, the biochemical changes induced by the toxin were examined using the same inhibitory enzymes as in the CNF1/DNT treated cultures. Activity inhibition showed similar results; this suggested that rPMT activates the p21rho protein and possibly a novel signal transduction pathway. This may be a method for rPMT to circumvent the need for integrin-mediated signals generated in adherent cells (Lacerda *et al* 1996). In addition, the group suggested that polymerization of the actin cytoskeleton (affected by rPMT-induced stress fibre formation) may have a role in the endocytosis of other bacteria by animal cells (Lacerda *et al* 1996).

1.4. Campylobacter spp Pathogenesis

1.4.1. Campylobacter spp pathogenic mechanisms.

It is now known that *Campylobacter jejuni* and related species are the most common bacterial causes of acute infective diarrhoea in the developed world and that, along with bacterial motility, adherence and invasion, toxin production plays an important role in the pathogenesis of bacterial infection. Therefore the identification and characterisation of these virulence factors produced by the bacteria is a major concern in the understanding of pathological mechanisms exhibited by the bacteria.

The first of these major virulence factors in *C. jejuni* infection is motility, which has been widely covered as a research field (Ageuro-Rosenfeld *et al* 1990; Black 1988; Walker *et al* 1986; Wallis 1994; Ketley 1997). The motility of *Campylobacter* requires the production of flagella and there have been a number of papers identifying genes expressing flagellin sub-units, *flaA and flaB* genes (Cervantes *et al* 2000; Taylor *et al* 1992; Bleumink-pluym *et al* 1999). These polar flagella, along with cell shape, combine to give *Campylobacter* an unusually high level of motility in viscous environments and it has been speculated that host cell adhesion is not even required if the bacteria can successfully colonise the mucus (Lee *et al* 1986).

Adherence of the bacteria on to the host cell surface is very important in colonisation and localisation of bacterial secretions. There have been a number of studies carried out on the process (Prasad *et al* 1996; Joe *et al* 2000) identifying target areas in the extracellular membrane, such as fibronectin (Steven *et al* 2000) and binding proteins, one of which (PEB1) has been cloned (Pei and Blaser 1993).

The third virulence factor is invasion and evidence such as inflammation and bacteraemia suggest that invasion is an important pathogenic mechanism. Research carried out on invasion have produced results including the identification of microtubules as a major target for host intestinal cell invasion (Hu *et al* 1999; Biswas *et al* 2000) and the identification of an invasive associated marker region in the bacterial genome using RAPD fingerprinting (Carvalho *et al* 2001). There have also been a number of cellular models used to study the invasiveness of *Campylobacter* (Babakhani *et al* 1993; Konkel *et al* 1992; Wooldridge *et al* 1996). However, invasion levels detected

in vitro have been shown to be less than 1% of applied bacterial on a mammalian cellular monolayer, with efficient intracellular bacterial killing taking place (Wassenaar 1997). Therefore toxin production can be regarded as an important virulence factor in *Campylobacter* enteritis.

Campylobacter spp are known to produce a number of toxins. There have been a few reviews on the toxins identified (Wassenaar 1997; Ketley 1997; Wallis 1994) but there is a lot of confusion about the identification and characterisation of these toxins. Proteinaceous toxins fall into two classes, depending on their primary mode of action: enterotoxins and cytotoxins. Enterotoxins are defined as "secreted proteins with a capacity to bind to a cellular receptor, enter the cell and elevate intracellular cyclic AMP levels". Cytotoxins are defined as "proteins that kill target cells" (Wassenaar 1997).

1.4.2. Campylobacter spp cytotoxins

There have been a number of cytotoxins reported in *Campylobacter jejuni* cultures, but because groups use different cell lines, different bacterial culture conditions and strains, with little attempt at comparing "new" discoveries with toxins already reported, the literature on cytotoxin production is muddled (Wassenaar 1997). In Trudy Wassenaar's review on *Campylobacter jejuni* toxin production, she defined six cytotoxins produced by the organism after consulting various reports of novel Campylobacter toxins. She concluded that the six cytotoxins were (a) a 70 kDa cytotoxin active on HeLa, CHO and other cells but inactive on Vero cells (Mahajan and Rodgers 1990; Mizuno *et al* 1994; Goossens *et al* 1985); (b) a cytotoxin active in Vero and Hela cells

(Moutinho-Fragoso *et al* 1996); (c) Cytolethal distending toxin (CDT) (see section 1.4.3); (d) a cytotoxin neutralised by Stx antitoxin (Moore *et al* 1988); (e) a cytotoxins(s) displaying haemolytic activity (see section 1.4.4); and (f) a hepatotoxin (Kita *et al* 1992).

A heat-stable toxin was identified by Lam (1993) when attempting to produce a rapid cytotoxicity assay for the detection of *C. jejuni* toxins. The assay was a chromium release assay, using ^{51}Cr-labelled chicken lymphocytes. The group found 1 human, 3 chicken and 4 turkey strains of bacteria which had a cytotoxic effect and, of these, strain C111 supernatant retained its cytotoxicity after being heated to 60°C for 1 hour and 100°C for 10 minutes. However, this is not a characteristic of known enterotoxins, which is defined in section 1.2 (Lam 1993).

More recent toxin discoveries have been reported by groups such as Lee *et al* (2000), who screened the culture filtrates from 10 *Campylobacter* species using CHO, HeLa, Vero, Int 407 and mouse macrophage cell lines. They detected a heat-stable cytotoxin in strain 81116, which was secreted during stationary phase that had its activity destroyed using proteolytic enzymes (Lee *et al* 2000).

Kawaguchi *et al* (1989) reported that enterotoxin and cytotoxin activity produced by *Campylobacter jejuni* C6 were mainly found in culture supernatant, but they observed different distribution between the two toxin types in other fractions. Cytotoxin activity was detected in LPS fractions, suggesting that LPS may play a role in the cytotoxic activity of the bacteria, with solubilisation experiments suggesting that cytotoxin should be produced

intracellularly in early log phase, but released after the stationary phase of growth (Kawaguchi *et al* 1989).

This theory is supported by the findings of Bacon *et al* (1999), who recently reported the presence of a cytotoxic porin-LPS complex in a clinical isolate of *Campylobacter jejuni*. Analysis of the complex by SDS-PAGE revealed a 45 kDa band and a high molecular weight carbohydrate moiety. Cytotoxic activity was heat labile at 70°C and resistant to inactivation with trypsin. N-terminus sequencing of the protein component revealed 97% homology with the outer-membrane porin protein from *C. jejuni* (Bacon *et al* 1999).

1.4.3 Campylobacter spp cytolethal distending toxin (CDT).

A recent advance in the understanding of protein involvement in the pathogenesis of *Campylobacter* was reported by Pickett *et al* (1996). The group cloned and sequenced DNA from *C.jejuni* which contained genes required for the production of cytolethal distending toxin (CDT). A CDT-like toxin was first reported to be produced by *Campylobacter* by Johnson and Lior (1986). The toxin, which was found in the bacterial culture supernatant after removal of cells by centrifugation, caused several cultured mammalian cell lines (such as HeLa and Vero cells) to become slowly distended after 2-4 days treatment, after which the cells lysed. The toxin activity was heat sensitive, trypsin sensitive and non-dialysable, confirming that it was a protein. Screening of known *Campylobacter jejuni* strains by Johnson and Lior, revealed that 41% produced CDT (Johnson and Lior 1988).

The genes responsible for CDT expression (*cdt* A/B/C) have been cloned and sequenced in *E. coli* (Picket *et al* 1994; Scott and Kaper *et al* 1994). The three genes are closely linked and encode for proteins predicted to have molecular weights of 25.5, 29.8 and 20.3 kDa, respectively. However, due to the presence of consensus leader sequences, the mature proteins are likely to be somewhat smaller (Scott and Kaper 1994). Investigation into the use of *cdt* primers is underway, with the aim of producing a detection kit for CDT expression in different enteropathogens. An example of this was reported by Eyigor *et al* (1999), who tested four different primer pairs for their ability to amplify *cdt* genes from a variety of *C. jejuni* and *C. coli* isolates. They reported that all four sets were useful and that *cdt* gene sequences were

relatively conserved within species, but that there was a divergence in gene sequences between species (*e.g.* identification of *C. jejuni* and *C. coli* using *Eco*RI digestion), which may be used to speciate *Campylobacter* in clinical tests (Eyigor *et al* 1999).

The three *cdt* genes themselves form a tripartite complex which is essential for CDT activity (Lara-Tejero and Galan 2002). *cdt*A and *cdtC* are thought to make up the heterodimeric B sub-unit required for delivery of the *cdtB* encoded holotoxin, which acts as the enzymatically active sub-unit of a proposed, fully functional CDT tripartite toxin (Lara-Tejero and Galan 2002). A recent HeLa cell assay by Purdy *et al* (1996) suggested that iron acquisition had a significant effect on CDT expression in some *Campylobacter* strains. The group are carrying out on-going investigations into promoter elements and putative regulatory regions across the *cdt ABC* gene complex (Purdy *et al* 2000).

Detection of CDT activity in cultured cells is a different matter, with a number of groups using different cell lines and culture conditions during assays. Schulze *et al* (1996) found the CHO-K1 cells gave the best results in cytotoxin detection. The cytotoxin caused the formation of large, rounded polymorphic and elongated cells, along with a weakened growth rate (Schulze *et al* 1996).

In vivo tests by Okuda *et al* (1997) were carried out using a suckling mouse model. The group studied diarrheagenicity in mice after intragastrically introducing CDT-containing bacterial culture supernatant. They reported induction of loose and/or watery faeces at a higher rate than in CDT- culture supernatant treated mice, after 24 hours exposure. They also detected tissue

damage (due to necrosis and reparative hyperplasia) in the descending colon (Okuda *et al* 1997).

A study on the effect of CDT on cultured cells was reported by Aragon *et al* (1997). Here, CDT induced a progressive accumulation of F-actin assemblies to form structures resembling actin stress fibres. Accumulation was accompanied by an apparent blockage of cell division (Aragon *et al* 1997).

Another mechanism of cell cycle arrest induced by CDT was reported by Lara-Tejero and Galan (2000), where transient expression of the CdtB sub-unit in cultured Cos 1 cells caused marked chromatin disruption, and microinjection of the sub-unit induced cytoplasmic distention and cell cycle arrest. The proposed features of the sub-unit were type I deoxyribonuclease-like (Lara-Tejero and Galan 2000).

1.4.4. Campylobacter spp Haemolysins.

A pathogenic action induced by *Campylobacter* infection is the infiltration and destruction of gut epithelial cells, resulting in bloody diarrhoea. This suggests a cytolytic process is involved. A study by Hossain *et al* (1993) detected the presence of a number of haemolysins produced by *C.jejuni* and secreted in the culture medium. These proteins were found to lyse erythrocytes from rabbits and other species. The group used freshly collected rabbit red blood cells along with human, mouse, rat and other species' erythrocytes in a haemolysis assay for the toxins. They found that the toxins were more prominent in bacterial cultures grown for 60 hours at 42°C. The secretion of the soluble haemolysins resulted in the highest amount of lysis in rabbit

erythrocytes and the least amount in chicken cells. This may be due to variations in molecular compositions of erythrocyte membranes in different species. The haemolysins were found to be heat-stable at 60°C, heat-labile at 100°C and were not detected in the log phase of bacterial cultures. The group concluded that the toxins used a two step mechanism of action, where a temperature-independent binding reaction preceded a temperature-dependent lytic stage. They also suggested a multi-hit lytic system, where more than one haemolysin molecule is required for the lysis of a single erythrocyte (Hossain *et al* 1993).

A study by Akan *et al* (2000) examined 20 strains of *C. jejuni* and *C. coli* taken from broiler chickens on rabbit, sheep and chicken erythrocytes cultured on agarose or in microplate wells. As in the above study, rabbit erythrocytes proved the most sensitive to haemolytic activity, which was best detected at 42°C under aerobic conditions, with 80 % of *C. jejuni* isolates showing positive haemolytic activity (Akan *et al* 2000).

1.4.5. Campylobacter spp cholera-like toxin (CLT).

In 1992, Collins *et al* detected CLT from a clinical sample of *C.jejuni*, and the toxin was investigated in an effort to determine structure and other molecular mechanisms of action that produce alterations of intestinal secretion. A number of methods were tried by this group for screening culture filtrates, including GM1 ELISA plates and cholera anti-toxin standard coated ELISA plates. The former is of interest because GM1 is the known gut receptor for the cholera toxin (Griffiths *et al* 1986; Lencer *et al* 1999a; Hirst *et al* 1998).

These ELISA techniques showed a positive result for the presence of a cross-reactive protein, with the antigen-coated ELISA producing the best detection results. A second approach used was the CHO assay (defined by Guerrant *et al* 1974; 1977), which relies on the ability of enterotoxins to produce a characteristic change in CHO cell morphology *e.g* elongation (Guerrant *et al* 1974). In the Collins study, concentrated culture filtrates of four isolates (from a total of eight) induced CHO cell elongation in over 50 % of cells. This was regarded as indicative of enterotoxigenic activity. The final assay used by Collins *et al* (1992), was the rat ileal loop assay. This relies on the ability of toxins to inhibit re-absorption of fluid introduced into the ileal lumen. After concentrate inoculation into ligated rat ileal loops, no induction of absorption alterations were detected (Collins *et al* 1992). Collins concluded from these studies that *C. jejuni* CLT is produced in small, possibly clinically insignificant quantities.

The molecular structure of the protein has not been firmly established and a number of equivocal reports have been made on the detection of CLT. Klipstein *et al* (1984) reported a *C. jejuni* produced CLT that bound to GM1 and possessed a subunit structure similar to CT (Klipstein *et al* 1984). McCardell *et al* (1984) reported an immuno-affinity purified CLT which had no subunit structure and a molecular weight of approximately 70 kDa (McCardell *et al* 1984). Goossens *et al* (1985) reported cholera-like enterotoxin production from 25 *C. jejuni* clinical isolates, with activity producing morphological changes in CHO cells being neutralised by antisera against cholera toxin and *E. coli* heat-labile enterotoxin, thus demonstrating a close immunological relation to the two toxins. Daikoku *et al* (1990) reported a

polypeptide observed on SDS-PAGE at 68 kDa after affinity purification using an anti-CT IgG column, but also detected two bands (68 and 54kDa) after affinity purification using a ganglioside (GM1) column. The group surmised that the CLT holotoxin consisted of at least two polypeptides of 68 and 54 kDa and that the large sub-unit should have antigenic sites similar to those of CT and LT. Both sub-units are responsible for binding to ganglioside receptors on the cell surface (Daitoku *et al* 1990).

In conclusion, there have been a number of toxins identified or detected, but to date, the exact roles that most of these toxins play in infections by *Campylobacter* are unknown. The majority of the toxins reported require further purification and characterisation at the molecular level in order to understand fully their potential role in the pathogenesis of *Campylobacter* enteritis.

1.4.6. Neurodegeneration As A Consequence Of Camplyobacter Infection.

A recent discovery in the context of *Campylobacter* infection research is the relationship between *Campylobacter* infection and the incidence of certain neurological disorders. The correspondence of endotoxins in the cerebrospinal fluid with the appearance of neurological symptoms, such as seizures and comas has resulted in increased research into the neurological results of bacterial infections that primarily target the gastrointestinal system. However, at present the pathogenic mechanism by which the endotoxins disturb neuronal function is unclear.

An example of *Campylobacter* infection resulting in neurological defects is the reported association with the acute monophasic polyneuropathy, Guillan-Barre Syndrome (GBS) (Allos 2001). GBS is an acute disease of the peripheral nerves that is characterised by the stripping away of myelin in a segmented fashion, resulting in rapid ascending paralysis which can lead to respiratory muscle compromise and death. Since the eradication of poliomyelitis in the western hemisphere, it has become the most common cause of acute neuromuscular paralysis in the developed world (Allos 1998). Patients show cranial nerve dysfunction and experience sensory abnormalities, especially in the lower extremities. It has been confirmed that between 50 and 75% of cases are preceded by a recognised acute infectious illness, although the mechanism by which infections may trigger GBS is unknown (Allos 1998).

Campylobacter infection was first reported as a potential trigger in 1982, when a patient developed GBS with irreversible neurological damage 2 weeks after a gastrointestinal illness caused by *Campylobacter* (Rhodes and Tattersfield 1982). From further reports it was learned that male cases outnumbered female cases 3 to 1 and that all *C.jejuni*-associated cases were more severe and more likely to produce axonal injury (Mishu and Baser 1993; Constant *et al* 1983; Molnar *et al* 1982).

In studies investigating the presence of *Campylobacter*, 8 to 50% of GBS patients had *Campylobacter* cultured from their stools soon after onset of neurological symptoms (Allos 1998). Along with serological studies, where one third of GBS patients showed serological evidence of antecendent *Campylobacter* infection, at least 30 to 40% of GBS patients had been

infected with *Campylobacter* in the 10-day to 2 week period before the onset of their neurological symptoms. Because of the lag phase between infection and neurological symptoms, these figures are more likely to be underestimates (Allos, 1998).

The association of infection with the presence of antibodies against gangliosides in patients with GBS and MFS (Miller Fisher Syndrome, a GBS variant) suggests that the antibodies are induced by the infection, since ganglioside-like epitopes are found in LPS of *C.jejuni* isolates from GBS patients (Jacobs *et al* 1997). Gangliosides are surface components of many cells, including peripheral nerves. They are glycolipids that contain one or more sialic acid residues (Allos 1998). Patients with GBS frequently have antibodies to gangliosides, such as GM1, GD1a, GD1b, GT1b, asialo-GM1, and LM1 (Allos 1998, Ilyas *et al* 1988; Willison *et al* 1993). The most common autoantibodies in GBS are against GM1 (Nobile-Orazio *et al* 1992), where as MFS is more commonly associated with antibodies against GQ1b (Chiba *et al* 1992).

Campylobacter spp possess outer membrane LPS, which are complex glycolipids but, unlike most other gram-negative bacteria, the LPS of *Campylobacter* resembles human glycoconjugates because they contain sialic acid (Moran *et al* 1991). Recent studies have indicated that the antigenic similarity between some regions of the bacterial LPS and human gangliosides may result in ganglioside-mimicry associated with the presence of the autoantibodies observed in GBS patients (Aspinall *et al* 1994). The cross-reactivity of GBS patient anti-GM1 IgG antibodies with GBS-associated *C. jejuni* supplies more evidence to support the hypothesis. From recent studies

it has been suggested that most of the anti-LPS IgG activity in GBS and MFS patients is due to antibodies against ganglioside-like epitopes in LPS, indicating that these epitopes are immunodominant in a humoral immune response against LPS (Jacobs *et al* 1997).

Reports of antibody production against peripheral nerve proteins, which were induced by immunisation of animals with *C. jejuni* proteins, also suggests that *C. jejuni* infection may induce cross-reactive antibodies against bacterial structures other than LPS (Fujimoto and Amako 1990). Further bacterial protein involvement in the immune response against LPS may include the induction of T cell activation by the production of a protein that binds to LPS, serving as a carrier protein for T cell help (Willison and Kennedy 1993). These proteins may be the enterotoxins produced by certain *C. jejuni* strains which can specifically bind to gangliosides. Therefore, if a strain of *C. jejuni* expresses both the enterotoxin and the ganglioside-like LPS, thus providing an epitope for the T cells, these will become activated and may skew B cells against LPS to switch to IgG1 and IgG3 production. These specific antibody subclasses have been shown to be induced by infection and high titres of these antibodies are characteristic for T cell dependent immune responses (Jacobs *et al* 1997).

The above evidence of a *Campylobacter* endotoxin resulting in activation of an auto-immune response may not be the only pathological mechanism of bacterial induction of these neurological disorders. Therefore investigation into the possible role of secreted toxins may help to improve understanding of the implications of *Campylobacter* infection and the onset of GBS/MFS.

1.5. Project Aims

In order to achieve a better level of understanding towards the role of toxins in *Campylobacter* infection the aims of this thesis were:

1. To determine the optimal toxin producing *Campylobacter jejuni* strain from the three strains made available (namely; 11351, 11322, 11168).

2. To optimise bacterial culture conditions, so that bacterial cells produced the highest amount of toxins possible, by determination of cytotoxic effects of bacterial culture extracts towards mammalian cells *in vitro*.

3. To initially characterise culture extracts with regards to their effects on cultured mammalian cells, specifically on cytoskeletal networks and associated cellular morphological changes as a result of exposure to sub-cytotoxic levels.

4. Purification of a novel *Campylobacter jejuni* toxin to the point of N-terminal sequencing, along with characterisation at the molecular level, in order to better understand its mechanism of action.

CHAPTER 2

2.1 Materials:

All chemicals used for buffers and media were of the highest quality and purchased from Sigma-Aldrich Ltd (Poole, UK) or BDH (Leicester, UK). All tissue culture plastic-ware was sterile and of the highest quality available from SLS (Nottingham, UK).

2.1.1 Reagents:

2.1.1.1 Bacterial Culture Extracts

All material obtained from bacterial cultures was supplied by Miss Kathryn Holmes, Microbiology section (Department of Life Sciences, Nottingham Trent University). Three *Campylobacter* strains were used; 11168, 11322 and 11351. These were grown in a number of culture environments in order to optimise toxin production. Bacterial cells were grown either on blood agar plates or in culture media flasks. Broths included either brain heart infusion (with [BHI+] or without [BHI-] yeast extracts) or brucella medium, with or without supplements (consisting of 0.9mM $FeSO_4$, 1.3mM NaS_2O_5 and 2.3mM sodium pyruvate). The cells were grown for a number of days in a microaerobic atmosphere, as indicated in the results sections and extracts were tested for cytotoxicity to establish the optimal growth period. In the case of flask cultures, the bacterial cells were collected by centrifugation and the supernatant samples were filtered through 0.2 μm pore filters to remove any remaining bacteria. All samples were stored at -20°C until required. In the case of agar plate cultures, the bacterial cells were scraped from the plates and washed in sterile PBS [137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4]. The cells were collected through centrifugation (5000 g) and 3 sterile PBS washes, finally re-suspended in 5ml sterile PBS.

2.1.1.2 Western Blotting Material

Immobilon P^{SQ} PVDF transfer blot (membrane pore size 0.1μm) and Optitran BA-S 83 reinforced nitrocellulose membrane filter (pore size 0.2 μm) were purchased from Schleicher and Schuell (Dassell, Germany). Whatman Number 1 Filter paper was purchased from Whatman (Kent, UK).

2.1.1.3 Primary Antibodies

Anti-NF-H (phosphorylation independent; clone number N52), anti-acetylated tubulin (clone number 6-11B-1) and anti-α-tubulin (B-5-1-2) were all bought from Sigma-Aldrich (Poole, UK).

Anti-Cholera Toxin β sub-unit (clone number 3D11) was purchased from Biodesign International (Maine, USA) and clone number 2/63 was supplied from Chemicon International Ltd (California, USA). Anti-Cholera toxin β sub-unit polyclonal antiserum was purchased from Sigma-Aldrich (Poole, UK).

2.2. Methods :

2.2.1 Tissue Culture Techniques

2.2.1.1. Mammalian cell lines

Mouse N2a neuroblastoma, rat C6 glioma, Chinese Hamster Ovary (CHO) and human umbilical cord endothelial cells (ECV 304) were obtained from the European Tissue Culture Collection (ETCC).

2.2.1.2. Growth and maintenance of mammalian cell lines

All cell lines were grown at 37°C in a Jouan IG150 incubator with a humidified atmosphere of 5 % (v/v) CO_2/95% (v/v) air. Cells were cultured in sterile growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) containing sodium bicarbonate and high glucose, and supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, penicillin G (100 units/ml) and streptomycin (100μg/ml).

The cells were maintained in the logarithmic growth phase and passaged at approximately 80% confluence. In the case of N2a and C6 cells, the monolayer of cells was detached from the flask surface by gently jetting growth medium over the monolayer and knocking the cells off with a Gilson pipette, otherwise cells were treated with 0.25% Trypsin/PBS for 5 minutes, cell detachment was stopped by the addition of equal volumes of complete growth medium. The cell suspension was then centrifuged for 5 minutes at

1,000 rpm in an MSE Centaur 2 benchtop centrifuge and resuspended in 1 ml fresh growth medium. One quarter of the re-suspended cells were then inoculated into a sterile T25 vented flask containing 10 ml fresh growth medium. To ensure sterility, all procedures were carried out in a Class II (Gelaire BSB 4) laminar flow hood at all times.

2.2.1.3. Cryopreservation of cell lines

As for normal cell passage the cells were taken off the flask surface and spun down for 5 minutes at 1,000 rpm. The supernatant was discarded and the cells were resuspended in 1 ml cell freezing medium [65 % (v/v) DMEM containing L-glutamine, penicillin G and streptomycin as before, and 25% (v/v) foetal bovine serum and 10 % (v/v) DMSO]. The cell suspension was then placed into a cryovial, lagged in tissue paper and incubated overnight at -70°C. The vial was then transferred to liquid nitrogen for permanent storage.

2.2.1.4. Restoration of mammalian cell lines stored in liquid nitrogen

The cryovial of cells was removed from the liquid nitrogen container and thawed quickly by incubation in a water bath at 37°C. The thawed cell suspension was immediately diluted in 10 ml fresh growth medium and centrifuged for 5 minutes at 1,000 rpm. The cells were then resuspended in 1 ml fresh growth medium and then inoculated into a sterile T25 vented flask containing 10 ml fresh growth medium (entire suspension volume). The cells were left in the incubator for 24 hours to allow recovery and the formation of a

monolayer, after which time the growth medium was replaced. As in normal growth and maintenance, the cells were passaged at approximately 80 % confluence. After a single passage the cells were deemed fit for experimentation.

2.2.1.5. Preparation of mammalian cell lines for bacterial toxin cytotoxicity assays

N2a and C6 cell lines were plated out at a density of 50,000 cells/ml, whereas the CHO and ECV 304 cell lines were plated out at a density of 25,000 cells/ml in sterile 24 well culture plates. Cell numbers were calculated using the trypan blue exclusion assay for determining cell viability (see section 2.2.1.6). Each well was seeded with 500 μl of cell suspension and incubated under standard conditions (given in section 2.2.1.2) for 24 hours to allow cell recovery and the formation of a monolayer. At this stage the cells were ready for exposure to bacterial culture extracts.

2.2.1.6. Trypan blue exclusion assay

Cells were taken at the resuspended stage during passage. 50 µl of cell suspension were added to 50 µl trypan blue (supplied ready to use) and mixed thoroughly. 10 µl of the resultant suspension were applied on to a haemocytometer chamber for counting (Neubauer haemocytometer 1/400 mm^2 (B.S.748)). Cell viability was assessed by the cell's ability to exclude trypan blue dye, and calculated using the following equation:

$$\text{Cell Viability} = \frac{\text{Live cells}}{\text{total number of cells}} \times 100$$

2.2.1.7. Preparation of bacterial culture extracts

Bacterial culture extracts from broth cultures were supplied as two separate states. The broth culture supernatants required no further preparation before their addition to cultured mammalian cells. Bacterial cells collected by centrifugation and suspended in sterile PBS were diluted to a fixed biomass concentration by adjusting resuspension absorbance at 650nm to a value of 1.2, then bath sonicated in a Kerry Pulsatron Sonics water bath containing water and ice (for suspension cooling) for 15 minutes. The cell suspension was then centrifuged for 30 minutes at 10,000 G. The supernatant was then taken off, requiring no further preparation before exposure to mammalian cell cultures. The remaining cell pellets were resuspended in PBS and stored at -20°C.

2.2.1.8. Exposure of mammalian cells to bacterial extracts

Mammalian cells were plated out and given a 24 hour recovery time as described in section 2.2.1.5. The growth medium was then replaced and previously prepared bacterial extracts were added. Initially, the extracts were added as a serial dilution to assess the minimal cytotoxic concentration, from 10 % down to 0.0625 % (v/v). In further experiments, values above the minimal cytotoxic concentration were added to cells. The mammalian cells were exposed to bacterial extracts for 48 hours, after which cell growth was assessed by the reduction of methyl blue tetrazolium (MTT) [see following section]. Control cells were treated with, in the case of broth cultures, fresh broth corresponding to the broth used in the culture flask, or sterile PBS for cell sonicates.

2.2.1.9. Measurement of cell viability by the reduction of MTT

After 48 hours exposure to bacterial extracts, 50 µl MTT (5 mg/ml in phosphate buffered saline) were added to each well and the cells were incubated for 45 minutes under standard conditions. The growth medium was then carefully removed without disturbing the cells and 1 ml dimethyl sulfoxide (DMSO) was added to each well. The plates were then gently agitated for 5 minutes and the absorbances read in a spectrophotometer set at a wavelength of 570 nm.

2.2.1.10. Heat and trypsin treatment of bacterial extracts

In order to determine whether any cytotoxins present in the extracts were heat resistant, bacterial culture extracts were incubated in a water bath at 100°C for 10 minutes. The samples were allowed to cool for 30 minutes before exposure to mammalian cell cultures in the above mentioned cytotoxicity assays. The possibility of the presence of protein cytotoxins was further studied by incubating the bacterial extracts with 0.1 % (w/v) trypsin at room temperature for 30 minutes. The trypsin was inactivated by heating the extracts at 100°C for 10 minutes. The extracts were allowed to cool for 30 minutes before treatment of mammalian cell cultures.

2.2.1.11. Induction of cell differentiation in the presence or absence of bacterial extracts

N2a and C6 cells were plated out at an initial density of 50,000 cells/ml, and CHO and ECV 304 cells were plated out at a density of 25,000 cells/ml in to sterile 24 well culture plates. The cells were grown for a 24 hours recovery period in growth medium prior to the induction of cell differentiation in the presence or absence of non-cytotoxic concentrations of bacterial culture extracts. N2a cell differentiation was induced by serum removal and the addition of 0.3 mM dibutyryl cyclic AMP in serum-free medium [DMEM containing L-glutamine, penicillin G and streptomycin at the concentrations mentioned above] (Shea *et al* 1991; Flaskos *et al* 1998; De Girolamo *et al* 2000; Fowler *et al* 2001). C6 cell differentiation was induced by serum

removal and the addition of 2 mM sodium butyrate in serum-free medium (Hargreaves *et al.*, 1989; Flaskos *et al.*, 1998). CHO and ECV 304 cell differentiation were induced by serum removal alone. Simultaneously, after the induction of cell differentiation, cells were exposed to 1% (v/v) bacterial extracts (previously assessed by MTT reduction assays as a non-cytotoxic concentration) for 24 hours.

2.2.1.12. Fixing and staining of differentiated cells

After a 24 hour exposure period, serum free medium was discarded carefully so as not to disturb the cells, which were then fixed at -20°C for a minimum of 30 minutes in a fixing solution containing 90% (v/v) methanol in Tris buffered saline [50 mM Tris, 200 mM NaCl; pH 7.4]. The fixed cells were then stained for 1 minute at room temperature with Coomassie brilliant blue dye (1.25 g Coomassie blue-R250, 10 % (v/v) glacial acetic acid, 40 % (v/v) methanol, 50 % (v/v) distilled water). The cells were then washed with distilled water and allowed to air-dry, after which they were ready to be viewed using an inverted light microscope.

2.2.1.13. Morphological assessment of differentiated cells

The total number of cells and the proportion of round versus flat cells were determined in each of 5 random fields per well. Neurites (cellular process produced on cell differentiation in neuroblastoma and glial cells) were also counted. In the case of N2a cells these were regarded as axon-like when

they were greater than 2 cell body diameters in length with an extension foot (Keilbaugh *et al* 1991; De Girolamo *et al* 2000; Fowler *et al* 2001). In the case of C6 cells the number of cellular extensions, all of which were greater than 2 body diameters in length was recorded (Flaskos *et al* 1998). Cell shape was divided into 2 different categories; round and flat cells. "Round" cells were typically spherical in shape and had a smooth morphology, whereas "flat" cells had a more flattened and irregular morphology. This helped to determine if the bacterial extracts had a cytotoxic effect on the cells causing the cells to round up. In the case of the CHO and ECV 304 cells only cell morphology was recorded, as neither cell line produced cellular extensions. Therefore, cell shape was divided into "round", "flat" and "elongated". This allowed the detection of putative cyto-lethal distending or cyto-lethal rounding toxins in the bacterial extracts (Schulze *et al* 1998; Macfarland *et al* 1992).

2.2.2 Gel electrophoresis and Western blotting analysis

2.2.2.1. Preparation of cell extracts for gel electrophoresis and Western blotting

Cells were seeded out at the same cell density as for culture well experiments (section 2.2.1.5) in to sterile T25 vented tissue culture flasks containing 10 ml growth medium. The cells were allowed to grow for 24 hours before differentiation was induced (as described earlier) in the presence and absence of bacterial culture extracts. After a 24 hour exposure time, the cell monolayer was rinsed gently with ice cold DMEM, taking care not to dislodge the cells. The cells were then solubilised in 1 ml of 0.1 % (w/v) SDS in PBS, boiled for 1 minute and placed in an Eppendorf tube. The cell suspension was then vortex-mixed for 1 minute and 50 μl samples were taken for protein estimation as described in section 2.2.2.3). Once the protein content had been estimated, 100 μl of cell suspension were added to 100 μl of double-strength electrophoresis sample buffer [0.1 M Tris-HCl, pH 6.8, 50 mM DTT, 3 % (w/v) sodium dodecyl sulphate (SDS), 20 % (w/v) glycerol, 0.1 % (w/v) bromophenol blue] and denatured by boiling for 10 minutes, and then centrifuged for 1 minute at 10,000 g. Denatured proteins were then stored at -20°C until required.

2.2.2.2. Protein Estimation

Protein estimation was performed using the method described by Lowry *et al* (1951). To produce a calibration curve, a set of BSA standards were prepared ranging from 0 μg to 100 μg of protein. The cell extracts and BSA standards were treated identically. To each Eppendorf tube, 1 ml of working reagent [2% (w/v) sodium carbonate in 0.1M sodium hydroxide containing 1% (w/v) sodium potassium tartrate] was added. The suspensions were vortex-mixed and incubated at room temperature for 15 minutes. A volume of 100 μl of Folin and Ciocalteau phenol reagent (diluted 1:1 (v/v) with distilled water) was then added to each suspension. The Eppendorf tubes were again vortex-mixed, then incubated at room temperature for 30 minutes. After incubation, absorbances were read in a spectrophotometer set at a wavelength of 690 nm.

2.2.2.3. Separation of proteins in cell extracts by gel electrophoresis

One-dimensional SDS-PAGE was carried out using a 7.5% (w/v), or a 10% (w/v) polyacrylamide resolving gel as indicated in the results chapters. These were overlaid with a 4 % polyacrylamide stacking gel (Laemmli, 1970). A BIORAD MiniProtean® II electrophoresis cell kit was used for protein separation. Gel plates were washed with absolute ethanol prior to assembly. Once the plates had been assembled and clamped to the gel casting stand, the resolving gel mixture was prepared as indicated below. Gel polymerisation was induced by the addition of 100 μl of 10 % (w/v) ammonium

persulphate (APS) and 10μl of N, N, N, N-tetramethyl-ethylenediamine (TEMED). When the resolving gel mixture had been poured, a layer of water-saturated butan-2-ol was gently layered above the polymerising mixture to prevent any gel shrinkage due to exposure to air. Once the resolving gel had polymerised, the layer of butan-2-ol was washed off using SDS-PAGE running buffer [25.6 mM Tris-base, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3]. The resolving gel was then overlaid with a 4 % (w/v) polyacrylamide stacking gel (prepared as indicated below) which was induced to polymerise by the addition of 50 μl APS and 20 μl TEMED. Once the stacking gel had been poured and polymerisation induced, a 10-toothed comb was placed in the gel mixture.

Resolving gel (10 ml):

Reagent	Volume (7.5 % gel)	Volume (10 % gel)
40 % (w/v) acrylamide (Biorad)	1.88 ml	2.5 ml
1.5 M Tris-HCl, pH 8.8	2.50 ml	2.5 ml
10 % (w/v) SDS	100 μl	100 μl
Distilled Water	5.52 ml	4.9 ml

4% Stacking gel (10 ml):

Reagent	Volume
40 % (w/v) acrylamide	1.00 ml
0.5 M Tris-HCl, pH 6.8	2.50 ml
10 % (w/v) SDS	100 µl
Distilled water	6.40 ml

Once the stacking gel had polymerised, the comb was removed and the gel assemblies were removed from the casting stand and loaded onto the inner cooling core of the electrophoresis tank and then into the tank. The gels were totally submerged in SDS-PAGE running buffer and air bubbles were excluded. Before cell extract loading was carried out, the previously prepared extract samples and molecular weight markers (SDS-PAGE pre-stained molecular weight standards ranging from 33,500 to 205,000 kDa) were boiled for 10 minutes and vortex-mixed. The samples were then loaded into the wells formed in the stacking gel at volumes previously calculated by protein estimation assays to ensure equal protein loading. The gels were run at 40 mA per gel, until the dye front had run to the end of the gel, using a BIORAD Power Pac 300. The gels were then either stained with Coomassie brilliant blue or Silver staining reagent to visualise protein bands and molecular weight markers. Alternatively, the proteins were electrophoretically transferred on to nitrocellulose membrane filters for Western blotting analysis.

2.2.2.4. Staining of electrophoresed proteins in polyacrylamide gels

2.2.2.4.1. Coomassie brilliant blue stain

The polyacrylamide gel was removed from the gel plates and placed into a glass tray containing 1.25 % (w/v) Coomassie blue-250 in a solution of 25 % (v/v) ethanol and 10 % (v/v) glacial acetic acid. The gel was placed on an orbital shaker, allowing the uptake of Coomassie blue (Orbital shaker SO1, Stuart Scientific, Loughborough, UK). After 24 hours incubation at room temperature, the background colour was removed by placing the gel in a destaining solution [25 % (v/v) ethanol, 10 % (v/v) glacial acetic acid] on the orbital shaker. After several hours in destain, protein bands on the gel were viewed on a light box.

2.2.2.4.2 Staining of proteins with the BIORAD Silver stain

The polyacrylamide gels were placed in a fixative solution [40% (v/v) methanol, 10% (v/v) glacial acetic acid] overnight at room temperature. The gels were then incubated in the presence of an oxidiser [10% (v/v) oxidiser in distilled water] for 10 minutes at room temperature on an orbital shaker. The oxidiser was removed and the gel was rinsed by three 10 minute washes in distilled water, or until all yellow colour had been removed from the gel. Silver reagent [10% (v/v) silver reagent in distilled water] was added to the gels for a period of 30 minutes. The silver reagent was then discarded and the gels were washed in distilled water for 2 minutes. Developer [8g developer in 250

ml distilled water] was then added and incubated for approximately 30 seconds or until the solution became cloudy. N.B. All solutions supplied as a kit from BIORAD. This was discarded and replaced with fresh developer solution. This procedure was repeated until protein bands were revealed. The developing reaction was stopped by flooding the gels with stop solution [5 % (v/v) glacial acetic acid in distilled water] (Merril *et al* 1981). The stained gels were viewed on a light box.

2.2.2.5 Western blotting of cell extracts separated by SDS-PAGE.

The separated proteins were transferred electrophoretically on to nitrocellulose membrane filters submerged in electroblotting buffer [39 mM glycine, 48 mM Tris-base, 0.0375 % (w/v) SDS, 20 % (v/v) methanol] (Towbin *et al.*, 1979). The protein transfer was performed using a BIORAD Trans-Blot electrophoretic transfer cell at 30 volts overnight at room temperature. The resultant western blots were, either dried between two pieces of clean filter paper and stored at room temperature, or probed with the relevant antibody solutions, as discussed in section 2.2.2.7.

2.2.2.6. Staining of Western blots using copper phthalocyanine 3, 4', 4'', 4''' tetrasulphonic acid (tetrasodium salt)

The efficiency of the protein transfer was assessed by staining the Western blot with copper phthalocyanine staining solution [0.05% (w/v) copper phthalocyanine 3, 4', 4'', 4''' tetrasulphonic acid (tetrasodium salt) in 12 mM

hydrochloric acid] for approximately 2 minutes. Destaining of blots was achieved by submerging them in a solution of 12 mM sodium hydroxide (Bickar and Reid, 1992). After destaining, blots were neutralised by incubation in TBS.

2.2.2.7. Immunoprobing of Western blots

Western blots were blocked at room temperature in 3 % (w/v) bovine serum albumin in TBS (BSA/TBS) for at least 1 hour before probing with primary antibodies.

Primary antibodies used to probe Western blots included:

1. Anti-actin rabbit polyclonal antibody (Sigma-Aldrich Ltd (Poole, UK)), diluted 1:100 in BSA/TBS.
2. Anti-acetylated tubulin (clone 611B1) and anti-tubulin (clone B512) mouse monoclonal antibodies, both diluted 1:2,000 in BSA/TBS.
3. Anti-NF-H (phosphorylation independent) (clone N52) mouse monoclonal antibodies, which was diluted 1:500 in BSA/TBS.
4. Anti-cholera toxin B sub-unit rabbit polyclonal antibody, diluted 1:5,000 in BSA/TBS.
5. Anti-cholera toxin B sub-unit (clone 2/63 from Chemicon International Inc; clone 3D11 from Biodesign International) mouse monoclonal antibody, diluted 1:20,000 in BSA/TBS (or in BSA/PBS when western blots were developed using enhanced chemiluminescence techniques, described in section 2.2.2.8).

Western blots were incubated, submerged in antibody solutions at 4°C overnight with shaking. The probed blots were then washed for three 20

minute periods in TBS containing 0.1% (v/v) Tween-20 (TBS/Tween) and then incubated for 2 hours at room temperature in either alkaline phosphatase-conjugated goat anti-mouse IgG (for blots probed with mouse primary antibodies), or alkaline phosphatase-conjugated goat anti-rabbit IgG (for blots probed with rabbit primary antibodies). Both secondary antibodies, which were purchased from Dako Ltd (Cambridge, UK), were diluted 1:1,000 in BSA/TBS. After three more 20 minute washes in TBS/Tween, followed by a brief wash in TBS, the blots were allowed to equilibrate in substrate buffer [0.75 M Tris-base, pH 9.5] for 5 minutes. Developer [33 μl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) [50 mg/ml in dimethyl formamide (DMF)] and 44 μl nitro blue tetrazolium (NBT) [75 mg/ml in 70 % (v/v) DMF] added to 20 ml substrate buffer] was then added to the blots. Blot development was carried out through incubation of the probed blots in developing solution at room temperature in the dark. The reaction was stopped by extensive washing with distilled water. The blots were then dried between 2 pieces of clean filter paper.

2.2.2.8 Enhanced chemiluminescence (ECL) development of immunoprobed Western blots

After overnight incubation in primary antibody solution the western blots were washed in PBS containing 0.1 % (v/v) Tween-20 (PBS/Tween) for three 20 minute periods. The blots were then incubated for 2 hours at room temperature submerged in horse-radish peroxidase-conjugated goat anti-mouse IgG, diluted 1/1,000 in BSA/PBS (Dako Ltd, Cambridge, UK). The

blots were then washed in PBS/Tween for three periods of 20 minutes at room temperature before being submerged in ECL plus solution (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK). [Solution A: ECL Plus substrate solution containing Tris buffer; Solution B: stock Acridan solution in dioxane and ethanol. Solution B was diluted 40:1 in Solution A [to a final volume of 0.1 ml/cm^2], which had been allowed to equilibrate to room temperature and agitated for 5 minutes. Excess ECL plus solution was removed from the membranes by dabbing the blot edge dry on clean tissue paper. The blots were then placed protein side up and wrapped in Saran wrap, with all air bubbles removed. The wrapped blots were then placed in an X-ray cassette. The rest of the development was carried out in a dark room under a safety light. A sheet of Hyperfilm^{TM}ECL autoradiography film was cut to the size of the blots and the X-ray cassette was opened allowing the film to be placed directly on top of the blots. The cassette was closed and pressure was placed on the top for a 15 second exposure period. The film was then removed from the cassette and placed in developing solution [GBX Developer/Replenisher purchased from Sigma] until protein bands were visualised. The film was then briefly washed in water for a period of 2 minutes, followed by incubation in fixing solution [GBX Fixing/Replenishing solution, purchased from Sigma] for a further 5 minutes, or until the film changed from white to a grey colour. Finally, the film was washed in water for 1 minute and hung up to air dry.

2.2.3 Indirect immunofluorescence assay of mammalian cell lines induced to differentiate in the presence or absence of bacterial extracts.

2.2.3.1 Preparation of mammalian cells for immunofluorescence and confocal laser fluorescence microscopy

Cell lines were inoculated at a final concentration of 1 million cells in 20 ml sterile growth medium, consisting of DMEM containing sodium bicarbonate and high glucose and supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, penicillin G (100 units/ml) and streptomycin (100 μg/ml). The cell suspension was added to a sterile Petri dish containing a glass slide (specifically treated for cell attachment with 2% Decon, acetone and 3-aminopropyltriethooxysilicone, before drying overnight). The cells were then given the usual 24 hour recovery period under standard *in vitro* culture conditions (see section 2.2.1.2) to form a monolayer, before serum removal and induction of cell differentiation (see section 2.2.1.11). Immediately after differentiation had been induced the cells were exposed to 1 % (v/v) bacterial culture extract/control solution for a 24 hour period.

2.2.3.2 Immunofluorescence probing of cells differentiated in the presence or absence of bacterial extracts

After 24 hours exposure to bacterial culture extracts/control solution the growth medium was discarded and the cells were submerged in fixing solution, containing 90 % (v/v) methanol in TBS at -20°C for 30 minutes. The

cells were then rinsed in TBS for 5 minutes at room temperature. The cells were then submerged in detergent solution [0.5 % (v/v) Triton-X 100 in Tris-buffered saline] for 10 minutes on a shaker. This was followed by three 20 minute periods of washing in TBS. The cells were then blocked in 3 % (w/v) BSA/TBS solution for 1 hour at room temperature. Before addition of primary antibodies could be carried out, the specific areas (wells) containing cells had to be circled with a hydrophobic pen (purchased from Dako Ltd, Cambridge, UK) to avoid any primary antibody cross-contamination. Primary antibodies added included: anti-actin rabbit polyclonal antibody (diluted 1:100 in 3 % (w/v) BSA/TBS) and anti-tubulin mouse monoclonal antibody (diluted 1:1,000 in BSA/TBS. The cells were incubated in primary antibody solution overnight at 4°C. After overnight probing, the primary antibody solutions were carefully discarded and the "wells" were washed individually in TBS for three 20 minute periods. The "wells" were then re-circled with the hydrophobic pen before the secondary antibody solutions were applied. In the case of the anti-actin primary antibody, the secondary antibody used was fluorescein isothiocyanate isomer 1 (FITC)-conjugated sheep anti-rabbit IgG (purchased from Dako Ltd, Cambridge, UK). For the case of the anti-tubulin monoclonal antibody, the secondary antibody used was FITC-conjugated goat anti-mouse IgG. Both secondary antibodies were diluted 1:40 in BSA/TBS. Due to the light sensitivity of the fluorescein probe, the Petri dishes containing the cells were covered in tin foil and incubated for 2 hours at room temperature. The cells were then washed in TBS/Tween for two 10 minute periods followed by one 10 minute period in TBS. The slides were then dried with tissue, around the ringed specimen areas without causing any damage to the cells. One drop of

Vectoshield mounting medium anti-fading agent (Vector Laboratories Ltd, Peterborough, UK) was added to each "well" and cover slips (of 0 grade thickness) were added. The cover slips were sealed around their edges with nail varnish. The cells were then visualised using a Leica TCS NT confocal microscope and pictures were scanned using a confocal laser scanner.

2.2.4 Partial purification of cholera-like protein toxin from bacterial culture extracts

2.2.4.1 Materials

Buffer A: 50mM (w/v) Tris-HCl, 1mM (w/v) ethylene diamine tetra acetic acid (EDTA) 1mM 2-mercaptoethanol, 10mM phenyl methyl sulphonyl fluoride (PMSF), pH 7.8.

Dialysis tubing: Cellulose membrane dialysis tubing, retaining all molecules of molecular weight 12,000 and greater; purchased from Sigma Aldrich Company Ltd (Poole, UK)

2.2.4.2 Preparation of bacterial culture extracts for protein separation by ion exchange chromatography

Initial treatment of bacterial culture extracts involved protein precipitation. This involved the gradual addition of 80 % (w/v) ammonium sulphate to culture extract suspensions at 4°C with gentle stirring. The suspension was then incubated for a peroid of 4 hours at 4°C with gentle stirring. The suspension was then centrifuged at 10,000 rpm in an MSE Centaur 2

benchtop centrifuge for 15 minutes at 4°C. The protein precipitates were re-suspended in Buffer A, giving a final volume 20 times more concentrated than the starting volume. Required lengths of dialysis tubing were cut and boiled in distilled water for 1 hour. The protein suspension was then equilibrated to ion exchange chromatography column running buffer through dialysis against Buffer A containing 0.1mM benzamidine at 4 °C with stirring overnight. Dialysed samples were then centrifuged at 1,000 g for 10 minutes at 4°C in order to remove any large aggregates which may have blocked the chromatography column and the supernatant was collected for application to the column.

2.2.4.3 Separation of proteins in bacterial extracts by ion exchange chromatography

The equilibrated samples were then passed through an Econo-Pac Q cartridge 5 ml sephadex syringe column, which had previously been equilibrated with low salt (0.01 M NaCl) buffer A at a rate of 2 ml/minute. The unbound proteins were flushed out with a high salt (0.1M NaCl) buffer A, before bound proteins were eluted from the column using an automatic Biorad Logic HR system. This produced a linear gradient of ions from 0.1M to 0.5M NaCl. Fractions were collected automatically in 3ml volumes at a rate of 3ml/minute using a Biorad Model 2128 Fraction Collector. Fraction protein absorbances were then read at a wavelength of 280nm using a Beckmann DV-7 spectrophotometer. Protein content was also assessed for each fraction using the micro Lowry protein estimation assay (described in section 2.2.2.2).

Fractions were then stored at 4°C overnight, or at -20°C for longer periods until required.

2.2.4.4 Dot-Blot analysis of ion exchange fractions

Once ion exchange column fraction protein content had been assessed, 50 μg protein from each fraction were analysed by a dot immunobinding assay using a SRC 96 D Minifold I Dotblotter (purchased from Schleicher and Schuell, Dassell, Germany). A sheet of Optitran BA-S 83 reinforced nitrocellulose (membrane pore size 0.2 μm) was placed on top of a clean piece of Whatman No. 1 filter paper in the manifold. The 96 well lid was then secured and the nitrocellulose was rinsed by adding 250 μl TBS to each well. This was drawn off using a Beckmann water vacuum. The required volume of each fraction (for addition of 50 μg protein) was then added to each well, and again, the excess solution was drawn off with vacuum suction. The wells were then rinsed twice with 250 μl TBS, before the blot was taken off and blocked in 3 % BSA/TBS for 1 hour at room temperature. The blots were then probed overnight at 4°C with either (a) rabbit anti-cholera toxin B sub-unit polyclonal antibody, diluted 1:5,000 in 3% BSA/TBS, or (b) with a mouse anti-cholera B sub-unit monoclonal antibody, diluted 1:20,000 in 3 % BSA/TBS overnight at 4°C. The blots were then washed for three 20 minute periods in TBS/Tween and incubated in either alkaline phosphatase-conjugated sheep anti-rabbit IgG (1:1,000) (for the polyclonal antibody probed blots), or alkaline phosphatase-conjugated goat anti-mouse IgG (1:1,000) (for the monoclonal antibody probed blots) for 2 hours at room temperature.

The blots were then washed again for three 20 minute periods in TBS/Tween. Finally the blots were washed in TBS for 10 minutes then equilibrated with substrate buffer [0.75 M Tris-base, pH 9.5] before addition of developing solution [contents described in section 2.2.2.1]. The developing reaction was stopped by extensive incubation in distilled water, before the blots were dried between 2 sheets of clean filter paper. The relative intensities of the dots were then assayed using Quantiscan software, in order to assess the reactivity of fractions with the anti-cholera toxin B sub-unit primary antibody, and the most reactive fractions were isolated for further investigation.

Samples of each anti-cholera toxin primary antibody-reactive fraction were taken and pooled. The pooled fractions were then dialysed against PBS for 4 hours at 4°C. The samples were then filter concentrated using Millipore Ultrafree®-15 Centrifugal Filters, containing a Biomax high-flux polysulphone membrane, which retained molecules of molecular weight 10,000 Daltons and above. A typical example would consist of 10 ml fraction pool placed in the filter concentrator unit and centrifuged at 2,000 g for 20 minutes, leaving a concentrated suspension of approximately 500 μl (concentration factor of 20). Protein estimations were then carried out on concentrated samples and their protein compositions were determined by SDS-PAGE and Western blotting techniques (sections 2.2.2.3, 2.2.2.4.1 and 2.2.2.5). Thereafter, the samples were ready to be used in mammalian cell cytotoxicity assays (see section 2.2.18) and neurotoxicity assays (section 2.2.1.11).

All fractions were stored at -20°C until required.

2.2.5 Separation of anti-cholera toxin B sub-unit primary monoclonal antibody-reactive proteins by affinity chromatography

2.2.5.1 Materials

Coupling buffer: 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3.

Blocking buffer: 0.2 M glycine, pH 8.0.

Acetate buffer: 0.1 M sodium acetate, 0.5 M NaCl, pH 4.0.

2.2.5.2. Methods

Fractions that showed reactivity against the anti-cholera toxin B sub-unit antibody were pooled and dialysed against PBS for 4 hours at 4°C. The pool was then applied to an anti-cholera B sub-unit polyclonal or monoclonal antibody-coupled affinity column. The affinity column consisted of a cyanogen bromide activated agarose resin comprising a cross linked 4 % beaded-agarose which, when hydrated for 15 minutes in 200ml 1mM hydrochloric acid hydrating solution per gramme freeze-dried powder, swelled from 3.5 ml/g resin. The re-hydrated resin was then added to a 15 ml sintered glass funnel with a glass-microfibre filter and Whatmann filter paper discs. The hydrating solution was drained off with the aid of a Buchner flask. 7 ml of coupling buffer (2ml/gramme resin) were added to 5 mg polyclonal antibody/monoclonal antibody (a maximum of 5mg protein/ml resin was recommended by the manufacturer to reduce steric hindrance between the active sites during coupling). This ligand/coupling solution was then added to the resin in the sintered funnel, in order to re-suspend the resin. The

suspension was then placed in a 20ml sterilin tube and spun end-over-end for 2 hours at room temperature using an automated tube shaker.

The suspension was then placed back into the sintered funnel and the coupling buffer was drawn off. This buffer was retained for protein to assess binding efficiency. 5ml blocking buffer were added to the antibody-coupled resin in the funnel. The sterilin tube was washed out with a further 5ml blocking buffer, which was then added to the funnel containing the resin. This was placed in a 20ml sterilin tube and spun end-over-end for 2 hours at room temperature. The suspension was then placed back into the sintered funnel and the blocking buffer was drawn off. The resin was then washed with 15 ml coupling buffer followed by 15ml acetate buffer. This was repeated alternately 4 times, drawing buffers off each time with the Buchner funnel.

The antibody-coupled resin was then added to a 2ml syringe housing which had been plugged with compacted glass wool. The column was washed through with 50ml PBS. At this point, the addition of fraction pools was carried out. Each pool was passed through the column a minimum of 5 times to allow complete binding of proteins to coupled ligands. The column was then washed with 10ml PBS to remove any non-specifically bound proteins, and fractions were collected in 1ml volumes. The bound proteins were then eluted off the column with 10ml 50mM Tris-HCl, pH 10.5. The fractions were collected in 1ml samples into 1.5ml Eppendorf tubes, containing 100µl neutralising buffer [10x PBS, pH 7.0]. Protein content was estimated in all fractions by absorbance measurements at a wavelength of 280nm and the micro Lowry assay. The composition of eluted fractions was assessed through separation of proteins by SDS-PAGE, along with Western blotting and

protein band probing with anti-cholera toxin β sub-unit monoclonal antibody as described before (sections 2.2.2.3, 2.2.2.5 and 2.2.2.7).

2.2.6. Further purification of bacterial toxins by SDS-PAGE and electroelution.

2.2.6.1. Preparation of ion exchange fractions for gel electrophoresis and electroelution

Bacterial proteins were initially separated by application to an ion exchange chromatography column, and eluted fractions were further characterised with regards to anti-cholera toxin B sub-unit antibody reactivity by application to a nitrocellulose membrane using a dotblot manifold. These fractions were then pooled and concentrated using Millipore centrifugal filter devices. The concentrated fraction pool proteins were then precipitated using 5 volumes of acetone at -20°C for 4 hours, before being re-suspended and boiled in 100μl electrophoresis sample buffer (see section 2.2.2.1). The proteins were then separated using SDS-PAGE on a 10% polyacrylamide resolving gel (see section 2.2.2.3). The protein samples were applied to the gel in numerous wells, along with two wells of molecular weight markers so that the gel could be split, leaving both pieces containing a lane of markers and a least one lane of sample. One half of the gel was then Western blotted and probed using the anti-cholera toxin B sub-unit monoclonal antibody, while the other half was stained using Biorad Copper Stain and Destain Kit for Electrophoresis.

2.2.6.2 Staining of polyacrylamide gels using Biorad Stain and Destain for Electrophoresis Kits

The gel was rinsed in distilled water for 3 to 5 minutes. The gel was then transferred to dilute copper stain (diluted one part copper stain to 9 parts distilled water, refer to BIORAD for details) and completely immersed for 5 minutes to allow development. The gel was then transferred to distilled water and rinsed for 3 minutes. The distilled water was then removed and replaced. The gel was then stored at 4°C until required.

2.2.6.3 Separation of proteins by electroelution

Once the Western blot of the other half of the gel had been developed the specific reactive protein band was marked on the copper stained gel and the area surrounding the band was excised using a sterile scalpel. The gel fragment was then minced into small pieces using the scalpel and transferred to dilute destain solution.

While the gel fragments were destaining, the electroelution tank was set up. This involved washing the membrane cap and silicone adapter in electroelution buffer [10mM CAPS buffer, 10% methanol, pH 11]. The frit was then placed on the end on the glass tube and made flush to the bottom of the tube. This was then immersed in electroelution buffer. The membrane cap was then fitted into the silicone adapter while submerged in electroelution buffer. Air bubbles were then expelled using a Pasteur pipette. The silicone adapter/membrane caps were then filled with electroelution buffer and fitted

onto the frit-end of the glass tube. This was then fitted into the grommet and made flush. All other grommets not in use were plugged and the inner and outer tank were filled with sufficient electroelution buffer to cover the electrical filament.

Once all dye had been removed from the gel fragments, they were carefully placed into the glass tubes in the electroblotting tank and the proteins were removed from the gel fragments by electroelution at 10mA/glass tube for 4 hours, with gentle stirring using a magnetic stirring rod at room temperature.

The glass tubes were then carefully drained and removed, after which the silicone adapters were taken off. The solution in the adapters contained the eluted proteins, which were placed in Eppendorf tubes. The adapters were then washed with 200µl fresh buffer, which were added to the Eppendorf tubes. The protein composition of the suspension was then assayed using the Lowry protein assay, before being used in mammalian cell cytotoxicity assays and separated by SDS-PAGE electrophoresis. The separated proteins were then western blotted onto Optitran BA-S reinforced nitrocellulose (membrane size 0.2µm) and Immobilon P^{SQ} PVDF transfer blot (membrane pore size 0.1µm) using 10mM CAPS, 10% (v/v) methanol electroblotting buffer. The nitrocellulose blots were probed with the anti-cholera toxin B sub-unit monoclonal antibody and developed as normal. The Immobilon blots were stained using amido black stain, then de-stained in 10% (v/v) methanol.

The reactive protein bands were then compared with the stained protein bands, and the corresponding band identified. The Immobilon blot was then taken to Queens Medical Centre for N-terminal sequencing analysis of the

toxin protein. This was carried out by Mr Kevin Bailey and involved the Sanger method of amino acid hydrolysis (Sanger *et al* 1977), followed by HPLC analysis of resultant amino acid fragments. The amino acid sequence was then compared to known amino acid sequences using the LALIGN sequence analysis database.

2.2.7. Further purification of bacterial extract proteins by hydrophobicity column chromatography.

After ion exchange, further protein purification was carried out by utilising the hydrophobic behaviour of enterotoxin proteins sought after. It was hypothesised that since the toxin(s) investigated had shown outer membrane protein properties that they would exhibit some degree of hydrophobicity (required to bind to the lipids in the outer membrane of the bacteria). Therefore a phenyl sepharose resin (Sigma-Aldrich Ltd (Poole, UK)) was used to further purify the required toxin proteins.

Firstly, ammonium sulphate saturation ranges required for optimal column binding and elution were determined. This was carried out by adding 1 ml of ion exchange column fraction pool to 500µl resin in an Eppendorf and vortex-mixing for 1 minute. The suspension was then allowed to stand at room temperature for 1 minute before being spun down in a microfuge at 10,000 g for 1 minute. The supernatant was then removed and any protein present was considered as unbound protein. A 50µl sample was then removed for toxin analysis. Ammonium sulphate was then added to the supernatant at the required weight to produce 10% saturation to the solution. This was vortex-

mixed for 1 minute, then allowed to stand at room temperature for 1 minute. The suspension was then spun down in a microfuge at 10,000g for 5 minutes to remove any protein precipitate. The solution was then added to the resin and vortex-mixed for 1 minute, before being allowed to stand at room temperature for 1 minute. The suspension was then spun down at 10,000 g for 1 minute and the supernatant removed. A 50µl sample was taken for toxin analysis. This method of ascending ammonium sulphate saturation was continued in 10% steps up to 80% saturation (which was the salt concentration used for protein precipitation prior to ion exchange chromatography) as above. This gave unbound protein samples that were assessed through dotblot analysis, probed with the anti-cholera toxin B sub-unit IgG as normal. Thus, the optimal salt concentration for column binding was determined.

For optimal elution salt concentration the bound protein/resin suspension was spun down at 10,000 g for 1 minute. The supernatant was then taken off and the resin was re-suspended in 70% ammonium sulphate saturated PBS. The suspension was vortex-mixed for 1 minute, then allowed to stand at room temperature for 1 minute. The suspension was then spun down at 10,000 g for 1 minute and the supernatant taken off. And so on down to 0% salt saturation. Again the supernatant samples were assayed by dotblot analysis with the anti-cholera toxin B sub-unit IgG, and the optimal elution salt concentration was determined.

The phenyl sepharose column was run using the Biorad Logic HR system as for the ion exchange chromatography. The column was equilibrated at a starting salt concentration of 40% or 60% ammonium sulphate saturation (in

PBS), for optimal protein binding. A linear reducing salt gradient was then automatically achieved by the programme. Fractions were collected in 1ml volumes at a rate of 2ml/minute automatically, using the Biorad Model 2128 Fraction Collector.

Fraction protein compositions were then assayed using the Biorad protein assay (since high ammonium sulphate concentrations were not compatible with the Lowry protein assay) and were analysed by dotblotting with the anti-cholera toxin B sub-unit IgG.

The fractions were then pooled and dialysed against PBS, before being filter concentrated using Millipore Ultrafree®-15 Centrifugal Filters (see section 2.2.4.3) to a volume of 500µl (a concentration factor of 20). Thereafter, they were ready to be used in mammalian cell cytotoxicity assays. Alternatively, toxin content was determined by gel electrophoresis and gel staining. All fractions were stored at -20°C until further required.

2.2.8 Isolation of porcine Brain Cytosol

The porcine brain was stripped of any red blood cell layers and weighed. The brain sample was then added to microtubule extraction buffer [MES: 100mM morpholinoethane sulphonic acid, 0.5mM magnesium chloride, 0.5mM EGTA, 1mM GTP] at 0.5ml buffer per gram of brain tissue. The sample was then homogenised in a blender for 10 seconds. The suspension was then homogenised by 4 passes of glass-teflon homogenisation at 400 r.p.m. The homogenate was then centrifuged at 100,000 G for 1 hour at 4°C. The

supernatant was then taken off, aliquoted into 1ml samples and stored at -20°C for further analysis.

Cytosol was incubated by centrifugation at 100,000 g for 45 minutes at 30°C. Pellets containing assembled microtubules were re-suspended and incubated for 30 minutes on ice in MES buffer, containing 0.1mM GTP and clarified by centrifugation at 5,000 g for 20 minutes at 4°C. The resultant supernatants of microtubule proteins were stored at -20°C for further analysis.

2.2.9 Effects of *Campylobacter jejuni* 11351 culture extracts on microtubule assembly.

The microtubule protein extracts were aliquoted into 200µl volumes and 2µl GTP (final concentration, 1mM) were added. A sub-cytotoxic toxin concentration of 1%(v/v) (*i.e.* 2µl culture extract) was added and microtubule disassembly was allowed to take place at 37°C for 30 minutes. Polymerised microtubules were then spun down in a Beckman benchtop ultracentrifuge at 120,000 g for 20 minutes at 35°C. The supernatant was dicarded and the protein pellets were re-suspended by boiling in 20µl electrophoresis sample buffer and stored at -20°C until separation by SDS-PAGE.

CHAPTER 3

3. Cytotoxicity Assays Results and Discussion

Three different strains of *Campylobacter.jejuni* were used: 11351, 11168, 11322. These were grown under a number of different conditions, as mentioned in the methods section (also shown in table 3.1). The bacterial culture extracts were tested for cytotoxicity at a number of concentrations, initially on two mammalian cell lines, mouse neuroblastoma cells (N2a) and human endothelial cells (ECV 304).

N2a cells were chosen as a non-target cell type for measuring a general cytotoxic effect. ECV endothelial cells were used as a likely target cell type for *Campylobacter* toxins.

The aim of these experiments was to find optimal bacterial growth conditions for cytotoxin production. In cases where results of cell viability assays indicated that bacterial culture treatments showed no significant cytotoxicity towards the mammalian cells, time was saved by excluding such extracts from further experimentation.

Table 3.1 Summary of culture conditions used to grow *Campylobacter jejuni* bacterial strains 11351, 11168 and 11322.

BACTERIAL CULTURE GROWTH MEDIUM
BHI minus yeast extracts (BHI-)
BHI plus yeast extracts (BHI+)
Brucella minus supplements (Brucella-)
Brucella plus supplements (Brucella+)
Blood agar plates (BA)

Table 3.1. The table shows the various culture conditions in which the bacteria were grown. From these cultures, supernatant and cell sonicates were tested.

3.1 Cytotoxicity of *Campylobacter jejuni 11351* culture extracts tested on human endothelial cells (ECV) and mouse neuroblastoma cells (N2a)

Extracts taken from cells grown under culture conditions shown in table 3.1 were assayed for their cytotoxicity, on both N2a and ECV cell lines.

Figure 3.1.1 shows an example of a dose response curve for the cytotoxic effects of three bacterial cell sonicates, taken from bacterial cultures stopped at different stages of growth on blood agar. The bacterial cell numbers were always equalised at a constant absorbance (see methods chapter, section 2.1.1.1). As shown in figure 3.1.1a, the 1 day old bacterial cell sonicates

showed significant cytotoxicity towards N2a cells only at a concentration of 10% (v/v) compared with the control treated cells ($p<0.05$). However, the 2 and 3 day old bacterial cell sonicates showed significant inhibitory effects at concentrations of 5% and 10% (v/v) ($p<0.05$), with the 3 day old cell sonicates showing inhibitory effects at 2.5% (v/v) ($p<0.05$). 1.25% (v/v) bacterial cell sonicates concentrations resulted in non-significant cytotoxicity, but the 2 and 3 day old cell sonicates produced a significant reduction in viability at a concentration of 0.625% (v/v) ($p<0.05$). The lowest concentration (0.32% (v/v)) of added cell sonicate from all samples did not produce a significant effect on N2a cell viability.

Figure 3.1.1b shows the cytotoxic effects of cell sonicates from bacterial cells grown on blood agar, on cultured ECV cells. However, as no cytotoxicity was observed in 2 day old bacterial cell sonicates (unlike that seen in N2a cells, figure 3.1a), cell sonicates taken from bacterial cells grown for longer periods were also tested. It can be observed from the graph that only bacteria aged 5 and 8 days show the expected dose response curve associated with a decrease in treatment concentrations. In these two cases, only the 8 day old bacterial cell extracts show any significant cytotoxicity at concentrations of 2.5% (v/v) ($p<0.01$) and 5% (v/v) ($p<0.005$) but not to the extent seen in the mouse neuroblastoma test cell line (figure 3.1.1a).

Figure 3.1.1. Cytotoxicity assay on cultured N2a and ECV cells treated with *Campylobacter jejuni* 11351 cell sonicates.

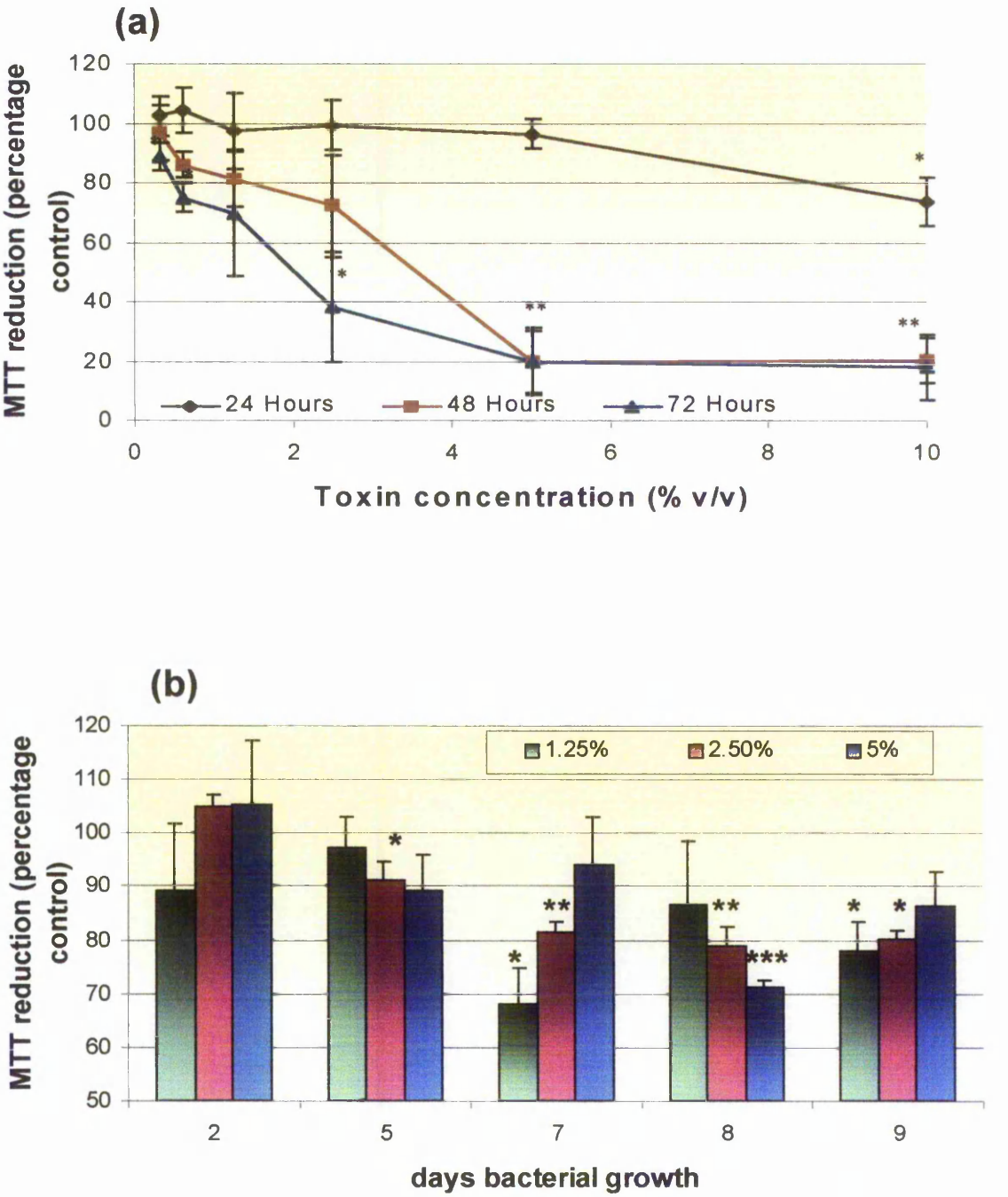


Figure 3.1.1. Cell sonicates were prepared from *C. jejuni* 11351 grown on blood agar plates for 1 to 9 days as indicated. Varying amounts of each extract were applied to (a) N2a and (b) ECV cell cultures for 48 hours, as indicated. Shown is the subsequent reduction of MTT by N2a and ECV cells, expressed as a percentage of control values ± standard deviation (n=5). Asterisks indicate a significance of differences compared to controls as follows: *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

As shown in figure 3.1.2a, the cytotoxic effects of the BHI- culture supernatant towards N2a cells was significant (*i.e* $p < 0.05$) at all concentrations down to 2.5% (v/v), in comparison with the broth control treated cells. The cytotoxic effects of the cell sonicates from the BHI- cultures were not as dramatic. None of the cell sonicate concentrations showed significant cytotoxicity compared to control treated cells ($p>0.05$).

By contrast, figure 3.1.2b shows that cell sonicates were more toxic than the BHI- broth culture supernatants towards ECV cells. In the case of the cell sonicates, all treatment concentrations except 1.25% (v/v) showed slight but significant cytotoxicity. The BHI- broth culture supernatant treatments showed no significant cytotoxicity towards ECV cells, which contrasts with the cytotoxic effects of the same treatments when tested on cultured N2a cells.

Figure 3.1.2. Cytotoxicity assay on ECV and N2a cells exposed to *C.jejuni* 11351 BHI- broths culture cell sonicates/culture supernatants.

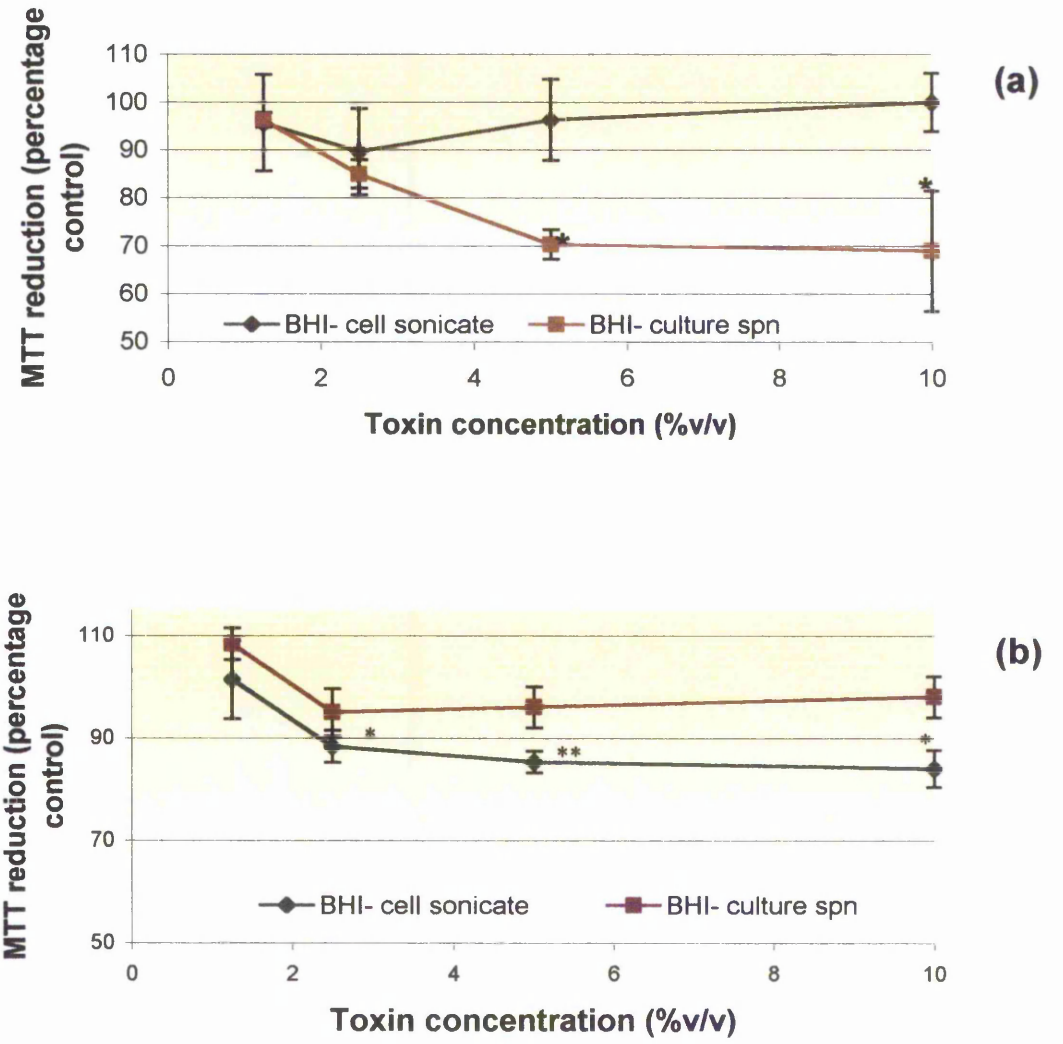
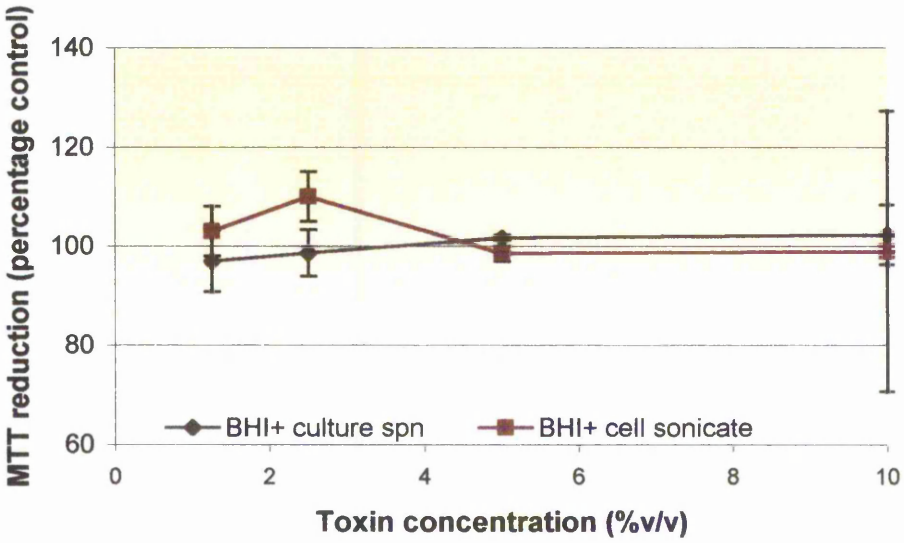


Figure 3.1.2. Cell sonicates and broth culture supernatants were prepared from *C. jejuni* grown in BHI- broth cultures for 3 days as indicated. Varying amounts of each extract were applied to (a) N2a cell cultures and (b) ECV cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a and ECV cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to controls as follows: *, $p<0.05$; **, $p<0.01$.

As shown in figure 3.1.3a BHI+ culture supernatant and cell sonicates had no significant effect on MTT reduction by N2a cells at any of the concentrations tested. However, the fact that two experiments produced significant cytotoxic effects in supernatant treated cells compared to control treated cells, and one experiment produced no significant effect (giving an overall non-significant cytotoxic effect when the three experiments were combined), suggests that there may be a toxin present in the culture supernatant and thus further work was carried out on this culture growth medium. However, as the cell sonicate consistently produced no significant cytotoxic effects, further experimentation with this extract was halted.

In contrast, figure 3.1.3b shows that there is slight but significant cyotoxicity exhibited towards ECV cells at concentrations of 2.5% and 5% (v/v) of the supernatant ($p<0.01$).

Figure 3.1.3. Cytotoxicity assay on ECV and N2a cells exposed to *C.jejuni* 11351 cell/culture supernatant extracts, taken from cultures grown in BHI+ broths.



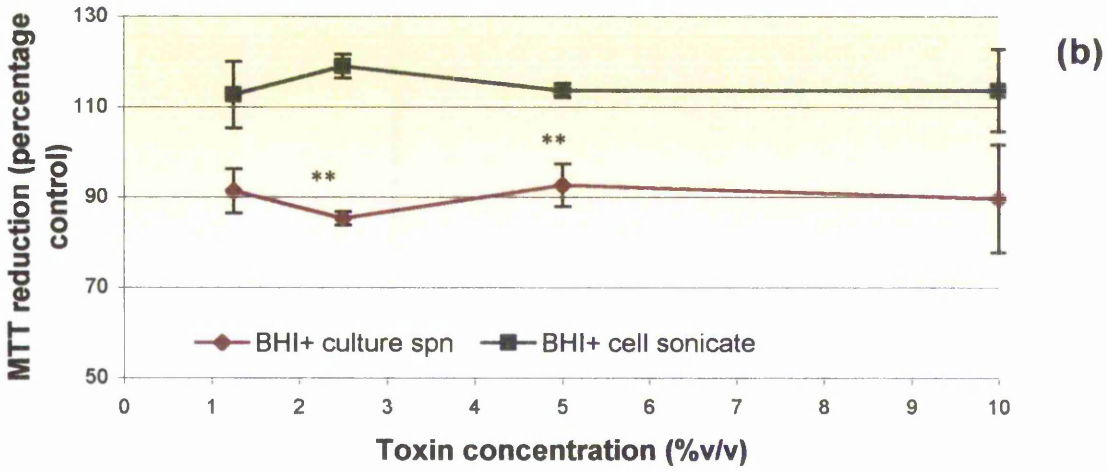


Figure 3.1.3. Cell sonicates and broth culture supernatants were prepared from *C. jejuni* grown in BHI+ broth cultures for 3 days as indicated. Varying amounts of each extract were applied to (a) N2a cell cultures and (b) ECV cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a and ECV cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to controls as follows: **, $p<0.01$.

Figure 3.1.4 shows the effects of treatment with culture supernatant and cell sonicates taken from Brucella plus supplement bacterial cultures. The cell sonicate treatments showed no significant cytotoxicity compared with control treated cells at any concentrations, but the culture supernatant treatments showed significant inhibitory effects at 5% (v/v) ($p<0.05$). Because of the low levels of cytotoxicity exhibited by Brucella culture extracts (also see figure 3.1.5) it was decided not to run assays on ECV cells, which had previously shown a reduced response to the bacterial culture extracts. This was a time/labour saving decision.

Figure 3.1.4. Cytotoxicity assay on N2a cells exposed to *C.jejuni* 11351 Brucella+ broth cell sonicates/culture supernatant.

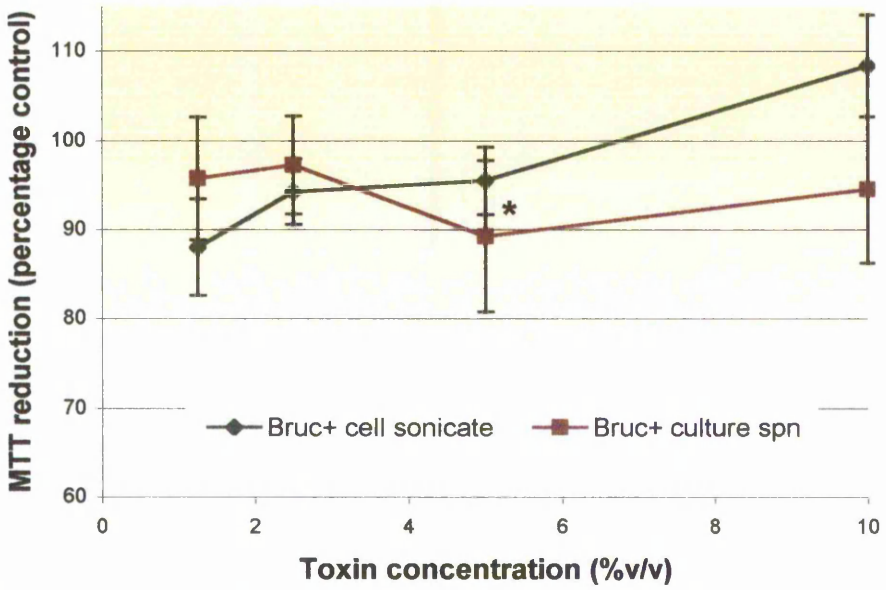


Figure 3.1.4. Cell sonicates and broth culture supernatants were prepared from *C. jejuni* grown in Brucella+ broth cultures for 3 days as indicated. Varying amounts of each extract were applied to N2a cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to controls as follows: *, $p<0.05$.

Figure 3.1.5 shows the effects of culture supernatants and cell sonicates taken from Brucella minus supplements bacterial cultures. In this case the cell sonicates showed significant cytotoxicity towards N2a cells at a treatment concentration of 1.25% (v/v), but showed no expected dose response pattern. Therefore the cytotoxic result at the lower concentration was considered an anomaly. The results for the culture supernatant showed no significant cytotoxicity at any concentration tested.

Figure 3.1.5. Cytotoxicity assay on N2a cells exposed to *C.jejuni* 11351 Brucella- broth cell sonicates/culture supernatants.

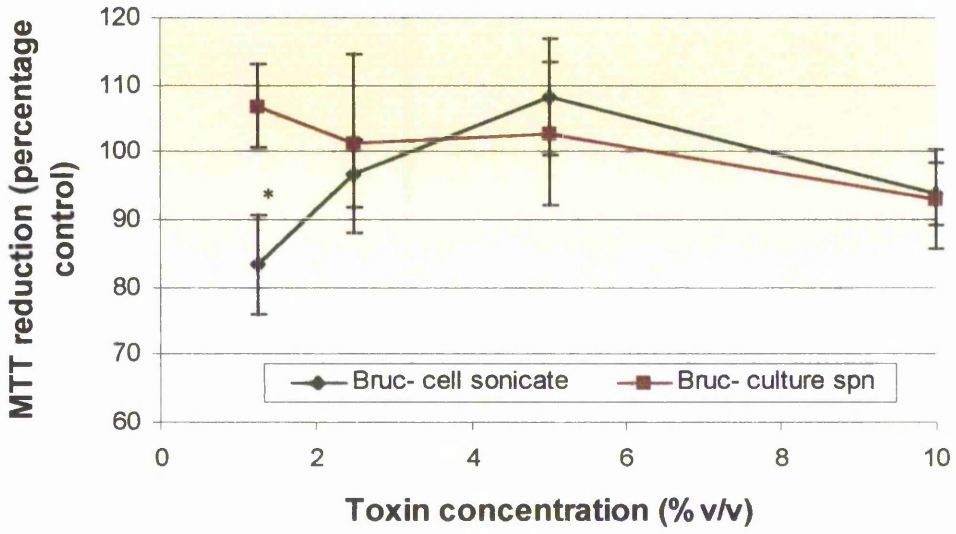


Figure 3.1.5. Cell sonicates and broth culture supernatants were prepared from *C. jejuni* grown in Brucella- broth cultures for 3 days as indicated. Varying amounts of each extract were applied to N2a cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to controls as follows: *, $p<0.05$.

Having tested a range of bacterial culture conditions for toxin production by strain 11351, the next step was to try other *Campylobacter jejuni* strains in order to assess the relative cytotoxicity of the 11351 strain. Therefore another 2 strains were tested for cytotoxicity using the same approach on the same cultured mammalian cell lines. Firstly, strain 11168 was assayed, the results of which can be seen in section 3.2.

3.2 Cytotoxic effects of *Campylobacter jejuni* 11168 on cultured mouse neuroblastoma (N2a) cells.

The cytotoxic effects of extracts from *Campylobacter* strain 11168 were not as great as those observed for strain 11351, therefore not all bacterial culture conditions were assayed as time was an ever present factor. The results in figure 3.2.1a show cytotoxicity for extracts taken from sonicated cells grown on blood agar plates, with bacterial age ranging from one to three days. The figure shows no significant cytotoxic effect produced by the one day old bacteria (as for strain 11351, Figure 3.1.1).

Treatments taken from bacteria grown for 2 and 3 days both show significant cytotoxic effects at certain concentrations (1.25% for the 48 hour bacteria, and 5% for the 3 day old bacteria), but the expected dose response of increasing cytotoxicity with increasing treatment concentrations was not observed in either of the treatments. This would suggest spurious cytotoxic effects at lower treatment concentrations.

Figure 3.2.1b shows the cytotoxic effects of *Campylobacter jejuni 11168* sonicated cell extracts taken from blood agar plate cultures on cultured ECV cells. The results show that there is no significant cytotoxicity at any concentration normally associated with cytotoxicity towards cultured N2a cells (5% (v/v) in the case of 3 day old bacterial cultures).

For this reason no further assays were carried out on cultured ECV cells using strain 11168, as it has already been shown to be a less cytotoxic strain than 11351.

Figure 3.2.1. Cytotoxicity assay on cultured N2a and ECV cells treated with *Campylobacter jejuni* 11168 cell sonicates.

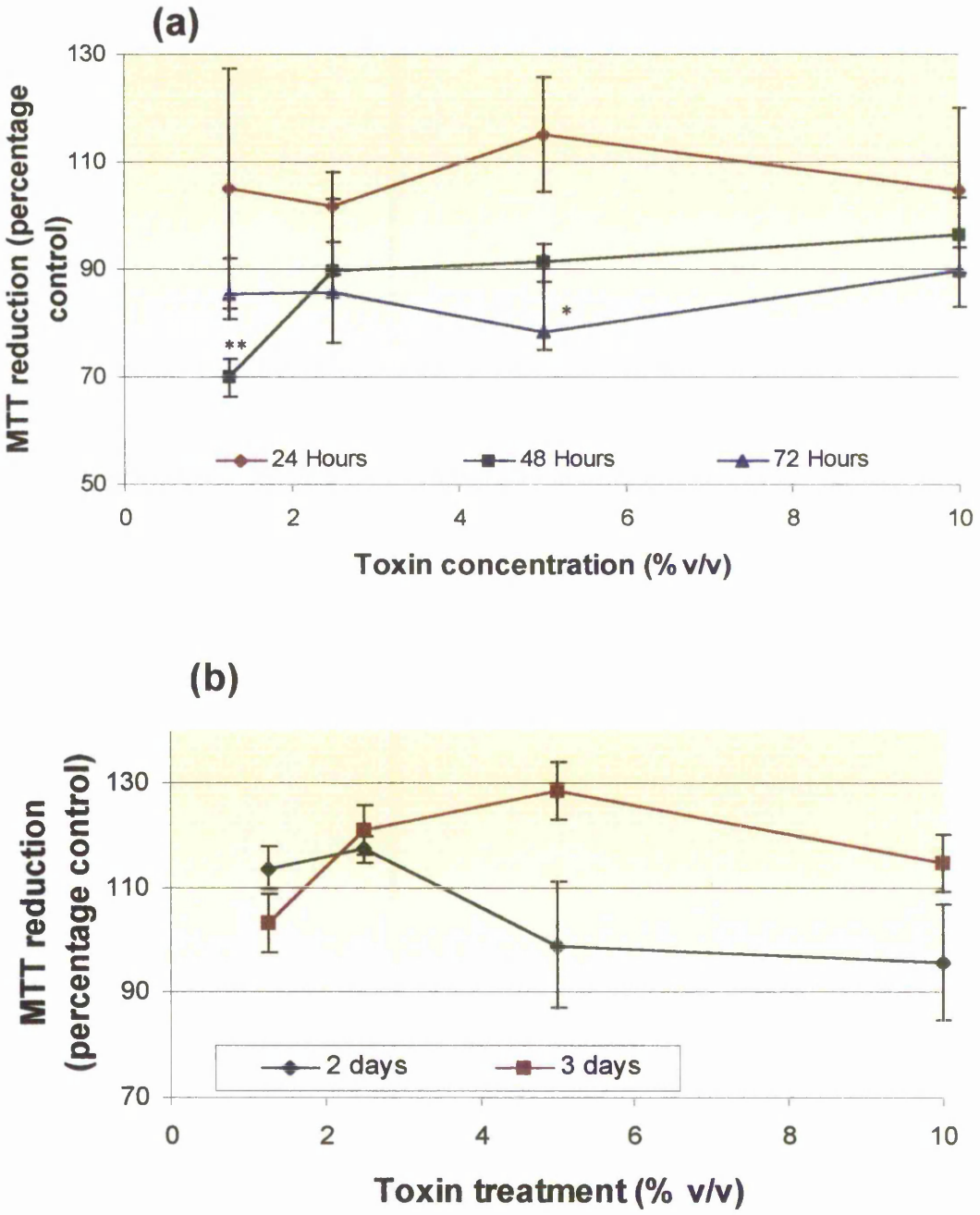


Figure 3.2.1. Cell sonicates were prepared from *C. jejuni* 11168 grown on blood agar plates for 1 to 3 days as indicated. Varying amounts of each extract were applied to (a) N2a and (b) ECVcell cultures for 48 hours, as indicated. Shown is the subsequent reduction of MTT by N2a and ECV cells, expressed as a percentage of control values ± standard deviation (n=5). Asterisks indicate significance of differences compared to controls as follows: *, $p<0.05$; **, $p<0.01$.

In figure 3.2.2, the cytotoxic effects of culture supernatant and sonicated cell extracts on cultured N2a cells can be observed. In this case, the supernatant shows a dose response pattern, but does not have the extent of cytotoxicity seen in the 11351 strain grown in BHI- broth (figure 3.1.2) with only toxin concentrations of 10% (v/v) showing slight but significant cytotoxicity ($p<0.05$). The extracts taken from sonicated cells showed significant cytotoxicity at 1.25% (v/v) treatment ($p<0.05$), but showed no significant toxicity at any of the higher concentrations, again suggesting a spurious result for the lower treatment concentration.

The reduced levels of cytotoxicity observed in this strain grown in BHI- broth compared with the 11351 strain was the basis for not studying cytotoxicity of 11168 grown in BHI+ broth, since it had already been observed in the more toxic 11351 strain, that the BHI- broth cultures produced more cytotoxic effects on the cultured mammalian cells (figure 3.1.2).

Figure 3.2.2. Cytotoxicity assay on N2a cells exposed to *C.jejuni* 11168 BHI- broths culture cell sonicates/culture supernatants.

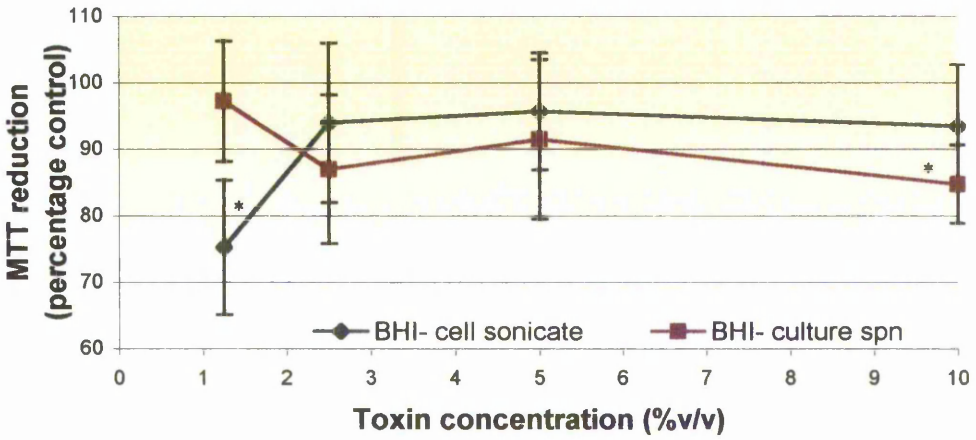


Figure 3.2.2. Cell sonicates and broth culture supernatants were prepared from *C. jejuni* grown in BHI- broth cultures for 3 days as indicated. Varying amounts of each extract were applied to N2a cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to controls as follows: *, $p<0.05$.

For the same reason that only bacteria grown in Brucella+ broth were assayed for cytotoxicity (seen in figure 3.2.3), since it had been previously shown that bacteria grown in this broth (figure 3.1.4) produced more cytotoxic effects than bacteria grown in Brucella- broth (figure 3.1.5).

In the case of *Campylobacter jejuni* 11168 grown in Brucella plus supplements (figure 3.2.3), it can be observed that there were no significant cytotoxic effects when the mammalian cells were exposed to bacterial culture supernatant. There was significant cytotoxicity at the 10% (v/v) and 1.25% (v/v) treatment concentrations in the case of the sonicated bacterial cell extracts ($p<0.05$). However, once again the over all cytotoxicity profile for the 11168 strain grown in the Brucella plus supplements culture broth was less

significant than in the case of bacteria grown in BHI culture broth, whether it be for the 11351 strain or the 11168 strain. These findings suggested that BHI culture broth (specifically BHI-) was the optimal culture broth of those tested for the production of cytotoxins.

Figure 3.2.3 Cytotoxicity assay on N2a cells exposed to *C.jejuni* 11168 Brucella+ broth cell sonicates/culture supernatant.

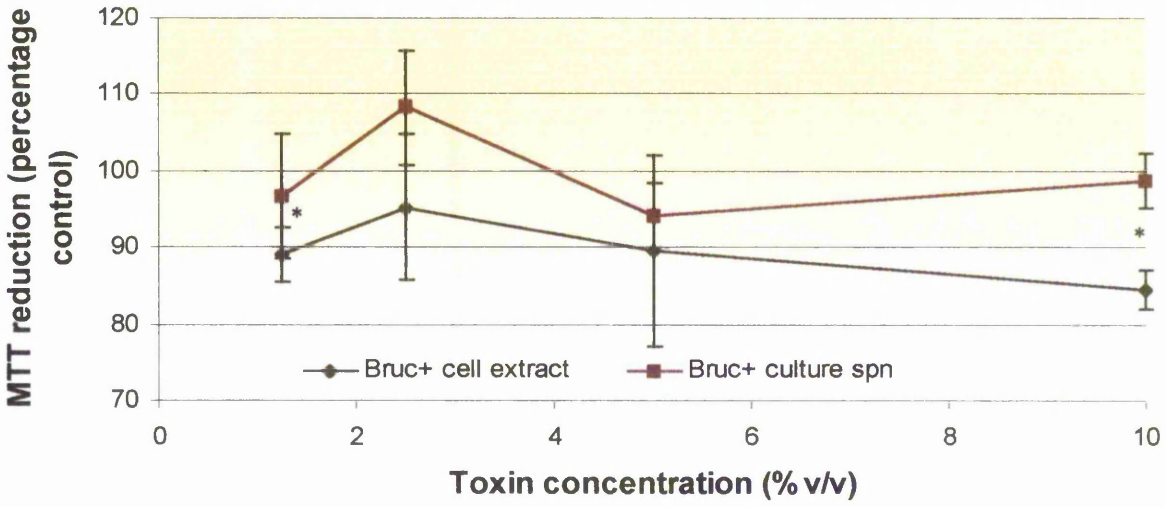


Figure 3.2.3. Cell sonicates and broth culture supernatants were prepared from *C. jejuni* grown in Brucella+ broth cultures for 3 days as indicated. Varying amounts of each extract were applied to N2a cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to controls as follows: *, $p<0.05$.

To further support this conclusion, a third *Campylobacter jejuni* strain, 11322, was assayed for cytotoxicity against the cultured mouse neuroblastoma (N2a) cells in an identical manner as the first two strains. The results can be see in the following section.

3.3 Cytotoxic effects of *Campylobacter jejuni* 11322 cell sonicates/culture supernatants on cultured mouse neuroblastoma (N2a) cells.

Figure 3.3.1 shows no significant cytotoxicity for any of the treatment concentrations in the case of cell sonicates from 24 hour cultures on blood agar. However, there is significant cytotoxicity at treatment concentrations of 10% and 5% (v/v) in the case of 2 and 3 day old bacterial cell sonicates. The cytotoxicity of these cell extracts is reduced to non-significant levels at concentrations of 2.5% (v/v) and below. Both ages showed the predicted dose response pattern and, although more cytotoxic than the corresponding bacterial extracts from the 11168 strain (Figure 3.2.1), they are still less cytotoxic than the 11351 strain bacterial extracts (Figure 3.1.1).

Figure 3.3.1b shows the cytotoxic effects of cell sonicates taken from strain 11322 blood agar plate cultures on cultured ECV 304 cells. Again, there is no significant cytotoxicity observed at any treatment concentrations, compared with the significant cytotoxicity observed in cultured N2a cells (see Figure 3.3.1). Because of the lack of cytotoxicity in this assay, no further work was carried out using this strain.

Figure 3.3.1. Cytotoxicity assay for N2a cells and ECV cells exposed to *Campylobacter jejuni* 11322 blood agar plate cell sonicates.

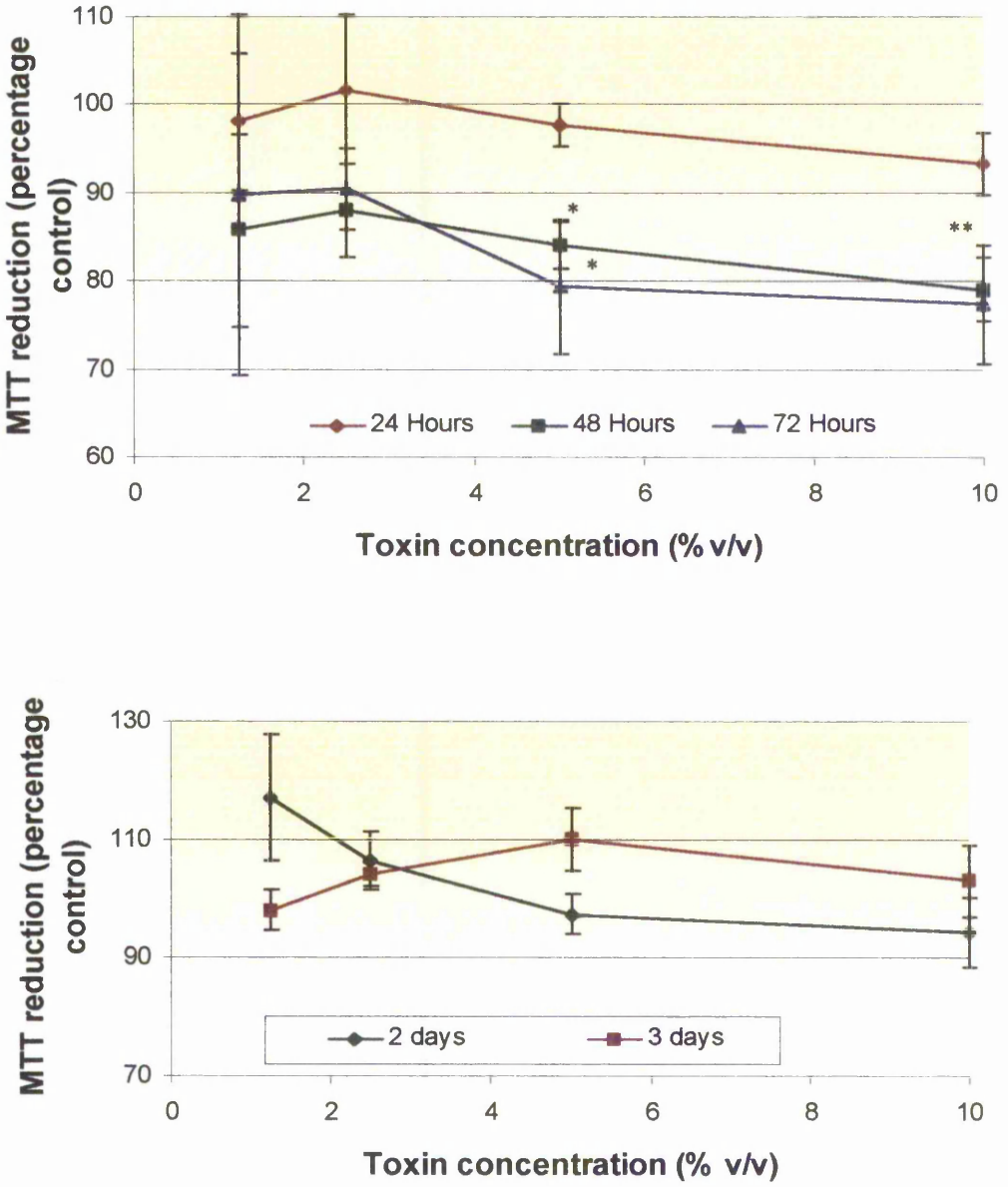


Figure 3.3.1. Cell sonicates were prepared from *C. jejuni* grown in blood agar cultures for 1 to 3 days as indicated. Varying amounts of each extract were applied to (a) N2a cell cultures and (b) ECV cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a and ECV cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to controls as follows: *, $p<0.05$; **, $p<0.01$.

Figure 3.3.2 shows the cytotoxic effects of culture supernatant and cell sonicates taken from BHI- broth cultures on N2a cells. The figure shows significant cytotoxic effects at treatment concentrations of 5% and 10% (v/v) in the case of the bacterial culture supernatant ($p<0.05$). Further dilutions in treatment concentrations showed no significant cytotoxic effects. The cell sonicates show significant cytotoxic effects at treatment concentrations of 1.25% (v/v) ($p<0.05$), but not at the higher concentrations.

Figure 3.3.2. Cytotoxicity assay on N2a cells exposed to *C.jejuni* 11322 BHI- broths culture cell sonicates/culture supernatants.

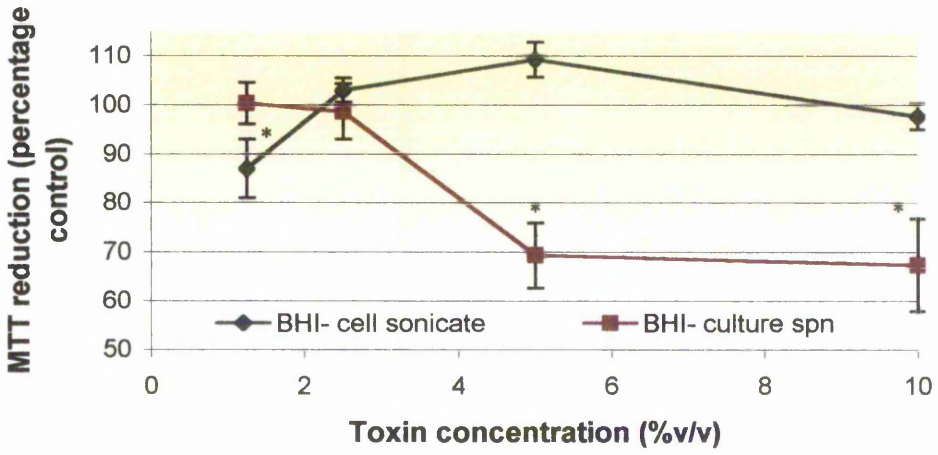


Figure 3.3.2 Cell sonicates and broth culture supernatants were prepared from *C. jejuni* grown in BHI- broth cultures for 3 days as indicated. Varying amounts of each extract were applied to N2a cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to controls as follows: *, $p<0.05$.

Figure 3.3.3 shows the cytotoxic effects of extracts from *Campylobacter jejuni* 11322 cells grown in Brucella plus supplements broth cultures. The graph

shows that there was no evidence of any significant cytotoxicity exhibited by either the cell sonicates or the cell culture supernatants. Therefore no further tests were carried out with either this particular culture medium, or with this strain, as the evidence shown clearly suggests a less consistent cytotoxic effect on cultured mammalian cells than that observed for the previous strains tested.

Figure 3.3.3 Cytotoxicity assay on N2a cells exposed to *C.jejuni* 11322 Brucella+ broth cell sonicates/culture supernatant.

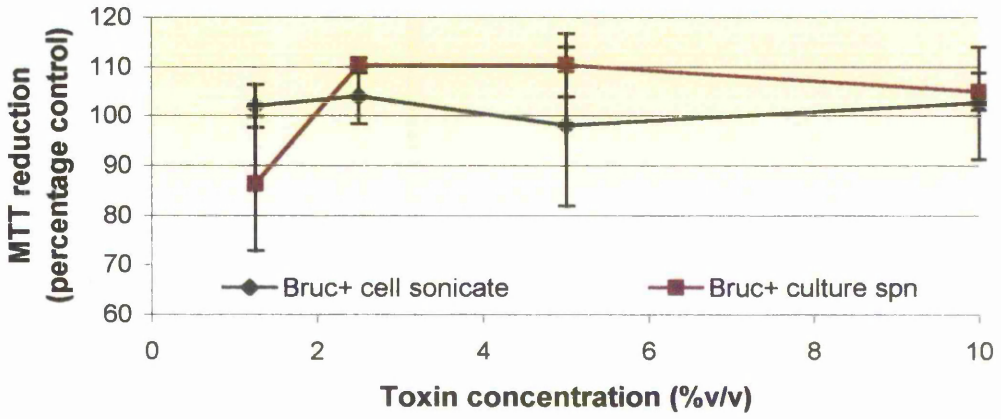


Figure 3.3.3. Cell sonicates and broth culture supernatants were prepared from *C. jejuni* grown in Brucella+ broth cultures for 3 days as indicated. Varying amounts of each extract were applied to N2a cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a cells, expressed as a percentage of control values ± standard deviation (n=3).

Therefore, through a progressively selective programme of investigative general cytotoxicity assays, a number of *Campylobacter jejuni* strains grown in different culture conditions have been tested on a number of cultured mammalian cell types. This resulted in the selection of strain 11351 and culture extracts from three culture conditions (blood agar plates and brain heart infusion culture broth with or without supplements). These bacterial extracts were taken forward and used as the source of *Campylobacter jejuni* toxin production in subsequent investigations.

3.4 Heat/trypsin treatment of *Campylobacter jejuni* culture extracts and the resultant effects on cytotoxicity towards cultured mammalian cells.

The aim of these experiments was to determine if the toxins responsible for the cytotoxicity shown in the previous sections were true protein toxins. A protein toxin would be expected to be heat and/or protease sensitive. But there is also a class of bacterial toxins known as "complex lipopolysaccharide/protein toxins" attached to the cell wall of gram negative bacteria (for example, *Enterobacteriaceae*) (Arbuthnott 1978). Such toxins might be heat sensitive, whereas a toxin composed of lipopolysaccharide only would be heat resistant (Lee, *et al* 2000; Kawaguchi, *et al* 1989).

Therefore, it was thought necessary to determine whether the toxins detected were true protein toxins, by exposing the culture extracts to temperatures of 100°C and trypsin treatment.

Figure 3.4.1 shows the effects of heat treatment on the cytotoxicity of cell sonicates taken from *Campylobacter jejuni* 11351 cells grown on blood agar plates when tested on cultured ECV and N2a cells. Figure 3.4.1a shows that there is no significant difference in cytotoxicity towards N2a cells of heated extracts compared with non-heated controls. This is supported by the results shown in figure 3.4.1b, where again there was no significant reduction in cytotoxicity after heat treatment when tested on cultured ECV cells.

Figure 3.4.1. Effects of heat treatment on the cytotoxicity of *Campylobacter jejuni* 11351 cell sonicates taken from blood agar plate cultures.

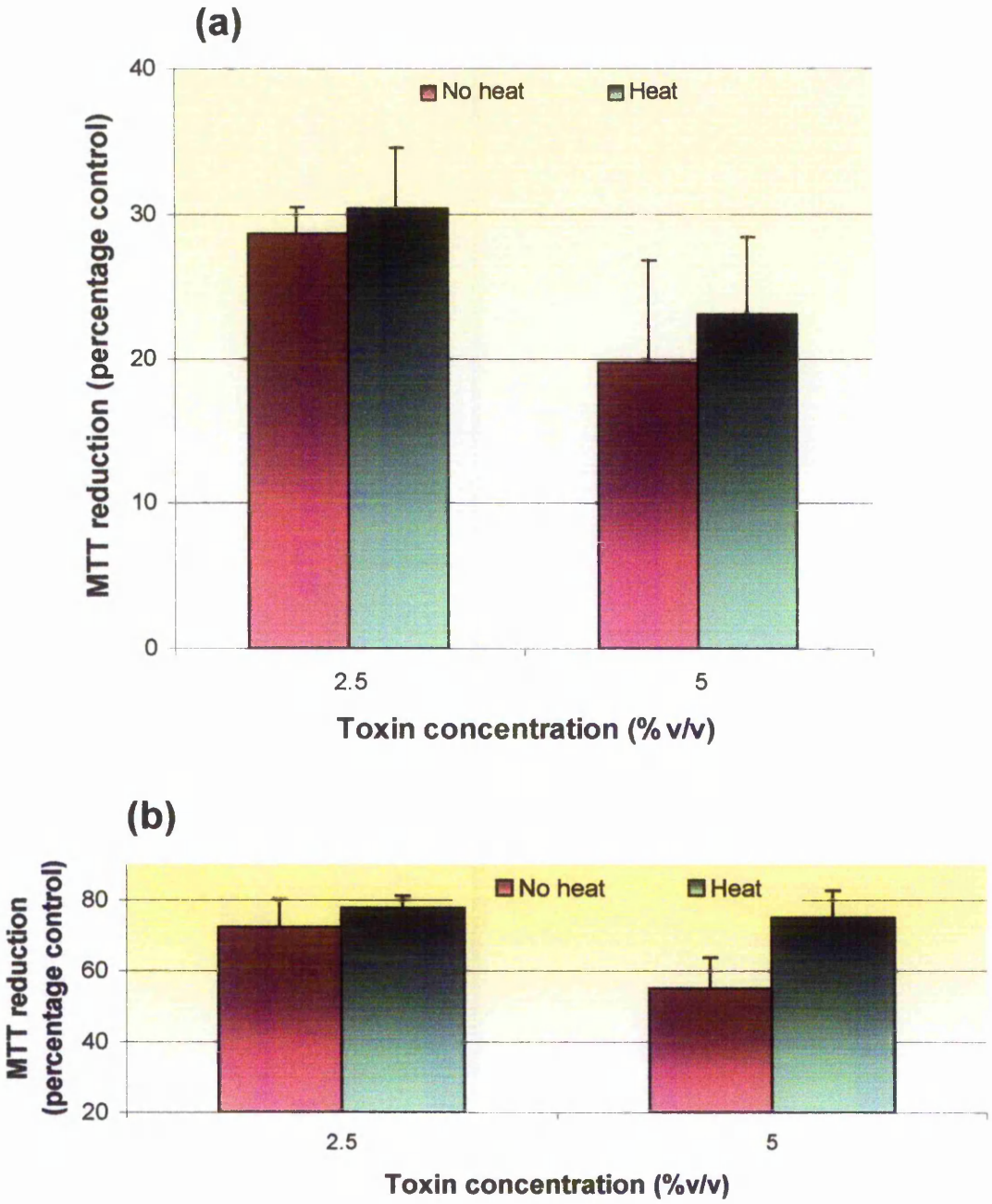
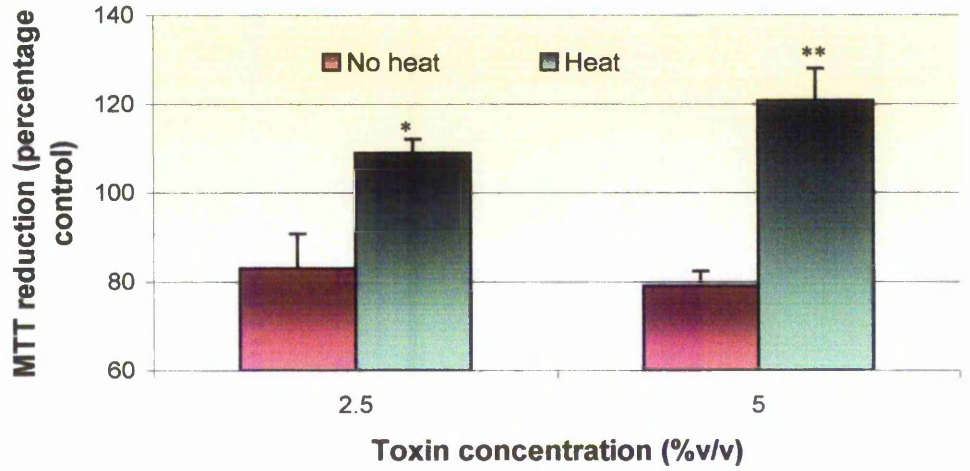


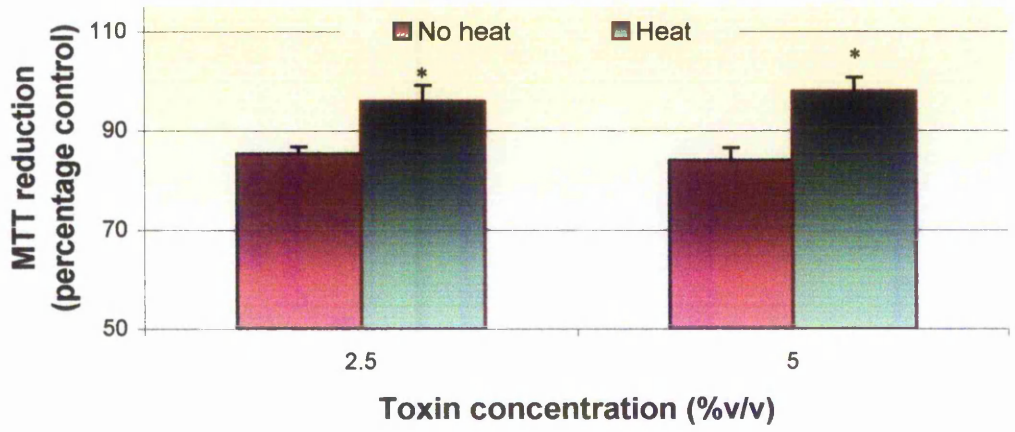
Figure 3.4.1. Cell sonicates were prepared from *C. jejuni* grown on blood agar plates for 3 days as indicated. The cell sonicates were heated to 100°C for 10 minutes to allow heat inactivation of protein toxins. Varying amounts of each extract were applied to (a) N2a and (b) ECV cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a cells, expressed as a percentage of control values ± standard deviation (n=3).

Figure 3.4.2 shows the effects of heat treatment on the cytotoxicity of culture supernatant taken from *Campylobacter jejuni* 11351 BHI- broth cultures, added to ECV and N2a cell cultures. Figure 3.4.2a shows a significant reduction in cytotoxicity after heat treatment at 2.5% (v/v) toxin concentration ($p<0.05$) and at 5% (v/v) toxin concentration ($p<0.01$). Figure 3.4.2b further supports these results, also showing a significant reduction in cytotoxicity after heat treatment at both toxin concentrations ($p<0.05$), although toxicity of non-heat treated supernatants was much weaker in ECV cultures.

Figure 3.4.2 Effects of heat treatment on the cytotoxicity of *Campylobacter jejuni* 11351 culture supernatants taken from BHI- broth cultures.



(a)



(b)

Figure 3.4.2. Cell culture supernatants were prepared from *C. jejuni* grown in BHI- broth cultures for 3 days as indicated. The culture supernatants were heated to 100°C for 10 minutes to allow heat inactivation of protein toxins. Varying amounts of each extract were applied to (a) N2a cell cultures and (b) ECV cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a and ECV cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to non-heated controls as follows: *, $p<0.05$; **, $p<0.01$.

Figure 3.4.3 shows the effects of heat treatment on the cytotoxicity of culture supernatant taken from a BHI+ broth culture added to cultured ECV and N2a cells. There was a significant reduction in cytotoxicity towards N2a cells at 5% (v/v) toxin concentration ($p<0.01$) (figure 3.4.3a). But Figure 3.4.3b shows a significant reduction in cytotoxicity towards ECV cells at both 2.5% (v/v) toxin concentration ($p<0.05$) and 5% (v/v) toxin concentration ($p<0.05$). The ECV graph shows less of a reduction in cytotoxicity as a result of heating, but the error bars were much smaller and therefore cytotoxin inhibition was significant ($p<0.05$).

Figure 3.4.3. Effects of heat treatment on the cytotoxicity of *Campylobacter jejuni* 11351 culture supernatant taken from BHI+ broth cultures.

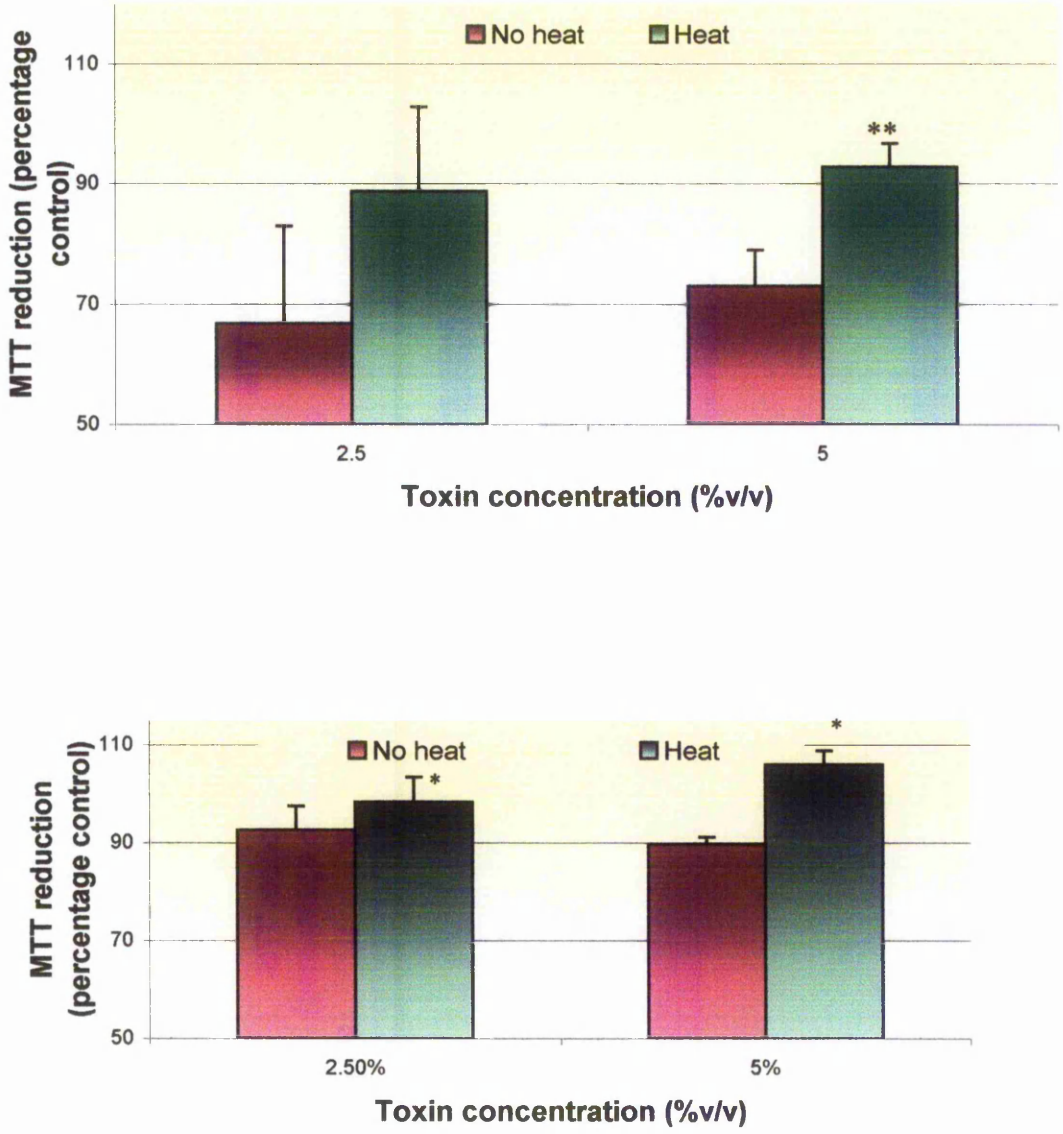


Figure 3.4.3. Cell culture supernatants were prepared from *C. jejuni* grown in BHI+ broth cultures for 3 days as indicated. The culture supernatants were heated to 100°C for 10 minutes to allow heat inactivation of protein toxins. Varying amounts of each extract were applied to (a) N2a cell cultures and (b) ECV cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a and ECV cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to non-heated controls as follows: *, $p<0.05$; **, $p<0.01$.

The reduction in cytotoxicity shown in the figures 3.4 2 and 3.4.3 is significant at the higher toxin concentrations (at least $p<0.05$ in all cases) and suggests cytotoxicity is mainly due to a true protein bacterial toxin(s). However, there does seem to be a level of cytotoxicity still present after culture supernatant heat treatment (seen in figure 3.4.3a).

For further evidence of protein cytotoxin involvement, cell sonicates/culture supernatants were treated with the proteolytic enzyme trypsin in order to inhibit activity. These assays were only carried out on N2a cells, because from the evidence of the heat treatment assays the ECV cells were not as susceptible to toxin treatment as the mouse neuroblastoma cells.

Figure 3.4.4 shows the effects of trypsin treatment on the cytotoxicity of blood agar cell sonicates. The graph shows that there was no evidence of any significant reduction in cytotoxic effects of cell sonicates after trypsin treatment. This supports the results shown in figure 3.4.1 and, taken together, these results suggest the presence (in blood agar cell sonicates) of a non-protein structure with cytotoxic effects on the cultured mammalian cells. This could take the form of an individual bacterial toxin or an active domain of a lipopolysaccharide/protein bacterial toxin.

Figure 3.4.4. Effects of trypsin treatment on the cytotoxicity of *Campylobacter jejuni* 11351 blood agar cell sonicates.

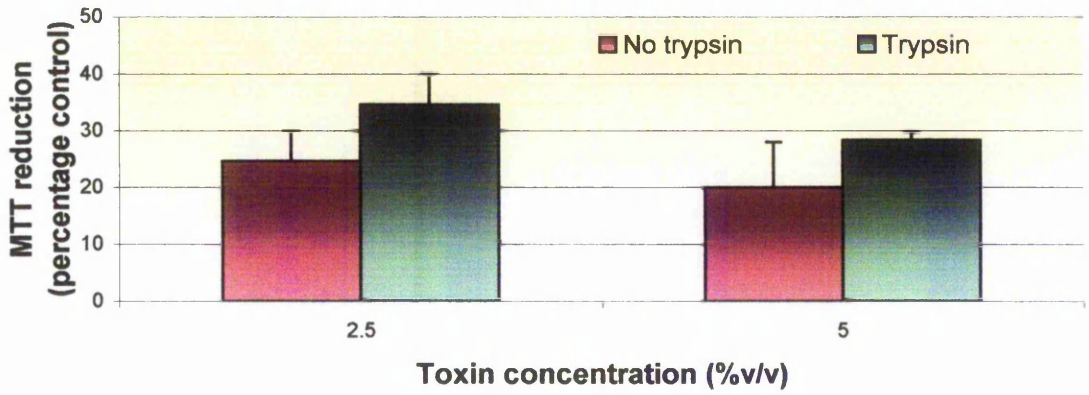


Figure 3.4.4 Cell sonicates were prepared from *C. jejuni* grown on blood agar plates for 3 days as indicated. The cell sonicates were trypsin treated for 10 minutes to allow protease inactivation of protein toxins. Trypsin was inactivated by heating to 100°C for 10 minutes. Varying amounts of each extract were applied to N2a cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a cells, expressed as a percentage of non-trypsin treated control values ± standard deviation (n=3).

Figure 3.4.5 shows the effects of trypsin treatment on the cytotoxicity of culture supernatants taken from BHI- broth cultures. Similar to the results for heat treatment (figure 3.4.2), the reduction in cytotoxicity after trypsin treatment is significant at the higher concentration ($p<0.05$).

Figure 3.4.5. Effects of trypsin treatment on the cytotoxicity of *Campylobacter jejuni* 11351 culture supernatant taken from BHI- broth cultures.

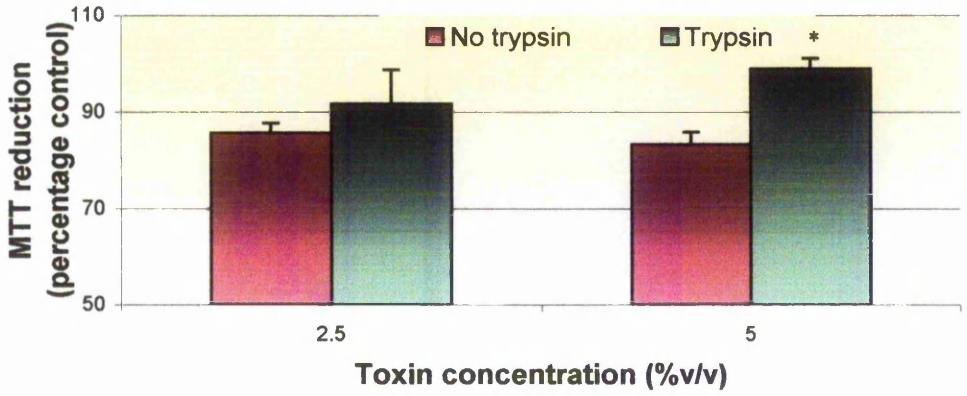


Figure 3.4.5 Cell culture supernatants were prepared from *C. jejuni* grown in BHI- broth cultures for 3 days as indicated. The culture supernatants were trypsin treated for 10 minutes to allow protease inactivation of protein toxins. Trypsin was inactivated by heating to 100°C for 10 minutes. Varying amounts of each extract were applied to N2a cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to non-trypsin treated controls as follows: *, $p<0.05$.

Figure 3.4.6 shows the reduction in cytotoxicity observed after trypsin treatment of BHI+ broth culture supernatants (figure 3.4.3b) was also detected after treatment with trypsin. The effects were found to be significant at 5% (v/v) toxin concentration ($p<0.05$) but not at the lower concentration.

Figure 3.4.6. Effects of trypsin treatment on the cytotoxicity of *Campylobacter jejuni* 11351 culture supernatant taken from BHI+ broth cultures.

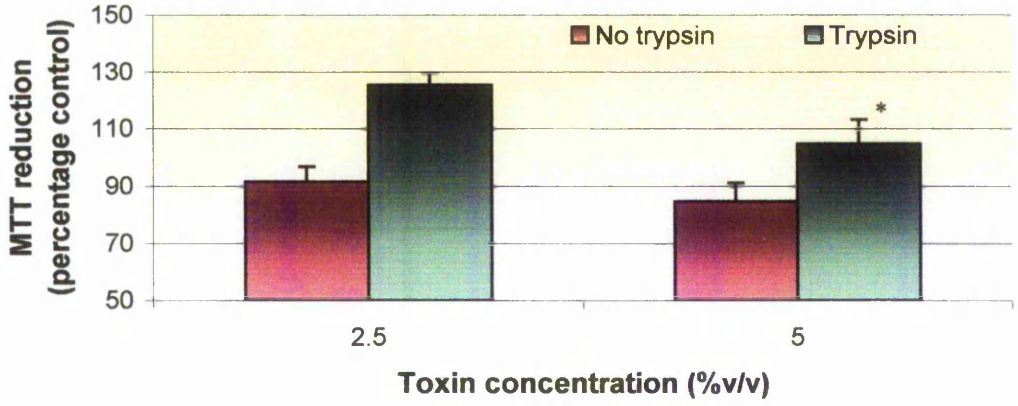


Figure 3.4.6 Cell culture supernatants were prepared from *C. jejuni* grown in BHI+ broth cultures for 3 days as indicated. The culture supernatants were trypsin treated for 10 minutes to allow protease inactivation of protein toxins. Trypsin was inactivated by heating to 100°C for 10 minutes. Varying amounts of each extract were applied to N2a cell cultures for 48 hours as indicated. Shown is the subsequent reduction of MTT by N2a cells, expressed as a percentage of control values ± standard deviation (n=3). Asterisks indicate significance of differences compared to non-trypsin treated controls as follows: *, $p<0.05$.

Overall, figures 3.4.2/3.4.5 along with 3.4.3/3.4.6 show a significant reduction in cytotoxicity when comparing results before and after exposure to both heat and trypsin, suggesting the presence of a protein cytotoxin in the BHI broth culture supernatants.

In summary, there is evidence for at least 2 cytotoxins present in the culture extracts of *Campylobacter jejuni* 11351 when tested on both cultured mouse neuroblastoma (N2a) and human endothelial cells (ECV), albeit with somewhat reduced sensitivity in the latter cell line. The optimal conditions for the production of cytotoxins appear to be cell sonicates taken from blood agar plate cultures and culture supernatants taken from both BHI -/+ broth cultures.

Of these cytotoxins, there appears to be a heat/trypsin resistant cytotoxin present in the extracts taken from sonicated cells grown on blood agar, and a heat/trypsin sensitive cytotoxin present in the supernatant extracts taken from the BHI broth cultures. The next step in the project was to take these cytotoxic culture supernatants/bacterial extracts and examine their effects on the morphology and the cytoskeleton of cultured mammalian cells, in order to investigate the mechanisms of toxin action on host cells and to attempt to reveal target proteins in the host.

3.5. Cytotoxicity Assays Discussion

From the cytotoxicity assay results shown in chapter 3, a number of situations were observed where mammalian cell cytotoxicity was in evidence. All three bacterial strains showed evidence of cytotoxin production but, of the three, strain 11351 seemed to be the most consistently cytotoxic, producing a mammalian cell toxin in most of the conditions under which it was grown. The observed presence of cytotoxin in BHI cell culture supernatant (figure 3.1.2 and 3.1.3) is in agreement with a number of studies using BHI broth as a culture medium for cytotoxin production in *Campylobacter* (Lam 1993; Coote and Arain 1996).

Evidence of cytotoxin production was most significant in extracts produced by cell sonication (figure 3.1.1) particularly in bacterial cells cultured on blood agar for 3 days. These findings are comparable with the detection of cytolethal distending toxin and cytolethal rounding toxin in a number of *Campylobacter jejuni* clinical isolates, where blood agar cell sonicates were tested for cytotoxicity on cultured CHO cells (Schulze *et al* 1998).

Production of cytotoxins in Brucella culture extracts was not convincingly demonstrated in cytotoxicity assays (figures 3.1.4 and 3.1.5). Although this was not considered a problem, as the other two culture conditions looked more promising, it was unexpected, because Brucella broth cultures are widely used in cytotoxin production in *Campylobacter* studies (Johnson and Lior 1986; Moutinho-Fragoso *et al* 1996; Misawa *et al* 1995; Bacon *et al* 1999;

Moore *et al* 1988). Perhaps the *Campylobacter* strains were not as suited to the Brucella culture medium as the strains used in these reports.

Of the three strains it was thought prudent to continue work with strain 11351 because, although there was evidence of cytotoxin production by strains 11168 (figures 3.2.1 to 3.2.3) and 11322 (figures 3.3.1 to 3.3.3), the results were not as consistent. By contrast, extracts from strain 11351 produced consistent reductions in mammalian cell viability after culture in BHI broth cultures and on blood agar plate cultures (figures 3.1.1 to 3.1.3). The reasons for inconsistent cytotoxicity between strains and growth media conditions were not investigated, but possibly the conditions tested were not optimal for toxin production in these strains.

Media selection proved to be a simple case of rejecting the culture growth medium which produced significantly less or inconsistent cytotoxicity compared to the other culture conditions. Therefore, the Brucella medium was rejected, as the results showed little cytotoxicity after mammalian cell exposure to the bacterial culture extracts. This left the BHI broths with and without yeast extracts and the blood agar plates as the chosen culture conditions for production of cytotoxins by strain 11351. Of these situations, the BHI- culture supernatant and the extracts from cell sonication after growth on blood agar plates for three days proved to be the most promising for the production of cytotoxins, as indicated by the higher yields of toxin activity (figures 3.1.1 and 3.1.2).

The results in chapter 3 suggest at least two types of cytotoxins were being produced by the bacteria. Firstly, the toxicity of BHI cell culture supernatants suggests that there was a secreted cytotoxin present in the BHI broth culture.

Secreted cytotoxins, or exotoxins are defined as "protein toxins synthesised and released into the medium by actively growing cells" (Raymond and Alouf 1970). There is no increase in such toxins during the stationary phase and there is very little evidence of intracellular presence of the exotoxin. The toxins can be released after growth or after a lag period (Raymond and Alouf 1970).

Evidence supporting the idea that this toxin is a protein, can be seen in figures 3.4.2 and 3.4.3, where heat inactivation experiments carried out on the culture supernatant resulted in significant reductions in its cyototoxic effects, suggesting that the cytotoxin was at least partially heat sensitive. These results are similar to a number of findings concerning heat sensitive cytotoxin production in *Campylobacter* (Misawa *et al* 1995; Mizuno *et al* 1994). Trypsin inactivation of toxicity gave further support for the existence of a secreted protein toxin (figures 3.4.5 and 3.4.6). A similar reduction in inhibitory effects was also detected in studies by Lee, *et al* (2000), using the *Campylobacter jejuni* strain 81116; and Mahajan and Rodgers (1990), using a virulent clinical *Campylobacter jejuni* strain.

The mechanisms of action of this type of cytotoxin are normally intracellular or through pore formation in target cells (Lee *et al* 2000). If the cytotoxins act intracellularly, they must bind to the cells and be internalised before reaching their target in the cytoplasm (Schmitt *et al* 1999). Cytotoxins which act intracellularly commonly consist of two sub-units, an A sub-unit with enzymatic activity and a pentamer of B sub-units which bind to specific sphingoglycolipid receptors on the host cell membrane (Popoff 1998). The two predominant mechanisms of toxicity are inhibition of cellular protein synthesis and inhibition

of actin filament formation, both leading to cell death (Aktories 1997). Pore formation in the target cells is carried out by cytotoxins classed as cytolysins and lytic activity in erythrocytes is carried out by haemolysins (Sillero and Almirall 1999; Hossain *et al* 1993; Akan *et al* 2000). The formation of pores results in the induction of a broad range of secondary responses, including cytokine release, cytoskeleton dysfunction, secretion of granule constituents and generation of lipid mediators (Wassenar 1997).

From the literature, and especially the report on *C.jejuni* toxin production by Wassenar (1997), it has been suggested that this organism produces several different cytotoxins (summarised in Chapter 1). The evidence of a secreted cytotoxin in this thesis therefore suggests that the toxin could be one of the those already discovered although the possibility that this may be a novel secreted cytotoxin cannot be ignored. Further toxin isolation/characterisation in terms of size, structure and mechanisms of virulence were required in order to determine whether or not the toxin matched the characteristics of an identified cytotoxin.

Evidence of a second type of cytotoxin can also be seen in figures 3.1.1 and 3.4.1/3.4.4. The extracts produced from blood agar cell sonication produced significant cytotoxicity when added to cultured mammalian cells. The lack of effect of heat and trypsin treatments on cytotoxicity suggested that the toxin was heat and trypsin resistant, pointing towards the production of an endotoxin by the bacteria. Endotoxins include antigenic complexes of protein, polysaccharide and lipids. The protein determines the antigenicity of the molecule, the polysaccharide determines the immunological specificity and the lipid the toxicity (Lagrange *et al* 1995). They are derived from and bound

to the bacterial cell wall and are characteristically more heat-stable than exotoxins (Raymond and Alouf 1970). The fact that the bacterial cell sonicate in this chapter was cytotoxic towards proliferating mammalian cells, suggests that the toxin was bound to the cell wall or outer membrane and had been released due to sonication.

All this information suggests a non-protein molecule, loosely bound to the bacterial cell wall, *i.e* an endotoxin. There is less published work on endotoxins compared with exotoxins; purification of toxins from bacterial culture medium seems to be the preferred method. However, investigations in to the effects of bacterial endotoxins on the immune system of the body and also central nervous system have been published. For example Koller *et al* (1994) investigated the effects of bacterial endotoxins on cultured astrocytes and neuronal cells. They found that the endotoxin treatment selectively impaired electrophysiological properties of cultured astrocytes at concentrations comparable to those found in the cerebrospinal fluid of patients with bacterial meningitis. By contrast, resting potential and membrane potential of cultured neurons was unchanged on treatment with the endotoxin (Koller *et al* 1994).

Other aspects of endotoxin toxicity have been described in numerous papers which investigated the effects of the bacterial endotoxins on the immune system and the induction of auto-antibodies being produced as a result of ganglioside mimicry by the endotoxins (specifically the LPS structures of the toxins). This auto-immune response is thought to result in the acute monophasic polyneuropathy GBS, which leads to a heterogenous range of motor and sensory deficits (Jacobs *et al* 1997).

In conclusion this chapter describes the evidence for at least two types of toxins, a heat and trypsin resistant cytotoxin present in cell sonicates from *Campylobacter jejuni* 11351 blood agar cultures and a heat and trypsin sensitive cytotoxin present in the culture supernatant from *Campylobacter jejuni* 11351 BHI broth cultures. Both toxins show consistent cytotoxicity and produced a significant reduction in mammalian cell viability at concentrations of less than 5% (v/v) compared to control cells.

The measurement of cytotoxicity is a non-specific end-point as a result of exposure to high levels of the toxins, but in order to discover the initial effects and identify specific primary toxin targets, investigation into the sub-cytotoxic effects of the toxins were required. Therefore further work on the effects of the toxins on the cytoskeletal networks and general cell morphological changes were studied, the results of which can be observed in the next chapter.

CHAPTER 4

4 Sub-Cytotoxic Effects Of Bacterial Culture Extracts On Cultured Mammalian Cells Results and Discussion.

4.1 General sub-lethal effects on cultured mammalian cell morphology.

Results from Chapter 3 show the presence of at least two cytotoxins in bacterial cell culture extracts capable of causing cell death.

Cell death is a very non-specific end point caused by exposure to relatively high levels of toxin. Studying effects of sub-lethal concentrations of toxins may help to reveal more subtle effects and identify specific/primary toxicity targets.

We chose to look at changes in cell morphology and associated cytoskeletal networks in order to try and determine mechanisms of toxicity in more detail.

Firstly three cell types, mouse neuroblastoma (N2a), human endothelial cell (ECV) and chinese hamster ovary cell (CHO), were stained with Coomassie blue in order to observe any general sub-lethal effects on cultured mammalian cell morphology after treatment with sub-cytotoxic levels of bacterial culture extract.

Figure 4.1.1 shows that there is a distinct elongation of bacterial culture extract treated CHO cells (figure 4.1.1b) when compared with the control treated CHO cells (figure 4.1.1a), indicating the possible presence of a cytolethal distending toxin (CLDT) like toxin. The CLDT has been identified and the gene sequence cloned (Johnson and Lior 1987; Pickett, *et al* 1996). Results from PCR assays using CLDT gene sequence-specific oligonucleotide probes on Campylobacter jejuni 11351 showed the presence

of DNA sequences showing homology to published CLDT gene sequences (Holmes, 2001).

Figure 4.1.1 Effects of *C. jejuni* 11351 BHI- cell culture supernatant on the morphology of CHO cells.

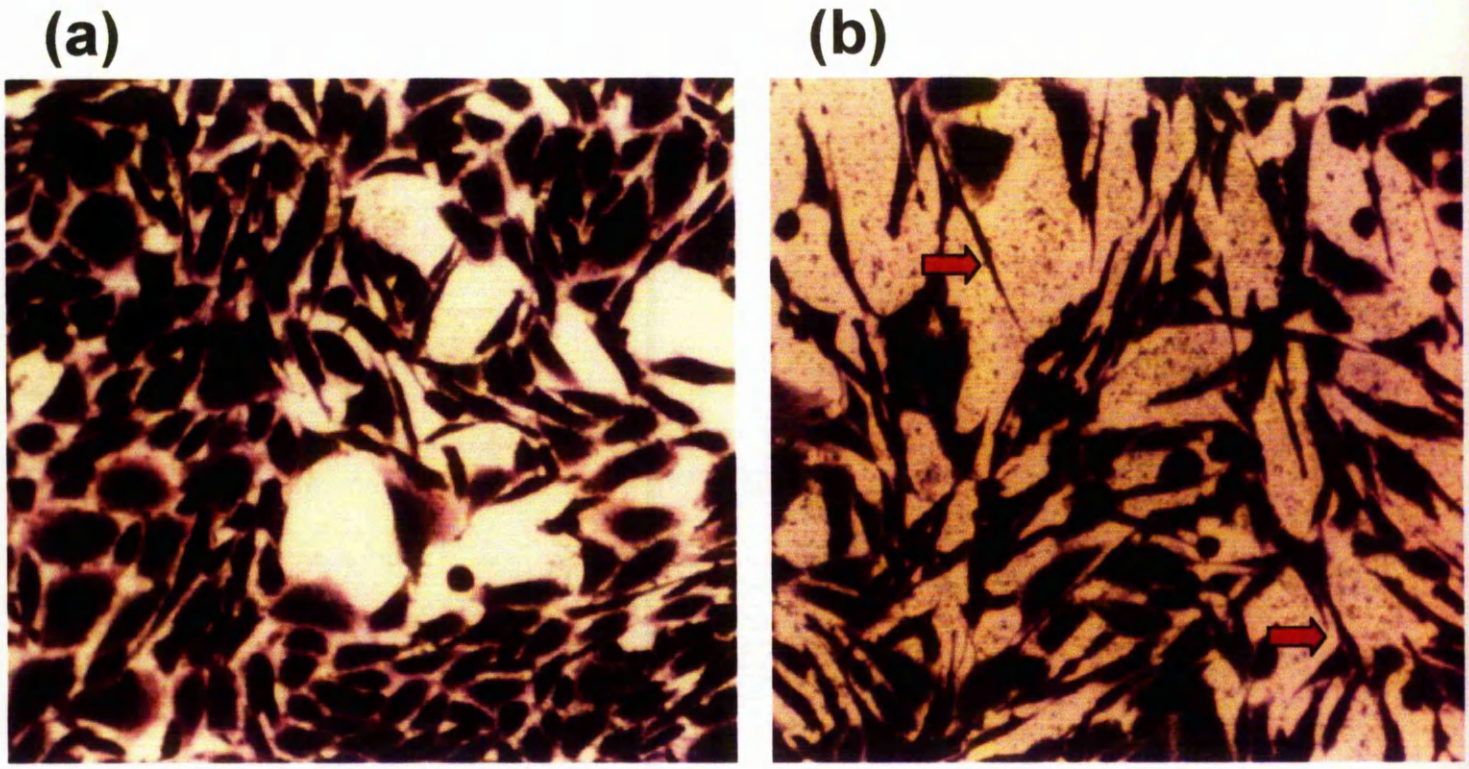


Figure 4.1.1. Images of Coomassie blue stained CHO cells. The cells were plated out into 24 well culture plates, then given 24 hours recovery period, before induction of differentiation by removal of serum. At the same time the cells were exposed to (a) BHI- broth control 1% (v/v) and (b) C. jejuni 11351 BHI- culture supernatant 1% (v/v) for 48 hours, before cells were fixed and stained. Distending cells are indicated by the red arrows.

Figure 4.1.2 shows the Coomassie blue staining of human endothelial cells (ECV) incubated with BHI- broth control (figure 4.1.2a) and BHI- culture supernatant (figure 4.1.2b). The difference in cell morphology compared to controls is less significant than in the CHO figures (figure 4.1.1). However, there are a number of cells that are more elongated compared to the control cells in figure 4.1.2b. This again suggests the presence of a cytolethal distending toxin, such as that described by Johnson and Lior (1987). But there are also a number of more rounded cell bodies in the treated cell figure, compared to the more flattened cells in the control cell figure. This suggests the presence of another toxin, previously identified as the cytolethal rounding toxin by Schulze, *et al* (1998). However, the morphological differences between control treated and bacterial culture extract treated are not as striking as those seen in the CHO (figure 4.1.1) or the N2a cells (figure 4.1.3).

Figure 4.1.2. Effects of *C. jejuni* 11351 BHI- cell culture supernatant on the morphology of ECV cells.

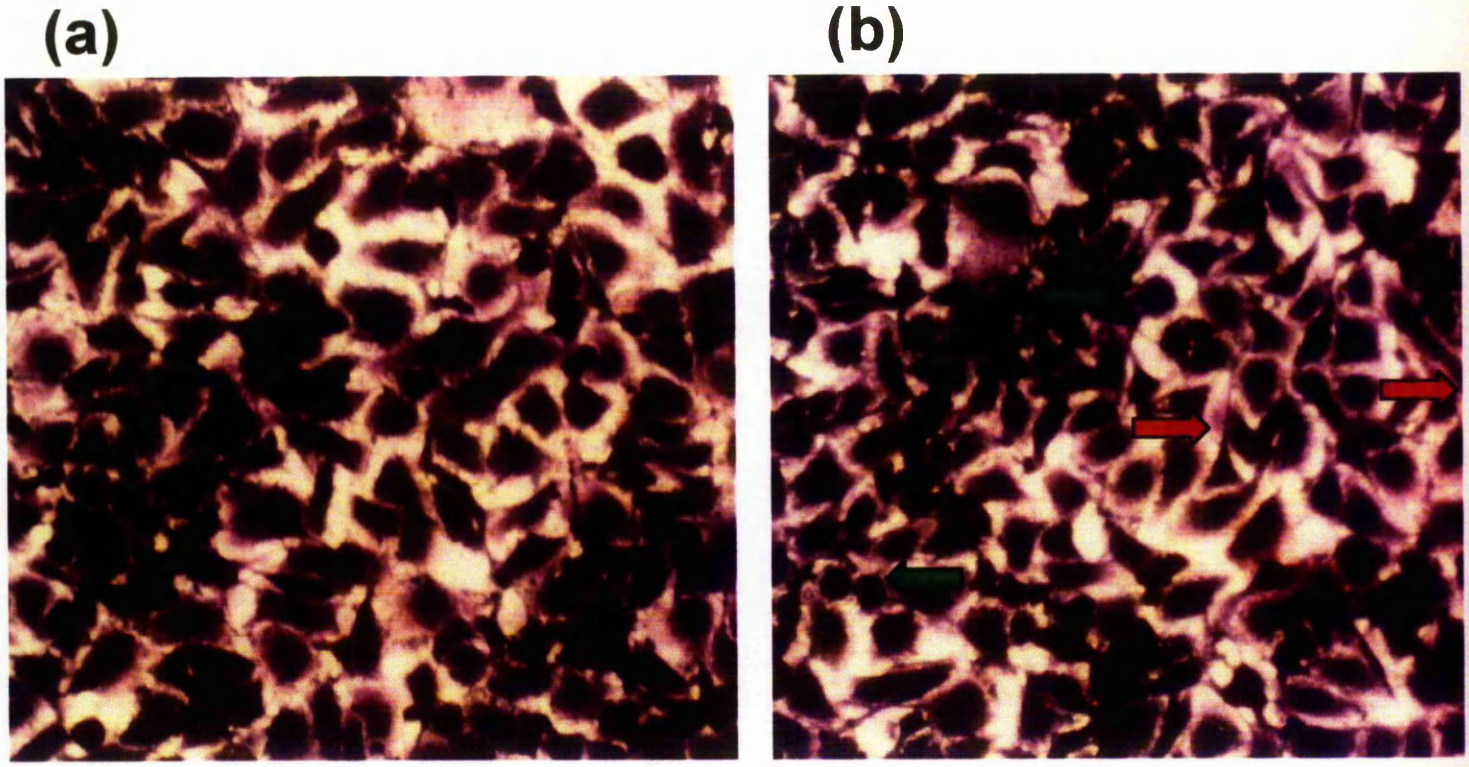


Figure 4.1.2. Images of Coomassie blue stained ECV cells. The cells were plated out into 24 well culture plates, then given 24 hours recovery period, before induction of differentiation by removal of serum. At the same time the cells were exposed to (a) BHI- broth control 1% (v/v) and (b) C. jejuni 11351 BHI- culture supernatant 1% (v/v) for 48 hours, before cells were fixed and stained. Distending cells are indicated by the red arrows. Rounded cells are indicated by green arrows.

Figure 4.1.3 shows the Coomassie blue staining of mouse neuroblastoma (N2a) cells, incubated with BHI- broth control (figure 4.1.3a) and BHI- culture supernatant (figure 4.1.3b). The difference in cell morphology compared to controls is more significant than in either the CHO figures (figure 4.1.1), or the ECV figures (figure 4.1.2). The cells show a distinct reduction in axonal outgrowths, in both number and size, when comparing bacterial culture supernatant treated (figure 4.1.3a) to control treated cells (figure 4.1.3b). This dramatic effect on cells at a sub-cytotoxic treatment level has previously been described by Flaskos *et al* (1998), when testing the toxic compound tricresyl phosphate on differentiating N2a cells.

Figure 4.1.3. Effects of *C. jejuni* 11351 BHI- cell culture supernatant on the morphology of N2a cells.

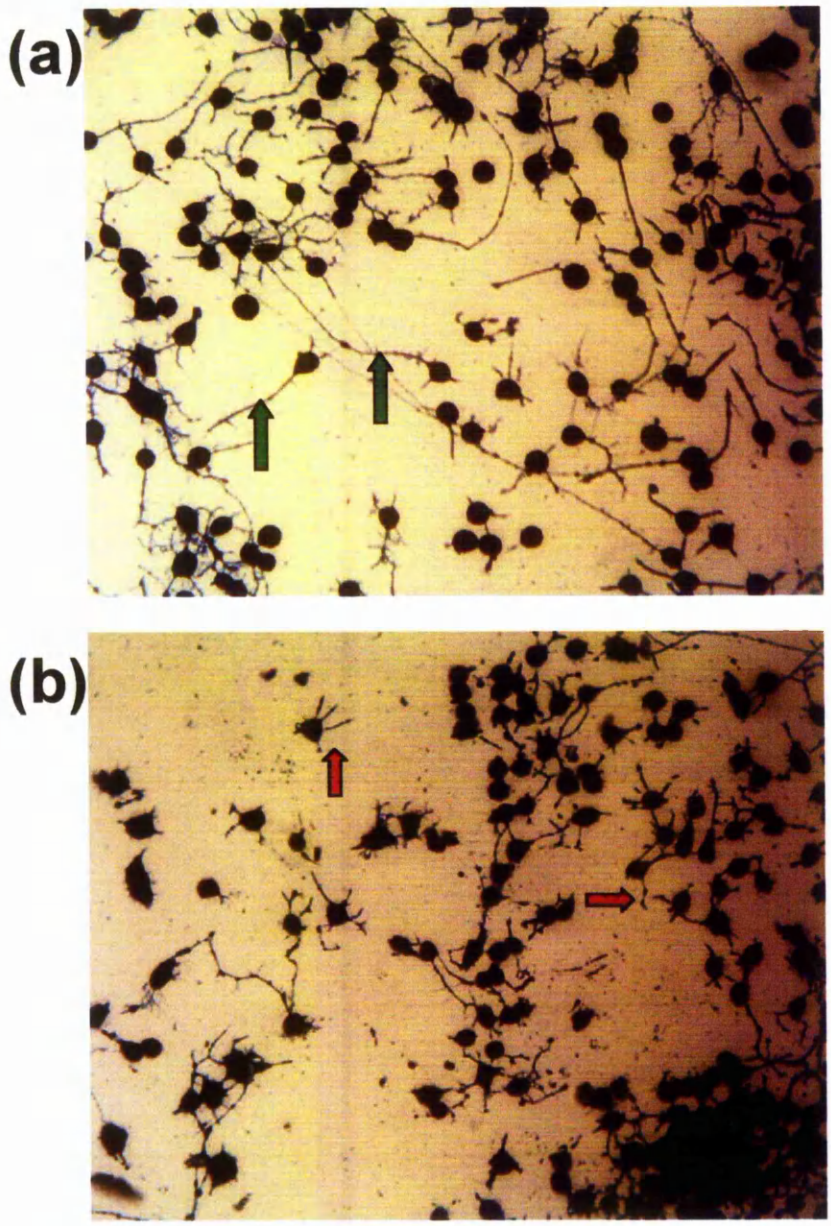


Figure 4.1.3. Images of Coomassie blue stained N2a cells. The cells were plated out into 24 well culture plates, then given 24 hours recovery period, before induction of differentiation by addition of sodium butyrate. At the same time the cells were exposed to (a) BHI- broth control 1% (v/v) and (b) C. jejuni 11351 BHI- culture supernatant 1% (v/v) for 48 hours, before cells were fixed and stained. Reductions in axon outgrowth size in treated cells are indicated by the red arrows. Normal axon outgrowth in control cells indicated by green arrows.

4.2.1 Effects of sub-lethal levels of toxin on N2a cells.

As the mouse neuroblastoma cells were the most sensitive to toxin treatment, it was of particular interest to study the effects of toxin on cell morphology in this cell type. Measurement of axon growth has been used successfully as a marker of sub-lethal effects for a range of toxins (Keilbaugh *et al* 1991; Shea *et al* 1991; Flaskos *et al*; 1998; Sachana *et al* 2001). Therefore, effects on axonal growth in N2a cells were used as a marker for sub-lethal effects of the toxins present in our bacterial culture extracts.

It should be noted however, that sub-cytotoxic treatment of CHO, ECV and C6 cell lines was carried out, but effects on cell morphology were not quantified sufficiently to include in the thesis.

Figure 4.2.1.1 shows the effects of a number of bacterial cell extracts taken from blood agar cultures stopped at different stages of growth. The 2 day and 7 day old bacterial extracts did not produce a significant sub-cytotoxic effect, but the 5 day old bacterial extracts produced a significant decrease in axon outgrowth compared with control cells ($p<0.01$). This suggests that different degrees of toxin expression or different toxin production occur at different stages of bacterial cell growth.

Figure 4.2.1.1. Sub-cytotoxic effects of *Campylobacter jejuni 11351* blood agar plate cell sonicates on differentiating mouse neuroblastoma (N2a) cells.

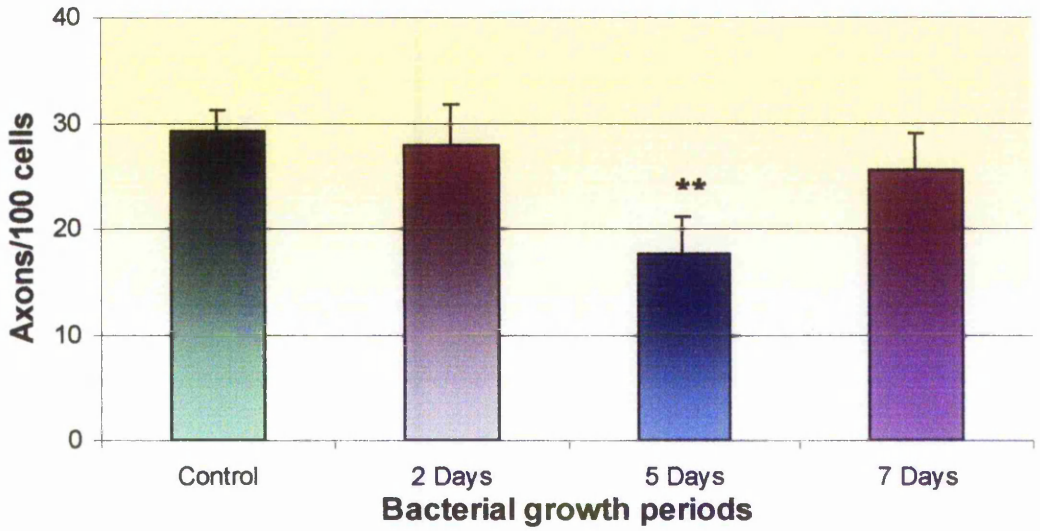


Figure 4.2.1.1. Shows the sub-cytotoxic effects of *C. jejuni* 11351 blood agar cell sonicates. Axon outgrowth was measured in the presence and absence of bacterial cell sonicates as described in Methods (section 2.2.1.12). Data are expressed as mean number of axons per 100 cells ± standard error (n=5). Asterisks indicate significance of differences compared to controls as follows: **, $p<0.01$.

Figure 4.2.1.2 shows the effects of sub-lethal concentrations of BHI- culture supernatant on axonal growth by differentiating N2a cells. Statistical analysis showed the reduction in axon growth to be extremely significant ($p< 0.001$).

Figure 4.2.1.2. Sub-cytotoxic effects of *Campylobacter jejuni 11351* BHI-culture supernatant on differentiating mouse neuroblastoma (N2a) cells.

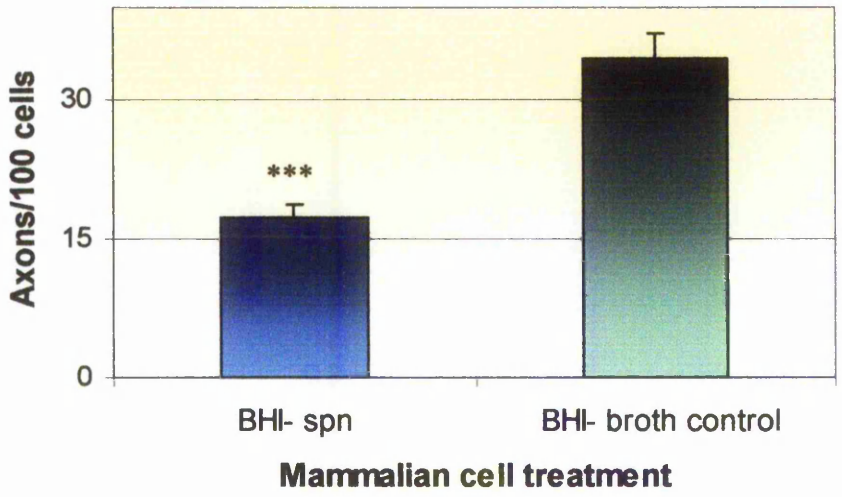


Figure 4.2.1.2 Shows the sub-cytotoxic effects of *C. jejuni* 11351 BHI- broth culture supernatant. Axon outgrowth was measured in the presence and absence of bacterial culture supernatant as described in Methods (section 2.2.1.12). Data are expressed as mean number of axons per 100 cells ± standard error (n=5). Asterisks indicate significance of differences compared to controls as follows: ***, $p<0.001$.

Figures 4.2.1.3 shows the sub-cytotoxic effects of BHI+ culture extract treatments on N2a cells. It can be seen that the level of axon growth in the BHI+ broth culture supernatant treated cells was significantly lower than the axon growth counted in control cells ($p< 0.001$).

Figure 4.2.1.3. Sub-cytotoxic effects of *Campylobacter jejuni 11351* BHI+ culture supernatant on differentiating mouse neuroblastoma (N2a) cells.

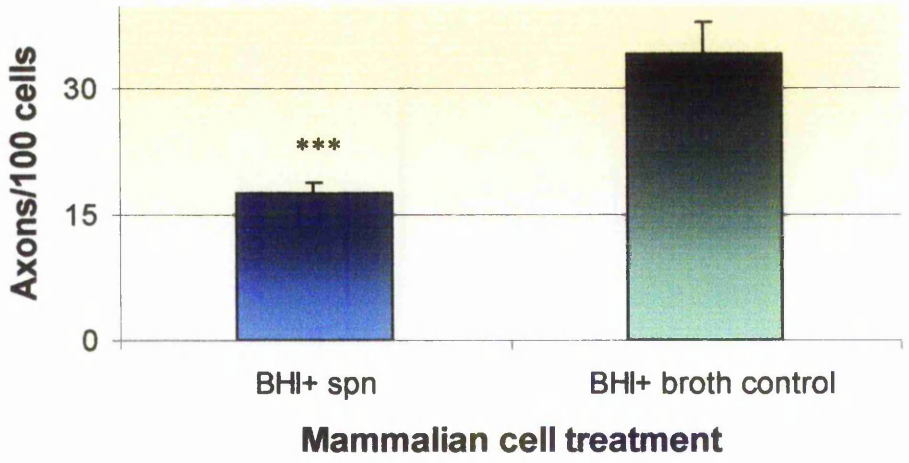


Figure 4.2.1.3 Shows the sub-cytotoxic effects of *C. jejuni* 11351 BHI+ broth culture supernatant. Axon outgrowth was measured in the presence and absence of bacterial culture supernatant as described in Methods (section 2.2.1.12). Data are expressed as mean number of axons per 100 cells ± standard error (n=5). Asterisks indicate significance of differences compared to controls as follows: ***, $p<0.001$.

4.2.2. Effects of heat on neurotoxicity of BHI culture supernatant.

Further investigation into the nature of these sub-cytotoxic effects can be seen in figures 4.2.2.1 and 4.2.2.2. Here, the effects of heat (100°C for 10 minutes) on the toxicity of culture supernatants are shown.

Figure 4.2.2.1 shows higher levels of axon outgrowth in N2a cells incubated with heat treated, compared to non-heat treated culture supernatants ($p<0.001$), suggesting inactivation of axon-inhibitory neurotoxins present in the extract. This shows that a heat-labile neurotoxin was present in the extract.

Figure 4.2.2.1 shows the effects of heating on the sub-lethal effects of culture extracts from *Campylobacter jejuni* cultured in BHI- broth, on cultured mouse N2a cells. The graph shows that there is a significant reduction in neurotoxicity after heat treatment of the extracts, when compared to axon out-growth in cells exposed to bacterial culture extracts without heat treatment ($p<0.001$). This suggests the presence of a heat-sensitive neurotoxin, which may therefore be proteinaceous in structure.

Figure 4.2.2.1. Sub-cytotoxic effects of *Campylobacter jejuni 11351* BHI- culture supernatant after heating on differentiating mouse neuroblastoma (N2a) cells.

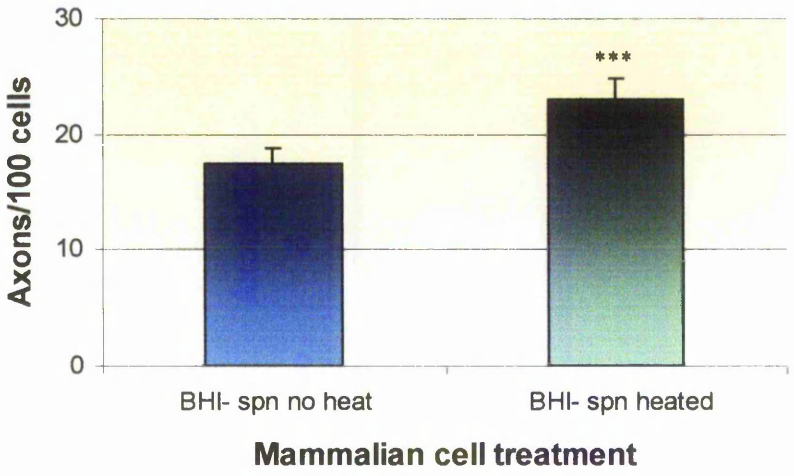


Figure 4.2.2.1. Shows the sub-cytotoxic effects of *C. jejuni* 11351 BHI- broth culture supernatant before and after heating. Axon outgrowth was measured in the presence of heat or non-heat treated bacterial culture supernatant as described in Methods (section 2.2.1.12). Data are expressed as mean number of axons per 100 cells ± standard error (n=5). Asterisks indicate significance of differences compared to controls as follows: ***, $p<0.001$.

Figure 4.2.2.2 shows the effects of heating on the sub-lethal effects of culture extracts from *Campylobacter jejuni* cultured in BHI+ broth, on cultured mouse

neuroblastomas. The graph shows that there is a significant reduction in neurotoxicity after heat treatment of the extracts, when compared to axon out-growth in cells exposed to bacterial culture extracts without heat treatment ($p<0.05$), again suggesting the presence of a heat-sensitive neurotoxin.

Figure 4.2.2.2. Sub-cytotoxic effects of *Campylobacter jejuni 11351* BHI+ culture supernatant after heating on differentiating mouse neuroblastoma (N2a) cells.

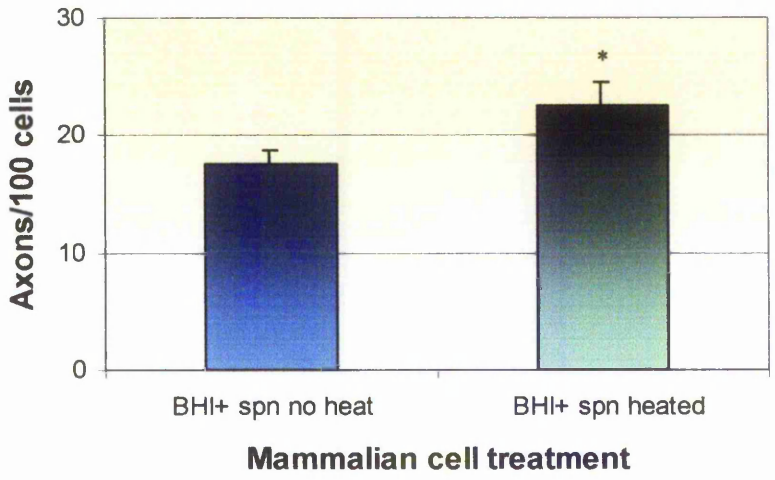


Figure 4.2.2.2. Shows the sub-cytotoxic effects of *C. jejuni* 11351 BHI+ broth culture supernatant before and after heating. Axon outgrowth was measured in the presence of heat or non-heat treated bacterial culture supernatant as described in Methods (section 2.2.1.12). Data are expressed as mean number of axons per 100 cells ± standard error (n=5). Asterisks indicate significance of differences compared to controls as follows: *, $p<0.05$.

The above effects observed on axon outgrowth suggest the presence of a toxin(s) which could cause changes in cell morphology at sub-cytotoxic levels.

Therefore the next step was to investigate the sub-cytotoxic effects on specific cytoskeletal proteins which are important in control of cell shape. These proteins can therefore be used as early markers of toxicity following exposure to bacterial toxin. Although this approach does not establish whether proteins are affected directly or indirectly, the assays do provide a quantitative measure of sub-cytotoxic effects on cytoskeletal protein levels after bacterial toxin exposure.

4.3 Western blot analysis

The intention was to compare the levels of specific cytoskeletal proteins present in the differentiated cells and compare their levels in treated and control cells under the same conditions used for measurement of axon growth. This would indicate which proteins were affected, either directly or indirectly, by the neurotoxins. As mentioned earlier, this was carried out using Western blotting techniques, the results of which can be seen in figures 4.3.1 to figure 4.3.3.

Figure 4.3.1 shows the sub-cytotoxic effects of blood agar cell sonicates from *Campylobacter jejuni* 11351 on the levels of cytoskeletal proteins in extracts from mouse N2a and human ECV cells.

As shown in figure 4.3.1a cross-reactivity of antibodies with α-tubulin reduced in toxin treated N2a cells compared to untreated control cells, suggests a decrease in tubulin levels after toxin exposure. By contrast the alpha tubulin levels in ECV cells showed an increase after toxin exposure. In either case,

the figure shows an effect on protein levels, either resulting in an increase or decrease when compared to control treated cell levels.

Figure 4.3.1b shows cross-reactivity of antibodies against acetylated alpha tubulin. In this figure the blot again shows a reduction in the levels of acetylated tubulin in the N2a cells, but, again there is a slight rise in acetylated tubulin levels in toxin treated ECV cells.

Figure 4.3.1c shows cross-reactivity of cell extracts with antibodies that recognise actin. Once again, in the case of cell extracts taken from blood agar plates and added to the mammalian cells, there is evidence of a decrease in actin protein levels when comparing treated to control N2a cells, but an increase in actin protein levels when comparing treated to control ECV cells. Thus, a change in cytoskeletal protein levels was detected in toxin treated cells, which implies a sub-cytotoxic effect due to cell extract exposure.

Figure 4.3.1. Effects of sub-cytotoxic levels of *C. jejuni* 11351 blood agar cell sonicates on mammalian cytoskeletal proteins.

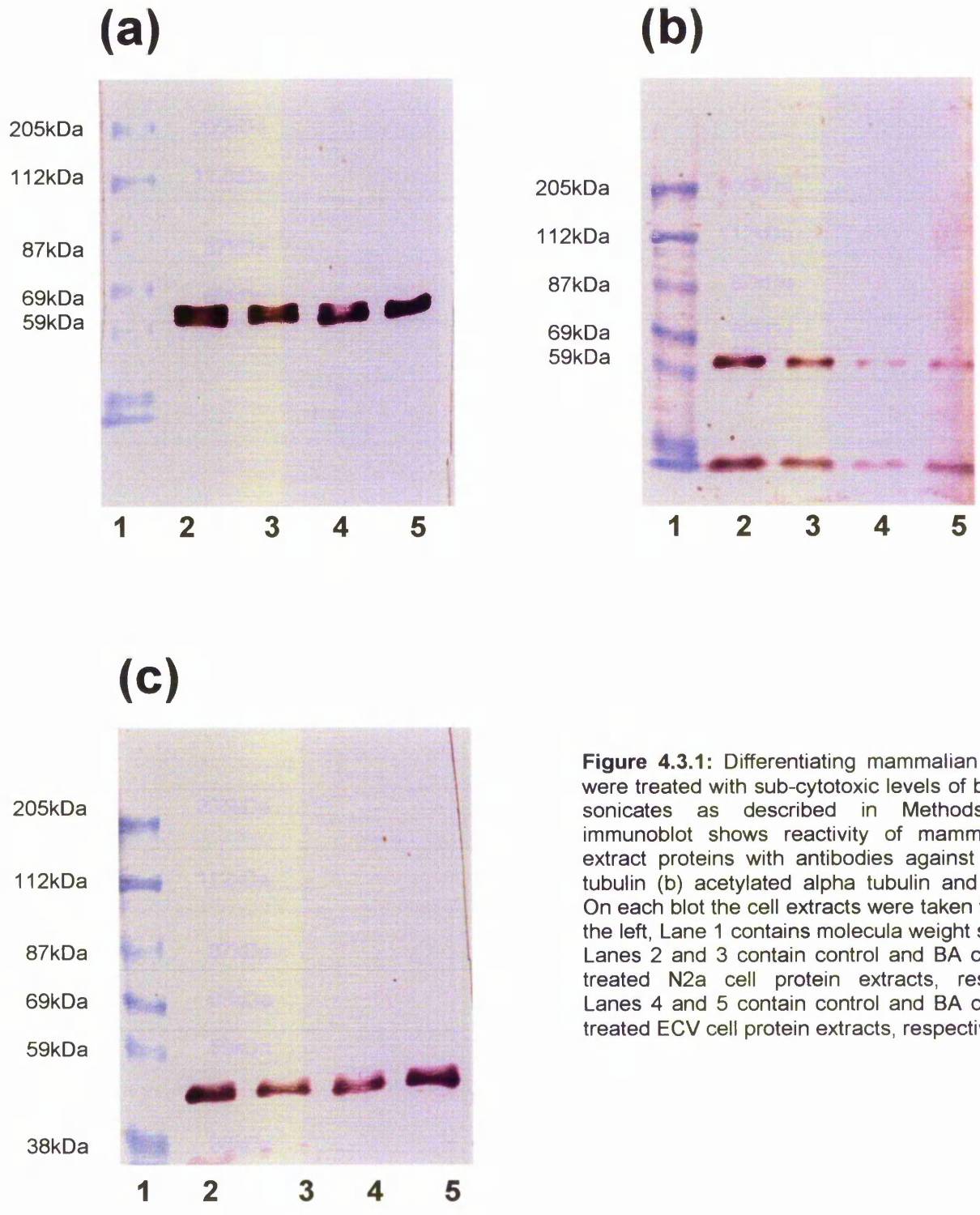


Figure 4.3.1: Differentiating mammalian cell lines were treated with sub-cytotoxic levels of blood agar sonicates as described in Methods. The immunoblot shows reactivity of mammalian cell extract proteins with antibodies against (a) alpha tubulin (b) acetylated alpha tubulin and (c) actin. On each blot the cell extracts were taken from: from the left, Lane 1 contains molecula weight standards. Lanes 2 and 3 contain control and BA cell extract treated N2a cell protein extracts, respectively. Lanes 4 and 5 contain control and BA cell extract treated ECV cell protein extracts, respectively.

Similar results were obtained on Western blotting analysis of proteins from N2a cells treated with BHI- culture supernatant (figure 4.3.2). There is a clear reduction in the cross reactivity of the antibody in toxin-treated cells, indicating that a reduction in the levels of tubulin is associated with the inhibition of axon growth.

Probing of lanes 4 and 5, which contain proteins taken from control and toxin treated ECV cells, shows that there is a slight decrease in tubulin level, but not as pronounced as in the N2a cells. Figure 4.3.2b shows cross-reactivity of cell extracts with monoclonal antibody 611B1, which recognises acetylated alpha tubulin, a post translationally modified form of tubulin associated with stable populations of axonal microtubules (Polevoda and Sherman 2002). This was done to investigate whether the neurotoxin was affecting the process of post translational tubulin modification. On the blot, lanes one and two again show proteins taken from control treated (lane 2) and culture extract treated (lane 3) N2a cell extracts. The results are similar to the reduction in protein levels seen in figure 4.3.2a. Lanes three and four show proteins taken from ECV 304 cells, again as a control to investigate whether the effects of the toxin are cell line specific. Again it can be observed that there is a definite decrease in acetylated-tubulin alpha sub-unit reactivity although the overall level of acetylated tubulin was lower than in the neuronal cells. This shows that the sub-cytotoxic effects on tubulin levels are not cell type specific.

Figure 4.3.2c shows cross-reactivity of cell extracts with an antibody that recognises the microfilament protein, actin. Again the treated cells (lane 3) showed a decrease in actin levels compared with the untreated control cells (lane 2). The same can be said for the actin protein levels seen in the ECV

cells (lanes 4 and 5), which also showed a significant decrease in toxin treated cells compared with control cells.

The probed blot shown in figure 4.3.2d shows cross-reactivity with the neurofilament heavy chain polypeptide (200kDa). The bands in lanes one and two are cross-reactive proteins in N2a cell extracts, treated as before. The bands show a reduction in levels of NFH in the toxin treated cells (lane 3) compared to control cells (lane 2). Lanes 4 and 5 contain proteins taken from the control (C6) cell line. This blot shows no presence of neurofilament protein bands, as the protein is cell type specific and is confined to neuronal cells. Since the C6 are glial cells, they make a good negative control for this antibody.

Figure 4.3.2. Effects of sub-cytotoxic levels of *C. jejuni* 11351 BHI- broth culture supernatant on mammalian cytoskeletal proteins.

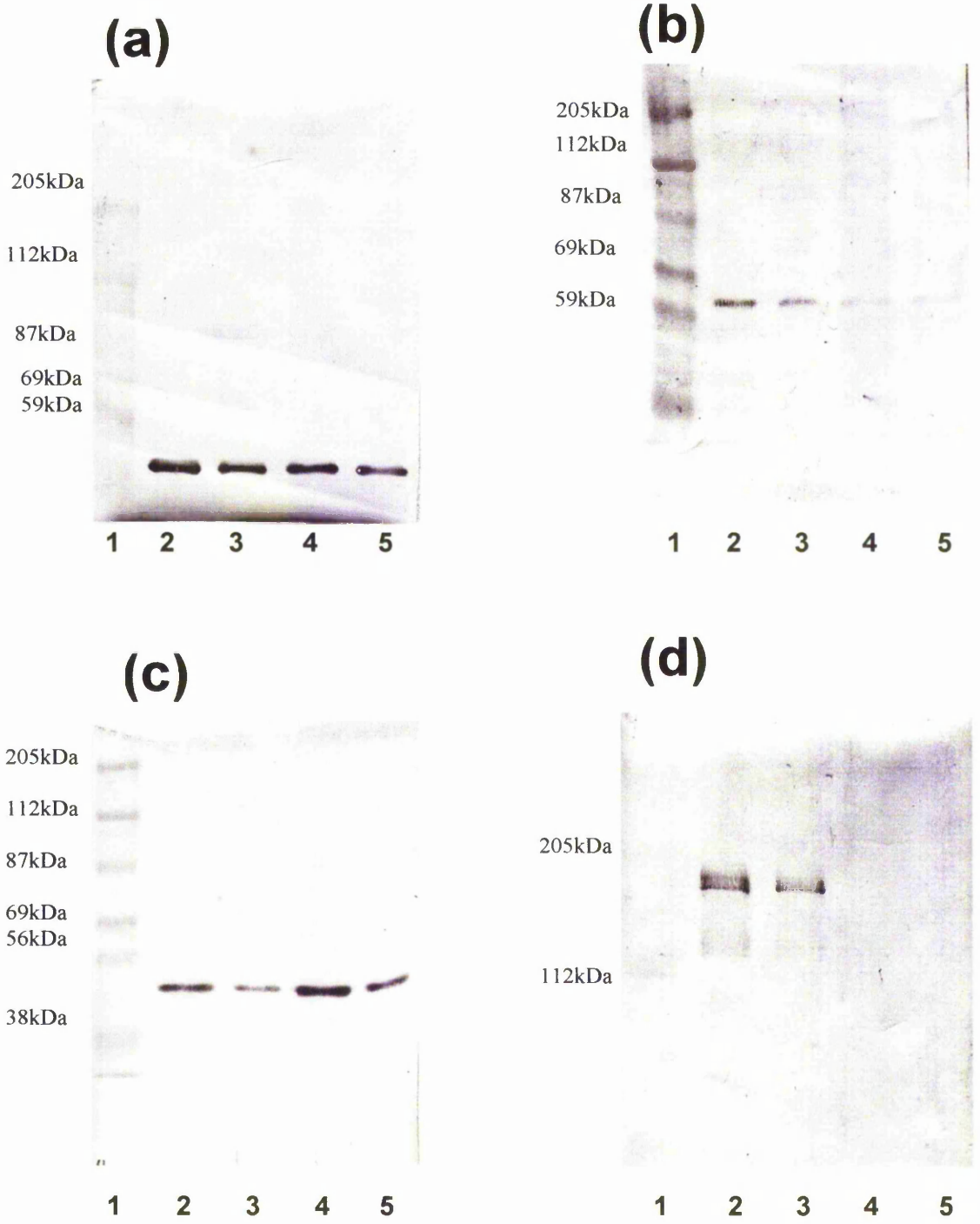


Figure 4.3.2. Differentiating mammalian cell lines were treated with sub-cytotoxic levels of BHI- broth culture superntatant as described in Methods. The immunoblot shows reactivity of mammalian cell extract proteins with antibodies against (a) alpha tubulin (b) acetylated alpha tubulin (c) actin and (d) neurofilament heavy chain. On each blot the cell extracts were taken from: from the left, Lane 1 contains molecular weight standards. Lanes 2 and 3 contain control and BHI- culture supernatant treated N2a cell protein extracts, respectively. Lanes 4 and 5 contain control and BHI- culture supernatant treated ECV cell protein extracts, respectively; except in figure (d), where lanes 4 and 5 contain C6 protein extracts.

Figure 4.3.3a shows cross-reactivity of extracts from BHI+ culture supernatant treated mammalian cells with anti-alpha tubulin. The immunoblot shows a marked reduction in tubulin levels after toxin treatment of both cell lines (as observed in the BHI- culture supernatant and blood agar sonicate treated cell samples), suggesting the production of toxin(s) with similar sub-cytotoxic effects on cytotoskeletal proteins.

Figure 4.3.3b shows cross-reactivity of BHI+ treated samples with monoclonal antibody, 611B1, against acetylated alpha tubulin sub-units. The figure shows that there was a significant reduction in acetylated tubulin levels in bacterial culture supernatant treated cells compared with control treated cells (comparable with figure 4.3.2b).

Figure 4.3.3c shows cross-reactivity with BHI+ treated samples with monoclonal antibodies against actin. Similar to the microtubule proteins, the actin levels are shown to decrease as a result of toxin treatment. This was detected in both cell types and supported the evidence for a BHI broth culture supernatant toxin which produces a decrease in microtubule, microfilament and intermediate filament cytoskeletal protein levels (figure 4.3.2 and 4.3.3).

Figure 4.3.3. Effects of sub-cytotoxic levels of *C. jejuni* 11351 BHI+ broth culture supernatant on mammalian cytoskeletal proteins.

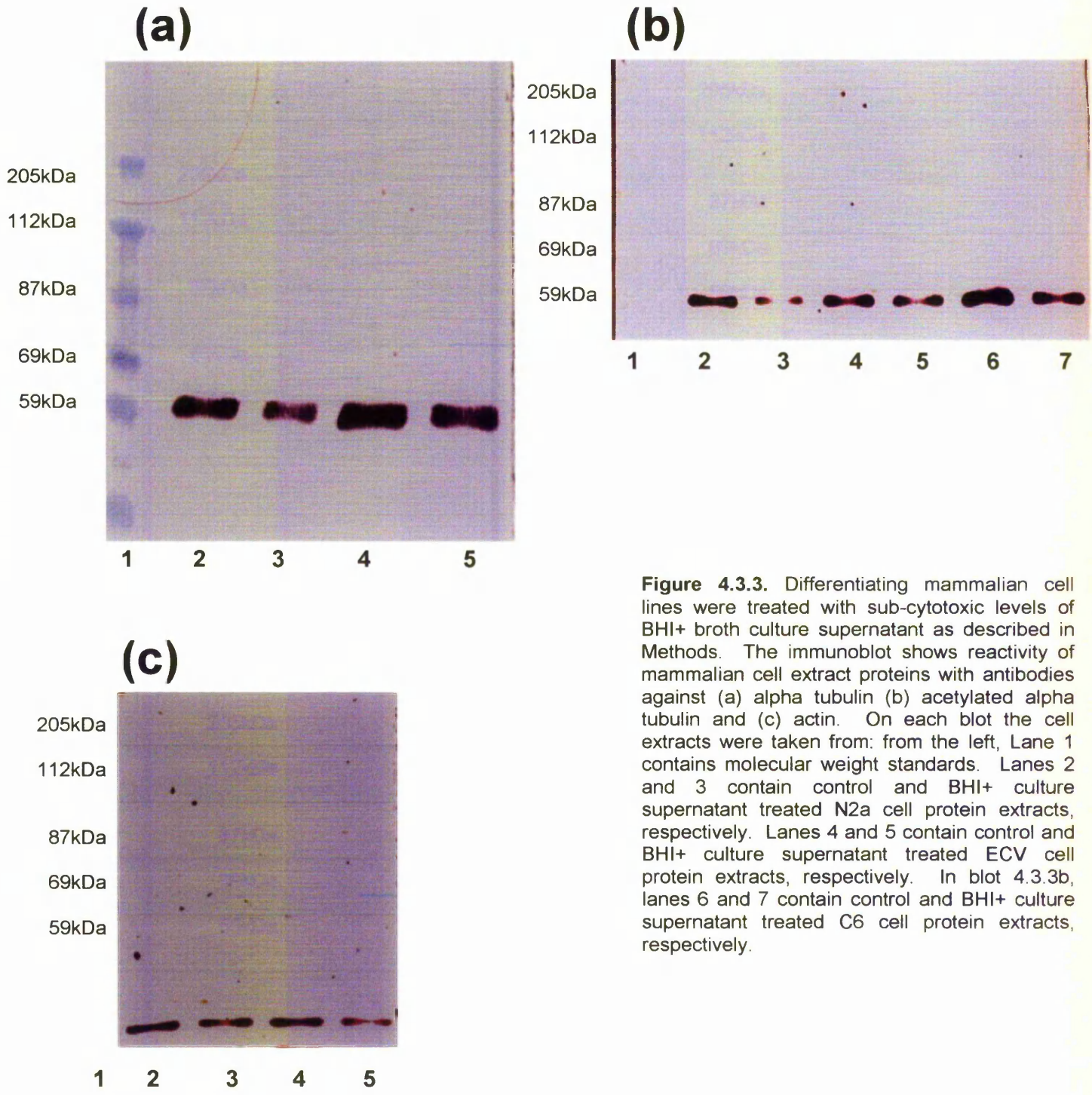


Figure 4.3.3. Differentiating mammalian cell lines were treated with sub-cytotoxic levels of BHI+ broth culture supernatant as described in Methods. The immunoblot shows reactivity of mammalian cell extract proteins with antibodies against (a) alpha tubulin (b) acetylated alpha tubulin and (c) actin. On each blot the cell extracts were taken from: from the left, Lane 1 contains molecular weight standards. Lanes 2 and 3 contain control and BHI+ culture supernatant treated N2a cell protein extracts, respectively. Lanes 4 and 5 contain control and BHI+ culture supernatant treated ECV cell protein extracts, respectively. In blot 4.3.3b, lanes 6 and 7 contain control and BHI+ culture supernatant treated C6 cell protein extracts, respectively.

Figure 4.3.4 shows cross-reactivity of cell extracts with a monoclonal that recognises heat shock protein-70. This protein was used as a "house-keeping" protein and used as a marker to show constitutive gene expression in treated and control treated cells.

The immunoblot shows that in all three cell lines, protein levels do not significantly alter when comparing treated with untreated cell samples. This suggests that any changes in cytoskeletal protein levels are not due to changes in protein loading or differences in protein expression between cell lines, but as a direct/indirect result of exposure to bacterial culture extracts.

Figure 4.3.4. Immunoblots of *Campylobacter jejuni* 11351 BHI- culture supernatant treated mammalian cells probed with antibody against heat shock protein-70.

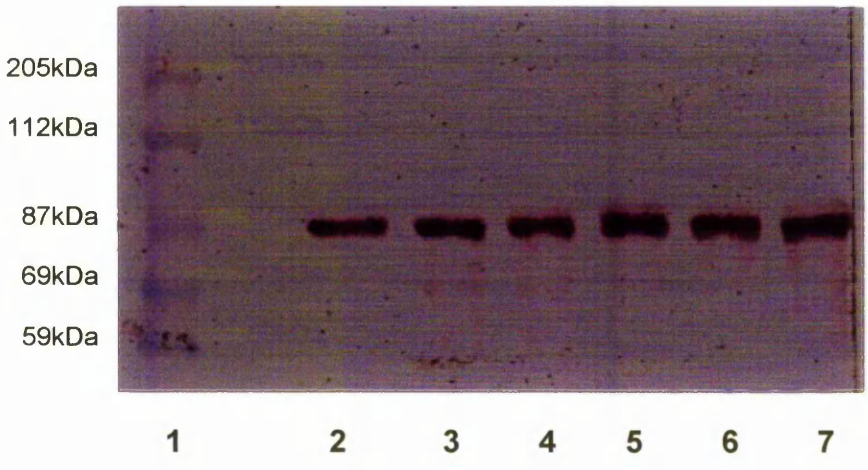


Figure 4.3.4. Differentiating mammalian cell lines were treated with sub-cytotoxic levels of BHI+ broth culture supernatant as described in Methods. The immunoblot shows reactivity of mammalian cell extract proteins with antibodies against heat shock protein 70. On each blot the cell extracts were taken from: from the left, Lane 1 contains molecular weight standards. Lanes 2 and 3 contain control and BHI- culture supernatant treated N2a cell protein extracts, respectively. Lanes 4 and 5 contain control and BHI- culture supernatant treated ECV cell protein extracts, respectively. Lanes 6 and 7 contain control and BHI- culture supernatant treated C6 cell protein extracts, respectively.

Since the probing of Western blots suggests that cytoskeletal protein levels were altered by exposure to toxin, it was of further interest to determine any effects of toxin on the intracellular distribution of the 3 cytoskeletal networks. Therefore N2a cells were induced to differentiate in the presence and absence of toxins and cytoskeletal networks were stained by indirect immunofluorescence. For these experiments, only the mouse neuroblastoma cell line (N2a) was used, as this particular cell line displayed the most prominent reductions in cytoskeletal protein levels as seen in the immunoblot figures above.

4.4 Confocal fluorescence microscopy of actin and tubulin distribution in differentiating N2a cells treated with bacterial extracts

The images of control and toxin treated neuroblastoma cells, which can be seen in figures 4.4.1 and 4.4.2 allow visualisation of any changes in the intracellular distribution of the proteins in the cytoskeleton following toxin exposure.

Figure 4.4.1 shows the distribution of tubulin in the control and toxin treated cells. The intensity of tubulin staining was relatively weak in the treated cells, which showed no distinct lines pattern when compared with control cells. Staining intensity of microtubules was much greater in the control treated cells, and the distribution of tubulin appeared to be more universal throughout the cell body and axonal extensions compared with the BHI- culture supernatant treated cells. In figure 4.4.1b, it can be observed that in BHI-culture supernatant treated cells, there is a relatively strong fluorescence

around the axonal extensions compared with the cell body cytoskeleton and there are also distinct "localised" areas of fluorescence around the cell body area.

Figure 4.4.1. Indirect immunofluorescence localisation of total tubulin in BHI- broth control (a) and culture extract (b) treated N2a cells.

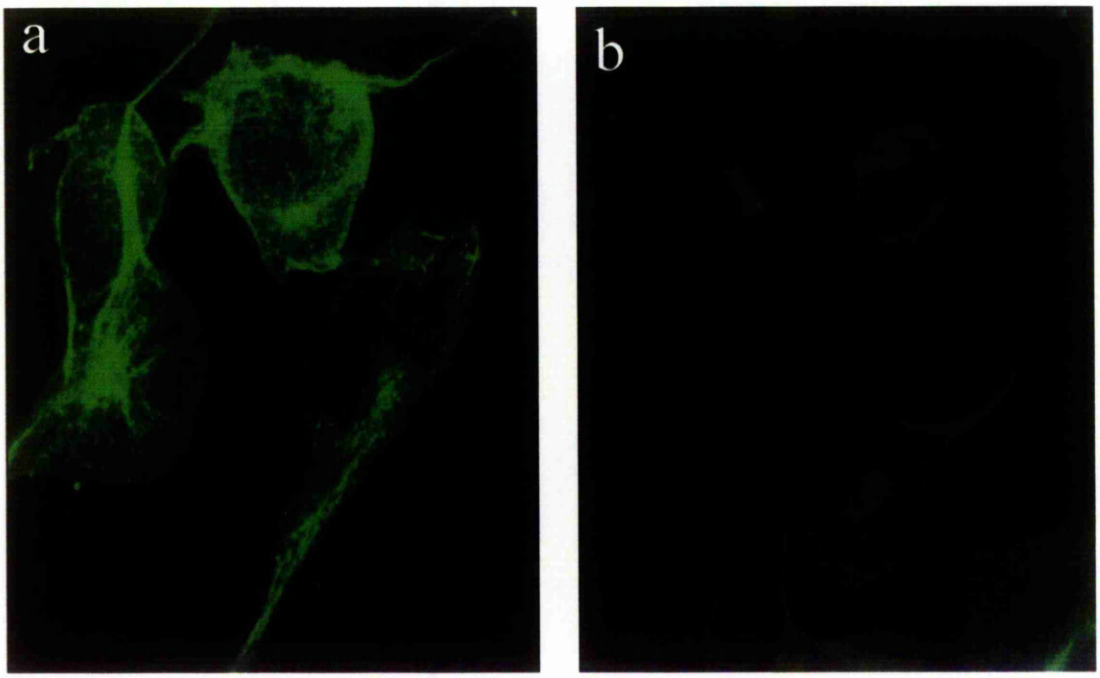


Figure 4.4.1. Shown are confocal images of microtubule networks stained by anti-alpha tubulin antibody B512, in control (a) and BHI- culture supernatant treated (b) N2a cells. The cells were grown overnight, induced to differentaite and exposed to the toxins for 48 hours. The cells were then fixed and microtubule proteins immunofluorescence stained. Images show a distinct reduction in toxin treated (b) cell protein immunostaining compared with control (a) treated cells.

Figure 4.4.2 shows the distribution of microfilaments in BHI- broth control treated (a) and *C. jejuni* 11351 BHI- culture supernatant treated (b) cells, again illustrating the decrease in protein order. Actin staining was detected throughout the cell body and axonal extensions of the control broth treated cells. However, in the culture extract treated cells, fluorescence was restricted mainly to the axonal extensions and to distinct regions around the cell body, suggesting protein tangling.

Again, fluorescence was relatively bright in the control broth treated cells, suggesting a reduction in actin-antibody reactivity, which agrees with the reduction in total cellular actin protein levels seen on Western blots.

Figure 4.4.2. Indirect immunofluorescence localisation of actin proteins in control and BHI- culture supernatant treated N2a cells.

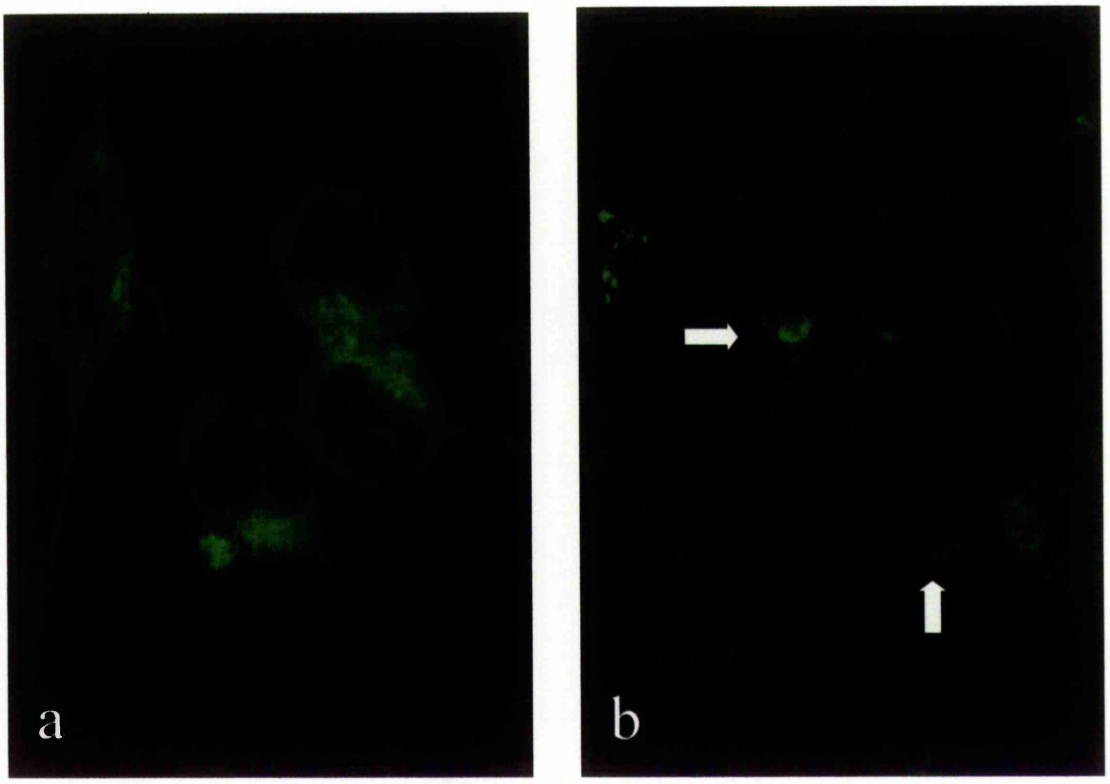


Figure 4.4.2. Shown are confocal images of microfilament networks stained by anti-actin antibody, in control (a) and BHI- culture supernatant treated (b) N2a cells. The cells were grown overnight, induced to differentaite and exposed to the toxins for 48 hours. The cells were then fixed and microfilament proteins immunofluorescence stained. Images show a distinct reduction in toxin treated (b) cell protein immunostaining compared with control (a) treated cells. Localisation of protein staining in toxin treated (b) cells is indicated with arrows.

Results from the Western blotting and indirect immunofluorescence assays suggests that the toxins are able to cause changes in the levels and intracellular distribution of tubulin, a number of assays were carried out to assess the effects of the same concentrations of *C. jejuni* 11351 blood agar cell sonicates and BHI culture supernatants, on microtubule assembly *in vitro*.

Axonal stability is dynamic and requires assembly of microtubules at one end of the axon and disassembly of microtubules at the other (Black 1994). This keeps the axon in a balanced regenerating state. Therefore one possible method of pathological interference by the bacterial toxins could be to disrupt the assembly of tubulin into microtubules.

Inhibition of microtubule assembly has been used as a method for detection of toxicity by a number of groups (Fowler *et al* 2001; Sachanna *et al* 2001; De Girolamo *et al* 2000) and so is a developed assay that is easily carried out.

4.5 Effects of *Campylobacter jejuni* 11351 culture extracts on porcine brain microtubule assembly *in vitro*

The effects of *C. jejuni* 11351 blood agar cell sonicates and BHI- culture supernatant on microtubule assembly were studied using porcine brain cytosol as a source of tubulin.

The samples were placed in centrifuge tubes on ice and a reaction mixture was added, including either 1% control or 1% bacterial culture extract (unpurified blood agar cell sonicate, or BHI- culture supernatant). Microtubule polymerisation was allowed to take place at 37°C for 30 minutes in the presence of 1mM GTP. Microtubule polymer was then spun down in an ultra-centrifuge and microtubule protein pellets were re-suspended in readiness for gel electrophoretic separation. Equal volumes of samples were loaded onto the gel and levels of protein were compared between control and bacterial culture extract treated samples.

Figure 4.5.1 shows a photograph of electrophoretically separated proteins from samples treated with BHI- broth control and BHI- culture supernatant extracts. The figure shows that 8μl and 6μl volumes overloaded the lane and so no more than 5μl were added to future gels.

It can be seen from the figure that there is a significant increase in staining intensity of microtubule proteins in the samples incubated with BHI- culture supernatant compared to controls. This suggests that there has been an increase in polymer yield in the presence of bacterial BHI- culture supernatant.

Figure 4.5.1. Optimisation of gel loading for assembly of porcine brain microtubules in the presence of BHI- broth control or BHI- culture supernatant.

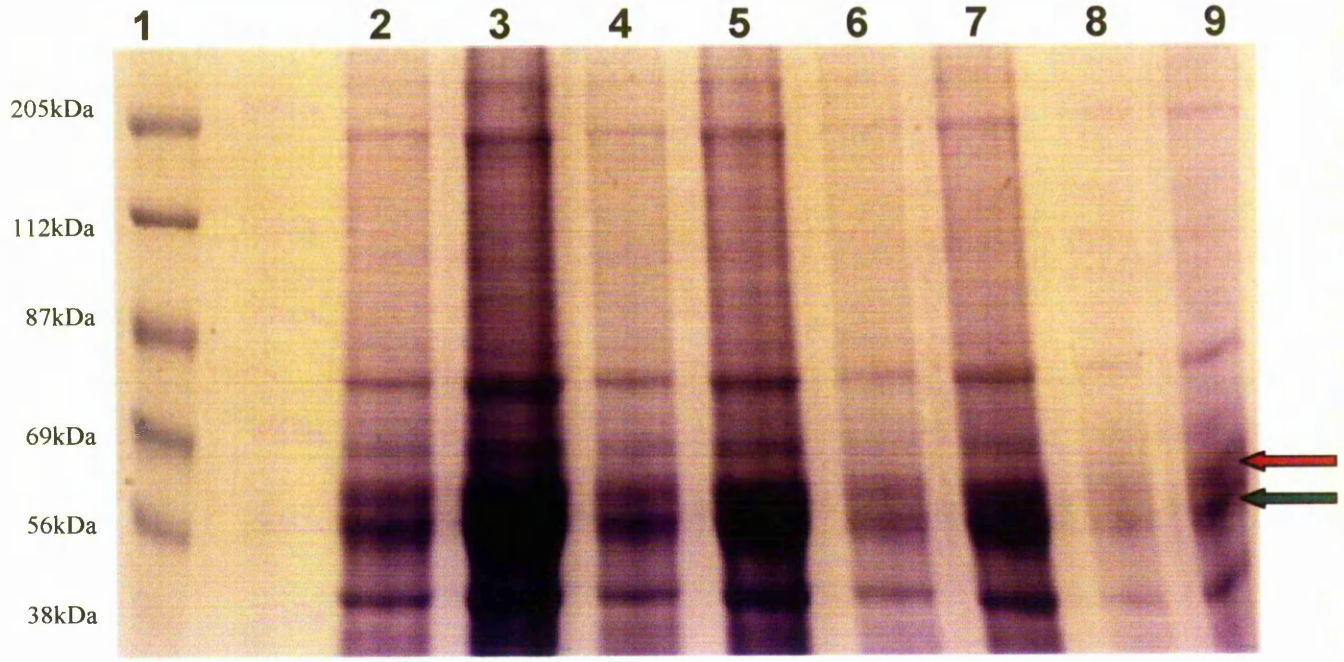


Figure 4.5.1. Image of protein samples after microtubule assembly in the presence of BHI- broth control (lanes 2,4,6 and 8), or BHI- culture supernatant (lanes 3,5,7 and 9). Protein loading was optimised by adding a number of volumes to the gel in order to visualise the differences in assembled microtubule proteins (lanes 2 and 3 = 8μl; lanes 4 and 5 = 6μl; lanes 6 and 7 = 4μl and lanes 8 and 9 = 2μl). The alpha and beta tubulin sub-units are indicated by arrows (Red arrow = alpha tubulin; Green arrow = beta tubulin).

Figure 4.5.2 shows an image of electrophoretically separated microtubule proteins after microtubule assembly in the presence of PBS control or in the presence of blood agar plate grown, *C.jejuni* cell sonicates. Again the protein loading volumes were the same as shown in figure 4.5.1, in order to optimise visualisation of protein level changes. The figure shows that microtubule

assembly in the presence of bacterial cell extracts results in an a slight increase in microtubule protein levels. This was not as pronounced as found in BHI- culture supernatant treated reactions (figure 4.5.1).

Figure 4.5.2. Optimisation of gel loading for assembly of porcine brain microtubules in the presence of buffer control or blood agar plate grown cell sonicates.

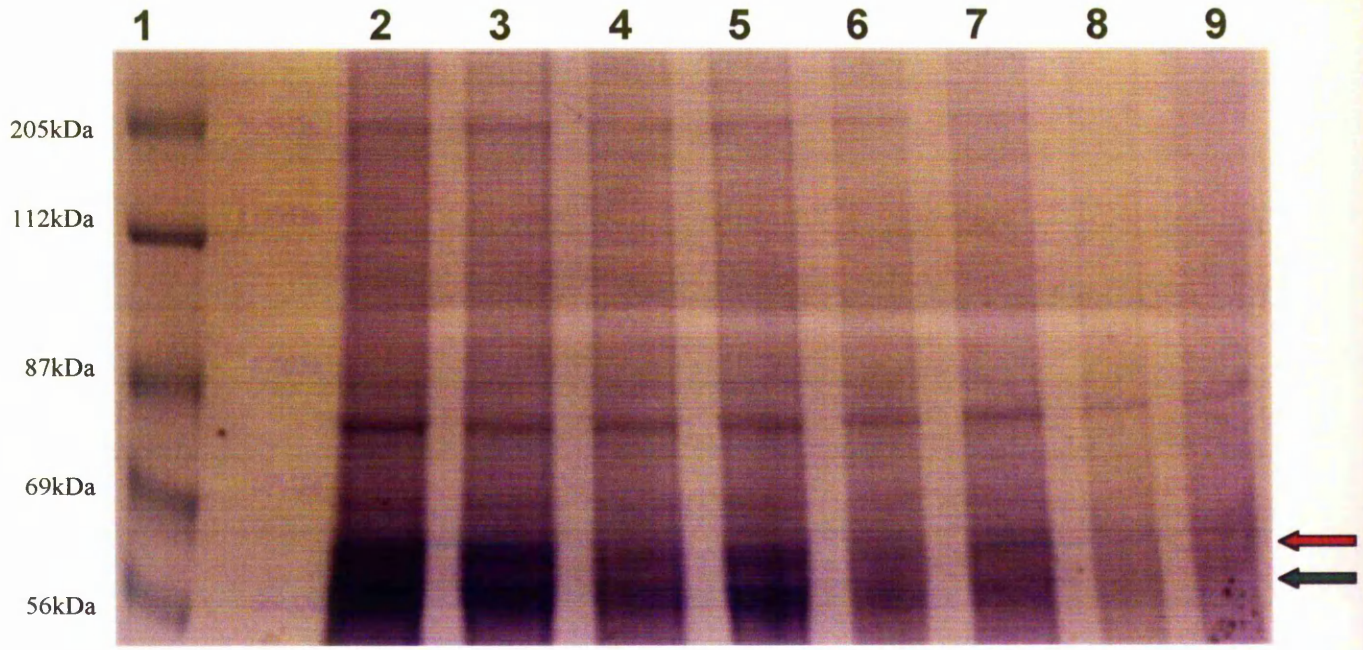


Figure 4.5.2. Image of protein samples after microtubule assembly in the presence of PBS buffer control (lanes 2,4,6 and 8), or Blood agar plate cell sonicates (lanes 3,5,7 and 9). Protein loading was optimised by adding a number of volumes to the gel in order to visualise the differences in assembled microtubule proteins (lanes 2 and 3 = 8μl; lanes 4 and 5 = 6μl; lanes 6 and 7 = 4μl and lanes 8 and 9 = 2μl). The alpha and beta tubulin sub-units are indicated by arrows (Red arrow = alpha tubulin; Green arrow = beta tubulin).

The two figures shown above were general qualitative measurements of differences in microtubule proteins sedimented by centrifugation after an assembly reaction in the absence/presence of bacterial culture extracts. A further step in the investigation of toxin targets was to assess the changes of particular tubulin sub-units in the protein samples after the reaction. This was achieved by electrophoretically separating the protein samples until the alpha and beta tubulin sub-units could be defined on the gel. The bands were then quantitatively assessed by measuring the density of each band on the digital image using Quantiscan software along with a digital camera. The results were then presented graphically for comparison with control treated tubulin sub-unit densities.

The image of the gel electrophoretic separation can be seen in figure 4.5.3a. The bacterial culture extracts used for the experiment shown in figure 4.5.3a were partially purified toxin fractions collected after the original culture extracts had undergone ammonium sulphate precipitation (to remove any non-protein components) followed by ion exchange chromatography. Fractions that tested positively for cytotoxicity and sub-cytotoxicity were then used in these assembly experiments. The purification process is described in depth in the next chapter, but for this chapter it is sufficient to concern ourselves with the effects on microtubule assembly and tubulin sub-unit composition.

Therefore, photographs of the separated stained tubulin bands were taken and the tubulin sub-unit densities were assessed using Quantiscan software. The results are presented as a percentage of control alpha and beta tubulin density. The results can be seen in figure 4.5.3b. The figure shows that, although in the initial assembly assays (figures 4.5.1 and 4.6.1) there was

evidence of a slight increase in microtubule proteins after assembly in the presence of bacterial culture extracts, closer inspection of the tubulin sub-units, shows there is an apparent increase in beta sub-units, but a slight decrease in the alpha sub-units.

Figure 4.5.3. Effects on microtubules assembled in the presence/absence of partially purified BHI- culture supernatant or blood agar cell sonicates.

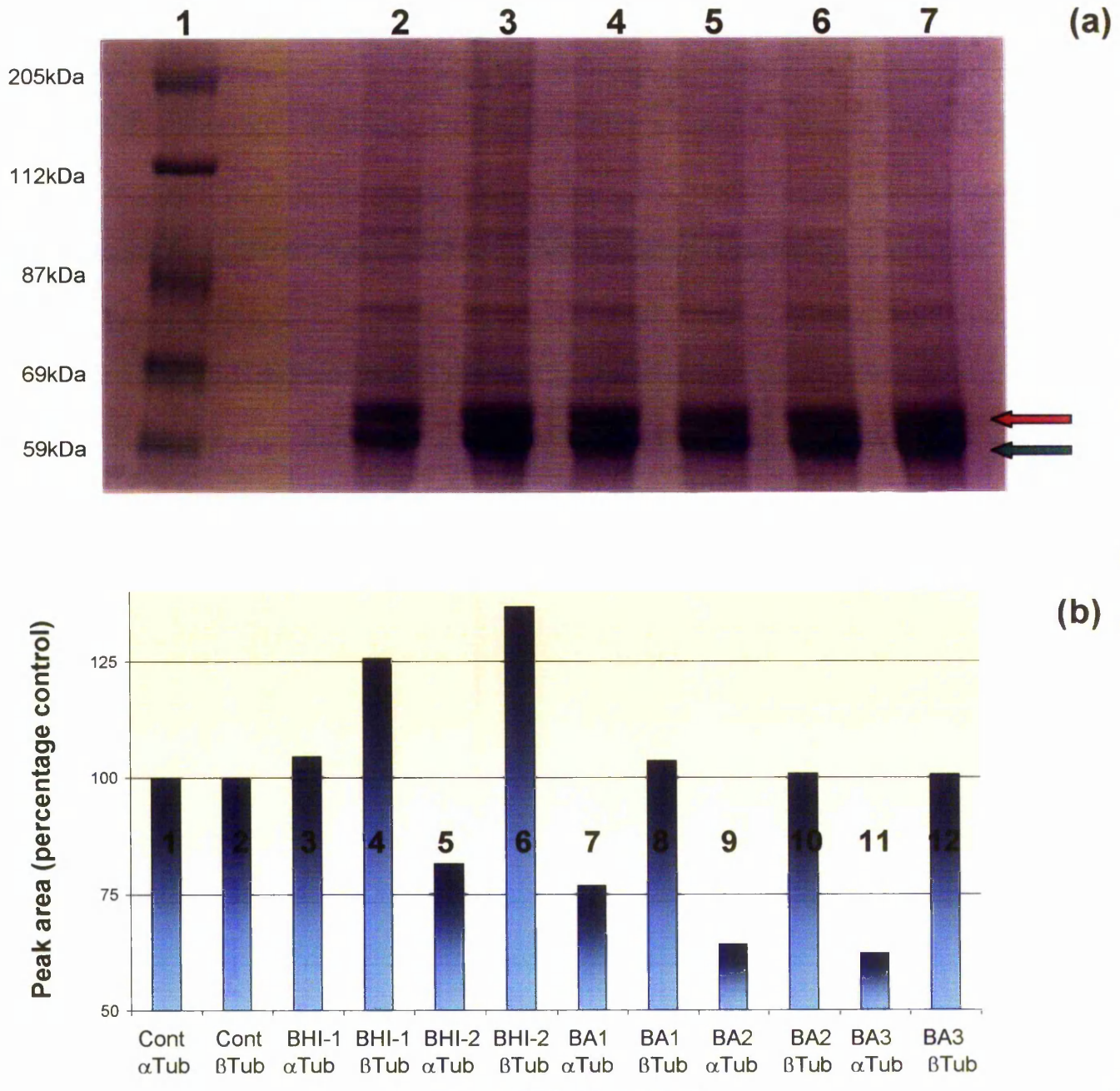


Figure 4.5.3. (a) Image of protein samples after microtubule assembly in the presence of buffer control (lane 2), BHI- culture supernatant IECfraction pools (lanes 3 and 4), blood agar sonicate IEC fraction pools (lanes 5, 6 and 7). Gels were run until the alpha and beta sub-units of tubulin proteins could be distinguished to a degree allowing digital image capture and analysis using Quantiscan software (b). The alpha and beta tubulin sub-units are indicated by arrows (Red arrow = alpha tubulin; Green arrow = beta tubulin). The graph (figure 4.5.3b) shows alpha and beta tubulin sub-unit density against microtubule assembly treatment. The graph shows the levels of alpha and beta tubulin sub-units present after densitometric analysis of the digital image shown in figure 4.5.4a.

The data presented in this chapter suggests that there is a definite sub-cytotoxic effect on axon outgrowth and cytoskeletal proteins (an early marker of sub-cytotoxic effects). These proteins are fundamental to cell membrane stability, and changes to specific cytoskeletal protein localisation within the cell membrane and axonal outgrowths may have been involved in changes observed in general cell morphology. Finally, sub-cytotoxic concentrations of bacterial culture extracts had an effect on microtubule assembly and on the relative amounts of alpha and beta tubulin sub-units.

In agreement with the results in chapter 3, we can conclude that there are a number of bacterial toxins present in these bacterial culture extracts. The next step in the research should therefore be to attempt to isolate, identify and characterise a specific novel bacterial toxin, the results of which are described in the following chapter.

4.6. Discussion of Sub-Cytotoxic Effects Of Bacterial Culture Extracts On Cultured Mammalian Cells.

The cytotoxins detected in Chapter 3 can be classified as cytopathogenic toxins, meaning they produce morphological cell alterations (which are visible using a light microscope) which may lead to cell death (cytotoxic/cytolethal). A number of these cytopathogenic effects are targeted to the disorganisation of the cytoskeletal framework, which may result in the inability of cells to proliferate due to the lack of a contractile ring (Thelestam and Florin 1994). Indeed, there are several reports of *Campylobacter* affecting cytoskeletal proteins (Hu *et al* 1999; Biswas *et al* 2000; Wooldridge *et al* 1996).

The experiments carried out in Chapter 4 aimed to investigate the effects of sub-cytotoxic levels of *Camplyobacter jejuni* culture extracts on mammalian cell morphology and cytoskeletal proteins in order to assess potential target cytoskeletal proteins and to evaluate the feasibility of a sub-cytotoxic assay as a marker of potential cytotoxicity at increased toxin concentrations; this might be more representative of *in vivo* toxin levels.

The general cell staining images shown in figures 4.1.1 and 4.1.2 and 4.1.3 showed morphological evidence for two types of toxins that have been reported in a number of studies and possibly a third, novel neurotoxin. Figure 4.1.1 shows general staining of CHO cells after treatment with control/toxin. The treated cells showed an elongated cell body, suggesting the presence of a cytolethal distending toxin (CLDT) identified in a number of *Campylobacter* toxin studies (Purdy *et al* 2000; Mooney *et al* 2000; Whitehouse *et al* 1998; Bag *et al* 1993). This particular toxin has also been reported to produce actin

stress fibre formation (Aragon *et al* 1997) which would support the findings shown in figure 4.4.2, in which major changes were also observed in actin distribution.

Figure 4.1.2 shows the general staining of human endothelial cells. Although sub-cytotoxic effects on general cell morphology are less obvious in this cell line, there does appear to be more of an increase in rounded cell shapes in the toxin treated cells. This suggests the presence of the cytolethal rounding toxin (CLRT) which has also been reported to be produced by a number of *Campylobacter jejuni* virulent clinical isolates (Schulze *et al* 1998; Hanel *et al* 1998). As cell rounding/elongation are likely to involve disruption of the cytoskeleton, a useful extension of this work would be to study the effects of toxin on cytoskeletal organisation in these cell lines, along with studies on toxin effects towards N2a cells. Because the N2a cell line was used to test cell type specificity of *Campylobacter* toxins, along with the fact that *Campylobacter* infection has been associated with the acute monophasic polyneuropathy, Guillian-Barre-Syndrome, it was decided to carry out a assessment of neurotoxicity. This would show the presence of any toxins capable of targeting neuronal cells, thus showing evidence of a second possible virulence factor of infection, along with ganglioside mimicry by *Campylobacter* LPS endotoxins which was reported to be the cause of the auto-immuno GBS (Ang *et al* 2002; Belkum *et al* 2001; Aspinall *et al* 1996; Allos 1998).

This theory was supported by general cell staining (seen in figure 4.1.3), which showed distinct reductions in both axon outgrowth number and size in bacterial culture supernatant treated cells. Similar results were observed

when testing the toxic compound tricresyl phosphate on differentiating N2a cells (Flaskos *et al* 1998).

A common quantitative neurotoxicity assay used at sub-cytotoxic toxin levels is to monitor the extension axon-like outgrowths by differentiating neuronal cell lines (Keilbaugh *et al* 1991; Shea *et al* 1991; Flaskos *et al* 1994 and 1998). The results shown in figures 4.2.1 to 4.2.2 suggest that there may be a neurotoxin present in BHI broth culture supernatants, which produced a significant reduction in axon outgrowth compared with control treated cells. This neurotoxin was also heat sensitive, with a significant decrease in such neurotoxic effects resulting from heating of bacterial culture supernatant before its addition to neuronal cell cultures. These results suggest the presence of a proteinaceous neurotoxin. This neurotoxin is presumably secreted by the bacteria into the culture medium (*i.e* an exotoxin) and able to reduce axon outgrowth at sub-cytotoxic concentrations or cell death at higher, cytotoxic levels.

Results from figure 4.2.2.1, on the other hand, suggest the presence of a cell bound neurotoxin (endotoxin) which also resulted in the significant reduction of axon outgrowth, but seems only to be expressed in 5 day old bacteria; cells have reached stationary phase of bacterial growth cycle at this time point (Holmes, 2001). The study of *Campylobacter* involvement in GBS has always lent towards the belief that ganglioside mimicry of the LPS complexes bound to the bacterial membrane results in the induction of self-immune responses (Saida *et al* 1996; Allos 1998; Jacobs *et al* 1997; Yuki *et al* 1999). Nothing has actually been done on the effects of *Campylobacter* toxins on target

neuronal cells. Therefore, this novel finding cannot be directly compared to the reports of general cytotoxicity or cholera-like auto-immuno response inducing endotoxins for which simultaneous production was reported in a study by Mizuno *et al* (1994). However, the finding does suggest research into toxin involvement in *Campylobacter* infection associated neuropathies may be of some value.

As the cytoskeleton is known to play a central role in the growth and maintanence of axons, it represents a potential target for toxin action. Therefore, protein components of each array (microtubules, intermediate filaments and microfilaments) were chosen as markers for cytoskeletal involvement in *Campylobacter* toxin cytopathogenicity.

The levels of these cytoskeletal protein in lysates taken from control/toxin treated cells can be seen on the probed Western blots in figures 4.3.1 to 4.3.3. The results show that exposure of N2a and ECV cells to BHI broth culture supernatants results in a reduction in the levels of the microtubule protein tubulin. The reduction in the levels of cross-reactivity of antibody 611B1 with acetylated alpha tubulin (figures 4.3.2 and 4.3.3) may reflect a possible effect on microtubule stability, since lower reactivity suggests a reduced pool of acetylated alpha tubulin, which in turn suggests a change in post-translational modification of alpha tubulin that is associated with stable bundles of microtubules. This suggests that at least some toxin targets are at the protein level. Microtubule involvement in *Campylobacter jejuni* invasion has been reported by Hu *et al* (1999), who showed evidence of bacterial co-localisation with microtubules using anti-tubulin IgG immunoprobing of human intestinal mucosa monolayers.

Figures 4.3.2c and 4.3.3c show that there were also reductions in actin levels resulting from treatment with the BHI culture supernatants. Sub-cytotoxic targeting of actin has been well documented in a number of bacterial infections. For example *C. botulinum* C2, *C. perfringens* iota, *C. spiroforme* toxins and the ADP-ribosyltransferase produced by *C. difficile* are all actin modifying toxins (Boquet *et al* 1998; Lacy and Stevens 1998; Balfanz *et al* 1996).

Figure 4.3.2d shows a reduction in total neurofilament heavy chain proteins in N2a cells treated with BHI- culture supernatant. This was expected, as neurite outgrowth and stabilisation may require the localised incorporation into the cytoskeleton of certain components, such as microtubule-associated proteins and neurofilaments (Shea *et al* 1991). Therefore the reduction in neurofilament proteins supports the drop in neurite outgrowth as a result of BHI broth culture supernatant treatment (figures 4.3.2 to 4.3.3). Similar results were observed when testing the toxic compound tricresyl phosphate on differentiating N2a cells (Flaskos *et al* 1998).

Similar experiments using cells treated with the blood agar cell sonicates indicate there was a general decrease in all of the cytoskeletal proteins in the neuroblastoma cell protein samples, but a general increase in the ECV cell protein samples (figure 4.3.1). The latter can still be considered as a sub-cytotoxic effect, since there have been many reports of bacterial toxins causing the aggregation of cytoskeletal proteins. For example, the cytotoxic necrotizing factors produced by *E.coli* produce an increase in actin stress fibre formation and focal contacts in cultured cells (Flatau *et al* 1997; Lacerda *et al*

1996) and similar results are produced by *Clostridium difficile* toxin A (Donelli and Fiorentini 1992).

The disruption of microtubule and microfilament protein immunoreactivity was further confirmed by visualisation of cytoskeletal protein distribution around the cell body and neurite outgrowths. Figures 4.4.1 and 4.4.2 show that there was a general disruption of tubulin and actin organisation in toxin treated cells showing altered localisation of antibody staining, possibly caused by changes in protein polymerisation and actin stress fibre formation, as reported by Flatau *et al* (1997) and Lacerda *et al* (1996). These images suggest there is a general disruption in microtubule and microfilament stability and organisation, which may represent an early marker of cytopathogenicity.

In order to study the possibility of an interaction between *Campylobacter* toxins and microtubule proteins, studies on microtubule assembly were carried out *in vitro*. The aim of these experiments was to investigate whether toxin treatment affected microtubule formation, since controlled assembly and dissasembly of microtubules is vital to the regulation of microtubule-dependent processes (Hargreaves 1997).

The results (figures 4.5.1 and 4.5.2) suggest increased levels of microtubule assembly in the presence of both BHI broth culture supernatant and blood agar cell sonicates. Although the apparent increase in microtubule assembly appears to contradict the decrease in tubulin staining intensities in immunofluorescence staining, it does show that the toxin is able to interact with microtubule proteins or enzyme activities/proteins that regulate microtubules. The decrease in levels of tubulin, *etc* in N2a cells may have arisen from either decreased expression or synthesis of tubulin. Alternatively,

exposure to the toxin may trigger events that stimulate proteolysis of these proteins in N2a cells. Increased levels of all proteins in ECV cells may be due to increased gene expression, increased protein synthesis, or decreased protein degradation and more work is needed to establish the molecular basis of these effects in more detail.

After the assembly assay, tubulin protein sub-units were sufficiently separated to allow quantitative measurement of the two sub-units (figure 4.5.3). Both figures show a distinct decrease in the alpha sub-unit in treated protein samples (this corresponds to the western blot results that showed decreases in the alpha sub-unit also). The apparent general increase in the tubulin protein levels was due to the much larger increases in the beta sub-unit proteins after exposure to the bacterial culture extracts. The changes seen in specific tubulin sub-unit levels, along with general tubulin distribution throughout the cell membrane and also the increase in total microtubule levels due to interference in assembly are all shown to coincide with each other as a result of exposure to the bacterial toxin. The interaction of toxins with microtubules is in agreement with the report of Hu *et al* (1999) who suggests that bacterial invasion of human intestinal cells is associated with microtubules and dynien cytoskeletal proteins. In their study, a reduction of 85% in bacterial invasion was observed after depolymerisation of microtubules in host cells using colchicine, vinblastine and vincristine. The findings of Hu *et al* (1999), along with results shown in chapter 4, are consistent with a direct interaction between a *Campylobacter* toxin and microtubule proteins, whether through direct bacterial cell contact during host intestinal cell invasion, or as a target or transport system for bacterial toxins.

CHAPTER 5

5.1. Novel *Campylobacter jejuni* 11351 Toxin Isolation And Characterisation Results

The results shown in the last two chapters were mainly obtained using whole bacterial culture extracts. The data suggest that there could be a number of toxins present in the extracts responsible for the various effects observed. In order to characterise the cytotoxic and sub-cytotoxic effects of a single (novel) bacterial toxin, isolation of that toxin from the crude bacterial culture extract was required. In order to do this, a number of methods were employed.

Of particular interest were protein toxins as a number of these have been described in the literature and N terminal sequence data of any protein toxin isolated could be used in a comparison with known bacterial protein sequences. Crude bacterial culture extract proteins were precipitated with 80% (w/v) saturated ammonium sulphate to concentrate proteins (see section 2.2.4.2). The protein extracts were then passed through an anion exchange column (Econo-Pac Q Sephadex cartridge, see section 2.2.4.3). This was carried out in order to separate proteins by differences in charge. All eluted fractions were assayed for protein content by their absorbance at 280nm. These fractions were then dialysed against PBS and tested for cytotoxicity on cultured ECV cells.

Figure 5.1.1a shows a profile of protein absorbance (read at a wavelength of 280nm) plotted against column fraction number. Note that on this particular column run the unbound proteins had not been fully removed from the column before the salt gradient was introduced, this resulted in contamination of bound protein with unbound protein in the initial salt gradient eluted fractions.

Therefore the expected decrease in protein levels just before the start of the elution of resin-bound protein fractions is not observed on the graph. That aside, the graph shows that there is an initial elution of unbound protein, followed by a dip in protein levels (around fractions 21 to 24) and finally there is a distinct bound protein peak (around fractions 25 to 29) coming off the column near the end of the run.

All column fractions were then dialysed against PBS and applied to cultured mammalian cells in order to determine the cytotoxicity of each fraction.

As shown in figure 5.1.1b, the cytotoxic effect generally followed the levels of protein in each fraction, showing an increase in cytotoxicity around the initial fractions, including the early bound fractions contaminated with unbound proteins. The protein peak around fractions 23 to 27 also showed an increase in cytotoxic activity. There was also a higher level of cytotoxicity in fractions collected near the end of the run. This was interesting because the fractions coming off the column at this stage contained relatively low levels of protein, suggesting the presence of a highly cytotoxic protein at low concentration levels. This was promising for purification purposes because, although high levels of toxin were desirable, low levels of protein suggest fewer contaminant proteins in the fraction, making isolation of the single toxic protein more feasible.

Figure 5.1.1. Protein elution/cytotoxicity profile of *Campylobacter. jejuni* 11351 BHI- culture supernatant separated by ion exchange chromatography

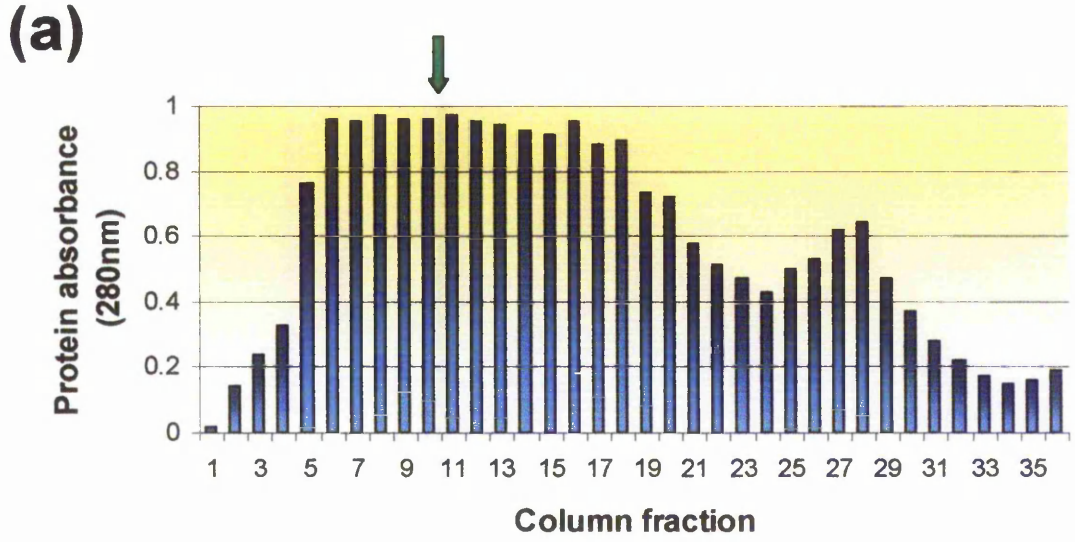


Figure 5.1.1a. Shown are the protein absorbance readings, taken at a wavelength of 280nm for the column fractions collected from a Q Sephadex ion exchange column. Sample applied to the top of the column was taken from an equilibrated protein sample of a *Campylobacter jejuni* 11351 BHI- broth culture. The graph shows a distinct bound protein peak eluted around fractions 25 to 29. Introduction of a salt gradient is indicated by the green arrow.

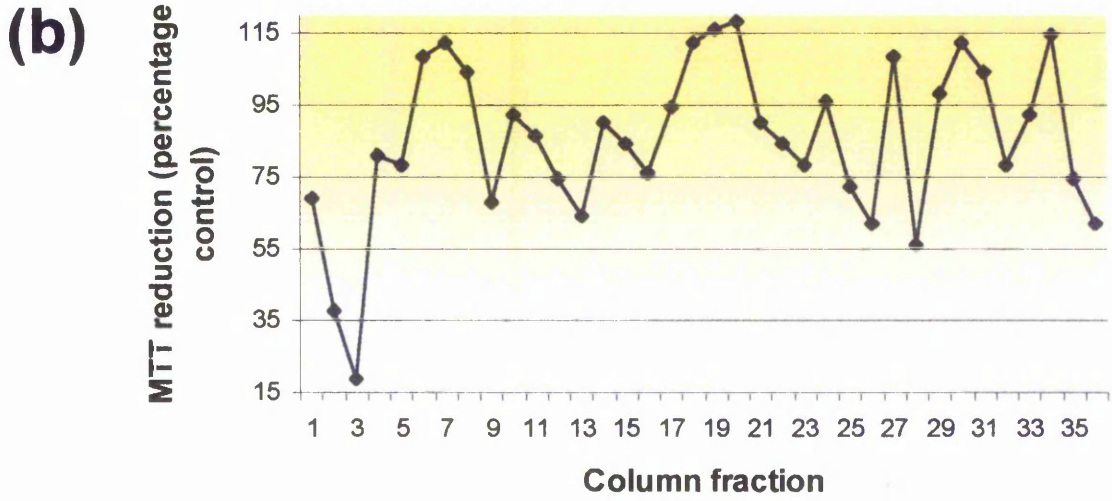


Figure 5.1.1b. Shown is the reduction of MTT by fraction-treated ECV cells, expressed as a percentage of control values. The graph shows cytotoxic effects on cultured cells corresponding with eluted bound protein fractions (fractions 23 to 27) and fractions corresponding to lower protein levels (fractions 35 to 36).

Cell sonicates from *Campylobacter jejuni* 11351 cells grown on blood agar plates were also applied to the ion exchange column in the same manner as the BHI- culture supernatants. The protein absorbance readings (280nm), which can be seen in figure 5.1.2a, show the expected removal of unbound protein (fractions 1 to 11), followed by an initial bound protein peak coming off (fractions 12 to 16) and then another drop in protein levels. A second protein peak eluted off the column near the end of the run (fractions 28 to 35). The fractions were then taken for cytotoxicity testing as in the previous column fractions the results of which can be seen in figure 5.1.2b.

Figure 5.1.2b shows a lack of cyotoxicity corresponding to the unbound fractions, which suggests no loss of toxic proteins in the wash. A group of cytotoxic fractions was detected corresponding with the first protein peak (fractions 11 to 18). There also appeared to be cytotoxic fractions just beyond those showing the main protein peak (fractions 11 to 17). This suggests elution of the toxic proteins after the larger amount of bound proteins came off the column. There was also a final cluster of cytotoxic fractions corresponding with the second protein peak (fractions 30 to 34), suggesting a second amount of cytotoxic proteins eluting from the column at higher salt concentrations. This may contain similar toxin proteins as those eluted in the first protein peak, but associated with other proteins, or an entirely different protein altogether.

Figure 5.1.2. Protein elution/cytotoxicity profile of *Campylobacter. jejuni* 11351 blood agar cell sonicates separated by ion exchange chromatography.

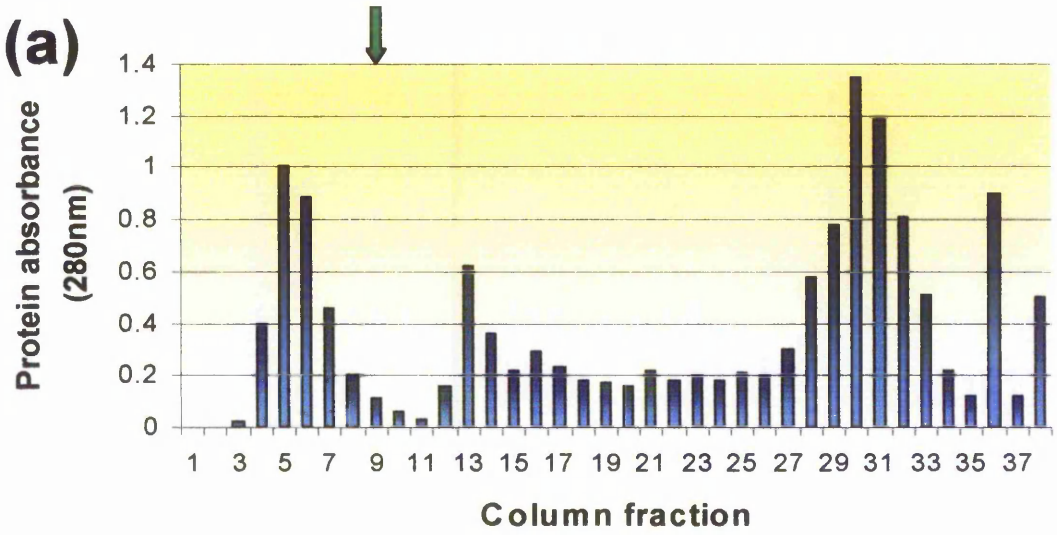


Figure 5.1.2a. Shown are the protein absorbance readings, taken at a wavelength of 280nm for the column fractions collected from a Q Sephadex ion exchange column. Sample applied to the top of the column was taken from an equilibrated protein sample of a *Campylobacter jejuni* 11351 blood agar cell sonicate. The graph shows a distinct bound protein peak eluted around fractions 12 to 16, followed by a second protein peak around fractions 27 to 35. Introduction of a salt gradient is indicated by the green arrow.

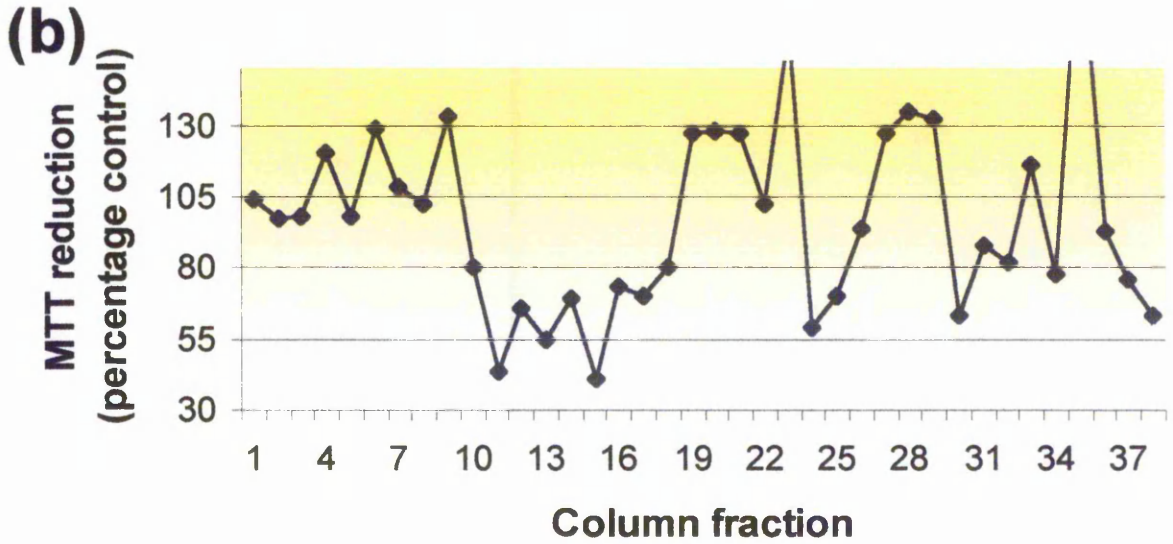


Figure 5.1.2b. Shown is the reduction of MTT by fraction-treated ECV cells, expressed as a percentage of control values. The graph shows cytotoxic effects on cultured cells corresponding with initial bound protein fractions (fractions 12 to 16). The protein peak eluted later in the run also corresponds to a group of cytotoxic fractions (fractions 30 to 34).

All column fractions from both culture extracts were then concentrated (20x) using poly ethylene glycol (PEG) and assayed for protein content.

As mentioned in the introduction, a cholera like toxin has been identified in *Campylobacter* strains, which can be neutralised by anti-cholera toxin antibodies (Daikoku, *et al* 1990; Goossens, *et al* 1985; McCardell, *et al* 1984). Daikoku, *et al* (1990) also suggested that a protein toxin showing sub-units of 68kDa, 54kDa and 45kDa after SDS-PAGE could be purified from a clinical *Campylobacter jejuni* isolate using an affinity column compromising immobilised anti-CT antiserum. Furthermore, as shown in figure 5.1.3, in our own preliminary work we also found that BHI- culture supernatant/blood agar cell sonicate column fraction toxicity towards ECV cells could be at least partially neutralised by a polyclonal anti-CT antiserum.

Figure 5.1.3. Neutralisation of *C. jejuni* 11351 cytotoxicity towards ECV cells by treatment with anti-cholera toxin antiserum.

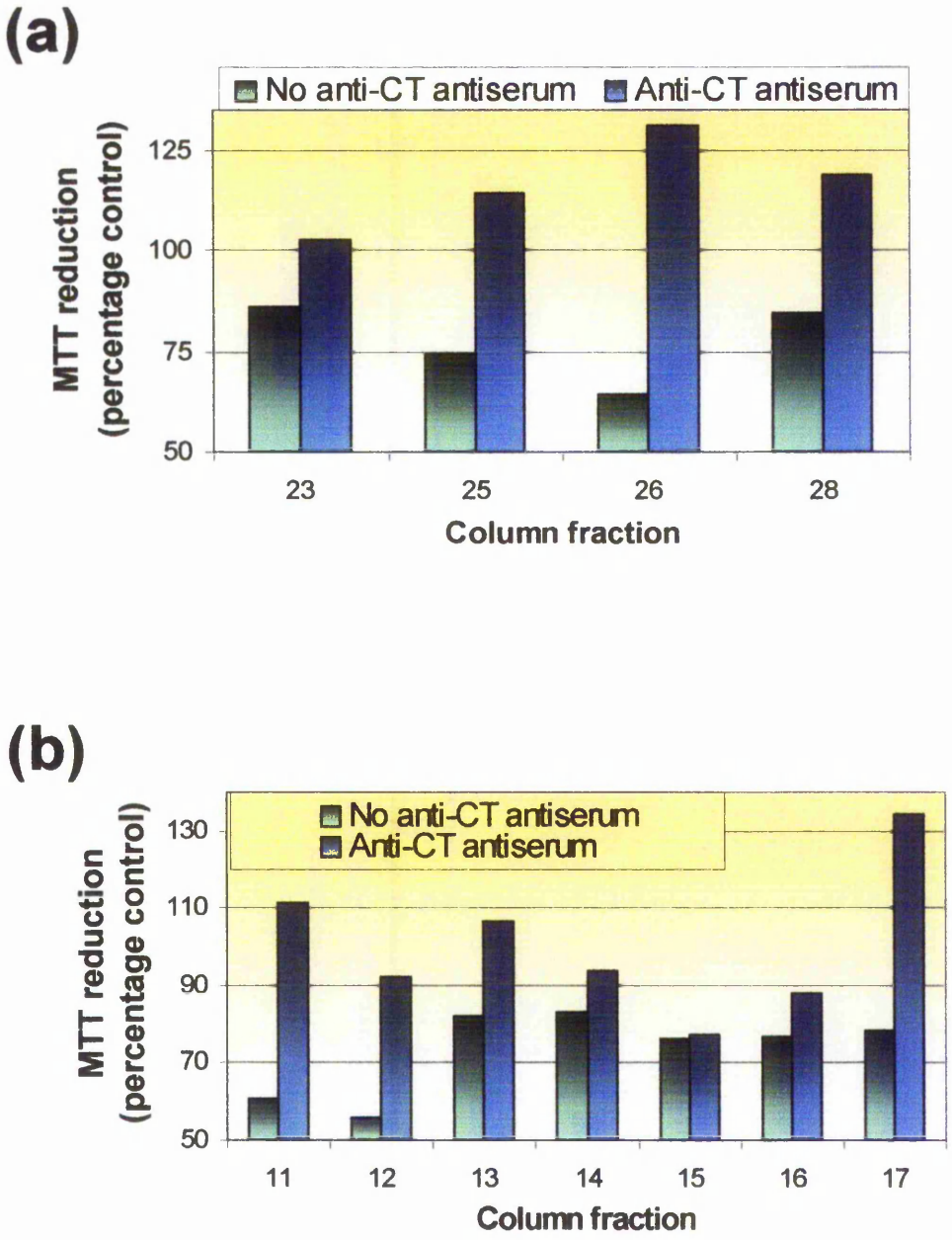


Figure 5.1.3. Shown is the reduction of MTT by ECV cells after treatment with (a) fractions 23 to 28 from ion exchange eluted *C. jejuni* 11351 BHI-culture supernatant and (b) fractions 11 to 17 from ion exchange eluted *C. jejuni* 11351 blood agar cell sonicates with/without anti-CT antiserum (Sigma), expressed as a percentage of control values.

It was therefore of interest to screen toxin fractions further by probing immuno dot blots with antibodies against cholera toxin to determine whether cross-reactivity corresponded with cytotoxicity. The fraction samples were dot-blotted onto nitrocellulose at 20μg protein per dot and immunoprobed with anti-cholera β sub-unit polyclonal antiserum (see section 2.2.4.4), the results of which can be seen in figure 5.1.4. Figure 5.1.4a shows the antibody reactivity for the BHI- column fractions. Dots A1 and A2 were buffer negative control and from A3 to C12 the column fractions were added, 1 to 34. The more intense reactivity can be seen in fractions 25 (C3) to 29 (C7). This corresponds to the protein peak and the increase in cytotoxic activity (seen in figure 5.1.1), suggesting the presence of a cholera-like protein toxin eluted at a lower salt concentration on the ion exchange column (figure 5.1.1).

Figure 5.1.4b shows the antibody reactivity for the blood agar plate cell extracts column fractions. Dots A1 to D3 contain 20μg samples from column fractions 1 to 39. Dots D4 to D12 contain buffer negative controls. The figure shows that, although less cross-reactive, the more intense dots were observed in column fractions 16 (B4) to 18 (B6). This corresponds to the first bound peak from the ion exchange column and to an increase in cytotoxicity on cultured ECV cells (figure 5.1.2).

The second protein peak from the column also has corresponding reactive fractions. These can be seen in dots C3 (fraction 27) to C10 (fraction 34). The corresponding increase in cyototoxicity was observed in fractions 30 to 34 in figure 5.1.2.

Figure 5.1.4. Anti-cholera toxin β sub-unit antiserum reactivity with BHI-culture supernatant and blood agar plate cell sonicate ion exchange fractions.

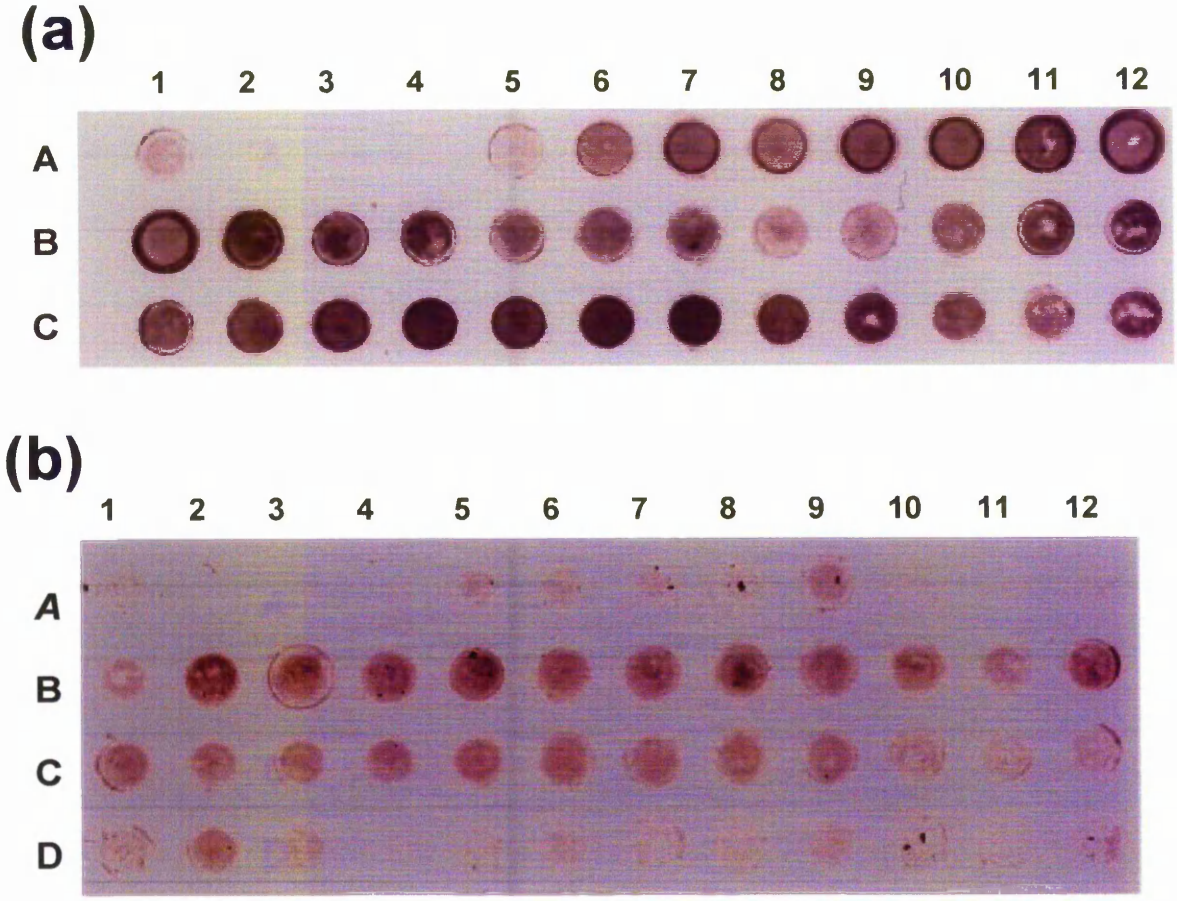


Figure 5.1.4. Anti-cholera toxin β sub-unit antiserum reactivity with (a) BHI- culture supernatant ion exchange fractions. Dots A1 and A2 were buffer negative controls. Dots A3 to C12 contained column fractions 1 to 34. Reactive fractions included fractions 25 to 29 (C3 to C7), which corresponded with increased cytotoxicity (figure 5.1.1b) and (b) blood agar cell sonicate ion exchange fractions. Dots A1 to D3 contain column fractions 1 to 39, with reactive fractions including fractions 16 to 18 (B4 to B6) and fractions 25 to 29 (C1 to C5). These fractions correspond with an increase in cytotoxicity seen when applied to cultured ECV cells (figure 5.1.2b).

The next step in the protein toxin identification was to separate the column fractions that were cross-reactive and cytotoxic by gel electrophoresis and Western blotting techniques. This allowed immunoprobing of blots with the anti-cholera antiserum and visualisation of the molecular weight of the cross-reactive proteins present, which can be seen in figure 5.1.5. Figure 5.1.5a shows the immunoblot of BHI- column cross-reactive fractions 25 to 30. The fractions contain reactive proteins around the 80kDa mark, but all fractions (albeit reduced in fraction 25) show reactive bands just above 40kDa.

Figure 5.1.5b shows the immunoblot for blood agar cell sonicate cross-reactive column fractions. Again the immunoblot shows reactive bands just above the 70kDa mark. But fractions 26 to 30 show reactive bands around the 40kDa mark, suggesting correspondence to the bands seen in figure 5.1.6a and those identified as the cholera like toxin by Diakoku, *et al* (1990) from a clinical *Campylobacter jejuni* isolate.

In order to further purify this cholera-like protein toxin, an affinity column was produced. The polyclonal antiserum to cholera toxin was coupled to a cyanogen bromide activated cross-linking agarose resin, which was set-up as a column in a 2ml housing unit (see section 2.2.5.2). The ion exchange purified cell sonicates from blood agar *Campylobacter jejuni* 11351 were then applied to the top of the affinity column and eluted by increasing pH (as described in section 2.2.5.2). The protein absorbance readings (280nm) were then recorded for the eluted column fractions and the results can be seen in figure 5.1.6.

Figure 5.1.5. Western blots of ion exchange column fractions of *C. jejuni* BHI- culture supernatant and blood agar cell sonicates immunoprobed with anti-cholera toxin β sub-unit antiserum.

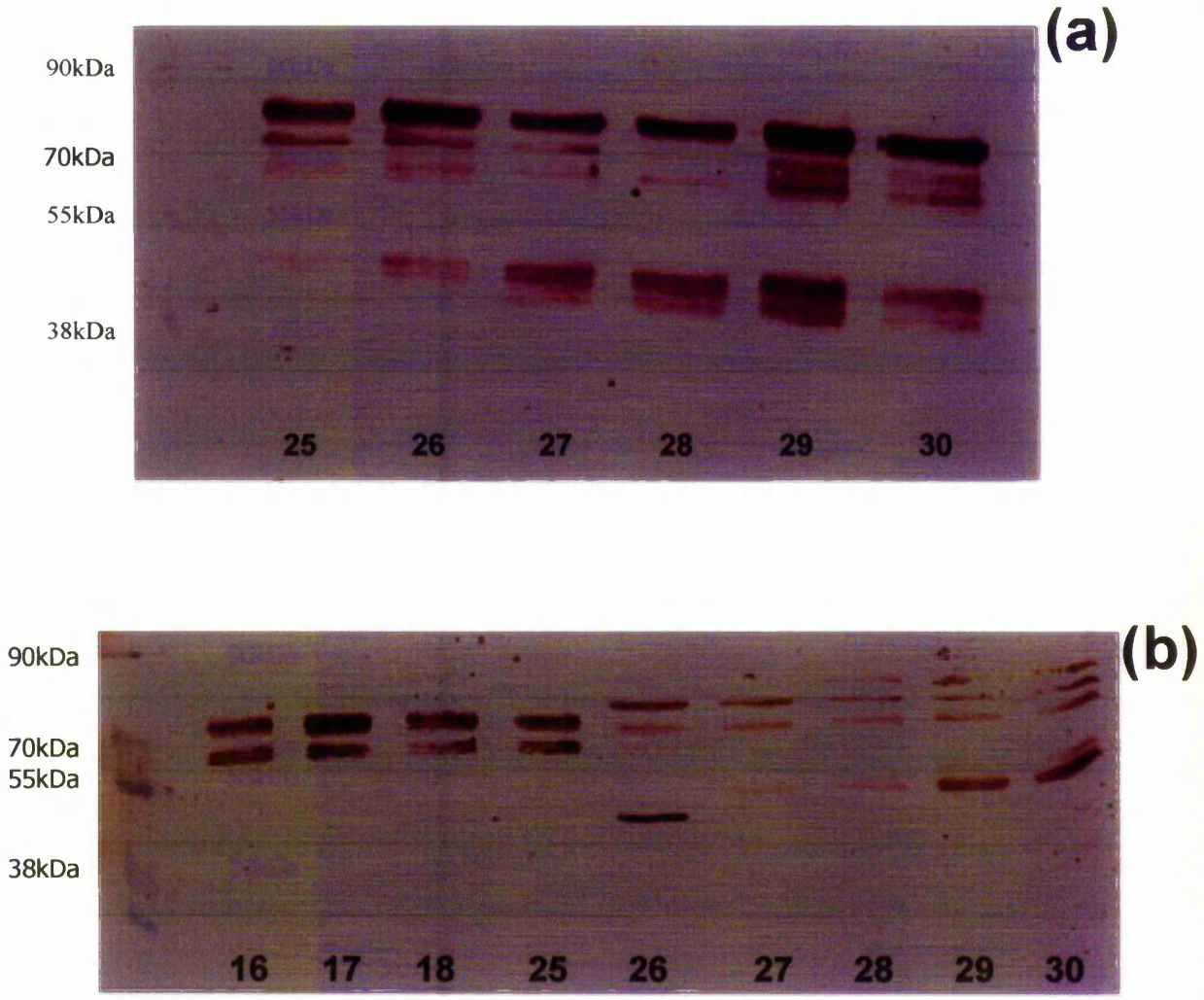


Figure 5.1.5. Samples added to column were taken from (a) *Campylobacter jejuni* 11351 BHI- broth culture supernatant. Lane 1 contains protein molecular weight standards. Lane 2 to 7 contain protein samples from fractions 25 to 30 (fractions showed antiserum reactivity on dotblot [figure 5.1.4]) and (b) *Campylobacter jejuni* 11351 blood agar plate cell sonicates. Lane 1 contains protein molecular weight standards. Lane 2 to 4 contain protein samples from fractions 16 to 18. Lanes 5 to 10 contain protein samples from fractions 25 to 30 (showed antiserum reactivity in dotblot [figure 5.1.4]).

The graph shows protein levels decreasing as the unbound proteins were flushed through the column, before bound proteins were removed with 50mM Tris-HCl buffer pH 10.5. The eluted proteins were collected in neutralising buffer to pH 7. The fractions containing the eluted protein peak appeared to be fractions 8 to 11, which were kept for further analysis.

Figure 5.1.6. Protein profile of *Campylobacter jejuni* 11351 blood agar cell sonicate affinity purification.

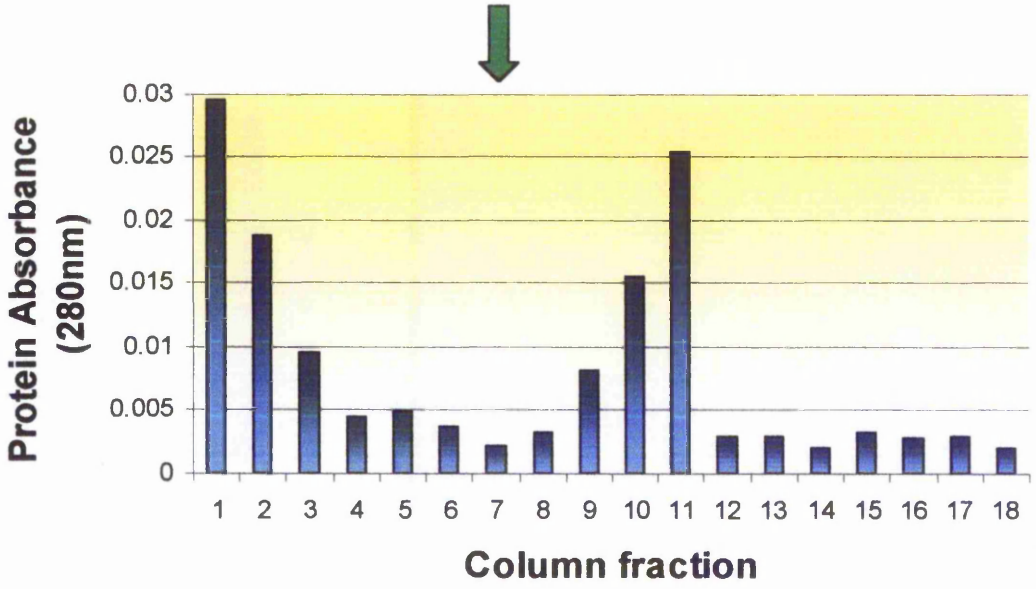


Figure 5.1.6. Shown are protein absorbance readings taken at 280nm for anti-cholera toxin affinity column fractions. Samples applied to the column were partially purified samples of blood agar plate grown *Camplyobacter jejuni* 11351 cell sonicates. The graph shows a bound protein peak in fractions 8 to 11 eluting off the column mid run. Introduction of increasing pH is indicated by green arrow.

The eluted protein peak fractions (8 to 11) were pooled and dialysed against PBS buffer. 20μg protein samples were then separated by gel electrophorsis and the protein bands stained with silver stain reagents. The image of the stained gel can be seen in figure 5.1.7. The figure shows affinity column bound protein around 70 kDa and just below 50 kDa, similar to those shown to cross react with anti-CT antiserum on the Western blots. There was also a strong protein band around 40 kDa, which may be explained by protein degradation.

Figure 5.1.7. Silver stained of gel electrophoretic separation of anti-cholera toxin affinity column binding fractions from *Campylobacter jejuni* 11351 blood agar cell sonicates.

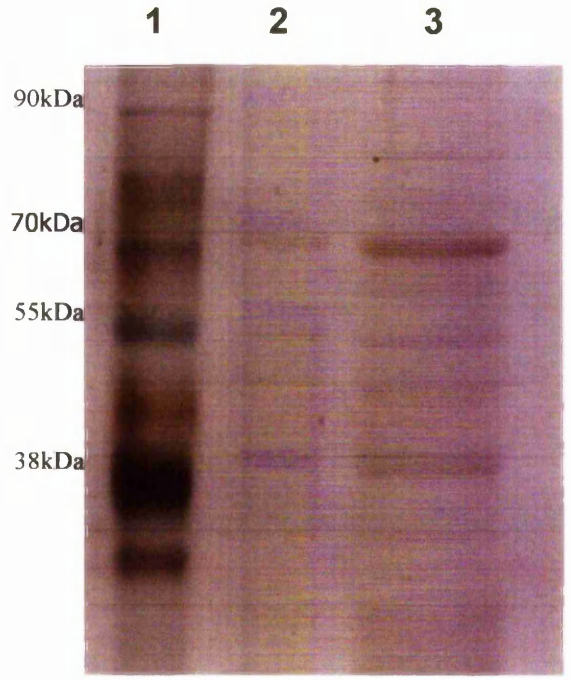


Figure 5.1.7. Lane 1 contains protein molecular weight markers (with a contaminant band between 55 and 38kDa. Lane 2 contains column wash fractions (unbound proteins). Lane 3 contains eluted bound protein peak fractions, pooled together. The bound protein fractions show protein bands at 70kDa, approx 45kDa and also another band at approx 40kDa.

The dialysed column bound protein fractions were then tested for cytotoxicity on cultured human endothelial cells. The results can be seen in figure 5.1.8. The figure shows reduction of MTT as a percentage of control cells after exposure to 5% (v/v) unbound protein fractions (green bar) and after exposure to 5% (v/v) bound protein fractions (blue bar). The wash fractions showed no cytotoxic effects compared with control cells, but the eluted fractions showed cytotoxicity compared to control cells.

Figure 5.1.8. Cytotoxic effects of anti-cholera toxin affinity chromatography fractions on cultured ECV cells.

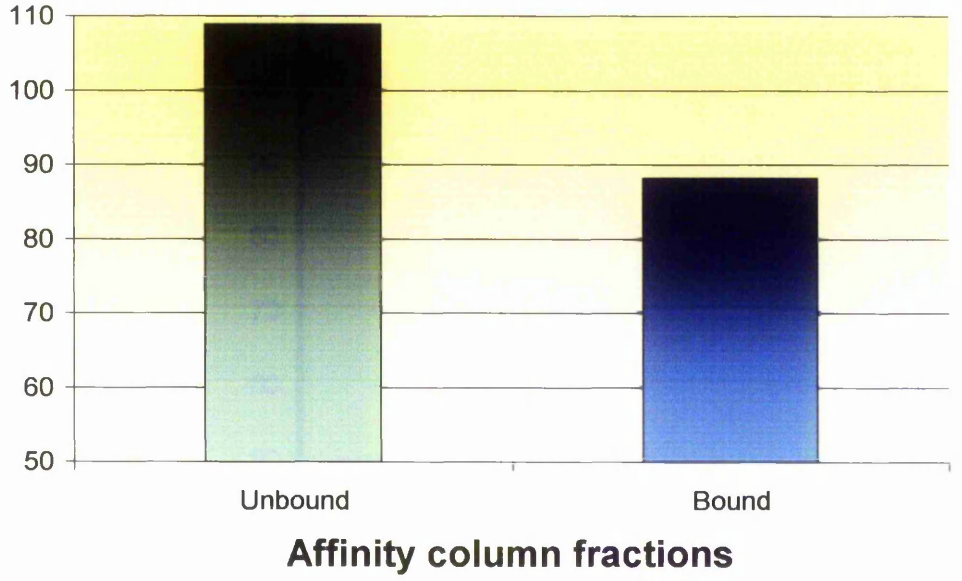


Figure 5.1.8. Unbound, wash protein fractions showed no cytotoxic effects compared to buffer treated control cells. Bound, eluted protein fractions showed cytotoxicity compared to control cells. The fractions had been pooled, dialysed against PBS and concentrated using a millipore centrifuge concentrator and added to cultured ECV cell at a concentration of 0.5%(v/v).

Unfortunately the protein levels of the fractions coming off the affinity column were very small and therefore were not sufficient to carry out further characterisation of any protein toxins present in the eluted fractions. Therefore the ion exchange chromatography process was repeated to produce larger volumes of culture extract samples for isolation of cholera-like protein toxins using the affinity column. It was also decided to concentrate on blood agar cell sonicates, since these proved the most promising source of cholera-like toxins and purification of both blood agar plate cell sonicates and BHI- culture supernatant was proving too time consuming. The protein absorbance readings (280nm) for the repeat ion exchange column fractions can be seen in figure 5.1.9. The figure shows an unbound protein peak coming off the column with the wash fractions, followed by an initial bound protein peak in fractions 28 to 31. A second bound protein peak eluted off the column straight after, in fractions 33 to 39. And lastly a minor bound protein peak came of the column near the end of the run in fractions 58 to 61.

Figure 5.1.9. Protein profile for *Campylobacter jejuni* 11351 blood agar plate cell sonicate ion exchange fractions.

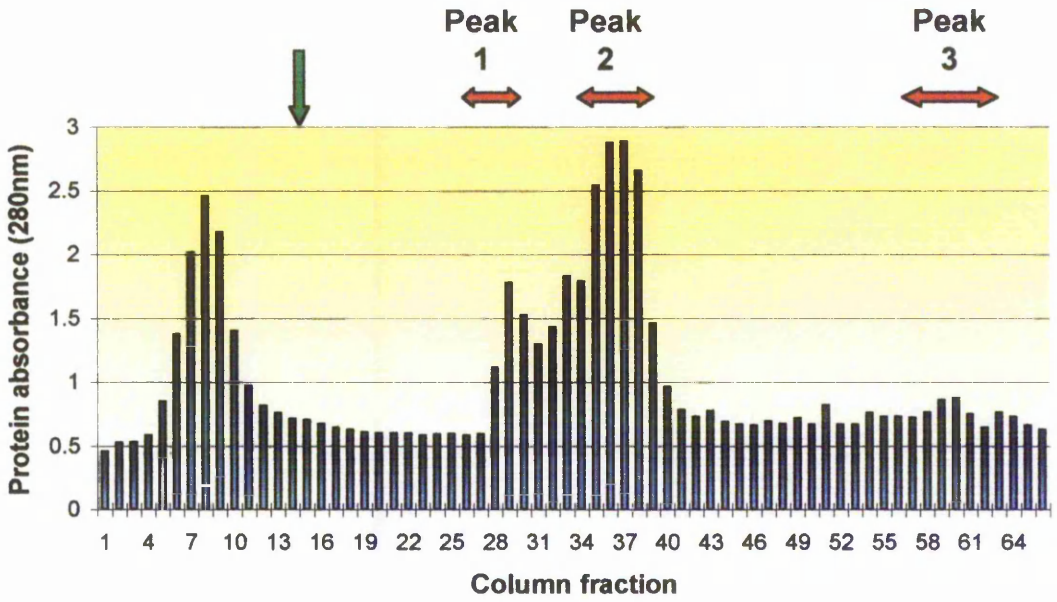


Figure 5.1.9. Shown is the protein absorbance readings (280nm) for *Campylobacter jejuni* 11351 blood agar plate cell sonicate ion exchange fractions. The figure shows an unbound protein peak (fractions 5 to 11), an initial bound protein peak (fractions 28 to 31) and a second bound protein peak (fractions 33 to 39). There was a final bound protein peak in fractions 58 to 61.

Once again the fractions were dialysed against PBS and dotblotted onto nitrocellulose, before being immunoprobed with anti-cholera toxin antiserum, the results of which can be seen in figure 5.1.10. The figure shows intense reactivity in fractions 29 to 36 (C5 to C12). There was no intense cross-reactivity evident in fractions corresponding with the third protein peak (figure 5.1.9), but there was an intense reactivity shown by fractions 4 to 11 (A4 to A11) which corresponded to the unbound protein elution peak (figure 5.1.9).

Therefore the column fractions were pooled into samples of protein containing fractions and non-protein containing fractions, as determined by A_{280}. Pool 1 contained unbound non-protein fractions 1 to 3; pool 2 contained unbound protein fractions 4 to 11; pool 3 included bound non-protein fractions 12 to 27; pool 4 comprised bound protein peak 1 fractions 28 to 31; pool 5 contained bound protein peak 3 fractions 32 to 39; pool 6 included bound protein peak 3 fractions 44 to 66. A 20μg protein sample from each pool was separated by gel electrophoresis and silver stained as before. The image of the stained gel can be seen in figure 5.1.11.

Figure 5.1.10. Anti-cholera toxin antiserum cross-reactivity with dot blots of *Campylobacter jejuni* 11351 blood agar cell sonicate ion exchange fractions.

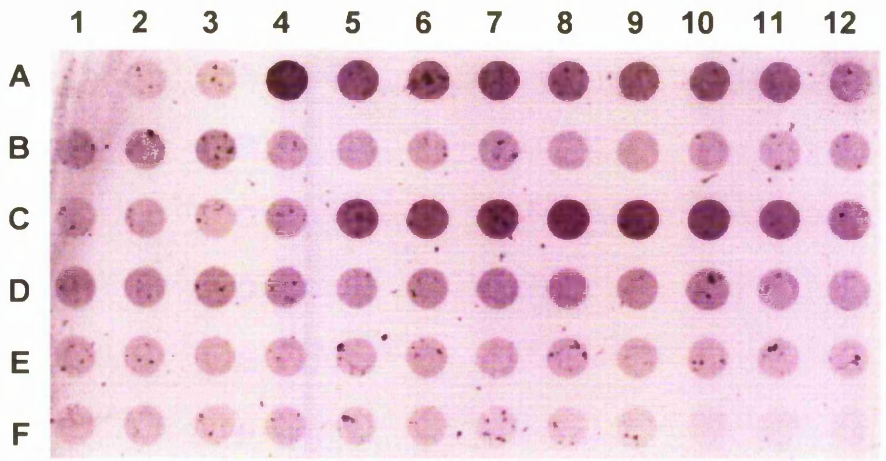


Figure 5.1.10. Dot blot of *C. jejuni* 11351 blood agar cell sonicate ion exchange fractions probed with anti-CT antiserum. Dots A1 to F6 contain protein samples of column fraction 1 to 66. Dots F7 to F12 were buffer negative control. The dot blot shows antiserum cross-reactivity in fractions A4 to A11 (unbound protein peak fractions). Also fractions 29 (C5) to 36 (C12). These fractions (29 to 35) correspond to the first and second bound protein peaks (figure 5.1.9).

Figure 5.1.11 shows that the unbound ion exchange protein peak contained a number of proteins coming off the column, including an intense band at 70kDa. Lane 5, containing pool 4 also showed a protein band at 70kDa, but also showed the two expected bands at 45 and 40kDa. Lane 6, containing pool 5, showed the protein band at 45kDa, but the 70kDa protein (indicated with a green arrow) was not detected on the silver stained gel. The third protein peak in lane 7 showed little evidence of any protein bands, suggesting that there was very little protein in the fractions.

Figure 5.1.11. Silver stain of gel electrophoretic separation of *Campylobacter jejuni* 11351 blood agar cell sonicate ion exchange fraction pools.

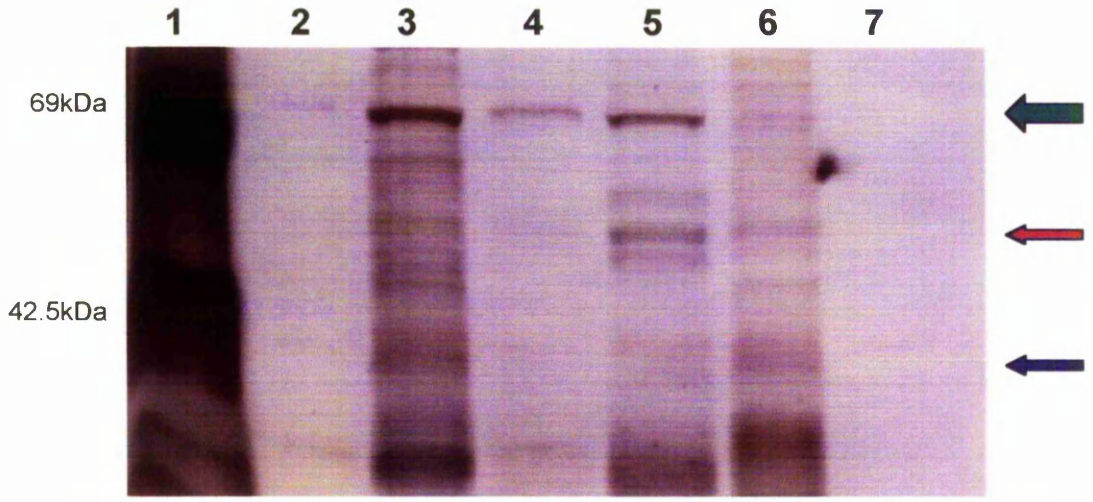


Figure 5.1.11. Fraction pools taken from *C. jejuni* 11351 blood agar cell sonicate ion exchange were separated by SDS-PAGE and stained using silver stain. Lane 1 contains protein molecular weight markers. Lane 2 to 7 contains pools 1 to 6, respectively, from the ion exchange column described in figure 5.10. 40kDa (indicated by a blue arrow) and 45kDa (indicated by a red arrow) proteins can be observed in lanes 5 and 6. 70kDa protein (indicated by a green arrow) can be seen in lanes 3, 4 and 5.

The fraction pools showing evidence of protein content (pools 2, 4 and 5) were then tested for cytotoxicity on cultured ECV cells as normal, the results of which can be seen in figure 5.1.12. The figure shows the unbound protein peak (pool 1; column fractions 6 to 11) was found to have a cytotoxic effect towards cultured ECV cells. The ion exchange resin bound protein peak 1 (pool 4; column fractions 28 to 31) and the second ion exchange resin bound protein peak (pool 5; column fractions 32 to 39) also had cytotoxic effects on the cultured ECV cells.

Figure 5.1.12. Cytotoxic effects of *Campylobacter jejuni* 11351 blood agar cell sonicate ion exchange fraction pools on cultured human endothelial cells.

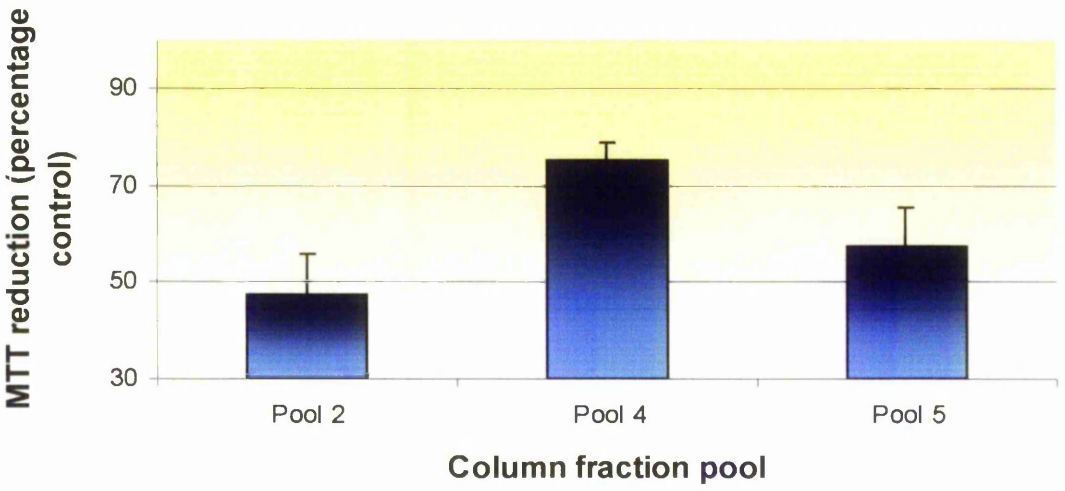


Figure 5.1.12. Samples applied to column were taken from *Campylobacter jejuni* 11351 blood agar plate grown cell sonicates. The graph shows cytotoxicity in all fraction pools. The removal of unbound protein toxins from the sample after ion exchange suggested the presence of a mixture of protein toxins present in the cell sonicate.

The results shown in figure 5.1.12 show evidence of a protein toxin in the unbound fractions after ion exchange chromatography. Therefore the unbound protein fraction pool was also stored for further analysis, along with the two bound protein peak pools.

The bound fraction pools were then combined and applied to an anti-cholera toxin affinity column. However, unlike the affinity column used previously (figure 5.1.6), this column was made up of a cyanogen bromide activated agarose resin coupled to an anti-cholera toxin β sub-unit monoclonal antibody (Chemicon). The change from polyclonal to monoclonal primary antibody was made in order to increase antigen specificity, hence increasing toxin purification efficiency by reducing non-specific binding. The affinity column purification was carried out as before, except that protein assays were done manually after fraction collection, and the protein content of column fractions can be seen in figure 5.1.13a. The figure shows fraction one containing unbound proteins and fractions 12 to 21 containing a peak of eluted bound proteins. Unlike the previous affinity column, the levels of absorbance did not subside in later fractions, resulting in termination of the elution before protein levels dropped (figure 5.1.13a). The eluted protein fractions were dialysed against PBS, and tested for cytotoxicity by application to cultured ECV cells, the results of which can be seen in figure 5.1.13b.

Figure 5.1.13b shows the levels of cytotoxicity in the anti-cholera toxin affinity column fractions, compared with the protein content (mg/ml) in the column fractions. The level of MTT reduction drops significantly compared to controls in ECV cells treated with material from fractions 11 to 19. These fractions correspond with the eluted bound protein peak (figure 5.1.13a), suggesting

that the isolation of the cholera-like protein toxin through affinity chromatography had been successful.

To check the molecular weight of the toxin and the purity of the fractions, they were separated by gel electrophoresis. The gels were silver stained to reveal proteins and then western blotted onto nitrocellulose and immunoprobed with anti-cholera toxin antibody to check molecular weights of cross-reactive bands (figures 5.1.14 and 5.1.15, respectively).

Figure 5.1.14 shows an image of a silver stained gel electrophoretic separation of the proteins contained in fractions 12 to 19 (bound proteins, seen in figure 5.1.13). The image shows a very faint doublet in all fractions running beside the 69kDa marker (lane 1). However the protein bands around 40kDa, previously observed after affinity column purification, were not detected.

Figure 5.1.13. Protein content/Cytotoxicity of anti-cholera toxin affinity column fractions from ion exchange partially purified C.*jejuni* 11351 blood agar cell sonicates.

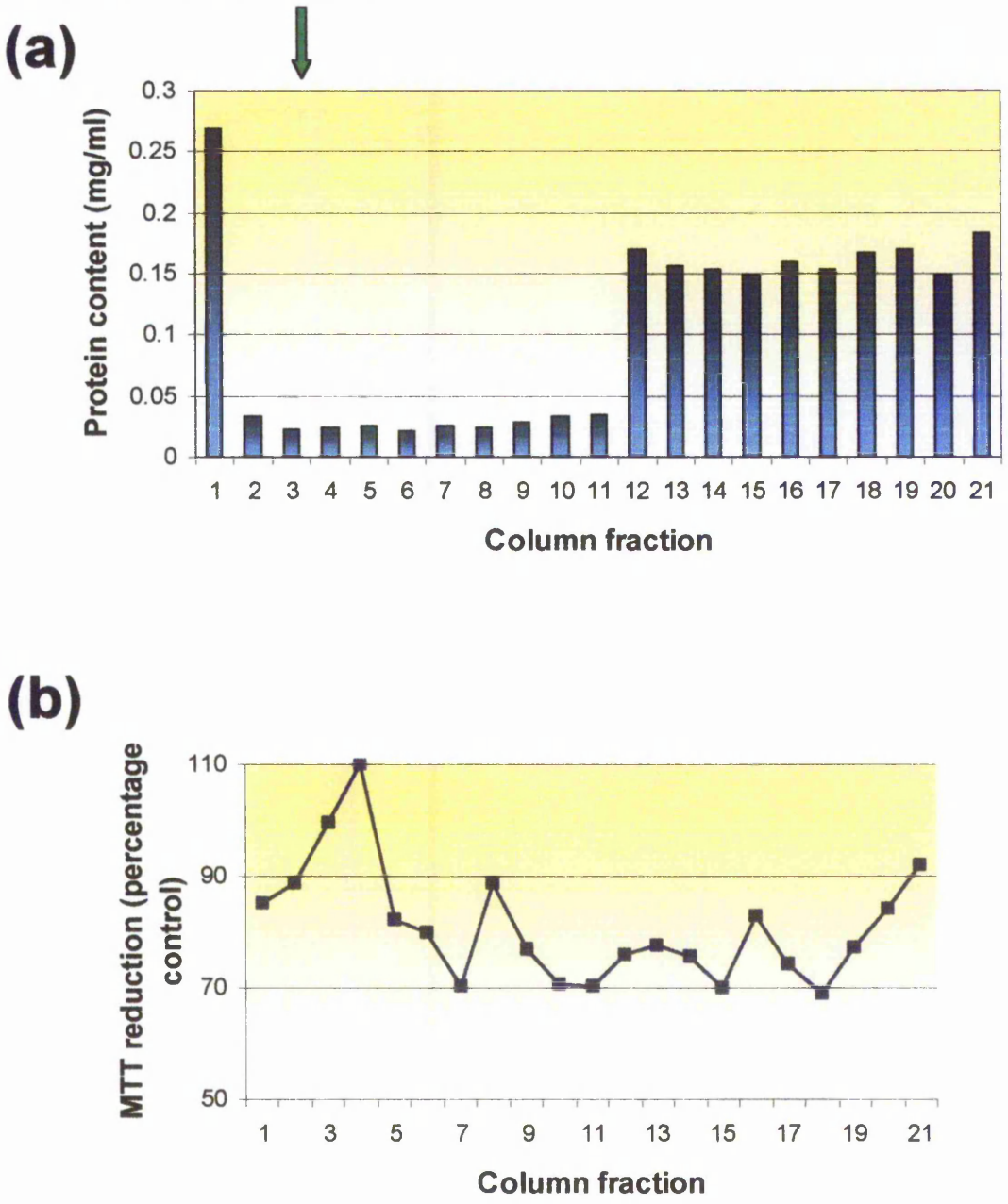


Figure 5.1.13. Protein content (a) and Cytotoxic effects (b) anti-cholera toxin affinity column fractions from *C. jejuni* 11351 blood agar cell sonicate. Figure 5.1.13a shows the removal of unbound proteins (fractions 1 and 2), before an increase in pH (indicated by green arrow), resulting in the elution of bound proteins (fractions 12 to 21). Figure 5.1.13b shows an increase in cytotoxic effects in fraction 5 to 19, which corresponds to the bound protein peak eluted from the affinity column.

Figure 5.1.14. Silver stain of gel electrophoretic separation of affinity column fractions containing bound proteins.

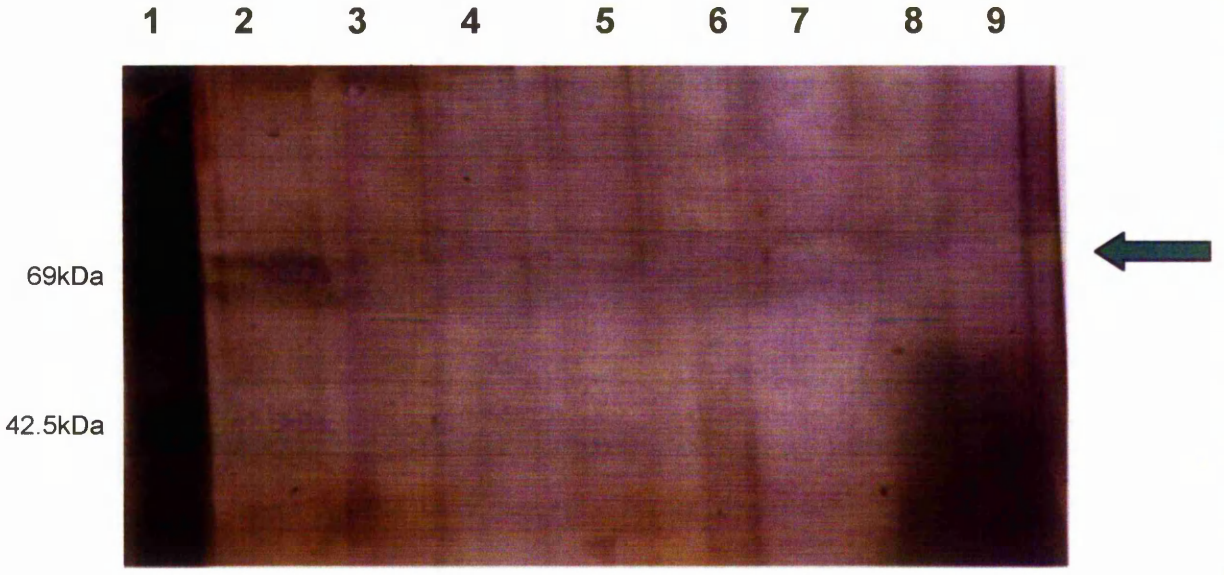


Figure 5.1.14. Affinity column fractions were separated by SDS-PAGE and the gels stained with silver stain. The image shows protein molecular weight standards in lane 1, along with fractions 12 to 19 (lanes 2 to 9). These were the fractions observed to contain the eluted protein peak. A protein band doublet at the 70kDa mark (indicated by a green arrow) was observed, but no expected bands at the 40kDa mark were detected.

Figure 5.1.15 shows the immunoblot of the same column fractions immunoprobed with anti-cholera toxin monoclonal IgG. Again the blot shows a triplet at the 70kDa molecular standard and no bands around the 40kDa mark. Despite this inconsistency with previous affinity column fractions, the proteins isolated looked pure enough for N-terminal amino acid sequencing. Therefore the fractions containing the protein from the column (fractions 12 to 19) were pooled, concentrated down using a millipore centrifugal concentrator and then electrophoretically separated, before blotting onto immobilin

membrane filters. The blot was reversibly stained with amido black and an image captured before destaining. The blot was sent to the Nottingham University Queens Medical Centre protein sequencing unit for analysis. The image of the stained immobilon membrane filter can be seen in figure 5.1.16.

Figure 5.1.15. Immunoblot of affinity column fractions, immunoprobed with an anti-cholera toxin monoclonal antibody.

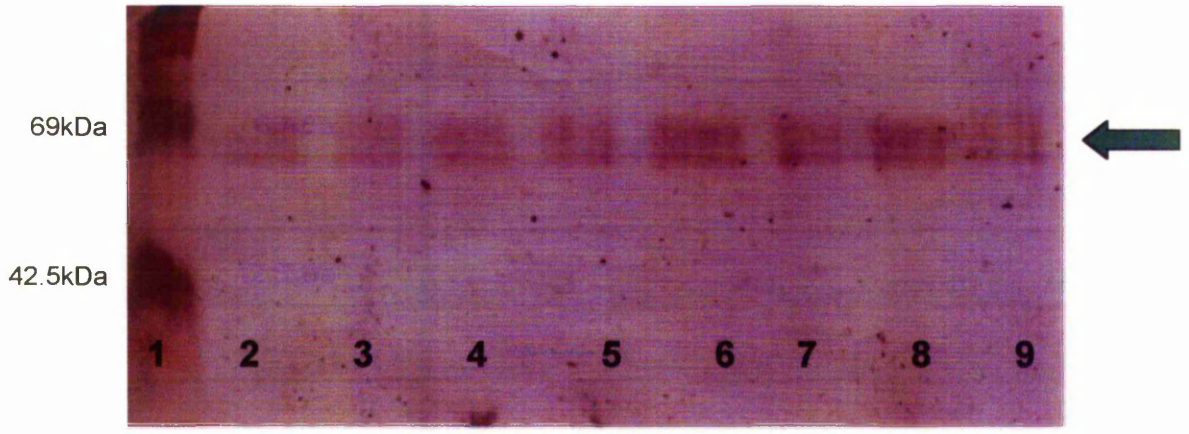


Figure 5.1.15. Immunoblot of affinity column fractions, immunoprobed with an anti-cholera toxin monoclonal IgG. The immunoblot shows protein molecular weight standards in lane 1, along with fractions 12 to 19 (lanes 2 to 9). These were the fractions observed to contain the eluted protein peak. The reactive protein bands were observed around the 70kDa mark (indicated by green arrow). This triplet was not expected, as previous blots had shown bands around the 50kDa mark.

Figure 5.1.16 shows the amido black stained immobilon blot with lane 1 containing kaleidoscope molecular weight markers and lane 3 containing a sample of the affinity column fraction pool. The pool was made up of fraction 12 to 19, which contained the main protein peak eluted from the affinity column. Stained bands were sequenced using an HPLC machine (out of department) and the band at the 32kDa mark was found to be mouse IgG light chain. The band just above the 43kDa mark was found to be mouse IgG

heavy chain. The band at the 69kDa mark was not pursued past the first 5 amino acids as no convincing results were obtained from the HPLC run (possibly due to the levels of protein in the band). This band may have been our protein toxin, but no convincing N-terminal sequence data was obtained and, more to the point, there was a possible contamination with the anti-cholera toxin IgG from the column. To confirm antibody contamination in the elution fractions the column was washed with 50mM Tris-HCl elution buffer and Western blots of the wash samples were probed with anti-mouse secondary antibody alone. The immunoblot can be seen in figure 5.1.17.

Figure 5.1.16. Amido black stain of immobilon blot containing anti-cholera toxin affinity column fractions from ion exchange purified *C. jejuni* 11351 blood agar cell sonicates.

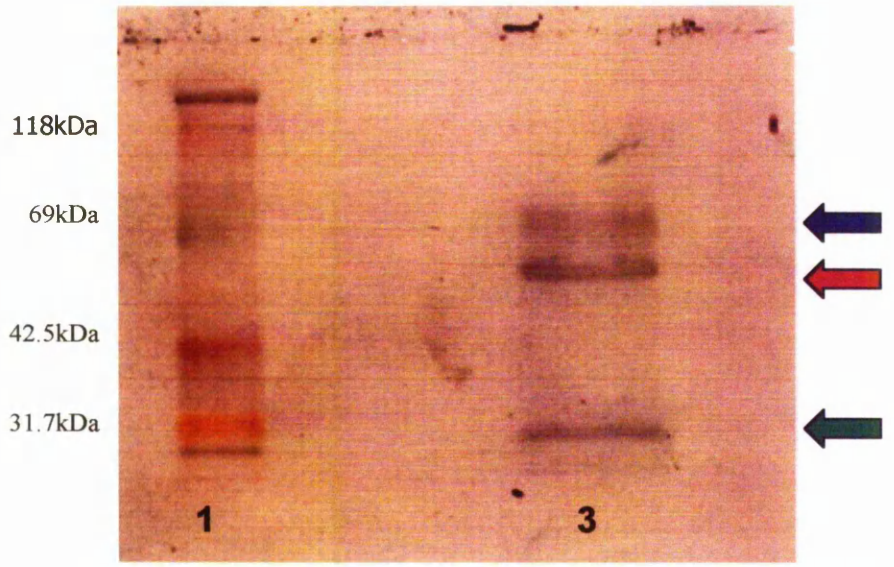


Figure 5.1.16. Amido black stain of immobilon blot containing anti-cholera toxin affinity column fractions. The blot shows a protein band at 30kDa (indicated by a green arrow), which was identified as mouse IgG light chain. The protein band at 50kDa (indicated by a red arrow) was identifed as mouse IgG heavy chain. The sequence of the protein band at 70kDa (indicated by a blue arrow) was not identified.

The immunoblot shown in figure 5.1.17 shows that the elution buffer contained some of the antibodies coupled to the resin column (with the IgG heavy chain showing up after secondary antibody probing only). This suggests that the elution conditions had damaged the coupled antibodies. Due to time constraints it was decided to try a different purification method to accumulate sufficient toxin for N-terminal sequencing..

Figure 5.1.17. Immunoblot of affinity column wash samples to check for antibody leaching.

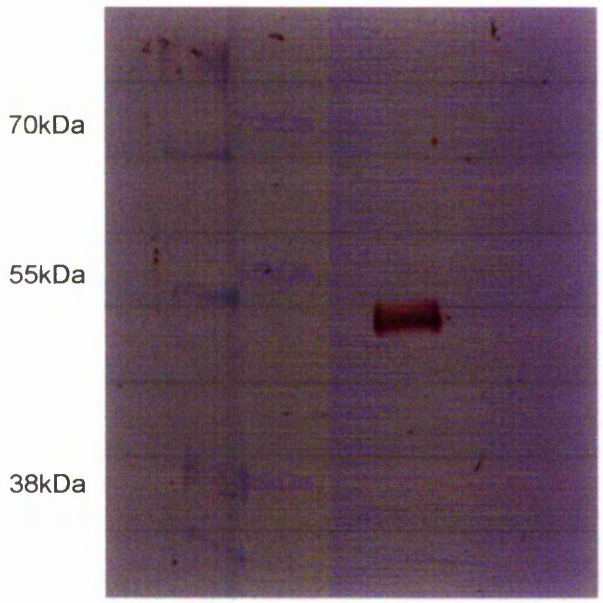


Figure 5.1.17. Immunoblot of affinity column wash samples to check for antibody leaching. The blot shows that even without primary antibody incubation, anti-mouse secondary immunoprobing reveals mouse IgG heavy chains present in the sample taken after washing the column with elution buffer alone.

The technique used for enrichment of anti-cholera toxin antibody reactive protein was electro-elution. The cross-reactive fraction pool from ion exchange chromatography was separated by gel electrophoresis and anti-cholera toxin antibody reactive protein bands were identified by probing Western blots, as described in section 2.2.6.3. The amido black stained immobilon blot used for sequencing and its corresponding probed Western blot can be seen in figure 5.1.18. Both blots showed reactive bands at the 50kDa position (*i.e.* just above the molecular weight of the cholera-like protein toxin identified by Diakoku, *et al* 1990). The protein bands were resolved better on the immobilon blot, the anti- CT antibody cross-reactive protein band was sequenced (see Appendix for full data). The results from N-terminal sequencing showed two possible protein sequences for the first nine amino acids. The two possible sequences were:

T E L K E A I K D

A P V E I G V V L

Taking the two sets of amino acids and placing them through the protein sequence LALIGN data-base (www.ch.embnet.org/software/LALIGN-form.html), which finds multiple matches for sub-segments in two sequences, a match was found for the combined sequence:

T P L E E A I K D

The above sequence matched the first nine amino acids of a major outer membrane protein found in *Campylobacter jejuni*, identified by Schroeder, *et al* (1997). The protein had previously been confirmed as a porin toxin produced by *Campylobacter jejuni*, which may have a role in the adhesion of

bacterial cells onto intestinal cells (Bolla, *et* al., 1995). A second match was found for the sequence combination of:

A E V K I G V V L

This sequence matched the nine amino acid sequence starting from position 19 of an ABC transport system periplasmic binding protein (Parkhill 2000).

Of the two, the most likely protein identified as the cytotoxin isolated was probably the porin, as the transport protein has not been reported to have cytotoxic effects on mammalian cells.

Figure 5.1.18. Immobilon (a) and probed nitrocellulose (b) blots for sequencing/identification of electro-elution isolated anti-cholera toxin IgG reactive proteins from *C. jejuni* 11351 blood agar cell sonicates.

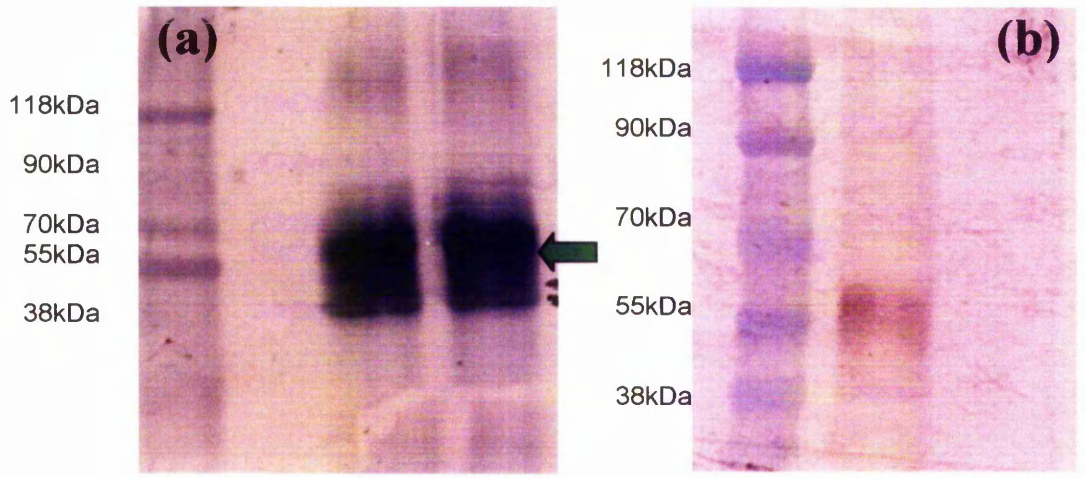


Figure 5.1.18 Images show separation of electro-eluted proteins, blotted and probed on (a) immobilon blot, for sequencing of electro-elution isolated anti-cholera toxin IgG cross-reactive proteins (indicated by a green arrow). Protein bands sequenced using a non-departmental HPLC. And (b) nitrocellulose blot, for identification of electro-elution isolated anti-cholera toxin IgG cross-reactive proteins. The reactive protein band did not resolve as expected (at 70kDa), but corresponded with the position of the protein band in the immobilon blot.

The affinity isolated protein toxin was then assayed for toxin enrichment, since the whole point in the purification process was to isolate/enrich the level of a specific, novel protein cytotoxin. Therefore the electro-eluted fraction samples were assayed for protein content and assayed for effects at cytotoxic and sub-cytotoxic effects as for the original ion exchange fractions. The results were then compared with effects observed in the original ion exchange culture fractions in order to estimate toxin enrichment. The results can be seen in figures 5.1.19 (cytotoxicity) and figure 5.1.20 (sub-cytotoxicity).

Figure 5.1.19 shows that at 5% and 10% (v/v) concentrations the electro-eluted toxin samples produce a 20% reduction in cell viability compared with control treated cells. This can be directly compared with a 30% (pool 4) and 45% (pool 5) reduction in cell viability produced by the ion exchange purified blood agar plate cell extracts, respectively. The fact that a much lower toxin protein concentration after electro-elution (0.095 mg/ml) than that of ion exchange fractions (7.8 mg/ml) produced only slightly less cytotoxicity, provides clear evidence of toxin enrichment.

Figure 5.1.19. Cytotoxic effects of electro-eluted cholera-like protein toxin on cultured human endothelial (ECV) cells.

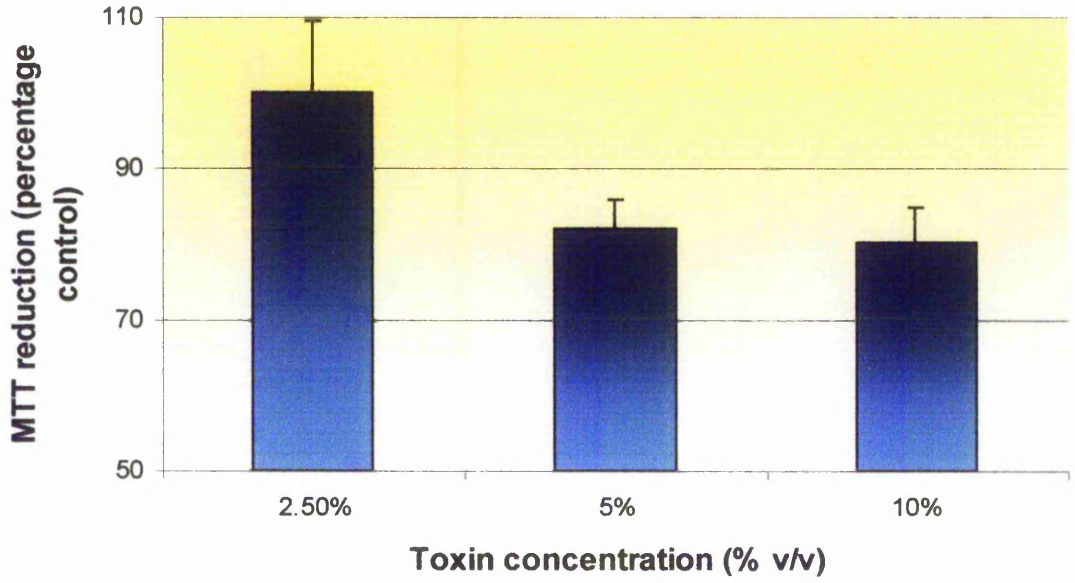


Figure 5.1.19. Electro-eluted cholera toxin antibody cross-reactive fractions were dialysed against PBS and tested for cytotoxicity on cultured ECV cells as described in methods. The graph shows a 20% decrease in cell viability for cells treated with 5% - 10% toxin, compared to PBS-treated control cells.

Figure 5.1.20 shows the effects of electro-eluted toxin on cultured N2a cells (model for sub-cytotoxic effects due to axonal outgrowth as marker system) at sub-cytotoxic concentrations. The graph shows that there is a significant decrease in axon outgrowth from toxin treated cells compared with control cells. This can be compared with the original cell sonicates, that produced a 40% decrease in axon outgrowth (from 25% outgrowth to 15%). This shows a definite enrichment of neurotoxin, since the reduction in protein content was significant (82% reduction in protein levels compared with fractions after ion exchange chromatography).

Figure 5.1.20. Sub-cytotoxic effects of electro-eluted cholera-like protein toxin on differentiating N2a cells.

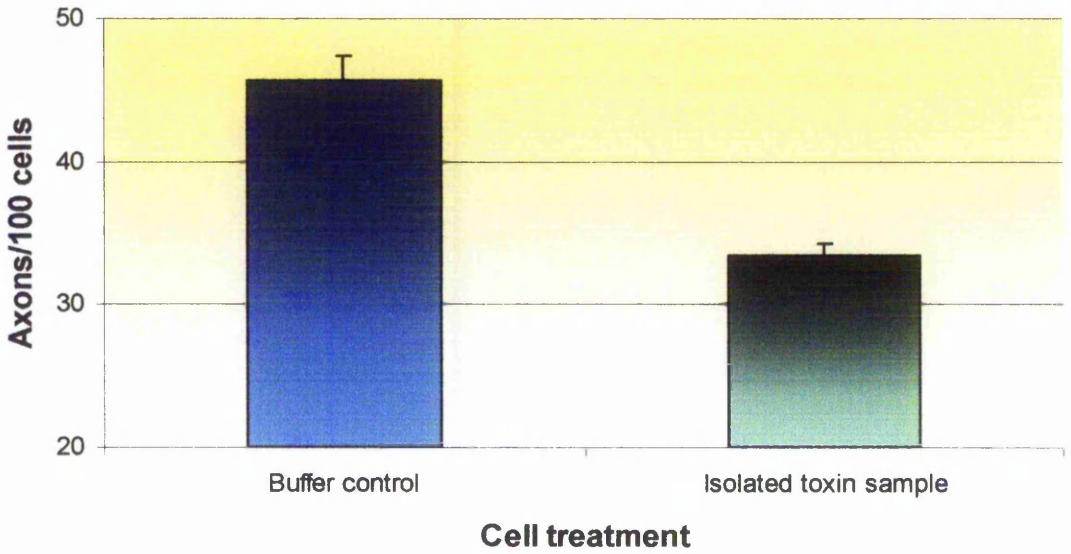


Figure 5.1.20. Sub-cytotoxic effects of enriched cholera-like protein toxin on differentiating N2a cells. The graph shows a 20% reduction in axon outgrowth in treated cells compared with control cells. This was directly comparable to the 40% reduction in axon outgrowth produced by the original bacterial cell sonicates, thus showing definite evidence of protein toxin enrichment.

Protein enrichment was tabulated and can be seen table 5.1.1. The table shows that if one unit of inhibition is taken as 10% reduction from control levels (either in MTT reduction or axons/100 cells) per mg protein, then the affinity isolated toxin shows a 21 fold enrichment in cytotoxicity and a 60 fold enrichment in sub-cytotoxicity compared to ion exchange purified toxin. This provides definite evidence of toxin enrichment through affinity purification.

Table 5.1.1. Enrichment of *C. jejuni* 11351 cholera toxin antibody cross-reactive toxin.

	Protein conc (mg/ml)			*Inhibition (units/mg protein)*	*Fold enrichment*
Ion exchange toxin	7.8	MTT reduction (% control)	80	1.026	-
		Axon outgrowth (% control)	37.9	0.49	-
Electro-eluted toxin	0.095	MTT reduction (% control)	20	21.052	21
		Axon outgrowth (% control)	28.3	29.5	60

Table 5.1.1. Showing toxin fold enrichment for samples taken from electro-eluted toxin compared with ion exchange isolated toxin. The right hand column shows a 21 fold enrichment in cytotoxicity and a 60 fold enrichment in sub-cytotoxicity.

The results shown in this chapter show definite toxin enrichment and give two possible identities to the toxin in question. Further work required for toxin purification and characterisation can be seen in Chapter 6.

5.2. Discussion of Novel *Campylobacter jejuni* 11351 Toxin Isolation and Characterisation.

The results from Chapters 3 and 4 showed evidence for the presence of at least two cytotoxins both with sub-cytotoxic effects on cultured mammalian cell morphology, and more specifically, the three cytoskeletal networks present in the cytoplasm. This work was carried out using crude bacterial culture supernatants and cell sonicates. In order to isolate and characterise a specific toxin, purification and isolation of that toxin was required. Initial purification steps involved culture supernatant/cell sonicate centrifugation, followed by protein precipitation through the addition of ammonium sulphate to 80% saturation. Partial purification through ion exchange chromatography was then carried out, using a Q-Sephadex cartridge. The protein elution profile in figure 5.1.1a shows that non-bound proteins contaminated initial bound protein fractions due to a mistake in the timing of the start of the salt gradient elution buffer during ion exchange chromatography of BHI- broth culture supernatant. This was corrected in the separation of blood agar cell sonicate (figure 5.1.2a). Cytotoxicity towards mammalian cells (figures 5.1.1b and 5.1.2b) corresponded well with protein peaks coming off the columns, suggesting the presence of cytotoxin in these fractions. During further ion exchange separations to scale up the amount of blood agar cell sonicate

toxin, two clear protein peaks were resolved on the salt gradient (figure 5.1.9). This was probably due to a more efficient salt concentration gradient (automatically produced). Cytotoxicity assays using the eluted protein peaks (figure 5.1.12), again showed toxin presence in fractions containing high levels of protein.

Although this initial purification step resulted in the removal of some non-cytotoxic proteins present in the culture extracts, further purification was required for the isolation and characterisation of a single protein toxin. Affinity chromatography using anti-cholera toxin antiserum was therefore decided upon because (a) it had been used in the partial purification of a cholera-like toxin from a *Campylobacter jejuni* clinical isolate by Daikoku *et al* (1990) and (b) anti-cholera toxin antiserum was found to partially neutralise cytotoxicity of crude culture supernatant and cell sonicate (figure 5.1.3). Furthermore, initial dot-blot analysis of fractions eluted from the initial ion exchange columns showed cross-reactivity in specific fractions (figure 5.1.4), along with some cross-reactivity in the unbound protein fractions (possibly due to column overload due to large sample volumes) in later separations (figure 5.1.10). This, together with the neutralisation of toxicity in crude extracts, suggests the presence of a cholera-like protein toxin in both culture supernatant and cell sonicates that was still present after ion exchange chromatography.

Probing of Western blots of ion exchange fractions with the anti-CT antiserum revealed cross-reactive protein bands at approximately 70kDa and 40kDa (figure 5.1.5), similar to cytotoxin protein molecular weights identified in reports by Diakoku *et al* (1990) and Bacon *et al* (1999), after isolation using anti-CT affinity chromatography. The presence of more than one band could

suggest either more than one toxin, or a complex of sub-units. Alternatively the lower molecular weight form could be a degradation product of the 70kDa band.

In order to isolate a large amount of cross-reactive protein, an affinity column was produced (as reported by Diakoku *et al* 1990), firstly, using a polyclonal anti-cholera toxin B sub-unit antiserum and secondly, using a monoclonal anti-cholera toxin B sub-unit IgG (as used by Diakoku *et al* 1990; Suzuki *et al* 1994; McCardell *et al* 1984) as antigens bound to a cyanogen bromide activated agarose resin. The column profile shown in figure 5.1.6 shows that a single protein peak was eluted mid-run from the antiserum affinity column, whereas the protein peak eluted from the monoclonal affinity column was eluted near the end of the run (figure 5.1.13a). This may be explained by binding specificity differences between the polyclonal and monoclonal affinity columns, resulting in the elution of bound proteins in a shorter time period by the less specific polyclonal column. The fact that the protein absorbance readings did not peak from the monoclonal column (figure 5.1.13a) suggests further elution was required for complete protein elution. Analysis of protein peaks by SDS-PAGE (figures 5.1.7 and 5.1.14) and cytotoxicity testing (figures 5.1.8 and 5.1.13b) indicated that the eluted protein fractions contained major protein bands at approximately 70kDa and 40kDa, which retained a cytotoxic effect (suggesting the presence of the 68kDa protein isolated by Diakoku *et al* 1990; Suzuki *et al* 1994; McCardell *et al* 1984). Indeed, the fact that the protein concentration was relatively low after purification suggested enrichment of toxin.

Immunoprobing to check reactive protein molecular weights (figure 5.1.15) resulted in a reactive doublet at the 70kDa marker (also observed in the initial antiserum blots, figure 5.1.5).

The fact that the 70kDa bands were retained from initial probing after ion exchange and through affinity purification, along with the presence of cytotoxicity, suggests the cross-reactive proteins present are important for toxin activity.

Affinity purified proteins were separated by SDS-PAGE and transferred onto Immobilon membrane filters for N-terminus sequencing. The results of the sequencing showed the protein bands (figure 5.1.16) corresponded to the anti-cholera IgG heavy chain (43kDa) and light chain (32kDa). The 69kDa band (of similar weight to band reported by Diakoku *et al* 1990); Suzuki *et al* 1994; McCardell *et al* 1984) was not resolved well enough for sequencing. This meant that the eluted column fractions were being contaminated by anti-cholera toxin IgG leaching off the column during elution. A possible reason was the stringency of the elution buffer and may be resolved by reducing the alkaline pH of the buffer, in order to preserve antigen/resin coupling.

Due to time constraints at this stage of the project, in order to accumulate sufficient toxin for sequencing, a different purification technique was devised, using a combination of western blotting along side protein electro-elution was used in order to purify the anti-cholera toxin IgG-reactive protein bands separated by SDS-PAGE (figure 5.1.18). N-terminal sequencing identified a nine amino acid sequence for an approximately 50kDa band (similar in size to band reported by Diakoku *et al* 1990; Suzuki *et al* 1994; McCardell *et al* 1984; Collins *et al* 1992). Analysis by BLAST search and LALIGN databases

produced two possible protein matches. The first was a major outer membrane protein, reported to be a cytotoxic porin, with a molecular weight of 43kDa (Bolla *et al* 1995; Schroder *et al* 1997; Amako, *et al* 1996). A similar toxin amino acid sequence was reported by Moutinho-Fragoso, *et al* (2000), working on the *Campylobacter coli* species, although their isolation techniques did not involve anti-cholera toxin IgG purification (Moutinho-Fragoso *et al* 2000).

The second match was for the ABC transport system periplasmic binding protein reported by Parkhill *et al* (2000). The group reported the full genome sequence for *Campylobacter jejuni* 11168, of which residues 1 to 369 produced a branched-chain amino-acid ABC system periplasmic binding protein. The nine amino acid sequence identified in this thesis matched a nine amino acid sequence starting from residue 18 of said transport protein. This was the least likely match of the two possible proteins as there was a 17 amino acid "tail" at the N-terminal before the sequence matched our identified protein which could be explained by N-terminal proteolytic cleavage of our protein, or post-translational modification of the Parkhill protein, as the sequence identified was based on nucleotide rather than amino acid sequence. Moreover, there is no evidence for cytotoxicity of this protein, which has been identified as a transport protein in the bacteria and therefore, logically would have no virulence activity outside the cell (Parkhill *et al* 2000).

Therefore it is more likely that the protein cytotoxin which has a molecular weight of approximately 50kDa and is immunologically similar to the cholera toxin, has the N-terminal amino acid sequence matching the major outer

membrane protein of *Campylobacter jejuni.* This protein has been characterised as a cytotoxic protein that plays an important part in *Campylobacter jejuni* virulence (Bolla *et al* 1995; Schroder *et al* 1997; Amako, *et al* 1996). After a LALIGN sequence homology search, it was found that the porin protein contained a number of amino acid sequence homologies with the cholera toxin. This would explain the cross-reactivity with the anti-CT monoclonal antibodies.

To summarise, a cholera toxin-like protein was partially purified, with N-terminal sequencing resulting in a match with the major outer membrane protein of *Campylobacter jejuni.* The purification procedure resulted in a toxin cytotoxicity enrichment of 21 fold and a sub-cytotoxicity enrichment of 60 fold compared to original culture samples. Further work on toxin isolation is required for homologous extraction of the protein, which would allow full characterisation into pathological mechanisms of action and identification of specific toxin targets present in mammalian cells.

CHAPTER 6

6. General Discussion and Future Work

6.1 General Discussion.

6.1.1. Campylobacter jejuni strain/culture condition selection.

It is now known that *Campylobacter jejuni* and related species are the most common bacterial causes of acute infective diarrhoea in the developed world. Along with bacterial motility, adherence and invasion, toxin production plays an important role in the pathogenesis of bacterial infection. Therefore the identification and characterisation of these virulence factors produced by the bacteria are of major concern in the understanding of pathological mechanisms exhibited by the bacteria.

The main aims of this thesis were; firstly, the investigation of three *Campylobacter jejuni* strains (11351, 11322, 11168) in order to determine the most effective cytotoxin producing strain; secondly, a number of bacterial culture conditions were tested in order to optimise bacterial growth conditions, for cytotoxin production; thirdly, once cytotoxic levels of bacterial culture extracts were determined, investigations into sub-cytotoxic effects on mammalian cell morphology and the cytoskeleton were carried out. Finally, attempts were made to isolate a novel toxin to a degree that allowed N-terminal sequencing and estimation of toxin identity (through comparisons with known sequences in BLAST and LALIGN databases), followed by characterisation of toxin mechanisms of action at the molecular level.

The general cytotoxicity from the studies carried out on *Campylobacter jejuni* 11351, 11168 and 11322 were sufficient for the selection of the most consistent cytotoxin producing strain. Experiments concerning bacterial growth conditions also resulted in two prominent culture conditions that promoted cytotoxin production. The bacterial strain selected for further work was *Campylobacter jejuni* 11351, grown in BHI +/- supplements, or on blood agar plates, due to the fact that BHI broth culture supernatants and the blood agar cell sonicates produced the most consistent cytotoxic effects on a number of cultured mammalian cell lines (N2a and ECV). These conditions have also been selected for a number of studies on *Campylobacter spp* toxicity (Lam 1993; Coote and Arain 1996; Schulze *et al* 1998), although the growth conditions that were rejected have also been used in a number of studies (Johnson and Lior 1986; Fagoso *et al* 1996; Misawa *et al* 1995). This variation in cytotoxin production by *Campylobacter spp* in similar growth conditions may be explained by inter-laboratory procedural differences or strain culture variations, but whatever the reason, there seems to be a constant variation in the results of *Campylobacter* toxicity research which does cause some overlap concerning the discovery of "novel" toxins (Wassenaar 1997).

From the results of cytotoxicity assays it was obvious that at least two types of toxins were being produced in the two bacterial culture conditions. Firstly, there was a secreted heat/trypsin labile exotoxin produced by bacteria grown in BHI broth cultures. Previously reported cytotoxins work intracellularly, after entry through the mammalian cell membrane via pore formation (Sillero and Almirall 1999; Hossain *et al* 1993; Akan *et al* 1993). Secondly, a heat/trypsin

stable cell-attached endotoxin was produced by bacteria grown on blood agar plates. Endotoxins have been reported to induce autoantibodies as a result of mammalian cell ganglioside mimicry, resulting in polyneuropathies such as GBS and MFS (Jacobs *et al* 1997). The endotoxin showed the highest level of cytotoxicity, suggesting *Campylobacter jejuni* 11351 grown on blood agar plates would prove the most fruitful in cytotoxin isolation.

6.1.2. Sub-cytotoxic effects on mammalian cell morphology/cytoskeleton.

The sub-cytotoxic effects of the bacterial broth culture supernatant included elongation of cell bodies in CHO cells. This suggests the presence of a cytolethal distending toxin (CDT) (Purdy *et al* 2000; Mooney *et al* 2000; Whitehouse *et al* 1998; Bag *et al* 1993). At the molecular level, the supernatant appeared to produce major redistribution of the actin network, from diffuse cytoplasmic staining to aggregates plus relatively high staining in neurites, in N2a cells (observed in immunofluorescence images of broth supernatant treated cells), which has also been associated with CDT (Aragon *et al* 1997). When introduced to ECV cells the cell body rounding was observed, suggesting the presence of a cytolethal rounding toxin (CRT) (Schulze *et al* 1998; Hanel *et al* 1998).

Experiments examining the possibility of neurotoxin production by *Campylobacter jejuni* 11351, revealed neurotoxic effects on N2a cells treated with BHI broth culture supernatant. The sub-cytotoxic effects were heat sensitive, suggesting a proteinaceous neurotoxic exotoxin, secreted into the culture supernatant. The results of treating N2a cells with sub-cytotoxic levels

of blood agar cell sonicate, showed the presence of a neurotoxin expressed at the stationary phase of the cell cycle. These findings require further investigation in order to assess the significance of neurotoxin production as a further virulence factor in the bacterial involvement in neuropathies, such as GBS and MFS.

Further investigation into sub-cytotoxic effects of bacterial culture extracts on cytoskeletal proteins revealed a reduction in microtubule proteins, including α tubulin sub-unit protein levels and also, post-translationally modified acetylated α tubulin sub-unit protein levels in a number of cultured mammalian cell lines. The latter results suggesting that sub-cytotoxic effects were directed at the protein level, as the data represents changes in protein levels in a post-translationally modified isomer. These results are supported by reports on microtubule involvement during *Campylobacter jejuni* invasion of human intestinal mucosa monolayers (Hu *et al* 1997).

Sub-cytotoxic effects on actin and neurofilament protein levels revealed toxin targeting of the intermediate filament and microfilament networks, showing a similar mode of action to a number of bacterial toxins; *C. botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* toxins and the ADP-ribosyltransferase produced by *C. difficile* are all actin modifying toxins (Boquet *et al* 1998; Lacy and Stevens 1998; Balfanz *et al* 1996).

Respective protein level increases observed in ECV cells treated with blood agar cell sonicates represents a different method of toxin sub-cytotoxic activity, but does still represent an effect, as a number of reports describe

toxin-induced protein level increases (Flatau *et al* 1997; Lacerda *et al* 1996; Donelli and Fiorentini 1992).

These changes in protein levels support immunofluorescence images that showed a general disruption of the microtubule and microfilament networks. These changes may be an early sign of cytopathogenicity.

6.1.3. "Novel" Campylobacter jejuni toxin isolation/identification.

Results from Chapters 3 and 4 suggest production of at least two different cytotoxins by *Campylobacter jejuni* 11351 (as described in section 6.1.1). Therefore, toxin isolation to the point of N-terminal sequencing was the next step taken. Initial purification of the BHI broth culture supernatant and blood agar cell sonicate involved protein precipitation followed by partial purification through ion exchange chromatography. These techniques resulted in the presence of a cytotoxin in the bound protein fractions eluted from the column (in both cases). Further purification was required before the toxin proteins were homogenous enough for N-terminal sequencing. Therefore, after initial neutralisation assays supported previous reports on production of a cholera-like toxin by *Campylobacter jejuni* (Daikoku *et al* 1990), affinity chromatography was attempted. Isolation of an immuno-reactive protein was contaminated with anti-cholera toxin β sub-unit monoclonal antibody heavy and light chains, which had leached from the column during elution. Therefore N-terminal sequencing of toxin proteins was not achieved using this method. The results did however, suggest the presence of an immuno-

reactive cytotoxin which could be isolated using anti-cholera toxin β sub-unit monoclonal antibodies.

Electro-elution isolated immuno-reactive protein bands were N-terminally sequenced, and compared with known protein sequences using BLAST and LALIGN databanks. Two possible protein matches were resolved. Firstly, a major outer membrane protein reported as a cytotoxic porin (Bolla *et al* 1995; Schroder *et al* 1997; Amako, *et al* 1996). Secondly, an ABC transport system periplasmic binding protein, which was reported to be a gene product from residues 1 to 369 of the *Campylobacter jejuni* 11168 genome (Parkhill *et al* 2000). Of the two, the outer membrane protein porin seems the most likely candidate, since the toxin sequenced was from cell sonicate, which may suggest an endotoxin, as part of the cell wall.

In order to fully determine the identity of the toxin further work is required in the purification process, along with further characterisation of the toxin activity at the molecular level.

6.2. Future Work.

As mentioned above further work is required for the purification of the toxin. To this end, in a preliminary study, ion exchange eluted protein fractions were pooled and applied to a phenyl sepharose resin hydrophobicity column after ammonium sulphate saturation ranges required for optimal column binding and elution were determined (section 2.2.7). At this late stage in the project, preliminary results showed immuno-reactive protein fractions, that after separation by SDS-PAGE, revealed 3 closely resolved protein bands (approximately 50kDa). But cytotoxicity assays showed no significant reductions in MTT reduction compared to control treated cells. Further work is required in this area, since the gels of separated immuno-reactive proteins looked the most promising compared with previous purification techniques.

Preliminary work on the actions of the toxins at the molecular level has also involved ADP-ribosylation activity assays. These assays involved treating mammalian cell lysates with bacterial culture extracts in the presence of thymidine to blocks the poly(ADP-ribose)polymerase, preventing consumption of NAD^+, which is required for quantitative ADP-ribosylation of actin. Therefore any ADP-ribosylation of actin must be induced by toxin activity. Unfortunately, preliminary results were inconclusive and further study of this potential toxin activity is required, along with assays concerned with the other major molecular mechanisms of action associated with bacterial pathogenesis, including deamidation, monoglycosylation and tyrosine phosphorylation.

For further understanding of the activities of the identified toxin, it is important to carry out experiments with cytoskeletal proteins (such as effects on protein levels, protein distribution within the cell body/extensions) and the resulting effect on cell body morphology using cells specifically treated with the homogenous toxin. Also, further investigation into the effect of the toxin on a range of neuronal cell lines would allow better understanding of the toxin and its role in the production of neuropathological syndromes associated with *Campylobacter jejuni* infections. Likewise, further studies on the changes in the organisation of the cytoskeleton in toxin-treated CHO and ECV cell lines would help to characterise the molecular basis of toxin action in non-neuronal cell lines.

Partial sequencing analysis of the toxin may be used to produce DNA probes that could be utilised in the identification of toxin presence in clinical cases of *Campylobacter spp* infection, and may also be used to discover any relationship between toxin production and the onset of any neurological disorders, such as GBS.

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APPENDIX

File Name: run 1725
In Folder: quad 650:Model 610A *f*:

14 cycles acquired on an Applied Biosystems, Inc. Model 473A
Run started on Friday, March 31, 2000 at 11:34 am

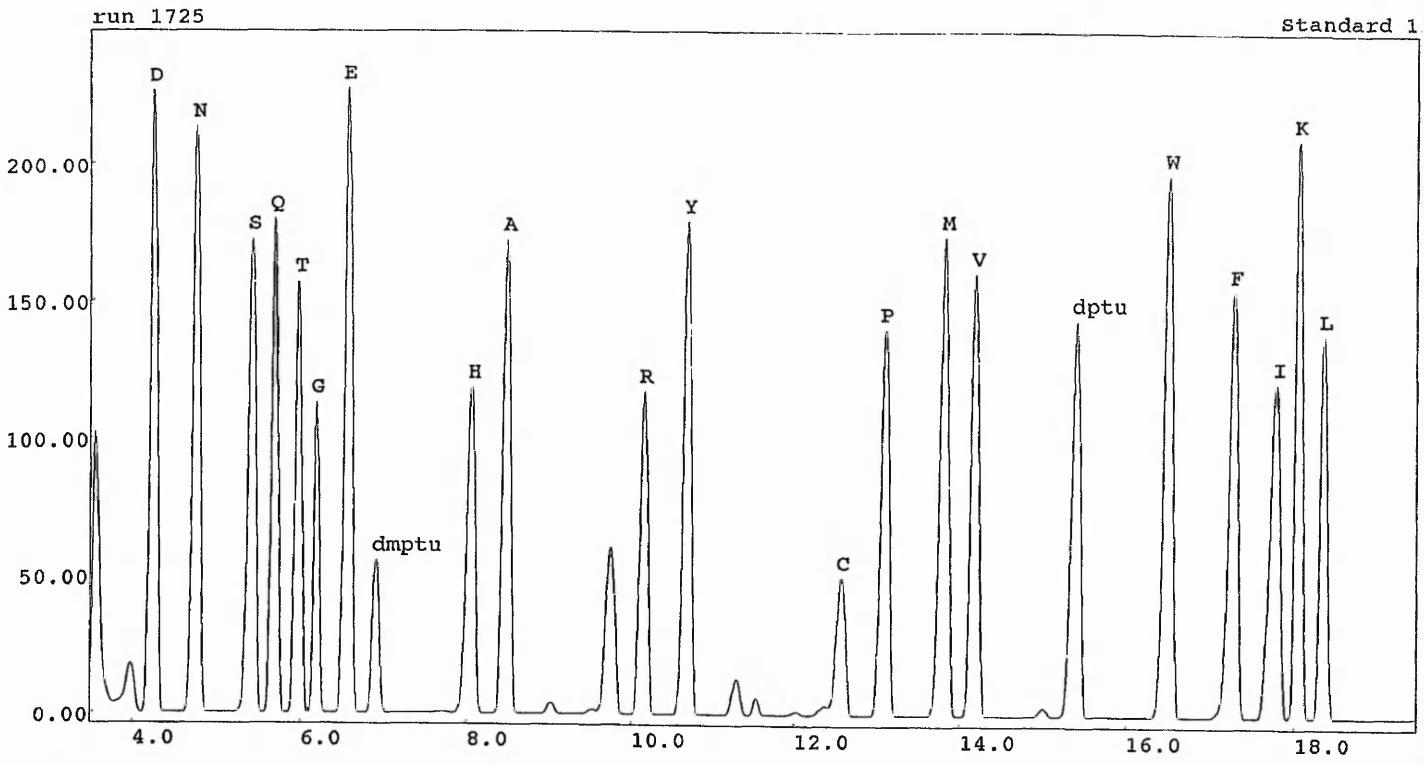
Sample Name:	run 1725
Reference ID:	K Spears, Trent Univ.
Sample Amount:	0 picomoles
Standard Amount:	50 picomoles
Sampling Rate:	2.0 Hertz
Detector Scale:	0.010 AUFS
User Comments:	Unknown band, lowest MW of a number present on blot (not well separated, some 'smearing'). Band of interest identified by customer.
Called sequence:	~~APLKBAIKK~~
User sequence:	mixed - see below

Analyzed Using Filtered Data, Smooth Degree 9, No Baseline

Analysis Limits:	1 - 9
Integration Limits:	3.50 - 20.00 min.
Baseline Limits:	0.10 - 1.00 min.
Peak Width:	0.01 min.
Peak Separation:	0.01 min.
Search Length:	0.30 min.
RT Factor:	1.95
Sensitivity:	1.00

EVIDENCE FOR FOLLOWING AMINO ACIDS:-

cycle	A.A.
1)	T + A
2)	E + P
3)	L + V
4)	K + E
5)	E + I
6)	A + G
7)	I + V
8)	K + V
9)	D + L



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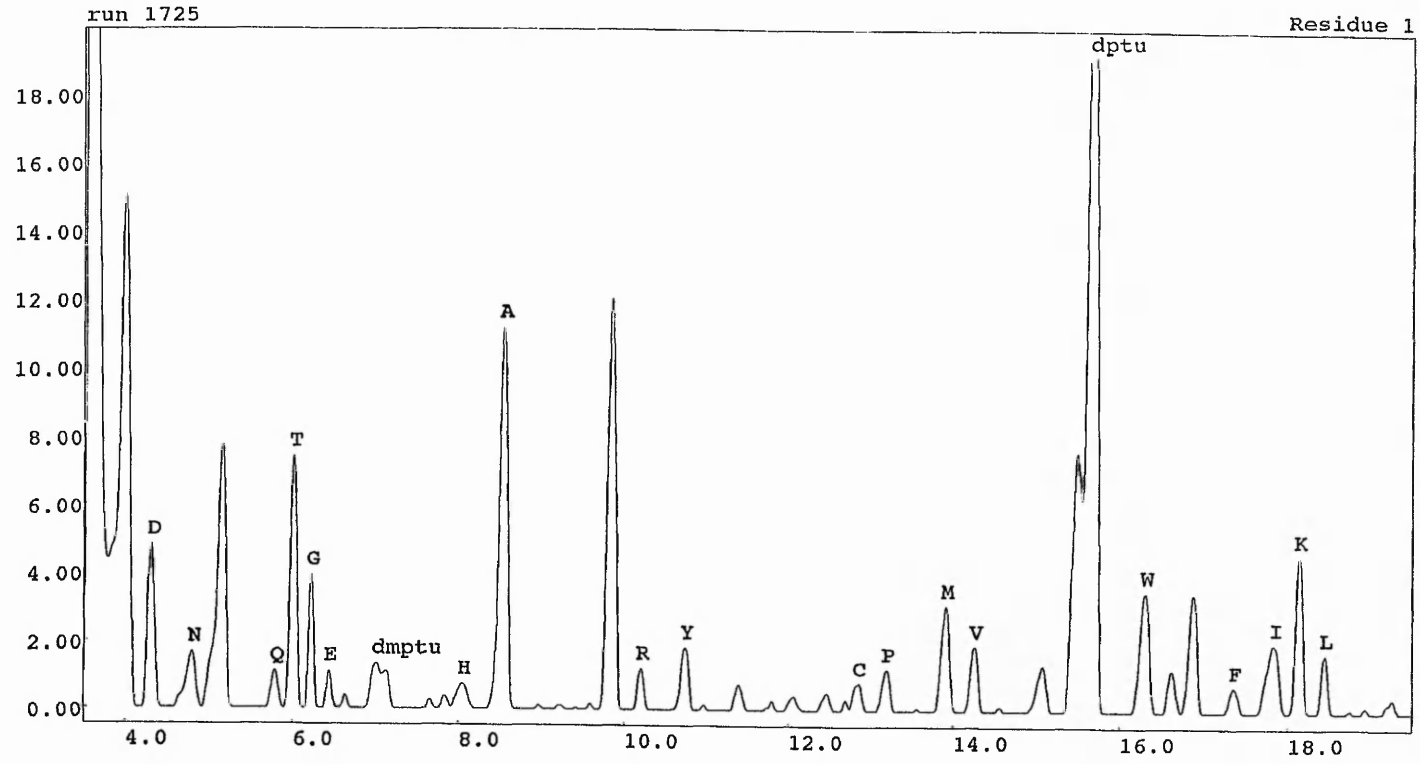
4	4.76	N	c	198095	50.00
5	5.42	S	c	159916	50.00
6	5.68	Q	c	164295	50.00
7	5.97	T	c	144692	50.00
8	6.18	G	c	99969	30.00
9	6.57	E	c	211965	50.00
10	6.90	dmptu		51350	50.00
11	7.68			483	50.00
12	8.05	H	c	111847	50.00
13	8.48	A	c	160730	50.00
14	9.01			3767	50.00
15	9.52			1441	50.00
16	9.74			58397	50.00
17	10.15	R	c	110410	50.00
18	10.69	Y	c	167992	50.00
19	11.29			12341	50.00
20	11.52			5481	50.00
21	12.02			1032	50.00
22	12.37			3424	50.00
23	12.56	C		47727	50.00
24	13.09	P	c	133167	50.00
25	13.82	M	c	164081	50.00
26	14.18	V	c	151166	50.00
27	14.99			2858	50.00
28	15.38	dptu		136164	50.00
29	16.50	W	c	186099	50.00
30	17.26	F	c	146667	50.00
31	17.77	I	c	116314	50.00
32	18.04	K	c	194417	50.00
33	18.34	L	c	129406	50.00
34	19.50			552	50.00



Residue 1, Filtered, No baseline

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4	4.78	N	c	1564	0.39
5	5.13			7230	0.00
6	5.78	Q	c	1029	0.31
7	6.00	T	c	6809	2.35
8	6.22	G	c	3377	1.01
9	6.44	E	c	956	0.23
10	6.63			327	0.00
11	7.00	dmptu		1293	1.26
12	7.64			213	22.05
13	7.82			334	0.00
14	8.04	H	c	712	0.32
15	8.53	A	c	10464	3.26
16	9.57			124	4.30
17	9.84			11295	9.67
18	10.20	R	c	1125	0.51
19	10.74	Y	c	1758	0.52
20	11.38			705	2.86
21	11.78			222	2.03
22	12.05			393	19.04
23	12.46			478	0.00
24	12.69			239	0.00
25	12.84	C		778	0.29
26	13.18	P	c	1156	0.43
27	13.90	M	c	2923	0.89
28	14.25	V	c	1813	0.60
29	15.07			1290	22.57
30	15.49			7348	0.00
31	15.67	dptu		27131	9.96
32	16.30	W	c	3367	0.90
33	16.62			1181	0.00
34	16.87			3241	0.00
35	17.34	F	c	698	0.24
36	17.83	I	c	1971	0.85
37	18.13	K	c	4250	1.09
38	18.43	L	c	1567	0.61
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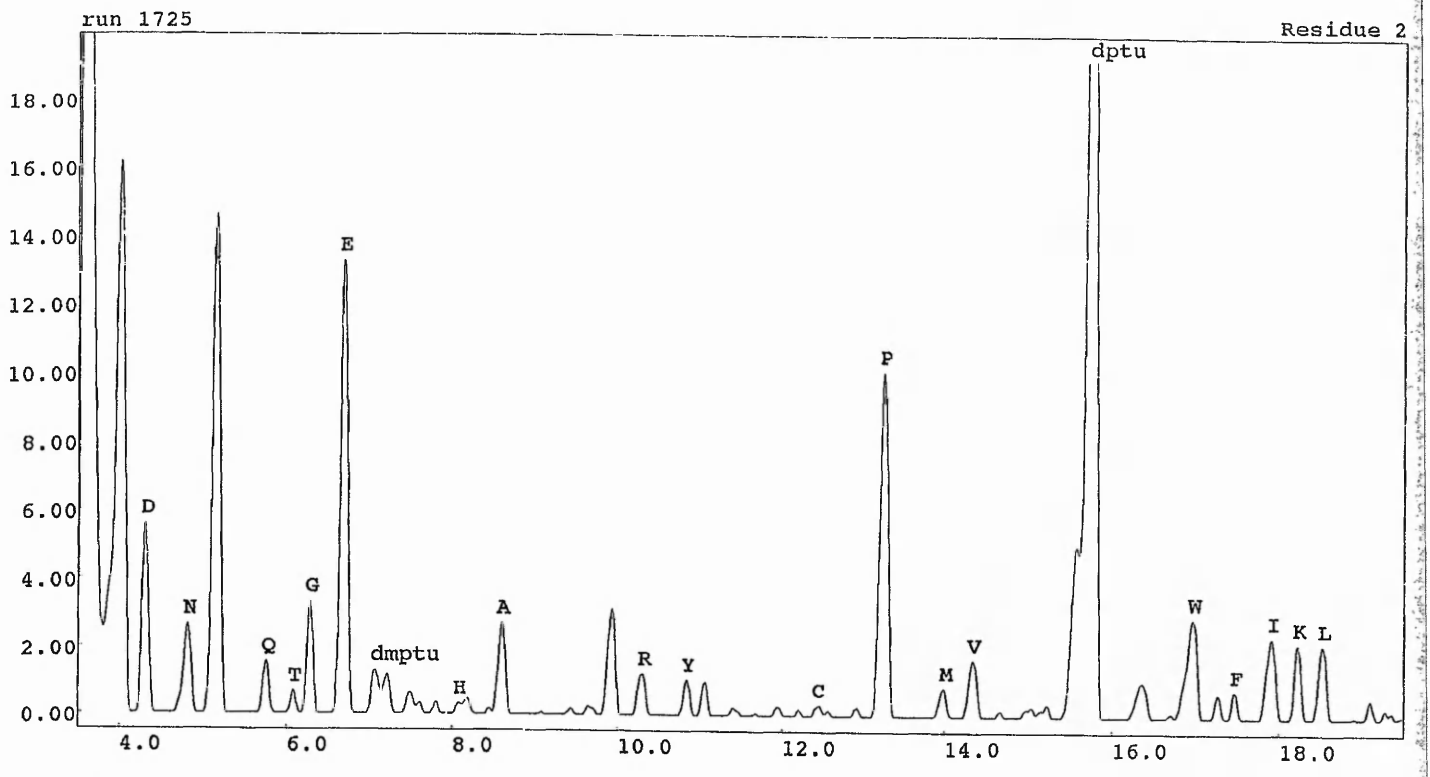

Residue 2, Filtered, No baseline

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4	4.81	N	c	2473	0.62
5	5.16			13681	0.00
6	5.75	Q	c	1400	0.43
7	6.07	T	c	571	0.20
8	6.28	G	c	2983	0.90
9	6.68	E	c	12307	2.90
10	7.06	dmptu		1219	1.19
11	7.20			1078	0.00
12	7.47			570	0.00
13	7.79			289	29.92
14	8.07	H	c	281	0.13
15	8.59	A	c	2517	0.78
16	9.63			198	0.17
17	9.93			2955	0.00
18	10.29	R	c	1153	0.52
19	10.83	Y	c	948	0.28
20	11.05			865	3.50
21	11.92			204	9.88
22	12.42	C		252	0.26
23	12.90			201	0.00
24	13.25	P	c	9544	3.58
25	13.98	M	c	760	0.23
26	14.33	V	c	1590	0.53
27	15.74	dptu		32378	11.89
28	16.34			1005	0.00
29	16.95	W	c	2810	0.96
30	17.25			600	0.00
31	17.45	F	c	681	0.23
32	17.90	I	c	2268	0.97
33	18.21	K	c	1996	0.77
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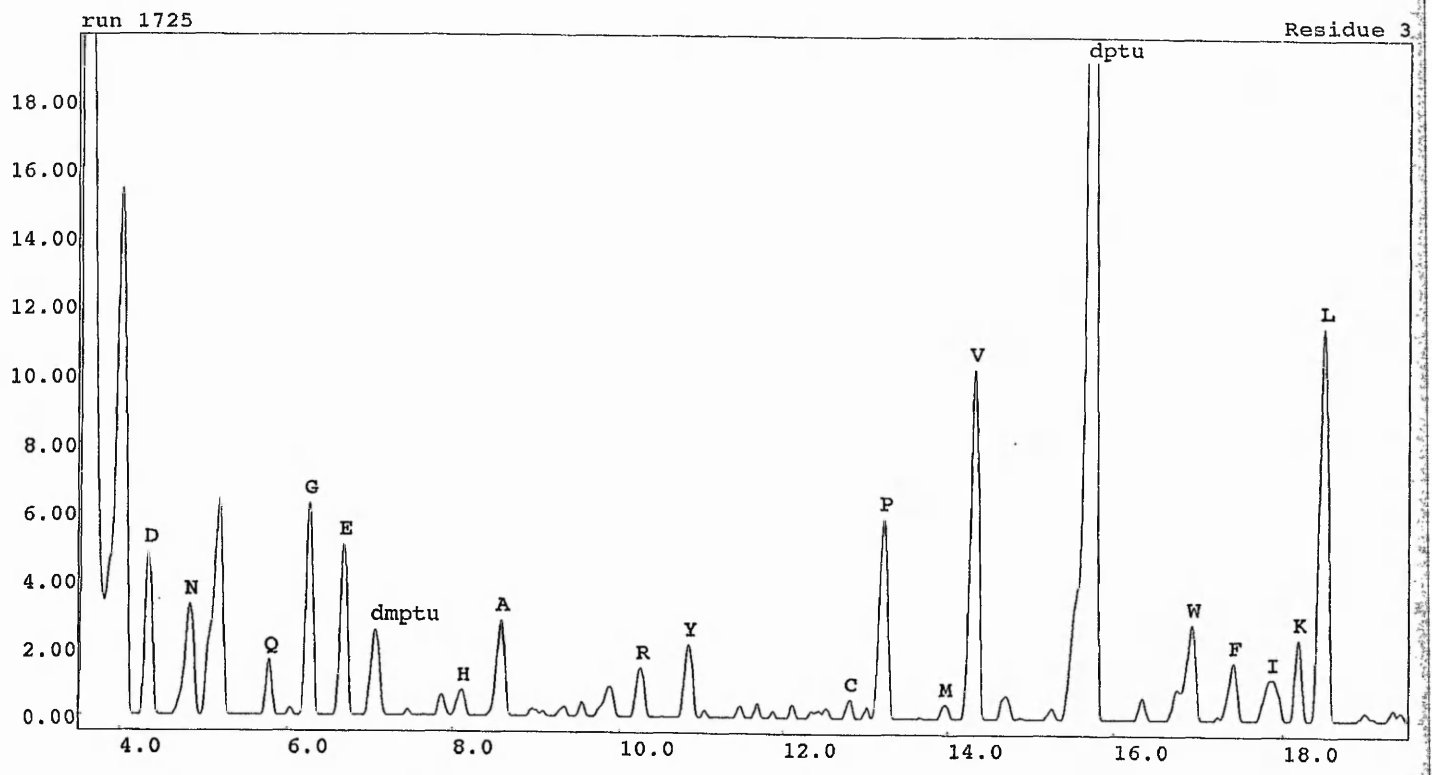

Residue 3, Filtered, No baseline

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4	4.82	N	c	3073	0.78
5	5.18			5976	0.00
6	5.77	Q	c	1461	0.44
7	6.26	G	c	5793	1.74
8	6.68	E	c	4641	1.09
9	7.05	dmptu		2389	2.33
10	7.86			543	56.21
11	8.10	H	c	712	0.32
12	8.57	A	c	2640	0.82
13	9.53			359	12.46
14	9.75			245	0.21
15	9.87			838	0.00
16	10.25	R	c	1372	0.62
17	10.82	Y	c	2031	0.60
18	11.45			284	2.59
19	11.68			345	0.00
20	12.11			322	15.60
21	12.82	C		489	0.51
22	13.02			266	0.00
23	13.23	P	c	5544	2.08
24	13.95	M	c	373	0.11
25	14.32	V	c	9639	3.19
26	14.68			646	0.00
27	15.23			265	0.00
28	15.72	dptu		38388	14.10
29	16.33			580	0.00
30	16.76			865	0.00
31	16.92	W	c	2638	0.90
32	17.41	F	c	1589	0.54
33	17.84	I	c	1196	0.51
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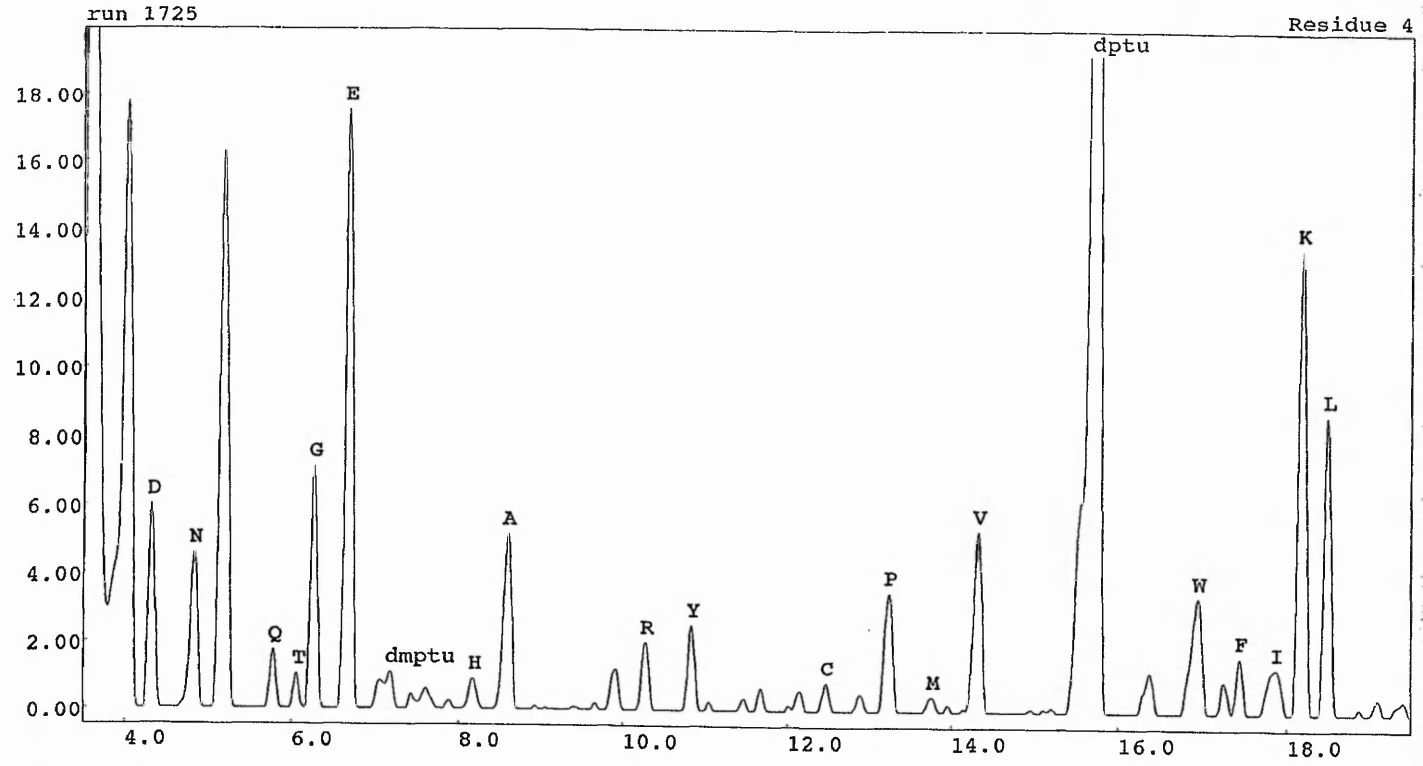

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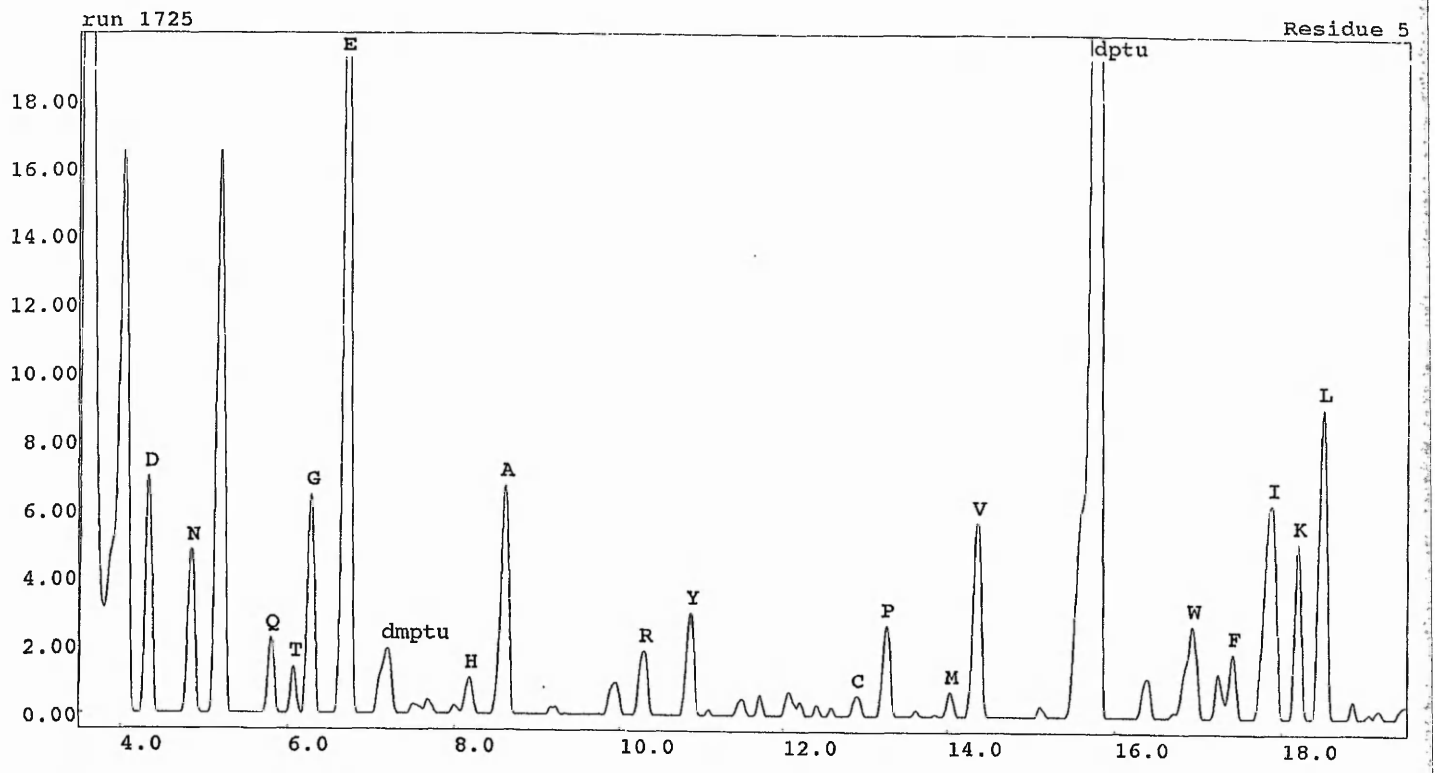
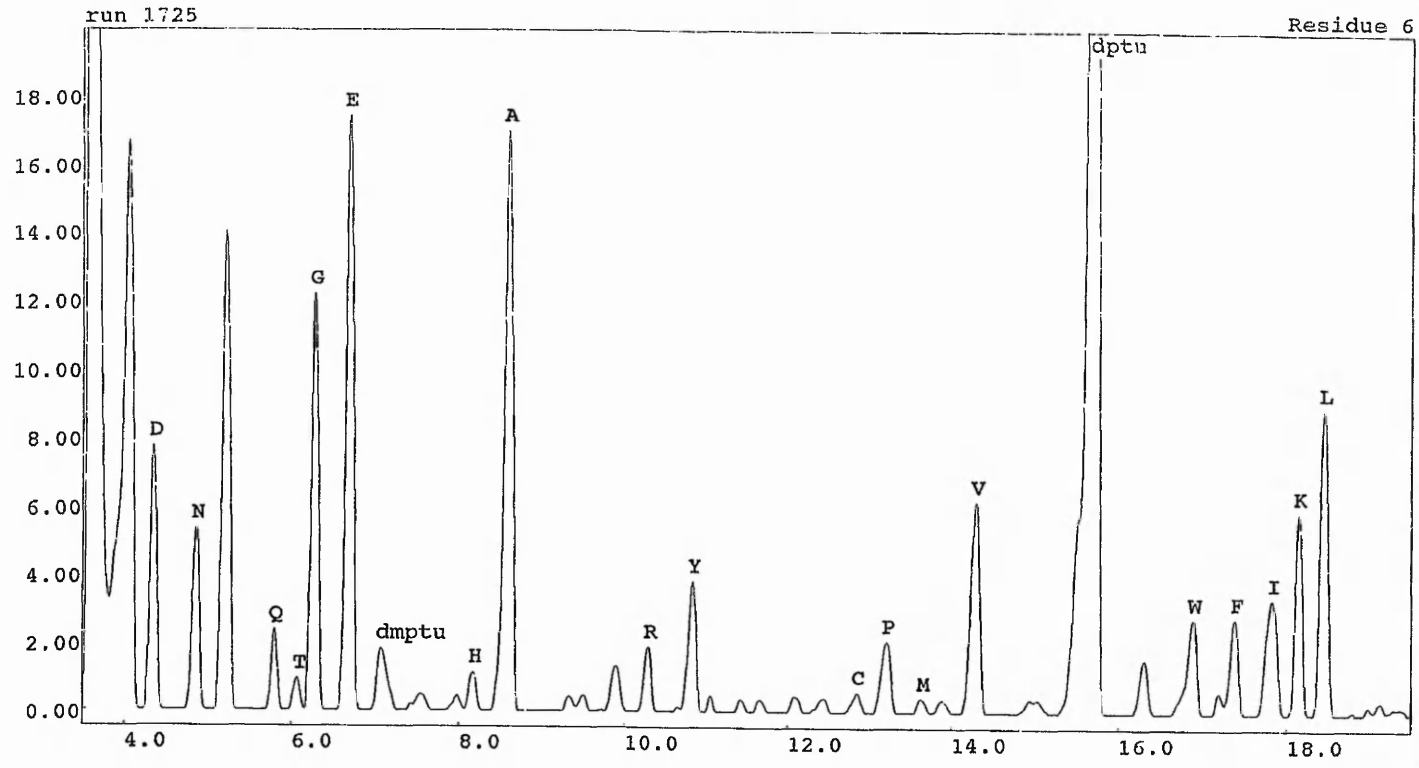
4	4.82	N	c	4236	1.07
5	5.18			15316	0.00
6	5.77	Q	c	1567	0.48
7	6.05	T	c	906	0.31
8	6.26	G	c	6535	1.96
9	6.68	E	c	16261	3.84
10	7.06			801	0.00
11	7.18	dmptu		1011	0.98
12	7.43			380	0.00
13	7.61			566	58.59
14	7.88			207	0.00
15	8.16	H	c	824	0.37
16	8.58	A	c	4845	1.51
17	9.65			158	0.14
18	9.90			1147	0.00
19	10.27	R	c	1845	0.84
20	10.82	Y	c	2303	0.69
21	11.03			208	0.84
22	11.46			312	2.85
23	11.67			597	0.00
24	12.00			136	6.59
25	12.13			552	0.00
26	12.46	C		766	0.80
27	12.88			470	0.00
28	13.23	P	c	3318	1.25
29	13.74	M	c	416	0.13
30	13.94			155	0.00
31	14.32	V	c	5010	1.66
32	15.72	dptu		51822	19.03
33	16.36			1122	0.00
34	16.91	W	c	3273	1.12
35	17.23			886	0.00
36	17.42	F	c	1520	0.52
37	17.84	I	c	1306	0.56
38	18.17	K	c	12733	3.27
39	18.48	L	c	8109	3.13
40	18.87			152	0.00
41	19.08			429	0.00
42	19.33			274	24.82
43	19.71			883	0.00



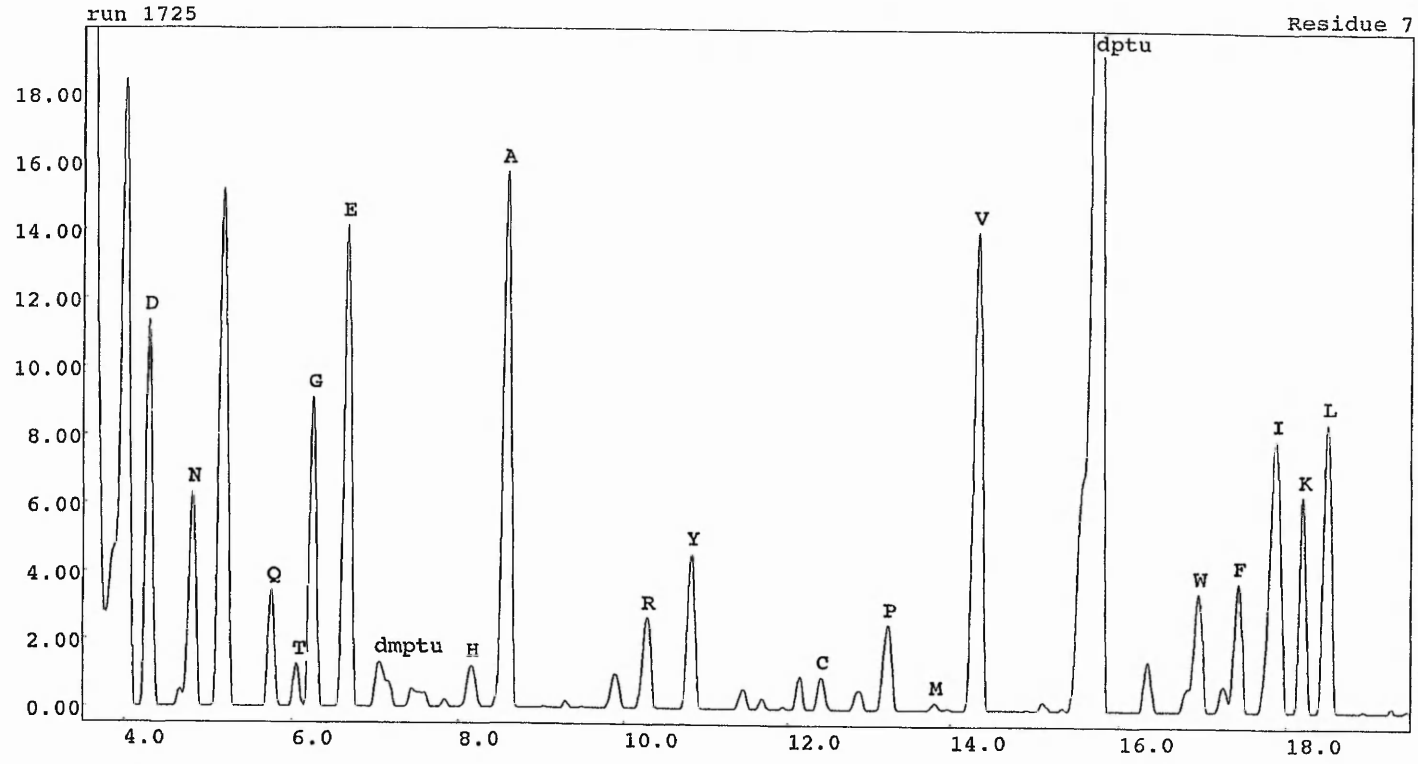
Residue 5, Filtered, No baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
1	3.58			86391	47.13
2	4.03			15592	46.22
3	4.33	D	c	6344	1.51
4	4.84	N	c	4433	1.12
5	5.18			15481	0.00
6	5.79	Q	c	1997	0.61
7	6.06	T	c	1209	0.42
8	6.28	G	c	5926	1.78
9	6.69	E	c	22318	5.26
10	7.17	dmptu		1809	1.76
11	7.49			254	0.00
12	7.68			355	36.75
13	8.00			213	0.00
14	8.18	H	c	983	0.44
15	8.61	A	c	6300	1.96
16	9.18			183	2.43
17	9.94			903	0.77
18	10.28	R	c	1787	0.81
19	10.85	Y	c	2811	0.84
20	11.07			125	0.51
21	11.48			450	4.11
22	11.70			522	0.00
23	12.07			652	31.59
24	12.40			258	0.00
25	12.58			205	0.00
26	12.90	C		551	0.21
27	13.26	P	c	2484	0.93
28	13.62			148	0.00
29	14.02	M	c	647	0.21
30	14.35	V	c	5382	1.78
31	15.11			262	4.58
32	15.75	dptu		50304	18.47
33	16.39			1086	0.00
34	16.71			139	0.00
35	16.92	W	c	2536	0.86
36	17.23			1175	0.00
37	17.41	F	c	1726	0.59
38	17.86	I	c	6093	2.62
39	18.18	K	c	4674	1.20
40	18.48	L	c	8511	3.29
41	18.83			432	0.00
42	19.14			215	0.00
43	19.45			344	31.16
44	19.67			715	0.00



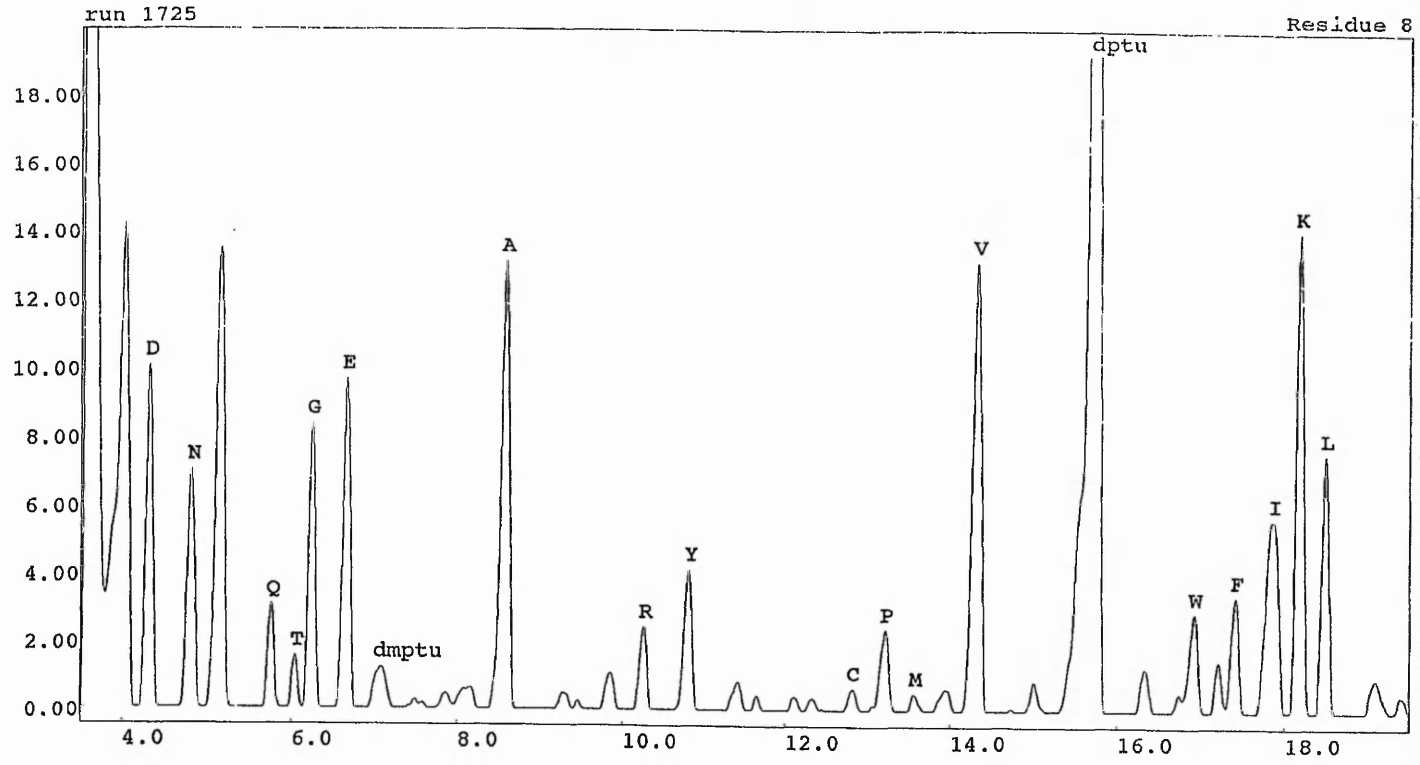
Residue 6, Filtered, No baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
1	3.60			84325	46.00
2	4.04			15754	46.70
3	4.33	D	c	7107	1.70
4	4.84	N	c	4951	1.25
5	5.21			13061	0.00
6	5.78	Q	c	2129	0.65
7	6.06	T	c	866	0.30
8	6.27	G	c	11276	3.38
9	6.69	E	c	16153	3.81
10	7.07	dmptu		1724	1.68
11	7.53			450	46.58
12	7.97			391	0.00
13	8.16	H	c	1053	0.47
14	8.58	A	c	15919	4.95
15	9.32			390	13.53
16	9.49			418	0.00
17	9.89			1267	1.08
18	10.28	R	c	1764	0.80
19	10.83	Y	c	3564	1.06
20	11.05			377	1.53
21	11.42			331	3.02
22	11.65			325	0.00
23	12.07			439	21.27
24	12.41			370	0.00
25	12.84	C		538	0.20
26	13.20	P	c	2014	0.76
27	13.62	M	c	390	0.12
28	13.88			334	0.00
29	14.28	V	c	5857	1.94
30	14.94			363	6.35
31	15.68	dptu		53551	19.66
32	16.30			1476	0.00
33	16.87	W	c	2600	0.70
34	17.17			556	0.00
35	17.36	F	c	2597	0.89
36	17.81	I	c	3252	1.40
37	18.13	K	c	5440	1.40
38	18.43	L	c	8393	3.24
39	18.96			188	0.00
40	19.10			324	0.00
41	19.60			721	65.31



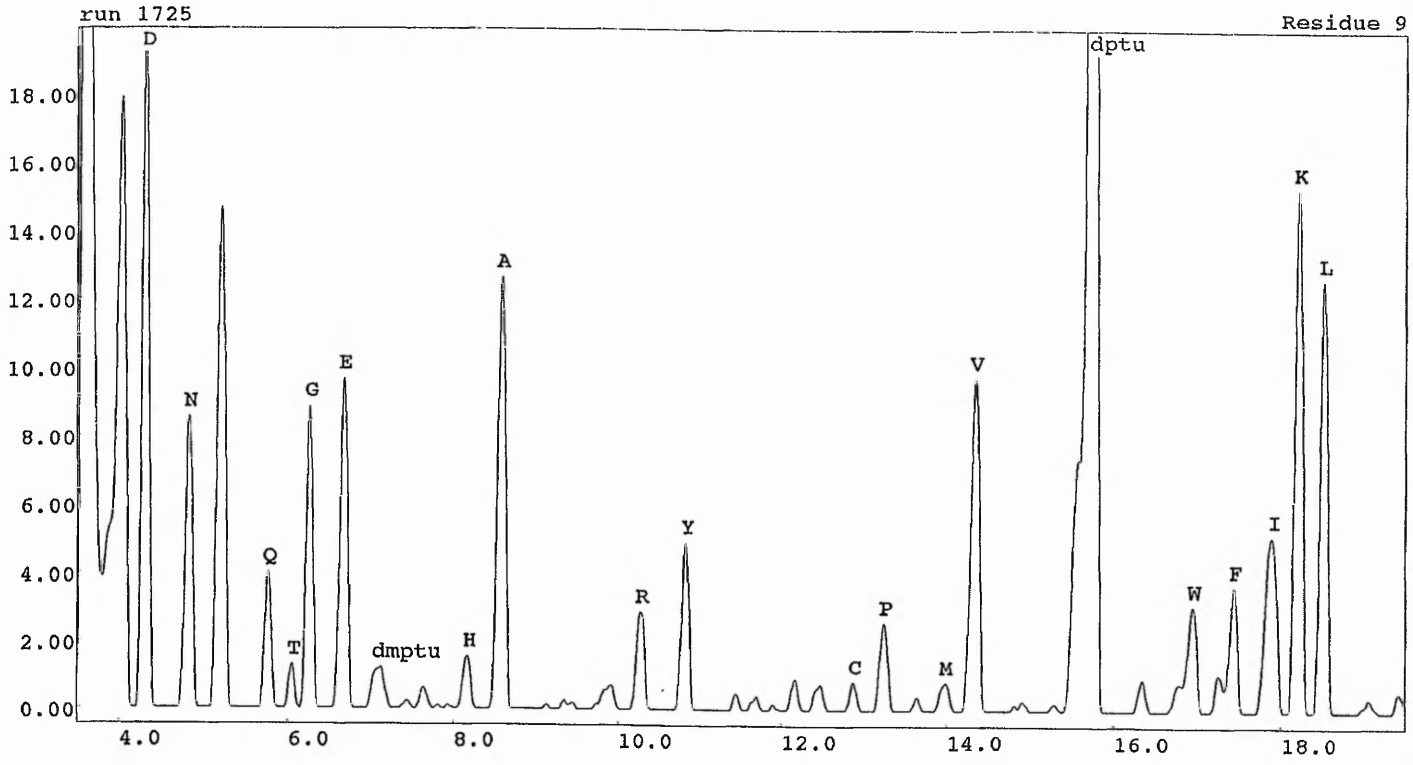
Residue 7, Filtered, No baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
1	3.57			83949	45.79
2	4.01			17350	51.43
3	4.29	D	c	10398	2.48
4	4.68			446	0.00
5	4.82	N	c	5801	1.46
6	5.18			14376	0.00
7	5.75	Q	c	3138	0.95
8	6.05	T	c	1126	0.39
9	6.24	G	c	8383	2.52
10	6.66	E	c	13107	3.09
11	7.03	dmptu		1264	1.23
12	7.44			497	0.00
13	7.82			184	19.05
14	8.15	H	c	1137	0.51
15	8.57	A	c	14722	4.58
16	9.28			161	2.14
17	9.89			937	0.80
18	10.27	R	c	2521	1.14
19	10.82	Y	c	4265	1.27
20	11.45			534	4.87
21	11.68			282	0.00
22	12.14			886	42.93
23	12.41	C		882	0.92
24	12.88			546	0.00
25	13.23	P	c	2395	0.90
26	13.79	M	c	182	0.06
27	14.32	V	c	13250	4.38
28	15.08			222	3.88
29	15.73	dptu		58051	21.32
30	16.33			1359	0.00
31	16.93	W	c	3298	1.12
32	17.23			713	0.00
33	17.41	F	c	3557	1.21
34	17.86	I	c	7672	3.30
35	18.19	K	c	5802	2.24
36	18.48	L	c	7979	3.08
37	19.24			117	0.00



Residue 8, Filtered, No baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
1	3.58			87460	47.71
2	4.02			13444	39.86
3	4.32	D	c	9239	2.20
4	4.81	N	c	6526	1.65
5	5.14			12632	0.00
6	5.74	Q	c	2788	0.85
7	6.03	T	c	1375	0.48
8	6.23	G	c	7687	2.31
9	6.66	E	c	8911	2.10
10	7.03	dmptu		1096	1.07
11	7.82			330	34.16
12	8.57	A	c	12269	3.82
13	9.26			401	5.32
14	9.84			1024	0.88
15	10.25	R	c	2274	1.03
16	10.80	Y	c	3883	1.16
17	11.39			743	3.01
18	11.63			345	3.15
19	12.09			361	17.49
20	12.82	C		583	0.61
21	13.22	P	c	2226	0.84
22	13.56	M	c	428	0.13
23	13.87			349	0.00
24	14.31	V	c	12400	4.10
25	15.00			766	13.40
26	15.71	dptu		53346	19.59
27	16.33			1191	0.00
28	16.73			463	0.00
29	16.90	W	c	2710	0.92
30	17.19			1343	0.00
31	17.39	F	c	3176	1.08
32	17.84	I	c	5558	2.39
33	18.17	K	c	13067	3.36
34	18.47	L	c	6983	2.70
35	19.05			894	0.00
36	19.38			431	39.04
37	19.67			746	0.00



Residue 9, Filtered, No baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
1	3.58			85288	46.52
2	4.03			16873	50.02
3	4.31	D	c	18264	4.36
4	4.82	N	c	7908	2.00
5	5.20			13823	0.00
6	5.76	Q	c	3689	1.12
7	6.04	T	c	1147	0.40
8	6.25	G	c	8137	2.44
9	6.67	E	c	8988	2.12
10	7.06	dmptu		1120	1.09
11	7.62			565	58.49
12	8.16	H	c	1456	0.65
13	8.57	A	c	11851	3.69
14	9.83			554	0.47
15	10.27	R	c	2754	1.25
16	10.82	Y	c	4576	1.36
17	11.43			426	3.89
18	11.63			238	0.00
19	12.15			839	40.65
20	12.44			692	0.00
21	12.87	C		768	0.29
22	13.23	P	c	2450	0.92
23	13.62			337	0.00
24	13.97	M	c	775	0.24
25	14.33	V	c	9201	3.04
26	15.73	dptu		52756	19.37
27	16.33			859	0.00
28	16.77			789	0.00
29	16.93	W	c	2962	1.01
30	17.24			1017	0.00
31	17.42	F	c	3436	1.17
32	17.88	I	c	5008	2.15
33	18.19	K	c	14271	5.51
34	18.49	L	c	11822	4.57
35	19.41			512	46.38
36	19.68			1076	0.00

Pmol

	A	D	E	F	G	H	I	K	L	M	N
1	3.26	1.05	0.23	0.24	1.01	0.32	0.85	1.09	0.61	0.89	0.39
2	0.78	1.21	2.90	0.23	0.90	0.13	0.97	0.77	0.79	0.23	0.62
3	0.82	1.06	1.09	0.54	1.74	0.32	0.51	0.56	4.21	0.11	0.78
4	1.51	1.31	3.84	0.52	1.96	0.37	0.56	3.27	3.13	0.13	1.07
5	1.96	1.51	5.26	0.59	1.78	0.44	2.62	1.20	3.29	0.21	1.12
6	4.95	1.70	3.81	0.89	3.38	0.47	1.40	1.40	3.24	0.12	1.25
7	4.58	2.48	3.09	1.21	2.52	0.51	3.30	2.24	3.08	0.06	1.46
8	3.82	2.20	2.10	1.08	2.31	0.00	2.39	3.36	2.70	0.13	1.65
9	3.69	4.36	2.12	1.17	2.44	0.65	2.15	5.51	4.57	0.24	2.00

	P	Q	R	S	T	V	W	Y
1	0.43	0.31	0.51	0.00	2.35	0.60	0.90	0.52
2	3.58	0.43	0.52	0.00	0.20	0.53	0.96	0.28
3	2.08	0.44	0.62	0.00	0.00	3.19	0.90	0.60
4	1.25	0.48	0.84	0.00	0.31	1.66	1.12	0.69
5	0.93	0.61	0.81	0.00	0.42	1.78	0.86	0.84
6	0.76	0.65	0.80	0.00	0.30	1.94	0.70	1.06
7	0.90	0.95	1.14	0.00	0.39	4.38	1.12	1.27
8	0.84	0.85	1.03	0.00	0.48	4.10	0.92	1.16
9	0.92	1.12	1.25	0.00	0.40	3.04	1.01	1.36

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DT   15-DEC-1998 (Rel. 37, Last annotation update)
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OC   Bacteria; Proteobacteria; epsilon subdivision; Campylobacter group;
OC   Campylobacter.
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RX   MEDLINE; 97306651.
RA   Schroeder W.F.K.J., Moser I.;
RT   "Primary structure analysis and adhesion studies on the major outer
RT   membrane protein of Campylobacter jejuni.";
RL   FEMS Microbiol. Lett. 150:141-147(1997).
RN   [2]
RP   SEQUENCE OF 1-31.
RC   STRAIN=85H;
RX   MEDLINE; 95362649.
RA   Bolla J.-M., Loret E., Zalewski M., Pages J.-M.;
RT   "Conformational analysis of the Campylobacter jejuni porin.";
RL   J. Bacteriol. 177:4266-4271(1995).
CC   -!- FUNCTION: ASSEMBLE TO FORM A FUNCTIONAL PORIN. MAY BE ONE OF THE
CC       STRUCTURES RESPONSIBLE FOR ADHESION TO INTESTINAL CELLS.
CC   -!- SUBUNIT: HOMOTRIMER AND MONOMER.
CC   -!- SUBCELLULAR LOCATION: OUTER MEMBRANE.
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Nucleotide

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ACCESSION CAB73274
PID g6968454
VERSION CAB73274.1 GI:6968454
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SOURCE Campylobacter jejuni.
ORGANISM Campylobacter jejuni
Bacteria; Proteobacteria; epsilon subdivision; Campylobacter group; Campylobacter.
REFERENCE 1 (residues 1 to 369)
AUTHORS Parkhill,J., Wren,B.W., Mungall,K., Ketley,J.M., Churcher,C., Basham,D., Chillingworth,T., Davies,R.M., Feltwell,T., Holroyd,S., Jagels,K., Karleyshev,A., Moule,S., Pallen,M.J., Penn,C.W., Quail,M., Rajandream,M.A., Rutherford,K.M., VanVliet,A., Whitehead,S. and Barrell,B.G.
TITLE The genome sequence of the food-borne pathogen Campylobacter jejuni reveals hypervariable sequences
JOURNAL Nature 403, 665-668 (2000)
REFERENCE 2 (residues 1 to 369)
AUTHORS Parkhill,J.
TITLE Direct Submission
JOURNAL Submitted (09-FEB-2000) Submitted on behalf of the Campylobacter sequencing team, Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA E-mail: parkhill@sanger.ac.uk
COMMENT Notes:
Details of C. jejuni sequencing at the Sanger Centre are available on the World Wide Web.
(URL, http://www.sanger.ac.uk/Projects/C_jejuni/).
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