UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

MICROBIOLOGY

STRUCTURAL AND BIOLOGICAL STUDIES ON THE SURFACE
ANTIGENS INVOLVED IN THE PATHOGENESIS OF CAMPYLOBACTER
GASTRO-ENTERITIS

by Harry Michael McBride

1985

ACKNOWLEDGEMENTS

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ABBREVIATIONS

BA=   Blood agar

BSA=   Bovine Serum Albumin

CFU=   Colony Forming Units

EDTA=   Ethylene-diaminetetraacetic acid

LPS=   Lipopolysaccharide

M.wt.=   Molecular weight

MOMP=   Major Outer Membrane Protein

OM=   Outer Membrane

OMP=   Outer Membrane Protein

PAGE=   Polyacrylamide gel electrophoresis

PBS=   Phosphate Buffered Saline (Oxoid Ltd, Basingstoke)

PBS+B=   Phosphate Buffered Saline plus calcium and magnesium

PCV=   Packed Cell Volume

RIPA=   Radioimmunoprecipitation assay

SDS=   Sodium Dodecyl Sulphate

TRIS=   Tris(hydroxymethyl) methylamine

On RIPAs and Immunoblots:

H= Homologous Serum i.e. Serum from the patient from which the bacterium was isolated

C= Control Human Serum i.e. Post natal serum

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ABSTRACT

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Master of Philosophy

STRUCTURAL AND BIOLOGICAL STUDIES ON THE SURFACE
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The outer membranes of C.jejuni and C.coli were isolated by two methods, spheroplast formation and detergent solubilization of the inner membrane. Membranes prepared by either procedure were essentially similar. Two main protein bands predominated in the outer membranes. A variable molecular weight protein of M.wt 43-46,000 represented the major band in total protein profiles, it was heat-modifiable and surface exposed. The second band had a M.wt in the range 62-64,000 it was not heat-modifiable and was identified as flagellin. An acid-extract from the surface of the C.jejuni cells contained 25K and 27K proteins. Staining of OM proteins with Schiff's reagent revealed staining in the 27-30K region and probably represented LPS. The techniques of radio-immunoprecipitation and immunoblotting were used to examine the antigenicity of C.jejuni proteins. Both the 43K Momp and the 63K flagellin proteins were immunogenic.
    Different flagellins elicited antibodies that showed either broad or limited cross-reactivites. The biological role of flagella was investigated by the isolation of an flagellate non-motile variant (SF1) and an aflagellate variant (SF2) from C.jejuni 81116. The amino-acid compositions of the flagellins were essentially identical to those of other known bacteria. Attachment studies to erythrocytes of various species revealed differences in the attachments of 81116 and the variants. It is proposed that attachment to RBC is through a surface adhesin and that active and to a lesser extent inactive flagellla blocked this attachment. Adsorption isotherm data supported this view. In contrast attachment to eukaryotic cells was considerably lower compared to RBCs. However it is possible that an adhesin resides on the flagella producing a tenuous attachment to cell surfaces. Specificity to a cell line of intestinal origin was observed. The three strains were examined in vivo using an infant mouse model for colonization. The possession of flagella and not motility was the major factor in successful colonization.

CHAPTER 1

INTRODUCTION

The recognition at the beginning of this century of unusual vibrio-like organisms associated with bovine and ovine abortion led to a considerable interest within the veterinary sciences that has been maintained to this day. However, over sixty years were to pass before this same group of organisms were to generate similar interest within the field of human medicine, ultimately leading to the formation of the entirely new genus Campylobacter.

During those sixty years a plethora of classification schemes produced considerable confusion within the scientific literature. An understanding of the history of campylobacters therefore requires an understanding of the development of this nomenclature.

1.1. Taxonomy.

Although campylobacters were first described by McFaydean and Stockman in 1913 the vibrio-like organisms were not named until 1919 when Smith and Taylor isolated them from aborted bovine fetuses and therefore designated them Vibrio fetus. The name Vibrio jejuni was given by Jones et al., (1931) to an organism associated with winter scours of cattle. These microaerophilic vibrios were cultured from lesions of the small bowel mucosa, and re-inoculation of healthy cattle with pure cultures of the organism reproduced the disease. Interestingly, however, winter scours is now considered to be due to a viral agent and not campylobacter (Campbell and Cookingham, 1978). A similar organism, isolated from cases of swine dysentery, was named Vibrio coli by Doyle in 1944, however as with Vibrio jejuni and winter scours, swine dysentery is now believed not to be due to Vibrio coli but to a spirochete Treponema hyodysenteriae (Glock & Harris, 1972 ; Smibert, 1978). Between 1953 and 1971 various classification schemes were proposed for C.fetus and these are shown in Table 1.1. In 1957 King, on

Table 1.1. Comparison of various classifications of Campylobacter
jejuni

Bryner et al 1962	Mohanty et al 1962	Florent 1953	Smibert 1974	Veron &Chatelain 1973	Berg 1971
Biotype 1	I	venerealis	fetus	venerealis	A-1
Biotype sub1	III	venerealis	fetus	venerealis	A-sub 1
Biotype2	II	intestinalis	intestinalis	fetus	A-2
Biotype2	II	intestinalis	intestinalis	fetus	B
			jejuni	C.jejuni	C
			jejuni	C.coli	C

comparing microaerophilic vibrios isolated from various sources, noted a group isolated from human blood cultures, that appeared to have a higher optimum growth temperature than similar vibrio-like organisms. The term "related vibrios" was applied, by King, to organisms exhibiting this characteristic. The formation of the new genus Campylobacter (Greek: Campylo meaning curved and Bacter meaning rod) was suggested by Sebald and Veron in 1963 for the V.fetus group because of fundamental differences when compared to the true vibrios, in particular DNA base compositions and fermentive abilities, C.fetus being proposed as the type species of the genus. A reclassification of C.fetus was suggested by Veron and Chatelain in 1973, one of the main changes being the redesignation of the "related vibrio" species of C.fetus as C.jejuni (from V.jejuni of Jones et al., 1931) and C.coli (from V.coli of Doyle, 1944, 1948). However, in the 1974 edition of Bergey's Manual the "related vibrios" were classified by Smibert as C.fetus subspecies jejuni. More recent evidence using biochemical tests and the powerful tool of DNA hybridization has proven that the separation of the "related vibrios" into C.jejuni and C.coli was justified (Skirrow and Benjamin, 1980a, 1980b ; Owen and Leaper, 1981 ; Belland and Trust, 1982). To clarify the considerable confusion that the various classification schemes had produced an internationally approved nomenclature was agreed in 1980 (Skerman et al., Approved lists of bacterial names, 1980) and this system has been reproduced in Fig 1.1.

1.2. Biotyping and Serotyping.

A number of biotyping and serotyping schemes have been published for the typing of veterinary and human isolates of campylobacters. The following account concerns only those schemes that have become generally accepted for the typing of the thermophilic group of campylobacters.

The differentiation of the genus Campylobacter as a whole depends on a large number of biochemical and nutritional tests (Manual of Clinical Microbiology ; 3rd

Fig 1.1. Classification of Genus Campylobacter

CATALASE +ve group

C.fetus $25^{\circ}C+; 43^{\circ}C-$

Sub. venerealis (sub.fetus[1]) — Infertility in cattle

Sub.fetus (Sub. intestinalis[1]) — Sporadic abortion in sheep and cattle; Uncommon invasive opportunist in man

Thermophilic group $25^{\circ}C-; 43^{\circ}C+$

C.jejuni biotype 1&2

C.coli — Intestinal commensal of birds and other animals. Sporadic abortion in sheep. Acute enterocolitis in man and some animals

NARTC[2] — Intestinal commensals of seagulls and other animals

CATALASE -ve group

C.sputorum

sub. sputorum — Mouth commensal

sub. bubulus — Commensal of bovine genital tract

sub. mucosalis[3] — Intestinal adenomatosis of pigs

Sub.= Subspecies

Nomenclature is that of Approved Lists of Bacterial Names with the exception of:

(1) Smibert, 1974.
(2) Nalidixic acid-resistant "thermophilic campylobacters", presumptive C.laridis. Skirrow and Benjamin (1980a).
(3) Lawson and Rowland (1974).
Adapted from Newell, D.G. (1982).

edition, 1980), however the biotyping of the thermophilic group of campylobacters depends on only a limited number of criteria which have been used together to form the basis of the biotyping scheme shown in Table 1.2. (Skirrow and Benjamin, 1980a, 1980b). An extended biotyping scheme for thermophilic campylobacters utilising a test for DNAase activity has recently been presented (Lior, 1983). The biotyping schemes separate the thermophilic campylobacters only into a limited number of groups which gives low discriminating power for epidemiological studies. For this reason two serotyping schemes have been developed for the investigation of environmental and clinical isolates. The first to be described was developed by Penner and Hennessy (1980) and involves the passive hemagglutination of sheep erythrocytes sensitized with soluble, heat-stable antigens from C.jejuni and C.coli. The sensitized erythrocytes are reacted against a panel of typing antisera prepared against formalised bacteria. To date forty-two antisera for C.jejuni and eighteen for C.coli form the Penner serotyping scheme. A second serotyping scheme (Lior et al., 1982) detects heat-labile antigens, and involves the slide agglutination of bacterial isolates with typing antisera prepared against formalised whole organisms. The typing antisera were absorbed with the homologous, heated bacterial suspensions to remove antibodies directed against heat-stable antigens. At present fifty-three serogroups have been identified. The use of these serotyping schemes, sometimes in conjunction with biotyping schemes has led to a much better understanding of the epidemiology of campylobacter infections.

1.3. Physiology, Nutrition and Coproculture.

Although the microaerophilic nature of campylobacters is now well recognised, the reasons for this oxygen toxicity are not totally understood. In 1978 George et al., found that sodium bisulphite and sodium pyruvate in combination with ferrous sulphate (FBP) greatly enhanced

Table 1.2. Identification chart for intestinal
campylobacters

	C.fetus	NARTC	C.jejuni biotype1	C.jejuni biotype2	C.coli
Growth at:25^{0}C	+	–	–	–	–
43^{0}C	–	+	+	+	+
Nalidixic acid (30µg disc)	R	R	S	S	S
Hippurate hydrolysis	–	–	+	+	–
H$_2$S production (FBP)	–	+	–	+	–

+=growth; –=no growth; R=resistant (0mm);
S=sensitive (>=6mm from edge of disc)

the aerotolerance of C.fetus. This aspect of enhanced aerotolerance was studied by Hoffman (1978) and Hoffman et al., (1979) the authors finding no evidence for FBP causing any physiological change in C.fetus that could satisfactorily account for the improved oxygen tolerance. It has been shown that FBP spontaneously decomposes hydrogen peroxide and superoxide radicals, raising the possibility that FBP supplement, at least in part, increases aerotolerance by reducing levels of these bactericidal agents (Hoffman, 1978).

The first identification of the energy sources used by V.fetus was made in 1957 by Alexander, who found tricarboxylic acid cycle intermediates, or such compounds that could be easily introduced into the cycle were being utilized, while sugars were found not to be suitable energy sources . Chemically defined media have since been developed that support the growth of the majority of campylobacter strains (Fletcher and Plastridge, 1963 ; Smibert, 1963 ; Tritz and Ogg, 1967), all the defined media containing an array of amino acids and vitamins. The respiratory physiology and energy conservation efficiency of C.jejuni has recently been studied by Hoffman and Goodman (1982) with hydrogen and formate being found the most energy efficient substrates of those examined.

A number of filtration techniques and selective media, using antibiotics and dyes, for use in the veterinary field were published between 1961 and 1976, and a review of the development of these procedures has been presented (Smibert, 1978). The first reported association of microaerophilic vibrios with human disease was made in 1946 by Levy, during a study of gastroenteritis affecting a large institution in Illinous. Vibrio-like organisms were seen in stained smears of faeces from 20% of the patients but were not cultured from faecal specimens although microaerophilic vibrios were recovered from blood cultures of 13 out of 39 patients. In the following twenty-five years further successful isolations of campylobacters were made from "clean sites" such as blood and synovial fluid (King, 1957 ; King and Bronsky, 1961 ;

Middelkamp and Wolf, 1961 ; Wheeler and Borchers, 1961; Mandel and Ellison, 1963 ; Darrel, Farrel and Mulligan, 1967 ; White, 1967 ; Fleurette, Flandrois and Diday, 1971). The first successful isolation of thermophilic campylobacters from stool specimens was achieved in Australia by Copper and Slee in1971 after they had noticed selective resistance of campylobacters to Cephalothin thereby allowing purification from other enteric organisms. However, the authors did not continue with this work and in 1972 Dekeyser et al., used selective filtration through a 0.65μm Millipore membrane filter to successfully isolate campylobacters from the stools of two adults with diarrhoea. In 1973 Butzler and colleagues isolated C.jejuni from diarrhoeal and non-diarrhoeal stools of humans using a selective medium containing antibiotics. In the following years a number of selective media formulations were described to aid isolation of campylobacters from human sources, notably those of Skirrow (1977) and of Blaser et al., (1979). It has been due to these developments in the culturing of campylobacters that has led to a rapid increase in the numbers of C.jejuni/coli being isolated from human infections, this dramatic growth reflecting the increasing numbers of laboratories now successfully cultivating the organism (Fig 1.1a.).

1.4. Diarrhoeal Diseases.

There can be little doubt that the acute diarrhoeal diseases caused by bacteria are a major cause of morbidity and mortality in developed and under-developed countries.(Grady and Keusch, 1971). It has been estimated that in any 24-hour period 200-million people have gastro-enteritis, most commonly diarrhoea and most likely bacterial in origin (Gorbach, 1970).

1.4.1. Diarrhoea and Morbidity.

The extent of the morbidity associated with diarrhoeal diseases is difficult to analyse. In many under-developed

GASTROINTESTINAL INFECTIONS : 1977-84
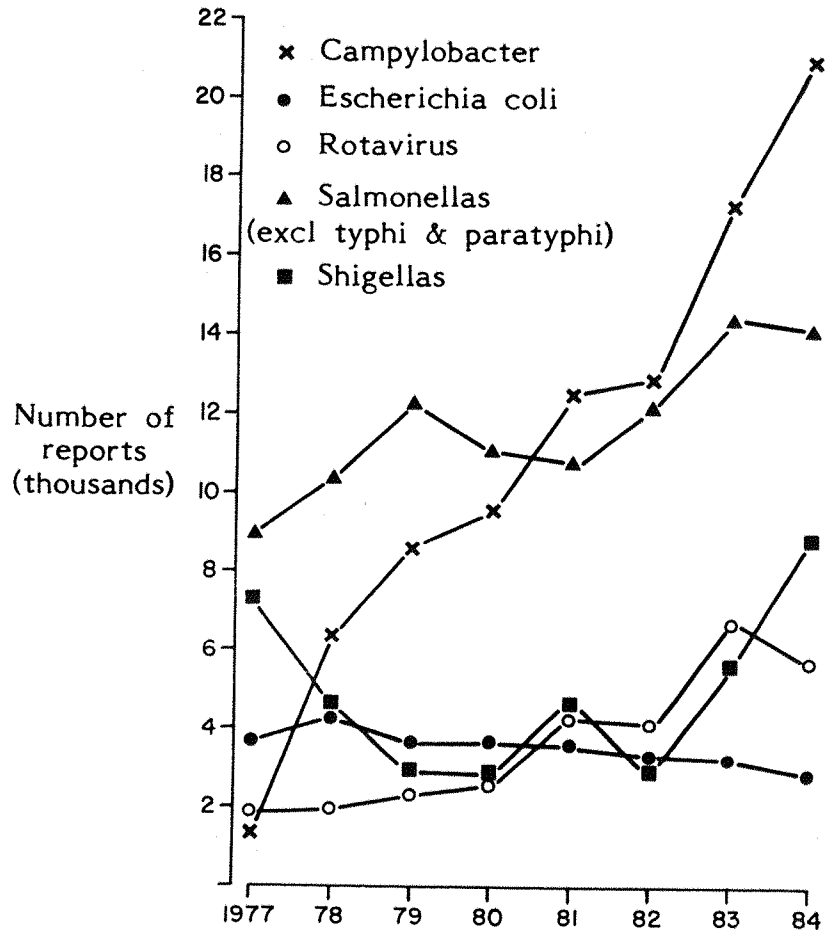
Figure 1.1a. Reproduced from Public Health Service (England and Wales) CDSC Report, unpublised.

countries "normal bowel function" can mean two or three watery or mushy stools per day, these populations seeking medical advice only for symptoms of severe dehydration or illness. In a recent study (Snyder and Merson, 1982) data from 24 published studies were analysed in order to estimate the annual morbidity and mortality from acute diarrhoeal disease in the developing world. In Asia, Africa and Latin America, for children under five years of age, the total number of episodes of diarrhoeal illnesses was found to be 744-1000 million per year.

Within the developed world diarrhoeal illness is also an important cause of morbidity, to such an extent that it has been found that within a defined population of Cleveland families, diarrhoeal disease was second only to the common cold in causing time lost from work (Dingle et al., 1964). In 1978 the estimated number of discharges from hospitals in England and Wales of people who had been admitted with an infection of the alimentary tract was 28,440 (D.H.S.S. office of population censuses and surveys). The average number of beds used daily was 562 with a mean duration of stay of between six and eight days.

1.4.2. Diarrhoea and Mortality.

It is upon the children of the under-developed countries that the diarrhoeal diseases can have the most tragic consequences. A little over ten years ago, within some developing countries, a child had only a 50% chance of reaching its seventh year, the majority of these deaths being due to gastro-enteritis (Kretchmer, 1969). With the advent of primary health care programmes for developing countries, whether implemented by the countries themselves or by international agencies such as the World Health Organisation, the fatalities due to diarrhoeal illnesses have been reduced. However, the total number of deaths attributable to bacterial gastro-enteritis within Asia, Africa, and Latin America for children under the age of five years, using 1980 population figures has been estimated at around 4.6 million per year (Snyder and Merson, 1982).

Even within the industrialised countries enteritis has been found amongst the ten leading causes of death of children aged one to four years of age, in half the North American, European and Oceanic countries studied (W.H.O. statistics report 27, 563-568, 1974). To what extent do infections caused by Campylobacter jejuni/coli contribute to this overall picture?

1.4.3. C.jejuni/coli Infections and Morbidity.

Data on the morbidity associated with campylobacter enteritis is difficult to obtain due to the few defined populations that have been studied. A further complication is the fact that although, in general, asymptomatic excretion of campylobacters is unusual in developed countries (Skirrow 1977; Pai et al., 1979) it is variable in the developing countries (Butzler et al., 1973 ; De Mol and Bosmans, 1978 ; Bokkenheuser et al., 1979 ; Blaser et al., 1980 ; Ringertz et al., 1980).

A recent study has given some indications of the morbidity associated with campylobacter infections in a developed country (Kendall & Tanner, 1982). The authors investigated a general practice population in Epsom, England over a period of three years, in order to assess the incidence and clinical features of campylobacter enteritis. Within their defined population the percentage of enteritis cases specifically due to campylobacters was found to be 20%, with an occurance of 14.9% in an unselected population based on samples from patients with diarrhoea in a similar catchment area, and sent to the laboratory by other practitioners. A relatively high infection rate was reported in children which is at variance to the argument put forward by other investigators who have suggested that the relatively high isolation rates of campylobacters from children is due to a disproportionate sampling of faeces from this age group, and that when the percentage of positive faeces is compared with age groups the highest incidence is within the adult population (Butzler and Skirrow, 1979). It is generally accepted now that the highest incidence is

within the young adult population, a population that makes a major contribution to the economic resources of a country. One estimate of the number of working days lost due to campylobacter enteritis has been calculated as approaching 600,000 cases of campylobacter enteritis per year within England and Wales (Skirrow, 1982). An in-patient study gave a mean hospital stay of eleven days (range 2-46 days) for campylobacter enteritis (Pentland, 1979). Recognising the fact that most campylobacter infections will be of a mild nature lasting no more than one or two days and only a small minority will require hospital admission, it is still apparent that campylobacter infections place a significant drain on the economic and health resources of a community.

1.4.4. C.jejuni/coli Infections and Mortality.

In comparison with the morbidity associated with campylobacter infections the mortality associated with the organism is insignificant, with the few reported cases usually involving old or compromised hosts. A 20% mortality associated with C.jejuni bacteremia has been reported (Guerrant et al., 1978), however only ten cases were studied 40% of which had some underlying disease. A study from the War Mamorial Childrens Hospital, Cape Town involving an investigation of ten children with C.fetus infections included two cases of children with a poor health status whom subseqently died. The authors stated that in the world literature there was a significant mortality of 10-20% associated with campylobacter infections (Schewitz and Roux, 1978). However, at this time only about 100 confirmed cases had been published and these probably represented the more severe manifestations of a massive underlying population of campylobacter infections. Dehydration and vomiting are not usually prominent in campylobacter enteritis but may occasionally be severe enough to contribute to death (Evans and Dadswell, 1967 ; Public Health Service (England and Wales) CDSC Report, unpublised, 1978/50). A review of campylobacter enteritis by Karmali and Fleming

(1979a) includes a number of references to deaths associated with the infections in compromised patients, and a second review has recently been presented in the relationship between campylobacter infections and abortion in humans (Coid and Fox, 1983).

1.5. The Evidence for Campylobacter Pathogenesis.

The preceding sections have assumed a pathogenic role for campylobacters, the evidence for this assumption is presented below, being based on a recent review (Karmali and Fleming, 1979a).

(1) Examination of symptomatic and asymptomatic patients has shown a high correlation between the presence of Campylobacter jejuni/coli in the stools and illness (Butzler et al., 1973 ; Skirrow, 1977).

(2) The organism disappears from the stools during convalescence (Karmali & Fleming, 1979b).

(3) C.jejuni/coli has been isolated from blood cultures of patients with diarrhoea (Levy, 1946 ; King, 1957 ; Middlekamp and Wolf, 1961 ; Wheeler and Borchers, 1961 ; King, 1962 ; Mandel and Ellison, 1963 ; Darrell et al., 1967 ; Evans and Dadswell, 1967 ; White, 1967 ; Dekeyser et al., 1972).

(4) Significant antibody titres develop in patients with campylobacter enteritis (Skirrow, 1977 ; Karmali and Fleming, 1979b).

(5) Clustering of symptomatic cases occurs (Levy, 1946 ; Cadranel et al., 1973 ; Hayek and Cruickshank, 1977 ; P.H.L.S. CDSC Report, unpublished, 1978/47 ; Ibid., 1978/50 ; Vanhoof et al., 1978 ; Commun Dis Scot Wkly Rep 4:, 1979).

(6) Human volunteer studies and accidental self-inoculation with pure campylobacter cultures has resulted in diarrhoea and abdominal pain (Prescott and

Karmali, 1978 ; Robinson, 1981 ; Black et al., 1983)

(7) Treatment with erythromycin, to which the organism is sensitive, results in rapid clearance of organisms from the stools and resolution of the symptoms. It should be noted however, that the diarrhoeal phase and excretion of C.jejuni/coli can be variable even in the absence of erythromycin (Karmali and Fleming, 1979b).

(8) Stools from patients with campylobacter enteritis usually contain no other known pathogens (Karmali and Fleming, 1979b) although cases of multiple infections have been reported (Butzler and Skirrow, 1979 ; McGechie et al., 1982).

(9) In developed countries isolation of C.jejuni/coli from asymptomatic controls is usually less than 2% (Rettig, 1979) however it should be noted that in the less well developed countries asymptomatic carriage may be more common (De Mol, 1982 ; Glass et al., 1982 ; Rajan and Mathan, 1982 ; Richardson et al., 1982)

1.5.1. Clinical Manifestations of Campylobacter Infections in Humans.

The usual presentation of campylobacter enteritis is an unpleasant but self-limiting attack of acute diarrhoea of two or three days duration, with abdominal pain and discomfort lasting up to a week or more. The description presented here of "classical" campylobacter enteritis is based on cases where the symptoms were severe enough to be presented to a physician.

1.5.2. Incubation Period and Prodromal Symptoms.

Due to the difficulty in pinpointing the time and source of campylobacter ingestion it is usually impossible to establish the incubation period before the organism produces clinical symptoms. From the analysis of environmental data linked to outbreaks of enteritis

(Brouwer et al., 1979 ; Itoh et al., 1980), and
experimental infections (Steele and McDermott, 1978 ;
Robinson, 1981 ; Black et al., 1983), an incubation
period of between two and ten days is possible, the
evidence suggesting three to five days as being the norm
(Butzler and Skirrow, 1979). Prodromal symptoms are often
encountered and may last between twenty-four and
ninty-six hours, the former being more common (Skirrow,
1977). Fever and malaise are the usual features with
headache, backache, aching of limbs and colicky abdominal
pains also being reported in some cases. A body
temperature of 40°C is often found and can lead to
delirium and confusion. Nausea may be present but
vomiting is rare being found in less than 30% of
patients, with dehydration also being unusual (Karmali
and Fleming, 1979a). The abdominal pain is periumbilical
and cramping, may occur before, during, and after the
diarrhoeal phase, and is often a more distressing feature
of the disease than the diarrhoeal episode itself.

1.5.3. Diarrhoeal phase.

The diarrhoeal stage may begin gradually or
explosively, but in all cases faeces become fluid,
foul-smelling and may contain bile, blood, mucus or
inflammatory cellular exudate. This acute stage may last
for 2 or 3 days with up to eight or more bowel movements
a day, at the end of which time the stools have normally
become watery in appearance. The frequency of bowel
movements slowly diminishes and the stools become
semi-formed, however the abdominal pain may persist for
one or two weeks and the taking of solid food too early
after the attack may precipitate a further episode.
Approximately 20% of patients may have a relapsing,
prolonged or severe illness (Blaser et al., 1979).

1.5.4. Complications.

Complications arising from the disease affect only a
small percentage of those with gastroenteritis, however

the large number of cases reported each year means complications occur to a significant number of patients. The types of complications arising from C.jejuni and C.coli infections are increasing every year as the involvment of these organisms in disease processes other than enteritis are recognised. The spectrum of these complications is too wide to be described here and the interested reader is referred to a recent review (Mandel, De Mol and Butzler, 1984).

1.5.5. Disease and other Campylobacters.

Four other species of campylobacter-like organisms can be isolated from humans, the first, C.sputorum sputorum, is found as part of the normal flora of the oral cavity and has not been associated with disease (Loesche et al., 1965). The second C.fetus fetus is pathogenic for man. This organism almost invariably attacks compromised hosts, the most common symptom of this opportunistic infection being bacteraemia without localized infection. A review of 102 examples of septicaemia or other non-enteric human infections revealed only sixteen cases were there was no underlying medical condition or environmental exposure (Rettig, 1979). In a study of ninety-one cases of campylobacter bacteraemias in compromised hosts, fifty were due to C.fetus fetus and only ten were due to C.jejuni or C.coli (Guerrant et al., 1978).
In general therefore, C.fetus fetus is an important pathogen of compromised and/or elderly patients while C.jejuni is a major pathogen causing disease in young and previously healthy individuals. The other two campylobacter-like organisms have only recently been recognised and their roles as pathogens have yet to be established. An organism has been isolated from the gastric antrum of patients suffering from active chronic gastritis (Warren and Marshal, 1983). Although by gram stain and growth conditions these organisms resemble campylobacters, they do possess certain morphological differences particularly with respect to their flagella. The other organisms are classified, at present, as

campylobacter-like organisms (CLO) and have been isolated from rectal swabs taken from homosexual men with, and in some cases without, gastro-intestinal illness. As with the previous organisms CLO's have many similarities with campylobacters but also differ in a number of respects (Quinn et al., 1983 ; Fennell et al., 1984). It is likely that a number of other campylobacter-like organisms may be isolated in the future now that culturing of the organisms has become a routine procedure.

1.6. Animal Models of Campylobacter Infections.

A variety of animal species have been examined in attempts to reproduce the disease experimentally, however few of these have met with total success. Many of these models have used animals that are unsuitable for use in the smaller research laboratories i.e. rhesus monkeys, calves etc. In general and with a few exceptions, when a disease process was observed in these animals it was of a mild nature with minor histological changes within the gastrointestinal tract. Further details of these animal models has been presented in a recent review article (Newell, 1984).

Small mammals offer a much better proposition for the development of in vivo models of campylobacter enteritis within the smaller laboratory and a number of species have been examined. Ferrets can be induced to produce diarrhoea and undergo long term colonisation by oral administration of C.jejuni (Ackerman et al, 1982). Experimental infections of young mink also produces diarrhoea and histopathological changes within the colonic mucosa (Hunter and Prescott, 1983). Short term colonisation of neonatal rats and rabbits is also possible although evidence of enteritis is not seen (Field et al., 1981).

Neonatal and adult mice have both been extensively studied as models of campylobacter infections. Although neonatal mice may be routinely colonized withC.jejuni(Field et al., 1981 ; Newell and Pearson,

1984) the colonisation of normal adult mice has proven more difficult to establish (Field et al., 1981). However, alterations in the microflora of the intestinal tract of adult mice by the use of tobramycin (Field et al., 1984), gnotobiotic mice (Blaser and Duncan, 1983 ; Fauchere, 1983), or direct inoculation into laparatomised mice (Merrel et al., 1982) can produce consistent colonisation. Neonatal mice (5-6 days old) become colonized for up to three weeks after intra-gastric inoculation of C.jejuni (Field et al., 1981 ; Newell and Pearson, 1984). The majority of organisms were recovered from the caecum and large intestine with lower numbers in the ileum and duodenum. The duration of colonisation and the extent of the mortality in the mice has been found to be dependent on the strain of organism used (Field et al., 1981). Enteritis was not seen in colonized infant mice although intestinal absorption of D-glucose and D-galactose has been found to be reduced in these animals (Madge, 1980). Enteritis without mortality in six-day old mice has been observed however after intra-gastric inoculation of animal-passaged C.jejuni (Kazmi et al., 1983). After intra-gastric inoculation of adult mice a transient bacteraemia occurs within the first twenty-four hours and organisms may be recovered from various sites in the body (Blaser et al., 1983a ; Blaser and Duncan, 1983). The organisms appear to invade the intestinal mucosa, are then endocytosed by enterocytes and grow within the intestinal lymphoid tissues, without invasion of the reticuloendothelial system (Fauchere et al., 1983 ; Newell, 1984). Enteritis has been reported after colonization of gnotobiotic and monoxenic adult mice (Fauchere et al., 1983) although as yet this has not been confirmed by other workers. A review of experimental studies of campylobacter enteritis has recently been presented (Newell, 1984). From the above description it would appear that the mouse models offer a useful and convenient in vivo system in which to study campylobacter virulence.

1.7. Pathogenic Mechanisms.

The initiation of bacterial diarrhoea is due to an interaction between the pathogen and host tissue, resulting in a disturbance of the gastro-intestinal tract. The mediators of this disturbance can be of a number of types including enterotoxins, cytotoxins and direct attachment to, and invasion of the mucosal surface.

1.7.1 Toxins.

One of the most common causes of bacterial diarrhoea is the elaboration of an enterotoxin or cytotoxin by the pathogen. Enterotoxins are exotoxins produced by an organism that affect biochemical pathways within the enterocytes leading to fluid secretion into the lumen of the intestinal tract. Cytotoxins are also exotoxins but their mode of action causes cell death and tissue damage. All bacterial enterotoxins so far identified have been shown to be proteins generally classified as either heat stable (ST) or heat labile (LT), are usually resistant to trypsin, inhibited at low pH and produce their effect on the small bowel (Giannella, 1981). There have been a number of laboratory tests developed for the identification of enterotoxins, heat labile enterotoxins are detected in tissue culture assays using mouse adrenal cells (Y-1), Chinese Hamster Ovary (CHO) or Vero cells (Donta et al., 1974 ; Guerrant et al., 1974 ; Speirs et al., 1977), while the intradermal injection of rabbits has been used to detect the vascular permeability factor associated with the LT toxins of E.coli and V.cholerae. Rabbit, pig and calf intestinal loops may be used for the detection of both LT and ST toxins, the latter also being active in the suckling mouse model (Dean et al., 1972). One of the most studied enterotoxins is that produced by V.cholerae, a heat labile protein of molecular weight 82,000-84,000 being composed of two subunits designated A and B. The B subunit is composed of five polypeptide chains and is responsible for the binding of the toxin to the ganglioside GM1 receptor on the surface of a susceptible cell. The A subunit consists of two

polypeptide chains termed alpha and beta, the alpha chain being the active component. The heat-labile enterotoxin produced by some E.coli strains shares almost complete identity with cholera toxin including its structure and mode of action (Evans et al., 1972 ; Dorner and Mayer, 1975). A second, heat stable type of enterotoxin is produced by some E.coli strains, this has a lower molecular weight, (4,400-5,500), than the heat labile toxin and produces fluid secretion via calcium uptake and activation of particulate guanylate cyclase (Field et al., 1978 ; Newsome et al., 1978).

Experimental evidence for the production of enterotoxins by the following organisms has been found; V.parahemolyticus, Bacillus cereus, Clostridia, Yersina,Staphylococci, Salmonella, Shigella, Enterocolitica, Streptococci, Klebsiellaand Pseudomonas (Reviewed by Giannella, 1981).

A number of bacteria have been shown to produce cytotoxins that are lethal to tissue culture cells, included in this group of organisms are Shigella dysenteriae (Keusch, 1973), Aeromones hydrophila (Donta and Haddow, 1978), Clostridia (Keusch and Donta, 1975), E.coli (Konowalchuk et al., 1977) and V.parahemolyticus (Carruthers, 1975). Little is known of the mechanism of these toxins although inhibition of protein synthesis may be involved, at least in the case of Shigella dysenteriae cytotoxin (Thompson et al., 1976).

1.7.2. Campylobacter Toxins.

The extent of toxin production in C.jejuni /coli is still far from clear, although until recently the evidence suggested that it was not a common occurance in these organisms (Guerrant et al., 1978 ; Butzler, 1981, 1982 ; Firehammer and Myers, 1981 and Manninen et al., 1982).

In opposition to the above findings a number of positive enterotoxin activities have recently been reported. In 1982 enterotoxin activity in the mouse adrenal cell system by three C.fetus fetus and two

C.jejuni isolates was reported (Gubina et al., 1982).
More recently evidence has accumulated that suggests some
clinical isolates of campylobacter do produce
enterotoxins and/or cytotoxins (Guerrant et al., 1978 ;
Johnson and Lior, 1983, 1984 ; McCardell et al., 1983b ;
Ruiz-Palacios et al., 1983 ; Yeen et al., 1983), with
both some evidence of immunological and pharmalogical
similarties to cholera toxin (McCardell et al., 1983b ;
Ruiz-Palacios et al., 1983) and some evidence against
(Olsvik et al., 1984). A net sodium secretory flux,
(plasma-lumen), and impaired glucose transport in adult
rat jejunal segments perfused with culture supernatants
of four C.jejuni strains has also been reported
(Ruiz-Palacios, 1982 ; Fernandez et al., 1983). Assuming
some strains of campylobacter are pathogenic by virtue of
enterotoxin(s) and/or cytotoxin(s) production, the
clinical presentation of some cases of campylobacterosis
strongly suggests that an invasive mechanisms is also
involved.

1.7.3. Mucosal Attachment and Invasion.

The mucosal surfaces of the human body can be an
inhospitable environment for a potential pathogen, with
mucin production, sloughing of cells, peristalsis and
antibody production all combining to prevent the organism
gaining access to the mucosal and sub-mucosal surfaces.
Despite these defences a number of organisms have been
shown to attach to epithelial cells in vivo and in vitro.

1.7.3.1. Attachment.

Examination of urethral exudates from cases of
gonorrhea reveal gonococci in close association with host
cells (Ward and Watt, 1972) and this cell attachment has
been confirmed in vitro using human fallopian tube organ
cultures (Ward et al., 1974). Similar findings have been
found in the comparision of the adherence of bacteria to
epithelial cells in vitro and their virulence in vivo
i.e. Group A Streptococci (Ramirez-Ronda, 1978 ; Scheld

et al., 1978), certain strains of E.coli (Jones and Rutter, 1974), Salmonella (Duguid and Old, 1980), Proteus mirabilis (Silverblatt, 1974 ; Silverblatt and Ofek, 1978), and Bordetella pertussis (Sato et al., 1979). A number of reviews of bacterial attachment have also been published which give further details of organisms showing evidence of this property (Watt, 1980 ; Beachey, 1981 ; Giannella, 1981 ; Sparling, Swanson and Puziss. Eds, 1983).

Certain bacterial cell surface structures have been shown to assist in the attachment of an organism to target cells. Common adhesins found on a large number of gram-negative organisms are the fimbriae (pili) which have been shown to function in the attachment of N.gonorrhea, E.coli, V.cholerae, Klebsiella, Serratia, Shigella, Enterobacter, Salmonella, Citrobacter, Proteus, Bordetella pertussis and Pseudomonas aeroginosa (reviewed by Beachey, 1981). The fimbriae from these organisms are not only capable of penetrating the electrostatic barrier around a cell (Heckels et al., 1976) but have also been shown to be involved in specific binding to the host cell surface via receptors on the eukaryotic cell (Jones and Rutter, 1974b ; McNeish et al., 1975 ; Beachey and Ofek, 1976 ; Burrows et al., 1976 ; Jones and Freter, 1976 ; Evans et al., 1978 ; Svanborg-Eden, 1978 ; Svanborg-Eden and Hansson, 1978 ; Sato et al., 1979 ; Pearce and Buchanan, 1980 ; Trust et al., 1980). Other surface components have also been associated with increased attachment to target cells, such as surface proteins of N.gonorrhea (Lambden et al., 1979 ; James and Heckels, 1980) ; Lipoteichoic acid-M protein of Streptococcus pyogenes (Ellen and Gibbons, 1972 ; Ofek et al., 1975) ; membrane protein(s) of Mycoplasma (Hu et al., 1977 ; Banai et al., 1980 ; Krause et al., 1983), the glycocalyx of certain bacteria (Costerton et al., 1978), and possibly some flagella (Guentzel and Berry, 1975 ; Eshdat et al., 1981 ; Attridge and Rowley, 1983).

Although the ability to attach to mucosal surfaces can contribute to the virulence of an organism many commensal organisms will also attach to cell surfaces without

causing an infection indicating that attachment alone will not produce disease.

1.7.3.2. Invasion.

A number of organisms are capable of initiating a disease process by mechanisms which involve attachment to, and in some cases invasion of the mucosal surface. In the case of V. cholerae attachment to the intestinal tract surface allows more efficient presentation of cholera toxin to the target cells, however, invasion does not occur subsequent to this initial attachment. Other organisms will invade the mucosal surface to proliferate locally or systemically, included in this group of pathogens are Shigellae, N.gonnorrhea, C.perifringes, V.parahaemolytica, Salmonella, Yersinia and certain strains of E. coli (reviewed by Giannella, 1981 ; McGee et al., 1981). Of these organisms, Shigellae and Salmonella are the most studied pathogens and the following description of invasion is based on work with these organisms. In the initial stages Salmonellae colonize the ileum and colon (Giannella, 1975 ; Formal et al., 1975) while Shigella colonize the ileum only transiently with a more stable population in the colon (Rout, 1975). Proceeding invasion the glycocalyx of the epithelial cells may be partially digested (Prizont, 1982), followed by degeneration of the brush border (Takeuchi, 1971 ; Polotskii et al., 1974). The organisms are then enfolded by an invagination of the cell membrane so that they initially lie within vacuoles inside the cytoplasm of the cell. Salmonella causing gastroenteritis remain localised in the mucosa while those responsible for a typhoid-like disease enter the lamina propria via the basal cell membrane. The organisms are then transported in the lymph and blood circulations to colonize the reticuloendothelial system (Hornick et al., 1970). Shigella appear to digest the vacuole membrane so that they lie free within the cytoplasm from where they invade adjacent epithelial cells. The overt signs of the disease process probably involves a number of factors. To

cause disease the organism must be capable of multiplying within the tissue (Formal et al., 1965), this may cause an acute inflammatory reaction leading to ulceration of the colonic mucosa in shigellosis and less severe ulceration of the ileum and colon with salmonella infections. The more severe ulceration of shigellosis may be due to a cytotoxin elaborated by the organism (Keusch and Jacewicz, 1975 ; Brown et al., 1980). In general it should be remembered that the overall disease process in these infections probably represents a combination of invasion induced inflammation and elaboration of cytotoxins and/or enterotoxins by the organisms (Sandefur and Peterson, 1976, 1977 ; Peterson and Sandefur, 1979 ; Eiklid and Olsnes, 1983). It is likely that viable virulent organisms are required for invasion to occur (Hale et al., 1979 ; Kihlstrom, 1980), and the fact that enterotoxigenic bacteria such as E.coli and V.cholerae and various nonpathogenic bacteria adhere to the mucosa without invading the epithelium suggests specific signal(s) may be required for internalisation. In order to invade a number of other requirements may also have to be fulfilled, in the case of S.flexneri a complete LPS coat is required (Gemski et al., 1972 ; Formal and Hornick, 1978), and it is likely that certain virulance factors are plasmid mediated (Jones et al., 1982 ; Sansonetti et al., 1982) in addition to those on the bacterial chromosome (Formal et al., 1971).

1.7.4. Campylobacter Attachment and Invasion.

Colonization of the mucus layer of the intestine by C.jejuni has been demonstrated recently by electron-microscopic studies using animal models (Merrel et al., 1982 ; Lee et al., 1983), and the in vivo adhesion of the organisms to mucosal surfaces has also been demonstrated (Fauchere et al., 1983 ; McCardell et al., 1983a ; Newell and Pearson, 1984), as has in vitro attachment to tissue culture cells (Newell and Pearson, 1984) and isolated brush borders (Taylor, 1982).
The clinical presentation of campylobacter enteritis

is suggestive of an invasive process as blood and leukocytes are frequently found in the stools (Karmali and Fleming, 1979a, 1979b), bacteraemia may be a frequent occurance (Guerrant et al., 1978), and histological examination reveals mucosal oedema, polymorphonuclear leukocyte dominated inflammatory infiltrates, crypt deformation and surface erosion together with haemorrhagic necrosis of the small intestine and numerous hyperplastic mesentric lymph nodes (Reviewed by Mandel et al., 1984). There are a number of systems available for the study of invasion, one of the most common procedures being the Sereny test. Live organisms are instilled into the conjunctival sac of Guinea-pigs, a positive test resulting in invasion of the corneal epithelial cells producing a keratoconjunctivitis which may correlate with intestinal invasion (La Brec et al., 1964). However, all attempts to demonstrate invasion by C.jejuni/coli in the Sereny test have so far proven negative (Guerrant et al., 1978 ; Gubina et al., 1981 ; Manninen et al., 1982). Invasion of primary in vitro cultures of ten day old chick embryo cells has been demonstrated, together with invasion of the gastro-intestinal tract of eight day old chicks (Butzler and Skirrow, 1979 ; Sanyal et al., 1983), and the chorioallantoic membranes of chick embryos (Davidson and Solomon, 1980). A number of animal models have also been used to demonstrate invasion in vivo (Taylor, 1982 ; Fauchere et al., 1983 ; McCardell et al., 1983a). Extensive studies on the pathogenicity of C.jejuni/coli (Manninen et al., 1982) included HeLa cell association and HeLa cell invasion-immunofluorescence studies. Attachment to and invasion of the HeLa cells was seen, however although strain differences existed, in general the attachment and invasion was lower than of that seen with Listeria or Yersinia control organisms. Invasion of HeLa and INT-407 cells has been demonstrated in electron-microscopic studies (Newell and Pearson, 1984). The role of attachment and invasion in the pathogenesis of campylobacters requires further study not only as a disease process but also as a more efficient way of presenting toxins to target cells.

From the above description it can be seen that a detailed study of the external surface of a pathogen can prove to be of fundamental importance in determining the virulence, pathogenic mechanisms and immunological characteristics of the organism. The following section therefore describes the general arrangement of the gram-negative bacterial surface.

1.8. General Structure of Gram-Negative Bacteria.

The general organisation of the membrane components of gram-negative bacteria is now fairly well understood. The cytoplasm is limited on its external surface by a 75Å wide plasma membrane (inner membrane). This membrane has the characteristic lipid bilayer structure and contains many proteins and enzymes embedded within it which are involved in active transportation, oxidative phosphorylation and the synthesis of some macromolecules. Immediately external to this inner membrane is a periplasmic region which contains certain hydrolytic enzymes and binding proteins. The next major component of the cell wall is peptidoglycan, an alternating backbone of N-acetylmuramic acid and N-acetylglucosamine residues in b-1,4 linkage substituted with a tetrapeptide of alternating L and D amino residues. Cross-linking the tetrapepetides are peptide bridges formed from the terminal carboxyl group of one tetrapeptide to an available group (usually a free NH_2 of diaminopimelate) of an adjacent tetrapeptide. This interlinking structure provides much of the relative rigidity and stability of gram-negative cells.

The outer membrane is morphologically and biochemically distinct from the inner membrane being a 75Å wide lipid bilayer with a relatively less complex structure compared to the inner membrane, containing less phospholipid, proteins and enzymes, but with an unique component lipopolysaccharide. A number of procedures have been developed to allow the separation of the inner and outer membranes of gram-negative bacteria. The initial step involves the preparation of membrane material, the subsequent step physically separating the inner and outer

membrane components. The first stage may be accomplished in a number of ways, the more commonly used procedures being ; (1) lysis of osmotically fragile cells produced by the formation of spheroplasts (Miura and Mizushima, 1968), (2) lysis of intact cells by the use of a french pressure cell (Schnaitman, 1970), and (3) ultrasonic disruption of intact cells (Churchward and Holland, 1976). the second stage may also be accomplished in a number of ways including separation of membranes on a sucrose gradient (Miura and Mizushima, 1968), separation within a preparative particle electrophorograph (Heidrich et al., 1970 ; White et al., 1972) or separation by selective use of detergents (Schnaitman 1971 ; Filip et al., 1973).

1.8.1. Outer Membrane Proteins.

The majority of the investigations into outer membrane proteins have involved studies of E.coli and S.typhimurium. From the data accumulated since the early 1970's a general picture of the arrangement of proteins in the outer membrane of these organisms has emerged. Of the major outer membrane protein components two can be found complexed non-covalently to peptidoglycan after whole cell membranes are solubilised in 2% SDS at 60° C (Rosenbusch, 1974). One of these bound proteins was identified as a lipoprotein (Braun and Rehn, 1969) and will be discussed later, the second has been described as matrix protein. Matrix proteins, although similar in their characteristics, are considered not to be a single protein species, some organisms having been shown to have a number of separate matrix proteins such as the OmpC and the OmpF proteins of E.coli K12 (Bragg and Hou, 1972), and the OmpC, OmpD and OmpF proteins of S.typhimurium (Ames, 1974). A particular matrix protein may be isolated in the absence of the other(s) (Nakamura and Mizushima, 1976 ; Schmitges and Henning, 1976) and each has been shown to have a separate gene locus (reviewed by Bachmann and Low, 1980). It is the names of these structural genes that have been used to establish the

standard nomenclature for these and other outer membrane proteins (Lee et al., 1979 ; Reeves, 1979). The matrix proteins of E.coli K12 have been shown to exist as oligomeric forms in vivo (Palva and Randell, 1976 ; Reithmeier and Braggs, 1977) and require to be heated above 70°C in SDS before monomeric forms can be isolated (Nakamura and Mizushima, 1976).The examination of the matrix protein-peptidoglycan complex by electron-microscopy reveals a hexagonal lattice which suggests three molecules of protein form one unit within the lattice (Steven et al., 1977). The incorporation of matrix protein into artificial LPS-phospholipid vesicles enhances their permeability to sucrose but excluded oligo- and poly- saccharides of molecular weights 900 or higher (Nakae and Nikaido, 1975 ; Nakae, 1976a, 1976b). It is likely that the matrix proteins form passive diffusion pores which allow the transfer of small molecular weight, hydrophilic molecules through the membrane and for this reason these proteins have been termed "porins" (Nakae, 1976b). As well as their function as porins the matrix proteins also contain receptors for phages (Datta et al., 1977 ; Verhoef et al., 1977).

Upon heating E.coli. outer membranes, solubilised in SDS, above 50°C a protein is observed on PAGE the apparent molecular weight of which increases over its molecular weight at lower temperatures (Inouye and Yee, 1973).This same protein is also susceptible to trypsin and pronase cleavage even when embedded within the membrane (Bragg and Hou, 1972 ; Inouye and Yee, 1972). The protein exhibiting these characteristics is called the Omp A protein and has a molecular weight of about 35,000 when solubilised above 50°C in SDS (Nakamura and Mizushima, 1976). The Omp A protein functions in F-pilus mediated conjugation (Skurray et al., 1974) and as a receptor for phages (Manning et al., 1976 ; Datta et al., 1977 ; Van Alpen et al., 1977).

A low molecular weight (about 7,000) lipo-protein was identified in E.coli outer membranes by Braun and Rehn in 1969. It consists of 58 amino acids, the e-amino group of the c-terminal lysine being covalently linked to the

carboxyl group of every tenth to twelfth
meso-diaminopimelic acid of peptidoglycan (Braun and
Sieglin, 1970 ; Braun and Bosch, 1972a, 1972b ; Hantke
and Braun, 1973). A free form of the lipoprotein (unbound
to peptidoglycan) is also found within the outer membrane
(Inouye et al., 1972). The exact function of lipoprotein
is still uncertain although roles as passive diffusion
pores and as an anchor between the peptidoglycan and the
outer membrane have been proposed (Inouye, 1974 ; Braun
et al., 1976).

In addition to these major components the outer
membrane may contain 10-20 minor proteins.Under certain
growth conditions some of these proteins may occur in
quantities approaching those of the major proteins. The
function of these minor proteins vary but include uptake
of nutritional substrates, DNA replication and cell
division and receptors for phage and colicins (Reviews:
Braun et al., 1976 ; Braun and Hantke, 1977 ; DiRienzo et
al., 1978).

1.8.2. Lipopolysaccharide.

Lipopolysaccharide is composed of a polysaccaride
chain of repeating sugar units linked to an acidic
oligosaccharide region (the core) which in turn is
covalently linked to lipid , (lipid A), embedded in the
outer membrane. The polysaccharide side chains vary
between bacterial species and strains (Luderitz et al.,
1966 ; Peterson and Quie, 1981) and are responsible for
the 'O' antigen specificity of the LPS (Kauffmann et al.,
1960, ; Staub, 1960 ; Westphal, 1960 ; Luderitz, Staub,
and Westphal, 1966). The LPS of bacteria is toxic and may
be associated with some of the manifestations of
gram-negative infections, in particular the lipid A
portion having been shown to be responsible for shock,
pyrogenicity, leukopenia, hypotension and the local
Schwartzman reaction (Galanos et al., 1972, 1977). In
addition to its endotoxin function LPS stimulates
specific antibody production and non-specific B-cell and
T-cell activation (Turner and Rowe, 1964 ; Rossen et al.,

1967 ; Coutinho et al., 1975 ; Oppenheim and Rosenstreich, 1976). Finally external to the outer membrane may be fimbriae, flagella and capsular components (Fig 1.3.). A number of these structures can play an important role in the pathogenesis and virulence of an organism and will be discussed in further detail.

1.8.3. Fimbriae.

Fimbriae are filamentous protein appendages that can be found in large numbers over the entire surface of some organisms. They are of a relatively simple construction being composed of a single protein arranged into a helix to form a long, hollow filament of 3-30nm in width and 0.2μm to a few micrometers in length (Brinton, 1966). It is these pili that are responsible for the pilus mediated attachment previously mentioned. Although pili have been shown to play an important role in the attachment of bacteria to various target sites in vivo and in vitro thay are not functionally or immunologically identical. A number of bacterial species are capable of producing type 1 pili that mediate mannose sensitive haemagglutination (Duguid et al., 1966) while a range of distinct pili have been shown to initiate mannose resistant haemagglutination of erthrocytes from various species (Jones and Rutter, 1974a, 1974b ; Burrows et al., 1976 ; Evans et al., 1977 ; Orskov et al., 1977). Included in the latter group are the human colonisation factor antigens CFA/1 and CFA/11 (Evans et al., 1975 ; Evans and Evans, 1978), the K88 antigen of swine specific ETEC (Jones and Rutter, 1974a, 1974b) and the K99 antigen of calf and sheep specific ETEC (Orskov et al., 1975).

1.8.4. Flagella.

Flagella are proteinous filamentary structures extending from the bacterial surface. They may occur at the ends of the cell (polar flagella) or over the entire cell surface (peritrichous flagella) and are responsible

Gram-Negative Cell Surface
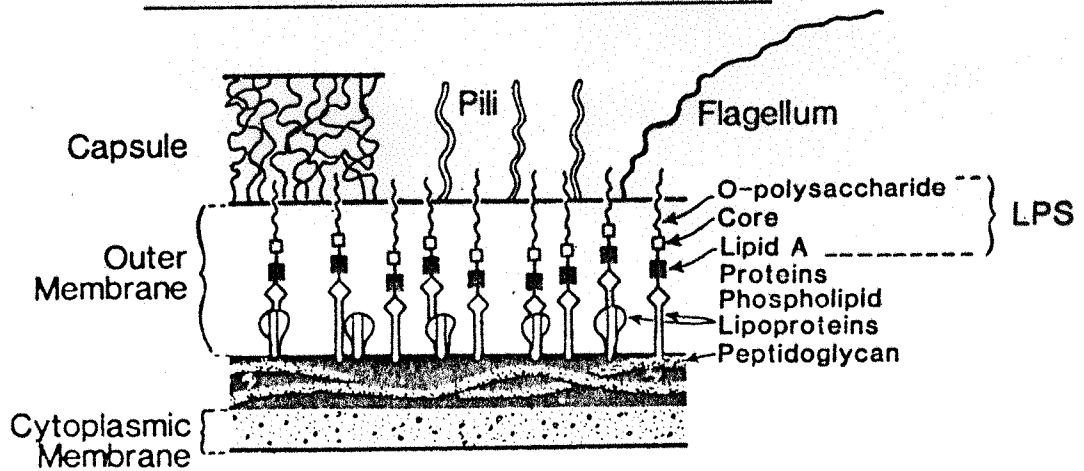
Figure 1.2. The general arrangement of the gram-negative cell surface. Note: not all the components shown are necessarily present on the surface of all gram-negative bacteria.

for bacterial motility. The bacterial flagella apparatus is composed of the basal body, flagellar hook and flagellar filament. The filament may be composed of a single type of protein subunit as in Salmonellae and E.coli (Parish and Ada 1969 ; Silverman and Simon 1972), from two polypeptides such as found in Caulobacter crescentus (Langenaur and Agabian, 1976) or surrounded by a sheath as in V.cholera (Yang et al., 1977). The molecular weight of the flagellin that forms the filament varies considerably between species, from 33,000 in Bacillus subtilus to 60,000 for E.coli (Iino, 1969 ; McDonough and Smith, 1976). The filament may be up to 20µm in length and 20nm in diameter (unsheathed) and takes the form of a semi-rigid helix (DePamphilis and Adler, 1971a ; Doetsch and Sjoblad, 1980). The hook region is a short (70-90nm) slightly curved structure having a diameter in excess of that found in the filament. The hook protein is different from flagellin and has a molecular weight range from 33,000 in B.subtilis (Dimmitt and Simon, 1971) to 42,000 in E.coli (Kagawa et al., 1976). The function of the hook is unknown but roles as a 'universal joint' (Silverman and Simon, 1977) or in correct orientation of the filament (Macnab, 1978) have been suggested.

The basal body is a complex structure consisting of a number of rings embedded into the cell wall of the organism (DePamphilis and Adler, 1971b). These rings have been named ; the 'L' ring associated with the LPS outer wall ; the 'P' ring associated with the peptidoglycan ; the 'S' ring lying in the periplasmic region and the 'M' ring attached to the inner membrane.

The increased virulence associated with the possession of flagella is now well recognised (Guentzel and Berry, 1975 ; Jones and Freter, 1976 ; Eubanks et al., 1977 ; Yancy et al., 1979 ; McManus et al., 1980 ; Caven and Montie, 1981) and is thought to be due to the ability to penetrate mucus layers and exhibit chemotactic responses.

1.8.5. Capsules.

These structures are usually composed of polysaccharides and have been shown to protect some pathogenic bacteria from phagocytosis (Smith, 1977 ; Gotschlich et al., 1978 ; Peterson et al., 1978 ; Van Dijk, 1979 ; Wilkinson et al., 1979a). The increased virulence of encapsulated bacteria is thought to be due to lack of opsonisation of this surface component and the failure of PMLs to recognise 'hidden' opsonins such as antibody and the complement component C3b (Wilkinson et al., 1979b).

1.9.Campylobacter Surface Components.

Although the surface composition of C.intestinalis has been studied little information is available on the outer membrane and associated structures of the C.jejuni and C.coli group. Electron-microscopy of a cross-section through C.intestinalis cells reveals a structural organisation typical of gram-negative cells (Werner, 1963 ; Ritchie et al., 1966). The peptidoglycan of intestinal and venereal strains of C.intestinalis has been isolated using hot SDS extraction. The disaccharide tetrapeptide was reported as GLc NAc-MurNAc-L-Ala-D-Glu-mesoDpm-D-Ala (Keeler et al., 1966 ; Winter et al., 1971). The outer membrane of C.intestinalis has been extracted in Triton X-100 and EDTA and by SDS. The Triton -EDTA extracted material contained four bands when examined by SDS-PAGE with molecular weights of 100,000, 45,000, 38,000 and 31,000. A major band of 41,000 was noted if the material was heated to 100°C before electrophoresis (McCoy et al., 1976b).

Detailed analysis of the C.jejuni/coli outer membrane has only recently begun to be studied (Logan and Trust, 1982 ; Naess and Hofstad, 1982). In the study by Logan and Trust (1982) outer membranes were isolated by sodium lauryl sarcosinate extraction and the polypeptides and LPS analysed by SDS-PAGE. As with other gram-negative organisms the outer-membrane was shown to contain relatively few proteins, a number of which were heat modifiable. The absence of Brauns lipoprotein was noted

as it had previously been for C.fetus (Winter et al., 1971). Association of protein with the peptidoglycan layer was only found for the major outer membrane protein of both jejuni and coli strains. Surface exposure of the proteins was determined by the use of 125I-lactoperoxidase with only three outer membrane proteins being labelled.

1.9.1. Campylobacter Fimbriae.

Although a large number of campylobacter species have been examined for the presence of fimbriae, there is no documented evidence for the possession of these structures (Dijs and Graaf, 1982). It would appear that this relatively common adhesin found on other gram-negative bacteria does not contribute to the virulence of campylobacters.

1.9.2. Campylobacter Flagella.

Campylobacters possess a single flagellum at one or both poles of the cell, the length of the flagellum being up to three times the length of the cell (Smibert, 1978). The only available detailed knowledge of campylobacter flagella relates to studies on C.intestinalis. The mean width of the flagellum varied between 18 to 21 nm depending on the phase of growth and although this width was unusually wide there was no evidence of a flagella sheath (McCoy et al., 1975b). The amino acid composition of C.intestinalis flagella has been determined with all the common amino acids being found except cysteine, whilst proline was present in trace amounts only (Ullmann, 1976).

1.9.3. Campylobacter Capsules.

A microcapsule has been demonstrated at the periphery of C.intestinalis cells. Unlike the polysaccharide capsules of Group A streptococci, N.meningitidis and E.coli the microcapsule of C.intestinalis is a

glycoprotein and in this respect resembles Y.pestis (Amies, 1951 ; Englesberg and Levy, 1954 ; Winter et al., 1978). However, as with the polysaccharide capsules the microcapsule of C.intestinalis has been shown to be responsible for an antiphagocytic activity. The chemical composition of the capsule has been analysed as containing 4% carbohydrate (hexose, pentose and methylpentose) and a protein component low in basic, aromatic and sulphur-containing amino acids. The capsular glycoprotein has been isolated in a glycine-HCl acid extract and found to have a molecular weight of 97K (McCoy et al., 1975a ; Winter et al., 1978).

There is only limited evidence of a microcapsule on C.jejuni or C.coli strains (Buck and Parshall, 1982 ; Merrell et al., 1982).

1.9.4.Campylobacter Lipopolysaccharides.

Isolated C.fetus lipopolysaccharide has an endotoxin activity producing temperature increase and generalised Shwartzermen reaction (Dennis, 1959). The LPS of C.jejuni and C.Coli strains has been isolated by the aqueous phenol method (Naess and Hofstad, 1982) and the following sugars found to be present ; glucose, galactose, L-glycero-D-manno-heptose and glucosamine, one strain also containing galactosamine. The LPS was also found to contain 2-keto-3-deoxy-octonate (KDO) and phosphorus.

At the present time detailed knowledge on the surface structures and antigens of C.jejuni and C.coli is lacking. As the outer membrane surface is probably the first component of an invading organism encountered by a host, and the resulting immune response is often directed against components of this membrane, and by analogy with other pathogens were surface components are involved in colonization, resistance to host defences and other aspects of pathogenesis, the study of the surface components of C.jejuni and C.coli organisms may shed some light on their role as pathogens.

CHAPTER 2

MATERIALS AND METHODS

Unless otherwise stated all general laboratory chemicals were purchased from BDH (Poole, Dorset) or Fisons (Loughborough, leicestershire), specialised chemicals from Sigma (Poole, Dorset) and bacteriolgical media from Oxoid Ltd (Basingstoke).

2.1. Bacterial strains

The strains used during this investigation are described in table 2.1. The human isolates, with the exception of 81116 were isolated from sporadic cases of campylobacter enteritis. Strain 81116 was isolated from a patient investigated as part of an outbreak of campylobacter enteritis at a school (Palmer et al., 1983), while the NCTC C.coli was originally isolated from a pig placenta.

2.1.1. Serotyping

Serotyping results are shown in table 2.1.

2.1.2. Biotyping

The biotyping of the organisms was achieved using the procedures of Skirrow and Benjamin (1980) and the results are shown in table 2.1. The test were performed as follows:

2.1.2.1. Oxidase reaction

Reagent: 1%(w/v)   N,N,dimethyl-p-phenylenediamine dihydrochloride.

Procedure: A piece of filter paper was placed in a petri dish and 2 or 3 drops of reagent soaked on to it. The

Table 2.1. Strains used in study.

Strain	Source	Biotype	Serotype	
			Penner	Lior
33817	HUMAN	C.jejuni 2	U.T.	N.T.
51180	HUMAN	C.jejuni 1	30	N.T.
63923	HUMAN	C.jejuni 1	2	N.T.
68879	HUMAN	C.jejuni 1	U.T.	N.T.
3571	HUMAN	C.coli	30	N.T.
11353	N.C.T.C.	C.coli	5,30	N.T.
SO7	HUMAN	N.T.	N.T.	1
SO8	HUMAN	N.T.	4	1
SO9	HUMAN	N.T.	N.T.	1
SO10	HUMAN	N.T.	4,50	1
SO11	HUMAN	N.T.	4,50	N.T.
SO12	HUMAN	N.T.	2	N.T.
SO13	HUMAN	N.T.	2	53
SO14	HUMAN	N.T.	1	1
SO15	HUMAN	N.T.	4	26
81116	HUMAN	C.jejuni 2	6	6

(1) Serotyping performed by
(a) Penner serotypes: Dr. J.L.Penner, Department of Microbiology, University of Toronto, Ontario, Canada; Dr. D.M. Jones, P.H.L.S., Manchester, U.K.
(b) Lior serotypes: Dr. B. Rowe, Division of Enteric Pathogens, Central Public Health Laboratory, London, U.K.
(2) U.T.= Untypeable
(3) N.T.= Not Typed

colony to be tested was smeared from FBP agar onto the filter paper using a platinum wire, a positive result was indicated by the production of a dark purple colour in 5-10 seconds and a weak positive result by colour production within 10-60 seconds.

2.1.2.2. Catalase reaction

Reagent: 3%(w/v) hydrogen peroxide.

Procedure: A drop of reagent was placed on the bacterial growth on FBP agar and a positive result recorded if effervescence was noted.

2.1.2.3. Hippurate hydrolysis

Reagents: (1) 1%(w/v) sodium hippurate

(2) 3.5%(w/v) ninhydrine dissolved in an equal mixture of acetone and butanol.

Procedure: A heavy inoculum of campylobacter was taken from blood agar (BA) and mixed into the sodium hippurate to give a milky suspension (approximately equal to an optical density at 540nm of 1.0). This suspension was incubated for two hours at $37^{\circ}C$ followed by the addition to the suspension of 0.2ml of the ninhydrin solution. The suspension was re-incubated at $20^{\circ}C$ for two hours and a positive result recorded if the formation of a deep purple colour was observed.

2.1.2.4. Hydrogen sulphide production.

Reagent: H_2S (FPB) broth

	grm/litre
(1) Nutrient broth base No2	25.0
(2) Davis agar	1.2
(3) Ferrous sulphate ($FeSO_4.7H_2O$)	0.5
(4) Sodium metabisuphite	0.5
(5) Sodium Pyruvate	0.5

This broth was autoclaved (103.5KPa for 5min) and aliquoted into sterial glass bijous.

Procedure: A loopful of growth from an 18h BA culture of bacteria was inoculated intact into the center of the semi-solid broth. The cap was replaced and the bijou incubated at 20^0C for 4h. A positive result was indicated by blackening around the inoculum.

2.1.2.5. Nalidixic acid sensitivity

Reagent: Nalidixic acid disc containing $30\mu g$

Procedure: An FBP agar plate was inoculated with the test organism in such a way as to give a lawn of growth. The disc was placed in the center of the plate which was incubated at 37^0C under microaerophilic conditions. A positive result was registered if a zone of no growth was observed around the disc.

2.2 Media and growth conditions

2.2.1. Blood agar plates (BA)

Constituents:

(1) Blood agar base No2   40g/litre
        (2) Defribrinated horse blood
             (Lab M, London)   50ml/litre
        (3) Davis agar   8g/litre

        Procedure: The blood agar base and Davis agar were
dissolved into distilled water and autoclaved (103.5KPa
for 15min). The media was allowed to cool to about 55^{0}C
and placed into a water bath at the same temperature for
30min. The horse blood was then added and the complete
media mixed gently to avoid frothing, the blood agar
being finally poured into 9cm petri dishes.

Use: (1) Recovery of stored bacteria from liquid
             nitrogen.
        (2) Subculture of bacteria before inoculation of
             other media.

2.2.2. BA-VPT plates (Skirrow's medium)

Constituents:

(1) Blood agar base No2   40g/litre
(2) Defribrinated lysed horse blood
    (Lab M, London)   50ml/litre
(3) Vancomycin   10mg/litre
(4) Polymixin B sulphate   2500I.U./litre
(5) Trimethoprim lactate   5mg/litre

Procedure: As for BA plates, the antibiotics being added
at the same time as the blood.

Use: (1) Isolation of campylobacters from human faecal
material.

2.2.2.1. 2% BA-VPT plates

Constituents:

As for BA-VPT except that agar concentration was increased to 2% by the addition of Davis agar (8g/litre).

Procedure: As for BA-VPT plates.

Use: (1) Isolation and viable counting of campylobacters in the intestinal segments and faecal material of mice.

2.2.3. FBP agar

Constituents:
(1) Blood agar base No2		40g/litre
(2) FBP supplement		
(a) Ferrous sulphite ($FeSO_4 .7H_2O$)		0.5g/litre
(b) Sodium metabisulphate		0.5g/litre
(c) Sodium pyruvate		0.5g/litre
(3) Davis agar		8.0g/litre

Procedure: Prepared as for BA plates the FBP supplement being added instead of the horse blood.

Use: (1) For the growth of bacteria that are to be used for outer membrane preparations.
(2) For the growth of bacteria prior to surface labelling of the cells with Iodine125.
(3) Growth of bacteria for immunisation of rabbits.

2.2.4. Southampton Campylobacter Nutrient Broth (SCNB)

Constituents:

(1) Nutrient broth base No2	25g/litre
(2) Yeast extract	
(Difco Laboratories, Surrey)	5g/litre
(3) L-cysteine hydrochloride	0.1g/litre
(4) Potassium nitrate	0.8g/litre
(5) FBP supplement (Oxoid Ltd)	1.0ml/litre
(6) Sodium bicarbonate (10% w/v)	20mls/litre

Procedure: The constituents were dissolved with the exception of the FBP supplement and the sodium bicarbonate. The solution was autoclaved (103.5KPa for 15min) and then allowed to cool before the supplement and bicarbonate were added.

Use: (1) Growth of bacteria for spheroplast preparations.
     (2) Growth of campylobacters for flagella preparations.
     (3) Growth of bacteria for acid extraction.

2.2.5. Microaerophilic atmosphere

Inoculated media with the exception of SCNB cultures were incubated in anaerobic jars without catalyst. The jars were evacuated to 600mm of mercury and refilled to atmospheric pressure using a 10% CO_2, 90% N_2 gas mixture (BOC special gases, crawley).

SCNB cultures consisted of one litre of media in a two litre flask, sealed by a non-absorbant cotton wool plug. The flasks were sealed within polyethylene bags (0.6m by 0.88m), the air was removed and the bags filled with the above gas mixture.

2.2.6. Incubation temperatures.

Primary isolation of campylobacters from human faecal material was carried out at $43^{\circ}C$. Subsequent subculture, resuscitation from liquid nitrogen storage and growth for experimental procedures was carried out at $37^{\circ}C$.

2.2.7. Subculture

Minimal subculturing from primary plates was carried out before storage of the organisms in liquid nitrogen. A single subculture from liquid nitrogen storage onto BA was used before inoculation of the media used in the experimental procedures.

2.2.8. Liquid nitrogen storage

Bacterial strains were stored as suspensions in glycerol broth (10% v/v glycerol in 1% w/v proteose peptone).

2.3. Cell culture and collection

The human epithelial cell lines HeLa 229 (carcinoma of cervix), HEp 2 (carcinoma of larynx), and Int 407 (carcinoma of intestine; all obtained from Flow Laboratories Ltd, Irvine) were grown as monolayers in Eagles Minimal Essential Medium (MEM, Gibco Europe Ltd, Paisley) containing foetal calf serum, (10%v/v), Benzyl penicillin, (100 units /ml), and Gentamicin,(10μg/ml). The cells were cultured at $37^{\circ}C$ in a humidified incubator in an atmosphere of 5%(v/v) carbon dioxide in air. The cells were passsaged by washing the monolayers three times in PBS followed by the addition of PBS containing 0.005%(w/v) trypsin (Gibco Ltd, Paisley). After exposure to the trypsin for thirty seconds, excess trypsin was removed and the cells re-incubated at $37^{\circ}C$ until they had been released from the flask surface. The cells were

resuspended in tissue culture medium and flasks inoculated at cell dilutions of 1:6 for HeLa and HEp 2 cells and 1:4 for Int 407 cells.

Prior to bacterial-epithelial cell attachment studies the monolayers were removed from the flasks using the above procedure and 24-well trays (LH Engineering Ltd, Stoke Poges) were seeded at 4×10^5 cells/well (HeLa and HEp 2 cells) or 5×10^5 cells/well (Int 407 cells). These cells were grown in antibiotic free medium for 18h at 37^0 C to give lightly confluent monolayers, using the same atmospheric conditions described above.

2.3.1. Collection of Erythrocytes

Fresh blood was obtained from several animal species and human volunteers into collection vials containing 10 I.U./ml of heparin. The erythrocytes were pelleted by centrifugation at 800xg for 10min and washed three times in a defined campylobacter growth medium (DCGM, 3.5.1.). The cell pellet was finally resuspended in DCGM to give a 10% packed cell volume as measured in a haematocrit.

2.4. Assay and Analysis procedures.

2.4.1. Protein estimation

Protein was determined by the use of the Folin-Ciocalteau phenol reagent as described by Lowry (1951).

Reagents: (1) 1%(w/v) Copper sulphate ($CuSO_4$ $5H_2O$)
         (2) 2%(w/v) Potassium tartrate
         (3) 2%(w/v) Sodium carbonate in 0.1M Sodium hydroxide
         (4) 1M Folin-Ciocalteau phenol reagent (BDH Ltd, Poole)

Standards: Bovine serum albumin in water ; 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml

Unknowns: Unknown protein solutions at approximately 1mg/ml.

Procedure: Equal volumes of the copper sulphate and potassium tartrate solutions were mixed and this mixture diluted to 1:50 in the alkaline sodium carbonate to give an alkaline copper reagent. To tubes containing 0.1ml of standard BSA solutions or 0.1ml of the unknowns was added 1ml of the alkaline copper reagent solution and, after mixing, the tubes incubated at 20^0C for 15min. To each tube 0.1ml of the Folin-Ciocalteau phenol reagent was added with immediate mixing and the tubes incubated at 20^0C for 30min. Samples were run in duplicate and were read at 750nm (SP 1800 spectrophotometer, Pye Unicam, Cambridge) against water blanks similarly treated. The unknown protein concentrations were determined by establishing a standard curve using the bovine serum albumin results.

2.4.2. Succinate dehydrogenase assay

Reagents:

VOLUME

(1) Potassium phosphate buffer
    (0.1M pH 7.6)   0.7ml

(2) Potassium cyanide
    (0.1M pH 7.0 with HCl)   0.05ml

(3) Sodium succinate (0.2M)   0.05ml

(4) 2,6,dichlorophenol indophenol (1.2mM)   0.05ml

(5) Phenazine methosulphate (5mg/ml)   0.05ml

(6) Sample (0.1ml of fractions
    or 0.05mg protein)   0.1ml

Procedure: Reactants were maintained at 37^0C and the reaction started by the addition of the phenazine methosulphate, the reduction in the 2,6, dichlorophenol indophenol being followed at 600nm using a SP1800 spectrophotometer connected to a Unicam AR25 linear chart recorder. Succinate dehydrogenase activity was expressed as μmoles 2,6, dichlorophenol indophenol reduced per minute at 37^0C, using the following equation:

$$\frac{\Delta E}{min} \times \frac{1}{2.1 \times 10^{-2} \mu mole\, cm^{-1}} \times \frac{\text{Total volume of reaction mixture}}{10^3} \times \frac{1}{\text{volume of sample}} = \text{units per ml of sample}$$

where $\Delta E/min$ = the average change in absorbance at 600nm per minute and 2.1×10^{-2} $\mu moles^{-1}$ cm^{-1} = the molar extinction co-efficient of dichlorophenol indophenol at pH 7.6

2.4.3. Lipopolysaccaride assay

Reagents:

(1) Carbocyanine dye:
(1-ethyl-2-(3-(ethylnaptho-(1,2d)-thiazolin-2-ylidene-2-
methylpropenyl)naptho(1,2d)thiazolium bromide:
Eastman Kodak)   10mg
(2) 1,4, dioxan   10ml
(3) Sodium acetate buffer (0.03M pH 4.05)   100ml
(4) Ascorbic acid (0.1M)   0.2ml

Standards: E.coli serotype 0127 b8 LPS in water ;
1,3,5,7,9,11,and 15µg/0.5ml.

Unknowns: Protein from membrane pellets in water ;
10µg/0.5ml.

Procedure: The carbocyanine dye was dissolved in the
dioxan and an equal volume of sodium acetate buffer, the
total volume was then adjusted to 100ml by the further
addition of the same buffer. To 10ml of this solution
were added the ascorbic acid, 7ml of sodium acetate
buffer and 17ml of water (LPS reagent). The standards (in
duplicate) and the unknowns (in duplicate) were placed in
test tubes and 0.5ml of LPS reagent was added to each
tube which were then incubated at 20°C for ten minutes in
the dark. The samples were measured at 472nm in a SP1800
spectrophotometer and the unknown LPS concentrations
calculated from a standard curve using the E.coli LPS.

2.4.4. Amino acid analysis

Reagents:

(1) Salt free loading buffer pH 2.1:
Citric acid   21g
Methanol   100ml
Thiodiglycol (25% w/w in H_2O)   2.5ml
Brij 35 (10% w/v)   3.5ml

H_2O to 1 Litre

(2) 2-mercaptoethane sulfonic acid   (3N)

(3) Hydrochloric acid   (6M)

(4) Nor-leucine   (0.1mg/ml)

(5) Lysozyme   (1mg/ml)

Samples: Campylobacter flagellin (50μg)

Procedure: The campylobacter flagella samples were placed
in acid washed glass ampoules together with 19.7μl of the
Nor-leucine solution. Other ampoules contained 50μl of
the lysozyme solution and 19.7μl of Nor-leucine. All
samples were freeze dried for 18h in a Piran 3 freeze
drier (Edwards,BOC Ltd Crawley). For HCl hydrolysis 100μl
of 6M HCl was added to the appropriate tubes whilst 50μl
of mercaptoethane sulphonic acid was added to the other
tubes. All tubes were heat sealed under vacuum and heated
at 109°C for 18h in the case of HCl hydrolysates or 24h in
the case of mercaptoethane sulphonic acid hydrolysates.
For HCl hydrolysates only, the tubes were broken open and
the samples evaporated to dryness in vacuo over KOH. A
drop of water was added to each sample which was then
re-evaporated and this procedure repeated untill the
hydrolysate had a neutral pH. For mercaptoethane
sulphonic acid hydrolysates the tubes were opened and 10N
NaOH added to neutralise the acid. In all cases the final
volume was adjusted to 150μl by the addition of salt free
buffer. The amino acid analysis was kindly carried out by
Ms T. Smythe (Southampton University chemical pathology
department) using a Chromaspek amino acid analyser (Rank
Hilger). The areas of the amino acid peaks were
calculated and this raw data used together with the
development of a computer program (appendix 1) to
determine the number of residues of each amino acid
present in the proteins.

2.5. SDS-PAGE

   A 10-25% linear gradient SDS-PAGE system was used to
examine the protein profiles of prepared material. The

SDS-PAGE system used has been described by Lambden et al (1979) and used the discontinuous buffer system of Laemmli (1970).

Reagents:

(1) Acrylamide solution, 50%(w/v) acrylamide ; 1.33%(w/v) bis-acrylamide, the solution being filtered after dissolving.
(2) Sodium dodecyl sulphate, (2%w/v).
(3) Separating gel buffer, (14.5%(w/v) Tris, pH 8.8 with HCl).
(4) Stacking gel buffer, (3.03%(w/v) Tris pH 6.8 with HCl) ; 0.08%(v/v) TEMED.
(5) Running buffer, 0.303%(w/v) Tris ; 1.44%(w/v) glycine ; 0.1%(w/v) SDS, pH 8.3.
(6) Ammonium persulphate solution, 10mg/ml.
(7) Dissociating buffer, 3.03%(w/v) Tris ; 4%(w/v) SDS ; 20%(v/v) glycerol ; 10%(v/v) 2-mercaptoethanol ; 0.002%(w/v) bromophenol blue, pH 6.8 with HCL and stored in the dark.
(8) Glycerol, 50%(v/v).
(9) NNN'N'-tetramethylethylenediamine (TEMED).

Procedure: A mould was formed from glass PAGE plates, spacers (1.5mm or 0.75mm thickness) and clips using vaseline to seal the spacers and joints. The following two solutions were then made;

(1) 25% Acrylamide solution:

Acrylamide solution	10ml
SDS solution	1ml
Separating gel buffer	5ml
50% glycerol solution	4ml
TEMED	10μl

(2) 10% acrylamide solution:

Acrylamide solution	5ml
SDS solution	1ml
Separating gel buffer	5ml
TEMED	10μl
H_2O	9.6ml

Both solutions were degassed on a vacuum line before use.

The mould was placed into the water bath and held level with clamps. To the 25% and 10% acrylamide solutions were added 0.2ml and 0.4ml of the ammonium persulphate solution respectively. A 10%-25% linear gradient gel was cast using a triple channal peristaltic pump (Pharmacia P3) to give progressive dilution of the 25% solution by the 10% acrylamide solution. The separating gel was over layered with iso-butanol and allowed to set. The following solution was made;

Stacking gel:

Acrylamide solution	0.6ml
SDS solution	0.5ml
Stacking gel buffer	5.0ml
H_2O	3.0ml

If thin spacers were used the acrylamide solution was increased to 0.8ml. The solution was degassed before use. The free liquid was removed from the separating gel which was rinsed with stacking gel buffer and dried. The

stacking gel solution was activated by the addition of 1ml of the ammonium persulphate solution and was then poured in over the separating gel, a comb was inserted and the gel allowed to set. the comb was removed and the wells rinsed with running buffer. The gel was placed into an electrophoresis tank and the lower reservoir filled with running buffer. The wells were loaded with derivatised samples (see 2.5.1) and the upper reservior also filled with running buffer. Electrophoresis was carried out for 20h in the case of protein separations and 5h in the case of carbohydrate (LPS) separations.

2.5.1. Derivatisation of samples

Samples containing 5 to 20µg of protein were re-suspended in dissociating buffer and heated at 100^0C for 5min or 37^0C for 2hr.

2.5.2. Staining of gels

2.5.2.1. Protein staining

Reagents:

(1) Stain:
    Glacial acetic acid   50ml
    Isopropanol   100ml
    PAGE blue 83   0.25g
    H_2O 350ml

The stain was filtered before use.

(2) Destain:
    Glacial acetic acid   50ml
    Isopropanol   50ml
    H_2O 400ml

(3) Storage solution:
    Glacial acetic acid   100ml
    H_2O 900ml

Procedure: The gel was placed into the stain for 18hr (thick gels) or 4hr (thin gels) and then transferred into the destain for a corresponding time. Once sufficiently destained the gels were maintained in storage solution.

2.5.2.2. Carbohydrate (LPS) staining

Reagents:

(1) Schiff's reagent:

(A) Pararosaniline hydrochloride solution colour index 42500 (Sigma)

(B) Sodium metabisulphite solution:

Sodium metabisulphite	0.4g
Glacial acetic acid	10ml
H_2O	190ml

(2) Fixing solution

Ethanol	40ml
Glacial acetic acid	5ml
H_2O	55ml

(3) Oxidising solution:

Periodic acid	1.4g
Glacial acetic acid	10ml
H_2O	190ml

(4) Destain:

Methanol	50ml
Glacial acetic acid	75ml
H_2O	875ml

Procedure: The gel was placed in fixing solution for 18hr and then transferred to the oxidising solution for 2hr. Staining was achieved by placing the gel into the metabisulphite solution for 2-3hr with one change of

solution after 30min followed by immersion in the paraosaniline solution for 18hr at 20^0C. Finally the gel was destained until the background was clear.

2.5.3. Molecular weight markers.

The proteins used as molecular weight markers are shown in table 2.2. Once the gels were destained a standard curve was plotted of the logarithm of molecular weight against distance migrated by the standard protein (Mr value, Weber and Osborn 1969). The molecular weights of unknown proteins were determined from their Mr values by extrapolation from the standard curve.

2.5.4. Gel Drying.

Thin gels were shrunk in 250ml of 50% (v/v) methanol for 3-4hr at 20^0C after which time they were transferred onto Whatman No.1 chromatography paper (Whatman, Maidstone, Kent). The gel was placed onto a gel drying board (Ravin Scientific, Haverhill, Suffolk) and covered with cling film, the board was then placed inside a plastic bag which was then sealed. The gel was placed down onto a photographic hot-plate at 60^0C and dried under vacuum for 3-4hr.

Thick gels were shrunk in 10% (v/v) glycerol, 50% (v/v) methanol in H_2O for 4-5hr and then mounted on a drying board in the same arrangement as for thin gels. The thick gels were dryed under vacuum at 20^0C for 24-48hr.

2.5.5. Autoradiography.

The dried gels were mounted on stiff card which was then encased in aluminium foil. Autoradiographic film (Kodak XAR-5 X-Ray sensitive film, Kodak, Hemel Hempstead, Herts) was then exposed to the gel in a cassette fitted with a Kodak X-Omatic intensifying screen. The cassette was placed at -70^0C for 16-48hr before the film was processed in a Kodak RP X-Omat

Table 2.2. SDS-PAGE Molecular Weight Markers

PROTEIN	Molecular Weight
B-GALACTOSIDASE	130K
BOVINE SERUM ALBUMIN	68K
OVALBUMIN	43K
TRYPSIN	23K
LYSOZYME	14K

(1) and (3) were used as markers for LPS gels
(2)-(5) were used as markers for protein gels

automatic developer (X-Ray department, Southampton General Hospital).

2.6. Radioiodination procedures.

2.6.1. Surface labelling of bacterial cells.

2.6.1.1. Lactoperoxidase procedure.

Reagents:

(1) Lactoperoxidase, 1mg/ml
(2) ^{125}I-sodium iodide, 1mCi/ml (Amersham international)
(3) H_2O_2 , 0.01M
(4) Cysteine, 5mM
(5) Bacterial suspension: $2x10^9$ cfu/ml, (equivalent to an $OD_{540}=10.0$) in PBS+B

Procedure: The bacterial cells were harvested from FBP agar and washed twice in PBS+B by centrifugation at 5,000g for 15min at 4^0C. The bacterial cell pellet was resuspended in PBS+B to the required optical density and 0.5ml of this suspension transferred to a 15ml polycarbonate centrifuge tube containing 5µl of the lactoperoxidase solution and 50µl of the ^{125}I-sodium iodide. The reaction was started by the addition of 50µl of H_2O_2 and maintained by further additions at 2.5min intervals. After 10min the reaction was stopped by the addition of 10ml of the cysteine solution and the bacterial cells recovered by centrifugation at 5,000g for 15min. After three washes in PBS the bacterial cell pellet was resuspended in distilled water. The incorporation of ^{125}I was determined by counting a sample in a Wilj 2001 Gamma counter (Wilj international, Ashford Kent) while a protein estimation was determined as described in 2.4.1.

2.6.1.2. Iodogen procedure

Reagents:

(1) Iodogen (1,3,4,6,Tetrachloro-3a,6a,diphenylclycouril; Pierce chemicals Ltd, Rockford,Ill., USA) dissolved in chloroform, 1mg/ml

(2) Potassium iodide, 0.1mM

(3) ^{125}I-sodium iodide in NaOH, 100mCi/ml (Amersham international, Ashford, Kent)

(4) Bacterial suspension: 7.5×10^9 cfu/ml in PBS

Procedure: The bottom of a glass test tube (76mmx9mm) was coated with 10μl of iodogen which was dried onto the tube under a stream of nitrogen. To this tube were added 40μl of the bacterial suspension, 5μl of the potassium iodide solution and 5μl of the ^{125}I-sodium iodide solution. This mixture was incubated at 20^0C for 10min with occasional shaking. The reaction was stopped by the addition of 1ml of PBS and transfer of the liquid phase to a microcentrifuge tube. Recovery of the bacteria was achieved by centrifugation at 12,000g for 90sec and the pellet was washed five times in PBS. The final pellet was resuspended and solubilised in RIPA buffer as described in 2.7.2.

2.6.2. Iodination of Protein A

Reagents:

(1) Protein A, 1mg/ml in PBS

(2) ^{125}I-sodium iodide, 100mCi/ml (Amersham International)

(3) Iodo-beads (Pierce Chemicals ltd, Rockford, Ill, USA)

Equipment:
Sephadex G25 (Pharmacia) column 8cmx1cm.

Procedure: To a glass test tube were added 100μl of the Protein A solution, 10μl ^{125}I-sodium iodide and 1 iodo-bead. The reagents were reacted on a shaker at 20^0C for 5min after which time they were removed from the test tube and applied to the top of the G25 column. The iodonated Protein A and free ^{125}I were eluted in that order

from the column with PBS and 0.5ml fractions collected.

2.6.3. Iodonation of purified flagella

Reagents:

(1) Methyl 3,5,di-^{125}I-iodohydroxybenzimidate in methanol, 1mCi/ml (Amersham international)
(2) flagella (0.363mg/ml) in Tris-HCl buffer (0.3M pH 8.5)

Procedure: The methyl 3,5, di-^{125}I-iodohydroxybenzimidate was added to a glass vial (0.5ml) and the methanol removed under a stream of nitrogen. To this vial were added 330µl of the flagella suspension and the reactants rotated on a Taab mixer at 37^0 C for 48h. The radio-labelled flagella were dialysed against 0.9%(w/v) NaCl for 3h at 4^0C and then transferred to a collodian bag (Sartorius instruments Ltd, Surrey) and concentrated to 0.5ml. The flagella were resuspended in RIPA buffer to the final concentration required (2.7.2.)

2.7. Immunochemical procedures

2.7.1. Antisera

2.7.1.1. Whole bacteria antisera.

Rabbits (New Zealand Whites) were immunised intramuscularly with formalin treated (0.3% formalin) bacterial suspension (OD_{540} =10) diluted 1:1 in Freunds complete adjuvant and boosted three times at 14-day intervals with a series of subcutaneous injections of the same antigen in Freunds incomplete adjuvant.

2.7.1.2. Flagella antisera

Anti-flagella antibodies were raised using the same protocol as used for the preparation of anti-bacterial cell antibodies with the exception that 20µg of purified

flagella was used as the antigen at each immunisation.

2.7.1.3. Outer membrane antisera

This antisera was prepared using the above protocol but substituting 20µg sarkosyl prepared outer-membranes as the antigen.

2.7.2. Radioimmunoprecipitation assay (RIPA).

Principle: Radiolabelled antigen was reacted with the test sera and antigen-antibody complexes precipitated out of solution by the addition of protein A-Sepharose 4B beads. Antigens were detected by autoradiographs of SDS-PAGE gels.

Reagents:

(1) Radiolabelled antigens: ^{125}I-labelled flagella (approximately 2,000cps) or ^{125}I-surface labelled bacteria solubilised in 0.1%(w/v) SDS, 1%(v/v) Empigen BB (Albright and Wilson Ltd, Whitehaven) in PBS at approximately 40,000cps.

(2) Antisera: Human sera or rabbit hyper-immune sera at 1:2 dilution in PBS.

(3) Protein A-Sepharose 4B beads (5mg) swollen at 4^0C in 100µl of PBS containing 1mg of BSA.

Procedure: The ^{125}I-labelled material was centrifuged at 10,000g for 1.5hr to remove insoluble material. From the supernatant 80µl was taken and mixed with 40µl of sera at 4^0C on a Taab mixer (Taab Laboratories Ltd) for 20min. At the end of this time 40µl of swollen Protein A-sepharose beads were added and the mixture re-incubated with mixing for 1hr at 20^0C. The protein A bound immune complexes were sedimented in a bench centrifuge at 12,000g for 30sec and washed five times in PBS at 4^0C before being resuspended in SDS-PAGE dissociating buffer in

preparation for analysis by SDS-PAGE.

2.7.3. Electroimmunoblotting

Principle: Polypeptides are electroblotted onto nitrocellulose paper prior to being reacted with sera (Towbin et al 1979). Any bound antibodies were detected with (a) ^{125}I-labelled protein A and the nitrocellulose paper used in autoradiography or (b) Peroxidase conjugated goat anti-rabbit IgG detected with O-dianisidine.

Reagents:

(1) Blotting buffer: Tris (20mM), Glycine (150mM) and Methanol (20%v/v).

(2) Blocking buffer: BSA (3%w/v), Tris (10mM), NaCl (0.9%w/v), pH 7.4.

(3) Incubating buffer: NaCl (150mM), EDTA (5mM), Tris-HCl (50mM pH 7.4), Gelatin (0.25%w/v) and NP40 (0.05%v/v).

(4) Wash buffer: NaCl (1M), EDTA (50mM), Tris-HCL (500mM pH 7.4), Gelatin (0.25%w/v) and Sodium lauroyl sarcosine (0.4%w/v).

(5) Peroxidase conjugated goat anti-rabbit IgG (Miles Re search Laboratories), diluted 1:1000 in incubating buffer.

(6) ^{125}I labelled protein A (See 2.6.2) diluted in incubating buffer to give approximately 1×10^6 cpm/ml.

(7) O-dianisidine substrate: 2.5mg O-dianisidine dissolved in 1ml methanol (acidified with 1 drop of 1M HCl) at 37^0C. Final volume adjusted to 100ml by the addition of Tris-HCL buffer (10mM pH 7.4) and 10µl of H_2O_2 added (30%w/v). Used immediatly.

(8) Human antisera diluted 1:10 in incubating buffer.

(9) Rabbit antisera diluted 1:100 in incubating buffer.

Procedure: The polyacrylamide gel was placed in contact with a nitrocellulose sheet (BA85, 0.45µm pore size: Schleicher and Schull, Dassel, W.Germany) and the two

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clamped together with three sheets of Whatman No1 chromatography paper and two non-absorbent foam pads on either side. This arrangement was placed into a blotting tank which was then filled with blotting buffer. The polypeptides were transferred at 60V for 5h or 50V for 18h at 4^0 C. After transfer un-occupied protein binding sites on the nitrocellulose sheet were blocked by incubating in blocking buffer for 2h at 37^0C. After a brief wash in PBS the blots were incubated in either rabbit or human antisera for 2h at 37^0C followed by a brief wash in PBS. If rabbit antisera had been used the nitrocellulose sheet was next incubated in the peroxidase conjugate for 2h at 37^0C while those blots using human antisera were incubated in the protein A solution for the same period. Unbound proteins were removed by extensive washing in wash buffer (at least five changes over 1h). Protein A blots were dried and autoradiographed (2.5.5.) while the peroxidase blots were incubated with O-dianisidine substrate at 37^0C for 1h and the resulting reaction stopped by extensive washing in distilled water.

2.8. Electron microscopy.

Bacteria and bacterial cell components were negatively stained with 2%(w/v) phosphotungstic acid (pH 7.0) for 2min after being absorbed onto carbon- formvar grids. Electron-micrographs were taken on a Philips 300 transmission electron microscope (Philips, Eindhoven, Netherlands).

CHAPTER 3

EXPERIMENTAL AND RESULTS

3.1. The preparation of outer membranes from C.jejuni/coli.

3.1.1. Spheroplast derived membrane preparations.

A description of the various techniques that have been developed in order to separate the inner and outer membranes of gram-negative bacteria has been presented in 1.8. From these possibilities initial attempts to separate the two membrane components were based on the lysis of osmotically fragial cells produced by the formation of spheroplasts, followed by the separation of the inner and outer membranes on a sucrose gradient. This procedure has the advantage of being a relatively gentle means of separating the membranes and avoids the use of denaturing detergents which may inactivate enzyme systems and cause the loss of proteins from the membrane.

3.1.1.1. Membrane isolation procedure.

The production of campylobacter spheroplasts and the subsequent isolation of membrane components was based on the modification of the techniques of Miura and Mizushima (1968) and Osborn and Munson (1974). Bacteria were harvested directly from FBP agar into cold 0.75M sucrose, 10mM Tris-HCl (pH 7.8) to give a final A_{540} of 10. Lysozyme (10mg/ml) was added to a final concentration of 200µg/ml followed by the slow addition of EDTA (4.5mM pH 7.5) to give a final concentration of 3mM. The EDTA was added by peristaltic pump over a period of 15min, the cell suspension being gently stirred on ice during this operation. The suspension continued to be stirred at room temperature for a total of 1.5h during which time

spheroplast formation was monitored by darkground microscopy. At the end of this time more than 80% of the bacterial cells had formed spheroplasts. spheroplast lysis and membrane separation was initiated by the addition of three volumes of cold distilled water containing 0.2%(w/v) Brij 58. To this solution were added the following agents to the stated final concentration: 3mM magnesium chloride, 14 units/ml DNAase 1 and 15units/ml RNAase. After incubation for 20min at 37^0C unlysed cells were removed by centrifugation (5,000g, 30min) and the membrane fraction was recovered from the supernatant solution (100,000g 2h) and finally resuspended in 1.2ml of 20% w/v sucrose, 5mM EDTA. A portion of the membrane fraction (0.5ml) was layered onto a 25% w/w to 55% w/w discontinuous sucrose gradient of the following composition: 1.5ml 25%, 2ml 30%, 2ml 35%, 1.5ml 40%, 1.5ml 45%, 2ml 50% and 1ml 55%, all sucrose solutions contained 5mM EDTA.

Centrifugation was carried out at 90,000g for 15h at 4^0 C in a 6x14ml swing-out rotor. Fractions (0.5ml) were collected by upward displacement of the gradient by slowly pumping in 65% w/w sucrose at the base of the gradient using a gradient fraction collector (MSE). The sucrose concentrations of the individual fractions were calculated from refractive index measurements using a refractometer (modell 1, Carl Zeiss, Jena), from which buoyant densities were determined (Handbook of Chemistry and physics. 53rd Edition, CRC Press, Cleveland, Ohio, USA.). The distribution of membrane components within the gradient was determined by the presence of protein as indicated by absorbance at 280nm and from SDH analysis (Fig 3.1.).

3.1.1.2. Analysis of separated membranes

Three main bands were observed within the gradient an upper, diffuse, amber-coloured band and two closely-spaced, lower, opaque white bands corresponding to the distribution shown in Fig 3.1. The upper band had a buoyant density of 1.148g/ml, the lower bands having
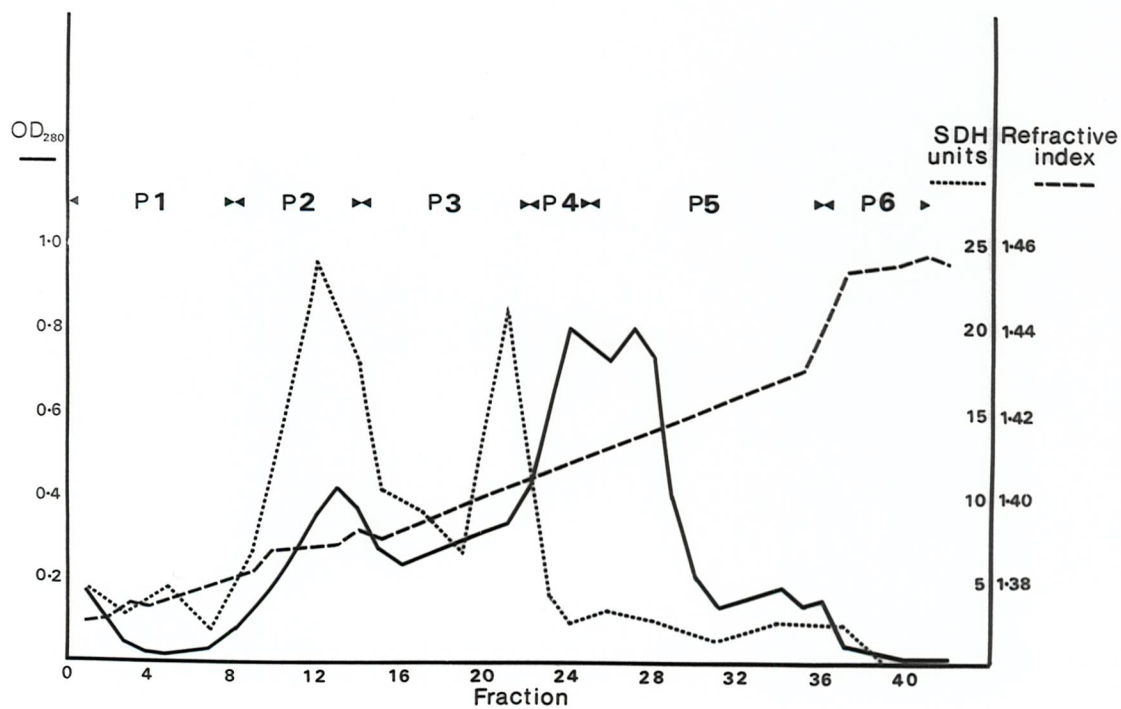
Figure 3.1. Seperation on a sucrose gradient of membrane material prepared by spheroplast formation of C.Jejuni 81116.

densities of 1.19 and 1.22g/ml respectively. The majority
of the SDH activity was associated with the upper band
(p=1.148g/ml), with a secondary peak further down the
gradient (p=1.182g/ml). The initial fractions were pooled
as indicated in Fig 3.1 to give fractions P1-P6 and
membranes recovered by centrifugation (100,000g 2h) and
subjected to further analysis, the results of which are
shown in table 3.1. It had been noted that no visible
pellets were obtained upon centrifugation of fractions P1
and P6 and further analysis indicated no detectable
protein within these two fractions, thus further analysis
were not performed. Pellet one (fraction P2) was a deep
amber colour and contained 63% of the total SDH recovered
and only 7% of the total LPS. There was a general
decrease in the total amounts of SDH and a general
increase in the total amounts of LPS present in pellets
with increasing bouyant density such that pellet four
(fraction P5) contained 7% of the total SDH and 46% of
the total LPS. An anomaly was noted in the total amounts
of SDH placed on, and recovered from the gradient, with
an apparent three-fold increase in the amounts recovered.
This phenomena had been noted before and is thought to be
due to greater accessibility of the enzyme for the
substrate upon exposure of the membranes to Brij 58 and
their separation within the gradient (Schnaitman 1970 ;
Rutberg and Hoch 1973). It was also noted that although
there was an apparent 33-fold increase in the specific
SDH activity between pellets one and four there was only
a corresponding 1.7-fold difference in LPS. This was
probably due to the non-specificity of the LPS assay
compounded by the presence of other, interferring,
carbohydrates.

The protein profiles of the recovered pellets are
shown in Fig 3.2 (Note: molecular weights on figures
represent M.wt. x 1,000). It can be seen that pellets one
and two contained considerably more protein bands than
pellets three and four. Pellet four contained several
bands which appeared specifically associated with this
fraction, the molecular weights of which were 89.1K,
84.1K, 82.2K, 63K, 55.5K, 53.1K, 43.4K, 42.2K, 35.5K and

Table 3.1. Analysis of Spheroplast Prepared Membrane

Material Separated on a Sucrose Gradient

Fraction	Total Protein (µg)	Total SDH (units $\times 10^2$)	Specific SDH (units/mg protein)	Total LPS (mg)	Specific LPS (mg/mg protein)
P2	222	8.37	3.77	0.071	0.32
P3	468	3.56	0.76	0.206	0.44
P4	368	0.48	0.13	0.243	0.66
P5	839	0.10	0.12	0.445	0.53
TIP	1897	12.51	-	0.97	-
TPG	3600	5.04	0.14	1.73	0.48

TIP= Total recovered in pellets

TPG= Total in unseparated membranes placed on gradient

28.8K. In addition a band of 14.3K was present which probably represented lysozyme from the preparative procedure. The differences in the SDH distribution and the protein profiles of pellets 1 and 4 indicated that pellet 1 was largely cytoplasmic membrane enriched material while pellet 4 represented outer membrane enriched material. Pellets 2 and 3 were intermediate membrane fractions with the former more closely resembling cytoplasmic membrane and the latter being similar to the outer membrane. The outer membrane (pellet 4) contained at least ten protein bands of which two are predominant (63K and 43.4K).

3.1.2. Sodium lauryl sarcosine extraction of membranes.

Because of the difficulty experienced in producing consistently good spheroplasts and the possibility of cross contamination of the outer membrane material with cytoplasmic membrane an alternative method of outer membrane preparation was sought based on the procedure of Filip et al (1973). The procedure involves the selective solubilisation of the inner membrane of gram-negative bacteria by the ionic detergent sodium lauryl sarcosinate (sarkosyl), and the subsequent recovery of the outer membrane by centrifugation. The initial preparation of membranes was achieved by the sonication of freshly harvested bacteria.

3.1.2.1. Membrane isolation procedure.

Bacteria were harvested from FBP agar directly into PBS+B at 4°C and the suspension was centrifuged at 5,000g for 5min before being resuspended in 0.05M Tris-HCl buffer pH 7.5. The bacteria were washed once before being resuspended to an OD_{540} =1.0 in the same buffer, the suspension was then sonicated on ice (4x30 sec periods with 30sec cooling intervals) at 10μamps (Soniprep 150, MSE Ltd). Cellular debris was removed by centrifugation at 5,000g for 20min and the total membranes recovered by centrifugation at 100,000g for 2h (Prepspin 50, MSE Ltd).
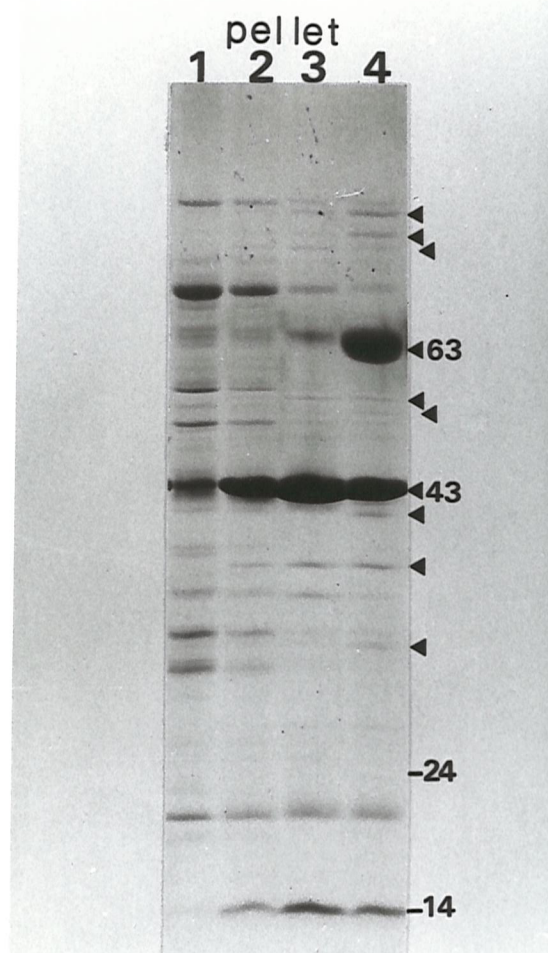
Figure 3.2. Protein profiles of spheroplast prepared
membrane material.

(1) ▶ Indicates bands specifically associated with outer
membrane enriched fraction.

(2) Pellets 1-4: spheroplast fractions P2-P5
respectively.

The pellet was resuspended in 1%w/v Sarkosyl in 7mM EDTA, pH 7.6 at $37^{o}C$ for 20min at a protein : detergent ratio of approximately 1:4 (w/w). The suspension was centrifuged at 100,000g for 2h and the pellet was re-extracted with detergent as above. The extracted membranes were washed three times in 0.05M Tris- HCl buffer, pH 7.5 before being resuspended in distilled H_2O and stored at $-20^{o}C$.

3.1.2.2. Comparision of spheroplast derived and sarkosyl extracted membranes

Comparison of the insoluble and soluble fractions with the pellets obtained from spheroplast prepared membranes is shown in Fig 3.3. The sarkosyl solubilised material had a complex protein profile whilst that of the sarkosyl insoluble material was relatively simple. The soluble material shared a number of bands with those observed in spheroplast pellet 1 however there were also considerable differences indicating that the two materials, while probably representing cytoplasmic membrane material, were not identical. A specific difference being the increased amount of the 43.4K band within the sarkosyl solubilised material. In contrast the sarkosyl insoluble material closely resembled spheroplast pellet 4, however of the ten main bands detected within spheroplast pellet 4, four (84.1K, 82.2K, 42.2K and 28.8K) were absent from the sarkosyl insoluble material. Proteins of these molecular weights were observed within the sarkosyl soluble material however. Approximately fourteen protein bands were present in the sarkosyl insoluble material, most if not all of which corresponded to bands seen in spheroplast pellet 4. Some of these protein bands had been considered to be cytoplasmic in origin from the spheroplast data, however it seems likely that these bands may be associated with the outer membrane and share similar molecular weights with inner membrane proteins.

Due to the clear similarities between sarkosyl extracted outer membrane material and the spheroplast prepared outer membrane enriched fraction, the relative
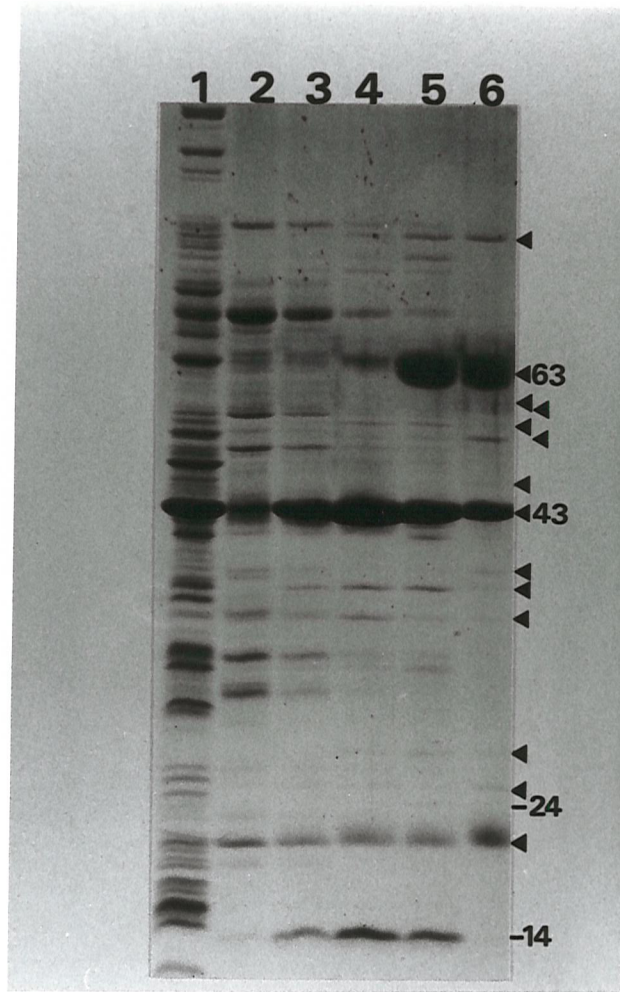
Figure 3.3. Comparison of the protein profiles of spheroplast prepared and detergent extracted membrane material.

(1) ▶ Indicates bands specifically associated with the outer membrane fraction of sarkosyl prepared material.

(2) Track 1: sarkosyl soluble material; Tracks 2-5: spheroplast pellets 1-4 respectively; Track 6: sarkosyl insoluble material.

ease and reproducability of the sarkosyl procedure
recommended the latter as the technique of choice for
further outer membrane preparations.

3.2. Comparison of the outer membranes of clinical isolates of C.jejuni and C.coli.

3.2.1. Outer membrane proteins

The whole cell protein and outer membrane profiles of
strain 81116 are shown in Fig.3.4a and are compared to
the whole cell protein profiles of six isolates of
campylobacter (4 jejuni and 2 coli) in Fig 3.4b. These
are typical of the profiles of the many C.jejuni and
C.coli organisms which were examined. Comparison of the
total protein profile and purified outer membrane (OM)
from 81116 revealed that the major 43K band identified in
both spheroplast and sarkosyl prepared OMs appeared
identical to the major band in the total protein profile
(Fig 3.4a). A comparable band of variable molecular
weight in the range 43-46K was present in the total
protein profiles of other clinical isolates (Fig 3.4b).
In contrast the second major band seen in spheroplast and
sarkosyl prepared OMs (63K band Fig 3.4a) was not easily
identifiable in all the total protein profiles.
Comparison of the C.jejuni (Fig 3.4b tracks 1,2,4 and 6)
and C.coli (Fig 3.4b tracks 3,5) proteins showed broad
similarities between the profiles, however a 35K band
present in the C.jejuni strains appeared to be absent in
the C.coli strains, the latter containing a 41K band
apparently absent in the C.jejuni profiles, although
33817 had a band of slightly lower molecular weight (40K
Fig 3.4b track 2). Unfortunately total protein profiles
are too complex to make such comparisons with any degree
of certainty, however examination by SDS-PAGE of the
simplified OM profiles can help resolve this problem.
Hence the sarkosyl prepared OMs of the six clinical
isolates were examined by SDS-PAGE and the results are
shown in Fig 3.5. As expected the profiles were
considerable simplified with approximately ten bands
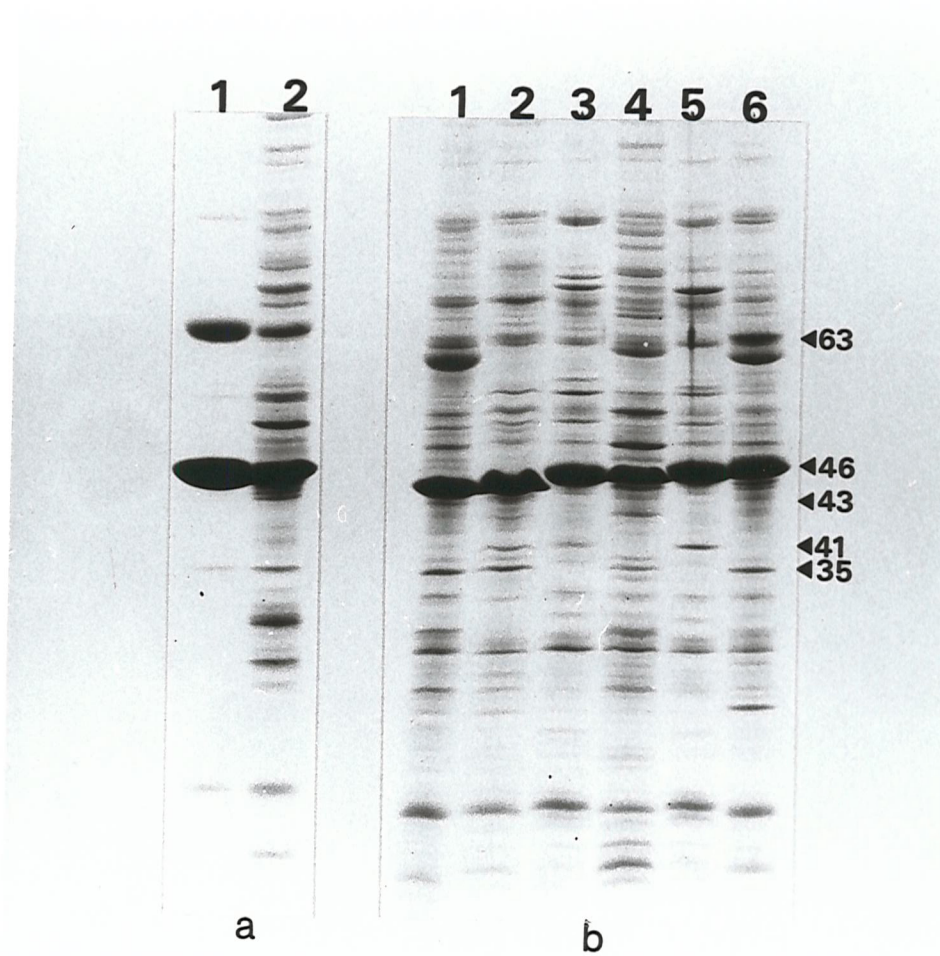
Figure 3.4. The total protein profiles of isolates of
C.jejuni and C.coli.
(1) Figure 3.4a. Track 1: outer membrane preparation of
81116; Track 2: total protein profile of 81116.
(2) Figure 3.4b. Track 1: isolate 63923; Track 2: isolate
33817; Track3: isolate 11353; Track4: isolate 68879;
Track5: isolate 3571; Track6: isolate 51180.
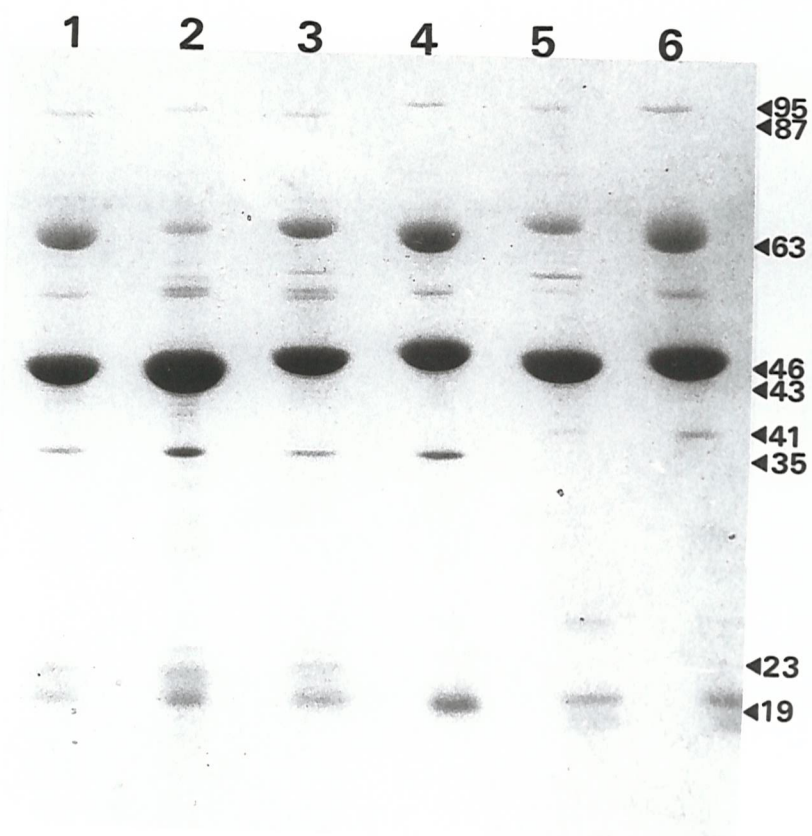
Figure 3.5. The outer membrane profiles of C.jejuni and C.coli isolates.

(1) Track 1: isolate 33817; Track 2: isolate 63923; Track 3: isolate 68879; Track 4: isolate 51180; Track 5: isolate 11353; Track 6: isolate 3571.

being easily identified. The 43-46K band appears to contribute the major proportion of the protein within these OM profiles. A major protein band with an approximate molecular weight of 63K was present as had been previously noted in strain 81116. It was evident that there was considerable variations in the intensity of this band making the comparison of molecular weights difficult. Nevertheless the molecular weights did seem to vary in the range 62-63K. The 35K band that was detected in the total protein profiles (Fig 3.4) was found to be specifically associated with the C.jejuni isolates examined and was apparently replaced in the two C.coli isolates by a 41K band. A minor band of relatively high molecular weight was observed having variable molecular weight between strains in the range 87-95K. A variable number of minor bands were observed in the OMs of the isolates within the molecular weight ranges 54-60K and 19-22K.

3.2.1.1. Heat modification of the outer membrane proteins.

Solubilisation of OM proteins in SDS and 2-mercaptoethanol at different temperatures (2.5.1) can reveal heterogeneity in behaviour of the proteins allowing easier comparisons and identifications. When C.jejuni (Fig 3.6) and C.coli (Fig 3.7) OMs were heated in the presence of SDS-2-mercaptoethanol at 37^0C for 2h or 100^0C for 5min a number of heat modifiable proteins were observed. The major 43-46K band was heat modifiable in both the C.jejuni and C.coli isolates, in both cases the bands having increased apparent molecular weights at the higher temperature. The 35K "C.jejuni" band and the 41K "C.coli" band also showed increased molecular weight at the higher dissociation temperature being 33K and 38K respectively at the lower value. The high molecular weight (87K-95K) bands were no longer detectable at 37^0C in the C.jejuni strains and were apparently replaced by bands in the molecular weight range of 110-117K, however in the case of the C.coli isolates the corresponding
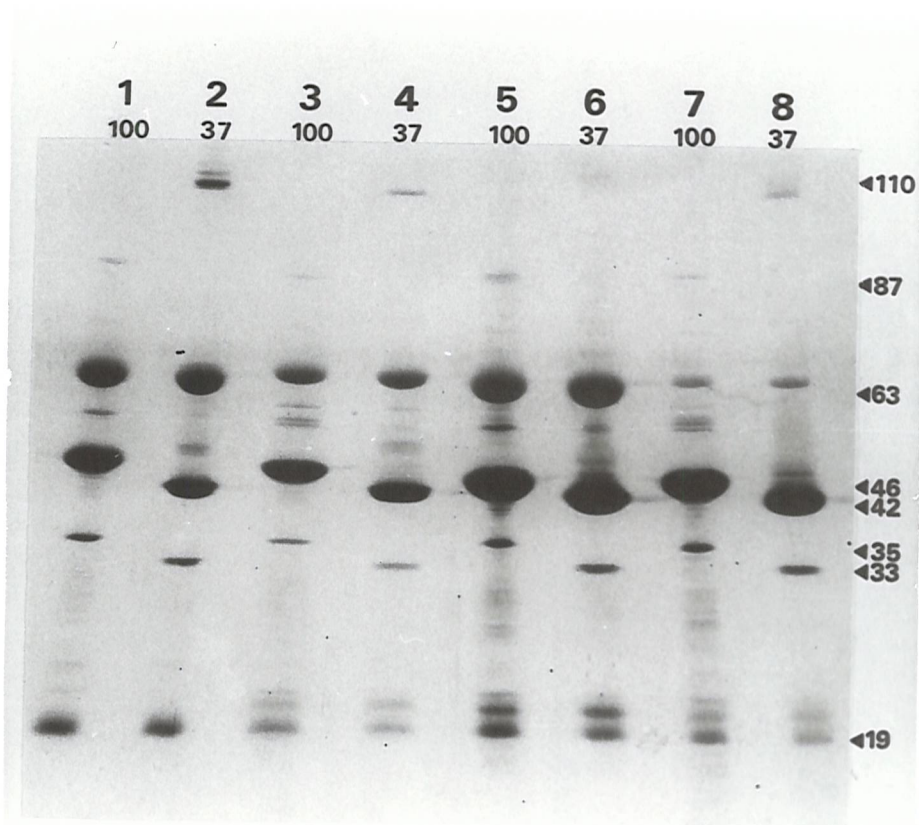
Figure 3.6. Heat modification of the outer membrane proteins of C.jejuni isolates.

(1) Temperatures at which outer membranes were heated in dissociation buffer are given below track numbers. Temperatures of 100°C were held for 5min while 37°C was held for 2h.

(2) Tracks 1&2: isolate 51180; Tracks 3&4: isolate 68879; Tracks 5&6: isolate 33817; Tracks 7&8: isolate 63923.
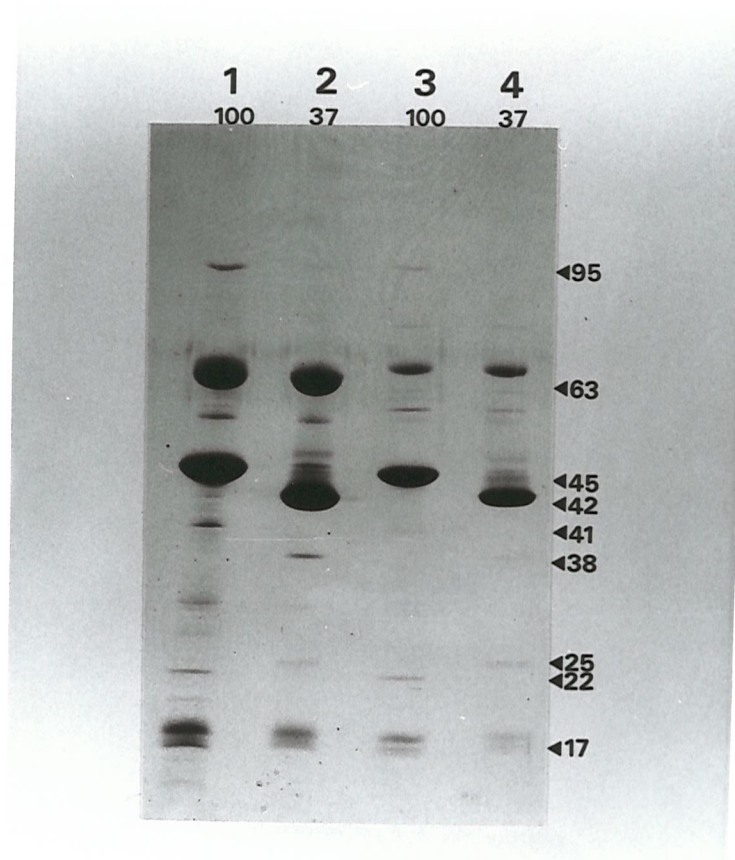
Figure 3.7. Heat modification of the outer membrane proteins of C.coli isolates.

(1) Temperatures at which outer membranes were heated in dissociation buffer are given below track numbers. Temperatures of 100°C were held for 5min while 37°C was held for 2h.

(2) Tracks 1&2: isolate 3571; Tracks 3&4: isolate 11353.

bands were no longer detectable in the gel at the lower temperature. The change in the molecular weights of the minor bands in the 54-60K molecular weight range was difficult to estimate as their new positions within the gel were not easily discernible, however new bands were often seen just above the major 43-46K band after the samples had been heated at 37^0 C for 2h. Three out of the four C.jejuni isolates (68879, 33817, and 63923) had a triplet of minor bands of molecular weights 19K, 21K and 23K the latter of which appeared to be lost from the gel at the lower dissociation temperature. The C.coli strains, while not possessing a triplet of low molecular weight bands, did contain a band of 21.5K that appeared to increase its molecular weight to 24.7K upon heating at 37^0 C. The major 62-63k band did not appear to be heat modifiable.

3.2.1.2. ^{125}I-surface labelling of the OM proteins of whole bacteria.

The radio-iodonation procedure has been described in 2.6.1. and the autoradiographs of the SDS-PAGE separated proteins of C.jejuni isolates (tracks 1-5) and one C.coli isolate (track 6) are shown in Fig 3.8.

The major variable molecular weight (43-46K) OM protein was surface labelled in the C.jejuni isolates as were four minor constant molecular weight proteins (72K, 64K, 35K and 27K). In addition a minor variable protein (70K-75K) was also ^{125}I- labelled. Iodination of surface proteins using the Iodogen technique produced a similar pattern to the lactoperoxidase procedure except an additional protein of 27.5K was also observed (autoradiograph not shown). Surface iodonation of C.coli 3571 (Fig 3.8 track 6) revealed a different pattern to the C.jejuni isolates. The major outer membrane band was surface labelled as were the 75K and 27K proteins, however the 72K, 64K, and 35K proteins were absent. A number of proteins in the C.coli isolate showed weak labelling including the 41K OM protein, the other proteins having molecular weights of 88.1K, 56.2K, 53.7K,
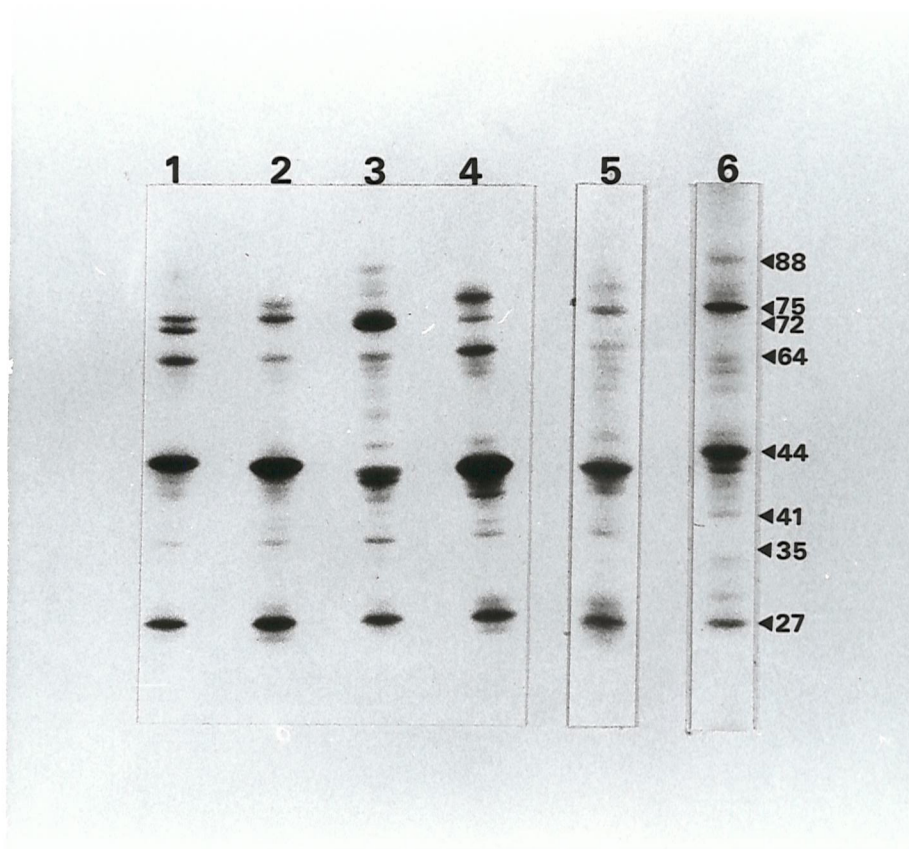
Figure 3.8. Autoradiograph of ^{125}I-surface labelled campylobacter isolates.
(1) Track 1: isolate 51180; Track 2: isolate 68869; Track 3: isolate 63923; Track 4: isolate 33817; Track 5: isolate 81116; Track 6: isolate 3571.

49.5K, 37.7K and 29.8K.

3.2.1.3. Acid extraction of the cell surface proteins of C.jejuni 81116

An acid extraction was prepared from C.jejuni 81116 by the method of McCoy et al (1975a). Bacteria were harvested from CNB by centrifugation at 5,000g for 20min and washed twice in distilled water, the bacteria being recovered at each step by centrifugation at 5,000g for 15min. The bacterial pellet (4g wet weight) was resuspended in 100ml of 0.2M glycine-HCl buffer pH 2.2 and stirred at 20°C for 15min. The cells were removed by centrifugation (10,000g 20min) and the supernatant was neutralised and dialysed against distilled water at 4°C for 18h. Examination of this supernatant by SDS-PAGE revealed that it contained approximately six protein bands (Fig 3.9 track 2). A band of 63K was present and corresponded to the 63K band seen in an OM preparation from the same strain (Fig 3.9 track 1). In contrast the major 43K protein present in OM preparations formed only a minor component of the acid extractable material, while the remaining bands in the acid extract had molecular weights of 38K, 27K, 25K and 17K. The 27K and 25K bands representing the major components of the glycine-HCl extract and were not present in the sarkosyl extracted outer membrane (Fig 3.9 track 1).

3.2.2. Carbohydrate (LPS) in outer membranes of C.jejuni and C.coli.

The presence of glycoproteins and/or LPS in the sarkosyl prepared OMs were detected as described in 2.5.2.2. As shown in Fig 3.10 when the gels were stained by Schiffs reagent at least two bands were revealed in each tract. The upper band stained faintly and had a molecular weight of about 130K however the lower band(s) stained more intensely and had variable molecular weights between isolates (range 27.4K-30.2K). Isolate 51180 contained a double band of 26K and 29K with diffuse
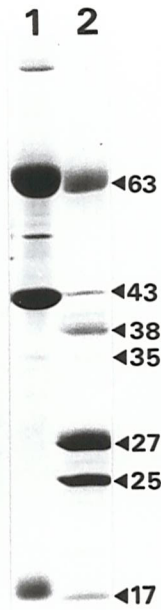
Figure 3.9. Acid extract of the surface of C.jejuni 81116.

(1) Track 1: outer membrane proteins; Track 2: acid extracted proteins.
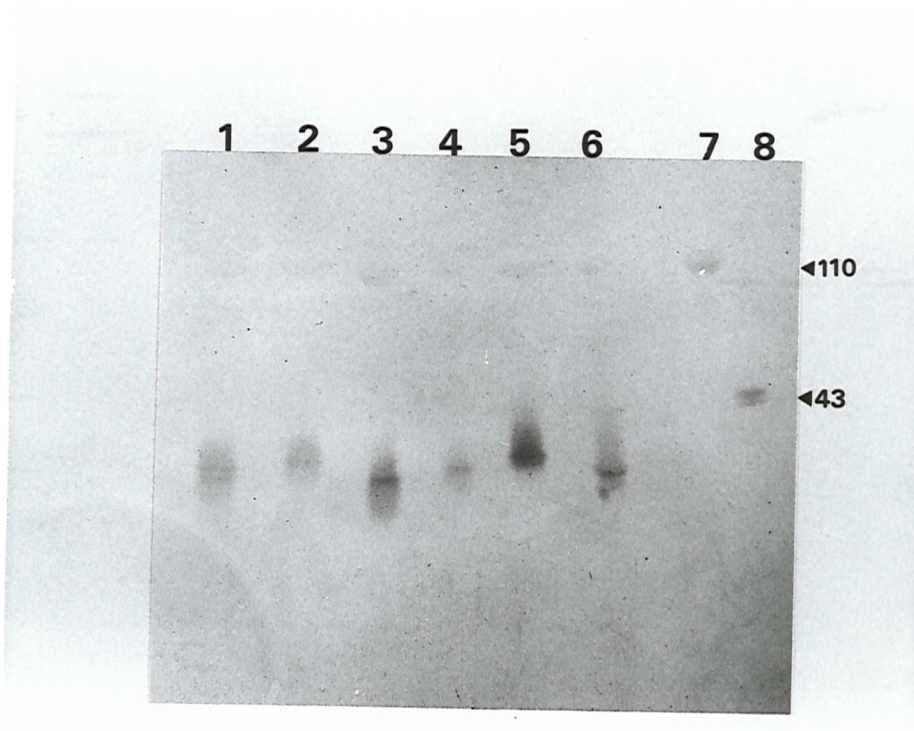
Figure 3.10. Detection of LPS and/or glycoproteins in outer membranes of C.jejuni and C.coli.
(1) Track 1: isolate 51180; Track 2: isolate 68879; Track 3: isolate 33817; Track 4: isolate 63923; Track 5: isolate 3571; Track 6: isolate 11353; Track 7: B-galactosidase; Track 8: ovalbumin.

staining between them.

3.2.3. Preparation of campylobacter flagella.

Examination by electron-microscopy of OM material from spheroplast and sarkosyl preparations revealed the presence of substantial numbers of flagella filaments. Therefore in order to identify the protein band that related to the flagella of campylobacters these locomotive structures were isolated.

Bacteria (5.5g wet weight) were harvested from SCNB by centrifugation (5,000g for 20min) and resuspended in 250ml of PBS+B at 4^0 C. The bacterial suspension was homogenized on ice in a laboratory blender fitted with disintegrating and axial flow heads (Silverson Machines, Chesham, UK) for three periods of 1min with 30sec cooling intervals. Isolation of the flagella was achieved by centrifugation at 5,000g for 30min to remove the cellular debris, followed by centrifugation of the supernatant at 100,000g for 2h. These centrifugation steps were repeated twice and the purity of the flagella was monitored by electron microscopy of negative-stained preparations and by SDS-PAGE. Attempts to further purify the flagella by acid dissociation followed by "seeding" the neutralised solution with flagella fragments produced only aggregated material with no true filaments. As the flagella were to be used for immunisation procedures it was decided to use the "native filaments" purified by differential centrifugation. The analysis by SDS-PAGE of the purified flagella from strain 81116 revealed a major 63K protein band and a minor 87K protein band (Fig 3.11 track 1). Comparison of these protein bands with the proteins of an OM preparation from the same isolate run in parallel (Fig 3.11 track 2) indicated that these flagella associated proteins appeared to be identical with the 63K and 87K proteins previously mentioned.

3.3. Antigenicity of C.jejuni proteins.

Isolation and part characterization of membrane
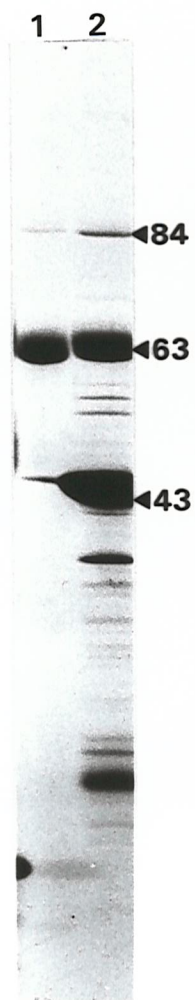
Figure 3.11. Flagella proteins of C.jejuni 81116.
(1) Track 1: purified flagella; Track 2: outer membrane
preparation.

specific proteins of clinical isolates of C.jejuni and C.coli enabled investigation of the antigenicity of these and other cellular proteins during human infections.

3.3.1. RIPA of ^{125}I-surface labelled C.jejuni with clinical sera from an outbreak.

RIPA was used, as described in 2.7.2., to examine the reaction of several human convalescent sera, obtained after an outbreak of campylobacter enteritis at a school (Palmer et al., 1983), to the ^{125}I-surface labelled proteins of a bacterial isolate from the same outbreak (Fig 3.12). Four sera studied precipitated the I-labelled 43K major OM protein band. In addition the homologous and one heterologous sera precipitated 49K and 27K proteins respectively. Eleven control sera investigated precipitated the 43K protein, but to a lesser extent than the convalescent sera. The flagella protein was not detected by any of the sera using this assay system.

3.3.2. Electroimmunoblot of C.jejuni flagella.

The apparent lack of activity of the clinical sera in 3.3.1. for the campylobacter flagella protein was not surprising since they had failed to label during surface labelling experiments using the lactoperoxidase and Iodogen procedures. To investigate the presence of anti-flagellin antibodies within the sera from this outbreak an electroimmunoblot experiment (as described in 2.7.3.) was performed using the sera from patient 81116 against proteins from his homologous isolate, purified flagellin from the homologous isolate and proteins of an aflagellate variant (SF2 see 3.4.) derived from the homologous isolate. The results (Fig 3.13.) showed the presence of antibodies reacting with a protein of 63K present in the original isolate and with purified flagellin but not with the aflagellate variant. In contrast to RIPA the blots failed to detect antibodies directed against the major OM protein, indeed only those

1 2 3 4 5

◄49

◄43

◄27
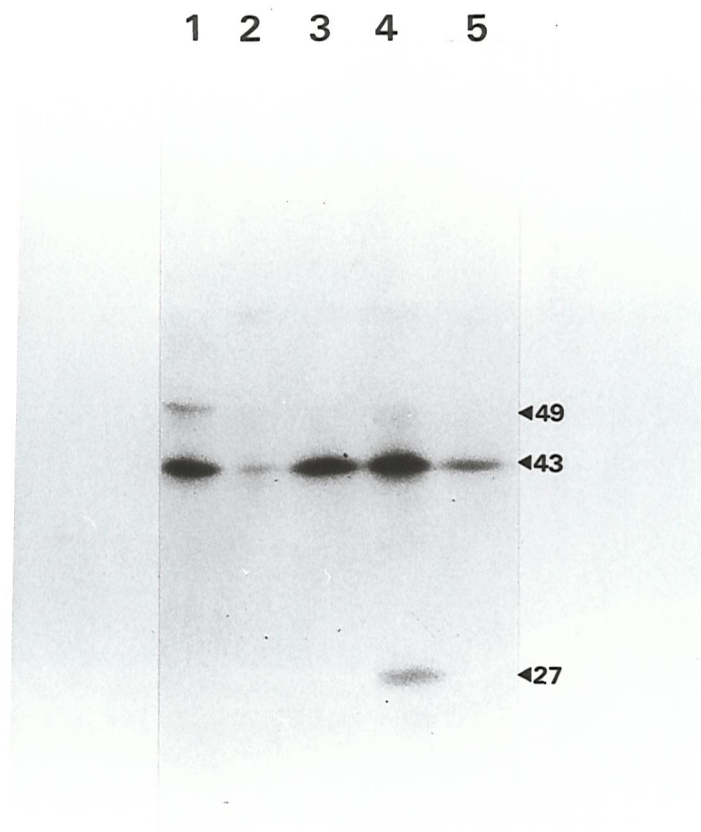
Figure 3.12. RIPA of ^{125}I-surface labelled proteins with human sera.

(1) Track 1: homologous convalescent serum (81116); Track 2: control human sera; Track 3,4,&5: heterologous convalescent human sera.
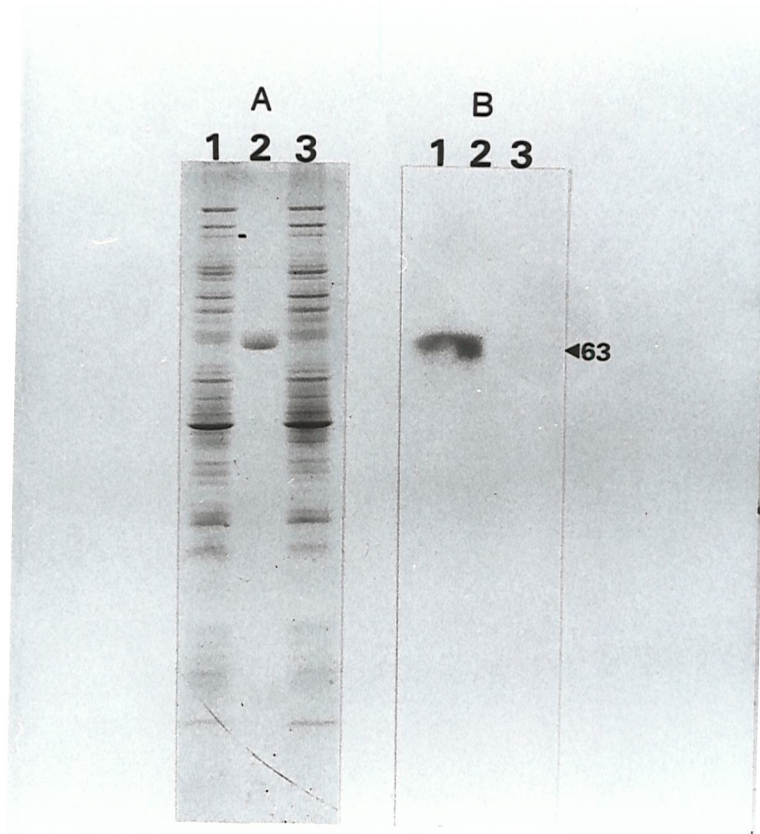
Figure 3.13. Human antibodies directed against campylobacter flagellin.

(1) Figure 3.13A: PAGE of proteins; Figure 3.13B: immunoblot of gel using serum 81116.

(2) Track 1: whole cell proteins of 81116; Track 2: purified flagellin of isolate 81116; Track 3: whole cell proteins of SF2 (aflagellate variant of 81116).

antibodies directed against the flagellin were detected.

3.3.3. RIPA of ^{125}I-labelled C.jejuni flagella.

In order to prepare ^{125}I-flagella labelling with iodohydroxybenzimadate, which reacts with free amino groups, rather than Iodogen,which reacts with tyrosine residues, was chosen (2.6.3). Flagella labelled with this reagent were obtained with a specific activity of 2μCi/mg of protein. They were then used in a RIPA, with human convalescent sera from the outbreak under investigation. The homologous and four heterologous sera precipitated the flagella band (Fig 3.14a) whilst eight control human sera only weakly precipitated this protein (Fig 3.14b). A small amount of the 43K protein was also precipitated indicating slight contamination of this particular flagella preparation with outer membrane material.

3.3.3.1. RIPA of ^{125}I-flagella by unrelated human convalescent sera.

To further investigate the presence of anti-flagella antibodies in human convalescent sera a RIPA was performed using ^{125}I-labelled campylobacter flagella (from isolate 81116) and nine unrelated human convalescent sera for which matched isolates were available. (Fig 3.15). Eight of the nine sera strongly percipitated the labelled flagella, whereas as previously, little or no reaction was detected with nine control sera.

3.3.4. Electroimmunoblots of clinical sera with campylobacter proteins.

Although RIPA,s can be a useful tool with which to study the immune responses to some antigens during infection the failure to detect antibodies to flagella when using the Iodogen labelling procedure revealed major limitations. Thus study of many sera would require the labelling of each individual isolate which would be a costly and time consuming process. Therefore a response
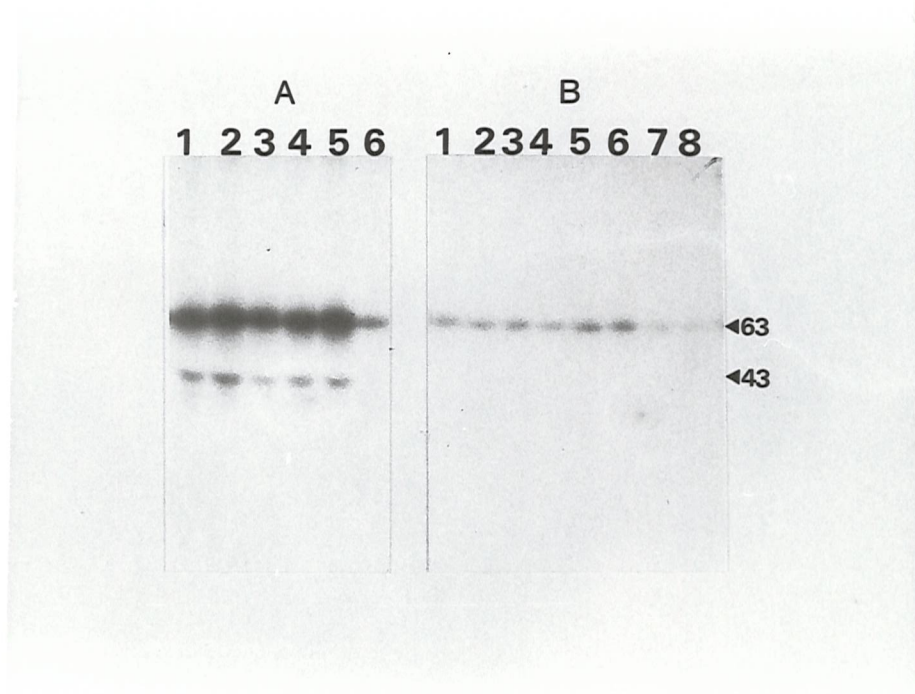
Figure 3.14. RIPA of ^{125}I-labelled flagella with human sera.

(1) Figure 3.14A. Tracks 1-5: human convalescent sera; Track 6: radiolabelled flagella.

(2) Figure 3.14B. human control sera.
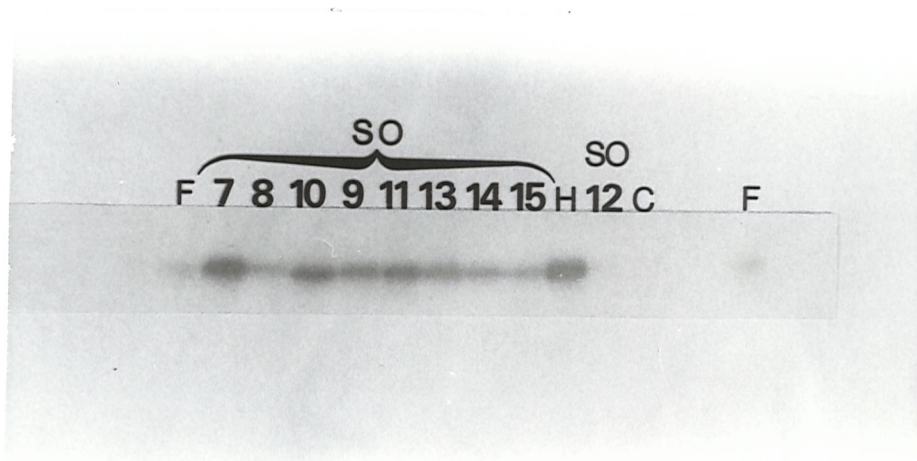
Figure 3.15. RIPA of ^{125}I-labelled campylobacter 81116 flagella by human convalescent sera.

(1) SO7-15 refers to sera from patients giving isolates SO7-15.

(2) H refers to homologous serum.

(3) C refers to human control serum.

(4) F refers to radiolabelled flagellin marker.

to flagellin was determined by immunoblotting each of the nine clinical sera described in 3.3.3.1. against all the matched isolates (Table 2.1).

Analysis of the resulting autoradiographs revealed a number of different patterns of reaction (Figs 3.16-3.20). One patient (SO7) had developed antibodies to a large number of proteins, these antibodies also cross-reacted with the proteins of the heterologous strains (Fig 3.16). The major band present in all but one strain corresponded with the molecular weight region of flagellin (62-63K), however antibodies were also present to a major band of 64K present in two isolates ,SO12 and SO13, the latter being the isolate lacking the 63K band. A second type of reaction pattern was demonstrated by serum from patients SO11 and SO15 (Fig 3.17). Once again the major band was of molecular weight 63K except in the case of strain SO15 in which the 64K band was detected in the abscence of the 63K band. In addition both sera gave the same pattern of reaction with a number of proteins in the 43K molecular weight region of all strains, although these bands did not have the same molecular weights in all isolates and did not correspond to the molecular weights of the major OM proteins.

The sera from patients SO9, SO10, SO13 and SO14 also reacted strongly with the 63K band (or the 64K band from strain SO15) with the exception of serum SO14 which failed to react with the 63K protein of isolate SO13 (Fig 3.18). Unlike SO7, SO11 and SO15 these sera reacted weakly with other proteins. The last two sera, SO8 and SO12, gave a distinctive reaction pattern (Fig 3.19) in which the sera reacted only with the 63K or 64K band from isolates SO8, SO 10, SO12, SO14 and SO15. There was indications of weak reactions with a limited number of other bands with these two sera, in particular serum SO8 reacting weakly with a 92K protein band in its homologous strain. This was the only occassion where a protein band of molecular weight greater than 64K was detected. In some cases there was an indication that the 62-64K region band in the same isolate had a slightly different molecular weight when blotted by different sera. An

Figure 3.16. Immunoblot of isolates SO7-SO15 using serum SO7.
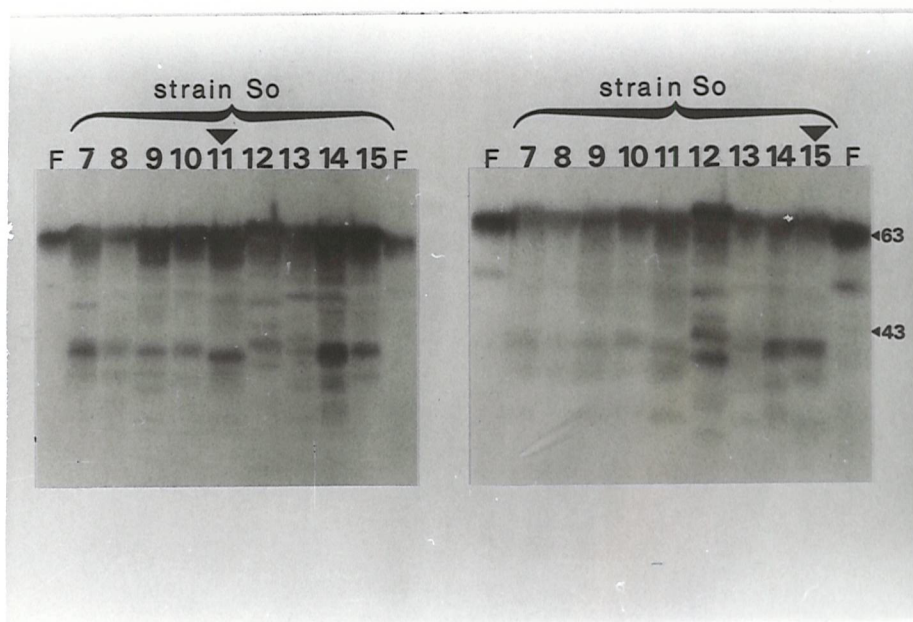
Figure 3.17. Immunoblot of isolates SO7-SO15 using sera
SO11 and SO15.

(1) Large arrow indicates serum used.
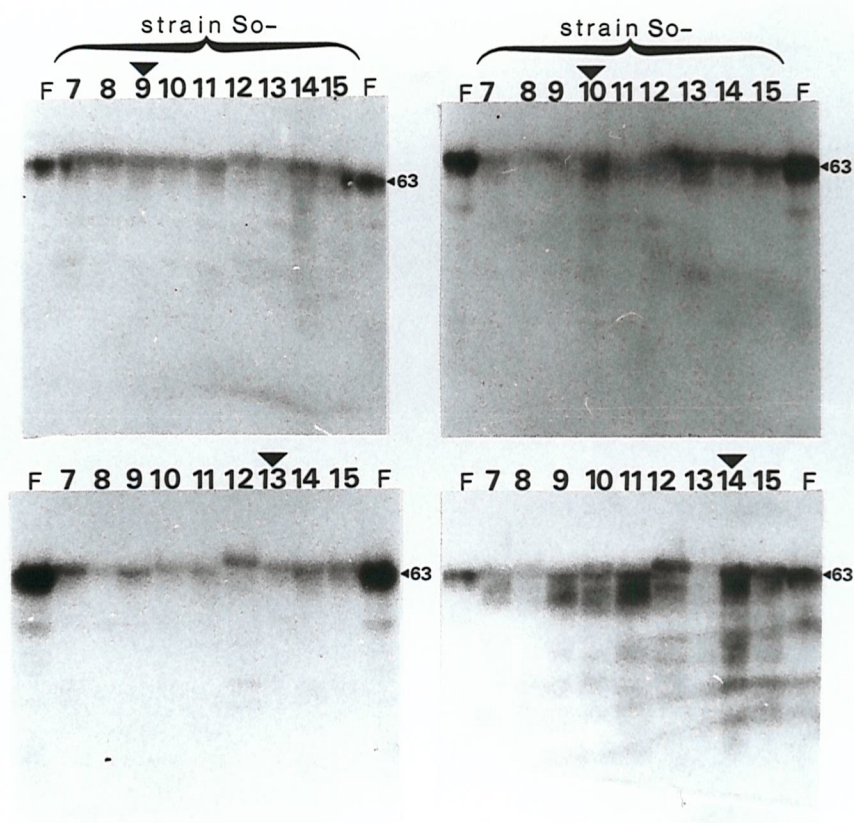
Figure 3.18. Immunoblots of isolates SO7-SO15 using sera
SO9, SO1Ø, SO13 and SO14.
(1) Large arrow indicates serum used.
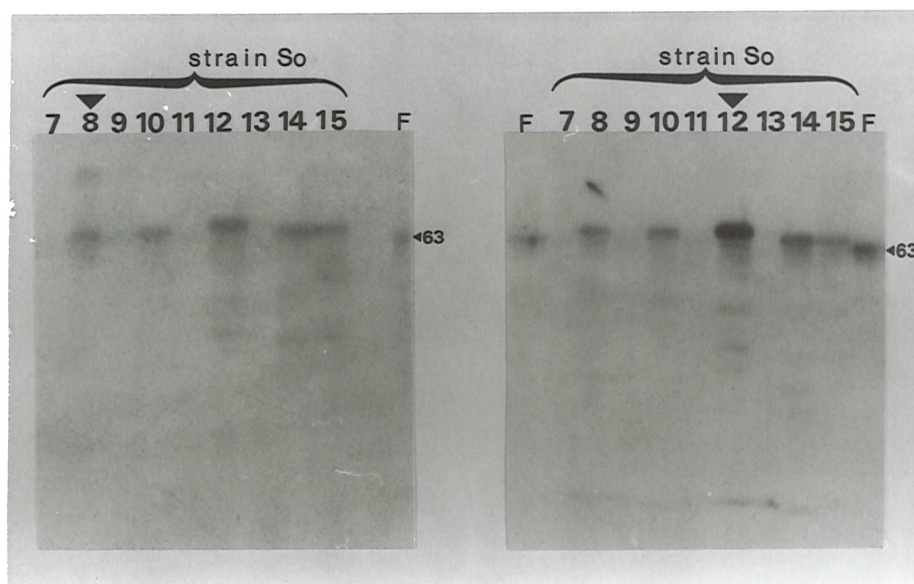
Figure 3.19. Immunoblots of isolates SO7-SO15 using sera SO18 and SO12.

(1) Large arrow indicates serum used.
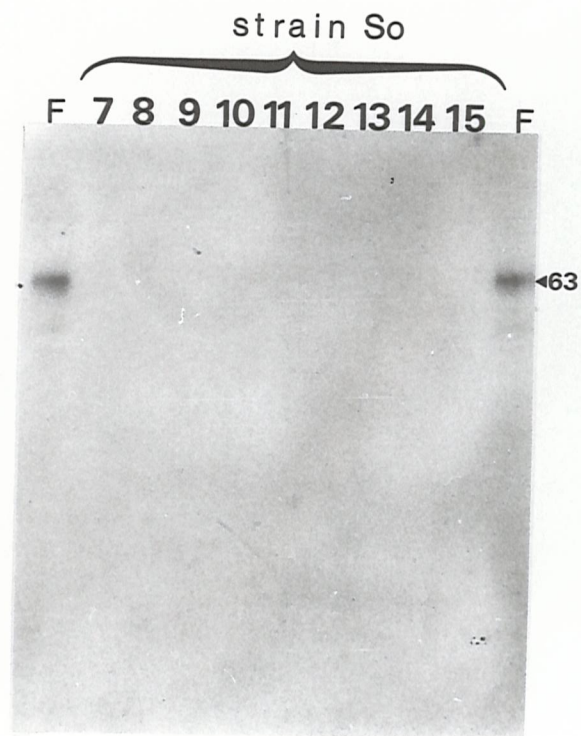
Figure 3.2Ø. Immunoblot of isolates SO7-SO15 using human control serum.

example of this can be seen in Fig 3.18 upon comparing this major band in isolate SO10 when blotted by sera SO9 and SO13. Two important points were noted, in no case was the major 43-46K OM protein detected and secondly serum from a control patient failed to react with any of the protein bands (Fig 3.20).

3.4. Flagella mutants of C.jejuni

To investigate the role of flagella as a virulence factor it was necessary to isolate mutants with disfunction or loss of this organelle.

3.4.1. Isolation of flagella mutants.

Nonmotile mutants were obtained after sequential enrichment on semi-solid media. Strain 81116 was inoculated onto nutrient gelatin agar (1% w/v peptone, 0.33% w/v yeast extract, 0.5% w/v NaCl, 0.8% w/v gelatin, and 0.75% w/v agar and an enriched population of non-motile bacteria was selected from the center of the colonies. The enriched population was inoculated onto a motility agar plate (Nutrient gelatin agar containing 1% w/v agar) to give approximately 100 cfu and after incubation at 37^{0}C for 18h the resulting colonies were replica-plated onto a nitrocellulose sheet. The endogenous bacterial peroxidase was eliminated by incubating the wet blots in methanol containing 0.5% w/v H_2O_2 (100 vol). The identification of those colonies containing flagella was achieved with a rabbit anti-flagella antiserum following the procedure described in 2.7.3. but using o-dianisidine as the substrate. Using this procedure it was possible to identify on the original motility plate those colonies of non-motile organisms containing flagella. Approximately 4% of the non-motile colonies in the enriched population were flagellated. A series of non-motile organisms were selected, cloned six times to confirm there stability and stored in liquid nitrogen. One flagellate non-motile (Fla+ Mot-) variant (SF1) and one aflagellate non-motile (Fla-

Mot⁻) variant (SF2) were selected for study in greater
detail.

3.4.2. Characterization of Mot⁻ variants.

The colony morphology of 81116, SF1 and SF2 when grown
on motility agar is shown in Fig 3.21. Strains SF1 and
SF2 gave discrete, small colonies whilst 81116 gave
colonies which spread to cover the entire plate. The
variants SF1 and SF2 conformed to the same biotype and
serotype as the wild-type strain 81116. The wild-type
organism had a characteristic darting motility when
viewed by dark-ground microscopy while both variants were
non-motile. Rarely a motile organism was observed in
suspensions of SF1. Flagella were visible on the surface
of 81116 and SF1 when examined by electron-microscopy but
not on SF2 (Fig 3.22a,b,c). The wild-type strain and SF1
gave identical sarkosyl extracted OM profiles when
analysed by SDS-PAGE. In contrast SF2 lacked the 62K and
84K protein bands (Fig 3.23.). Examination of OM
preparations from 81116 and SF1 by electron-microscopy
revealed the presence of disc-like structures resembling
those reported by Curry et al., 1983 (Fig 3.24.). Similar
observations of OM material from SF2 failed to detect
these structures. These observations were confirmed by
A.Curry and A.J.Fox (PHLS Manchester).

The antigenic profiles of 81116, SF1 and SF2 were
examined by Dr D.G. Newell (PHLS Southampton) who
supplied Figs 3.25. and 3.26. for which I am grateful.
Whole cell SDS-PAGE profiles were electroblotted onto
nitrocellulose, incubated with rabbit antisera raised
against C.jejuni 81116, SF2 or purified flagella and
immunostained with goat anti-rabbit IgG conjugated to
peroxidase (2.7.3.). The antigenic profiles of 81116 and
SF2 are shown in Fig 3.24. Antisera raised against 81116
and SF2 when blotted against total cell protein profiles
gave complex reaction patterns from which it was
impossible to unequivocally identify the flagella protein
band (Fig 3.25A&B tracks 1 and 3). Interestingly,
antisera raised against purified flagella, while reacting

strongly with 81116 and purified flagella (Fig 3.25C tracks 1 and 2) also reacted weakly with protein bands in the 63K region from SF2 (Fig 3.25C track 3). Antisera raised against SF2, however, failed to react with purified flagella (Fig 3.25B track 2). The presence of low levels of flagella specific antibodies in antisera raised against SF2 was investigated by RIPA (2.7.2.) using the same rabbit antiserum and ^{125}I-flagella (Fig 3.26). ^{125}I-flagella were precipitated by antisera raised against 81116 organisms and purified flagella, but was also achieved with antisera raised against SF2 (Fig 3.26. Track 3). The possibility of radioactive material spilling over from adjacent tracks was eliminated by confirming that the SF2 antisera had precipitated significantly more counts than control antisera.

3.4.3. Amino acid analysis of the flagella from strains 81116 and SF1.

Flagella were isolated and purified from 81116 and the Fla$^+$ Mot$^-$ variant as described in 3.2.3. and an amino acid analysis performed as described in 2.4.4. The relative numbers of residues of each amino acid are shown in table 3.2. The slight variation in certain amino acid residues between the two bacterial strains is probably due to experimental variation.

Analysis of the types of residues present shows a high proportion of hydrophobic residues (42%) followed by acidic residues (22%), polar residues (21%), basic residues (8%) and aromatic residues (5%). The amino acid methionine accounted for 2% of the residues while histidine, tryptophane, proline, cysteine, and cystine were absent.

3.5. The biological role of campylobacter flagella.

Biological assays of bacterial-cell attachment and intestinal tract colonization were investigated using isolate 81116 and the derived variants SF1 and SF2 to assess the potential importance of flagella as a
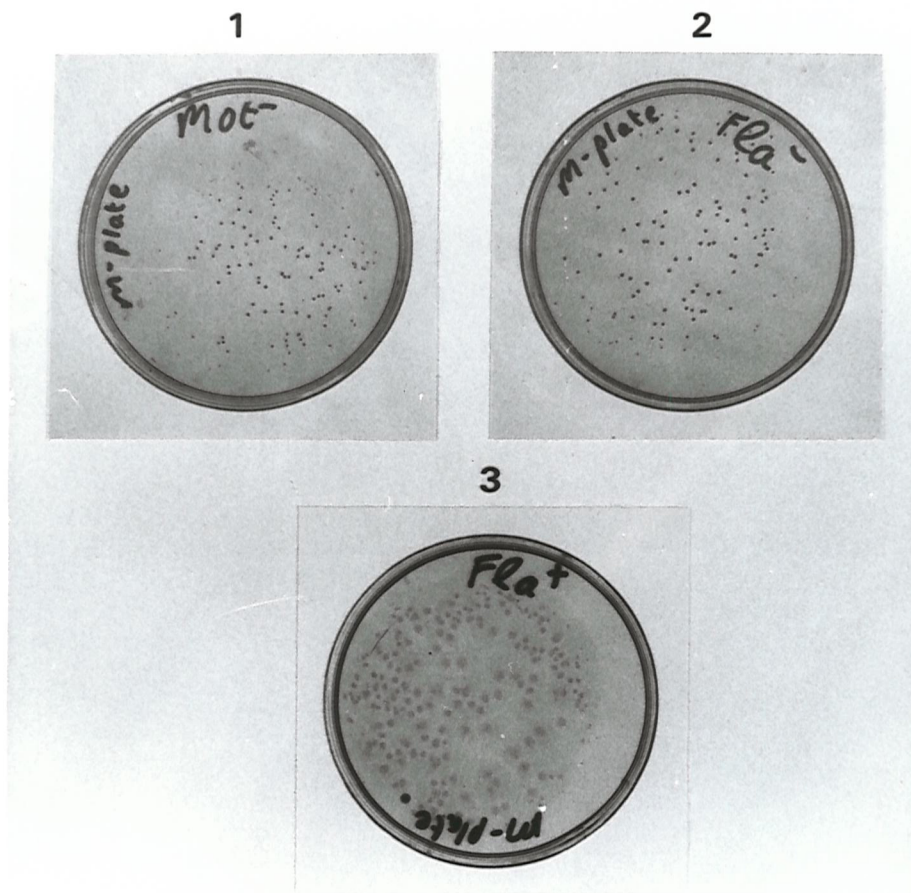
Figure 3.21. Motility of 81116, SF1, and SF2.
(1) Plate 1: SF1; Plate 2: SF2; Plate 3: 81116.
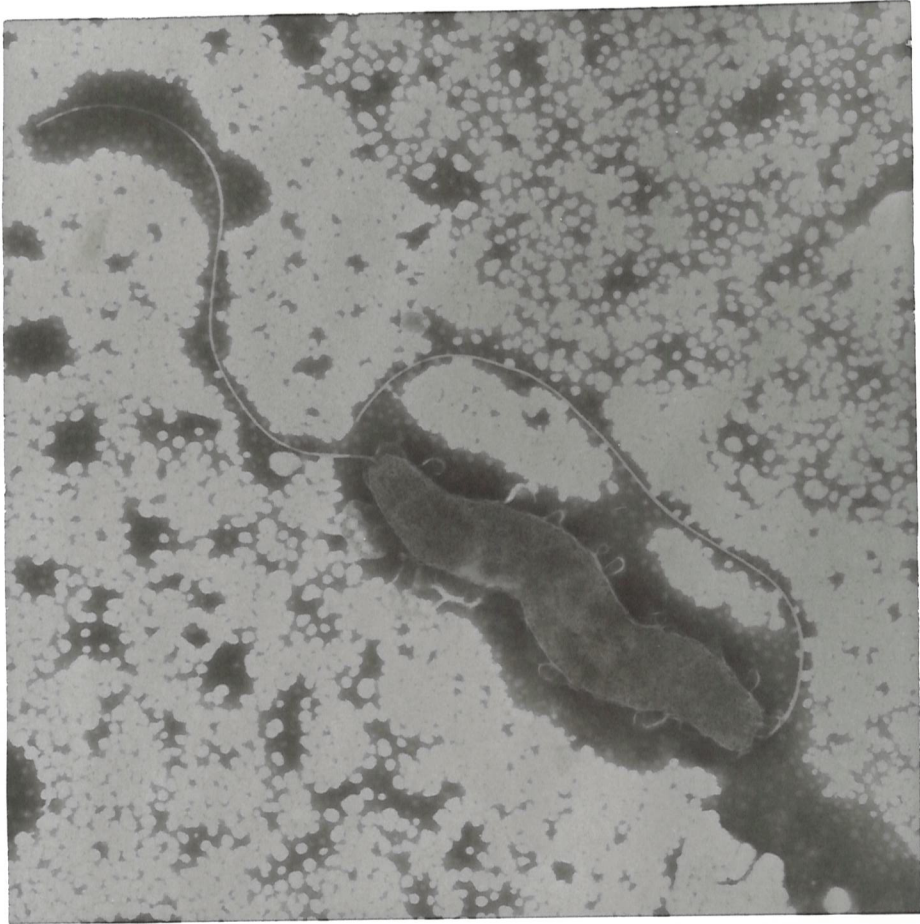
Figure 3.22a.   Electron-micrograph of 81116.
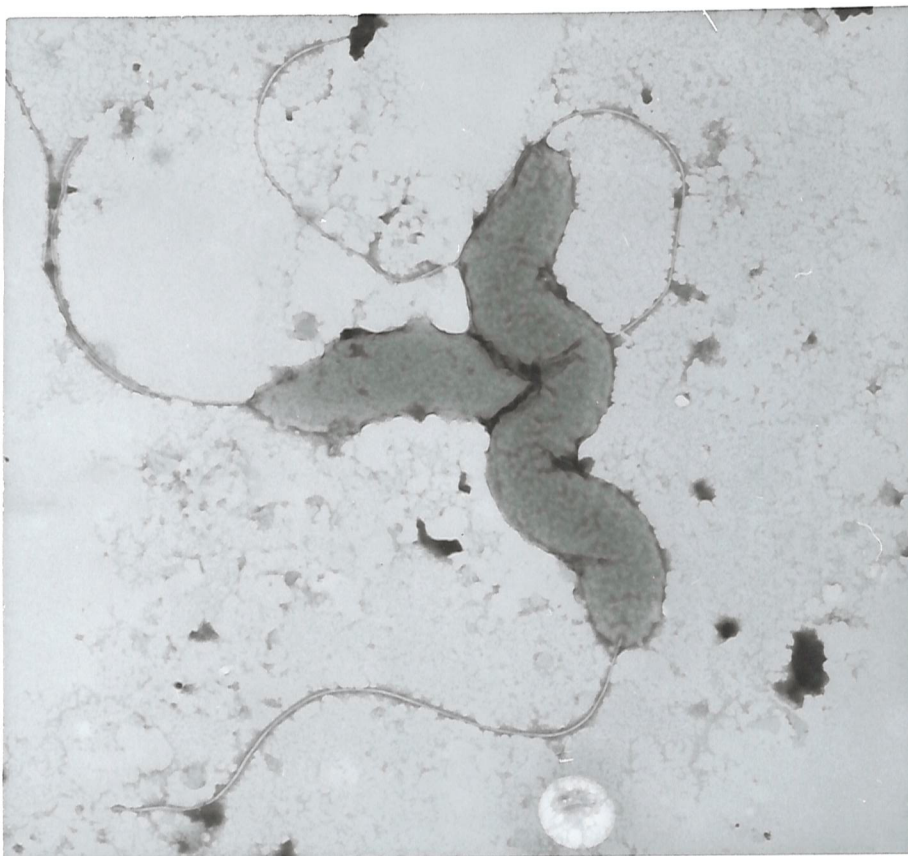
Figure 3.22b. Electron-micrograph of SF1.

Figure 3.22c. Electron-micrograph of SF2.
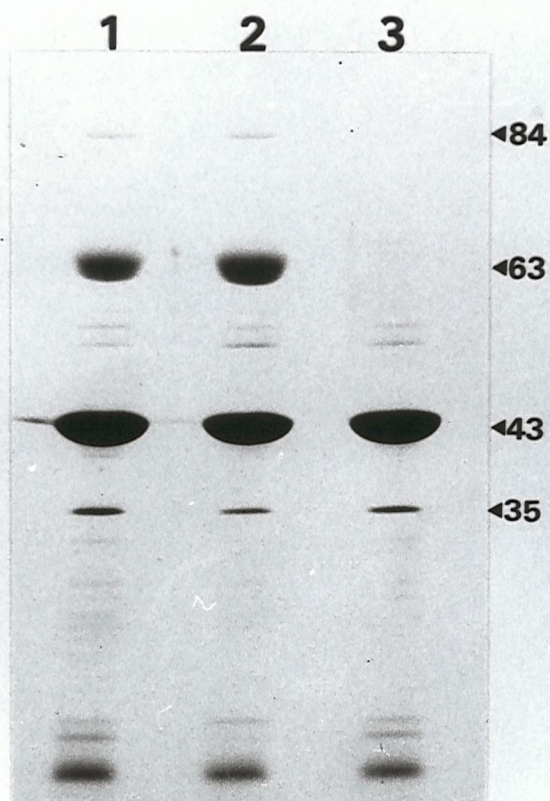
Figure 3.23. Outer membrane profiles of 81116, SF1, and SF2.

(1) Track 1: 81116; Track 2: SF1; Track 3: SF2.
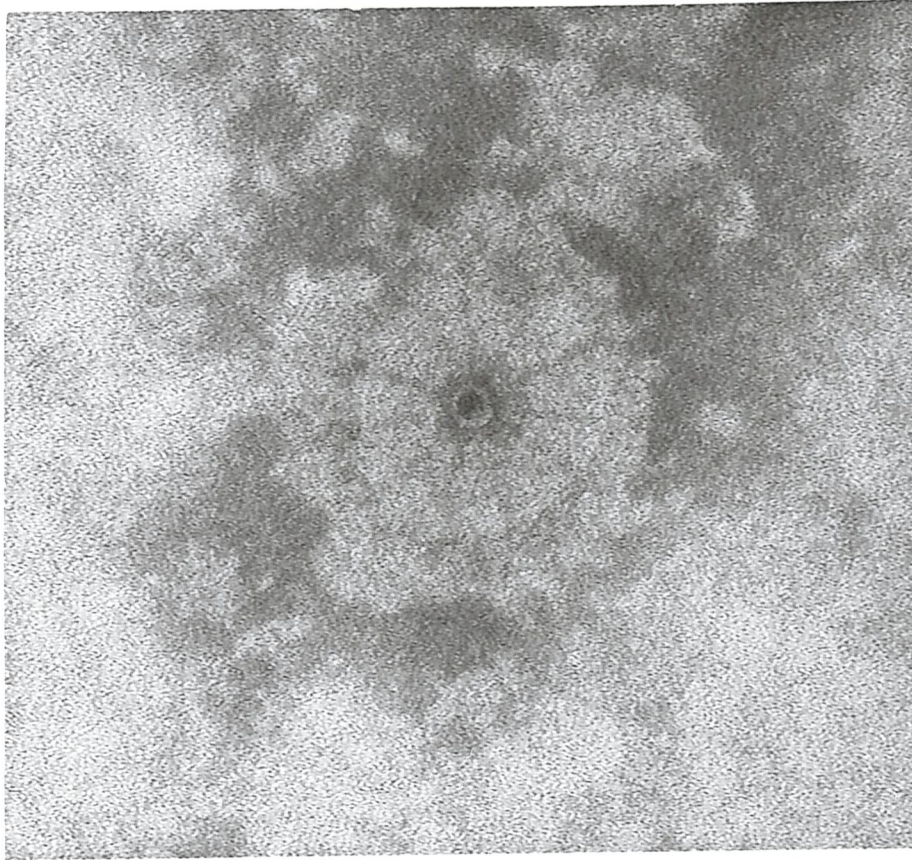
Figure 3.24. Flagella basal body of campylobacters 81116
and SF1.
(1) Magnification 380,000.
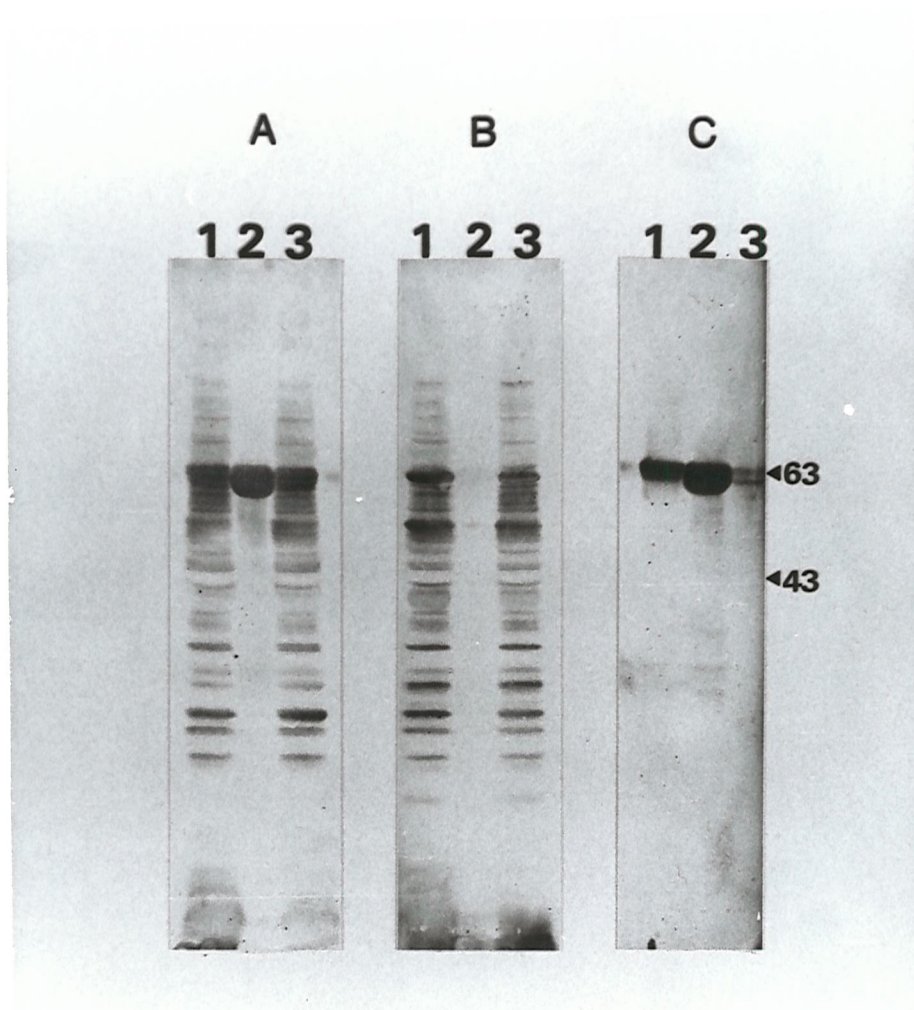
Figure 3.25. Antigenic profiles of 81116, SF1, and SF2 using rabbit antisera.
(1) Tracks 1A,1B&1C: 81116 proteins; Tracks 2A,2B&2C: purified 81116 flagella; Tracks 3A,3B&3C: SF2 proteins.
(2) Figure 3.25A: Antisera raised against 81116; Figure 3.25C: antisera raised against purified flagella; Figure 3.25B: antisera raised against SF2.
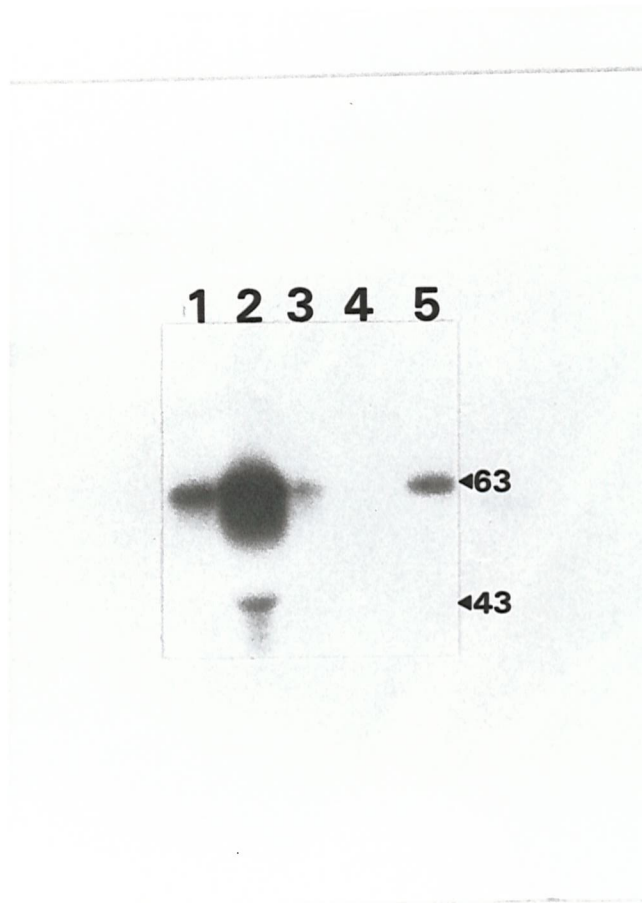
Figure 3.26. Ripa of ^{125}I-labelled flagella with hyper-immune rabbit antisera.

(1) Track 1: Rabbit antisera raised against purified flagella; Track 2: Rabbit antisera raised against 81116; Track 3: Rabbit antisera raised against SF2; Track 4: Pre-immunisation rabbit antisera; Track 5: Radiolabelled flagella.

Table 3.2. The amino acid composition of flagella from

C.jejuni 81116 and SF1

	Mole %[b]		No.residues/molecule[c]	
	81116	SF1	81116	SF1
Aspartic acid	16.5	15.9	90	87
Theronine	8.8	8.3	55	52
Serine	9.8	10.8	71	78
Glutamic acid	9.9	9.6	48	47
Glycine	6.4	7.3	71	81
Alanine	7.7	7.6	68	67
Valine	5.4	5.2	34	33
Methionine[a]	2.3	2.3	11	11
Isoleucine	8.6	7.7	48	43
Leucine	7.0	7.0	39	39
Tyrosine	2.3	2.6	9	10
Phenylalanine	4.5	4.9	19	21
Lysine	6.7	6.5	33	32
Histidine	0	0	0	0
Arginine	4.0	4.2	16	17
Half-cystine[a]	0	0	0	0
Tryptophan[a]	0	0	0	0
Proline	0	0	0	0

(a) Determined by hydrolysis with 3N 2-Mercaptoethane sulfonic acid

(b) Results represent the average of six hydrochloric acid hydrolysis for 81116 and two hydrochloric acid hydrolysis for SF1

(c) Calculations assumed a molecular weight of 62K

virulence factor.

3.5.1. Intrinsic radiolabelling of campylobacter cells.

In order to monitor and quantify the adherence of campylobacter organisms to target cells it was decided to intrinsically radiolabel viable bacterial cells.

The first requirement in order to achieve this was the development of a culture medium which would allow sufficient incorporation of the radio-label into the cell. Preliminary experiments indicated that tissue culture medium was capable of supporting the growth of C.jejuni 81116. As eukaryotic-bacterial cell attachments were to be investigated it was decided to develop a defined campylobacter growth medium (DCGM) based on Dulbecco's modification of Eagle's medium (Smith et al., 1960), and having the following basal composition :

	mg/ml
$CaCl_2 : 2H_2O$	265.0
$Fe(NO_3)_3 : 9H_2O$	0.1
KCl	400.0
$MgSO_4 : 7H_2O$	200.0
NaCl	6400.0
$NaH_2PO_4 : 2H_2O$	162.5
D-Ca pantothenate	4.0
Choline chloride	4.0
Folic acid	4.0
Inositol	7.2
Nicotinamide	4.0
Pyridoxal HCl	4.0
Riboflavin	0.4
Thiamine HCl	4.0

This basal solution was made at x10 strength before being filter sterilised (0.22µm filter) and stored at 4° C. In order to determine which amino acids were essential for the growth of the organism individual amino acids were omitted from the basal solution after it had been diluted to single strength. The remaining amino acids

were added to a final concentration of 200µg/ml. Prior to being inoculated with the organisms sodium bicarbonate (0.37% w/v) and sodium pyruvate (0.025% w/v) were added and the media adjusted to pH 7.2.

The bacteria were harvested from BA into PBS, and the OD_{260} of the suspension was adjusted to 1.0 and 10µl of this suspension was inoculated into 5ml of defined media. The media were incubated at 37^0 C with shaking under a microaerophilic atmosphere for 18h. At the end of the incubation period the OD_{540} of each media was measured against a defined media blank. The results of this experiment are shown in table 3.3. Analysis of the results indicated that the removal of glutamine and isoleucine had caused a reduction in growth, while the removal of methionine resulted in a total absence of growth. The examination of the morphology of these organisms by dark-ground microscopy revealed an essentially normal appearance with some elongation of cells with the omission of the majority of the amino acids but that the removal of glutamine, isoleucine and methionine produced spheroplast-like structures. From this data it was decided to optimise the content of methionine within the media (leaving all other amino acids at 200µg/ml) with the intention of using ^{35}S-methionine as a label. A preliminary experiment indicated that the limiting concentration of methionine lay below an initial concentration of 10µg/ml. Therefore a second experiment was performed with a range of methionine concentrations between 0 and 10µg/ml (Fig 3.27).

This experiment indicated that the minimal essential methionine concentration was approximately 7µg/ml. It was therefore decided to standardise the methionine concentration of the media to this value. A second important criteria was the time at which to harvest the organisms, and to determine this a growth curve was establised (Fig 3.28). Analysis of the morphology of the organisms by dark-ground microscopy during this growth curve indicated significant elongation of the organisms after 14h of growth. It was decided, therefore, to

Table 3.3. Determination of the essential amino acids

for growth of C.jejuni 81116

Amino acid omitted	Growth %
Arginine	88
Cysteine	77
Cystine	70
Glutamine	26
Glycine	114
Histidine	75
Isoleucine	21
Leucine	69
Methionine	0
Phenyalanine	68
Serine	73
Threonine	93
Tryptophan	118
Tyrosine	96
Valine	87
All amino acids	0
no amino acids	100

(1) % Growth determined as:

$$\frac{OD_{540} \text{ test media at 18h}}{OD_{540} \text{ complete media at 18 h}} \times 100$$
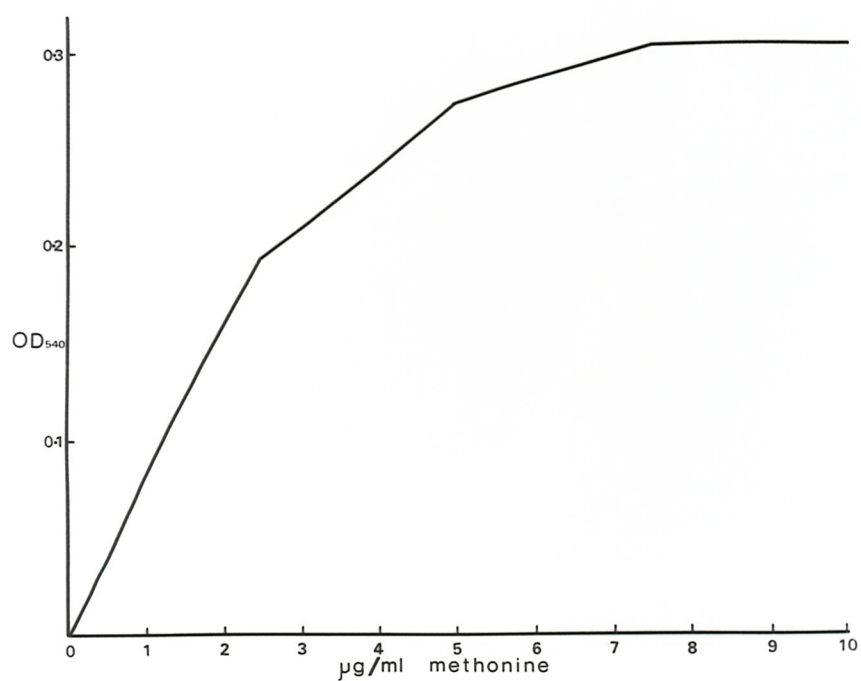
Figure 3.27. Optimisation of methionine level for growth
of C.jejuni 81116 in defined media.
(1) OD_{540} was determined after 18h growth.
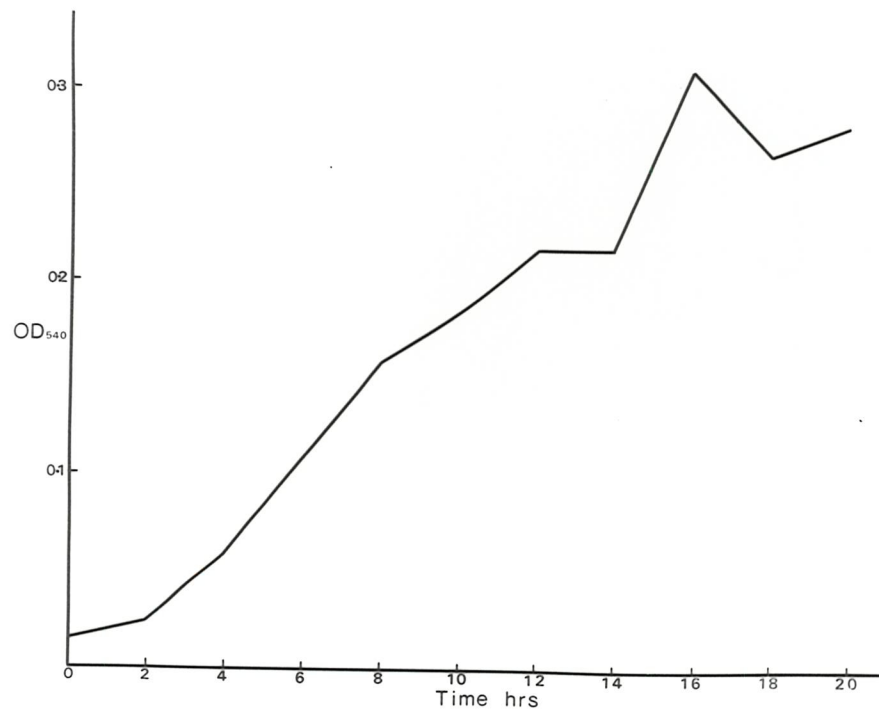
Figure 3.28. Growth curve for C.jejuni 81116 in defined media.

harvest the organisms after 12h of growth. In order to
radio-label the bacteria L-^{35}S-methionine (Specific
activity approximately 105mCi/mMol; Amersham
international) was added to the DCGM to a final
concentration of 10µCi/ml. This gave a total methionine
concentration of 16µg/ml, the specific activity of the
bacteral suspension (OD_{260} =0.1) after harvesting and
washing being between 0.05 and 0.1 µCi/ml. The analysis
of the total protein profiles of these radio-labelled
organisms by SDS-PAGE revealed normal protein patterns
(Gel not shown).

3.5.2. Campylobacter attachment to erythrocytes.

Erythrocytes were collected and prepared for
attachment experiments as descriped in 2.3.1. The
bacteria were radio-labelled as described in 3.5.1. and
after harvesting were washed once in cold DCGM and
recovered by centrifugation at 5,000g for 20min. The
bacterial pellet was resuspended in DCGM to the required
cfu/ml (measured at A_{260} in a spectrophotometer) and 1ml
of this suspension added to 1ml of erythrocytes at a PCV
of 10%. Preliminary experiments indicated that maximal
attachment to the erythrocytes was achieved at 2h of
incubation under constant gentle mixing. All experiments,
therefore, were carried out for 2h after which the
erythrocytes and attached bacteria were separated from
unattached bacteria by centrifugation through a cushion
of Dextran 110 (Lambden et al., 1979). The erythrocyte
pellet was then lysed by the addition of 200µl of 0.25M
NaOH and decolourised with 100µl of H_2O_2 (30% w/v). To
this solution 5ml of scintillation fluid was added
(Scintillator 299 , Packard) and the radioactivity
measured in a liquid scintillation counter (1211 mini-
beta, LKB). A one tailed students t-test was used to
indicate significance (Appendix 2).

The percentage attachment of campylobacter strains
81116, SF1 and SF2 to various animal erythrocytes are
shown in table 3.4. The aflagellate variant (SF2)
attached to the red cells better than either SF1 or

TABLE 3.4 Attachment of bacterial strains to erythocyte suspensions

Erythrocytes	Experiment 1			Experiment 2		
	% Attachment (± S.D)[a] SF-2	81116	Statistical significance	% Attachment (± S.D)[a] SF-2	SF-1	Statistical significance
Rabbit	23.0 ±2.6	6.0 ±0.3	p < 0.01	27.7 ±4.7	15.2 ±0.7	p < 0.01
Mouse	21.4 ±1.4	2.2 ±0.2	p < 0.01	24.6 ±4.4	8.0 ±0.9	p < 0.01
Guinea-pig	11.0 ±1.8	1.5 ±0.06	p < 0.01	25.0 ±4.1	8.8 ±0.6	p < 0.02
Rat	3.0 ±0.4	0.5 ±0.02	p < 0.01	11.0 ±1.4	4.1 ±0.4	p < 0.01
Chicken	1.4 ±0.2	N.D.	–	2.3 ±0.6	0.6 ±0.03	p < 0.01
Goat	0.8 ±0.09	0.5 ±0.06	p < 0.01	0.8 ±0.07	0.9 ±0.1	N.S.
Sheep	0.8 ±0.1	0.7 ±0.1	N.S.	1.0 ±0.03	2.1 ±0.2	p < 0.01
Human A	4.7 ±0.8	0.5 ±0.02	p < 0.01			
Human B	5.8 ±0.9	0.7 ±0.04	p < 0.01			
Human O	2.7 ±0.5	0.5 ±0.02	p < 0.01			

[a] Standard deviation of the mean calculated from 4 replicates.

N.S. Not significant

81116, and although there was no direct comparison, SF1 appeared to have a higher percentage attachment than the wild-type strain 81116. Differences were also noted in the attachment of the strains to erythrocytes from different species in particular the attachment to human, rat, goat, sheep and chicken erythrocytes being significantly lower than attachment to rabbit, mouse or guinea-pig red blood cells. Additionally there was a small difference in the attachment to human erythrocytes of the three different blood groups tested, with attachment of SF2 to group B better than to group A or group O.

To gain some further insight into the attachment of these bacteria to erythrocytes an adsorption isotherm was constructed using 81116 and SF2 with rabbit erythrocytes. A standard attachment assay was performed as described above with the exception that various concentrations of bacteria were added to the standard suspension of erythrocytes. To analyse the results it was assumed that the total numbers of bacteria added to the assay system was directly proportional to the dpm added. A plot of the total dpm added to the erythrocytes versus the total number of dpm bound to the cells gives a linear relationship (Fig 3.29). It is evident from this plot that the cell surface was not saturated even at the highest number of bacteria added. One of the most commonly used equations in the study of bacterial attachment to surfaces is the Langmuir adsorption isotherm (Shaw 1970). In its original form a number of assumptions are made : (1) There are a finite number of identical sites for attachment per unit surface area (2) Bacterial cells approach the surface without steric hindrance (3) The adsorption process is reversible and (4) Adsorption is limited to a mono-molecular layer. With these assumptions the equation has the following form (Gibbons et al., 1976)

$$C/Q = 1/KN + C/N \qquad \text{(Equation 1)}$$

where

C= The concentration of free bacteria at equilibrium

Q= The maximum number of bacteria adsorped onto the erythrocytes

K= K_1/K_2 where K_1 = The specific rate constant for adsorption and K_2 = The specific rate constant for desorption and

N= The maximum number of receptor sites on the erythrocytes.

If the model follows the above equation then a plot of C/Q verses C will be linear. However if it is assumed that the attachment of the bacteria to the erythrocytes is non-reversible then a second equation will apply having the following form (Fletcher 1977).

$$(X)s = \frac{(X)s\ (X)ads}{K'} + \frac{R}{K} \qquad \text{(equation 2)}$$

where

R= The rate of bacterial adsorption

(X)s= The total number of bacteria present at equilibrium

(X)ads= The total number of bacteria adsorbed

K relates the rate of adsorption to (X)s and the number of vacant adsorption sites i.e. K represents the intensity of adsorption. K' is a limiting constant and is dependent upon the adsorption capacity of the surface. If the model applies a plot of (X)s against (X)s(X)ads should be linear with the slope giving K' and the intercept R/K.

When the data was substituted into equation 1 a non-linear response was obtained having linear regression correlates for 81116 and SF2 of 0.66 and 0.68 respectively. However when the same data was substituted into equation 2 a curvilinear response was seen with linear regression correlates for 81116 and SF2 of 0.95 and 0.94 respectively (linear regression plots shown in

Fig 3.30). From these graphical plots the values of K' and R/K were as follows

81116 : K'= 2.9 x 10^4 bacteria   R/K= 5.8 x 10^4 bacteria

SF2   : K'= 9.1 x 10^4 bacteria   R/K= 5.5 x 10^4 bacteria.

3.5.3. Campylobacter attachment to epithilial cells.

To establish an attachment model system that more closely resembles the attachment of campylobacters to intestinal epithlial cells, immortal epithlial cell lines were used as targets for radio-labelled bacteria.

Epithlial cells were cultured as described in 2.3. and radio-labelled bacteria prepared as described in 3.5.1. The bacteria were harvested and washed as described in 3.5.2. and resuspended to an OD_{260} equal to 0.2 in DCGM. The antibiotic-free medium was removed from the cell monolayer and 0.5ml of the bacterial suspension added to each well. The trays were returned to a CO_2 incubator and maintained at 37^0C in an atmosphere of 5%CO_2 for a given incubation period. The bacterial suspensions were then removed from the monolayers which were washed three times with Hanks balanced salt solution. The monolayers were solubilised in 1% w/v SDS in 0.1M NaOH and liquid scintillation counted as described in 3.5.2. The percentage attachment was determined from the dpm bound to the monolayer divided by the dpm added to the monolayer x 100. Each experiment contained six replicates for each test, and a one-tailed students t-test was used to indicate significant differences.

An initial experiment was performed using the attachment of SF1 to INT 407 cells at various time intervals in order to determine an appropriate incubation time. There was a steady increase in attachment with time followed by a rapid decline after five hours of incubation (Fig 3.31). This marked decline was assumed to be due to cell death and subsequent loss from the monolayer and increasing dilution of ^{35}S-labelled
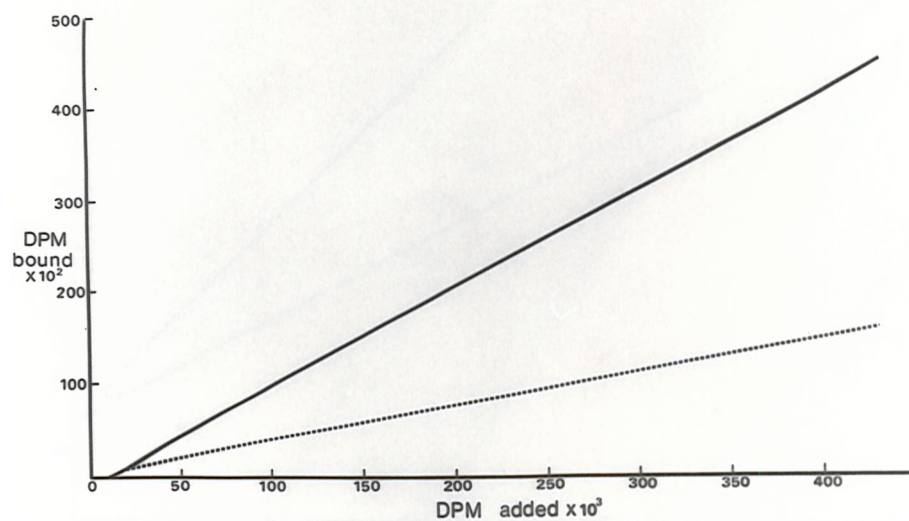
Figure 3.29. Campylobacter attachment to erythrocytes. Relationship between radiolabelled bacteria added and bacteria bound.

(1) ⋯⋯⋯⋯ 81116

⎯⎯⎯ SF-2
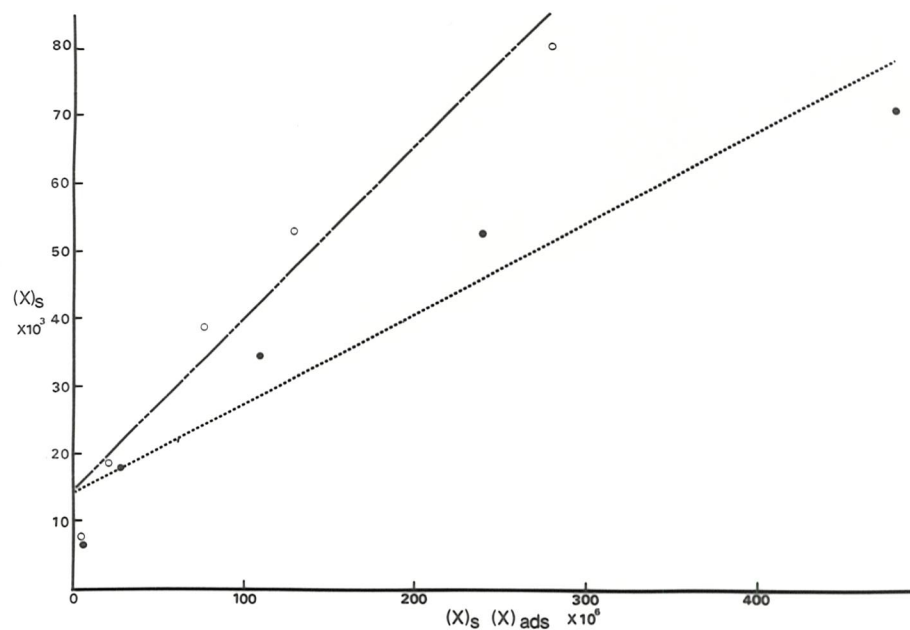
Figure 3.30. Adsorption isotherms (non-reversible binding) for 81116 and SF-2.
(1) ▬▬▬▬81116
▪▪▪▪▪▪▪▪▪▪SF-2

bacteria. A 4h incubation period was therefore chosen for subsequent assays. A comparison of the attachment of 81116, SF1 and SF2 to the three epithlial cell lines chosen was undertaken and the results are shown in table 3.5. For each cell line examined the bacterial strain SF1 attached significantly better ($p < 0.01$) than 81116 or SF2. These differences were most noticeable in the attachment to INT 407 cells with SF1 achieving up to a four-fold increase compared to 81116 and SF2.

The effect of various carbohydrates on the attachment of ^{35}S-labelled SF1 to INT 407 cells was also investigated. A standard attachment experiment was performed with the exception that the bacteria were suspended in DCGM containing the carbohydrates at the required concentration. The results of these experiments are shown in table 3.6. The greatest inhibition was caused by mannose and the least by fucose with the non-sugar carbohydrate glucitol also causing some inhibition. The inhibition by mannose and to a lesser extent glucitol was concentration dependent.

3.6. Colonization of infant mice.

The role of flagella in the attachment of C.jejuni to target cells in vitro may not reflect the situation in vivo and therefore the infant mouse model described in 1.6. was used to compare the abilities of 81116, SF1 and SF2 to colonize the gastro-intestinal tract.

These experiments were performed in collaboration with Dr.J.Dolby of the Clinical Research Center, Northwick Park Hospital, Harrow, London to whom I am most grateful. The colonization procedure was based on that used by Field et al (1981) and was performed on five-day old Balb/c mice. The bacterial strains were grown and diluted to the required cfu/ml by the addition of PBS or boiled cows milk. The PBS suspensions were intragastrically inoculated while milk suspensions were administered orally. The method of inoculation did not appear to affect the colonization. Initially infant mice were
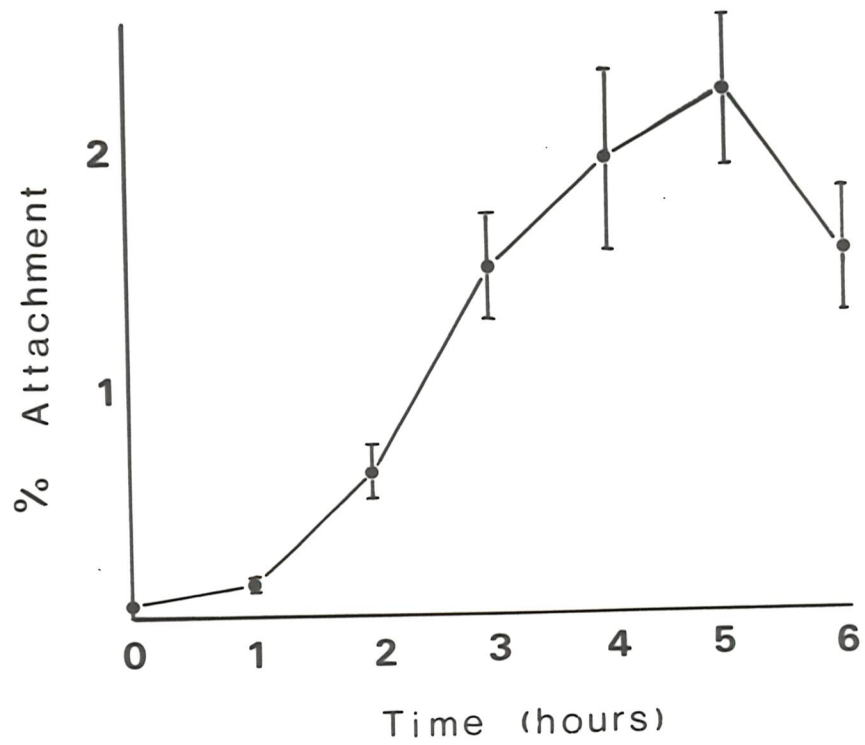
Figure 3.31. Time course of the attachment of SF-1 to Int-407 cells.

(1) Bars represent standard deviations from the mean for six replicates.

Table 3.5. The attachment of bacterial strains to tissue
culture cell monolayers

Cell line	81116	SF-1	SF-2
Int 407	0.23 ± 0.006	1.60 ± 0.3	0.2 ± 0.02
HEp 2	0.21 ± 0.02	0.26 ± 0.02	0.16± 0.03
Int 407	0.33 ± 0.03	1.05 ± 0.04	0.31± 0.02
HeLa	0.30 ± 0.04	0.45 ± 0.04	0.02± 0.02

(1) Standard deviation of the mean calculated from six
replicates

Table 3.6. The inhibition of attachment of SF-1 to
                Int 407 cell monolayers

Treament	Concentration (mM)	% attachment	%Inhibition
Control	-	1.38±0.11	-
Mannose	0.1	1.19±0.06	14
Mannose	1.0	1.12±0.06	19
Mannose	10.0	0.90±0.03	35
Mannose	50.0	0.65±0.02	53
Galactose	50.0	0.91±0.07	34
Glucose	50.0	0.82±0.06	40
Fucose	50.0	1.04±0.02	24
N-acetyl-glucosamine	50.0	0.81±0.07	41
N-acetyl-galactosamine	50.0	0.94±0.08	32
Glucitol	0.1	1.19±0.07	14
Glucitol	1.0	1.06±0.11	23
Glucitol	10.0	1.02±0.08	26

(1) Standard deviation of the mean calculated from six
replicates

separated from the dams for periods of 2h prior to and after inoculation, however it was found that unseparated mice gave similar results to the separated infants and therefore later experiments utilised unseparated mice. The inoculumn was 1×10^4 - 1×10^7 cfu of the wild-type strain or the variants. At specific times mice were sacrificed by decapitation and segments of the intestinal tract removed and homogenised in PBS using a stomacher (Lab-blender 80, Seward Laboratory, London, U.K.). Samples of the homogenate were diluted in PBS and plated onto 2% VPT plates for viable counting. The results were expressed as the total numbers of organisms present within that segment of intestine. For long term studies (in excess of 14 days) the number of organisms present were determined by total counts of faecal pellets similarly treated to intestinal seqments. The results were expressed as the total number of bacteria per gram of faecal material. Recovered organisms were monitored for motility on nutrient gelatin agar (3.4.1) and for flagella by transmission electron-microscopy. Preliminary experiments using 81116 gave consistent results in terms of the distribution of bacteria through-out the intestinal tract and a typical response is shown in fig 3.32. Bacteria were reduced to low numbers or eliminated from the stomach, duodenum, upper ileum and middle ileum within three days post-inoculation. The lower ileum, caecum and colon contained large numbers of organisms for in excess of ten days. For comparative studies between 81116, SF1 and SF2 the total numbers of organisms in the entire gastro-intestinal tract were used for the first fourteen days and faecal pellet bacterial counts for colonizations in excess of fourteen days. These comparisons are shown in Fig 3.33. For the first fourteen days each point represents the average bacterial count for at least four and generally six mice while the bars indicate the range of counts observed in those animals. In excess of fourteen days each point represents the bacterial count per gram of faecal material for individual mice.

The wild-type strain colonized the gastro-intestinal

tract with up to a ten-fold increase in the number of organisms recovered compared to the inoculmn (Fig 3.33A). There were no indications of illness or diarrhoea in the mice and the distribution of organisms within the intestinal tract followed the same trend as shown in Fig 3.32. There were detectable organisms in faecal pellets from these mice for up to thirty-one days post-inoculation and colonization could be achieved with as few as 3×10^4 cfu per mouse. In contrast the aflagellate variant, SF2, was cleared from the intestinal tract by day seven post-inoculation (Fig 3.33B). There was never an increase above the inoculum of organisms within the intestinal tract and in contrast to 81116 the variant was only recovered from the caecum and lower ileum of the infant mice.

The second, flagellate and non-motile, variant SF1 colonized the middle and lower ileum as well as the caecum and colon although, in general, the colonization lasted for shorter a period (up to fourteen days) compared to 81116 (Fig 3.33C). However, approximately half the mice showed evidence of a revival of colonization between 18 and 22 days post-inoculation. In contrast to 81116 the numbers of organisms recovered varied considerably between mice although they were often higher than that seen with 81116. At the most pronounced state of colonization up to one-hundred fold more organisms were present within the intestinal tract compared with the initial inoculum. As with 81116 an inoculum as low as 1×10^4 was capable of initiating colonization.

In all cases the motilities (as discerned by growth on motility agar) and flagellation of the recovered organisms was identical to the initial inoculum.
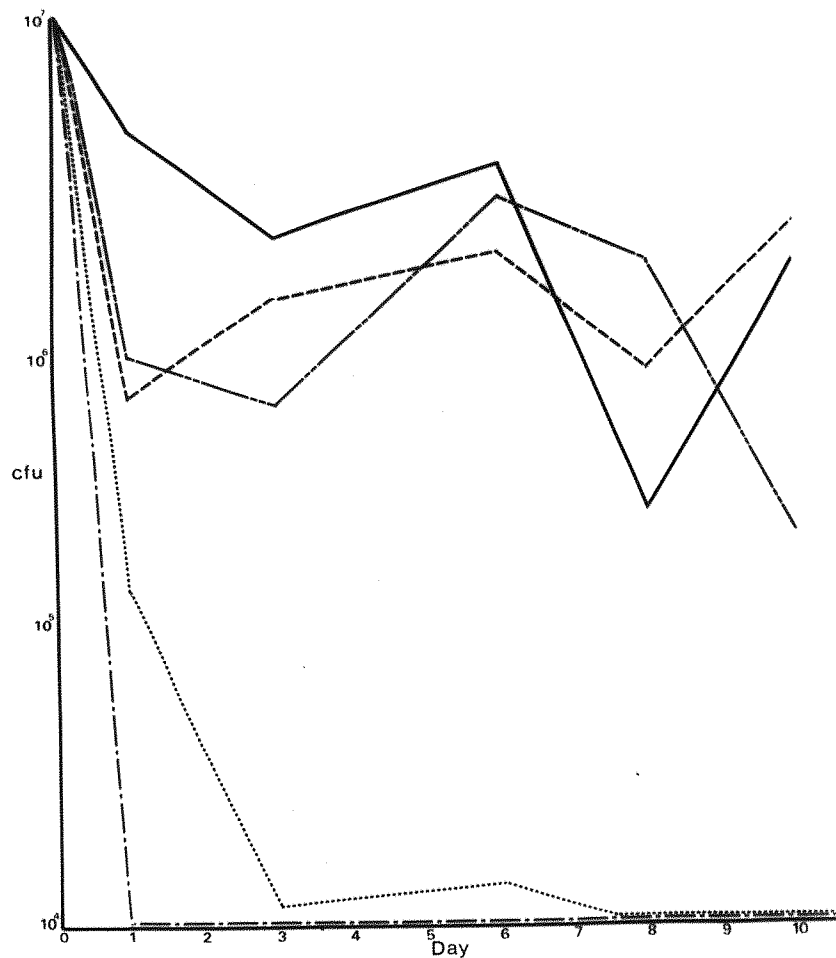
Figure 3.32. Colonization of the intestinal tract of five-day old mice by C.jejuni 81116.

(1)

━ ‧ ━ ‧ ━ ‧ ━ Stomach, duodenum and upper ileum.

▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪▪ Middle ileum.

━━━━━━━━━ Lower ileum.

━ ━ ━ ━ ━ Caecum.

━━━━━━━━━ Colon.
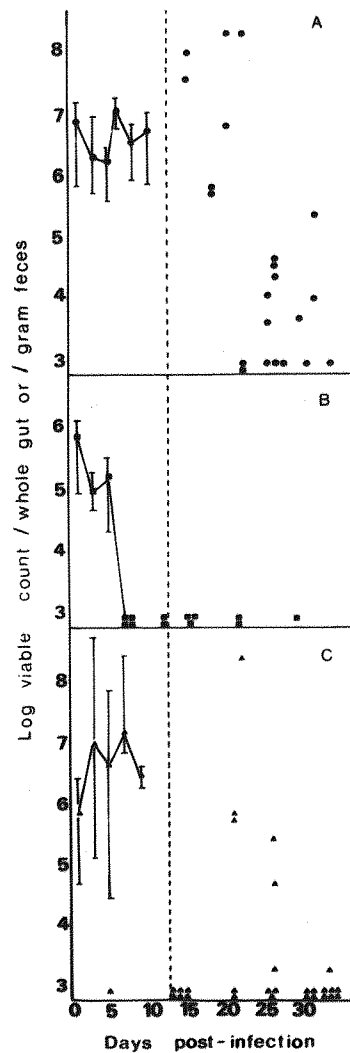
Figure 3.33. Colonization of the intestinal tract of five-day old mice by 81116, SF-1 and SF-2.
(1) figure 3.33A: 81116; Figure 3.33B: SF-2; Figure 3.33C: SF-1.

CHAPTER 4

DISCUSSION

The outer membranes of C.jejuni and C.coli organisms have been isolated and examined. Initial experiments were aimed at identifying a reliable procedure for the isolation of these membranes and to gain some insight into the effect the isolation procedures had on their protein composition. The development of a spheroplasting procedure with campylobacter cells and the subsequent isolation of the inner and outer membranes on a sucrose gradient proved difficult to achieve with any acceptable level of reproducability. This problem is not unique to campylobacters, similar findings having been reported for B.pertusis cells (Ezzell et al., 1981, Robinson and Hawkins, 1983).

The biochemical analysis of the membranes prepared by spheroplast formation indicated a good separation of inner and outer membranes when specific SDH activity was used as the marker for purity. This degree of purity was confirmed by the protein profiles of the various fractions collected from the gradient. However, considerable contamination of the cytoplasmic membrane by LPS was observed. In addition to the problems associated with interference of the LPS assay system already described another possible explanation is suggested by the observation that adhesion zones exist between the inner and outer membranes of many gram-negative bacteria which serve as sites for export of LPS, capsule polysaccharides and major outer membrane proteins (Reviewed by Bayer et al., 1982). Indeed the identification of these adhesion zones has placed doubt on the quantitative separation of these membranes by sucrose density centrifugation. The cells of campylobacter organisms can shed considerable amounts of membrane vesicles from their surface during growth. The extent of this shedding is similar to Neisseria organisms

and it has been proposed that the lack of lipoprotein within the membrane may account for this degree of vesicle release (DeVoe, 1982). The released membranes are enriched in LPS while, in the case of Campylobacter jejuni, there is evidence that the amount of the presumptive matrix protein is reduced (Logan and Trust, 1982). It may be possible that a certain amount of the adhesion zone membranes may contaminate the inner membrane fraction on a sucrose gradiant and thereby increase the amount of LPS detected within that fraction.

A recent analysis of sarkosyl soluble and insoluble membrane material (Blaser et al, 1983) showed that while all SDH activity was removed by solubilisation of the inner membrane there existed considerable variations in the enrichment of LPS (strictly KDO levels) in the insoluble membranes isolated from different strains (range 1.3 to 11 fold). This confirms the analysis presented here where the enrichment of LPS in spheroplast prepared outer membranes was 1.7 fold above the inner membrane. Comparison between these spheroplast prepared outer membranes and sarkosyl-insoluble membranes revealed essentially identical membrane protein profiles, however the absence of 84.1K, 82.2K, 42.2K and 28.8K bands in the sarkosyl prepared outer membrane was noted. This loss of outer membrane proteins during sarkosyl extraction has recently been confirmed by other investigators (Blaser et al, 1983., Mills and Bradbury, 1984), the latter authors also comparing against spheroplast prepared membranes and noting the loss of 80K, 56K, 53-54K, 40K and 34K protein bands in the sarkosyl extracted outer membranes. It was decided therefore that further comparisons of outer membranes from campylobacter organisms could be made using the more reliable procedure of sarkosyl solubilisation of inner membranes.

In spheroplast and sarkosyl prepared membranes two main bands predominate. The first had a variable molecular weight between isolates within the range 43-46K, it was heat modifiable, surface exposed and represented the major band in total protein profiles of the organisms. These characteristics suggest it is the

same as protein band e recently described by Logan and Trust (1982), subsequently named Omp 1 by the same authors (1984b), and the 41-45K protein reported by Blaser et al., (1983) and Mills and Bradbury (1984). Differences were noted however in the degree of mobility of this particular protein reported by the various investigators. Logan and Trust, for instance, reported three molecular weights for band e depending on the solubilisation temperature chosen; an oligomeric form of >92K, a globular form of 33K and a linear form of 45K. When solubilised at $37^{o}C$ all three forms where noted in contrast to the data presented here were only the globular form was observed. This difference is explained by the different times of solubilisation used, Logan and Trust solubilising for 3min while the present work solubilised for 2h. The characteristics of this particular protein, together with its peptidoglycan association (Logan and Trust 1982), make it likely that this represents the matrix or porin protein that is found in other gram-negative organisms and therefore is important as a structural component of the outer membrane and as a functional protein forming diffusion channels through the membrane.

The second major protein band in the outer membranes of C.jejuni and C.coli had a variable molecular weight in the range of 62-64K. The purification of flagella from campylobacter organisms indicated that this 62-64K protein was flagellin. It was not heat-modifiable nor surface labelled using the lactoperoxidase procedure. Confirmation of a 63K flagellin protein present in C.jejuni cells that did not label with 125I using lactoperoxidase has recently been presented, (Logan and Trust, 1983). The proportion of flagellin within outer membranes preparations varied considerably between isolates indicating differences in the flagellation of different isolates or differences in the susceptability to detergent extraction. Although the molecular weight for this flagellin was higher than reported for most other bacteria the amino acid composition was not significantly different from other known flagellins in

terms of mole percentage of the individual residues (Parish and Ada, 1969 ; Maruyama et al., 1978). Although the overall amino acid compositions of the flagellins from 81116 and its non-motile variant SF1 were similar there were significant differences in the amounts of serine and glycine detected. In the case of serine the differences amounted to seven residues and for glycine ten residues. These differences, when compared to the total number of residues present, may be due to the experimental variability inherent in the technic used, however the existence of an actual difference between the two flagellins can not be excluded. In terms of the overall amino acid composition of the flagellins there was a slightly higher content of polar residues (183-187) compared to hydrophobic residues (157-160).

Although the amino acid analysis gives information on the overall composition of the proteins a more important consideration is the conformation of the polypeptide chains. The presence of a high content of hydrophobic residues at the surface of the flagella could allow more efficient binding to the eukaryotic cell surface while, conversely, a high content of acid residues could reduce binding to the negatively charged eukaryotic surface. There was no specific data obtained that would allow any determination of the conformation of the flagellin of Campylobacter jejuni, however the inability to radio-label flagella using lactoperioxidase or iodogen may indicate that the nine or ten tyrosine residues present in the protein may be internalised in native flagella filaments.

Although certain physical characteristics of the other minor outer membrane proteins were determined it was not possible to place any biological activity on these proteins. A 35K protein was present in the outer membranes of C.jejuni and was apparently replaced by a 41K protein in C.coli strains. These proteins were heat modifiable and surface exposed. These characteristics are the same as the protein f reported recently by Logan and Trust (1982) and the 30K band reported by Blaser et al.,

(1983). These authors did not report any observation of a molecular weight difference between C.jejuni and C.coli isolates. A possible explanation resides in the different SDS-PAGE systems employed as Logan and Trust used 12.5% linear gels while Blaser et al used 10% gels. This may have reduced the resolution sufficiently to obscure these differences. Although this may be the explanation one can not exclude the possibility that there were no differences in the C.jejuni and C.coli isolates examined by these other authors and that the differences observed would have been found not to be a consistent feature had a greater number of isolates been examined. This question as to whether the 35K and 42K proteins represent a means of differentiation between C.jejuni and C.coli remains to be answered.

The protein band observed in the 87-95K weight region probably represents the 92K protein (protein a) as recently reported by Logan and Trust (1982). However whereas this protein was found to be heat modifiable in the present study, this behaviour was not reported by the latter authors. It should be noted however that at a dissociation temperature of $37^{o}C$ in the Logan and Trust gels the complexes of protein e obscure the high molecular weight region and it is possible that the heat modifiability of protein a was not observed by the authors for this reason. This 87-95K protein was not surface exposed as has been noted for protein a. In the present study a doublet band in the 72-75K molecular weight region was clearly present in radio-labelled surface proteins of C.jejuni isolates, these bands were not visible in outer membranes prepared by sarkosyl extraction, however a doublet was present in spheroplast material (Fig 3.3). Recently doublet bands in the 75K region of sarkosyl extracted membranes has been reported (band b ; Logan and Trust, 1982 ; Mills and Bradbury, 1984). These authors also found these proteins to be surface exposed. It seems likely therefore that these two proteins may be only weakly associated with the outer membrane and that the extraction procedure employed during this study removed the proteins from the outer

membranes. A number of minor outer membrane bands were identified in the present study principally in the 56-60K and 19-23K regions. These bands almost certainly have there equivalents in the 55K protein d and 20K protein g recently described (Logan and Trust, 1982) and in the minor proteins identified by Mills and Bradbury (1984). The differences in the number of minor bands reported by the various authors probably represent differences in the resolution of the various SDS-PAGE systems used.

Outer membrane material separated on PAGE gels and stained with Schiffs reagent revealed intense staining in the 27-30K molecular weight region. There was no evidence of 'step-ladder' staining characteristic of 'O' polysaccharide side chains, although this may have reflected a lack of sensitivity in the staining procedure used. However in a recent study using silver staining it was reported that C.jejuni LPS lacks 'O' side-chains and that a step-ladder effect at high loading concentrations was due to aggregation of LPS molecules (Logan and Trust 1984). The LPS was described as having a fast migrating species of ca. 14K and a slow migrating species of ca. 20K. The quite significant differences in these molecular weights compared to those found in the present study reflects differences in the SDS-PAGE systems used which influence the molecular weight values observed for LPS to an even greater extent than of that seen with proteins. The nature of the 130K band revealed by Schiffs staining was not determined but may represent aggregates of LPS or a high molecular weight glycoprotein.

Having identified the surface proteins of C.jejuni and C.coli the antigenic properties of these proteins were investigated using RIPA and immunoblotting procedures. The MOMP band (43-46K) was immunogenic during human infections as revealed by the RIPA procedure. However there were low levels of antibodies directed to this or a similar cross-reacting protein in human control sera. This back-ground level of antibodies may be due to to a low level of antibodies directed to the MOMP of thermophilic campylobacters, not unexpected considering the frequency of infections due to this organism, or due

to cross-reacting antibodies formed against the MOMP of other gram-negative bacteria, also not unexpected considering the highly conserved nature of the amino acid sequence of porins proteins (Hofstra and Dankert 1979 ; Overbeeke et al 1980). This low level of antibodies reacting to C.jejuni MOMP has also been recently reported by other workers (Logan and Trust, 1983 ; Blaser et al 1984 ; Mills and Bradbury, 1984 ; Nachamkin and Hart, 1985 ; Wenman et al 1985). Similar RIPA reactions of the MOMP with sera from homologously and heterologously immunised rabbits and with human convalescent sera has recently been reported (Blaser et al., 1984).

A number of workers have reported the detection of C.jejuni MOMP using immunoblots with rabbit sera (Logan and Trust, 1983 ; Blaser et al., 1984 ; Wenman et al., 1985) and human acute and convalescent sera (Blaser et al., 1984 ; Mills and Bradbury 1984 ; Nachamkin and Hart 1985 ; Wenman et al., 1985). This is in direct contrast with the present work in which the MOMP was not detected using the immunoblotting procedure. The most likely explanation for reactions within RIPA's and not immunoblots is that the epitope responsible for the reaction is defined by the three-dimensional structure of the folded molecule(s) and not by a primary amino acid sequence on the polypeptide chain. However as these separate investigators have reported the reaction of the MOMP during immunoblotting there would appear to be some other reason for this discrepancy. At present the reason(s) are unknown. The results of these other investigators suggest that the antibodies produced in response to a campylobacter infection and directed at the MOMP cross-react with the MOMP of thermophilic campylobacters strains unrelated to the infecting strain. Variations in patients responses to the MOMP have also been reported (Nachamkin and Hart, 1985).

Antibodies were readily detected using iodohydroxybenzimidate labelled flagella and human convalescent sera. Human control sera weakly precipitated the flagella band indicating low levels of specific or

cross-reacting antibodies. All the human convalescent sera from an outbreak precipitated the purified flagella from the homologous isolate as did eight out of nine sera from unrelated cases indicating substantial cross-reactivities. There have been no previous reports of the detection of C.jejuni flagella using the RIPA procedure and human sera, principally due to the use of ineffective labelling procedures by other investigators. During the present study confirmation of the presence of flagellin specific antibodies in a human convalescent serum was achieved by immunoblotting against the homologous strain and an aflagellate derivative. The studies on the surface antigens of C.jejuni were extended by immunoblotting nine matched isolates and sera from cases of campylobacter enteritis. There was considerable variation in the number of proteins to which antibodies were produced. While one patients serum was capable of reacting with a wide range of C.jejuni proteins other sera cross-reacted only with flagellin. Whether these different responses were host, disease process or pathogen related remains to be answered but have recently been confirmed by independent investigators (Nachamkin and Hart, 1985). Clearly from the immunoblots various flagellins are present within the isolates examined.

There was limited evidence that some isolates may contain more than one species of flagellin, and that these flagellins may be preferently recognised by the cross-reactions of heterologous sera since the reactions of serums SO9 and SO13 with isolate SO10 produced flagellin bands of slightly different molecular weights (Fig 3.18). Because of the diffuse nature of the flagellin band in SDS-PAGE and the immunoblots together with the limited number of reactions examined it was impossible to prove that a single isolate did contain more than one flagellin , however the present development of monoclonal antibodies to the flagellin molecule should supply the necessary finese with which to answer this question.

The present work does not supply sufficient data to explain the reactions seen in the immunoblots. For

example while most sera cross-reacted with most, if not all the flagellins two sera (SO8 and SO12) reacted with only half the isolates. One possible explanation however is that campylobacter flagellin contains both distinct common antigenic determinants and type specific determinants. Most of the hosts produced antibodies to the common and variable determinants while two produced antibodies only to the latter. In this form the hypothesis resembles that proposed to explain the immunological structure of N.gonorrhea pili (Heckels and Virji, 1984). It should be noted that competitive immunoassays using monoclonal antibodies raised against 81116 flagellin indicates the presence of at least four epitopes (Newell, 1983).

The antigenicity of C.jejuni flagellin using hyper-immune rabbit sera and immunoblotting has recently been presented (Logan and Trust, 1983 ; Blaser et al., 1984 ; Wenman et al., 1985). In all cases the flagellin was found to be both strongly immunogenic and antigenic and the resulting antibodies cross-reacted with all the other strains of thermophilic campylobacters examined confirming the presence of conserved antigenic determinants. A number of workers have also produced data on the antigenicity of flagellin using human sera in immunoblots. In one such study IgA, IgG and IgM antibody responses were noted in patients recovering from campylobacter enteritis. The antibodies produced cross-reacted strongly with the flagellins of other C.jejuni strains (Blaser et al., 1984). In an investigation into the production of antibodies during the course of a campylobacter infection a steady rise in flagellin and outer membrane protein specific antibiodies was reported (Mills and Bradbury, 1984). Once again cross-reacting antibodies were detected reacting with the flagellins of other thermophilic campylobacters. The identification of flagellin as a major surface antigen has been further confirmed by more recent work (Nachamkin and Hart, 1985 ; Wenman et al., 1985). The former authors also confirmed evidence presented here on the variability of the flagellin molecular weights between isolates.

To summarise: the evidence presented here and by other investigators on the antigenicity of the MOMP and flagellin of C.jejuni is as follows. The MOMP and flagellin constitute the two major protein antigens during campylobacter infections. Although both the MOMP and flagellin are the major antigens, the flagellin tends to induce greater antigen-antibody binding in immunoblots and are more consistently found in immunoblots compared to the MOMP. Normal human sera can react with the MOMP and flagellin to a limited extent. Antibodies produced in response to specific MOMP and flagellins can cross-react with MOMP's and flagellins of C.jejuni and C.coli strains unrelated to the infecting strain. These cross-reactions are more often found between flagellins of C.jejuni/coli than between the MOMP's, this is especially true when comparisons are made between C.jejuni/coli and more distantly related species such as C.fetus and C.laridis (Wenman et al., 1985). Thus campylobacter flagellins are stronger interspecies antigens than the MOMP suggesting greater conservation of the amino acid structure.

The immunoblot of serum SO7 with the nine isolates demonstrates the range of proteins that can be immunogenic. In a RIPA of strains and serum from the same outbreak (Fig 3.12) proteins of 49K and 27K were precipitated by homologous and heterologous sera respectively. A 27K protein was a major component of a glycine-HCl acid extract from C.jejuni. The characteristics of this particular protein, acid extractibility, surface exposure, lack of presence in sarkosyl extracted outer membranes, immunogenicity with a molecular weight of around 27K gives apparent identity with proteins of 29-30K recently described (Logan and Trust, 1983 ; Blaser et al., 1984 ; Mills and Bradbury, 1984 ; Wenman et al., 1985). Interestingly, a hyper-immune rabbit antiserum raised against one specific C.jejuni strain by the former authors detected a 31K protein in all the C.jejuni and C.coli strains examined. In the present study only one heterologous human convalescent sera reacted with the 27K protein despite

the fact that surface radiolabelling data suggests this protein is common to all C.jejuni and C.coli strains. This may reflect differences in the sensitivity of hyper-immune rabbit antisera tests compared with convalescent human sera or in the different immune responses associated with different animal species and/or antigen presentation. The detection of a 30K band in RIPA using human convalescent sera has recently been presented (Blaser et al., 1984). In the present study a 92K protein was detected by one serum to its homologous strain (Fig 3.19, serum SO8). An outer membrane protein of 92.5K has been recently reported as conferring heat-label antigenic specificity when hyper-immune rabbit antisera was used (Logan and Trust, 1983 ; Wenman et al., 1985). However Mills and Bradbury (1984) have reported a 92K OM protein in a strain which was detected using unrelated serum, suggesting that this specificity may be wider than at first thought.

The foregoing discussion has demonstrated the major part played by flagella as a surface structure and antigen on C.jejuni cells. Subsequent studies therefore concentrated on the biological role of C.jejuni flagella. Two variants were prepared from isolate 81116. All available data confirmed that variant SF1 possessed paralysed flagella and SF2 was aflagellate. The nature of the mutation in SF1 is not known but the production of flagellin by SF2 despite the lack of flagella may suggest a block at the insertion of the flagellin into the membrane or alteration of the flagellin molecule itself making polymerization into flagella impossible. The lack of the basal disc structure described by Curry et al (1983) suggests the former as a likely mechanism. The 'M' ring of Aquaspirillum serpens has regularly spaced fibrils emanating from it (Coulton and Murry, 1978) and it has been suggested these act as anchorage structures. This radial spacing is reminiscent of the patterns seen on the basal disc of C.jejuni. The basal disc has also been suggested as an important requirement in the assembly and function of flagella (reviewed by Doetsch

and Sjoblad, 1980).

The attachment of radiolabelled 81116, SF1 and SF2 to erythrocytes indicated that variations existed which were dependent not only on the state of flagellation of the isolates but also on the animal species from which the erythrocytes were obtained. In this assay the bacteria were mixed with the erythrocytes and it is proposed that the general order of attachment of SF2>SF1>81116 can be explained by hinderence of an outer membrane adhesin by the flagella of SF1 and 81116. The active flagella of 81116 causing greater inhibition than the non-active flagella of SF1. There were not only differences in attachment when erythrocytes from different species were used but also when erythrocytes from the same species but different animals were used. This examplifies the problems associated with biological assays of this sort. The problem may be resolved by using fixed erythrocytes which can be stored for longer periods. However careful experimentation would be required to eliminate the possibility of the fixation process changing the nature of the attachment. The reasons for the differences in attachment to erythrocytes from various animals is unknown but may reside in variations of the sugar moieties associated with the erythrocyte membrane. The adsorption isotherm data suggested that the attachment of SF2 and 81116 to rabbit erythrocytes was non-reversible. However a note of caution is required. As previously explained adsorption isotherm analysis depends on a number of assumptions, and even the attachment of streptococci to an inert support such as a hydroxylapatite surface requires careful analysis to take into account such variables as low affinity non specific binding, high affinity specific binding and multiple receptor binding (Staat and Peyton, 1984). However if it is assumed that the results obtained did reflect the true situation then the main difference between the binding of 81116 and SF2 does not reside in the intensity of adsorption (R/K value) but in the adsorption capacity of the surface (K') such that the erythrocyte surface was able to accommodate three times as many SF2 bacteria

compared to 81116 bacteria. As the erythrocyte surface itself was constant this would suggest that as bacteria were bound to the erythrocyte surface they altered that surface. This would support the hypothesis that the active flagella of 81116 cause inhibition of attachment by blocking access of other bacteria to the binding sites.

The attachment assays of 81116, SF1 and SF2 binding to tissue culture cells of human origin, in contrast to the binding to erythrocytes did not involve mixing of the two cell populations and therefore contact between the bacteria and cell surface depended on passive contact or active motility. The procedure used to wash the cell monolayer has been employed to study the high affinity attachment of S.typhimurium to HeLa cells (Jones et al., 1981) and it is likely that bacteria remaining attached to the cell surface after this procedure are held by strong multi-focal binding. Although the relative order of abilities of these three strains to attach to the different eukaryotic cell surfaces were consistent the percentage attachments were quite low. Nevertheless similar levels of attachment have been recorded for the attachment of S.typimurium to HeLa cells (Jones et al., 1981) and of V.parahaemolyticus to INT-407 cells (Gingras and Howard, 1980). In both the latter cases the attachments studied were of high affinity binding which could not be disturbed by fluid shear across the cell surface. A number of possibilities exist for low level adherence : (1) All the bacteria within the population exhibit weak, low affinity attachment (2) only a minority of the bacteria within the population possess the necessary adhesins and (3) the eukaryotic cell posses only a limited number of receptors for the bacterial adhesin. At present the reasons for this low attachment are unknown, however the fact that these cells are transformed may mean that the bacterial adhesin receptors have been modified or reduced in numbers on these cells and further experimentation would be required to show if this, or some other reason, were responsible.

It is perhaps of interest to note the specificity

exhibited in the attachment of SF1 to cells of intestinal origin as compared to Hela and Hep-2 cells. The greater ability of SF1 to attach to INT-407 cells as compared to 81116 and SF2 could be explained by the presence of specific adhesins associated with the campylobacter flagellum. These adhesins may be responsible for a tenuous attachment to the eukaryotic surface such that the presence of active flagella on the bacterial cell can cause the disassociation of the bacteria from that surface. This sort of mechanism could be considered a disadvantage in vivo but the model used does not take into account such factors as chemotaxis and physical damping of the flagella by the mucus layer of the intestinal tract.

The data from the attachment models used during this study can be used to propose a hypothetical mechanism for the attachment of C.jejuni in vivo. The active flagella allow the organism to penetrate the mucus layers and to approach the epithlial surface. The flagella (having small radius of curvature) penetrates to the cell surface to overcome the repulsive effects of the diffuse double layers and forms a tenuous attachment. Permanent attachment occurs through specific bacterial surface adhesins. The disease process proceeds through invasion and/or cytotoxin and/or endotoxin production. The non-specificity of the inhibition of attachment in the presence of various sugars may reflect a receptor composed of a complex of sugar residues or, alternatively, a number of receptors having different sugar residues, however inhibition was also exhibited by the non-sugar carbohydrate glucitol suggesting that this inhibition of attachment was of a more general nature. The greater inhibition of mannose compared to fucose is at variance to a recent study of the adherence of campylobacter species to INT-407 cells were fucose was found to be the most efficient inhibitor of attachment (Cinco et al., 1984). The reasons for this discrepancy are not known, however sufficient differences exist in the different media and conditions used to store and cultivate the organisms to made it possible that

modification of the adhesin(s) had occured.

Having establised the effect of flagella variations in the _in vitro_ assays the wild-type strain and its two variants were examined _in vivo_ using the infant mouse model. Although the aflagellate variant was rapidly cleared from the intestinal tract of the infant mice, 81116 and SF1 were both capable of successfully colonizing the lower portion of the intestinal tract for in excess of thirty days. This indicates that the possession of flagella and not active motility is the major deciding factor in successful colonization. Previous experimentation on the role of flagella in the adherence of bacteria to cell surfaces has involved non-flagellate mutants and therefore have not separated the possible roles of flagella associated adhesins and bacterial motility (Guentzel and Berry, 1975 ; Jones and Freter, 1976). A recent report on the role of _V.cholerae_ flagella in adherence used antibodies specific for LPS which were capable of immobilising the organisms. These paralysed bacteria also retained their ability to adhere to intestinal seqments (Attridge and Roweley, 1983).

It is clear that much still has to be learned about the pathogenicity of the thermophilic campylobacters despite the advances of recent years. From the data produced during this study it appears that campylobacter flagella are a major surface antigen to which the human host produces antibodies during an infection. These antibodies cross-react to other strains of _C.jejuni/coli_ although not universally. What effect do anti-flagella antibodies have and could a flagella vaccine be effective? There is evidence available from animal model studies that flagella vaccines can offer protection against _V.cholerae_ (Eubanks _et al._, 1977 ; Yancy _et al._, 1979), _Pseudomonas aeruginosa_ (Montie _et al._, 1982) and _Clostridium chauvoei_ (Tamura and Tanaka, 1984). There effect appears to be due to immobilisation of the organism and/or aggregation allowing removal from the target site. Immobilisation has been observed with antiflagella sera raised against _C.fetus_ (McCoy _et al._, 1976). It may not be necessary to use orally administered vaccines to stimulate the mucosal system as

intravenously administered <u>Salmonella</u> flagella selectivly localise in bronchial and intestinal lamina propria (Hunter, 1972).

The requirement for a vaccine effective against <u>C.jejuni/coli</u> would be a debatable issue but the current research into the biochemistry, immunology and pathogenesis of this common pathogen will at least supply the necessary knowledge on which to make these decisions.

```
 10 REM Amino Acid Analysis
 35 REM November 1983
 36 REM name$(DIM) now in effect!
 38 REM add a PROC for printer.
 40 :
 50 *TV255
 52 *TV 0,1
 55 MODE 6:VDU 28,0,23,39,1,12
 60 :
 90 PROCkeys
100 PROCdim
110 PROCtitle
120 :
180 PROCmenu
190 IF jump%=TRUE PROClist:GOTO 260
200 PROCinputs
210 PROClist
250 PROCarea
260 PROCcheckarea
300 PROCarea2
350 PROCspillage
400 PROChydrolysis
450 PROCresidue_ng
500 PROCanswer
515 :
520 *FX 220,27
522 REM Escape key re-enabled.
530 REM VDU 30:PRINT'" Results :-":VDU 13,11
540 GOTO 180
550 END
560 :
600 DEF PROCmenu
610 LOCAL x%,y%,count%
620 RESTORE 630
630 DATA Save final data to disc,Print results,Read
internal DATA statements,Type in new data,Load data from
```

disc,Modify current data,Verify last disc operation,Exit
from program

```
  650 CLS:x%=6:y%=2:maxmenu%=8:jump%=FALSE:save%=FALSE
  660 FOR count%=1 TO maxmenu%
  670    READ menu$
  680    PRINTTAB(x%,y%+count%);count%;") ";menu$
  690    NEXT
  700 PRINTTAB(x%,y%+maxmenu%+3)"Select option:";
  705 choice%=GET-48:IF choice%<0 OR choice%>maxmenu%
GOTO 705 ELSE PRINT;choice%
  710 ON choice% GOTO 721,722,723,724,725,739,726,738
  721 save%=TRUE:PROCdiscsave:GOTO 620
  722 GOTO 620:REM PROCprinter:GOTO 620
  723 opt%=1:ENDPROC
  724 opt%=2:ENDPROC
  725 opt%=3:save%=FALSE:PROCdiscsave:ENDPROC
  726 ON ERROR PROCerror
  727 *LOAD file$ 8000
  728 ON ERROR OFF:ENDPROC
  738 PRINT''''"Program now completed.Type RUN to
resume":VDU30:END
  739 jump%=TRUE
  740 ENDPROC
  895 :
  900 DEF PROCwait
  910 VDU 28,0,24,39,24,12
  920 PRINTSPC(5)"Press SPACEBAR to continue";
  940 REPEATUNTILGET=32
  950 VDU 12,28,0,23,39,1
  955 ENDPROC
  999 :
 1000 DEF PROCdim
 1002 VDU 23,245,102,102,102,102,126,96,192,128
 1003 VDU 23,231,62,102,102,62,6,60,0,0
 1004 VDU 23,220,48,48,48,54,3,6,12,15
 1005 aamax%=20
 1006 DIM name$(20)
 1010 REM *DIM g(20):?not required
 1020 DIM cS(20,2),cU(20),cL(20)
 1030 DIM LL(20),LU(20)
```

```
1040 DIM gL(20),fL(20),kL(20)
1050 DIM mU(20),pU(20),rU(20)
1060 DIM tU(20),pL(20),w1(20),sT(20)
1070 DIM w2(20),ssr(8)
1075 DIM head$(4)
1080 red=1:green=2
1085 head$(1)="Std(red)"
1086 head$(2)="Std(green)"
1087 head$(3)="Lysosyme"
1088 head$(4)="Unknown"
1090 file$=""
1095 *FX220,9
1096 REM The TAB key is now ESCAPE
1100 ENDPROC
1199 :
1200 DEF PROCmemory
1205 VDU 28,0,1,39,0,12
1210 DIM P%-1:PRINT"Memory left...";~HIMEM-P%;"(Hex),
";:PRINT;HIMEM-P%;"(Decimal)";
1215 VDU 28,0,23,39,1,12
1240 ENDPROC
1245 :
1250 DEF PROCinputs
1260 CLS
1265 PRINTTAB(31,2)"1270 PRINTTAB(0,4)"Amount LYSOSYME
added.......<   ";CHR$ 245;CHR$ 231;"> 1280
PRINTTAB(0,8)"Amount N-LEUCINE added.......<   nM> 1290
PRINTTAB(0,11)"Amount STANDARD amino acids..<   nM> 1292
PRINTTAB(0,13)"(Cystine is calculated at ";CHR$ 220;"
above value)"
1295 PRINTTAB(0,17)"Estimated M.Wt. of protein   <
,000>"'TAB(36)" 1300 INPUTTAB(30,4) f%
1310 IF f%=0 THEN f%=50:PRINTTAB(31,4);f%
1320 INPUTTAB(30,8) n
1330 IF n=0 THEN n=10:PRINTTAB(31,8);n
1340 INPUTTAB(30,11) d%
1350 IF d%=0 THEN d%=10:PRINTTAB(31,11);d%
1360 INPUTTAB(30,17) v%
1370 IF v%=0 THEN v%=63:PRINTTAB(31,17);v%
1375 TIME=0:REPEATUNTILTIME=100
```

```
1380 v%=v%*1000
1390 *FX 21,0
1391 REM flush keyboard buffer
1400 ENDPROC
1995 :
2000 DEF PROClist
2005 CLS
2006 @%=3
2010 RESTORE 8000
2050 FOR count%=1 TO aamax%
2060    READ name$(count%)
2065    REM name$=LEFT$(name$,3)
2070    PRINTTAB( 0,count%)count%;" ";name$(count%)
2080    NEXT
2085 @%=10
2090 ENDPROC
2095 :
2100 DEF PROCarea
2110 IF opt%=3 ENDPROC
2114 col1%=0:col2%=13
2115 PROCclearRT
2116 RESTORE9201
2118 IF opt%=2 PRINTTAB(col1%,0)"Std(RED)   Std(GREEN)";
2120 FOR aa%=1 TO aamax%
2125    IF opt%=1 READ cS(aa%,red):GOTO2150
2130    INPUTTAB(col1%,aa%) cS(aa%,red)
2140    INPUTTAB(col2%,aa%) cS(aa%,green)
2150    NEXT
2152 IF opt%=1 PROCreadCSGREEN
2155 RESTORE9301
2158 IF opt%=2 CLS:PRINTTAB(col1%,0)"Lysosyme   Unknown";
2160 FOR aa%=1 TO aamax%
2165    IF opt%=1 READ cL(aa%):GOTO2180
2170    INPUTTAB(col1%,aa%) cL(aa%)
2180    NEXT
2185 RESTORE9400
2190 FOR aa%=1 TO aamax%
2195    IF opt%=1 READ cU(aa%):GOTO2210
2200    INPUTTAB(col2%,aa%) cU(aa%)
2210    NEXT
```

```
2212 ENDPROC
2213 :
2214 :
2215 DEF PROCcheckarea
2216 FOR count%=1 TO 4
2217   alter%=FALSE
2219   PROCclearRT
2220   FOR aa%=1 TO aamax%
2225     PROCscreen(head$(count%),count%,0)
2242     NEXT
2243   PROCbadarea(count%)
2248   IF alter%=TRUE THEN count%=count%-1
2250   NEXT
2255 VDU 26,12
2260 ENDPROC
2495 :
2500 DEF PROCbadarea(n%)
2515 line%=0 : x%=1
2518 VDU 28,30,22,39,1,12
2519 PRINT"Line no.";
2520 FOR aa%=1 TO aamax%
2530   PRINTTAB(0,aa%)" ";aa%;
2540   NEXT
2560 VDU 28,0,24,39,22,12
2570 PRINT'" Give line number for corrections <  >"'"
 or press RETURN to continue.";
2580 INPUTTAB(35,1) line%
2590 IF line%<0 OR line%>aamax% GOTO 2580
2600 VDU 12:IF line%=0 ENDPROC
2602 alter%=TRUE:PRINTTAB(1,1)"Give correct value
for";name$(line%);
2605 VDU 28,18,22,39,1
2608 PRINTTAB(0,line%);"> .    <";
2610 INPUTTAB(x%,line%) newval$
2614 IF newval$="" ENDPROC
2615 newval=EVAL(newval$)
2616 IF n%=1 cS(line%,red)   =newval
2620 IF n%=2 cS(line%,green)=newval
2630 IF n%=3 cL(line%)   =newval
2640 IF n%=4 cU(line%)   =newval
```

```
2700 ENDPROC
2995 :
3000 DEF PROCarea2
3050 FOR aa%=1 TO aamax%
3060    IF cU(aa%)<0 THEN
LU(aa%)=(ABS(cU(aa%))/cS(aa%,green))*d% ELSE
LU(aa%)=(cU(aa%)/cS(aa%,red))*d%
3065    IF aa%=8 THEN LU(aa%)=LU(aa%)/2
3080    IF cL(aa%)<0 THEN
LL(aa%)=(ABS(cL(aa%))/cS(aa%,green))*d% ELSE
LL(aa%)=(cL(aa%)/cS(aa%,red))*d%
3085    IF aa%=8 THEN LL(aa%)=LL(aa%)/2
3088    NEXT
3090 ENDPROC
3095 :
4000 DEF PROCspillage
4010 PROCtitle
4020 spillfactorU=n/LU(13)
4022 spillfactorL=n/LL(13)
4025 CLS:PRINTTAB(5,6)"NOR-leucine correction
factor"'TAB(5) STRING$(29,"_")
4026 PRINT''TAB(5)"1. 'Unknown'  =";n;"/";LU(13)
4027 PRINT'TAB(19)"=";spillfactorU
4028 PRINT''TAB(5)"2. 'Lysosyme' =";n;"/";LL(13)
4029 PRINT'TAB(19)"=";spillfactorL
4030 FOR aa%=1 TO aamax%
4040    pU(aa%)=LU(aa%)*spillfactorU
4050    pL(aa%)=LL(aa%)*spillfactorL
4060    NEXT
4070 PROCwait
4090 ENDPROC
4095 :
4100 DEF PROChydrolysis
4120 RESTORE 8100
4140 FOR aa%=1 TO aamax%
4150    READ gL(aa%)
4160    kL(aa%)=gL(aa%)*f%
4170    mU(aa%)=kL(aa%)/pL(aa%)
4180    rU(aa%)=mU(aa%)*pU(aa%)
4190    NEXT
```

```
4195 ENDPROC
4199 :
4200 DEF PROCresidue_ng
4205 CLS:PROClist:PROCclearRT
4210 RESTORE 8301
4215 totall=0
4220 FOR aa%=1 TO aamax%
4230   READ sT(aa%)
4240   tU(aa%)=rU(aa%)*sT(aa%)
4250   totall=totall+tU(aa%)
4255   PROCscreen(" tU(aa%)",5,1)
4260   NEXT
4265 PROCwait
4270 ENDPROC
4295 :
4300 DEF PROCanswer
4305 VDU 26,12:PROCtitle
4310 x=v%/totall
4320 FOR aa%=1 TO aamax%
4330   w1(aa%)=x*rU(aa%)
4340   w2(aa%)=INT(w1(aa%)+0.5)
4350   NEXT
4405 PROClist:PROCclearRT
4415 PRINTTAB(0,0)"Total number residues"
4420 FOR aa%=1 TO aamax%
4430   PRINTTAB(0,aa%);w2(aa%),w1(aa%)
4440   NEXT
4450 PROCwait
4490 ENDPROC
4495 :
4600 DEF PROCreadCSCREEN:**
4610 RESTORE 9250        :**
4620 FOR aa%=1 TO aamax%:**
4630   READ cS(aa%,green) :**
4640   NEXT              :**
4650 ENDPROC:REM**********
4985 :
4990 REM #### PROCscreen( , , ) ####
4995 :
5000 DEF PROCscreen(variable$,type%,side%)
```

```
5005 REM PROCclearRT
5010 PRINTTAB(0,0) variable$
5040 sred%=1:sgreen%=11
5041 IF type%=1 ssr(1)=cS(aa%,red)
5042 IF type%=2 ssr(2)=cS(aa%,green)
5043 IF type%=3 ssr(3)=cL(aa%)
5044 IF type%=4 ssr(4)=cU(aa%)
5045 IF type%=5 ssr(5)=tU(aa%)
5048 REM FOR aa%=1 TO aamax%
5050 PRINTTAB(sred%,aa%); ssr(type%)
5052 IF side%=2 PRINTTAB(sgreen%,aa%); ssr(type%)
5055 REM IF ssg<>FALSE PRINTTAB(sgreen%,aa%); ssg
5060 REM NEXT
5200 ENDPROC
5295 :
5300 DEF PROCclearRT
5310 VDU 28,18,22,39,1,12
5320 ENDPROC
5995 :
6000 DEF PROCdiscsave
6002 ON ERROR PROCerror:GOTO 180
6010 VDU 12:PRINTTAB(9,1)"";
6012 VDU 28,0,12,39,2,12
6015 IF save%=TRUE PRINT"SAVING DATA ON DISC"' ELSE
PRINT"RETRIEVING FILE FROM DISC"'
6020 VDU14:*CAT
6025 VDU15
6030 VDU 28,0,23,39,12,12
6035 IF save%=FALSE PROCdiscin:PROCfilename:VDU
26,12:PROCtitle:ENDPROC
6040 PROCfilename
6050 :
6155 PRINTTAB(z%,y%+2) "ddmmyy";:INPUTTAB(z%,y%+2) date$
6156 IF VAL(date$)=0 OR LEN(date$)<>6 GOTO 6155
6157 year%=EVAL(RIGHT$(date$,2)):IF year%<83 OR
year%>100 GOTO 6155
6158 year$=CHR$(year%-18) : date$=LEFT$(date$,4)
6159 ?&25A=&20 :REM set CAPS-lock
6160 PRINTTAB(z%,y%+4) SPC(3):INPUTTAB(z%,y%+4) intl$
6165 IF LEN (intl$) >3 OR LEN (intl$) <1 GOTO 6160
```

```
6170 PRINTTAB(z%,y%+6) SPC(20):INPUTTAB(z%,y%+6) expt$
6172 IF LEN(expt$)>20 GOTO 6170
6180 PRINTTAB(z%,y%+8) SPC(2):INPUTTAB(z%,y%+8) trace$
6181 IF VAL(trace$)>26 OR trace$="0" GOTO 6180
6182 IF VAL(trace$)<>0 trace$=CHR$(VAL(trace$)+64)
6184 IF LEN(trace$)<1 OR LEN(trace$)>1 GOTO 6180
6185 PRINTTAB(z%,y%+10) SPC(2);:INPUTTAB(z%,y%+10) try%
6186 IF try%<1 OR try%>26 GOTO 6185
6187 try$=CHR$(try%+64)
6190 VDU 28,13,23,39,2,12
6200 PRINTTAB(0,12);date$''intl$''expt$''trace$''try$;
6210 PROCwait
6215 :
6220 file$=RIGHT$(trace$+date$+year$+try$,7)
6225 data=OPENOUT(file$)
6230 PRINT #data,date$,intl$,expt$,trace$,try$,aamax%
6235 PRINT #data,f%,n,d%,v%
6240 FOR count%=1 TO aamax%
6250    PRINT
#data,cS(count%,1),cS(count%,2),cL(count%),cU(count%),w2(
count%)
6260    NEXT
6290 CLOSE #data
6300 ON ERROR OFF
6390 ENDPROC
6395 :
6400 DEF PROCerror
6410 ON ERROR OFF
6420 CLOSE #0
6430 CLS:PRINTTAB(5,2)"Error condition
(";ERR;")"'TAB(5)"during disc access"''TAB(5);
6440 REPORT
6480 PROCwait
6490 ENDPROC
6495 :
6995 :
7000 DEF PROCdiscin
7005 CLOSE#0
7010 PRINTTAB(0,0)"Type exact title of disc file"'"you
require <        >"
```

```
7020 INPUTTAB(14,1) file$
7030 IF LEN(file$) <>7 GOTO 7010
7040 input=OPENUP(file$)
7050 INPUT #input,date$,intl$,expt$,trace$,try$,aamax%
7060 INPUT #input,f%,n,d%,v%
7070 FOR count%=1 TO aamax%
7080    INPUT
#input,cS(count%,1),cS(count%,2),cL(count%),cU(count%),w2
(count%)
7085    NEXT
7090 CLOSE #input
7095 ENDPROC
7096 :
7100 PROCfilename
7110 VDU 28,13,23,39,2,12
7120 PRINTTAB(0,12);date$''intl$''expt$''trace$''try$;
7130 PROCwait
7140 ON ERROR OFF
7290 ENDPROC
7395 :
7400 DEF PROCtitle
7410 VDU 28,0,1,39,0,12
7420 PRINTTAB(10,0)"AMINO ACID ANALYSIS"
7430 VDU 28,0,23,39,1
7440 ENDPROC
7490 :
7500 DEF PROCfilename
7510 x%=2:y%=0:z%=x%+12
7520 VDU 28,0,23,39,12,12
7530 IF file$<>"" PRINT"Previous title was
'";file$;"'.";
7540 PRINTTAB(x%,y%+2)"Expt date  <ddmmyy>"
7550 PRINTTAB(x%,y%+4)"Initials   <   >"
7560 PRINTTAB(x%,y%+6)"Expt title <";SPC20;">"
7570 PRINTTAB(x%,y%+8)"Trace no.  <   >   (1 to 26)";
7580 PRINTTAB(z%+5,y%+9)"(  or   )";
7590 PRINTTAB(x%,y%+10)"Correction <  >   (A to Z )";
7600 ENDPROC
7990 :
7995 :
```

```
8000 DATA Aspartic
acid,Threonine,Serine,Glutamine,Proline,Glycine,Alanine,C
ystine,Valine,Methionine,Isoleucine,Leucine,NOR-leucine,T
yrosine,Phenylalanine,Histidine,Lysine,Arginine,Tryptopha
n,Cysteine
8085 :
8090 REM Data for Theoretical nM of aa's...( gL(aa%) )
8100 DATA 1.46538
8101 DATA 0.48846
8102 DATA 0.6978
8103 DATA 0.34886
8104 DATA 0.13956
8105 DATA 0.83736
8106 DATA 0.83736
8107 DATA 0.27912
8108 DATA 0.41868
8109 DATA 0.13956
8110 DATA 0.41868
8111 DATA 0.55824
8112 DATA 0
8113 DATA 0.20934
8114 DATA 0.20934
8115 DATA 0.06978
8116 DATA 0.41868
8117 DATA 0.76758
8118 DATA 0.41868
8120 DATA 1:REM cysteine
8299 :
8300  REM DATA for MWt of aa residues    ( sT(aa%) )
8301 DATA 115.1
8302 DATA 101.12
8303 DATA  87.09
8304 DATA 129.13
8305 DATA  97.13
8306 DATA  57.07
8307 DATA  71.09
8308 DATA 103.16
8309 DATA  99.15
8310 DATA 131.21
8311 DATA 113.18
```

```
8312 DATA 113.18
8313 DATA 0
8314 DATA 163.19
8315 DATA 147.19
8316 DATA 137.16
8317 DATA 128.19
8318 DATA 156.2
8319 DATA 186.23
8320 DATA 0
8999 :
9195 REM *** vary the following data*** 9196 :
9200 REM Data for AREA of STANDARD (red)
9201 DATA 1.151
9202 DATA 1.338
9203 DATA 1.386
9204 DATA 1.277
9205 DATA 0.816
9206 DATA 1.483
9207 DATA 1.3899
9208 DATA 2.01
9209 DATA 1.274
9210 DATA 1.588
9211 DATA 1.296
9212 DATA 1.712
9213 DATA 1.465
9214 DATA 1.544
9215 DATA 1.578
9216 DATA 1.207
9217 DATA 1.743
9218 DATA 1.026
9219 DATA 1.297
9220 DATA 1
9249 REM Data for AREA of STANDARD (green)
9250 DATA 0.36,0,0,0,0,0,0,0,0,0,0,0,0.629,0,0,0,0,0,0,0
9300 REM Data for AREA of LYSOSYME
9301 DATA -2.058
9302 DATA 2.465
9303 DATA 3.577
9304 DATA 2.3
9305 DATA 0.47
```

```
9306 DATA 4.416
9307 DATA 4.111
9308 DATA 1.803
9309 DATA 1.776
9310 DATA 0.792
9311 DATA 1.962
9312 DATA 3.569
9313 DATA 1.856
9314 DATA 1.155
9315 DATA 1.3216
9316 DATA 0.399
9317 DATA 2.303
9318 DATA 3.132
9319 DATA 2.995
9320 DATA 1:REM cysteine
9395 :
9399   REM DATA FOR   UNKNOWN
9400 DATA 3.125
9401 DATA 2.175
9402 DATA 3.063
9403 DATA 2.453
9404 DATA 0.061
9405 DATA 3.144
9406 DATA 2.597
9407 DATA 0
9408 DATA 1.156
9409 DATA 0
9410 DATA 1.8012
9411 DATA 2.044
9412 DATA 2.044
9413 DATA 0.12
9414 DATA 0.968
9415 DATA 0.09
9416 DATA 1.524
9417 DATA 0.589
9418 DATA 0
9419 DATA 0
```

$\bar{x}=$ mean attachment to cells by bacteria A

$\bar{y}=$ mean attachment to cells by bacteria B

$d=$ Ø i.e. we test $\mu_1 = \mu_2$

$\varepsilon=$ level of significance

$\Phi=$ degree of freedom

Test for difference d of means

We test null hypothesis Ho; $\mu_1 - \mu_2 = d$ for the difference of means of two normal populations.

$$x = \left\{ x_1, x_2 \cdots\cdots, x_{n1} \right\} \quad , \bar{x} = \frac{1}{n1} \sum_{i=1}^{n_1} x_i$$

$$y = \left\{ y_1, y_2 \cdots\cdots, y_{n2} \right\} \quad , \bar{y} = \frac{1}{n2} \sum_{i=1}^{n_2} y_i$$

$$t = \frac{\bar{x} - \bar{y} - d}{\sqrt{\dfrac{1}{n1} + \dfrac{1}{n2}} \; \sqrt{\dfrac{\Sigma x_i^2 - n_1 \bar{x}^2 + \Sigma y_i^2 - n_2 \bar{y}^2}{n_1 + n_2 - 2}}}$$

For $t\phi\left(\dfrac{\epsilon}{2}\right)$ of $\phi = n_1 + n_2 - 2$

Ho is accepted when $-t\phi\left(\dfrac{\epsilon}{2}\right) < t < t\phi\left(\dfrac{\epsilon}{2}\right)$.

Ho is rejected otherwise.

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