ENTEROTOXIGENIC *Escherichia coli* INFECTION OF PIGS: EXPRESSION, EXTRACTION, PURIFICATION AND THE ADHESIVE PROPERTIES OF THE K88 FIMBRIAL ADHESIN

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Doctor of Philosophy

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The ability of *Escherichia coli* to express the K88 fimbrial adhesin was satisfactorily indicated by the combined techniques of ELISA, haemagglutination and latex agglutination. Detection of expression by electron microscopy and the ability to metabolize raffinose were unsuitable.

Quantitative expression of the K88 adhesin was determined by ELISA. Expression was found to vary according to the *E.coli* strain examined, media type and form. In general it was found that the total amount was greater, while the amount/cfu was less on agar than in broth cultures. Expression of the K88 adhesin during unshaken batch culture was related to the growth rate and was maximal during late logarithmic to early stationary phase.

A combination of heat extraction, ammonium sulphate and isoelectric precipitation was found suitable for both large and small scale preparation of purified K88ab adhesin. Extraction of the K88 adhesin was sensitive to pH and it was postulated that this may affect the site of colonisation of by ETEC *in vivo*.

Results of haemagglutination experiments were consistent with the hypothesis that the K88 receptor present on erythrocytes is composed of two elements, one responsible for the binding of K88ab and K88ac and a second responsible for the binding of the K88ad adhesin.

Comparison of the haemagglutinating properties of cell-free and cell-bound K88 adhesin revealed some differences probably indicating a minor conformational change in the K88 adhesin on its isolation. The K88ab adhesin was found to bind to erythrocytes over a wide pH range (pH 4-9) and was inhibited by αK88ab and αK88b antisera. Inhibition of haemagglutination was noted with crude heparin, mannan and porcine gastric mucin, chondrosine and several hexosamines, glucosamine in particular. The most potent inhibitor of haemagglutination was n-dodecyl-β-D-glucopyranoside, one of a series of glucosides found to have inhibitory properties. Correlation between hydrophobicity of glucosides tested and degree of inhibition observed suggested hydrophobic forces were important in the interaction of the K88 adhesin with its receptor.

The results of Scatchard and Hill plots indicated that binding of the K88ab adhesin to porcine enterocytes in the majority of cases is a two-step, three component system. The first K88 receptor (or site) had a K_a of $1.59 \times 10^{14} M^{-1}$ and a minimum of 4.3×10^4 sites/enterocyte. The second receptor (or site) had a K_a of $4.2 \times 10^{12} M^{-1}$ with a calculated 1.75×10^5 sites/enterocyte.

Attempts to inhibit binding of cell-free K88 adhesin to porcine enterocytes by lectins were unsuccessful. However, several carbohydrates including trehalose, lactulose, galactose 1→4 mannopyranoside, chondrosine, galactosamine, stachyose and mannan were inhibitory. The most potent inhibitor was found to be porcine gastric mucin. Inhibition observed with n-octyl-α-D-glucopyranose was difficult to interpret in isolation because of interference with the assay, however, it agreed with the results of haemagglutination inhibition experiments.

KEY WORDS: *Escherichia coli*, K88 adhesin/receptor, expression, purification, haemagglutination.

For my Mother and Father

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ABBREVIATIONS

ABTS	2'2'azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cfu	colony forming units
cmc	critical micellar concentration
°C	degrees centigrade
dH_2O	distilled water
DNA	deoxy-ribonucleic acid
EHEC	enterohaemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ETEC	enterotoxigenic *Escherichia coli*
g	gramme
g/l	grammes per litre
HA	haemagglutination titre
HAP	haemagglutination titre of purified K88 adhesin
hr	hour
IS	insertion sequence
$K88^-$	K88 negative
$K88^+$	K88 positive
KDal	Kilodaltons
l	litre
M	Molar mass
MDal	MegaDalton
mg	milligramme
min	minute
ml	millilitre
mRNA	messenger ribonucleic acid
MR	mannose resistant
MS	mannose sensitive
NCTC	national collection of type cultures
NRS	normal rabbit serum
nm	nanometre
OD	optical density
OMP	outer membrane protein
PAGE	polyacrylamide-gel electrophoresis
PBS	phosphate buffered saline
PBS+M	phosphate buffered saline and 2% w/v D-mannose.
PBST	phosphate buffered saline and tween-20
pI	isoelectric point
sd	standard deviation
SDS	sodium dodecylsulphate
se	standard error
sem	standard error of the mean
TBS	Tris-buffered saline
t_d	doubling time
TTBS	tween Tris-buffered saline
Tris	hydroxymethyl methylamine
v/v	volume per volume
w/v	weight per volume
α	anti (serum)
μ	growth rate
μg	microgramme
μl	microlitre
♥,♣,☺,☻,♪, ☞,☜,☼,*,$	identifying symbols

1 INTRODUCTION

1.1 Scope of introduction.

The area of bacterial adhesin/receptor interaction has been constantly expanding since it was first discovered that bacteria could adhere to cell surfaces. Adhesin/receptor systems have been recognised for a wide range of bacterial genera (Beachey 1981, Jones and Isaacson 1983, Ofek and Sharon 1990). It would be impracticable to give a satisfactory introduction to all these systems. Therefore, this introduction will concentrate on the K88 adhesin/ receptor system for *Escherichia coli*. Where of relevance other *E.coli* adhesin/receptor systems will be described.

1.2 General.

1.2.1 Origin and use of the term fimbriae.

Since their independent discovery in 1949 by Anderson and Houwink, non-flagellate, filamentous bacterial appendages have been subsequently referred to as "filaments", "bristles" "fimbriae", "pili" or "cilia". The term fimbriae (singular fimbria) was first proposed by Duguid in 1955 who felt that the original term, filament, proposed by Houwink and van Iterson was inadequate and indistinctive (Ottow 1975). Subsequently, Brinton urged that the term fimbriae should be replaced by the designation pili (Latin for hair or hair-like structure) since the term fimbriae (Latin for thread, fibre or fringe) was linguistically incorrect (Ottow, 1975). Today, the term fimbriae is generally used because of priority of usage while the term pili is usually taken to indicate filamentous, non-flagella appendages involved in conjugation (Jones and Isaacson 1983).

With the finding that at least two morphological types of fimbriae could be discerned by electron microscopy, the term fimbrillar fimbriae has come into use. This term is used to signify those fimbriae that are very thin , flexible and have a diameter of between 2-5nm. The majority of fimbriae from *E. coli*

however are rigid, have a diameter of ~7nm, an apparent axial hole and as a group retain the term fimbriae (Mooi and De Graaf 1985).

1.2.2 Terms of reference used in adhesin/receptor systems.

The term adhesin is used to denote any substance on the bacterial surface that mediates the attachment of the bacterium to the mammalian cell surface. The term lectin is also used to describe some adhesins. Haemagglutinin is used to describe any component of the bacterial surface that causes the agglutination of erythrocytes. Because of their similarity in function, adhesins and haemagglutinins are often synonyms. Early experiments showed that the activity of many agglutinins/adhesins could be inhibited with α-mannosides and were accordingly termed mannose-sensitive (MS). The activity of others was not inhibited by α-mannosides and these were termed mannose-resistant (MR) (Duguid *et al.* 1955). Receptors are those components of the animal cell surface that react specifically with bacterial adhesins and haemagglutinins, and are responsible for the adhesion of bacteria to animal cell surfaces (Jones 1977, Jann and Hoschutzky 1990).

1.2.3 Why do bacteria adhere?

In the past two decades, it has become evident that bacterial adhesion, in most cases mediated by specific fimbriae, is a prominent if not essential feature in the pathogenicity not only of *E.coli* but of all bacteria that infect epithelial surfaces (Candy 1980, Beachey 1981, Mooi *et al.* 1984, Klemm 1985, Jann 1987, Jann and Hoschutzky 1990). However, it is not only pathogenic bacteria that benefit from adhesion since many commensals also adhere (Sugarman 1980, Klemm 1985). For both pathogenic and commensal bacteria, adhesion allows persistence in an area from which under normal circumstances they would be removed e.g. by peristalsis (Jones 1977, Candy 1980, Bijlsma 1985a, Jann 1987, De Graaf 1988, Jann and Hoschutzky 1990, Svanborg-Eden *et al.* 1990). In addition, the close positioning to an epithelial

surface allows for improved acquisition of nutrients and avoidance of the host immune response (Jones 1977, Jann 1987, Jann and Hoschutzky 1990). For pathogenic bacteria, adherence also allows for more efficient delivery of both exo- and endotoxins, colonisation of specific tissues or sites and, in the case of invasive organisms enhances access to the underlying tissues (Beachey 1981, Klemm 1985, Jann 1987, Jann and Hoschutzky 1990, Svanborg-Eden *et al.* 1990).

1.2.4 Adherence is not always beneficial to the bacterium.

Epithelial cell surface layers are continually shed. Over a period of time, this would substantially reduce the microbial population if all cells remained firmly attached to the shed surface (Jones 1977). In addition, the adherence to polymorphonuclear cells would lead to the inadvertent destruction of the bacterium (Beachey 1981, Svanborg-Eden *et al.* 1984, Rodriguez-Ortega *et al.* 1987). The most obvious method available to the bacterium to overcome these disadvantages is to modify its adhesive ability (Jones 1977). Indeed in many bacterial species the expression of organs of attachment is regulated e.g. phase variation (see Section 1.5.4) of type 1 fimbriae in *E. coli* (De Graaf 1990).

1.2.5 Physicochemical aspects of adhesion.

An important factor to consider is that before bacteria can interact with eukaryotic cells they must overcome the natural forces of repulsion due to the sum negative surface charge present on both cell types (De Graaf and Mooi 1986). The theory of interactions between like-charged particles at a long distance has been considered in terms of the colloidal theory. Known as the DLVO theory after its originators (Deryagin, Landau, Verwejj and Overbeek) it predicts the sum contribution of attractive and repulsive forces with distance based on van der Waals and electrostatic attractions (Deryagin and Landau 1941, Verweij and

Overbeek 1948). Figure 1.1 shows the sum forces of attraction and repulsion as predicted by the DLVO theory.

As seen in Figure 1.1 there are two energy minima (5-8 and <2nm) and a single energy maximum. It may be hypothesized that as a bacterium approaches a cell it enters the secondary minimum with weak reversible binding occurring. However, due to the energy maximum the bacterium cannot approach any closer. It has been demonstrated that the forces of both attraction

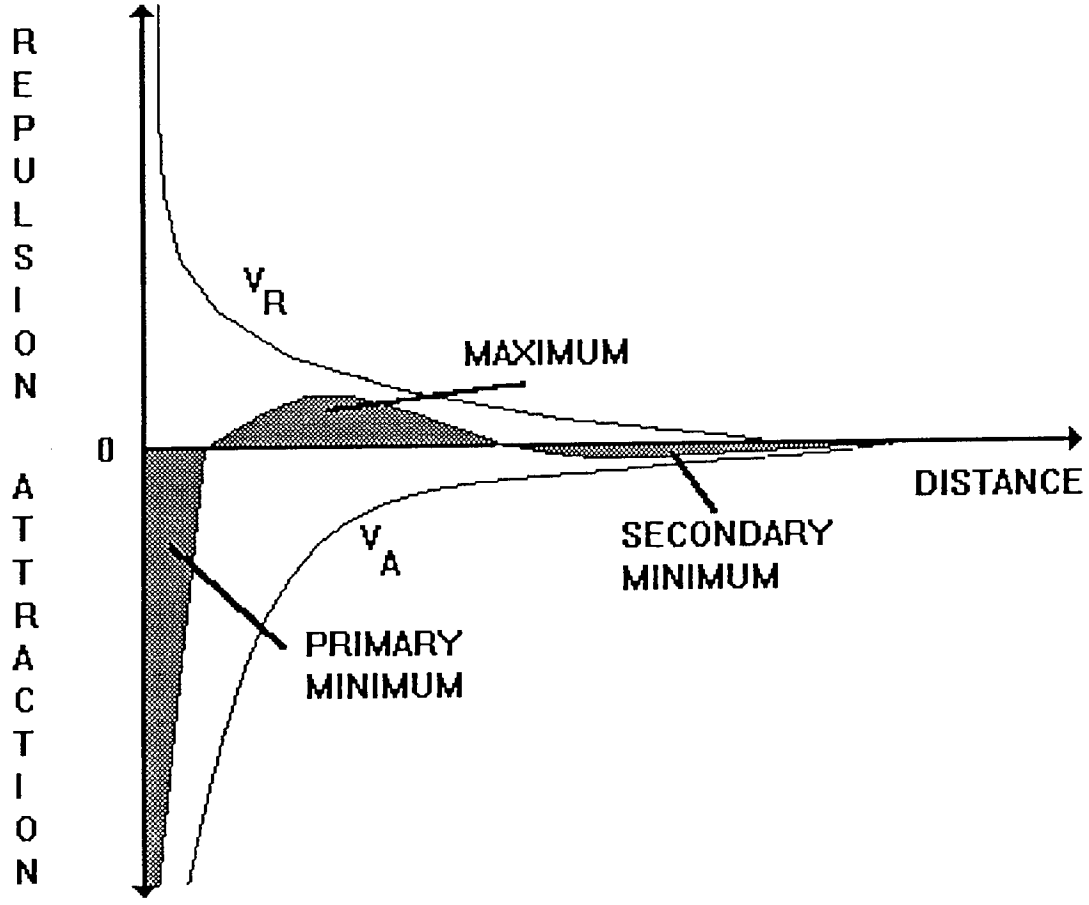


Figure 1.1 *Schematic diagram of the total interaction energy V_T (shaded area) between two particles e.g. a bacterium and epithelial cell, as the sum of the electrostatic energy of repulsion (V_R)and the energy of attraction (V_A).*

After De Graaf 1986

and repulsion decrease with the decreasing radii of curvature of the particles concerned. The degree of reduction however, is greater for the forces of repulsion than those of attraction i.e. the energy maximum decreases. Thus individual fimbria with considerably smaller radii of curvature than the bacterial cell are able to penetrate the energy maximum, bind within the primary energy minimum and irreversibly attach a bacterium to a cell. Note that the bacterium itself remains in the secondary energy minimum (Weiss and Harlos 1972).

Interactions between two particles, in this case a bacterium and epithelial cell at a short distance have also been described in terms of surface hydrodynamics (Absolom *et al.* 1983). Here, non-specific short-range interactions are considered as in systems where the effect of electric charges and specific biochemical reactions may be disregarded. This latter theory concludes that where the surface tension of the bacterium is greater than that of the medium, adhesion is greater to substrata with a high surface tension. When the surface tension of the bacterium is lower than that of the medium, adhesion will occur to substrata with a low surface tension. In the case of bacterial adhesion to epithelial cells the surface tension of the bacterial extracellular materials (e.g. polysaccharides) that surround the organism should be considered and not the surface tension of the bacterium itself (De Graaf and Mooi 1986).

Both of the above theories do not adequately describe adhesin/receptor reactions which occur at close range (Jones 1977). Short range forces that may be involved here include electrostatic bonds, hydrogen bonds, van der Waals bonds, hydrophobic interactions and perhaps covalent bonds (De Graaf and Mooi 1986).

Finally, it should be stressed that any theory that attempts to explain bacterial adhesion should take into account hydrodynamic shear forces. In the case of animals, these forces are generated by the flow of mucus, saliva, urine, and intestinal contents through a restricted passage. For a given velocity of flow

of solvent, the hydrodynamic force acting on an attached particle has been shown to be roughly proportional to the third power of the dimensions of that particle (Dahneke 1975). In practice, the hydrodynamic forces generated at low solvent velocities are quite able to displace bacteria bound in the secondary minima predicted by the DLVO theory (De Graaf and Mooi 1986).

1.2.6 Further considerations for the mechanism of bacterial adhesion.

As more information has been gathered on the adherence of bacteria to mucosal surfaces it has become apparent that the process is multi-factorial and not just dependent on the simple binding of bacteria to epithelial cells. It has been proposed (Freter 1981) that the following major steps are involved:-

a Chemotactic attraction of motile bacteria to the mucus gel lining the various body tracts

b Penetration of and trapping within the mucus gel (which may be passive or actively promoted by bacterial motility and/or chemotaxis).

c Adhesion to receptors in the mucus gel or to mucosa-associated layers of the indigenous microflora.

d Adhesion to the epithelial cell surfaces

e Multiplication of the mucosa-associated bacteria

Each of the above steps can be modified or reversed entirely by substances such as taxins, inhibitors of adhesion and substances for bacterial growth that are present in the mucosal micro-environment (Freter 1981).

Association with the bacterial mucosa is often important for bacterial colonisation but can also lead to more effective elimination from the host (Freter 1981). The role of mucin is complicated since it is becoming apparent that its variability allows it to act as a receptor mimic (Neutra and Forstner 1987). Mucin receptors have been found for a wide range of organisms such as *Streptococcus mutans*, influenza virus, *S.sanguis*, *Bacteroides spp.*, and enterotoxigenic *E.coli* (ETEC) while other organisms such as *Shigella flexneri*, *Treponema spp.* and *Vibrio cholerae* have been shown to bind mucin (Neutra and Forstner 1987, Metcalfe 1991). In general it seems that where mucin is strongly bound to the underlying epithelial cells it may promote attachment, while where it is loosely bound it can lead to specific and non-specific removal of an organism from the host (Isaacson 1977, Parsons and Mulholland 1978, Freter 1981, Neutra and Forstner 1987, for more details of the structure and function of mucin see Section 1.6.3).

1.2.7. *Escherichia coli* types involved in gastrointestinal disorders.

E.coli responsible for intestinal disorders can be classified into enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEC) and enterohaemorrhagic (EHEC) types. The ETEC strains are characterised by the production of heat stable (ST) and/or heat-labile (LT) enterotoxin and by their ability to adhere to the intestine (De Graaf and Mooi 1986). EPEC are strains that are able to cause diarrhoea but that do not produce either ST or LT and are not invasive by the Sereny test. The pathogenic mechanism of diarrhoea caused by EPEC has yet to be established, although enterotoxins distinct from LT and ST have been identified in some EPEC strains (Knutton *et al.* 1987). EIEC strains are associated with a dysentery-like diarrhoeal disease. They are able to adhere to cultured HEp-2 cells and human enterocytes. *In vivo*, EIEC adhere and subsequently invade the deeper tissues by an unknown mechanism (De Graaf and Mooi 1986, De Graaf 1990). EHEC are strains associated with

outbreaks of haemorrhagic colitis. They are not invasive and do not produce either LT or ST, however, they do produce elevated levels of a shiga-like cytotoxin. Recently, it has been found that some strains possess a 60 Megadalton plasmid which codes for fimbriae enabling them to adhere to cultured Henle-407 intestinal cells (Karch *et al.* 1987).

The first fimbrial adhesin associated with ETEC was described in 1961 (Orskov *et al.* 1961). Termed K88 it was subsequently found that this adhesin was essential for the pathogenicity of the strains that possessed it (Williams-Smith and Linggood 1971, Williams-Smith and Huggins 1978). The remainder of this introduction will describe in detail the distribution, function, structure, location, biogenesis, expression and receptor interaction of what is probably the most widely researched fimbria associated with enteric disease, K88.

1.2.8. Occurrence of adhesins on enterotoxigenic *Escherichia coli.*

In 1893 while investigating an outbreak of calf scour Jensen demonstrated the association of *E.coli* with diarrhoea and suggested that an analogous disease occurred in piglets (Bijlsma 1985a). From their location in electron microscopic studies, it was first suggested that fimbriae serve as organs of attachment (Houwink 1949). The results of haemagglutination studies further suggested that fimbriae act as organs of adhesion (Duguid *et al.* 1955). In 1961 a new K antigen (K88) was described which was common to *E.coli* strains causing diarrhoea in neonatal pigs (Orskov *et al.* 1961). Subsequently it was shown that K88 was fimbrial in nature (Stirm *et al.* 1967a,b), was an adhesin (Williams-Smith and Linggood 1971, Bertschinger 1972, Jones and Rutter 1972, Gibbons *et al.* 1975, Burrows *et al.* 1976) and was essential for the pathogenesis of ETEC possessing it (Williams-Smith and Linggood 1971, Gibbons *et al.* 1975). As shown in Table 1.1 several fimbrial adhesins have now been described. The fimbriae listed are also known as host specific fimbriae since strains expressing them are usually only found associated with

Table 1.1 Fimbrial adhesins associated with enteric disease

ADHESIN	HOST SPECIES	YEAR OF DESCRIPTION	REFERENCE
K88	PIGLET	1961	Orskov *et al.* 1961
K99	CALF,LAMB, PIGLET	1972	Smith and Lingood 1972
CFA1	MAN	1975	Evans *et al.* 1975
987P	PIGLET	1976	Nagy *et al.* 1976
CFA2	MAN	1978	Evans and Evans 1978
F41	CALF	1980	Morris *et al.* 1980
F(Y)	CALF	1980	Morris *et al.* 1985
AH25	CALF	1982	Morris *et al.* 1985
E8875	MAN	1982	Thomas *et al.* 1982
RDEC-1	RABBIT	1983	Cheney *et al.* 1983
F165	PIGLET	1986	Fairbrother *et al.* 1986
CS31A	CALF	1988	Girardeau *et al.* 1988

one or a limited number of host species (De Graaf and Mooi 1986). In addition to these so-called host specific adhesins, most ($\sim 70^{\%}$) pathogenic and commensal strains express a common fimbrial adhesin known as type 1 (Jones 1977, Levine *et al.* 1983, Orskov and Orskov 1983, Klemm 1985, Wold *et al.* 1988, Oudega and De Graaf 1988). This illustrates a general point that many *E.coli* strains can express more than one fimbrial type (Schneider and To 1982, Orskov and Orskov 1983, Klemm 1985, Evans *et al.* 1986, De Graaf and Mooi 1986, Gander and Thomas 1987, Finlay and Falkow 1989, Korhonen *et al* 1990, Moon 1990).

1.3 Mode of infection by ETEC expressing the K88 fimbria.

1.3.1 Incidence of infection by ETEC expressing the K88 antigen.

ETEC expressing the K88 antigen are the most prevalent form of *E.coli* infection found worldwide wherever pigs are raised in high numbers (Rapacz and Hasler-Rapacz 1986, Wilson and Francis 1986). It has been estimated that $K88^+$ ETEC cause 50% of the 10 million piglet deaths each year (Walters and Sellwood 1982). As can be readily appreciated, much research effort has been concentrated on the factors affecting the incidence and control of a pathogen of such economic importance.

1.3.2 Historical perspective and characteristics of infection mediated by ETEC expressing the K88 antigen.

During a serological analysis of a number of *E.coli* types frequently isolated from oedema disease and enteritis in pigs it was found that many of the strains isolated expressed a previously unrecognised antigen termed K88 (Orskov *et al.* 1961). With one of the strains used by Orskov *et al.* (1961) it was found that diarrhoea could be induced in hysterectomy-derived, 1 day-old pigs (Kramer and Nderito 1967). When using a non-pathogenic strain, even 10 times the dose had no effect. The authors reported that colonization of the intestine started 7hr after oral administration of 10^2 pathogenic *E.coli* in the ileum and large intestine. Diarrhoea always appeared concurrently with the detection of pathogenic microorganisms in the duodenum (Kramer and Nderito 1967).

Arbuckle (1970) investigated the distribution of pathogenic and non-pathogenic *E.coli* strains after experimental challenge and in natural infection of pigs. Fluorescent staining showed that adhesion was first apparent 6-7hr after challenge in the posterior small intestine at the base of villi. Bacterial colonisation appeared to start at the base and gradually progressed towards the

villi tips. The author postulated that the initial site of colonisation may be influenced by the comparatively low flow rate of gut contents both at the base of villi and in the posterior small intestine. It was further suggested that fimbriae may act as adhesins *in vivo*. In a similar study, Bertschinger *et al.* (1972) found that after challenge of pigs, $K88^+$ bacteria were characteristically found along the villi from tip to base and contiguous to the brush border but not in the crypts of the small intestine. Using electron microscopy the authors reported an electron-lucent region that separated the bacteria from microvilli in ultrathin sections. One of several possible explanations for this region was that it was occupied by the K88 antigen. Jones and Rutter (1972) presented evidence that the K88 antigen was produced *in vivo* and that $K88^+$ bacteria adhered to the mucosa of the small intestine . Adhesion of both $K88^+$ bacteria and cell free K88 antigen to small intestinal tissue was demonstrated *in vitro*. This adhesion was inhibited by anti-K88 antiserum and in the case of $K88^+$ bacteria did not occur when the organisms were cultivated at 18°C. The authors concluded that the K88 antigen was responsible for the adhesion of $K88^+$ bacteria to the wall of the small intestine, and that adhesion was essential for the virulence of $K88^+$ bacteria in conventionally reared piglets.

Convincing evidence of the role of the K88 antigen as a virulence determinant came from the studies of Smith and Linggood (1971). Here, the role of the K88 antigen as an adhesin in promoting colonization of the intestine was studied either by implanting the plasmid encoding the K88 antigen and a plasmid encoding the production of enterotoxin into non-pathogenic strains of *E.coli* or by removal of these plasmids from pathogenic strains and subsequently feeding such modified strains to neonatal pigs. The implantation of the K88 and enterotoxin plasmids into a non-pathogenic *E.coli* strain resulted in the ability of the modified strain to colonize the small intestine and to cause the development of diarrhoea. A $K88^-$ *E.coli* strain derived from a $K88^+$ enteropathogenic *E.coli* failed to proliferate in the anterior small intestine and

did not cause diarrhoea in newborn pigs. Introduction of a K88 plasmid from another *E.coli* strain back into a strain previously cured of the K88 plasmid restored the strains ability to produce diarrhoea. An important point to note is that the introduction of both the K88 plasmid and the plasmid coding for enterotoxin production into a K12 *E.coli* did not result in a strain capable of causing diarrhoea. This latter finding emphasizes that the ability of ETEC to cause disease is more complicated than the simple possession of an adhesin and the ability to produce enterotoxin (Chan *et al.* 1982, Gaastra and De Graaf 1982). More recent infection experiments with cloned K88 plasmids have confirmed that possession of the K88 antigen alone confers adhesiveness and is not facilitated by some other plasmid encoded factor (Dougan *et al.* 1983, 1986).

Although colonisation starts in the posterior small intestine, the possession of the K88 antigen enables *E.coli* to proliferate throughout the small intestine (Kramer and Nderito 1967, Williams-Smith and Huggins 1978, Sellwood 1981, Moon 1990). Colonisation of the anterior small intestine of the pig, where the speed of chyme flow is greatest, is characteristic of ETEC possessing K88 fimbriae since ETEC expressing either 987P or K99 fimbriae are associated with the colonisation of the posterior small intestine. (Williams-Smith and Huggins 1978). It has been suggested that a possible explanation for this observed difference in colonisation pattern reflects the comparatively greater "efficiency" of the K88 fimbrial adhesin.(Williams-Smith and Huggins 1978). Alternatively, the difference in colonisation pattern may be attributable to the enhanced stability of the K88 fimbria at acid pH (Payne *et al.* 1991). The ability to colonize the anterior small intestine results in severe diarrhoea because of the comparatively high susceptibility of this region to the effects of enterotoxin activity.

Infection studies have shown that the K88 antigen is associated with both neonatal and post-weaning disease in pigs. This is also unlike *E.coli* expressing

either K99 or 987P fimbrial antigens which are associated with neonatal disease only (Runnels *et al.* 1980, Gaastra and De Graaf 1982, Wilson and Francis 1986, Lindahl et al 1987). Experiments with isolated porcine intestinal epithelial cells have confirmed that the adhesion of $K88^+$ *E.coli* is independent of age while the adhesion of $K99^+$ bacteria decreases with the age of the pig (Runnels *et al.* 1980). However, in the case of bacterial infection with $K99^+$ ETEC, the loss of the ability to adhere to isolated epithelial cells does not correlate with the age at which resistance to ETEC induced disease becomes apparent (Runnels *et al.* 1980). It therefore seems that additional factors are involved in determining susceptibility to ETEC expressing K99. In the case of ETEC expressing the K88 antigen it has been shown that there is a marked correlation between the genetic status of an individual pig and its susceptibility to bacterial infection. Rutter *et al.* (1975) challenged piglets with a virulent $K88^+$ *E.coli* strain and at autopsy determined whether they possessed brush borders which bound the same bacterial strain or not. Those which bound $K88^+$ bacteria were designated "adhesive" and those that did not were designated "non-adhesive". It was found that 91% of those piglets designated "adhesive" and 4% of those designated "non-adhesive" died or showed clinical symptoms of diarrhoea. More recent research has demonstrated that the view that pigs can be divided into "adhesive" and "non-adhesive" phenotypes is too simplistic (Bijlsma and Bouw 1985). There is also some evidence suggesting that the ability of porcine epithelial cells to bind $K88^+$ organisms may decrease with age (Walker and Nagy 1980). A further complication is the finding that porcine mucin can bind to the K88 antigen and that the amount of this mucus "receptor" increases with age (Conway *et al.* 1990). Despite such complexity, it is apparent that in order to be infectious in pigs, ETEC expressing K88 must be able to bind to the small intestinal mucosae. However, further factors such as enterotoxin production, avoidance of host immune response, resistance to proteolytic enzymes, resistance to acid pH and other unknown factors may

enhance or diminish the pathogenic potential of such strains (Bijlsma 1985a, Finlay and Falkow 1989).

1.3.3 Relationship between serotype and incidence of ETEC expressing K88.

E.coli strains are usually classed according to their serogroup which itself is determined by the sum of the surface antigens of a particular strain. These surface antigens are divided into three main types: the O antigens determined by the polysaccharide side chains of the lipopolysaccharide, the capsular or microcapsular polysaccharide K antigens and the proteinaceous flagellar H antigens which are all chromosomally determined (Orskov and Orskov 1978). With the discovery of fimbrial antigens (mostly plasmid encoded) a fourth additional type of antigen is included in some serotypes to indicate their presence eg. K88. Out of an estimated 10,000 possible combinations of O,K,H antigens only a restricted number are associated with fimbrial antigens (Klemm 1985, Bijlsma 1985a, see Table 1.2).

Table 1.2 O-serotypes associated with fimbrial antigens

FIMBRIAL ANTIGEN	ORIGIN	O-SEROTYPE
K88	PIGLET	O8, O45, O138, O141 O147, O149, O157
987P	PIGLET	O9, O20, O141
K99	CALF, LAMB	O8, O9, O20, O101
K99	PIGLET	O64, O101
F41	CALF	O9, O101
CFA/1	HUMAN	O15, O25, O63, O78
CFA/2	HUMAN	O6, O8

Why only a limited number of *E.coli* strains support a restricted number of fimbrial types is not known although the reason is probably multifactorial (Klemm 1985). One possible explanation is that of plasmid compatibility i.e. plasmids bearing fimbrial genes are only stable in a limited number of serogroups (Evans *et al.* 1977). Alternatively, only certain serogroups may have the necessary cellular infrastructure to express and assemble fimbriae correctly (Bijlsma 1985a). Finally, the ability of only a restricted number of serogroups to express additional virulence factors may restrict the number of serogroups that could benefit from the adhesive ability of fimbriae (Klemm 1985).

1.3.4 Relationship of K88 expression and raffinose metabolism.

Genes coding for the ability to metabolize raffinose (6-O-α-D-galactopyranosyl-1α-D-glucoside-2β-fructofuranoside) are frequently found located on the same plasmids that code for the production of K88 fimbriae (Williams-Smith and Parsell 1975, Shipley *et al.* 1978, De Graaf 1990). The raffinose determinant contains four proteins involved in raffinose metabolism, an α-galactosidase, a permease, an invertase and a regulatory protein (Schmid and Schmitt 1976). In strains where raffinose and K88 production are not found together, only the ability to express K88 is usually lost, probably the result of a point mutation (Shipley *et al.* 1978). It is rare to detect a strain which has lost the ability to metabolize raffinose while still expressing K88 (Shipley *et al.* 1978). The ability to metabolize raffinose has therefore been used as a test for the presence of K88 fimbriae (Williams-Smith and Parsell 1975, Guinee and Jansen 1979). Strains which have lost both the ability to metabolize raffinose and express K88 are found relatively frequently (Shipley *et al.* 1978). Genetic analysis has shown that this corresponds to the deletion of a 30Kbp stretch of DNA (Shipley *et al.* 1978, Mooi et al 1979). This DNA segment contains both determinants and is flanked by IS1 insertion sequence repeats giving it the

ability to be translocated by a rec-A independent mechanism (Schmitt *et al.* 1979).

Why raffinose metabolism and K88 expression should be closely linked is unknown. For instance, the colonising ability of ETEC strains possessing the K88 antigen is not affected by the presence of raffinose utilising ability (Williams-Smith and Huggins 1978). Even so it has been suggested that raffinose enzymes are involved in the degradation of sugars that compete with the intestinal receptor for the K88 adhesin (Mooi and De Graaf 1985, De Graaf and Mooi 1986). Alternatively high concentrations of raffinose in the porcine gut may be used by invading ETEC strains to enhance cell wall production and its concomitant protection from phagocytosis (Gaastra and De Graaf 1982, De Graaf and Mooi 1986).

1.4 Properties of fimbriae.

1.4.1 Structural.

A bacterium may possess up to ~1,000 fimbriae peritrichously located on its surface (Klemm 1985). Each fimbria consists of an array of between 100-1,000 protein subunits with an assembled diameter of 2-7nm and a length of between 0.2-2.0μm (Klemm 1979, Pearce and Buchanan 1980, De Graaf and Mooi 1986, Hacker 1990). The majority of the subunits are identical with molecular weight ranging from 14-30 Kdal (Klemm 1979). Known as the major subunits these form the structural framework of the fimbriae. Other subunits (minor) are present in limited numbers, often located at the tips and in some cases representing the functional or adhesin component of the fimbriae (Beachey and Abraham 1987, Lund *et al.* 1987, Abraham *et al.* 1988b, Hinson and Williams 1989, van Zijderveld 1990). It has been suggested that a tip location places the adhesin away from the influence of lipopolysaccharide O-side chain (Lindberg *et al.* 1987). Intact isolated fimbriae have molecular

weights ranging from $1x10^5$-$6x10^6$ daltons under non-denaturing conditions (Evans *et al.* 1979, Ferreiros and Criado 1983, Karhanis and Bhogal 1986, Hinson and Williams 1989).

The structure of type 1 fimbriae has been studied in detail by means of a combination of electron microscopy, crystallography and X-ray diffraction. They are thought to consist of a rigid, right-handed array of subunits with a 2.3nm repeat distance corresponding to 3.125 subunits per turn (Brinton 1965). Comparatively little work has been done on fimbrillar fimbriae of which K88 is a typical example. However, they are thought to consist of a loosely helical array of subunits without an apparent axial hole (De Graaf *et al.* 1980a, De Graaf and Mooi 1986). The K88 fimbria has an estimated molecular weight of $>10^6$ which corresponds to >37 major subunits (Jones 1977). However, it has been noted that as many as 100 subunits are present (Klemm 1981). A total of three minor fimbrial components have also been detected in the intact K88 fimbrial structure (Oudega *et al.* 1989 van Zijderveld 1990). The K88 fimbria has a length of 0.2-1.3μm and a diameter of 2.1nm (Wadstrom *et al.* 1979).

1.4.2 Chemical.

Chemical analysis of fimbriae has shown that the protein components of some are subject to post-translational modification. For instance, type 1 fimbriae have been shown to contain one residue of reducing sugar per subunit. Amino-sugar residues have been detected in 987P fimbriae and in some P fimbriae (associated with strains of *E.coli* causing urinary tract infections). The function of these post-translational modifications is unknown but they may play a role in stability or solubility of the fimbriae, enhanced protection against proteolytic degradation or a role in receptor recognition (De Graaf and Mooi 1986). For the K88ab fimbriae in particular, it has been suggested that post-translational modification might be necessary for receptor recognition (Mooi *et al.* 1983).

Intact fimbriae are generally acidic proteins with pI's of between 3.5-5 (see Table 1.3). An exception is the K99 fimbria (pI 10.1). At physiological pH therefore most fimbriae are negatively charged which may contribute to the net repulsive forces between a bacterium and the eukaryotic surface. However, it may be that the charge density of the fimbria is very low or alternatively ionic bridges may shield the negative charge (De Graaf 1986). In the case of K88 fimbriae it has been reported that divalent metal ions are important for adhesion (Sugarman *et al.* 1982). This suggests that ionic bridges may play a role in the adhesion of K88 fimbriae.

The amino acid composition of several major fimbrial subunits is given

Table 1.3 Some characteristics of fimbrial adhesins of enterotoxigenic *E.coli* (ETEC)

FIMBRIAE	DIAMETER (nm)	MORPHOLOGY	*(pI)	LOCATION OF GENES	INTESTINE COLONIZED
** TYPE 1	7.0	RIGID	3.9	$ CHROM	-
K88	2.1	FLEXIBLE	4.2	PLASMID	PIG
987P	7.0	RIGID	3.7	CHROM	PIG
CS31A	2.0	FLEXIBLE	-	PLASMID	PIG
K99	4.8	FLEXIBLE	10.1	PLASMID	PIG,LAMB, CALF
F41	3.2	FLEXIBLE	4.6	CHROM	PIG,LAMB, CALF
CFA/1	7.0	RIGID	4.6	PLASMID	HUMAN
CFA/2-CS1	7.0	RIGID	-	PLASMID	HUMAN
CFA/2-CS2	7.0	RIGID	-	PLASMID	HUMAN
CFA/2-CS3	2.0	FLEXIBLE	-	PLASMID	HUMAN

*Where:- *pI=Isoelectric point; $=Located on chromosome; -=not known.*
*** Please note that type 1 fimbriae are included for comparison. The role of type 1 fimbriae as pathogenic adhesins is controversial.*

in Table 1.4. It should be noted that most subunits contain two cysteine residues, the exception being the K88 major subunit (De Graaf and Mooi 1986). The cysteines form intrasubunit di-sulphide bonds, the fimbria as a whole being held together by non-covalent intersubunit forces (Klemm 1985).

1.4.3 Primary structure.

The primary structure of a number of fimbrial subunits have been partially or completely resolved (De Graaf and Mooi 1986). The three K88 serotypes, K88ab, K88ac and K88ad (Orskov *et al.* 1964, Guinee and Jansen 1979) have all had their primary sequences determined (Gaastra *et al.* 1981,1983, Josephsen 1984, Klemm 1981). The K88 major subunit is synthesized as a 285 amino acid long precursor, with subsequent processing cleaving 21 amino acids to form the mature protein (Gaastra *et al.* 1981). There are between 20 and 30 differences between the protein sequences of the major subunits present in the three main serotypes of K88 fimbria corresponding to 10-15% of the total protein sequence. The differences between the three K88 fimbria variants are not restricted to a particular part of the major subunit sequence, although differences do tend to come in clusters. One particularly variable region is found between residues 162-175, a region which may code for the variable "b","c" and "d" antigenic factors of the K88 fimbria (Josephsen 1984). Highly conserved sequences are also found within the three variants (Josephsen 1984). For example, the first 37 and last 28 amino-acid residues are completely identical. A large stretch of amino-acid residues showing complete homology is also found between residues 106-133. Two antigenic determinants have been predicted within these conserved sequences which possibly represent the "a" determinant. The conserved nature of the N and C-termini and other parts of the K88 sequence may reflect a role in fimbrial assembly, stability and possibly receptor recognition (Josephsen 1984, De Graaf and Mooi 1986).

Table 1.4 Amino acid composition of various ETEC fimbrial subunits

AMINO ACID	FIMBRIAL TYPE					
	TYPE 1	K88	987P	K99	F41	CFA/1
Aspartic acid /asparagine	19	30	34	22	27	12
Threonine	19	29	28	21	18	15
Serine	10	18	23	16	33	17
Glutamic acid /glutamine	13	17	16	6	24	11
Proline	2	5	8	4	11	7
Glycine	16	36	26	18	39	10
Alanine	31	28	26	16	16	19
Cysteine	2	0	2	2	ND	0
Valine	15	21	15	6	17	19
Methionine	0	3	1	3	3	3
Isoleucine	4	13	12	8	10	5
Leucine	10	20	17	7	14	12
Tyrosine	2	10	1	5	13	4
Phenylalanine	7	11	3	7	9	2
Histidine	2	0	0	3	6	1
Lysine	3	11	10	7	12	8
Hydroxylysine	0	0	0	0	10	0
Arginine	3	8	2	2	4	1
Tryptophan	0	4	1	1	ND	1
TOTAL	158	264	225	159	266	147
* Mwt ($ KDal)	15.7	27.5	23.0	16.0	29.5	15.0

*Where:- ND=not determined; *=Molecular weight; $=Kilodalton.*

Please note that the composition of type 1A and of K88ab is given.

1.4.4 Genetics.

The K88 antigen was at first suspected and then proven to be expressed by a transmissible plasmid (Orskov *et al.* 1961, Orskov *et al.* 1964, Jones and Rutter 1974). Early experiments indicated that the K88 plasmid contained at least two elements, the K88 antigen itself and a transfer factor (Bak *et al.* 1972). Subsequently, because of inconsistency of correlation between donor capability and K88 possession it was considered that the K88 antigen and transfer factor were on different plasmids (Orskov and Orskov 1966, Bak *et al.* 1972). The transfer factor was found to determine the expression of an F-like pilus (Meynell *et al.* 1968, Bak *et al.* 1972).

The size of the K88 plasmid is 50 MDal. (Gaastra and De Graff 1982). Such a large amount of DNA is not amenable to cloning. Therefore the plasmid was at first partially digested and the smallest DNA fragments still showing K88 expression were subcloned and physical maps established (Mooi *et al.* 1979). Gene products were elucidated by their expression in minicells, mutational inactivation and their reaction with specific antisera (Kehoe *et al.* 1981, Gaastra *et al.* 1981, Mooi *et al.* 1981, Dougan *et al.* 1983). The genetic organisation of the K88 gene cluster and other representative fimbrial gene clusters is given in Figure 1.2 (De Graaf 1990). A point to note about the K88 fimbrial gene cluster is that, compared to the other fimbrial gene clusters, the roles of FaeC and FaeG have been reversed. For instance, FimA, FanC and PapA are all major constituents of the structure of type 1, K99 and Pap fimbriae respectively, whereas, at the equivalent position, FaeC is a minor fimbrial component (Oudega *et al.* 1989). In the K88 operon FaeG is the major fimbrial component and thought also to act as the K88 adhesin (Oudega *et al.* 1989). It has been suggested that since *fae*C and *fae*B are only separated by 2bp, the mutational inactivation of the Shine-Dalgarno box has resulted in this unique, at present, orientation of the K88 operon (Oudega *et al.* 1989).

1.4.5 Biogenesis of fimbriae.

Fimbrial subunits are some of the very few proteins of *E.coli* that are transported across both the inner and outer membranes. After transport across the membranes the individual subunits are then assembled into the mature fimbriae, whose length are regulated and which are in turn anchored to the cell surface (Oudega and De Graaf 1988, De Graaf 1990). In addition, bacteria have the ability to regenerate fimbriae that have been physically removed from their surface which at least partly involves the mobilisation of a pool of "spare"

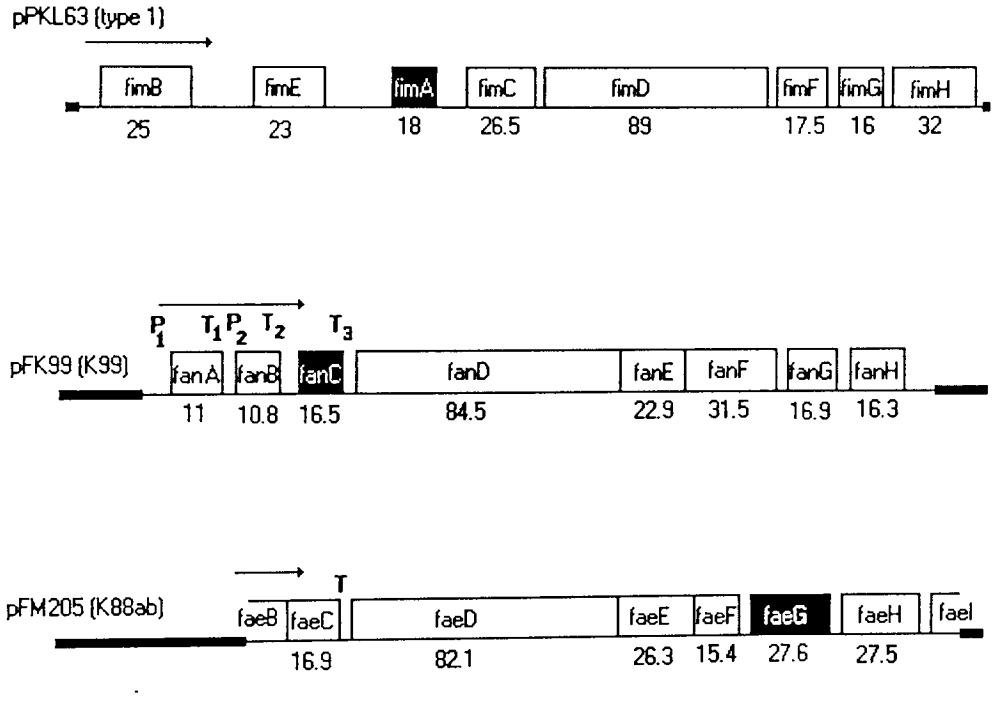


Figure 1.2 *Structural organisation of the gene clusters involved in the biogenesis of fimbrial adhesins. The location of the genes are indicated by boxes while the molecular weight in KDal is indicated directly below them. The location of known promoter and terminator sequences are indicated by* ***P*** *and* ***T*** *respectively. Arrows indicate the direction of transcription. The genes coding for the major structural genes are in black. Note that the K88ab structural gene is located at the distal and not proximal end of the respective gene cluster.*

After De Graaf 1990

cytoplasmic fimbrial subunits (van Verseveld 1985, Oudega et al 1989, Klemm 1985). It has been shown that the assembly of type 1 can take as little as three minutes at 37°C (Dodd and Eisenstein 1984).

Functionally, the capacity of fimbriae to adhere to their respective receptors on the intestinal mucosa or the underlying enterocytes may be encoded by either major or minor fimbrial components (Oudega and De Graaf, De Graaf 1990). Many of the fimbriae where minor subunits act as adhesins have them located at their tips (Abraham *et al.* 1988b, Beachey and Abraham 1987, Finlay and Falkow 1989, Oudega *et al.* 1989, Moch *et al.* 1987). Fimbrial biosynthesis must then allow for this polarisation of function.

Analysis of the gene clusters responsible for the biosynthesis of various fimbriae (see Figure 1.2) has shown that these clusters, as expected, contain much more information than that required simply for the production of the major fimbrial subunit. In general at least five or six auxiliary or "helper" proteins with distinct functions are encoded within the various gene clusters (Baga *et al.* 1984, Klemm 1985, De Graaf and Mooi 1986, Hacker 1990, De Graaf 1990). Much information on the role of these additional proteins has been obtained by their mutational inactivation and by the experimental determination of their subcellular location (Mooi *et al.* 1981, Mooi *et al.* 1984, De Graaf and Mooi 1986, De Graaf 1990). Detailed models for the biosynthesis of fimbriae have been proposed (Mooi and De Graaf 1985, De Graaf and Mooi 1986, De Graaf 1990).

1.5 The expression of fimbriae.

1.5.1 Why regulate fimbrial expression?

An ecological reason for the regulation of fimbrial expression becomes apparent when it is considered that fimbrial biogenesis may account for 1-2% of total protein synthesis in a cell (Klemm 1985). Since host specific fimbriae

are only functional *in vivo* i.e. at 37°C, their regulation under non-physiological conditions would provide a considerable energy saving for the bacterium concerned (Smyth 1986). However, control of fimbrial expression is more than an all or nothing expression of fimbrial genes. This becomes apparent because even in the absence of fimbrial assembly (e.g. when cultured at 20°C) the bacterium seems to maintain fimbrial subunit synthesis but not fimbrial assembly (Isaacson 1983). As a consequence a pool of unassembled subunits builds up in the cytoplasm (Oudega *et al.* 1989, Klemm 1985, van Verseveld 1985). Such a pool would presumably allow for a more rapid fimbrial assembly time on entering a favourable environment than would protein synthesis *per se* (van Verseveld 1985).

1.5.2 Effect of media on fimbrial expression.

Most information on the importance of media on fimbrial expression concerns the biosynthesis of either type 1 or K99 fimbriae. In the case of type 1 fimbria, the long established view is that maximal expression is favoured by serial culture in broth media (Orskov and Orskov 1983, Orskov *et al.* 1980) and that there is little or no expression when cultured on agar has been questioned recently (Jann 1987, Gander and Thomas 1987). Experiments based on specific monoclonal antibodies and on the ability to agglutinate yeast cells, a generally used marker for type 1 pili have shown that for some strains the converse is true (Jann 1987, Gander and Thomas 1987). In addition it was shown that for some *E.coli* strains there was a marked reduction in the expression of type 1 fimbriae when cultured at 20°C. Previously it was thought that type 1 production was independent of temperature of cultivation (Duguid and Old 1980, Dodd and Eisenstein 1984, Gander and Thomas 1987). It seems likely therefore that generalisations concerning culture medium and temperature effects on type 1 fimbrial expression do not apply to all strains and that multiple mechanisms of type 1 fimbrial regulation may exist (Gander and

Thomas 1987).

Investigations into the production of K99 antigen by means of slide agglutination tests, enzyme-linked immunosorbent assay (ELISA), and hydrophobic interaction chromatography (HIC) showed that temperature of cultivation, media and O serogroup all had an effect (De Graaf *et al.* 1980b,c). Maximal expression occurred at 37°C with little production below 30°C. Minimal salt medium with glucose and semisynthetic Minca medium (see Appendix 1) were most suitable for K99 production. In complex media the production of K99 was strongly reduced probably due to inhibition by L-alanine (De Graaf *et al.* 1980b). The inhibitory ability of L-alanine can be partly reversed by the presence of L-isoleucine and L-threonine (Isaacson 1983, van Verseveld 1985). Production of K99 by strains bearing antigen O101 was ~10 times more than strains bearing O8, O9, or O20. Plasmid transfer experiments indicated that the phenotypic expression of the K99 antigen is most probably related to the cell wall composition of the host (De Graaf *et al.* 1980c). Other experiments have shown that acidic pH strongly represses K99 production. In this case no fimbriae were detected after growth at pH 5.5 or lower while the same strains when grown at pH 6.5 expressed K99 fimbriae in abundance (Francis *et al.* 1989).

Glucose and other fermentable sugars suppress the synthesis of type 1 fimbriae in unshaken, aerobic broth cultures (De Graaf 1986). It seems probable that this is attributable to the selective outgrowth of non-fimbriated cells rather than catabolite repression as originally postulated (Eisenstein and Dodd 1982). The ability of type 1 fimbriated cells to carry out oxidative metabolism after forming a pellicle at the broth/air interface gives them a selective advantage when cultured statically. Such an advantage disappears on the addition of glucose which enables the outgrowth of non-fimbriated bacteria (through fermentative metabolism) which do not bear the metabolic cost of fimbriation (Eisenstein and Dodd 1982).

Information on glucose-mediated control of K99 production is contradictory (De Graaf 1986). It has been reported that absence or presence of glucose-mediated repression of K99 production was dependent on the cultivation media. In addition, low concentrations of cyclic AMP (cAMP) seemed to derepress K99 synthesis after catabolite induced repression had occurred, while higher cAMP concentrations were associated with a lower K99 synthesis. Further studies with an adenyl cyclase (cya) mutant have indicated that glucose-induced catabolite repression affected the synthesis of K99 subunits but not the assembly of K99 fimbriae. It has been suggested that strains producing K99 can be divided into two groups; in the first, production of K99 appeared to be constitutive while in the other, K99 expression was glucose dependent (van Verseveld 1985).

Little definitive work has been done on the effect of media on the expression of K88 fimbriae. It has been reported that K88 production is independent of the pH of the media used to cultivate the host strain (Francis *et al.* 1989). The results of seroagglutination tests suggested that blood agar was optimum for promoting the expression of both K88 and 987P by many strains (Francis *et al.* 1982). It has also been stated that K88 is preferentially expressed on solid agar but not after growth at 18°C or in broth (Levine 1981, Levine *et al.* 1983). Indirect evidence has also been obtained that K88 expression is maximal on solid media (Guinee and Jansen 1979).

Generally, the expression of fimbriae other than type 1 is thought to be favoured by growth on agar-based media (Jann and Hoschutzky 1990).

1.5.3 Expression of fimbriae during the growth cycle.

The expression of K88, K99 and F41 as a function of the growth rate (μ) has been determined (Jacobs and De Graaf 1985, Isaacson 1980, Isaacson 1983, van Verseveld 1985). These fimbriae are synthesized during the exponential growth phase (Jacobs and De Graaf 1985, Isaacson 1980). The expression of

K99 and F41 appeared to occur only at high μ ($>0.2h^{-1}$) in growth rate domain 1 (see Figure 1.3, van Verseveld 1985). At the end of the exponential phase the biomass continued to increase while the concentration of fimbriae either stabilised (van Verseveld 1985) or was strongly repressed (Jacobs and De Graaf 1985). The production of K99, F41 and probably K88 in growth rate domain 1 suggests that the expression of fimbriae occurs only at low concentrations of

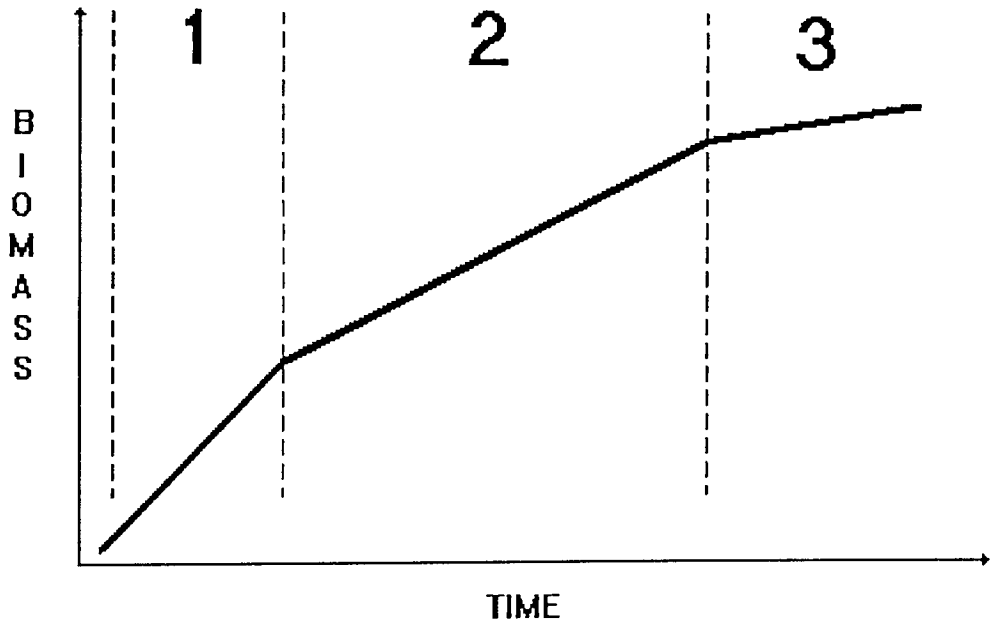


Figure 1.3 *Growth domains in bacteria. The use of the recycling fermentor has established the occurrence of three growth domains during the culture of bacteria. Each domain is characterized by the biomass yield on the limiting substrate and the range of μ (growth rate) it encompasses. Domain 1 consists of μ values higher than $0.05hr^{-1}$ or mass doubling times (Fd) shorter than 14hr where highest yield values are obtained. Domains 2 and 3 consist, respectively, of μ values between 0.05 and $0.01hr^{-1}$ and lower than $0.01hr^{-1}$ or mass doubling times of between 14 and 69hr or higher than 69hr. Domains 2 and 3 are additionally characterized by elevated levels of guanosine 5'-diphosphate 3'-diphosphate. In domain 3 unbalanced growth occurs and the stringent response instigated.*

After Van Verseveld et al. (1985)

guanosine polyphosphates and cAMP and is arrested at high levels of these regulatory nucleotides (the stringent response, De Graaf and Mooi 1986). However, there is evidence that fimbrial subunit biosynthesis is unaffected by growth rate, only fimbrial assembly being controlled during the growth cycle of bacteria expressing K99 fimbriae (Isaacson 1983, van Verseveld 1985). The expression of the K88, K99 and F41 fimbriae has been shown to be equivalent during both anaerobic and aerobic growth in media buffered at pH 7 (van Verseveld 1985, Jacobs and De Graaf 1985).

1.5.4 Phase variation.

Phase variation is the non-random switching of a bacterium from a fimbriated to non-fimbriated phase and *vice versa* (Brinton 1959, De Graaf 1990). The probability of switching from one phase to the other is strongly influenced by environmental conditions having a frequency of about 1:1,000 per bacterium, per generation (Eisenstein 1981, Eisenstein and Dodd 1982). Phase variation has been noted for type 1, 987P and some P fimbriae, which all have chromosomally located genes (De Graaf 1990). However, phase variation has also been noted for CFA/1 expression whose gene cluster is plasmid located. Phase variation has not been described for any of the other fimbrial systems.

1.5.5 Temperature dependent expression of fimbriae.

Expression of the K88 antigen and many if not all other host specific fimbriae is maximal at 37°C (De Graaf and Mooi 1986, Levine 1981). As the temperature is lowered there is a gradual decrease in fimbrial expression until at 18°C production is negligible (Orskov 1964, Levine 1981, Gaastra and De Graaf 1982, De Graaf 1990). Such control of fimbrial expression is thought to be in part controlled by temperature-dependent promoter activity. For instance, the activity of the P_1 promoter in the K99 operon (see Figure 1.2) is regulated by temperature (Roosendaal 1987). Alternatively or additionally, the

biosynthesis of several fimbriae (K88, K99 and F41) is regulated by the specific growth rate. In this case, lower fimbrial production at lower temperature may be attributed to a slower growth rate since their biosynthesis is directly proportional to growth rate (Isaacson 1980, van Verseveld *et al.* 1985, Jacobs and De Graaf 1985). Such thermoregulatory effects have been noted in *E.coli* K12 hosts into which wild-type plasmids have been transferred or mobilised and in such hosts containing recombinant plasmids bearing the genes for K88 biogenesis (Mooi *et al.* 1979, Isaacson 1980). It should be stressed that in the latter transconjugants even though expression is largely mediated by a plasmid-encoded promoter, temperature-regulated expression of fimbrial production still occurs (Mooi *et al.* 1979).

1.5.6 Genetic control of fimbrial expression.

There is only a limited amount of information available concerning how the various genes within a gene cluster interact during the expression of fimbriae. In particular, how fimbrial expression is quantitatively controlled is virtually unknown. The qualitative control of fimbriation during phase variation is however well understood and a theory explaining temperature controlled expression of K88 and K99 fimbriae has also been proposed (Mooi and De Graaf 1985, Hacker 1990).

The genetic control of phase variation involves the interaction of several genes and seems to occur in both clinical isolates and laboratory strains of *E.coli* (Eisenstein 1981, Freitag *et al.* 1985, Abraham *et al.* 1985, Abraham *et al.* 1986). The region which regulates the transcription of type 1 fimbrial determinants is located at the proximal (5') end of the *fim* gene cluster, upstream from the fimbrial structural gene *fim*A (see Figure 1.2). An invertible DNA element of 314 base pairs located immediately upstream of the *fim*A gene is responsible for the ability of fimbrial expression to turn "on" or "off". This element which is flanked on each side by a 9 base pair inverted repeat

sequence, carries the *fim*A specific promoter region. Depending on the direction of integration of this promoter, the *fim*A gene, together with the whole determinant, will be either transcribed or not. Two genes, *fim*E and *fim*B, located upstream of the invertible element are also necessary for phase variation and seem to be transcribed by their own promoters. The respective proteins of 23 and 25 Kdal. seem to bind to the invertible DNA region and switch the promoter element "on" (*fim*B) or "off" (*fim*E). Both proteins are highly basic and have a high degree of homology to each other (Klemm 1986).

Additional modulation of phase variation is controlled by a trans-acting protein, the integration host factor (IHF). This protein is encoded by the *E.coli* genes *him*A and *hip/him*D and is normally essential for phage integration and excision. Mutants in these genetic loci reduce the frequency of switch events from 10^{-2}-10^{-3} to 10^{-5} per cell per generation (Eisenstein *et al.* 1987). In addition, a seven-fold reduction of the transcriptional activity of the *fim* determinant was observed in IHF-negative strains when compared to strains carrying intact IHF coding genes (Dorman and Higgins 1987). Sequence homology studies have shown that there are two IHF consensus sequences present in the type 1 regulatory region. This has led to suggestions that the IHF protein shows recombinase activity during the fimbrial switch (Klemm 1986, Eisenstein *et al.* 1987, Dorman and Higgins 1987). Recently, another protein was found to regulate the expression of the type 1 determinant. Mutations in this gene led to the over-production of the adhesin protein FimH and the generation of so called "fibrosomes". The gene responsible for FimH is located downstream of *fim* A and is therefore thought to encode a repressor protein (Abraham *et al.* 1988a).

As mentioned previously (see Section 1.5.1), the expression of K88 and K99 fimbriae along with many others is repressed at low temperatures, even where fimbrial expression is controlled by a plasmid borne promoter (Mooi *et al.* 1979). This suggests that fimbrial assembly at least is strongly repressed at

low temperature and that a step subsequent to the initial transcription of the fimbrial operon is responsible for the thermoregulatory control of fimbrial expression (De Graaf and Mooi 1986). One theory to explain this is based upon the finding of a region of dyad symmetry between either *fan*C and *fan*D (the K99 operon) or *fae*C and *fae*D (the K88 operon). In both cases it is possible to predict the formation of a mRNA hairpin stem and loop structure by a transcript of this region (Mooi and De Graaf 1985). It was thought that the stabilisation of mRNA secondary structure at low temperature was responsible for the observed non-expression at $<20°C$. In the case of the K99 operon, this proposed terminator (T_3) has an efficiency of 90% (Roosendaal et al 1984). Such "translational attenuation" was in agreement both with the observed temperature regulation and with the fact that the structural subunit (FanC) was produced in far greater amounts than the auxiliary proteins downstream (FanD-FanH). This suggests that one or more of the proteins downstream from FanC (i.e. FanD-FanH) are responsible for temperature regulation.

A complication to this explanation is the observation that in the K88 operon the major structural gene (*fae*G) is located downstream from the postulated region of dyad symmetry. For the K99 operon the opposite is true. Additionally, the stability of the stem and loop structure formed by the K88 operon is much lower than that of the K99 operon (Mooi and De Graaf 1985). The region of dyad symmetry in the K88 operon contains the 3' end of the *fae*C gene, the ribosome binding site of *fae*D, and the translation initiation codon of the latter gene (Mooi *et al.* 1986, Roosendahl 1987). Translation of *fae*C is terminated within the ribosome binding site preceding the start codon of *fae*D. Therefore it is conceivable that a ribosome terminating translation of *fae*C could reinitiate translation of *fae*D without being released from the mRNA molecule (translational coupling). It has been shown that if the translation of the 3' end of *fae*C is prevented, the synthesis of FaeD is also decreased (Mooi and De Graaf 1985, De Graaf 1990). Therefore it seems that the alternative position

of the major subunit and the decrease in the stability of secondary mRNA formation in the stem and loop structure may be related. A common link between the K88 and K99 operons is that the activity of the genes *fae*D and fanD respectively, corresponds to the expression of fimbriae or not. This along with previous data indicating that proteins coded for by these genes reside in the bacterial outer membrane suggests that they are central to the ability to modulate fimbrial assembly (van Doorn *et al.* 1982, De Graaf 1990).

1.5.7. An hypothesis for the genetic control of fimbrial expression in the K88 and K99 fimbrial operons.

It may be postulated that originally, as is still the case with the K99 operon, the K88 adhesin was encoded for by the gene immediately prior to the region of dyad symmetry (*fae*C) and that the protein encoded by *fae*G was only a minor component of the mature fimbria. In this scenario, the relative expression of genes within the K88 operon was controlled by "translational attenuation" mediated by secondary mRNA structure between *fae*C and *fae*D while fimbrial assembly was controlled by the level of expression of the protein encoded for by *fae*D. Thus at low temperature (low growth rates), a pool of FaeC would accumulate and no fimbrial assembly would occur. At higher temperatures (high growth rates), secondary mRNA would be destabilised with both fimbrial subunit synthesis and assembly occurring. Subsequently while probably under intense selective pressure, the roles of FaeC and FaeG were reversed. To accommodate this while still responding to the thermoregulatory control of fimbriation, the secondary mRNA structure that could be formed between *fae*C and *fae*D became extended but weakened. At low temperatures, the synthesis of both FaeC and FaeD is greatly reduced and fimbrial expression repressed, however a pool of FaeG is synthesised in preparation for a favourable environment by "translational coupling". At higher temperatures (> growth rates) mutational inactivation of the Shine-Dalgarno box preceding *fae*C

prevents its efficient transcription, while the expression of both *Fae*D and *Fae*G results in the formation of intact functional fimbriae on the bacterial cell surface.

1.6 Binding properties of fimbriae.

Fimbriae confer adhesive properties on the ETEC that possess them. Fimbriae are able to adhere to mammalian cell surfaces partly because their shape enables them to penetrate charge clouds on the cell surface and partly because their configuration allows them to recognise and subsequently bind cell surface glycolipid or glycoprotein (De Graaf and Mooi 1986, De Graaf 1986). Several *in vitro* assays have been developed to investigate what is precisely involved in this binding to cell surface glycoconjugates. These have been facilitated by the ability of certain isolated epithelial cells and erythrocyte species to bind fimbriae in the same specific manner as is thought to occur *in vivo*.

1.6.1 Haemagglutination.

Haemagglutination was originally described by Guyot in 1908, Rosenthal in 1943 and Kauffmann in 1948 (referenced in Duguid *et al.* 1955). It is based on the ability of cell-bound/free adhesin to bind to many erythrocytes and cause them to agglutinate. Only cell-free adhesins such as type 1 with several binding sites or bacterial cells with many adhesive appendages can therefore cause haemagglutination (Jones and Isaacson 1983). Since the original studies, the ability to haemagglutinate has become recognised as a simple method for testing bacterial cultures and their cell free products for adhesive activities (Jones and Isaacson 1983). Once the ability to haemagglutinate has been discovered, inhibition tests are then usually applied to determine the nature of the adhesin receptor present on the erythrocyte. By implication the nature of receptors present on erythrocytes are thought to reflect the natural receptor for the

adhesin (Jones and Isaacson 1983).

In 1967 it was shown that two *E.coli* strains expressing K88 had the ability to agglutinate guinea pig (GP) erythrocytes (Stirm *et al.* 1967b). Later, conclusive proof was presented demonstrating that the K88 antigen was responsible for MR haemagglutination of GP erythrocytes at 4°C. At 37°C the erythrocytes dissipated and the term mannose-resistant and eluting (MRE) was introduced to describe this type of haemagglutination (Jones and Rutter 1974). The authors reported that erythrocytes from cows, goats, sheep, chickens and humans were agglutinated inconsistently by the 3 strains of $K88^+$ *E.coli* used. Only 2 out of 6 erythrocyte preparations from chickens were agglutinated by all 3 *E.coli* strains used. All $K88^+$ cultures agglutinated guinea pig erythrocytes while none agglutinated horse erythrocytes. Since this original study on erythrocyte types capable of binding the K88 fimbria, several similar studies have been carried out (Guinee and Jansen 1979, Parry and Porter 1978, Porter and Linggood 1983, Bijlsma *et al.* 1985, Cox and Houvenaghel 1987, Jacobs *et al.* 1987c) the most comprehensive of which was in 1978 (Parry and Porter 1978) before the discovery of the K88ad serotype (Guinee and Jansen 1979). Parry and Porter (1978) investigated the ability of 15 erythrocyte types to bind the ab and ac serotypes of K88. Of the 15, only chicken, guinea pig and pigeon erythrocytes were able to bind K88. In the case of hen and pigeon erythrocytes only the presence of the K88ab antigen resulted in their agglutination. More recent studies have concentrated on the haemagglutination attributed to all three serotypes of K88. In particular, erythrocytes from guinea pigs, chickens and pigs have been investigated. It was hoped that the erythrocyte receptor expressed by pigs would correlate with the natural receptor they express on their enterocytes, unfortunately this was not found to be the case (Cox and Houvenaghel 1987).

Gibbons *et al.* (1975) attempted to determine the nature of the K88 receptor on guinea pig erythrocytes by means of inhibition with glycoproteins

and fractions from sow colostrum. By selectively cleaving sugar residues from terminal positions of glycoproteins the authors found that unsubstituted β-D-galactosyl residues were important for the binding of K88. However, the binding reaction was found to be complicated by the finding that one of the inhibitory glycoproteins studied did not possess a β-D-galactosyl structure. To explain this the authors postulated that the K88 adhesin may react in a non-specific manner to some complex high molecular weight glycoproteins found in mucus secretions. In a more recent report, workers found that oligosaccharides obtained from glycolipid fractions of the pig small intestine inhibited the agglutination of guinea pig erythrocytes by K88 (Nilsson and Svenson 1983). Additionally, they found that α-D-Gal-(1-3)-D-Gal and α-D-Galp-(1-3)-α-D-Galp-(1-3)-D-Gal both inhibited this haemagglutination. An interesting point to note was the finding that milk fat globules or fat globule membranes could inhibit haemagglutination by K88 and K99 antigens (Reiter and Brown 1976). This may indicate that milk has a non-immune anti-adhesive potential.

In an attempt to localise the binding site of the K88 adhesin, experiments were carried out on the inhibition of both chicken and guinea pig haemagglutination by specific anti- K88a, K88b or K88c sera (Parry and Porter 1978). The results indicated that the K88b determinant was responsible for the agglutination of chicken erythrocytes since anti-K88a inhibited the reaction poorly. This is unlike the inhibition of the binding of K88 fimbriae to enterocytes where specific αK88a antibodies are good inhibitors. However, the authors suggested that the K88/chicken erythrocyte reaction was a good model system since, like the binding of K88 fimbriae to enterocytes but unlike the binding of K88 to guinea-pig erythrocyte it was stable at physiological temperatures (Wilson and Hohmann 1974, Parry and Porter 1978).

1.6.2 Intestinal epithelial cells.

Studies on the binding properties of bacteria bearing the K88 antigen were originally directed to determining the expression of K88 *in vivo* and where in the intestine the bacteria bound (Jones and Rutter 1972). It was found that $K88^+$ bacteria adhered to the mucosa of the small intestine, whereas $K88^-$ bacteria did not attach and were distributed throughout the lumen (Bertschinger *et al.* 1972). An *in vitro* method was developed for determining the binding of bacteria bearing K88 to isolated tissue slices (Jones and Rutter 1972). This method was found to be rather laborious and was replaced by a method based on the binding of $K88^+$ bacteria to isolated intestinal brush border cells (Wilson and Hohmann 1974, Sellwood *et al.* 1975). Results showed that the ability to adhere to these cells was dependent on the presence of the K88 antigen (Wilson and Hohmann 1974).

By using an *in vitro* assay based on binding to isolated brush borders, Sellwood *et al.* (1975) were able to show that there were at least two phenotypes of pig brush border. They found that bacterial binding was an all or none phenomenon i.e. either bacteria expressing K88 covered the whole brush border surface (designated adhesive) or there was no binding (designated non-adhesive). The existence of adhesive and non-adhesive phenotypes was confirmed in experimental infection studies (Rutter *et al.* 1975). With the discovery of a third K88 serotype (Guinee and Jansen 1979) binding studies revealed that there were at least 4 (Rapacz and Hasler-Rapacz 1986) or 5 (Bijlsma and Bouw 1985) major K88 receptor phenotypes (see Table 1.5). To account for this observed multiplicity of receptor phenotypes and based on segregation data, Rapacz and Hasler-Rapacz (1986) suggested that the adhesion receptor(s) for the K88ab and K88ac antigens were specified by an autosomal complex codominant gene at the K88 adhesion locus, or by genes at two very closely linked loci. No theory concerning the interrelation of the K88ad antigen

receptor was given by Rapacz and Hasler-Rapacz (1986) because of insufficient data for analysis, however, an hypothesis to account for the several observed phenotypes of the K88 receptors has been proposed (Bijlsma and Bouw 1985). Here it is thought that expression of the K88 receptor(s) is controlled by two genes. At one gene locus the ability to bind K88 serotypes ab and ac is determined by two alleles "S" (receptor expression) and "s" (no receptor expressed). This represents the original idea concerning the expression of the K88 receptor (Sellwood *et al.* 1975, Gibbons *et al.* 1977). At the other gene locus, the ability to bind the K88ad serotype is determined. Again two alleles are possible, "D" (receptor-positive) and "d" (receptor negative).

A further complication is the finding of a "weak" adhesive phenotype where an average of only 5 bacteria adhere to each brush border as opposed to the normal 10 or more (Sellwood 1980b, Sellwood 1981, Bijlsma and Bouw 1985, Rapacz and Hasler-Rapacz 1986). This variation in receptor efficacy suggests that other unknown factors, in addition to a simple gene-receptor relation are active (Rapacz and Hasler-Rapacz 1986) and it has been suggested

Table 1.5 Pig phenotypes relating to adhesion of K88ab-, K88ac-, and K88ad-positive *E.coli* strains to brush borders.

PHENOTYPE	ADHESION OF *E.COLI* STRAINS BEARING			NUMBER OF PIGS TESTED
	K88ab	K88ac	K88ad	
A	+	+	+	18
B	+	+	-	13
C	+	-	+	10
D	-	-	+	5
E	-	-	-	19

After Bijlsma (1985c)

that epistatic gene(s) may modify the K88 receptor(s) (Bijlsma and Bouw 1985). This modification seems especially prevalent to the "D" receptor and is thought to control the finding of the 15 minor K88 receptor phenotypes in addition to the 5 major phenotypes (Bijlsma and Bouw 1985, Cox and Houvenaghel 1987). It has been calculated that $\sim 12^{\%}$ of brush borders have their "D" receptors modified whereas modification of the "S" receptor (which is responsible for the binding of K88ab and K88ac) seems rare (Rapacz and Hasler-Rapacz, 1986).

Epidemiological data shows that the incidence of K88-associated diarrhoea in pigs occurs over a wide age span, from neonatal to post-weaning piglets (Runnels *et al.* 1980). Experiments to determine the ability of intestinal cells from pigs of various ages to bind K88-bearing bacteria have shown that unlike bacteria expressing K99 fimbriae there is no age-related decline in the number of adherent bacteria (Runnels *et al.* 1980). More recent experiments however, have suggested that the expression of the K88 receptor(s) may be age related to a degree (Bijlsma *et al.* 1985, Rapacz and Hasler-Rapacz 1986). It was found that some brush borders isolated from some pigs were of a "mixed adhesive" phenotype (Rapacz and Hasler-Rapacz 1986). As the term suggests, these brush border preparations were found to contain both K88 adherent and non-adherent cells. Among the negative enterocytes of the mixed phenotype both cuboidal and columnar epithelial cells were observed. The cuboidal cells, however, formed the major portion of the negative cells. The authors postulated that the cuboidal cells, as younger crypt enterocytes, did not yet express K88 receptors whereas the older columnar cells did (Rapacz and Hasler-Rapacz 1986). The mixed adhesion phenotype, although mainly associated with the type D phenotype was also found amongst the other 3 major adhesive brush border phenotypes (see Table 1.5). In another study, expression of the D phenotype was associated only with older pigs (8 months), suggesting that the expression of the "D" receptor for K88 was age related (Bijlsma *et al.* 1985).

In order to understand the nature of the K88 receptor, several new or

modified assays have been developed. The first such assay was based on the enhancement or not of the binding of radiolabelled, purified K88 to pig brush border plasma membranes (Kearns and Gibbons 1979). During the preparation of brush border plasma membranes it was found that 78% of the K88 receptor activity was associated with supernatant fractions. By incubating these fractions in the presence of plasma membranes and K88 it was found that only supernatant fractions obtained from receptor-positive brush border plasma membranes subsequently enhanced the binding of K88 to plasma membranes (Kearns and Gibbons 1979). A similar assay was employed to determine the binding of purified radiolabelled K88 to porcine brush borders (Sellwood 1980a). In this case brush borders were treated with 1% formaldehyde to abolish non-specific binding. Repeated washing was used to separate bound from unbound K88 antigen (Sellwood 1980a). Scatchard plot analysis of the results obtained suggested the existence of two types of brush border binding sites, one of high affinity (Ka $9x10^{12}M^{-1}$) with $8.5x10^5$ sites/brush border and another, extremely high in number but of very low affinity. The non-linearity of the plot was thought most likely due to negatively cooperative binding (i.e. the affinity of binding decreases with increasing adhesin concentration) of the purified K88 antigen (Sellwood 1980a). Anderson, *et al.* (1980) used differential filtration to separate bound from unbound purified K88. This assay was made possible by the careful selection of a filter pore size which, while retaining the majority of the porcine brush border membranes used, allowed most of the free K88 to pass through the filter. Another approach has been to radiolabel pig intestinal brush borders, detergent solubilize them, centrifuge to remove particulate material and then fractionate the resultant mixture by chromatography (Staley and Wilson 1983). Each fraction was then assayed for the ability to bind $K88^+$ bacteria, unbound membrane material being removed by washing. Specificity of binding was confirmed by inhibition with anti-fimbrial antisera, purified fimbriae and non-labelled solubilized brush borders. Based on the binding of

radiolabelled $K88^+$ bacteria, the most recent approach has involved the immobilization of brush borders to polystyrene plates used in conjunction with a modification of Western blotting (Laux *et al.* 1986).

Using the assays above, five basic methods of approach have been used to characterize the K88 receptor on porcine intestinal cells:-

A Chemical treatment. This has usually entailed the oxidation of brush borders by sodium metaperiodate. Several experimenters have shown that this treatment abolishes the binding ability of the K88 receptor (Sellwood 1980a, Laux *et al.* 1986). Although other cell membrane components are also susceptible to oxidation, sensitivity to periodate is strongly indicative of the presence of carbohydrate as part of the K88 receptor structure (Sellwood and Kearns 1979).

B Enzyme treatment. Reduction in bacterial adhesion subsequent to enzyme treatment of the substratum has been used to identify receptors e.g. the classical detection of the receptor for influenza virus (Gottschalk 1972). Treatment of porcine brush borders with pronase inhibits their binding of K88 while treatment with trypsin inhibited binding in one report and had no effect in another (Sellwood 1980a, Laux *et al.* 1986). Combined with its sensitivity to metaperiodate this suggests that the K88 receptor is glycoprotein in nature (Anderson *et al.* 1980, Sellwood 1980a, Staley and Wilson 1983, Laux *et al.* 1986). Treatment with α-fucosidase, Helix pomatia digestive juice, β-galactosidase and α-mannosidase had no effect on the binding of radiolabelled K88 by porcine brush borders (Sellwood 1980). Small increases in binding of K88 were noted after the brush borders were treated with Trichomonas foetus extract and neuraminidase suggesting that further K88 binding sites were uncovered, allowing increased binding (Sellwood 1980a).

C Inhibition with carbohydrates. Inhibition here is assumed to be due to

competition between the inhibitor and the natural receptor for the adhesin and therefore to indicate structural similarity of the inhibitor to the natural receptor (Jones and Isaacson 1983). The finding that the K88 receptor is likely to be a glycoprotein has prompted several researchers to attempt to inhibit the binding of the K88 adhesin by simple mono- and disaccharides (Anderson *et al.* 1980, Laux *et al.* 1986, Sellwood 1980a, Staley and Wilson 1983). Although many simple sugars have been investigated for their inhibitory potential only a few have been shown to inhibit the K88 adhesin/receptor interaction. Of the inhibitory sugars found, none are good inhibitors as compared to the inhibition of the binding of type 1 fimbriae by D-mannose (Ofek and Sharon 1990). Inhibitory sugars noted are, D-galactosamine (Laux *et al.* 1986, Sellwood 1980a, Staley and Wilson 1983), D-manosamine, D-glucosamine (Sellwood 1980a), N-acetylgalactosamine (Anderson *et al.* 1980), N-acetylglucosamine (Anderson *et al.* 1980, Staley and Wilson 1983), galactose, glucose (Staley and Wilson 1983), stachyose and galactan (Sellwood 1980a). The significance of several sugars with free amino groups acting as inhibitors is not apparent since it has been reported that other compounds with free amino groups, e.g. ethanolamine, Tris (2-amino-2-(hydroxymethyl) propane-1,3-diol) and glycine were also found to inhibit to a similar degree (Sellwood 1980a).

D Inhibition by lectins. Lectins are reactive ligands which function in an analogous way to bacterial adhesins i.e. they specifically recognise a single or limited number of sugar residues. The inhibition of an adhesin/receptor interaction by a lectin is thought to indicate a similar specificity between the lectin and adhesin. A drawback to the use of lectins is that many are large molecules and may sterically hinder the function of a receptor without being actually bound to it. Only one study has examined the effect of a limited number of lectins on the K88 adhesin/receptor interaction (Sellwood 1980a). Of 4 lectins tested only one, a fucose-binding lectin was found to be inhibitory.

Mannose-specific, D-galactose-specific and N-acetyl-D-galactosamine and D-galactose-specific lectins were all non-inhibitory (Sellwood 1980a).

E Direct isolation of presumptive receptor. Initial work on the isolation of the K88 receptor suggested that it was glycolipid in nature (Kearns and Gibbons 1979). The authors postulated that a polar glycolipid which they isolated from brush borders was either the receptor for the K88 antigen or possibly formed part of a receptor complex (Kearns and Gibbons 1979). It was subsequently reported that a wide range of molecular weight components (25-690 KDal) isolated from pig intestinal brush borders were capable of binding $K88^+$ bacteria (Staley and Wilson 1983). Using affinity chromatography followed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of a reducing agent the authors identified two major bands. With molecular weights of 23 and 32-35 KDal it was proposed that these bands corresponded to subunits of a K88 receptor which in its native state existed in multi-meric forms (Staley and Wilson 1983). More recently, it has been reported that murine brush borders contain three proteins (molecular weights 57, 64, and 91-Kdal) with affinity for radiolabelled $K88^+$ bacteria (Laux *et al.* 1986).

1.6.3 Intestinal mucin.

Gastrointestinal mucus is a viscous, slippery gel that covers most of the mucosal surfaces of the gastrointestinal tract. Composed of a complex mixture of glycoproteins, water, various serum and cellular macromolecules, electrolytes, microorganisms and sloughed cells, mucus has a unique capacity to protect delicate epithelial surfaces. Its unusual consistency is due to its major gel-forming glycoprotein component called mucin, which is synthesized and secreted by specialized "mucous cells". (Neutra and Forstner 1987). Mucin is a polydisperse glycoprotein which in its native form is composed of large

glycopeptide subunits that vary in size (250-2x10^3 KDal) and in the composition of their oligosaccharide side chains (Neutra and Forstner 1987). Containing up to 80% carbohydrate, each glycoprotein subunit consists of oligosaccharide side-chains (having an average of 6-8 sugar residues for intestinal mucins) that are linked via O-glycosidic bonds to serine or threonine residues of a peptide backbone. Individual glycopeptide subunits may be linked directly by disulphide bonds with others or they may bind to "link" peptides that bridge the gap between two or more glycopeptides during the formation of high molecular weight mucin polymers (Neutra and Forstner 1987).

Interest has arisen in mucin partly because of its location i.e. it has the potential to act both as a potentiator and barrier to invading microorganisms (Isaacson 1977, Parsons and Mulholland 1978, Freter 1981, Neuta and Forstner 1987, Drumm 1988) and partly because of the recent development of a simple *in vitro* assay which can characterize its binding ability (Laux *et al.* 1986). Using an *in vivo* rabbit model, Parsons and Mulholland (1978) suggested that mucin acts as a non-specific inhibitor of bacterial adhesion to the bladder and postulated that this may apply to other mucous surfaces. In the case of ETEC expressing the K88 antigen it has been shown that they bind to specific glycoproteins present in both murine and porcine mucus. It was demonstrated that this adhesion was inhibited by trypsin, pronase, sodium metaperiodate and D-galactosamine (Laux *et al.* 1986). Further work indirectly indicated that a 40-42 Kdal glycoprotein was responsible for the observed binding (Metcalfe *et al.* 1991). Other experimenters have presented evidence indicating that the expression of K88-receptor in porcine ileal mucus was age-related (Conway *et al.* 1990). These researchers have suggested that the comparatively large amount of mucus K88 receptor present in older pigs may block the binding of $K88^+$ bacteria to the underlying epithelial cells. Specific mucus receptors have now been reported for the adhesins K88, K99, 987P, F41, and AF/R1 (Metcalfe *et al.* 1991).

1.6.4 Nature of the adhesin receptor binding site.

It has been suggested that hydrophobic interactions may be important in enabling bacteria to overcome the natural charge repulsion between themselves and the mammalian cell surface (Wadstrom *et al.* 1980). Indeed it has been shown that fimbriated bacteria, including those expressing K88, have both a reduced surface charge and enhanced hydrophobic adsorptive properties compared to non-fimbriated ones (Wadstrom *et al.* 1979,1980). In experiments quantifying the hydrophobic nature of K88 variants it was found that bacteria expressing K88ab bound more strongly to phenyl and octyl sepharoses than those expressing K88ac fimbriae (Wadstrom *et al.* 1979).

The principal component of K88 fimbriae is the major subunit FaeG although there are minor subunits present (van Zijderveld 1990). Since in the case of K88 fimbriae, the major subunit is thought to represent the adhesin, attention has concentrated on determining the site on FaeG which binds to the K88 receptor (Oudega and De Graaf 1988). Jacobs *et al.* (1987c) chemically and then enzymatically cleaved the intact K88ab (FaeG) protein to determine which fragments then inhibited the adherence of K88 fimbriae to erythrocytes or porcine intestinal epithelial cells (Jacobs *et al.* 1987c). Active peptides were isolated and corresponded to the tripeptides Ser-148-Leu-Phe-150 and Ala-156-Ile-Phe-158. These peptides form part of the conserved regions in the primary structure of the K88 variants. The authors postulated that the receptor binding domain of the K88 fimbria could be a hydrophobic cleft in the major subunit which encompasses a conserved amino acid sequence containing either or both the inhibitory tri-peptides. They suggested that modifications in the variable regions surrounding such a hydrophobic cleft could account for the observed differences in the inhibition profiles they observed with the K88 variants. Further, they suggested that the relatively high concentrations of tripeptide required to inhibit the haemagglutinating ability of K88ad fimbriae indicated

that differences in receptor molecules exist, in particular between the receptors for K88ad and K88ab or K88ac (Jacobs *et al.* 1987c). This is in agreement with the results obtained with the brush border binding assay reported elsewhere (Bijlsma and Bouw 1985). Additional experiments using oligonucleotide-directed site specific mutagenesis have confirmed that Phe-150 is important in the binding of K88 fimbriae (Jacobs *et al.* 1987b).

1.6.5 Why do bacterial adhesins bind carbohydrate residues?

A common theme in bacterial adhesin/receptor interactions is that all the adhesins are protein in nature while almost exclusively all the receptors they recognize are carbohydrates (Jones and Isaacson 1983, Hinson and Williams 1989, Finlay and Falkow 1989). The carbohydrates concerned are usually present on the surface of epithelial cells in the form of either glycolipids or glycoproteins (Hinson and Williams 1989). However, as is shown by the binding abilities of mucin, carbohydrate that is more loosely associated with epithelial cells may also be involved. Since epithelial cells line all of the mammalian body tracts and the body surface, the strategic placement of carbohydrate means that it is the most likely collision partner for a bacterium (Holgersson *et al.* 1985). With this initial proviso, tissue tropism of bacterial infection may be controlled at least in part by variability in carbohydrate structure. That the selectivity/specificity of bacterial infection can be controlled by carbohydrate is illustrated by the K88 adhesin/receptor system. In this case the genetic non-expression of the carbohydrate-based K88 receptor results in pigs which are resistant to infection by ETEC expressing the K88 adhesin (Jones and Isaacson 1983).

1.6.6 A possible role for multivalent binding in adhesin/receptor systems.

A theoretical attempt has been made to distinguish between binding

systems with high affinity (e.g. between type 1 fimbriae and its receptors) or those with low affinity (Holgersson *et al.* 1985). In the latter category, multivalent interactions are necessary to allow bacteria to bind to a surface. Experimentally, the two types can be distinguished in inhibition studies. In the case of high affinity systems, comparatively low concentrations of soluble inhibitors (usually low molecular weight sugars) can inhibit binding e.g. 0.01-0.5% w/v D-mannose inhibits the binding of type 1 fimbriae (Ofek and Sharon 1990). In the case of low affinity binding systems only multivalent inhibitors are active, soluble inhibitors having little or no effect. An example of the latter is the binding of Shiga toxin to cultured Hela cells (Holgersson *et al.* 1985). In this case, binding could not be inhibited by the disaccharide Galα1→4βGal up to a concentration of 4mg/ml whereas the same sugar coupled to albumin gave 50% inhibition at a concentration of about 75μg/ml conjugate. It is interesting to note that Gibbons *et al.* (1975) reported that the only way to remove completely the inhibitory activity of pig submaxillary mucin on the haemagglutination of guinea-pig erythrocytes by the K88 antigen was to cleave its heterosaccharide side chains. This cleavage would not affect the heterosaccharide side chain structure but would effectively convert a multivalent inhibitor to a monovalent one. The observed results suggest that multivalent binding may be important in the binding of $K88^+$ ETEC. A role for multivalent binding may be to avoid or reduce inhibition by secreted sugars or cell degradation products (Holgersson *et al.* 1985).

1.7 Aims of this study.

The correlation between the expression of K88 fimbriae by enterotoxigenic *E.coli* and the incidence of diarrhoea in pigs is well established (Smith and Linggood 1971, Rutter *et al.* 1975). The possession of K88 fimbriae enables bacteria to adhere to the porcine small intestine, facilitating colonisation and the ensuing infection (Wilson and Hohmann 1974, Rutter *et al.* 1975). Various methods have been used for the preparation of the purified adhesin component of K88 fimbriae (Stirm *et al.* 1967a, Parry and Porter 1978, Mooi and De Graaf 1979, Anderson *et al.* 1980, Guinee *et al.* 1980). However, little is known about the levels of expression of K88 fimbriae during culture. Several studies have been performed with the aim of characterising the receptor with which the K88 adhesin interacts (Gibbons *et al.* 1975, Kearns and Gibbons 1979, Anderson *et al.* 1980, Sellwood 1980a, Staley and Wilson 1983, Laux *et al.* 1986). Despite this effort, characterising the K88 receptor on both erythrocytes and enterocytes has been problematical.

A general aim of this study was to further characterise the factors affecting the interaction of the K88 fimbrial adhesin with its receptor. This was to include studying the effect of media type and form on the expression of K88 fimbriae and the determination of suitable methods for both the small and large scale preparation of purified K88 adhesin. Subsequent work was directed at analysing the adhesive properties of both the purified K88 adhesin and the cell-bound K88 fimbriae. A large number of erythrocyte species were to be screened for the ability to bind the K88 adhesin and the reaction analyzed. In addition, a new assay was to be developed for the determination of the binding properties of the purified K88 adhesin. This assay was then to be used in the further characterisation of the K88 receptor present on porcine enterocytes.

2 MATERIALS AND METHODS.

Addresses of suppliers and manufacturers are given in Section 2.3.11.

2.1 Bacterial strains.

A total of nine strains of *Escherichia coli* were used in this study. On receipt, the strains were immediately cultured statically in nutrient broth overnight at 37°C. Dilutions in sterile phosphate buffered saline (PBS, Dulbecco A, see Table 2.1) were then in turn cultured overnight on nutrient agar plates and typical colonies cultured as above in nutrient broth. 0.5ml aliquots were removed and 0.5ml sterile glycerol aseptically added. These stock cultures were then stored at -20°C. Details of strains used are given in Table 2.2. Experiments concerning the characterisation of the strains used in this study are given in Chapter 3.

Table 2.1 Composition of phosphate buffered saline used in study.

COMPONENT	CONCENTRATION (g/litre)
NaCl	8.00
KCl	0.20
Na_2HPO_4	1.15
KH_2PO_4	0.20

All PBS was sterilised for 10min at 115°C, 10 psi.

2.2 Chemicals and growth media.

All chemicals were of analar grade or equivalent and were obtained from either Sigma chemical company Ltd., BDH Ltd. or Bio-Rad Labs. Ltd. unless otherwise stated. All solutions were made up with distilled deionised water at pH7 unless otherwise stated. Nutrient broth or nutrient agar (Oxoid) were routinely used for bacterial culture. All media were routinely sterilised at 121°C for 15min. For media containing either glucose or raffinose sterile, filtered

Table 2.2 Details of E.coli strains used in experimental work.

SEROTYPE	COMMENTS	SOURCE
K12	-	Dr R.Sellwood AFRC
K12:K88ab	above strain with K88ab plasmid from serotype O8:K87:K88ab:H19	"
O8:K87:K88ab:H19	G7, ST^+,LT^+	"
O8:K87:K88ac:H19	G205, R986 LT^+,	"
K88ad	JM, LT^+,	"
O149:K91:H10	J2, cured variant of strain below	"
O149:K91:K88ac:H10	Abbotstown, W1	NCTC 10650
O147:K89:K88ac:H19	-	NCTC 10758
O157:K88ac:H19	-	NCTC 10964

Where :- AFRC = Agricultural and Food Research Council, Institute of Animal Health, Compton, Newbury, Berks. NCTC = National Collection of Type Cultures, 61 Colindale Avenue, London. NW9 5HT. G7, G205, R986, JM, J2, Abbotstown, W1 are alternative designations for the strains. LT and ST are heat-labile and heat-stable enterotoxins respectively.

(0.2μm) sugar solution was aseptically added to the other media constituents which had been heat sterilized. Experiments involving the study of raffinose metabolism used a minimal media (De Graaf *et al.* 1980c). Experiments investigating the expression of the K88 adhesin and the growth characteristics of several *E.coli* strains used several different media (see Appendix 1).

2.3 General methods.

2.3.1 Growth conditions.

Bacteria used for the expression of the K88 adhesin were routinely cultured statically for 16hr at 37°C in broth. Culture was usually carried out with 100ml of broth in sterile 250ml Erlenmeyer (conical) flasks. Unless

otherwise indicated media were usually aseptically inoculated by sterile loop from stock cultures. Non-expression of K88 fimbriae was induced by growth in unshaken broth for 72hr at 37°C.

2.3.2 Measurement of bacterial numbers.

In the majority of experiments where required, bacterial concentration was determined by viable counting. Bacterial cultures were subject to 100-fold serial dilutions up to $1x10^{-6}$ and 10-fold serial dilutions from $1x10^{-6}$ to $1x10^{-8}$ in PBS. Agar plates were dried in an oven for ~30min and 250μl of bacterial suspension carefully added to the centre of the plate. The plates were slowly rotated to spread the suspension and then incubated upside down at 37°C overnight. Viable counts were determined in triplicate from plates having between 30 and 250 colonies and were recorded as colony forming units (cfu).

Where required total counts were determined by diluting the bacterial suspension in PBS and counting using a haemocytometer. Here, the number of bacteria present in at least 5 of the small squares (multiplication factor $2.5x10^5$) were counted. Arbitrarily, bacteria overlapping with the top and left-hand lines of the square were included while those overlapping the bottom and right hand-side lines were ignored.

In the experiments conducted to deduce the expression of the K88 adhesin, cell suspensions were standardised or monitored according to their absorbance at 600nm. An absorbance value of 1.00 at 600nm was found to equate to ~5-$10x10^8$ cfu/ml (see Figure 4.1). Unless stated otherwise, bacterial growth was monitored at 600nm.

2.3.3 Determination of viability.

This was calculated by dividing the number of viable bacterial cells by the total count determined and was expressed as a percentage.

2.3.4 Protein assays.

Two protein assays were employed, one based on Coomassie brilliant blue and the other based on the absorbance of protein solutions at 280nm.

Protein microassay.

This procedure was based on an existing large scale assay (Bio-Rad Ltd. protein assay) where the absorbance maximum of an acidic solution of Coomassie brilliant blue G-250 shifts from 465 to 595nm when protein binding occurs. The method has been scaled down for use with 96-well microplates (Dynatech immulon 2) and modified slightly to reduce bubble formation when mixing reagents.

A series of bovine serum albumin (BSA) standards using the same diluent as in the samples were prepared with final protein concentrations varying between 50μg and 200μg/ml. Triplicates of each standard (100μl/well) and negative control (100μl of diluent) were added to the microplate. The test sample itself was serially diluted in triplicate down a 96-well plate, changing the pipette tips after each dilution. 50μl of the dye reagent concentrate was then carefully added to each well. The final step was to add 50μl of distilled water (dH_2O) to mix the reactants. The absorbance at 620nm was then measured 30min later and a standard curve constructed.

Assay based on absorbance at 280nm.

As for the above assay, a series of BSA standards in the sample diluent were prepared and their absorbance at 280nm monitored in order to construct a standard curve. Doubling dilutions of the sample were then prepared and the concentration of the stock solution calculated.

2.3.5 Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE was performed according to the method of Laemmli (1970) with either a 12 or 15% (pH 8.8) acrylamide running gel, a 4% (pH6.8) stacking gel and an acrylamide/N'N'-Bis-methylene-acrylamide ratio of 37.5:1. Final sample preparations were diluted 1:4 with sample buffer (2% w/v SDS, 5% β-mercapto-ethanol) boiled for 5min and run at a constant 200 volts for 40min using a Protean 2 mini-gel system (Bio-Rad Ltd.). Gels were stained with Coomassie blue (0.02% w/v Coomassie blue R-250 in 25% v/v ethanol and 10% v/v acetic acid in dH_20) either undiluted for 30min or as a 1:4 dilution overnight. Gels were destained with repeated washes in 25% v/v ethanol and 10% v/v acetic acid in dH_20) Where greater sensitivity was required gels were silver stained using potassium dichromate/nitric acid as oxidiser and sodium carbonate/paraformaldehyde as the developing agent according to a regime given in a commercial kit (Bio-Rad Ltd).

2.3.6 Gel densitometry.

Coomassie blue stain intensity is proportional to the amount of protein present over the range 1-15μg (Ragan 1986). Coomassie blue stained SDS-PAGE gels were examined by gel densitometry for the semi-quantitative determination of the concentration of the K88 major structural protein (FaeG) present in extracts. Analysis was performed with the aid of an Ultrascan XL laser densitometer (LKB) connected to an Olivetti M24 personal computer running Gelscan XL software. Gels were scanned with a line beam and absorbance graphs constructed based on a peak width of 3, using signal integration and a common base line. Where necessary, a gel track containing the purified K88 preparation was also scanned as a standard.

2.3.7 Western blotting.

The method used was based on Bio-Rad's immunoblot procedure with a few modifications. Reagents used were, transfer buffer consisting of Tris 2.4g/l, glycine 11.7g/l and methanol 20% v/v. The Tris was dissolved in the methanol/water before adding glycine. If necessary, the pH was adjusted to 8.3 with 1mM NaOH. Tris-buffered saline (TBS) was prepared containing 2.4g/l Tris and 29.3g/l NaCl. Usually the Tris and NaCl were dissolved in ~1.5 litres of dH_2O and the pH adjusted to 7.5 before being made up to 2 litres. Tween Tris-buffered saline (TTBS) containing 0.25ml/l of Tween-20 was suspended in TBS. Blocking solution used contained 1% w/v BSA in TTBS. Primary antibody (αK88ab) which had been raised in rabbits and from which non-specific antibodies had been removed (see Section 2.6) was diluted 1:200 in TBS before use. Secondary antibody consisting of goat anti-rabbit peroxidase conjugate was diluted 1:1,000 in TBS. Finally the horseradish peroxidase (HRP) colour reagent consisted of 4-chloro-1-naphthol (50mg) and 60μl of hydrogen peroxide (30% w/v) suspended in 100ml of TBS.

An SDS-PAGE running gel was prepared as normal. The stacking gel however was shaped with the aid of a single tooth comb rather than the normal 12-tooth comb. The gel was loaded with 100μl of the sample (in sample buffer) and the gel run as normal. Following electrophoresis, the gel was carefully transferred to a plastic dish and equilibrated in transfer buffer for 25min. During this equilibration step a piece of 0.45μM nitrocellulose sheet was cut to the approximate gel size. Orientation of the nitrocellulose sheet relative to the gel was recorded by using a marker pen to draw a red line across the top of the sheet. The nitrocellulose membrane was then wetted by slowly sliding it in at a 45 degree angle into transfer buffer and allowing it to soak for 20min. While handling membranes gloves were worn. At the same time as soaking the nitrocellulose, two fibre pads and filter paper sheets were also soaked in

transfer buffer. The gel cassette holder was assembled in the order, fibre pad, presoaked filter paper, gel, presoaked filter paper and finally fibre pad. At each stage of assembly all components were soaked in transfer buffer with the formation of air bubbles being avoided. After the second piece of filter paper was added, the gel sandwich was carefully rolled with a glass test tube to remove any trapped air bubbles. After complete assembly, the gel cassette holder was inserted into the gel buffer tank, the cooling unit added and the buffer unit filled with approximately 400ml of transfer buffer. The complete unit was run at 100 volts for 1hr with continuous stirring of buffer. After transfer, the nitrocellulose membrane was removed and soaked in TBS for 10min before being removed and carefully covered in blocking solution. After equilibrating for 30min the blocking solution was removed and the membrane cut up into parallel strips running from the top to the bottom of the membrane. Each strip was then placed in separate chambers of an incubation tray (Bio-Rad Ltd.) and 40ml of primary antibody solution added. After incubation at room temperature for 2hr the primary antibody was removed and the strips washed by rinsing once briefly in 40ml dH_2O and then twice with 40ml of TBS. 40ml of secondary antibody was then added and the membrane incubated at room temperature for a further hour. Again the membrane was washed in 40ml of dH_20 and two 10min soaks in 40ml of TBS. Finally, the HRP colour reagent was added and the strips allowed to develop for approximately 30min after which the reaction was terminated by several washes in 40ml of dH_2O. As a negative control, the primary antibody was omitted from the first incubation step.

2.3.8 Study of raffinose metabolism.

This was based on the use of a minimal media supplemented with either glucose or raffinose (see Appendix 1).

10μl of *E.coli* stock was used to inoculate 10ml of minimal medium supplemented with glucose (MM+G) as carbon source. After incubation at 37°C for 16hr the cells were pelleted by centrifugation at 6,500rpm for 5min (MSE Microcentaur) before being washed three times and finally being resuspended in PBS to their initial volume. Sterile minimal medium supplemented with either raffinose or glucose was then added to the wells of honeycomb plates (Labsystems Ltd., 200μl/well). Honeycomb plates are specially modified microplates with optically clear bottoms and lids produced for use in the bioscreen system (Labsystems Ltd. see bottom of page⊛). After the media had been prewarmed for 30min 10μl loops were then used to inoculate the wells of the honeycomb plates which were then incubated at 37°C without shaking. Any changes in absorbance due to growth were then monitored at 580nm with the aid of bioscreen. Typically, cultures were incubated at 37°C for 60hr with 20 seconds of shaking before any measurements were taken (every 30min).

2.3.9 Electron microscopy.

Bacteria were examined using a Phillips EM400T transmission electron microscope operated at 80kV on either formvar or carbon coated 3mm copper grids. The specimen was transferred from suspension to the copper grid using a platinum loop heat sterilised between transfers. The specimen was left on the

⊛*The bioscreen is an automated system for the monitoring of absorbance, in particular for the monitoring of growth during bacterial culture. Honeycomb plates are placed on a platform within a computer controlled environment. Control parameters which can be set include wavelength to be monitored, temperature and time of incubation and the degree and time of shaking. The bioscreen is connected to a personal computer where results are recorded. When using unshaken cultures, the microplate under test is briefly shaken to disperse any bacteria present before measurements are taken.*

grid for between 30-60 seconds which was then blotted with filter paper to remove excess fluid. ☞Grids were then carefully washed four times with dH_2O to remove any traces of medium☜. The sample was stained for between 2-5 seconds with either 1% sodium silicotungstate or 2% phosphotungstic acid. Carbon coated grids which were subsequently to be stained with 2% ammonium molybdate were exposed to ultraviolet illumination for 5min (carbon-coated side away from source) before the grids were floated on top of a 1:1 mixture of stain and bacterial suspension (in PBS) for 30 seconds. In all cases excess stain was quickly removed with filter paper and grids left to dry in a desiccator for 15min before being examined. In some cases, specimens were shadowed with platinum rather than negatively stained before examination.

To examine the purified K88 fimbrial preparation for intact fimbriae the urea present was removed by dialysis against PBS and the concentration of K88 adjusted to 0.75mg/ml. Specimens were treated as above except for the omission of the washing step (between ☞ and ☜) and stained with 1% sodium silicotungstate.

2.3.10 Equipment used.

Centrifuges	Sorvall RC-5B refrigerated superspeed centrifuge Beckman l8-70M ultracentrifuge MSE microcentaur Jouan CR411
Electrophoresis	Bio-Rad mini-protean 2 and accessories Bio-Rad model 500/200 power supply
Western blotting	Bio-Rad Mini Trans-blot electrophoretic transfer cell Biometra fast-blot
Water baths	Jencons scientific type SB-55 Grant instruments Ltd. type JB2
Spectrophotometer	Cecil CE272 series 2 linear UV spectrophotometer LKB Ultrospec plus UV/VIS spectrophotometer

pH meter	Kent industrial measurements Ltd. EIL 7020 Gallenkamp pH stick
Suspension mixer	Luckham model 802
Dialysis	Pierce microdialyser systems 100+500
Plate washer	Dynatech Ultrawash 2
Plate readers	Dynatech MR 700 microplate reader Titertech Multiskan MCC
Sonicator	Braun labsonic 2,000 fitted with 16mm probe
Homogeniser	Tri-R instruments Ltd. model K41
Whirlimixer	Gallenkamp spinmix
Microscopes	Zeiss ICM 405 Olympus BH-2 fitted with Olympus C-35AD-2 camera and exposure control unit using Ilford FP4 film.
Cameras	As above and Polaroid MP-4 land camera using either Polaroid 667 or 665 film.
Rotary mixer	Stuart scientific blood tube rotator SB1
Growth monitor	Labsystems bioscreen

2.3.11 Addresses of manufacturers and suppliers.

Aldrich Chemical Company Ltd., The Old Brickyard, New Road, Gillingham. Dorset.

Anachem Ltd., Charles Street, Luton. Beds.

BDH Chemicals Ltd., Broom Road, Poole. Dorset.

Beckman RIIC Ltd., Turnpike road, Cressex industrial estate, High Wycombe. Bucks.

Becton Dickinson UK Ltd., Between Towns Road, Cowley. Oxford. Oxon.

Bio-Rad Labs. Ltd., Caxton way, Watford. Herts.

Cambio, 34 Millington Road, Cambridge. Cambs.

Camlab, Nuffield Road, Cambridge. Cambs.

Cambridge Research Biochemicals Ltd., Button End, Harlston. Cambridge. Cambs.

CP Instrument Company Ltd., Po Box 22, Bishops Stortford. Herts.

Difco Laboratories Ltd., Po Box 14B, Central Avenue, East Molesey. Surrey.

Dynatech Labs Ltd., Daux Road, Billingshurst. Sussex.

Flow Labs., Woodcock hill, Harefield Road, Rickmansworth. Herts.

Gallenkamp, Belton Road, West Loughborough, Leis.

Gibco Ltd. Trident House, Po Box 35, Renfrew Road, Paisley. Renfrewshire.

ICN Biomedicals Ltd. Lincoln Road, Cressex Industrial Estate, High Wycombe. Bucks.

Jencons (Scientific) Ltd. Cherrycourt Way Industrial Estate, Stanbridge Road, Leighton Buzzard. Beds.

Labsystems (UK) Ltd., 12 Redford Way, Uxbridge. Middx.

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

Luckham Ltd., Burgess Hill. W.Sussex.

Millipore (UK) Ltd., 11-15 Peterborough Road, Harrow. Middx.

MSE Scientific Instruments, Sussex Manor Park, Crawley, Sussex.

Oxoid Ltd., Wade Road, Basingstoke. Hamps.

Philip Harris Scientific., 618 Western Avenue, Park Royal, London.

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

Polaroid (UK) Ltd. Ashley Road, St. Albans.

Sigma Chemical Co. Ltd., Fancy Road, Poole. Dorset.

The Central Veterinary Laboratory, New Haw, Weybridge. Surrey.

Whatman Labsales Ltd., St Leonards Road, 20120 Maidstone. Kent.

Zeiss, Woodfield Road, Welwyn Garden City. Herts.

2.4 Preparation and purification of the K88 adhesin.

2.4.1 Preparation of outer membrane proteins (OMPs).

Each of 24 sterile, cotton topped Erlenmeyer flasks containing 100ml of nutrient broth were inoculated with 200μl of freshly grown bacterial suspension. The culture was then incubated at 37°C for 7hr and the optical density (OD) monitored at 590nm (final OD usually between 0.45 and 0.50). Each 200ml of suspension was spun at 2,000g for 5min and each pellet washed twice in 100ml PBS. Resultant pellets were pooled and suspended in a total of 200ml PBS containing 0.1mM phenylmethylsulphonylfluoride (PMSF). The suspension was then sonicated (Braun labsonic 2,000 with 16mm probe) at 1500 watts for a total of 5min in 30 second bursts with 1min in between for cooling. The resulting sonicate was then centrifuged at 5,000g for 5min and the pellet discarded. 5ml of 22% w/v sarkosyl detergent was added to the turbid supernate and left at 20°C for 30min. After solubilization the supernate was centrifuged at 40,000g for 45min (at 4°C). The supernate obtained was discarded and the pellet suspended in 5ml of 2% w/v sarkosyl containing 0.1mM PMSF. Again, the supernate was centrifuged at 40,000g for 45min at 4°C. Here only the pellet was retained and suspended in 5ml of 10mM Tris-HCl buffer (pH8) containing 2% w/v SDS at 20°C and then centrifuged at 100,000g for 45min (at 4°C). Both the final supernate and pellet were stored at -20°C before use, the latter in 5ml of Tris/SDS buffer as above.

2.4.2 Large-scale preparation of the K88 fimbrial adhesin.

- Method 1

This method was adapted from that originally given in Mooi and De Graaf (1979).

24 sterile, cotton topped Erlenmeyer flasks containing 100ml of nutrient

broth were each inoculated with 10μl of stock suspension and incubated unshaken for 16hr at 37°C. Twelve 200ml batches of the resulting suspension were centrifuged at 5,000g for 5min and the supernates discarded. Each pellet was suspended in 75ml of 50mM Tris-HCl buffer (pH7.4), and the contents of three centrifuge tubes pooled and centrifuged at 2,000g for 5min (at 4°C). These pellets were then suspended in 45ml of the above buffer supplemented with 1M NaCl to a total of 180ml. This suspension was then sonicated (see Section 2.4.1) for 10x 30 second with 1min intervals between each burst for cooling. The sonicate was centrifuged at 2,000g for 5min and the pellet discarded. The supernate was spun at 50,000g for 60min (at 4°C) and the resultant supernatant carefully decanted and retained. Protein present in the supernate was then precipitated overnight at 4°C by the addition of 32g of ammonium sulphate (60% saturation). The white precipitate was then pelleted by centrifugation at 5,000g for 10min, the supernate being discarded. The pellet remaining was suspended in 20ml of 100mM Tris-HCl buffer (pH7.4) in the presence of 2M urea. This solution was then dialysed against 1 litre of the same buffer with stirring at 4°C for 4hr with a change of buffer after 2hr.

Method 2

This method was adapted from that by De Graaf *et al.* (1980).

Each of 24 sterile, cotton topped Erlenmeyer flasks were inoculated with 10μl of bacterial stock suspension and cultured unshaken at 37°C for 16hr. Twelve, 200ml aliquots of the resultant suspension were then pelleted by centrifugation at 2,000g for 5min. After discarding the supernates, each of three pellets were sequentially suspended in 200ml of buffer A (50mM Na_2HPO_4, 2M urea, pH7.0) and the process repeated for the remaining nine pellets. ☺These suspensions were incubated at 60°C for 20min and then centrifuged at 30,000 for 15min (at 4°C). The resultant supernates were then

precipitated by the addition of ammonium sulphate (60% saturation) with gentle stirring overnight at 4°C. Precipitates were then spun down at 5,000g for 10min (again at 4°C) and suspended in 20ml buffer A. Solutions were then dialysed against buffer A for 4hr with one change of buffer after 2hr. K88 adhesin preparations were stored in cryotubes at -20°C.

Method 3

This procedure used here was basically similar to that of method 2 except that the bacterial cells were cultured on nutrient agar rather than broth. The change in media form was to repress the expression of type 1 fimbriae (Orskov *et al.* 1980).

10ml of nutrient broth was inoculated with 10μl of bacterial stock culture and then incubated unshaken at 37°C overnight. 90ml of PBS was aseptically added to the overnight culture and 5ml of the resultant suspension used to seed Roux bottles containing 150ml of nutrient agar. The Roux bottles were then incubated at 37°C for 16hr and the cells harvested with 25ml of cold sterile buffer A. Harvested suspensions were then pooled and 30ml aliquots dispensed into 50ml polycarbonate tubes. The suspensions were then processed as from ☺ in method 2 above.

2.4.3 Further purification of fimbriae.

Fimbrial preparations produced by the third method given above were subjected to further purification based on the insolubility of the K88 adhesin near its isoelectric point (Stirm *et al.* 1967a, Parry and Porter 1978). Preliminary experiments showed that the following procedure produced the most pure final preparation (as judged by SDS-PAGE). Briefly, 20ml of fimbrial preparation was dialysed against 1 litre of 50mM Na_2HPO_4 (pH8) for a total of 4hr at 4°C with one change of buffer after 2hr. The pH of the

solution was then adjusted to 4.2 by the addition of concentrated glacial acetic acid and left overnight at 4°C. Using a microcentrifuge (MSE microcentaur) the isoprecipitate was spun down at 6,500rpm for 5min, washed 3 times in PBS (pH4.2) and finally suspended in PBS (pH5.3), leaving at 4°C for 4hr. Again, the isoprecipitate was pelleted by centrifugation at 6,500rpm (MSE microcentaur) for 5min and washed 3 times in PBS (pH5.3) before finally being suspended in PBS, 2M urea (pH8). This preparation was regarded as the purified K88 fimbrial adhesin (see Figure 5.3. for photograph demonstrating purity).

2.4.4 Small scale method for the preparation of the K88 fimbrial adhesin.

This was an adaptation of the second large scale method outlined above. Briefly, 12x 1ml aliquots of broth culture (or cell suspension) were pelleted at 6,500rpm for 3min (MSE microcentaur) in Eppendorf tubes. The supernates were discarded and each pellet resuspended in 1ml of buffer A (see above). After incubation at 60°C for 20min the tubes were centrifuged at 13,000rpm for 20min. The resultant supernates were pooled and the protein present precipitated by the addition of 5g ammonium sulphate (60% saturation). After 2hr at 4°C, the precipitate was pelleted at 13,000rpm for 7min and the supernate discarded. Each pellet was then serially diluted in 200μl of buffer A. Dialysis against buffer A was then performed using a microdialyser (Pierce chemical company) with one change of degassed buffer after 2hr and a total dialysis time of 4hr.

2.4.5 Effect of temperature, time of incubation at 60°C, buffer composition and pH on the small-scale extraction of the K88 adhesin.

To determine the effect of temperature, bacterial suspensions were incubated at either 40°C or 50°C for 20min and otherwise treated as in the standard small-scale procedure. Temperatures lower than 40°C were not investigated since it was assumed that the K88 fimbria was stable at physiological temperatures and below. To determine the effect of incubation time, bacteria were incubated at 60°C for 5, 10, 20 and 60min otherwise the procedure was carried out as normal. Alternative buffers based on buffer A were also used: 50mM Na_2HPO_4; 2M urea; 2M urea, 50mM Na_2HPO_4, 0.8M NaCl, all at pH7. These substitute buffers were only used during the initial incubation step; buffer A was used for all subsequent steps. The pH was altered by adding either 3M NaOH or HCl to buffer A. Again, buffers with altered pH were only used during the initial incubation step. In all experiments comparing the effects of various treatments, aliquots of the same nutrient broth grown culture were used.

2.5 Preparation and use of porcine intestinal epithelial cells.

2.5.1 Source.

Intestine samples were obtained from pork weight pigs reared at the AFRC Institute of Animal Health at Compton in Berkshire. Immediately after the slaughter of the pigs, lengths of intestine containing little or no chyme were excised from the small intestine, labelled and stored on ice for transport back to Porton. On arrival, the intestines were stored at 4°C before processing (within one week).

2.5.2 Preparation.

The procedure used was a slight modification of that given by Sellwood *et al.* 1975. Krebs-Henseleit (KB) buffer containing NaCl 7g/l, KCl 1g/l, $NaHCO_3$ 2.1g/l and KH_2PO_4 was used, supplemented with either sucrose 102.7g/l (KB+sucrose) or EDTA 1.46 g/l(KB+EDTA).

The intestines were cut into 35-40cm lengths, each segment then being processed individually. To wash through any gut contents, one end of the segment was fixed with a screw-threaded clip and then filled with 35ml of KB+EDTA. After ~30 seconds the clip was released and the segment then filled with 50ml of the same buffer before being clipped at both ends. To prevent the segment drying it was then immersed in KB+sucrose and incubated at room temperature for 20min. The segment was then removed from the KB+sucrose buffer before being gently rubbed between forefinger and thumb for ~1min. The contents were then emptied and retained before the segment was refilled with 50ml of KB+EDTA buffer and incubated for a further 20min at room temperature. The intestinal segment was then rubbed again to remove loosely bound epithelial cells, the contents retained and the segment then discarded. The above process was repeated until all the intestine from a particular pig had been processed. The epithelial cells were then collected by centrifugation at 900g for 10min, the supernate carefully discarded and 8 volumes of KB+EDTA added. The cells were then homogenised with a teflon-tipped tissue grinder (clearance 0.015-0.023cm) running at 11,000rpm (Tri-R model K41). The resultant homogenate was then centrifuged at 900g for 10min and the supernate discarded. Again the pellet was resuspended in 8 volumes of KB+EDTA buffer and homogenised. This cycle of centrifugation, homogenisation and resuspension was repeated until the viscosity of the suspension was much reduced (~5-6 times). At this stage the pellet produced

after centrifugation usually gave the impression of being fibrous. The epithelial suspension (mostly brush borders) was then washed twice in PBS before being filtered through glass wool. To facilitate storage, glycerol was added to a concentration of 50% v/v and the epithelial cells were stored at -20°C. Epithelial cells stored in this manner were active for at least six months (Bijlsma *et al.* 1985b). In all cases before use epithelial cell suspensions were washed twice in PBS.

2.5.3 Porcine intestinal epithelial cell adhesion test.

This test was performed as outlined by Sellwood *et al.* (1975). Stock suspensions of porcine intestinal epithelial cells (mostly brush border cells although the distribution varied between preparations) were centrifuged at 10,000g for 10min, washed twice in PBS and adjusted to $1x10^6$/ml. Fresh cultures of *E.coli* were pelleted at 6,500rpm (MSE Microcentaur) and resuspended in PBS to a concentration of $1x10^9$/ml. 100μl of the epithelial cell suspension ($1x10^6$)was then added to an equal volume of bacterial suspension and incubated at room temperature on a rotary mixer for 30min. 10μl aliquots were then spotted onto a microscope slide and examined by phase contrast microscopy under oil (1,000x magnification).

2.5.4 Effect of the K88 adhesin on the adhesion test.

As outlined in Sellwood *et al.* (1975), 100μl of doubling dilutions of purified K88 adhesin (stock concentration = 0.76mg/ml) were incubated at 37°C with an equal volume of porcine epithelial cells ($1x10^6$) for 30min on a rotary mixer. 100μl of bacterial suspension ($1x10^9$ in PBS) was then added and the whole mixture again incubated for 30min on a rotary mixer at room temperature. Samples were then spotted onto a microscope plate before being examined by phase microscopy.

2.5.5 Examination of the ability of purified K88 adhesin to agglutinate bacterial suspensions.

100μl of bacterial suspensions ($1x10^9$ in PBS) were added to an equal volume of K88 adhesin (0.5mg/ml in PBS) and incubated at room temperature on a rotary mixer for 30min. Samples were spotted onto microscope slides before being examined by phase microscopy.

2.5.6 Effect of K88 antisera on the adhesion test.

This was performed according to Sellwood *et al.* (1975). 100μl of undiluted antisera was added to an equal volume of bacterial suspension (strain K12:K88ab; $1x10^9$/ml) and incubated at ambient temperature for 1hr. Suspensions of epithelial cells were added and mixed on a rotary mixer at room temperature for 30min. Samples were examined by phase contrast microscopy.

2.6 Preparation of specific polyclonal α(anti)k88 antiserum.

2.6.1 Method of antiserum production.

The K88ab antigen was prepared from the K12:K88ab transconjugant strain as outlined in the second large scale method for the isolation of the K88 adhesin (see Section 2.4.2). A 0.3mg/ml solution was emulsified with an equal volume of Freund's complete adjuvant with a small homogeniser (Tri-R model K41). Two New Zealand white rabbits were sub-cutaneously injected with 1ml of the emulsion at 4-6 sites. After 4 weeks, the rabbits were boosted with a similar schedule except that the antigen was emulsified with a similar volume of incomplete Freund's adjuvant. Six weeks after the original inoculations the rabbits were bled, while non-immune serum was obtained from a control rabbit.

2.6.2 Preparation of absorbed antiserum.

Blood obtained from immunised rabbits was stored at 4°C for 1hr and then centrifuged at 2,000rpm (Jouan CR411) for 10 min at 4°C to remove cell debris. 200μl-1,000μl aliquots of the supernate was then centrifuged at 6,500rpm (MSE Microcentaur) for 5min, the serum retained and the pellet discarded. Antiserum at this stage was regarded as crude. Absorption of antisera was based on the method of Jones 1972. *E.coli* strain K12 was cultured overnight on nutrient agar, the cells harvested in PBS and pelleted at 5,000g for 10min. Serum obtained above was diluted 1:5 in saline and added to the bacterial pellet (wet weight 4.28g). After brief shaking, the mixture was incubated at 37°C for 2hr and then overnight at 4°C. Bacteria were pelleted at 10,000g for 1hr and the supernate filtered twice through a 0.2μm membrane filter (Gelman sciences). After subsequent analysis (see 5.2) the antiserum at this stage was regarded as specific anti-K88ab (αK88ab) serum. To obtain anti-K88a (αK88a) serum, 2.5ml of a 1:5 dilution of the αK88ab serum was absorbed with 1.27g of nutrient broth grown *E.coli* serotype K88ad for 1hr at 37°C. Bacterial cells were pelleted at 13,000rpm for 5min (MSE Microcentaur) and the supernate filtered twice (0.2μm pore size). All antisera were stored at -20°C before use. The specificity of the K88ab antisera was determined by ELISA, Ouchterlony and Western blotting.

2.6.3 Determination of antiserum specificity by enzyme-linked immunoabsorbent assay (ELISA).

The ELISA detailed below was for use in detecting both cell free and cell-bound K88 adhesin. In the case of the detection of purified K88 fimbrial adhesin antigen, the final concentrations of adhesin and antisera were both determined in preliminary experiments. For the detection of cell bound K88 adhesin, overnight agar cultures (at 37°C) were harvested and suspended in PBS

to an optical density of 1.00 at 600nm. The remainder of the ELISA was performed as below with the bacterial suspension being substituted for the adhesin solution.

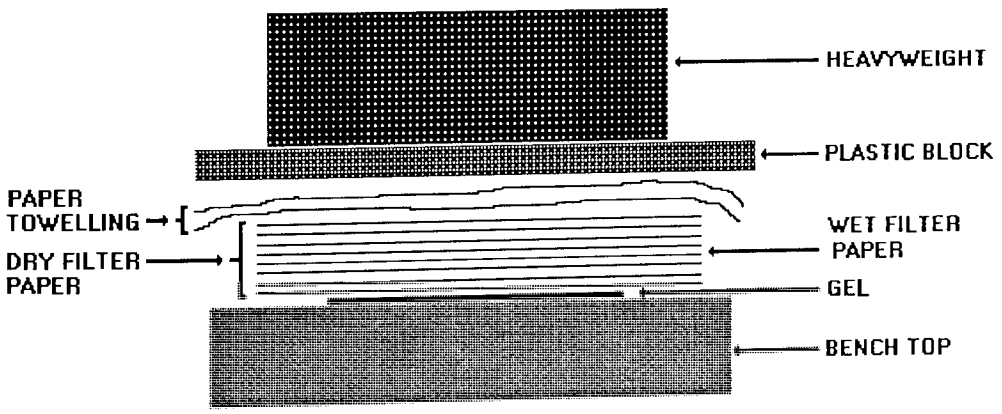
A 96-well Dynatech Immulon 2 microplate was coated with 100μl/well of purified K88ab adhesin at a concentration of 7.5μg/ml which had been diluted from stock in PBS. The plate was then incubated overnight at 4°C before the unbound adhesin was removed and the plate tapped onto tissue paper several times. 100μl/well of blocking agent (1% w/v dried skimmed milk powder in PBS) was then added before incubating the plate at 37°C for 1hr. The plate was then washed three times with 0.02% Tween-20 in PBS. 100μl of a 1:100 dilution of antiserum in blocking agent was then added to each well and the plate again incubated at 37°C for 1hr. At this stage a 1:100 dilution of non-immune serum was included as a negative control. After washing three times in PBS/Tween as above, 100μl of detection antibody (1:1,000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate in blocking agent) was then added to each well and the plate incubated at 37°C for 1hr. The plate was then washed five times in PBS/Tween and 100μl of ABTS (55mg 2,2 azinobis-3-ethylben-thiazoline sulfonic acid in 0.2M phosphate/citrate buffer pH4.3 containing 0.04%v/v H_2O_2) substrate added. When a strong colour had developed (15-30min) the reaction was stopped by the addition of 100μl/well of 0.01% sodium azide in 0.1M citric acid. The plates were then read at either 414nm (Titertech multiskan MCC) or at 410nm (Dynatech MR-700). All tests were performed in duplicate.

2.6.4 Determination of antiserum specificity by double diffusion (Ouchterlony).

A 1% solution of agarose-M (LKB) in either Tris-barbital buffer (containing barbitone 22.4g/l, Tris 44.3g/l, calcium lactate 0.53g/l and sodium

azide 0.65g/l) or dH_2O was dissolved by vigorous stirring and boiling. 10ml of this solution was carefully added to 84x94mm gelbond films (LKB) with the hydrophilic side facing upwards. The agarose was allowed to set before a size 2 cork borer was used to cut an array of either hexagonal or square holes surrounding a centre well. A plugged Pasteur pipette connected to a vacuum line was then used to remove the agarose from the wells. A 0.75mg/ml solution of purified K88 in PBS (pH 8) was then heated at 60°C for 5min before 20μl was added to the centre well. 20μl of either αK88ab antisera or non-immune antisera were then added to the surrounding wells (between neat and 1:8 dilutions of the antisera were found suitable). The gel was then placed in a covered container containing some wetted paper towelling and left overnight at

Figure 2.1. Apparatus used for the drying of double diffusion (Ouchterlony) gels.



Gels were alternatively washed and then compressed by the apparatus above to compact them for examination and storage. The final stage of the preparation involves the drying of the gel with a hairdryer.

37°C. The gel was then placed on a flat bench and a series of filter papers, towelling and finally a heavy weight added (see Figure 2.1). The gel was compressed in this manner for 15min, washed in saline on a suspension mixer for 10min and this cycle of compression and then washing in saline repeated. A further compression of the gel was conducted before a final wash in dH_2O for 10min. The gel was then stained with Coomassie blue (see 2.3.5 for recipe) for 15min before being destained in a mixture of 40% methanol, 10% ethanol in dH_2O until background staining disappeared. As a last step the gels were dried before being examined over a light box.

2.7 Characterisation of the binding of the K88 adhesin to epithelial cells.

2.7.1 Standard assay for K88 receptor.

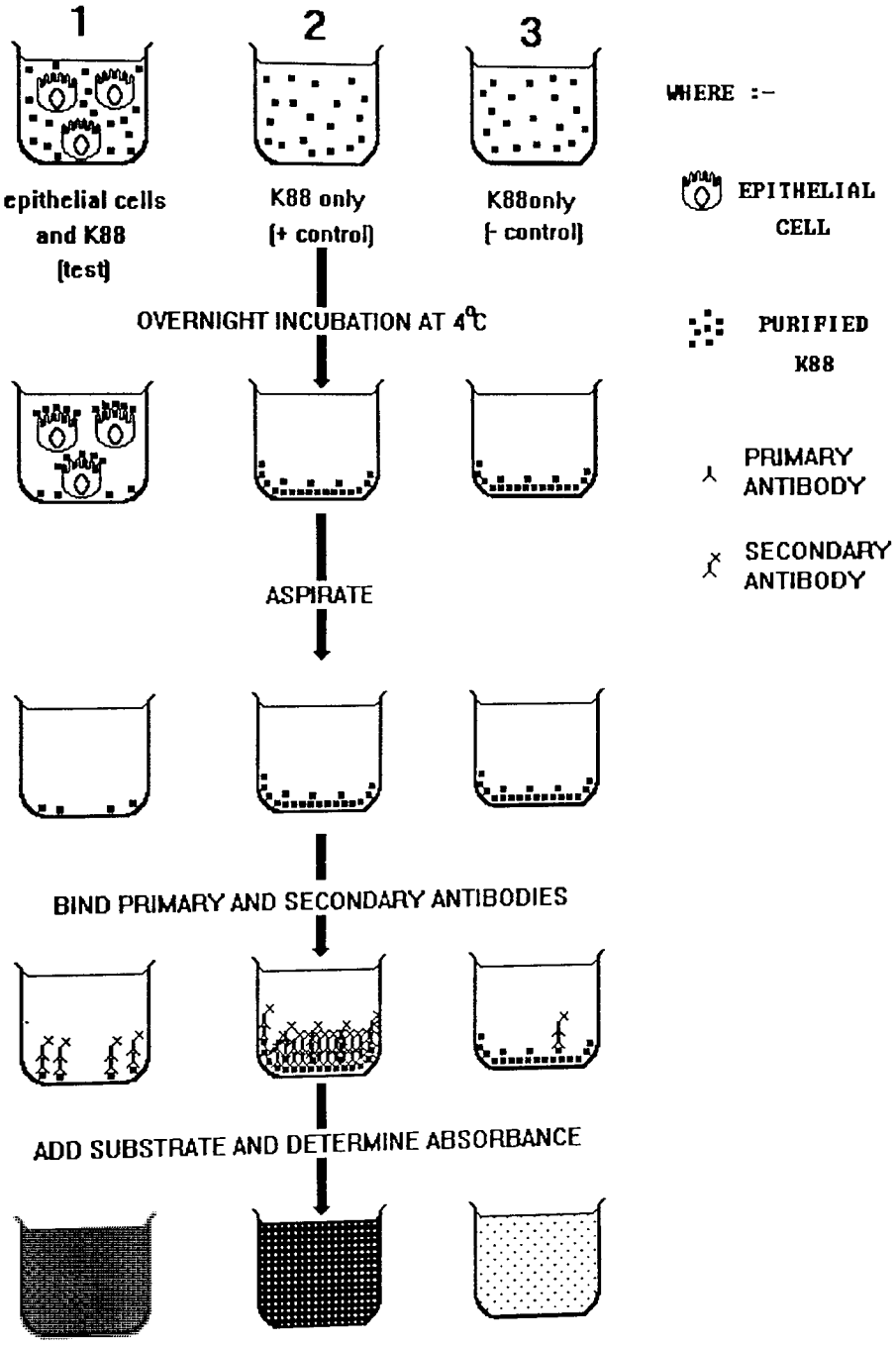
This assay was developed to quantitate the binding of purified K88ab adhesin to the K88 receptor(s) present on porcine epithelial cells. Preliminary experiments were first conducted to determine an appropriate concentration of purified K88 adhesin to be used in the assay (see Section 2.7.2). In addition an appropriate concentration of intestinal epithelial cells to be used was determined. In this case, doubling dilutions of epithelial cells (stock concentration = $1x10^6$) in PBS were prepared down the columns of a 96-well plate. An equal volume (100μl) of purified K88 (15ng/ml) was added to each well and the plate incubated overnight at 4°C. The plate was processed as outlined from the position marked ☻ below. Controls used consisted of 100μl PBS and 100μl of purified K88 (15ng/ml) with either αK88 antiserum (positive control) or non-immune serum (negative control) as the primary antibody in the ELISA. For further details of the standard assay see Figure 2.2.

Doubling dilutions were prepared of a stock solution of purified K88 (75 or 15ng/ml in PBS) down each column of a 96-well microplate (Dynatech

Immulon 2). An equal volume (100μl) of epithelial cells ($5x10^5$/ml) were then added to the first five columns. To five further columns 100μl of PBS was added to each well. Three of these latter columns were for use as positive controls (αK88 antiserum to be added subsequently) and two as negative controls (non-immune rabbit serum to be added subsequently). After overnight incubation at 4°C ☻ the plate was carefully aspirated, tapped dry onto tissue paper and 100μl/well blocking agent (1% dried skimmed milk powder in PBS) added to block unoccupied attachment sites left in the wells. After incubation for 1hr at 37°C the plate was washed three times in PBS containing 0.02% Tween-20 (PBST). 100μl of a 1:1,000 dilution of αK88 antiserum (in blocking agent) was added to the first eight columns while 100μl of a 1:1,000 dilution of normal rabbit serum was added to each of the remaining wells. The plate was again incubated at 37°C for 1hr and then washed three times in PBST. 100μl of goat α-rabbit horseradish peroxidase conjugate in blocking agent was then added to each well and the plate incubated at 37°C for 1hr. After incubation the plate was washed five times in PBST before 100μl substrate ((1mM 2,2'Azino-bis(3-Ethylbenz-thiazoline-6-sulfonic acid)) in 0.2M phosphate, 0.2M citrate 0.002%v/v hydrogen peroxide buffer (ABTS) was added to each well. After ~1hr the absorbance at either 414 or 410nm was determined.

Figure 2.2 (opposite). *The standard assay for the K88 receptor(s) was based on the enzyme-linked immunoabsorbent assay (ELISA). The initial stages of the ELISA were modified so that the amount of purified K88 adhesin present in the plate wells could be quantitatively measured after its incubation with a known concentration of porcine epithelial cells. In 1 epithelial cells were incubated with a known quantity of the K88 adhesin which then bound during the overnight incubation step. The epithelial cells were removed from the system along with the bound K88. To quantitate the amount of K88 adhesin remaining in 1, doubling dilutions of the K88 adhesin (2) were simultaneously analyzed with 1 and used to calculate a standard curve of absorbance (414nm) against K88 adhesin concentration. The standard curve was used to determine the amount of K88 adhesin bound to the microplate well. K88 adhesin bound to the microplate well is the equivalent to "free" K88 adhesin since it represents K88 adhesin which did not bind to the epithelial cells during the overnight incubation step. By subtracting the amount of free K88 adhesin determined from the initial amount added it was possible to determine the amount of K88 adhesin which had bound to the epithelial cells. A negative control (3) is included to allow for any non-specific absorbance in 1+2 and its value was subtracted from 1 and 2 before the construction of the standard curve and the determination of the amount of bound/free K88 adhesin.*

Figure 2.2


1
2
3
epithelial cells and K88 (test)
K88 only (+ control)
K88only (- control)
OVERNIGHT INCUBATION AT 4°C
ASPIRATE
BIND PRIMARY AND SECONDARY ANTIBODIES
ADD SUBSTRATE AND DETERMINE ABSORBANCE
WHERE :-
EPITHELIAL CELL
PURIFIED K88
PRIMARY ANTIBODY
SECONDARY ANTIBODY

2.7.2 Assay to determine the inhibition of the K88 receptor(s) by various carbohydrates.

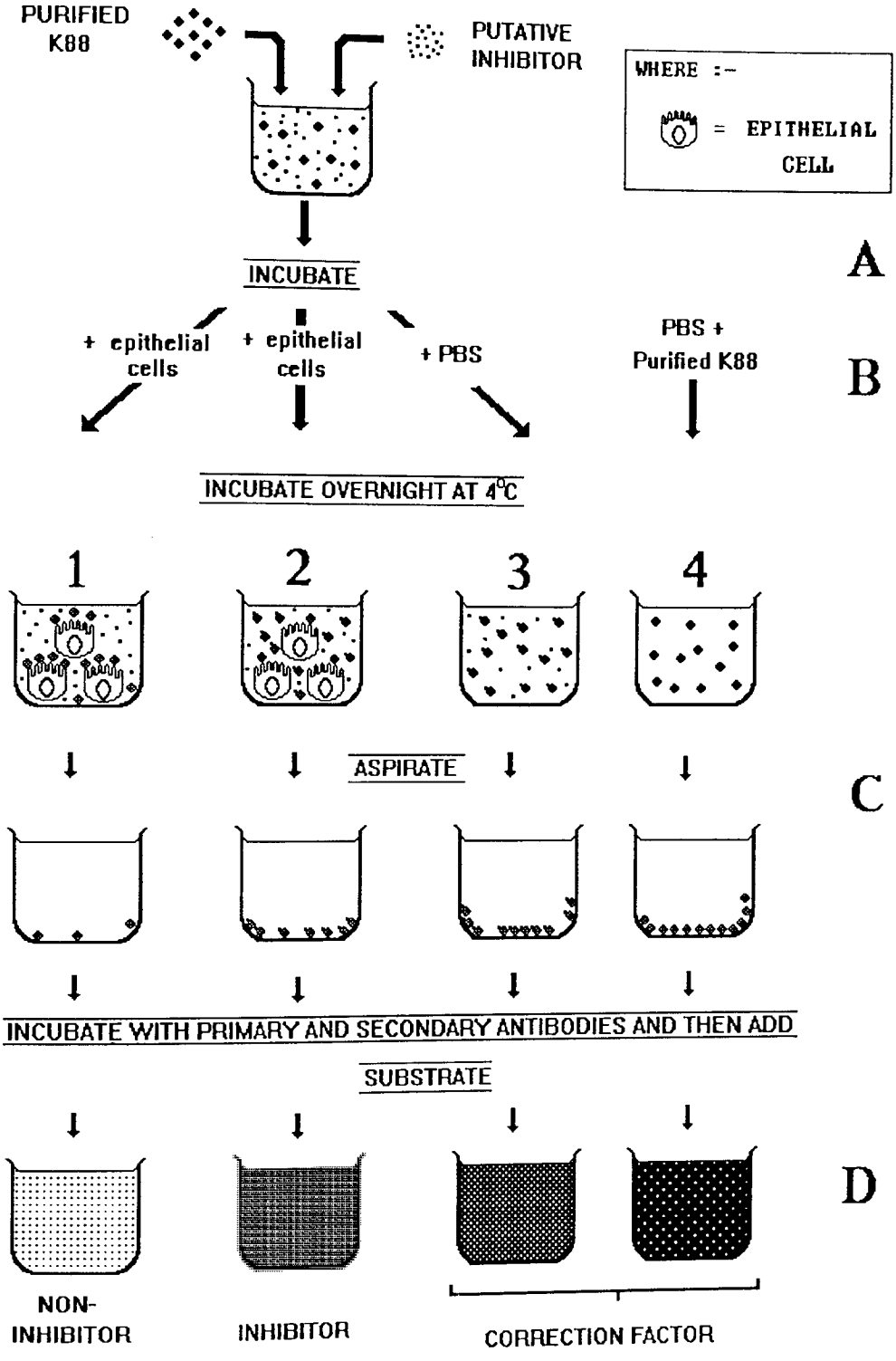
A preliminary experiment was conducted to determine the reaction profile of the ELISA with various concentrations of purified K88 adhesin. Doubling dilutions of purified K88 (stock concentration of 0.76mg/ml) were prepared across a 96-well plate. The plate was incubated overnight at 4°C and processed as outlined in Section 2.7.1 (from ☻) with the same controls. Final values were obtained by subtracting the value of the negative control from that of the test. All samples were performed in triplicate.

The inhibitory potential of carbohydrates was based on the standard assay with a pre-incubation step as follows (see Figure 2.3):-

500μl of carbohydrate (in PBS) was mixed with 500μl of purified K88 (30ng/ml in PBS) and incubated at 37°C for 1hr. 200μl of this mixture was then added to an equal volume of epithelial cells ($5x10^5$/ml), briefly mixed and 3x 100μl aliquots incubated overnight at 4°C in a microplate. The remainder of the assay was performed as for the standard assay. Several control wells were processed on each plate including a control to determine the maximum K88 adhesin which could bind to the epithelial cells consisting of 50μl of epithelial cell suspension ($5x10^5$/ml) and 50μl of purified K88 adhesin (15ng/ml). A correction factor was also determined by comparing the results obtained in the presence (50μl of sugar/K88 adhesin mixture and 50μl PBS/well) or absence (50μl of K88 adhesin [15ng/ml] and 50μl of PBS) of the sugar concerned (see Figure 2.3) and was necessary to allow for any non-specific inhibition of the binding of the purified K88 adhesin to the microplate by the carbohydrate. For the calculation of final absorbance values, the absorbance obtained with the negative control consisting of 50μl K88 adhesin (15ng/ml) and 50μl PBS/well was first subtracted. All tests were carried out in triplicate, all controls in duplicate.

Figure 2.3 (opposite). *Schematic diagram of assay developed to determine inhibition of the K88 receptor(s) present on intestinal epithelial cells isolated from pigs. The assay was based on determining the amount of K88 adhesin remaining in the microplate wells after various pretreatments. The putative inhibitor was incubated with purified K88 adhesin for 1hr at 37°C to allow enough time for recognition and binding to occur if appropriate (A). Aliquots of this mixture either had a known concentration of porcine epithelial cells or PBS added. A positive control was included consisting of a dilution of K88 adhesin in PBS. A further control consisted of porcine epithelial cells and K88 adhesin in PBS only (not shown). This latter control was to quantitate the maximum amount of K88 adhesin which could be bound by the epithelial cells. The result obtained here was similar to that in 1 where the carbohydrate used was not inhibitory and the maximum amount of K88 adhesin was retained by the epithelial cells and thus removed from the system (during C). In 2, the carbohydrate has bound to the K88 adhesin which is therefore prevented from binding to the epithelial cells. In this case, less K88 adhesin is removed from the system when the epithelial cells are removed (C) resulting in a higher final signal (see 2). 3 and 4 were included on each plate to allow for any direct inhibition a particular test substance may have on the binding of the K88 adhesin to the microplate. After D, the correction factor was calculated by dividing the result of 4 by the result of 3. The final values of 1 and 2 were then obtained by multiplying them by the correction factor.*

Figure 2.3


PURIFIED K88
PUTATIVE INHIBITOR
WHERE :-
= EPITHELIAL CELL
A
INCUBATE
+ epithelial cells
+ epithelial cells
+ PBS
PBS + Purified K88
B
INCUBATE OVERNIGHT AT 4°C
1
2
3
4
ASPIRATE
C
INCUBATE WITH PRIMARY AND SECONDARY ANTIBODIES AND THEN ADD SUBSTRATE
D
NON-INHIBITOR
INHIBITOR
CORRECTION FACTOR

2.7.3 Assay to determine inhibition of the K88 receptor(s) by lectins.

Again this was based on the standard assay outlined in Section 2.7.1. The lectins were obtained freeze-dried in ampoules from the supplier (Sigma Chemical Company Ltd.). Lectins were suspended in PBS to a final concentration of 0.4mg/ml. In the case of lectins requiring metal ions for activity, ions at the appropriate concentration were included in the PBS before the lectins were suspended. Experimentally, 100μl of lectin was mixed with 300μl of epithelial cells ($5x10^5$/ml) and incubated at 37°C for 30min. 100μl of this mixture was then added to an equal amount of K88 adhesin (15ng/ml) in the well of a microplate and incubated overnight at 4°C. To assess the absorbance value in the absence of inhibition a control consisting of 100μl of BSA and 300μl of enterocytes was processed as the test sample above with 100μl of this mixture being incubated with an equal amount of K88 adhesin (15ng/ml). A positive control consisting of 100μl purified K88 + 50μl BSA (0.4mg/ml) + 50μl PBS was also prepared. 100μl purified K88 + 100μl PBS with non-immune rabbit serum used as the primary antibody in the subsequent ELISA was used as the negative control. The mean absorbance difference between the positive control wells and the wells containing BSA substituted for the lectins was regarded as corresponding to 100% binding. Before determination of the degree of inhibition by individual lectins all wells were corrected for the absorbance of the mean of the negative control wells. All samples and controls were carried out in triplicate, all plates were performed in duplicate.

2.7.4 Formalinization of epithelial cells.

Epithelial cells ($5x10^5$) were suspended in 1% formaldehyde for 7 days at 4°C according to the method of Sellwood (1980a). Epithelial cells were washed three times in PBS before use in the standard assay.

2.8 Characterisation of the binding of the K88 adhesin to erythrocytes.

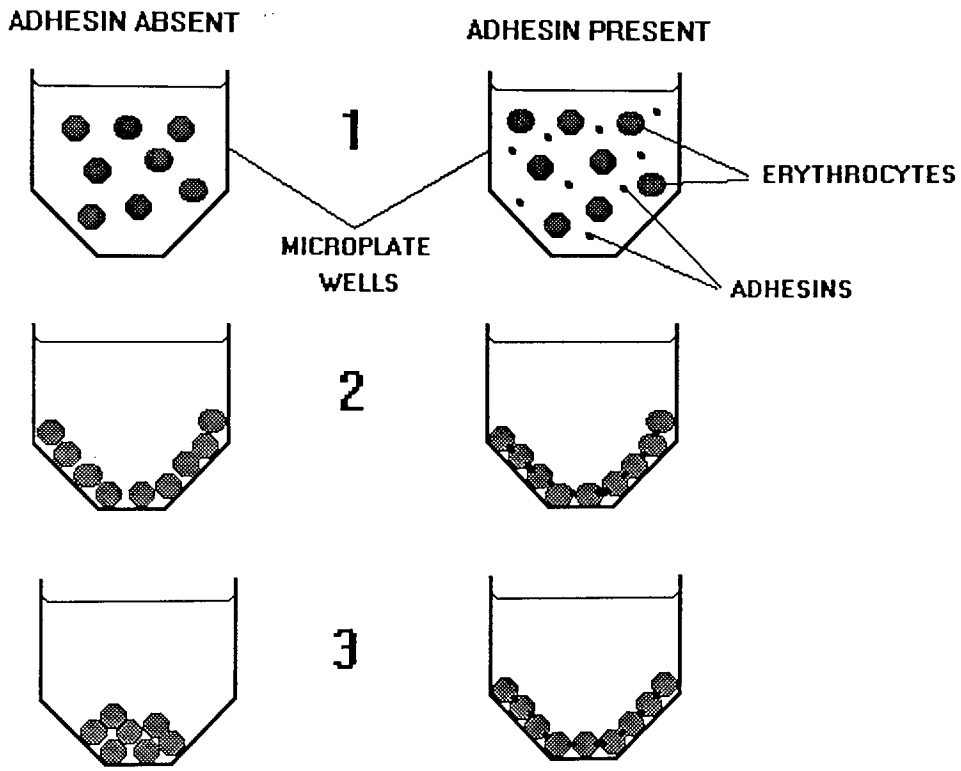
2.8.1 Microhaemagglutination method.

This was based on the method of Jones (1972). Erythrocytes were collected and immediately stored in heparinised tubes. The erythrocytes were then washed repeatedly in 2 volumes of either saline (if erythrocytes were subsequently to be used in pH based studies) or PBS until supernate was clear (centrifugation at 500g for 10min). Erythrocyte cell numbers was determined by dilution (~1:500 was usually suitable) and counting in a haemocytometer. Erythrocytes were usually stored at 4°C at a cell concentration of 1-10x10^9/ml in PBS. Suspensions were washed daily in PBS and under these conditions could be used for up to 1 week with no detectable change in properties. Before use, erythrocyte suspensions were diluted as required in PBS containing 2% w/v D-mannose (PBS+M). A concentration of 2x10^8/ml was found to be roughly equivalent to a 1% v/v erythrocyte suspension. A schematic diagram describing the microhaemagglutination method is given in Figure 2.4.

2.8.2 Determination of the ability of bacterial strains to express the K88 adhesin and cause haemagglutination.

Nutrient broth cultures (30ml) were centrifuged at 5,000g for 10min, washed once in PBS and then resuspended in 2ml of PBS. 1ml of this bacterial suspension was adjusted to an absorbance of 1.00 at 600nm and subjected to an ELISA as in Section 2.9.5. A 100μl aliquot of the remaining suspension was used to determine the viable count of bacteria. The concentration of bacteria was then adjusted to 1x10^{10} cfu/ml by pelleting the bacteria (6,500rpm for 3min, MSE microcentaur) and resuspending them in PBS. 25μl of the adjusted suspension was added to the well of a 96-well round- bottomed plate (Sterilin) on ice. 25μl of erythrocyte suspension (guinea-pig, hen, or rabbit at 5x10^8/ml)

Figure 2.4 Schematic diagram of the mechanism of the microhaemagglutination method.



The initial step of haemagglutination is the mixing of erythrocytes with the suspected adhesin (1). After the initial mixing, the reactants are incubated on ice. During this incubation step the erythrocytes settle to the bottom of of the well of the microplate under the influence of gravity (2). Upon further incubation, the erythrocytes, in the absence of an adhesin form a pellet at the bottom of the well. In the presence of an adhesin, pellet formation is prevented by the crosslinking of the erythrocytes by the adhesin (3). Note that the above applies to both cell-bound and cell-free adhesin.

in 2% w/v D-mannose was then added and the plate incubated at 4°C for ~1hr. A negative control consisting of 25μl PBS and an equal amount of erythrocyte suspension was also incubated on each plate. All tests were performed in duplicate.

2.8.3 Determination of the effect of pH on the haemagglutination of guinea-pig and hen erythrocytes.

This was based on the microhaemagglutination method outlined above. Overnight broth cultures of *E.coli* strain K12:K88ab were pelleted at 5,000g for 10min, washed once in saline and then resuspended to $3.75x10^9$ cfu/ml. Before use the washed suspensions were stored at 4°C. Bacterial dilutions were prepared across the rows of a 96-well round bottomed plate as above. The stock solution of erythrocytes ($1x10^9$/ml) were then diluted 1:5 in PBS+M buffers (range pH 3-10). 25μl of each of these suspensions were then added to the equal volume of bacterial suspension in the wells of the 96-well plate. The plate was then incubated on ice for ~1hr before being examined for haemagglutination.

2.8.4 Determination of the haemagglutination titre (HA).

To determine the haemagglutination titre (HA) of bacterial suspensions, doubling dilutions were prepared in saline across the rows of a 96-well round-bottomed plate (Sterilin). An equal volume (25μl) of erythrocytes (guinea-pig, $2x10^9$/ml; chicken, $5x10^8$/ml; rabbit, $1x10^9$/ml; marmoset $5x10^8$/ml and pig, $1x10^9$/ml) were then added to the bacterial dilutions while the plate was on ice. The plate was then briefly shaken (5-10 seconds) on a plate shaker (Bioblock Scientific) before being incubated on ice for ~1hr and then examined. The HA was recorded as either the highest dilution of bacterial suspension or actual bacterial concentration which resulted in a complete mat of aggregated

erythrocytes on the plate well surface. Where more accurate determinations of the haemagglutination titre were required, initial dilutions of 1/3 and 1/5 of the stock bacterial suspension ($1x10^{10}$) were used to prepare doubling dilutions. Initially a negative control consisting of *E.coli* serotype K12 was used on each plate as a $K88^-$ bacterial suspension. In subsequent experiments, the bacterial suspension was replaced by PBS in the negative control. Figure 2.5 examines the theoretical mechanism and implications of determining haemagglutination titres.

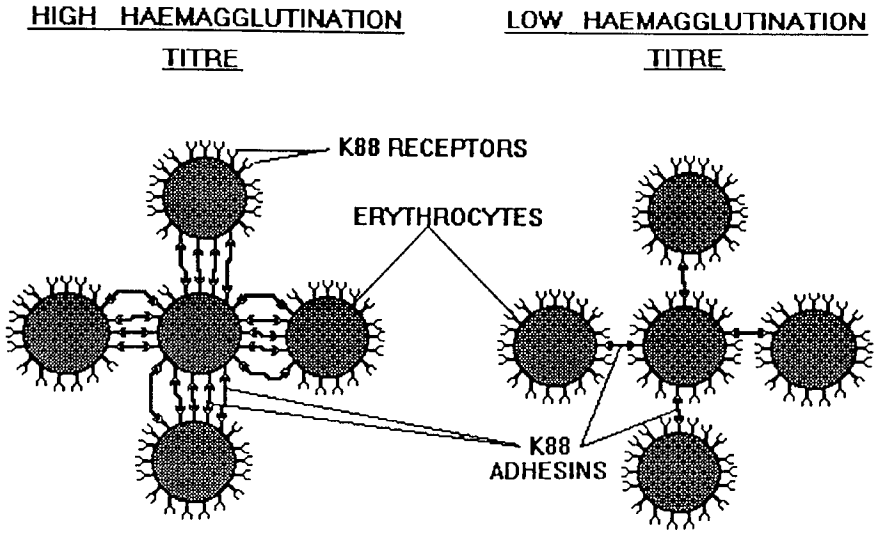
2.8.5 Determination of the haemagglutination titre of the purified K88 adhesin (HAP).

The haemagglutination titre of the purified K88ab adhesin preparation (HAP) was determined in a similar way to the HA. In this case, doubling dilutions (also if required 1/3 and 1/5 dilutions) of the stock K88 (0.76mg/ml) were prepared across the 96-well plate while on ice. The plate was then processed as for bacterial suspensions. See also Figure 2.5.

2.8.6 Determination of the inhibitory properties of various carbohydrates on K88 adhesin-induced haemagglutination.

The basic technique was based on that of Jones 1972, Gibbons *et al.* 1975 and Ono *et al.* 1989. The inhibition of the agglutination of both guinea-pig and hen erythrocytes was investigated. Erythrocytes were washed and prepared as in Section 2.8.1. The various carbohydrates were suspended in PBS to a stock concentration from which doubling dilutions were prepared. 25μl of the dilutions of the putative inhibitor were added to the wells of a column of 96-well round bottomed plates, with the highest concentrations at the top. 25μl of purified K88ab (12.5μg/ml for guinea-pig, 750μg/ml for hen) was then added to each of the wells and the plate incubated at room temperature for 1hr

Figure 2.5 Consideration of the mechanism for the determination of haemagglutination titre.



The determination of the haemagglutination titre involves finding the lowest amount of adhesin that can prevent pellet formation in a microplate well. A low haemagglutination titre (see above) indicates that the adhesin has a comparatively high affinity for the complementary receptor present on the erythrocyte. In effect comparatively few bonds are formed between adhesin and receptor and many receptor sites are unoccupied. If a high haemagglutination titre is recorded it indicates that the affinity of the adhesin for the receptor is comparatively low. In this case many adhesin/receptor bonds are required to prevent the erythrocytes from pelleting and more receptor sites are occupied (see above). Note that the haemagglutination titre recorded is only an approximation of the affinity of an adhesin since at low concentrations of adhesin the system is unlikely to have achieved equilibrium in the time between the mixing of the reactants and the pelleting of the erythrocytes. In addition, comparison of haemagglutination titres achieved by the same adhesin serotype with differing erythrocytes is only approximate because the number/ availability of K88 receptors expressed by those erythrocytes is likely to vary.

with continuous gentle shaking. 25μl of 1% (~2x10^8 cells/ml) erythrocyte suspension was added to each plate while on ice and then mixed briefly. The plate was then incubated at 4°C for 2hr or covered and incubated overnight at 4°C before being examined for haemagglutination. As a positive control, PBS was substituted for the putative inhibitor in the initial incubation at room temperature. Two negative controls were employed, in one, PBS was substituted for the purified K88 adhesin in the initial incubation step while in the other 50μl of PBS was mixed with the erythrocyte suspension.

2.8.7 Inhibition of haemagglutination by specific antiserum.

This was performed largely as in Parry and Porter 1978. Eight times the HA and HAP were determined from the results of Sections 2.8.4 and 2.8.5. Each round-bottomed 96-well plate was divided into three sections of four columns, each 1/3 used for either guinea-pig, hen or rabbit erythrocytes. Doubling dilutions of stock solutions of either specific αK88ab, αK88b or non-immune rabbit serum were prepared. 25μl of either 8 HA or HAP was added to the first three columns of each section. 25μl of the dilutions of either αK88ab or αK88b serum was added in duplicate to the first two columns of each section. 25μl of the dilutions of non-immune serum were added to the third column in each section. 25μl of the appropriate specific serum and 25μl of PBS was added to the fourth column in each section. The plate was then briefly shaken and incubated at room temperature for 30min. After incubation, 25μl of guinea-pig erythrocytes (2x10^9/ml in 2% w/v D-mannose) were added to each well in the first set, 25μl of hen erythrocytes (5x10^8/ml in 2% w/v D-mannose) were added to the middle set and finally 25μl of rabbit erythrocytes (1x10^9/ml in 2% D-mannose) were added to the last set. The plate was then briefly shaken and incubated on ice for ~1hr before being examined for haemagglutination.

2.8.8 Determination of the temperature stability of the haemagglutination reaction.

This experiment was designed to determine the effect of temperature on the K88 adhesin/erythrocyte reaction. The temperature stability of the erythrocyte mat formed by all three serotypes of both cell bound and cell-free K88 with guinea-pig, hen and rabbit erythrocytes was determined. Initially 25μl of either 8 HA or HAP was added to the wells of a 96-well round-bottomed plate and 25μl of erythrocyte suspension (guinea-pig and hen $5x10^8$/ml, rabbit $1x10^9$/ml in 2% w/v D-mannose) added. The plate was briefly shaken and incubated on ice for 1hr. A negative control of 25μl PBS and 25μl erythrocyte suspension was also employed on each plate. The plate was then floated in a water bath at 10°C for 15min and the stability of the erythrocyte mat determined while the plate was placed on ice. The plate was then floated in a water bath at 15°C for 15min and the stability of the erythrocyte mat again determined. The process of floating the plate in the water bath, determining the erythrocyte mat stability while the plate was on ice was repeated in 5°C divisions up to a maximum of 50°C (for proposed mechanism see Figure 2.6).

2.9 The growth and expression of the K88 adhesin by *E.coli* strains using various media and differing cultural conditions.

2.9.1 Growth curves of bacterial strains in various broth media.

Growth was monitored using bioscreen (Labsystems Ltd.) at 600nm. Individual wells of a 100-well honeycomb plate (Labsystems Ltd.) were filled with 400μl of various broth media (see Appendix 1). Individual colonies from nutrient agar plates were aseptically suspended in 10ml of PBS. 25μl of a 1:10 dilution of this suspension was used to inoculate the wells of the honeycomb

Figure 2.6 (opposite). *Temperature stability of the K88 adhesin/ erythrocyte interaction. In the presence of excess K88 adhesin all the K88 receptors are occupied and the erythrocytes are crosslinked (1). Increasing the temperature increases thermal agitation, breaking low affinity K88 adhesin/receptor bonds (2) and resulting in pellet formation (3). High affinity bonds remain unbroken (2) and the erythrocytes remain crosslinked in a mat of cells (3).*

Figure 2.6

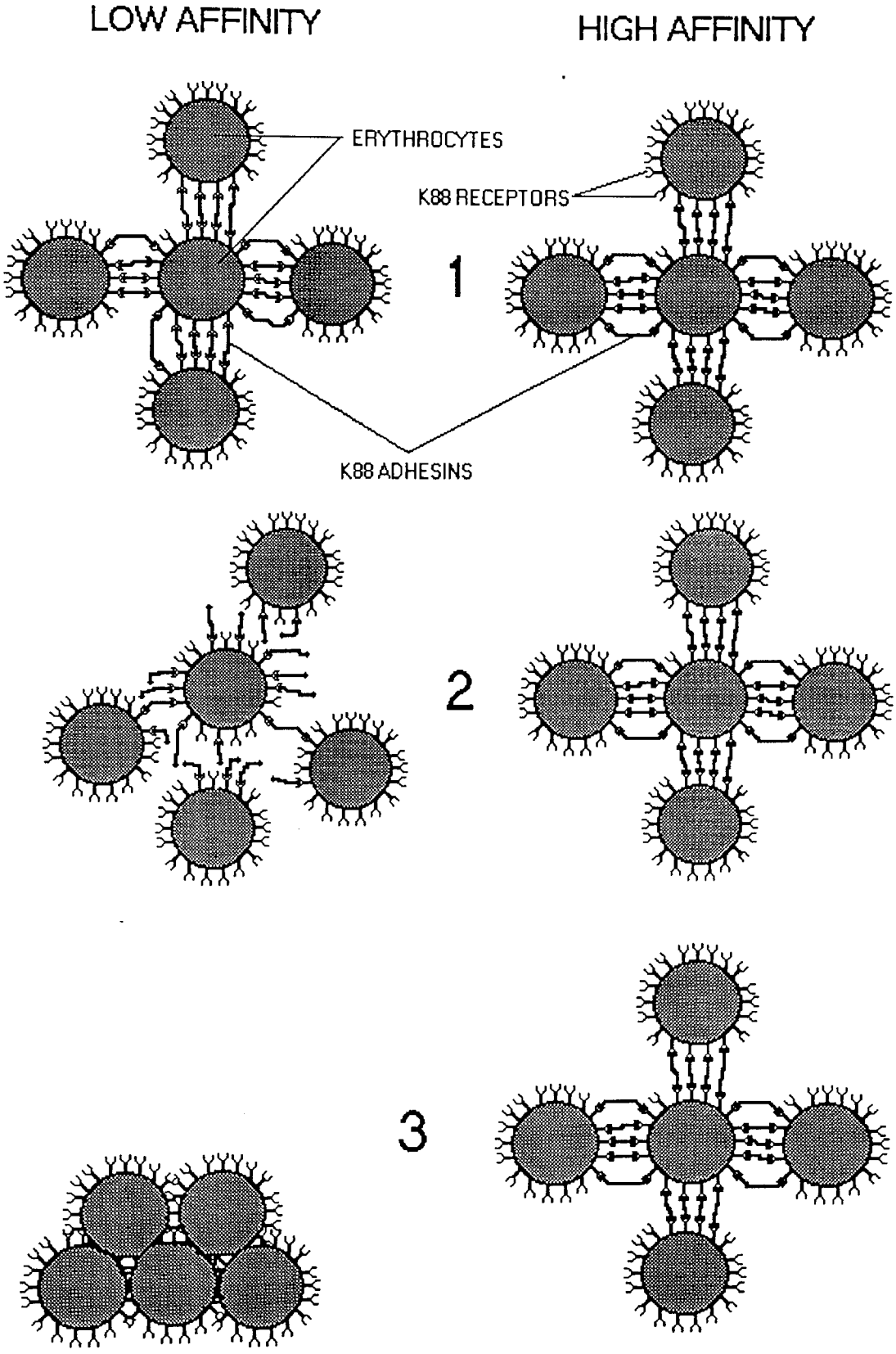

LOW AFFINITY
HIGH AFFINITY
ERYTHROCYTES
K88 RECEPTORS
1
K88 ADHESINS
2
3

plate. Readings were taken every 30min and the cultures incubated unshaken at 37°C for 20hr.

2.9.2 Non-quantitative determination of the expression of the K88 adhesin.

The non-quantitative determination of the ability of *E.coli* strains under examination was determined in several ways. The ability or not of a culture to express the K88 adhesin was indicated by the ability to metabolize raffinose (see 2.3.8), direct extraction of a protein of approximately the correct molecular weight (see 2.4.2) and haemagglutination (see 2.8.2). The ability of individual colony forming units (cfu) to express the K88 adhesin was determined by dot blotting (2.9.3) and using the Fimbrex K88 kit (2.9.4).

2.9.3 Dot blotting of bacteria.

Two similar methods were employed. In the first, individual colonies were chosen and suspended in PBS before being spotted on to a nitrocellulose membrane. In the second method, colony material was directly blotted onto the same type of membrane before further processing.

Method 1

Serial dilutions (in PBS) of bacterial cultures were prepared and used to inoculate thoroughly dried nutrient agar plates. After overnight culture at 37°C, discrete colonies were removed with a loop which was then briskly rotated in a cryotube (Nunc) containing 100μl of 0.1% BSA in PBS with 10mM sodium azide (Buffer A). A gridded millipore type HA membrane filter (0.45μm pore size) was then briefly soaked in dH_2O before being allowed to dry in air for 15min. 1μl of bacterial suspension was then spotted on to the membrane. A positive control consisting of 1μl of 9.25, 1.85, 0.37, 0.07 and 0.02μg/ml concentrations of purified K88 in buffer A was run concurrently as a positive

control. Small cuts in the membrane were used to determine orientation. The membrane was then dried in a warming cabinet (set at 40°C) for 15min before it was saturated in 5% BSA in PBS containing 10mM sodium azide for 30min at 37°C. ♪ The membrane was then washed 3 times for 5min in buffer A before 20ml of a 1:1,000 dilution of αK88 in buffer A was added and the membrane incubated with constant agitation at room temperature for 1hr. The membrane was again washed three times in buffer A for 5min before the membrane was incubated in 20ml of the secondary antibody (1:1,000 goat anti-rabbit AuroProbe BL plus, in 5% v/v gelatin, 0.1% w/v BSA, 10mM sodium azide) for 2hr. The membrane was then washed twice in buffer A for 5min and once in dH_2O for 1min before 30ml of a 1:1 mixture of enhancer and initiator (Janssen) was used to cover the membrane. Development was facilitated by gentle mixing until a strong signal had appeared (15-40min). At this point the reaction was stopped by three washes in excess dH_2O for 10min, and the membrane dried between filter paper.

Method 2

A gridded type HA membrane filter (as above) was immersed in dH_2O and dried in a warming cabinet for 5min. At this stage the membrane was still damp. The membrane was then carefully placed on a nutrient agar plate containing well-isolated colonies and gently pressed. The membrane was then carefully removed and placed in a drying cabinet set at 40°C for 30min. 20ml of 5% BSA, PBS, 10mM sodium azide was then added to the membrane before incubation at 37°C for 30min. The remainder of the procedure was carried out as in method 1 (marked ♪).

2.9.4 The Fimbrex K88 kit.

This is a commercial kit (supplied by the Central Veterinary Laboratory,

Weybridge) based on a rapid agglutination test which uses coloured latex coated with monoclonal αK88 antibody to detect the K88 fimbrial adhesin on *E.coli* grown on solid medium. The test was performed as the instructions supplied with the kit.

2.9.5 Quantitative determination of the expression of the K88 adhesin.

The quantitative expression of the K88 adhesin by a variety of bacterial strains was determined either directly by ELISA or indirectly by protein extraction and ELISA.

By direct ELISA☺

Preliminary experiments were carried out in order to determine a suitable concentration of bacteria (cfu/ml) for use in the ELISA. 240ml of *E.coli* strain K12:K88ab culture in brain heart infusion broth (N°1, Difco) was pelleted at 5,000g for 10min washed once and resuspended in 10ml of PBS. The viable count of this suspension determined (see Section 2.3.2) and a doubling dilution series prepared from neat to 1:16,384. The optical density (at 600nm), final ELISA absorbance (see below ♥) and equivalent K88 adhesin concentration of each dilution was determined. From the results it was decided to standardise the absorbance to 1.00 at 600nm for bacterial suspensions in PBS.

♥ Doubling dilutions of purified K88ab (100μg/ml) were prepared in plastic universals. Duplicate aliquots of each K88 concentration were added (100μl) to the columns of a 96-well plate (Dynatech Immulon 2). These were used both as positive controls and in order to quantitate the amount of K88 adhesin detected in the sample wells. Eight 100μl samples of the bacterial suspensions under test were added to each column of the plate and the plate

incubated overnight at 4°C. Column 1 and 12 of each plate were not used as it was found that these were often not uniform in optical properties. The remainder of the ELISA was performed as in Section 2.6.3 except that two of the sample wells were used as negative controls where non-immune rabbit serum was used as the primary antibody. To quantitate the results, a standard curve was constructed from the results of the purified K88 standards. The sample value was calculated by subtracting the value of the negative control and comparing the remaining result with the standard curve.

This term is used to indicate the stage at which an ELISA was used to detect the K88 adhesin. It should not be confused with the type of ELISA used which was indirect (ie. the binding of a secondary antibody is responsible for the final signal) in all cases.

By protein extraction and ELISA

Here, 12ml samples were taken and the K88 adhesin extracted as in Section 2.4.4. The final extract was diluted either 1:10 or 1:20 in PBS and subjected to an ELISA as above.

2.9.6 Determination of the expression of the K88 adhesin after culture with various media.

Stock cultures were used to inoculate nutrient broth which was then incubated unshaken overnight at 37°C. Serial dilutions were prepared in PBS and 0.25ml used to inoculate nutrient agar plates which were in turn incubated overnight at 37°C. Well separated individual colonies were then transferred by sterile loop to 10ml of PBS. The suspension was then briefly spun in a vortex mixer. Broth cultures (20ml) were then aseptically inoculated with 10μl of bacterial suspension and incubated unshaken at 37°C for 16hr. For agar cultures, 1ml of bacterial suspension was added to one petri dish (90mm diameter containing 20ml of medium) and again cultivated for 16hr at 37°C.

Cells were harvested from the plates with 10ml of PBS. Both the bacterial cultures and suspensions were centrifuged at 5,000g for 10min and the pellets washed in 10ml of PBS. Suspensions were again pelleted and resuspended this time in 2ml of PBS. Each suspension was then monitored at 600nm and its absorbance adjusted to 1.00 by the addition of PBS. At least three colonies were analyzed for each combination of bacterial strain, medium type and medium form. All suspensions were analyzed by direct ELISA as in Section 2.9.5.

2.9.7 Expression of the K88 adhesin during growth in batch culture

Individual colonies on nutrient agar plates of dilutions of stock cultures were suspended in 1ml of PBS. 10μl of this suspension was then used to inoculate 500ml of the various media in 1 litre glass Duran bottles. The cultures were then incubated statically at 37°C. To cover the full 48hr time period required for each strain/medium combination, identical cultures were inoculated with a 12hr time delay between inoculations. 20ml samples were aseptically removed after 7,8,9,10,11,12hr from the first culture and at 15,18,24,28,32,48hr intervals from the second culture. To determine the amount of K88 expressed, 1ml of each sample was pelleted at 6,500rpm (MSE microcentaur) for 5min, washed once in 1ml PBS repelleted as before and resuspended in 300μl of PBS. 100μl of suspension was added to each of three wells of a 96-well microplate (Dynatech Immulon 2) and the amount of K88 adhesin present quantitated as in Section 2.9.5. In addition the amount of K88 present was quantitated for *E.coli* strain O8:K87:K88ab:H19 by its initial extraction from 12ml of broth and subsequent analysis by ELISA again as in Section 2.9.5.

3 CHARACTERISATION OF *E.COLI* STRAINS USED IN THIS STUDY

3.1 Introduction.

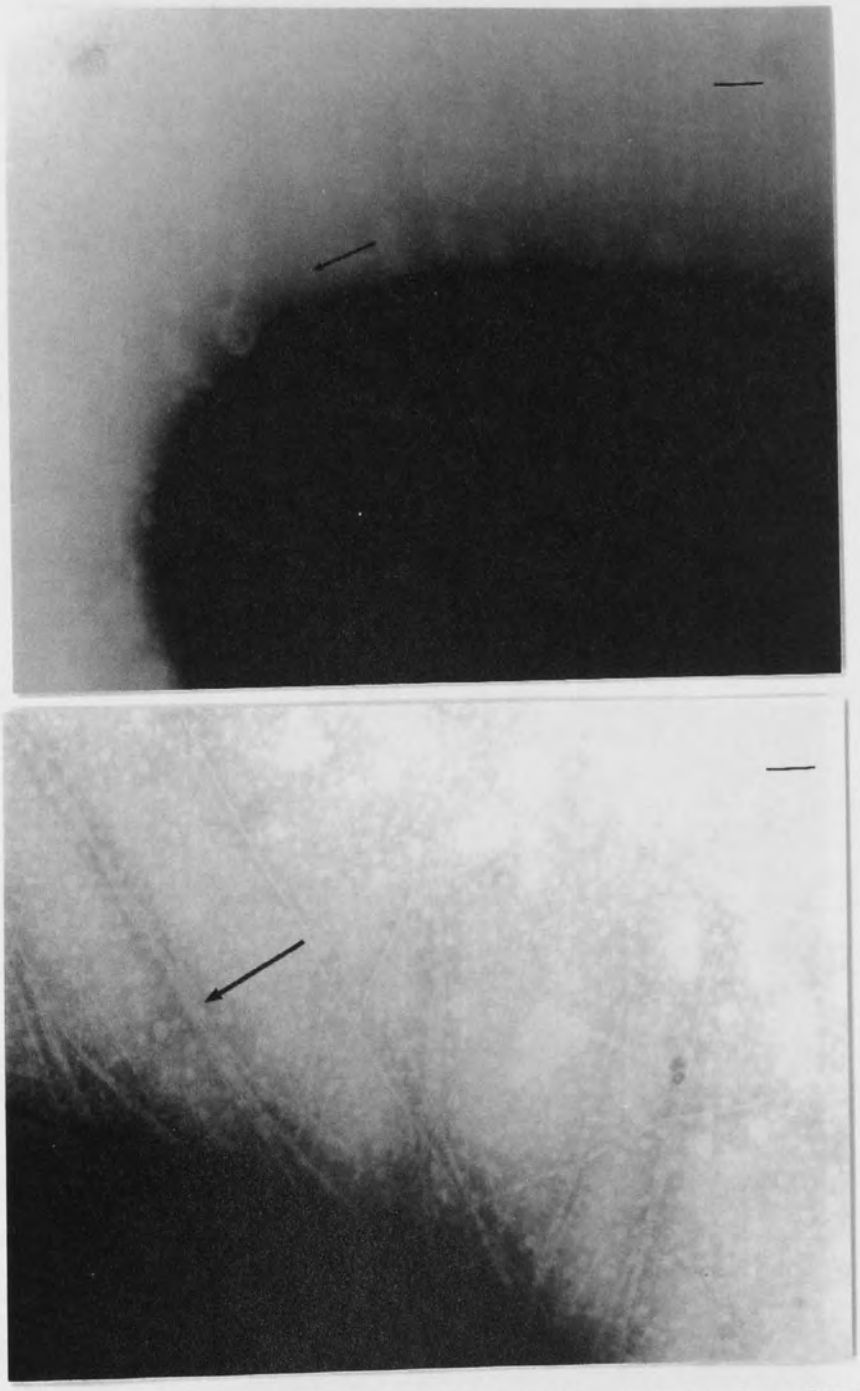
Before proceeding with more detailed investigations of the K88 adhesin\receptor system it was necessary to characterise the *E.coli* strains to be used. In particular, it was important that the ability or not to express the K88 adhesin should be investigated. Since in later experiments it was envisaged that other cell surface components may have an influence on their results e.g. the K88-mediated binding of whole bacteria may be sterically hindered by the presence of larger fimbriae, it was also important to examine the strains to be used for other fimbrial types in addition to K88.

3.2 Electron microscopy

There are very few published photographs of intact K88 fimbriae (Stirm *et al.* 1967, Wadstrom *et al.* 1979). One possible reason may be because the fimbria is so narrow (~2nm) it is at the limit of the resolution of transmission electron microscopy (Singleton and Sainsbury 1981, Chan *et al.* 1982). However, larger fimbriae are easily visible by electron microscopy. Most researchers have used either ammonium molybdate (Wadstom *et al.* 1980), phosphotungstic acid (Orskov, Orskov and Birch Anderson 1980) or uranyl acetate (Kallenius *et al.*. 1980) to stain preparations for subsequent examination. All these are negative stains which are electron dense in comparison to the specimen. Fimbriae therefore show up as light structures against a dark background.

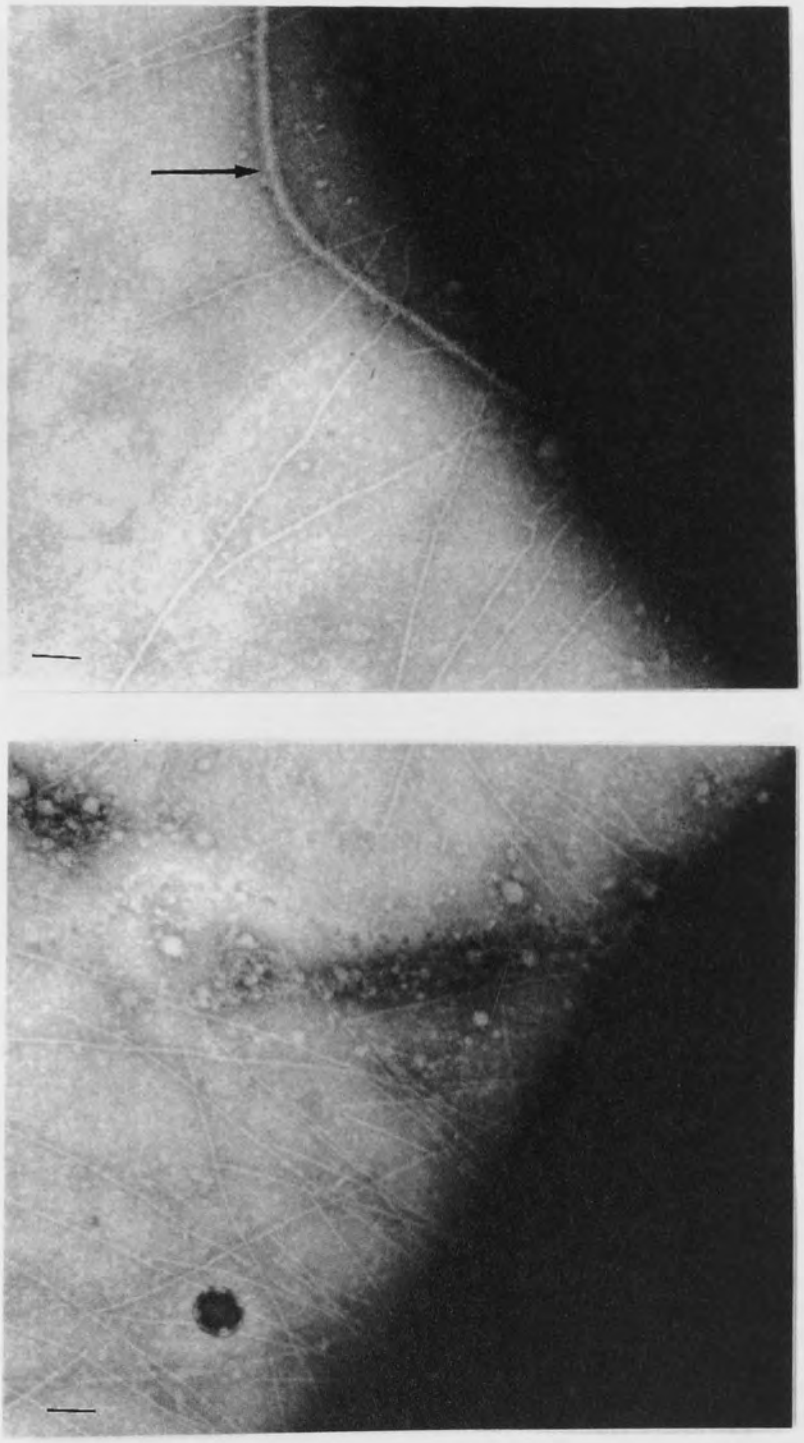
Initial electron microscope studies were unsuccessful in demonstrating the K88 fimbrial adhesin on any of the bacterial strains used (Figures 3.1-3.5). However, they did demonstrate that other fimbrial types were present (diameter 5-7nm). The degree of fimbriation between different strains was found to be variable. For instance, serotypes O8:K87:K88ab:H19 (Figure 3.4), O149:K91

Figure 3.1



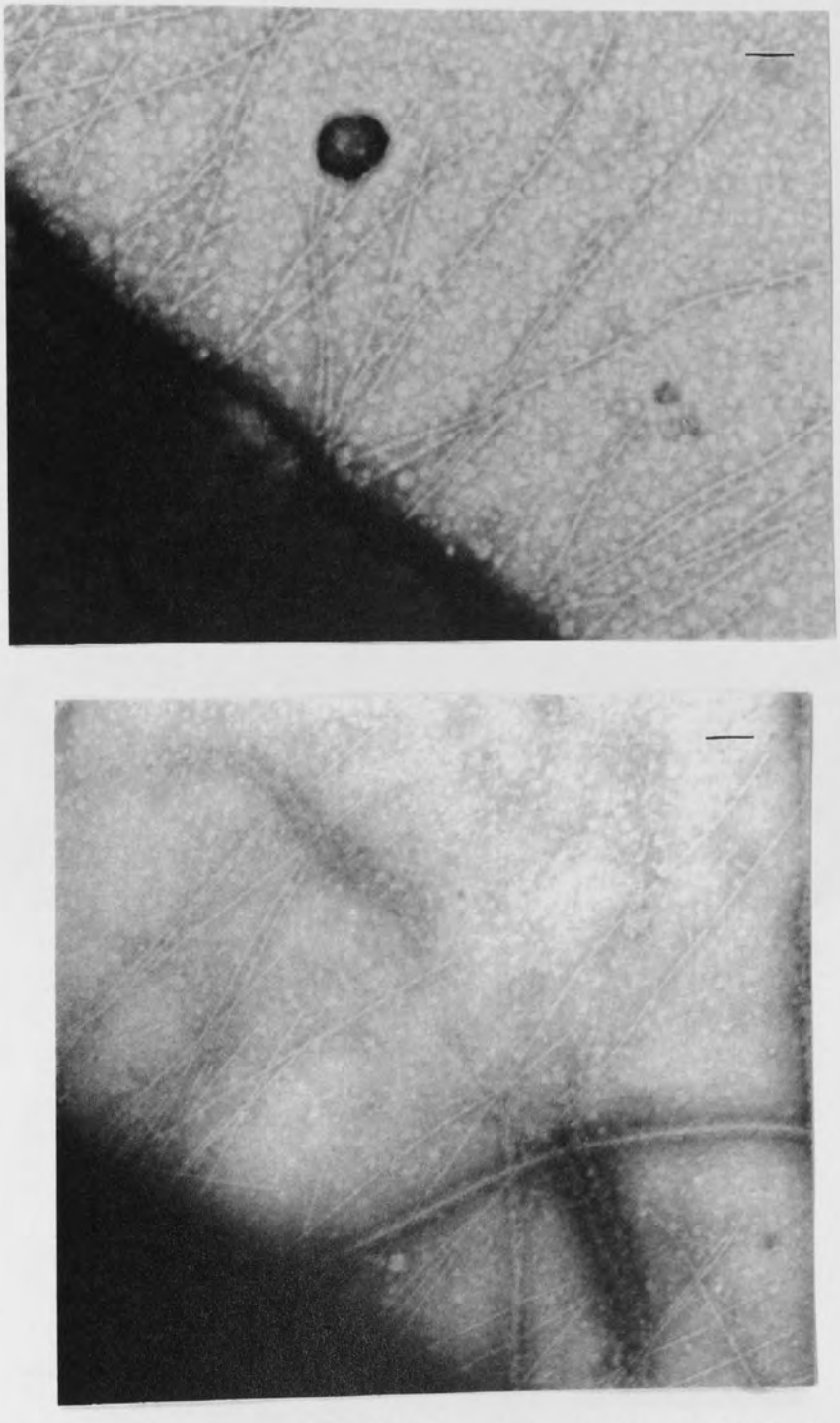
E.coli strain K12 (top). Bacteria were cultured in broth at 37°C. Approximately 90% of the bacteria were non-fimbriated. This particular bacterium had a few fimbriae (diameter ~7nm, arrowed), bar represents 100nm. E.coli strain K12:K88ab (bottom) was again cultured in broth at 37°C. For this strain ~90% of the bacteria were fimbriated (4-5nm). The fimbriae present had a tendency to form bundles (arrowed). Bar represents 60nm.

Figure 3.2



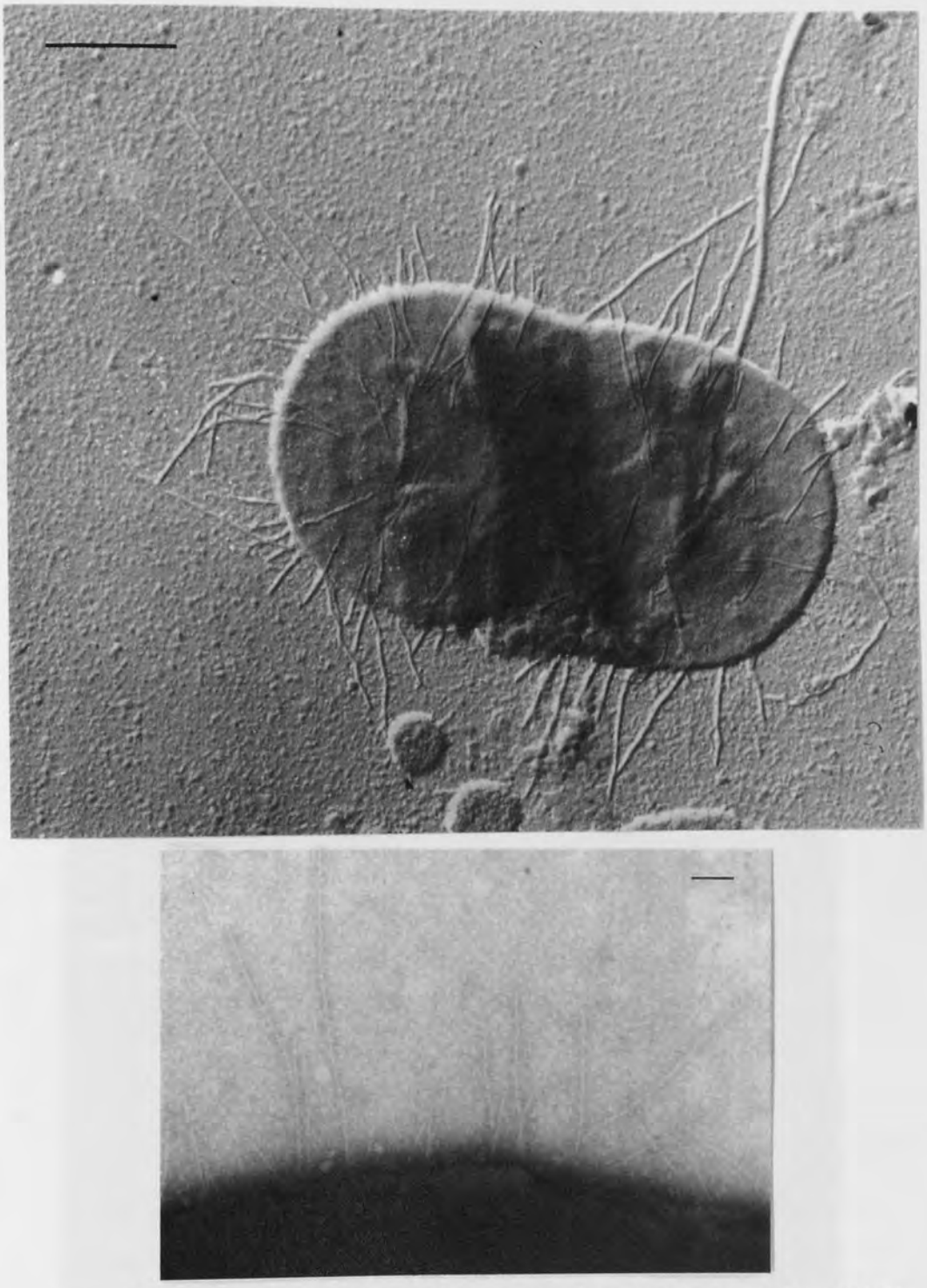
E.coli strain O149:K91:H10 cultured in broth at 37°C. >90% of the bacteria were fimbriated (diameter ~7nm). Of those bacteria that were fimbriated, half expressed fimbriae with a kinked appearance (top photograph) while half expressed fimbriae that were straight (bottom photograph). Note the thicker flagella (arrowed) present in the top photograph. Bar represents 60nm.

Figure 3.3



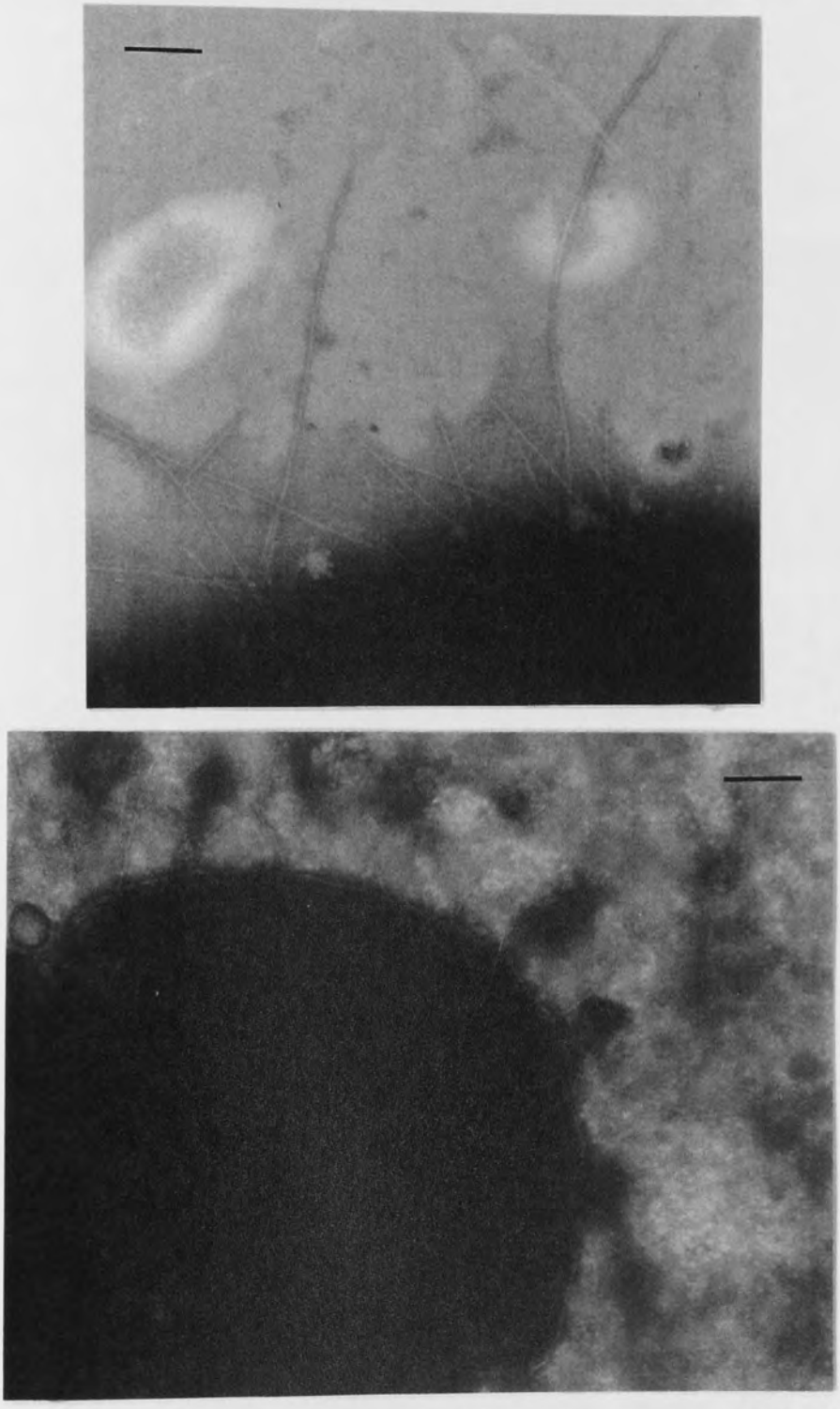
E.coli strain O149:K91:H10 cultured in broth at 37°C. Specimens examined on carbon coated grids had a more granular background (top photograph), however, fimbriae were visualised more clearly than those examined on formvar coated grids (bottom photograph). 1% sodium silicotungstate was used as negative stain. Bar represents 60nm.

Figure 3.4



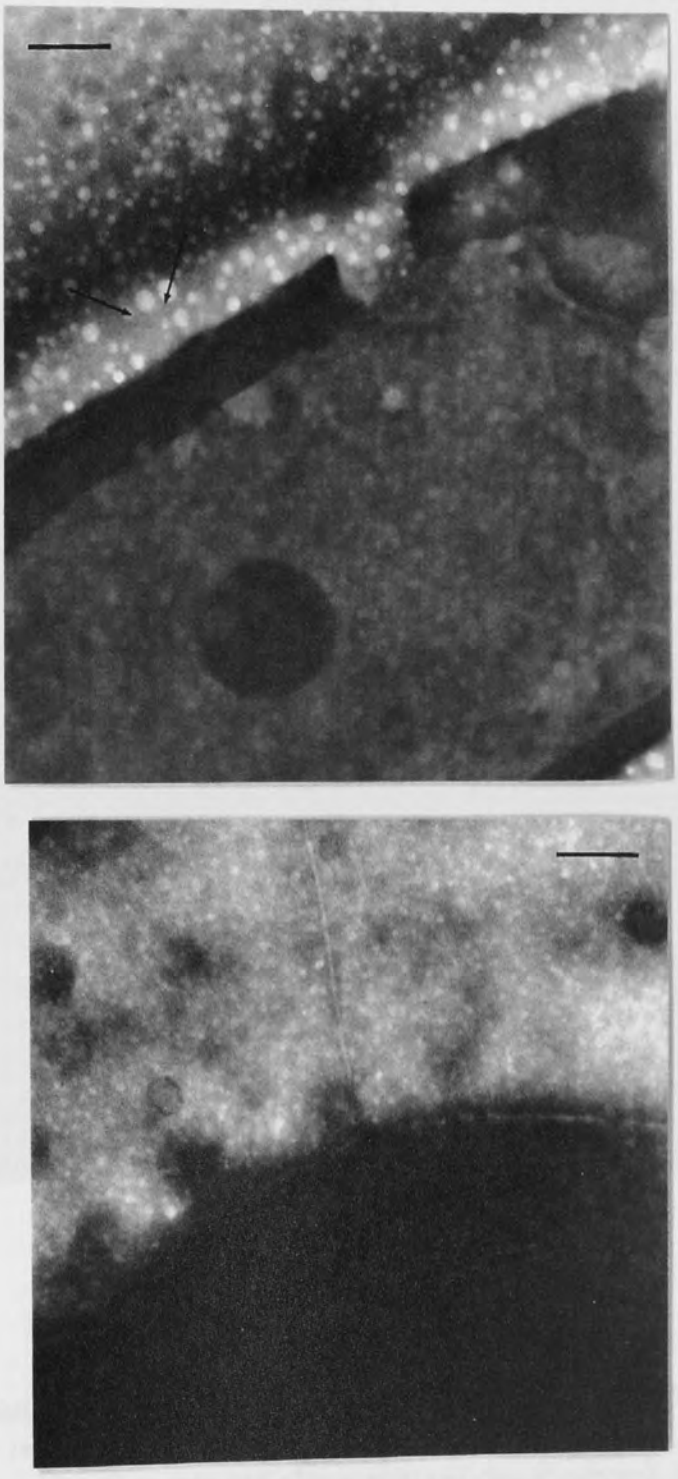
E.coli strain O8:K87:K88ab:H19 cultured in broth at 37°C. Metal shadowing was found to be suitable for demonstrating the three dimensional arrangement of fimbriae on the bacterial surface (top photograph). By metal shadowing fimbriae appeared thicker than when subject to negative staining (bottom photograph). Bar represents 420nm (top) or 40nm (bottom).

Figure 3.5



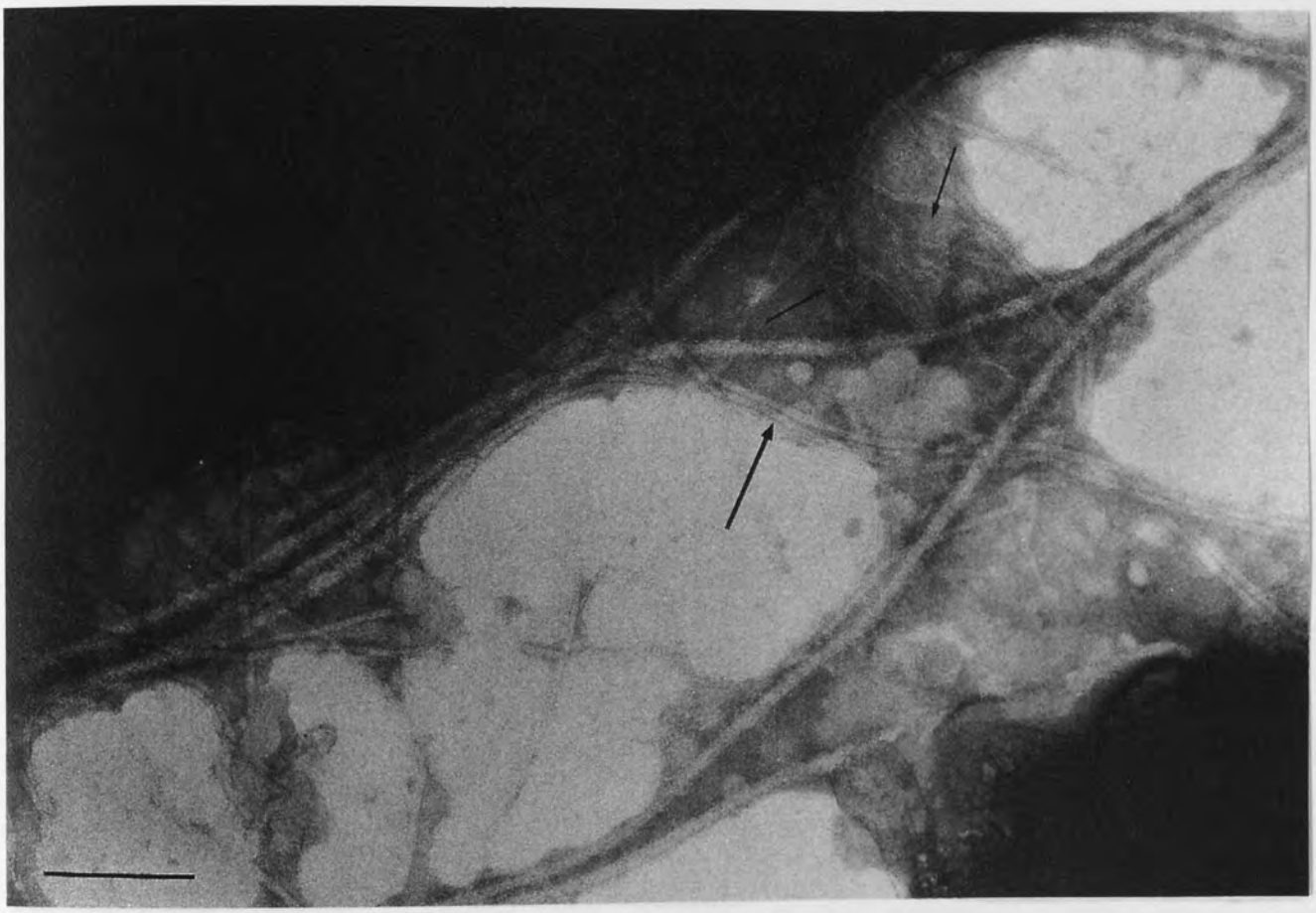
E.coli strain O8:K87:K88ac:H19. Cultivation temperature had little affect on the expression of fimbriae. Bacteria were cultivated unshaken in broth either at 37°C (top photograph) or at 18°C (bottom photograph). Mean diameter of fimbriae in both cases was 7nm. Bar represents 180nm (top) or 110nm (bottom).

Figure 3.6



E.coli strain K88ad. This strain did not show obvious signs of being fimbriated when cultured at either 37°C (top photograph) or 18°C. There was some evidence of the expression of fimbriae with a morphology similar to that reported for K88 (arrowed). Preparations were stained with 1% sodium silicotungstate. Bar represents 180nm (top) or 110nm (bottom).

Figure 3.7



E.coli strain K12:K88ab culture unshaken in broth at 37°C. Fimbriae corresponding in morphology to K88 are present (fine arrows). In addition, fimbriae with a wider diameter are also expressed (thick arrow). Bacteria were stained with 2% ammonium molybdate. Bar represents 100nm.

:H10 (Figures 3.2 and 3.3) and K12:K88ab (Figures 3.1 and 3.7) were highly fimbriated while serotype O8:K87:K88ac:H19 (Figure 3.5) was variable in terms of fimbriation and serotypes K12 (Figure 3.1) and K88ad (Figure 3.6) were poorly fimbriated.

The nature of the fimbriae that were present was difficult to discern. However, nutrient broth culture at 18°C (see Figures 3.5 and 3.6) did not seem to affect the fimbriation observed suggesting that they were not host specific fimbriae which are repressed at low temperature (De Graaf and Mooi 1986). This suggests that the fimbriae present are type 1 since their expression has been reported to be independent of temperature of cultivation (De Graaf and Mooi 1986). However, examination of bacteria cultivated on agar (which is thought to repress the expression of type 1 fimbriae, Jann 1987) at 37°C did not reveal any differences to those grown in broth. In addition, the effect of mannose on the haemagglutinating ability of three of the strains suggested that only strain 08:K87:K88ac:H19 possessed type 1 fimbriae (see Table 3.1).

Since a recent study (Gander and Thomas 1987) has indicated that multiple mechanisms of type 1 regulation may exist, it seems quite likely that the fimbriae present on serotype 08:K87:K88ac:H19 are type 1 fimbriae. The fimbriae present on serotypes 08:K87:K88ab:H19 and K88ad are type 1 related in terms of morphology, and are expressed at 20°C. However, these fimbriae are also expressed when cultivated on agar and do not agglutinate guinea-pig erythrocytes as would be expected by type 1 fimbriae. Therefore, since these characteristics do not conform to any of the known fimbriae it does not seem possible from the experiments performed to identify them. The expression of fimbriae by the remaining strains was unaffected by temperature and media of cultivation. In addition, the majority were found to conform to type 1 fimbriae in terms of morphology, the exception being the "kinked" fimbriae (Figure 3.2) present on 50% of serotype O149:K91:H10. The relevance of these "kinked"

Table 3.1 Effect of D-mannose on the haemagglutination of guinea pig erythrocytes by *E.coli* strains possessing the K88 adhesin

D-mannose and its derivatives selectively inhibits the binding of type 1 fimbriae to guinea pig erythrocytes. Because of this, the effect of D-mannose on haemagglutination is thought to indicate the presence or not of type 1 fimbriae (Truszczynski and Osek 1987).

fimbriae is unknown. An interesting point to note was the finding that the transconjugant strain K12:K88ab was heavily fimbriated (Figures 3.1 and 3.7) unlike its K12 parent strain. Thus it seems that the K88ab plasmid contains not only information for the expression of the K88 fimbria but also the information required for the expression of a further unidentified fimbrial structure. It should be noted that the K88 plasmid is often associated with a transfer factor coding for a F-like pili (Bak *et al.* 1972, Orskov and Orskov 1966). Although conforming in structure to some of the unidentified fimbriae in the *E.coli* strains used there are usually only one or two present per bacterial cell (Singleton and Sainsbury 1981, Orskov and Orskov 1983). Therefore, the unidentified fimbriae are unlikely to be associated with a transfer factor.

In only a few experiments was it possible to demonstrate fimbriae with the published morphology of the K88 fimbria (see Figures 3.6 and 3.7). In the latter figure ammonium molybdate was used as negative strain and this seemed better able to demonstrate the presence of the K88 fimbria than the other

negative stains used. A possible explanation for this difficulty in detection of the K88 fimbriae was that normally the fimbriae lie flat across the surface of the bacteria. Therefore only when another bacterial cell was juxtaposed and the surface of the K88-positive bacteria disturbed was the presence of the K88 fimbria exposed (see Figure 3.7). A previously published photograph also shows K88 fimbriae between two nearby bacteria (Wadstrom *et al.* 1979).

3.3 Determination of the expression of the K88 fimbrial adhesin

The ability to express the K88 fimbrial adhesin was determined by a number of procedures. Initial experiments relied on the extraction of a protein of the correct molecular weight by a method known to extract the K88 protein (see Chapter 5) and the observation of a typical haemagglutination pattern when the strains were tested with several erythrocyte types. Subsequently, the availability of specific antisera allowed the development of an ELISA and the availability of commercial kit (Fimbrex) was used to confirm the expression of the K88 adhesin.

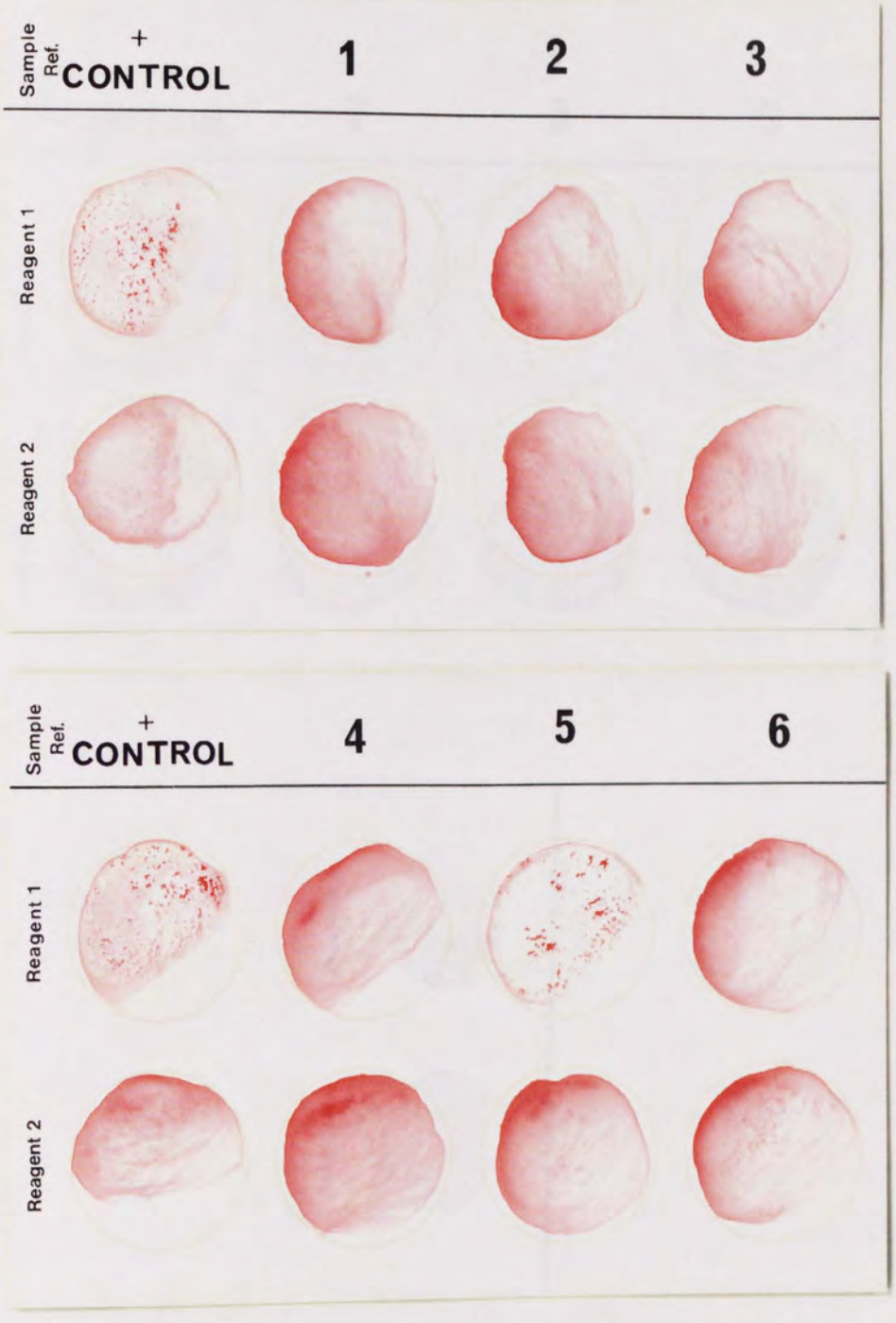
The cards obtained through the use of the Fimbrex K88 kit are presented on pages 121 and 122. A positive result is achieved when there is evidence of precipitation with reagent 1 (contains monoclonal α(anti)-K88 reactive antibody) but not with reagent 2 (contains non-reactive antibody). Precipitation relies on the cross-linking of latex beads by a multivalent antigen in a similar manner to the haemagglutination of erythrocytes (Jones and Isaacson 1983). However, unlike haemagglutination, precipitation of the latex beads is not facilitated by the purified adhesin (see Figure 3.9). Why there is a difference is not apparent because the affinity of the monoclonal antibody for the adhesin is at least as high as the affinity of the adhesin for the erythrocyte receptor. This can be assumed because antibody induced agglutination of K88-positive strains is stable at room temperature whereas agglutination by e.g. guinea pig erythrocytes is

unstable at room temperature and yet agglutination of erythrocytes by purified adhesin occurs (at 4°C). A possible explanation may be that the monoclonal antibody recognises an epitope on the intact adhesin which is not present in the purified adhesin. The results obtained with the Fimbrex K88 kit are presented in Figures 3.8, 3.9 and Table 3.2.

Several studies have investigated the haemagglutination properties of the K88 fimbrial adhesin (Jones and Rutter 1974a, Parry and Porter 1978, Bijlsma *et al.* 1985, Cox and Houvenaghel 1987, Jacobs *et al.* 1987c). These have shown that the K88 adhesin characteristically agglutinates both guinea-pig and chicken erythrocytes. Experiments to be presented later (Chapter 6) also indicated that the K88 adhesin could agglutinate rabbit erythrocytes. ELISA-based tests for the K88 adhesin has been used previously (Mooi *et al.* 1979, Hone *et al.*. 1988). The ELISA used in this study relies on K88 bound from intact bacteria to the microplates used and its subsequent detection by specific αK88ab antibody as in Section 2.6.3. The results of both the haemagglutination studies and ELISA are presented in Table 3.2.

The results obtained by the Fimbrex K88 kit, by ELISA and by haemagglutination were all found to be in agreement. It appeared that none of the NCTC sourced bacterial strains had the ability to express the K88 adhesin. No attempt was made to serotype the NCTC strains but it seems unlikely that all three of the strains used and only those three strains were contaminated. The remaining strains all appeared to react according to their serotypes. Although the results achieved were not quantitative, the Fimbrex K88 kit seemed especially suited for the rapid determination of the ability to express the K88 adhesin although unable to detect cell free adhesin.

Figure 3.8



The fimbrex K88 kit was used for the determination of the expression of the K88 adhesin by the E.coli strains used in this study. A positive result is attributed to the agglutination of pink latex beads caused by the binding of monoclonal antibodies to the K88 adhesin. Where :- 1=O149:K91:K88ac:H10; 2=O147:K89: K88ac:H19; 3=O157:K88ac:H19; 4=K12; 5=K12:K88ab and 6=O149:K91:H10.

Figure 3.9

The fimbrex K88 kit was used to identify E.coli strains that expressed the K88 fimbriae. However, the kit was unable to identify purified cell-free K88ab adhesin (0.75mg/ml, lower strip). Where :- 7=O8:K87:K88ab:H19; 8= O8: K87:K88ac:H19; and 9=K88ad.

Table 3.2 Determination of the expression of the K88 fimbrial adhesin by various strains of *E.coli*

STRAIN	HAEMAGGLUTINATION			FIMBREX	ELISA
	CHICKEN	GUINEA PIG	RABBIT		
K12	NO	NO	NO	-ve	0.00
K12:K88ab	YES	YES	YES	+ve	1.47
O8:K87:K88ab:H19	YES	YES	YES	+ve	1.65
O8:K87:K88ac:H19	YES	YES	YES	+ve	0.86
K88ad	YES	YES	YES	+ve	0.52
O149:K91:H10	NO	NO	NO	-ve	0.00
O149:K91:K88ac:H10	NO	NO	NO	-ve	0.00
O147:K89:K88ac:H19	NO	NO	NO	-ve	0.00
O157:K88ac:H19	NO	NO	NO	-ve	0.00

Bacterial suspensions in PBS were adjusted to an absorbance of 1.00 at 600nm before use in the ELISA. ELISA results are given only in terms of final absorbance at 410nm and are semi-quantitative. For haemagglutination, the number of cfu/ml was determined and the suspension adjusted in PBS to $1x10^{10}$/ml before use. The results obtained with the Fimbrex K88 kit were determined for several individual colonies of each bacterial strain.

3.4 Determination of the ability to metabolize raffinose

Historically it was found that genes coding for the ability to metabolize raffinose were frequently associated with the ability to express the K88 adhesin (Gaastra and de Graaf 1982). Subsequently it was found that the genes for raffinose metabolism and K88 fimbrial production were located on the same non-conjugative plasmid (Gaastra and de Graaf 1982). Since the ability to metabolize raffinose and the expression of the K88 adhesin are closely linked, the former has been used to indicate the latter (Williams-Smith and Parsell

1975, Guinee and Jansen 1979). Thus the selection of K88-positive transconjugants after mating experiments has been based on their ability to metabolize raffinose (Williams-Smith and Parsell 1975, Guinee and Jansen 1979). Such transconjugants when grown on agar supplemented with raffinose form large colonies while no growth occurs or tiny colonies are formed by the non-transformants (Williams-Smith and Parsell 1975). This difference in growth was used as the basis of the present series of experiments on the metabolism of raffinose by the *E.coli* strains under study here.

The growth curves of the strains used in this study are presented in Figures 3.10 and 3.11.

Using the absorbance as an indicator of cell mass it was possible to calculate both the mean generation time (t_d) and the growth rate (μ) for the log phase of growth (Stanier *et al.* 1983). The growth rate was calculated from the formula:

$$\text{Slope (log)} = \mu/2.303$$

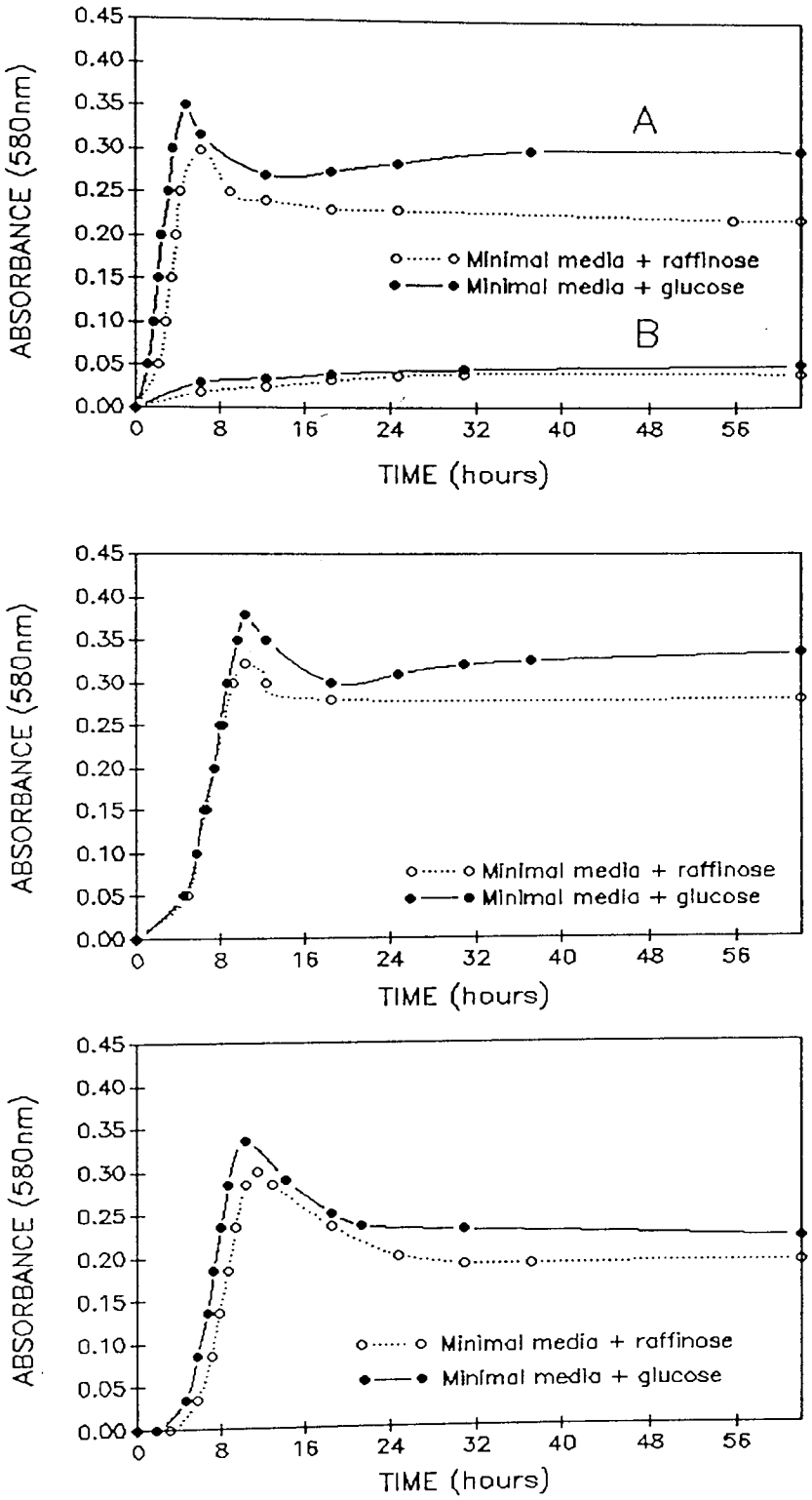
The mean generation time was calculated from the formula:-

$$\mu = \ln 2/t_d$$

The values obtained for μ and t_d are given in Table 3.3.

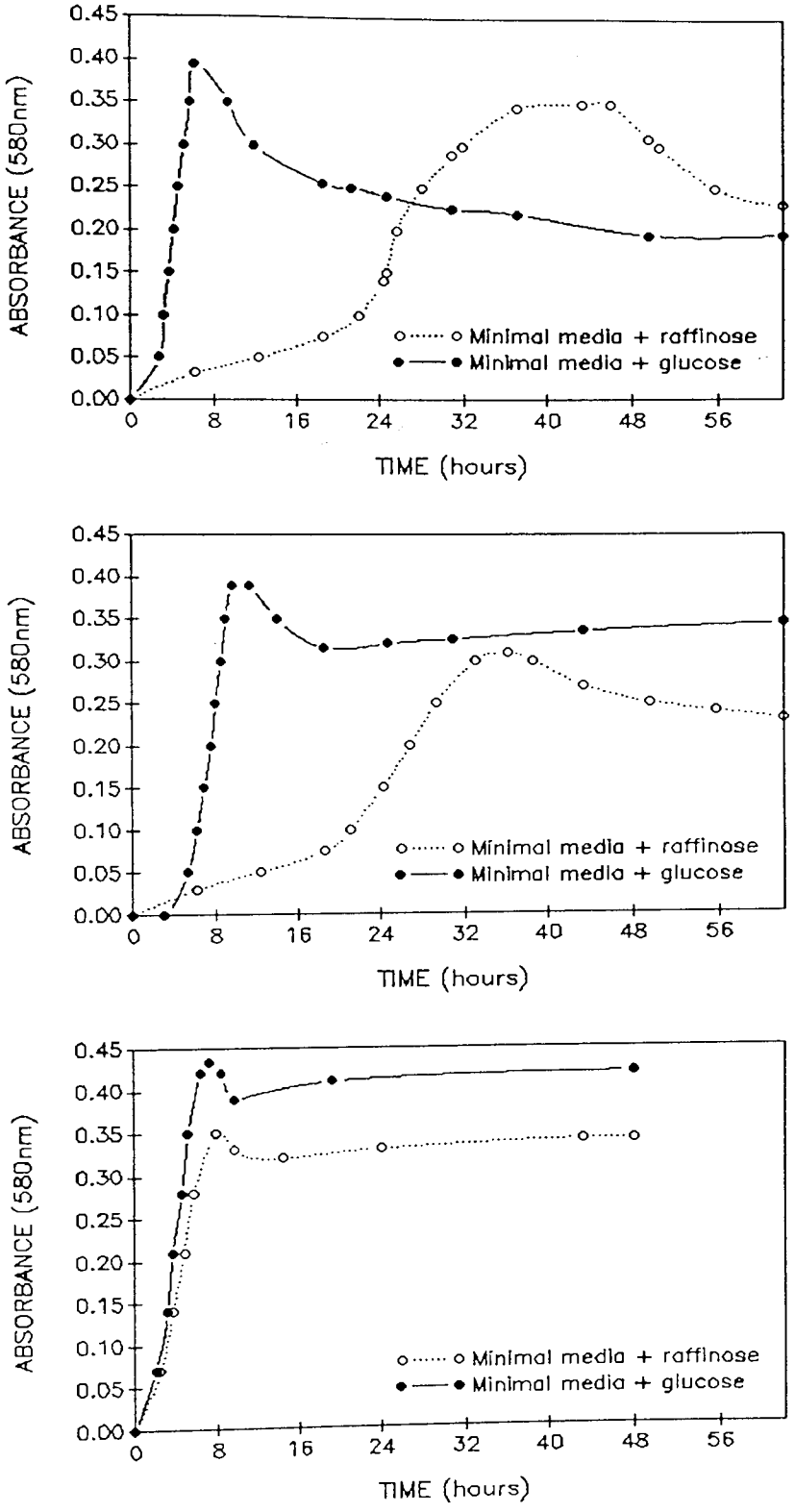
Despite repeated attempts, *E.coli* strain K12 was found to grow poorly on the minimal media used (see Figure 3.10). *E.coli* K12 strains possess an α-galactosidase specified by the *mel* operon which can metabolize raffinose. Despite this, the presence of raffinose is not sufficient for the induction of the specific transport and hydrolase activities required for its own efficient utilisation by *E.coli* K12 strains (Schmid and Schmitt 1976). Therefore the inability of the K12 strain to grow on minimal media supplemented with raffinose was expected and has indeed been used in K12 transformation

Figure 3.10



The ability of various strains of E.coli to grow on minimal media supplemented with either raffinose or glucose. Growth curves were monitored by Labsystems bioscreen. Where 1A=K12:K88ab; 1B=K12; 2=O8:K87: K88ab:H19 and 3=K88ad.

Figure 3.11



The ability of various strains of E.coli to grow on minimal media supplemented with either raffinose or glucose. Where 4=O149: K91:H10; 5=O8:K87:K88ac:H19; and 6=O157:K88ac:H19.

Table 3.3 Calculated values of t_d and μ for various strains of *E.coli* when cultivated on minimal media supplemented with glucose or raffinose.

STRAIN	MINIMAL MEDIA SUPPLEMENTED WITH RAFFINOSE		MINIMAL MEDIA SUPPLEMENTED WITH GLUCOSE	
	μ (h^{-1})	t_d(min)	μ(h^{-1})	t_d(min)
K12:K88ab	0.77	54	0.75	55
O149:K91:H10	0.07	605	0.67	62
O8:K87:K88ab:H19	0.41	102	0.42	100
O8:K87:K88ac:H19	0.08	556	0.52	80
K88ad	0.45	92	0.51	81
O157:K88ac:H19*	0.43	96	0.50	84

** This strain corresponds to NCTC strain 10964 and is representative (in terms of growth) of the remaining two NCTC strains used in this study.*

experiments as a marker to identify transformants (Shipley *et al.* 1978). The inability of the K12 strain to grow on simple media supplemented with glucose was unusual and could not be explained (Buchanan and Gibbons 1975). An interesting point to note was that the K12:K88ab transconjugant strain was able to grow on minimal media supplemented with glucose suggesting that the K88ab plasmid contains genes for the catabolism of glucose.

Apart from the K12 strain all the other bacteria examined were able to grow well on minimal media supplemented with glucose. The absence of a noticeable lag phase was thought to be attributable to a combination of a comparatively large inoculum, pre-warming of the media and an inoculum which was itself still in the log phase of growth. However, differences in growth were readily apparent when bacteria were cultured on minimal media supplemented with raffinose. In this case, the growth rate of strains O149:K91:H10 and O8:K87:K88ac:H19 was between 5 and 11-fold lower than

that observed for the remaining strains which were largely unaffected by the change in substrate (see Table 3.3). Since there is a strong correlation between the ability to metabolize raffinose and the ability to express the K88 fimbrial adhesin this suggests that the two strains above do not express the K88 adhesin while the remaining strains do.

Transfer from minimal media supplemented with glucose to one supplemented with raffinose did not cause an appreciable lag phase for the majority of the strains used. This is probably attributable to the rapid induction of the enzymes necessary for the metabolism of raffinose (induction of the enzymes responsible for lactose metabolism in *E.coli* takes 2-3 minutes, Strickberger 1985). The finding of renewed logarithmic growth in strains O149:K91:H10 and O8:K87:K88ac:H19 after ~20hr was interesting and may represent the mobilisation of previously inactive genes for the metabolism of raffinose. However, since the delay in this renewed growth corresponded to ~2 doubling times a more fundamental change in the culture may be involved e.g. a change in the permeability of the bacteria or possibly the release of degradative enzymes during cell lysis which converted raffinose into a metabolisable form.

The results obtained on the ability to metabolise raffinose contradict those given in Section 3.2. For instance by ELISA, haemagglutination and according to the Fimbrex K88 kit serotype O8:K87:K88ac:H19 does express the K88 fimbrial adhesin while the results of raffinose metabolism indicates that it does not. However, the basic assumption at the outset of the raffinose experiments was that the ability to metabolise raffinose was a good indicator of the presence of the K88 adhesin and not an absolute one. Although rare, strains which possess the K88 adhesin and yet do not metabolize raffinose are found (2 out of 21 strains tested, Guinee and Jansen 1979). In addition, there is some evidence that the storage of strains for long periods can lead to the loss

of the ability to metabolize raffinose (Williams-Smith and Parsell 1975). Thus it seems that strain O8:K87:K88ac:H19 does express the K88 adhesin but cannot metabolize raffinose. The three NCTC sourced strains used all have the ability to metabolize raffinose and yet do not express the K88 adhesin according to the results of ELISA, haemagglutination and the Fimbrex K88 kit. There are at least three possible explanations for this finding. The first is that a mutation had occurred in the K88 plasmid resulting in the loss of K88 production but not raffinose metabolism. In support of this it has been reported that such mutants can be readily isolated usually containing a point mutation (Shipley *et al.* 1978). Alternatively, the ability to metabolize raffinose may be chromosomally encoded and not dependent on plasmid genes in these strains. It has been reported that 14 of 57 enteropathogenic strains of *E.coli* did not express the K88 adhesin but were able to metabolize raffinose without being able to transmit this property (Williams-Smith and Parsell 1975). Finally, none of the NCTC sourced strains were serotyped to confirm their identity. Therefore, it is possible that the NCTC strains examined were either incorrectly supplied or represented contaminants with the ability to metabolize raffinose.

3.5 Conclusions

Detection of K88 fimbriae by electron microscopy was found to be difficult and not a viable method of determining their expression. However, the results of electron microscopy did indicate that several of the strains to be used in this study expressed fimbriae in addition to K88. Attempts to use the ability to metabolize raffinose to indicate the presence of K88 fimbriae proved unreliable since one of the *E.coli* strains used was seemingly unable to utilise raffinose while still expressing K88 fimbriae. The combined results of a K88-specific ELISA, haemagglutination and the Fimbrex K88 kit were, however, effective in determining the expression of the K88 fimbrial adhesin.

4 GROWTH AND THE EXPRESSION OF THE K88 FIMBRIAL ADHESIN BY *E.COLI.*

4.1 Introduction.

Little definitive work has been done on the effect of media on the expression of K88 fimbriae. This is despite the cloning of the genes responsible for the regulation, transport and assembly of K88 fimbrial subunits into the mature fimbria (Kehoe *et al.* 1981, Gaastra *et al.* 1981, Mooi *et al.* 1981 and Dougan *et al.* 1983). The aim of the work undertaken here was to investigate the effect of media type and form on the expression of the K88 fimbrial adhesin. It was envisaged that the results would enable the recommendation of media for future work. Quantitation of the amount of fimbrial protein expressed was facilitated by the development of a simple ELISA-based assay.

4.2 Growth curves of *E.coli* strains.

The ability of six strains of *E.coli* (see Table 4.1) to grow in eight different media (see Table 4.2) was investigated. Previous experiments (Chapter 3) had indicated that four of the *E.coli* strains used expressed the K88 adhesin while the remaining two were used as controls. The media chosen (or similar) have all been used previously in experiments investigating the properties of the K88 fimbrial adhesin.

The investigation of the growth curves was conducted as an initial screening experiment to determine the ability of the various broth media under test to support the growth of the *E.coli* strains being examined. In addition, the effect of the possession of the K88 plasmid on growth characteristics was to be observed. In particular, it had already been noted that the transconjugant strain K12:K88ab was able to metabolise glucose while its parent strain could not (see Chapter 3). Whether this would potentiate the growth of strain K12:K88ab on the various media tested was of interest.

Table 4.1 *E.coli* strains used during growth curve experiments.

STRAIN	ABBREVIATION
K12	K12
K12:K88ab	K88
O149:K91:H10	K88$^-$
O8:K87:K88ab:H19	K88ab
O8:K87:K88ac:H19	K88ac
K88ad	K88ad

Table 4.2 Media used during growth curve experiments.

MEDIA	ABBREVIATION	REFERENCE
Brain heart infusion No1	BHI 1	Laux *et al.* 1986
Brain heart infusion No2	BHI 2	Stirm *et al.* 1967a
Tryptone soya broth	TSB	♣
NZY	NZY	£
Nutrient broth No2	NB 2	Jones and Rutter 1972
Minimal media and casamino acids	MINCA	Gaastra *et al.* 1981
Tergitol	TERG	Jones and Rutter 1974a
Nutrient broth	NB	Jones and Rutter 1974a

Where :- ♣ *=used as a substitute for trypticase soy broth (Jacobs and De Graaf 1985)*

£=modification of NY media (Josephsen et al. 1984)

Table 4.3 reveals that there was considerable variation in the parameters of growth depending on the media and *E.coli* strain concerned. However, only the K12 strain was found unable to grow on some of the media used (TERG and MINCA). This agrees with the inability of the strain to grow on the

Table 4.3 ♠Summary of the growth curves obtained with various media and *E.coli* strains.

STRAIN	MEDIA	LAG PHASE (min)	μ (h^{-1})	T_d (min)	LENGTH OF LOG PHASE (min)	* OD AT END OF LOG PHASE	* OD AFTER 20hr
K12	BHI 1	160	0.77	54.0	133	0.66	0.58
K88	BHI 1	200	0.79	52.8	120	0.59	0.96
K88-	BHI 1	360	1.18	35.3	76	0.53	1.08
K88ab	BHI 1	240	0.83	50.4	67	0.60	0.89
K88ac	BHI 1	218	1.30	31.9	76	0.62	0.93
K88ad	BHI 1	293	0.95	43.9	108	0.60	1.10
K12	BHI 2	160	0.85	48.7	120	0.66	0.60
K88	BHI 2	240	0.76	54.8	122	0.59	0.92
K88-	BHI 2	360	1.04	40.0	89	0.56	1.10
K88ab	BHI 2	253	1.13	36.8	67	0.60	1.00
K88ac	BHI 2	218	1.09	38.0	89	0.62	0.93
K88ad	BHI 2	293	1.09	38.0	89	0.60	1.14
K12	TSB	184	0.59	70.8	189	0.40	0.34
K88	TSB	173	0.67	61.8	278	0.54	0.78
K88-	TSB	360	1.04	40.0	49	0.28	0.96
K88ab	TSB	222	1.24	33.4	129	0.30	0.72
K88ac	TSB	231	0.54	77.0	169	0.55	0.72
K88ad	TSB	293	0.73	56.7	104	0.43	0.96
K12	NZY	200	0.80	52.3	78	0.18	0.16
K88	NZY	240	0.32	129.7	140	0.33	0.66
K88-	NZY	373	0.84	49.4	89	0.42	0.94
K88ab	NZY	253	0.65	64.4	76	0.48	0.72
K88ac	NZY	218	1.02	40.7	111	0.34	0.63
K88ad	NZY	293	1.11	37.4	85	0.27	0.80

Where:- *=*Determined at 600nm,* ♠ =*Results given are the means of triplicates of a typical experiment.*

Table 4.3 (cont.)

STRAIN	MEDIA	LAG PHASE (min)	μ (h^{-1})	T_d (min)	LENGTH OF LOG PHASE (min)	* OD AT END OF LOG PHASE	* OD AFTER 20hr
K12	NB 2	200	0.33	124.8	209	0.05	0.06
K88	NB 2	240	0.71	58.8	98	0.12	0.42
K88-	NB 2	373	0.44	95.4	151	0.36	0.56
K88ab	NB 2	266	1.08	38.4	73	0.12	0.34
K88ac	NB 2	178	0.86	48.6	94	0.12	0.47
K88ad	NB 2	316	0.50	82.7	131	0.36	0.63
K12	MINCA	-	-	-	-	-	-
K88	MINCA	302	0.52	79.8	196	0.24	0.32
K88-	MINCA	471	0.75	55.8	78	0.32	0.40
K88ab	MINCA	289	0.69	60.4	178	0.26	0.31
K88ac	MINCA	253	0.55	76.2	240	0.28	0.44
K88ad	MINCA	316	0.20	92.1	191	0.24	0.36
K12	TERG	-	-	-	-	-	-
K88	TERG	302	0.53	78.1	120	0.24	0.34
K88-	TERG	480	0.72	57.7	120	0.24	0.33
K88ab	TERG	311	0.76	54.6	182	0.30	0.43
K88ac	TERG	289	0.70	59.8	138	0.31	0.39
K88ad	TERG	316	0.44	94.9	302	0.32	0.34
K12	NB	200	1.01	41.2	85	0.08	0.06
K88	NB	277	0.50	83.3	120	0.12	0.28
K88-	NB	373	0.74	56.2	164	0.19	0.32
K88ab	NB	266	0.94	44.2	73	0.10	0.32
K88ac	NB	178	0.86	48.6	138	0.12	0.29
K88ad	NB	316	0.20	92.1	191	0.24	0.36

Where:- *=*Determined at 600nm*

minimal media used in raffinose experiments (Chapter 3). The introduction of the K88ab plasmid into this strain did not appreciably effect the growth rate with BHI 1, BHI 2, or TSB media. With NZY and NB media the growth rate with strain K12:K88ab was substantially lower while with NB 2 substantially higher than the K12 strain. Under non-limiting growth conditions the metabolic cost of fimbriation (Klemm 1985, Smyth 1986) is probably not significant and this may reflect the comparable growth rates observed with BHI 1, BHI 2 and TSB. Under more demanding conditions, non-fimbriated bacteria would be expected to outgrow their fimbriated counterparts as occurs during the growth of bacteria capable of expressing type 1 fimbriae (Eisenstein and Dodd 1982). An analogous situation may be occurring during the growth of K12:K88ab bacteria when grown on NZY and NB media (see Table 4.3). Where nutrients become more limiting, the possession of plasmid encoded genes may enable growth under conditions where the plasmid-less strain grows poorly or not at all. This may explain the poor growth of strain K12 and the comparatively good growth of strain K12:K88ab on NB 2, MINCA and TERG media. A common factor for all the media used was the finding that the final bacterial population supported was higher with strain K12:K88ab than with the parent strain K12. This was especially apparent in the post-logarithmic growth and may reflect the adoption of a predominantly oxidative metabolism by the fimbriated strain. Indeed this extended post-logarithmic growth was typical of all the fimbriated strains used. The ability to adopt an oxidative metabolism during unshaken culture has been postulated to explain the outgrowth of fimbriated organisms in mixed cultures (Eisenstein and Dodd 1982).

It is interesting to make comparisons between the two K88$^-$ strains used. In the first case, the K12 strain is poorly fimbriated while strain O149:K91:H10 although not possessing K88 fimbriae was heavily covered with other fimbriae (see Chapter 3). A general finding with the O149:K91:H10 strain was that there

was a long lag phase followed by a comparatively short log phase followed by extended post-logarithmic growth. On the richer media (BHI 1, BHI 2 and TSB) the comparatively extended log phase of the K12 strain resulted in higher OD readings at the end of the log phase for the K12 strain than for the O149:K91:H10 strain. On the poorer media, the growth rates and duration of the log phases were much more comparable for the two strains. However, the OD obtained at the end of the log phase by strain O149:K91:H10 was invariably higher than that obtained by the K12 strain. Additionally, the O149:K91:H10 strain unlike the K12 strain was quite capable of growing on the MINCA and TERG media. Overall it appeared that under unrestricted growth conditions the K12 strain was capable of outgrowing the O149:K91:H10 strain however, under limiting conditions the O149:K91:H10 strain possessed additional properties that gave it the advantage.

For the remaining three K88-positive strains the growth characteristics were similar to each other and the K12:K88ab strain in the various media. As might be expected the lowest growth rates were apparent in the MINCA media although the OD at the end of log phase and after 20hr were similar to those achieved with TERG and NB. For the remaining media, particular strains seemed to favour particular media although the OD achieved at the end of log phase were generally similar since strains showing a lower growth rate tended to compensate by having a longer log phase (e.g. in BHI 1). On all the media except MINCA and TERG strain K88ad had a higher OD after 20hr than the other K88-positive strains. Whether this reflects the generally poor fimbriation (apart from K88, see Chapter 3) of this strain is not apparent.

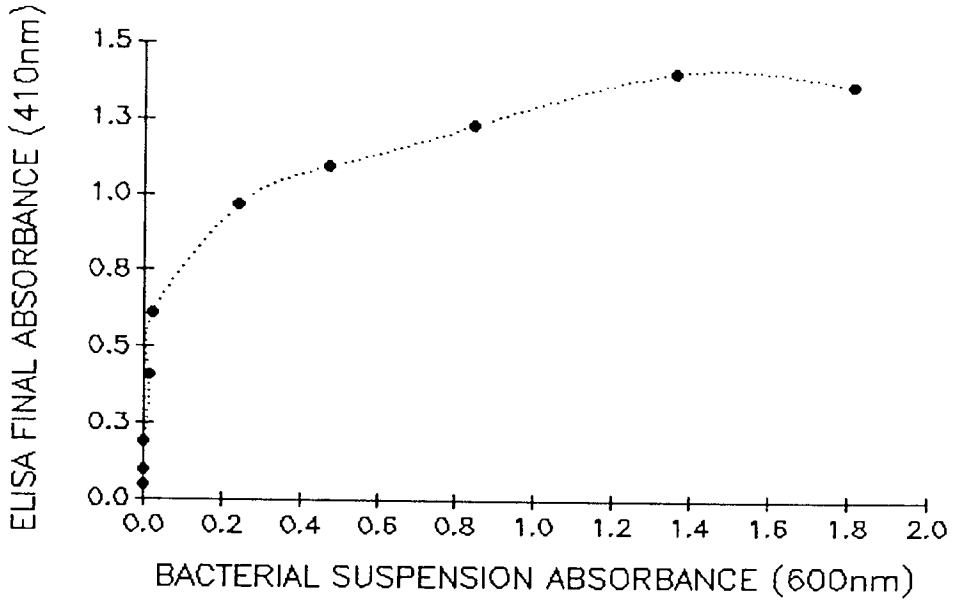
4.3 Comparison of the expression of the K88 fimbrial adhesin by *E.coli.*

The quantitative determination of the K88 fimbrial adhesin was based on an ELISA assay. Several workers have used ELISA-based assays for the

quantitative determination of fimbrial adhesins (Mooi *et al.* 1979, Isaacson 1980, De Graaf *et al.* 1980c, Eisenstein and Dodd 1982, Van Verseveld *et al.* 1985, Hone *et al.* 1988). The most common technique is based on the sonication of the bacterial suspension and the subsequent determination of the amount of K88 adhesin in the extract (De Graaf *et al.* 1980b,c, Van Verseveld *et al.* 1985). Where many samples are to be processed this method can be laborious and time-consuming. As an alternative a competitive ELISA has been developed (Eisenstein and Dodd 1982, Hone *et al.* 1988). This assay is based on the inhibition of the binding of a known concentration of anti-adhesin antibody to ELISA plates pre-coated with purified adhesin. Any adhesin molecules present in a sample bind to the specific antibody and result in its removal from the system. The resultant drop in the final ELISA reading is quantitated by the incubation of the specific antibody with known concentrations of the purified adhesin and the construction of a standard curve. This latter technique has been used to quantitate both cell-free and cell-bound K88 fimbrial adhesin (Hone *et al.* 1988).

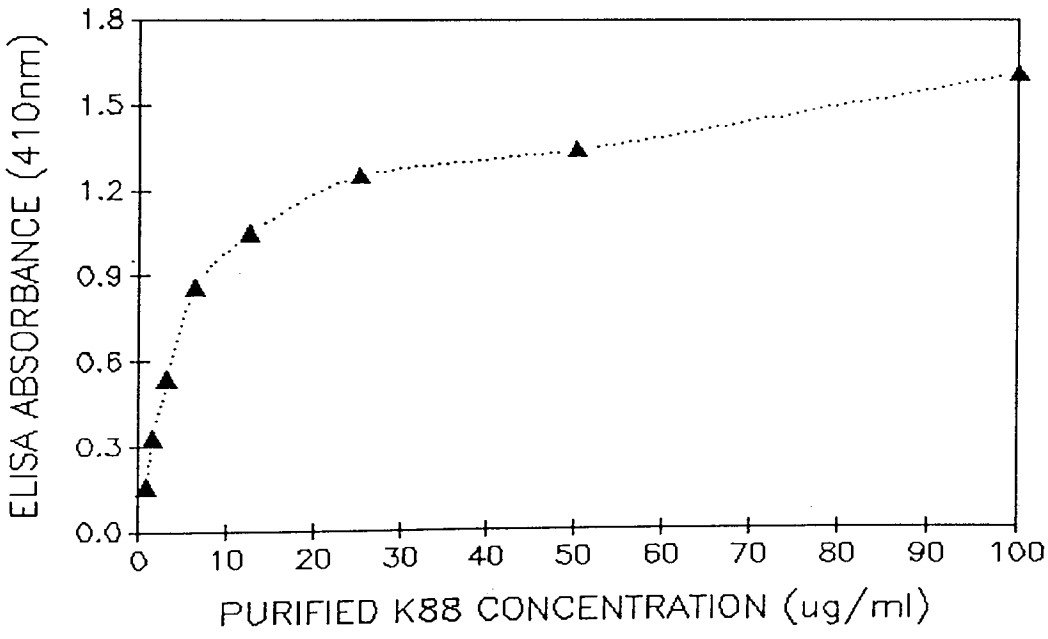
The ELISA results presented in Chapter 3 demonstrated that it was feasible to detect the K88 adhesin after the overnight incubation of bacterial suspensions in ELISA microplates. In order to modify this assay to determine the expression of the K88 fimbrial adhesin by bacterial suspensions several preliminary experiments were performed. Firstly, a standard curve was constructed where the absorbance of the bacterial suspension (at 600nm) was compared with the final absorbance obtained by ELISA (see Figure 4.1). From the standard curve it was decided to standardise bacterial suspensions to an absorbance of 1.00 (at 600nm). Secondly, to quantitate the results it was important to run a range of standards of the appropriate concentration consecutively with the test suspensions. To determine the appropriate range, doubling dilutions of a known concentration of purified K88ab preparation were

Figure 4.1 Standard curve comparing bacterial absorbance with the absorbance determined by ELISA.



E.coli strain K12:K88ab was cultured unshaken at 37°C for 16hr in BHI 1. An ELISA absorbance value of 1.00 corresponded to ~5x10^8 cfu/ml while the detection limit by ELISA was ~1x10^6 cfu/ml. Values plotted are the mean of triplicates.

Figure 4.2 Standard curve of purified K88ab adhesin detected by ELISA.



Dilutions of the purified K88 fimbrial protein extracted from strain K12:K88ab were added to the wells of a 96-well microplate. After overnight incubation at 4°C, the K88 adhesin was detected as outlined in Section 2.9.5. Results are the means of triplicates.

examined by ELISA and a standard curve of concentration against final absorbance produced (see Figure 4.2). From the results of the standard curve it was decided to use doubling dilutions of an initial 100μg/ml concentration to assess the amount of K88 fimbrial adhesin present in test wells. Thirdly, in order to present accurate data on the expression of K88 adhesin/cfu the viability of the bacterial suspensions when cultured with different media was determined (see Table 4.4).

Whether the assay detects cell-free, cell-bound or both types of K88 fimbrial adhesin is unknown. However, it was assumed that the non-specific affinity (K_a $2x10^{12}M^{-1}$, see Chapter 7) of the K88 fimbrial adhesin antigen for the microplate was sufficient either to remove it from the bacterial surface or for the bacteria to remain firmly bound to the ELISA plate. The detection efficiency (i.e. to what degree the calculated concentration of the K88 adhesin reflected the actual concentration of K88 present) of the assay was unknown.

Table 4.4 Viability of *E.coli* strain K12:K88ab after 16hr growth in various broth media.

MEDIA	% VIABILITY	MEDIA	% VIABILITY
BHI 1	97.9	NB 2	100.0
BHI 2	100.0	MINCA	100.0
TSB	100.0	TERG	24.8
NZY	100.0	NB	100.0

After growth, cells were washed once in PBS before being resuspended to their original volume in PBS. When tergitol-grown suspensions were examined the cells appeared much longer than when grown in other media. Since tergitol is a detergent it seems that cell division may be affected by its presence resulting in the low viability observed.

Therefore, the results presented (see Tables 4.4-4.7) are in terms of the equivalent concentration of the K88 adhesin.

Only one previous study has accurately determined the amount of K88 fimbrial adhesin per bacterium (Hone *et al.* 1988). These workers presented data stipulating the amount of K88/cfu with a concentration range of $1.5x10^{-15}$ to $3.1x10^{-16}$g on nutrient agar plates formulated with blood based agar (Difco) and supplemented with 0.05% w/v galactose. The levels of expression (range $2x10^{-15}$ to $5.4x10^{-14}$ g/cfu) found with strains O8:K87:K88ac:H19 and K88ad agree with these figures although the values obtained (range $1.1x10^{-14}$ to $3x10^{-13}$ g/cfu) with strains O8:K87: K88ab:H19 and K12:K88ab are somewhat higher. Differences in the levels of expression reported here and by Hone *et al.* (1988) are probably attributable to a combination of different media, strains examined and method of assay.

Unlike the remaining strains, comparable levels (K88/cfu) of the K88 fimbrial adhesin were detected on both agar and broth media for strain K12:K88ab (see Table 4.5). The only exceptions here were cultivation on or in NZY or NB media where the amount of K88 detected in broth was 2 or 3-fold higher respectively than the equivalent agar culture. The combined findings of a high and comparable expression/cfu irrespective of media form may indicate that expression is not being regulated in this strain and is proceeding at its maximal rate. Hyperfimbriation in *E.coli* strain expressing type 1 fimbriae has been found to be attributable to the inactivation of a repressor gene (*fim* E/*hyp* mutants, Hinson and Williams 1989). Whether an analogous situation could occur in the K88 operon is uncertain since originally only part of the K88 operon was cloned (Mooi *et al.* 1982, Kehoe *et al.* 1983). However, recently the complete K88ab determinant was cloned and found to expresses 10-fold the amount of K88 fimbriae as previous clones. This confirms that mechanisms for the regulation of K88 fimbriae do exist (De Graaf 1990). For strains

Table 4.5 The expression of the K88 adhesin by *E.coli* strain K12:K88ab when cultivated with various media.

MEDIA	TOTAL DETECTED (mg)		* K88/cfu ($gx10^{-13}$)	
	AGAR	BROTH	AGAR	BROTH
BHI 1	6.9 ± 1.4	3.0 ± 0.05	1.6 ± 0.4	2.9 ± 0.6
BHI 2	7.3 ± 1.9	2.5 ± 0.06	1.0 ± 0.3	1.4 ± 0.3
TSB	6.3 ± 0.5	0.6 ± 0.01	1.5 ± 0.2	3.0 ± 0.6
NZY	12.1 ± 0.4	0.5 ± 0.02	1.4 ± 0.1	1.0 ± 0.2
NB 2	10.9 ± 0.4	1.2 ± 0.04	2.2 ± 0.4	1.5 ± 0.3
$ MINCA	6.2	2.4	1.2	2.0
TERG	3.6 ± 0.1	1.1 ± 0.02	NA	NA
NB	2.7 ± 0.2	0.5 ± 0.01	0.9 ± 0.1	2.7 ± 0.7

Table 4.6 The expression of the K88 adhesin by *E.coli* strain O8:K87:K88ab:H19 when cultivated with various media.

MEDIA	TOTAL DETECTED (mg)		* K88/cfu ($gx10^{-14}$)	
	AGAR	BROTH	AGAR	BROTH
BHI 1	7.5 ± 2.10	0.50 ± 0.03	12.9 ± 3.4	4.3 ± 0.3
BHI 2	4.9 ± 0.83	0.26 ± 0.02	6.5 ± 0.8	1.7 ± 0.1
TSB	1.2 ± 0.06	0.89 ± 0.03	1.1 ± 0.1	12.5 ± 1.8
NZY	2.6 ± 0.19	0.57 ± 0.02	5.7 ± 0.5	21.8 ± 3.5
NB 2	3.6 ± 0.53	1.02 ± 0.08	7.3 ± 0.5	13.1 ± 0.6
$ MINCA	3.7	1.08	7.2	9.0
TERG	8.2 ± 0.04	1.49 ± 0.02	NA	NA
NB	0.8 ± 0.06	0.29 ± 0.03	2.8 ± 0.2	33.6 ± 2.5

Where *=*the amount of K88 fimbrial adhesin determined per cfu; $=this result was determined in only one experiment; NA=not applicable.*

Table 4.7 The expression of the K88 adhesin by *E.coli* strain O8:K87:K88ac: H19 when cultivated with various media.

MEDIA	TOTAL DETECTED (μg)		* K88/cfu ($gx10^{-15}$)	
	AGAR	BROTH	AGAR	BROTH
BHI 1	311 ± 45	64 ± 7	4.5 ± 1.5	7.7 ± 1.2
BHI 2	220 ± 55	57 ± 7	3.6 ± 1.6	4.3 ± 1.0
TSB	57 ± 7	244 ± 19	4.0 ± 1.0	47.0 ± 6.4
NZY	74 ± 4	31 ± 1	2.8 ± 0.9	10.8 ± 0.7
NB 2	112 ± 15	66 ± 6	6.4 ± 3.8	24.0 ± 5.0
$ MINCA	300	67.2	ND	6.2
TERG	105 ± 9	74 ± 3	NA	NA
NB	11 ± 1	19 ± 1	2.0 ± 0.6	15.0 ± 2.0

Table 4.8 The expression of the K88 adhesin by E.coli strain K88ad when cultivated on various media.

MEDIA	TOTAL DETECTED (μg)		* K88/cfu ($gx10^{-15}$)	
	AGAR	BROTH	AGAR	BROTH
BHI 1	206 ± 40	230 ± 38	3.1 ± 0.8	23.1 ± 10.3
BHI 2	160 ± 26	196 ± 23	3.0 ± 0.4	10.2 ± 3.7
TSB	363 ± 26	68 ± 9	15.1 ± 0.9	3.6 ± 1.0
NZY	105 ± 16	78 ± 5	7.0 ± 1.7	8.5 ± 0.4
NB 2	143 ± 37	107 ± 7	3.7 ± 0.7	22.0 ± 11.3
$ MINCA	211	107	6.8	8.9
TERG	428 ± 95	128 ± 9	NA	NA
NB	65 ± 5	54 ± 8	53.7 ± 10.6	20.0 ± 5.1

Where *=*the amount of K88 fimbrial adhesin determined per cfu; NA=not applicable; ND=not determined; $=this result was determined in only one experiment.*

O8:K87:K88ab:H19 and K88ad the relative expression of K88 on agar or in broth was largely dependant on the media concerned (see Tables 4.6 and 4.8). However, for the majority of media, K88 expression/cfu was greater for broth grown cells than those cultured on agar. In the case of all media tested for the expression of the K88 adhesin by serotype O8:K87:K88ac:H19 the amount of K88 detected/cfu was greater for broth rather than agar-grown cells (see Table 4.7). It was not possible to determine the expression of K88/cfu after growth on the TERG media because the viability of the strains when cultured with this media was low (see Table 4.4). Under these condition the number of cfu was not regarded as an accurate description of the number of bacteria present.

The results obtained with *E.coli* strains K12:K88ab and O8:K87:K88ab:H19 (see Tables 4.5 and 4.6) demonstrate that the total amount of K88 adhesin detected on agar is much higher than that in broth. In the case of strain K12:K88ab the total amount of K88 detected with NZY agar was 24-fold higher than with the equivalent broth. For strain O8:K87:K88ab:H19 the amount of K88 adhesin detected on BHI 2 was 19-fold higher than the equivalent broth. Although the differences were less it was generally found that the total amount of K88 detected on agar was greater than that in broth for the remaining two strains (see Tables 4.7 and 4.8). The exceptions were TSB and NB with serotype O8:K87:K88ac:H19 and BHI 1 and BHI 2 with serotype K88ad. In all these cases, the difference could be attributed to a comparatively greater expression of K88/cfu by the broth-grown cells. While the total amount of K88 fimbrial adhesin detected was greater on agar than the equivalent broth cultures, the difference was found attributable to the greater bacterial population supported by the agar media rather than increased expression. In fact, as detailed above, for the majority of cases the amount of K88 fimbrial adhesin/cfu was either equal or greater in broth than on the equivalent agar media.

For the purposes of this present study, fimbrial expression is regarded as the amount of fimbrial adhesin/cfu. A generally held belief is that the expression of K88 fimbriae along with others such as K99, CFA/1 and CFA/2 is favoured by cultivation on agar (Guinee and Jansen, 1979; Levine 1981; Levine *et al.* 1983). From the results presented above however, it can be seen that expression of the K88 fimbrial adhesin is favoured by broth rather than agar cultivation for the majority of strains and media.

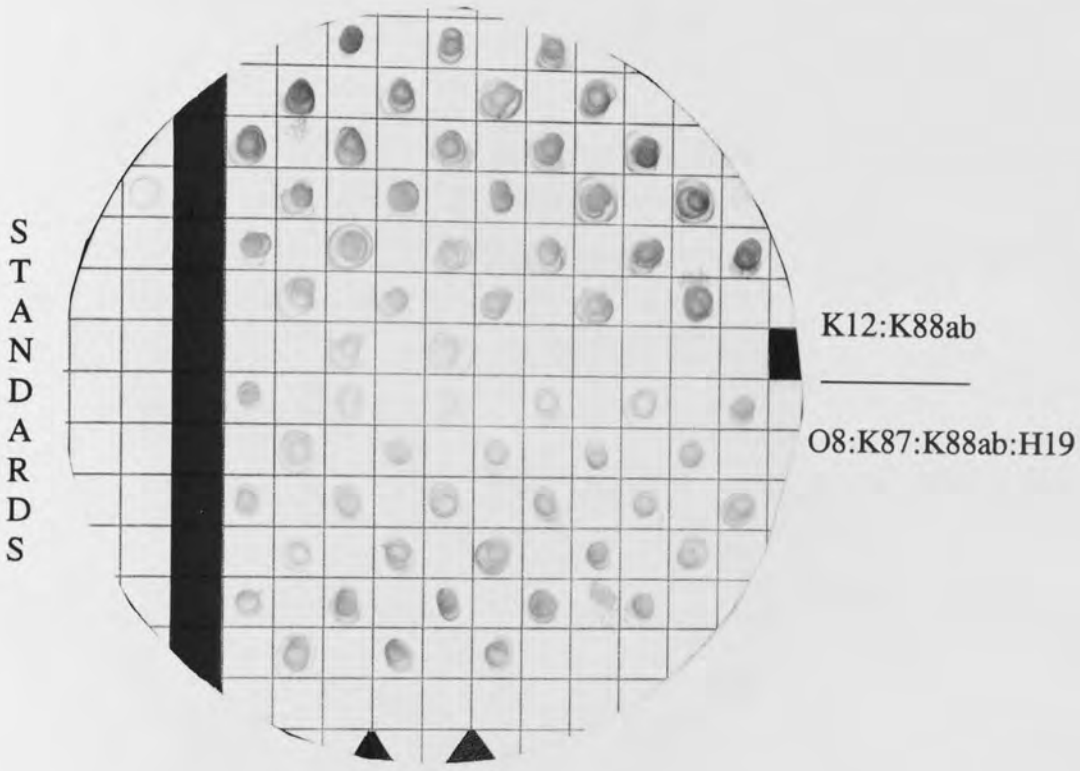
For K99 fimbriae, expression is inhibited by culture with complex media, probably due to the presence of L-alanine (De Graaf *et al.* 1980b). In addition, it has been found that the expression of the K99 fimbria was ~10-fold greater with strains bearing the O101 antigen than those bearing O8, O9 or O20. Plasmid transfer experiments indicated that the phenotypic expression of the K99 adhesin was most likely related to the cell wall composition of the host (De Graaf *et al.* 1980c). In agreement with previous findings (De Graaf *et al.* 1980b) no evidence was obtained in this study to suggest that the expression of the K88 fimbriae was inhibited in complex media. Similarly, even though the total amount of K88 adhesin detected and the amount/cfu varied considerably this did not seem attributable to the O-serotype of the strain concerned. For instance, the total amount of K88 adhesin detected and the amount/cfu on agar for serotype O8:K87:K88ac:H19 was on average 27 and 16-fold less and in broth 10 and 9-fold less than serotype O8:K87:K88ab:H19 respectively, despite the fact that they have the same O type. An alternative explanation for the observed differences in expression of the K88 adhesin may be attributable to the plasmid encoded nature of the K88 fimbrial adhesin (Orskov and Orskov, 1966) since differences in the level of expression may be due to differences in the copy number of the plasmid concerned. A further, less likely possibility may be that the level of expression is controlled by the strength of the gene promoters involved (Old and Primrose, 1987).

The expression of several fimbrial types is subject to a form of modulated expression known as phase variation. Depending on environmental conditions the number of bacteria within a population expressing a particular fimbrial adhesin varies (Brinton 1959, Nagy *et al.* 1977, Eisenstein and Dodd 1982). Since the amounts of K88 adhesin detected on the various *E.coli* strains were so variable (range = $3x10^{-13}$ to $2x10^{-15}$g/cfu) it was thought that this may reflect the fact that there was variation in the number of the bacteria expressing K88 fimbriae. To examine this possibility, dilutions of cultures of the various *E.coli* strains were plated out onto nutrient agar plates. The ability of individual colonies to express the K88 fimbrial adhesin was then determined as in Section 2.9.3. From the results presented in Figures 4.3-4.6 it was apparent that all the colonies of strains capable of expressing the K88 fimbrial adhesin did indeed express it.

Based on the assumptions of 100 fimbrial subunits per intact fimbria (Klemm, 1981) and a subunit molecular weight of 27.5KDal (Klemm, 1985) it can be calculated that 66,000 fimbriae are present on the surface of each bacterium of serotype K12:K88ab when cultivated in TSB broth. This is the maximum number of fimbriae detected for any strain/media combination. The minimum detected (438) was for strain O8:K87:K88ac:H19 when grown on nutrient agar. The calculated values of fimbrial numbers compares to a value of 100-1,000 previously quoted for fimbriae in general (Klemm, 1985). The comparatively high values of fimbrial numbers reported may be attributed to the fimbrillar nature of the K88 adhesin (Mooi and de Graaf 1985).

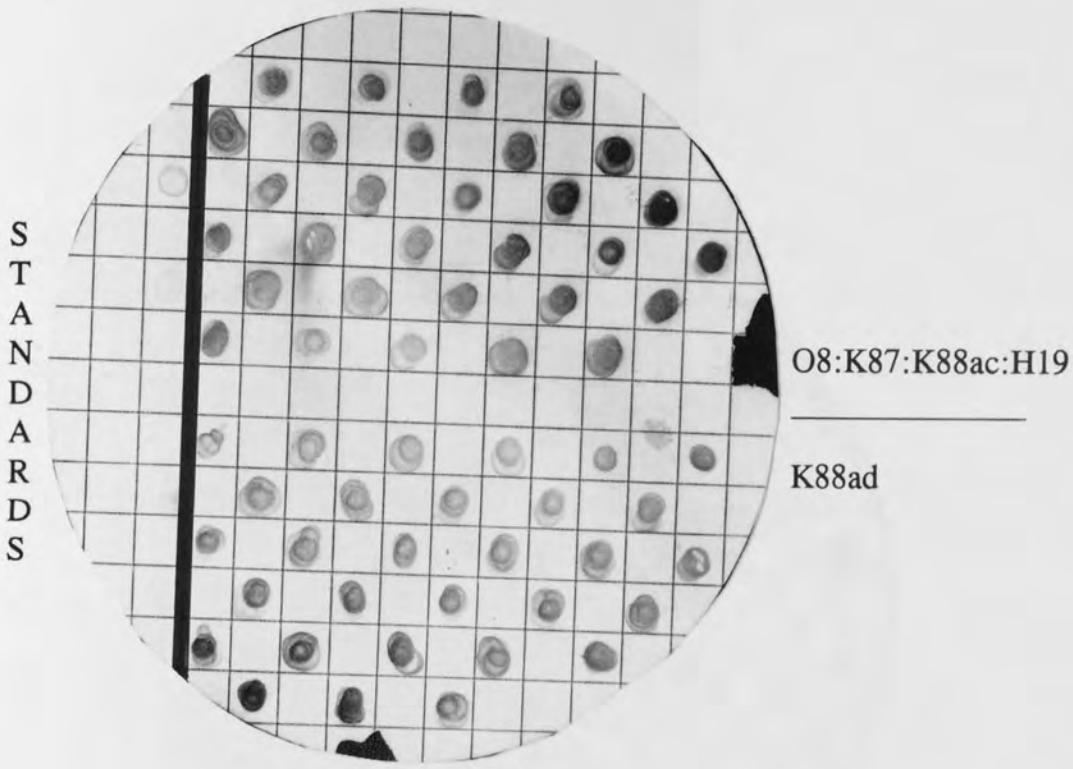
Determination of the quantitative expression of the K88 fimbrial adhesin is important since it allows the recommendation of the most appropriate media to be used for cultivation. In particular, determining which media/strain combination results in the greatest expression of K88 fimbriae indicates which combination is most suitable for the subsequent extraction of the adhesin since

Figure 4.3 Dot blotting of *E.coli* strains for the detection of the K88 fimbrial adhesin.



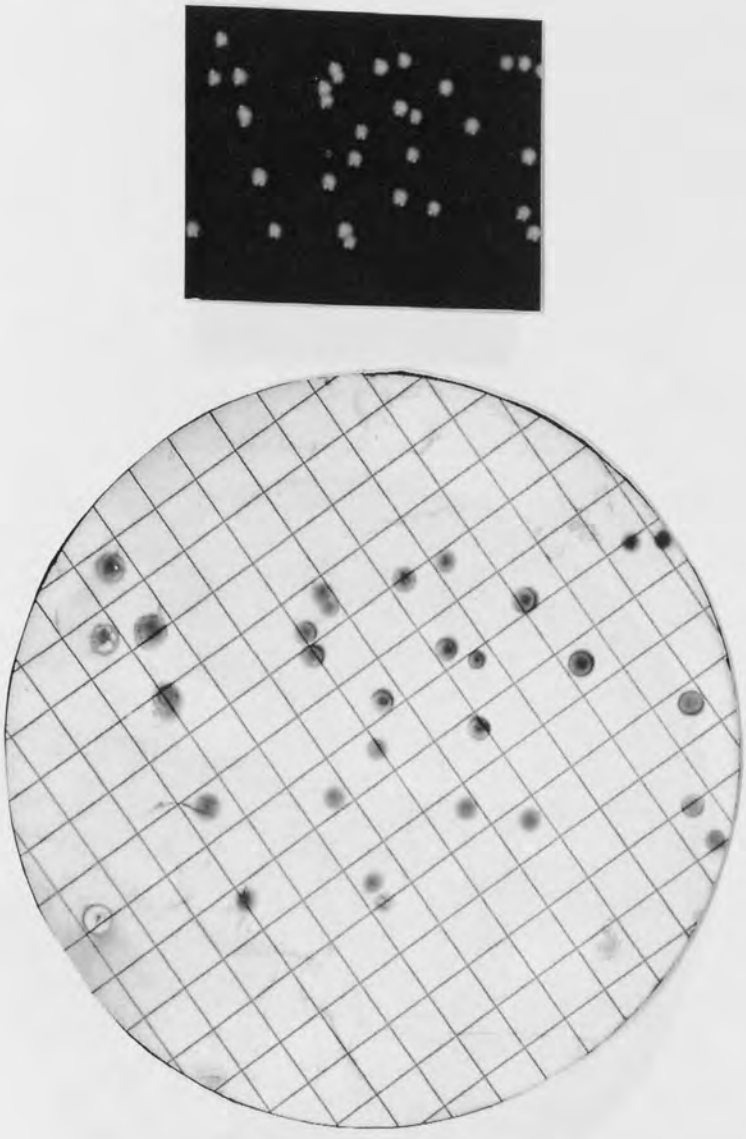
Using dot blotting method 1 (see 2.9.3) thirty colonies from each of the E.coli strains under examination were tested for the ability to express the K88 fimbrial adhesin. For E.coli strains K12 and O149:K91:H10 no colonies expressing the K88 adhesin were detected (not shown). The results of the strains K12:K88ab and O8:K87:K88ab:H19 are given above. The top half of the membrane was strain K12:K88ab and the bottom half strain O8:K87:K88ab: H19. As a positive control dilutions of purified K88ab were spotted on the left-hand side of the blue line at concentrations of 9.25, 1.85, 0.37, 0.07 and 0.02μg/ml (from top to bottom).

Figure 4.4 Dot blotting of E.coli strains for the detection of the K88 fimbrial adhesin.



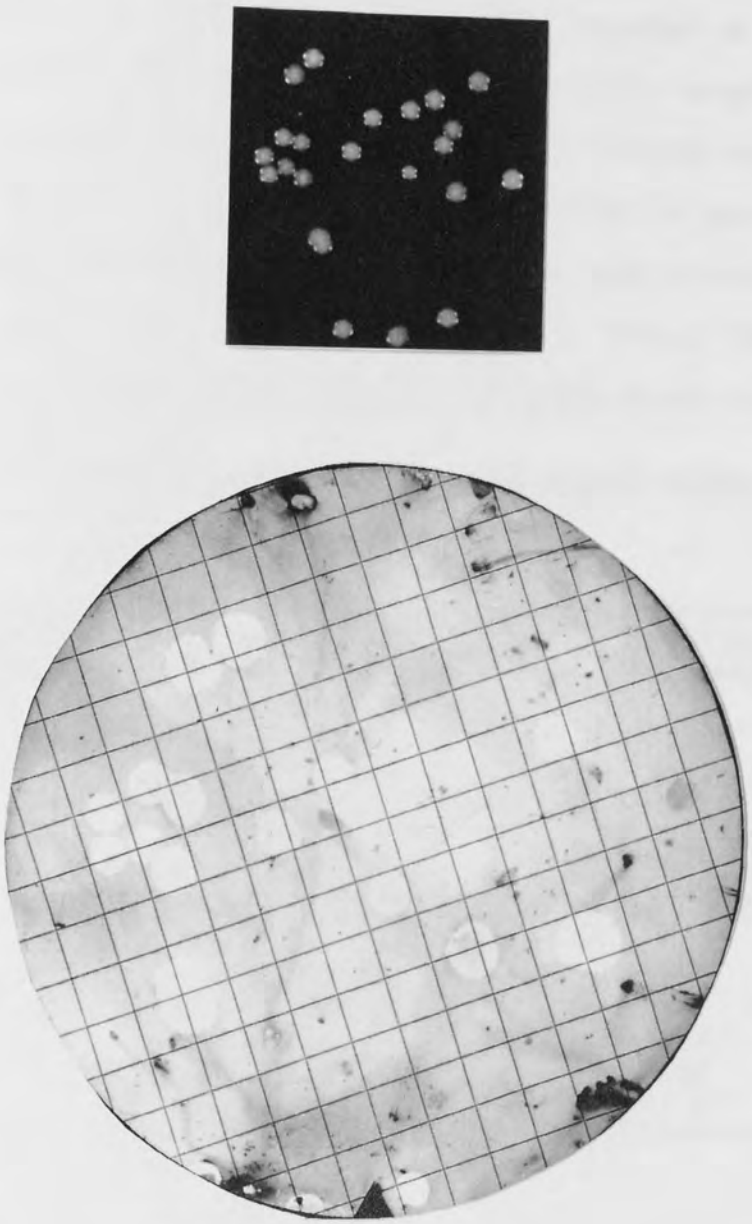
The results of the dot blotting of strains O8:K87:K88ac:H19 and K88ad are given above. As for figure 4.3 thirty colonies of each E.coli strain were examined for the ability to express the K88 fimbrial adhesin antigen. The membrane was divided as shown above. The positive control used was as in figure 4.3.

Figure 4.5 Dot blotting of E.coli O8:K87:K88ac:H19 colonies from agar plates.



The pattern of colonies on the original agar plate (top photograph) was compared to the pattern obtained after the use of a specific αK88 antiserum to detect blotted K88 adhesin on a nitrocellulose membrane (bottom photograph). As can be seen, the pattern of colonies is identical indicating that all the colonies on the original plate were expressing the K88 fimbrial adhesin. Similar results were obtained with E.coli strains K12:K88ab, 08:K87:K88ab: H19 and K88ad.

Figure 4.6 Dot blotting of *E.coli* O149:K91:H10 colonies on agar plates.



Agar-grown E.coli strain O149:K91:H10 was subject to dot-blotting as outlined in section 2.9.3. In this case, no colonies expressing K88 fimbriae were detected. Similar results were obtained with E.coli strain K12.

maximising yield is important. On the other hand since the adhesive capacity of a bacterium is presumed to be related to the number of fimbriae per cell (Isaacson 1980, Van Verseveld *et al.* 1985), the optimum strain/media combination for adhesion experiments was regarded as the combination resulting in the highest quantity of K88/cfu. From this viewpoint differences in expression of the K88 adhesin on either agar or in broth were related to both the *E.coli* strain and media concerned. However, in general, the level of expression of the K88 adhesin decreased in the order of strain K12:K88ab > O8:K87:K88ab:H19 > O8:K87:K88ac:H19 ≃ K88ad. Table 4.9 outlines media recommended for the culture of the various *E.coli* strains.

Table 4.9 Media recommended for the cultivation of strains capable of expressing the K88 adhesin.

PURPOSE	STRAIN	SPECIFIC MEDIA	GENERAL MEDIA
Preparation of the K88 fimbrial adhesin	K12:K88ab	NZY AGAR	BHI 1 AGAR
	O8:K87:K88ab:H19	TERG AGAR	
	O8:K87:K88ac:H19	BHI 1 AGAR	
	K88ad	TERG AGAR	
Adhesion studies	K12:K88ab	TSB BROTH	NB BROTH
	O8:K87:K88ab:H19	NB BROTH	
	O8:K87:K88ac:H19	TSB BROTH	
	K88ad	NB AGAR	

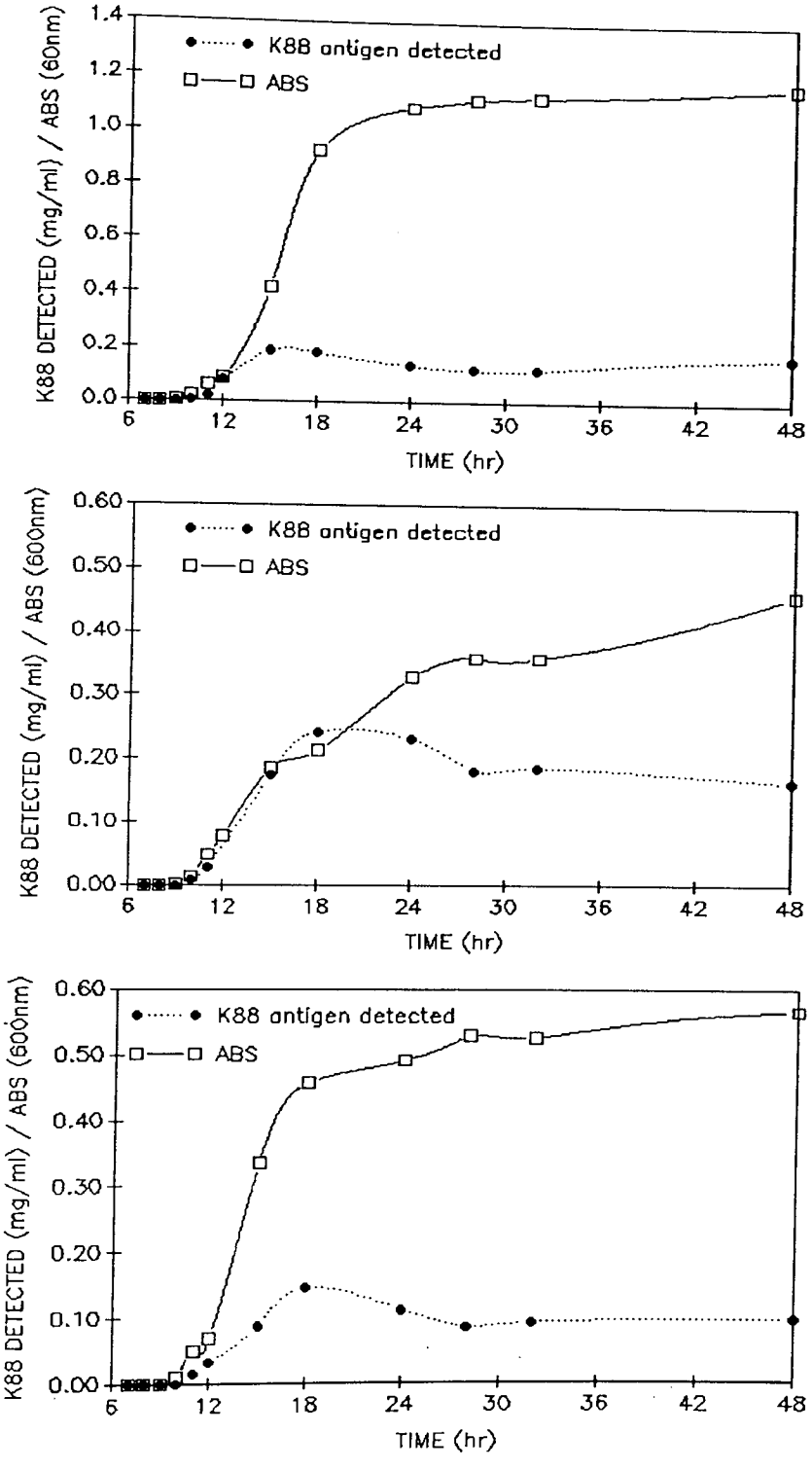
4.4 Expression of the K88 fimbrial adhesin during batch culture.

Little is known about the production of K88 fimbriae by *E.coli* strains during their growth. Studies using steady state chemostat experiments and a recycling fermentor have shown that the production of K99 and F41 fimbriae was correlated with growth rate under both aerobic and anaerobic conditions (Van Verseveld *et al.* 1985). In addition it has been reported that during batch culture, the biosynthesis of K88 as well as K99 and F41 fimbriae is dependant on growth phase (Jacobs and De Graaf 1985, Isaacson 1980). The purpose of this section was to quantitate the expression of the K88 fimbrial adhesin during batch culture. The results obtained were used to determine the optimum time at which to harvest cells before extraction of the K88 adhesin.

The production of the K88 adhesin by four strains of *E.coli* on three different broth media was monitored. The media were chosen because they represented media on which growth was comparatively high (BHI 1), medium (NB 2) or low (NB, see Table 4.3). Expression was determined by direct ELISA (Figures 4.7-4.10). In addition, the expression of the K88 adhesin by strain O8:K87:K88ab:H19 on the three media and strain K12:K88ab on NB 2 media was determined by the heat extraction of the K88 adhesin followed by ELISA (see Section 2.9.5 and Figures 4.11-4.12).

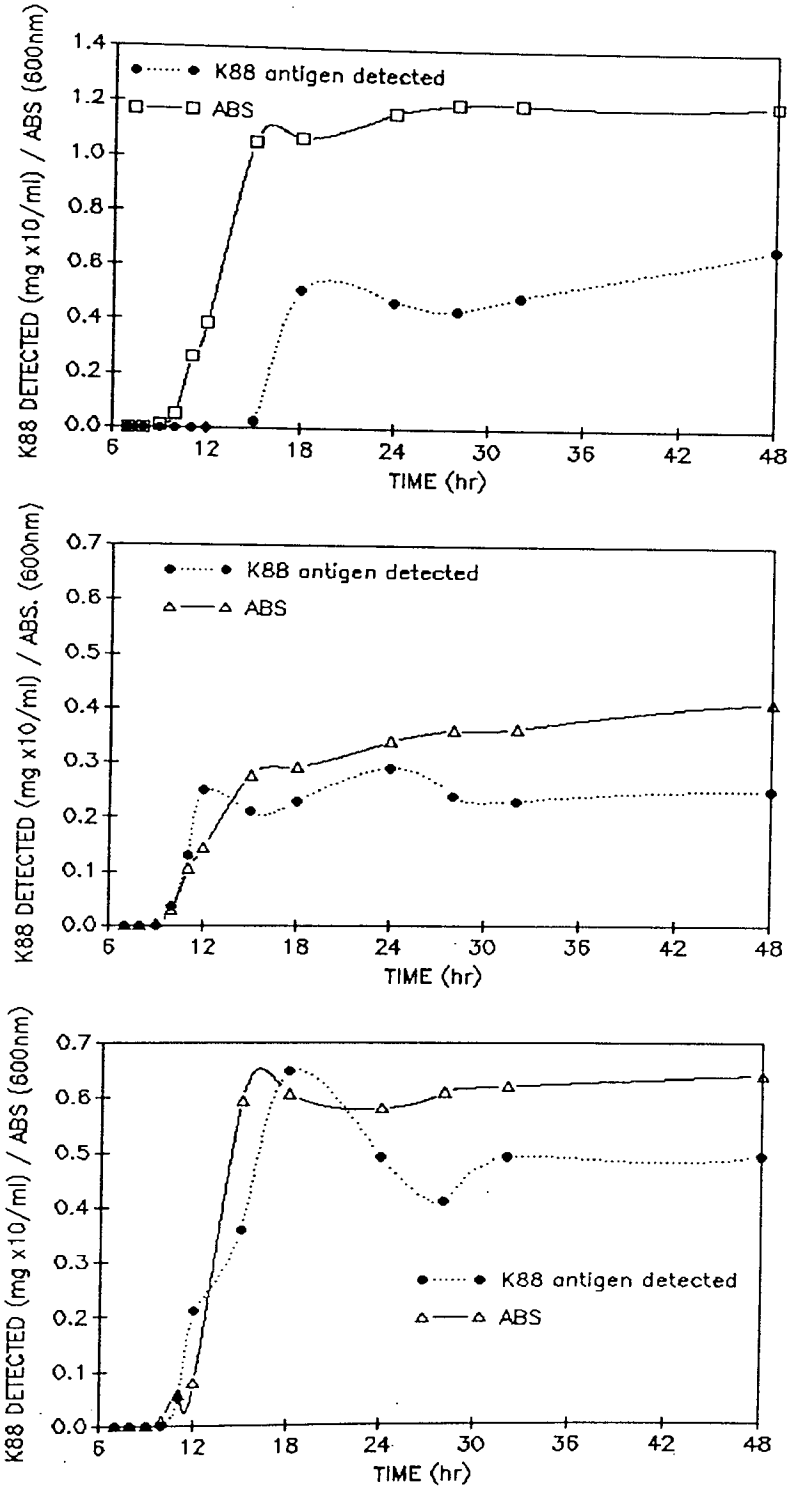
Comparing the amount of K88 adhesin detected on the four strains confirms the results obtained previously for the extraction of the K88 fimbrial adhesin after 16 hours of culture (Section 4.3). Again it is apparent that the level of expression of strain K12:K88ab is high compared to the other strains in particular strains O8:K87:K88ac:H19 and K88ad. Exact comparison of the of the amount of K88 adhesin expressed by the same strain on the various media is difficult because it is not known what effect the difference in culture

Figure 4.7 Expression of the K88 fimbrial adhesin by *E.coli* strain K12:K88ab during unshaken batch culture at 37°C.



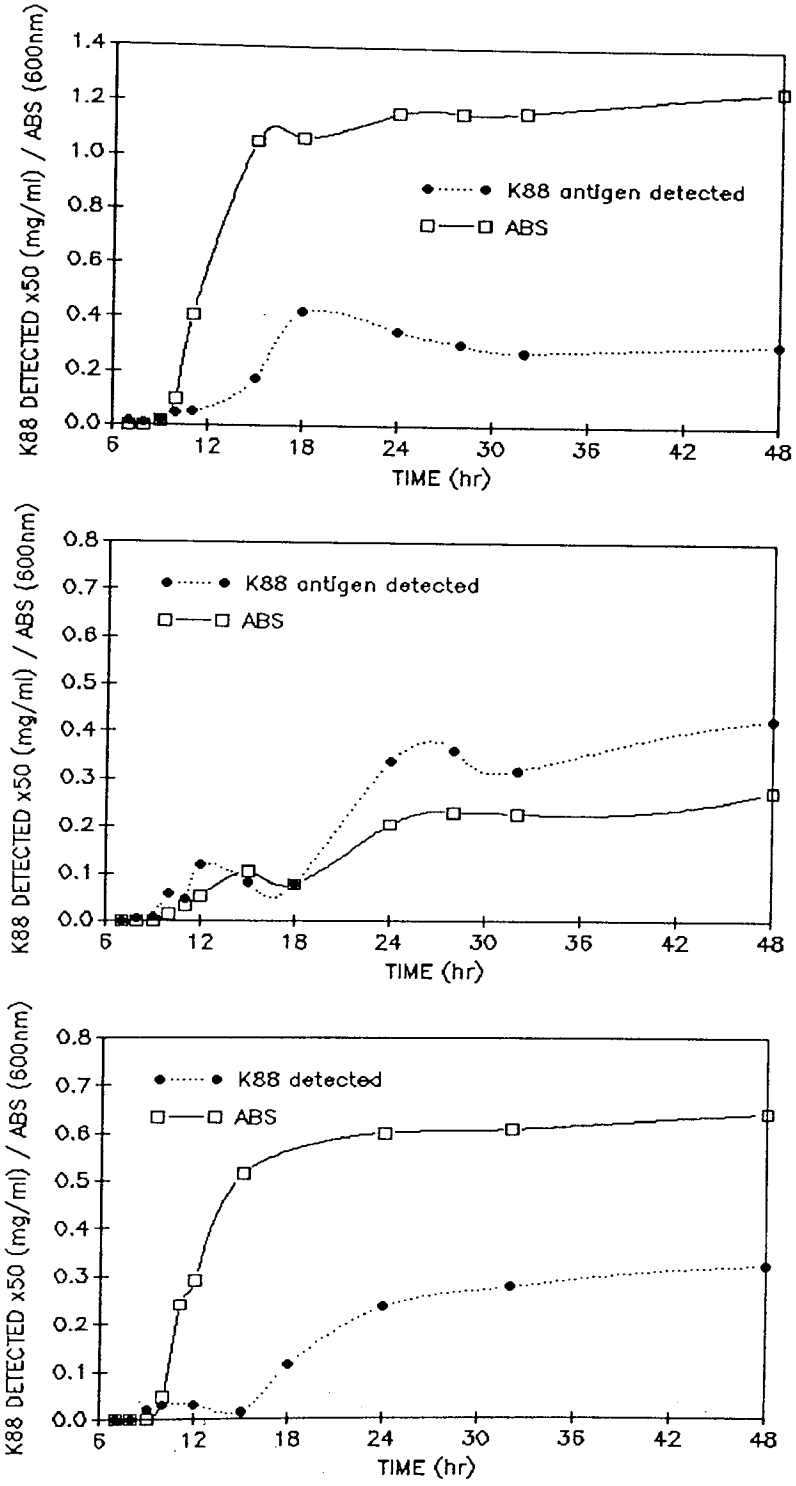
The expression of the K88 fimbrial adhesin was determined by ELISA. Media used for culture was BHI 1(top), NB(middle) and NB 2 (bottom). Where ABS=Absorbance.

Figure 4.8 Expression of the K88 fimbrial adhesin by *E.coli* strain O8:K87: K88ab:H19 during unshaken batch culture at 37°C.



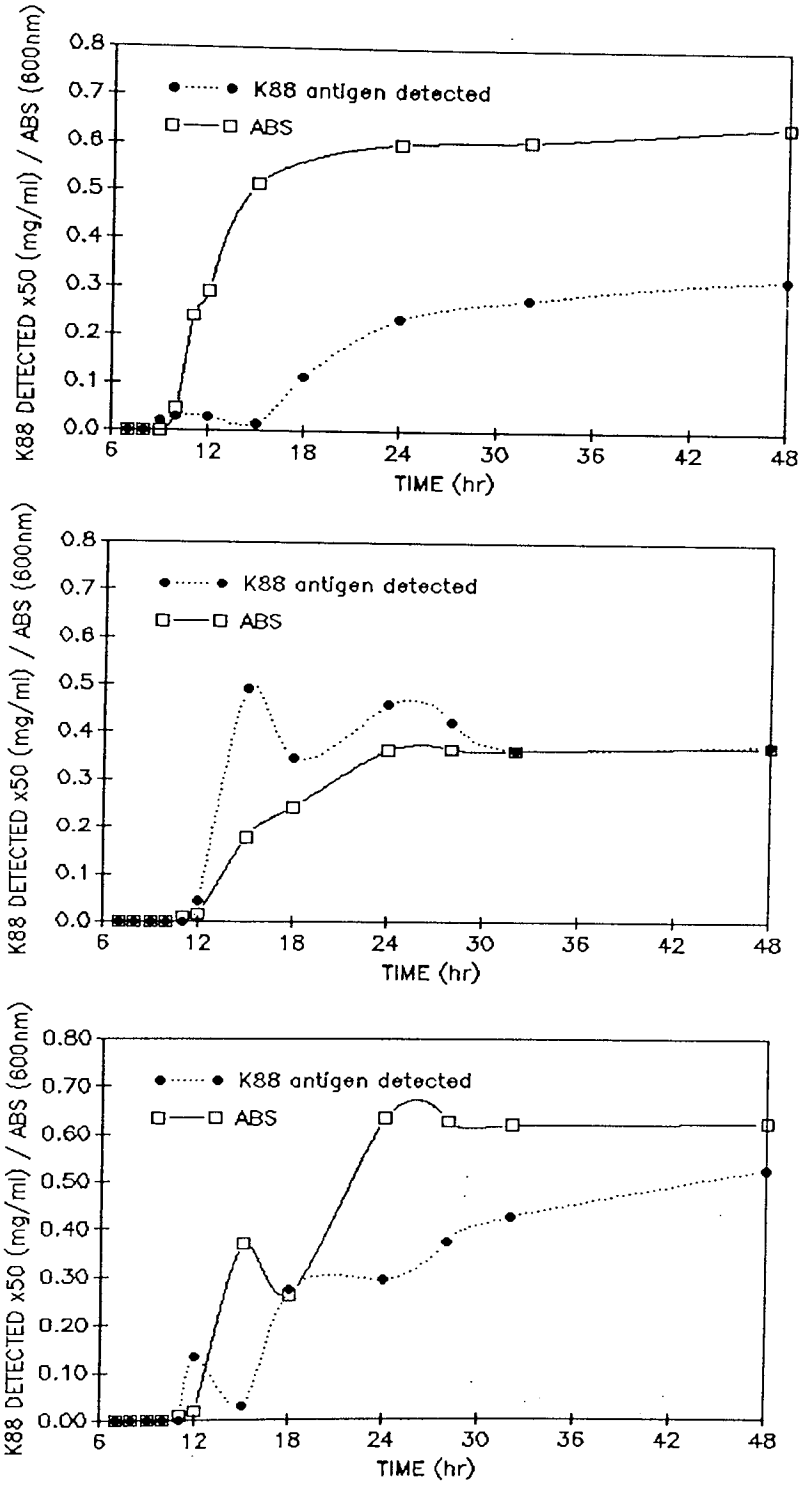
The expression of the K88 fimbrial adhesin was determined by ELISA. Media used for culture was BHI 1(top), NB(middle) and NB 2(bottom). Where ABS=Absorbance.

Figure 4.9 Expression of the K88 fimbrial adhesin by *E.coli* strain O8;K87; K88ac:H19 during unshaken batch culture at 37°C.



The expression of the K88 fimbrial adhesin was determined by ELISA. Media used for culture was BHI 1(top), NB(middle) and NB 2 (bottom). Where ABS=Absorbance.

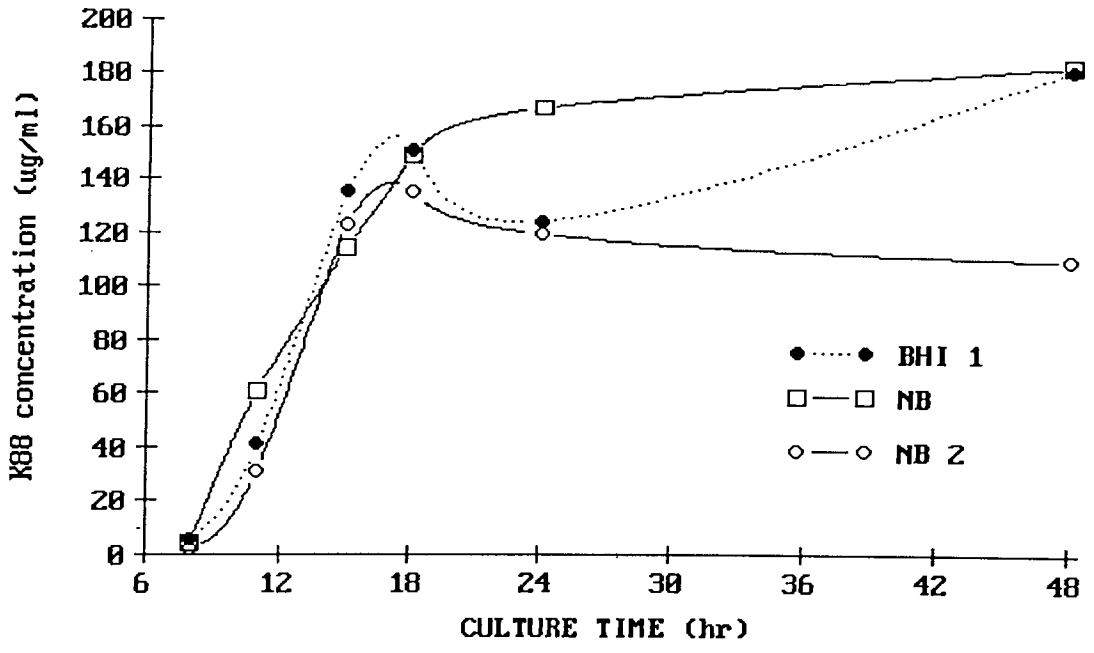
Figure 4.10 Expression of the K88 fimbrial adhesin by *E.coli* strain K88ad during unshaken batch culture at 37°C.



The expression of the K88 fimbrial adhesin was determined by ELISA. Media used for culture was BHI 1(top), NB(middle) and NB 2 (bottom) Where ABS=Absorbance.

density will have on the amount detected. This was not a problem in the experiments presented in Section 4.2 since the bacterial suspensions were adjusted to the same optical density. However, comparison of the results presented in Figure 4.8 with those obtained after the extraction of the K88 fimbrial adhesin (Figure 4.11) do reveal similar dynamics. Such agreement between two different assays is good evidence for the accuracy of both. The amount of K88 fimbrial adhesin detected after heat extraction was between 2

Figure 4.11 Quantitative determination of the expression of the K88 adhesin by *E.coli* strain O8:K87:K88ab:H19 during batch culture.

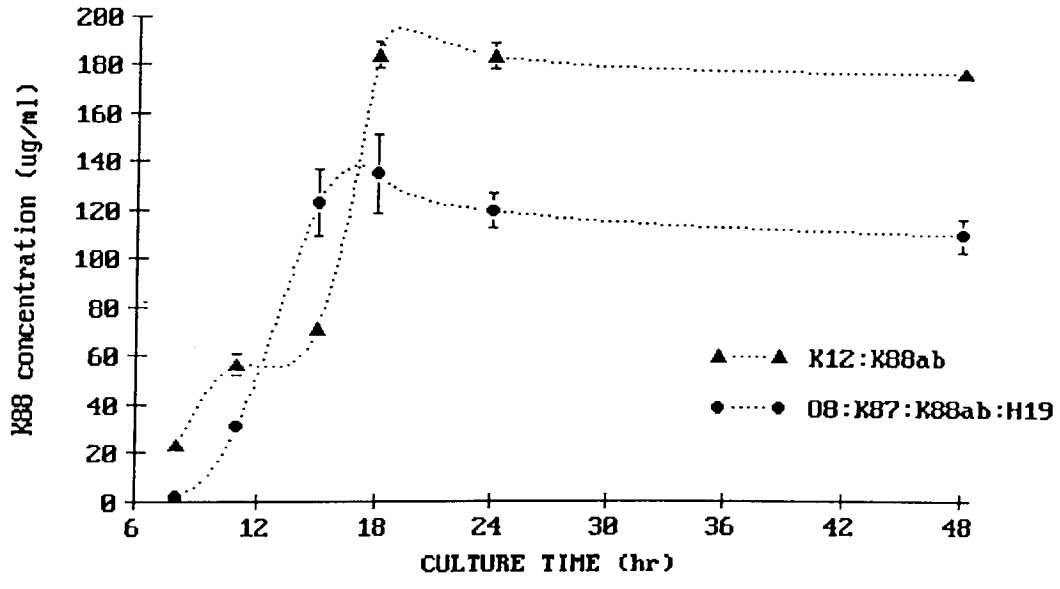


Bacteria were cultured unshaken at 37°C with samples being obtained at specified time intervals during batch culture and subjected to the small-scale method for the preparation of the K88 fimbrial adhesin (see Section 2.4.4). The K88 adhesin present in the extracts was then quantitated by specific ELISA (see Section 2.9.5). Key indicates media used. The results plotted are the means of triplicates.

to 6-fold higher than that detected directly. This difference is presumed to reflect a difference in the efficiency of the release of the K88 adhesin when either subjected to heat extraction or allowed to bind to wells of a microplate respectively. Hone *et al.* (1988) detected 1.4 to 1.8-fold more K88 adhesin after heat extraction than on the bacterial surface.

A general finding with all the media/strain combinations was that the expression of the K88 adhesin peaked after 15-18hr of culture. This corresponds to late exponential phase to early stationary phase and agrees with previous findings for the K88 adhesin (Jacobs and de Graaf 1985). Similar results have been reported for the expression of the F41 and K99 fimbrial adhesins (Isaacson 1980, Jacobs and De Graaf 1985). It is interesting to note

Figure 4.12 Expression of the K88 adhesin by *E.coli* strains K12:K88ab and O8:K87:K88ab:H19 during batch culture on NB 2 media.



Bacteria were cultured unshaken in broth at 37°C and samples taken at specified time intervals. The K88 fimbrial adhesin was extracted and quantitated by ELISA. (Results are given as the means of triplicates ± standard error.

that where growth curves were shown have two peaks that the expression of the K88 adhesin likewise had two peaks (see Figures 4.10 and 4.11).

After the expression of the K88 fimbrial adhesin peaked it was found that in most cases the amount detected dropped and then either stabilised or increased or decreased slightly. Using an enzyme-linked antibody centrifuge assay, Isaacson (1980) reported similar results during the expression of the K99 fimbrial adhesin.

4.5 Conclusions

E.coli strains capable of expressing the K88 fimbrial adhesin antigen are able to grow on a wide range of media. During growth in unshaken batch culture, post-logarithmic growth may be facilitated by fimbriae (K88 included) since the only strain not to show such a growth characteristic was poorly fimbriated. The level of expression of the K88 adhesin was not inhibited by complex media and varied according to both the *E.coli* strain and the media under examination. Comparison of the levels of expression of the K88 adhesin detected revealed that in general the total amount was greater while the amount/cfu was less on agar than in broth cultures. The general media found appropriate for use in the extraction/preparation of the K88 fimbrial adhesin was BHI 1 agar while the general media suitable for binding studies was found to be NB broth. Comparison of the levels of the expression of the various *E.coli* strains suggests that adhesive ability decreases in the order K12:K88ab > O8:K87:K88ab:H19 > O8:K87:K88ac:H19 $\simeq$ K88ad. Studies on the K88 adhesin during unshaken batch culture indicated that expression was related to the growth rate and was maximal during late logarithmic to early stationary phase depending on the strain/media combination.

5 PRODUCTION, PURIFICATION AND IDENTIFICATION OF THE K88 FIMBRIAL ADHESIN.

5.1 Introduction.

An early problem in this study was the preparation of purified K88 adhesin protein. A complication was that no specific αK88 antiserum was available initially to confirm the identity of isolated protein. Two approaches were used to solve this problem. Firstly, it had previously been reported that heat extraction followed by isoelectric precipitation resulted in highly purified K88 fimbrial protein. Purity had been demonstrated by immunoprecipitation, analytical centrifugation and moving-boundary electrophoresis (Stirm *et al.* 1967a). Additionally, the characteristic molecular weights of the various serotypes of the K88 adhesin after SDS-PAGE were known (Mooi and De Graaf 1979). Thus, it was regarded that a highly purified protein of characteristic molecular weight (by SDS-PAGE) produced by a method based on heat extraction and isoelectric precipitation from strain K12:K88ab was likely to be the K88ab adhesin. Secondly, it has been shown previously that the K88 adhesin haemagglutinates a restricted number of erythrocyte species (Parry and Porter 1978). Therefore, use was made of the known haemagglutination pattern of the K88 and other adhesins to further characterise the isolated protein.

For final confirmation of the identity of the isolated, purified K88 adhesin protein, rabbits were immunised and mono-specific putative αK88ab antiserum produced. The properties of this putative αK88ab antiserum were determined by immunoprecipitation, Western blotting, ELISA and by comparison with control αK88-specific serum which by that time had become available.

The aim of this section of work was three-fold. Firstly, there was a requirement for a comparatively large amount (>100mg) of purified K88

fimbrial protein for future work. Secondly, specific αK88 antisera was required as a molecular tool e.g. for inhibition studies. Finally, it was envisaged that the development of a small-scale method for the extraction of the K88 adhesin protein would facilitate work on both the mechanism of the heat extraction and on the rapid production of small amounts of purified K88 adhesin.

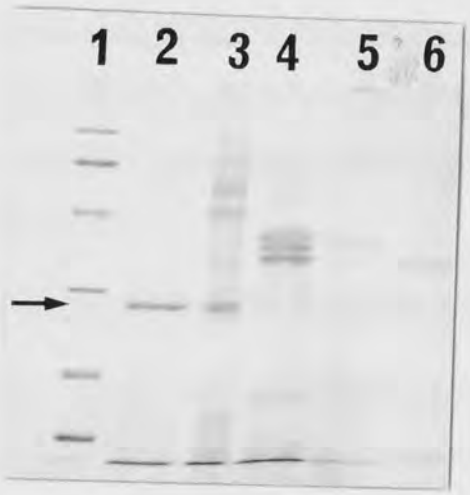
5.2 Production of the K88 fimbrial adhesin protein.

Several methods have been used for the preparation of fimbriae or their subunits based variously on sonication (De Graaf *et al.* 1980a), homogenisation (Stirm *et al.* 1967a, Mooi and De Graaf 1979, Guinee *et al.* 1980, Kuzuya *et al.* 1988) and heat extraction (Stirm *et al.* 1967a, Parry and Porter 1978, Anderson *et al.* 1980, De Graaf *et al.* 1980, Karkhanis and Bhogal 1986). In the case of K88 fimbriae further purification has been based on isoelectric precipitation only (Anderson *et al.* 1980), isoelectric precipitation followed by ultracentrifugation (Stirm *et al.* 1967a) or isoelectric focusing (Parry and Porter 1978), affinity chromatography (Kuzuya *et al.* 1988), ammonium sulphate precipitation followed by column chromatography, SDS treatment and further column chromatography (Guinee *et al.* 1980), and finally, ammonium sulphate precipitation followed by column chromatography, lyophilization and further column chromatography (Mooi and De Graaf 1979).

Initial experiments were conducted to investigate preparations of K88 fimbriae obtained by sonication or heat treatment. These were compared to a method which was not specific for K88 fimbriae but rather for outer membrane proteins. Comparison of the methods was based on the purity and quantity of a band in a SDS-PAGE gel (see Figure 5.1), which corresponded to the molecular weight of the K88 fimbrial subunit (27.5 KDal, Mooi and De Graaf 1979, Gaastra and De Graaf 1982). It was apparent that only a faint band of the appropriate molecular weight was present in the OMP preparation and that this

method was unsuitable for the preparation of K88 fimbriae. Both heat treatment and sonication resulted in similar amounts of K88 fimbriae being released as determined by SDS-PAGE. However, the sonicated preparation contained many more contaminating proteins than the one obtained by heat treatment. Accordingly, heat treatment was regarded as the method of choice for the initial extraction of K88 fimbriae.

Figure 5.1 Comparison of the methods for the extraction of the K88 fimbrial protein.



E.coli serotype K12:K88ab cultured in unshaken nutrient broth was subjected to various procedures to determine the most appropriate for the extraction of the K88 fimbrial protein. SDS-PAGE was used to monitor the protein profiles of resultant extracts. Where:- lane 1=marker proteins, from top to bottom, rabbit muscle phosphorylase b (97), bovine serum albumin (66), hen white ovalbumin (43), bovine carbonic anhydrase (31), soyabean trypsin inhibitor (21.5) and hen egg white lysozyme (14); lane 2=heat extract; lane 3= sonication extract; lane 4=outer membrane protein (OMP) extract pellet; lane 5=OMP extract supernate; lane 6=OMP extract pellet. Molecular weights (in KDal) of marker proteins are indicated in brackets. The position of the K88 fimbrial protein is indicated by an arrow.

5.3 Purification of the K88 fimbrial adhesin protein.

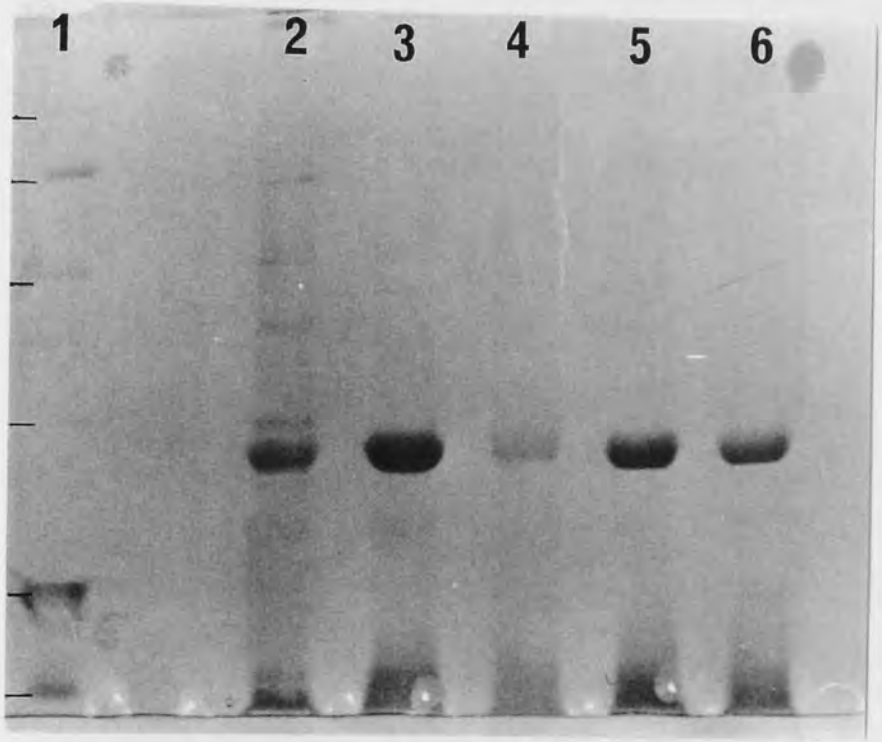
The simplest previous method for the purification of a crude extract of K88 fimbriae had been based on isoelectric precipitation (Stirm *et al.* 1967a, Parry and Porter 1978, Anderson *et al.* 1980). It was originally intended to optimise the conditions of isoelectric precipitation (see Figure 5.2) and then to further purify the preparation by column chromatography as had been employed for the K99 fimbrial adhesin (De Graaf *et al.* 1980a). However, the inclusion of the ammonium sulphate precipitation step (as noted but not used by Stirm *et al.* 1967a) before the isoelectric precipitation step was sufficient to produce a highly purified preparation without any further treatment (see Figure 5.2). On heavy overloading of SDS-PAGE gels it was possible to demonstrate a few very minor contaminants which were calculated to represent $<5\%$ of the total protein present (see Figure 5.3).

5.4 Identification of the K88 fimbrial adhesin protein

The isolated K88 adhesin has been found to have haemagglutinating properties (Jones and Rutter 1974a, Parry and Porter 1978). In the absence of specific antisera use was made of these properties to confirm the identity of the protein isolated as outlined above. Comparison of the haemagglutination properties of the isolated protein with those known for the 4 main types of fimbriae associated with ETEC strains of porcine origin (see Table 5.1) showed a strong correlation with the K88 fimbrial adhesin. This finding, along with the characteristic molecular weight of the isolated, purified protein was regarded as strong evidence confirming the identity of the protein.

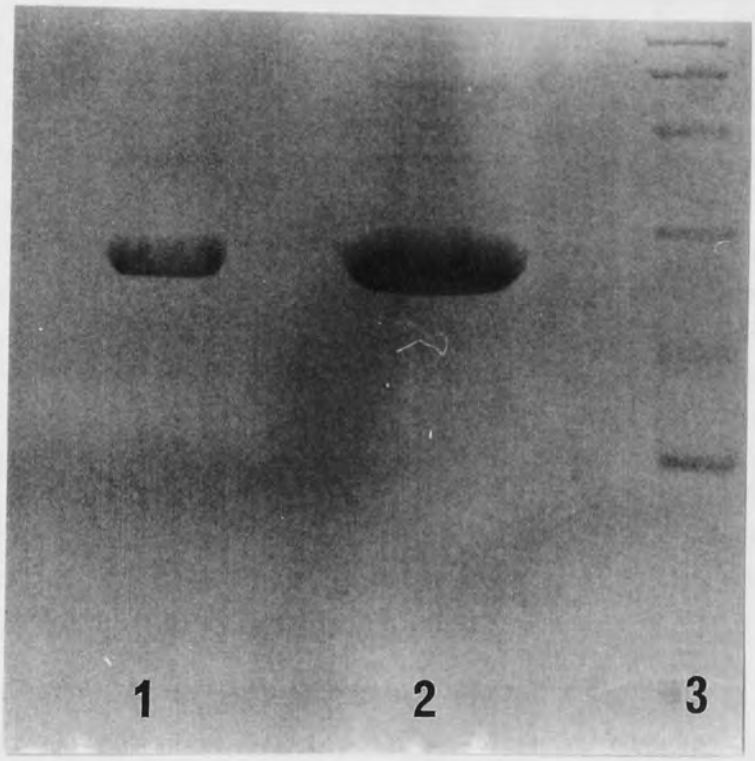
Further confirmation of the nature of the putative purified K88ab adhesin was based on the raising of specific antiserum to it. Rabbits were immunised and non-specific antibodies removed from the resultant sera by absorption with

Figure 5.2 Use of isoelectric precipitation in the purification of the K88 fimbrial adhesin protein.



Isoelectric precipitation was used for purification of the K88 fimbrial adhesin protein (pI 4.2) during its large scale preparation. The method was initially optimised by comparing the SDS-PAGE profiles of crude K88 fimbrial protein when subjected to various isoelectric precipitation procedures. The gel presented above was silver stained to demonstrate contaminants in the preparation. Lane 1=marker proteins (1:10 dilution of stock in sample buffer, otherwise as for figure 5.1); lane 2=crude K88 protein; lane 3=single isoelectric precipitation at pH 5.3; lane 4=single isoelectric precipitation at pH 4.2; lane 5=three isoelectric precipitations at pH 5.3; lane 6=three isoelectric precipitations at pH 4.2. Before samples were run on the gel their pH was adjusted to 8 by the dropwise addition of 1M NaOH. Isoprecipitates formed at pH 4.2 were denser than those at pH 5.3 and less protein was lost during washing stages (see section 2.4.3). Precipitates formed at both pH were virtually free of contaminating protein.

Figure 5.3 SDS-PAGE gel showing final preparation of K88 fimbrial adhesin protein after isoelectric precipitation.



The above is presented to demonstrate the purity of the K88 fimbrial adhesin protein preparation used in this thesis. With heavy overloading a few high molecular weight contaminants were visible (see lane 2). K88 adhesin protein was extracted and purified from E.coli strain K12:K88ab cultured on agar at 37°C for 16hr. Where Lane 1=5µl of purified extract; lane 2=20µl of purified K88 extract; lane 3=marker proteins (as for figure 5.1).

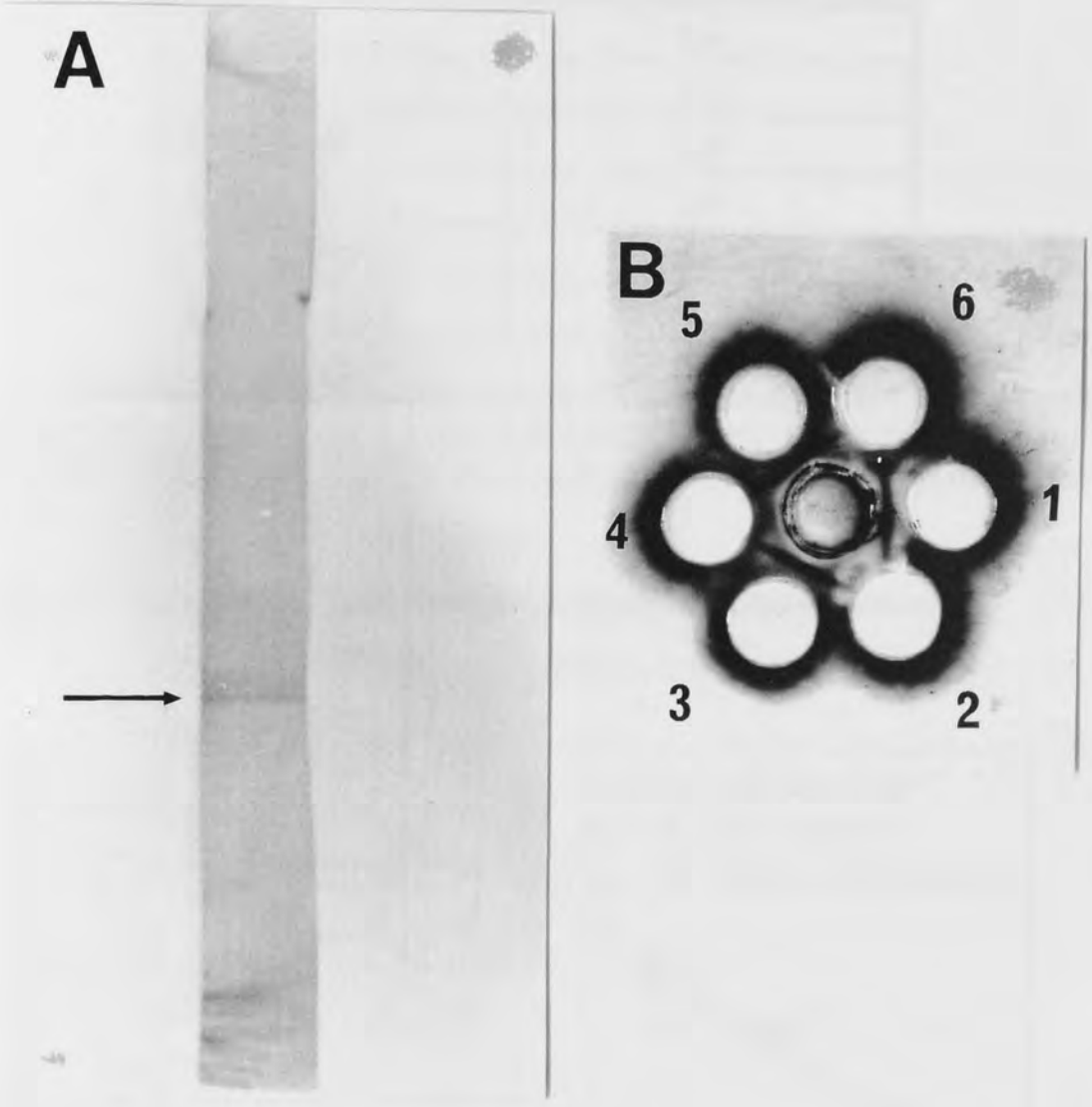
Table 5.1 Haemagglutination profiles of fimbrial adhesins.

RED BLOOD CELL TYPE	AGGLUTINATED BY PURIFIED K88	REPORTED HAEMAGGLUTINATION PROFILE			
		K88	987P	K99	F41
COW	NO	NO	NO	NO	NO
GUINEA PIG	YES	YES	NO	NO	YES
HEN	YES	YES	NO	NO	NO
HORSE	NO	NO	NO	YES	YES
PIG	YES	?	NO	NO	NO
SHEEP	NO	NO	NO	YES	YES

The ability of the purified K88 preparation to cause haemagglutination was monitored in a similar manner to that described in Section 2.8.5 except that no dilutions of stock (0.76mg/ml) were prepared. Data on 987P, K99 and F41 fimbriae was from Mooi and De Graaf 1985. The haemagglutinating properties of the purified K88 adhesin preparation were later examined in more detail (Chapter 6). Where:- ?=Reports on the agglutination of pig erythrocytes by K88ab have been contradictory. Parry and Porter (1978) found no agglutination, Cox and Houvenaghel (1987) found that agglutination was dependant on the number of washes of bacterial suspensions while Bijlsma et al. (1985) reported agglutination in all cases.

E.coli serotype K12 (see Section 2.6). Western blotting and immuno-precipitation were used to check that the sera were specific for the immunogen (see Figure 5.4). For uniformity of results, only one of the putative αK88ab antiserum samples was used in the subsequent experiments. The ability of this antiserum to detect K88 adhesin was then determined by ELISA (see Table 5.2). Of the six strains examined only the four capable of expressing the K88 adhesin were recognised by the putative αK88ab antiserum. Finally, the putative K88ab adhesin was used to coat microplate wells and the ability of αK88 antiserum raised in this study and αK88 antiserum kindly supplied by Dr R. Sellwood (AFRC Compton, Newbury. Berks.) to recognise it tested by ELISA (see Figure 5.5). Overall, the results presented in this section demonstrate that the protein isolated by a combination of heat extraction,

Figure 5.4 Ability of putative αK88 antiserum to recognise its corresponding immunogen by Western blotting and immunoprecipitation (Ouchterlony).



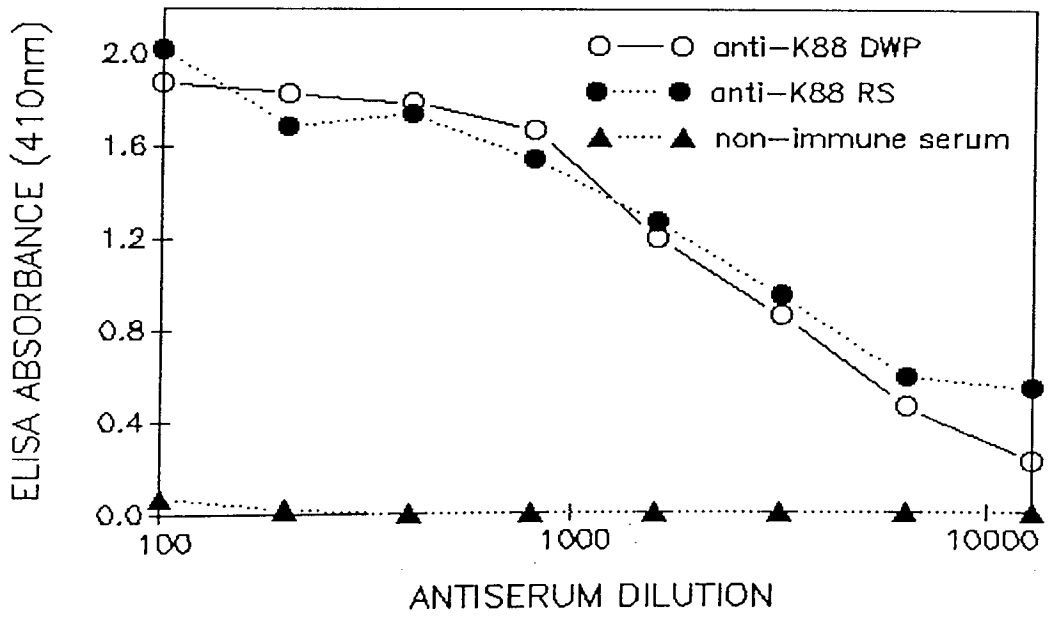
For Western blotting (A), SDS-PAGE gels were loaded with the purified K88ab preparation which was subsequently detected by antiserum prepared against the same adhesin (arrowed). No band was detected by control serum (not shown). Lines of precipitation were apparent by immunoprecipitation (B) with the αK88ab antiserum (wells 1,2,3) but not observed with control serum (wells 2,4,6). During immunoprecipitation, it was important to maintain the K88ab protein added to the centre well (0.75mg/ml) pH 8 otherwise it tended to precipitate.

Table 5.2 Specificity of antiserum raised against the purified preparation of the K88 fimbrial adhesin.

STRAIN	OD READING (410nm)
K12	0.00
K12:K88ab	0.89
O149:K91:H10	0.00
O8:K87:K88ab:H19	0.51
O8:K87:K88ac:H19	0.81
K88ad	0.35

Bacterial cells from various E.coli strains were harvested after growth on nutrient agar, washed, and suspended in PBS to an OD of 1.00 (600nm). An ELISA was then performed on the various suspensions as outlined in Section 2.6.3.

Figure 5.5 Comparison of ELISA results obtained with putative αK88 antiserum and antiserum of known specificity.



The ELISA was performed as outlined in Section 2.6.3 with 100μl of purified K88 (10μg/ml) used to initially coat the plates. Values obtained with the specific antisera were compared to those obtained with non-immune (control) serum. Note that anti-K88ab DWP was the serum prepared in this study (see Section 2.6) while anti-K88RS was the serum kindly supplied by Dr R. Sellwood.

ammonium sulphate precipitation and isoelectric precipitation is the K88ab adhesin and that the antiserum raised in this study was specific for it.

5.5 Small scale method for the rapid extraction of the K88 fimbrial adhesin protein.

5.5.1 Use of the small-scale extraction method.

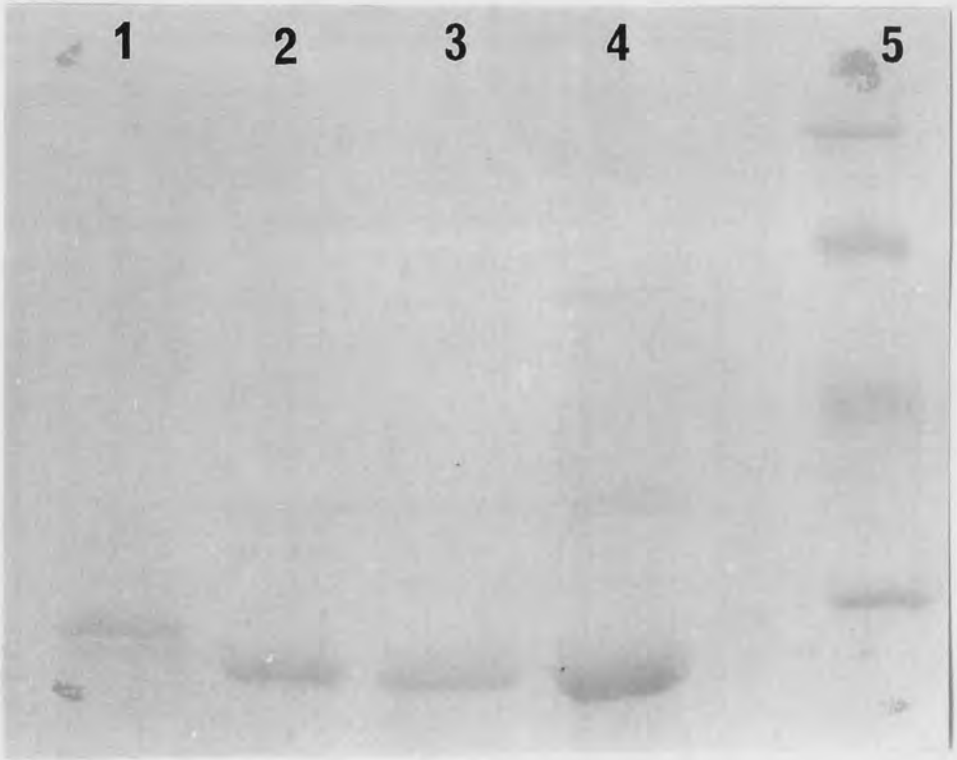
The small-scale method for the extraction of the K88 adhesin was based on the large scale method 2 (see Section 2.4.2). A typical yield obtained from 12ml of nutrient broth culture with strain K12:K88ab using the small-scale method was 0.15mg (Payne *et al.* 1991). No intact fimbriae were detected by electron microscopy after staining with 1% sodium silicotungstate. The method was found to extract with a high degree of purity all three serotypes of the K88 adhesin from various *E.coli* strains (see Figure 5.6). The small-scale extraction method was also used to demonstrate which *E.coli* strains expressed the K88 adhesin and that expression was repressed at 18°C (Orskov *et al.* 1961, De Graaf and Mooi 1986, see Figure 5.7).

5.5.2 Mechanism of the small-scale extraction method.

Heat extraction, followed by ammonium sulphate and isoelectric precipitation resulted in a remarkably pure preparation of the K88 fimbrial protein suggesting that it was facilitated by a specific characteristic of the K88 fimbria itself. To investigate further, the conditions of the small-scale method were altered and their effect on the extraction noted. Preliminary experiments were based on altering the extraction times at 60°C (see Section 2.4.5). No significant quantitative or qualitative differences were observed even when heat extraction was only continued for 5min. In subsequent experiments, both the buffer composition and the temperature at which the heat extraction was conducted were altered (see Figure 5.8). Again no significant differences in the

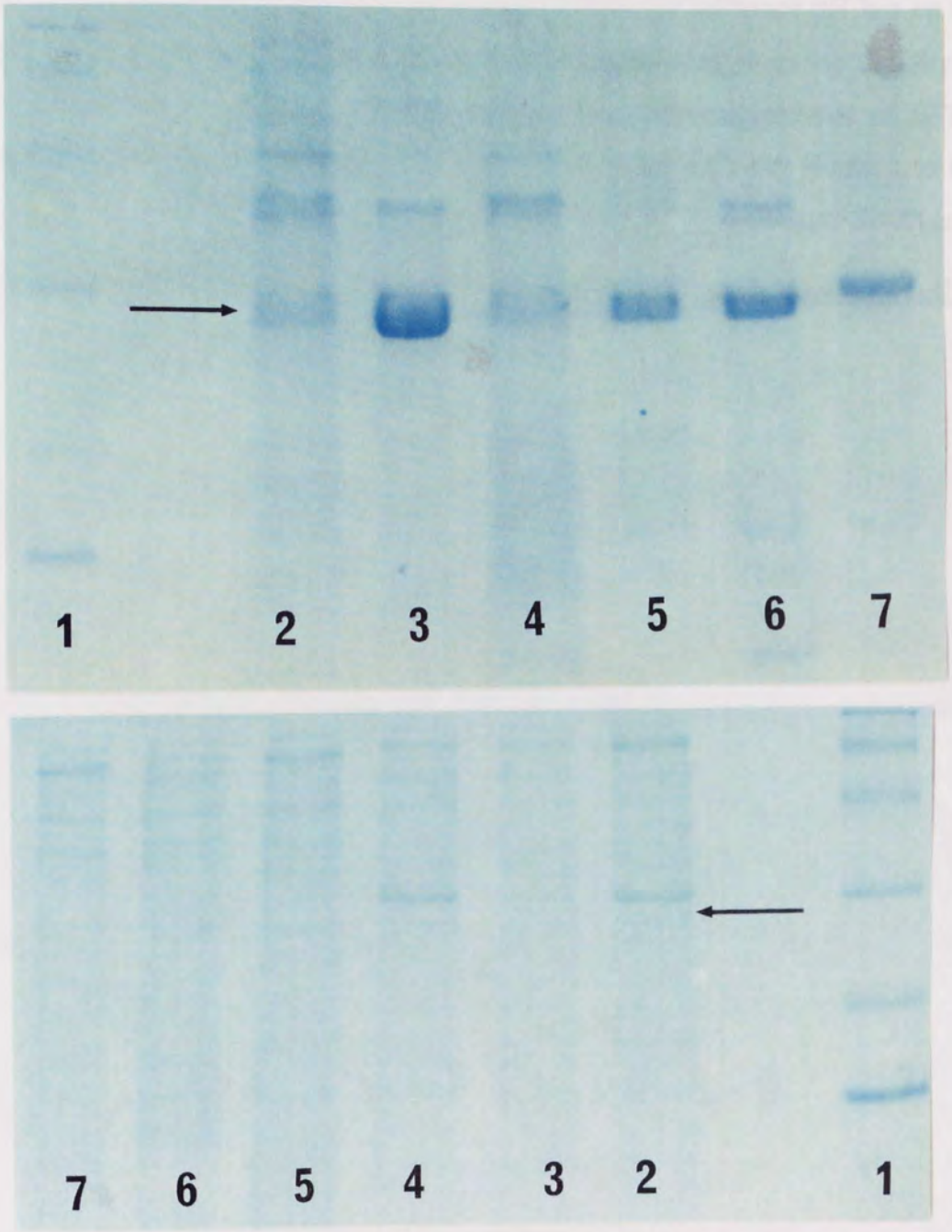
extraction were noted. However, in later experiments where the pH of the buffer used during the heat extraction process was varied there was a marked difference in the amount of K88 adhesin extracted depending on the pH (see Figure 5.10 also Payne *et al.* 1991). At pH values 9-10 the efficiency of extraction was double that at pH 7. Conversely, at pH 2-4 virtually no K88 was extracted.

Figure 5.6 Use of the small-scale method in the extraction of the three serotypes of the K88 adhesin protein.



The above SDS-PAGE gel demonstrates the ability of the small-scale method to effectively extract all three serotypes of the K88 adhesin. The K88 fimbrial protein was extracted from nutrient agar grown E.coli strains cultured at 37°C for 16hr. The gel was stained with coomassie blue. Where:- Lane 1= serotype K88ad; lane 2=serotype O8:K87:K88ab:H19; lane 3=serotype O8: K87:K88ac:H19; lane 4=serotype K12:K88ab; lane 5=marker proteins (as for top four markers in figure 5.1).

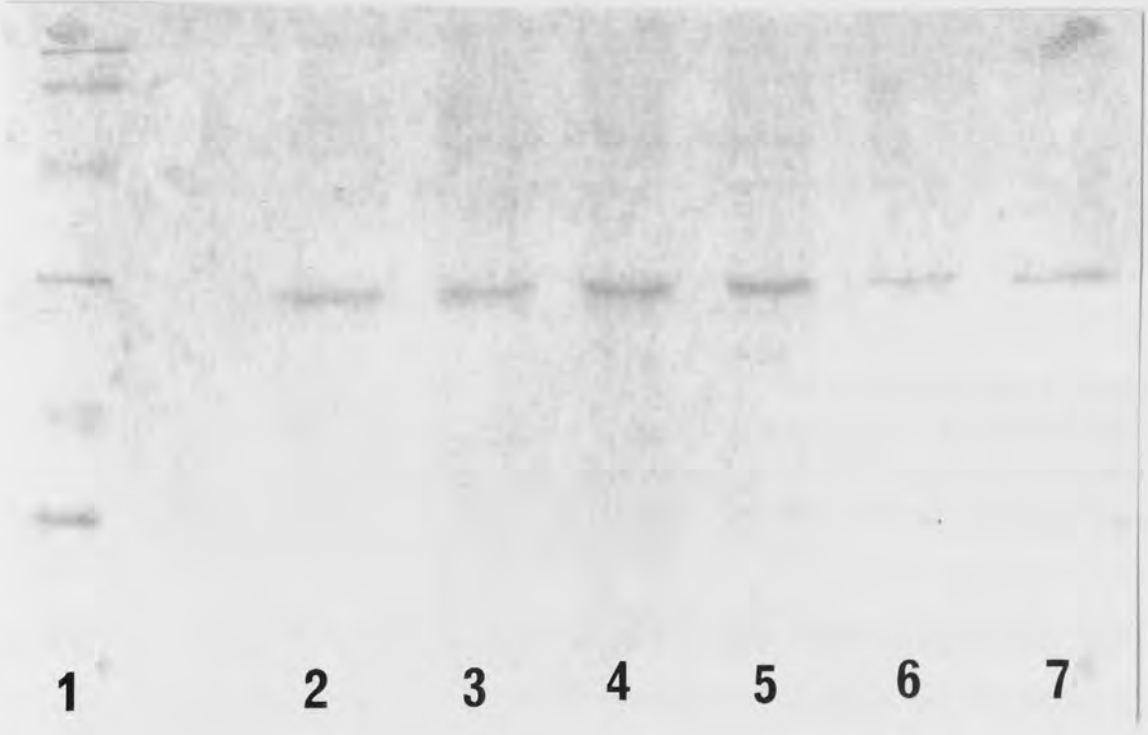
Figure 5.7 Use of the small-scale method to demonstrate the expression of the K88 fimbrial adhesin.



Extracts produced by the small-scale method for the extraction of the K88 fimbrial adhesin were analysed by SDS-PAGE. The above photographs of gels demonstrate the expression of the K88 adhesin by K88+ strains and the repression of this expression after culture at 18°C. Bacteria were cultured on agar at either 37°C (top photograph) for 16hr or at 18°C for 72hr (bottom photograph) and stained with coomassie blue. Similar results were obtained with broth grown cultures. Where:- 1=marker proteins (as in Figure 5.1), 2=K12, 3=K12:K88ab, 4=O149:K91:H10, 5=O8:K87:K88ab:H19, 6=O8: K87:K88ac:H19 and 7=K88ad. The position of the adhesin is shown by arrows.

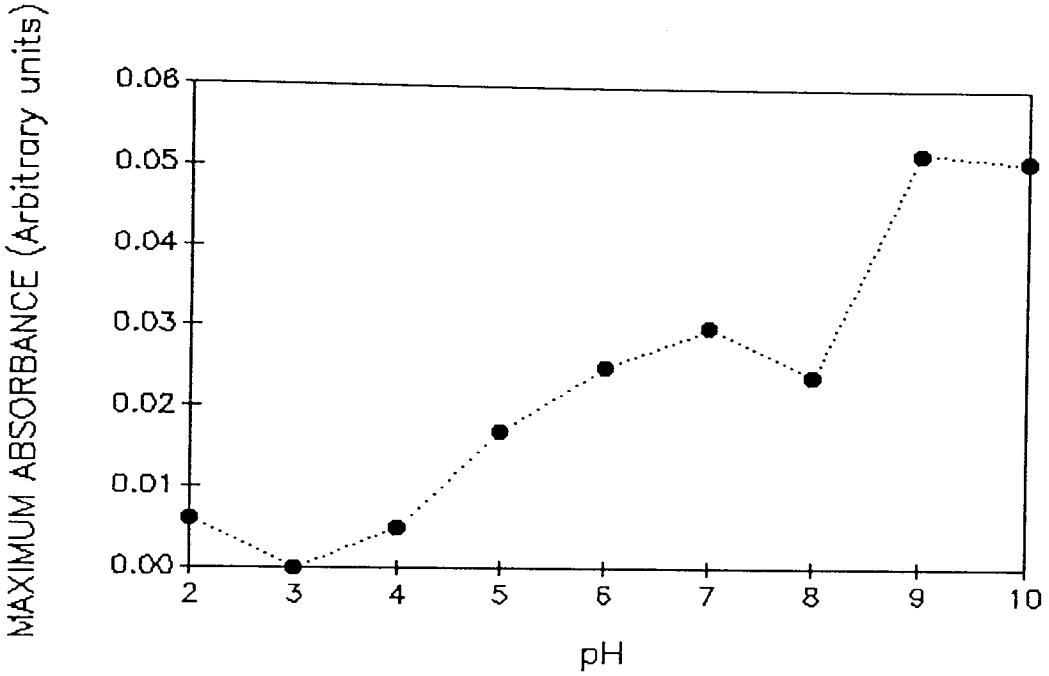
The finding of the striking correlation between pH and the efficiency of the extraction of the fimbrial protein is presumed to reflect the influence pH has on the stability of the K88 fimbrial structure. Gross pH-induced changes in the stability of the K88 fimbrial structure are likely to play a role in the pathogenesis of ETEC expressing K88 fimbriae. This is apparent since the mean pH from stomach to the lower intestine in piglets varies over a range of 3.5-7.2 (Williams-Smith and

Figure 5.8 Effect of buffer composition and temperature on the heat extraction of the K88 fimbrial protein.



The K88 fimbrial protein was extracted from E.coli stain K12:K88ab which had been cultured unshaken at 37°C overnight in nutrient broth. All extractions were performed on aliquots of the same batch cultures. Samples were run on a 12% SDS-PAGE gel and stained with Coomassie blue. Where:- Lane 1=marker proteins (as in Figure 5.1); lane 2=extracted at 40°C; lane 3= extracted at 50°C; lane 4=extracted at 60°C; lane 5=50mM Na_2HPO_4; lane 6=2M urea; 2M urea, 50mMNa_2HPO_4, 0.8M NaCl. All buffers were at pH 7. For more details of the method see Section 2.4.5.

Figure 5.9 Variation in the amount of K88 fimbrial protein detected after heat extraction at various pH values.



The K88ab fimbrial protein was subjected to heat extraction from strain K12:K88ab at various pH. Samples were then separated on a 12% SDS-PAGE gel and the amount of K88 adhesin present determined in a semi-quantitative manner by gel densitometry. The position of the K88 fimbrial protein was confirmed by the simultaneous running of a standard.

Linggood 1971). It can be postulated that at comparatively alkaline pH's, K88 fimbriae binding bacteria to intestinal surfaces are sheared by the force of peristalsis due to the instability of the fimbriae under these conditions. Conversely, acidic pH stabilises the fimbrial structure. It would be expected therefore that pH-related fimbrial stability would promote colonisation at the more acidic sites in the intestine. Infection of pigs by ETEC bearing K88 fimbriae is associated with the colonisation of the whole of the small intestine (Sellwood 1981, Moon 1990). This is unlike ETEC expressing 987P or K99 fimbriae which are associated with the colonisation of only the posterior portion

of the small intestine (Williams-Smith and Huggins 1978). The greater "efficiency" of the K88 adhesin has been previously postulated as responsible for the colonisation of the anterior small intestine where the speed of chyme flow is greatest (Williams-Smith and Huggins 1978). However, the results presented here indicate that enhanced stability of K88 fimbriae at acid pH may be responsible or at least aid colonisation at this site since the average pH of the chyme in the anterior portion of the small intestine of piglets is 5.8 (Williams-Smith and Linggood 1971).

5.6 Conclusions

A combination of heat extraction, ammonium sulphate precipitation and isoelectric precipitation was found to suitable for the large scale preparation of purified K88ab adhesin protein. By SDS-PAGE a single homogeneous band was noted with an apparent molecular weight of 27.5KDal. The results of haemagglutination studies were consistent with the known agglutination pattern of the K88ab adhesin. Antiserum raised against the putative K88ab adhesin recognised both it and only those strains of *E.coli* capable of expressing the K88 adhesin. Control αK88 antiserum was found to recognise the isolated purified putative K88 adhesin. By ELISA both the αK88ab antiserum raised in this study and the control αK88 antiserum were found to have similar titres while non-immune serum did not recognise the isolated putative K88ab adhesin. In summary, >100mg of purified K88ab (single band by SDS-PAGE and Coomassie blue staining) was obtained and used to produce mono-specific αK88ab antiserum. Various characteristics and tests of the purified K88ab adhesin and its corresponding antiserum confirmed their identity and specificity respectively.

Adaptation of the large-scale method for the production of purified K88 adhesin led to the development of a small-scale version. This latter method was

comparatively rapid and was shown to be capable of extracting all three serotypes of the K88 adhesin with a high degree of purity. Investigation of the mechanism of the small-scale method led to the discovery of the pH-related stability of K88 fimbriae. It was postulated that the comparative stability of K88 fimbriae at acidic pH contributes to the characteristic association of $K88^+$ ETEC with the colonisation of the anterior small intestine.

6 HAEMAGGLUTINATION PROPERTIES OF THE K88 ADHESIN.

6.1 Introduction.

Haemagglutination is an easily assayable characteristic of many bacterial adhesins (Jones and Isaacson 1983). To agglutinate erythrocytes, adhesins can either be cell-free or cell-bound providing that they can bind to several neighbouring erythrocytes (Jones and Isaacson 1983). The exact nature of the driving force behind the haemagglutination mechanism is unknown although low ionic strength favours the reaction suggesting that electrostatic forces are important (Lindahl *et al.* 1987). The introduction of inhibitors into adhesin/ erythrocyte systems has provided information on the complex interactions which are occurring. In the case of several *E.coli* adhesins, information gained through haemagglutination experiments has provided information on their *in vivo* receptors e.g. type 1 (Neeser *et al.* 1986), K99 (Lindahl *et al.* 1987), P (Leffler and Svanborg-Eden 1980) and CFA (Neeser *et al.* 1988). However, despite this it must be emphasised that adhesin receptors present on erythrocytes are analogues which are probably similar rather than identical to the *in vivo* receptor (Moon 1979).

It has become increasingly apparent that the binding of the K88 adhesin to its epithelial cell receptor is more complex than originally thought (Sellwood *et al.* 1975). Studies have revealed that there are multiple porcine enterocyte phenotypes for the adhesion of *E.coli* expressing K88 fimbriae while the K88 receptor(s) present on their surface may be modified by epistatic genes (Bijlsma and Bouw 1985, Rapacz and Hasler-Rapacz 1986). In addition, with the finding of minor fimbrial components in the intact K88 fimbrial structure it has been suggested that both one of these (either FaeC or FaeF), and the major structural subunit (FaeG) have adhesive properties (Oudega *et al.* 1989, De Graaf 1990).

The aims of this Chapter were as follows:-

A It has been suggested that porcine epithelial cells can express up to five major K88 receptor phenotypes (Bijlsma and Bouw 1985). By screening a large number of erythrocyte types and determining the adhesive ability of the three K88 adhesin serotypes information was sought confirming that analogous receptor phenotypes were present on erythrocytes.

B There has been no comprehensive attempt to quantitate the haemagglutinating ability of all three serotypes of the K88 adhesin and erythrocyte species. Earlier experiments (Chapter 4) had indicated that the expression of the K88 adhesin varied according to the *E.coli* strain examined. Information was sought to determine whether the relative expression of cell-bound K88 adhesin correlated with haemagglutinating ability.

C No previous study has detailed how the adhesive ability of cell-bound or free K88 adhesin are related. Several studies on the properties of K88 fimbriae have been based on the adhesion of isolated cell-free adhesin (Kearns and Gibbons 1979, Anderson 1980, Sellwood 1980a). In this study, the comparative haemagglutinating abilities of the two forms of the K88 adhesin were calculated and used to determine whether the adhesive ability of the isolated K88 adhesin reflected that of the intact fimbria.

D A final aim was to confirm and extend information on the haemagglutinating characteristics of the K88 adhesin (Jones 1972, Gibbons *et al.* 1975, Parry and Porter 1978, Sellwood and Kearns 1979). In particular, much of the available information concerned K88-mediated agglutination of guinea pig erythrocytes and not of the agglutination of other erythrocyte species. The temperature stability of the K88 adhesin/chicken erythrocyte reaction suggests that this is more comparable with the natural K88 adhesin/enterocyte system than the guinea pig erythrocyte/K88 adhesin system

(Parry and Porter 1978). Therefore the haemagglutinating characteristics of both chicken and guinea pig were investigated.

6.2 Requirements for haemagglutination.

Very little published information on the mechanism of haemagglutination is available (see Section 6.1). The microhaemagglutination method used in these studies was developed empirically by Jones (1972) as an improvement over the earlier tile agglutination test (Duguid and Gillies 1967) for the detection of the K88 adhesin. Because of the lack of definitive information, the haemagglutination reaction is presumed to be dependant on the following factors:-

A Presence of both adhesin and receptor. This is self explanatory, if either the receptor or adhesin is absent then the erythrocytes have no means of interacting with each other and therefore haemagglutination does not occur.

B Affinity of adhesin\receptor interaction. The affinity of an adhesin for its receptor is analogous to that of an antibody for one determinant of an antigen. It reflects the strength with which the adhesin and receptor interact and is influenced by such factors as the size of binding site, the closeness of fit and the nature and strength of the intermolecular forces (e.g. Van der Waals and hydrophobic forces) concerned. Where many adhesins interact in concert with an erythrocyte (e.g. bacteria expressing many fimbriae) or an adhesin with several binding sites (e.g. cell-free fimbriae) interacts with an erythrocyte the overall binding strength of the bacterial or fimbrial/erythrocyte reaction is important rather than the affinity of individual adhesin/receptor bonds.

C Suitable environment. This should not be confused with factors affecting the affinity of the receptor\adhesin reaction when they have both adopted a

favourable conformation. It does concern chemical or physical properties which may prevent the adoption of complementary molecular structures. For instance, extremes of pH may favour a different structure to that stable at physiological pH. In studies using isolated cell-free adhesins, stabilising additional proteins may be required for the characteristic binding properties of the intact adhesin to be realised. Another example is the need for the presence of ions that affect the conformation of the adhesin, receptor or both.

D Absence of inhibitors. This is in part connected to C above. However, it concerns the prevention of haemagglutination after both adhesin and receptor have adopted complementary conformations. The presence of either adhesin or receptor analogues would effectively interfere with the process of haem-agglutination. If either the concentration or the affinity of the adhesin/ receptor analogue were high enough then no haemagglutination would be apparent. This is the basis of inhibition experiments, where the nature of inhibitors is thought to reflect that of the receptor inhibited. A special case, is inhibition mediated by antisera. Since any inhibition observed by antisera is likely to be attributable to steric hindrance (except in exceptional circumstances), then the properties of the inhibitor do not reflect those of the receptor inhibited.

E Concentration of adhesin and receptor. The microhaemagglutination test is based on the passage of a mixture of erythrocytes and proteins through a diluent under the force of gravity. There is a limited time for interaction between the mixing of reagents and the formation of a pellet of erythrocytes at the bottom of the well as occurs in negative controls. Within this time it is likely that a critical number of erythrocytes would have to be cross-linked to enable the formation of an erythrocyte mat and so prevent pellet formation (as would occur in a positive control). The fulfilment of a critical number of cross-

linked bacteria must be limited by the number of adhesins/receptors present since, below a certain concentration of either or both a sufficient number of cross-links would not be formed.

More details of the postulated mechanism of the microhaemagglutination mechanism is given in Figures 2.4-2.6.

6.3 Investigation of the ability of the K88 adhesin to agglutinate various erythrocyte types.

6.3.1 Interpretation of the haemagglutination results

The majority of fimbriae expressed by *E.coli* consist of a major subunit protein (usually referred to as the structural subunit) which forms the "backbone" of the fimbrial structure. At intervals along the length and\or at the tips of fimbriae are minor protein subunits (Krogfelt 1991). In several cases it has been found that one of the minor proteins acts as the adhesin and not the major structural protein (Lund *et al.* 1987, Schmoll *et al.* 1989, Krogfelt *et al.* 1990). Providing that only one adhesin is involved, haemagglutination reactions can provide valuable information on the nature of the receptor e.g. the nature of the receptor for P fimbriae was first indicated by its specific reaction with the Pk antigen present on human erythrocytes (Kallenius *et al.* 1980). As in other *E.coli* fimbrial systems it has become apparent that the intact K88 fimbria contains not only the major structural component, FaeG, but also the minor protein components FaeC, FaeF and FaeH (Van Zijderveld 1990). Unlike other systems, however, it has been reported that two of the proteins present in K88 fimbriae have adhesive properties. In particular, FaeG is thought to be responsible for the agglutination of guinea-pig erythrocytes (Jacobs *et al.* 1987b,c) while it has also been reported that FaeC or FaeF is responsible for

the agglutination of chicken erythrocytes (De Graaf 1990).

Strong evidence against the existence of two adhesins being present in the K88 fimbrial structure was presented by Jacobs *et al.* (1987a). These workers reported that two tripeptides isolated by enzymatic digestion of FaeG were able to inhibit the agglutination of both guinea pig and chicken erythrocytes by isolated K88 fimbriae. This contradicts the idea that independent adhesins are responsible for the agglutination of guinea pig and chicken erythrocytes since it seems unlikely that a tripeptide isolated from one adhesin would inhibit agglutination by the second adhesin. If two adhesins were present in the isolated K88 fimbriae, one responsible for the agglutination of guinea pig and the other chicken erythrocytes, then a possible explanation of the ability of these isolated tripeptides to inhibit both is that the receptor binding sites of the adhesins were extremely similar. If this were so, then the results of Jacobs *et al.* (1987a) clearly demonstrate that from a functional viewpoint there is no difference between the two adhesins present. This is a circular argument since if there is no functional difference between the adhesins then they would both agglutinate the same erythrocyte species. Therefore, although there is some controversy over the number and identity of the K88 adhesin, and in the absence of direct experimental evidence to the contrary, the analysis of the data presented in this study is based on the assumption that FaeG is the only adhesin present in the K88 fimbria (Oudega *et al.* 1989).

6.3.2 Previous information on erythrocyte species agglutinated by the K88 adhesin.

Several studies have investigated the haemagglutinating ability of the K88 fimbrial adhesin antigen (Jones 1972, Jones and Rutter 1974, Parry and Porter 1978, Bijlsma *et al.* 1985, Jacobs *et al.* 1987c and Cox and Houvenaghel 1987). The reported ability of the K88 adhesin protein to agglutinate

erythrocyte types reported in these studies is summarised in Table 6.1. In addition, Table 6.1 gives information on the method of assay used in previous studies and the methods used for the preparation of the cell-free K88 adhesin.

As outlined in Table 6.1 previous results obtained with all three K88 serotypes, whether cell-bound or cell-free, have been identical. Similarly, the results obtained with guinea-pig and chicken erythrocytes have been in complete agreement. However, the results obtained with porcine erythrocytes have been variable, a finding which is attributable to the inconsistent ability of the K88ab adhesin to agglutinate porcine erythrocytes. Cox and Houvenaghel (1987) noted that the agglutination of porcine erythrocytes by the K88ab adhesin was dependant on whether cultures were used directly for haemagglutination or whether the bacteria were first washed. In the former case, agglutination was not apparent whereas in the latter it was. The authors did not offer an explanation for the observed difference although the results they obtained suggested that a component of the media used (tryptone soy broth or agar) was responsible (Cox and Houvenaghel 1987). In agreement with this explanation, Jones and Rutter (1974) found that nutrient broth inhibited haemagglutination by the K88 adhesin. However, Parry and Porter (1978) found that cell-free K88ab adhesin did not agglutinate porcine erythrocytes despite being suspended in phosphate buffered saline. An alternative explanation was suggested by Bijlsma *et al.* 1985 who observed that the haemagglutination tests of Parry and Porter (1978) were carried out at room temperature while their tests were carried out at 0°C. Cox and Houvenaghel (1983) who also reported that porcine erythrocytes were agglutinated by the K88ab adhesin performed their haemagglutination studies at 4°C. Evidence in support of the temperature sensitivity of the agglutination of porcine erythrocytes by the K88ab adhesin is presented in Section 6.4.

Table 6.1 Reported haemagglutination profiles of K88 adhesins.

ERYTHROCYTE TYPE	FORM OF K88 ADHESIN						FORM OF ASSAY	METHOD OF PREPARING CELL FREE ADHESIN	REFERENCE
	CELL FREE			CELL BOUND					
	ab	ac	ad	ab	ac	ad			
PORCINE	+	-	+	+	-	+	Microhaemagglutination	Agar grown, sheared, ammonium sulphate precipitation.	Bijlsma *et al.* 1985
BOVINE	-	-	-	-	-	-			
GUINEA-PIG	+	+	+	+	+	+			
CHICKEN	+	-	-	+	-	-			
OVINE	-	-	-	-	-	-			
EQUINE	-	-	-	-	-	-			
GUINEA PIG	N/D	N/D	N/D	+	+	N/D	Microhaemagglutination	Agar grown, sheared, isoelectric precipitation, centrifugation	Jones and Rutter 1974
* CHICKEN	N/D	N/D	N/D	+	+	N/D			
GUINEA-PIG	+	+	+	N/D	N/D	N/D	Microhaemagglutination	Broth grown, sonicated extracts no further purification	Jacobs *et al.* 1987c
CHICKEN	+	-	-	N/D	N/D	N/D			
PORCINE	-	-	+	N/D	N/D	N/D			
PORCINE	N/D	N/D	N/D	+/-	-	+	glass tube	N/A	Cox and Houvenaghel 1987
CHICKEN	+	-	N/D	+	-	N/D	Microhaemagglutination	Agar grown, ab heat extracted, ac sheared then purified by centrifugation and isoelectric precipitation	Parry and Porter 1978
PIGEON	+	-	N/D	+	-	N/D			
PORCINE	-	-	N/D	-	-	N/D			
GUINEA-PIG	+	+	N/D	+	+	N/D			

Where:- N/D= Not determined; N/A= Not applicable;= the given results were obtained in 2 out of 6 samples of erythrocytes tested, the remaining 4 samples were not agglutinated by the K88 adhesin; +/-= the result obtained depended on whether the bacterial culture was washed or not before assay respectively; the microhaemagglutination assay developed by Jones and Rutter (1972) was used in this study.*

6.3.3 Erythrocyte species agglutinated by the K88 adhesin in this study.

Preliminary experiments with guinea-pig and chicken erythrocytes indicated that the haemagglutination titre observed with cell-free K88 adhesin and presumably cell-bound adhesin was related to the concentration of erythrocytes used. It was found that the higher the concentration of erythrocytes then the greater was the observed haemagglutination titre. This finding was thought to be attributable to the greater number of cross-links required to form a stable erythrocyte mat at higher erythrocyte concentrations. Since the size of erythrocytes varies between species, preliminary experiments were carried out to determine the erythrocyte concentrations which resulted in similar-sized pellets in negative controls (for concentrations used see Section 2.8.4). This was regarded as a more appropriate method of standardisation than erythrocyte concentration since distinct pellets were then observed with all erythrocyte types and results were more clear-cut.

The results obtained in this present study are given in Table 6.2. Of the 11 erythrocyte types tested 5 were agglutinated in the presence of two or more of the three K88 serotypes. Each serotype of cell-free K88 adhesin agglutinated at least three of the erythrocyte types. Since agglutination requires the adhesin structure to have several binding sites (Jones and Isaacson 1983) it seems possible that intact or at least partially intact fimbriae were present. This is despite the fact that fimbrial structures were not apparent when both large and small scale preparations of the K88 adhesin were examined by electron microscopy. The results obtained with guinea-pig erythrocytes are in complete agreement with those obtained previously (see Table 6.1) and emphasise the ability of guinea-pig erythrocytes to bind all three serotypes of the K88 fimbrial adhesin. The results obtained with chicken erythrocytes are in agreement with

Table 6.2 Observed haemagglutination titres of various erythrocyte species.

ERYTHROCYTE TYPE	NUMBER EXAMINED	HAEMAGGLUTINATION TITRE							
		CELL FREE ADHESIN (ng/ml)				CELL BOUND ADHESIN ($x10^7$)			
		£ K88ab	* K88ab	* K88ac	* K88ad	K88ab	K88ab	K88ac	K88ad
GUINEA PIG	2	100	720	4,300	1,840	7.9	6.3	50	6.3
CHICKEN	1	390	720	4,300	>59,000	7.9	6.3	25	>100
RABBIT	5	25-50	360	540	230	7.9	6.3	6.3	3.2
MARMOSET	1	3,125	5,750	>34,000	>59,000	3.2	3.2	6.3	6.3
PIG	3	390-1,560	11,500	4,300-8,500	920	13-25	13-25	>1,000	32-63

*Haemagglutination titres (HA) were determined by the microhaemagglutination assay in the presence of 0.5% w/v D-mannose. Erythrocyte concentrations were adjusted to give a similar sized pellet in negative controls. All titres were determined at 0-4°C. Other erythrocyte types examined with no detectable haemagglutination were:- RAT(1), MOUSE(1), MONKEY(1), OVINE(2), EQUINE(2) and BOVINE(2). Numbers in brackets indicates number of samples examined. Based on a K88 fimbrial molecular weight of $1x10^6$ Daltons (Jones 1977) it can be calculated that a minimum of between 30 and 6,900 fimbriae bind to each erythrocyte for agglutination to occur with cell-free adhesin while for the cell-bound adhesin between 2 and 32 erythrocytes are bound by each bacteria. Where:- *= Total protein concentration in small-scale extracts was determined. The purity of these extracts was such (see Figure 5.6) that this was regarded as representative of the concentration of K88 adhesin itself present. £=The adhesin here was extracted and purified from E.coli strain K12:K88ab as outlined in Section 2.4.*

those obtained previously with K88 adhesin of serotypes K88ab and K88ad. However, it was consistently noted that *E.coli* strain O8:K87:K88ac:H19 and the adhesin protein extracted from it agglutinated chicken erythrocytes in agreement with Jones and Rutter (1974) but in disagreement with more recent findings (see Table 6.1). A possible reason for this disagreement is the variability in properties of the K88ac adhesin protein. For instance, there are several minor serological types of the K88ac adhesin (Smyth 1986). In addition it has been reported that there are anodic and cathodic forms of the K88ac adhesin demonstrable by their direction of migration during immuno-electrophoresis through noble agar (Guinee and Jansen 1979). A functional difference between anodic and cathodic forms of K88ac adhesins was reported in porcine brush border K88 receptor blocking experiments by Bijlsma *et al.* (1982). Thus it may be that agglutination of chicken erythrocytes by the K88ac adhesin is dependent on the exact source and nature of the K88ac adhesin concerned.

The results obtained with porcine erythrocytes and cell-bound K88 adhesin are in complete agreement with Bijlsma *et al.* (1985) and Cox and Houvenaghel (1987). Unlike Parry and Porter (1978), Bijlsma *et al.* (1985) and Jacobs *et al.* (1987c) it was noted that cell free K88ac adhesin was able to agglutinate porcine erythrocytes. A possible explanation here was the method of preparation of the cell-free K88ac adhesin. All former workers have used homogenisation as the basis of their extraction of the K88 adhesin. Heat extraction was the basic method used in this study for the preparation of the K88ac adhesin and it may be that homogenisation denatures the K88ac adhesin or alters its conformation in such a way that it can no longer bind porcine erythrocytes effectively.

The finding that marmoset and rabbit erythrocytes are agglutinated by the K88 adhesin has not been noted previously. Both erythrocyte types are

agglutinated by all three serotypes of the cell bound K88 adhesin. In this respect they resemble guinea pig erythrocytes, however, unlike guinea pig erythrocytes, marmoset and rabbit erythrocytes show roughly equal haem-agglutination titres with the three serotypes of cell-bound K88 adhesin. With cell-free K88 adhesin, rabbit erythrocytes show a low haemagglutination titre with all three K88 serotypes (range 25-540ng/ml). Marmoset erythrocytes on the other hand show high haemagglutination titres (range 3,125-5750ng/ml) towards cell-free K88ab adhesin, and are not agglutinated by cell-free K88ac or K88ad adhesin.

Finally, it should be noted that the erythrocyte receptor(s) for the K88 adhesin may not be an immutable component of the erythrocyte surface of a particular species. Variability in the composition of erythrocytes may be influenced by the age and strain of the donor animal possibly explaining the differing K88 adhesin-mediated haemagglutination profiles reported in this and other studies. Indeed, in agreement with Parry and Porter (1978), the haemagglutination titres observed with guinea-pig erythrocytes were variable (see Table 6.3). Variation in the haemagglutinating ability of chicken erythrocytes has also be noted previously (Jones and Rutter 1974). Therefore, analysis of the haemagglutinating abilities of the K88 adhesin should allow for the effect of intraspecies variability and age of the donor animal on the composition of erythrocytes used.

Table 6.3 Variability in haemagglutination titre of *E.coli* serotype K12:K88ab and guinea pig erythrocytes.

	ERYTHROCYTE SAMPLE NUMBER					
HAEMAGGLUTINATION TITRE ($x10^7$)	1	2	3	4	5	6
	7.9	7.9	23.0	23.0	23.0	12.0

6.3.4 K88 adhesin-binding phenotypes: evidence for the action of two separate genetic elements.

Using the three known K88 serotypes, either 4 or 5 adhesion phenotypes have been demonstrated to be present on porcine enterocytes (Bijlsma and Bouw 1985, Rapacz and Hasler-Rapacz 1986, see also Table 1.5). Depending on the adhesion phenotype, none, one, two or all three of the K88 serotypes bind to isolated porcine enterocytes (brush borders). To account for the observed multiplicity of K88-receptor phenotypes Rapacz and Hasler-Rapacz (1986) suggested that the K88 receptor(s) were specified by either two or three closely linked genetic loci or alternatively, alleles. K88 receptors corresponding to these two or three genetic loci/alleles would be found on the surface of enterocytes. Segregation data obtained by Rapacz and Hasler-Rapacz (1986) was in agreement with the hypothesis that there are three separate K88 receptors each recognising a different adhesin serotype. Furthermore this data indicated that the receptor genes for the K88ab and K88ac adhesins were very closely linked while that for the K88ad adhesin was more distant and possibly separate. Based primarily on phenotypic segregation data Bijlsma and Bouw (1985) assumed that the K88 receptor(s) was coded for at two genetic loci. The first locus coded for the receptor responsible for the binding of the K88ab and K88ac adhesins while the second coded for the K88ad adhesin receptor. Two allelic forms of the gene at each loci were thought to code for receptor expression or not. In addition to segregation data, Bijlsma and Bouw (1985) argued that K88 receptor blocking experiments (Bijlsma *et al.* 1982) indicated that receptor sites for the K88ab and K88ac adhesins were more similar (if not identical) than the receptor sites for the K88ad adhesin.

One simple method of determining the number of types of K88 receptor and by inferrment the number of controlling genetic elements is by finding the

number of receptor phenotypes (Rapacz and Hasler-Rapacz 1986). With two independent elements the maximum number of phenotypes is 4 while with three independent elements the maximum number is eight. At first hand, the finding of five different receptor phenotypes by Bijlsma and Bouw (1985) could be regarded as indicating that three independent elements control the expression of the K88 receptor. However, in 343 out of 345 pigs Rapacz and Hasler-Rapacz (1986) found only four K88 receptor phenotypes, none showing segregation between the adhesion of the K88ab and K88ac adhesins. The K88 receptor phenotype found by Bijlsma and Bouw (1985) but not Rapacz and Hasler-Rapacz (1986) showed adhesion by the K88ab and K88ad adhesins but not the K88ac adhesins. An intriguing finding in this present study was the cryptic ability of porcine erythrocytes to bind the K88ac adhesin (see Table 6.2). It was found that although *E.coli* serotype O8:K87:K88ac:H19 did not agglutinate porcine erythrocytes, the cell-free adhesin isolated from it did. An analogous situation in the binding of the K88 adhesin to porcine enterocytes would explain the finding of an "extra" K88 receptor phenotype by Bijlsma and Bouw (1985) who used cell bound adhesin in their studies. With none of the five erythrocyte species that the K88 adhesin was found to agglutinate was there any evidence of the complete separation of the receptor(s) for the K88ab and the K88ac adhesins. However, the results obtained with chicken erythrocytes (not agglutinated by either cell-free or bound K88ad adhesin) do indicate a separation between the receptor(s) for K88ab/K88ac and the receptor(s) for the K88ad adhesin. Thus the data from haemagglutination studies is in agreement with the hypothesis that the K88 receptor responsible for the binding of the K88 adhesin is composed of two elements. The first binds the K88ab and K88ac adhesins while the second is bound by the K88ad adhesin. The simplest explanation for the genetic basis of this dichotomy is that, as first suggested by Bijlsma and Bouw (1985), the K88 receptor is coded for at two genetic loci.

It should be noted that the results of receptor blocking experiments (Bijlsma et al. 1982, Bijlsma and Bouw 1985) indicate that despite the genetic separation of the elements of the K88 receptor, their final products are intimately associated.

6.3.5 K88 adhesin-binding phenotypes: evidence for the action of epistatic genes.

During their investigation of the binding of the K88 adhesin, Bijlsma and Bouw (1985) found that in addition to 5 major K88 receptor phenotypes there was evidence for the existence of several minor phenotypes. The authors postulated that epistatic genes could influence the expression of the K88 receptor(s). Strictly speaking, epistatic genes prevent the expression of other genes (Suzuki *et al.* 1981). However, the minor phenotypes noted by Bijlsma and Bouw (1985) where characterised by the weak adhesion of certain K88 adhesin serotypes rather than their complete elimination. Thus modifier genes where the effect of a gene is "diluted" rather than prevented may affect the expression of the K88 receptor(s) on porcine enterocytes (Suzuki *et al.* 1981).

Comparison of the haemagglutination titres obtained with erythrocyte species (guinea pig, rabbit, marmoset and pig) with the potential of recognising all three serotypes of the K88 adhesin reveals that there is considerable variation in the haemagglutination titres observed. For instance rabbit and marmoset erythrocytes are all agglutinated by a minimum of between 3.2-7.9x10^7 bacteria regardless of adhesin serotype. The minimal bacterial concentration for the agglutination of guinea pig by the cell-bound K88ab adhesins and the K88ad adhesin also fall within a similar range. However, the minimum bacterial concentration of serotype O8:K87: K88ac:H19 for the agglutination of guinea pig erythrocytes is 50x10^7 while that for the agglutination of pig erythrocytes varies from 13-63x10^7 for the cell-bound

K88ab and K88ad adhesins to not at all by the cell-bound K88ac adhesin. Such variation of haemagglutination titres is reminiscent of the minor K88 receptor phenotypes present on enterocytes reported by Bijlsma and Bouw (1985). It is interesting to speculate whether the same modifier genes that affect the expression of the K88 receptor(s) on porcine enterocytes also affect the expression of K88 receptor analogues present on erythrocytes.

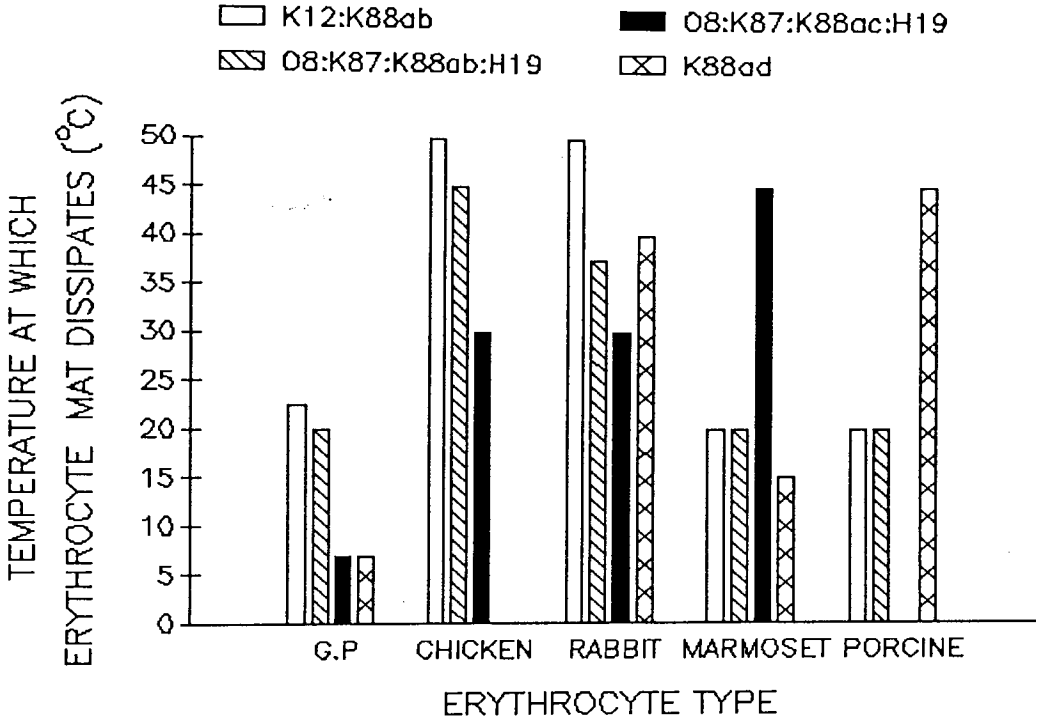
6.4 Comparison of the haemagglutinating properties of cell-free and cell-bound K88 adhesin.

The results presented in Table 6.2 reveal that for guinea-pig, chicken and rabbit erythrocytes the haemagglutination titres obtained with cell-free adhesin reflect those obtained with cell-bound K88 adhesin. For instance, the haemagglutination titres observed with both the cell-free (390 and 720ng/ml) and cell-bound (7.9 and $6.3x10^7$ respectively) K88ab adhesins are roughly equivalent with chicken erythrocytes. The increased haemagglutination titre with cell-free K88ac adhesin and chicken erythrocytes (4,300 ng/ml) is also reflected by a comparable increase in the haemagglutination titre observed with cell-bound K88ac adhesin ($25x10^7$). In addition, neither the cell-free or the cell-bound forms of the K88ad adhesin were found able to agglutinate chicken erythrocytes. Despite this similarity (between adhesin forms) in haemagglutination titres with guinea pig, chicken and rabbit erythrocytes, the temperature stability (see Figures 6.1 and 6.2) of adhesin/receptor complexes formed with these erythrocytes do suggest differences. In general, the temperature stability of cell-bound adhesin/erythrocyte mats was greater or equal to those observed with cell-free K88 adhesin. For instance, the adhesin/erythrocyte complex formed by bacteria of serotype K12:K88ab and chicken erythrocytes was stable at 50°C while the adhesin/erythrocyte complex formed by the adhesin isolated from the same bacterial strain was only stable

up to 25°C. Further evidence of the differing properties of the K88 adhesin depending on its form is given by the haemagglutination titres and temperature stability results obtained with porcine and marmoset erythrocytes. In the case of marmoset erythrocytes, the change in adhesin form from cell-bound to cell-free results in the abolition of the ability of the K88ac and K88ad adhesins to haemagglutinate. Even the haemagglutinating ability of cell-free K88ab adhesin is much reduced with marmoset erythrocytes when compared to the cell-bound forms of the adhesin. For instance the haemagglutination titre with marmoset erythrocytes and *E.coli* serotype O8:K87:K88ab:H19 bacteria was $3.2x10^7$ which was half of the haemagglutination titre ($6.3x10^7$) observed with chicken erythrocytes and the same bacterial serotype. However, with the K88ab adhesin isolated from serotype O8:K87:K88ab:H19 the haemagglutination titre with marmoset erythrocytes was 5,750ng/ml while that observed with chicken erythrocytes was 720ng/ml. Such changes in the observed haemagglutination titres is thought to indicate a conformational change in the K88 adhesin structure when removed from the bacterial surface. In the case of the agglutination of marmoset erythrocytes this conformational change results in a decrease not only in the haemagglutination titre but also a decrease in the stability of adhesin/ erythrocyte mats indicated by their reduced temperature stability (see Figures 6.1 and 6.2).

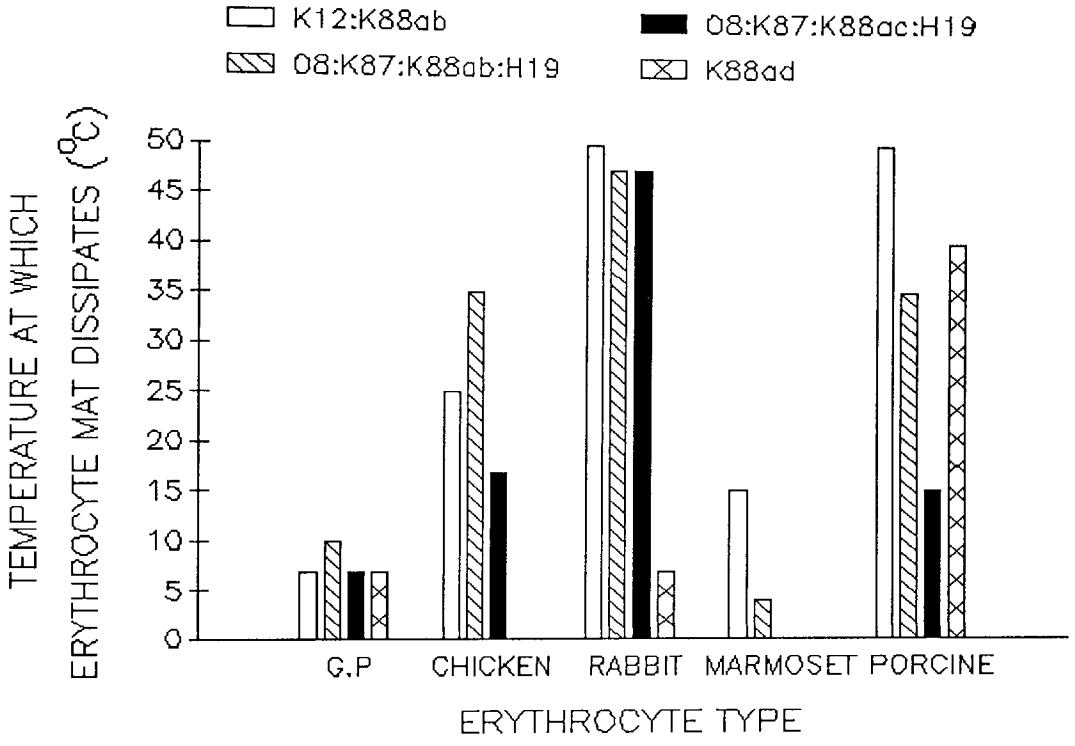
In contrast to results obtained with marmoset erythrocytes, those with porcine erythrocytes indicate that cell-free K88 adhesin has a greater affinity for the K88 receptor analogue present than the cell-bound form. In particular, porcine erythrocytes were not agglutinated by the highest concentration of serotype O8:K87:K88ac:H19 used ($1,000x10^7$) but were agglutinated by the cell-free adhesin isolated from this strain (haemagglutination titre=4,300-8,500ng/ml). Temperature stability results emphasise this finding since they show that particularly for the K88ab and K88ac adhesins there is a marked

Figure 6.1 Temperature stability of erythrocytes cross-linked by various serotypes of cell-bound K88 adhesin.



During haemagglutination, a number of cross-links are formed between bacteria (in this case with the aid of cell-bound K88 fimbriae) and the erythrocytes they react with. The formation of bacterial/erythrocyte complexes results in a mat of erythrocytes across individual wells of round-bottomed microplates. Increasing the temperature at which this bacterial/erythrocyte complex is incubated, increases thermal agitation and weakens the cross-links. If the temperature is raised further, sufficient numbers of the cross-links between the bacteria and erythrocytes are disrupted and a pellet is formed in the bottom of the microplate well. The above bar chart demonstrates the maximum temperature at which the bacterial/erythrocyte complex is stable. In the majority of cases when the maximum temperature is exceeded the erythrocytes immediately pellet. In two cases however (E.coli strains O8:K87:K88ac:H19 and K88ad with rabbit erythrocytes), disruption of the mats occurred gradually over a 10°C range. Bacteria/erythrocyte mats could be incubated at 50°C for 15min, remixed and incubated on ice for pellet formation three times without affecting the temperature stability of the subsequent mats formed. All experiments were performed in triplicate.

Figure 6.2 Temperature stability of erythrocytes cross-linked by various serotypes of cell-free K88 adhesin.



The formation of cell-free adhesin\erythrocyte complexes occurs in an analogous fashion to cell-bound adhesin\erythrocyte complexes (see Figure 6.1). The above bar chart indicates the maximum temperature at which cell-free adhesin\erythrocyte mats are stable. On reaching a critical temperature the mats disperse and form pellets at the bottom of microplate wells. In contrast to cell-bound adhesin\erythrocytes mats, those formed by cell-free adhesin were not resistant to heating to 50°C. In fact, after two incubations at 50°C for 15min none of the erythrocyte/cell-free adhesin combinations would re-agglutinate. This suggests that cell-free K88 adhesin is more susceptible to denaturation than the cell-bound adhesin. All experiments were performed in triplicate.

increase in the stability of cell-free adhesin/erythrocyte complexes when compared to the cell-bound equivalent. The comparatively poor temperature stability of porcine erythrocytes agglutinated by cell-bound K88ab adhesin may explain the inability of Parry and Porter (1978) to detect haemagglutination by this adhesin since their experiments were carried out at room temperature.

6.5 Relationship of K88 fimbrial expression and haem-agglutination.

It has been suggested previously that the adhesive capacity of a bacterium is related to the number of fimbriae expressed (Isaacson 1980, Van Verseveld *et al.* 1985). The quantification of the expression of the K88 adhesin by the strains used in this study was presented in Chapter 4. Table 6.4 gives the results obtained for *E.coli* cultured unshaken at 37°C for 16hr in nutrient broth as used in haemagglutination studies.

If the adhesive capacity of K88-expressing bacteria was solely dependant on the amount of K88 adhesin expressed then it would be expected for *E.coli* of serotypes K12:K88ab and 08:K87:K88ab:H19 to show equal adherence which was greater than that of serotypes O8:K87:K88ac:H19 and K88ad. In fact most of the haemagglutination titres and temperature stability results indicate that there is a rough correlation between observed adhesiveness and the amount of K88 expressed. For instance, the haemagglutination titre and

Table 6.4 Expression of the K88 fimbrial protein by *E.coli* strains examined by haemagglutination.

	E.coli SEROTYPE			
	K12:K88ab	O8:K87:K88ab:H19	O8:K87:K88ac:H19	K88ad
K88 DETECTED (g/cfu x 10^{-14})	**27.0**	**33.6**	**1.5**	**2.0**

temperature stability of cell-bound K88ab adhesin whether expressed by serotype K12:K88ab or O8:K87:K88ab:H19 are similar. In addition for all five erythrocyte types, the haemagglutination titres for the K88ab serotypes are either similar or less than the K88ac serotype, while for four of the five serotypes the temperature stability is higher. However, comparisons with serotype K88ad are more difficult since, for instance with rabbit erythrocytes, despite expressing only $2x10^{-14}g$ of K88 adhesin/cfu compared to the $33.5x10^{-14}g$/cfu expressed by serotype O8:K87:K88ab:H19, the haemagglutination titre of serotype K88ad is half that of O8:K87:K88ab:H19. Similar results were achieved with guinea-pig, marmoset and porcine erythrocytes and suggest that the binding characteristics of the K88ad adhesin are significantly different to K88ab and K88ac. Haemagglutination inhibition experiments have previously shown that differences in K88 receptor molecules exist in particular between the receptor for K88ad and that of K88ac and K88ab (Jacobs *et al.* 1987c) and the results obtained here are further evidence of these differences.

6.6 Characterisation of the K88 adhesin\receptor interaction during the agglutination of erythrocytes.

6.6.1 Effect of pH on the agglutination of guinea pig and chicken erythrocytes by the K88 adhesin.

Infection by ETEC expressing K88 fimbriae is associated with colonisation throughout the whole small intestine of the pig (Williams-Smith and Huggins 1978). Obviously this implies that the K88 receptor/adhesin interaction is stable over the pH range encountered (pH 5.1-7.7) in the small intestine of the pig (Williams-Smith and Jones 1963). Jones (1972) reported that the haemagglutination of guinea-pig erythrocytes by bacterial suspensions expressing the K88 adhesin was equivalent between pH 5.6-8.0. However, the titres observed were four-fold less at pH <5.6 and completely abolished at a

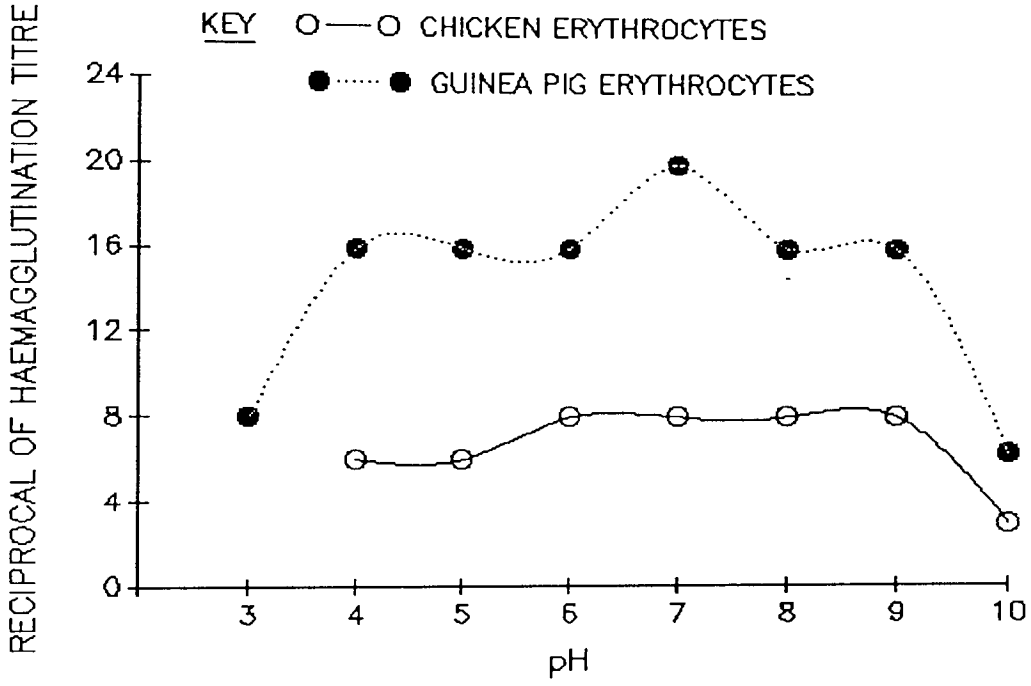
pH of 8.0 or greater. A more recent study indicated that the binding of radiolabelled K88 adhesin to porcine brush borders occurred over a wide pH range although the observed binding decreased markedly below pH 5 and above pH 8 (Sellwood and Kearns 1979). In agreement with Jones (1972) this present study reports that not only do bacteria expressing K88 fimbriae bind over a wide pH range to guinea-pig erythrocytes but that this also applies to chicken erythrocytes (see Figure 6.3). In addition, this binding has similar characteristics to the binding of cell-free K88 adhesin as reported by Sellwood and Kearns (1979). The wide pH activity of the K88 adhesin/receptor reported here compares with a pH maxima for K99 and F41 adhesins with mucin of between 4.75 and 5.25 with values being 4-fold less at <pH3 and >pH6 (Mouricout and Julien 1987). Earlier data (see Section 5.5.2) had indicated that there was a marked correlation between pH and the stability of the K88 fimbrial structure as a whole. A possible speculation is that the combined effect of pH on fimbrial stability and adhesin activity may explain or at least influence the distribution of bacteria during the infection of pigs by ETEC expressing the K88 adhesin.

The similarity in the pH profiles of brush borders binding the K88 adhesin and erythrocytes binding cell-bound K88 adhesin is regarded as further evidence of the similarity between the K88 receptor(s) present on porcine epithelial cells and guinea-pig and chicken erythrocytes.

6.6.2 Inhibition of the agglutination of guinea pig, chicken and rabbit erythrocytes by the K88ab adhesin with specific antisera.

These experiments were performed to demonstrate that the K88 adhesin was responsible for the observed agglutination of erythrocytes. They were based on the assumption that specific αK88 antiserum would inhibit haemagglutination mediated by the K88 adhesin. It has previously been

Figure 6.3 The effect of pH on the binding of cell-bound K88 adhesin to chicken and guinea pig erythrocytes.



The haemagglutination titre was determined as the highest dilution of stock bacterial suspension ($3.8x10^9$) that formed a complete mat of erythrocytes on the microwell plate bottom. To minimise any possible generalised deterioration in erythrocyte structure at pH extremes, suspensions were made from stock directly before use. However, storage of the erythrocytes overnight at 4°C did not reveal any detectable (by light microscopy) damage of guinea-pig erythrocytes although there was slight increase in the amount of cell lysis at acid pH. Chicken erythrocytes were similar except that at pH 3 they spontaneously formed a gel-like consistency and could not be used. In a trial experiment the pH of the bacterial/erythrocyte mixture was checked by spotting it on to litmus paper. The result given for guinea-pig erythrocytes is the mean ± standard error of four samples while that of hen erythrocytes is of a single chicken erythrocyte sample. All results were carried out in duplicate.

Table 6.5 Inhibition of the agglutination of various erythrocyte species by K88ab adhesin using αK88ab antiserum.

ANTISERUM SPECIFICITY	ERYTHROCYTE TYPE					
	GUINEA PIG	CHICKEN	RABBIT	GUINEA PIG	CHICKEN	RABBIT
K88ab	128,000	800	256,000	400	200	200
NRS	640	40	640	20	<20	<20

The above table gives the haemagglutination inhibition titres obtained with αK88ab serum and normal rabbit serum (NRS). The shaded area represents the values obtained with purified cell-free adhesin prepared from E.coli serotype K12:K88ab while the unshaded area represents the values obtained with cell-bound adhesin of the same serotype. For further details of the specificity of the αK88 antiserum see Chapter 5.

demonstrated that the agglutination of guinea-pig erythrocytes by both cell-free and cell-bound K88 adhesin was inhibited by specific antisera (Jones 1972). In addition Parry and Porter (1978) demonstrated that the agglutination of chicken erythrocytes by cell-free K88ab adhesin was inhibited by K88ab and K88b specific antisera. The results given in Table 6.5 show that mono-specific αK88ab serum inhibits the haemagglutination of chicken, guinea pig and rabbit erythrocyte species by both cell-free purified adhesin and cell-bound adhesin. In agreement with Parry and Porter (1978), αK88b antibodies specifically prevent the haemagglutination of chicken, guinea-pig and rabbit erythrocytes by the homologous cell-bound adhesin only (see Table 6.6). It should be stressed that anti-active site antibodies are important for binding inhibition since it has been shown that anti-structural unit monoclonal antibodies do not inhibit haemagglutination (Moch *et al.* 1987).

Variation in the haemagglutination inhibition titres observed with cell-

free adhesin is likely to be attributable to the method of assay. In particular, 8x the haemagglutination titre of the cell-free adhesin was used to agglutinate the erythrocytes. Since the haemagglutination titre for the erythrocyte species varies (see Table 6.2) then in effect the amount of adhesin present that the antiserum blocks also varies. eg. 3,120ng/ml of purified K88 adhesin was used to agglutinate the chicken erythrocytes while only 200ng/ml was used to agglutinate the rabbit erythrocytes.

Table 6.6 Inhibition of the agglutination of erythrocytes by cell-bound K88 adhesin using αK88b antiserum.

CELL-BOUND ADHESIN TYPE	HAEMAGGLUTINATION INHIBITION TITRE		
	GUINEA PIG	CHICKEN	RABBIT
K88ab	>128	>128	>128
K88ac	16	16	16
K88ad	4	N/A	8

Where:- N/A = not applicable, chicken erythrocytes are not agglutinated by the K88ad adhesin.

6.6.3 Inhibitory ability of various carbohydrates on the agglutination of guinea pig and chicken erythrocytes by cell-free K88ab adhesin.

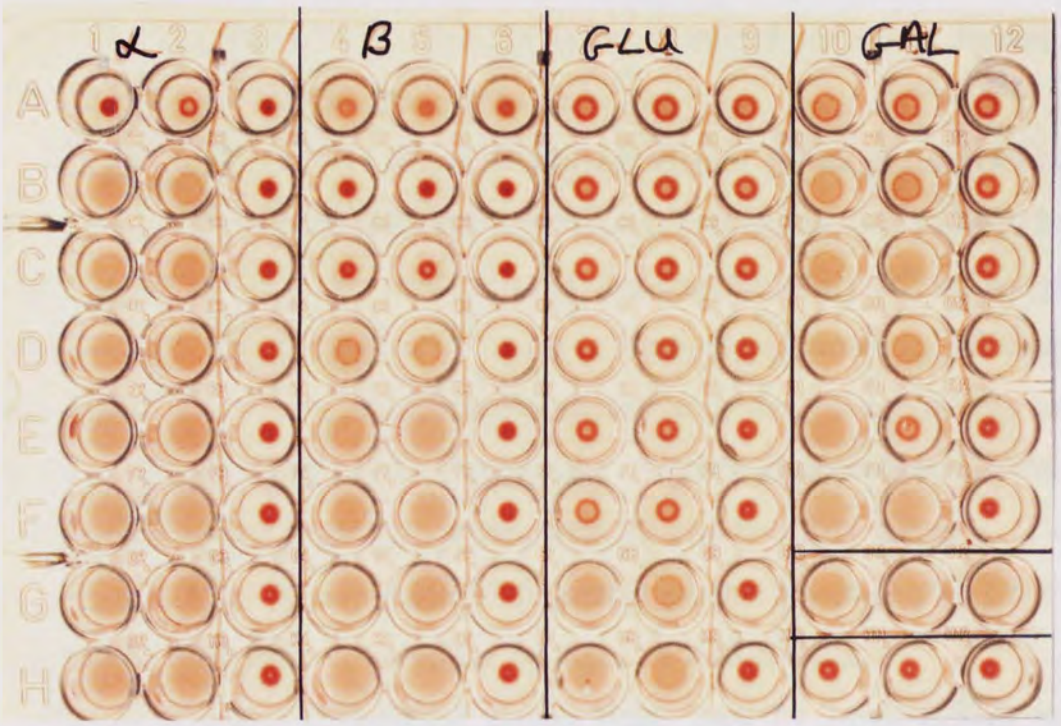
Previous studies have shown that glycosaminoglycans, glycogen, simple monosaccharides and glycosides do not inhibit the haemagglutination of guinea-pig erythrocytes (Jones 1972, Gibbons et al 1975). More recently however, it has been reported that α-D-Galp-(1-3)-D-Gal and α-D-Galp-(1-3)-α-D-Galp-(1-3)-D-Gal can inhibit the haemagglutination of guinea-pig erythrocytes (Nilsson and Svensson 1983). Both glycoproteins and glycolipids have been shown to

inhibit the agglutination of guinea-pig erythrocytes by the K88 adhesin and it has been suggested that β-D-galactosyl residues are important (Jones 1972, Gibbons *et al.* 1975, Nilsson and Svenson 1983). No studies have been carried out on the inhibition of the binding of the K88 adhesin to chicken erythrocytes.

Previous experiments have been based on the inhibition of agglutination mediated by 4-8 haemagglutinating doses (one haemagglutinating dose is regarded as being equivalent to the haemagglutination titre, HA) of either cell-free or cell-bound K88 adhesin (Jones 1972, Gibbons 1975). More recently in the analogous K99/horse erythrocyte receptor system Ono *et al.* (1989) used only 2 haemagglutination doses in their inhibition experiments. Preliminary experiments indicated that the number of haemagglutinating doses used affected the haemagglutination inhibition titre observed. For instance, using 4HA the minimum inhibitory titre for porcine gastric mucin was 73μg/ml, however, when using 1HA the minimum inhibitory titre was 18μg/ml. Hence, to maximise the sensitivity of the method all haemagglutination inhibition assays were performed with 1HA. Initial experiments were carried out to determine the inhibitory potential of the various compounds under test. Further experiments were then carried out to determine the minimal inhibitory concentrations of particular compounds (see Figure 6.4). Table 6.7 lists all inhibitors noted.

Compounds tested and not found to inhibit the agglutination of either guinea pig or chicken erythrocytes were: mannose (750mM), ribose (750mm), fucose (750mM), fructose (750mM), melibiose (750mM), lactulose (750mM), trehalose (750mM), stachyose (750mM), N-acetylglucosamine (750mM), N-acetylgalactosamine (750mM), esculin (4.2mM), cellobiose (150mM), heparin (partially purified 56.2 mg/ml), chondroitin sulphate A (30mg/ml), chondroitin sulphate B (20mg/ml), chondroitin sulphate C (6mg/ml), inulin (1mg/ml),

Figure 6.4 Determination of the minimal inhibitory concentration required to inhibit the cell-free K88ab agglutination of guinea-pig erythrocytes.



After determining in initial experiments that a compound could inhibit the agglutination of erythrocytes by cell-free K88 adhesin doubling dilutions of the inhibitor were used to determine the minimal inhibitory concentration. The above microplate was used to determine the minimal inhibitory concentration of four inhibitors with decreasing concentration from top to bottom. Where:- Columns 1-3=α-Octyl α-D-glucopyranoside (3.3mg/ml); Columns 4-6=α-Octyl β-D-glucopyranoside (19.2mg/ml); Columns 7-9=glucosamine (750mM) and Columns 10-12,rows A-F=galactosamine (750mM). Every third column was used as a negative control (PBS instead of adhesin). Row G 10-12 was used as a positive control (PBS instead of inhibitor) and row H 10-12 used as a second negative control (PBS instead of inhibitor and adhesin).

Gal1→4Man (228mM). In addition, Tris (750mM) and glycine (750mM) did not inhibit the agglutination of guinea-pig erythrocytes. The concentration used is given in brackets. It was not possible to determine the inhibitory potential of ethanolamine with guinea pig erythrocytes or of glucosamine and galactosamine with chicken erythrocytes because the negative control concerned formed a erythrocyte mat in the absence of adhesin.

An efficient inhibitor of both the agglutination of guinea-pig and chicken erythrocytes by the K88ab adhesin was porcine gastric mucin. Jones (1972) had previously noted that the inhibition of haemagglutination of guinea-pig erythrocytes by porcine gastric mucus was incomplete, however, inhibition noted in this study was always complete and clear-cut. Jones (1972) and Gibbons *et al.* (1975) also noted that porcine intestinal glycoproteins inhibited the agglutination of guinea-pig erythrocytes by cell-free K88 adhesin. This present study is the first to report that porcine gastric mucin can inhibit the agglutination of chicken erythrocytes by cell-free K88 adhesin.

How the porcine intestinal glycoproteins used by Jones (1972) and Gibbons *et al.* (1975) relate to the porcine gastric mucin used in this study is not known since no detailed information is available on the composition of the commercially available (BDH) porcine gastric mucin used here. The ability of porcine mucin to bind the K88 adhesin has been reported in several studies (Laux *et al.* 1986, Conway *et al.* 1990, Metcalfe *et al.* 1991). However, the component of mucin responsible for the binding of the K88 adhesin has not been conclusively identified. Metcalfe *et al.* (1991) suggested that a 40-42 Kdal glycoprotein was responsible while Conway *et al.* (1990) found that 50% of K88 binding activity was associated with a large (sedimentable at 26,000g for 9hr) ileal mucus component.

An interesting finding is that K88-mediated haemagglutination is susceptible to inhibition by some forms, although not all, of heparin. Heparin

Table 6.7 Inhibition of the K88ab adhesin-mediated agglutination of guinea pig and chicken erythrocytes.

CARBOHYDRATE/ RELATED COMPOUND	MINIMAL INHIBITORY CONCENTRATION	
	GUINEA-PIG	CHICKEN
Heparin (crude)	161.3 mg/ml	80.7 mg/ml
Heparin (low Mwt)	NI (100 mg/ml)	50.0 mg/ml
Mannan	28.1 mg/ml	37.5 mg/ml
Pig gastric mucin	0.018 mg/ml	< 1.25 mg/ml
Chondrosine	& 35.1mM	NI (23.4mM)
Mannosamine	750 mM	NI (750mM)
Galactosamine	750 mM	ND
Glucosamine	35.2 mM	ND

Where:- NI=no inhibition; &=no inhibition observed at 23.4mM.

is a member of a class of complex polysaccharide molecules known as acidic glycosaminoglycans generally found in animal connective tissue. Heparin in particular is a polysaccharide characterized by the presence of glucosamine and α-D-glucopyranosyluronic acid and L-idopyranosyluronic acid residues with O-sulphate, N-sulphate and N-acetyl groups attached (Jeanloz 1970). Previously it had been found that heparin acted as a non-specific inhibitor of the adhesion of *E.coli* to the bladders of rabbits (Parsons *et al.* 1979). Buchanan *et al.* (1978) found that heparin and other acidic polymers to a lesser extent interfered with binding of gonococcal fimbriae to human buccal cells. It is not apparent from the results of the present study whether the observed inhibition with some forms of heparin is specific or not since it occurred at high concentration (161.3 and 80.7 mg/ml with guinea pig and chicken erythrocytes respectively).

At these high concentrations it is conceivable that impurities may be

responsible for the observed inhibition. For instance, the presence of free or even bound glucosamine in the preparation may explain the inhibition observed since glucosamine is inhibitory (see Table 6.7). Three other glycosamino-glycans tested (chondroitin sulphate A,B and C) did not inhibit haem-agglutination. Unusually though a degradation product of chondroitin sulphate (chondrosine, see Figure 6.5) was found to be inhibitory. As for heparin, the presence of glucosamine in the structure of chondrosine may explain the inhibition observed. Glucosamine was found to be inhibitory down to a minimum concentration of 35mM. Inhibition at this level compares with that noted for D-mannose (0.56-28mM) and the haemagglutination of erythrocytes mediated by type 1 fimbriae (Ofek and Sharon 1990). Previously it has been suggested that the inhibition of the binding of cell-free K88 adhesin to porcine brush borders was attributable to the possession of a free amino-group (Sellwood 1980a). However, this does not apply to the haemagglutination mediated by cell-free K88ab adhesin since neither Tris or glycine which like galactosamine possess a free amino group, are inhibitory. Additionally, the inhibition by glucosamine did appear specific since inhibition by the similar monosaccharides, mannosamine and galactosamine was at 20-fold higher concentrations.

The finding that mannan inhibits agglutination of guinea-pig and chicken erythrocytes by cell-free K88 adhesin was unexpected. Mannan is a polymer of D-mannose, a sugar which is recognised by type 1 fimbriae present on many Enterobacteriaceae. D-mannose itself does not inhibit haemagglutination of guinea-pig and chicken erythrocytes. It has been reported that some high molecular weight glycoproteins non-specifically inhibit haemagglutination (Gibbons *et al.* 1975) and it may be that mannan acts in an analogous manner.

The most potent inhibitor of the agglutination of guinea-pig erythrocytes by the cell-free K88ab adhesin was found to be one of a range of glucosides

tested (see Table 6.8). The basic structure of a glucoside is given in Figure 6.6. Glucosides are obtained by the reaction of alcohol with glucose with the non-sugar component being termed an aglycon (Finar 1986). It is readily apparent that the degree of inhibition observed is proportional to the length of saturated chain substituted into the glucose ring. In addition, the introduction of a thiol group into n-octyl-β-D-glucopyranose resulted in a >four-fold increase in its inhibitory capacity. Thus it seems that the ability of glucosides to inhibit the K88 adhesin is dependant both on the nature of the head group and attached aglycon. One characteristic of glucosides that is related to the length of the alkyl chain present is the net hydrophobicity of the molecule (Brown *et al.* 1970). Increasing the length of the alkyl chain produces an increase in the net hydrophobicity of glucosides and a corresponding decrease in the critical micellar concentration (cmc) value (Brown *et al.* 1970). Several workers have previously found that hydrophobic forces are important in the binding of ETEC expressing K88 fimbriae (Smyth *et al.* 1978, Wadstrom *et al.* 1979, Wadstrom *et al.* 1980). In particular, it has been suggested that a small hydrophobic cleft in the K88 adhesin encompassing the conserved amino acid sequences Ser-148-Leu-Phe-150 and/or Ala-156-Ile-Phe-158 forms the receptor binding domain. (Jacobs *et al.* 1987c, De Graaf 1986). Jacobs *et al.* (1987c) further suggested that variations in the amino sequence surrounding the above conserved sequences could account for differences in the haemagglutination spectrum observed with different adhesin serotypes. Inhibition by glucosides may therefore be attributable to interference with the hydrophobic interactions between the K88 adhesin and its receptor and may not necessarily be specific. In this respect, it is interesting to note that the sugar head group of glucosides play an important role in the hydrophobic interactions of glycosides (Brown *et al.* 1970). Thus the observed increase in the inhibitory potential after the substitution of a thiol group into octyl-β-D-glucopyranose may be due to an

Figure 6.5 Structure of chondrosine.

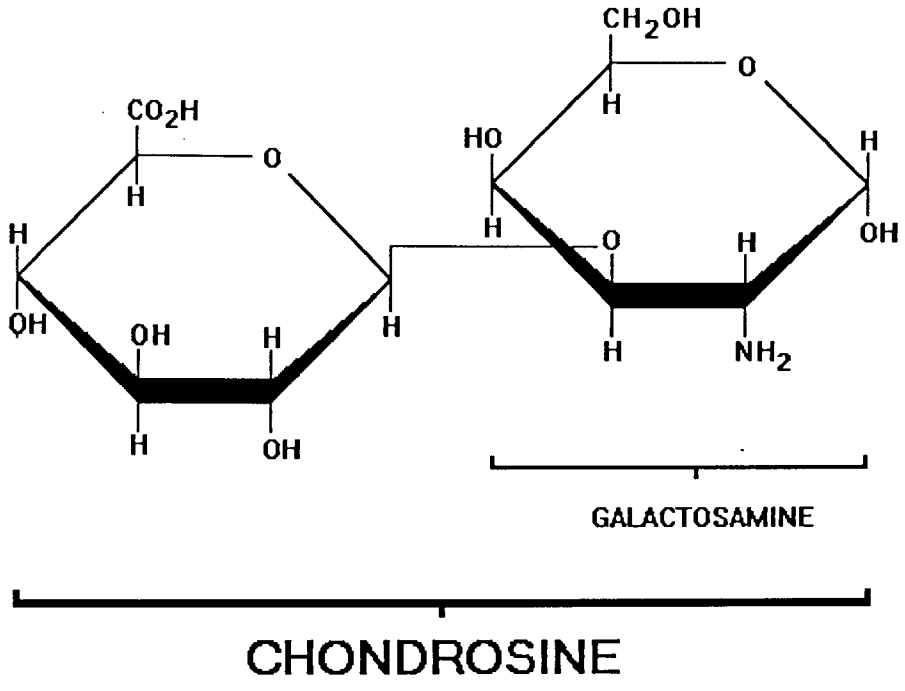


Figure 6.6 Basic structure of a glucoside.

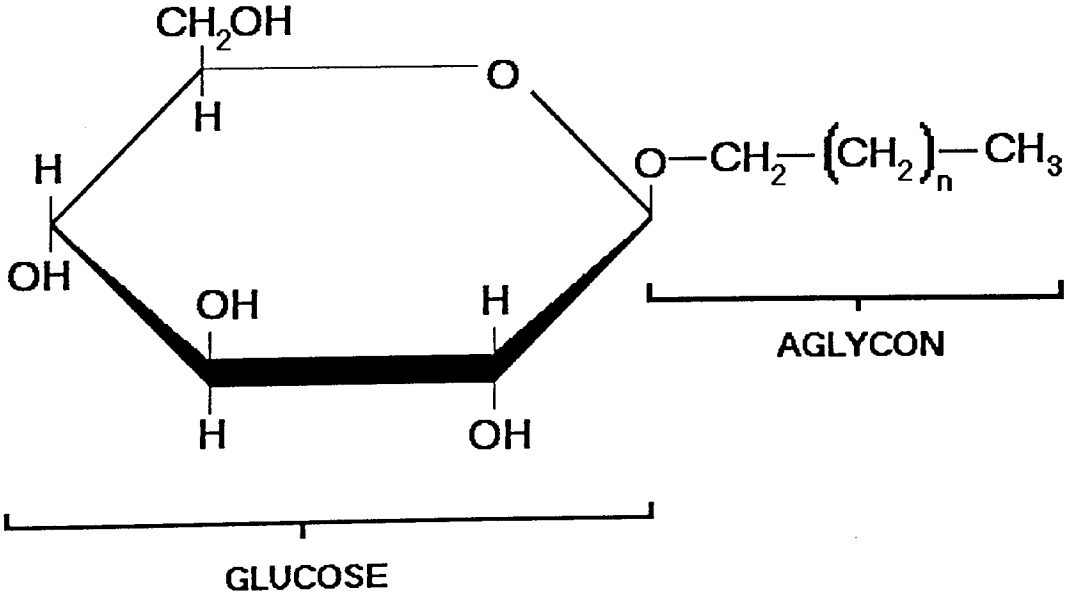


Table 6.8 Inhibitory activity of various glucosides on the agglutination of guinea pig erythrocytes by cell-free K88ab adhesin.

GLUCOSIDE	MINIMAL INHIBITORY CONCENTRATION (mM)
n-Hexyl-β-D-glucopyranoside	>41.00
n-Heptyl-β-D-glucopyranoside	34.00
α-octyl-α-D-glucopyranose	11.00
α-octyl-β-D-glucopyranose	16.00
n-Octyl-β-D-thioglucopyranoside	<4.10
n-Nonyl-β-D-glucopyranoside	8.20
n-Dodecyl-β-D-glucopyranoside	0.72

enhancement in the hydrophobicity of the resultant molecule and not because of increased similarity to the K88 receptor.

An alternative explanation for the observed inhibition of the K88 adhesin/receptor interaction may be attributable to the detergent properties of glucosides. It is possible that inhibition was due to the solubilisation of the K88 receptor from the erythrocyte membrane. However, no haemolysis was observed during haemagglutination experiments to suggest membrane disruption. A further point is that the observed inhibititory concentrations of n-hexyl-β-D-glucopyranoside, α-octyl-α-D-glucopyranose and α-octyl-β-D-glucopyranose correspond approximately to their observed cmc values or greater (Brown *et al.* 1970). Since detergents are only active in solubilising membranes at below their cmc values (Houslay and Stanley 1983), at the concentrations used in the haemagglutination studies the glucosides would have been inactive as detergents. Therefore solubilisation of the K88 receptor does not explain the inhibition of haemagglutination observed by glucosides.

Results presented in Chapter 7 indicate that two receptors or sites are involved in the binding of the K88ab adhesin to porcine enterocytes. This may explain the difficulty that several researchers have found in finding comparatively simple sugars which inhibit the adhesion of the K88ab adhesin to both erythrocytes (providing that two receptors/sites are also present) and porcine enterocytes (Gibbons *et al.* 1975, Sellwood 1980a). The ability of glucosides to interfere with the driving force (i.e. hydrophobic interaction) behind the binding of the K88 adhesin may explain the ability of the relatively simple glucosides to inhibit both receptors/sites present on erythrocytes/ enterocytes.

One possible use of n-dodecyl-β-D-glucopyranoside would be as an effective antagonist of the K88 adhesin\receptor interaction enabling the determination of non-specific binding. This would enable the accurate calculation of the binding characteristics of the K88 adhesin to erythrocytes, porcine mucin and intestinal epithelial cells. In addition, n-dodecyl-β-D-glucopyranoside could be used to confirm the nature of isolated putative K88 receptors.

6.7 Conclusions.

Investigation of the haemagglutinating ability of erythrocytes from eleven different species revealed that guinea-pig, chicken, porcine, marmoset and rabbit erythrocytes were agglutinated by at least two of the three K88 adhesin serotypes. Of these five, the ability of marmoset and rabbit erythrocytes to be agglutinated by the K88 adhesin had not been reported previously. Each of the three K88 adhesin serotypes tested was able to agglutinate at least three of the five K88 receptor-positive erythrocyte types. It was apparent that during the analysis of K88-mediated haemagglutination consideration should be given not only to the existence of both anodic and cathodic forms of the K88ac adhesin

but also to the likely variability in the K88 receptor present on the erythrocytes used.

Comparison of the K88 adhesin binding phenotypes of various erythrocytes with those reported for porcine intestinal cell revealed that phenotypes corresponding to three of the four major intestinal phenotypes are also observed on erythrocytes. It was found that porcine erythrocyte had a cryptic ability to bind the K88ac adhesin, only noted with the cell-free form of the adhesin. No separation was found between the haemagglutinating ability of the K88ab and K88ac forms of the adhesin. However, the K88ad form of the adhesin was unable to haemagglutinate chicken erythrocytes unlike both the K88ab and K88ac forms. This is consistent with the hypothesis that the K88 receptor is composed of two elements, one responsible for the binding of the K88ab and K88ac forms of the adhesin and one responsible for the binding of the K88ad adhesin. Previous results of K88 receptor blocking experiments suggest that the two elements are intimately associated in the final structure (Bijlsma *et al.* 1982, Bijlsma and Bouw 1985). The simplest explanation for the genetic basis of the hypothesis above is that the K88 receptor is coded for at two loci. Variation in the haemagglutination titre and temperature stability of the three erythrocyte species that bound all forms of the K88 adhesin showed similarities with the proposed ability of epistatic genes to modify the porcine intestinal K88 receptor. It is possible that similar modifier genes affect both the *in vivo* K88 receptor and its analogues present on certain erythrocyte species.

The results suggested that there was a rough correlation between the degree of the expression of the K88 adhesin and the observed ability to haemagglutinate. However, other factors such as the affinity and availability of the adhesin receptors must also be important as it was found that despite being comparatively poorly expressed, the adhesive ability of the cell-bound K88ad adhesin was the equivalent or better than the highly expressed K88ab adhesin

in many cases. This finding, along with previous data (Jacobs *et al.* 1987c, see above) is again consistent with the hypothesis that the K88 receptor is composed of two elements, one binding the K88ab and K88ac adhesins and the other binding the K88ad adhesin.

Comparison of the properties of cell-free and cell-bound K88 adhesins revealed that there were some differences in their haemagglutination ability. The results suggested that there was a minor, probably conformational change in the structure of the K88 adhesin on its isolation. In the majority of cases this did not affect the apparent affinity that the K88 adhesin had for the receptor analogues present on the erythrocyte surface. However, in the case of the haemagglutination of marmoset erythrocytes the apparent affinity of the K88 adhesins for their receptor analogues decreased on the change in adhesin form from cell-bound to cell-free. Overall, it was regarded that the properties of the cell-free adhesin did reflect those of the intact fimbria especially in the case of the K88ab adhesin which was largely unaffected by its form.

The K88ab adhesin was found to bind to K88 receptor analogues present on both guinea pig and chicken erythrocytes over a wide pH range (pH 4-9). Binding of all three serotypes of the K88 adhesin to guinea pig, chicken and rabbit erythrocytes was effectively inhibited by mono-specific αK88ab antiserum. Inhibition of haemagglutination by αK88b serum however, was restricted to the binding of the homologous adhesin. This suggests that there is some differentiation in the receptor binding sites between the three adhesins.

Attempts to inhibit the K88 receptors present on chicken and guinea-pig erythrocytes showed that they are similar, with crude heparin, mannan and porcine gastric mucin inhibiting both. However, inhibition noted with the various forms of heparin may have been attributable to impurities because of the high concentrations involved. Further studies on the K88 receptor present on guinea-pig erythrocytes revealed that chondrosine and several hexosamines,

in particular glucosamine were inhibitors. However, the most potent defined inhibitor was found one of a series of glycosides whose inhibitory potential was determined. N-dodecyl-β-D-glucopyranoside was found to inhibit the agglutination of guinea-pig erythrocytes at a concentration of 0.72mM. A correlation was noted between the length of the alkyl chain present in the structure of the glucosides tested and the degree of inhibition. Since the length of alkyl chain present in the glucoside is directly proportional to the overall hydrophobicity of the molecule it was postulated that hydrophobic forces were important in the binding of the K88ab adhesin. Thus inhibition noted was thought to be attributable to interference with the hydrophobic interactions of the K88 adhesin and its receptor and may not involve a more specific interference with binding. Despite the uncertainty over the precise mode of action of n-dodecyl-β-D-glucopyranoside at the present time, it is envisaged that it should prove useful in the further analysis of the K88 adhesin/receptor interaction e.g. in the determination of non-specific binding.

7 ENTEROCYTE BINDING PROPERTIES OF THE K88 FIMBRIAL ADHESIN PROTEIN.

7.1 Introduction

In general, investigations of *in vivo* phenomena *in vitro* is limited by the inability to reproduce *in vitro* accurately the important criteria for the *in vivo* phenomenon of interest. However, because of the comparative ease of performance and the ability to manipulate variables much research effort has been directed towards the development of *in vitro* models that reflect the *in vivo* situation. As further demonstrated in Chapter 6, initial and latter work on the K88 receptor has been based on the presence of analogues on certain erythrocyte species (Jones 1972, Gibbons *et al.* 1975, Parry and Porter 1978, Cox and Houvenaghel 1987). An alternative approach has been to either isolate porcine tissue (Jones and Rutter 1972) or intestinal epithelial cells from the normal site of colonisation by ETEC expressing the K88 adhesin and then to examine the *in vivo* receptor for the K88 adhesin *in vitro*.

Because of its laborious nature, the method of investigating the binding of the K88 adhesin to cell slices was rapidly superseded by a method based on the binding of ETEC expressing the K88 adhesin to isolated porcine intestinal epithelial cells (Wilson and Hohmann 1974, Sellwood *et al.* 1975). This approach led to the discovery of the genetic basis of the K88 receptor(s) and the methods efficacy was confirmed by animal infection studies (Rutter *et al.* 1975). Susceptibility to experimental challenge by ETEC expressing the K88 adhesin was found to be directly related to the ability of the ETEC strain to adhere or not to epithelial cells isolated from the animals concerned as determined in an *in vitro* adhesion test (Rutter *et al.* 1975).

More recent work has refined the genetic basis of porcine susceptibility to infection attributable to ETEC expressing the K88 adhesin. With the finding of a third K88 serotype it has been proposed that there are either four or five main K88 receptor phenotypes expressed by porcine intestinal epithelial cells

and a variety of minor phenotypes attributable to the action of epistatic (modifying) genes which may be responsible for the observed "weak" adhesive phenotype (Bijlsma and Bouw 1985, Rapacz and Hasler-Rapacz 1986).

Recent suggestions of the presence of two adhesins in the intact K88 fimbrial structure (as discussed in Chapter 6) has complicated the interpretation of binding studies on the K88 adhesin. However, several studies have been performed on the binding of both cell-free and cell-bound K88 adhesin to porcine intestinal cells and mucin. Along with the results from haemagglutination studies these have suggested a role for carbohydrate in the structure of the K88 receptor. This has directed attempts at inhibiting the K88 adhesin/receptor by various glycoproteins, glycolipids, individual sugars and related compounds. Despite intensive efforts, however, attempts to identify and isolate the K88 receptor have so far proved inconclusive.

The aims of this present study were as follows:-

A Microscopic adhesion studies were performed to investigate the binding of the various K88 serotypes to porcine enterocytes and to confirm the presence of the K88 receptor.

B Development of an ELISA-based assay to investigate the binding characteristics of cell-free K88 adhesin to porcine brush borders.

C Modification of the ELISA assay to determine the ability of a large number of carbohydrates, related compounds and lectins to inhibit the binding of the K88 adhesin to porcine enterocytes.

7.2 Microscopic properties of the binding of *E.coli* expressing the K88 adhesin to porcine intestinal epithelial cells.

7.2.1 Preparation of porcine intestinal epithelial cells (enterocytes).

A total of 11 porcine enterocyte preparations were made, one gave a poor yield and was not investigated further, the cell numbers determined of those remaining is given in Table 7.1. The method used in their preparation

was based on that given by Sellwood *et al.* (1975) and is very similar to that described by Rapacz and Hasler-Rapacz (1986) for the preparation of porcine brush borders.

Table 7.1 Final concentration of porcine enterocyte preparations

Preparation number	Cells/ml ($x10^6$)	Preparation number	Cells/ml ($x10^6$)
D1201	1.4	D1195	20.0
D1201	9.4	D1199	1.4
D1192	4.7	D1190	4.6
D1193	1.7	D2022	0.5
D1194	1.6	D2405	0.8

The term porcine enterocyte was preferred to brush border cell despite the finding that most preparations contained >90% enterocytes with columnar morphology, distinct nuclei and brush borders. This was thought a more accurate general description of the cells present because in some preparations a significant number (~20%) of cuboidal cells were present in addition to other cell debris (5-10%). However, the presence of non-brush border cells in a preparation is unlikely to affect interpretation of results achieved during the study of the binding of the K88 adhesin to brush borders and enterocytes since they have been shown to have comparable adhesive properties (Rapacz and Hasler-Rapacz 1986).

7.2.2 Enterocyte adhesion test

A microscopic assay of the binding of bacteria to porcine intestinal epithelial cells is a simple assay that has been used by several investigators of the K88 adhesin/receptor system (Wilson and Hohmann 1974, Sellwood *et al.* 1975, Bijlsma *et al.* 1982,1985, Rapacz and Hasler-Rapacz 1986). In agreement

with Wilson and Hohmann (1974), in none of the preparations were there any obvious endogenous bacteria present although inoculating aliquots onto nutrient agar plates did reveal the presence of varying degrees of bacterial contamination. The nature of these contaminating bacteria was not pursued since they did not affect the adhesion assay.

Table 7.2 gives the results of the eight porcine enterocyte preparations which were fully phenotyped for the adhesion of the three serotypes of the K88 adhesin. No attempt was made to check the expression of the K88 adhesin by the bacterial suspensions used since the stock strains concerned had previously been fully characterised (see Chapter 3). Preliminary experiments had showed that broth cultures could be used directly without apparently affecting the adhesion profile. However, all results given were performed with washed nutrient broth suspensions.

Storage of epithelial cells in 50% glycerol at -20°C (as used in this study) for up to six months has been used previously without affecting the adhesion pattern observed (Bijlmsa *et al.* 1982, Rapacz and Hasler-Rapacz 1986). Experiments investigating the effect of storage in 50% v/v glycerol for 2 months on the adhesion pattern of porcine intestinal epithelial cells did not reveal any qualitative differences when compared to the adhesion pattern of the cells when freshly isolated. However, in the adhesion test a greater amount of cell debris was visible and the epithelial cells clumped to a greater extent when using preparations that had been stored compared to same preparations when they were freshly isolated. In addition, very few isolated epithelial cells were generally present and it was difficult to focus on the cells and therefore count the adherent bacteria due to the excessive clumping. Preliminary experiments had indicated that even without washing the presence of glycerol did not affect the adhesion pattern. It therefore seems that the increased difficulty in determining the number of bound bacteria was not due to residual glycerol

Table 7.2 Adhesion phenotypes of porcine enterocyte preparations.

ENTEROCYTE PREPARATION	RECEPTOR PHENOTYPE		
	K88ab	K88ac	K88ad
D1201	+*	+*	+
D1202	+*	-	+*
D1190	+	+	+
D1192	+*	+*	+
D1193	+	+	+
D1194	+	+	+
D1195	+*	+*	-
D1199	+	+	-

*Where:- +=adhesion present, -=adhesion absent, *=the adhesion noted here was of the weak adhesive type (see text). The K88ab phenotype was obtained using serotype O8:K87:K88ab:H19, the K88ac phenotype was obtained using O8:K87:K88ac:H19 and the K88ad phenotype using serotype K88ad. All results were obtained either with fresh enterocytes either without the addition of glycerol or washed cells that had been stored in 50% glycerol for a short period of time. The criteria used for determining adherence was as outlined by Rapacz and Hasler-Rapacz (1986). Epithelial cells were defined as resistant to adherence when at least 95% of them were free of bacteria and the remaining exhibited no more than three bacteria attached to the surface of a single epithelial cell. Preparations showing at least 5% positive brush borders with a minimum of four bacteria attached per cell were considered positive and carrying adhesive receptors. The same bacterial suspension of a particular serotype was used throughout. The preparations D2022 and D2405 (not included in table) were only checked for the adhesion of the K88ab adhesin for which they were both positive. In all cases adherence of E.coli K12 was negative, however, serotype O149:K91:H10 reproducibly bound to epithelial preparation D1190.*

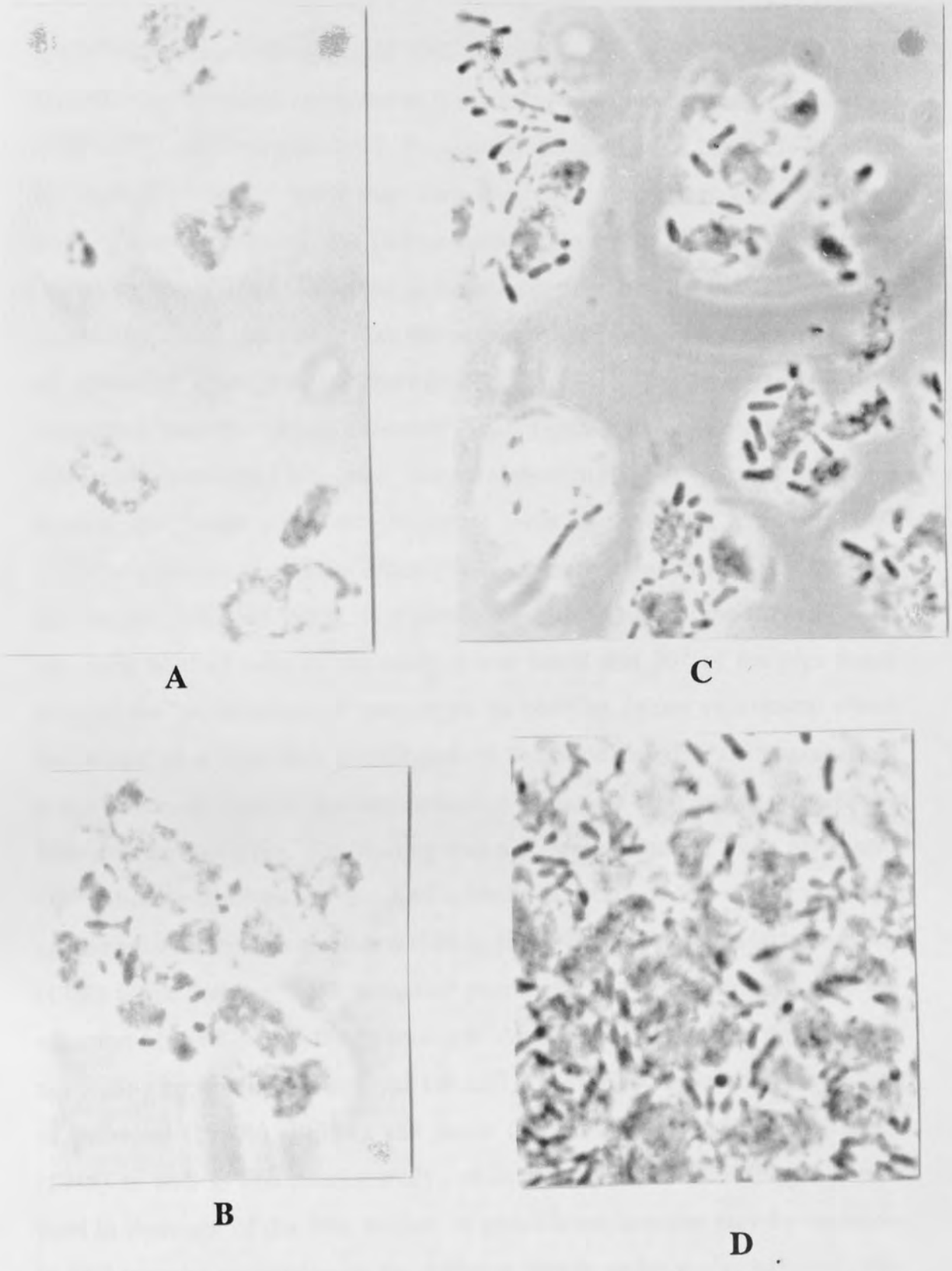
being present after the washing steps but rather an inherent deterioration in the epithelial cells themselves.

Since in certain circumstances there is mannose-sensitive attachment of *E.coli* to porcine brush borders (Sellwood 1980b) preliminary experiments were carried out which demonstrated that there was no qualitative difference in the results of the adhesion test when performed in the presence of 5% w/v D-mannose, although there was a slight reduction in the degree of epithelial cell clumping observed. For serotypes O8:K87:K88ab:H19 and K88ad this was not unexpected since the results of haemagglutination experiments had indicated that these serotypes did not express mannose sensitive adhesins. However, even serotype O8:K87:K88ac:H19 where mannose-sensitive adhesins had been detected, the adhesion test was not significantly affected by the presence or not of 5% w/v D-mannose.

In a large study, Rapacz and Hasler-Rapacz (1986) phenotyped 345 pigs for adhesion by all three of the K88 serotypes and found that 46.7% of those examined did not bind the K88 adhesin. In this present study none of the enterocyte preparations examined were resistant to adhesion by all three serotypes of the K88 adhesin. This may partly be explained because the enterocytes phenotyped were not selected completely at random but rather six of the preparations were from the same litter. However, it has been reported previously that the adhesive phenotypes can dominate (Sellwood *et al.* 1975, Gibbons 1977) and in one particular herd it was found that only 12.2% of the pigs in a commercial herd were of a non-adherent phenotype (Snodgrass *et al.* 1981).

Examples of the adhesion events observed in the cell adhesion assay are given in Figure 7.1. In several cases a variation in the number of adherent bacteria attached to a single epithelial cell was noted. Only uniformly high numbers (10 or more) of bacteria were reported to adhere to susceptible

Figure 7.1 K88 adhesin-mediated adhesion to porcine enterocytes



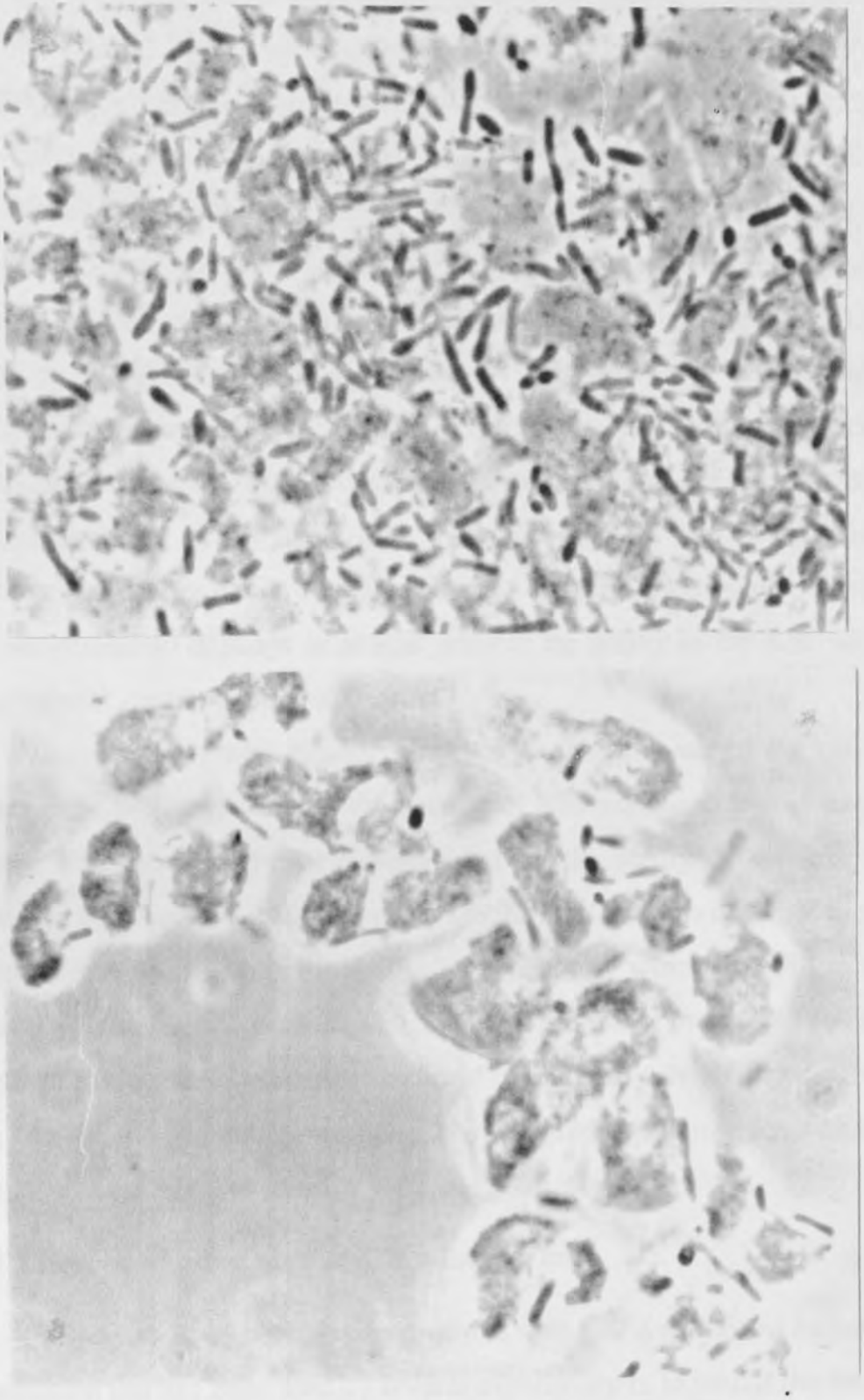
Where:- A=epithelial cells without any added bacteria; B=serotype O8:K87:K88ac:H19 with enterocyte preparation D1202, adhesion to this preparation was poor; C=demonstrates that only bacteria bound to well isolated enterocytes could be counted accurately; D=majority of adhesive cells formed clumps. Phase contrast x1,000 magnification.

epithelial cells by Sellwood *et al.* 1975 and Bijlsma *et al.* 1982 however, other reports have indicated variations in the number of adherent bacteria (Sellwood 1980b,1981, Bijlsma *et al.* 1985, Rapacz and Hasler-Rapacz 1986). The finding of enterocytes with a lower than normal number of adherent bacteria during phenotyping experiments was first noted by Sellwood in 1980b. Known as the "weak adhesive", this phenotype is characterised by only 2-4 bacteria adhering to each epithelial cell rather than the normal 10-15^{+} and by the poor clumping of epithelial cells present (Sellwood 1980b,1981). Previous studies have suggested that the "weak adhesive" phenotype was dominant to the non-adherent phenotype. However, animal infection studies showed that pigs bearing the "weak adhesive" phenotype were also resistant to infection by ETEC expressing the K88ac adhesin in the same manner as the fully resistant phenotype (Sellwood 1981). In a previous report using porcine material from the same herd as used in this study it was found that 30$^{\%}$ of the pigs tested showed the "weak adhesive" phenotype. In addition, in one experiment where the whole of a litter was phenotyped (R.Sellwood personal communication) every littermate showed the weak adhesive phenotype with one or more of the K88 adhesin serotypes. The finding that the "weak adhesive" was associated with all three serotypes of the K88 adhesin as found in this study is also in agreement with Bijlsma and Bouw (1985). However, Rapacz and Hasler-Rapacz (1986) found that the "weak adhesive" phenotype was associated only with the adhesion of the K88ad adhesin serotype. An explanation for this difference is not readily apparent since serotype O8:K87:K88ab:H19 was used in the studies of Sellwood (1980b), Bijlsma and Bouw (1985), Rapacz and Hasler-Rapacz (1986) as well as this present study, while serotype O8:K87:K88ac:H19 was used in three out of the four studies. A possible explanation may be variation in K88 receptor expression by the differing breeds under study, however, this seems unlikely due to the large number of breeds examined by Rapacz and Hasler-Rapacz (1986).

7.2.3 Inhibition of the binding of bacteria expressing the K88 adhesin by cell-free K88 adhesin.

The ability of cell-free K88 adhesin to inhibit the binding of bacteria expressing the K88 adhesin was used initially as evidence confirming the specificity of the latter when binding to isolated porcine brush borders (Sellwood *et al.* 1975). More recently workers have investigated the differing ability of the various serotypes of cell-free adhesin to inhibit the binding of bacteria expressing the various K88 serotypes as a means of differentiating the binding abilities of the three serotypes (Bijlsma *et al.* 1982). Figure 7.2 shows the effect of the addition of purified cell-free K88ab adhesin on the binding of bacteria expressing the same adhesin. As can be seen in Figure 7.2 addition of purified cell-free adhesin not only reduces the degree of clumping of enterocytes in the presence of bacteria expressing the K88 adhesin but also prevents the adhesion of those bacteria. In agreement with Bijlsma *et al.* (1982, 1985) the pre-incubation of enterocytes with purified K88ab adhesin prevented the binding of bacteria expressing each of the three K88 serotypes. However, it was not apparent by what mode of action the cell-free adhesin prevented bacterial adhesion because it was found that in the presence of cell-free K88ab adhesin, five of the six *E.coli* strains examined formed large aggregated bacterial masses (see Figure 7.3). Thus it could not be determined whether prevention of bacterial adhesion was due to blocking of the K88 receptor sites or to bacterial agglutination. Previous workers (Sellwood *et al.* 1975, Bijlsma *et al.* 1982, Bijlsma *et al.* 1985) on the inhibition of bacterial binding by cell-free adhesin have not noted the occurrence of bacterial agglutination although it is known that at least some of the bacterial strains (including O8:K87:K88ac:H19 as used in this study) expressing the K88 adhesin do autoagglutinate (Orskov *et al.* 1964, Chandler *et al.* 1986). Previously it has

Figure 7.2 Effect of cell-free K88 adhesin on the binding of bacteria expressing the K88 adhesin.



Where:- top photograph shows the adherence of E.coli serotype K12:K88ab to enterocyte preparation D1190 causing clumping; bottom photograph as above except the enterocyte preparation was pre-incubated with purified K88ab (0.75mg/ml) adhesin before the addition of bacteria. Note that only a few bacteria are now adherent. Phase contrast x1,000 magnification.

been postulated that the autoagglutinability of K88$^+$ strains is associated with the hydrophobic properties of piliated bacteria (Chandler *et al.* 1986). A possible reason for the observed bacterial agglutinating ability in this study but not in previous reports is the differing method of preparation of the cell-free K88 adhesin. Heat extraction was the basic nethod used in this study followed by further purification with ammonium sulphate and isoelectric precipitation. Both the methods of Sellwood *et al.* (1975) and Bijlmsa *et al.* (1982,1985) were based on an initial homogenisation step followed either by isoelectric precipitation or ammonium sulphate precipitation for further purification respectively. Since the K88 fimbrial protein preparation used in this study is homogeneous by SDS-PAGE and ~95-99% pure it is possible that a conformation change either within or between the fimbrial subunits, triggered by homogenisation destroys or at least reduces the ability of the K88 adhesin to agglutinate certain strains of *E.coli*.

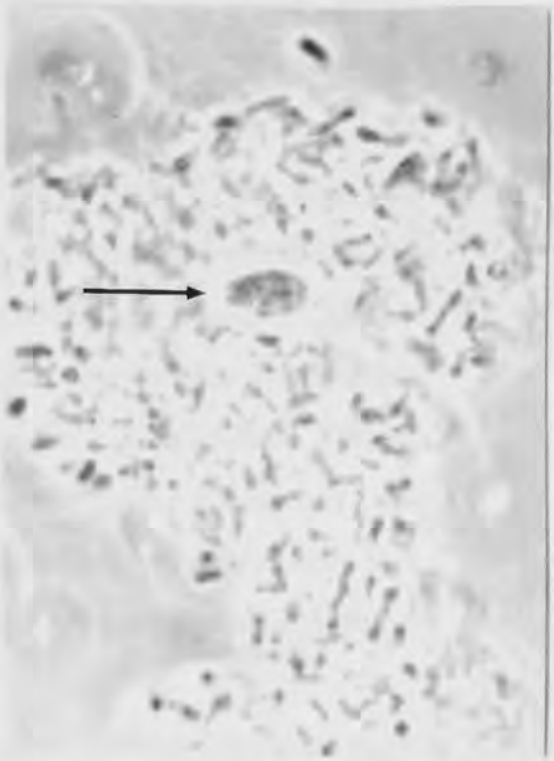
The degree of agglutination observed in the presence of the cell-free adhesin does not correlate with the amount of K88 expressed by the bacteria present. This is most vividly demonstrated by the ability of cell-free K88 adhesin to agglutinate *E.coli* serotype O149:K91:H10 which does not express any detectable K88 adhesin itself. The mechanism by which the K88 adhesin causes agglutination of bacteria is unknown, however, *in vivo* it could be postulated that bacterial clumping mediated by the K88 adhesin has a survival value as it would aid protection from degradative enzymes, immune destruction, acid pH and possibly facilitate colonisation.

7.2.4 Effect of antisera on the K88 adhesion test.

Preincubation of bacterial suspensions with αK88 antiserum before the cell adhesion test has been used by several experimenters to inhibit the binding of bacteria to porcine enterocytes (Wilson and Hohmann 1974, Sellwood *et al.*

Figure 7.3 Agglutination of *E.coli* by purified K88ab adhesin.

E.coli SEROTYPE	DEGREE OF AGGLUTINATION
K12	-
K12:K88ab	+
O149:K91:H10	+++
O8:K87:K88ab:H19	++
O8:K87:K88ac:H19	+++
K88ad	++



KEY

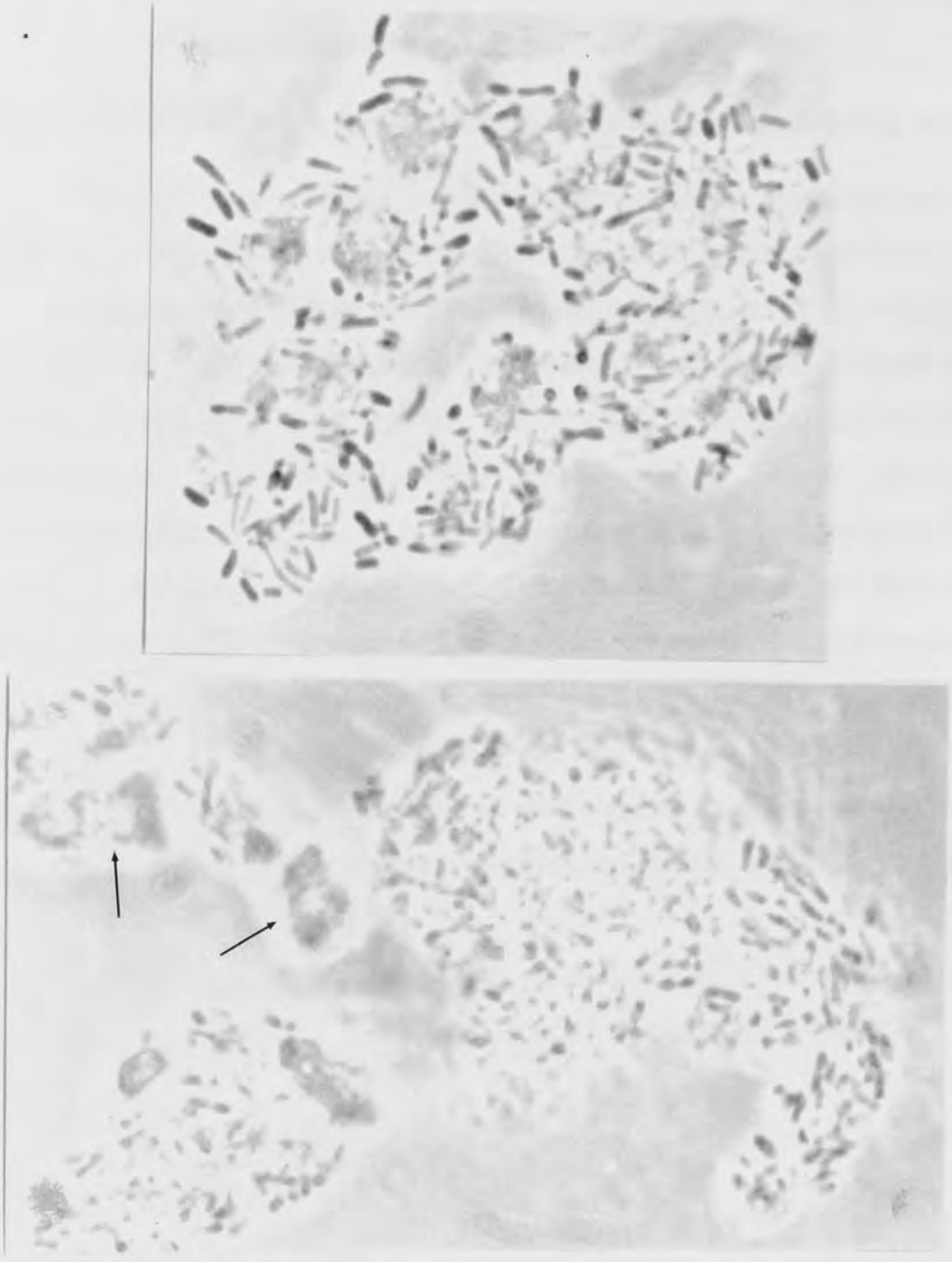
Designation	% Bacterial agglutination
-	<5
+	5-20
++	20-50
+++	>80

Pre-incubation of purified K88ab adhesin with porcine enterocytes prevents the binding of bacteria that express each of the three serotypes of the K88 adhesin. However, it was difficult to determine whether this inhibition of adhesion was attributable to blocking of receptor sites or to bacterial agglutination. Incubation of bacteria alone with purified adhesin results in the agglutination to different degrees of 5 of the 6 strains examined (see above left). In the cell adhesion assay the ability of the purified K88 adhesin to agglutinate bacteria was not always associated with the presence of the corresponding adhesin being present on the bacteria. For instance when used in the adhesion assay, serotype O149:K91:H10 was agglutinated by the purified K88 adhesin (see photograph above, arrow indicates brush border without any attached bacteria surrounded by agglutinated bacteria).

1975, Parry and Porter 1978). In all cases, the appropriate αK88 antiserum inhibited binding, while normal rabbit serum, antisera specific for O and H antigens, antiserum against K12 *E.coli* and antiserum from which αK88-specific antibodies had been absorbed were all non-inhibitory. In this study, αK88 serum was prepared in a similar manner to that of Sellwood *et al.* 1975. Purified, cell-free K88ab adhesin extracted from serotype K12:K88ab was used to inoculate rabbits and non-specific antibodies removed from the resultant serum by absorption against *E.coli* K12. This antiserum effectively inhibited the agglutination of several erythrocyte species by the K88ab adhesin (see Chapter 6). However, despite repeated attempts the αK88ab serum like the control serum did not inhibit the binding of *E.coli* serotype K12:K88ab to D1190 enterocytes. The unabsorbed (ie. the crude αK88 serum before absorption with K12 bacteria) αK88 serum was inhibitory although bacterial agglutination was visible and this was thought responsible for the lack of bacterial binding and the absence of enterocytes clumping.

A possible explanation for why the αK88 serum was not inhibitory may be that *E.coli* strain K12:K88ab possesses an adhesin in addition to the K88 adhesin. Consequently in inhibition experiments bacterial binding was unaffected because not all of the adhesins present on the surface of the bacteria were neutralised by the mono-specific αK88 antiserum. The presence of receptors in addition to those for the K88 adhesin on the surface of the enterocyte preparation (D1190) used in these experiments was indicated by the reproducible ability of *E.coli* serotype O149:K91:H10 to bind in microscopic assays (see Figure 7.4). The nature of the putative non-K88 adhesins found on *E.coli* serotypes K12:K88ab and O149:K91:H10 is unknown although fimbriae in addition to K88 were expressed by them (see Chapter 3).

Figure 7.4 Binding of *E.coli* serotype O149:K91:H10 to enterocyte preparation D1190 and the effect of cell-free K88ab adhesin.



Enterocyte preparation D1190 was unusual among those examined since it was found that serotype O149:K91:H10 bacteria adhered well to it (top photograph). Preincubation of enterocytes with cell-free K88ab adhesin resulted in the rapid agglutination of the bacteria present upon their addition leaving the enterocytes bare (arrowed in bottom photograph). However, this was attributable to bacterial agglutination and not the blocking of receptors that may of been recognised by an adhesin present on serotype O149:K91:H10.

7.3 Characterisation of the interaction of the K88 adhesin with porcine enterocytes.

7.3.1 A brief theoretical background for the analysis of binding data.

This section is intended as an introduction to the large amount of literature on the analysis on the interaction of ligands with macromolecules, for more detailed explanations the reader is referred to Dahlquist (1978), Weilland and Molinoff (1981), Klotz (1982), Zivin and Waud (1982), Munson and Rodbard (1983) and Laudron (1984). The equations to be described here are applicable in their various forms to describing enzyme kinetics, antibody/antigen interactions and the interaction of ligands with receptors for hormones, neurotransmitters and drugs (Dalquist 1978, Weiland and Molinoff 1981, Fersht 1985). The use of these analyses for the binding of bacterial adhesins to their receptors has been poorly applied with very little information being available on the affinity of binding and other characteristics. However, information on the binding isotherms of whole *E.coli* to mucus material has been published and it is anticipated that interest in this area will increase (Mouricout and Julien 1987).

In its simplest form the binding of a ligand to a receptor can be represented by the following equation (Weiland and Molinoff 1981).

$$[L] + [R] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [RL]$$

Where:- [L]=Concentration of free ligand; [R]=Concentration of unoccupied receptor sites; [RL]=Concentration of ligand-receptor complex; k_1=Rate constant of forward reaction (association rate constant, k+1); k_2=Rate constant of back reaction (dissociation rate constant, k-1).

At equilibrium the rate of formation of the receptor/ligand complex is equivalent to the rate of its breakdown and this allows for the calculation of the equilibrium dissociation constant K_d.

$$K_d = k_1/k_2$$

With the assumption that the total number of receptor sites has a finite limit ie.

$$[R] = [R_T]-[RL]$$

it is possible to derive the equation :-

$$\frac{[RL]}{[L]} = \frac{[R_T]-[RL]}{K_d}$$

Where:- R_T=Total concentration of receptors present.

Known as the Scatchard equation (Scatchard 1949), the above formula results in a straight line when [RL]/[L] is plotted against [RL] and enables the calculation of R_T (intercept on the X-axis) and K_d (which equals -1/slope). The advantages of the Scatchard plot are that it determines K_d directly and the value of R_T by extrapolation under non-saturation conditions ie. where not all of the receptor sites in a sample are occupied provided the reaction has reached equilibrium (Dahlquist 1978, Weiland and Molinoff 1981). Under saturating conditions it is possible to calculate K_d since it is equivalent to the concentration of ligand that occupies 50% of the receptors in a given sample. The affinity that a ligand has for its receptor is related to the equation given below:-

$$\text{AFFINITY } (K_a) = 1/K_d$$

Therefore the higher the K_d value the lower is the affinity that the ligand has for its receptor. For the above equations to remain valid at least three

assumptions are made :-

A A single molecule of ligand interacts with a single molecule of receptor.

B The initial binding of ligand does not influence the binding of subsequent molecules.

C The binding reaction between the ligand and its receptor is at equilibrium.

If one or more of these assumptions is not valid then a curvilinear Scatchard plot will result. One example of this is where the initial binding of ligand influences the subsequent binding of ligand. This is termed "cooperation" and is regarded as negative if the apparent affinity of the ligand decreases after the first ligand molecule has bound and positive if the apparent affinity of secondary (or more) ligands increases (Weiland and Molinoff 1981). A method of determining the presence and degree of cooperation is based on the construction of a Hill plot. This plot is based on the log transformed Hill equation as shown below:-

$$\log \frac{[RL]}{([R_T]-[RL])} = n \log[L] - \log K'_d$$

Where K'_d=is an overall constant consisting of all the K_d's of the interacting binding sites; n=the Hill coefficient.

A plot of log ($[RL]/[R_T]-[RL]$) against log [L] is a straight line with a slope equal to the Hill coefficient and an intercept on the X-axis equivalent to K'_d (Weiland and Molinoff 1981). If the slope is greater than unity then the plot

indicates that positive cooperation is occurring and if less than unity it indicates negative cooperation. A drawback of the Hill plot is that it requires that R_T be known, however, in most practical situations the value of R_T can be determined from a Scatchard plot (Weiland and Molinoff 1981).

7.3.2 Development of ELISA-based assay for the investigation of the K88 adhesin/receptor interaction.

Several *in vitro* assays have been developed to examine and quantitate the binding of the K88 adhesin to its receptor present on porcine epithelial cells (Kearns and Gibbons 1979, Sellwood 1980a, Anderson *et al.* 1980, Staley and Wilson 1983). All have been based on the radiolabelling either of the purified cell-free K88 adhesin or of isolated porcine brush borders. The assay developed for use in this study is based on the ELISA technique (Tijssen 1985). The general advantages of enzyme assays are summarised in Table 7.3.

Table 7.3 Advantages of enzyme immunoassays.

-Very high sensitivity, detectability and specificity are possible.
-Equipment required is relatively cheap.
-Assays may be very rapid and simple.
-Reproducibility is high and evaluation is objective.
-Feasible under field conditions.
-No radiation hazards.
-Reagents are cheap and generally of long shelf-life.
-Versatility of assays may be significantly increased by the great variety and specific properties of enzymes.
-Full advantage of the properties of monoclonal antibodies may be achieved with enzyme immunoassay (EIA).

After Tijssen 1985

A specific advantage of the ELISA-based assay used in this study to investigate the binding of the K88 adhesin to porcine enterocytes is that non-specific binding does not play a significant role. This is important since during the subsequent analysis of the binding the inability to allow for non-specific binding would severely restrict the accuracy of calculations. For binding studies in general a control experiment is usually performed simultaneously with the test experiment to allow for non-specific binding of ligands (Billard *et al.* 1984, Kilpatrick *et al.* 1986). For the K88 adhesin/receptor system use has been made previously of the availability of non-adherent brush border cells to characterise non-specific binding (Sellwood 1980a). It was found that despite the fact that *E.coli* expressing the K88 fimbriae did not bind to these non-adherent brush borders in the microscopic adhesion test they were still capable of binding a significant quantity of cell-free radiolabelled K88 adhesin (Sellwood 1980a). In this previous study, treatment of the brush borders with 1% formaldehyde was found to completely abolish non-specific binding while leaving the specific binding largely unaffected. Subsequently for all K88 adhesin binding studies all brush borders were treated with formaldehyde before use (Sellwood 1980a). The ELISA-based assay here avoids non-specific binding of the K88 adhesin by incubating it in a "competitive" situation. The incubation is competitive since both the enterocytes and the microplate wells in which the cell-free adhesin is incubated have an affinity for the K88 adhesin. The affinity that the adhesin has for the microplate well can be calculated from the concentration required to saturate the binding sites present in the microwell plate. The concentration of adhesin at which 50% of the binding sites on the microwell plate are occupied with K88 adhesin is equivalent to the K_d of the reaction. For the microwell-plates used in this study this corresponds to a K_a of $2x10^{12} \pm 0.9M^{-1}$(sem). As can be readily appreciated this represents a high affinity (most enzyme/substrate systems have K_a's of less than $1x10^8M^{-1}$, Fersht

1985). Therefore in order for the cell-free adhesin to bind to the enterocytes the affinity of the interaction must exceed the affinity that the K88 adhesin has for the microwell plate. Any non-specific affinity that the cell-free K88 adhesin has for porcine enterocytes is likely to be several orders of magnitude lower and therefore would not be detected in the ELISA-based assay. On the other hand, it has been reported (Sellwood 1980a) that the affinity of the K88 adhesin/brush border receptor is $9x10^{12}M^{-1}$ and would therefore be readily detectable.

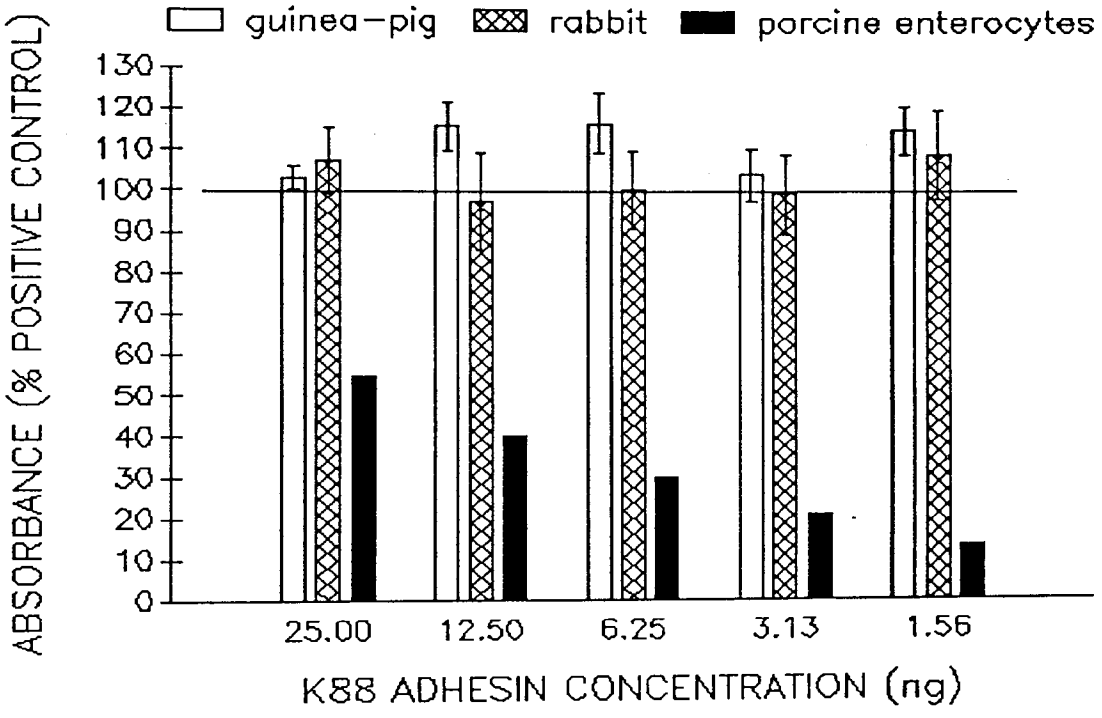
In an attempt to demonstrate that only specific binding would be detected in the ELISA-based assay, a total of 10 enterocyte preparations were screened to identify a non-adhesive phenotype (see Section 7.2). However, none of the enterocyte preparations examined were found to be non-adherent. With the same aim, use was made of the finding that treatment with 1% formaldehyde abolishes the non-specific binding of the K88 adhesin (Sellwood 1980a). However, after treatment with 1% formaldehyde for 7 days at 4°C and despite repeated washing, enterocytes treated in such a manner adhered to the microplate wells. The adherence of the formaldehyde-treated enterocytes to the microplate wells led to highly variable and erroneous results. Therefore formaldehyde treated enterocytes could not be used. As a further alternative, erythrocytes from several species were substituted for the enterocytes in the ELISA-based assay which was then performed as normal. It was found that no detectable K88 adhesin bound to erythrocytes (see Figure 7.5), even those (guinea-pig and rabbit) that are agglutinated by the K88 adhesin in haemagglutination assays (see Chapter 6). This finding demonstrated that even specific binding of the K88 adhesin is not detected in the ELISA-based assay if it is not of sufficient affinity (ie. $>2x10^{12}M^{-1}$). It was regarded therefore that non-specific binding could be ignored in the ELISA-based assay. The inability of the receptor analogues on erythrocytes to bind the K88 adhesin in the ELISA-based assay emphasises that the receptors present on porcine enterocytes

and erythrocytes are similar but not identical.

Although the ELISA-based assay used had the major advantage that non-specific interactions could be ignored it also had a few limitations. A primary limitation was that the range over which the adhesin concentration could be accurately determined was limited. This is attributable to the nature of the binding of the K88 adhesin to the ELISA plate. With increasing concentration, the binding of the K88 adhesin follows a rectangular hyperbola (see Figure 7.6). Thus only the range of concentration up to the saturating concentration could be measured accurately. In practical terms, under the conditions used in this present series of experiments this was equivalent to a range of ~0-50ng/ml.

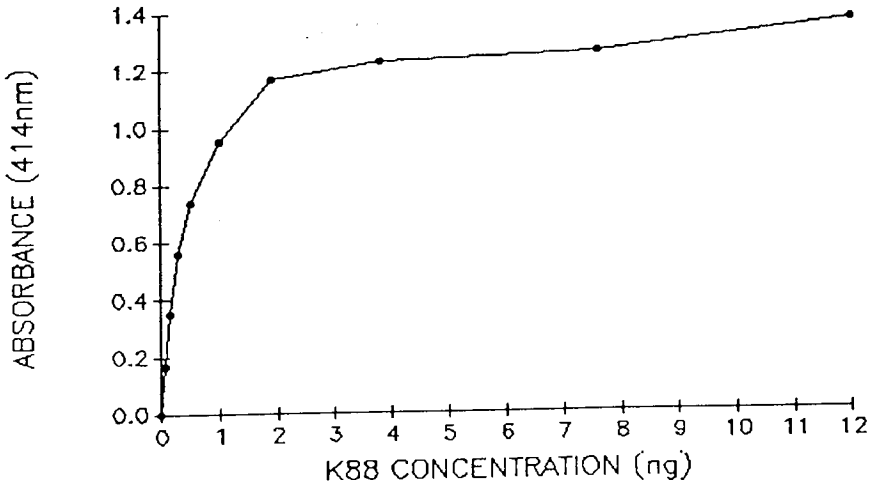
A second limitation of the assay became apparent when attempts were made to perform the assay in the presence of 0.1% w/v BSA. BSA has been used previously in the initial incubation mixture of epithelial cells and cell-free purified K88 adhesin, presumably to minimise non-specific binding (Sellwood 1980a). Use of BSA in the assay used in this study resulted in an apparent decrease in the amount of K88 present. This was attributed to competition between the K88 adhesin and BSA for binding sites on the microplate well surface since the BSA was present in excess (10mg/well compared to 3ng/well of K88 adhesin). Thus the presence of BSA effectively reduced the detectability (Tijssen 1985) of the K88 adhesin and the range over which binding data could be obtained. Because of this BSA was not included in the final assay employed in this study. The absence of BSA was not regarded as significant in the inhibition studies since several other workers investigating both the K88 adhesin (Anderson *et al.* 1980, Staley and Wilson 1983, Laux *et al.* 1986) and other adhesins (Mouricout and Julien 1987) have not included BSA or its equivalent in adhesion assays. However, as discussed below (see Section 7.3.3), the presence of BSA in the buffer used in adhesion assays may be important in the determination of binding constants.

Figure 7.5 Ability of various erythrocyte species to bind the K88 adhesin.



Erythrocytes were substituted for enterocytes in the ELISA-based adhesion assay. Not even those erythrocytes that were specifically agglutinated by the K88 adhesin in haemagglutination tests were able to bind and remove significant amounts of the K88 adhesin. In the above figure a horizontal line has been drawn to indicate the value of the positive control. Erythrocytes were used at a concentration of 1x10^6/ml. The equivalent experiment with porcine enterocytes (D2405) is included for comparison. For the erythrocytes examined results are given as the mean ± standard deviation.

Figure 7.6 The binding of the purified cell-free K88 adhesin to a microplate.



Later experiments investigated the ability of various sugars to inhibit the K88 adhesin/receptor interaction. Here, the finding that the apparent amount of K88 adhesin binding to microplate wells was reduced was likely to be attributable to either one or both of two possible mechanisms. The first is that the binding of the putative inhibitor to the K88 adhesin in turn inhibited the binding of the adhesin to the microplate. An important consideration here is that both the binding of the K88 adhesin to its receptor and the binding of proteins to plastic are likely to be facilitated by hydrophobic interactions (Smyth *et al.* 1978, Cantarero *et al.* 1980, Wadstrom *et al.* 1980, Jacobs *et al.* 1987c). Thus the binding of an inhibitor to the K88 adhesin would be expected at least in part to be dependent on hydrophobic interaction and once bound, an inhibitor may not only affect the ability of the K88 adhesin to bind to the K88 receptor on the enterocytes present but also its ability to bind to the microplate well. In addition carbohydrates or related compounds which do not themselves recognise the binding site where the K88 adhesin interacts with the K88 receptor may sterically hinder that interaction by binding to a nearby site on the K88 adhesin even though the apparent inhibitor does not reflect the K88 receptor. This latter scenario seems unlikely since the majority of compounds tested for inhibitory properties are small molecules ($<1{,}000$ Mwt) and even if bound to the K88 adhesin are unlikely to interfere with the K88 adhesin/receptor interaction unless bound within the adhesins binding site and therefore be a "true inhibitor".

A second mechanism to allow for the decreased absorbance observed was that the direct binding of sugars/related compounds to the microplate wells was interfering with the binding of the purified K88 adhesin to the microplate. No information on the binding of sugars to microplates was available in the literature and no attempt was made to determine the ability of any of the putative inhibitors to bind to the microplates used. It is possible that sugars,

especially those substituted with alkyl chains e.g. glucosides do bind to microplates through hydrophobic forces.

To circumvent the above two mechanisms during experiments on inhibition, control wells containing both the K88 adhesin and the putative inhibitor under test and the K88 adhesin only were compared and what was termed the "correction factor" calculated (see Section 2.7.2). This correction factor was an indication of the ability of the putative inhibitor to prevent the binding of the K88 adhesin by either mechanism but does not differentiate which is operative. Evidence that the use of the correction factor was effective was apparent because without it the majority of test wells containing adhesin, putative inhibitor and enterocytes gave lower final absorbances than control wells containing adhesin and enterocyte only. Inclusion of the correction factor resulted in equivalent final absorbances in test and control wells in the majority of cases (ie. with non-inhibitors) while in those remaining it highlighted the difference in absorbance between control and test wells.

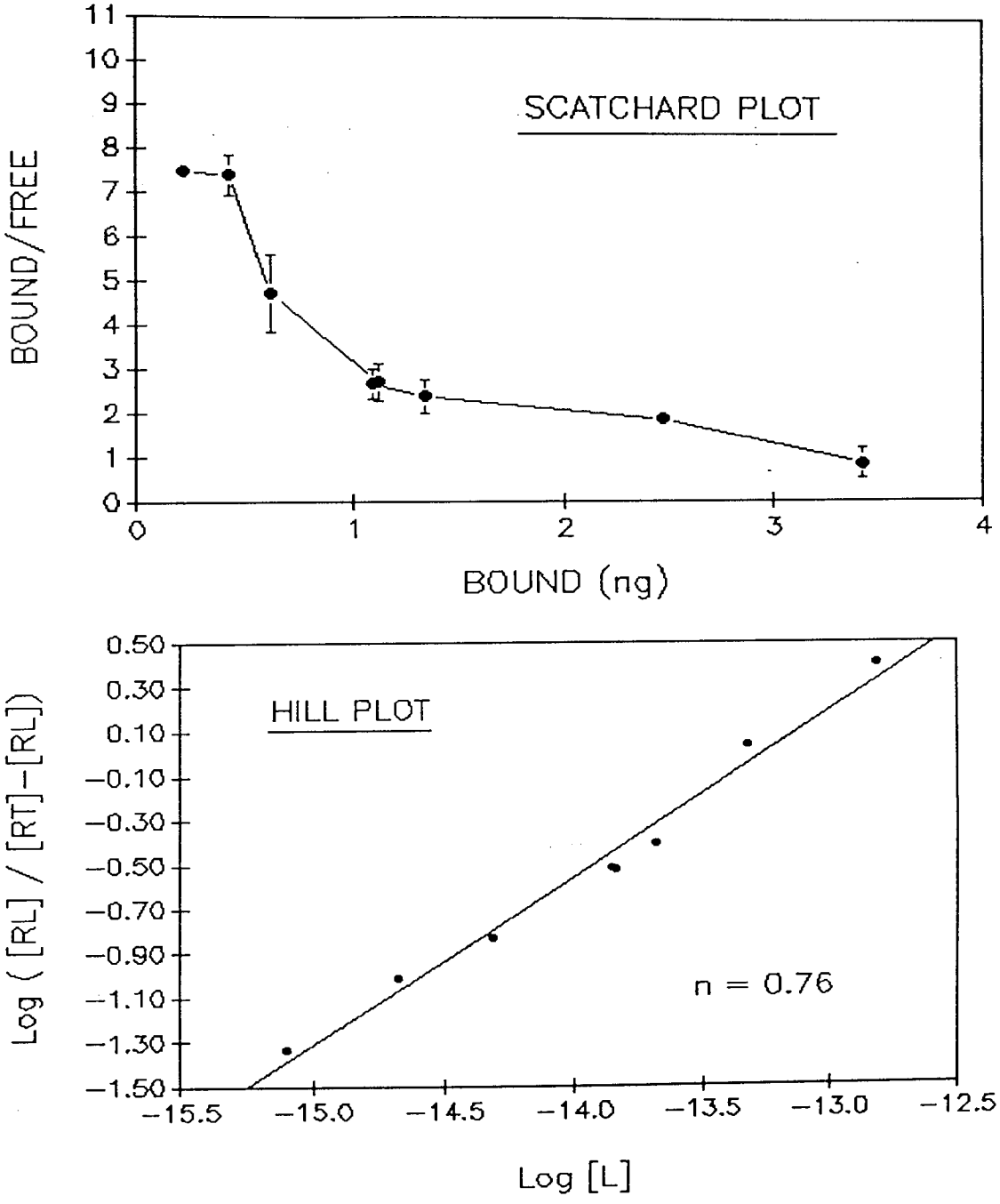
The inability to differentiate between inhibition attributable to competition for the adhesin binding site and apparent inhibition due to the binding of the putative inhibitor to the microplate well emphasises the need for caution in the interpretation of the inhibition tests. However, the assay does not result in "false negatives" ie. all compounds identified as non-inhibitors are correct although there may be some "false positives" ie. compounds identified as inhibitors of the K88 adhesin/porcine enterocyte interaction which in fact only interfere with the binding of the K88 adhesin to the microplate well. Accordingly, the inhibitory potential of compounds detected in the ELISA-based assay was confirmed where possible either by haemagglutination inhibition experiments (see Chapter 6) or in the literature.

7.3.3 Characteristics of the binding of the K88 adhesin to its receptor present on porcine enterocytes.

A typical example of the Scatchard and Hill plots obtained for the binding of the K88 adhesin to porcine enterocytes are given in Figure 7.7. The Scatchard plot obtained is curvilinear with upward concavity corresponding with a Hill coefficient of 0.76 and K'_d of $7.85x10^{-14}M$ ($K'a = 1.27x10^{13}M^{-1}$). With linear Scatchard plots it is possible to calculate K_d directly from the slope (Weiland and Molinoff 1981, Zivin and Ward 1982). However, derivation of data from a curvilinear Scatchard plot must be treated with caution and some reservation (Dalhquist 1978, Klotz 1982). With this in mind it is still possible to obtain some information from the initial slope of a Scatchard plot providing its limitations are realised (Dahlquist 1978). For instance, determination of the initial slope allows calculation of the apparent equilibrium dissociation constant K_{diss} rather than the actual K_d. In addition, determination of the number of binding sites by extrapolation from the initial slope of the plot to the X-axis gives a value for the minimum number of sites present with the calculated K_{diss} (Dahlquist 1978). For the binding of the K88 adhesin to porcine enterocytes the calculated K_{diss} is $6.3x10^{-15}M$ corresponding to an apparent association constant K_{app} of $1.59x10^{14}M^{-1}$ and a minimum of $4.3x10^4$ receptor sites/enterocyte. Thus K_{app} is approximately 10-fold greater than K'_a. This is to be expected since the former is based on values obtained at low K88 adhesin concentrations (equivalent to the "high" affinity binding present) only while the latter is based on both the "high" and comparatively "low" affinity binding present (ie. at high bound adhesin concentrations).

The K_{app} value calculated in this study is approximately 17-fold higher than the value ($9x10^{12}M^{-1}$) for K_a reported by Sellwood (1980a). An explanation for the difference may be the high bound/free ratios of K88 adhesin observed in

Figure 7.7 Scatchard and Hill plots of the binding of the purified K88 adhesin to porcine enterocytes.

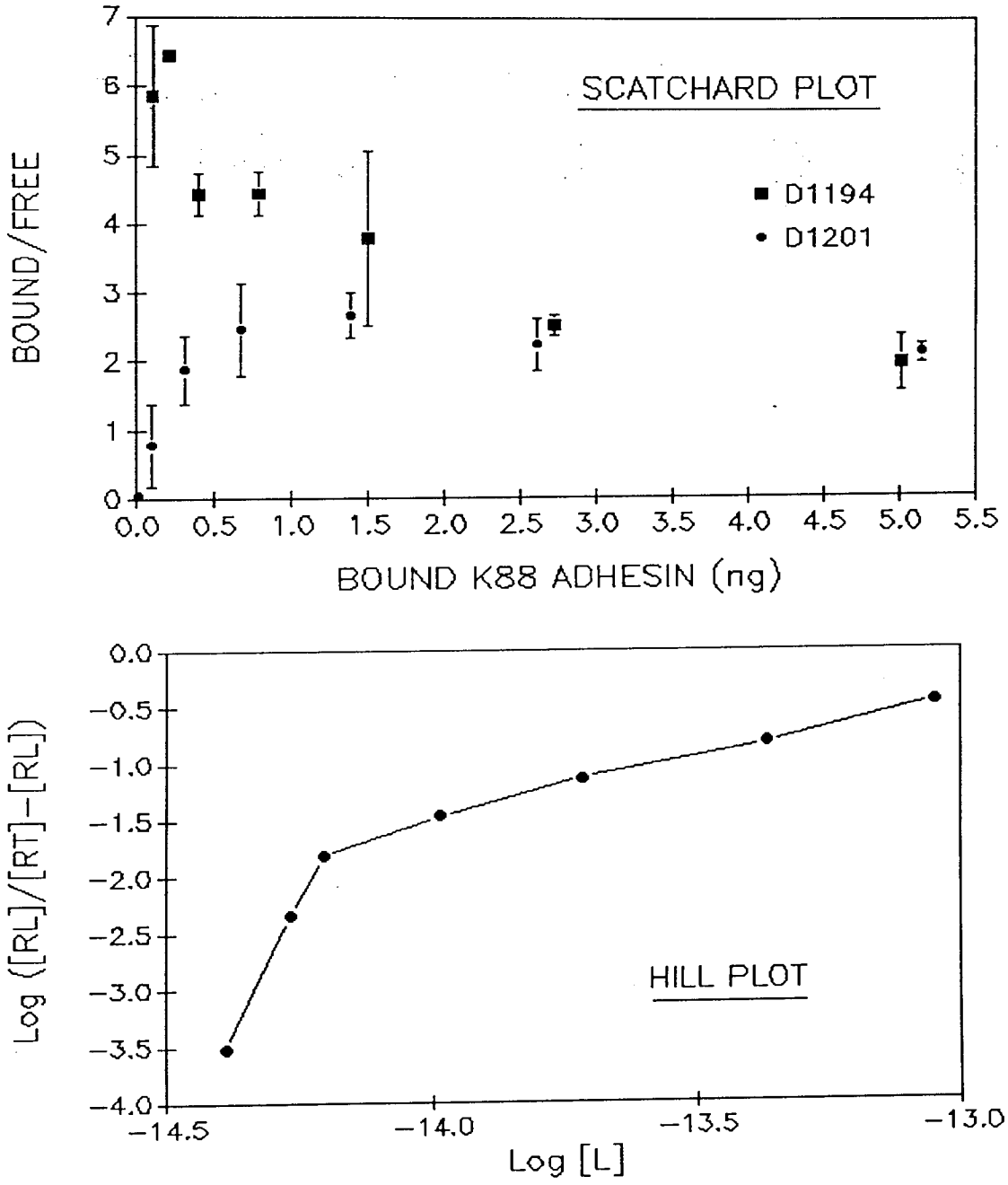


Scatchard analysis of the binding of the K88 adhesin to porcine enterocytes gives a curvilinear plot (upper graph, mean ± sd). Extrapolation of the initial slope of the curve to the Y-axis by linear regression allows the calculation of the minimum number of receptor sites present and the apparent equilibrium dissociation constant K_{diss}. Based on a molecular weight of 27,500 for the K88 adhesin and a value of $6.02x10^{23}$ for Avogadro's constant then K_{diss} is $6.3x10^{-15}M$ with a minimum of $4.3x10^{4}$ receptor sites per enterocyte. Using the same data a Hill coefficient of 0.76 and K'd of $7.85x10^{-14}M$ was calculated (lower graph). Data was obtained from 3 experiments.

this study. Compared to the values noted by Sellwood (1980a) the results found in this study are approximately 20-fold greater at the same bound concentration of K88 adhesin. This reflects the problem that although the calculations for the determination of K_d and consequently K_a are based on the assumption that the K88 adhesin acts as a monovalent ligand in reality as demonstrated in the haemagglutination studies the K88 adhesin used in this study is multivalent. It is presumed that either the inclusion of BSA in the adhesion assay or the method of preparation of the cell-free K88 adhesin by Sellwood (1980a) avoided its multivalent nature. Attempts to incorporate BSA into the ELISA assay were unsuccessful since the BSA interfered with the binding of the K88 adhesin to the microplate (see Section 7.2.2).

Apart from non-specific binding there are several possible explanations for a curvilinear Scatchard plot including ligand-ligand interactions, affinity heterogeneity of either the adhesin or its receptor, negative cooperation or a two-step/three component binding reaction (Dahlquist 1978, Sellwood 1980a, Weiland and Molinoff 1981). Without detailed kinetic analysis and the use of statistically complex methods of analysing the equilibrium binding isotherm it is usually difficult to differentiate between these possibilities (Weiland and Molinoff 1981). However, during the analysis of one of the 10 enterocyte preparations it was found that the binding isotherm had a different form to those others examined (see Figure 7.8). The binding of cell-free K88 adhesin to enterocyte preparation D1201 exhibited positive cooperation i.e. the apparent affinity of the adhesin increased with increasing concentration. The simplest explanation for this departure from the "normal" binding isotherm may be that the porcine enterocytes concerned do not possess the comparatively high affinity K88 receptor found in the remaining

Figure 7.8 Scatchard and Hill plots of the binding of the K88 adhesin to enterocyte preparation D1201.



One of the enterocyte preparations examined in the ELISA-based binding assay (D1201) gave completely different binding characteristics to the remaining nine examined. In the Scatchard plot (top) enterocyte preparation D1201 appeared to exhibit positive cooperation in the binding of the K88 adhesin. Using linear regression analysis the number of receptor sites/enterocyte was calculated at $1.75x10^5$. Construction of a Hill plot (bottom) gave a Hill coefficient of 1.07 with a K'_d of $2.1x10^{-13}M$ corresponding to a K'_a of $4.8x10^{12}M^{-1}$. The binding data (top) of enterocyte preparation D1194 was included for comparison (mean ± sd).

enterocyte preparations. Accordingly, it is suggested that the majority of enterocytes express two forms of the K88 receptor (or possibly two sites on the same receptor) and that the binding of the K88 adhesin to porcine enterocytes is a two-step/three component system.

As was found in this present study, Sellwood (1980a) also obtained a curvilinear binding isotherm for the interaction of the K88 adhesin with porcine enterocytes. On the basis of dissociation experiments Sellwood (1980a) concluded that site-site interactions of the negatively cooperative type were responsible for the observed binding isotherm of the K88 adhesin/receptor system. However, it was also noted by Sellwood (1980a) that at low concentrations the binding of radiolabelled K88 adhesin was increased by the addition of small amounts of unlabelled adhesin. This would be difficult to explain if the binding of the K88 adhesin was based solely on a negatively cooperative mechanism since this finding is characteristic of positively cooperative binding. However, if as suggested above the binding of the K88 adhesin was a two step process, one step of which exhibits positive cooperation, then enhancement of binding at low K88 adhesin concentrations would be expected.

Although enterocyte preparation D1201 exhibited the "weak adhesive" phenotype in the microscopic assay with the K88ab adhesin this does not correspond with the absence of the "high" affinity receptor since several enterocyte preparations exhibiting the "weak adhesive" phenotype possessed both the "high" and "low" affinity K88 receptors for the K88ab adhesin (see Table 7.2). A possible explanation of the weak adhesive phenotype is that it is determined by receptor availability but not expression. Although speculative it may be possible that modification of the enterocyte surface may prevent or interfere with the approach of whole bacteria resulting in the "weak adhesive" phenotype while as in the ELISA assay isolated fimbriae or fimbrial subunits

can still approach and bind.

7.3.4 Inhibition of the K88 adhesin/porcine enterocyte interaction by lectins.

Lectins have been defined as "carbohydrate-binding proteins of non-immune origin that agglutinate cells or precipitate polysaccharides or glycoconjugates" (Goldstein *et al.* 1980). In this present study it was attempted to inhibit the binding of the K88 adhesin by the preincubation of enterocytes in the presence of lectins which had been previously characterised for their carbohydrate binding ability. To maximise the sensitivity of the assay a concentration of the K88 adhesin was used equivalent to approximately the K_d of the microplate being used (see Figure 7.5).

None of the lectins used were effective inhibitors of the K88 adhesin/enterocyte reaction (see Table 7.4). Of those showing above 10% inhibition only the recognition of galactose by the peanut and pagoda tree lectins were complimentary. The most potent lectin tested was the asparagus pea lectin (obtained from Tetragonolobus purpureas) which primarily recognises α-L-Fucose, however, even here the inhibition observed was poor (22.14%). In contrast, Sellwood (1980a) found that a lectin recognising fucose residues was a potent inhibitor (>70%) of the K88 adhesin/porcine epithelial cell reaction. Unfortunately, the source of the fucose-binding lectin was not specified and as shown by the comparatively poor inhibitory action of the fucose-specific lectin from gorse used in this study the source of the lectin is important (see Table 7.4).

There are at least three possible reasons why the observed inhibition by lectins was poor. Firstly, the enterocyte preparation used (D2405) was one of the preparations postulated previously (Section 7.3.3) to express 2 receptors for the K88 adhesin. Thus even if a lectin were capable of binding to one site this

Table 7.4 Ability of various lectins to inhibit the binding of the K88 adhesin to its receptor(s) on porcine enterocytes.

LECTIN NAME/SOURCE	SPECIFICITY	% INHIBITION
PEANUT	D-Galactose	10.71
BAUHINA PURPUREA	N-Acetyl-D-galactosamine, D-Galactose, Lactose	5.00
BANDEIRAEA SIMPLICIFOLIA BS-1	Methyl-D-galactopyranoside, D-Galactose, Melibiose, Raffinose, D-Fucose	6.43
ULEX EUROPEUS 2	N,N'-Diacetylchitobiose	2.86
GORSE	L-Fucose	7.14
ASPARAGUS PEA	L-Fucose	22.14
LENTIL	Methyl-mannopyranoside	15.00
PAGODA TREE	N-Acetyl-D-galactosamine, D-Galactose	18.89
HORSE GRAM	N-Acetyl-D-galactosamine	3.33
ROMAN SNAIL	N-Acetyl-D-galactosamine	0.00
LIMULUS POLYPHEMUS	Fetuin	6.67
CONCANAVALIN A TYPE 3	Methyl-D-mannopyranoside, D-Fructose, D-Mannose	0.00
CONCANAVALIN A TYPE 5	Methyl-D-mannopyranoside, D-Fructose, D-Mannose	5.56

may have been largely masked by the ability of the K88 adhesin to bind to a second uninhibited site. Secondly, the affinity of the K88 adhesin for the receptors present on porcine enterocytes is very high (K'a 1.27×10^{13}). The concentration of lectins used (0.4mg/ml) may have been insufficient to effectively compete with the K88 receptor(s) present and inhibit the binding of the K88 adhesin despite similar specificities of lectin and adhesin. Finally, none of the lectins used may have recognised an important determinant for the binding of the K88 adhesin.

7.3.5 Inhibition of the K88 adhesin/porcine enterocyte interaction by carbohydrates and related compounds.

In several systems, either certain inhibitors (Gibbons *et al.* 1975), or K88 receptor analogues (Laux *et al.* 1986,Metcalfe *et al.* 1987, 1991), or the K88 receptor(s) itself (Sellwood 1980a) has been shown to be susceptible to treatment with periodate. Periodate acts as an oxidising agent that disrupts the bonds between carbon atoms carrying free hydroxyl or unacetylated amino groups (Hancock and Poxton 1988). Although other cell membrane components are susceptible to periodate oxidation, sensitivity to periodate suggests that at least part of the K88 receptor is formed by carbohydrate residues (Sellwood and Kearns 1979). Therefore, in common with other workers (Anderson *et al.* 1980, Staley and Wilson 1983, Sellwood 1980a, Laux *et al.* 1986) an attempt was made in this study to inhibit the K88 adhesin/receptor interaction with carbohydrates or related compounds. Out of a total of 98 compounds tested (see Appendix 2) only 14 gave what was regarded as significant inhibition ($>30^{\%}$, see Table 7.5).

No significant inhibition was observed with any of the pentoses, hexoses, heptoses, tri-saccharides, thio-sugars, deoxy-sugars or sugar alcohols tested. Of the 13 disaccharides tested 5 were shown to be inhibitory, lactulose (4-O-β-D-galactopyranosyl-α-D-fructose, $46.4^{\%}$ at 250mM), melibiose (6-O-α-D-galactopyranosyl-D-glucopyranose, $48.4^{\%}$ at 250mM), trehalose (1-O-α-D-glucopyranosyl-α-D-glucopyranoside, $50.8^{\%}$ at 250mM), 4-O-β-D-galactopyranosyl-D-mannopyranose ($32.2^{\%}$ at 73mM) and chondrosine (2-amino-2-deoxy-3-O-β-D-glucopyranurosyl-D-galactose, $100^{\%}$ at 50mM). Of these 5, two (melibiose and trehalose) have been tested previously and been found to not inhibit the binding of the K88 adhesin to porcine brush borders (Sellwood 1980a). The inhibitory ability of melibiose is further confused by

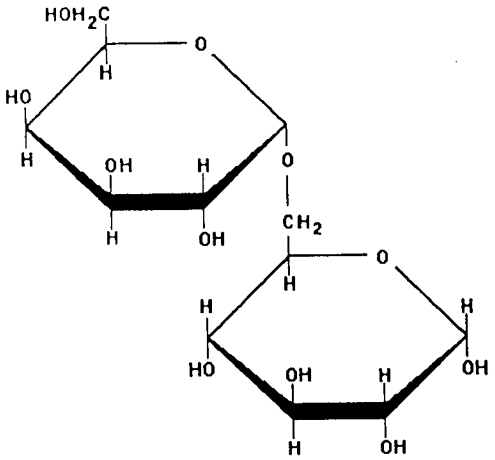
Table 7.5 Inhibitors of the K88 adhesin/porcine enterocyte interaction.

SUGAR/ COMPOUND	C.F.	CONCENTRATION
D-GALACTOSAMINE	1.1	250mM
MELIBIOSE	2.4	250mM
TREHALOSE	3.2-5.9	250mM
LACTULOSE	2.0	125mM
GAL 1→4 MAN	2.4-3.3	73mM
CHONDROSINE	8.6-9.6	50mM
STACHYOSE	2.4-2.9	94mM
CHONDROITIN SULPHATE B	1.8-3.0	100mg/ml
HEPARIN	2.8	25mg/ml
MANNAN	2.45	0.5mg/ml
PORCINE GASTRIC MUCIN	8.1	0.25mg/ml
BACITRACIN	74.1-76.2	31mM
ESCULIN	30	25mM
n-OCTYL-α-D-GLUCOPYRANOSE	44.9-51.3	25mM

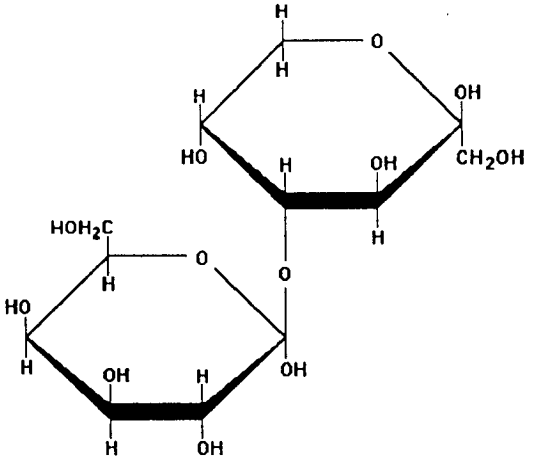
A total of 98 compounds were tested for their ability to inhibit the K88 adhesin/porcine enterocyte interaction. Of these a total of 14 showed greater than 30% inhibition at the minimal concentration given above. The results given are the mean of triplicates. Inhibition was confirmed by the performance of at least two separate experiments on the individual putative inhibitors. Inhibition observed was found to be dose dependant, with inhibition observed being roughly equivalent above a certain minimum concentration. Where:- C.F. = correction factor.

the finding in this study that melibiose obtained from one supplier was inhibitory while that obtained from another was non-inhibitory (see Appendix 2). Accordingly, the inhibitory potential of melibiose is treated with caution. An explanation for the ability of trehalose to inhibit in this study and not previously may be attributed to the higher (2x) final concentration used here. Of the four clearly identified disaccharide inhibitors of the K88 adhesin/porcine enterocyte interaction it is difficult to detail common inhibitory components from their simple structures (see Figures 7.9 and 7.10).

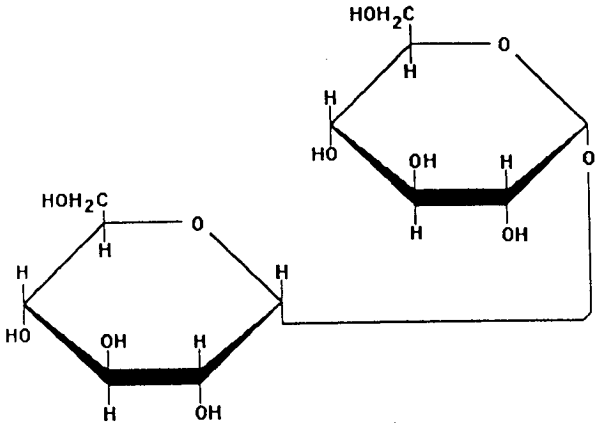
One residue which is present in two of the inhibitors is D-galactosamine. Not only is the free sugar inhibitory but so is chondrosine, a disaccharide which can be obtained from chondroitin sulphate by mild acid hydrolysis (see Figure 7.10, Jeanloz 1970). Previous investigators (Sellwood 1980a, Staley and Wilson 1983) have found D-galactosamine to inhibit the binding of the K88 adhesin to porcine brush borders. In the study by Sellwood (1980a) it was difficult to determine whether the inhibitory ability of D-galactosamine was specific or attributable to the possession of a free amino groups since similar levels of inhibition were observed with ethanolamine, Tris and glycine. This was despite the finding that the inhibition observed with D-galactosamine was >2x that observed with either D-mannosamine or D-glucosamine (Sellwood 1980a). In this present study inhibition observed with Tris was minimal (6.6% at 250mM) while inhibition observed with D-galactosamine was significant (47.8 at 250mM) and approximately 3x that observed with either D-mannosamine or D-glucosamine or four other amino sugars tested (see Appendix 2). The finding that chondrosine is inhibitory is interesting since it has a similar structure to the putative inhibitor (β-D-galactosyl 1→4 N-acetylhexosamine) previously postulated to be present in the mucus of the piglet small intestine by Jones (1972).



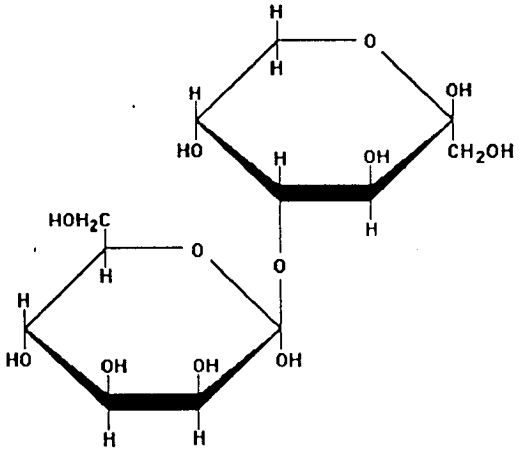
MELIBIOSE



LACTULOSE

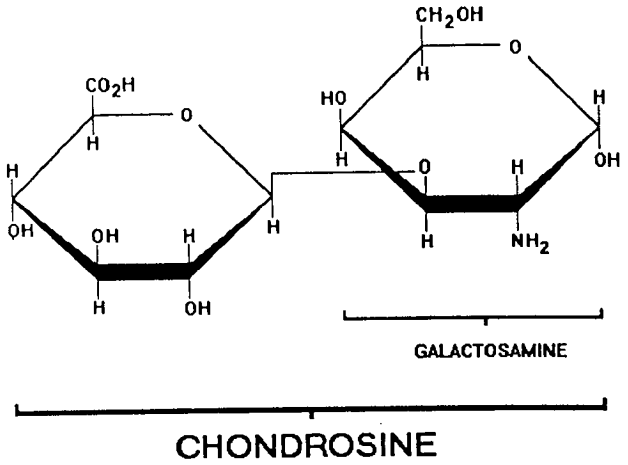


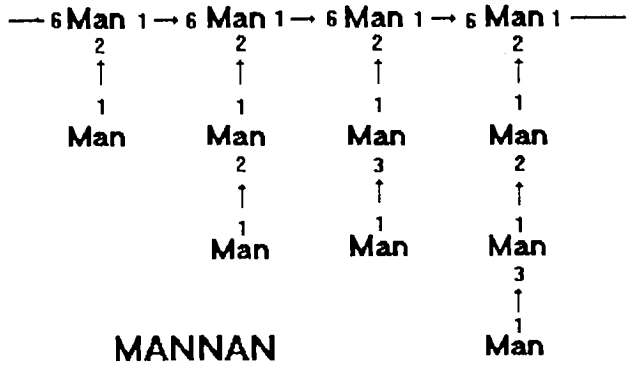
TREHALOSE



D-GALACTOPYRANOSYL 1-->4 MANNOSE

Figure 7.9 *Structures of inhibitors of the K88 adhesin/porcine enterocyte interaction.*





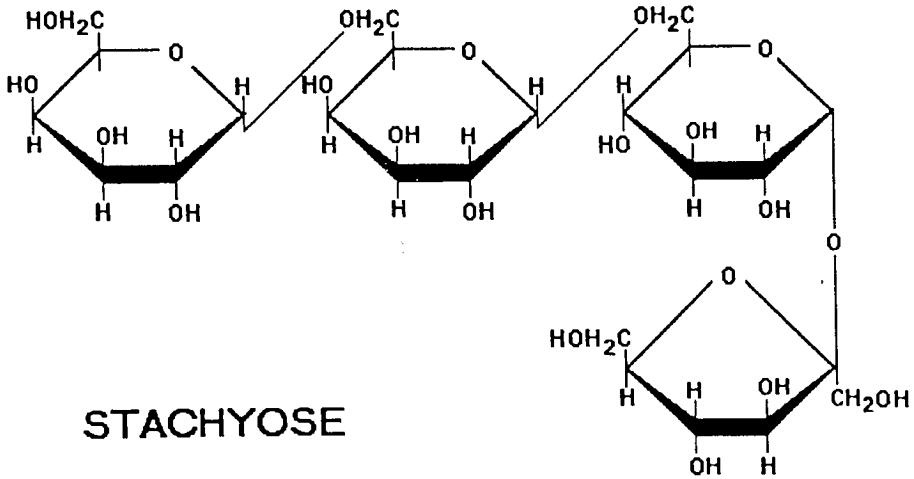


Figure 7.10 *Structures of inhibitors of the K88 adhesin/porcine enterocyte interaction.*

Two glycosylaminoglycans (heparin and chondroitin sulphate B) were found to be inhibitors, albeit only at high concentration (see Table 7.5). In the study of Buchanan and co-workers (1978) it was concluded that the binding of gonococcal pili to buccal cells was dependant on hydrophobic interactions and decreases in repulsive charged forces were important in the process. It was found that in the gonococcal pili/buccal cell system that heparin and to a lesser extent other acidic polymers inhibited binding while attachment was promoted by highly positively charged polymers such as protamine sulphate. Although no conclusive evidence of the importance of charge in the binding of *E.coli* expressing K88 fimbriae to enterocytes has been reported (note that calcium ions were found to enhance the binding of $K88^+$ *E.coli* to pig intestinal brush borders [Staley and Wilson 1983] while binding was found to be unaffected by ionic strength by other workers [Sellwood and Kearns 1979]) a role for hydrophobic interactions in the binding of K88 and other *E.coli* is well established (Wadstrom *et al.* 1979, 1980). Therefore, heparin and chondroitin sulphate B may inhibit the binding of the K88 adhesin to porcine enterocytes in an analogous manner to the inhibition of the binding of gonococci to buccal cells by heparin i.e. by interference with hydrophobic interactions.

The ability of stachyose (α-D-galactopyranosyl-1→6-α-D-galactopyranosyl-1→6-α-D-glucopyranosyl-1→2-β-D-fructose) to inhibit the K88 adhesin/porcine enterocyte interaction has been reported previously (Sellwood 1980a). In this present study stachyose was a comparatively effective inhibitor with 44.1% inhibition noted at a concentration of 94mM. However, inhibition observed with raffinose which comprises 3 out of the 4 stachyose sugar residues was not significant (21.9% at 250mM), neither was inhibition observed with sucrose which comprises 2 of the 4 stachyose residues (19.7% at 250mM). Since inhibition observed with melibiose was doubtful while the highest concentration of Gal-1→6-Gal that was used (7.3mM) may not of been

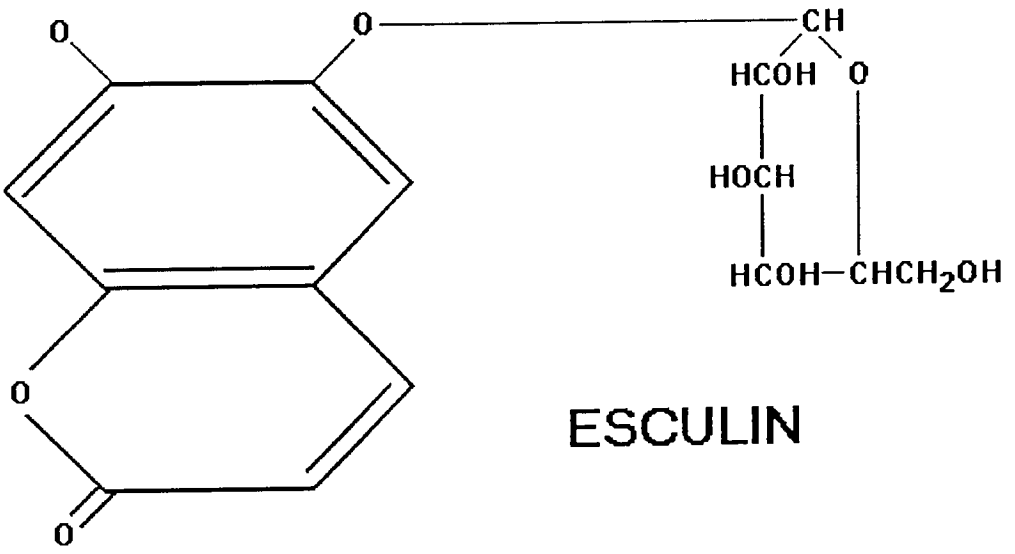
sufficient to show inhibitory potential (both of which comprise 2 out of the 4 residues of stachyose) then it was not possible to assign inhibitory ability to a particular moiety of stachyose.

Perhaps the most unexpected result was the finding that mannan was an effective inhibitor (50% at 0.5mg/ml) of the K88 adhesin/porcine enterocyte interaction. Mannan is a homopolymer of D-mannose linked either 1→6, 1→3 or 1→2 (see Figure 7.10). Attempts to inhibit the binding of the K88 adhesin to enterocytes with D-mannose were unsuccessful (13.1% at 250mM). No previous information on the inhibitory ability of mannan has been reported. However, it has been noted previously that galactan (a homopolymer of D-galactose) inhibits the K88 adhesin/ enterocyte interaction (Sellwood 1980a). In this present study no significant inhibition was noted with galactan although this may be attributable to the 10-fold lower concentration. Eight other polysaccharides tested were non-inhibitory. The ability of mannan to inhibit the binding of the K88 adhesin was confirmed in haemagglutination experiments (see Chapter 6). Inhibition observed in haemagglutination experiments was, however, at approximately 60-fold higher concentrations than those observed in the ELISA-based inhibition assay. Since mannan is the only one of 10 polysaccharides tested that was inhibitory, and also inhibits the haemagglutination of both guinea-pig and chicken erythrocytes then it seems that a specific mechanism is involved and must reflect in an unknown way the binding of the K88 adhesin to its receptor(s).

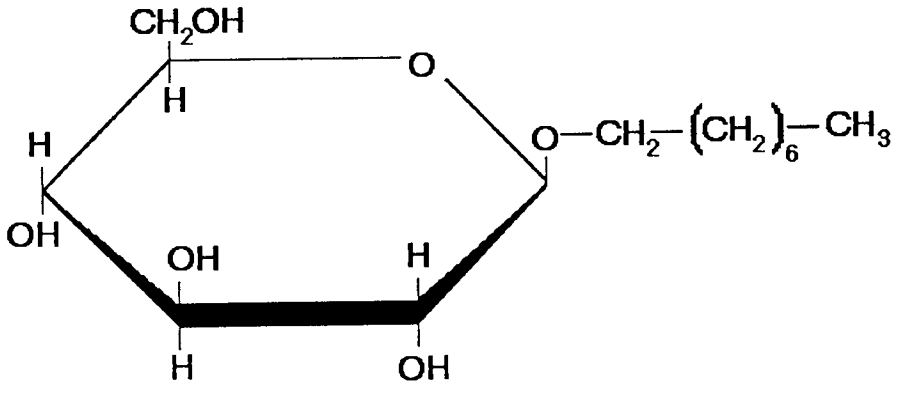
The remaining three inhibitors of the K88 adhesin/porcine enterocyte interaction are bacitracin (39.1% at 31mM), esculin (50.8% at 25mM) and n-octyl-α-D-glucopyranose (100% at 25mM). All these gave high correction factors and in the case of bacitracin and esculin, variable inhibition values. Bacitracin is a dodecapeptide antibiotic which probably binds to the microplate via its peptide component, inhibiting the binding of the K88 adhesin to the

microplate well because of its comparatively high concentration (31mM compared to $0.1x10^{-9}$mM for the K88 adhesin). Inhibition noted with esculin and n-octyl-α-D-glucopyranose (for structures see Figure 7.11) was difficult to interpretate in isolation, however, at least with n-octyl-α-D-glucopyranose this present finding agrees with the results of earlier haemagglutination experiments (see Chapter 6).

Figure 7.11 Structures of inhibitors of the K88 adhesin/porcine enterocyte interaction.



ESCULIN



n-OCTYL-α-D-GLUCOPYRANOSE

Difficulty in determining a common or indeed a potent inhibitor of the K88 adhesin/enterocyte interaction was probably multifactorial. Firstly, analysis of binding data has showed that the K88 adhesin has an extremely high affinity for porcine enterocytes (K'a of $1.27x10^{13}M^{-1}$), for an inhibitor to block or displace such powerful binding would require either a high concentration or a similar affinity. Secondly, the binding data also suggested that at least two receptors for the K88 adhesin were present on the enterocyte preparation used in the inhibition experiments. Thus an inhibitor mimicking one receptor may not prevent or perhaps at best only partially interfere with the binding of the adhesin to the second K88 receptor present. Finally, inhibition noted with chondroitin sulphate B and heparin may be attributable to non-specific mechanisms or contaminants due to the high concentrations used and probably do not reflect the structure of the K88 receptor(s).

7.4 Conclusions.

Investigation of the ability to bind cell-bound K88 adhesin revealed that all 10 porcine enterocyte preparations examined were able to bind the K88ab form of the adhesin. Eight of the enterocyte preparations were completely phenotyped for the adhesion of the three K88 adhesin serotypes. Four of the preparations examined showed weak adhesion characterised by the adhesion of 2-4 bacteria/enterocyte and poor clumping of the enterocytes present. This weak adherence phenotype was observed with various enterocyte preparations for all three of the K88 adhesin variants. Attempts to block the adhesion of bacteria expressing the K88 adhesin to porcine enterocytes by the addition of cell-free purified K88ab adhesin to the reaction mixture were complicated by the finding that 5 of the 6 strains of *E.coli* used (including serotype

O149:K91:H10 which does not express any detectable K88 adhesin) were spontaneously agglutinated by the cell-free K88ab adhesin. Therefore, although inhibition of the binding was apparent it was not possible to determine whether this was attributable to the blocking of receptor binding sites or simply the agglutination of the bacteria present. The inability of mono-specific αK88 antiserum to block the adhesion of *E.coli* serotype K12:K88ab to the porcine enterocyte preparation examined was thought to be due to the possession of a second adhesin (in addition to the K88 adhesin) by this strain.

The use of an ELISA-based assay for the examination of the binding characteristics of the K88ab adhesin/enterocyte gave valuable information. The Scatchard plot of the binding data was curvilinear in form with an overall affinity constant (K'_a) of $1.27x10^{13}M^{-1}$. Further analysis of the binding data was consistent with the hypothesis that the interaction of the K88ab adhesin with the majority of porcine enterocytes was a two-step three component system. The first receptor (or site) has an affinity (K_a) of $1.59x10^{14}M^{-1}$ with a calculated $4.3x10^4$ sites/enterocyte. The second receptor (or site) exhibits positive cooperation (Hill coefficient of 1.07) and has an overall affinity (K'_a) of $4.8x10^{12}M^{-1}$ with a calculated $1.75x10^5$ sites/enterocyte. One of the enterocyte preparations examined possessed only the receptor site showing positive cooperation. However, this did not correlate with any binding differences when examined by the microscopic adhesion assay using cell-bound K88 adhesin.

Attempts to inhibit the binding of the purified cell-free K88 adhesin to porcine enterocytes by lectins were largely unsuccessful with the most potent inhibitor, the asparagus pea lectin (which recognises α-L-Fucose residues) only inhibiting the reaction by 22.14%. Attempts to inhibit the same reaction with carbohydrates or related compounds were more successful. The amino-sugar galactosamine, the disaccharides trehalose, lactulose, Gal-1→4-Man and chondrosine, the tetrasaccharide stachyose and the polysaccharide mannan were

all inhibitors. Two glycosylaminoglycans (heparin and chondroitin sulphate B) were also found to be inhibitors while the most potent inhibitor was found to be porcine gastric mucin. Inhibition observed with esculin and n-octyl-α-D-glucopyranose was difficult to confirm in isolation since they interfered with the binding of the K88 adhesin to the microplate wells in which the experiments were performed.

8 CONCLUDING REMARKS.

Enterotoxigenic *E.coli* expressing the K88 fimbrial adhesin are associated with neonatal and post-weaning diarrhoea in swine (Runnels *et al.* 1980, Wilson and Francis 1986). Production of enterotoxins and probably other unknown virulence factors by ETEC expressing K88 fimbriae leads to a disease of major economic importance (Gaastra and De Graaf 1982, Walters and Sellwood 1982, Rapacz and Hasler-Rapacz 1986, Wilson and Francis 1986). Since K88 fimbriae confer the fundamental property of adherence (Smith and Linggood 1971, Jones and Rutter 1972), much research effort has been directed towards the characterisation of the binding of ETEC expressing the K88 fimbriae. Early work was directed at determining the expression of the K88 fimbriae *in vivo* and the site of colonisation. Subsequently, the binding of bacteria expressing K88 fimbriae to porcine intestinal slices, isolated intestinal cells, erythrocytes and mucin has been investigated. Several binding assays have used purified K88 adhesin obtained from intact K88 fimbriae by a variety of procedures. However, despite the work of several studies and unlike the receptors for e.g. K99 and P fimbriae the receptor for K88 has remained enigmatic.

This present study was conceived to further elucidate the factors affecting both the expression of the K88 fimbriae and its complementary receptor. Initially as an adaption of several existing methods a technique was developed for the extraction and purification of the K88 adhesin. The resulting, highly purified preparation was subsequently used in several experiments e.g. in the quantification of cell-bound K88 adhesin, haemagglutination and enterocyte binding. Previously, the ability of ETEC expressing K88 fimbriae to colonise the anterior small intestine unlike ETEC expressing other fimbriae was thought attributable to the greater "efficiency" of the K88 fimbriae. The development and use of a small-scale method in this study led to the finding that the extraction of the K88 fimbriae was sensitive to pH. Since the mean pH from stomach to lower intestine in piglets varies over a range of 3.5-7.2 (Williams-Smith and Linggood 1971) it was postulated that the colonisation site

of ETEC expressing K88 fimbriae is influenced by the pH stability of the fimbrial structure. The K99 fimbriae is subject to heat extraction in a similar manner to the K88 fimbriae but unlike those expressing K88 fimbriae, ETEC expressing K99 fimbriae are not associated with the colonisation of the anterior small intestine of pigs. It would be interesting to determine whether the difference in colonisation pattern between ETEC expressing K88 and those expressing K99 fimbriae would be reflected in their relative sensitivity to pH during heat extraction.

In the last decade it has become apparent that the seemingly simple adhesion of *E.coli* expressing K88 fimbriae to the porcine mucosa is more complicated than originally thought. The intact K88 fimbrial structure not only contains the major structural subunit protein (FaeG) but also several minor proteins (FaeC, FaeF and FaeH, Van Zijderveld 1990). In several other fimbrial systems e.g. type 1 it has been found that minor components are the adhesins present and not the major structural subunit. Indeed in the case of K88 fimbriae it has been suggested that one of the minor fimbrial components is responsible for the agglutination of chicken erythrocytes (De Graaf 1990). Even the properties of the K88 receptor have become increasingly complicated. Originally, it was thought that K88 receptor expression or not was controlled at one locus with one dominant gene resulting in its expression and one recessive gene resulting in non-expression (Sellwood *et al.* 1975). Subsequently, using all three known serotypes of the K88 adhesin it was found that 4 or 5 phenotypes were observed on porcine brush borders suggesting that at least 2 genetic loci were involved (Bijlsma and Bouw 1985, Rapacz and Hasler-Rapacz 1986). Even more puzzling was the observation of minor K88 adhesin binding serotypes where a mixture of the main phenotypes was found, one usually dominating the other. It was suggested that modifying genes were interacting with the loci for the K88 receptor, altering their adhesive phenotype (Bijlsma and Bouw 1985). A further difficulty in elucidating precisely how

ETEC expressing K88 fimbriae interact with the small intestine mucosa is the finding that mucin components are effectively bound by these bacteria (Conway *et al.* 1990, Metcalfe *et al.* 1991). Thus, to determine the factors involved in the adhesion of ETEC expressing K88 fimbriae allowance has to be made for differentiation of structural and adhesive components in the fimbrial structure, mucin interaction and variation both in the actual expression and possible modification of the K88 receptor present on the epithelial cells in the small intestine.

One approach to simplifying the study the adhesive properties of K88 fimbriae is to monitor the agglutination of erythrocytes by them. In this system, the effects of mucin interactions can be ignored and receptor variation minimised. In this study, a wide range of erythrocytes were examined for the ability of both cell-free and cell-bound K88 fimbriae/adhesin to agglutinate them. It was found that out of a total of 11 erythrocyte species examined, 5 were agglutinated by either or both of the cell-bound or cell-free K88 adhesin, including rabbit and marmoset erythrocytes which have not been previously noted. For reasons outlined in Chapter 6 it was assumed that the observed agglutination was attributable to FaeG the major structural component of K88 fimbriae. Previously it has been argued that K88 receptor analogues present, at least on guinea pig erythrocytes do not reflect the actual K88 receptor present on porcine cells because agglutination is unstable above 4°C. However, for several of the erythrocyte species found agglutinated by the K88 adhesin here, binding was found to be relatively insensitive to temperature, particularly that observed with rabbit erythrocytes. It seems therefore that the K88 receptor found on rabbit erythrocytes is a "better" analogue than that found on guinea pig erythrocytes for instance. However, the results of experiments presented in Chapter 7 indicate that the affinity that cell-free K88 adhesin has for the K88 receptor analogue present on rabbit and other erythrocyte species is somewhat less than the affinity it has for the receptor(s) present on porcine enterocytes.

This finding emphasized that the K88 receptor analogues present on erythrocytes are similar (to a greater or lesser degree) to the K88 receptor(s) present on enterocytes but are not identical.

In this study, the results of haemagglutination experiments supported the view that the K88 receptor is coded for by two genetic elements. None of the erythrocyte species examined showed a separation of the adhesive properties of the K88ab and K88ac adhesins although there were some quantitative differences observed. However, the agglutination of chicken erythrocytes by only the ab and ac forms of the K88 adhesin demonstrated that the adhesive properties of the K88ad adhesin was different. This finding was supported by the correlation shown by the ab and ac forms but not by the K88ad adhesin between the degree of expression and the haemagglutination titre observed with cell-bound adhesin. These results, in conjunction with previous experiments on the blocking of bacteria expressing the K88 adhesin by cell-free adhesin (Bijlsma *et al.* 1982) and those investigating the inhibitory properties of tri-peptides isolated from the K88 major fimbrial subunit FaeG (Jacobs *et al.* 1987c) are strong evidence for the separation of the binding sites for the ab/ac forms and the K88ad form of the K88 adhesin.

Attempts to inhibit the agglutination of guinea pig and chicken erythrocytes revealed that porcine gastric mucin was effective. Presumably, inhibition noted here was related to the presence of receptors for the K88 adhesin found in mucin *in vivo*. Since the mucin used in this study is available in large quantities commercially, then fractionation studies could be readily used to identify the nature of the inhibitor(s) present and possibly confirm the results of Metcalfe *et al.* (1991) who found that a 40-42KDal glycoprotein identified in porcine mucin was bound specifically by the K88 adhesin. Inhibition of the agglutination of erythrocytes by mannan, a polymer of mannose agreed with the observation that the binding of the K88 adhesin to enterocytes was also inhibited by mannan. No inhibition of either

haemagglutination or the binding of the K88 adhesin to enterocytes was observed with mannose. Neither was the binding of the K88 adhesin to enterocytes inhibited by lectins that recognise mannose. No inhibition of the binding of the K88 adhesin was noted with any of nine other polysaccharides tested. Thus, it seems that the polymeric nature of mannan confers inhibitory properties not present in the monomer, D-mannose or any of the other polysaccharides tested. It would be interesting to determine whether the K88 adhesin could bind to mannan directly.

The most potent inhibitor of haemagglutination mediated by the K88 adhesin was a glucoside, n-dodecyl-β-D-glucopyranoside. Other glucosides were also found to be inhibitory to a lesser degree, one of which was also implicated in preventing the binding of K88 adhesin to porcine enterocytes. It was postulated that the inhibitory properties of the glucosides was attributable to interference with the hydrophobic interaction of the K88 adhesin with its complementary receptor. From the number of glucosides tested it was difficult to determine whether more specific interactions with the K88 adhesin were involved. However, it may be possible to use maltosides and other detergents to determine whether more specific interactions are involved.

Previous experiments on the binding of the K88 adhesin to its *in vivo* receptor(s) has involved the formalinization of enterocytes to prevent non-specific binding (Sellwood 1980a). In this present study, the inability to obtain non-adhesive enterocytes led to the development of an ELISA-based assay for the binding of the K88 adhesin. Here, non-specific interactions could be ignored because of competition between porcine enterocytes and the microplates used for the binding of the K88 adhesin (see Chapter 7). Analysis of binding data revealed that the K88 adhesin had a very high affinity for the receptor(s) present on enterocytes. One of the enterocyte preparations examined had a different binding isotherm to the others examined. It was suggested that the simplest explanation was that the majority of porcine enterocytes have at least

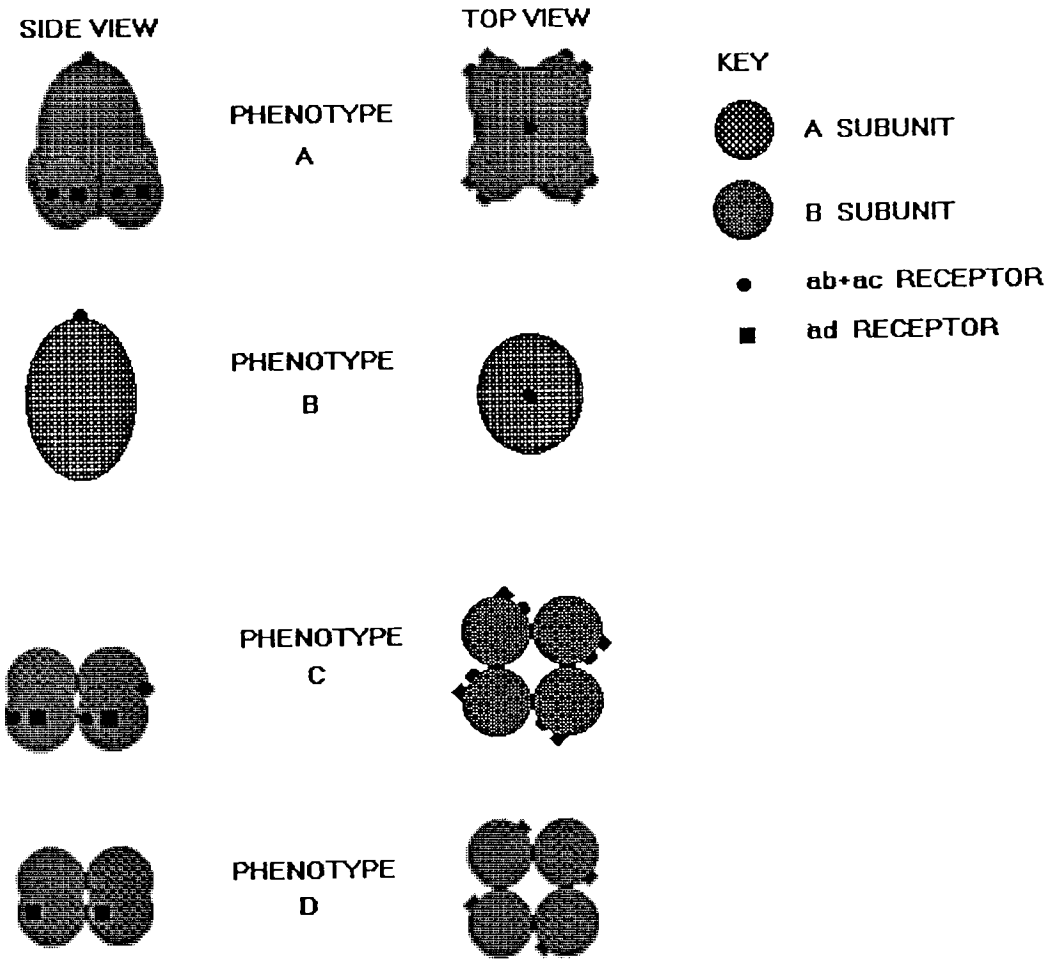
two receptors or sites for the K88ab adhesin. This may explain, at least in part, the difficulty that several workers have had in identifying a potent inhibitor of the K88 adhesin. Incubation of the K88 adhesin with a single receptor mimic may only prevent adhesion to one of the sites on the K88 receptor to which the adhesin binds. Only those inhibitors that contain both of the putative receptor mimics or interfere with the mechanism by which the K88 adhesin interacts with its receptor will be recognised as inhibitors. Thus for instance, porcine gastric mucin may contain two K88 receptor mimics that enables it to interfere with the adhesive properties of K88 fimbriae. An alternative mechanism may be that porcine mucin interferes with the hydrophobic interaction of the K88 adhesin with its receptor(s) in a similar manner postulated to occur with glucosides.

Evidence in support of the hypothesis that there are two K88ab receptors comes from the work of Staley and Wilson (1983). Using affinity chromatography and SDS-PAGE these workers isolated two major subunits from porcine enterocytes with the ability to bind $K88^+$ *E.coli*. Staley and Wilson (1983) concluded that the intestinal receptor for the binding of $K88^+$ *E.coli* was a glycoprotein that in its native state existed in multimeric forms. In studies involving the binding of radiolabelled K88 adhesin to formalinized porcine enterocytes Sellwood (1980a) found evidence for the presence of two K88 receptors. As mentioned above, based on the study of binding phenotypes, Bijlsma and Bouw (1985) stated that there were two receptors for the K88 adhesin. Further, the results of adhesion blocking experiments indicate a segregation between the receptor sites for the ab and ac forms and the ad form of the K88 adhesin.

Taking into account evidence obtained in this study and known characteristics of the K88 receptor(s) previously known, a tentative schematic diagram of the K88 receptor(s) thought possible is given in Figure 8.1.

Figure 8.1 (opposite). *Schematic diagram of the postulated K88 receptor complex present on porcine enterocytes. The K88 adhesin interacts with porcine enterocytes in a complex manner. The schematic diagram of the postulated relationship between enterocyte phenotype, adhesin binding site and receptor constitution given is an attempt to collate all the known characteristics of the K88 receptor(s). As described in the text direct isolation of the K88 receptor has indicated that it consists of two subunits (Staley and Wilson 1983). Genetic evidence obtained by Bijlsma and Bouw (1985) suggested that the K88 receptor was coded for by two elements, one responsible for the binding of the ab and ac forms and another responsible for the ad form of the K88 adhesin. Evidence obtained by Sellwood (1980a) indicated that the K88 receptor had two binding sites for the K88 adhesin. In agreement, this present study found that there were two binding sites for the K88ab adhesin. Evidence presented by Bijlsma et al. (1982) demonstrated that it was possible in a specific manner to block the adhesion of bacteria bearing K88 fimbriae by pre-incubation of the enterocytes with the various serotypes of the K88 adhesin. The enterocyte phenotypes described by Bijlsma et al. (1982) are indicated in the schematic diagram. Phenotype A consists of one type A subunit surrounded by a tetramer of type B subunits. The relative ratio of the A and B subunits is based on the finding in this thesis that the two postulated binding sites present on the porcine enterocytes were in a ratio of 4.07:1. The presence of K88ab and K88ac sites being present on both subunits while the K88ad adhesin binding site is present on only 1 subunit is to explain the results of cell-blocking experiments and the finding in this study that the K88ab adhesin binds to two sites. For instance pre-incubation with the K88ab adhesin blocks the subsequent adhesion of bacteria expressing all three serotypes of the K88 adhesin. This is attributable to the blocking of binding sites on both the A and the B subunits. Pre-incubation with the K88ad adhesin was found to block only the adhesion of bacteria expressing the homologous adhesin. In this case, even if the binding of the K88ad adhesin sterically hindered the subsequent binding of bacteria expressing the K88ab and K88ac adhesins to the B subunit they were still able to bind to the A subunit. With phenotype B enterocytes, only the A type K88 receptor subunit is present. Therefore only the ab and ac forms of the K88 adhesin can bind. The K88 receptor present on chicken erythrocytes may be analogous to the one present on phenotype B enterocytes. With phenotype C enterocytes only the B subunit is expressed. However, there has been a slight conformational change in the arrangement of the B subunits when compared to that found with phenotype A enterocytes. This results in the partial obstruction of the binding site of the ab and ac K88 adhesins but leaves the binding of the K88ad adhesin unaffected. Cell bound K88ac adhesin is unable to bind to this phenotype in a similar manner to its inability to bind to porcine erythrocytes. However, as evidenced by the ability of cell-free K88ac adhesin to bind to porcine erythrocytes, cell-free K88ac adhesin may also be able to bind to phenotype C enterocytes. Phenotype D enterocytes represent an alternative conformation of the B subunits. In this case, the K88ab and K88ac binding sites are completely obstructed and only bacteria expressing the K88ad form of the adhesin are able to bind. In the study of Bijlsma et al. (1985a) results were obtained suggesting that the K88ad binding site of phenotype A enterocytes may have two configurations in a similar manner to the two configurations of the B subunit postulated here.*

Figure 8.1


SIDE VIEW
TOP VIEW
KEY
PHENOTYPE A
A SUBUNIT
B SUBUNIT
ab+ac RECEPTOR
ad RECEPTOR
PHENOTYPE B
PHENOTYPE C
PHENOTYPE D

To further expand the work presented here and to confirm the nature of the K88 receptor(s) it is suggested that the following approaches could be used:-

1 Attempted inhibition of the agglutination of erythrocytes by the K88ad adhesin. Since it is postulated that the K88ad adhesin only binds to one receptor then it is more likely to be inhibited by simple sugars etc. than the K88ab adhesin which binds to two receptors.

2 A consequence of the ability of only the ab and ac forms of the K88 adhesin being able to agglutinate chicken erythrocytes is that the K88ad receptor analogue is either absent or altered. In effect, the ab and the ac forms of the K88 adhesin are binding to only one receptor. As in 1 above agglutination of chicken erythrocytes by K88ab or K88ac may be more amenable to inhibition by simple sugars etc.

3 Using a similar principle and reasoning to 1 and 2 above attempt to inhibit the binding of the K88ad adhesin to porcine enterocytes in general or the binding of the ab and ac forms of the K88 adhesin to B phenotype (Bijlsma 1982) enterocytes.

The work presented in this thesis was directed at elucidating the factors affecting the expression, extraction, production and purification of the K88 fimbrial adhesin and its interaction with its complementary receptor and analogues present on porcine enterocytes and various erythrocyte species respectively. Information gained and techniques developed should be directly transferable to other fimbrillar fimbriae at least e.g. the use of the small-scale method for the extraction of the K99 fimbriae. Apart from advancement in the determination of mucin components capable of binding the K88 adhesin (Laux *et al.* 1986, Metcalfe *et al.* 1991) little work on the nature of the K88 receptor(s) present on porcine enterocytes has been done since the studies of Sellwood (1980a) and Staley and Wilson (1983). The results presented here are

in agreement with the complex interaction of the K88 adhesin with its receptor(s). However, in the future careful consideration of the adhesin serotype and enterocyte phenotype may lead to the isolation of the K88 receptor complex and its subsequent characterisation.

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Please note

The majority of the abbreviations for the journals listed here are in accordance with those used in Index Medicus 1991 published by the National Institute of Health No91-267. Washington D.C. USA. Where abbreviations were not listed in the Index Medicus either the full name of the journal is given or if available an abbreviation as used in a later publication was used as a substitute.

APPENDIX 1 Media recipes

All media were sterilised at 121°C, 15psi for 15min.

20ml of media was added per Petri dish.

90mm Petri dishes were used.

Where relevant, sterile glucose was aseptically added to media when hand hot.

All media were made with Milli-Q water.

For agar equivalent, broth were supplemented with either Difco bacto-agar (BHI No1, BHI No2 and NZY, 15g/l) or agar technical No3 (M9, MINCA, NB, NB 2 and TERG, 15g/l)

Brain heart infusion No1 (BHI No1) **Difco**

Calf brains, infusion from	200g/l
Beef heart, infusion from	250g/l
Proteose peptone	10g/l
Dextrose	2g/l
Sodium chloride	5g/l
Disodium phosphate	2.5g/l

Media rehydrated according to manufacturers instructions 37g/l.

pH 7.3

Brain heart infusion and supplements (BHI No2) **Difco**

as Brain heart infusion No1 except supplemented with:-

Bacto peptone	10g/l
NaCl	3g/l
$NaHPO_4$	2g/l

pH 7.5

Minimal media and casamino acids (MINCA)

KH_2PO_4	1.36g/l
Na_2HPO_4	10.10g/l
Trace salts	1ml
Casamino acids	1.00g/l
0.1% w/v glucose	1.00g/l

pH 7.5

Trace salts

$MgSO_4.7H_2O$	10g/l
$MnCl_2.4H_2O$	1g/l
$FeCl_3.6H_2O$	0.135g/l
$CaCl_2.2H_2O$	0.4g/l

Nutrient broth (NB) **Oxoid**

Lab-lemco powder	1g/l
Yeast extract	2g/l
Peptone	5g/l
NaCl	5g/l

pH 7.4

Nutrient broth No2 (NB 2) **Oxoid**

Lab-lemco powder	10.00g/l
Peptone	10.00g/l
NaCl	5.00g/l
KH_2PO_4	0.45g/l
Na_2HPO_4	0.81g/l

Glucose 0.1% w/v Final concentration

pH 7.5

NZY

NaCl	5g/l
$MgSO_4.7H_2O$	2g/l
Yeast extract (Difco)	5g/l
Casein hydrolysate	10g/l

pH 7.5

Tergitol-7 (TERG) **Difco**

Proteose peptone No3	5.00g/l
Bacto-yeast extract	3.00g/l
Bacto-lactose	10.00g/l
Tergitol-7	0.01g/l
Bacto-brom thymol blue	0.02g/l

pH 6.9

Tryptone soya **Oxoid**

Pancreatic digest of casein	17.0g/l
Papain digest of soyabean meal	3.0g/l
Sodium chloride	5.0g/l
Dibasic potassium phosphate	2.5g/l
Dextrose	2.5g/l

pH 7.3

APPENDIX 2 **Results of inhibition studies on the K88 adhesin/porcine enterocyte interaction.**

SUGAR/COMPOUND	CONCENTRATION	% INHIBITION
PENTOSES		
1 D-arabinose	250mM	13.1
2 L-arabinose	250mM	15.7
3 D-lyxose (P)	250mM	11.5
4 L-lyxose	250mM	20.2
5 D-ribose (P)	250mM	26.2
6 D-xylose	250mM	7.5
7 L-xylose (P)	250mM	8.9
HEXOSES		
8 D-allose	139mM	20.3, 28.3
	28mM	7.1, 7.7
9 L-allose	139mM	11.9, 19.6
	28mM	10.7, 0.0
10 dextrose (P)	250mM	23.4
11 D-fructose	250mM	27.9
12 D-galactose	250mM	19.7
13 α-D-glucose	250mM	13.1
14 β-D-glucose	250mM	14.8
15 D-mannose	250mM	13.1
16 L-mannose	250mM	0.0
17 L-sorbose	250mM	17.2
18 D-sorbose	139mM	5.1, 4.4
19 glucose-6-phosphate	250mM	14.5

HEPTOSES		
20 D-glucoheptose	250mM	9.7
21 D-mannoheptose	250mM	11.2
DISACCHARIDES		
22 cellobiose	250mM	0.0
23 Gal 1→3 Ara	24mM	14.0, 1.7
	3mM	0.0
24 lactose hydrate	250mM	16.4
25 lactulose	250mM	53.7, 47.4
	125mM	46.4
	63mM	17.3
	22mM	8.5, 8.8, 0.0
	4mM	0.0
26 melibiose (P)	250mM	48.4
	125mM	29.6
	63mM	25.9
	32mM	19.1
	22mM	10.5, 5.1
	7mM	0.0, 1.0
27 melibiose	250mM	6.7
	125mM	11.8
	63mM	10.6
	32mM	13.6
28 maltose	250mM	26.2

29 sucrose	250mM	19.7
30 trehalose	250mM	50.8, 80.3
	125mM	28.2
	63mM	16.4
	32mM	18.5
	23mM	10.5, 8.5
	7mM	4.1, 0.0
31 turanose	250mM	14.2
32 Gal 1→4 Gal	31mM	10.5, 3.4
	7mM	15.5, 0.0
33 Gal 1→4 Man	73mM	63.2, 32.2
	7mM	11.3, 4.0
34 Gal 1→6 Gal	7mM	1.0, 0.0
TRISACCHARIDES		
35 melezitose	250mM	19.3
36 raffinose	250mM	21.9
37 Gal→Gal→Glu	4mM	0.0
TETRASACCHARIDES		
38 stachyose	94mM	44.1, 82.6
	19mM	16.1, 12.8
39 stachyose tetradecacetate	5mM	0.0
POLYSACCHARIDES		
40 α-cyclodextrin	0.25mg/ml	0.7
41 β-cyclodextrin	0.25mg/ml	0.0
42 glycogen type 3	2.5mg/ml	0.0
43 glycogen type 3	2.5mg/ml	0.0
44 inulin	2.5mg/ml	0.0

45 pullulan	2.5mg/ml	14.8
46 starch	2.5mg/ml	0.0
47 xylan	2.5mg/ml	0.0
48 mannan	2.5mg/ml	93.4, 80.3
	1.0mg/ml	67.2
	0.5mg/ml	50.0
	0.25mg/ml	17.7
	0.13mg/ml	13.4
49 galactan	10mg/ml	23.3, 9.1
<u>THIO-SUGARS</u>		
50 thio-β-D-galactopyranose	250mM	9.6
51 thio-β-D-glucose	250mM	6.7
52 thio-digalactoside	125mM	19.1, 14.7
<u>AMINO-SUGARS</u>		
53 N-acetyl-D-galactosamine	250mM	16.4
54 N-acetyl-D-glucosamine	250mM	14.8
55 D-galactosamine	250mM	47.8
	125mM	31.8, 27.3, 16.7
	63mM	18.0
	32mM	11.3
	25mM	1.5
56 D-glucosamine	250mM	16.0
	125mM	5.6, 11.1
	25mM	0.0
57 D-mannosamine	250mM	14.5
	125mM	7.9, 4.0
	25mM	0.0
58 D-lyxoslamine	250mM	17.8

		References
59 N-acetyl-β-D-mannosamine	250mM	11.9
60 α-D-galactosamine-1-phosphate	250mM	7.4
DEOXY-SUGARS		
61 6-deoxy-D-glucose	250mM	6.7
62 2-deoxy-D-glucose (P)	250mM	21.2
63 2-deoxy-D-galactose	152mM	0.0, 1.1
64 2-deoxy-D-ribose (P)	250mM	10.6
65 D-fucose	250mM	10.9, 3.1, 1.5, 0.0
66 L-fucose (P)	250mM	14.0, 3.1, 0.0
67 L-rhamnose (P)	20mM	19.7
SUGAR-ALCOHOLS		
68 D-arabinitol (P)	250mM	14.2
69 L-arabinitol (P)	250mM	2.7
70 erythritol (P)	250mM	0.0
71 galactitol (P)	250mM	0.0
72 inositol (P)	250mM	10.6
73 D-mannitol	250mM	9.8
74 ribitol (P)	250mM	3.1
75 sorbitol (P)	250mM	6.3
76 xylitol (P)	250mM	0.0
GLYCOSIDES		
77 methyl-α-D-galactopyranoside	152mM	0.0
78 1-O-methyl-β-D-galactopyranoside	250mM	0.0, 0.0
79 methyl-α-D-glucopyranoside	250mM	12.4, 1.5
80 methyl-β-D-glucopyranoside	125mM	27.0, 19.1

81 3-O-methyl-D-glucopyranose	125mM	9.0, 0.0
82 methyl-α-D-mannopyranose (P)	250mM	7.8
83 methyl-α-D-xylopyranoside (P)	250mM	9.4
84 methyl-β-D-xylopyranoside	250mM	6.3
85 n-octyl-α-D-glucopyranose (P)	125mM	100.0, 100.0
	25mM	100.0, 100.0
	5mM	5.1, 5.7
86 n-octyl-β-D-glucopyranoside	saturated soln.	2.3, 0.0
87 salicin (P)	250mM	10.9
88 6-O-methyl-D-galactose	250mM	3.1, 0.0

GLYCOSYLAMINOGLYCANS (or derivatives)

89 heparin	56mg/ml	58.1, 63.6
	25mg/ml	70.2, 81.4
	11mg/ml	19.6, 5.9
90 heparin Lmwt	100mg/ml	11.6, 0.0
91 chondroitin sulphate A	100mg/ml	0.0, 0.0
92 chondroitin sulphate B	100mg/ml	16.3, 45.5
	20mg/ml	26.1, 23.5
93 chondrosine	50mM	100.0, 79.7

MISCELLANEOUS

94 porcine gastric mucin	2.5mg/ml	82.0, 80.3
	1.0mg/ml	82.0
	0.5mg/ml	100.0
	0.25mg/ml	40.3
	0.13mg/ml	22.4
95 mucic acid	10mg/ml	23.3, 13.6
96 polymyxin B sulphate	7mg/ml	6.8, 15.2
97 bacitracin	31mM	40.7, 39.1

98 esculin	250mM	59.4
	125mM	100.0, 100.0
	63mM	31.8
	32mM	76.2
	25mM	100.0, 50.8
	5mM	0.0, 0.0

All results given are the mean of triplicates in individual experiments. In all cases where significant inhibition was detected experiments were repeated and dose dependant results determined. All compounds tested were obtained from Sigma chemical company unless noted. Where (P) = supplied by Pfanstiel labs. inc., heparin Lmwt = low molecular weight heparin (approx. 4-6KDal).