Disease mechanism of enteropathogenic Escherichia coli.

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by

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To:

My wife Alison.

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CONTENTS

ABSTRACT

Colonization of gut mucosal surfaces by enteropathogenic *Escherichia coli* (EPEC) elicits a severe persistant diarrhoea in infants and young children without elaboration of enterotoxins or tissue invasion. The formation of a distinct ultrastructural lesion involving intimate adherence to cell surfaces, loss of microvilli and membrane perturbations, however, has for some time been recognized as an important component of pathogenesis, but the processes involved in lesion formation and its relevance to the disease state remained obscure.

In this study intimate adherence of EPEC to surfaces of cultured cells has been shown to elicit specific changes reminiscent of cellular activation by various hormones and growth factors via signal transduction pathways. The formation of two second messengers, 1,4,5-inositol trisphosphate and diacylglycerol by the activity of phospholipase C (PLC) on membrane phosphatidylinositol lipids is known to promote release of calcium from intracellular stores and to activate protein kinase C (PKC) respectively. Therefore the observed intracellular calcium elevation, calcium-dependent actin accretion at sites of bacterial attachment, release of glycosyl-phosphatidylinositol (GPI) anchored proteins, eventual loss of host cell viability, and phosphorylation of target cell proteins on EPEC infection of cultured cells, are consistent with a mechanism of diarrhoeagenesis that involves activation of a host cell signal transduction pathway terminating in PLC activity.

I propose that the rapid onset of severe secretory diarrhoea associated with EPEC infections is induced by phosphorylation of particular ion transport proteins in the microvillus membrane, mediated by calcium-dependent kinases and PKC. In addition reduction in the surface area of infected regions of the gut, which may itself enhance hypersecretion by preventing absorption, can be explained by calcium-dependent breakdown of the microvillus cytoskeleton.

Chapter 1. GENERAL INTRODUCTION.

The work presented in this thesis concerns the diarrhoeagenic mechanism of enteropathogenic *Escherichia coli*, an important cause of infant morbidity and death throughout the world. I concentrate on physiological and biochemical changes in host cells induced by bacterial attachment, and have developed an integrated model of the diarrhoeal mechanism based upon the experimental findings. In this Introduction I give an overview of the long history of diarrhoeal diseases with particular emphasis on severe protracted enteritis of infants and the identification of the aetiological agent, and conclude with a description of the pathogenic mechanism as known at the start of this project.

1:1. *Historical overview of diarrhoeal disease.*

Perhaps one of the earliest records of severe diarrhoeal disease affecting a population comes from a graphic description in the Bible (**II Chronicles, chapter 21, verses 14-19**) of an epidemic that raged through the country of Judah around 900 BC during the reign of King Jehoram. From an examination of the narrative it appears that the writer is describing classical dysentery affecting men, women, children and animals, which was described as "having great sickness by disease of thy bowels, until thy bowels fall out by reason of the sickness day by day". The fact that this example from 3,000 years ago may appear stilted to a few readers in this early seventeenth century English translation, and would be an impossibility for the majority of us in its original ancient Hebrew, highlights some of the problems when looking at records from a different era. Firstly, the conventions governing the style of writing change as the language evolves with time; secondly, different geographical regions adopt diverse terminologies to describe similar events, which causes problems in translation; and thirdly, descriptions will always change as a greater scientific understanding of the event becomes available. A description of a similar event occurring today in the latter half of the twentieth century would obviously read very differently, thanks in the main

to the advances in scientific knowledge, but also to the fact that it would be contemporary to both reader and writer, and would almost certainly be reported to interested persons in a scientific journal, written in English (the scientific standard language). These points are considered in the next section which appraises the severity of infantile diarrhoea in the past by looking at information sources from the 17th, 18th, and 19th centuries. The fact that scientists and physicians were the authors in no way guarantees consistency of terms between authors of different times and places. For example, bacterial names and symptom descriptions have changed enormously even over the last 45 years. Reading the early editions of Topley and Wilson or Bergy we discover that many of the familiarities of modern bacteriology, such as *Escherichia coli*, are either absent, or incognito under a previous name.

Recent advances in the study of infantile diarrhoea come at the end of a long history of a disease that all but obliterated the young population of Europe and the New World during the second half of this millenium. Records from the 17th century clearly convey the enormity of the problem (**Creighton, 1975**), with parts of London showing more burials of infants than baptisms during the summer months of several years (baptisms being compulsory at that time, the baptismal registers gave a better indication of births than some of the other official documentation). In London during the period 1667 to 1720, there were 328,231 recorded infant deaths resulting from "summer diarrhoea", an average of over 6,000 per year. Eight week periods in August and September for six hot summers during this time showed that in each case a quarter or more of the annual deaths from infantile diarrhoea occurred within these two summer months. However, over the 53 years the percentage of the annual deaths of infants by diarrhoea during this two month period showed a downward trend. By looking at the figures for the weeks either side of this narrow eight week window, it appears that by 1700 the high summer incidence of infantile diarrhoea had broadened out to include most of July. Whether this reflects climate changes or is simply the result of a growing population is uncertain.

In roughly the first half of this period one of the major causes of mortality was recorded in the death registers as "griping of the guts" which from various sources appeared to be an entry used for infants or young children that had died from cholera-like diarrhoea (**Creighton, 1975**). This condition cannot be attributed to true cholera, however, since there was no increase in the incidence of cholera-like diarrhoea among the adult population as would be expected; likewise dysentery (which also has distinguishable symptoms) can be excluded. The incidence of "griping of the guts" in registers reduced dramatically from 1692 to be replaced by the entry "convulsions". The age range and seasonal incidence of the entries suggests that this reflects a change in terminology rather than the decline of one disease and the coincident rise of another (**Creighton, 1975**). By the hot summers of 1717 to 1729 infant mortality was as high as ever but the register showed almost complete replacement of "griping of the guts" by "convulsions". These two terms were purely descriptive of the disease in question, in the same way as "lock jaw" and "consumption" describe diseases arising from infection with *Clostridium tetani* and *Mycobacterium tuberculosis* respectively. With no effective treatment, severe protracted diarrhoea in infants led to malnutrition, dehydration, and fever ultimately leading to convulsions and death (**Bacon-Wood, 1847**), which explains to some extent the two terms used for the same condition.

Around this time the American physician Benjamin Rush recognized the correlation between warm weather and the increased incidence of severe infantile diarrhoea (**Rush, 1789**) and dismissed the common notion that it was associated with dentition. In the view of many physicians of the day, the change in temperament associated with the discomfort experienced as teeth break through the gum could often predispose infants to convulsions and diarrhoea. Although this notion cannot really be taken seriously, it is conceivable that the increase in oral investigation with dirty fingers at this time may be a significant cause of infection. Rush noted that the disease was common in all the big towns and cities of America, but only at warmer times of year. Southern towns such as Charleston, South Carolina, experienced the onslaught during April and May, hence the name "April and May disease", while in more

northerly towns the disease was virtually absent until June or July, but was most common in September.

Some of the treatments recommended by Rush to alleviate the suffering of victims appear to be based on sound medical practice, even by today's standards. Many of his infusions, for example, would have been beneficial by restoring the electrolyte balance of the patient, effecting a rapid improvement in condition. Rush also noted with some interest the craving of some patients for salted foods and the subsequent apparent improvement in their condition. The use of opiates, which today are known to be antagonists of certain electrolyte transport processes was also strongly recommended by Rush as an effective remedy for many sufferers when used in conjunction with infusions (**Rush, 1789**). Whether the improvement of his patients was coincidental or whether his treatments did effect relief or cure is not certain, although many of his remedies are reminiscent of modern rehydration therapy. Cure or coincidence, Benjamin Rush was one of the first to make a comprehensive study of the causes and cures of infant diarrhoea and drew attention to the need for sanitation and cleanliness in the fight against it.

By the middle of the nineteenth century another switch in reporting practice had occurred in England, this time bringing clarity to the real cause of many of the childhood deaths; entries of "death by diarrhoea" increased while those under "death by convulsions" showed a coincident decline. The year 1846 was marked by an unusually high incidence of adult death by cholera, and an even higher number of infant deaths by diarrhoea. This high level of diarrhoeal deaths among infants, unlike those of adults, was maintained well into the latter half of the nineteenth century. Clearly this was not due to an annual cholera epidemic restricted to infants; more likely it indicates a change in the aetiology of summer diarrhoea (**Creighton, 1975**) resulting from increases in the population with worsening sanitation and housing.

Examination of the diarrhoeal death rates and employment profiles for the main English cities during the latter half of the nineteenth century reveals that during the decade 1871 to 1881 many provincial towns were expanding to accommodate their fast

growing industrial populations, producing housing environments which in some cases were worse than in parts of London. Prior to this period London, the largest urban complex, suffered more than one diarrhoeal death per 1000 head of population, mostly among infants under one year of age, a rate far higher than the rest of England and Wales. Following major industrialisation of towns and cities elsewhere in England and Wales, infantile summer diarrhoea and accompanying deaths rose in these areas to rival London. The number of deaths per thousand births of infants under one year of age due to summer diarrhoea showed a correlation with the percentage of women employed in the industries of the town (**Creighton, 1975**). Cities such as Leicester and Preston with high infantile death rates attributed to diarrhoea also showed high numbers of women employed in factories (**Creighton, 1975**), the majority of whom were of child-bearing age, and presumably mostly married with children. Also, working women did not breast feed their infants, preferring bottle feeding for convenience, with all the problems of maintaining sterility that this entails (**Creighton, 1975**). The increased popularity of bottle feeding in early industrial Britain, along with the major hygiene problems of communal nurseries, no doubt increased diarrhoeal deaths among infants but it was by no means the only factor involved; children of post-nursing age also suffered summer diarrhoea, although death rates were lower (**Creighton, 1975**). Many writers of the time attributed the large number of infant deaths during summer and autumn months solely to city living. However it appears that rural areas experienced a similiar, but smaller, rise in diarrhoeal deaths in warmer months.

Up until the end of the nineteenth century, annual country-wide figures for deaths by infantile diarrhoea averaged around 15,000. Notable exceptions were years with hot summers (1880, 1884, 1886, and 1893) when this figure doubled. From 1900 to the beginning of the First World War, the number of infant deaths by diarrhoea per thousand live births decreased from 41.3% to 28% (**Taylor, 1954**). This decline continued after the First War down to 10.9% by the start of the Second World War, after which it decreased further to 0.8% by 1951. There were still quite large numbers of

cases, but the survival rate was much higher. Up until the 1960's summer diarrhoea was prevalent and a cause for serious concern, but with the improved treatment and sanitation, death rates and incidence rates were much lower than had been previously known. Within the last 20 years there have been several outbreaks or epidemics of infantile diarrhoea but these have generally been confined to hospitals and institutions. At present the normal incidence of infantile diarrhoea (for which the aetiological agent has now been identified, **see sections 1:2 and 1:3**) is comparatively low, with small regional hospital laboratories identifying as few as 60 episodes per year.

1:2. *The causative agent of summer diarrhoea.*

When in 1939 Dr. John S.B. Bray started work at the Hillingdon Hospital, Middlesex, as an emergency medical service pathologist, his interest in infantile diarrhoea was aroused by the large number of bottle fed infants among his subjects (**Finlay, 1973**). These children died of severe gastroenteritis (or, as it was termed then, "summer diarrhoea", "cholera infantum", or "disease of the season") which was characterised by apparently stable subjects rapidly becoming critically ill and on the point of death within hours. Bacteriological and pathological examinations showed little or no difference between gastroenteritis patients and healthy individuals, except for a characteristic seminal smell associated with the more severe cases. Culture plates from such diarrhoea patients with apparently normal faecal flora also had this characteristic seminal smell and were termed "smeller" cultures. In an attempt to discover clinically important differences between apparently pathogenic bacteria and their innocuous counterparts, Bray undertook extensive sugar fermentation profiles for "smeller" and "non-smeller" cultures. The only difference was that "smeller" bacteria isolated from cases of severe infantile diarrhoea fermented maltose slowly while strains from healthy individuals gave no indication of maltose fermentation.

Bray went on to raise antibodies to one of these strains by inoculation of his pet rabbit Snowy with the so-called Wickens strain, isolated from an infant with severe protracted diarrhoea who later died. Over a four year period many strains isolated

from healthy and diseased infants, as well as from human adults and animals, were tested with this antiserum to determine whether agglutination could be observed, and the degree of its specificity for the slow maltose fermenters. Eventually Bray was able to state conclusively that his antiserum was specific for *Bacterium coli var. neapolitanum* isolates from human infants with severe protracted diarrhoea. This organism was also isolated from healthy infants, but these were later explained by the fact that the infants had either recently had an episode of diarrhoea or that they soon developed the disease. The initial results published in 1945 showed that antigenically homogeneous *Bacterium coli* strains were associated with outbreaks of summer diarrhoea among infants (**Bray, 1945**). The Wickens antiserum agglutinated bacteria isolated from stool cultures of 95% of infants with severe summer diarrhoea, compared to only 4% of controls without diarrhoea. Almost half of the summer diarrhoea cases encountered by Bray were considered to have been acquired in hospital, which caused considerable concern, not least because 28-30% of affected infants died. A later article elaborated the procedure of slide agglutination used to detect this group of enteric pathogens (**Bray and Beavan, 1948**), which is fundamentally the same procedure used routinely today. These findings were soon corroborated by groups all round the world, establishing *Bacterium coli* (or *E. coli* as it is now known) as the causative agent of summer diarrhoea in infants.

1:3. *Limited serogroups of E. coli isolated from infantile diarrhoea.*

The development of a serotyping system for *E. coli* (**Kauffmann, 1947**) enabled a systematic categorisation of isolates based on the presence of heat stable lipopolysaccharide (LPS) O antigens on the cell surface. O antigen specific antisera were prepared by immunization of rabbits with bacteria heated at 100°C for 2 h to remove heat labile surface antigens, revealing the stable O antigens of the LPS. Accurate O serogrouping of clinical isolates was achieved by mixing dilutions of antisera with bacterial isolates heated at 100°C for 2 h to obtain end point titres.

For full serotyping of strains, the heat labile flagellar H antigen type was also determined using antisera raised against the whole organism with the O specific agglutinins absorbed out. The presence of other heat labile surface antigens comprising an envelope or capsule around the bacteria was initially determined by the inability of O specific antisera to directly agglutinate certain isolates of *E. coli* without first being heated at 100°C (**Kauffmann, 1947**). These antigens, designated K, occurred in three forms, A, B, or L. The majority of *E. coli* possessed K antigens of type L, which were easily removed by the heat treatment at 100°C. *E. coli* isolated from outbreaks of infantile diarrhoea however, were found to possess a K antigen of the B type. Consequently these isolates were for some time given a B serotype distinct from the normal K antigens. Eventually the B antigens were incorporated into the K numbering system, but still the full serotype of infantile diarrhoea isolates often contained the B as well as K serotypes. More recently it has been discovered that the B antigens are not in fact part of the K antigen group, but rather consist of a heat sensitive portion of the O antigen of LPS (**Levine and Edelman, 1984**).

Shortly after Bray's publication (**Bray, 1945**), two serogroups were recognised as being associated with infantile diarrhoea, O55 and O111. Attempts to show pathogenicity of these strains for young animals initially failed, leading some workers to turn to human subjects. The morally dubious step of infecting a two month old child having multiple congenital defects with 10^8 *E. coli* of serogroup O111, led to the development of severe diarrhoea, convincing many workers of their pathogenicity for young humans (**Neter and Shumway, 1950**). With later adult volunteer studies, healthy individuals infected with O111 *E. coli* strains isolated from a cases of neonatal enteritis, were also shown to develop diarrhoea (**Kirby et al., 1950**). Larger studies, in which healthy adults were infected with O55 and O111 *E. coli* strains isolated from a cases of severe protracted infantile diarrhoea, showed that the severity of symptoms was related to the size of inoculum administered (**Ferguson and June, 1952**). Around this time, observations that cultures of frozen faeces from nosocomial outbreaks of infantile diarrhoea during 1947 in New York State, were predominantly of the serogroup O111

gave the first indication that *E. coli* enteropathogens belonged to a limited set of serogroups (**Neter et al., 1953**). The term enteropathogenic *E. coli* (EPEC), coined to describe strains incriminated with infantile diarrhoea (**Neter et al., 1955**), were by 1957 known to be associated with at least 13 O serogroups (**Dulaney and Michelson, 1935; Bray, 1945; Biering-Sorensen et al., 1947; Bray and Beavan, 1948; Giles et al., 1949; Charter and Taylor, 1952; Taylor and Charter, 1952; Ewing et al., 1955; Orskov, 1955; Taylor and Charter, 1955; Charter, 1956; Ewing et al., 1956; Ewing et al., 1957**), including O18ab, O18ac, O26, O44, O55, O86, O111, O114, O119, O125, O126, O127 and O128. By the early 1980's two additional serogroups were cited as being associated with infantile diarrhoea, O142 and O158 (**Orskov et al., 1960; Rowe et al., 1974; Rowe, 1979**).

Between 1950 and 1961 it was realised that different serotypes within incriminated serogroups were not equally pathogenic and that the ability to cause diarrhoea in infants was associated with particular O:H serotypes (**Ewing et al., 1963**). Each EPEC serogroup could have up to four H types which were associated with ability to cause disease (**Rowe et al., 1974; Rowe, 1979; Robins-Browne, 1987**). Some serogroups of *E. coli*, however, were rarely found in healthy individuals (e.g. O55) and could be regarded as potentially pathogenic without the need for full serotyping (**Robins-Browne, 1987**). Other serogroups commonly found in normal intestinal flora had only one serotype implicated in diarrhoeal disease (e.g. O86:H34) and required full serotyping for identification (**Ewing et al., 1963; Orskov, 1954**).

1:4. *Pathogenic mechanisms of E. coli enteropathogens.*

By the early 1970's *E. coli* strains had been identified which elaborate soluble enterotoxins responsible for intestinal hypersecretion in infected patients (**Gorbach et al., 1971; Levine, 1987**). Producing strains, termed enterotoxigenic *E. coli* (ETEC), colonise the small bowel causing a secretory type diarrhoea mediated by two toxin types (**Sack et al., 1980**), heat labile enterotoxin (LT), which has structural and antigenic similarities to cholera toxin (**Levine et al., 1983**), and two heat stable enterotoxins (STa and STb). LT promotion of diarrhoea involves modification of the host cell adenylate

cyclase through ADP-ribosylation of the N_s regulatory protein (**Kahn and Gilman, 1984**). Elevation in cytoplasmic levels of cyclic adenosine monophosphate (cAMP) causes activation of cAMP-dependent protein kinases (**Kahn and Gilman, 1984**), leading to phosphorylation of several intestinal brush border membrane proteins thought to be involved in electrolyte and water efflux (**de Jonge, 1984; Donowitz et al., 1984**). STa and STb are stable to both heat and extremes of *p*H by virtue of the abnormally high proportion of cysteine residues they contain. The genes for STa and STb, which show no apparent sequence homology (**So and McCarthy, 1980; Moseley et al., 1983**), are carried on phage genomes resident in toxigenic strains of *E. coli*. As well as being structurally and genetically diverse, STa and STb also exhibit different mechanisms of diarrhoea production. STa binds to unique intestinal receptors in segments of the small and large intestine (**Giannella et al., 1983; Frantz et al., 1984**), and activates the host particulate form of guanylate cyclase (**Guerrant et al., 1980; Rao et al., 1980**); this leads to elevated cytosolic levels of cyclic-guanosine monophosphate (cGMP), and increased cGMP-dependent protein kinase activity (**de Jonge, 1976; de Jonge, 1981; de Jonge, 1984a**). STb, on the other hand, promotes diarrhoea by a mechanism which at present remains obscure but seems to independent of cyclic nucleotides.

Enteroinvasive *E. coli* (EIEC), recognised in 1971 as a cause of invasive dysenteric diarrhoeal disease are physiologically and pathogenically similar to *Shigella* (**DuPont et al., 1971; Kopecko et al., 1985**). Both contain large plasmids carrying genes essential for cell attachment, invasion and spread to adjacent cells (**Harris et al., 1982; Hale et al., 1985; Makino et al., 1986; Rennels and Levine, 1986**). *Shigella* require three regions of the chromosome for full virulence linked to the histidine locus (*his*) at 44 min, the purine E locus (*purE*) at 12 min, and the arginine-mannitol region (*arg-mtl*) at 74 to 90 min (**Sansonetti et al., 1983; Derbyshire et al., 1989**). Experiments where segments of the *Shigella flexneri* chromosome were conjugally transfered to K-12 strains of *E. coli* revealed the identity of these determinants, with the *his* locus associated with the O antigen of LPS (**Sansonetti et al., 1983**), the *purE* locus with provocation of

keratoconjunctivitis in the guinea pig eye (Sereny test, **Sansonetti et al., 1983**) and the *arg-mtl* region with the aerobactin system of iron uptake (**Derbyshire et al., 1989**). The aerobactin system in *Shigella* probably constitutes an important virulence determinant as it does in other invasive organisms, and with minor differences it is very similar to the equivalent system in *E. coli* pathogens (**Derbyshire et al., 1989**). The production of high levels of the potent Shiga or Shiga-like cytotoxin, which kills cells by inhibiting host protein synthesis through cleavage of the 60S ribosomal subunit (**Thompson et al., 1976; Reisbig et al., 1981**), is also important in virulence.

Identification of EPEC as an enteropathogen prompted enormous interest in the diarrhoeal mechanism of these organisms, and yet no obvious virulence determinants could be identified (**Levine and Edelman, 1984**). Nevertheless, the pathogenicity of EPEC strains shown by their capacity to cause diarrhoea in volunteers was convincing (**Kirby et al., 1950; Ferguson and June, 1952**). The inability of EPEC to invade or produce any recognisable enterotoxin caused a number microbiologists to consider that EPEC isolates had lost plasmids encoding the genes for pathogenic determinants during storage (**Gangarosa and Merson, 1977**). Others suggested that EPEC comprised a group of enteropathogens with a diarrhoeagenic mechanism distinct from cell invasion or soluble toxin production (**Gross et al., 1976; Gurwith et al., 1977**). The situation was finally resolved by showing that EPEC (including those that had been in storage for some time) were able to cause a severe diarrhoea in adult volunteers despite the fact that they were unable to invade or produce the recognised enterotoxins LT and ST, thus confirming their pathogenicity, and its independence of all recognised diarrhoeagenic mechanisms (**Levine et al., 1978**). Some EPEC strains were discovered to produce low levels of a Shiga-like toxin when grown in iron-depleted medium (**O'Brien et al., 1982; Echeverria et al., 1987a; Echeverria et al., 1987b**), but its relevance to the overall diarrhoea process was inconclusive since there was no correlation between the amount of toxin produced and the ability of strains to cause disease (**Levine et al., 1978; O'Brien et al., 1982**).

1:5. *Pathogenic mechanism of EPEC.*

1:5:1. The attaching and effacing (AE) lesion of EPEC. The first ultrastructural examination of EPEC infected gut tissue, carried out on piglets infected with *E. coli* O55:H7, revealed a series of characteristic changes in the absorptive cells of the gut, including lengthening and loss of microvilli at sites of bacterial adherence, followed by invagination and thickening of the plasma membrane and increased electron density in the adjacent cytoplasm (**Staley et al., 1969; Figure 1.1**). These observations were later confirmed using rabbit ileal loops (**Polotsky et al., 1977**) and suckling rabbits (**Peeters et. al., 1984b**) as models of infection. The term attaching and effacing *E. coli* (AEEC) was coined to describe more fully EPEC which cause these morphological changes in infected gut tissue (**Moon et al., 1983; Peeters et al., 1984a**). Following initial attachment to gut microvilli, EPEC adhere intimately to intestinal cell surfaces, leading ultimately to localized loss of microvilli and gross ultrastructural changes in the cell (**Moon et al., 1983**). The plasma membrane swells around the attached bacteria to form what has become known as the pedestal, which corresponds with the previously described regions of increased electron density in the cytoplasm (**Knutton et al., 1987b**).

Ultrastructural studies of biopsy material from human patients with EPEC diarrhoea confirmed the formation of the attaching and effacing (AE)-lesion *in vivo*, with effacement of microvilli from the enterocyte surface in the vicinity of attached bacteria and cupping of the plasma membrane around bacteria forming a pedestal (**Ulshen and Rollo, 1980; Rothbaum et al., 1982; Rothbaum et al., 1983; Taylor et al., 1986**). Many of the strains isolated during the course of these studies were later confirmed to be of the AEEC group by the fact that they induced similar ultrastructural changes in the guts of experimental animals (**Moon et al., 1983; Tzipori et al., 1985**).

It is evident from gut biopsies of children with protracted EPEC infections that gross histological damage occurs to the gut surface with almost complete destruction of the absorptive area (**Rothbaum et al., 1982, Rothbaum et al., 1983**). Examination of

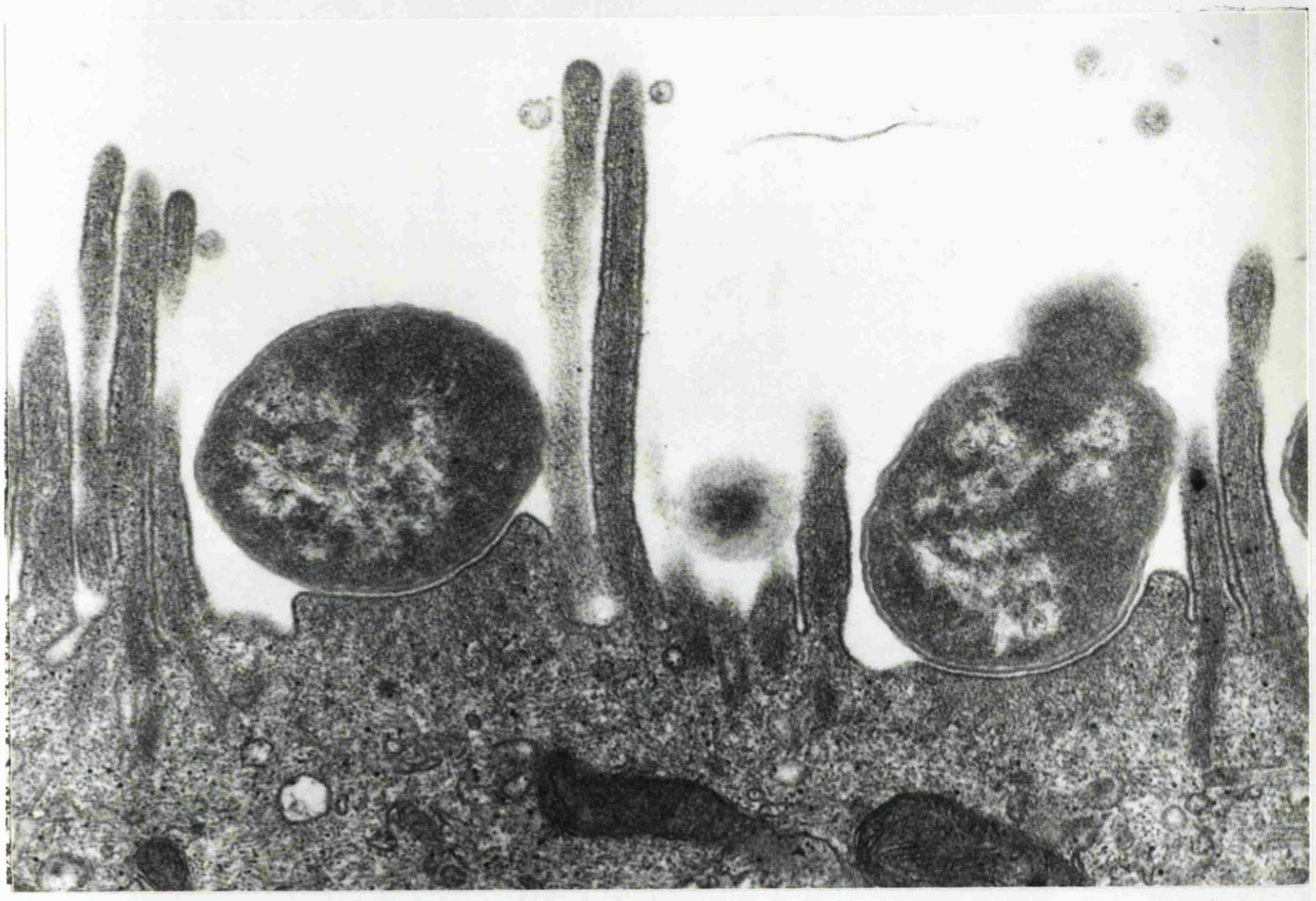


Figure 1.1. Ultrastructure of EPEC infected human gut tissue. Human ileal biopsies infected with EPEC strain E2348-69 for 3 h and observed by transmission electron microscopy shows characteristic lesion formation, consisting of effacement of microvilli, pedestal formation, and accretion of densely staining material beneath attached bacteria, (electron-micrograph kindly supplied by S. Knutton, institute of child health, Birmingham). Magnification 100,000 X.

histological sections of jejunal and ileal biopsy material from patients with severe and protracted diarrhoea (**Rothbaum et al., 1983**) in some cases reveals extensive villus atrophy and thinning of the mucosal lining wherever colonisation is heavy. Ultrastructural examination of crypt cells demonstrates distinctive vacuolization of the cytoplasm and loss of the normal basal position of the nucleus (**Rothbaum et al., 1983**). Regions of the lamina propria also show evidence of cellular infiltration by lymphocytes, plasma cells, and eosinophils, but with virtual absence of polymorphonuclear leukocytes (**Rothbaum et al., 1983**). A similar situation is seen in the colon as a consequence of overgrowth, with edematious lamina propria and moderate lymphocyte and plasma cell infiltration (**Rothbaum et al., 1982**). Changes in cell organelles indicative of intracellular damage are also seen in heavily colonised enterocytes and are thought to precede the eventual death and loss of cells from the villus surface (**Rothbaum et al., 1983**).

Biopsies infected *in vitro* with known diarrhoeagenic EPEC strains also exhibit the AE-lesion (**Figure 1.1**), with extensive vesiculation of the microvillus membrane and pedestal formation identical to that seen in patients (**Knutton et al., 1987b**). A development in this *in vitro* technology allowed significant advances in the study of EPEC virulence with the discovery that an apparently identical pedestal lesion is formed on the surface of the tissue culture cell line HEp-2 (**Knutton et al., 1987a; Knutton et al., 1989**), the colon carcinoma derived cell line CaCo-2 and primary human embryonic lung fibroblasts HEL, (**Knutton et al., 1989**). As with gut tissue, EPEC adhere initially by a putative fimbrial adhesin, followed by intimate contact between the plasma membrane of the host cell and the bacterial envelope. The pedestal forms at the position of bacterial contact, and incorporates a dense pad of filaments which can be visualised as an electron dense area directly beneath the plasma membrane (**Knutton et al., 1987a**).

The similarities of the EPEC lesion to the early stages of the invasion process seen with *Shigella* and EIEC has prompted speculation concerning possible mechanistic similarities between EPEC pathogenesis and the initial stages of invasion. As

mentioned previously EPEC are not invasive when assayed using the Sereny test, but do show a low level of intracellular penetration when adhering to cultured cells (**Andrade, 1986**), and production of endocytosis-inducer adhesins (**Andrade and Da Silva, 1987**) which are thought to mediate attachment to human infant enterocytes.

1:5:2. HEp-2 adherence. 80% of *E. coli* strains belonging to the traditional EPEC O serogroups were found to attach to cells of the tissue culture cell lines HEp-2 and HeLa (**Cravioto et al., 1979**). This contrasted with only 19% of nonpathogenic *E. coli* strains isolated from heathy individuals. Adherence of EPEC strains to tissue culture cells was mannose resistant and not associated with any of the known colonization factors (**Cravioto et al., 1979**). The system developed by Cravioto and coworkers formed the basis of an *in vitro* test for EPEC adherence which has been extensively used in the characterisation of EPEC isolates (**Baldini et al., 1983a; Scaletsky et al., 1985**). Suggestions that adherence of EPEC to host cells was not dependent on the presence of fimbriae (**Scotland et al., 1983**) have been questioned since rod-like structures have been observed to be involved in the non-intimate attachment of bacteria to gut cell microvilli (**Knutton et al., 1987a**), but to date no fimbriae have been conclusively identified to mediate tissue culture cell adherence.

Isolates of EPEC serogroups were found to adhere to HEp-2 and HeLa cells in one of two distinct patterns, termed localised adherence (LA), where the bacteria form clumps or microcolonies (usually 1 or 2 per cell) on the cell surface, and diffuse adherence (DA), where bacteria adhere evenly over the cell (**Scaletsky et al., 1984**). The genes for the DA adhesin have recently been cloned and DNA probes made, which are specific for DA strains (**Benz and Schmidt, 1989**). LA strains were termed class I EPEC while isolates with different adherence patterns were described as class II EPEC (**Nataro et al., 1985a; Nataro et al., 1985b**). Neither type of adherence was inhibited by the presence of mannose, but bacterial cell viability and the presence of calcium ions in the medium during bacterial growth have more recently been found to affect LA

(**Andrade and Santa Rosa, 1986**). The host cell receptor specific for the LA adhesin is suggested to be a surface glycoprotein, since addition of N-acetylgalactosamine inhibits adherence, presumably by competition with receptor proteins (**Scaletsky et al., 1988**).

Some of the isolates previously classed as DA (**Nataro et al., 1985b**) were later re-classified in the new category of aggregative adherers (AA), due to their ability to attach in clumps to the glass cover slips on which the tissue culture cells were grown (**Appendix 4; Nataro et al., 1987a; Gomes et al., 1989**). Variability in the procedures used by various groups for HEp-2 adherence assays also led some workers to confuse AA with LA (**Levine et al., 1988**), emphasising the need to agree a standard assay protocol and possibly establish standard LA, DA, and AA strains to be used as controls in screening new isolates (**Appendix 4.**).

The ability of diarrhoeagenic *E. coli* isolates of EPEC serogroups to form the characteristic AE lesion in gut tissue and cultured cells (**Polotsky et al., 1977; Rothbaum et al., 1982; Rothbaum et al., 1983; Knutton et al., 1987a; Knutton et al., 1987b**) initially seemed to correlated only with those strains that displayed LA to HEp-2 cells. Recent evidence however shows that lesion-forming ability is not limited to LA strains or to isolates of classical EPEC serogroups (**this study; S. Knutton, personal communication**). Moreover strains which would be classed as nonadherent under standard assay conditions have been shown to adhere to HEp-2 cells and form lesions when incubation is extended (**this study**).

A 94,000 molecular weight protein, initially reported to be encoded on a large conjugative plasmid (**Chart et al., 1988**) has also been identified in many EPEC strains. Convalescent serum from patients and adult volunteers contain antibodies which predominantly recognize this protein, suggesting that it is expressed at the surface of bacteria and may be involved in adherence. Attempts to isolate the genes for this protein have been hampered however, by the apparent inability of laboratory strains to express the 94,000 protein when transformed with natural vector plasmids (**Chart et al., 1988; McConnell et al., 1989**). This was at first understood as expression of the protein requiring factors from the EPEC genetic background, which are missing in laboratory

strains, but observations that plasmid cured derivatives of EPEC strains are still able to express very low amounts of the 94,000 protein, suggests a chromosomal location for the 94,000 protein genes, and a plasmid encoded regulatory function.(**Chart et al., 1988**)

1:5:3. The genetics of localised adherence. In a study where plasmid DNA from 32 *E. coli* isolates of EPEC serotypes were analysed, 31 were found to contain large 80-180 kb plasmids (**Baldini et al., 1983a; Laporta et al., 1986**). In the case of one of these strains, E2348-69 (O127), the genes for LA were found to be encoded on the single 120 kb plasmid pMAR2 by curing the strain of plasmid DNA (**Nataro et al., 1987b**). The resulting plasmidless derivative MAROO1 had lost the ability for LA to HEp-2 cells under standard assay conditions, but with extended incubation, limited adherence and lesion formation were observed (**Knutton et al., 1987a**). This suggested a two stage adherence mechanism for EPEC, the first mediated by plasmid encoded factors facilitating LA, and the second controlled by chromosomal genes promoting intimate adherence and lesion formation.

The regions of the pMAR2 plasmid required for LA adherence were determined by deleting sections of a Tn*801* tagged derivative pMAR7, and isolating self replicating regions carrying the Tn*801* antibiotic resistance (**Figure 1.2**), and screening transformants for LA (**Baldini et al., 1986**). A 40 kb region of the original plasmid, representing a 60% deletion, was isolated (pMAR15) and shown to encode LA to HEp-2 cells. The determinants for LA were found to span one of the two *Sal*I sites of pMAR15 by inserting DNA at each, and showing that insertion at only one site abolished LA. With this information a 1 kb *Bam*H-*Sal*I fragment was isolated and used as a DNA probe (EAF probe) for screening of clinical isolates for the LA determinant. Southern blot analysis of LA EPEC isolates using the EAF probe revealed a high degree of sequence conservation in several plasmids (**Nataro et al., 1987b**).

More recently it has been reproted that the genes for localized adherence and lesion formation are encoded on a single 95 kb plasmid in at least one O111 EPEC strain (**Batt et al., 1988**). A large conjugative plasmid from a lesion-forming O111 strain of *E.*

Figure 1.2. Construction of plasmid pMAR15 from the EPEC adherence plasmid pMAR2. The natural EPEC plasmid pMAR2 was tagged with the ampicillin resistance determinant of Tn*801*, yielding pMAR7. This was deleted by 60% ligated, transformed into HB101 and selected for ampicillin resistance and LA. The resulting plasmid was termed pMAR15

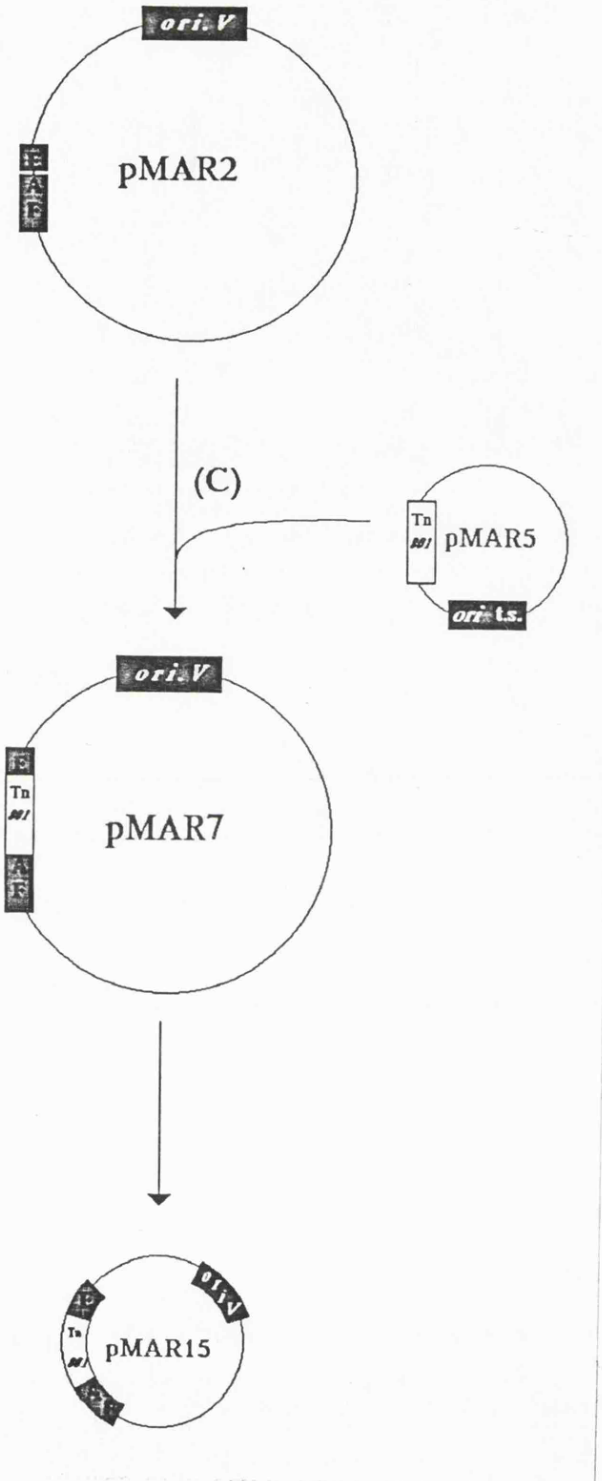


Figure 1.2. Construction of plasmid pMAR15 from the EPEC adherence plasmid pMAR2.

coli was transferred to a laboratory strain, which aquired LA, the ability to form the characteristic attaching and effacing lesion and O111 LPS. Since that first report there has been no subsequent data presented. Other groups working with the same strain have not been able to corroborate the findings, which suggests that some error in interpreting the results may have been made (**S. Knutton, personal communication**). It is possible for instance that during the conjugal transfer of plasmid, DNA segments of the chromosome may also have been mobilized into the recipient, or a mutant donor strain selected for.

1:5:4. Iron uptake. The concentration of free iron in secretions and other body fluids (e.g. serum) of higher animals is maintained at bacteriostatically low concentrations (typically 10^{-18} M) by the synthesis of iron chelating molecules such as lactoferrin and transferrin (**Crosa, 1984**). Bacteria assimilate 10^6 ferric ions each generation when dividing every 30 min, and therefore require free iron at a concentration of 0.4 to 4 μM in order to avoid iron deprivation (**Weinberg, 1978**). This has prompted the assertion that reduction of free iron to low levels constitutes an effective host defence mechanism against bacterial infection (**Weinberg, 1978**), a notion that is supported by observations that the pathogenicity of otherwise poorly infectious bacteria is greatly enhanced when administered in conjunction with iron salts (**Bullen and Griffiths, 1987**). Bacteria produce iron binding molecules called siderophores which sequester iron with greater efficiency than host chelators, enabling it to be used for bacterial growth (**Rogers, 1973; Bindereif and Neilands, 1983**). Generally siderophores are low molecular weight molecules produced by bacteria in response to iron stress; they remove iron from host chelator molecules and are then actively taken up by the producer organism via a specific outer membrane receptor protein (**Crosa, 1984**). The synthesis of the phenolic siderophore enterochelin is widely distributed among *E. coli* strains, but is considered to have no role in virulence since even apparent nonpathogens are competent in its synthesis. However many *E. coli* pathogens are also able to produce the hydroxamate siderophore aerobactin (**Bindereif and Neilands, 1985a; Linggood et al., 1987**). Initially identified by its association with ColV plasmids in invasive bacteria (**Williams, 1979**),

loss of the ability to make aerobactin from normally pathogenic organisms, or its acquisition by poorly pathogenic strains is associated with a corresponding change in virulence, related to their reduced or enhanced iron uptake abilities (**Williams and George, 1979**).

The genes for the aerobactin system are located on plasmids or the chromosome in different strains of *E. coli*. They were initially cloned from the plasmid ColV-K30 (**Bindereif and Neilands, 1983**), and were found to consist of 5 genes organised in a single transcriptional unit (**Carbonetti and Williams, 1984; Gross et al., 1984; Bindereif and Neilands, 1985b**). The proximal four genes *iucA-D* encode the polypeptides responsible for biosynthesis of the aerobactin molecule, and the distal gene *iutA* codes for the outer membrane receptor protein (**Carbonetti and Williams, 1984**). The aerobactin genes of plasmid ColV-K30 and several other plasmids are flanked by IS*1* like sequences (**Perez-Casal and Crosa, 1984; Roberts et al., 1986; Waters and Crosa, 1986**), suggesting that the aerobactin system may be part of a transposon, although until recently there was no evidence that the IS*1* sequences were active (**Lorenzo et al., 1988**). The relevance of an active transposon, carrying an important virulence determinant like aerobactin can be appreciated in the light of pathogen evolution. Since the aerobactin genes have been discovered on the chromosome in some isolates and on a variety of plasmids in others, it is likely that the evolution of these strains involved transposition of the aerobactin genes from an ancestral vector plasmid onto the chromosome or resident plasmid(s). Transposition onto plasmids would further increase the spread of the system by allowing the passage of the plasmid containing the genes into other related hosts, where they would be free to transpose again onto resident plasmids or chromosomal locations. Mapping of the aerobactin region from various sources indicated that the divergence of the system is not very great (**Waters and Crosa, 1986**), suggesting a fairly recent spread of the system to these bacteria. However aerobactin systems have been identified more recently which have little homology at the DNA level compared with the ColV-K30 system (**Carbonetti et al., 1986: Linggood et al., 1987: Crosa et al., 1988**). Such divergent systems have been

identified in *E. coli* associated with extraintestinal infections (**Carbonetti et. al., 1986**), *E. coli* from farm animals (**Linggood et al., 1987**), and human meningitic strains of *Enterobacter cloacae* (**Crosa et al., 1988**). In the case of *Enterobacter cloacae*, the aerobactin molecule produced was identical with that from strains carrying ColV-K30, even though the biosynthesis genes showed no homology. Also the receptor protein reacted with antisera raised against the 74,000 dalton protein of ColV-K30, indicating a significant amount of homology at the protein level (**Crosa et al., 1988**).

The genes for aerobactin uptake and biosynthesis in *Shigella* show a high degree of homology with the equivalent system in *E. coli* ColV-K30, except that the receptor protein in *Shigella* shows an apparent molecular size of 76,000 compared to 74,000 for *E. coli* (**Griffiths et al., 1985**). Both proteins show iron regulated expression and are competent in iron-aerobactin uptake. The aerobactin receptor also acts as the receptor for cloacin DF13 of *Enterobacter cloacae*, once bound to the aerobactin receptors, it is internalized and causes inhibition of protein synthesis and cell death by the cleavage of ribosomal RNA. The aerobactin receptor of *Shigella* also acts as a receptor for cloacin DF13, but normally, strains are less sensitive compared to equivalent aerobactin positive *E. coli* (**Derbyshire et al., 1989**). This was initially considered to reflect differences in the receptor proteins, but has since been shown to be due to the masking properties of LPS encoded by the *his*-linked locus of the *Shigella* chromosome (**Derbyshire et al., 1989**).

1:6 *Enterhaemorrhagic E. coli.* In 1982 a widespread outbreak of haemorrhagic colitis in the U.S.A. (characterized by copious bloody diarrhoea) was found to be associated with *E. coli* O157:H7, a serotype not previously recognized as a cause of diarrhoeal disease in humans (**Riley et al., 1982**). The absence of faecal leukocytes and fever distinguished it from classical dysentery caused by *Shigella* or EIEC (**DuPont et al., 1971**), and suggested that the causative agent may be a new diarrhoeagenic agent. This group of *E. coli* enteropathogens, now termed enterohaemorrhagic *E. coli* (EHEC), were shown to elaborate potent cytotoxins active against HeLa and Vero cells, one of

which, the Shiga-like toxin 1, was neutralized by antibodies raised against the Shiga toxin from *S. dysenteriae,* suggesting that the two toxins were very similar if not identical (**Brown et al., 1982: Eiklid and Olsnes, 1983; O'Brien and Holmes, 1987**). Many strains of this type also produce a second cytotoxin, Shiga-like toxin 2, which is not neutralized by antibodies raised against Shiga toxin (**Scotland et al., 1985**).

Ultrastructural examination of gut tissue infected with EHEC demonstrated that bacteria attach and cause effacement of microvilli in a manner identical with that seen with classical EPEC (**Moon et al., 1983; Tzipori et al., 1985; Tzipori et al., 1986**). However EHEC colonise only the colon causing a severe lesion with little or no leukocyte infiltration, while EPEC colonise the entire intestine, causing a less severe lesion with some evidence of leukocyte infiltration (**Levine, 1987**).

E. coli of the the serotype O26:H11 were originally considered to be a member of the EPEC group since it was evident from gnotobiotic piglet studies that they form the AE-lesion characteristic of EPEC (**Levine, 1987**). Isolates of this serotype also produce high levels of Shiga-like toxin (**O'Brien and Holmes, 1987**) and induce a diarrhoea similar in quality to that caused by EHEC of serotype O157:H7 (**Wade et al., 1979; O'Brien and LaVeck, 1983**). For these reasons they have more recently been reclassified as EHEC (**Levine, 1987**).

E. coli strains of serotype O157:H7 possess a 90 kb plasmid encoding the genes for a fimbrial adhesin which does not hybridise with the EAF probe of EPEC, but mediates the attachment of bacteria to cultured Henle-407 gut-derived epithelial cells (**Karch et al., 1988**). Plasmid-cured derivatives lack fimbriae, and coversely plasmid DNA from a clinical EHEC isolate transformed into a nonfimbriated laboratory strain of *E. coli* resulted in the aquisition of adhesive properties and the expression of fimbriae (**Karch et al., 1988**). EHEC strains of serotype O26:H11 also possess a large 90 kb plasmid (**Williams et al., 1978**), which shows no hybridisation with the EAF probe, but encodes an adhesive factor which mediates the attachment of bacteria to gut tissue. With the development of EHEC-adherence specific DNA probes it has been shown that

90 kb plasmids in both O157:H7 and O26:H11 strains carry the genes for the same adhesive factors (**Levine, 1987**).

1:7. *Aims of project.*

At the start of the project the aims were outlined as the isolation and characterisation of chromosomal and plasmid genes responsible for lesion formation and diarrhoeagenesis, identification of protein products and their function in disease, and the elucidation of the mechanisms involved in pathogenesis. Initial attempts at cosmid cloning from EPEC strains 2036-80, E2348-69 and MAR001, highlighted difficulties in the identification of clones containing the desired DNA sequences without reliable screening protocols based on the pathogenic mechanism. Enriching for clones which have aquired adherence factors, was unsuccessful since attachemnt of EPEC to cell surfaces was extremely inefficient in the absence of plasmids containing the EAF genes. Detection of lesion formation in gut and cultured cells infected with individual clones using electronmicroscopy was considered impractical for the large scale screening required. Therefore it was decided to concentrate initially on the processes of the disease with the intention, primarily of elucidating the pathogenic mechanism employed by EPEC as a group. Screening protocols developed from understanding the disease processes would then be used to identify and isolate clones, containing DNA sequences important for the development of diarrhoea and formation of the lesion.

Chapter 2. LESION FORMATION AND THE CYTOSKELETON.

2:1. *Introduction.*

As described in detail in chapter 1, EPEC infected gut tissue results in a characteristic attaching and effacing lesion at the sites of bacterial attachment (**Polotsky et. al., 1977; Rothbaum et. al., 1982; Moon et. al., 1983; Rothbaum et. al., 1983; Knutton et. al., 1987b**). Initial stages of EPEC colonization of gut tissue is associated with extensive breakdown of the actin core and vesiculation of the unsupported membranes of microvilli (**Knutton et. al., 1987b**). Subsequent stages involving adherence to the cell surface, and formation of the characteristic pedestal structure (**Polotsky et. al., 1977; Rothbaum et. al., 1982; Moon et. al., 1983; Rothbaum et. al., 1983; Knutton et. al., 1987a; Knutton et. al., 1987b**), is accompanied by the less frequently described accumulation of a dense filamentous pad within the pedestal(**Figure 2.1**), directly beneath the sites of bacterial attachment (**Staley et al., 1969; Knutton et al., 1987a; Knutton et al., 1987b**). Since loss of microvilli and other characteristic cytoskeletal changes seen in EPEC infected gut cells necessitates enormous rearrangements in the structure of cellular actin, it was proposed that the filamentous pad visualised within the pedestal constitutes the actin remnants of localized breakdown of the cytoskeleton. A similar lesion with clearly visible pedestal filaments in EPEC infected HEp-2 cells (**Knutton et al., 1987a**) allowed this proposal to be investigated further using a fluorescein-conjugated actin binding phallotoxin (**Wehland et. al., 1977; Wulf et. al., 1979; Barak et. al., 1980**) to identify regions of the cell containing filamentous actin.

2:2. *Materials and methods.*

2:2:1. Stock solutions. Chemicals and reagents were obtained from Sigma or BDH unless otherwise stated in text, and were stored according to the manufacturer's instructions governing temperature, light, and humidity. Aqueous solutions were

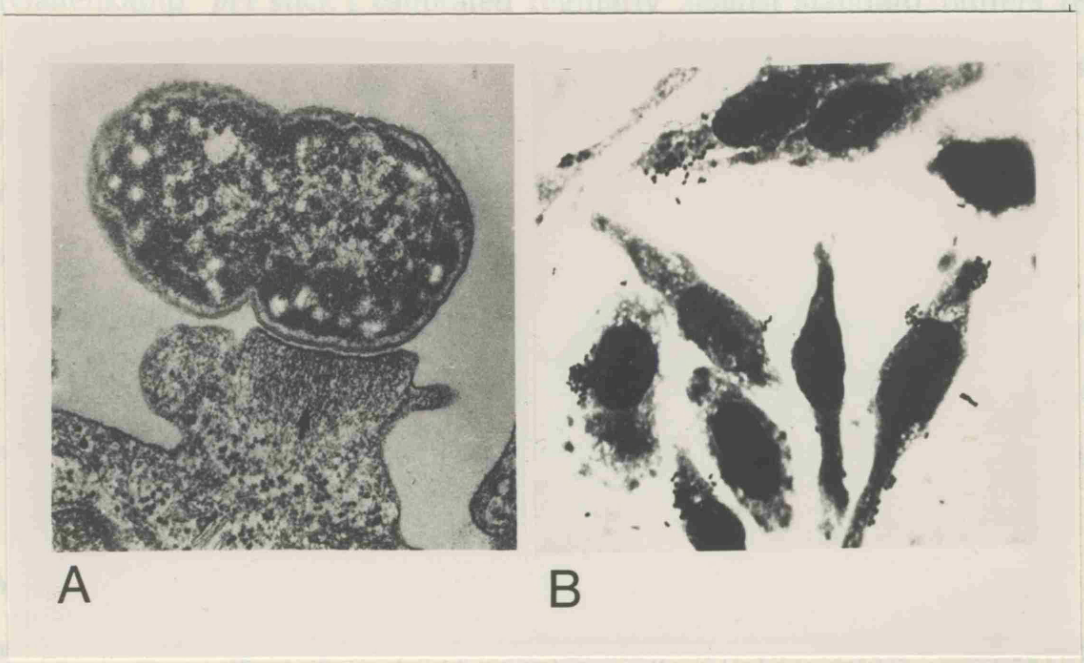


Figure. 2.1. Intimate adherence of EPEC to cell surfaces is associated with formation of a characteristic pedestal lesion containing a dense filamentous pad within the pedestal. (**A**) Electronmicrograph, showing attachment of EPEC to HEp-2 cells with pedestal formation and filamentous pad indicated by arrow (Dr. S. Knutton, Institute of child health, Birmingham). Magnification 50,000 X. (**B**) Light micrograph of EPEC infected HEp-2 cells showing typical localized adherence to cultured HEp-2 cells. Magnification 1,000 X.

prepared as stocks in distiled water, and where a required *p*H is indicated were adjusted to this value using either HCl or NaOH, as monitored by a hand held *p*H meter (Gallenkamp '*p*H stick') calibrated regularly against standard buffers (Fisons standard *p*H tablets). Non-aqueous solutions were prepared in sterile dimethylsulphoxide (DMSO), unless otherwise stated. Generally stock solutions were sterilised by autoclaving at 1 kg/cm^2 for 15 min. Sugar solutions were autoclaved at 0.7 kg/cm^2 for 10 min. Solutions of heat labile compounds (proteins, antibiotics and some amino acids) were sterilised by ultrafiltration through sterile nitrocellulose filters with 0.2 μm pore size (Millipore). Glass bottles (Schott, 'Duran'), 30 ml plastic universals (sterilin), or 1.5 ml microcentrifuge tubes (Eppendorf) were used to store solutions.

2:2:2. Media for bacterial growth. Luria broth (LUB; **Bertani, 1951; Lennox, 1955**) contained the following, 1% (w/v) tryptone (Oxoid), 0.5% (w/v) yeast extract (Oxoid), and 0.5% (w/v) NaCl, adjusted to *p*H 7.0 with 1 *N* NaOH. Where indicated in the text, 0.2% (w/v) glucose (LUB-glucose) was added, as were appropriate antibiotics. Luria agar (LUA) consisted of LUB solidified with 1.5% (w/v) agar (Difco). Stock LUA (400 ml) was sterilised by autoclaving at 1 kg/cm^2 for 15 min, and stored at room temperature. When required, LUA was re-melted using a microwave oven (400 W for 15 min), allowed to cool to approximately 55^{o}C before adding glucose and filter sterilised antibiotics and poured (20 to 25 ml) into 90 ml plastic petri dishes (Sterilin). The agar was allowed to set at room temperature before being dried at 37^{o}C and stored inverted at 4^{o}C.

2:2:3 Bacterial strains, growth and storage. Origin and details of strains used in this study are presented in **Appendix 1**. Agar plates were inoculated to give single colonies and incubated inverted in a 37^{o}C constant temperature room, or in incubators set to the appropriate temperature, for 16 to 48 h. Liquid cultures in 20 ml glass test-tubes, 20 ml plastic universals (Sterilin) or glass baffled conical flasks were grown with aeration by shaking at 100 rpm (New Brunswick reciprocating table) in a constant temperature

room of the appropriate temperature (normally 37°C), or in shaking water baths (New Brunswick) at 200 rpm set at temperatures between 18 and 45°C for 16 h (overnight cultures) or for any other required times (see text). Relative cell densities were routinely estimated by measuring the culture absorbance at wavelengths of 480 or 600 nm (A_{480} or A_{600}; Bausch and Lomb Spectronic 20 spectrophotometer). The absolute cell density of a given culture, when required, was obtained by performing a viable count (serial dilutions of the culture plated onto agar) of a fresh liquid culture. To ensure purity of stocks, bacteria were twice streaked out for single colonies on agar plates. Pure stocks were tested for phenotypic characteristics and were routinely stored on agar plates at 4°C for up to 2 months. As required, fresh stock plates were made by streak-purification from frozen stocks maintained at -20 and -80°C in glycerol (made by resuspending pelleted bacteria from fresh overnight cultures in 1 ml of sterile glycerol freezing solution containing 40% (v/v) glycerol and 60% (v/v) LUB and placed in plastic screw top cryo-vials (Nunc)).

2:2:4 Tissue culture cells, growth and storage. Primary cell cultures of human embryonic lung fibroblast (HEL) were obtained from Leicester Royal Infirmary Department of Virology, while the transformed (malignant) cell lines HEp-2 (originally derived from human laryngeal carcinoma tissue; **Toolan, 1954**) and CaCo-2 (of colonic carcinoma origin; **Fogh et al., 1977**) were initially obtained from Birmingham's Institute of Child Health. All cell lines were cultured as monolayers in tissue culture flasks (Sterilin), at 37°C in Dulbecco's Modified Eagles Medium without pyruvate (DMEM; Gibco), containing 10% heat inactivated, mycoplasma screened foetal calf serum (FCS; Gibco) (20% FCS for CaCo-2 cells) and 2 mM glutamine (Gibco) under an atmosphere of 5% CO_2 in air. Monolayers were washed twice with 10 ml phosphate buffered saline (PBS; containing 10 mM phosphate and 0.14 M NaCl, *p*H 7.4), 1 ml of 0.05% (w/v) trypsin, 0.02% (w/v) EDTA (Gibco) was added, and flasks were incubated at 37°C for 1 to 3 min. Cells were removed from the monolayer with a firm tap against

the palm of the hand. DMEM containing 10% FCS (10 ml) was added to quench trypsin action and the cells were evenly dispersed by gentle agitation. The cell suspension was used to seed fresh flasks containing 20 ml of DMEM with 10% FCS, at maximum split ratios of 1:6 for HEp-2 cells, 1:4 for CaCo-2 cells, and 1:3 for HEL cells. Flasks were incubated at 37°C under 5% CO_2 in air until confluent, with changes of medium every 2 days. For coverslip cultures, cell suspensions were used to seed bacteriological petri dishes (Sterilin) containing 12 mm^2 autoclave-sterilised coverslips in 25 ml DMEM containing 10% FCS. Cultures were incubated to the required degree of confluence (either 40, 60, or 100%) at 37°C under 5% CO_2 in air. For storage of cell lines, cells from suspensions were gently pelleted by centrifugation at 800 rpm for 2 min in 10 ml conical bottomed plastic tubes (Sterilin). Pellets were resuspended in 1 ml of sterile freezing medium (10% FCS, and 10% DMSO in DMEM) by gentle agitation, transferred to 2 ml screw top plastic cryo-tubes (Nunc) and placed in an insulated freezing box (with cotton wool insulation to a depth of 2.5 cm all round). The temperature was reduced by 1°C/min to a final value of -80°C by placing the box at -20°C for 1 h and then at -80°C for 1.5 h. Tubes were finally stored under liquid nitrogen for up to 4 years. Cells were revived from storage by removing cryo-tubes from the liquid nitrogen and quickly thawing at 37°C for 20 min. Flasks containing 20 ml of DMEM with 10% FCS were seeded using the thawed cell suspensions and incubated at 37°C under 5% CO_2 in air for 24 h to allow attachment. Medium was replaced with fresh every 2 days until monolayers reached confluence.

2:2:6. Actin staining. Subconfluent HEp-2 monolayers on 12 mm^2 glass cover slips were placed in the wells of a multiwell tissue culture plate in 2 ml of PIPES (*p*H 7.2)-buffered DMEM containing 2% FCS and 0.5% D-mannose), and infected with 10^8 bacteria (grown overnight in LUB-glucose at 37°C without shaking). Infected monolayers were incubated at 37°C for 3 h, washed three times in medium and either fixed for 20 min in 3% formalin or incubated for a further 3 h before fixing. Cells were permeabilised with 0.1% Triton X-100 in PBS for 5 min, washed three times in PBS and stained for 20 min in

the dark with 5 μg/ml fluorescein isothiocyanate-phalloidin (Sigma) in PBS. Cover slips were washed three times in PBS and mounted in glycerol-PBS containing 1 mg/ml *p*-phenylenediamine (stored in the dark at -20°C). Specimens were examined and photographically recorded by incident light fluorescence and phase contrast microscopy using a Zeiss D-7082 fluorescent microscope.

2:3. *Results.*

2:3:1. Identification of pedestal pad protein. Sub-confluent HEp-2 monolayers infected with EPEC strains 2036-80 and E2348-69 for 3 h were stained for filamentous actin using a commercially available fluorescent derivative of the potent actin binding phallotoxin, phalloidin (**Wehland et. al., 1977; Wulf et. al., 1979; Barak et. al., 1980**). Examination of stained monolayers by fluorescent microscopy revealed a network of stress fibres in both uninfected and infected cells. Infected cells however, exhibited intense regions of actin-specific fluorescence associated with individual attached bacteria (**Figure 2.2**). Careful adjustment of the microscope focus indicated that the accretion of actin had occured beneath adherent bacteria, presumably within the pedestal to which they were attached (**Figure 2.3**). By superimposing phase and fluorescent images of infected cells it was confirmed that it was the part of the host cell beneath attached bacteria and not the bacteria themselves that was fluorescent. The fluorescence associated with positions of bacteria adherence was very intense, indicating a high concentration of filamentous actin within the pedestal that cannot be explained solely on the basis of accumulation of cytoskeletal debris. Rather it is thought likely to result from a localized burst of actin filament polymerization in the vicinity of each attached bacterium.

2:3:2. Specificity of the Fluorescent Actin Staining (FAS) test. To determine whether accretion of actin as detected by this method was specific for lesion-forming EPEC isolates, fluorescent staining was carried out on monolayers infected with strains

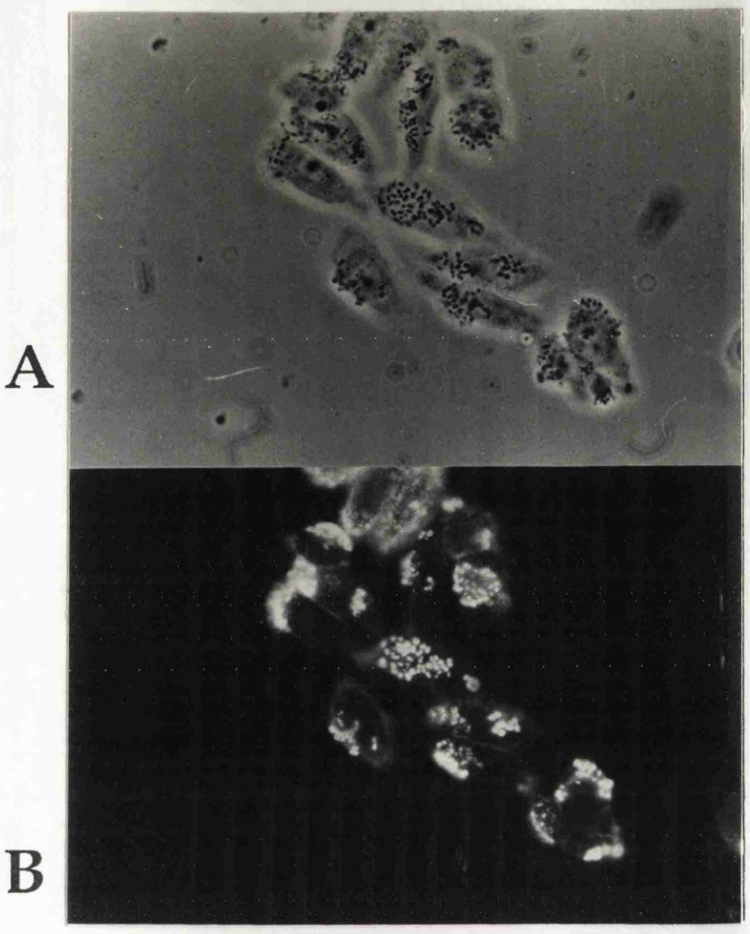
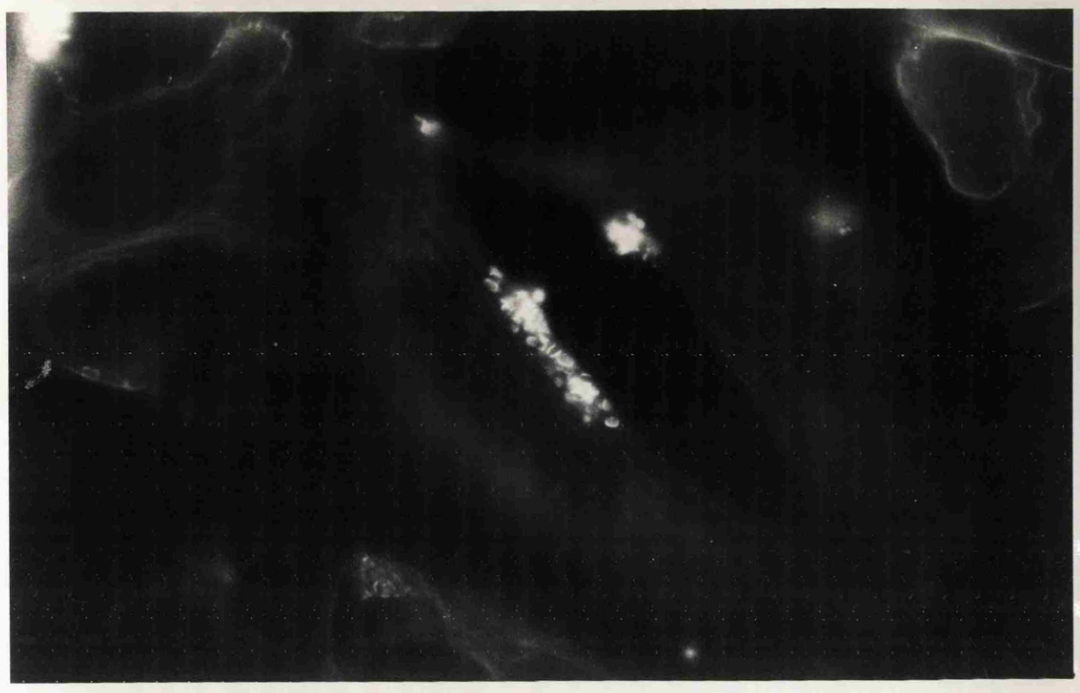


Figure 2.2. HEp-2 monolayers infected for 3 h with 10^8 *E. coli* of EPEC strain 2036-80 were stained with phaloidin to detect fialmentous actin. (**A**) phase micrograph, localized adherence of EPEC to HEp-2 cells. (**B**) Fluorescence micrograph, intense regions of actin fluorescence associated with indavidual attached bacteria. Magnification 500 X.

Figure 2.3. Accretion of actin occurs at the inside surface of the plasma membrane. HEp-2 monolayers infected for 3 h with 10^8 *E. coli* of EPEC strain 2036-80 were stained with phaloidin to detect fialmentous actin. (A) Fluorescence micrograph, focussed on the attached bacteria and accreted actin. (B) Fluorescence micrograph, focussed on the cytoskeleton inside the cell. Magnification 2,000 X.



A

B

Figure 2.3. Accretion of actin at the inside surface of the plasma membrane.

whose adherence pattern to HEp-2 cells and lesion-forming ability have been well characterised (**Table 2.1**). The results show that only known lesion-forming EPEC isolates gave conclusive FAS test positive results. In contrast, nonlesion forming strains that adhered to HEp-2 cells in a diffuse (**Figure 2.4**) or aggregative pattern gave no indication of actin accumulation (**Table 2.1**).

2:3:3. Lesion formation on tissue culture cell lines other than HEp-2. To reduce the likelihood of missing lesion-forming isolates unable to adhere to HEp-2 cells two additional cell lines (HEL and CaCo-2) were used in the evaluation of the FAS test for clinical isolates. An additional advantage of using primary HEL cells, which spread in culture forming thinner cells, is clearer visualization of the pedestal actin-fluorescence against the cell background. Also since CaCo-2 cells differentiate at confluence producing a brush border, they more closely approximate to the natural target of enteric pathogens. EPEC infection of HEL (**Figure 2.5**) and CaCo-2 monolayers showed identical actin staining characteristics to that seen with HEp-2 cells, and in addition caused the effacement of brush border microvilli from CaCo-2 monolayers with the concomitant formation of pedestal structures (**Knutton et al., 1989**). The clarity of the FAS test carried out using HEL cells enabled strains which exhibit low levels of adherence, to be screened for lesion-formation. MAR001, the plasmid cured derivative of E2348-69, which shows limited adherence to all cell lines, was shown to be FAS test positive after extended incubation for 6 hours (with washing in fresh medium every 2 h). (**Table 2.1**). Individual attached bacteria could be easily seen and scored for actin accretion. This in combination with the observation that infection with HB101(pMAR15) (the laboratory strain transformed with the pMAR2 derivative PMAR15), showed no actin fluorescence associated with the attached bacteria (**Table 2.1**), is consistent with the previous findings outlined in chapter 1 that development of the lesion is independent of bacterial adherence encoded by the EAF region of plasmid pMAR2, but at the same time formation of the disease state is greatly enhanced by the presence of plasmids encoding enteroadhesins (**Levine et al., 1985**).

Knutton et al., 1989.

Bacterial strains	HEp-2 adherence	FAS test result (+/-)	Lesion formation[a]	Bio-type
2036-80	localized	positive	positive	EPEC
E2348-69	localized	positive	positive	EPEC
MAR001[b]	localized	positive	positive	EPEC
660-79	localized	positive	positive	EPEC
182-83	localized	positive	positive	EPEC
E851	localized	positive	positive	EPEC
DPO95	diffuse	negative	negative	EPEC
135	diffuse	negative	negative	EPEC
JPN10	aggregative	negative	negative	EPEC
136	aggregative	negative	negative	EPEC
469-3[c]	diffuse	negative	negative	?
E32511	localized	positive	not done	EHEC
E47784	localized	positive	not done	EHEC
1457	---	(-)[e]	negative	EIEC
HB101[d]	localized	negative	negative	----

Table 2.1. Correlation between the ability of strains to form the attaching and effacing lesion on gut tissue and the FAS test on HEp-2 monolayers.

[a] Lesion formation determined by S. Knutton.

[b] MAR001 is a plasmid cured derivative of E2348-69.

[c] 469-3 does invade cells of monolayers.

[d] Laboratory strain HB101 containing the localized adherence plasmid pMAR15 (a deletion derivative of pMAR2 from E2348-69).

[e] A diffuse fluorescence was observable around internalized bacteria.

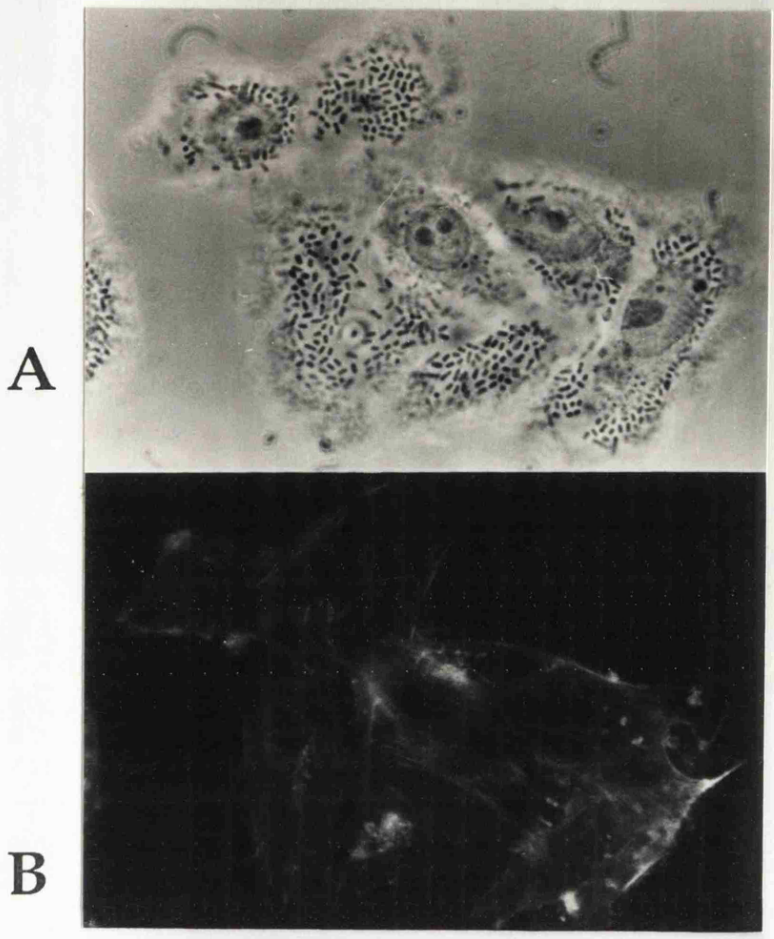


Figure 2.4. Actin distribution in HEp-2 cells infected with non-lesion forming strains of *E. coli*. HEp-2 monolayers infected with 10^8 *E. coli* of strain 135 for 3 h were stained for actin using phaloidin. (**A**) Phase micrograph, diffuse adherence of bacteria to HEp-2 cells. (**B**) Fluorescence micrograph, no actin accretion associated with the positions of bacterial attachment. Magnification 500 X.

Figure 2.5. Actin accretion in HEL cells at the sites of bacterial attachment. HEL cell monolayers infected with 10^8 *E. coli* of EPEC strain 2036-80 for 3 h were stained for actin accretion using phalloidin. (**A**) Phase micrograph, normal microcolony development on HEL cells. (**B**) Fluoresence micrograph, actin accretion associated with indavidual attached bacteria. Magnification 2,000 X.

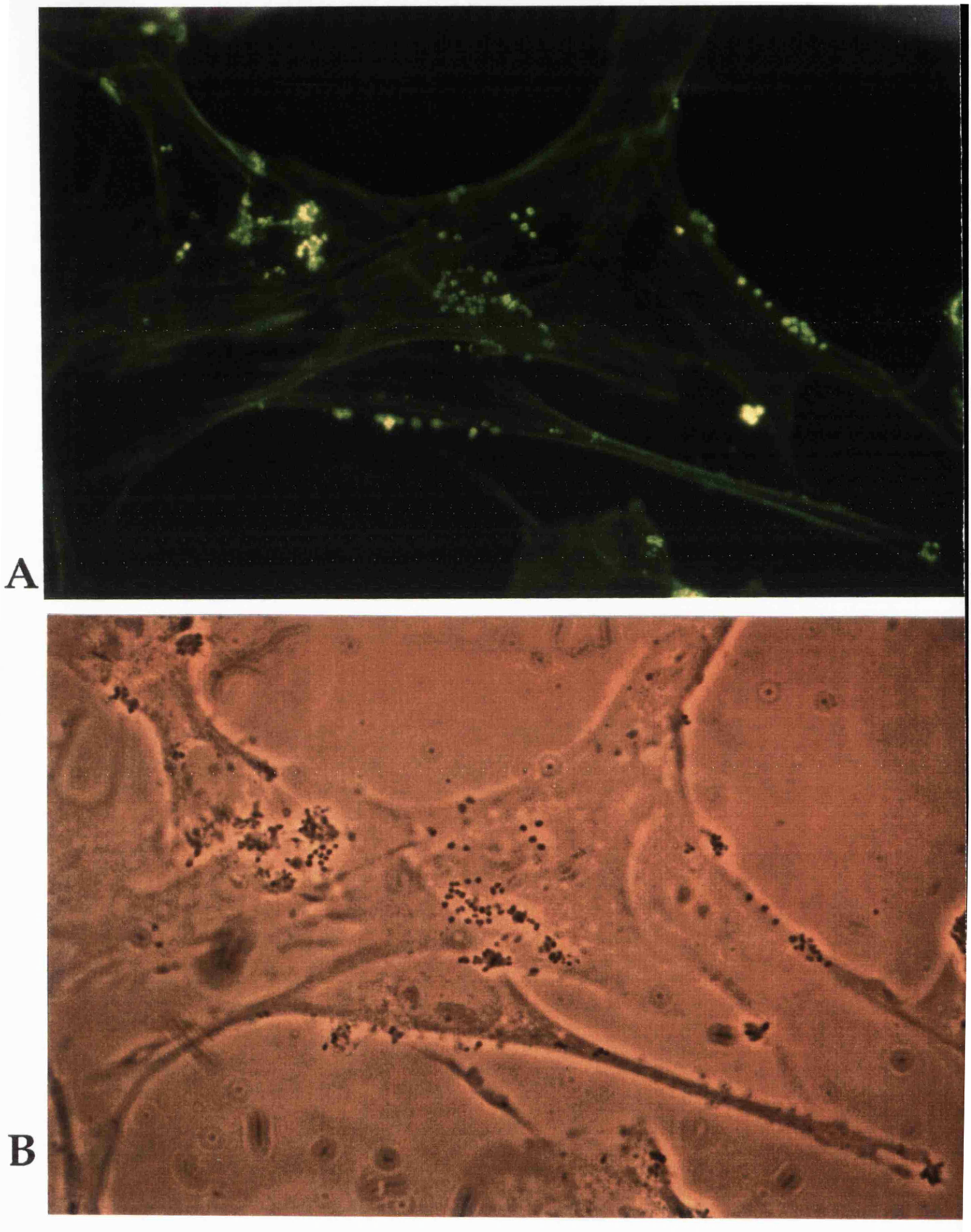


Figure 2.5. Actin accretion in HEL cells at the sites of bacterial attachment.

Enterohaemmorrhagic *E. coli* (EHEC) isolates have been shown to produce an attaching and effacing lesion in the large bowel (**Tzipori et. al., 1986**) identical to that seen with classical EPEC strains in the small intestine. Also the ultrastructure of the EPEC membrane lesion in many ways resembles the initial stages of invasion by EIEC. Therefore the actin distribution in both HEp-2 and HEL cells infected with EHEC and EIEC was examined using extended incubation. EHEC caused extensive actin accretion at the sites of bacterial adherence in both cell types (**Table 2.1**) indicating the presence of pedestal formation on the cell surface. EIEC on the other hand were seen to elicit a diffuse and less well pronounced actin fluorescence associated with the internalized bacteria (**Table 2.1**).

2:4. *Conclusions and discussion.*

Until now, O serogrouping of clinical isolates was the only routinely used test for the identification of EPEC. The development of DNA probes specific for the LA genes (EAF) has been invaluable in the identification of many EPEC strains. But not all isolates capable of forming an attaching and effacing lesion on gut tissue and cultured cells contain DNA that hybridises with the EAF probe. The definitive virulence property of all EPEC is the formation of the attaching and effacing membrane lesion, which up to this time could only detected using electronmicroscopy. Since the filamentous pad formed within the lesion pedestal has been identified as actin, the lesion is for the first time readily detected using simple light microscopy. The intense actin fluorescence associated with the positions of bacteria attachment not only implies an active accretion process being involved, but also makes the FAS test extremely sensitive for poorly adherent bacteria able to form an attaching and effacing lesion. The FAS test was found to be specific for isolates of the EPEC and EHEC groups of enteropathogens (both of which form identical membrane lesions on gut tissue). EIEC, were easily distinguished form EPEC and EHEC by the qualitatively different type of actin accretion, giving a diffuse actin distribution around the internalised bacteria.

Chapter 3. ACTIN ACCRETION AT THE SITES OF BACTERIAL ATTACHMENT.

3:1. *Introduction.*

The visualization of intense filamentous actin-dependent fluorescence in EPEC infected tissue culture cell lines (the FAS test), as developed by S. Knutton and myself (**Knutton et al., 1988; Knutton et al., 1989**) is now becoming recognized as a simple protocol for the reliable detection of lesion forming clinical isolates. As mentioned in chapter 2, the density of actin within the pedestal was considerably greater than would be expected if it had resulted purely from the accumulation of actin debris resulting from cytoskeletal breakdown. Indicating that *de novo* actin filament polymerization is occurring at the sites of bacterial attachment. Procedures known to elevate intracellular calcium and cause loss of microvilli in gut and renal cells form an ultrastructurally similar lesion to that seen in EPEC infected gut tissue (**Matsudaira and Burgess, 1982; Goligorsky et al., 1986**), suggesting that the EPEC induced cytoskeletal actin rearrangements seen during infection may result from an elevation in the intracellular calcium of target cells. Modulation of cytoskeletal structure in eukaryotic cells in part relies on actin changes resulting from the activities of a set of related, calcium-dependent actin filament severing proteins (**Schilwa, 1981; Matsudaira and Janmey, 1988**). Interestingly the proteins responsible for actin severing have dual functions, also promoting actin assembly into F-actin filaments under certain conditions (**Burgess and Prum, 1982; Glenney and Glenney, 1985; Yin, 1988; Matsudaira and Janmey, 1988**). Four such proteins have been identified in eukaryote cells, gelsolin and villin which are only found in cells from higher eukaryotes, and severin and filamin which have been found in the lower eukaryotes *Dictyostelium* and *Physarum* respectively (**Matsudaira and Janmey, 1988**), and certain species of yeast. All have similar functions as regard actin severing and polymerization, and indeed show a high degree of homology at the protein level (**Arpin et al., 1988; Bazari et al., 1988**).

The first of these proteins to be discovered was gelsolin, a protein of 85 KDa located in the cytoplasm of the cell which showed a calcium dependent association with filamentous actin (**Coue et al., 1985**). At high calcium concentrations gelsolin associates with, and severs actin fibres (**Figure 3.1**) by a nonproteolytic and nonenzymic process (**Yin and Stossel, 1979; Matsudaira and Janmey, 1988; Yin, 1988**). Gelsolin binds to adjacent monomers in actin filaments and introduces a break between them (**Matsudaira and Janmey, 1988; Yin, 1988**). The protein remains associated with the barbed end of the filament (the end to which monomers are preferentially added) forming a cap, and preventing filament elongation by further addition of actin monomers (**Yin, 1988**). The activities of gelsolin and similar proteins are regulated at two levels, by calcium ions, and by the phosphatidylinositol-lipid (PI-lipid) content of the membrane (**Yin, 1988**). A lowering in calcium levels causes a reduction in severing activity but does not dissociate gelsolin from the severed filament end (**Coue and Korn, 1985; Yin, 1988**). However, in low calcium conditions, binding of the gelsolin cap to PI-lipids in the membrane (in particular phosphatidylinositol 4,5-diphosphate [PIP_2]), causes dissociation of gelsolin from the filament end (**Yin, 1988**), allowing elongation by monomer addition (**Figure 3.2**).

It is postulated that localized elevation in intracellular calcium concentration resulting from cellular activation by various hormones or growth factors (**Conrad and Rink, 1986; Goligorsky et al., 1986; Taylor et al., 1988c; Gukovskaya et al., 1989**), may mediate changes in cell morphology (**Wilkinson et al., 1988; Zimmermann, et al., 1988**) through regulation of gelsolin (**Yin, 1988**). Return of the cell to the resting state, would involve a decrease in calcium concentration allowing PIP_2 mediated dissociation of gelsolin from actin filament ends, and fascilitating an explosive burst of actin polymerization (**Ankenbauer et al., 1988; Yin, 1988**). In normal circumstances cells may undergo cycles of stimulation during which calcium at any location in the cell may only transiently exceed critical concentrations (>1 μM) for gelsolin severing activity. Also processes involved in restoring cells to the prestimulation state (down-regulation) will further reduce calcium concentrations and increase the PIP_2 content of the plasma

Figure 3.1. Diagramatic representation of the process of actin filament cleavage by gelsolin (the sol stage). In low calcium conditions gelsolin is free in the cytoplasm and unassociated with actin (**A**). As the local intracellular calcium concentration rises, gelsolin associates with filamentous actin by binding to adjacent monomers (**B**). Once the local calcium concentration reaches a critical level of 1 μM, gelsolin mediates nonproteolytic cleavage of actin filaments between monomers and remains associated with one end (C). The overall effect of calcium-activated gelsolin cleavage of actin, is a reduction in the local viscosity of the immediate cytoplasm.

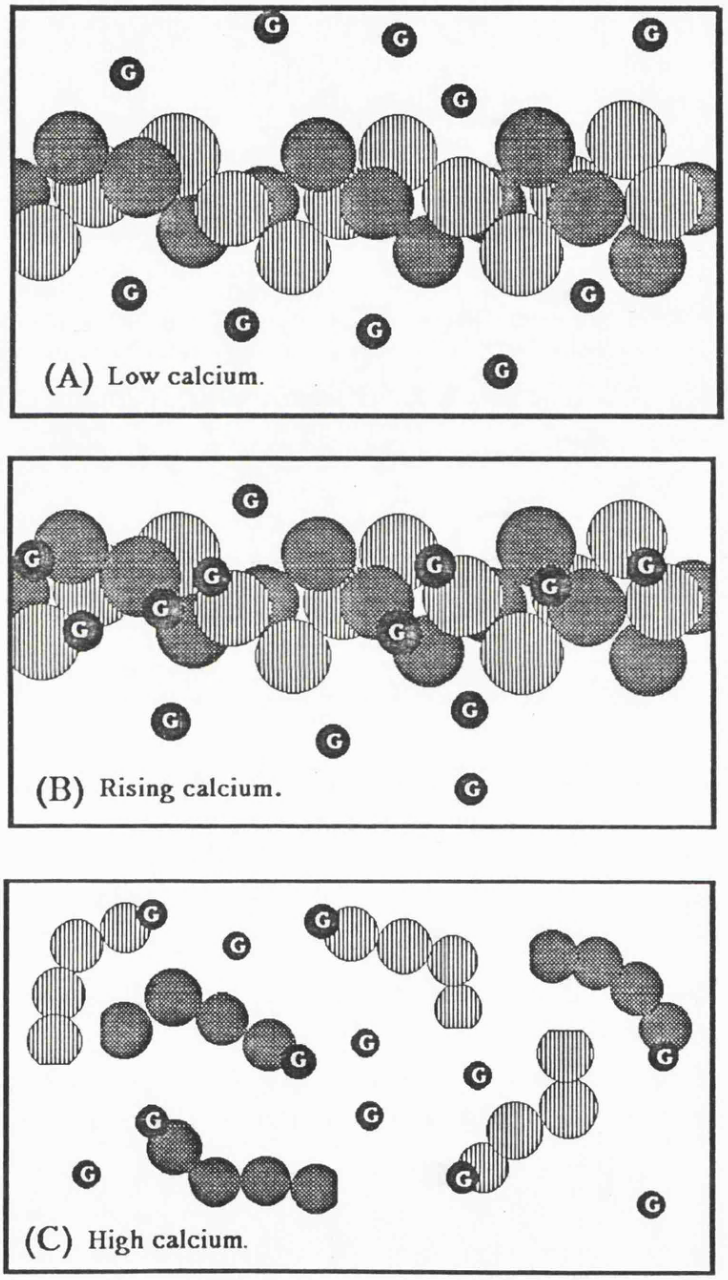


Figure 3.1. Diagramatic representation of the process of actin filament cleavage by gelsolin (the sol stage).

Figure 3.2. Diagramatic representation of PI-lipid dependent dissociation of gelsolin from actin fragments and the nucleation of actin filament polymerization (the gel stage). Lowering of local intracellular calcium concentrations either by diffusion, sequestering by storage organelles or down regulation processes facilitates uncapping of actin fragments by binding to membrane PI-lipids (A). Low calcium conditions stimulates a burst of actin polymerization by addition of monomers to the vacant barbed end (**B**), and a dense network of newly formed actin fibres is produced by association of actin filaments (C). The formation of a dense network of actin filaments increases the local viscosity of the cytoplasm, yeilding an actin gel.

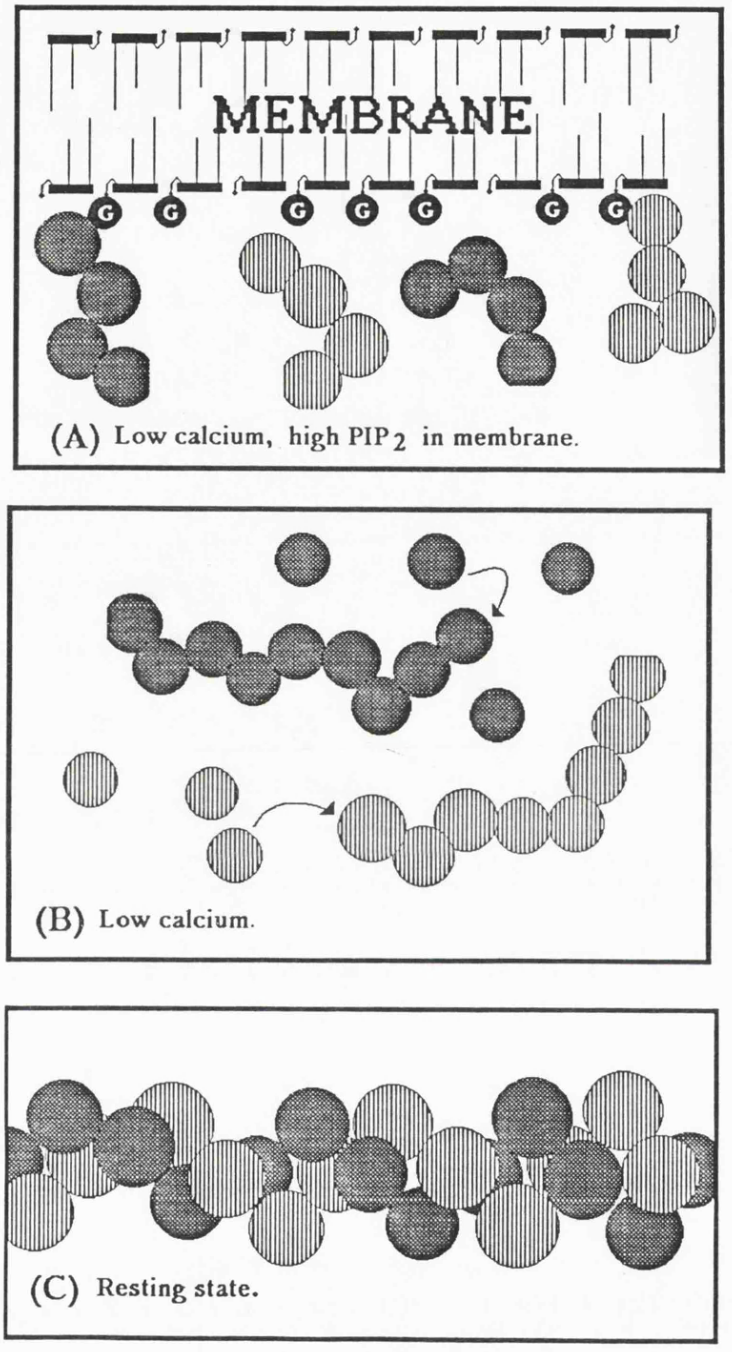


Figure 3.2. Diagramatic representation of PI-lipid dependent dissociation of gelsolin from actin fragments and the nucleation of actin filament polymerization (the gel stage).

membrane (**Ase, 1988; Bouscarel et al., 1988**). The outcome of such cycles of calcium increase and fluctuation in PIP_2 content of the membrane would be dramatic changes in local viscosity of the cytoplasm over very short time periods (**Yin and Stossel, 1979; Yin, 1988**). Termed gel sol cycles, these may be the driving force for the membrane movements observed in many stimulated cells. The explosive burst of actin polymerisation which seems to occur in close proximity to the inside surface of the plasma membrane (**Yin, 1988**), could cause outward surges of the membrane, forming cell processes, ruffles, and pseudopodia (**Weeds, 1982**).

Analogous properties are seen with villin a 95 KDa actin modulating protein found exclusively in the microvillus (**Bretscher and Weber, 1979**), except that at low calcium concentrations it exhibits a bundling function by virtue of an additional actin binding site in the head region of the molecule, allowing for cross linking of actin filaments into large fibres (**Arpin et al., 1988; Bazari et al., 1988; Drenckhahn and Rolf, 1988**).

3:2. *Materials and Methods.*

3:2:1. Fluorescence actin staining (FAS). The distribution of filamentous actin in HEp-2 cells was determined using 5 μg/ml fluorescein isothiocyanate (FITC)-phalloidin (Sigma) in PBS, as previously described in Chapter 2. Solutes requiring nonpolar solvents were dissolved in DMSO to give a stock solution at least 100 x the working concentration.

3:3. *Results.*

3:3:1. Kinetics of lesion formation. A series of experiments were conducted to evaluate 1) the time scale of lesion development, 2) at what point if any during infection, the cell is committed to lesion formation, and 3) whether the lesion is reversible.

showing attached bacteria was lower, and microcolony size smaller, but that attachment could still be observed at an infection time of as little as 0.5 h. Lesion formation was defined as a visible increase in actin fluorescence associated with individual attached bacteria. Cells infected for 1 h or more showed obvious fluorescence around attached bacteria, whereas infections maintained for less than 1 h appeared as uninfected controls. At the 1 h time point the quality of actin fluorescence associated with individual attached bacteria was different from that seen at later time points. The tight, discrete fluorescence of 3 h infections was not apparent; instead there was a hazy, diffuse fluorescence which I term the "pro-lesion state".

The point of commitment for lesion formation was determined by killing attached EPEC at various times after infection using high concentrations of streptomycin, which had previously been determined to give the quickest killing of EPEC with the lowest toxicity for HEp-2 cells (**Table 3.1**). After washing with fresh antibiotic free DMEM at 37°C, incubation was continued in fresh DMEM growth medium containing 10% FCS for a total time of 4 h. Monolayers treated with streptomycin at or before 1 hour after infection showed complete inhibition of lesion formation, whereas shortly after 1 h of infection diffuse actin fluorescence could be observed around some attached bacteria. Cells infected for 2 hours or more before killing developed a normal FAS positive result (**Table 3.2**). Once the full lesion or the diffuse pro-lesion were evident, killing of attached bacteria did not reverse the accretion of filamentous actin. Also attached bacteria killed upto 1 hour post-infection did not go on to develop the lesion even when incubation was continued for a further 24 hours.

Monolayers infected with EPEC for 3 hours and then treated with streptomycin, showed no reversal of the FAS result when incubated for a further 24 hours in fresh medium without antibiotics. The inviability of the attached bacteria was shown by the fact that microcolonies did not increase in size and a negligible viable count was obtained for infected HEp-2 cells plated onto nutrient agar.

Streptomycin conc. (μg/ml)	colony forming units/ml
0	100,000,000[a]
5	100,000,000[a]
10	1000,000[a]
25	55,200
50	21,300
100	10,150
200	4,200
250	3,800
300	3,000
320	1,900
340	1,000
360	250
370	50
380	45

Table 3.1. KIlling of EPEC strain 2036-80 by streptomycin. A mid log phase culture of EPEC strain 2036-80 in LUB-glucose was diluted to approximately 10^8 bacteria/ml with DMEM comtaining 10% foetal calf serum. 1 ml aliquots were dispensed into 2.5 cm plastic tissue culture plates and the streptomycin concentration adjusted to the appropriate values. Plates were incubated at 37°C for 10 min, after which time viable counts were carried out by serial dilution onto non-selective agar plates. Viability of the various cultures were expressed as colony forming units /ml. [a] Approximate figures.

Infection time (min or h.	Lesion formation FAS test (+/-)
0	-
15 min	-
30 min	-
45 min	-
1 h	-/+[a]
1.25 h	+
3 h	+

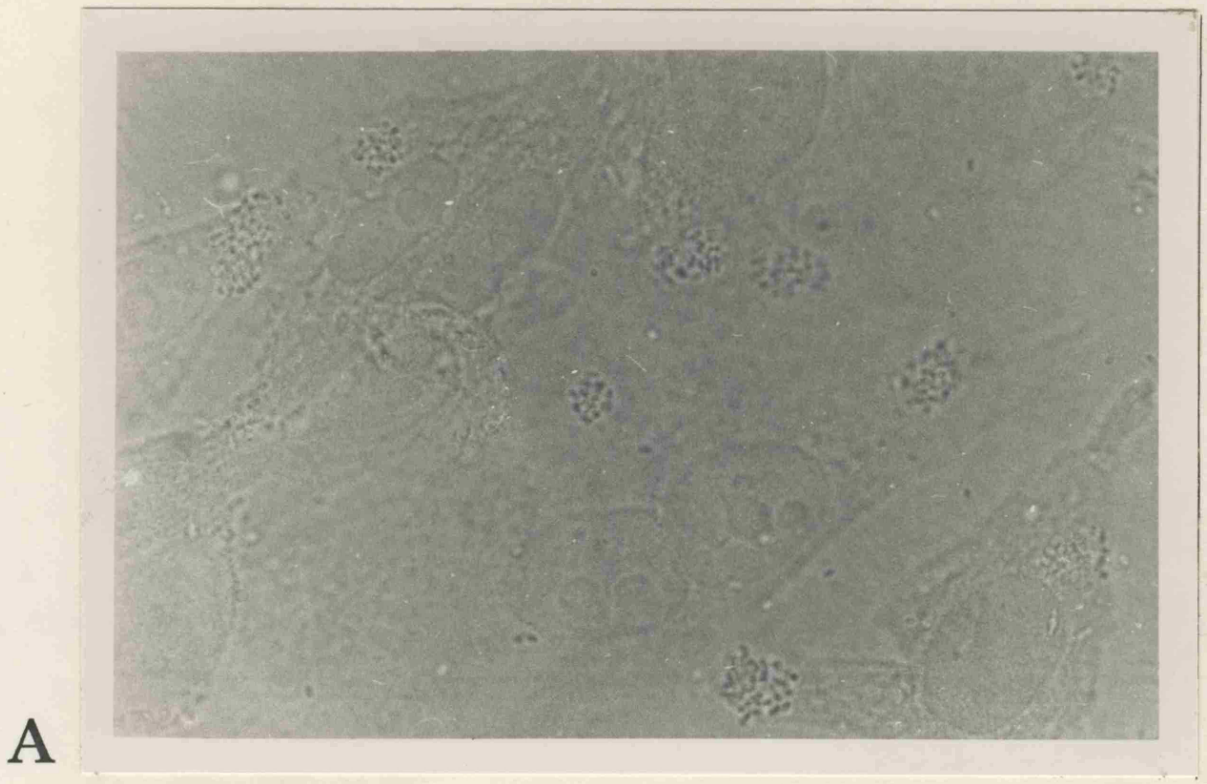
Table 3.2. Time course of lesion formation on HEp-2 cell monolayers infected with EPEC strain 2036-80. Confluent monolayers on glass cover slips were infected with 10^8 bacteria for various times, fixed and stained for filamentous actin with fluorescein conjugated phalloidin (FAS test). Lesion formation was scored as the development of a distinctive fluorescence associated with attached bacteria.

[a] Time point at which the diffuse pro-lesion associated with attached bacteria was easily identified.

3:3:2. Involvement of calcium in actin accretion. Since the structure of cellular actin is regulated *in vivo* by the concentration of intracellular calcium, it's role in the development of the EPEC-lesion was investigated using the FAS test. HEp-2 cells at 50-60% confluence were placed into low calcium medium containing the calcium chelator EGTA (4 mM). Monolayers were infected with EPEC and incubated for 3 hours, after which time lesion formation was scored using the FAS test (**Figure 3.3**). Actin accretion at the site of bacterial attachment appeared to be unaffected by limitation of extracellular calcium. The experiment was repeated with the ionophore ionomycin present (1 μM) to allow any calcium mobilized from internal stores to escape into the medium. Again actin accretion was observed at the sites of bacteria attachment. One possible reason for this could be the localized nature of the lesion, possibly resulting from mobilization of intracellular calcium stores in close proximity to the sites of bacterial attachment, causing local calcium increases in this region of the cytoplasm. It is unlikely that such localized regions of high calcium would disperse efficiently by treatment with ionophores, before actin breakdown and accretion had occurred.

The mobilization of calcium from intracellular stores has been reported to be inhibited by the drug dantrolene (**Danko et al., 1985**); therefore this drug was used to determine whether actin accretion could be inhibited or reduced by lowering available calcium in internal stores. Dantrolene treated EPEC infected cells showed a reduction in the intensity of the actin fluorescence similar to the pro-lesion seen in cells infected with EPEC for 1 hour (**figure 3.4**). This fluorescence could not be reduced further by increasing the dantrolene concentration or by altering the time of addition. It is possible that not all intracellular calcium stores are sensitive to dantrolene inhibition to the same extent. So that local concentrations of calcium may be transiently elevated even in the presence of dantrolene, leading to partial break down of actin but not allowing the explosive burst of actin accretion to occur. Another possibility is that any transient elevation in calcium may bind to calmodulin or similar calcium binding proteins which might be involved in the initial stages of lesion formation. This would in effect reduce

Figure 3.3. Effect of extracellular calcium on lesion formation. HEp-2 cell monolayers infected with 10^8 *E.coli* of EPEC strain 2036-80 in DMEM containing 4 mM EGTA for 3 h. and stained for filamentous actin. **(A)** Phase micrograph, showing microcolony formation on cells of the monolayer. **(B)** Fluorescence micrograph, indicating intense actin accretion associated with individual attached bacteria. Magnification 2,000 X.



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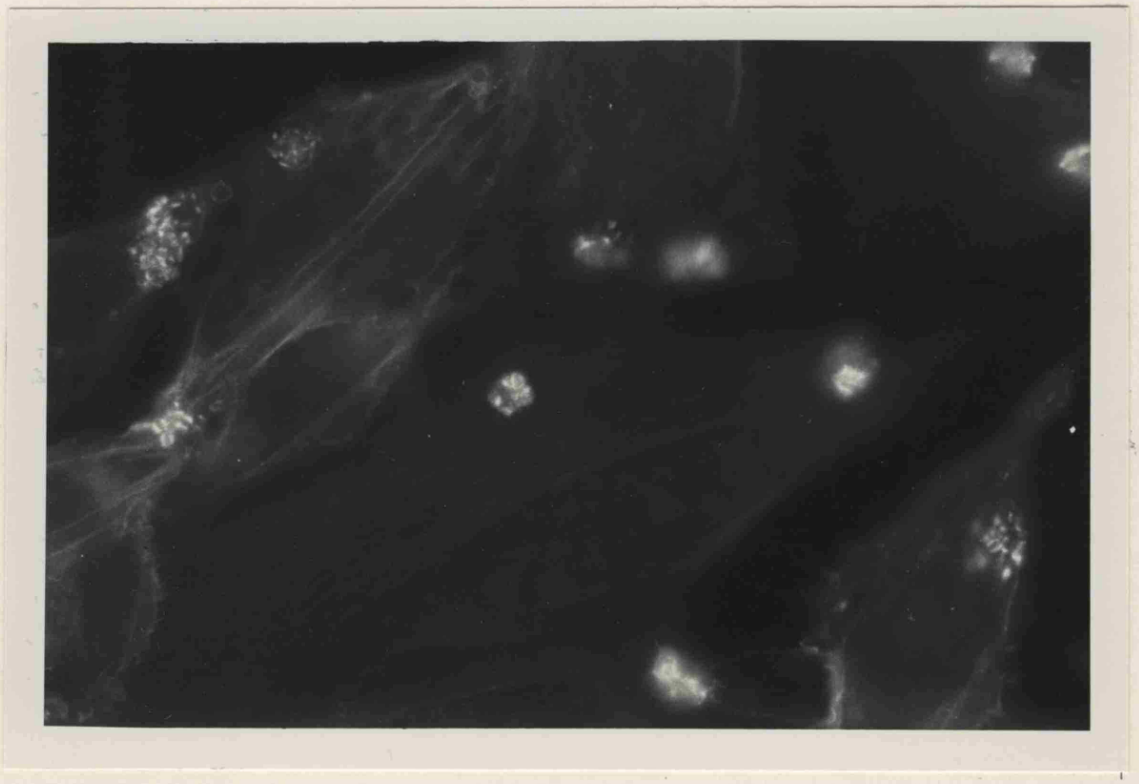

B

Figure 3.3. Effect of extracellular calcium on lesion formation.

Figure 3.4. Effect of dantrolene on lesion formation. HEp-2 monlayers pre-treated with 50 μM Dantrolene were infected with 10^8 bacteria of EPEC strain 2036-80 and screened for accretion of filamentous actin at the sites of bacterial attachment by staining with phalloidin. (A) Phase micrograph, showing normal microcolony formation on cells of the monolayer. (B) Fluorescence micrograph, indicating diffuse actin fluorescence associated with sites of bacterial attachment. Magnification 2,000 X.

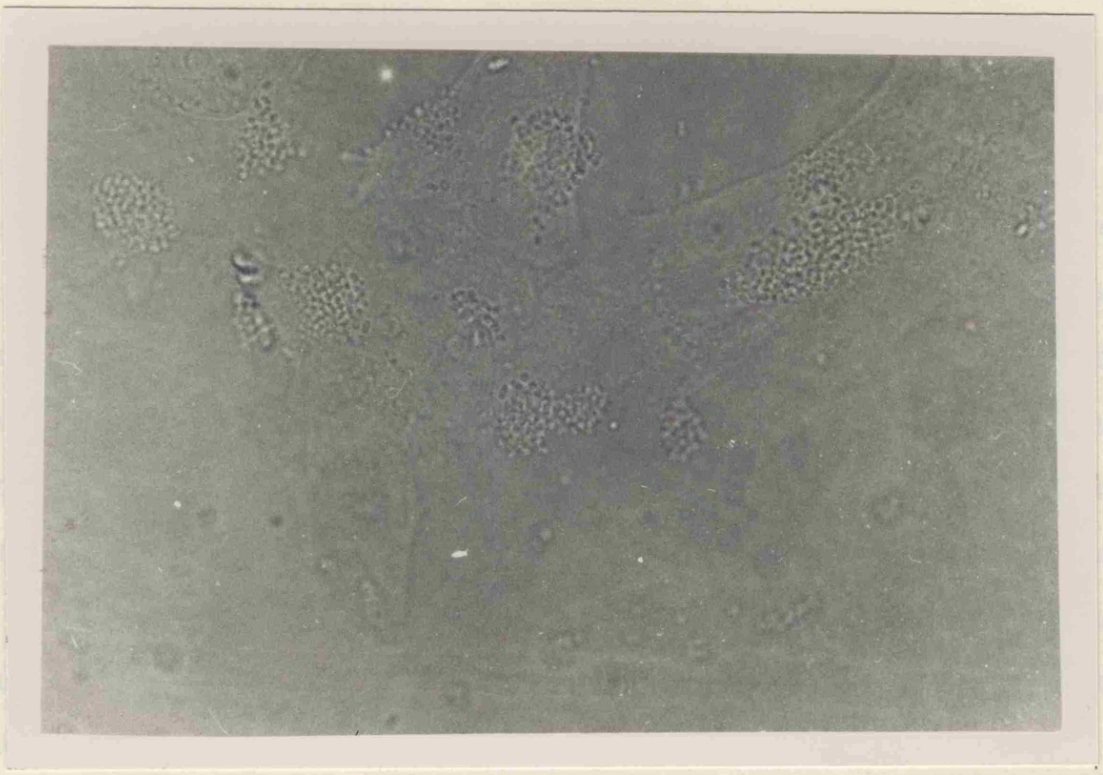

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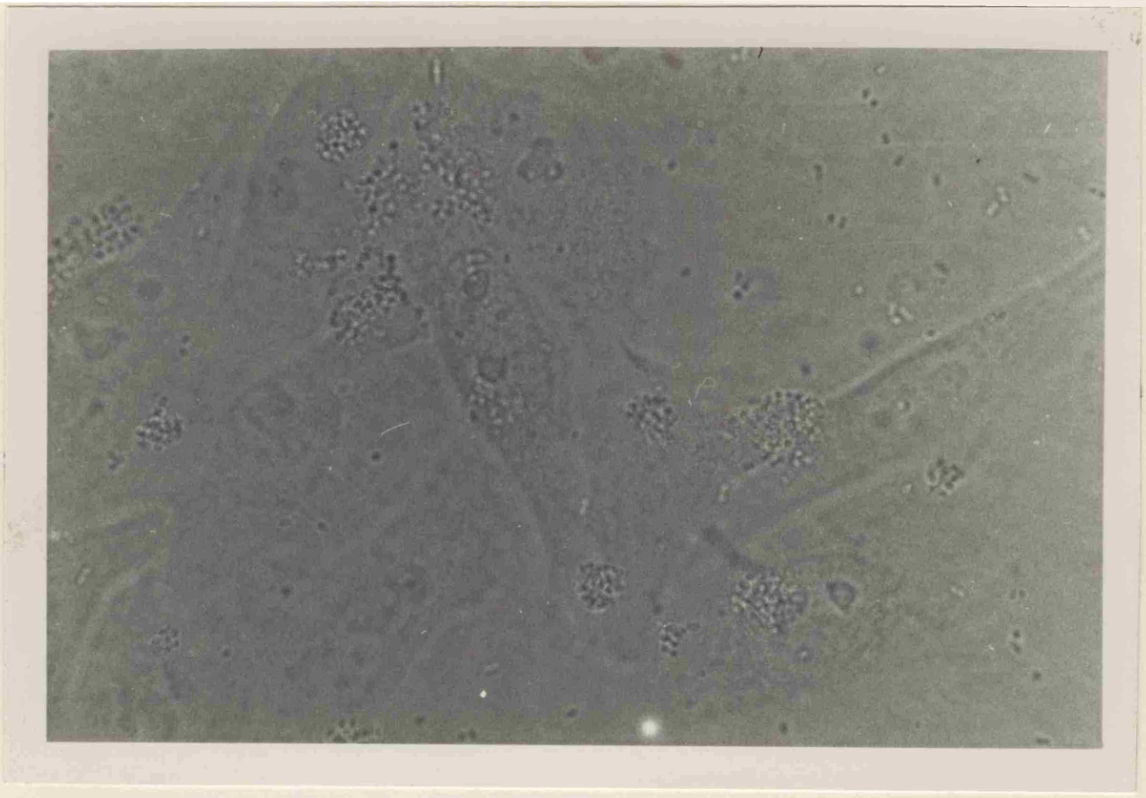
Figure 3.4. Effect of dantrolene on lesion formation.

the concentration of free calcium back down to normal levels and possibly prevent further actin accretion.

Inhibition of calmodulin action might therefore be expected to block this putative initial stage, thereby preventing the development of the pedestal. Pre-treatment of HEp-2 cells with low concentrations (0.5 μM) of the calmodulin inhibitors compound R24571 and 48/80 (Sigma) did not inhibit the accumulation of actin at sites of bacterial adherence (**Figure 3.5**, and **Figure 3.8**). But at higher concentrations (5 μM) partial inhibition was seen with 48/80, in the case of compound R24571 the host cells showed marked vacuolization of the cytoplasm, and disruption of stress fibres. compound R24571 also caused some damage to bacteria, causing normally nonfluorescent bacteria to exhibit a low level of phalloidin fluorescence, possibly due to leakage of phalloidin into the bacterial cells. With 48/80 treatment, however, complete inhibition of actin accretion in infected cells was seen with little damage to bacteria or host cells. In an attempt to overcome the problem of cells being exposed for long periods to the cytotoxic compound compound R24571, cells were also treated with the inhibitors at the time of infection. Actin accretion was inhibited as before but the cytotoxic effects though still evident were less severe..

HEp-2 cells pre-loaded with the cell-permeable calcium chelator quin-2 were infected with EPEC to determine whether any inhibition of lesion formation could be detected in cells buffered for calcium. The results (**Figure 3.9**) revealed that artificial calcium buffering did not greatly affect normal lesion formation in infected cells. This may be a result of the localized nature of the lesion or possibly because an element involved in lesion development is able to remove calcium from the quin-2 molecule, so overcoming the buffering effect.

Figure 3.5. Effect of low concentrations of the calmodulin inhibitor compound R24571 on lesion formation. HEp-2 monolayers were pre-treated with 0.5 μM R24571 for 0.5 h and infected for 3 h with 10^8 EPEC strain 2036-80. **(A)** Phase micrograph, normal adherence of bacteria to cells as microcolonies. **(B)** Fluorescence micrograph, actin accretion unaffected by treatment with actin fluorescence associated with indavidual attached. Magnification 2,000 X.



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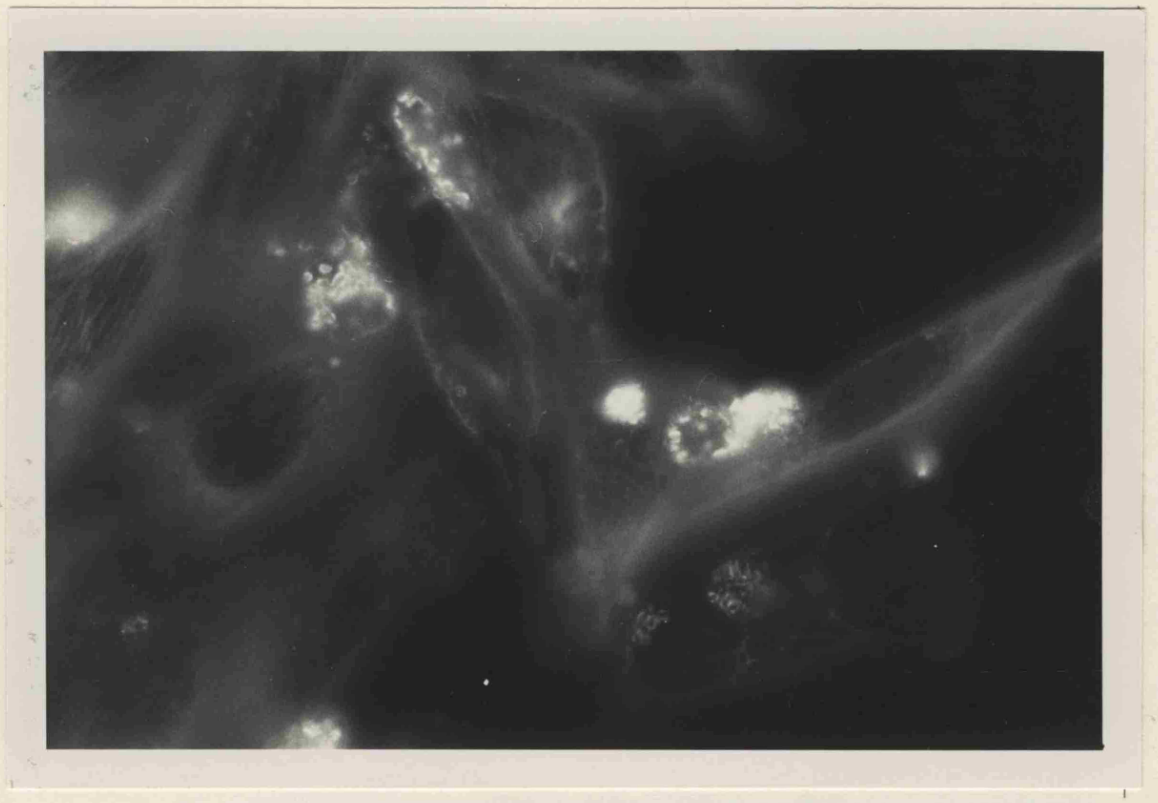

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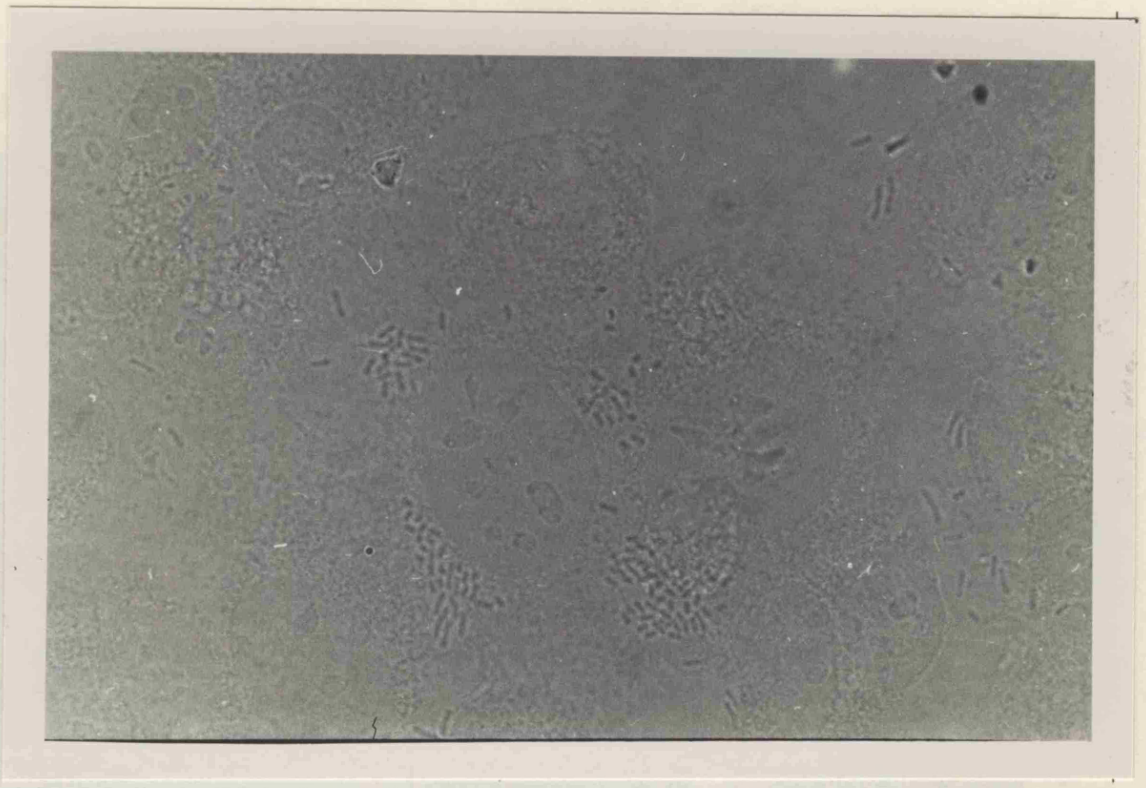
Figure 3.5. Effect of low concentrations of the calmodulin inhibitor compound R24571 on lesion formation.

Figure 3.6. Effect of high concentrations of the calmodulin inhibitor compound R24571 on lesion formation. HEp-2 monolayers were pre-treated with 5 μM R24571 for 0.5 h and infected for 3 h with 10^8 EPEC strain 2036-80. **(A)** Phase micrograph, normal adherence of bacteria to cells as microcolonies. **(B)** Fluorescence micrograph, no accretion of actin observed in association with attached bacteria. Magnification 2,000 X.



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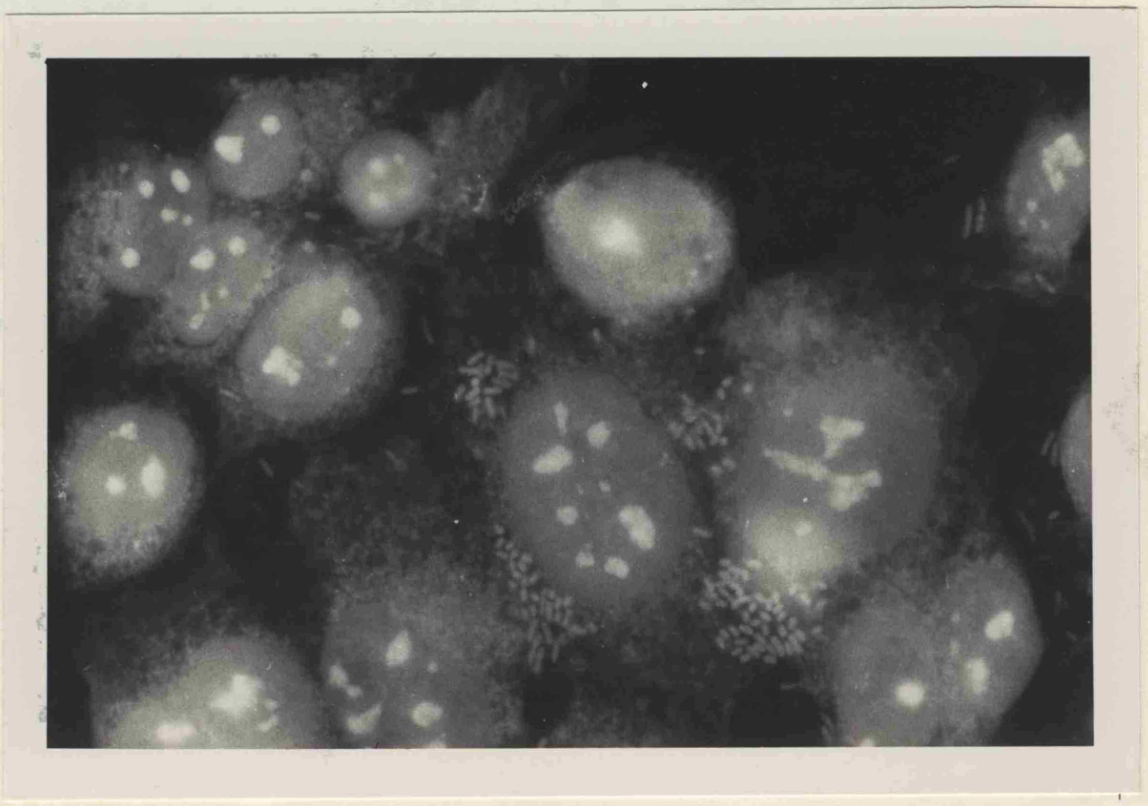

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Figure 3.6. Effect of high concentrations of the calmodulin inhibitor compound R24571 on lesion formation.

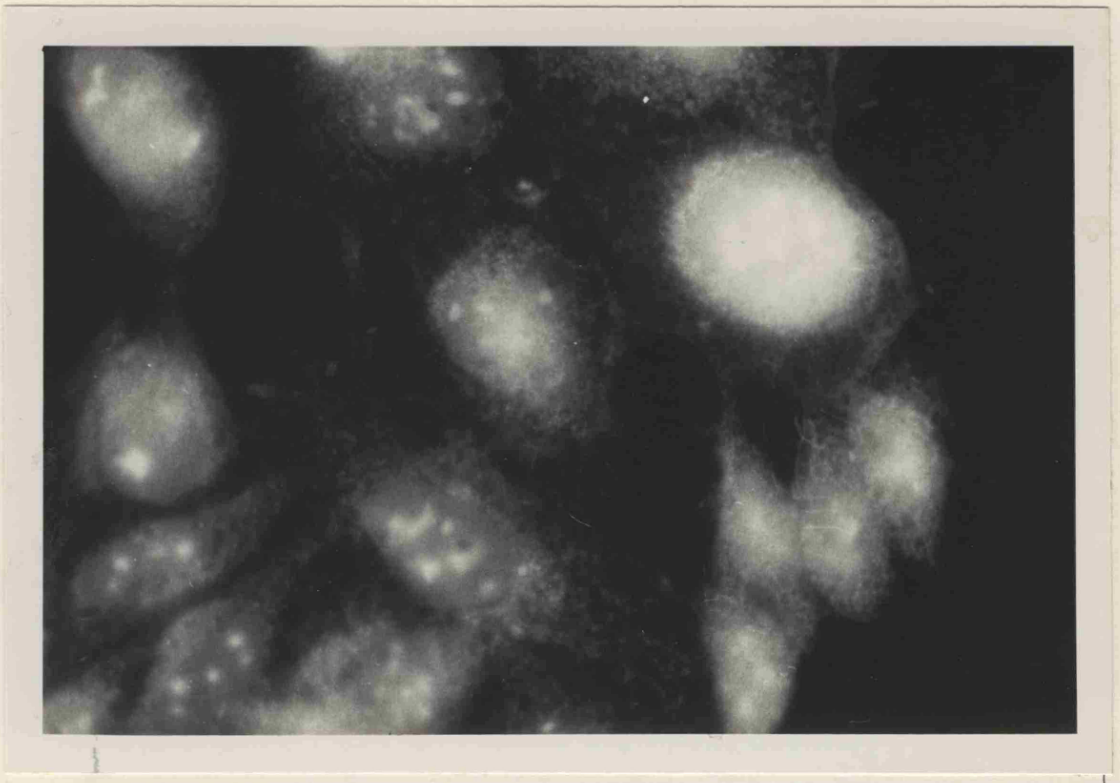
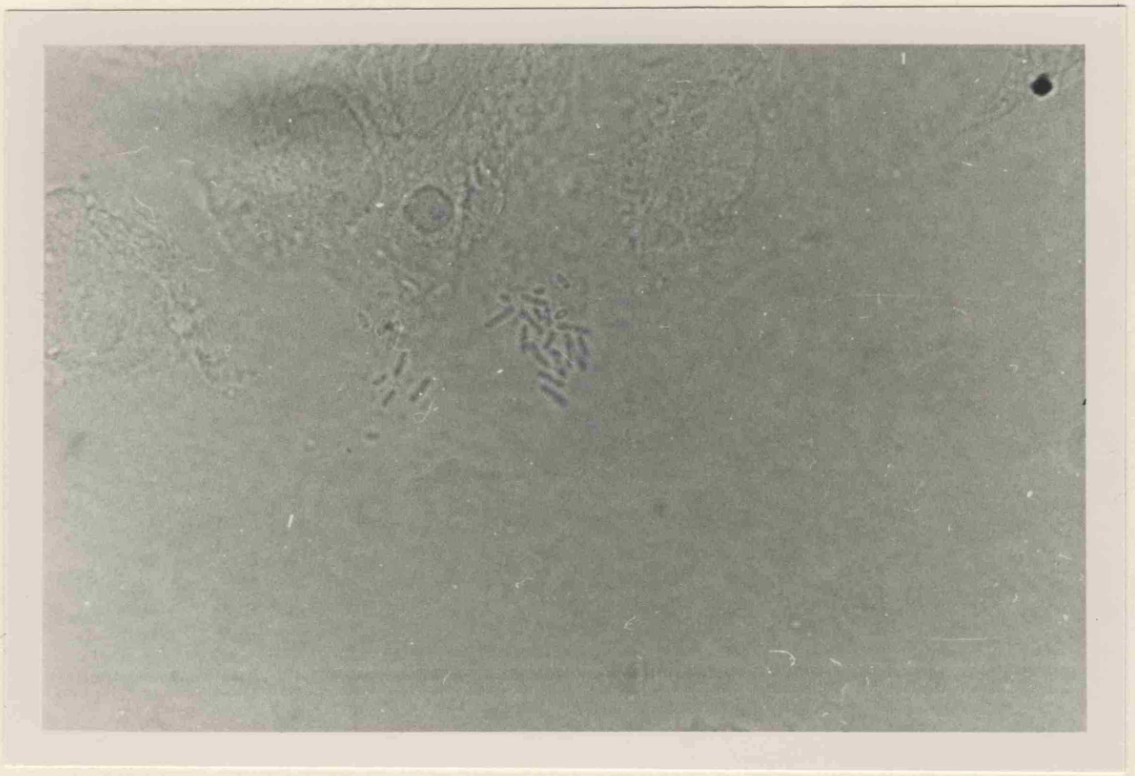


Figure 3.7. Effect of high concentrations of compound R24571 on cytoskeletal integrity. Uninfected HEp-2 monolayers pre-treated with 5 μM compound R24571 for 0.5 h and stained for filamentous actin. Disruption of the cytoskeleton and intense regions of fluorescence in the nucleus suggests cells were in a distressed state. Magnification 2,000 X.

Figure 3.8. Effect of low concentrations of the calmodulin inhibitor 48/80 on lesion formation. HEp-2 monolayers were pre-treated with 0.5 μM 48/80 for 0.5 h and infected for 3 h with 10^8 EPEC strain 2036-80. (A) Phase micrograph, reduced microcolony formation, and slight elongation of bacteria. (B) Fluorescence micrograph, reduced diffuse actin fluorescence associated with regions of bacterial attachment. Magnification 2,000 X.



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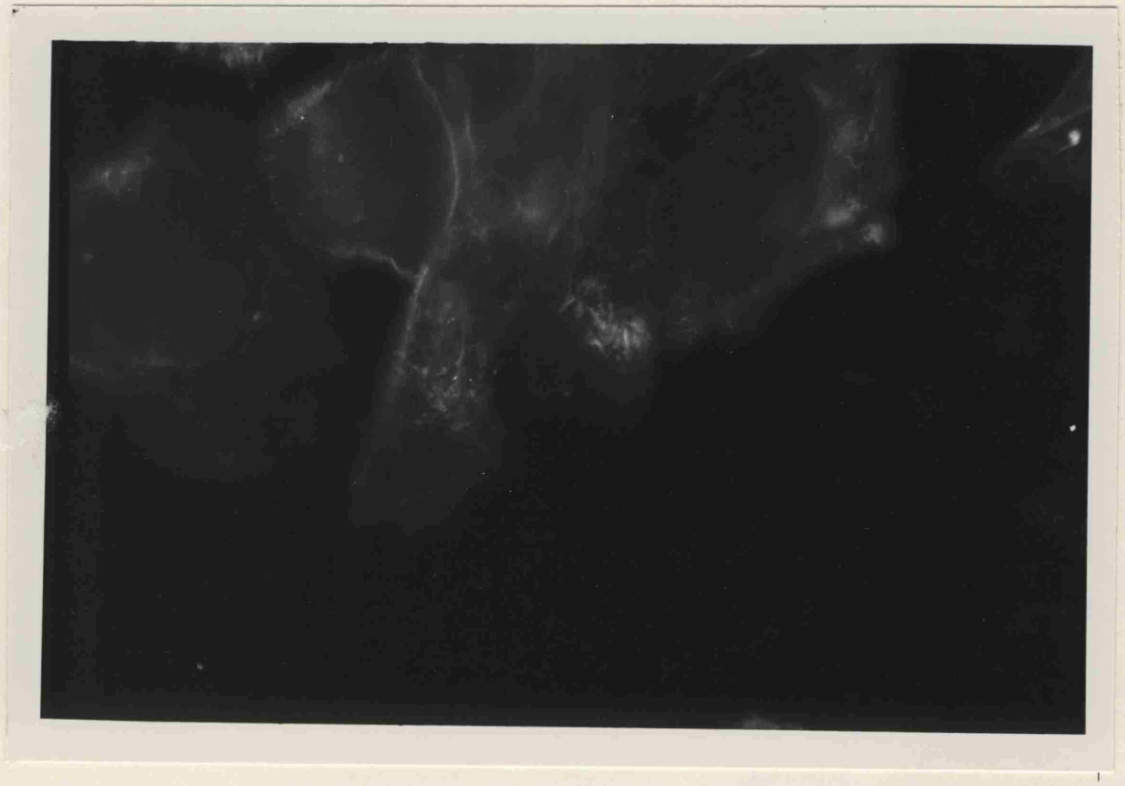
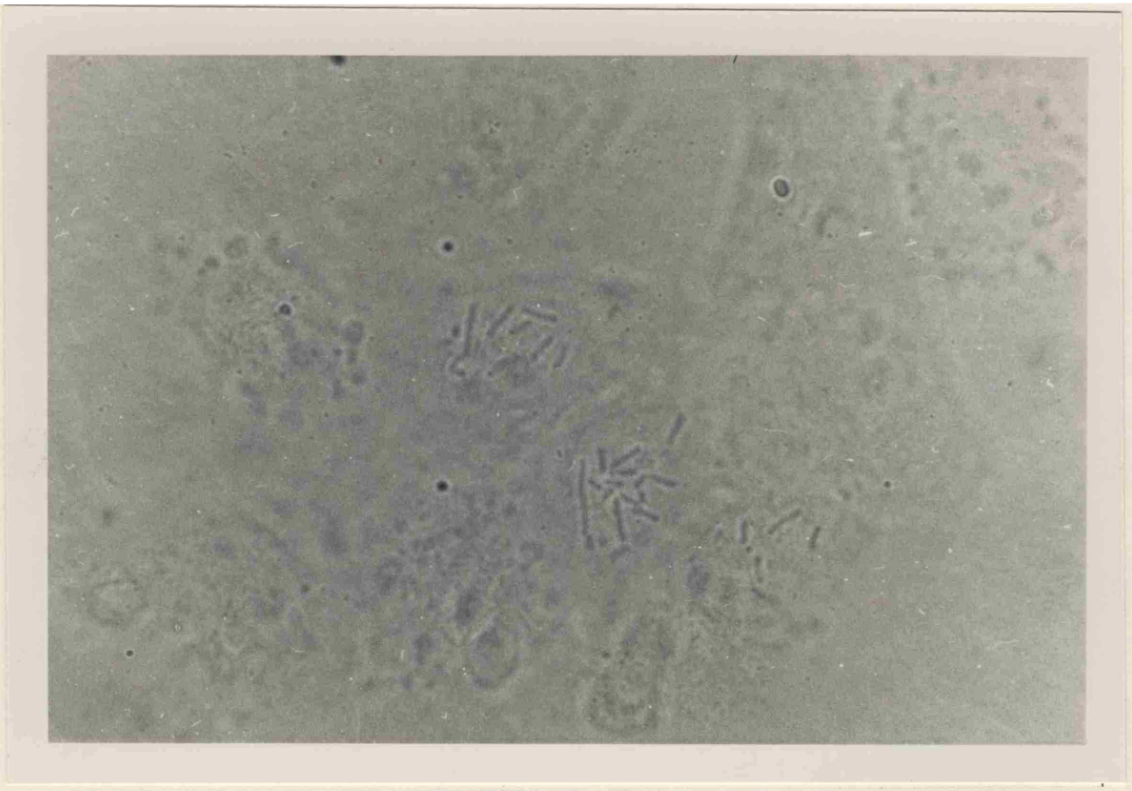


B

Figure 3.8. Effect of low concentrations of the calmodulin inhibitor 48/80 on lesion formation.

Figure 3.9. Effect of high concentrations of the calmodulin inhibitor 48/80 on lesion formation. HEp-2 monolayers were pre-treated with 5 μM 48/80 for 0.5 h and infected for 3 h with 10^8 EPEC strain 2036-80. (A) Phase micrograph, reduced microcolony formation, and elongation of bacteria. (B) Fluorescence micrograph, increased general fluorescence of cells and no actin fluorescence associated with regions of bacterial attachment. Magnification 2,000 X.



A

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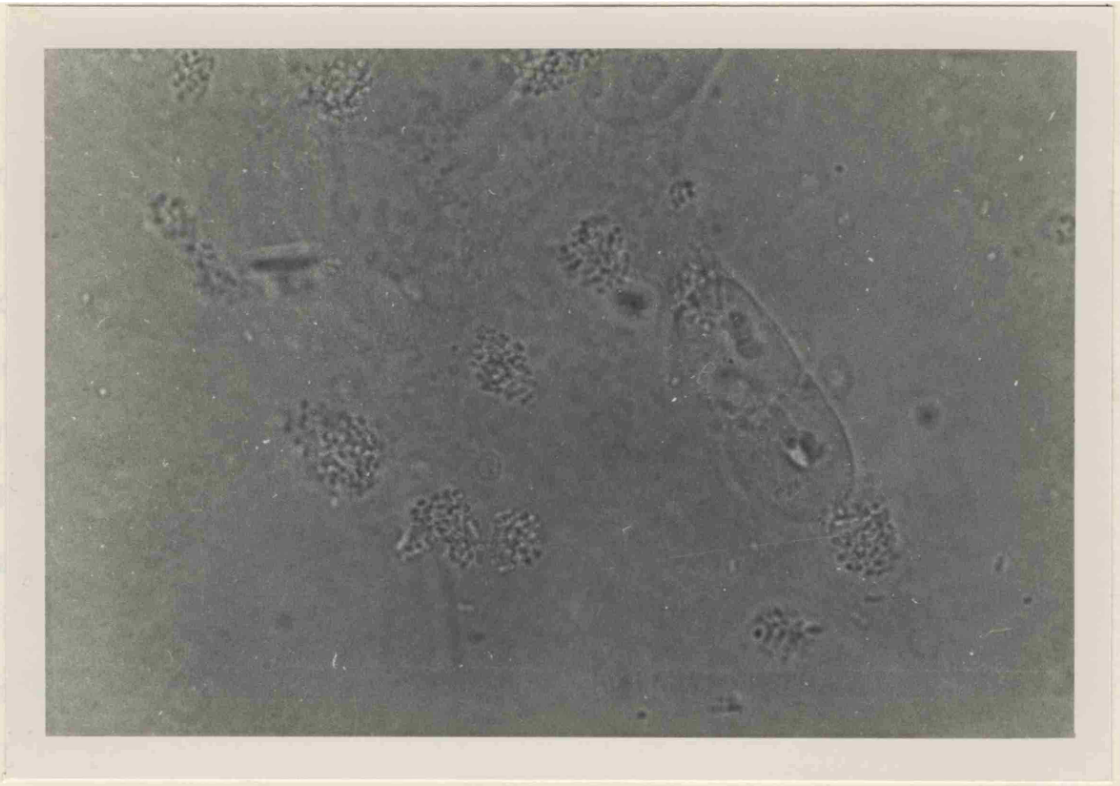
Figure 3.9. Effect of high concentrations of the calmodulin inhibitor 48/80 on lesion formation.

Figure 3.10. Effect of chelation of intracellular calcium with quin2 on lesion formation. HEp-2 cell monolayers pre-loaded with 12.5 μM quin2 were infected with 10^8 bacteria of EPEC strain 2036-80 for 3 h. (A) Phase micrograph, normal microcolony formation. (B) Fluorescence micrograph, slight reduction in actin fluorescence associated with attached bacteria. Magnification 2,000 X.



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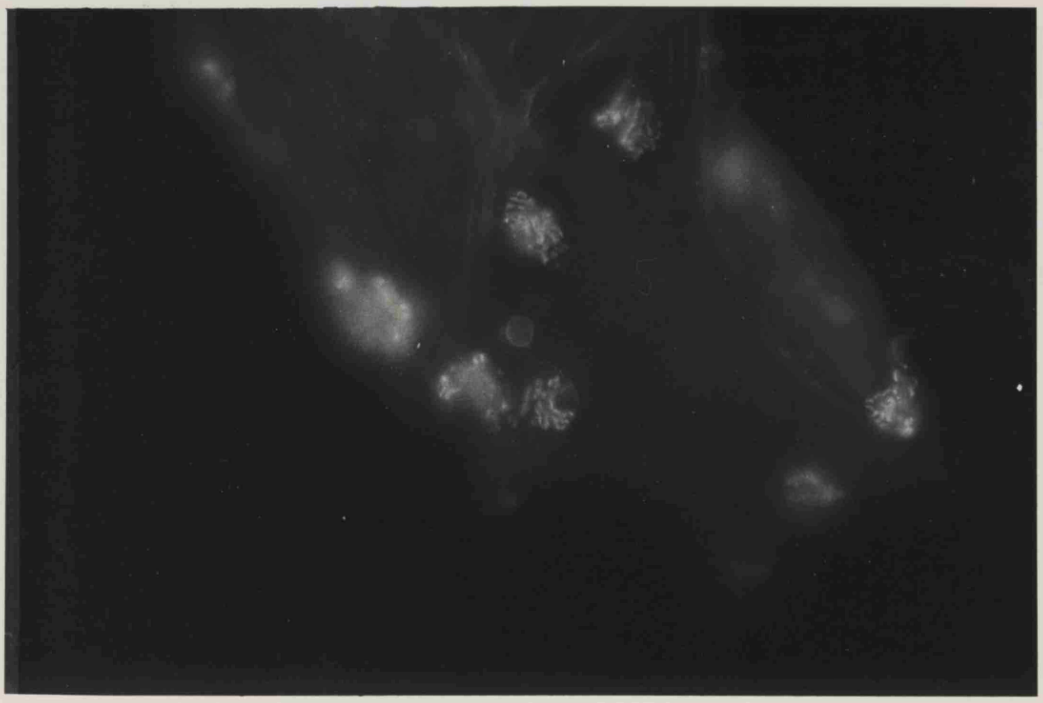

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Figure 3.10. Effect of chelation of intracellular calcium with quin2 on lesion formation.

3:3. *Discussion and conclusions.*

The accretion of actin within the pedestal may either be considered as a cause of, or coincident with lesion pedestal formation. As mentioned previously it is unlikely that the dense pad of actin seen in the pedestal forms purely as a result of actin breakdown elsewhere in the cell, (e.g. in the microvilli of gut enterocytes). The density and localized nature of actin accretion suggests that *de novo* actin filament polymerization is occurring. It has been noted previously that accretion of actin is associated with the mobility of invasive bacteria such as *Shigella* around the cytoplasm, even causing membrane extrusions containing the bacteria surrounded with short actin filaments (**P. Sansonetti, personal communication**). In this way actin accretion acts as a wave pushing bacteria and membrane outwards, facilitating the lateral movement of the invading organism into adjacent cells. In a similar way the breakdown products of calcium dependent actin solation by villin in the microvilli and gelsolin in the body of the cell may form nucleation sites for actin accretion analogous to the gel sol cycle of actin. Since major membrane movements are required for pedestal formation, it is postulated that substantial accretion of filamentous actin just below the membrane at the site of bacterial attachment forms a localized actin gel which forces the membrane and attached bacteria out from the body of the cell. The initial breakdown of the microvillus cytoskeleton most likely results from the calcium activation of the actin severing protein villin, while the accreted actin and the resulting pedestal arise as a consequence of gelsolin activity in the cytoplasm. The loss of microvilli would allow the attachment of incoming EPEC to the plasma membrane surface of the cell, possibly causing an elevation in the local calcium concentration in the cytoplasm. Any changes in calcium concentration in the vicinity of the attached bacteria would promote local actin filament severing by gelsolin (**Figure 3.1**). Consequently as the calcium disperses and local concentrations declined the large number of short gelsolin capped actin fragments would associate with PIP_2 in the membrane. The ensuing uncapping of the fragment's barbed end would promote a burst of actin polymerization leading to a local

actin gel (**Figure 3.2**) which would push the membrane out in the direction of its formation.

The fact that the actin pad formation as detected using the FAS test could not be totally inhibited using procedures designed to limit the changes in cytosolic calcium, is thought to reflect the difficulties of working with such a localized phenomenon, rather than the possibility that calcium rises play no part in the fate of actin during EPEC infection. The use of calmodulin inhibitors, however, has shown that pedestal formation can be prevented, indicating a role for calmodulin-calcium in the early stages of the lesion. However as yet, other effects of these inhibitors on the cell cannot be ruled out as the cause of the observed FAS test inhibition.

Chapter 4. VIABILITY OF EPEC INFECTED HEP-2 CELLS.

4:1. *Introduction.*

EPEC infection of gut tissue is characterised by major ultrastructural changes in the host cell, involving breakdown and redistribution of cytoskeletal actin (Chapters 2 and 3). In addition, prolonged infection of patients with EPEC causes gross histological dammage to the intestinal mucosa resulting from extensive cell death in regions of colonisation (Chapter 1). These observations suggest that a cytopathic effect is associated with EPEC, and it would therefore be expected that extended infection of tissue culture cells with EPEC isolates would be accompanied by loss of host cell viability.

The processes which eventually lead to cell death can be split into two categories, those that are reversible, characterised by pronounced morphological changes in the cell including bleb formation at the cell surface, chromatin clumping, condensation of the mitochondrial matrix, and dilation of the endoplasmic reticulum, and those that are irreversible, including disruption in the integrity of the cell membrane, mitochondrial swelling, DNA fragmentation, and in some cases mitochondrial calcification (**Trump and Berezesky, 1985; Herman et al., 1988; Kaminskas and Li, 1989**). In many instances the most obvious sign of cellular crisis is vesiculation and blebbing of the plasma membrane (**Trump and Berezesky, 1985; Lemasters et al., 1987; Herman et al., 1988**) which has led to its use as an indicator of impending cell death. Hepatocytes in which anoxia (depletion of the ATP pool) is mimicked by treatment with potassium cyanide and iodoacetate show extensive blebbing of the plasma membrane surface (**Herman et al., 1988; Nicotera et al., 1989**), and loss of cell viability within 3 hours (**Herman et al., 1988**). Opinion is split, however, concerning the underlying cause of membrane blebing, some groups have reported an increase in intracellular calcium concentration of individual cells (as measured using the calcium indicator dye fura2) as an early event in the process of cell death, and

coincident with bleb formation (**Trump et al., 1981; Trump and Berezesky, 1985; Trump and Berezesky, 1987; Nicotera et al., 1989**). Evidence given in support of this notion shows that preloading cells with the non-fluorescent calcium buffer BAPTA, (bis-[*O*-aminophenoxy]-ethane-N,N,N',N'-tetracetic-acid), dampened the rise in cytosolic calcium, abolished bleb formation and extended the viability of the cells by several hours (**Trump and Berezesky, 1985; Nicotera et al., 1989**). The role of calcium in the loss and viability and distortions in the plasma membrane is unclear and contentious, but appears to relate to alterations in cytoskeletal elements and their association with the plasma membrane, (**Trump et al., 1981; Nicotera et al., 1989**), and the activation of certain proteases, which themselves modulate cytoskeletal structure (**Nicotera et al., 1989**). Other groups maintain that increases in intracellular calcium are not associated with bleb formation, or the early stages of viability loss (**Lemasters et al., 1987; Herman et al., 1988**); but rather changes in *p*H, cell volume and membrane-cytoskeleton interactions (**Trump et al., 1981**) contribute to disturbances and blebing of the plasma membrane. It is agreed that cell death almost certainly relates to the breakdown of plasma membrane integrity resulting directly from formation of numerous blebs on the cell surface (**Lemasters et al., 1987; Herman et al., 1988; Nicotera et al., 1989**). During the early stages of cellular distress, blebs are small, but with time they coalesce to form larger balloonings of the plasma membrane (**Herman et al., 1988**). Loss of viability results from a rapid and irreversible rupture of the larger membrane balloons causing complete loss of membrane integrity (**Lemasters et al., 1987; Herman et al., 1988; Nicotera et al., 1989**). Therefore the two stages of cell death (reversible and irreversible) are separated by the actual point of death caused by membrane rupture.

In the preceeding two chapters it has been shown that EPEC infection of tissue culture cells causes important biological changes, including cytoskeletal dammage, alterations in local actin densities, and plasma membrane blebbing. These are also characteristics of cells which are in distress, and may eventually lead to cell death. Therefore the viability of EPEC infected tissue culture cells was investigated at different

times after infection, using two vital staining procedures to determined whether loss of viability could be detected in the early stages of EPEC infection.

4:2. *Materials and methods.*

The viability of EPEC infected tissue culture cells was determined using two methods which rely on the uptake of fluorescent dyes into different cellular compartments. The first involves staining with acridine orange and ethidium bromide, and relies on the selective exclusion of one dye, giving a visible colour difference between viable and non-viable cells. Both acridine orange and ethidium bromide bind to DNA and therefore will tend to selectively stain the nucleus. In viable cells acridine orange readily enters the cell, staining the nucleus green under fluorescence, while ethidium bromide is excluded from the cell. In nonviable cells both dyes are able to enter the cell through loss of membrane integrity, causing the nucleus to appear yellow under fluorescence. (**Parks et al., 1979**) The second protocol uses rhodamine 123 staining, which acts as a specific probe for mitochondrial membrane potential (**Johnson et al., 1980; Johnson et al., 1981**). Entry and accumulation of this dye into the mitochondrial matrix depends on the presence of a high potential difference across the mitochondrial membrane, which itself depends on the metabolic activity of the cell.

4:2:1. Ethidium bromide and acridine orange staining. HEp-2 cells were grown to 50% confluence on glass cover slips as described in Chapter 2. Monolayers were infected with 10^8 bacteria for the stated times, and washed every 2 h to remove excess bacteria. At the end of this time monolayers were rinsed three times with fresh DMEM medium at 37°C and stained with 1 μg/ml each of ethidium bromide and acridine orange solution (in a final volume of 20 μl). The monolayers (still in the staining solution) were mounted inverted on glass slides using a small cushion of silicone grease at each corner of the cover slip, and observed immediately at low (x160) and medium (x400) power under fluorescence and phase with a Zeiss D-7082 fluorescent microscope. Dead cell

controls were obtained by treating monolayers with 3% formaldehyde for 1 h, or 34 mg/ml chloramphenicol for 24 h.

4:2:2. Rhodamine 123 staining. HEp-2 cell monolayers at 50% confluence were infected for appropriate times, washed 3 times in fresh DMEM medium at 37°C, and stained in DMEM medium containing 10 μg/ml rhodamine 123 at 37°C for 10 min. After washing 3 times in DMEM without rhodamine 123, cover slips were mounted in 10 μl of fresh DMEM on glass slides and observed under phase and fluorescence using a Zeiss D-7082 fluorescent microscope.

4:3. *Results.*

4:3:1. Acridine orange/ethidium bromide staining. Control live and control dead HEp-2 cell monolayers showed observable differences in staining using the dyes ethidium bromide and acridine orange. Live cells fluoresced a distinctive green, while dead cells had a uniform orange fluorescence throughout. When HEp-2 monolayers were infected with *E. coli* isolates consisting of EPEC and non-EPEC strains for various times, and were then stained for viability using ethidium bromide and acridine orange, striking differences in the killing ability of the various strains were observed. With the lesion-forming EPEC strains 2036-80 and E2348-69, the percentage viability of infected cells had dropped to about 20% by 6 h (**Table 4.1**) and to about 10% by 9 h. For the invasive strain 469-3 and the diffuse adhering strain 135 no reduction in viability was detected over these times, the percentage viability remaining comparable with the contols (**Table 4.1**). Culture supernatant filtrates of the two EPEC strains caused no reduction in viability of monolayers even after 9 hours at 37°C. In addition the involvement of an intracellular calcium increase in the apparent viability loss of EPEC (2036-80) infected cells, was indicated by the observation that loading of monolayers prior to infection with the calcium chelator quin2 extended the viability by 2-3 h (**Table 4.1**).

Table 4.1. Viability of confluent HEp-2 cell monolayers grown in DMEM containing 10% foetal calf serum on 12 mm^2 treated with EPEC and non-EPEC *E. coli* strains for various times. Viability was determined using two vital staining protocols, etidium bromide and acridine orange (Eth/Ao), and rhodamine 123.

[a] Known localized adhering, lesion-forming EPEC isolates.

[b] Monolayers loaded with 12.5 μM quin2 prior to infection. Viability using rhodamine not determined.

[c] Monolayers treated with filter sterilised culture supernatants

[d] Diffuse adhering, invasive, non-lesion-forming isolate.

[e] Diffuse adhering, non-invasive, non-lesion-forming isolate.

[f] Control laboratory strain HB101 carrying the localized adherence plasmid pMAR15.

[g] Uninfected control monolayer.

ND:- Not Done

Bacterial strains	Time hours	% Viability (Eth/Ao)	% Viability (rhodamine)
Uninfected[g]	---	100	100
2036-80[a]	1 h	100	100
2036-80[a]	2 h	88	95
2036-80[a]	4 h	66	69
2036-80[a]	6 h	17	20
2036-80[a]	9 h	6	3
2036-80[b]	1 h	100	ND
2036-80[b]	2 h	100	ND
2036-80[b]	4 h	95	ND
2036-80[b]	6 h	60	ND
2036-80[b]	9 h	26	ND
2036-80[c]	6 h	100	100
2036-80[c]	9 h	100	100
E2348-69[a]	1 h	100	100
E2348-69[a]	2 h	90	93
E2348-69[a]	4 h	68	65
E2348-69[a]	6 h	20	23
E2348-69[a]	9 h	8	8
E2348-69[c]	6 h	100	100
E2348-69[c]	9 h	100	100
469-3[d]	1 h	100	100
469-3[d]	4 h	100	100
469-3[d]	6 h	100	100
469-3[d]	9 h	100	90
135[e]	1 h	100	100
135[e]	6 h	100	100
135[e]	9 h	100	100
HB101[f]	4 h	100	100
HB101[f]	6 h	100	100
HB101[f]	9 h	100	100

4:3:2. Rhodamine 123 staining. Using the alternative method of viability assessment which relies on the uptake of rhodamine 123 by functional mitochondria, live cells were seen to give a characteristic granular, green fluorescence, which at higher magnifications was identified as being associated with mitochondria. Dead cells on the other hand presented very little fluorescence. A similar time scale of HEp-2 cell viability loss was seen after infection with strains 2036-80 and E2348-69 (**Table 4.1**), a significant porportion of the cell population about 80% being inviable by 6 hours. Again, strains 469-3 and 135, and the culture supernatant filtrates from 2036-80 and E2348-69 caused no appreciable loss of HEP-2 viability after 9 hours infection (**Table 4.1**).

4:4. *Discussion and conclusions.*

Although these vital staining methods are useful for the evaluation of effects on the cell of infection with EPEC, it must be realised that a nonviable result using these dyes does not necessarily mean that the condition of the cell is irreversible. As mentioned in the introduction to this chapter the point of actual cellular death forms a boundary between reversible and irreversible phases. During the reversible phase (termed here cell distress) transport mechanisms and permeability of the plasma and mitochondrial membranes are bound to be affected. Therefore the vital staining protocols described above will reflect this by giving staining patterns indicative of viability loss. The point of actual irreversible cellular death cannot be distinguished using these methods unless they are carried out in conjunction with reversibility tests, which at this time is not possible with EPEC infected cells. Therefore the times given in this chapter should be taken as the point where cells enter the reversible phase of the death process, with actual loss of viability occuring some time after this point. With this in mind, the figures of percentage loss of viability after a given time should be regarded as the number of cells in distress at that time, death may result some considerable time later.

By 3 h infection with the two EPEC strains 2036-80 and E2348-69 no appreciable loss of HEp-2 cell viability could be detected using either of the methods described above. By 9 h however, the percentage viability of the HEp-2 monolayer appeared reduced to below 10%, indicating a cytopathic effect of EPEC for cultured cells.

The two procedures used for the evaluation of viability demonstrate that both the proper functioning of the plasma membrane, and the membrane potential of mitochondria are affected within a short period of time subsequent to infection with lesion forming EPEC isolates. This apparent loss of viability appears to be associated with attachment of the bacteria to the cell surface, since the culture supernatant filtrates from 2036-80 and E2348-69 caused no observable reduction in HEp-2 cell viability. The involvement of calcium in this process was indicated by the increased survival of 2036-80 infected cells, previously loaded with the calcium buffer quin2. The nonlesion forming, FAS test negative strains 135 and 469-3 were not associated with loss of host cell viability in this time scale. However, A slight decrease in the number of apparently viable cells seen at 9 h infection with the invasive strain 469-3, using rhodamine 123, may suggest that the invading organism or one of its products is able to affect the functioning of the host cell mitochondria, which may eventually lead to loss of viability after a longer period of incubation.

As mentioned previously the process of cell death is in most cases attributed to the eventual breakdown of membrane integrity resulting from rupture of large blebs on the cell surface (**Herman et al., 1988**). This in turn is probably preceded by elevation in intracellular calcium levels and the formation of smaller blebs in distressed cells (**Nicotera et al., 1989**). Prior to membrane rupture (which would allow the uncontrolled influx of calcium) this elevation is thought to result from a controlled influx and/or the release of calcium from intracellular storage sites (i.e. the endoplasmic reticulum and mitochondria) in response to cellular signals (**Trump et al., 1985; Trump et al., 1987**). The fact that quin2 loading delays the effects of EPEC on viability staining, and that calcium elevation seems to precede cell death (**Trump et al., 1985; Trump et al., 1987;**

Nicotera et al., 1989), suggests that intracellular calcium elevations may have a role in the pathogenesis of EPEC isolates.

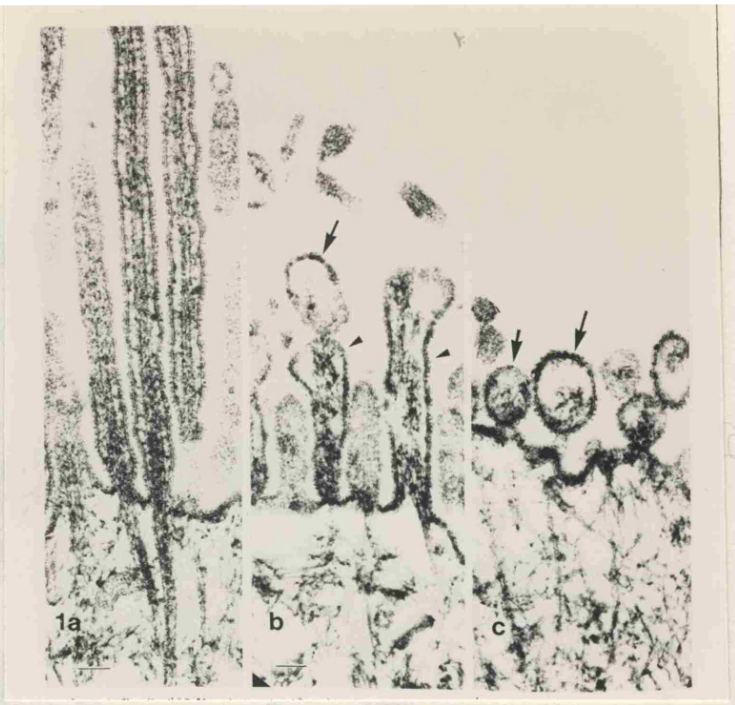
Chapter 5. INTRACELLULAR CALCIUM IN EPEC-INFECTED HEp-2 CELLS.

5:1. *Introduction.*

The characteristic lesion of EPEC infected gut cells (**Polotsky et al., 1977; Knutton et al., 1987b**) has been previously described as showing remarkable similarities to the observed loss of microvilli when permeabilized enterocytes are treated with a high concentration of calcium (**Mooseker et al., 1980b; Boedeker, 1982; Burgess, and Prum, 1982; Matsudaira and Burgess, 1982; Knutton et al., 1987b; [Figure 5.1]**). Influx of calcium ions from outside the cell mediates the activation of villin (an actin severing protein), causing breakdown of the actin component of the microvillus core, leading to eventual loss of microvilli through membrane vesiculation (**Mooseker et al., 1980a; Mooseker et al., 1980b; Matsudaira and Burgess, 1982; Glenney and Glenney, 1985**). A similar effect is seen in canine renal proximal tubule cells (**Figure 5.2**), treated in ways which are known to elevate intracellular calcium by two different mechanisms (**Goligorsky et al., 1986**), i.e. with the agonists parathyroid hormone and angiotensin II, or with calcium ionophores. Binding of certain agonists to their respective receptors elicits mobilization of intracellular calcium stores (**Goligorsky et al., 1986; Mahadevan et al., 1987; Mattila et al., 1989; Tsuda et al., 1985**) through a signal transduction process, in which membrane associated PLC is activated via a coupled GTP binding G-protein (**Imboden and Weiss, 1987; Inoue et al., 1988; Meyer and Stryer, 1988; Schnefel et al., 1988; Taylor et al., 1988a; Aiyar et al., 1989; Baldassare et al., 1989**). PLC mediates the hydrolysis of membrane phosphatidylinositol 4,5-diphosphate (PIP_2) forming the second messenger molecules 1,4,5-inositol trisphosphate (1,4,5-IP_3) and diacylglycerol (DAG) (**Berridge, 1984; Berridge, 1987; Berridge and Irvine, 1984; Exton, 1988**). This process constitutes an important pathway for the conveyance of hormone generated signals across the cell membrane (**Figure 5.3**). 1,4,5-IP_3 is released into the cytoplasm, and functions as the second messenger for the mobilization of calcium from intracellular stores (**Irvine et**

Figure 5.1. Similarity between calcium dependent loss of microvilli from the surface of permeabilized enterocytes treated with high calcium medium (**Matsudaira and Burgess, 1982**), and the EPEC lesion. (A) Permeabilized chicken enterocytes treated with high calcium. (a) Untreated enterocytes. (b) and (c) Enterocytes treated with high calcium medium (1 μM) showing progressive loss of microvilli through membrane vesiculation. Magnification 40,000 X. (B) Typical vesiculation of microvillus membrane during early stages of EPEC infection of gut tissue (Dr. S. Knutton, Institute of child health, Birmingham). Magnification 30,000 X.

A



B

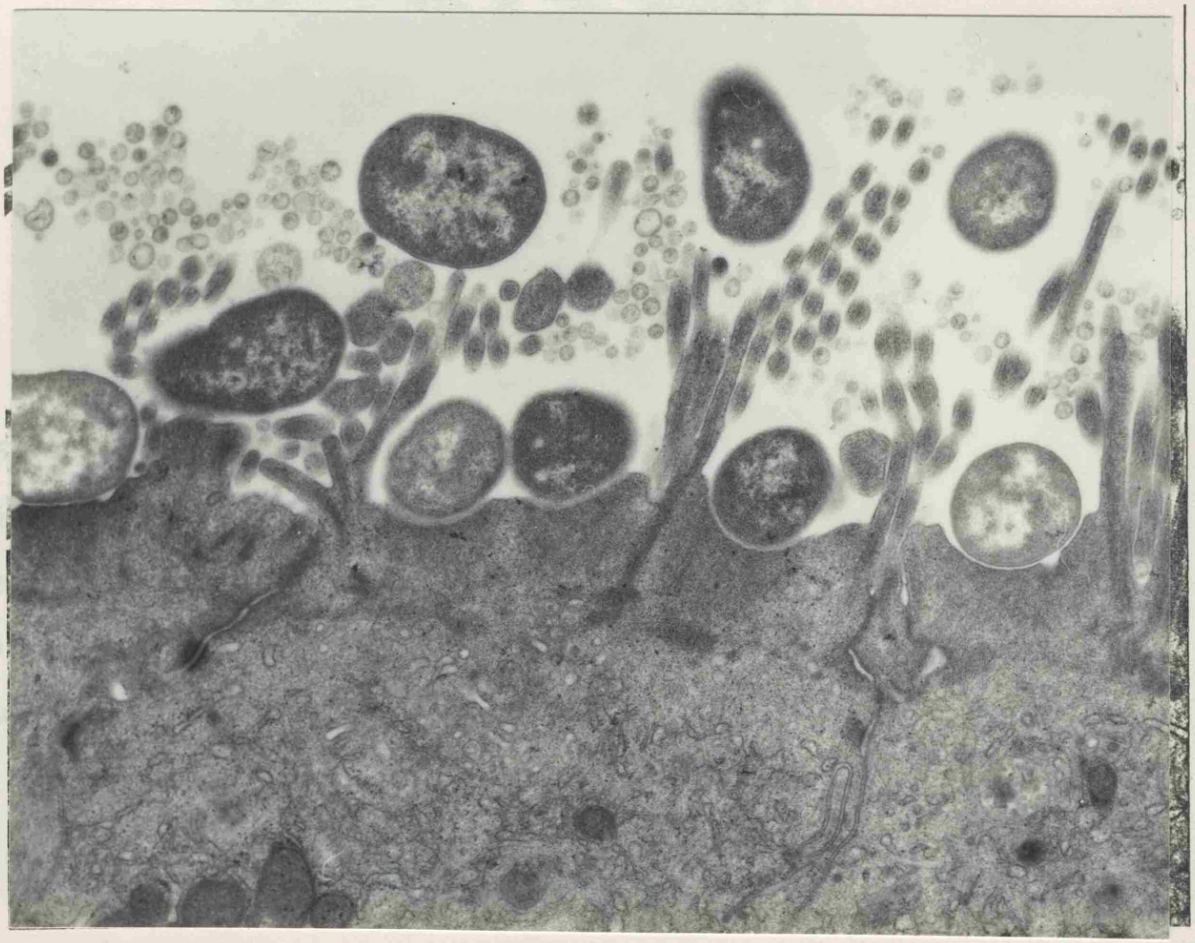


Figure 5.1. Similarity between calcium dependent loss of microvilli from the surface of permeabilized enterocytes treated with high calcium medium (**Matsudaira and Burgess, 1982**), and the EPEC lesion.

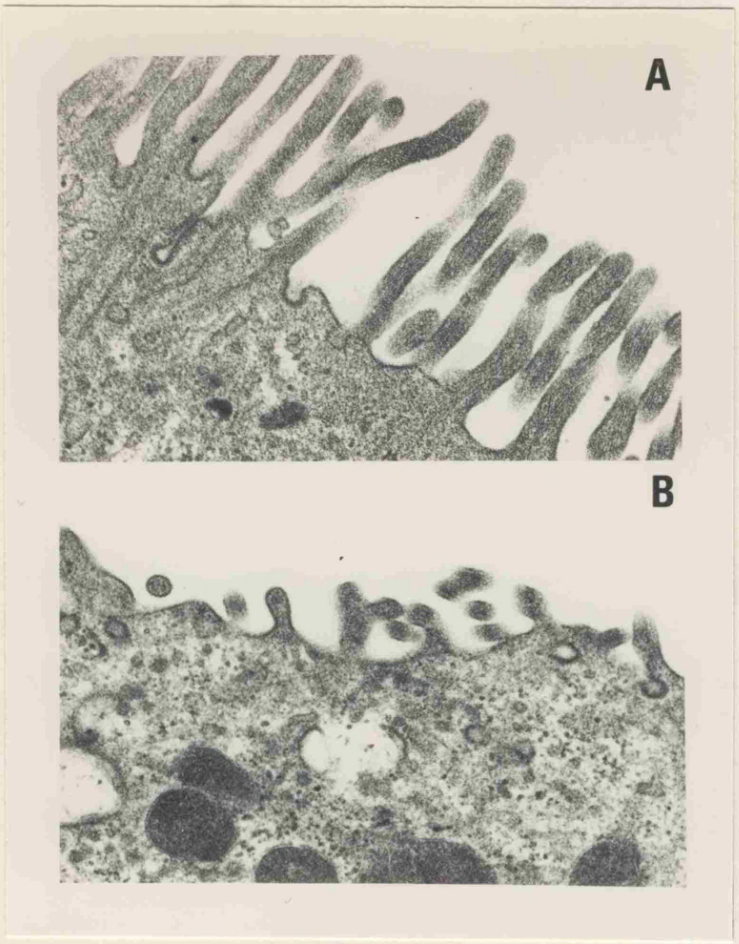


Figure 5.2. Cells derived from canine renal proximal tubule cells treated with calcium mobilizing, parathyroid hormone (**Goligorsky et al., 1986**). (**A**) Untreated cells. (**B**) Cells treated with parathyroid hormone, showing loss of microvilli through membrane vesiculation producing a lesion similar to that seen with EPEC infection of gut tissue. Magnification 30,000 X.

Figure 5.3. Diagram of hormone induced signal transduction pathway. Binding of hormone to receptors causes activation of phospholipase C (PLC) via intermediary GTP binding G-proteins (Gp). PLC activity mediates the hydrolysis of membrane phosphatidylinositol lipids (PIP_2), generating the two second messengers 1,4,5-inositol trisphosphate (1,4,5-IP3) and diacylglycerol (DAG). 1,4,5-IP3 triggers release of calcium from intracellular stores in the endoplasmic reticulum and DAG activates membrane associated protein kinase C (PKC).

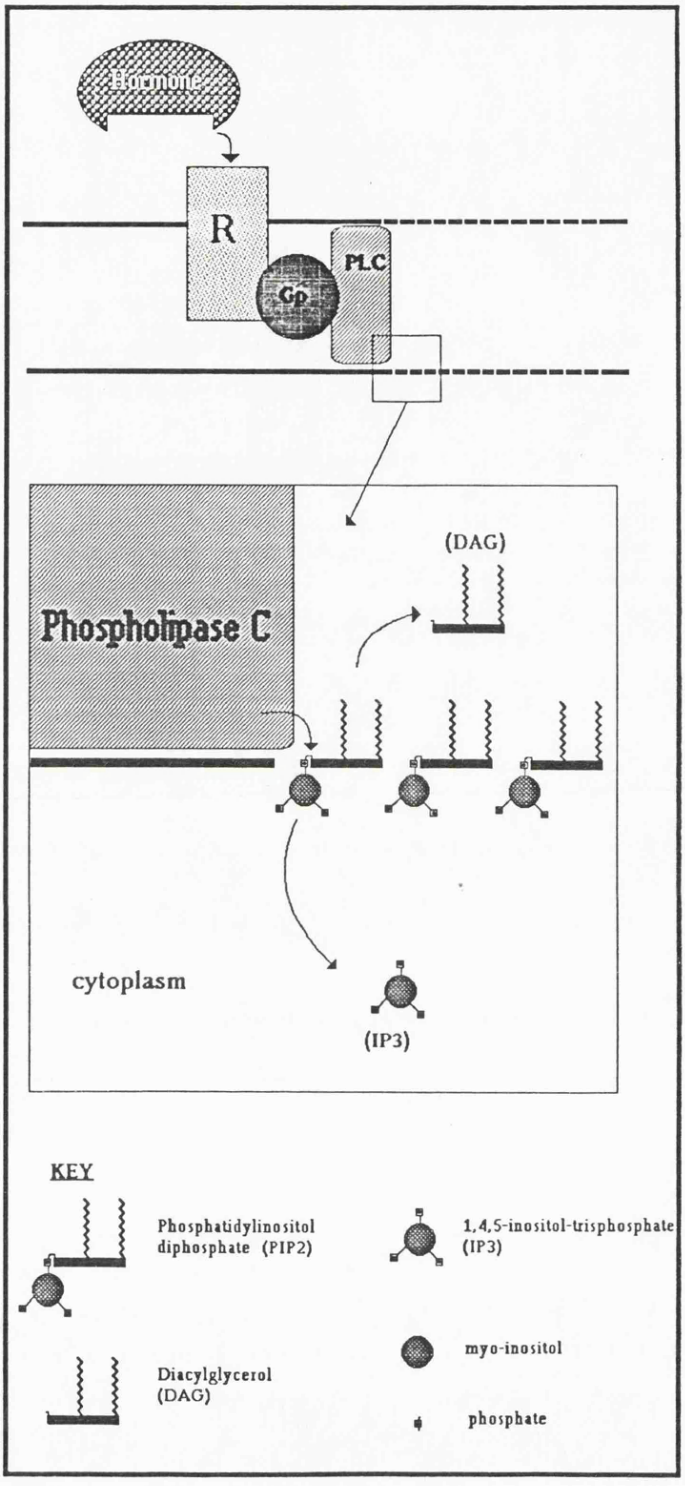


Figure 3.3 Model of signal transduction pathway.

Figure 5.3. Diagram of hormone induced signal transduction pathway.

al., 1986), thought to reside in the endoplasmic reticulum, and DAG remains within the plane of the membrane activating the calcium/phospholipid dependent protein kinase C (PKC) (**Berridge, 1984**). Calcium ionophores on the other hand allow direct influx of calcium from the extracellular medium (**Rink, 1983**) by rendering the plasma membrane specifically leaky to divalent metal ions (**Figure 5.4**).

The aforementioned similarity of morphological changes arising from elevated intracellular calcium in microvilliated cells to that seen during EPEC infection of gut tissue (Chapter 1) strongly suggested the involvement of an intracellular calcium rise in the EPEC induced cytoskeletal actin rearrangements of the lesion (Chapter 2 and 3). In addition, as described in Chapter 4, sustained elevation of intracellular calcium precedes loss of cell viability (**Lemasters et al., 1987; Trump and Berezesky, 1987**), further implicating a calcium rise in EPEC infected cells. Proposed mechanisms by which such a calcium rise could be achieved include influx from outside the cell by activation of membrane channels or membrane damage, and mobilization of intracellular stores (**Conrad and Rink, 1986; Exton, 1988; Penner et al., 1988**).

The intracellular calcium concentration of many cell types has been measured using the fluorescent calcium binding indicator dyes quin2 (**Tsien et al., 1982; Tsien et al., 1984**) and fura2 (**Grynkiewicz et al., 1985**). These compounds, based on EGTA (**Figure 5.5**), bind calcium ions and undergo a shift in the peak fluorescence wavelength and intensity when excited at 340 nm (**Tsien et al., 1982; Cobbold and Rink, 1987**). Acetoxymethyl-ester derivatives of quin2 and fura2 are more lipophilic than the free acids and therefore more readily enter the cell (**Tsien, 1981**), where endogenous esterases cleave the ester groups from the molecules, trapping them in the cytoplasm where they will bind calcium (**Tsien et al., 1982**). The intensity of the free acid peak fluorescence is proportional to the level of bound calcium, and therefore to the actual cytosolic concentration (**Tsien et al., 1982**). Calculation of the intracellular concentration of free calcium using these dyes in the conditions recommended depends on the intensity of the peak fluorescence, the level of dye loading in the cells, the

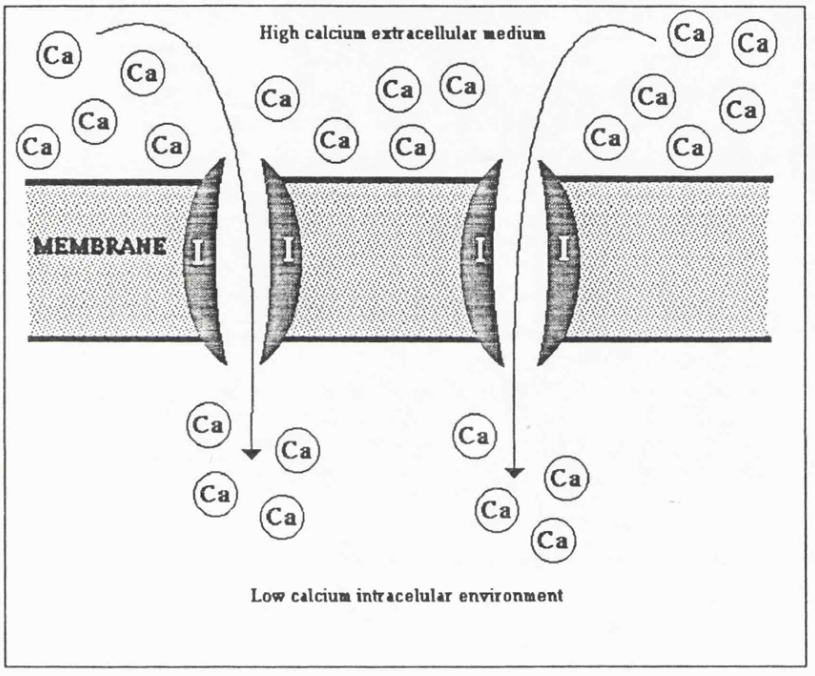


Figure 5.4. Diagram of the action of calcium ionophores A23187 and ionomycin. Ionophore molecules (I) dissolve in the membrane of the cell forming divalent metal ion specific pores, allowing the influx of calcium, magnesium, and manganese ions from the external environment.

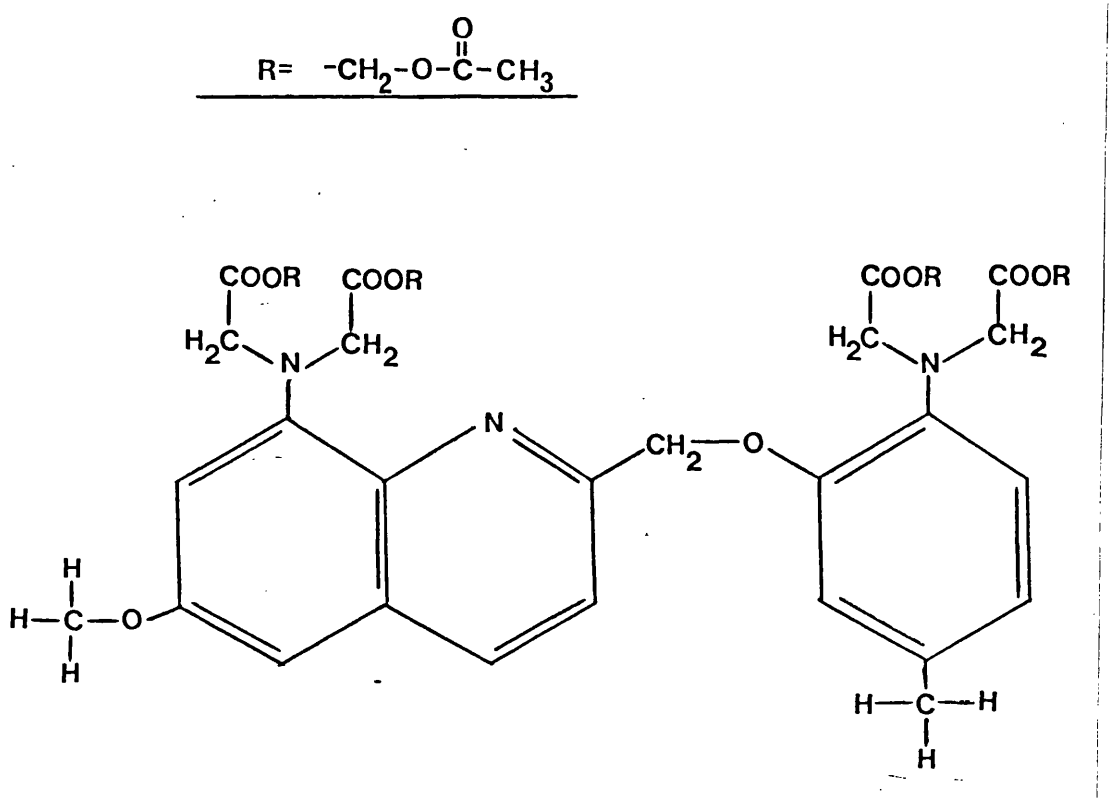


Figure 5.5. Structure of quin2-AM, based on the calcium chelator EGTA. The four carboxyl groups blocked by acetoxymethyl ester groups (R) enable the molecule to enter the cell membrane where endogenous esterases remove the R groups, trapping it in the cytoplasm. Quin2 binds calcium with a stoichiometry of 1:1 and a dissociation constant of 115 nM in physiological conditions. The calcium ion binds as a ligand, coordinated to the four carboxyl groups of the free quin2 molecule.

endogenous fluorescence of the dye without bound calcium in intracellular conditions, and the dissociation constant of the dye for calcium (**Tsien et al., 1982**).

The choice of indicator dye used for calcium measurements depended on the loading and retention characteristics of the cells used. Certain cell types produce extracellular esterases which cleave the ester groups from the dye before they enter the cell, effectively reducing the concentration of membrane permeable dye available for loading (**Maruyama et al., 1987**). This problem has been largely overcome by the use of esterase inhibitors. Other cells seem less able to retain one or other of the dyes due to excessive breakdown, leakage from the cell or incomplete hydrolysis of ester groups (**Oakes et al., 1988**). Initial experiments showed that the fluorescent calcium dye quin2 was loaded and retained in HEp-2 cells monolayers far more satisfactorily than the new generation dye fura2, and for this reason all calcium measurements were carried out using quin2 loaded cells. But because fura2 responses to calcium fluctuations are reported to be better than quin2 (**Grynkiewicz et al., 1985**), it is considered important to develop calcium measuring protocols in future to include fura2, possibly by changing the cell line used to one which responds better to fura2 loading. Potential candidate cell lines for future use include HEL and CaCo-2 cells.

5:2. *Materials and Methods.*

5:2:1. Intracellular calcium. To assess the effect of bacterial attachment on the calcium balance of infected HEp-2 cells the fluorescent calcium indicator quin2 (2-{[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-aminoquinoline; **Figure 5.5**) was used as a probe of intracellular free calcium. Quin2 complexed with calcium exhibits maximum fluorescence at 492 nm after excitation at 340 nm, while free quin2 acid and quin2 complexed with other divalent cations such as Mn^{2+} show minimal fluorescence at this wavelength (**Tsien et al., 1982**). As described previously, quin2 loading of cells was achieved using the acetoxymethyl ester derivative quin2-AM.

HEp-2 cells were grown to confluence on 12 mm^2 glass cover slips in DMEM containing 10% FCS as described in Chapter 2. Cells were infected as required by the addition of 200 µl of unshaken, overnight Luria broth bacterial cultures to 3 ml DMEM in Petri dishes containing several cover slips. After incubation at 37°C for 90 min, monolayers were loaded with quin2 by addition of 3.7 µl of a 10 mM stock solution of quin2-AM in DMSO, giving a 12.5 µM final concentration. After incubation in the dark at 37°C for 1 hour to allow uptake of the dye, monolayers were washed and incubated in fresh medium for a further 15 min to allow complete conversion of quin2-AM to the free acid. Single cover slips were then placed in 3 ml plastic cuvettes, and fluorescence measured by excitation at 340 nm with emission measurements at 492 nm using a Perkin-Elmer 204A fluorescence spectrophotometer.

Three readings were recorded for each sample (**Figure 5.6**). The inherent fluorescence of quin2-loaded cells (F) depended on the intracellular free calcium concentration of the monolayers. The maximum fluorescence (F_{max}), possible with the particular level of quin2 loading was measured by permeabilizing cell monolayers to extracellular calcium with 1 µM final concentration ionomycin (Calbiochem) added from a 0.6 mM stock solution in DMSO. Minimum fluorescence (F_{min}), equating to effective absence of calcium, was obtained by displacement of calcium from quin2 by addition of excess Mn^{2+} (4 mM final concentration). Values of F, F_{max} and F_{min} were used to calculate intracellular free calcium levels using the relationship

$$[Ca^{2+}]_I = K(F - F_{min})/(F_{max} - F),$$

where K is the effective dissociation constant (115 nM at 1 mM Mg^{2+}) of the Ca^{2+}/quin2 complex. Monolayers loaded with quin2 were checked for resting calcium levels as described above, as an indication of the health and condition of the cells, and the integrity of the cell membrane. A batch of monolayers were considered to be in the right condition if the sample tested in this way gave a pronounced rise in fluorescence to F_{max} and the value for the intracellular calcium concentration was between 50 and 80 nM.

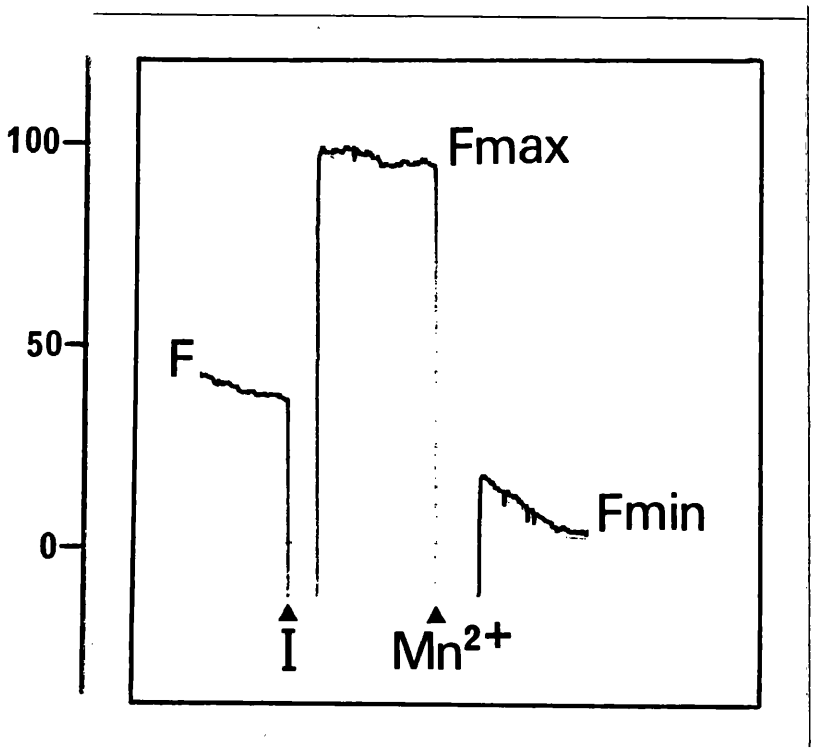


Figure 5.6. Measurement of intracellular calcium concentrations in HEp-2 monolayers loaded with quin2. Excitation of loaded monolayers at 340 nm. gives a characteristic fluorescence (F) at 492 nm, whose intensity relates directly to the average intracellular calcium concentration of the monolayer. Permeabilizing cells to divalent metal ions with ionophore allows the influx of extracellular calcium, giving the maximum fluorescence (F_{max}). Minimum fluorescence (F_{min}; corresponding to absence of calcium) is achieved by treating ionophore permeabilized monolayers with excess manganese, which displaces calcium from quin2 effectively quenching the quin2/calcium fluorescence.

5:3. *Results.*

5:3:1. Quin2 loading of cells. Quin2 loading of HEp-2 monolayers on glass cover slips was followed by spectral measurements over the range 400-500 nm (**Figure 5.7**) by excitation at 340 nm using a Perkin-Elmer 204A fluorescence spectrophotometer. A characteristic peak fluorescence for trapped quin2/calcium was observed at 492 nm, which was larger than the peak at this wavelength corresponding to autofluorescence of unloaded monolayers (**Figure 5.8**). In freshly loaded cells a fluorescence peak at 430 nm was also observed (**Figure 5.7**), which declined when incubation was continued in fresh medium lacking quin2-AM for 15-20 min (**Figure 5.7**) and was thought to represent uncleaved ester bound to the cell surface (**Tsien et. al., 1982**). Because of the variability between different batches of monolayers, only those which gave the correct responses to ionomycin and manganese ions (as described in materials and methods) were used in calcium measurements (**Figure 5.6**).

5:3:2 EPEC-induced intracellular calcium elevation. EPEC infected HEp-2 cell monolayers showed an increase in quin2 fluorescence (**Figure 5.7**) corresponding to an intracellular calcium concentration up to 8 times higher than that seen with uninfected monolayers (**Table 5.1**). Addition of sufficient EGTA (4mM) to reduce the concentration of extracellular free calcium to sub-micromolar levels had no effect on the EPEC-induced rise (**Table 5.1**), suggesting that the observed increase was not dependent on influx from the medium. This was corroborated by the observation that measurement of F_{max} values for quin2 loaded monolayers required the presence of the ionophore ionomycin to allow calcium influx (**Figure 5.9**); also excess manganese did not quench the quin2/calcium fluorescence of infected cells unless they had been treated with ionophore (**Figure 5.9**).

Figure 5.7. Loading of quin2 into HEp-2 cell monolayers. Confluent HEp-2 cell monolayers on 12 mm^2 glass cover slips in growth medium (DMEM containing 10% foetal calf serum) were loaded with quin2 by incubating in growth medium containing 12.5 μM quin2-AM at 37°C for 1 h. **(A)** Emmission spectrum of HEp-2 monolayers freshly loaded with quin2, excited at 340 nm. Peak fluorescences observed at 430 nm and 492 nm, correspond to bound quin2-AM and cytoplasmic free quin2 acid, respectively. **(B)** Emmission spectrum of quin2 loaded HEp-2 monolayers incubated in quin2-AM free growth for 15 min at 37°C. Loss of the peak fluorescence at 430 nm and increased fluorescence at 492 nm corresponds to complete cleavage of the quin2-AM ester groups, and release of quin2 into the cytoplasm. **(C)** Time course of quin2-AM cleavage, generating free quin2 acid. **(a)** 0 min, **(b)** 5 min, **(c)** 15 min, incubation in quin2-AM free growth medium..

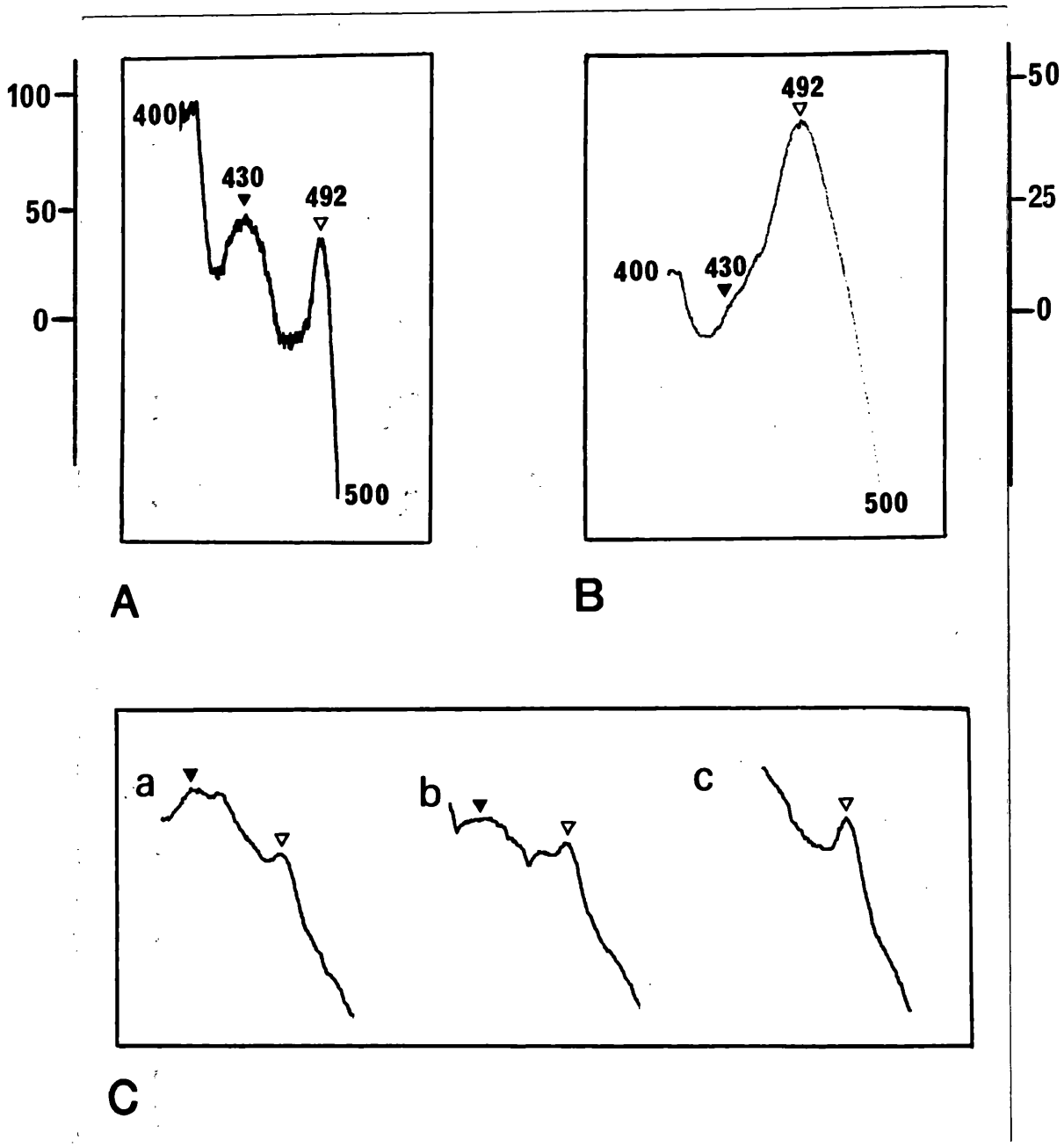


Figure 5.7. Loading of quin2 into HEp-2 cell monolayers.

Figure 5.8. Alterations in fluorescence intensity at 492 nm for HEp-2 monolayers. (a) uninfected, HEp-2 monolayers, (b) HEp-2 monolayers infected with EPEC strain 2036-80 for 3 h, (c) uninfected HEp-2 monolayers loaded with quin2, (d) HEp-2 monolayers loaded with quin2 and infected with HB101(pMAR15) for 3 h, (e) and (f) quin2 loaded HEp-2 monolayers infected with EPEC strains 2036-80 and E2348-69 respectively for 3 h.

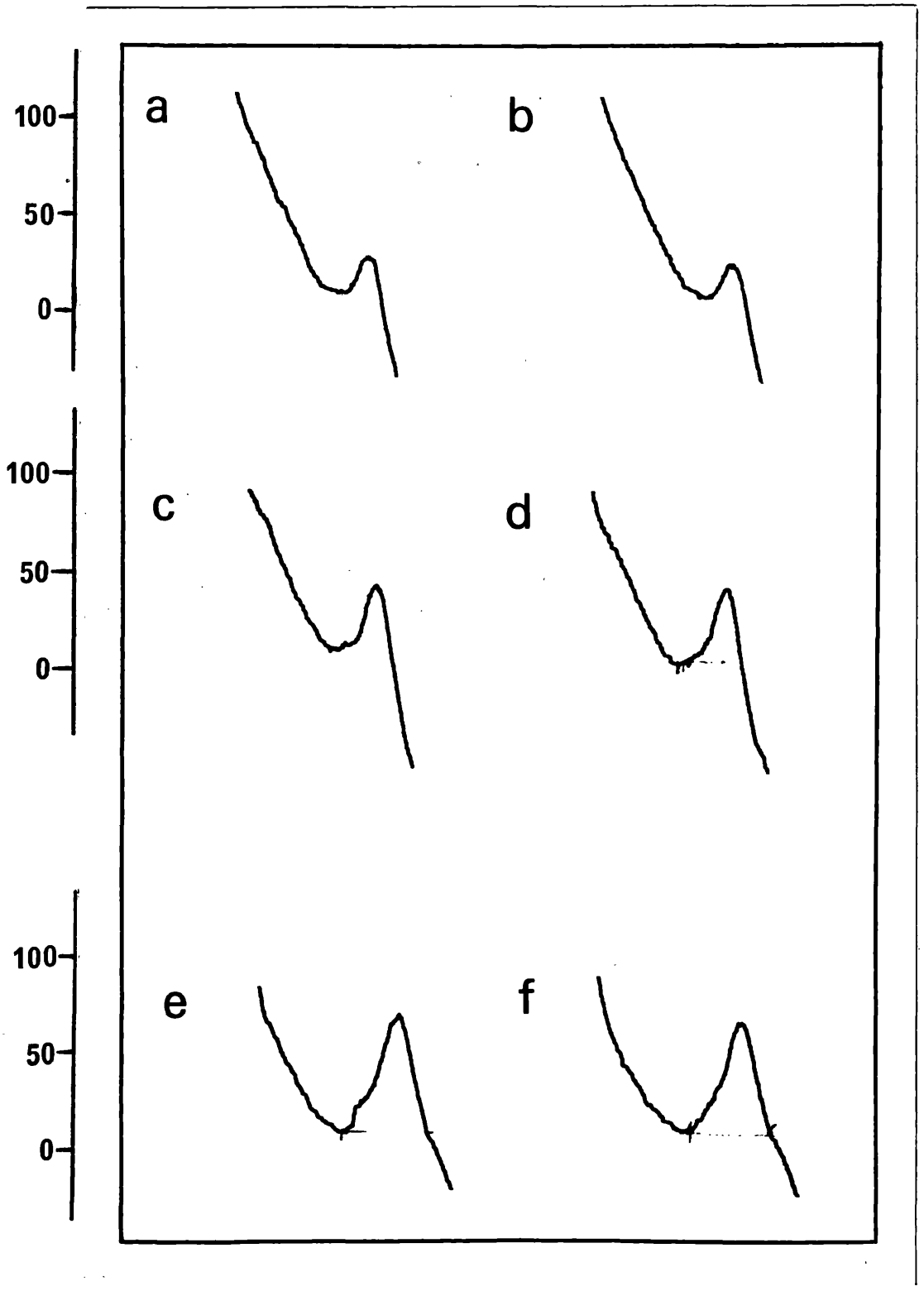


Figure 5.8. Alterations in fluorescence intensity at 492 nm for HEp-2 monolayers.

Bacterial strains	Ca^{2+} conc. (nM)	EGTA treatment
uninfected	61	no
uninfected	56	yes
E2348-69	252	no
E2348-69	238	yes
2036-80	230	no
2036-80	225	yes
HB101(pMAR15)	61	no
HB101(pMAR15)	60	yes

Table 5.1. Effect of extracellular calcium on the intracellular rise induced during EPEC infection of HEp-2 monolayers. Extra cellular calcium was reduced to sub nM concentrations by the addition of 4 mM EGTA, and the intracellular calcium concentration of EPEC infected cells determined after 3 h infection.

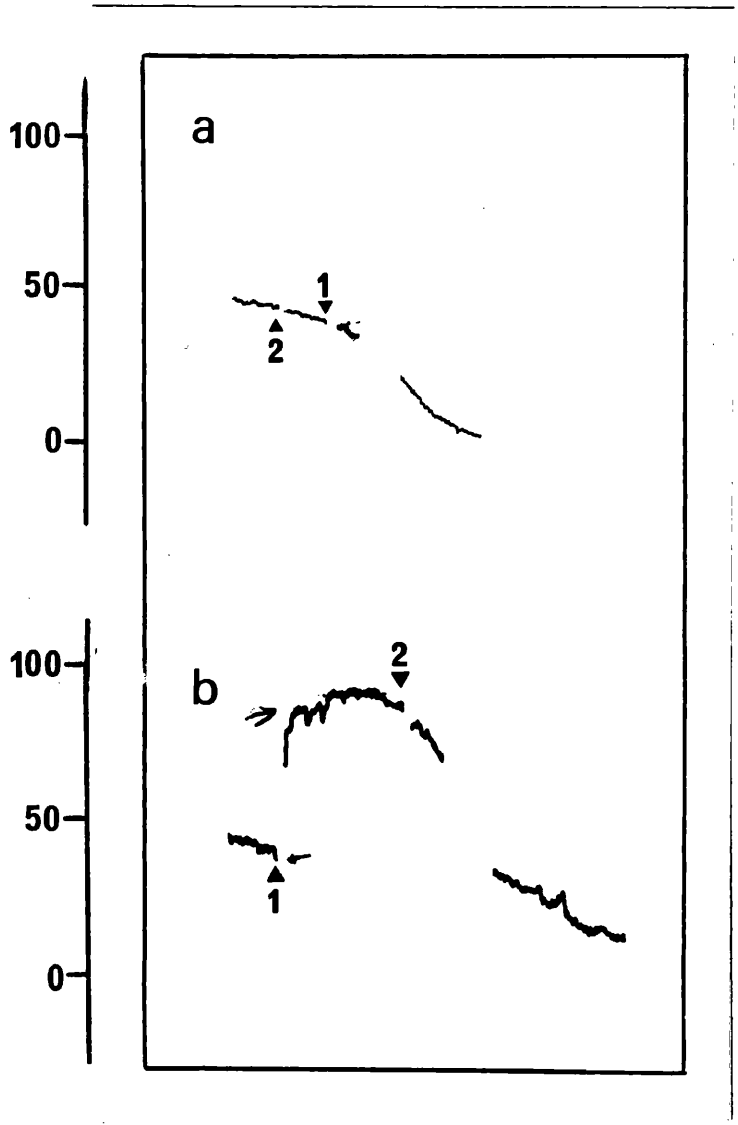


Figure 5.9. Integrity of the plasma membrane in EPEC infected HEp-2 cells. (a) HEp-2 monolayers infected with EPEC strain 2036-80 for 3 h, treated with 4 mM manganese ions (2) and subsequently 1 μM ionomycin (1). Manganese ions were unable to quench the intracellular quin2/calcium fluorescence until the HEp-2 plasma membrane was made permeable to divalent metal ions with ionomycin. (b) Permeabilization of HEp-2 cell plasma membranes with ionomycin (1), mediated influx of calcium and a maximum fluorescence (F_{max}) of trapped quin2. Quin2/calcium fluorescence was subsequently able to be quenched with 4 mM manganese ions.

5:3:3. Source of intracellular free calcium. The other possible sources of the additional calcium is mobilizable stores within the host cell. The drug dantrolene, normally used for the treatment of spastic conditions and malignant hyperthermia (**Ohnishi et al., 1986**) has been reported to prevent mobilization of calcium from stores in the endoplasmic reticulum by inhibiting the releasing action of 1,4,5-IP3 (**Van Winkle, 1976; Danko et al., 1985**). Intestinal ion efflux in the rabbit ileum, and the action of hormones such as angiotensin II (known to induce calcium elevations via 1,4,5-IP3 formation) are both reduced in the presence of dantrolene (**Donowitz et al., 1983; Ward, 1987**). The rise in intracellular calcium associated with EPEC infected HEp-2 monolayers was abolished by pre-treatment of monolayers with dantrolene (50 μM) for 30 min (**Table 5.2**). The bacteria were seen to attach and grow normally on dantrolene-treated cells forming microcolonies that were indistinguishable from those on untreated cells.

5:3:4. Time course of calcium elevation. Three lesion forming EPEC isolates were used to determine the time course of calcium elevation in infected cells (**Table 5.3**). The size of microcolony and percentage infection varied slightly from strain to strain, which may explain the slight differences in the intracellular calcium concentrations seen. Elevation in calcium concentration was detected with all three strains after 1 h of infection, increasing steadily throughout the total infection time of 4 h (**Table 5.3**). Final intracellular calcium concentrations were 5-8 times those seen with uninfected HEp-2 monolayers (50 nM) or with monolayers infected with the non-lesion forming control strain HB101(pMAR15).

5:3:5. Lesion formation and calcium elevation. For a small collection of HEp-2 adherent isolates of *E. coli*, intracellular calcium concentrations in infected monolayers were elevated only by lesion forming strains (**Table 5.4**), indicating a possible relationship between elevated intracellular calcium and loss of microvilli during infection. For confirmation of this, it was considered essential to carry out a more

Bacterial strains	Ca^{2+} conc. (nM)	Dantrolene treatment
uninfected	61	no
uninfected	55	yes
E2348-69[b]	252	no
E2348-69[b]	65	yes
2036-80[b]	230	no
2036-80[b]	57	yes
HB101(pMAR15)[a]	70	no
HB101(pMAR15)[a]	66	yes

Table 5.2. The effect of dantrolene on EPEC-induced intracellular calcium rises. Intracellular calcium concentrations of confluent HEp-2 monolayers, either untreated or pre-treated with 50 μM dantrolene and infected with EPEC strains 2036-80 and E2348-69 for 3 h, were measured using the fluorescent calcium indicator dye quin2. Values were compared with similarly treated control monolayers either uninfected or infected with the negative control strain HB101(pMAR15). Figures were obtained from the average of three experiments.

[a] Laboratory strain HB101 containing the EPEC adherence plasmid pMAR15, conferring LA.

[b] EPEC strains known to produce the AE-lesion

Bacterial strains	CALCIUM CONCENTRATION (nM) AT 0 h	1 h	2 h	3 h	4 h
uninfected	56	--	52	--	58
HB101[a]	50	51	51	59	54
E2348-69[b]	54	105	--	200	322
2036-80[b]	51	110	--	250	310
1923-77[b]	50	--	90	280	316

Table 5.3. Time course of the intracellular rise in calcium observed during infection of HEP-2 monolayers with lesion-forming EPEC.

[a] Laboratory strain HB101 containing the EPEC adherence plasmid pMAR15, conferring LA.

[b] EPEC strains known to produce the AE-lesion

Bacterial strains	HEp-2 adherence	Lesion formation	Calcium conc. (nM)
uninfected	---	---	57
HB101[a]	localized	negative	76
2036-80[b]	localized	positive	285
E2348-69[b]	localized	positive	320
1923-77[b]	localized	positive	316
469-3[c]	diffuse	negative	72
135[d]	diffuse	negative	65

Table 5.4. Intracellular calcium concentrations in HEp-2 monolayers infected with adherent *E. coli* isolates.

[a] Laboratory strain HB101 containing the EPEC adherence plasmid pMAR15, conferring LA.

[b] EPEC strains known to produce the AE-lesion.

[c] Invasive strain.

[d] Diffuse adhering strain.

extensive screening of lesion forming and non-lesion forming isolates, but technical difficulties arising from the procedure precluded its use in large scale screening. Other procedures were considered, among which the most promising would be fluorescent digital image analysis of individual infected cells to determine localized calcium concentrations. Such equipment was unavailable at Leicester, and the length of time required to set up and calibrate our model system excluded the possibility of approaching any of the limited number of institutions in Great Britain with this equipment.

5:3:6. Rapid screening of isolates for calcium elevation. The recently developed fluoresceine conjugated calcium indicator dye FLUO-3 (**Kao et al., 1989; Minta et al., 1989**) is reported to give an emission in the visible region of the spectrum on calcium binding. A rapid screening procedure requiring only a fluorescence microscope was therefore devised for the large scale screening of isolates, but time did not permit more than a preliminary trial experiment. HEp-2 monolayers grown on glass cover slips were infected, and loaded with 12.5 μM FLUO-3 in the same way as described for quin2 loading. Monolayers were mounted on glass microscope slides in 10 μl of DMEM + 10% FCS and observed using a fluorescent microscope. Uninfected monolayers showed a uniform low fluorescence, whereas monolayers treated with ionophore to allow influx of calcium were visibly more fluorescent. From observations of HEp-2 monolayers infected with the EPEC strains 2036-80 and E2348 it was evident that infected cells showed a higher level of fluorescence compared to their uninfected neighbours, but attempts to record these differences photographically were unsuccessful because of the low light levels. Further work is required to determine the optimum conditions for loading, and the concentration of FLUO-3 which gives the best level of fluorescence for photographic recording.

5:3. *Discussion and conclusions.*

5:3:1. The EPEC induced calcium rise. EPEC infection of HEp-2 monolayers caused a significant increase in the calcium dependent fluorescence of trapped quin2, indicating a rise in intracellular free calcium to 200-322 nM after 3 h infection. Because of the localized nature of the lesion, however, it is likely that this reflects considerably greater local increases in the vicinity of the attached bacteria. The observed calcium increase was not dependent on extracellular calcium, since effectively removing free calcium from the medium by chelation with EGTA did not affect the levels of intracellular calcium recorded. This suggested that it was not due simply to nonspecific influx resulting from the death of infected cells, a view which was confirmed by the observation that added manganese was unable to quench the quin2/calcium fluorescence unless the cells had been permeabilized with ionomycin. Moreover there appeared to be a controlled elevation of intracellular calcium, implied by the fact that complete inhibition of the calcium rise could be achieved using the drug dantrolene, suggesting the involvement of second messenger formation. The accepted mode of action of dantrolene, inhibition of calcium release from the endoplasmic reticulum by the second messenger molecule 1,4,5-IP_3 (**Ohnishi et al., 1986; Ohnishi, 1987; Danko et al., 1985; Donowitz et al., 1983**), strongly suggests the involvement of a signal transduction pathway and the formation of second messengers in the observed calcium rise (**Berridge, 1984; Berridge and Irvine, 1984; Volpe et al., 1985; Wollheim, and Biden. 1986; .Exton, 1988**). Normally any rise in calcium as a result of 1,4,5-IP_3 controlled release is transient (**Berridge et al., 1988; Meyer and Stryer, 1988**), rapidly returning to basal levels by a process of down regulation, involving degradation of intracellular signal molecules and desensitisation of receptors through phosphorylation (**Woods et al., 1987; Bouscarel et al., 1988; Harnett and Klaus, 1988; McDonough et al., 1988; Troyer et al., 1988; Tyagi et al., 1988; Martin and Harden, 1989**). However in EPEC infected cells calcium levels remained elevated for at least 3 h; this may be explained by the dynamic and localized nature of EPEC infection, in which bacteria

attach and grow throughout this period and therefore maintain stimulation of the infected cells, or additional processes may be involved in the sustained elevation. It has been reported that sustained rises in intracellular calcium are achieved in non-excitable cells by two mechanisms, formation of 1,3,4,5-inositol tetrakisphosphate (1,3,4,5-IP_4) from 1,4,5-IP_3 which replenishes the 1,4,5-IP_3 sensitive store, prevents calcium being sequestered from the cytoplasm and inhibits hydrolysis of 1,4,5-IP_3 (**Joseph et al., 1987**). The second mechanism proposed for maintenance of high cytosolic calcium levels involves an interaction between cAMP and calcium in cells activated by 1,4,5-IP_3-dependent calcium mobilization. An increased chloride current formed in response to the action of the two second messengers promotes a prolonged influx of calcium from the extracellular environment (**Penner et al., 1988**). The mechanisms by which cAMP and calcium signals are coupled to form two interacting pathways of cell stimulation is not fully understood at this time.

From a limited screening of adherent *E. coli* strains, it appears that induction of a rise in intracellular calcium is specific for lesion-forming isolates. Strains which were previously shown to form an attaching and effacing lesion by electron-microscopy gave calcium elevations in the range 285-320 nM, while cells infected with strains that were unable to form the AE-lesion showed calcium levels comparable to uninfected monolayers. The FLUO-3 method of screening for calcium elevation in monolayer cells seems promising. Initial experiments showed a significant but small increase in the level of calcium dependent fluorescence in cells infected with EPEC strains, suggesting sub-optimum loading rather than inability of the equipment to detect FLUO-3/calcium emissions. I am confident that with further development of this procedure, optimum conditions of loading to give satisfactory levels of fluorescence for photography can be achieved, so providing a quick and simple screening method for bacterial induced rises in intracellular free calcium.

From the experiments reported here it seems likely that EPEC induce significant increases in cytosolic free calcium concentrations via the generation of second messenger molecules in a signal transduction pathway, as seen during such processes

as hormone and neurotransmitter stimulation. A review of present concepts of signal transduction and cellular regulation seems pertinent at this point, to enable likely models of processes involved in the EPEC-induced calcium rises to be understood in a wider context.

5:3:2. Inositol phosphate metabolism. Within the membranes of mammalian cells are three major PI lipid types which differ from one another in the degree of inositol phosphorylation (**Majerus et al., 1988**). The conversion of unphosphorylated-PI lipids into the higher phosphorylated forms (phosphatidylinositol 4-monophosphate [PIP], and PIP_2), is dependent on the activity of specific kinases (**Blowers and Trewavas, 1988; Majerus et al., 1988**). All three PI lipids act as a substrates for PLC, yielding DAG and any of six different inositol phosphate products, including inositol 1-monophosphate (1-IP), inositol 1,4-bisphosphate (1,4-IP_2), and 1,4,5-IP_3, and the 1:2-cyclic derivatives of each of these (**Majerus et al., 1988**). The flexibility of a system which allows one enzyme to recognize three substrates and produce six potentially active products is enormous, and although there are different forms of PLC in various tissues each is able to produce the six inositol phosphates from the three substrates (**Majerus et al., 1988**). Since *myo*-inositol has six hydroxyl groups capable of phosphate substitution (**Figure 5.10**), 63 possible *myo*-inositol phosphates can be formed, in addition to the three known cyclic derivatives. The cellular activity of most inositol phosphates is obscure; many may simply be breakdown products resulting from down regulation processes, although it is conceivable that even these may have functions. At present about 20 of these have been detected in one or more cell types (**Majerus et al., 1988**), but only a few have been shown to elicit specific cellular activities, such as calcium mobilization by 1,4,5-IP_3 (**Berridge and Irvine, 1984; Velasco et al., 1986**). The majority of the 20 forms so far discovered are the result of modifications to the basic six formed through PLC activity. A key enzyme in this process is the 45 KDa protein 5-phosphomonoesterase, which degrades 1,4,5-IP_3 to 1,4-IP_2 by cleaving the 5′ phosphate group (**Majerus et al., 1988**). Its activity is regulated by PKC-induced phosphorylation at multiple sites, giving

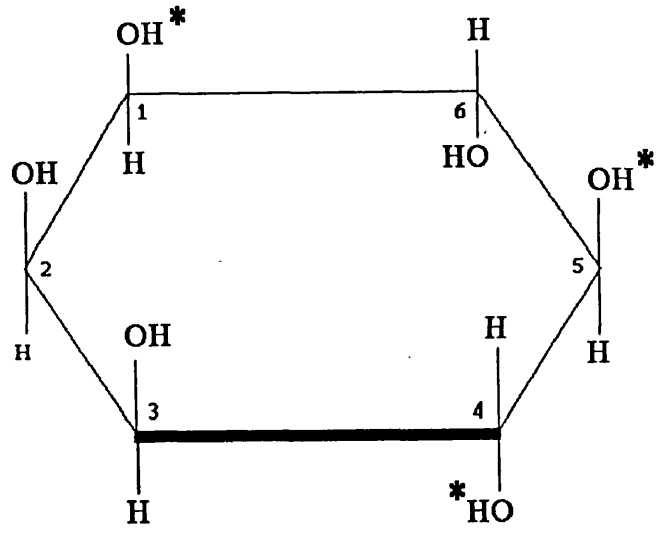


Figure 5.10. Structure of *myo*-inositol. The six hydroxyl groups of *myo*-inositol can each be substituted by phosphate to yield 63 possible inositol phosphates in addition to the three known cyclic inositol phosphates. The symbol * represents the position on the inositol ring where phosphate substitution occurs in PIP_2, which yields 1,4,5-IP_3 upon PLC cleavage.

a severalfold increase in activity with respect to each of the three known substrates, 1,4,5-IP_3, cyclic 1:2,4,5-IP_3, and 1,3,4,5-IP_4 (**Majerus et al., 1988**).

In activated cells the phosphorylation of 1,4,5-IP_3 at the 3′ position yields 1,3,4,5-IP_4, which in turn can be dephosphorylated to give 1,3,4-IP_3 by removal of the 5′ phosphate (**Doughney et al., 1988, [Figure 5.11]**). As mentioned above 1,3,4,5-IP_4 increases the duration of calcium elevation (**Joseph et al., 1987**), in part by binding to the membrane of the endoplasmic reticulum and stimulating the influx of calcium into 1,4,5-IP_3 sensitive stores (**Figure 5.12**) (**Bradford and Irvine, 1987; Imboden and Weiss, 1987**). Evidence suggesting compartmentalisation of the endoplasmic reticulum into different calcium storage areas (**Nilsson et al., 1987**), sensitive to either 1,4,5-IP_3 or a cyclic derivative of IP_3 (possibly cyclic 1:2,4,5-IP_3) indicates that several mechanisms are available in the cell to mediate prolonged elevations of intracellular calcium (**Muallem et al., 1988**). This may be of particular interest bearing in mind that EPEC cause an extended elevation of intracellular calcium in infected cells. As part of the down-regulation of cell activation, inositol phosphate second messengers are removed from the cell by progressive dephosphorylation via lithium sensitive enzyme reactions (**Ragan et al., 1988**), to ultimately yield *myo*-inositol, which is fed back into the synthesis of PI-lipids (**Figure 5.11**).

5:3:3. Role of calcium in cellular functions. Calcium has important regulatory roles in the cell, e.g. activation of specific protein kinases (**Adelstein, 1982; Manalan and Klee, 1984; Londesborough and Nuutinen, 1987; Ryazanov, 1987; Colbran et al., 1989**), modulation of actin and cytoskeletal architecture (**Bretscher and Weber, 1980; Mooseker et al., 1980b; Adelstein, 1982; Burgess and Prum, 1982; Manalan and Klee, 1984; Glenney and Glenney, 1985**), cytoplasmic fluidity (**Weeds, 1982; Matsudaira and Janmey, 1988; Yin, 1988**), cell division and growth control (**Means and Dedman, 1980**), ion transport (**Frizzell, 1977; Donowitz, 1983; Windhager and Taylor, 1983; Donowitz and Welsh, 1986; Fondacaro, 1986; Liedtke, 1989**), and the activity of certain proteases (**Waelkens et al., 1985; Wang et. al;., 1988; vonRuecker et al., 1988; Alonso et al., 1989**),

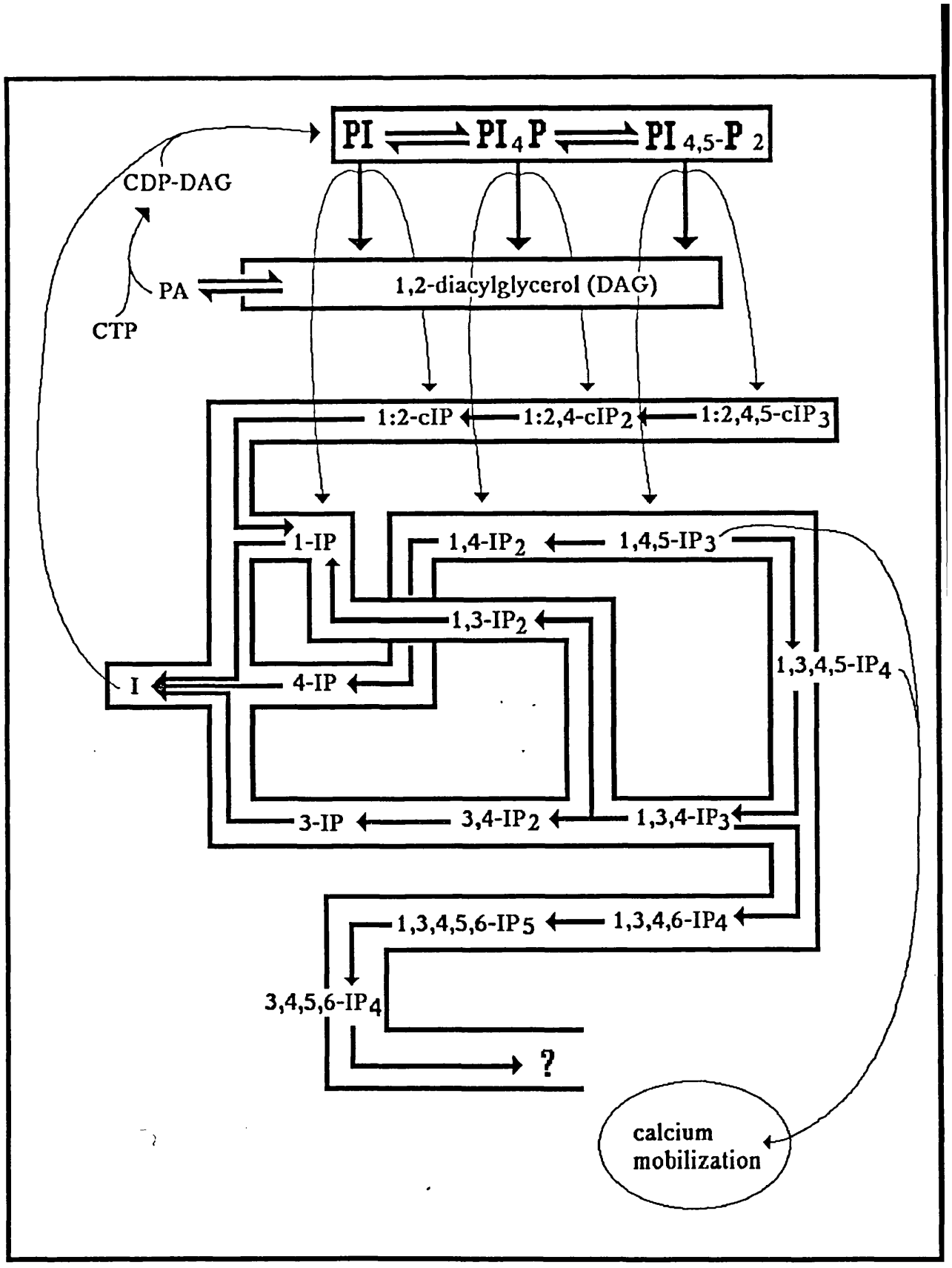


Figure 5.11. Proposed pathway of inositol phosphate synthesis and breakdown in the cell.

Figure 5.12 Effect of 1,3,4,5-IP_4 on intracellular calcium status of the cell. 1,3,4,5-IP_4 produced by phosphorylation of 1,4,5-IP_3 mediates the influx of extracellular calcium directly into 1,4,5-IP_3 sensitive stores in the endoplasmic reticulum. It may also mediate controlled, direct influx of calcium from the extracellular medium in to the microvilli of absorptive cells of the gut and kidney, causing cytoskeletal breakdown and loss of microvilli **(Goligorsky et al., 1986).**

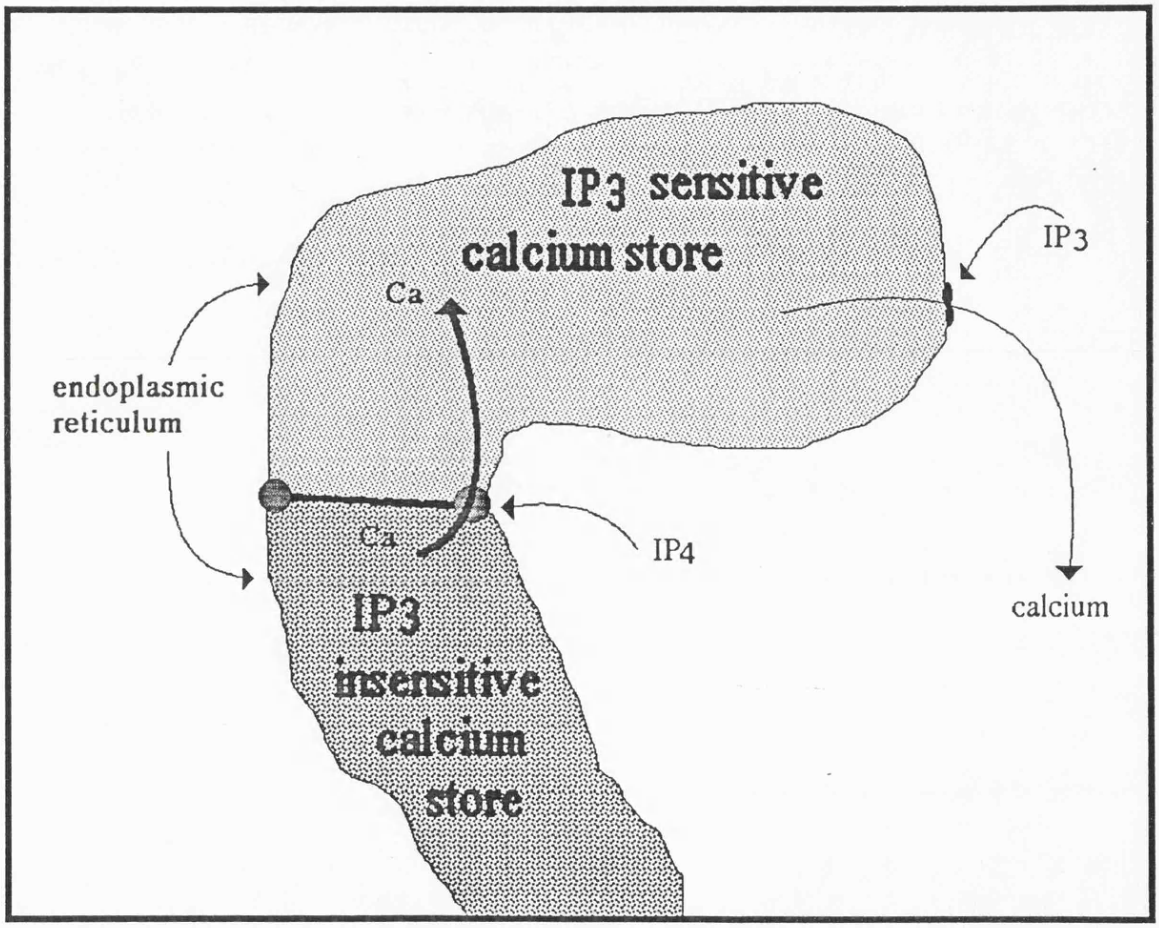


Figure 5.12 Effect of 1,3,4,5-IP_4 on intracellular calcium status of the cell.

to name a few. Therefore a rise in intracellular calcium promoted by the formation of 1,4,5-IP_3 will have wide ranging effects on cell function. Indeed it is difficult to find a process which is totally independent of calcium, highlighting its pivotal role in normal cellular functioning, and possibly stressing its potential as one of the major effectors of cellular activation by agonists.

The control of intestinal ion transport is a complex field of study, impinging on such areas as pharmacology, gastrointestinal physiology and microbiology. The major regulators of water and ion transport in intestinal epithelium, in response to both neurohormonal stimulation and microbial toxins, are the second messenger molecules cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP),and ionic calcium (**Donowitz and Welsh, 1986**). The heat stable enterotoxin STa of enterotoxigenic *E. coli* induces its pathogenic effect by activating host guanylate cyclase (**Epstein et al., 1986**), causing elevated cytosolic levels of cGMP, stimulation of a cGMP dependent protein kinase, and possible phosphorylation of ion transport proteins (**Evered and Whelan, 1985**). *E. coli* heat labile enterotoxin (LT) is of two types (I and II), both cause substantial increases in cytosolic levels of cAMP (**Chang et al., 1987; Holmgren, 1981**), leading to activation of a cAMP-dependent protein kinase and phosphorylation of membrane proteins thought to regulate ion transport (**Evered and Whelan, 1985**). It has also been suggested however that both STa induced elevation of cGMP levels and LT stimulated cAMP increase may promote or augment diarrhoea by elevating intracellular levels of free calcium ions (**Evered and Whelan, 1985**), a situation which implies the existence of complex interactions between second messenger pathways.

Several studies using intact intestinal epithelium have indicated that cAMP mediated electrolyte transport and STa activity are attended by a rise in intracellular calcium (**Frizzell et al., 1979; Ilundain and Naftalin, 1979**). Evidence for actual coupling between different second messenger generating systems has been discovered for nonintestinal cells (**Arshad and Holdsworth, 1980; Small et al., 1986; MacNeil, 1987; Prentki et al., 1987**), although there are differences between different cell types.

For example, cAMP elevates intracellular calcium concentrations by promoting calcium entry through voltage sensitive channels in the plasma membrane of the pancreatic cell line HIT T15 (**Prentki et al., 1987**), whereas in liver tissue cAMP formation causes release of calcium from mitochondrial stores (**Arshad and Holdsworth, 1980**). Calcium dependent regulation of guanylate cyclase activity has been observed in quite diverse cell types, from *Dictyostelium* to human platelets (**Small et al., 1986; MacNeil, 1987**) but as yet there is little firm evidence for cGMP control of intracellular calcium concentration except for an apparent coincident rise in calcium when some cells are treated with STa (**Evered and Whelan, 1985**).

The guanine nucleotide GTP elicits a range of activities which effect the calcium concentration of the cytoplasm. Studies reporting the effects of GTP on calcium elevation appear to be of four types. Firstly, GTP added to permeabilized Swiss 3T3 cells promotes calcium elevation by stimulating the G-protein coupled to PLC, thereby provoking hydrolysis of PI-lipids and the formation of the second messengers 1,4,5-IP_3 and DAG (**O'Rourke et al., 1987; Taylor et al., 1988a**). Secondly, GTP seems to have an inherent ability to increase both the calcium releasing and accumulating capability of the endoplasmic reticulum in saponin permeabilized macrophages (**Kimura et al., 1988**). The accumulating activity of GTP treated cells is inhibited by the presence of polyethylene glycol (PEG) or bovine serum albumin (BSA), whereas the releasing activity remains unchanged. This suggests that, in macrophages at least, GTP stimulates simultaneous calcium release from, and accumulation in the endoplasmic reticulum, and the predominance of the releasing activity at any time, in normal conditions possibly depends on the existence of an unknown factor whose effect is mimicked by PEG and BSA. Thirdly, GTP increases the amount of 1,4,5-IP_3-released calcium from the endoplasmic reticulum of hepatocytes by enabling the movement of calcium from 1,4,5-IP_3-insensitive to 1,4,5-IP_3-sensitive stores in the endoplasmic reticulum (**Thomas, 1988**). This function is similar to that seen with 1,3,4,5-IP_4 and may relate to a GTP-dependent phosphorylation of 1,4,5-IP_3 at the 3' position to yield 1,3,4,5-IP_4 (**Thomas, 1988**). Fourthly, in cells derived from guinea pig parotid gland, GTP and

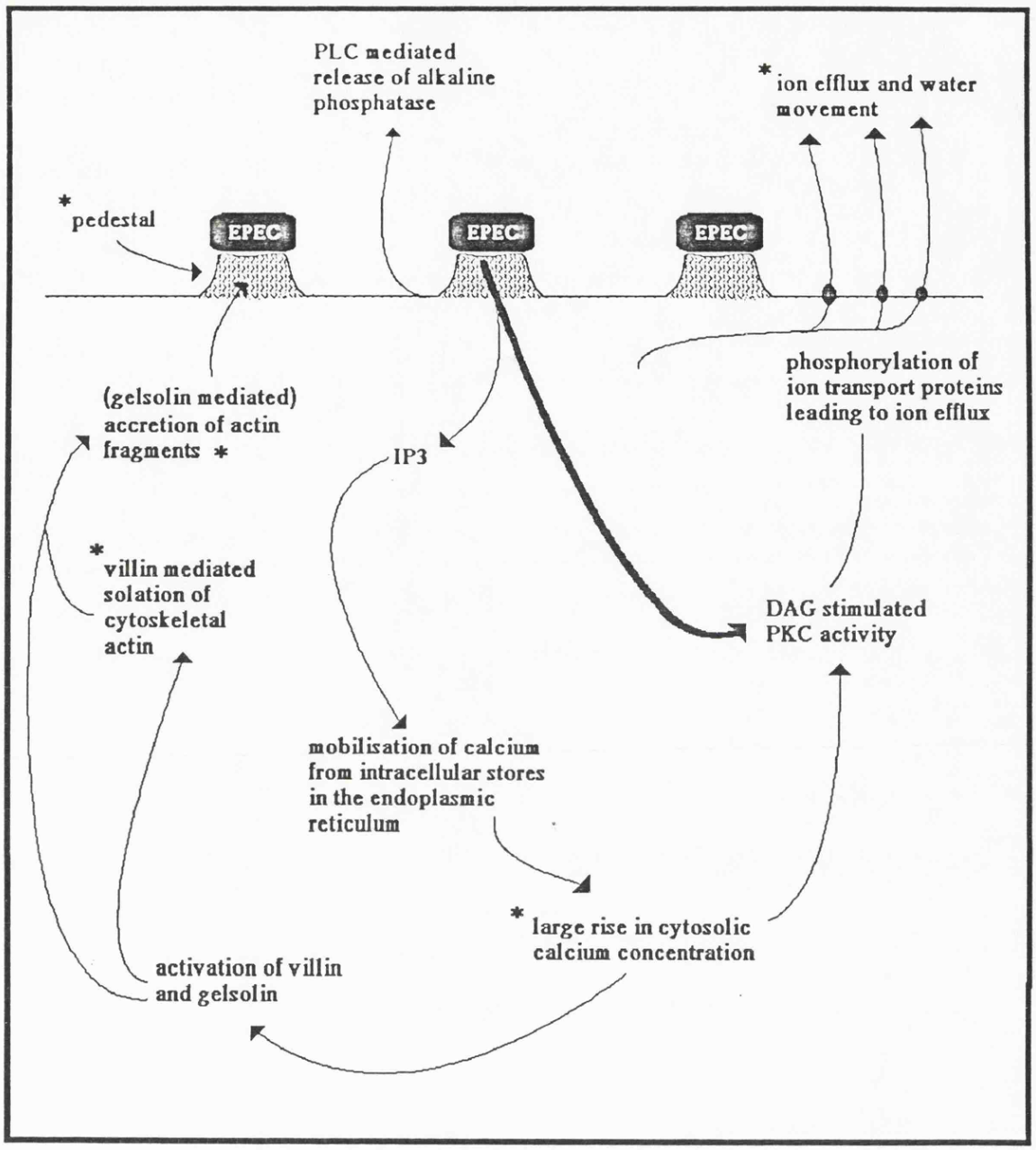


Figure 5.13. Initial putitive model of calcium elevation in EPEC infected HEp-2 cells

1,4,5-IP_3 release calcium, from different intracellular compartments in the rough endoplasmic reticulum and the smooth endoplasmic reticulum (in close proximity to the plasma membrane), respectively (**Henne and Sloing, 1986; Henne et al., 1987**).
Calcium itself appears to have a considerable role in the normal control of intestinal ion transport (**Donowitz and Welsh, 1986**). Active transport of sodium (Na^+) and chloride (Cl^-) ions in the small intestine, and Na^+, Cl^-, and potassium (K^+) ion transport in the colon have been shown to be regulated by
the calcium levels in the cell (**Donowitz, 1983; Donowitz et al., 1984b**). The absorption of neutral NaCl in the rat colon and rabbit ileum is considerably reduced when the cellular concentration of calcium is increased (**Bolton and Field, 1977; Donowitz, 1983; Donowitz et al., 1984b**), whereas in similar conditions the secretion of Cl^- and K^+ is increased in the rabbit ileum and colon (**Bolton and Field, 1977; Zimmerman et. al., 1983**). Trapping calcium in intracellular stores located in the endoplasmic reticulum using the drug dantrolene significantly increases Na^+ and Cl^- absorption in the ileum, inhibiting the effects of carbachol on ion transport but not those of cAMP or serotonin (**Donowitz and Welsh, 1986**). From this evidence it seems reasonable to assume that there are at least two calcium stores involved in the regulation of ion transport in the ileum. The first is mobilizable in response to PIP_2 hydrolysis, while the second seems dependent on elevation of cAMP (as mentioned previously).

5:3:4. Model for the mechanism of EPEC-induced calcium rises. The discovery that EPEC cause an elevation in the intracellular calcium concentration of infected cells by mobilizing intracellular stores, possibly through the formation of 1,4,5-IP_3, allows the development of a rudimentary model of the biochemical consequences of EPEC infection on host cells (**Figure 5.13**). This model involves EPEC-induced activation of PIP_2-specific PLC and subsequent formation of 1,4,5-IP_3 and 1,3,4,5-IP_4 leading to sustained mobilization of calcium stores. It predicts several discrete activities that should be present in EPEC infected cells, including changes in actin distribution resulting from calcium activation of villin and gelsolin, PKC activity and the

phosphorylation of specific protein substrates, activation of the neutral serine protease calpain, the release from the cell surface of certain ectoproteins (such as alkaline phosphatase) with PLC sensitive anchors, eventual host cell death resulting directly from prolonged calcium elevation, PKC-mediated activation of certain cellular oncogenes, and alkalinization of host cell cytoplasm resulting from PKC activation of proton efflux. In an attempt to confirm this model, a series of experiments were conducted to determine whether any of the predicted events actually occur in EPEC infected cells.

Chapter 6. RELEASE OF GLYCOSYL-PHOSPHATIDYLINOSITOL ANCHORED ECTOPROTEINS FROM HEP-2 CELL SURFACES.

6:1. *Introduction.*

The membrane of the eukaryotic cell constitutes a permeability barrier against free exchange of water soluble molecules between the cytoplasm and the external environment. Cellular requirements for the uptake of nutrients, disposal of waste products, and sensing environmental changes are facilitated by specific mechanisms in the plasma membrane, which allow controlled crossing of the membrane barrier. During the last 20 years it has become apparent that these processes are regulated by membrane associated proteins (**Low, 1989a; Low, 1989b**), anchored to the lipid bilayer in several different ways (**Ferguson and Williams, 1988**). Membrane anchors can be divided into three main groups, (1) association between hydrophobic regions of the membrane protein and lipid bilayer, (2) covalent attachment via amino groups of proteins to the carboxyl groups of fatty acids in the membrane, and (3) non-protein anchors consisting of glycosyl-phosphatidylinositol (GPI) attached to ethanolamine (**Low, 1989b**).

Ectoproteins bound to the cell surface via GPI linkages (**Figure 6.1**), are of considerable interest because they are released from the cell surface in response to specific phospholipase activity (**Low and Prasad, 1988**). Indeed a number of bacterial toxins with phospholipase C activity (i.e. which cleave PIP_2 yielding DAG and IP_3) cause the release of specific proteins from cell surfaces by hydrolysis of GPI linkages. This has led to speculation that eukaryotic cells may themselves have endogenous phospholipases specifically for the purpose of releasing GPI anchored proteins. The function of these ectoproteins is at present not fully understood, but since their activities are diverse, the functions of the group as a whole are undoubtedly heterogeneous. Proteins attached in this way are numerous and can vary according to cell type and species of origin (**Low and Zilversmit, 1980; Ferguson and Williams,**

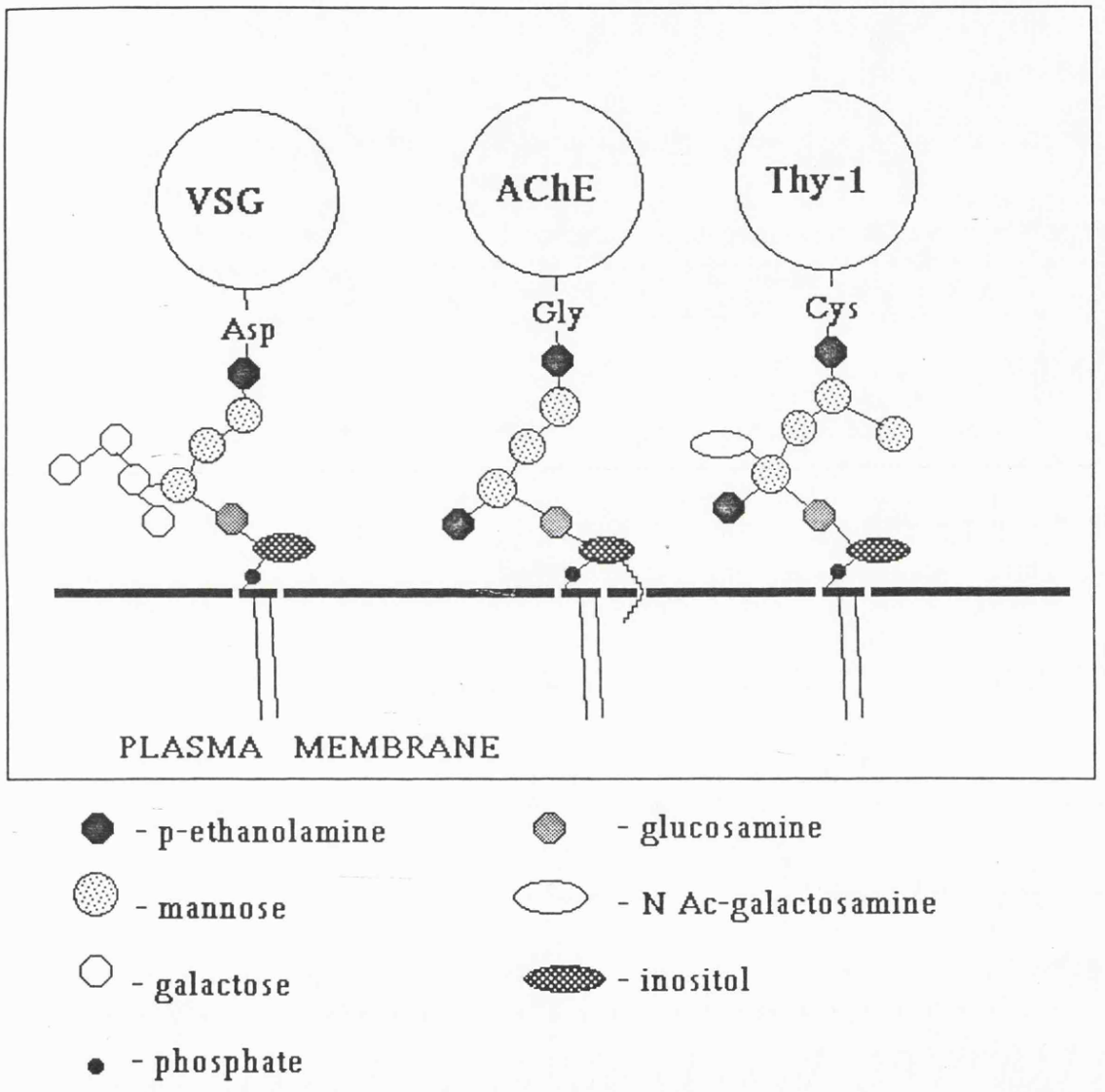


Figure 6.1. Structure of glycosyl-phosphatidylinositol (GPI) linkages and the slight variations in side groups associated with different GPI anchored proteins.

1988); one such protein found on the surface of most mammalian cells is the partially heat stable placental type alkaline phosphatase (PLAP), which is abundant in placental tissue (**Low and Zilversmit, 1980**).

Elevated alkaline phosphatase activity has been detected in the serum of pregnant women and of patients suffering from certain cancers, suggesting that these two conditions may involve induction of phospholipase activity, possibly through hormonally activated signal transduction (**Tsuda et al., 1985; Meyer and Stryer, 1988; Schnefel et al., 1988; Taylor et al., 1988b; Aiyar et al., 1989; Baldassare et al., 1989**). Another group of releasable ectoproteins that have been detected in the serum of pregnant women are the pregnancy-specific β1-glycoproteins ($PS_{\beta}G$, **Watanabe and Chou, 1988**) derived from the placenta. They are detectable during the first 2 to 3 weeks of pregnancy, and by term may reach concentrations as high as 400 μg/ml. The same protein is also detectable in the serum of patients suffering from hydatidiform mole, invasive mole, and choriocarcinoma (**Watanabe and Chou, 1988**), and indeed is used to monitor a patient's recovery from choriocarcinoma. In normal biological conditions the release of PLAP and other proteins anchored in the same manner is proposed to be dependent on, and characteristic of, the activation of endogenous or the presence of exogenously added PLC or PLD (**Low, 1989a**). Obviously the effect and result of such a release depends in part on the nature of the GPI anchored protein and its effect on target cells or processes.

Cells activated by extracellular signals through a signal transduction pathway involving the activation of PLD release GPI anchored proteins, leaving the lipid moiety of the anchor (thought to be PA) associated with the cell (**Saltiel et al., 1987**). Hormones such as insulin stimulate the formation of DAG in certain cell types through a system which has been found to be independent of phosphatidylinositol phosphate metabolism. Therfore, cell stimulation is not accompanied by 1,4,5-IP_3 formation and elevated calcium (**Saltiel et al., 1987**). This raises the possibility that with some hormones binding to receptors terminates in the activation of phospholipases which can recognise GPI anchors but not PIP_2.

Liver cell membranes contain a GPI specific phospholipase which cleaves non-phosphorylated phosphatidylinositol (PI) lipids of the anchors, yielding DAG and two compounds thought to regulate the activity of enzymes involved in phosphate-glycan modulation (**Fox et al., 1987**). The enzyme is active as a monomer with an apparent molecular weight of 62,000, is calcium independent (unlike PLC) and seems to be the mediator of insulin action (**Fox et al., 1987**). The activation of the GPI dependent phospholipase and the ensuing activation of parallel signal transduction cascades is specifically linked to the release of alkaline phosphatase in BC3H1 myocyte cells (**Romero et al., 1988**). Treatment with insulin causes the release of alkaline phosphatase and the formation of DAG. A similar process has also been demonstrated in osteoblastic cells (**Levy, et al., 1986**).

One possible function of a system which allows for the release of alkaline phosphatase (and possibly other proteins) through agonist binding and signal transduction is in the maintainence of receptors in a desensitised state to allow down regulation of the cell (**Stadel et al., 1988**). The process of down regulation involves desensitisation of the proteins involved in signal transduction (i.e. receptor, G-protein, and phospholipase) through phosphorylation by PKC (**Chapters 5 and 7**). The presence of alkaline phosphatase on the surface of the cell continually removes phosphate groups from the phosphorylated receptor molecules, leading to their eventual sensitisation once more (**Stadel et al., 1988**). Removal of alkaline phosphatase from the surface of the cell through PLC or PLD cleavage would allow for the desensitisation of one pathway to remain while another is activated through *de novo* DAG production from the GPI anchors. This enables a high degree of flexibility in respect to the pathways that are active at any time.

6:2. *Materials and methods.*

6:2:1. Alkaline phosphatase preparation. Phospholipase C activity was scored by the ability of EPEC strains to release alkaline phosphatase from the surface of HEp-2 cells.

Figure 6.2. Standard curve of units of alkaline phosphatase activity against absorbance at 410 nm (A_{410}). The formation of product (*p*-nitrophenol from *p*-nitrophenol phosphate) was followed by absorbance measurements at A_{410}. For each sample, units of enzyme activity was calculated and the standard curve polotted.

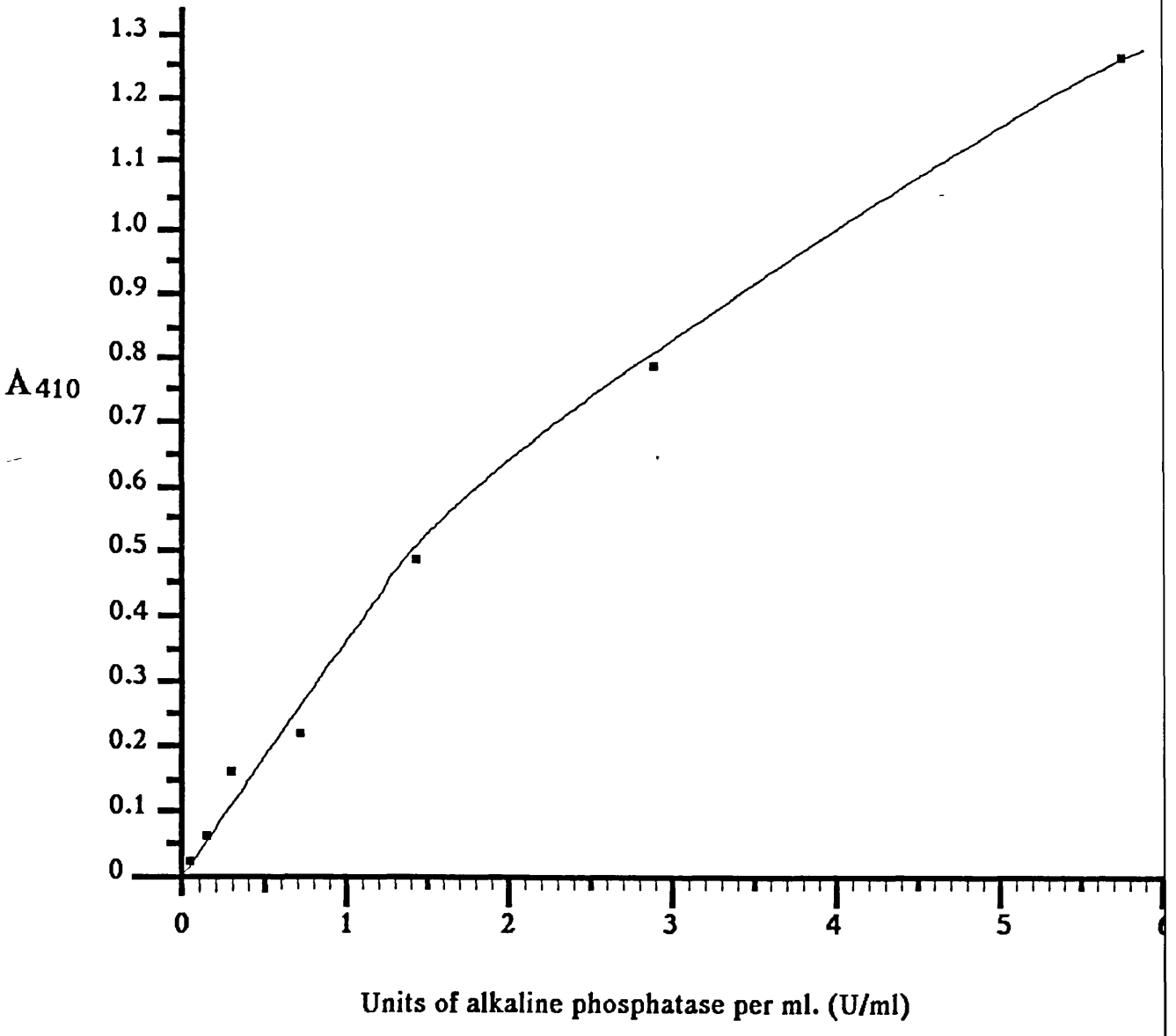


Figure 6.2. Standard curve of units of alkaline phosphatase activity against absorbance at 410 nm (A_{410}).

Monolayers were grown to confluence in tissue culture flasks, and infected with 10^9 bacteria grown to A_{600}= 0.7 in LUB-glucose without shaking at 37°C. Infected monolayers were incubated at 37°C for 1 h, washed twice in PBS (37°C), and incubation continued at 37°C in Hanks balanced salts (1.3 mM $CaCl_2$, 5.4 mM KCl, 0.4 mM KH_2PO_4, 1 mM $MgSO_4$, 140 mM NaCl, 0.31 mM Na_2HPO_4, 20 mM PIPES, *p*H 7.2, 0.1% D-glucose) containing 1% FCS for a further 3 h. Culture supernatants were collected, bacteria and cell debris pelleted by centrifugation in a Du Pont Sorval HB4 rotor at 12,000 rpm for 15 min at 4°C, and membrane fragments pelleted by centrifugation in a Beckman L5-65 ultracentrifuge and Ti75 rotor at 38,500 rpm (100,000 x g) for 1 h at 4°C. Supernatants were adjusted to *p*H 9.0 with 2 M Tris *p*H 10, filter sterilised and stored at -20°C.

6:2:2. Alkaline phosphatase assay. To 2 ml culture supernatants diluted 1:10 with Hanks balanced salts (*p*H 9.0) were added 20 µl of a 500 mM stock of *p*-nitrophenolphosphate solution in Hanks. Incubation was at 37°C for 2.5 min and the reaction was stopped by adding 5 µl of 2 M HCl and placing on ice until needed. A_{410} measurement of samples were made, and the amount (µM) of *p*-nitrophenol released obtained from a standard curve (**Figure 6.2**). Units of enzyme (defined as the amount of enzyme required to release 1 µM of product at 37°C in 1 min) present per ml of supernatant were calculated.

6:3. *Results.*

6:3:1. EPEC induced release of PLAP from HEp-2 cells.

The evidence that intimate adherence of EPEC to target cells causes mobilization of calcium from intracellular stores, and promotes cytoskeletal actin rearrangements suggested the involvement of a phosphatidylinositol specific phospholipase (PLC or PLD), either as part of a signal transduction pathway or possibly as a surface component of the bacteria. Release of the GPI anchored protein PLAP in response to

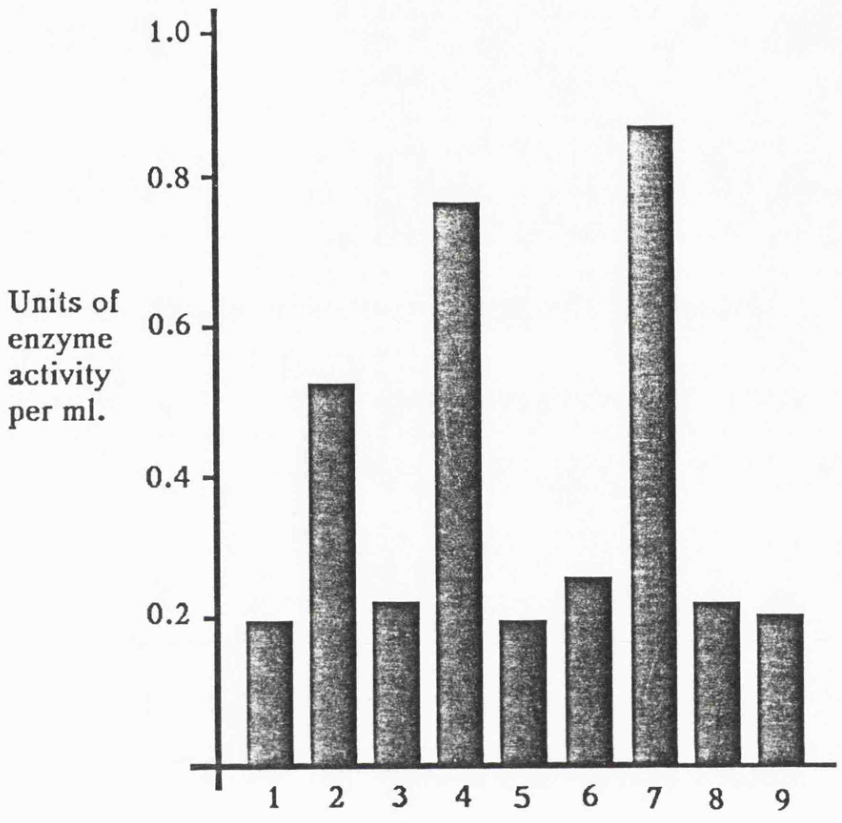
EPEC infection was monitored as an indication of phosphatidylinositol specific phospholipase activity.

The culture medium from HEp-2 monolayers was tested for the presence of PLAP and the activity calculated as units of enzyme activity present/ml of medium. Monolayers infected with EPEC strain 2036-80 released up to 6 times more PLAP activity than uninfected control cells (**Figure 6.2**), strongly suggesting the activity of a PI specific phospholipase. The culture medium from EPEC strain 2036-80 cultures did not promote additional release of PLAP activity, arguing against the possibility that a soluble toxin with PLC/D activity is associated with at least this EPEC isolate (**Figure 6.2**).

6:3:2. Screening of *E. coli* isolates for PLAP releasing ability. The ability of four well characterised strains (**Appendix 1**) to release PLAP from the surface of HEp-2 cells was assayed (**Figure 6.2**). Like 2036-80 the true AE lesion-forming isolate E2348-69, also released significantly more PLAP as compared to uninfected HEp-2 controls, and HEp-2 monolayers infected with the negative control strain HB101(pMAR15). DA strain 135 and the invasive strain 469-3 released little more PLAP than controls. These data provide strong evidence that lesion-forming EPEC strains promote breakdown of phosphatidylinositol phosphate lipids in the host cell membrane.

6:3:3. Hormone induced release of PLAP. To determine whether PLC/D activated through hormone-induced signal transduction could release PLAP, the culture fluids from HEp-2 monolayers treated with the hormone angiotensin II (1 μg/ml for 2 h) were tested for activity (**Figure 6.2**). Angiotensin II, which is known to activate receptor-coupled PLC causing the breakdown of PI lipids (**Chapter 5**), released 3 times more PLAP activity than controls cells. This was lower than activity released during EPEC infection but was nevertheless considered to be significant. A possible reason is that there are few receptors for angiotensin II on the surface of HEp-2 cells. These data

Figure 6.2. Release of alkaline phosphatase from the surface of HEp-2 cells in response to infection with EPEC or treatment with hormones. (1) Uninfected HEp-2 monolayers, (2) monolayers treated with angiotensin II (1 μg/ml) for 2 h, (3) monolayers infected with the laboratory strain HB101 carying the EPEC plasmid pMAR15, for 3 h, (4) monolayers infected with EPEC strain 2036-80 for 3 h, (5) monolayers treated with filter sterilized culture supernatants from EPEC strain 2036-80 for 3 h, (6) HEp-2 monolayers down-regulated with TPA (50 nM) for 24 hours then infected with EPEC strain 2036-80 for 3 h, (7) monolayers infected with EPEC strain E2348-69 for 3 h, (8) monolayers infected with the DA, nonlesion-forming strain 135 and (9) monolayers infected with the DA invasive strain 469-3.



KEY

1) HEp-2 control
2) Angiotensin II
3) HB101 (pMAR15)
4) 2036-80
5) 2036-80 supernatant
6) 2036-80 TPA treated
7) E2348-69
8) 135
9) 469-3

Figure 6.2. Release of alkaline phosphatase from the surface of HEp-2 cells in response to infection with EPEC or treatment with hormones.

indicate that activation of PLC through signal transduction is able to release significant amounts of GPI anchored ectoproteins, although it is difficult to imagine the precise mechanism involved since there is no evidence that both the anchor and the coupled PLC are in the same leaflet of the membrane. However, the fluid structure of the membrane suggests that it is possible for conformational change to bring the GPI anchor into contact with activated PLC; alternatively activation of cells by hormones and the accompanying calcium rise might activate a GPI specific phospholipase in the same membrane plane as the anchor.

6:3:4. Characterisation of PLAP. PLAP is reported to be partially heat stable (**Low and Zilversmit, 1980; Webb and Todd, 1988**). The activity released during EPEC infection was tested to determine whether it conforms to these criterion. Activity was assayed after incubation at various temperatures for 15 minutes and compared to the results obtained for other commercially available alkaline phosphatases (**Figure 6.3**). EPEC released PLAP showed half maximal activity after incubation at 45°C, which was reduced to 20% at 65°C and zero at 100°C. Heat sensitive calf intestinal alkaline phosphatase (CIP) showed complete inhibition at 45°C and heat stable bacterial alkaline phosphatase (BAP) showed full activity at 65°C, although this was lost by incubation at 100°C. PLAP released from HEp-2 cells during EPEC infection can therefore be considered to be "partially heat stable".

6:3:5. Effect of downregulation on PLAP release. HEp-2 monolayers were treated with the phorbol ester TPA (50 nM) for a prolonged period of 24 hours to downregulate signal transduction pathways involving receptor coupled PLC. They were then treated with either EPEC for 3 h or angiotensin II for 2 h, and culture media were assayed for PLAP activity (**Figure 6.2**). In both cases PLAP activity was considerably lower than that seen with normal HEp-2 monolayers. It appears that downregulation of host cell signal transduction systems by long term activation of PKC reduces although does not inhibit totally, release of PLAP from the surface of HEp-2 cells, in response to the

Figure 6.3. Heat stability of different alkaline phosphatases. Graphs of enzyme activity against temperature for (+) PLAP, (o) CIP, and (*) BAP. Showing the intermediate heat stability of PLAP compared to heat stable BAP and heat labile CIP.

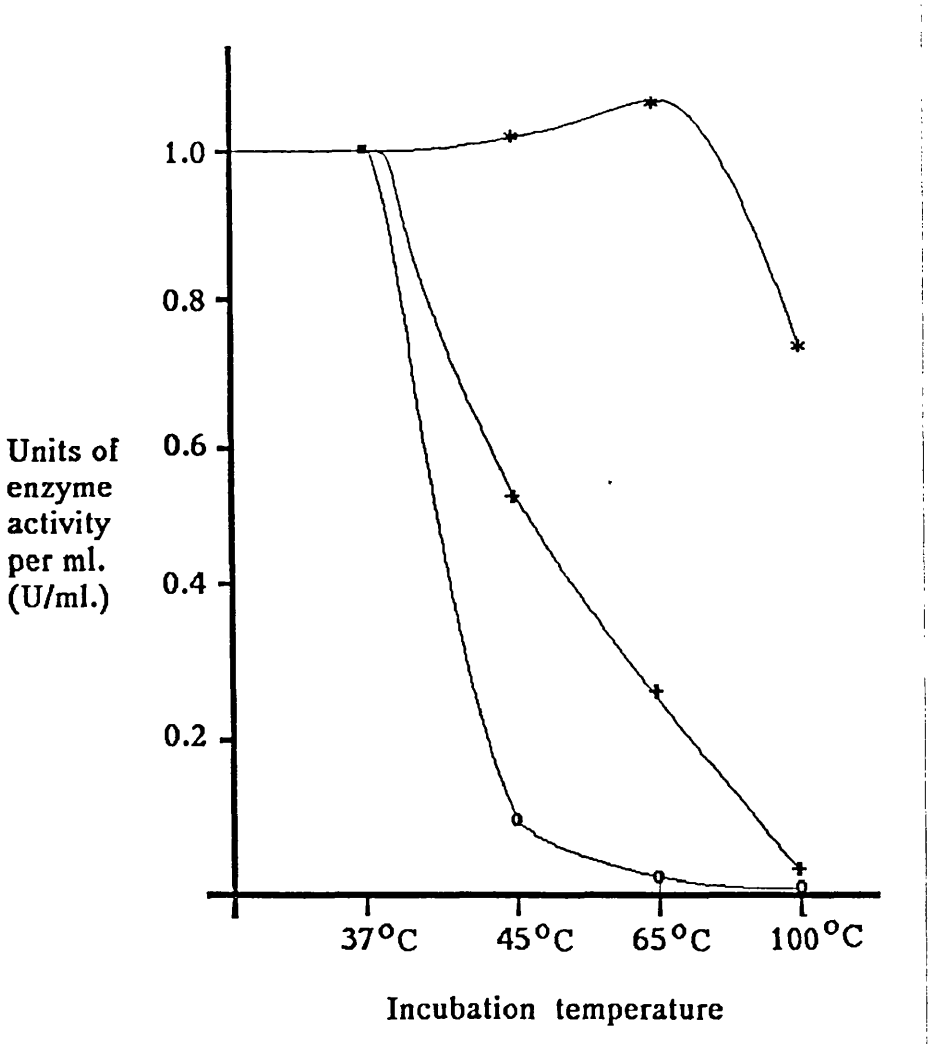


Figure 6.3. Heat stability of different alkaline phosphatases.

angiotensin II and EPEC suggesting that PLC is activated by signal transduction in response to EPEC infection.

6:4. *Discussion and Conclusions.*

The release of PLAP from the surface of HEp-2 cells in response to infection with known lesion forming strains of *E. coli* strongly indicates an associated PI-specific phospholipase activity responsible for the cleavage of GPI protein anchors. The fact that the release of PLAP from infected cell surfaces could be reduced to basal levels by prior treatment with TPA, suggests that EPEC may mediate host cell activation via a signal transduction pathway. Treatment of cells with TPA for extended periods effectively prevents cells responding to external signals. It is speculated that PKC activity interupts signal transduction by phosphorylating one or more of the transducing proteins thus inhibiting PI-phospholipase activity (**Rodriguez-pena and Rozengurt, 1984**), or by controling the removal of second messengers from the cytoplasm.

All lesion forming isolates tested caused release of a similar enzyme activity, in addition the culture supernatent from one strain (2036-80), did not release of PLAP from the surface fo HEp-2 monolayers tested no PLAP releasing activity was found in culture supernatants. This suggests that the EPEC component responsible for the release of PLAP is not an exported soluble toxin, but rather is associated with the bacterial cell, causing release of PLAP in response to intimate adherence to the host cell surface.

The significance if any, of PLAP released from cell surfaces during natural infections is obscure. Since several of the proteins anchored by GPI linkages are known to be involved in cell to cell adhesion (**Low,1989b**), cell activation and the release of GPI anchored proteins would eventually result in the breakdown of the affected tissue; a situation seen during prolonged EPEC infection of villus surfaces (**Rothbaum et al., 1982**). Although highly speculative, it seems reasonable that since the lipid moiety of the anchor is biologically active (**Low, 1989a; Low, 1989b**), release of GPI anchored

proteins may itself activate additional pathways of cell stimulation which may increase and diversify the host cell responses.

Chapter 7. PROTEIN PHOSPHORYLATION IN HEp-2 CELL MONOLAYERS.

7:1. *Introduction.*

Protein phosphorylation plays a crucial role in the regulation of a seemingly ever increasing number of cellular processes (**Edelman et al., 1987; Hunter, 1987**). In several cases the transport of ions and organic molecules across biological membranes is known to involve protein kinase activity (**de Jonge and Lohmann, 1985; Fondacaro and Shlatz-Henderson, 1985; Fondacaro, 1986; Henriksen et al., 1989; Raymond et al., 1989**). By studying the pathogenesis of enterovirulent micro-organisms such as *Vibrio cholerae,* and ETEC (LT and ST producers) the formation of second messenger molecules has been implicated in the activation of specific host cell protein kinases that may be involved in ion transport and the diarrhoea process (**Field, 1979a; Field, 1979b; Holmgren, 1981; Giannella, 1983**). EPEC do not elaborate LT or ST, nor do they promote elevation of either cAMP or cGMP levels (**Levine et al., 1978; Long-Krug et al., 1984; Law et al., 1987; Law, 1988**), and yet they produce diarrhoea of comparable severity to *Vibrio cholerae,* which is unlikely to result solely from a calcium regulated reduction in the absorptive area of the gut through effacement of microvilli. Since cAMP and cGMP are known to activate specific protein kinases as part of the diarrhoeal response, it seemed likely that a protein kinase activity linked to the EPEC induced formation of another set of messenger molecules was possible. The observed increase in intracellular calcium (**Chapter 5**), which itself has second messenger properties (**Conrad and Rink, 1986**), would not only mediate changes in the cytoskeleton and architecture of the cell, but would probably also activate a set of calcium-dependent protein kinases (**Manalan and Klee, 1984; Means and Dedman, 1980**) which may mediate intestinal ion efflux. Also the proposed mechanism of calcium elevation by IP_3 mediated release from intracellular stores requires that protein kinase C should be activated through the

concomitant formation of DAG (**Berridge, 1984; Berridge, 1987; Berridge et al., 1988; Berridge and Irvine, 1984; Exton, 1988**).

PKC first identified as a proteolytically activated protein kinase (**Nishizuka, 1986**), can also be activated by a mechanism that does not require proteolysis. PKC has an absolute requirement for calcium and phospholipid (normally phosphatidylserine) for its activity (**Castagna et al., 1982; Molleyres and Rando, 1988; Orita et al., 1985; Rando, 1988; Severin et al., 1988; Verkest et al., 1988**). Other phospholipids activate PKC in the presence of DAG or other diol lipids by substituting for phosphatidylserine, including the phospho-inositides, phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol 4,5-diphosphate (PIP_2) (**O'Brian et al., 1987**). DAG is the natural endogenous activator of PKC (**Molleyeres and Rando, 1988; Severin et al., 1988**), produced by PLC action on PIP_2; it increases the affinity of PKC for calcium thereby rendering it fully active without a net increase in the cytosolic concentration (**Rando, 1988**). For this reason PKC is considered to be biochemically dependent on calcium but under certain conditions, physiologically independent (**Nishizuka, 1986; Rando, 1988**). Activation of PKC by calcium and phospholipid is also dependent on the substrate protein (**Bazzi and Nelsestuen, 1987**). Binding of various substrates will alter the requirements of PKC for calcium and phospholipids, giving differential phosphorylation of substrates in identical conditions.

PKC is ubiquitous, being found in all eukaryote tissues looked at (**Fondacaro and Shlatz-Henderson, 1985; Orita et al., 1985; Nishizuka, 1986; Fan et al., 1988; Hughes and Ashcroft, 1988; Shimomura et al., 1988; Tsunoda et al., 1988; Yoshida et al., 1988; Lampe and Johnson, 1989; Mattila et al., 1989; Peyron et al., 1989; Uhing et al., 1989; Wolfson et al., 1989**), although different isozymic forms may be present in different cell types (**Huang et al., 1988b; Makowske et al., 1988**). The location of PKC in cells has been difficult to establish. Using immunolocation techniques and screening sub-cellular fractions, it seemes that PKC is absent from the nucleus (**Nishizuka, 1986**), but evidence also exists that the nucleus contains proteins which can act as PKC substrates (**Nishizuka, 1986**), which has led some to speculate that there may be a nuclear form of

PKC which is as yet unrecognized. However, since the substrates of PKC are in many cases the substrates for other kinases as well, it may simply be fortuitous that proteins in the nucleus are recognized as substrates by PKC *in vitro*. More commonly the presence of PKC and its activity in cells, is screened for by identification of marker phosphoproteins thought to be phosphorylated only by PKC (**Rodriguez-pena and Rozengurt, 1986; Brooks et al., 1987**)

In unstimulated cells of all types, except those derived from brain tissue, PKC is found as an inactive cytoplasmic form (**Burgoyne, 1989**). The normal process of PKC activation in cells other than those derived from brain tissue depends on the mobilisation of the inactive form from the cytoplasm to the inside surface of the plasma membrane by what is considered to be a calcium dependent process (**Siess and Lapetina, 1988; Burgoyne, 1989; Phillips et al., 1989**). However, mobilisation alone is insufficient for PKC activation; it also requires the presence of DAG in the membrane formed by the hydrolysis of PI-lipids by activated PLC (**Berridge, 1987; Berridge and Irvine, 1984**). The formation of 1,4,5-IP_3 causes an elevation in cytosolic calcium which enables PKC to be mobilized to the membrane where it binds phosphatidylserine and is activated by membrane associated DAG.

PKC is also activated by the presence of certain compounds termed phorbol esters, the majority of which are potent activators of PKC and powerful tumour promoting agents (**Castagna et al., 1982**), although recent reports have suggested that not all effects of TPA are mediated by PKC (**Ryves et al., 1989; Sha'afi, 1989**). The most widely used phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), activates PKC by dissolving in the membrane and mimicking the action of DAG (**Aitken, 1986; Aitken, 1987; Brooks et al., 1987**). The observation that TPA alone is able to increase to amount of PKC associated with the membrane, while at the same time reducing the cytosolic content (**Persaud et al., 1989**), has been used to argue against the involvement of calcium in PKC mobilization. One possible explanation for the increase in membrane associated PKC in response to TPA is that inactive cellular PKC is in equilibrium between the cytosol and the plasma membrane, with the equilibrium strongly in favour

of the cytosolic location. TPA introduced into the membrane of the cell would act as a receptor for PKC, thereby altering the equilibrium in the direction of the membrane, causing a calcium-independent increase in the PKC content of the membrane. All PKC activating molecules including TPA have similar structures to DAG, which is considered to be important for their activating ability (**Molleyres and Rando, 1988; Rando, 1988; Leli et al., 1990**). However TPA, unlike DAG, is extremely resistant to degradation in the membrane, and cells treated with TPA experience an unusually long term PKC activation (**Rando, 1988**), which may result in artificial or exaggerated responses in the cell.

A recent report has shown that TPA activation of PKC is associated with the *de novo* formation of DAG by phospholipase D mediated cleavage of phosphatidylcholine (**Cabot et al., 1989**). This reveals interesting possibilities, that in certain cells activated by PLC mediated hydrolysis of PIP_2, PKC activity may result in the formation of DAG from phospholipid sources other than PI-lipids thereby extending PKC activity by circumventing normal processes of down regulation. Also, this provides evidence of coupling between presumably different systems of cell activation.

In the normal situation DAG is rapidly phosphorylated to phosphatidic acid (PA) by diacylglycerol kinase (**Jeng et al., 1988; Yamada et al., 1989**), and removed from the membrane by further degradation (**Nishizuka, 1984; Rando, 1988**). The products of DAG degradation are either recycled to form new membrane lipids via CMP-phosphatidate (**Godfrey, 1989**), or syphoned off as precursors for the synthesis of prostaglandins and leukotrienes in inflammatory cells such as neutrophils and macrophages (**Chilton, 1989; Gukovskaya et al., 1989**). These mediators of inflammation are produced in response to cell stimulation by various agents including bacterial LPS, and are an important part of the host defence mechanism against invading organisms. The binding of LPS to receptors and consequent activation of G-proteins and coupled PLC or phospholipase D (PLD) increases the membrane DAG content and stimulates PKC as well as causing a rise in intracellular calcium (**Daniel-Issakani et al., 1989**). This causes proliferation of B-lymphocytes and the release of

arachidonic acid metabolites and interleukin 1 from macrophages (**Daniel-Issakani et al., 1989**). Prostaglandins and leukotrienes in turn stimulate calcium mobilization and PKC activation in target cells which is believed to result from the induction of PLC and the characteristic hydrolysis of PIP_2 (**Davis et al., 1987**).

Phophatidic acid itself has properties which suggests that it may act as a second messenger. Cells treated with PA in some instances respond as they would to growth factors, showing an elevated cytosolic calcium concentration resulting from the mobilization of intracellular stores, increased PKC activity and an associated elevation in cytoplasmic *p*H (**Moolenaar et al., 1984; Moolenaar et al., 1986; Grinstein et al., 1988; Ladoux et al., 1988**). Evidence suggests that PA itself is not responsible for the mobilisation of calcium or the activation of PKC, but rather it stimulates PIP_2 metabolism and the formation of 1,4,5-IP_3 and DAG (**Moolenaar et al., 1986**). This may result from the ability of PA to form non-bilayer domains in membranes at high calcium concentrations, causing perturbations in membrane structure (**Eklund et al., 1988**) rendering PIP_2 susceptable to, PLC attack (**Moolenaar et al., 1986**).

The binding of several agonists (hormones and growth factors) to their receptors promotes PKC activation (**Nishizuka, 1986; Mahadevan et al., 1987; Bouscarel et al., 1988; Fan et al., 1988; Huckle et al., 1988; Johnson and Baglioni, 1988; Kazlauskas and Cooper, 1988; Slivka and Insel, 1988; Tyagi et al., 1988; Augert et al., 1989; Mattila et al., 1989; Raymond et al., 1989; Uhing et al., 1989**). In most cases this is the result of PLC activity and the formation of 1,4,5-IP_3 and DAG. The activation of PKC can also have a regulatory role on other responses of cells to the hormone that caused stimulation, or to agonists that bind to receptors subsequently. For example the activation of PKC by phorbol ester mediates serine phosphorylation of the insulin receptor, thereby decreasing its tyrosine kinase activity, altering the cells reaction to insulin stimulation (**Takayama et al., 1988**). This observation highlights the fact that cell stimulation by particular agonists does not exist in isolation of all other routes of activation (**Taylor et al., 1988b**), and that care should be taken when evaluating the consequences of any induced activity in the cell, in case interactions between pathways

are overlooked. Recently the toxic shock syndrome toxin I (TSST-1), a 22 kDa protein of *Staphylococcus aureus* with mitogenic properties, has been shown to initiate inositol phosphate turnover, calcium mobilization and PKC translocation to the plasma membrane in T cells (**Chatila et al., 1989**). These events may form part of the pathogenic mechanism of the syndrome, possibly initiating fever by a PKC-dependent increase in expression of interleukin-1 (IL-1) in macrophages and monocytes, and interleukin-2 (IL-2) in T cells. The observation that TSST-1 enhanced expression of IL-1 appears to have at least part of its mechanism in common with endotoxin activation (**Ikejima et al., 1989**), not only reaffirms the above argument for pathway interactions, but further implicates the importance of PKC activation, since lipid A is also known to cause activation of PKC (**Stott and Williamson, 1978; Weiel et al., 1986; Ellis et al., 1987; Daniel-Issakani et al., 1989**). In addition, since PKC is involved in the control of gene expression (**Morley and Traugh, 1989; Tuazon et al., 1989**), and cell division and mitogenesis (**Collins and Rozengurt, 1984; Rozengurt et al., 1984, Sawada et al., 1990**) the role of PKC activity in this condition seems pivotal.

Other hormones and agonists seem to stimulate the formation of PA directly without prior 1,4,5-IP_3 and DAG production, through a signal transduction pathway which terminates in the activation of PLD rather than PLC (**Bocckino et al., 1987; Godfrey, 1989**). Since PLD is not specific for PI phospholipids, it will elicit the formation of PA (and therefore eventually DAG) from a range of other phospholipids, including phosphatidylcholine (**Bocckino et al., 1987; Cabot et al., 1988**), causing the activation of PKC and mobilisation of calcium without PIP_2 turnover (**Augert et al., 1989**). Much data previously thought to be contradictory, in which PKC activation by certain agonists was seen without the "statutory PIP_2 breakdown" have been reconsidered in the light of this evidence.

Cellular PKC activity is strictly regulated by feed back systems (down-regulation) thought to involve the inactivation or removal of key proteins of the signal transduction pathway directly or indirectly by phosphorylation (**Nishizuka, 1986**). There are at least three theoretical foci of down-regulation the receptor, the G-protein,

and coupled PLC (**Collins and Rozengurt, 1984; Jaiswal et al., 1988; Troyer et al., 1988**), all of which would effectively prevent on-going synthesis of second messengers in response to continued cell stimulation. Any of these mechanisms of down-regulation would not on their own remove PKC activity from the cell in a sufficiently short time to be of significance in cessation of cell stimulation. For the complete abeyance of PKC activity it is essential that pre-formed signals are eliminated, phosphate groups are removed from substrates, and PKC activity and location returned to that of the resting state. Pre-formed DAG is removed from the plasma membrane as PA through phosphorylation by DAG kinase, which itself is phosphorylated by PKC which presumably controls its activity (**Nishizuka, 1986; Kanoh et al., 1989**), and further broken down to give arachidonic acid (**Krishnamurthi and Joseph, 1989**). The enzyme activities associated with the dephosphorylation of PKC substrates, including autophosphorylation of PKC (**Mitchell et al., 1989**) has recently been identified as type I and type 2A phosphatases, which are characteristically inhibited by okadaic acid (**Aitken personal communication; Cohen and Cohen, 1989**). This is a complex fatty acid polyketal produced by various marine dinoflagellates found in the digestive glands of shell fish and marine sponges such as *Halichondria okadaii* (**Cohen and Cohen, 1989**). It is the cause of the majority of shell fish poisoning cases and has been found to be a potent tumour promoting agent (**Cohen and Cohen, 1989**). At first okadaic acid was considered to be a PKC activator since treated cells showed increased phosphorylation of known PKC substrates. It has since become apparent, however that okadaic acid prevents the dephosphorylation of substrates phosphorylated by the low level of endogenous PKC activity present in unstimulated cells (**Cohen and Cohen, 1989**). Active PKC is a monomeric protein with a molecular weight of 80,000 (**Edelman et al 1987**) and is removed from the plasma membrane as part of the down-regulation process by the calcium activated neutral endoproteinase calpain. An increase in cytosolic calcium concentration activates and mobilizes calpain to the plasma membrane where it cleaves active-membrane associated PKC to yield two fragments. One of 30,000 molecular weight remains associated with the plasma membrane and

constitutes the regulatory domain containing the binding sites for calcium, phosphatidylserine, and DAG: The second fragment of 50,000 molecular weight, contains the catalytic domain, and is released into the cytosol (**Edelman et al., 1987**) where it continues phosphorylating cytosolic substrates independently of calcium, phosphatidylserine or DAG. This activity has become known as protein kinase M (PKM) (**Edelman et al., 1987**); it is extremely short lived in the cytoplasm and is further broken down by other proteases as part of the down-regulation process. Interestingly PKC activity itself appears to be intimately involved in the down-regulation of its own activity and most if not all signal transduction pathways involving PI-lipid hydrolysis (**Rodriguez-pena and Rozengurt, 1984; Berridge, 1987**). Treatment of many cell types with TPA for extended periods will result in the eventual loss of PKC activity from the cell (**Rodriguez-pena and Rozengurt, 1984**). This situation is further complicated by the apparent differential down-regulation of isozymic subspecies of PKC (**Ase et al., 1988**), potentially allowing one aspect of PKC activity to remain in operation while others are inactivated by down-regulation. The nature of the differential regulation of different isozymes of PKC is at present not understood.

PKC appears to be the controlling factor in several ion transport pathways (**Fondacaro, 1986; Cohn, 1990**), which is of particular interest to this study since it indicates a potential role of PKC in diarrhoea. Oral administration of TPA to conscious rats provokes profuse secretory diarrhoea and intestinal hypersecretion of comparable severity to that seen with administered cholera toxin (**Fondacaro, 1986; Fondacaro and Shlatz-Henderson, 1985b**). TPA also increases chloride ion secretion from isolated rabbit intestinal tissue (**Donowitz et al., 1985; Fondacaro et al., 1985a; Fondacaro et al., 1985b**) and raises intracellular *p*H in various mammalian cell types due to efflux of protons (**Moolenaar et al., 1984**). The evidence presented in previous chapters that EPEC induce several distinct changes in infected cells, including elevation of calcium by mobilization of intracellular stores, major changes in cell shape and actin distribution, release of GPI anchored ectoproteins, and loss of cell viability within the time scale of the other events, indicates the activation of a signal tranduction process involving PLC.

This in turn suggests that activation of PKC should accompany EPEC infection, causing phosphorylation of protein substrates identical to those mediated by known activators of PKC.

7:2. *Materials and Methods.*

7:2:1. Polyacrylamide gel electrophpresis (PAGE).(Laemmli, 1970; Maniatis et. al., 1982)

7:2:2. Coomassie blue staining and autoradiography. Gels were fixed and stained for proteins with staining solution (25% v/v propan-2-ol, 10% v/v glacial acetic acid, 0.5 mg/ml Coomassie brilliant blue R [Sigma]) for 2 h, placed into destain (25% v/v propan-2-ol, 10% v/v glacial acetic acid) for 6 h with frequent changes of destain and dried onto 3MM paper (Whatman) by vacuum and heat (Bio Rad 1125 slab gel drier). Phosphoproteins were detected by autoradiography for 8 to 16 h using X-ray film (Kodak 'X-Omat S').

7:2:3. Activation of protein kinase C. HEp-2 and CaCo-2 cells were grown to confluence in 5 ml tissue culture Petri dishes (Nunc). HEp-2 monolayers were maintained for 1 or 2 days in DMEM containing 0.5% FCS, and CaCo-2 monolayers were maintained for 2 or 7 days in DMEM containing 10% FCS. Prior to phosphorylation experiments, confluent cell monolayers were incubated for 4 hours in 2ml phosphate-free DMEM (Gibco) containing 0.5% FCS and 50 μCi of carrier free ^{32}Pi phosphate (Amersham) to label the intracellular ATP pool. Cells were treated with bacteria or known activators of protein kinase C (PKC) for the appropriate times. To terminate phosphorylation reactions cells were rapidly washed 3 times in ice cold PBS and cells scraped off using a rubber policeman into 50 μl of ice cold sonication buffer (50mM Tris. HCl *p*H 5.0, containing 50 mM benzamidine HCl, 50 mM NaF, 2.5 mM sodium pyrophosphate, 5 mM glycerophosphate, 2 mM EDTA, 20 μg/ml leupeptin, 20 μg/ml pepstatin A, 1.5% Triton X-100, 0.1% SDS) and placed into 1.5 ml Eppendorf

tubes containing 10 µl of a DNase/RNase solution (100 µg/ml DNase 1, 50 µg/ml RNase A, 0.5 M Tris. HCl, *p*H 7.0, 50 mM $MgCl_2$), and rapidly frozen. Cells were lysed by sonicating thawed cell suspensions for 30 sec on ice, and proteins solubilized by boiling in an equal volume of sample buffer (62.5 mM Tris. HCl, *p*H 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) for 5 min. Protein preparations were subjected to high speed centrifugation to pellet cell debris, and equal volumes of solubilized proteins were electrophoresed on 10 or 12.5%_polyacrylamide gels containing SDS. With CaCo-2 monolayers, and occasionally with HEp-2 monolayers, cells were resuspended in sonication buffer without Triton or SDS. Lysates were then separated into membrane and soluble fractions by centrifugation (100,000 x g for 45 min) and solubilized proteins from each were loaded onto gels which were electrophoresed over night.

7:2:4. Mobilization of protein kinase C. HEp-2 cell monolayers grown to confluence in tissue culture Petri dishes were infected with 10^9 bacteria for 1 h at 37°C, washed in medium and incubated for a further 2 h. Monolayers were removed in 1 ml of PBS containing 20 mM Tris. HCl, *p*H 7.5, 2 mM EDTA, 20 µg/ml leupeptin, 20 µg/ml pepstatin A, to 1.5 ml microfuge tubes and sonicated for 30 sec on ice (3x 10 sec bursts). Cell sonicates were removed to thick walled micro-ultracentrifuge tubes and membranes pelleted by centrifugation (100,000 xg for 1 h at 4°C). Membrane pellets were extracted with 1 ml sonication buffer containing 1% Triton X-100 by sonicating for 5 sec on ice (**Hauschildt et al., 1988**). Insoluble membrane fractions were pelleted by centrifugation as above and the Triton soluble membrane fractions removed to 1.5 ml microfuge tubes. All fractions were kept for assay at -20°C and protein concentrations determined by the method of Bradford (**1976**) just prior to assay. Protein kinase C was assayed basically as described by **Hauschildt et al. (1988)**, by incubating 4 µg of protein from the Triton soluble membrane fraction for 15 min at 37°C in 250 µl of 20 mM Tris. HCl (*p*H 7.5) containing 5 mM $MgCl_2$, 0.4 mg/ml Histone III-S (Sigma), 40 µg/ml phosphatidylserine (Sigma), 0.8 µg/ml 1,2-diolein or 100 nM TPA, 500 µM $CaCl_2$, and 10 µM ^{32}P-γ-ATP (specific activity 3000 µCi/mmol). The reaction was terminated by the

addition of 250 µl of cold Bovine serum albumin (BSA, 1 mg/ml) and 2 ml of cold 25% trichloroacetic acid (TCA). After 20 min on ice the precipitate was pelleted by centrifugation in a microcentaur microfuge at 6,000 rpm for 5 min, dissolved in 1 *N* KOH, precipitated again as above, and collected on glass fibre filters (Whatman GFC 2.5 cm discs) by suction. Filters were washed twice with cold 25% TCA and once with cold acetone. ^{32}P phosphorylated Histone III-S was quantified by liquid scintillation using Optiphase scintillant.

7:2:6. Characterisation of PKC substrates. Membrane and cytosolic fractions from EPEC infected HEp-2 monolayers were prepared by sonication (3x 10 sec on ice) and centrifugation (100,000 xg for 30 min). Membrane pellets were resuspended in an equivalent volume of sonication buffer, and proteins were solubilized by addition of an equal volume of solubilising buffer and boiling for 5 min. Proteins were loaded onto 12.5% polyacrylamide electrofocussing tube-gels and run until the dye front was 25% from the end (first dimension). Gels were removed from the tubes, and set in placed at the top of a 12.5% polyacrylamide *p*H gradient gel (*p*H 8 to *p*H 2) using polyacrylamide *p*H 8. Gels were run until the dye front was 25% from the end, at which time gels were removed from the electrophoresis tank, fixed as described above and stained with Coomassie blue. The position of ^{32}P labeled phosphoproteins was detected by autoradiography of the dried gels as described above.

7:3. *Results.*

7:3:1. EPEC induced protein kinase activity (Baldwin et al., 1990). To determine whether any EPEC mediated phosphorylation events could be detected subsequent to attachment, HEp-2 monolayers loaded with ^{32}Pi were infected with sufficient lesion forming EPEC to give 90%-99% infection after 3 hours at 37°C. Proteins isolated from infected monolayers were analysed by PAGE and autoradiography for the presence of any additional phosphoproteins. Originally nine new phosphoprotein species were

identified in monolayers infected with the lesion-forming, diarrhoeagenic EPEC strain, 2036/80 (**Figure 7.1**). Of these, two proteins of molecular weights 21,000 and 29,000 (P_{21} and P_{29} respectively) were clearly the major phosphorylated species; the former appeared diffuse and heavily labelled suggesting a glycoprotein or multiple protein band. The other minor phosphoprotein species identified had molecular weights of approximately 23,000, 27,000, 30,000, 34,000, 36,000, 40,000 43,000 (P_{23}, P_{27}, P_{30}, P_{34}, P_{36}, P_{40} and P_{43} respectively

Prior to labelling, the HEp-2 monolayers were maintained in low serum medium to reduce cell growth, which is thought to enhance the activity of certain protein kinases. Phosphorylation of two additional proteins of molecular weight 15,000 and 19,000 (P_{15} and P_{19}) and P_{27} and P_{29} were affected by the length of time spent in low serum medium. Cells maintained in low serum medium for 40 to 50 h prior to infection showed a reduction in labelling of P_{27} and P_{29} when compared to infected cells that had been maintained in low serum for only 30 to 40 hours. The opposite was true for the phosphoproteins P_{15} and P_{19}, which were only apparent in cells that had been maintained in low serum for 40 h or more. For this reason cells were usually maintained on low serum medium for 40 hours, and proteins P_{21} and P_{29} used as landmarks for EPEC-induced phosphorylation events to try to eliminate variation due to growth status of the cells used.

Phosphoprotein profiles from HEp-2 cells infected with other adherent *E. coli* strains which do not form an attaching and effacing lesion were compared with those from known lesion-forming EPEC. Nonlesion-forming strains have either an aggregative (AA) or diffuse (DA) pattern of adherence to HEp-2 cells. DA strains 135 and DPO95, caused no additional phosphorylation events as compared to HEp-2 controls, while the AA strains 136 and JPN10 stimulated the phosphorylation of major protein species similar in size to P_{21} and P_{29} (**Figure 7.2**).

7:3:2. Identification of the protein kinase involved (Baldwin et al., 1990). To determine whether the second messengers cAMP and cGMP were involved in the

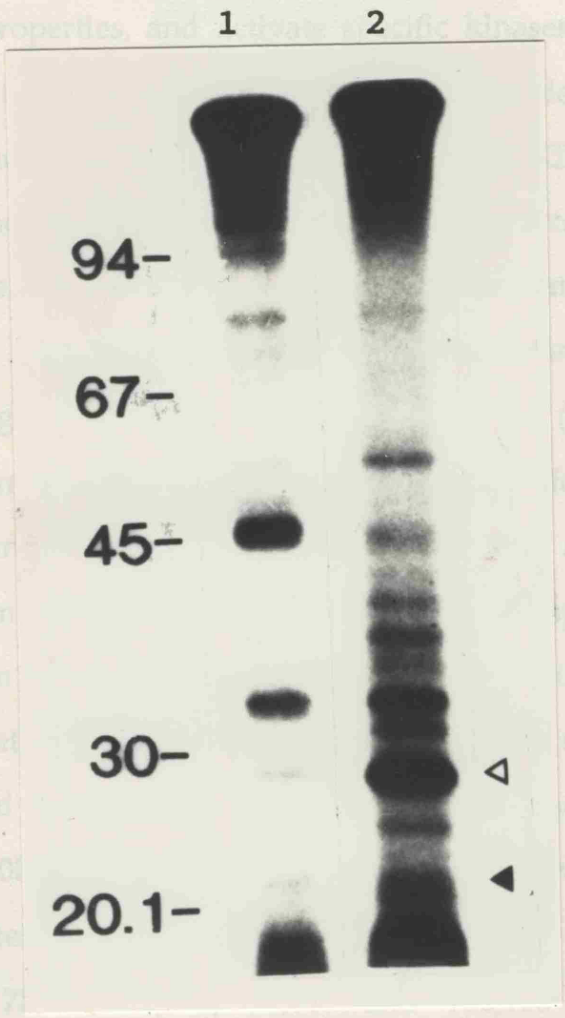


Figure 7.1. Protein kinase activity induced in HEp-2 monolayers infected with lesion forming *E. coli* isolate 2036-80. SDS-polyacrylamide (10% [w/v]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from uninfected HEp-2 cells (lane 1) and HEp-2 cells infected with 10^8 *E. coli* of EPEC strain 2036-80 (lane 2) Arrow heads indicate P_{21} and P_{29}. Molecular weight markers were phosphorylase b (94,000), albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and lactalbumin (14,300).

phosphorylation events observed in EPEC infected HEp-2 cells, proteins were prepared from monolayers treated with dibutyryl derivatives of cAMP and cGMP (Db.cAMP and Db.cGMP respectively). These are able to enter cells by virtue of their lipophilic membrane soluble properties, and activate specific kinases. Phosphoprotein profiles from cells treated in this way were compared with those derived from EPEC infected cells. Db.cAMP induced the phosphorylation of an 80 KDa. protein species which correlated with none of the EPEC-induced phosphoproteins, whereas Db.cGMP treatment of HEp-2 cells induced no additional phosphorylation events as compared to untreated cells. The observation that EPEC cause elevation of cytosolic calcium concentrations through mobilisation from internal stores (**Chapter 5**) suggested the possibility that calcium ions may act as a second messenger for the activation of calcium dependent protein kinases. To determine whether calcium alone was involved in the activation of a calcium/calmodulin type protein kinase phosphoprotein profiles of cells treated with a calcium ionophore were compared with cells infected with EPEC (**Figure 7.3**). The phosphorylation profile of ionophore treated cells showed some similarity to the profiles obtained from EPEC infected cells. Phosphoproteins of approximate molecular weight 36,000, 40,000 and 43,000 appeared to correspond to the minor EPEC induced phosphoproteins on polyacrylamide gels. However four additional proteins of molecular weights 75,000, 80,000, 90,000 and 100,000 which showed elevated phosphorylation could not be correlated with any of the EPEC induced phosphoproteins.

The calcium/phospholipid dependent protein kinase, protein kinase C was implicated as the cause of the major phosphorylation events by comparing the profiles derived from EPEC infected cells with those obtained from cells treated with the phorbol ester TPA, a known activator of PKC. Cells maintained for 30 to 40 h in low serum medium and then either infected with EPEC or treated with TPA showed a similar phosphoprotein profile (**Figure 7.3**). TPA promoted phosphorylation of proteins identical to the EPEC induced phosphoproteins P_{21}, and P_{29} and of several other proteins similar in size to some of the minor phosphoproteins seen with EPEC infected

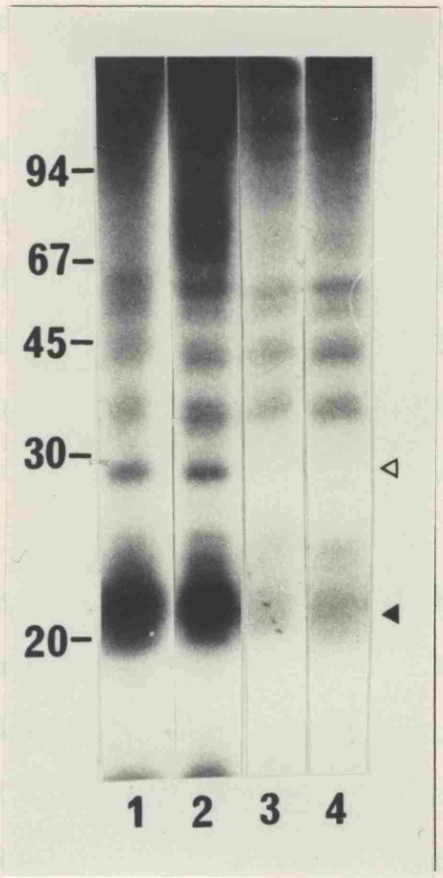


Figure 7.2. Protein kinase activity induced in HEp-2 monolayers infected with non-lesion forming *E. coli* isolates. SDS-polyacrylamide (12% [w/v]) gel electrophoresis of $_{32}$P-labeled total-cell phosphoproteins prepared from HEp-2 cells incubated for 3 h at 37°C with non-lesion forming AA strains 136 (lane 1) and JPN10 (lane 2), and DA strains 135 (lanes 3) and DPO95 (lane 4). Arrow heads indicate P_{21} and P_{29}. Molecular weight markers are as described in **Figure 7.1.**

cells (i.e. P_{23}, P_{27}, P_{34}, P_{36}, P_{40}, and P_{43}). Reports that purified rat brain PKC is activated by the lipid A component of bacterial lipopolysaccharide (LPS) (**Ellis et al., 1987**), suggested that lipid A might be involved in the observed PKC activation. HEp-2 cells treated with purified lipid A-containing LPS showed the characteristic phosphorylation of P_{21}, and P_{29}, (**Figure 7.3**) but only after a longer incubation time compared to EPEC and TPA treatment (phosphorylation was not detected until at least 3 hours after addition of LPS). This, and the fact that other *E. coli* strains such as HB101(pMAR15), 135 and DPO95 (which also contain lipid A in their LPS) adhere to HEp-2 cell surfaces but do not activate PKC, indicates that the phosphorylation events observed in EPEC infected HEp-2 cells are not simply due to surface lipid A.

7:3:3. Kinetics of PKC activation (Baldwin et al., 1990). The dose response of HEp-2 cells to increasing numbers of added bacteria revealed that phosphorylation of P_{21} and P_{29} was detectable after 3 hours incubation with 10^5 bacteria/ml (**Figure 7.4**), sufficient for only 10% of the HEp-2 monolayer to have detectable microcolonies. This may in part be due to the lack of sensitivity in the procedure for estimating the percentage infection, which cannot easily detect cells infected with 1 or 2 bacteria, but which may still activate the cell.

HEp-2 monolayers infected with EPEC for various times showed that phosphorylation of P_{21} and P_{29} could be detected after 1 hour incubation (**Figures 7.5** and **7.6**). Cells treated with TPA also showed a comparable time scale of phosphorylation to that seen with EPEC infection, allowing detection of the P_{21} and P_{29} phosphoproteins after 1 hour.

Bacteria killed by heat (65°C for 5 minutes) or antibiotics (200 μg/ml streptomycin final concentration) caused no alteration in the phosphoprotein profile as compared to HEp-2 controls (**Figure 7.5**). Which suggests that the viability of the infecting cells is essential for PKC activation. In addition supernatant filtrates from EPEC cultures, added to HEp-2 cell monolayers gave phosphoprotein profiles equivalent to uninfected controls, indicating the abscence of a soluble factor exported from EPEC (**Figure 7.5**).

7:3:4. Mechanisms of PKC activation (Baldwin et al., 1990). Several routes of PKC activation may be proposed including production of an exported phorbol ester-like compound, elaboration of an exported toxin capable of indirectly activating PKC through one of the many receptor linked signal transduction pathways used by hormones and growth factors, or intimate attachment of EPEC to the host cell surface activating PKC indirectly through a receptor-linked signal transduction pathway. Since the characteristic phosphorylation events observed were not mediated through a soluble or exported product of lesion-forming EPEC strains (**Figure 7.5**), it was suggested that intimate attachment to target cells was involved in PKC activation. Interestingly, culture filtrates from the AA strains JPN10 and 136 did cause PKC activation and the phosphorylation of P_{21} and P_{29}; heat treatment (70°C for 10 min) of the culture filtrates abolished this activity, giving phosphoprotein profiles identical to uninfected controls (**Figure 7.7**).

Some hormones neurotransmitters and bioactive peptides are known to activate PKC and elevate intracellular calcium concentrations through signal transduction pathways involving the breakdown of PI containing lipids. Comparing the phosphoprotein profiles from EPEC infected cells and cells treated with hormones known to use the PI signal transduction pathways was hampered by the fact that not all cell types have the required receptors. Pilot experiments with the hormone angiotensin II, which does use the PI signal transduction system, showed that HEp-2 cells responded to by phosphorylating P_{15}, P_{19}, P_{21}, and P_{29} (**Figure 7.3**).

Addition of phosphatidic acid (PA) (100 nm), the product of PLD activity on membrane phospholipids, to HEp-2 monolayers promoted the rapid phosphorylation of P_{21} and P_{29} by 15 min (**Figure 7.3**), compared with 1 hour for TPA and EPEC. Exogenously added PLC (1 μg/ml) also caused the same phosphoprotein profile as seen with other PKC activators in a comparable time scale (**Figure 7.3**).

In an attempt to distinguish between PKC activation through signal transduction and other potential mechanisms, HEp-2 cells pre-treated with GDPβS (an inhibitor of receptor G-protein coupled phospholipase activation) were infected with EPEC and the

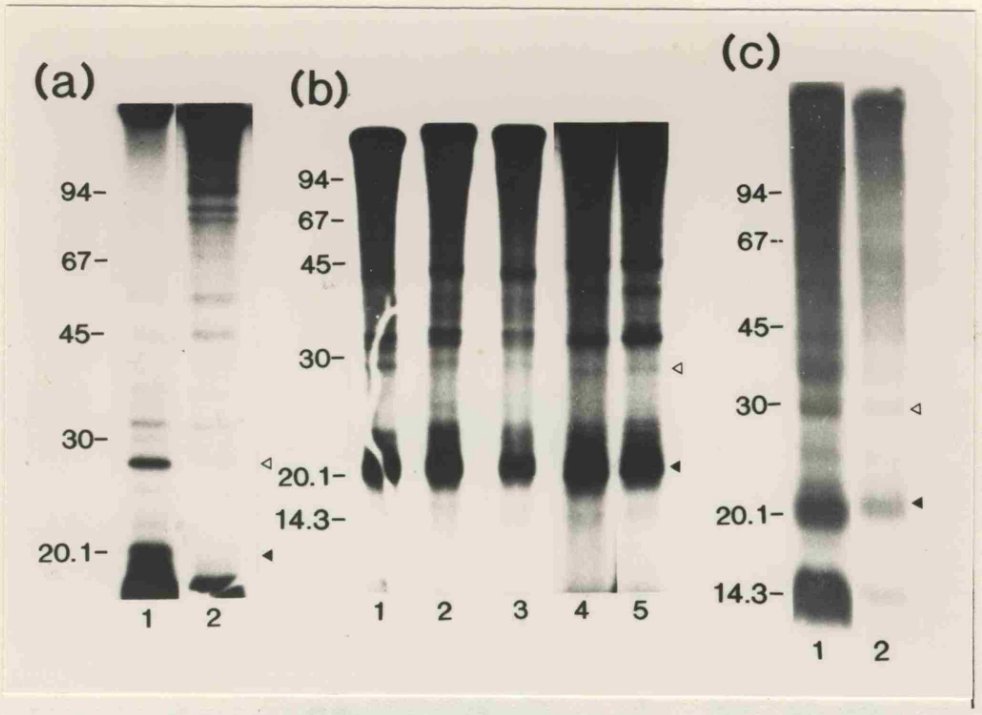


Figure 7.3. Effect of known protein kinase activators on HEp-2 cell protein phosphorylation. (a) SDS-polyacrylamide gel (10% [w/v]) electrophoresis of ^{32}P-labeled total cell phosphoproteins prepared from HEp-2 cell monolayers incubated for 3 h with 10^8 EPEC strain 2036-80 (lane 1), or treated with 12.5 μM A23187 for 1 h (lane 2). (b) SDS-polyacrylamide (12% [w/v]) gel electrophoresis of ^{32}P-labeled total cell phosphoproteins prepared from HEp-2 monolayers infected with 10^8 EPEC strain 2036-80 for 3 h (lane 1), treated with (10 nM) TPA for 2 h (lane 2), bacterial LPS serotype O111 (20 ng/ml) for 4 h (lane 3), phosphatidic acid (10 nM) for 15 min (lane 4), or phospholipase C (1 μg/ml) (lane 5). (c) SDS-polyacrylamide (12% [w/v]) gel electrophoresis of ^{32}P-labeled total cell phosphoproteins prepared from HEp-2 monolayers incubated with 10^8 EPEC strain 2036-80 for 3 h (lane 1) or for 3 h with 1 μg/ml angiotensin II (lane 2). Arrow heads indicate P_{21} and P_{29}. Molecular weight markers are as described in **Figure 7.1.**

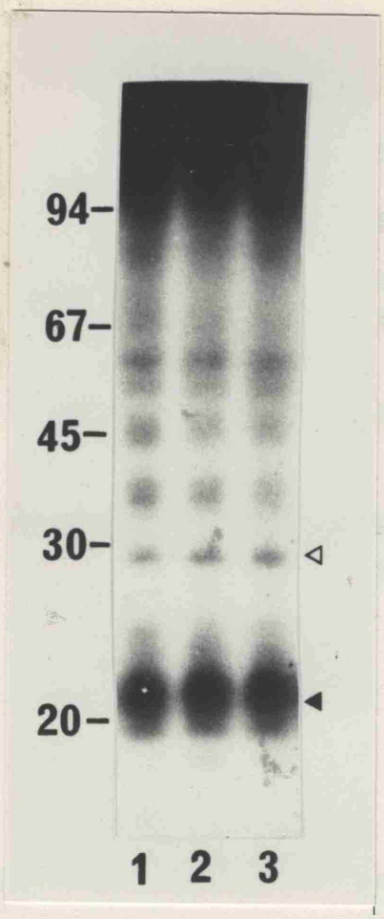


Figure 7.4. Activation of PKC in HEp-2 monolayers infected with increasing doses of bacteria. SDS-polyacrylamide (12% [w/v]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers infected with EPEC strain 2036-80, 10^5 bacteria (lane 1), $5x10^6$ bacteria (lane 2) and 10^8 bacteria (lane 3). Arrow heads indicate P_{21} and P_{29}. Molecular weight markers are as described in **Figure 7.1.**

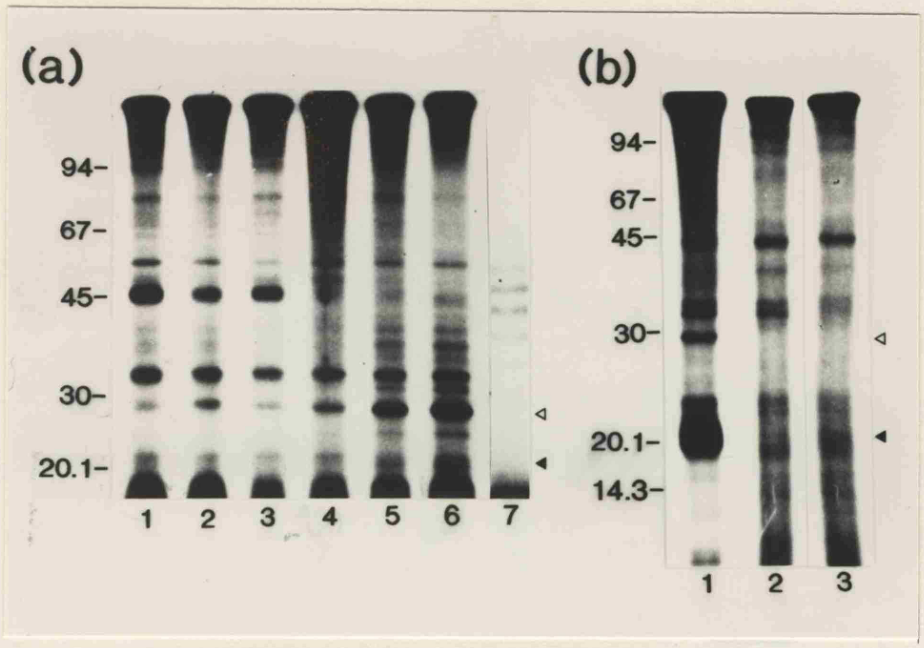


Figure 7.5. Time course of PKC activation in EPEC infected HEp-2 monolayers, and its dependence on bacterial attachment and viability. (a) SDS-polyacrylamide (10% [w/v]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers after incubation with bacteria. Lane 1, Uninfected HEp-2 control; lane 2, infected with laboratory strain HB101 containing EPEC adherence plasmid pMAR15; lanes 3 through 6, infected with EPEC strain 2036-80 for 0.5, 1, 2, and 3 h, lane 7, phosphorylated species derived from 10^8 EPEC strain 2036-80 grown for 3 h in similar conditions in the absence of HEp-2 cells. (b) SDS-polyacrylamide (12% [w/v]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers incubated for 3 h with 10^8 live EPEC strain 2036-80 (lane 1), heat killed (65°C, 7 min) 10^8 EPEC strain 2036-80 for 3 h (lane 2), or filter-sterilized culture supernatents of EPEC strain 2036-80 (lane 3). Arrow heads indicate P_{21} and P_{29}. Molecular weight markers are as described in **Figure 7.1.**

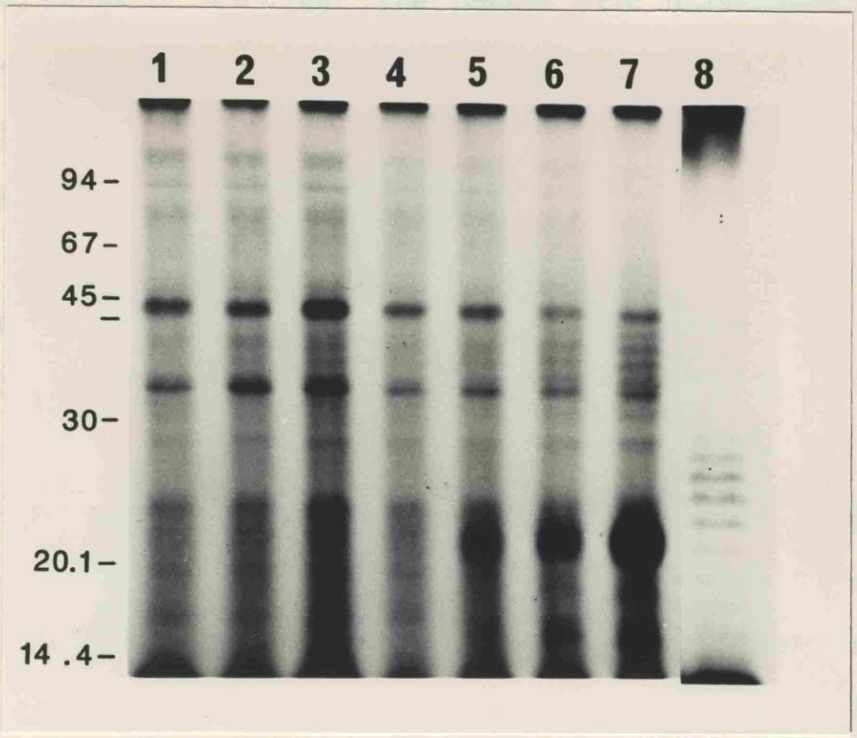


Figure 7.6. Time course of PKC activation and P_{21} phosporylation in EPEC infected HEp-2 monolayers. SDS-polyacrylamide (12% [w/v]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers after incubation with bacteria. Lane 1, Uninfected HEp-2 control; lane 2, infected with laboratory strain HB101 containing EPEC adherence plasmid pMAR15; lane 3, infected with old overnight culture of HB101(pMAR15); lanes 4 through 7, infected with EPEC strain 2036-80 for 0.5, 1, 2, and 3 h, lane 8, phosphorylated species derived from 10^8 EPEC strain 2036-80 grown for 3 h in similar conditions in the absence of HEp-2 cells. Arrow heads indicate P_{21} and P_{29}. Molecular weight markers are as described in **Figure 7.1.**

phosphoprotein profile compared to untreated cells. GDPβS only inhibits PKC activation as a result of PI cleavage through a signal transduction pathway, and not through other routes of activation. The results were inconclusive (**Figure 7.8**). Phosphorylation of P_{21} and P_{29} was certainly lowered, but not fully inhibited by GDPβS as would have been expected if the mechanism involved susceptible signal transduction pathways.

7:3:5. Screening for the ability to induce PKC (Baldwin et al., 1990). To determine whether EPEC isolates in general have the ability to activate PKC, 8 known lesion forming strains of different serotypes and from different parts of the world, and 4 nonlesion-forming strains were used to infect HEp-2 monolayers (**Figure 7.9**). The phosphoprotein profiles obtained clearly show that all 8 of the lesion-forming isolates cause phosphorylation of the phosphoproteins P_{21}, and P_{29}, whereas the nonlesion-forming strains gave profiles identical to the uninfected controls. In addition it can be seen that P_{15} and P_{19} were also prominent in this case because the monolayers used were maintained on low serum for 45 h instead of the normal 30-40 h.

7:3:6. *In vitro* PKC mobilisation assay. One of the characteristics of PKC activation is mobilisation of the enzyme from the cytoplasm, where it is in an inactive form, to the plasma membrane, where it is activated by binding of phosphatidylserine (PS) and DAG. Because monitoring phosphorylation of known PKC substrates in HEp-2 cells is a lengthy procedure a quick reliable assay was developed for the mobilisation of PKC to the plasma membrane where it could be isolated and its activity assayed in desired conditions. The basic assay consisted of incubating the Triton X-100 soluble fraction from the plasma membrane with phosphatidylserine, calcium, TPA or DAG, HI histone, and ^{32}P-ATP. PKC activity is therefore measured as ^{32}P incorporation into HI histone. The assay was first characterized using the known activator of PKC TPA, and the ionophore A23187 to mimic calcium mobilisation. HEp-2 monolayers treated with TPA for 1 hour showed increased PKC activity associated with the plasma membrane,

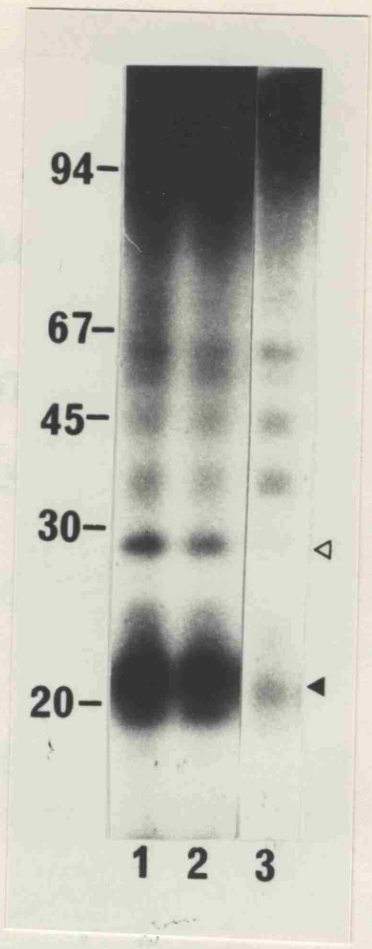


Figure 7.7. A soluble, heat labile PKC activating factor associated with AA strains of *E. coli*. SDS-polyacrylamide (12% [w/v]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers incubated with 10^8 of the AA strain JPN10 for 3 h (lane 1), culture supernatent filtrates for 3 h (lane 2), or heated culture supernatent filtrate (65°C, 10 min) for 3 h (lane 3). Arrow heads indicate P_{21} and P_{29}. Molecular weight markers are as described in **Figure 7.1.**

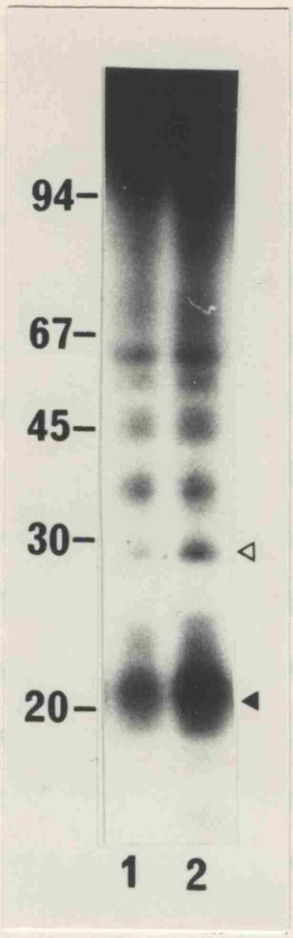


Figure 7.8. Effect of inhibiting G-protein action with GDPβS, on PKC activation by EPEC. SDS-polyacrylamide (12% [w/v]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from HEp-2 monolayers incubated with 10^8 EPEC strain 2036-80 (Lane 1), 10^8 EPEC strain 2036-80 and 1 *m*M GDPβS (lane 2). Arrow heads indicate P21 and P29. Molecular weight markers are as described in **Figure 7.1.**

while treatment with A23187 for the same time showed no increase above the controls. When monolayers were treated with both TPA and A23187 for 1 hour a massive decrease in the plasma membrane associated PKC activity was observed (**Figure 7.10**). An indication of the mechanism involved in this dramatic depletion of membrane PKC activity came from the observation that both leupeptin and the anti-rejection drug cyclosporin A inhibited this process (**Figure 7.10**). These agents are known to inhibit the calcium activated neutral endoproteinase calpain which has PKC as one of its substrates. The results show that elevated calcium possibly by activating calpain, causes removal of PKC activity from the membrane leaving a membrane depleted of PKC. EPEC infected HEp-2 cells mimicked the effect seen with TPA and A23187, showing depleted PKC in the Triton soluble membrane fraction of infected cells compared to uninfected cells (**Figure 7.10**). The EPEC-induced depletion of membrane associated PKC activity was also inhibited by the addition of leupeptin or cyclosporin A showing that the previously detected calcium elevation activates calpain. Diffuse adhering strains on the other hand caused no depletion in the PKC activity of the membrane. While the aggregative strains showed a similar decrease to that seen with EPEC strains (**Figure 7.10**).

7:3:7. Characterisation of the P21 phosphoprotein. Attaching and effacing EPEC mediate the phosphorylation of several HEp-2 proteins, the most prominent of which is a diffuse, heavily labeled, species of about 21 KDa. The diffuse nature of the band suggests that it could represent a glycoprotein, a multiple band of dissimilar proteins, or one protein with different degrees of phosphorylation. Initial 2-dimensional PAGE in which the first dimension resolved molecules by size, and the second by pH_i (pH of the proteins isoelectric point, i.e. the pH of the protein) were not clear, but nevertheless showed two phosphoproteins of size 21 KDa. Later 2D gels carried out on similar samples from EPEC and TPA treated cells confirmed the presence of at least two P_{21} phosphoproteins with pH_i values of 3.5 to 4.0 (**Figure 7.11**).

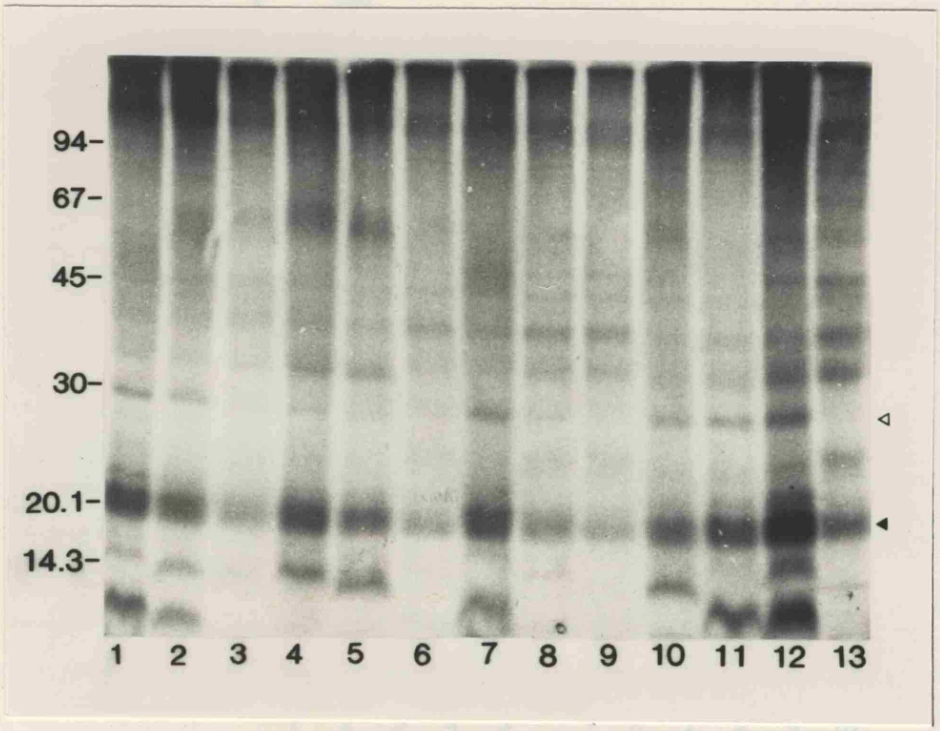
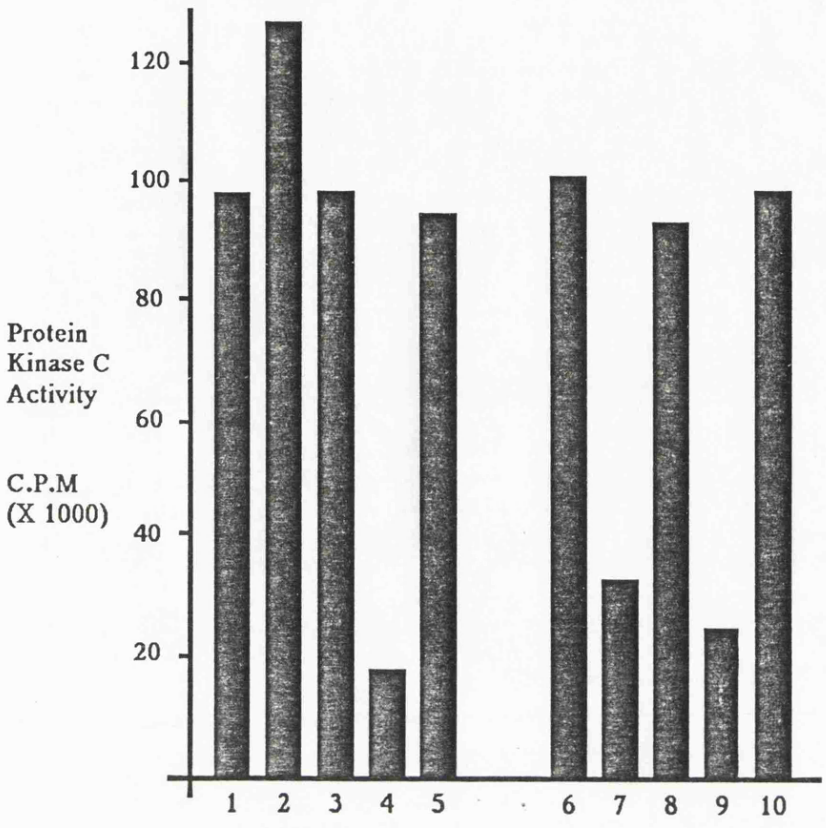


Figure 7.9. Screening of EPEC isolates for ability to activate PKC in HEp-2 monolayers. SDS-polyacrylamide (12% [w/v]) gel electrophoresis of ^{32}P-labeled total cell phosphoproteins prepared from HEp-2 monolayers infected for 3 h with 10^8 EPEC serogrouped, FAS test positive clinical isolates 2036-80 (lane 1), E2348-69 (lane 2), E1621-1 (lane 4), E57107 (lane 5), E57106 (lane 7), E851 (lane 10), BCH2/82 (lane 11), and 182/83 (lane 12). HEp-2 cells were also infected with 10^8 FAS test-negative clical isolates DPO95 (lane 3), 135 (lane 6), and 469-3 (lane 9). Lanes 13 and 8 are phosphoprotein profiles from uninfected HEp-2 monolayers and monolayers infected with 10^8 HB101(pMAR15) for 3 h. Arrow heads indicate P21 and P29. Molecular weight markers are as described in **Figure 7.1.**



KEY

1) HEp-2 control
2) TPA 1 hour
3) A23187 1 hour
4) TPA + A23187 1 hour
5) TPA + A23187 + cyclosporin 1 hour
6) HB101
7) 2036-80
8) 2036-80 + cyclosporin
9) E2348
10) 135

Figure 7.10. Location of PKC activity in EPEC infected HEp-2 cells. The Triton soluble membrane fractions of HEp-2 monolayers treated with EPEC strains or protein kinase activators were assayed for PKC activity by ^{32}P incorporation into HI histone. Lane 1,

Through collaborative work with Dr. Alistair Aitken at NIMR, Mill Hill, it has been possible to further characterize and purify this protein with the aim of obtaining sequence data that can be used to identify the protein by comparison with sequences in data bases. To date this work has shown that P_{21} is a cytoplasmic protein phosphorylated on threonine residues, with no indication of any phosphoserine or phosphotyrosine. On native gels P_{21} is associated (in the phosphorylated form) with a complex 90-100 KDa in size, which dissociates upon treatment with guanidiniumchloride or when the complex is subsequently run on SDS gels, giving the characteristic 21 KDa phosphoprotein. There is some evidence of another minor phosphoprotein of 40-45 KDa associated with the complex; whether this is one of the other proteins seen during EPEC infection is not unknown. Attempts at sequencing have been hindered by low protein yields and by the fact that initial sequencing attempts revealed that the protein was blocked at the N-terminal end. Recently P_{29} has been found to be a membrane associated protein, and attempts to obtain sequence data is presently underway.

7:3:8. EPEC induced PKC activation in the tissue culture cell line CaCo-2. CaCo-2 cell monolayers maintained in growth medium containing 10% FCS for 7 days to allow differentiation of cells, and infected with EPEC or treated with TPA or okadaic acid, gave similar phosphoprotein profiles to that seen in HEp-2 cells (**Table 7.1**). The major phosphoprotein species in each case had molecular weights of 21,000 and 29,000, however with the two EPEC strains 2036-80 and E2348-69 an additional band of 12,000 molecular weight was apparent. Interestingly, infection of CaCo-2 cells with the DA strain 135 induced phosphorylation of a protein similar in size to P_{21}. Since this strain was previously unable to induce phosphorylation in HEp-2 cells it is likely that receptors for a toxin component of strain 135 are present in CaCo-2 cells but absent in HEp-2 cells. When the length of time that the CaCo-2 monolayers were allowed to differentiate was increased from 7 to 14 days, those infected with EPEC gave the same profiles as before (**Figure 7.12**), whereas monolayers treated with TPA or okadaic acid

Figure 7.11. Two dimensional (2D) polyacrylamide gel electrophoresis of ^{32}P-labeled total-cell proteins from HEp-2 monolayers infected with EPEC strain 2036-80. **(A)** Coomassie stained 2D gel showing two distinct acidic proteins of molecular weight about 21,000. **(B)** Autoradiograph of 2D gel of ^{32}P-labeled proteins prepared from HEp-2 monolayers infected with EPEC strain 2036-80. 21,000 molecular weight acidic proteins are heavily labeled with ^{32}P, indicated by star.

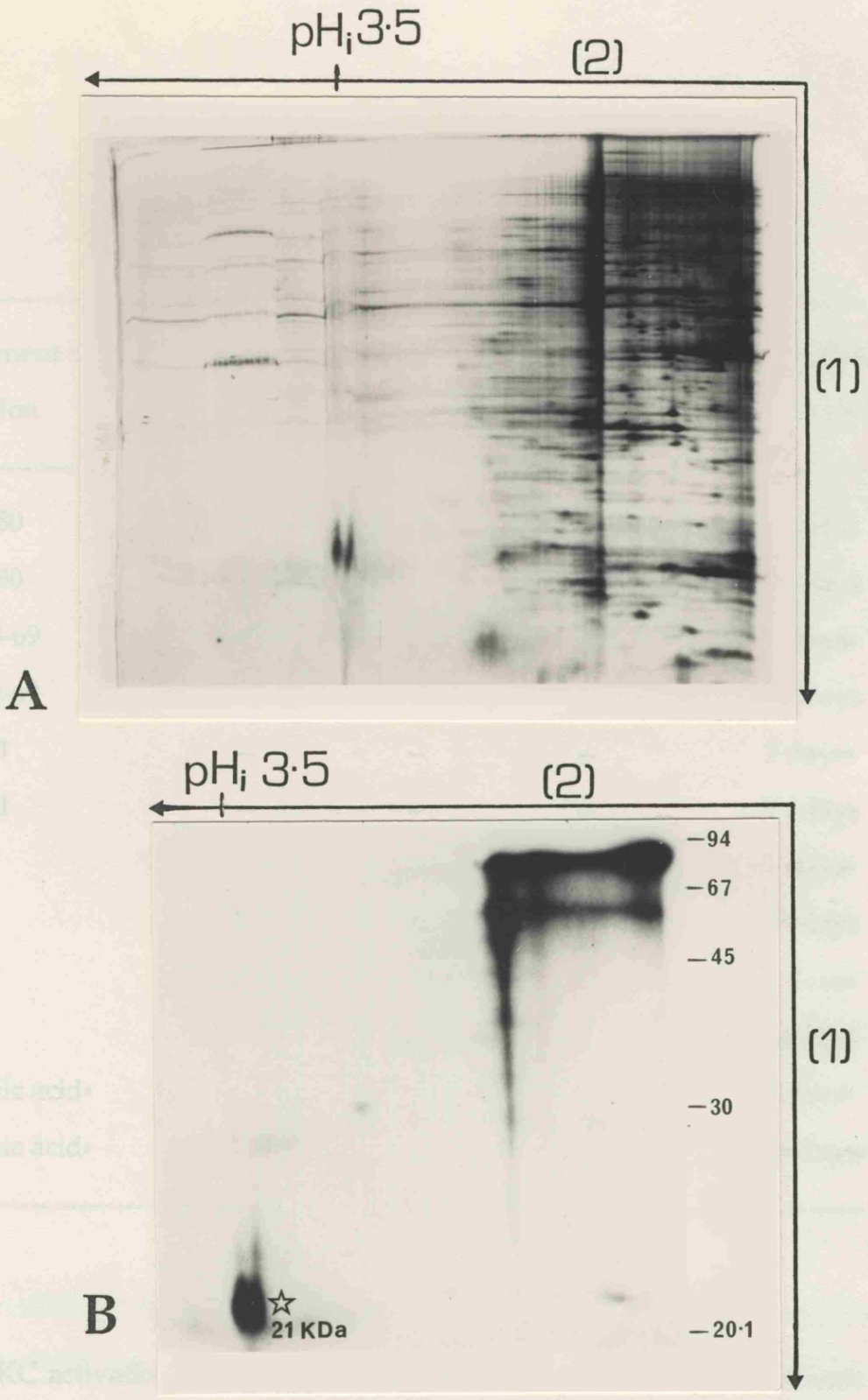


Figure 7.11. Two dimensional gel electrophoresis of ^{32}P-labeled total-cell proteins from HEp-2 monolayers infected with EPEC strain 2036-80.

Treatment or infection	PHOSPHOPROTEINS 29,000	21,000	12,000	Days in in GM
2036-80	++	++	+	7 days[a]
2036-80	+++	+++	+	14 days
E2348-69	+++	++++	+++	7 days[a]
E2348-69	++++	+++++	++++	14 days
HB101	--	--	--	7 days[a]
HB101	--	--	--	14 days
135	-/+	++	-/+	7 days[a]
135	+	+++	+	14 days
TPA[a]	+++	+++	--	7 days[a]
TPA[a]	--	--	--	14 days
okadaic acid[a]	+++	+++	--	7 days[a]
okadaic acid[a]	--	--	--	14 days[a]

Table 7.1. PKC activation in CaCo-2 monolayers in response to infection with *E. coli* strains and treatment with known inducers of PKC activity. [a] Data provided by Angel Hernandez of NIMR, Mill Hill, London.

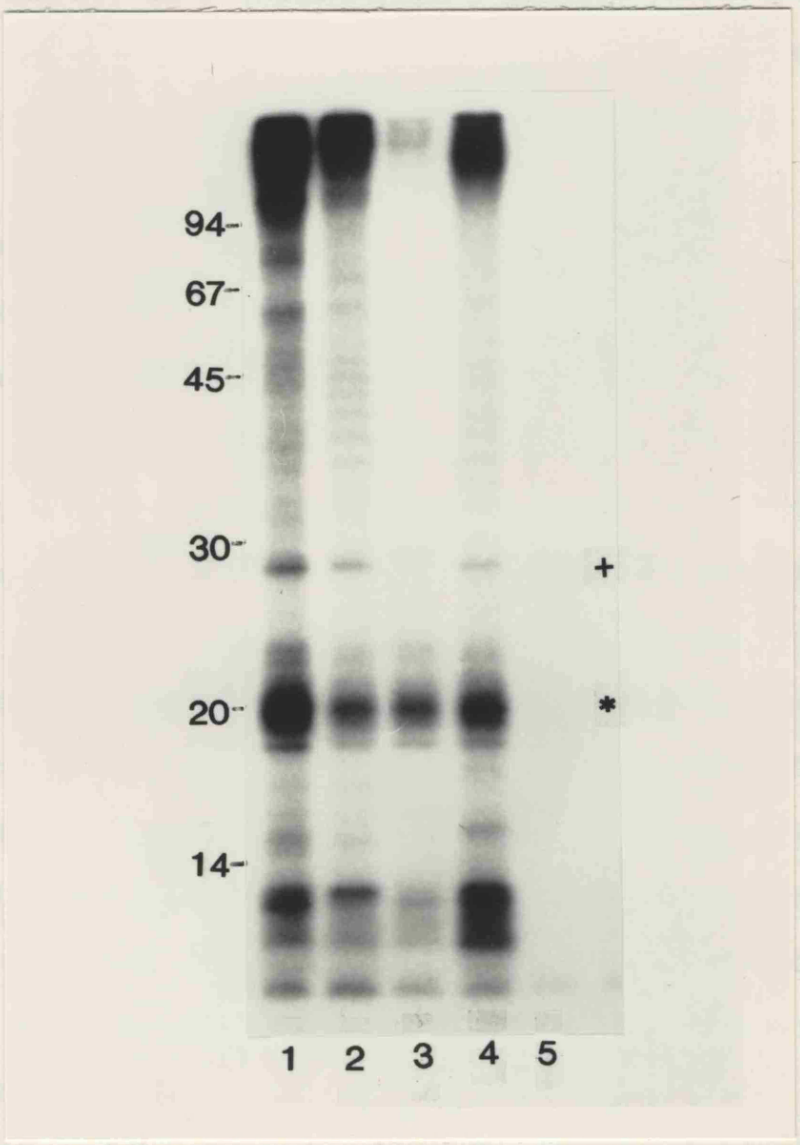


Figure 7.12. Activation of PKC in CaCo-2 monolayers infected with *E. coli* isolates for 2 h. Monolayers were incubated in growth medium for 14 days once confluence was reached, to allow differentiation of cells. SDS-polyacrylamide (12% [w/v]) gel electrophoresis of ^{32}P-labeled total-cell phosphoproteins prepared from CaCo-2 monolayers infected with (lane 1), E2348-69 (lane 2) 135, (lane 3) HB101(pMAR15), (lane 4) 2036-80, (lane 5) filtered culture supernatants from 2036-80. Arrow heads indicate P_{12}, P_{21} and P_{29}. Molecular weight markers are as described in **Figure 7.1.**

gave completely different phosphoprotein profiles (**Table 7.1**). Monolayers infected with EPEC strains 2036-80 and E2348-69 showed phosphorylation of protein species with molecular weights of 12,000, 21,000 and 29,000, whereas monolayers treated with TPA or okadaic acid showed no additional protein phosphorylation above background (**Table 7.1**). In all conditions the laboratory strain HB101 containing the EPEC adherence plasmid pMAR15 did not increase protein phosphorylation.

7:4. *Conclusions.*

EPEC isolates 2036-80 and E2348 and the nonlesion-forming AA strains JPN10 and 136 activated a cellular protein kinase, causing phosphorylation of a number of protein species in HEp-2 cells. The nonlesion forming DA strains 135, DPO95 and PES25, however, showed no evidence of protein kinase activity above background. The protein kinase activity associated with HEp-2 cells infected with EPEC was shown to be independent of the cyclic nucleotides cAMP and cGMP. Elevated cytosolic calcium levels, mediated by the calcium ionophore A23187, caused the phosphorylation of proteins corresponding in size to a number of the minor phosphoproteins identified during EPEC infection. The phosphorylation of the major protein species (P_{21} and P_{29}) were unaffected by this treatment suggesting the activation of two protein kinases in EPEC infected cells. Since elevation of cytosolic calcium activates calmodulin it is postulated that one of these kinases induced during EPEC infection is a calmodulin dependent protein kinase.

The mediator of the major phosphorylation events was identified as PKC, a protein kinase which is characteristically activated in response to cell stimulation resulting from the break down of PI lipids in the membrane by PLC, and the formation of DAG. EPEC infection of HEp-2 cells was accompanied by the phosphorylation of 11 protein substrates, of which the two major species (P_{21} and P_{29}) were located in the cytoplasmic and membrane fractions of cell sonicates respectively. Twelve EPEC isolates known to form the AE lesion all caused similar phosphorylation of proteins including P_{21}, and P_{29}, indicating activation of PKC.

Phosphorylation events were not mediated by a soluble toxin in EPEC strains, since culture supernatant filtrates had no effect on the phosphorylation state of HEp-2 proteins. Filtrates from AG strains on the other hand were shown to contain a soluble factor which promoted identical phosphoprotein profiles to those observed with whole cultures. The putative toxin of AA strains was inactivated by heating 70°C for 10 min, suggesting that it is protein in nature. The fact that both EPEC and AG strains of *E. coli* mediate PKC activation in host cells (albeit by different mechanisms) appears to endorse the view that PKC activity is not specific to lesion formation, but rather may simply correlate with the ability of the organism to cause secretory diarrhoea (enterovirulence). It is generally accepted that AA strains of *E. coli* are enteropathogens with a diarrhoeagenic mechanism distinct from ETEC, EIEC, and EHEC (**Levine, 1987**), (unlike DA isolates for which there is no evidence for enterovirulence); AA strains are also assumed to have an alternative mechanism from EPEC since they do not form an attaching the effacing lesion. Here I show however that these two groups of diarrhoeagenic *E. coli* isolates activate the same protein kinase in target host cells by apparently distinct mechanisms, EPEC via intimate adherence, and AA strains through a soluble proteinaceous toxin. It seems possible that PKC activity in infected cells mediates the diarrhoea process by the phosphorylation of ion transport proteins in the plasma membrane.

PKC activity could be detected in EPEC infected cells after 1 hour, and showed no sign of decrease even after 4 hours, a comparable time scale to the observed calcium increases and actin accumulation discussed in previous chapters. The viability of EPEC at the time of infection was also essential; no PKC activity could be detected in cells infected with killed bacteria, even after prolonged incubation. This may relate to the inability of dead bacteria to make intimate contact with the plasma membrane. Inoculum size had little effect on the time course of PKC activation, bacterial numbers sufficient for 20 or 90% infection of HEP-2 cells as determined by the number of cells supporting detectable microcolonies after 3 h, gave similar degrees of protein phosphorylation. This is interpreted as PKC induction in individual cells requiring the

adherence of only 1 or 2 bacteria, which are virtually undetectable using normal HEp-2 adherence assays. With the more sensitive FAS test (**Chapter 2**) individual attached bacteria can be visualized and be seen to have formed an active lesion. As discussed previously, lesion formation results in the calcium dependent redistribution of actin, which in turn is mediated by the PLC-specific hydrolysis of PI lipid and the formation of second messengers. Therefore it seems reasonable to assume that if individual attached bacteria cause cytoskeletal rearrangements they will also activate PKC through DAG formation. Therefore, although only 20% of the cells will have detectable microcolonies, a large proportion of the remaining cells may have one or two attached bacteria capable of activating PKC.

Many routes may lead to PKC activation in cells. A number of hormones and growth factors have been found to activate PKC by binding to PLC coupled receptors (**Berridge and Irvine, 1984; Nishizuka, 1986**). Several bacterial and fungal toxins resemble PLC and may promote PKC activity in treated cells (**Ikezawa et al., 1976; Imamura and Horiuti, 1979; Berka and Vasil, 1982; Vasil et al., 1982**). Even the lipd A component of bacterial LPS has been shown to stimulate PKC *in vitro* by substituting for phosphatidylserine (**Ellis et al., 1987**). The effect of a number of potential PKC stimulating agents on HEp-2 cells was investigated in order to assess the likely involvement of each in the phosphorylation events observed with EPEC. Cells treated with the hormone angiotensin II or a PLC bacterial toxin gave identical phosphoprotein profiles to those seen with TPA or EPEC infection. These observations revealed that HEp-2 cells are able to be stimulated both by hormone induced signal transduction pathways and the external hydrolysis of membrane PI lipids. In each case the time course of PKC stimulation was comparable to that seen with EPEC. This was not the case, however, with the DAG derivative phosphatidic acid (PA) and bacterial LPS. Both gave identical phosphorylation patterns as compared to EPEC infected cells (indicating PKC stimulation), but on vastly different time scales. PA treated HEp-2 cells showed a greater level of protein phosphorylation in 15 min than EPEC infection induced in 3 h.

On the other hand, high concentrations of bacterial LPS took 4 to 5 h to induce the same level of protein phosphorylation as seen at EPEC infection times of 2 h.

The structure of PA (a DAG moeity with an additional phosphate group attached) would seem to suggest both a hydrophilic and a hydrophobic character. The former would seem to exclude it from the hydrophobic membrane environment, while the latter would promote membrane association. This seemed to be confirmed by the observation that aqueous solutions of PA readily form stable emulsions, presumably resulting from micelle formation. PA added to cells may be incorporated into the cell membrane where it could be dephosphorylated to yield DAG or have other effects on the cell which would result in PKC stimulation (**Moolenaar et al., 1984**). It has been previously noted that addition of PA to artificial membrane bilayers promotes major disturbances in the membrane structure (**Eklund et al., 1988**), which in the plasma membrane of cells has been speculated to promote PLC mediated PIP_2 hydrolysis, resulting in the mobilization of calcium and activation of PKC. As stated above, PA formation results from the PLD specific hydrolysis of PI lipids, or from the phosphorylation of DAG formed by the action of PLC.

Activation of host cell PLC can be inhibited by GDPβS, which binds to and inactivates the G-protein coupling receptor to PLC. Phosphorylation of P_{21} and P_{29} in EPEC infected cells was certainly lowered but not fully inhibited by GDPβS as would have been expected if the mechanism involved susceptible signal transduction pathways. An explanation for the incomplete inhibition observed could be that the cells had not taken up enough GDPβS to inhibit all pathways and therefore would required longer pre-incubation with a greater concentration of GDPβS to give conclusive results. However the fact that phosphorylation was diminished slightly is considered to be an indication that a signal transduction pathway is involved.

Cells maintained in low serum medium for varying times showed slightly different patterns of protein phosphorylation, with some phosphoproteins (P_{15} and P_{19}) only evident in quiescent cells, while others (P_{21} and P_{29}) were present under all conditions (at longer times in low serum the levels of P_{21} and P_{29} was reduced). From

studies with purified P_{21} it appears that P_{15} and P_{19} are products of P_{21} proteolytic cleavage, since purified P_{21} stored for any length of time was partially degraded to give two additional protein bands on polyacrylamide gels of similar size to P_{15} and P_{19} **(Alistair Aitkin, personal communication)**. This finding suggests that either a protease is co-purified with P_{21} (which seems unlikely since the unusual properties of P_{21} are used for its purification, and no additional protein bands can be seen on polyacrylamide gels), or that P_{21} has an endogenous protease activity under particular conditions. In cells maintained in low serum for times greater than 40 hours this protease activity seems to be induced, forming P_{15} and P_{19} from P_{21}, both of which remain PKC substrates. This may also explain why P_{21} is less prominent in quiescent cells compared to those which are slowly growing. These are areas of research are being continued by the group at Mill Hill.

The function (if any) of these proteins during the development of diarrhoea is uncertain at present; moreover the fact that HEp-2 cells bear little resemblance to the normal host tissue of EPEC, the distal intestinal mucosa, may cause problems in interpreting the phosphorylation results in terms of function during disease. For this reason experiments were initiated using the colon derived cell line CaCo-2 which bears greater similarity to the natural host cell of EPEC by producing microvilli at confluence. EPEC infection of CaCo-2 cells maintained in growth medium containing 10% FCS for 7 days (which is not long enough to facilitate full differentiationof cells in the monolayer) showed phosphorylation of identical proteins to P_{21} and P_{29} of HEp-2 cells. Incubation of confluent monolayers for 14 days in medium containing 10% FCS considerably reduced the number of phosphoproteins present in the profiles but still revealed the presence of the characteristic P_{21} and P_{29} phosphoproteins. When, however CaCo-2 monolayers of different degrees of differentiation (ie. 7 days and 14 days in growth medium post-confluence) were treated with TPA, a known activator of PKC, major differences between the phosphoprotein profiles were seen. Cells maintained in growth medium for 7 days and then treated with TPA, stimulated the phosphorylation of a number of proteins including P_{21} and P_{29}. In contrast the same experiment carried out

with fully differentiated monolayers showed no increase above background for any protein. This suggests that kinase expression may be dependent on the growth or differentiation state of the cells. It is possible that in undifferentiated cells, PKC mediates the phosphorylation events observed, while in differentiated cells a protein kinase with similar substrate specificities (ie. P_{21} and P_{29}) is responsible for the observed phosphorylation events. Since EPEC have been shown to cause major cytoskeletal actin rearrangements it seems likely that other cytoskeletal proteins, such as myosin will also be involved. Preliminary evidence from 2D gels, size and physical characteristics, suggests that P_{21} may be myosin light chains which is a substrate for both PKC and the calcium activated myosin light chain kinase. Therefore I propose that in mature enterocytes myosin light chain kinase is initially activated in the microvillus by the elevation in calcium which causes the phosphorylation of myosin light chains thus aiding the dissolution of the actin cytoskeleton and effacement of the microvilli. Subsequently attachment of EPEC to the body of the cell may cause a secondary activation of PKC which mediates electrolyte efflux and diarrhoea.

From the observation that a rise in calcium accompanies EPEC infection, it was predicted that PKC activity should be removed from the membrane to the cytoplasm as a result of calcium activation of the neutral endoproteinase calpain. When monolayers were treated with both TPA and A23187 for one hour to activate PKC and elevate intracellular calcium levels, a massive decrease in the plasma membrane associated PKC activity was observed. This effect was inhibited by leupeptin and cyclosporin A, both inhibitors of calpain (**vonRuecker et al., 1988**). An identical response seen during EPEC infection confirmed that calpain is activated in infected cells. Calpain cleavage of membrane associated PKC and the release of the catalytic fragment into the cytoplasm as active PKM possibly explains the phosphorylation of cytoplasmic proteins such as P_{21} during EPEC infection.

2D gels carried out on samples from EPEC and TPA treated HEp-2 cells revealed that there were at least two P_{21} phosphoproteins with pH_i values of 3.5 to 4.0. The extremely acidic nature of P_{21} may be a consequence of a high degree of

phosphorylation rather than an endogenous characteristic of the unmodified protein alone, an idea which can be verified once the protein has been purified and antibodies have been raised for immunoblotting. The P_{21} doublet as shown by 2D gel electrophoresis may be the result of different degrees of phosphorylation of the one protein rather than the existence of two distinct proteins of roughly the same size and characteristics. The former explanation seems the most likely since phosphoamino acid analysis of the two proteins showed that only phosphothreonine was present.

The activation of PKC during EPEC infection has important potential consequences on the cell. Compounds such as phorbol esters which are known to activate PKC are also powerful tumourigenic agents, suggesting a role for PKC in the control of cell transformation possibly through activation of cellular oncogenes. PKC has been implicated in the expression of certain oncogenes and the modification of several oncogene products, in addition cells transformed by Ha-*ras* shows elevated levels of DAG and PKC activity (**Lacal et al., 1987**). The real possibility that EPEC infection may result in the activation of cellular oncogenes is a cause for concern especially in prolonged infections which are common in infants, as prolonged exposure to active phorbol esters in known to promote transformation. It seems unlikely that mature differentiated villus enterocytes are capable of full transformation. However colonization of the crypts during prolonged infection may cause activation of PKC in enterocyte progenitor cells giving the potential for transformation in these cells and genetic change through activated oncogenes or their products.

Chapter 8. ADHESION OF EPEC TO HEp-2 CELL MONOLAYERS.

8:1. *Introduction.*

The onset of EPEC mediated diarrhoea is dependent on the successful bacterial colonisation of the gut mucosal epithelium (**Sherman et al., 1989**). As described in chapter 1, this is greatly enhance in most EPEC strains by the presence of a plasmid encoded putative fimbrial type adhesin, termed EPEC adherence factor (EAF). Effective screening of clinical isolates for gut attachment is hampered, however, by difficulties in obtaining healthy gut tissue of human origin, which has led to the development of an *in vitro* model of bacterial attachment using the tissue culture cell line HEp-2 (**Cravioto, 1979**). It has been reported that most if not all attaching and effacing *E. coli* isolates adhere in a localized fashion to cells in HEp-2 monolayers (**Nataro et al., 1987a; Nataro et al., 1987b; Tavendale and Old, 1986**), and contain DNA sequences that hybridise with the 1 Kb EAF DNA probe derived from the plasmid pMAR15. As described in Chapter 1, pMAR15 was constructed from pMAR7 (**Baldini et al., 1986**), a Tn*801* tagged derivative of pMAR2 (**Appendix 3**). Tn*801* itself was obtained from pMR5, a temperature sensitive replication mutant of RP1 (**Nataro et al., 1987b**). Note the two ampicillin resistance plasmids RP1 and RP4, which reportedly contain the transposons Tn*801* and Tn*1* respectively are in fact the same plasmid (**Muster et al., 1983**), and therefore Tn*801* and Tn*1* are identical. Thus the availability of extensive restriction maps of Tn*1* allow comparisons of pMAR15 and the parent pMAR2 to be made, enabling the location and orientation of Tn*801* to be determined. The published maps of the restriction endonuclease sites contained within pMAR15 (**Baldini et al., 1986; Nataro et al., 1987b**) however give information for only three enzymes, which is insufficient for reliable identification of the EAF region and structural analysis of the pMAR15 construct. Therefore detailed restriction mapping using the seven restriction enzymes *Bam*H1, *Eco*R1, *Hind*III, *Kpn*1, *Pvu*II, *Sal*1, and *Xho*1 was considered important for a more complete understanding of these plasmids.

The expression of many fimbrial type adhesins is temperature regulated (**Goransson and Uhlin, 1984; Eisenstein, 1988**); adhesion to target cells occurs at 37°C but is abolished by bacterial growth at lower temperatures. For a number of adhesins, regulation has been determined to occur at the transcriptional level (**Goransson and Uhlin, 1984; Williams and Hinson, 1987**). Whether the adhesin responsible for localized adherence of EPEC is temperature regulated is unknown.

Since the proposal that the EPEC adhesin responsible for LA to HEp-2 cells was not fimbrial in nature (**Scotland and Rowe, 1983**), structures similar to recognised fimbriae have been observed, aparrently mediating adherence of bacteria to brush border cells in gut biopsies infected with EPEC (**Knutton et al., 1987b**). This and the observations that initial colonisation of target cells is nonintimate (**Knutton et al., 1987a; Knutton et al., 1987b**), and that transfere of pMAR15 into the normally nonfinbriated laboratory strain HB101 results in the expression of LA and nonintimate adherence to HEp-2 cells (**Baldini et al., 1986; Knutton et al., 1987a**), suggests that adherence of EPEC is in the first instance mediated by fimbrial like structures

8:2. *Materials and Methods.*

8:2:1. Adherence of bacteria to HEp-2 cells. HEp-2 cells were grown to 50% confluence on sterile 12 mm^2 cover slips in DMEM containing 10% FCS, and 0.5% D-mannose. They were infected with 10^8 bacterial cells for 1 hour at 37°C, washed 3 times in PBS, and incubation was continued for either 2 or 5 hours in fresh DMEM with 10% FCS and 0.5% D-mannose. Coverslips were washed twice in PBS (37°C) with agitation, fixed for 10 min in 70% methanol, and stained for 20 min in 10% Giemsa. Excess stain was removed by repeated washing in distilled water and coverslips air dried and mounted on slides in DPX resin. HEp-2 adherence patterns were observed and photographed.

8:2:2. Visualisation of fimbriae by negative staining. Electron microscopy was carried out at the Institute of Child Health, Birmingham, under the expert supervision of S.

Knutton. 10 µl bacterial cultures (grown without shaking overnight at 37°C) in 1.5 ml microfuge tubes were gently pelleted by centrifugation and washed twice in sterile PBS. The pellet was finally resuspended in 0.5 ml of water containing 150 µg/ml of the wetting agent bacitracin and 2% ammonium molybdate *p*H 7.0. A total of 10 µl was applied to carbon-coated copper grids for 2 min and the excess removed with filter paper. Specimens were air dried and examined by transmission electon microscopy. To prepare copper grids, dust-free glass slides were flooded with a 0.25% solution of formvar in ethyl acetate, excess solution was tipped off and the celoidin film dried under a 40 W lamp for 10 min. The dry celoidin film was scored with a sharp scalpel and floated off onto the surface of dust-free water. Copper grids were placed on the film, which was removed from the water surface onto 3 MM paper. Once dry the copper grids were carbon coated and the formvar membrane removed by placing the grids onto 3 MM paper soaked in ethyl acetate, they were then ready for use.

8:2:3. Plasmid DNA preparation. Plasmid pBR322 in strain HB101 was obtained from E. Griffiths (Department of Bacteriology, National Institute for Biological Standards and Control [NIBSC], South Mimms, Herts.). A restriction map of the cloning vector pBR322 is presented in **appendix 2.**

Protocols for bulk and small scale preparations of large (>30 kb) low copy number and small multi-copy plasmids were adapted from the methods of **Birnboim and Doly (1979)** and **Ish-Horowicz and Burke (1981).**

Large-scale preparation of multi-copy plasmid DNA. A 10 ml LUB overnight culture of the plasmid carrying strain was added as inoculum to a 5 l flask containing 2 l of LUB-glucose with appropriate antibiotics and incubated at 37°C with shaking until the absorbance at 600 nm (A_{600}) reached 0.5. 10 ml of a stock chloramphenicol solution (34 mg/ml in ethanol) was added to a final concentration of 170 µg/ml, and incubation continued at 37°C for a further 16 h. Bacterial cells were harvested by centrifugation (Du Pont Sorval GS3 rotor at 9,000 rpm for 15 min at 4°C), resuspended in 24 ml of

TEG-lysozyme buffer (25 mM Tris. HCl, *p*H 8.0, 10 mM EDTA, 50 mM glucose, 0.1 mg/ml lysozyme), and incubated on ice for 30 min. 32 ml of lysis solution (0.2 *N* NaOH, 1% SDS) were added, mixed, and incubated for a further 5-10 min on ice (or until culture became opalescent), followed by 24 ml of cold potassium acetate (3 M K^+, 5 M $acetate^-$, *p*H 4.8) with thorough mixing, and tubes returned to ice for 1 h. The precipitate was pelleted by centrifugation (Du Pont Sorval GS3 rotor at 9,000 rpm for 20 min at 4°C) and supernatants decanted into 3 polypropylene centrifuge tubes (approximately 20 ml/tube). DNA was precipitated by adding an equal volume of pre-cooled propan-2-ol and incubating at -20°C for at least 15 min, and pelleted by centrifugation (Du Pont Sorval HB4 rotor at 12,000 rpm for 20 min at 4°C). DNA pellets were rinsed twice in 70% ethanol (-20°C), drained, vacuum dried, and each resuspended in 5 ml sterile TE buffer (10 mM Tris. HCl, *p*H 7.5, 1 mM EDTA) at room temperature. 1 g of CsCl and 0.05 ml of ethidium bromide stock solution (10 mg/ml) were added to each ml of DNA solution, and thoroughly mixed to dissolve CsCl. The solutions were transferred to Beckman Quickseal tubes and ultracentrifuged (Beckman L5-65 ultracentrifuge and Ti75 rotor) at 68,000 rpm for 16 h at 18°C. Plasmid bands were visible in daylight as red bands and fluoresced under UV light; they were removed to 20 ml sterile plastic universal bottles (Sterilin) using a 1 ml syringe and large bore needle passed down the side of the tube from the top. Ethidium bromide was removed from the gradient fractions by repeated extraction with CsCl saturated propan-2-ol, and the DNA was precipitated by adding 2 volumes of sterile distilled water, then two volumes of absolute ethanol, and incubating at -20°C for 10 min. DNA was pelleted by centrifugation in an HB4 rotor as above, and precipitates washed with 70% ethanol, drained, vacuum dried and resuspended in 1 ml of TE buffer. DNA purity and yield were assessed by agarose gel electrophoresis and restriction analysis. Stock DNA solutions were maintained at 4°C and -20°C.

Large-scale preparation of large plasmids. The procedure used for bulk isolation of plasmids greater than 30 kb, was essentially the same as above, except chloramphenicol amplification was omitted and all manipulations were undertaken with extreme care to

avoid shearing large DNA molecules. A phenol/chloroform extraction step in phenol resistant tubes (Oak Ridge) was included just prior to the final propan-2-ol precipitation. Phenol/chloroform mix (50% w/v phenol, 2% v/v Iso-amyl alcohol, 0.05% w/v 8-hydroxyquinoline in chloroform) was added in equal volumes to the supernatant and mixed gently until homogeneous. Phases were allowed to separate at 4°C, and the upper aqueous phase removed to an Oak Ridge tube containing an equal volume of chloroform. Phenol was removed into the chloroform phase by gently mixing until homogeneous, then allowing phases to separate, and removing the upper aqueous layer to another Oak Ridge tube containing an equal volume of diethylether. The phases were mixed and allowed to settle; the lower aqueous layer was removed to a fresh tube, propan-2-ol precipitated and CsCl density gradient purified as described above.

Small-scale plasmid preparation. For mini-plasmid preparation of both large and small plasmids the procedure was essentially a scaled down version of the large scale preparation. Bacteria pelleted in microfuge tubes (Eppendorf) from 2 ml of a 10 ml overnight LUB-glucose culture were resuspended in 24 µl of TEG-lysozyme buffer, incubated on ice for 30 min, and lysed by adding 32 µl of lysis solution. Incubation on ice was continued for a further 5 min with occasional gentle mixing, or until cells were fully lysed. 12 µl of cold potassium acetate solution was added, thoroughly mixed, incubated on ice for 1 h, and the precipitate pelleted by centrifugation (Microcentaur Microfuge at 16,000 rpm for 10 min at room temperature). Plasmid DNA intended for restriction enzyme analysis was then phenol/chloroform, chloroform, and diethylether extracted before precipitation of DNA with an equal volume of cold propan-2-ol at -20°C for 20 min. Precipitates were collected by centrifugation (Microcentaur Microfuge at 16,000 rpm for 20 min at room temperature), pellets were washed in 70% ethanol, drained, vacuum dried and resuspended in 20 µl of TE buffer.

8:2:4. Restriction endonuclease digestion. For single enzyme digestions, reaction mixtures contained 0.1 to 1 unit (U) of enzyme (BRL and Anglian) and 1 µg of DNA in 1

x "react" buffer (BRL, specific for the enzyme). Incubation in microfuge tubes at the recommended temperature (usually 37°C) was for 1 h. The enzyme reaction was stopped by incubation at 65°C for 10 min, followed on occasion by elutip extraction (see section 8:2:7) and propan-2-ol precipitation. For double digests, high, medium and low salt buffers were used as suggested (**Maniatis et al., 1982**), and incubation at the recommended temperature extended to 1.5 h. Restriction fragments were analysed by agarose gel electrophoresis.

8:2:5. Agarose gel electrophoresis. Agarose (FMC SeaKem HGT) was dissolved to 0.4-2% (w/v) in TAE buffer (40 mM Tris, 2 mM EDTA, adjusted to *p*H 7.4 with glacial acetic acid) using a microwave oven. Ethidium bromide was added from a 10 mg/ml stock solution to give a final concentration of 0.01 μg/ml. The agarose solution was allowed to cool to hand hot before casting gels of sizes depending on the dimensions of the glass plate mould used). Gel moulds were made by placing tape along the edges of glass plates to give a water tight container and wells formed with a plastic comb suspended 1-2 mm above the glass on large paper clips. The edges of the gel mould were sealed with 10 ml of molten agarose pipetted around the bottom of the tape, which was allowed to fully set before casting the gel. The gel surface was flamed using a bunsen burner to remove any air bubbles and the gel allowed to set. The gel comb and the tape walls were carefully removed and the gel (on its glass support) placed in horizontal perspex electrophoresis tanks containing TAE buffer sufficient to cover gel by 2 mm. DNA samples were mixed with one-tenth volume of agarose loading buffer (50% v/v glycerol, 0.5% w/v bromophenol blue, in TAE buffer), and layered into the wells. Gels were electrophoresed at 1 V/cm overnight or 10 V/cm for 1-2 h (Kingshill 10A05C power supply), and examined using a short wavelength ultraviolet (UV) transluminator (Fotodyne) through an orange filter box. Gels were photographed with Kodak plus-Pan Professional film.

8:2:6. Electroelution of DNA from agarose gels. Required bands were identified on the gel and incisions made directly in front of the leading edge of each band using a sharp scalpel. Dialysis membrane attached to 3 MM paper (soaked in gel buffer) was inserted into the incision with the 3MM paper towards the DNA band, ensuring that no air bubbles were trapped. Electrophoresis was continued until the required band was captured on the 3 MM paper, which was withdrawn and placed in a 400 µl microfuge tube with a hole pierced in the bottom. The tube was placed inside a 1.5 ml microfuge tube and centrifuged for 15 sec, the eluate in the outer tube was collected and kept. 100 µl of elution buffer (50 mM Tris. HCl, *p*H 7.6, 200 mM NaCl, 1 mM EDTA, 1% SDS) were added and the eluted DNA collected in the outer tube by centrifugation for 15 sec. This process was repeated twice and all eluate fractions pooled; the DNA was propan-2-ol precipitated, washed in 70% ethanol, vacuum dried and resuspended in 10 µl TE.

8:2:7. Elutip purification of DNA. Elutip mini affinity columns (Anderman) were rinsed with 5 ml high salt buffer II (20 mM Tris. HCl, *p*H 7.4, 1 M NaCl, 1 mM EDTA) followed by 10 ml of low salt buffer I (20 mM Tris. HCl, *p*H 7.4, 200 mM NaCl, 1 mM EDTA). DNA solutions were made up to 2 ml with buffer I and slowly passed down the column (discarding the eluate). The column was washed with 5 ml of buffer I and the DNA eluted from the column in 0.5 ml of buffer II. The DNA was propan-2-ol precipitated, washed in 70% ethanol, vacuum dried and resuspended in 10 µl TE.

8:2:8. Dephosphorylation of DNA restriction fragments. Removal of the 5'-phosphate groups from DNA ends was performed by bacterial alkaline phosphatase (BAP; BRL). The DNA was propan-2-ol precipitated, washed in 70% ethanol, vacuum dried and resuspended in an equal volume of Tris buffer (*p*H 8.5). BAP was added at $6.1X10^{-3}$ U/µg of DNA and incubated at 68°C for 1 h. Removal of BAP was accomplished by electroelution of the DNA from an agarose gel or elutip extraction of the reaction mixture.

8:2:9. Ligation of DNA. Approximately 30 units of T4 DNA ligase (BRL) were added per μg of DNA in 1 x ligase buffer (5 x ligase buffer stock obtained form BRL) and incubated at 18°C for 16 h. Ligations were checked by agarose gel electrophoresis.

8:2:10. Preparation and transformation of competent cells. Methods were adapted from **Maniatis et al., 1982.** An overnight LUB-glucose culture of recipient bacteria was used as inoculum for 10 ml LUB-glucose pre-warmed to 37°C to give A_{600}= 0.06, incubation was continued at 37°C with shaking until mid log phase of growth was reached (A_{600}= 0.4). The culture was chilled on ice for 5 min and divided into 1.5 ml aliquots. Cells were harvested by centrifugation for 30 sec, resuspended in 1.5 ml of ice-cold 100 mM $MgCl_2$ and incubated on ice for 20 min. After centrifugation cells were resuspended in ice-cold 100 mM $CaCl_2$, incubated on ice for 20 min, harvested and resuspended in 1 ml lots of ice-cold 100 mM $CaCl_2$. Competent cells were maintained on ice for up to 1 h before transformation. DNA (0.1 to 1 μg) in 10 μl of TE and 3 μl of sterile DMSO were added to 200 μl of competent cells and incubated on ice for 30 min. The tubes were then heated at 45°C for 1 min, placed on ice for 2 min, followed by addition of 1 ml of LUB-glucose and incubation at 37°C for 1.5 h to allow expression of antibiotic resistance. Serial dilutions were made of transformed bacterial suspensions and 100 μl of the appropriate dilutions spread on selective LUA plates and incubated overnight at 37°C.

8:2:11. DNA/DNA Hybridisation. Circular nylon filters (Amersham Hybond-N) carefully placed on the surface of agar plates with colonies to be blotted were marked for orientation and left in contact with the colonies for 1 min. The filters were carefully removed and placed colony side up on 3 MM paper soaked with 0.5 M NaOH, 1.5 M NaCl for 7 min (to denature DNA) and neutralised for 3 min on 3 MM pads soaked in 0.5 M Tris. HCl, *p*H 7.2, 1.5 M NaCl, 1 mM EDTA. Neutralisation was repeated using 3 MM pads soaked in fresh solution for 3 min. Filters were then washed with brisk agitation in PBS to remove cell debris, and air dried at 37°C on 3 MM paper. DNA was

covalently cross-linked to the filter by short wave UV irradiation and filters stored at 4°C or used immediately for hybridisation.

Probe hybridization. Filters were pre-wetted by floating on the surface of the hybridization solution (0.5 M Na_2HPO_4 buffer, *p*H 7.2, containing 1 mM EDTA, 7% SDS). The fully wetted filters were pre-hybridized for 5 min at 65°c, in Perspex hybridisation tanks followed by addition of the radiolabeled probe and hybridization at 65°C overnight. Unbound probe was removed at 65°C by 3 x 5 min washes 40 mM Na_2HPO_4 buffer *p*H 7.2, containing 1% SDS), followed by 3 x 5 min washes in 0.1 x SSC (0.88 mg/ml NaCl, 440 μg/ml sodium citrate, adjusted to *p*H 7.0 with NaOH), containing 1% SDS. Radioactively labelled colonies were detected by autoradiography of the dried filters for 8-16 h using X-ray film (Kodak X-Omat S).

8:2:12. Purification and labelling probe fragments (adapted from, **Feinberg and Vogelstein, 1984**). Plasmids containing probe fragments were cleaved with appropriate restriction enzymes. The resulting fragments were separated on a 0.8% low melting point agarose gel containing 0.01% ethidium bromide. The probe fragment was identified by UV illumination and removed using a scalpel, weighed and sterile distilled water added at a ratio of 1.5 ml of water to 1 g of gel. The agarose gel slice was melted in a boiling water bath for 7 minutes and equilibrated to 37°C for 15 minutes before use or stored at -20°C until needed. Stock solutions were made up as follows. Solution A was 610 mM Tris. HCl, *p*H8.0, 61 mM $MgCl_2$, 1 μM 2-mercaptoethanol, 0.244 mM dCTP, 0.244 mM dTTP, 0.244 mM dGTP, this was stored at -20°C. Solution B was 2 M HEPES titrated to *p*H 6.6 with NaOH, and stored at 4°C. Solution C was Pharmacia hexadeoxyribonucleotides dissolved in 3 mM Tris. HCl, *p*H 7.0, 0.2 mM EDTA, to give a solution of 90 OD_{660} units/ml; this was stored at -20°C. Solutions A, B, and C were mixed in the ratio 2:5:3 to give "oligolabelling buffer" (OLB, [**Feinberg and Vogelstein, 1984**]) which was stored at -20°C for a maximum of 3 months. Bovine serum albumin was made as a 10 mg/ml solution and the Klenow fragment of DNA polymerase I was diluted to give a final concentration of 1 unit/ml. [$^{32}P_i$]-α-dATP (3000 Ci/mmol, 10

μCi/ml was obtained from Amersham. To 15-25 ng of DNA (adjusted to approximately 20 μl) was added, in the following order, 1.6 μl sterile distiled water, 2 μl OLB, 2.4 μl BSA, 0.5 μl ^{32}P-α-ATP, 1 μl klenow, and incubated either at 37^{0}C for 3 h, or at room temperature overnight. Labelled DNA was used directly in the hybridisation reactions.

8:3. *Results.*

8:3:1. Plasmid content of *E. coli* of EPEC serogroups. Plasmids are vectors for many virulence determinants, including toxins, antibiotic resistance, and adherence factors. Within the group of *E. coli* recognized as EPEC by serogroup, at least three distinct adhesins have been identified and at least two of these are carried on large plasmids (**Baldini et al., 1983a; Laporta et al., 1986; Benz and Schmidt, 1989**). Although there is no reason why large plasmids should be the sole repository for adhesin genes in the EPEC group, the size of most adhesin gene complexes dictates that a plasmid carrying genetic determinants for fimbrial attachment to eukaryote cells must be larger than about 15 kb (**Shipley et al., 1981; Willshaw et al., 1983; Baldini et al., 1986; Morrissey and Dougan, 1986**).

A small collection of 23 isolates known to adhere to Hep-2 cells by either LA, DA, or AA were screened for the presence of large plasmids using the small scale preparative protocol for plasmid DNA. Plasmid profiles revealed that all strains examined except 5582 (O128) contained large plasmids greater than 80 kb in size (**Figure 8.1**). In addition to the large low copy number plasmids the majority of strains looked at also possessed at least one small, multi-copy plasmid (**Figure 8.1**). Two strains (2036-80, and E12801) were estimated to contain 5 or more, with sizes in the range 2 to 20 kb. Six isolates (1923-77, 135, 136, 182-83, IQBAL(2), and JPN10), apparently contained no small plasmids, but only one strain was completely devoid of plasmid DNA.

Figure 8.1. Plasmid profiles of *E. coli* clinical isolates of EPEC serogroups. Gel (**A**), HB101 (lane **1**), 3605/73 (lane **2**), H19 (lane **3**), 184-83 (lane **4**), 1923-77 (lane **5**), 1367-86 (lane **6**), IQBAL 1 (lane **7**), 136 (lane **8**), 135 (lane **9**), 177-83 (lane **10**), QEHH1/86 (lane **11**), 189 (lane **12**), E12801 (lane **13**), lambda DNA 50 kb marker (lane **14**), pUN121 4 kb marker (lane **15**). Gel (**B**), MAR001 (lane **1**), E2348-69 (lane **2**), 1092-80 (lane **3**), 2311 (lane **4**), 182-83 (lane **5**), 5582 (lane **6**), IQBAL2 (lane **7**), 64 (lane **8**), 764 (lane **9**), JPN10 (lane **10**), HB101(pJPN8), (lane **11**), 2036-80 (lane **12**), HB101(pMAR15) (lane **13**), lambda DNA 50 kb marker (lane **14**), pUN121 4 kb marker (lane **15**). All strains examined except 5582 (O128) contained large plasmids greater than 80 kb in size.

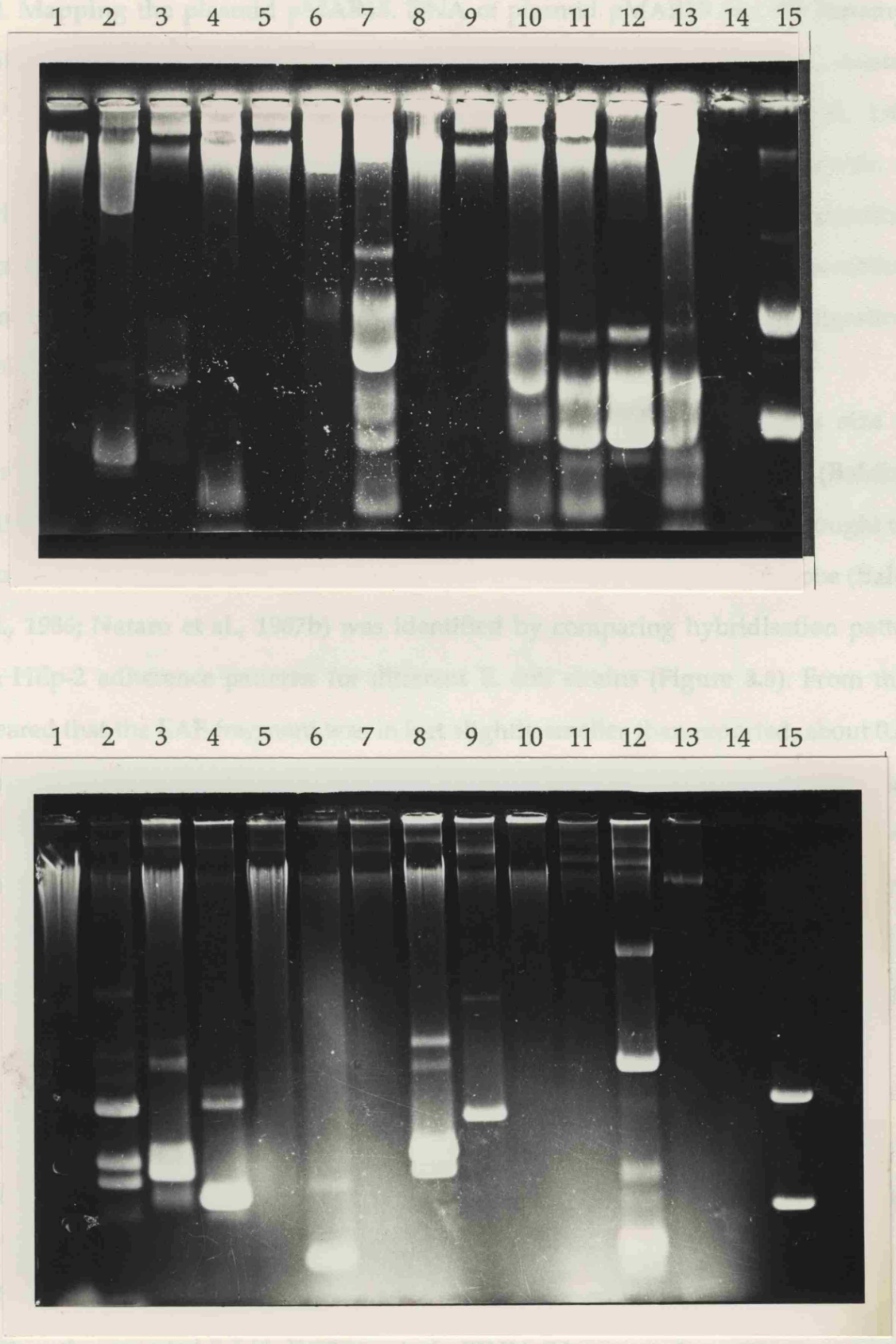


Figure 8.1. Plasmid profile of *E. coli* clinical isolates of EPEC serogroups.

8:3:2. Mapping the plasmid pMAR15. DNA of plasmid pMAR15 and the kanamycin resistant derivative of pMAR2, pJPN11 (pMAR2 with a 1.4 kb kanamycin resistance [Km^r] insert at the *Sal*1 site distal to the region important for LA [**Nataro et al., 1987**]), was isolated using the large scale preparative procedure for large plasmids, and restriction endonuclease digests were performed according to the suppliers conditions. Fragment sizes were determined after separation on agarose gels, and the orientation of fragments in the plasmid molecules was determined by double and triple digestion of the plasmid DNA or isolated fragments.

From the restriction map obtained for pMAR15 (**Figure 8.2**), its size was determined to be about 43 kb. The map confirmed previously reported data (**Baldini et al., 1986; Nataro et al., 1987b**), but gave a more detailed map of the region thought to be important for LA. The 1 kb *Bam*H1/*Sal*1 fragment used as the EAF DNA probe (**Baldini et al., 1986; Nataro et al., 1987b**) was identified by comparing hybridisation patterns with HEp-2 adherence patterns for different E. coli strains (**Figure 8.3**). From this it appeared that the EAF fragment was in fact slightly smaller than reported, about 0.8 kb (**Figure 8.4**). The presence of an internal *Eco*R1 site however enabled it to be reliably distinguished from an other *Bam*H1/*Sal*1 fragment of similar size. An 8.3 kb *Hind*III fragment (*H*8.3) encompassing the EAF probe was easily identified in pJPN11. *H*8.3 was isolated and used as a probe for all subsequent EAF screenings. pMAR15 did not contain *H*8.3 the equivelent DNA is part of a larger 12.5 kb *Hind*III fragment (**Figure 8.2**).

By comparison of the restriction maps for pMAR15 and pJPN11 the transposon Tn*801* in pMAR15 was shown to be located adjacent to the EAF probe fragment, between two regions considered essential for LA, and in the orientation shown (**Figure 8.2**). The transposon had inserted into the fragment corresponding to the *H*8.3 fragment of pJPN11 and pMAR2, forming the larger fragment of 12.5 kb observed in pMAR15. In addition, the reported 1.4 kb Km^r insert of pJPN11 (**Nataro et al., 1987b**) was found to 2.2 - 2.3 kb; whether this was due to an original underestimate of the insert size at the time of construction is unknown.

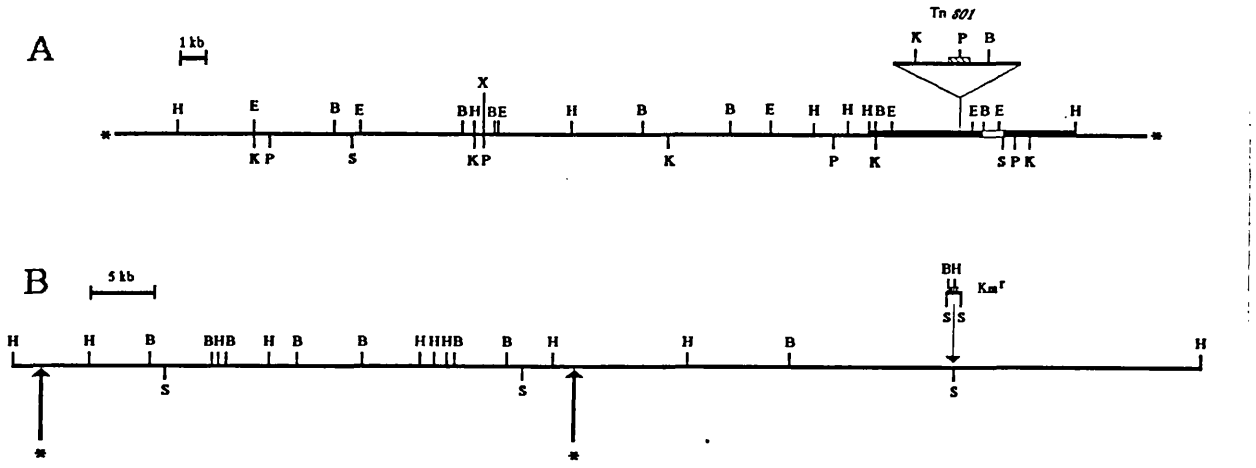


Figure 8.2. Restriction maps of EPEC adherence plasmids. (**A**) pMAR2 and the kanamycin tagged derivative pJPN11, (**B**) pMAR15. Restriction enzymes used, H is *Hind* III, B is *Bam*H1, K is *Kpn*1, P is *Pvu*II, S is *Sal*1, X is *Xho*1, E is *Eco*R1.

Bacterial strains	HEp-2 adherence	EAF hybrid.	1.3 Kbp hybrid.	0.8 Kbp hybrid.
E2348-69	LA	+	+	+
MAR001	LA[a]	-	-	-
HB101(pMAR15)	LA	+	+	+
2036-80	LA	+	-	+
E851	LA	+	-	+
660-79	LA	+	-	+
JPN10	AG	-	-	-
136	AG	-	-	-
135	DA	-	(+)[b]	-
DPO95	DA	-	-	-
469-3	DA	-	-	-

Table 8.3. Hybridisation profiles for two DNA fragments approximately 1 kb in size derived from a *Bam*H1/*Sal*1 double digest of pMAR15. The two fragments had sizes of 1.3 kb and 0.8 kb. Hybridisations were carried out with known EAF positive and negative strains. [a] MAR001 gave a localized pattern of adherence if incubation was continued for 6 h with washes every 2 h. [b] Hybridisation was positive but weaker than positive controls.

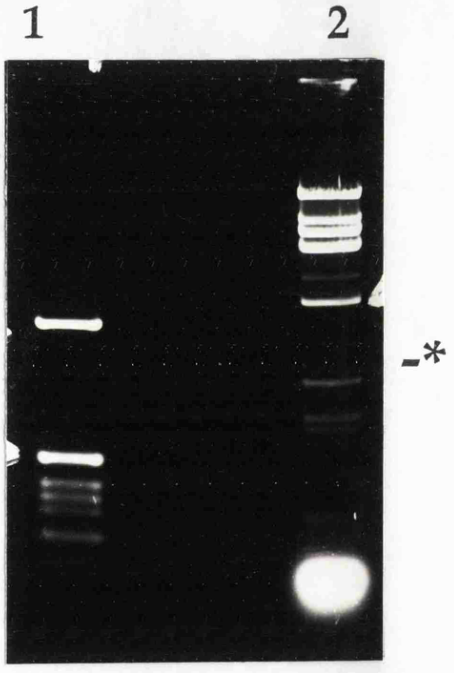


Figure 8.4. Identity of the 1 kb EAF probe fragment. A *Bam*H1/*Sal*1 digest of pMAR15 was run on a 1% agarose gel. Fragments of around 1 kb were identified by comparrison with molecular weight standards in (**lane 1**) (*Hinf* 1 digest of pBR322). EAF probe fragment of 0.8 kb in (**lane 2**) is indicated by (-*).

8:3:3. Cloning of the 1 kb EAF DNA probe fragment. pMAR15 DNA doubly digested with *Bam*H1 and *Sal*1 was ligated into the cloning vector pBR322 which had been digested to completion with *Bam*H1 and *Sal*1, and dephosphorylated with bacterial alkaline phosphatase. A total of 1 μg of ligated DNA was used to transform competent *E. coli* DH5-α, and transformants selected on Luria agar plates containing 100 μg/μl ampicillin, and IPTG and Xgal. Ampicillin resistant colonies which were blue after 24 h incubation at 37°C, were picked and screened by colony hybridisation for the presence of EAF DNA sequences using the *H*8.3 EAF fragment derived from pJPN11 as as probe.

Three clones were identified which hybridised with *H*8.3 DNA. Plasmid DNA was extracted from each clone and cleaved with *Bam*H1 and *Sal*1 yielding two fragments (4 kb and approximately 0.8 kb) was thought to correspond with the vector and insert respectively (**Figure 8.5**).

8:3:4. Effect of temperature on EPEC adherence to HEp-2 cells. *E. coli* isolates of EPEC serogroups grown at 37°C in Luria broth without shaking exhibited normal LA, DA, or AA to HEp-2 cells when incubated at 37°C in DMEM containing 10% foetal calf serum, 1 mM glutamine and 0.5% D-mannose. In all but one strain (the AA strain 136) adherence was abolished when incubation was carried out at 27°C, whether the bacteria had been grown at 27 or 37°C (**Figure 8.6**). In most LA strains, however, a low level of adherence could be observed at 27°C when incubation was continued for longer than 3 h, which is thought to reflect the direct attachment of bacteria to the cell surface by intimate adherence. The fact that both EPEC strains 2036-80 and E2348-69 ony displayed LA when grown and incubated at 37°C suggests that not only could adhesin expression be temperature regulated, but also, that attachment to cells itself is a temperature sensitive function. Whether this is through a conformational change in either adhesin or receptor, or loss of adhesin from fimbriae is an area for further investigation.

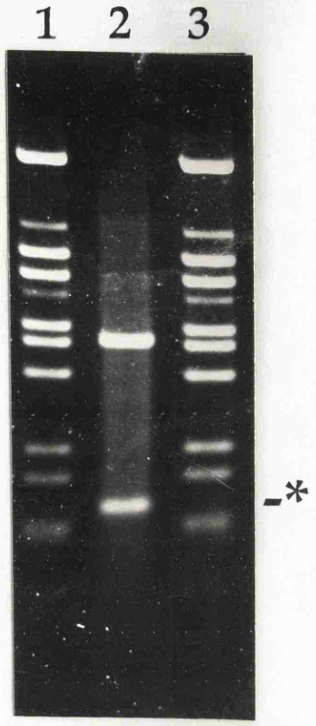


Figure 8.5. Clone 1 was digested with *Bam*H1 and *Sal*1 yielding two fragments of 4 kb and 0.8 kb (**lane 2**). Molecular weight standards (**lanes 1 and 2**) were Lambda DNA digested with *Bst*EII. The 0.8 kb fragment (indicated by -*)gave an identical hybridisation profile as previously demonstrated in **Figure 8.3.**

growth temp.	bacterial strains	incubation temp.	HEp-2 adherence
37oC	2036-80	37oC	LA
37oC	2036-80	27oC	NA
27oC	2036-80	37oC	NA
27oC	2036-80	27oC	NA
37oC	E2348-69	37oC	LA
37oC	E2348-69	27oC	NA
27oC	E2348-69	37oC	NA
27oC	E2348-69	27oC	NA
37oC	1923-77	37oC	LA
37oC	1923-77	27oC	NA
37oC	DPO97	37oC	LA
37oC	DPO97	27oC	NA
37oC	177-83	37oC	LA
37oC	177-83	27oC	NA
37oC	JPN10	37oC	AA
37oC	JPN10	27oC	NA
37oC	136	37oC	AA
37oC	136	27oC	AA
37oC	135	37oC	DA
37oC	135	27oC	NA
37oC	DPO95	37oC	DA
37oC	DPO95	27oC	NA

Figure 8.6. Sensitivity of HEp-2 adherence to growth and incubation temperature. *E. coli* strains were grown at stated temperatures, and incubated with HEp-2 cells for 3 h at either 37 or 27oC. HEp-2 adhesion assays were carried out as described in materials and methods.

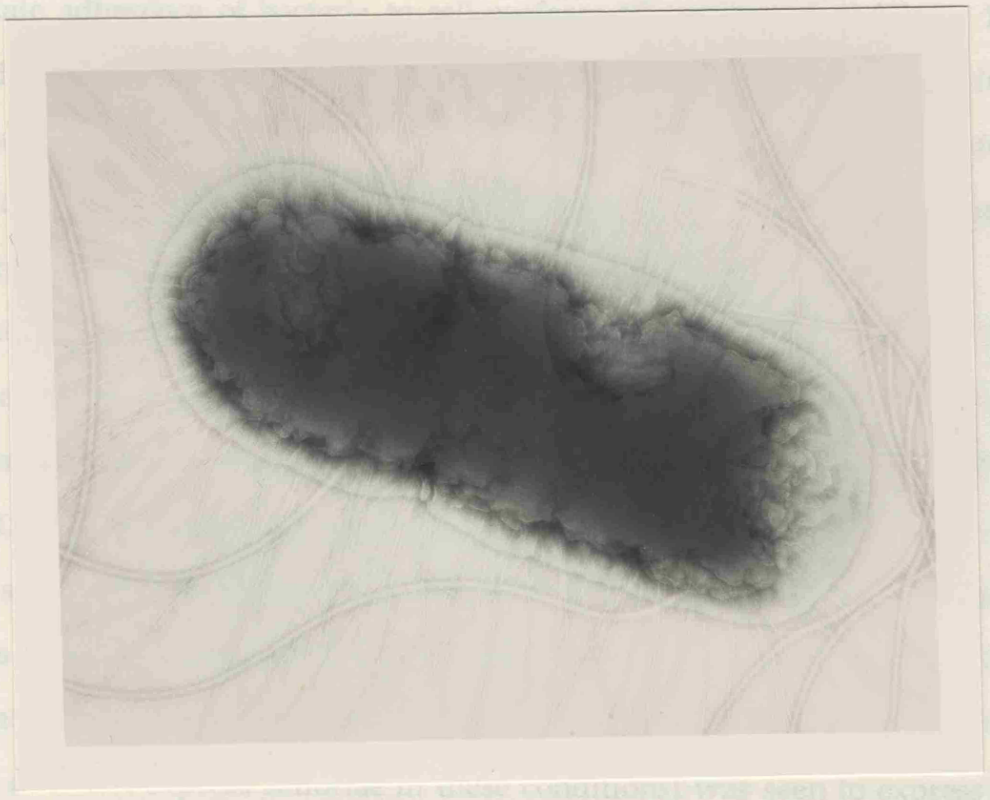


Figure 8.7. Negative staining of fimbriae on the surface of EPEC strain 2036-80. Bacteria were grown in Luria broth with glucose over night without shaking at 37°C. Magnification 80,000 X.

8:3:5. Detection of fimbriae. Bacterial attachment to HEp-2 cells in culture occurred only when bacteria were grown and incubated at 37°C and not at 27°C, suggesting temperature regulation of expression of the adhesin responsible for HEp-2 adherence. Many adhesins form part of fimbriae, rod-like structures which facilitate the nonintimate adherence of bacteria to cell surfaces (**Goransson and Uhlin, 1984**). As discussed in chapter 1, the evidence for fimbrial adherence is still inconclusive for EPEC, and remains an area of speculation. Ruthenium red staining of EPEC attached to gut enterocytes, however, reveals rod like appendages which appear to be responsible for bacterial attachment (**Knutton et al., 1987b**).

The expression of fimbriae by EPEC isolates grown at 37°C and 27°C was determined using negative staining and transmission electron microscopy. At 37°C all EPEC examined produced fine rod-like appendages radiating from the cell surface (**Figure 8.7**), with some variation in the length and diameter apparent among the different strains. The same bacteria grown at 27°C gave no indication of fimbriae-like structures being present. MAR001, a plasmid cured derivative of E2348-69, was unfimbriated at both growth temperatures, while the laboratory strain HB101 (which normally does not express fimbriae in these conditions) was seen to express fimbriae when transformed with the plasmid pMAR15 and grown at 37°C. Again expression of fimbriae was abolished in HB101(pMAR15) grown at 27°C.

8:4. *Discussion and Conclusions.*

Large plasmids are common among EPEC isolates; this study shows them in at least 95% of strains. Furthermore 71% of strains contained one or more small multi-copy plasmids, and only 5% did not contain any plasmid DNA. To date all genes for localized adherence have been located on large low copy number plasmids (**Baldini et al., 1983a; Laporta et al., 1986**). Therefore the likelihood that the large plasmids of the LA strains encode adhesin genes is high.

The plasmid pMAR15, which was constructed from pMAR2 and carries the genes for LA, was shown to carry a Tn*801* insert between the regions of the plasmid

required for attachment to HEp-2 cells (**Baldini et al., 1986; Nataro et al 1987b**). It has previously been observed that the efficiency of adherence of pMAR15-containing bacteria is considerably lower than that of the parent (this study). Which suggests that expression from the ampicillin resistance gene promoter in Tn*801* may interfere with expression of the LA phenotype, possibly by transcriptional overrun into regions essential for full LA expression.

In the published description of the EAF probe (**Nataro et al., 1985a; Nataro et al., 1985b; Nataro et al., 1987a**) it was stated that a 13 kb *Hind*III fragment containing the EAF fragment was cloned from plasmid pMAR2 into the tetracycline and chloramphenicol resistant vector pAYC184 and screened for aquisition of ampicillin resistance; the resulting plasmid was termed pMAR22. From the more complete restriction map of pMAR15 and pMAR2 described here it is obvious that the *Hind*III fragment encompassing the EAF probe region in pMAR2, is 8.3 kb and not 13 kb. As described above transposition of Tn*801* on to pMAR2 (to give pMAR7) occured at the region adjacent to the EAF probe. This event increased the size of the *Hind*III fragment encompassing the EAF probe from 8.3 kb to 12.5 kb. This new 12.5 kb *Hind*III fragment now contained the EAF probe and the entire Tn*801* sequence including the ampicillin restance gene. This, and the fact that pMAR2 does not carry ampicillin resistance suggests that pMAR22 was constructed from pMAR15 or pMAR7 and not pMAR2. Furthermore the construct pMAR22 was also confusingly described as pMAR2 by Nataro and coworkers, most likely resulting from a simple typographical error (**Nataro et al., 1985a**).

The phenotypes of LA, DA, and to some extent AA to HEp-2 cells was shown to be temperature regulated, with full expression only apparent at 37°C. This may reflect mechanisms for the conservation of energy reserves in the bacterium, only allowing the expression of energy expensive adhesins when they would be of some use, i.e. in the gut of the host organism. The nature of these putative adhesins remains obscure in many cases, but by electron microscopy it has been shown that, at the temperature which allowed attachment of bacteria to HEp-2 cells, fimbrial like structures could be

detected on all LA strains. For the EPEC isolate E2348-69, LA was indirectly shown to be facilitated by fimbrial structures since the plasmidless derivative MAR001 did not display fimbriae at either temperature, while HB101 acquired fimbrae at 37°C when transformed with pMAR15.

Chapter 9. CHARACTERISATION OF EPEC ISOLATES.

9:1. *Introduction.*

As outlined previously, EPEC are routinely identified from patients under two years of age with severe and persistant diarrhoea solely on the basis of *E. coli* serogrouping (**Levine,1987; Robins-Browne, 1987**). With increasing work loads and diminishing resources many PHLS laboratories no longer screen for EPEC serogroups among the *E. coli* isolates they obtain because it is costly in terms both of materials and of man hours. Additionally, although the importance of EPEC as an agent of disease among young children is recognised (**Levine and Edelman, 1984; Levine,1987; Robins-Browne, 1987**), confidence in the screening protocols used by laboratories is low. Diagnostic procedures based on the detection of localized adherence to HEp-2 cells, either directly (**Cravioto et al., 1979; Baldini et al., 1983; Nataro et al., 1987a; Nataro et al., 1987b; Tavendale and Old, 1986; Levine et al., 1988**), or indirectly using DNA probes for the LA genes (**Nataro et al., 1985a; Baldini et al., 1986; Nataro et al., 1987b; Levine et al., 1988**), are unreliable because it is not known whether all EPEC strains actually harbour the plasmid which carries them. Plasmids convey an evolutionary selective advantage on groups of organisms, not only because of the genes they encode (which may facilitate adherence to particular cell types or resistance to particular antibiotics) but also because their transmissibility, ease of loss or acquisition, and apparent ability (in some cases) to cause recombination events in the chromosome gives a highly adaptive and responsive fluidity to the genetic pool. This very fact precludes HEp-2 adherence as a reliable test for the EPEC group of enteric pathogens, since it is likely that it will absent in some EPEC strains and possibly present in some non lesion-forming *E. coli* isolates. The likelihood is that there are several plasmid or chromosomally encoded adhesins associated with EPEC as there are with ETEC for instance, and the use of one criterion which is distinct from the actual mechanism of diarrhoea may simply limit the range of organisms deemed to be EPEC.

An ideal screening procedure would have as its basis a stably maintained (chromosomally encoded) function which is inextricably coupled to the pathogenic mechanism. The ultrastructural changes of the attaching and effacing lesion in gut tissue (**Staley et al., 1969; Polotsky et al., 1977; Knutton et al., 1987b**) is the best example of such a criterion since it defines the EPEC group and the basis of the disease mechanism, but it is of limited use in screening protocols because of the cost, time, and technical expertise required to obtain diagnostic grade electron-micrographs. The FAS test described in chapter 2 (**Knutton et al., 1989**) is more suited for this task since it is designed to detect the lesion in tissue culture cells rapidly and with a simple fluorescence microscope. A weakness of the FAS test which is shared with the HEp-2 adherence assays of course, is the use of tissue culture cells in place of human gut tissue, which constitutes an artificial system since it is fortuitious that gut pathogens should adhere to a cell line derived from laryngeal carcinoma. However the possible occurrence of initial adhesins in EPEC strains distinct from the established EAF determinant will tend to limit the usefulness of the test. To reduce this, the modified procedure which increases infection time and uses different culture cell lines, was used to detect isolates which in normal conditions would be missed through poor adherence to HEp-2 cells. Extended incubation times proved effective in detecting enterohaemorrhagic strains of *E. coli* (e.g. O26 strains) which are known to be competent in lesion formation (**Knutton et al., 1989**), but the possibility remains that pathogenic isolates are being missed because they are not adherent even in these conditions.

A collection of routinely identified EPEC strains from the Portsmouth area health authority were used in an evaluation of the standard laboratory procedures used for EPEC isolation by using the FAS test as an indication of lesion formation. This was in order to establish the reliability of routine serogrouping in the identification of lesion forming *E. coli* isolates. In addition these Porsmouth EPEC serogrouped (PES) strains were fully serotyped and tested for their HEp-2 adherence pattern, possession of EAF

localized adherence genes, plasmid profiles, and iron uptake systems to determine whether other virulence determinants are associated with EPEC.

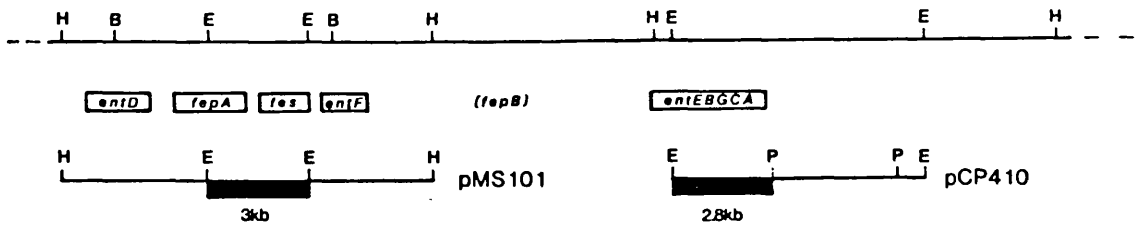
9:2. *Materials and methods.*

9:2:1. Siderophore bioassays. Overnight 10 ml cultures of *E. coli* indicator strains AN1937, for detection of enterochelin production, and LG1522, for detection of aerobactin production, were diluted to A_{600}= 0.1 with PBS and spread on iron restricted M9 minimal agar plates (**Maniatis et al., 1982**), made by adding 10 ml of filter sterilised solution B (200 mM $MgSO_4$, 20% glucose, 10 mM $CaCl_2$) to 1 liter of solution A (6 mg/ml Na_2HPO_4, 3 mg/ml KH_2PO_4, 0.5 mg/ml NaCl, 1 mg/ml NH_4Cl, *p*H adjusted to 7.4 and autoclaved), media was solidified by addition of 1.5% agar before autoclaving and iron restricted with 100 μM dipyridyl. Once dry, test strains were stabbed into the agar and incubated at 37°C for 16 hours. Siderophore producing test strains were indicated by growth of the indicator strain around the point of inoculation.

9:2:2. Colony blot hybridisations. Colony blot hybridisations were carried out for LA and aerobactin genes as described in Chapter 8. DNA probes for the EAF region (*H*8.3, Chapter 8) and the aerobactin biosynthesis and receptor genes (**Figure 9.1**) were prepared, and labelled with ^{32}P by oligolabelling as described in Chapter 8.

9:2:3. Colicin preparation and sensitivities. Colicins are antibacterial products of *E. coli* and related enterobacteriacae which bind to identifiable protein receptors on the bacterial surface. Colicins were purified (**De Graaf et al., 1969; Van Tiel-Menkveld et al., 1981; Krone et al., 1986**) by inoculating 500 ml of LUB-glucose in a 2 l baffled flask with 10 ml overnight LUB-glucose culture of a producer strain (**Appendix 1**), and incubating at 37°C to A_{600}= 0.6. Mitomycin C was added to a final concentration of 1 μg/ml from a 2 mg/ml stock solution, and incubation continued for 20 min with shaking. Bacterial cells were harvested by centrifugation in a Du Pont Sorval GS3 rotor

(a) Enterochelin



(b) Aerobactin

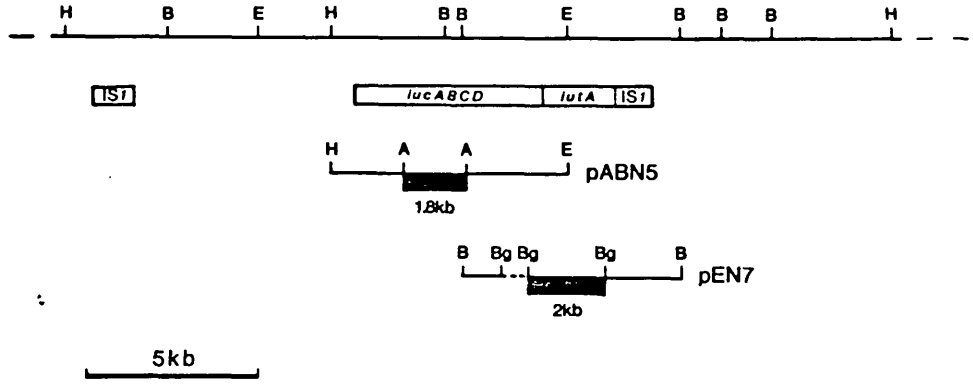


Figure 9.1. Genetic and restriction maps of the areobactin and enterochelin iron uptake systems. (a) Enterochelin gene probes are indicated by the blocked in regions, representing receptor gene probe derived from pMS101, and biosynthesis gene probe derived from pCP410. (b) Aerobactin gene probes are indicated by blocked in regions, representing biosynthesis gene probe derived from pABN5 and receptor gene probe derived from pEN7.

at 6,000 rpm for 15 min at room temperature, resuspended in 500 ml of fresh LUB-glucose pre-warmed to 37°C, and incubated for a further 3-16 h (depending on the yield required) at 37°C. Cells were removed from the culture medium by centrifugation in a Du Pont Sorval GS3 rotor at 6,000 rpm for 15 min at 4°C and discarded. To the culture medium were added 121.5 g of $(NH_4)_2SO_4$, giving a 40% saturated solution, which was incubated at 4°C overnight with stirring. The precipitate was collected by centrifugation in a Du Pont Sorval GS3 rotor at 9,000 rpm for 20 min at 4°C, dissolved in 10 ml 50 mM Tris (*p*H 8.0), 20 mM EDTA, and stored at -20°C until needed. The culture medium was increased to 60% saturation by adding 66 g more of $(NH_4)_2SO_4$, and incubation was continued at 4°C overnight with stirring. The precipitate was again collected by centrifugation as above, and dissolved in 10 ml Tris buffer. All dissolved precipitate fractions were dialysed overnight at 4°C against 4 l of Tris buffer, filter sterilised and finally stored at -20°C. The activity of each fraction was determined using standard sensitive and resistant strains. The sensitivity of *E. coli* strains to colicins and therefore the presence of specific outer-membrane proteins in that strain, was determined using the filter disc patch test. The test strain was streaked on to LUA-glucose plates and a 9 mm filter disc dipped in colicin solution placed on the surface of the plate, which was incubated overnight at 37°C. Zones of clearing around the filter disc indicated sensitivity of the test strain to the colicin and therefore the presence of the particular receptor protein.

9:2:4. Verotoxin. Filter sterilised culture supernatants from bacteria grown in LUB-glucose and LUB-glucose containing 0.31 mg/ml of the iron chelator Desferal were diluted 1:10 with fresh medium and their cytopathic effects observed on Vero cell and HEp-2 cell monolayers by adding supernatants to give a 1:10 dilution in tissue culture medium. Cytopathic effects were scored as rounding up of cells.

9:3. *Results.*

9:3:1. HEp-2 adherence patterns of PES strains. 13 of the 25 PES strains tested 52% were non adherent to HEp-2 cells in standard assay conditions, but with prolonged incubation, 11 of these showed weak localized adherence. Only 8 strains displayed localized adherence after 3 hours incubation, but with a much reduced microcolony size as compared to the control strains 2036-80 and E2348-69. Of the remaining 4 strains, one was a true diffuse adherer and 3 showed a normal aggregative pattern of adherence (**Table 9.1**).

9:3:2. EAF probe hybridisation. The 25 PES strains were screened for possession of the localized adherence EAF genes by colony blot hybridisation using the two probes which encompass the EAF region (1 kb and 8.3 kb fragments of pMAR15). Even after repeated attempts with different hybridisation conditions in which control strains behaved as expected, none of the 25 isolates showed any hybridisation with either probe as compared with controls (**Table 9.1**).

9:3:3. Detection of the aerobactin and enterochelin iron uptake systems. Aerobactin production was detected by bioassay in only 7 of the 25 PES strains screened (28%) (**Table 9.1**). Of these 5 were non adherent to HEp-2 cells in 3 h, and 1 each were diffuse and aggregative adherers. Confirmation of this was obtained by colony blot hybridisation with the DNA probes (**Figure 9.1**) for the aerobactin biosynthesis genes (*Iuc*) and the aerobactin receptor gene (*Iut*). In each case the strains that were positive for the bioassay also hybridised with the two DNA probes (**Table 9.1**). The aerobactin receptor protein was also shown to be present in those strains which hybridised with the *Iuc* and *Iut* gene probes, and were able to cross feed the indicator strain LG1552 in the aerobactin bioassay, by their sensitivity to cloacin DF13, which kills sensitive cells by binding to the aerobactin receptor (Chapter 1).

Table 9.1. Characterization of the Portmouth EPEC serogrouped (PES) strains. PES strains were isolated over several years from the Portsmouth area, predominantly from infants with diarrhoea. All except one (PES 25) were from children under 2 years of age, and were either hospitalized (not necessarily due to diarrhoea) or outpatients with diarrhoea. The strains were identified as EPEC using standard pooled sera as part of the laboratory routine. All isolates were fully serotyped (carried out by F. and I Orskov), and screened using the FAS test for lesion formation, HEp-2 adherence pattern, EAF hybridisation, and presence of the aerobactin and enterochelin systems of iron uptake using gene probes (*iut*- aerobactin receptor gene, *iuc*- aerobactin biosynthesis genes, *fep*A- enterochelin receptor gene, *ent*D- enterochelin biosynthesis gene), colicin sensitivities (cloacin DF13 for aerobactin receptor, colicin D for enterochelin receptor), and bioassays for both aerobactin and enterochelin production. All isolates gave positive results for enterochelin (gene probes, colicin B and D sensitivity, and bioassay) data not shown. All strains that were positive for aerobactin gene probes and bioassay were also sensitive to cloacin DF13, data not shown. No strain produced detectable amounts of vero toxin. Shaded areas represent those strains which were FAS positive.

A	B	C	D	E	F	G	H	I	J
STRAIN NUMBER (PES)	SEROTYPE O, H, AND K	AERO-BACTIN BIO-ASSAY	IUT +/-	IUC +/-	EAF +/-	FAS TEST +/-	HEP-2 ADHERE. LA,DA NA,AG	AGE MON-THS	D OR S AND I OR O
PES 7	O6:K5:H1	+	+	+	-	-	NA	13	D/O
PES 25	O18ac:H-	+	+	+	-	-	DA	29	D/O
PES 16	O26:H-	-	-	-	-	+	NA	NK	NK
PES 17	O26:H-	-	-	-	-	+	NA	12	D/O
PES 18	O26:H-	-	-	-	-	+	NA	NK	NK
PES 10	O51:H49	-	-	-	-	-	LA	1	D/I
PES 11	O55:H-	-	-	-	-	-	NA	10	D/O*
PES 12	O55:H-	-	-	-	-	-	NA	14	D/O
PES 13	O55:H7	-	-	-	-	-	NA	8	D/I
PES 8	O55:H34	-	-	-	-	-	AG	23	D/O
PES 24	O86:H?	-	-	-	-	-	NA	22	D/O
PES 3	O111:H25	-	-	-	-	-	LA	7	S/I
PES 4	O111:H-	+	+	+	-	-	NA	16	D/I
PES 5	O111:H25	-	-	-	-	-	AG	14	NK/I
PES 6	O111:H-	+	+	+	-	-	NA	NK	NK
PES 15	O111:H25	+	+	+	-	-	NA	10	D/O
PES 2	O114:H-	+	+	+	-	-	NA	19	D/O**
PES 14	O119:H4	+	+	+	-	-	LA	6	D/I**
PES 26	O124:H25	-	-	-	-	-	LA	NK	D/NK
PES 19	O126:H27	-	-	-	-	-	AG	16	D/O
PES 20	O127:H40	-	-	-	-	+	LA	5	D/O
PES 21	O127:H-	-	-	-	-	-	LA	10	S/I
PES 22	O127:H40	-	-	-	-	+	LA	NK	D/O
PES 23	O127:H40	-	-	-	-	+	LA	2	D/O
PES 1	O128ab:H12	-	-	-	-	-	NA	NK	D/O

Table 9.1. Characterization of the Portmouth EPEC serogrouped (PES) strains.

As expected all 25 PES strains produced enterochelin in the bioassay, and hybridised with the enterochelin receptor gene probe (*fep*A) and the biosynthesis gene probe (*ent*D). The presence of the enterochelin receptor protein was confirmed for all strains by their sensitivity to colicins B and D

9:3:4. Lesion formation using the FAS test. Of the 25 PES strains 6 were FAS test positive representing only 24% of isolates traditionally classified as EPEC by serogrouping in this study (**Table 9.1**). Of these, three were of the EPEC serotype O26:H- and three of O127:H40. Routine screening of *E. coli* isolates by serogroup alone, from this, seems extremely inefficient for the identification of lesion forming strains. Use of the FAS test, however, identifies only those strains able to form an attaching and effacing lesion and therefore is more effective as a screening method for EPEC. Four of the six FAS test positive isolates (i.e. three O127:H40 isolates and one O26:H-) were known to have originated from outpatients under 2 years of age with severe persistant diarrhoea. For the remaining two *E. coli* O26:H-, no patient details were available, yet they were almost certainly isolated from cases of severe diarrhoea, since serogrouping of *E. coli* isolates were only carried out on out patients with these symptoms. None of the FAS test positive strains produced aerobactin or harboured the genes for its biosynthesis or uptake which perhaps suggests that this virulence determinant is of less importance for pathogenicity of EPEC than it is in other *E coli* pathogenic groups (i.e. enteroinvasive *E. coli*).

9:4. *Discussion and conclusions.*

These results indicate that *E.coli* of classical EPEC serotypes as routinely identified in PHLS laboratories are not necessarily EPEC on the basis of lesion formation. This perhaps highlights the main problem with the routine screening of *E. coli* isolates by serogroup, that is that many of the strains isolated will not be EPEC by pathogenesis or may not, in some cases be diarrhoeagenic at all. This also raises the intriguing possibility alluded to before (Chapter 8) that many isolates of tradition EPEC

serogroups may utilise a novel pathogenic mechanism distinct from that seen with lesion forming strains.

A similar study carried out by Stuart Knutton further clarified the situation, as far as serogrouping is concerned. Stool samples from diarrhoea patients collected over a short period of time were directly screened using the FAS test for lesion-forming bacteria. A high proportion of the FAS positive isolates were not of classical EPEC serotypes (**S. Knutton, Personal communications**). Indicating that serogrouping is failing to detect many of this class of enteropathogens. It seems reasonable to assume from this evidence that EPEC are not restricted to a small range of serogroups, since lesion formers have been found outside this traditionally accepted range. The Portsmouth study described here in which only 24% of clinical isolates of traditional EPEC serogroups apparently utilise this pathogenic mechanism, suggests that there is little benefit in screening *E. coli* solely on the basis of O serogroup. Routine use of the FAS test would provide a simple and effective means for the identification of EPEC, and for the first time furnish those interested in this group of enteropathogens with a reliable indication of their prevalence.

The aerobactin system of iron uptake, another important virulence determinant of many *E. coli* pathogens, seems to be of little importance in the pathogenesis of the EPEC isolates in this study. Interestingly, none of the 25 PES strains looked at contained DNA sequences that hybridised with the EAF probe for localized adherence genes, although eight were found to adhere to HEp-2 cells and form microcolonies. It was observed that the quality of localized adherence was different for these strains, compared to the EAF positive controls 2036-80 and E2348-69, which formed distinct microcolonies after only 2 h incubation. PES strains which were categorized as LA required at least 4 h to produce comparable sized microcolonies, and before this time very little adherence could be detectedat all. These observations raise the possibility that another adhesin apart from EAF is utilised by some lesion forming *E. coli* strains, which may only be recognised by the use of the FAS test with longer incubation.

Chapter 10. GENERAL DISCUSSION.

Early endeavours to elucidate the diarrhoeagenic mechanisms of EPEC tended, perhaps rather naively, to assume that they would be based on those mechanisms which had already been described. This is reflected by repeated unsuccessful attempts by many groups to show LT and ST enterotoxigenic properties associated with EPEC strains (**Robins-Browne 1987; Long-Krug et al., 1984; Law et al., 1987; Law, 1988**). Obviously it is important to establish similarities between EPEC and other diarrhoeagenic organisms. However, since adult volunteer studies of the late 1970's showed that EPEC diarrhoeagenesis was independent of all previously known virulence factors (**Levine et al., 1978**), it is clear that the EPEC group comprises enteropathogens which employ novel pathogenic mechanisms (**Ulshen and Rollo, 1980**).

From the evidence presented in this thesis, a model of the pathogenic mechanism of EPEC has been constructed (**Figures 10.1, 10.2,** and **10.3**) which attempts to explain the major changes observed in EPEC infected cells. It cannot claim to be fully comprehensive, and there will undoubtedly be parts whose significance is not immediately apparent, but this most likely reflects the fact that our understanding of the subject is incomplete rather than invalidating the entire model. However, any proposed model of EPEC diarrhoeagenesis must at least attempt to explain the major events of EPEC infection, (1) rapid onset of secretory diarrhoea in patients, (2) absence of soluble toxins, (3) localized effacement of microvilli, (4) pedestal formation at sites of bacterial attachment, and (5) gross histological damage associated with prolonged infection of patients. Models also have to be predictive, indicating probable additional events or stages and possible approaches for their confirmation. The model presented here to explain some of the important events seen during EPEC infection of target cells was developed from the predictions of an initial model based on preliminary evidence available at the start of the project.

I propose that initial colonization of the intestinal brush border is facilitated by rod like fimbriae which also mediate LA to cultured HEp-2 cells. Intimate bacterial adherence to receptor proteins in the microvillus membrane follows dissolution or contraction of fimbriae, thus overcoming the natural repulsive forces between the two cell surfaces while maintaining attachment of bacteria. These receptor proteins may be similar to or identical with, receptors for binding of hormones which regulate the absorptive area of the gut by reducing the microvillus length (**Goligorsky et al., 1986**). Binding of bacteria to these receptors induces a host cell signal transduction pathway involving the activation of PLC probably via an intermediary G-protein (**Figure 10.1**). PLC activity would cause breakdown of membrane PI-lipids (in particular PIP_2) and concomitant formation of two second messenger molecules (**Figure 10.1**), DAG (which remains in the plane of the membrane) and 1,4,5-IP_3 (which moves into the cytoplasm). 1,4,5-IP_3 normally causes mobilization of intracellular calcium stores in the endoplasmic reticulum. However, since the cellular organization of epithelial cells such as HEp-2 (used as a model of infection in this study) is different from that of mature enterocytes, where the endoplasmic reticulum does not extend beyond the terminal web at the base of microvilli (**Figure 10.2**), it is unlikely that 1,4,5-IP_3 will have a significant effect on calcium release from the endoplasmic reticulum at this stage. However phosphorylation of 1,4,5-IP_3 at the 3 position produces 1,3,4,5-IP_4 which would mediate influx of calcium from the gut lumen into the microvilli. Many effects on the structure and functioning of the microvillus would accompany elevated calcium concentrations. Firstly, PKC is mobilized to the membrane where it is activated by DAG, causing phosphorylation of membrane associated proteins involved in anchoring cytoskeletal elements to the microvillus membrane (**Bennett et al., 1988; Huang et al., 1988**), regulation of cytoskeletal structure (**Huang et al., 1988; Zimmermann et al., 1988**), and ion transport (**Moolenaar et al., 1984; Fondacaro and Shlatz-Henderson, 1985; Grinstein et al., 1988; Ladoux et al., 1988**). Secondly, activation of the calcium dependent protease calpain would lead to cleavage of membrane bound PKC, producing the cytosolic active form PKM; this may mediate phosphorylation of

Figure 10.1. Initial stages of EPEC infection of the gut mucosal surface. Bacteria initially adhere via fimbriae. contraction of fimbriae allows intimate adherence of bacteria to cell surface receptors, which in turn activates a host signal transduction pathway terminating in PLC. Formation of the second messengers DAG and 1,4,5-IP_3, and the subsequent phosphorylation of 1,4,5-IP_3 to yield 1,3,4,5-IP_4, mediates breakdown of microcvillus core and ion hypersecretion by inducing PKC and raising calcium. Microvilli are lost by vesiculation, enabling the bacteria to adhere to the cell.

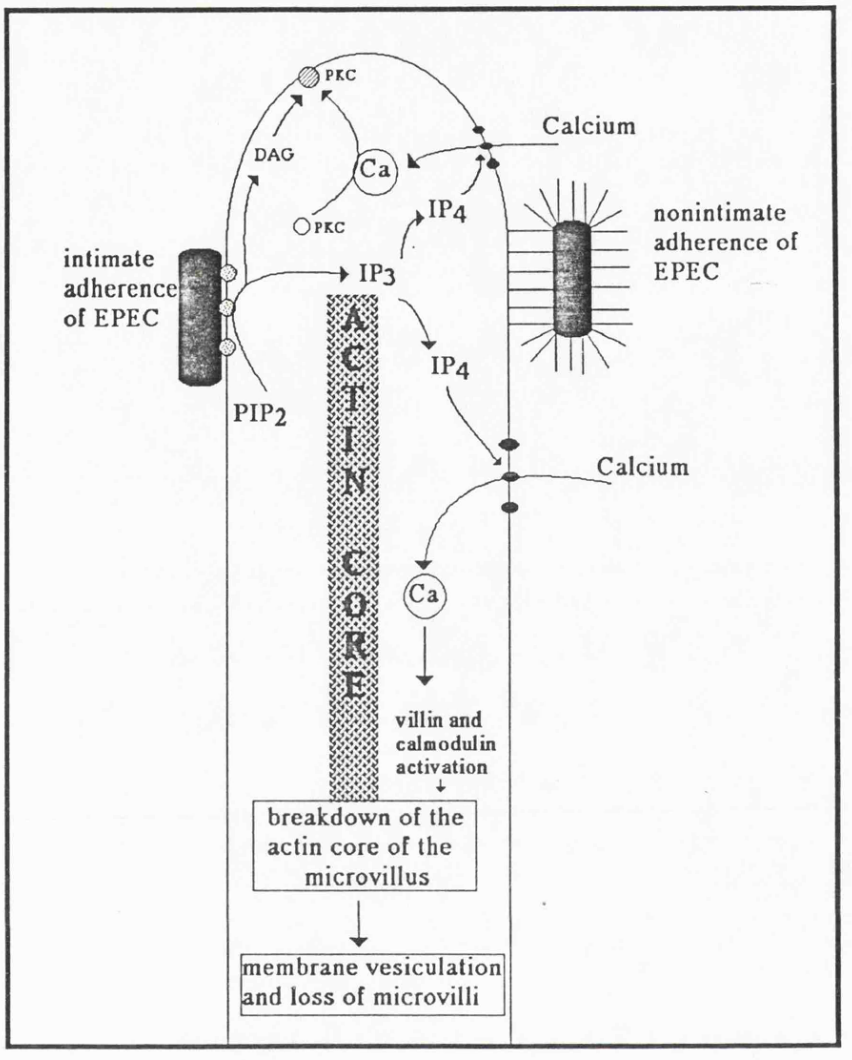


Figure 10.1. Initial stages of EPEC infection of the gut mucosal surface.

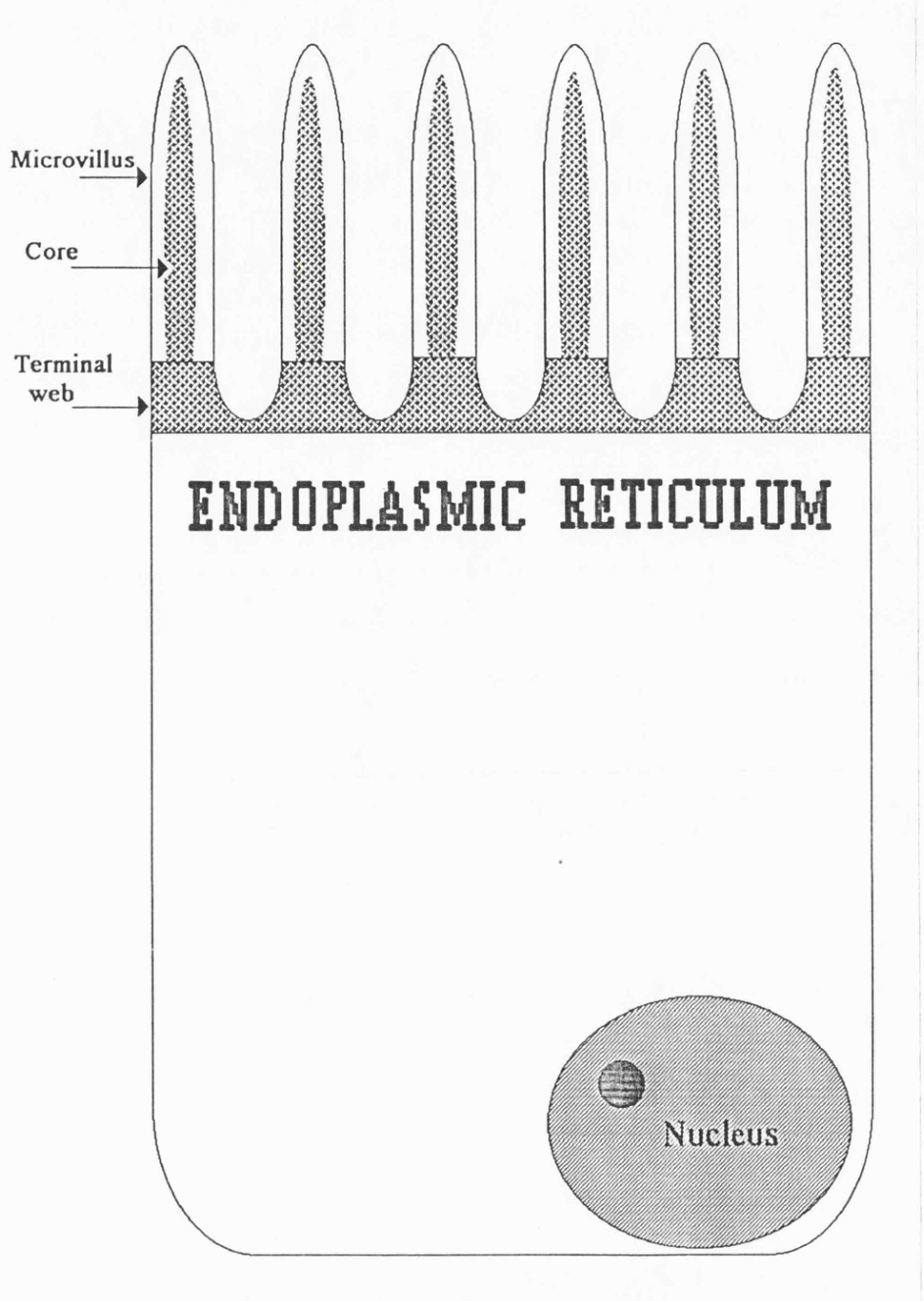


Figure 10.2. The basic structure of intestinal enterocytes. Unlike undifferentiated epithelial cells the highly specialised brush border microvilli do not contain any regions of endoplasmic reticulum since it does not extend beyond the terminal web at the base of the microvillus core. Therfore 1,4,5-IP_3 is able to initially mobilize calcium from storage regions in the endoplasmic reticulum.

Figure 10.3. Intimate attachment of bacteria to the surface of enterocytes. Loss of microvilli through calcium mediated core breakdown and membrane vesiculation enables bacteria to adhere to the body of the enterocyte where activation of receptor coupled PLC again produces the two second messengers DAG and 1,4,5-IP_3. The ensuing activation of PKC and mobilization of calcium from intracellular stores mediates major cytoskeletal rearrangements, and profuse intestinal hypersecretion through the activation of ion transport proteins in the plasma membrane.

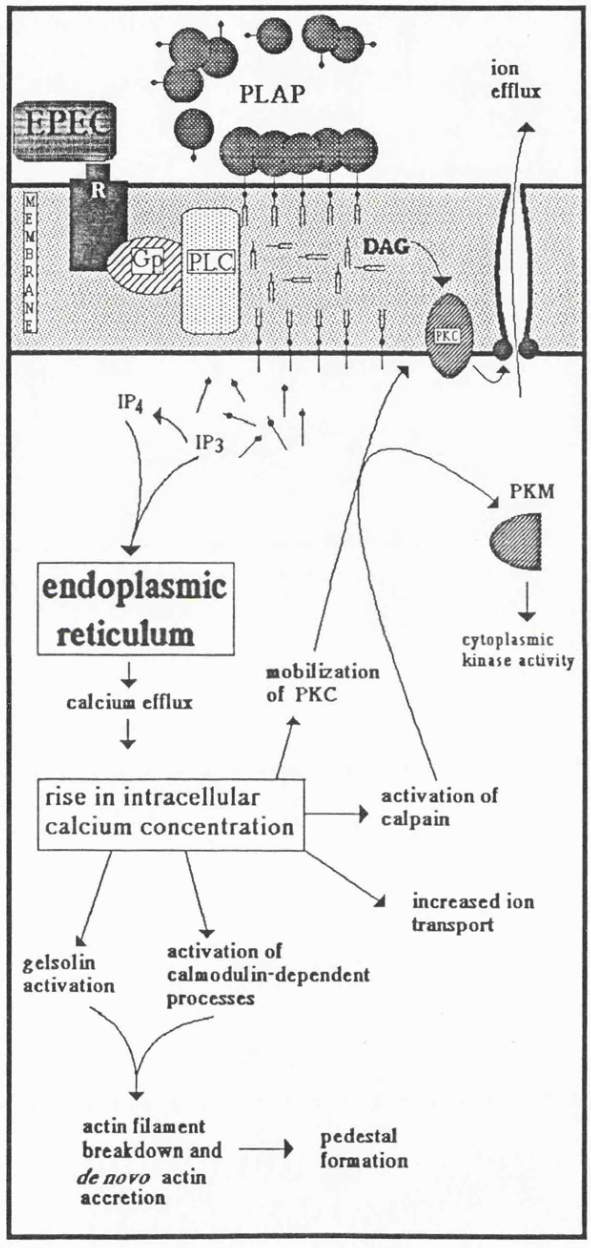


Figure 10.3. Intimate attachment of bacteria to the surface of enterocytes.

proteins involved in maintaining core structure of the microvilli. Thirdly, increased calcium will saturate calmodulin, a major calcium binding protein of the microvillus core involved in many cellular processes, including calcium buffering and the active maintenance of cytoskeletal integrity. Fourthly, the actin-severing function of villin, another major cytoskeletal protein essential for the stability of the microvillus core structure, will be induced, mediating rapid breakdown of filamentous actin in the core, and causing loss of rigidity and vesiculation of the microvillus membrane. This process may be enhanced by the probable activation of calcium-dependent and PKC-activated ion transport systems in the microvillus membrane. The overall result of this initial stage of EPEC infection would therefore be effacement of microvilli from enterocyte surfaces causing considerable reduction in the absorptive area of infected regions of the gut (**Figure 10.1**). It is unlikely, however, that this would fully explain the rapid onset of diarrhoeal disease seen during experimental infections of adult volunteers, since only a limited area of the gut would be affected. Thus additional functions are involved in the rapid onset of disease. It seems likely that, since calcium and PKC are important regulators of ion transport processes, this early stage of infection may also be characterized by a rapid secretory response in the gut as a result of PKC and calcium activation of ion transport systems.

Following loss of microvilli, bacteria intimately adhere to the body of the cell possibly via an intermediate fimbrial attachment stage as described above. In this case (**Figure 10.3**) activation of PLC and second messenger formation will cause mobilization of calcium stores from regions of the endoplasmic reticulum in close proximity to the adherence sites of the bacteria, promoting localized increases in intracellular calcium concentration. This in turn will activate the actin-severing properties of cytoplasmic gelsolin, facilitating rapid local fragmentation of cytoplasmic actin filaments to yield a high concentration of short gelsolin capped actin fragments. Subsequent dispersal of initially high localized calcium concentrations by diffusion would promote binding of the gelsolin cap to PI-lipids of the plasma membrane and uncapping of the actin fragments. Uncapped actin would then form nucleation sites for

monomer addition, causing a rapid burst of actin polymerization and a substantial increase in the concentration of filamentous actin directly beneath sites of bacterial adherence. The effect of rapid actin gel formation would be upthrusting of the plasma membrane at the position of gel formation (i.e. beneath the attached bacteria), producing the characteristic pedestal of the EPEC lesion. Local calcium changes would also mobilize PKC to the plasma membrane and activate calpain causing major phosphorylation events in both cytoplasm and membrane. These in conjunction with calcium may promote further cytoskeletal changes and mediate hypersecretion of electrolytes and water from infected enterocytes mediating the profuse diarrhoea characteristic of EPEC infection.

A consequence of PLC activity is the cleavage of GPI anchors of several protein types. The role (if any) of these during the course of the disease is uncertain, but one possibility is that release of GPI anchored proteins involved in cell-to-cell adherence would eventually begin to break down the structure of the villus. Cells experiencing prolonged stimulation as described above would eventually lose viability and be removed from the villus surface by loss of cell-to-cell adherence, possibly by cleavage of GPI anchored proteins. With prolonged infection characteristic of neonatal diarrhoea, continual cleavage of GPI anchors would eventually lead to extensive histological damage to the mucosal surface. The release of GPI anchored proteins by PLC would also extend the period of time that cells experience the effects of stimulation. In normal circumstances activation of cells via signal transduction pathways is transient, being rapidly returned to pre-stimulation conditions by processes of down-regulation. However, the region of GPI anchors containing DAG or PA which remain in the membrane are also biologically active, and will continue to stimulate cells by activation of PKC and induction of PLC after the signal transduction pathway has been down regulated by phosphorylation of intermediate G-proteins; the DAG moiety would stimulate PKC activity, and the phosphorylated derivative of DAG, PA, would promote calcium mobilization, by activation of PLC and PIP_2 hydrolysis independent of receptor binding. This may play a role in the extended PKC

activation and intracellular calcium rise observed in EPEC infected HEp-2 cells. Other factors which may contribute to extended activation in infected cells are the replenishment of 1,4,5-IP_3 sensitive stores by 1,3,4,5-IP_4-dependent influx of extracellular calcium directly into 1,4,5-IP_3 sensitive regions of the endoplasmic reticulum and the dynamic nature of EPEC infection of target cells, which would mediate activation of new regions of the cell as microcolony size increases through bacterial growth.

In conclusion, infection with EPEC in many ways mimics the effects of various hormones and growth factors, in stimulating host cell signal transduction processes. Prolonged infections contributes to the severity of the disease by artificially extending activation of normally transient processes. No doubt EPEC diarrhoea results from the consequences of two effects of extended cellular activation, (1) reduction in the absorptive area of the gut (possibly more important in later stages of the disease), and (2) secretory diarrhoea mediated by elevated calcium and activation of PKC. The two branches of the model, calcium and PKC, would have many interactive functions during disease, particularly in intestinal hypersecretion and cytoskeletal arrangement, but it seems reasonable to assume that many more consequences of PKC activity and calcium elevation await discovery.

Proposed future work includes cloning of the virulence genes responsible for these processes, the identification of the protein products, and their action on cells. Since there is similarity between the initial stages of cell invasion with *Shigella* and EIEC and the EPEC lesion, the genes and protein products thereof may show some relatedness. This, and the fact that AA strains of *E. coli* have been shown in this study to activate PKC via a soluble factor or toxin will require a more extensive comparison of EPEC and other pathogenic mechanisms of *E.coli.*

The activation of PKC and substantial rise in intracellular calcium observed in EPEC infected cells raises the real possibility that the expression of certain cellular oncogenes (in particular *ras* and *src*) may be affected. This is of particular interest since prolonged PKC activation and oncogene expression has been implicated in

transformation processes and raises the worrying prospect that extended EPEC infection may lead to genetic change and transformation in the enterocyte progenitor cells of the crypts.

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APPENDIX 1.

Bacterial strains.

Strain name	Serotype	Biotype	HEp-2 adh.	Reference.
HB101	rough	C/B	NA	**Boyer and Dussoix (1969).**
E2348-69	O127	EPEC	LA	**Knutton et al., 1989.**
MAR001	O127	EPEC	LA	**Knutton et al., 1989.**
2036-80	O119	EPEC	LA	**Knutton et al., 1989.**
660-79	O55	EPEC	LA	**Knutton et al., 1989.**
182-83	O128ac	EPEC	LA	**Knutton et al., 1989.**
E851	O142	EPEC	LA	**Knutton et al., 1989.**
1923-77	O111	EPEC	LA	**Knutton et al., 1989.**
DPO95	O18ac	EAEC	DA	**Knutton et al., 1989.**
135	O18ac	EAEC	DA	**Knutton et al., 1989.**
JPN10	O44	EAEC	AA	**Knutton et al., 1989.**
136	O86	EAEC	AA	**Knutton et al., 1989.**
469-3	O21	EIEC	DA	**Hinson et al., 1987.**

Strain N3406, cloacin DF13 producer. *E. coli* F205 containing plasmid pJPN73 which is pClo.DF13 of *Enterobacter cloacae* tagged with Tn*901* confering ampicillin resistance. Provided by Dr. E. Griffiths NIBSC, South Mimms, Herts.

Strain BZB2102 (K12), colicin B producer. *E. coli* W3110 *gyr*A containing the plasmid pColB of K260. Provided by Dr. E. Griffiths NIBSC, South Mimms, Herts.

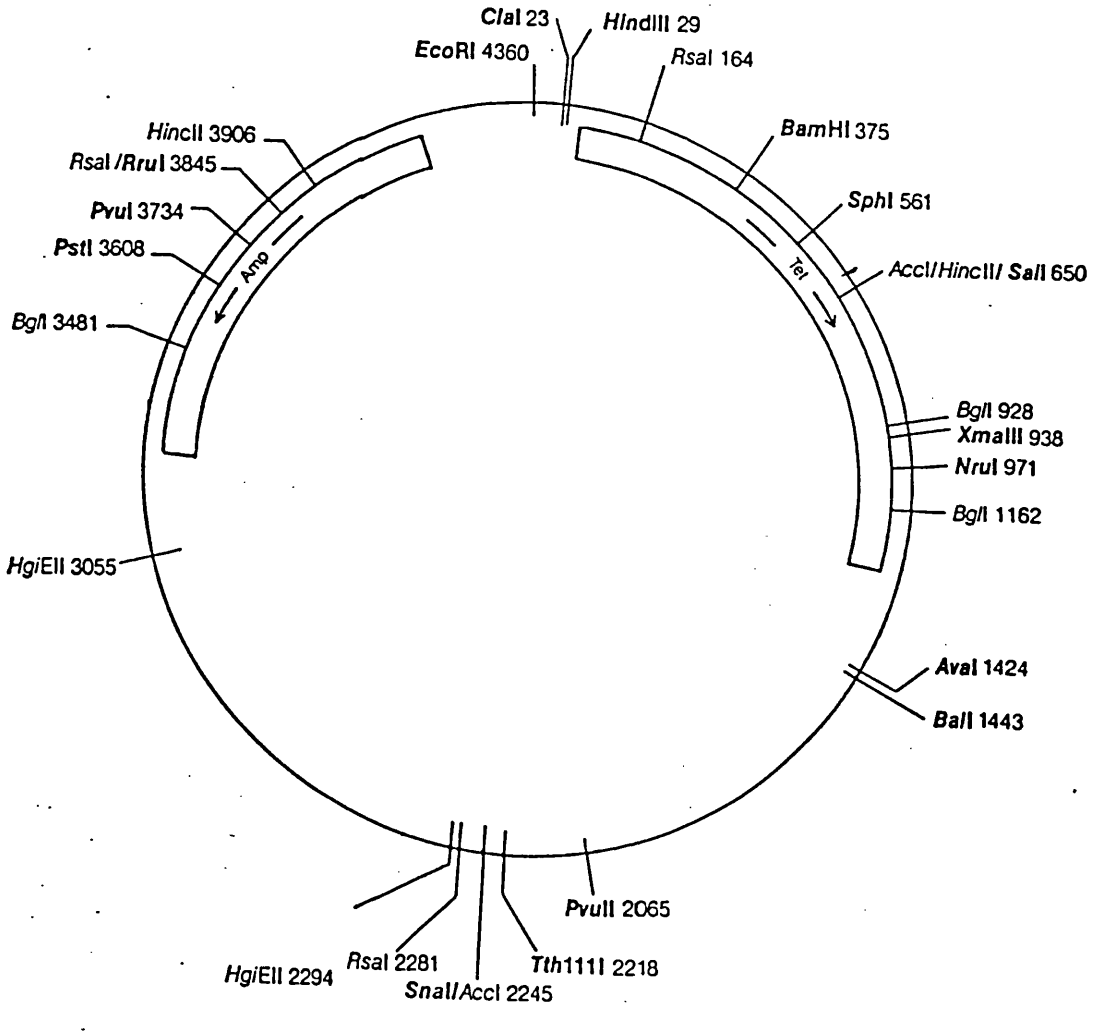
Strain BZB2103 (K12), colicin D producer, *E. coli* W3110 *gyr*A containing the plasmid pCold of CA23. Provided by Dr. E. Griffiths NIBSC, South Mimms, Herts.

PES (Portsmouth EPEC serogrouped) *Escherichia coli* strains were isolated and provided by Dr. R. Lowe and Mrs. D.L. Roberts, St. Mary's Hospital, Portsmouth.

PLASMIDS

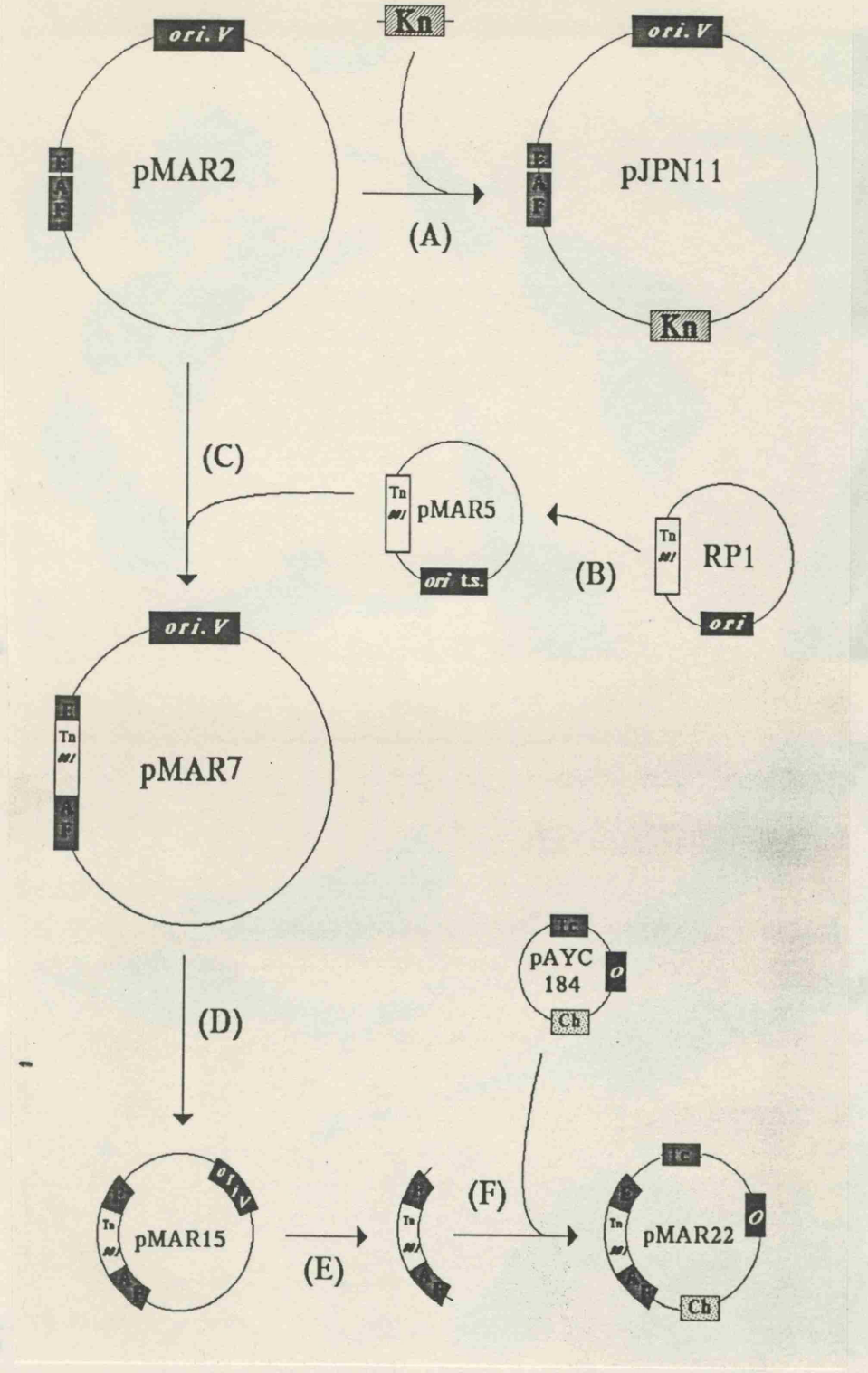
pBR322 (cloning vector), pCP410 (enterochelin biosynthesis genes), pMS101 (enterochelin uptake genes), pEN7 (aerobactin receptor gene) were supplied by Dr. E. Griffiths NIBSC, South Mimms, Herts. pABN5 (aerobactin biosynthesis genes) this laboratory, Dr. P.H. Williams.

APPENDIX 2



Restriction map of cloning vector pBR322.

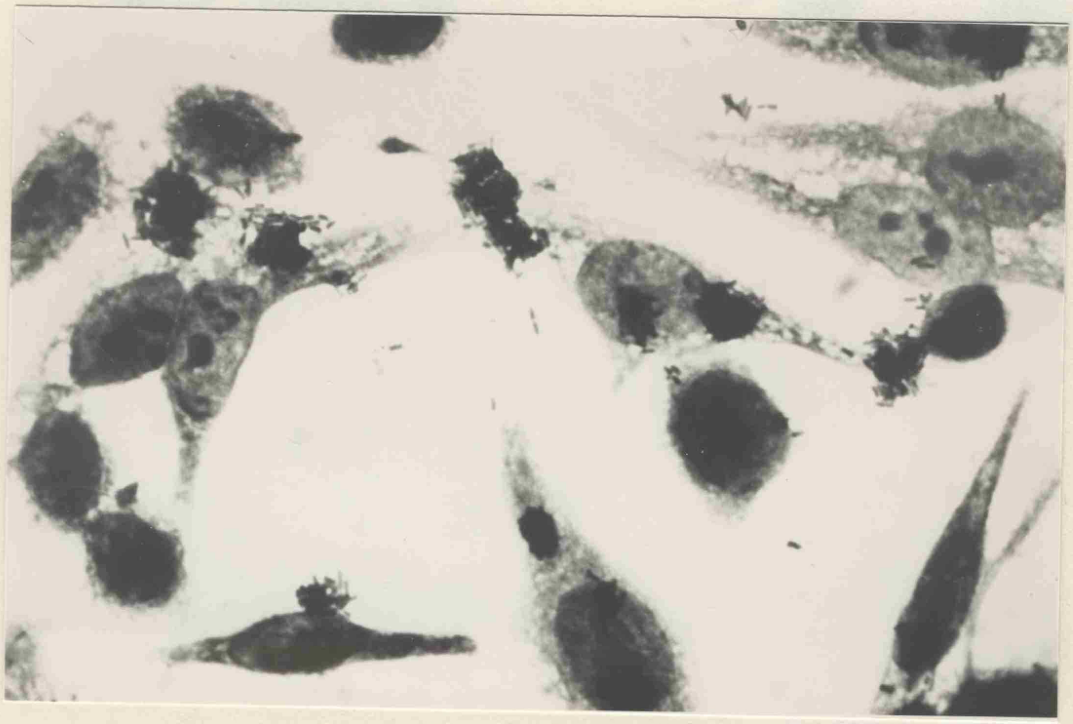
APPENDIX 3



The construction of pMAR15 from pMAR2 and the origin of all genetic elements

APPENDIX 4

A



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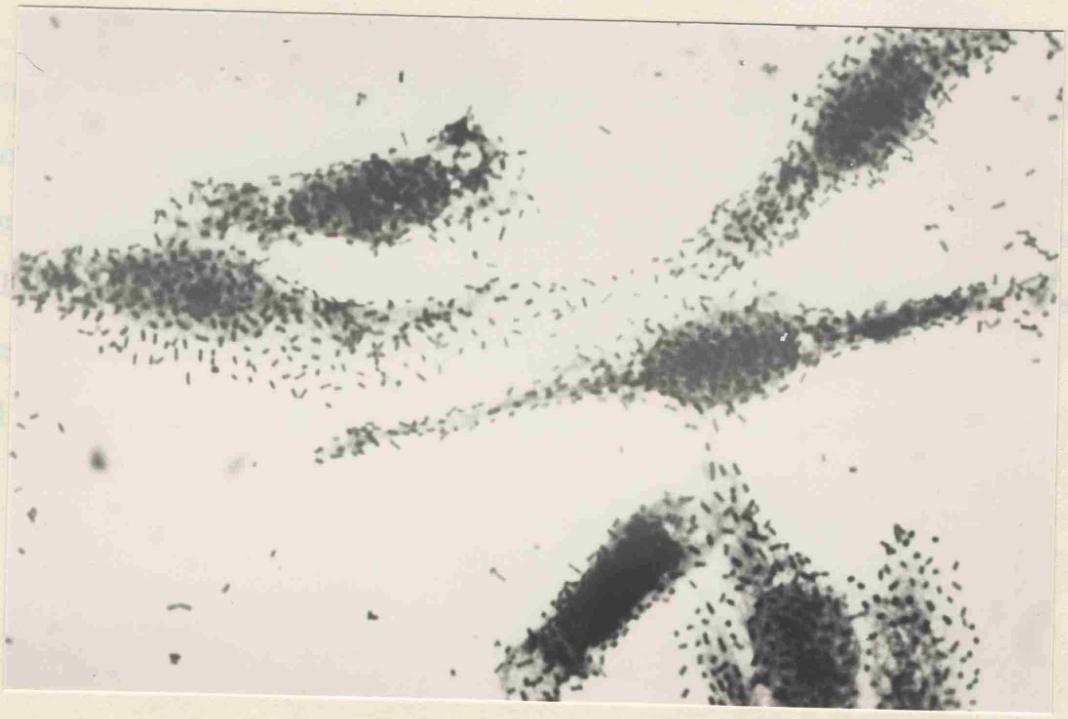
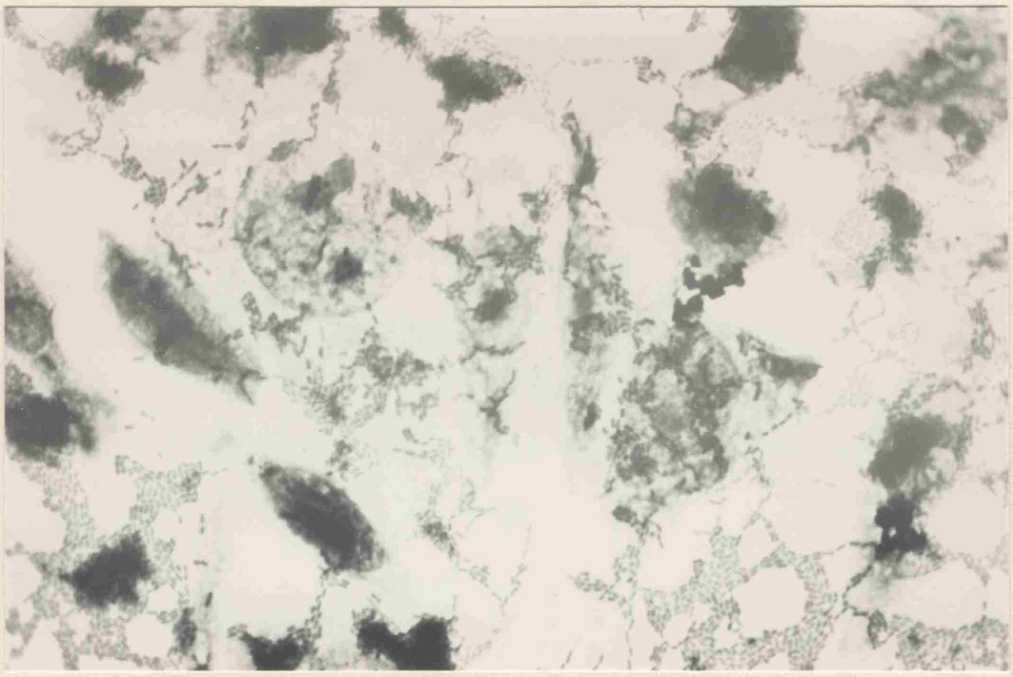


C



Adherence patterns EPEC serogroup *Escherichia coli* isolates to cultured HEp-2 cells. HEp-2 monolayers were grown to 50% confluence and then infected with 10^8 *E coli*. Incubation was continued for 3 h at 37°C after which time monolayers were washed 3 times with sterile PBS and then fixed and stained with Giemsa. Three main patterns of adherence can be identified, **(A)** Localized adherence (LA), **(B)** Diffuse Adherence (DA), and **(C)** Aggregative Adherence (AA). Magnification 2,000 X.

APPENDIX 5.

Abreviations

A	absorbance
AA	aggregative adherence
AE	attaching and effacing
ADP	adenosine diphosphate
-AM	Acetoxymethyl ester derivative of compound
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BAPTA	bis-[*O*-aminophenoxy]-ethane-N,N,N',N'-tetracetic-acid
BSA	bovine serum albumin
Ca^{2+}	calcium ions
$[Ca^{2+}]_I$	intracellular calcium concentration
CaCo	Colonic Carcinoma
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
Cl-	chloride ions
cm	centimetre
CMP	cytosine monophosphate
DA	diffuse adherence
DAG	Diacylglycerol
Db.cAMP	dibutyryl cyclic AMP
Db.cGMP	dibutyryl cyclic GMP
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethylsulphoxide
DNase 1	deoxyribonuclease 1
EDTA	ethylenediaminetetra acetic acid

EGTA	ethyleneglycol-bis(β-aminoethy ether) N,N,N′,N′, tetra acetic acid
EHEC	enterohaemorrhagic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EPEC	enteropathogenic *Escherichia coli*
ER	endoplasmic reticulum
ETEC	enterotoxigenic *Escherichia coli*
F	fluorescence
F_{max}	maximum fluorescence
F_{min}	minimum fluorescence
FAS	Fluorescent Actin Staining
FCS	Foetal Calf Serum
FITC	fluorescein isothiocyanate
g	gravitational constant
GDP	guanosine diphosphate
GDPβS	guanosine 5′-O-(thiodiphosphate)
Gp	GTP binding protein
GPI	glycosyl-phosphatidylinositol
GTP	guanosine triphosphate
h	hours
HEL	Human Embryonic Lung
HEp	Human Epithelium
I	*myo*-inositol
IL-1	interleukin-1
IL-2	interleukin-2
1-IP	1-inositol monophosphate
1,4-IP_2	1,4-inositol bisphosphate
1,4,5-IP_3	1,4,5-inositol trisphosphate
1,3,4,5-IP_4	1,3,4,5-inositol tetrakisphosphate
K	dissociation constant
K^+	potassium ions

kb	kilobase
kDa	kilodalton
kg	kilogram
LA	localized adherence
LPS	lipopolysaccharide
LT	heat labile enterotoxin
LUA	Luria agar
LUB	Luria broth
μCi	microcuries
min	minutes
mg	milligram
μg	microgram
Mg^{2+}	magnesium ions
ml	millilitres
μl	microlitre
mm	millimetres
μm	micromitre
mM	millimolar
μM	micromolar
Mn^{2+}	Manganese ions
N	normal
Na^{+}	sodium ions
nm	nanometres
PA	Phosphatidic acid
PBS	phosphate buffered saline
PEG	polyethylene glycol
PI	Phoshatidylinositol
PIP	phosphatidylinositol 4-monophosphate
PIP_2	phosphatidylinositol 4,5-diphosphate
PKC	protein kinase C
PKM	protein kinase M

PLAP	placental type alkaline phosphatase
PLC	phospholipase C
PLD	phospholipase D
PNPP	*p*-Nitrophenylphosphate
PS	phosphatidylserine
PSβG	pregnancy-specific β1-glycoproteins
quin2	2-{[2-bis-(carboxymethyl)-amino-5-methyl -phenoxy]-methyl}-6-methoxy-8-bis-(carboxymethyl)-aminoquinoline
RNase A	ribonuclease A
rpm	revolutions per minute
SDS	sodium dodecylsulphate
sec	seconds
ST	heat stable enterotoxin
TCA	trichloroacetic acid
TPA	12-O-tetradecanoylphorbol 13-acetate
TSST-1	toxic shock syndrome toxin I
v	volume
w	weight
W	watts
2D	two dimentional

APPENDIX 6.

SUPPLIERS

Reagents were supplied (unless otherwise stated) by:-

inorganic reagents	--	**Fisons plc**, Loughborough, LE11 ORG. England. and **BDH Chemicals Ltd.**, Broom Road, Poole, Dorset, BH12 4NN. England.
Bacteriological media	--	**DIFCO Laboratories Ltd.**, Central Avenue, East Molesey, Surrey, KT8 OSE. England.
Restriction enzymes	--	**GIBCO BRL**, Longbridge Way, Uxbridge, Middlesex UB8 2UG. England.
Organic reagents	--	**SIGMA Chemical Company Ltd.**, Fancy Road, Poole, Dorset, BH17 7NH. England.
Calcium ionophores, and fluorescent dyes.	--	Calbiochem. **Novabiochem (UK) Ltd.**, University Boulevard, Nottingham, NG7 2QJ.

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Sodium Dantrolene was a generous gift from **Norwich Eaton Pharmaceuticals, Inc.**, Norwich, New York, 12815-0191. USA.