THE *IN VITRO* ATTACHMENT OF *ESCHERICHIA COLI* TO HUMAN UROEPITHELIAL CELLS: INVESTIGATION OF HOST AND BACTERIAL CELL FACTORS.

A THESIS PRESENTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN MICROBIOLOGY AT MASSEY UNIVERSITY,
PALMERSTON NORTH, NEW ZEALAND.

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1982

TO

MUM AND DAD

ACKNOWLEDGEMENTS

I am indebted to the Department of Microbiology and Genetics, Massey University, New Zealand for providing me with the opportunity and facilities for this study. In particular, I thank Dr. Heather J.L. Brooks for her encouragement and expertise, and for the use of her collection of *E.coli* isolates. Also, many thanks to Professor D.F. Bacon, Head of Department, for his friendly and scientific guidance.

I acknowledge the grateful receipt of a Rotary International Foundation Scholarship, and the support of a Medical Research Grant of New Zealand.

Thanks to Drs. Svanborg Edén, Parsons, Beachey, Ørskov and Sugarman for their kind donation of reference strains. Also to Dr. K.A. Bettelheim, National Health Institute, Wellington, for serotyping a number of isolates, and to Dr. A.A. Corbett, Grey Street Medical Laboratory, for use of his facilities.

Special thanks to the following people:

Mr Mervyn J. Birtles and Irene Hall in the Department of Physiology and Anatomy, for friendly advice on histology and for assistance with a reproducability study.

Mr Doug H. Hopcroft and Mr R. Bennett of the Electron Microscope laboratory, D.S.I.R. and Mr Nick Ellison, for assistance with sectioning of samples and for providing photographs.

Dr. Ian G. Andrews for donation of compounds.

Mr W.D. Stirling for patient direction of statistical analysis.

The Immunohaematology Department, Palmerston North Hospital, for supplying human erythrocytes.

My many true friends, especially Catherine, Kerry, Pat, Kay, Lesley, Sarah, John, Davie, Craig, Dougie, Ronnie Scruff, Dr. Roy Thornton, Drs. Vaughan and Cathy Crow, and the staff of the Department of Microbiology and Genetics.

I am indebted to Kirsty Stalker for her patience and diligence in typing this manuscript.

Finally, to the women who so ably filled the bottles: in order of volume, Catherine, Wendy, Margaret, Fiona, Jayne, Sarah, Lesley, Isabel, Jocelyn, Mrs C,G,H and Z, plus the single donors! This study would not have been possible but for their drinking habits the night before!

ABSTRACT

Escherichia coli is the main causative organism in urinary tract infections (UTI) in adult women. Attachment of E.coli to the uroepithelium is believed to be an important step in the onset of infection, as it enables the bacteria to resist hydrokinetic clearance mechanisms. In the present study, a reproducible *in vitro* test system was used to assay E.coli attachment to uroepithelial cells harvested from the urine of premenopausal women.

Type specific antisera and an indirect fluorescent antibody technique were also used to detect E.coli attached to uroepithelial cells. The results from this study were comparable to those obtained using methylene blue as a bacterial stain. The fluorescent antibody technique was applied to the study of uroepithelial cells from women with symptomatic UTI, and E.coli were seen attached in varying numbers to these cells. Uroepithelial target cells with large numbers of E.coli attached, were found to exist within any given cell population. The capacity to attach to uroepithelial cells appeared to be a property held by the majority of E.coli strains isolated from urinary tract infections.

Receptivity of uroepithelial cells to attachment of certain E.coli strains varied over the menstrual cycle in a repetitive, cyclical pattern, similar to expected oestrogen levels. Highest attachment values were obtained between days 9 and 13, 18 and 28, and lowest values between days 14 and 17 of the menstrual cycle. Uroepithelial cells collected from six women in the first few months of pregnancy were highly receptive to E.coli attachment; whereas cells collected in the later months were less receptive. These results did not follow expected oestrogen levels.

The bladders of a pig, a lamb and a group of mice were examined for the presence of mucopolysaccharides. Periodic acid Schiff's

(PAS) reagent and alcian blue were used to identify a wide range of mucus substances. Several techniques were devised incorporating these two stains in combination with viability or bacterial stains to examine the mucopolysaccharides coating human uroepithelial cells from the urine sediment. These double-staining techniques proved to be highly reproducible. Significantly more viable than nonviable uroepithelial cells were coated with mucus. Attachment of one E.coli strain was greater to mucus coated than to non-coated cells; while attachment of a second strain was apparently unaffected by the presence of a mucus coat.

A proportion of E.coli strains produce a mannose sensitive haemagglutinin (MSHA) detected with guinea pig erythroyctes; while a proportion of E.coli strains produce a mannose resistant haemagglutinin (MRHA) detected with human erythrocytes. The role of these bacterial haemagglutinins in the attachment of E.coli to uroepithelial cells was investigated. The expression of a MSHA was in some cases affected by storage of E.coli for 12 months; while expression of a MRHA was not affected by storage. The presence of a MSHA did not enhance attachment to uroepithelial cells. However, after growth on trypticase soy agar, a number of strains expressed a MRHA and attached in significantly greater numbers to uroepithelial cells. Several experiments were designed to investigate the nature of the MRHA.

Electron microscopy was used to examine E.coli cells for the presence of pili. Strains expressing a MSHA were highly piliated; strains possessing a MRHA were highly piliated, poorly piliated or non-piliated. Further studies were carried out using transmission electron microscopy and scanning electron microscopy to examine bacterial attachment to the uroepithelial cell surface. In the former, ruthenium red was used in one instance to stain the polysaccharides which coat the bacterial and uroepithelial cells.

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INTRODUCTION

SECTION 1.1

THE EPIDEMIOLOGY AND PATHOGENESIS OF URINARY TRACT INFECTIONS

Urinary tract infections (UTI) are a common cause of illness in early childhood, infancy, sexually active women and in the elderly (Black and Moore, 1969; Savage, 1971; Stamey, 1978; Siegel *et al*. 1980). In males, urinary infections rarely occur before the age of 50, in the absence of instrumentation. It has been estimated that some 10 to 20% of all women acquire a UTI at some time in their lives (Stamey, 1978). These infections can be termed "specific" or "non-specific" (Smith, 1978). The non-specific urinary tract infections can be further classified as either complicated or uncomplicated. The former refers to bacterial invasion of the urinary tract leaving residual inflammatory changes; and also refers to infections related to an obstruction of the urinary tract (Kunin, 1979). Uncomplicated infections have no underlying structural or neurological lesions. These generally represent the first few episodes of UTI in women, and tend to respond well to chemotherapy. It has been estimated that *Escherichia coli* accounts for over 80% of the species recovered from cases of uncomplicated UTI (McFadyen *et al*. 1973), whereas, species of *Proteus*, *Klebsiella*, *Enterobacter*, *Enterococci*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* are more commonly recovered from patients with previous history of infection and/or instrumentation (Kunin, 1979).

Several types of UTI may be diagnosed. Significant *Bacteriuria* is the term given to infections diagnosed by the presence of a

significant number of viable bacteria in the urine, (> 10^5 Bacteria per ml urine) (Brumfitt and Hamilton-Miller, 1981). The incidence of bacteriuria increases with age, and is often as high as 10% in elderly women. Routine cultures of pregnant women and sexually active, non-pregnant women have revealed that up to 7% have significant asymptomatic bacteriuria (Waltzer, 1981). Untreated bacteriuria leads to clinically apparent acute pyelonephritis in 40% of cases in later stages of pregnancy. (Kass, 1960,a). The term Cystitis refers to infection of the bladder, causing symptoms of frequency, dysuria, urgency and suprapubic discomfort (Smith, 1978). Pyelonephritis has been broadly defined as an inflammatory process of the kidney and its adjacent structures, and is usually associated with vesicoureteral reflux. Acute pyelonephritis is a clinical term consistent with symptoms of fever, flank pain, tenderness and the presence of leukocytes and red blood cells in the urine (Kunin, 1979). An increased incidence of this disease has been reported in pregnant women (Whalley, 1967). Chronic pyelonephritis is a term which implies that bacteria or bacterial antigens are persistant in the kidney, giving rise to a continuing inflammatory response (Kunin, 1979). Asymptomatic bacteriuria is a term given to a UTI where significant numbers of viable organisms are present in urine, without the presence of clinical symptoms of an infection (Stamey, 1978). The "Urethral Syndrome" is a term given to an ailment in patients with symptoms of lower UTI, in the absence of significant bacteriuria. This condition is common in up to 50% of women who present themselves to their general practitioner with symptoms of UTI. After discussion with a Consultant Urologist at Palmerston North Hospital, it appears that in certain clinical cases squamous metaplasia is diagnosed in patients with the urethral syndrome. Cautorization of the squamous cells coating the bladder trigone often results in a loss of UTI symptoms.

Recently, this syndrome has been re-named "dysuria and frequency without significant bacteriuria" as reported by Brumfitt and Hamilton-Miller (1981). It is possible that organisms such as mycoplasma species are involved in patients with this syndrome. Birch et al., (1981) have isolated Ureaplasma urealyticum in patients with "sterile pyelonephritis", after urine collection by suprapubic puncture. Other organisms may be found to be involved though alternative causes of this syndrome may be related to physiological disturbances or psychological disorders. These possibilities are at present a matter of great interest and discussion.

Recurrent urinary tract infections are an important clinical problem. Approximately 80% of these are reinfections of the urinary tract with different organisms at each occurrence (Stamey, 1975). The importance of recurrent infections has lead to their inclusion in the clinical classification of UTI: these categories are (a) first infection, (b) unresolved bacteriuria, (c) bacterial persistence in recurrent infections and, (d) reinfection of the urinary tract in recurrent infections. (Brumfitt and Percival, 1964; Stamey, 1975; Cicmanec and Evans, 1980).

1.2 BACTERIAL ENTRY INTO THE URINARY TRACT.

Four main pathways of bacterial entry into the urinary tract have been proposed by Smith (1978); namely, the ascending route of infection, hematogenous spread, lymphatogenous spread and direct extension from another organ. It is believed that the ascending route is the most common in UTI, particularly in women as they have a shorter urethra as compared to men (Brumfitt and Percival, 1964). The infecting organisms are widely believed to be of faecal origin. These bacteria sequentially colonize the colon, vaginal vestibule, periurethral region and distal urethra, before ascending into the

bladder (Kunin, 1979). Colonization of the urethral and periurethral areas by potential uropathogens has been reported to occur to a greater degree in women susceptible to UTI than in healthy females. (Cox, 1966; Hinman, 1966; Cox et al., 1968; Elkins and Cox, 1974; Kallenius and Winberg, 1978; Bollgren et al., 1979; and Marrie et al., (1980,b). Furthermore, pathogenic and nonpathogenic bacteria have been found colonizing areas deep within the urethra (Cox, 1966). The normal urinary tract from the bladder neck to the glomerulus is sterile (Stamey et al., 1965). Introduction of bacteria from these externally colonized areas into the bladder may not necessarily lead to infection (Kunin, 1978). It is generally accepted that the main reason that the bladder is inherently resistant to infection is the intermittant cleansing action of voiding urine, which in effect removes any bacteria from the bladder. For an organism to initiate infection, it must firstly resist this urine outflow, possibly by attaching to the bladder lining. The importance of this bacterial attachment will be discussed later.

1.3 THE HOST IMMUNE RESPONSE.

As in most infections, the host defence mechanisms are activated in response to an attack of UTI. Serum antibody responses have been reported in patients with renal and bladder infections (Bremner et al., 1969; Mattsby-Baltzer et al., 1981). A local antibody response of IgG and secretory IgA antibodies has been detected in urine from children with UTI (Sohl Akerlund et al., 1979). Antibodies in combination with the wash-out effect of urine may prevent colonization of the urinary tract. The adhesion of E.coli to uroepithelial cells has been shown to be inhibited by IgG and secretory IgA antibodies isolated from children with acute pyelonephritis. (Svanborg Edén and Svennerholm, 1978). Initial

colonization of the vaginal introitus may also be prevented by IgG and/or IgA in the vaginal fluid (Stamey and Howell, 1976).

Significantly higher numbers of Gram-negative bacteria have been reported by Hofmann et al., (1981) to colonize the periurethral area of young girls prone to UTI or suffering an acute attack compared to healthy children. This finding was thought to be related to a local weakness in the host defence mechanism of the children susceptible to infection. Riedasch et al. (1981,b) have reported that the rat bladder mucosa is capable of mounting a local immune response against bacterial invasion. By using a rat model, Kaijser et al., (1978) have investigated the use of vaccination to protect against ascending E.coli pyelonephritis. The possibility of protecting against ascending pyelonephritis by vaccination has also been considered by Silverblatt and Cohen (1979) using antipili antibody, and by Brooks et al., (1977) using formalin-treated E.coli cells. Further investigations on the host immune response to infection should prove useful, and may assist with attempts to induce a protective response in patients with a history of recurrent infections, by vaccination.

1.4 THE USE OF ANTIMICROBIAL AGENTS IN THE TREATMENT OR PREVENTION OF URINARY TRACT INFECTIONS.

Uncomplicated urinary tract infections are often effectively resolved with the use of antimicrobial agents. The range of antibiotics used in relation to the treatment of various forms of UTI have been well documented and reference is made to three reviews (Kunin, 1979; Stamm, 1980; Sugarman and Pesanti, 1980). In clinical practise, most primary infections are cleared with an orally absorbed drug, given for a period of 7 to 10 days. Single-dose therapy is also applied in some patients, and

has been shown to be effective. Prophylaxis with drugs such as trimethoprim-sulfamethoxazole or nitrofurantoin has been shown to prevent emergence of drug resistant E.coli, and prevent recurrence of infection in susceptible women (Stamm et al., 1980). Urinary tract infections caused by antibiotic resistant E.coli strains have been known to occur. Lincoln et al., (1970) have suggested that these bacteria initially attain their resistance in the intestine, following treatment of the patient with antibiotics. These drug resistant bacteria then ascend to the urinary tract and give rise to infection. The bacteria present in the bladder may ascend to the kidneys and give rise to acute retrograde pyelonephritis. Bille and Glauser (1981) have reported that this infection can be prevented by aminoglycoside treatment, which results in an accumulation of antibiotic in the renal tissues. Although it is outwith the scope of the present study to examine antimicrobial therapy in great detail, one area of study is worthy of discussion.

Recent reports have investigated the effect of subinhibitory concentrations of antibiotics on bacterial attachment to epithelial cells. (Beachey et al., 1981; Scheld et al., 1981; Tylewska et al., 1981). Eisenstein et al., (1979;1981) have reported that sublethal concentrations of streptomycin significantly reduce E.coli mannose mediated binding to epithelial cells. Beachey et al., (1981) found that sublethal concentrations of penicillin prevented the surface expression of the mannose-specific adhesins of E.coli, by distorting cell wall biosynthesis. However, once the adhesins had been formed, their activity was not affected by the low antibiotic concentration. It appears that the penicillin affected the production of type 1 pili, which mediated the E.coli attachment to the human buccal cells. The aberrant fimbrial protein did not produce the mannose sensitive haemagglutination of guinea pig

erythrocytes characteristic of the presence of type 1 pili. In a study by Frimodt-Møller et al., (1981) the ability of E.coli to adhere to the bladder wall of rats was found to significantly decrease after intravenous administration of mecillinam. Inhibition of a mannose resistant haemagglutinin (MRHA) of E.coli by tetracycline has been reported by Roland and Heelan (1979). They suggest that antibiotics may prevent E.coli adherence to the intestinal mucosa, or may detach the bacteria, once adherence has occurred. In these experiments, human group 0 erythrocytes were tested with E.coli strains, in the presence of each of thirty-two antibiotics. Investigations which attempt to inhibit the mannose resistant haemagglutination reaction are of importance, particularly as this MRHA is thought to be involved in mediating attachment of certain E.coli strains to uroepithelial cells (Ørskov et al., 1980,b). In the present study, a number of compounds were tested for inhibition of the MRHA; although no antibiotics were used. With the present debate over the widespread use of antibiotics and the existence of drug resistant pathogens in the environment, it is perhaps wise to investigate other compounds for inhibition of bacterial attachment to mucosal surfaces. Such a compound would be particularly useful if it could inhibit production of the "bacterial adhesin" and also prevent attachment to the epithelial surface.

1.5 UROPATHOGENIC PROPERTIES OF E.COLI ISOLATED FROM CASES OF URINARY TRACT INFECTION.

The word pathogenicity refers to the capacity of an organism to produce disease. This capacity is possessed by relatively few microbial species, and in these cases to varying degrees (Smith, 1977). The complexity of the infectious process emphasises that

many factors are involved in the bacterial cell to host cell interaction. As suggested by Boyce and Edwards (1960), the importance of host susceptibility should not be overlooked, especially as only a small proportion of women appear to be susceptible to recurrent UTI. However, it may be that differences between bacterial populations are partly responsible for the varied severity of UTI in patients. These differences may be due to possession of a number of uropathogenic properties. Several research workers have investigated these properties of E.coli strains isolated from cases of UTI (Bettelheim and Taylor, 1968; Gruneberg and Bettelheim, 1968; Brooks et al., 1980; 1981; Hanson et al., 1981; Van den Bosch et al., 1981,b). From these publications, the following conclusions can be drawn: Of the E.coli strains causing UTI, 80% belong to common O-antigen groups O1, O2, O4, O6, O7, O8, O9, O18, O25, O68 and O75. Strains with high K-antigen titre ($\geq$ 32) are more commonly isolated from urinary tract infections than from the periurethral area or stools of healthy individuals. Certain K-antigens, especially K1, are more commonly found in E.coli urinary isolates. Bacteria causing acute pyelonephritis are resistant to the bactericidal activity of normal serum (Lidin-Janson et al., 1977). This resistance has been associated with the amount of K-antigen present on these strains (Glynn et al., 1971). The presence of this capsular material on E.coli cells may increase the invasiveness and virulence of these strains particularly in the kidney. A large number of E.coli urinary strains ferment dulcitol and salicin, though the significance of this has not been fully established. The prevalence of haemolytic strains in UTI may suggest that haemolysin production is related to the ability of E.coli to invade and infect the urinary tract. The α-haemolysin is a cell-free, filterable protein, commonly produced by these E.coli urinary isolates (Dudgeon et al., 1921; Williams Smith, 1963;

Zwadyk and Snyder, 1971; Cooke and Ewins, 1975; Minshew et al., 1978). The β-haemolysin is cell associated (Williams Smith, 1963). Recently, Van den Bosch et al., (1981,a) reported that haemolytic, virulent E.coli strains killed mice more rapidly than non-haemolytic, virulent strains. From experiments using this mouse model, it was concluded that haemolysin production by E.coli is a decisive virulence factor in nephropathogenicity.

Current attention is being centred on the inclusion of the capacity of bacteria to attach to uroepithelial cells as an additional uropathogenic property. It appears that the virulence of E.coli strains in the urinary tract, may be related to the possession of one or more of these uropathogenic characteristics.

SECTION 2.1

THE PHENOMENON OF ATTACHMENT.

The phenomenon of attachment is important in the pathogenicity of a variety of microorganisms, especially bacteria. Different aspects of the subject are at present under considerable investigation and several worthwhile reviews have recently been published. (Gibbons and van Haute, 1975; Smith, 1977; Ellwood *et al*., 1979; Bitton and Marshall, 1980, Beachey, 1981; Elliot *et al*., 1981). It is apparent from these reports that most infections begin on the mucosal membranes of the alimentary, respiratory and urogenital tracts. As these areas are bathed by a mucosal layer, moving lumen contents and often by commensal microorganisms, attachment may be a means by which pathogens colonize these tissues. This adhesiveness is thought to involve a specific interaction between bacterial surface ligands and receptors in the epithelial cell surface (Elliot *et al*., 1981). The importance of bacterial attachment has resulted in a number of studies with a variety of organisms and tissue material. Rather than discuss each study at length, a list is presented in Table 1A; this gives representative examples of studies carried out on microbial attachment to mucosal surfaces, for organisms apart from *E.coli*. Reference can be made to the reviews described above, with additional references being included in the table itself. It is apparent from the size of the table, that the phenomenon of attachment is under widespread examination. A separate review of each area of investigation would be impracticable. However, within the confines of this present study, it is worthwhile discussing the importance of a number of *E.coli* strains in relation to attachment properties. Enterotoxigenic *E.coli* (ETEC) are an important cause of diarrhoea in humans and animals. It is believed that these strains require additional

TABLE 1A

EXAMPLES OF STUDIES ON MICROBIAL ATTACHMENT TO MUCOSAL SURFACES.

ORGANISM	SITE OF ATTACHMENT	ADDITIONAL REFERENCES
Vibrio cholerae	epithelium of small intestine	
Vibrio parahaemolyticus	human fetal intestinal (HFI) cells	
Neisseria gonorrhoeae	urethral, cervical epithelium, tissue cultures, buccal cells	Trust et al., 1980
Neisseria meningitidis	buccal epithelial and posterior pharyngeal cells, erythrocytes	Salit, 1981 Salit and Morton, 1981
Oral Streptococci sp.	oral, pharyngeal cells, teeth, tongue, hydroxyapatite beads, tissue cultures	Kilian et al., 1981
Streptococcus sanguis	canine heart valves, human fibrin and platelets	Ramirez-Ronda, 1980 Scheld et al., 1981
Proteus mirabilis	uroepithelial, buccal cells, tissue cultures	Sugarman, 1980 Peterson et al., 1981
Staphylococcus aureus	nasal mucosal cells, pharyngeal cells	Fainstein & Musher, 1979 Aly et al., 1980
Pseudomonas aeruginosa	human buccal, tracheal cells	Johanson et al.,1979 Ramphal et al., 1980
Shigella species	intestinal epithelium	
Salmonella typhimurium	ileal mucosa of mice, human buccal cells, human and animal erythrocytes and uroepithelial cells.	Duguid et al., 1966 Tannock et al.,1975
Bordetella pertusis	tissue cultures	
Klebsiella pneumoniae	human buccal cells, erythrocytes, rat bladder cells	Fader et al., 1979 Sugarman & Donta, 1979,b
Enterobacter aerogenes	human buccal cells	Sugarman & Donta, 1979,b

TABLE 1A Cont'd

ORGANISM	SITE OF ATTACHMENT	ADDITIONAL REFERENCES
Haemophilus sp.	pharyngeal cells, erythrocytes	Scott & Old, 1981
Serratia marcescens	erythrocytes, pharyngeal cells	Duguid *et al.*, 1966 Fainstein & Musher, 1979
Yersinia enterocolitica	mammalian epithelial cells	Okamoto *et al.*, 1980 Old & Robertson, 1981
Clostridium perfringens	pig intestine	
Bacteroides sp.	rat peritoneal mesothelium, erythrocytes, human buccal cells	Onderdonk *et al.*, 1978 Okuda *et al.*, 1981
Corynebacterium renale	bovine bladder epithelial cells	Takai *et al.*, 1980
Eikenella corrodens	human buccal epithelial cells	Yamazaki *et al.*, 1981
Actinomyces naeslundii	human buccal epithelial cells	Saunders & Miller, 1980
Veillonella sp. *Lactobacilli* sp.	teeth, tongue, buccal cells	
Candida albicans	human uroepithelial, vaginal, buccal cells	Marrie & Costerton, 1981 Sobel *et al.*, 1981,a
Mycoplasma pneumoniae	ciliated respiratory epithelium, erythrocytes	Sobeslavsky *et al.*, 1968
Chlamydia trachomatis	conjunctival, urethral epithelium, cell cultures	
Entamoeba histolytica	erythrocytes, bacteria, intestinal epithelial cells	
Leishmania donovani	hamster macrophages	Giannini *et al.*, 1981
Adenovirus	various mucosal epithelia	
Influenza virus	bronchia and tracheal epithelium	

virulence properties, along with enterotoxin, for the manifestation of disease (Levine, 1981). Various ETEC isolates have been found to possess piliated antigens. The K88 and K99 antigens are plasmid-mediated and enable E.coli to attach to the intestine of piglets (K88) and to the intestine of calves and lambs (K99) respectively, giving rise to diarrhoea (Ørskov and Ørskov, 1966; Ørskov et al., 1975; Levine, 1981). Colonization factor antigens (CFA) I and II were later identified from cases of human diarrhoea. These piliated antigens are also plasmid-mediated and promote *in vitro* adhesion of ETEC strains to epithelial cells (Evans et al., 1975: Evans and Evans, 1978; Levine, 1981). Strains possessing the K88, K99 antigens, or the CFA/I or CFA/II all exhibit a mannose resistant haemagglutination pattern with various groups of erythrocytes. (Jones and Rutter, 1974; Burrows et al., 1976; Evans et al., 1980,b; Levine, 1981). The importance of bacterial haemagglutinins will be discussed later. Finally, a new pilus antigen, named 987p, has been found on ETEC strains which lack a K88 or K99 antigen. This pilus mediates colonization of the piglet ileum (Isaacson et al., 1978).

The phenomenon of bacterial attachment to mucosal surfaces has been one of the most fruitful areas of investigation in infectious diseases. This mechanism of attachment appears to be important in UTI, because E.coli attached to the uroepithelium might be able to resist hydrokinetic clearance by voided urine. Once attached, the bacteria may then multiply and give rise to infection (Mackintosh et al., 1975). Studies of E.coli attachment to uroepithelial cells are therefore a great importance, particularly when host cell and bacterial cell factors are considered. In the present report, investigations were carried out on several important areas concerning the attachment of E.coli to uroepithelial cells, using a modified *in vitro* assay system, first reported by Svanborg Edén et al., (1976).

2.2 *IN VITRO* ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

At present, no suitable experimental model has been described for studying the role of bacterial attachment in animals naturally prone to UTI. Tissue culture models suitable for attachment studies are also rare, although Salit and Gotschlich (1977) reported that E.coli attached to monkey kidney (vero) cells on a monolayer culture. Several *in vitro* models have been described following the initial work of Gibbons and van Haute (1971) who studied bacterial attachment to oral epithelial surfaces.

In 1976, Svanborg Edén et al. reported that E.coli attached *in vitro* to uroepithelial cells from human urinary sediment. This ability to attach was believed to be related to the severity of the UTI produced *in vivo*. This *in vitro* model employs uroepithelial cells harvested from the urines of healthy individuals, and assayed with E.coli isolated from various types of urinary tract infections. The technique involves visual counting of bacteria attached to the uroepithelial cells: Reproducability studies were later reported illustrating the technique's effectiveness (Svanborg Edén et al., 1977; Svanborg Edén, 1978). However, variations were discovered in the receptivity of uroepithelial cells from different samples, to E.coli attachment. These variations were not adequately investigated by Svanborg Edén. Using radiolabelled bacteria, Schaeffer et al., (1979) reported that uroepithelial cell variation correlated with hormonal changes, known to arise over the menstrual cycle. The importance of this cyclical uroepithelial cell variation has not been adequately investigated since this report. As this is in need of further clarification, an investigation was carried out in the present study to determine E.coli attachment to uroepithelial cells collected from urine samples at intervals over consecutive

menstrual cycles. A similar study was also carried out using uroepithelial cells collected from urine samples of pregnant women, to investigate whether receptivity to E.coli attachment varied during pregnancy. Other reports have investigated bacterial attachment to vaginal epithelial cells (Mardh and Weström, 1976; Fowler and Stamey, 1977; 1978; Botta, 1979; 1981; Parsons et al., 1979,a; Parsons and Schmidt, 1980). These studies are of importance in relation to the pathogenesis of urinary infections for two reasons. Firstly, the genital and urinary systems are embryologically related (Tanagho, 1978). Secondly, when the route of infection is ascending, uropathogens are believed to attach to the vaginal introitus prior to onset of infection in the urinary tract. (Stamey, 1978). In the same sense, studies on attachment to periurethral cells can also be regarded as important (Kallenius and Winberg, 1978; Kallenius et al., 1980,b).

Uroepithelial cell samples used in the *in vitro* system contain transitional cells representative of the bladder and ureter epithelium, and squamous cells from the bladder trigone and urethra. Morphologically the bladder epithelium can be regarded as an extension of the kidney tubules, and in some respects is comparable to the epithelia of the human mouth, oesophagus and vagina (Hicks, 1975). Although transitional cell turnover is slow, there are sufficient numbers of cells present in the sediment of mid-stream urine samples to carry out the *in vitro* attachment assay. Svanborg Edén et al.,(1976) incorporated a trypan blue stain in their assay. This dye is taken up by nonviable epithelial cells at Stage 3 of cell death (King et al., 1959). Erythrosine B can also be used in this way, to distinguish viable cells from the nonviable cells which do not respire or carry out glycolysis (Phillips and Andrews, 1959). The incorporation of a viability stain and a bacterial stain would certainly assist with identification

of bacteria attached to viable epithelial cells. Such a double-staining combination was devised for the *in vitro* assay system used in the present study. The Gram stain and indirect fluorescent antibody staining technique have been used by Tannock et al., (1975) to illustrate Salmonella typhimurium attached to the ileal mucosa of mice. Stains have been used in a number of studies to illustrate bacteria attached to cell surfaces. The use of fluorescent antibody is extremely worthwhile as it enables precise identification of bacteria attached to epithelial cells. This technique was incorporated in the present study: This is the first known report which detects E.coli attached to uroepithelial cells, with the use of a fluorescent antibody technique.

A histological study of the human bladder has shown that mucopolysaccharides coat the luminal surface(Monis and Dorfman, 1967). Although studies on mucopolysaccharides have been carried out using rabbit bladders (Parsons et al., 1975; Shrom et al., 1977, Parsons et al., 1979,b) no such study has been attempted on uroepithelial cells from the urinary sediment. Such an investigation would be of interest particularly if the mucus coating on cells could be related to cell viability and to the numbers of bacteria attached after completion of an *in vitro* assay. If mucus can be identified on uroepithelial sediment cells, it could be argued that these are adequately representative of cells lining the bladder cavity *in vivo*. Several double staining techniques were devised in the present study, to investigate the presence of mucopolysaccharides on the uroepithelial cell surface. Stains such as periodic acid Schiff's(PAS) reagent and Alcian blue were used to detect the presence of mucopolysaccharides on the uroepithelial cell surface. The PAS reaction is a sensitive one, detecting proteoglycans, neutral mucosubstances, glycolipids and certain other polysaccharides. Alcian blue is a basic dye which forms links with anionic groups in

glycosaminoglycans (GAG). The nature of these dyes and their application has been reported elsewhere (Drury and Wallington, 1967; Lamanna et al., 1971; Culling, 1974). The possibility that mucopolysaccharides on the uroepithelial cell surface have a role in attachment of E.coli, is worthy of investigation.

2.3 THE IMPORTANCE OF BACTERIAL AND HOST CELL PROPERTIES IN THE ATTACHMENT PROCESS.

The reports of Svanborg Edén and Hansson (1978) and Schaeffer et al., (1979) suggested that attachment of E.coli to uroepithelial cells is mediated by type 1 pili present on the bacterial surface. However, more recently, Ørskov et al., (1980,a;1980,b) suggested that type 1 pili mediate adherence to urinary slime, Tamm-Horsfall glycoprotein, and not to uroepithelial cells. The presence of Type 1 pili is indicated by the possession of a mannose sensitive haemagglutinin (MSHA). This haemagglutinin is detected with guinea pig erythrocytes, and is inhibited in the presence of α D mannose. A large proportion of E.coli urinary isolates possess a MSHA suggesting that this is an additional uropathogenic property (Brooks et al., 1981). The role of type 1 piliated strains inducing a MSHA, in the attachment of E.coli to uroepithelial cells is in need of further clarification. Several experiments are described in the present report, which investigated the role of a MSHA in E.coli attachment to uroepithelial cells. Of greater importance in the attachment process may be the possession of a mannose resistant haemagglutinin (MRHA) by E.coli isolates. This factor is detected with human erythrocytes and is not inhibited by the presence of α D mannose. The MRHA is commonly found on E.coli strains isolated from patients with UTI (Brooks et al., 1981). Expression of a MRHA is in some cases associated with the presence

of pili other than type 1 (Duguid et al., 1979). Of the 19 MRHA+ | MSHA-strains examined by Duguid et al., 12 strains were piliated, while the other 7 strains were non-piliated. Attachment to uroepithelial cells of strains possessing a MRHA has been recently reported by Korhonen et al., (1980,a). These authors have emphasized the importance of a pili-mediated attachment mechanism. However, there is a high incidence of non-piliated strains of E.coli which possess a MRHA (Ip et al., 1981). Whereas the MSHA is best expressed on bacteria grown aerobically in static broth cultures, the MRHA is best expressed after growth on a solid agar surface (Duguid et al., 1979; Ørskov et al., 1980,b; Fein, 1981). It is important that these growth conditions are taken into account when studying bacterial haemagglutinins. Further investigations are needed to determine the role of the MRHA on piliated and non-pilated E.coli strains in the attachment to uroepithelial cells. In the present study, a group of E.coli strains were examined for the presence of pili, using electron microscopy. The presence or absence of pili on these stains was correlated with their haemagglutination properties, and their capacity to attach to uroepithelial cells. Other electron microscopy techniques were also used in this study to investigate bacterial attachment to uroepithelial cells. These methods incorporated the use of the transmission and the scanning electron microscopes.

Several studies have recently reported the haemagglutination properties of pathogenic bacteria other than E.coli; namely Vibrio cholerae eltor (Bhattacharjee and Srivastava, 1978), Yersinia enterocolitica (Maclagan and Old, 1980), Aeromonas hydrophila (Atkinson and Trust, 1980), Neisseria meningitidis (Salit, 1981) and Haemophilus species (Scott and Old, 1981). These reports emphasize the importance of bacterial haemagglutinins in relation

to a wide variety of pathogenic interactions. Clearly the haemagglutination properties of E.coli and other pathogens deserve further investigation. In the present report, several experiments were designed to investigate the role of bacterial haemagglutinins in the attachment of E.coli to uroepithelial cells.

As well as possession of uropathogenic properties by E.coli strains, the susceptibility of host cells to bacterial attachment should be considered of great importance in the pathogenesis of UTI. It appears that target cells exist within a host cell population, to which large numbers of bacteria attach (Gibbons and van Haute, 1971; Svanborg Edén, 1978; Parsons et al., 1979, a) The nature of these target cells has yet to be determined. Recently, globoseries glycolipids were proposed as one type of receptor molecule on the uroepithelial cell for E.coli attachment (Leffler and Svanborg Edén, 1980;1981). Hicks (1975) has reported that the luminal bladder membrane is comprised of one third protein and two thirds lipid, of which a large proportion is cerebroside. This cerebroside may be related to the glycosphingolipid receptor site for E.coli attachment. It is possible that the density or nature of glycolipids or other receptor sites on the uroepithelial cell, is important in confering susceptibility of certain women to UTI.

Susceptibility to bacterial attachment appears to involve a multitude of host and bacterial factors, but it may reflect the host susceptibility to UTI. By studying bacterial attachment to uroepithelial cells from women with symptomatic UTI, it should be possible to relate *in vitro* findings with the *in vivo* situation. Such studies may initiate additional investigations which may, in time, determine why certain women suffer recurrent urinary tract infections while others do not even acquire a first infection. An examination of bacteria attached to uroepithelial cells collected from women with symptomatic UTI, is reported in the present study.

MATERIALS

A. BACTERIA

A total of 145 E.coli urinary isolates and 38 E.coli faecal isolates were stored in 2 cm^3 brain heart infusion (BHI) broth with 0.4 cm^3 glycerol at -10°C. The E.coli urinary strains were as follows:

STRAIN NUMBER	DETAILS
104, 178, 226	Isolated from children with acute pyelonephritis.
27 TO 313	Isolated, 1978 to 1979 from female, domiciliary patients aged 16 or more years with symptomatic urinary infection.
314 TO 319	Isolated 1975 from female, domiciliary patients aged 16 or more years with symptomatic urinary infection. Stored at 4°C on agar slopes.
320	Isolated from a woman with acute pyelonephritis, Donated by Dr. C. Svanborg Edén, Sweden.
321	Supplied by Dr. C. Lowell Parsons,U.S.A.
322	Strain C1212-77 reported by Ørskov et al. (1980,b).
323	Strain C1214-77 reported by Ørskov et al. (1980,b).
324 TO 326	Donated by Dr. Barrett Sugarman, U.S.A.
327	Donated by Dr. E.H. Beachey, U.S.A.

Strains 320 to 327 were obtained as reference strains known to attach to epithelial cells. The E.coli faecal strains were isolated from the stools of healthy women and stored in 2 cm^3 BHI broth with 0.4 cm^3 glycerol at -10°C. All strains were serotyped for O, H and Kl antigens by Dr. H.J.L. Brooks, Massey University and Dr.K.A. Bettelheim, National Health Institute, Wellington.

B. BACTERIAL GROWTH MEDIA

The media used to culture E.coli included brain heart infusion (BHI) broth, BHI agar, beef extract broth (Bacto, Difco, U.S.A.), trypticase soy broth, trypticase soy agar (TSA), (BBL, U.S.A.) nutrient broth, nutrient agar (Gibco, U.S.A.) and cysteine lactose electrolyte deficient (CLED) agar (Oxoid, U.K.).

C. UROEPITHELIAL CELLS

Uroepithelial cells were harvested from mid-stream urine (m.s.u.) samples supplied by healthy, premenopausal women whose ages ranged from 19 to 40 years. No samples were collected from women using oral contraceptives, antibiotics or other medicines. The samples were transported to the laboratory and used without delay. A few were discarded when found to contain an excess of urinary mucus, crystalline material, amorphous debris, erythrocytes or indigenous bacteria.

D. BUFFER SOLUTIONS

Various buffer solutions were used in the study. Buffered saline was made up per 1000 cm^3 distilled water, at a concentration of 0.01 M. The pH was recorded at room temperature. Sodium chloride was added to the buffers to attain isotonicity with the uroepithelial cells. The standard buffer used was phosphate buffered saline (PBS) at pH 7.1, and osmolality of 300 m osmoles per litre.

1. Buffered saline pH 3.0

NaCl	8 g
Citric Acid Anhydrous	1.67g
$Na_2HPO_4.2H_2O$	0.29g

2. Buffered saline pH 3.8

NaCl	8 g
Citric Acid Anhydrous	1.02 g
$Na_2HPO_4.2H_2O$	0.73 g

3. Buffered saline pH 4.7

NaCl	8 g
Citric Acid Anhydrous	0.854g
Trisodium citrate dihydrate	1.632g

4. Buffered saline pH 5.8

NaCl	8 g
$NaH_2PO_4.H_2O$	1.4 g
$Na_2HPO_4.2H_2O$	0.12 g

5. Buffered saline pH 6.4

NaCl	8 g
$NaH_2PO_4.H_2O$	1.15 g
$Na_2HPO_4.2H_2O$	0.38 g

6. Buffered saline pH 7.1 = Standard PBS

NaCl	8 g
$NaH_2PO_4.H_2O$	0.34 g
$Na_2HPO_4.2H_2O$	1.21 g

7. Buffered saline pH 8.0

NaCl	8 g
$NaH_2PO_4.H_2O$	0.08 g
$Na_2HPO_4.2H_2O$	1.35 g

8. Buffered saline pH 9.6

NaCl	8 g
Na_2CO_3	1.06 g
$NaHCO_3$	0.756g

9. Buffered saline pH 10.1

NaCl	8 g
Na_2CO_3	0.636g
$NaHCO_3$	0.336g

10. PHILLIPS PBS pH 7.0

NaCl	8.42 g
KCl	0.3 g
$CaCl_2$	0.12 g
$MgSO_4.7H_2O$	0.25 g
$NaH_2PO_4.H_2O$	0.04 g
$Na_2HPO_4.2H_2O$	0.31 g

11. JARVINEN PBS pH 6.9

NaCl	8 g
KCl	0.2 g
$CaCl_2$	0.1 g
$MgCl_2.6H_2O$	0.1 g
KH_2PO_4	0.2 g
$Na_2HPO_4.2H_2O$	1.15 g

All buffers were autoclaved at 121^{o}C for 20 mins. and stored at room temperature or 4^{o}C. Details regarding the use of buffers have been reported by Dawson et al.(1979) and Cruickshanks et al. (1975).

Cacodylate buffer (pH 7.2) was prepared by dissolving cacodylic acid. (SIGMA, U.S.A.) to the appropriate concentration in deionized water.

E. ERYTHROCYTES

Human A and 0 Rhesus positive erythrocytes were supplied by the Department of Immunohaematology, Palmerston North General Hospital. Guinea pig red blood cells were collected by cardiac puncture of healthy animals and 10% v/v sodium citrate (3.8% solution in distilled water) was added to prevent clotting. Sterile defibrinated horse erythrocytes were supplied by Gibco, New Zealand. Erythrocytes were stored at 4°C and used for haemagglutination tests within five days.

F. STAINS

A number of stains were used in the study to distinguish bacterial and uroepithelial cells. These stains are classified as follows with the concentration used for each being reported in the methods sections.

STAIN	DETAILS	BRAND NUMBER	MANUFACTURER
Methylene Blue	In PBS to pH 6.8	CI 52015	British Drug Houses (BDH)
Safranin	In Distilled Water	CI 50240	BDH
Erythrosine B	In PBS to pH 7.1	Batch 2844,CI 45430	Gurr, London.
Trypan Blue	In PBS to pH 7.5	CI 23850	BDH
Giemsa	In Glycerol 1:1 Methanol	NO.34034	BDH
Methyl Green	In Distilled Water	CI 42590	Sigma, USA
Malachite Green	In Distilled Water	CI 42000	BDH
Crystal Violet	In Distilled Water	CI 42535	Hopkins and Williams, U.K.
Ruthenium Red	Practical Grade in Cacodylate Buffer	R-2751	Sigma, USA

G. CHEMICALS USED

The product, batch or serial numbers are given with the manufacturer's name.

1. 17β oestradiol-3-methyl benzoate, E-9000, Sigma U.S.A.
2. Formaldehyde: 10 cm^3 formalin, 1.5 cm^3 distilled methanol to prevent polymerization, used as 0.5% solution in PBS (pH 7.1).
3. α-methyl-D-mannoside, M3752, Sigma.
4. α D mannose, M4625, Sigma.
5. Mannitol,Batch 94197, AJAX Chemicals, Australia.
6. Concanavalon A, Grade III, C-2631, Sigma.
7. D(+) galactose, G-0625, Sigma.
8. α L(+) arabinose, 683200, BDH.
9. α D(+) fucose, F-2127, Sigma.
10. α D(+) xylose, 30590, BDH.
11. α D ribose, 38058, BDH.
12. rhamnose, 590041, Difco, U.S.A.
13. α D raffinose, 38056, BDH.
14. dulcitol, D-0256, Sigma.
15. salicin, S-0625, Sigma.
16. Sorbitol, 30242, BDH.
17. galacturonic acid, 38029, BDH.
18. D-glucuronic acid, grade 1, Sigma.
19. α D-glucosamine HCl, Koch Light laboratories, England.
20. D-galactosamine HCl, Batch 40023, Koch Light.
21. N-acetyl-D-glucosamine, Batch 59785, Koch Light.
22. 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside (a glycoside of N-acetyl-galactosamine), Batch 67054, Koch Light.
23. N-acetyl neuraminic acid (sialic acid), A-2501, Sigma.
24. D fructose, 28433, BDH.
25. D(+) glucose.

26. sucrose.

27. α-D-kojibiose, Batch 52513, Koch Light.

28. α-D-melibiose, M-5500, Sigma.

29. lactose, Batch 80759, Koch Light.

30. D(+) melezitose H_2O, M-5375, Sigma.

31. D(+) maltose, 15718, Riedel-De Haënag Seelze, Hannover, Germany.

32. L-alanine, 37006, BDH.

33. L-leucine, L-8000, Sigma.

34. DL-serine, S-4375, Sigma.

H. MATERIALS FOR TISSUE CELL CULTURE

1. BHK Eagles Medium (Wellcome, England).

Filter sterilised (0.22 μm filter).

338 cm^3 standard medium with additions of

(i) penicillin, streptomycin, 10^6 units in 2 cm^3 saline.

(ii) 20 cm^3 bicarbonate buffer; a stock solution was made up with 22g $NaHCO_3$ and 12.5g 0.4% phenol red solution dissolved in 500 cm^3 of distilled water, saturated with CO_2, dispensed, autoclaved at 121°C for 15 minutes and stored at 4°C.

(iii) 40 cm^3 fetal calf serum, i.e. 10% of total volume. A 5% solution was used for maintenance cultures.

The medium was saturated with CO_2 and the final pH was 7.6.

2. Trypsin/versene: 8g NaCl, 0.4g KCl, 1g glucose, 0.58g $NaHCO_3$, 0.2 cm^3 1% phenol red solution, 0.5g trypsin, 0.2g versene in 1000 cm^3 distilled water. A stock solution was stored in 2 cm^3 aliquots at -20°C and 18 cm^3 distilled water was added for use.

3. Formal Saline: a 10% solution was used consisting of 90 cm^3 of 0.85% saline, 10 cm^3 formaldehyde stock.

4. T 199 medium (Wellcome, England).

5. Phosphate buffered saline (pH 5.8) as in Part D of materials.

METHODS

SECTION 1.

ATTACHMENT OF E.COLI CELLS TO HUMAN URINARY TRACT EPITHELIAL CELLS *IN VITRO*

1.1. ASSAY METHOD FOR *IN VITRO* ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

The method used to assay attachment of E.coli to uroepithelial cells was a modification of that described by Svanborg Edén et al. (1976).

BACTERIA: (1) GROWTH IN BROTH:- a loopful of bacteria taken from a stock culture was inoculated into 10 cm^3 BHI broth and incubated at 37°C for 16 hours. The bacterial suspension was then centrifuged at 1500 x g for 12 mins., resuspended in phosphate buffered saline (PBS) at pH 7.1 to a concentration of 10^{10} organisms per cm^3 PBS and further incubated at 37°C for 30 mins. to regenerate pili (Novotny et al. 1969).

(2) GROWTH ON AGAR:- a loopful of bacteria taken from a stock culture was inoculated onto a plate containing trypticase soy agar (TSA) and incubated at 37°C for 16 hours. Bacteria from the plate culture were suspended in PBS (pH 7.1) to a concentration of 10^{10} organisms per cm^3 PBS and incubated at 37°C for 30 mins.

A Neubauer haemocytometer (Weber, England) was used to count the number of bacteria in each suspension.

UROEPITHELIAL CELLS: The uroepithelial cells were harvested from early morning, mid-stream urine specimens by centrifugation at 250 x g for 12 mins., washed three times in PBS (pH 7.1) and

resuspended in 4 cm^3 PBS. To four drops of cell suspension was added one drop of erythrosine B (0.4% in PBS, pH 7.1); a red viability stain which is taken up by nonviable cells. One drop of this suspension was placed in a haemocytometer to assess total uroepithelial cell numbers and the number of viable cells present. Samples which had less than 20% cell viability or a total count of less than 1×10^5 cells were not used in the assay.

ATTACHMENT ASSAY: A test tube containing 1×10^8 bacteria and 1×10^5 uroepithelial cells in approximately 1 cm^3 PBS, pH 7.1 was incubated at 37°C for 60 minutes at 20 revs per minute in a water bath with a shaker. The suspension was washed by centrifugation five times in PBS at 250 x g for 12 minutes to remove unattached bacteria. After the second wash, 2 drops of 0.4% erythrosine B were added to the suspension. Subsequent washing removed excess stain and left the nonviable uroepithelial cells stained red and the viable cells unstained. The final suspension of cells in 0.5 cm^3 PBS was air dried onto a glass slide. To clearly distinguish attached bacteria, preparations were counterstained with methylene blue (0.4% in PBS, pH 7.1) rinsed in distilled water, air dried and examined at 1000 x magnification by light microscopy. Viable uroepithelial cells had a blue nucleus and light blue cytoplasm and nonviable cells had a dark purple nucleus and purple cytoplasm. Attached bacteria were dark blue and clearly distinguished on all parts of the cells. (Plates 1 and 2).

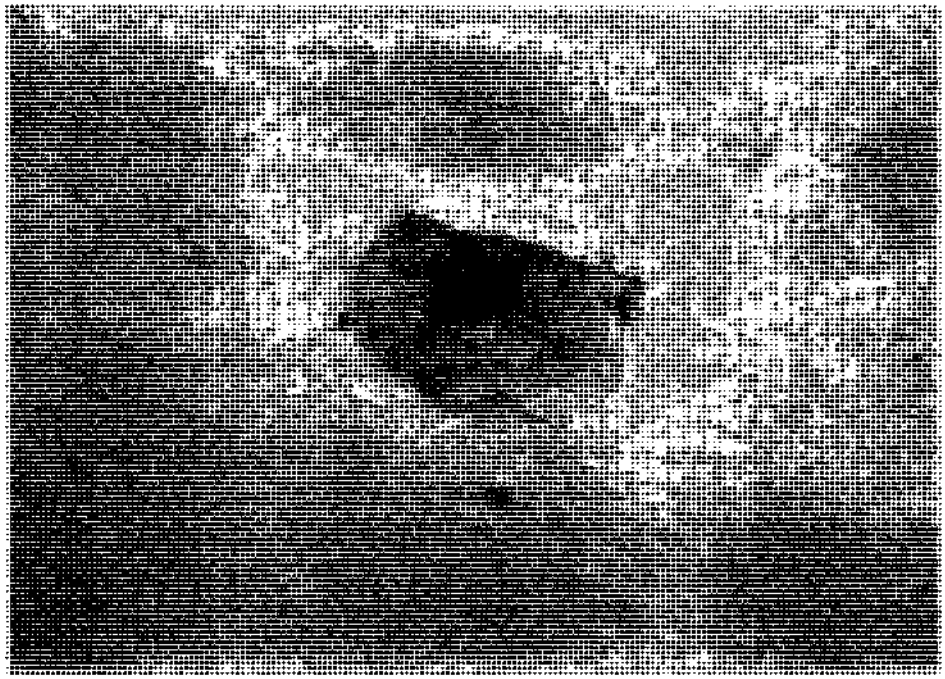
Forty viable uroepithelial cells (20 squamous and 20 transitional cells) were examined and the numbers of E.coli attached were recorded. Cells which appeared folded, clumped together or damaged were disregarded. Each slide was viewed from the top left corner in a systematic way to avoid examination of uroepithelial cells more than once.

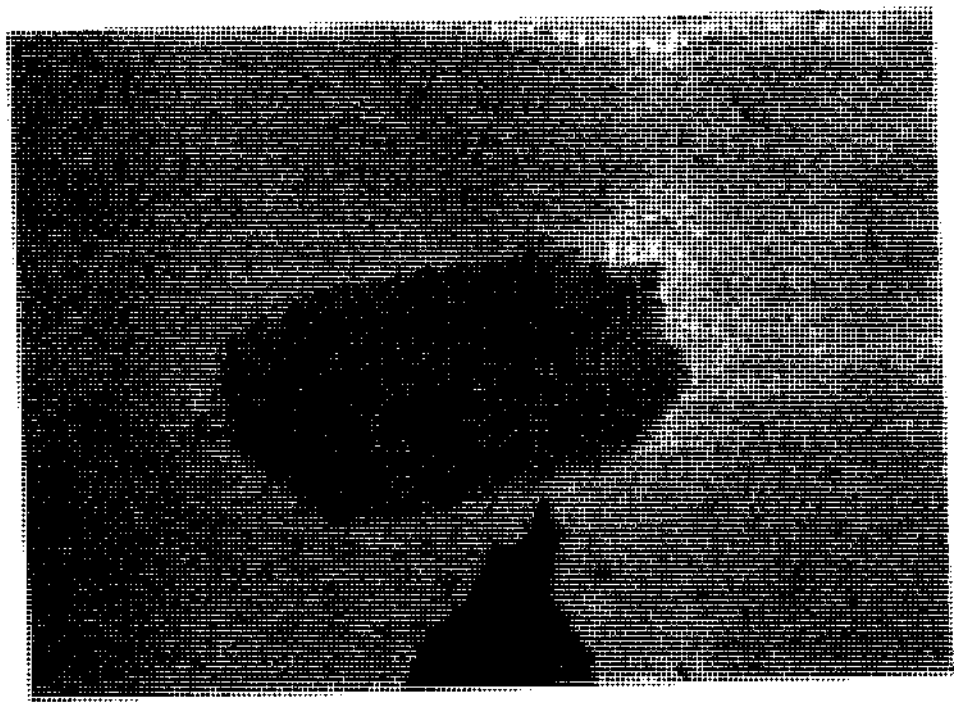
PLATE 1.

Nonviable transitional uroepithelial cell with no E.coli attached: stained with erythrosine B and methylene blue. (Magnification Ca x 360).

PLATE 2.

Viable squamous uroepithelial cell with E.coli strain 73 attached, following bacterial growth on TSA: stained with erythrosine B and methylene blue. (Magnification Ca x 360).





Controls consisted of uroepithelial cells in PBS without E.coli added, to assess any indigenous flora present on the cells. The mean number of E.coli-like organisms attached to 40 viable uroepithelial cells (20 squamous cells and 20 transitional cells) in the controls was deducted from the experimental values to give a net attachment figure for each test.

The pH of each urine sample was measured and samples were regularly monitored for asymptomatic urinary tract infections by culturing on cysteine lactose electrolyte deficient (CLED) agar. Some urine samples contained small amounts of urinary mucus. This was removed in the washing process as the mucus sedimented on centrifugation at a higher level than the uroepithelial cells. Between washes the cell samples were mixed on a vortex mixer to free cells from any mucus that was present in the cell sediment.

1.1.1 STATISTICAL ANALYSIS

Throughout the study several statistical methods were used to analyse the results. These methods can be referred to in "Fundamental Research Statistics" edited by J.T. Roscoe (1969).

1.2 DETECTION OF E.COLI ATTACHED TO UROEPITHELIAL CELLS BY AN INDIRECT FLUORESCENT ANTIBODY TECHNIQUE

A further experiment was carried out to determine the reproducability of the attachment assay. An indirect antibody technique was used to detect E.coli attached to uroepithelial cells. The mean values were compared with those obtained from double-stained preparations to verify that the latter technique, used in the assay, was effective in detecting E.coli attached to uroepithelial cells. In addition, this experiment would confirm that any indigenous

bacteria present on the uroepithelial cells do not interfere with E.coli attachment.

The preparation of type specific antisera for five strains of E.coli with serotypes 01, 04, 06:K1, 07 and 075 was similar to that described by Edwards and Ewing (1972). The E.coli were inoculated from stock cultures onto agar slopes then to 10 cm^3 BHI broth and incubated overnight at 37°C. Each culture was formalized by the addition of 0.05 cm^3 formalin and injected into the marginal ear vein of a rabbit. The first dose was 0.3 cm^3, the second 0.5 cm^3, the third 1 cm^3 and the 4th, 5th and 6th were 2 cm^3 with a four to five day interval between injections. Six to eight days after the final injection the rabbits were bled from the ear once weekly for three weeks. Up to 15 cm^3 of blood was collected on each occasion in a universal bottle, incubated at 37°C for 1 hour and placed at 4°C overnight. The serum was collected, centrifuged at 250 x g for 15 mins. to remove erythrocytes, dispensed in 2 cm^3 amounts into bijou bottles and stored at -20°C.

The titres of the E.coli antisera used in the test were determined as follows: One drop from an E.coli suspension of 10^{10} organisms per 1 cm^3 in PBS was added to each of 10 areas of a glass slide. The bacteria were air dried and fixed for 10 mins. with acetone. An antiserum to an E.coli strain was diluted twofold in PBS (pH 7.1) and one drop from each dilution was placed on a separate area of the slide and incubated in a moist atmosphere at 37°C for 30 mins. The slide was rinsed in PBS flooded with 1 cm^3 fluorescent-labelled antirabbit immunoglobulin (1:8 dilution in PBS) (Wellcome, England) and incubated for a further 30 mins. in a moist atmosphere at 37°C. The slide was rinsed in PBS, mounted in phosphate buffered glycerol (pH 9.2) and examined under the ultra violet light from the mercury lamp source of a Polyvar microscope (Reichert Jung). The antiserum titre was taken as the highest dilution where fluorescent

bacteria could be seen. The procedure was repeated for each of the five antisera.

A group of 20 E.coli strains were selected with common 0 serotypes 01, 04, 06, 07 and 075 and assayed for attachment to uroepithelial cells from pooled urine samples using the method described in section 1.1. On completion of the assay incubation step, the cell suspensions were each divided into two portions. Cells in one portion were washed and double-stained as in section 1.1, while cells in the remaining portion were washed five times in PBS, air dried on a glass slide and stained with fluorescent-labelled antibody in the following manner.

Each slide was fixed for 10 minutes in acetone, rinsed in PBS and incubated with the appropriate 0 serotype antiserum diluted in PBS for 30 minutes at 37^{o}C in a moist atmosphere. The slide was then rinsed in PBS with 3 buffer changes over 30 minutes. Each slide was incubated with fluorescent-labelled antirabbit immunoglobulin (1 in 8 dilution in PBS) for 30 minutes at 37^{o}C in a moist atmosphere, rinsed in 3 changes of PBS, mounted in phosphate buffered glycerol and examined under the epiluminescent ultra violet light source. The outline of the uroepithelial cells was evident without the need for a counterstain and 40 uroepithelial cells (20 squamous and 20 transitional cells) were evaluated for E.coli attachment. (Plates 3 to 6).

A control with uroepithelial cells and no E.coli added was included in the test. After the incubation period of the attachment assay, one portion of the suspension was washed and double-stained as in section 1.1. The other portion was air dried onto a slide, fixed for 10 minutes in acetone and flooded with 1 cm^{3} antiserum from a pool which consisted of 4 drops of each antiserum dilution. The slide was washed in 3 changes of PBS then stained with fluorescent antibody as described above. The number of E.coli

attached to 40 uroepithelial cells was evaluated.

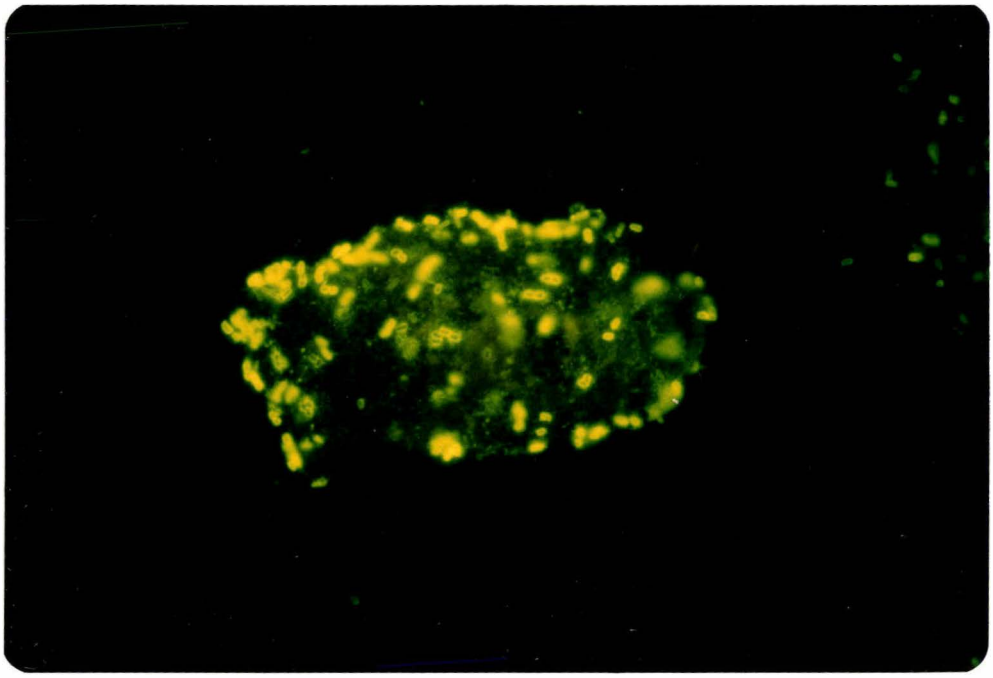
The mean values were calculated and compared for both staining techniques, using the two way analysis of variance.

PLATE 3.

Squamous uroepithelial cell with E.coli strain 289 (01:H7) attached, following bacterial growth on TSA: stained with fluorescent antibody (Magnification Ca x 360).

PLATE 4.

Squamous uroepithelial cell with E.coli strain 322 (06:K2:H1 attached, following bacterial growth on TSA: stained with fluorescent antibody (Magnification Ca x 360).



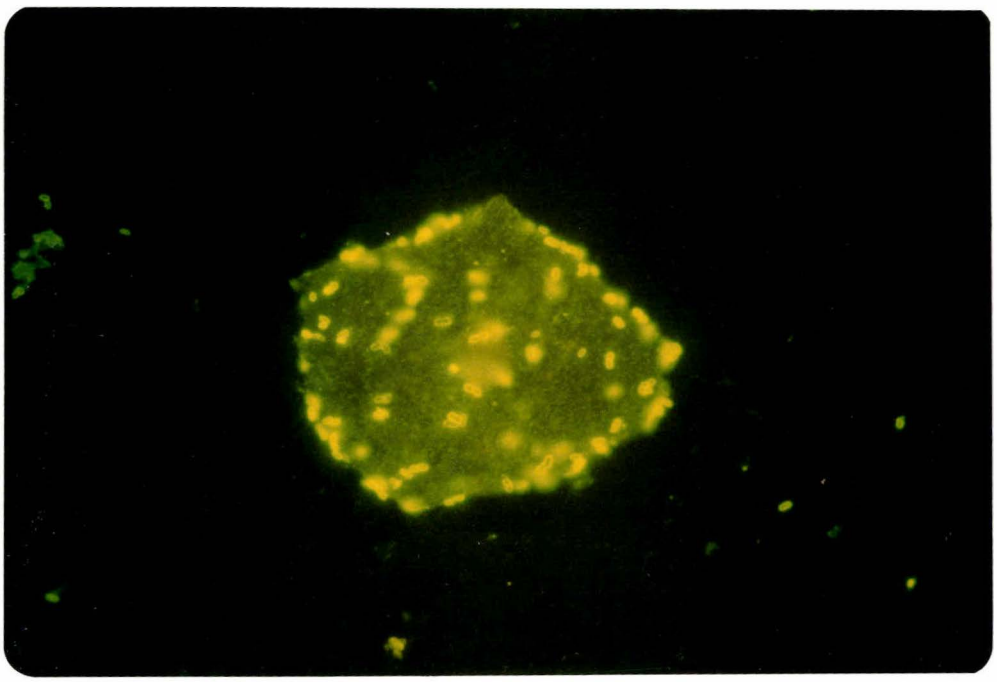
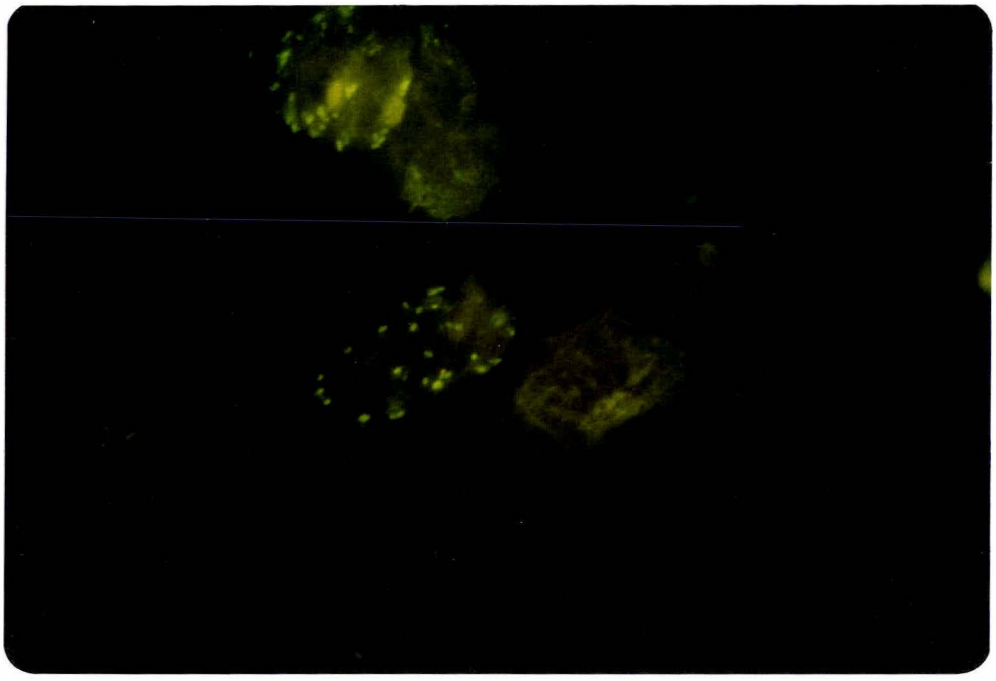


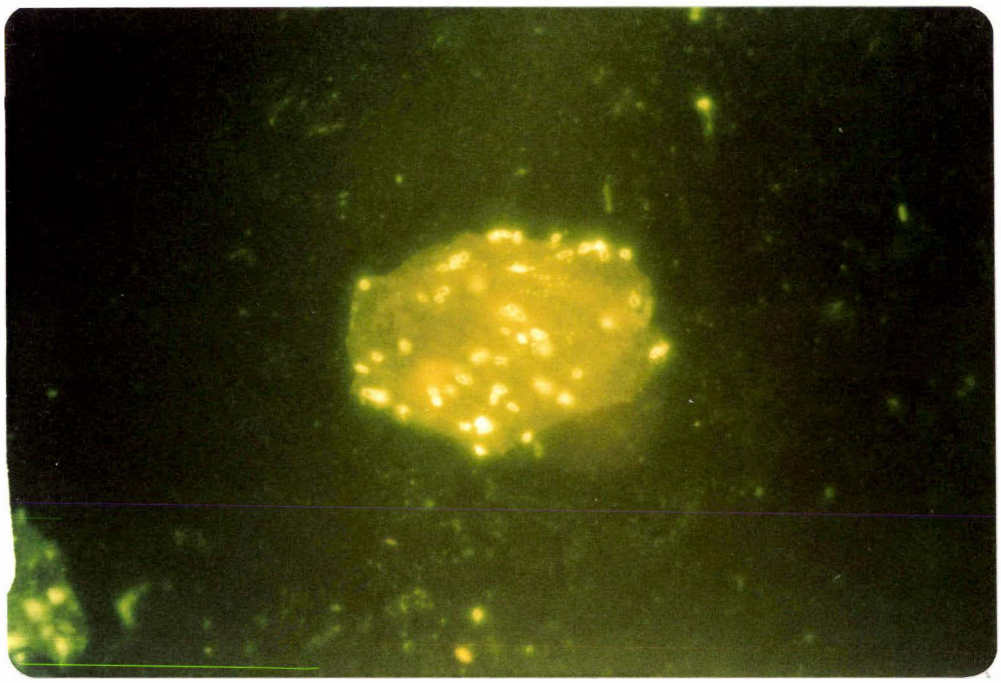
PLATE 5.

Squamous and transitional uroepithelial cells with and without E.coli strain 87 (075:HR) attached, following bacterial growth in BHI broth: stained with fluorescent antibody (Magnification Ca x 145).

PLATE 6.

Squamous uroepithelial cell with E.coli strain 207 (075:H-) attached, following bacterial growth on TSA: stained with fluorescent antibody (Magnification Ca x 290).





METHODS

SECTION 2.

HISTOLOGICAL STAINING TECHNIQUES USED TO INVESTIGATE MUCOPOLYSACCHARIDE-COATED EPITHELIAL CELLS

2.1 DETECTION OF MUCOPOLYSACCHARIDES ON SECTIONS FROM LAMB AND PIG BLADDERS.

Two histological stains were selected which identified a wide range of mucopolysaccharide substances. Periodic acid Schiff's stain (PAS) was used to distinguish proteoglycans, neutral mucosubstances, glycolipids and certain polysaccharides (Drury and Wallington, 1967). These substances were stained a distinctive magenta colour. Alcian blue (AB) was used to detect the presence of glycosaminoglycans which stained a blue colour. (Drury and Wallington,1967). Two urinary bladders, one from a lamb and one from a pig were removed at time of slaughter and processed as follows.

On removal each bladder was injected with Bouin's fluid to retain its shape and placed in a bottle containing Bouin's fluid for fixation at room temperature for 24 hours. The bladders were dehydrated in 70% alcohol for 18 hours and processed in an automatic tissue processor overnight. The bladders remained for a preset time in the processor beakers of 70%, 100%, 100%, 100% alcohol, chloroform, xylene,xylene and finally two beakers of paraffin wax heated to 140^{o}C. The specimens were removed, embedded in 60^{o}C molten wax and cut into blocks using a heated knife. A Reichert sliding microtome was used to section the specimens to 5 µm thickness and the sections were floated onto the surface of an electrothermal water bath. Selected sections were then placed

on glass slides which were coated with a thin layer of egg albumin to aid adherence to the slide. The slides were placed at 37^{o}C for 1 hour to dry the sections and then passed through xylene to remove excess wax. Each section was stained in one of two ways.

The first staining was with alcian blue, haematoxylin and eosin (H & E). The haematoxylin stained the cell nucleus and cytoplasmic basophilia black and eosin stained the cytoplasmic acidophilia pink to red (Culling, 1974). The use of the latter two stains gave better contrast to the sections but still allowed the alcian blue coated cells to be distinguished.

STAGES

1. Sections brought to water via 100%, 100%, 70% alcohol rinses.
2. Stained for 10-15 mins. in Alcian blue (0.3% in 3% aqueous acetic acid).
3. Rinsed in tap water.
4. Stained in aqueous Mayer's Haemulum (Haematoxylin) for 10 mins.
5. Washed in tap water.
6. Rinsed in Scott's tap water to blue for 2 mins.
7. Washed in tap water.
8. Stained in 1% aqueous eosin Y for 2 mins.
9. Rapidly washed in water.
10. Dehydrated in 70%, 100%, 100% alcohol, cleared in xylene, mounted in DPX.

The second method used PAS and haematoxylin. No eosin was used as the pink to red colouring would have interfered with the magenta of PAS positive areas of the section. The haematoxylin stain gave contrast to the section. (Culling, 1974).

STAGES

1. Sections brought to water via 100%, 100%, 70% alcohol rinses.
2. Oxidised for 10 mins. in 1% aqueous periodic acid.
3. Washed in running tap water for 5 mins.
4. Rinsed three times in distilled water.
5. Specimens covered with Schiff's reagent for 15 mins.
6. Rinsed in running tap water for 15 mins.
7. Stained in Mayer's Haemulum (haematoxylin) for 7 mins.
8. Washed in tap water.
9. Rinsed in Scott's tap water to blue for 2 mins.
10. Dehydrated in 70%, 100%, 100% alcohol, cleared in xylene, mounted in DPX.

Sections were viewed under light microscopy at 400 x and 1000 x magnification. Photographs were taken to illustrate the stained sections. (Plates 7,8).

2.2 DOUBLE STAINING TECHNIQUES TO DETERMINE THE RELATIONSHIP BETWEEN VIABILITY OF UROEPITHELIAL CELLS AND THE PRESENCE OF A MUCOPOLYSACCHARIDE COAT.

In preliminary investigations uroepithelial cells were stained with PAS and alcian blue, and several double and treble staining procedures were attempted to combine viability, bacterial and mucopolysaccharide stains. Two double-staining techniques were devised to determine whether the presence of a mucopolysaccharide coat was related to the viability of uroepithelial cells. The methods have been reported elsewhere (Reid & Brooks, 1982).

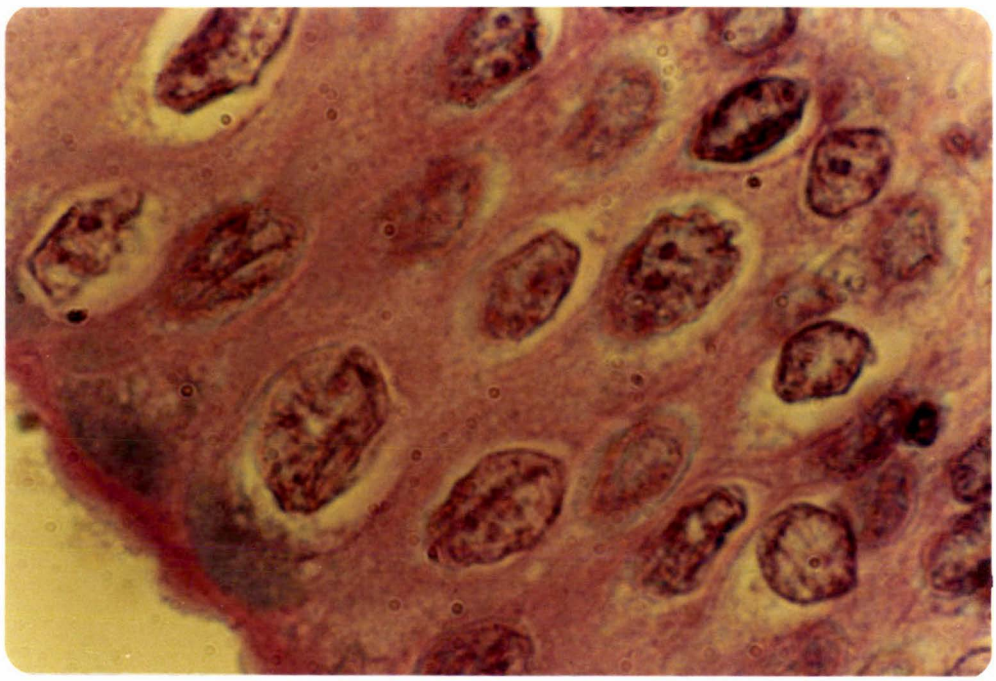
To assess the viability of the uroepithelial cells erythrosine B (C.I. 45430, Batch 2844, Gurr,London) and trypan blue (C.I. 23850, British Drug Houses) were used as vital stains (PHILLIPS, 1973).

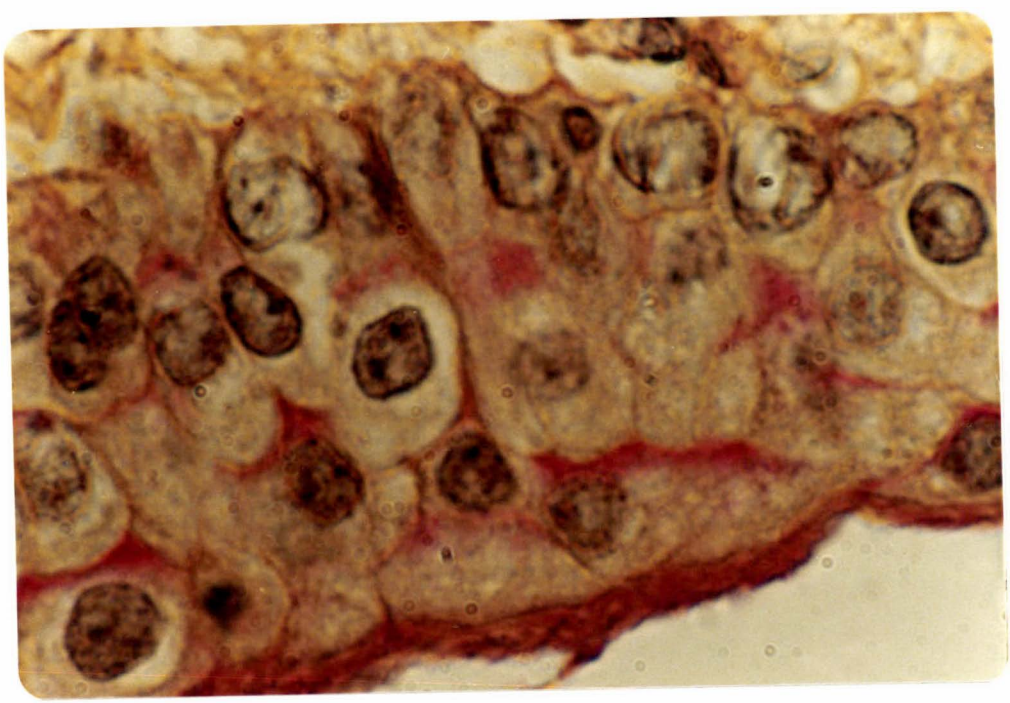
PLATE 7.

Transverse section of pig bladder with mucopolysaccharides particularly evident at peripheral surface: stained with alcian blue, haematoxylin and eosin (Magnification Ca x 360).

PLATE 8.

Transverse section of lamb bladder with mucopolysaccharides particularly evident at peripheral surface: stained with PAS reagent and haematoxylin (Magnification Ca x 360).





Uroepithelial cells were harvested by centrifugation at 250 x g for 12 mins. from early morning, mid-stream urine samples from healthy women. The cells were washed three times in phosphate buffered saline (PBS) pH 7.1 and resuspended in PBS to a volume of 1.0 cm^3. Then, 0.05 cm^3 erythrosine B (0.8% in PBS) was added to half the suspension and 0.05 cm^3 trypan blue (0.8% in PBS) to the remaining half. Both suspensions were left for 30 seconds at room temperature, then washed twice in 10 cm^3 PBS to remove excess stain and air dried onto separate glass microscope slides.

(a) ERYTHROSINE B/ALCIAN BLUE STAINING

Erythrosine B preparations were stained with alcian blue 8GX (0.3% in 3% aqueous acetic acid, pH 3.5) for 10 mins., then rinsed in running tap water and air dried. (Plate 9).

(b) TRYPAN BLUE/PERIODIC ACID SCHIFF'S STAINING

For Schiff's reagent, 400 cm^3 distilled water was heated to 37^{o}C, 2g parafuchsin added and the mixture shaken until dissolved. To this was added 7.6g sodium metabisulfite with 2 cm^3 concentrated HCl and the solution shaken and left overnight. The following day, 2g of activated charcoal was added and the solution was shaken and filtered before use.

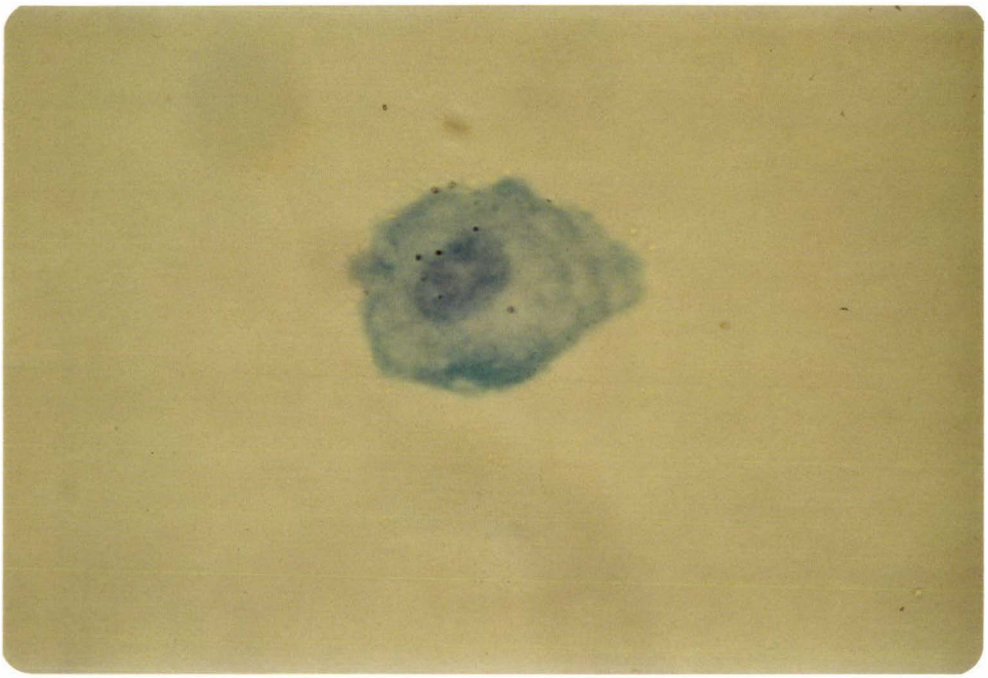
Trypan blue preparations were flooded with 1% periodic acid for 6 mins. then placed in a staining rack, rinsed in running tap water for 5 mins. and finally rinsed three times with distilled water. Schiff's reagent was applied for 10 mins., the slides rinsed for a further 10 mins. in running tap water and finally air-dried at room temperature. (Plate 10).

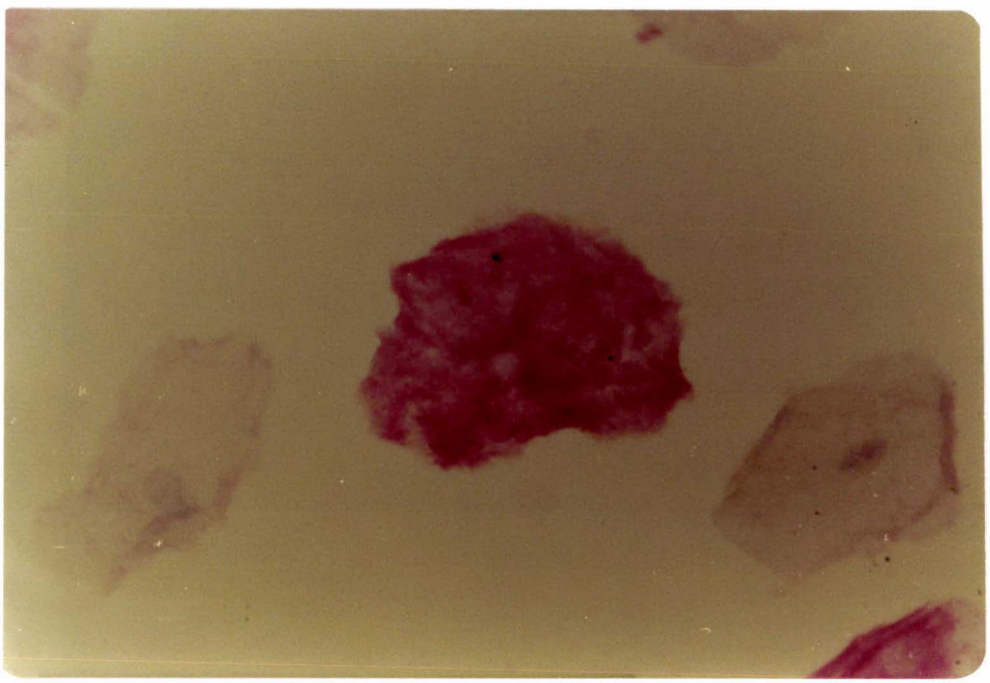
PLATE 9.

Nonviable transitional uroepithelial cell coated with mucopolysaccharides: stained with erythrosine B and alcian blue (Magnification Ca x 360).

PLATE 10.

Three squamous uroepithelial cells. In centre is a viable cell coated with mucopolysaccharides; cells at far left and right are nonviable and not coated with mucopolysaccharides: stained with trypan blue and PAS reagent (Magnification Ca x 290).





(c) REPRODUCABILITY TESTING OF VIABILITY AND MUCOPOLYSACCHARIDE STAINING TECHNIQUES.

To assess the reproducability of the viability and mucopolysaccharide staining techniques, three individuals evaluated a group of 10 slides prepared from one uroepithelial cell suspension. Five of the slides were stained with erythrosine B and alcian blue, and five with trypan blue and PAS. The evaluators included an experienced histologist and a junior technician. For the viability assays each evaluator randomly viewed 25 squamous and 25 transitional uroepithelial cells and scored them viable or nonviable for each of the 10 slides. To monitor the presence or absence of a mucopolysaccharide coat, each evaluator randomly viewed 50 viable and 50 nonviable uroepithelial cells. Of the 50 cells, 25 were squamous and 25 transitional. The slides were examined by each individual at separate times and the results were independently tabulated on completion of all the evaluations to avoid bias due to knowledge of previous results. Each group of results was statistically analysed using the one-way analysis of variance method.

2.3 DOUBLE STAINING TECHNIQUES TO DETERMINE THE RELATIONSHIP BETWEEN CELL MUCOPOLYSACCHARIDE COAT AND BACTERIAL ATTACHMENT.

Two further double-staining techniques were devised to determine attachment of *E.coli* to mucopolysaccharide coated and noncoated cells as reported elsewhere (Reid & Brooks, 1982). Two *E.coli* strains were used, strain 87 grown in broth and strain 207 grown on trypticase soy agar (TSA). The broth cultures were centrifuged at 1500 x g for 10 mins. and resuspended in PBS to a concentration of 10^{10} organisms per cm^3. The TSA cultures were suspended in PBS to a similar concentration as determined by haemocytometer counts.

Each strain was incubated at 37°C for 30 mins. to allow regeneration of pili then assayed for attachment to 10 uroepithelial cell samples prepared as outlined in section 1.1, with the exception that the erythrosine B and methylene blue staining steps were not included.

(a) ALCIAN BLUE/SAFRANIN STAINING

Air dried slides of cells with attached E.coli were stained by immersion in alcian blue 8GX (0.3% in 3% aqueous acetic acid, pH 3.5) for 10 mins. then rinsed in tap water and flooded with 1% aqueous safranin (C.I. 50240, BDH) for 1 min. to stain the bacteria. The slides were rinsed in tap water and immersed for 2 seconds in 70% alcohol and twice in 100% alcohol to dehydrate the cells and remove excess stain and finally cleared through xylene to prevent further loss of stain. (Plate 11).

(b) PERIODIC ACID SCHIFF'S/METHYLENE BLUE STAINING

Uroepithelial cells with attached E.coli were stained with PAS as described earlier then flooded for 30 seconds with methylene blue (C.I. 52015, BDH, 0.04% in PBS), rinsed in tap water, dehydrated with alcohol and cleared through xylene. (Plate 12).

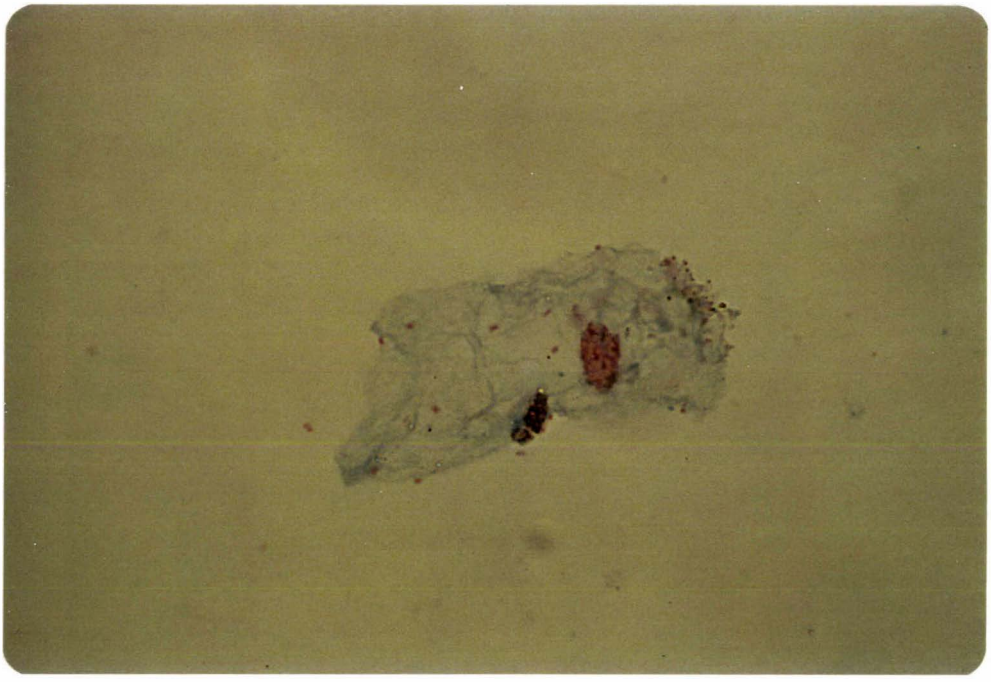
The number of bacteria attached to mucopolysaccharide coated and noncoated uroepithelial cells were assessed by examining 40 coated and 40 noncoated cells at random. In each case the 40 cells comprised 20 squamous and 20 transitional uroepithelial cells. For each test, duplicate slides were prepared and assessed. Control slides consisted of uroepithelial cells without added E.coli to observe any indigenous flora present on the cells. All slides were studied by light microscopy at 1000 x magnification, and photographs were taken of the preparations. The results were statistically analysed using two-way analysis of variance.

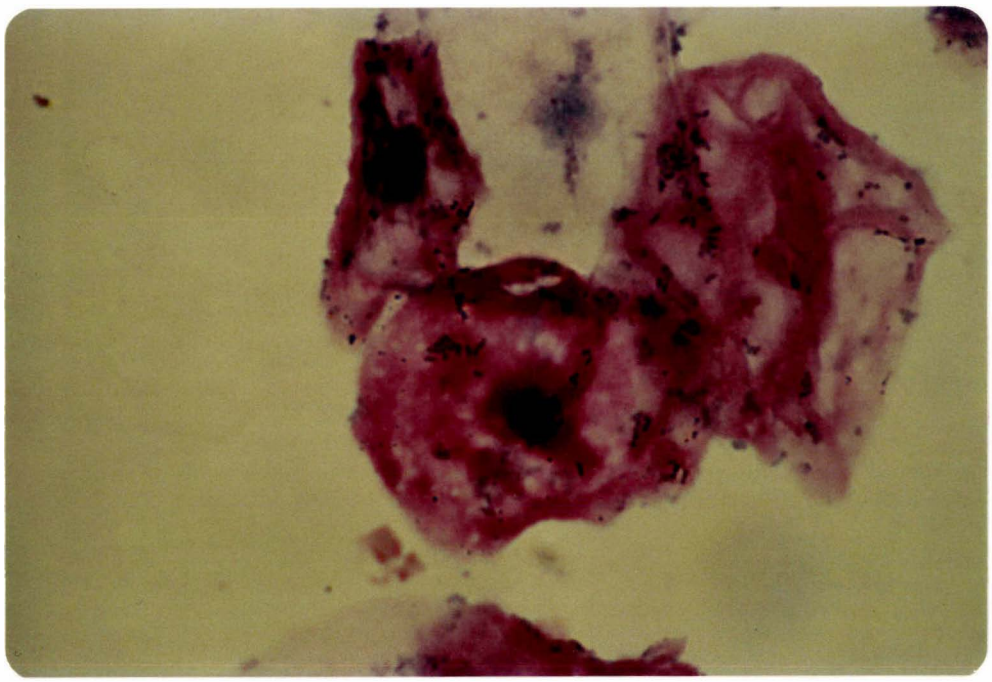
PLATE 11.

Mucopolysaccharide coated squamous uroepithelial cell with *E.coli* strain 322 attached, following bacterial growth on TSA: stained with alcian blue and safranin (Magnification *Ca* x 360).

PLATE 12.

Squamous uroepithelial cells with *E.coli* strain 207 attached, following bacterial growth on TSA. Cell at top centre is not mucus coated, other three cells are mucus coated: stained with PAS reagent and methylene blue (Magnification *Ca* x 360).





2.4 EFFECT OF OESTROGEN ON BLADDER MUCUS COAT

Previous experiments suggested that attachment of E.coli strains grown in BHI broth, to uroepithelial cells was influenced by factors related to menstrual cycle changes. (see section 1.4). This variation roughly corresponded to the expected normal urine and blood levels of oestrogen, especially to the plasma levels of 17 β oestradiol. Further experiments established that animal bladder cells and human urinary sediment cells were coated with mucopolysaccharides. This coating may influence the attachment of certain E.coli strains to the uroepithelial cell surface.

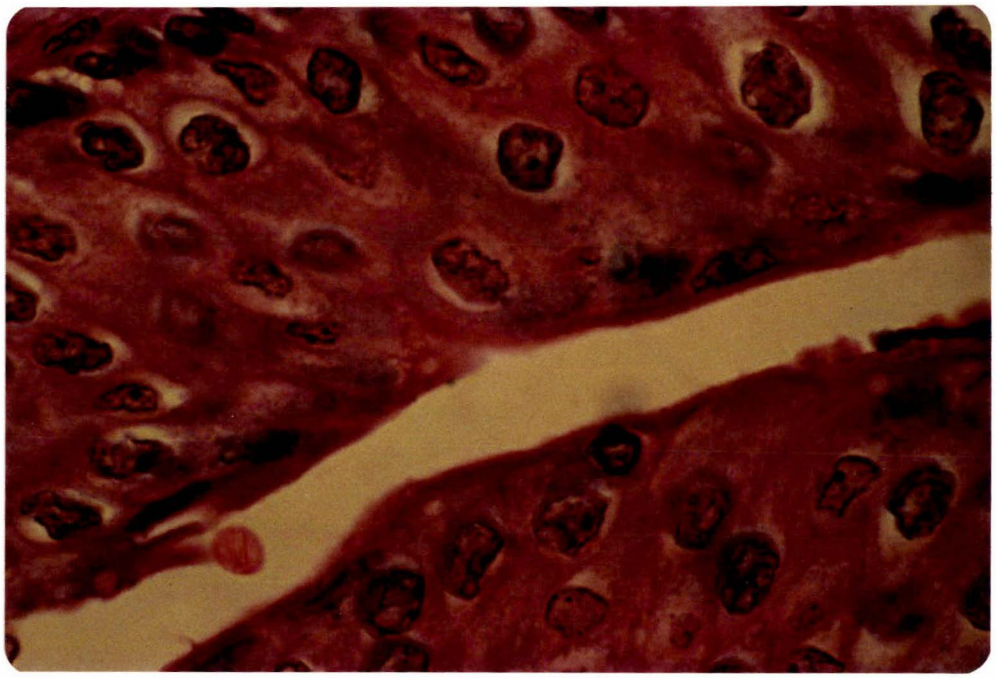
A mouse model was used to investigate whether raised blood levels of oestradiol noticeably altered the mucopolysaccharide lining of the bladder. Sixteen weaned female mice were placed in 4 groups. The first group of five mice were injected sub-cutaneously (s/c) with 0.1µg 17β oestradiol-3-methyl benzoate in 0.05 cm^3 peanut oil. The second group of five mice were injected s/c with 0.05 µg 17β oestradiol-3-methyl benzoate in 0.05 cm^3 peanut oil. Of the remaining six mice, three were injected s/c with 0.05 cm^3 peanut oil and three were left untouched. The mice were ear-marked, left in cages with food and water ad libitum for four days then sacrificed. The bladders were removed, emptied, refilled with Bouin's fixation fluid, processed, embedded, sectioned and stained using the same method as described in section 2.1 for lamb and pig bladders. Each section was carefully examined to determine whether bladders of the oestrogen treated mice had a noticeably altered mucus coated epithelium compared to the epithelium of untreated and peanut oil treated mice. As this was a visual comparison, the services of an experienced histologist were called upon to confirm the examinations. (Plates 13, 14).

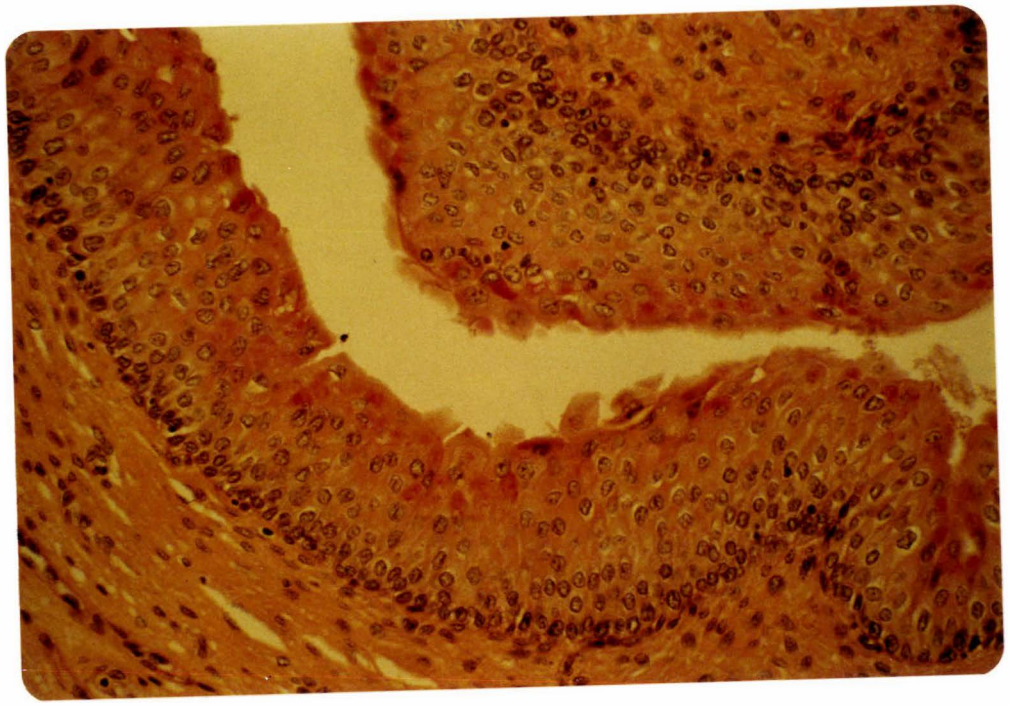
PLATE 13.

Transverse section of mouse bladder with mucopolysaccharides particularly evident at peripheral surface, after treatment with 17 β oestradiol: stained with alcian blue, haematoxylin and eosin (Magnification Ca x 360).

PLATE 14.

Transverse section of mouse bladder with mucopolysaccharides particularly evident at peripheral surface, without oestroge treatment: stained with PAS reagent and haematoxylin (Magnification Ca x 145).





METHODS

SECTION 3.

THE ROLE OF BACTERIAL HAEMAGGLUTININS IN THE *IN VITRO* ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

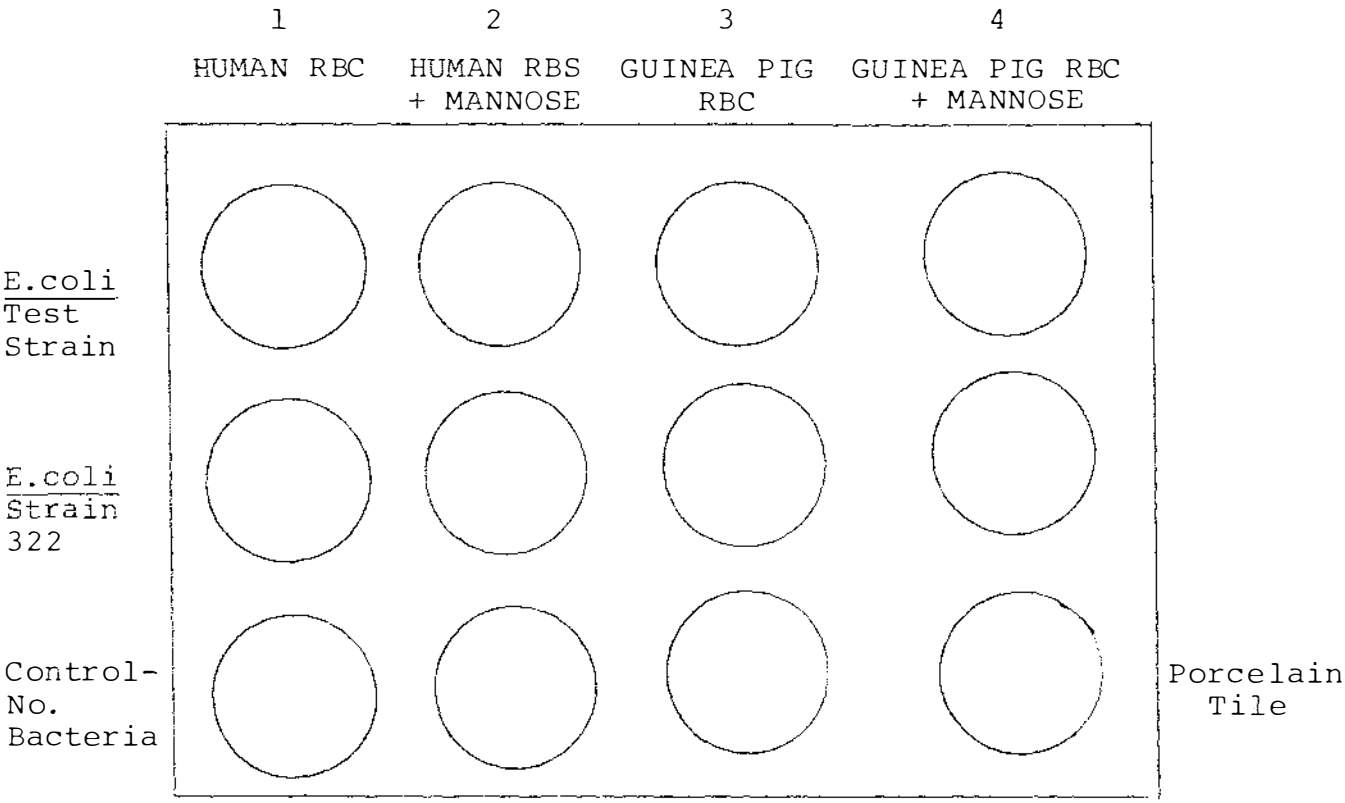
3.1 HAEMAGGLUTINATION TEST.

The haemagglutination test used was similar to that described by Kallenius et al. (1980,b). Bacterial suspensions of 10^{10} organisms per cm^3 in PBS were used; 50 µl were added to four wells in a white porcelain tile. Fresh, human, group A erythrocytes and guinea pig erythrocytes were washed four times in PBS and resuspended to 3% (v/v) in PBS; 50 µl of human A erythrocytes were added to wells 1 and 2, and 50 µl of guinea pig erythrocytes were added to wells 3 and 4. To wells 1 and 3 was added 50 µl PBS, and to wells 2 and 4 was added 50 µl of PBS containing 1% α D mannose. Each E.coli strain was thus tested for both a mannose sensitive haemagglutinin, detected using guinea pig erythrocytes and a mannose resistant haemagglutinin, detected using human A erythrocytes. In each batch of tests, two controls were included. E.coli strain 322 is known to possess a MRHA and a MSHA after growth in broth, and it was used to confirm that the test was working and sensitive. A second control of PBS without bacteria was incorporated to confirm that the erythrocytes were not autoagglutinating. Figure 1A presents a summary of the test. The porcelain tiles were gently rocked then placed at $4^{o}C$ for 60 minutes, after which time the haemagglutination patterns were read under a direct light source.

The haemagglutination of human and guinea pig erythrocytes appeared as large granular clumps of red blood cells which settled to the centre of the wells and became dispersed on rocking the tiles. A weak haemagglutination reaction was seen when fine granular

FIGURE 1A.

HAEMAGGLUTINATION TEST



Each well is 26 mm in Diameter.

1% α D Mannose in PBS

3% Human A Rhesus Positive Erythrocytes in PBS

3% Guinea Pig Erythrocytes in PBS

10^{10} Bacteria per cm^3 from BHI broth or TSA cultures

Total Volume Per Well = 150 µl

Incubation $4^{o}C$, 60 minutes.

MRHA = Agglutination in Wells 1 and 2

MSHA = Agglutination in Well 3 and no agglutination in Well 4.

Control - No Bacteria = No. Agglutination.

N.B. A MRHA can also be detected in some instances by Agglutination of Guinea Pig erythrocytes in the presence of mannose.

A MSHA can also be detected in some instances by Agglutination of Human erythrocytes, inhibited in the presence of mannose.

clumps appeared after several minutes of rocking the tiles. A negative haemagglutination reaction was seen when no granularity appeared and a smooth red blood cell suspension was evident as a dense pattern throughout the depth of the well. This negative reaction was seen in the presence of α D mannose for E.coli strains possessing a MSHA. The haemagglutinating reaction was not inhibited by D mannose for E.coli strains possessing a MRHA. (Plates 15-19).

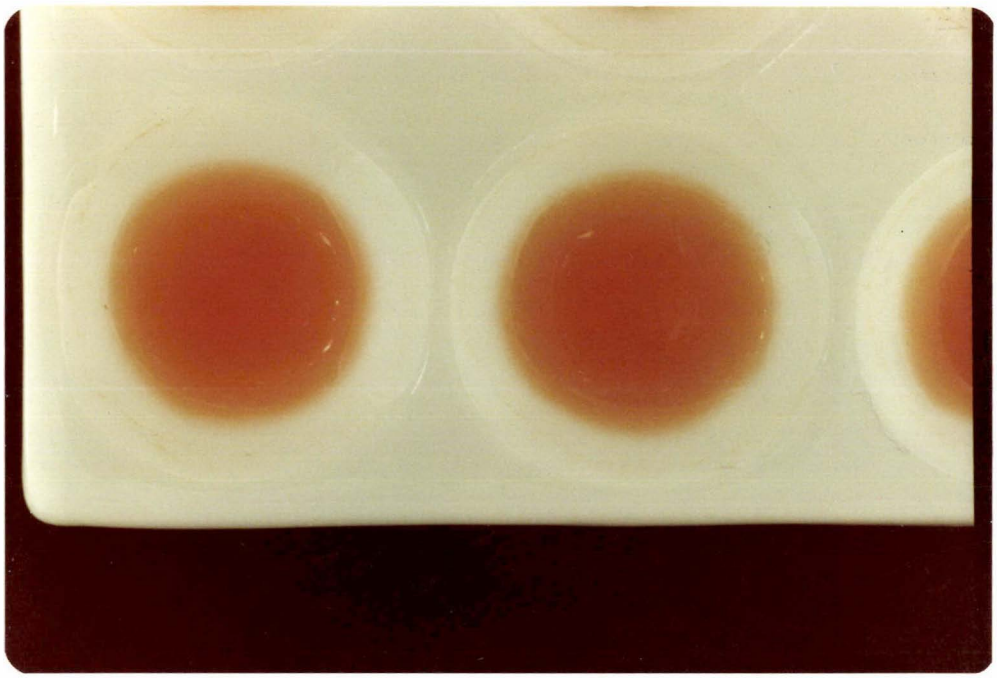
The 50 µl amounts used in the test gave an approximate ratio of between 20 and 70 bacteria to 1 erythrocyte, which was considered sufficient by Duguid et al. (1979) to give visible clumping.

PLATE 15.

Negative haemagglutination reaction for human group A erythrocytes: left side well with PBS and right side well with PBS and α D mannose.

PLATE 16.

Haemagglutination of guinea pig erythrocytes by BHI broth grown E.coli strain 67: right side well with PBS and left side well with PBS and α D mannose (ie. expression of a MSHA).



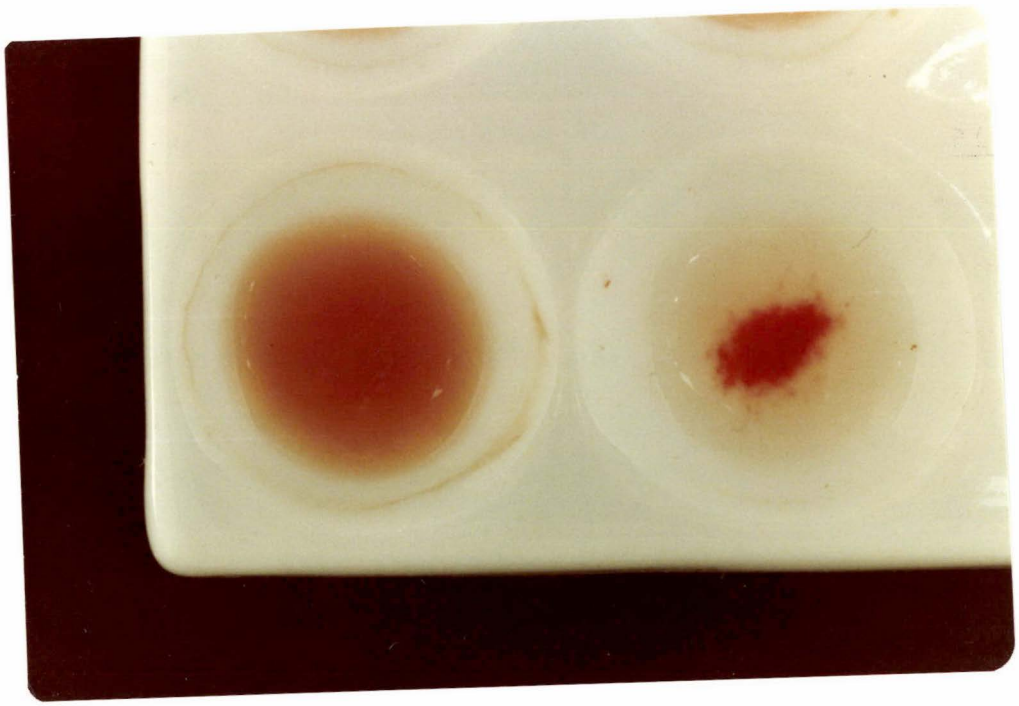
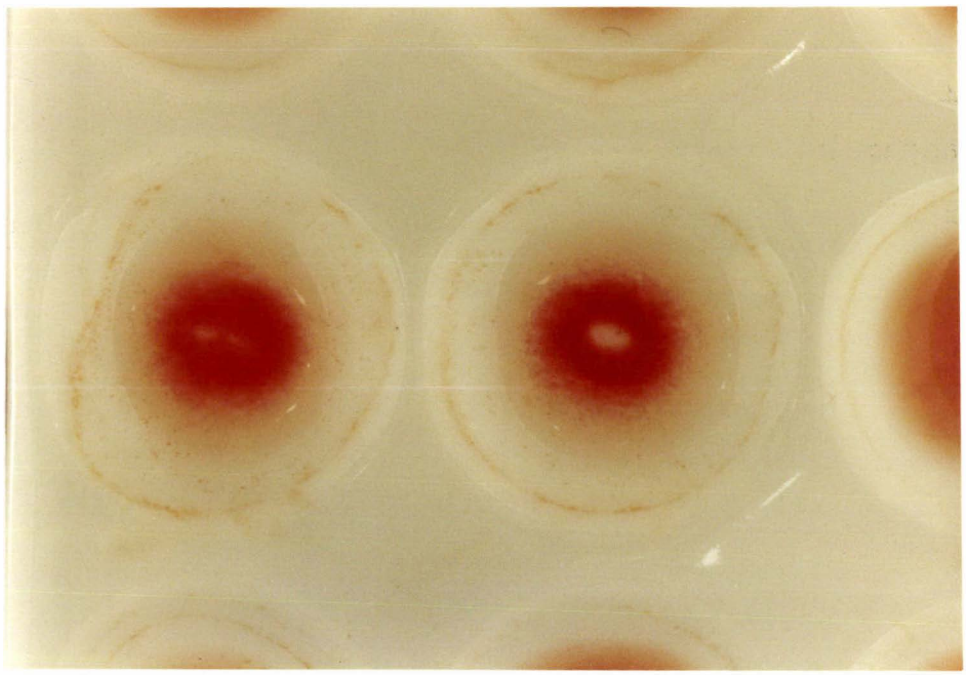


PLATE 17.

Haemagglutination of human group A erythrocytes by TSA grown E.coli strain 158: left side well with PBS and right side well with PBS and α D mannose (ie. expression of a MRHA).

PLATE 18.

Haemagglutination of human group A erythrocytes by TSA grown E.coli strain 158 in presence of PBS with α D mannose. (ie. expression of a MRHA).



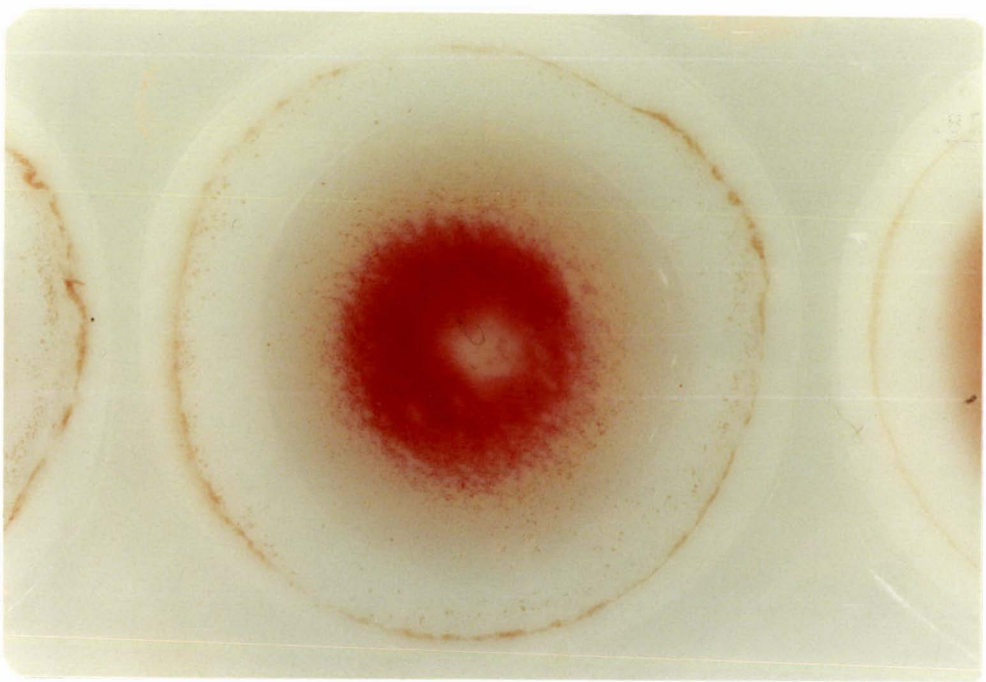
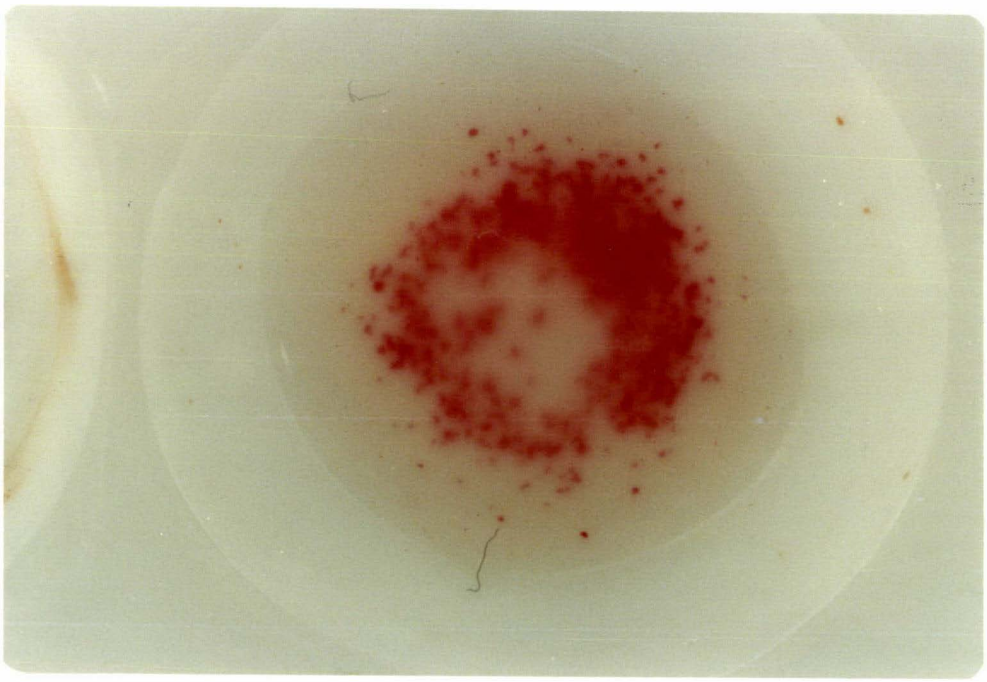


PLATE 19.

Haemagglutination of human group A erythrocytes by TSA grown E.coli strain 322, in presence of PBS with α D mannose. (ie. expression of a MRHA).



METHODS

SECTION 4.

THE USE OF ELECTRON MICROSCOPY TO INVESTIGATE ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

4.1 INTRODUCTION

Several techniques were used to investigate the attachment of E.coli to uroepithelial cells by electron microscopy. The phosphate buffer used throughout the study was 0.1M, pH 7.2, (2.51g $Na_2HPO_4.12H_2O$, 0.41g KH_2PO_4 in 1000 cm^3 distilled water). The uranyl acetate stain used on sectioned material was dissolved in 50% ethanol and stored in a dark bottle. The lead citrate stain was made up with 0.025g lead citrate, 10 cm^3 distilled water, 0.1 cm^3 10N NaOH, and shaken until dissolved. All other materials and instruments used are included in the methodology.

4.2 THE USE OF A NEGATIVE STAINING TECHNIQUE FOR DETECTION OF PILI ON E.COLI CELLS.

(1) In the first experiment, 24 E.coli strains were cultured on TSA at 37°C overnight and suspended in distilled water to a concentration of 10^{10} organisms per cm^3, and incubated for 30 mins at 37°C. Approximately 25 µl of the E.coli suspension were placed on a formvar-coated copper grid of 3 mm diameter and left for 1 minute. Blotting paper was used to remove excess suspension before one drop of staining agent was placed onto the grid and left at room temperature for several minutes. The stain solution comprised 4 Pasteur pipette drops of 2% phosphotungstic acid, 4 drops of 2% ammonium molybdate and 1 drop of 0.5% bovine serum albumin which

acted as a wetting agent. Blotting paper was used to remove excess stain and gently dry the grid which was then transferred to a PHILIPS EM 200 electron microscope (E.M.) for examination. The E.coli were examined at 12,000 to 14,000 X magnification and 50 clearly distinguishable organisms were assessed for the presence of pili. Each strain was examined in duplicate.

(2) The second experiment consisted of three groups of strains cultured on TSA or BHI broth overnight at 37°C. The TSA cultures were suspended in distilled water to a concentration of 10^{10} organisms per cm^3 while the broth cultures were centrifuged at 1500 x g for 10 mins. then resuspended to a concentration of 10^{10} organisms per cm^3 distilled water. Both sets of suspensions were incubated at 37°C for 30 mins. then stained and assessed as in Part (1).

(3) In the third experiment, 20 E.coli strains were grown on TSA at 18°C for 48 hrs, resuspended to a concentration of 10^{10} organisms per cm^3 in distilled water and tested for the presence of pili as described in Part (1).

Selected E.M. fields were recorded photographically. The results from this study and all those related to the use of electron microscopy, are presented in results section 5. (Plates 20 to 25).

4.3 THE USE OF TRANSMISSION ELECTRON MICROSCOPY TO STUDY ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

There are many ways to process specimens for examination under transmission electron microscopy (T.E.M.). For this reason, each step in the procedure used for this study will be carefully detailed.

PLATE 20.

Negatively stained preparation of non-piliated E.coli strain 207, possessing a MRHA after growth on TSA. (Magnification x 34,000 : BAR = 1µm).

PLATE 21.

Negatively stained preparation of flagellated, non-piliated E.coli strain 66, not possessing a haemagglutinin after growth on TSA. (Magnification x 22,500 : BAR = 1µm).

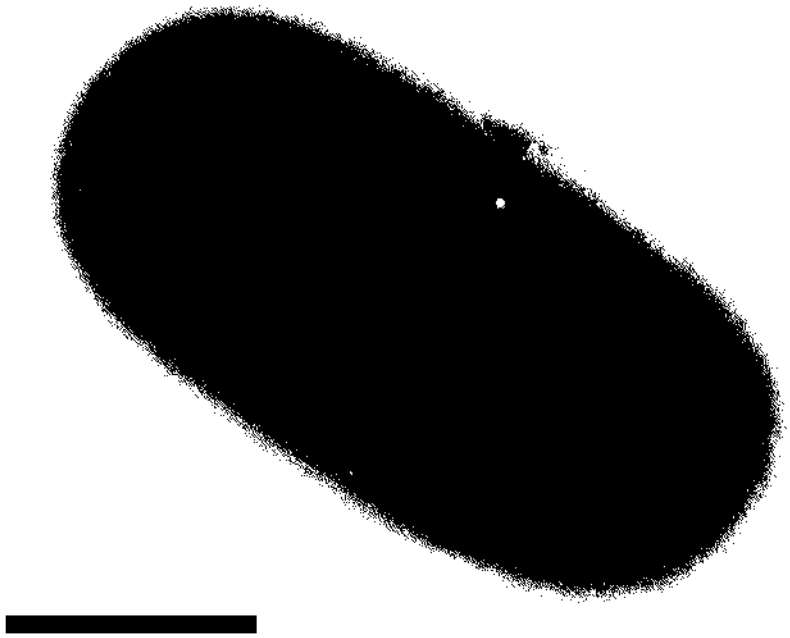
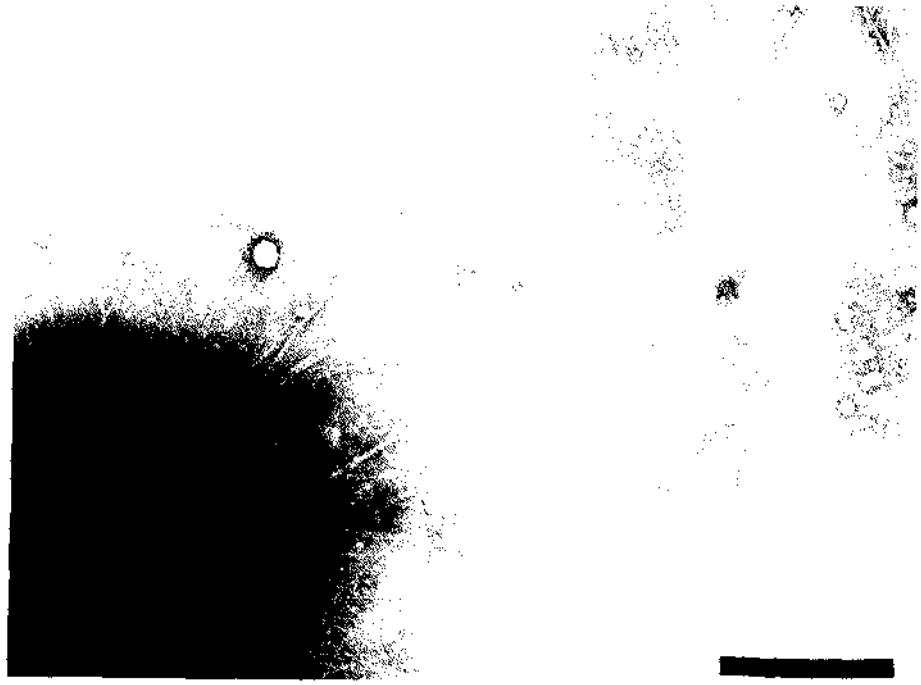


PLATE 22.

Negatively stained preparation of type 1 piliated E.coli strain 323, possessing a MSHA after growth in BHI broth. (Magnification x 46,000 : BAR = 0.5μm).

PLATE 23.

Negatively stained preparation of piliated E.coli strain 158, possessing a MRHA after growth on TSA. (Magnification x 25,000 : BAR = 1μm).



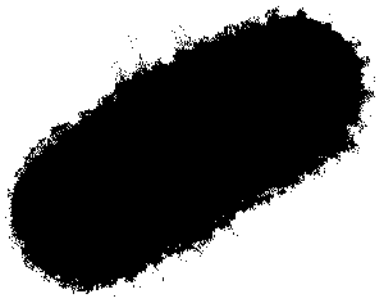


PLATE 24.

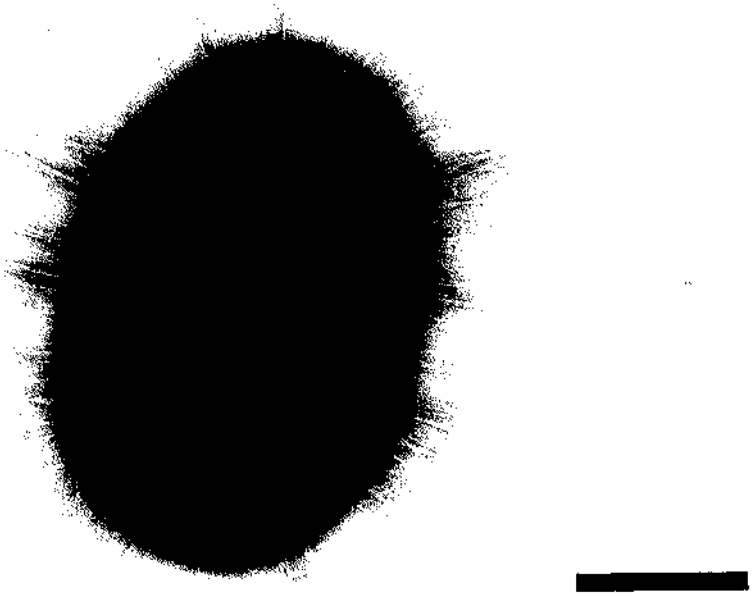
Negatively stained preparation of piliated *E.coli* strain 104, possessing a MRHA after growth on TSA.

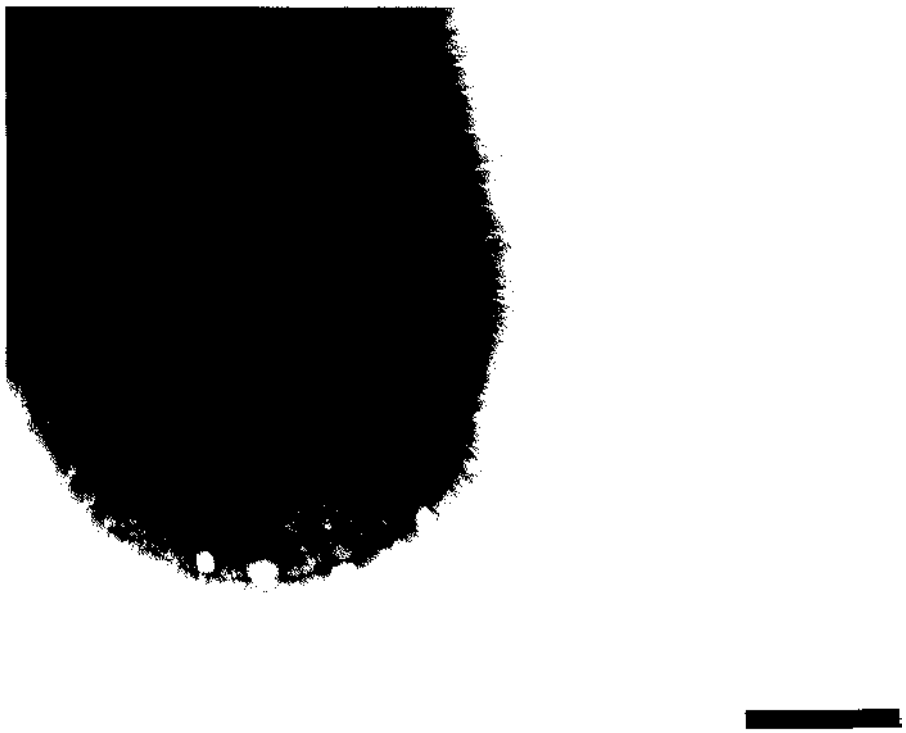
(Magnification x 46,000 : BAR = 0.5µm).

PLATE 25.

Negatively stained preparation of piliated *E.coli* strain 158, possessing a MRHA after growth on TSA.

(Magnification x 82,500 : BAR = 0.25µm).





4.3.1 COLLECTION OF SPECIMENS.

In order to have a sufficiently large pellet suitable for sectioning, the attachment assay was carried out as described in section 1.1 but using 4 times the quantities of bacteria and uroepithelial cells. Following adsorption and removal of unattached bacteria by washing, the uroepithelial cells were left as a pellet in the test tube.

4.3.2 PRIMARY FIXATION.

The pellet was covered carefully with a fixitive described by Karnovsky in(1967),(2% Formaldehyde, 3% glutaraldehyde in 0.1M phosphate buffer) and was left at $4^{o}C$ for 2 hours. The cells were washed 3 times in 10 cm^3 phosphate buffer (P.B.) and the supenatant removed. To form a more solid pellet, 1 drop of 2% molten agar was carefully mixed in with the cells and allowed to set. The agar coated pellet was removed from the tube, excess agar cut off and the pellet dissected into specimens of about 1 mm^3 using a scalpel blade.

The sections were placed in a glass bijou bottle, covered with Karnovsky's fixitive for 2 hours at $4^{o}C$, then the fixitive was replaced with phosphate buffer. Three buffer rinses were applied in which the sections were held in fresh buffer for 10 mins., 10 mins., and 20 mins. at room temperature.

4.3.3 SECONDARY FIXATION.

The specimens were post-fixed in osmium tetroxide (OsO_4), a secondary fixitive which has great value as an "electron stain" due to its high metallic density. Osmium tetroxide in solid and aqueous

phase is volatile and was therefore handled in a ventilated fume cupboard. A 1% solution was made up in phosphate buffer and added to the specimen bottle. With the lid intact the bottle was placed at $4^{o}C$ for 1 hour. The OsO_4 was removed in the fume cupboard, discarded into a separate waste container, and the specimens were suspended in PB and held for 10 mins. at room temperature to remove the remaining OsO_4. A further 2 rinses of PB for 10 and 20 mins. completed the washing process.

4.3.4 DEHYDRATION.

After fixation, specimens were dehydrated to render them stable in the electron beam and to facilitate the infiltration of the non-aqueous embedding media. This was achieved by taking the specimens through a graded ethanol series then through propylene oxide which was used as the resin diluent for infiltration.

The ethanol was added to the specimen bottle in several steps: a 25% solution for 15 mins., a 50% solution for 15 mins., a 75% solution overnight, a 95% solution for 15 mins., a 100% solution for 15 mins. and a 100% solution for 1 hour. On removal of the ethanol, 100% propylene oxide was added in the fume cupboard and left covering the sections for 15 mins., after which a fresh 100% solution was added and held for a further 15 mins. at room temperature. The propylene oxide was removed and the sections further processed.

4.3.5 INFILTRATION AND EMBEDDING.

Specimens must ultimately be embedded in a support which is firm enough to permit the cutting of thin sections of approximately 60-120 nm. Epoxy resins are most commonly used as they give

satisfactory tissue preservation and section cutting quality.

For the infiltration step, FLUKA resin was used which contained 10.94g epoxy resin, 9.58g hardener, 0.4 cm^3 accelerator and 0.2 cm^3 plastizer. A stock solution was made up containing 20 cm^3 resin and 60 cm^3 propylene oxide. The specimens were covered with the solution and the bottles were left in the fume cupboard overnight with their lid removed; this allowed the propylene oxide to evaporate leaving a 25% resin solution. The following day this resin was replaced with a 100% solution of resin and the bottle placed on an agitator and gently rocked for 8 hours at 37^{o}C, to maximise infiltration. The resin was finally removed and carefully disposed of and the specimens placed in wells on a small rubber tray (10 cm x 6 cm).

Durcapan ACM resin mixture was used to cover the specimens and the embedding process was continued for 36 hours at 60^{o}C. Once embedded the specimens were stored in glass bijou bottles at room temperature to await sectioning.

4.3.6 SECTIONING OF SPECIMENS.

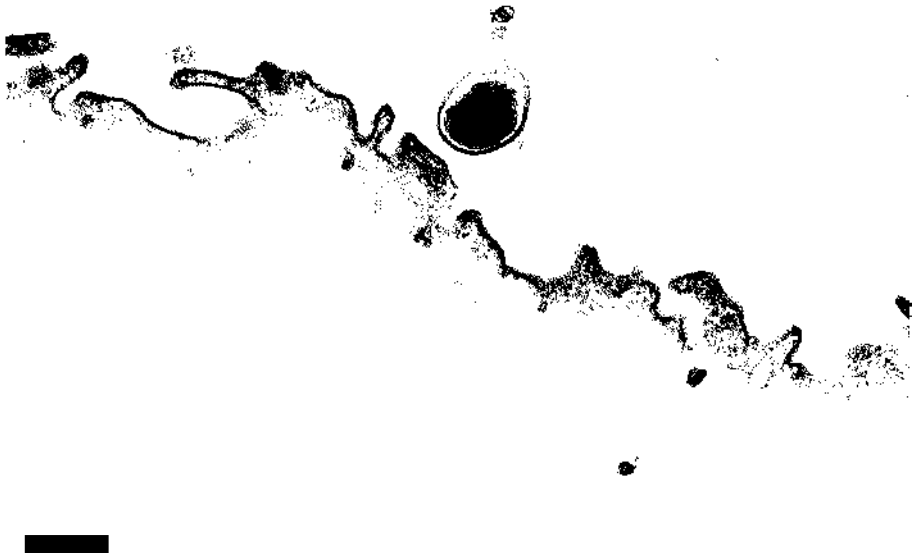
Glass knives were cut using an LKB-7800 knifemaker. These knives were used to make the mesa, with initial cuts of 4 μm then 2 μm. An LKB-1180 ultramicrotome was used to prepare sections that could be analysed under the T.E.M.. With the trimmed specimen in place, sections were cut and floated onto a water-boat which had been fixed in place using masking tape. Sections which appeared tinted with silver were 60 to 90 nm thick while gold coloured sections were 90 to 150 nm thick. Both thicknesses were deemed satisfactory for T.E.M. analysis and samples were each placed on unsupported copper grids of 3 mm diameter.

PLATE 26.

Transmission electron micrograph of uroepithelial cell from control sample: indigenous bacterium close to surface of uroepithelial cell (Magnification x 22,500 : BAR = 0.5μm).

PLATE 27.

Transmission electron micrograph of uroepithelial cell from control sample: indigenous bacterium close to surface of uroepithelial cell (Magnification x 82,500 : BAR = 0.25μm).



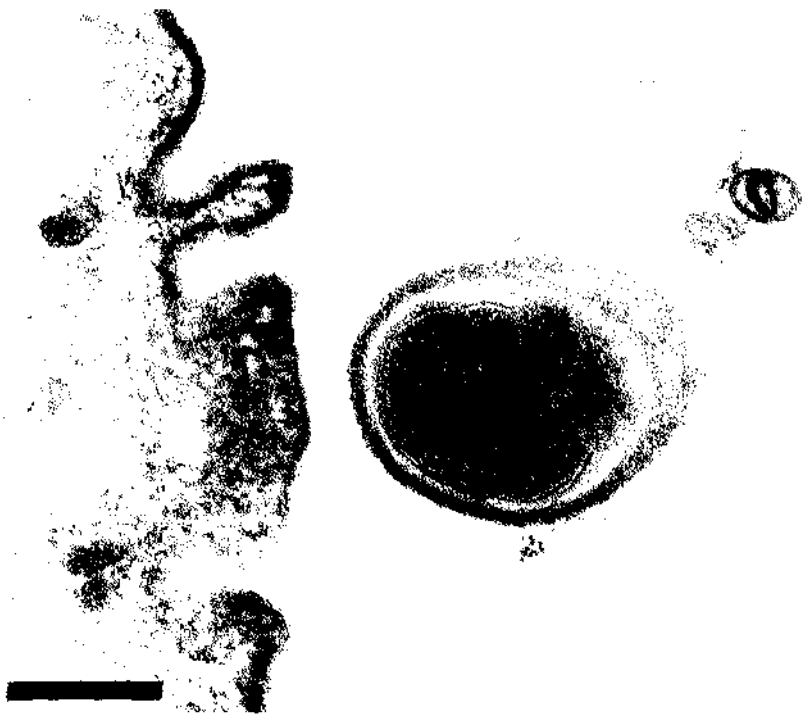


PLATE 28.

Transmission electron micrograph of uroepithelial cell from control sample: indigenous bacteria close to surface of uroepithelial cell (Magnification x 34,000 : BAR = 0.5µm

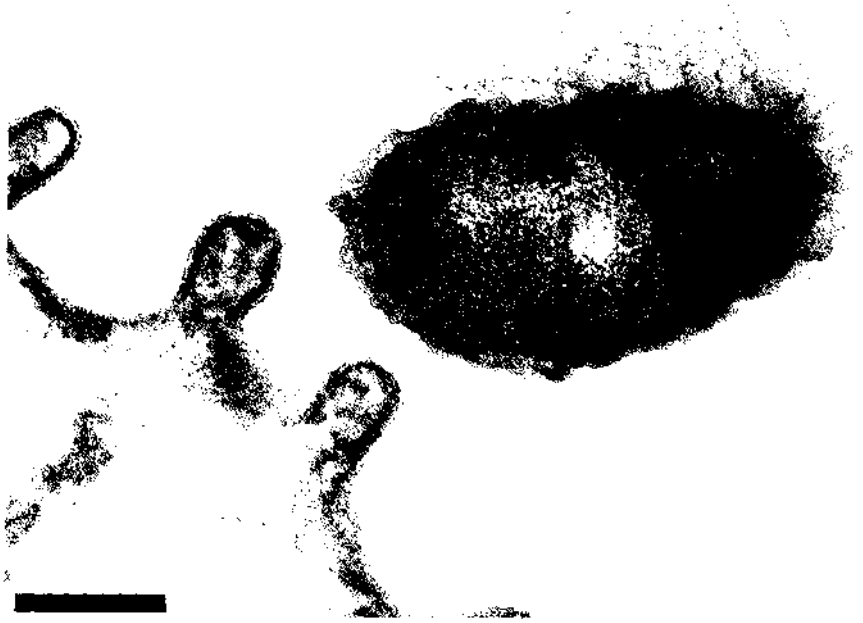
PLATE 29.

Transmission electron micrograph of uroepithelial cell from control sample: indigenous bacteria close to and in contact with the surface of uroepithelial cell. (Magnification x 34,000 : BAR = 0.5µm).

PLATE 30.

Transmission electron micrograph of uroepithelial cell from a cell sample incubated with BHI broth grown E.coli strain 67: bacterium close to surface of uroepithelial cell.

(Magnification x 82,500 : BAR = 0.25μm).



4.3.7 STAINING AND E.M. ANALYSIS.

The sections on the copper grids were stained at room temperature by immersing the grid in a drop of uranyl acetate for 6 minutes followed by lead citrate for 6 minutes. The grids were placed in the Philips EM 200 or EM 201C for observation; selected E.M. fields were recorded photographically. (Plates 26 to 30).

4.4 RUTHENIUM RED STAINED PREPARATIONS FOR TRANSMISSION ELECTRON MICROSCOPY.

Ruthenium red is an electron-dense stain specific for poly-anions such as acidic polysaccharides, and for most extracellular carbohydrate fibres (LUFT, 1971). When staining for these polysaccharides, specific antibodies can be used to stabilize against collapse and condensation which may occur in the dehydration process (MACKIE et al., 1979). Aldehyde fixitives are used in the procedure, and because of its solubility Ruthenium red is maintained in all aqueous washes as suggested by Costerton, (1980).

The procedure was as follows:

1. The attachment assay was carried out as described in section 1.1, using E.coli and uroepithelial cells.
2. After incubation at 37^{o}C for 1 hour at 20 rpm in a shaker, the uroepithelial cells were washed five times in PBS, pH 7.1, to remove unattached bacteria. The cells were then resuspended in 0.5 cm^{3} PBS. To this was added 0.5 cm^{3} of type specific antiserum diluted in PBS. The antisera were prepared from E.coli with O serotypes 01, 04, 06, 07 and 075 as described in methods section 1.2. The uroepithelial cells were incubated with the antiserum at 37^{o}C for 1 hour.

3. The cells were washed twice in fresh cacodylate buffer (pH 7.2), and resuspended in 0.5 cm^3 buffer.
4. 0.5 cm^3 cacodylate buffer containing 0.05% ruthenium red was added to the cell suspension and left for 15 seconds at room temperature.
5. 1 cm^3 of 0.5% glutaraldehyde fixitive in cacodylate buffer containing 0.15% ruthenium red was then added to the cell suspension.
6. After 1 hour at room temperature, the uroepithelial cells were centrifuged in 10 cm^3 buffer. The buffer was removed and 2 drops of 2% molten agar were added to enrobe the cells. These cells were then left overnight at $4^{\circ}C$, covered with 1 cm^3 cacodylate buffer containing 0.013% ruthenium red, to prevent evaporation.
7. The following day, the buffer was removed and the blocks of cells trimmed to approximately 2 mm^3 sizes. These blocks were placed in glass vials and covered with 5% glutaraldehyde in buffer containing 0.05% ruthenium red.
8. After 2 hours at room temperature, the blocks were washed in 5 x 10 min. changes of buffer containing 0.05% ruthenium red.
9. Post-fixation was carried out for 4 hours at $4^{\circ}C$ in 2% $0s0_4$ in buffer containing 0.05% ruthenium red.
10. The blocks were washed for 10 mins. in each of 5 changes of buffer containing 0.05% ruthenium red.
11. The blocks were dehydrated at room temperature as follows:

    30% acetone in buffer containing 0.05% ruthenium red - 30 min.

    50% acetone in buffer containing 0.05% ruthenium red - 30 min.

70% acetone in distilled water	- 2 min.
90% acetone in distilled water	- 2 min.
100% acetone	- 2 min.
100% acetone	- 2 min.
Propylene oxide	- 30 secs.
Propylene oxide	- 30 secs.

The exposure time to acetone concentrations ≥ 70% and to propylene oxide was shorter than that normally used. This was to prevent loss of the ruthenium red stain which may have occurred with longer exposure. Due to the softness of the tissue, infiltration of these solutions still occurred.

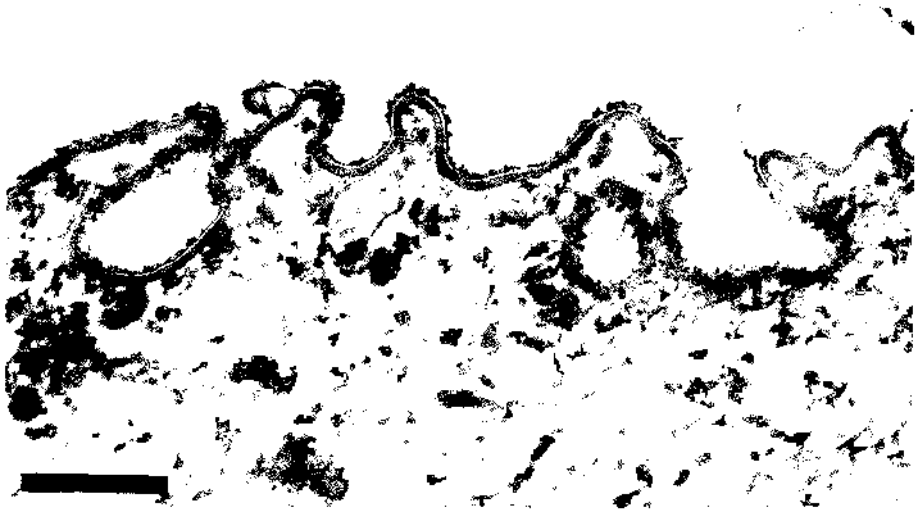
12. The blocks were soaked in propylene oxide - Fluka epoxy resin mixture (3:1) and left overnight at room temperature. By the next day, 100% resin was present due to propylene oxide evaporation.
13. Fresh resin was added to infiltrate the blocks over an 8 hour period at room temperature.
14. Fresh Durcapan resin was used to cure and embed the blocks at 60^{o}C for 68 hours.
15. The blocks were trimmed to approximately 60 to 120 nm sections and placed on formvar coated copper grids.
16. The sections were stained for 6 minutes with uranyl acetate (in 50% ethanol), washed in 50% ethanol and distilled water, stained with lead citrate, washed in distilled water, blotted dry and viewed in a Philips EM 200. (Plates 31 to 36).

PLATE 31.

Transmission electron micrograph of ruthenium red stained preparation: electron-dense glycocalyx surrounding the uroepithelial cell surface. (Magnification x 77,000 : BAR = 0.25μm).

PLATE 32.

Transmission electron micrograph of ruthenium red stained E.coli strain 207 bacterium from a TSA grown culture. (Magnification x 48,000 : BAR = 0.5μm).



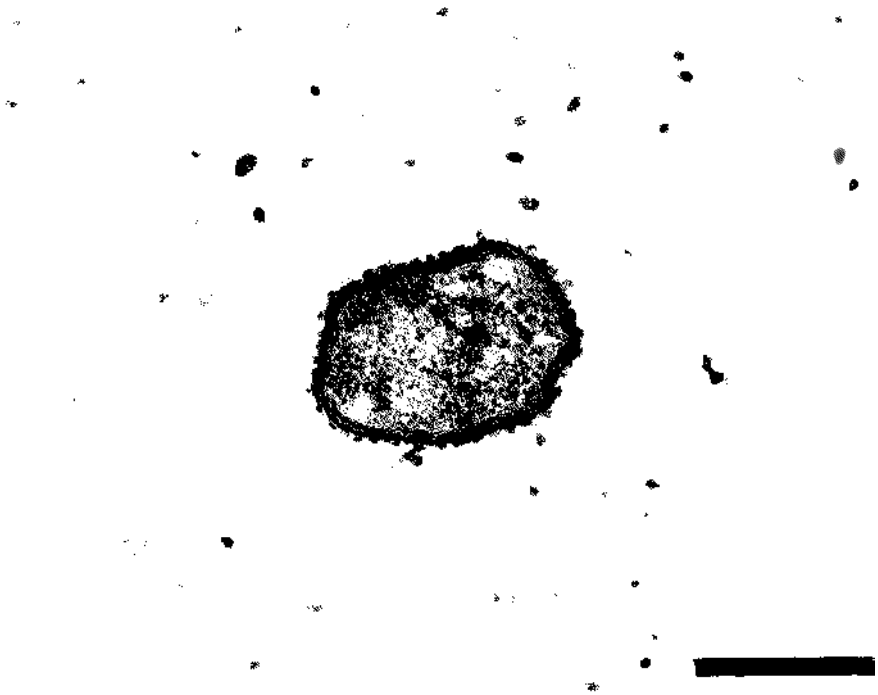
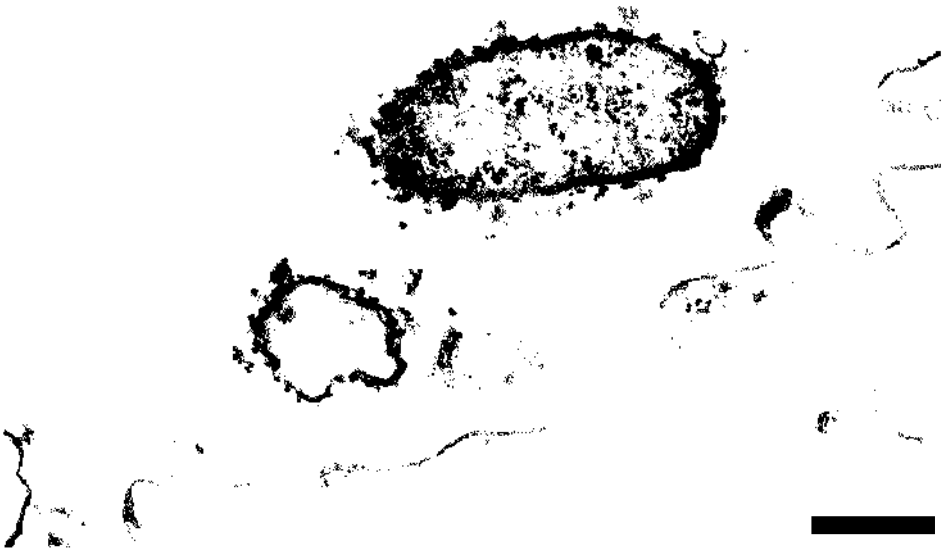


PLATE 33.

Transmission electron micrograph of ruthenium red stained preparation following assay of uroepithelial cells with E.coli strain 207, grown on TSA: bacteria close to uroepithelial cell surface (Magnification x 33,400 : BAR = 0.5µm).

PLATE 34.

Transmission electron micrograph of ruthenium red stained preparation, following assay of uroepithelial cells with E.coli strain 207, grown on TSA: bacteria attached to and close to uroepithelial cell surface (Magnification x 33,400 : BAR = 0.5µm).



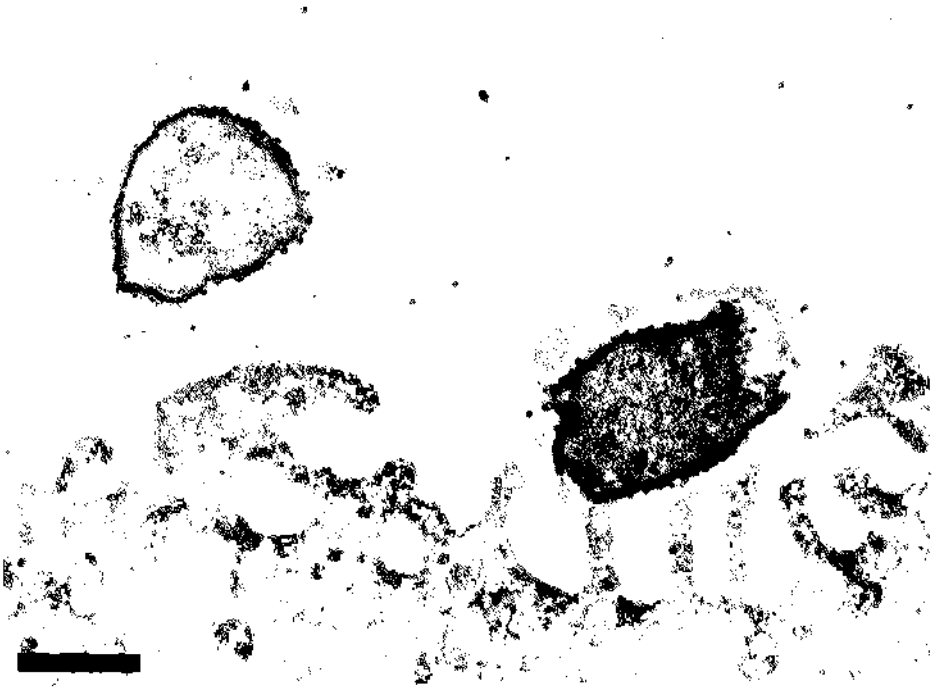
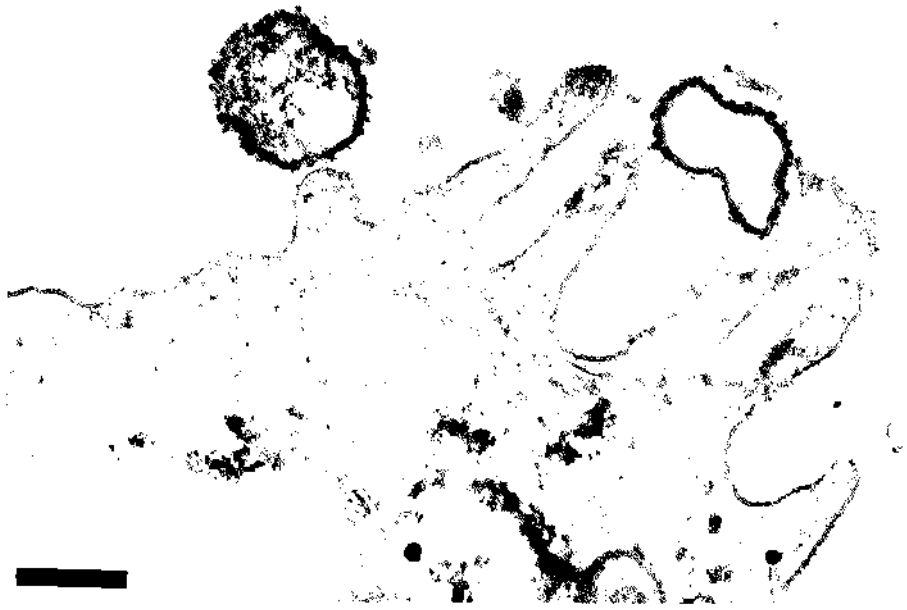


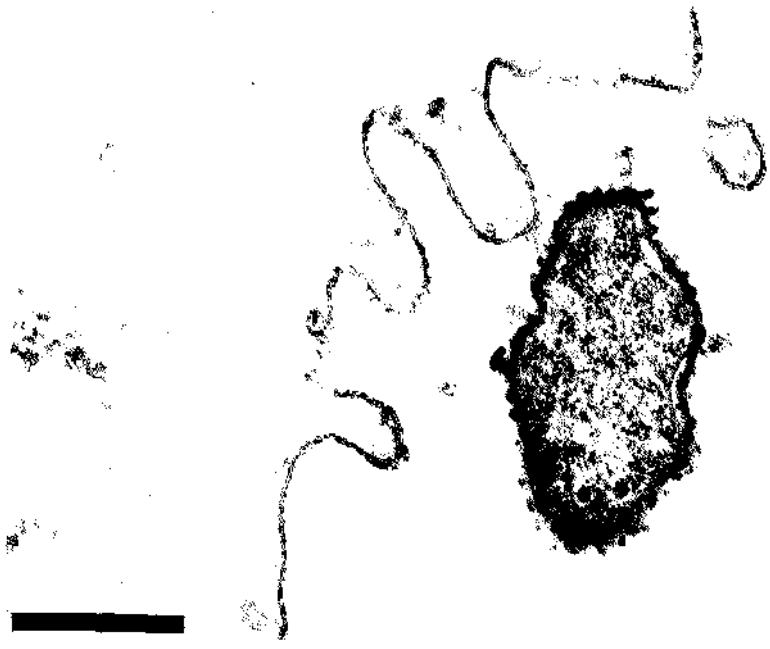
PLATE 35.

Transmission electron micrograph of ruthenium red stained preparation following assay of uroepithelial cells with E.coli strain 207, grown on TSA: bacterial attachment to cell surface. (Magnification x 30,000 : BAR = 0.5µm).

PLATE 36.

Transmission electron micrograph of ruthenium red stained preparation following assay of uroepithelial cells with E.coli strain 207, grown on TSA: bacterial attachment to cell surface (Magnification x 45,000 : BAR = 0.5µm).





4.5 THE USE OF SCANNING ELECTRON MICROSCOPY TO STUDY ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

A scanning electron microscope (S.E.M.) was used to investigate the attachment of E.coli to the surface of uroepithelial cells.

Using E.coli strains grown on TSA or in BHI broth overnight at 37^{o}C, the attachment assay was carried out with uroepithelial cells as described in section 1.1. After incubation and removal of unattached bacteria the cells were resuspended in 0.25 cm^{3} distilled water without being stained with erythrosine B or methylene blue. Two Pasteur pipette drops of the sample were placed on a polycarbonate membrane filter (0.6 μm) and fixed with Karnovsky's fixitive at 4^{o}C for 2 hours, followed by 3 washes in 0.1M phosphate buffer (pH 7.2) and 2 washes in distilled water. Secondary fixation was carried out using a 1% solution of osmium tetroxide made up in phosphate buffer; this was added to the filter and held for 1 hour at 4^{o}C, and followed by 3 washes in phosphate buffer. The specimens were dehydrated through 25%, 50%, 70% and 100% alcohol and dried in a POLARON E 3000 critical point-drier. The specimen membrane was placed on an aluminium stub and sputter coated with gold to approximately 10 nm thickness in a POLARON E 5100.

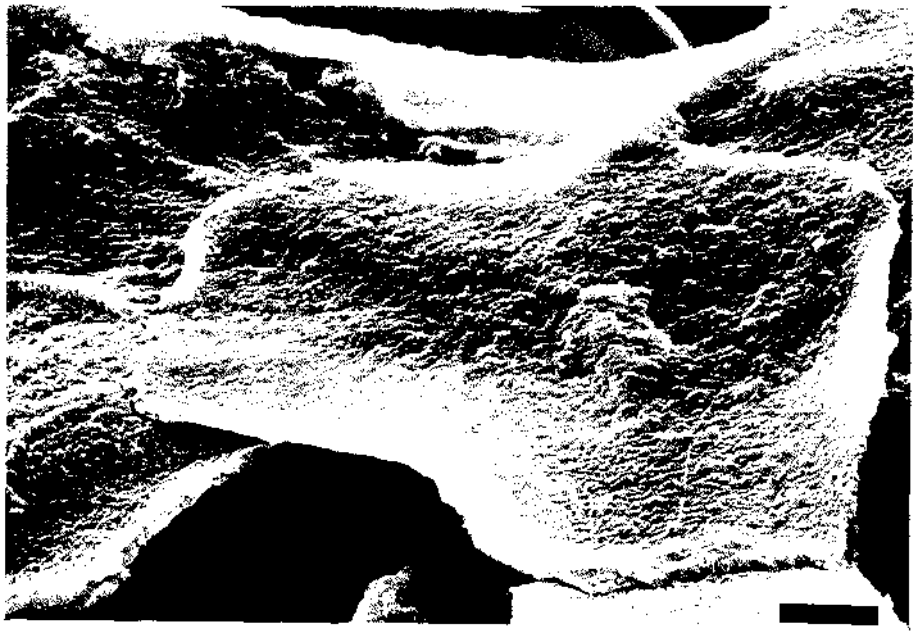
The specimens were viewed at 16 Kv and photographed to record the results (Plates 37 to 43).

PLATE 37.

Scanning electron micrograph of squamous uroepithelial cell (Magnification x 650 : BAR = 20μm).

PLATE 38.

Scanning electron micrograph of microvilli protruding from the surface of a uroepithelial cell (Magnification x 8000 : BAR = 2μm).



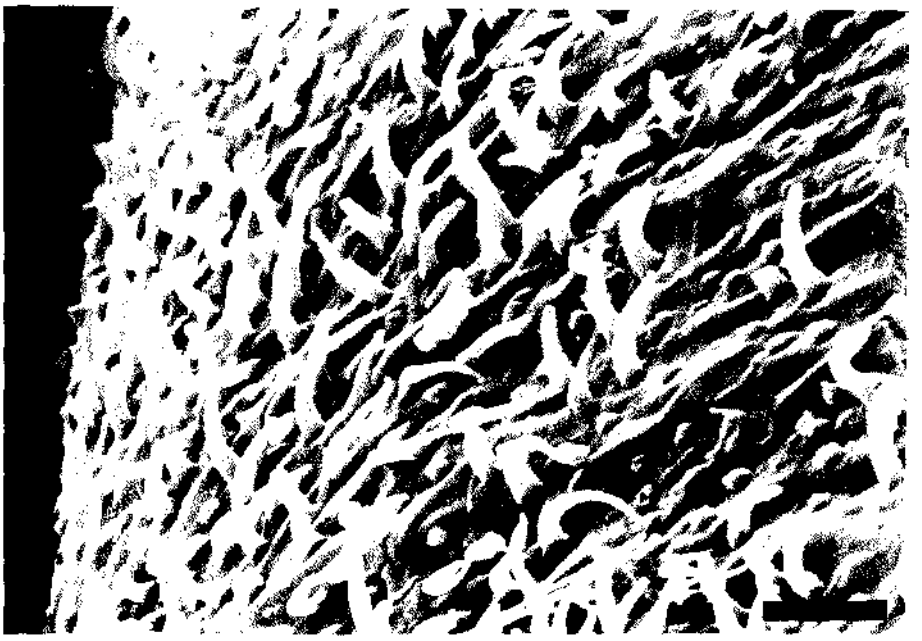
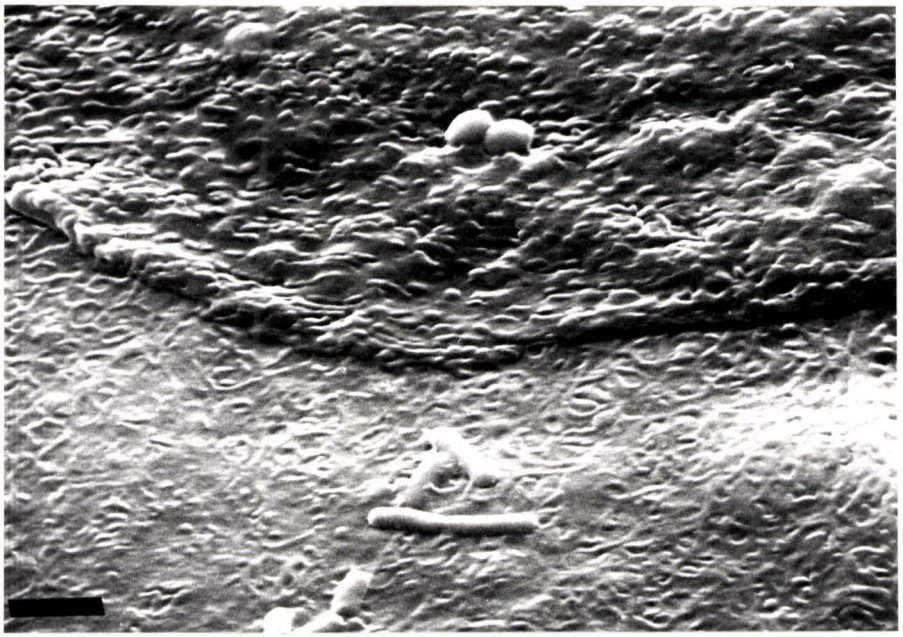


PLATE 39.

Scanning electron micrograph of uroepithelial cells which were assayed in the absence of E.coli: indigenous bacteria present on the uroepithelial cell surface. (Magnification x 6000 : BAR = 2μm).

PLATE 40.

Scanning electron micrograph of uroepithelial cells from a sample which was assayed in the absence of E.coli : indigenous bacterium present on the uroepithelial cell surface which is covered with microridges and microvilli. (Magnification x 2000 : BAR = 5μm).



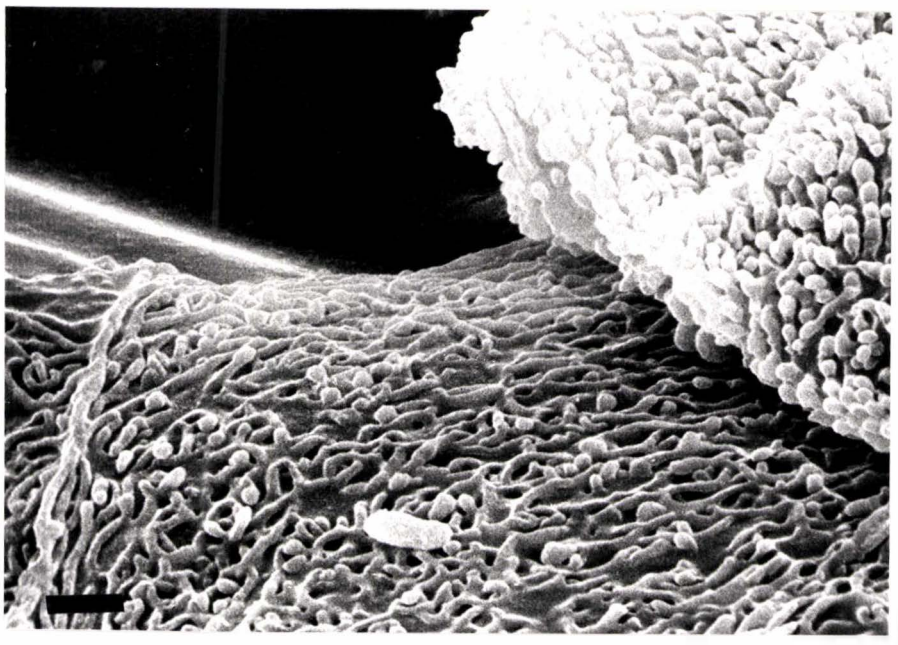
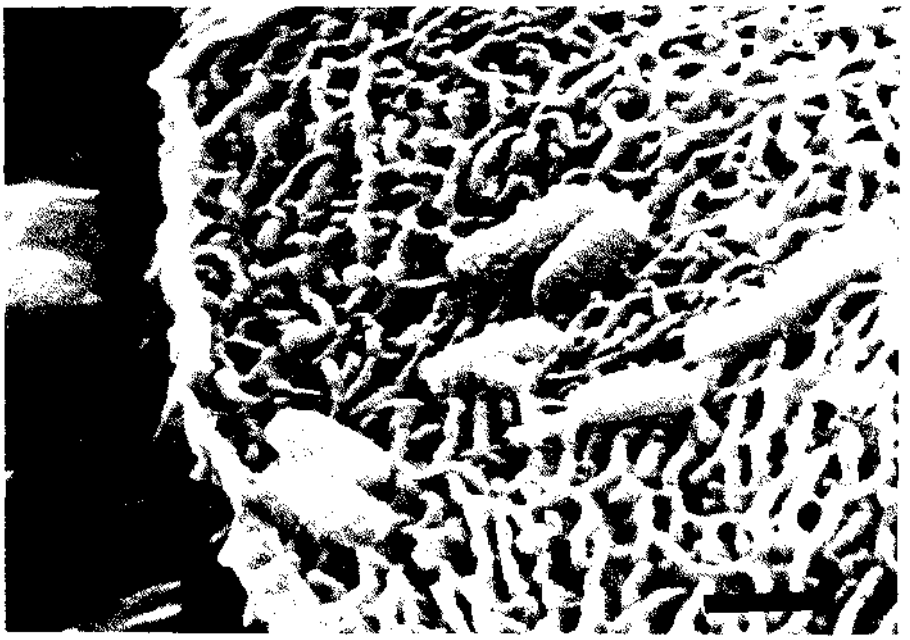


PLATE 41.

Scanning electron micrograph of uroepithelial cell from a sample which was assayed in the presence of BHI broth grown E.coli strain 87: E.coli-like organisms present on the uroepithelial cell surface (Magnification x 3,400 : BAR = 5μm).

PLATE 42.

Scanning electron micrograph of uroepithelial cell from a sample which was assayed in the presence of BHI broth grown E.coli strain 87: E.coli-like bacterium present on the uroepithelial cell surface (Magnification x 8,500 : BAR = 2μm).



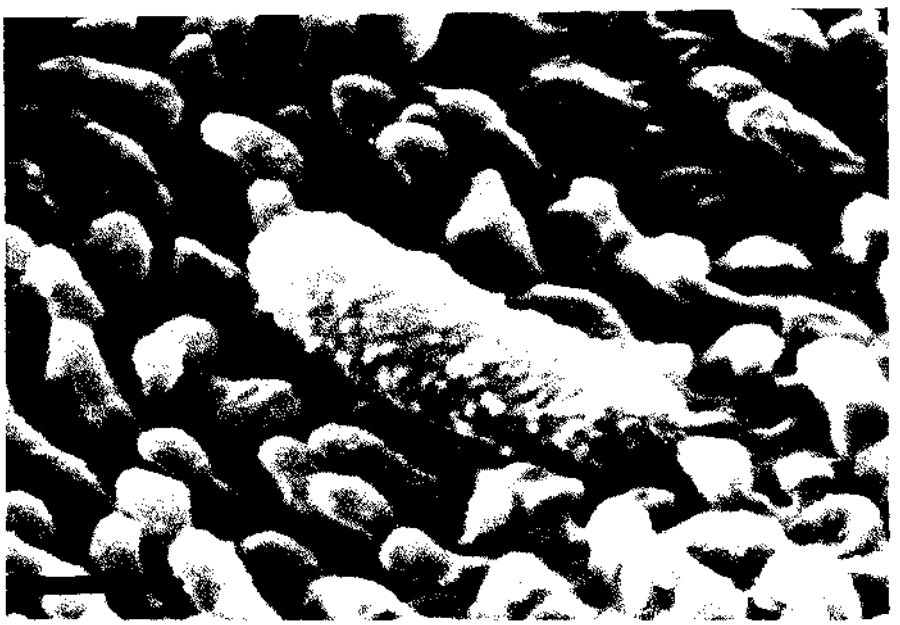
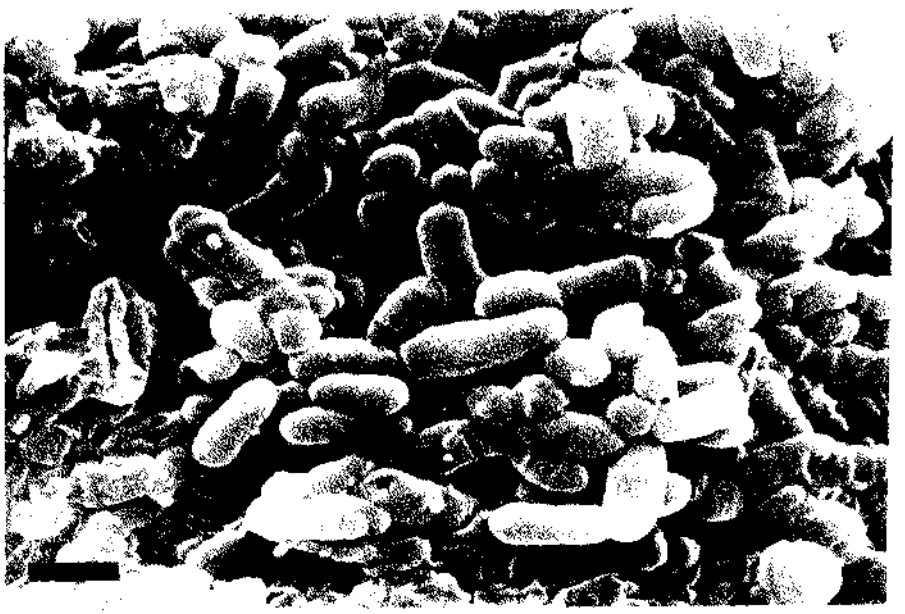


PLATE 43.

Scanning electron micrograph of uroepithelial cell from a sample which was assayed in the presence of TSA grown E.coli strain 207: uroepithelial cell surface covered with E.coli- like organisms.
(Magnification x 3000 : BAR = 4μm).



METHODS

SECTION 5.

THE USE OF CELL CULTURE TECHNIQUES TO STUDY E.COLI ATTACHMENT.

In order to investigate attachment of E.coli urinary strains to cells cultured in a monolayer, African Green Monkey kidney cells (VERO) were used. This standard kidney cell line was chosen because of the known propensity of E.coli to infect kidney tissue *in vivo*.

5.1 ATTACHMENT OF E.COLI TO TISSUE CULTURE VERO CELLS.

Vero cells, from a stock held at -70°C, were grown as a monolayer in 20 cm^3 BHK Eagles medium with 10% fetal calf serum, penicillin, streptomycin and bicarbonate buffer. The glass tissue culture flasks were held at 37°C for 5 days in a 5% CO_2 enriched atmosphere. When the growth was confluent the tissue cultures were trypsinized twice with 2 changes of 5 cm^3 trypsin at 37°C, suspended in BHK Eagles medium and the cells counted in a haemocytometer. For the passage of cells, a suspension of 3 x 10^6 cells were suspended in 20 cm^3 BHK Eagles and incubated as a monolayer in a tissue culture flask.

For the attachment assay, 1 x 10^6 trypsinized cells were added to a circular, cell culture dish (35 mm diameter) containing a 22 mm^2 coverslip. The cells were incubated in 2 cm^3 of antibiotic-free BHK Eagles medium at 37°C for 18 hours in air, enriched with CO_2 to 5%. The monolayer was washed with three volumes of PBS (pH 5.8) and a sample of cells tested for viability by determining exclusion of erythrosine B (0.4% w/v in PBS). The coverslip was placed cell-side upwards

in a fresh cell culture dish and 1 x 10^9 E.coli added in 2 cm^3 PBS. The bacteria had been grown on TSA overnight at 37°C and suspended to a concentration of 10^{10} organisms per cm^3 PBS. The cell monolayer was incubated with E.coli at 37°C for 90 minutes, and the monolayer washed with 7 changes of PBS to remove unattached bacteria.

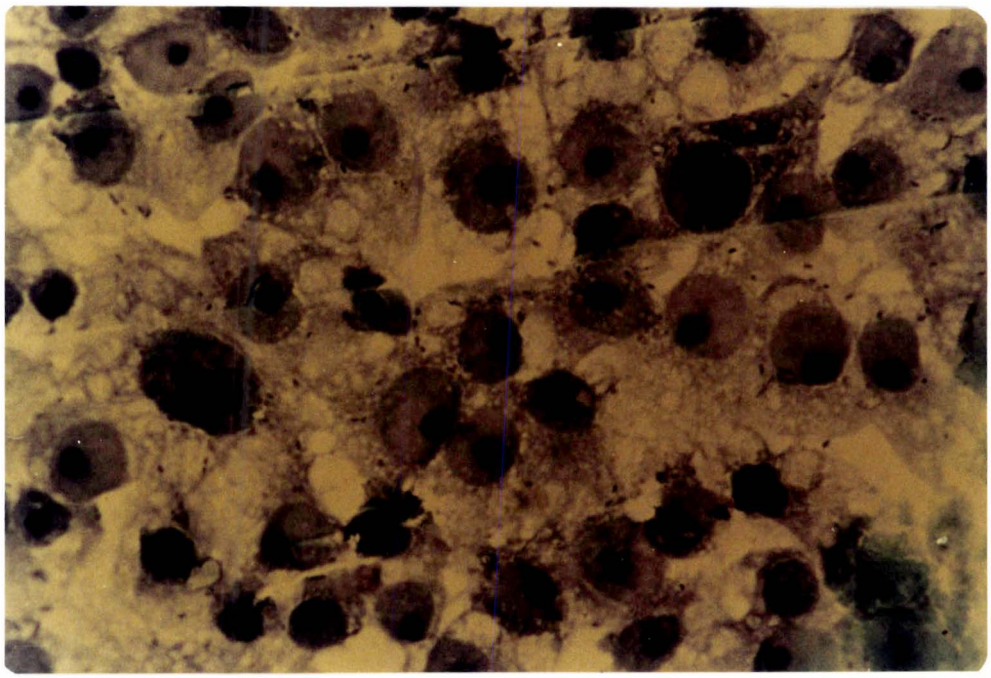
The coverslip was mounted with DPX on a glass microscope slide cell side upwards and the cell monolayer fixed in 10% formal saline for 5 minutes, washed in PBS, air dried, stained with methylene blue (0.04% w/v in PBS) for 30 seconds, rinsed in distilled water, air dried and viewed at 1000 x magnification. The number of E.coli attached to 40 tissue culture cells was then counted. A total of 42 E.coli urinary strains grown on TSA were tested in duplicate for attachment to tissue culture cells. Counts were expressed as mean numbers of attached E.coli per cell. The control for each test consisted of a coverslip with a tissue culture cell monolayer incubated at 37°C for 90 minutes in PBS without E.coli added. These cells were also monitored for the presence of bacteria by assessing 40 cells at random. (Plate 44).

5.2 ATTACHMENT OF E.COLI TO TISSUE CULTURE CELLS DERIVED FROM NEW BORN MOUSE KIDNEY.

In addition to vero cell cultures, cells from the kidneys of 12 new born mice were grown as a monolayer. The surgically removed kidneys were dissected using sterile scalpel and forceps and the tissue trypsinized in 5 to 10 cm^3 trypsin (0.5% solution). The suspension was centrifuged at 200 x g for 10 minutes in PBS (pH 7.1) to remove tissue debris, and the cellular material in 1 cm^3 PBS was resuspended in 99 cm^3 T199 medium supplemented with 10% fetal calf serum. The cells were grown as a monolayer in

PLATE 44.

Monkey kidney (Vero) cell monolayer with TSA grown *E.coli* strain 116 attached to the cells: stained with methylene blue (Magnification *Ca* x 290).



plastic culture flasks and passaged every 2 to 3 days.

To assay attachment, seven E.coli strains were selected which attached to uroepithelial cells *in vitro* and to some extent to Vero cell culture monolayers. These strains were grown on TSA or BHI broth, suspended in PBS to a concentration of 10^{10} organisms per cm^3 and assayed for attachment to cell monolayers from new born mouse kidneys, using the technique described in 6.1 for Vero cells. The number of E.coli attached to 40 cells was assessed and a mean value calculated for each strain.

RESULTS

SECTION 1.

ATTACHMENT OF E.COLI CELLS TO HUMAN URINARY TRACT EPITHELIAL CELLS *IN VITRO*.

1.1 ASSAY METHOD FOR *IN VITRO* ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

The assay method proved effective in detecting the attachment of E.coli to uroepithelial cells. The double staining technique facilitated the differentiation of viable and nonviable uroepithelial cells with bacteria attached. Throughout the study, uroepithelial cell viability in samples ranged from 25 to 89% viable cells. The mean value for the urine pH of 100 samples from 10 non-pregnant women was pH 5.75 and for the urine of 79 samples from 6 pregnant women was pH 6.11. In all tests the control cell samples incubated without E.coli added, gave a mean of 0 to 5 E.coli-like organisms per uroepithelial cell.

Unless it is stated otherwise the E.coli strains used in the next series of experiments were strains 67 and 87 grown in BHI broth and strains 125 and 207 grown on TSA.

VARIABILITY OF ASSAY METHOD:

1.1.1 UROEPITHELIAL CELL VIABILITY DURING THE ASSAY.

An experiment was performed to determine whether the conditions of the attachment assay affected uroepithelial cell viability. Twenty uroepithelial cell samples were tested for viability using erythrosine B (0.4% w/v PBS). For each sample 100 uroepithelial

TABLE 1

UROEPITHELIAL CELL VIABILITY DURING ASSAY

PERCENTAGE VALUES

SAMPLE	VIABILITY AT START	VIABILITY AT END
1	41	40
2	55	53
3	25	23
4	35	33
5	21	20
6	25	25
7	25	27
8	63	60
9	40	38
10	30	27
11	32	36
12	27	29
13	29	33
14	33	29
15	43	40
16	40	40
17	59	59
18	63	60
19	44	40
20	70	69
	MEAN = 40	MEAN = 39.05

cells (50 squamous cells and 50 transitional cells) were assessed. The results were statistically interpreted using two-way analysis of variance. The results are presented in Table 1. There was no significant difference between uroepithelial cell viability at the start and the end of the attachment assay ($P > 0.05$).

1.1.2 VARIATION IN ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS WITHIN ANY ONE SAMPLE.

This experiment was carried out to obtain a value for the variation in attachment of E.coli which occurred within a given uroepithelial cell sample.

Nine separate uroepithelial cell samples were assayed for attachment with E.coli strains 43, 67, 73, 87, 125, 153 and 207 as described in section 1.1. The attachment values for 40 viable uroepithelial cells were obtained and a standard error of the mean was calculated. A percentage value was then calculated to represent the variation of the mean within the 40 uroepithelial cells for any given sample.

The results are presented in Table 2. The variation within cell samples for attachment of E.coli ranged from 6.95% to 14.82%. This level of variation was present even allowing for up to 420 E.coli attaching to one uroepithelial cell in sample number 7. The mean value for this sample was 65 E.coli per cell and the large range of counts stressed the fact that uroepithelial "target cells" which are highly receptive to E.coli attachment, are present in cell samples. These "target cells" will be discussed later.

From the results of this experiment, a 15% level of variation was taken to represent the expected variability in attachment of E.coli to a given uroepithelial cell sample.

TABLE 2

VARIATION IN ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS WITHIN ANY ONE SAMPLE.

ROEPITHELIAL CELL SAMPLE	STRAIN	GROWTH CONDITIONS	E.COLI ATTACHED TO 40 UROEPITHELIAL CELLS			PERCENTAGE VARIATION OF MEAN
			MEAN	S.E.M.	RANGE OF COUNTS	
1	43	Broth	6	0.753	0 to 25	12.55
2	67	Broth	12	0.83	3 to 29	6.95
3	87	Broth	15	1.84	4 to 48	12.26
4	153	TSA	37	4.5	4 to 108	12.19
5	125	TSA	59	8.06	11 to 321	13.66
6	207	TSA	62	4.87	10 to 140	7.85
7	125	TSA	65	10.14	12 to 420	14.82
8	207	TSA	69	10.14	16 to 400	14.7
9	73	TSA	78	11.05	12 to 370	14.2

1.1.3 VARIATION BETWEEN DUPLICATE EXPERIMENTS USING THE SAME SUSPENSIONS EACH OF E.COLI AND UROEPITHELIAL CELLS.

An experiment was designed to determine whether a significant variation occurred when attachment was assayed using duplicate samples of E.coli and uroepithelial cells.

Twenty E.coli strains were assayed for attachment to 20 separate uroepithelial cell samples, using the method described in section 1.1. The results, presented in Table 3 show that no statistical difference was present between mean attachment values of duplicate samples, after analysis by two-way variance ($p > 0.1$). Although some subsequent experiments incorporated duplicate samples, these results suggest that there may not be a need for these duplications.

1.1.4 VARIATION BETWEEN UROEPITHELIAL CELL SAMPLES FROM DIFFERENT WOMEN ON THE SAME DAY.

This experiment investigated whether uroepithelial cell samples from five women varied in receptivity to E.coli attachment. The uroepithelial cells were assayed for attachment with E.coli strains 67, 87, 125 and 207 as in section 1.1.

The results are presented in Table 4. One-way analysis of variance was used to compare attachment to the 40 uroepithelial cells evaluated in each test sample. There was no significant difference between the attachment values for strains 125 and 207 grown on TSA. ($p > 0.1$). However, there was a highly significant difference between the attachment values for both strains 67 and 87 grown in BHI broth, when results for the five women were compared ($p < 0.001$).

TABLE 3

VARIATION BETWEEN DUPLICATE EXPERIMENTS USING THE SAME SUSPENSIONS EACH OF *E.COLI* AND UROEPITHELIAL CELLS.

SAMPLE NUMBER	MEAN NUMBER OF *E.COLI* PER UROEPITHELIAL CELL	
	TEST 1	TEST 2
1	71	68
2	65	64
3	61	62
4	61	60
5	59	60
6	42	41
7	36	36
8	34	32
9	17	16
10	16	17
11	17	17
12	15	16
13	13	12
14	13	14
15	13	13
16	14	13
17	10	9
18	10	11
19	8	8
20	7	8
	M = 29.1	M = 28.85

TABLE 4

VARIATION BETWEEN UROEPITHELIAL CELL SAMPLES FROM DIFFERENT WOMEN ON THE SAME DAY.

SAMPLE	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL			
	BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
	STRAIN 67	STRAIN 87	STRAIN 125	STRAIN 207
1	10	11	56	60
2	16	16	65	69
3	5	6	53	54
4	12	13	59	66
5	8	7	56	56
	$p < 0.001$	$p < 0.001$	$p > 0.1$	$p > 0.1$

TABLE 5

VARIATION BETWEEN UROEPITHELIAL CELL SAMPLES FROM ONE WOMAN ON DIFFERENT DAYS.

SAMPLE	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL			
	BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
	STRAIN 67	STRAIN 87	STRAIN 125	STRAIN 207
1	16	14	61	69
2	12	11	59	64
3	10	10	58	62
4	10	10	55	57
5	4	6	52	55
	$p < 0.001$	$p < 0.001$	$p > 0.1$	$p > 0.1$

No such difference was observed for TSA grown cultures.

1.1.5 VARIATION BETWEEN UROEPITHELIAL CELL SAMPLES FROM ONE WOMAN ON DIFFERENT DAYS.

This experiment investigated whether there was variation in E.coli attachment to uroepithelial cells collected from one woman on five different days. The attachment assay described in section 1.1 was used for E.coli strains 67, 87, 125 and 207 and uroepithelial cells collected from one woman at weekly intervals.

The results of this study are presented in Table 5. One way analyis of variance was used to compare attachment to the 40 uroepithelial cells evaluated in each test sample. There was no significant difference between the attachment values for strains 125 and 207 grown on TSA ($p > 0.1$). However, there was a highly significant difference between the attachment values for both strains 67 and 87 grown in BHI broth, when results for the 5 samples were compared ($p < 0.001$). No such difference was observed for TSA grown cultures.

1.1.6 COMPARISON OF E.COLI ATTACHMENT TO VIABLE AND NONVIABLE Uroepithelial Cells.

Uroepithelial cells from 10 urine samples were assayed for attachment with E.coli strains 67, 87, 125 and 207 as described in section 1.1. The number of E.coli attached to 40 viable and 40

nonviable uroepithelial cells was determined for each test. The mean attachment values were compared using two-way analysis of variance. The frequency distributions of E.coli attached to 100 viable and 100 nonviable uroepithelial cells were noted for BHI broth grown and TSA grown cultures.

From the results in Table 6 it appeared that E.coli attached in greater numbers to viable than to nonviable uroepithelial cells. This difference was highly significant both for BHI broth grown cultures and for TSA grown cultures ($p < 0.001$). These results emphasized the importance of using the double staining technique devised for the assay. This technique permitted evaluation of E.coli attached to viable as well as nonviable uroepithelial cells. Therefore, highest attachment values were found for viable uroepithelial cells, and in subsequent assays only these cells were evaluated unless stated otherwise.

The frequency distributions are illustrated in Figure 1, Parts A and B. The attachment of E.coli to uroepithelial cells did not appear to follow a normal distribution. For BHI broth grown cultures, 50% of the viable cells appeared to be highly receptive to E.coli attachment (> 10 E.coli per uroepithelial cell) compared to only 21% of the nonviable cells. The mean attachment values for the 200 uroepithelial cells were 12.1 E.coli per viable cell and 8.2 E.coli per nonviable cell.

For TSA grown cultures, 56% of the viable cells were highly receptive to E.coli attachment (> 50 E.coli per uroepithelial cell) compared to only 20% of the nonviable cells. The mean attachment values for the 200 uroepithelial cells were 58.9 E.coli per viable cell and 33.1 E.coli nonviable cell.

It is apparent that the concept of uroepithelial "target cells" is different and depends on the nature of the E.coli strain concerned. In the case of strains 67 and 87 grown in BHI broth,

TABLE 6

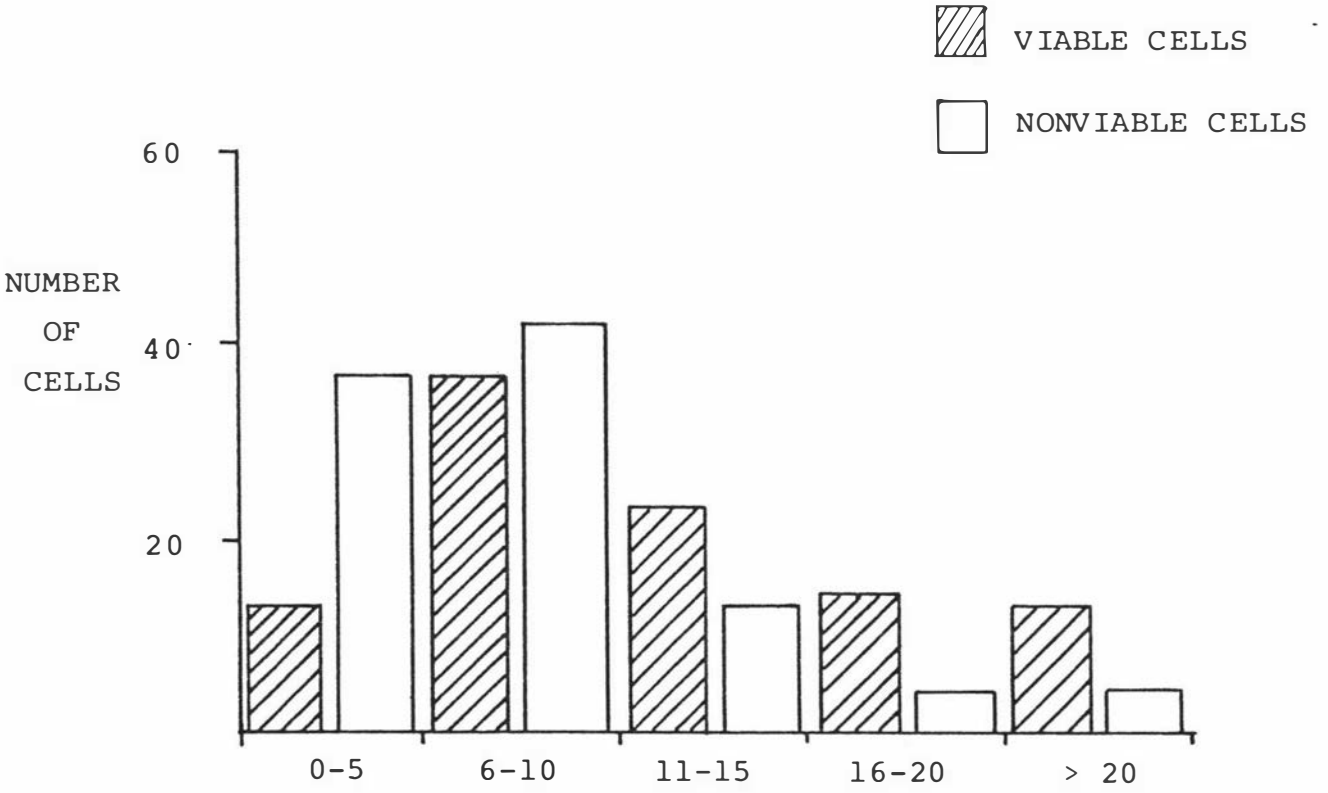
COMPARISON OF E.COLI ATTACHMENT TO VIABLE AND NONVIABLE UROEPITHELIAL CELLS.

SAMPLE	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL			
	BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
	VIABLE CELLS	NONVIABLE CELLS	VIABLE CELLS	NONVIABLE CELLS
1	14	12	59	38
2	21	14	65	52
3	13	9	62	51
4	14	7	60	38
5	13	8	61	40
6	10	7	58	39
7	10	5	57	48
8	9	5	57	32
9	7	5	54	35
10	20	15	66	49
	MEAN = 13.1	MEAN = 8.7	MEAN = 59.9	MEAN = 42.2
	$p < 0.001$		$p < 0.001$	

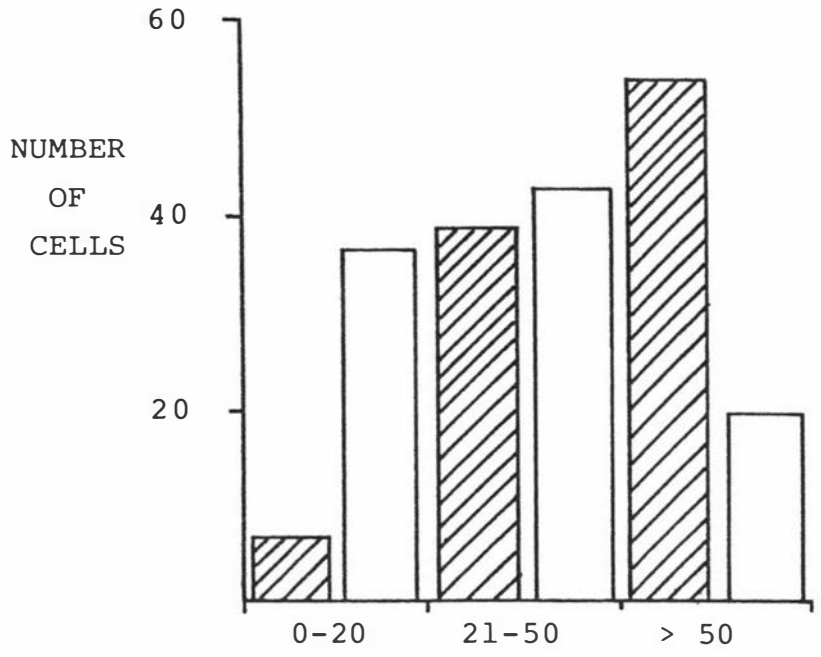
FIGURE 1

FREQUENCY DISTRIBUTIONS FOR COMPARISON OF E.COLI ATTACHMENT TO 100 VIABLE AND 100 NONVIABLE UROEPITHELIAL CELLS.

A. ATTACHMENT OF STRAINS 67 and 87 GROWN IN BHI BROTH



B. ATTACHMENT OF STRAINS 125 AND 207 GROWN ON TSA



the "target cells" were those to which more than 10 E.coli attached whereas for strains 125 and 207 grown on TSA, the "target cells" were those to which more than 50 E.coli attached. The results suggest that E.coli strains may have different mechanisms for attachment to the uroepithelial cell surface.

1.1.7 COMPARISON OF E.COLI ATTACHMENT TO SQUAMOUS AND TRANSITIONAL UROEPITHELIAL CELLS.

Two types of uroepithelial cells were distinguished in a given cell suspension; namely squamous cells known to originate from the bladder trigone, urethral and periurethral areas, and transitional cells from the epithelium of the ureters and bladder. To compare E.coli attachment to the two cell types, 10 uroepithelial cell samples were assayed with strains 67, 87, 125 and 207 as described in section 1.1. The mean attachment values were calculated and compared by two-way analysis of variance. Each mean value represented 40 viable squamous or 40 viable transitional cells. The frequency distributions of E.coli attached to 100 squamous and 100 transitional cells were noted for BHI broth grown and TSA grown cultures.

The results are presented in Table 7. Overall, BHI broth grown strains attached in greater numbers to squamous than to transitional cells and the difference was highly significant ($p < 0.001$). This overall difference was also significant for TSA grown cultures which also attached in larger numbers to squamous cells ($p < 0.01$). However, attachment of E.coli to transitional cells was in three cases higher than attachment to squamous cells.

The frequency distributions are presented in Figure 2, Parts A and B. The attachment of E.coli to both uroepithelial cell types

TABLE 7

COMPARISON OF E.COLI ATTACHMENT TO SQUAMOUS AND TRANSITIONAL UROEPITHELIAL CELLS.

MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL

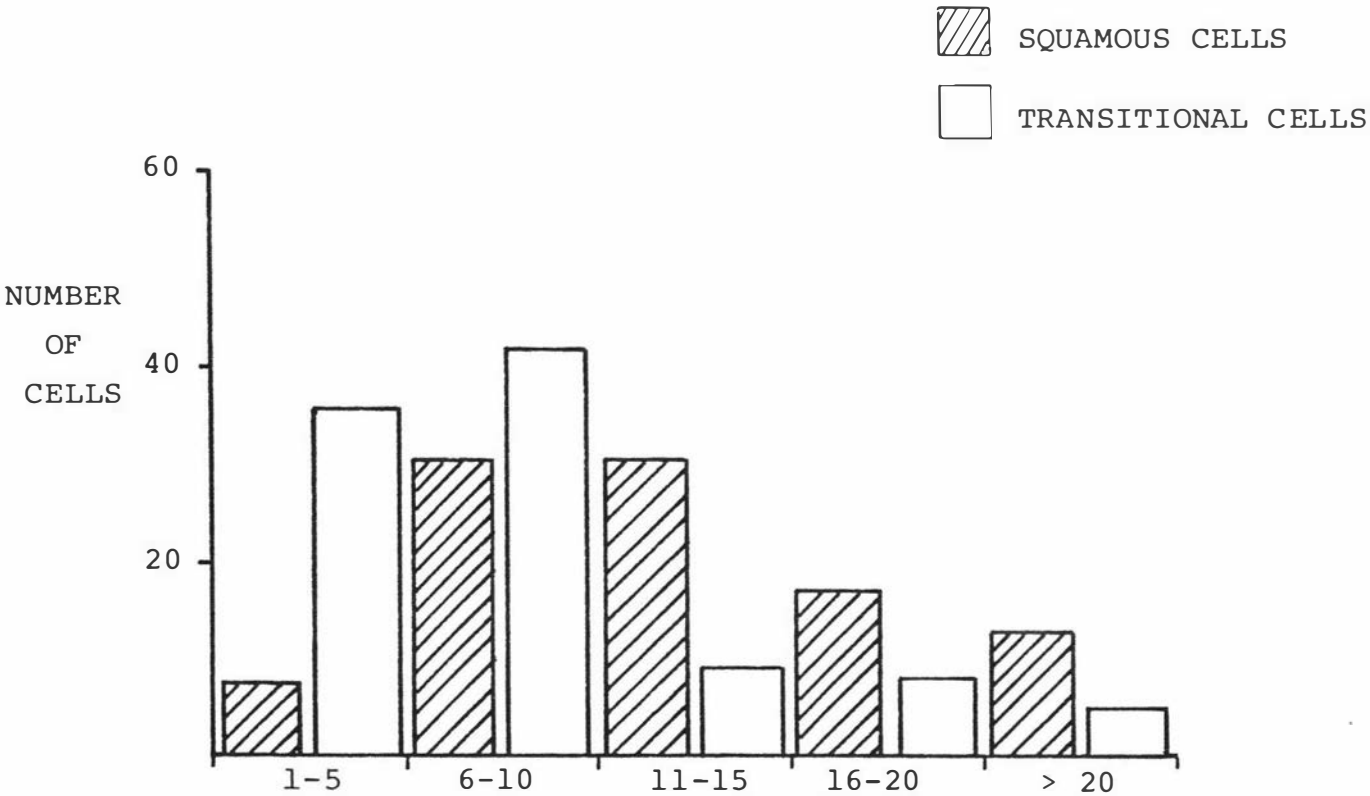
SAMPLE	BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
	SQUAMOUS CELLS	TRANSITIONAL CELLS	SQUAMOUS CELLS	TRANSITIONAL CELLS
1	12	9	65	47
2	21	13	72	59
3	6	5	53	48
4	18	9	65	54
5	13	16	67	64
6	14	9	67	49
7	16	10	59	60
8	14	11	59	62
9	12	8	58	49
10	11	5	59	46
	MEAN = 13.7	MEAN = 9.5	MEAN = 62.4	MEAN = 53.8

$p < 0.001$ $p < 0.01$

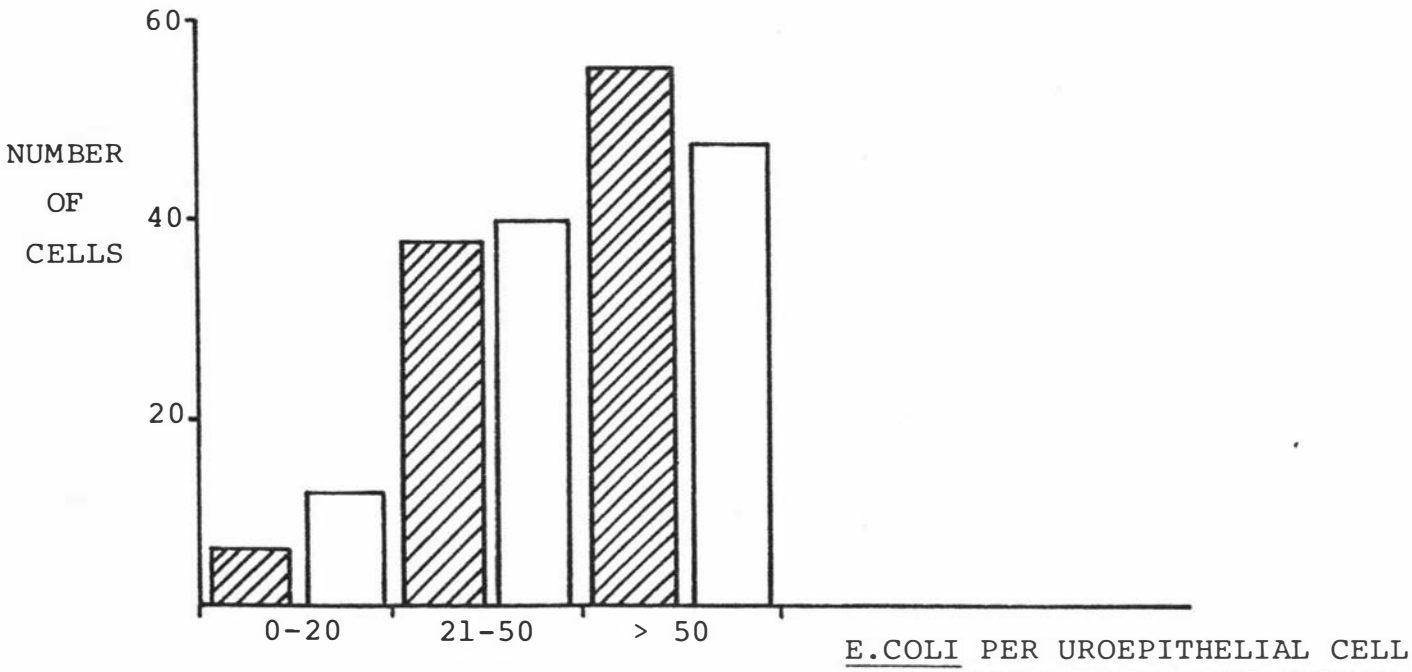
FIGURE 2

FREQUENCY DISTRIBUTION FOR COMPARISON OF *E.COLI* ATTACHMENT TO 100 SQUAMOUS AND 100 TRANSITIONAL UROEPITHELIAL CELLS.

A. ATTACHMENT OF STRAINS 67 AND 87 GROWN IN BHI BROTH



B. ATTACHMENT OF STRAINS 125 AND 207 GROWN ON TSA



did not follow a normal distribution. For BHI broth grown cultures, 61% of the squamous cells and only 22% of the transitional cells were highly receptive to E.coli attachment (> 10 E.coli per uroepithelial cell). The mean attachment values for the 200 cells were 13.2 E.coli per squamous cell and 8.2 E.coli per transitional cell.

For TSA grown cultures, 56% of the squamous cells and 48% of the transitional cells were highly receptive to E.coli attachment (> 50 E.coli per uroepithelial cell). The mean attachment values for the 200 cells were 64.2 E.coli per squamous cell and 53.7 E.coli per transitional cell.

Clearly the transitional cells were more receptive to attachment of strains 125 & 207 grown on TSA, than to attachment of strains 67 and 87 grown in BHI broth. The differences between these four strains will be discussed in section 3.

REPRODUCABILITY OF ASSAY METHOD:

The results presented in these sections provide evidence that the assay method is both reliable and reproducible.

1.1.8 DETERMINATION OF E.COLI ATTACHED TO UROEPITHELIAL CELLS WITH HIGH OR LOW NUMBERS OF INDIGENOUS BACTERIA ON THE CELL.

In all uroepithelial cell populations, a proportion of the cells was observed to have indigenous bacteria adsorbed to the surface. The use of a double staining technique made it possible to distinguish E.coli-like organisms from non E.coli-like indigenous bacteria attached to viable uroepithelial cells. An experiment was designed to confirm that E.coli attachment to uroepithelial

cells was being measured rather than attachment of indigenous bacteria, using the attachment assay described in section 1.1. In addition, it was hoped to determine whether E.coli attached to the same degree to uroepithelial cells with high numbers of indigenous bacteria present on the surface as compared to cells with low numbers.

E.coli strains 87 grown in BHI broth and 207 grown on TSA were assayed for attachment to five uroepithelial cell samples, as described in 1.1. The mean number of E.coli-like organisms attached to 40 viable uroepithelial cells with low numbers of indigenous bacteria ($\leq$ 10 bacteria per cell) was determined. The results were compared with the mean for E.coli attached to 40 cells with high numbers of indigenous bacteria (> 30 bacteria per cell). Statistical analysis was carried out using two-way analysis of variance. A control with uroepithelial cells and no E.coli added was also incorporated to give a net result for E.coli attachment.

The results of this experiment are presented on Table 8. There was no significant difference between the number of E.coli-like organisms attached to viable uroepithelial cells which had low or high numbers of non E.coli-like indigenous bacteria present on the cells ($p > 0.1$). E.coli-like organisms were also seen attached to indigenous non E.coli-like bacteria on some cells. The controls with PBS and uroepithelial cells gave a mean of 0 to 2 E.coli-like organisms per cell whether low or high numbers of indigenous bacteria were present. The Table also shows the mean number of non E.coli-like organisms present in the uroepithelial cells which were examined.

TABLE 8

DETERMINATION OF E.COLI ATTACHED TO UROEPITHELIAL CELLS WITH HIGH OR LOW NUMBERS OF INDIGENOUS BACTERIA ON THE CELL.

STRAIN 87

SAMPLE	MEAN NUMBER OF E.COLI-LIKE ORGANISMS PER UROEPITHELIAL CELL		MEAN NUMBER OF NON E.COLI-LIKE ORGANISMS PER UROEPITHELIAL CELL	
	LOW FLORA	HIGH FLORA	LOW FLORA	HIGH FLORA
1	9	8	6	39
2	5	5	3	49
3	8	8	6	73
4	12	11	3	52
5	10	12	4	61
	$\bar{M}$ = 8.8	$\bar{M}$ = 8.8	$\bar{M}$ = 4.4	$\bar{M}$ = 54.8
	$p > 0.1$			

STRAIN 207

SAMPLE	MEAN NUMBER OF E.COLI-LIKE ORGANISMS PER UROEPITHELIAL CELL		MEAN NUMBER OF NON E.COLI-LIKE ORGANISMS PER UROEPITHELIAL CELL	
	LOW FLORA	HIGH FLORA	LOW FLORA	HIGH FLORA
1	57	55	0	50
2	60	59	8	34
3	54	56	4	41
4	63	65	6	62
5	61	62	7	42
	$\bar{M}$ = 59	$\bar{M}$ = 59.4	$\bar{M}$ = 5	$\bar{M}$ = 45.8
	$p > 0.1$			

1.1.9 NUMBER OF UROEPITHELIAL CELLS ASSESSED FOR E.COLI ATTACHMENT.

This experiment was carried out to determine whether the number of uroepithelial cells evaluated for E.coli attachment affected the mean attachment values.

Using the method of section 1.1, E.coli strains 67, 87, 125 and 207 were assayed for attachment to uroepithelial cells from three urine samples. The mean number of E.coli attached to 20, 30, 40 and 50 uroepithelial cells was determined for each strain, and the results are presented in Table 9.

The mean values for assessment of 20, 30, 40 or 50 uroepithelial cells did not differ outside the 15% variation present within a cell sample as reported in section 1.1.2. The differences between mean values for broth grown cultures was ± 1 E.coli per cell and ± 6 E.coli per cell for TSA grown cultures. Subsequent experiments continued the assessment of 40 viable uroepithelial cells for E.coli attachment.

1.1.10 NUMBER OF WASHES REQUIRED TO REMOVE UNATTACHED E.COLI FROM UROEPITHELIAL CELLS.

This experiment was carried out as in section 1.1, for assaying E.coli strains 67 and 87 grown in BHI broth, and uroepithelial cells from pooled urine samples. Following the assay incubation of 60 min, at 37^{o}C and 20 r.p.m. in a shaker, the suspensions were washed in PBS, pH 7.1 from 1 to 10 times.

The mean number of E.coli attached to 40 viable uroepithelial cells is reported for each sample in Table 10.

The removal of unattached E.coli from the preparations was

TABLE 9

NUMBER OF UROEPITHELIAL CELLS ASSESSED FOR E.COLI ATTACHMENT.

SAMPLE	NUMBER OF UROEPITHELIAL CELLS EVALUATED	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL			
		BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
		STRAIN 67	STRAIN 87	STRAIN 125	STRAIN 207
1	20	9	10	60	58
	30	9	10	59	55
	40	9	10	62	57
	50	9	11	59	56
2	20	8	8	58	56
	30	7	7	58	58
	40	8	8	59	62
	50	8	7	59	59
3	20	11	14	59	57
	30	11	13	62	57
	40	10	14	59	60
	50	10	14	58	59

TABLE 10

NUMBER OF WASHES REQUIRED TO REMOVE UNATTACHED E.COLI FROM UROEPITHELIAL CELLS.

NUMBER OF WASHES IN BUFFER	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	
	STRAIN 67	STRAIN 87
1	22	24
2	19	20
3	15	15
4	14	14
5	13	14
6	13	14
7	14	13
8	12	14
9	13	13
10	13	14

only effective after 4 washes in buffer. After 1, 2 or 3 washes there were unattached bacteria still evident in the suspensions. These unattached bacteria gave rise to elevated mean attachment counts of 15 to 24 E.coli per uroepithelial cell. The difference between the mean values obtained after 4 to 10 buffer washes, was not outside the 15% variation present in a cell sample, as reported in section 1.1.2. In subsequent tests, five buffer washes were incorporated to remove unattached bacteria.

1.1.11 ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS SUBJECTED TO A VARYING NUMBER OF WASHES, PRIOR TO ASSAY.

Uroepithelial cells were harvested from urine samples and suspended in PBS, pH 7.1. Aliquots of 1×10^5 cells were dispensed into separate tubes, washed 0,1,2,3,4,5 and 10 times in PBS at 250 x g for 12 minutes and assayed for attachment of E.coli strains 67, 87, 125 and 207 as described in section 1.1.

The results are presented in Table 11. There was no difference between the mean attachment values other than the 15% variation known to be present within a cell sample, as reported in section 1.1.2.

In subsequent experiments, uroepithelial cells were prewashed three times in PBS, pH 7.1, to remove small amounts of urinary mucus or amorphous debris that might be present in the urine sample.

1.1.12 ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS DERIVED FROM ALIQUOTS OF SINGLE URINE SPECIMENS.

A sample of urine was divided into three and the cells harvested and washed. Uroepithelial cells from each of the three suspensions were assayed for attachment with E.coli strains 67, 87, 125 and 207,

TABLE 11

ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS SUBJECTED TO A VARYING NUMBER OF WASHES PRIOR TO ASSAY.

NUMBER OF WASHES IN PBS FOR UROEPITHELIAL CELLS PRIOR TO ASSAY	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL			
	BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
	STRAIN 67	STRAIN 87	STRAIN 125	STRAIN 207
0	11	13	58	60
1	12	15	57	62
2	13	15	59	61
3	13	15	61	65
4	13	14	60	64
5	13	14	62	66
10	12	15	64	67

as in section 1.1. The experiment was carried out in triplicate.

Analysis of the results on Table 12 failed to detect any significant difference between the mean attachment values for aliquots A, B and C. The small variations were less than the 15% variation present within a given cell sample, as reported in section 1.1.2. The results for the three samples confirmed the reproducability of the experiment. The results suggested that urine samples could be divided and uroepithelial cells used for the assay without a significant variation in the mean attachment values occurring.

1.1.13 ASSESSMENT OF E.COLI ATTACHMENT TO UROEPITHELIAL CELLS ON FOUR SEGMENTS OF A PREPARED SLIDE.

This experiment was carried out to determine whether random assessment of uroepithelial cells on four segments of a prepared slide, would give rise to similar mean attachment values. Uroepithelial cells were assayed for attachment with E.coli strains 67, 87, 125 and 207, as in section 1.1. Each resultant slide was divided into four segments using a marker pen. The number of E.coli attached to 40 viable uroepithelial cells in segments A, B, C and D was assessed. The tests were carried out in triplicate.

The results are presented in Table 13. There was little difference observed between counts of E.coli attached to uroepithelial cells from any of four regions on the slides. The small differences between mean attachment values could be explained by the 15% variation reported in section 1.1.2.

1.1.14 ASSESSMENT OF E.COLI ATTACHED TO UROEPITHELIAL CELLS FOR PREPARATIONS EXAMINED ON THREE OCCASIONS.

This experiment was designed to determine whether a slide

TABLE 12

ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS DERIVED FROM ALIQUOTS OF SINGLE URINE SPECIMENS.

SAMPLE	ALIQUOT	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL			
		BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
		STRAIN 67	STRAIN 87	STRAIN 125	STRAIN 207
1	A	5	6	56	55
	B	5	7	56	57
	C	5	6	60	56
2	A	7	10	57	64
	B	8	9	58	69
	C	8	10	59	65
3	A	12	11	54	62
	B	11	10	56	59
	C	12	10	56	64

TABLE 13

ASSESSMENT OF E.COLI ATTACHMENT TO UROEPITHELIAL CELLS ON FOUR SEGMENTS OF A PREPARED SLIDE.

SLIDE	SEGMENT	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL			
		BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
		STRAIN 67	STRAIN 87	STRAIN 125	STRAIN 207
1	A	7	10	53	57
	B	7	10	58	57
	C	7	9	53	61
	D	6	10	55	58
2	A	8	12	57	62
	B	9	12	57	61
	C	8	12	54	63
	D	8	11	58	64
3	A	7	9	55	55
	B	7	9	56	56
	C	7	10	56	55
	D	7	10	58	58

preparation examined on three separate occasions gave rise to similar mean values for E.coli attachment to uroepithelial cells. E.coli strains 67, 87, 125 and 207 were assayed for attachment to three uroepithelial cell samples, using the method described in section 1.1. Each resultant slide was evaluated for E.coli attachment to 40 viable uroepithelial cells on three different occasions.

The results are presented in Table 14. The mean values for attachment of each E.coli strain were similar for preparations counted on each of three occasions. There did not appear to be a counting bias on any one occasion. The small differences between the mean values could be explained by the 15% variation occurring within a cell sample, as in 1.1.2.

EFFECT OF VARYING THE CONDITIONS OF THE ATTACHMENT ASSAY.

Several parameters were tested to verify that the assay conditions described in section 1.1 were optimal for E.coli attachment to uroepithelial cells. Each test was carried out in duplicate using a uroepithelial cell suspension prepared from pooled urine samples.

1.1.15 VARIATION OF BACTERIAL GROWTH TIME.

(i) The growth phase of E.coli strains 67 and 87 was monitored by inoculating BHI broth cultures from stocks and assessing the optical density and the total and viable counts of the bacterial suspensions at regular intervals for 72 hours. Samples for viable counts were plated in triplicate onto BHI agar while total cell counts were monitored on a haemocytometer.

The growth curves obtained for E.coli strains 67 and 87 confirmed that 4 hour cultures were in the log phase, 8 hour cultures were late log to early stationary phase and that 12 and 16 hour

TABLE 14

ASSESSMENT OF E.COLI ATTACHED TO UROEPITHELIAL CELLS FOR PREPARATIONS EXAMINED ON THREE OCCASIONS.

SLIDE	OCCASION OF EVALUATION	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL			
		BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
		STRAIN 67	STRAIN 87	STRAIN 125	STRAIN 207
1	1ST	6	8	52	58
	2ND	6	8	55	58
	3RD	6	7	52	58
2	1ST	6	9	55	59
	2ND	7	9	56	62
	3RD	7	9	57	64
3	1ST	8	7	59	56
	2ND	7	7	57	58
	3RD	8	8	57	57

cultures were in the stationary growth phase.

(ii) In the second experiment E.coli cells were cultured in BHI broth for 72 hours at 37°C. After 4, 8, 12, 16, 20, 24, 48 and 65 hours, 1 x 10^8 bacteria were removed and assayed for attachment to 1 x 10^5 uroepithelial cells as described in section 1.1.

Strain 207 was grown overnight at 37°C in BHI broth then inoculated from this to TSA and incubated at 37°C. After 5, 7, 12, 16, 18, 24, 40 and 48 hours incubation a sweep of growth was suspended in PBS, pH 7.1 and adjusted to 10^{10} organisms per 1 cm^3 PBS. These bacteria were then assayed with uroepithelial cells using the method of section 1.1.

The results from this second experiment are presented in Table 15. An incubation time of 16 hours was optimal for BHI broth grown cultures giving highest mean attachment to uroepithelial cells. This incubation time was used in subsequent experiments.

After growth in BHI broth, strain 207 gave a mean attachment value of 11 E.coli per uroepithelial cell. However,after 12 hours on TSA this level of attachment increased to 55 E.coli per cell. In subsequent experiments a 16 hour incubation time was used for TSA grown cultures. An investigation of the difference in attachment levels for strain 207 grown in BHI broth and on TSA will be dealt with in section 3.

1.1.16 GROWTH OF E.COLI USING DIFFERENT MEDIA.

This experiment was designed to select an appropriate growth medium which would result in high levels of E.coli attachment to uroepithelial cells. E.coli strains 67, 87, 207 and 320 were cultured overnight at 37°C in each of 12 media, then assayed for attachment to uroepithelial cells, as described in section 1.1.

TABLE 15

VARIATION OF BACTERIAL GROWTH TIME.

BHI BROTH GROWN CULTURES			TSA GROWN CULTURES	
TIME OF INCUBATION AT 37°C	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL		TIME OF INCUBATION AT 37°C	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL
	STRAIN 67	STRAIN 87		STRAIN 207
4	6	7	(16 HRS - BHI BROTH)	11
8	8	6	5	11
12	7	9	7	35
16	12	12	12	55
20	7	8	16	55
24	9	7	18	53
48	3	3	24	48
65	3	3	40	45
			48	38

Each strain was grown aerobically at 37°C in BHI broth, trypticase soy broth, nutrient broth, beef extract broth, trypticase soy agar with and without 5% horse erythrocytes, BHI agar, nutrient agar and CLED agar. In addition, BHI broths and TSA plates were incubated anaerobically in jars containing CO_2/H_2 gas paks (BBL,USA). In order to encourage pili production, each strain was incubated for 24 hours at 37°C in 25 cm^3 BHI broth at low oxygen tension in a 28 cm^3 universal, and subcultured six times before being assayed with uroepithelial cells.

The results are presented in Table 16. Aerobic growth in BHI broth was appropriate for E.coli strains 67 and 87 giving high mean attachment values to uroepithelial cells. Strains 207 and 320 attached to give high mean values after growth on agar media. In subsequent experiments aerobic growth on TSA was used to culture bacteria on a solid surface prior to the attachment assay.

1.1.17 VARIATION IN THE DENSITY OF E.COLI CELLS EMPLOYED IN THE ASSAY FOR ATTACHMENT TO 1 x 10^5 UROEPITHELIAL CELLS.

This experiment was designed to determine an appropriate bacterial cell density in the attachment assay. E.coli strains 87 grown in BHI broth and 207 grown on TSA were suspended to a concentration of 10^4, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10} and 10^{11} organisms per 0.5 cm^3 in PBS, pH 7.1. For each concentration 0.5 cm^3 was added to 1 x 10^5 uroepithelial cells in 0.5 cm^3 PBS and assayed for attachment as in section 1.1.

Although the attachment levels of strain 87 and strain 207 were significantly different, the graphs presented in Figure 3 give comparable patterns. Reduced levels of attachment were observed when less than 10^8 E.coli cells were present in the assay: mean values of 0 to 7 E.coli per cell for strain 87 and 0 to 16 E.coli

TABLE 16

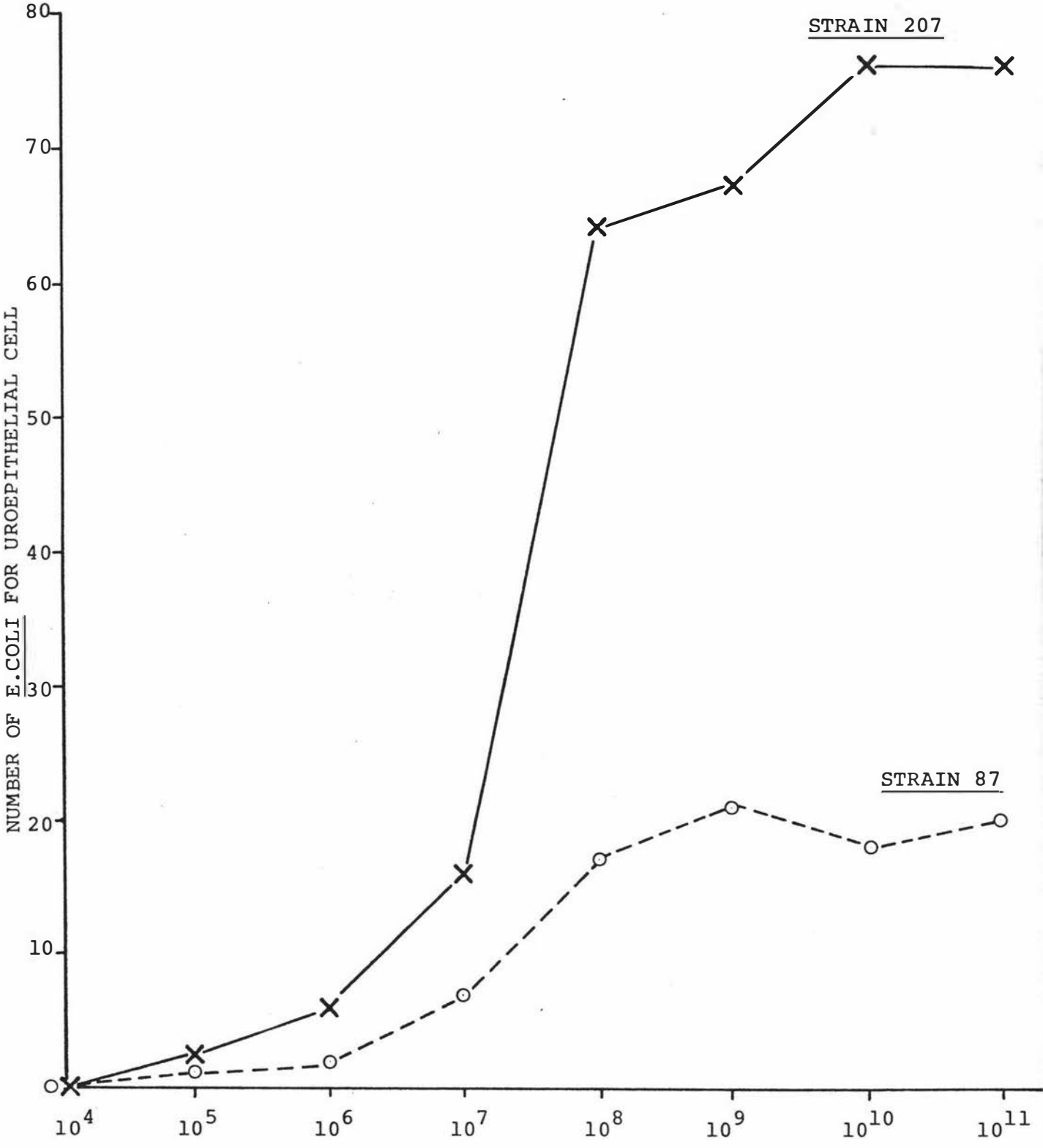
GROWTH OF E.COLI USING DIFFERENT MEDIA.

GROWTH MEDIUM	STRAIN 67	STRAIN 87	STRAIN 207	STRAIN 320
BHI BROTH	12	14	11	12
TRYPTICASE SOY BROTH	8	9	16	10
NUTRIENT BROTH	8	11	NT	NT
BEEF EXTRACT BROTH	5	6	NT	NT
BHI BROTH - ANAEROBIC	5	8	14	15
BHI BROTH - 6 SUB-CULTURES	3	7	7	9
TRYPTICASE SOY AGAR	5	13	62	25
TSA WITH 5% HORSE BLOOD	9	13	66	22
TSA - ANAEROBIC	10	10	66	26
BHI AGAR	6	11	63	28
NUTRIENT AGAR	9	11	47	24
C.L.E.D. AGAR	7	15	46	28

NT = Not Tested.

FIGURE 3

VARIATION IN THE DENSITY OF E.COLI CELLS EMPLOYED IN THE ASSAY FOR ATTACHMENT TO 1 x 10^5 UROEPITHELIAL CELLS.



per cell for strain 207. However, at a level ≥ 1000 E.coli to 1 uroepithelial cell in 1 cm^3 PBS, the mean attachment values rose to 17 to 21 E.coli per cell for strain 87 and 64 to 76 E.coli per cell for strain 207. In subsequent assays, cell densities of 1×10^8 E.coli cells and 1×10^5 uroepithelial cells in a total volume of 1 cm^3 were adopted as standard assay procedure.

1.1.18 VARIATION OF THE CONDITIONS OF INCUBATION FOR THE ATTACHMENT ASSAY.

Three parameters of the attachment assay incubation period were varied in the following set of experiments. E.coli strains 67 and 87 grown in BHI broth and strain 207 grown on TSA were assayed with uroepithelial cells as in section 1.1.

I. TIME OF INCUBATION : Uroepithelial cells were incubated with E.coli at 37°C, 20 r.p.m. in a shaker for 0, 30, 60, 90, 120, 180 and 240 minutes.

The results presented in Table 17 suggested that an incubation time of at least 60 minutes was appropriate to obtain maximal attachment of E.coli to uroepithelial cells. The differences in mean values for 60 minutes to 240 minutes incubation was less than the 15% variation level known to be present in a cell sample, as reported in section 1.1.2. In subsequent experiments a 60 minute incubation time was used.

II. TEMPERATURE OF INCUBATION : Uroepithelial cells were incubated with E.coli for 60 minutes at 20 r.p.m. in a shaker at 4°C, 22°C, 37°C and 44°C.

The results on Table 17 showed that attachment of E.coli to uroepithelial cells does occur at 4°C, 22°C, and 44°C. However, for all three strains, attachment appeared to be highest at 37°C; this temperature was used in subsequent assays.

TABLE 17

VARIATION OF THE CONDITIONS OF INCUBATION FOR THE ATTACHMENT ASSAY

	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL		
	BHI BROTH GROWN CULTURES		TSA GROWN CULTURES
I TIME OF INCUBATION (minutes)	STRAIN 67	STRAIN 87	STRAIN 207
0	3	4	13
30	6	7	32
60	8	9	64
90	9	9	65
120	8	9	60
180	9	10	63
240	9	10	69
II TEMPERATURE OF INCUBATION (^{o}C)			
4	3	4	39
22	6	5	39
37	8	9	64
44	6	5	35
III SPEED OF SHAKING WATER BATH (r.p.m.)			
0	5	5	49
20	10	9	65
50	5	6	55

III.SPEED OF SHAKER : Uroepithelial cells were incubated with E.coli at 37°C for 60 minutes in a shaker at 0, 20 and 50 r.p.m..

It appeared from the results on Table 17 that maximal attachment of E.coli to uroepithelial cells occurred with a 20 r.p.m. gentle mixing of the cell suspension. This speed was used in subsequent assays.

1.1.19 VARIATION OF THE BUFFER pH.

For this experiment, E.coli strains 67 and 87 were grown in BHI broth and strains 125 and 207 were grown on TSA. The bacteria were suspended at 10^{10} organisms per cm^3 buffer with pH values ranging from 3.0 to 10.1. Uroepithelial cells were harvested from pooled urine samples and suspended in tubes of buffer with pH values between 3.0 and 10.1. Each of the cell suspensions was washed three times in buffer of the appropriate pH value and finally suspended to a concentration of 5×10^5 uroepithelial cells per cm^3 buffer. The attachment assay was carried out as in section 1.1 but with buffer of the appropriate pH value being used in place of standard PBS (pH 7.1).

The results are presented in Table 18. The attachment of all four E.coli strains to uroepithelial cells was highest for buffer pH 6.4 and 7.1. There were no viable uroepithelial cells present on completion of the tests using buffer pH 3.0 and 3.8. Cell viability was reduced in buffer pH 4.7, 5.8, 9.6 and 10.1. In subsequent experiments standard PBS (pH 7.1) was used for the assay.

1.1.20 COMPARISON OF THREE DIFFERENT CATION CONTAINING BUFFER SOLUTIONS FOR AN EFFECT ON ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

Attachment of E.coli to uroepithelial cells was compared

TABLE 18

VARIATION OF THE BUFFER pH.

MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL

BUFFER pH VALUE	BHI BROTH GROWN CULTURES		TSA GROWN CULTURES	
	STRAIN 67	STRAIN 87	STRAIN 125	STRAIN 207
3.0	NT	4	5	4
3.8	4	5	6	4
4.7	5	7	9	5
5.8	7	13	24	27
6.4	13	15	56	60
7.1	13	16	58	61
8.0	7	14	34	31
9.6	7	12	28	22
10.1	3	7	12	10

NT = Not Tested

using three different buffers. The first was standard PBS, pH 7.1. The second was Phillip's buffer (Phillips and Andrews, 1959) and the third was Jarvinen's buffer (Jarvinen and Sandholm, 1980). As indicated from the formulae in the materials section, these latter two buffers contain the physiological levels of calcium and magnesium ions known to be present in urine (Keele and Neil, 1965).

Ten E.coli strains were grown either in BHI broth or on TSA and assayed with uroepithelial cells as in section 1.1. The results are presented in Table 19. After statistical analysis by two-way variance, there was found to be no significant difference between the ten mean attachment values for the three buffer solutions ($p > 0.1$). The attachment levels of the 10 E.coli strains varied, but the overall mean values were similar; namely 32.0, 30.4 and 29.8 E.coli per cell. It appeared that buffers containing calcium or magnesium ions did not significantly affect the attachment of E.coli to uroepithelial cells. In subsequent assays, PBS (pH 7.1) was used.

1.1.21 COMPARISON OF ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS SUSPENDED IN URINE AND IN PBS.

This experiment was designed to compare the level of attachment of E.coli to uroepithelial cells suspended in urine with that for cells harvested from the same urine sample and washed three times in PBS. By inoculating bacteria into the urine samples containing uroepithelial cells it was felt that this may partly resemble the *in vivo* situation of bacteria entering the bladder. It was of interest to enquire if under such conditions, E.coli attached to the uroepithelial cells and if in fact attachment was as high as when the bacteria and cells were incubated in 1 cm^3 PBS.

TABLE 19

COMPARISON OF THREE DIFFERENT CATION CONTAINING BUFFER SOLUTIONS FOR AN EFFECT ON ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

STRAINS	GROWTH CONDITIONS	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL		
		STANDARD PBS	PHILLIPS BUFFER	JARVINEN'S BUFFER
43	TSA	4	6	5
61	BHI BROTH	6	6	4
67	TSA	5	6	6
73	TSA	82	77	75
87	BHI BROTH	8	8	7
122	TSA	70	66	67
207	TSA	57	53	56
289	TSA	69	64	60
320	BHI BROTH	9	9	8
321	BHI BROTH	10	9	10
		MEAN = 32.0	MEAN = 30.4	MEAN = 29.8

$p > 0.1$

Six early morning urine samples were collected from healthy, premenopausal women and pooled in a 2000 cm^3 flask. The urine was mixed, the pH was determined and the pooled sample divided into two equal amounts. One portion of approximately 600 cm^3 was centrifuged and the uroepithelial cells washed three times and resuspended in 7 cm^3 of PBS (pH 7.1). To each of seven test tubes was added 1 x 10^5 uroepithelial cells in 1 cm^3 PBS. Six of the tubes were inoculated with 1 x 10^8 E.coli suspended in 0.01 cm^3 PBS. The E.coli comprised of six strains grown either in BHI broth or on TSA. The seventh tube served as a control with 1 x 10^5 uroepithelial cells and 0.01 cm^3 PBS. The attachment assay was carried out as in section 1.1.

The remaining portion of the pooled urine was further divided into 7 equal aliquots of approximately 85 cm^3 and placed in 250 cm^3 flasks. Each flask was inoculated with 1 x 10^8 E.coli (in 0.01 cm^3 of PBS) prepared from the same 6 E.coli strains as for the first part of the test. The seventh flask had 0.01 cm^3 PBS added to serve as a control. The suspensions were incubated for 60 minutes at 37°C at 20 r.p.m. in a shaker. The urine was then centrifuged at 200 x g for 12 minutes and the uroepithelial cells were treated as described in section 1.1. The mean attachment values were calculated for both sets of the experiment after evaluation of E.coli attached to 40 uroepithelial cells (20 squamous cells and 20 transitional cells).

A total of 24 E.coli strains were tested by this method and the mean attachment values were statistically compared for cells in urine and for cells in PBS by the two-way analysis of variance. Strain 87 grown in BHI broth and strain 207 grown on TSA were tested with each urine sample, to serve as a reference for attachment to uroepithelial cells. Consequently the test was carried out on 6 consecutive days using urine samples from the same group of women,

TABLE 20

COMPARISON OF ATTACHMENT OF *E.COLI* TO UROEPITHELIAL CELLS SUSPENDED IN URINE AND IN PBS.

STRAIN	GROWTH CONDITIONS	MEAN NUMBER OF *E.COLI* PER UROEPITHELIAL CELL	
		CELLS IN URINE	CELLS IN PBS
43	BHI BROTH	5	7
49	BHI BROTH	5	10
61	BHI BROTH	5	8
66	BHI BROTH	8	9
73	TSA	34	73
74	BHI BROTH	6	6
87	BHI BROTH	11	16
89	TSA	32	53
104	TSA	32	47
122	TSA	42	72
125	TSA	33	56
129	TSA	23	47
130	BHI BROTH	6	6
132	BHI BROTH	7	7
159	TSA	50	52
173	TSA	40	39
178	TSA	41	60
190	BHI BROTH	6	8
207	TSA	51	55
231	BHI BROTH	6	7
242	BHI BROTH	7	8
284	TSA	58	53
288	BHI BROTH	5	6
289	TSA	50	63

$p < 0.01$

assayed with 4 strains of E.coli and with the two reference strains.

The results for this experiment are listed in Table 20. The mean attachment values were comparable for cells suspended in urine and in PBS. The statistical difference ($p < 0.01$) was due to lower attachment values for cells suspended in urine. This was probably due to the large volume of urine in which the uroepithelial cells and bacteria were incubated. The urine pH ranged from 5.5 to 5.8. Over the set of experiments, the reference strain 87 gave a mean attachment value of ± 1 E.coli per uroepithelial cell to those reported in Table 20. Strain 207 gave a mean attachment value of ± 4 E.coli per uroepithelial cell to those values in Table 20. As the variation between these samples was small, the results obtained on each day for the other strains were not altered.

These results suggested that urine could be used as a suspending fluid in place of phosphate buffered saline, for E.coli attachment to uroepithelial cells. The attachment of four strains to uroepithelial cells suspended in urine was to a non-specific, basal level as reported in the next section 1.1.22. Nevertheless, 20 E.coli strains were shown to attach to uroepithelial cells suspended in 85 cm^3 of urine. The high attachment levels for 12 strains grown on TSA will be investigated in section 3.

1.1.22 ATTACHMENT OF NON-URINARY BACTERIA TO UROEPITHELIAL CELLS: COMPARISON WITH ATTACHMENT OF URINARY E.COLI STRAINS TO DEFINE A NON-SPECIFIC BASAL LEVEL FOR THE ATTACHMENT ASSAY.

In 1978, Svanborg Edén reported that an arbitrary value for non-adhering strains of E.coli was < 10 bacteria per uroepithelial cell. This value was not based on experimental findings.

In order to investigate whether a non-specific, basal level of attachment to uroepithelial cells existed, two strains of bacteria

were selected. Laboratory strains of Bacillus subtilis and Salmonella typhimurium were taken as representative of bacteria not normally isolated from the urogenital tract. Both strains were cultured at 37°C for 16 hours in BHI broth and on TSA. E.coli strain 87 was grown in BHI broth and strain 207 was grown on TSA. All four bacterial species were then assayed for attachment to uroepithelial cells as in section 1.1.

The results are given in Table 21. The non-urinary bacteria attached to uroepithelial cells giving a net mean value of less than 5 bacteria per cell. The urinary strains 87 and 207 gave appropriate attachment to uroepithelial cells suggesting that the cell suspension was receptive to bacterial attachment.

The frequency distributions of each preparation were analysed. Only 20% of the uroepithelial cells had between 6 and 10 non-urinary bacteria attached, with one cell having 10 bacteria attached to the surface. However, urinary E.coli strain 87 attached to 90% of the uroepithelial cells in numbers greater than 5 bacteria per cell, and E.coli strain 207 attached to 100% of the uroepithelial cells in numbers greater than 5 bacteria per cell. These results lead to the definition of a non-specific, basal level for the attachment assay. The value adopted was that of a mean of 5 bacteria per uroepithelial cell. Subsequently, E.coli strains attaching to uroepithelial cells after growth in BHI broth and after growth on TSA, to a level of $\leq$ 5 bacteria per cell are described as "non-attachers". Such attachment represents the non-specific, basal level for the attachment assay.

1.2 DETECTION OF E.COLI ATTACHED TO UROEPITHELIAL CELLS BY AN INDIRECT FLUORESCENT ANTIBODY TECHNIQUE.

The titres of E.coli antisera used in this experiment were 1:10

TABLE 21

ATTACHMENT OF NON-URINARY BACTERIA TO UROEPITHELIAL CELLS : COMPARISON WITH ATTACHMENT OF URINARY E.COLI STRAINS TO DEFINE A NON-SPECIFIC BASAL LEVEL FOR THE ATTACHMENT ASSAY.

STRAINS	MEAN NUMBER OF BACTERIA PER UROEPITHELIAL CELL	
	BHI BROTH GROWN CULTURES	TSA GROWN CULTURES
SALMONELLA TYPHIMURIUM	3.7	2.3
BACILLUS SUBTILIS	4.3	3.7
E.COLI STRAIN 87	13.3	NT
E.COLI STRAIN 207	NT	58.5

NT = Not Tested

for serotype 01, 1:320 for serotypes 04 and 06:K1 and 1:160 for serotypes 07 and 075. The use of an indirect fluorescent antibody technique proved to be successful in detecting attachment of E.coli to uroepithelial cells (Plates 3 to 6).

The results are presented in Table 22, which compared the mean attachment values obtained using the double staining technique with those from the fluorescent antibody technique. There was no significant difference between the two columns of mean values when analysed by two-way variance ($p > 0.1$). The control samples gave mean attachment values of 3 E.coli per cell detected by both methods.

The use of fluorescent antibody facilitated specific identification of attached E.coli on the surface of uroepithelial cells. However, the procedure was time consuming and more expensive than the double staining technique due to usage of antisera and commercial fluorescent antibody. In subsequent experiments, the double staining technique was used to detect E.coli attached to viable uroepithelial cells, unless otherwise stated.

1.3 THE PRESENCE OF ATTACHED E.COLI ON UROEPITHELIAL CELLS COLLECTED FROM WOMEN WITH SYMPTOMATIC URINARY TRACT INFECTIONS: DETECTION BY USE OF FLUORESCENT-LABELLED IMMUNOGLOBULIN AND BY USE OF METHYLENE BLUE.

(a) During the course of study a morning urine sample was collected from a healthy premenopausal woman and the uroepithelial cells washed three times in PBS. On examining the sample prior to use in the assay, it was discovered that unattached E.coli-like organisms were present in the suspension, and the uroepithelial cells had similar organisms attached to the surface. The cell suspension was double-stained with erythrosine B and methylene blue. The mean number of E.coli-like organisms attached to 30 viable uroepithelial cells was 8 bacteria per cell. The urine was cultured

TABLE 22

DETECTION OF E.COLI ATTACHED TO UROEPITHELIAL CELLS BY AN INDIRECT FLUORESCENT ANTIBODY TECHNIQUE : COMPARISON WITH DOUBLE STAINING TECHNIQUE.

STRAIN	SEROTYPE	GROWTH CONDITIONS	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL: DOUBLE STAINING TECHNIQUE	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL: FLUORESCENT ANTIBODY TECHNIQUE
33	04:K1:HR	TSA	13	9
44	06:K1:H-	TSA	6	5
63	06:H1	TSA	26	27
68	01:H7	TSA	19	20
87	075:HR	BHI BROTH	17	17
105	07:H-	TSA	18	20
107	04:H5	TSA	19	17
114	04:H-	TSA	11	15
143	01:H7	BHI BROTH	15	15
158	04:H5	TSA	55	56
166	075:H-	TSA	39	50
199	06:K1:H-	TSA	5	6
207	075:H-	TSA	57	59
218	01:H-	TSA	3	3
242	075:H5	TSA	18	15
249	07:K1:H-	BHI BROTH	11	9
283	07:H-	TSA	39	43
289	01:H7	TSA	83	78
305	07:H6	TSA	5	6
322	06:K2:H1	TSA	34	36
			MEAN = 24.65	MEAN = 25.3

$p > 0.1$

on CLED agar and an E.coli urinary tract infection was discovered (> 10^5 organisms per cm^3 urine). The organism was identified using the API system, and the strain was labelled No. 290 in our collection. Symptoms of cystitis arose 7 days later. The woman visited her general practitioner and after a 5 day course of Septrin antibiotics the infection was cleared. Examination of uroepithelial cells from a urine sample taken on completion of treatment demonstrated that no E.coli were present in the urine or attached to the uroepithelial cells.

(b) It was decided to further investigate the levels of E.coli attachment to uroepithelial cells from women with symptomatic urinary tract infections. The study was carried out at Grey Street Medical Laboratory in Palmerston North. Fresh mid-stream urine samples were collected here from women with symptoms of urinary infection. The laboratory technician checked each sample for the presence of white cells, red blood cells, bacteria and uroepithelial cells by microscopic examination. Each sample was inoculated on CLED agar to test for significant growth. At the same time, 10 cm^3 of selected samples were removed, centrifuged at 250 x g for 10 minutes, washed thrice in PBS and the uroepithelial cells resuspended in 1 cm^3 PBS. If sufficient numbers of uroepithelial cells were present the experiment was continued; samples were discarded if few cells or excess amorphous debris were present. In general it appeared that a greater number of uroepithelial cells were present in the urine samples from women later found to have urinary infection than from urine samples later found to be uninfected.

The washed cell suspension was divided equally, and 0.5 cm^3 was placed on each of two glass slides and air dried. On identification of an E.coli urinary infection by significant growth on CLED agar and biochemical analysis using the Microbact system, the appropriate

slides were either discarded or further processed.

The first of the 2 slides was stained with methylene blue (0.4% w/v in PBS) for 30 secs and washed in distilled water. The mean number of E.coli-like organisms attached to 40 uroepithelial cells (20 squamous cells and 20 transitional cells) was calculated. The second slide for each laboratory sample was fixed for 10 minutes in acetone and rinsed in PBS. The slide was then flooded with 1 cm^3 pooled type specific antisera raised against five E.coli serotypes 01, 04, 06, 07 and 075. Each slide was then rinsed in PBS and flooded with fluorescent-labelled anti-rabbit immunoglobulin; this procedure is fully described in methods section 1.2. The mean number of E.coli attached to 40 uroepithelial cells (20 squamous cells and 20 transitional cells) was calculated and compared with the values obtained with methylene blue staining.

The results are presented in Table 23. In 18 of the 37 samples the mean values obtained with the methylene blue staining were similar to those obtained using fluorescent antibody as analysed by two-way variance ($p > 0.1$). It was found that E.coli were attached to both squamous and transitional cell types. In the other 19 samples, the mean values obtained with the fluorescent antibody technique ranged from 0 to 5 E.coli per cell and were substantially smaller that the values obtained with methylene blue. As only five type specific antisera preparations were incorporated in the staining process, it was felt that these 19 samples were likely to have E.coli of other 0 serotypes attached to the surface.

Eleven urine samples from healthy women were examined using both staining procedures: mean values of between 0 and 4 E.coli per cell were obtained using the fluorescent antibody technique and mean values of between 0 and 4 E.coli-like organisms per cell using methylene blue.

TABLE 23

THE PRESENCE OF ATTACHED *E.COLI* ON UROEPITHELIAL CELLS COLLECTED FROM WOMEN WITH SYMPTOMATIC URINARY TRACT INFECTIONS : DETECTION BY USE OF FLUORESCENT-LABELLED IMMUNOGLOBULIN AND BY USE OF METHYLENE BLUE.

Laboratory Sample Number	Mean number of *E.coli*-like organisms per uroepithelial cell — Methylene Blue Stain	Mean number of *E.coli* per uroepithelial cell — Fluorescent Antibody	Laboratory Sample Number	Mean number of *E.coli*-like organisms per uroepithelial cell — Methylene Blue Stain	Mean number of *E.coli* per uroepithelial cell — Fluorescent Antibody
550	12	1	2001	81	86
637	10	5	2048	16	1
643	15	0	2105	23	21
711	5	4	2127	20	19
799	6	6	2157	7	7
930	21	20	2186	26	0
961	15	1	2277	33	31
1549	16	0	2352	17	17
1571	19	19	2951	10	0
1609	7	0	2992	24	21
1691	9	0	3105	11	11
1698	18	16	3272	7	8
1706	14	11	3316	7	0
1725	22	19	3336	20	0
1793	5	0	3376	40	0
1907	17	1	3390	28	1
1924	75	1	3478	22	1
1974	21	18	3584	8	10
			3594	85	0

1.4 VARIATION IN UROEPITHELIAL CELL RECEPTIVITY OVER THE MENSTRUAL CYCLE.

From results obtained in section 1.1.4 and 1.1.5 it appeared that uroepithelial cells varied significantly in their receptivity to the attachment of E.coli strains grown in BHI broth but not those grown on TSA. This variation occurred between cell samples from different women and from the same woman on different days.

It was decided to investigate the variation in uroepithelial cell receptivity for E.coli attachment, using urine samples collected from five women every 2 to 3 days over the menstrual cycle. Uroepithelial cells from each sample were assayed for attachment with E.coli strains 67 and 87 grown in BHI broth, using the method of section 1.1. The exact days of the menstrual cycles were not disclosed by the women until two complete cycles had elapsed, in order to avoid subjective bias. The mean number of E.coli cells attached to 40 viable uroepithelial cells was determined and subsequently plotted against the day of the menstrual cycle with which it had coincided. Day 1 was taken as the first day of menstrual bleeding.

From the results it appeared that a cyclical, repetitive pattern emerged. There appeared to be two peak times in each cycle when uroepithelial cells were most receptive to E.coli attachment and one trough period when cells were least receptive. Depending on the length of the menstrual cycle for any individual, the peak values were recorded between days 9 to 13 and 18 to 28 while the lowest values were recorded between days 14 to 17. The results for the mean attachment of E.coli to uroepithelial cells are presented in Table 24. By use of one-way analysis of variance, the mean values and the values for the 40 cells evaluated for each test were compared for peak and trough results. The difference was highly

TABLE 24

VARIATION IN UROEPITHELIAL CELL RECEPTIVITY OVER THE MENSTRUAL CYCLE

	DAYS OF CYCLE	SUBJECT	MEAN NUMBER OF *E.COLI* PER UROEPITHELIAL CELL	
			STRAIN 67	STRAIN 87
1ST PEAK	9 to 13	1	11	13
		2	9	8
		3	8	9
		4	8	9
		5	8	9
			$\bar{M}$ = 8.8	$\bar{M}$ = 9.6
TROUGH	14 to 17	1	4	3
		2	4	3
		3	2	4
		4	4	5
		5	2	3
			$\bar{M}$ = 3.2	$\bar{M}$ = 3.6
2ND PEAK	18 to 28	1	10	8
		2	10	9
		3	8	9
		4	10	10
		5	10	13
			$\bar{M}$ = 9.6	$\bar{M}$ = 9.8

TABLE 25

FREQUENCY DISTRIBUTIONS OF E.COLI ATTACHED TO 100 UROEPITHELIAL CELLS ON TROUGH AND PEAK DAYS OF THE MENSTRUAL CYCLE.

RANGE OF E.COLI ATTACHED PER UROEPITHELIAL CELL	NUMBER OF UROEPITHELIAL CELLS			
	TROUGH DAYS		PEAK DAYS	
	STRAIN 67	STRAIN 87	STRAIN 67	STRAIN 87
0 - 5	73	88	22	26
6 - 10	23	12	44	34
11 - 20	4	0	30	30
> 20	0	0	4	10

significant ($p < 0.001$). The mean attachment values obtained on trough days were similar to the non-specific, basal attachment levels defined in section 1.1.22.

The frequency distributions are presented in Table 25. The difference between peak and trough day attachment values corresponded with an increase in the number of target cells (> 10 E.coli per cell as defined in section 1.1.6) on peak days and a decrease in the number of target cells on trough days. On peak days, approximately 30 to 40% of the uroepithelial cells were target cells highly receptive to E.coli attachment, whereas only 0 to 4% were target cells on the trough days.

The results of the study were compared with plasma and urine oestrogen levels known to be maintained over the menstrual cycle in normal healthy women. These oestrogen levels peak around day 12 prior to a dramatic decrease around day 15 following ovulation, and then they rise to a second peak around day 20 of the cycle (Shearman, 1972). The oestrogen levels of the women in this study were not measured. Nevertheless, the attachment assay results correlated with the cyclical, repetitive pattern of the oestrogen hormones, especially plasma levels of 17β oestradiol, normally found in premenopausal women.

These results highlighted the variation in uroepithelial cell receptivity to attachment with BHI broth grown E.coli strains. This stresses the importance of using pooled urine samples and carrying out each set of experiments on one day to minimise variability between cell samples.

1.5 VARIATION IN UROEPITHELIAL CELL RECEPTIVITY DURING PREGNANCY.

To date, uroepithelial cells from pregnant women had not been studied for attachment of E.coli. In order to determine whether

variation in uroepithelial cell receptivity for E.coli attachment occurred during pregnancy, the following experiments were carried out.

Uroepithelial cells were collected from six pregnant subjects at 2 to 3 weekly intervals, with first samples collected as early in pregnancy as was possible and the last samples at 1 and 2 months post partum. As it was known that two women were attempting to conceive, uroepithelial cells were collected and assayed for E.coli attachment at weekly intervals, prior to conception and during pregnancy until 2 months post partum. E.coli strains 67 and 87 were grown in BHI broth and assayed with each uroepithelial cell sample as described in section 1.1. The mean number of E.coli attached to 40 viable uroepithelial cells was calculated for each test, and the results were analysed on completion of all the experiments.

The data presented in Table 26 gives the weeks of pregnancy for each subject when mean attachment of E.coli to uroepithelial cells was at high and low levels. The uroepithelial cell receptivity for E.coli attachment appeared to differ for each subject. In general, the highest mean attachment values were obtained with uroepithelial cells, collected in the early part of pregnancy. By comparison, only low attachment values were obtained with cells collected in the latter part of pregnancy and up to two months post partum.

The trend became more evident when all the results were pooled. These are presented graphically in Figure 4. The highest mean attachment values of between 10 and 16 E.coli per cell were obtained in weeks 1 to 16. The levels fell between weeks 17 to 20 to an apparently non-specific, basal level of 4 to 6 E.coli per cell until 8 weeks post partum. The differences between high and low attachment values were statistically significant when the counts

TABLE 26

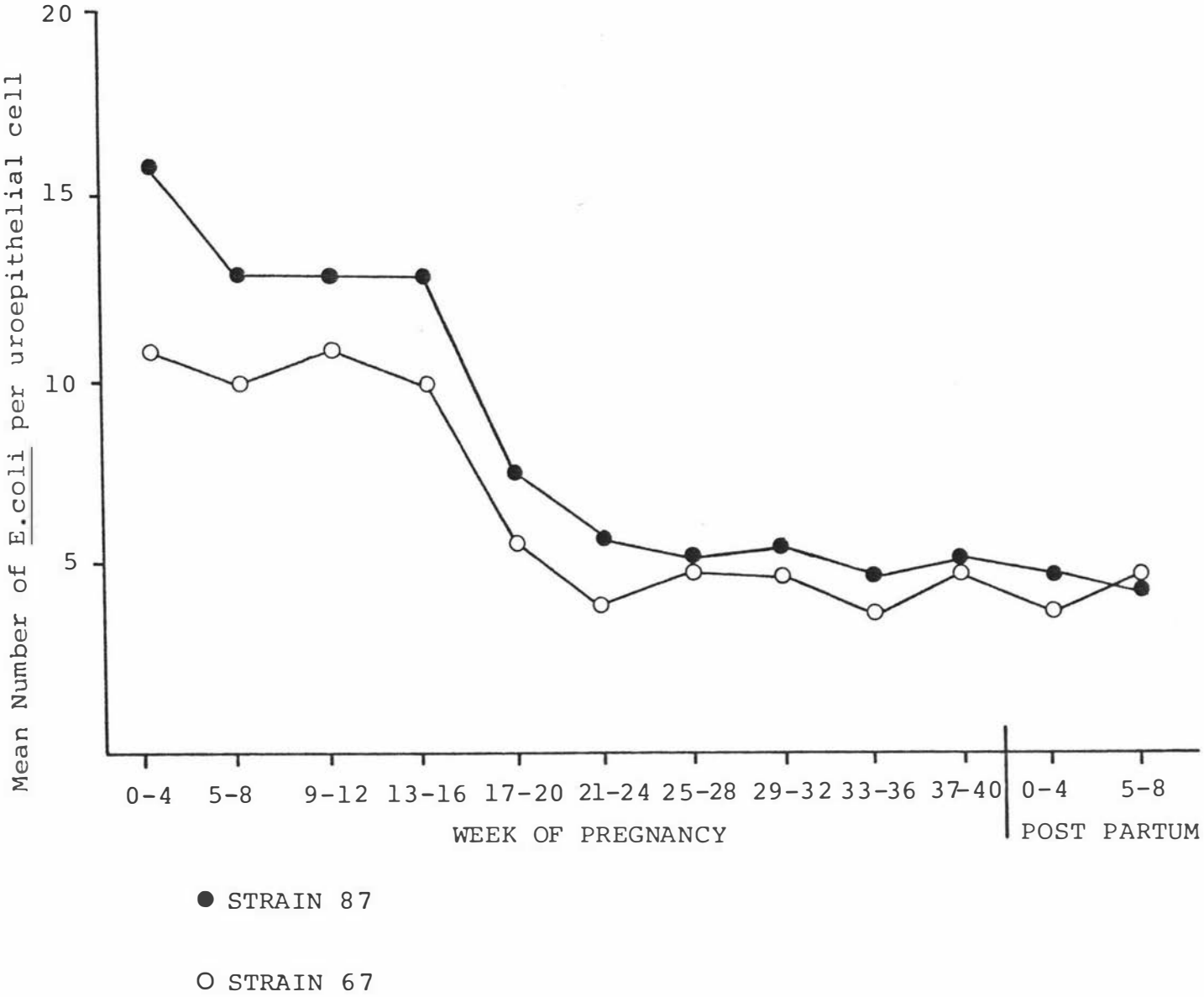
VARIATION IN UROEPITHELIAL CELL RECEPTIVITY DURING PREGNANCY.

PREGNANT SUBJECT	DEGREE OF ATTACHMENT	STRAIN 67 RANGE FOR MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	STRAIN 67 WEEKS OF PREGNANCY	STRAIN 87 RANGE FOR MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	STRAIN 87 WEEKS OF PREGNANCY
1	HIGH	23-24	6-16	25-34	6-15
	LOW	3-8	17-35	4-10	17-35
2	HIGH	10-16	12-16	12-16	12-16
	LOW	3-6	18-39	4-7	18-39
3	HIGH	10-12	7-13	15	7-12
	LOW	4-5	15-39	4-7	15-39
4	HIGH	9-14	8-14	9-11	8-14
	LOW	2-6	17-38	4-6	17-38
5	HIGH	8-9	11-20	10-17	11-20
	LOW	2-6	21-39	3-6	21-39
6	HIGH	8-12	1-15	9-25	1-15
	LOW	1-5	17-40	4-6	17-40

FIGURE 4

SUMMARY OF MEAN E.COLI ATTACHMENT VALUES

FOR 6 STUDIES WHICH USED UROEPITHELIAL CELLS COLLECTED FROM 6 PREGNANT WOMEN AT 2 TO 3 WEEKLY INTERVALS.



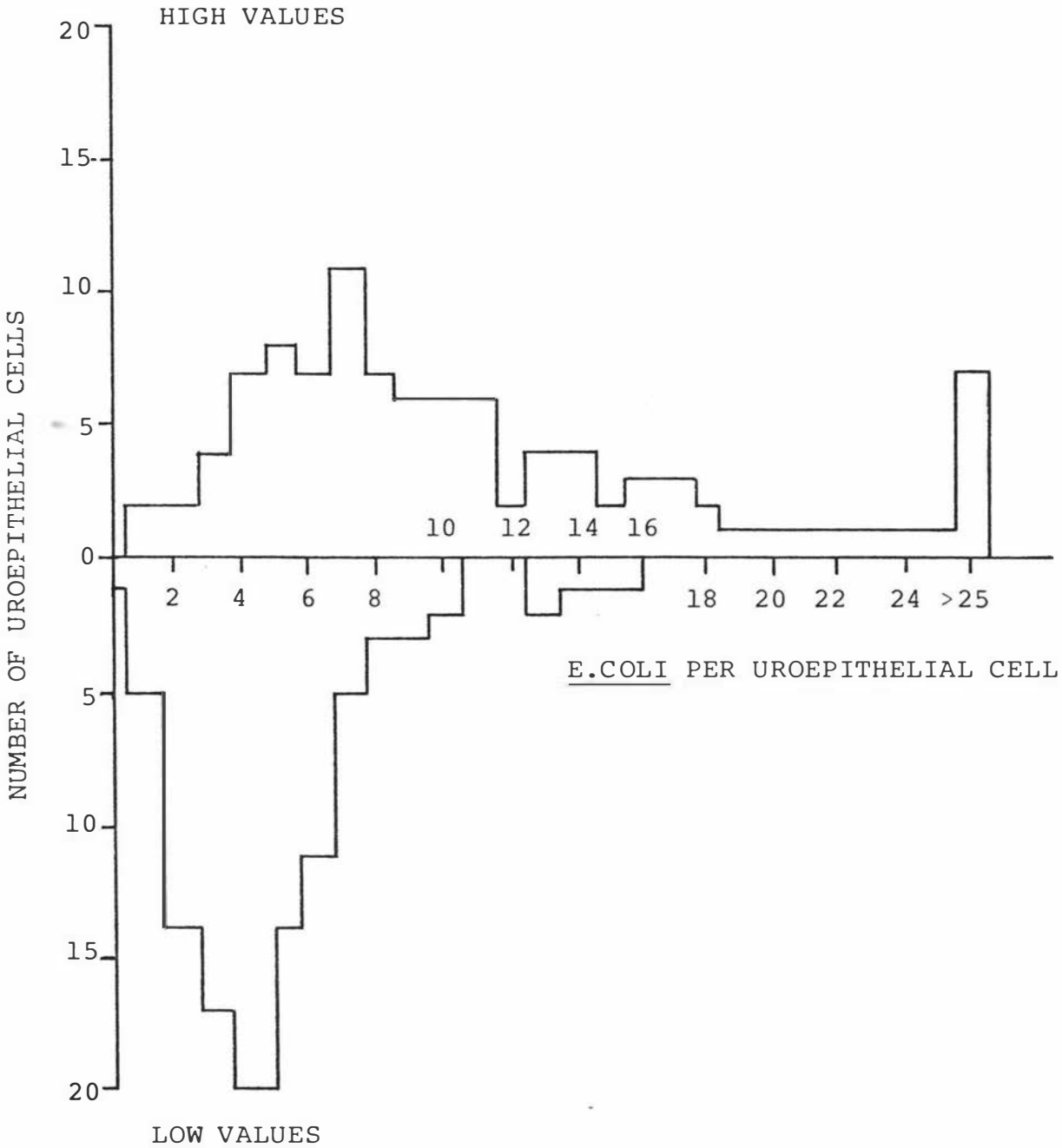
of 40 uroepithelial cells were analysed using one-way variance ($p < 0.001$).

The frequency distributions are presented in Figure 5. The attachment of E.coli to 100 uroepithelial cells resulting in a low mean value of 4.8 E.coli per cell, resembled a normal distribution curve (Roscoe, 1969). However,the distribution for 100 cells with a mean of 12.3 E.coli per cell was skewed and did not follow an obvious pattern. From the graphs,it was calculated that 40% of the uroepithelial cells with the high mean attachment, were target cells (> 10 E.coli per cell as defined in section 1.1.6), whereas only 5% of the cells with the low mean value were target cells. Therefore, in the early part of pregnancy there appeared to be more target cells in the uroepithelial cell population to which large numbers of E.coli attached. And in the later part of pregnancy there were relatively few target cells.

Variation in the receptivity of uroepithelial cells from non-pregnant subjects was shown to roughly coincide with the two oestrogen peaks and the fall off in oestrogen in the menstrual cycle (as in section 1.4). However, no relationship between oestrogen levels and receptivity to E.coli attachment was found in the pregnant subjects. The total plasma oestrogen level is known to increase at week 16 up to a maximum level at week 40 (Shearman, 1972). Although no hormone levels were measured for the women in this study, from published hormone levels observed in pregnancy (Shearman, 1972) only plasma human chorionic gonadotropin (HCG) corresponded with the E.coli attachment results. The HCG levels are highest from weeks 3 to 12, falling dramatically around week 16 to a low level which is maintained up to week 40.

FIGURE 5

FREQUENCY DISTRIBUTIONS OF E.COLI ATTACHED TO 100 UROEPITHELIAL CELLS ON DAYS WHEN RECEPTIVITY WAS HIGH OR WAS LOW DURING PREGNANCY.



RESULTS

SECTION 2.

HISTOLOGICAL STAINING TECHNIQUES USED TO INVESTIGATE MUCOPOLYSACCHARIDE-COATED EPITHELIAL CELLS.

2.1 DETECTION OF MUCOPOLYSACCHARIDES ON SECTIONS FROM LAMB AND PIG BLADDERS.

The PAS and alcian blue stains identified a thin mucopolysaccharide coat lining the periphery of the lamb and pig bladder cavities. The mucus coating was most evident at the apex of cells on the outermost epithelial layer and was seen to vary in thickness on different cells. A smaller proportion of cells in a deeper epithelial region, before the connective tissue, were sparsely coated with mucus. The cells in the sections were clearly distinguishable and were stained with the cytoplasmic and nuclear stains. Photographs were taken to record the results (Plates 7, 8).

2.2. DOUBLE STAINING TECHNIQUES TO DETERMINE THE RELATIONSHIP BETWEEN VIABILITY OF UROEPITHELIAL CELLS AND THE PRESENCE OF A MUCOPOLYSACCHARIDE COAT.

Attempts to combine viability, bacterial and mucopolysaccharide stains proved unsuccessful, mainly due to the masking effect of bacterial stains. The double staining techniques devised were successful in distinguishing viable and nonviable uroepithelial cells with and without a mucopolysaccharide coat. Descriptions of these cells are given in Table 27. The mean results of the reproducability study are presented in Table 28. All results are expressed as

TABLE 27

DESCRIPTIONS OF VIABLE AND NONVIABLE UROEPITHELIAL CELLS WITH AND WITHOUT MUCOPOLYSACCHARIDE COATING

STAIN	COLOUR OF VIABLE CELLS	COLOUR OF NONVIABLE CELLS
Alcian blue Positive	Light blue nucleus Light blue cytoplasm	Deep purple nucleus Blue/purple cytoplasm
Alcian blue Negative	Unstained	Red nucleus Clear/pink cytoplasm
PAS Positive	Unstained nucleus Magenta cytoplasm	Dark purple nucleus Magenta cytoplasm
PAS Negative	Unstained	Dull blue nucleus Clear/light blue cytoplasm

TABLE 28

REPRODUCABILITY TEST RESULTS

DOUBLE STAIN	MEAN VALUES AS PERCENTAGE OF TOTAL COUNT		
	Evaluator 1	Evaluator 2	Evaluator 3
Erythrosine B/Alcian Blue			
(a) Number of viable cells	24.4	25.2	26.8
(b) Viable mucus coated cells	86.4	83.6	87.2
(c) Non-viable mucus coated cells	44.4	44.8	41.8
Trypan blue/PAS			
(d) Number of viable cells	26.4	26.0	24.0
(e) Viable mucus coated cells	82.4	85.6	81.6
(f) Non-viable mucus coated cells	22.4	20.0	21.2

No statistical difference between the evaluators in each group tested : $p > 0.10$.

percentage values and represent the mean of five slide evaluations. Statistical analysis was carried out for each group separately (a, b, c, d, e and f) using one-way analysis of variance. There was no significant difference between the results obtained by the three evaluators in their analysis of the viability and mucopolysaccharide coating of the uroepithelial cell preparations ($p > 0.1$). In addition, there was no significant difference between the proportion of viable and nonviable cells detected by the use of either erythrosine B or trypan blue (Table 28, groups a and d) ($p > 0.1$). Of 4,500 cells examined in total, only 8 cells were scored as being equivocal.

2.3 DOUBLE STAINING TECHNIQUES TO DETERMINE THE RELATIONSHIP BETWEEN CELL MUCOPOLYSACCHARIDE COAT AND BACTERIAL ATTACHMENT.

The double staining techniques (alcian blue/safranin and PAS/methylene blue) clearly distinguished bacteria attached to all parts of mucopolysaccharide coated and noncoated uroepithelial cells. Descriptions of the resultant staining are presented in Table 29. Coated and noncoated cells were clearly distinguishable and their detection was unaffected by the bacterial stains used. A few cells which showed faint non-specific PAS colouring were scored as noncoated.

The results of the experiments using E.coli strains 87 and 207 are presented in Table 30. It appeared that strain 87 attached in greater numbers to mucus coated uroepithelial cells than to noncoated cells. Statistical analysis by the two-way variance test proved this difference to be highly significant for PAS stained cells over unstained cells ($p < 0.001$) and significant for alcian blue stained cells over unstained cells ($p < 0.01$). After analysis of the results for strain 207, there was no statistically

TABLE 29

DESCRIPTION OF MUCOPOLYSACCHARIDE COATED AND NON-COATED UROEPITHELIAL CELLS WITH ATTACHED BACTERIA:STAINED WITH ALCIAN BLUE/SAFRANIN OR PAS/METHYLENE BLUE

STAIN	COLOUR OF EPITHELIAL CELL	COLOUR OF BACTERIA
Alcian blue Positive	Purple nucleus Blue/purple cytoplasm	Red
Alcian blue Negative	Red nucleus Clear/pink cytoplasm	Red
PAS Positive	Blue/purple nucleus Magenta cytoplasm	Dark blue
PAS Negative	Blue nucleus Unstained cytoplasm	Dark blue

TABLE 30

DOUBLE STAINING TECHNIQUES TO DETERMINE THE RELATIONSHIP BETWEEN CELL MUCOPOLYSACCHARIDE COAT AND BACTERIAL ATTACHMENT.

MEAN NUMBER OF *E.COLI* STRAIN 87 PER UROEPITHELIAL CELL

SAMPLE	PAS POSITIVE STAINED CELLS	PAS NEGATIVE STAINED CELLS	ALCIAN BLUE POSITIVE STAINED CELLS	ALCIAN BLUE NEGATIVE STAINED CELLS
1	8	7	9	7
2	11	8	8	5
3	9	7	10	9
4	12	3	9	7
5	11	8	9	8
6	12	6	12	11
7	15	10	14	14
8	10	7	10	9
9	12	8	12	11
10	11	7	11	11
	MEAN = 11.1	MEAN = 7.1	MEAN = 10.4	MEAN = 9.2

$p < 0.001$ $p < 0.01$

TABLE 30 Cont'd

MEAN NUMBER OF *E.COLI* STRAIN 207 PER UROEPITHELIAL CELL

SAMPLE	PAS POSITIVE STAINED CELLS	PAS NEGATIVE STAINED CELLS	ALCIAN BLUE POSITIVE STAINED CELLS	ALCIAN BLUE NEGATIVE STAINED CELLS
1	39	39	38	38
2	45	45	46	43
3	50	46	52	55
4	42	35	34	34
5	46	55	47	45
6	51	54	51	51
7	58	59	58	52
8	48	49	49	50
9	41	40	40	37
10	44	39	40	43
	MEAN = 46.4	MEAN = 46.1	MEAN = 45.5	MEAN = 44.8

$p > 0.1$ $p > 0.1$

significant difference between attachment to PAS stained and PAS unstained cells ($p > 0.1$) or again to alcian blue stained and alcian blue unstained cells ($p > 0.1$).

It therefore appeared that attachment of the E.coli strain 87 grown in BHI broth, was influenced by the presence of a mucopolysaccharide coat on the uroepithelial cell surface, but that attachment of the E.coli strain 207 grown on TSA was not apparently affected by the presence or absence of a mucus coating.

2.4 EFFECT OF OESTROGEN ON BLADDER MUCUS COAT.

The injections of 17ß oestradiol-3-methyl benzoate were sufficient to stimulate vaginal opening and significant uterus enlargement in all the treated mice. The six control mice each had a closed vagina and small uterus. Examination of the sections after staining for mucopolysaccharides showed no visible difference in the structure of the bladder epithelium. There was no apparent difference in the thickness of the mucus coating on the epithelial cells lining the bladder wall of treated and untreated mice. The histologist confirmed the results and no further investigations were carried out.

RESULTS

SECTION 3.

THE ROLE OF BACTERIAL HAEMAGGLUTININS IN THE *IN VITRO* ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

A large proportion of E.coli urinary isolates have been reported to possess a mannose sensitive haemagglutinin (MSHA) detected using horse or guinea pig erythrocytes. Agglutination is characteristically inhibited in the presence of α D mannose (Duguid and Gillies, 1957; Evans et al. 1980,b and Brooks et al., 1981). E.coli urinary isolates have also been reported to possess a mannose resistant haemagglutinin (MRHA) detected using human A or 0 Rhesus positive erythrocytes. In this case, agglutination is not affected by the presence of α D mannose (Duguid and Gillies, 1957; Kallenius et al., 1980,b and Brooks et al., (1981). The MRHA was best observed after growth of E.coli on agar, while the MSHA was best observed when the organisms were grown in broth. This was also reported by Duguid and Gillies, (1957), Fein, (1981) and Ørskov et al., (1980,b). It was decided to investigate the role of bacterial haemagglutinins in the *in vitro* attachment of E.coli to uroepithelial cells.

3.1 HAEMAGGLUTINATION TEST.

The results obtained from this test were easily read and consistent for all strains tested on at least two occasions. The key describing the haemagglutination results should be referred to for all tables where haemagglutination patterns are reported. (see also Plates 15 to 19).

KEY FOR HAEMAGGLUTINATION TEST RESULTS

Human = Human Group A Rhesus Positive Erythrocytes

Guinea Pig = Guinea Pig Erythrocytes.

MRHA = Presence of a Mannose Resistant Haemagglutinin .
The reaction is not inhibited by α D Mannose.

MSHA = Presence of a Mannose sensitive Haemagglutinin .
The reaction is inhibited by α D Mannose.

\- = No Haemagglutination reaction.

MRHA± OR MSHA± = Weak Haemagglutination reaction.

3.2 EFFECT OF UROEPITHELIAL CELL STORAGE AT 4°C AND RECEPTIVITY TO E.COLI ATTACHMENT.

In 1980, Kallenius et al. (b), reported that periurethral cells could be stored at 4°C for up to 10 days without affecting attachment of E.coli. It was decided to investigate the effect of uroepithelial cell storage at 4°C on receptivity to E.coli attachment. It was considered that cells collected over a few days from the same group of women, and then stored at 4°C, could be used to perform experiments requiring large numbers of uroepithelial cells.

In this experiment, uroepithelial cells from four urine samples were harvested, washed and resuspended in PBS. Four aliquots of 1×10^5 cells were assayed immediately for the attachment of E.coli strains 73 and 207 grown on TSA and strains 43 and 87 grown in BHI broth. The attachment assay was carried out as in section 1.1 and the mean attachment values calculated for each strain. The remaining uroepithelial cells were suspended in PBS and stored at 4°C for 7 days and then assayed for attachment of the same four E.coli strains as described above. The mean attachment values were again calculated and compared with those on day 1, using two-way analysis of variance. The haemagglutination pattern of each strain was tested as described in section 3.1.

The results are presented in Table 31. There was no signficant difference between the attachment values obtained with cells tested on day 1 and those with cells tested after 7 days storage at 4°C ($p > 0.1$). It is therefore feasible to collect uroepithelial cells and store them at 4°C for several days. This permits the establishment of a large pool of cells which can be used in attachment assays. The differences noted between the attachment levels of the four E.coli strains tested, will be discussed later.

TABLE 31

EFFECT OF STORAGE ON RECEPTIVITY OF UROEPITHELIAL CELLS TO E.COLI ATTACHMENT.

E.COLI STRAIN	GROWTH MEDIUM	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES G.PIG	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL DAY 1	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL DAY 7
43	BROTH	-	-	2	4
87	BROTH	-	MSHA	13	11
73	TSA	MRHA	MSHA	74	72
207	TSA	MRHA	-	57	53
				MEAN = 36.5	MEAN = 35.0

$p > 0.1$

3.3 ATTACHMENT TO UROEPITHELIAL CELLS OF E.COLI STRAINS POSSESSING A MSHA, A MRHA, OR NEITHER HAEMAGGLUTININ.

Fourteen E.coli strains were selected for this experiment on the basis of their different haemagglutinating properties. Strains 43, 52, 94, 132, 159 and 242 were placed in Group A: they did not produce a haemagglutinin after a single subculture in 10 cm^3 BHI broth, statically at 37°C for 16 hours, but did produce a MSHA after six subcultures in 10 cm^3 BHI broth, statically at 37°C for 24 hours.

Strains 40, 94, 116, 125, 166 and 207 were placed in Group B: they did not produce a haemagglutinin after a single subculture in 10 cm^3 BHI broth, statically at 37°C for 16 hours, but did produce a MRHA after subculture on trypticase soy agar. Three strains served as controls in Group C, namely strains 43, 74 and 322. Of these, strain 43 did not produce a haemagglutinin after a single subculture in BHI broth or on TSA, but did produce a MSHA after six subcultures in BHI broth; strain 74 did not produce a haemagglutinin under any of the three growth conditions; and strain 322 produced a MRHA and a MSHA after subculture in both BHI broth conditions, but only produced a MRHA after subculture on TSA.

Growth of E.coli in static broth cultures and in cultures grown serially in static broth has been reported to encourage production of a MSHA (Duguid et al., 1955; Brinton, 1978). The possession of a MSHA has been associated with the presence of filamentous appendages "fimbriae" or "pili" as reported by Duguid and Anderson (1967). Growth of E.coli on an agar medium has been shown to encourage production of a MRHA (Duguid et al., 1979; Ørskov et al., 1980,b).

In this experiment, the E.coli strains were grown under the appropriate conditions, then assayed for attachment to uroepithelial

TABLE 32

ATTACHMENT TO UROEPITHELIAL CELLS OF E.COLI STRAINS POSSESSING A MSHA, A MRHA OR NEITHER HAEMAGGLUTININ.

Group A Strains	SINGLE SUBCULTURE IN 10 cm^3 BHI BROTH				SIX SUBCULTURES IN 10 cm^3 BHI BROTH			
	Number of E.coli per Uroepithelial Cell		Haemagglutination Erythrocytes		Number of E.coli per Uroepithelial Cell		Haemagglutination Erythrocytes	
	PBS	PBS + 2.5% α D Mannose	Human	Guinea Pig	PBS	PBS + 2.5% α D Mannose	Human	Guinea Pig
43	3	3	-	-	3	3	-	MSHA
52	4	4	-	-	4	3	-	MSHA
94	5	5	-	-	5	4	-	MSHA
132	8	8	-	-	6	5	-	MSHA
159	9	9	-	-	9	6	-	MSHA
242	8	7	-	-	8	4	-	MSHA
	MEAN = 6.17	MEAN = 6.0			MEAN = 5.83	MEAN = 4.17		

TABLE 32 Cont'd

Group B Strains	SINGLE SUBCULTURE IN 10 cm^3 BHI BROTH				SINGLE SUBCULTURE ON TSA			
	Number of E.coli per Uroepithelial Cell		Haemagglutination Erythrocytes		Number of E.coli per Uroepithelial Cell		Haemagglutination Erythrocytes	
	PBS	PBS + 2.5% α D Mannose	Human	Guinea Pig	PBS	PBS + 2.5% α D Mannose	Human	Guinea Pig
40	5	4	-	-	35	36	MRHA	-
94	5	5	-	-	33	34	MRHA	-
116	10	10	-	-	36	40	MRHA	-
125	9	9	-	-	57	58	MRHA	-
166	7	7	-	-	26	28	MRHA	-
207	8	7	-	-	58	59	MRHA	-
	MEAN = 7.33	MEAN 7.0			MEAN = 40.8	MEAN = 42.5		

TABLE 32 Cont'd

Control Group C Strains	SINGLE SUBCULTURE IN 10 cm^3 BHI BROTH				SIX SUBCULTURES IN 10 cm^3 BHI BROTH				SINGLE SUBCULTURE ON TSA			
	Number of E.coli per Uroepithelial Cell		Haemagglutination Erythrocytes		Number of E.coli per Uroepithelial Cell		Haemagglutination Erythrocytes		Number of E.coli per Uroepithelial Cell		Haemagglutination Erythrocytes	
	PBS	PBS + 2.5% α D Mannose	Human	Guinea Pig	PBS	PBS + 2.5% α D Mannose	Human	Guinea Pig	PBS	PBS + 2.5% α D Mannose	Human	Guinea Pig
43	3	3	-	-	3	3	-	MSHA	4	3	-	-
74	6	6	-	-	4	4	-	-	4	3	-	-
322	16	15	MRHA	MSHA	11	9	MRHA	MSHA	26	26	MRHA	-
	MEAN = 8.3	MEAN = 8.0			MEAN = 6.0	MEAN = 5.3			MEAN = 11.3	MEAN = 10.67		

cells harvested from pooled urine samples, as described in the methods section 1.1. Each strain was also tested for its haemagglutination activity as described in the methods section 3.1. For each assay, duplicate samples were used and α D mannose (2.5% w/v in PBS) was added to determine whether attachment to uroepithelial cells, particularly with strains possessing a MSHA, was inhibited. Mean attachment results were calculated and compared using two-way analysis of variance.

The results are presented in Table 32. The analysis of variance established that the presence of 2.5% α D mannose had no significant effect on the levels of attachment ($p > 0.1$). Cultural conditions which encouraged production of a MSHA in Group A strains, did not give rise to significantly different attachment values ($p > 0.1$). However,after growth on TSA the six strains in Group B produced a MRHA and attached to uroepithelial cells in significantly greater numbers than when no MRHA was produced after a single subculture in BHI broth ($p < 0.001$).

The results for Group C control strains suggested that the growth conditions were not themselves a contributing factor in the attachment process. Had this been so, strains 43 and 74 grown on TSA, would have been expected to attach in large numbers to uroepithelial cells, rather than in the non-specific manner observed. Strain 322 possessed both a MRHA and a MSHA after one and six subcultures in BHI broth. However, attachment of this strain to uroepithelial cells was highest when only a MRHA was expressed after growth on TSA.

3.4 THE LEVELS OF ATTACHMENT TO UROEPITHELIAL CELLS OF A LARGER GROUP OF E.COLI STRAINS WITH DIFFERENT HAEMAGGLUTINATION PROPERTIES.

The role of bacterial haemagglutinins in attachment of E.coli

to uroepithelial cells was further investigated using sixty E.coli strains. Of these, 20 strains produced a MRHA alone after growth on TSA; 20 strains produced a MSHA alone after growth in 10 cm^3 BHI broth and 20 strains did not produce a haemagglutinin. One exception, strain 52, produced a MSHA only after six subcultures in 10 cm^3 BHI broth. Each strain was assayed in duplicate for attachment to uroepithelial cells harvested from pooled urine samples, using the method described in section 1.1. On the day of the test, the haemagglutination pattern of each E.coli strain was confirmed using the method in section 3.1. Results were analysed using one-way analysis of variance.

The results of this experiment are presented in Table 33. The 20 E.coli strains grown on TSA and which were shown to have expressed a MRHA, attached in significantly greater numbers to uroepithelial cells than the 20 strains grown in BHI broth and shown to carry a MSHA ($p < 0.001$). The 20 strains grown on TSA or in BHI broth and not possessing a haemagglutinin, gave similar levels of attachment to uroepithelial cells as those strains possessing a MSHA ($p > 0.1$). This demonstrated that the bacterial growth conditions alone were not responsible for the differences in attachment levels for the three groups of strains.

The results support the findings of the previous experiment. The presence of a MRHA after growth on TSA was shown to enhance attachment of E.coli strains to uroepithelial cells. These findings also help to explain why differences had occurred between the attachment values of E.coli strains grown in BHI broth and on TSA, as reported in section 1.

TABLE 33

THE LEVELS OF ATTACHMENT TO UROEPITHELIAL CELLS, OF A LARGER GROUP OF E.COLI STRAINS WITH DIFFERENT HAEMAGGLUTINATION PROPERTIES.

STRAINS GROWN ON TSA POSSESSING A MSHA		STRAINS GROWN IN BHI BROTH POSSESSING A MSHA		CONTROL STRAINS POSSESSING NEITHER A MRHA OR MSHA		
Strain No.	Mean no. E.coli per Uroepithelial Cell	Strain No.	Mean no. E.coli per Uroepithelial Cell	Strain No.	Mean no. E.coli per Uroepithelial Cell	Media
289	69	87	14	207	8	BROTH
178	59	67	12	125	9	BROTH
207	58	49	11	284	16	BROTH
125	57	35	11	159	9	BROTH
284	57	73	11	116	10	BROTH
104	51	33	10	40	5	BROTH
159	49	44	9	94	5	BROTH
63	44	47	9	146	8	BROTH
153	39	269	9	239	11	BROTH
116	36	159	9	166	7	BROTH
40	35	42	8	242	10	TSA
94	33	242	8	132	8	TSA
146	33	130	7	61	6	TSA
158	30	288	7	316	6	TSA
239	28	132	6	315	5	TSA
232	28	274	6	74	4	TSA
206	27	81	6	43	4	TSA
166	26	69	5	318	4	TSA
177	24	285	5	317	3	TSA
262	21	52	4	314	2	TSA
	MEAN = 40.2		MEAN = 8.35		MEAN = 7.0	

$p > 0.1$

3.5 ATTACHMENT TO UROEPITHELIAL CELLS OF E.COLI STRAINS WHICH POSSESS THE K1 ANTIGEN.

The amount of negatively charged capsular polysaccharide (K antigen) present on the E.coli surface has been found to be significantly greater in urinary isolates than in faecal isolates (Brooks et al., 1980; 1981). Studies have shown that about 84% of E.coli strains causing neonatal meningitis and 36% of those causing neonatal sepsis contain K1 capsular antigen (Robbins et al., 1974, and Sarff et al., 1975). Additionally, a recent study of adult bacteremias has shown the E.coli K1 serotype to be associated with a higher morbidity than non K1 E.coli strains. (Weinstein and Young, 1978).

The following experiment was designed to determine whether the K1 antigen of E.coli urinary strains played a direct role in attachment to uroepithelial cells.

From E.coli stock cultures, 21 urinary isolates, which possessed the K1 antigen, were grown in BHI broth and on TSA and assayed in duplicate for attachment to uroepithelial cells from pooled urine samples as in section 1.1. The haemagglutination pattern for each strain was confirmed using the method described in section 3.1.

Strain 207 grown on TSA and strain 87 grown in BHI broth were used as reference strains to test the receptivity of the uroepithelial cell suspension to E.coli attachment.

All results presented in Table 34 were analysed by one-way or two-way analysis of variance. It appeared that the K1 antigen of E.coli urinary strains did not play a direct role in attachment to uroepithelial cells. As in section 3.3 and 3.4, the results showed that strains grown on TSA, expressing a MRHA attached to uroepithelial cells to significantly higher levels than when these

TABLE 34

ATTACHMENT TO UROEPITHELIAL CELLS OF E.COLI STRAINS,

WHICH POSSESS THE K1 ANTIGEN.

		BHI BROTH GROWN CULTURES			TSA GROWN CULTURES		
		MEAN NO. E.COLI PER	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NO. E.COLI PER	HAEMAGGLUTINATION ERYTHROCYTES	
STRAIN	SEROTYPE	UROEPITHELIAL CELL	HUMAN	GUINEA PIG	UROEPITHELIAL CELL	HUMAN	GUINEA PIG
73	08:K1:H14	11	-	MSHA	82	MRHA	MSHA
173	018ab:K1:H-	8	-	MSHA	41	MRHA	MSHA
153	07:K1:H-	10	-	MSHA±	39	MRHA	-
232	06:K1:H1	6	-	MSHA±	28	MRHA	-
282	ONT:K1:H1	12	-	MSHA	26	MRHA	-
249	07:K1:H1	10	-	MSHA±	22	MRHA	MSHA
83	02:K1:H6	7	-	MSHA	21	MRHA	MRHA
181	07:K1:H-	7	-	MSHA	20	MRHA	MSHA
243	02:K1:H7	6	-	MSHA±	18	MRHA±	-
		MEAN = 8.56			MEAN = 33.0		

Cont'd ...

TABLE 34 Cont'd

		BHI BROTH GROWN CULTURES			TSA GROWN CULTURES		
		MEAN NO. E.COLI PER	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NO. E.COLI PER	HAEMAGGLUTINATION ERYTHROCYTES	
STRAIN	SEROTYPE	UROEPITHELIAL CELL	HUMAN	GUINEA PIG	UROEPITHELIAL CELL	HUMAN	GUINEA PIG
172	02:K1:H6	10	-	MSHA	12	MSHA±	MSHA
240	02:K1:H6	6	-	MSHA	6	-	MSHA
95	051/111:K1:H4	7	-	MSHA	10	-	MSHA
97	06:K1:H31	8	-	MSHA	10	MSHA·	MSHA
33	04:K1:HR	10	-	MSHA	9	-	-
130	02:K1:H1	7	-	MSHA	7	-	MSHA
42	02:K1:H6	8	-	MSHA	7	-	MSHA
233	02:K1:H1	7	-	MSHA	7	-	MSHA
44	06:K1:H-	9	-	MSHA	6	-	MSHA
43	0R:K1:H-	3	-	-	4	-	-
74	ONT:K1:H1	6	-	-	4	-	-
285	017:K1:HNT	5	-	MSHA	4	-	MSHA
		MEAN = 7.17			MEAN = 7.17		

$\frac{9}{11}$ $p > 0.1$

same strains were grown in BHI broth ($p < 0.001$), and when other strains not expressing a MRHA were grown in BHI broth or on TSA ($p < 0.001$). There was no significant difference between the attachment values of the first 9 strains grown in BHI broth (mean 8.56 E.coli per cell) and the second group of 11 strains grown in BHI broth (mean 7.17 E.coli per cell), ($p > 0.1$).

Strain 207 served as a reference for the level of attachment, and gave a mean of 57 E.coli per uroepithelial cell after growth on TSA. Strain 87 grown in BHI broth gave a mean attachment of 14 E.coli per uroepithelial cell. This suggested that the uroepithelial cell sample, used in this experiment, was highly receptive to E.coli attachment under the conditions of the test.

3.6 ATTACHMENT OF E.COLI FAECAL ISOLATES TO UROEPITHELIAL CELLS

Strains of E.coli isolated from stools of healthy adult females have been found to possess a MSHA and/or a MRHA significantly less frequently than strains isolated from women with symptomatic urinary tract infections (Brooks et al., 1981). The following experiment was carried out to investigate the role of a MSHA and a MRHA in the attachment of E.coli faecal strains to uroepithelial cells.

A collection of E.coli faecal isolates had been isolated by Dr. H.J.L. Brooks from the stools of healthy women in Palmerston North. From these, twenty strains were selected which expressed a MSHA, a MRHA, both haemagglutinins or neither haemagglutinin. Each strain was grown both in BHI broth and on TSA,and assayed in duplicate for attachment to uroepithelial cells harvested from pooled urine samples, as described in section 1.1. On the day of the experiment, each strain was retested for haemagglutination of guinea pig and human erythrocytes as in section 3.1.

ABLE 35

ATTACHMENT OF E.COLI FAECAL ISOLATES TO UROEPITHELIAL CELLS.

STRAINS	BROTH GROWN CULTURES			TSA GROWN CULTURES		
	MEAN NO. E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NO. E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES	
		HUMAN	GUINEA PIG		HUMAN	GUINEA PIG
Co 32	11	-	-	36	MRHA	MSHA
Co 1	6	-	-	25	MRHA	-
Co 37	9	-	MSHA±	23	MRHA±	MSHA±
Co 20	17	-	-	22	MRHA	MRHA
Co 9	8	-	-	18	MRHA±	-
Co 24	13	MRHA	MSHA	18	MRHA	MSHA
Co 4	10	-	MSHA±	17	MRHA	MSHA±
Co 27	11	MSHA	MSHA	17	MRHA	MSHA
Co 31	8	-	MSHA±	17	MRHA	MSHA±
Co 2	8	MRHA±	MSHA	16	MRHA±	-
	MEAN = 10.1	$p < 0.001$		MEAN = 20.9		
Co 23	11	-	MSHA	11	-	MSHA
Co 25	8	-	-	10	-	-
Co 29	7	-	MSHA	8	-	MSHA
Co 3	9	-	MSHA	7	-	MSHA
Co 6	5	-	MSHA	7	-	MSHA
Co 10A	6	-	-	7	-	-
Co 21	7	-	-	6	-	-
Co 7C	3	-	MSHA	5	MSHA	MSHA
Co 17A	5	-	MSHA	4	-	MSHA
Co 18B	5	-	MSHA	4	-	MSHA
	MEAN 6.6			MEAN = 6.9		

$p > 0.25$

Urinary Strain 207 grown on TSA and strain 87 grown in BHI broth were used as reference strains to test the receptivity of the uroepithelial cell suspension to E.coli attachment.

All results presented in Table 35 were analysed by one-way and two-way analysis of variance. The 10 strains grown on TSA, and expressing a MRHA attached significantly higher to uroepithelial cells than strains grown in BHI broth ($p < 0.001$). Lower attachment values were found for the second group of 10 strains not expressing a MRHA after growth on TSA or in BHI broth ($p < 0.001$). Growth of this second group of strains on TSA or in BHI broth resulted in similar attachment levels ($p > 0.25$). Urinary strains 87 and 207 served as a reference for the level of attachment. Strain 207 grown on TSA, gave a mean of 58 E.coli per uroepithelial cell, and strain 87 grown in BHI broth, gave a mean of 14 E.coli per cell. This suggested that the uroepithelial cell sample was highly receptive to E.coli attachment, under the conditions of the test.

The attachment of faecal strains grown on TSA and expressing a MRHA was not as high as the levels reported in section 3.4 for E.coli urinary strains. Nevertheless, the results support the hypothesis that the presence of a MRHA after growth on TSA, enhances attachment of E.coli urinary and faecal strains to uroepithelial cells. Faecal strains not expressing a haemagglutinin or with only a MSHA did not attach as well to the uroepithelial cells.

3.7 PRODUCTION OF A MRHA BY E.COLI STRAIN 207 GROWN ON TSA.

This experiment was related to the findings presented in section 1.1.15 which indicated that attachment of strain 207 to uroepithelial cells varied depending on the growth conditions used for this strain. In the course of that experiment, the haemagglutina-

tion pattern was determined for strain 207 after each incubation interval by the method outlined in section 3.1. In this way, it was possible to determine the growth phase on TSA at which this E.coli strain produced a MRHA. This finding could then be related to the attachment levels to uroepithelial cells.

After a 16 hours incubation interval in BHI broth, strain 207 did not produce a MRHA nor a MSHA; and the resultant mean attachment was 11 E.coli per uroepithelial cell. After inoculation onto TSA, and 5 hours incubation, no haemagglutinin was detected and the mean attachment was 11 E.coli per cell. A weak MRHA reaction was noted after 7 hours incubation on TSA and this coincided with a mean attachment value of 35 E.coli per cell. A strong MRHA reaction was observed after 12 and up to 48 hours of incubation on TSA, and the resultant attachment values rose to 55 E.coli per cell after 12 to 16 hours of incubation then decreased to 38 E.coli per cell after 48 hours of incubation. (see Table 15).

It therefore appears that a MRHA was produced, at the level of detection by the haemagglutination test, during the first 7 hours growth on TSA. Production of the MRHA coincided with a dramatic increase in the level of attachment of E.coli to the uroepithelial cells.

RESULTS

SECTION 4.

FURTHER ANALYSIS OF *E.COLI* URINARY AND FAECAL STRAINS WITH PARTICULAR REFERENCE TO THE ROLE OF BACTERIAL HAEMAGGLUTININS IN THE ATTACHMENT OF *E.COLI* TO UROEPITHELIAL CELLS.

4.1 ANALYSIS OF 145 *E.COLI* URINARY ISOLATES AND 38 FAECAL ISOLATES FOR ATTACHMENT TO UROEPITHELIAL CELLS AND HAEMAGGLUTINATION OF HUMAN AND GUINEA PIG ERYTHROCYTES.

The haemagglutination pattern of 145 *E.coli* urinary and 38 faecal strains was determined using human A Rhesus positive and guinea pig erythrocytes by the method in section 3.1. Each strain was tested on two or more occasions following growth in BHI broth and on TSA. Of these strains 111 urinary isolates and 23 faecal isolates were tested in duplicate for attachment to uroepithelial cells using the methods described in section 1.1 after growth in BHI broth and growth on TSA. The strains were tested in groups of up to 20 strains per day, using uroepithelial cells harvested from pooled urine samples. In every batch of strains tested, strains 87 grown in BHI broth and 207 grown on TSA were included and served as references for attachment. The mean number of *E.coli* attached to 40 viable uroepithelial cells was calculated for each strain. The results were adjusted to allow for variation between uroepithelial cell samples. This was achieved by calculating the overall mean for the reference strains, 87 and 207, and multiplying by the mean value of the test strain divided by the mean value of the reference strain obtained on the day of test. Adjusted mean values for broth grown cultures were calculated using the results

for reference strain 87 grown in BHI broth; adjusted mean values for TSA grown cultures were calculated using the results for reference strain 207 grown on TSA.

The results of this study, for adjusted mean attachment values are presented in Appendix 1 and are summarised in Tables 36 and 37. The haemagglutination results are also presented.

4.1.1. ATTACHMENT OF *E.COLI* TO UROEPITHELIAL CELLS

The overall mean value for strain 87 grown in BHI broth was 14 *E.coli* per cell and for strain 207 grown on TSA 58 *E.coli* per cell. The highest individual mean result for strains grown in BHI broth, was 17 *E.coli* per cell. The overall adjusted mean values for strains grown in BHI broth and possessing a MRHA, was marginally higher than for strains grown in BHI broth and not possessing a MRHA. However, only a small proportion of strains produced a MRHA when grown in broth and the number tested was insufficient for statistical analysis. For cultures grown on TSA, the overall adjusted mean values indicated that strains possessing a MRHA attached more highly to uroepithelial cells than other strains. The range of individual mean counts for *E.coli* urinary isolates possessing a MRHA was 13 to 82 *E.coli* per cell; but only 2 to 16 *E.coli* per cell for strains without a MRHA. The *E.coli* faecal isolates gave a similar pattern though attachment to uroepithelial cells for the strains tested, was not as high.

The results summarised in Table 36 indicate that a MRHA was expressed particularly during growth on TSA. Of the 111 urinary strains tested, only 12 produced a MRHA after growth in BHI broth, and these gave a net mean attachment of 13.08 *E.coli* per uroepithelial cell. Only 7 of the 111 strains (6% of the total) attached to uroepithelial cells in numbers $\leq$ 5 *E.coli* per cell, after growth

TABLE 36

SUMMARY OF MEAN NUMBER OF URINARY AND FAECAL E.COLI ATTACHED TO UROEPITHELIAL CELLS AFTER TESTING 111 URINARY STRAINS AND 23 FAECAL STRAINS.

HAEMAGGLUTINATION ERYTHROCYTES		BHI BROTH GROWN CULTURES *					
		URINARY			FAECAL		
HUMAN	GUINEA PIG	NOS. TESTED	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	RANGE OF ALL INDIVIDUAL MEAN COUNTS	NOS. TESTED	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	RANGE OF ALL INDIVIDUAL MEAN COUNTS
MRHA	MSHA	6	13.83	11 to 17	2	10.5	8 to 13
MRHA	-	6	12.5	10 to 17	-	-	-
-	MSHA	58	8.43	4 to 15	14	7.79	3 to 11
-	-	39	7.79	2 to 16	7	8.43	6 to 17
		TRYPTICASE SOY AGAR GROWN CULTURES *					
MRHA	MSHA	12	28.67	13 to 82	7	19.86	17 to 36
MRHA	-	47	34.06	17 to 70	6	19.33	15 to 25
-	MSHA	35	6.89	3 to 12	7	6.57	4 to 11
-	-	17	6.53	2 to 16	3	7.67	6 to 10

* The mean values represent the figures reported in Appendix 1 which were adjusted in relation to reference strains 87 and 207 used throughout the study.

in BHI broth or on TSA. This level of attachment was termed "non-specific", following the results obtained from the experiment described in section 1.1.22. Of the 7 strains here with non-specific attachment, none had an O serotype commonly found in urinary tract infections in Palmerston North, namely O1, O2, O4 O6, O7, O68 and O75 (Brooks et al., 1981). It appeared that 93.7% of the urinary strains and 87% of the faecal strains tested in this study, attached specifically to uroepithelial cells *in vitro*.

4.1.2 HAEMAGGLUTINATION RESULTS.

Analysis of the haemagglutination properties of 145 E.coli urinary strains and 38 E.coli faecal strains is presented in Appendix 1 and is summarised in Table 37. The haemagglutination followed one of the four patterns on Table 37. A few strains expressed a MRHA detected with guinea pig erythrocytes, but these were included under the heading of Human $MRHA^+$, Guinea Pig -ve . A few strains expressed a MSHA detected with human erythrocytes, but these were included under the heading of Human -ve, Guinea Pig $MSHA^+$. It should be noted that the co-expression of a MSHA and a MRHA would not be detected with the same group of erythrocytes: the MRHA would mask the mannose sensitive reaction.

The results of the present study were confirmed two or more times for each strain. The overall results indicated that growth in BHI broth was suitable for expression of a MSHA, and growth on TSA was suitable in particular for expression of a MRHA.With regard to haemagglutinating activity, the majority of E.coli strains fell into the following categories:

(i) possessed a MSHA following culture in BHI broth.

(ii) possessed a MRHA following culture on TSA

(iii) did not possess a MSHA or a MRHA following BHI broth culture but did possess a MRHA after growth on TSA.

TABLE 37

SUMMARY OF HAEMAGGLUTINATION TESTS FOR 145 E.COLI URINARY STRAINS AND 38 E.COLI FAECAL STRAINS.

HAEMAGGLUTINATION PATTERNS		BHI BROTH GROWN CULTURES				TRYPTICASE SOY AGAR GROWN CULTURES			
ERYTHROCYTES		URINARY		FAECAL		URINARY		FAECAL	
HUMAN	GUINEA PIG	NO.TESTED	PERCENTAGE	NO.TESTED	PERCENTAGE	NO.TESTED	PERCENTAGE	NO.TESTED	PERCENTAGE
MRHA	MSHA	8	5.5	2	5.3	17	11.7	7	18.4
MRHA	-	8	5.5	0	0	49	33.8	6	15.8
-	MSHA	83	57.3	22	57.9	55	37.9	16	42.1
-	-	46	31.7	14	36.8	24	16.6	9	23.7

(iv) possessed neither a MSHA nor a MRHA.

Only 16 urinary and 2 faecal strains possessed a MRHA following growth in BHI broth. Of all faecal strains, 34.2% possessed a MRHA after growth on TSA while 57.9% possessed a MSHA following growth in BHI broth. By comparison, 45.5% of the urinary isolates possessed a MRHA following growth on TSA, while 57.3% possessed a MSHA after growth in BHI broth. The possession of these two haemagglutinins appears to be relatively common among E.coli urinary and faecal isolates.

4.2 PROPERTIES OF E.COLI URINARY STRAINS RELATED TO POSSESSION OF A MRHA OR A MSHA.

4.2.1 ANALYSIS OF SIX PROPERTIES.

A large number of the E.coli strains used in this study had previously been analysed for certain properties by Dr. H.J.L. Brooks and associates, the details of which have been published elsewhere (Brooks et al., 1981). These included O and H serotyping, determination of K antigen titre, fermentation of dulcitol, salicin and sucrose, detection of haemolysin with horse, sheep and human group O erythrocytes, phospholipase activity and detection of a MRHA and a MSHA. An analysis was undertaken to determine the possible role of these properties in attachment of E.coli strains to uroepithelial cells. In total, the properties were analysed for 39 urinary strains possessing a MRHA alone after growth on TSA, and 47 urinary strains which possessed a MSHA alone after growth in BHI broth. The result for each property was compared for strains with a MRHA and those with a MSHA using the X^2 test as described by Roscoe (1969). The properties of 38 faecal isolates were not analysed due to the small

number of strains which would have been present in each group. The haemagglutination pattern of the strains was determined from results of the study in section 4.1.

The results from part (a) of this study are presented in Appendices 2 and 3, with a summary on Table 38.

Of the 39 strains with a MRHA after growth on TSA, 11 possessed a MSHA after growth in BHI broth. Therefore the separation of the two groups of strains being analysed was not as categorical as would have been preferred. Nevertheless, the first group of strains all possessed a MRHA after growth on TSA, and the second group all possessed a MSHA after growth in BHI broth. Statistical analysis using the X^2 (CHI squared) test failed to detect any significant difference between the properties of the two groups of strains. The significance values were as follows: $p > 0.1$ for dulcitol fermentation; $p > 0.1$ for salicin fermentation; $p > 0.05$ for sucrose fermentation; $p > 0.5$ for haemolysin activity and $p > 0.5$ for phospholipase activity. Of the strains tested for K antigen titre, 77% of those with a MRHA alone were K rich (reciprocal titre ≥ 32), and 57.4% of those with a MSHA alone were K rich. However this difference between these two values was not statistically significant ($p > 0.05$).

From this analysis it is not possible to associate any of these properties directly with attachment of E.coli to uroepithelial cells nor with the possession by these strains of either a MRHA or a MSHA. However, it was apparent that a large percentage of the strains tested, fermented dulcitol, salicin and sucrose, showed haemolysin activity and possessed a high K Antigen titre.

TABLE 38

SUMMARY OF THE PROPERTIES OF E.COLI URINARY STRAINS RELATED TO POSSESSION OF A MRHA OR A MSHA.

PROPERTIES	RESULTS EXPRESSED AS PERCENTAGE OF TOTAL IN EACH GROUP: 39 STRAINS WITH MRHA ALONE AFTER GROWTH ON TSA.	47 STRAINS WITH MSHA ALONE AFTER GROWTH IN BHI BROTH OR ON TSA.	STATISTICAL ANALYSIS
Dulcitol Fermentation	94.88	87.23	$p > 0.1$
Salicin Fermentation	79.5	70.21	$p > 0.1$
Sucrose Fermentation	41.03	57.45	$p > 0.05$
Haemolysin Activity:-			
Horse Erythrocytes	41.03	38.3	$p > 0.5$
Sheep Erythrocytes	10.26	8.5	$p > 0.5$
Human 'O' Erythrocytes	35.9	31.9	$p > 0.5$
Phospholipase Activity	7.69	12.77	$p > 0.5$
K Antigen Reciprocal Titre:-			
$\geq$ 1024	23.1	17.0	
512	5.1	8.5	
256	15.4	6.4	
128	12.8	10.6	
64	10.3	6.4	
32	10.3	8.5	
16	5.1	4.3	
8	2.6	2.1	
4	5.1	4.3	
2	2.6	0	
Nil	7.6	31.9	

4.2.2 COMMON O SEROTYPES

Seven E.coli O serotypes have been commonly found in cases of urinary tract infection in New Zealand namely O1, O2, O4, O6, O7, O68 and O75 as reported by Brooks et al., 1981. The results from the previous study of section 4.1, were used in the analysis of haemagglutination properties and attachment values of urinary strains grown on TSA which had common or uncommon O serotypes. This study was carried out to determine the percentage of strains with a MRHA in both groups and to ascertain whether E.coli strains with a common O serotype which possessed a MRHA, attached in higher numbers to uroepithelial cells than strains which also possessed a MRHA, but had an O serotype not commonly found in cases of urinary tract infection. The results were analysed using a X^2 test, and are presented in Table 39. It was apparent that significantly more strains of common O serotypes possessed a MRHA after growth on TSA, than did strains with uncommon O serotypes ($p < 0.001$). However, attachment to uroepithelial cells was equally high for E.coli with a MRHA regardless of the O serotype, ($p > 0.1$). Strains which did not possess a MRHA gave rise to significantly lower attachment values to uroepithelial cells ($p < 0.001$). Many of the strains exhibiting the highest attachment values were serotype O75. However, there were insufficient numbers to compare these strains with strains of other serotypes.

4.2.3 MOTILITY.

In order to determine the effect of motility on attachment of E.coli to uroepithelial cells, 38 urinary isolates were selected. Each strain was cultured in BHI broth, centrifuged at 1500 x g for 10 minutes, then resuspended in 1 cm^3 PBS. The suspensions were

TABLE 39

E.COLI STRAINS WITH 0 SEROTYPES COMMONLY FOUND IN UTI, AND STRAINS WITH OTHER 0 SEROTYPES NOT COMMONLY FOUND IN UTI: COMPARISON OF HAEMAGGLUTINATION PROPERTIES AND ATTACHMENT TO UROEPITHELIAL CELLS.

CLASSIFICATION	HAEMAGGLUTINATION OF HUMAN ERYTHROCYTES		MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	
	NOS.TESTED	% WITH A MRHA	STRAINS WITH MRHA	STRAINS WITHOUT MRHA
COMMON 0 SEROTYPES (01,2,4,6,7,68,75)	75	60	33.1	7.8
OTHER 0 SEROTYPES	70	31.4	33.6	5.69
		$p < 0.001$		

TABLE 40

EFFECT OF MOTILITY ON ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

MOTILE STRAINS GROWN IN BHI BROTH				NON-MOTILE STRAINS GROWN IN BHI BROTH			
STRAIN	SEROTYPE	MOTILITY	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	STRAIN	SEROTYPE	MOTILITY	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL
52	06:H1	+/-	6	40	075:H-	-	5
63	06:H1	+	17	47	0R:H-	-	9
65	02:H4	+	10	86	075:H0	-	9
73	08:K1:H14	+	11	94	075:H-	-	5
83	02:K1:H6	+	7	122	075:H-	-	11
89	075:H5	+/-	8	129	075:H-	-	13
90	ONT:H1	+	13	153	07:K1:H-	-	10
104	02:H4	+/-	10	166	075:H-	-	10
116	ONT:HR	+	10	173	018ab:K1:H-	-	8
125	ONT:H1	+	9	178	o75:H-	-	7
146	O6:H5	+	8	181	07:K1:H-	-	7
158	04:H5	+	10	207	075:H-	-	8
159	07:HNT	+/-	9	215	075:H-	-	10
177	06:H1	+	15	239	06:H-	-	11
206	06:H1	+	10	249	07:K1:H-	-	10
232	06:K1:H1	+	6	284	0R:H-	-	16
262	06:H1	+/-	15	301	075:H-	-	8
282	ONT:K1:H1	+	12	313	0NT:H-	-	6
312	02:H1	+	12	321	04:H-	-	11
			MEAN = 10.42				MEAN = 9.16

\+ Denotes over 75% of culture was motile.

+/- Approximately 50% of culture was motile.

\- Denotes no motile organisms. $p > 0.1$.

diluted 1 in 10^4 using PBS and one drop was placed in a haemocytometer. Approximately 100 organisms were assessed for motility. A (+) denoted over 75% motility; a (+/-) denoted approximately 50% motility and a (-) denoted a non-motile culture. Each strain was assayed for attachment to uroepithelial cells from a pooled sample, as described in section 1.1. The results are presented in Table 40, and were analysed by one-way analysis of variance. The motile strains of E.coli attached to uroepithelial cells to the same degree as non-motile strains ($p > 0.1$). The presence of flagella did not appear to enhance or inhibit the capacity of E.coli strains to attach to uroepithelial cells.

4.3 EFFECT OF STORAGE OF E.COLI ISOLATES ON HAEMAGGLUTINATION ACTIVITY AND ATTACHMENT TO UROEPITHELIAL CELLS.

4.3.1 ATTACHMENT OF FRESHLY ISOLATED E.COLI STRAINS.

This experiment was carried out to compare attachment to uroepithelial cells using freshly isolated E.coli urinary strains and E.coli isolates stored at $-10^{\circ}C$ for several years. Sixteen E.coli strains were isolated at a local clinic, and numbered 290 to 313 for this study. Within 5 days of isolation these strains were grown in BHI broth and assayed for attachment to uroepithelial cells, using the method described in section 1.1. Each strain was also tested after growth on TSA. Haemagglutination tests were carried out in duplicate for all strains, using the method described in section 3.1.

Strain 303 was also tested for attachment to uroepithelial cells harvested on days of the menstrual cycle when receptivity of cells to E.coli attachment was at peak or trough levels, as

described in section 1.4. Strain 87 was simultaneously tested to serve as a reference for attachment.

Strain 290 was originally isolated from a woman working in the Department of Microbiology and Genetics. Two weeks after treatment of her urinary infection, her urine culture was negative and her uroepithelial cells did not have E.coli-like organisms attached. These cells were then tested for *in vitro* attachment of strain 290, and again on days when cell receptivity was at peak and trough levels. This woman became pregnant 1 year later and her uroepithelial cells were tested for attachment of E.coli strains 67 and 87; strain 290 was also assayed for attachment during weeks 7, 9, 17 and 39 of the pregnancy. This strain was also assayed for attachment to cells from four other women to compare the results with cells from the subject from whom the strain was isolated.

The results of the study on freshly isolated E.coli strains are presented in Appendix 1. The mean attachment values were similar to the range of values obtained from strains stored at $-10^{\circ}C$. Strain 303 gave similar results to those reported in section 1.4 for strains 67 and 87 over the menstrual cycle. The highest mean values for strain 303 were 8, 9, 10, 11, and 15 E.coli per cell,for cells collected between days 9 to 13 and 18 to 28 of the cycle. The lowest values of 2, 3, 4 and 5 E.coli per cell were recorded between days 14 to 17 of the cycle.

Strain 290 grown in BHI broth, attached to uroepithelial cells from various women with mean values between 6 and 10 E.coli per cell, on days of the menstrual cycle when receptivity of the cells was high. This strain's capacity to attach to uroepithelial cells was not high. When assayed with cells harvested during pregnancy, mean attachment values of between 9 and 11 E.coli per cell were recorded at weeks 7 and 9, while mean values of 2, 3 and

4 E.coli per cell were recorded at weeks 17 and 39. The results for strain 290 indicate that its capacity for attachment was not increased or decreased by storage at -10°C for well over a year.

4.3.2 STORAGE OF FOUR E.COLI STRAINS OVER 12 MONTHS.

The following experiment was designed to determine whether storage conditions affected haemagglutination properties and attachment to uroepithelial cells of four E.coli urinary strains. Strains 67, 87, 320 and 322 were stored for 12 months (a) on an agar slant (2% peptone, 0.5% NaCl, 2% agar) at room temperature and (b) in 2 cm^3 BHI broth with 0.4 cm^3 glycerol at -10°C. At regular intervals of approximately 4 to 6 weeks over 12 months, these cultures were inoculated into BHI broth, and onto TSA, grown at 37°C for 16 hours and tested for attachment to uroepithelial cells pooled from the same group of women each time. The method in section 1.1 was used and the haemagglutination properties were assessed using the method in section 3.1. Strain 87, stored at -10°C in 2 cm^3 broth was also inoculated into five BHI broths on 11 occasions over 12 months, then assayed for attachment to uroepithelial cells. This experiment was designed to determine whether the stock cultures contained a stable population of bacteria whose capacity to attach to uroepithelial cells did not vary on any one day of testing.

Tables 41 A,B,C,D present the results obtained for E.coli strains 67, 87, 320 and 322 stored in 2 different ways and assayed after growth in BHI broth. The probability values given under each table represent results from a two-way analysis of variance which compared the 11 attachment results in each column. Strain 67 showed some variation in attachment with lower values obtained from subcultures of E.coli stored on agar at room temperature ($p > 0.001$).

TABLE 41

EFFECT OF STORAGE OF E.COLI STRAINS OVER 12 MONTHS :

STRAINS 67 AND 87 GROWN IN BHI BROTH PRIOR TO ASSAY.

A STRAIN 67

DAYS FROM START OF TEST	(a) AGAR SLANT; ROOM TEMPERATURE			(b) $2cm^3$ BHI BROTH - $10^{\circ}C$		
	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES	
		HUMAN	GUINEA PIG		HUMAN	GUINEA PIG
3	7	-	MSHA	8	-	MSHA
69	8	-	MSHA	10	-	MSHA
108	9	-	MSHA	9	-	MSHA
146	10	-	MSHA	9	-	MSHA
172	9	-	MSHA	10	-	MSHA
205	8	-	MSHA	10	-	MSHA
235	9	-	MSHA	10	-	MSHA
275	11	-	MSHA	11	-	MSHA
304	9	-	MSHA	10	-	MSHA
331	8	-	MSHA	10	-	MSHA
359	8	-	MSHA	9	-	MSHA

$p > 0.001$

TABLE 41 Cont'd

B STRAIN 87

DAYS FROM START OF TEST	(a) AGAR SLANT; ROOM TEMPERATURE			(b) $2cm^3$ BHI BROTH - $10^{\circ}C$		
	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES	
		HUMAN	GUINEA PIG		HUMAN	GUINEA PIG
3	11	-	MSHA	11	-	MSHA
69	10	-	-	10	-	MSHA
108	17	-	MSHA	16	-	MSHA
146	14	-	-	13	-	MSHA
172	15	-	-	15	-	MSHA
205	14	-	-	15	-	MSHA
235	11	-	-	13	-	MSHA
275	15	-	-	14	-	MSHA
304	13	-	-	13	-	MSHA
331	13	-	-	13	-	MSHA
359	13	-	-	13	-	MSHA

$p > 0.1$

TABLE 41

EFFECT OF STORAGE OF E.COLI STRAINS OVER 12 MONTHS:

STRAINS 320 AND 322 GROWN IN BHI BROTH PRIOR TO ASSAY.

C STRAIN 320

DAYS FROM START OF TEST	(a) AGAR SLANT; ROOM TEMPERATURE			(b) $2cm^3$ BHI BROTH - 10^oC		
	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES	
		HUMAN	GUINEA PIG		HUMAN	GUINEA PIG
3	9	MRHA	MSHA	9	MRHA	MSHA
69	10	MRHA	MSHA	11	MRHA	MSHA
108	16	MRHA	MSHA±	15	MRHA	MSHA
146	11	MRHA	-	12	MRHA	MSHA
172	13	MRHA	-	14	MRHA	MSHA
205	14	MRHA	-	15	MRHA	MSHA
235	11	MRHA	-	13	MRHA	MSHA
275	15	MRHA	-	13	MRHA	MSHA
304	12	MRHA	-	11	MRHA	MSHA
331	13	MRHA	-	14	MRHA	MSHA
359	12	MRHA	-	12	MRHA	MSHA

$p > 0.01$

TABLE 41 Cont'd

D STRAIN 322

DAYS FROM START OF TEST	(a) AGAR SLANTS; ROOM TEMPERATURE			(b) $2cm^3$ BHI BROTH - $10^{o}C$		
	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES	
		HUMAN	GUINEA PIG		HUMAN	GUINEA PIG
3	15	MRHA	MSHA	15	MRHA	MSHA
69	13	MRHA	MSHA	14	MRHA	MSHA
108	18	MRHA	MSHA	17	MRHA	MSHA
146	16	MRHA	MSHA±	16	MRHA	MSHA
172	17	MRHA	-	16	MRHA	MSHA
205	17	MRHA	MSHA±	17	MRHA	MSHA±
235	18	MRHA	-	18	MRHA	MSHA
275	15	MRHA	-	18	MRHA	MSHA
304	14	MRHA	-	16	MRHA	MSHA
331	14	MRHA	-	15	MRHA	MSHA
359	13	MRHA	-	14	MRHA	MSHA

$p > 0.1$

There was no evidence that the levels of attachment of the other three strains varied in response to the different maintenance conditions.

The difference between each of the 11 rows of tables A,B, C and D was significant when analysed using two-way variance ($p < 0.001$). This suggested that over the 12 months, attachment to uroepithelial cells varied for the 11 tests carried out. When all 4 strains were similarly analysed for variation between test days within storage condition (a), then (b) there was strong evidence once again suggesting variability ($p < 0.001$ for each test) between test days.

In short, there was little variation for attachment to uroepithelial cells for strains stored under the two conditions, but attachment to uroepithelial cells varied over the 11 tests. This variation may have reflected uroepithelial cell variation known to occur between sample days and known to affect attachment of certain E.coli strains grown in BHI broth (see sections 1.1.4, 1.1.5).

It was not possible to conclude that the bacterial strains had not varied in their ability to attach to uroepithelial cells, over the 12 months. However, when comparing attachment values at the start and the completion of the study, there was no downward or upward trend apparent. In addition, in several cases, attachment to uroepithelial cells was high for each strain regardless of the storage conditions, such as on days 108, 172, 235 and 275. It would be most unlikely that variation due solely to the bacterial cultures would have simultaneously occurred in each culture on these 4 days. Such results suggest that variation between the 11 sample day results was due to the uroepithelial cell suspensions rather than the bacterial cultures.

The haemagglutination properties of the four strains tested

did not vary for the bacteria stored in BHI broth at -10°C. However, the production of a MSHA by strains 87, 320 and 322 was affected after prolonged storage on agar slants at room temperature. The MSHA was not detected after approximately 146 to 205 days storage. The loss of a MSHA did not affect the attachment of these strains to uroepithelial cells.

Table 41 E presents the results for strain 87 grown overnight at 37°C in BHI broth five times from the stock culture held at -10°C, and assayed for attachment to uroepithelial cells. Over 12 months, there was no detectable effect on expression of a MSHA, as this was present in each test. Haemagglutination of human erythrocytes was tested each time, but was always negative. The mean attachment figures for the five tests on one day did not vary more than 12.9% of the overall mean attachment figure for that day. This variation could be explained by the 15% variation known to occur within a uroepithelial cell sample at any one time, as reported in section 1.1.2. It was concluded that an inoculum from the stock culture could be taken at random and used to inoculate BHI broth cultures, without any large variation being found in the subsequent *in vitro* assay results for attachment to uroepithelial cells. There was no upward or downward trend in the attachment levels, though highest mean figures were recorded on Day 172 and lowest on day 359. This variation was thought to be due to variation in uroepithelial cell receptivity to the E.coli attachment.

Table 42 A,B,C,D presents the results of the final study involving E.coli strains stored over 12 months. This experiment was first commenced after 84 days storage when it was decided to investigate the effect of subculturing the strains onto TSA prior to the attachment assay. Statistical analysis was carried out using two-way analysis of variance to analyse the differences

TABLE 41 Cont'd

EFFECT OF STORAGE OF E.COLI STRAINS OVER 12 MONTHS

E. STRAIN 87 TESTED FIVE TIMES ON EACH OCCASION.

DAYS FROM START OF TEST	TEST 1		TEST 2		TEST 3		TEST 4		TEST 5	
	A	B	A	B	A	B	A	B	A	B
5	12	MSHA	14	MSHA	12	MSHA	13	MSHA	11	MSHA
70	13	MSHA	15	MSHA	15	MSHA	15	MSHA	14	MSHA
109	13	MSHA	12	MSHA	14	MSHA	14	MSHA	14	MSHA
146	13	MSHA	14	MSHA	14	MSHA	15	MSHA	13	MSHA
172	15	MSHA	15	MSHA	15	MSHA	15	MSHA	16	MSHA
205	15	MSHA	14	MSHA	15	MSHA	16	MSHA	14	MSHA
235	13	MSHA	13	MSHA	12	MSHA	11	MSHA	12	MSHA
275	14	MSHA	13	MSHA	13	MSHA	12	MSHA	13	MSHA
304	12	MSHA	13	MSHA	13	MSHA	12	MSHA	12	MSHA
331	13	MSHA	13	MSHA	12	MSHA	12	MSHA	12	MSHA
359	13	MSHA	12	MSHA	12	MSHA	11	MSHA	11	MSHA

A = Mean No. of E.coli per Uroepithelial Cell.

B = Haemagglutination of guinea pig erythrocytes.

between the storage on agar at room temperature and in BHI broth at -10°C; and between the days of storage to determine whether attachment to uroepithelial cells varied over the 12 months. The probability values beneath each table show the evidence for differences between the columns of results. There did not appear to be a difference for strains 67 and 87 stored on agar or in BHI broth ($p > 0.1$) but there was evidence of a difference for strains 320 and 322 with resultant highest attachment to uroepithelial cells in cultures stored at -10°C.

The difference between attachment levels on each day of test was insignificant with probability levels of $p > 0.1$ for strain 67; $p > 0.01$ for strain 87; $p > 0.01$ for strain 320 and $p > 0.05$ for strain 322. There were several days, namely 84, 135, 235, 275 and 304 when attachment values were marginally higher, but this was thought to be due to increased receptivity of the uroepithelial cell suspension to E.coli attachment rather than to variation within the E.coli cultures.

The results from the haemagglutination tests showed that a MRHA was present in strains 320 and 322 regardless of storage conditions, over the 12 months. For these strains, no MSHA was detected after growth on TSA. Strain 87 did not agglutinate human or guinea pig erythrocytes after growth on TSA, regardless of the storage conditions. Strain 67 did not agglutinate human erythrocytes but did produce a MSHA after growth on TSA, regardless of the storage conditions, with the exception of one result on day 235 when the agar stored culture subbed onto TSA did not express a MSHA. The reason for this exception is uncertain however, it is possible that experimental error may have been responsible.

TABLE 42

EFFECT OF

STORAGE OF E.COLI STRAINS OVER 12 MONTHS:

STRAINS 67 AND 87 GROWN ON TSA PRIOR TO ASSAY

A STRAIN 67

	(a) AGAR SLANT; ROOM TEMPERATURE			(b) $2cm^3$ BHI BROTH - 10^oC		
DAYS FROM START OF TEST	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	GUINEA PIG	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	GUINEA PIG
84	5	-	MSHA	4	-	MSHA
135	5	-	MSHA	5	-	MSHA
174	3	-	MSHA	5	-	MSHA
205	4	-	MSHA	5	-	MSHA
235	4	-	-	4	-	MSHA
275	6	-	MSHA	5	-	MSHA
304	6	-	MSHA	5	-	MSHA
331	5	-	MSHA	5	-	MSHA
359	4	-	MSHA	5	-	MSHA

$p > 0.1$

TABLE 42 Cont'd

B STRAIN 87

DAYS FROM START OF TEST	(a) AGAR SLANT; ROOM TEMPERATURE			(b) $2cm^3$ BHI BROTH - $10^{\circ}C$		
	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES	
		HUMAN	GUINEA PIG		HUMAN	GUINEA PIG
84	14	-	-	16	-	-
135	14	-	-	18	-	-
174	18	-	-	17	-	-
205	19	-	-	18	-	-
235	18	-	-	19	-	-
275	17	-	-	17	-	-
304	17	-	-	17	-	-
331	13	-	-	14	-	-
359	14	-	-	15	-	-

$p > 0.1$

TABLE 42 Cont'd

C STRAIN 320

DAYS FROM START OF TEST	(a) AGAR SLANT; ROOM TEMPERATURE			(b) $2cm^3$ BHI BROTH - $10^{o}C$		
	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES	
		HUMAN	GUINEA PIG		HUMAN	GUINEA PIG
84	23	MRHA	-	26	MRHA	-
135	23	MRHA	-	25	MRHA	-
174	20	MRHA	-	24	MRHA	-
205	21	MRHA	-	23	MRHA	-
235	20	MRHA	-	22	MRHA	-
275	21	MRHA	-	24	MRHA	-
304	23	MRHA	-	23	MRHA	-
331	20	MRHA	-	21	MRHA	-
359	20	MRHA	-	21	MRHA	-

$p > 0.001$

TABLE 42 Cont'd

D STRAIN 322

DAYS FROM START OF TEST	(a) AGAR SLANT; ROOM TEMPERATURE			(b) $2cm^3$ BHI BROTH - $10^{\circ}C$		
	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES	
		HUMAN	GUINEA PIG		HUMAN	GUINEA PIG
84	35	MRHA	-	34	MRHA	-
135	32	MRHA	-	31	MRHA	-
174	28	MRHA	-	30	MRHA	-
205	29	MRHA	-	30	MRHA	-
235	31	MRHA	-	33	MRHA	-
275	29	MRHA	-	30	MRHA	-
304	25	MRHA	-	32	MRHA	-
331	26	MRHA	-	30	MRHA	-
359	27	MRHA	-	30	MRHA	-

$p > 0.025$

4.4 TWO CULTURE CONDITIONS REPORTED TO AFFECT THE EXPRESSION OF A MRHA FOR E.COLI STRAINS.

In 1981, Hagberg et al. reported that growth of E.coli by subculturing twice in 5 cm^3 broth in a 100 cm^3 flask and incubating for 48 hours at 37°C on each occasion encouraged expression of a MRHA, and resulted in high attachment levels to uroepithelial cells. Twenty E.coli strains were subsequently cultured using this technique and assayed for attachment to uroepithelial cells as described in section 1.1. The haemagglutination pattern of these strains was also tested using the method of section 3.1.

A second experiment was carried out following reports by Duguid et al. (1955), and more recently by Evans et al. (1978) who discussed a MRHA of enterotoxigenic E.coli. These authors proposed that production of a MRHA was inhibited following growth of E.coli on agar at a lowered temperature. The same 20 E.coli strains as above, were cultured on TSA at 18°C and assayed for attachment to uroepithelial cells from the same pool of cells as above.

As a comparison, these E.coli strains were also assayed after growth on TSA and in BHI broth at 37°C. The mean attachment values and haemagglutination patterns were compared to those obtained from the first two parts of this study. The uroepithelial cells used were taken from a single pooled sample. The mean attachment values were calculated and compared using analysis of variance methods described by Roscoe (1969). The results are presented in Table 43.

There did not appear to be a significant difference between attachment of the first sample of 10 strains to uroepithelial cells ($p > 0.25$), for the four culture conditions. However, there was a highly significant difference when the results of the second group of ten strains were analysed ($p < 0.001$). This difference

TABLE 43

TWO CULTURE CONDITIONS REPORTED TO AFFECT THE EXPRESSION OF A MRHA FOR E.COLI STRAINS:

HAEMAGGLUTINATION PROPERTIES AND ATTACHMENT TO UROEPITHELIAL CELLS FOR E.COLI STRAINS GROWN UNDER THESE TWO CONDITIONS COMPARED WITH RESULTS OBTAINED AFTER STANDARD GROWTH IN BHI BROTH AND ON TSA.

STRAIN	STANDARD BHI BROTH GROWN CULTURES			STANDARD TSA GROWN CULTURES			5 cm^3 BHI BROTH IN 100 cm^3 SUBCULTURED 37°C, 48 HRS			TSA CULTURES 18°C, 8 HRS		
	A (i)	A (ii)	B	A (i)	A (ii)	B	A (i)	A (ii)	B	A (i)	A (ii)	B
43	-	-	5	-	-	4	-	MSHA	5	-	-	4
61	-	-	6	-	-	6	-	-	10	-	-	7
66	-	-	9	-	-	12	MSHA±	MSHA	11	-	-	12
74	-	-	5	-	-	4	-	-	3	-	-	5
87	-	MSHA	14	-	-	16	-	MSHA	11	-	-	13
132	-	-	8	-	-	8	-	MSHA	6	-	-	8
190	-	MSHA	10	-	MSHA	7	-	MSHA	7	-	MSHA	8
231	-	MSHA	13	-	MSHA	8	MSHA±	MSHA	7	-	MSHA	8
242	-	-	8	-	-	10	-	MSHA	5	-	-	9
288	-	MSHA	7	-	-	4	-	MSHA	6	-	-	4
			$\bar{M} = 8.5$			$\bar{M} = 7.9$			$\bar{M} = 7.1$			$\bar{M} = 7.8$
89	-	-	8	MRHA	-	54	MRHA±	MSHA	13	-	-	9
104	MRHA	-	10	MRHA	-	51	MRHA	MSHA	30	-	-	8
122	-	-	11	MRHA	-	50	MRHA±	MSHA	20	-	-	10
125	-	-	9	MRHA	-	57	MRHA±	MSHA±	11	-	-	11
129	-	-	13	MRHA	-	39	MRHA±	MSHA	15	-	-	13
159	-	-	9	MRHA	-	49	MRHA±	MSHA	14	-	-	9
178	-	-	7	MRHA	-	59	MRHA±	MSHA	14	-	-	9
207	-	-	8	MRHA	-	58	MRHA	MSHA	14	-	-	8
284	-	-	16	MRHA±	-	57	MRHA±	-	10	-	-	13
289	MRHA±	-	17	MRHA	-	69	MRHA	MSHA	13	-	-	10
			$\bar{M} = 10.8$			$\bar{M} = 56.3$			$\bar{M} = 15.3$			$\bar{M} = 10.0$

A = Haemagglutination of (i) Human erythrocytes (ii) Guinea Pig erythrocytes

B = Mean E.coli per Uroepithelial Cell.

$\bar{M}$ = Mean

was apparently due to high attachment to the uroepithelial cells of strains grown on TSA expressing a MRHA. Indeed, there was also a signficant difference when the results from the first, third and fourth columns were analysed ($p < 0.025$). This was due to the higher attachment values obtained after subculturing the organisms twice in 5 cm^3 BHI broth in 100 cm^3 for 48 hours at 37°C. However, this cultural condition did not give rise to as high attachment levels as those obtained after growth on TSA ($p < 0.001$). When the results from growth in standard BHI broth were compared to those from TSA cultures at 18°C, there was no evidence of a difference ($p > 0.25$).

The results from the haemagglutination tests proved to be of interest. For the first group of ten strains the presence or absence of a MSHA did not affect attachment, though growth in 5 cm^3 BHI broth in 100 cm^3 flask appeared to be appropriate for expression of a MSHA. In the second group of strains both a MSHA and a MRHA were expressed after growth under these conditions, in 9 of the 10 strains. By comparing the second group of results, it appeared that growth in standard BHI broth did not encourage haemagglutinin production; growth in TSA at 37°C encouraged MRHA expression and this appeared to have been repressed or inhibited after growth on TSA at 18°C. These results support the hypothesis growth on TSA at 37°C is appropriate for the sole expression of a MRHA, and that this results in enhanced attachment to uroepithelial cells.

4.5 ATTACHMENT OF *E.COLI* URINARY STRAINS TO BUCCAL EPITHELIAL CELLS.

Two experiments were undertaken to investigate the attachment of *E.coli* urinary strains to buccal epithelial cells.

The buccal cells were collected by rubbing a sterile cotton wool swab against the cheeks of the buccal cavity. The swab was

immersed in a tube containing 5 cm^3 PBS, pH 7.1, and the buccal cells resuspended by shaking the swab. The cells were then washed three times in PBS and resuspended to a concentration of 1×10^5 cells per 0.5 cm^3 PBS. In the first experiment, cells were harvested from three individuals and separately assayed for attachment with 10 E.coli urinary strains, grown in BHI broth or on TSA, as in section 1.1.

The results are presented in Table 44. Statistical analysis using two-way variance showed that overall, there was no significant difference between the mean attachment values obtained with cells from 3 individuals: $p > 0.05$ for strains 49 to 67 and $p > 0.1$ for strains 122 to 178. However, there were small differences between individuals with strains 67, 122 and 207. The haemagglutination properties of each strain are also presented and illustrate that strains grown on TSA and possessing a MRHA attached significantly better to buccal epithelial cells than strains possessing a MSHA.

In the second experiment, buccal cells were pooled together from three individuals. Twenty E.coli urinary strains were assayed for attachment to these buccal cells and simultaneously assayed with uroepithelial cells harvested from pooled urine samples, using the method in section 1.1. The haemagglutination test described in section 3.1 was used to confirm the presence of bacterial haemagglutinins.

Table 45 presents the results of this experiment. The first set of 10 strains did not express a MRHA and attachment to buccal cells did not differ significantly from that to uroepithelial cells, after analysis by two-way variance ($p < 0.1$). However, attachment to buccal cells was marginally lower than attachment to uroepithelial cells.

For strains grown on TSA and expressing a MRHA, attachment was significantly higher to uroepithelial cells than to buccal

TABLE 44

ATTACHMENT OF E.COLI URINARY STRAINS TO BUCCAL EPITHELIAL CELLS FROM THREE INDIVIDUALS.

STRAIN	GROWTH CONDITION	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER OF E.COLI PER BUCCAL EPITHELIAL CELL: CELLS FROM INDIVIDUAL 1	CELLS FROM INDIVIDUAL 2	CELLS FROM INDIVIDUAL 3
49	BROTH	-	MSHA	4	5	4
87	BROTH	-	MSHA	4	5	4
130	TSA	-	MSHA	7	3	4
130	BROTH	-	MSHA	9	4	5
67	TSA	-	MSHA	9	3	4
				$\bar{M} = 6.6$	$\bar{M} = 4.0$	$M = 4.2$
122	TSA	MRHA	-	13	24	17
104	TSA	MRHA	-	14	19	17
289	TSA	MRHA	-	14	15	11
207	TSA	MRHA	-	23	34	36
178	TSA	MRHA	-	24	21	18
				$\bar{M} = 17.6$	$\bar{M} = 22.6$	$\bar{M} = 19.8$

TABLE 45

COMPARISON OF ATTACHMENT OF E.COLI URINARY STRAINS TO BUCCAL AND UROEPITHELIAL CELLS.

STRAIN	GROWTH CONDITION	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER EPITHELIAL CELL: BUCCAL CELLS	MEAN NUMBER E.COLI PER EPITHELIAL CELL: UROEPITHELIAL CELLS
43	BROTH	-	-	5	5
49	BROTH	-	MSHA	4	11
61	BROTH	-	-	4	6
66	BROTH	-	-	12	9
67	TSA	-	MSHA	5	5
74	BROTH	-	-	5	5
87	BROTH	-	MSHA	4	14
130	TSA	-	MSHA	5	7
130	BROTH	-	MSHA	6	7
132	BROTH	-	-	3	8
				$\bar{M} = 5.3$	$\bar{M} = 7.7$
				$p < 0.1$	
89	TSA	MRHA	-	20	54
104	TSA	MRHA	-	17	51
122	TSA	MRHA	-	18	70
125	TSA	MRHA	-	45	57
129	TSA	MRHA	-	19	39
159	TSA	MRHA	-	33	49
178	TSA	MRHA	-	21	59
207	TSA	MRHA	-	31	58
284	TSA	MRHA±	-	15	57
289	TSA	MRHA	-	13	69
				$\bar{M} = 23.2$	$\bar{M} = 56.3$

$p < 0.001$

TABLE 46

FREQUENCY DISTRIBUTION OF E.COLI STRAIN 207 GROWN ON TSA, ATTACHED TO 100 VIABLE BUCCAL EPITHELIAL CELLS AND 100 VIABLE UROEPITHELIAL CELLS.

RANGE OF E.COLI ATTACHED PER EPITHELIAL CELL	NUMBER OF BUCCAL EPITHELIAL CELLS	NUMBER OF UROEPITHELIAL CELLS
0	0	0
1 to 20	62	7
21 to 50	24	39
> 50	14	54

$p < 0.001$

epithelial cells ($p < 0.001$). Nevertheless, attachment to buccal cells was highest for the group of strains possessing a MRHA, rather than the strains possessing a MSHA or no haemagglutinin.

Table 46 presents the frequency distributions of E.coli strain 207 grown on TSA and expressing a MRHA, attached to 100 viable buccal cells and 100 viable uroepithelial cells. There was a highly significant difference between the distributions after analysis using a chi-squared method, reported by Roscoe (1969). There were substantially more uroepithelial target cells (54%) than buccal target cells (14%) to which large numbers of E.coli attached (> 50 E.coli per cell). These experiments indicated that E.coli have the capacity to attach to epithelial cells of different origin. However, the attachment of urinary strains appeared to be more specific for uroepithelial cells than for buccal epithelial cells.

Finally, buccal cells from several samples were double-stained with trypan blue and PAS reagent, and with erythrosine B and alcian blue to determine the presence of mucopolysaccharides on viable and nonviable cells, using the method described in section 2.2. In total, 100 viable and 100 nonviable cells were assessed for the presence of a mucus coat. Of the buccal cell population, 30% of the cells were viable, and of these, only 15% stained with PAS reagent and 10% stained with alcian blue. Of the nonviable cells, only 5% stained with PAS reagent and 5% with alcian blue. It was evident that most of the buccal epithelial cells harvested from the mouth and used in these investigations, were not coated with mucopolysaccharides.

4.6 ALTERNATIVE HAEMAGGLUTINATION TESTS USED TO DETECT THE MRHA OF E.COLI ISOLATES.

A further five haemagglutination test systems were used to

verify the results from earlier studies. Each system was used to detect the haemagglutination pattern of 43 E.coli isolates, following growth on TSA plates.

METHOD A This was similar to that described by Duguid et al. (1955) and Scott and Old (1981). One Pasteur pipette drop of bacterial suspension (10^{11} organisms per cm^3 in PBS) was added to the wells of a white porcelain tile, with one drop of human 0 Rhesus positive erythrocytes (0.3% v/v in PBS) and one drop of PBS with or without α D mannose (0.5% w/v in PBS).

Haemagglutination was noted after 15 minutes at 4°C on ice, then 30 minutes at 20°C and after 60 minutes at 50°C.

METHOD B This method was similar to that described by Jones and Rutter (1974) and Smyth et al. (1978). From an E.coli suspension of 5 x 10^{10} organisms per cm^3 in PBS, 25 µl was removed and added to the well of a plastic microtiter tray with 25 µl PBS human 0 Rhesus positive erythrocytes (3% v/v in PBS) and 25 µl PBS with or without α D mannose (0.5% w/v in PBS). Haemagglutination was read after 1 hour at 4°C, then 1 hour at 20°C.

METHOD C This test system utilised glass microscope slides (Powell and Finkelstein, 1966; Ljungh et al. 1979). From an E.coli suspension of 10^{10} organisms per cm^3 in PBS, one Pasteur pipette drop was added to a slide with one drop of human A Rhesus positive erythrocytes (3% v/v in PBS) with or without α D mannose (1% in PBS). The results were noted after gently rocking the slide at room temperature for several minutes then placing the slide on ice at 4°C for 1 hour.

METHOD D White porcelain tiles were again used in a method described by Duguid et al. (1979). From an E.coli suspension of 10^{12} organisms per cm^3 in PBS, 50 µl was removed and added to a well

with 50 µl of human 0 Rhesus positive erythrocytes (3% in PBS), with or without α D mannose (2% in PBS). Haemagglutination was read at 20°C room temperature then after 15 minutes at 4°C on ice.

METHOD E The method described by Van Den Bosch (1980) was used; 100 µl of bacterial suspension (10^9 organisms per cm^3 in PBS)and 100 µl of human A Rhesus positive erythrocytes (0.5% v/v in PBS) were added to a well on a Disposo-tray with or without α D mannose (1% w/v in PBS). The tray was gently agitated on an Adam's Nutator for 30 minutes at 37°C. Haemagglutination was read at this point and again after the tray was left overnight at 4°C.

All the tests were carried out on one day, with haemagglutination being recorded as described in section 3.1. Control strains were grown on TSA and consisted of strain 322 known to possess a MRHA, strain 172 which possessed a MSHA and strain 43 which did not possess a MRHA or a MSHA. In addition, each test contained a control with PBS, erythrocytes and no E.coli added to ensure that any haemagglutination observed was not due to the buffer solution or to the erythrocytes exhibiting autoagglutination.

A. STRAINS WHICH EXPRESSED A MRHA UNDER THE STANDARD TEST CONDITIONS.

The results given in Table 47 indicate that a MRHA in the 22 E.coli strains under test could be detected by any of the five alternative haemagglutination methods. A MRHA was detected for every strain after a period of time at 4°C or 37°C, and in most cases at 22°C, with exceptions for methods C and D where some strains gave a negative or a weak mannose resistant haemagglutination. The elution of a MRHA at 50°C was evident in all but three strains, as noted under method A results. The MRHA of strains 104, 153 and

TABLE 47

ALTERNATIVE HAEMAGGLUTINATION TESTS FOR DETECTION OF THE MRHA.

A. STRAINS WHICH EXPRESSED A MRHA UNDER THE STANDARD TEST CONDITIONS.

HAEMAGGLUTINATION WITH HUMAN A OR O ERYTHROCYTES

STRAINS	METHOD A			METHOD B		METHOD C		METHOD D		METHOD E	
	4°C	20°C	50°C	4°C	20°C	20°C	4°C	20°C	4°C	37°C	4°C
40	MRHA	MRHA	-	MRHA	MRHA	MRHA±	MRHA	-	MRHA±	MRHA±	MRHA
63	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA
73	MRHA±	MRHA±	-	MRHA	MRHA	MRHA±	MRHA	-	MRHA±	MRHA	MRHA
86	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA	MRHA±	MRHA	MRHA	MRHA
89	MRHA	MRHA	-	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA±	MRHA
104	MRHA	MRHA	MRHA±	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA
116	MRHA	MRHA	-	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA	MRHA
122	MRHA	MRHA	-	MRHA	MRHA	-	MRHA	MRHA±	MRHA	MRHA	MRHA
125	MRHA	MRHA	-	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA	MRHA
129	MRHA	MRHA	-	MRHA	MRHA	-	MRHA	MRHA±	MRHA	MRHA	MRHA
146	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA
153	MRHA	MRHA	MRHA±	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA
158	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA
159	MRHA±	MRHA	-	MRHA	MRHA	-	MRHA	MRHA±	MRHA±	MRHA	MRHA
173	MRHA	MRHA	MRHA±	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA
178	MRHA	MRHA	-	MRHA	MRHA	MRHA±	MRHA	MRHA	MRHA	MRHA	MRHA
207	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA	MRHA±	MRHA	MRHA	MRHA
215	MRHA	MRHA	-	MRHA	MRHA	-	MRHA	MRHA±	MRHA	MRHA±	MRHA
232	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA	MRHA
284	MRHA	MRHA	-	MRHA	MRHA	MRHA±	MRHA	MRHA±	MRHA	MRHA	MRHA
289	MRHA	MRHA	-	MRHA	MRHA	MRHA±	MRHA	MRHA	MRHA	MRHA	MRHA
322	MRHA	MRHA	-	MRHA	MRHA	MRHA	MRHA	MRHA±	MRHA	MRHA±	MRHA

173 was weaker after 60 minutes at 50°C.

The control strain 322 expressed a MRHA at 4°C, 22°C and 37°C; strain 172 expressed a MSHA at 4°C, 22°C and 50°C as indicated in Table 48, strain 43 did not express a MRHA or a MSHA as indicated in Table 48, and the control with PBS and no E.coli added gave a negative haemagglutination result.

B. STRAINS WHICH DID NOT EXPRESS A MRHA UNDER THE STANDARD TEST CONDITIONS.

The results of this study are presented in Table 48. From these it is apparent that no MRHA was detected using the five alternative haemagglutination methods, for each of the 21 E.coli strains which had been grown on TSA. This confirmed the results from the standard haemagglutination test, reported in Appendix 1, which showed that none of these strains possessed a MRHA. However, of the 21 strains tested in this experiment, only strain 44, 49, 81 and 172 expressed a MSHA active with human erythrocytes at 4°C, 20°C, 37°C or 50°C. The reaction was weak in most cases.

Using the standard haemagglutination test described earlier 13 of the 21 strains were shown to express a MSHA active with human erythrocytes. However, using the 5 tests described here, a MSHA active with human erythrocytes was only detected with 4 of the 21 strains. The reason for this conflicting result was not apparent. Nevertheless previous results have shown that the presence of a MSHA is detected most efficiently using guinea pig erythrocytes, rather than human erythrocytes.

In conclusion, haemagglutination was easiest to detect when white porcelain tiles were used, and the system described in section 3.1 was found to be appropriate for detection of a MRHA and a MSHA on E.coli strains.

TABLE 48

ALTERNATIVE HAEMAGGLUTINATION TESTS FOR DETECTION OF THE MRHA.

B. STRAINS WHICH DID NOT EXPRESS A MRHA UNDER THE STANDARD TEST CONDITIONS.

STRAINS	HAEMAGGLUTINATION WITH HUMAN A OR O ERYTHROCYTES										
	METHOD A			METHOD B		METHOD C		METHOD D		METHOD E	
	4°C	20°C	50°C	4°C	20°C	20°C	4°C	20°C	4°C	37°C	4°C
33	-	-	-	-	-	-	-	-	-	-	-
42	-	-	-	-	-	-	-	-	-	-	-
43	-	-	-	-	-	-	-	-	-	-	-
44	-	-	-	MSHA±	-	-	-	-	-	-	-
49	-	-	-	-	-	-	-	-	-	MSHA±	-
66	-	-	-	-	-	-	-	-	-	-	-
67	-	-	-	-	-	-	-	-	-	-	-
69	-	-	-	-	-	-	-	-	-	-	-
74	-	-	-	-	-	-	-	-	-	-	-
81	MSHA±	MSHA±	MSHA±	MSHA	MSHA	-	MSHA±	-	MSHA±	-	-
87	-	-	-	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-	-	-	-
130	-	-	-	-	-	-	-	-	-	-	-
132	-	-	-	-	-	-	-	-	-	-	-
172	-	MSHA±	MSHA±	MSHA±	MSHA	-	MSHA±	-	MSHA±	-	-
226	-	-	-	-	-	-	-	-	-	-	-
242	-	-	-	-	-	-	-	-	-	-	-
269	-	-	-	-	-	-	-	-	-	-	-
274	-	-	-	-	-	-	-	-	-	-	-
285	-	-	-	-	-	-	-	-	-	-	-
288	-	-	-	-	-	-	-	-	-	-	-

4.7 EFFECT OF TEMPERATURE, pH AND FORMALDEHYDE TREATMENT ON THE MRHA OF E.COLI ISOLATES.

The following set of experiments were carried out to investigate the properties of a MRHA produced by E.coli urinary isolates. The results are presented in Table 49. All the experiments were carried out on the same day.

I. STANDARD HAEMAGGLUTINATION TEST

Twenty three E.coli strains, grown on TSA were tested for haemagglutination of human group A erythrocytes as described in section 3.1. The results are presented in Table 49. All strains expressed a MRHA, except strain 172 which possessed a MSHA and strain 43 which did not agglutinate the red blood cells.

II. EFFECT OF TEMPERATURE ON THE MRHA

From the same TSA cultures as in part I the E.coli strains were suspended in PBS to a concentration of 10^{10} organisms per cm^3 : 1 cm^3 of each suspension, was placed in a test tube and incubated at 65°C for 1 hour, before the organisms were tested for haemagglutination of human group A erythrocytes as in section 3.1.

The results on Table 49 indicate that MRHA activity was lost on incubating the 23 E.coli suspensions at 65°C for 60 minutes. The MSHA activity of strain 172 was only slightly inhibited by the heat treatment.

The heat treatment resulted in loss of viability of the E.coli as detected by lack of growth of 3 strains cultured on TSA plates on completion of the experiment.

III. EFFECT OF BUFFER pH ON THE MRHA

The 25 E.coli strains as above, were grown on TSA and suspended

TABLE 49

EFFECT OF TEMPERATURE, pH AND FORMALDEHYDE TREATMENT ON THE MRHA OF *E.COLI* ISOLATES.

STRAINS	STANDARD HAEMAGGLUTINATION TEST	65°C,60 MINS.	BUFFER PH 3.0	BUFFER PH 10.1	PBS CONTAINING 0.5% FORMALDEHYDE
40	MRHA	-	-	-	-
63	MRHA	-	-	MRHA	MRHA
73	MRHA	-	MRHA±	-	-
86	MRHA±	-	-	MRHA±	-
89	MRHA	-	-	MRHA±	-
104	MRHA	-	-	MRHA	MRHA
116	MRHA	-	-	MRHA	MRHA±
122	MRHA	-	-	-	-
125	MRHA	-	-	MRHA±	-
129	MRHA	-	-	MRHA±	-
146	MRHA	-	-	MRHA	MRHA±
153	MRHA	-	-	MRHA	MRHA±
158	MRHA	-	-	MRHA	MRHA
159	MRHA	-	-	MRHA±	-
166	MRHA	-	-	MRHA±	-
173	MRHA	-	-	MRHA	MRHA±
178	MRHA	-	-	MRHA±	-
207	MRHA	-	-	MRHA±	-
215	MRHA±	-	-	-	-
232	MRHA	-	-	MRHA	-
284	MRHA±	-	-	MRHA±	-
289	MRHA	-	-	MRHA±	-
322	MRHA	-	MRHA±	MRHA	MRHA
172	MSHA±	MSHA±	-	MSHA	MSHA±
43	-	-	-	-	-

in buffers of pH 3.0 and of pH 10.1 to a concentration of 10^{10} organisms per cm^3. The buffers were made up as described in the materials section. Each strain was incubated in buffer for 30 minutes at 37°C and then for 60 minutes at 37°C in a shaker at 20 r.p.m. to simulate the conditions of the *in vitro* attachment assay described in section 1.1. The E.coli strains were then tested for haemagglutination of human group A erythrocytes as in section 3.1.

The extreme acid pH 3.0 inhibited expression of a MRHA on all but 2 of the strains tested, and for these strains a weaker haemagglutination reaction was recorded. The expression of a MSHA was also inhibited for control strain 172.

The alkaline pH (10.1) appeared to partially inhibit expression of a MRHA with loss of expression in 4 strains and a weaker haemagglutination reaction in 8 strains. The expression of a MSHA in control strain 172 was apparently unaffected by buffer pH 10.1.

Viability of three E.coli strains grown on TSA was not apparently affected by treatment in buffer at pH 3.0 or 10.1.

IV. EFFECT OF FORMALDEHYDE TREATMENT ON THE MRHA.

From the same 25 TSA cultures as previously used, E.coli were suspended to a concentration of 10^{10} organisms per cm^3 in PBS containing 0.5% formaldehyde, and incubated at 37°C for 4 hours. The haemagglutination test described in section 3.1 was carried out to determine the effect of formaldehyde treatment on the MRHA.

The results in Table 49 indicate that formaldehyde inhibited expression of a MRHA in 15 E.coli strains and partially inhibited expression of a MRHA in 4 strains. The expression of a MRHA in strains 63, 104, 158 and 322 was not inhibited by formaldehyde

treatment. These strains were then incubated at 37°C for 4 hours in PBS containing 1% formaldehyde. However, expression of a MRHA was still not inhibited. The formaldehyde treatment did not inhibit expression of a MSHA by strain 172. The treatment resulted in a loss of viability of the bacteria as detected by lack of growth of three E.coli strains on TSA.

For all experiments, the control of PBS and erythrocytes without E.coli added, resulted in a negative haemagglutination reaction.

4.8 EFFECT OF FORMALDEHYDE TREATMENT ON THE ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS .

From the previous experiment, part IV section 4.7, it was observed that in some E.coli strains, the expression of a MRHA was affected by the presence of 0.5% formaldehyde. In order to investigate whether formaldehyde treatment of E.coli affected attachment to uroepithelial cells, the following experiment was carried out.

Ten E.coli urinary strains were grown on TSA at 37°C overnight, suspended in PBS, (pH 7.1) and divided into three aliquots. The first and third portions were left untreated at 37°C for 4 hours, while the second was incubated at 37°C for 4 hours in the presence of PBS containing 0.5% formaldehyde. Uroepithelial cells were harvested from pooled urine samples and also divided into three aliquots, with the first and second being left untreated at 37°C for 4 hours while the third portion was incubated at 37°C for 4 hours in the presence of PBS containing 0.5% formaldehyde. After incubation, 1×10^5 uroepithelial cells from samples 1, 2 and 3 were assayed for attachment with 1×10^8 E.coli from the corresponding bacterial samples (1,2 and 3) using the method described in section 1.1. Formaldehyde treated samples were washed once in PBS.

TABLE 50

THE EFFECT OF FORMALDEHYDE ON *E.COLI* ATTACHMENT TO UROEPITHELIAL CELLS.

STRAINS	EFFECT OF PBS CONTAINING 0.5% FORMALDEHYDE ON HAEMAGGLUTINATION OF HUMAN ERYTHROCYTES	MEAN NUMBER OF *E.COLI* PER UROEPITHELIAL CELL		
	HAEMAGGLUTINATION	CELLS UNTREATED *E.COLI* UNTREATED	CELLS UNTREATED *E.COLI* IN PBS CONTAINING 0.5% FORMALDEHYDE	CELLS IN PBS CONTAINING 0.5% FORMALDEHYDE *E.COLI* UNTREATED
73	-	70	27	26
125	-	58	39	33
178	-	49	28	21
207	-	57	38	38
284	-	56	18	24
		MEAN = 58	MEAN = 30	MEAN = 28.4
104	MRHA	47	15	15
153	MRHA±	33	16	11
158	MRHA	39	19	16
173	MRHA±	36	24	21
322	MRHA	31	23	23
		MEAN = 37.2	MEAN = 19.4	MEAN = 17.2

Between Columns $p < 0.001$.

The mean number of E.coli attached to 40 viable uroepithelial cells was determined. The results from samples 1, 2 and 3 were compared using analysis of variance, described by Roscoe (1969). The results are presented in Table 50. There was a decrease in the number of E.coli attached to uroepithelial cells after treatment of either the bacteria or the cells with PBS containing 0.5% formaldehyde. This decreased attachment was evident for strains whose MRHA was either sensitive or relatively insensitive to formaldehyde treatment. For the first group of five strains, the net decrease in attachment was 51% while that of the second group of strains was 54% when compared with untreated preparations.

Two-way analysis of variance confirmed that there was a highly significant difference between the three columns in Table 50. ($p < 0.001$). Further analysis comparing the results from treated preparations suggested that attachment of E.coli to uroepithelial cells was equally inhibited whether bacteria or uroepithelial cells were treated in formaldehyde ($p > 0.1$).

It is possible that this inhibition of attachment is not directly related to possession of a MRHA. After formaldehyde treatment, the attachment of strains 125, 178, 207 and 284 was approximately three times as high as when the same strains were grown on TSA at 18^{o}C (see Table 43), although in both instances haemagglutination was totally inhibited. Had the formaldehyde treatment specifically inhibited expression of a MRHA, it may have been expected that attachment levels would have been as low as those for cultures grown at 18^{o}C.

4.9 TITRATION AND INHIBITION OF THE MRHA.

4.9.1 TITRATION OF THE MRHA.

The MRHA titre was determined for 12 E.coli urinary strains

which had previously given strong to weak haemagglutination reactions and which had attached to uroepithelial cells to varying degrees. Each strain was grown on TSA at 37°C for 16 hours and suspended to a concentration of 10^{10} organisms per cm^3 in PBS with or without 1% α D mannose. These suspensions were subjected to a series of twofold dilutions in PBS with or without 1% α D mannose. The haemagglutination test was then carried out using human group A Rhesus positive erythrocytes as in section 3.1. The highest dilution which gave a positive haemagglutination result was taken as the end point.

The end points for titration of MRHA in 12 E.coli strains are reported in Table 51. There did not appear to be a correlation between MRHA titre and attachment of the E.coli strains to uroepithelial cells. Five strains with a reciprocal titre of $\geq$ 16 gave a mean attachment value of 51.2 E.coli per uroepithelial cell whereas the seven strains with a lower titre gave a net mean of 34.6 E.coli per cell. However, this difference was not found to be statistically significant after the results of the 12 strains were compared using one-way analysis of variance ($p > 0.1$). The two strains 284 and 215 which gave weak haemagglutination reactions also had a low reciprocal titre of 4 and 8. However, their mean attachment values were 57 and 54 E.coli per uroepithelial cell.

A reciprocal titre of 32 represented approximately 1.6×10^7 E.coli and a reciprocal titre of 4 represented approximately 1.25×10^8 E.coli. Of all the E.coli urinary strains tested throughout this study only 9 strains gave weak MRHA reactions and of these, 7 were tested for attachment to uroepithelial cells. The mean results as reported in Appendix 1, ranged from 13 to 57 E.coli per cell while the mean attachment results for strains expressing a strong MRHA reaction was 15 to 82 E.coli per cell. Therefore, the strength of the haemagglutination reaction for any one strain

TABLE 51

TITRATION OF THE MRHA

STRAINS	MEAN NUMBER OF *E.COLI* PER UROEPITHELIAL CELL	HAEMAGGLUTINATION HUMAN A ERYTHROCYTES	RECIPROCAL HAEMAGGLUTINATION TITRE
301	70	MRHA	32
289	69	MRHA	16
207	58	MRHA	8
284	57	MRHA±	4
215	54	MRHA±	8
63	44	MRHA	16
129	39	MRHA	16
322	34	MRHA	32
83	21	MRHA	4
27	19	MRHA	8
278	18	MRHA	4
300	15	MRHA	8

could not be directly related to the ability of E.coli to attach to uroepithelial cells.

4.9.2 INHIBITION OF THE MRHA.

This experiment was designed to investigate inhibition of the MRHA expressed by 7 E.coli urinary strains.

Thirty one compounds were tested for inhibition of the haemagglutination reaction and these included the following: (a) the carbohydrates arabinose, xylose, rhamnose, mannose, glucose, fructose, galactose, sucrose, maltose, lactose, raffinose, mannitol, dulcitol, sorbitol and salicin which are known to be variously fermented by E.coli ; (b) Ribose, galactosamine, glucosamine and fucose, which are found in the outer membrane lipopolysaccharide layer of enterobacteriaceae; (c) the lectin concanavalon A, which forms a precipitate with α D mannose and its derivatives ; (d) Neuraminic acid, which is the basic component of the K1 capsular antigen possessed by certain E.coli strains; (e) glucuronic acid, which is a component of the K5 capsular antigen possessed by certain E.coli strains. Hexuronic acids in general are a major constituent of human urinary glycosaminoglycans, which also contain serine, leucine and alanine (Endo, et al. 1980,a,b,) All these compounds were thus chosen because of their relationship with the cell structure of E.coli or because of their relationship with compounds found in the urinary tract. Five other compounds were added to the list: namely Kojibiose, Melibiose, Melezitose, N-acetyl-D-glucosamine and the glycoside of N-acetyl-D-galactosamine.

Each compound was suspended to a concentration of 1% or 2% w/v in PBS (pH 7.1). The two exceptions were galacturonic acid and D-glucuronic acid which gave a net pH of 3.0 in standard PBS, and were therefore suspended in Tris-HCl buffer to give a net pH 7.2.

Seven E.coli urinary strains were selected to represent 0 serotypes commonly found in urinary tract infections. These included strains with 0 serotypes 1, 2, 4, 6, 7 and 75, with an additional 07:K1 strain which was included as the K1 antigen is known to be composed of neuraminic acid residues (Stevens et al, 1980), and this compound was one of the 31 tested.

Each E.coli strain was suspended to a concentration of 10^{10} organisms per cm^3 in PBS (pH 7.1) and dispensed into 32 wells of porcelain tiles. To one well was added 50 µl PBS, to a second well was added 50 µl PBS containing 1% α D mannose and to the other wells was added 50 µl of each compound being tested. The haemagglutination test was carried out using human A Rhesus positive erythrocytes as described in section 3.1. A positive haemagglutination reaction was termed HA; a weakly positive reaction was termed HA±; and a negative reaction termed -ve.

The results are presented in Table 52. With only two exceptions, none of the compounds appeared to inhibit the expression of the MRHA for the seven E.coli strains tested. The exceptions were galacturonic acid and D-glucuronic acid which partially inhibited the haemagglutination of strains 159 and 207. A further 20 E.coli strains which expressed a MRHA after growth on TSA, were tested for inhibition of the haemagglutination reaction in the presence of these two glycuronic acids. Only the expression of a MRHA of strains 40 and 125 was partially inhibited. The significance of this finding has not been established.

In one additional test, 0.5 cm^3 of the mannose, rhamnose, galactose and glucosamine solutions were added to a test tube and mixed together. Then 50 µl of this solution was added to 50 µl of the suspension containing strain 207 (serotype 075:H-) and 50 µl of human erythrocytes on a porcelain tile. The haemagglutination test was carried out as in section 3.1, to determine whether these

four sugars, known to be present in the neutral 0 serotype polysaccharide layer of strains with an 075 serotype, inhibited the MRHA expression. However, the haemagglutination was unaffected by the presence of these four carbohydrate compounds.

TABLE 52

INHIBITION OF THE MRHA

COMPOUNDS TESTED	CONCENTRATION	HAEMAGGLUTINATION RESULTS WITH HUMAN A ERYTHROCYTES: E.COLI STRAINS AND SEROTYPE						
		207 O75:H-	322 O6:K2:H1	320 O2:KNT:H4	158 O4:H5	289 O1:H7	159 O7:HNT	153 O7:K1:H-
α D Mannose	1%	HA	HA	HA	HA	HA	HA	HA
Mannitol	2%	HA	HA	HA	HA	HA	HA	HA
Concanavalon A-Lectin	2%	HA	HA	HA	HA	HA	HA	HA
D Galactose	2%	HA	HA	HA	HA	HA	HA	HA
L Arabinose	2%	HA	HA	HA	HA	HA	HA	HA
α D Fucose	2%	HA	HA	HA	HA	HA	HA	HA
D Xylose	2%	HA	HA	HA	HA	HA	HA	HA
D Ribose	2%	HA	HA	HA	HA	HA	HA	HA
Rhamnose	2%	HA	HA	HA	HA	HA	HA	HA
α D Raffinose	2%	HA	HA	HA	HA	HA	HA	HA
Dulcitol	2%	HA	HA	HA	HA	HA	HA	HA
Salicin	2%	HA	HA	HA	HA	HA	HA	HA
Sorbitol	2%	HA	HA	HA	HA	HA	HA	HA
Galacturonic acid	2%	HA±	HA	HA	HA	HA	HA±	HA
D-Glucuronic acid	2%	HA±	HA	HA	HA	HA	HA±	HA
α D Glucosamine	2%	HA	HA	HA	HA	HA	HA	HA
D Galactosamine	1%	HA	HA	HA	HA	HA	HA	HA
N-Acetyl-D-Glucosamine	2%	HA	HA	HA	HA	HA	HA	HA
N-Acetyl-D-Galactosamine Glycoside	1%	HA	HA	HA	HA	HA	HA	HA
N-Acetyl-Neuraminic acid	1%	HA	HA	HA	HA	HA	HA	HA

TABLE 52 Cont'd

COMPOUNDS TESTED	CONCENTRATION	HAEMAGGLUTINATION RESULTS WITH HUMAN A ERYTHROCYTES: E.COLI STRAINS AND SEROTYPE						
		207 075:H-	322 06:K2:H1	320 02:KNT:H4	158 04:H5	289 01:H7	159 07:HNT	153 07:K1:H-
D Fructose	2%	HA	HA	HA	HA	HA	HA	HA
Glucose	2%	HA	HA	HA	HA	HA	HA	HA
Sucrose	2%	HA	HA	HA	HA	HA	HA	HA
α-D-Kojibiose	2%	HA	HA	HA	HA	HA	HA	HA
α-D-Melibiose	2%	HA	HA	HA	HA	HA	HA	HA
Lactose	2%	HA	HA	HA	HA	HA	HA	HA
D-Melezitose	2%	HA	HA	HA	HA	HA	HA	HA
D Maltose	2%	HA	HA	HA	HA	HA	HA	HA
L-Alanine	2%	HA	HA	HA	HA	HA	HA	HA
L-Leucine	2%	HA	HA	HA	HA	HA	HA	HA
DL-Serine	2%	HA	HA	HA	HA	HA	HA	HA

HA = Haemagglutination

HA± = Weak Reaction

RESULTS

SECTION 5.

THE USE OF ELECTRON MICROSCOPY TO INVESTIGATE ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS

Refer to methods section 4 for the details concerning these experiments.

5.1 THE USE OF A NEGATIVE STAINING TECHNIQUE FOR DETECTION OF PILI ON E.COLI CELLS.

All results presented in this study were averages of duplicate tests carried out for each E.coli strain. The results presented in Tables 53 to 55 relate the presence of pili to the results reported in Appendix 1 for attachment of E.coli to uroepithelial cells and the haemagglutination properties of these strains after growth on TSA.

The numbers of pili per cell were counted to an approximate value and were seen to differ for bacterial cells within any given preparation. Some bacteria were highly piliated while others in the preparation had only small numbers of pili per cell. The overall mean values are presented in Tables 53 to 55. (See also Plates 20 to 25).

(1) The results of the first experiment are presented in Table 53. All the strains expressed a MRHA after growth on TSA, and of these 38% were well-piliated (> 70% piliation), 33% were poorly piliated (1 to 50% piliation) and 29% were non-piliated. There was no apparent correlation between possession of a MRHA and presence of pili. In addition there was no apparent correlation between the possession of pili and the degree

TABLE 53

THE USE OF A NEGATIVE STAINING TECHNIQUE FOR DETECTION OF PILI ON E.COLI CELLS:

EXPERIMENT 1.

STRAIN	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	GUINEA PIG	% PILIATED	AVERAGE NUMBER OF PILI PER CELL
63	44	MRHA	-	100	64
104	51	MRHA	-	100	38
232	28	MRHA	-	100	25
322	34	MRHA	-	100	96
116	36	MRHA	-	96	16
158	30	MRHA	-	92	42
321	23	MRHA	-	84	64
159	49	MRHA	-	84	16
320	25	MRHA	-	72	43
125	57	MRHA	-	36	47
146	33	MRHA	-	14	15
40	35	MRHA	-	12	23
122	70	MRHA	-	8	14
129	39	MRHA	-	8	13
86	29	MRHA±	-	4	10
153	39	MRHA	-	4	20
313	62	MRHA	-	4	30
89	54	MRHA	-	0	0
166	26	MRHA	-	0	0
178	59	MRHA	-	0	0
207	58	MRHA	-	0	0
284	57	MRHA±	-	0	0
289	69	MRHA	-	0	0
301	70	MRHA	-	0	0

to which any strain attached to uroepithelial cells. The net mean attachment value for well-piliated strains was 35 E.coli per uroepithelial cell; 45.5 E.coli per cell for poorly piliated strains, and 56.1 E.coli per cell for non-piliated strains. One-way analysis of variance did not show up any significance between these mean values for the 24 strains tested ($p > 0.1$).

The nature of the pili present on the E.coli cells was not fully established, though haemagglutination tests did not detect a MSHA which is indicative of the presence of type 1 pili reported to be 7 nm in diameter by Brinton (1965) and Ørskov et al. (1980,b). Electron-microscope observations of 10 strains, suggested that the pili were approximately 6 nm in diameter, and up to 2 and 3 μm in length.

(2) The results of the second experiment are presented in Table 54. The non-haemagglutinating strains tested were all non-piliated, with the exception of strain 74 which had only 4% piliation. The six strains expressing a MSHA, after a single subculture in 10 cm^3 BHI broth, were highly piliated, though mean attachment to uroepithelial cells was only between 3 and 14 E.coli per cell.

When the results presented in Tables 53 and 54 were compared for strains 63, 104, 116 and 158, the following points were noted: (i) strain 158 grown in 10 cm^3 BHI broth was 62% piliated, yet after growth on TSA, this strain was 92% piliated. In both cases only a MRHA was detected and 44 and 42 pili per cell, respectively were evident. (ii) strain 104 grown in 10 cm^3 BHI broth was 78% piliated but was 100% piliated after growth on TSA. In both cases only a MRHA was detected and 35 and 38 pili per cell respectively, were evident. (iii) when strain 63 was grown on TSA, only a MRHA was produced and the cultures were 100% piliated. However, when grown in 10 cm^3 BHI broth, strain 63 produced a MRHA and a MSHA,

TABLE 54

THE USE OF A NEGATIVE STAINING TECHNIQUE FOR DETECTION OF PILI ON E.COLI CELLS:

EXPERIMENT 2.

STRAINS	MEDIA	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		% PILIATED	AVERAGE NUMBER OF PILI PER CELL
			HUMAN	GUINEA PIG		
43	TSA	4	-	-	0	0
66	TSA	12	-	-	0	0
132	TSA	8	-	-	0	0
242	TSA	10	-	-	0	0
74	TSA	4	-	-	4	28
87	BROTH	14	-	MSHA	96	83
323	BROTH	3	-	MSHA	92	43
49	BROTH	11	-	MSHA	80	68
130	BROTH	7	-	MSHA	80	59
67	BROTH	12	-	MSHA	79	46
232	BROTH	6	-	MSHA±	84	35
158	BROTH	10	MRHA	-	62	44
104	BROTH	10	MRHA	-	78	35
63	BROTH	17	MRHA	MSHA	75	51
116	BROTH	10	-	-	18	9

yet was only 75% piliated. The reason for this reduction in piliation could not be explained from the results presented, (iv) when strain 116 was grown on TSA, only a MRHA was expressed and 96% piliation was evident. However, after growth in 10 cm^3 BHI broth, no haemagglutinin was detected for strain 116, and piliation dropped to only 18%. This suggested that the pili present after growth on TSA at 37°C, may be associated with the expression of a MRHA.

(3) The results for detection of pili on cultures grown at 18°C on TSA are presented in Table 55, and are compared to previous results for each strain cultured at 37°C on TSA. The mean number of E.coli attached to 40 viable uroepithelial cells is also presented along with the haemagglutination properties of each strain reported in Appendix 1.

For the first five strains it was evident that growth at 18°C on TSA resulted in decreased piliation, fewer pili per cell, as well as loss of expression of a MRHA and decreased attachment to uroepithelial cells, compared to cultures grown at 37°C. The second group of strains did not produce pili at 18°C or 37°C. However, no MRHA was expressed after 18°C growth and decreased attachment to uroepithelial cells was noted compared to that obtained after growth on TSA at 37°C. Of the five strains which expressed a MSHA on TSA at 37°C, only strain 67 produced a weak reaction for a MSHA after growth at 18°C, with a 21% decrease in piliation of the cells. The other four strains did not produce a MSHA at 18°C, and gave piliation of between 0 and 36%, compared to 52 and 80% at 37°C. In addition there were fewer pili per cell recorded at the lower temperature of growth for these four strains. Attachment to uroepithelial cells was low whether the strains were grown at 18° or 37°C.

TABLE 55

THE USE OF A NEGATIVE STAINING TECHNIQUE FOR DETECTION OF PILI ON E.COLI CELLS:

CULTURES GROWN AT 37°C ON TSA.

EXPERIMENT 3.

STRAIN	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	% PILIATED	AVERAGE NUMBER OF PILI PER CELL
63	44	MRHA	-	100	64
104	51	MRHA	-	100	38
322	34	MRHA	-	100	96
116	36	MRHA	-	96	16
232	28	MRHA	-	100	25
	$\bar{M}$ = 38.6			$\bar{M}$ = 99.2	$\bar{M}$ = 47.8
207	58	MRHA	-	0	0
178	59	MRHA	-	0	0
89	54	MRHA	-	0	0
166	26	MRHA	-	0	0
284	57	MRHA±	-	0	0
	$\bar{M}$ = 50.8			$\bar{M}$ = 0	$\bar{M}$ = 0
42	7	-	MSHA	80	34
44	6	-	MSHA	70	36
67	5	-	MSHA	76	49
69	4	-	MSHA	52	36
130	7	-	MSHA	74	45
	$\bar{M}$ = 5.8			$\bar{M}$ = 70.4	$\bar{M}$ = 40
43	4	-	-	0	0
66	12	-	-	0	0
74	4	-	-	4	28
132	8	-	-	0	0
242	10	-	-	0	0
	$\bar{M}$ = 7.6			$\bar{M}$ = 0.8	$\bar{M}$ = 5.6

TABLE 55 Cont'd

CULTURES GROWN AT 18°C ON TSA

STRAIN	MEAN NUMBER OF E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES		% PILIATED	AVERAGE NUMBER OF PILI PER CELL
		HUMAN	GUINEA PIG		
63	7	-	-	12	12
104	8	-	-	22	12
322	5	-	-	48	27
116	6	-	-	68	27
232	5	-	-	16	10
	$\bar{M}$ = 6.2			$\bar{M}$ = 33.2	$\bar{M}$ = 16.8
207	8	-	-	0	0
178	9	-	-	0	0
89	9	-	-	0	0
166	6	-	-	0	0
284	13	-	-	0	0
	$\bar{M}$ = 9			$\bar{M}$ = 0	$\bar{M}$ = 0
42	7	-	-	0	0
44	1	-	-	26	22
67	6	-	MSHA±	60	31
69	4	-	-	0	0
130	6	-	-	36	33
	$\bar{M}$ = 4.8			$\bar{M}$ = 24.4	$\bar{M}$ = 17.2
43	4	-	-	4	20
66	12	-	-	0	0
74	5	-	-	4	20
132	8	-	-	0	0
242	9	-	-	0	0
	$\bar{M}$ = 7.6			$\bar{M}$ = 1.6	$\bar{M}$ = 8

$\bar{M}$ = Mean Value

The last group of five strains did not express a MRHA or a MSHA either at 37°C or at 18°C and gave almost identical mean attachment values to uroepithelial cells. The frequency of piliation ranged from 0 to 4%, with the temperature of growth apparently making little difference to the results for each of these strains. The 4% piliation of strains 43 and 74 after growth at 18°C was not thought to be significant.

5.2 THE USE OF TRANSMISSION ELECTRON MICROSCOPY TO STUDY ATTACHMENT OF *E.COLI* TO UROEPITHELIAL CELLS.

The sectioned material was examined successfully under the transmission electron microscope. In the first experiment, ruthenium red stain was not applied to the preparations. Uroepithelial cells which had been incubated in the absence of bacteria or in the presence of *E.coli* strain 67, were examined. Examination of these specimens showed rod-shaped and coccal-shaped bacteria close to the uroepithelial cell surface (Plates 26 to 30). There was a gap of approximately 0.03 to 0.2 μm between the bacterial cell and the uroepithelial cell. This gap may be an artefact caused by the preparation of the samples, or it may be due to reasons not yet established. The coccal-shaped bacteria represent the indigenous bacteria present on the uroepithelial cells, and also noted on cells examined by scanning electron microscopy (see later). It cannot be fully ascertained that the rod-shaped bacteria seen were *E.coli* cells. Unless uroepithelial cells free from indigenous bacteria can be assayed with *E.coli*, then examined under the transmission electron microscope, the resultant specimens must be interpreted with caution.

In the ruthenium red stained preparations, the uroepithelial cells were seen to have a thin, uneven layer of electron dense

material around the outer surface. (Plates 31, 33 to 36). This material is comprised of acidic mucopolysaccharides which form a glycocalyx around the cell (Marrie et al. 1980,a). These mucopolysaccharides were only evident on preparations which employed ruthenium red staining. Bacteria of E.coli strain 207 were also seen to be coated with a ruthenium red stained mucus coat (Plate 32). It is possible that the antiserum added to the preparation, stabilised this fibrous matrix around the bacterial cell. Preparations of uroepithelial cells incubated with E.coli strain 207 were also examined. Rod-shaped bacteria were seen attached in some cases to the mucus coat of the uroepithelial cells (Plate 35); in other cases, a gap of approximately 0.03 µm was present between the bacteria and the uroepithelial cell's mucus layer. Similar results were obtained by Marrie et al., (1980,a) who incorporated ruthenium red staining in the examination of bacteria attached to uroepithelial cells. Costerton et al., (1981 a,b) also found similar results for bacteria attached to tissue from the bovine rumen, emphasizing the importance of the host and bacterial cell glycocalyx in the attachment process.

5.3 THE USE OF SCANNING ELECTRON MICROSCOPY TO STUDY ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

Uroepithelial cells were examined by scanning electron microscopy. Most of the cells had microvilli and/or microridge structures on the cell surface (Plates 37 to 42). In control samples without E.coli added, only a few uroepithelial cells had small numbers of rod-shaped and/or coccal-shaped bacteria attached to the surface (Plates 39, 40). Uroepithelial cells incubated with E.coli strains 87 and 207 were seen to have rod-shaped bacteria

attached to the surface (Plates 41 to 43).A proportion of the cells in each of these preparations was covered with large numbers of bacteria: this was particularly evident for cells with E.coli strain 207 attached (Plate 43). In some instances, bacteria appeared to be attached together on the cell surface. These results confirmed the findings obtained from light microscopy examination of uroepithelial cell preparations.

Kjaer et al.,(1976) and Davies and Hunt (1981) have also noted the "cobblestone" appearance of cells from the human uroepithelium following examination by scanning electron microscopy. Colleen et al., (1980) have reported that a proportion of target cells with > 10 bacteria attached to the surface, were present on the urethral mucosa of patients with urinary tract infections. Therefore, the results of the present study are similar to those reported elsewhere.

RESULTS

SECTION 6.

THE USE OF CELL CULTURE TECHNIQUES TO STUDY E.COLI ATTACHMENT.

6.1 ATTACHMENT OF E.COLI TO TISSUE CULTURE VERO CELLS.

The experimental procedure has been outlined in methods section 5.

Counts from 20 monolayer cultures showed that approximately 88% of the vero cells were viable. There were no bacteria evident on vero cells which had been incubated without added E.coli. The results of the attachment assay are presented in Table 56, and are compared to the *in vitro* attachment of these E.coli strains to uroepithelial cells as recorded in Appendix 1. The haemagglutination properties of these strains is also presented. The highest levels of attachment were obtained with E.coli strains expressing a MRHA after growth on TSA. However, the range of values for these strains was between 2 and 23 E.coli per cell, compared to a range of between 0 and 6 E.coli per cell for strains expressing a MSHA, or no haemagglutinin. These results did not correspond to the *in vitro* attachment levels. For example, strain 289 gave a mean attachment of 69 E.coli per uroepithelial cell but only 2 E.coli per vero cell. Strain 249 attached to a greater degree to vero cells with a mean of 16 E.coli per cell. However, this strain only gave a mean of 22 E.coli per uroepithelial cell for the *in vitro* test. Similarly, in both groups, strains which gave high attachment values to uroepithelial cells did not necessarily attach in large numbers to vero cells (Plate 44).

TABLE 56

ATTACHMENT OF E.COLI TO TISSUE CULTURE VERO CELLS.

COMPARISON WITH ATTACHMENT TO UROEPITHELIAL CELLS *IN VITRO*.

STRAIN	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER VERO CELL
322	34	MRHA	-	23
249	22	MRHA	MSHA	16
158	30	MRHA	-	15
153	39	MRHA	-	12
63	44	MRHA	-	11
207	58	MRHA	-	11
104	51	MRHA	-	10
232	28	MRHA	-	10
146	33	MRHA	-	9
116	36	MRHA	-	8
83	21	MRHA	MRHA	7
89	54	MRHA	-	7
27	19	MRHA	-	5
215	54	MRHA±	-	5
40	35	MRHA	-	4
125	57	MRHA	-	4
129	39	MRHA	-	4
178	59	MRHA	-	3
73	82	MRHA	MSHA	2
159	49	MRHA	-	2
289	69	MRHA	-	2
69	4	-	MSHA	6
172	12	MSHA'	MSHA	6
43	4	-	-	4
49	7	-	MSHA	3
33	9	-	-	2
61	6	-	-	2
67	6	-	MSHA	2
87	16	-	-	2
226	8	-	MSHA	2
285	4	-	MSHA	2
290	5	-	MSHA	2
42	7	-	MSHA	1
44	6	-	MSHA	1
74	4	-	-	1
130	7	-	MSHA	1
132	8	-	-	1
233	7	-	MSHA	1
269	7	-	MSHA	1
95	10	-	MSHA	0
242	10	-	-	0
288	4	-	-	0

$\bar{M} = 8.1$ $\bar{M} = 1.9$

A cell culture model would have been useful if high attachment values were obtained and if these corresponded to results from *in vitro* studies using uroepithelial cells. As neither of these characteristics were evident using vero cells as a monolayer, it was decided not to carry out further studies with this model.

6.2 ATTACHMENT OF E.COLI TO TISSUE CULTURE CELLS DERIVED FROM NEW BORN MOUSE KIDNEY.

The cell lines obtained from new born mouse kidneys remained viable with a confluent monolayer of cells for 4 passages. When these cells were assayed for attachment with 7 E.coli strains, the resultant mean attachment values varied from 0 to 2 E.coli per cell. No further assays were carried out due to these low attachment levels.

DISCUSSION

Discussion of the results is divided into two sections. The first deals with the attachment assay and significance of E.coli attachment to uroepithelial cells. The second section discusses the possible importance of bacterial haemagglutinins in the attachment process.

SECTION 1.

1.1 ASSAY METHOD FOR *IN VITRO* ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

The assay method used in this study was a modification of that described by Svanborg Edén et al., (1976). The modifications were incorporated following preliminary studies in which three major difficulties were encountered with the original assay method:

(1) bacterial attachment was not clearly differentiated using only a trypan blue viability stain and phase contrast microscopy;

(2) indigenous, non E.coli-like flora could not be distinguished from E.coli attached to the uroepithelial cells;

(3) no account was taken of E.coli-like organisms which formed part of the normal flora and could be found attached to uroepithelial cells derived from the urethra and periurethral area.

The use of methylene blue was introduced to stain the bacteria attached to viable and nonviable cells, which were differentiated with erythrosine B. In this way, the number of E.coli-like

organisms attached to viable and nonviable uroepithelial cells was readily determined. The inclusion of a control of uroepithelial cells in PBS without added E.coli, enabled the number of E.coli-like indigenous organisms per cell to be determined and a net E.coli attachment value to be obtained. Several double-staining combinations were initially tested. The use of trypan blue and neutral red has recently been reported by Albright et al., (1979). However, the use of erythrosine B and methylene blue provided the most satisfactory combination for the present study. (Plates 1, 2).

Other test systems have been reported elsewhere for assaying the attachment of E.coli to uroepithelial cells, periurethral cells and vaginal epithelial cells. Radioactively-labelled E.coli were used in a number of studies (Schaeffer et al., 1979; Sugarman and Donta, 1979, a; 1979 b; and Parsons et al., 1979,a). The number of attached E.coli were assessed by passing the uroepithelial cell suspensions through a scintillation counter. Parsons et al., (1979,a) preferred this method and suggested that other techniques contained significant sources of error. One criticism was that the visual assessment of bacteria attached to epithelial cells gave rise to large deviations in the counts obtained: some cells had a small amount of bacteria attached, while other cells had extremely large numbers attached. However, observations in the present study support the hypothesis that these variations are due to the presence of target cells within the uroepithelial cell population to which large numbers of E.coli attach. Only by visual assessment, can these cells be detected. Although the use of radiolabelled-bacteria, which allows assessment of up to 50,000 epithelial cells can be advantageous, the technique does not distinguish nonviable cells, broken cells, folded or clumped cells. The importance of assessing viable uroepithelial cells has been established in the present study. Significantly more E.coli attach to viable cells than to nonviable

cells, and a greater proportion of viable cells are coated with mucopolysaccharides. In addition, uroepithelial cell viability for a given cell suspension was not found to be altered by the assay technique. The monitoring of E.coli attached to squamous and transitional cells was also shown to be of importance, and only by visual examination can these cell types be distinguished.

A number of authors have employed membrane filters in their attachment assays (Mardh and Westrom, 1976; Fowler and Stamey, 1977; and Kallenius and Winberg, 1978). Generally, membrane filters are used to remove unattached bacteria from the cell suspension. The epithelial cells are retained on the filter surface, then resuspended in buffer and air dried onto a glass slide. Bacterial stains such as methylene blue, Gram's stain, May-Grunwald/Giemsa stain or safranin have been applied to these preparations, though not in conjunction with cell viability stains. One argument in favour of these techniques has been that by not using centrifugation, the uroepithelial cell structure remains intact. However, in most cases epithelial cells were harvested by centrifugation prior to the assay. A second argument has been that centrifugation on completion of the assay incubation period may either remove attached bacteria, or increase the number of bacteria attached by forming an epithelial cell sediment which has previously unattached bacteria lodged between the cells. From the studies described in section 1.1.11, extensive washing of uroepithelial cells does not greatly alter the number of E.coli attached to uroepithelial cells. It is possible that the suction used in membrane filtration deposits unattached bacteria on the uroepithelial cells rather than removing them through the membrane pores. Recently, Chick et al., (1981), have reported a modified technique to assay bacterial attachment to uroepithelial cells. They employed a toluidine blue and borax staining technique to differentiate urinary mucus from uroepithelial

cells, and claim that E.coli possessing a MSHA adhere to mucus, while strains possessing a MRHA adhere to uroepithelial cells. In the present study, free urinary mucus was carefully removed in the washing of uroepithelial cells prior to assay and the use of toluidine blue and borax is thought to be unnecessary. However, attachment levels for strains possessing a MRHA were higher than for strains possessing a MSHA, and this finding is discussed in section 2.

E.coli attachment to uroepithelial cells was unaffected by the presence of indigenous bacteria on the cell surface. Indigenous organisms could not be removed from the cell surface by vortex mixing, contrary to the findings of Fowler and Stamey (1977). By using a double staining technique, non E.coli-like indigenous bacteria could be differentiated from E.coli-like organisms attached to the uroepithelial cell surface. In some instances, the E.coli-like organisms were attached closely together on the cell and often in association with the non E.coli-like organisms colonizing the cell surface. Results from scanning electron microscopy later confirmed this close association. (Plate 43). Marrie et al.,(1980,a) have investigated uroepithelial cells from the urine of patients with urinary tract infections and reported the presence of bacterial microcolonies attached to the cell surface. They suggested that the infecting bacteria and the normal urethral flora were attached to contiguous areas of the uroepithelial cells. These results appear to be in agreement with those obtained in the present study. The importance of an association between infecting bacteria and indigenous organisms on the cell surface is in need of further study. It is possible that potential uropathogens adhere to indigenous organisms present on urethral and periurethral cells, prior to infecting the bladder. The inclusion of a control in every experiment, consisting of uroepithelial cells without added E.coli, ensured that the final attachment values reported in the

present study were not significantly altered by the presence of indigenous E.coli-like organisms.

1.2 VARIABILITY OF THE ATTACHMENT ASSAY

The results obtained in the present study are compared with those reported by Svanborg Edén (1978) on adherence of E.coli to uroepithelial cells and by Cornelius et al., (1979) on adherence of E.coli to vaginal epithelial cells.

In the present study, E.coli were not washed in buffer prior to assay with uroepithelial cells partly as this step was regarded as unnecessary, and also because the two reports mentioned above had found that washing bacteria decreases their attachment capacity.

After analysis of several standard error of the mean (S.E.M.) values, it was considered that variation of less than 15% occurred for E.coli attachment to 40 uroepithelial cells from any given sample. This variation was higher than the 8.7% variation reported by Svanborg Edén. Her percentage value was only obtained from one set of results (where the mean attachment value was 90 E.coli per uroepithelial cell), whereas the 15% variation obtained in the present study included E.coli strains attaching to uroepithelial cells with varying degree of efficiency. The variation between duplicate samples was not significant, in agreement with the results of Svanborg Edén and Cornelius et al.. However, there was significant variation for E.coli attached to uroepithelial cells collected from different women on the same day, and from the same woman on a number of days. This variation was calculated by Svanborg Edén to be 20% and by Cornelius et al. to be 19%. Rather than merely express such variation as a percentage value, the present report investigated the variability of uroepithelial cell receptivity to E.coli attachment. Experimental results demonstrated a highly significant difference in attachment of BHI

broth grown E.coli strains to uroepithelial cells from different urine samples. The highest mean value was 16 E.coli per cell and the lowest, 4 E.coli per cell. There was no significant difference in attachment of TSA grown E.coli strains to uroepithelial cells from different urine samples. The highest mean value was 69 E.coli per cell, and the lowest was 52 E.coli per cell. It is possible that these results reflect two different mechanisms whereby E.coli may attach to uroepithelial cells:

(1) Attachment largely depends on the receptivity of the uroepithelial cell population which varies from sample to sample. Receptivity may be influenced by the number of receptor sites present on the cell surface and the proportion of "target" cells, i.e. the proportion of highly receptive cells. Bacterial factors in this instance may be of secondary importance.

(2) Attachment is largely dependant on bacterial factors. "Target" cells exhibiting high susceptibility to attachment exist but are in relatively constant proportions in different samples, so that variations between uroepithelial cell samples do not occur.

Higher attachment levels were found for viable than for nonviable uroepithelial cells. On death of the cell, the metabolic processes controlling the membrane are known to break down and allow entry of vital stains (Phillips, 1973). Uroepithelial cells have been reported to remain viable for long periods of time (Lewis and McCoy, 1922); this was also confirmed in the present study. Furthermore, uroepithelial cells were equally receptive to E.coli attachment after 7 days storage at $4^{o}C$. The fact that fewer bacteria attach to nonviable cells may suggest that E.coli receptor sites are membrane associated. A small proportion of cells at the early stages of death may take up a vital

stain and be termed nonviable, yet may have an intact membrane to which large numbers of E.coli attach. This would explain why a proportion of nonviable cells have equally large numbers of E.coli attached, compared to viable uroepithelial cells.

With regard to the existence of uroepithelial "target" cells, these have been arbitrarily defined as cells with > 10 E.coli attached or > 50 E.coli attached depending on the properties of the E.coli strains attaching to the cells. Similar variations in attachment of bacteria to uroepithelial cells have also been noted *in vivo*. Colleen et al., (1980) and Marrie et al., (1980,a) have reported that a proportion of epithelial cells on the human urethral surface were more heavily colonized by bacteria. This suggests that the existence of "target" cells is not an artefact of the *in vitro* attachment assay, but rather a reflection of the *in vivo* situation. Further experiments in the present study have shown that transitional as well as squamous uroepithelial cells are highly receptive to E.coli attachment. This may further indicate that bladder and urethral cells are not homogeneous in their receptivity to E.coli. Thus, it is possible that certain areas of the urinary tract may be more susceptible to E.coli attachment. Women susceptible to recurrent urinary tract infection may have a higher proportion of "target" cells within these areas, and this may partly explain why some women are reinfected by uropathogens. Furthermore, the size, nature and distribution of these receptive areas in the urinary tract may be influenced by host factors such as hormones, urine contents or other factors possibly associated with a lowering of the host immune response. Not all women are prone to urinary tract infection and this may be due to lack of adequate bacterial receptor sites in their bladder. Some women may only be prone to infection

for a period of time relative to the presence of "target" cells in certain areas of the urinary tract. These possibilities are in need of investigation and it is expected that the results obtained from such studies would be of great importance in understanding the pathogenesis of urinary tract infections.

1.3 REPRODUCABILITY OF THE ATTACHMENT ASSAY

The results presented in Table 10 indicate that there was little difference between mean attachment values obtained when 20, 30, 40 or 50 uroepithelial cells were evaluated. This was perhaps surprising in view of the uneven distribution of attached bacteria in any cell sample. It was expected that counting bacteria attached to only 20 uroepithelial cells would yield a more inaccurate result compared to evaluating 40 or 50 cells per sample. However, this did not occur and the results are in agreement with those reported by Svanborg Edén(1978) who found that E.coli attachment varied by only 1% when 40 to 120 uroepithelial cells were evaluated. For experiments in the present study, it was considered expedient to count bacteria attached to at least 40 uroepithelial cells in any sample.

Although four buffer washes were found to be sufficient to remove unattached bacteria, it was decided to use five washes to be certain that no unattached bacteria remained in the cell suspensions. The washing of uroepithelial cells in buffer prior to the assay, removed urinary mucus and amorphous debris without affecting receptivity to E.coli attachment. Results shown in sections 1.1.12 to 1.1.14 indicate that the technique was highly reproducible using aliquots of the same bacterial and uroepithelial cell preparations; and significant bias was not introduced during evaluations of the cell preparations.

1.4 EFFECT OF VARYING CONDITIONS OF THE ATTACHMENT ASSAY.

Highest attachment of E.coli to uroepithelial cells was obtained using post log-phase bacterial cultures. This finding is discussed later. When four strains were grown in broth and on agar media, attachment to uroepithelial cells was found to be significantly different. Increased attachment occurred only with 2 of the 4 strains (207 and 320) when they were cultured on an agar surface. These observations led to the conclusion that the attachment of E.coli to uroepithelial cells by at least one mechanism is influenced dramatically by the bacterial growth conditions.

The results presented in Figure 3 concerning the influence of bacterial density on attachment, were similar to those reported by Svanborg Edén (1978). The optimal bacterial density was $> 10^8$ bacteria with 1×10^5 uroepithelial cells in 1 cm^3 final volume. This density resulted in high attachment values for E.coli to uroepithelial cells. Attachment of E.coli was affected by temperature: a temperature of 37^oC appeared to be optimal and this may be relevant to attachment *in vivo* as the temperature of the human body is also approximately 37^oC.

A phosphate buffered saline solution, pH 7.1, appeared to be the most appropriate for the attachment assay. This buffer has an osmolality of 300 mosmoles per litre, which is similar to the osmolality of early morning urine specimens from females, as reported by Asscher et al.,(1966). Buffer pH may affect attachment of some strains of E.coli: for example, strains 67, 125 and 207 attached to uroepithelial cells less well when suspended in buffer at pH 5.8, than in buffer at pH 6.4 (Table 18). Both these pH values fall within the physiological range of urine.

Kaye (1967) reported that urine sometimes inhibits bacterial

growth and can be bactericidal to small inocula of E.coli. However, this antibacterial property of urine has not been fully substantiated (Stamey and Mihara, 1980). In the present study attachment of E.coli to uroepithelial cells suspended in urine was generally comparable to those results obtained when the cells were suspended in PBS. The slightly lower attachment values for cells in urine may be explained by the lower density ratio of E.coli to uroepithelial cells. The experiment suggests that E.coli introduced into a bladder containing approximately 80 cm^3 urine, have the capacity to attach to uroepithelial cells within one hour. Depending on fluid intake, the time between micturitions is normally greater than one hour. At night, when fluid intake is low, urine is produced at a slow rate and a small bacterial inoculum in the bladder may multiply and reach a sufficient concentration to allow attachment to the uroepithelium, before voiding occurs. Perhaps the efficacy of the "washout theory" (hydrokinetic clearance of bacteria which retains bladder sterility) may be related to the dilution of bacteria to a point where attachment is sub-optimal. Therefore, depending on the frequency of micturition, and the size and nature of the bacterial inoculum from the urethra into the bladder, an infection may or may not arise.

1.5 THE USE OF FLUORESCENT ANTIBODY TO DETECT E.COLI ATTACHED TO UROEPITHELIAL CELLS.

Type specific antisera and an indirect fluorescent antibody staining method were used to detect E.coli attached to uroepithelial cells. The results were comparable to those obtained using methylene blue as a bacterial stain. This is the first report to confirm E.coli attachment to uroepithelial cells using such specific techniques. The fluorescent antibody binds to the rabbit antibody

which in turn binds to the E.coli attached to the uroepithelial cell surface. When the ultra-violet light source is applied, the fluorescing E.coli are clearly visible (Plates 3 to 6). These results refute any argument that the counting procedure is not accurate using only methylene blue as a bacterial stain.

When the fluorescent antibody technique was applied to the study of cells collected from infected urine samples, interesting results were obtained. E.coli were found to be attached to the uroepithelial cells harvested from 37 women with symptomatic urinary tract infections. The wide range of attachment values (5 to 86 E.coli per cell) were similar to those obtained in the *in vitro* studies reported in Appendix 1. This binding suggests that the *in vitro* assay reflects the attachment levels found *in vivo*. It could perhaps be argued that the E.coli attached to uroepithelial cells were part of an indigenous flora on the cells, and were not responsible for the urinary infection. This argument is difficult to refute, though the fact that transitional cells from the bladder were colonized with E.coli does suggest that these bacteria were not part of the normal flora, because the bladder is normally sterile. In addition, the five serotypes used to prepare antisera were those most commonly isolated from urinary infections. To verify that the E.coli attached were responsible for the infection, a simple experiment could have been carried out. The E.coli strain from each infection could have been isolated then reacted with the pooled type specific antisera to ascertain the 0 serotype. A positive agglutination reaction would confirm that the infecting strain was a similar serotype to the E.coli strain attached to the uroepithelial cells. A small number of attached E.coli and E.coli-like organisms seen in some preparations from healthy women with uninfected urines, were probably part of the normal urethral and periurethral flora. Cox (1966) isolated E.coli and other Enterobacteriaceae from the urethra of healthy women. In conclusion, the results of the present

study support the hypothesis that E.coli attachment to uroepithelial cells occurs in the infection process.

1.6 POSSIBLE INFLUENCE OF CATIONS IN ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

There was no significant difference between attachment of E.coli to uroepithelial cells suspended in standard buffer and buffers containing physiological levels of calcium and magnesium. In 1967, Williams and Wacker pointed out that the five ions H^+, Na^+, K^+, Mg^{++} and Ca^{++} are the major, mobile inorganic cations of biological systems. In the present study, physiological levels of Ca^{++} and Mg^{++} present in buffer solutions, did not appear to affect attachment of E.coli to uroepithelial cells. Sugarman (1980) carried out an adherence assay with E.coli and buccal epithelial cells suspended in buffered, commercially obtained, heavy metal salts. He reported that zinc and iron salts, but not sodium, calcium, lithium, magnesium or potassium salts, increased adherence of E.coli to buccal cells. The uroepithelial cells used in the present assay would not be expected to be highly metabolically active. Therefore, should cell metabolic activity be necessary for cations to mediate E.coli attachment, then the results of the experiment are not surprising. On the other hand, the buffered saline used throughout the assay would have contained traces of a number of different cations as well as large amounts of Na^+, and it is possible that the experiment was not sensitive enough to detect cationic involvement. Such cations may have already been present on the uroepithelial cell surface where they may have acted as cofactors in the attachment process. The presence of futher cations in a buffer solution may not have affected the overall cationic charge of these uroepithelial cells. Alternatively, the

presence of cations may have no effect on the attachment of E.coli to uroepithelial cells.

Costerton et al., (1978; 1981, a; 1981, b,) have proposed that a divalent cation may act as a bridge between the negatively charged glycocalyx of bacteria and of animal cells. The bridging would overcome electrostatic repulsive forces and may be involved in the initial step in the adherence of bacteria to mucosal cell surfaces. As the glycocalyx is comprised of a largely anionic polysaccharide matrix, the linkage between bacterium and epithelial cell may be mediated by a lectin-like protein on the bacterial cell and terminal carbohydrates on the host cell. The presence of divalent cations has been shown by Hoover et al., (1980) to be important in attachment of polymorphonuclear leukocytes to the endothelial lining of blood vessels. Johanson et al., (1979) have reported that divalent cations are required for firm attachment of Pseudomonas aeruginosa and Klebsiella pneumoniae to respiratory tract epithelial cells. Heckels et al., (1976) have reported that alteration of the negative surface charge of Neisseria gonorrhoeae by divalent cations, facilitated attachment to negatively charged amnion cells. It is also possible that similar cations may be important in certain bacterial cell/epithelial cell interactions. Such cations may be influential in enzymic reactions at the host cell surface or in creating conformation changes, that in turn create fewer or greater numbers of bacterial receptor sites. Further investigations need to be carried out on metabolically active uroepithelial tissues to fully substantiate the influence of cations in bacterial colonization. Such studies may involve the use of chelators which would remove Mg^{++} or Ca^{++} ions that may be present in trace amounts on the cell surface and which may be involved in the attachment process.

1.7 THE POSSIBLE INFLUENCE OF HORMONES ON THE ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

Variation in the susceptibility of uroepithelial cells to E.coli attachment corresponded with the expected rise and fall in blood and urine oestrogen levels over the menstrual cycle. Highest attachment levels were obtained for uroepithelial cells assayed between days 9 and 13, and 18 and 28, in the menstrual cycle; lowest levels were obtained for uroepithelial cells assayed between days 14 and 17 of the cycle. The possibility that variations in hormone levels over the menstrual cycle may influence bacterial attachment to epithelial cells has been discussed by Schaeffer et al., (1979). They found that E.coli adhered to uroepithelial cells in a cyclical, repetitive pattern with counts of up to 16 E.coli per cell in the early phase of the menstrual cycle and counts as low as 0 E.coli per cell in the late phase. The E.coli strain used possessed a MSHA and the degree of attachment observed was similar to the results of the present study using E.coli strains 67 and 87 which expressed a MSHA after growth in BHI broth. Had Schaeffer et al. more carefully analysed their results, they would have noted a secondary peak of attachment, around day 20 of the cycle. With more frequent sampling, it is possible that Schaeffer et al. would have obtained similar results to those presented here.

In an early report, Svanborg Edén (1978) did not investigate the reasons for the 20% variability between uroepithelial cell samples in their receptivity to E.coli attachment. However, following the report of Schaeffer et al., (1979), Svanborg Edén et al., (1980) reported that E.coli attachment to squamous and transitional cells did not vary significantly with the menstrual cycle of the cell donor. This latter report is subject to criticism on several points. The assay was only carried out on days 8, 15, 16, 17, 27, and 28

of the menstrual cycle of one woman. In the present study, samples were assayed at 2 to 3 day intervals over consecutive menstrual cycles of five women. Secondly, it is possible that the peak values of 10 and 16 E.coli per cell, reported by Svanborg Edén et al., represent a cyclical peak which the present study found to occur at two times over each menstrual cycle. The actual day in which these peak values occur may vary with each individual and may depend on the length of the menstrual cycle. Had these authors sampled more regularly, they may also have obtained two peaks of attachment over consecutive menstrual cycles. Thirdly, Svanborg Edén et al. did not consider that attachment values of 1 E.coli per cell on one occasion and 16 E.coli per cell on another occasion were significantly different. Statistical analysis of these peak and trough values would almost certainly have established a highly significant difference between attachment of E.coli to cells from these two samples. In Svanborg Edén's studies, uroepithelial cells are harvested from one female donor. Unless these cells are pooled from several urine samples prior to assay, it is possible that certain differences between attachment values reported by Svanborg Edén are due to changes in receptivity of the uroepithelial cells collected at various times over the menstrual cycle, rather than due to differences in the attachment capacity of bacterial strains. The levels of attachment in Svanborg Edén's report of 1980, differ markedly from those previously reported by her. Although the same E.coli strains were used and the same uroepithelial cell donor was apparently used, earlier studies obtained mean attachment values of up to 125 E.coli per cell (Svanborg Edén et al., 1976). Mean values of 51.2 E.coli per squamous cell and 33.4 E.coli per transitional cell were also reported (Svanborg Edén et al., 1977). While her recent report presents mean values of between 2 and 17 E.coli per squamous and transitional cell. These large discrepancies have not been explained by the author.

In order to support her theory that the menstrual cycle does not affect E.coli attachment, Svanborg Edén referred to the work of Kallenius et al., (1980,b). This latter study proposed that variations in epithelial cell receptivity to bacterial attachment are not related to menstrual cycle hormone levels. This conclusion is based on the results of 17 periurethral samples collected and assayed with E.coli during 12 months of study. This is an average of only 1 test every 21 days. From such a poorly designed study, it is impossible to relate results to a menstrual cycle pattern. Kallenius et al. concluded that variability in attachment was due to variations in bacterial properties caused by storage over 12 months. In fact, analysis of their results suggests that the variability was due to differences in periurethral cell receptivity rather than bacterial cell differences. They further reported that attachment levels of up to 900 E.coli per cell were obtained. Close examination of the photograph in Figure 1 of their paper, suggests that large numbers of unattached bacteria may not have been removed by the membrane filtration process and may have given rise to falsely enhanced attachment figures.

A recent report by Riedasch et al., (1981, a) found no correlation between attachment of E.coli to uroepithelial cells collected at different times in the menstrual cycle. These results once again were based on a limited number of assays with uroepithelial cells collected on days 4, 10, 15 and 22 of the cycle. Other studies on human vaginal epithelial cells have failed to agree whether or not attachment of E.coli and Group B Streptococci is influenced by the menstrual cycle. (Botta, 1979; Forslin and Danielsson, 1980; Sobel et al., 1981, b; Zawaneh et al., 1981).

Botta (1981) has reported fluctuations in E.coli attachment to vaginal and uroepithelial cells in accordance with menstrual cycle changes. Attachment levels were highest in the third week of the

cycle and Botta proposed that E.coli utilize a receptor(s) whose synthesis or accessibility fluctuates during the menstrual cycle. Such changes in receptor sites may be a reflection of hormonal influences mediated via the urine and/or blood supply. Botta et al, (1981) have further studied vaginal and urethral swabs from a group of healthy women and a group of women with recurrent bacteriuria,at weekly intervals. They report that colony counts and the number of women with positive cultures of Enterobacteriaceae were higher at ovulation in the infected patients that in the control group of healthy women. They found a correlation between colonization of the vaginal and urethral areas and the peak of oestrogen serum levels. In women with recurrent bacteriuria, the period of high oestrogen levels lasted longer than in women from the control group. In addition, the colony counts from swabs taken from two patients with a history of cystitis, increased steadily during the menstrual cycle and cystitis recurred immediately after menses. This report has great value as the serum hormone levels of the women were tested at weekly intervals over two consecutive cycles. It therefore remains a possibility that bacterial attachment to vaginal, urethral and uroepithelial cells is influenced by hormones such as oestrogen. It may be that only certain strains of E.coli, which do not express a MRHA,are affected by the changing receptivity of these cells over the menstrual cycle. Further investigations are needed to verify this.

In 1980, Rud proposed that oestrogen affects the physiology of the urinary tract and increases the urethral pressure. In the present study, 17β oestradiol was found to affect the urogenital tract of mice, but it had no apparent affect on the mucopolysaccharide layer of the bladder. Oestradiol 17β is the most potent of the natural oestrogens, and its effect on the uroepithelium, if any, may be to increase phospholipid and glycogen content, as has been

found in studies of the uterus (Jensen and De Sombre, 1972). Following studies on E.coli growth in oestrogen treated mouse kidneys, Harle et al., (1975) suggested that oestrogen may predispose to the development of kidney infection. Larson et al., (1977) found that the vaginal microflora of rats fluctuated when 17β oestradiol was administered to ovariectomized rats. Therefore, although there was no apparent quantitative difference in the mucopolysaccharide layer of oestradiol treated mice in the present study, hormone treatment may have affected the animals in other ways, possibly making them more susceptible to infection. Further experiments with hormone treated animals may prove to be of interest, particularly if bacterial attachment to the bladder is simultaneously tested. Such experiments could test compounds other than oestrogen. Stitch et al., (1980) described a non-steroid hormone which showed bi-phasic excretion in the urine over the menstrual cycle, reaching a maximum in the luteal phase, and maximum excretion during the first trimester of pregnancy. Such a compound may increase susceptibility to attachment by changing the number of surface receptor sites, surface charge or mucopolysaccharide coat of the uroepithelial cells. This possibility would be worthy of investigation using a tissue culture model and an animal model.

1.8 VARIATIONS IN THE ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS COLLECTED FROM PREGNANT WOMEN.

Uroepithelial cells collected in the first few months of pregnancy were highly receptive to E.coli attachment. Whereas, uroepithelial cells collected in the later part of pregnancy were less receptive to E.coli attachment. The results did not follow the expected oestrogen hormone levels which rise around week 12 and reach a maximum at week 40; but roughly correlated

with the expected rise and fall of human chorionic gonadotropin (HCG) levels (Shearman, 1972). Bahl (1969) showed that HCG comprises two chromatographically homogeneous glycopeptides with a carbohydrate composition as follows: N-acetylneuraminic acid (α fucose) - β galactose - β-N-acetylglucosamine - α mannose; with sialic acid or fucose at the non-reducing ends of the chains. It is possible that these glycopeptides effect receptor sites on the uroepithelial cells and increase susceptibility to E.coli attachment.

In pregnant women, bacteriuria is present in approximately 7 to 14% of women as detected on their first visit to an antenatal clinic (Savage et al., 1967; Van Rooyen, 1969; and McFadyen et al., 1973). The most common organism causing bacteriuria is E.coli, and 30 to 40% of pregnant women with untreated infections in early pregnancy develop pyelonephritis later in pregnancy (Kass, 1960,a; and Kincaid-Smith, 1968). Kass (1960,b) proposed that hormonal changes during pregnancy may act as predisposing factors which increase the incidence of bacteriuria in pregnancy. It is possible that these changes include an increase in receptivity of the uroepithelium to E.coli attachment. Once attached, E.coli may readily become established in the urinary tract, as pregnancy urine has an increased nutrient content (Roberts and Beard, 1965), and exhibits an increased pH which is invariably favourable to growth of E.coli (Asscher et al., 1966). In the present study, urines from pregnant subjects had higher pH values than those from non-pregnant subjects. The possibility that hormones may affect bacterial attachment to uroepithelial cells during pregnancy is interesting and in need of further investigation.

1.9 THE POSSIBLE ROLE OF UROEPITHELIAL CELL MUCOPOLYSACCHARIDES IN THE ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

The mucopolysaccharides present on the bladder lining of pigs, lambs and mice were stained with PAS reagent and alcian blue. The mucus was most concentrated at the apex of the peripheral epithelial cells and appeared to form a surface coat lining the bladder wall (Plates 7, 8, 13, 14). Epithelial cells several layers below the surface showed traces of mucopolysaccharide material. From this present study, it appeared that the epithelial cells were producing the mucopolysaccharides, though further investigations would be needed to confirm this. Monis and Dorfman (1967) have shown that the transitional epithelium of man can be stained with PAS reagent and alcian blue. In other studies, Thompson et al., (1979) have reported that oestradiol treatment lowered the height of rat ventral prostate epithelial cells and reduced the number of cell organelles and secretory bodies. The vaginal epithelium is also influenced by cyclical changes in oestrogen levels, and at ovulation the size of vaginal squamous cells has been shown to increase (Boon and Rietveld, 1980). However, in the present study, the thickness of the bladder mucopolysaccharide coat was not seen to differ between mice treated or untreated with 17β oestradiol. It is possible that a qualitative change in the uroepithelium occurs which alters the nature of potential bacterial receptor sites. Such a change may not be detected using conventional mucopolysaccharide stains.

Double staining techniques were devised to detect the presence of mucopolysaccharides coating viable and nonviable uroepithelial cells, and to detect bacteria attached to mucus coated and non-coated cells. These staining methods are believed to be the first reported to investigate the presence of mucopolysaccharides on the surface of human uroepithelial cells from the urinary sediment

(Reid and Brooks, 1982). The results of this report are described in the present study. Over 80% of viable uroepithelial cells were coated with mucopolysaccharides, whereas only between 20 and 45% of nonviable cells were mucus coated (Plates 9, 10). The viable cell population may be more representative of cells on the peripheral layer of the urinary tract, as this region is known to be coated with mucopolysaccharides. This mucus coat may be lost in later stages of cell death when the cell membrane structure is altered. The presence of a mucus coat containing glycosaminoglycans, proteoglycans, neutral mucosubstances, glycolipids and certain polysaccharides may partly explain why more E.coli have been found to attach to viable than to nonviable uroepithelial cells.

In a second set of experiments using double staining techniques, E.coli strain 87, grown in BHI broth, attached in significantly greater numbers to mucus coated than to noncoated uroepithelial cells. However, strain 207, grown on TSA, attached in equally high numbers to coated and to non-coated uroepithelial cells (Plates 11, 12). The mechanism of attachment of these two strains appears to be different. The attachment of one strain was affected by the presence of a mucus coat and the attachment of the other strain was not.

Alcian blue is a cationic dye binding to negatively charged acidic mucopolysaccharides. Urinary glycosaminoglycans are negatively charged and contain galactosamine, hexuronic acid, xylose, galactose, and L-fucose (Endo et al., 1980,a; 1980, b). These glycosaminoglycans bind strongly to cations (Chakrabarti and Park, 1980), and it is possible that these mucopolysaccharides have a role in E.coli attachment to the uroepithelial cell surface. The mucosubstances stained by PAS reagent may also be important in the attachment process, and may contain receptor sites for the E.coli cells. Despite the fact that oestrogen did not appear to change the mucopolysaccharide layer of the mouse bladder, (Plates 13, 14) the possibility exists that these substances are influenced by

hormones in humans (Mulholland, 1979). It has been reported by Zachariae (1958) that a secretion, which may be stained with PAS reagent,is produced in the human oviduct epithelial cell surface at the time of ovulation; and secretion is maximal between days 22 and 24 of the menstrual cycle. This area is in need of further investigation.

The possible role of mucopolysaccharides in the pathogenesis of urinary infections has been discussed for a number of years. Cox and Hinman (1961) reported that the normal bladder resists infection by mechanical voiding and by an antibacterial factor(s) inherent in the bladder and not contained in the urine. Mulholland et al., (1966) did not find that the bladder mucosa had any antibacterial action. However, subsequent studies by Norden et al., (1968) on guinea pigs and by Parsons et al., (1975) on rabbits, suggested that the bladder mucosa may be a primary, antibacterial defence barrier that prevents microbial attachment to the epithelial surface. Mucopolysaccharides stained with PAS reagent, alcian blue and colloidal iron (stains hyaluronic acid) have been shown to inhibit E.coli attachment to the bladder wall. (Shrom et al., 1977; Parsons et al., 1977; Parsons and Mulholland, 1978). Although these three studies also demonstrated that clearance of bacteria from the rabbit bladder is 99% efficient, it could be argued that the numbers of bacteria not removed were sufficient to give rise to an infection.

The work of Parsons and associates included removal of bladder mucopolysaccharides by 0.4 N HCl. Following this treatment, E.coli, Klebsiella pneumoniae and Staphylococcus aureus were able to attach to the bladder epithelial cells in equally large numbers(Parsons and Mulholland, 1978). This suggests that these organisms have similar receptor sites on the bladder wall. Alternatively, the acid treatment may have altered the receptivity of the bladder

epithelial cells and caused a large, non-specific attachment to take place. The glycosaminoglycan compound Heparin has since been shown to inhibit E.coli adherence to acid treated bladder surfaces (Hanno et al., 1978; Parsons et al., 1979, b; Parsons et al., 1980,a; 1980,b). It was proposed that glycosaminoglycans place a water barrier between the transitional cells and the environment, masking the highly charged moieties of the cell surface (Parsons et al., 1980,b). The electrochemical charge of the mucus layer may be important in blocking bacterial adherence to the transitional cells (Parsons et al., 1978). These reports conclude that the ability of E.coli to adhere to the bladder is inhibited by the mucopolysaccharides coating the vesical surface and that these mucus substances have a protective role. From the results of the present study, there is no evidence that mucopolysaccharides have a protective role in the bladder. Rather, the presence of a mucopolysaccharide coat enhanced attachment to uroepithelial cells in one strain of E.coli grown in BHI broth, and had no discernable effect on the attachment of a second strain, grown on TSA. It would be of interest to use this second strain to investigate attachment to acid treated and non-treated bladder cells. It may be discovered in the future that the mucopolysaccharide layer in the bladder of women susceptible to urinary tract infections, differs from that of non-susceptible women.

1.10 THE RELATIONSHIP BETWEEN *IN VITRO* ATTACHMENT RESULTS AND THE PATHOGENESIS OF URINARY TRACT INFECTIONS.

In 1965, Stamey et al. proposed four reasons why urinary tract infections are fourteen times more common in women than in men. These were; differences in urethral length, contamination of the female urethra by pathogenic bacteria from the vagina and rectum, incomplete emptying of the bladder and displacement of the

short female urethra during sexual intercourse allowing passage of bacteria into the bladder. The importance of sexual intercourse in urinary infections has been placed in perspective by Kunin (1978). Although bacteria may enter the bladder during intercourse it has not been shown that this alone is sufficient to give rise to infection. While the risk of infection is increased if a pathogenic organism colonizing the periurethral area is introduced into the bladder, infection is not inevitable.

It has been well demonstrated that bacteria isolated from urinary infections are of faecal origin. Gruneberg (1969) reported that E.coli serotypes commonly found in urinary infections are also isolated from the rectal, vaginal and periurethral flora of women. If attachment of E.coli to uroepithelial cells is to be considered an important step in the onset of a urinary tract infection, then faecal E.coli isolates would be expected to attach to uroepithelial cells. This was found to be so in the present study and the results in Appendix 1 establish that faecal E.coli strains attach to uroepithelial cells with varying degrees of efficiency. Introital colonization with E.coli has been found to be significantly higher in patients susceptible to urinary infections, than in healthy premenopausal women (Marsh et al., 1972; Stamey and Sexton, 1975; Pfau and Sacks, 1977; Schaeffer and Stamey, 1977). This introital colonization varies from hour to hour and from day to day, and is only regarded as significant if potential urinary pathogens are isolated four or more consecutive times over 48 hours (Seddon et al., 1976). The adherence of E.coli to vaginal epithelial cells has also been demonstrated in a number of *in vitro* studies (Mardh and Westrom, 1976; Fowler and Stamey, 1977; 1978; Parsons and Schmidt, 1980). It appears that potential uropathogens from faecal origin possess the ability to colonize the vaginal introitus, prior to infecting the urinary tract. No studies of E.coli attachment to

vaginal epithelial cells were carried out in this report. However, it is of interest to note that vaginal target cells, to which many E.coli attached, were found by Fowler and Stamey (1978) and Parsons and Schmidt (1980). Therefore, comparisons may be made between the study of vaginal cell attachment and uroepithelial cell attachment where target cells also appear to exist.

Under normal circumstances, bacteria must overcome a mid-urethral pressure zone to enter the bladder (Mayo and Hinman, 1973). It has not yet been ascertained whether sequential attachment along the urethral meatus and urethra takes place. Cooper et al., (1980) have suggested that the ability of the organism to ascend the urethra is a more decisive step than colonization of the periurethral area. It is possible that possession of flagella enables bacteria to enter the bladder along the mucosal surface. In this study, flagellated bacteria did not attach to uroepithelial cells to a greater degree than nonflagellated bacteria. Nevertheless, the role of flagella in the infectious process cannot be overlooked.

Once inside the bladder, bacteria may multiply in urine. Anderson et al.,(1979) reported that E.coli strains isolated from cases of urinary tract infections, have a smaller generation time in urine than less common urinary pathogens. These bacteria may then attach to the urinary tract cells. In the present study, bacteria in both log and stationary phases of growth attached to uroepithelial cells, though post log-phase cultures tended to attach in highest numbers. Mackintosh et al.,(1975) investigated hydrokinetic clearance of bacteria from the bladder and reported that a constant number of organisms were present in infected urines during frequent voiding. These voided bacteria were thought to have originated as progeny shed by bacteria already attached to the uroepithelium. Therefore, it is highly likely that bacterial

attachment to the uroepithelium is an important step in the onset of urinary tract infections.

Although the ability of each E.coli to attach to uroepithelial cells has been found to differ, the presence of target cells on the uroepithelium may enable a sufficient number of bacteria to attach and give rise to infection. Strains cultured in BHI broth may resemble bacteria multiplying in urine (Cox and Hinman, 1961). Strains cultured on agar may more closely resemble bacteria growing in microcolonies on the mucosa of the bladder, urethra and periurethral area. Therefore, it is important when carrying out *in vitro* studies, to culture bacteria in broth as well as on agar, prior to assaying with uroepithelial cells. Although *in vitro* studies cannot totally simulate conditions in the human urinary tract,certain basic conditions have been included in the assay to take account of the *in vivo* situation. The 37^{o}C incubation temperature is the same as the physiological body temperature; and a shaker speed of 20 rpm for the assay partly takes account of urine movement which would occur inside the human bladder, allowing the bacteria to come into contact with the uroepithelial cells.

The study of E.coli attachment to uroepithelial cells collected from women with symptomatic urinary tract infections was also of importance, and it enabled comparison of *in vitro* and *in vivo* results. Furthermore, the finding that E.coli attached to uroepithelial cells from one woman 7 days prior to her developing symptoms of cystitis, supports the hypothesis that bacterial attachment is important in the pathogenesis of urinary tract infections. This attachment would be expected to occur following entry of bacteria into the bladder from a urethral or periurethral source. Once inside the bladder, bacterial attachment would depend on a number of factors including the bacterial strain's capacity to

attach, and the susceptibility of the host uroepithelium. In time, an infection may ensue and in some cases give rise to the symptoms of cystitis.

SECTION 2.

In this section, the possible importance of bacterial haemagglutinins in E.coli attachment to uroepithelial cells, is discussed.

2.1 THE ROLE OF A MANNOSE SENSITIVE HAEMAGGLUTININ IN THE ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

A MSHA is indicated by the presence of Type 1 pili or fimbriae on the E.coli surface (Duguid et al., 1966; Ottow 1975). The haemagglutination reaction of E.coli with horse or guinea pig erythrocytes is inhibited by the presence of 0.5% to 2.0% w/v α-D-mannose or methyl-α-D-mannoside. (Plate 16). The α-configuration at the C-1 position in D-mannose was found to be important in this inhibition (Old, 1972). The high incidence of Type 1 piliated strains among E.coli urinary isolates suggests that this factor may be one of several properties important in the establishment of urinary tract infections (Brooks et al., 1980; 1981).

Analysis of 145 E.coli urinary isolates indicated a high incidence of the MSHA. However, from the results of the present study, it appears that presence of a MSHA does not enhance E.coli attachment to uroepithelial cells. Of the 111 strains tested for attachment capacity, 58 possessed a MSHA alone after growth in BHI broth, but the highest adjusted mean attachment value was 15 E.coli per uroepithelial cell, as presented in Table 36. Six of these strains were examined by electron microscopy and found to be well-piliated. Nevertheless, attachment to uroepithelial cells was not great, and was not significantly higher than those results obtained using E.coli strains which did not express a MSHA or a MRHA. Therefore, it appears that possession of Type 1 pili

does not enhance attachment to uroepithelial cells.

Several studies have proposed that a MSHA mediates attachment of E.coli to uroepithelial cells, urinary mucus, buccal epithelial cells, candida yeast cells, porcine intestinal epithelial cells, rat bladder cells and monkey kidney cells in tissue culture. (Salit and Gotschlich, 1977; Isaacson, et al., 1978; Ofek and Beachey, 1978; Svanborg Edén et al., 1978; Schaeffer et al., 1979; 1980; Ørskov et al., 1980, b; Van Den Bosch et al., 1980; Avots-Avotins et al., 1981; Ofek et al., 1981). The attachment levels reported by Schaeffer et al., (1979;1980), were similar to those obtained in the present study. However, Schaeffer et al., found that D-mannose inhibited the attachment process, contrary to the results in Table 32 of the present report. Several publications by Svanborg Edén and associates have further confused the issue. In 1978, Svanborg Edén and Hansson reported that a MSHA mediated attachment of E.coli to uroepithelial cells. Mean adhesion figures of between 69 and 130 E.coli per cell were obtained. Between 36 and 70% of bacteria in each of these suspensions possessed Type 1 pili. However, attachment to uroepithelial cells for these strains was not inhibited by the presence of D-mannose or its derivatives. Nevertheless, Svanborg Edén and Hansson proposed that the Type 1 pili mediated attachment and reported that poorly piliated and non-piliated strains attached to uroepithelial cells with lower mean values of between 0 and 14 E.coli per cell. More recently, in conjunction with Hagberg et al.,(1981), Svanborg Edén reported that strains expressing a MSHA gave overall mean attachment values of 9 E.coli per uroepithelial cell which was decreased to 3 E.coli per cell in the presence of D mannose. Clearly, this result is contrary to those reported in 1978, and suggests that Type 1 pili mediates low levels of attachment to uroepithelial cells, and that this attachment is inhibited by D mannose. In addition,

the attachment values in the 1981 report are significantly lower than those presented in the earlier report. From the results of the present study, it appears that strains possessing a MSHA attach to uroepithelial cells in fairly low numbers, and that this attachment is not inhibited by D mannose. Furthermore, when growth conditions were used to encourage production of a MSHA, the attachment values obtained were not significantly different from those for the same E.coli strains grown under conditions which did not result in production of a MSHA (see Table 32). Analysis of a larger number of strains indicated that attachment levels were not significantly different between strains which only produced a MSHA and those which did not produce a detectable haemagglutinin. These experiments indicate that Type 1 pili are not apparently involved in the attachment process.

It is possible that Type 1 pili are an important factor in the invasion of the urinary tract by mediating bacterial attachment to urinary slime. Ørskov et al., (1980, a; 1980,b) have reported that an E.coli strain (C1214-77) expressing a MSHA was trapped by urinary mucus which comprised the Tamm Horsfall protein secreted by the kidney tubules. This urinary glycoprotein is composed of mannose, galactose, glucose, fucose, sialic acid, N-acetylglucosamine and N-acetylgalactosamine (Hunt et al., 1980). It seems likely that the mannose residue was the receptor for the Type 1 pili of strain C1214-77. This strain was used in the present study (labelled 323) and attached in low numbers to uroepithelial cells, confirming the observations of Ørskov and associates. However, urinary mucus was carefully removed in the preparation of uroepithelial cell suspensions used in the present study, therefore, it cannot be confirmed that strain C1214-77 was prevented from attaching to uroepithelial cells because of urinary slime. Perhaps an even lower attachment value would have been obtained if the assay had been

carried out in the presence of urinary mucus and uroepithelial cells. The possibility remains that strains such as C1214-77 are able to give rise to a urinary tract infection by colonization of urinary mucus, which may not be fully removed by the hydrokinetic clearance of voided urine. Therefore, low *in vitro* attachment values to uroepithelial cells may reflect an alternative *in vivo* mechanism by which E.coli strains invade the urinary tract. This may explain why a number of urinary and faecal isolates did not attach to uroepithelial cells in large numbers *in vitro*. Possibly *in vivo*, these strains invade the urinary tract by Type 1 pili-mediated attachment to urinary mucus. Although this is in need of further investigation, the hypothesis is supported by the finding that a large proportion of urinary and faecal isolates produce Type 1 pili. Analysis of uroepithelial cells, urinary mucus and bacterial strains isolated from women with symptomatic urinary tract infections may provide some answers to this question.

2.2 THE ROLE OF A MANNOSE RESISTANT HAEMAGGLUTININ IN THE ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

The present study has established that attachment to uroepithelial cells is enhanced when E.coli strains are grown on TSA and possess a MRHA. Analysis of 145 urinary isolates indicated that 45.5% produced a MRHA after growth on TSA, while only 11% produced a MRHA after growth in BHI broth. Of the 38 faecal isolates examined, 34.2% produced a MRHA after growth on TSA, while only 5.3% produced a MRHA after growth in BHI broth (see Table 37). Clearly there is a high incidence of the MRHA on urinary and faecal isolates, but in most cases, growth on an agar surface is essential for its production. The results also establish that a MRHA is in some instances co-produced with a MSHA.

When a number of urinary and faecal strains were tested for *in vitro* attachment to uroepithelial cells, it was apparent that possession of a MRHA after growth on TSA, enhanced the attachment capacity. (See Table 36). These experiments emphasize the need to culture bacteria using different media, when examining properties such as presence of haemagglutinins. Of the strains tested, a proportion did not appear to produce a MRHA or a MSHA. Attachment to uroepithelial cells for these strains ranged between 2 and 17 E.coli per cell after growth on TSA or in BHI broth. It is possible that an additional haemagglutinin was produced by some or all of these strains, and not detected by the haemagglutination tests used in this study.

It was puzzling to find that strains possessing a MRHA after growth in BHI broth, did not attach to uroepithelial cells in as large numbers as when the same strains were grown on TSA, and found to produce a MRHA. The possibility that an additional "attachment factor" is coexpressed with the MRHA only after growth on an agar surface cannot be dismissed. This possibility will be discussed later. The presence of the MRHA in a number of E.coli strains isolated from patients with urinary tract infections, has been reported by other research workers (Minshew et al., 1978; Ljungh et al., 1979; Brooks et al., 1981). The importance of the MRHA as a virulence factor in E.coli strains causing primary pyelonephritis has been suggested by Vaisanen et al., (1981). Svanborg Edén has recently reported that E.coli strains possessing a MRHA attached in large numbers to human uroepithelial and buccal cells (Hagberg et al.,1981; Korhonen et al., 1981). The results are a little surprising in view of her earlier declaration that the MSHA mediates attachment to uroepithelial cells. However, the results of the most recent reports are in agreement with the results of the present study, and with those reported by Kallenius et al., (1980,b) for E.coli attachment

to periurethral cells. Parsons and Schmidt (1980) have also shown that E.coli attach to vaginal epithelial cells. A strain used in that study was also tested in the present report; labelled 321, this strain produced a MRHA after growth on TSA and attached to uroepithelial cells with a mean of 23 E.coli per cell. It is possible that production of a MRHA enhances the capacity of certain E.coli strains to attach to vaginal, periurethral and uroepithelial cells *in vivo*. As the route of infection is believed to be ascending, in most cases of UTI, attachment of E.coli to the lining of the urethra, periurethral area and vaginal introitus is probably highly important in colonization of these areas. It is not yet known whether E.coli expresses haemagglutinins *in vivo*, though it is possible that a MRHA is expressed in certain E.coli strains which colonize these mucosal surfaces. Once inside the bladder, some E.coli strains may then express a MSHA during growth in urine. These important questions remain a matter of speculation for the time being. However, the fact that not all strains produce a MRHA, indicates that other attachment mechanisms may be involved in E.coli attachment to epithelial surfaces. Further investigations on the role of the MRHA in the attachment process may be necessary. These may incorporate the use of enzyme treatments, such as trypsin, on the bacterial or uroepithelial cells to assess the net effect on attachment, and on expression of a MRHA.

Not all E.coli strains appear to be capable of producing a MRHA, and some of those that did not, were found to attach to uroepithelial cells in low numbers. This suggests that either attachment is not a prerequisite for infection in all cases of UTI, or low-level attachment is still sufficient for infection to occur. Previous work on the importance of hydrokinetic clearance mechanisms supports the first hypothesis. For example , attachment may not be a necessary prerequisite for infection in patients with significant bladder residues, provided there are sufficient bacteria in residual urine to counteract the wash-out effect of voided urine (Hinman, 1966;

Hinman and Cox, 1966). The results of the study on women with symptomatic bacteriuria indicate that low-level attachment occurs in some cases, so that the second hypothesis may also be true. If this is the case, then the hormonal status of the donor and the presence or absence of a mucopolysaccharide coat on uroepithelial cells may be of utmost significance in influencing the susceptibility of the host.

2.3 FURTHER ANALYSIS OF E.COLI STRAINS POSSESSING A MRHA.

Results of the present study clearly indicate that almost all E.coli isolates attach *in vitro* to uroepithelial cells to some degree. Investigations using bacteria which are not normally urinary tract pathogens, led to the definition of a non-specific, basal level of attachment for the *in vitro* assay: i.e. $\leqslant$ 5 bacteria per cell. Of all the urinary isolates tested, 93.7% attached to uroepithelial cells to a greater level than 5 bacteria per cell, and 100% of the strains possessing a MRHA after growth on TSA attached with a mean of > 5 E.coli per cell. Of all the faecal isolates tested, 87% attached to uroepithelial cells at a level of greater than 5 bacteria per cell, and 100% of the strains possessing a MRHA after growth on TSA, attached with a mean of >5 E.coli per cell. These results establish that E.coli attachment to uroepithelial cells, particularly for strains possessing a MRHA after growth on TSA, is a specific process and not a basal level of attachment which may be a function of the assay technique.

The E.coli urinary strains used in the present report had previously been investigated for several properties thought to be important in pathogenicity for the urinary tract (Brooks, et al., 1981). An attempt was made to correlate these properties with haemagglutination patterns. There was a high incidence of the MRHA in strains with 0 serotypes commonly found in urinary tract infections. These strains may have greater infectivity due to the presence of

a MRHA which may assist attachment to the uroepithelium. This may explain why these strains are more commonly isolated from patients with UTI. The results also indicated a high incidence of dulcitol and salicin fermentors, though the significance of this has not been established. There was a fairly high incidence of haemolytic strains and it is possible that presence of a haemolysin or the ability to ferment dulcitol and salicin is important in the infection process, after attachment to the uroepithelium has taken place.

2.3.1 IMPORTANCE OF THE K1 ANTIGEN IN ATTACHMENT TO UROEPITHELIAL CELLS.

A large number of K rich strains were found among the E.coli isolates. These had a reciprocal K antigen titre of > 32, as described by Brooks et al., (1981). However, the results presented in Table 34 of the present study indicate that possession of a K1 antigen was not directly related to attachment capacity to uroepithelial cells. Only K1 strains producing a MRHA after growth on TSA, attached in large number to uroepithelial cells. It has been reported that K antigen is found in a greater proportion and in significantly greater amounts among E.coli urinary isolates than periurethral or faecal isolates of healthy individuals. (Glynn et al., 1971; Kaijser, 1973; McCabe et al., 1975; Brooks et al., 1980; 1981; Nimmich et al., 1980; Van Den Bosch et al., 1981, b). The K1 antigen is a sialic acid homopolymer (Stevens et al., 1980). Recently, Evans et al., (1981) have reported a close correlation between E.coli strains possessing a K1 capsular antigen and a MRHA. The K1 antigen may assist invasion of the renal tissues and enhance resistance to phagocytosis and serum killing. (Ørskov et al., 1971; Smith and Kaijser, 1976; Ørskov, 1978; Weinstein and Young, 1978; Gemski et al.,

1980). Therefore, K antigen and particularly the K1 may be an important virulence factor in upper UTI; though the presence of the K1 antigen on bacterial cells does not enhance attachment to uroepithelial cells.

2.3.2 IMPORTANCE OF BACTERIAL MOTILITY IN ATTACHMENT TO UROEPITHELIAL CELLS.

The results presented in Table 40 suggest that bacterial motility does not enhance E.coli attachment to uroepithelial cells. However, it could be argued that the flagellated organisms are able to maintain motility across the moist mucosal surface of the urinary tract, and also enable distribution in the bladder urine. Thus, flagellated bacteria may have a greater chance of encountering uroepithelial target cells *in vivo*, to which the bacteria may then attach and give rise to infection.

2.3.3 CULTURE CONDITIONS WHICH AFFECT THE PRODUCTION OF A MRHA.

The results from Table 43 establish that a MRHA is not produced when E.coli strains are grown on TSA at 18^{o}C. Attachment to uroepithelial cells was also found to be significantly reduced for these strains. Duguid et al., (1979) have also reported loss of MRHA production at lower growth temperatures of 20 to 25^{o}C. Evans et al. (1978) reported that enterotoxigenic E.coli (ETEC) strains, isolated from adults with diarrhoea, produced a MRHA after growth on agar at 37^{o}C, but not at 18^{o}C.

In the present study, a second experiment established that E.coli attachment to uroepithelial cells was not greatly enhanced when the bacteria were grown at 37^{o}C in a small volume of BHI broth, spread over the bottom of a large flask. This method of culturing

E.coli had been reported by Hagberg et al. in (1981). Their results indicated that a large proportion of their strains possessed a MRHA and a MSHA. Had they chosen to use an agar medium, they may have found that a MRHA alone was produced. It is important to grow E.coli strains under conditions which allow expression of the bacterial haemagglutinins. This is particularly important when carrying out attachment studies, or when determining the incidence of haemagglutinins among E.coli isolates.

2.4 EFFECT OF STORAGE OF E.COLI STRAINS OVER 12 MONTHS.

The storage of four E.coli urinary isolates over 12 months did not significantly alter the capacity to attach to uroepithelial cells. Expression of a MRHA was not apparently affected by the storage conditions. Loss of MSHA production was noted, particularly for strains stored at room temperature on agar slopes. However, attachment to uroepithelial cells was not correspondingly affected. Cornelius et al. (1979) reported that E.coli isolated from patients with bacteriuria banked on agar slopes at room temperature for 2 to 3 days, had substantially greater adherence capacity to vaginal epithelial cells, than strains banked for 15 months or more. Combining these results with findings of the present study, it is evident that E.coli strains should be stored in BHI broth and glycerol at -10^{o}C rather than on agar slants at room temperature, if attachment studies are to be carried out.

The loss of MSHA production may have been due to phase variation. The expression of a MSHA is chromosomally controlled, and Swaney et al. (1977) have reported that E.coli phase variants contain the genetic information to produce Type 1 pili. The presence of Type 1 pili endow E.coli strains with mannose-sensitive, haemagglutinating activity. Piliation can be lost by one of four

reversible mechanisms: phase variation, plasmid loss or gain, pil gene mutation or reversion, and quantitative regulation. Swaney et al. (1977) described phase variation of an E.coli K12 strain and found that the presence or absence of Type 1 pili varied, and the rate of change from one phase to another was influenced by environmental conditions. It is possible that similar phase variation of Type 1 pili occurred in the present study, resulting in the loss of a MSHA in strains 87, 320 and 322 which were stored on agar at room temperature.

Expression of a MRHA was apparently stable when strains 320 and 322 were stored for 12 months in BHI glycerol broth and on agar slopes. It remains to be established whether or not E.coli urinary isolates carry a plasmid which mediates expression of a MRHA. A study of this nature would prove to be worthwhile, and may lead to the determination of the genes which code for the MRHA on the bacterial cell.

It could be argued that strains stored for long periods of time are no longer representative of virulent strains capable of producing infection *in vivo*. However, when freshly isolated E.coli strains were assayed for attachment to uroepithelial cells, the range of mean values were similar to those obtained using E.coli strains stored for longer periods of time. An alternative experiment could have been devised to test the virulence of E.coli isolates. By using an appropriate animal model, it may have been possible to establish whether E.coli strains caused damage to areas of the urinary tract. These strains could have been tested immediately after isolation from patients with UTI, then again after 12 months storage in glycerol broth at -10^{o}C. A system such as this has been reported by Van Den Bosch et al. (1979) using a mouse model to test differences in nephropathogenicity between human E.coli isolates. Depending on the effect each strain had on the mouse, the E.coli

isolates were classified into one of three virulence groups. However, it could be argued that the use of human bacterial isolates on the tissues of mice does not constitute an appropriate model for testing bacterial virulence.

The experiments reported in the present study were primarily designed to investigate the effect of storage on the capacity of E.coli to attach to uroepithelial cells. The results have indicated that this capacity, as well as the ability to produce haemagglutinins, is preserved when E.coli isolates are stored in BHI glycerol broth at -10^{o}C.

2.5 SPECIFICITY OF E.COLI ATTACHMENT TO EPITHELIAL CELLS.

The specificity of E.coli attachment was investigated by comparing attachment to uroepithelial cells, buccal epithelial cells and tissue culture cells. Attachment to uroepithelial cells was significantly higher than to buccal cells, particularly for E.coli strains possessing a MRHA after growth on TSA. The frequency distributions indicated that fewer buccal target cells existed to which large numbers of E.coli attached. The proportion of uroepithelial target cells within a given population had been previously shown to be higher. Similar attachment results have been reported by Ørskov et al. (1980, b). These authors found that strain C1212-77 grown on agar and possessing a MRHA, attached in larger numbers to uroepithelial cells than to buccal cells. This strain, labelled 322, was also used in the present study, and was found to attach to uroepithelial cells with a mean of 34 E.coli per cell, following culture on TSA. Studies by Schaeffer et al. (1981) have investigated attachment of E.coli urinary strains to buccal and vaginal epithelial cells. They propose that changes in adhesive characteristics of epithelial cells are associated with

susceptibility to urinary tract infections. Sugarman and Donta (1979, b) found that buccal cell receptor sites were saturated when less than 10% of the available surface was occupied. Their results also indicated that the number of bacterial receptor sites present on the buccal cell surface varied from cell to cell. With regards to the levels of E.coli attachment to buccal cells, the observations of Sugarman and Donta (1979, b) and Schaeffer et al. (1981) are comparable to those reported here. It is possible that lower attachment values are related to the apparent absence of mucopolysaccharides on the buccal cell surface. Only 10 - 15% of the viable cells tested in the present study were coated with mucopolysaccharides which stained using PAS reagent or alcian blue.

Attachment of E.coli to monkey kidney (vero) cells in tissue culture did not correlate with the results obtained from *in vitro* studies. Highest attachment figures were obtained using E.coli strains grown on TSA and possessing a MRHA (Plate 44). However, strains which attached in large numbers to uroepithelial cells *in vitro* did not correspondingly attach in large numbers to vero cells. Overall, attachment to vero cells was not great for the strains tested (see Table 56). Salit and Gotschlich (1977) reported that Type 1 piliated E.coli K12 attached to vero cells with a mean of 14.2 bacteria per cell, while non-piliated bacteria attached with a mean of 0.17 bacteria per cell. In the present study, strains possessing a MSHA, indicative of the presence of Type 1 pili, attached to vero cells with mean values of 0 to 6 E.coli per cell. Strains which did not express a haemagglutinin attached with mean values of 0 to 4 E.coli per cell. These results do not confirm the findings of Salit and Gotschlich. In addition, these authors did not investigate attachment of strains possessing a MRHA after growth on TSA. In the present study, the attachment levels of these strains ranged from 2 to 23 E.coli per vero cell.

Drasar et al. (1980) have stated that *in vitro* attachment results should be compared to those obtained from a cell culture model and from the natural diseased animal. Uroepithelial cells obtained from women with UTI have been examined for E.coli attachment in the present study, as discussed earlier. However, the comparison of *in vitro* results with those obtained using a vero cell culture model has not proved to be successful. Further investigations were attempted using a mouse kidney cell culture model. However, the attachment results obtained were not sufficiently large to warrant the use of this system. An appropriate cell culture model would be beneficial, particularly if fresh human kidney or bladder cells could be used. The attachment of E.coli to these cells may help determine whether factors such as a MRHA, are particularly effective in mediating attachment. Additional experiments could be undertaken to determine the influence of hormones, mucopolysaccharides, target cells, bacterial pili and other factors on the attachment of E.coli to metabolically active tissue culture cells.

Recently, there have been several encouraging reports on the use of primary human urinary cells in tissue culture (Felix et al., 1980; Sack et al., 1980;Colleen and Mardh, 1981). The attachment of group A Streptococci to the HEp-2 cell line has been reported by Grabovskaya et al. (1980). In 1973, Buonassisi reported the use of a cell line from the lumen of the rabbit aorta, which synthesized and secreted various species of sulfated mucopolysaccharides. There are signs that a suitable tissue culture system may be devised to investigate bacterial attachment related to UTI.

2.6 ALTERNATIVE HAEMAGGLUTINATION TESTS FOR DETECTION OF THE MRHA.

A number of haemagglutination test systems were used to detect the presence of a MRHA in E.coli strains. A MRHA was detected at 4°C, 22°C and 37°C, with the 4°C temperature being optimal (see Table 47). There was no apparent difference between agglutination of human group A or group 0 erythrocytes, and the haemagglutination reaction was most easily seen using white porcelain tiles. The results using method A supported the finding of Duguid (1964) that the mannose resistant haemagglutination reaction is eluted at 50°C.

The fact that the MRHA was detected in method E at 37°C was not surprising as it is produced after growth on TSA at 37°C. The results in Table 48 show that the second group of strains did not express a MRHA. These findings confirmed the earlier results obtained using the standard haemagglutination test of section 3.1. It appears that this standard test is indeed effective in detecting the presence of a MRHA in E.coli strains.

Evans et al. (1980,b) have proposed that bacterial haemagglutinins should be classified as part of the routine typing of isolates. Their studies employed bovine, chicken, monkey,human and guinea pig erythrocytes. Only the latter two types were used in the present study; human group A blood was used in preference to groups B or 0, as recommended by Vosti (1979). However, the results in Table 47 of the present study, have indicated that the use of group A or group 0 erythrocytes is satisfactory for these tests. Analysis of the results in Appendix 1 shows that E.coli strains 83,300 and Co20 grown on TSA, expressed a MRHA which agglutinated guinea pig erythrocytes. For these strains, co-production of a MSHA would be undetected using guinea pig erythrocytes, as the MRHA would mask the effect. Strains 97, 160, 183 and 199 grown on TSA and strain 220 grown in

BHI broth, expressed a MSHA which agglutinated human group A erythrocytes. These unusual haemagglutination reactions were not investigated further. It would be interesting to establish the reasons for the different haemagglutination reactions of these strains. In particular whether the haemagglutination test results described above, reflect a difference in the nature of the MRHA or the MSHA of certain E.coli strains.

The haemagglutination reactions of bacterial isolates have been widely used as an index of the adhesive capacity of pathogenic microorganisms. However, Ellwood et al. (1979)have questioned the relevance of this and suggest it unlikely that the simple cell surface of erythrocytes reflects the surface of mucosal epithelial cells, which are physiologically more complex. This is certainly a point worth remembering. In addition, the conditions used to test for haemagglutinins may not be appropriate for detection of certain types of haemagglutinins. Alternatively a "co-factor" responsible for adhesiveness may be expressed with a MRHA or a MSHA, but may not as yet be detected by the simple haemagglutination tests. This may explain the differences between E.coli attachment to uroepithelial cells for strains possessing a MRHA after growth on TSA and in BHI broth: the adhesive "co-factor" may only be expressed after growth on TSA while the MRHA is expressed after growth under both conditions. Further investigations on this matter are needed.

2.7 EFFECT OF TEMPERATURE, pH AND FORMALDEHYDE TREATMENT ON THE MRHA OF E.COLI ISOLATES.

When a group of E.coli strains were incubated at 65^{o}C for 60 mins., the MRHA activity was abolished, though the MSHA activity of the reference strain 172 was only slightly decreased (see Table 49). This confirmed the findings of Duguid et al. (1979); and those of

Evans et al. (1978) who reported that a MRHA of ETEC strains was destroyed on heat treatment. A drop in buffer pH to pH 3.0 was sufficient to destroy a MRHA of 21 of the 23 E.coli strains and the MSHA of reference strain 172. The haemagglutination activity of these strains was in most cases, reduced in buffer of pH 10.1. Individual E.coli strains may have differed in their susceptibility to changes in buffer pH. Nevertheless, these two extreme buffer pH values did appear to affect the activity of the MRHA on the strains tested. Treatment in 0.5% formaldehyde destroyed or decreased the MRHA activity of 19 of the 23 E.coli strains tested, confirming the finding of Duguid et al. (1979). The expression of a MSHA was not affected by formaldehyde, and reference strain 43 did not produce a MRHA or a MSHA under any of the test conditions. Formaldehyde binds to protein molecules, but does not affect carbohydrates or lipids (Millonig and Marinozzi, 1968). Further experiments in the present study indicated that formaldehyde treatment of uroepithelial cells and bacteria significantly decreased E.coli attachment to uroepithelial cells. It is possible that the MRHA-mediated attachment to uroepithelial cells involves a protein molecule(s) which is affected by formaldehyde. If the MRHA is protein in nature, it would explain the effect of temperature, pH and formaldehyde on the activity of this haemagglutinin. Banai et al. (1981) have recently studied attachment of Mycoplasma gallisepticum to human erythrocytes. This report states that treatment of the mycoplasmas by heat (55^{o}C), glutaraldehyde (0.05% in PBS) or prolonged exposure to low pH 5.0, drastically reduced or abolished attachment. These results supported the theory that the mycoplasma membrane components responsible for specific binding to the sialoglycoprotein receptors on the erythrocytes, were protein in nature. The possibility exists that a protein molecule mediates attachment of E.coli strains possessing a MRHA to uroepithelial cells and/or to human erythrocytes.

2.8 TITRATION AND INHIBITION OF THE MRHA.

When the titre of the MRHA of 12 E.coli strains was determined, there was no correlation with the degree to which these strains attached to uroepithelial cells. High attachment levels were not obtained in every case for strains with a high MRHA titre. However, in general,strains with a MRHA reciprocal titre above 16 attached in greater numbers to uroepithelial cells, than strains with a lower MRHA titre. These results are in agreement with the findings of Kallenius et al. (1980,b) and Van Den Bosch et al. (1980). Further investigations to establish an explanation for these results would prove to be worthwhile. Although the presence of a MRHA on TSA grown E.coli strains enhances attachment to uroepithelial cells, other factors may also determine the degree to which this attachment takes place.

No inhibition of the MRHA activity was found when a large group of compounds was tested with 7 E.coli strains. There was a marginal decrease in the haemagglutination activity of 4 out of 27 strains in the presence of galacturonic acid or glucuronic acid. However, the significance of this finding was not established. It would have been of great interest to have found an inhibitor of the haemagglutination reaction. Such a compound may then have been used to test for inhibition of attachment to uroepithelial cells. In addition, it could have been tested with a wide range of E.coli extraintestinal isolates possessing a MRHA, and this may have established whether the MRHA of these strains had the same haemagglutination mechanism.

The possibility that carbohydrates inhibit bacterial adherence to mucosal surfaces has been previously investigated (Rivier and Darekar, 1975; Salit and Gotschlich, 1977; Ofek and Beachey, 1978; Ofek et al., 1978; Vosti, 1979; and Schaeffer et al., 1980).

However, these studies have only been related to inhibition of a MSHA. De Graaf et al. (1980) have recently reported that production of K99 surface antigen was decreased by 95% when L-alanine was present in the minimal growth medium. This K99 antigen is common among ETEC strains isolated from calves and lambs suffering from diarrhoea, and it expresses a MRHA active with bovine erythrocytes. Alanine did not inhibit the MRHA of the urinary isolates in the present study, although incorporation of this amino acid or any other compound, in the growth medium, was not attempted.

From the results presented, there was no single compound which inhibited the MRHA of the E.coli strains tested. It is possible that a number of compounds added together might inhibit the MRHA reaction. When this was attempted using E.coli strain 207 and mannose, rhamnose, galactose and glucosamine together, no inhibition was noted.

2.9 THE PRESENCE OF PILI ON E.COLI STRAINS EXPRESSING A MRHA.

The results of this study were obtained using negatively stained preparations examined by transmission electron microscopy. All 24 E.coli strains initially tested produced a MRHA after growth on TSA. Of these, 9 strains were well-piliated, 8 were poorly piliated and 7 were non-piliated. These results confirm the findings of Duguid et al. (1979) and Ip et al. (1981) that not all strains exhibiting a MRHA are piliated (Plates 20 to 25). The growth of E.coli on TSA tends to discourage production of type 1 pili (Ørskov et al. 1980,b) and none of the 24 strains tested here exhibited a MSHA with human group A or guinea pig erythrocytes. The nature of the pili was not established from these experiments, though it is unlikely that they were type 1 pili as no MSHA was detected in the haemagglutination reactions. The possibility exists

that pili similar to the F7 pilus reported by Ørskov et al. (1980,b) were present on these strains. Further investigations are needed to determine the nature of these pilus structures. Korhonen et al. (1980,a; 1980,b) have purified pili from E.coli strains isolated from the urine of patients with symptomatic UTI. They claim that mannose-resistant pili from E.coli strain 3048 are mediators of attachment to human uroepithelial cells. However this strain (3048) expressed a MRHA and a MSHA, and therefore type 1 pili were present on the cells. Perhaps it would have been more fruitful to have studied a piliated E.coli strain which produced only a MRHA. Kallenius et al. (1980,b;1980,c;1981,a) support the view that pili mediate attachment of E.coli to erythrocytes and uroepithelial cells. These recent reports have investigated the occurrence of P-fimbriated E.coli strains in patients with acute pyelonephritis. Of these isolates, 91% possessed a newly defined P-fimbriae; though the evidence for these structures was based on haemagglutination reactions. Further studies may be required to categorise this fimbrial antigen in the light of previous reports by Duguid et al. (1966) and Ørskov et al. (1980,b) which defined seven types of fimbrial antigens. From the results of the present study, there was some evidence indicating that pili present on strains possessing only a MRHA, were 6 nm. in diameter, similar to the F7 pilus reported by Ørskov et al. (1980,b) and distinct from the type 1 pilus which has a diameter of 7 nm. (Brinton, 1965; Duguid and Anderson, 1967). However, these observations are not sufficient proof of the type of pili present on the strains examined. It is possible that more than one type of pilus is present on the bacterial cells of strains exhibiting a MRHA (Jann et al., 1981).

The numbers of pili counted for each bacterial cell, can only be taken as an approximation. Even at high magnification and by analysing enlarged photographs of the cells, the exact numbers of pili

present were in some cases difficult to estimate. Some pili may have been sheared off in the preparation of the samples, while others may not have been visible at the magnifications used. No pili were detected on non-haemagglutinating E.coli strains.

The strains 63, 104 and 158 expressed a MRHA alone after growth in BHI broth but pili production was 30% lower than after growth on TSA. In addition, attachment to uroepithelial cells after growth in broth was significantly lower than after growth on TSA. It is possible that the pili found on strains expressing a MRHA, are best produced on an agar surface, rather than in broth. Further investigations are needed on this and also to determine whether the pili found on strains expressing a MRHA, are involved in attachment of E.coli to uroepithelial cells. One such investigation would be to carry out an *in vitro* assay with a piliated E.coli strain and uroepithelial cells, then to examine sectioned material by transmission electron microscopy. This may show whether the bacterial pili mediate the attachment to the uroepithelial cell surface.

Growth of E.coli at 18°C was found to inhibit pili production. This correlated with loss of production of a MRHA and a significant decrease in attachment to uroepithelial cells (see Table 55). Strains which did not produce pili at 37°C or 18°C also lost their MRHA and attached in lower numbers to uroepithelial cells after growth at the lower temperature. Suppression of pili production at lower temperatures, has also been reported by Duguid et al. (1979) for strains possessing a MRHA, and by Freer et al. (1978) for ETEC strains possessing a MRHA. Production of a MRHA at 37°C on an agar surface suggests that these organisms may be able to produce a MRHA when colonizing areas of the human urogenital tract, which are also at this temperature. Expression of this haemagglutinin may be one of several uropathogenic properties which enable these organisms

to infect the urinary tract. This suggestion is in agreement with Evans et al. (1980,a) who have proposed that a MRHA may be an important virulence factor in extraintestinal infections caused by E.coli. It appears that growth of E.coli on TSA at 37°C encourages production of a MRHA and in some cases an unidentified type(s) of pili is(are) also produced.

2.10 POSSIBLE NATURE OF THE MANNOSE RESISTANT HAEMAGGLUTININ.

In the present study, experiments have shown that the MRHA is produced by a proportion of E.coli strains after growth on TSA at 37°C. Expression of a MRHA was inhibited by growth at 18°C but not at 37°C in a CO_2/H_2 atmosphere. This haemagglutinin was destroyed by heating to 65°C, by treatment in formaldehyde, and was affected by acid and alkaline buffer pH extremes. The mannose resistant haemagglutination reaction was not inhibited by simple carbohydrates or amino acid compounds. The expression of a MRHA was stable after 12 months storage, and did not appear to be related to the O, H or K serotypes of E.coli strains. In some instances, a MRHA was found in conjunction with a MSHA. The presence of pili has been noted in a proportion of strains which only express a MRHA. The presence of a MRHA enhanced E.coli attachment to mucopolysaccharide coated and non-coated uroepithelial cells. Results have suggested that uroepithelial "target cells" exist to which many E.coli attach. It is possible that these "target cells" contain receptors for the MRHA. The process of attachment appeared to be specific, and buccal cell or tissue culture cell attachment values were found to be lower than values for attachment to uroepithelial cells. The titre of the MRHA did not appear to be directly related to the degree of attachment to uroepithelial cells.

It seems likely that the MRHA is a complex structure. The

data presented suggests that the MRHA may be a biologically active lectin-like protein or glycoprotein, which has complex carbohydrates as terminal residues. Lectins have been found to agglutinate erythrocytes and other cell types, by binding to sugar molecules on the cell surface (Sharon and Lis, 1972; Ochoa, 1979). If the MRHA is a lectin-like protein,it may be expected to interact with the carbohydrate moieties on glycolipids, glycoproteins or other such mucosal surface compounds acting as receptor sites. Such a protein might be affected by growth temperature, heat treatment, formaldehyde treatment and acid or alkaline treatment. The haemagglutination of human erythrocytes would only be inhibited by the presence of complex carbohydrate molecules similar to the terminal residues on the protein. In some E.coli strains this MRHA may be present as proteinaceous pili, while in other strains the protein may be present on the cell surface.

The possibility also exists that a second factor is co-expressed with the MRHA, and is important in attachment to uroepithelial cells. This factor may only be expressed after growth on an agar surface and may be similar in chemical nature to the MRHA.

At present these proposals are simply hypothetical. However, the nature of the E.coli receptor site on erythrocytes and epithelial cells has been recently investigated. Kallenius et al. (1980,a) have proposed that an α-D-Gal ρ-(1-4)-β-D-Gal ρ-(1-4)-D-Glc, carbohydrate moiety of the P^k glycosphingolipid (trihexosyl ceramide) is the receptor on human erythrocytes, for attachment of a uropathogenic E.coli strain possessing a MRHA. This P^k blood group antigen has an α-D-galactose terminal, non-reducing sugar. The agglutination of P_1 type A Rhesus positive erythrocytes by an E.coli strain possessing a MRHA, was not inhibited by D-galactose. This indicated that the size of the erythrocyte receptor was larger than a monosaccharide. This finding may explain why monosaccharides did

not inhibit the MRHA in the present study. Leffler and Svanborg Edén (1980; 1981) have also proposed that glycosphingolipids of the globoseries are receptors on human erythrocytes and uroepithelial cells for E.coli strains possessing a MRHA. Similar findings have been reported by Kallenius et al. (1981 b,c). Evidence for a glycolipid receptor for E.coli on the bladder epithelium has also been reported by Davis et al., (1981). In 1981, Lomberg et al., further suggested that individuals without a P_1 blood group antigen are at less risk of acquiring recurrent urinary infections than P_1 positive individuals.

A glycolipid receptor on pig intestinal brush borders for the K88 antigen of E.coli has been reported by Kearns and Gibbons, (1979). Faris et al. (1980) have further suggested that both glycolipids and glycoproteins may be involved in the erythrocyte receptor for CFA/I and K99 antigens of ETEC strains. The importance of the bacterial and epithelial cell glycocalyx should not be overlooked. Costerton (1981,b), has indicated that the bacterial glycocalyx contains uronic acids and other sugar compounds which may be involved in the attachment of bacteria to mucosal surfaces.

This research on receptors should perhaps be seen in perspective. The results suggest that glycolipids are receptor sites for piliated E.coli strains expressing a MRHA, but do not take account of the receptor sites for non-piliated strains expressing a MRHA. As Roland (1981) pointed out, the pathogenesis of urinary tract infections is an extremely complex subject. The existence of glycolipid receptor sites for bacterial attachment may therefore only be important in a small proportion of bacterial to epithelial cell interactions which give rise to UTI.

Nevertheless, the determination of the chemical and structural nature of the MRHA will be an important step in understanding bacterial attachment to erythrocytes and epithelial cell surfaces.

It is possible that the nature of this MRHA will be discovered in the very near future.

2.11 THE USE OF ELECTRON MICROSCOPY TO INVESTIGATE ATTACHMENT OF E.COLI TO UROEPITHELIAL CELLS.

2.11.1 TRANSMISSION ELECTRON MICROSCOPY

The use of transmission electron microscopy permitted close analysis of bacterial attachment to the uroepithelial cell surface. The photographs taken during these studies show that there was some direct interaction between bacteria and the uroepithelial cell. (Plates 26 to 30). In some instances a gap of approximately 0.03 to 0.2μm was evident which may have been due to an electrostatic repulsion between the two cell types. Alternatively it may have been caused by a condensation of mucosal material at the cell surface leaving the impression that a gap was present.

The use of ruthenium red stain enabled the acidic mucopolysaccharides coating the uroepithelial cells to be stained. (Plate 31). This layer on the outside of the cell surface was not evident using standard methods. The ruthenium red stained mucus coat was present all along the surface of the cells examined, though in some areas condensation of the material may have occurred. Bacteria were also seen to be coated with a ruthenium red stained mucus coat (Plate 32). This fibrous matrix around the bacterial cell may have been stabilised by the antibody added in the preparation of these samples. In some cases bacteria were seen to interact with the uroepithelial cell surface, while in other cases there was a small gap between the two cell types. (Plates 33 to 36). Ruthenium red stain has been used to investigate colonization of animal tissues by autochthonous and pathogenic

bacteria (Costerton et al.,1978; 1981,a; 1980,b; Cheng et al.,1981). Marrie et al. (1980,a) have studied adherence of E.coli to uroepithelial cells using a ruthenium red staining technique. They reported bacteria with a fibrous matrix adhering to uroepithelial cells which had a polysaccharide glycocalyx stained with ruthenium red. As well as Costerton and associates, Jarvinen and Sandholm (1980) have also proposed that the uroepithelial cell glycocalyx is important in the attachment of E.coli to the uroepithelium. The results of the present report indicate that the use of stains such as ruthenium red to identify mucus substances under electron microscopy, is important in studies of bacterial and epithelial cell interactions. The possibility exists that molecules on the glycocalyx of bacteria and uroepithelial cells are sometimes involved in the attachment process. Further investigations may determine whether the receptor sites for bacterial attachment to uroepithelial cells are contained within the glycocalyx layer of the two cell types.

Similar staining techniques have been used to study bacteria associated with rabbit kidney cells (Johnston and Latta, 1978) and rat bladder cells (Fukushi et al., 1979). Alcian blue was used in section 2 of the present study to stain glycosaminoglycans on uroepithelial cells. This stain has also been used to stain the "cell coat" of various rat tissues (Behnke and Zelander, 1970). Using alcian blue in conjunction with transmission electron microscopy, these authors obtained results comparable to those obtained using ruthenium red as an electron dense stain. Parlanti and Monis (1980) have also used alcian blue to study histological sections of the vaginal mucosa of rats. The luminal glycocalyx contained a glycoprotein rich in sulfate and was stained with the alcian blue. These investigations also used ruthenium red stain in conjunction with electron microscopy. The well developed glycocalyx was seen on sections from the upper half of the rat vagina. Hormone treatment

of these mice was found to increase the glycocalyx layer. This latter finding is of interest. It is possible that the sections from an oestrogen treated mouse bladder, examined in the present study using histological techniques, may have stained with ruthenium red. Subsequent analysis by electron microscopy may have more conclusively indicated whether the bladder mucus coat was affected by oestrogen treatment. The application of electron dense stains and the use of transmission electron microscopy are clearly of importance in studying bacterial attachment to epithelial cells.

2.11.2 SCANNING ELECTRON MICROSCOPY.

The scanning electron microscope was used to examine uroepithelial cell samples with and without bacteria attached. (Plates 37 to 43). Rod shaped bacteria were seen on the surface of uroepithelial cells which had been assayed with E.coli. A proportion of "target cells" were noted to which large numbers of bacteria were attached. Bacteria were in some cases packed together on the surface of uroepithelial cells (Plate 43). Often, differently shaped organisms were closely associated on the cell surface. This was thought to be an association between E.coli and indigenous bacteria. These bacteria may have been present in microcolonies on the uroepithelial cell surface, as also suggested by Marrie et al. (1980,a). Many of the uroepithelial cells were covered with microridges,and microvilli structures giving them a "cobblestone" appearance as described by Davies and Hunt (1981). In that study and in the present study, bacteria were seen attached to microridges and microvilli, of some cells. In the control samples, with no E.coli added, many of the uroepithelial cells were found to be devoid of bacteria, though a few cells had several rod-shaped or coccal shaped bacteria on the surface (Plate 39).

Bacteria have also been found to attach to urethral cells examined by scanning electron microscopy after removal from patients with urinary tract infection (Colleen et al., 1980). These authors also report the presence of microvilli structures on the cell surface and the existence of target cells to which > 10 E.coli were found to be attached. Ferenczy (1977) has investigated the human uterine epithelium at various stages in the menstrual cycle, and found that 17β oestradiol stimulated the formation and development of a large number of cilia and microvilli on the epithelial cell surface. It remains a possibility that hormones such as 17β oestradiol alter the surface of uroepithelial cells over the menstrual cycle, and thereby, affect the receptivity of these cells to E.coli attachment. A scanning electron microscope study of uroepithelial cells collected at regular intervals over the menstrual cycle, particularly from women susceptible to UTI, may uncover interesting results.

APPENDIX 1

ANALYSIS OF 145 E.COLI URINARY ISOLATES AND 38 FAECAL ISOLATES FOR ATTACHMENT TO UROEPITHELIAL CELLS AND HAEMAGGLUTINATION OF HUMAN AND GUINEA PIG ERYTHROCYTES.

(RESULTS ADJUSTED TO REFERENCE STRAIN 87 FOR BHI BROTH GROWN CULTURES AND STRAIN 207 FOR TSA GROWN CULTURES).

URINARY ISOLATES

STRAIN	SEROTYPE	BHI BROTH GROWN CULTURES			TSA GROWN CULTURES		
		HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL
27	06:H1	-	-	4	MRHA	-	19
32	06:H1	-	-	6	MRHA	-	18
33	04:K1:HR	-	MSHA	10	-	-	9
35	016:HNT	-	MSHA	12	MRHA±	MSHA	18
37	0153:H5	MRHA	MSHA±	NT	MRHA	MSHA	NT
40	075:H-	-	-	5	MRHA	-	35
42	02:K1:H6	-	MSHA	8	-	MSHA	7
43	0R:K1:H-	-	-	5	-	-	4
44	06:K1:H-	-	MSHA	9	-	MSHA	6
47	0R:H-	-	MSHA±	9	MRHA	-	18
49	023:H45	-	MSHA	11	-	MSHA	7
50	038:K1:H-	MSHA±	MSHA	NT	MSHA	MSHA	NT
52	06:H1	-	-	6	MRHA	-	20
53	0NT:K1:H-	MSHA±	MSHA	NT	MRHA±	MSHA	NT
54	06:H1	MRHA	-	12	MRHA	-	22
59	015:H-	MRHA±	MSHA	NT	MRHA	MSHA±	NT
61	025:H1	-	-	6	-	-	6
63	06:H1	MRHA	MSHA	17	MRHA	-	44
65	02:H4	-	-	10	MRHA±	-	20
66	02:H4	-	-	9	-	-	12
67	02:H6	-	MSHA	12	-	MSHA	5
68	01:H7	-	MSHA	9	MRHA	-	19
69	0NT:H-	-	MSHA	5	-	MSHA	4
73	08:K1:H14	-	MSHA	11	MRHA	MSHA	82

APPENDIX 1 Cont'd

STRAIN	SEROTYPE	BHI BROTH GROWN CULTURES			TSA GROWN CULTURES		
		HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL
74	ONT:K1:H1	-	-	5	-	-	4
75	ONT:H14	-	MSHA±	NT	MRHA	MSHA	NT
81	06:K1:H-	-	MSHA	6	MSHA	MSHA	9
83	02:K1:H6	-	MSHA	7	MRHA	MRHA	21
84	02:K1:H6	-	MSHA	10	-	MSHA	10
86	075:H-	-	-	9	MRHA±	-	29
87	075:HR	-	MSHA	14	-	-	16
88	0107:H27	-	MSHA	NT	-	MSHA	NT
89	075:H5	-	-	8	MRHA	-	54
90	ONT:H1	-	-	13	MRHA	-	19
94	075:H-	-	-	5	MRHA	-	23
95	051/111:K1:H4	-	MSHA	7	-	MSHA	10
97	06:K1:H31	-	MSHA	8	MSHA	MSHA	10
104	02:H4	MRHA	-	10	MRHA	-	51
105	07:H-	MRHA	MSHA±	15	MRHA	MSHA±	18
107	04:H5	-	MSHA	9	MRHA	MSHA	19
114	04:H-	-	MSHA	11	-	MSHA	11
116	ONT:HR	-	-	10	MRHA	-	36
120	ONT:H16	-	MSHA	NT	-	-	NT
122	075:H-	-	-	11	MRHA	-	70
125	ONT:H1	-	-	9	MRHA	-	57
127	ONT:H14	-	MSHA	NT	-	MSHA	NT
129	075:H-	-	-	13	MRHA	-	39
130	02:K1:H1	-	MSHA	7	-	MSHA	7
132	048:H-	-	-	8	-	-	8
135	0133:H1	MSHA±	MSHA	NT	-	MSHA	NT
136	ONT:H8	MSHA±	MSHA	NT	MSHA±	MSHA	NT
140	021:H33	-	MSHA	NT	-	MSHA	NT
142	09:K1:H19	-	MSHA	NT	-	MSHA	NT
143	01:H7	-	MSHA±	15	MRHA	-	39
146	06:H5	-	-	8	MRHA	-	33
148	088:K1:H5	MSHA±	MSHA	NT	MSHA	MSHA	NT
151	09:HNT	-	-	NT	-	-	NT
153	07:K1:H-	-	MSHA±	10	MRHA	-	39
158	04:H5	MRHA	-	10	MRHA	-	30
159	07:HNT	-	-	9	MRHA	-	49

APPENDIX 1 Cont'd

STRAIN	SEROTYPE	BHI BROTH GROWN CULTURES			TSA GROWN CULTURES		
		HAEMAGGLUTINATION ERYTHROCYTES HUMAN	GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL
160	O103:K1:HNT	-	MSHA	NT	MSHA±	MSHA	NT
163	O68:HNT	-	MSHA±	NT	-	MSHA±	NT
166	O75:H-	-	-	10	MRHA	-	26
168	O36:H4	-	-	NT	MRHA	-	18
169	OR:H-	-	MSHA±	NT	-	-	NT
172	O2:K1:H6	-	MSHA	10	MSHA±	MSHA	12
173	O18ab:K1:H-	-	MSHA	8	MRHA	MSHA	41
177	O6:H1	-	MSHA	15	MRHA	-	24
178	O75:H-	-	-	7	MRHA	-	59
181	O7:K1:H-	-	MSHA	7	MRHA	MSHA	20
183	O6:H1	-	-	4	MSHA	MSHA	7
184	O36:H39	-	MSHA	NT	-	MSHA±	NT
186	O45:HR	-	-	NT	-	-	NT
190	O2:K1:H6	-	MSHA	10	-	MSHA	7
199	O6:K1:H-	-	MSHA	9	MSHA	MSHA	5
200	O68:HNT	-	MSHA	NT	-	MSHA	NT
202	O102:K1:H14	-	MSHA	NT	MRHA	MSHA	18
206	O6:H1	MRHA±	-	10	MRHA	-	27
207	O75:H-	-	-	8	MRHA	-	58
208	ONT:H-	MRHA±	-	NT	MRHA	MSHA	NT
210	O14:H-	-	MSHA	NT	-	MSHA	NT
215	O75:H-	-	-	10	MRHA±	-	54
218	O1:H-	-	-	12	-	-	3
219	ONT:H4	-	MSHA±	NT	-	MSHA	NT
220	O22:K1:H1	MSHA±	MSHA	NT	-	MSHA	NT
226	ONT:H17	-	MSHA	7	-	MSHA	8
227	ONT:H39	-	-	NT	MRHA	-	NT
231	O2:H6	-	MSHA	13	-	MSHA	8
232	O6:K1:H1	-	MSHA±	6	MRHA	-	28
233	O6:K1:H1	-	MSHA	7	-	MSHA	7
234	ONT:H21	MRHA±	MSHA	NT	MRHA	-	NT
235	O5:H21	MSHA±	MSHA	NT	-	MSHA	NT
239	O6:H-	-	-	11	MRHA	-	28
240	O2:K1:H6	-	MSHA	6	-	MSHA	6
242	O75:H5	-	-	8	-	-	10
243	O2:K1:H7	-	MSHA±	9	MRHA±	-	18

APPENDIX 1 Cont'd

STRAIN	SEROTYPE	BHI BROTH GROWN CULTURES			TSA GROWN CULTURES		
		HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL
248	O2/111:K1:H7	-	-	NT	-	-	NT
249	O7:K1:H-	-	MSHA±	10	MRHA	MSHA	22
250	OR:HNT	-	MSHA±	NT	-	MSHA±	NT
255	O20:K1:H31	MSHA	MSHA	NT	MSHA	MSHA	NT
261	O91:K1:H10	-	MSHA	NT	-	MSHA	NT
262	O6:H1	MRHA	-	15	MRHA	-	21
263	ONT:K1:H7	-	MSHA	NT	-	MSHA	NT
269	O18ab:H-	-	MSHA	9	-	MSHA	7
274	O6:H31	-	MSHA	6	-	-	6
275	O68:H-	-	-	NT	MRHA±	-	NT
278	O6:H1	-	MSHA	6	MRHA	-	18
279	ONT:H7	-	-	NT	-	-	NT
282	ONT:K1:H1	-	MSHA	12	MRHA	-	26
283	O7:H1	-	MSHA±	12	MRHA	MSHA±	39
284	OR:H-	-	-	16	MRHA±	-	57
285	O17:K1:HNT	-	MSHA	5	-	MSHA	4
286	O6:K1:H1	MSHA	MSHA	6	MSHA	MSHA	7
288	O15:H-	-	MSHA	7	-	-	4
289	O1:H7	MRHA±	-	17	MRHA	-	69
290	O12:H4	-	MSHA	6	-	MSHA	5
299	O9:H19	-	MSHA	6	-	MSHA	9
300	O54:H21	-	MSHA	8	MRHA	MRHA	15
301	O75:H-	-	-	8	MRHA	-	70
302	O2:H1	-	MSHA	6	MSHA	MSHA	4
303	O18ac:H-	-	MSHA	8	MSHA	MSHA	8
304	O15:H2	-	MSHA	12	-	MSHA	7
305	O7:H6	MSHA±	MSHA	7	MSHA±	MSHA	7
306	O6:H1	-	-	7	MRHA	-	17
307	O6:H12	MSHA±	MSHA	6	MSHA	MSHA	8
308	ONT:H19	-	MSHA	6	-	MSHA	5
309	ONT:H-	-	MSHA±	9	-	-	9
310	O51/147:H4	MSHA	MSHA	4	-	MSHA	3
311	ONT:H9	MSHA±	MSHA	6	MSHA	MSHA	6
312	O2:H1	MRHA	MSHA	12	MRHA	MSHA	33
313	ONT:H-	-	-	6	MRHA	-	62
314	OR:H10	-	-	2	-	-	2

APPENDIX 1 Cont'd

STRAIN	SEROTYPE	BHI BROTH GROWN CULTURES			TSA GROWN CULTURES		
		HAEMAGGLUTINATION ERYTHROCYTES HUMAN	GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL
315	012:HNT	-	-	3	-	-	5
316	075:HNT	-	-	7	-	-	6
317	08:H9	-	-	6	-	-	3
318	0111:H12	-	-	7	-	-	4
319	088:HNT	-	-	3	MRHA±	MSHA±	13
320	02:KNT:H4	MRHA	MSHA	12	MRHA	-	25
321	04:H-	MRHA	MSHA	11	MRHA	-	23
322	06:K2:H1	MRHA	MSHA	16	MRHA	-	34
323	06:K2:H1	-	MSHA	6	-	MSHA	3
324	06:H1	-	-	10	MRHA	-	20
325	051:H15	-	MSHA	7	-	MSHA	3
326	06:H-	-	MSHA	6	MSHA±	MSHA	9
327	016:H48	MSHA	MSHA	6	MRHA	MSHA	21

APPENDIX 1 Cont'd FAECAL ISOLATES

STRAIN	SEROTYPE	BHI BROTH GROWN CULTURES			TSA GROWN CULTURES		
		HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	HAEMAGGLUTINATION ERYTHROCYTES GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL
Co 1	OR:H-	-	-	6	MRHA	-	25
Co 2	01:H33	MRHA±	MSHA	8	MRHA±	-	16
Co 3	06:H1	-	MSHA	9	-	MSHA	7
Co 4	062:K1:H6	-	MSHA±	10	MRHA	MSHA±	17
Co 6	050:H5	-	MSHA	5	-	MSHA	7
Co 7C	OR:HNT	-	MSHA	3	MSHA	MSHA	5
Co 8A	015:K1:H6	-	MSHA	NT	-	MSHA	NT
Co 8B	02:H4	-	MSHA±	8	MRHA±	-	15
Co 9	0142:K1:H21	-	MSHA±	8	MRHA±	MSHA±	18
Col0A	OR:H-	-	-	6	-	-	7
Col0C	025:HNT	MSHA±	MSHA	NT	MSHA±	MSHA	NT
Col1A	OR:K1:H-	-	-	NT	-	-	NT
Col1B	04:K1:H5	-	MSHA	8	MRHA	MSHA	16
Col2A	015:K1:H6	-	MSHA	NT	-	MSHA	NT
Col2B	0112ab:H-	-	-	NT	-	-	NT
Col3	020:H-	-	-	NT	-	-	NT
Col4	OR:H4	-	MSHA	NT	-	MSHA	NT
Col5A	071:H48	-	-	NT	-	-	NT
Col7A	OR:H-	-	MSHA	5	-	MSHA	4
Col8A	092:H33	-	-	NT	-	-	NT
Col8B	011:K1:H15	-	MSHA	5	-	MSHA	4
Col9	OR:H7	-	-	6	MRHA±	-	19
Co20	0128:K1:HR	-	-	17	MRHA	MRHA	22
Co21	OR:H10	-	-	7	-	-	6
Co22	015:K1:H17	MSHA±	MSHA	NT	MSHA±	MSHA	NT
Co23	06:H1	-	MSHA	11	-	MSHA	11
Co24	01:K1:H33	MRHA	MSHA	13	MRHA	MSHA	18
Co25	OR:K1:H-	-	-	8	-	-	10
Co26	0128:K1:H12	-	MSHA	NT	-	MSHA	NT
Co27	0141:K1:H7	MSHA	MSHA	11	MRHA	MSHA	17
Co28A	038:HNT	MSHA±	MSHA	NT	-	MSHA	NT
Co28B	038:HNT	-	-	NT	-	-	NT
Co29	OR:H8	-	MSHA	7	-	MSHA	8
Co30	070:H4	MSHA±	MSHA	NT	MSHA±	MSHA	NT

APPENDIX 1 Cont'd

STRAIN	SEROTYPE	BHI BROTH GROWN CULTURES			TSA GROWN CULTURES		
		HAEMAGGLUTINATION ERYTHROCYTES HUMAN	GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL	HAEMAGGLUTINATION ERYTHROCYTES HUMAN	GUINEA PIG	MEAN NUMBER E.COLI PER UROEPITHELIAL CELL
Co31	07:H-	-	MSHA±	8	MRHA	MSHA±	17
Co32	069/79:H-	-	MSHA	11	MRHA	MSHA	36
Co33	0111/140:HNT	-	MSHA	NT	-	MSHA	NT
Co37	021:K1:H-	-	-	9	MRHA±	-	23

LEGEND FOR APPENDIX 1

06 = 0 Serotype 6

H1 = Flagellar Antigen 1

K1 = Capsular Antigen 1

ONT and HNT = Not Typable

OR and HR = Resistant Type

H- = Non Flagellate

051/111 = Antigenically similar

NT = Not Tested.

APPENDIX 2

PROPERTIES OF E.COLI URINARY STRAINS RELATED TO THE POSSESSION OF A MRHA.

	FERMENTATION			HAEMOLYSIN			PHOSPHOLIPASE	K. ANTIGEN RECIPROCAL TITRE
STRAIN	DULCITOL	SALICIN	SUCROSE	HORSE	SHEEP	HUMAN O		
27	+	+	-	+	-	+	-	512
32	+	+	-	+	-	+	-	64
40	+	+	+	+	-	-	-	16
47	+	+	-	-	-	-	-	≥1024
52	+	+	-	+	-	+	-	32
54	+	+	-	+	-	+	+	128
63	+	+	-	+	-	+	-	16
65	+	-	+	-	-	-	-	512
68	+	+	+	-	-	-	-	64
86	+	+	+	-	-	-	-	≥1024
89	+	+	+	-	-	-	-	64
90	+	+	-	+	-	+	-	≥1024
94	+	+	+	-	+	-	-	32
116	+	+	+	-	-	-	-	64
122	+	+	+	-	-	-	-	256
125	+	+	+	-	-	-	+	256
129	+	+	+	-	-	-	-	≥1024
143	+	-	-	+	+	-	+	256
146	+	+	-	+	-	+	-	256
153	+	-	+	-	-	-	-	4

Cont'd

APPENDIX 2 Cont'd

STRAIN	FERMENTATION			HAEMOLYSIN			PHOSPHOLIPASE	K. ANTIGEN RECIPROCAL TITRE
	DULCITOL	SALICIN	SUCROSE	HORSE	SHEEP	HUMAN O		
158	-	+	-	+	-	+	-	256
159	+	-	-	-	-	-	-	2
166	+	+	+	-	-	-	-	≥1024
168	+	-	-	-	-	-	-	NIL
177	+	+	-	+	-	+	-	128
206	+	+	-	+	-	+	-	8
207	+	+	+	+	-	-	-	≥1024
215	+	+	+	-	-	-	-	≥1024
227	+	+	+	-	-	-	-	256
232	+	+	-	+	+	+	-	4
234	+	+	-	-	-	-	-	≥1024
239	+	+	-	+	-	+	-	128
243	+	+	-	-	-	-	-	32
262	+	+	-	+	-	+	-	32
275	+	-	-	-	-	-	-	NIL
278	+	+	-	-	-	+	-	128
282	-	+	-	-	-	-	-	NIL
284	+	-	+	-	+	-	-	128
289	+	-	-	-	-	-	-	≥1024

APPENDIX 3

PROPERTIES OF E.COLI URINARY STRAINS RELATED TO THE POSSESSION OF A MSHA

STRAIN	FERMENTATION			HAEMOLYSIN			PHOSPHOLIPASE	K. ANTIGEN RECIPROCAL TITRE
	DULCITOL	SALICIN	SUCROSE	HORSE	SHEEP	HUMAN O		
33	+	+	-	+	-	+	-	≥1024
42	+	+	+	-	-	-	-	128
44	+	+	+	+	-	+	+	512
49	+	-	+	-	-	-	-	NIL
50	+	-	-	-	-	-	-	NIL
67	+	+	+	-	-	-	-	NIL
69	+	-	-	-	-	-	-	NIL
81	+	+	+	+	-	+	+	256
84	+	+	+	+	-	+	-	32
87	+	+	+	-	-	-	-	32
88	-	+	-	-	-	-	-	≥1024
95	+	+	+	-	-	-	+	128
97	+	+	+	+	-	+	-	≥1024
114	+	+	-	+	-	-	-	4
120	+	+	+	-	-	-	-	512
127	+	-	+	-	-	-	-	64
130	+	+	-	+	-	+	+	256
135	-	-	-	-	-	+	-	256
136	+	+	+	+	-	-	-	NIL
140	-	-	+	-	-	-	-	NIL
142	+	+	+	-	-	-	-	NIL
148	+	+	-	-	-	-	-	512
160	+	+	+	-	+	-	-	NIL
163	+	-	-	-	-	-	-	≥1024

Cont'd

APPENDIX 3 Cont'd

STRAIN	FERMENTATION			HAEMOLYSIN			PHOSPHOLIPASE	K. ANTIGEN RECIPROCAL TITRE
	DULCITOL	SALICIN	SUCROSE	HORSE	SHEEP	HUMAN O		
169	+	-	+	-	+	-	-	32
172	+	+	+	+	-	+	-	16
183	+	+	-	-	-	-	-	16
184	+	+	-	+	+	-	-	8
190	+	+	+	-	-	-	-	512
199	+	+	+	+	-	+	-	128
200	+	-	-	-	-	-	-	128
210	+	+	-	-	-	-	-	≥1024
219	+	+	-	-	-	-	-	NIL
220	+	+	-	+	-	+	-	NIL
231	+	+	+	-	-	-	-	4
233	+	-	+	-	-	-	+	≥1024
235	-	-	-	+	-	-	-	≥1024
240	+	+	+	+	-	+	-	64
250	+	-	-	+	+	+	-	NIL
255	+	+	+	+	-	+	-	64
261	+	+	+	-	-	-	+	NIL
263	-	+	+	-	-	-	-	NIL
269	-	-	-	-	-	-	-	NIL
274	+	+	+	+	-	+	-	128
285	+	-	+	-	-	-	-	NIL
286	+	+	-	+	-	+	-	≥1024
288	+	+	-	-	-	-	-	32

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A Wee Poem

You don't catch it off your granny,
Or because your cousin's mummy
Has had it twice
And she's only 33.

But you get it, why we don't know,
In the sun, the rain and the snow
And it might come back
As a painful little wee.

Having showers and beer unrelented,
May very well help prevent it,
As long as kidney stones
Don't get to block the way.

But as for sex; that's another story,
Though I dare say, not quite so gory,
As some of the things that
You hear some people say.

So if you're female, then beware dear
Coz you're more likely to get snaired dear
Though the drugs may cure
Your ills and pains quite fast.

Still, if you're lucky, in the long run,
And us Scientists find the right gun,
Then we may prevent it,
Or destroy it - to the last.

Gregor Reid
1980