EXPERIMENTAL *ESCHERICHIA COLI* URINARY TRACT INFECTIONS IN THE MOUSE

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The cellular immune response to E.coli in mice was examined by the delayed hypersensitivity response in the footpad. Viable bacteria, as present during an acute urinary tract infection, were required to sensitize the mice. However, as all mice could not be infected to the same degree, the delayed hypersensitivity reaction was not easily standardised. Results with inbred mice indicate that this response is not correlated with immunity to infection.

Strains of inbred mice varied in their response to urinary tract infections; some were susceptible to infection and others were resistant. Crossing a sensitive strain with a resistant strain resulted in an F_1 generation of mice that were susceptible to urinary tract disease.

Inducing a diuresis in mice by adding 5% glucose to their drinking water increased the susceptibility of the animals to kidney infection. The osmolality of urine determined whether E.coli grew or was killed in vitro. The relevance of this finding and the results above to the course of E.coli urinary tract infections in man are discussed.

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CHAPTER 1

INTRODUCTION

Over 100 years ago Louis Pasteur observed that human urine could support the growth of bacteria (Pasteur, 1863). Since then a considerable amount of scientific attention has been concentrated on the study of urinary tract infections. Epidemiological studies have shown that this type of infection is prevalent throughout the human population, race and geographical location having little effect on the incidence of the disease (Kunin and McCormack, 1968; Levy and Kass, 1970). Women appear to be more susceptible to infection than men and the incidence of the disease rises in women with increasing age, sexual activity and parity (Kunin and McCormack, 1968; Kass, 1960). Urinary tract infections can also lead to complications during pregnancy (Brumfitt *et al*, 1967).

Escherichia coli is by far the most common cause of urinary tract infections. In otherwise healthy women outside of hospital *E.coli* is responsible for more than 90% of urinary tract infections (Gillespie *et al*, 1971). The symptoms and severity of the acute disease vary markedly from person to person as does the degree of kidney involvement. Recurrent infections are also a problem. Anatomical abnormalities of the urinary tract greatly increase the susceptibility to infection but renal involvement occurs frequently in the absence of any obstructions or dysfunction.

Study of the factors involved in the pathogenesis of the disease could therefore be useful in the treatment and prevention of urinary tract infection.

Experimental models of infection

To investigate urinary tract infections *in vivo* a number of experimental methods of inducing urinary tract infection have been utilised. It is possible to produce infection in many small animals with most of the common urinary tract pathogens. Experimental models vary as to the route by which bacteria are introduced into the urinary tract, and whether obstruction or trauma are used.

Haematogenous infections can be initiated by injection of bacteria into the bloodstream. Rapid clearance of bacteria from the circulation occurs and some of the organisms become lodged in the kidney. Direct injection of bacteria into the kidney tissue will also produce kidney disease. In both cases for infection of the kidney to occur, temporary ligation of the ureter, massage of the kidney or local injury to the organ is necessary (Braude *et al*, 1955; Kaijser and Olling, 1973). An ascending infection can be induced by injection of bacteria into the bladder.

The pathological differences between haematogenous and ascending pyelonephritis were investigated by Heptinstall (1965). He produced haematogenous infections in rats by the intravenous injection of E.coli and temporary occlusion of the ureter and ascending infection by injection of bacteria into the bladder. Reflux of organisms up the ureters was assisted by squeezing the bladder several times with the external meatus compressed. In some experiments the ureters were obstructed during the induction of ascending infection.

Different patterns of infection were noted for the two types of disease. The haematogenously induced disease gave widespread lesions throughout the kidney whereas the ascending type lesions appeared earlier and were initially confined to the fornices and perihilar regions.

Obstruction of the ureter after bladder inoculation of bacteria resulted in a blood borne infection pattern. In this case it was suggested that a low dose of organisms probably entered the blood stream during bladder massage and lodged in the obstructed kidney. The different patterns of infection produced by the two methods suggests that they must be treated as two separate and unrelated means of induction of disease.

The route of infection in humans is generally thought to be ascent from the urethra to the bladder and from there the kidneys are infected by movement of bacteria up the ureters. It is therefore this mode of infection which has been most widely imitated in experimental infections. Reflux of bacteria up the ureters to the kidney will result in renal disease as shown by Heptinstall (1965). However the intact bladder of experimental animals is very difficult to infect and injected bacteria are cleared rapidly.

Modification of the bladder by the introduction of a foreign body such as a glass bead or removal of a portion of the bladder wall greatly facilitates establishment of infection (Rocha and De Almeida, 1965; Rocha and Barros, 1965). The mouse model of infection that we have used relies on the insertion of two small silk stitches into the anterior bladder wall. These act as a foreign body within the bladder. Bacteria injected into the lumen of the bladder rapidly multiply and some ascend the ureters to infect the kidneys. Infections have been shown to persist in the urinary tract for at least 50 days (Nicholson and Glynn, 1975).

No surgical intervention was needed in rats to produce infection, if the rats were subjected to a diuresis 3 to 5 days prior to bladder inoculation of bacteria (Freedman, 1967). Diuresis was accomplished by allowing the rats free

access to a solution of 5% glucose in tap water. Infection in the rats undergoing diuresis rapidly spread from the bladder to the kidneys. In rats with infected kidneys due to injection of bacteria directly into the cortex of one kidney, diuresis resulted in rapid spread of the bacteria to the bladder and then to the other kidney.

Keane and Freedman (1967) producedpyelonephritis in mice drinking 5% glucose in water, upon bladder inoculation of E.coli. In contrast Andriole (1970) demonstrated in haematogenous infections in rats that reduction of water intake increased the susceptibility of the kidney to infection, while diuresis had a protective effect. This anomaly of the opposite effects of diuresis for the two types of infection is presumably due to inherent differences in the two models. Dilution of the urine may provide bacteria injected into the bladder with a better medium for growth, whereas dilution of urine in the kidney could lower the tonicity of the kidney and thus permit body defence mechanisms such as phagocytosis better conditions for dealing with the invading blood borne organisms. Thus diuresis is an aid to the establishment of ascending infections, but protective against haematogenous infections.

Many methods are thus available to initiate urinary tract infections in experimental animals. It is difficult to be certain which of these best simulate the infection in human beings. As infection in people is generally thought to

be of the ascending type, an experimental infection with blood borne bacteria in combination with renal massage is not necessarily a true representation of the human disease.

It is hoped that our model of ascending infection, in which the only aid to bacterial colonisation is the presence of a foreign body ,is a reasonable model of the human disease. Reservations must still be made however when it comes to interpreting results obtained. Direct extrapolation from the mouse model to the human patient may not always be possible due to differences in the anatomy and functioning of the urinary tracts of mice and people.

In spite of their limitations, however, animal models have been widely used to test hypotheses that evolve from the observation of human urinary tract infections. Experimental infections have been performed in order to attempt to characterise the host response to infection. These and our own studies using the mouse model of infection will be discussed later.

Characteristics of strains of E.coli that cause urinary tract infections

Aerobic gram-negative bacilli are responsible for the majority of urinary tract infections although occasionally other micro-organisms such as enterococci or staphylococci are encountered. *E.coli* is, however, the predominant

pathogen. Gillespie, Lee and Linton in 1971 showed that in otherwise healthy women, outside of hospital, E.coli was responsible for more than 90% of urinary tract infections.

Many strains of E.coli can be serologically characterised by their possession of three different types of antigen: the flagella or H antigen, the cell wall or O antigen, and the capsular or K antigen.

Approximately two-thirds of uncomplicated asymptomatic urinary tract infections are due to E.coli strains belonging to just a few O serotypes; O types 1, 2, 4, 6, 7, 25, 50 and 75 are those most frequently found in urinary tract disease (Bergstrom et al, 1967; Grüneberg et al, 1968; Rantz, 1962; Turck and Petersdorf, 1962; Vahlne, 1945; Vosti et al, 1964). Rantz (1962) proposed that these E.coli with the common serotypes were those found most often causing urinary tract disease because the possession of these O antigens conferred invasive properties on the bacteria. Thus, these serotypes had a special pathogenicity for the urinary tract.

Further investigation of the distribution of the common serotypes revealed that these types also predominated in the faecal flora. Thus these serologically identifiable O types are found more often in urinary tract infections because they are more prevalent among the organisms of the faeces.

The faecal flora is the source of organisms for an ascending infection and as these serotypes are the most common in the bowel they are also found with the greatest frequency in bacteriuria. The O antigen would therefore not appear to confer any special pathogenic properties on E.coli strains as regards their infectivity for the urinary tract (Brumfitt, 1969; Turck and Petersdorf, 1962).

Most E.coli possess surface located K antigen, often in the form of a distinct capsule. The K antigen of E.coli was first described by Kaufmann over 30 years ago (Kaufmann, 1943). It was designated the K antigen from the German word for capsule : Kapsel.

Three types of K antigens have been distinguished and labelled L, A and B (Kaufmann, 1966). The L antigens are thermolabile, i.e. lose their immunogenicity after heating to 100°C, while the more common B antigen although losing immunogenicity at 100°C is still capable of being bound to antibody after this treatment. The A antigens are resistant to boiling but are inactivated by heating to 120°C (Ørskov and Ørskov, 1970). The A and B antigens are usually acid polysaccharides but some of the L antigens have been shown to be of a protein nature (Hungerer et al, 1967).

Many investigators have been interested in the importance of K antigen as a bacterial virulence factor. Vahlne (1945) and Sjösted (1946) found that capsulated forms of E.coli were more frequently detected in clinical isolates than in normal faeces. These types were also capable of growth outside the bowel such as in the peritoneum and urine and could haemolyse red blood cells. This suggested that they had special pathogenic properties.

The presence of K antigen was shown by Glynn and Howard (1970) to influence the sensitivity to complement of strains of E.coli. The amount of K antigen present, as measured by the ability to inhibit the agglutination of sheep red blood cells by rabbit haemolysin,was directly related to the complement resistance of a particular strain. When injected into mice strains of E.coli rich in K antigen were less readily phagocytosed than strains of E.coli with little or no K antigen (Howard and Glynn, 1971a). The presence of K antigen also increased the virulence of E.coli for mice when injected intracerebrally.

Sjösted in 1946 first suggested that the K antigen may confer properties on E.coli which enhance the pathogenicity of the bacteria for the urinary tract. Glynn, Brumfitt and Howard (1971) examined strains of E.coli isolated from the urine of patients with bacteriuria. They compared them with faecal strains from antenatal patients

and with faecal specimens from randomly selected hospital patients. A significantly greater proportion of K rich strains was found in the urine isolates compared with either set of faecal controls. This excess of K rich strains in the urine isolates was due to patients in whom the kidneys were infected and who thus had both renal and bladder infections. Thus, while strains of E.coli reach the bladder in proportion to their frequency in the faecal flora, strains rich in K antigen would appear to be more likely to succeed in subsequently invading the kidneys.

In contrast, Mabeck, Ørskov and Ørskov (1971) were unable to correlate individual O, K and H antigens of the infecting strain with renal pathogenicity. Three serotypes O2:K1:H4, O4:K12:H5 and O6:K2a;2c:H1 were found to be significantly more common in cases of pyelonephritis than other strains. However no quantitation of the K antigen present was made.

Strains of E.coli with serotypes O1, O2, O6 and O7 were tested for the presence of K2a:2c, K13 and K1 antigen by Kaijser (1973). Bacteria were obtained from schoolgirls with urinary tract infections and the stools of healthy schoolchildren. K antigen was present more often and in significantly greater amounts in E.coli strains from the urine of

children with pyelonephritis than among the strains isolated from cases of cystitis, or from the stools of healthy schoolchildren.

Recently McCabe et al (1975) have confirmed that K rich strains are more frequently isolated from urine than from faeces. They could not however detect any correlation between the K antigen content of strains isolated from 100 bacteraemic patients with the severity and outcome of bacteraemia. The amount of K antigen possessed by the blood culture isolates was not significantly greater than that of the faecal isolates, but was significantly lower than that of the urinary E.coli isolates. The possession of K antigen therefore does not enhance the virulence of E.coli in bacteraemia.

This finding is in opposition to the results of Roantree and Pappas (1960). Twenty-four and forty-eight hours after the intravenous inoculation of bacteria into rabbits, the bacterial content of the blood was determined. In each of 6 experiments in which bacteria were found at 24 hours, the complement resistant strains outnumbered the complement sensitive strains by about 10 to 1. Thus the complement resistant strains had better survived the bactericidal effect of rabbit serum and extrapolating back to the human patient would be expected to cause more cases of bacteraemia than

complement sensitive strains. This confirmed the clinical findings of Roantree and Rantz (1960) where bacteria isolated from the blood of bacteraemic patients were found to be more likely to be resistant to the bactericidal action of serum than those isolated from the stools and urine.

As no determination of the K antigen content of these "complement resistant" strains was made it may not be valid to compare these findings with those of McCabe et al. (1975) Taylor (1975) has shown that the K antigen of E.coli is not the only factor determining complement resistance. It would appear however from the findings of Glynn et al (1971), Kaijser (1973), and McCabe et al (1975) that the K antigen of E.coli if not confering pathogenic properties on bacteraemic isolates, does enable strains of E.coli possessing this capsular antigen to successfully invade kidney tissue.

Myerowitz et al (1972) found that out of 1,535 urinary tract isolates, 20 strains of E.coli and 1 of Pseudomonas aeruginosa cross reacted with the capsular polysaccharides of pneumococci, meningococci, and Haemophilus influenzae type B. These cross reactive antigens appeared to be acidic polysaccharides resembling E.coli K antigens. Acidic polysaccharides confer invasive properties on pyogenic bacteria and Myerowitz et al therefore suggested that the invasiveness of certain faecal flora for the urinary tract was related to

the possession of this type of surface antigen. They did not however differentiate between strains causing renal infection and those responsible for infection limited to the bladder; the cross reacting bacteria were also only a small proportion of all the urinary tract isolates.

The H antigen of E.coli is the flagella of the bacteria. Thus only motile strains of E.coli will possess H antigen. Non-motile strains have no flagella and are H antigen negative. Both motile and non-motile E.coli can cause urinary tract infections.

Weyrauch and Bassett (1951) in experiments using an artificial urinary tract were able to demonstrate that motile bacilli could move in the opposite direction to a flowing column of fluid. In contrast, even a stagnant column was not ascended by non-motile bacteria. If motile and non-motile organisms were present together,the motile bacteria carried the non-motile bacteria with them when ascending the fluid. More recently Boyarsky and Labay (1969) have reported that retrograde instillation of E.coli cultures, killed cultures and endotoxin into the bladder, stops ureteral peristalsis.

The experiments of Weyrauch and Bassett did not obviously include all the factors involved in the transmission of bacteria up the ureters to involve the kidneys. Non-motile bacteria will infect the upper urinary tract and

possibly reflux of urine up the ureters from the bladder aids their passage. If peristalsis is stopped by the action of endotoxin, as postulated by Boyarsky and Labay, this will aid the ascent of both motile and non-motile bacteria alike.

The host response to infection

The immune response to urinary tract infections due to E.coli probably involves both cell-mediated and humoral types of response. Cell-mediated immunity to infection has only recently been studied, whereas humoral immunity in the form of serum antibody levels has been investigated extensively for many years.

Measurements of the cellular immune response in persons suffering from urinary tract infection can be made by evaluating the activity of circulating or local lymphocytes. Soltys and Brody (1968) studied the adherence of bacteria to the circulating lymphocytes of persons with pyelonephritis. Less adherence occurred in patients with pyelonephritis than in control non-infected persons. The explanation for this was that either the reactive lymphocytes were sequestered by the infected renal parenchyma and thus the circulating pool was reduced in pyelonephritic persons, or that the cells had become tolerant to the antigen during the infection and no longer reacted against it.

The incorporation of ^{3}H-thymidine into the circulating and kidney lymphocytes of rabbits with haematogenously induced E.coli pyelonephritis was evaluated under basal conditions and in response to T cell mitogens, and specific E.coli antigens by Sanford and Barnett (1965). The kidney lymphocytes were found to be derived from the circulatory pool ,and enhanced lymphocyte reactivity to antigen in the kidney occurred at a time when the synthesis of local antibody increased. This increase in antibody coincided with the infecting bacteria losing their viability. Thus lymphocyte responsiveness may be an important determining factor on the course of an infection.

Whether the active lymphocyte represents T or B cells has not been established. Lymphocytes stimulated by lipopolysaccharide in mice have been shown to be exclusively B cells (Moller et al, 1973). It is possible that both kinds of lymphocyte are required at the site of infection to synthesise all types of antibody. Recently Smith (1975) has shown that the lipid of lipopolysaccharide is necessary for the activation of B lymphocytes.

Serum antibodies against the infecting bacteria can often be detected in persons with urinary tract disease. The O antibody response in patients with E.coli infection can be diagnostically useful. Serum antibody measurement

can help to establish if the infection involves the kidneys or is limited to the lower urinary tract. Infection of the renal parenchyma gives an O antibody titre rise, whereas as a rule bladder infection results in agglutinating titres not significantly different to those normally found (Needell et al, 1955; Percival et al, 1964; Brumfitt et al, 1969).

The role of antibody produced during a urinary tract infection is questionable. Despite having high serum antibody titres people with renal disease still have active infections. Recurrence of infection is also a feature of urinary tract disease. Re-establishment of infection is often accompanied by a secondary immune response, as shown by increased antibody production (Vosti et al, 1965). Thus high serum antibody titres against the infecting organism would not appear to confer any protection.

The cellular immune response and the sequence in which specific antibodies were produced was followed in rats with experimental retrograde pyelonephritis (Spencer and Fairhead, 1972). IgM and IgA immunocytes were found in large numbers in the spleen, lymph glands and urinary tract within 48 hours of the initiation of infection. The IgM response reached a peak at 5-10 days and then declined. This coincided with a gradual and persistent increase in IgG immunocyte infiltration.

The peak IgM and IgA response occurred at the same time as a rapid decrease in bacterial cell numbers was observed. Remission of infection often occurred at this point. In contrast the appearance of the IgG response was associated with continuing infection.

If the IgM response is protective but is inhibited by the IgG response, infection may persist by negative feed-back of the IgG on IgM antibody production. Thus the production of antibodies may not necessarily protect against infection. The IgG response appears to inhibit the synthesis of specific antibody and may be a factor in the establishment of chronic infection.

Bluestone et al (1973) infected mice, undergoing a diuresis, by direct bladder inoculation of E.coli. Increased serum antibody levels were only found in animals that developed pyelonephritis. The marked hypergammaglobulinaemia of these animals afforded no protection against infection, as is found in humans.

Mice infected by our method show a rapid rise in circulating antibody levels, as measured by direct agglutination of both live and heat killed bacterial suspensions. The peak serum titre is reached by 7 days and high serum levels then persist for many weeks. Serum antibody would therefore not appear to be protective.

Attempts to prevent infection in experimental animals by immunisation prior to bacterial challenge have given varying results. Kaijser and Olling (1973) hyperimmunised rabbits with E.coli 06:K2a,2c;H1, or E.coli 022:K13:H1 or E.coli 02:K2ab:H1. After ligature of the ureter for 24 hours,rabbits were infected by intravenous injection of E.coli 06:K13:H1. Rabbits with antibodies to 06 or K13 were protected against infection. The H1 antibodies afforded no protection. Thus immunisation of rabbits reduced the incidence of haematogenous kidney infection.

Subcutaneous immunisation of mice with heat killed E.coli by Montgomerie et al (1972) protected mice from death due to endotoxin shock and from haematogenous pyelonephritis, but produced little or no protection from ascending infection in mice subjected to a diuresis. Thus immunisation would appear to protect against haematogenous but not ascending infections. As serum antibody produced during an infection has no apparent effect on an ascending infection,it is also possible that serum antibody has no protective effect on the infection initiated after immunisation has taken place. This could explain why many infections recur despite high serum titres against E.coli. As most human infections are thought to be of the ascending type, immunisation against the most common serotypes causing urinary tract disease would not be expected to be of any use in preventing people from contracting cystitis and pyelonephritis.

Antibody present within the urinary tract can be demonstrated by the technique of Thomas et al (1974). Immunglobulins secreted into the urinary tract coat the bacteria against which they are directed. Reacting bacteria obtained after centrifugation of infected urine samples with fluorescein conjugated immunoglobulin to the host immunoglobulin present on the surface results in a fluorescent complex. This can be viewed by normal fluorescent microscopy techniques. Thomas et al (1974) found fluorescent staining bacteria in the urine of 34 out of 35 patients with clinically defined pyelonephritis but in only 1 out of 20 patients with acute cystitis. All types of immunoglobulin could be detected by this method although IgG antibodies gave the strongest reaction. This test can be used as a diagnostic tool to identify the extent of urinary tract disease; people with a positive fluorescent antibody test are more likely to be suffering from pyelonephritis than people whose infected urine does not contain antibody coated bacteria.

The susceptibility of persons to urinary tract disease probably has some genetic basis. People vary in their resistance to infection and may often succumb to infection for no apparent reason. Genes would thus be expected to control the host response to infection.

The mouse model of ascending urinary tract infection, due to E.coli, has been used to investigate host and bacterial factors involved in the pathogenesis of the disease.

CHAPTER 2

THE ROLE OF BACTERIAL FACTORS IN THE PATHOGENESIS OF E.COLI URINARY TRACT INFECTIONS

INTRODUCTION

Glynn *et al* (1971) and Kaijser (1973) have provided evidence that the possession of the K antigen is related to the ability of strains of *E.coli* to cause kidney infection. The role of K antigens and other factors which may help to determine the virulence of *E.coli* for the urinary tract have been investigated in a mouse model of infection.

MATERIALS AND METHODS

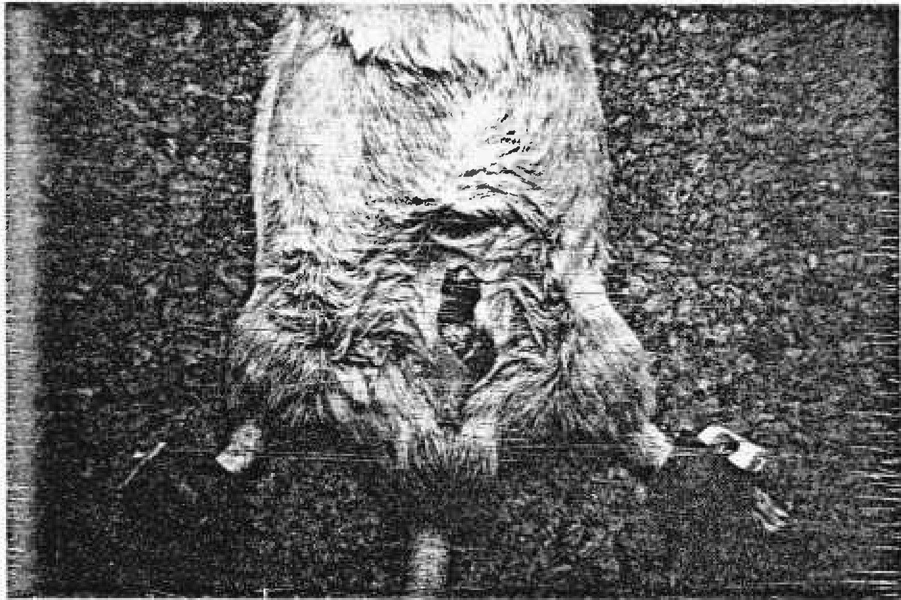
1) Experimental urinary tract infections in mice

The method of inducing urinary tract disease was used for all experiments (unless otherwise indicated) and was a modification of that suggested by Mr. M. Snell. Adult mice (3 to 6 months of age) were anaesthetised with veterinary Nembutal (Abbot Laboratories Ltd., Queenborough, Kent, ME11 5EL), by intraperitoneal injection of 0.6 mg/10 g mouse body weight and unconsciousness was maintained throughout the operation with ether.

Mice were secured to a cork board by electric cable clips covered with rubber tubing (see Plate 1) and the skin of the abdomen was swabbed with 70% alcohol to reduce infection at the site of the operation. The bladder was exposed

Plate I

Infective procedure. The bladder has been emptied and two small silk stitches have been inserted into the bladder wall. Injection of *E. coli* into the bladder lumen initiates the infection.



by a suprapubic incision and any urine present aspirated. It was essential to empty the bladder before injection of bacteria as overdistension of the bladder caused immediate micturation and loss of the inoculum.

Two small silk stitches (Mersutures Silk 5/0 B.P.C. Mersutures Ethicon Ltd., Scotland) were inserted into the upper anterior bladder wall, to act as a foreign body within the bladder (Plate 1). A dose of bacteria in a volume of 0.025 ml was injected into the bladder lumen with a microsyringe and 30 gauge needle. The peritoneum was closed with Catgut (Mersutures Plain 3/0 B.P.C.) and the skin layers held together with 9 mm Autoclips (Clay Adams, Parsipanny, N.J. 07054, U.S.A.). Appropriate aseptic precautions were taken throughout the procedure. Recovery of the mice from anaesthesia was aided by placing them in a box in a warm (30°C) room. Most mice survived the operative procedure; deaths within 24 hours of the operation were attributable to post-operative shock or adverse reaction to the anaesthetic.

2) Assessment of the infection

The mice were assessed for infection after three or more days. The animals were killed by cervical dislocation, and a 10 µl urine sample was taken for quantitative culture.

The kidneys were removed aseptically, weighed and homogenised for 2 minutes in 10 ml of 0.15 M saline using a Colworth Stomacher 80 (A.J. Seward & Co.Ltd., London).

Viable bacterial counts were made by one of two methods:-

1) Kidney and urine samples were diluted in 10% broth in saline, and 0.1 ml aliquots were plated in duplicate onto MacConkey agar plates, or

2) Samples were diluted into molten MacConkey agar and plated out as 0.1 ml drops by use of the Colworth Droplette (A.J. Seward & Co.Ltd.). 0.1 ml of the kidney homogenate was also plated onto MacConkey agar plates. The Droplette technique is a new method designed to economise on plastic petri dishes and agar growth medium (Sharpe and Kilsby , 1971; Sharpe et al, 1972).

It was found to be essential to add an extra 1% of agar to MacConkey agar used in the Droplette technique to counteract the loss of surface tension,due to the bile salts in the media, exerting a detergent action on the liquid drops. Unless the agar was added to the media 0.1 ml drops spread too far on contact with the petri dish and were too flat to be seen properly on the Droplette viewer. The two methods of viable counting gave comparable results, after incubation of all plates overnight at 37°C.

3) Bacteria

The strains of Escherichia coli with which the mice were infected have been previously used in this department (Table 1) (C.M. Milne, 1966; C.J. Howard, 1970). The K antigens of E.coli are capable of inhibiting the agglutination of sheep red blood cells by rabbit haemolysin. The agglutination inhibiting activity (AIA) of a strain of E.coli is a measure not only of the amount of K antigen the strain possesses but also of the biological activity of that K antigen. The resistance of E.coli to phagocytosis and complement killing, and the virulence of the bacteria for mice, is related to the K antigen content of the strain (Howard and Glynn, 1971a;Glynn and Howard, 1970).

Bacteria were grown overnight in glucose nutrient broth (Digest Broth + 0.1% glucose, Southern Group Laboratory, Hither Green Hospital, London), washed and resuspended in 0.15 M saline at the desired concentration as measured by optical density. The viable counts of inocula were confirmed by quantitative culture.

Table 1

E. COLI STRAINS

E.coli strain	Serotype	Agglutination inhibiting activity	Source	Complement sensitivity CH50 killing 50% of bacteria
WF82	O117:K? :H27	64	Piglet	29.0
WF41	O17:K16:H18	0	Stool	<0.7
WF96	O7:K1 : H6	2	Urine	0.8
WF98	O6:K13:H16	16	Urine	8.0
WF60	O6:K13: H1	32	Baby	9.5
WF 8	O6:K13: H^-	32	Urine	>32.4
WF26	O6:K13:H31	64	Urine	>32.4
WF 6	O6:K13: H^-	64	Urine	12.0

Data taken from C.M. Milne
Thesis "Lysozyme and immune bacteriolysis"
Bristol 1966 and C.J. Howard Ph.D. Thesis
" The biological effects and properties of
K antigens of *Escherichia coli*" London 1970

RESULTS

1) The effect of K antigen

Porton male mice weighing 30-40 g were infected with strains of *E.coli* of differing K antigen content. The first seven strains of *E.coli* (Table 1) were assessed for their ability to infect mouse kidneys. Four of the strains have the same serotype, 06:K13, but differ in the amounts of K antigen present.

Mice were infected in groups of twenty-four for each strain of *E.coli*. Three different dilutions of the bacteria were used and thus 8 mice were infected with each dose. The infections were assessed at 3 days.

The viable counts obtained from the urine and kidneys of mice infected with the different *E.coli* strains are analysed in Tables 2 and 3. Urines were said to be infected if they contained more than 1,000 organisms per ml and the recovery of 100 bacteria from a kidney indicated an infection of the organ with *E.coli*.

The majority of the urine samples were found to be infected (Table 2) and the geometric mean count recovered for each dose injected tended to rise with increasing inoculum size (Table 3). There was no relation between the geometric mean urine count and the K antigen content of the strain.

Table 2

E. COLI INFECTIONS OF MICE

Strain	AIA	Log dose injected into bladder	Mice No.	Mice Infected	Mice %	Urines No.	Urines $>10^3$/ml	Urines %	Kidneys No.	Kidneys $>10^2$	Kidneys %
WF82	64	3.63	9	2	22	9	6	66	18	2	11
		4.79	7	2	28	7	4	57	14	4	28
		7.09	7	4	57	7	6	86	14	6	43
WF41	0	5.46	10	3	30	10	4	40	20	4	20
		6.94	9	3	33	9	9	100	18	4	22
		7.42	7	4	57	(6)	6	100	14	7	50
WF96	2	4.44	8	1	12	8	6	75	16	2	12
		6.32	8	4	50	8	7	87	16	6	37
		7.44	8	4	50	8	7	87	16	6	37
WF98	16	4.09	8	2	25	8	7	87	16	4	25
		6.22	8	3	37	8	8	100	16	6	37
		7.53	7	5	71	(6)	6	100	14	10	71
WF60	32	4.40	8	3	37	8	6	75	16	4	25
		6.11	9	8	89	9	7	78	18	14	77
		7.70	7	5	71	(6)	5	83	14	10	71
WF8	32	4.44	8	1	12	8	5	62	16	1	6
		6.16	8	5	62	8	7	87	16	8	50
		7.74	8	7	87	(7)	7	100	16	14	87
WF26	64	2.97	8	3	37	8	2	25	16	5	31
		5.14	8	6	75	8	6	75	16	10	62
		7.23	8	8	100	8	8	100	16	13	81
Total			168	83	49	164	129	79	336	140	41

( ) Urine samples not obtained from all mice investigated

$$\frac{\text{Mice with 1 kidney infected}}{\text{Mice with renal involvement}} = 31\%$$

Table 3

GEOMETRIC MEAN BACTERIAL COUNTS RECOVERED FROM URINES & KIDNEYS OF MICE INFECTED WITH E. COLI

Strain	AIA	Log. dose injected into bladder	Urines Log. mean ±SD of mean	Kidneys Log. mean ±SD of mean
WF 82	64	3.63	4.59 ± 1.91	2.21 ± 0.27
		4.79	4.70 ± 0.65	3.24 ± 1.38
		7.09	5.90 ± 0.99	4.09 ± 0.98
WF 41	0	5.46	4.53 ± 1.26	2.76 ± 1.15
		6.94	4.43 ± 1.41	3.10 ± 0.18
		7.42	4.41 ± 1.56	3.79 ± 0.83
WF 96	2	4.44	5.39 ± 1.93	2.95 ± 1.10
		6.32	5.18 ± 1.67	4.83 ± 1.49
		7.44	6.41 ± 1.20	3.76 ± 1.60
WF 98	16	4.09	5.42 ± 1.53	2.74 ± 0.80
		6.22	5.15 ± 1.38	4.23 ± 1.91
		7.53	5.91 ± 2.04	3.00 ± 1.33
WF 60	32	4.40	5.10 ± 1.24	3.50 ± 1.80
		6.11	6.46 ± 1.80	4.54 ± 1.47
		7.70	6.76 ± 1.18	4.53 ± 1.65
WF 8	32	4.44	4.30 ± 0.65	2.18
		6.16	5.54 ± 0.75	3.19 ± 0.53
		7.74	5.61 ± 1.61	3.82 ± 1.40
WF 26	64	2.97	6.04 ± 0.80	3.22 ± 1.67
		5.14	5.64 ± 2.30	3.39 ± 1.22
		7.23	5.96 ± 1.50	3.38 ± 1.24

The number of kidneys infected by the E.coli strains was variable (Table 2). The results are best presented as number of kidneys rather than number of mice since both kidneys of a mouse were not always involved; 31% of infected mice had a unilateral infection. When the percentage of kidneys infected is plotted against the dose of bacteria injected into the bladder, the various strains of E.coli gave different dose response curves (Fig. 1). Strains of E.coli with a high K antigen content, i.e. WF26, WF60 and WF8, infected a greater percentage of mouse kidneys, and at lower doses than the poor K content strains WF98, WF96 and WF41. The piglet strain WF82 was not very infective for mouse kidneys. The dose response curve of this strain has a shallow slope; only a few more kidneys were infected by a high dose of the bacteria than by a low dose inoculum.

The dose of bacteria that would lead to 50% of the kidneys becoming infected, the ID_{50}, was calculated for each strain directly from the graph. When the ID_{50} is plotted against the agglutination inhibiting activity (AIA), the measure of the K antigen content of a strain, a straight line is obtained. This shows that for the six strains of human origin the ID_{50} is inversely proportional to the AIA (Fig. 2). Thus the infectivity of these six strains for mouse kidneys is dependent on their K antigen content. The more K antigen the strains possess, the greater their infectivity for mouse kidneys.

Figure I

Percentage of kidneys infected related to dose and strain of E. coli

E.coli infections in mouse kidneys

—— High 'K' strain
– – Low 'K' strain
······ High 'K' piglet strain

WF 26
WF 8
WF 50
WF 98
WF 41
WF 82
WF 96

% kidneys infected

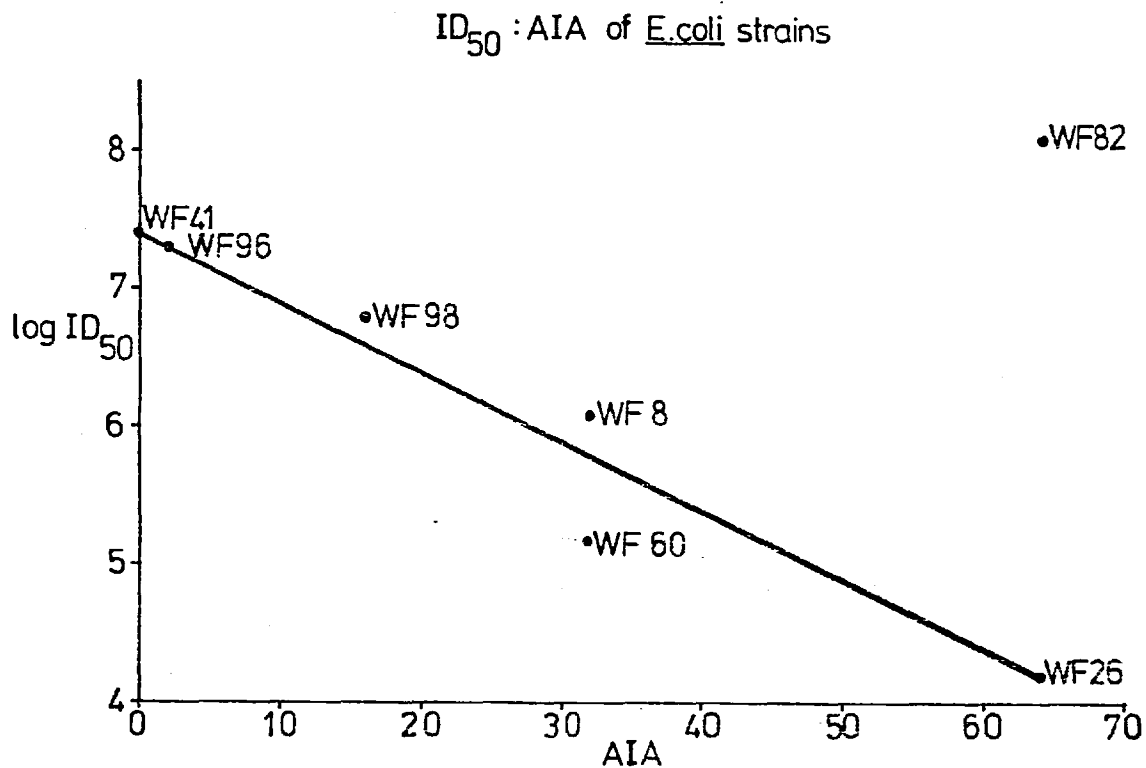
0 10 20 30 40 50 60 70 80 90

3 4 5 6 7 8

log dose

Figure 2

Agglutination inhibiting activity of E. coli strains related to their ID_{50} as calculated from Figure I



The geometric mean count recovered from the kidneys also tended to rise with increasing inoculum size (Table 3) but as in the case of the geometric mean count recovered from the urine, no relation existed between this value and the AIA of the E.coli strain.

2) The effect of flagella

Porton male mice were infected with a non-motile strain of E.coli WF6 (Table 1) and the dose response curve compared with those of two strains from the previous experiment, one of which is motile, E.coli WF26, and the other non-motile, E.coli WF8, (Figure 3 and Table 4).

The dose response curves for E.coli WF6 and E.coli WF8 are very similar and indicate that non-motile strains are capable of infecting mouse kidneys. The agglutination inhibiting activity and serotype of E.coli WF6 are the same as those of E.coli WF26. E.coli WF26 is much more virulent for mouse kidneys than is E.coli WF6, especially at lower doses. This suggests that although a strain of E.coli does not have to be motile in order to cause upper urinary tract disease, motility may help the organisms to colonise the kidneys.

Fig. 3 DOSE RESPONSE CURVES OF MOTILE AND NON-MOTILE STRAINS OF _E. COLI_

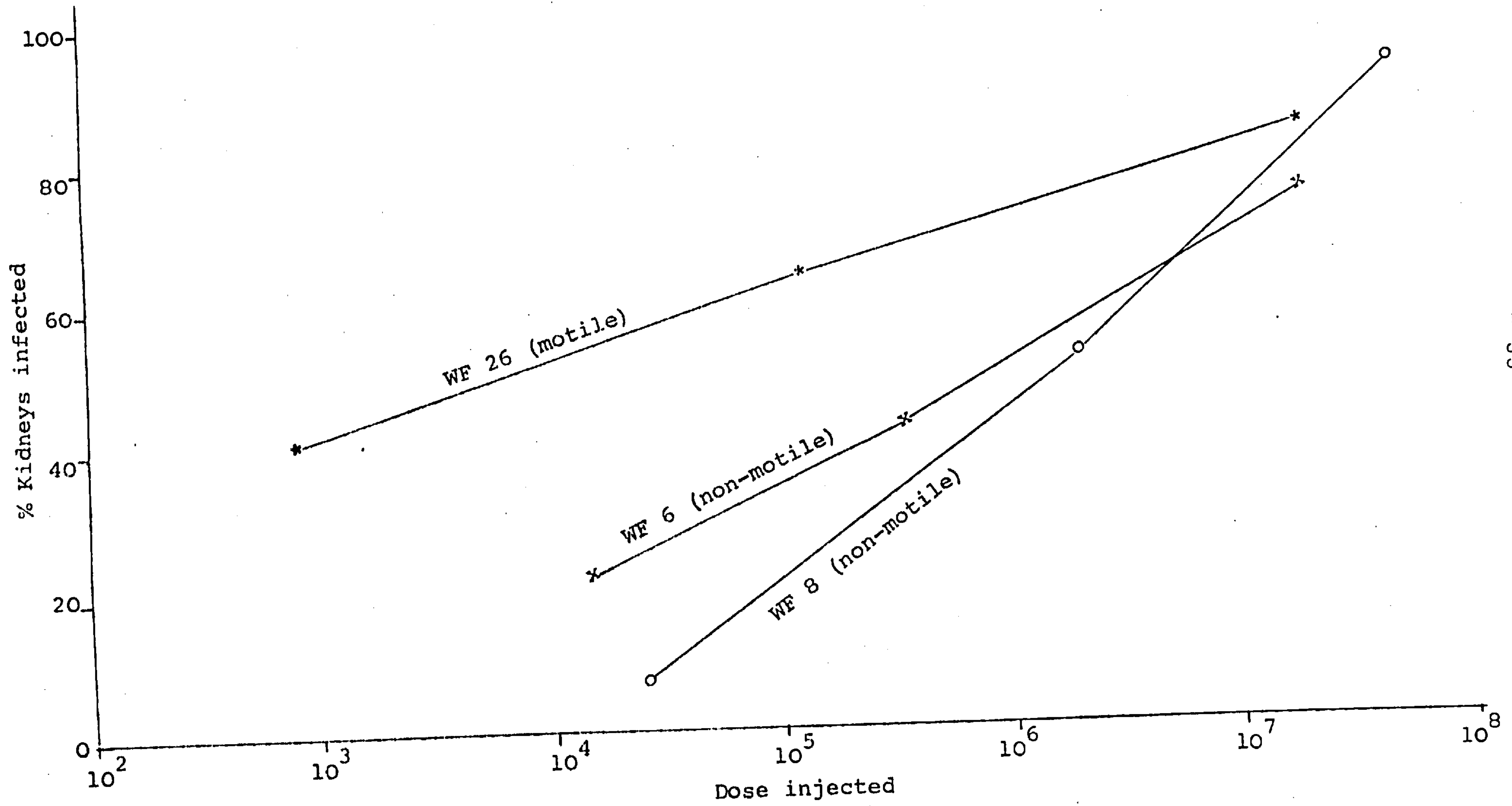


Table 4

DOSE RESPONSE CURVES OF MOTILE AND NON-MOTILE STRAINS OF *E. COLI*

Strain	Log dose injected	No. kidneys infected with $>10^2$ bacteria/kidney	% kidneys infected
WF26 (motile)	2.97 5.14 7.23	5/16 10/16 13/16	31 62 81
WF8 (Non -motile)	4.44 6.16 7.74	1/16 8/16 14/16	6 50 87
WF6 (Non -motile)	4.10 5.54 7.18	3/14 5/12 6/8	21 41 75

3) The effect of not emptying the bladder prior to injection of bacteria into the bladder lumen

Groups of 20 Porton male mice were infected with four strains of E.coli:- WF41, WF96, WF82 and WF26 (Table 1). The bladder was not emptied prior to injection of 1.25 x 10^6 bacteria into the bladder lumen.

The infection rates for kidneys of the four strains used are presented in Table 5. When these results are compared with the rates of kidney involvement that would have occurred in emptied bladders (by taking readings from Fig. 1 for the percentages of kidneys infected with 1.25 x 10^6 bacteria), the infection rates for non-emptied bladders are much higher. No correlation with the K content of the strain exists for the non-emptied bladders. It is possible that the inoculation of bacteria into the full bladders has resulted in reflux of bacteria up the ureters.

4) An attempt to increase the virulence of E.coli by passage

Three Porton male mice were infected with E.coli WF98 (Table 1), by injection of 5 x 10^6 bacteria into the bladder lumen. After 3 days the mice were killed and all six kidneys homogenised in 10 ml 0.15 M saline for 2 minutes in the Stomacher. A sample of the homogenate was taken for a viable count to be made. Ten ml of saline was added to the homogenate and the mixture was incubated overnight at 37°C. This

Table 5

INFECTIONS PRODUCED BY THE INJECTION OF 1.25×10^6 E.COLI INTO EMPTIED OR NON-EMPTIED BLADDERS

	E.coli strain			
	WF41	WF96	WF82	WF26
Agglutination inhibition activity	0	2	64	64
% kidneys infected:				
bladder non-emptied	62.5	82	60	55
bladder empty	30	37.5	43	80
[read from dose response curve]				

resulted in growth of the E.coli present in the kidney tissue. Slow centrifugation of the incubated mixture, 400 g for 10 minutes, deposited the organ debris and left the bacteria in suspension. The supernatant was decanted off from the sediment and washed twice with 0.15 M saline. The bacteria were finally resuspended in 2 ml of saline, a sample was taken to assess the viable count and the purity of the suspension (Table 6, Fig. 4). A further 3 mice were then infected by injection of the passaged bacteria into the bladder lumen.

The bacteria were passaged by these means 6 times. Bacteria recovered from the last passage were used to infect a group of 8 male Porton mice. A control group of Porton mice were infected with 5 x 10^6 of the original strain, E.coli WF98.

The passaged bacteria infected fewer mouse kidneys than did the normal E.coli WF98 (Table 7). The recovery per kidney for the passaged bacteria was however higher than that obtained with the normal E.coli WF98. This suggests that although the ability of E.coli WF98 to infect mouse kidneys has not been increased, once the kidneys have become infected the passaged bacteria grow better in that environment than do normal E.coli WF98. This has probably been achieved by the overnight incubation of the recovered bacteria in kidney homogenate, rather than by the serial passage of the bacteria through mice.

Table 6

EFFECT OF PASSAGE ON THE VIRULENCE OF *E. COLI* WF98

Passage	Bacteria/ml to initiate infection	Viable count	
		Non-incubated homogenate/ml	Per kidney
1	2.0×10^{8}	2.0×10^{3}	6.66×10^{3}
2	3.0×10^{7}*	$<10^{3}$	-
3	8.0×10^{8}*	7.8×10^{2}	2.6×10^{3}
4	3.8×10^{8}*	7.0×10^{3}	2.33×10^{4}
5	3.0×10^{8}*	5.2×10^{4}	1.73×10^{5}
6	3.1×10^{8}	1.0×10^{1} †	3.3×10^{1}

* Bacteria obtained by incubation of homogenate from previous passage with 10 ml saline overnight at 37° and separated by differential centrifugation from kidney debris

† Bacteria giving count of 5.0×10^{8}/ml after overnight incubation. Used to infect test mice.

Fig. 4 GROWTH OF E. COLI DURING PASSAGE IN MICE

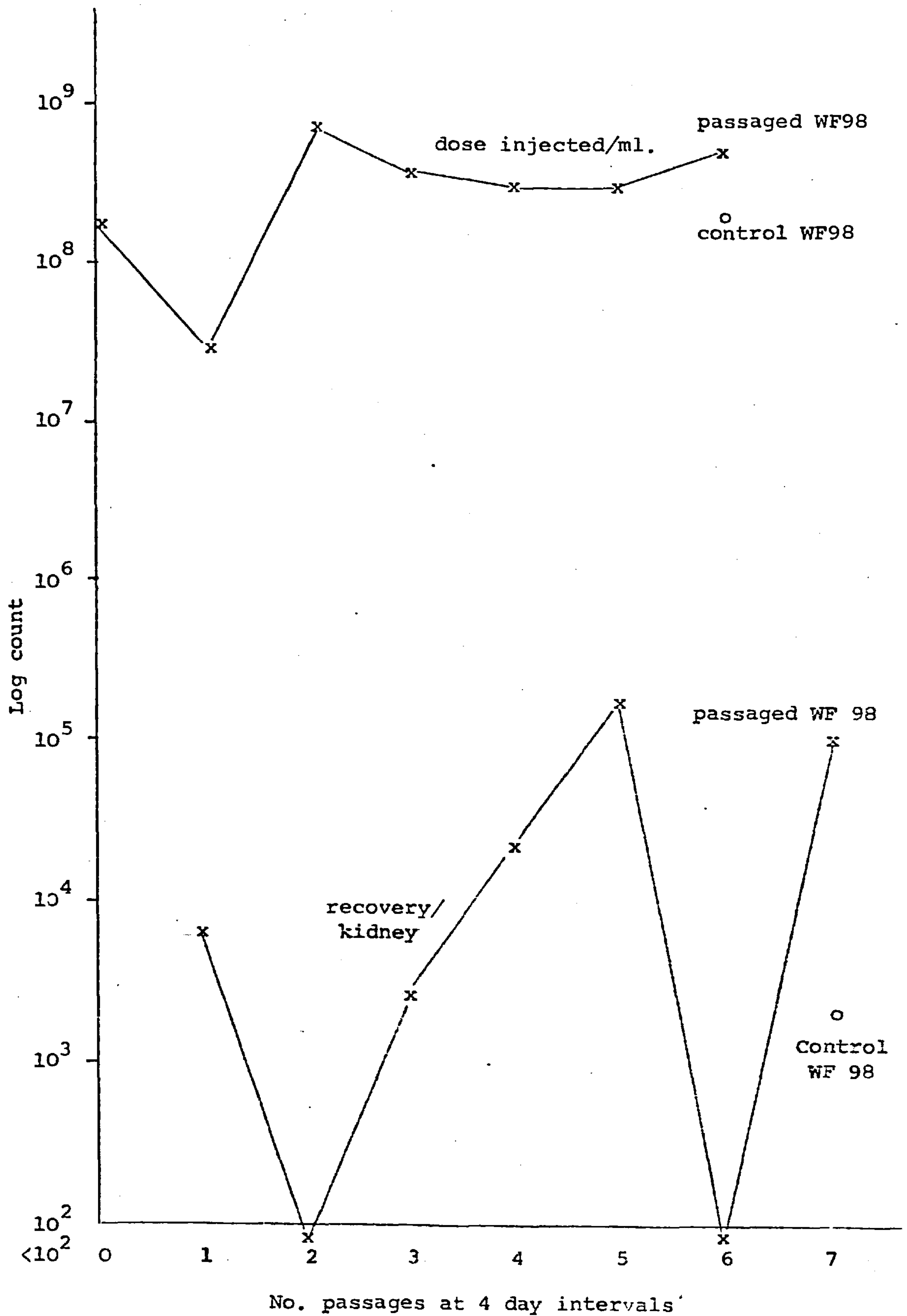


Table 7

INFECTIVITY OF PASSAGED *E. COLI* IN KIDNEYS

	E. coli WF98 $>10^2$	
	Passaged	Normal
No. kidneys infected	3/12 (25%)	7/14 (50%)
Recovery/kidney	1×10^5	2.1×10^3

5) The time course of an E.coli urinary tract infection

Fifty male Porton mice were infected on Day 0 with 2.5×10^6 E.coli WF8. Groups of mice were then killed at intervals of time up to 50 days and assessed for infection of the kidneys and urine (Table 8). At the end of the experiment the mice were still infected. The urines were nearly always found to be infected and the proportion of diseased kidneys did not vary to any great extent as time progressed. A peak of maximum geometric mean bacterial counts recovered from the infected kidneys and urines appeared to occur between 17 and 21 days, but this phenomenon may be a reflection of the small numbers of animals studied at each point in time. The infection produced in mice by the method described here would thus appear to be a chronic infection, and may be similar to that in humans with obstruction or dysfunction of the urinary tract, where infection may persist for long periods.

DISCUSSION

The mouse model of infection has been used to investigate the importance of bacterial factors in determining the virulence of E.coli for mouse kidneys. It is important to empty the bladder prior to infection as otherwise reflux of bacteria up the ureters results in the kidneys becoming

Table 8

MAINTENANCE OF AN *E. COLI* WF 8 URINARY TRACT INFECTION BY MALE PORTON MICE FOR 50 DAYS

Day	Urines $>10^3$/ml	Kidneys $>10^2$	Kidneys $>10^3$	Log mean urines ± SD	Log mean kidneys $<10^2$ ±SD
3	5/5	3/10	3/10	6.44 ± 1.53	6.00 ± 0.69
7	5/5	4/10	2/10	7.40 ± 0.93	3.88 ± 2.13
10	5/5	1/10	1/10	5.83 ± 2.16	3.65
14	5/5	5/10	2/10	6.43 ± 0.93	3.26 ± 0.79
17	5/5	4/10	4/10	7.11 ± 0.76	4.49 ± 1.04
21	5/5	5/10	5/10	7.48 ± 1.55	4.79 ± 0.82
28	3/5	4/10	1/10	6.88 ± 2.13	2.92 ± 0.64
35	4/4	2/ 8	2/ 8	5.69 ± 1.27	4.90 ± 0.47
42	4/4	5/ 8	4/ 8	7.67 ± 1.28	3.71 ± 0.66
50	4/4	5/ 8	5/ 8	7.85 ± 0.78	4.32 ± 1.02

infected irrespective of the strain of organism used. Over-distension of the bladder could also result in injury of the bladder mucosa and subsequent release of bacteria into the bloodstream. Heptinstall (1965) postulated that a kidney could become infected even if the ureter was obstructed, by escape of bacteria into the bloodstream upon violent injection of bacteria into the bladder lumen. Bacteria were in this case carried to the kidney by the blood stream. Injection of bacteria into an emptied bladder is unlikely to result in any significant degree of trauma to the bladder walls.

The ability of strains of E.coli to infect the kidneys of mice was related to the activity of their K antigens. K rich strains more readily infected mouse kidneys than did strains with little or no K antigen. This confirms expectations based on the knowledge that K antigen enhances the virulence of E.coli by increasing the resistance of the organisms to host defence mechanisms such as phagocytosis and antibody mediated complement killing (Glynn and Howard, 1970; Howard and Glynn, 1971a). The result is in accordance with the findings of Kaijser (1973) and Glynn et al (1971) who found more K rich strains of E.coli in isolates from patients with pyelonephritis than in those where infection was limited to the bladder, or in faecal isolates. The K antigen of E.coli therefore confers nephropathogenic properties on the bacteria. Attempts to determine exactly how

K antigen acts, within the environment of the urinary tract, are described in subsequent chapters.

If it were possible to serotype strains of E.coli isolated from urinary tract infections, and to assess their K antigen content, persons at risk from kidney disease could be identified. However until more rapid methods of determining the K content of strains can be developed, this finding is not likely to be of any diagnostic or prognostic use.

Peristaltic waves propel urine from the kidney pelvis to the bladder. Reflux of urine from the bladder into the ureter is prevented by the oblique insertion of the ureter into the bladder, at the site of the vesicoureteral valve. Inflammation during a bladder infection may reduce the competence of the vesicoureteral valve and allow infected urine to reflux up the ureter (Hutch et al, 1963). Boyarsky and Labay (1969) caused a cessation of ureteral peristalsis in dogs by the instillation of cultures of E.coli, killed E.coli, and endotoxin into the bladder.

The combined effects of reflux and cessation of peristalsis probably result in E.coli ascending the ureter to infect the kidneys. This would explain how non-motile strains of E.coli reach the kidney pelvis. The motile strain of E.coli however was more infective for mouse

kidneys than the non-motile strains. This may be directly related to the motility of the bacteria or may be a consequence of unknown factors which determine the virulence of E.coli. Ascent of a moving column of fluid has been demonstrated by Weyrauch and Bassett (1951). However for motility to be an important factor in determining the virulence of E.coli for kidney tissue, it would be necessary for there to be a continuous fluid film in the ureter between the kidney and bladder and perhaps for the bacteria to be attracted towards kidney tissue by chemotaxis.

The attempt to increase the infectivity of E.coli for mouse kidneys was unsuccessful. Fewer kidneys were infected by the passaged bacteria than by the original strain. However those kidneys that were infected with the passaged strain contained more bacteria than the control infected kidneys. The significance of this latter finding is unknown but is not relevant to the ability of strains to infect mouse kidneys.

The time course experiment showed that mice infected by the method described here will retain the infection for at least 50 days. Three days has been chosen as the time to assess the acute infection but data regarding the infectivity of individual strains of E.coli for the urinary

tract of mice probably holds true over longer periods of time, as shown by the fact that the proportion of kidneys infected by E.coli WF8 did not show any drastic variation with advancing time.

The infectivity of E.coli for the urinary tract is thus determined by several factors. Strains of E.coli have an inherent ability to invade kidney tissue, which is not increased by passage, but is enhanced if the strains are rich in K antigen. The role of motility is questionable as both motile and non-motile strains will cause renal disease. Other, perhaps unknown factors are probably involved but these as yet have to be determined. The mouse model of infection is an experimental means of inducing an ascending infection and results obtained relating the K antigen content of strains to their infectivity for kidneys confirm the observations made on human urinary tract disease.

CHAPTER 3

THE ROLE OF MOUSE FACTORS IN THE PATHOGENESIS OF E.COLI URINARY TRACT INFECTIONS

INTRODUCTION

The previous chapter has shown that the course of an acute *E.coli* urinary tract infection may be influenced by characteristics of the bacteria themselves. Probably as important in determining the outcome of infection was the host response to the disease. In order to determine if the host response to a urinary tract infection is an inheritable reaction, inbred strains of mice were infected with *E.coli*, as previously described.

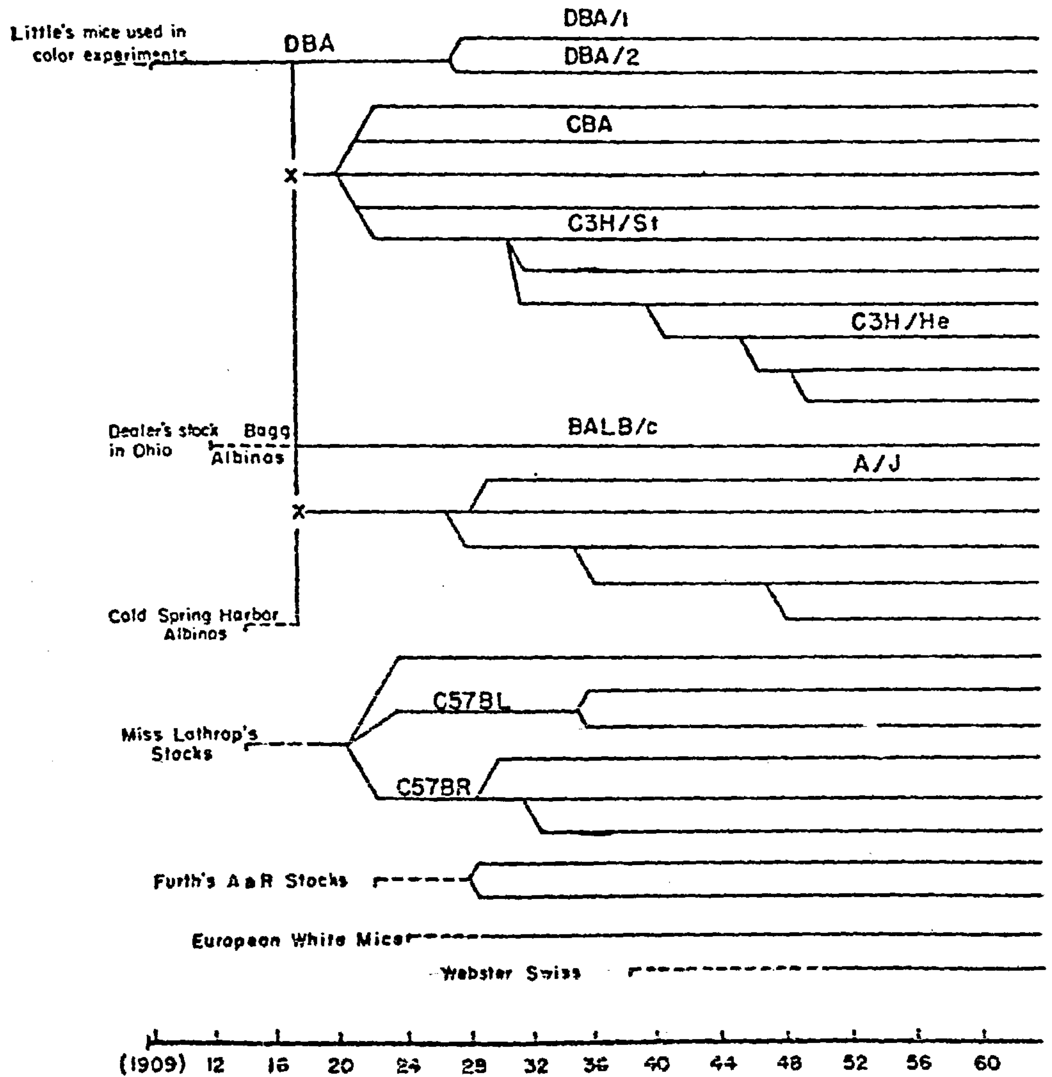
MATERIALS AND METHODS

1) *Mice*

Inbred strains of mice were obtained from lines raised in the St. Mary's Hospital Medical School Animal House. The origins of some of the strains are illustrated in Fig. 5.

F_1 mice of the crosses (Balb/c x CBA) and(B10D$_2$ newlinexC3H)were bred; these crosses being designed to obtain hybrid mice from parent strains of mice with differing susceptibilities to *E.coli* urinary tract infection.

Fig. 5 ORIGINS OF SOME INBRED STRAINS



In Biology of the Laboratory Mouse by The Staff of The Jackson Laboratory. 1966 P. 2
McGraw-Hill Book Company: New York

High and low responder mice were obtained from Dr. G. Biozzi in January 1974. These mice have been bred from random matched Swiss white mice by selective breeding for the amplitude of agglutinin production against heterologous erythrocytes (Biozzi et al, 1968; Biozzi et al, 1970). A progressive separation of the mice into two distinct lines occurred; the high responder line give a good antibody response and the low responder line give a poor antibody response against sheep red blood cells. Colonies of high and low responder mice have been bred in the Animal House from the mice obtained from Dr. Biozzi.

2) Experimental infections

Groups of inbred mice and high and low responder mice were infected with 7.5×10^6 E.coli WF98. This dose is the ID_{50} inoculum of E.coli WF98, as determined in Porton mice (Chapter 2). The infections were assessed after 3 days. The dose responses of $B_{10}D_2$ newline, C3H and F_1 ($B_{10}D_2$ newline x C3H) male mice to E.coli WF98 were investigated by inoculation of varying concentrations of bacteria into the bladders of small groups of the mice.

RESULTS

1) E.coli urinary tract infections in inbred strains of mice

Infecting different strains of inbred mice with a dose of 7.5 x 10^6 E.coli WF98 gave the results as presented in Table 9. A different response was obtained for each strain of mouse; some of the strains were much more susceptible to E.coli kidney infection than others.

When a positive infection was taken to be the recovery of more than 10^3 organisms from a kidney, the strains DBA/1, CBA, DBA/2 and C3H/He were all observed to have more than 40% of their kidneys infected; the strains $B_{10}D_2$ newline, Balb/c,C57 black and A/Jax had less than 15% infected. The different strains of inbred mice therefore vary considerably in their susceptibility to E.coli kidney infection and can be ranked in increasing order of the percentage of infected kidneys recovered.

The viable counts recovered from the urines and kidneys of the inbred mice are illustrated in Figs. 6 and 7. The geometric means of the counts obtained in the kidneys and urines were calculated. As the count of non-infected urines could actually be between zero and 9.0 x 10^2, and 10^3/ml is the minimum count using the methods described previously, an arbitrary value of 10^1 organisms per ml of urine was assigned to the non-infected urines for the purposes of calculating the mean urine concentration of bacteria

Table 9

E.COLI WF98 URINARY TRACT INFECTIONS IN INBRED MICE

Strain	H2 type	Kidneys infected $>10^2$	(%)	$>10^3$	(%)
B10D2 new line	d	8/26	(31)	0/26	(0)
Balb/C	d	6/32	(19)	1/32	(3)
C57 black	b	11/32	(35)	4/32	(12)
A/Jax	a	18/30	(60)	4/30	(13)
DBA/1	q	19/24	(80)	11/24	(46)
CBA	k	28/32	(87)	14/32	(44)
DBA/2	k	25/30	(83)	22/30	(74)
C3H/He	d	26/28	(93)	23/28	(82)
F1 Balb/c	x CBA	12/30	(40)	5/30	(17)
F1 B10D2 new line	x C3H	28/32	(87.5)	22/32	(71)

<u>Fig.6</u> VIABLE COUNTS RECOVERED FROM THE URINES OF INBRED MICE

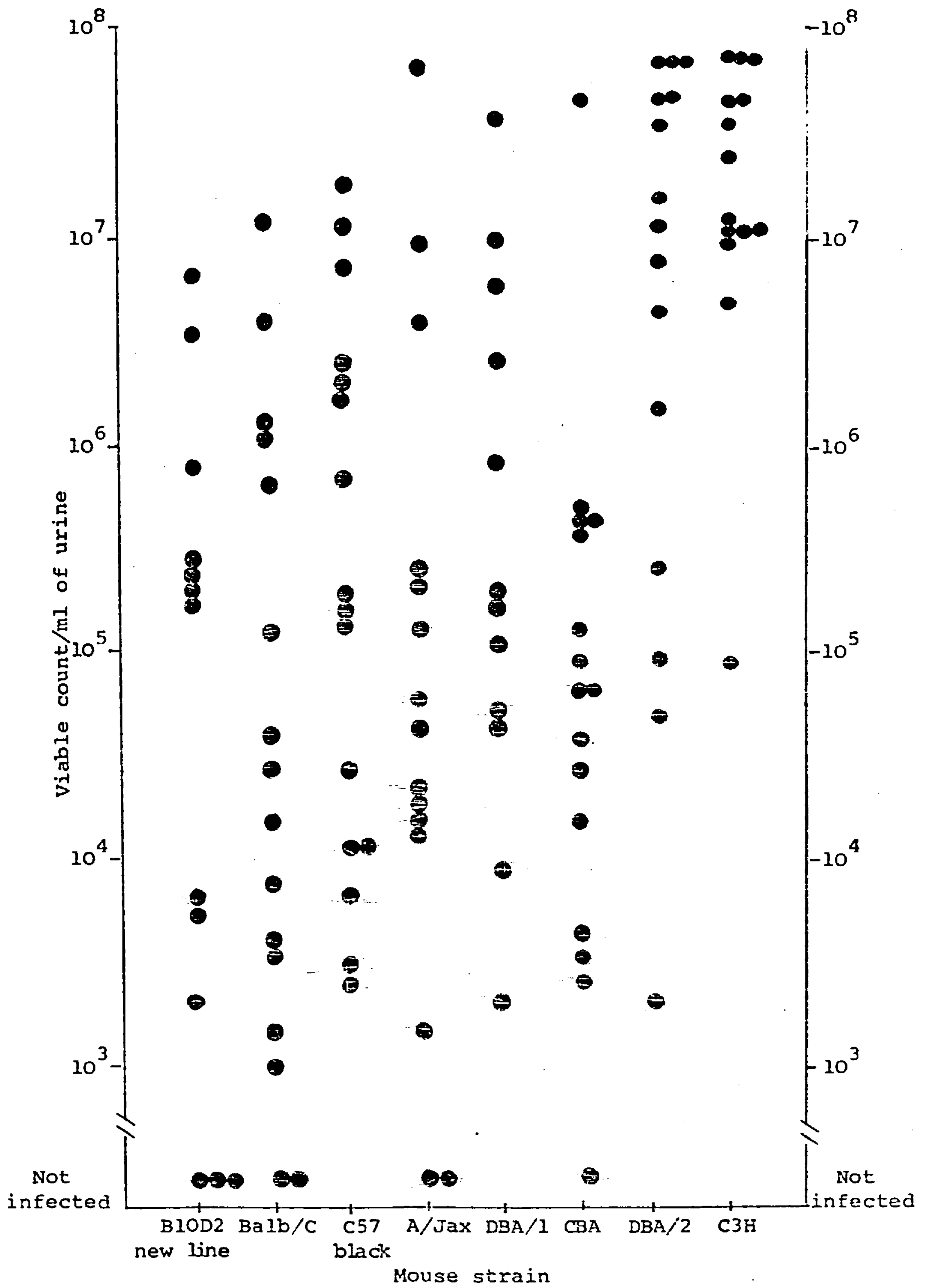
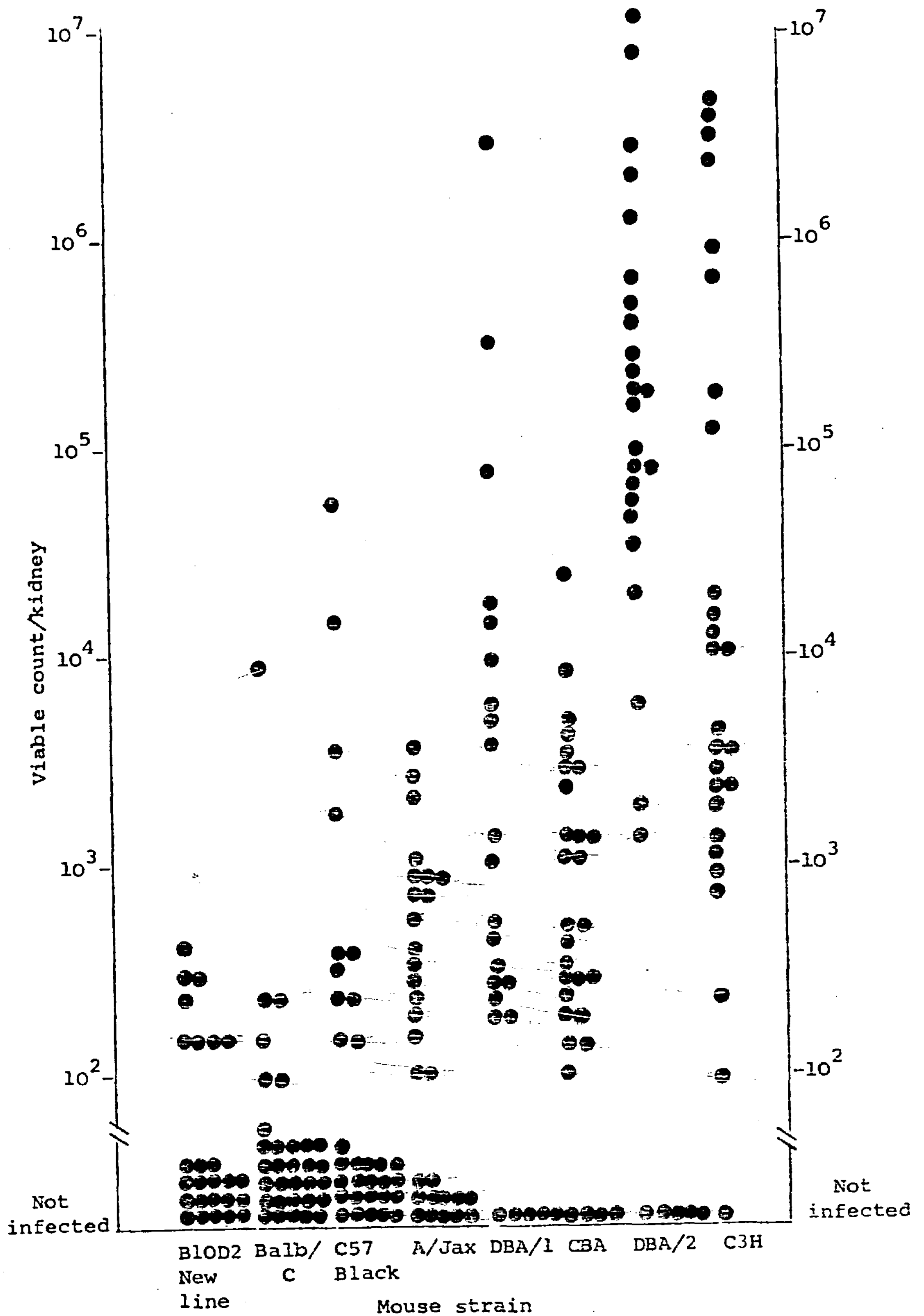


Fig. 7 VIABLE COUNTS RECOVERED FROM KIDNEYS OF INBRED STRAINS OF MICE



for each inbred strain. Likewise as non-infected kidneys could contain from zero to 9.0 x 10^1 organisms, without detection by our method of assessment, a value of 10^1 organisms was assigned to each non-infected kidney. Thus the mean urine or kidney count represents the mean of all the urines and kidneys of each strain of mouse. Justification of the choice of these arbitrary values is shown in Table 10.

The histogram of the mean urine and kidney counts (Fig. 8) showed a gradual change across the strains in the magnitude of the mean kidney count obtained. The inbred strains C3H and DBA/2 were much more susceptible to E.coli WF98 urinary tract infections than the other strains. The inbred strains $B_{10}D_2$ newline and Balb/c were highly resistant to kidney disease compared with the other strains of mice. The variation in the mean urine counts was much less pronounced than the variation observed in the mean kidney counts.

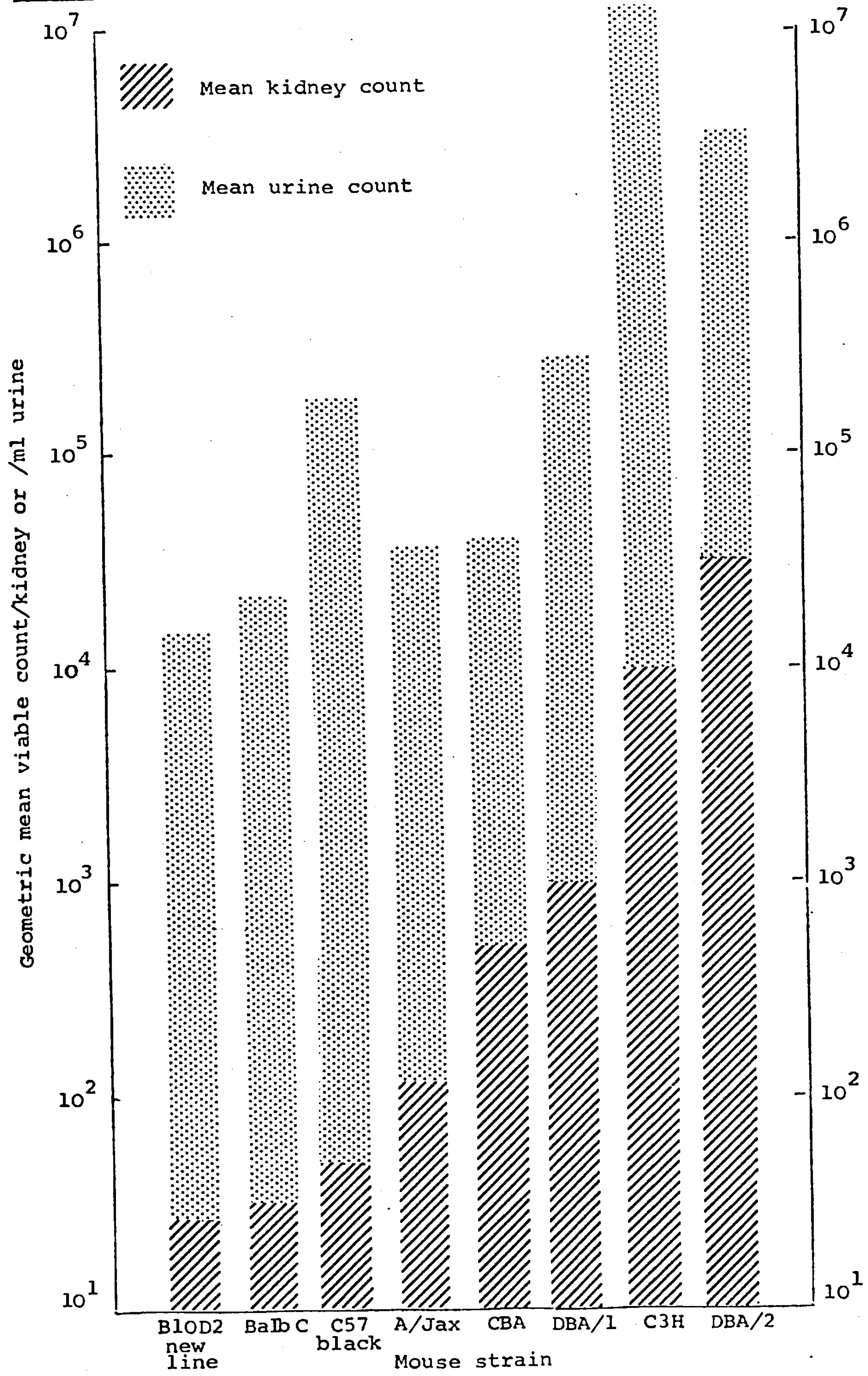
Inbred strains of mice therefore vary in their susceptibility to E.coli urinary tract infection, as produced by the method described in the previous chapter.

Table 10

EFFECT OF CHANGING THE ARBITRARY VALUE GIVEN TO NON-INFECTED URINES AND KIDNEYS ON THE GEOMETRIC MEAN COUNT OBTAINED

Arbitrary value	Log. geometric mean - kidney count obtained Balb/C mice		DBA/2 mice	
	Urines	Kidneys	Urines	Kidneys
0	4.20	0.48	6.52	4.36
5	4.26	1.04	6.52	4.48
1×10^1	4.30	1.28	6.52	4.52
5×10^1	4.39	1.85	6.52	4.64
10^2	4.43	2.10	6.52	4.69

Fig. 8 GEOMETRIC MEAN VIABLE KIDNEY AND URINE COUNTS



2) Genetics of the variation in susceptibility to E.coli urinary tract infections

In order to see if susceptibility to E.coli urinary tract infections was inherited, F_1 generation male mice were infected with the ID_{50} dose of E.coli WF98. The F_1 cross ($B_{10}D_2$ newline x C3H) was a cross between a highly susceptible and a very resistant inbred strain of mouse, in contrast to the F_1 (Balb/c x CBA) mice which were the products of the mating of two inbred strains of mice with intermediate susceptibility.

Infection of male ($B_{10}D_2$ newline x C3H) F_1 mice resulted in 71% of the kidneys containing more than 1,000 E.coli (Table 9). This was very similar to the infection rate of 82% observed in C3H mice, and completely different to the zero infection rate observed with the $B_{10}D_2$ newline parent type mice. The F_1 ($B_{10}D_2$ newline x C3H) mice thus behave like the parent inbred strain, C3H mice.

Infection of male F_1 (Balb/c x CBA) mice with the same infective dose gave a kidney infection rate of 17%, which is intermediate between the rates in the parent strains. The F_1 (Balb/c x CBA) mice therefore do not act like either of the two parent types as regards susceptibility to E.coli urinary tract disease. The viable counts obtained in the kidneys of the F_1 crosses are compared with those of the parent strains (Figs. 9 and 10).

Fig.9 VIABLE COUNTS RECOVERED FROM KIDNEYS OF BALB/C, CBA, AND F1(BALB/C x CBA) MICE

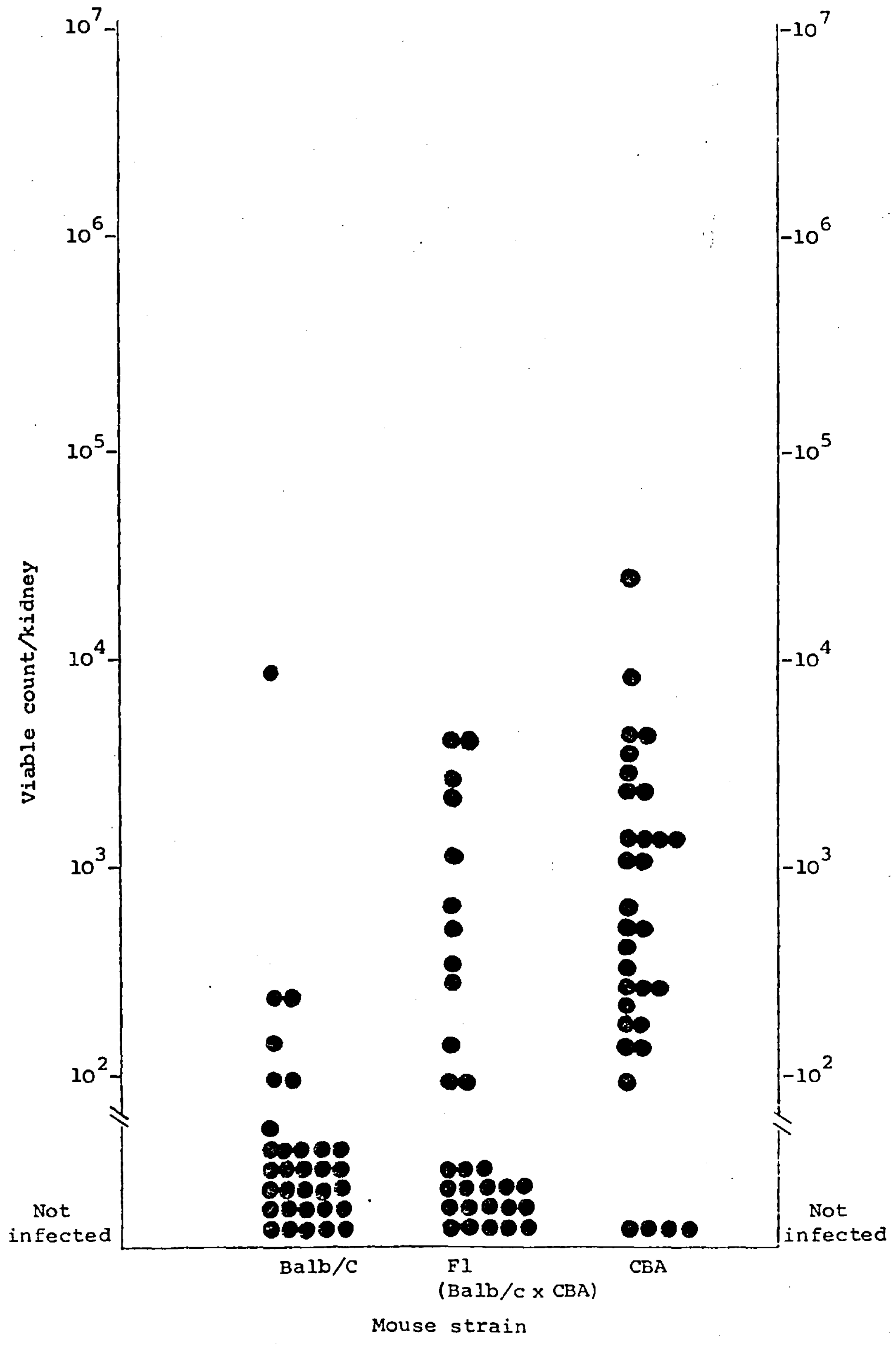
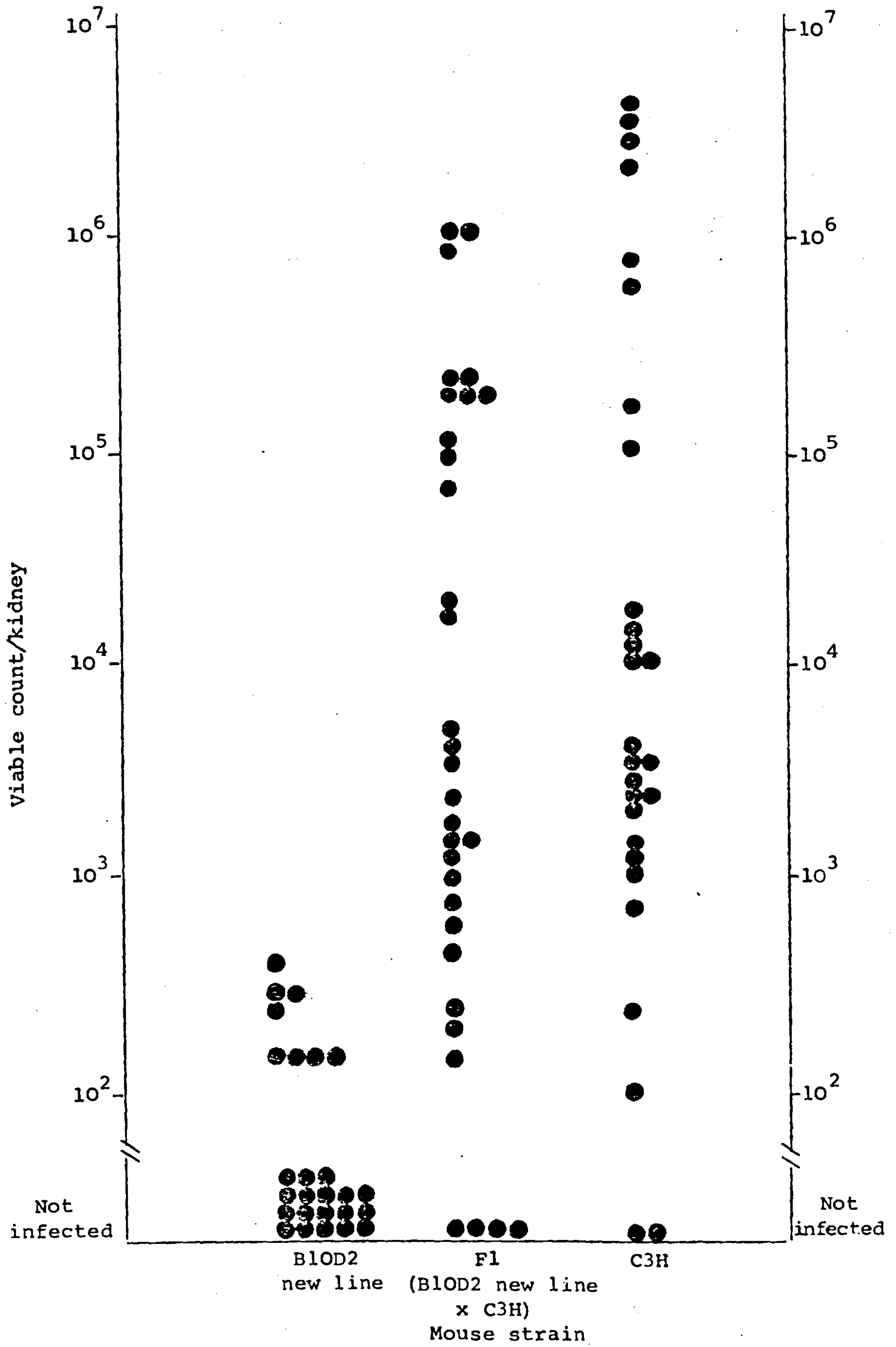


Fig.10 VIABLE COUNTS RECOVERED FROM KIDNEYS OF B10D2 NEW LINE, C3H AND F1(B10D2 NEW LINE x C3H) MICE



The geometric mean kidney and urine counts were calculated as before and compared with those of the parent mouse strains for each of the F_1 crosses (Figs. 11 and 12). The F_1 ($B_{10}D_2$ newline x C3H) mice behaved like the C3H mice in contrast to the F_1 (Balb/c x CBA) mice which had a mean kidney count intermediate between those of the parent strains.

The dose response curves of $B_{10}D_2$ newline, C3H and F_1($B_{10}D_2$ newline x C3H),male mice are illustrated by Figs. 13 and 14.Fig.13 shows the dose responses based on the numbers of kidneys containing more than 100 organisms, and Fig. 14 is similar except that the basal limit of infection was taken to be 1,000 organisms. A similar pattern of dose response curves was obtained in each case.

The ID_{50} dose for each strain was calculated; the ID_{50} being that dose of E.coli which would result in 50% of the kidneys containing more than 100 organisms. It was not possible to calculate the ID_{50} at the higher level of infection. The ID_{50} dose for the C3H mice was 5×10^3, for the F_1($B_{10}D_2$ newline x C3H) mice 10^3, and for the $B_{10}D_2$ newline mice 10^8 E.coli per mouse. The F_1 ($B_{10}D_2$ newline x C3H) and the C3H mice are therefore at least 10,000 times more susceptible to E.coli urinary tract infection than $B_{10}D_2$ newline mice.

<u>Fig.11</u> GEOMETRIC MEAN VIABLE KIDNEY AND URINE COUNTS OF BALB/C, CBA AND F1 (BALB/C x CBA) MICE

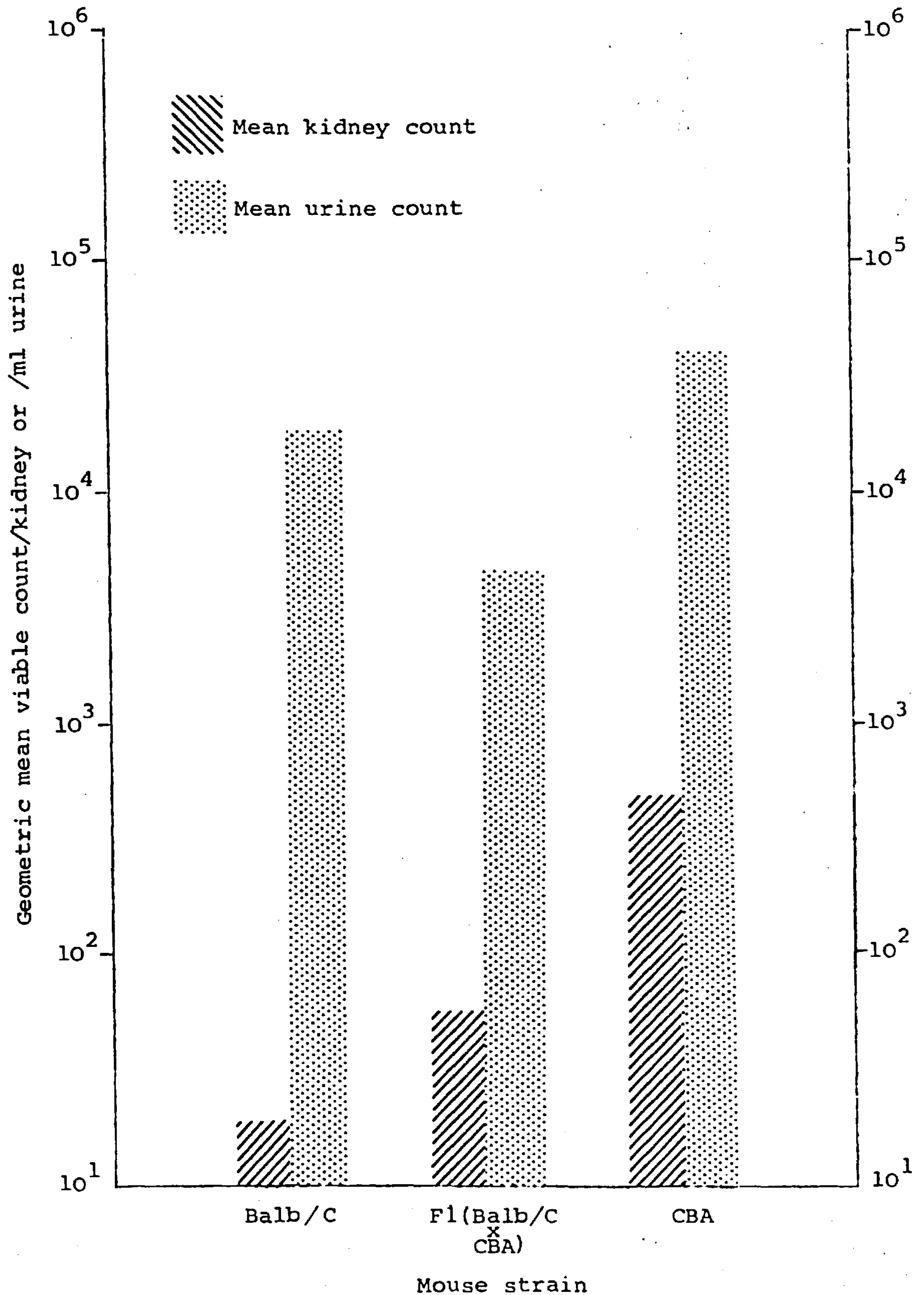


Fig.12 GEOMETRIC MEAN VIABLE KIDNEY AND URINE COUNTS B10D2 NEW LINE, C3H AND F1(B10D2 NEW LINE X C3H) MICE

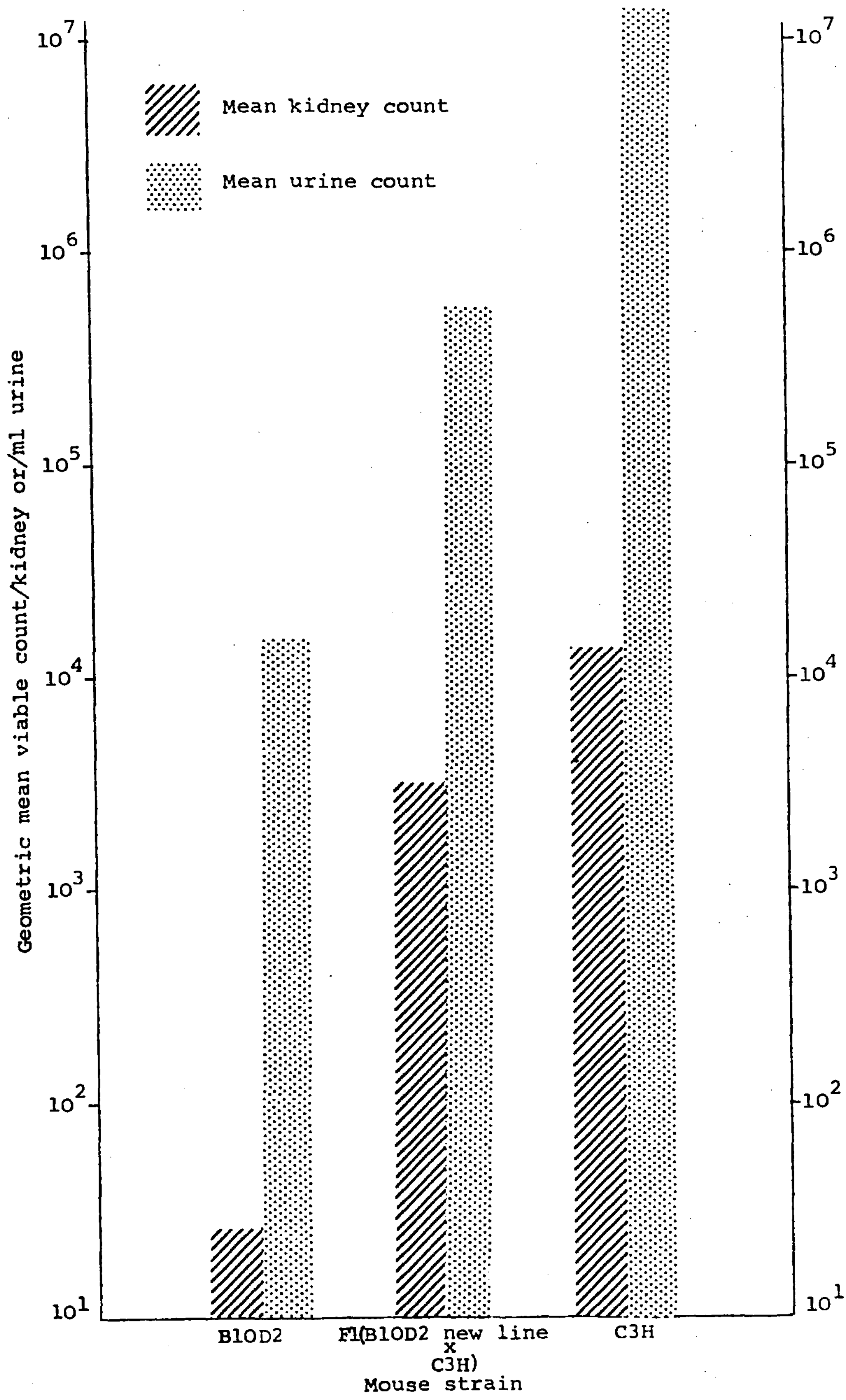


Fig.13 DOSE RESPONSE CURVES OF B10D2 NEW LINE, C3H AND F1(B10D2 NEW LINE x C3H)MICE. INFECTION TAKEN AS >10^2/KIDNEY

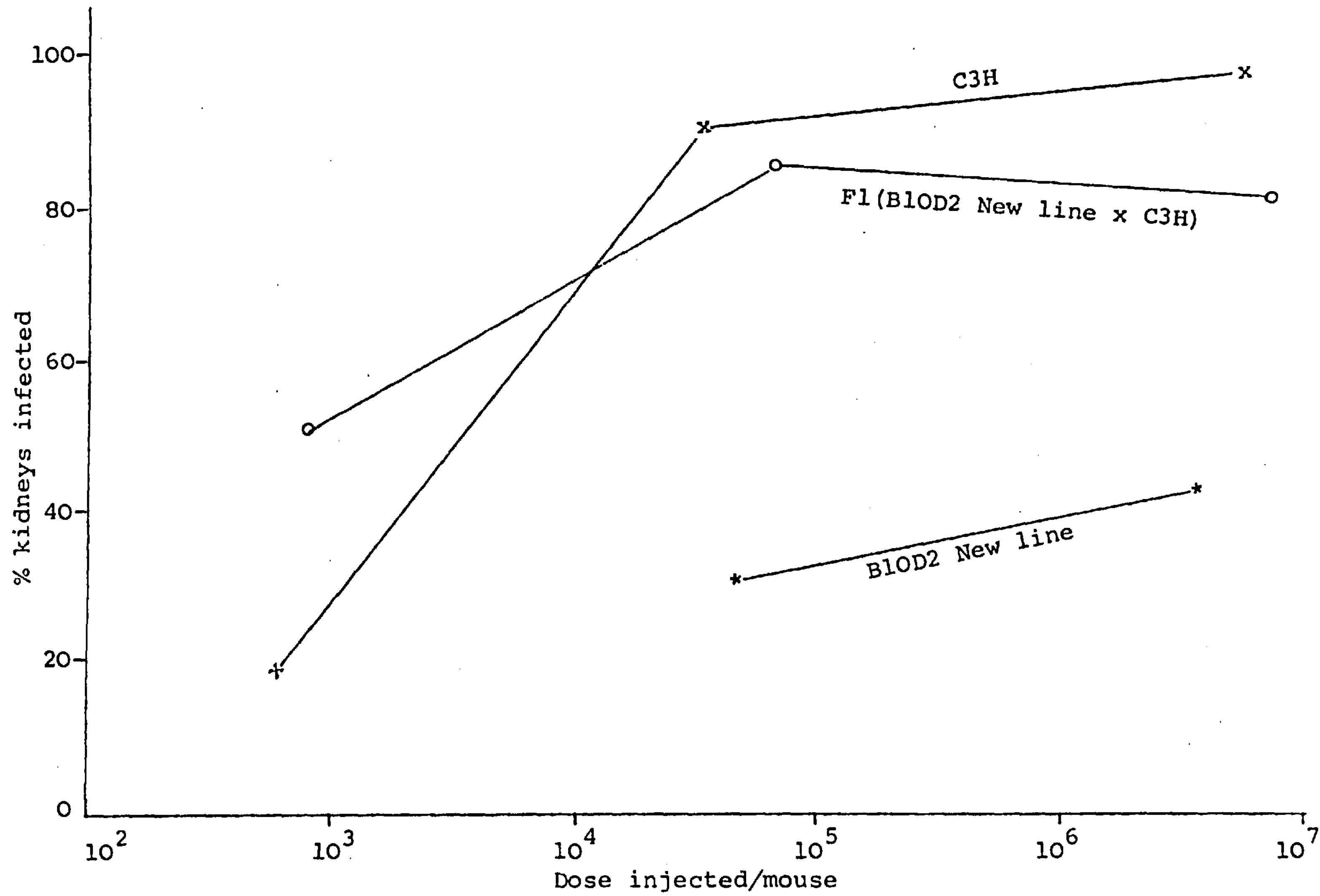
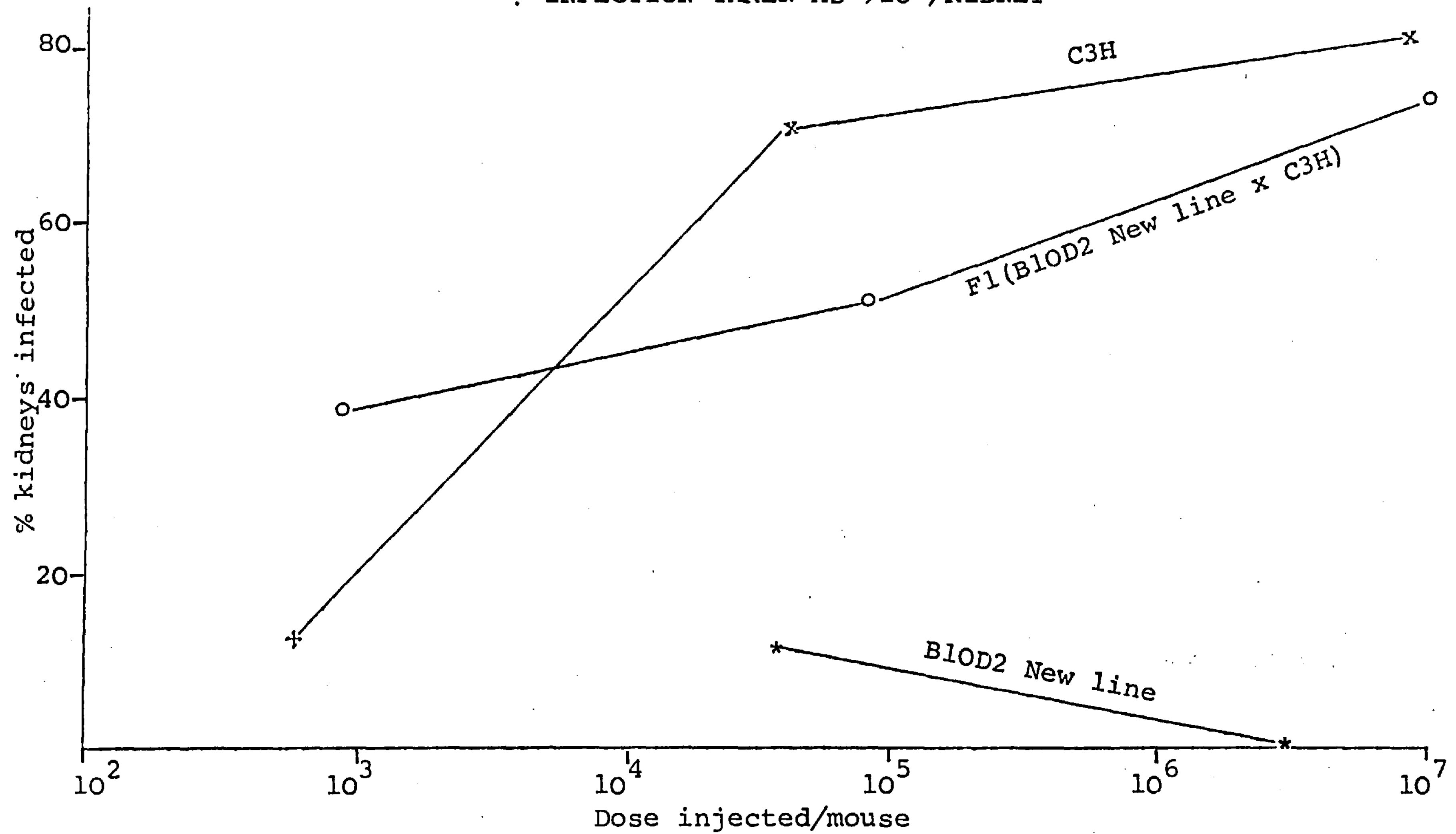


Fig.14 DOSE RESPONSE CURVES OF B10D2 NEW LINE, C3H, AND F1(B10D2 NEW LINE x C3H) MICE

INFECTION TAKEN AS $>10^3$/KIDNEY



3) E.coli urinary tract infections in Biozzi high and low responder mice

Male Biozzi high and low responder mice were infected with the ID_{50} dose of E.coli, as calculated in Porton mice. The high responder mice were very susceptible to infection with 60% of their kidneys containing more than 1,000 organisms, whereas the low responder mice were resistant with only 12.5% of their kidneys being infected with more than 10^3 bacteria (Table 11). Differentiation of an outbred population of white mice into two lines of mice on the basis of their antibody response to sheep red blood cells has thus resulted in the production of two lines of mice with widely different susceptibilities to E.coli urinary tract disease.

DISCUSSION

The reaction in the host during an infection of the urinary tract with E.coli is of great importance in determining the outcome of infection. The injection of the same dose of E.coli into the bladders of inbred mice has shown that some strains of mice are more susceptible than others to an infection of the urinary tract. A progressive change from resistance to susceptibility occurs across the range of inbred mice and the inheritance of the response to urinary tract infection cannot be explained on the basis of

Table 11

E. COLI KIDNEY INFECTIONS IN BIOZZI HIGH AND LOW RESPONDER MICE

	High line	Low line
Kidney with $>10^2$ bacteria		
no.	25/34	13/32
%	74	40
Kidney with $>10^3$ bacteria		
no.	21/34	4/32
%	60	12.5

Comparison of numbers of kidneys infected with $>10^3$ /kidney of high and low lines $\chi^2=14.97$ n=1 P=<0.1 (using four-fold tables and Yates correction)

Reference: Bradford Hill, A. (1961) Principles of Medical Statistics. P. 172 Lancet: London

simple genetics. These findings indicate that the host response to infection is probably governed by a large group of genes, rather than by a small group or a single gene.

The variation amongst the strains is in contrast to the response observed when inbred strains of mice are infected subcutaneously with Salmonella typhimurium C5 (Plant and Glynn, 1974; 1976). With this infection the strains can be divided into two quite distinct groups; Balb/c, C57 black, and DBA/1 strains of mice died after injection of less than 100 organisms in contrast to strains DBA/2, C3H/He, A/Jax and CBA whose LD_{50} was greater than 10^5 Salmonella typhimurium C5. An F_1 generation of mice of the cross of a resistant and susceptible strain, the (Balb/c x CBA) F_1,was resistant to infection. In this case resistance to infection was a dominant characteristic and it was suggested that the response of inbred mice to this infection was controlled by a single gene or small group of genes.

Different mechanisms of resistance to infection are certainly involved for these two infections, and there is no correlation between the response to Salmonella typhimurium C5 infection and the reaction to an infection of the urinary tract with E.coli. Therefore different groups of genes would appear to code for the host responses to each of these two infections.

If a large group of genes is involved in the host reaction to E.coli urinary tract disease, then the variation across the range of inbred mouse strains may be explained by postulating that each inbred strain of mice has a different mixture of the genes involved. A lack of the dominant genes which determine resistance to disease could explain the high susceptibility of some strains to E.coli urinary tract infections. It is possible that individual genes, within the large group of genes involved, act at different points in the urinary tract. The responses governed by the genes probably include humoral and cellular immunity, and the susceptibility of the mucosal surfaces of the urinary tract to bacterial adhesion.

The genes which determine humoral and cellular immunity are known as 'Immune response' or Ir genes. Some Ir genes are linked to histocompatibility antigens, others are not (Benacerraf, 1974). The H-2 type of the inbred strains are given in Table 10. No correlation between the H-2 type and resistance or susceptibility to infection can be seen, and therefore the genes involved in immunity to E.coli urinary tract disease are not H-2 type linked.

The response of people to E.coli urinary tract infection may be under a similar kind of genetic control to that observed in these inbred mice. Inherited susceptibility to infection could explain why some people succumb more readily

than others to urinary tract disease, even in the absence of any abnormality of the urinary tract and other predisposing factors. If the genetically controlled mechanisms which determine the response to infection could be identified in these mice, then this might lead to a fuller understanding of the disease that occurs in people.

Whitmore and Haughton (1975) have recently demonstrated the genetic control of the susceptibility of mice to Rous sarcoma virus induced tumours, and Webster (1937) found that mice resistant to B.enteritidis infection were susceptible to viral encephalitis and that both factors were inheritable. The mechanisms under genetic control which enable piglets to resist E.coli enteric infections have been characterised by Sellwood et al (1975). The ability to resist the adhesion of E.coli to the brush borders of the small intestine made piglets resistant to enteritis. Phenotypic characterisation of the piglets showed that the adhesion or non-adhesion of E.coli was a property of the brush borders, and was inherited by the animals in a simple Mendelian fashion. Subsequent chapters of this thesis describe the cellular and humoral immune responses of mice during E.coli urinary tract infections. These immune responses were investigated in an attempt to characterise the mechanisms of resistance involved in immunity to E.coli urinary tract disease.

Biozzi high responder mice were found to be susceptible to E.coli renal infection and low responder mice were resistant. The susceptibility of the high responder animals to E.coli urinary tract infection appears to be linked to the ability of these animals to make large amounts of antibody to sheep red blood cells. This suggests that the production of copious amounts of antibody may not be beneficial to the host. The antibody response of Biozzi high and low responder animals during an E.coli urinary tract infection is discussed in a later chapter.

Recently it has been suggested that the difference between the high and low responder mice resides in the functioning of their macrophages rather than in their antibody producing cells (Howard et al, 1974). Macrophages from the low responder line mice are hyperactive compared with the macrophages of high responder mice, as regards the the endocytosis and intracellular digestion of antigen (Wiener and Bandieri, 1974). Thus antigen injected into low responder mice is rapidly rendered non-immunogenic, whereas antigen in the high responder mice is not degraded so fast and results in the increased antibody levels of the high line mice. Branched dextrans and native levans which give an equivalent response from both lines of mice (Howard et al, 1974) are either macrophage independent in stimulating B cells to produce antibody or they are independent of the difference between the macrophages of the two lines.

The difference between the Biozzi high responder and low responder mice thus appears to reside in the ability of their macrophages to ingest and degrade antigen. When infected via the bladder with E.coli WF98, high responder mice were susceptible to infection whereas the low responder animals were resistant. If the macrophages of the low line mice are as effective at destroying live E.coli as they are at degrading other antigens then this could possibly explain the resistance of these mice to E.coli urinary tract disease. Experiments to assess macrophage degradation of E.coli by cells from the two lines may help to prove or disprove this hypothesis.

CHAPTER 4

THE ROLE OF HUMORAL IMMUNE FACTORS DURING AN E.COLI URINARY TRACT INFECTION

INTRODUCTION

The role of humoral immune factors in an infection of the urinary tract with E.coli was investigated. The K antigen is known to make the bacteria more resistant to antibody mediated complement killing and to phagocytosis. Immunisation would therefore be expected to enhance resistance to infection and depletion of serum complement might render mice more susceptible.

MATERIALS AND METHODS

1) Measurement of serum antibody levels to E.coli

Bacteria of the strain to which the antibodies had been raised were grown overnight in glucose nutrient broth. If the antibodies to be determined were directed against the O antigen of the bacteria, the cells were killed by steaming for 1 hour. Live E.coli were necessary for determination of the anti-OK titre of the serum and cells diluted in 0.5% formalin in 0.15 M saline were used to assess the anti-H titre. All cells were washed three times in 0.15 M saline after harvesting from the broth; this removed all free K antigen from the heat killed cells. Heated,formalin killed and live bacteria were finally resuspended in 0.15 M saline to give a suspension of bacteria of OD 2.0.

Mice were bled from the tail vein into heparinised capillary tubes (Gelman Hawksley Ltd., Lancing, Sussex) and the tubes were sealed with Christaseal (Gelman Hawksley Ltd.). The tubes were centrifuged at 400 g to separate the serum from the blood. Breaking the capillary tube just above the 'buffy coat' allowed the serum to be removed from the tube in 10 μl aliquots by means of an Eppendorf pipette and plastic tip.

The agglutinations were carried out in Microtitre trays with rounded bases (Cooke Microtitre System, Sterilin polystyrene U form Microtitre plates, Sterilin Ltd., Britain). 40 μl of 0.15 M saline was added to each well of the first row of the tray and all other wells were filled with 25 μl of 0.15 M saline. 10 μl of the serum was added to the 40 μl of saline in the first row to make an initial 1/5 dilution. Subsequent 1/2 dilutions along the row were made by means of the 25 μl Titertek Multidiluter (Flow Laboratories Ltd., Irvine, Ayrshire, Scotland). The last row of the tray was not used for the dilutions but left as a control row to check that the bacterial cell suspension was not self-agglutinating.

25 μl of the appropriate bacterial suspension was added to each well of the tray. After incubation at 37°C for 1 hour the trays were kept at 4°C overnight. Agglutination

was read using a Microtitre viewer (Cooke Instruments Ltd., England). The antibody titre was taken to be that dilution of serum at which agglutination of the bacteria was present in conjunction with non-agglutinated cells which gave a small 'button' at the bottom of the well. In the absence of agglutinating titres of serum all the bacterial cells settled at the bottom of the wells to form 'buttons'.

2) Measurement of serum antibody levels to E.coli lipopolysaccharide

Antigen coated sheep erythrocytes were prepared freshly before each determination of antibody titre. A 1 mg/ml solution of E.coli 0127:B8 Westphal lipopolysaccharide (Difco Laboratories, Detroit, Michigan, U.S.A.) in phosphate buffered saline (PBS) was boiled for 1 hour. To 0.5 ml of this solution 0.1 ml of packed sheep red blood cells, previously washed 3 times in PBS, and 1.4 ml of PBS were added (Oxoid sheep red blood cells in Alsevers solution). The mixture was left to incubate for 1 hour at 37°C and was continuously stirred by placing the container on a shaker (New Brunswick Shaker, New Brunswick Scientific Co.Inc., New Brunswick, N.J., U.S.A.) at low speed.

The coated cells were then washed 6 times with PBS. Spinning to sediment the cells during the washing procedure was only at 400 g, in order to avoid the cells sticking to

each other. The cells were finally resuspended to a volume of 10 ml in PBS to give a final 1% solution of coated erythrocytes. Non-coated sheep red blood cells were prepared in parallel with the coated cells by adding 0.5 ml of PBS to the incubation mixture instead of 0.5 ml of lipopolysaccharide solution.

Agglutinations were carried out by adding 25 µl of PBS to each well, except those wells in the first row, of microtitre plates. A 1/5 dilution of mouse serum was made in the first row of wells by adding 10 µl of serum to 40 µl of PBS. Doubling dilutions along the rows were then prepared as for E.coli agglutinations. 25 µl of the coated or non-coated sheep red blood cell suspensions were added to each well and after incubation at 37°C for 1 hour, the microtitre trays were placed overnight in the refrigerator. The titre was read in the same manner as for bacterial agglutination.

3) Measurement of serum antibody levels to sheep red blood cells

The antibody titre to sheep red blood cells was determined by agglutinating a 1% suspension of uncoated sheep erythrocytes with mouse serum.

4) Immunisation of mice with *E.coli*

Mice were immunised with *E.coli* WF8 (Table 1), a non-motile strain containing the same O and K antigens as *E.coli* WF26, the O6 and K13 antigens. Heat killed *E.coli* WF8 were prepared by steaming the bacteria for 1 hour,then washing them 3 times with 0.15 M saline to remove all traces of K antigen.

The mice were injected intraperitoneally with 1×10^7 heat or formalin killed bacteria suspended in 0.15 M saline. The number of bacteria present in the suspensions was determined by optical density readings. Three or six doses of the antigen were given to each mouse at 5 days intervals.

5) Immunisation of mice with *E.coli* "extract"

An "extract" of *E.coli* WF26 was made by concentrating and purifying the supernatant fluid from a culture of the organisms. *E.coli* WF26 was grown at 37°C for 48 hours in Cohn's Minimal Salt Medium (Cohn, 1963). The bacteria were separated from the resulting culture fluid by centrifugation for 30 mins at 7,000 g in an MSE 18,000 centrifuge. Any residual bacteria were removed from the supernatant by Seitz filtration. The extract was then concentrated in an Amicon membrane filtration unit using a PM 10 filter (Amicon Corporation,Lexington, Mass 02173, U.S.A.). After dialysis

in the cold against 0.15 M saline for 48 hours, the extract was centrifuged (MSE 75,000) at 200,000 g for 2 hours to remove any lipopolysaccharide present. The protein content of the extract was determined by the method of Lowry et al (1951) and the concentration was adjusted to a final volume of 1 mg/ml. The extract was used at this protein concentration in all experiments.

The extract was originally prepared for the purposes of eliciting a delayed hypersensitivity response to E.coli in the footpads of mice (Chapter 5). It was however noted that the extract induced an antibody response in mice and therefore the protective capacity of these antibodies was tested. Male Porton mice were immunised with the E.coli WF26 extract following the protocol described in Table 13.

6) Reduction of serum complement levels

Serum complement levels were reduced in mice by the injection of either zymosan (ex Saccharomyces cerevisiae. Koch Light Laboratories) or cobra venom factor (Cordis Laboratories, Roden, Holland) according to the dosage schedules of Easmon and Glynn (1976).

The reduction in the complement levels of zymosan treated mice was assessed either by the method of Rosenberg and Tachibana (1962) or by equating complement levels in the

serum of mice with the ability of the mice to mount an inflammatory response. 0.025 ml of a 5% turps in oil mixture was injected into the hind right footpad of the mice and the depression of the inflammatory response of the zymosan treated mice compared with the normal mice was taken to indicate the extent to which zymosan had reduced the complement of the treated animals (Ward and Cochrane, 1965; Willoughby et al, 1969).

The dosage of cobra venom factor used would be expected to result in complete reduction of the serum haemolytic complement levels (Cochrane et al, 1970; Easmon and Glynn, 1976).

RESULTS

1) Antibody titres of mice during an acute infection of the urinary tract

The antibody titres of Porton male and Biozzi high and low responder female mice to live and heat killed *E.coli* WF98 were determined. Mice were then infected with 1.25×10^7 bacteria. Their antibody response to the infecting organisms was assessed on days 3, 5, 7, 10 and 14 after the initiation of the infection.

Antibodies to the infecting bacteria were rapidly produced by all three types of mice but the response of the Biozzi high responder mice was 4 times greater than that of the Porton or Biozzi low responder mice (Fig. 15). The response of the Biozzi low responder mice closely follows that of the Porton mice. After 5 days the response of all the mice reached a peak and all animals had circulating antibody titres to both O and the O plus K antigen of E.coli of at least 640. Experiments in which mice have been infected for a long period of time have shown that the antibody titre to both live and heated E.coli remains elevated for many weeks. A Porton male mouse with an E.coli WF82 urinary tract infection had a titre of 160 against live WF82 after 8 weeks and a titre of 64 6 months after the infection had commenced. The infection persisted in the animal for at least 9 months.

2) Antibody titres of mice against E.coli lipopolysaccharide

Porton, Biozzi high responder and Biozzi low responder male mice were injected intraperitoneally with 5 µg of E.coli O127:B8 Westphal lipopolysaccharide. The antibody responses to the lipopolysaccharide are shown in Fig. 16.

The high and low responder Biozzi mice gave identical responses with a peak titre of 1280 on Day 7. The titres of Porton mice were similar or slightly higher. Titres against uncoated sheep red blood cells were negligible.

Fig. 15 ANTIBODY RESPONSE TO E. COLI WF 98 OF PORTON, BIOZZI, HIGH AND BIOZZI LOW RESPONDER MICE WITH AN E. COLI WF 98 URINARY TRACT INFECTION

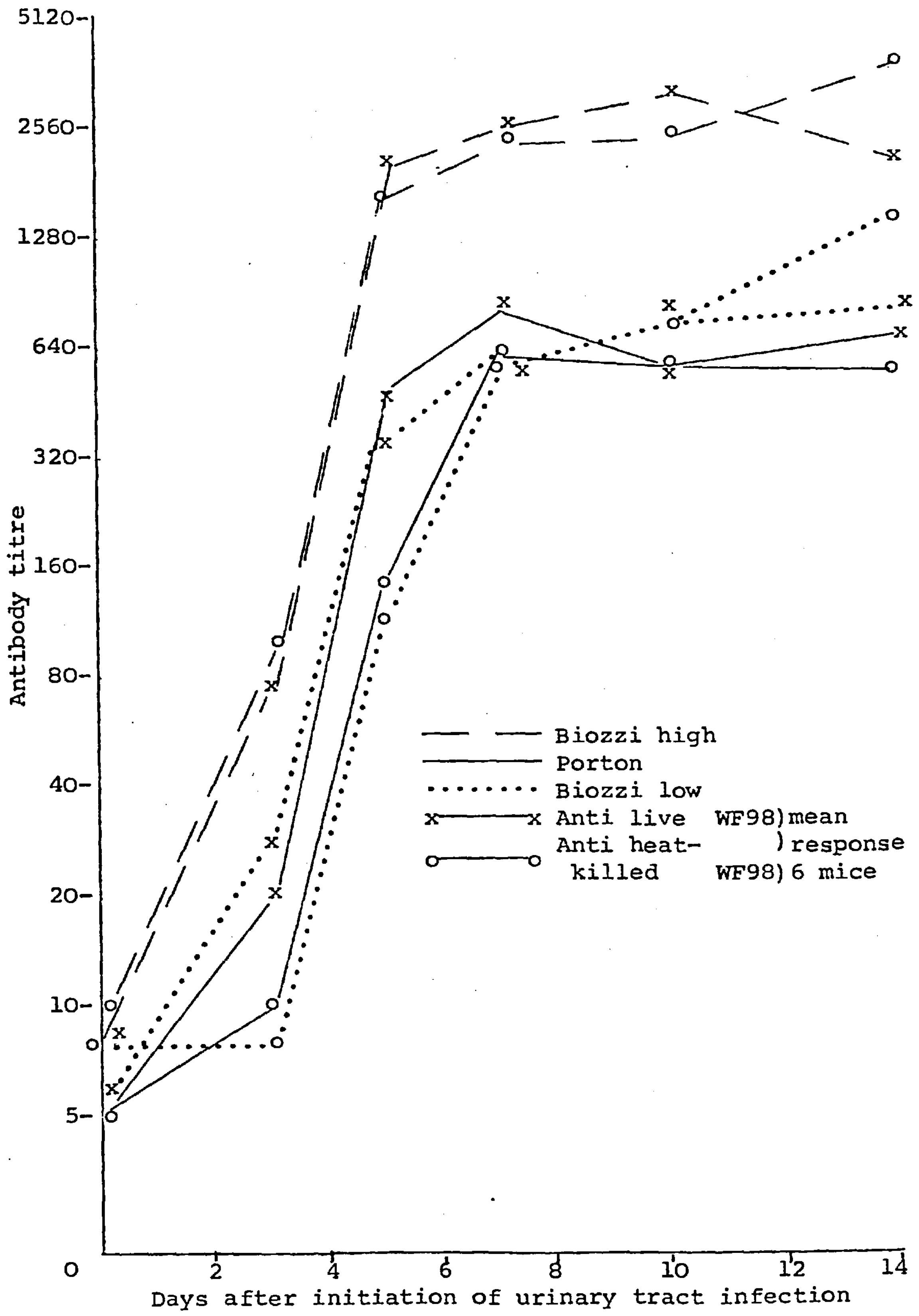
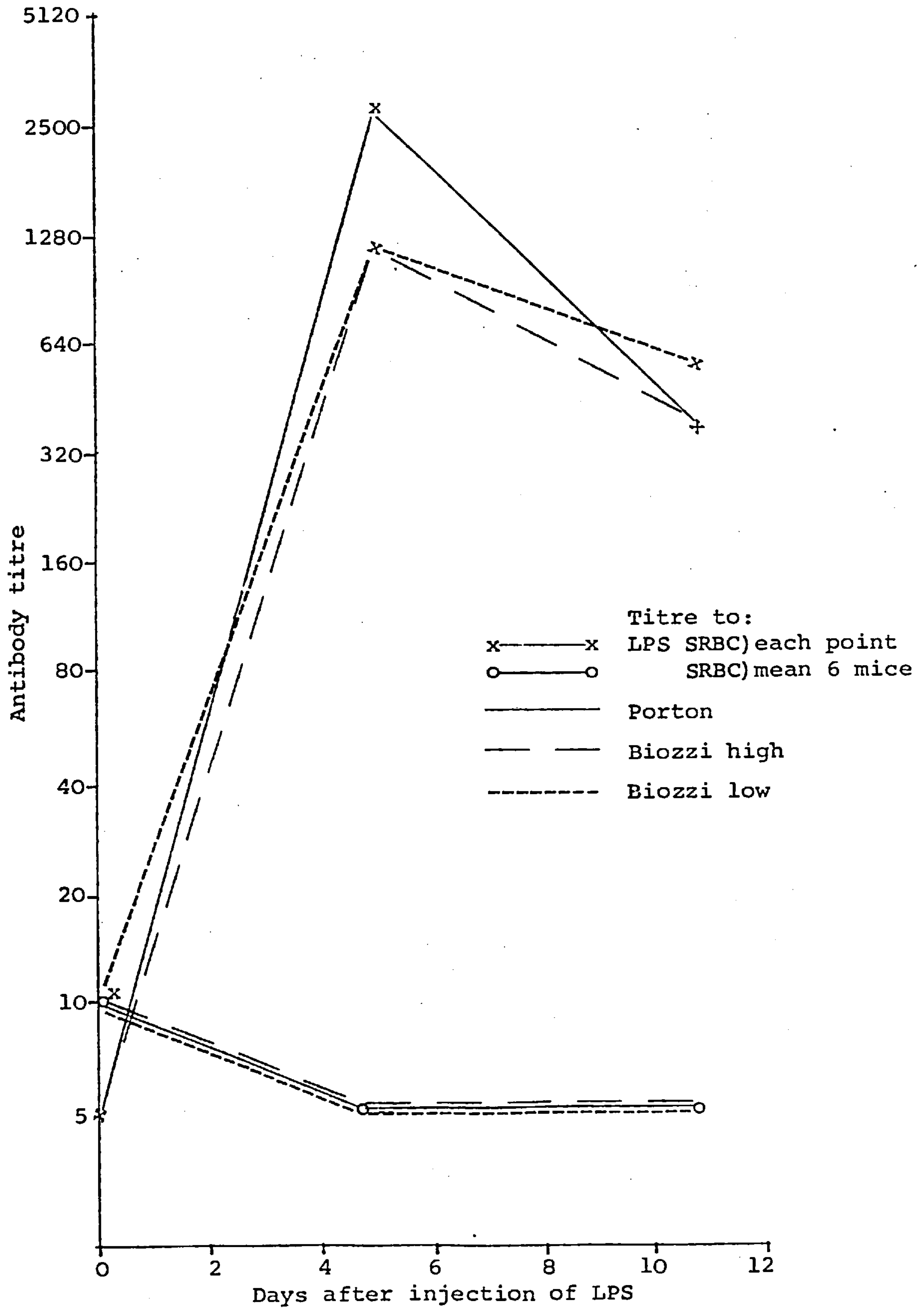


Fig.16 ANTIBODY RESPONSE TO E.COLI LIPOPOLYSACCHARIDE OF PORTON, BIOZZI HIGH AND BIOZZI LOW RESPONDER MICE



3) Antibody titres of Biozzi high and low line mice to sheep red blood cells

In order to check that the lines of mice bred here still differed in the magnitude of their antibody response to sheep red blood cells, Biozzi high and low responder mice were injected intravenously with 10^8 sheep red blood cells. The results (Fig. 17) show a very clear difference between the lines.

4) Urinary tract infections in mice immunised with E.coli

The antibody titres of immunised Porton mice to *E.coli*, were determined. The mice were then challenged with an *E.coli* WF26 urinary tract infection.

The infections produced in the mice gave no consistent pattern of results (Table 12); the total kidney infection rates however were very similar in both immunised and normal mice. Thus circulating antibody, produced by immunisation, neither protected mice against an *E.coli* urinary tract infection nor enhanced the infectivity of the organisms for the kidney.

5) Urinary tract infections in mice immunised with E.coli extract

Mice immunised with *E.coli* WF26 extract were assessed for their antibody response to live and heat killed *E.coli* WF26. The animals were then given an *E.coli* WF26 urinary tract infection.

Fig.17 ANTIBODY RESPONSE TO SHEEP RED BLOOD CELLS OF BIOZZI HIGH AND LOW RESPONDER MICE

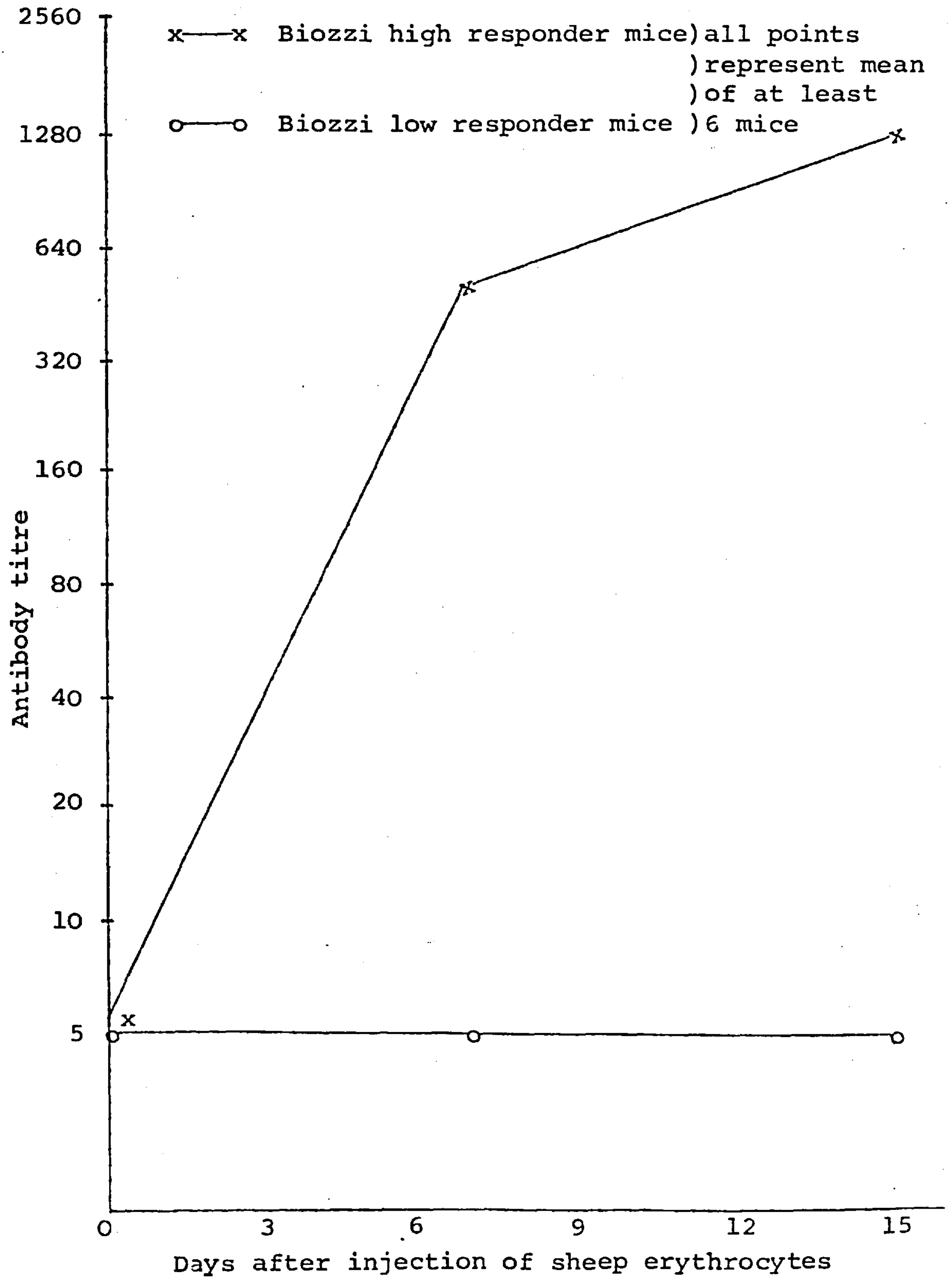


Table 12

E. COLI URINARY TRACT INFECTIONS IN MICE IMMUNISED WITH HEAT AND FORMOLIN KILLED E. COLI

	Controls		Mice immunised with E. coli					
			Heat killed			Formalin killed		
	Kidneys infected $>10^2$	Kidneys infected $>10^3$	Mean titre	Kidneys infected $>10^2$	Kidneys infected $>10^3$	Mean titre	Kidneys infected $>10^2$	Kidneys infected $>10^3$
Experiment a) challenge = 7.25×10^6 6x immunised	9/16	7/16	320	5/16	4/16	160	3/12	3/12
Experiment b) challenge = 3.88×10^6 3x immunised	9/16	4/16	2560	7/16	5/16	40	8/14	8/14
Experiment c) challenge = 4.38×10^6 3x immunised	8/16	4/16	2560	12/16	8/16	320	8/16	6/16
Total Kidneys infected	26/48	15/48		24/48	17/48		19/42	17/42
% kidneys infected	54	31		50	35		45	40

χ^2 test on total infected immunised kidneys with $>10^3$ bacteria/kidney (heat killed and formolin killed) compared with controls

$\chi^2 = 1.17 \quad n = 1 \quad P = <0.3 >0.20$ i.e. not significant

(χ^2 test using fourfold tables and Yates correction)

No protection by antibody was demonstrated, despite some of the mice having titres of 160 against the bacteria (Table 13). When infection was taken to be more than 10^3 organisms per kidney, the immunised animals appeared to be more susceptible to urinary tract disease than the control mice. The differences observed however were not significant, as shown by the χ^2 test.

6) Effect of passive transfer of antibodies to E.coli on an E.coli urinary tract infection in the recipients

Antisera to E.coli WF26 was obtained by bleeding out male Porton mice with raised antibody titres to the organism. Antibodies had been raised in these mice by either injecting the footpads of the mice with E.coli WF26 extract or by infecting the urinary tracts of the mice with E.coli WF26. Serum from several mice was pooled and the antibody titre against E.coli WF26 was determined (Table 14).

Male Biozzi low responder mice were injected subcutaneously with 0.25 - 0.5 ml of the pooled antiserum 3 hours prior to the induction of a urinary tract infection. All mice were assessed for disease after 3 days.

The transfer of immune serum against E.coli WF26 to Biozzi low line mice resulted in the first experiment in three mice dying and the surviving mice being highly infected. In

Table 13

URINARY TRACT INFECTIONS IN MICE IMMUNISED WITH *E. COLI* EXTRACT AND INFECTED WITH 4.2×10^7 *E. COLI* WF26 ON DAY 0

	Immunised x3	Immunised x2	Immunised x1	Controls
25 µl dose Day 4	1x		1x	Not immunised
" 14	2x	2x		
Average antibody titre Day 1:				
Heated *E.coli* WF26	160	80	10	5
Live " "	160	40	20	<5
Kidneys infected				
$>10^2$ *E.coli*/kidney	5/6	15/16	7/8	13/16
$>10^3$ " "	2/6	8/16	4/8	3/16
% kidneys infected $>10^3$ *E.coli*/kidney	33	50	50	19
Total kidneys containing $>10^3$	14/30			3/16

χ^2 test on total immunised infected kidneys containing $>10^3$ *E.coli* / kidney compared with controls:

$\chi^2 = 2.39$ n = 1 P = <0.2 >0.1 i.e. not significant

(using fourfold table and Yates correction)

Table 14

PASSIVE TRANSFER OF *E. COLI* WF26 ANTIBODIES ON AN *E.COLI* WF26 INFECTION IN BIOZZI LOW LINE MICE

	Experiment	Mice	
		Antibody treated	Control
Titre antisera	a	320	Not
	b	640	immunised
Dose	a	0.5 ml	-
	b	0.25 "	-
Infecting dose	a	1×10^7	1×10^7
	b	1×10^7	1×10^7
Deaths < 24 h after infection	a	3/7	0/4
	b	0/10	0/4
Infected kidneys [$>10^3$/kidney]	a	7/8	1/8
	b	8/20	5/8
Total $>10^3$ infected kidneys		15/28 = 53%	6/16 = 38%

χ^2 test using fourfold tables and Yates correction

$\chi^2 = 0.51$ and $P < 0.5 > 0.3$ i.e. not significant

the second experiment no deaths occurred and no enhancement of the virulence of the infecting organism was observed. Although the titre of the antiserum with which the mice had been injected was higher in the second experiment than in the previous experiment, the volume of serum given to the animals was lower.

The χ^2 test on the total results shows no significant difference in the number of kidneys infected in antisera treated mice compared with control mice.

7) Effect of decomplementing mice with zymosan on E.coli urinary tract infections

In experiment I measuring the haemolytic serum complement levels of control and zymosan treated mice by the method of Rosenberg and Tachibana, resulted in the anomolous finding that the zymosan treated mice had a higher complement titre than the normal mice (Table 15). Depletion of complement by injection of zymosan on Day 0 may have stimulated complement synthesis and resulted in overcompensation.

In the subsequent experiments zymosan was administered daily. A separate experiment to assess the effect of zymosan on the inflammatory response in the footpad had shown that the maximum reduction of footpad swelling occurred when zymosan was administered at the same time as the

Table 15

E. COLI URINARY TRACT INFECTIONS IN MICE DECOMPLEMENTED WITH ZYMOSAN

Experiment	I	II	III	IV	Total
Dose zymosan mg/mouse	5	5	3	3	
Timing	-2 h	Day -1,0,1	Day -1,0,1	Day -1,0,1	
C'H 50's/ml					
Day 1 zymosan	100	-	-	-	
control mice	58	-	-	-	
% inhibition footpad swelling Day 1	-	52	48	30	
Infective dose	1.8×10^4	1.76×10^5	8.0×10^6	2.88×10^4	
E. coli WF	98	98	41	26	
Kidneys $>10^2$/kidney					
zymosan	4/12	9/16	8/18	11/18	32/64 50%
control	3/16	7/16	7/16	8/16	25/64 39%
Kidneys $>10^3$/kidney					
zymosan	4/12	7/16	2/18	5/18	18/64 28%
control	2/16	3/16	3/16	5/16	13/64 20%

χ^2 test on $>10^3$ infected using fourfold table + Yates

χ^2 test on $>10^3$ infected using fourfold table + Yates correction

$\chi^2 = 0.68$ $n = 1$ $\therefore P = <0.50>0.30$

i.e. not significant

inflammatory agent (Fig. 18). However, giving two doses of zymosan 48 and 24 hours prior to the injection of the turps in oil suspension had an almost maximal effect on the degree of footpad swelling. Injecting mice with zymosan as indicated in Table 15 would therefore have been expected to keep the circulating complement levels at a minimum throughout the three day infection; this was checked by measuring the inflammatory response in the footpad one day after the induction of the infection.

A lower dose of zymosan was administered in experiments III and IV to prevent mice developing a Schwartzman-like reaction after the second and third injections of zymosan. This reaction had been observed in mice injected with repeated 5 mg doses of zymosan.

Although zymosan depleted the complement levels of the mice, the process did not have any consistent effect on the number of infected kidneys recovered from decomplemented mice (Table 15). The χ^2 test on the total results indicates that the small increase in the total numbers of infected kidneys obtained in zymosan treated mice compared with normal mice is not significant. Thus depleting mice of complement by administering zymosan has no effect on the outcome of an acute E.coli urinary tract infection. (Figs.19,20).

Fig.18 PERCENTAGE SWELLING OF MOUSE FOOTPADS 4 H AFTER INJECTION OF A TURPENTINE/OIL MIXTURE AS AN INFLAMMATORY AGENT INTO THE RIGHT HIND FOOTPAD; MICE PREVIOUSLY TREATED AT DIFFERENT TIMES WITH 5 MG/MOUSE ZYMOSAN OR ANTI-C'3 SERUM

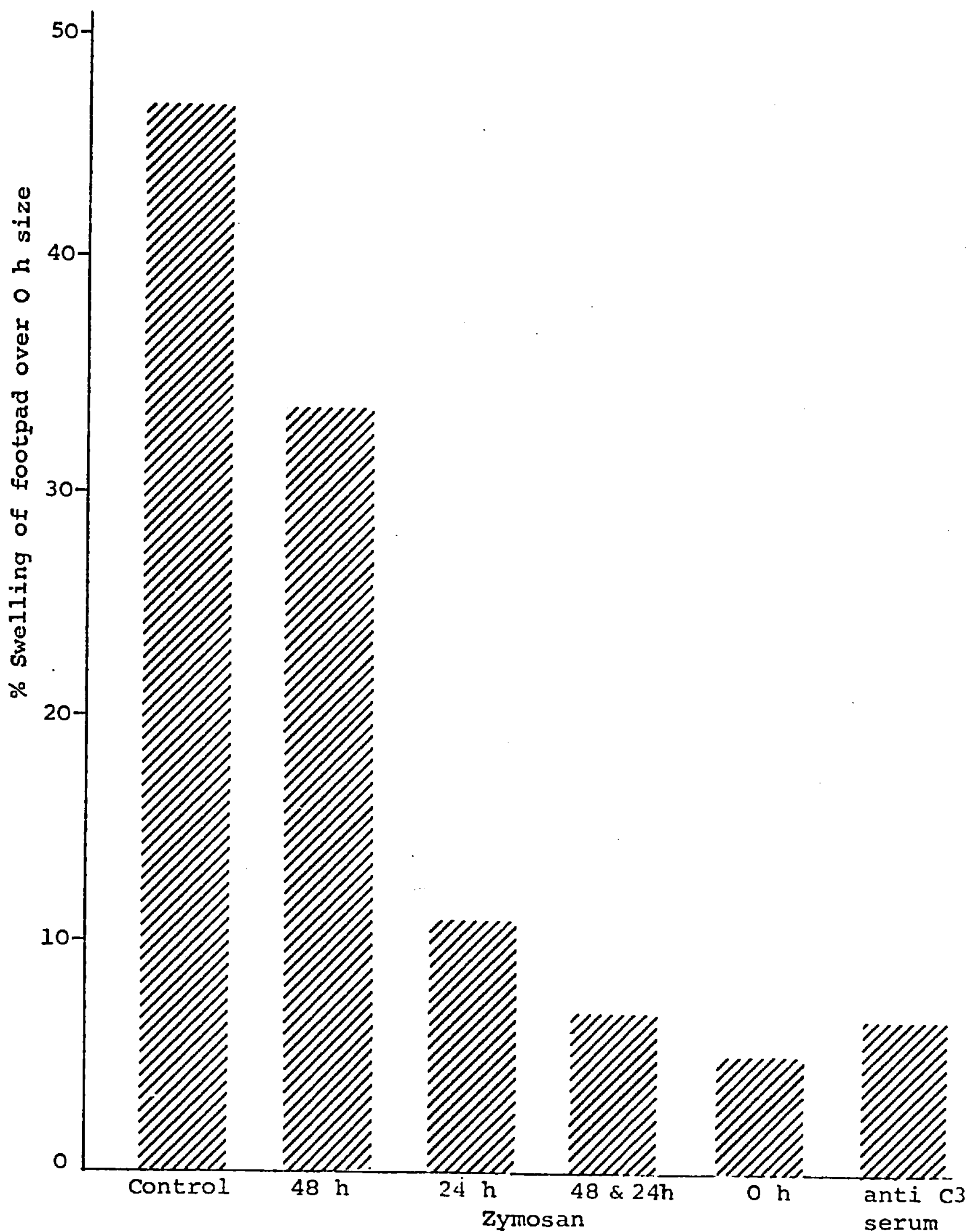


Fig.19 VIABLE COUNTS OBTAINED FROM THE URINES OF CONTROL AND AND ZYMOSAN TREATED MICE WITH E.COLI URINARY TRACT INFECTION

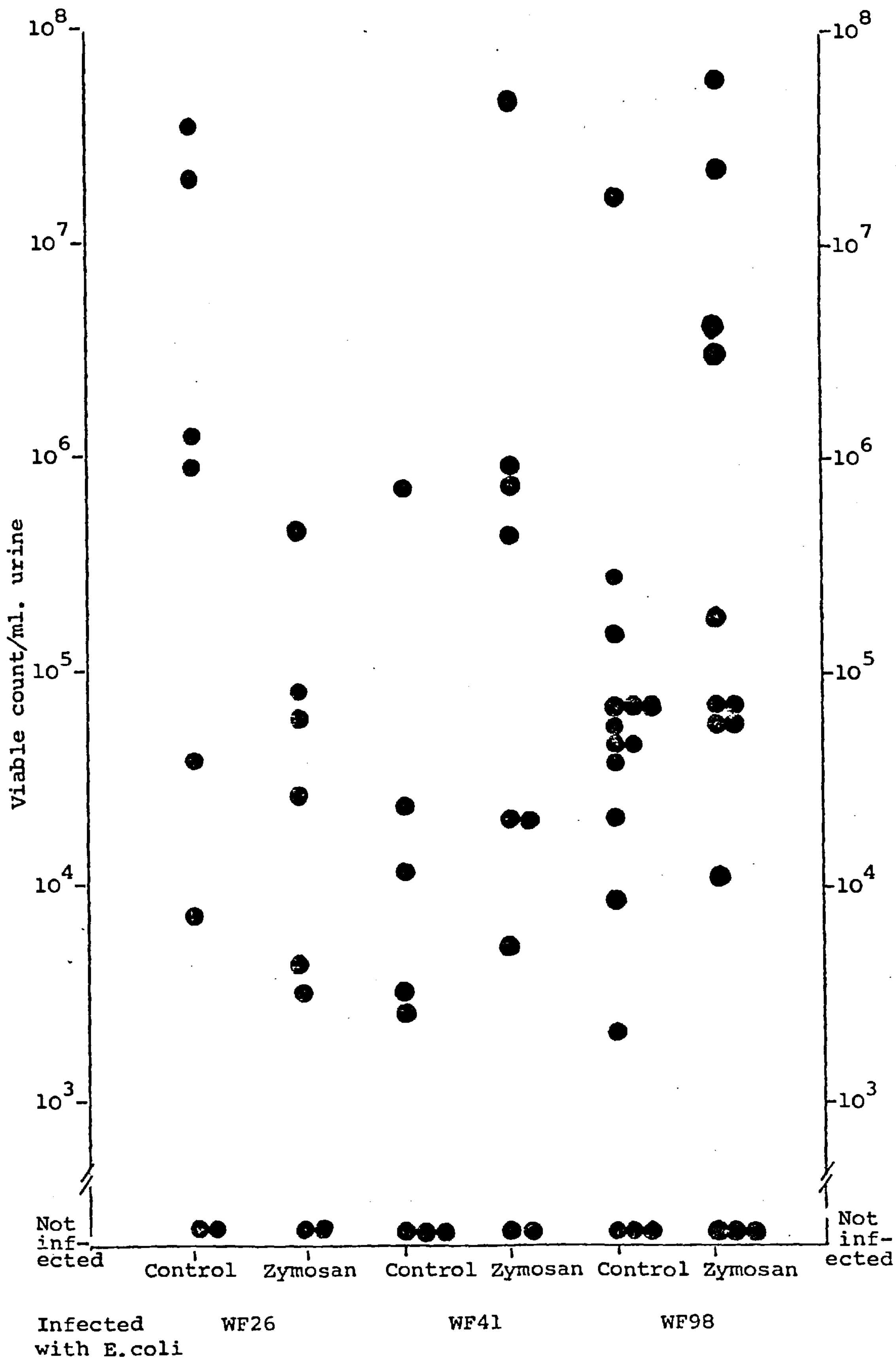
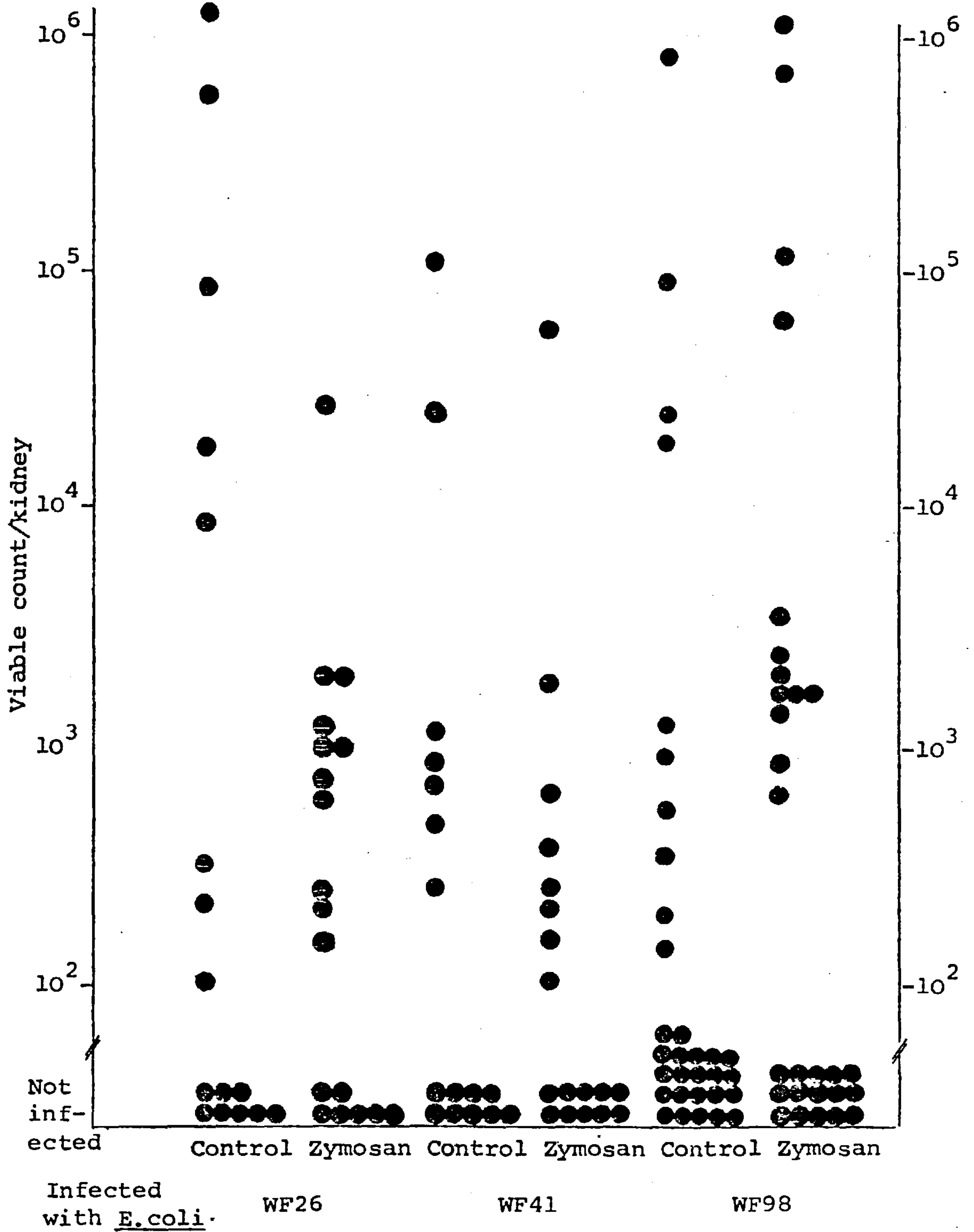


Fig. 20 VIABLE COUNTS OBTAINED FROM THE KIDNEYS OF CONTROL AND ZYMOSAN TREATED MICE WITH *E.COLI* URINARY TRACT INFECTIONS



8) Effect of decomplementing mice with cobra venom factor on E.coli urinary tract infections

Cobra venom factor is a potent anticomplementary agent. One unit of cobra venom factor is defined as the quantity of cobra venom factor in 0.1 ml that reduces the haemolytic capacity of a 1/20 dilution of normal human serum by 50% (Cochrane et al, 1970). Treatment of the mice with the doses as indicated in Table 16 was therefore expected to result in the complete depletion of circulating complement in these animals.

Control and treated mice were infected with E.coli WF98 and the number of infected kidneys was determined after 3 days. In experiments I and IV many mice died within 24 hours of the induction of the infection; the number of control mice that died was within expected limits. A possible explanation of this is that the bacteria injected into the decomplemented mice leaked from the bladder into the bloodstream and caused a fatal septicaemia.

Reducing the infective dose of bacteria or decreasing the amount of cobra venom factor administered to the mice resulted in a negligible number of deaths in the other experiments, but variable findings as to the outcome of the acute infection in the decomplemented mice. No definite trend either to increased or decreased susceptibility to infection was established in the cobra venom factor treated mice. (Figs.21,22).

Table 16

E.COLI URINARY TRACT INFECTIONS IN MICE DECOMPLEMENTED WITH COBRA VENOM FACTOR

Experiment:	I	II	III	IV	V	Total
CVF units/20 g body wt. i.p.	2	2	2	2	1, then 2	
Day	-1	-1, 1	-1, 1	-1, 1	-1, 1	
Infective dose *E. coli* WF98	1.3×10^7	2.1×10^6	5.6×10^6	6.0×10^6	5.4×10^6	
Deaths < 24 h after infection						
CVF	6/10	0/ 8	0/10	7/11	1/10	14/49
controls	1/10	0/ 8	1/10	0/10	1/ 9	3/47
$>10^2$/kidney						
CVF	5/ 8	1/16	14/20	3/ 8	6/18	29/70 =40%
controls	3/10*	6/16	6/18	7/10*	4/16	26/70 =36%
$>10^3$/kidney						
CVF	3/ 8	1/16	8/20	1/ 8	4/18	17/70 =24%
controls	3/10	4/16	5/18	5/10	3/16	20/70 =28%

χ^2 on $>10^3$ results using fourfold tables and Yates correction

$\chi^2 = 0.23$ n = 1 P = <0.7>0.5 i.e. not significant

* Only 5 surviving animals assessed for infection

Fig.21 VIABLE COUNTS RECOVERED FROM THE URINES OF CONTROL(C) AND COBRA VENOM FACTOR(CVF) TREATED MICE, WITH AN E. COLI WF98 URINARY TRACT INFECTION

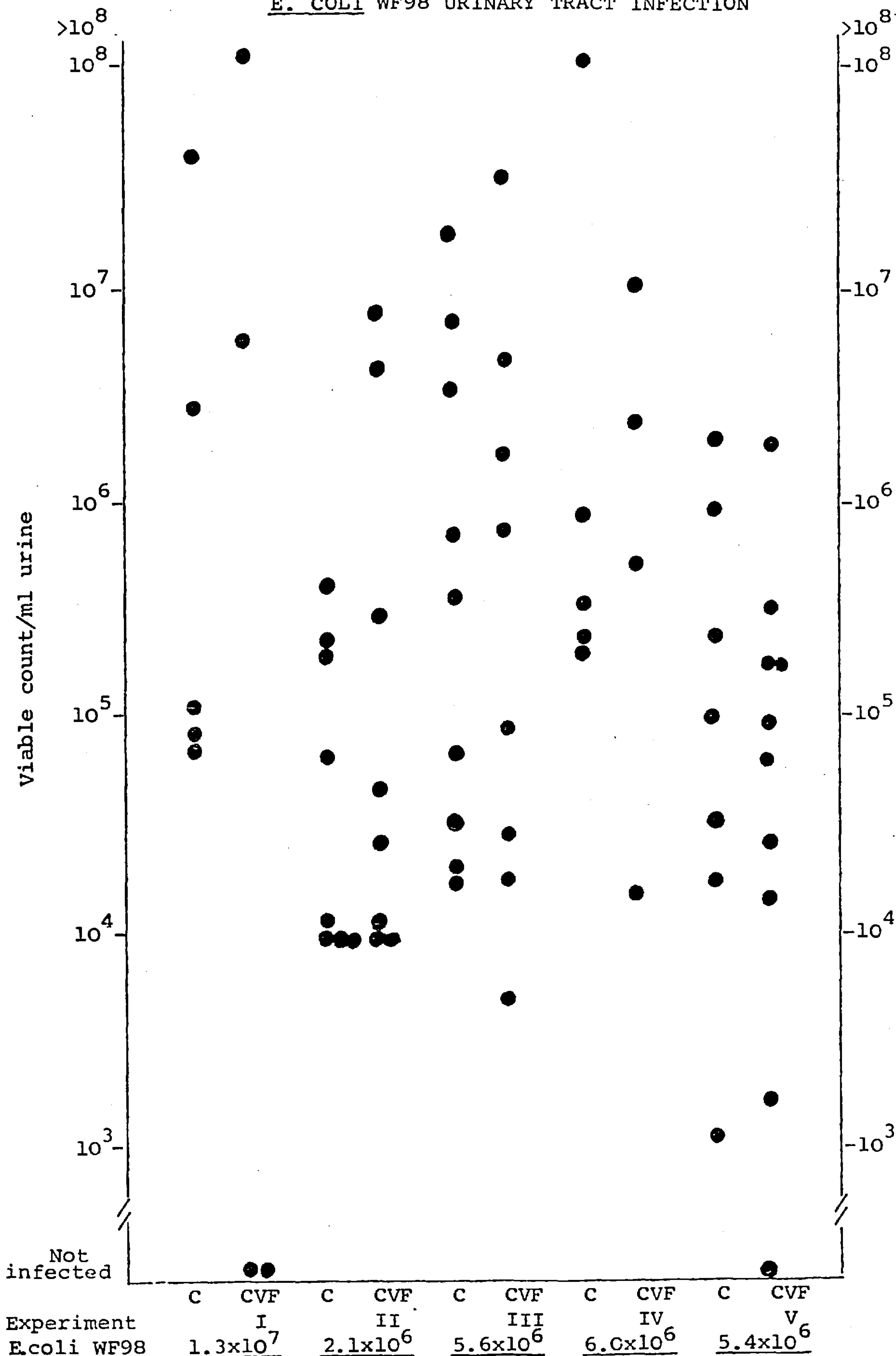
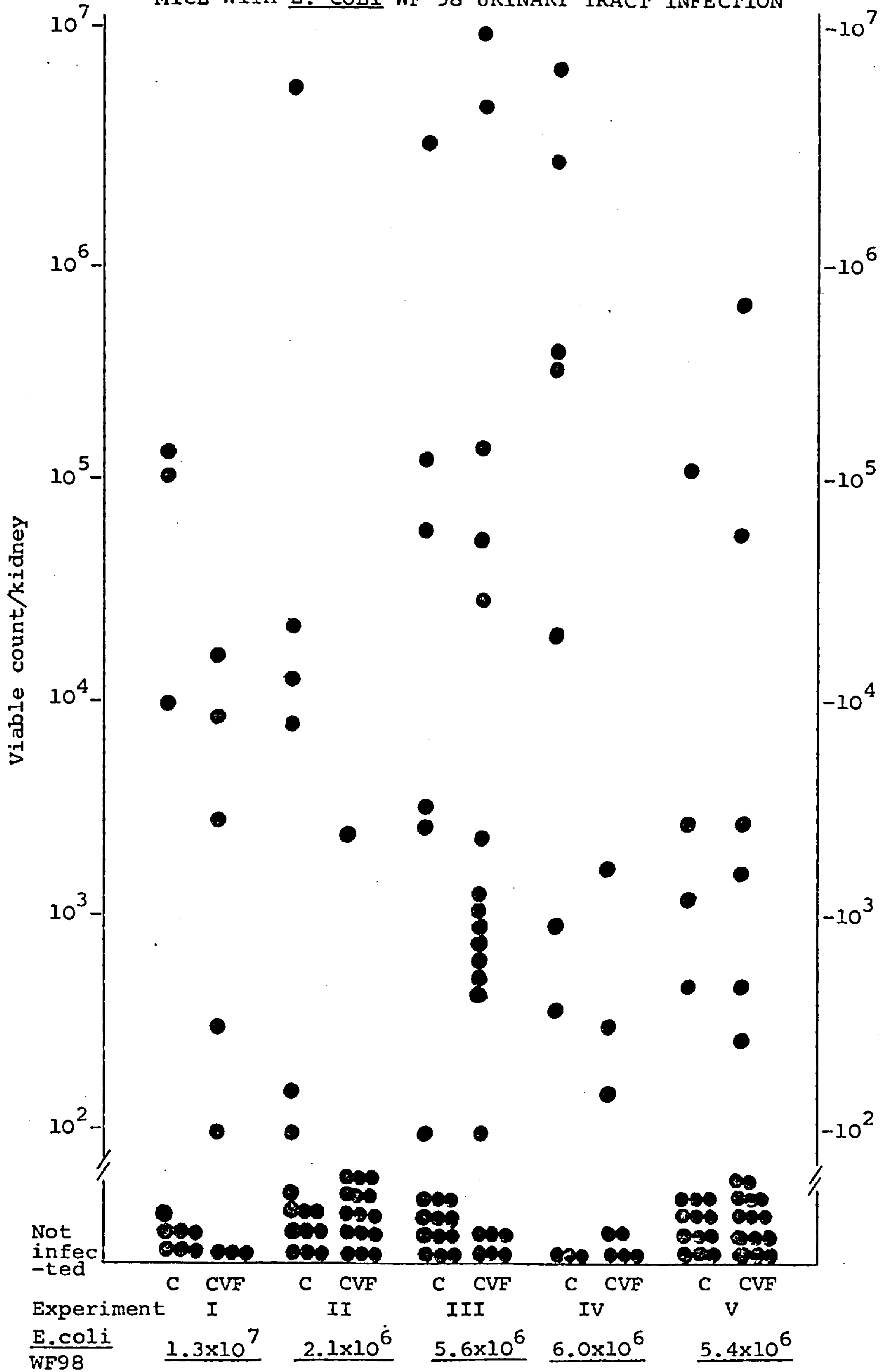


Fig.22 VIABLE COUNTS RECOVERED FROM THE KIDNEYS OF CONTROL(C) AND COBRA VENOM FACTOR(CVF) TREATED MICE WITH E. COLI WF 98 URINARY TRACT INFECTION



9) Comparison of $B_{10}D_2$ old line mice with $B_{10}D_2$ newline mice

$B_{10}D_2$ old line mice have a deficiency in their complement pathway at C5 (Nilsson and Müller-Eberhard, 1967). Thus if the whole complement sequence is involved in the immune response to infection, these deficient mice might be expected to be more susceptible to urinary tract disease than $B_{10}D_2$ newline mice which have a normal complement system.

A group of nine mice from each line were infected with 3.12 x 10^6 E.coli WF98, and assessed for infection after 3 days (Table 17). (Figs.23,24).

The number of infected kidneys obtained for each line did not differ significantly ($X^2 = 0.371$, $p = < 0.7 > 0.5$). This again indicates that complement is not involved in E.coli infections of the urinary tract.

DISCUSSION

A. Antibody

The Biozzi high responder mice gave a good antibody response to sheep red blood cells and the low responder mice only a poor one; the Biozzi mice kept here are therefore breeding true for the selected characteristics. In contrast both lines of Biozzi mice gave a similar reaction to live

Table 17

E.COLI URINARY TRACT INFECTIONS IN B10D2 OLD LINE AND B10D2 NEW LINE MICE

		B10D2 old line	B10D2 new line
Infected urines	$>10^3$/ml	9/9	7/7
Kidneys	$>10^2$/kidney	6/18	7/16
"	$>10^3$/ "	2/18 (11%)	4/16 (25%)

No. infected kidneys compared with 10^3 kidney :-

$\chi^2 = 0.371$ $n = 1$ $P = <0.7>0.5$ i.e. not significant

Fig.23 VIABLE COUNTS RECOVERED FROM THE URINES OF B10D2 NEW LINE AND B10D2 OLD LINE MICE WITH AN *E. COLI* WF98 URINARY TRACT INFECTION

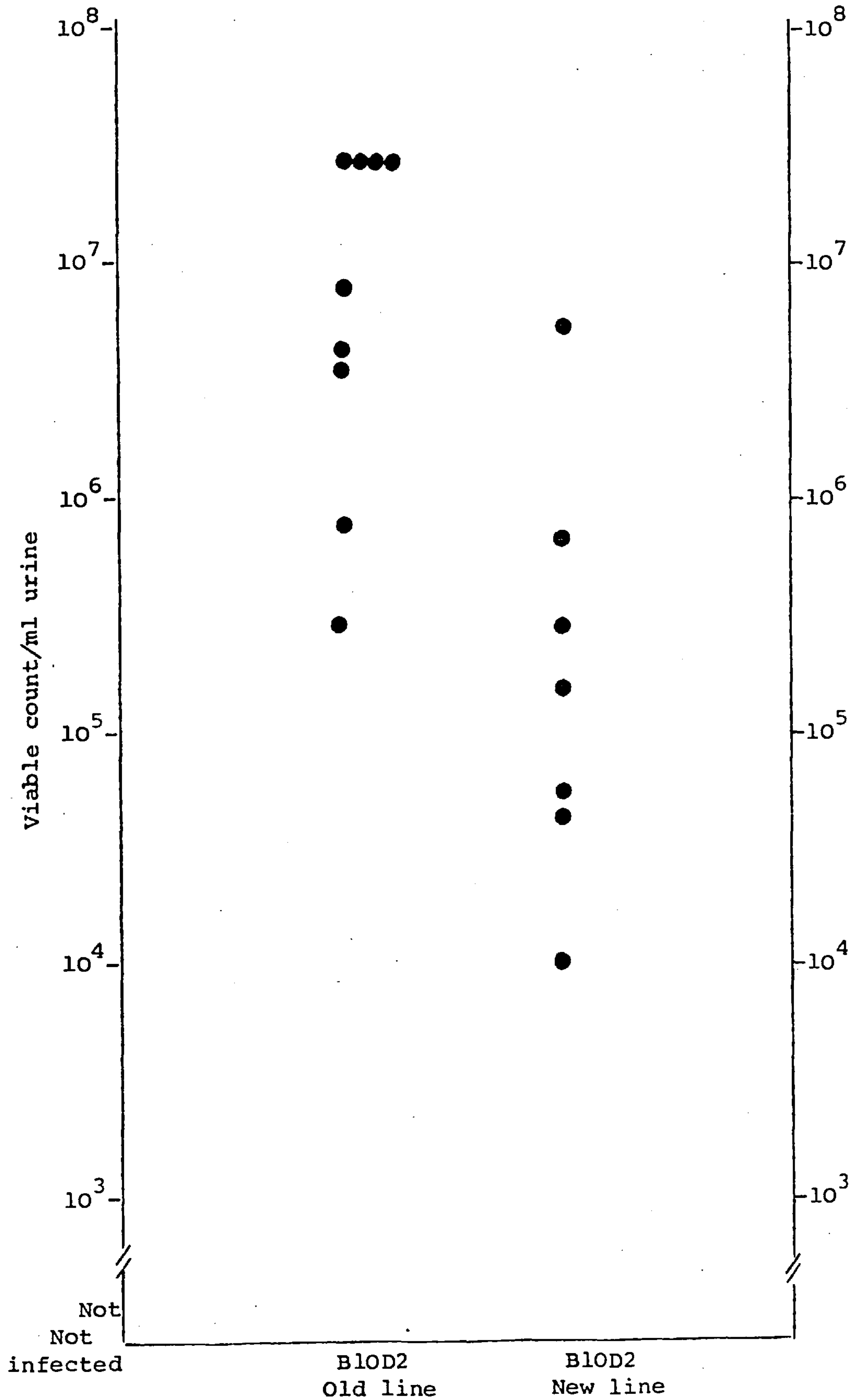
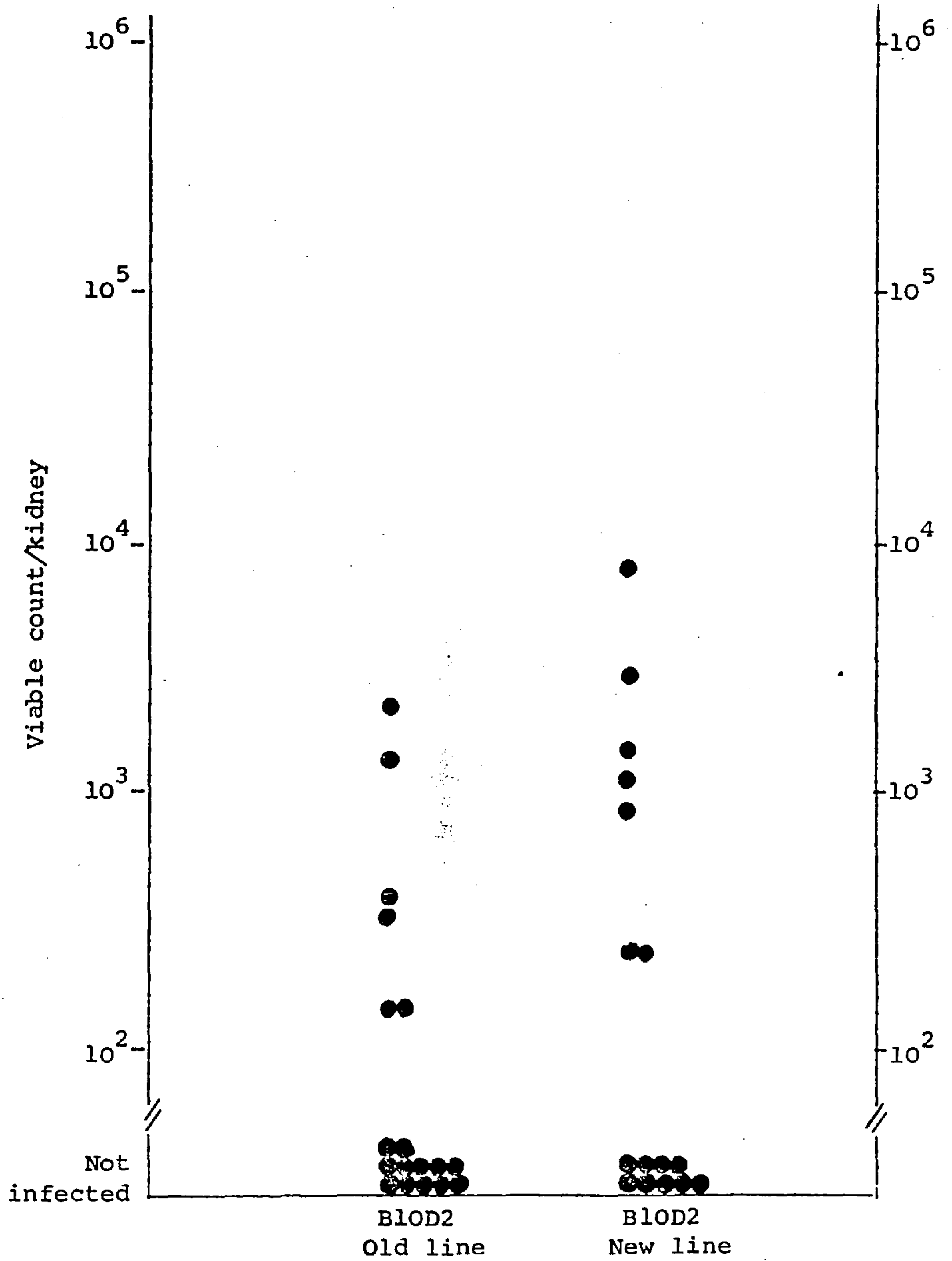


Fig.24 VIABLE COUNTS RECOVERED FROM THE KIDNEYS OF B10D2 NEW LINE AND B10D2 OLD LINE MICE WITH AN E. COLI WF 98 URINARY TRACT INFECTION



E.coli and to *E.coli* lipopolysaccharide, antibodies being produced by each line. Howard *et al* (1974) found that branched dextran and native levan gave an equivalent antibody response in both lines of mice. They suggested that these antigens did not have to be degraded by the macrophages of the mice in order for stimulation of the B cells to occur. The lipopolysaccharide of *E.coli* is a potent B cell mitogen and thus a T independent antigen.

It is possible that the stimulation of Biozzi high and low responder mice to produce antibodies against *E.coli* is also macrophage independent. If there were no differences in the B cells of the mice, direct stimulation of these cells by the *E.coli* would result in equivalent antibody production by the two lines. The elevation of the high line antibody response compared to the low line antibody response (Fig. 15) observed during an infection of the urinary tract may have been due to more bacteria being present in the urinary tract of the susceptible high responder mice than in the resistant low responder animals.

The difference in the susceptibilities of the two lines of Biozzi mice to urinary tract disease remains to be explained. Wiener and Bandieri (1974) showed that an important difference between the two lines of mice was in the rate at which their macrophages rendered antigens non-immunogenic. Degradation of the antigen occurred faster in

the macrophages of the low responder mice than in the macrophages of the high responder animals. This may also apply to the phagocytosis and destruction of *E.coli*. Hyperactive macrophages in the low line mice might be responsible for protecting the mice from upper urinary tract disease, while high line mice succumb more readily to disease because clearance of the invading bacteria is not carried out very effectively by their phagocytic cells. This hypothesis could probably be tested by *in vitro* studies of the degradation of radioactive labelled *E.coli* by the macrophages of the two lines by a method similar to that of Wiener and Bandieri (1974). The clearance of *E.coli* from the bloodstreams of high and low line Biozzi mice is discussed in Chapter 5.

An infection in the urinary tract resulted in a rapid rise in circulating antibody levels. The peak titres were reached within 5 days of the induction of infection, and the continued high levels after this time were probably due to the persistence of the antigen within the urinary tract. This is compatible with the observations made on people with urinary tract disease. The presence of high antibody titres to the infecting organism in people with active disease is often indicative of upper urinary tract involvement (Reeves and Brumfitt, 1968; Winberg *et al*, 1963). The one dose

stimulation of mice with bacterial lipopolysaccharide also resulted in a rapid rise in antibody titre but the peak response was followed by a decline in circulating antibody levels. Ahlstedt *et al* (1973) found that mice responded to *E.coli* lipopolysaccharide by rapidly producing antibodies and that after reaching a maximum the production of immunoglobins declined. Either specific antibody inhibited further synthesis or catabolism of the immunogen removed the stimulus from the antibody producing cells.

Immunising mice with killed *E.coli* or with an extract of live bacteria did not enhance the resistance of the mice to urinary tract infection. Uehling and Constant (1970) also failed to show any protection against ascending infection by serum antibody. Montgomerie *et al* (1972) immunised mice by subcutaneous injection of heat killed *E.coli*. This procedure protected mice from endotoxin induced death and haematogenous pyelonephritis but produced little or no resistance to ascending infections. Immunisation against the O and K antigens of *E.coli* by Kaijser and Olling (1973) protected rabbits against haematogenous pyelonephritis and Sanford *et al* (1962) and Kyriakos and Ikari (1969) prevented urinary tract infections in rats by antiserum to whole *E.coli* or to the O antigen alone. As most infections in people are of the ascending type, the ability to protect against a haematogenous infection may not be of great clinical importance.

Immunisation procedures therefore appear to protect animals against experimental haematogenous kidney disease but not against an ascending infection of the urinary tract.

Serum antibodies may well protect against haematogenous infections by aiding the opsonisation and destruction of bacteria in the bloodstream before they infect the kidney. The role of antibody within the urinary tract is not known. Antibody coated bacteria in the urine can be detected by the method of Thomas *et al* (1974) and a positive result has been shown to be indicative of kidney involvement.

Passive transfer of antibodies, directed against *E.coli*, to low responder Biozzi mice slightly increased the susceptibility of the recipients to urinary tract infection, although the result was not significant. Mackaness *et al* (1974b) have shown that immune complexes resulting from the interaction of antibody and antigen can depress the delayed hypersensitivity reaction. If cellular immune mechanisms are involved in the resistance of Biozzi low responder mice to *E.coli* urinary tract infection, then the passive transfer of antibody may have depressed delayed hypersensitivity and enhanced the infection. (The delayed hypersensitivity response of Biozzi high and low responder mice is discussed in Chapter 5.)

Alternatively the infections have been made worse in the low line mice by the passively transferred antibody inducing an immediate inflammatory response to the invading bacteria. Several mice died in the first experiment and this suggested an immediate reaction by the mice against E.coli. Death may have been caused by shock initiated by the presence of immune complexes within the body.

B. Complement

Zymosan and cobra venom factor deplete complement by activating the alternative complement pathway, and thus causing depletion of C3 (Pillemer et al, 1954; Götze and Müller-Eberhard, 1971). Easmon and Glynn (1976) measured the haemolytic complement titre of mice treated with the same doses of zymosan and cobra venom fector as described here and found these substances to be highly effective at depleting complement. Mice with artificially reduced or naturally low complement levels readily succumbed to an intraperitoneal infection with Staphylococcus aureus. Depleting serum complement levels however had no effect on the outcome of an acute E.coli urinary tract infection, and this suggested that the complement system of defence against infection does not act in the environment of the kidney or bladder.

Beeson and Rowley in 1959 incubated homogenates of body tissues with complement and either E.coli bacteria or sensitised erythrocytes. The kidney tissue was found to inhibit both the bactericidal and the haemolytic action of complement and this activity was not localised to any one site of the organ. The component of complement inactivated was C4 and this suggested that the kidney exerts its anti-complementary nature by the formation of ammonia, C4 being inactivated by this chemical. Complement is therefore unable to act in kidney tissue.

$B_{10}D_2$ old line mice are a complement deficient strain of mice as they have no C5 activity. These mice are therefore unable to complete the sequence C5-C9 and cannot give complement induced cytolytic responses (Nilsson and Müller-Eberhard, 1967). When E.coli was injected into the bladder the resulting urinary tract infection was no different in these mice than in the normal $B_{10}D_2$ newline mice. Thus a natural deficiency in the complement system has not altered the susceptibility of the mice to urinary tract infection. Complement is therefore not involved in the host defence against E.coli urinary tract infection.

E.coli urinary tract infections in mice were thus not affected by humoral immune mechanisms. This suggests that immunity to *E.coli* urinary tract disease may be cell mediated and this point is investigated in Chapter 5.

The K antigen of *E.coli* confers nephropathogenic properties on the bacteria (see Chapter 2) and also helps to determine their resistance to phagocytosis and antibody mediated complement killing (Glynn and Howard, 1970). As neither antibody nor complement acts in the urinary tract, the ability to resist these substances cannot be the determining property of the K antigen which enables K rich strains of *E.coli* to invade the upper urinary tract. The K antigen of K rich *E.coli* must therefore confer some other property on the bacterial cells which enables them to infect the kidney with a far greater efficiency than *E.coli* bacteria with little or no K antigen. The ability of *E.coli* strains to grow in urine is investigated in Chapter 6.

CHAPTER 5

THE ROLE OF CELLULAR IMMUNE FACTORS DURING AN E.COLI URINARY TRACT INFECTION

INTRODUCTION

Resistance to *E.coli* urinary tract disease in mice would not appear to involve humoral immunity. Resistance to this infection might possibly be controlled by cellular mechanisms. The ability of mice to exhibit a delayed hypersensitivity response to *E.coli* and to remove foreign particles from the circulation was investigated in order to establish if immunity to *E.coli* urinary tract disease is indeed controlled by cellular means.

MATERIALS AND METHODS

1) Sensitisation of mice to E.coli WF26

Mice were sensitised to *E.coli* WF26 by the following means:-

a) Urinary tract infection - Mice were infected with 7.5×10^6 *E.coli* by direct inoculation of the bacteria into the bladder.

b) Heat killed bacteria - Mice were injected subcutaneously or intraperitoneally with heat killed bacteria in Freund's complete Adjuvant (Difco Ltd.). Various concentrations of bacteria were injected in order to determine the optimal sensitising dose.

c) Extract of bacteria - An extract of the bacteria E.coli WF26 was prepared as described in Chapter 4. Mice were sensitised by injection of 25 µl aliquots of the extract into the hind footpads.

d) Subcutaneous infection - Silicon rubber tubing or polyurethane tubing chambers were inserted under the skin of anaesthetised mice. Each mouse was given two chambers, one each side of the spine, under the loosened skin of the flank. The incision in the skin was held together with Autoclips. The chambers filled with a clear sterile fluid, and injection of 1×10^5 E.coli WF26 resulted in a subcutaneous E.coli WF26 infection. This method is an adaptation of the method of Arko (1972).

2) Measurement of the delayed hypersensitivity response to E.coli WF26

The thicknesses of the right and left hind feet of control non-sensitised and sensitised mice were measured on a Tesameter Micrometer (Tesa S.A., Renens, Switzerland). Three measurements were made on each footpad and the mean of the determinations was taken to be the thickness of the footpad at that point in time.

25 µl of E.coli WF26 was injected subcutaneously into the right hind footpad and 25 µl of 0.15 M saline was injected likewise into the left paw. The left hind paw was a control for non-specific swelling of the footpad upon injection of

25 µl of fluid. Control mice were injected in order to correct the determination for any response to the extract due to non-specific sensitisation of the mice. The foot-pads were measured 4, 24 and 48 hours after stimulation of the mice with the extract.

Results were calculated by taking the mean value, deducting the 0 hour mean measurement, then correcting for the control left paw response in that animal, to obtain the swelling present in the paw due to extract at any point in time (as illustrated by Fig. 25). The final response is obtained by deducting the response in control mice due to non-specific swelling from this value. This gives a figure in millimetres which is indicative of the swelling in the footpad due to delayed hypersensitivity to E.coli WF26.

An attempt was made to elicit the delayed hypersensitivity response with live E.coli WF26. 2.5×10^4 live E.coli WF26 were injected into the footpad in a volume of 25 µl of saline, instead of 25 µl of E.coli extract.

3) Potentiation of the delayed hypersensitivity response with cyclophosphamide

Mice were injected intraperitoneally with 200 mg/Kg body weight cyclophosphamide (Cyclophosphamide - Endoxana. Ward Blenkinsop & Co.Ltd., London) two days before sensitisation to E.coli WF26. Lower doses, 100 mg/Kg and 150 mg/Kg, were also assessed for their ability to potentiate delayed hypersensitivity.

Fig. 25 CALCULATION OF FOOTPAD SWELLINGS AT TIME t_x

t_o - Right foot - 3 measurements   Left foot - 3 measurements

a, b, c   Mean d_{Rto} e, f, g   Mean d_{Lto}

t_x - Right foot - 3 measurements   Left foot - 3 measurements

a_1, b_2, c_2 Mean d_{Rtx} e_1, F_1, g_1 Mean d_{Ltx}

Swelling in right foot at $t_x = d_{Rtx} - d_{Rto} = D_{Rtx}$

Swelling in left foot at $t_x = d_{Ltx} - d_{Lto} = D_{Ltx}$

Swelling due to E.coli response alone $= D_{Rtx} - D_{Ltx} = X_{tx}$

Average response of n mice in the group at time $t_x = \frac{\sum_1^n X_{tx}}{n}$

This calculation is made for both the sensitised and the control groups of mice at 4 h, 24 h, and 48 h. As all measurements are made in mm, the units of the average response are also mm.

4) Potentiation of the delayed hypersensitivity response with B.C.G.

Mice were injected intravenously with 8×10^5 units B.C.G. (Glaxo, Batch B121) 14 days prior to sensitization with *E.coli* WF26.

5) Clearance of bacteria from the bloodstream of mice

Mice were anaesthetised with 0.6 mg/10 g body weight Nembutal and bacteria were injected into the tail vein. Blood samples were taken by retro-orbital puncture into heparinised capillary tubes at times zero and thereafter every 3 minutes. 50 µl aliquots of the blood were lysed in 2 ml of 0.1% sodium carbonate solution and viable counts made as in previous experiments. This is the method of Benacerraf *et al* (1959). The phagocytic index K was calculated from the formula:-

$$K = \text{log count at time 0} - \text{log count time x} / t_x - t_o$$

6) Clearance of carbon from the bloodstream of mice

The clearance of carbon from the bloodstream of mice was investigated according to the methods of Halpern *et al* (1953) and Biozzi *et al* (1953). The phagocytic index K was calculated from the formula:-

$$K = \log C_o - \log C_t \,/\, t_o - t_t$$

where C_o and C_t are the concentrations of carbon at times zero and 't' minutes.

RESULTS

1) Measurement of delayed hypersensitivity to E.coli WF26 in mice with an E.coli WF26 urinary tract infection

Male Porton mice were infected with E.coli WF26 and their delayed hypersensitivity response was elicited in a different group of animals each week for four weeks.

Mice sensitised to E.coli WF26 by these means exhibited a delayed hypersensitivity reaction in the footpad (Table 18). Antibody to E.coli WF26 is produced during the course of an acute infection (see Chapter 4) and this antibody was perhaps involved in the inflammatory response observed at 4 hours. The reaction at 4 hours increased with increasing time between the initiation of infection and the measurement of delayed hypersensitivity. When a large footpad response was obtained at 4 hours, it was difficult to establish if the 24 and 48 hour readings were due to the inflammatory response subsiding or the subsequent appearance of the delayed hypersensitivity reaction.

Mice with an 8 day E.coli WF26 urinary tract infection exhibited a minimal 4 hour reaction and a delayed hypersensitivity response at 24 and 48 hours. An 8 day urinary tract infection thus gave the clearest delayed hypersensitivity reaction.

Table 18

DELAYED HYPERSENSITIVITY TO E. COLI WF 26 IN PORTON MALE MICE WITH AN E. COLI WF 26 URINARY TRACT INFECTION

Delayed hyper - sensitivity response	Control response controls (no U.T.I) mm hours			Test response mm hours			Test - control mm hours		
Day	4	24	48	4	24	48	4	24	48
8 Expt. a)	0.28	0.11	0.23	0.25	0.49	0.44	-0.03	0.38	0.21
Expt. b)	0.03	0.05	0.02	0.23	0.38	0.25	0.20	0.33	0.23
15	-0.03	0.38	-0.025	0.84	0.24	0.32	0.87	-0.14	0.32
21	-0.02	0.005	0.004	0.28	0.22	0.31	0.30	0.22	0.31
28	-0.01	?	0.035	1.08	?	0.43	1.09	?	0.39

The responses obtained in the control mice were variable, perhaps due to natural sensitisation to *E.coli* WF26.

2) Measurement of delayed hypersensitivity in mice sensitised by means other than an active urinary tract infection

The previous experiment showed that mice produced a delayed hypersensitivity response to *E.coli* WF26 after sensitisation to the organism by means of an active urinary tract infection. The degree to which any individual mouse could be infected was not controllable as after injection of a known inoculum into the bladder, subsequent infection of the upper urinary tract was extremely variable (see Chapter 2). It was thus not possible to sensitise each mouse to *E.coli* to the same extent.

An attempt was therefore made to find a method of sensitising mice uniformly to *E.coli* WF26. Groups of mice were injected either subcutaneously or intraperitoneally with various doses of heat killed bacteria in Freund's complete adjuvant, at different times prior to footpad testing. The reaction obtained in the footpad, however, was not as good as that observed in mice with an 8 day urinary tract infection (Table 19).

Table 19

DELAYED HYPERSENSITIVITY TO E. COLI WF 26 IN PORTON MICE SENSITIZED BY MEANS OTHER THAN AN E. COLI URINARY TRACT INFECTION

Sensitization E. coli WF 26	Day	Control mm. 4 h	Control mm. 24 h	Control mm. 48 h	Test mm. 4 h	Test mm. 24 h	Test mm. 48 h	Test-control mm. 4 h	Test-control mm. 24 h	Test-control mm. 48 h
Heat killed in FCA (CHALLENGE EXTRACT)										
10^4 i.p.	7	-0.03	0.07	0.07	-0.02	0.20	0.20	0.01	0.13	0.13
10^4 i.p.	14	0.04	0.15	0.12	0.02	0.13	0.20	0.02	-0.02	0.08
10^4 s.c.	7	-0.03	0.07	0.07	0.12	0.15	0.07	0.15	0.08	0
10^4 s.c.	14	0.04	0.15	0.12	0.06	0.34	0.17	0.02	0.19	0.05
10^5 i.p.	4)				0.05	0.24	0.11	0	0.10	-0.12
10^5 i.p.	6)				0.05	0.10	0.10	0	-0.04	-0.13
10^6 i.p.	4)				0.08	0.23	0.19	0.03	0.09	-0.04
10^6 i.p.	6)	0.05	0.14	0.23	0.16	0.35	0.34	0.11	0.21	0.11
10^7 i.p.	4)				0.09	0.22	0.21	0.04	0.08	-0.02
10^7 i.p.	6)				0.13	0.19	0.20	0.08	0.05	-0.03
10^8 i.p.	4)				0.12	0.21	0.17	0.07	0.07	-0.06
10^8 i.p.	6)				0.03	0.23	0.21	-0.02	0.09	-0.02
10^6 i.p. *	5	0.27	0.04	0.02	0.24	0.14	0.10	-0.03	0.10	0.08
S.C. chamber infections (CHALLENGE EXTRACT)										
Silicon	10	0.11	0.25	0.10	0.22	0.39	0.24	0.11	0.14	0.14
Polyurethane	10	0.11	0.25	0.10	0.08	0.25	0.19	-0.03	0	0.09
Silicon	6	0.13	0.22	0.26	0.20	0.41	0.32	0.07	0.19	0.06
Extract										
2 x 25µl	10	-0.05	0.15	0.09	0.15	0.28	0.23	0.20	0.13	0.14

* Challenge 2.5 x 10^4 E. coli WF 26

Elicitation of the response in mice, sensitized with 10^6 heat killed E.coli WF26 by the injection of 2.5×10^4 live E.coli WF26 instead of E.coli WF26 extract, only produced a minimal reaction (Table 19). The extract is therefore better for eliciting the response than live bacteria.

Sensitisation of mice by injection of 2 doses of extract produced a reaction in the footpad after elicitation with extract, but the response was poor (Table 19).

It therefore seemed probable that live bacteria were needed for adequate sensitisation of the mice against E.coli WF26. Mice were infected with E.coli in subcutaneous chambers and the delayed hypersensitivity response was elicited with extract. Mice infected with E.coli in silicon rubber chambers gave the best response but high control responses indicated that the mice were already sensitized to E.coli (Table 19). The response obtained however was still not as good as that obtained with an active E.coli urinary tract infection, despite the presence of large numbers of bacteria within the chambers.

As Lagrange et al (1974b) had shown that cyclophosphamide potentiates the delayed hypersensitivity response of mice to sheep red blood cells, Porton mice were injected with 200 mg/Kg cyclophosphamide 2 days prior to sensitisation

to E.coli. After 4 or 6 days the footpad response was elicited. Within 2 hours of the injection of extract the mice treated with cyclophosphamide appeared shivery and hunched up. Their abdomens looked 'pinched in', and shortly afterwards they died. Death was thought to be due to endotoxin shock, this substance being present in small amounts in the extract. The one mouse that survived showed a good response at 48 hours (Table 20).

Lowering the dosage of cyclophosphamide to 150 mg/Kg and 100 mg/Kg resulted in fewer deaths, but no potentiation of the delayed hypersensitivity response. Thus cyclophosphamide cannot be used to enhance the delayed hypersensitivity reaction to E.coli.

Mackaness et al (1974a) potentiated the delayed hypersensitivity response of mice to sheep red blood cells with B.C.G. However, no enhancement of the delayed hypersensitivity reaction to E.coli WF26 was demonstrable in B.C.G. treated Porton mice (Table 20).

Thus, a standard method of sensitising Porton mice to E.coli has not been found. The reactions obtained with the various methods investigated were all poorer than the reactions observed in mice with an 8 day E.coli urinary tract infection, and cyclophosphamide and B.C.G. did not enhance the response.

Table 20

ENHANCEMENT OF DELAYED HYPERSENSITIVITY TO E. COLI WF 26 BY CYCLOPHOSPHAMIDE AND B.C.G.

Enhancement procedure * (before sensitization)	Sensitization procedure † (before elicitation) of heat-killed E. coli i.p. in FCA	Control response mm hours 4	24	48	Test response mm hours 4	24	48	Test-control mm hours 4	24	48
Cyclophos-phamide 2 days *	10^4 E. coli WF 26									
Dose	Day †									
200 mg/Kg	4	0.08	0.08	0.22	0.21	0.15	0.50	0.13	0.07	0.28
					3/4 died					
200 mg/Kg	6	-0.02	0.17	0.01	all died					
200 mg/Kg	-	0.08	0.08	0.22	-0.04	-0.02	0.12	-0.12	-0.10	-0.10
-	4	0.08	0.08	0.22	0.02	0.11	0.14	-0.06	0.03	-0.08
-	6	-0.02	0.17	0.01	0.01	0.16	0.11	0.03	-0.01	0.10
150 mg/Kg	4	0.09	0.18	0.20	-0.03	0.14	0.14	-0.12	-0.04	-0.06
					3/5 died					
100 mg/Kg	4	0.09	0.18	0.20	0.07	0.14	0.20	-0.02	-0.04	0
BCG i.v. 14 days*	10^6 E. coli WF 26									
Units										
$8x10^5$	4				0.02	0.12	0.03	0.04	0.14	0.01
$8x10^5$	-	0.02	0.02	0.02	0.01	0.14	0.10	0.03	0.16	0.08
-	4				0.03	0.23	0.11	0.05	0.25	0.09

3) Delayed hypersensitivity response of inbred mice to E.coli WF26

Balb/c and CBA male mice were sensitised to E.coli WF26 by injection of 10^4 heat killed E.coli WF26 either intraperitoneally in Freund's complete adjuvant or subcutaneously in saline. The Balb/c mice demonstrated a slight response after sensitization with the intraperitoneally administered antigen. The CBA mice, in contrast, gave no response with both means of sensitisation (Table 21).

Sensitisation of inbred mice with an E.coli urinary tract infection resulted in the demonstration of good delayed hypersensitivity by most of the inbred strains of mice resistant to E.coli urinary tract infections (Table 22). Strains of inbred mice, highly susceptible to urinary tract infection,only gave poor responses. This suggested that the ability of mice to resist a urinary tract infection with E.coli was dependent on their capicity to mount a delayed hypersensitivity reaction against the invading organism.

This hypothesis however would not appear to be true as the F_1 ($B_{10}D_2$ newline x C3H) mice, although highly susceptible to E.coli,gave a good delayed hypersensitivity response to E.coli.

Table 21

MEASUREMENT OF DELAYED HYPERSENSITIVITY TO E. COLI WF 26 IN BALB/C AND CBA MICE SENSITISED BY 10^4 HEAT KILLED E. COLI WF 26 IN FCA OR SALINE

Strain	Sensitised Day -4 by	Control mm. 4 h	24 h	48 h	Test mm. 4 h	24 h	48 h	Test-control mm. 4 h	24 h	48 h
Balb/C	10^4 heat killed E.coli in FCA i.p.	0.10	0.05	0.05	-0.006	0.21	0.20	0.10	0.16	0.15
	10^4 heat killed E.coli in saline s.c.				0.03	0.03	0.15	-0.07	-0.02	0.10
CBA	10^4 heat killed E.coli in FCA i.p.	0.14	0.19	0.18	0.02	0.25	0.20	-0.12	0.06	0.02
	10^4 heat killed E.coli in saline s.c.				0.11	0.09	0.24	-0.03	-0.10	0.06

Table 22

MEASUREMENT OF DELAYED HYPERSENSITIVITY TO E. COLI WF 26
IN INBRED MICE WITH AN 8-DAY E.COLI WF26 URINARY TRACT INFECTION

	STRAIN Males		CONTROLS MM.			TEST MM.			TEST-CONTROL MM.		
			4 h	24 h	48 h	4 h	24 h	48 h	4 h	24 h	48 h
RESISTANT TO INFECTION	B10 D2 new line	a)	0.01	0.09	0.23	0.54	0.92	0.48	0.53	0.83	0.25
		b)	0.07	0.06	0.38	0.27	0.29	0.55	0.20	0.23	0.17
	Balb/C	a)	0.02	-0.02	0.14	0.15	0.34	0.22	0.13	0.36	0.08
		b)	0.08	0.15	0.05	0.08	0.41	0.21	0	0.26	0.16
	A/Jax	a)	0.07	0.13	0.18	0.06	0.51	0.48	-0.01	0.38	0.30
		b)	0.025	0.09	0.15	0.50	0.58	0.92	0.48	0.49	0.77
	C57 black	a)	0.09	0.14	0.39	0.09	0.35	0.12	0	0.21	-0.27
		b)	0.15	0.185	0.25	0.53	0.79	0.56	0.38	0.60	0.31
	CBA	a)	-0.06	0.06	0.11	0.39	0.65	0.97	0.45	0.59	0.86
		b)	-0.01	0.04	0.15	0.13	0.17	0.56	0.14	0.13	0.41
SUSCEPTIBLE TO INFECTION	DBA/1		0.23	0.20	0.25	0.08	0.21	0.07	-0.15	0.01	-0.18
	C3H/He	a)	-0.02	0.02	0.07	0.13	0.04	0.03	0.15	0.02	-0.04
		b)	0.025	0.11	0.17	0.09	0.28	0.36	0.07	0.17	0.19
	DBA/2		0.05	0.12	0.15	0.18	0.31	0.23	0.13	0.19	0.08
B10D2 x C3H F1 mice			0.125	0.08	0.11	0.44	0.48	0.35	0.315	0.40	0.24

4) Delayed hypersensitivity response of Biozzi high and low responder mice to E.coli WF26

Biozzi high and low responder mice were sensitised to *E.coli* WF26 either by an infection of the urinary tract or by intraperitoneal injection of 10^6 heat killed *E.coli* WF26 in FCA. The infected mice were assessed for their delayed hypersensitivity response after 15 days and the mice sensitised with dead bacteria were challenged with extract 6 days after injection of the bacteria.

A very poor response to *E.coli* WF26 was obtained with both lines of mice for each of the two methods of sensitisation (Table 23). The low responder control mice did, however, show a good response, whereas a minimal response was obtained in the high line controls. The low line mice would appear to have an inherent degree of natural sensitivity to *E.coli*, probably induced by *E.coli* present in the gut flora.

5) Clearance of bacteria from the bloodstream of mice

Porton and Wright Fleming Institute mice rapidly cleared *E.coli* WF26 from the bloodstream (Table 24). The mean value obtained for K was 0.140. Most of the bacteria injected into the tail vein were deposited in the liver and spleen and less than 1% were recovered from the kidneys.

Table 23

DELAYED HYPERSENSITIVITY RESPONSE OF BIOZZI HIGH AND LOW RESPONDER MICE TO E. COLI WF 26. RESPONSE TESTED WITH E. COLI WF 26 EXTRACT

Responder mice	Sensitised by	Average swelling mm. Control 4 h	24 h	48 h	Test 4 h	24 h	48 h	Test -control 4 h	24 h	48 h
High	E. coli WF 26 U.T.I. Day -15	0.09	0.08	0	0.42	0.17	0.11	0.33	0.09	0.11
	10^6 heat killed E. coli WF 26 in FCA i.p. Day -6	"			0.14	0.03	0.14	0.05	-0.05	0.14
Low	E. coli WF 26 U.T.I. Day -15	0.17	0.14	0.26	0.02	0.08	0.11	-0.15	-0.06	-0.15
	10^6 heat killed E. coli WF 26 in FCA i.p. Day -6	"			0.04	0.14	0.21	-0.13	0	-0.05

Table 24

CLEARANCE OF *E. COLI* WF 26 FROM THE BLOOD OF WRIGHT-FLEMING INSTITUTE AND PORTON MICE

Strain	Dose i.v.	Viable counts/g after 20 min. Liver	Spleen	R.Kidney	L.Kidney	Phagocytic Index K
	Per mouse					
Wright-Fleming Institute	1.5×10^8	1.3×10^7	2.1×10^7	5.0×10^5	-	0.161
	"	6.2×10^7	4.2×10^7	3.8×10^4	4.7×10^4	0.091
	"	-	-	-	-	0.151
	"	-	-	-	-	0.149
	/10g Body weight					
Porton	5×10^7	1.2×10^8	1.0×10^8	8.4×10^5	1.21×10^6	0.127
	"	1.55×10^8	2.0×10^8	3.75×10^5	4.25×10^5	0.157
	"	6.1×10^7	1.32×10^8	3.7×10^5	4.6×10^5	0.142
	"	7.7×10^7	1.32×10^8	1.02×10^6	4.35×10^4	0.146
Geometric mean		6.39×10^7	8.22×10^7	3.62×10^5	3.44×10^5	Mean=0.140

E.coli WF82 was cleared more slowly from the circulation than was E.coli WF26 (Table 25). This suggested that E.coli WF26 was less resistant to the bactericidal powers of complement and to phagocytosis than had previously been noted for this strain (Howard and Glynn, 1971).

Comparison of the rate of clearance of E.coli WF98 from the blood of two different strains of inbred mice showed that Balb/c mice cleared the bacteria faster than CBA mice (Table 26). The students't'test on the K values gave a probability of these values occurring by chance of < 0.01. The difference observed between the two lines of mice is therefore statistically significant. The geometric mean viable counts recovered from the spleens, livers and kidneys were very similar in both Balb/c and CBA mice. In contrast the geometric mean lung count was significantly higher in the CBA than in the Balb/c mice, the students 't' test in the actual counts giving a value of t of 5.4 and a probability of < 0.01.

No difference was found in the rate of clearance of E.coli WF98 by Biozzi high responder and low responder mice (Table 27). The geometric mean counts recovered from the livers and spleens were similar for the two lines. The low line kidneys, however, contained more bacteria than the high line kidneys and this difference was shown to be statistically significant by use of the students 't' test ($t_{right\ kidneys} = 2.33$, $P < 0.05 > 0.01$; $t_{left\ kidneys} = 2.86$, $t < 0.01$).

Table 25

CLEARANCE OF E. COLI WF 82 FROM THE BLOOD OF MALE PORTON MICE

Dose i.v.	VIABLE COUNTS Liver	Spleen	R.kidney	L.kidney	K phagocytic index
$1.0x10^8$/10g body weight	n.d.	n.d.	n.d.	n.d.	0.04
	n.d.	n.d.	n.d.	n.d.	0.02
	$9.3x10^7$	$4.65x10^7$	$4.0x10^6$	$3.94x10^6$	n.d.

Table 26

CLEARANCE OF E. COLI WF 98 FROM THE BLOOD OF BALB/C AND CBA MICE

All mice injected i.v. with 0.1 ml/10 g body weight 2.5 x 10^9/ml. E. coli WF 98 in log phase

Strain	Mouse	Viable count/gm recovered after 20 min.					K phago-cytic index
		Liver	Spleen	Lung	R.kidney	L.kidney	
Balb/C	1	4.6 $x10^8$	8.4 $x10^8$	2.77$x10^7$	4.4 $x10^6$	3.76$x10^6$	0.189
	2	3.15$x10^8$	1.28$x10^8$	1.61$x10^7$	2.12$x10^7$	1.92$x10^7$	0.189
	3	2.63$x10^8$	2.71$x10^8$	2.0 $x10^7$	5.65$x10^6$	4.96$x10^6$	0.136
	4	2.35$x10^8$	9.0 $x10^7$	6.4 $x10^6$	1.8 $x10^6$	2.42$x10^6$	0.153
	5	2.5 $x10^8$	1.42$x10^8$	3.1 $x10^7$	4.25$x10^6$	3.75$x10^6$	0.172
	6	3.45$x10^8$	5.1 $x10^7$	3.35$x10^6$	3.1 $x10^6$	2.25$x10^6$	0.178
CBA	1	5.45$x10^8$	1.28$x10^8$	1.05$x10^9$	2.56$x10^7$	2.85$x10^7$	0.136
	2	5.2 $x10^8$	1.06$x10^9$	2.04$x10^8$	7.8 $x10^6$	6.6 $x10^6$	0.074
	3	4.45$x10^8$	3.2 $x10^8$	1.03$x10^8$	1.35$x10^7$	1.01$x10^7$	0.077
	4	3.4 $x10^8$	2.85$x10^8$	3.22$x10^7$	4.55$x10^6$	5.7 $x10^6$	0.088
	5	2.4 $x10^8$	6.2 $x10^8$	4.2 $x10^7$	5.8 $x10^6$	6.0 $x10^6$	0.120
	6	3.25$x10^8$	7.4 $x10^8$	5.1 $x10^7$	6.2 $x10^6$	6.9 $x10^6$	0.099
Geometric mean counts							Mean
Balb/C		2.9 $x10^8$	1.6 $x10^8$	1.3 $x10^7$	4.7 $x10^6$	4.3 $x10^6$	0.170
CBA		3.8 $x10^8$	4.1 $x10^8$	1.05$x10^8$	8.6 $x10^6$	8.6 $x10^6$	0.099

Students 't' test on K values gives $t = 5.4$ n.d.f. = 10
Therefore $P < 0.01$ i.e. highly significant

Students 't' test on mean lung counts gives $t = 2.85$ n.d.f. = 10
Therefore $P < 0.01$ i.e. highly significant

Table 27

CLEARANCE OF *E. COLI* WF 26 FROM THE BLOOD OF BIOZZI HIGH AND LOW RESPONDER MICE

Mice injected i.v. with 0.1 ml/10 g body weight 1×10^{10}/ml *E. coli* WF 26

Responders	Viable count/g.				K
	Liver	Spleen	R.kidney	L.kidney	
High					
1	-	-	-	-	0.100
2	-	-	-	-	0.142
3	5.7×10^{8}	3.5×10^{8}	6.7×10^{6}	8.6×10^{6}	0.126
4	5.4×10^{8}	9.3×10^{7}	2.5×10^{6}	2.8×10^{6}	0.144
5	1.0×10^{9}	4.7×10^{8}	1.28×10^{7}	1.5×10^{7}	0.164
6	4.65×10^{8}	3.6×10^{8}	6.3×10^{6}	3.0×10^{6}	0.219
7	6.4×10^{8}	2.65×10^{8}	7.0×10^{6}	6.3×10^{6}	-
8	3.3×10^{9}	2.7×10^{8}	2.5×10^{6}	2.6×10^{6}	-
Low					
1	-	-	-	-	0.186
2	-	-	-	-	0.080
3	1.16×10^{9}	3.6×10^{8}	2.15×10^{8}	2.25×10^{8}	0.128
4	9.4×10^{8}	5.25×10^{8}	1.88×10^{7}	2.5×10^{7}	0.130
5	1.05×10^{9}	7.7×10^{8}	2.7×10^{7}	3.7×10^{6}	0.144
6	3.25×10^{8}	3.7×10^{8}	1.78×10^{7}	1.49×10^{7}	0.144
7	3.65×10^{8}	7.4×10^{7}	6.2×10^{6}	5.0×10^{6}	-
8	5.4×10^{8}	8.4×10^{7}	7.1×10^{6}	1.02×10^{7}	-
Geometric mean					Mean:
High	8.13×10^{8}	2.7×10^{8}	5.36×10^{6}	5.13×10^{6}	0.149
Low	6.45×10^{8}	2.6×10^{8}	2.09×10^{7}	2.60×10^{7}	0.137

Students 't' test on mean kidney counts gives:-

t right kidney = 2.33 ndf = 10

Therefore P = $<0.05>0.01$ i.e. significant

t left kidney = 2.84 ndf = 10

Therefore P = <0.01 i.e. highly significant

6) Clearance of carbon from the blood of Balb/c and CBA mice

Balb/c mice cleared carbon from the circulation at a faster rate than that observed in CBA mice (Table 28). A proportional difference between the strains appears to exist for both carbon particles and E.coli. The K values for Balb/c mice were 0.170 and 0.0158 for E.coli and carbon respectively and 0.099 and 0.0098 respectively for CBA mice. This suggests an inherent difference in the phagocytic capacities of the reticuloendothelial systems of these mice which is independent of the particles involved.

DISCUSSION

As humoral immunity did not have any effect on the outcome of acute E.coli urinary tract infections in mice, it seemed likely that resistance to infection was controlled by other mechanisms, possibly of a cellular nature. Many bacterial infections have been shown to involve cellular immune reactions.

Delayed type hypersensitivity can be demonstrated following many infections. A response can be obtained after infections in both man and experimental animals with brucellosis (Spink, 1956), diphtheria bacilli and streptococci (Moen, 1936) and vaccinia viruses (Turk *et al*, 1962).

<u>Table 28</u>

CLEARANCE OF CARBON FROM THE BLOOD OF BALB/C AND CBA MICE

Strain	Mouse	O.D. Blood sample 3	6	9	12	15	K	Mean K
		minutes						
Balb/C	1	0.600	0.528	0.462	0.21	0.31	0.024	0.0158
	2	0.16	0.153	0.069	-	0	0.10	
	3	0.600	0.592	0.384	0.502	0.44	0.0111	
	4	0.682	0.642	0.405	0.492	0.484	0.0123	
CBA	1	0.805	0.742	0.652	0.538	0.480	0.0187	0.0098
	2	0.815	0.879	0.768	0.765	0.763	0.00242	
	3	0.378	0.804	0.696	0.635	0.592	0.0117	
	4	0.715	0.718	0.666	0.641	0.602	0.00625	

The acquisition of resistance to infection with *Listeria* and *Brucella* was shown by Turk *et al* (1962) to coincide with the acquisition of delayed type hypersensitivity. Mackaness (1967) equated the two events and suggested that immunity to the infection was mediated by the ability to mount a delayed hypersensitivity response. More recently Plant and Glynn (1974) have shown that strains of inbred mice resistant to infection with *Salmonella typhimurium* C5 are better at mounting a delayed hypersensitivity reaction to the infecting organism than strains of mice susceptible to this mouse pathogen.

Mice with an active *E.coli* urinary tract infection will give a delayed type hypersensitivity response after injection of a concentrated culture supernatant into their footpads. The optimum time between initiation of infection and measurement of the response was found to be 8 days. Lagrange *et al* (1974a) initiated a delayed type hypersensitivity response in mice by injecting them intravenously or subcutaneously with sheep red blood cells. No adjuvant was necessary and 10^5 sheep red blood cells intravenously was the optimal sensitizing dose. Increasing the inoculum of erythrocytes reduced and eventually abolished the response. The reaction was mediated by θ bearing lymphocytes and after being elicited once the mice became refractory to

further stimulation. Splenectomy had a profound effect on the observed responses; far higher doses of sheep red blood cells were needed to sensitize the mice and splenectomised animals were no longer refractory upon a second encounter with the antigen.

Mackaness et al (1974b) showed that as the sensitizing dose of sheep red blood cells was increased, the serum haemagglutinin titre also rose. As the antibody titre became elevated, the delayed hypersensitivity response diminished. This inverse relationship between antibody titre and the prevailing level of delayed hypersensitivity indicated a direct inhibitory effect of antibody on the production of the delayed hypersensitivity response. The complex formed by antibody and antigen was thought to block the activated T cells, which mediated the delayed hypersensitivity reaction, and thus the response was inhibited.

The delayed hypersensitivity response to E.coli has probably been initiated with a very low dose of antigen. The optimum response was obtained 8 days after the initiation of an E.coli urinary tract infection. Antibody synthesis after the infection of the urinary tract with E.coli is rapid (Chapter 4, Fig. 16) and optimum titres are obtained within 6 days of the initiation of the disease. These high circulating titres probably inhibit the delayed

hypersensitivity response after 8 days. The presence of high titres of antibody was suggested in many of the results obtained; inflammation 4 hours after the injection of extract was probably due to antibody-antigen stimulation of the Arthus reaction.

Inbred strains of mice resistant to E.coli urinary tract disease gave a good delayed hypersensitivity response to E.coli when sensitised to the organism by an 8 day urinary tract infection, in contrast to susceptible inbred strains which only exhibited a poor response. This suggested that the ability to mount a good delayed hypersensitivity reaction to E.coli protected the resistant strains of mice from urinary tract disease.

The F_1 ($B_{10}D_2$ newline x C3H) mice, however, were able to mount a good delayed hypersensitivity response to E.coli despite being susceptible to infection. Thus resistance to disease is not related to the ability to produce a good cellular reaction to E.coli present in the urinary tract.

By analogy to the results of Lagrange et al (1974), the production of a delayed hypersensitivity response to E.coli may be very dependent on the dose of the sensitising antigen administered; both too low a dose and too high a dose of the antigen would fail to prime the delayed hypersensitivity response. Although the inbred strains of mice were

all infected with a standard inoculum of E.coli,the subsequent proliferation of bacteria within the urinary tract is dependent on the susceptibility of the mouse strain to the disease. Thus the stimulatory dose of antigen would vary from strain to strain and the very susceptible strains of mice may have been unable to give a delayed hypersensitivity reaction,as the sensitising dose of bacteria they had encountered was probably too large. If all strains of inbred mice could be given a standard sensitising dose of antigen,then the natural ability of the strains to mount a delayed hypersensitivity reaction to E.coli could be examined. The Biozzi high and low responder lines of mice both failed to give a delayed hypersensitivity response to E.coli,probably because the reaction was inhibited by the antibody which is rapidly produced by both lines of mice in response to an E.coli urinary tract infection (Chapter 4, Fig. 16).

Mice were sensitised with known doses of heat killed bacteria with or without Freund's complete adjuvant in order to find a method for priming mice with a standard dose of E.coli, so that their natural ability to mount a delayed hypersensitivity reaction could be determined. All methods tried only produced weak reactions. This suggested that live bacteria are needed for adequate sensitisation of the animals to occur. Priming mice against live E.coli

with organisms growing in a subcutaneous chamber, however, was not successful. Again the problem of standardising the sensitising dose of antigen is encountered. Thus until mice can be given a known dose of antigen, which probably needs to contain a factor present only in live cells, the ability of the mice to mount a delayed hypersensitivity reaction cannot be monitored with any accuracy. Conclusions concerning the role of delayed hypersensitivity in an acute urinary tract infection can thus not be drawn at present.

The delayed hypersensitivity response of mice to sheep red blood cells can be enhanced if the antibody response to the erythrocytes is suppressed by the cytotoxic drug cyclophosphamide (Lagrange et al, 1974b). Attempts to boost the delayed hypersensitivity of mice sensitised to E.coli with heat killed bacteria by cyclophosphamide resulted in the mice dying within a few hours of the injection of the challenge dose of E.coli extract into the hind footpad. It is thought that the mice died either from endotoxin shock or from a reaction similar to tuberculin shock.

Pieroni et al (1970) decreased the LD_{50} dose of E.coli endotoxin for mice from 425 µg to 0.001 µg by first injecting the mice with 25 µg of actinomycin D. The mice died from ocular haemorrhage, weight loss and other pathological symptoms of endotoxin shock. Actinomycin D, like cyclophosphamide, is an anti-metabolic drug. Actinomycin D

rapidly and completely blocks the synthesis of RNA or DNA templates and at high concentrations blocks DNA synthesis (Goldberg and Reich, 1964). Cyclophosphamide exerts its effect in rapidly dividing cells and these cells are possibly the same cells as those affected by actinomycin D. If this is so,it may render the mice treated with cyclophosphamide many times more susceptible to endotoxin than normal mice, the E.coli extract being known to contain traces of endotoxin. The effect observed is mediated by cyclophosphamide because with decreasing doses of cyclophosphamide fewer mice died from this reaction. No deaths occurred in the sheep red cell primed mice of Lagrange et al (1974b) because these cells presumably contain no endotoxin.

The reaction observed in the mice may also be likened to tuberculin shock. It was possible that the cyclophosphamide had indeed potentiated the delayed hypersensitivity response of the mice. The one mouse that survived the treatment showed a good footpad response to E.coli. Tuberculin shock ensues when a highly sensitized guinea pig is injected with a large amount of dead tubercle bacilli. The animal becomes prostrated after 3 to 4 hours, the body temperature drops and death may follow in 5 to 30 hours. These symptoms are very similar to those observed in the E.coli sensitized, cyclophosphamide treated mice.

As the mice died soon after the elicitation of the footpad response from either one or both of the above types of reaction, no measurement of any potentiation in the delayed hypersensitivity response was possible.

Mackaness et al (1974a) infected mice with B.C.G. before sensitising them to sheep red blood cells. The factors normally formed by antigen-antibody complexes which inhibit the T cells involved in the delayed hypersensitivity response did not appear to function normally in the B.C.G. infected mice. The delayed hypersensitivity response to sheep red blood cells was enhanced despite the presence of high circulating titres of antibody. When the delayed hypersensitivity response to E.coli in B.C.G. infected mice was examined, no potentiation of the footpad reaction occurred. This suggested that either the experimental conditions were not favourable to demonstrate an enhanced response or that the mechanisms controlling the delayed hypersensitivity response of mice to the bacteria E.coli were different to those involved in the cellular immune reaction to sheep red blood cells.

The passive transfer of antibody to Biozzi low responder mice (see Chapter 4) resulted in a slightly increased rate of kidney infection in the treated animals. This suggested that naturally occurring delayed hypersensitivity to E.coli can be inhibited by antibody directed

against the bacteria. Sensitisation to E.coli has probably been mediated by E.coli strains present in the bowel flora. Thus delayed hypersensitivity may possibly have a protective function against E.coli disease of the urinary tract.

The ingestion of material by macrophages may be the prelude to an immune response or an essential step in the manifestation of acquired resistance to an invading organism. The ability of the cells of the reticulo-endothelial system to remove foreign substances from the circulation may be indicative of the degree of cellular immunity that the animal can display when confronted with infectious agents within its body. The rate at which intravenously injected materials are cleared from the circulation is an estimate of the phagocytic activity of the macrophages of the reticulo-endothelial system of the animal (Benacerraf et al, 1957).

The rate of clearance of E.coli WF26 from the circulation of mice was much faster than the rate obtained for E.coli WF82. The K value for WF26 was similar to the value obtained by Howard and Glynn (1971a) for E.coli WF26 in the presence of antibody and to the value of K that would be obtained for a complement sensitive strain of E.coli. This suggested that either the mice used here had naturally occurring antibody which opsonised E.coli WF26 or that the

strain WF26 was no longer complement resistant but had become complement sensitive. The K value obtained for E.coli WF82 was similar to that obtained by Howard and Glynn (1971a) for this strain in the absence of agglutinating antibody.

E.coli WF98 was cleared from the circulation of Balb/C mice significantly faster than from the circulation of CBA mice. Organ counts, including those of the kidneys ,were similar in each strain, with the exception of the counts recovered from the lungs. Nearly ten times more bacteria were found to be deposited in the lungs of the CBA mice than in the lungs of the Balb/c mice. Thus, despite a slower total rate of clearance of bacteria from the circulation in CBA mice, the alveolar macrophages of these mice appeared to be more active than the corresponding cells in Balb/c mice.

When carbon was injected into inbred Balb/c and CBA mice, the difference between the strains observed initially with E.coli was again apparent. The CBA mice cleared carbon from the circulation much more slowly than the Balb/c mice. The proportional difference in the rates between Balb/c and CBA mice was very similar for both E.coli WF98 and carbon stabilised by gelatin. This suggested an inherent difference between the mouse strains in the ability of their macrophages

to phagocytose any type of foreign material. Janet Plant (personal communication) found that Balb/c mice have bigger spleens and also more white cells throughout the body than CBA mice. This could explain the greater efficiency of the Balb/C reticulo-endothelial system at removing particles from the circulation. If this difference between the strains is also true in the environment of the urinary tract, this could possibly explain why Balb/c mice are more resistant to E.coli urinary tract infection than CBA mice.

The clearance of E.coli WF98 from the circulation of Biozzi mice occurred to the same extent in both high and low responder lines of mice. However, significantly more viable bacteria were recovered from the kidneys of the low responder mice than from the kidneys of the high responder mice. Thus, although the reticulo-endothelial system of the two lines of mice appeared to have a similar function, the actual number of bacteria carried to the kidneys in the blood was greater in the low line mice than in the high line mice. If the increased number of bacteria in the low line kidneys is due to a greater flow of blood to the low line kidneys than to high responder kidneys, then this could possibly explain the resistance of the low line mice to ascending E.coli urinary tract infection. A good blood flow to the kidneys would ensure that blood-borne means of immunity were rapidly transferred to the kidneys during an infection of the urinary tract.

In conclusion, it would appear that cellular immune mechanisms are involved in the host response to E.coli urinary tract infection. A delayed hypersensitivity reaction can be elicited in mice with an active E.coli infection. The role of delayed hypersensitivity in immunity to urinary tract disease is uncertain. Initial experiments indicate that the ability to mount a delayed hypersensitivity response against the invading organism may be protective, but until a standard method of sensitising animals to E.coli can be found, the natural ability of mice to produce this reaction cannot be accurately determined. If antibody regulates the delayed hypersensitivity response to E.coli, then further investigation of the effect of passive transfer of antibody on a urinary tract infection may prove to be of interest. The increased ability of the Balb/c reticulo-endothelial system, compared with that of CBA mice, to clear carbon and E.coli bacteria from the circulation perhaps indicates that macrophages which can readily phagocytose foreign materials are an important part of the host defences against urinary tract disease.

CHAPTER 6

FACTORS AFFECTING THE SURVIVAL OF E.COLI IN URINE

INTRODUCTION

E.coli bacteria may grow in urine. The persistence of *E.coli* strains in the bladder will be affected by how well they grow in the urine. It is known that mouse urine has a higher osmolality than human urine. The ability of strains of *E.coli* with differing K antigen contents to grow in urine or solutions of known osmolality was therefore examined.

MATERIALS AND METHODS

1) Determination of urine osmolalities

Urine osmolalities were measured automatically on an Osmette A machine in the Chemical Pathology Department here (Osmette A.Precision Instruments Ltd., Newton, Massachussets, U.S.A.). The machine determines the osmolality by measuring the freezing point of the solution. The depression of the freezing point from that obtained with pure water is a function of the osmolality of a solution. For a non-ionic substance dissolved in water the osmolality is equivalent to the molality. Urines of high osmolality were diluted with distilled water prior to osmolality measurement on the machine.

2) Alteration of the osmolality of urine samples

The osmolality of human female urine was modified by the addition of urea (B.D.H. Chemicals Ltd., Poole, England). 0.3 g of urea added to 10 ml of urine raised the osmolality from 469 mOsm/Kg to 1020 mOsm/Kg (Table 30). The osmolality of all modified urines was determined using a 1/10 dilution of the sample in distilled water.

3) Measurement of the growth of E.coli in urine

A measured volume of mouse, human, or modified human urine was dispensed into a sterile capped container and incubated at 37°C in a water bath. Strains of E.coli were grown overnight in Nutrient Broth, were washed in 0.15 M saline and resuspended in 0.15 M saline to a known concentration by O.D. The urine samples were inoculated with a small volume of the bacterial suspension in order to avoid changing the osmolality of the urines significantly by the addition of a large volume of 0.15 M saline.

An immediate sample of the inoculated urines was taken for the 't_o' determinations of the viable counts. The samples were incubated at 37°C in a water bath without agitation. Further samples were taken over a period of time for the determination of the numbers of viable bacteria present in the urine. All determinations of bacterial counts were made, as previously described, using the Droplette technique.

4) Induction of a diuresis in mice

A water diuresis was initiated in male Porton mice by substitution of their drinking water with a sterile solution of 5% glucose in tap water. Food was allowed freely. After 4 days the mice were infected either by the inoculation of bacteria into an unstitched bladder or by intravenous injection of *E.coli* bacteria. The diuresis was continued until the mice were assessed for infection of the kidneys.

RESULTS

1) The osmolality of human and mouse urines

Mouse urine samples were collected by catching the urine in a petri dish. The urines from several mice of one inbred strain were pooled and both male and female mouse urines were included. The samples were diluted in distilled water and the osmolalities were determined. Human female urine (source A.N.) samples were also assessed for their osmolality.

The mouse urines were very much more concentrated than the human urine (Table 29). The inbred strains of mice produced the most concentrated urine but no differences between the strains related to their susceptibilities to

Table 29

MEASUREMENT OF THE OSMOLALITY OF HUMAN AND MOUSE URINE

Strain	% Kidneys $>10^3$ E.coli WF 98	Osmometer reading (mOsm/Kg)	Dilution in distilled water	Osmolality (mOsm/Kg)
Mice				
B1OD2 new line	0	1351	1/2	2702
Balb/C	3	1806	1/2	3612
A / Jax	13	1093	1/2	2186
C57 black	12	1009	1/2	2018
CBA	44	1204	1/2	2408
DBA/1	46	683	1/3	2049
C3H/He	82	1207	1/2	2400
DBA/2	74	768	1/3	2284
Biozzi high responder	60	826	1/2	1652
Biozzi low responder	12	559	1/3	1677
Porton	25	421	1/3	1263
Human A.N. 2/12/75		360	Undiluted	360
17/11/75		865	Undiluted	865

E.coli urinary tract disease were found. The osmolality of urine from the Biozzi high or low responder lines of mice was similar for each line. This suggests that the susceptibility of inbred mice to E.coli urinary tract infection is not related to the osmolality of the urine they produce.

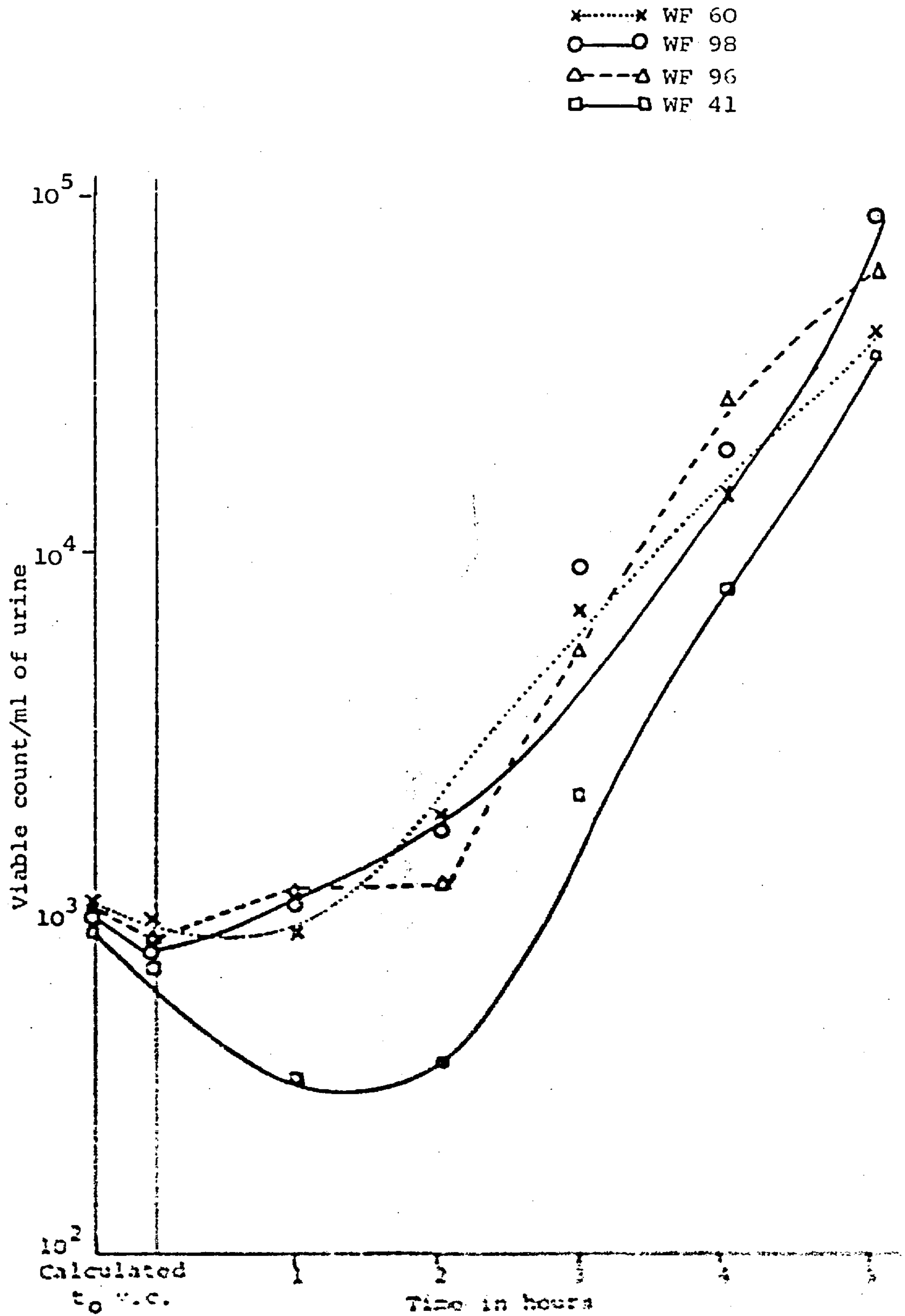
The osmolality of human urine was variable but the values obtained were much lower than those obtained with mouse urine. As people are much more susceptible to urinary tract infection than mice it is possible that the osmotic strength of urine helps to determine the degree of survival of invading bacteria within the bladder.

2) Growth of strains of E.coli in human female urine

Samples of sterile human urine were inoculated with E.coli strains of differing K antigen content to give an initial concentration of 10^3 bacteria/ml. The osmolality of the urine was 865 mOsm/Kg and the bacterial strains were E.coli WF60, WF98, WF90 and WF41.

After an initial lag phase of approximately 2 hours, the E.coli strains WF60, WF98 and WF96 grew readily in the non-shaken urines (Fig. 26). The low K antigen content strain E.coli WF41 however appeared to decline in numbers before entering a phase of logarithmic growth. Thus human female urine readily supports the growth of E.coli.

Fig. 26 INCUBATION OF *E. COLI* STRAINS WF 60 (AIA = 32), WF 98 (AIA = 16), WF 96 (AIA = 2) AND WF 41 (AIA = 0) IN HUMAN FEMALE URINE OF OSMOLALITY 865 mOsm/Kg



3) Growth of strains of *E.coli* in mouse urine

Urine was collected from male and female mice of the inbred strains C57 black, C3H and CBA. 1 ml aliquots of the urines were inoculated with 10^6 *E.coli* of strains WF26, WF98 and WF96. Incubation of the bacteria with the urines resulted in death of the organisms, rather than growth as observed in the human female urine (Fig. 27 - 29). The degree to which the urines were bactericidal did not appear to vary between the mouse strains, but the *E.coli* strains each gave an individual response. The high K antigen content strain *E.coli* WF26 was more resistant to the killing effect than *E.coli* WF96, a low K strain, but the medium K antigen strain was the most sensitive of all the strains to the killing effect. Mouse urine, which is more concentrated than human female urine, therefore, does not support the growth of *E.coli*.

4) Comparison of the growth kinetics of *E.coli* in male and female mouse urine

Urine was collected from various strains of inbred mice. Samples from female mice were all pooled together, as were the urines from male inbred mice. The osmolalities of the pooled samples were determined. 1 ml aliquots of the urines were inoculated with 10 µl of 2×10^8/ml *E.coli* WF26 or *E.coli* WF96.

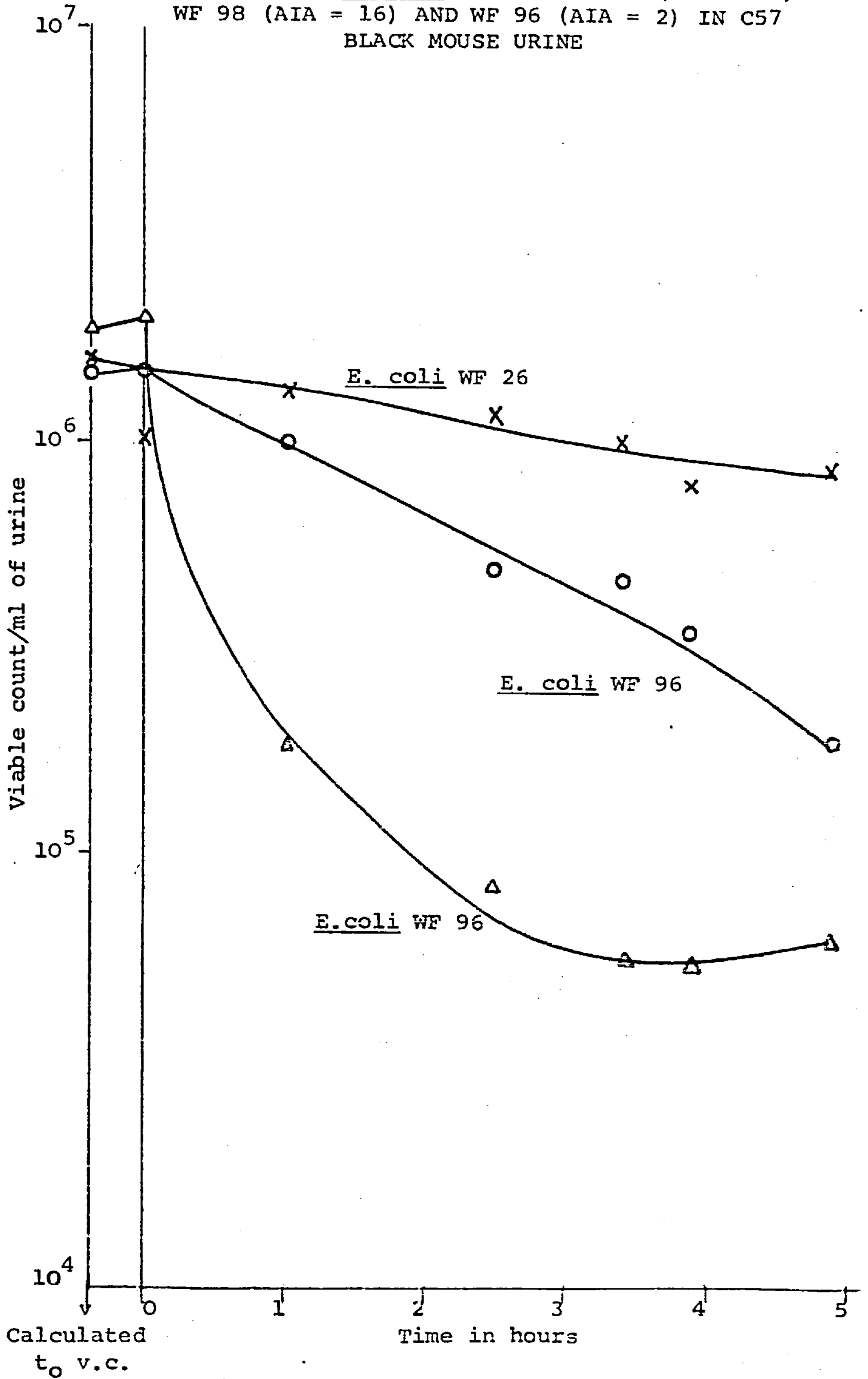


Fig. 27 INCUBATION OF E. COLI STRAINS WF 26 (AIA = 64) WF 98 (AIA = 16) AND WF 96 (AIA = 2) IN C57 BLACK MOUSE URINE

Fig. 28 INCUBATION OF *E. COLI* WF 26 (AIA = 64), WF 98 (AIA = 16) AND WF 96 (AIA=2) IN C3H MOUSE URINE

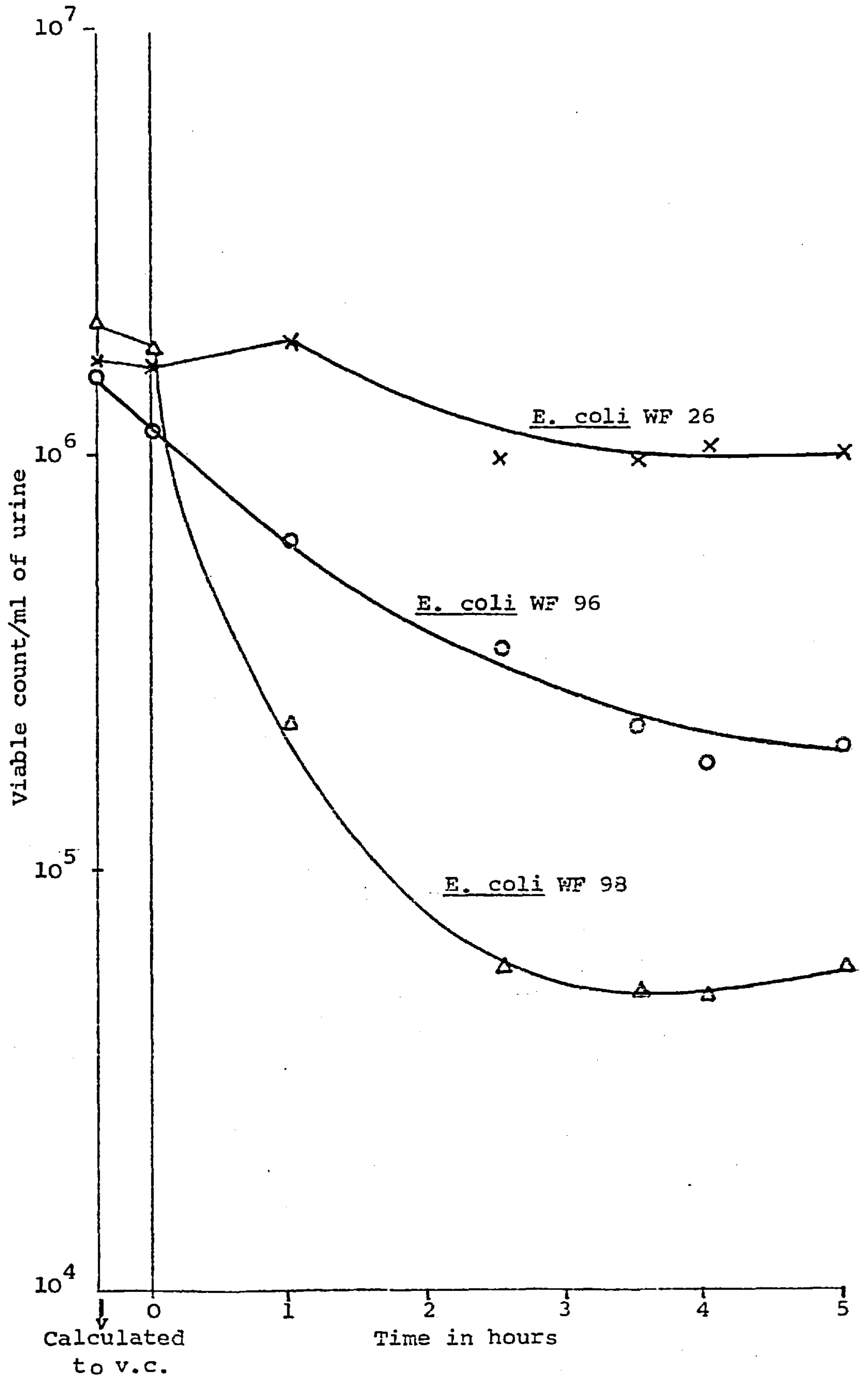
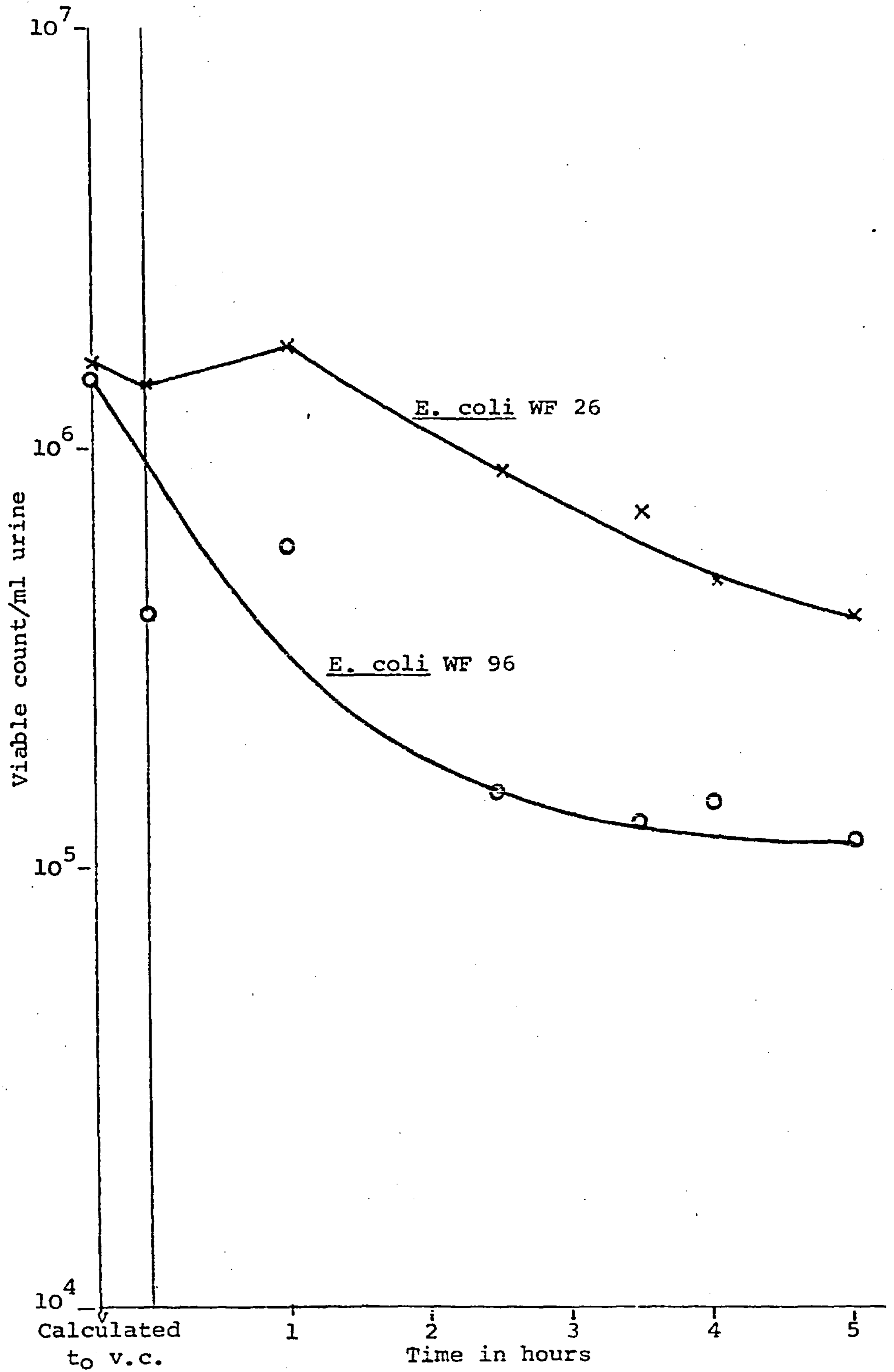


Fig. 29 INCUBATION OF E. COLI WF 26 (AIA = 64) AND E. COLI WF 96 (AIA = 2) IN CBA MOUSE URINE



The osmolalities of the male and female urine pools were very similar and very little difference was found in the bactericidal effect exhibited by the two pools on the E.coli strains (Fig. 30). Thus male and female mouse urine have the same bactericidal effect on E.coli bacteria. The use of urine from both male and female mice in the previous experiments thus gave results unaffected by the sex of the mice from which the urine was derived.

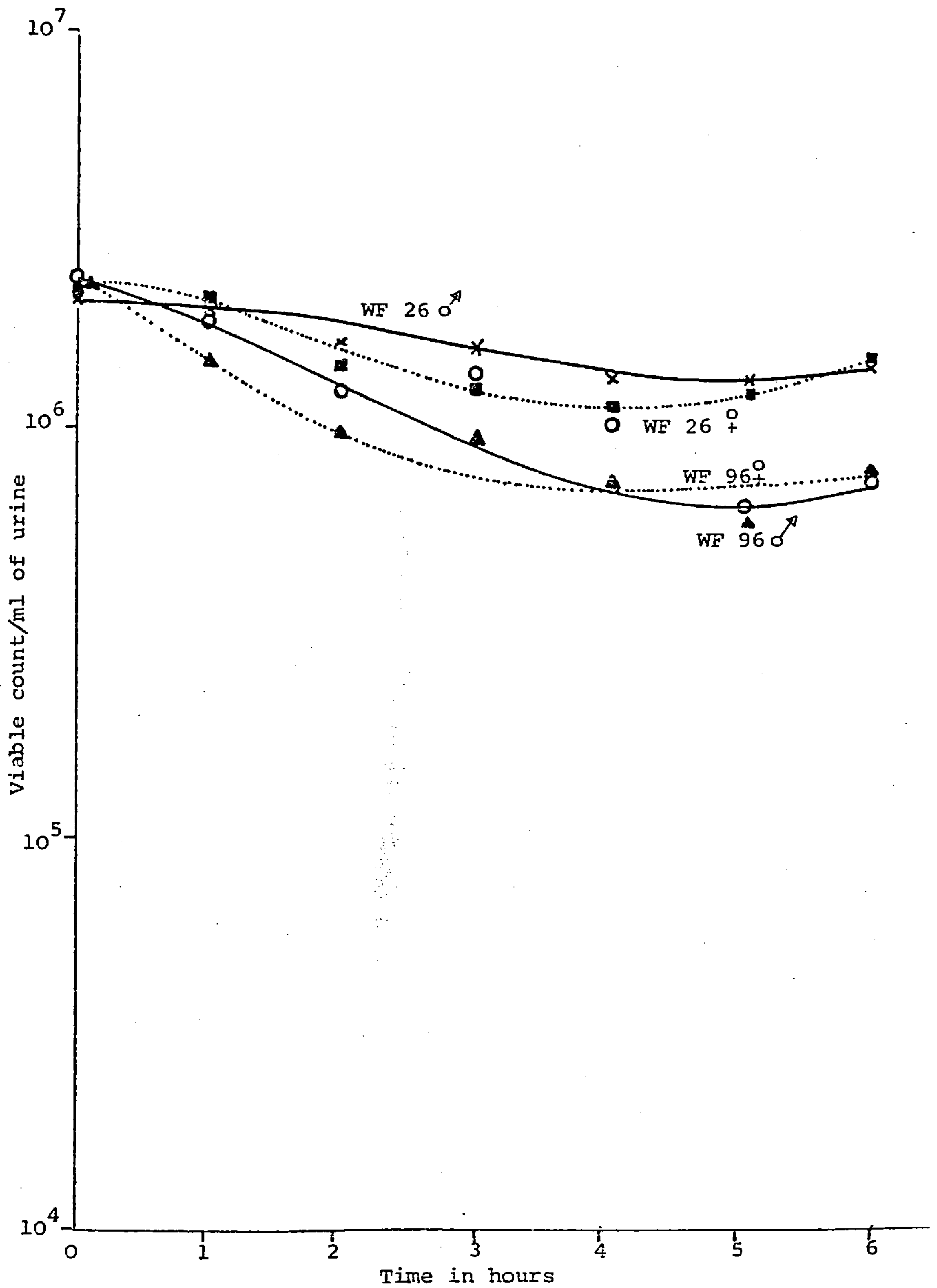
Again the difference in the response of the E.coli strains can be observed. E.coli WF26 was more resistant to the killing effect of mouse urine than E.coli WF96.

5) Growth of E.coli in human female urine of modified osmolality

Mouse urine was noted to have a higher osmolality than human urine and the incubation of E.coli in the two media resulted in growth of the organisms in human urine and death of the bacteria in mouse urine. It was therefore necessary to investigate the effect of osmotic pressure on the growth kinetics of E.coli in urine.

In a preliminary experiment human female urine (source A.N.) was collected and sterilised by millipore filtration, The addition of urea to 10 ml samples of the urine altered the osmolality of the medium. The modified urines were inoculated with E.coli WF26 or WF98. With increasing

Fig.30 INCUBATION OF *E. COLI* WF 26 (AIA = 64) AND *E. COLI* WF 96 (AIA = 2) IN MALE (OSMOLALITY = 2630 mOsm/Kg) AND FEMALE (OSMOLALITY = 2600 mOsm/Kg) MOUSE URINE



osmolality the urine changed from being a medium which supported bacterial growth to a medium which was bactericidal (Figs. 31,32) (Table 30). The curves obtained for both strains of E.coli were similar but there was a suggestion that the higher osmolality urines were less bactericidal for the high K antigen strain E.coli WF26 than they were for lower K antigen content strain E.coli WF98. Thus with increasing osmolality, urine becomes first inhibitory to growth, then actively bactericidal.

In a second experiment six strains of E.coli were incubated in urine that had been sterilised by autoclaving. The osmolality of the urine was modified with urea (AR urea, BDH Chemicals Ltd.) (Table 31). This sample of urine appeared to be more bactericidal than that used in the previous experiment as even at osmolalities of about 750 mOsm/Kg, the bacteria were inhibited from growing. Thus the osmolality of urine which is dependent on the urea concentration would not appear to be the only factor determining the growth kinetics of E.coli in this medium.

The strains E.coli WF82, WF60, WF96 and WF26 were less readily killed by the modified urines of low osmolality than were the lower K antigen content strains, E.coli WF98 and WF41. With increasing osmolality this difference was still apparent, although by the time the osmolality was increased to approximately 2500 mOsm/Kg, the modified urines were rapidly bactericidal for all the strains.

Fig. 31 GROWTH OF *E. COLI* WF 26 (AIA = 64) IN HUMAN FEMALE URINE OF MODIFIED OSMOLALITY

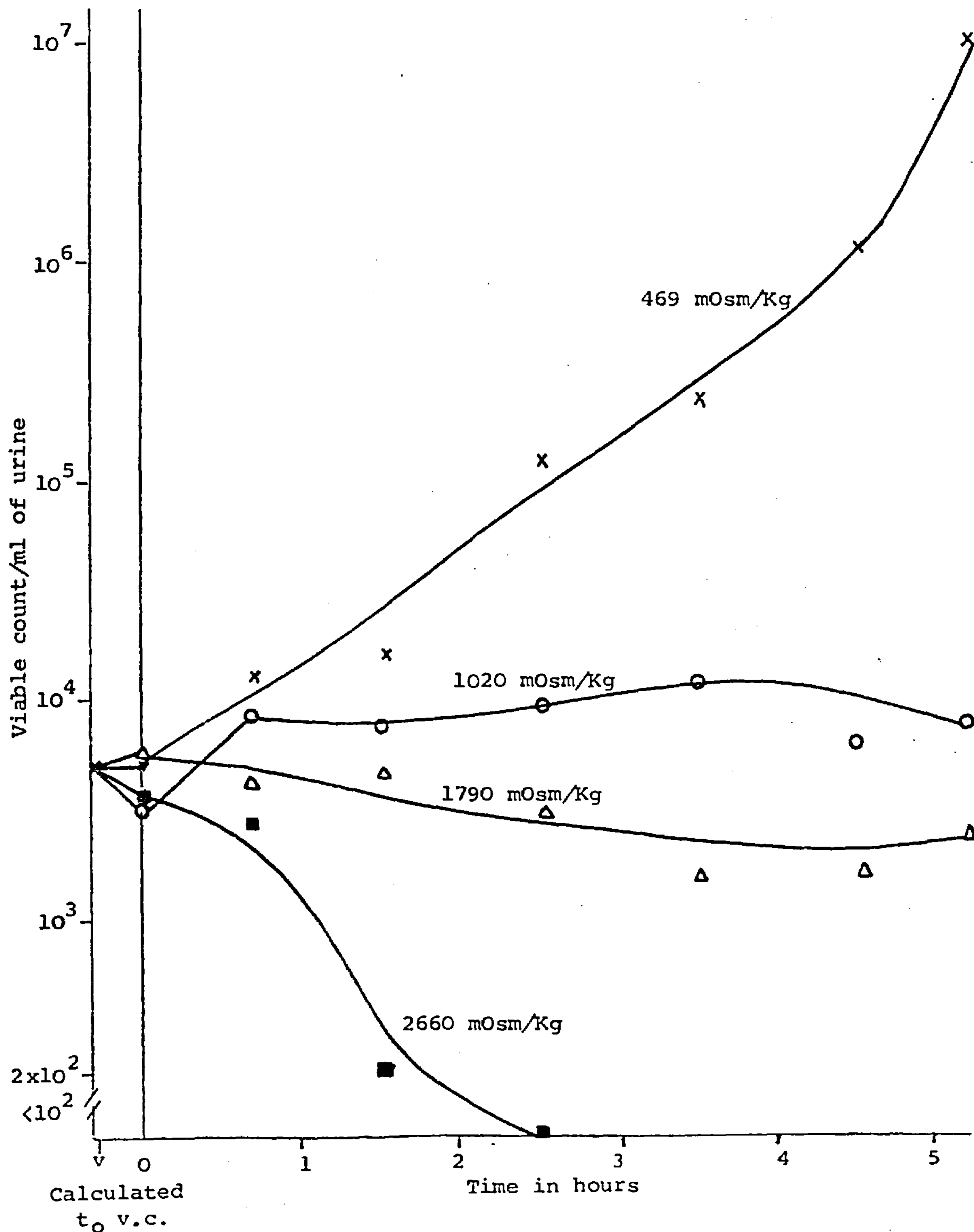


Fig. 32 E. COLI WF 98 (AIA=16) IN HUMAN FEMALE URINE OF MODIFIED OSMOLALITY

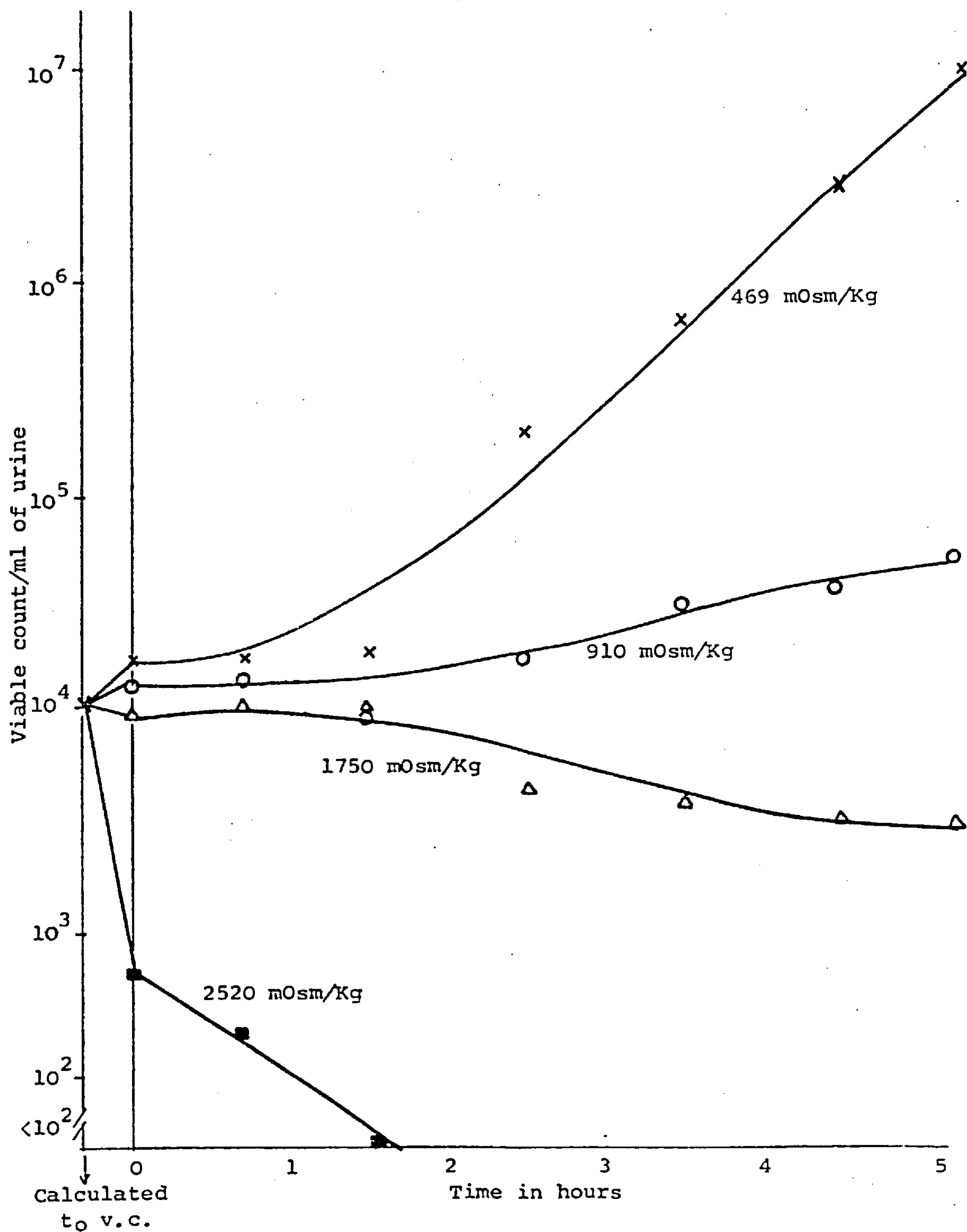


Table 30

GROWTH OF E. COLI IN HUMAN FEMALE URINE OF MODIFIED OSMOTIC PRESSURE

Experiment 1) E. coli strains WF 26 and WF 98

SAMPLE	LOG VIABLE COUNTS							
	A	B	C	D	E	F	G	H
Time h								
0	3.69	4.22	3.49	4.10	3.77	3.99	3.81	2.78
3/4	4.11	4.25	3.94	4.15	3.62	4.06	3.43	2.51
1 1/2	4.19	4.27	3.88	3.97	3.66	4.03	2.30	1.78
2 1/2	5.10	5.30	3.94	4.24	3.48	3.63	1.78	<1.0
3 1/2	5.37	5.83	4.09	4.51	3.18	3.58	2.00	<1.0
4 1/2	5.08	6.45	3.83	4.58	3.24	3.50	1.78	<1.0
5 1/4	7.05	6.96	3.93	4.71	3.41	3.48	1.30	<1.0
G urea/10 ml.	0	0	0.3	0.03	0.9	0.9	1.5	1.5
Osmolality mOsm/Kg estimated	600	600	1100	1100	2100	2100	3100	3100
Osmolality mOsm/Kg actual	469	469	1020	910	1790	1750	2660	2520
E. coli WF	26	98	26	98	26	98	26	98

Table 31

OSMOLALITIES OF MODIFIED URINES - EXPERIMENT II

G urea/10ml urine	0.3	0.7	1.1	1.5	2.0
		Osmolality (mOsm/Kg)			
E. coli strain WF					
82	740	1440	1910	2600	3430
98	870	1520	1940	2360	3280
41	870	1540	2120	2520	3020
26	730	1350	2120	2690	-
60	760	1240	1950	2480	-
96	850	1500	1870	2730	-

From the graphs (Fig. 33 to 38) the initial log drop in numbers of bacteria per minute of time was calculated for each strain for the lower two or three urea concentrations. The values obtained were plotted against the osmolality of the modified urine (Fig. 39). The curves obtained are an indication of the rate at which death of the organisms accelerates when the osmolality of the urine plus urea medium is increased. The steeper the slope of the line, the more susceptible are the organisms to the killing effect of high osmotic pressure solutions.

The curves obtained with the high K antigen strains E.coli WF26 and E.coli WF82 were flatter than those of the other strains of bacteria. Thus these strains are more resistant to killing by high osmolality solutions than the low K antigen content strains WF41, 96, 98 or 60. Only two points are shown for E.coli WF98. This was because the third solution was so rapidly bactericidal for this strain that no count was obtained at 40 minutes and thus no determination of the slope of this line could be made. Presumably the line on Fig. 39 would have been very nearly vertical and to the left of the high osmolality line of E.coli WF96 if it had been possible indeed to calculate it. The strains WF96 and WF41 may be seen to be very susceptible to the killing effect as even at low osmolalities the slopes of the lines obtained are greater than those obtained for the resistant strains.

Fig. 33 INCUBATION OF *E. COLI* WF 82 (AIA = 16) IN HUMAN FEMALE URINE OF MODIFIED OSMOLALITY

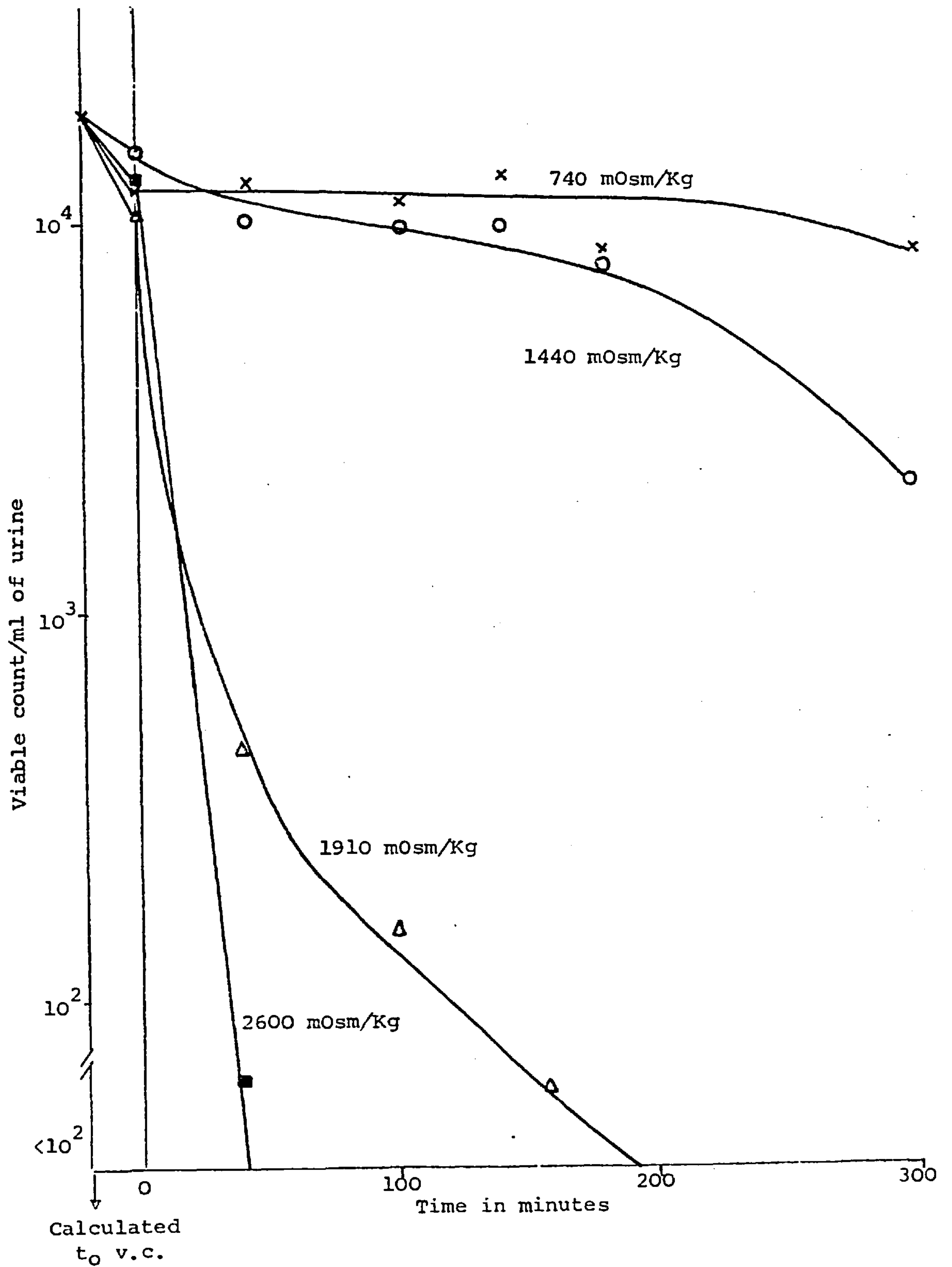


Fig.34 INCUBATION OF *E. COLI* WF 98 (AIA = 16) IN HUMAN FEMALE URINE OF MODIFIED OSMOLALITY

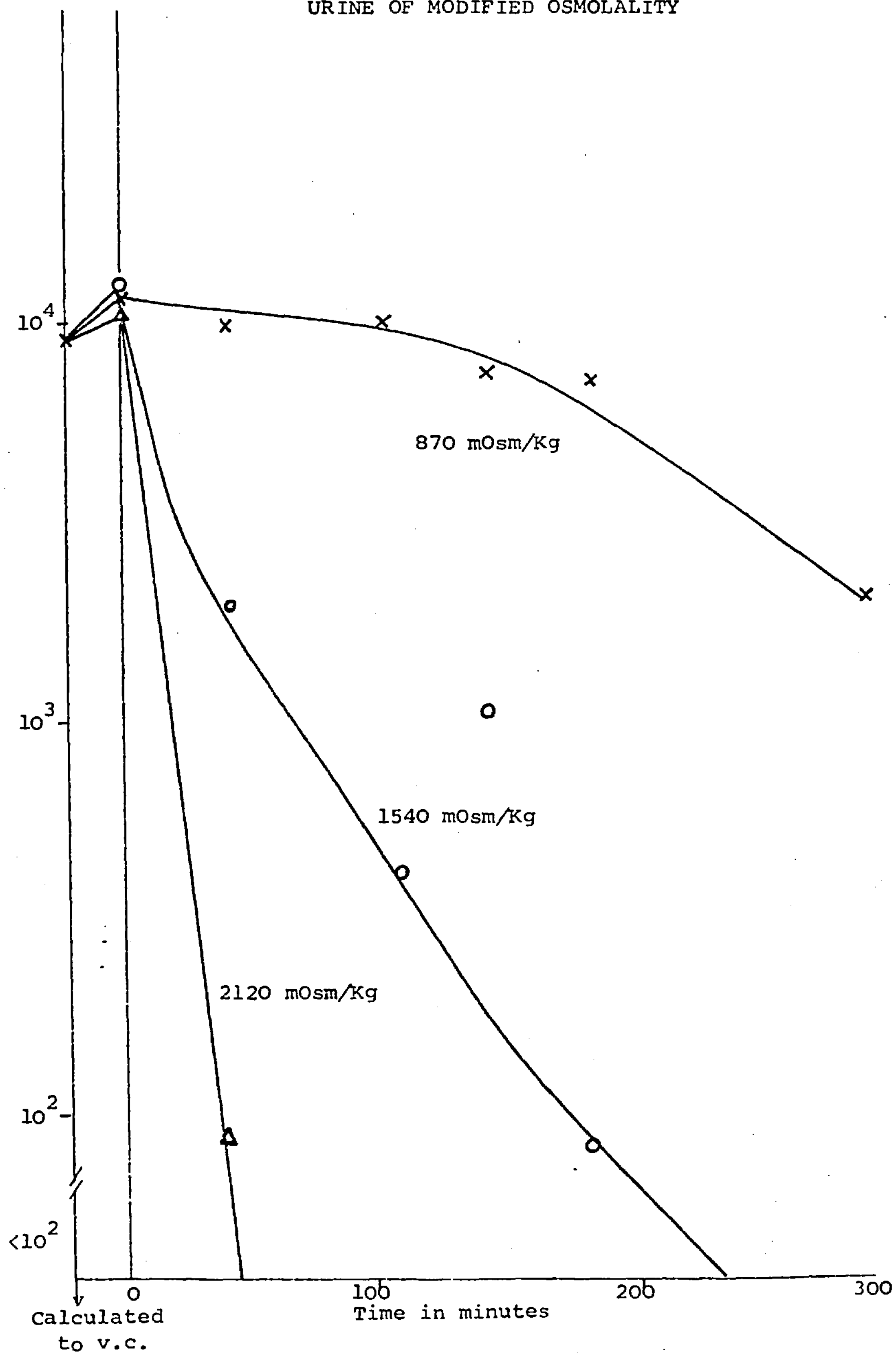


Fig. 35 INCUBATION OF E. COLI WF 41 (AIA = O) IN HUMAN FEMALE URINE OF MODIFIED OSMOLALITY

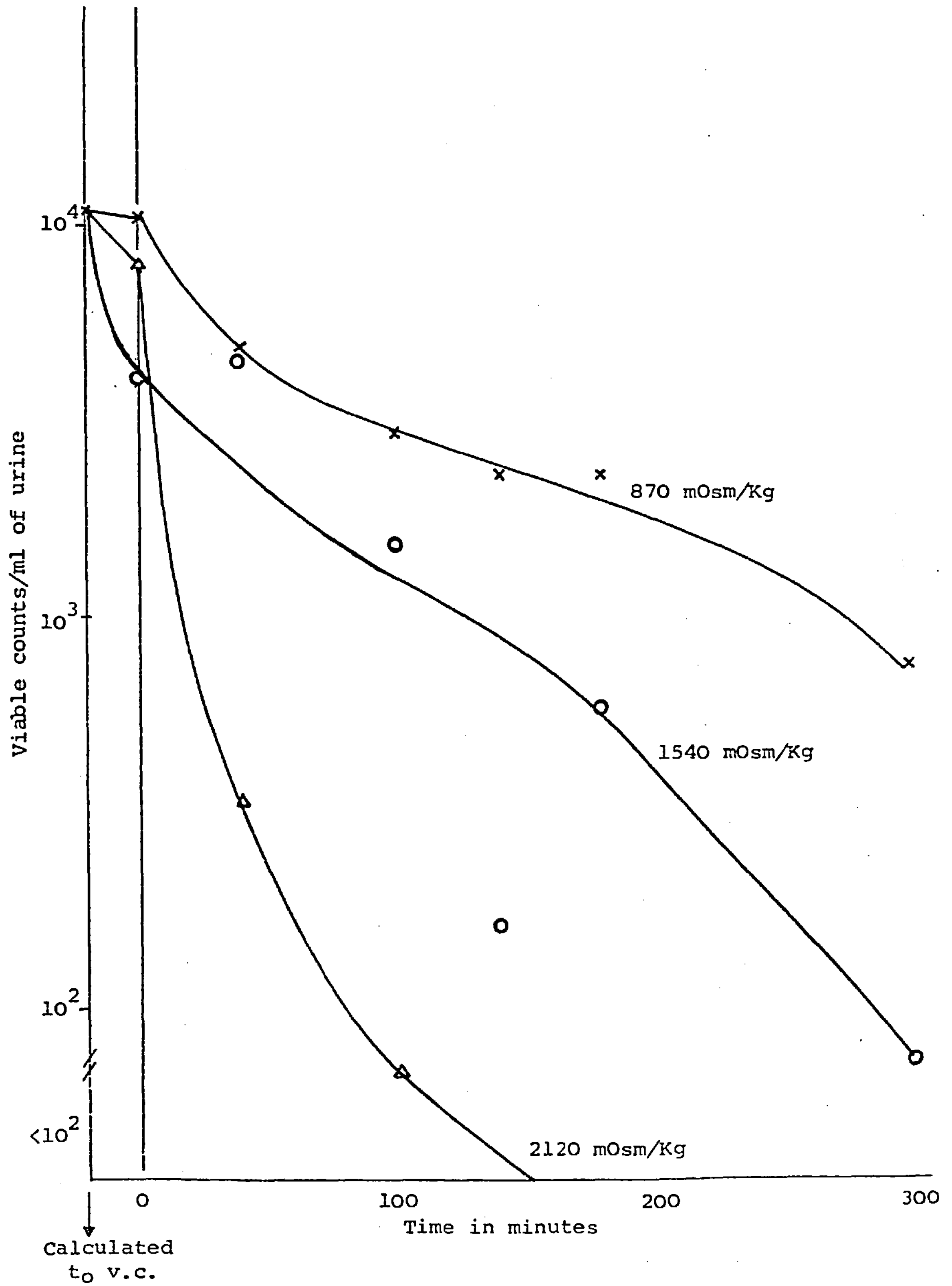


Fig.36 INCUBATION OF E.COLI WF 26 (AIA 64) IN HUMAN FEMALE URINE OF MODIFIED OSMOLALITY

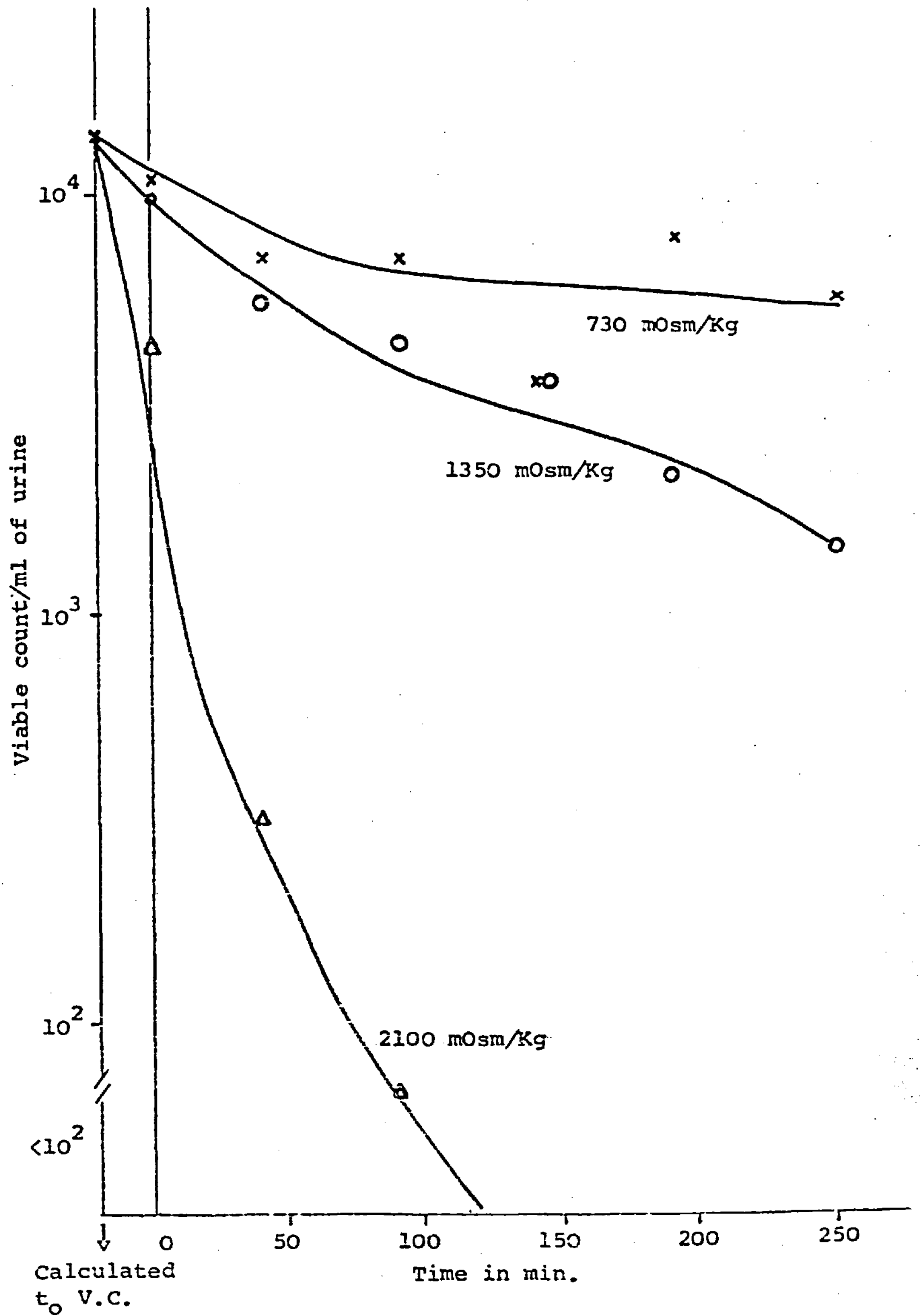


Fig. 37 INCUBATION OF E. COLI WF 60 (AIA = 32) IN HUMAN FEMALE URINE OF MODIFIED OSMOLALITY

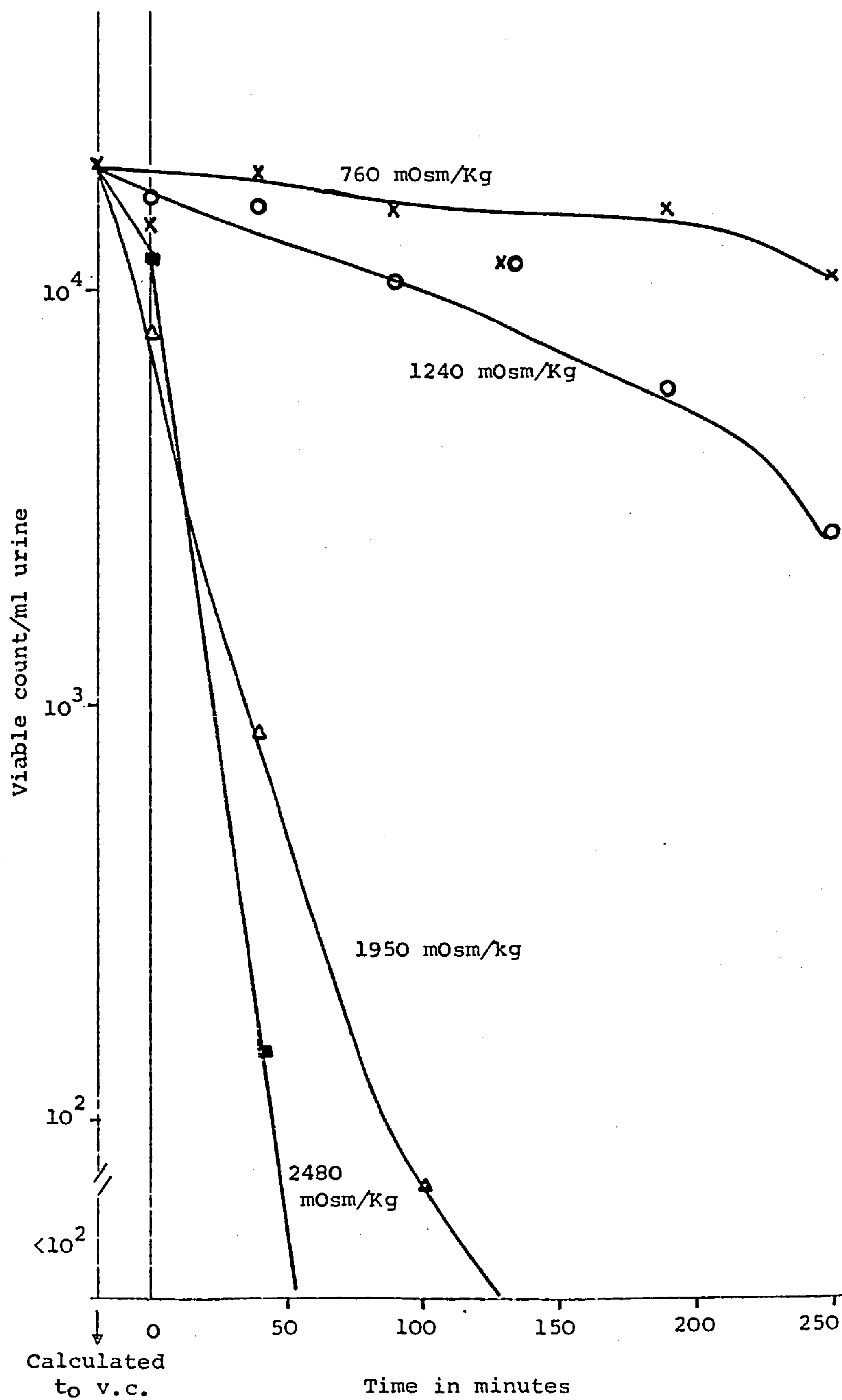


Fig.38 INCUBATION OF *E.COLI* WF 96 (AIA = 2) IN HUMAN FEMALE URINE OF MODIFIED OSMOLALITY

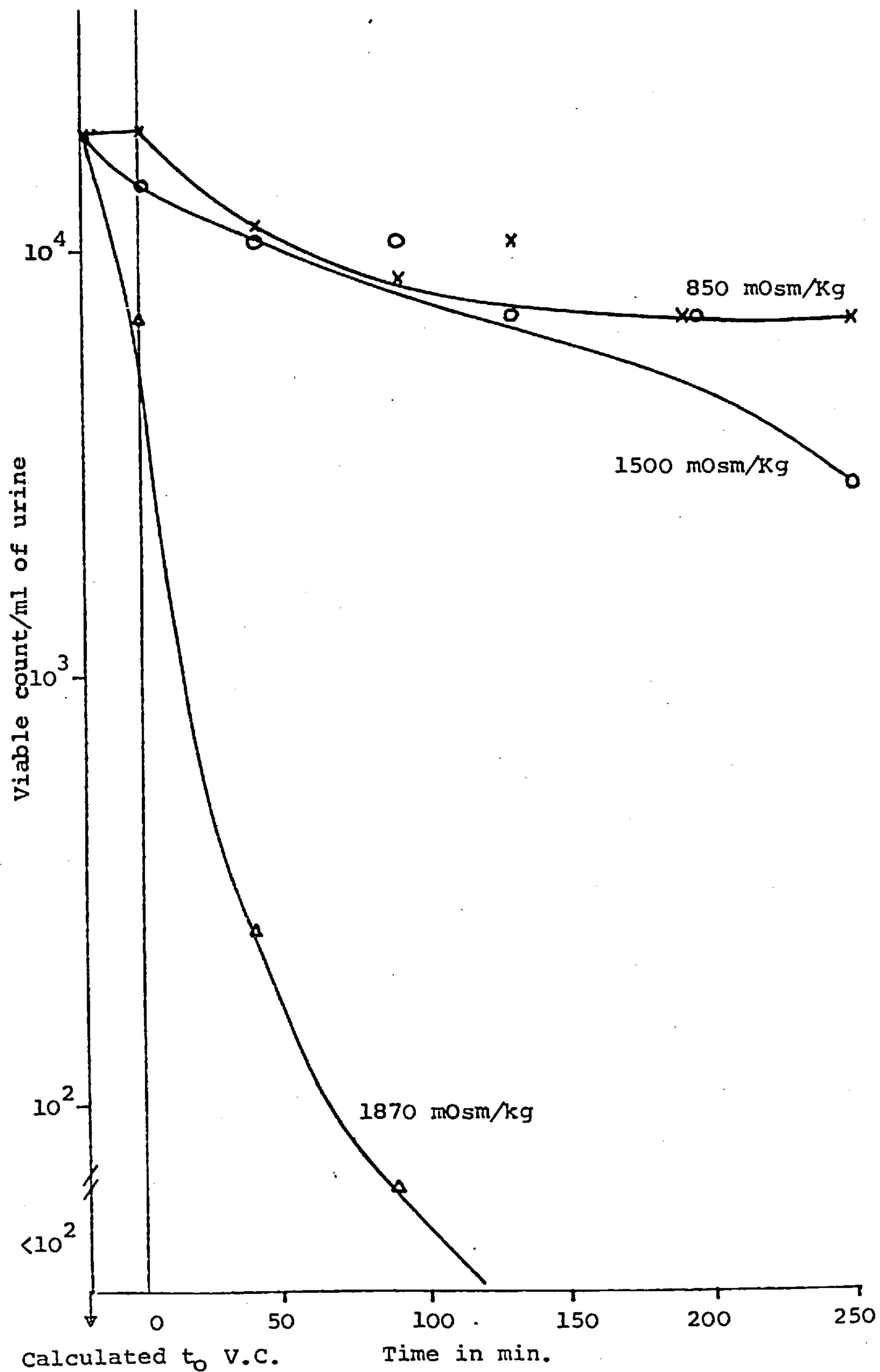
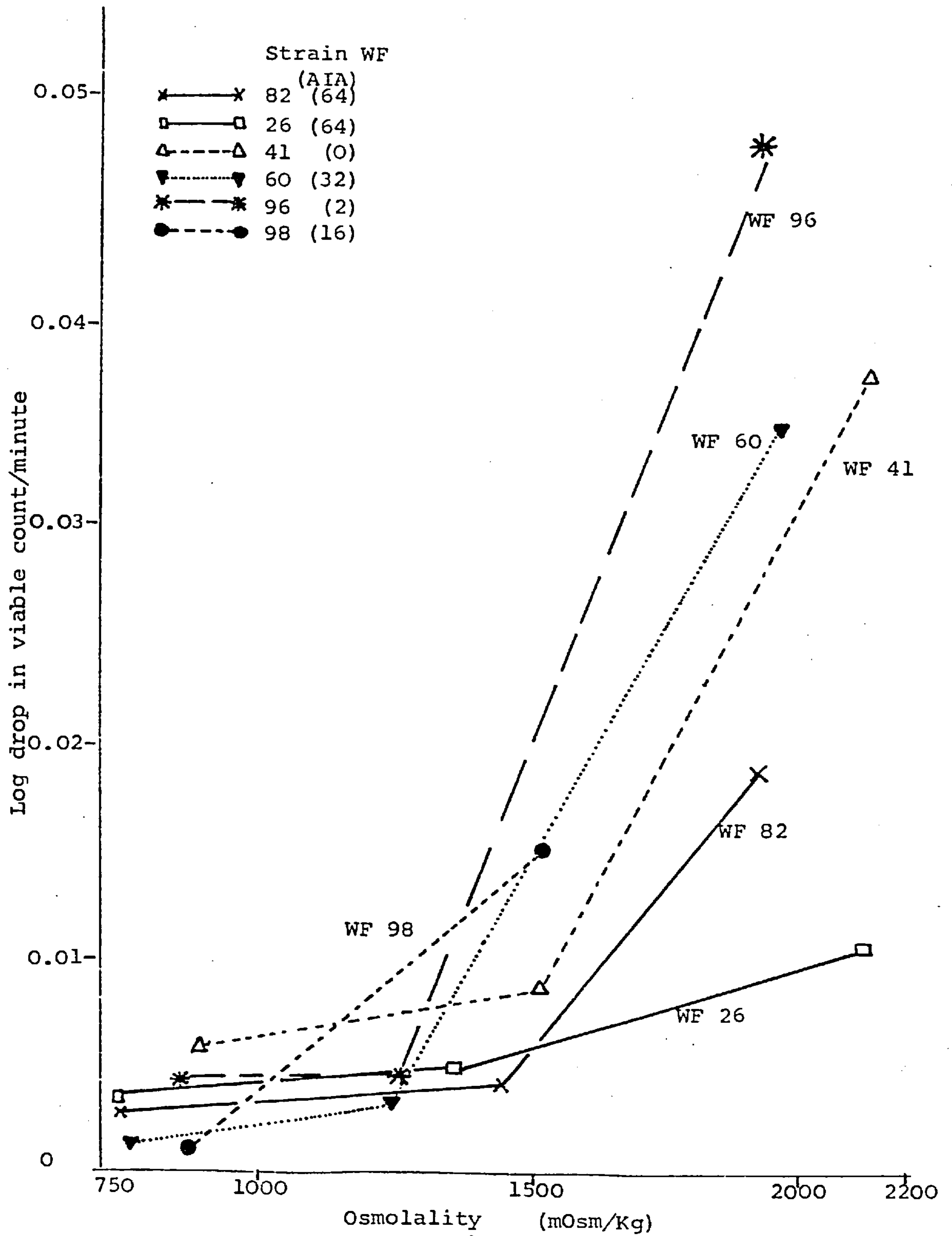


Fig. 39 RATE OF DECLINE OF CULTURES OF E. COLI WITH CHANGE IN OSMOLALITY OF THE URINE MEDIUM



A correlation with the K antigen content of the strains is apparent as the high K content strains are more resistant to the killing effect of high osmolality urines than are strains of E.coli with little or no K antigen. The correlation is not so perfect with the strains of lower agglutinating inhibiting activities but this may be a reflection of the large jumps in osmolality that were used to investigate the effect in these experiments. Further experiments of this kind may help to elucidate the conditions under which the phenomenon of killing by high osmolality operates in both mouse and human urine.

6) The effect of water diuresis on the susceptibility of mice to E.coli urinary tract infection

As the susceptibility of strains of E.coli to the killing effect of high osmotic pressures is increased if the osmolality is raised, then decreasing the osmolality of a high osmotic pressure urine should increase the viability of E.coli bacteria present in that medium.

The osmolality of Porton mouse urine was decreased by initiating a diuresis in the animals. After 4 days the animals were infected by inoculation of bacteria into the bladder. No stitches were placed in the bladder wall. The diuresis was continued for a further 3 days. The mice were then assessed for kidney infection. The weights of the mice, their urine osmolality and blood sugar levels were also determined.

The induction of a water diuresis in the mice resulted in the increased susceptibility of the animals to E.coli urinary tract infection (Tables 32, 33). Infection was produced in the absence of stitches in the bladder wall. The viable counts obtained (Figs. 40, 41) indicated that the urines and kidneys of the mice undergoing a water diuresis contained more bacteria than the urines and kidneys of the control mice.

The mice subjected to a diuresis weighed more than the control mice. This was probably due to water retention in these animals combined with the ingestion of a high carbohydrate content fluid for seven days. The osmolality of the urine of the test mice was lower than that of the normal mice. The induction of a water diuresis thus reduced the osmolality of the urine of these mice, as expected. No difference was found in the blood sugar levels between the experimental and control mice and no glucose was found in the urine after testing with "Uristix" (Ames, England).

In the experiment with E.coli WF26, several mice died. A very high dose of bacteria had been injected into the bladder and death was probably due to septicaemia. Although the urinary tract of mice undergoing a water diuresis is more susceptible to infection than the urinary tract of normal mice, the inoculation of bacteria into the unstitched bladder

Table 32

EFFECT OF WATER DIURESIS ON THE SUSCEPTIBILITY OF MALE PORTON MICE TO *E. COLI* WF 98 URINARY TRACT INFECTION

Treatment	Wt. of 8 mice g	Urine from 8 mice ml	Urines $>10^3$ bacteria/ml	Kidneys $>10^3$ /kidney
Diuresis	350	2.5	8/8	3/16
Control	283	1.0	1/8	0/16

χ^2 using fourfold table and Yates correction = 1.47

$\therefore$ $P<0.3>0.2$ i.e. not significant but only very small numbers

Table 33

EFFECT OF WATER DIURESIS ON THE SUSCEPTIBILITY OF MALE PORTON MICE TO *E. COLI* WF 26 URINARY TRACT INFECTION

Treatment	Wt. of 8 mice g.	Osmolality of pooled urines mOsm / Kg	Blood sugar -mean ±S.D.	Urines $>10^3$ bacteria /ml	Kidneys $> 10^3$ bacteria /kidney
Diuresis	336	868	102 ± 22 mg/100ml	6/6 2 died	9/12
Control	285	1222	93 ± 13 mg/100ml	3/5 3 died	3/10

χ^2 using fourfold tables and Yates correction = 2.824

P = <0.1>0.05   i.e. significant but only very small numbers

Fig. 40 VIABLE COUNTS RECOVERED FROM THE KIDNEYS OF NORMAL MICE (N) AND MICE SUBJECTED TO DIURESIS (D) E.COLI INJECTED INTO UNSTITCHED BLADDERS

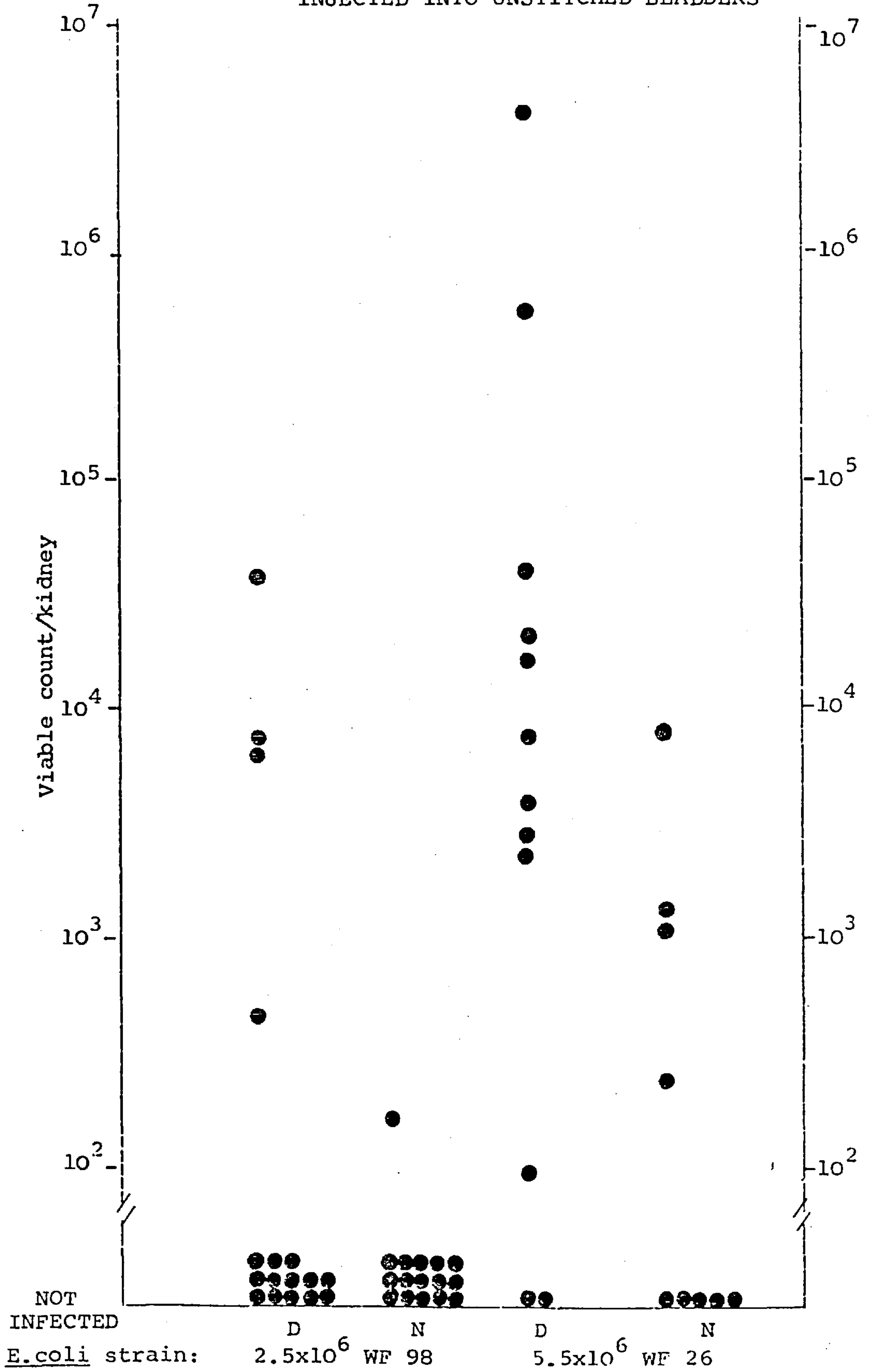
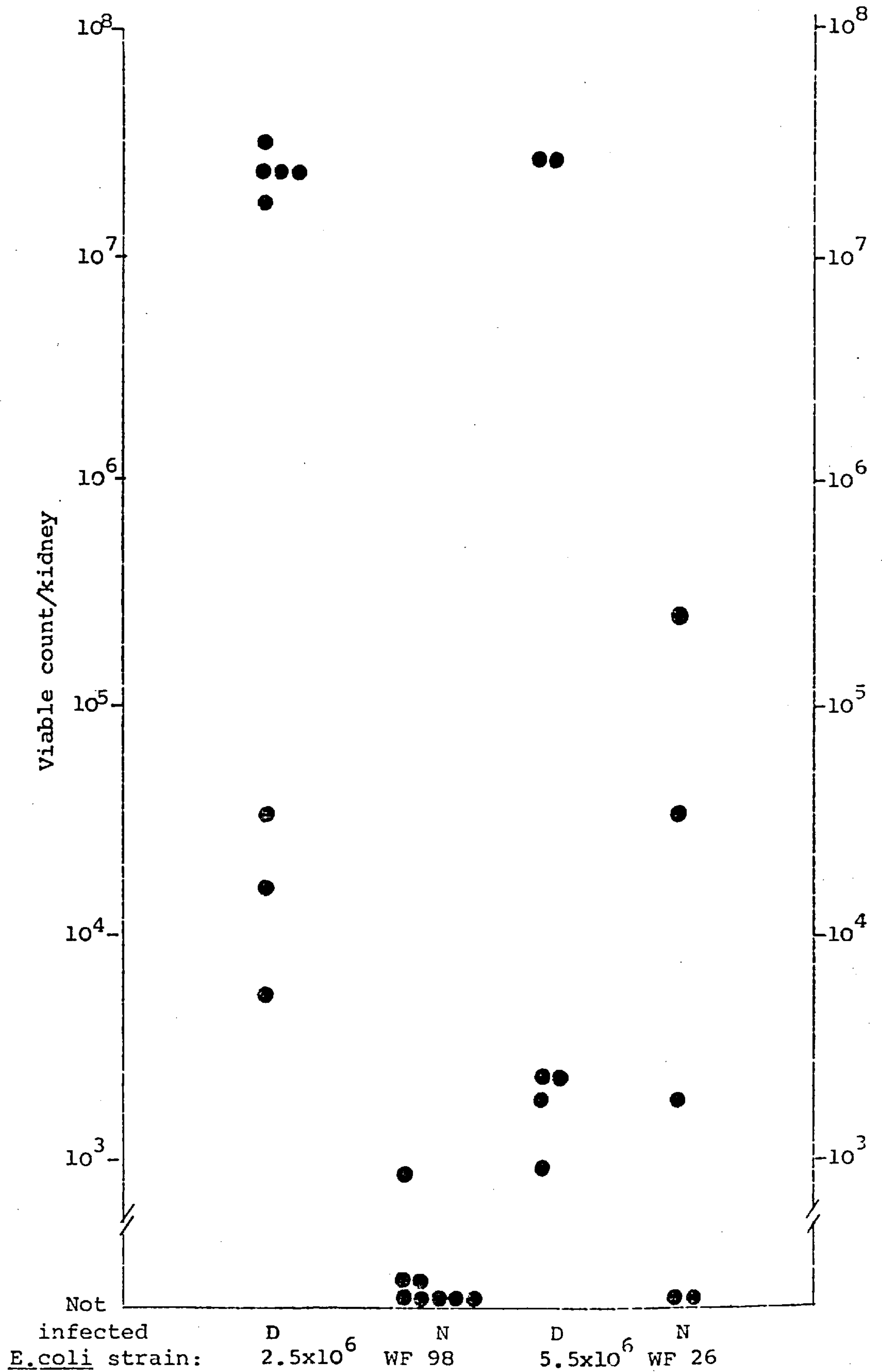


Fig. 41 VIABLE COUNTS RECOVERED FROM THE URINES OF NORMAL MICE (N) & MICE SUBJECTED TO DIURESIS (D) E. COLI INJECTED INTO THE UNSTITCHED BLADDER



of water-loaded mice is not as efficient a method of producing urinary tract disease as is the method of infection (described in Chapter 2) involving two stitches in the bladder wall of normal mice. Much higher doses of E.coli WF26 are needed to infect diuresed mice than normal mice with stitched bladders.

The effect of diuresis on a haematogenously induced kidney infection was also investigated. Normal Porton male mice and Porton male mice who had been drinking 5% glucose solution for 4 days were injected intravenously, into the tail vein, with 1.2×10^8 E.coli WF98. Groups of mice were killed at intervals up to 3 days after the initiation of the infection, and viable counts were made on the kidneys and livers of the animals.

The control mice were found to have cleared most of the bacteria from their organs by 3 days (Table 34). In contrast the water-loaded mice were unable to free their organs from infection. On days 1, 2 and 3 more bacteria were present in the kidneys of the test mice compared with the control mice. Thus a diuresis would appear to increase the susceptibility of male Porton mice to a haematogenously induced kidney infection.

Table 34

TIME COURSE OF A HAEMATOGENOUSLY INDUCED *E. COLI* WF98 URINARY TRACT INFECTION IN MICE UNDERGOING DIURESIS AND CONTROL MALE PORTON MICE

Viable counts obtained/organ

	DIURESIS			CONTROL		
	R.kidney	L.kidney	Liver	R.kidney	L.kidney	Liver
1/2 h	$<10^4$	1.0×10^4	n.d.	1.0×10^4	1.5×10^4	n.d.
	$<10^4$	1.0×10^4	n.d.	$<10^4$	$<10^4$	n.d.
	$<10^4$	$<10^4$	n.d.	1.5×10^4	2.0×10^4	n.d.
	1.5×10^4	2.0×10^4	n.d.	1.0×10^4	$<10^4$	n.d.
1 h	$<10^4$	$<10^4$	n.d.	$<10^4$	$<10^4$	n.d.
	$<10^4$	$<10^4$	n.d.	$<10^4$	$<10^4$	n.d.
	$<10^4$	$<10^4$	n.d.	1.5×10^4	$<10^4$	n.d.
	$<10^4$	$<10^4$	n.d.	$<10^4$	1.5×10^4	n.d.
24 h	3.75×10^6	5.0×10^6	3.8×10^5	4.4×10^6	2.3×10^6	4.0×10^5
	6.6×10^5	2.75×10^6	1.4×10^5	5.6×10^3	6.2×10^3	3.0×10^6
	2.03×10^6	$<10^2$	n.d.	1.8×10^5	4.0×10^4	n.d.
48 h	$<10^3$	4.3×10^4	1.5×10^3	$<10^3$	$<10^3$	2.5×10^6
	6.2×10^7	3.17×10^7	1.6×10^4	8.4×10^6	1.9×10^6	3.25×10^5
	$<10^3$	$<10^3$	n.d.	$<10^3$	$<10^3$	n.d.
72 h	3.7×10^4	$<10^2$	1.3×10^3	$<10^2$	$<10^2$	$<10^2$
	$<10^2$	$<10^2$	1.5×10^2	$<10^2$	$<10^2$	7.5×10^2
	8.6×10^4	9.0×10^2	n.d.	$<10^2$	$<10^2$	n.d.
	-	-	-	$<10^2$	1.05×10^3	n.d.

n.d.=not done

The viable counts recovered from the livers of the mice undergoing a diuresis were, however, lower than those obtained from the livers of the control mice. This suggests a role for blood flow in the results obtained. Increased blood flow to the kidneys of diuresed mice results in more bacteria being carried to the kidneys and fewer to the liver. Increased blood flow occurs in response to diuresis in order to promote the excretion of the extra fluid ingested by the animals. It is also possible that the increased volume of fluid passing through the kidneys reduces the osmotic pressure of that environment and allows bacteria lodged there to grow better, in much the same way as they would if they had reached the kidney from the ureter.

The induction of a diuresis, by decreasing the osmolality of the urine, results in the increased susceptibility of mice to both ascending and haematogenously induced kidney disease.

DISCUSSION

Human female urine readily supported the growth of *E.coli* bacteria. Only small numbers of bacteria were inoculated into the urine in order to represent an ascending infection, where the inoculum of bacteria from the urethra is probably even smaller than this. *E.coli* strains WF60,

WF98 and WF96 grew in the urine, after an initial lag phase, but the low K antigen strain E.coli WF41 entered a decline phase before entering the period of logarithmic growth. This suggests that E.coli WF41 is less able to adapt to growth in urine than other strains of E.coli.

Increasing the osmolality of human female urine by addition of urea altered the growth kinetics of the bacteria. With increasing osmolality growth of the bacteria ceased and then with high concentrations of urea the medium became inhibitory for growth. Kaye (1968) followed the fate of small numbers of Escherichia coli inoculated into urine from human volunteers. Urine from normal individuals was often inhibitory and sometimes bactericidal for growth. The anti-bacterial activity was not related to lack of nutrient material but to the osmolality, urea concentration, and ammonium concentration of the urine. The urea concentration was more important than the osmolality or the ammonium concentration and addition of urea to a non-inhibitory urine made it inhibitory, as has been noted here.

The various strains of E.coli used by Kaye demonstrated marked differences in susceptibility to the inhibitory and bactericidal activity of urine. Incubation of WF strains of E.coli in human female urine of modified osmolality has also shown strain differences. Kaye serotyped his strains but

made no estimation of their K antigen content. The results presented here indicate that K rich strains of E.coli are better able to survive the bactericidal effect of high osmotic pressure solutions than are those strains of E.coli with little or no K antigen.

This finding may help to explain why K rich strains are more likely to cause kidney infection than E.coli strains containing small amounts of K antigen (Chapter 2). Firstly, the resistant strains, the K rich strains, would better survive the initial inoculation into urine, especially if the urine was one that was normally bactericidal for sensitive bacteria. Secondly, the organisms would be resistant to the high osmotic pressures encountered in the kidney tissue.

The counter current theory of the production of urine relies on zones of differing osmotic pressure throughout the structure of the kidney. This is in order for the concentration of urine to be controlled and for the salts needed by the body to be conserved. The innermost parts of the medulla have the highest osmotic pressures and Bray (1960) showed that the papillary regions of the medulla of rats have osmotic pressures up to 2000 mOsm/Kg. Thus, as invading bacteria from the ureters come first into contact with these regions of high osmolality, the strains must be able to resist the bactericidal effect of this environment in order

for colonisation of the kidney tissue to occur. If the possession of K antigen is related to resistance to high osmotic pressure environments, then this could well explain the greater propensity of K rich strains to infect mouse and human kidneys.

The K antigen of E.coli is correlated with the resistance of the organism to complement killing and phagocytosis. Howard and Glynn (1971b) showed that both the amount of K antigen present and its degree of polymerisation are important in determining a strain of E.coli's resistance to phagocytosis and complement killing. If the steric arrangement of these acidic polysaccharides on the surface of the bacteria is important in determining the physical resistance of the organisms to these events, then a surface coating of such molecules may also protect the bacteria against the ravages of a high osmotic pressure environment. Thus the K antigen of E.coli, by a purely physical means, may determine the ability of strains of E.coli to invade the upper urinary tract. Further experiments in modified human urine, using one strain of E.coli grown at different temperatures and on different media to alter the K antigen content, may help to substantiate this hypothesis.

Mouse urine was bacteriostatic or bactericidal for strains of E.coli. No graded difference was observed in the osmolalities of the urines of inbred mice, which might have corresponded to their ability to resist urinary tract infection (Chapter 3). The urine from male and female inbred mice had a very similar effect on the strains of E.coli and therefore the use of male mice in most of the experimental work presented here would not have been affected by any bactericidal factor present in male mouse urine that is not present in female mouse urine. In man the situation is probably different. Male human urine contains a bactericidal prostatic factor (Stamey et al, 1968). This is thought, in conjunction with other differences of an anatomical nature, to be responsible for the lower incidence of urinary tract infections in men than in women.

The mouse urines exerted different effects on the strains of E.coli. The K rich strains, as before, were more resistant to the bactericidal action of the urine than were the other strains. The finding that diuresis increased the susceptibility of mice to urinary tract infection is in accordance with the above results. A reduction in the osmolality of the urine produced by the mice, due to an increased water intake, allowed the bacteria to survive better in the mouse urinary tract. Levitin et al (1962) measured the concentrations of solutes in water in various

parts of the dog kidney, during both water diuresis and hydropenia. Water diuresis was associated with a reduction in the quantity of sodium present, and an increased rate of turnover of sodium in the medulla compared to the cortex. The counter current theory of the production of urine within the tubules continued to operate despite the lowered osmolality of the kidney tissue. However, the efficiency of the system was decreased during water diuresis. At the height of a water diuresis the urine osmolality was 100 mOsm/Kg, the mean plasma osmolality was 285 mOsm/Kg, while the papilla and medulla were still distinctly hypertonic in comparison to the plasma with osmolalities of 381 and 401 mOsm/Kg respectively.

If the same situation exists in mice then the osmolality of the kidney tissue will be reduced during water diuresis. Therefore bacteria will readily invade the urinary tracts of these mice because they are less inhibited by the more dilute urine and they are better able to survive in the lower solute environment of the kidney tissue.

Miller and North (1960) fed rats either on a high (50%) protein diet or on a low (5%) protein diet. The high protein diet caused the urinary urea concentration to rise and these rats had a significantly reduced growth of Streptococcus faecalis in the cortex,medulla,and papilla of the kidney,

compared with rats fed on a low protein diet. In vitro tests showed that urea concentrations, such as those found in the urine of the high protein diet rats,was bactericidal for Str.faecalis, and the killing effect of high urea concentrations was thought to explain the lower bacterial counts obtained in the kidneys of these rats. Phagocytosis of Str.faecalis by leucocytes was suppressed in vitro by an osmolality greater than 1000 mOsm/Kg but it was thought that this effect was not the dominant factor determining the rate of growth of Str.faecalis in kidney tissue. Thus the tonicity of kidney tissue would appear to be an important determinant of the susceptibility of the organ to bacterial infection.

The osmolality of human urine is much lower than that found in rats and mice. If the kidneys of people have a lower tonicity than that observed in these animals, it might explain why women readily succumb to urinary tract infection even in the absence of any pathological condition that would predispose to disease.

The osmolality of urine may be important in preventing disease of the urinary tract by ensuring that bacteria entering the bladder lumen do not grow before they are voided from the bladder by micturition. The results presented here suggest that the practice of encouraging people with a urinary

tract infection to drink copious amounts of fluid may not necessarily be beneficial. Unless the osmolality of the kidney tissue and urine is lowered to such an extent that it will not support bacterial growth, lowering the osmolality of fluids in the urinary tract may reduce the inhibitory effect of the urine and kidney tissue and thus promote rather than cure the infection. Increased flushing of the bacteria from the bladder due to increased urine output will act in opposition to this effect.

Dontas et al (1974) induced a diuresis in 60 hospital patients with symptoms of a urinary tract infection. They found that although some people were cured of their infection, other patients with a previously negative urine culture had bacteria present in the urine after diuresis. The change from negative to positive urine cultures was indicative of the patient having upper urinary tract disease. Dontas and colleagues concluded that a rapid increase in urine flow does not necessarily decrease the bacterial content of the urine and may in fact convert a previously concentrated urine free of organisms into a dilute infected urine.

Diuresis would be thought to wash out all the bacteria from the bladder lumen. This sometimes occurs but often after an initial drop in the bacterial count of the urine, a state is reached when the bacterial count remains almost constant.

Mackintosh and colleagues (1975a and b) have investigated this phenomenon using mathematical equations and a computer to predict the effects of diuresis, hourly micturition, residual volume and the binding of bacteria to the bladder wall, on the clearance of organisms from the urinary tract. Their theoretical analysis led to the conclusion that the initial rapid fall in the bacterial concentration was a function of diuresis and that the subsequent constant output originates from bacteria bound to multiplication sites on the uroepithelium. These bound organisms shed their progeny into the urine and thus maintain the steady state concentration of organisms.

The dynamics of the growth of bacteria in the urinary tract is therefore a complex situation, being influenced by the osmolality of the urine, the degree of binding of the organisms to the bladder wall, and the rate of clearance of infected urine from the bladder. Mice used in our study have infected urinary tracts despite having high osmotic pressure urine. The presence of two stitches in the bladder wall must obviously disturb the normal mechanisms of defence which keep the bladders of these animals free from naturally occurring infections. The urinary tracts of the control mice in the diuresis experiments only had a minimal degree of infection 3 days after the initiation of the infection in the unstitched bladder. The only trauma to the bladder was in this case, the aspiration of the contents of the bladder and then the injection of bacteria into the lumen of the pouch.

The finding that diuresis increased the susceptibility of mice to an ascending urinary tract infection is in accordance with the results of Keane and Freedman (1967). After the injection of bacteria into the bladder, normal mice were found to clear the organisms, whereas in mice undergoing water diuresis,the bacteria were able to persist and multiply, and caused the development of severe pyelonephritis and papillary necrosis.

Freedman (1967) produced pyelonephritis in rats drinking 5% glucose in water and found that as few as 10 viable bacterial units were capable of survival and multiplication in the bladder lumen. A millionfold increase in susceptibility to chronic bacteriuria was claimed but this is a much greater efficiency of inducing infection than the results presented here in mice would predict. However, the use of animals with urinary tract infections, induced by a state of diuresis, has been made use of to provide a means for studying the relationship of bacteriuria to the chronic manifestations of urinary tract disease. Montgomerie *et al* (1972) used diuresing mice as a model of *E.coli* pyelonephritis to determine the role of systemic and local immunity in the natural history of urinary tract infections. Mice were immunised by the subcutaneous, intravenous and intravesical routes in an attempt to protect them from both ascending and

haematogenous infection. Subcutaneous injection of heat killed bacteria prevented pyelonephritis produced by the intravenous route but had no significant effect on an ascending infection.

The effect of water restriction and ammonium chloride acidosis on the course of E.coli pyelonephritis was determined in non-obstructed rat kidneys by Andriole (1970). Water restriction and ammonium chloride acidosis were found to increase the incidence of pyelonephritis, and a water diuresis was claimed to have a protective effect. The results are the opposite to those presented here. Humoral and cellular defence mechanisms, which are usually inhibited by high osmotic pressures, were thought by Andriole to be more active in the tissues of the diuresing animals than normal animals, and thus they prevented the infection developing in the water-loaded animals. The increase of medullary blood flow, in response to the need to excrete the excess ingested water in the animals undergoing diuresis, aided protection by promoting the faster delivery of phagocytes and humoral means of defence to the kidneys.

Why these results are the opposite to the other results is presumably a feature of the experimental conditions used. The rats were infected by the haematogenous route with approximately 10^8 organisms, acidosis was produced by forced ingestion of a 300 mM solution of ammonium chloride, and diuresis

following ammonium chloride was accomplished by allowing the rats free access to tap water. The osmolalities and salt concentrations found in the kidneys of these animals are presumably different to those found in animals undergoing diuresis by drinking 5% glucose in tap water. If this is so, it may explain the anomalous results obtained under the differing experimental conditions.

In conclusion, the osmolality and urea concentration of urine and of kidney tissue appear to be important determinants of the susceptibility of the urinary tract to bacterial infection. Mice normally produce very high osmolality urine and this may explain the natural resistance of the unobstructed, unmanipulated mouse urinary tract to infection. Conversely, human urine has a much lower concentration of solutes than mouse urine and the susceptibility of certain individuals to urinary tract infection may be dependent on this. Diuresis increased the kidney infection rate in mice but whether or not diuresis is beneficial in human cases of urinary tract disease is still open to debate. Increasing the osmolality of human female urine by the addition of urea converted a urine capable of supporting bacterial growth into a medium inhibitory or even bactericidal for E.coli. The results from these experiments suggested that strains of E.coli

rich in K antigen were better adapted to resist the killing effects of high osmotic pressure solutions than strains of *E.coli* with little or no K antigen. This may explain the greater propensity of K rich *E.coli* to infect the upper urinary tract.

CHAPTER 7

GENERAL DISCUSSION

Experimental E.coli urinary tract infections were induced in mice by the injection of bacteria into the stitched bladder. Host and bacterial factors of importance in determining the outcome of the acute infection were studied by manipulation of the experimental conditions under which the mice were infected.

The possession of K antigen by E.coli strains determined the virulence of the bacteria for the upper urinary tract. The K antigen has the property of making E.coli bacteria resistant to phagocytosis and complement killing. However, as humoral means of immunity were shown not to be involved in protecting mice from infection, these characteristics of K antigen could not therefore influence the virulence of E.coli within the urinary tract. The virulence determining factor of E.coli appears to reside in the ability of K rich strains of E.coli to resist the killing effects of solutions of high osmolality.

The host response to an infection of the urinary tract with E.coli involves both cellular and humoral mechanisms. Infected mice rapidly produced antibody but no protective role could be attributed to this or to antibody induced by immunisation procedures. The passive transfer of antibody to E.coli into Biozzi low responder mice rendered the animals

slightly more susceptible to kidney disease. These findings suggest that antibody produced during an infection of the urinary tract in people may also serve no protective function and could indeed be a determining factor in the maintenance of the chronic infection.

Delayed hypersensitivity to E.coli can be demonstrated in mice with an E.coli urinary tract infection. However, until the sensitization procedure has been standardised,it will not be possible to determine if this cellular reaction has any protective function. The interactions of cellular and humoral mechanisms of immunity deserve further consideration. The study of the reactions of T and B lymphocytes, and macrophages during a urinary tract infection, by depletion experiments and cell activity determinations, may give additional information about the role of cellular immunity in E.coli urinary tract infections.

Inbred mice have proved to be a useful tool for studying the host response to infection as all the mice within a strain are genetically identical. The strains of inbred mice varied in their susceptibility to E.coli urinary tract disease; some strains were susceptible to infection and others were resistant. The inheritance of the susceptibility to disease did not follow simple Mendelian genetics. These findings indicated that the host reaction to an infection of the urinary

tract is governed by a large number of genes. When the mechanisms, governed by these genes which determine the host response to infection have been identified it will probably be found that the ability of inbred mice to mount these reactions will be directly correlated to their susceptibility to E.coli urinary tract disease.

The response of people to E.coli urinary tract infections is also presumably under a similar kind of genetic control to that observed in inbred mice. Inherited susceptibility to infection could explain why some people succumb more readily than others to urinary tract disease, even in the absence of any abnormality of the urinary tract. If the genetically controlled mechanisms which determine the host response to infection can be identified in mice, and they are the same as those in people, then it might be possible to treat or prevent infections of the human urinary tract by direct manipulation of the host response to disease.

A few possible clinical applications can be drawn from the results obtained. The finding that diuresis increases the susceptibility of mice to E.coli kidney disease may have implications on the practice of encouraging people with E.coli urinary tract infections to drink copious amounts of water. It is possible that lowering the osmolality of the urine in this way results in the increased risk of the kidney becoming

infected with E.coli from the bladder. A study of the ability of E.coli to grow in normal and very weak osmolality urines and the determination of the actual osmolalities encountered in the human urinary tract during normal fluid intake and diuresis may help to resolve this point.

The finding that K rich strains of E.coli are more virulent for the kidney than E.coli strains with little or no K antigen could prove to be of diagnostic and prognostic use. People with a urine infection due to an E.coli strain of high K antigen content are more likely to be suffering from, or may more readily succumb to kidney infection than persons with a low K antigen urine isolate of E.coli. However, for the determination of K antigen content to become a routine test in diagnostic laboratories, it is necessary to find more rapid methods of assessing the K content of E.coli strains than those available at present.

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APPENDIX

Br. J. exp. Path. (1975) 56, 549

INVESTIGATION OF THE EFFECT OF K ANTIGEN IN *ESCHERICHIA COLI* URINARY TRACT INFECTIONS BY USE OF A MOUSE MODEL

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Summary.—Urinary infection could be produced in mice by the inoculation of *Escherichia coli* into the bladder, provided that sutures had first been inserted into the bladder wall to act as foreign bodies. While the bladder was almost always infected, the kidneys were involved less often. The frequency of kidney infection was directly proportional to the amount and activity of the K antigen of the infecting strain of *E. coli*. The significance of K antigens in relation to host defence mechanisms and tissue invasion is discussed.

The most frequent type of urinary tract infection is that due to *Escherichia coli*, the source of which is generally thought to be the patient's own faecal flora. The route of infection is ascending, *via* urethra and bladder to the kidneys. Spread of infection to the kidneys is not invariable but is more likely in pregnant women or where there is any cause of urinary stasis. Because of the undoubted importance of such host factors, insufficient attention has been paid to the possible virulence of the infecting organisms. One such factor is the K antigen. Strains of *E. coli* rich in K, *i.e.* in which the antigen is present in large amounts or in particularly active form, or both, are better than K-poor strains at resisting phagocytosis or killing by complement (Glynn and Howard, 1970; Howard and Glynn, 1971). Because of this Glynn, Brumfitt and Howard (1971) examined strains of *E. coli* from women with urinary tract infection. There was a significantly higher proportion of K-rich *E.coli* in urine from patients with infection than in strains from the faecal flora. On further analysis the excess of K-rich strains was found to be in those patients with renal involvement. There was no increase in such strains in patients in whom the infection was confined to the bladder. Mabeck, Ørskov and Ørskov (1971) found 3 serotypes of *E. coli*, 02.K1.H4, 04.K12.H5 and 06.K2a. 2c.H1 to be particularly common in their series of patients with pyelonephritis but they did not quantitate the K antigen. Kaijser (1973) found more K-rich strains in schoolgirls with pyelonephritis than in those with cystitis. If K-rich strains are more likely than others to invade the kidney from the urine, this could prove to be of prognostic value.

Kaijser and Olling (1973) claimed that antibodies could protect rabbits against experimental pyelonephritis. However, infection was by the haematogenous route. In order to study further the bacterial factors involved in renal infection, we have established an ascending infection in mice using a method suggested to us by Mr M. Snell. As a first step, the role of K antigens in such infections in mice was examined.

MATERIALS AND METHODS

Mice.—Outbred albino male Porton mice weighing 30–40 g were used.

Bacteria.—Seven strains of *E. coli*, 6 of human and 1 of piglet origin, were used. Details of

TABLE I.—*Properties of Strains of* Escherichia coli *Used*

Stain of *E. coli*	Source	Serotype	Titre of agglutination inhibiting activity	Complement sensitivity (No. of CH_{50} units killing 50% of *E. coli* under standard conditions)
WF 82	Piglet	0117 :K?:H27	64	29
WF 41	Stool	017 :K16:H18	0	<0·7
WF 96	Urine	07 :K1:H6	2	0·8
WF 98	Urine	06 :K13:H16	16	8·0
WF 60	Baby	06 :K13:H1	32	9·5
WF 8	Urine	06 :K13:H⁻	32	>32·4
WF 26	Urine	06 :K13:H31	64	>32·4

(Data taken from C. M. Milne, M.Sc. Thesis, Bristol, 1966; C. J. Howard, Ph.D. Thesis, London, 1970)

their properties, including serotypes and the agglutination inhibiting activities of their K antigens (Glynn and Howard, 1970) are given in Table I. The strains were chosen to give a range of K activity and hence of complement sensitivity. More particularly they include 4 strains all of serotype 06.K13 but with differing K activities.

Bacteria were grown overnight in glucose nutrient broth, washed and resuspended in 0·15 mol/l saline at the desired concentration as measured by optical density and subsequently confirmed by quantitative culture.

Infecting mice.—Mice were anaesthetized with intraperitoneal Nembutal (pentobarbitone sodium 0·6 mg/10 g mouse body weight) and ether.

The bladder was exposed by a suprapubic incision and any urine was removed by a syringe and 30-gauge needle. Two silk stitches (Mersutures Silk 5/0 *B.P.C.*, Mersutures Ethicon Ltd, Scotland) were put in the upper anterior bladder wall and the bacterial suspension was injected in a constant volume of 0·025 ml using a micro syringe. It was essential to empty the bladder before injecting the bacteria or irregular results were obtained. The peritoneum was closed with catgut (Mersutures Plain 3/0 *B.P.*) and the skin clipped with 9 mm Autoclips (Clay Adams, Parsipanny N. J. 07054 U.S.A.). Mice were left in the warm (30°) until they had recovered from the anaesthetic. Food and water were allowed *ad libitum.*

For each strain of *E. coli* a minimum of 24 mice were infected, different doses being given to groups of 8. Occasionally, 1 mouse per group died soon after operation.

Examination of mice for kidney infection.—Mice were killed 3 days after being infected. A urine sample was taken for quantitative culture. The kidneys were removed aseptically, weighed, homogenized in 10 ml 0·15 mol/l NaCl using a Colworth Stomacher 80 (A.J. Seward and Co. Ltd, London) and viable counts made.

RESULTS

Preliminary studies suggested that 3 days was a convenient time to examine the kidneys for an acute infection. At 3 days the urine was nearly always infected, no matter which strain of *E. coli* had been given (Table II). The viable bacterial count in the urine, although dose dependent, varied widely with means of between $10^{4 \cdot 3}$ and $10^{6 \cdot 7}$.

TABLE II.—*Course of Infection in 50 Mice given* $2 \cdot 5 \times 10^6$ (*approximately* 1 ID_{50}) *of* E. coli *WF 8*

Day	No. of infected urines	No. of infected kidneys
3	5/5	3/10
7	5/5	4/10
10	5/5	1/10
14	5/5	5/10
17	5/5	4/10
21	5/5	5/10
28	3/5	4/10
35	4/4	2/8
42	4/4	5/8
50	4/4	5/8

Kidneys containing more than 100 viable organisms were regarded as infected though counts of up to 10^6 viable organisms per kidney were seen. Provided a kidney was infected at all, its bacterial content was related to the size of the original infecting dose but not to the strain used. However, the number of kidneys infected though also dose dependent varied significantly between different strains of *E. coli*. Results are presented

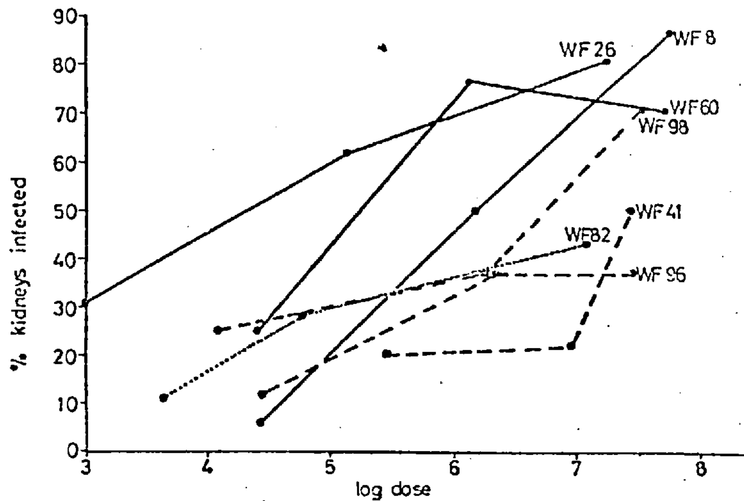

FIG. 1.—Percentage kidneys infected related to dose and strain of *E. coli*. Each point represents 7–10 mice, *i.e.* 14–20 kidneys. Results are given as percentage to even out small differences in sample size. ———— K rich strain *E. coli*, – – – – – – K poor strain *E. coli*, ... K rich piglet strain *E. coli*.

as number of kidneys infected rather than number of mice since both kidneys were not always involved. In 26 of 83 mice with renal involvement the infection was unilateral. The 7 strains of *E. coli* gave dose-response curves which differed in slope and in the level of infectivity at any given dose (Fig. 1). The dose of each strain infecting 50% of kidneys (ID_{50}) was calculated and plotted against the agglutination inhibiting activity (AIA) of the strains's K antigen (Fig. 2). For the human strains the ID_{50} fell proportionally as the K activity rose. The piglet strain WF82 clearly did not fit into the same curve having both an active K and a high ID_{50}.

An alternative way of expressing the results is by the infectivity of each strain at a given dose. Thus, for an infecting

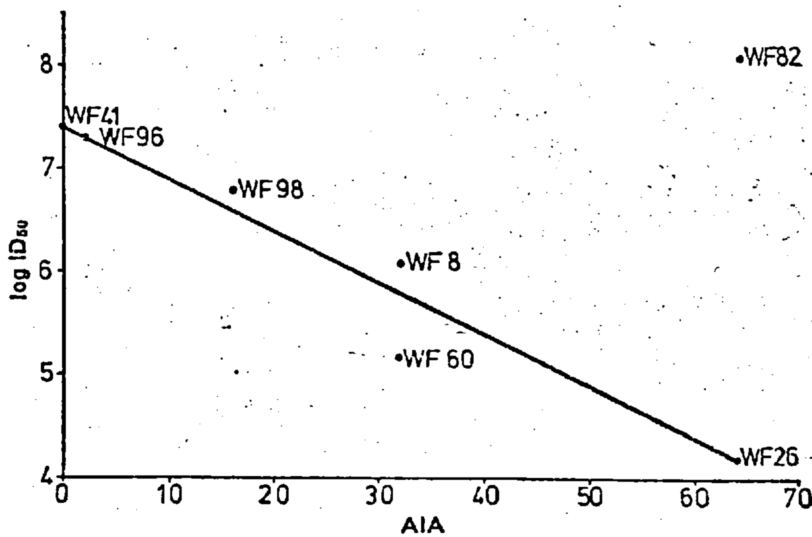

FIG. 2.—Agglutination inhibiting activity (K activity) of *E. coli* strains related their ID_{50} calculated from Fig. 1.

dose of $1 \cdot 25 \times 10^7$ viable bacteria the least active strain, WF41, infected 30% of kidneys and the most active, WF26, 80%.

In another experiment, 50 mice were given approximately 1 ID_{50} ($2 \cdot 5 \times 10^6$ bacteria) of strain WF8 and then examined in groups of 4–5 at intervals (Table II). Urinary infection was present in all the mice and persisted throughout the experiment, *i.e.* 50 days. About half the kidneys were infected and, allowing for the small numbers in each group, the proportion did not change with time.

DISCUSSION

In mice with a foreign body, in this case a suture in the bladder, urinary infection was readily established by several strains of *E. coli*. The ability of such strains to involve the kidney was related to the activity of their K antigens. This confirms expectations based on the effect of K antigens on defence mechanisms such as phagocytosis and complement killing (Glynn and Howard, 1970; Howard and Glynn, 1971) and fits in with the findings in pregnant women (Glynn *et al.*, 1971) and schoolgirls (Kaijser, 1973).

Recently, Kalmanson *et al.* (1975) found no differences in the presence of K antigens between strains of *E. coli* from upper and lower urinary tract infections in women. However, they do not seem to have taken account of the amount and activity of K antigen present.

In a study of *E. coli* strains from urine, faeces and the blood of patients with bacteraemia, McCabe *et al.* (1975) found a significant increase in the amount and activity of K antigens in the urinary isolates compared with those from faeces, but no significant difference in K antigen content of the blood culture isolates. Unfortunately they did not distinguish between upper and lower urinary tract infections but did notice a bimodal distribution of K antigen activity in the urinary strains which would fit with the general concept presented here. They therefore suggested that K antigen activity might predispose to renal pathogenicity but not to a general increase in virulence. If this is so, it would be necessary to explain the propensity of K-rich strains to invade the kidney in some way other than their resistance to phagocytosis and complement killing since these defence mechanisms are probably more active in the blood stream than in the kidney.

Roantree and Pappas (1960) found significantly more complement resistant strains of *E. coli* in the blood of patients with bacteriaemia than in faeces. They did not measure K antigen and while this is one determinant of complement resistance, there may well be others. However, Ørskov *et al.* (1971) found a predominance of acidic K antigens in strains of *E. coli* associated with bacteraemia, and acid groups on the O antigen of a few locally invasive strains associated with a dysentery-like syndrome. In spite of the findings in bacteraemia of McCabe *et al.* (1975), it is tempting to believe that surface acidic groups, particularly K antigens, may favour tissue invasion. Myerowitz *et al.* (1972) have pointed out that many urinary *E. coli* have antigens cross-reacting with capsular polysaccharides in pneumococci, meningococci and *H. influenzae* b and they, too, suggest a general role in pathogenicity for acidic polysaccharides. However, there are numerous cross-reacting polysaccharide determinants in nature and, while such speculation is stimulating, the understanding of virulence mechanisms is best based on the careful analysis of individual factors.

The aetiological importance of factors in the host is well known clinically and is implicit in our method of producing infection. At present we are using the mouse model to examine possible genetic and immunological factors in renal infection. It is worth considering whether a systematic survey of K activity in strains of *E. coli* isolated from human urinary infections could be of diagnostic or prognostic value in relation to the problem of renal involvement.

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