A STUDY OF THE PATHOGENESIS
AND IMMUNOBIOLOGY OF PYELONEPHRITIS

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SHORT ABSTRACT

The experiments carried out during this investigation have studied the pathogenesis and immunobiology of pyelonephritis. Two experimental models of the disease were developed in laboratory animals and several new analytical procedures for studying biological changes during the course of the disease were established. In particular, methods for the determination of glomerular filtration rates in small animals, the automated analysis of antibacterial antibody and the in-vitro determination of the response of rat lymphocytes to phytohaemagglutinin (PHA) were established.

The effect of chronic active pyelonephritis on renal function was studied and the effect of antibiotic treatment and elimination of infection on the pathological changes in pyelonephritis was determined. Eradication of infection did not affect the gross pathology and histopathological changes found at autopsy. These experiments have also investigated the role of bacterial interference as a determinant in the epidemiology of renal infection. It was shown that mixed renal infections with \textit{E. coli} were uncommon and that the pattern of infection was determined by the resident pathogen. The relationship between bacterial infection of the renal parenchyma with \textit{E. coli} and the establishment of pathological lesions was investigated and the conclusion reached that infection of the kidney is not always associated with pathological changes. The term "acquiescent infection" was then used to describe the host parasite relationship in which active persistent bacterial infection is not associated with pathological lesions.
Antigen presentation as a factor in the protective immune response to renal infection was also studied and experiments carried out which demonstrated that immunological memory to the somatic antigen of *E. coli* persisted for at least six months after primary immunization and appeared to be carried by the B lymphocytes. The distribution of B lymphocytes in the peripheral blood and lymphoid sites and the functional capacity of T cells during the course of pyelonephritis were also investigated. Lymphocytes forming the lymphocytic infiltrate in the kidney were identified as thymus derived lymphocytes by their surface labelling characteristics but further experiments showed that T lymphocytes in the kidney, which would normally respond to stimulation with PHA in-vitro, were non-responsive and presumably non-functional. A unique factor associated with renal cells was found to be capable of blocking the PHA response of T lymphocytes, suggesting that ablation of cell-mediated immune mechanisms in the kidney may contribute to the persistence of infection in pyelonephritis. This concept was investigated by studying the effect of a selective deficiency of thymus derived lymphocytes on the course of pyelonephritis. The experiments showed, however, that ablation of T lymphocytes did not appreciably alter the course of the disease. Further experiments were then carried out investigating the role of B lymphocytes in the immune response by manipulation of the hosts immune capacity using immunosuppressive drugs. Animals with pyelonephritis did not produce antibacterial antibody but were able to eliminate organisms more readily from the infected kidney than untreated animals with a normal immune response. This suggests that blocking of the phenomenon of immunologic
enhancement may explain these unexpected results and that the immune response to renal infection could have an enhancing role, protecting the bacterial cell from otherwise effective host defense mechanisms.

Clinical aspects of antimicrobial resistance in pyelonephritis were also investigated in a study of urinary tract pathogens from 120 patients. The results showed that standard methods for testing antibiotic sensitivity may reject potentially useful antibiotics and that up to 75% of microorganisms considered resistant to individual antibiotics may in fact be sensitive to concentrations of antibiotic attainable in the tubular lumen. In certain circumstances the determination of minimum inhibitory concentration of selected antibiotics may be of considerable value in patient management.
CHAPTER 1

INTRODUCTION
CLINICAL OBSERVATIONS

Infections of the urinary tract are common in both children and adults, but information on the natural history of chronic pyelonephritis has been difficult to obtain. Part of the problem lies in the fact that no definite pathological or clinical pattern has emerged and the "silent" clinical course of many patients developing severe renal disease after bacterial infection has been well documented (Angell, Relman & Robbins, 1968; Kimmelstiel et al., 1961; Andersen et al., 1973). As a result, the characteristics of the disease have been variously defined by clinicians, radiologists, pathologists and microbiologists.

Factors such as age and sex are known to be important and affect the pathogenesis and progress of urinary tract infections. Neonatal infections have been found to occur predominantly in boys and are believed to be haematogenous in origin (Bergstrom et al., 1972a) whereas after the first year of life, urinary tract infections in girls outnumber boys by a factor of 30 and ascending infections are thought to be more important (Smallpiece, 1968; Kunin, Deutscher & Paquin, 1964).

One alarming feature of infections in children is the high rate of recurrence and in a prospective study of 80 neonatal urinary tract infections Cohen (1972) found a recurrence rate of 40% and 60% following first and second infections respectively. Most occur within two months and the indications are that a simple recurrence is highly suggestive of increased susceptibility to infection and the likelihood of a continuing problem with recurrent infections.

On a longer term basis, other investigators have found evidence
for progressive disease in 19% of girls hospitalized for renal infections and reinvestigated 15 to 25 years later (Lindblad & Ekengren, 1969).

The pathogenesis and prognosis of urinary tract infection in adults has been studied in considerable detail, but because terminal renal failure from chronic pyelonephritis is not common (Kimmelstiel et al., 1961; Heptinstall, 1969; Barnes et al., 1972), the relationship of this condition to bacterial invasion of the kidney has been seriously questioned (Freedman, 1967; Freeman, 1973). The high morbidity from infections of the urinary tract remains unquestioned, however, and a large number of patients each year consult their general practitioner because of urinary tract symptoms. A figure of 12 per 1,000 females at risk has been given by Milne et al. (1969). Bacteriuria is also common in New Zealand with proven infections of the urinary tract accounting for 1.8% of all new consultations in a survey of general practitioners in this country, compared with 0.2% for all other diseases of the kidney (Gallagher, personal communication, 1973). Autopsy studies in this country, using strict criteria, have also confirmed the frequency with which pyelonephritis may be found and an incidence of 6.9% was found in a study of 174 cases (Robinson & Herdson, 1973).

Young women with intercourse-induced bouts of cystitis comprise the largest single group seen in general practice. Asymptomatic urinary tract infections can be found in approximately 1% of infants, school girls and nuns, but this increases to 5 to 10% in the former group of young women.

Between 30 and 50% of the patients presenting with symptoms
suggestive of urinary tract infection, however, do not have bacteriuria (Fairley et al., 1971; Steensberg et al., 1969; Gallagher, Montgomerie & North, 1965) and it is probable that most adult patients who present with symptomatic and proven infections will have a relatively benign course. This comment is supported by several long-term prospective studies where asymptomatic bacteriuria in non-pregnant women, renal function in patients with radiological pyelonephritis and the effect of urinary tract infections on renal function in domiciliary patients were studied (Asscher et al., 1973; Guttman, 1973; Gower, 1973). The collective finding from these studies was that there was little evidence of bacteriuria leading to an increase in blood pressure, serum urea, kidney scarring or deterioration of renal function over a period of up to ten years. The observation that progressive parenchymal damage has been more frequently recorded in patients without overt infection (Fairley & Butler, 1970; Rolleston, 1970; Andersen et al., 1973) suggests that vesico-ureteric reflux and occult renal infection may play an important role in parenchymal scarring and the loss of renal function.

The close association of ureteric reflux with chronic pyelonephritis (reflux nephropathy) has been effectively argued by Bailey (1973) although some authors consider that renal atrophy does not occur in the absence of urinary tract infection (Govan et al., 1974) and there is no evidence that reflux alone will lead to functional renal changes (Uehling, 1971; Fritjofsson & Sundin, 1966).

There is increasing evidence, however, that considerable renal
damage may result from vesico-ureteric reflux alone (Rolleston, Shannon & Utley, 1970). Some investigators now believe that the pyelotubular back flow which occurs in gross vesico-ureteric reflux may itself lead to an arrest of renal growth. Scar formation then occurs at the site of intra-renal reflux as Hodson has demonstrated in experimental animals in the absence of continued renal infection (Hodson, 1972). MacGregor (1970) has expressed the viewpoint that progressive renal destruction of the type labelled chronic pyelonephritis can progress irrespective of continuing bacterial infection, but the role of infection in the initial pathological changes in an individual kidney cannot be stated with certainty.

The consensus of opinion seems to be that a high incidence of renal scarring does occur after urinary tract infection in childhood, but that progressive loss of renal function from renal infection in later life is unusual (Bullen & Kincaid-Smith, 1970; Asscher, 1972; Freedman, 1972) although it can occur (Bailey, Little & Rolleston, 1969; Little, McPherson & deWardener, 1965; Davies, McLachlan & Asscher, 1972). These conclusions have been supported in a recent study by Parker and Kunin (1973) who selected women between the age of 15 and 35 with severe clinical renal infection and followed their progress over a period of 10 to 20 years. The basis of the study was that, as this form of urinary tract infection represented a severe clinical expression of the disease, it would provide a yardstick by which the effect of less serious infections could be measured. Repeated episodes of infection occurred within three years of follow up and 23% were reinfected within
a six-month period. At the times of follow up, however, there was little to suggest progressive loss of renal function in most patients, but there was justification for the close supervision of patients with a history of pyelonephritis. The question of recurrent urinary tract infection is important both from a management and epidemiological point of view. Studies by McGeachie (1966) on a group of 49 patients showed that 90% of the recurrences occurred up to six months after the last infection, and that of these, 80% of the strains were different from the strain isolated from the previous infection. Gruneberg (1970) and Pryles and Glagovsky (1965) have reported similar figures with 71% and 85% respectively of recurrent infections being caused by a different organism. The effect of chemotherapy on the faecal and periurethral flora is related to this topic and a number of authors have documented the correlation between faecal bacteria and the organism causing urinary tract infection (Lincoln, Lidin-Janson & Winberg, 1970; Gruneberg, 1969). Winberg et al. (1973) in a series of observations of the relationship between chemotherapy and faecal and periurethral flora, have shown that changes in the bacterial flora of the latter regions precede the appearance of the corresponding bacteria in the urinary tract. It was shown that sulphonamide therapy had a selective effect on the faecal flora and that the resident intestinal \textit{E.coli} were replaced by sulphonamide resistant \textit{E.coli} within a few days of commencing sulphonamide therapy. With these comments in mind, it would seem important to consider the effect of antimicrobials used in the treatment of urinary tract infection, not only on the
causative organism, but also on the host flora. Experiments along these lines have already been carried out by Gruneberg, Smellie & Leakey (1973).

SOME FACTORS AFFECTING THE ESTABLISHMENT OF URINARY TRACT INFECTION

Bacteria enter the bladder through the short female urethra despite its high pressure zone (Mayo & Hinman, 1973) and the presence of urethral mucus (Hutch, 1969). There is evidence that bacteria can pass up the female urethra during micturition (Hinman, 1966) and that sexual activity may be a significant contributing factor (Kunin & McCormack, 1968). The male bladder, on the other hand, is protected not only by the length of the urethra, but also by the antibacterial properties of prostatic fluid which is capable of killing most strains of bacteria within six hours both in the dog and in man (Stamey et al., 1968). The close relationship between introital and vulval bacteria and the presence of the same strains of bacteria in the urinary tract (Gruneberg, 1969; Cox, Lacy & Hinman, 1968; O'Grady et al., 1970) of the female provides further evidence for the portal of entry of bacteria and an explanation for the high incidence of urinary infection in the female, compared with the male. The bladder is likely to be infected frequently but because of an efficient host defence system, eradication of bacteria occurs in most instances. The spontaneous cure of one-third of the patients with asymptomatic bacteriuria (Asscher et al., 1969) and the remarkable ability of the healthy bladder to expel and destroy bacteria introduced into urine in the bladder (Cox & Hinman, 1961) illustrate the
effectiveness of this mechanism.

The kinetics of the mechanical removal of bacteria from the urinary tract have been studied in vivo (Cox & Hinman, 1961) and in vitro (O'Grady et al., 1973) and show the importance of complete emptying of the bladder on the elimination of bacteria from the urinary tract. Once infection is established, a residual urinary volume as small as 10 ml. makes elimination of bacteria from the bladder less efficient (Shand et al., 1970).

Contrary to the generally held view, urine is not a good culture medium and several characteristics of freshly voided urine such as a comparatively low pH and an osmolality that is above optimum for growth, serve to restrict bacterial growth (Asscher et al., 1966). Urinary urea in high concentrations is inhibiting to bacterial growth (Kaye, 1968) and a high protein diet has been shown to reduce bacterial growth in the renal medulla and cortex of rats with enterococcal pyelonephritis (Miller & North, 1966). In man, a high protein diet results in concentrated urine with a high urea content which has been shown to be a less effective culture medium.

The bladder wall, too, has been shown to have considerable bactericidal activity (Norden, Green & Kass, 1968) but the precise mechanism of this bactericidal effect has not finally been elucidated. One study indicated that bacteria were killed during phagocytosis by polymorphonuclear leucocytes (Cobbs & Kaye, 1967).

When bacteriuria is established in the bladder, the vesico-ureteric valve and the ureter present further mechanical barriers to the spread of infection. In experimental studies
it has been found that ascending infection can be established with relative ease in the rat, compared with most animal species. The rat lacks an effective vesico-ureteric valve and reflux can be shown to occur at each normal voiding (Mahoney & Persky, 1963). In dogs, infection is uncommon even when intravesical injection of bacteria leads to the induction of vesico-ureteric reflux; therefore, other factors may also be involved (Schoenberg et al., 1964). In vitro and in vivo studies have shown that ureteric smooth muscle contractibility is affected by common pathogens (Teague & Boyarsky, 1968; King & Cox, 1972) and Tsuchida, Hiroatsu and Shigeru (1973) have suggested on the basis of experiments on dogs that both vesico-ureteric reflux and abnormal ureteric peristalsis are necessary conditions for renal infection to become established. Recent studies have shown that the ureter has an intrinsic nerve supply (Aung-Khin, 1973; Gosling, 1970) which controls local peristalsis and that destruction of the nerves may be associated with ureteric atony and dilation (Boyarski, Labay & Glenn, 1968). Application of these experimental procedures to a study of ascending infection may resolve the role of ureteric function in preventing upper urinary tract invasion.

A widespread belief that has influenced the investigation of host defence mechanisms is that the renal medulla is unusually susceptible to infection and is the site of bacterial persistence in chronic infection. Phrases such as the "unique susceptibility" and the "exquisite sensitivity" of the medulla to infection appear in the literature on pyelonephritis, but are inconsistent with much experimental evidence. One mechanism that has been proposed to explain the apparent susceptibility of
the medulla to infection is that the hypertonic region of the medulla causes a delayed granulocytic response that allows infection to become established in the medulla (Rocha & Fekety, 1964). However, subsequent events involving the spread of infection beyond the original lesion show that mobilisation of phagocytic cells into adjacent infected but undamaged tissue does not occur and that the polymorphonuclear infiltrate remains confined to the initial inflammatory foci. Thus, the short delay in the appearance of phagocytic cells in the inflammatory thermal lesion of the medulla compared to cortex is probably not an important feature in the establishment of infection. A more important defect may be the poor chemotactic capacity of Escherichia coli, both in the cortex and in the medulla, resulting in an ablated rather than a delayed phagocytic cell response. Evidence of neutrophilic exudation being mediated through the complement system (Gewurz et al., 1967) which is inactivated by renal tissue (Beeson & Rowley, 1959) is consistent with the deficient response to infection of the phagocytic cell.

Bacterial persistence has been investigated experimentally in the rat kidney where the vascular cortical tissue can readily be differentiated macroscopically from the medulla and from the papilla. When care is taken to separate cortex from the medulla, there is no suggestion that bacteria either proliferate preferentially in medullary tissue or persist in that region; the consistent increase in bacteria in the papilla may simply represent concentration of luminal bacteria (Miller & North, 1966).

Although the mobilisation of polymorphonuclear leucocytes
during renal infection has been studied, the function of other inflammatory cells forming the interstitial infiltrate has not been well characterised. Components of the host's defence in the kidney are likely to include non-specific phagocytosis, cell-mediated immune responses and specific antibody production. In both acute and chronic renal infection the small lymphocyte has been found to be the predominant inflammatory cell even when numerous polymorphonuclear leucocytes are present in luminal casts. There is ample evidence documenting the movement of antibody-forming cells into the infected kidney (Miller & North, 1971; Spencer & Fairhead, 1972) but the significance of these remain to be defined. As yet, there is no evidence for a significant cell-mediated immune response in pyelonephritis, although cellular immunity with macrophage activation induced by stimulation of thymus-derived lymphocytes has been described in several non-renal bacterial infections (Lane & Unanue, 1972).

HOST IMMUNE RESPONSE TO URINARY TRACT INFECTION
In acute symptomatic urinary tract infection, high temperature with loin pain and tenderness indicate renal parenchymal involvement but symptoms restricted to the lower urinary tract do not exclude the possibility of upper urinary tract infection (Kass & Zinner, 1969). Many laboratory tests have been devised to provide a definitive diagnosis of renal involvement and the immune response of the host to infection has been investigated in great detail in man and experimental animals (Holmgren & Smith, 1975).

The immune response to urinary tract infection is partly age
dependent. The studies of Winberg et al. (1963) on patients with acute infections judged by lowered capacity to concentrate urine, showed a marked and rapid antibody response except in patients below the age of two months. In contrast, patients with normal concentrating capacity showed no definite change in antibody titre. Andersen et al. (1965) and Hanson et al. (1970) have also found similar results which were also substantiated in an investigation by Jodal (1974). Only 2 of 40 patients with cystitis (5%) but 48 of the 54 pyelonephritis patients (89%) had increase serum titres when compared with 643 healthy children.

In adults, equally encouraging results have been reported by Reeves and Brumfitt (1968) who found a good correlation between positive ureteric urine cultures and antibody titres but only when direct bacterial agglutination was used. Bacterial agglutination was also used by Percival, Brumfitt & deLouvois (1964) to study the antibody response of 293 patients with urinary tract infections. In this study, 93% of the 41 patients with acute pyelonephritis showed specific agglutination titres that were higher than those observed in a control group of 20 normal subjects. Furthermore, in patients where early detection of infection was possible, rising antibody titres were observed in 19 out of 21 cases. In the same series of 86 patients with asymptomatic bacteriuria or symptoms confined to the urinary tract, only 15 showed agglutination titres above the normal range and in 7 of these, renal infection was confirmed. Similar results were reported by Vosti, Monto & Rantz (1965) who studied the antibody response in 60 patients with urinary
infections by measuring 0 specific antibody in serum. An immune response was noted during the first and second week of the disease and reached a peak in the third to fourth week before returning to baseline levels by the second to third month. In this study, a significant rise in the antibody titre against the infecting strain occurred in 14 of 17 patients with acute pyelonephritis, but in only 2 out of 33 episodes of cystitis.

The majority of investigators have reported total antibody titres in their studies without determining the immunoglobulin class of the antibody. The distribution of the 0 specific antibodies of serum and urine among the immunoglobulin classes could clearly be of importance and Vosti and Remington (1968), in a study of the physico-chemical properties of serum antibody in urinary tract infections, found three patterns of 7S and 19S immunoglobulin production. Normal controls were found to have low serum antibody levels primarily in 19S immunoglobulins class. Patients with acute pyelonephritis on the other hand, showed a response characterized by a significant change in the level of both 7S and 19S specific antibody. A more complex picture was seen in those patients with cystitis and asymptomatic bacteriuria. In these cases, acute and convalescent sera contained both 7S and 19S antibody, the levels of which remained constant during the course of the infection.

The available data therefore provides clear evidence that a significant increase in 0 specific agglutinins may occur during the course of pyelonephritis and although a similar response can also occur in cystitis, it is relatively uncommon in the latter condition. The magnitude of the immune response most
probably reflects the degree of antigenic stimulation and while this is considerable in acute parenchymal infection, it is usually insufficient to stimulate significant antibody response when infection is confined to the bladder. This effect has been used to differentiate parenchymal involvement from cystitis and although a single antibody titre is of limited use, a significant change in the antibody titre may be of considerable diagnostic value.

More recently, it has been possible to determine the site of infection by ureteral catheterisation and to follow the antibody response in patients who have had infections localised to the upper or lower urinary tract. In general, patients with renal infections had higher titres of antibody than patients with infection localised in the bladder, but because of the wide range of titres, determination of circulating antibody levels was only of limited use in predicting the site of infection (Clark, Ronald & Turck, 1971; Boutros, Mourtada & Ronald, 1972; Fairley et al., 1971). One reason for discrepancies between the reports of various groups may be the type of analytical procedure used to measure the immune response to the invading organism. Both bacterial and passive haemagglutination procedures measure predominantly IgM antibodies while precipitation techniques measure IgG antibodies. In both techniques the visual end point is a secondary manifestation of the antigen-antibody reaction and a recent method termed the Elisa assay (enzyme-linked-immuno-absorbent assay) may provide more detailed information as it detects primary binding between antigen and antibody. The method has already been used to determine the
antibody content and immunoglobulin class of serum from individuals with urinary tract infection (Jodal, 1974). Ahlstedt et al. (1972) have shown that laboratory results may also be affected by the avidity of the antibodies present. High avidity antibodies tended to give lower titres and as repeated infections would be expected to result in the elaboration of high avidity antibody, agglutination methods may not be the most appropriate methods of analysing serum in this situation.

Urinary antibody has also been used as a diagnostic aid and "O" antigen specific antibodies were detected in all 14 patients with recurrent urinary tract infections but only three out of eight following an initial attack of pyelonephritis (Hanson et al., 1971). Technical problems concerned with the concentration of urinary samples have largely been responsible for the limited use made of this analysis, but these have been overcome and the application of the Elisa procedures to the determination of urinary antibody using unconcentrated urine has already yielded useful results (Jodal, 1974).

EXPERIMENTAL RENAL INFECTIONS

The pathological changes in chronic pyelonephritis in man including scarring, interstitial and pelvic inflammation and tubular atrophy, are a combination of inflammatory and post-inflammatory changes, none of which is specific and which do not usually give an indication of the aetiology, bacterial or otherwise. In clinical investigation it has not been practical, apart from isolated studies in small groups of patients, to correlate the associated histopathological changes in the kidney with bacteriological information obtained during individual
episodes of infection. Because of the limitations of human studies, experimental studies in the rat, rabbit and dog have been used to investigate the host response to urinary tract infection. Spontaneous renal infection does not occur with any frequency in experimental animals and it has usually been necessary to manipulate the urinary tract in order to predispose the kidney to infection. Complete ureteral obstruction (Guze & Beeson, 1956), partial ureteral obstruction (Guze, Hubert & Kalmanson, 1965), renal scarring (Rocha, 1963), renal massage (Braude, Shapiro & Siemienski, 1955), acidifying agents (Freedman & Beeson, 1961), potassium depletion (Woods et al., 1960) and renal vein constriction (Brumfitt & Heptinstall, 1959) have all been used by various workers to establish infection.

Although most investigators have induced pyelonephritis with E.coli, other organisms have also been used. In particular, Streptococcus faecalis has been used extensively by Guze and his associates to study the pathogenesis, pathology and host-parasite relationships in pyelonephritis. Staphylococci (Foster, 1965), proteus strains (Cotran, 1963) and pseudomonas have also been used but infections with the latter organisms tend to produce renal abscesses rather than pyelonephritis, and their relevancy to the clinical disease is doubtful.

The majority of the experimental models mentioned previously have involved a haematogenous challenge with the infecting organism but models utilizing a retrograde challenge have also been described. The first successful model for ascending pyelonephritis was described by Vivaldi et al. (1959) where
renal infection occurred in 60 to 90% of rats following the injection of a proteus strain into the bladder. The ascending route was confirmed in later experiments by Cotran et al. (1963) who gave details of the pathological bacteriological and immunological features. *E. coli* has also been used to induce retrograde infections but, in contrast to proteus pyelonephritis, the infections are milder and few animals become infected. Ascending Klebsiella infections in rats (Anderson & Jackson, 1961), also produced infection in approximately 60% of animals but in contrast to infection with *E. coli*, inflammation of the renal pelvis increased progressively and parenchymal lesions were observed more frequently.

One feature of experimental ascending pyelonephritis has been that the renal lesions have shown a wide spectrum of severity from mild pyelitis to severe necrotizing pyelonephritis. The introduction of 0.2 ml. of inoculum containing $10^6$ *E. coli* leads to a mild pyelitis but the severity of the lesions can be increased by the insertion of a foreign body into the bladder. On the other hand, when 1.5 ml. containing $10^8$ bacteria were injected (Heptinstall, 1964) gross pyelonephritic lesions developed, especially when the bladder was compressed at the time of introduction. In most experimental animals, acute inflammation lasts for about three weeks, but thereafter the lesions heal spontaneously with sterilization of the renal tissue and scar formation. In some models, particularly enterococcal infection, active renal infection may occur for more than a year and the lesions show a persistent interstitial infiltrate, tubular destruction and advanced fibrosis which may sometimes be periglomerular. Histological changes in blood
vessels, however, are not common.

The factors involved in the resolution of experimental renal infection have been investigated in detail, particularly as they relate to an important problem in human pyelonephritis. The persistence of infection in relation to non-specific resistance (Jackson, Arana & Kozij, 1970), acquired immunity (Sanford, Hunter & Souda, 1962), persistence and distribution of bacterial antigen (Cotran, 1963), the role of protoplasts and L forms (Guze, 1968) and repetitive infection (Arana, Kozij & Jackson, 1965; Sommers et al., 1964) have all been studied. One of the problems in relating these features to chronic progressive pyelonephritis in man has been that so far there is little evidence in any experimental animal of a progressive disease. Kalmanson, Sommers & Guze (1965) did report progression of the lesion in the absence of bacteria in ascending enterococcal pyelonephritis in the rat but progression was defined in terms of histological alterations and there was no indication of an extension of the gross scarring or a deterioration of renal function.

LOCAL IMMUNE RESPONSE IN URINARY TRACT INFECTIONS

A local immune response to infection occurring in the kidney and bladder has also been investigated in some detail. In an early study, Cotran (1963) utilized an immunofluorescence procedure to demonstrate cells containing immunoglobulin in frozen sections of kidneys from pyelonephritic animals but more recently it has been possible to study the in vitro synthesis of proteins by pyelonephritic tissue using a tissue culture system containing $^{14}$C labelled amino-acids. Newly synthesized
immunoglobulins containing the radioactive amino acids were isolated using ion exchange chromatography, and then precipitated with specific antisera to allow the quantitation of the IgM, IgG and IgA produced in culture. With this analytical approach, it has been possible to study the sequence of antibody production during the local immune response to infection (Lehmann et al., 1968). Previous studies in this laboratory have shown that the cellular kinetics of the immune response to infection could be followed using an assay system that allowed the detection and quantitation of antibody forming cells producing antibacterial antibody (Miller & North, 1971). With modification, it has been possible to extend the investigation and demonstrate the association between areas of the pyelonephritic kidney containing antibody producing cells and histological evidence of pyelonephritis. The same procedure was used to study pyelonephritis in rabbits, which have a more defined pelvic space, where it was shown that antibody synthesized in the kidney accumulated in the urine of the pelvic space and ultimately contributed to urinary antibody (Miller & North, 1973). More recently the application of the Elisa technique to the study of the local immune response in pyelonephritis has promised an even more sensitive and specific methodology (Engvall & Perlmann, 1972).

In contrast to studies of serum antibody, most of the data concerned with the local immune response has been derived from experiments using animal models. Haematogenous induction of pyelonephritis in rabbits led to a significant increase in immunoglobulin synthesis by the infected kidney which was found to be primarily IgG but IgA was also present (Lehmann et al.,
1968). In an extension of this study, Smith, Hand and Sanford (1972) identified the newly synthesized IgA as secretory IgA. Since secretory IgA was synthesized late in the inflammatory response in pyelonephritis and could not be demonstrated during the mild pyelonephritis produced by the retrograde route, the authors considered that extensive tubular epithelial destruction was necessary for the synthesis of secretory IgA in pyelonephritis. No specific antibody activity could be shown when the IgA fraction was examined. More recent studies again using the Elisa procedure have shown that locally synthesized immunoglobulins of the IgG, IgA and IgM class all contained antibacterial antibody (Smith et al., 1974). One particularly interesting finding was that the early antibody synthesized was IgG and IgA whereas IgM antibodies appeared later in the antibody response but no increase in avidity of locally produced antibody was found as infection progressed.

Immunoglobulin and specific antibody synthesis has also been investigated in rabbits with lower urinary tract infections (Hand et al., 1970). It was possible to show an increase in immunoglobulin and specific antibody synthesis, although the increase in immunoglobulin was almost entirely IgG. The role of local synthesis of antibody in the bladder on the clearance of bacteria by the bladder was also investigated by the same authors but the clearance of bacteria appeared to be independent of local antibody synthesis suggesting that antibacterial antibody may not be an important factor in antibacterial mechanisms operating on the bladder mucosal surface.

In the clinical studies too, increased amounts of IgA and IgG
have been found in concentrated urine from 14 children (Jodal et al., 1974) with recurrent urinary tract infections, and three out of eight with a first attack of pyelonephritis. Similar results have been reported by Kaufman, Katz & McIntosh, (1970) and Uehling and Steihm (1971) and with the application of the Elisa procedure, it was possible to demonstrate antibody against E.coli K1 antigen in 11 patients, all with asymptomatic bacteriuria (Jodal et al., 1974).

Holmgren and Smith (1975) have discussed in detail several mechanisms which may account for the increase in immunoglobulin and antibody in urinary tract infection. Local synthesis in the kidney could certainly account for an increase in the level of IgA and IgG in the urine, but so could impaired tubular function. The physico-chemical characteristics of IgA may contribute to an increase in urinary immunoglobulin during infection as this immunoglobulin is known to be selectively transported into secretions. The presence of increased quantities of IgG in urine from individuals with repeated infections could be due to a cumulative effect of several factors. Firstly, an increased local synthesis of IgG as has been discussed, and possibly a specialized subclass of IgG with diminished tubular reabsorption could contribute to increased levels of IgG in the urine of individuals with pyelonephritis. Furthermore, the recent demonstration in two separate laboratories that individuals with renal infection had antibody capable of coating bacteria in the urine (Thomas, Shelekov & Forland, 1974; Jones, Smith & Sanford, 1974) in the absence of a similar serum antibody, suggests that antibody with a high specificity for antigen may be selectively transported into the urine.
IMMUNOBIOLOGY OF EXPERIMENTAL PYELONEPHRITIS

Most experimental investigations of the immunobiology of pyelonephritis have been concerned with determining whether circulating antibody or other components of the host immune response may have a protective role in preventing reinfection. Earlier studies by Sanford, Hunter & Souda (1962) and Hunter, Akins & Sanford (1964) showed that immunization with heat killed organisms did protect the host against renal damage when animals were challenged by the haematogenous route. Protection was also evident following passive immunization. Studies by Arana et al. (1965) also showed some initial protection resulting from a previous infection with E.coli when the rats were challenged by the retrograde route but with repeated challenges little protection was found. In related experiments, mice immunized subcutaneously with heat killed E.coli were protected against haematogenous pyelonephritis but were not protected against ascending or retrograde infection. There was no demonstrable protection when heat killed E.coli were injected into the bladder but the mice were protected from early endotoxic death (Montgomerie et al., 1972). Radford et al. (1974) studied active immunization against E.coli retrograde infection using heat killed organisms as the immunizing agent. Immunization did not prevent bacterial invasion of the kidney nor was the incidence or severity of the renal scarring affected despite the presence of a high titre of antibody to the somatic antigen. Fourteen days after immunization with a formalin killed vaccine, very high titres of IgM and IgG anti-K-antigen antibodies were found and although immunization reduced the severity of the disease, the frequency of renal scarring following ascending E.coli infection was not
affected. Fitzpatrick and Girard (1968) on the other hand, found that the incidence of Pr. mirabilis and Ps. aeruginosa infection of the kidney could be significantly reduced when mice were immunized by a variety of methods. More recently, Kaijser, Holmgren & Hanson (1972), using an intraperitoneal challenge in mice, was able to demonstrate the protective effect of passively transferred 19S and 7S antibodies directed against E.coli 0 and K antigens. Low avidity antibodies have been found to be less protective than antibodies of higher avidity (Ahlstedt, Holmgren & Hanson, 1974) and hyperimmunization of rabbits was shown to give rise to anti 0 and anti K antibodies which were protective against experimental haematogenous pyelonephritis (Kaijser & Olling, 1973).

Since the observation by Glynn, Brumfitt & Howard (1971) that a disproportionate number of strains of E.coli infecting the urinary tract and particularly the upper urinary tract were rich in K antigen, the protective capacity of antibodies to this antigen has been investigated in some detail. The importance of K antigen as a virulence factor has already been shown by Ewertse(n (1946), and more recently by Glynn & Howard (1970) and Howard & Glynn (1971).

Limited research has been carried out on the response of the host to K antigens of E.coli but Holmgren (1970) has shown that bacteria containing K antigen were capable of stimulating both an IgM and an IgG antibody response to the K antigen but that considerable variation in the responsiveness of individual rabbits was found. A similar variation occurred in a clinical study reported by Kaijser et al. (1971). Considerable interest
in the K antigens of \textit{E. coli} has also been raised by the possibility of using this antigen as an immunizing antigen to stimulate the production of host-protective antibodies, particularly in recurrent urinary tract infections. Earlier studies have considered only the effect of antibody against the O antigen but more recently the protective effect of antibody against the K antigens has also been evaluated. Wolberg \& DeWitt (1969) showed that K antibody and to a lesser extent, O antibody, could protect against lethal infection in mice and Kaijser, investigating this question in rabbits, found that both O and K antibody but no H antibody was protective (Kaijser et al., 1972; Kaijser \& Olling, 1973).

Brooks (1974) and co-workers also commented on the role of K antigen in retrograde pyelonephritis. Vaccination with heat killed or formalinized \textit{E. coli} 0111 or 06 prevented retrograde \textit{E. coli} infection being established when animals were challenged with the homologous strain. No protection was found against a second and more invasive strain of \textit{E. coli} 06 and since all three strains possessed the same amount of K antigen, the differences in results were not attributed to its presence. The authors did not measure anti K antibody however, and in view of the demonstrated variability of the individual animals to respond to K antigen stimulation, this conclusion may not be warranted.

One problem in producing protective immunization in clinical practice is that recurrent infections of the urinary tract are usually due to reinfection with a different strain of \textit{E. coli} than isolated on previous occasions. Thus, even if an effective
immune response to one serological strain arose, this would not be likely to be protective during subsequent infections. Kunin (1963) described an antigen common to E.coli, proteus and salmonella strains but which the common antigen was present in an immunogenic form in only three strains of E.coli (014-056-0124). Some experiments have been carried out to assess the protective effect of immunization with the common antigen but with variable results. Domingue et al. (1970) was able to show that the immune response to common antigen from salmonella species protected rabbits from pyelonephritis when challenged with Pr. mirabilis. McCabe and Greely (1973), on the other hand, found that although active immunization of mice with E.coli 014 gave rise to high titres of antibody against common antigen, that mice were not protected from a challenge by Klebsiella pneumoniae or E.coli. In a similar study, however, both active and passive immunization with another cross reactive antigen, the Re determinant of Gram negative bacilli, did afford significant protection to mice immunized with Re 595 of S.minnesota and challenged with heterologous smooth Gram negative bacilli (McCabe, 1972).

In man, antibody mediated protection against urinary tract infection has not been substantiated and cases have been reported of recurrent urinary tract infection in the face of high antibody titres (Vosti et al., 1965; Williamson et al., 1964). These studies measured only anti 0 antibodies but now that reliable methods are available for determining the level of anti K antibody, some reassessment will be necessary.

The persistent lymphocyte infiltrate found in pyelonephritis has
stimulated a number of investigations into the immunobiology of renal infection and clinical studies have shown that kidneys may frequently be sterile in pyelonephritis, a finding that suggests that the progression of active chronic pyelonephritis may not require the presence of viable bacteria (Angell et al., 1968; Aoki et al., 1969). The possibility that an autoimmune process might be involved in the pathology of the chronic pyelonephritic lesion was suggested by Holmgren's observation of an antigenic relationship between kidney tissue and certain strains of \textit{E. coli} (Holmgren et al., 1971). Kovats (1961) has also demonstrated the antigenicity of endotoxin altered tissue. When antikidney antibodies have been searched for in both patients and experimental animals with pyelonephritis, none have been found (Kalmanson & Guze, 1963; Kramer et al., 1961; Montgomerie, Kalmanson & Guze, 1969). It is possible, however, that the kidney absorbed any circulating antikidney antibody in a similar way to the absorption of antikidney antibody in glomerulonephritis (Lerner, Glassock & Dixon, 1967). Previous studies have demonstrated the local synthesis of antibody to heterologous kidney fixing antibody in nephrotoxic serum nephritis (Lehmann et al., 1969) suggesting that antikidney antibody might be produced locally in pyelonephritis. Studies have been carried out to investigate this but no newly synthesized antibody directed against normal or infected kidney could be demonstrated (Miller et al., 1970).

With the identification of two major subgroups of functionally distinct lymphocytes, attempts have been made to identify the nature, and significance of the lymphocytic infiltrate in
pyelonephritis. Coles et al. (1974) depleted rats of lymphocytes using anti-lymphocyte-globulin and neonatal thymectomy but found the course of the disease to be unaffected by the absence of T lymphocytes. No attempt, however, was made to assay the extent of the T cell depletion so that immune status of the animals at the time of challenge can only be inferred. Other investigators have attempted to disclose an immune component of the pyelonephritis lesion by manipulation of the immune response using immune suppressive agents. Both Hageman et al. (1973), using a combination of immuno-suppressive and antimicrobial therapy and Strong, Lawson and Hodges (1974), using azothioprine were able to favourably alter the course of chronic pyelonephritis.

An immunopathological component in pyelonephritis has also been suggested by the results of Beregi's (1974) examination of renal tissue with fluorescein labelled antisera to selected serum components. Immune deposits were found on the glomerular basement membrane in 7 out of 25 cases of pyelonephritis. IgG and complement were detected on the glomerular basement membrane in all seven cases with the additional finding of IgM in five cases and fibrin in one. The results confirmed the findings of Kobayashi (1966) who performed renal biopsies on 14 children with pyelonephritis and found IgG and complement deposition in three cases.

In the first table, a summary of the more recent reports related to the immunobiology of pyelonephritis have been arranged in chronological order.
TABLE 1. Chronological summary of representative papers which have contributed to the recent literature on the immunobiology of experimental renal infections.
<table>
<thead>
<tr>
<th>Subject</th>
<th>Animal Host</th>
<th>Route of Infection and Bacterial Species</th>
<th>Lead Author and Reference</th>
<th>Synopsis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunity in the pathogenesis of haematogenous pyelonephritis.</td>
<td>Rat</td>
<td>Haematogenous infection with E.coli, Kl.pneumoniae and Pr. mirabilis.</td>
<td>Sanford (1962) J.exp. Med. 115, 383.</td>
<td>Immunity was shown to have a significant role in the pathogenesis of pyelonephritis only in the absence of obstruction.</td>
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<tr>
<td>Urinary immunoglobulin excretion in urinary tract infection.</td>
<td>Rat</td>
<td>Urethral installation of E.coli.</td>
<td>Uehling (1972) Invest. Urol. 9, 408.</td>
<td>A protective role for urinary IgG in the rat was not found.</td>
</tr>
<tr>
<td>Amount and avidity of antibody in relation to protective capacity.</td>
<td>Mice</td>
<td>Intraperitoneal injection with E.coli.</td>
<td>Ahlstedt (1973) Immunology 24, 191.</td>
<td>Protective capacity of the immune response related to antibody level.</td>
</tr>
<tr>
<td>K antigen of E.coli and urinary tract infection.</td>
<td>Clinical study</td>
<td>Natural infection.</td>
<td>Kaijser (1973) J. infect. Dis. 127, 670.</td>
<td>K antigen was found more often and in greater amounts in urinary tract pathogens compared with random strains of E.coli.</td>
</tr>
<tr>
<td>The antibody response and its protective capacity.</td>
<td>Rabbit</td>
<td>I.V. challenge with E.coli. and ligation of the ureter.</td>
<td>Kaijser (1973) J. infect. Dis. 128, 41.</td>
<td>Antibodies to 06 and KL3 antigen but not H1 were protective against hematogenous pyelonephritis.</td>
</tr>
<tr>
<td>Immunization with common antigen.</td>
<td>Mice</td>
<td>Intravenous challenge with K1.pneumoniae or E.coli.</td>
<td>McCabe (1973) Infect. Immunity 7, 386.</td>
<td>High titres of antibody to common antigen failed to enhance resistance.</td>
</tr>
<tr>
<td>Local immune response to pyelonephritis.</td>
<td>Rabbit</td>
<td>Direct inoculation of E.coli into the kidney.</td>
<td>Miller (1973) J. infect. Dis. 128, 195.</td>
<td>Antibody forming cells in the kidney were confined to areas with histological evidence of pyelonephritis. Antibody also accumulated in the renal pelvis.</td>
</tr>
<tr>
<td>Study Title</td>
<td>Organism</td>
<td>Method Description</td>
<td>Reference</td>
<td>Key Findings</td>
</tr>
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<tr>
<td>Role of T cells in experimental pyelonephritis.</td>
<td>Rat</td>
<td>Retrograde infection with E. coli.</td>
<td>Coles (1974) Clin.exp. Immunol. 16, 629</td>
<td>Renal scarring and the histological appearance of the lesions were unaffected by presence or absence of T cells.</td>
</tr>
<tr>
<td>Immunization and ascending pyelonephritis.</td>
<td>Rat</td>
<td>Retrograde infection with E. coli.</td>
<td>Radford (1974) J.Path. 112, 169</td>
<td>High titre antibody did not prevent invasion of the kidney or affect the severity of scarring.</td>
</tr>
<tr>
<td>Immunologic mechanism as a factor in the genesis of chronic pyelonephritis.</td>
<td>Rat</td>
<td>Direct inoculation of heat killed E. coli.</td>
<td>Strong (1974) Invest. Urol. 11, 479</td>
<td>Histological changes identical with chronic pyelonephritis prevented by the administration of azathioprine.</td>
</tr>
</tbody>
</table>
In this section the relationship between bacterial infection and the pathology of acute and chronic renal infection has been examined. Two experimental models of pyelonephritis were developed in the rat, both of which displayed many of the characteristic features of the disease in man. In the first, pyelonephritis was induced by the direct inoculation of bacteria into the kidney; this model proved to be particularly useful in the study of chronic infection. In the second model, an infection of the kidney was established with *E. coli* using a retrograde challenge without the necessity of trauma, forced reflux or ureteric obstruction. A method for the determination of glomerular filtration rate in rats was also developed and this analysis allowed the estimation to be carried out following the injection of $^{51}$Cr.EDTA and the sampling of a single blood specimen. The procedure was used in a study of the effect of chronic infection on renal function. Finally a previously unreported host-parasite relationship has been disclosed and named "acquiescent renal infection". Data has been presented describing a host-parasite relationship in which active and persistent infection is not associated with pathologic changes.
CHAPTER 2

EXPERIMENTAL PYELONEPHRITIS: A METHOD FOR INDUCING PYELONEPHRITIS IN THE RAT BY THE DIRECT INOCULATION OF E.COLI INTO THE KIDNEY
INTRODUCTION

One of the problems in studying acute and chronic experimental pyelonephritis has been to induce a renal infection that resembles the natural disease in man. Both the upper and lower urinary tract of most experimental animals are extremely resistant to infection and it has been necessary to obstruct the urinary tract (Guze & Beeson, 1956), produce a focal injury with obstruction (Roche et al., 1958) or manipulate the kidney with firm massage (Braude, Shapiro & Siemierski, 1955) before an infection can be established. Models utilizing these principles have been used in investigations into the pathogenesis of renal infection.

Although it has been possible to induce pyelonephritis in an unobstructed renal tract with an intravenous injection of Streptotoccus faecalis (Guze, Goldner & Kalmanson, 1961), this has not been achieved with E. coli. A new method of inducing pyelonephritis by the direct inoculation of bacteria into the kidney was developed to provide an experimental model for the present studies. The bacteriological and histological characteristics of the model are described in this chapter.

MATERIALS AND METHODS

Animals

Female white Wistar rats from a random bred strain were used.

Bacterial strain

A strain of E. coli 075 previously used in a number of studies of experimental pyelonephritis and obtained originally from
Dr. J.P. Sanford's laboratory in the Department of Internal Medicine at Southwestern Medical School, Dallas, Texas, was used in these experiments. The culture was maintained as a freeze dried preparation and a vial reconstituted every 14 days. After an overnight broth culture, subcultures were made onto blood agar and single colonies used to inoculate a further broth culture. This culture was washed in sterile saline and then stored at -10°C in 14 aliquots. One aliquot was plated on blood agar daily and provided a culture for the following day's experiments.

**Induction of pyelonephritis**

The kidney was first exposed and brought to the exterior skin surface. The bacterial inoculum was introduced into three sites in the exposed kidney using a fine glass capillary formed from a standard 1 mm. O.D. micro-haematocrit capillary tube, pulled out to give a fine tip with an external diameter of 0.25 mm.

The capillary was pushed through a rubber stopper in the end of a glass tube and movement of the contents of the capillary controlled with a rubber bulb. One μl of the diluted broth culture containing $3 \times 10^5$ organisms was injected centrally and into each pole deep in the kidney parenchyma. The glass capillary was inserted into the kidney with a firm thrust and the bacterial inoculum expelled as the capillary was withdrawn (Figure 1).
FIGURE 1.

Experimentally induced pyelonephritis in the rat. The kidney was exposed and the bacterial inoculum introduced under direct vision through a glass micropipette. The white spot on the surface of the kidney is a reflection artifact.
Determination of the distribution of bacteria within the kidney

Animals were killed in a CO₂ chamber and both left and right kidneys removed using a sterile procedure and then decapsulated in a sterile petri dish. The medulla and attached papilla was removed from each kidney using a hollow cylindrical blade of 8 mm. internal diameter. Cortical tissue was readily distinguished from medullary tissue in the kidney core and was removed with a sharp blade. The papilla was then removed from the medullary segment. The crescent of renal cortical tissue remaining after removal of the medullary core was trimmed to remove residual medullary tissue and added to the cortical tissue previously removed from the extremes of the medullary core (Figure 2).

Each piece of tissue was weighed and homogenized in physiologically normal saline using a teflon tissue homogenizer. Agar pour-plates of ten-fold dilutions of the homogenate were made to obtain the bacterial count per gram of tissue. Pour-plate dilutions of homogenates of spleen, liver, peri-renal nodes, and lumbar nodes were also carried out to determine the bacterial content. Quantitative bacterial counts were performed on aspirated urine from the bladder. Blood samples previously taken by heart puncture at specified intervals were cultured in a biphasic culture medium bottle and examined for growth, daily, over a period of seven days.

Biochemical analysis

Blood samples were taken by heart puncture at death and the serum analysed, using a Technicon autoanalyser, for urea,
FIGURE 2.

Dissection of the kidney to give clearly defined samples of cortical, medullary and papillary tissue. A hollow cylindrical blade was used to remove a core of renal tissue as illustrated on the left hand side. The central section illustrates the region of the kidney sampled. Cortical tissue was readily distinguished from medullary tissue in the core of kidney tissue (lower, right) and was removed with a sharp blade. The papilla was then removed from the medullary segment. The crescent of renal cortical tissue remaining after removal of the medullary core was trimmed to remove residual medullary tissue and added to the cortical tissue previously removed from the outer aspects of the medullary core.
sodium, CO₂, protein, bilirubin and creatinine.

Histology
Tissue for histological examination was placed directly into a 0.1% solution of cetylpyridinium chloride in 10% formaldehyde. After routine processing and cutting, sections were stained with haematoxylin and eosin.

Experimental design
A unilateral pyelonephritis was produced in animals in groups of four to six and the animals sacrificed at various intervals. The bacterial distribution in the kidney and the bacterial numbers present in the spleen, liver, blood, urine and peripheral lymphoid tissue were determined. Other animals were sacrificed and blood samples were taken for biochemical and histological studies.

RESULTS
Gross changes in infected kidneys
Direct inoculation of bacteria into the kidney with a glass micro-pipette resulted in a consistent and reproducible infection in the renal parenchyma. All of the animals challenged showed during the next 100 days histological and bacteriological evidence of renal infection, and in most cases, all three sites inoculated produced similar wedge-shaped lesions originating in the medullary region and extending to the cortex. Both the gross and histological changes were confined to the lines of inoculation (Figure 3). As the infection progressed, pitted and scarred areas characteristic of chronic pyelonephritis in the human developed (Figure 4).
FIGURE 3.

Haematoxylin and eosin stained section of a pyelonephritic kidney. The histological changes and cellular infiltrate are confined to the line of inoculation.

FIGURE 4.

Gross appearance of a rat kidney with experimentally induced pyelonephritis. Typical scarring seen 6 months after the initiation of infection.
Histopathology

The earliest changes in the infected kidneys were found three days after challenge with the appearance of areas of acute inflammation confined to the line of inoculation but extending down into the papilla (Figure 5). At five days, an acute interstitial inflammation was found that extended from the calyceal epithelium to the capsule (Figure 6). Patchy tubular necrosis, many tubular pus casts, and some proteinaceous tubular casts were also present. Polymorphonuclear neutrophils were also present in the interstitial infiltrate but were never found as the predominant inflammatory cell in the acute lesion. Seven days after infection, the round cell infiltrate consisted of small lymphocytes and plasma cells which by ten days had become a major component of the inflammatory infiltrate (Figure 7). In addition to numerous tubular casts containing pus and proteinaceous material, some early interstitial fibrosis was seen. The glomeruli were unaffected but some of the small arteries in the area of involvement showed minor intimal and medial proliferation.

As the infection progressed, the presence of a marked lymphocytic infiltrate in the kidney became a feature of the histopathology although leucocyte casts could still be found in the distal and collecting tubules up to six months after the initial challenge. Cortical depressions (Figure 8) with glomerular crowding and interstitial fibrosis (Figure 9) were first seen one month after challenge but when a further group of animals were examined six and nine months later, there was no evidence to suggest progression of the lesions.
FIGURE 5.

Wedge-shaped area of inflammation in the papilla of an animal with experimental pyelonephritis. The tubules are filled with polymorphonuclear leucocytes.

FIGURE 6.

Extension of the inflammatory lesion to involve the cortex.
Predominant lymphocytic infiltrate in the kidney of animals with experimental pyelonephritis. Although lymphocytes accounted for most of the cells in the cellular infiltrate, polymorphonuclear leucocyte casts continued to be found at all stages of the disease.

FIGURE 8.

Histological section from a kidney with healed pyelonephritis showing cortical depression and glomerular crowding.
FIGURE 9.

Glomerular crowding and interstitial fibrosis in the kidney of an animal with chronic experimental pyelonephritis.
Quantitation of the pathological changes

In several of the studies that follow, it was necessary to
quantitate the pathological changes in the kidney to assess
the effects of a number of biological manipulations. The
gross changes and degree of scar formation were assessed on a
0-10 scale with each point on the scale representing one tenth
of the surface of the kidney (Figure 10). In some experiments,
the term "residual infection" appears. This term was used to
indicate foci of incompletely healed lesions within scarred
areas. The histopathological assessment of the degree of
renal damage was also scored on a 0-10 scale. Ten represented
lesions involving 25% or more of the section. Lesser degrees
of damage were scored on a 0-10 scale where each unit
represented 2.5% of the total area of the kidney section
(Figure 11). The degree of lymphocytic infiltration was
also quantitated in some cases (Figure 12).

Bacteriology

The number of bacteria in the kidney, spleen, liver, blood,
urine and lymph nodes were determined at intervals from 5
minutes to 100 days after challenge. With $3 \times 10^5$ organisms
approximately 30% of the inoculum remained in the kidney 5
minutes after challenge and bacteria were also recovered from
the liver, blood, spleen, and peri-renal nodes immediately
after challenge. After an initial drop the number of bacteria
in the cortex, medulla and papilla of the kidney increased
sharply over the first 24 hours and remained constant for about
5 days. Over the next 30 days the bacterial content of the
kidney, particularly the cortex and medulla, declined, but
Quantitation of the degree of scar formation. Assessment was made on a 0-10 scale with each point on the scale representing one tenth of the surface of the kidney. The kidney illustrated in (a) was scored as 6, (b) as 4, (c) as 2 and (d) as 1.
FIGURE 11.

Histopathologic assessment of the degree of renal damage. On the scale used, 10 represented lesions involving 25% or more of the area of the section. Lesser degrees of damage were scored on a 0-10 scale where each unit represented 2.5% of the total area of the kidney section. The kidney section (x8) illustrated in (a) was scored as 10 and (b) as 2. (c) and (d) are photomicrographs taken under lower power magnification (x40).
Quantitation of the lymphocytic infiltrate in the kidney. An arbitrary 0-10 scale was used. In the figure, (a) represented an infiltrate scored as 8, (b) as 6, (c) as 4 and (d) as 2.
increased again over the period between 30 and 100 days (Figure 13). The weight of the tissue sampled is given in Table 2. The results confirmed previous findings that bacteria persist for extended periods in the kidney during certain types of experimental renal infections in the rat (Miller and North, 1971). In contrast to the kidney, bacteria are progressively cleared from the blood, spleen and liver although bacteria continued to be found in small numbers in both the renal and lumbar nodes later in the course of the disease. The bacterial content of the urine in challenged animals remained consistently high and an average urine bacterial count of $1.5 \times 10^4$ organisms/ml was found throughout the study.

**Numbers of bacteria required to establish infection**

Ten-fold dilutions of the bacterial inoculum were prepared and unilateral pyelonephritis induced in a further series of animals which were killed eight days later. Four rats were used for each dilution. Infection was uniformly established in all kidneys challenged with an inocula from $3 \times 10^5$ to $3 \times 10^2$. No gradient in the number of bacteria recovered from the kidney after challenge with the various inocula was found and histological changes were also remarkably uniform (Table 3).

**Biochemistry**

The blood biochemistry of the pyelonephritic animals showed no major changes over the interval studied (Table 4). Serum urea values remained normal although a marginal increase in
FIGURE 13.

Distribution of bacteria in the spleen, kidney, lymph nodes and liver in animals with *E.coli* pyelonephritis. Animals were studied at intervals from 5-100 days after challenge with a total of $3 \times 10^5$ *E.coli* 075 inoculated directly into the kidney. Both the liver and the spleen were sterile 7 days after infection whereas bacterial infection persisted in the kidney for extended periods.
TABLE 2. Comparative weights of cortex, medulla and papilla used for bacterial counts.
<table>
<thead>
<tr>
<th></th>
<th>Cortex</th>
<th>Medulla</th>
<th>Papilla</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of samples</td>
<td>148</td>
<td>148</td>
<td>148</td>
<td>74</td>
<td>74</td>
</tr>
<tr>
<td>Mean wgt. (mg.)</td>
<td>575</td>
<td>155</td>
<td>24</td>
<td>381</td>
<td>559</td>
</tr>
<tr>
<td>Std. Deviation (mg.)</td>
<td>133</td>
<td>58</td>
<td>12</td>
<td>97</td>
<td>200</td>
</tr>
</tbody>
</table>
TABLE 3. Relationship of the number of bacteria inoculated directly into the kidney to the incidence of pyelonephritis in the rat.

Ten-fold dilutions of the bacterial inoculum were prepared and unilateral pyelonephritis induced in a series of animals which were killed 8 days later. Six rats were used for each dilution. Infection was uniformly established in all kidneys challenged with an inoculum of $3 \times 10^5$ to $3 \times 10^2$ organisms. No gradient in the number of bacteria recovered from the kidney after challenge with the various inocula was found and the histological changes were also remarkably uniform.
<table>
<thead>
<tr>
<th>Number of organisms inoculated</th>
<th>No. of animals</th>
<th>Bacteria per gram of kidney</th>
<th>Bacteria per ml of urine</th>
<th>Occurrence of pyelonephritis</th>
</tr>
</thead>
<tbody>
<tr>
<td>$3 \times 10^5$</td>
<td>6</td>
<td>$7.7 \times 10^5$</td>
<td>$5.9 \times 10^3$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.9 \times 10^6$</td>
<td>$8.9 \times 10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$9.1 \times 10^5$</td>
<td>$6.6 \times 10^4$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$9.9 \times 10^4$</td>
<td>$2.0 \times 10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$7.4 \times 10^5$</td>
<td>$4.6 \times 10^3$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$6.4 \times 10^2$</td>
<td>$4.0 \times 10^1$</td>
<td>+</td>
</tr>
<tr>
<td>$3 \times 10^4$</td>
<td>6</td>
<td>$6.5 \times 10^4$</td>
<td>$1.2 \times 10^2$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$6.9 \times 10^3$</td>
<td>$1.7 \times 10^3$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$5.6 \times 10^3$</td>
<td>$3.0 \times 10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4.7 \times 10^5$</td>
<td>$1.6 \times 10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$7.2 \times 10^5$</td>
<td>$5.6 \times 10^2$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$5.2 \times 10^5$</td>
<td>$2.6 \times 10^5$</td>
<td>+</td>
</tr>
<tr>
<td>$3 \times 10^3$</td>
<td>6</td>
<td>$6.2 \times 10^2$</td>
<td>$6.0 \times 10^1$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$3.2 \times 10^5$</td>
<td>$2.2 \times 10^6$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$3.0 \times 10^5$</td>
<td>$1.9 \times 10^7$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.1 \times 10^6$</td>
<td>$6.3 \times 10^3$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.4 \times 10^5$</td>
<td>$1.0 \times 10^5$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$9.0 \times 10^5$</td>
<td>$4.0 \times 10^5$</td>
<td>+</td>
</tr>
<tr>
<td>$3 \times 10^2$</td>
<td>6</td>
<td>$8.5 \times 10^5$</td>
<td>$3.8 \times 10^5$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2.2 \times 10^6$</td>
<td>$1.7 \times 10^6$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$6.3 \times 10^6$</td>
<td>$0$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.5 \times 10^4$</td>
<td>$2.9 \times 10^6$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$8.8 \times 10^4$</td>
<td>$1.3 \times 10^4$</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$8.2 \times 10^4$</td>
<td>$2.3 \times 10^4$</td>
<td>+</td>
</tr>
<tr>
<td>$3 \times 10^1$</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE 4. Selected biochemical values measured during the course of experimental pyelonephritis.

Although the animal studies had only a unilateral pyelonephritis, their serum biochemistry was followed during the course of the disease to determine whether any changes in blood constituents, particularly urea and creatinine indicating failing renal function, could be found. No major changes were found with advancing disease.
<table>
<thead>
<tr>
<th>Days after Infection</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>7</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine mg/100ml</td>
<td>0.8</td>
<td>0.8</td>
<td>0.6</td>
<td>0.7</td>
<td>0.6</td>
<td>0.7</td>
<td>0.7</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Serum urea mg/100ml</td>
<td>40</td>
<td>45</td>
<td>47</td>
<td>39</td>
<td>48</td>
<td>45</td>
<td>38</td>
<td>40</td>
<td>44</td>
<td>43</td>
<td>43</td>
<td>39</td>
<td>37</td>
</tr>
<tr>
<td>Serum sodium mEq/L</td>
<td>146</td>
<td>150</td>
<td>148</td>
<td>150</td>
<td>149</td>
<td>145</td>
<td>149</td>
<td>144</td>
<td>141</td>
<td>142</td>
<td>147</td>
<td>144</td>
<td>146</td>
</tr>
<tr>
<td>Serum protein g/100ml</td>
<td>5.8</td>
<td>5.8</td>
<td>6.3</td>
<td>5.6</td>
<td>6.0</td>
<td>8.1</td>
<td>6.8</td>
<td>6.5</td>
<td>6.3</td>
<td>6.5</td>
<td>6.5</td>
<td>6.5</td>
<td>6.4</td>
</tr>
<tr>
<td>Serum bilirubin mg/100ml</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
<td>0.2</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
<td>Serum CO₂ mEq/L</td>
<td>18</td>
<td>17</td>
<td>21</td>
<td>25</td>
<td>23</td>
<td>21</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>21</td>
<td>23</td>
<td>22</td>
<td>24</td>
</tr>
</tbody>
</table>
serum creatinine occurred over the latter stages of the disease. This was to be expected with the induction of unilateral pyelonephritis.

**DISCUSSION**

Induction of experimental pyelonephritis in the rat by the direct inoculation of bacteria into the kidney produced an infection similar to the wedge-shaped lesion seen in other experimental models (Beck et al., 1961-62; Braude, Shapiro and Siemienski, 1959) and in man.

Resolution of the macroscopic acute inflammatory lesions had begun by ten days and healed cortical scars were found in all animals studied from 30 days onwards. Over the next three months, the scars showed considerable contracture confined to the original areas of infection but the pathological lesions were self-limiting and there was no evidence of superimposed retrograde infection. The histological appearances were similar to those seen in human pyelonephritis and wedge-shaped areas of interstitial inflammation developed with a base in the cortex and the apex extending down into the medulla. Similar appearances have been noted in staphylococcal pyelonephritis in animals with intra-renal hydronephrosis (Beck et al., 1961-62).

Leucocyte tubular casts were seen after five days and infected kidneys showed extensive acute interstitial inflammation confined to the pyelonephritic lesion. During the early stages of infection the predominant cells seen in the inflammatory lesion were small lymphocytes, mononuclear cells and plasma
cells. Polymorphonuclear leucocytes were also present in the interstitium but were concentrated in the leucocyte casts in the tubular lumen. Braude et al. (1955) also found the great majority of cells comprising the inflammatory infiltrate to be mononuclear cells and consider that the polymorphonuclear cells accumulate in the tubular lumen on account of their greater mobility. Leucocyte casts were not seen in spontaneous haematogenous pseudomonas pyelonephritis but in this case the morphology was that of a necrotizing interstitial inflammatory reaction followed eventually by necrosis of the tubular cells (Teplitz et al., 1964). Relman (1960) has stressed the importance of the leucocyte cast in the diagnosis of chronic pyelonephritis while other data from pyelonephritis induced with Streptococcus faecalis suggest that leucocyte casts result from an acute inflammatory process (Breslau et al., 1964).

Our study, which is supported by the bacteriological data, confirms the former view that leucocyte casts continue to be formed with a continuous low grade infection. In the resolving and chronic pyelonephritic lesions, although the inflammatory infiltrate consisted primarily of lymphocytes, plasma cells and reticulum cells, leucocyte casts were regularly demonstrated.

In contrast to other experimental models where hyalinization of the glomeruli has been noted (Cotran, 1963), the glomeruli in the current model remained normal even when the pyelonephritic scars showed large numbers of glomeruli clustered together because of tubular loss. This finding is consistent with other reports on experimental pyelonephritis in rats where the glomeruli persisted in the scars of the chronic pyelonephritic
kidney with only slight morphological changes (Heptinstall, Kissane & Still, 1963). In man, chronic pyelonephritis is characterized histologically by dilated atrophic tubules containing colloid casts, interstitial fibrosis and a lymphocytic infiltrate. These histological features have been found in our model in the rat although the lymphocytic infiltrate remained the predominant feature. The consistent presence of leucocyte casts in the present model together with persistent renal infection suggested a continuing pyelonephritic process. In this model, however, there was no evidence of a progressive lesion involving lateral spread within the kidney. Progression has been noted in experimental pyelonephritis induced with *klebsiella* species where the lesion was considered to develop in proportion to the duration of infection and was characterized by the progressive peripheral extension of chronic inflammation in both cortex and medulla (Anderson & Jackson, 1962).

The distribution of bacteria within the pyelonephritic animal and in particular within the kidney, confirmed the ability of infecting organisms to persist for extended periods in renal tissue (Guze et al., 1961). A transient bacteraemia that followed the injection of bacteria into the kidney was cleared within 30 minutes and the small number of bacteria that lodged in the spleen and liver were progressively eliminated during the first week. Bacteria were evenly distributed throughout the kidney 5 minutes after inoculation but the bacterial content of the cortex dropped most sharply during the next 30 minutes. Multiplication, however, occurred in the cortex and
24 hours after infection, the bacterial content of cortex, medulla and papilla were similar. As the infection progressed, the bacterial content of the papilla was generally higher than that of cortex and medulla. The difference between the bacterial content of the cortex and medulla was not as pronounced as other reports have suggested (Guze et al., 1961). Similar results confirming the even cortical and medullary bacterial content were found in enterococcal pyelonephritis when the papilla was separated from the cortical and medullary tissue (Miller & North, 1966).

Although there is good evidence that the medulla is more susceptible to infection when the nephrons are obstructed, (Freedman & Beeson, 1958) the unobstructed medulla is relatively resistant to infection and enormous numbers of bacteria may be present in both the papilla and the pelvis of the kidney without establishing further lesions. The widely accepted question of preferential bacterial persistence in the medulla is less convincing when detailed methods are used to dissect the kidney in experimental models.

An explanation for the ease with which direct inoculation of bacteria into the kidney induces pyelonephritis is not readily apparent. Intra-renal hydronephrosis with its attendant susceptibility to infection resulting from the insertion of the micro-pipette seems most likely although in other studies the infection rate has shown only a marginal increase when obstruction has been incomplete (Guze & Beeson, 1958). Complete obstruction, however, resulted in infection almost without exception and although in the present case, the obstruction is
confined to the needle track, sufficient alteration of urine flow must occur to lower the resistance of renal tissue to infection.

A consistent infection was induced even when the inoculum was diluted considerably and less than $3 \times 10^2$ bacteria introduced into the kidney. This suggests that very few organisms are required to establish infection in the presence of tissue injury and in similar experiments carried out in rabbits it has been shown that less than ten organisms were required to establish infection when introduced into the medulla (Freedman & Beeson, 1958).

Use of the direct inoculation to induce pyelonephritis has a number of advantages over experimental models currently available. Many of these utilize some form of extra-renal obstruction which results in a diffuse pyelonephritic process involving the entire renal substance. In contrast, the method described induced a pyelonephritic lesion confined to the specific area of the kidney challenged with bacteria and produced a renal lesion with many features of the disease in man.
CHAPTER 3

EXPERIMENTAL PYELONEPHRITIS: THE SPECIFICITY OF THE INFLAMMATORY LESION
In Chapter 2 a method was described for the induction of experimental pyelonephritis by the direct inoculation of bacteria into the surgically exposed kidney using a fine glass capillary. In the experiments that follow, the procedure has been used to initiate pyelonephritis in several hundred animals with a success rate of approximately 95%. Prat has drawn my attention to his independent observations in the Czechoslovakian literature in which similar results were found (Prat & Hatala, 1973).

As most methods of inducing infection involve some damage to the kidney to form a focus around which infection is established, the resultant histological changes may not represent the response of the host to infection alone. In the previous chapter the specificity of the histopathological changes associated with pyelonephritis were not differentiated from the non-specific inflammatory response and subsequent reparative changes which would be produced by any form of trauma.

In this chapter, it has been shown that there are quantitative and qualitative changes in the cellular infiltrate specifically associated with active infection which are quite distinct from, and additional to, the inflammatory infiltrate and reparative process common to tissue injury.
MATERIALS AND METHODS

Animals
Adult male, random bred Wistar rats weighing 250 g. were used in these studies.

Renal Injury
Pyelonephritis was induced as previously described in Chapter 2 by the direct inoculation of E.coli 075 into one kidney of each animal, the other serving as its control. A glass micro-pipette was used to introduce a total of \(3 \times 10^5\) organisms into three separate sites.

Killed bacterial antigen - A broth culture of E.coli 075 prepared as for the active infection was boiled for two hours and then washed three times before being resuspended in phosphate buffered saline. Procedures similar to those described above for active infection were used to introduce a similar number of killed bacteria into the kidney.

Thermal injury - A length of 22 gauge nichrome wire was heated in a bunsen flame and immediately plunged into the exposed kidney so that the point approached the papillay region. This was repeated at two other sites to produce three separate areas of thermal burn in each kidney.

Residual irritant - Two pieces of non chromic 4/0 catgut were passed through each pole of the left kidney on a cutting needle and remained in situ for the duration of the experiment.

Carrageenin - 0.5 ml of 1% sterile carrageenin was injected into three sites in the kidney through a #26 gauge needle.
Two groups, each of five animals were used to assess the pathological changes with each type of renal injury. One group was killed 7 days after injury and the other after 30 days.

Histological Techniques
The normal and treated kidneys from each animal were fixed in 10% neutral formalin for one week after removal. After fixation and processing serial 6 μm sections were cut through the entire kidney. After mounting, groups of sections from each block were stained with haematoxylin and eosin, and a series of connective tissue, glycoprotein and mucopolysaccharide specific stains, including the Masson trichrome, (Masson, 1929), colloidal iron (Mowry, 1963), alcian blue/periodic-acid Schiff (PAS) and alcian blue/aldehyde fuchsin stains (Spicer, Horn & Leppi, 1967).

RESULTS
All the experimental kidneys showed some evidence of damage both macroscopically and histologically.

Macroscopically, the most severe lesions were seen in the infected kidneys as deep cortical scars, whereas in the other groups small pitted areas at the site of inoculation were the only evidence of trauma.

Microscopically, there were qualitative and quantitative differences in the cellular infiltrate in the pyelonephritic kidneys as compared with the other forms of renal trauma.

In the pyelonephritic kidney, the affected area extended from the line of inoculation to involve the adjacent renal
parenchyma, producing zones of damage up to 4 mm. wide. In the case of the sterile trauma, however, the parenchymal damage was confined to the immediate inoculum site and was generally less than 0.2 mm. wide and never exceeded 0.5 mm. Examination of sections stained by the haematoxylin and eosin technique showed that the cellular infiltrate was much more intense in the infected animals, and consisted primarily of lymphocytes in both the 7 and 30 day lesions. Neutrophils were only seen in pyelonephritic animals, but were never the predominant cell, and in some cases were only found in the tubular lumen. The most striking difference seen on H & E sections of the various groups was the presence of large numbers of plasma cells in the pyelonephritic animals. In some cases these cells were aggregated into lymphoid follicles. These cellular changes were present in animals, both at 7 and 30 days after infection, but interstitial fibrosis was more apparent in the 30-day lesions. These comparisons are tabulated in Table 5. In Figure 14 the histological basis upon which the quantitative assessment was made is shown.

When sections which had been stained with the connective tissue and mucopolysaccharide stains were examined, several other features which were specific to the infected lesions were noted.

Whereas tubular cell necrosis, thickening of the PAS staining basement membrane and tubular dilatation were common to the various traumatic procedures, the infected lesions alone contained focal accumulations of large histiocytes 14-20 μm in diameter which stained green with the Masson trichrome
TABLE 5. Histopathological changes in the kidney following renal infection and various forms of sterile injury.
<table>
<thead>
<tr>
<th>Degree of Renal Damage (histological assessment)</th>
<th>Infection 7 days</th>
<th>Infection 30 days</th>
<th>Killed bacteria 7 days</th>
<th>Killed bacteria 30 days</th>
<th>Cat gut 7 days</th>
<th>Cat gut 30 days</th>
<th>Thermal burn 7 days</th>
<th>Thermal burn 30 days</th>
<th>Carrageenin 7 days</th>
<th>Carrageenin 30 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>+++++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cellular Infiltrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils</td>
<td>++</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>+++++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>+++++</td>
<td>++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Histiocytes containing PAS staining material</td>
<td>+++</td>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Histopathological changes. The degree of renal damage was assessed on a five plus scale.

(a) is a photomicrograph (x40) H/E of a pyelonephritic lesion which was scored as five plus.

(b) is a lesion from a thermal injury which was scored as one plus.

The lymphocytic infiltration was assessed on a similar basis.

(c) was a typical infiltrate scored as five plus, and

(d) as one plus.
procedure, blue with the colloidal iron stain and purple or pink with the alcian blue/PAS combination, indicating that they contained glycoprotein and/or acid mucopolysaccharide material.

These cells which were specific to the infected lesions were found either in clumps in the area of lymphocytic infiltration, or as rosettes around the periphery of degenerating tubules (see also Chapter 16).

In the carrageenin granulomata, collections of macrophages densely staining blue with the colloidal iron stain were seen, but the morphology, distribution and staining characteristics of these cells were markedly different from those of the histiocytes in the infected areas. Peritubular rosettes were seen only in the infected lesions. Numerous foreign body giant cells were found around the buried catgut sutures, but there were no histiocytes of the type seen in the pyelonephritic lesions.

DISCUSSION

Many of the histopathological changes seen in pyelonephritis can be produced by various forms of renal trauma (Heptinstall, 1969) and previous reports have emphasized the difficulty in distinguishing the cellular response to active infection from the response to tissue injury (Freedman et al., 1961; Freedman, 1966).

The current studies have demonstrated, however, that there are distinct qualitative and quantitative differences in the histopathologic response to infection and other forms of trauma.
Although scarring was common to all the experimental groups, it was most severe in the kidneys of infected animals. Infection also invoked a characteristic cellular response and the lymphocytic infiltrate containing sheets of plasma cells was specifically associated with infected areas and was not seen in the other forms of renal trauma.

Collections of histiocytes containing glycoprotein and mucopolysaccharide material were found in the interstitium, and as characteristic rosettes around the degenerating renal tubules, only in the kidneys of animals with active infection. These cells have also been described by Tan & Heptinstall (1969) and Cotran (1969) who both considered it likely that bacteria played some role in the aggregation of PAS positive interstitial histiocytes. The significance of observations that histiocytes may also form rosettes around renal tubules is not known, but as this phenomenon was only seen in pyelonephritic animals, it may be useful in implicating a bacterial aetiology in the differential diagnosis of interstitial nephritis.

Although these latter results were of considerable interest, the most important feature of the present study was to establish that the inflammatory response of the host to renal infection was markedly different from the response to sterile injury to the kidney.
CHAPTER 4

DETERMINATION OF GLOMERULAR FILTRATION RATE IN THE RAT
USING $^{51}$Cr-EDTA AND A SINGLE BLOOD SAMPLE
Conventional methods for measuring glomerular filtration rate (G.F.R.) are not suitable for small laboratory animals and single dose injection methods that use radioactive materials and avoid the problem of urine collections have been developed. Even these procedures do have disadvantages and the experimental procedures may be detailed and elaborate.

Recently Bryan et al. (1972) proposed a new formula for calculating the G.F.R. which eliminated the lengthy calculations involved using the external body counting method but required a knowledge of the distribution volume of the isotope for the experimental animal being used. In this experiment the distribution volume for $^{51}$Cr-EDTA in the rat was determined and Bryan's formula used to calculate the G.R.F. from a single blood sample following the administration of $^{51}$Cr-EDTA.

A comparison of this method with the results obtained using the standard two-compartment external body counting procedure has shown that the determination of the G.F.R. using single blood sampling is a convenient and reliable analysis of this function in the rat.
MATERIALS AND METHODS

Determination of G.F.R. using the two compartment external body method

The method of Johnson and Gollan (1968) was used to determine the G.F.R. using the two compartment method of Sapirstein et al., 1955. A scintillation probe, connected to a Picker ratemeter and a chart recorder, was placed over the head and neck region of the rat and background radioactivity recorded on the chart. At zero time a weighed amount (about 10 μCi) of $^{51}$Cr-EDTA solution was injected into the femoral vein. External body counting was continued for 70 - 90 minutes until the level of radioactivity was decreased to near background. Ten minutes after the injection of isotope a 200 μl blood sample was collected from the tail vein into capillary tubes and the plasma separated. 50 μl of the plasma was then counted in an Echo gamma counter and the concentration of isotope in the plasma determined from a standard preparation of the stock $^{51}$Cr-EDTA. The plasma count was then used to calibrate the external body counts recorded by the probe and the decay curve obtained plotted on semi-logarithmic graph paper. The glomerular filtration rate was then calculated according to the method of Sapirstein (1955).

Determination of distribution volume

Nine Wistar rats of both sexes with weights ranging from 220 - 350 g. were bilaterally nephrectomized. Twenty-four hours later 10 μCi of $^{51}$Cr-EDTA was injected into the femoral vein of each rat and plasma samples taken subsequently from the tail at 30 minute intervals for up to 7½ hours. By analysis of
variance, the time over which the plasma isotope levels were constant was found and the mean plasma concentration determined. The distribution volume was calculated from the formula

\[ V = \frac{I}{P_{\text{stable}}} \]

where \( V \) = distribution volume
\( I \) = injected dose
\( P \) = plasma concentration of isotope

Glomerular filtration rate using \(^{51}\text{Cr}-\text{EDTA}\) and the formula of Bryan et al.

Individual rats were weighed and anaesthetized with ether. Ten \( \mu \text{Ci} \) of \(^{51}\text{Cr}-\text{EDTA}\) were injected into the femoral vein and 200 \( \mu \text{l} \) of blood sampled from the tail vein 60 minutes later. The time of sampling was noted accurately and 50 \( \mu \text{l} \) of the plasma obtained was counted in an Echo gamma-counter.

The clearance of the \(^{51}\text{Cr}-\text{EDTA}\) was then calculated from the formula

\[ C = V \ln \left( \frac{P_0}{P_t} \right) \]

where \( C \) = the clearance in \( \text{ml./min.} \)
\( V \) = the distribution volume experimentally determined
\( P_0, P_t \) = the plasma concentration of isotope at time zero and time \( t \)

\( P_t \) is determined from counting a plasma sample and \( P_0 \) calculated from \( P_0 = I/V \), where \( I \) is the original dose.
RESULTS

Estimation of G.F.R. using the two compartment external body counting procedure

Clearance of $^{51}$Cr-EDTA was determined in a group of 30 Wistar rats of both sexes including a group of ten unilaterally nephrectomized rats. The G.F.R. for normal rats was found to be $0.54 \pm 0.16$ ml./min.100 g. (Table 6). The G.F.R. for unilaterally nephrectomized rats was $0.40 \pm 0.11$ ml./min./100 g., significantly different from the normal value ($p < .01$).

Reproducibility

To determine the reproducibility of the method, the G.F.R. was determined three times each on four normal male rats over a period of one month. The coefficients of variation ranged from 12.5 to 16.6% with a mean of 14.0%.

One compartment approximation

Because the calculation of G.F.R. by the two compartment method of Sapirstein is time consuming, the results of 45 determinations were re-calculated using the simpler one compartmental formula

$$C = \frac{I}{A \gamma}$$

where $I$ is the injected dose

$\gamma$ is the slope of the regression line for the plasma activity plotted on semilogarithmic graph paper against time

$A$ is the intercept of this line with the ordinate.

Sapirstein's formula introduces an extra term for the "inter-compartmental equilibration" that occurs during the
TABLE 6. Measurement of the glomerular filtration rate using the two compartment external body counting method.
<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Renal Condition</th>
<th>Sex</th>
<th>Weight (g.)</th>
<th>Clearance ml./min.</th>
<th>Clearance ml./min./100g.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal</td>
<td>M</td>
<td>262</td>
<td>0.98</td>
<td>0.37</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>M</td>
<td>236</td>
<td>2.21</td>
<td>0.94</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>M</td>
<td>255</td>
<td>1.73</td>
<td>0.68</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>M</td>
<td>311</td>
<td>1.34</td>
<td>0.43</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>M</td>
<td>360</td>
<td>2.05</td>
<td>0.57</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>M</td>
<td>406</td>
<td>2.37</td>
<td>0.58</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>M</td>
<td>359</td>
<td>1.36</td>
<td>0.38</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
<td>M</td>
<td>387</td>
<td>1.39</td>
<td>0.36</td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>M</td>
<td>342</td>
<td>1.85</td>
<td>0.54</td>
</tr>
<tr>
<td>10</td>
<td>&quot;</td>
<td>M</td>
<td>369</td>
<td>2.21</td>
<td>0.60</td>
</tr>
<tr>
<td>11</td>
<td>&quot;</td>
<td>M</td>
<td>247</td>
<td>1.16</td>
<td>0.47</td>
</tr>
<tr>
<td>12</td>
<td>&quot;</td>
<td>M</td>
<td>230</td>
<td>0.88</td>
<td>0.38</td>
</tr>
<tr>
<td>13</td>
<td>&quot;</td>
<td>M</td>
<td>325</td>
<td>2.22</td>
<td>0.68</td>
</tr>
<tr>
<td>14</td>
<td>&quot;</td>
<td>M</td>
<td>365</td>
<td>1.38</td>
<td>0.52</td>
</tr>
<tr>
<td>15</td>
<td>&quot;</td>
<td>F</td>
<td>272</td>
<td>0.80</td>
<td>0.29</td>
</tr>
<tr>
<td>16</td>
<td>&quot;</td>
<td>F</td>
<td>266</td>
<td>1.63</td>
<td>0.61</td>
</tr>
<tr>
<td>17</td>
<td>&quot;</td>
<td>F</td>
<td>266</td>
<td>1.28</td>
<td>0.48</td>
</tr>
<tr>
<td>18</td>
<td>&quot;</td>
<td>F</td>
<td>252</td>
<td>2.04</td>
<td>0.81</td>
</tr>
<tr>
<td>19</td>
<td>&quot;</td>
<td>F</td>
<td>272</td>
<td>1.35</td>
<td>0.50</td>
</tr>
<tr>
<td>20</td>
<td>Nephrect.</td>
<td>M</td>
<td>319</td>
<td>1.47</td>
<td>0.46</td>
</tr>
<tr>
<td>21</td>
<td>&quot;</td>
<td>M</td>
<td>288</td>
<td>1.01</td>
<td>0.36</td>
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<tr>
<td>22</td>
<td>&quot;</td>
<td>M</td>
<td>290</td>
<td>1.19</td>
<td>0.41</td>
</tr>
<tr>
<td>23</td>
<td>&quot;</td>
<td>M</td>
<td>289</td>
<td>0.80</td>
<td>0.28</td>
</tr>
<tr>
<td>24</td>
<td>&quot;</td>
<td>M</td>
<td>286</td>
<td>0.52</td>
<td>0.18</td>
</tr>
<tr>
<td>25</td>
<td>&quot;</td>
<td>M</td>
<td>342</td>
<td>1.27</td>
<td>0.37</td>
</tr>
<tr>
<td>26</td>
<td>&quot;</td>
<td>M</td>
<td>264</td>
<td>1.08</td>
<td>0.41</td>
</tr>
<tr>
<td>27</td>
<td>&quot;</td>
<td>M</td>
<td>270</td>
<td>1.40</td>
<td>0.52</td>
</tr>
<tr>
<td>28</td>
<td>&quot;</td>
<td>M</td>
<td>285</td>
<td>1.54</td>
<td>0.54</td>
</tr>
<tr>
<td>29</td>
<td>&quot;</td>
<td>M</td>
<td>290</td>
<td>1.27</td>
<td>0.44</td>
</tr>
</tbody>
</table>

For normal animals G.F.R. = 0.54 ± 0.16 (N = 19).
For nephrectomised animals G.F.R. = 0.40 ± 0.11 (N = 10)
By t-test there is a significant difference between rates for normal and nephrectomized rats (P < .01).
equilibration of the isotope between the vascular and the extravascular compartment.

His formula

$$C = \frac{I \gamma_A \gamma_B}{A \gamma_B + B \gamma_A}$$

applies

where $\gamma_A$ and $\gamma_B$ are the slopes of the regression lines for renal excretion and intercompartmental equilibration and $A, B$ are their respective intercepts with the ordinate.

In the simplified one compartment formula, however, the intercompartmental equilibration is considered to be rapid and the renal excretion thus to be rate limiting. The results calculated by both methods are shown in Figure 15. The correlation is understandably good as expected from the similarity between the two formulae although the one compartment method over-estimates the two compartmental results by 20%, a figure similar to that observed by Chantler in man (Chantler et al., 1969).

Distribution volume

Since it is known that different substances distribute themselves in the extracellular fluid in different and not necessarily proportional volumes in different species (Ladegaard-Pederson & Engell, 1972), it was necessary to determine the distribution volume in rats experimentally. A total of nine determinations made with $^{51}$Cr-EDTA gave a distribution volume of $24.3 \pm 0.9\%$ of body weight. This figure compares favourably with the figure of $26.9 \pm 1.7\%$ obtained by Bryan et al. (1972) using $^{125}$I-iothalamate.

Glomerular filtration rate using the $^{51}$Cr-EDTA and the formula of Bryan et al.

To establish the optimum time for plasma sampling following the
FIGURE 15.

Comparison of the one compartment with the two compartment method, using $^{51}$Cr-EDTA and external body monitoring

$r = 0.994$. Regression equation $Y = 1.192 X - 0.038$
administration of $^{51}\text{Cr-EDTA}$, blood samples were collected at intervals after the administration of $^{51}\text{Cr-EDTA}$ to a group of Wistar rats. Clearance rates were then determined simultaneously by the external body monitoring method and by the formula of Bryan. The correlation coefficients from the correlation of the Bryan formula with the two compartmental external body monitoring method were plotted against the blood sampling times. Maximum correlation was found at 60 minutes after administration of the isotope and this sampling time was used in further experiments. The glomerular filtration rate obtained using Bryan's formula following the analysis of a single 60 minute blood sample and the result of the two compartmental external body method were then compared in 33 individual experiments (Figure 16). A regression line of the former on the latter was calculated ($Y = 0.739 + 0.093; r = 0.83$) and in subsequent experiments this equation was used to correct the results obtained using Bryan's formula and a single 60 minute blood sample, to that expected from the two compartment body monitoring method.

**Confirmatory analysis using $^{51}\text{Cr-EDTA}$ and a single blood sample**

To confirm the efficacy of the method, the glomerular filtration rate was determined in a group of 11 hooded rats following the injection of $^{51}\text{Cr-EDTA}$ and an analysis of a single 60 minute blood sample. The rats were then unilaterally nephrectomized and the glomerular filtration rate again measured 2 and 7 weeks later. The results (Table 7) show a highly significant difference between the pre and post nephrectomy glomerular filtration rates.
Comparison of clearances by the timed plasma method (Bryan et al.), with the two compartment external body monitoring method.

$r = 0.828$. Regression equation $Y = 0.739 + 0.093$
GFR by single plasma sample method (ml/min/100g)

GFR by 2 compartment external body method (ml/min/100g)
TABLE 7. Glomerular filtration rate using $^{51}$Cr-EDTA and a single blood sample taken 60 minutes after administration. The results show a highly significant difference between the pre and post nephrectomy glomerular filtration rates.
### Glomerular Filtration Rate (ml./min./100g)

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Pre Nephrectomy</th>
<th>2 Weeks Post Nephrectomy</th>
<th>7 Weeks Post Nephrectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.93</td>
<td>0.51</td>
<td>0.62</td>
</tr>
<tr>
<td>2</td>
<td>0.63</td>
<td>0.47</td>
<td>0.46</td>
</tr>
<tr>
<td>3</td>
<td>0.75</td>
<td>0.38</td>
<td>0.59</td>
</tr>
<tr>
<td>4</td>
<td>0.75</td>
<td>0.48</td>
<td>0.45</td>
</tr>
<tr>
<td>5</td>
<td>0.72</td>
<td>0.69</td>
<td>0.39</td>
</tr>
<tr>
<td>6</td>
<td>0.99</td>
<td>0.74</td>
<td>0.51</td>
</tr>
<tr>
<td>7</td>
<td>0.50</td>
<td>0.53</td>
<td>0.47</td>
</tr>
<tr>
<td>8</td>
<td>0.81</td>
<td>0.59</td>
<td>0.58</td>
</tr>
<tr>
<td>9</td>
<td>0.67</td>
<td>0.50</td>
<td>0.49</td>
</tr>
<tr>
<td>10</td>
<td>0.72</td>
<td>0.71</td>
<td>0.60</td>
</tr>
<tr>
<td>11</td>
<td>0.75</td>
<td>0.58</td>
<td>0.32</td>
</tr>
<tr>
<td>Mean</td>
<td>0.74</td>
<td>0.56</td>
<td>0.52</td>
</tr>
<tr>
<td>SD</td>
<td>0.13</td>
<td>0.11</td>
<td>0.07</td>
</tr>
</tbody>
</table>

**By t-test**

- Pre vs 2 weeks post: \( t = 4.31 \) \( P < .0025 \)
- Pre vs 7 weeks post: \( t = 6.29 \) \( P < .0005 \)
- 2 weeks post vs 7 weeks post: \( t = 1.05 \) Not Significant
DISCUSSION

These studies have shown that an intravenous dose of $^{51}$Cr-EDTA followed by a single timed plasma sample may be used to determine the G.F.R. in small laboratory animals. Many studies have confirmed the suitability of $^{51}$Cr-EDTA clearance for the determination of glomerular filtration and results within 10% of the standard inulin clearance have been obtained (Chantler et al., 1969).

The principal advantages of $^{51}$Cr-EDTA are its low cost, high stability, availability as a radiopharmaceutical and its chemical characteristics, both in vitro and in vivo. Binding to plasma proteins is less than 2% (Stacey & Thorburn, 1966) in contrast to iothalamate which may be protein bound up to 27% (Maher & Tauxe, 1969). Two other reports, however, have claimed the plasma binding of iothalamate to be less than 3% (Sigman, Elwood & Knox, 1966; Pixberg, Hilger & Zielske, 1969). Extrarenal excretion of $^{51}$Cr-EDTA has also been found to be negligible (Garnett, Parsons & Veall, 1967; Brochner-Mortensen, Giese & Rossing, 1969; Downes & McDonald, 1964) whereas iothalamate is known to be excreted through the gall bladder (Bryan et al., 1972). The data of Bryan, Jarchow & Maher (1972) showed a mean extra renal excretion component of 0.34 ml./min. which represents a mean value of 13% of the total clearance in their rats. Variation in the extrarenal excretion of iothalamate may explain some of the differences in the variance of distribution volumes between iothalamate and $^{51}$Cr-EDTA in anephric rats. When $^{51}$Cr-EDTA was used in these experiments, the coefficient of variation for an equal sample size was less than half that
obtained by Bryan et al. (1972) using iothalamate even though in the latter case the bile ducts had been tied. $^{51}$Cr-EDTA was not taken into cells over the period of the current experiments. Garnett et al. (1967) found that after one hour incubation of the isotope with fresh heparinized whole blood there was no detectable uptake of activity by the erythrocytes. These results were confirmed in the current studies with anephric animals where the plasma activity remained constant for 5 to 6 hours after an initial equilibration.

Although inulin remains the preferred reference substance for renal clearance measurements, it is not practicable to use the standard chemical method for inulin in studies on small animals. Inulin has been radioactively labelled but some doubt remains whether or not the radioactive material has renal clearance characteristics identical to natural inulin (Chen, Terepka & Lane, 1963; Gennari, Cortell & Schwartz, 1970). Berglund and co-workers (1969) have also cast doubts on the suitability of inulin as an index substance for measurement of G.F.R. in the rat by suggesting that individual animals have varying pore sizes which are proportional to the animal mass. If this is correct, a clearance substance such as $^{51}$Cr-EDTA would be a better indicator of G.F.R. than the larger inulin molecule although the situation has not been investigated further.

Methods using external body counting provide an infinite number of points derived from the recorder chart for the construction of plasma decay curves but the data analyses are laborious and unsuitable for repetitive G.F.R. determinations. Serial plasma
sampling methods similarly have the disadvantage that five or six blood samples are required so that the animal must be anaesthetized or else held in a restraining cage. Bryan's formula used in these experiments makes the assumption that the intravenously injected isotope is instantaneously distributed throughout the distribution volume and that the equilibration of the extravascular with the intravascular compartment is rapid compared with the renal excretion. This assumption was confirmed in our present studies using Sapirstein's formula where the mean half-time of the intercompartmental equilibration was 6.8 min. and the half-time for the renal excretion was 53.9 min. Plasma concentration of $^{51}$Cr-EDTA also reached a maximum at 1 to 3 minutes, suggesting a rapid equilibration with the extravascular compartment.

Glomerular filtration rates calculated with Bryan's formula and obtained from the analysis of a single blood sample after $^{51}$Cr-EDTA administration, were shown to have a good correlation with G.F.R.'s determined by the two-compartment method. The distribution volume experimentally determined for the $^{51}$Cr-EDTA was also similar to other reported figures and analyses of unilaterally nephrectomized animals confirmed the suitability of using $^{51}$Cr-EDTA and a single blood sample analysis for the determination of the G.F.R.

During the course of these experiments, the procedure has been found to be reproducible and reliable and has provided a satisfactory procedure for the repetitive determination of G.F.R. in small animals.
CHAPTER 5

EXPERIMENTAL PYELONEPHRITIS: THE EFFECT OF CHRONIC ACTIVE PYELONEPHRITIS ON RENAL FUNCTION
INTRODUCTION

The association of continued renal infection with chronic progressive pyelonephritis has been accepted for many years. More recently, however, a number of reports have shown that chronic lesions may develop in the absence of viable bacteria (Angell et al., 1968; Aoki et al., 1969; Kalmanson et al., 1965) and an increasing number of investigators have begun to question the validity of this relationship.

Most experimental studies have searched for morphological evidence of progressive lesions during chronic renal infection but, because of the extreme variability and the well recognised difficulties in quantitating histological changes, this approach has several limitations. In these experiments the effect of continuing E.coli renal infection on renal function has been followed by determining the glomerular filtration rate, blood urea, and urinary concentrating capacity in antibiotic treated and untreated rats with pyelonephritis. With this protocol it was possible to assess quantitatively the degree of renal damage throughout the course of the disease and finally to compare the results of functional tests with the pathological details found at autopsy.
MATERIALS AND METHODS

Induction of pyelonephritis
Renal infection was produced by the direct inoculation of E. coli 075 into the surgically exposed kidney. Details of the procedure have been given in Chapter 2.

Animal strain
Female rats weighing between 220 and 235 g. were obtained from an inbred strain of hooded rat.

Serum urea
Serum samples were collected at regular intervals by venepuncture of the dorsal tail vein. Serum urea analyses were carried out in the Technicon SMA 12 autoanalyzer.

Measurement of maximum urine concentrating capacity
All animals had food and water removed 24 hours before being placed in a metabolic cage specifically designed for urine collection. At the end of the 24 hour period animals were induced to micturate, using a whiff of ether in a nose cone, before being placed in a metabolic cage still without food or water. All urine passed overnight was collected in a tube held in an ice bath. At 9 a.m. micturition was induced again and this urine was added to the overnight collection. Urinary osmolality was measured in a Knauer osmometer after preparing a 1:4 dilution of the urine in distilled water.

Bacterial content of renal tissue
Nutrient agar pour plates of serial ten fold dilutions of homogenized kidney were made to obtain the bacterial count per gram of wet renal tissue.
Histological processing

Tissue for histologic examination was placed directly into a 0.1% solution of cetylpyridinium chloride in 10% formaldehyde. After routine processing and cutting, sections were stained with haematoxylin and eosin.

Measurement of glomerular filtration rate (G.F.R.)

The glomerular filtration rate was measured by determining the clearance of a single intravenous injection of $^{51}$Cr-EDTA using the formula proposed by Bryan et al. (1972). This formula eliminates the lengthy calculations involved using the two-compartment external body counting method but does require a knowledge of the distribution volume of $^{51}$Cr-EDTA in the rat. Full details of the method have been given in the previous chapter.

Eradication of infection with antibiotic treatment

Renal infection was eliminated by combined intramuscular therapy with gentamicin (20 mg./kg./day) and carbenicillin (150 mg./kg./day). Antibiotic therapy was given for ten days commencing one week after the initiation of infection and repeated two months later. Gentamicin was administered in the morning and carbenicillin was given in the late afternoon of each day.

Experimental protocol

These investigations were carried out as two separate experiments to cope with the logistics of performing renal function analyses on the number of animals involved. In the first experiment pyelonephritis was established in one group whose renal function over a 36-week period was compared with an unmanipulated control group. This experiment established that there were some
differences of interest.

In the second experiment the effect of antibiotic treatment and the elimination of infection from the kidney on the preservation of renal function was determined by comparing the renal function of two groups of pyelonephritic animals, one of which was treated with antibiotics.

In all of the experiments, unilaterally nephrectomized animals were used so that the pathological changes occurring in the kidney of an animal with unilateral pyelonephritis would not be masked by compensatory mechanisms in a contralateral normal kidney.

RESULTS

Effect of chronic renal infection on renal function

Two groups each of 12 rats were unilaterally nephrectomized and pyelonephritis induced in the remaining kidney of one group three weeks later. The other group served as a control. Glomerular filtration rate, blood urea nitrogen and urinary concentrating capacity were determined on all animals in both groups over a 36-week period. Glomerular filtration rates were similar in the two groups of animals prior to the induction of pyelonephritis (Figure 17). A compensatory increase in the G.F.R. of the control non-infected animals resulted in a significant difference in the G.F.R. between the two groups which decreased as the compensatory increase in the G.F.R. in the control group declined. In contrast, the G.F.R.'s in the pyelonephritic animals decreased during the first month after challenge but during the latter period of the study they were
FIGURE 17.

Glomerular filtration rates in control and pyelonephritic animals over a 36-week period following the induction of renal infection.
not significantly different from the baseline values.

Blood urea levels were also similar in the control and infected groups prior to infection but a significant increase in the blood urea in the infected animals was apparent up to eight weeks after challenge (Figure 18). An inexplicable increase in the blood urea of two of the control animals reduced the significance of any changes over the latter period of the study.

The effect of renal infection on the urinary concentrating capacity was also determined and highly significant differences were found between the pyelonephritic and control groups (Figure 19). The loss of concentrating capacity in the pyelonephritic group was maximal during the first month but a further slow determination of concentrating ability occurred during the following 32-week period.

Nine months after establishing infection both groups of animals were killed and the kidneys removed for gross, histopathological and bacteriological examination. As expected, there were major differences between the two groups. All the pyelonephritic animals showed extensive cortical scarring, whereas kidneys from the control groups were normal.

The degree of damage in the pyelonephritic kidneys, assessed by histopathological examination of serial sections through the kidney, confirmed the gross appearances and showed considerable loss of functional renal parenchyma. Pathological changes were most extensive in the cortex and a marked lymphocytic infiltrate, focal areas of interstitial fibrosis, tubular dilation and colloid casts were found in all the kidneys examined.
FIGURE 18.

Blood urea levels in control and pyelonephritic animals over a 30-week period after the induction of renal infection.
- = control
□ = pyelonephritic

Blood urea (mg/100 ml)

Weeks after renal infection

0.025 < p < 0.05

p < 0.01

p < 0.001

p < 0.2

Weeks after renal infection

Weeks after renal infection

Weeks after renal infection

Weeks after renal infection
FIGURE 19.

Urine concentrating capacity in control and pyelonephritic animals over a 36-week period after the establishment of renal infection.
Weeks after renal infection

Max. urine conc. capacity mOsm/kg

- control
- pyelonephritic

$p < 0.3$ $p < 0.01$ $p < 0.01$ $p < 0.01$ $p < 0.01$
Glomerular and vascular alterations were minimal except in scarred areas where glomerular crowding was commonly seen. Calyceal abnormalities were not usually present although lesions in the medulla and papilla were frequently found. Heavy bacterial infection had also persisted in all the pyelonephritic kidneys whereas the control kidneys were sterile.

Renal function and pathology in antibiotic treated and non-treated animals

Two groups of 20 animals were unilaterally nephrectomized and pyelonephritis induced in the remaining kidney three weeks later. Seven days after challenge, one group of rats were treated with gentamicin and carbenicillin for ten days and a similar course of treatment administered two months later. This treatment schedule had been previously shown to be capable of eliminating infection from the kidney of animals with pyelonephritis.

The glomerular filtration rate, blood urea and urinary concentrating capacity was determined in individual animals from both groups over a period of 36 weeks. The G.F.R.'s of both groups were similar before challenge and there were no significant differences between the two groups over the first eight weeks of the study (Figure 20). Although considerable variation in the range of G.F.R.'s were found as time progressed, the G.F.R.'s in some of the untreated animals declined more rapidly than the treated groups. Blood ureas in treated and non-treated pyelonephritic rats were also compared but no significant differences between the two groups were found over the 36-week period (Figure 21). When the urinary concentrating
capacities of the two groups were compared, marked differences were found (Figure 22). One month after challenge a highly significant difference was found between the treated groups. A marked reduction in the concentrating capacity of the pyelonephritic but untreated group was demonstrated but the concentrating capacity of the treated group of pyelonephritic animals, although significantly reduced from baseline values, was not affected to the same extent. Over the following 8 month interval, however, the concentrating capacity of the treated group continued to decline slowly, whereas after the initial sharp decrease little change occurred in the concentrating capacity of the non-treated group over the same period.

At the time of sacrifice 36 weeks after challenge, the gross pathology, histopathological and bacteriological features of renal infection in the treated and non-treated animals were compared (Figure 23). Differences in the degree of damage assessed histopathologically were not particularly marked despite the fact that active infection persisted in the untreated group over the entire period of the study (P = 0.1 for the gross and histopathological assessments). Similar results were found when several prominent features of the histopathology in the treated and non-treated animals were compared (Table 8).

Cortical lesions did not commonly extend into the renal parenchyma, and the histopathological examination confirmed the gross appearance of extensive but relatively non-penetrating scar formation (Figure 24). There was evidence of glomerular crowding (Figure 25), interstitial fibrosis and tubular atrophy in both the cortex and medulla and a lymphocytic
FIGURE 20.

Glomerular filtration rates in antibiotic treated and non-treated pyelonephritic animals over a 36-week period. Antibiotics were administered for 10 days, one week and subsequently two months after challenge. The kidneys from the pyelonephritic but treated animals were sterile (one exception) at autopsy.
or
non
treated

□ = antibiotic treated
△ = non treated

Gomelar filtration rate (mL/min/100g)

0.05 < p < 0.25
0.1 < p < 0.05
p = 0.08
p = 0.02
0.01 < p < 0.025

0 6 12 26 36
Weeks after renal infection
FIGURE 21.

Blood urea levels in antibiotic treated and non-treated pyelonephritic animals over a 36-week period after challenge.
A = antibiotic treated
☐ = non-treated
FIGURE 22.

Urine concentrating capacity in antibiotic treated and non-treated pyelonephritic animals.
weeks after renal infection
Comparative bacteriology, gross pathology and histopathology of antibiotic treated and non-treated pyelonephritic animals at autopsy 36 weeks after the initial challenge.

Gross pathology: assessment was made on a 0-10 scale with each point on the scale representing one-tenth of the surface of the kidney (see Chapter 2).

Degree of histopathologic damage: on the scale used, 10 represented lesions involving 25% or more of the area of the section. Lesser degrees of damage were scored on a 0-10 scale where each unit represented 2.5% of the area of the kidney section.
FIGURE 24.

Cortical lesions in the scarred area of a kidney from an animal with chronic pyelonephritis.

FIGURE 25.

Glomerular crowding in a scarred and contracted cortical lesion 9 months after the establishment of infection.
**TABLE 8.** Assessment of the effect of antibiotic therapy on several histopathological features of chronic pyelonephritis.
<table>
<thead>
<tr>
<th></th>
<th>Number of animals</th>
<th>Degree of (1) renal damage</th>
<th>Mononuclear (2) infiltration</th>
<th>Lesion in the (3) medulla and papilla</th>
<th>Colloid (4) casts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyelonephritis</td>
<td>15</td>
<td>4.0 = 1.6</td>
<td>3.3 = 1.3</td>
<td>4.3 = 2.3</td>
<td>3.2 = 2.0</td>
</tr>
<tr>
<td>(treated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyelonephritis</td>
<td>25</td>
<td>4.73 = 2.1</td>
<td>3.9 = 1.7</td>
<td>5.4 = 2.0</td>
<td>3.3 = 2.2</td>
</tr>
<tr>
<td>(non-treated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wilcoxon analysis</td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>N.S.D.</td>
<td>N.S.D.</td>
</tr>
</tbody>
</table>

(1) Degree of renal damage: on the scale used, 10 represented lesions involving 25% or more of the area of the section. Lesser degrees of damage were scored on a 0-10 scale where each unit represented 2.5% of the area of the kidney section (see Chapter 2).

(2) Degree of mononuclear infiltration: quantitative 0-10 scale used to make a comparative assessment of the degree of mononuclear infiltrate (see Chapter 2).

(3) Lesions in the medulla and papilla: a comparative 0-10 scale was used. Mild, focal, collecting duct hyperplasia and an increase in connective tissue represented minor changes while extensive fibrosis, tubular dilation with blunting and deformation of the papillary tip were considered major pathological changes.

(4) Colloid cases: quantitative scale where each unit was equal to the average number of colloid casts seen per kidney section.

(5) N.S.D.: no significant difference when the two groups were compared by the Wilcoxon sum of ranks analysis for non-parametric data.
infiltrate which was particularly marked in the renal cortex. Colloid casts were present in all of the kidneys examined but glomerular and vascular abnormalities were not found apart from areas of scarring where degenerate glomeruli were common (Figure 26). Despite the sometimes extensive involvement of renal cortical tissue there was little evidence to suggest that tissue destruction was progressive. In most cases the lesions were circumscribed and reflected the extent of the initial infectious process established by the direct inoculation of bacteria into the kidney.

The involvement of the medullary and particularly the papillary tissue was of interest. Dilation of the collecting tubules and interstitial fibrosis were commonly found in the papilla (Figure 27) and in several cases marked papillary deformation and blunting of the papillary tip was seen (Figure 28).

**Bacterial infection in relation to pathological changes**

The results from the previous experiment showed that treatment with antibiotics reduced the effect of renal infection on the concentrating capacity although in a related study it has been shown that treatment did not affect the gross or histopathologic features over the same period (Chapter 14). This suggested that infection alone may affect the ability of the kidney to form a concentrated urine. In this experiment the possibility of renal infection occurring in individual kidneys distinct from pathologic changes was investigated.

Pyelonephritis was induced in the upper segment of six kidneys and the infection allowed to proceed for 14 days. At sacrifice the gross pathology in both upper and lower segments of each
FIGURE 26.

Degenerate glomeruli in a scarred and contracted area of a kidney from an animal with chronic pyelonephritis.

FIGURE 27.

Tubular dilatation and interstitial fibrosis in the papilla of an animal with chronic pyelonephritis.
FIGURE 28.

Left frame: deformation and blunting of the tip of the papilla in chronic experimental pyelonephritis.

Right frame: papilla from a litter mate control animal included for comparison.
kidney was assessed before the kidneys were cut in half and quantitative bacterial counts carried out. In an identical experiment the histological changes in the challenged and non-challenged segments were quantitated. The experiment has shown that major gross and histopathological changes occurred in the challenged segment of the kidney associated with infection. In contrast, the gross and histopathological appearances were normal in the non-challenged lower segment although bacterial infection was present in the lower segment of all kidneys (Figure 29).

DISCUSSION
The effect of pyelonephritis on renal function has been reported by other authors using a variety of experimental models and modes of investigation. The objective in these experiments was to study the effect of chronic active E.coli pyelonephritis on renal function and to determine the effect of antibiotic treatment and elimination of infection on the gross pathology, histopathology and renal function of animals with chronic pyelonephritis.

In considering the significance of these results, and their relationship to data from other investigators, the features of the experimental model need emphasizing. Renal lesions were established in all the animals challenged with E.coli and the gross and histopathological changes in the experimental animals were found to parallel closely the course of chronic pyelonephritis in man. Renal infection in the untreated pyelonephritic animals persisted throughout the entire experiment whereas all
Infection of the renal parenchyma without associated pathological changes. Kidneys of experimental animals were challenged in the upper segment and the bacteriological and histological features of both the upper and lower segments determined 14 days later.
the treated animals had sterile lesions at autopsy. The sequential determination of renal function on the same group of animals also eliminated problems associated with the comparison of functional tests in groups of animals challenged and sacrificed at different intervals.

These studies have shown that a marked loss of concentrating capacity was demonstrable when the maximum urinary osmolality of a group of animals with unilateral pyelonephritis were compared with normal control animals. A severe loss of concentrating capacity occurred during the first month but the subsequent loss of concentrating capacity over a further three week period was minimal. A compensatory increase in the glomerular filtration rate in the control non-challenged group occurred after nephrectomy but no comparable compensation in the infected group was found. Blood urea levels in the pyelonephritic animals were not significantly different from the unchallenged control groups despite the extent of the pathological damage in the former group.

Antibiotic therapy had a marked effect on the urinary concentrating capacity in pyelonephritic animals and the defect in concentrating ability was significantly less in the treated animals during the first 30 days after challenge. Infection again prevented a compensatory increase in the G.F.R. of pyelonephritic animals but this was not reversed by antibiotic therapy. Blood urea levels in treated and non-treated groups were not significantly different nor did antibiotic therapy and the eradication of infection appear to affect the gross pathology and histopathological changes found at autopsy.
Several other studies of experimental pyelonephritis have demonstrated a urinary concentrating defect with results similar to those reported here. Kaye & Rocha (1970) also observed a concentrating defect in experimentally induced pyelonephritis but considered that as it was readily reversed, it was not associated with permanent renal damage. Gonick et al. (1965) were also able to demonstrate a reduced maximal concentrating capacity as early as one week after the induction of enterococcal pyelonephritis although repetitive injections of bacteria were necessary to maintain a depressed concentrating capacity.

One of the most interesting observations was that treatment of infection with antibiotics limited the degree of depression of concentrating ability during the initial 30 days after challenge. In related experiments, it has been repeatedly shown that the gross and histopathologic changes in animals examined 30 days after challenge were not affected by antibiotic therapy even when treatment commenced as early as seven days after induction of infection (Chapter 14). This strongly suggests that the severe depression of urine concentrating capacity was associated with active bacterial infection as well as the loss of functional nephrons in the pyelonephritic lesions.

Other investigators have also suggested that a loss of concentrating ability in experimental pyelonephritis may be caused by infection alone. Kaye & Rocha (1970) found a poor correlation between histopathologic changes and the maximum urine concentrating ability and Beck et al. (1961), in a study of staphylococcal pyelonephritis found that resolution of the
lesion was associated with the restoration of renal function but persistent active infection resulted in a continued impairment.

Related experiments documenting acquiescent infection in animal models have also shown that a reduction in urinary concentrating capacity can occur in the absence of associated pathological changes (Chapter 8). There seems little doubt that the bacteriological status of the kidney is an important factor determining the concentrating capacity of the pyelonephritic kidney although the mechanism by which infection reduces urinary osmolality has not been defined. An increase in medullary blood flow by bacterial metabolic products has, however, been demonstrated in man and is likely to have led to the lowered urinary osmolality found in the present experiments (Mitchie & Mitchie, 1957; Lathem, 1956). The ability of antibiotic therapy to restore urinary concentrating capacity in individuals with renal infection also suggests that acquiescent infection may occur in man and provides an explanation for the efficacy of appropriate therapy in restoring normal renal function (Winberg, 1959; Clark et al., 1969).

One factor limiting the appreciation of the significance of infection alone as a determinant affecting renal function has been the belief that active bacterial infection is confined to regions of the kidney with evidence of inflammatory changes. This is almost certainly not the case as has been shown in the present experiments.

The observation of Kaye and Rocha that the loss of concentrating capacity found in acute pyelonephritis was reversible
(Kaye & Rocha, 1970), was not confirmed. The concentrating capacity was permanently impaired and even in the treated group a slow decline in concentrating ability occurred over a period of many weeks. The present experiments have shown clearly that the concentrating defect had a composite aetiology of which pathological damage and bacterial infection were individual components. The effects of pathological damage were not reversible although early treatment with antibiotics could diminish or eliminate the effect of infection alone.

The analysis of the pathological changes in the treated and unilateral animals at autopsy 36 weeks after the initiation of infection established that major parenchymal destruction occurred during the acute infection but the continued renal infection did not result in a significant progression of the pyelonephritic lesion when compared with untreated animals. The findings are in broad agreement with Glassock et al., who also found that the events occurring early in infection determined the eventual functional and histopathological characteristics of the disease (Glassock et al., 1974).

These results underscore the doubts raised by a number of workers regarding the role of continued infection in the aetiology of chronic progressive pyelonephritis (Little et al., 1965; Bailey, 1973; MacGregor, 1970). The high morbidity carried by infections of the urinary tract is unquestioned yet terminal renal failure from chronic pyelonephritis is not common despite the frequency of urinary tract infections (Freedman, 1966; Parker & Kunin, 1973; Heptinstall, 1969; Kimmelstiel et al., 1961). Although the host-parasite
relationship in chronic pyelonephritis has been subjected to intensive research, many details clearly remain to be defined.
CHAPTER 6

EXPERIMENTAL PYELONEPHRITIS:
INDUCTION OF RETROGRADE RENAL INFECTION
It is now generally accepted that renal infection is initiated by the reflux of infected urine from the lower urinary tract into the renal parenchyma. Many of the factors concerned with the mechanism by which bacteria enter the urinary tract and establish renal infection have been investigated using experimentally induced infections but the models in current use have several limitations.

The most significant of these is that renal infection with *E. coli* has not readily been established in the intact kidney and to initiate an acute lesion it has been necessary to either damage the kidney, force infected urine into the kidney or obstruct the flow of urine. These manipulations lead to the development of acute pyrogenic suppuration which does not reflect the natural history of the disease and may obscure the relevance of important features of the natural infection.

In the course of investigating factors influencing the establishment of renal infection, a method for producing infection in the intact and unmanipulated kidney was developed. This was accomplished by placing a reservoir of the bacterial inoculum in agar in the bladder using a small glass capsule which did not obstruct the flow of urine. Subsequent leaching of the inoculum into the bladder lumen led to the reflux of infected urine and the establishment of renal infection in 85% of the animals challenged.
MATERIALS AND METHODS

Animal strain
Female Wistar rats from a random bred strain, and weighing 230 to 250 g. were used.

Retrograde infection
A glass capsule, 6 mm. in length was prepared from a piece of capillary tubing 1.5 mm. external diameter. One end was almost completely sealed and a 1 cm. length of surgical cotton passed through the hole and secured to the sealed end of the capsule. The cotton length was used later to secure the capsule to the dome of the bladder. The bacterial inoculum was prepared by centrifuging a 6½ hour broth culture of E. coli 08 containing $1 \times 10^9$ organisms/ml. The deposited bacteria were then recovered, washed twice in two 10 ml. volumes of saline, resuspended in 0.5 ml. of broth and added to 1.5 ml. of 2.5% agar held at 42°C. Agar containing the inoculum was then inserted into the lumen of the capillary using a warmed syringe and a 26 gauge needle and then allowed to solidify. A suprapubic incision was made and the rat's bladder was exteriorized. A capsule was then inserted into the bladder lumen through a small slit in the dome of the bladder and the cotton, attached to the capsule, used to suture the slit and to retain the capsule in the dome to prevent obstruction of urine flow (Figure 30). No further manipulation of the kidney or urinary tract was required.

Bacteriology
Animals were sacrificed 3, 7, 14 and 21 days after challenge and the kidneys removed using an aseptic technique. The bacterial
FIGURE 30.

Insertion of the glass capsule, containing the bacterial inoculum in agar, into the bladder through a slit in the dome of the bladder. The suture attached to the capsule is used to close the slit and secure the capsule to the dome.
content per gram of tissue was determined by homogenizing the kidneys and preparing ten-fold dilutions of the homogenate in saline. 1 ml. aliquots of the homogenate were incorporated into nutrient agar pour plates. In an extension of this experiment, the distribution of bacteria in the cortex, medulla and papilla of kidneys from a further group of animals was determined. Details of the dissection of the kidney have been given in Chapter 2. Blood cultures of 0.3 ml. of blood obtained from the dorsal tail vein by venepuncture were prepared by diluting the sample in 5 ml. of broth and spreading this over a series of blood agar plates.

Serum antibody titres

Serum antibody levels were determined by a passive haemagglutination procedure using microtitre equipment. Full details of the sensitization of the sheep-red-blood cells are given in Chapter 10.

RESULTS

Bacteriology

Three days after insertion of the capsule both kidneys of all the animals challenged were infected, although gross changes were not seen at this stage. Visible lesions were seen in the kidneys of individual animals 5 days after infection and subsequently in those sacrificed up to 21 days after challenge. Blood cultures taken at regular intervals up to two hours after insertion of the capsule were sterile.

Counts of viable bacteria showed that infection was established in 85% of the kidneys challenged and then sacrificed over a
21-day period (Figure 31). The degree of infection varied considerably but the highest counts were found during the first week after challenge. Thereafter the number of bacteria per gram of kidney declined slowly but considerable numbers were still present 21 days later. When the distribution of bacteria in the infected kidneys was determined, similar bacterial counts were found in the cortex, medulla and papilla.

**Histological examination**

Histological evidence of renal infection was found in 35% of the animals with infected kidneys when serial sections through the kidneys were examined. Evidence of renal infection was first seen seven days after challenge and in the occasional animal there was evidence of gross infection with lesions extending from the papilla to the cortex.

Single, focal lesions were more commonly found and consisted of a mild lymphocytic infiltrate sometimes associated with an inflammatory polymorphonuclear infiltrate. By 14 days, acute inflammatory areas had progressed in a wedge-shaped extension of the early lesion in the papilla (Figures 32 and 33) to involve the cortex (Figure 34).

Leucocyte casts were seen throughout the lesion but the interstitial infiltrate consisted predominantly of small lymphocytes and an occasional group of plasma cells. In sections from some kidneys there was clear histologic evidence that the lesions had originated in the fornices while in other kidneys the lesion appeared to have been initiated in the papilla and there was no evidence of an inflammatory lesion in the fornices even when serial sections through the kidney were examined. Twenty-one
FIGURE 31.

Number of organisms in the kidney and the kidney cortex, medulla and papilla after retrograde challenge with *E. coli* 08. Groups of animals were sacrificed 3, 7, 14 and 21 days after challenge.
To follow the development of bacterial growth in various regions of the kidney, the graphs below depict the log number of bacteria per gram at different times after challenge.

- **3 days after challenge**
- **7 days after challenge**
- **14 days after challenge**
- **21 days after challenge**
FIGURE 32.

Initiation of an inflammatory lesion in the papilla after retrograde challenge with *E. coli* 08.

FIGURE 33.

Polymorphonuclear leucocytes in the interstitium and forming casts in acute retrograde infection.
Extension of the lesion in the papilla to involve the cortex.
FIGURE 35.

Serum antibody titre 3, 7, 14 and 21 days after retrograde challenge. Each point is the mean from 8 animals.
days after challenge, resolution of the acute infection was well advanced and interstitial fibrosis was seen in most lesions.

**Serum antibody response**

Serum antibody titres were determined on a further group of seven animals which were bled before challenge and at regular intervals after the insertion of the capsule. All animals showed an increase in serum antibody titre 3 days after challenge which reached a peak 7 days later. The antibody titre then declined slowly over a period of 21 days (Figure 35).

**DISCUSSION**

These studies have established that renal infection and pyelonephritis can be produced following the insertion into the bladder of a small capsule containing bacterial inoculum in an agar plug. Other investigators have found that even a highly virulent strain of *E. coli* could not produce infection in the normal kidney (Fierer et al., 1971) unless such a large bladder infusion was given that pyelovenous reflux occurred. In the current model, manipulation of the urinary tract was not essential for the establishment of infection. A reservoir of the infecting organism, however, rather than a transient challenge, was considered essential.

A number of experimental models of renal infection have relied upon the production of renal abscesses as a criteria for the establishment of pyelonephritis. Renal infections of this degree of severity are not common in clinical medicine and the similarity of many of the experimentally induced infections to
clinical disease is clearly in doubt. On the other hand, the severity of infection found in the present experiments approximates many renal infections encountered clinically and the model gains relevance in view of the fact that infection was established without trauma to the upper urinary tract.

The observation that severe infection occurred in many kidneys without the development of gross or histopathological lesions has also been reported by other investigators and is believed to represent reflux of infected urine into the pelvic space (Brooks et al., 1974). This phenomenon has been investigated in more detail in Chapter 8 with a different conclusion being drawn regarding the host-parasite relationship in this situation.

Although there is ample evidence for the occurrence of vesico-ureteric reflux in the rat (Mahoney & Persky, 1963; Sommer, 1961), and the transfer of infected bladder urine into the pelvic space of the kidney, the route by which bacteria enter the renal parenchyma has not been finally determined. Pyelo-venous reflux does not seem likely in the present case in view of the failure to demonstrate a bacteraemic phase and also the fact that renal trauma, which is usually required to localize a blood borne inoculum, was not used in these experiments.

Several studies have indicated, however, that the renal lymphatics may be involved in the intrarenal spread of infection (Murphy et al., 1958; Corriere & Murphy, 1967; Murphy & Schoenberg, 1960) and that bacteria are likely to enter the renal interstitium through the fornices and then spread to the cortex and medulla along the arcuate lymphatics.
Although the precise details of the mechanism of infection have not been determined, the model has proved to be of considerable value during investigations into the pathogenesis of renal infection.
CHAPTER 7

BACTERIAL INTERFERENCE AS A FACTOR IN RENAL INFECTION
INTRODUCTION

The ability of microorganisms of one species to interfere with the growth and survival of unrelated species has been recognised for many years. Even within species, bacterial interference has been found and in the case of neonatal infection, colonisation with an avirulent strain of staphylococcus has been used to control epidemics caused by pathogenic staphylococci (Light, Sutherland & Schott, 1965).

Microbiological aspects of renal infection have been studied in detail and although mixed infections with different strains are common where structural abnormalities of the urinary tract exist, the majority of infections appear to be due to a single strain of E.coli (Stamey, Govan & Palmer, 1965; Vosti, Monto & Rantz, 1962). The epidemiology of urinary tract infection has also been investigated and the predominant patterns of infection established by serological typing of the organisms isolated. Recurrent infections have been divided into relapses, where an initial infection is followed by a relapse of disease due to the same strain, and re-infection where the initial infection is eliminated but followed by a further infection with a different serotype of E.coli. A number of host factors have been investigated as contributing to this pattern, but there has been no satisfactory explanation to account for the majority of infections being caused by a single serotype of E.coli.

In these experiments the role of bacterial interference in determining patterns of renal infection caused by E.coli has
been investigated. Genetic markers were used to identify the
E. coli strains and allowed the ready identification and
quantitation of individual organisms from mixed infections.
The experiments have established that patterns of infection and
reinfection were determined by the resident pathogen in the
infected kidney and that an invading pathogen can create a
micro-environment in the kidney that prevents the establishment
of a second even closely related pathogen. These data provide
a ready explanation for the infrequent occurrence of mixed in-
fecctions of the kidney and suggest that bacterial interference
may be an important and unrecognized determinant in the epidemi-
ology of renal infection.

MATERIALS AND METHODS

Animals
Female rats weighing 220 to 250 g. were obtained from a random-
bred strain of Wistar rat.

Production of renal infection
Retrograde pyelonephritis was induced by the implantation of a
small glass capsule containing bacterial inoculum in agar, into
the dome of the surgically exposed bladder as described in the
previous chapter. In some experiments pyelonephritis was in-
duced by the inoculation of E. coli into the renal parenchyma
under direct vision as described in Chapter 2. Antibiotic
treatment was carried out with an intramuscular injection of
gentamicin at 10 mg. per kilogram per day for each rat for a
10 day period starting on the seventh day after challenge. This
treatment schedule was designed to be marginally effective and
to eliminate renal infection in approximately half the animals treated.

**Bacterial content of renal tissue**

Nutrient agar pour plates of serial ten-fold dilutions of homogenized kidney were made to obtain the bacterial count per gram of kidney tissue.

**Differentiation of E. coli 075 from E. coli 08 in mixed infection**

Details of the methodology utilizing stable genetic markers have been given in this section under bacterial strains.

**Bacterial strains**

Two strains of *E. coli* isolated from cases of clinical renal infection were characterized for use in these experiments.

<table>
<thead>
<tr>
<th>Serological type</th>
<th>Arabinose utilization</th>
<th>Streptomycin sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 1</td>
<td>075</td>
<td>-</td>
</tr>
<tr>
<td>Strain 2</td>
<td>08</td>
<td>+</td>
</tr>
</tbody>
</table>

Both strains had similar growth characteristics in broth and could not be differentiated by colonial morphology on conventional media. Confirmation of the identity of isolates from bacterial challenges with individual strains and with mixed strains were carried out by preparing templates of individual colonies on a nutrient agar plate. Fifty colonies were subcultured onto a 9 cm. Petri-dish containing 25 ml. of nutrient agar and incubated at 37°C for 16 hours. Replica sub-cultures using a fine felt cloth and a circular aluminium block were made onto a further three Petri-dishes containing nutrient agar, nutrient agar plus 100 µg. of Streptomycin per ml. and MacConkey
agar base to which 1% arabinose had been added. Replica plated Petri-dishes were then incubated overnight at 37°C, and the growth characteristics on the differential media used to confirm the identity of the individual isolates.

RESULTS

Pyelonephritis

Histologic evidence of renal infection was first seen seven days after challenge. Lesions consisted predominantly of wedge-shaped areas of involvement extending from the papilla to the outer cortex. Isolated focal lesions were also found and consisted of a mild lymphocytic infiltrate sometimes associated with a polymorphonuclear infiltrate. Fourteen days after challenge the interstitial infiltrate consisted predominantly of small lymphocytes and an occasional group of plasma cells and by 21 days resolution of the acute infection was well advanced with evidence of fibrosis in most lesions.

Simultaneous inoculation of E.coli 075 and E.coli 08 into the renal pelvis

To confirm that the two strains of E.coli were capable of establishing a mixed infection in the kidney, an inoculum of $4 \times 10^5$ organisms containing equal numbers of both strains were introduced directly into the renal parenchyma. Animals were sacrificed at intervals from 2 hours to 21 days after induction of renal infection and the degree of infection determined quantitatively. Two hundred colonies were subcultured onto agar templates which were then replicated onto the differential media to identify the individual isolates. The
total number of organisms identified as E. coli 075 and E. coli 08 in each kidney was then calculated. The results (Figure 36) show that the introduction of an inoculum containing the two strains of E. coli into the kidney resulted in a mixed infection containing both E. coli 075 and 08. Two hours after challenge, the two serotypes were present in approximately equal numbers and although some variation in the percentage of the individual serotypes was subsequently found, mixed infections persisted in all cases.

Retrograde renal infection following the introduction of E. coli 075 and 08 simultaneously into the bladder. The previous experiment established that both strains of E. coli were capable of proliferation when introduced directly into the kidney. In this experiment mixed retrograde infections of E. coli 08 and 075 were initiated in several groups of rats using equal numbers of both organisms. The bacterial inoculum was retained in the capillary in an agar gel and retrograde renal infections followed the surgical insertion of the capillary in the rats' bladders. Animals were sacrificed at intervals from 3 to 21 days after challenge and the bacterial content of individual kidneys determined. Further colonies were again subcultured onto nutrient agar and typed after replication onto differential media.

Although mixed infections were again found, renal infection caused by E. coli 08 alone (Figure 37) was the predominant pattern after a dual retrograde challenge. This finding was in marked contrast to the results of the previous experiment where mixed infections of E. coli 08 and 075 were invariably
FIGURE 36

Number of bacteria in individual kidneys 2 hours, 7, 14 and 21 days after the simultaneous inoculation of *E.coli 075* and *E.coli 08* into the renal parenchyma. Differentiation of the two strains of *E.coli* was made using stable genetic markers.
log No of bacteria per g of kidney
FIGURE 37.

Number of each strain of *E. coli* in individual kidneys 3, 7, 14 and 21 days after renal infection had been induced by the simultaneous introduction of *E. coli* 075 and 08 into the bladder.
seen.

**Ability of individual strains to establish retrograde renal infection**

The results of the previous experiment could be explained if the **E. coli 075** strain had a lesser capacity to induce retrograde renal infection than **E. coli 08**. In a further experiment, animals were infected in groups of three so that the infectivity of the two individual **E. coli** strains could be compared with the results of the dual infection established under similar conditions. Retrograde challenge was initiated in individual groups of animals with **E. coli 08** and **075**. A mixed culture, prepared from aliquots of the inocula from the individual infections, was then used to infect a further group with a dual **E. coli 08** and **075** challenge. Animals were sacrificed 3, 7, 14 and 21 days after challenge and the bacterial content of individual kidneys determined. Two hundred individual colonies from each kidney were examined on differential media to confirm the identity of the single challenges and to determine the proportion of **E. coli 08** and **075** present in kidneys from animals challenged with the dual infection.

The pattern of renal infection that emerged confirmed the previous experiments and established that both strains of **E. coli** were equally nephropathogenic. The ability of **E. coli 08** to preferentially establish renal infection and exclude **E. coli 075** was consistently demonstrated under the carefully standardized conditions. In all the experiments **E. coli 075** showed an invasiveness equivalent to **E. coli 08** when animals were challenged individually with **E. coli 08** and **075** (Figure 38). When the
FIGURE 38.

Capacity of \textit{E.coli 075} and \textit{E.coli 08} to establish retrograde renal infection. All animals in the 3 groups studied at each time interval were challenged simultaneously with \textit{E.coli 08}, \textit{E.coli 075} and mixed inoculum containing both organisms.
log No. of bacteria per g of kidney

- 14 days
- 3 days
- 21 days
- 7 days
results of the dual experimental challenges with both E.coli 075 and 08 were analysed, complete exclusion of E.coli 075 by
the E.coli 08 inoculum was found 3 days after infection. Similar results were also found 7, 14 and 21 days after chal-
lenge where E.coli 08 was still the predominant organism isolated. The two linked points 21 days after infection were
from the kidneys of a single animal; one kidney was infected solely with E.coli 08 and the other with E.coli 075.

Ability of resident renal pathogen to determine the pattern of recurrent infection
In the previous experiments we observed that although both
strains of E.coli were equally nephropathogenic, E.coli 08 was
able to prevent E.coli 075 from invading the kidney. The ex-
periments did not determine whether bacterial interference took
place in the bladder or in the kidney itself. In this experi-
ment unilateral pyelonephritis was induced in a group of 24
rats by the direct inoculation of E.coli 08 into the left kid-
ney. Once infection was established, animals were treated
from day 7 to 17 with a marginally effective antibiotic regimen
that eliminated the infection in some animals but left residual
E.coli 08 in others. A sterile thermal injury to the contra-
lateral kidney was carried out and served as a control, simula-
ting the damage produced by the pyelonephritic lesion. Thirty
days after infection all the animals were rechallenged by the
retrograde route with E.coli 075. The effect of residual renal
infection with E.coli 08 on the pattern of infection by the
challenging E.coli 075 was determined. The results have shown
that mixed infections in kidneys previously infected with
E.coli 08 were not encountered and the outcome of a retrograde
challenge by a second invading organism was dictated by the presence of an existing infection (Figure 39). In the group of animals sacrificed seven days after retrograde challenge, four of the six pyelonephritic left kidneys were still infected with *E. coli 08* after marginally effective antibiotic therapy. In the retrograde challenge *E. coli 075* was not able to establish infection in any of the *E. coli 08* infected kidneys but in contrast *E. coli 075* infection was readily established in five of the six contralateral uninfected right kidneys. Similarly *E. coli 075* infection was established in the two previously infected left kidneys where *E. coli 08* infection had been eliminated following antibiotic therapy (rats 5 and 6). Rat number 2 was of particular interest in that the right kidney was also infected with *E. coli 08* apparently following reflux of infected bladder urine from the initial infection and *E. coli 075* was excluded from both kidneys.

A similar pattern of infection was seen 14 days after retrograde challenge with *E. coli 075* where again *E. coli 075* infection was excluded from kidneys already infected with *E. coli 08* (left kidneys of rats 1, 2 and 3) but was readily established in the contralateral uninfected kidney of all six animals as well as the previously infected left kidneys from which infection had been eliminated (rats 4, 5 and 6). Twenty-one and 28 days after retrograde challenge, the same basic pattern of infection was still found and mixed infections in kidneys with a resident pathogen were not encountered.

Reciprocal experiments were then carried out where *E. coli 075* was used as a resident pathogen and the animals challenged with
Bacterial numbers in individual kidneys after pyelonephritis had been induced in the left kidney of all animals with *E. coli* 08 and treated with an antibiotic schedule that sterilized some kidneys but left others still infected with *E. coli* 08. Animals were then infected with a retrograde challenge of *E. coli* 075. The experiments have demonstrated the ability of a resident pathogen to determine the pattern of recurrent infection.
E. coli 08. The results confirmed the previous data demonstrating the ability of the resident pathogen to determine the pattern of infection.

In vitro analysis of bacterial antagonism between E. coli 08 and E. coli 075.

The previous experiments established that bacterial interference was a factor determining the localization and persistence of E. coli 08 and 075 in the kidney. The phenomenon was further investigated in vitro in an attempt to disclose the possible mechanism. Two individual 10 ml. samples of broth were inoculated with E. coli 075 and 08 and the growth determined by sampling the individual broth cultures 1 to 7 hours after inoculation. In a further experiment a broth sample was inoculated with a mixed culture of E. coli 08 and 075 and the growth curve for the mixed culture compared with E. coli 075 alone. In addition individual colonies from the mixed growth were subcultured and the ratio of E. coli 075 and 08 determined.

Growth curves for both the E. coli strains were very similar (Figure 40, left hand figure, top curves) and even when a mixed inoculum of E. coli 075 and 08 was used a growth curve similar to E. coli 075 alone was obtained (Figure 40, left hand figure, bottom curves). The ratio of E. coli 08 and 075, however, was altered considerably during the period under study and demonstrated the capacity of E. coli 08 to suppress the growth of E. coli 075 in vitro as well as in vivo. One hour after inoculation equal numbers of E. coli 08 and 075 were present in the broth but subsequently preferential replication of E. coli 08 occurred. At the end of the seven hour period, 74% of the
FIGURE 40.

In vitro analysis of antagonism between *E. coli* 08 and 075. Individual bacterial growth curves for *E. coli* 08 and 075 (left hand figure, top growth curves), and in another experiment *E. coli* 075 plus 08 and *E. coli* 075 alone for comparison (left hand figure bottom curves). The ratio of *E. coli* 08 to *E. coli* 075 showed that preferential growth of *E. coli* 08 had occurred in the mixed cultures (right hand figure) which initially contained equal numbers of both organisms.
organisms present were *E. coli* 08 (Figure 40).

**Effect of pH on bacterial growth**

To investigate the possibility that an adverse pH concentration might be responsible for the restricted growth of *E. coli* 075 found in the previous experiment, the pH of broth cultures inoculated with *E. coli* 08, 075 and 08 plus 075 was followed over a period of several hours. Ten ml. of unbuffered nutrient broth samples were inoculated with $1 \times 10^6$ viable bacteria and the pH of the samples determined at specified intervals after the cultures had been rapidly centrifuged at $4^\circ$C. to remove bacteria. Although growth of *E. coli* 08 did result in the production of a more acid medium than 075 (Figure 41) the differences between *E. coli* 075 and the mixed 08, 075 culture were not sufficient to explain the degree of preferential growth found in the preceding experiment.

**Colicin production as a factor determining restrictive growth in mixed infections.**

A final explanation considered was the possibility that one or even both *E. coli* strains carried a colicin active against the other strain. The suggestion is particularly relevant in the present situation as these antimicrobial substances are reactive only with strains of the same or closely related *E. coli* species. In these experiments, the putative colicin producer strain was stab inoculated into a Petri-dish containing nutrient agar. The plates were then incubated for 48 hours at $37^\circ$C. and the bacterial inoculum killed by exposing the Petri-dish to ether vapor at room temperature for one hour. A dilution of the indicator strain being tested for colicin susceptibility and
FIGURE 41.

The pH of broth cultures inoculated with E.coli 08, E.coli 075 and a mixed inoculum of E.coli 08 and 075.
containing $10^5$ bacteria was prepared in 0.9% nutrient agar and layered over the surface of the incubated Petri-dish. After incubation for 24 hours at $37^\circ$C., the confluent lawn of growth from the indication strain was examined for areas of lysis around the inoculum site of the producer strain. As a positive control a culture of \textit{E.coli} containing B and V colicins was tested using \textit{E.coli} K 12 as an indicator strain and zones of growth inhibition of up to 1 mm. diameter were found around the inoculum site of the colicin-containing strain. When the \textit{E.coli} strains used in the present experiments were tested against each other in a reciprocal producer-indicator situation there was no evidence of colicin production by either strain.

**DISCUSSION**

In these experiments, the role of bacterial interference as a factor affecting the epidemiology of urinary tract infection was investigated. When two unrelated strains of \textit{E.coli} 08 and 075 were introduced simultaneously into the kidney parenchyma by direct inoculation, mixed infections were found in the kidney up to 21 days after challenge. Animals challenged with a retrograde infection containing a mixed culture of the same organisms presented a different pattern and renal infection was most commonly caused by the 08 strain alone. Subsequent experiments showed that both strains of \textit{E.coli} were equally invasive as individual pathogens, but in a dual retrograde challenge with an inoculum containing both organisms, the 08 strain was the predominant organism isolated and mixed renal infections
with both *E. coli* 08 and 075, were rarely found. Further investigations showed that bacterial interference by a resident pathogen in the kidney dictated the pattern of retrograde infection. When a unilateral *E. coli* 08 infection was induced in one kidney prior to retrograde challenge with 075 it was found that 075 infection was established only in the contralateral uninfected kidney or in 08 infected kidneys where infection had been eliminated with an antibiotic. Infection with *E. coli* 075 was not found in kidneys already infected with *E. coli* 08. These results were confirmed in a reciprocal experiment where *E. coli* 075 was the resident pathogen and *E. coli* 08 the challenging organism.

Under experimental conditions, bacterial interference could have taken place in the glass capsule holding the inoculum, in the lower urinary tract or in the kidney. It was considered unlikely that bacterial interference occurred when the two *E. coli* strains were held as a mixed inoculum inside the glass capsule as we were not able to demonstrate any lytic activity of one strain for the other during a search for colicin-like antagonistic factors. Furthermore, equal numbers of organisms were found when the contents of the capsules containing a mixed inoculum were examined up to 48 hours after incubation at 37°C. and in the initial experiment both strains were found to be compatible when introduced into the kidney simultaneously. Some degree of bacterial interference occurring within the bladder has not been excluded but in view of the absolute correlation between the ability of an invading organism to establish infection and the bacteriologic status of the kidney,
bacterial interference within the kidney must be considered the prime mechanism determining the pattern of infection. This viewpoint is strengthened by the reciprocal relationship that was demonstrable in experiments where both *E. coli* 075 and *E. coli* 08 were established individually as resident pathogens in the kidney. In these experiments when an existing pathogen was resident in the kidney a second invading organism was excluded. However, uninfected kidneys or previously infected kidneys which had been sterilized by antibiotic treatment were readily invaded.

Other investigators have studied the effect of challenges with mixed organisms in experimental pyelonephritis. Guze, Hubert & Kalmanson (1969) used a mixed infection of *Streptococcus faecalis* and *Staphylococcus aureus* which are both capable of producing acute and chronic pyelonephritis after intravenous injection into manipulated animals. These results, following the induction of pyelonephritis by the simultaneous injection of both organisms, were similar to the results of the present experiments using different strains of *E. coli* introduced directly into the kidney. In both experiments mixed infections were established in the infected kidney.

In related experiments, however, it has not been possible to induce mixed infections even with the simultaneous inoculation of unrelated organisms. Rocha & De Almeida (1965) attempted to produce mixed infections using *E. coli*, *Pseudomonas aeruginosa*, and a coagulase negative staphylococcus but were unable to induce renal infection with more than one of the challenging species.
Gorrill (1958), studying the development of staphylococcal abscesses in mouse kidneys also found that a mixed challenge of two different strains of staphylococcus, identifiable by phage type, did not commonly cause a mixed abscess and one strain of staphylococcus had the ability to eliminate or exclude the other.

There is therefore adequate evidence of supporting bacterial interference as a factor influencing the establishment of infection in the renal environment, but because of the bacterial strains and the experimental models used, it has been difficult to relate many experiments to clinical renal infection. In our experiments, however, a model for retrograde infection was used which did not involve trauma to the upper urinary tract and closely approximates the natural history of the clinical disease.

A similar experimental situation has been investigated by Braude using a mixed infection with a colicin sensitive and a colicin producing strain of *E. coli* (Braude & Siemienski, 1968). When infection was induced in one kidney with the colicin sensitive strain and in the other kidney with the colicin producing strain, the colicin sensitive strain was eliminated from the urine in 25% of the animals and the degree of infection reduced in at least half of the animals. The experiments showed that one bacterial strain could eliminate the other from the bladder but had no effect on the course of infection in the kidney. In our experiments, however, there was no evidence to suggest that colicins were involved in restricting bacterial growth in the kidney although we were able to demonstrate in vitro, an
inhibitory effect of E. coli 08 on E. coli 075 when the two organisms were grown together in broth. A more likely explanation for the predominance of one strain and the failure to find mixed infections was that each organism was able to establish a micro-environment in the kidney that was unfavourable to the other. The effect that a member of a species may have on the growth characteristics of another organism of the same species in vivo has been infrequently studied on account of the obvious problem of identifying the individual organisms in mixed cultures. In the current experiments we were able to differentiate E. coli 075 and E. coli 08 using stable genetic markers so that the identification of up to 1,000 individual organisms from any one experiment could be performed without difficulty. With this typing procedure it was possible to study the effect of active renal infection with a micro-organism on a subsequent challenge with another organism of the same species.

An interesting clinical report has recently provided support for the concept of bacterial interference in urinary tract infection. In this case bilateral renal infection was confirmed by ureteric catheterization and a different strain of E. coli isolated in pure culture from each kidney (Koutsaimis & Roberts, 1971). The authors considered that the composition of the ureteric urine was the pathogen selective factor favouring the growth of the individual strains. The present studies have shown that this explanation is probably not correct but that a highly pathogen-selective micro-environment can be established within the kidney itself by a resident renal pathogen. A more likely explanation, therefore, is that renal invasion by the individual strains of E. coli occurred during separate episodes
of urinary tract infection. Subsequently, although the urine contained both pathogens the resident pathogen in the individual kidneys prevented a superimposed infection by the other strain.

Many studies have been carried out on the epidemiology of urinary tract infection and factors determining the biology of infection and re-infection have been investigated in depth. The current experiments were of interest as the pattern of infection was apparently determined by the resident pathogen infecting the kidney. This is clearly relevant in view of the persistence of infection in pyelonephritis and suggests that bacterial interference may be an important but unrecognised determinant in the epidemiology of urinary tract infection.
THE IMMUNOBIOLOGY OF PYELONEPHRITIS
In the introduction to these studies, a number of papers that have contributed to knowledge of the biology of pyelonephritis have been summarized and tabulated. In several of the more recent reports, investigators have addressed themselves to answering questions relating to the basic immunology of the immune response to renal infection. The writer's interest in the immunobiology of renal infection stems from observations made in Dr. Jay Sanford's laboratory by Drs. Lehmann and Smith demonstrating for the first time that a local immune response to infection occurred within the infected kidney. In subsequent experiments, the writer used a localized-haemolysis-in-gel procedure to study the cellular kinetics of the specific immune response to infection and related this response to circulating antibody and the local immune response within the kidney (Figures 42 and 43). A continuing interest in the biology of the immune response to renal infection was stimulated by the observation of small lymphocytes and plasma cells migrating into the kidney through the renal blood vessels. Histologically the perivascular cuffing in the kidney resembled the peri-arteriolar lymphocyte sheaths seen in the spleen and provided an encouraging morphological basis for investigating the immunobiology of lymphocyte function during the local immune response to renal infection (Figures 44 and 45). Other factors that have maintained interest in this topic have been the development of procedures for differentiating B and T lymphocytes and the development of in vitro methods for quantitating the potential functional capacity of T
lymphocytes. In the experiments that follow, several aspects of the immunobiology of pyelonephritis have been investigated.
FIGURE 42.

Plaque forming areas of antibody-producing cells in cryostat sections of normal and pyelonephritis kidney. 10 μ sections were laid on agarose containing complement and SRBC sensitized with *E. coli* 075 endotoxin.

(A) A section of whole kidney from a normal rat showing a faint but definite impression of the kidney underlying the section.

(B) Confluent haemolysis due to the presence of circulating antibody in kidney sections during the acute phase of infection.

(C) Wedge shaped sections of antibody forming cells that coincide with areas of lymphocytic infiltrate in pyelonephritic kidneys during the subacute and chronic stages of the disease.

(D) *H & E* stained cryostat sections of pyelonephritic kidney adjacent to the previous section showing the clearly defined areas of pyelonephritis and lymphocytic infiltrate.
FIGURE 43.

Plaque-forming areas in the kidneys of rabbits with locally induced bacterial infection demonstrating antibody forming cells synthesizing specific antibody. Note in particular the accumulation of antibody in the pelvic space of the kidney.
FIGURE 44.

Peri-arteriolar-lymphocyte-sheaths in the spleen.
(x 100, 400, 600 from top to bottom frame).
FIGURE 45.

Lymphocyte mobilization into the pyelonephritic kidney. The perivascular lymphocyte cuffing in these photomicrographs resembles that seen in the spleen in the previous plates (x 100, 400 and 600). Perivascular cuffing was only found within the lesion or immediately adjacent to the pyelonephritic wedge. In these cases streaming of the lymphocytes from the blood vessel towards the infected region was a notable feature.
ACQUIESCENT INFECTION AS A FACTOR IN PYELONEPHRITIS
INTRODUCTION

The persistence of active infection in the kidney is a characteristic feature of pyelonephritis in both human disease and some experimentally induced renal infections. Despite early convictions that chronic renal infection led to chronic pyelonephritis, the association of continuing infection with progressive loss of renal function has been challenged (Bailey, 1973; Angell, Relman & Robbins, 1968; Beeson, 1971). Recent procedures that allow the localization of infection have been of value in determining the site of infection, but details of the relationship between the presence of an infectious organism and the development of pathological lesions have been difficult to acquire. When the effect of infection on renal function has been followed, the results have shown that infection in an otherwise normal urinary tract does not result in a deterioration of renal function (Bullen & Kincaid-Smith, 1970; Gower, 1972).

As has been mentioned earlier, there is evidence in the literature that infection of the kidney is not synonymous with pathological changes, although this has been difficult to prove in man because of the patchy distribution of the lesions and the inconclusiveness of a normal renal biopsy. In these experiments the relationship between the establishment of infection and the development of pathological changes in the kidney have been examined, using an animal model.

A host-parasite relationship has been disclosed where the organism established an active infection in the renal
parenchyma, but infection alone did not usually lead to pathological changes in the kidney. It is suggested that the term "acquiescent infection" be used to describe the host-parasite relationship in this situation where a balance is maintained between the host and the invading organism.

**MATERIALS AND METHODS**

**Animal strain**

Female rats weighing 220 to 230 g. were obtained from an inbred HS (hooded) strain of rat obtained from Dr. Barbara F. Heslop, University of Otago Medical School, Dunedin, New Zealand, and maintained in this Department by consecutive brother-sister mating. The decision to use an inbred rat strain was made to eliminate the occasional non-immune-responder rat encountered in the random bred Wistar strain.

**Bacterial strain**

The strain of *E. coli* 075 used in these experiments was the same as that used in the previous studies.

**Production of renal infection**

Pyelonephritis was induced by the direct inoculation of *E. coli* into the surgically exposed kidney using a glass micro-capillary. Details of the method have been given previously in Chapter 2.

**Bacterial content of renal tissue**

Nutrient agar pour plates of serial ten-fold dilutions of homogenized kidney were made to obtain the bacterial count per gram of wet renal tissue.

**Histological processing**

Tissue for histological examination was placed directly into a
0.1% solution of cetylpyridinium chloride in 10% formaldehyde. After routine processing and cutting, sections were stained with haematoxylin and eosin.

Collection of blood for antibody titres
Blood was collected from the tail of lightly anaesthetized rats directly into micro-centrifuge tubes. After centrifugation, the serum was removed and stored at -20°C. until analyzed.

Determination of serum antibody in the autoanalyzer
Serum antibody levels were determined by an automated procedure using a Technicon autoanalyzer adapted to determine the level of antibacterial antibody. The method is essentially a passive haemagglutination procedure using sheep-red-blood-cells coated with endotoxin as a source of "O" antigen. With this procedure antibody titres are reported on a continuous scale and are not restricted to the limitations of serial dilution end points. The results recorded in Table 9 are the reciprocal of the highest dilution of serum showing detectable agglutination in the autoanalyzer. Full details of the method are given in Chapter 15.

Dissection of the kidney into cortex, medulla and papilla
The medulla with attached papilla was removed from each kidney with a hollow cylindrical blade of 7 mm. internal diameter. Cortical tissue on each side of the medullary cylinder was removed and the remaining tissue cut at the line of demarcation between medulla and papilla. The crescent of the cortex left after removal of the medullary cylinder was then trimmed to remove residual medullary tissue (see Chapter 2 for details). Each piece of tissue was weighed and homogenized in
physiologically normal saline. Agar pour plates of dilutions of the homogenate were made to obtain the bacterial count per gram of tissue.

**Measurement of maximum urine concentrating capacity**

All animals had food and water removed 24 hours before being placed in a metabolic cage specifically designed for urine collection. At the end of the 24 hour period, animals were forced to micturate, using a whiff of ether in a nose cone, before being placed in the metabolic cage still without food or water. All urine passed overnight was collected under paraffin to prevent evaporation. At 9 a.m. the following morning, they again micturated and this urine was added to the overnight collection. Urinary osmolality was measured in a Knauer osmometer after preparing a 1:4 dilution of the urine in water.

**Antimicrobial therapy**

When necessary, infection in the kidney was eliminated by treatment with gentamicin for ten days at a dosage of 20 mg./kg./day. In some experiments, intragastric nalidixic acid treatment was used at a dosage of 250 mg./kg./day for ten days. The *E. coli* 075 strain used in these experiments was sensitive to 1.0 μg./ml. of gentamicin and 1.6 μg./ml. of naladixic acid.

**RESULTS**

**Experimental pyelonephritis**

Unilateral pyelonephritis was induced in 34 rats by the direct inoculation of *E. coli* into one kidney and the animals maintained under normal laboratory conditions for up to six months. Characteristic features of the experimental infection in the
pyelonephritic kidney have been described in Chapter 2. The natural history of the infection in the contralateral unmanipulated kidney was studied in two groups of animals which were sacrificed 2 and 6 months after the induction of unilateral infection. At the time of death, the gross pathology, histopathological changes and bacteriological features of infection in the contralateral and pyelonephritic kidney were compared. The most notable feature was the lack of correlation between bacterial growth, the gross pathology and histopathological changes in the kidney (Figure 46). Although the method of inducing pyelonephritis involved minor local trauma, when killed bacteria were introduced instead of viable organisms, both the gross and histopathological changes were minimal and were confined to the microcapillary tract.

In the pyelonephritic kidney into which bacteria had been directly introduced, scarred and contracted kidneys were found showing the characteristic features of pyelonephritis. Similar numbers of bacteria were present in the contralateral kidney but in contrast the pathological changes were minimal. The term "acquiescent infection" has been introduced to describe the situation where bacterial invasion of the kidney is not associated with pathologic changes.

Histopathological changes in the pyelonephritic kidney consisted of depressed cortical scars with glomerular crowding, an intense lymphocytic infiltrate and interstitial fibrosis. In contrast, histopathological changes in the contralateral kidney were only occasionally seen despite the presence of bacterial infection. These changes consisted of minor
FIGURE 46.

The gross pathology, histopathological and bacteriological features of infection in the pyelonephritic and contralateral kidney 2 to 6 months after the induction of a unilateral infection in the pyelonephritic kidney only. Quantitation of the degree of renal damage formation. Assessment was made on a 0-10 scale with each point on the scale representing one-tenth of the surface of the kidney.

Histopathological assessment of the degree of renal damage. On the scale used, 10 represented lesions involving 25% or more of the area of the section. Lesser degrees of damage were scored on a 0-10 scale where each unit represented 2.5% of the area of the kidney (see Chapter 2).
inflammatory foci confined to the fornices, but in two animals, these foci extended to the cortex to produce a fine wedge-shaped lesion.

Determination of the distribution of bacteria in the pyelonephritic kidney and the contralateral kidney

Unilateral pyelonephritis was induced in a further group of six animals and the infection allowed to proceed for six months. At the time of sacrifice, both the pyelonephritic and the contralateral kidney were dissected into cortical, medullary and papillary tissue and the bacterial content of each region determined. Macroscopic differences between cortical, medullary and papillary tissues were quite distinct so that the spongy medulla and papilla could readily be separated from cortical tissue.

In these experiments bacteria were found to be evenly distributed throughout the contralateral kidney and were not confined to the renal pelvis or medulla (Figure 47). The presence of equivalent numbers of organisms in renal cortex, medulla and papilla of the contralateral kidney suggested that the invading organisms had penetrated into the interstitium of the kidney.

The effect of acquiescent infection on the renal concentrating capacity

Unilateral pyelonephritis was induced in a group of nine animals and the infection allowed to proceed for eight weeks. A further group of 12 matched animals was used as controls. The pyelonephritic kidney and an unmanipulated kidney in the control group was then removed and the urine concentrating capacity of the remaining determined two and three months later.
FIGURE 47.

Distribution of bacteria within the pyelonephritic and contralateral kidney.
The ability to form a concentrated urine was significantly impaired in the animals with acquiescent infection ($P < 0.001$) when this function was compared with control animals with a sterile kidney (Figure 48).

When the animals were sacrificed, two of the nine animals from the acquiescent infection group were found to be sterile. One of these had a concentrating capacity of 1988 mOsm./kg. and the other a maximum of 1,600 mOsm./kg. The remaining animals in the group were all infected and an average bacterial count of $2.3 \times 10^5$ organisms per gram of kidney was found. All kidneys from the control group were sterile.

**Effect of luminal sterilization on bacterial numbers in the pyelonephritic and contralateral kidney**

To investigate the intrarenal location of bacteria in the contralateral kidney, unilateral pyelonephritis was induced in three groups of six animals and the infection allowed to proceed for three months. At the end of this period, one group was treated daily for ten days with the urinary antiseptic, nalidixic acid, and the animals sacrificed seven days after the end of treatment. A further group of animals was treated with gentamicin, 20 mg./kg./day for ten days, a treatment schedule that was known to eliminate effectively infection from the renal parenchyma. The third group of animals was left untreated and served as a control group. The kidneys of all three groups of animals were examined for residual infection and the bacterial content of the pyelonephritic and contralateral kidneys determined.
FIGURE 48.

Maximum urine concentrating capacity in animals with acquiescent renal infection compared with control animals, 2 and 3 months after unilateral nephrectomy.
Two months

Three months

Control - Acquiescent infection

Control - Acquiescent infection

Max. urine conc. capacity mOsm / kg
In these experiments, gentamicin was able to eradicate an established infection while nalidixic acid had no effect on the number of bacteria in either the pyelonephritic or the contralateral kidney although the infecting organism was sensitive to less than 2 \( \mu g./ml \) of nalidixic acid (Figure 49).

**Induction of bacterial proliferation in the contralateral kidney following renal injury**

Data reported above has shown that large numbers of bacteria were present in the contralateral unmanipulated kidney although the pathologic changes were minimal. Further experiments were carried out to determine whether the host-parasite relationship could be altered by intrarenal obstruction. Unilateral pyelonephritis was induced in two groups of 18 rats and three months after the initiation of infection, the contralateral kidneys of one group were thermally injured in three sites using 23 gauge hot nichrome wire. When the effect of thermal injury on the bacteriological status of the contralateral kidney was examined seven days after injury, a remarkable increase in bacterial numbers had occurred in the thermally injured kidney. Whereas the number of organisms in the pyelonephritic kidney and the unmanipulated contralateral kidney in the first group were again found to be similar, bacterial numbers in the thermally injured contralateral kidney of the second group were increased up to 100 times that of the non-manipulated contralateral kidney (Figure 50).

Histologic sections of the thermally injured kidney were also examined. Although most of the thermally injured kidneys had shown a marked increase in bacterial proliferation, there was
FIGURE 49. The effect of sterilization of the renal tubular lumen with nalidixic acid treatment on bacterial numbers in the pyelonephritic and contralateral kidney.
Induction of bacterial proliferation in the contralateral kidney by renal trauma. Animals were sacrificed 7 days after thermal injury and the bacterial numbers determined in the thermally injured contralateral kidney, pyelonephritic kidney and a group of manipulated contralateral kidneys.
BACTERIOLOGY

![Graph showing bacterial load in different kidney conditions.](image)

- **Pyelonephritic kidney**
- **Contralateral kidney**
- **Contralateral kidney**

- **Direct infection**
- **Non manipulated**
- **Thermal injury**

Log No bacteria per g of kidney
histologic evidence of a pathologic lesion being established in only five of the 15 kidneys with bacterial proliferation. Kidneys from the remaining 13 animals with no inflammatory foci discernible histologically showed only a mild non-specific response to trauma similar to that seen following thermal injury to sterile control kidneys.

Immunologic status of the host during the activation of acquiescent infection

Serum antibody levels in rats with chronic renal infection remain at a raised level for prolonged periods after the initial infection has subsided (Miller & North, 1971), but the effect of activation of bacterial proliferation on the concentration of serum antibody is not known. To investigate this question, unilateral pyelonephritis was induced in two groups of ten animals and the source of the disease allowed to proceed for three months. Serial determinations of serum antibody levels were carried out, using the automated procedure, prior to the induction of bacterial replication in the contralateral kidney of one group by thermal injury. Serum samples were obtained from both groups 3, 7 and 10 days after the activation of infection and serum antibody levels again determined in the autoanalyzer.

Although increased bacterial proliferation occurred in all animals with thermal injury to the contralateral kidney there was no consistent increase in serum antibody levels. Five out of ten individual animals showed an increase in serum antibody after thermal injury but similar increases were seen in three control animals so that the significance of the increase in
TABLE 9. Immune response of the host during the activation of acquiescent infection
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<th>post-thermal trauma (days)</th>
<th>assessment*</th>
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<td>10</td>
<td>521</td>
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*NSI = no significant increase after thermal injury

SI = significant increase. An increase in serum antibody concentration of 500 or more titre units, between the final pre-thermal trauma-post-thermal trauma serum antibody concentration was taken as a significant increase.
the five test animals remains doubtful (Table 9).

**DISCUSSION**

A cause and effect relationship between infection and the pathologic changes characteristic of chronic pyelonephritis was accepted for several decades. Early studies claiming that up to 16% of patients examined at post-mortem had histological evidence of pyelonephritis (Macdonald et al., 1957) have been challenged (Pawlowski, Bloxdorf & Kimmelstiel, 1963) and the relationship of bacterial infection alone to chronic progressive pyelonephritis remains in doubt (MacGregor, 1970). The frequency of bacterial invasion of the kidney (Fairley et al., 1971; Stamey, Govan & Palmer, 1965) and the relative infrequency of associated chronic pathologic lesions illustrate the outcome of a host-parasite relationship that has been difficult to investigate in man and has not been clearly defined in the experimental animal.

Views of the host-parasite relationship in renal infection may well be influenced by the current experiments where unilateral pyelonephritis was induced by the direct inoculation of bacteria into one kidney only. Subsequent events leading to the establishment of infection in the contralateral unmanipulated kidney provided a unique model in that no manipulation of the contralateral kidney or the urinary tract was necessary. Under these circumstances, the establishment of infection in the contralateral kidney, it is believed, represents the natural history of renal infection in these animals and mimics many of the clinical and pathologic features of renal infection in man.
Using this model, it has been possible to investigate the relationship of the invading organism to the host and the association between the presence of viable bacteria in the kidney and the development of pathologic lesions. The most striking feature was the poor correlation between bacterial infection and the degree of pathologic changes in the kidney. Although renal tissue must still be considered unusually susceptible to persistent infection, the association of renal infection with consistent pathologic lesions is not tenable.

The finding that a high level of infection was readily established in the contralateral unmanipulated kidney was at variance with our previous studies using a unilateral pyelonephritis where the contralateral kidney remained sterile (Miller & North, 1971). The original experiments were carried out using a random bred strain of Wistar rat and the capacity of the organism to establish an acquiescent infection was only disclosed when hooded rats were used in an attempt to establish the experimental model in an inbred strain of rat.

With this inbred strain, acquiescent infection has been found in 100 out of 114 contralateral unmanipulated kidneys examined to date, and clearly demonstrate that this is a consistent feature of renal infection in these animals. The strain of hooded rat used in these experiments is widely used in transplantation studies but does not have any specific characteristics relevant to the present experiments. The phenomenon is not unique, however, and although this is the first occasion where such a host-parasite relationship has been described in detail, there are a number of reports in the literature recording
similar findings. Freedman (1967) noted that it was remarkable that large numbers of *E. coli* could be recovered from experimentally infected kidneys without evidence of pathological changes and the results of studies by Prat et al. (1964), Freedman & Beeson (1961), Carone et al., (1959) and Woods et al. (1960) all contain evidence of acquiescent renal infection in animals challenged by the retrograde route. Continuing investigations have also established that acquiescent infections can be found when strains of *E. coli* other than the one used in the present experiments are used to induce pyelonephritis.

From the evidence available, bacteria in the contralateral kidney appeared to have penetrated into the renal parenchyma and were not simply in the tubular lumen. Nalidixic acid was selected as a urinary antiseptic to sterilize the luminal contents on account of its protein binding (93%), negligible activity before excretion in the urine (Portmann et al., 1966) and the sensitivity of the infecting organism to low concentrations of nalidixic acid (1.6 μg./ml.). The failure of nalidixic acid therapy to affect bacterial numbers in the kidney strongly suggests that the bacterial cells are present in the renal interstitium in both the pyelonephritic and contralateral kidney and are consequently inaccessible to urinary antiseptics. Gentamicin, on the other hand, is rapidly and uniformly distributed in blood and tissue (Chisholm, Calnan & Waterworth, 1968) and although the sensitivity of the *E. coli* strain to gentamicin was similar (1 μg./ml.) to nalidixic acid, infection was readily eliminated from the kidney with gentamicin treatment. Most experimental retrograde models involve distension of the bladder with a heavy suspension of bacteria which is then forced
up the ureter into the kidney by pressure on the bladder. Vesico-ureteric reflux, however, occurs normally in the rat and the acquiescent infection established in the current experiments is more likely to have occurred as a result of constant but gentle reflux of infected urine.

Once a pyelonephritic lesion is established in the kidney, the host responds with a vigorous and well characterized immune response which appears neither to control the infection nor the result in an extension of the pathological process. The host seems to have become tolerant to a state of continuing infection in a normally sterile tissue and is incapable of eliminating the invading organism.

The term "tolerance" has a well established immunological meaning and it is suggested that the term "acquiescent infection" be used to describe the host-parasite relationship in this situation where the balance is maintained between the host and the invading organism, without local pathological lesions.

Although a causal relationship between bacterial infection of the urinary tract and the development of radiological changes of chronic pyelonephritis as documented by Smellie & Normand (1968) is generally recognised, there are many inconsistencies. Where patients with persistent renal infection have been followed, deterioration of renal function has not occurred and even where upper urinary tract infection has resulted in a concentrating defect this has been reversible both in man (Clark et al., 1969) and experimental animals (Kaye & Rocha, 1970).
A similar lack of correlation between bacterial invasion and renal pathology was found in a study of renal disease in diabetics (Halverstadt, Leadbetter & Field, 1966) and the normal renal function in most patients with ureterosigmoidostomy (Jacobs, 1967) and patients with indwelling catheters for up to 15 years (Talbot, Mahoney & Jaffee, 1959) supports the concept of an altered host-parasite relationship.

Acquiescent infection resulted in a substantial reduction in maximum urine concentrating capacity although it was initially believed that the acquiescent state represented a relatively benign condition. In attempting to explain the biological basis for an acquiescent state in renal infection, the role of the specific immune response has been re-examined. One possibility is that the invading organism may be utilizing the immune response to induce an acquiescent state and so escape host defence mechanisms. This pathogenic mechanism of immunologic enhancement has been well studied in tumour immunology (Feldman, 1972) although it has not yet been applied to microbial persistence. Immunological enhancement as a factor in acquiescent infection has been investigated in the current experiments by manipulating the immune response of the host with the immunosuppressive drug cyclophosphamid (Chapter 14). A remarkable inverse relationship between the immunocompetence of the host and the ability to clear bacteria from the kidney was found and the data suggested that the immune response led to the establishment of an acquiescent state that protected the invading micro-organism from normally effective host defence mechanisms.

The effects of activation of acquiescent infection on the
immune status of the host was of interest in view of the reliance that has been placed on serum antibody levels to indicate infection of the renal parenchyma in man (Percival, Brumfitt & de Louvois, 1964). In these experiments a consistent antibody titre was maintained for several weeks before the thermal injury and the activation of infection. Although increased bacterial proliferation was observed in all animals, there was no consistent increase in serum antibody levels. The results support earlier reports where circulating antibody levels did not reflect the course of the disease in chronic infection (Miller & North, 1971; Miller & North, 1973) and provide a ready explanation for the poor correlation between clinical renal infection and serum antibody response (Fairley et al., 1971).
CHAPTER 9

THE IN VITRO STIMULATION OF RAT LYMPHOCYTES

BY PHA AND OTHER MITOGENS
Although normal lymphocytes from a number of species have been cultured successfully, the maintenance of rat lymphocytes in culture has not been readily achieved. Schrek & Rabinowitz (1963) noted that while human lymphocytes could be stimulated to form typical blast cells by phytohaemagglutinin (PHA), rat lymphocytes did not respond. Similarly, Ling & Husband (1964) showed that staphylococcal protein induced a PHA-like transformation in human peripheral blood lymphocytes, but rat lymphocytes were again non-responsive.

Recently described conditions for the mitogenic stimulation of mouse lymphocytes (Adler et al., 1970) were also unsuccessful in our hands when applied to rat lymphocytes, and from other reports it appears that even when lymphocyte cultures from rats have been established, massive amounts of PHA are required to stimulate mitogenesis (Meuwissen, Van Alten & Good, 1969).

In planning the experiments in this section, it became evident that some quantitative measure of potential T cell function was essential to substantiate the experimental manipulations of T lymphocyte population. No reliable method was found in the literature and in this chapter, a method of culturing rat lymphoid cells in vitro and of measuring the response to phytohaemagglutinin and other mitogens is described.

MATERIALS AND METHODS

Tissue culture medium: throughout these experiments, the
tissue culture employed for culturing the cells was a complete medium designated Roswell Park Memorial Institute (RPMI) 1640 obtained from Grand Island Biological Company, Grand Island, New York. All media contained 100 units of penicillin and 100 μg of streptomycin per ml. Two preparations of the RPMI 1640 were used in the culture system. One termed the "insert" medium was supplemented with rat serum and the other, the reservoir medium, consisted of RPMI medium alone. To prepare a stock solution of the medium at two times normal concentration, a 5-litre pack was dissolved in distilled water with prolonged stirring and made up to a final volume of 2,500 ml. CO₂ was bubbled through the preparation until the medium was yellow and the medium was then filtered through a 0.45 μm cellulose acetate filter. To prepare the reservoir medium, 500 ml. of the two times concentrated RPMI 1640 medium were added to 429 ml. of sterile distilled water and the pH adjusted with 71 ml. of sterile 2.8% sodium bicarbonate. The "insert" medium for routine use was prepared by adding 38 ml. of water, 7 ml. of 2.8% sodium bicarbonate and 5 ml. of rat serum to 50 ml. of the two times concentrated RPMI medium. Eagle's minimal essential medium buffered with HEPES to a final pH of 7.2 (HEPES-MEM) was used for the preparation of lymphocytes prior to their being resuspended in "insert" medium.

Culture vessel: an in vitro tissue culture system essentially as described by Marbrook was used (Marbrook, 1967). The insert tube consisted of a 100 mm. piece of glass tubing of 9 mm. internal diameter, one end of which was sealed with a cellulose dialysis membrane (Visking Co., Union Carbide Corp.) held in place with a ring made from non-toxic silicone rubber
tubing. The tube fitted into a rubber bung which was inserted into a glass reservoir tube. An 18 gauge needle was pushed through the bung into the interior of the culture tube and acted as a breather inlet. All glassware was soaked overnight in a solution of Pyroneg (Diversey Wallace Ltd.) and rinsed several times in running tap water, deionized water and distilled water before drying and assembling.

Preparation of lymphocyte suspensions: lymphocytes were obtained by removing the spleen, thymus and lymph-nodes under sterile conditions into HEPES-MEM. Each tissue was gently teased through a 60 mesh stainless steel sieve. After two washes in HEPES-MEM the lymphocytes were suspended in the insert medium and 1 ml. containing 5 x 10^6 lymphocytes pipetted into the insert tube. 10 ml. of the reservoir medium was then added to the reservoir vessel and the cultures incubated in an atmosphere of 10% CO_2 and air. All cultures were prepared in triplicate.

Mitogenic stimulation: phytohaemagglutinin (Wellcome Reagents Ltd.) was used as supplied and suspended in 5 ml. of sterile physiological saline. 15 μl were added per tube except where indicated in the text. In some experiments pokeweed mitogen (PWM) (Grand Island Biological Co.), tuberculin (PPD) (Commonwealth Serum Lab., Melbourne, Australia) and a lipopolysaccharide extracted from selected Gram negative bacilli (LPS) essentially as described by Webster et al. (1955), was used as a mitogen.

Tritiated (³H) thymidine incorporation assay: 1.0 μCi of ³H thymidine (The Radiochemical Centre, Amersham) was added to
the insert tube of each culture for the final 16 hours of incubation. At the same time, the reservoir medium was discarded to prevent dilution of the isotope. At termination, the contents of the insert tube were washed through a Whatman GFC 25 mm. fibreglass disc held in a machined metal block. The contents of the insert were rinsed with a further volume of distilled water and finally with 5% and 10% trichloracetic acid to precipitate macromolecules retained on the fibreglass filter. Filters were transferred to glass vials for scintillation counting and dried at 37°C for 24 hours. 10 ml. of scintillation fluid (8 g. PPO, 200 mg. POPOP, 600 ml. of Triton X 100, 1400 ml. of Toluene) was then added and the vials counted in a Beckman Model B liquid scintillation spectrometer. The amount of $^3$H thymidine incorporation was finally reported as disintegrations per minute per $10^6$ lymphocytes (Figure 51).

Production of T cell depleted experimental animals by thymectomy and sublethal irradiation

Female rats weighing between 150 g. and 175 g. were thymectomized under direct vision using a split sternum approach and maintenance of respiration with a rodent respirator. After a two-week recovery period, the thymectomized animals were given 250 rads from a $^{60}$Co source at 150 rads per minute and the dose repeated at intervals of two weeks until a total of 1,000 rads had been given. Experiments were initiated two weeks after the final irradiation (Harding et al., 1971).
FIGURE 51.

Details of the method used to culture rat lymphocytes in vitro and determine the ability of the lymphocytes to respond to P.H.A. in culture.
RESULTS

Optimum time during culture to add $^3$H thymidine
Cultures containing $5 \times 10^6$ splenocytes and 15 μl of PHA were prepared in triplicate and incubated at intervals from 24 to 96 hours before the addition of $^3$H thymidine to the culture. Sixteen hours later, the cultures were processed in the routine manner and the amount of $^3$H thymidine incorporation determined. The results are shown in Figure 52. The highest rate of incorporation was found when PHA stimulated cultures were incubated for 72 hours before the addition of tritiated thymidine.

Optimum amount of PHA for lymphocyte stimulation
Increasing amounts of PHA from 2.5 μl to 25 μl. were added to each culture of $5 \times 10^6$ splenocytes. $^3$H thymidine was added after 72 hours incubation and the amount of incorporation determined. All the concentrations of PHA used resulted in a considerable mitogenic response although the greatest response was seen with highest concentration of PHA (Figure 53). In view of the plateau achieved with the lower concentrations of PHA, 15 μl. was chosen for routine use.

Incorporation of $^3$H thymidine
Further cultures containing $5 \times 10^6$ splenocytes were incubated for 72 hours before the addition of 1.0 μCi. of $^3$H thymidine. Incorporation of the isotope was allowed to continue for intervals of 4 up to 24 hours before being terminated. The amount of isotope incorporated reached a peak after eight hours, but because the 16-hour period was more convenient and gave the same degree of incorporation, this interval was used in future
Optimum point during culture for the addition of $^3$H thymidine. 1 μCi of $^3$H thymidine was added to cultures of $5 \times 10^6$ splenic lymphocytes at intervals from 24 to 96 hours after the culture was established.
FIGURE 53.

Optimum amount of PHA for lymphocyte stimulation in vitro. Increasing amounts of PHA from 2.5 to 25 μl. were added to each culture of 5 x 10^6 splenocytes.
Optimum number of lymphocytes for the culture vessel

Increasing numbers of splenocytes from $1.25 \times 10^6$ to $10 \times 10^6$ were cultured for 72 hours before adding $^3$H thymidine. The amount of incorporation over 16 hours was then determined. Incorporation of $^3$H thymidine increased in proportion to the number of cells added to the culture vessel and the ratio of $^3$H thymidine incorporation per $10^6$ lymphocytes remained steady (Figure 55). For the purpose of routine analyses, $5 \times 10^6$ lymphocytes were used in subsequent experiments.

Concentration of rat serum

Cultures containing $5 \times 10^6$ splenic lymphocytes were established in insert medium containing increasing concentrations of homologous rat serum from $1.25\%$ to $20\%$. $^3$H thymidine was added 72 hours later and the amount of incorporation over a 16-hour period was determined (Figure 56). The results have shown that a maximum response to PHA stimulation was obtained over a wide range of rat serum concentrations and for routine use a $5\%$ concentration of rat serum was used.

Confirmatory experiments

To confirm that the assay was a quantitative measure of T cell function, the PHA response of two groups, each of seven rats, was determined. Thymectomized and T cell depleted animals were prepared and a group of sham thymectomized and unmanipulated animals were also analyzed as controls. The mean PHA responsiveness of splenocytes from T cell depleted animals was only $4.2\%$ of the control group whereas splenocytes from sham thymectomized animals gave a mean response which was $93\%$
Optimum time for the incorporation of $^3$H thymidine. Incorporation of the isotope by cultures of $5 \times 10^6$ splenocytes was allowed to continue for intervals of 4 to 24 hours before being terminated. The filled circles are the results of control cultures which lacked PHA.
FIGURE 55.

Number of lymphocytes per culture. Increasing numbers of splenocytes from $1.25 \times 10^6$ to $10 \times 10^6$ were cultured. In the figure on the left, the incorporation of $^3$H thymidine per culture is presented while on the right hand side, the results from each culture have been normalized to give DPM/$10^6$ lymphocytes.
FIGURE 56.

Concentration of rat serum in insert medium. 5 x 10^6 splenocytes were cultured in insert medium containing concentrations of rat serum from 0.3 to 20%.
FIGURE 57.

Response of splenic lymphocytes to phytohaemagglutinin (PHA), pokeweed mitogen (PWM), tuberculin (PPD) and the lipopolysaccharides (LPS) of *E.coli* and *S.marcescens*. 
of the control group.

Response to mitogens other than PHA
PHA has been well characterized as a T cell mitogen whereas B cells in some strains of mice have been shown to respond to lipopolysaccharides from the gram negative bacilli and to PPD. A series of concentrations of lipopolysaccharides from E. coli 075 and S. marcescens and two other mitogens (PWM, PPD) were used in an attempt to stimulate splenocytes and establish the responsiveness of lymphocytes from our rodent strain to these agents. The results are shown in Figure 57. PHA and PWM both resulted in considerable stimulation of splenic lymphocytes, but the addition of the bacterial LPS and PPD in varying concentrations failed to stimulate a significant blastogenic response.

Variability of the PHA response
Forty-one cultures of splenic lymphocytes were carried out in triplicate from normal animals to determine the variation of the PHA response. The mean of the three cultures from the individual animals was taken as the PHA response of the individual animal. An average of 58,000 (SD, 18,000) DPM/10^6 lymphocytes was found and a similar variation was found when 15 cultures, again in triplicate, were carried out using splenic lymphocytes from one animal only.

DISCUSSION
The assessment of the response of lymphoid cells to PHA and other mitogens can best be carried out by culturing the cells in vitro and observing the effects of the mitogen on the
incorporation of tritiated thymidine by the dividing lymphocytes. Rat lymphocytes, however, have been difficult to culture and in these experiments, a simple but reliable method for the culture and mitogenic stimulation of these cells has been developed. Confirmatory experiments have also been carried out using lymphoid cells from thymus cell ablated animals which show that the method does measure T cell function.

Three factors seemed to be of particular importance and the first of these concerns the culture vessel. A variety of convenient systems such as disposable test tubes and microtitre plates have been used for culturing lymphocytes from other species, but we believe that the membrane and reservoir system devised by Marbrook (1967) provides the optimum conditions for the survival and replication of rat lymphocytes. This culture vessel is inexpensive and can be reused, although care must be taken to ensure that it is adequately washed and carefully assembled. The source of the serum supplement added to the insert medium is also important and a poor PHA response was found when either foetal calf serum or human serum was used to replace homologous rat serum. In all our experiments the serum used in the culture medium was obtained from the rat whose lymphocytes were being cultured but this was not necessary and pooled rat serum was successfully used on occasions. The greatest PHA responses were obtained with RPMI medium and consistently satisfactory cultures were obtained throughout this study and in subsequent experiments using this medium.

The interval between the establishment of the culture and the addition of $^3$H thymidine was also important as was the number
of lymphocytes added to the culture vessel when the amount of $^3$H thymidine incorporated per culture was a consideration. The amount of $^3$H thymidine incorporated per $10^6$ lymphocytes, however, was not influenced by the number of lymphocytes cultured and $5 \times 10^6$ cells were used routinely.

Other factors such as the most appropriate amount of PHA to add, the length of time to allow for $^3$H thymidine uptake and the amount of rat serum in the insert medium were not critical and a plateau was reached after optimum concentrations had been attained.

Mitogens other than PHA were added to cultures of the rat lymphocytes. The addition of pokeweed mitogen resulted in considerable blastogenensis but the bacterial lipopolysaccharides from several Gram negative bacilli and PPD failed to stimulate lymphocytes from our rodent strain. The response of rodents to LPS has been shown to be under genetic control (Di Pauli, 1972; Sultzer, 1972) and this could also be a factor determining the ability of lymphocytes to respond to stimulation in culture.

Confirmation of the validity of the methodology for determining T cell function was adequately shown in experiments where functional T cells were reduced by adult thymectomy followed by serial sublethal irradiation to eliminate residual T cells. The PHA response of lymphocytes from the T cell ablated group was reduced to 3% of the normal response while the B cell response to thymus independent antigens remained unaffected (Chapter 13).
The reliability of the system has been established over the last 18 months when 6,000 cultures have been carried out. During this period, minor and intermittent contamination of individual cultures has been the only problem encountered.