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LEPTOSPIRA INTERROGANS SEROTYPE POMONA INFECTION :

A STUDY IN HOST-PARASITE RELATIONSHIPS

A thesis presented in partial fulfilment of the requirements  
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at

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## SUMMARY

A study has been made of the host-parasite relationship that exists between Leptospira interrogans serotype pomona and a mammalian host as exemplified by the sheep. Tissue culture studies provide evidence that serotype pomona is capable of elaborating a cytotoxin at a low level. Surprisingly there is no indication that the organism produces either lecithinase or hyaluronidase and it appears that the organism enters and migrates within host tissue mainly by direct mechanical insinuation. The organism does not appear to commonly penetrate cells either in tissue culture or the host and thus avoids exposure to lysosomal enzymes. Migration is mainly between cells and the organism is capable of passing between tight junctions. Although exotoxin production does not appear to be of major importance in the migration of the organism, ultrastructural studies of the kidney in both mice and sheep indicate that tubular epithelial changes result from toxic rather than mechanical damage. Limited experimental studies into the possible roles of delayed type hypersensitivity and allergy in the pathogenesis of leptospirosis gave no indication that these are of any importance.

Haemolytic anaemia is a characteristic clinical feature of leptospirosis caused by serotype pomona. This is due to a haemolysin of large molecular weight which is not a lecithinase and acts independently of complement and specific antibody.

After the organism enters the animal body there is a period of rapid multiplication within the blood stream; the so called leptospiraemic phase. During this time the animal experiences a variable pyrexia. Serotype pomona can be observed in renal capillaries and renal interstitium quite early in the leptospiraemic phase. It is not clear whether there is

a specific tropism for renal tissue but there is no doubt that once the organism reaches the tubular lumen that it is free to multiply in a prolific manner. In this situation it appears to be protected from circulating antibody and organisms are passed out into the urine in large numbers for prolonged periods at a time when the animal may have a high level of circulating antibody.

Detailed investigations have been carried out into the nature of humoral antibody activity. It has been shown that there is a distinct, specific leptospiracidal antibody which is independent of agglutinating antibody. Leptospiracidal antibody is produced by certain immunogenic fractions of pomona cultures which can be separated by chromatographic methods.

Ultrastructural studies have revealed that leptospiracidal antibody strips the outer sheath from the organism. It acts independently of complement and is an IgG class antibody. The discovery of leptospiracidal antibody has provided a new concept in the study of immunity in leptospirosis. It is likely that it is the major factor in the termination of the leptospiraemic phase of the disease and it also should be expected to play a major role in preventing infection.

The application of this knowledge to the elaboration and evaluation of new vaccination procedures appears to hold great promise.

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## INTRODUCTION

This study is an attempt to investigate the relationship between the bacterial pathogen, Leptospira interrogans serotype pomona and some of its mammalian hosts and their cells. Using tissue culture techniques and other in vitro systems, attempts are made to define the parasite - host cell relationship and subsequently the study is extended to the whole mammalian host as exemplified by experimental infections in the sheep and suckling mice.

Of particular interest are the mechanisms by which L. interrogans serotype pomona produces disease and also the method by which it arrives and colonises the renal tubules at a time when specific antibody is at a high level in the host.

An indirect benefit of this work has been the accumulation of a considerable amount of new information concerning experimental leptospirosis in sheep.

## CHAPTER 1

### REVIEW OF THE LITERATURE AND AIMS OF THE PRESENT INVESTIGATION

#### CLASSIFICATION AND NOMENCLATURE OF THE LEPTOSPIRES

The designation Leptospira (Noguchi 1917, 1918) is the name of a morphologically characterised genus of the order Spirochaetales. The genus Leptospira includes all the organisms morphologically identical with the type organism icterohaemorrhagiae. This organism was found in 1915 to be the cause of infectious jaundice (Weil's disease) in man (Inada et al., 1916).

For many years the genus Leptospira has been divided into two main groups; the pathogens and the saprophytes. It has been recommended by the Taxonomic Subcommittee on Leptospira (1963) that they be regarded as two distinct species, namely Leptospira interrogans for the parasitic strains and Leptospira biflexa for the saprophytic organisms. More recently however, Borg-Peterson (1966) and a WHO expert group (WHO, 1967) have proposed that Leptospira interrogans be the only species recognised until sufficient valid taxonomic data has been collected upon which to base a sound classification. Whether or not the introduction of two species is favoured, it cannot be denied that there are at least two major complexes within the genus Leptospira and these are usually designated interrogans and biflexa. The differentiation of the leptospire within these two complexes is by serological tests and the subspecific categories are named serotypes.

The individuality of the serotypes is established on the evidence provided by cross agglutination-absorption tests. Two organisms are considered to belong to the same serological types if, after

cross-absorption with adequate amounts of a known (test) antigen, less than 10% of the titre regularly remains in the antisera of both the known and the unidentified serotypes. Serogroups are comprised of serotypes which cross-agglutinate to high titres with one another's antisera.

In spite of the fact that the serotypes of Leptospira have not been regarded as species for many years, the binomial convention of nomenclature is persistently being used as if they were.

In this study the various serotypes mentioned will be given their full names, e.g. Leptospira interrogans serotype pomona when they are first mentioned and subsequently they will be designated as either serotype pomona or simply pomona. This follows the nomenclature practice suggested by Turner (1967) and WHO (1967).

#### NATURAL DISEASES DUE TO LEPTOSPIRA INTERROGANS SEROTYPE POMONA

The organism used in this study derives its name from a small town in a dairy farming district in North Queensland. The name was proposed by Derrick (1942), one of the investigators of an outbreak of seven-day fever in that township. The leptospiral organism isolated from one of his patients was found to be different antigenically from any known Leptospira and so it was concluded that it represented a new serotype.

In most countries where pomona infections of man have been reported, it is associated with those who handle pigs, but in Australia and New Zealand the incidence is highest amongst dairy farmers, (Johnson, 1950; Faine and Kirshner, 1955). Leptospirosis was first recorded as occurring in cattle and sheep in New Zealand in 1950-51 (Anon, 1951).

Young calves infected with pomona exhibit a frequently fatal acute febrile illness characterised by haemoglobinuria, and sometimes jaundice. Kirschner et al. (1952) confirmed that pomona was the serotype responsible for redwater of calves and that damage to the udder of milking cows could also result from infection by the same organism. The significance of pomona infections as a cause of abortion in cows in New Zealand was first considered by Te Punga and Bishop (1953) and Salisbury (1954). This aspect of the disease is now considered to be of major importance in New Zealand.

Naturally occurring "spirochaetosis" of sheep had been suspected but had not been confirmed prior to the New Zealand report. However in order to demonstrate that such infections were possible both Melandi et al. (1933) and Semschow (1941) experimentally infected sheep with serotype icterohaemorrhagiae. Hartley (1952) reported on the 1950 outbreak in sheep giving the post mortem findings and histological evidence upon which the diagnosis was based. Sera from recovered sheep gave high agglutination titres (1:2,000) to pomona. Clinically the disease in sheep is characterised by a transient haemoglobinuria with a severe jaundice affecting the young lambs. The morbidity rate in lambs may be almost 100% with a mortality rate of around 18%, whereas the signs of infection in older sheep are mild or nonexistent (Webster and Reynolds, 1955).

Because the disease condition of calves known as redwater was well known to farmers in New Zealand it can be assumed that the 1950-51 outbreaks of leptospirosis did not represent the introduction of a new disease but rather the uncovering of one that had long existed in this country.

## PATHOLOGY OF LEPTOSPIROSIS

The disease of leptospirosis in domestic animals is characterised by one, two or all three of the following features; anaemia, icterus and haemoglobinuria. Both the haemoglobinuria and the icterus probably result from intravascular haemolysis (Jubb and Kennedy, 1970). The localisation of the leptospire in the kidney, following leptospiraemia, produces a focal or diffuse interstitial inflammation of the kidney with acute transient tubular degeneration. Localisation of the infection may occur in the pregnant uterus of many domestic animals and may result in abortion. Such abortions have been reported both experimentally and in field cases from cattle, pigs and sheep (Ryley and Simmons, 1954; Ryley, 1956; Ferguson et al., 1957; Smith et al., 1960). Abortions are not commonly recognised as being due to leptospiral infections in sheep, although in an early report (Beamer et al., 1953) the diagnosis of leptospirosis was made in a flock in which both death and abortions in ewes had occurred.

By far the most characteristic gross lesions associated with leptospirosis are those found in the kidney. The surface of this organ usually has several discrete grey to white foci from 1 to 5 mm in diameter (Reinhard and Hadlow, 1954; Fennestad, 1963; Michna and Campbell, 1969). These foci may be confined to the subcapsular cortex or they may extend as a narrow wedge deeper into the cortex. The above description probably applies to the lesions due to leptospirosis occurring in any mammalian kidneys. Variations from this general description are reported, but probably reflect the severity and duration of the infection and the infecting serotype. In pigs, for instance, a definite margin of hyperaemia is seen around the white focal lesion and this is interpreted as being a more acute reaction (Michna and Campbell, 1969). The renal cortical lesions may also be slightly elevated above the capsular surface

as reported in calves by Reinhard and Hadlow (1954).

In most mammalian kidneys infected with pomona there is an infiltration of lymphocytes, primarily in the cortex. Within these lymphocytic foci are remnants of tubules, together with a few tubules which have remained intact. In many studies of mammalian leptospirosis (Reinhard, 1951; Reinhard and Hadlow, 1954; Michna and Campbell, 1969; Fennestad, 1963; Martino et al., 1969) little or no change is reported in the glomeruli when examined by the light microscope. Visible changes of the glomerulus seen by light microscopy however have been reported by others (Brito et al., 1968; Sitprija and Evans, 1970) and it is possible that these may also depend on the severity of the infection and the infected serotype.

A number of electron microscopy studies have been undertaken using biopsy material from human cases of leptospirosis (Brito et al., 1965; Sandborn et al., 1966; Brito et al., 1967(a); Brito et al., 1967(b); Sitprija and Evans, 1970) and one of the most outstanding features of this research work has been the recognition of quite extensive glomerular lesions (Brito et al., 1965; Sitprija, 1968) which had not been detected under the light microscope. Although the glomerular lesions have been described, it is not known when these lesions appear in relation to the time of infection and the underlying causes of the lesions are as yet unexplained. These electron microscopic studies have provided only limited observations concerning tubular lesions. The degree to which tubular damage is due, to the migration of leptospores to the tubular site or the result of colonisation of the tubules, has not been investigated.

Of particular significance is the route by which the leptospores get from the blood stream to the proximal convoluted tubules; a site which enables them to survive for many months protected from bactericidal antibodies and in a medium which sustains growth.

## PATHOGENESIS

The role of haemolysis in the pathogenesis of leptospiral infections has still to be given a perspective along with other possible factors capable of inducing tissue damage; namely toxin, cold reactive antierythrocyte antibody, and antibody both with and without complement acting upon sensitised red blood cells. The action of antigen-antibody complexes on kidney tissues has been postulated as a mechanism responsible for much of the tissue damage occurring in the kidney (Taylor et al., 1970).

Because of the absence of demonstrable haemolysin in the plasma of anaemic sheep, the role of this in the pathogenesis has been questioned (Decker et al., 1970), especially as a cause of the haemolytic crisis which occurs in some cases of leptospiral infections of sheep due to pomona.

When considering the pathogenesis of leptospiral infections, there are four "organs" of the body worthy of special consideration. These <sup>"organs"</sup> are the blood, kidneys, liver and uterus and their damage may be the result of the action of the same or different mechanisms.

Firstly, there are the haemodynamic changes seen in young calves and sheep especially when infected with serotype pomona, and in man when infected with icterohaemorrhagiae. Kidney damage can be considered as a universal finding, although the degree of damage in subclinical cases may be slight. The third "organ" upon which the leptospiral organisms act to produce demonstrable damage is the uterus (Dozsa and Sahu, 1970) together with the foetus if it is gravid (Smith et al., 1960; Smith et al., 1966(a); Smith et al., 1966(b)). Finally, the liver of some species of animals is damaged by infection. Lambs infected with pomona may show massive invasion

of the liver by the spirochaete. In human infections, due to icterohaemorrhagiae, liver damage may be a distinct feature (Arean, 1962).

Many theories as to the cause of the haemodynamic changes have been postulated (Ferguson et al., 1957; Morse et al., 1957; Ringen, 1966; Decker et al., 1970; Sitprija and Evans, 1970; and Bhasin et al., 1971). The haemolysin produced by the organism when grown in vitro, obtained both from culture supernatant and following the disruption of washed cells, has been studied by Kemenes (1958), Valentine et al. (1964), Ringen (1966) and Alexander et al. (1971).

Cold haemagglutinin (CHA), occurring in sheep after experimental infection with serotype pomona, was first described by Decker et al. (1970) and its properties further investigated by Bhasin et al. (1971). These authors believe that this antierythrocyte antibody may be one of the mechanisms responsible for the anaemia which occurs in sheep.

Antibody and leptospiral haemolysin and their interactions have been ascribed varying responsibilities in the production of haemolysis. Ferguson et al. (1957) suggested that leptospiral antibody together with complement were responsible for the lysis of leptospiral organisms with the consequent release of haemolysin. Kanich et al. (1968), on the other hand, consider that antibody and complement bring about a lysis of red blood cells previously sensitised with leptospiral antigens. Haemolysis of red blood cells, with haemolysin per se as the dominant factor, is the hypothesis supported by Bauer and Morse (1958) and Bauer et al. (1961).

The contribution of the anoxia resulting from these haemodynamic changes to the overall tissue damage has been debated by Reinhard (1951), Arean (1962), Sitprija et al. (1965) and Sitprija and Evans (1970). It has been concluded from a study of leptospiral liver and kidney lesions that a toxin or, perhaps more correctly, an endotoxin is responsible for

the tissue damage produced (Stavitsky, 1945; Arean, 1962; Sitprija and Evans, 1970). The part played by anoxia or hypoxia in contributing to lesions in these organs is considered and given varying importance by Arean (1962) and Sitprija and Evans (1970). Murphy and Jensen (1969) believe the anoxia is all important in bringing about the death and eventual expulsion of the foetus in bovine leptospirosis due to pomona. Whilst haemodynamic changes occurring in both the dam and the foetus probably contribute to the expulsion of the foetus, Murphy and Jensen (1969) believe the invasion of the foetus by the leptospiral organism is of greatest importance. This foetal invasion and the subsequent haemolysis of its circulating blood is believed to cause the death of the foetus due to anoxia.

Other suggestions have been made as to the cause of leptospiral abortion and Te Punga and Bishop (1953) have postulated that pyrexia and the systemic reaction of the dam may be important factors in the causation of abortion. These authors consider a third possibility; that as a result of localized lesions in the placentomes the transfer of metabolites may be interfered with. Although the hypothesis of foetal anoxia held by Murphy and Jensen (1969) best fits their experimental findings, the other postulates may have some validity when the variable findings in field outbreaks of leptospiral abortion are considered.

OUTLINE OF THE PRESENT INVESTIGATION

The question of whether or not the damage to kidney cells is primarily due to a toxin, has been investigated by a number of workers (Harrington and Sleight, 1966; Parmas and Pinkiewicz, 1966; Stalheim, 1967; Miller et al., 1970; Yam et al., 1970), however the answer still

remains equivocal. The section of this study in which tissue cultures were used, was an attempt to determine if a cytotoxic substance was produced by the organism and whether it could be assayed using primary kidney cell cultures or any other readily available cell line.

Investigations were also carried out to determine if any bacterial extra cellular enzymes are released by leptospire which might contribute to their ability as pathogens.

As the sheep was to be used as one of the experimental animals in this study, it was considered necessary to collect some clinical data relevant to the infection in this animal. The findings reported here are in substantial agreement with those reported since the commencement of this study by Decker et al. (1970).

Using the kidneys from the experimentally infected sheep, the ultrastructural pathology was studied to try and deduce the possible pathogenesis of the lesions seen in this organ. Because the sheep were allowed to survive until their infection was well established, the possible sequence of the changes that occurred in the kidney during the establishment of the infection can only be guessed at. Mice were therefore used to try to elucidate how the leptospire become established in the convoluted tubules and at what point in the infection the glomerular and tubular damage is manifest.

The possibility that an immunological response is wholly or in part responsible for the clinical and pathological manifestations of this disease has also been considered, and the role of a possible hypersensitivity reaction investigated.

## CHAPTER 2

### TISSUE CULTURE INVESTIGATIONS

#### INTRODUCTION

Inhibition of cellular growth and degeneration with apparent cell death, were the cytopathic effects found in the tissue culture systems used in investigations by Harrington and Sleight (1966), Parnas and Pinkiewicz (1966), Stalheim (1967), Miller et al. (1970) and Yam et al. (1970). This evidence suggests that a toxin may be produced by pomona and that this toxin could be responsible for the tissue changes observed in leptospirosis.

This chapter describes experiments designed to study the effect on cells in culture of New Zealand field isolates of pomona. Five experiments were carried out. In the first, (Exp. 2a) cultures of rabbit kidney (RK) cells and foetal lamb kidney (FLK) cells were infected with the organisms and the microscopic changes in the developing monolayers were observed. A second experiment (Exp. 2b) used FLK cells infected with pomona and its aim was to determine by electron microscopy the intracellular changes occurring in pomona infected cells. Experiments 2c, 2d and 2e were designed to assay the level of toxicity, if any, of three ammonium sulphate precipitated fractions prepared from the supernatant of a pomona culture: In these experiments FLK cells and mouse L cells were used as indicator systems.

#### MATERIALS AND METHODS

##### Tissue culture primary cells

Similar procedures were used for preparing both primary rabbit

and foetal lamb kidney cultures. For the former cultures, young white rabbits in good condition were used. In the case of foetal lamb cultures, the foetuses were collected from the slaughter house and the kidneys removed within 2 hours. The kidneys were removed aseptically from the respective animals and placed in sterile petri dishes. All manipulations to the kidneys were performed under a hood equipped with germicidal ultra violet light. Working surfaces to benches and under the hood were cleaned prior to use, with 70% alcohol.

The capsule was stripped from the kidney which was then cut in half transversely. Using a sharp pointed pair of scissors, the medulla was removed and discarded. The cortex was cut into small pieces and these were washed twice using sterile phosphate buffered saline (PBS). The kidney pieces were then placed into a 250 ml fluted sidearm trypsinizing flask to which 30-40 ml of 0.25% trypsin and a sterile bar magnet were added. Trypsinization was done at 37°C for 15 minutes on a heated magnetic stirrer. After this the trypsin, along with any fine suspension of kidney tissue released by the trypsin, was decanted through the sidearm. Fresh trypsin was added and stirring continued for a further 20 minutes. The cells released by this trypsinization were collected and sedimented by centrifugation at 900 g for 8 minutes. The cells were washed twice in PBS and finally suspended in medium. Sufficient medium was added to give a final concentration of approximately 100,000 cells per ml. After gaining some experience at counting cells, it was found that estimations by eye produced just as good results and therefore counting was discontinued. Further trypsinizations of the tissue fragments were sometimes done when very large yields of cells were required. The cells suspended in medium were dispensed in 100 ml flat medical bottles and these were placed in a horizontal position in a 37°C incubator. In this way the cells were allowed to settle on the large flat surface of the bottle which rested on the incubator shelf.

### Tissue culture established line cells

Monolayers of mouse L cells were disaggregated with 0.25% trypsin, resuspended in TC 199 (Difco\*) and dispensed into 100 ml flat medical bottles. Incubation of these cultures was in the horizontal position at 37°C. A more detailed description of the trypsinizing technique used for this cell line is given in Appendix I.

All dispensing of liquids, for use with either primary cultures or those of established lines, was done by a simple pouring technique unless accuracy in measurement was required in which case a pipette with a rubber bulb was used.

### Preparation of tissue culture medium

For the initial establishment of all primary cultures, a Hank's based medium was used. The Hank's solution was a granulated preparation \* and to this was added 10% calf serum, 5% lactalbumin hydrolysate\*\*, tryptose phosphate broth\*\* and 5% yeast extract\*\*. If a breakdown in sterility was suspected, due either to difficulty experienced in removing the kidneys or a time lag between collection of the material and the processing for tissue culture, then penicillin and streptomycin were added to the medium. Streptomycin sulphate\*\*\* was added at 100 mcg/ml and Crystapen (benzylpenicillin)\*\*\* at the rate of 100 units/ml.

Adjustment of the pH of the medium was made using 4.4% sodium bicarbonate and the medium was maintained at approximately pH 7.4.

For the experiments using mouse L cells, the medium employed was

---

\* Difco Laboratories, Detroit, Michigan U.S.A.

\*\* Oxoid Division of OXO Limited, London.

\*\*\* Glaxo Laboratories (NZ) Limited, Palmerston North.

TC-199. This was reconstituted from a dehydrated preparation to which was added 10% calf serum and sodium bicarbonate. (For more detailed information on the tissue culture medium refer to Appendix I).

Glassware preparation

All glassware, including that used for the storage of media, phosphate buffered saline or trypsin, was washed thoroughly according to the method described in Appendix I.

Leptospiral cultures

For the first experiments in this study, where rabbit kidney cultures and foetal lamb kidney cell cultures were infected with leptospire, a recent field strain of pomona isolated from a pig was used. The isolate was maintained by weekly passage in Stuarts' medium\* enriched with 10% rabbit serum. The medium was dispensed in 5 ml amounts into 16 x 100 mm screw capped glass tubes. Incubation was at 27°C.

In the second series of experiments, in which ammonium sulphate precipitated fractions from culture supernatant were tested against tissue culture cells, the isolate was from one of the sheep in experiment 4b. The isolation in this case had been made in Fletcher's medium\* plus 10% rabbit serum. The organism was then subcultured into Stuart's medium with 10% rabbit serum and it was from this culture that fractions I, II and III were prepared.

---

\* Difco Laboratories, Detroit, Michigan, U.S.A.

### Fractionation of culture supernatant using ammonium sulphate

A seven day culture of pomona in 250 ml of Stuart's medium with 10% rabbit serum was sonicated\* for 10 minutes. The preparation was kept cool during sonication by placing the vessel containing the culture of leptospire in a mixture of ice and acetone. The sonicator probe was sterilized by dipping in alcohol and flaming. The sonicated culture was then subjected to 6,000 g for 30 minutes at 4°C using a Beckman Spinco model L centrifuge\*\*. The supernatant was collected in sterile flasks and examined by darkfield microscopy for the presence of leptospire.

Fractional precipitation of culture supernatant fluid was carried out using ammonium sulphate resulting in 3 fractions as described in Table 2.1.

### Preparation of control fractions

Uninoculated Stuart's medium was used to prepare control ammonium sulphate precipitated fractions and the same dialysis and centrifugation procedures were used for the supernatant as those shown in Table 2.1.

### Phosphate buffered saline (pH 7.4)

This was prepared as described in Appendix I.

### Trypsin solution

Trypsin was prepared as a 0.25% solution using a 1 : 250 dehydrated trypsin powder\*\*\* which was reconstituted in PBS. Phenol red indicator was added and the solution used at pH 7.4.

---

\* Dawe Sonicator, Dawe Instruments Limited, London.

\*\* Beckman Instruments Inc., Spinco Division, Palo Alto, Calif., U.S.A.

\*\*\* Difco Laboratories, Detroit, Michigan, U.S.A.

TABLE 2.1

7 DAY CULTURE OF LEPTOSPIRES IN 250 ml STUART'S MEDIUM

SONICATED FOR 10 MINUTES

CENTRIFUGED AT 6,000 g FOR 30 MINS.

SUPERNATANT

40%  $(\text{NH}_4)_2\text{SO}_4$

STAND AT 4°C OVERNIGHT

CENTRIFUGE 6,000 g 30 MINS

SUPERNATANT I

70%  $(\text{NH}_4)_2\text{SO}_4$

STAND AT 4°C OVERNIGHT

CENTRIFUGE 6,000 g 30 MINS

SUPERNATANT II

100%  $(\text{NH}_4)_2\text{SO}_4$

STAND AT 4°C OVERNIGHT

CENTRIFUGE 6,000 g 30 MINS

SUPERNATANT III

DISCARDED

SEDIMENT I

RESUSPENDED IN 15 ml OF  
STERILE P.B.S.

DIALYSED AGAINST STERILE  
TAPWATER

12 HOURS AT 4°C

WATER CHANGE DIALYSIS  
CONTINUED

24 HOURS AT 4°C

DIALYSED AGAINST STUART'S  
MEDIUM

12 HOURS AT 4°C

FRACTION I

SEDIMENT II

SAME DIALYSIS PROCEDURE

AS FOR SED. I.

FRACTION II

SEDIMENT III

SAME DIALYSIS AS FOR

SED., I & II.

FRACTION III

All tissue culture media were made in double glass distilled deionized water and sterilized using 0.22u cellulose acetate filters\*.

#### Calf serum

This was collected directly from the cut jugular vein of young calves at the time of slaughter. The blood flowed into a sterile stainless steel bucket and subsequently was kept as free as possible from contaminating bacteria. After the serum was separated it was stored at  $-20^{\circ}\text{C}$  in 50 ml aliquots. The serum was thawed and added to the final medium just prior to filtration.

Towards the end of the study a second and more satisfactory method was devised which enabled the removal of large quantities of sterile blood from young bovines held at the University. For this method the jugular vein region of a suitable animal was shaved and swabbed with alcohol. A 13 gauge needle was used for the bleeding procedure, the needle being attached by a rubber hose to a  $\frac{1}{2}$  gallon glass container. The complete apparatus had previously been sterilized by autoclaving. To facilitate the flow of blood from the jugular vein, a slight negative pressure was produced inside the bottle by means of water vacuum pump. The blood was allowed to stand overnight and after retraction of the clot within the glass bottle, the serum was decanted off into aliquots and stored at  $-20^{\circ}\text{C}$ . Although this serum was sterile, it was added to the medium prior to its sterilization by filtration.

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\* Millipore Corporation, Bedford, Mass., U.S.A.

### Staining for light microscopy

Coverslip cultures were fixed in 10% formal saline for 10 minutes and stained with haematoxylin and eosin using Mayer's haemalum (Culling, 1963).

For staining of the coverslip cultures with Oil Red O., methyl green pyronine, Feulgen and periodic acid-Schiff, modifications of the methods used for paraffin sections (Culling, 1963) were employed.

Detailed descriptions of these staining techniques are given in Appendix I.

### Direct staining technique for detection of mycoplasmas in cultured cells

Coverslip cultures were placed in an 0.6% sodium citrate solution and 1 ml of distilled water added by drops to make the final concentration of sodium citrate 0.45%. The coverslip was left in this solution for 10 minutes before an equal volume of Carnoy's fixative was added slowly. This fluid was then poured off and replaced with 2 ml of undiluted Carnoy's fixative. Ten minutes was allowed for fixation. The coverslip cultures were air dried, and when absolutely dry stained for 5 minutes in Orcein\* made up as a 2% solution in 60% glacial acetic acid. Following staining, the cells were washed three times in absolute alcohol and the slide mounted with DPX\*\* mountant. All the procedures were carried out at room temperature.

### Preparation of tissue culture cells for electron microscopy

The cells were removed from the glass surface by exposure to 0.25% trypsin for 2 minutes. The trypsin was then neutralised by the addition of about 5 times the volume of tissue culture medium complete with

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\* G.T. Gurr Limited, London, England.

\*\* British Drug Houses Limited, Poole, England.

calf serum. The suspended tissue culture cells were then centrifuged at 900 g for 8 minutes and the cells washed in PBS, centrifuged again and then resuspended in 3% glutaraldehyde and 2% formaldehyde in phosphate buffer pH 7.2. Primary fixation was complete in four hours and, following two washes in cold phosphate buffer, a secondary fixative, osmium tetroxide, was introduced for two hours. To ensure complete removal of all the osmium tetroxide, the cells were washed twice in phosphate buffer. They were then resuspended in a small quantity of molten agar. When the agar had gelled, it was removed from the tube and cut into 1 mm cubes. Dehydration was effected by holding the cubes for at least ten minutes in a graded series of ascending strengths of ethyl alcohol. The dehydrated cells were then infiltrated with Durcupan A.C.M. Fluka\* by placing them in a graded series of increasing strength of the resin. Agitation of the vials containing the tissue cubes and resin was performed continuously by mechanical means to aid the infiltration process. The final embedding was in Durcupan to which had been added 2% accelerator, 10% hardener and di-n-butyl phthalate as a plasticiser. Individual pieces of tissue were placed in size '0' gelatin capsules which had been filled with Durcupan. The capsules were carefully placed in an upright position and polymerised in an oven at 60°C for 2 days. Sections were cut on an LKB\*\* ultramicrotome and mounted on formvar coated grids.

## RESULTS

Infected cultures of both rabbit kidney and foetal lamb kidney cells showed distinct changes when compared with their respective controls.

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\* Fluka A.G. Chemische Fabrik Buchs. S.H.G.

\*\* L.K.B. Produkter A.B. FACK Stockholm Bromma 1, Sweden.

The responses of these two cell types were quite different. The rabbit kidney cytopathic effect (CPE) was characterised by death of many of the cells in the monolayer, with the few remaining viable cells maintaining their original morphology (Fig. 2.1). The foetal lamb kidney cells, on the other hand, did not show such a marked depletion of the monolayer in terms of actual numbers of cells, but the remaining cells all took on an elongated spindle shape (Fig. 2.2).

No significant additional information was obtained when the monolayers were stained by PAS, methyl green pyronine, Feulgen, or oil red O, and examined microscopically.

#### Cells examined by electron microscopy

##### Control cells

There were no noteworthy ultrastructural differences between control cells of rabbit or sheep origin. Both polyribosomes and free ribosomes were abundant and closely packed in the cytoplasm around the periphery of the cell and more sparse in the fibrillar or fibrous zone (Kochhar, 1968) in the central region of the cell. Profiles of invaginations between pseudopodal processes on the surface of the cells were seen, indicating pinocytotic activity. The endoplasmic reticulum was around the periphery of the cells, with the nucleus and a circumnuclear fibrillar area making up varying proportions of the cell (Fig. 2.3). Usually this zone made up about 50% of the area of the cell when seen in section. The fibrillar zone consisted of a ground substance made up of a dense mat of intracytoplasmic fibrils containing the Golgi apparatus, a few ribosomes and some elements of endoplasmic reticulum. Fat globules were prominent in both the fibrillar zone and the outer zone amongst the densely packed ribosomes (Fig. 2.4). These globules were not uniformly numerous in all

cells, and were absent in some cells. A common feature was the presence of dense membrane-bound bodies in the cytoplasm (Fig. 2.5). These 'dense bodies' contained either crystals, a number of other membrane-bound bodies or dense unrecognisable material.

The mitochondria were of irregular elongate, or rounded outline and with normal appearing cristae (Fig. 2.4). The nuclei had generally rounded outlines with very few, if any, shallow indentations.

Only a few vesicles and vacuoles were present in the cytoplasm of the control cells.

#### Infected cells

The Golgi apparatus was more prominent and extensive in these cells and consisted of many flattened sacs, vacuoles and vesicles of varying sizes. The Golgi complex was not surrounded by the extensive fibrillar zone which was so clearly visible in the control cells. Variations in the extent of the fibrillar area located around the nucleus between the noninfected and infected cells was marked. This area being well defined in the noninfected cells but smaller and less recognisable in the infected cells. Many vesicles and vacuoles were present throughout the cytoplasm and many of these structures had the appearance of degenerate mitochondria (Fig. 2.8) whilst others had been formed by the expansion of the endoplasmic reticulum (Fig. 2.8).

The inclusions contained within the cytoplasm of these cells were larger than any seen in the control cells and their contents had a less discernable structure (Fig. 2.6). The vacuoles and vesicles were more numerous in the cytoplasm of the infected cells and in some cells the vacuolation was excessive. Some of the vacuoles contained remnant material

(Fig. 2.7) while others were empty (Fig. 2.8). The mitochondria of infected cells were smaller with a denser appearance and the cristae were still visible although greatly thickened (Fig. 2.9). The nuclei of infected cells had deep indentations and some nuclei had complex shapes.

#### Treatment with protein fractions

The results after treatment of both mouse L and foetal lamb kidney cells with ammonium sulphate precipitated fractions I, II and III were difficult to interpret, particularly as the control cultures treated with sterile medium gave some degree of CPE. The only difference seen was in the appearance of the cells treated with fraction I. CPE occurred in these, one doubling dilution greater than with the control cells after 7 days incubation. This difference, although consistent, was not very marked. No differences could be detected between the fraction II and III treated cells and those treated with the control fractions. There was no difference in sensitivity between the mouse L cells and the foetal lamb kidney cells.

## DISCUSSION

#### Tissue culture study

A major difficulty experienced in the experiments dealing with the direct effect of pomona organisms on tissue culture cells was the differing environmental requirements for the leptospire and tissue culture cells. Conditions provided in this study tended to favour the tissue culture cells. Later work by Yam et al. (1970) has shown that leptospire will grow satisfactorily in Eagle's minimal essential medium (MEM) with 5% bovine serum or 10% ovine serum, a medium which was designed to support

tissue culture growth. Eagle's medium has not been tried in this study.

A criticism which can be levelled at many tissue culture assay studies is the possible contributing effect of mycoplasmas. These organisms have been found to infect many tissue cell cultures (Robinson et al., 1956; Edwards and Fogh, 1960; Carski and Shepard, 1961; O'Connell et al., 1964). In this study, in order to ensure that mycoplasmas were not present in sufficient numbers to produce any untoward effects, they were looked for by a direct staining technique (Fogh and Fogh, 1964). No mycoplasmas were seen in any of the primary tissue cultures. However as with many established cell lines the mouse L cells were infected but the level of infection was so low that it was not considered great enough to appreciably influence the responses of the cells.

Because the tissue culture studies were performed over an extended period of time, different isolates of pomona were used for the two parts of the experiment 2a. This may provide an explanation for the differences in response between the RK cells and the FLK cells. The rapid loss of toxin production thought to occur when leptospire are maintained in laboratory cultures (Turner, 1970) prompted the author to reisolate a field strain of pomona prior to the repetition in FLK cells of the work done with RK cells.

It cannot be stated with certainty the reason for the final appearance of the FLK cells. The decrease in total number of cells and the increase in the percentages of spindle shaped cells seen in the infected monolayers may have been due to death of all the epithelial type cells with survival and subsequent multiplication of the fibroblast cells, or conversion of some of the epithelial type cells into fibroblastic cells. Others have noted the damaging effect of leptospire on tissue culture cells (Harrington and Sleight, 1966; Yam et al., 1970; Parnas and Pinkiewicz, 1966) so in

this respect the New Zealand isolates of pomona are no different from those reported from other countries. The question that remains unresolved is whether or not this damaging effect is due to a 'toxin' or to a physical disruptive effect produced by this very motile organism or to a combination of them both. It has been shown that excess intracytoplasmic growth of bacteria can cause death of cultured cells. (Richardson, 1959; Shepard, 1957; Strauss and Hendee, 1959). No evidence of such growth was observed in this study. Toxin induced CPE in cultured cells due to other bacteria, particularly Shigella dysenteriae and Corynebacterium diphtheriae have been reported (Strauss and Hendee, 1959; Kato and Pappenheimer, 1960; Vicari et al., 1960; Bonventre, 1961). In 1970 Yam et al. prepared three protein fractions by ammonium sulphate precipitation and dialysis of the supernatant of a pomona culture. The first fraction, they claimed, produced CPE on the second day after inoculation and this reaction reached its maximum by the sixth day, the controls remaining intact. The second and third fractions produced no CPE. The work carried out in this study did not fully agree with these findings. Although the second and third fractions were similarly shown to be inactive there was only one dilution difference between the CPE noted with the first fraction in the experimental coverslip cultures and those of the controls on the sixth day. One major technical difference which could account for this disparity in results is the different controls used. Yam et al. (1970) used uninoculated medium in their control monolayers, whereas in this study the uninoculated medium was given exactly the same treatment of precipitation and dialysis as the inoculated medium. The dialysis was sufficient to remove all the ammonium sulphate, therefore the concentration of the medium and its serum component must in itself have been toxic for both FLK cells and mouse L cells. The use of Eagle's MEM for both the growth of the tissue culture cells and the leptospirae would have made this experiment much more satisfactory. The Stuart's medium

used was unsuitable for the maintenance of tissue cultures unless diluted by at least 50% with a good tissue culture medium. For this reason, the lowest possible dilution of protein fraction to which the tissue culture cells could be subjected was a 1 : 1 dilution and in fact a 1 : 2 dilution was the lowest used. It can be argued that this 'toxicity' possessed by the concentrated Stuart's medium masked the toxicity due to the leptospiral metabolic products present in the medium and that the Eagle's MEM was not toxic when concentrated by precipitation and dialysis. Another explanation for the disparity between Yam et al's (1970) results and those of this study is the possible lack of, or low level of production of toxin by leptospire in Stuart's medium compared with the production in Eagle's MEM. This, however, seems unlikely as Stalheim (1967) has claimed the existence of a toxic factor in the supernatant from a culture of leptospire in Stuart's medium as measured by a macrophage cytotoxicity test. Nevertheless the current experiments have produced some evidence of cytotoxin elaboration by pomona. The fact that this appeared to be quantitatively small does not necessarily invalidate the finding in relation to the in vivo pathogenesis of leptospirosis since the experimental system employed almost certainly represents a poor model of the in vivo relationship between the parasite and host cells.

#### Electron microscopy

The mitochondria are most sensitive indicators of cell damage and, according to Kochhar (1968), the density of mitochondrial matrix is a most sensitive indicator of adverse conditions within the mitochondria. There was definitely an increase in the density of the mitochondria of the infected tissue culture cells when prepared for electron microscopy but this was due completely to thickening of the mitochondrial cristae rather than any change in the matrix itself. The mitochondria appeared smaller

in size but no less numerous. The dense bodies seen in the cytoplasm of both infected and noninfected cells, but which were larger in the infected cells, were interpreted as autophagic inclusions, the contents of these being, amongst other things, autolysing mitochondria. It would be tempting to speculate that the contents of some of the vacuoles were degenerate leptospiral fragments but there was not sufficient clarity to the structure of these fragments to make any such claims (Fig. 2.7). It is not possible to speculate on any direct mechanical disruptive effect of pomona upon the tissue culture cells.

#### SUMMARY AND CONCLUSIONS

1. Continuous and primary kidney cell cultures have been employed in experiments to determine means whereby pomona damages cells.
2. In spite of technical difficulties associated with maintaining optimum environmental conditions for both tissue culture cells and the leptospire, these experiments have shown a cytopathic effect of whole cultures upon tissue culture cells.
3. One of three ammonium sulphate precipitation fractions prepared from sonicated whole cultures of pomona showed a slight cytopathic activity.
4. Ultrastructural studies of tissue culture cells exposed to whole cultures of pomona revealed numerous cytopathic vesicles and mitochondrial changes but provided no clear evidence of intracytoplasmic survival of these organisms. No definite evidence was obtained which would enable a full assessment of any direct mechanical disruption of the mammalian cells by the motile pomona organisms.

5. It was concluded that under experimental conditions, pomona does not produce large amounts of cytotoxin but it is realised that the experimental systems employed are a poor model of the intimate dynamic relationship between the parasite and host cell in vivo.

Fig. 2.1 Top: Rabbit kidney monolayer tissue culture of 5 days after infection with Leptospira interrogans serotype pomona. As compared with the control (below) the monolayer shows pyknosis, shrinkage and detachment of many cells. Few cells retain their normal morphology.

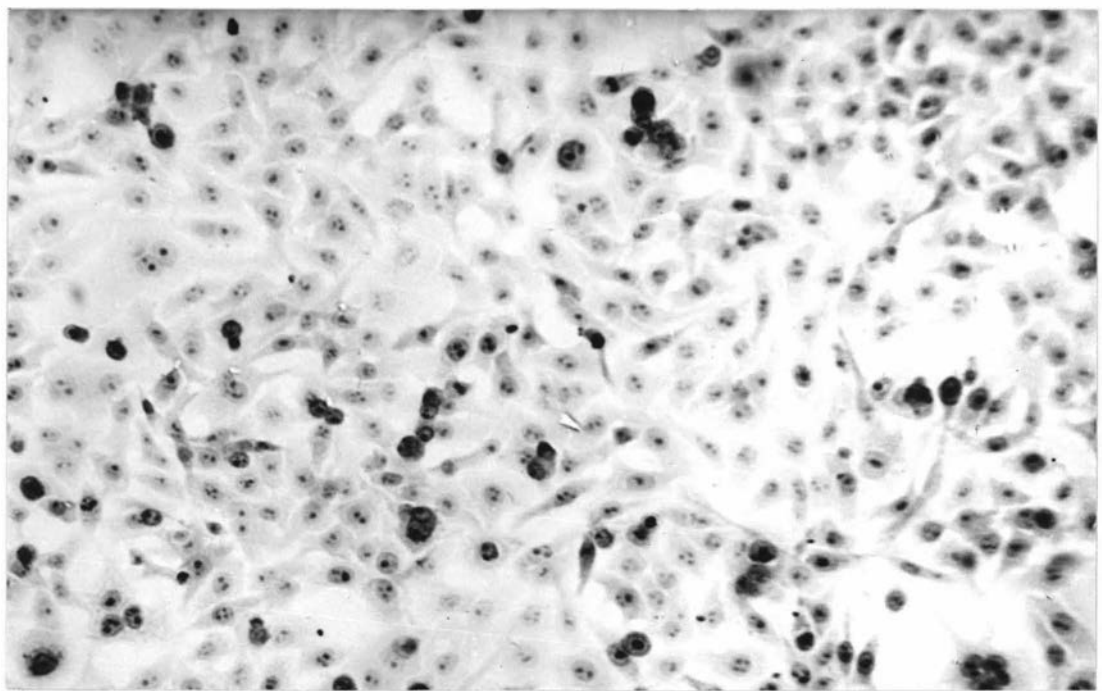
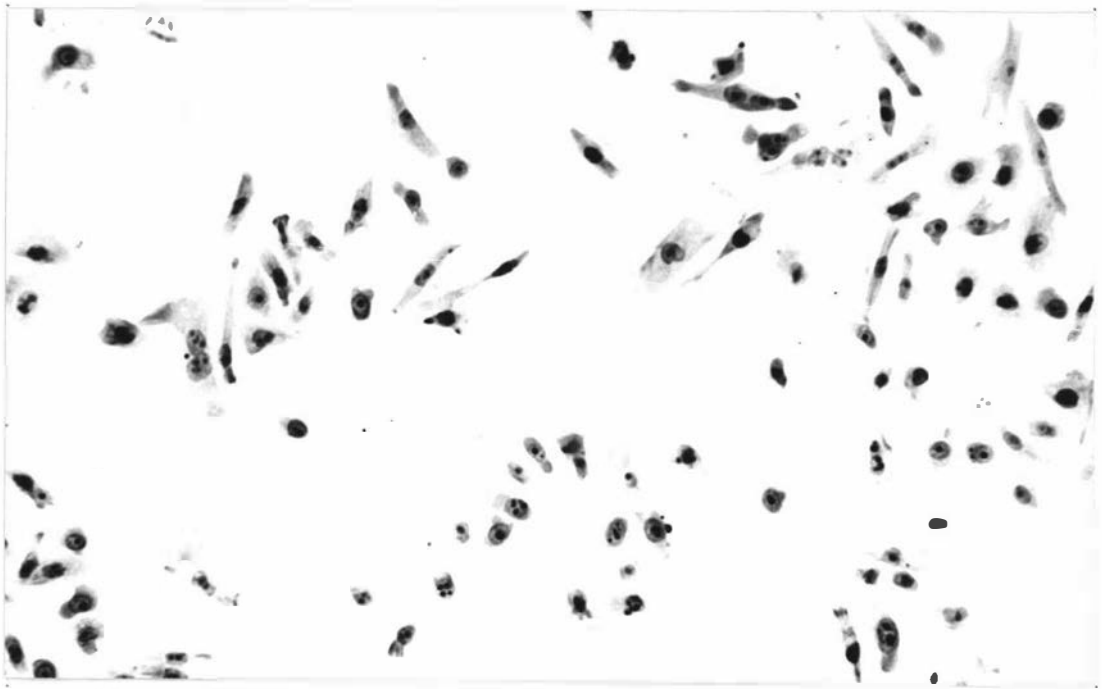


Fig. 2.2 Top: Foetal lamb kidney monolayer 5 days after infection with Leptospira interrogans serotype pomona. As compared with the control (below) many cells have assumed an elongated spindle shape.

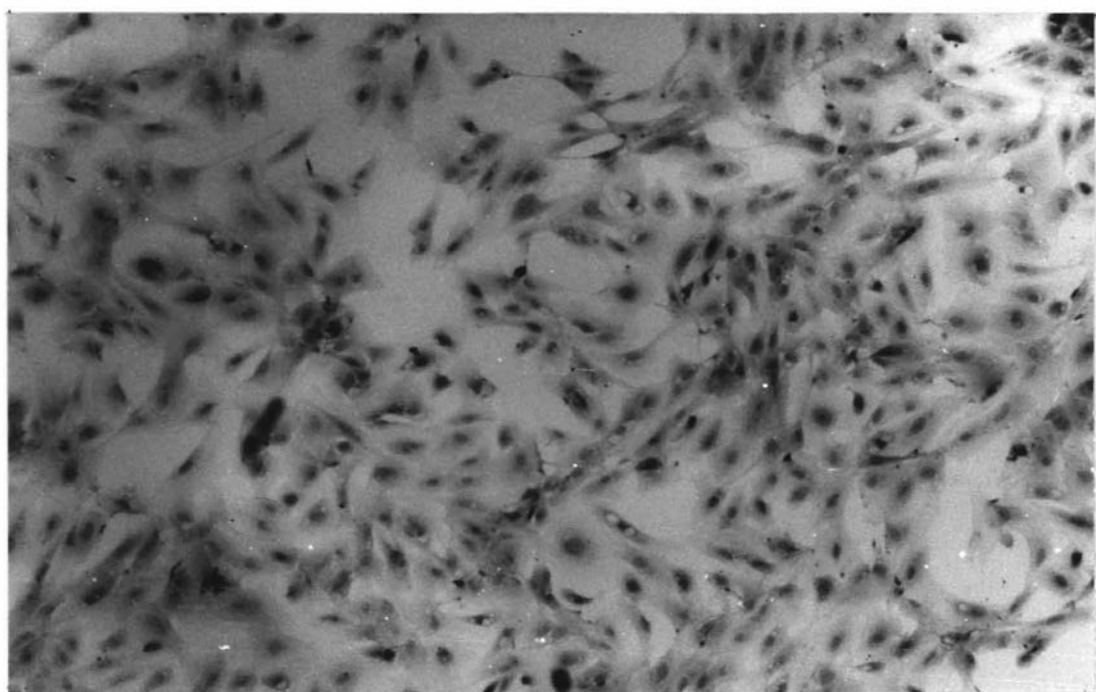
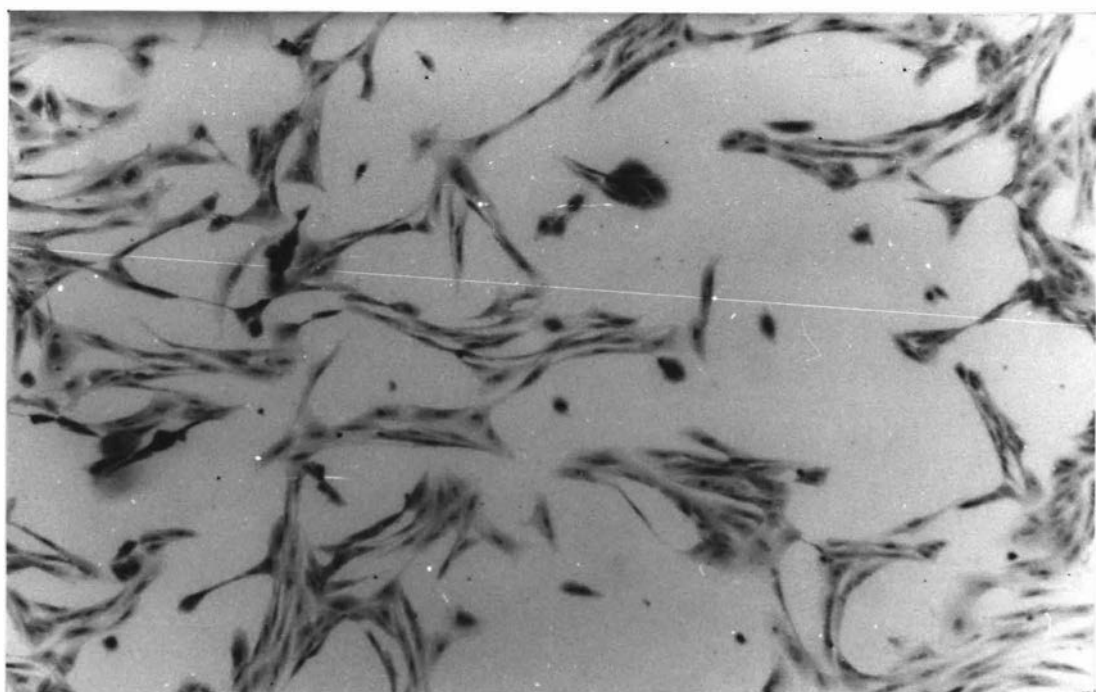


Fig. 2.3 Cell from a normal foetal lamb kidney tissue culture. Note that the endoplasmic reticulum (ER) is confined to the periphery of the cell. The nucleus (N) and circumnuclear fibrillar area (F) occupies more than half of the cell. EM x 10,000.

Fig. 2.4 Fat globules (L) and mitochondria (M) within the cytoplasm of a normal rabbit kidney cell in tissue culture. EM x 48,000.

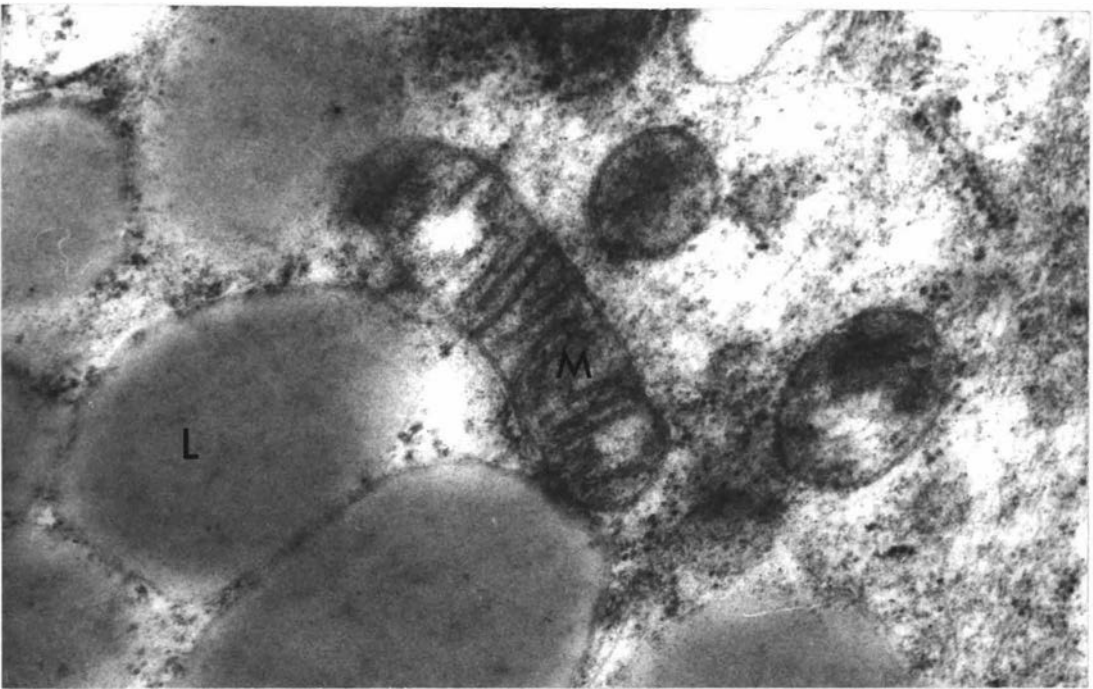
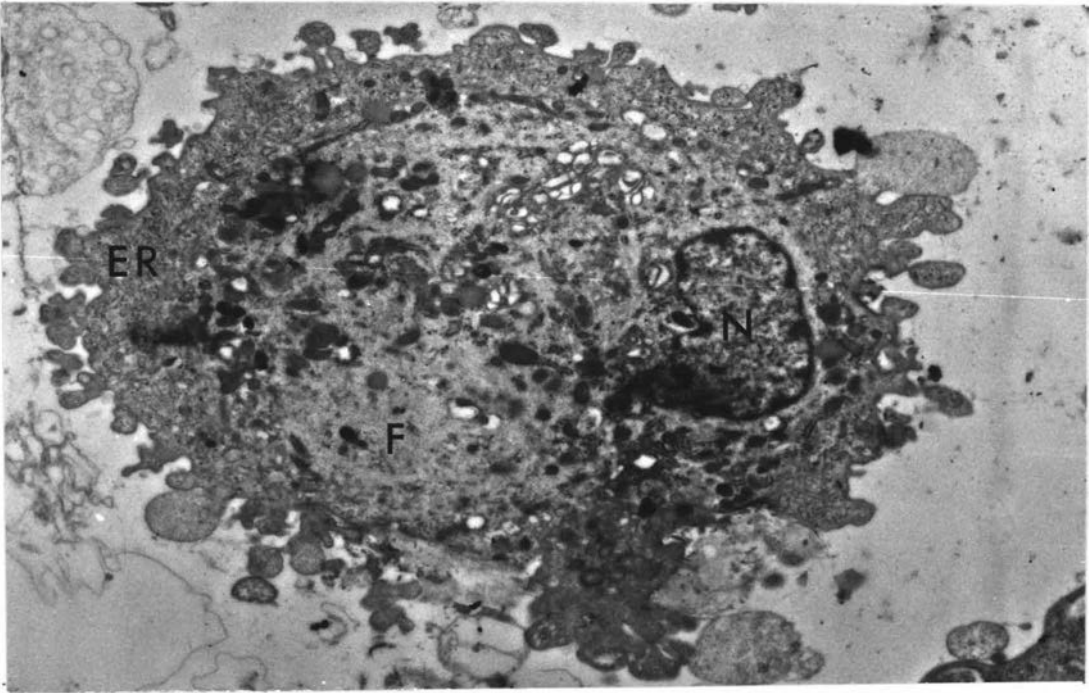


Fig. 2.5 Small dense membrane bound bodies (D) within the cytoplasm of normal foetal lamb kidney cells in monolayer culture. EM x 28,000.

Fig. 2.6 Large dense inclusion body (I) within the cytoplasm of a foetal lamb kidney cell 5 days after infection with pomona. EM x 16,500.

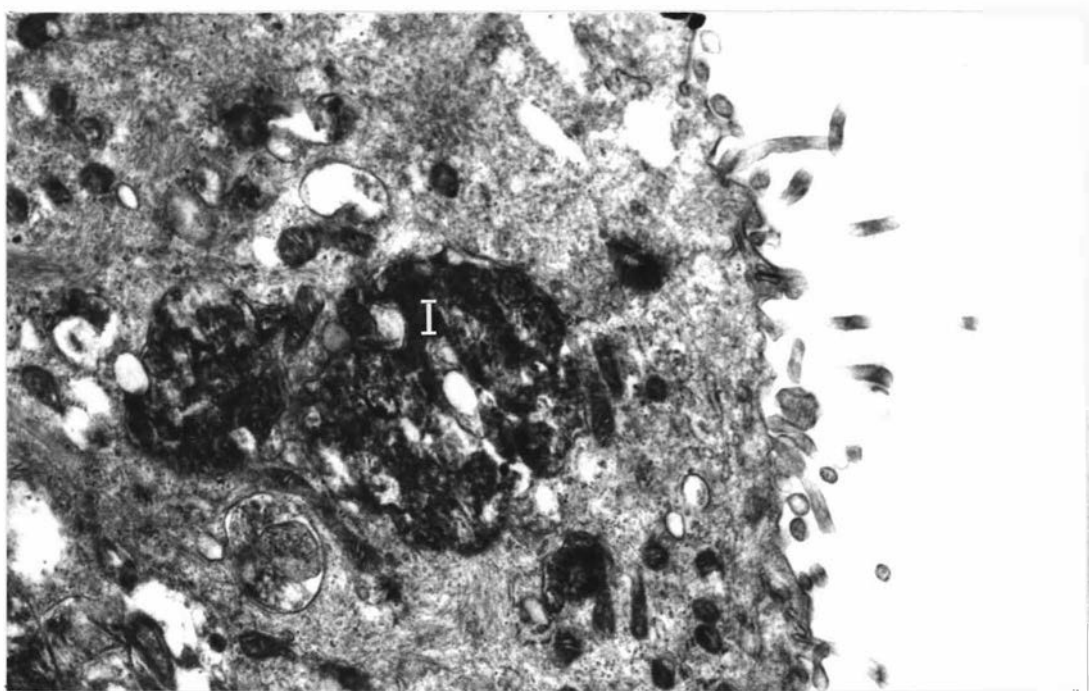
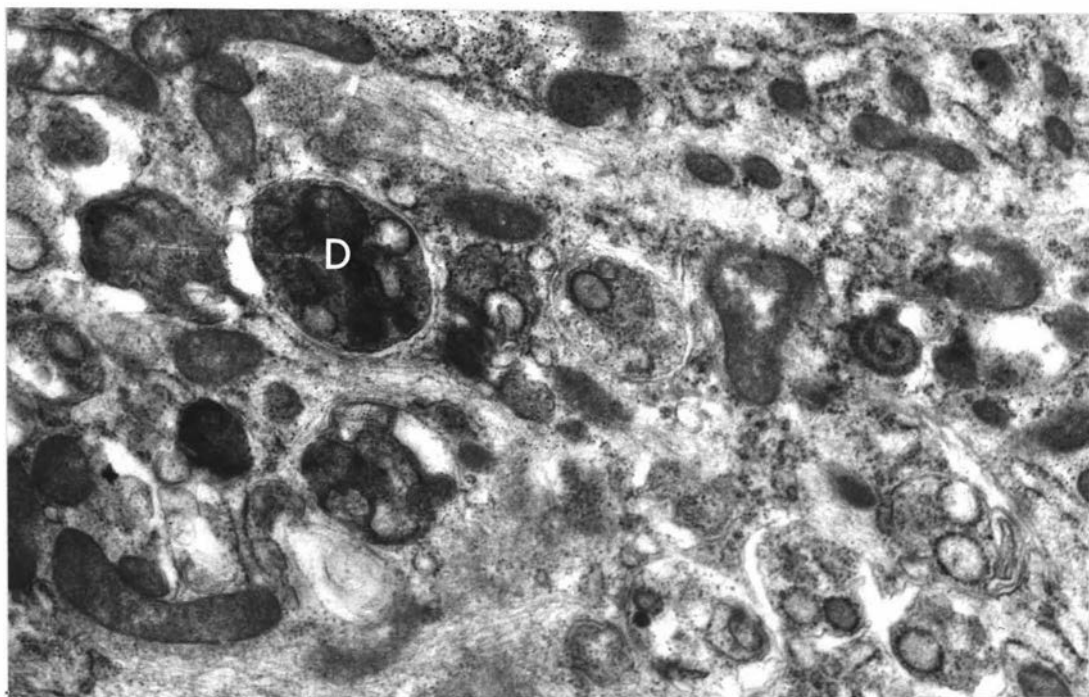


Fig. 2.7 Leptospira interrogans serotype pomona infected foetal lamb kidney tissue culture cell showing vacuolated cytoplasm. Within a vacuole there is material which could possibly be a remnant of a leptospire (arrow).  
EM x 35,000.

Fig. 2.8 Foetal lamb kidney tissue culture cell infected with pomona demonstrating excessive vacuolation of the cytoplasm.  
EM x 16,500.

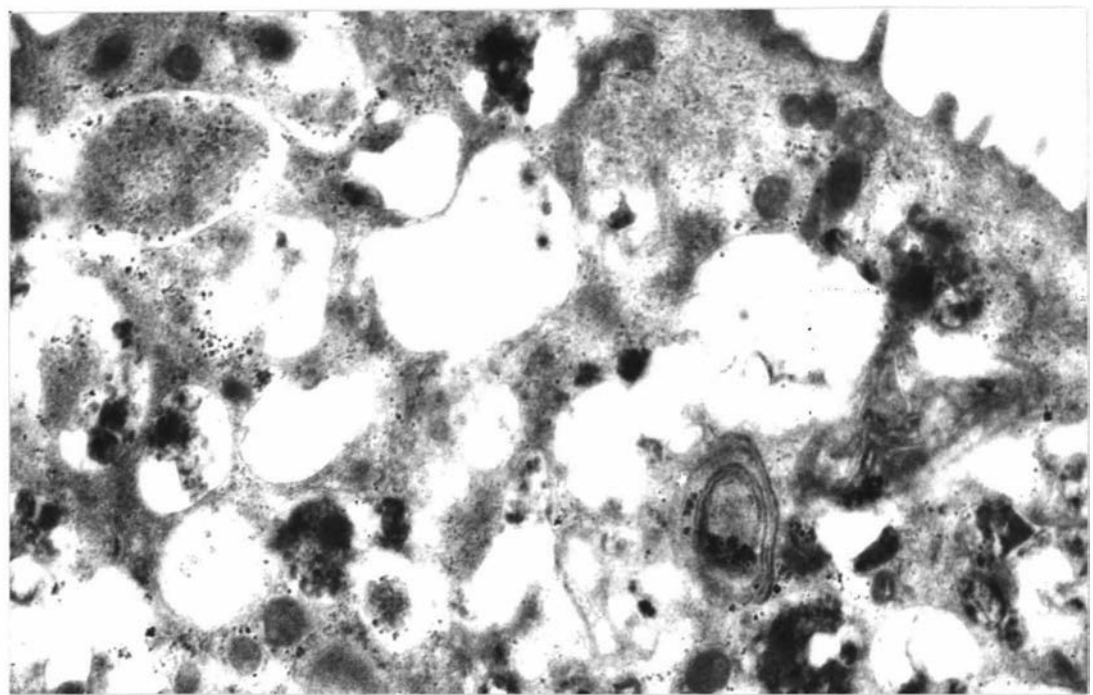
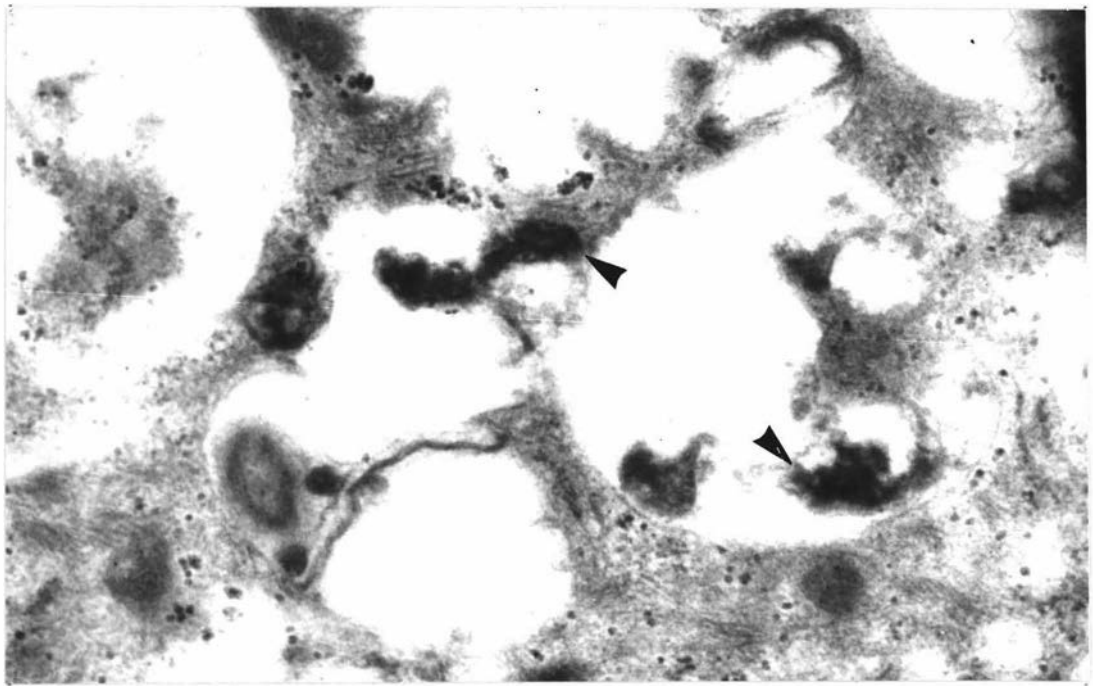
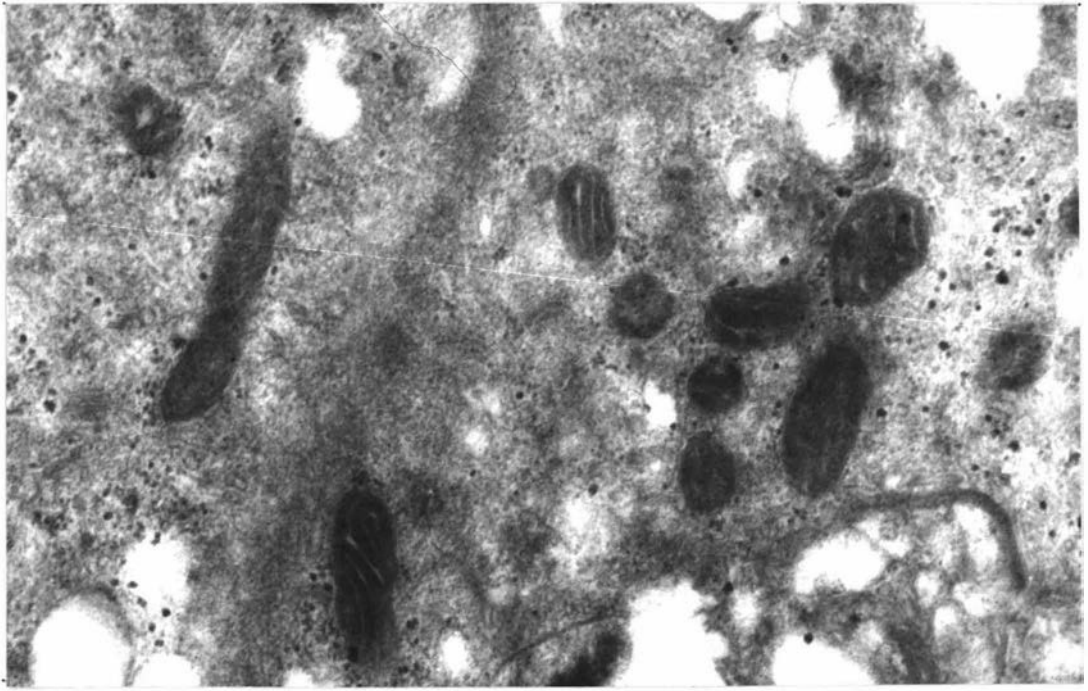


Fig. 2.9 A foetal lamb kidney cell grown in tissue culture and infected with pomona, showing smaller denser mitochondria with greatly thickened cristae. EM x 35,000.



CHAPTER 3

## THE IMPORTANCE OF HAEMOLYSINS AND ENZYMES IN PATHOGENESIS

## INTRODUCTION

The production of particular enzymes by some pathogenic bacteria explains very well why these organisms are capable of producing disease, whilst their close relatives without these attributes remain nonpathogenic. For example the potentially pathogenic staphylococci produce deoxyribonuclease and coagulase whereas the nonpathogenic staphylococci do not. No such differences have been recognised between the pathogenic leptospire and those making up the saprophytic group. Some other differences between the two groups have been observed, such as the relative resistance of the pathogenic group to 8 azoguanine, malachite green and basic fuchsin and the action of bivalent copper ions, their oxidase reactions, lipolytic activities, susceptibility to the leptospiricidal action of serum, their salt tolerances and temperature tolerances (W.H.O. 1957). Many of these differences have not been substantiated by workers in different laboratories neither have they applied in all strains of the same serotype, therefore their significance in conferring pathogenic properties on certain serotypes is in doubt (loc. cit.).

Described in this chapter is an account of the efforts made to look for other properties possessed by serotype pomona which could account for the pathogenic properties in this organism.

A few leptospiral serotypes, pomona included, produce a haemolysin which is capable of acting upon the red cells of different species. It was considered that a better appreciation of the importance of the haemolysin as a pathogenic factor might be obtained if its physical characteristics

were more closely defined. Accordingly in this study various fractions were prepared by chromatographic and precipitation methods and these were investigated for haemolytic activity.

## MATERIALS AND METHODS

### Fractionation of sonicated pomona cultures using column chromatography

A seven day culture of pomona grown in Stuart's medium with 10% rabbit serum was sonicated for 10 minutes and 1 ml of this preparation was fractionated on a Sephadex G200\* column equilibrated with Tris-HCl buffer, pH 8.0, 0.2M (Appendix II). Five millilitre fractions were collected using an Isco fraction collector. The protein concentration of the collected fractions was determined by measuring their absorbency at 280nm. The fractions were concentrated by dialysing against polyethylene glycol.

### Fractionation of sonicated pomona cultured by ammonium sulphate precipitation

Methods employed were those described in Chapter 2.

### Testing for haemolysin

To determine which of the chromatographic fractions contained haemolysin, 0.2 ml of 4% washed sheep red blood cells in normal saline was added to 0.2 ml from each fraction collected. The fractions were incubated overnight at 37°C and then centrifuged at 1,000 g to remove red cells. The supernatant was added to 3.6 ml of distilled water and visually examined for the presence of haemoglobin.

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\* Pharmacia, Upsala, Sweden.

To test leptospiral cultures and ammonium sulphate precipitated fractions for haemolysin, blood agar plates were prepared with a layer of salt agar, overlaid with 10% blood agar. The total thickness of the agar was 5 mm. Blood from many different species was used: human, chicken, cow, goat, sheep, rabbit, cat and dog. Wells were produced by depressing a sterile cork borer into the agar and then removing the agar plug so produced. The bottom of the wells were sealed with a drop of agar. In the first experiment, four wells were prepared in 10 cm plates of blood agar and into each well was delivered 3 drops of a leptospiral culture. The cultures to be tested had been subcultured 7 days previously. One was a recent pomona isolate and was the same strain as used in one of the experiments in Chapter 4. It was compared with laboratory strains of pomona and icterohaemorrhagiae. The fourth well contained sterile Stuart's culture medium as used to grow all the subcultures tested.

In a second experiment, the three fractions obtained by ammonium sulphate precipitation and dialysis as described in Chapter 2 were tested for haemolysin by the tube method and with blood agar plates.

The third experiment entailed a titration of fraction I from the ammonium sulphate precipitation. Normal saline was used as the diluent. In order to determine if specific neutralisation of haemolytic activity occurred a further series of dilutions of the same fraction I were made using as the diluent a high M.A.T. titre (1/10,000) antiserum collected from one of the sheep during the experiments conducted in Chapter 4. These were tested in blood agar wells.

To record the zones of haemolysis on the blood agar plates, measurements in millimetres were made from the edge of the well to the edge of the haemolytic zone when viewed with good underneath lighting. The zone sizes were read after 3 days incubation at 37°C.

#### Testing for the presence of deoxyribonuclease in cultures of leptospire

Four petri dishes of DNA agar\* were prepared and five wells made in the agar using a cork borer. The wells were spaced equidistant from one another and 2 cm from the outer edge of the plate. The wells contained 3 drops of a 7 day culture of a laboratory strain of pomona, an experimental strain of pomona, and a laboratory strain of icterohaemorrhagiae. Uninoculated Stuart's medium and a culture of Staphylococcus aureus were put in wells as negative and positive controls.

After incubating the plates for 48 hours at 37°C they were flooded with normal HCl. Clear zones appearing around a well were interpreted as a sign of deoxyribonuclease activity.

#### Testing for lecithinase activity in leptospiral cultures

Four Nagler plates were prepared using 10% egg yolk in blood agar base. Wells were prepared and filled with the same test materials as above except that the Staphylococcus aureus culture was replaced by a Robinson's meat broth\* culture of Clostridium perfringens. An opalescent zone appearing around a well after 24 hours incubation at 37°C denoted lecithinase production.

#### Testing for the production of hyaluronidase production by leptospiral cultures

Horse synovial fluid was collected from the fetlock joints of a normal horse, using a vacuum collecting tube.

A preliminary test was performed to determine the concentration at which the synovial fluid should be used for this experiment.

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\* Difco Laboratories Limited, Detroit, Michigan, U.S.A.

Doubling dilutions of horse synovial fluid were prepared in phosphate buffered saline (PBS) in 0.5 ml volumes. The dilutions were prepared in agglutination tubes and to each of these tubes 1.5 ml of PBS was added and mixed by inversion. The tubes were incubated in a 37°C water bath for one hour. Before the test was carried out, the tubes were cooled rapidly in an ice bath and 0.4 ml of an 0.5% solution of congo red added to each tube. The congo red was mixed with the synovial fluid dilutions by inverting the tubes several times. The tubes were then allowed to stand for five minutes before testing.

A level petri dish was filled with acid alcohol solution (for details see Appendix II) to a depth of 4.5 mm. Using a pasteur pipette with an internal diameter of 1 mm a quantity of test fluid was removed from each agglutination tube in turn. One drop from each tube was allowed to fall from a height of 10 mm into the acid alcohol. The results were read from the appearance of the patterns formed. The end point was taken as the highest dilution of substrate giving a distinct mass on the bottom of the dish. That dilution was said to contain one indicating dose of substrate.

The initial titration of horse synovial fluid used in this experiment gave an end point such that one indicating dose was equal to a 1 : 16 dilution. Therefore, for the purposes of this experiment to test for hyaluronidase a 1 : 2 dilution was used. Because of possible interference from the serum contained in most leptospiral culture media, the one chosen for this experiment was EMJH\* which is serum free (see Appendix II). A heavy seven day culture of pomona in EMJH was used.

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\* Difco Laboratories Limited, Detroit, Michigan, U.S.A.

Doubling dilutions of EMJH medium alone and doubling dilutions of pomona culture in EMJH were made. To each dilution was added 0.5 ml PBS and 0.5 ml of horse synovial fluid which had been diluted so that it contained 8 indicating doses. The end point was the tube containing the least amount of substrate from which the drop spread freely. Controls using buffer and substrate only were always included in the test.

## RESULTS

### Haemolysin production

Results are summarised in Table 3.1. Pomona (1) is the laboratory strain, pomona (2) is a fresh isolate. The largest zone of haemolysis occurred in the agar plates containing bovine red blood cells. In the plates using avian red cells the relatively large zone around the well containing a fresh pomona isolate, was noteworthy especially when compared to the laboratory strain. In the plate with sheep red blood cells the well containing uninoculated medium gave an unexplained very slight zone of haemolysis.

### Titration of haemolysin and its neutralization by homologous antiserum

Only a sonicated whole culture of a recent pomona isolate was used for titration. The last well showing a measurable zone of haemolysis was taken as the endpoint.

The pomona culture had a titre of 1 : 5. Complete neutralization of the haemolysin occurred with antiserum in all dilutions including the lowest which was at 1 : 2.

### Haemolytic activity in chromatographic fractions of whole cultures

The elution profile of the sonicated pomona preparation shows the

TABLE 3.1

HAEMOLYTIC ACTIVITY OF LEPTOSPIRAL CULTURES WITH RED BLOOD CELLS  
FROM VARIOUS SPECIES

Species of RBC	Width of zone of haemolysis (mm)			
	<u>pomona</u> (1)*	<u>pomona</u> (2)**	<u>icterohaem.</u>	Stuart's medium
Chicken	1.0	5.0	0	0
Sheep	4.5	4.5	1.0	1.0
Human	0.5	0	0	0
Goat	2.5	2.5	0	0
Cow	8.0	7.0	2.0	0
Cat	3.0	3.0	0	0
Rabbit	0	0	0	0
Dog	0	0	0	0

\* pomona (1) a laboratory strain of pomona.

\*\* pomona (2) a fresh isolate of pomona from a sheep.

protein concentration of the fractions collected from the Sephadex column (Fig. 3.1). Also in this figure the fractions in which haemolytic activity was detected are shown and the height of the shaded area is proportional to the amount of haemolysin subjectively estimated.

#### Lecithinase production

No evidence of lecithinase production in the form of an opalescent zone was demonstrated, however a narrow zone of clearing of the opaque medium was observed around the well containing the poimona culture.

#### Hyaluronidase production

There was no difference in the titrations for hyaluronidase activity between those using whole cultures and the negative controls. There was therefore no evidence of hyaluronidase production by poimona.

#### Deoxyribonuclease production

There was no evidence of any production of this enzyme by the method employed.

### DISCUSSION

The haemolytic activity of poimona is well known and haemolytic anaemia is in large part responsible for the deaths of poimona infected calves (Reinhard, 1951; Bauer and Morse, 1958; Bauer et al., 1961).

Four hypotheses have been advanced to explain the pathogenesis of leptospiral haemolytic anaemia. Each hypothesis ascribes a different role to antibody:-

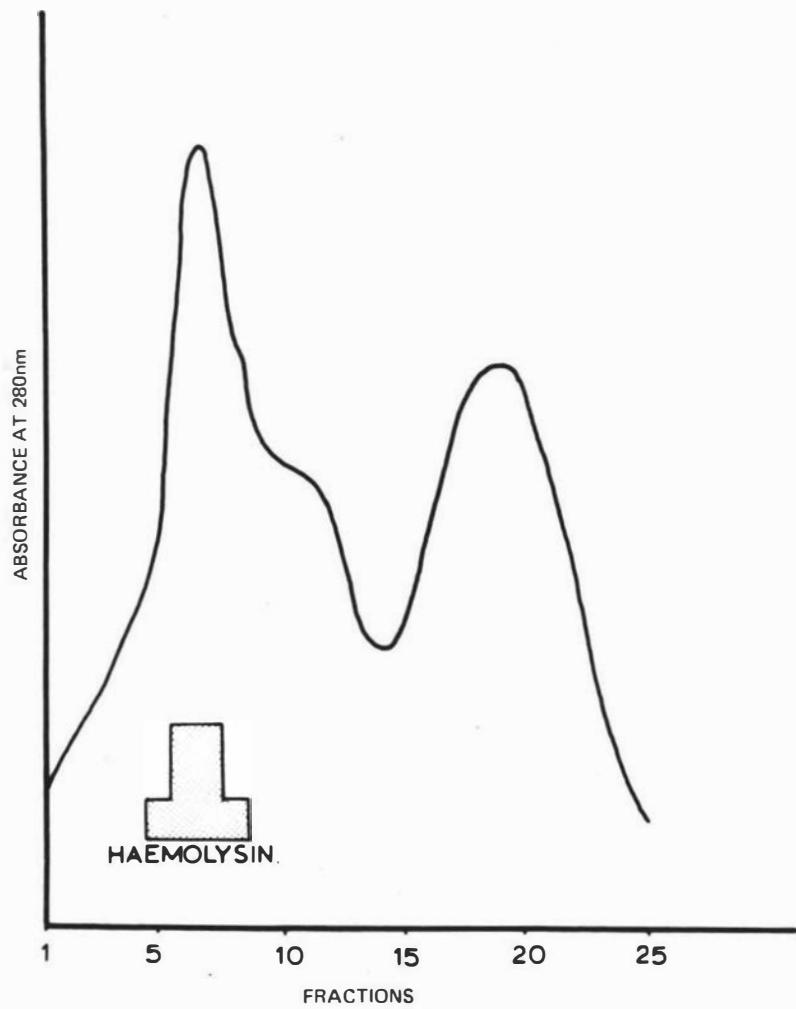


FIG 31 HAEMOLYSIN RELATED TO THE ELUTION PROFILE OF A SONICATED POMONA CULTURE.

The first hypothesis put forward by Ferguson et al. (1957) suggests that antileptospiral antibody, together with complement, lyses the leptospire and releases haemolysin. This hypothesis is not substantiated by the experiments reported here. The haemolytic activity of whole cultures has been demonstrated using red cells from a wide range of species and incorporated in agar, a system in which antibody could play no part. It could, however, be argued that this was an artificial situation in which a large number of organisms had lysed and that this situation is not really comparable to an in vivo one. Results from both these experiments and those of others (Decker et al., 1970) demonstrate, however, that haemolytic anaemia of varying degrees can occur before any demonstrable antibody titre. Also, the use in this experiment of washed red blood cells (RBC) to detect the degree of haemolysis indicates that complement is not necessary for this reaction to take place.

The second hypothesis, that of Kanich et al. (1968), suggests that antibody and complement bring about lysis of red blood cells previously sensitized with leptospiral antigen. In the present studies the RBC's were thoroughly washed therefore no antibody or complement could be present. These findings therefore completely rule out this second hypothesis.

In those studies that ascribe a role to antileptospiral antibody as a factor in the bringing about of haemolysis, an assumption is made that the production of this antibody is measurable by the microscopic agglutination test. This assumption is not warranted as the existence of an independent leptospiricidal antibody is reported in Chapter 8. Furthermore, any hypothesis that ascribes a role to agglutinating antibody as a factor in haemolysis is incompatible with time relationships that exist between the appearance of antibody and the commencement of intravascular haemolysis,

see Chapter 4, Bauer and Morse (1958) and Bauer et al. (1961). It has also been shown that serum with a high titre of agglutinating antibody inhibits haemolysis (Alexander et al., 1971).

The third hypothesis invokes the action of cold haemagglutinins and has been put forward by Decker et al. (1970) and Bhasin et al. (1971). The absence of any haemolytic activity by this antibody at normal body temperature, makes its significance in the genesis of leptospiral haemolytic anaemia hard to understand.

An earlier theory, put forward by Bauer and Morse (1958) and Bauer et al. (1961), maintained that haemolysin per se released by leptospiral organisms was the dominant factor in the development of the haemolytic anaemia. The results of this study are in agreement with their findings.

The chemical nature of the haemolysin is not known. Little can be said about its structure, however its appearance in fraction I prepared by precipitation with 40%  $(\text{NH}_4)_2\text{SO}_4$  as described in Chapter 2, together with its presence in the earlier fractions eluting from the Sephadex G200 column suggest that it is of a high molecular weight. That it is probably not lecithinase is indicated by the negative results of the egg yolk experiment.

The absence in pomona of any deoxyribonuclease activity detectable by the method used in this study, suggests that this enzyme is not responsible for conferring the property of pathogenicity on this serotype.

Another enzyme system possessed by some pathogenic micro-organisms is that of hyaluronidase production. This enzyme aids the organism in its intercellular spread by breaking down hyaluronic acid, a major component in intercellular ground substance. It is perhaps surprising that no detectable amount of hyaluronidase was to be found associated with sonicated cultures of pomona.

## SUMMARY AND CONCLUSIONS

1. Haemolysin per se appears to be the dominant factor responsible for the haemolytic anaemia which occurs in pomona infections.
2. The haemolysin produced by pomona has a fairly large molecular weight as deduced from its precipitation with 40% ammonium sulphate and its elution pattern using a Sephadex column.
3. There is no evidence which implicates either agglutinating antibody or complement in the development of leptospiral haemolytic anaemia.
4. The haemolysin produced by pomona is not a lecithinase.
5. Experiments to establish the elaboration by pomona of either hyaluronidase or deoxyribonuclease gave negative results.

CHAPTER 4

## EXPERIMENTAL LEPTOSPIROSIS IN SHEEP

## INTRODUCTION

Any in vitro studies into the host-parasite relationship of pomona infections must sooner or later be extended and related to the disease in the whole animal.

In choosing an experimental animal for these investigations it became clear that the sheep presented a considerable number of practical advantages as a test species. They are readily available, they are easy to observe and handle and the frequent collection of urine and blood samples presents little problem. The fact that sheep become naturally infected has long been appreciated (Anon, 1951). However, the clinical manifestations of pomona infections in sheep have not been studied in detail in New Zealand and only to a limited extent overseas (Morse et al., 1957; Decker et al., 1970 and Bhasin et al., 1971). The experiments described in this chapter therefore have provided significant new information on the disease in sheep. Such information should have considerable value in future investigations to determine the role of sheep in the epidemiology of leptospirosis in this country.

The main purpose of the present experiments was to observe the course and character of the experimental disease due to serotype pomona and relate these to the presence of the organism in the circulation and in the urine. In parallel to this, studies were made of the humoral antibody response to infection.

## MATERIALS AND METHODS

Two experiments were carried out infecting sheep with pomona and measuring various parameters of the blood and urine as well as making a clinical assessment of the animals.

The first experiment was in the nature of a pilot experiment. The second experiment incorporated control animals which in most respects acted as controls to the first experiment. Experience gained in the first experiment allowed an alteration in the schedule of specimen collection in the second experiment, to give a closer monitoring of parameters at expected times of change.

In Experiment 4a four Romney Marsh ewes, each with a lamb at foot, were purchased and all eight animals were experimentally infected.

Eight Romney ewes, each with a lamb at foot, were obtained for Experiment 4b. Four of the ewes together with their lambs were infected with pomona; the remaining four ewes and their lambs served as noninfected control animals.

All animals were apparently healthy and there was no history of previous disease. Prior to inoculation with leptospiral organisms the sera of the sheep were tested for agglutinins against the following serotypes of leptospire; pomona, grippotyphosa, bataviae, biflexa, pyrogenes, ballum, hardjo, hyos, australis, icterohaemorrhagiae, autumnalis, canicola and serjroe. All gave a negative reaction at dilutions of 1 : 10 and higher.

The sheep were kept indoors for a period of at least one week before inoculation as well as throughout the entire experimental period.

They were fed twice daily with good quality hay and once daily with sheep nuts. The control sheep were kept in a separate pen from the infected sheep. The pens had independent drainage and the chances of the control sheep becoming infected was minimal. The control sheep were always fed first and their pens cleaned out and samples of blood and urine taken before the infected sheep pens were entered.

The sheep were observed in the pre-inoculation period for clinical signs of disease, their rectal temperatures were taken twice daily and a urine sample taken once daily from each animal. A haematological examination was also made of each animal prior to inoculation of the experimental sheep with leptospire.

Following the inoculation of the experimental sheep, a clinical appraisal of the animals was made each morning. At the same time their rectal temperatures were taken using a clinical thermometer and mid-stream urine samples collected in 1 oz Universal containers. Urination was stimulated by holding the mouth and nose of the sheep firmly closed with the hand.

At the termination of the experiments at 31 days (Experiment 4a) and 34 days (Experiment 4b) the animals were subjected to a routine necropsy procedure. In both experiments data were collected up to and including the 31st day after inoculation.

#### Inocula

Recent isolates of poimona are believed to be more pathogenic than strains which have been maintained for any length of time in the laboratory. Accordingly the two strains used in these experiments were recently isolated from pig kidneys obtained from a local abattoir. The organisms were grown

in Fletcher's medium and were then subcultured once into Stuart's medium for use as the inoculum. The cultures took three weeks from the time of their isolation to reach a sufficient concentration for use.

The sheep were injected intraperitoneally with approximately  $46 \times 10^6$  leptospire/ml in 2 ml of Stuart's medium. An estimate of the number of organisms in the culture, prepared for inoculation, was obtained using the Petroff-Hausser counting chamber\* under the darkfield microscope. All the darkfield microscopy examinations were made using a Leitz Ortholux microscope\*\* equipped with a dry darkfield condenser, a 12.5 x objective and 10 x eyepieces.

#### Examination of blood

In Experiment 4a, samples of blood were taken from the jugular vein of each animal on postinfection days (P.I.D.) -3, -2, +3, +7, +10, +14, +17, +21, +24, +28, +31. In Experiment 4b these collections were made on P.I.D. -1, +1, +3, +8, +10, +13, +15, +17, +20, +22, +24, +27, +29, +31. The blood was collected in vacuum tubes both with and without E.D.T.A. as an anticoagulant. A drop of the blood containing E.D.T.A. was mixed with 1 ml of physiological saline in an agglutination tube and centrifuged at 2,000 r.p.m. for 5 minutes. The supernatant was examined directly by darkfield microscopy for the presence of leptospire. The samples collected in the E.D.T.A. vacuum tubes were also used to obtain both haemoglobin and haematocrit values and also for differential white cell counts.

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\* C.A. Hausser and Son, Philadelphia, U.S.A.

\*\* (Leitz Wetzlar) Ernest Leitz G.M.B.H. Wetzlar, Germany.

Haemoglobin estimations were made using the cyanmethaemoglobin method described by Dacie and Lewis (1968), and the values read directly on an EEL\* haemoglobinometer. The haemoglobinometer was standardized using commercially prepared standards\*\*. Leucocyte counts were made according to the method of Dacie and Lewis (1968) and differential counts were made on smears stained by a tetrachrome stain (MacNeal)\*\*\*. Haematocrit values were obtained using an International Microcapillary Reader\*\*\*\*.

The blood samples without E.D.T.A. were allowed to clot and the serum separated for serology. Using the technique detailed in Chapter 6 all serum samples were titrated within two days for agglutinating antibody to serotype pomona. If not titrated the same day as collected, the serum were stored at  $-15^{\circ}\text{C}$  overnight. The maximum dilution of each serum sample tested was 1 : 10,000.

#### Examination of urine

Darkfield microscopic examination for leptospire was carried out within one hour of collection of the urine samples. At the same time 0.2 ml of urine was added to one tube each of Stuart's and Fletcher's medium containing 100 mcg/ml of 5-fluorouracil\*\*\*\*\* to retard the growth of microbial contaminants (Johnson and Rogers, 1964). The medium was contained in 120 x 20 mm screw capped glass tubes. These tubes were incubated at  $27^{\circ}\text{C}$  and examined at regular intervals by darkfield microscopy.

\* Evans Electroselenium Limited, Halstead, Essex, England.

\*\* Diagnostic Reagents Limited, Thame, Oxon, England.

\*\*\* G.T. Gurr, London, England.

\*\*\*\* International Equipment Co., Needham Heights, Mass., U.S.A.

\*\*\*\*\* Hoffman - La Roche & Co., Basle, Switzerland.

Tests on urine for pH, protein, glucose, ketones, bilirubin and blood were carried out using Bili-Labstix\*. The manufacturers directions were strictly adhered to while using this product. Technical information as to the sensitivity of the reagents used in the Bili-Labstix are given in Appendix III. Casts and crystals were identified by their appearance and shape when urinary sediment was viewed under a regular light microscope.

## RESULTS

The results of both experiments 4a and 4b are illustrated graphically in Figs. 4.1 to 4.6. More detailed data are presented in Appendix III.

### Experiment 4a

Three lambs (594, 595, 596) in Experiment 4a showed an elevation of temperature, reaching a peak on postinoculation day (P.I.D.) 9, 7 and 6 respectively. Only one of the ewes (599) had any significant rise in temperature and her highest temperature ( $40.5^{\circ}\text{C}$ ) was recorded on P.I.D. 7 (Fig. 4.1). The highest temperature recorded from any of the sheep in this experiment was that of lamb 594 with a reading of  $41.3^{\circ}\text{C}$  (Fig. 4.2).

Haemoglobin levels started to fall in most cases three days after inoculation with leptospire and in the lambs the levels were at their lowest average level 10 to 14 days after inoculation. The haemoglobin levels in the ewes dropped to their lowest level 15 days after inoculation. The lowest haemoglobin level recorded was in lamb 594 on P.I.D. 14 at  $5.2 \text{ mg}/100 \text{ ml}$ .

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\* Ames Company, Division Miles Laboratories Inc., Elkhart, Indiana, U.S.A.

The overall haematocrit levels for both ewes and lambs were at their lowest between P.I.D. 10 to 14, the lowest haematocrit was 18% below the mean control value in Experiment 4b and occurred on P.I.D. 14 in lamb 594.

#### Experiment 4b

Infected lambs in this experiment began to show a rise in temperature on P.I.D. 4 when the average temperature for the infected lambs was 40.7°C; no corresponding rise in temperature was noted in the ewes. At no other time during the experiment did the infected lambs average temperatures approach the peak that occurred on P.I.D. 4. The highest temperature recorded for an individual animal was that of lamb 389, with a temperature of P.I.D. 4 of 41.6°C (Fig. 4.3).

Mean haemoglobin levels reached their lowest recorded level on P.I.D. 3, although no samples were taken on any of the days between P.I.D. 4 and 7. On P.I.D. 8 the levels were showing some recovery (Fig. 4.6). The average level of haemoglobin for the lambs was less than that recorded for the ewes until towards the conclusion of the experiment when the levels in the ewes dropped for reasons believed to be unrelated to the experiment.

The lowest individual haemoglobin level recorded was 9.3 g/100 ml in both a lamb (391) and a ewe (387), both of these were recorded on P.I.D. 3. On P.I.D. 8 the average haemoglobin values for the control lambs dropped to a level equal to that of the experimental lambs on P.I.D. 3. This was due to a drastic fall in the haemoglobin level of one control animal to 9.0 g/100 ml. The reason for this fall in level was not determined, but this lamb did not develop any titre to pomona and at no time could leptospire be demonstrated or cultured. The possibility of accidental leptospiral infection of this control animal could therefore be excluded. Average haematocrit levels in the experimental animals were consistently slightly lower than

those of the controls and the maximum difference occurred on P.I.D. 3 when the average for the control sheep was 38.4% while that of the experimental sheep was 34.7% (Fig. 4.6).

Neutrophil and lymphocyte counts, on an average, showed a change only over a period from P.I.D. 1 to 8, the neutrophils showing a biphasic fall in numbers over this period. The lymphocytes showed a corresponding double peaked rise in count (Fig. 4.6).

The sheep in both of these experiments gave little sign of illness except for a slight lethargy and an increased rate of respiration during the febrile period. One animal (599) in Experiment 4b had a much less obvious transient haemoglobinuria. Leptospire were first seen directly by darkfield microscopy in the urine on P.I.D. 14 and thereafter intermittently in one or more of the sheep until the termination of the experiment (Fig. 4.1).

In Experiment 4b the leptospire were cultured from one or more of the animals' blood on P.I.D. 1 to 6 inclusive. The first urine to give a positive culture was collected on P.I.D. 14 from animal 389. This same animal became a heavy shedder of organisms from P.I.D. 19 until her death at the termination of the experiment. This lamb also experienced the highest body temperature of any of the animals during the experiment.

Variations in the specific gravity, pH or quantity of protein present in the urine were either inconclusive or the existence of abnormal measurements within the control group made the interpretations difficult. For this reason these parameters have not been included in this chapter but may be found in Appendix III.

Immunological data in regard to these experiments is detailed and discussed in Chapter 6.

## DISCUSSION

The general pattern of experimental disease produced in the two experiments reported here can be regarded as broadly similar. The few minor variations in time scale of the changes in some parameters, can probably be explained as being due either to normal variations or to slight differences in pathogenic characteristics of the two different pomona isolates used.

The haemolytic anaemia which occurred in these experiments was less severe and most evident three days later than the marked haemolytic anaemia reported by Decker et al. (1970). The reasons for these differences is a matter of conjecture but two possible explanations are offered: Firstly a difference in the pomona strain used, and secondly better maintenance of virulence by use of an inoculum consisting of leptospiraemic hamster or guinea pig blood (Decker et al., 1970). In the experiments described here there is no doubt that the sheep were successfully infected. The kidneys tubules became colonised by leptospire and some of the animals became heavy shedders of organisms. This shedding of organisms was first noted on P.I.D. 14 both by direct examination and as a result of culturing of urine. Unlike the situation in Decker's experiments, many organisms were seen to be shed in the urine of our experimental animals and one animal did so intermittently from P.I.D. 14 until its death at the termination of the experiment on P.I.D. 31. This same animal was detectably leptospiraemic from P.I.D. 1 to 3 and again on P.I.D. 6. Gross examination of the kidney at the end of the experiment revealed many lesions similar to those found in field cases of leptospirosis. Not all the animals in the experiment were so severely infected although there was serological and histological evidence that all the inoculated animals did in fact become parasitized by the organism.

Explanations of the pathogenesis of leptospirosis in sheep can be drawn from the sequence of events observed when sheep are experimentally infected with leptospire. Morse et al. (1957) have suggested that the build up of circulating antibodies following leptospiraemia may bring about active lysis of the organisms, releasing endotoxin or haemolysin. The endotoxin or haemolysin may then cause the disruption of erythrocytes and a consequent haemoglobinuria.

Another explanation could be that the leptospire may elaborate sufficient haemolysin in vivo to lyse erythrocytes in large numbers without the intervention of antibody. Neither of these possibilities is excluded in the current experiments although experiments reported in Chapter 3 clearly indicate the great capacity of leptospire to produce haemolysin in vitro.

Personal communications indicate that the severe haemolytic crises reported by Decker et al. (1970) may not represent the typical situation that is found in New Zealand sheep with leptospirosis. A number of flocks have been found with a high incidence of kidney lesions typical of leptospirosis. Confirmation that these are in fact leptospirosis cases has been possible in a few instances, both by culture and histopathological examination. These infected flocks have had no observed clinical signs of ill health. This lends support to the contention that some outbreaks of leptospirosis may be of a more benign variety, similar clinically to that described in this study. Recognition and hence reporting of outbreaks of leptospirosis in sheep tends to be dependent on the clinical manifestation of the haemolytic crises type being present. Reports in the literature are therefore usually in regard to florid outbreaks of this type.

## SUMMARY AND CONCLUSIONS

1. Experiment leptospirosis initiated in sheep by fresh isolates of pomona has proved clinically less severe than the limited number of reports in the literature would indicate.
2. The following parameters have been noted in the experimental disease:
  - (a) Elevated temperatures occur between P.I.D. 4 and 9.
  - (b) The lowest haemoglobin levels are recorded between P.I.D. 3 and 15.
  - (c) Haematocrit levels were depressed during the period between P.I.D. 3 and 14.
  - (d) Leptospiraemia may be evident between P.I.D. 1 and 6.
  - (e) Leptospire may be detected in the urine of sheep as early as P.I.D. 14 and most animals became leptospiruric from P.I.D. 21 to at least P.I.D. 31.
3. It is likely that benign infections, similar to the experimental disease, may be more common in the field than is generally appreciated.

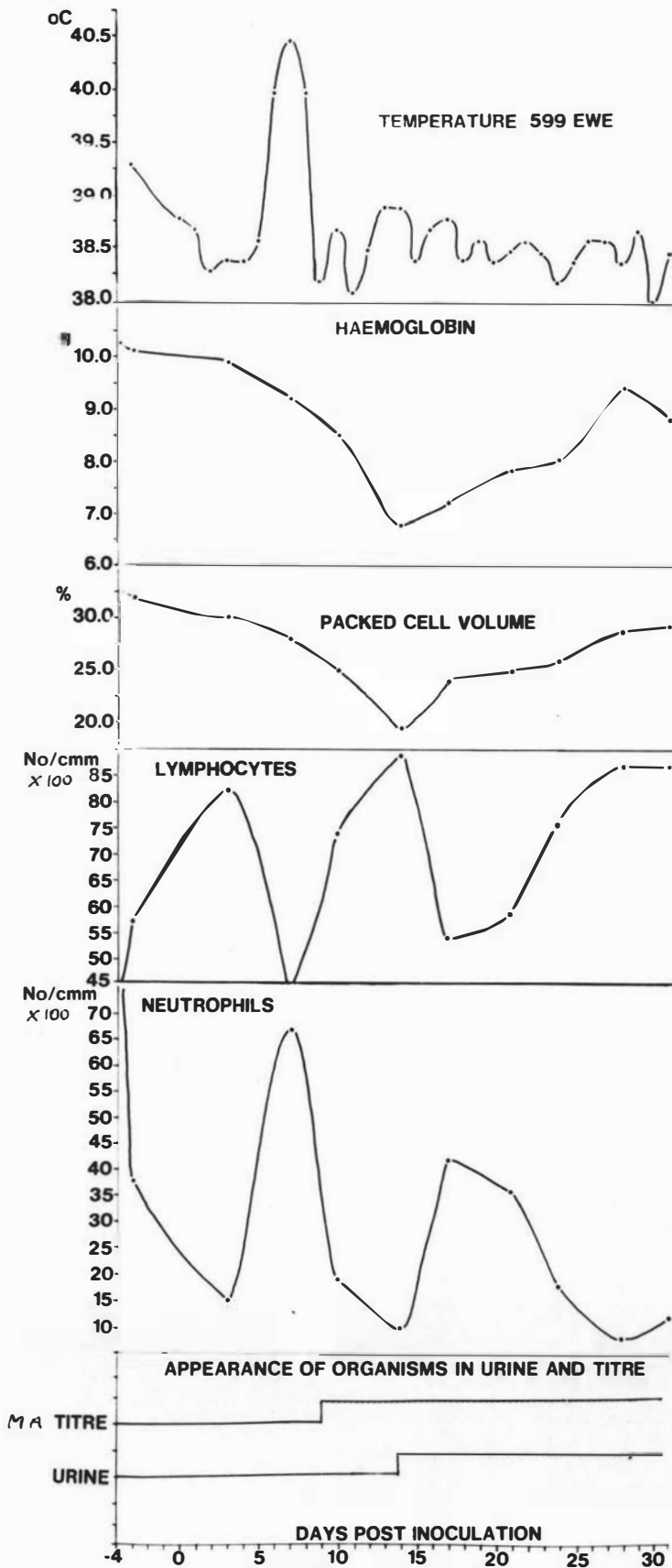


Fig. 4.1 Data collected from experimental ewe 599 (Experiment 4a) after infection with pomona.

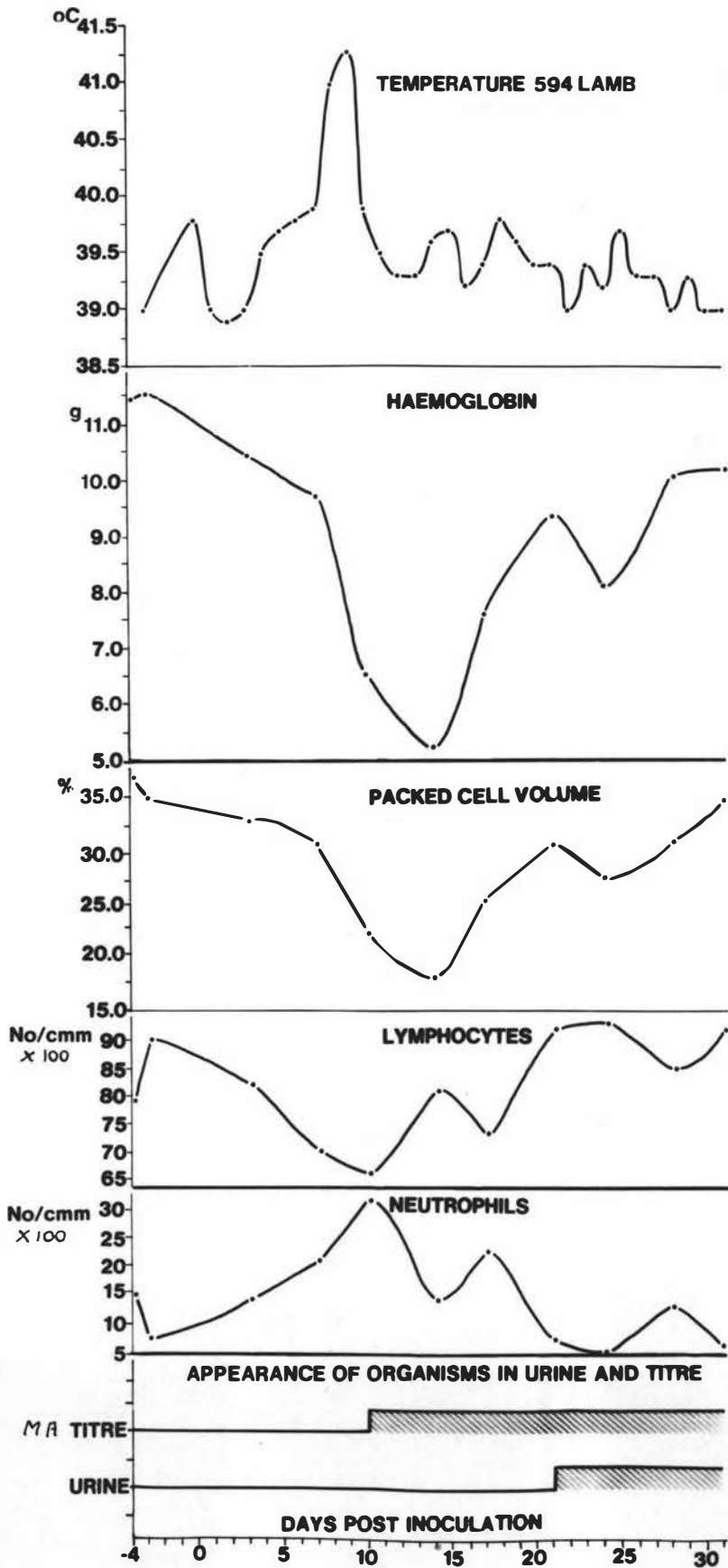


Fig. 4.2 Data collected from experimental lamb 594 (Experiment 4a) after infection with pomona.

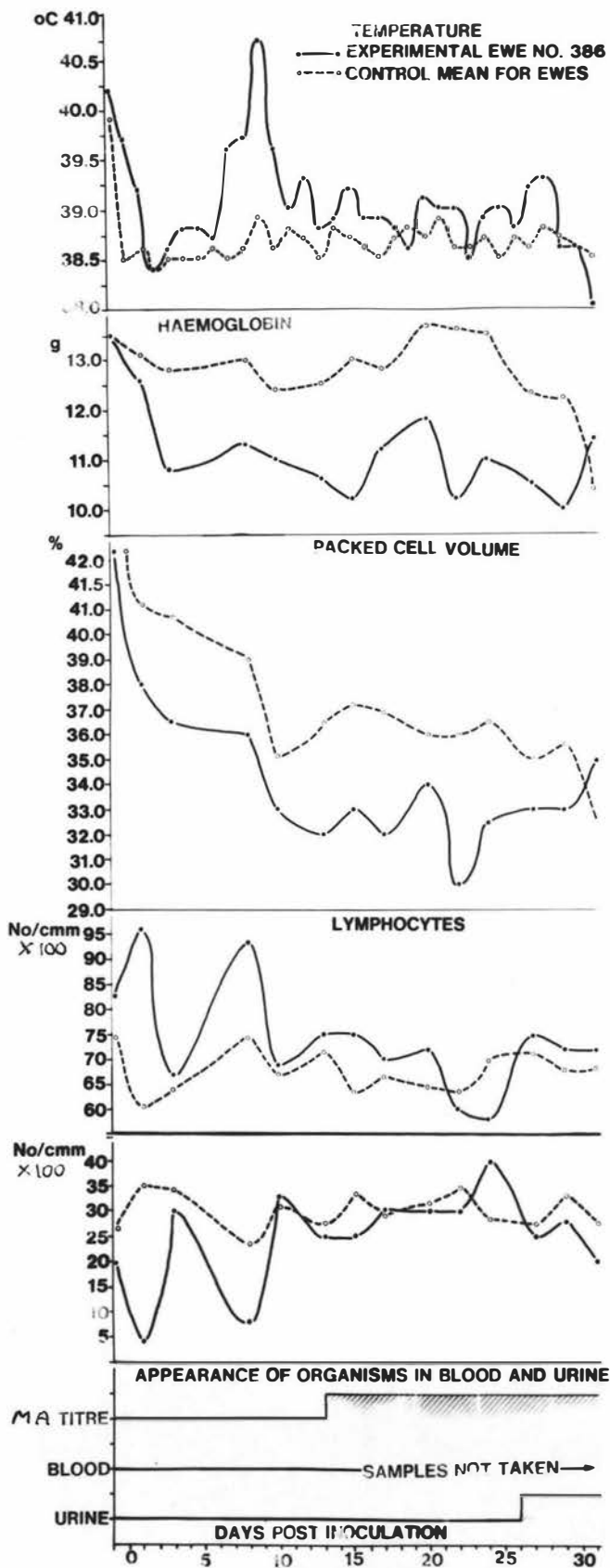


Fig. 4.3 Data collected from experimental ewe 386 after infection with pomona as compared with the mean values from 4 control ewes (Experiment 4b).

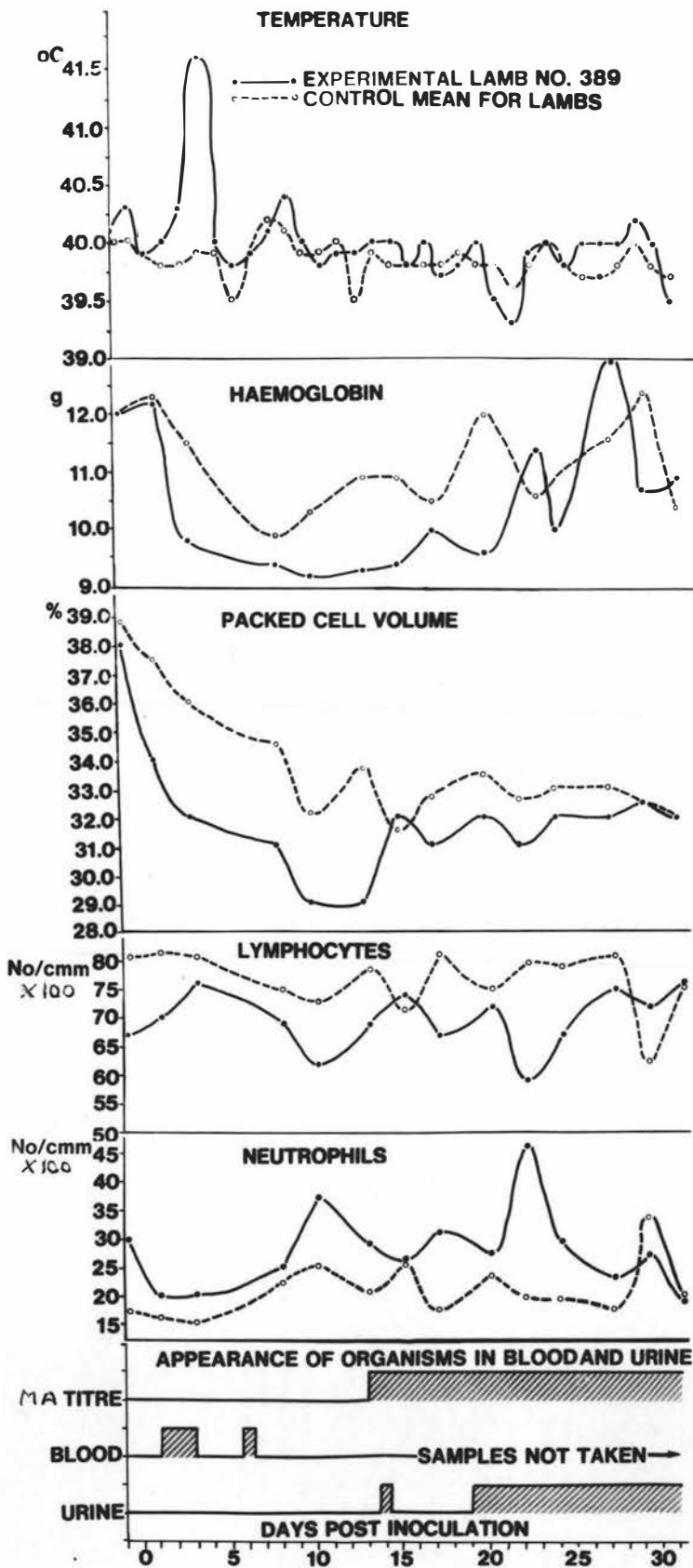


Fig. 4.4 Data collected from experimental lamb 389 after infection with pomona as compared with the mean values from 4 control lambs (Experiment 4b).

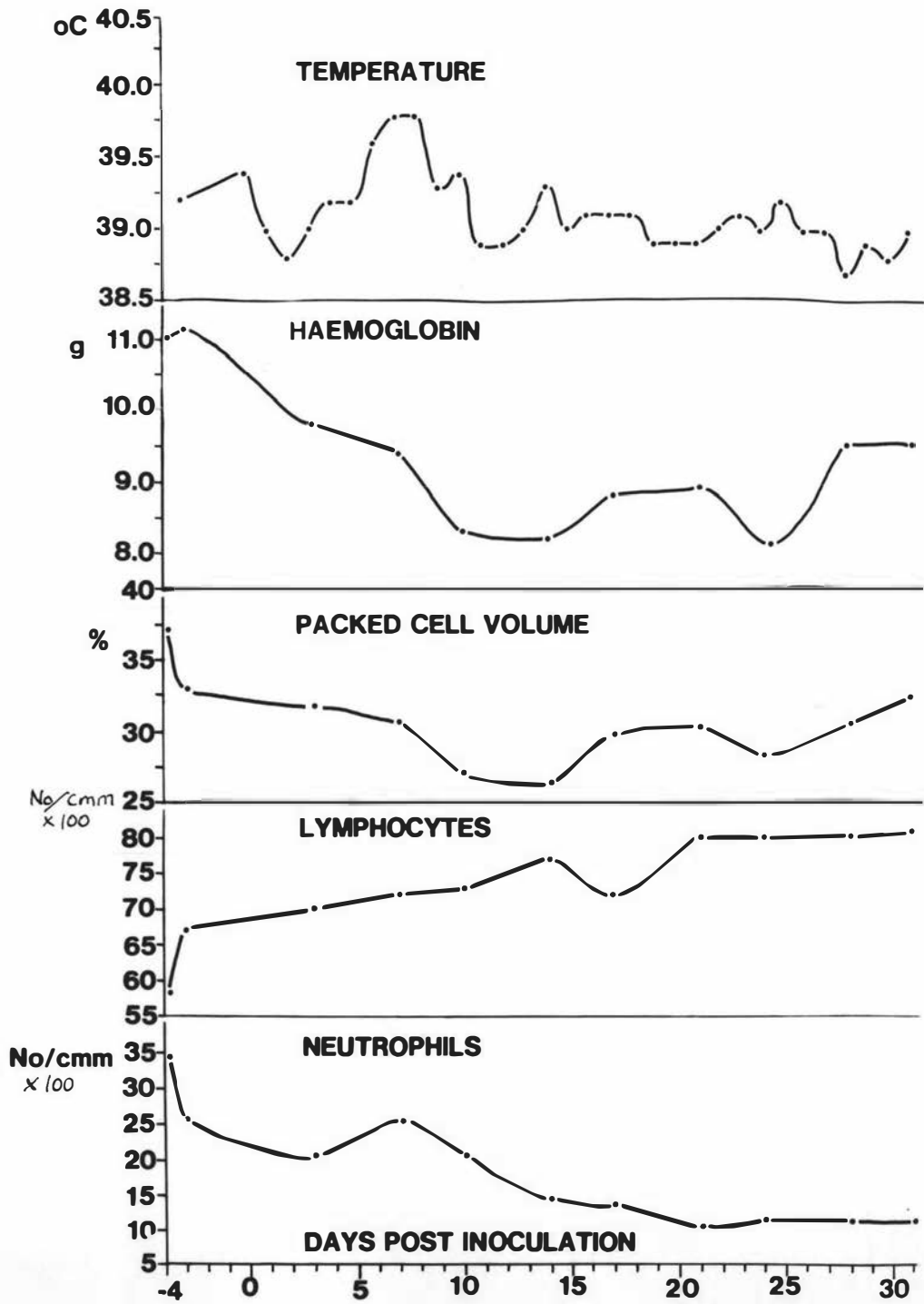


Fig. 4.5 Mean values for temperature, haemoglobin, PCV, lymphocyte and neutrophil counts, calculated from the readings taken from 6 experimental sheep in experiment 4a.

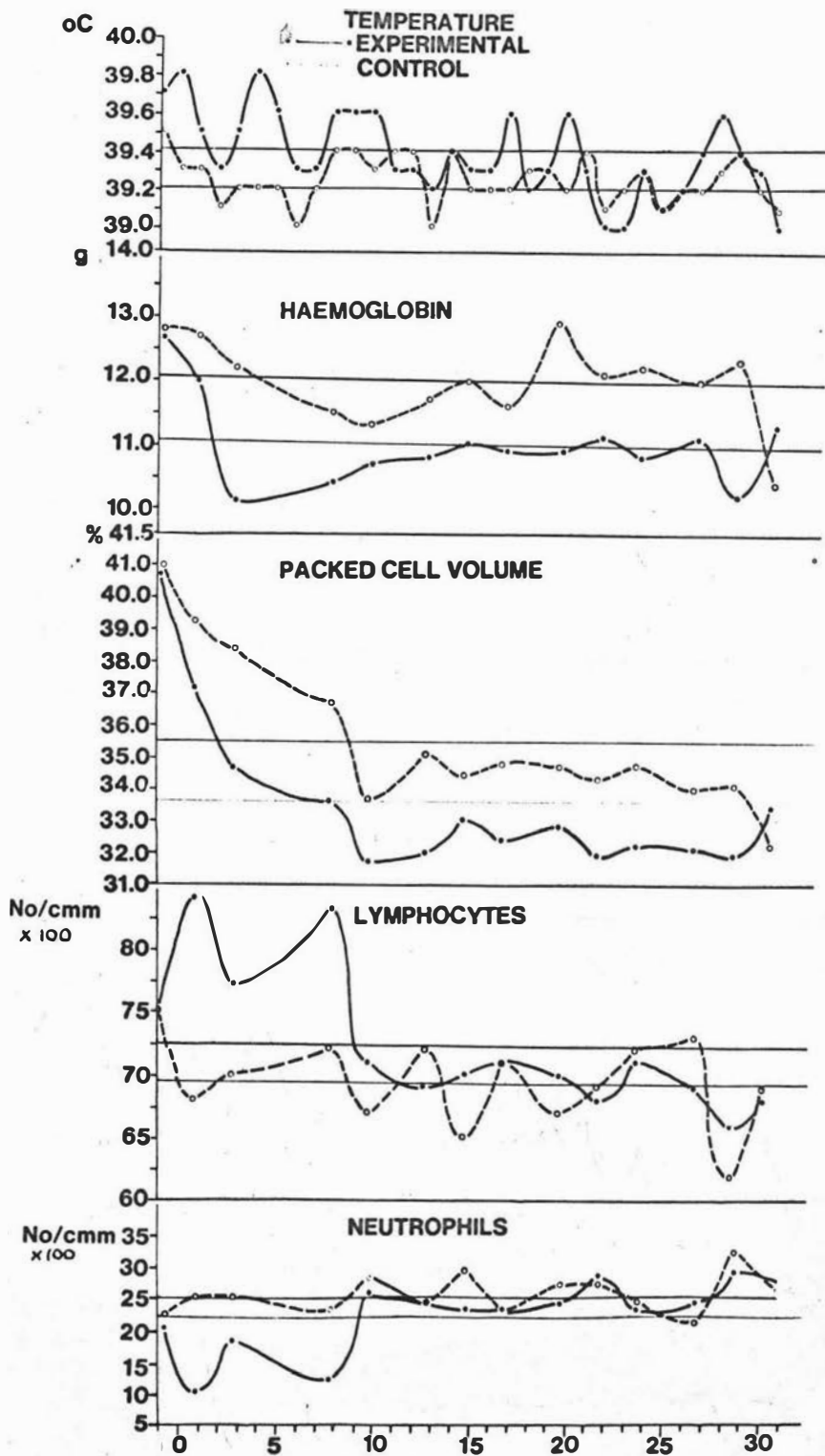


Fig. 4.6 Mean values for temperature, haemoglobin, PCV, lymphocyte and neutrophil counts, calculated from the readings taken from 8 experimental and 8 control sheep used in Experiment 4b.

## CHAPTER 5

### MORPHOLOGICAL CHANGES OF LEPTOSPIROSIS IN SHEEP WITH PARTICULAR REFERENCE TO THE KIDNEY

#### INTRODUCTION

The only noteworthy gross lesions found in the experimentally infected sheep (Chapter 4) at necropsy were those seen in the kidney and detailed morphological studies were confined to this organ. Colonisation of the kidney by leptospire has long been known as an outstanding feature of the post-acute phase of leptospirosis and an intensive study of the kidney including electron microscopy appeared to be the best approach to a fuller understanding of the spatial relationship between the parasitic leptospire and host cells.

In this study much use has been made of montages of electron micrographs to enable a wider field to be compared with a similar field from a serial section examined by the light microscope.

Up to the present time ultrastructural studies of the kidney in leptospirosis have concentrated upon human biopsy material (Brito et al., 1965; Sitprija and Evans, 1970). These infections have been due to a number of different serotypes.

One report in animals is of a spontaneous leptospiral infection in a rat; the rat was being used for ultrastructural studies of a different nature. This case enabled the authors (Martino et al., 1969) to describe the morphologic changes associated with the localisation of this unidentified serotype in the cells and lumen of the proximal tubules of the rat. Brito and co-workers (1966) have described the pathology of the guinea pig kidney when experimentally infected with icterohaemorrhagiae and Miller and Wilson

(1967) have described the kidney changes found in both acute and chronic pomona infections in hamsters.

## MATERIALS AND METHODS

### Collection of specimens

Immediately after the sheep from experiment 4a and 4b were killed by exsanguination, the right kidneys were removed. Selected portions from both cortex and medulla were placed into cold 3% glutaraldehyde with 2% formaldehyde in 0.1 M phosphate buffer pH 7.2, and cut into 1 mm cubed fragments. A portion of the remaining piece of kidney was fixed in 10% formalin to be embedded later in paraffin. The left kidney was removed for a more detailed examination of its gross morbid anatomy and for photography of the intact specimen.

### Methods for electron microscopy

Specimens were fixed in glutaraldehyde and formalin and embedded in Durcupan by the methods described in Chapter 2. Ultrathin sections were cut on an L.K.B. ultramicrotome, mounted on formvar coated grids and stained with uranyl nitrate (Watson, 1958) for 5 minutes and lead citrate (Reynolds, 1963) for 5 minutes. The sections were examined using a Philips 200 electron microscope.

### Preparation of specimens for light microscopy

After fixation in 10% formalin was complete, the pieces of kidney intended for light microscopic examination were trimmed and embedded in paraffin. Sections were cut at 5  $\mu$ m, routinely stained with haematoxylin and eosin and, to enable the leptospire to be visualized, by Warthin-Starry stain (Armed Forces Institute of Pathology, 1960).

### Cutting and staining of Durcupan embedded sections for light microscopy

Durcupan embedded sections were cut at 1  $\mu$ m on an ultramicrotome for light microscopy. The sections were collected from a dry knife and placed on a drop of water on a microscope slide which was then placed on a heating element and a gentle heat applied until all the water had evaporated. The sections were stained by a haematoxylin phloxine B method (Shires et al., 1969).

## RESULTS

### Gross lesions

In all cases within the group of experimentally infected sheep, lesions were visible on the cortical surface of the kidney. They were in the form of 1 to 3 mm, roughly circular, often ill-defined, white spots, level with the surface of the kidney (Fig. 5.1), except in one case in the second experiment where they were raised above the kidney surface (Fig. 5.2). The lesions in all cases were present in both kidneys in approximately equal size and numbers and distributed evenly throughout the cortex.

### Light microscopy

The lesions typically present in the kidneys of infected lambs and ewes consist of foci of interstitial infiltration by lymphocytes and plasma cells. These foci are seen at all levels within the cortex in the region of the corticomedullary junction and occasionally in the medulla. Neutrophil and eosinophil leucocytes are noteworthy components of the cellular infiltration in individual cases. In some foci the cellular infiltration is only one or a few cells thick between tubules (Fig. 5.3) but more frequently the infiltration is massive, one to three millimetres

in diameter, and only a few intact tubular elements can be identified within the focus (Fig. 5.4). Granules of haemosiderin within convoluted tubular epithelium are a fairly constant finding in the infected kidneys.

Adjacent to or within the inflammatory foci there is usually some evidence of tubular damage and regeneration. Tubules showing regeneration have an epithelium which is more flattened than normal, the cytoplasm tends to be basophilic and nuclei of the epithelial cells are more numerous and crowded one upon the other. Subjectively the epithelium of these tubules also appears to have an abnormal increase in mitotic figures.

In some instances proximal convoluted tubular lumina contain what appear to be individual necrotic tubular epithelial cells, but more commonly the tubules contain amorphous, presumably proteinaceous, material. The distal and collecting tubules and the loops of Henle are mostly normal.

Some glomeruli in both control and affected sheep are hypercellular but this is a more frequent finding in the affected kidneys, and glomeruli adjacent to the inflammatory foci are clearly more affected than elsewhere.

In Durcupan embedded sections there is basophilic material at the periphery of the lumina of some proximal convoluted tubules (Fig. 5.5).

Warthin-Starry stained paraffin sections show many leptospire (Fig. 5.6). All appear to be within tubular lumina.

#### Electron microscopy

The basophilic material around the periphery of the lumen of the proximal tubules, as seen by light microscopy of Durcupan embedded sections, is composed of a concentration of leptospire which are in close contact and intermingling with the brush border (Fig. 5.7). These organisms are in

some cases concentrated at the apical plasma membrane, close to the tight junction, and in a few cases even penetrate into the intercellular space. The other material filling the tubules appears to be of a proteinaceous nature. Many of the infected tubules have a complete or almost complete absence of brush border, whilst in others the brush border is intact in spite of the presence of large numbers of organisms. Outstanding in some of the electron micrographs, taken during this study, are the bulbous ends to the microvilli (Fig. 5.8, 5.9). These may reach a size of  $0.5\ \mu\text{m}$  in diameter while still remaining attached by their basal stalk to the apex of the epithelial cell. Some even larger sized bulbous structures were observed, but without serial sections it is not clear whether these are free in the lumen of the tubules or remain attached to the microvilli (Fig. 5.8). In some sections an estimated 70% of the microvilli show some degree of bulbous swelling to their ends. A few of the microvilli have actually increased in diameter more or less evenly along their whole length. In figures 5.9 and 5.10 a swelling of the whole apical region of individual cells can be seen and this apical region protrudes well into the lumen of the tubule. These protrusions of apices into the lumen are all devoid of microvilli. Some of the epithelial cells show an increased number of cytosomes together with an occasional autotrophic inclusion (Fig. 5.7). There is a reduction in the number of apical tubules and vesicles within the cytoplasm of the tubular epithelium.

The nuclei of the infected tubular epithelial cells are normal in appearance. Mitochondria, lysosomes, pieces of endoplasmic reticulum and remnants of brush border, together with much unrecognisable cellular debris may also be present in the tubular lumen (Fig. 5.12).

Individual leptospores, even when packed within the tubules and in close association with the brush border, are surrounded by a clear zone (Fig. 5.11). The micro-organisms are easily recognisable and can be seen

to be helical. Their diameter is 150 nm and they are surrounded by two envelopes, an outer membrane 7 to 10 nm and a cytoplasmic membrane 6 to 8 nm in thickness. The cytoplasm of the organism is of variable electron density and contains an occasional granule of approximately 15 nm diameter.

In the glomerulus there is a degree of foot process fusion, seen only in the kidneys of the infected sheep (Fig. 5.13). In many glomeruli, from the experimentally infected sheep, there was a quite extensive formation of new microvillus-like structures on the epithelial cells (Fig. 5.13, 5.14, 5.15). The mesangial hyperplasia, suspected as a result of light microscopic examination, is even better appreciated by electron microscopy as an extensive proliferation of mesangial matrix. Oedema of the capillary endothelial cells (Fig. 5.16), together with the proliferation of the mesangium, has caused almost complete obliteration of the capillary lumina in some glomeruli and in places the capillary lumina are only discernable because of the presence of a few remaining red blood cells (Fig. 5.15). The urinary spaces often contain 'balloons' of proteinaceous material identical in appearance to material seen in the lumen of affected convoluted tubules.

The glomeruli range from being completely normal, both by their light microscopic and electron microscopic appearance, to the degree of damage described above. This degree of damage seems to depend on the proximity of the glomerulus to infected proximal convoluted tubules.

#### DISCUSSION

The glomerulus, like any other organ or tissue, has only a limited number of responses to a variety of pathologic stimuli. The responses in this study can best be described in terms of changes to the capillary walls,

the mesangium, the basement membrane and to the glomerular epithelial cells.

Focal foot process fusion with extensive formation of microvillus-like structures from the epithelial cells as in this study (Fig. 5.13, 5.14, 5.15) has also been described in human cases of leptospirosis (Sitprija and Evans, 1970). Both foot process fusion and microvillus formation are common features of glomerular disease and are usually accompanied by proteinuria; the extent of the change being roughly proportional to the degree of proteinuria. The body of the podocyte cells may respond to disease stimuli by becoming enlarged with either a diffuse or focal swelling. The change in the infected sheep is of a diffuse nature and the rough surfaced endoplasmic reticulum within the podocytes is more prominent (Fig. 5.14), a change which may be related to an increased turnover of basement membrane (Simon and Chatelarat, 1969). Areas of basement membrane enlargement are seen occasionally in the glomeruli of the infected sheep, but this is not a consistent finding and occurs only in isolated areas within particular glomeruli. It would be unwise to consider this an important primary response to a leptospiral infection. In most cases the basement membrane is entirely normal in appearance.

Within the kidneys of the sheep experimentally infected with leptospire, swelling of the glomerular capillary endothelium is clearly seen (Fig. 5.16). The swollen cytoplasm of these cells, together with mesangial matrix proliferation, have severely reduced the size of the capillary lumen and in some cases it has been completely obliterated. This change is not peculiar to leptospirosis and represents a response of the endothelial cells to injury.

Changes in the endothelial cells and mesangial matrix in infected kidneys can be seen readily with the electron microscope. These changes

are most pronounced in glomeruli in close proximity to the infected tubules and it is possible that they are connected directly to these infected tubules. The full extent of the glomerular changes are not appreciated when sections are studied by the light microscope and they have apparently gone undetected in many pathological studies of leptospirosis.

The large 'balloons' of proteinaceous material seen within urinary space appear to be identical to those found in the proximal convoluted tubules (Fig. 5.15). These 'balloons' may possibly have reached the glomerulus by reflux from the proximal convoluted tubules. That this type of reflux can occur is supported by the observation by Waugh and Baschel (1961), who found detached tubular brush border in the urinary space. Artifactual protrusion of epithelial material from the proximal convoluted tubule is also reportedly common in cats and dogs (Bloom, 1954). Examination of the material occurring in the urinary space in this study did not suggest that it was of epithelial origin.

The mesangial cells in the glomeruli of infected sheep in this study have undergone proliferation together with quite extensive secretion of additional matrix material. An increase in the number of organelles and free ribosomes occurs during the proliferation of mesangial cells and this proliferation is accompanied by a secretion of mesangial matrix.

Thus it can be said that structural changes are present in podocytes, mesangium and capillary endothelium but not in basement membrane. However, it is necessary when studying the pathology of the glomerulus to consider the changes as a whole instead of focusing too much attention on modifications of individual elements. It is likely that the glomerular changes seen in this study are not specific to leptospirosis and probably are mainly a reflection of events elsewhere in the nephron and an altered glomerular filtration function.

Compared with knowledge of changes in the glomerulus, a lot less is known of the ultrastructural changes which occur in the tubules in response to injury. It is of paramount importance, when looking for pathological changes in the tubules, to take into account the method of fixation employed. Differences in fixation can lead to differences in appearance of the electron-micrographs, a point well made in many ultrastructural studies of the mammalian kidney and well documented by Sinclair (1962) and Osvaldo et al. (1965). The speed and efficiency of fixation is important because, if fixation has not been effective or has been too slow, swelling and rupture of the mitochondria may occur and this change could be mistaken for a reaction due to the insulting agent used in the experiment (Rouiller, 1969).

Perfusion techniques were not employed in these experiments but it was felt that, provided the artifacts likely to occur were kept in mind, and comparisons made with control material, immersion fixation was quite acceptable. Most of the ultrastructural studies of leptospiral kidney infections have been with human biopsy material (Sitprija and Evans, 1970; Brito et al., 1965) using the immersion technique, consequently a more direct comparison can be made between their findings and those in this study.

The tubular changes seen in the experimental sheep are to some extent those related to tubular epithelial cell degeneration and death, but of particular interest are the alterations in structure of the brush border of proximal convoluted tubules.

The "loss" of the microvillousities of the brush border may be due either to a general swelling of the apex of the cell or to a local detachment and or destruction of the microvilli with a rejoining of the plasma membrane at the base of the villi. A third possible explanation is that the absence or sparcity of microvilli may indicate that regeneration of the cells is

occurring and that the replacement cells are less well differentiated (Chatelanaat and Simon, 1969).

In the proximal convoluted tubules of sheep infected with pomona the microvilli can be seen in various forms. It can be postulated that these changes in the shape of the microvilli form part of a sequence of events leading up to the detachment of these microvillosities.

Those electron micrographs showing a reduction in the height and spacing of the microvilli at the apex of the proximal convoluted tubular epithelium are difficult to interpret but might be explained by any one of the aforementioned postulates. On the other hand, it is not well documented just what changes occur in respect to the distribution and appearance of microvilli as one approaches the thick loop of Henle. It is possible that an intermediate zone exists, consisting of epithelial cells with a mixture of normally appearing proximal convoluted tubular cells and cells approaching in appearance those found in the thick loop of Henle. However an observation that suggests that this is truly a pathological response by the cells to the presence of the leptospire, is that cells of this appearance were never seen in control material and were only seen in tubules in which the organism was present.

Necrotic cell debris and large 'balloons' of proteinaceous material, identical in appearance to that found in the urinary space, occurred on the lumina of many tubules. Although some 'balloons' were seen in the tubular lumina of normal kidneys, these latter structures are probably the result of poor fixation. The greater size and frequency of occurrence of the proteinaceous 'balloons' in pathological material make it a significant finding.

At no time in this study were leptospire seen to have actively invaded an otherwise normal cell. They were identified in the cytoplasm

and nucleus of necrotic cells lying free in the tubule (Fig. 5.17) but it is assumed that they entered the cell after its death and detachment. In support of this assumption, obviously necrotic cells can be seen (Fig. 5.7) in position in the tubular epithelium and these cells show no evidence of contained leptospire in spite of the presence of large numbers of organisms in the adjacent lumen.

From the evidence considered above it now becomes difficult to invoke an active mechanical disruptive action by leptospire on cells as an explanation of tubular damage. On balance it appears much more likely that such damage is due to the elaboration of a substance or substances which have a toxic effect on the epithelial cells.

#### SUMMARY AND CONCLUSIONS

1. It is considered likely that structural changes seen in some glomeruli in leptospirosis of sheep are indirect consequences of changes in filtration function and changes occurring in more distal parts of the nephron.
2. The changes believed to be specifically related to the presence of leptospire are those occurring in the tubules.
3. Proteinaceous 'balloons' fill many of the tubules including those containing leptospire. These 'balloons' were also seen in the urinary space of some glomeruli, but the possibility that their presence there was due to reflux could not be discounted.

4. The microvilli making up the brush border in the tubules colonised by leptospire were both sparse and in some tubules had bulbous ends. Both the changes in shape of the microvilli and the protrusions of the proximal convoluted tubular epithelium probably form part of a sequence of events leading to the detachment of the microvilli.

5. Necrotic epithelial cells were seen both before detachment and free in the lumen of the tubules. The presence of leptospire in these necrotic cells only after they were released into the lumen suggests that their necrosis was brought about by the toxic effects of the organism rather than mechanical disruption.

Fig. 5.1 The kidney of ewe 599 removed at necropsy 31 days after inoculation with Leptospira interrogans serotype pomona. One 3 mm ill-defined white spot is visible, level with the surface of the kidney (arrow).

Fig. 5.2 The kidney of lamb 390 necropsied 34 days after inoculation with Leptospira interrogans serotype pomona. Numerous raised lesions can be seen on the surface of the kidney.

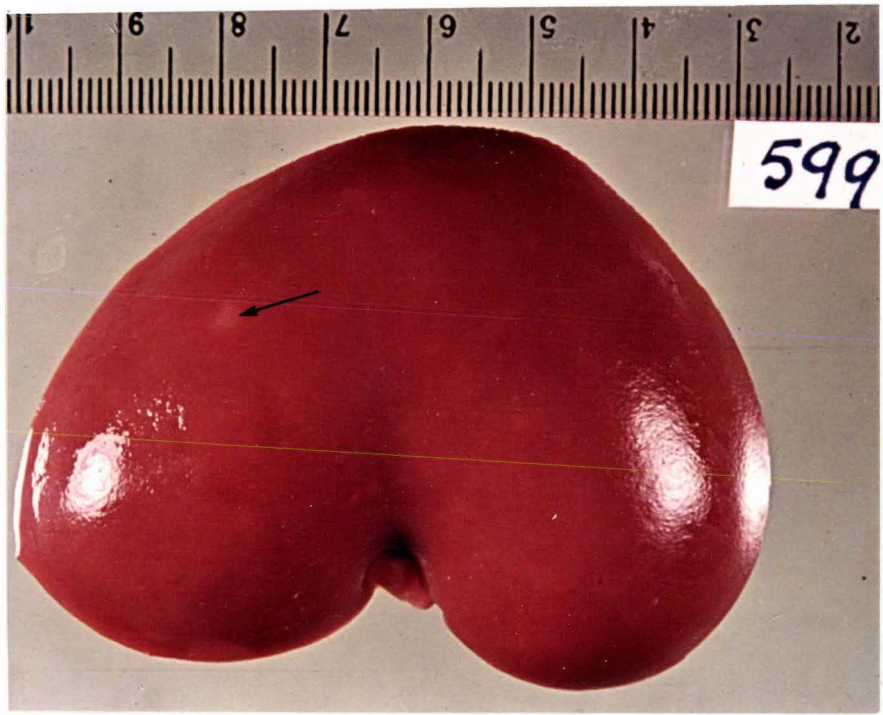


Fig. 5.3 The corticomedullary region of a sheep kidney showing a mild degree of inflammatory cellular infiltration between tubules. HE x 250.

Fig. 5.4 Section of pomona infected sheep kidney in which the infiltration of cells has taken on a follicular form with only a few intact tubular elements identifiable within the focus. HE x 25.

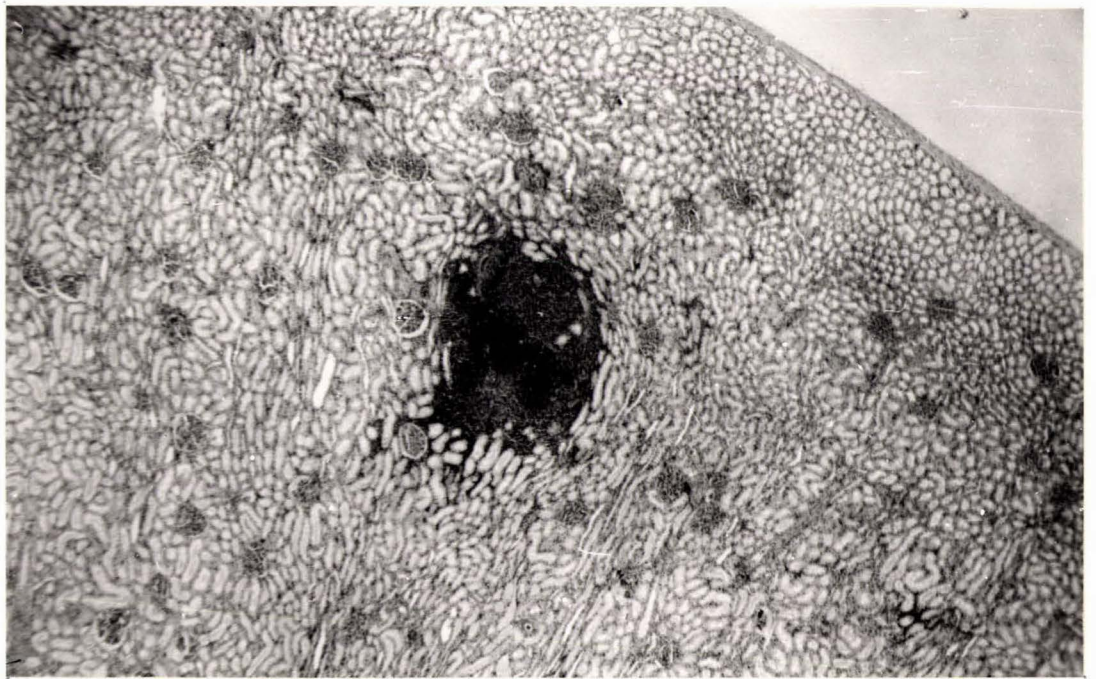
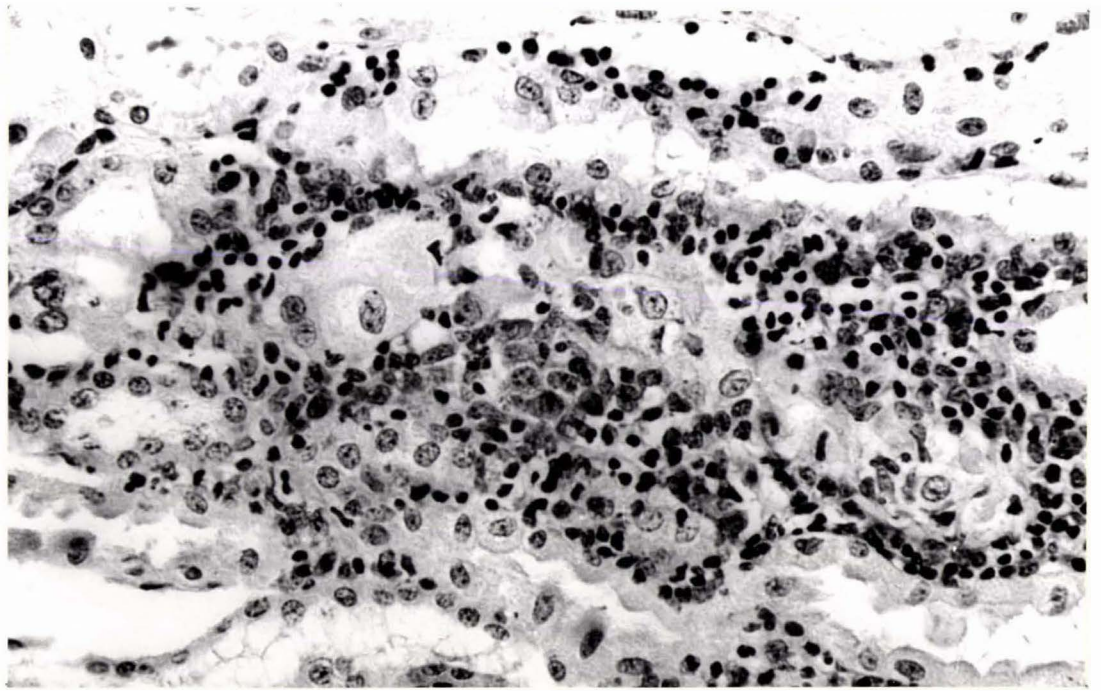


Fig. 5.5 A Durcupan embedded section of an infected sheep kidney. The darkly stained material (arrow) around the periphery of the lumina of some proximal convoluted tubules was subsequently identified as being a mass of leptospire. Haematoxylin Phloxine B x 600.

Fig. 5.6 Infected sheep kidney with many leptospire in a tubular lumen. Warthin-Starry x 600.

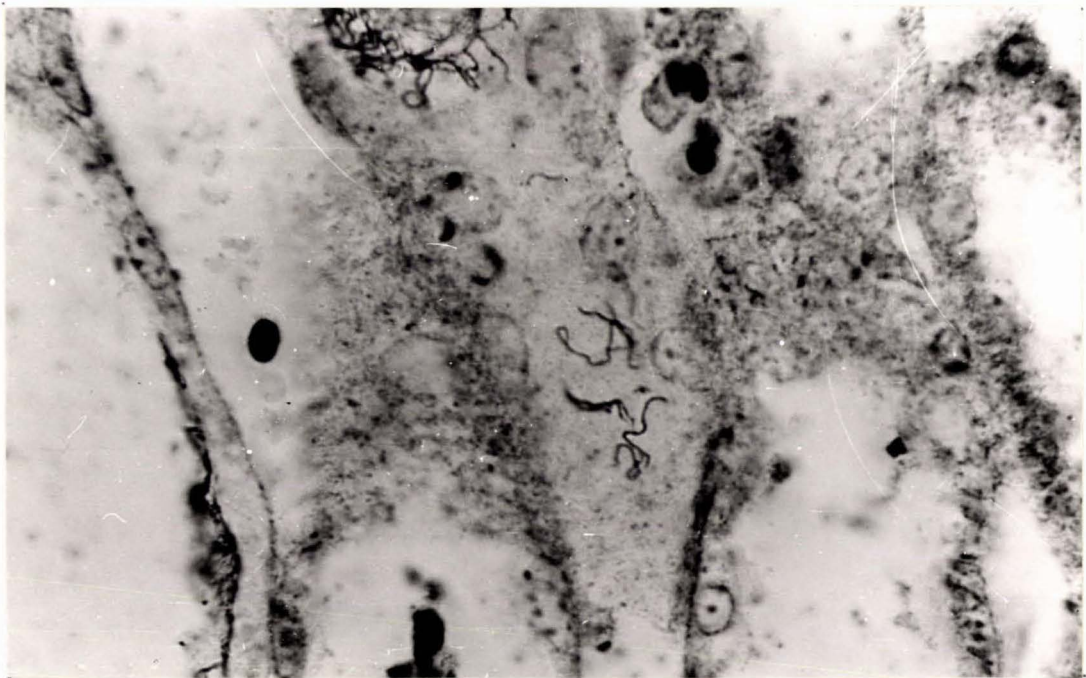
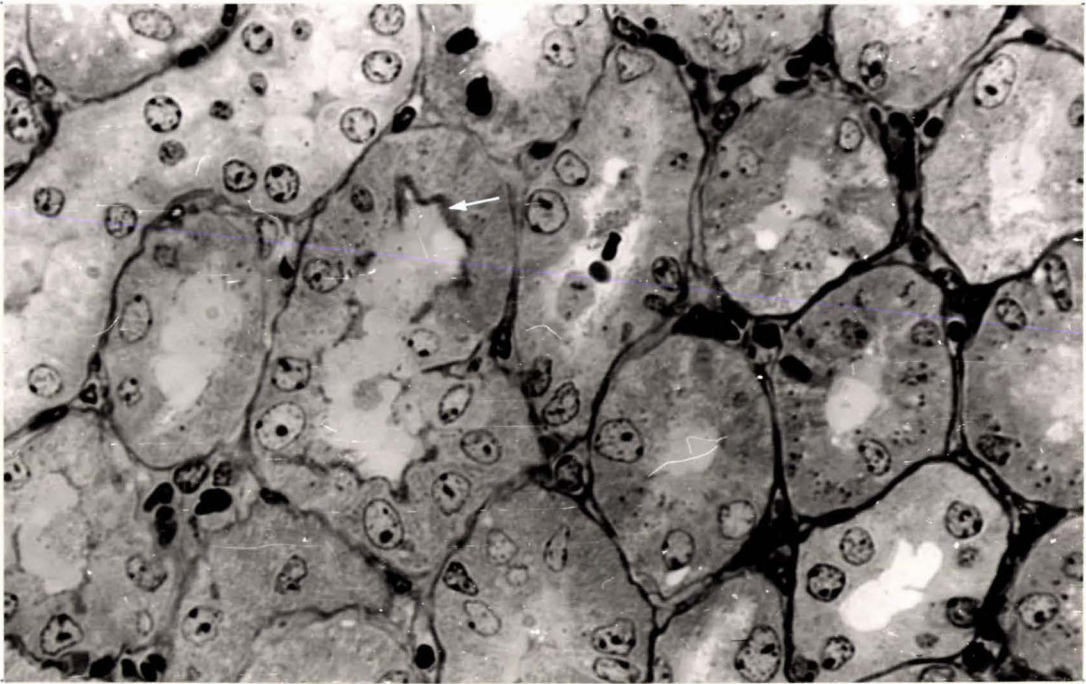


Fig. 5.7 A montage showing the concentration of leptospire around the periphery of the lumen of a proximal convoluted tubule. The organisms are intermingled with the brush borders. The centre of the lumen is filled with a proteinaceous material. One epithelial cell shows distinct apical necrosis (A) and another contains a dense autotrophic inclusion (I) reminiscent of the dense inclusions seen in infected tissue culture cells (cf Fig. 2.6).  
EM x 6,500.

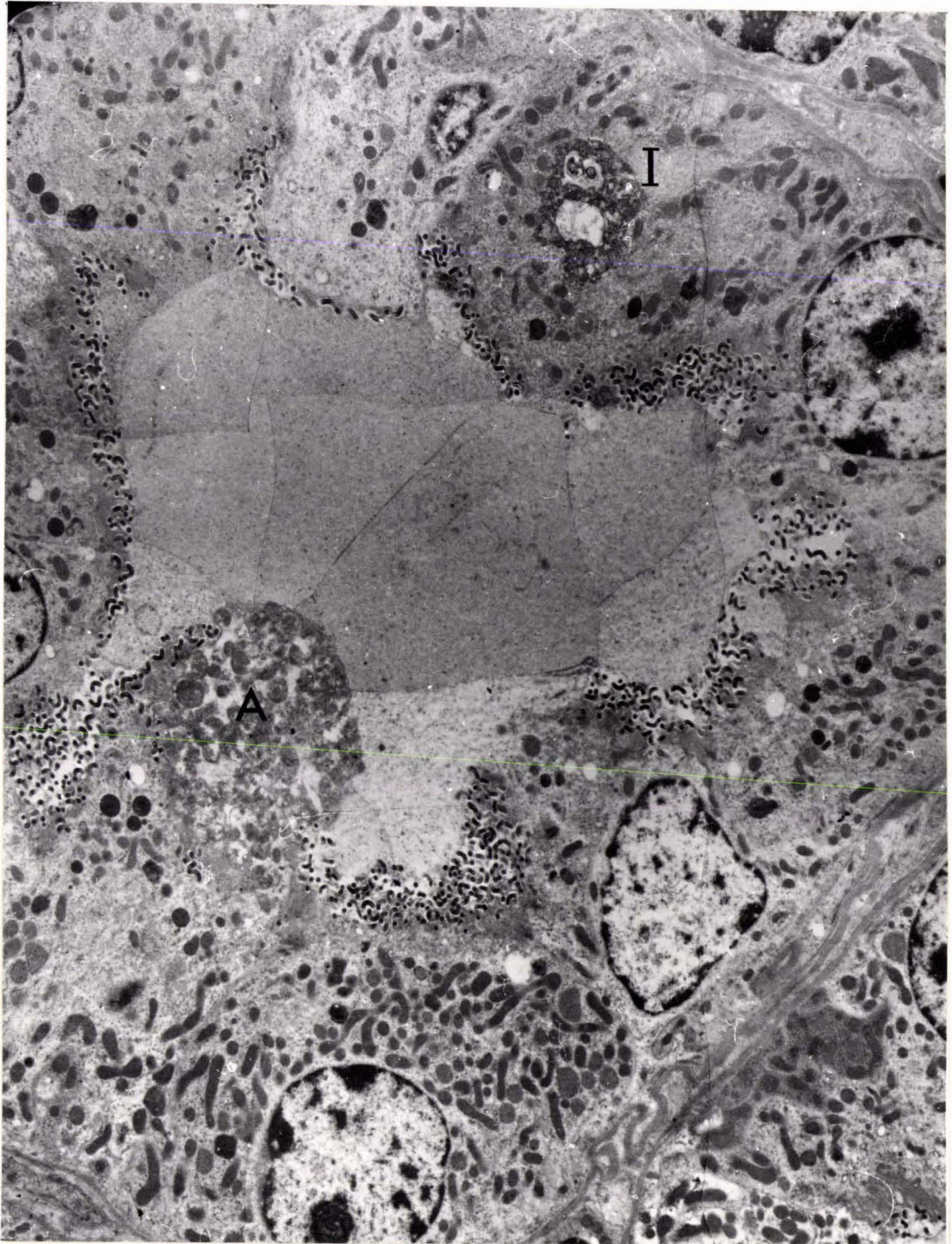


Fig. 5.8 Numerous leptospire in the tubular lumen (TL) and in association with a brush border which shows microvilli which have terminal bulbous swellings (Mv). EM x 12,600.

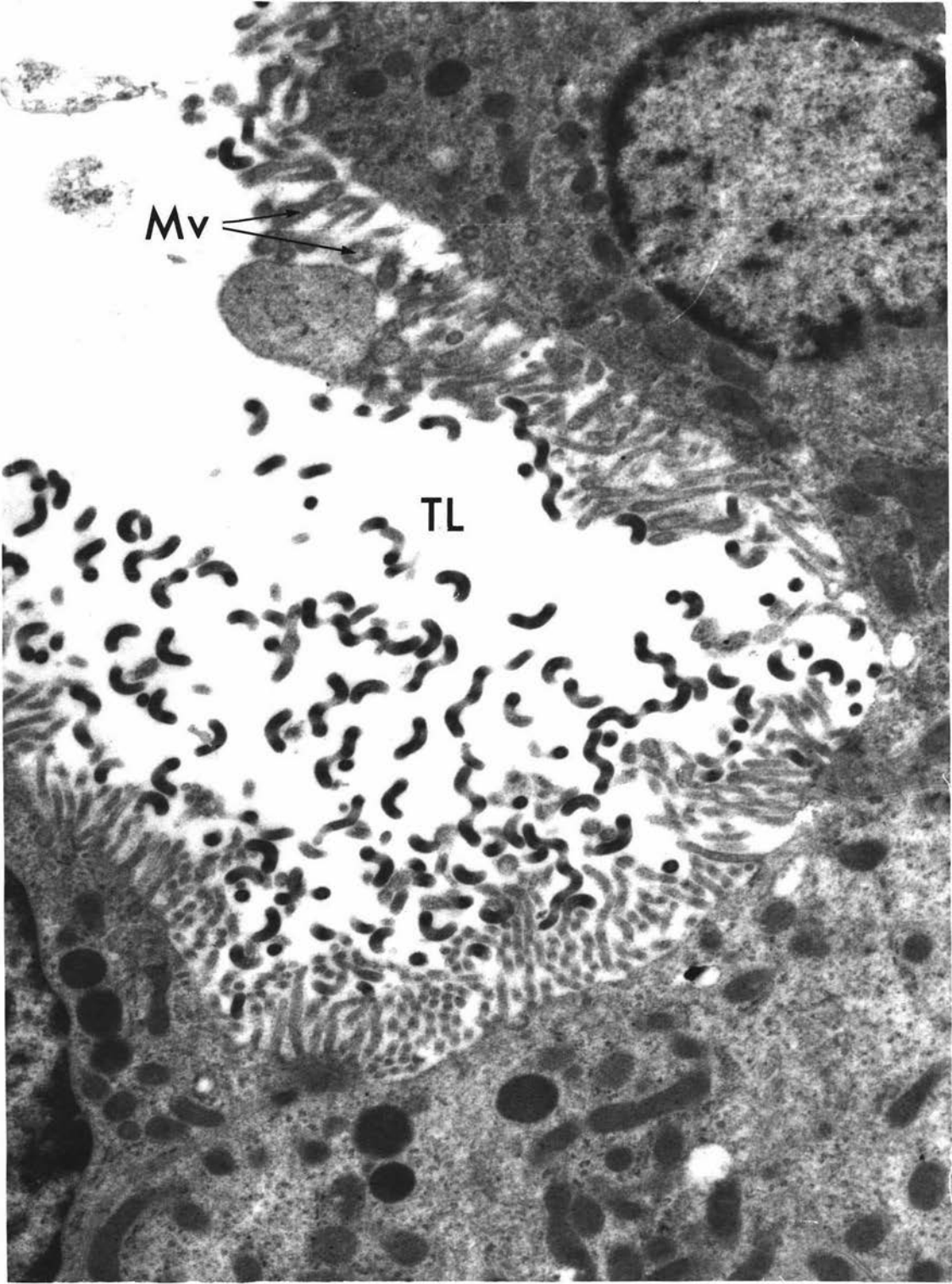


Fig. 5.9 Sheep kidney tubular lumen (TL) with microvilli showing bulbous swellings (Mv). The apical region of one cell (Ap) is seen to be protruding into the lumen and there is an absence of microvilli on the surface of the protruded portion of the cell. Within the tubular lumen is an accumulation of cell debris and free organelles (A). EM x 12,600.

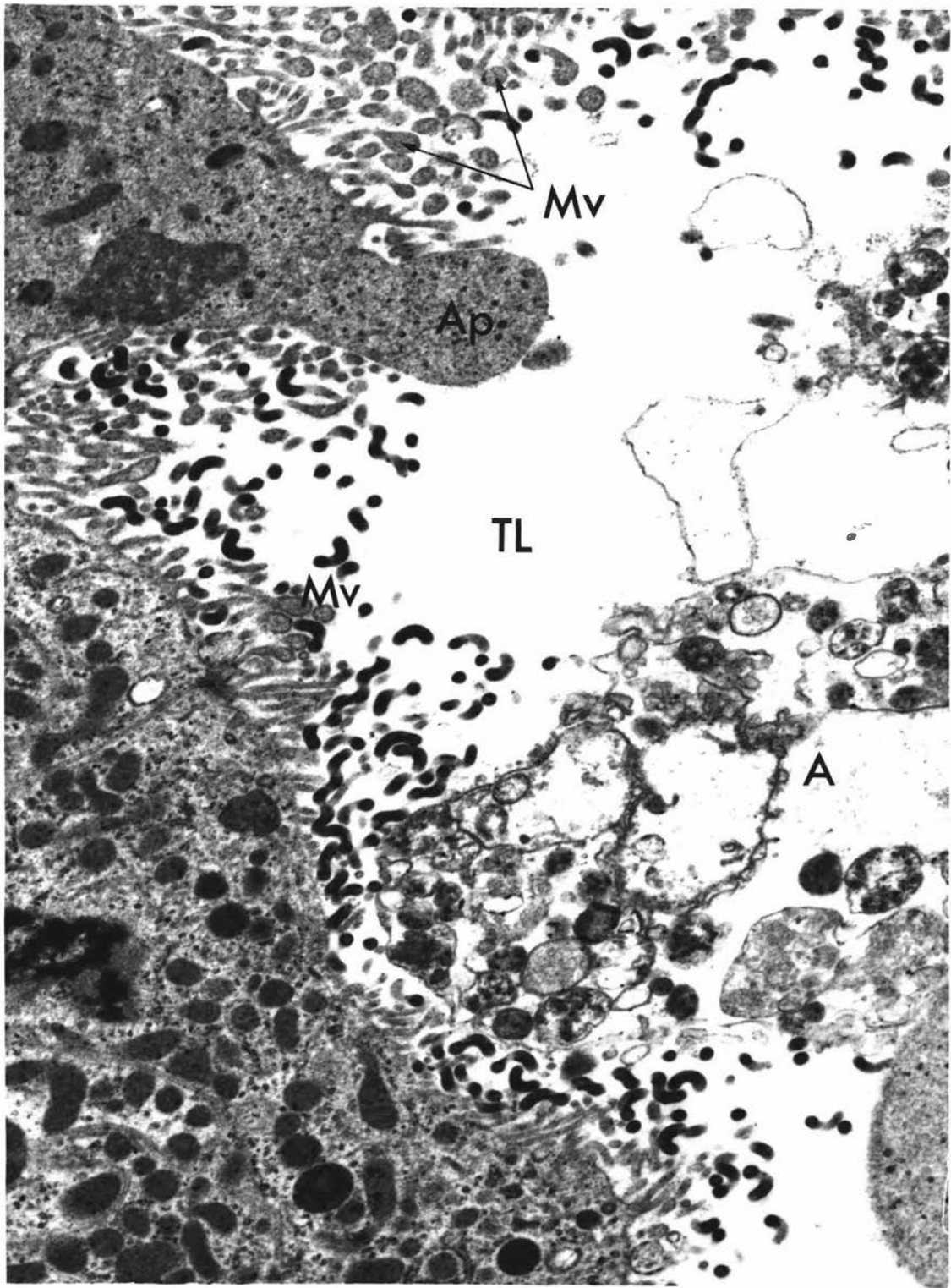


Fig. 5.10 Montage of a colonised convoluted tubule showing protrusion of a cell apex which contains a dense inclusion (I). The tubular lumen contains cellular debris (D) and a proteinaceous "balloon" (B). EM x 8,000.

Fig. 5.11 Individual leptospire closely associated with the brush border (BB). In some cases the axostyle (Ax) of the organism is discernable. EM x 65,000.

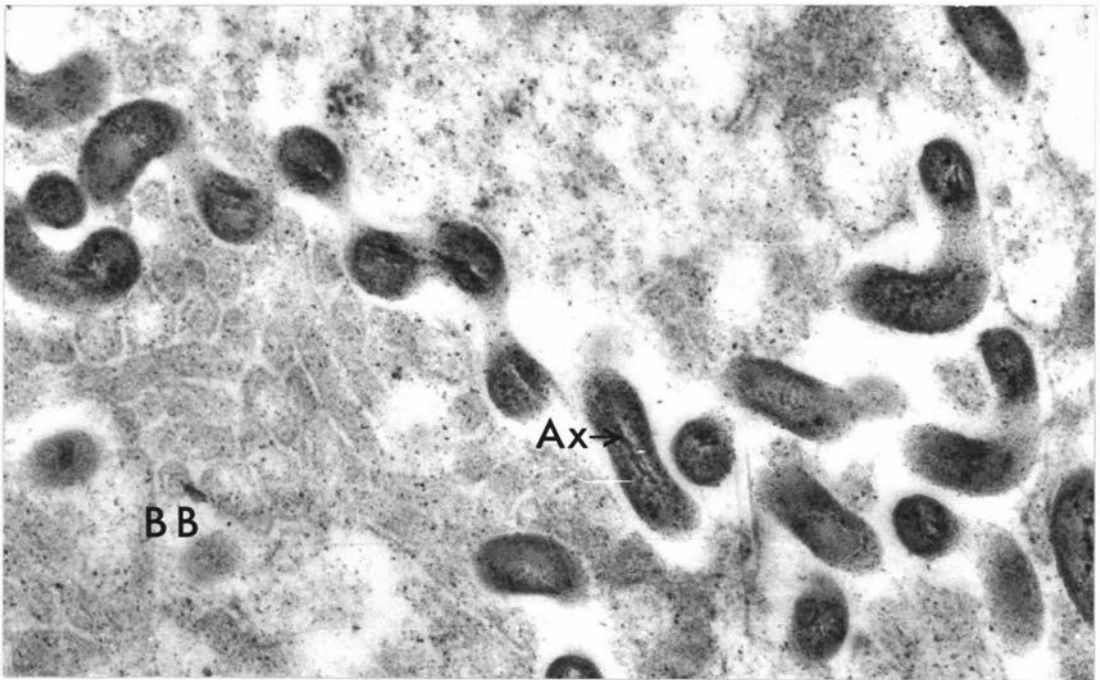
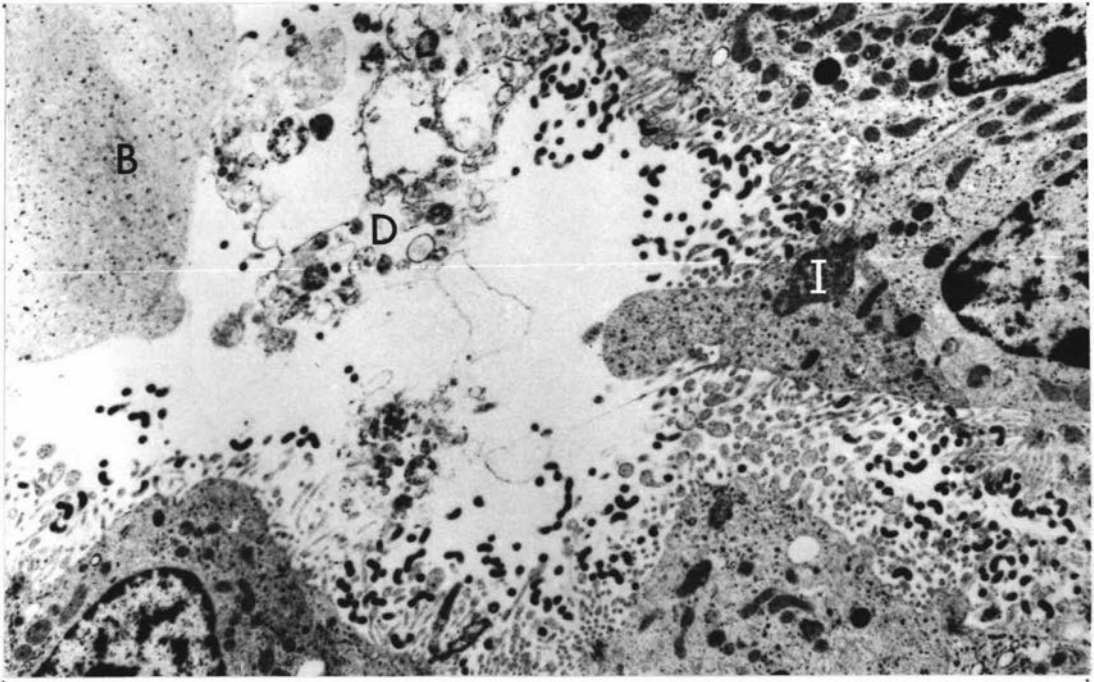


Fig. 5.12 The tubular lumen (TL) of an infected sheep kidney. The lumen contain mitochondria (M), lysosomes (Ly) and pieces of brush border (BB) together with other unrecognisable cellular debris. EM x 21,000.

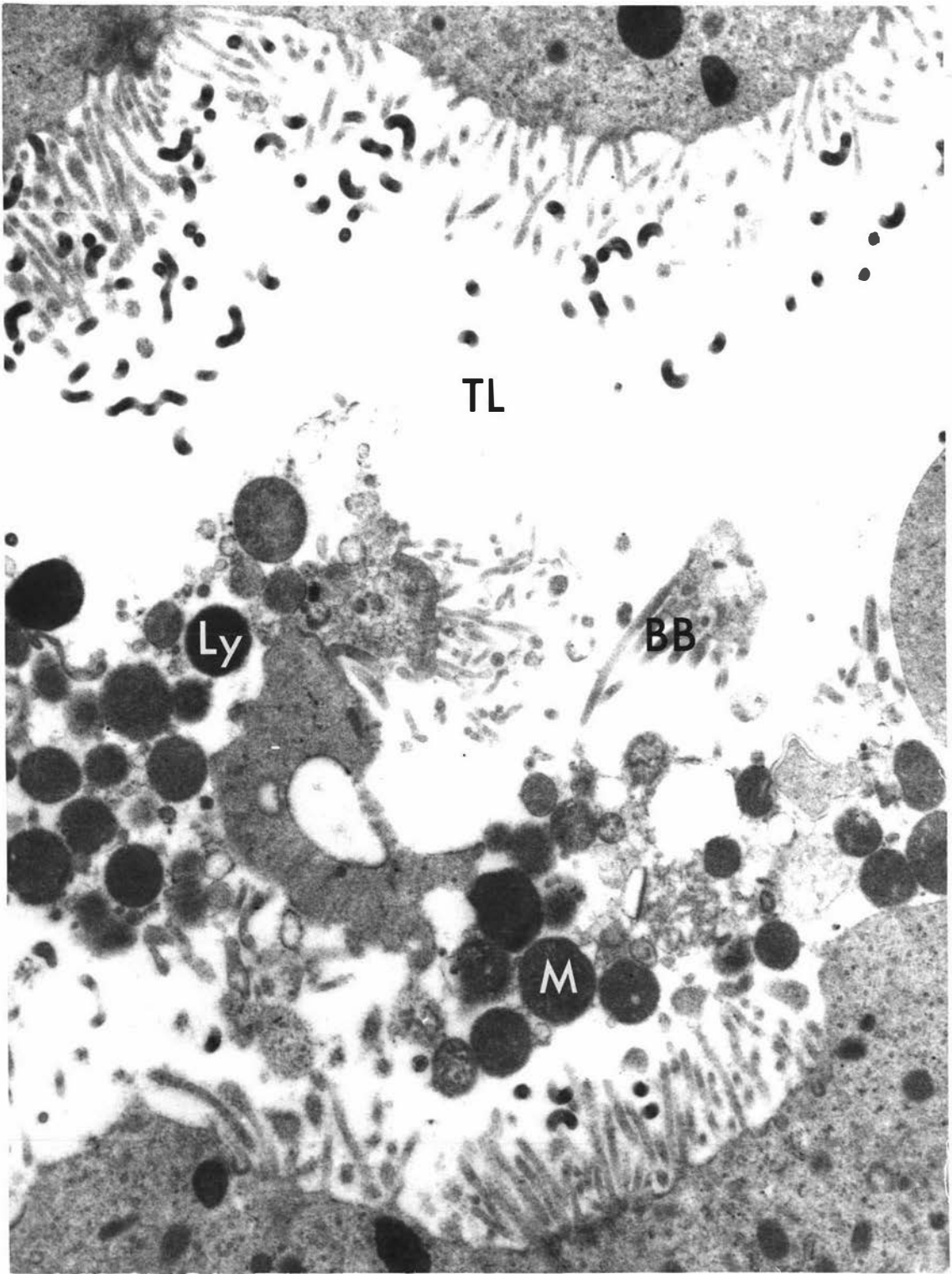


Fig. 5.13 Glomerulus of infected sheep illustrating focal foot process fusion (F) and extensive formation of microvillus-like structures (X) from the epithelial cells (Ep). EM x 12,600.

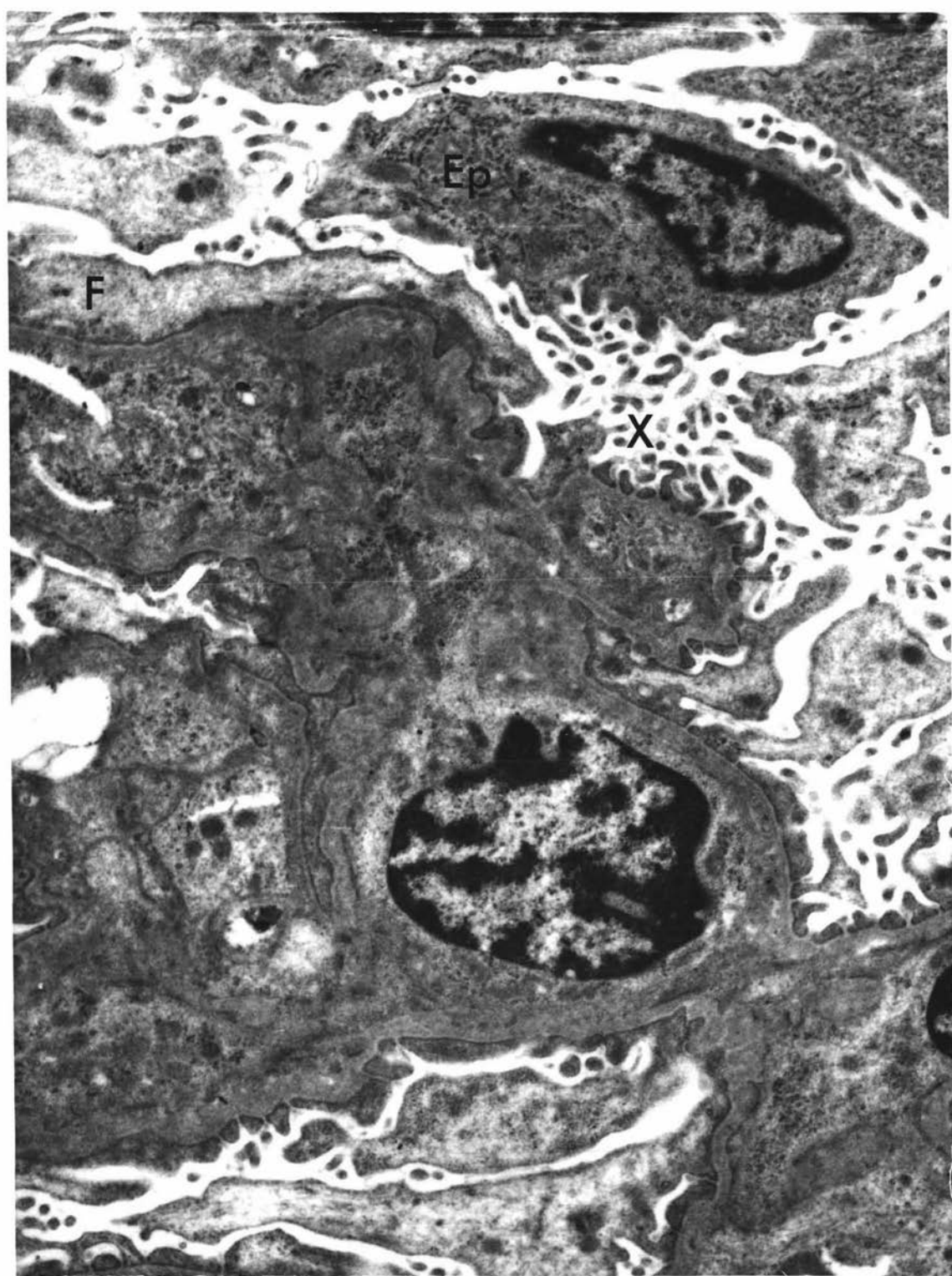


Fig. 5.14 Glomerular epithelial cells (Ep) in an infected sheep kidney showing microvillus-like structures (X), foot process fusion (Ff) and prominent endoplasmic reticulum (ER) within the podocyte. In some areas foot processes (FP) are normal. EM x 12,600.

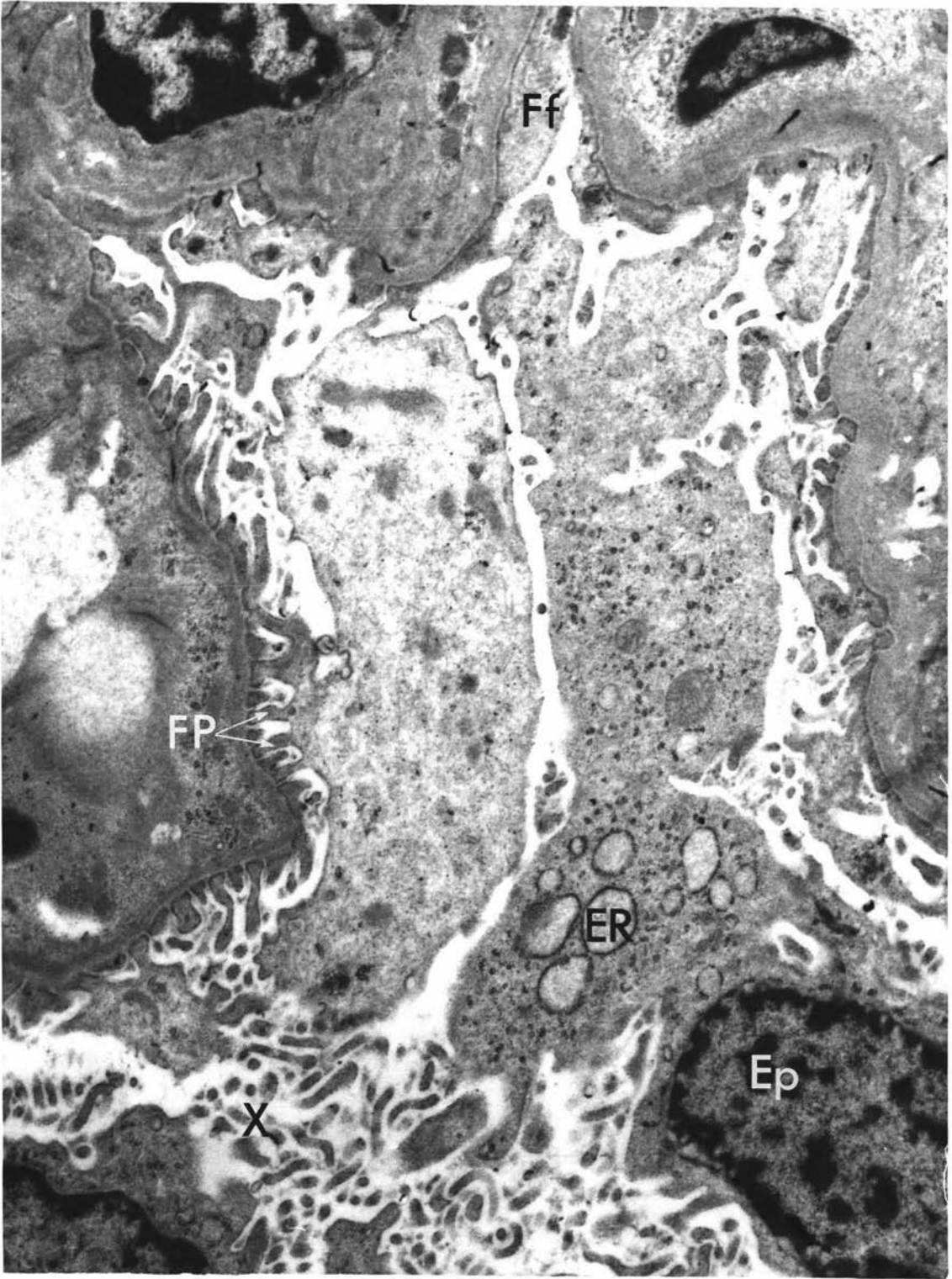


Fig. 5.15 The glomerulus of an infected sheep in which there is an almost complete obliteration of the capillary lumen, which is only discernable because of the presence of a red blood cell (RBC). Prominent and almost filling the urinary space are several large "balloons" (B) of proteinaceous material. EM x 11,000.

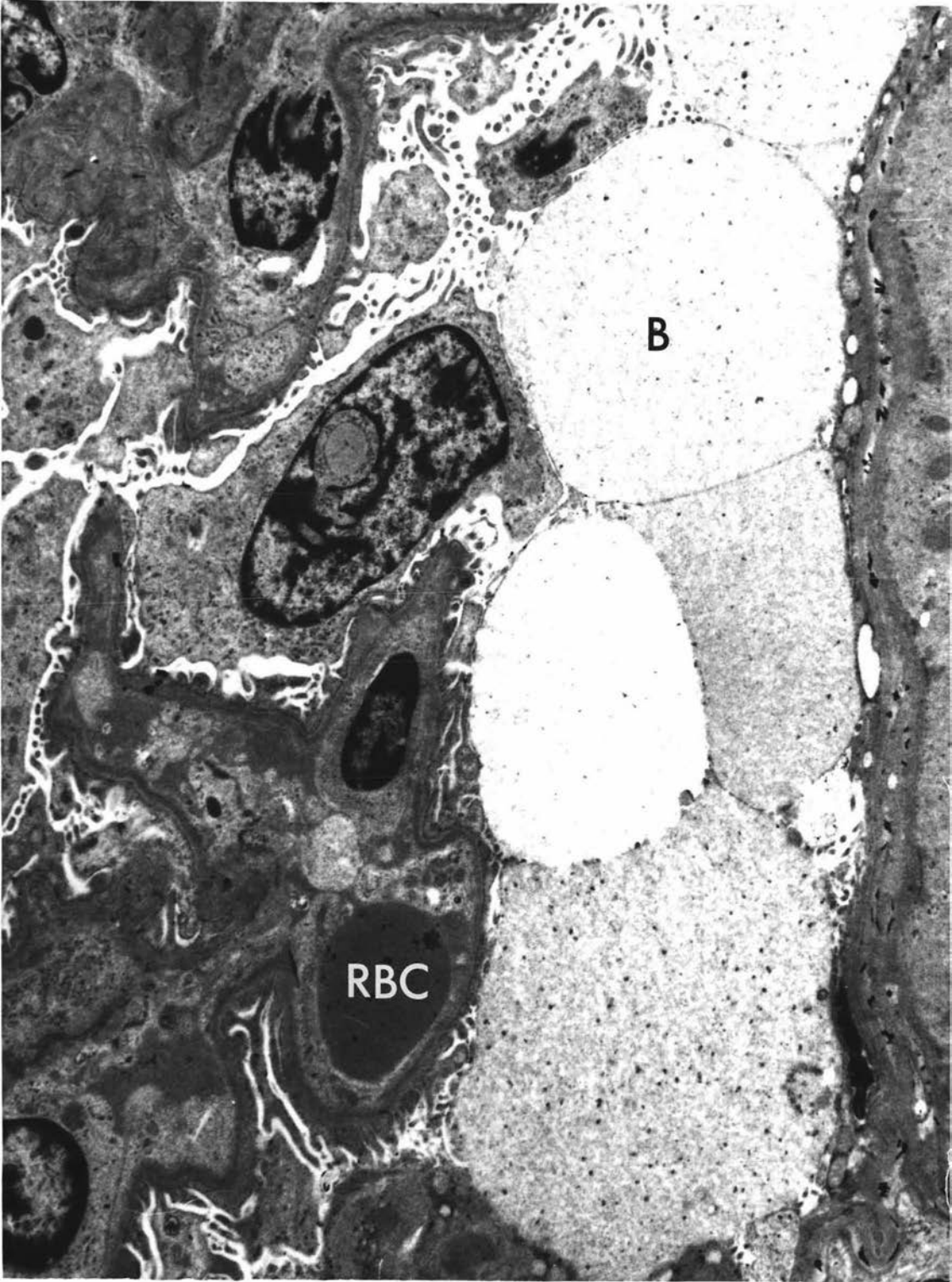


Fig. 5.16 Oedema (Oe) of the glomerular capillary endothelial cells in an infected sheep kidney.  
EM x 12,600.

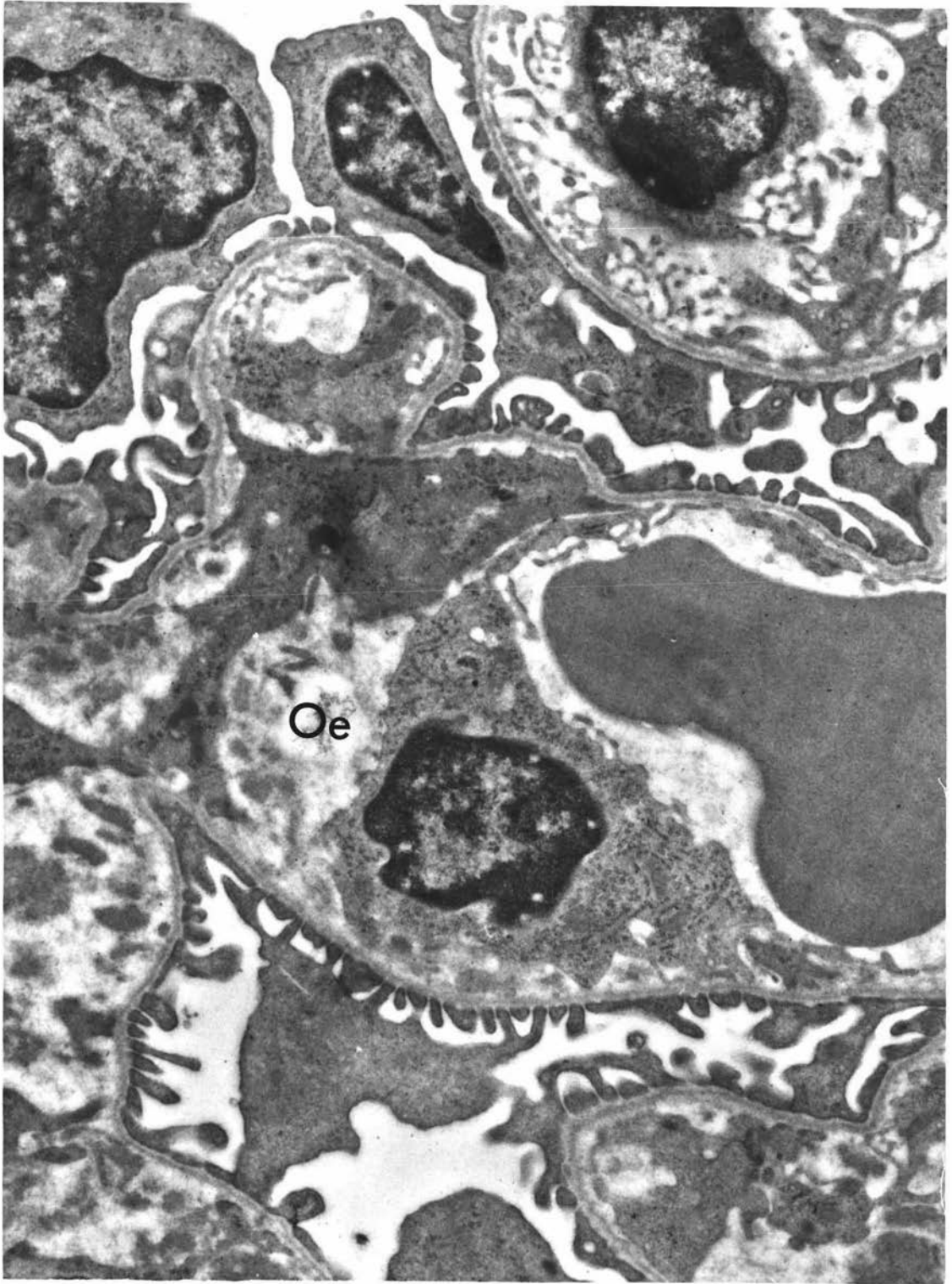
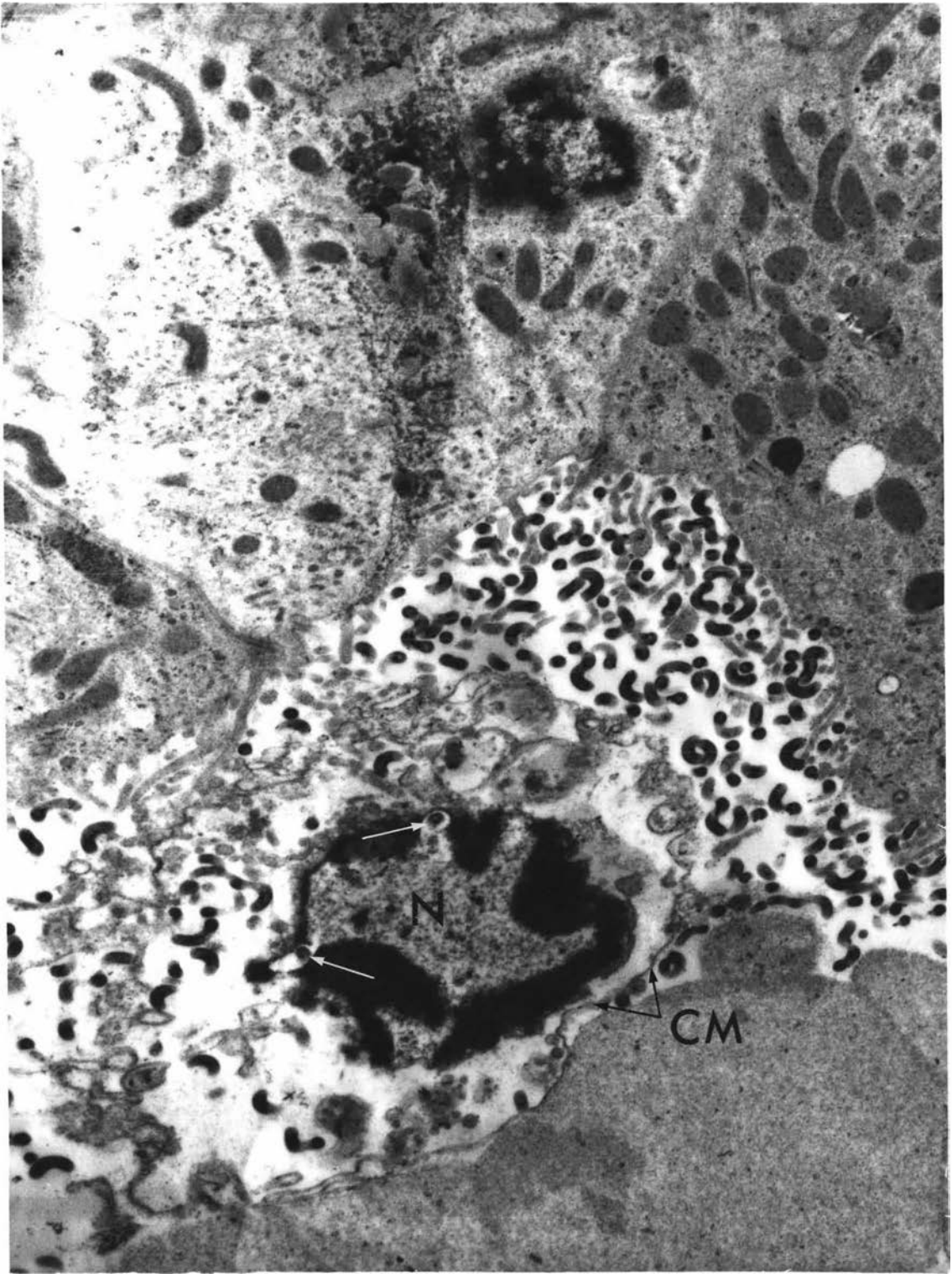


Fig. 5.17 A convoluted tubule of an infected sheep kidney containing a necrotic cell and many leptospires. Organisms (arrows) can be seen within the nucleus (N) and within the cell membrane (CM) of the necrotic cell. EM x 21,000.



CHAPTER 6

## SOME IMMUNOLOGICAL FACTORS IN THE PATHOGENESIS OF LEPTOSPIROSIS

## INTRODUCTION

It has been suggested by several authors that immune mechanisms may play a role in the pathogenesis of leptospirosis (Morter et al., 1960; Sleight et al., 1960; Ben-Efraim and Torten, 1969; Michna and Campbell, 1969; and Jubb and Kennedy, 1970). Michna and Campbell (1969), consider that the cytology of the inflammatory reaction in infected pig kidneys, with its lymphoreticular, and at times even follicular, structure, may be due to an immunological response.

In addition to the role of antibody in the pathogenesis of leptospirosis, it has been claimed that cell mediated reactions may be involved (Morter et al., 1960; Sleight et al., 1960; Ben-Efraim and Torten, 1969; Jubb and Kennedy, 1970). The presence of intense congestion around lesions in the kidney is given as evidence of a hypersensitivity reaction by Morter et al., (1960), and Sleight et al., (1960), whilst Jubb and Kennedy (1970) give delayed type hypersensitivity as an explanation for post-leptospiral ophthalmia. Using dogs and guinea pigs infected with Leptospira interrogans serotype canicola and grippotyphosa, Ben-Efraim and Torten (1969) obtained an ocular reaction with soluble extracts of the homologous strain of leptospire. The ocular reaction occurred 24 to 32 hours after injection and was, the authors claim, strikingly similar to post leptospiral ophthalmia.

The detection of cell mediated immunity by lymphocyte transformation has been demonstrated by Lehner (1967) and his technique was the one used in this study.

The influence of both antibodies and cell mediated immunity was tested by the following experiments:

1. Antibodies: (a) Correlation between the progress of the disease and the occurrence of agglutinins  
(b) Passive cutaneous anaphylaxis (PCA) reaction for reaginic antibody
2. Cell mediated immunity: Lymphocyte transformation.

## MATERIALS AND METHODS

### Microscopic agglutination test (MAT)

The microscopic agglutination test was performed on all sera from the experimental sheep detailed in Chapter 4 using as antigen 7 - 10 day old cultures of the appropriate leptospiral serotype. Before the commencement of the experiment all sera were examined for agglutinins to serotype pomona, grippotyphosa, bataviae, biflexa, pyrogenes, ballum, hardjo, tarrasovi, australis, icterohaemorrhagiae, autumnalis, canicola, and sejroe. All gave negative reactions at dilutions of 1 : 100 and higher. Subsequent testing of sera used the pomona serotype only. A detailed account of the MAT is given in Appendix IV.

### Homologous passive cutaneous anaphalaxis (PCA) test

Serum samples which had been collected from the experimental sheep and which showed high MAT titres were used in this PCA test. The serum samples used were from No. 386 (ewe) collected on post inoculation (PI) day 27, No. 389 (lamb) on PI day 27, and as a control, one serum sample collected on the same day from an uninfected sheep No. 392 (ewe).

The antigen for the homologous PCA and lymphocyte transformation experiments was prepared from a heavy 7 day old culture of pomona grown in Stuart's medium, centrifuged at 10,000 g and washed in sterile physiological saline. After recentrifugation and suspension in sterile saline, the mixture was sonicated until the organisms had been disrupted as judged by direct darkfield microscopy. During the sonication procedure the suspension of leptospire was kept cool by placing the container in an ice bath. Every effort was made to keep the antigen sterile. The mixture was covered at all times, even during sonication, and the sonicator probe was sterilised in absolute alcohol and flaming prior to use. The prepared antigen was stored at  $-15^{\circ}\text{C}$ .

A healthy Romney ram was shorn on the side of the chest so as to expose an area of skin about 30 x 30 cm. Twenty four hours after removal of the wool, multiple injections of the serum samples (0.5 ml at each site) were given intradermally. Seventy two hours after the skin injections were given, 5 ml of leptospira antigen together with 5.0 ml of 1% Evans blue dye in phosphate buffered saline were injected intravenously. The animal was examined over the next 30 minutes for evidence of an intradermal reaction and then at  $\frac{1}{2}$  hourly intervals for the next 3 hours.

#### Lymphocyte transformation

Lymphocyte transformation experiments were carried on PI days 17, 27 and 31. The same experimental and control animals were used throughout. To represent the experimental group, animals with the highest recorded MAT titres at PI day 17 were chosen. The technique employed was a modification of that used by Lehner (1967).

For the preparation of lymphocyte cultures whole blood was collected from the jugular vein of test animals into heparinised vacuum collecting tubes. From this collected blood 1 ml was withdrawn and placed

in a sterile universal bottle containing TC 199 with penicillin 100 u/ml and streptomycin 100 mg/ml. To this was added either 0.2 ml of antigen, phytohaemagglutinin (PHA) or normal saline and it was left to absorb for 1 hour at room temperature. To each culture was then added 1 ml of homologous plasma obtained by spinning the remaining heparinised blood at 3,000 r.p.m. for 10 minutes. The tops of the universal containers were screwed on tightly and the cultures incubated at 37°C. After 48 hours 0.1 µc of <sup>14</sup>C thymidine (at 35 mc/m M Amersham U.K.) was injected into each culture and these were then left for a further 24 hours.

In harvesting the lymphocytes the cultures were transferred to centrifuge tubes and spun at 1,000 r.p.m. to remove the supernatant. The centrifuged cells were resuspended in 10 ml of a working solution of hypotonic potassium chloride (see Appendix IV) and incubated at 37°C for 5 minutes. A further centrifugation (120 g) was performed to remove the supernatant and the cells washed 3 times in saline, twice in 5% trichloroacetic acid and 3 times in methanol, all at 4°C. The centrifuged deposits were dissolved separately in 0.5 ml of formamide and then transferred to counting vials to which 8 ml of Bray's scintillating medium was added (Bray, 1960).

Radioactivity was assayed in a Packard Tri-carb liquid scintillation counter\*. The counts were corrected for colour and chemical quenching by the channel ratio method and expressed as counts per 10 minutes at 100% efficiency. The amount of background radiation was determined for every series of tests by setting up a control counting vial containing Bray's solution and formamide only. The system was tested for nonspecific adherence of <sup>14</sup>C thymidine to cultures of cells from both infected and control ewes and lambs which had neither PHA nor antigen added to them.

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\* Packard Instruments Inc., U.S.A.

## RESULTS

### MAT titres

Serum collected on PI day 8 was the first sample in which antibodies were demonstrated in the experimental animals by microagglutination test. This was in animal 387 which showed a titre of 1/100. The second animal to develop a titre was sheep 388 on PI day 10, and by PI day 13 seven out of the eight experimental animals had titres of 1/1,000 or greater. The only animal not to have developed a demonstrable titre at this time was sheep 384 and the first recorded titre for this ewe was on PI day 17. This particular animal never had demonstrable antibodies at a serum dilution greater than 1/1,000 whereas all the others within the experimental group had titres to 1/10,000. Details of the MAT titres are given in Table 6.1 and similar results from a pilot study are in Appendix IV.

### Homologous PCA test

No significant reactions were noted at the injection sites in this experiment and the homologous PCA test was therefore negative.

### Lymphocyte transformation experiments

Counts were greatly elevated in PHA stimulated cultures but there were no noteworthy differences in  $^{14}\text{C}$  thymidine uptake between the cultures exposed to leptospiral antigen and their appropriate controls (Table 6.2). Leptospiral antigen did not therefore stimulate the transformation of lymphocytes in culture.



TABLE 6.2

## TEN MINUTE SCINTILLATION COUNTS OF LYMPHOCYTE CULTURES

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	PI day 17	PI day 27	PI day 31
Control ewe and antigen	204	227	211
Control ewe and antigen	194	255	237
Control lamb and antigen	216	225	272
Control lamb and antigen	254	224	233
Infected ewe and antigen	707	562	291
Infected ewe and antigen	253	237	228
Infected lamb and antigen	194	241	256
Infected lamb and antigen	187	199	212
Control ewe and PHA	30327	6014	32539
Control lamb and PHA	11434	10052	16125
Infected ewe and PHA	35487	28034	31485
Infected lamb and PHA	41847	6647	17148
Control ewe	259	366	362
Control lamb	327	263	291
Infected ewe	295	270	444
Infected lamb	207	214	255
Bray's sol plus formamide only	201	231	250

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## DISCUSSION

Homologous passive cutaneous anaphylaxis

In the homologous PCA test sensitisation is produced by intradermal injection of antibody and the antigen is injected at a later time intravenously so that it is presented uniformly and simultaneously to the whole sensitised area. The response consists of a marked increase in the permeability to large molecules of capillaries at the site of intradermal injection. This is demonstrated by a leakage of the Evan's blue dye into the tissue space which permits an evaluation of the intensity of the reaction (Brocklehurst, 1967).

This test has been used by Gell and Coombs (1968) to demonstrate in vivo the presence in sera of reaginic (homocytotropic) antibody. For this test, the serum samples are injected intradermally into the same species of animal, in this case the sheep. That sheep can produce a homocytotropic antibody which will give a positive homologous PCA response has been shown by Hogarth-Scott (1969) in his studies on Ostertagia infections. The results of the experiment described in this present study to show homocytotropic antibody were entirely negative. Leptospiral antigens do not therefore appear to act as allergens in sheep.

Lymphocyte transformation

The blast formation and cell proliferation which follow the reaction between lymphocytes from immunized animals and specific antigen are assumed to represent a memory response (Dutton, 1967; Vischer and Stastney, 1967). It has been suggested that the transformation response is triggered by the reaction between antigen and specific antibodies located on the lymphocyte cell surface (Dutton, 1967). Forbes (1965)

showed that lymphoblastic transformation is associated with specific antibody formation directed against the stimulating antigen.

The usefulness of lymphocyte transformation as a correlate in vitro of delayed hypersensitivity was shown by Mills (1966) and Oppenheim (1969). Peripheral lymphocytes from animals which, when immunized, produced only circulating antibodies failed to respond (Mills, 1966). It must be pointed out however that this correlation between lymphocyte transformation and delayed hypersensitivity is not complete. Lymphocyte transformation can occur in individuals lacking delayed hypersensitivity, e.g. undergoing secondary antibody responses or having reaginic sensitisation. Conversely it may not occur in individuals known to possess delayed hypersensitivity in certain antigens (Bloom, 1971).

Recently it has been shown by Johnson et al. (1972) that PHA mitogen activity is augmented by the presence of erythrocytes in the lymphocyte culture and it is suggested by authors that such a mitogen is unsuitable for experiments to determine immunological defects. They recommend the use of non-erythrocyte agglutinating mitogens such as BW-PHA or Pokeweed mitogen (PWM). However, because of the careful controls used during the experiment reported here and because of the similarity and very low level of  $^{14}\text{C}$  take up by the negative controls and those stimulated by leptospiral antigen, it is felt that the augmentation of activity resulting from the presence of erythrocytes would not interfere with the results of these experiments in any significant way. The negative results obtained therefore appear to be entirely valid.

## SUMMARY AND CONCLUSIONS

1. The usual pattern for leptospirosis in sheep is for MAT titres of 1 : 100 or greater to appear between the 10th and 13th day after infection. High titres are maintained at least until the 31st day after infections.

2. There was no evidence that allergy or delayed hypersensitivity to leptospiral antigens occur in pomona infection in sheep.

CHAPTER 7ELECTRON MICROSCOPIC OBSERVATIONS ON EXPERIMENTAL LEPTOSPIROSIS  
IN SUCKLING MICE

## INTRODUCTION

It became clear from the experiments on sheep reported in Chapter 5 that, although valuable observations had been made of the proximal convoluted tubules with well established colonies of leptospire, it was still not clear as to how the organisms reached the tubule and what kidney damage, if any, resulted from their migration to this site. To fully investigate these aspects and to obtain more detailed chronological data it became apparent that sequential studies of experimental infection would be necessary. It was not practical to carry out sequential killing of large numbers of sheep for this purpose and accordingly a switch was made to the suckling mouse as an experimental animal.

Most of the observations of leptospiral infections in the kidney have been made with the disease at the nephritic phase. Michna and Campbell (1969) however, include in their study a photomicrograph of a Levaditi stained paraffin section of a pig kidney in the pre-nephritic phase. This photograph is alleged to show very large numbers of leptospire crossing the epithelium. From this they postulate that the leptospire travel by way of "the intertubular spaces, the epithelial cells or their junctions and enter the tubules." Miller and Wilson (1967) describe the formation of lysosomal vacuoles in the tubular epithelial cells and Michna and Campbell (1969) infer that it is probably inside such a vacuole that the organism may migrate from the intertubular space to the tubular lumen.

It was to investigate further these observations and to obtain a more accurate assessment of the time taken for this migration that the study was undertaken. Additional information as to the damage evoked by pomona at the early stages of infection, was also obtained.

## MATERIALS AND METHODS

Two sets of experimental infections were initiated. In the first (Experiment 7a) the mice were killed at weekly intervals to study the overall pattern of the disease. Subsequently, in a second experiment, (Experiment 7b) the mice were killed at intervals of 2 days for more precise studies of the earlier stages of infection.

### Experiment 7a

In this experiment, twenty four, three week old mice were injected by the intraperitoneal route (I/P) with 0.1 ml of a culture of pomona in Fletcher's medium. This inoculum was a three week old culture, prepared by inoculating 10 ml of Fletcher's medium with urine from ewe 386 (from Experiment 4b of the previous chapter) during leptospirosis. The culture was uncontaminated and had not been subcultured. The inoculum contained  $4 \times 10^6$  organisms, however this count is subject to a greater degree of error than is usual because the Fletcher's medium is semi-solid. The count was estimated using a Petroff-Hauser counting chamber.

To act as controls, twelve three week old mice were injected intraperitoneally with sterile Fletcher's medium.

At weekly intervals six infected and three control mice were perfused with a fixative consisting of 3% glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer at pH 7.2. The perfusion was performed by giving

approximately 0.5 ml of the fixative I/P to bathe the kidneys in situ. The glutaraldehyde-formaldehyde mixture was then injected by the intracardiac (I/C) route until the mouse died. The quantity given (I/C) was within the range 0.2 - 0.3 ml. Following the death of the mouse, the right kidney was removed as quickly as possible and cut into 1 mm cubed fragments. These were immersed in 10 ml of cold glutaraldehyde-formaldehyde fixative and were then held at 4°C. The preparation of the kidney fragments for electron microscopy was the same as that applied to the sheep kidney fragments; see Materials and Methods Chapter 4.

#### Experiment 7b

Eighteen, two week old mice were used in this experiment. Twelve of these were experimentally infected and six used as controls. The inoculum was a three week old culture of pomona isolated from the kidney of a lamb infected during a field outbreak of leptospirosis. The volume of culture inoculated I/P into the experimental mice was 0.1 ml and contained  $7 \times 10^6$  organisms. The method of counting the organisms was the same as for the preceding experiment and was subject to the same degree of error. The control mice were injected with 0.1 ml of sterile Fletcher's medium by the I/P route.

Two infected mice together with one control were killed every second day from P.I.D. 2 - P.I.D. 12 inclusive. The perfusion of the mice and the collection and processing of the specimens was done in the same way as for Experiment 7a.

#### RESULTS

By the second day following inoculation of the mice with a pomona culture, organisms are seen both within capillaries and within "vacuoles"

within the capillary endothelial cell (Fig. 7.1). From the fourth to the eighth day following infection the organisms are mainly located in the interstitial tissue; in many of the sections taken at this period the leptospire is seen surrounded by a clear area and this is interpreted as oedematous transudate (Fig. 7.2). The interstitial tissue generally from the second day of infection shows a degree of oedema which becomes more severe with time (Fig. 7.3). No changes are observed in the uriniferous tubules and the glomeruli at this stage of the disease. In a few sections taken on the eighth day an infiltration of leucocytes into the interstitium has taken place.

Ten days after inoculation leptospire is seen between cell junctions of adjoining epithelial cells of the proximal convoluted tubules (Fig. 7.5). In spite of examining a very large number of sections this was the only site at which leptospire was observed en route between interstitial tissue and tubular lumina. At no time were they seen invading or within epithelial cells. Where the organisms are found close to tight junctions they are contained within small pockets and surrounded by electron lucent material (Fig. 7.5). In some sections the organisms are seen within the infoldings at the base of the proximal convoluted tubules (Fig. 7.4).

By the fourteenth day leptospire is within the proximal convoluted tubular lumen (Fig. 7.7); although some organisms are still to be seen between the epithelial cells and even in the interstitial spaces. In some sections at P.I.D.4 to P.I.D.8 vacuoles containing portions of leptospire can be identified within epithelial cells of the proximal convoluted tubule but on no occasion do these organisms appear intact or viable (Fig. 7.6).

A summary of the events occurring within the mouse kidney infected with poimona is given in the diagram in Fig. 7.8.

At no time in either of the mice experiments were glomerular lesions observed.

#### DISCUSSION

One of the earliest changes seen in the mouse kidney is interstitial oedema. This is present on the second day and most probably is a reflection of endothelial damage by the leptospire although the organisms are also present in the interstitium at this time and it could be that the oedema is due to some extent to an inflammatory response provoked by leptospiral toxins. This oedema probably aids the passage of the organism through the tissue to the base of the proximal convoluted tubules. The basement membrane structure around the cells of the proximal convoluted tubules becomes thicker and less dense. This alteration in density of basement membrane structure probably indicates that it is a less effective barrier to the movement of leptospire. Having reached the base of the cells of the proximal convoluted tubules the organisms go between cell junctions creating small pockets by parting the otherwise tightly opposed cell membranes. The tenth day is the earliest time at which leptospire can be seen in this intercellular site between proximal convoluted tubular epithelial cells and at no stage have the organisms been observed penetrating between cells junctions in other parts of the tubule. The forces attracting the spirochaetes towards the lumen of the proximal convoluted tubules are at this stage inexplicable. Although the passing of the organism through the tight junction has not actually been observed it must be hypothesised that these junctions relax sufficiently to allow the organism to get through to the tubular lumen. In a very few of the electron micrographs of tissues taken on P.I.D.4 to P.I.D.3 structures can be seen within cells of the proximal tubules that could be said to resemble fragments of leptospiral organisms. These

structures are inside lysosomes, suggesting that the organisms may on occasion actively penetrate the cells, becoming engulfed and digested by the lysosomal enzymes. However, it must be emphasized that these structures are not sufficiently well delineated to make identification conclusive. An alternative explanation is that a few organisms die within the cell junctions and become engulfed by the tubular epithelial cells.

The absence of glomerular lesions in the mice at any stage during the period under observation up to and including P.I.D.14 was noteworthy and excludes the glomerulus as a possible route by which the leptospire arrive within the lumen of the proximal convoluted tubule. It is reasonable to assume that the passage of organisms through the glomerulus would result in some visible ultrastructural changes.

#### SUMMARY AND CONCLUSIONS

1. Within the first four days after infection leptospire migrate from within capillary lumina of the mouse kidney to the interstitial tissue and in so doing produce a degree of capillary endothelial damage and an interstitial oedema.
2. By the tenth day leptospire start to migrate between the epithelial cell junctions of the proximal convoluted tubules. Many organisms have arrived within the tubular lumen by the fourteenth day and those within the lumen have started to multiply.
3. At no time during the first fourteen days of infection are lesions observed in the glomeruli and this structure is unlikely to be a route of entry of leptospire into the tubular lumen.

Fig. 7.1 A leptospire (L) in a vacuole within a capillary endothelial cell of a mouse kidney two days after experimental infection with Leptospira interrogans serotype pomona. EM x 47,000.

Fig. 7.2 Leptospires surrounded by an oedematous space (Oe) in the interstitial tissue. CL = Capillary lumen. EM x 35,000.

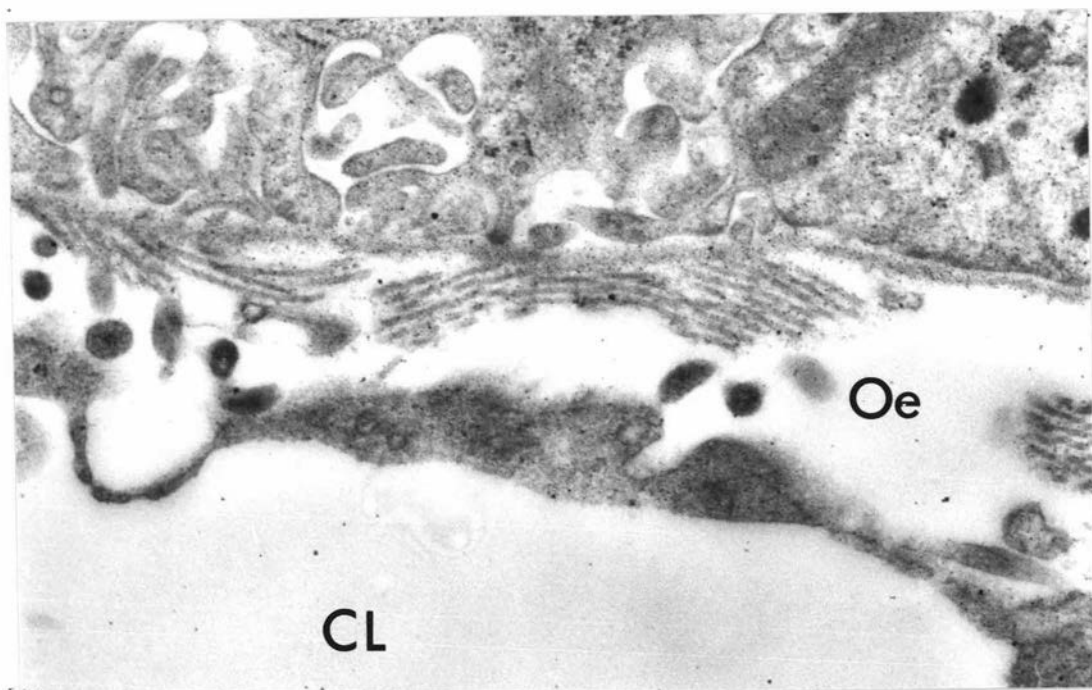
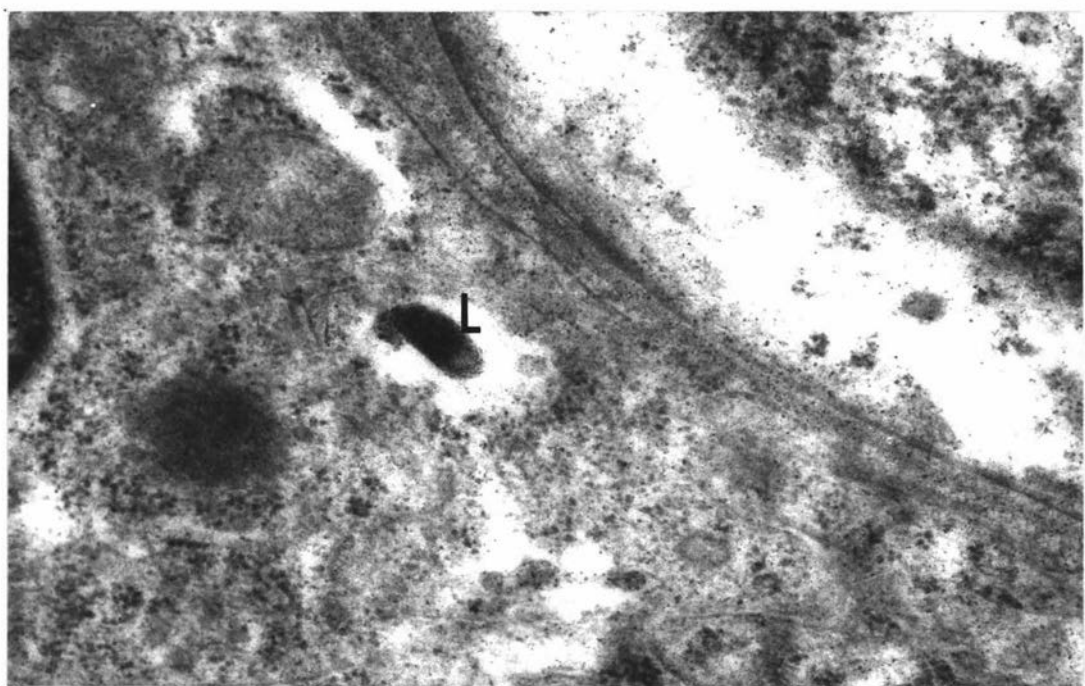


Fig. 7.3 Severe interstitial oedema (Oe) in a mouse kidney two days after infection with pomona.  
EM x 10,000.

Fig. 7.4 A leptospire (L) within an infolding at the base of a proximal convoluted tubular cell. The mouse had been infected 10 days previously. EM x 27,700.

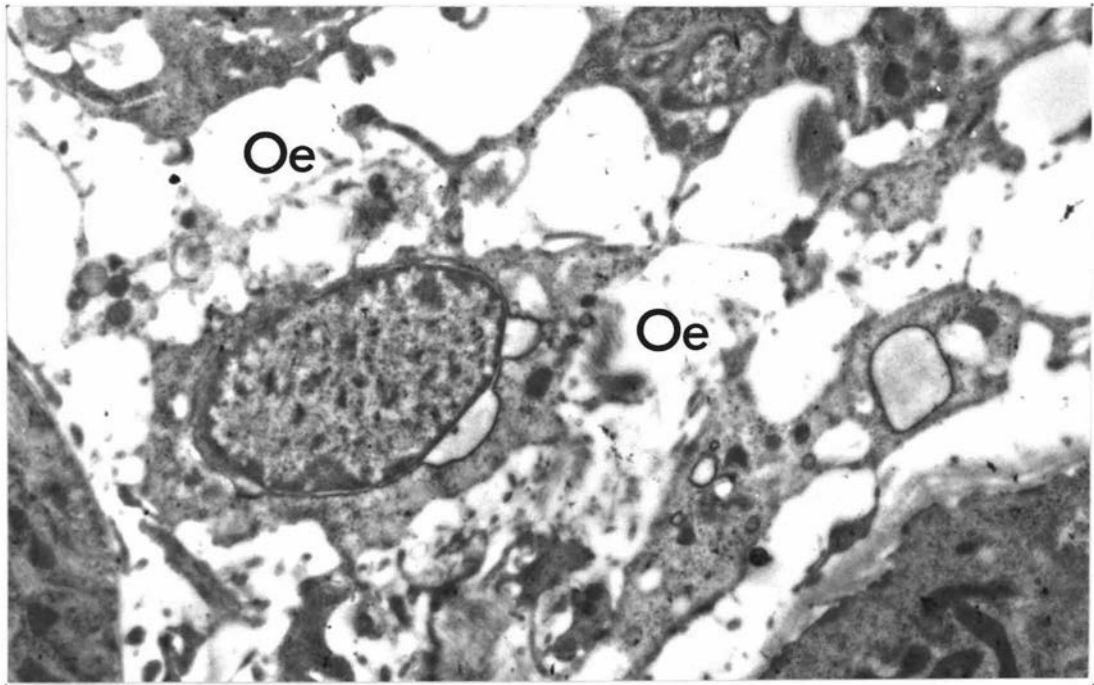


Fig. 7.5 A leptospire (L) migrating between adjoining convoluted tubular epithelial cells of the mouse kidney 10 days after infection with pomona. The organism is contained within a pocket of electron lucent material and is lying close to a tight junction (TJ) and the brush border (B<sub>2</sub>). EM x 47,600.

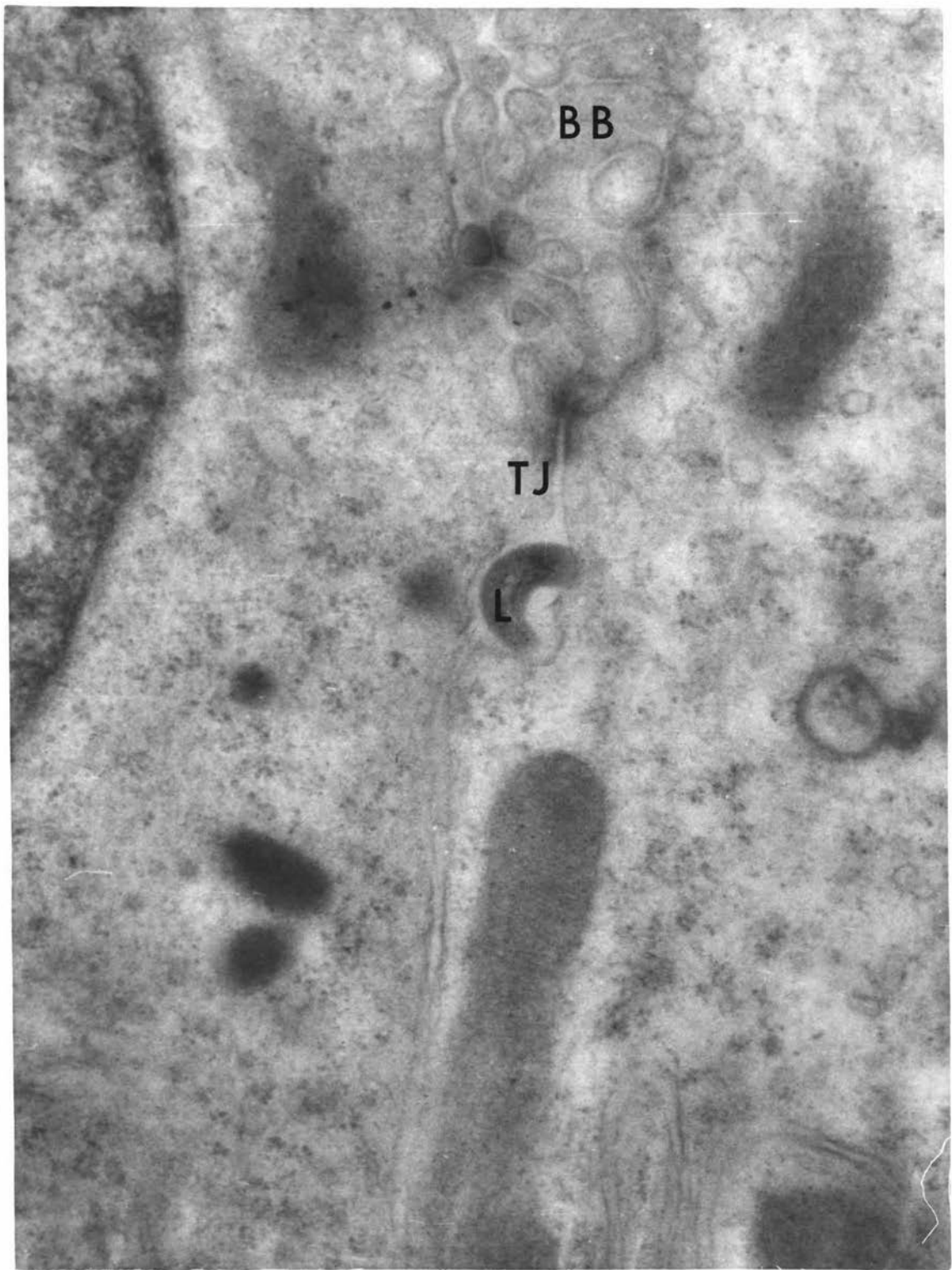
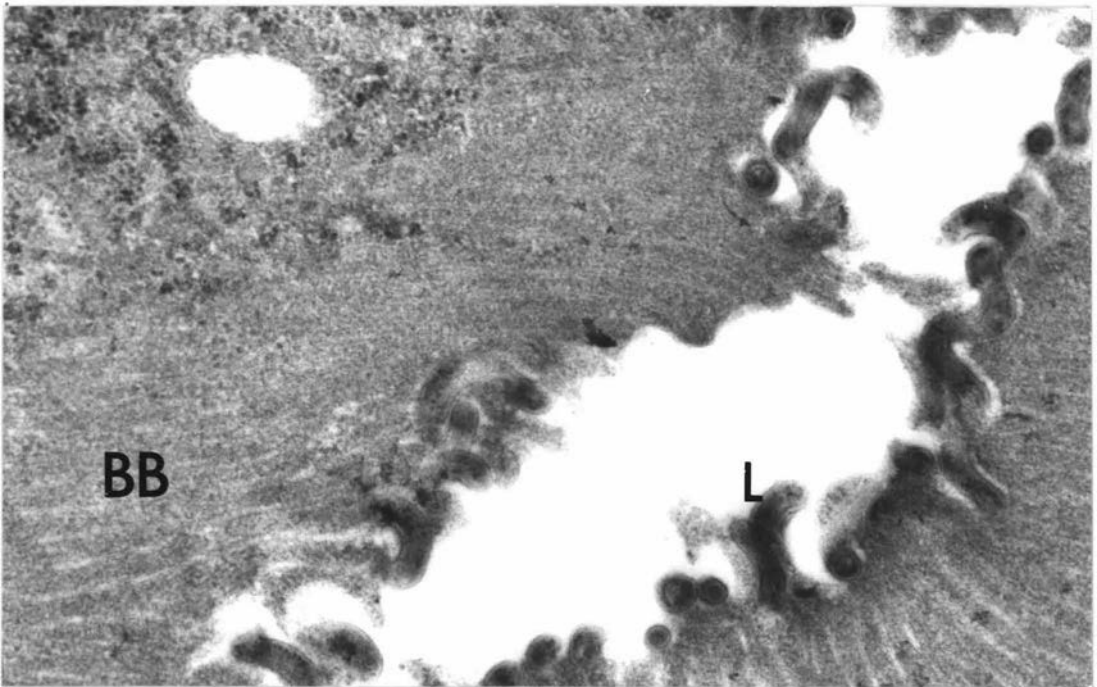
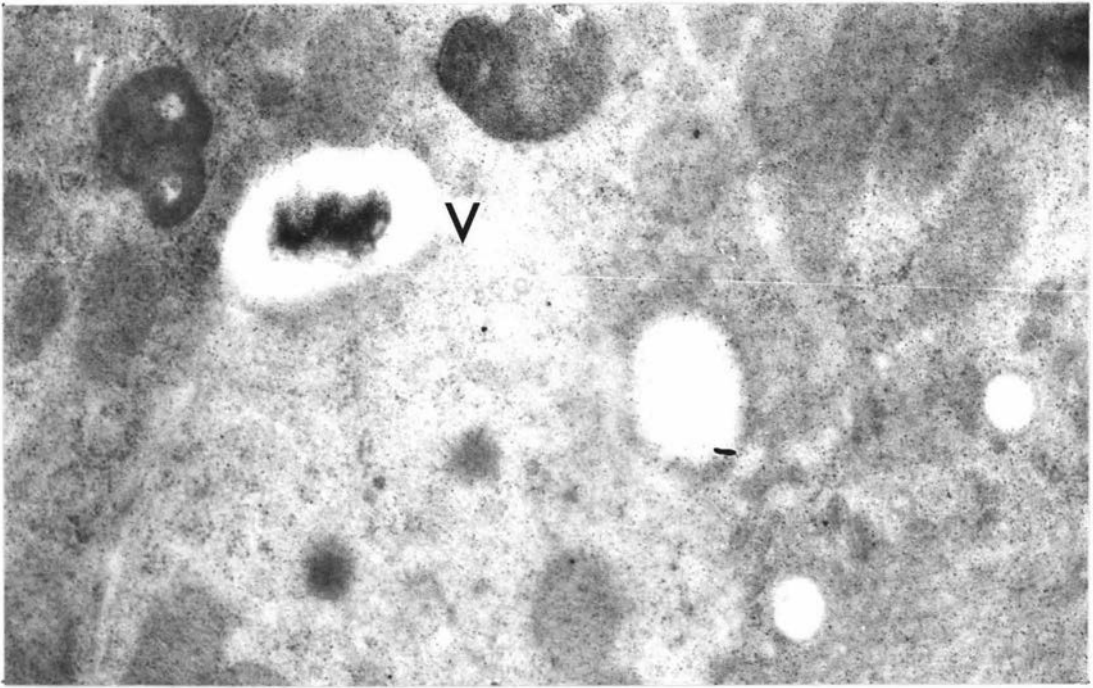


Fig. 7.6 A vacuole (V) containing what is possibly a remnant of a leptospire within the epithelium of a convoluted tubule. The mouse was infected 8 days previously. EM x 30,000.

Fig. 7.7 Mouse kidney 14 days after infection with pomona. Organisms (L) are well established within the tubular lumen and are closely associated with the brush border (BB) which appears normal. EM x 40,000.



The migration of leptospires within a mouse kidney

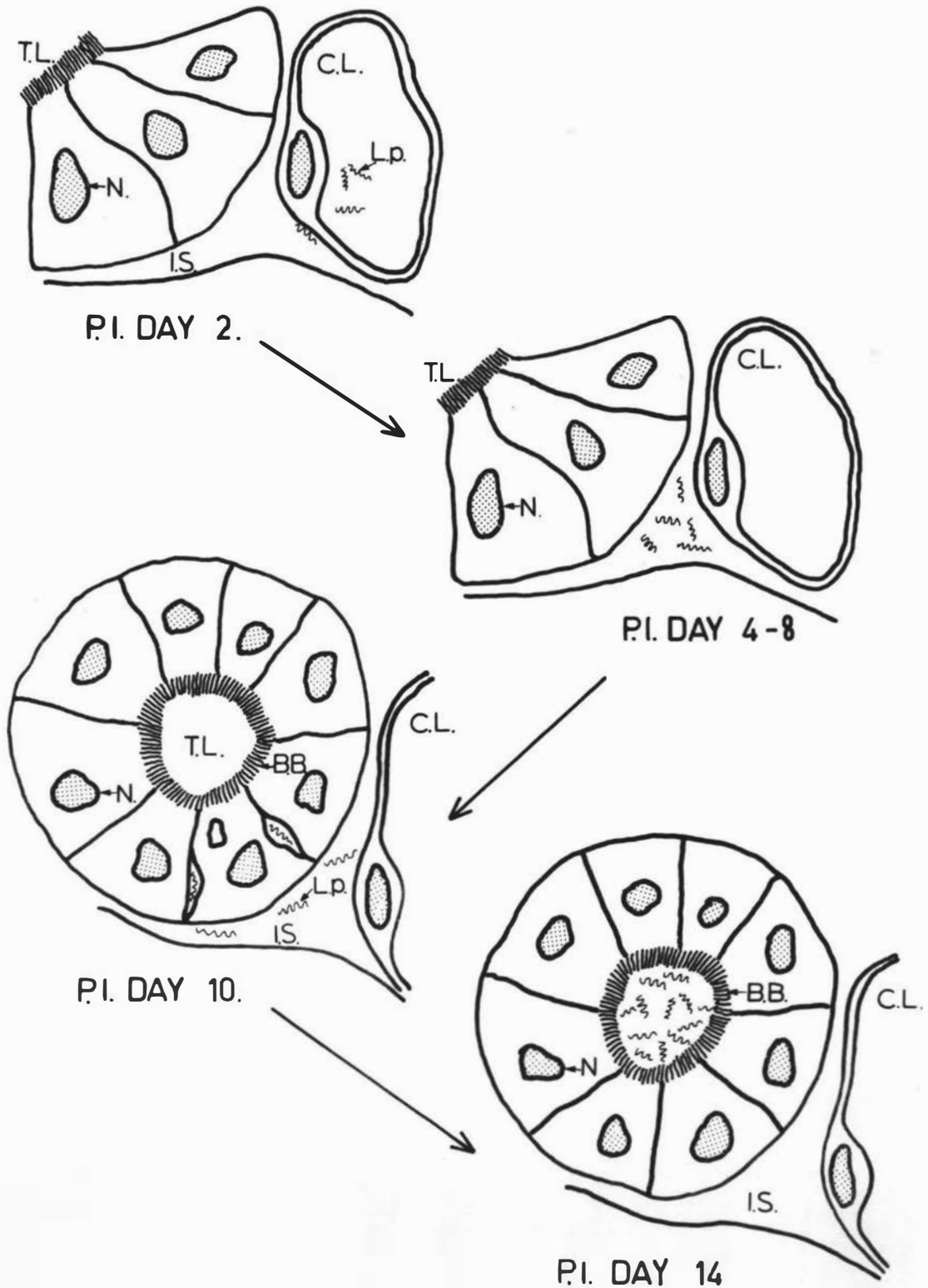


Fig. 7.8 A diagram showing the migration of leptospires (Lp) which occurs between post inoculation day 2 and 14. Starting from the capillary lumen (CL) the organism passes through the interstitium (IS) finally arriving within the tubular lumen (TL).

CHAPTER 8

## LEPTOSPIRACIDAL ANTIBODIES

## INTRODUCTION

Many of the investigators who have carried out studies on the pathogenesis of leptospiral infections in animals, have noted that an increasing level of agglutinating antibody as measured by the MAT is coincidental with the disappearance of the leptospiral organism from the blood (Morse et al., 1957; Bauer et al., 1961; Decker et al., 1970; and Bhasin et al., 1971). There is an implied assumption in many of these investigations that it is the agglutinins that are directly responsible for the termination of leptospiraemia. It has also been inferred that the agglutinating antibodies are responsible for the failure of leptospire to grow in serum-enriched culture medium when the serum used has a measurable level of homologous agglutinins (Turner, 1970). There is little information in the literature on the possible modes of action by which the agglutinins could bring about the death of leptospiral organisms and there have been no definitive in vitro studies. However a possible explanation for the lethal effect in vivo has been given by Faine (1964) who believes that it is due to an enhancement of phagocytosis. Faine demonstrated this opsonic effect using serum with high levels of agglutinins but did not establish conclusively that the enhanced phagocytosis was due solely to ~~these type of~~ antibodies.

This chapter describes experiments which demonstrate that certain immunogenic fractions of pomona preparations provoke distinct highly specific leptospiracidal antibodies. Also reported are in vitro investigations into the mode of action of leptospiracidal antibodies upon individual leptospire.

The significance of these antibodies and the manner in which they may affect the course of the natural and experimental disease is discussed.

## MATERIALS AND METHODS

### Experiments to investigate the possible existence of a leptospiracidal antibody

A seven day culture of pomona in Stuart's medium was fractionated on a Sephadex G200\* column and the fractions obtained were pooled as described below, each injected into rabbits in order to produce specific antisera. The antisera were tested individually for their ability to support growth of pomona when they were incorporated into Stuart's medium at the normal percentage for serum enrichment.

### Preparation of fraction by column chromatography

The fractions were prepared on a Sephadex G200 column from a sonicated seven day culture of pomona in Stuart's medium enriched with 10% normal rabbit serum. The collected fractions were pooled into five groups according to their presumed molecular weight and around obvious protein peaks. All the haemolysin containing fractions were included in one group (group 2). The pooled fractions were dialysed against polyethylene glycol until the original volume of the culture was obtained. These fractions were numbered 1 to 5 starting from the fraction with the largest molecular weight.

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\* Pharmacia, Upsala, Sweden.

### Immunisation regime

The five fractions were injected into individual rabbits at 10 day intervals. The first injection was 0.5 ml of dialysed fraction emulsified in 0.7 ml Freund's complete adjuvant\* and the three subsequent injections were emulsified in Freund's incomplete adjuvant. All injections were given intramuscularly. Two weeks elapsed from the time of the last injection until the harvesting of the antiserum from the rabbit.

### Growth studies

In order to determine the relative abilities of the antisera to support leptospiral growth, studies were made of each, by incorporating 0.5 ml of serum into 5 ml of Stuart's medium. Three 20 x 125 mm screw capped tubes of medium were prepared for each serum sample. The tubes of serum-enriched medium were sterilised individually by millipore filtration.

Each tube was inoculated with 0.25 ml of a seven day culture of leptospire. These were incubated at 27°C and daily counts of leptospires made using a Petroff-Hausser darkfield counting chamber. Evaluations were made of the growth sustaining capabilities of all the antisera. Comparisons were made between pre- and post-immunisation sera as well as between the sera prepared against the five different immunogens. Those sera which proved to inhibit the growth of pomona were also tested against the serotypes, icterohaemorrhagiae, bataviae, ballum, pyrogenes and canicola.

### The relationship of leptospiracidal antibody to agglutinating antibody

To determine the relationship, if any, between leptospiracidal antibody and agglutinating antibody as measured by the MAT, it was appropriate to test the prepared antisera for agglutinins. The MAT was

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\* Difco, Detroit, U.S.A.

performed according to the method described in Chapter 6. Since leptospiracidal titres of antisera (e.g. the antiserum against fraction 4) proved to be of the order of  $1/32$ , one run of MAT titration was carried out with fraction 4 antiserum to determine the effect of time on this titre. Doubling dilutions were used and samples from each dilution were examined microscopically for agglutination or lysis at 30 minute intervals up to  $6\frac{1}{2}$  hours and again at 26 and 50 hours.

#### Morphological changes in leptospire subjected to leptospiracidal antibody

In order to visualise the effect of leptospiracidal antibody upon pomona organisms, an electron microscopic study was undertaken. A  $1/16$  dilution of a leptospiracidal antiserum (fraction 4 antiserum) was allowed to react with a pomona culture for two hours and grids prepared by adding two drops of the culture antiserum mixture on to a formvar-carbon coated copper grid and negatively stained as described by Anderson and Doane (1972). A pomona culture added to both normal saline and normal rabbit serum were used as controls. The prepared grids were examined with a philips EM200 microscope.

#### Experiments to determine if leptospiracidal antibody enhances the haemolytic activity of pomona

Fraction 4 antiserum (1.0 ml) was added to 5 ml of an established culture of pomona in Stuart's medium. The antiserum was left in contact for two different time intervals, 2 hr and 12 hr. Control cultures with the same volume of normal rabbit serum were treated in the same way. After the appropriate time had elapsed the cultures were centrifuged at 6,000 g for 30 minutes and the supernatant titrated for haemolysin activity as described in Chapter 3.

Trials to determine possible methods to titrate leptospiracidal antibody in antiserum

1. Passive haemagglutination test

A modified version of the indirect haemagglutination test (Boyden, 1951; Garabedian et al., 1957) was employed. Sensitisation of erythrocytes was effected by adding 19 ml of a 1 : 40,000 dilution of tannic acid in saline to 1 ml of packed sheep red blood cells which had been washed three times in normal saline. The mixture was placed in a water-bath at 37°C for 10 minutes. At the end of this time the erythrocytes were deposited by centrifugation at 455 g for 3 minutes, the tannic acid solution removed by suction and the cells washed once with 25 ml physiological saline. The cells were again deposited and the saline removed and 2 ml of fraction 5 was added to the tanned cells. The cells were evenly suspended and left for 30 minutes at room temperature. After this time the now sensitized cells were removed by centrifugation, washed once with saline and finally suspended in 19 ml of 0.2 per cent gelatin in isotonic saline.

For the experiments using untanned cells the tanning procedure was omitted otherwise the procedure was the same.

Doubling dilutions of antisera were made in 0.025 ml volumes in plastic "microtitre" trays\* using physiological saline as the diluent. All antisera had been inactivated by heating at 56°C for 30 minutes. Sensitized erythrocytes (0.025 ml) were added to each serum dilution and mixed by carefully agitation. Included in each test series were control tubes containing (a) untreated erythrocytes, (b) tanned erythrocytes, and (c) sensitized erythrocytes and diluent. In addition to the test serum,

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\* Cooke Engineering Co., Virginia, U.S.A.

antiserum from other fractions and normal rabbit serum were also titrated.

The test remained undisturbed at room temperature for three hours before being read. The patterns of the sedimented erythrocytes were interpreted as recommended by Stavitsky (1954a, b).

## 2. Titration of leptospiricidal activity by growth studies

For this experiment doubling dilutions of unheated fraction 4 antiserum were prepared in normal rabbit serum and added to Stuart's medium so as to form 10% of the final medium. Each tube was then inoculated with 0.1 ml of a seven day pomona culture. Two tubes were used at each dilution. The results were read after 5 days by darkfield microscopy. Control tubes with normal rabbit serum were used for comparison.

## Leptospiricidal antibody, complement and heat stability

In order to see if complement was necessary for lysis of leptospire by leptospiricidal antibody, growth studies were made at doubling dilutions according to the method described immediately above except that the antiserum was treated to 56°C for 30 minutes prior to use. In addition similar studies were made in which the antiserum was heated to 65°C for 15 minutes in order to destroy any IgM class antibodies (Locke and Segre, 1965).

## RESULTS

### Growth studies

An inhibitory effect, specific for pomona was demonstrated by the growth studies and is graphically represented in Fig. 8.1 and also in Table 8.1 and 8.2. There was a marked decline in the number of organisms

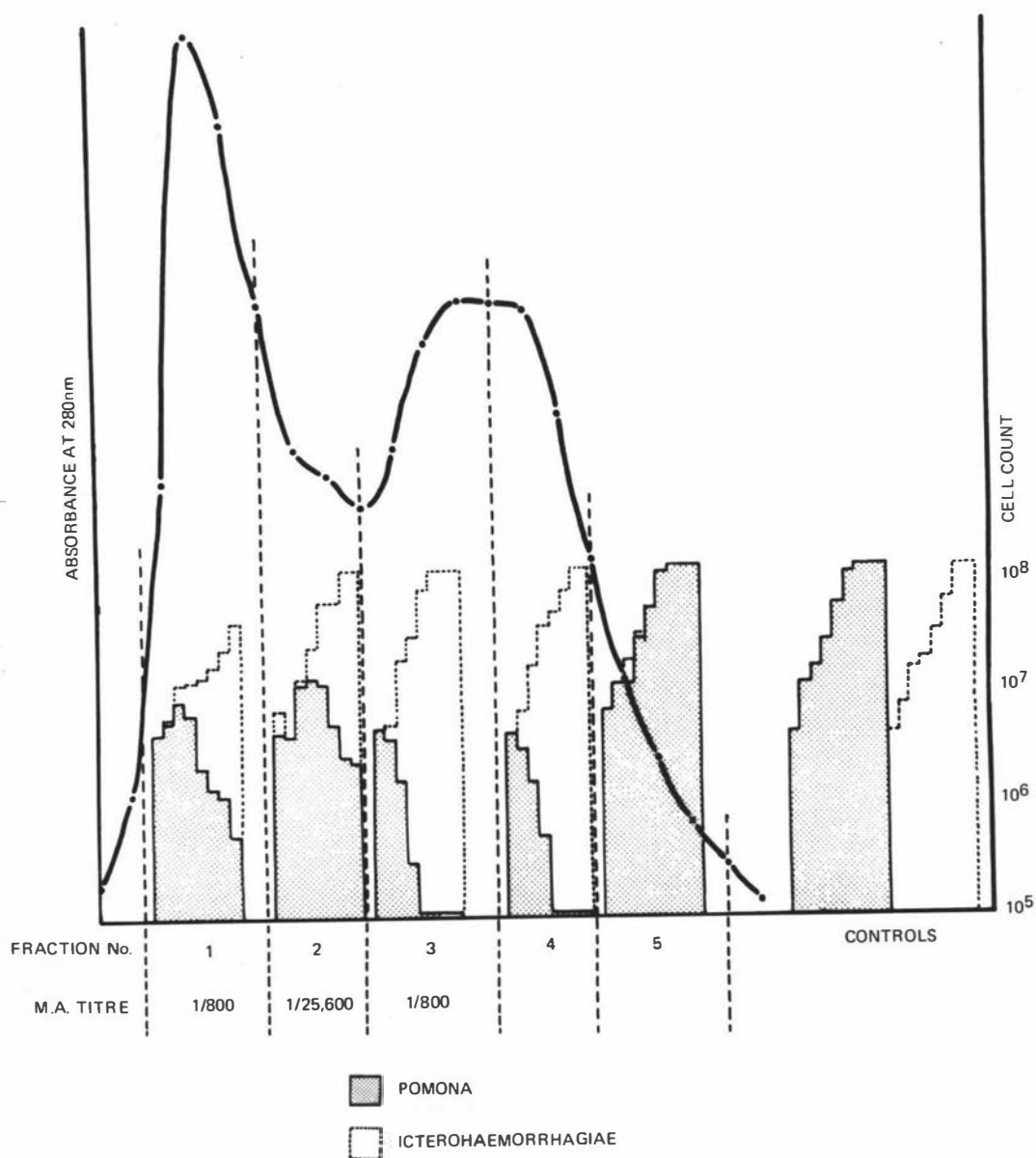


Fig. 8.1 Elution profile (—•—) superimposed upon histograms representing daily cell counts of leptospires growing in the presence of sera prepared against five immunogenic fractions of pomona obtained by chromatography in a Sephadex G200 column. The MA titres of each serum are given. Note that there is considerable suppression of growth of pomona by sera prepared against fractions 3 and 4 and that none of the sera had any adverse effect upon serotype icterohaemorrhagiae.

TABLE 8.1

MEAN COUNTS OF LEPTOSPIRES WHEN GROWN IN THE PRESENCE OF ANTISERA PREPARED  
AGAINST FIVE IMMUNOGENIC FRACTIONS OF POMONA

	*Fr 1 A/S	Fr 2 A/S	Fr 3 A/S	Fr 4 A/S	Fr 5 A/A	**NRS
DAY 0	$6.75 \times 10^6$	$6.62 \times 10^6$	$7.00 \times 10^6$	$6.50 \times 10^6$	$8.75 \times 10^6$	$6.75 \times 10^6$
1	$7.87 \times 10^6$	$6.50 \times 10^6$	$6.25 \times 10^6$	$5.12 \times 10^6$	$1.00 \times 10^7$	$1.12 \times 10^7$
2	$9.62 \times 10^6$	$1.00 \times 10^7$	$2.75 \times 10^6$	$2.25 \times 10^6$	$1.70 \times 10^7$	$2.60 \times 10^7$
3	$8.25 \times 10^6$	$1.74 \times 10^7$	$5.00 \times 10^5$	$7.5 \times 10^5$	$4.78 \times 10^7$	$4.94 \times 10^7$
4	$3.62 \times 10^6$	$1.47 \times 10^7$	N.O.S.***	N.O.S.	$7.75 \times 10^7$	$8.25 \times 10^7$
5	$1.87 \times 10^6$	$7.37 \times 10^6$	N.O.S.	N.O.S.	$1.07 \times 10^8$	$1.07 \times 10^8$
6	$1.12 \times 10^6$	$4.50 \times 10^6$	N.O.S.	N.O.S.	$1.25 \times 10^8$	$1.25 \times 10^8$
7	$7.50 \times 10^5$	$4.25 \times 10^6$	N.O.S.	N.O.S.	$1.25 \times 10^8$	$1.25 \times 10^8$

\* Fr 1 A/S = Fraction 1 antiserum

\*\* NRS = Normal rabbit serum

\*\*\* N.O.S. = No organisms seen

TABLE 8.2

MEAN LEPTOSPIRAL COUNTS OF DIFFERENT SEROTYPES IN PRESENCE OF FRACTION  
4 ANTISERUM AND NORMAL RABBIT SERUM

		FRACTION 4 ANTISERUM				
	ICTERO*	BATAVIAE	PYROGENES	BALIUM	CANICOLA	
DAY 0	$6.12 \times 10^6$	$6.62 \times 10^6$	$8.74 \times 10^6$	$9.00 \times 10^6$	$8.00 \times 10^6$	
3	$6.16 \times 10^7$	$6.87 \times 10^6$	$8.02 \times 10^7$	$5.12 \times 10^7$	$6.65 \times 10^7$	
5	$9.37 \times 10^7$	$7.00 \times 10^7$	$1.25 \times 10^8$	$7.72 \times 10^7$	$1.25 \times 10^8$	
7	$1.25 \times 10^8$	$8.50 \times 10^7$	$1.25 \times 10^8$	$1.06 \times 10^8$	$1.25 \times 10^8$	
		NORMAL RABBIT SERUM				
	ICTERO*	BATAVIAE	PYROGENES	BALIUM	CANICOLA	
DAY 0	$6.50 \times 10^6$	$7.75 \times 10^6$	$8.5 \times 10^6$	$9.87 \times 10^6$	$9.00 \times 10^6$	
3	$3.15 \times 10^7$	$2.51 \times 10^7$	$7.31 \times 10^7$	$5.21 \times 10^7$	$5.87 \times 10^7$	
5	$8.62 \times 10^7$	$6.20 \times 10^7$	$1.25 \times 10^8$	$1.15 \times 10^8$	$1.25 \times 10^8$	
7	$1.25 \times 10^8$	$1.05 \times 10^8$	$1.25 \times 10^8$	$1.25 \times 10^8$	$1.25 \times 10^8$	

\* Serotype icterohaemorrhagiae

in the cultures in which fraction 3 and 4 antiserum were used as the enrichment. In tubes containing antisera against fractions 1 and 2 the effect was less dramatic and in medium containing fraction 5 antiserum growth was the same as the normal rabbit serum controls. The inhibitory effect is clearly seen by the fourth day (Table 8.1) where growth in excess of  $3.6 \times 10^6$  was seen in all cultures except those containing fraction 3 and 4 antiserum and in these, no organisms were visible.

#### The relationship of leptospiracidal antibody to agglutinating antibody

Agglutinating and leptospiracidal antibodies appear to be quite distinct. The maximum MAT titre (1/25,600) was detected in fraction 2 antiserum and fraction 4 and 5 antiserum had no demonstrable agglutinins in the lowest dilution of 1/100. The results of this test are shown in Fig. 8.1.

The leptospiracidal effect of antisera appears to be rapid and to show little increase with time. When the modified MAT test was carried out with fraction 4 antiserum the initial titre was 1/32 and the end point was characterised by more than 50% lysis of the organisms without any evidence of agglutination. Subsequent examinations of the test at 30 minute intervals, revealed an increased lysis titre to 1/64 at 1½ hours but this showed no further increase up to and including the last reading at 50 hours.

#### Morphological changes in leptospire subjected to leptospiracidal antibody

The electron micrographs of pomona which had been exposed to fraction 4 antiserum, are characterized by a preponderance of organisms without their outersheaths. Many recognisable fragments of outersheath are to be seen lying free (Fig. 8.2 and 8.3). Some discernable fragments of outersheath are seen adhering to the leptospiral organism (Fig. 8.3). A majority of leptospire which have been in contact with the specific anti-

serum, exhibit numerous dense intracellular bodies (Fig. 8.4). Although an occasional outersheath fragment is seen in the control samples, there is quantitatively a great difference between the control and experimental preparations (Fig. 8.5). The overall surface of the experimental grids is covered with a debris consisting of variously sized roughly round fragments ranging from 35 mm to 250 mm in diameter.

#### Titration of leptospiracidal antibody using the indirect haemagglutination test

The results for this test were negative when using both untanned and tanned erythrocytes and this test therefore proved unsatisfactory.

#### Titration of leptospiracidal antibody by growth inhibition

Employing fraction 4 antiserum in doubling dilutions the titre by this method proved to be 1/16. This reduction in titre when compared with 1/64 in the modified MAT is probably due to continued growth of surviving organisms detected at five days which would not be apparent at 50 hours in the modified MAT. The end-point in this titration was very clear with only 4-10 organisms visible per microscope field compared with an uncountably large number in the next dilution.

#### Leptospiracidal antibody, complement and heat stability

The titre of leptospiracidal antibody in fraction 4 antiserum was unchanged after heating at 56°C for 30 minutes and therefore complement appears to play no part in the activity of the antibody. Similarly the titre remained unaffected after heating to 65°C for 15 minutes which shows that the antibody is not IgM since this class of immunoglobulin is inactivated by this treatment.

## DISCUSSION

This study has shown that certain leptospiral immunogens provoke a highly specific leptospiracidal antibody. This antibody is distinct from the antibody that is responsible for microscopic agglutination.

It appears logical that leptospiracidal antibody is likely to be of great importance in controlling the course of leptospiral infections. Previous attempts to explain the course of the disease in relation to agglutination titres of the host animal have not been entirely satisfactory. When more information can be obtained on the time of development and duration of leptospiracidal antibody titres, it may be possible to provide a better explanation of the pattern of events around the time of the cessation of the leptospiraemic phase of the disease. Clearly an important experimental step to be taken at this stage is the setting up of passive protection tests in which animals in the early leptospiraemic phase are given either specific leptospiracidal antibody or specific agglutinating antibody.

If leptospiracidal antibodies prove to be protective this finding would have implications upon the development of efficient leptospiral vaccines. A vaccine prepared from immunogens which provoke leptospiracidal antibodies would have the advantage of not stimulating agglutinating antibodies. These latter antibodies have been responsible for difficulties in the diagnostic interpretation of serological tests in vaccinated animals.

Leptospiral vaccines have not enjoyed a good reputation for effectiveness. The discovery of this immunogenic fraction now offers a considerable chance for improving the situation. Not only could vaccine be prepared from immunogens which will provoke greater titres of protective

antibody but with the development of growth study titration of leptospiracidal antibody reported here, it now becomes possible to use this technique in the evaluation of newly developed vaccines.

Although the present work has achieved a satisfactory separation of the immunogenic fraction of pomona responsible for provoking leptospiracidal antibody it has not been possible to pursue studies to locate the site of this immunogen on or in the intact organism. If this information could be obtained it would doubtless provide more information on the relationship of the organism to the host.

The titration of leptospiracidal antibody has demonstrated that complement is not needed for this antibody to destroy pomona. By heating the antiserum to 65°C for 15 minutes prior to the titration, no change in its level of activity is detected and from this it is inferred that the antibody is not IgM and is probably of the IgG class.

Electron microscopy studies have shown that this antibody which acts in the absence of complement brings about lysis of the organism by first completely disrupting the outersheath. The lytic action of immune serum is in agreement with the findings of Marshall (1966) and contrary to those of Lawrence (1955) who claimed that the agglutination-lysis test as it was then known, involved agglutination only and proposed that all reference to lysis should be dropped from the name. It is for this reason that the test became known as the MAT but it now seems that the previous name was more correct.

At least in the in vitro experiments carried out in this study the leptospiracidal antibody does not seem to contribute significantly to the enhancement of the haemolytic activity possessed by a culture of pomona. Perhaps the problem here revolves around the question of whether leptospiracidal

when disrupted release more haemolysin than do those that remain intact. A difficulty in solving this, is the lack of a very sensitive method of measuring haemolysin release.

Also demanding further attention and experimentation is the finding that this antibody apparently brings about lysis of the organism in the absence of complement. The whole question of the mechanism by which this antibody causes such a sudden disruption of the outersheath and eventual lysis of the organism is of considerable interest especially as this is perhaps the only bacterial lytic antibody so far described which acts without the need for complement. The exact manner in which leptospiracidal antibody brings about lysis remains a matter for speculation. It is possible that the antibody acts by specific metabolic inhibition or that its effect is more physical in nature. In this case antibody might be deposited on the outside of the organism and thus interfering with the passage of metabolites to and from the culture medium. Sheath disruption could be a consequence of cell death and lysis of the organism a natural sequel.

#### SUMMARY AND CONCLUSIONS

1. An immunogenic fraction of pomona capable of provoking a highly specific leptospiracidal antibody has been isolated. It is quite distinct from the fraction responsible for the production of serotype specific agglutinins.
2. The effect of this leptospiracidal antibody on the homologous serotype is rapid and strips the organism of its sheath and eventually brings about lysis of the organism.

3. Leptospiracidal antibody acts independently of complement and is probably an IgG class antibody.
4. Leptospiracidal antibody does not appear to enhance the haemolytic activity of pomona.
5. The discovery of a specific leptospiracidal antibody has considerable implications upon the development and testing of new leptospiral vaccines.

Fig. 8.2 Leptospira interrogans serotype pomona after 2 hours exposure to leptospiracidal antibody. The outer sheath (S) together with fragments of axostyle (Ax) and electron dense bodies (D) associated with the sheath can be identified. In the background material are other small fragments of electron dense material. Negatively stained with PTA EM x 35,000.

Fig. 8.3 Leptospira interrogans serotype pomona 2 hours after exposure to leptospiracidal antibody. One organism (L) is almost entirely devoid of its outer sheath and alongside is a completely detached outer sheath (S). Negatively stained with PTA EM x 35,000.

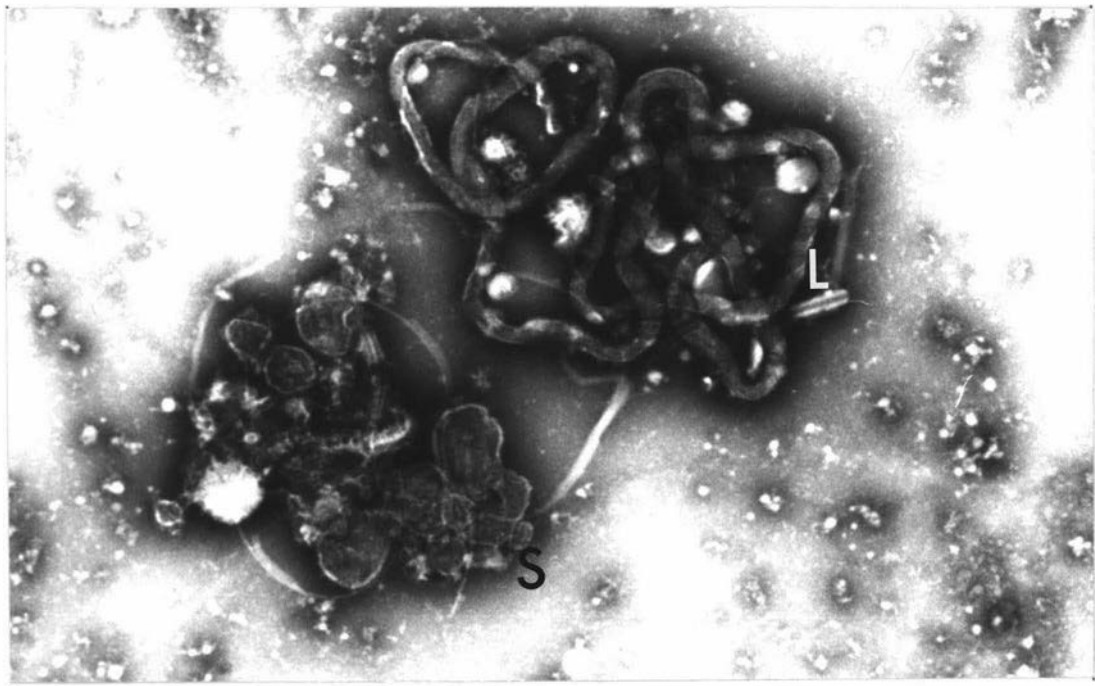
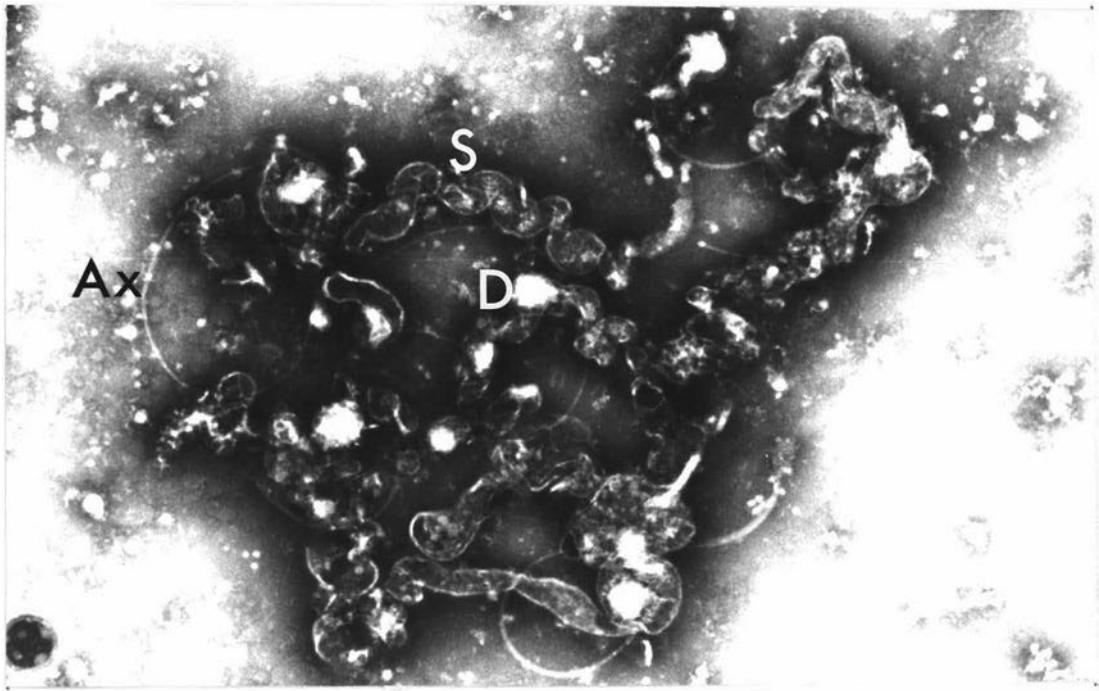
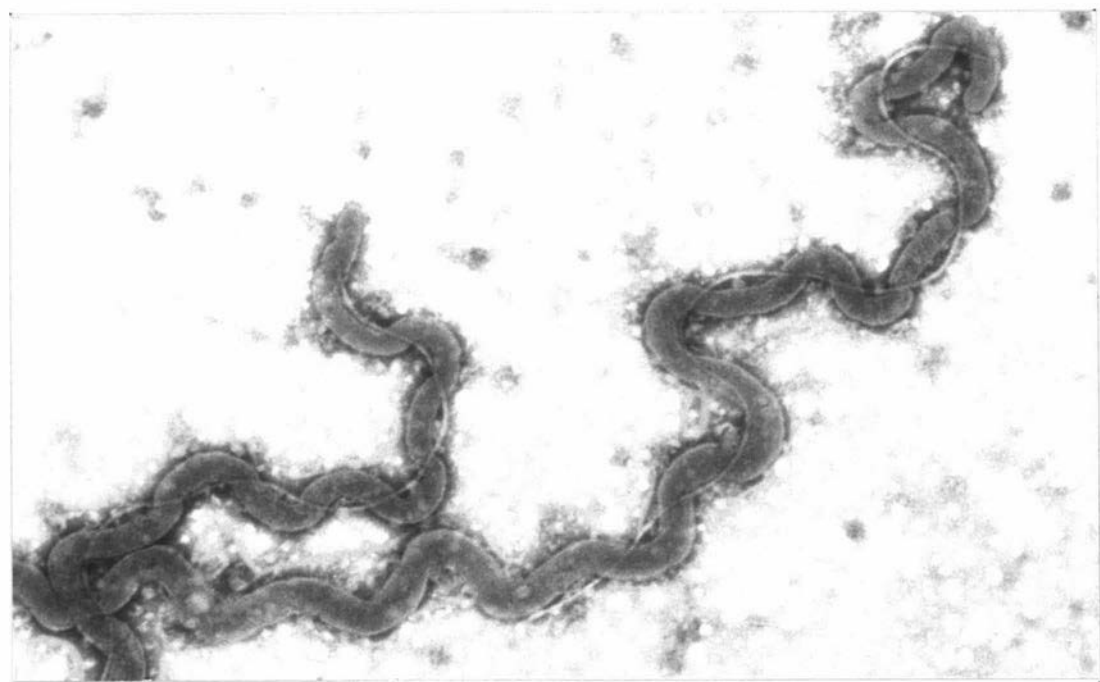
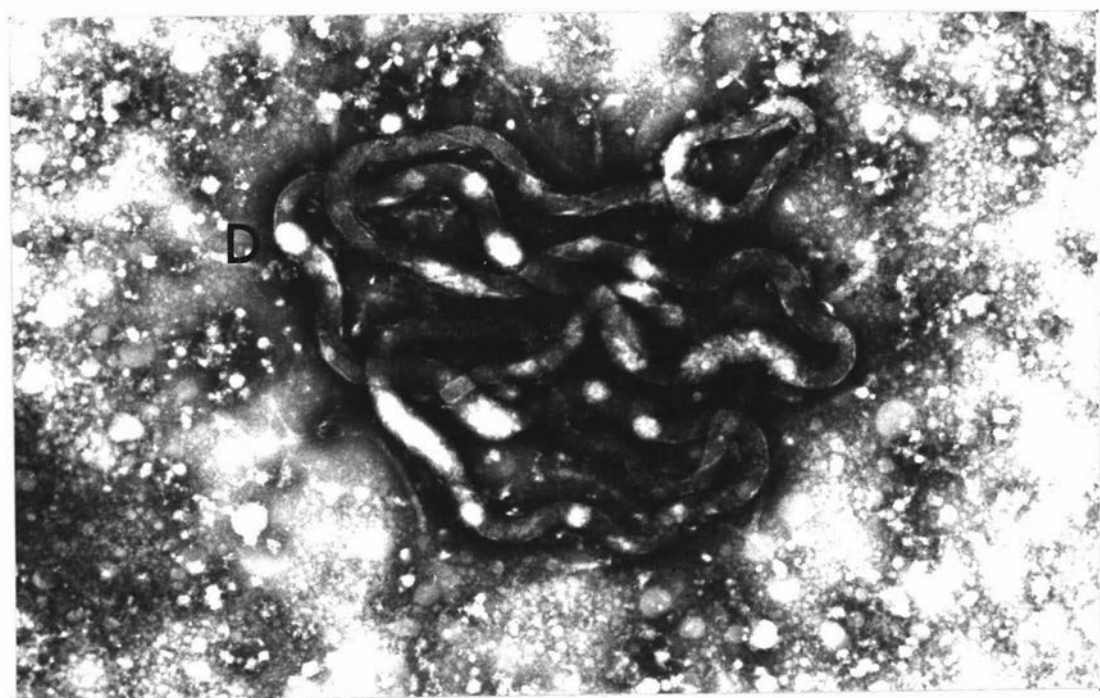


Fig. 8.4 A leptospiral organism after treatment with specific leptospiracidal antibody, exhibiting dense bodies (D) within the cytoplasmic cylinder. Negatively stained with PTA EM x 35,000.

Fig. 8.5 Pomona organisms in normal rabbit serum. Note the freedom from background debris of this preparation as compared with Fig. 8.4 Negatively stained with PTA EM x 35,000.



CHAPTER 9

## GENERAL DISCUSSION

Much of the experimental work reported here has been based on in vitro studies and the final application of any knowledge so gained must be in relation to the disease of leptospirosis as it occurs in the whole mammalian host.

Although mice have been very profitably used as experimental animals, the main emphasis of the work has been to explain the host parasite relationship between pomona and a naturally infected host such as the sheep. The hazards in applying findings from rather static in vitro situations to the dynamic state of the whole animal are appreciated and there are dangers in detailed extrapolation of results gained in the mouse to another species such as the sheep. However, it is believed that the findings are of a sufficiently basic nature that this approach seems justified.

The manner in which leptospores enter the body under natural conditions is an extremely difficult thing to investigate. It has been generally believed that the organisms' usual route of entry is via mucous membranes such as the conjunctivae, or through skin which has lost its integrity as a result of abrasions or maceration. It has not been established whether the actual mode of entry is by direct mechanical entry alone or occurs with the aid of enzymes. Although no direct studies into this were undertaken it appears that some of the findings reported here might well be applied in providing an understanding of how the organism enters the host. No evidence has been found that pomona is a significant producer of either

hyaluronidase or lecithinase; two enzymes which one might expect to be present if enzymic breakdown of cells or intercellular substance is an important feature of the entry process. The mouse kidney studies have shown that the organisms are capable of penetrating between tight junctions and it seems likely that organisms enter the body in this way at mucous surfaces or skin. Having gained entry into the body it is assumed that they rapidly move to capillary lumina and thence into the general circulation. One can only speculate upon the path followed by leptospire in making their entry into capillaries. Mouse studies have shown a migration path from capillaries into the interstitium of the kidney and one might assume that the organisms are therefore capable of moving in a reverse direction from interstitial tissue into a capillary.

Having entered the blood stream the organism undergoes a period of rapid multiplication which initiates the leptospiraemic phase of the disease. Under experimental conditions the timing and duration of this phase is somewhat variable and could depend upon factors such as the size and route of the infecting dose and the sensitivity and reliability of the methods used to detect organisms in the blood. The factors which bring about the termination of the leptospiraemia have not yet been fully established. To date it has been assumed that agglutinating antibodies are mainly responsible but the discovery of a non-agglutinating leptospiracidal antibody has provided a more plausible explanation for the sudden disappearance of leptospire from the blood. It has been shown (Marshall, 1973) that leptospiracidal antibody is detectable in infected sheep between 8 and 10 days post infection and using present test methods precedes the appearance of agglutinins by at least 2 days.

A feature of pomona infection in calves and, to some extent, in sheep is the development of haemolytic anaemia. For reasons discussed in Chapter 3 this anaemia has not been an outstanding feature of the

experimental infections described here. The haemolytic anaemia is characterised by a sudden drop in haemaglobin level followed by a gradual return to normal levels over a prolonged period. Of the several theories that have been proposed to explain the haemolytic episode, the one receiving most support from the experimental evidence given in Chapter 3 is that of Bauer and Morse (1958) who have concluded that the haemolysin which can be demonstrated in vitro is per se the major factor. The haemolysin is a relatively large molecule that acts independently of complement. Contrary to suggestions in the literature, no evidence could be found to involve an antigen-antibody reaction in the pathogenesis of intravascular haemolysis. Apart from the fact that the haemolysin is not a lecithinase, little can be said of its chemical nature or of the means whereby it produces haemolysis.

Although it has been generally accepted that the colonisation of renal tissue is an event which follows leptospiraemia, until sequential studies were carried out it was not known that these phases of the disease may overlap. That such overlap exists argues against the concept that kidney colonisation is a consequence of rising circulating antibody. Perhaps a more logical explanation is that the arrival of leptospores in the kidney is a chance event and that their continued multiplication within the tubular lumen is possible because they are protected from circulating antibody. There still remains a very distinct possibility that leptospores are specifically attracted to renal tissue by some as yet undefined mechanism. This is an aspect calling for further study.

Within the first four days leptospores migrate from the capillaries of the kidney into the interstitial tissue and in so doing damage capillary endothelium either by mechanical disruption or by means of a toxin. Along with this migration of organisms, and presumably as a result of capillary endothelial damage, is the formation of an interstitial oedema. By the

tenth day the leptospire are found between the cell junctions of the proximal convoluted tubules. Although it was never demonstrated, it is assumed that tight junctions of adjoining cells relax sufficiently to allow the organisms to continue their journey into the tubular lumen. By day fourteen the organisms are within the lumen and, judging by numbers, multiplication has already begun. The absence of hyaluronidase secretion by the organism was at first unexpected in view of the intercellular migration they undertake. On reflection, however, when one observes the distinctive motility of leptospire in semi-solid agar media, it is apparent that the organism is well fitted to insinuate itself between adjacent cells without the need for this enzyme.

The glomerular changes reported in the kidneys of sheep one month after infection (Chapter 5) are probably of little significance in the early stages of the disease. Apart from the qualifying fact that they were present in some of the controls the balance of evidence indicates that they may be an indirect consequence of haemoglobinuria or of changes in more distal parts of the nephron. This view is supported to some extent by the observation that no glomerular lesions were present in the kidneys of infected mice up to the fourteenth day. Haemoglobinuria was not seen at any time in these mice.

Changes that are observed in the convoluted tubules are due to the direct effects of pomona. The weight of evidence favours the suggestion that the lesions seen in the convoluted tubular epithelium are a result of toxins rather than of mechanical disruption. Although tissue culture experiments demonstrated only a low level of toxin production by pomona, this approach is a poor model of the in vivo situation where very large numbers of organisms are in close proximity to the host cells. A great deal of effort would be required to concentrate the cytotoxic element in order to reproduce

the situation that exists in vivo. The absence of any evidence of intracytoplasmic survival of pomona supports the hypothesis that these organisms migrate by an entirely intercellular route, thus enabling them to avoid contact with the lysosomal enzyme systems.

Among the earliest changes seen in pomona infected tubular epithelium is a reduction in the number of microvilli in the brush border. In some instances the microvilli swell at their free ends while still remaining attached to the apical surface of the cell by a basal stalk. In other cases the whole apical region of the cell protrudes into the tubular lumen. It is possible that this protrusion of the cell apex gives rise to proteinaceous "balloons" that are frequently seen in the lumen of infected tubules, but the complete absence of any organelles within these "balloons" puts this in doubt. A degree of necrosis of tubular cells is seen but this is of a low order and although some of the necrotic cells lying free in the tubular lumen contain recognisable leptospire, those that remain attached do not. This observation tends to preclude the theory that mechanical damage by this motile spirochaete is responsible for cellular necrosis. Other theories that have been advanced to explain kidney cell damage incriminate various types of immune reaction. The absence of IgE class antibody and failure of leptospiral antigen to transform lymphocytes suggests that allergy and delayed type hypersensitivity can be excluded as important factors in the production of lesions, at least in the sheep. However, this assertion leaves unexplained the fact that chronically infected kidneys are typically infiltrated by large numbers of lymphoid cells and often these take on a configuration which suggest a follicular arrangement. Jubb and Kennedy (1970) have suggested that much of the interstitial reaction is due to tubular damage allowing the escape of tubular contents. It seems reasonable that tubular fluid could be irritant to the interstitium in this

way but another possibility which might be considered is that tubular damage could provoke an autoimmune reaction to tubular epithelium or basement membrane.

Many of the tubules in a kidney may be colonized with pomona organisms and these become washed down with tubular contents into the bladder; from here they are voided in the urine. The shedding of organisms in the urine is known as the leptospiruric phase and in the sheep this can begin as early as the fourteenth day. The maximum time for shedding the organism by sheep has not been established but several experimental animals were still actively voiding organisms on the 31st day. It must be assumed that the organisms do not come in contact with significant levels of leptospiracidal antibody in any part of the urinary tract.

The demonstration of an antibody lethal for pomona in serum and the discovery that it is unrelated to the agglutinins normally measured by the MAT has questioned the aptness of measuring agglutinins to determine the degree of protection possessed by an animal after natural infection or vaccination. A growth inhibition titration would be a more logical and rewarding procedure. Some initial investigations not reported here have been undertaken into the time of onset, titre and duration of leptospiracidal antibody following experimental infections. Further studies are planned to measure these parameters, both in naturally infected animals and in animals vaccinated either with commercial vaccines or with purified immunogens.

It is difficult not to be enthusiastic about the importance of leptospiracidal antibody in protection of animals against leptospirosis, but much work remains to be done. Perhaps one of the first priorities should be the setting up of passive protection trials in highly susceptible hosts receiving varying amounts of specifically leptospiracidal antiserum. An animal such as the hamster, in which experimental leptospirosis is a

frequently fatal disease would appear to be ideal for this purpose.

A matter of considerable theoretical as well as practical importance is the question of which structural part of the leptospire contains the immunogens which stimulate leptospiracidal antibody. Once this is defined it will facilitate the preparation of more efficient vaccines, but perhaps more importantly it should provide an understanding of the sequence of events in the animal which expose its reticuloendothelial cells to significant quantities of the immunogen.

It has not been possible to invoke host cells per se as important facets of the immune process in leptospirosis. However, it is not considered that the current experiments have by any means fully excluded delayed type hypersensitivity as having some role. Improved methodology such as the use of purified immunogenic fractions might well be more fruitful in this regard. The observation of Faine (1964), that leptospiral antiserum with a high MAT titre has an opsonic effect, now calls for further investigation using antisera prepared against various immunogenic fractions.

Whatever further information may come to light, the basic fact remains that the sera from convalescent animals is specifically antagonistic to the growth of leptospire and it seems likely that this is a significant protection mechanism.

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APPENDIX I

## METHOD FOR TRYPSINIZING TISSUE CULTURE MONOLAYERS

1. Pour off tissue culture medium using sterile technique.
2. Replace with sterile warmed trypsin (0.25%).
3. Place in incubator (37°C) for 10 minutes.
4. When cells start to round off and cell sheet leaves the surface of the glass, pour the trypsin and cells into a sterile screw capped centrifuge tube.
5. Centrifuge at 80 g for 5 minutes.
6. Pour off supernatant and resuspend in P.B.S.
7. Recentrifuge, pour off supernatant and resuspend in medium.
8. Completely disaggregate by sucking up and down several times using a rubber bulb and 10 ml pipette.
9. Dispense into sterile glass bottles and add medium so that a final concentration of 100,000 cells/ml is achieved.
10. Incubate (37°C) with bottle in the horizontal position, marking the top surface of the bottle.

## TISSUE CULTURE MEDIUM

1.	Hank's solution	340 mls.
2.	Calf Serum	50 mls.
3.	5% Lactalbumin Hydrolysate	50 mls.
4.	Tryptose Phosphate Broth	50 mls.
5.	5% Yeast Extract	5 mls.
6.	Antibiotic Solution	2 mls.

1. Hank's solution

Using Oxoid Hank's Solution Granules Code BR19a.

Reconstitute 'A' in 1 litre glass distilled de-ionized water.  
Reconstitute 'B' in 1 litre glass distilled de-ionized water.

To dilute for making media:- take 34 mls 'A' and 34 mls 'B' and make up to 340 ml with glass distilled de-ionized water.

2. Calf serum

1. Blood collected from abattoirs in sterile stainless steel buckets.

2. Serum obtained in normal manner by centrifuging and stored unsterile in polythene containers in deep freeze.

### 3. Lactalbumin hydrolysate

Obtained from Nutritional Biochemical Corp.

1. Made by placing 25 grams of Lactalbumin hydrolysate into 500 mls glass distilled de-ionized water.

2. The mixture is heated until it dissolves but is not allowed to boil.

3. Mixture is then stored unsterile in 50 ml lots in polythene containers and stored in deep freeze.

### 4. Tryptose Phosphate Broth

Obtained from Difco. and made according to directions, i.e.

1. 29.5 grams are dissolved in 1,000 mls glass distilled de-ionized water and heated to dissolve.

2. Mixture is then dispensed into 50 ml quantities in polythene bottles and stored in the deep freeze.

### 5. Yeast extract

Obtained from 'Difco'.

1. A 5% solution is made up using glass distilled de-ionized water and heating to dissolve.

2. Media placed in 5 ml quantities in ½oz bottles and stored in deep freeze.

### 6. Antibiotic solution

Using 1 gram vials Streptomycin Sulphate and 1 mega vials Crystapen Benzylpenicillin (Sodium Salt) (Glaxo).

1. 1 vial of each is dissolved in 20 ml sterile glass distilled de-ionized water and placed in a sterile Universal bottle and stored in the deep freeze.

## TO MAKE FINAL MEDIA

1. Take ingredients out of deep freeze and thaw gently in 56°C water bath leaving for only about 10 minutes.

2. Lactalbumin hydrolysate placed in the flask first and heated until it becomes clear and precipitate dissolved. All other ingredients are then added and mixed.

3. Mixture is then filtered through a sterile millipore filter (15 lbs for 30 mins) using filter holder with filter diameter of 142 mm. (Millipore Filter Corporation, Bedford, Massachusetts, U.S.A.)

4. The media is filtered using sterile precautions into sterile prescription bottles.
5. Media incubated overnight in 37°C incubator and then stored in refrigerator.

#### TRYPsin SOLUTION

NaCl	4 gms.
KCL	0.1 gm.
Na <sub>2</sub> HPO <sub>4</sub>	0.575 gm.
KH <sub>2</sub> PO <sub>4</sub>	0.10 gm.
glass distilled de-ionized water	800 mls.
Phenol red indicator	0.02 gm.
Trypsin 1:250 (Difco)	1 gm.

1. The first 6 ingredients are weighed and measured and heated to dissolve the salts.
2. When the solution has cooled the trypsin is added and the solution heated again, gently to dissolve trypsin (never above 45°C).
3. Media Millipore filtered using 47 mm filter into 50 ml amounts in sterile prescription bottles and stored in deep freeze.
4. While trypsin is being used it may be kept at 4°C but discarded after a week.

#### PERIODIC-ACID-SCHIFF REACTION

##### Solutions

##### Periodic acid:

Periodic acid (HIO <sub>4</sub> )	0.6 g.
Distilled water	100 ml.
Nitric acid conc.	0.3 ml.

##### Schiff's Reagent

Basic fuchsin	0.5-1.0 g.
Distilled water	85.0 ml.
Sodium metabisulphate	1.9 g.
N HCL	15.0 ml.

Place Schiff's reagent in bottle with approximately 50-60 ml of free air space. Shake at intervals for at least 2 hours or overnight.

Add 200 mg activated charcoal: 2 minutes; shake occasionally. Filter. Store Schiff's reagent in a bottle with a minimum of air space above the solution and keep in the refrigerator. This will decrease the loss of SO<sub>2</sub>.

#### Sodium Bisulphate

Sodium metabisulphate	0.5 g.
Distilled water	100 ml.

#### Procedure

1. Treat with periodic acid, aqueous: 5 minutes.
2. Wash in running water: 5 minutes.
3. Treat with Schiff's reagent 10 minutes.
4. Transfer through sulphate solution, 3 changes 1-2 minutes in each.
5. Wash in running water: 5 minutes.
6. Counterstain, if desired.
7. Dehydrate, and mount.

#### MAYER'S HAEMALUM STAIN

1. Coverslips put in racks and fixed for at least 10 mins in 10% formal saline.
2. Wash in tap water.
3. 10 minutes in Celestian blue\*.
4. Wash in tap water.
5. Mayer's Haemalum\*\* for 10 mins (filtered).
6. Wash in tap water.
7. Blued in Scott's tapwater for 2 minutes.
8. Wash in tap water
9. Eosin\* for 2 minutes (filtered, then add 5 mls 1% acetic acid to the eosin).

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\* G.T. Gurr.

\*\* Edward Gurr.

10. Quick wash in water.
11. Up through the alcohols and finally into xylol.
12. Coverslips mounted onto slides, and labelled.

#### OIL RED O

A saturated solution of oil red O (0.5%) in isopropyl alcohol is kept in stock. For use, dilute 6 ml of stock solution with 4 ml of distilled water. Allow to stand for 5-10 mins, then filter.

This solution does not keep for more than 1-2 hrs.

#### Method

1. Wash coverslip culture well in water.
2. Place in fat stain in a closed container for 10-15 mins.
3. Differentiate in 60% alcohol to clear background.
4. Wash in water.
5. Counterstain nuclei lightly with haematoxylin for 1 min.
6. Blue in Scott's tapwater.
7. Mount in a water mounting medium.

#### Results

Lipids - Bright red.

Nuclei - Blue.

#### METHYL GREEN-PYRONIN

5% aqueous pyronin Y	17.5 ml.
2% aqueous methyl green (washed)	10 ml.
Distilled H <sub>2</sub> O	250 ml.

Dilute with an equal quantity of acetate buffer pH 4.8 before use.

#### Method

1. Bring coverslip cultures to water.
2. Pour on prepared staining solution and leave for 15 minutes.
3. Rinse quickly in distilled water and blot on non-fluffy filter paper.

4. Flood slide with acetone, leave for a second or 2, and flood again with acetone.
5. Flood slide with acetone-xylol (= parts).
6. Flood with xylol; leave until clear. Should this take unduly long, the section may be blotted and fresh xylol poured on.
7. Mount in neutral mountant and D.P.X.

### Results

DNA - green  
RNA - red.

### FEULGEN REACTION

1. Bring coverslip cultures down to aqueous distilled H<sub>2</sub>O and rinse briefly in cold NHCl.
2. Place in N HCl preheated to 60°C, for the optimum time of hydrolysis, about 10 minutes.
3. Rinse briefly in cold N HCl then in distilled water.
4. Transfer to Schiff's reagent for 45 minutes.
5. Drain and rinse in 3 changes of dilute sulphurous acid, rinse for 2 mins in each change.
6. Rinse in running water 30 mins.
7. Lightly counterstain with a 95% alcoholic solution of fast green.
8. Dehydrate and mount.

### GLASSWARE WASHING

All glassware was soaked in a 20% solution of Decon 75\* overnight and then washed in a Heinicke Typhoon\*\* glasswasher using a 9 minute wash cycle, a 9 minute warm water rinse and a 2 minute distilled water rinse. The washing powder used in the washing machine was Pyroneg\*\*\*. All glassware was then rinsed in 1N HCl and then three times with glass distilled water. The glassware was sterilized at 121°C for 15 minutes in an autoclave.

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\* Medical Pharmaceutical Developments Ltd, Brighton, U.K.

\*\* Heinicke Co., Hollywood, Florida, U.S.A.

\*\*\* Diversey Wallace Ltd, Papatoetoe, N.Z.

APPENDIX II

## E.M.J.H. MEDIUM

Concentrated Salt Solution

NH <sub>4</sub> Cl	5.35 gm
MgCl <sub>2</sub> ·6H <sub>2</sub> O	3.72 gm
NaCl	38.5 gm
Sterile distilled H <sub>2</sub> O	1000 ml

Buffer Solution

Na <sub>2</sub> HPO <sub>4</sub>	16.6 gm
KH <sub>2</sub> PO <sub>4</sub>	2.172 gm
Sterile distilled H <sub>2</sub> O	1000 ml

Magnetic stirring bar and magnetic rotator.

To 700 ml of distilled water add 50 ml of the concentrated salt solution and 40 ml of the buffer solution. To this is added 2.4 mg CuSO<sub>4</sub>·5H<sub>2</sub>O, 3.2 mg ZnSO<sub>4</sub>, 40.0 mg FeSO<sub>4</sub>·7H<sub>2</sub>O and 180.0 mg L-cystine; the medium is shaken then filtered, 4 Vitamin B<sub>12</sub> and thiamine are added and the volume adjusted to 1000 ml. All this is sterilized for 15 minutes at 121°C and after cooling 20% by volume of Oleic Albumin Complex (Difco) added.

Vit B <sub>12</sub>	160 μg
Thiamine HCl	160 μg

## ACID ALCOHOL

3% hydrochloric acid

95% methyl alcohol

## TRIS BUFFER

Tris	24.22 g
NaCl	23.4 g
H <sub>2</sub> O to	1000 ml
HCl 1.0N to pH 8.0	

Makes up to 2000 ml.

APPENDIX III

## BILI-LABSTIX TECHNICAL INFORMATION

pH

Impregnated with methyl red and bromthymol blue.

Sensitivity

Permits differentiation of pH values to half a unit within the range 5-9.

Specificity

pH is not affected by the urinary buffer concentration. Bacterial growth in a specimen may cause a marked alkaline shift and render it unsuitable for testing.

Protein

Impregnated with tetrabromphenol blue buffered to an acid pH. This area is yellow in the absence of protein but, at the same pH, changes to a shade of green depending on type and concentration of protein present.

Sensitivity

Five to 20 mg. of albumin per 100 ml. urine may be detected as a "Trace" result. The test area is more sensitive to albumin than to globulin, hemoglobin, Bence-Jones protein and mucoprotein.

Specificity

Although buffered adequately for most urines, exceptionally alkaline and/or highly buffered urines may give positive results in the absence of significant proteinuria; e.g., patients on alkaline medication, contaminated specimen container, stale urine. The test is unaffected by urine turbidity, X-ray contrast media, most drugs or their metabolites and urine preservatives which occasionally affect other protein tests.

Glucose

Impregnated with glucose oxidase which, with atmospheric oxygen, oxidizes glucose to gluconic acid and hydrogen peroxide. The latter, in the presence of peroxidase, oxidizes the chromogen system to a shade of purple.

Sensitivity

Approximately 0.1% glucose in urine is detectable. Sensitivity is influenced by inhibiting substances, temperature and pH of urine. One cause of inhibition is a large urinary concentration of ascorbic acid from therapeutic doses of vitamin C or parenteral preparations using vitamin C as a reducing agent; e.g., tetracyclines.

Specificity

Specific for glucose; no substance excreted in urine other than glucose is known to give a positive result. Reagent area does not react with

lactose, galactose, fructose nor reducing metabolites of drugs; e.g., salicylates and nalidixic acid. This test may be used to check whether the reducing substance found in urine is glucose.

### Ketones

Impregnated with sodium nitroprusside, glycine and buffer.

#### Sensitivity

The reagent area detects 5 to 10 mg. acetoacetic acid per 100 ml. urine, is less sensitive to acetone and does not react with beta-hydroxybutyric acid.

#### Specificity

This test reacts with acetoacetic acid and acetone in urine.

### Bilirubin

This area, impregnated with a stabilized diazotized 2,4- - dichloroaniline, is buff coloured and turns brownish when moistened with urine containing bilirubin. The intensity of brown colour is roughly proportional to the amount of bilirubin present.

#### Sensitivity

Field studies indicate that most observers will not read positive (+) results below 0.2 mg. % in urine.

#### Specificity

This test is specific for bilirubin in biological materials.

### Blood

Impregnated with a buffered mixture of organic peroxide and orthotolidine.

#### Sensitivity

Reagent area is more sensitive to free hemoglobin and myoglobin than to intact erythrocytes. Complements the microscopic examination. Sensitivity is reduced by high urinary concentrations of ascorbic acid (cf. Glucose).

#### Specificity

Hemoglobin, myoglobin and erythrocytes give a positive reaction; However, certain oxidizing contaminants, such as hypochlorites, may produce a false positive result.

384 EWE

P.I.D.	-1	1	3	8	10	11	12	13	15	17	20	22	24	27	29	31
DATE	19/10	21/10	23/10	28/10	30/10	31/10	1/11	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11
URINE. S.G.	1.070	1.052	1.050	1.050	1.051			1.046	1.050	1.051	1.050	1.053	1.051	1.020	1.047	1.048
pH	8.5	7.5	6.0	8.0	8.0	8.0	8.0	7.5	8.0	8.0	8.0	8.0	8.5	6.0	6.5	8.0
PROTEIN mg/100 ml	-	-	TRACE	-	TRACE	TRACE	TRACE	30 +	TRACE	-	TRACE	TRACE	-	-	-	-
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMOUNT CENTRIFUGED	INSUF. SAMPLE	10 ml	10 ml	10 ml	15 ml			10 ml	10 ml	6 ml	10 ml	10 ml	10 ml	10 ml	10 ml	10 ml
AMOUNT DEPOSIT		0.2 ml	-	-	-			-	-	-	-	-	-	-	-	-
CASTS. HYALINE	RARE					N.T.	N.T.									
FINELY GRANULAR	FEW	RARE	FREQUENT	FEW				FEW		OCCASIONAL	OCCASIONAL			FEW		
COARSELY GRANULAR																
WAXY																
CELLULAR				RED CELL CASTS				SMALL EPITH. CASTS	LARGE EPITH. CASTS							LARGE EPITH. CASTS
CELLS. LEUCOCYTES		1/HPF	FEW	4/HPF				FEW	FEW	FEW						FREQUENT
LARGE EPITHELIAL	FEW	OCCASIONAL	OCCASIONAL	OCCASIONAL			OCCASIONAL	OCCASIONAL	OCCASIONAL	FREQUENT						NUMEROUS
SMALL EPITHELIAL			FEW	FREQUENT			FREQUENT	FREQUENT	NUMEROUS							FREQUENT
R.B.C.			5/HPF		FEW											OCCASIONAL
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>		FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW LARGE TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>
BLOOD. Hb	13.6	12.2	10.8	10.0	13.4		13.4	13.5	13.0	11.9	12.6	12.2	11.8	10.8	11.8	11.8
HAEMOTOCRIT	44.5	40.0	38.0	35.0	33.0		33.0	35.0	35.0	34.0	32.0	32.0	32.0	31.5	34.0	34.0
W.B.C.	10,200	7,300	8,200	7,850	9,150		7,300	9,250	7,050	8,700	8,900	7,500	7,800	12,100	9,350	9,350
NEUTROPHILS	12	14	6	6	23		26	34	25	23	25	22	22	31	47	47
BANDS								1	1	2	2	2	2			
EOSINOPHILS	6	6	4	4	2		2	6	3	4	5	8	2	3	3	3
BASOPHILS			1					1	6	2	2	1	1			
LYMPHOCYTES	82	80	89	90	75		71	58	69	69	65	71	67	66	49	49
MONOCYTES							1	2	1	1	2	2	2	1	1	1
TITRE. TO L. POMONA	-	-	-	-	-	N.T.	N.T.	-	1/100	1/1,000	1/1,000	1/1,000	1/100	1/100	1/100	1/100
DIRECT BLOOD	-	-	-	-	-			N.T.	-	-	-	-	-	-	-	-
CULTURE BLOOD	-	-	-	-	-			-	-	-	-	-	-	-	-	-
DIRECT URINE	-	-	-	-	-			-	-	-	-	-	-	-	-	-
CULTURE URINE	-	-	-	-	-			-	-	-	N.T.	-	-	-	-	-

KEY  
 N.T. NOT TAKEN  
 HPF HIGH POWER FIELD  
 RARE FEW OCCASIONAL  
 FREQUENT NUMEROUS

285 EWE

P.I.D.	-1	1	3	8	10	13	15	17	20	22	24	27	29	31
DATE	19/10	21/10	23/10	23/10	30/10	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11
URINE. S.G.	1.034	1.043	1.042	1.001	1.040	1.037	1.004	1.022	1.020	1.006	1.020	1.011	1.010	1.004
pH	6.0	7.5	8.0	6.5	6.5	6.0	7.0	8.5	6.5	7.0	9.0	8.0	8.5	7.0
PROTEIN mg/100 ml	-	-	TRACE	-	-	-	-	-	-	-	-	-	-	-
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CASTS. HYALINE														
FINELY GRANULAR	FREQUENT	OCCASIONAL	OCCASIONAL							OCCASIONAL				
COARSELY GRANULAR	OCCASIONAL	FREQUENT						FEW						
WAXY														
CELLULAR														
CELLS. LEUCOCYTES	FEW		FEW			1/HPF	FEW	RED CELL CAST		SM. EPITH CAST		LG. EPITH CAST		
LARGE EPITHELIAL	1/HPF		OCCASIONAL	NUMEROUS		NUMEROUS	FREQUENT	NUMEROUS		OCCASIONAL		NUMEROUS	FEW	
SMALL EPITHELIAL	RARE	FEW					FEW			FREQUENT		RARE		
R.B.C.	FEW	NUMEROUS	FEW	FEW		1/HPF	1/HPF	FEW	FEW	FREQUENT	FEW	FEW	FEW	FEW
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>
BLOOD. Hb.	12.5	12.5	10.0	10.7	10.4	10.3	10.2	11.4	11.2	10.3	10.7	10.5	7.4	11.5
HAEMOCRIT	43.0	39.0	30.0	35.0	32.0	32.0	33.5	35.0	33.5	33.0	34	33.0	34.0	34.0
W.B.C.	6,600	8,050	8,250	8,700	6,450	6,000	6,650	6,000	6,700	8,450	7,200	7,550	9,200	6,250
NEUTROPHILS	15	5	24	3	23	37	30	27	25	21	27	33	42	20
BANDS			2			4		4	4	4	1	1		
EOSINOPHILS	5	3	4		1	5	3	10	2	10	5	5	3	9
BASOPHILS	1	1	1	1		1	1	3	3	1				
LYMPHOCYTES	79	91	69	96	73	53	65	59	66	63	67	61	54	71
MONOCYTES					1	1	1			1	1	1	1	
DIRECT BLOOD	-	-	-	-	-	-	N.T.			BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION				
CULTURE BLOOD	-	-	-	-	-	-								
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CULTURE URINE	-	-	-	-	-	-	-	-	-	N.T.	-	-	-	-

**KEY**  
 N.T. NOT TAKEN  
 HPF HIGH POWER FIELD  
 SM. SMALL  
 LG. LARGE  
 EPITH EPITHELIAL

RARE FEW OCCASIONAL  
 FREQUENT NUMEROUS

P.I.D.	-1	1	3	8	10	11	12	13	15	17	20	22	24	27	29	31			
DATE	19/10	21/10	23/10	28/10	30/10	31/10	1/11	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11			
URINE. S.G.	1.017	1.001	1.027	1.005	1.006	N.T.	N.T.	1.007	1.006	1.011	1.010	1.010	1.014	1.007	1.006	1.005			
pH	7.0	7.6	8.0	8.0	6.5	8.0	8.0	7.5	7.5	7.5	7.0	8.0	8.0	8.0	7.0	7.5			
PROTEIN mg/100 ml	-	-	-	-	-	100++	TRACE	-	-	-	-	-	-	-	-	-			
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
OCCULT BLOOD	-	-	MODERATE	-	-	LARGE	MODERATE	-	-	-	-	-	-	-	-	-			
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
AMOUNT CENTRIFUGED	7.5 ml	3 ml	10 ml	3.5 ml	6 ml	N.T.	N.T.	10 ml	10 ml	5.0 ml	10 ml	10 ml	10 ml	10 ml	10 ml	9.1 ml			
AMOUNT DEPOSIT	0.1 ml	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
CASTS. HYALINE	RARE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
FINELY GRANULAR	FEW	FREQUENT	-	-	-	-	-	-	RARE	-	-	-	-	-	-	-			
COARSELY GRANULAR	-	-	-	-	FREQUENT	-	-	-	-	FEW	RARE	OCCASIONAL	OCCASIONAL	-	OCCASIONAL	-			
WAXY	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
CELLULAR	OCCASIONAL RED CELL CASTS	RED CELL CASTS	-	-	LARGE & SMALL EPITH CASTS	-	-	LARGE & SMALL EPITH CASTS	-	LEUCO. EPITH CAST. RED CELL CASTS	-	LARGE EPITH. CAST	LARGE EPITH. CASTS	-	LEUCO. CASTS	RARE FEW OCCASIONAL			
CELLS. LEUCOCYTES	FEW	-	-	1/HPF	-	-	-	-	-	FREQUENT	-	OCCASIONAL	-	-	OCCASIONAL	-			
LARGE EPITHELIAL	FEW	OCCASIONAL	-	NUMEROUS	NUMEROUS	-	-	FREQUENT	NUMEROUS	FREQUENT	OCCASIONAL	NUMEROUS	FREQUENT	OCCASIONAL	FREQUENT	OCCASIONAL			
SMALL EPITHELIAL	10/HPF	FEW	-	-	NUMEROUS	-	-	-	-	NUMEROUS	-	NUMEROUS	FREQUENT	-	FREQUENT	-			
R.B.C.	FEW	NUMEROUS	-	-	-	-	-	-	FEW	OCCASIONAL	-	NUMEROUS	FREQUENT	FEW	FREQUENT	-			
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	-	-	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW SMALL TRIPLE PO <sub>4</sub>	FEW SMALL TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW LARGE TRIPLE PO <sub>4</sub>			
BLOOD. Hb.	13.5	12.6	10.8	11.3	11.0	-	-	10.6	10.2	11.2	11.8	10.2	11.0	10.5	10.0	11.4			
HAEMOCRIT	42.5	38.0	36.5	36.0	33.0	-	-	32.0	33.0	32.0	34.0	30.0	32.5	33.0	33.0	35.0			
W.B.C.	8,400	7,100	6,550	7,850	9,100	-	-	8,500	4,750	6,350	8,200	9,000	7,200	7,100	7,600	5,900			
NEUTROPHILS	20	4	30	8	33	-	-	25	25	30	30	30	40	25	28	20			
BANDS	-	-	-	2	-	-	-	-	-	-	-	2	4	-	-	2			
EOSINOPHILS	2	3	7	2	2	-	-	4	4	5	3	8	6	3	3	10			
BASOPHILS	1	1	1	1	1	-	-	1	1	1	1	1	1	1	1	1			
LYMPHOCYTES	77	92	62	88	64	-	-	70	70	65	67	55	53	70	67	67			
MONOCYTES	-	-	-	-	-	-	-	1	1	-	-	4	1	2	-	1			
TITRE. TO L. POMONA	-	-	-	-	-	N.T.	N.T.	1/10,000	1/10,000	N.T.	N.T.	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000			
DIRECT BLOOD	-	-	-	-	-	-	-	-	BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION								-	-	-
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+			
CULTURE URINE	-	-	-	-	-	-	-	-	-	-	-	N.T.	-	-	-	-			



388 LAMB

P.I.D.	-1	1	3	8	10	11	12	13	15	17	20	22	24	27	29	31				
DATE	13/10	21/10	23/10	28/10	30/10	31/10	1/11	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11				
URINE. S.G.	N.T.	1.052	1.052	1.055	NO SAMPLE	N.T.	NO SAMPLE	1.024	1.048	1.041	1.046	1.055	1.050	1.050	1.053	1.047				
pH	6.0	6.5	6.0	5.5		6.0		8.5+	8.5+	8.5	7.5	8.5	9.0	9.0	8.0	9.0				
PROTEIN mg/100 ml	-	30+	30-	30+		30+		-	30+	30+	TRACE	30+	30+	100++	TRACE	30+				
GLUCOSE	-	-	-	-		-		-	-	-	-	-	-	-	-	-				
OCCULT BLOOD	-	TRACE	-	SMALL		-		-	-	-	-	-	-	-	-	-				
KETONE BODIES	-	-	-	-		-		-	-	-	-	-	-	-	-	-				
BILE PIGMENTS	-	-	-	-		-		-	-	-	-	-	-	-	-	-				
AMOUNT CENTRIFUGED	INSUP. SAMPLE	5.2 ml	6.0 ml	4 ml		N.T.		INSUP. SAMPLE	9.5 ml	4.0 ml	10 ml	10 ml	7.5 ml	3.5 ml	3.3 ml	2.4 ml				
AMOUNT DEPOSIT	-	0.7 ml	-	0.3 ml		-		-	0.3 ml	0.1 ml	0.3 ml	0.2 ml	0.2 ml	0.2 ml	0.2 ml	-				
CASTS. HYALINE																				
FINELY GRANULAR		OCCASIONAL		OCCASIONAL				NUMEROUS	FREQUENT	FREQUENT	NUMEROUS	FREQUENT				FREQUENT				
COARSELY GRANULAR		OCCASIONAL						OCCASIONAL				FREQUENT								
WAXY														FEW	NUMEROUS					
CELLULAR		NUMER. RED CELL CASTS	RED CELL CAST	RED CELL CASTS				LG. EPITH CAST	LG. EPITH CAST 3/HPF			LG. EPITH CAST								
			FEW	SM. EPITH CAST																
CELLS. LEUCOCYTES																				
LARGE EPITHELIAL			OCCASIONAL	FREQUENT				OCCASIONAL	OCCASIONAL	FREQUENT	FREQUENT	OCCASIONAL	NUMEROUS	FEW	FREQUENT	NUMEROUS				
SMALL EPITHELIAL			FEW	FREQUENT				OCCASIONAL			FEW	OCCASIONAL	FREQUENT							
R.B.C.		NUMEROUS	FEW	NUMEROUS									FEW	NUMEROUS						
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>				TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>				
BLOOD. Hb.	13.0	11.6	11.1	11.2	12.6			11.0	12.2	12.2	11.2	12.2	11.8	11.9	12.8	11.5				
HAEMOCRIT	43.0	38.0	35.0	35.0	35.0			35.0	35.0	33.0	35.0	32.0	34.0	33.0	34.0	35.0				
W.B.C.	7,900	6,650	6,000	8,500	5,600			8,100	8,350	8,300	9,200	9,950	9,150	8,500	15,000	6,750				
NEUTROPHILS	21	12	16	21	23			27	21	12	19	23	10	14	25	25				
BANDS	1	1	2					2							1					
EOSINOPHILS	1	2		5					2	3	2		1	4		1				
BASOPHILS				1	1			1	2		2		2							
LYMPHOCYTES	77	85	82	73	76			70	75	85	76	76	87	82	74	72				
MONOCYTES										1	1	1	1			2				
TITRE TO L. POMONA	-	-	N.T.	-	1/100	N.T.	N.T.	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000				
DIRECT BLOOD	-	-	-	-	-	-	-	-	N.T.	BLOOD SAMPLES NOT TAKEN FOR CULTURE OR DIRECT EXAMINATION							-	-	-	-
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
DIRECT URINE	-	-	-	-	N.T.	N.T.	N.T.	-	-	-	-	-	-	-	-	-	-	-		
CULTURE URINE	-	-	-	N.T.	N.T.	N.T.	N.T.	-	-	-	-	N.T.	-	-	-	-	-	-		

KEY  
 N.T. NOT TESTED  
 HPP HIGH POWER FIELD  
 SM. SMALL  
 LG. LARGE  
 EPITH. EPITHELIAL  
 RARE  
 FEW  
 OCCASIONAL  
 FREQUENT  
 NUMEROUS

389 LAMB

P.I.D.	1	3	8	10	11	12	13	15	17	20	22	24	27	29	31				
DATE	19/10	21/10	23/10	26/10	30/10	31/10	1/11	2/11	4/11	6/11	9/11	12/11	13/11	16/11	16/11	20/11			
URINE. S.G.	1.048	1.020	1.030	1.019	1.034	N.T.	N.T.	1.024	1.027	1.025	1.015	1.011	1.029	1.036	1.031	1.007			
pH	6.0	6.0	8.0	5.5	7.0	8.0	8.0	7.0	8.5	7.5	7.5	7.5	8.5	7.5	6.0	6.5			
PROTEIN mg/100 ml	-	TRACE	-	30+	-	-	TRACE	-	-	-	-	-	-	TRACE	-	-			
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
OCULT BLOOD	-	-	-	SMALL	-	-	-	-	-	-	-	-	-	-	-	-			
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
AMOUNT CENTRIFUGED	1.6 ml	3.3 ml	INSUF. SAMPLE	0.5 ml	3.8 ml	N.T.	N.T.	4.5 ml	0.7 ml	4.5 ml	2.6 ml	10 ml	10 ml	10 ml	10 ml	9 ml			
AMOUNT DEPOSIT	0.05 ml	-	-	-	-	-	-	-	0.05 ml	-	-	-	0.2 ml	0.1 ml	-	-			
CASTS. HYALINE																			
FINELY GRANULAR	RARE	OCCASIONAL	FEW		OCCASIONAL				FREQUENT							FREQUENT			
COARSELY GRANULAR		FEW						NUMEROUS	OCCASIONAL			NUMEROUS	RARE						
WAXY																			
CELLULAR		WHITE CELL CASTS	LG. EPITH CASTS		SM. EPITH CAST		LG. EPITH CASTS	LG. EPITH & RED CELL CASTS	LG. EPITH CAST		SM. EPITH CAST		SM EPITH CAST	LG. EPITH & LEUCO. CASTS	LEUCOCYTE CASTS				
CELLS. LEUCOCYTES	FEW	8/HPF	3/HPF						FEW				FEW	NUMEROUS	FREQUENT				
LARGE EPITHELIAL	FREQUENT	NUMEROUS	FREQUENT	FREQUENT	FREQUENT			FREQUENT	NUMEROUS	OCCASIONAL	FREQUENT	VERY NUMER.	OCCASIONAL	NUMEROUS	NUMEROUS				
SMALL EPITHELIAL	FEW			OCCASIONAL	FREQUENT				OCCASIONAL		FEW								
R.B.C.	NUMEROUS			FREQUENT				1/HPF	FEW		FEW								
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>		FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW LG. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	LG. TRIPLE PO <sub>4</sub>	MANY TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>				
BLOOD. Hb.	12.0	12.2	9.8	9.4	9.2		9.3	9.4	10.0	9.6	11.4	10.0	13.0	10.7	10.9				
HAEMOCRIT	38.0	34.0	32	31.0	29.0		29.0	32.0	31.0	32.0	31.0	32.0	32.0	32.5	32.0				
W.B.C.	8,400	8,050	6,250	10,350	9,750		8,350	8,950	9,150	10,900	8,300	9,250	10,150	13,700	8,750				
NEUTROPHILS	30	20	20	25	31		29	26	31	27	46	29	23	27	19				
BANDS		2																	
EOSINOPHILS	1	7	3	6			1				1	1	2	2	1	1			
BASOPHILS			1				1					2							
LYMPHOCYTES	67	70	76	69	62		69	74	67	72	59	67	75	72	76				
MONOCYTES	2	1			1				2						4				
TITR TO L. POMONA	-	-	-	-	-	H.T.	N.T.	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000				
DIRECT BLOOD	-	-	-	-	-	-	-	N.T.	BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION										
CULTURE BLOOD	-	+	+	-	-	-	-	-	-	-	+	+	+	+	+	+			
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-			
CULTURE URINE	-	-	-	N.T.	-	-	-	-	-	-	-	N.T.	+	-	-	-			

KEY  
 N.T. NOT TESTED  
 HPF HIGH POWER FIELD  
 SM. SMALL  
 LG. LARGE  
 NUMER NUMEROUS  
 EPITH EPITHELIAL  
 LEUCO LEUCOCYTES

RARE FEW OCCASIONAL  
 FREQUENT NUMEROUS

390 LAMB

P.I.D.	-1	1	3	3	10	11	12	13	15	17	20	22	24	27	29	31		
DATE	19/10	21/10	23/10	25/10	30/10	31/10	1/11	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11		
URINE. S.G.	1.050	1.060	1.045	1.045	1.046	N.T.	N.T.	1.040	1.047	1.052	N.T.	1.049	1.053	1.052	1.052	1.049		
pH	6.5	6.5	8.0	7.0	8.5	8.0	8.0	8.0	8.5	8.5	8.5	8.5+	9.0	9.0	8.0	7.5		
PROTEIN mg/100 ml	30+	30+	TRACE	-	30+	-	TRACE	-	30+	TRACE	-	30+	30+	TRACE	TRACE	TRACE		
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
OCCULT BLOOD	-	-	SMALL	-	-	-	-	-	-	-	-	-	-	-	-	-		
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
AMOUNT CENTRIFUGED	9.0 ml	2.4 ml	INSUF. SAMPLE	10 ml	4.0 ml	N.T.	N.T.	10 ml	5.5 ml	1.7 ml	10 ml	7.2 ml	10 ml	3.0 ml	10 ml	10 ml		
AMOUNT DEPOSIT	0.3 ml	0.1 ml		0.3 ml	0.1 ml			0.2 ml	0.2 ml	0.1 ml	0.4 ml	0.3 ml	0.3 ml	0.2 ml	0.3 ml	0.2 ml		
CASTS. HYALINE				FEW														
FINELY GRANULAR			OCCASIONAL															
COARSELY GRANULAR			OCCASIONAL										OCCASIONAL			FREQUENT		
WATY																		
CELLULAR								LG. EPITH CASTS		LG. EPITH CAST	LG. EPITH CAST	LG. EPITH CAST		LG. EPITH CASTS	LG. EPITH & LEUCO CASTS	LEUCO CASTS		
CELLS. LEUCOCYTES	NUMEROUS																	
LARGE EPITHELIAL		FREQUENT		FREQUENT	OCCASIONAL		OCCASIONAL	OCCASIONAL	NUMEROUS	OCCASIONAL	NUMEROUS	FREQUENT	FEW	OCCASIONAL	FREQUENT	OCCASIONAL		
SMALL EPITHELIAL	3/HPF	FEW		OCCASIONAL			FEW		FEW		FEW				FEW			
R.B.C.	NUMEROUS								FEW		FEW							
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	MULTER. TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>		FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>		
BLOOD. Hb.	11.8	10.8	9.6	9.7	8.2		9.4	9.2	9.8	9.7	10.8	10.8	9.8	10.5	10.8			
HAEMOCRIT	38.1	34.0	31.5	32.5	26.0		28.0	29.0	32.0	31.0	31.0	30.0	30.0	28.5	32.0			
W.B.C.	8,000	5,700	9,100	8,050	8,900		7,300	7,950	8,350	8,450	7,150	7,800	8,500	7,000	6,150			
NEUTROPHILS	12	11	7	8	14		13	13	13	14	25	11	17	25	24			
BANDS																		
EOSINOPHILS	2	4	5	8	5		3	6	3	2			1	1				
BASOPHILS		3		1	1		1	2										
LYMPHOCYTES	85	82	88	84	80		83	78	83	82	73	87	80	74	75			
MONOCYTES	1						1	1	1	2	2	1	2		1			
TITRE TO L. PONOHA	-	-	-	-	-	N.T.	N.T.	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000		
DIRECT BLOOD	-	-	-	-	-	-	-	N.T.	BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION								-	-
CULTURE BLOOD	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+		
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
CULTURE URINE	-	-	-	N.T.	-	-	-	-	-	-	N.T.	-	-	-	-	-		

KEY  
 N.T. NOT TESTED  
 HPF HIGH POWER FIELD  
 SM. SMALL  
 LG. LARGE  
 NUMER. NUMEROUS  
 EPITH. EPITHELIAL  
 LEUCO. LEUCOCYTES

391 LAB

P.I.D.	-1	1	3	8	10	11	12	13	15	17	20	22	24	27	29	31	
DATE	19/10	21/10	23/10	28/10	30/10	31/10	1/11	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11	
URINE. S.G.	1.050	1.060	1.007	1.050	1.042	N.T.	N.T.	1.036	1.034	1.044	1.042	1.035	1.015	1.040	1.045	1.040	
pH 6.0	6.0	7.0	7.0	8.0	8.0	8.0	8.0	8.0	8.5+	8.5	8.0	9.0	8.0	8.5	8.0	8.0	
PROTEIN mg/100 ml	-	30+	-	30+	TRACE	30+	TRACE	30+	30+	30+	-	100++	-	TRACE	TRACE	TRACE	
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
AMOUNT CENTRIFUGED	2.3 ml	3.3 ml	3.5 ml	3 ml	2.3 ml	N.T.	N.T.	4 ml	5 ml	6.0 ml	10 ml	1.5 ml	10 ml	10 ml	6.4 ml	10 ml	
AMOUNT DEPOSIT	-	0.2 ml	-	0.1 ml	0.05 ml	-	-	0.1 ml	0.1 ml	0.05 ml	-	-	-	0.2 ml	0.2 ml	0.1 ml	
CASTS. HYALINE												RARE					
FINELY GRANULAR	FEW				OCCASIONAL					OCCASIONAL		FREQUENT		FEW			
COARSELY GRANULAR	FREQUENT				OCCASIONAL			RARE								FREQUENT	
WAXY																	
CELLULAR		WHITE CELL CASTS	RED CELL CASTS									LG. EPITH CAST					
CELLS. LEUCOCYTES	FEW	FREQUENT	OCCASIONAL					FEW	OCCASIONAL			FEW	NUMEROUS	NUMEROUS			
LARGE EPITHELIAL	OCCASIONAL	NUMEROUS		FREQUENT	FEW		FREQUENT	FREQUENT	OCCASIONAL	FREQUENT		NUMEROUS	NUMEROUS	NUMEROUS	FREQUENT	FREQUENT	
SMALL EPITHELIAL		OCCASIONAL			OCCASIONAL		FEW	OCCASIONAL	FEW	OCCASIONAL		NUMEROUS	NUMEROUS	FEW			
R.B.C.	OCCASIONAL		FREQUENT	FEW	FEW							FREQUENT	FEW				
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	LG. TRIPLE PO <sub>4</sub>		NUMER. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	MANY TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	
BLOOD. Hb.	13.1	12.2	9.3	9.8	10.4		11.3	10.8	9.4	12.2	10.8	10.2	9.8	10.0	11.2		
HAEMOCRIT	41.6	37.0	35	33.0	31.0		34.0	32.5	31.5	33.0	33.0	31.0	31.0	31.5	31.5		
W.B.C.	8,200	8,250	7,600	9,250	7,500		N.T.	N.T.	8,450	9,400	9,350	9,150	10,250	9,400	12,800		
NEUTROPHILS	17	13	35	19	14		16	12	9	19	9	23	23	31	27		
BAKES									1								
EOSINOPHILS	1	4	8	1	2		6	2	3	8	1	2	1	2			
BASOPHILS		2	1	3			1	3		1							
LYMPHOCYTES	82	80	56	75	84		78	82	87	70	87	74	74	67	71		
MONOCYTES				2						2	3	1	2		2		
TITRE TO L. POMONA	-	N.T.	-	-	-	N.T.	N.T.	1/1,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	1/10,000	
DIRECT BLOOD	-	-	-	-	-	-	-	-	N.T.	BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION							-
CULTURE BLOOD	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
CULTURE URINE	-	-	-	N.T.	-	-	-	-	-	-	-	N.T.	-	-	-	-	

KEY  
 N.T. NOT TAKEN  
 HPP HIGH POWER FIE  
 SM. SMALL  
 LG. LARGE  
 NUMER. NUMEROUS

RARE FEW OCCASIONAL  
 FREQUENT NUMEROUS

392 FWE (CONTROL)

P.I.D.	-1	1	3	8	10	13	15	17	20	22	24	27	29	31
DATE	19/10	21/10	23/10	28/10	30/10	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11
URINE S.G.	1.050	1.050	1.046	1.021	1.029	1.028	1.014	1.020	1.018	1.031	1.022	1.018	1.005	1.012
pH	8.0	8.0	8.5	8.0	8.0	8.5+	8.0+	8.0	8.0	8.0	8.5	8.5	6.5	7.5
PROTEIN mg/100 ml	-	TRACE	TRACE	-	-	-	-	-	-	TRACE	-	-	-	-
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMOUNT CENTRIFUGED	10 ml	10 ml	1.2 ml	10 ml	6.9 ml	0.7 ml	10 ml	8.0 ml	10 ml	10 ml	9 ml	9.5 ml	10 ml	10 ml
AMOUNT DEPOSIT	NEGLECTIBLE	0.2 ml	-	-	-	-	-	-	0.1 ml	-	-	0.1 ml	-	-
CASTS - HYALINE	RARE													
FINELY GRANULAR			OCCASIONAL					OCCASIONAL		OCCASIONAL		FEW		
COARSELY GRANULAR		OCCASIONAL												OCCASIONAL
WAXY														
CELLULAR														
CELLS - LEUCOCYTES	FREQUENT	FEW						FREQUENT						
LARGE EPITHELIAL	FREQUENT		NUMEROUS	FREQUENT	OCCASIONAL		FEW	NUMEROUS	OCCASIONAL					
SMALL EPITHELIAL	FEW	OCCASIONAL		OCCASIONAL	RARE	FEW		FEW	FEW					
R.B.C.	FEW	OCCASIONAL	RARE	FEW	FEW			FEW						
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>
BLOOD Hb.	13.4	13.0	13.8	13.4	12.4	11.4	12.6	15.2	13.8	13.4	13.4	11.9	11.9	9.8
HAEMOCRIT	43.0	41.0	40	39.0	36.5	34.0	37.0	36.5	35.0	34.0	37.0	35.0	35.0	32.0
W.B.C.	11,100	9,650	11,900	8,650	12,150	11,750	13,400	11,400	10,650	10,300	9,250	12,050	12,800	7,650
NEUTROPHILS	39	52	34	10	51	34	48	49	47	47	55	48	51	34
BANDS					5	4	3		4		4			
EOSINOPHILS	4	4	4			1		2	2	3	3	2	3	3
BASOPHILS				1	1	1	2							
LYMPHOCYTES	57	44	62	88	43	60	44	47	47	50	40	49	46	60
MONOCYTES				1			3	2		1	2	1		3
TITRE TO L. POMONA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT BLOOD	-	-	-	-	-	-	-	BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION						
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CULTURE URINE	-	-	-	-	-	-	-	-	-	N.T.	-	-	-	-

KEY  
 N.T. NOT TAKEN  
 HPP HIGH POWER FIELD  
 SM. SMALL  
 LG. LARGE  
 LEUCO. LEUCOCYTES

RARE FREW OCCASIONAL  
 FREQUENT NUMEROUS

393 EME (CONTROL)

P.I.D.	-1	1	3	8	10	13	15	17	20	22	24	27	29	31
DATE	19/10	21/10	23/10	26/10	30/10	2/11	4/11	6/11	3/11	11/11	13/11	16/11	18/11	20/11
URINE S.G.	1.038	1.044	1.045	1.022	1.012	1.011	1.025	1.044	1.026	1.015	1.084	1.012	1.037	1.030
pH	7.0	8.0	8.0	8.0	6.5	2.0	8.0+	9.0	7.5	9.0	9.0	7.5	8.5	8.0
PROTEIN mg/100 ml	-	-	TRACE	-	-	-	-	30+	-	-	-	-	TRACE	-
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMOUNT CENTRIFUGED	10 ml	5 ml	INSUP. SAMPLE	10 ml	3.5 ml	0.4 ml	10 ml	0.4 ml	10 ml	INSUP. SAMPLE	INSUP. SAMPLE	10 ml	4.5 ml	5 ml
AMOUNT DEPOSIT	-	0.05 ml	-	-	-	-	-	-	-	-	-	-	-	-
CASTS - HYALINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FINELY GRANULAR	-	FREQUENT	-	FEM SM. CASTS	OCCASIONAL	-	-	-	-	-	-	-	-	-
COARSELY GRANULAR	FEW	-	-	-	-	RARE	-	-	-	-	-	-	OCCASIONAL	-
WAXY	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CELLULAR	-	LG. EPITH CAST.	-	-	-	-	-	-	-	-	-	-	-	-
CELLS - LEUCOCYTES	-	-	-	-	-	-	FEW	-	-	-	-	-	-	-
LARGE EPITHELIAL	OCCASIONAL	FREQUENT	-	RARE	OCCASIONAL	FEW	RARE	OCCASIONAL	-	-	OCCASIONAL	FEW	OCCASIONAL	-
SMALL EPITHELIAL	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R.B.C.	FEW	FEW	OCCASIONAL	-	-	-	-	-	-	-	-	-	-	-
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	VERY FEW TRIPLE PO <sub>4</sub>	FEW LG. TRIPLE PO <sub>4</sub>	-	FEW LG. TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW LG. TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>
BLOOD Hb.	13.7	15.0	13.5	13.4	11.2	13.8	13.0	13.2	12.5	15.7	13.6	12.1	12.0	10.7
HAEMOTOCRIT	44.9	41.0	42	40.0	36.0	38.5	37.0	38.0	34.0	39.0	36.0	34.0	35.5	34.0
W.B.C.	9,300	7,450	7,600	7,200	7,600	5,800	6,750	7,000	6,250	8,250	7,000	6,350	7,100	6,250
NEUTROPHILS	36	46	34	29	31	25	21	17	32	27	16	22	22	32
BANDS	-	3	2	-	-	-	2	-	-	3	-	-	-	-
EOSINOPHILS	2	7	9	7	4	4	4	4	7	6	6	6	1	4
BASOPHILS	1	2	1	1	1	1	1	1	1	1	1	2	1	1
LYMPHOCYTES	59	41	54	63	65	71	71	76	61	62	77	70	74	62
MONOCYTES	-	-	-	-	-	-	1	3	-	2	-	-	-	1
TITRE TO L. POMONA	-	-	-	-	-	-	-	-	-	-	-	1/100	-	-
DIRECT BLOOD	-	-	-	-	-	-	N.T.	-	BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION					
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT URINE	-	-	-	-	-	-	N.T.	-	-	-	-	-	-	-
CULTURE URINE	-	-	-	-	-	-	-	-	-	N.T.	-	-	-	-

**KEY**  
 N.T. NOT TAKEN  
 HPF HIGH POWER FIELD  
 SM. SMALL  
 LG. LARGE

RARE FEW OCCASIONAL  
 FREQUENT NUMEROUS

394 EWE (CONTROL)

P.I.D.	-1	1	3	8	10	13	15	17	20	22	24	27	29	31
DATE	19/10	21/10	23/10	28/10	30/10	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11
URINE. S.G.	1.050	1.034	1.030	1.020	1.031	1.039	1.026	1.035	1.021	1.032	1.040	1.033	1.042	1.014
pH	8.0	9.0	7.5	8.0	8.5	8.0	8.5	8.5	8.0	8.5	9.0	9.0	9.0	7.5
PROTEIN mg/100 ml	-	-	-	-	-	-	-	30+	-	TRACE	TRACE	TRACE	-	-
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMOUNT CENTRIFUGED	6 ml	2.4 ml	8.1 ml	3 ml	0.5 ml	10 ml	3.0 ml	6.5 ml	10 ml	10 ml	INSUF. SAMPLE	5.0 ml	INSUF. SAMPLE	10 ml
AMOUNT DEPOSIT	0.1 ml	0.05 ml	-	-	-	-	-	-	-	-	-	-	-	-
CASTS. RYALINE														
FINELY GRANULAR		FEW			FREQUENT	OCCASIONAL		OCCASIONAL	FREQUENT	OCCASIONAL				
COARSELY GRANULAR			OCCASIONAL			RARE						OCCASIONAL		FREQUENT
WAXY														
CELLULAR			RED CELL CASTS											LG. EPITH CAST
CELLS. LEUCOCYTES						2/HPF					OCCASIONAL			
LARGE EPITHELIAL		FREQUENT	NUMEROUS	OCCASIONAL	FREQUENT	NUMEROUS	FEW	FREQUENT	FREQUENT				VERY NUMER.	FREQUENT
SMALL EPITHELIAL		OCCASIONAL	FREQUENT			FEW		FEW	FEW				FREQUENT	
R.B.C.	OCCASIONAL				FEW		FEW		FEW		FEW			
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	VERY FEW TRIPLE PO <sub>4</sub>	LARGE TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>		TRIPLE PO <sub>4</sub>
BLOOD. Hb.	14.8	11.8	12.3	13.2	14.8	13.2	14.8	12.0	15.0	12.1	16.0	12.4	12.2	11.1
HAEMOTOCRIT	44.8	42.5	41	38.0	35.0	36.0	38.0	38.0	39.0	37.0	37.0	36.0	38.0	33.0
W.B.C.	7,000	7,600	9,700	11,600	8,550	8,100	8,000	7,800	7,850	8,600	8,800	6,650	7,200	7,750
NEUTROPHILS	9	24	33	34	26	23	37	26	22	40	15	11	26	29
BANDS							2						5	3
EOSINOPHILS		3	2	9	10	8	4	10	11	5	6	9	4	3
BASOPHILS					2	2		2				2		
LYMPHOCYTES	90	73	59	57	62	67	56	61	64	54	78	78	65	65
MONOCYTES			1				1	1	3					1
TITRE TO L. POMONA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT BLOOD	-	-	-	-	-	-	N.T.	BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION						
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT URINE	-	-	-	-	-	-	N.T.	-	-	-	-	-	-	-
CULTURE URINE	-	-	-	-	-	-	-	-	-	N.T.	-	-	-	-

**KEY**  
 N.T. NOT TAKEN  
 HPF HIGH POWER FIELD  
 SM. SMALL  
 LG. LARGE  
 NUMER. NUMEROUS

RARE FEW OCCASIONAL  
 FREQUENT NUMEROUS

295 EWE (CONTROL)

P.I.D.	-1	1	3	8	10	13	15	17	20	22	24	27	29	31
DATE	19/10	21/10	23/10	25/10	30/10	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11
URINE S.G.	1.050	1.053	1.047	1.000	1.036	1.025	1.050	1.031	1.026	1.032	1.053	1.020	1.040	1.010
pH	7.5	8.0	8.5	6.5	8.0	8.0	9.0	8.5	8.0	8.0	9.0	9.0	8.0	7.0
PROTEIN mg/100 ml	-	30+	300+++	-	30+	-	30+	TRACE	TRACE	TRACE	30+	-	TRACE	-
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BL. & PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMOUNT CENTRIFUGED	2.4 ml	6.5 ml	10 ml	10 ml	6.2 ml	5 ml	0.7 ml	3.0 ml	10 ml	10 ml	2 ml	4.0 ml	9.5 ml	9 ml
AMOUNT DEPOSIT	0.1 ml	0.1 ml	0.1	-	-	-	-	-	0.1 ml	-	0.1 ml	0.1 ml	-	-
CASTS. HYALINE														
FINELY GRANULAR	OCCASIONAL		FEW							FREQUENT		FREQUENT		OCCASIONAL
COARSELY GRANULAR					RARE									OCCASIONAL
WAXY														
CELLULAR			LG. EPITH CAST			LG. EPITH & LEUCO. CASTS			SM. & LG. EPITH CASTS	LG. EPITH CAST	SM. EPITH CASTS		LG. EPITH CAST	LG. EPITH & LEUCO. CASTS
CELLS. LEUCOCYTES	FEW					3/HPF		NUMEROUS			OCCASIONAL		FEW	OCCASIONAL
LARGE EPITHELIAL	FREQUENT	OCCASIONAL	FREQUENT	FEW		FREQUENT		NUMEROUS	NUMEROUS	FREQUENT	NUMEROUS	FREQUENT	FEW	NUMEROUS
SMALL EPITHELIAL	FEW	FREQUENT						FREQUENT	NUMEROUS	OCCASIONAL	NUMEROUS	NUMEROUS		FEW
R.B.C.			FEW		FEW				FEW SM. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	OCCASIONAL	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	SMALL TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>
BLOOD. Hb.	12.2	12.6	11.6	12.2	11.2	11.6	11.6	11.8	13.6	13.2	11.0	12.7	12.7	10.2
HAEMOTOCRIT	40.8	40.0	40	39.0	33.5	37.5	37.0	35.0	36.0	34.0	36.0	35.0	34.0	31.0
W.B.C.	8,500	9,450	7,550	10,700	7,350	7,650	6,450	6,350	7,700	8,550	9,800	10,900	8,500	5,100
NEUTROPHILS	21	19	31	21	16	29	27	27	26	25	27	29	33	16
BANDS							3	3			2			
EOSINOPHILS	7	17	8	10	5	3	6	6	6	4	6	2		14
BASOPHILS					1		1	1			1	1		
LYMPHOCYTES	72	64	61	69	78	68	62	62	67	69	64	68	67	66
MONOCYTES							1	1	1	2	2			4
TITRE TO L. POMONA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT BLOOD	-	-	-	-	-	-	-	-	BLOOD SAMPLES NOT TAKEN FOR CULTURE OR DIRECT EXAMINATION					
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CULTURE URINE	-	-	-	-	-	-	-	-	-	N.T.	-	-	-	-

KEY

N.T.	NOT TESTED
HPF	HIGH POWER FIELD
SM	SMALL
LG	LARGE
EPITH	EPITHELIAL
LEUCO	LEUCOCYTES

RARE	FEW	OCCASIONAL
FREQUENT	NUMEROUS	

396 LAMB (CONTROL)

P.I.D.	-1	1	3	8	10	13	15	17	20	22	24	27	29	31	
DATE	19/10	21/10	23/10	28/10	30/10	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11	
URINE. S.G.	1.030	1.038	1.047	1.046	1.039	1.022	1.046	1.047	1.037	1.036	1.047	1.013	1.046	1.050	
pH	8.0	8.0	8.5	6.5	8.0	8.0	9.0	8.5+	8.5	8.5+	9.0	8.5	9.0	8.5	
PROTEIN mg/100 ml	-	TRACE	100++	30+	TRACE	TRACE	30+	30+	30+	-	TRACE	-	TRACE	TRACE	
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
AMOUNT CENTRIFUGED	2 ml	10 ml	6.4 ml	1.8 ml	1.1 ml	4.3 ml	1.3 ml	3.8 ml	10 ml	5.3 ml	5 ml	10 ml	2.2 ml	2 ml	
AMOUNT DEPOSIT	0.05 ml	0.15 ml	0.1 ml	0.1 ml	-	0.1 ml	-	0.2 ml	-	0.1 ml	0.1 ml	0.1 ml	0.1 ml	-	
CASTS. HYALINE															
FINELY GRANULAR	OCCASIONAL			OCCASIONAL		FEW	OCCASIONAL	FEW	FEW	NUMEROUS		FEW	NUMEROUS	FREQUENT	
COARSELY GRANULAR				FEW		NUMEROUS		FEW		NUMEROUS	FREQUENT		NUMEROUS		
WAXY															
CELLULAR						LG. EPITH CAST		LG. EPITH CAST		LG. EPITH CAST	LG. EPITH & LEUCO. CASTS	LG. EPITH CASTS	LG. EPITH CASTS	LEUCO CAST	
<u>CELLS. LEUCOCYTES</u>		RARE				FEW		FEW		FEW		FEW			
LARGE EPITHELIAL	FEW	NUMEROUS	OCCASIONAL	OCCASIONAL	FREQUENT	FREQUENT	NUMEROUS	NUMEROUS	FREQUENT	NUMEROUS		VERY NUMER.	VERY NUMER.	FREQUENT	
SMALL EPITHELIAL					OCCASIONAL	NUMEROUS		OCCASIONAL	OCCASIONAL			FEW	OCCASIONAL	OCCASIONAL	
R.B.C.		FEW		FEW			FEW	FEW		FREQUENT		FEW	FEW	FEW	
CRYSTALS	NUMER. TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	SMALL TRIPLE PO <sub>4</sub>
BLOOD. Hb.	12.2	13.0	12.0	12.0	11.4	12.3	11.6	12.8	13.3	11.7	11.2	13.8	13.8	11.7	
HAEMOTOCRIT	42.0	39.0	37	37.5	35.0	37.0	36.0	37.0	39.0	37.0	35.0	37.0	34.5	33.5	
W.B.C.	15,300	16,550	18,200	15,050	14,400	9,550	11,300	14,500	12,700	12,900	12,600	10,050	11,500	7,250	
NEUTROPHILS	9	11	19	22	23	18	20	21	20	19	16	23	27	20	
BANDS							1								
EOSINOPHILS	1	1	5	4		1	1	1	1			2	9	1	
BASOPHILS												1			
LYMPHOCYTES	90	88	76	72	76	81	77	76	77	81	80	77	63	77	
MONOCYTES	1			2			1		2		1		1	2	
TITRE TO L. POMONA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
DIRECT BLOOD	-	-	-	-	-	-	-	-	BLOOD SAMPLES NOT TAKEN FOR CULTURE OR DIRECT EXAMINATION						
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
CULTURE URINE	-	-	-	-	-	-	-	-	-	N.T.	-	-	-	-	

KEY	
N.T.	NOT TAKEN
HPF	HIGH POWER FIELD
SM	SMALL
LG	LARGE
EPITH	EPITHELIAL
LEUCO	LEUCOCYTES
NUMER	NUMEROUS

297 LAMB (CONTROL)

P.I.D.	-1	1	3	8	10	13	15	17	20	22	24	27	29	31
DATE	19/10	21/10	23/10	28/10	30/10	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11
URINE. S.G.	1.034	1.034	1.034	1.014	1.011	1.028	1.021	1.009	1.016	1.042	1.011	1.032	1.032	1.039
pH	8.0	6.0	5.5	5.5	5.0	5.5	8.0	7.5	8.0	9.0	8.0	7.5	8.0	8.0
PROTEIN mg/100 ml	-	TRACE	-	-	-	TRACE	TRACE	-	TRACE	TRACE	-	TRACE	-	-
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMOUNT CENTRIFUGED	1.7 ml	2.1 ml	INSUF. SAMPLE	0.6 ml	7.7 ml	2.5 ml	5.0 ml	10 ml	10 ml	10 ml	5.8 ml	10 ml	4.6 ml	8.0 ml
AMOUNT DEPOSIT	0.1 ml	-	-	-	-	-	-	-	0.1 ml	0.2 ml	-	0.1 ml	-	-
CASTS. HYALINE														
FINELY GRANULAR		FEW	RARE			OCCASIONAL		OCCASIONAL	FEW	NUMEROUS				
COARSELY GRANULAR		OCCASIONAL							FREQUENT		FREQUENT	RARE	FREQUENT	FEW
WAXY														
CELLULAR						LG. EPITH CASTS	LG. EPITH CASTS		LG. EPITH CASTS		LEUCO. CAST		LG. EPITH CASTS	LG. EPITH CASTS
CELLS. LEUCOCYTES	FEW							FREQUENT			FREQUENT		OCCASIONAL	
LARGE EPITHELIAL	OCCASIONAL	FREQUENT		OCCASIONAL	FREQUENT	NUMEROUS	NUMEROUS	NUMEROUS	NUMEROUS	OCCASIONAL	NUMEROUS	FREQUENT	NUMEROUS	NUMEROUS
SMALL EPITHELIAL							OCCASIONAL	FEW	FEW	OCCASIONAL		OCCASIONAL		
R.B.C.	NUMEROUS					FEW	1/HPF							
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW SM. TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>
BLOOD. Hb.	14.0	13.0	12.3	9.4	10.8	11.6	10.4	9.9	12.3	10.8	11.4	12.2	12.8	9.8
HAEMOCRIT	40.3	39.0	37	35.5	33.5	34.0	29.0	32.0	33.5	32.0	32.5	33.0	32.5	32.5
W.B.C.	7,500	8,000	6,850	12,750	13,400	8,250	10,200	10,150	12,500	10,200	10,800	10,200	13,300	10,000
NEUTROPHILS	29	37	28	24	38	21	31	20	36	25	25	12	49	23
BANDS		1					2						3	3
EOSINOPHILS			2			1	2	1	1			3	2	1
BASOPHILS		1	1				1							
LYMPHOCYTES	71	60	69	76	62	76	64	77	63	75	74	85	46	73
MONOCYTES		1				2	1				1			
TITRE TO L. POMONA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT BLOOD	-	-	-	-	-	-	-	-	BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION					
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CULTURE URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-

KEY

N.T.	NOT TAKEN
HPF	HIGH POWER FIELD
SM	SMALL
LG	LARGE
EPITH	EPITHELIAL
LEUCO	LEUCOCYTES

RARE	FEW	OCCASIONAL
FREQUENT	NUMEROUS	

398 LAMB (CONTROL)

P.I.D.	-1	1	3	8	10	13	15	17	20	22	24	27	29	31	
DATE	19/10	21/10	23/10	28/10	30/10	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11	
URINE. S.G.	1.045	1.046	1.050	1.039	1.050	1.056	1.049	1.053	1.051	1.052	1.050	1.042	1.050	1.050	
pH	6.0	8.0	9.0	8.0	8.5	7.0	8.0+	8.5	8.5	9.0	9.0	9.0	8.0	8.5	
PROTEIN mg/100 ml	-	TRACE	-	-	TRACE	30+	30+	30+	30+	30+	TRACE	30+	30+	TRACE	
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
AMOUNT CENTRIFUGED	1.3 ml	1.2 ml	INSUF. SAMPLE	3 ml	2.1 ml	2.6 ml	9.5 ml	1.6 ml	2.4 ml	1.0 ml	10 ml	7.4 ml	5.4 ml	1.8 ml	
AMOUNT DEPOSIT	-	0.05 ml	-	-	0.2 ml	0.2 ml	0.1 ml	0.1 ml	-	0.3 ml	0.3 ml	0.3 ml	-	-	
CASTS. HIALINE															
FINELY GRANULAR		OCCASIONAL		OCCASIONAL	NUMEROUS			FEW		OCCASIONAL	NUMEROUS	FREQUENT		FREQUENT	
COARSELY GRANULAR				RARE			OCCASIONAL								
WAXY															
CELLULAR										LG. EPITH & LEUCO CASTS		LG. EPITH CAST			
CELLS. LEUCOCYTES					FEW	1/HPF	FEW			FREQUENT	OCCASIONAL			OCCASIONAL	
LARGE EPITHELIAL		OCCASIONAL	RARE	FREQUENT	OCCASIONAL	NUMEROUS	NUMEROUS	FREQUENT	FREQUENT	NUMEROUS	NUMEROUS	FREQUENT	FREQUENT	FREQUENT	
SMALL EPITHELIAL		FREQUENT		FEW	FREQUENT	OCCASIONAL	NUMEROUS		FEW		FREQUENT		FEW		
R.B.C.		FEW		FEW			FEW				FEW			FEW	
CRYSTALS	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	FEW LG. TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	SMALL TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>
BLOOD. Hb.	10.8	11.3	9.7	9.4	8.6	10.0	11.2	9.8	10.4	9.9	10.4	11.0	11.6	10.9	
HAEMATOCRIT	36.0	35.0	34	33.0	29.0	32.0	29.0	30.0	32.0	31.5	32.5	32.0	32.0	32.0	
W.B.C.	7,900	7,600	23,650	8,850	10,350	10,100	10,100	8,850	8,900	11,000	10,150	10,450	9,700	9,350	
NEUTROPHILS	6	6	8	28	22	23	31	15	19	19	21	14	20	15	
BAND													1		
EOSINOPHILS			2	4	3				1	2	3	1	1	5	
BASOPHILS		1										1	1		
LYMPHOCYTES	93	92	88	68	74	77	69	85	80	78	76	83	77	77	
MONOCYTES	1	1	2		1					1		1		3	
TITRE TO L. POMONA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
DIRECT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
CULTURE URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

**KEY**  
 N.T. NOT TAKEN  
 HPF HIGH POWER FIELD  
 SM SMALL  
 LG LARGE  
 EPITH EPITHELIAL  
 LEUCO LEUCOCYTES

RARE FEW OCCASIONAL  
 FREQUENT NUMEROUS

BLOOD SAMPLES NOT TAKEN FOR CULTURE OR DIRECT EXAMINATION

399 LAMB (CONTROL)

P.I.D.	-1	1	3	8	10	13	15	17	20	22	24	27	29	31
DATE	19/10	21/10	23/10	28/10	30/10	2/11	4/11	6/11	9/11	11/11	13/11	16/11	18/11	20/11
URINE S.G.	1.048	1.044	1.047	1.030	1.031	1.052	1.055	1.053	1.037	1.053	1.054	1.044	1.054	1.046
pH	7.5	7.5	9.0	8.0	8.5	8.5+	8.5+	8.5+	8.5	8.5	9.0	8.5	8.5	8.5
PROTEIN mg/100 ml	TRACE	30+	TRACE	TRACE	TRACE	100++	100++	100+++	TRACE	TRACE	TRACE	-	100++	TRACE
GLUCOSE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
OCCULT BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KETONE BODIES	-	-	-	-	-	-	-	-	-	-	-	-	-	-
BILE PIGMENTS	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AMOUNT CENTRIFUGED	10 ml	5.8 ml	2 ml	4.5 ml	5.3 ml	4.7 ml	9.0 ml	4.0 ml	10 ml	10 ml	10 ml	10 ml	9.0 ml	1.2 ml
AMOUNT DEPOSIT	0.3 ml	0.1 ml	0.1 ml	0.1 ml	-	0.2 ml	0.3 ml	0.2 ml	0.1 ml	0.4 ml	0.3 ml	0.2 ml	0.3 ml	0.1 ml
CASTS - HYALINE														
FINELY GRANULAR		FREQUENT					NUMEROUS						NUMEROUS	FEW
COARSELY GRANULAR			NUMEROUS			FREQUENT								
WAXY														
CELLULAR		LG. EPITH CAST	LG. EPITH CASTS	RED CELL CAST	LG. EPITH CAST				LG. & SM. EPITH CASTS	LG. EPITH CAST	LEUCO CAST		LEUCO CASTS	
CELLS - LEUCOCYTES														
LARGE EPITHELIAL	FREQUENT	OCCASIONAL	FREQUENT	NUMEROUS	FEW	FREQUENT	NUMEROUS	FREQUENT	NUMEROUS	NUMEROUS	FREQUENT	RARE	FREQUENT	FEW
SMALL EPITHELIAL	FEW				FREQUENT	FEW	OCCASIONAL			FEW		OCCASIONAL		
R.B.C.		FREQUENT	FREQUENT		FEW			FEW		FEW				
CRISTALS	NUMER. TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	NUMER. TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>	FEW LG. TRIPLE PO <sub>4</sub>	TRIPLE PO <sub>4</sub>
BLOOD - Hb.	11.1	12.0	12.2	9.0	10.4	9.6	10.4	9.4	12.2	10.0	10.5	9.5	11.4	9.4
HAEMOCRIT	37.0	37.0	36	32.0	31.0	32.0	32.0	32.0	30.5	30.0	32.0	30.0	31.0	30.0
W.B.C.	10,000	20,150	13,900	10,150	10,750	9,900	13,150	9,900	9,900	12,450	9,850	12,000	12,500	7,550
NEUTROPHILS	27	11	7	15	17	19	20	13	17	15	14	20	30	21
BANDS	2							1					4	
EOSINOPHILS	2	2	2		2		3					1		3
BASOPHILS	1								1				2	
LIMPHOCYTES	68	86	90	84	80	80	76	86	80	85	86	79	64	76
MONOCYTES		1	1	1	1	1	1		2					
TESTE TO L. POMONA	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT BLOOD	-	-	-	-	-	-	-	-	BLOOD SAMPLES NOT TAKEN FOR CULTURING OR DIRECT EXAMINATION					
CULTURE BLOOD	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DIRECT URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CULTURE URINE	-	-	-	-	-	-	-	-	-	-	-	-	-	-

KEY

N.T.	NOT TAKEN
HPF	HIGH POWER FIELD
SM.	SMALL
LG.	LARGE
LEUCO	LEUCOCYTES
EPITH	EPITHELIAL

RARE	FEW	OCCASIONAL
FREQUENT	NUMEROUS	

## APPENDIX IV

## MICROSCOPIC AGGLUTINATION TEST

Procedure

0.9 ml of physiological saline is added to each of three test tubes. To the first tube is pipetted 0.1 ml of a 1:5 dilution of the serum to be tested, this is well mixed. Serial tenfold dilutions are made in the next 2 tubes by transferring 0.1 ml. This gives dilutions of 1:50, 1:500, 1:5000 in the three tubes. From each dilution 0.1 ml is transferred to an agglutination tube, so that there is one agglutination tube for each antigen at each dilution. To each of these agglutination tubes is added an equal volume (0.1 ml) of the appropriate antigen grown in Stuart's medium enriched with rabbit serum. The antigen must be a well grown 7 day culture. The final dilutions of 1:100, 1:1000, 1:10000 are thus obtained. Positive and negative serum and saline controls are included in each test run. The serum-antigen mixtures are incubated at 27°C for 2 hours and read by examining a small drop of each dilution by darkfield microscope. The best magnification is achieved using a 12.5X objectives and 10X eyepieces. Serum samples are designated positive when 50% or more of the leptospire have agglutinated in one or more of the dilutions. Agglutinated leptospire appear as bright white masses of tightly entangled organisms. In some cases the field may be almost empty it is assumed that in these cases the agglutinated organisms have sedimented to the bottom of the tube.

## HYPOTONIC KCl

Stock 7.4 g/1000 ml water

Working Solution, make up just before use.

7.5 ml Stock KCl in 100 ml water.