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LEPTOSPIROSIS : PATHOGENESIS AND RED CELL DESTRUCTION

A thesis presented in partial fulfilment of
the requirements for the
Degree of Doctor of Philosophy
at Massey University
Palmerston North
New Zealand

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1983

ABSTRACT

A study was made of the morphological changes in red blood cells (RBC's) from hamsters and calves during the development of haemoglobinaemia following infection with *Leptospira interrogans* serovars *ballum* and *pomona* respectively.

The major changes seen by scanning electron microscopy of RBC's from the haemoglobinaemic animals were spherocytosis and surface pitting. The major change seen by transmission electron microscopy was vacuolation of abnormally shaped RBC's with some vacuoles containing a small amount of a fine granular material. Few RBC's showed evidence of haemoglobin loss even though the animals from which the RBC's came were severely haemoglobinaemic. Those RBC's which did show haemoglobin loss contained membrane-bound dense granular inclusions in addition to the vacuoles observed in the fully haemoglobinated RBC's. The spherocytes from the haemoglobinaemic animals probably arose from echinocytes which were seen in prehaemoglobinaemic hamsters. Echinocytes seen in calves injected with 'toxin' can probably be considered as equivalent to echinocytes seen in the prehaemoglobinaemic hamsters. These echinocytes had membrane-bound portions of cytoplasm segregated from the remainder of the cytoplasm. It is thought that these portions of cytoplasm are defective and subsequently become digested in autophagocytic vacuoles with complete digestion resulting in the empty vacuoles or those containing a small amount of fine granular material as seen in the fully haemoglobinated RBC's. Inability of the cell to either fully digest or expel material within autophagocytic vacuoles may explain dense granular inclusions seen within partially haemoglobinated RBC's which are considered the most severely affected RBC's.

Present studies support other work that a 'toxin' elaborated by the organisms rather than mechanical damage is responsible for the

lesions observed. The original lesion is thought to be biochemical although biochemical studies were beyond the scope of the present work. This biochemical lesion is likely to be similar in all affected tissues. Sufficient biochemical and physiological differences exist between adults and neonates and between individuals of similar age of the same species, and between different animal species to explain the differences in susceptibility of RBC's to leptospiral 'toxins'.

RBC's from cattle, hamsters and humans suspended in non-immune plasma and incubated with *ballum* and *pomona* were never haemolysed while those suspended in saline were always haemolysed. Normal plasma thus has a protective effect. The protective action of plasma demonstrated *in vitro* required reconciliation with some conflicting findings of parallel studies *in vivo* in which RBC's were destroyed resulting in haemoglobinaemia. It therefore appears that another mechanism may be responsible for RBC destruction *in vivo*. Because RBC sequestration resulting in lowering of the PCV and haemoglobin occurred in the prehaemoglobinaemic animals, involvement of the reticulomacrophage system appeared likely. Other workers have suggested that RBC's which already have an abnormality may be further damaged or lysed within the splenic circulation. Thus in leptospiral infections, leptospiral 'toxins' may induce changes in RBC's leading to their sequestration within the spleen resulting in further damage and ultimately lysis and haemoglobinaemia.

The ground is now set for further studies to identify the putative biochemical lesions which would pave the way for development of new therapeutic regimes to prevent the more severe clinical features of the disease.

ACKNOWLEDGEMENTS

The opportunity to work for this thesis was provided by the Department of Veterinary Pathology and Public Health and made possible by the support of the Phyllis Irene Grey Fellowship.

I would like to thank my initial chief supervisor Dr R.H. Sutton for his assistance, interest and encouragement in the early stages of this research. I would also like to thank his successor, Dr A.C. Johnstone for his assistance in later experiments and for reading the drafts of this thesis. Professor B.W. Manktelow gave valuable advice and encouragement in completing the final copy of this thesis. In addition I would like to express my gratitude to Dr R.B. Marshall who was always willing to discuss experiments and results with me and provide help and encouragement.

Funds and facilities for the research were provided by the Department of Veterinary Pathology and Public Health, Veterinary Research Fund and Glaxo Laboratories (NZ) Ltd. The electron microscopy was carried out with the help and advice of members of the Electron Microscopy Unit, DSIR, Palmerston North and in particular, Mr D. Hopcroft. The Radiotherapy Department of the Palmerston North Public Hospital and Mr Trott provided facilities for the irradiation of hamsters. I would also like to thank the staff of the Clinical Pathology Department of the Palmerston North Hospital for the use of their autoanalyser and Mr B. Riddler of the Department of Agricultural Economics and the staff of the No 4 Dairy Unit for providing neonatal calves and some older cattle for blood sampling.

The hamsters were provided by the National Health Institute and maintained by Dr L.M. Schollum. In addition Dr Schollum provided the cultures of leptospires used in these experiments and assisted with the microscopic agglutination titres. Histological slides were prepared by Mrs P.M. Slack and Miss S.M. Malloch and culture media were prepared by Mrs J.L. Schramer. Preparation of photographs was done with the assistance of Mr T. Law.

I also wish to thank the staff of the Massey University Library for their prompt and efficient assistance in obtaining references. Mrs F.S. Wicherts typed this thesis and I would like to thank her for her excellent advice on thesis preparation.

Finally I would like to thank my husband, Ghee Yong, for his support and understanding over the years of working for this thesis.

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INTRODUCTION

Red blood cell (RBC) destruction resulting in haemoglobinaemia occurs in some species of animal infected with certain serovars of *Leptospira interrogans*. While the pathogenesis of the destruction has not been established, it has previously been considered that the pathogenetic mechanisms involved must be different to those which result in hepatic, renal and endothelial cell lesions.

The main purpose of the present work is to elucidate the pathogenesis of RBC destruction using morphological studies of RBC's from hamsters and calves infected with *Leptospira interrogans* serovars *ballum* and *pomona* respectively. Because the RBC cannot be studied in isolation, other organs such as bone marrow, spleen, liver and kidney which may be involved in RBC formation and destruction are also studied. In addition leptospiral infections which result in haemoglobinaemia are compared with those which do not. This is necessary for determining which facets of the disease are due to the leptospiral organisms *per se* and which are due to hypoxia consequent upon severe RBC loss. In addition, brains and the middle and inner ears are also examined in order to determine the cause of the apparent nervous signs sometimes observed in experimental and naturally occurring leptospirosis.

The major emphasis of the work is on RBC destruction *in vivo*. This is because it is considered that the RBC cannot be adequately studied in isolation from the full range of its environment in the animal body. However, some *in vitro* work is reported upon in order to provide additional information on specific points such as the effects of the proportion of foetal haemoglobin and the presence or absence of plasma in determining the degree of RBC destruction.

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CHAPTER 1

LITERATURE REVIEW

Leptospires may be divided into two species on the basis of their ability to infect animals. *Leptospira biflexa* are saprophytic organisms and *Leptospira interrogans* are parasitic organisms which infect animals causing the disease leptospirosis.

This literature review deals with the pathogenesis and pathology of leptospirosis in domestic animals with particular reference to red blood cell (RBC) destruction. Primarily epidemiological and serological studies and those reporting abortions are not considered as they have been dealt with in other reviews (Blackmore, 1981; Blackmore & Hathaway, 1980; Hathaway, 1981). The references on the pathogenesis and pathology of leptospirosis in domestic animals which are referred to in this review are summarized in Table 1-1.

1.0 PATHOGENESIS OF LEPTOSPIRAL INFECTIONS

In vitro investigations have shown differences in the biochemical activities between saprophytic and parasitic leptospires (Kasarov, 1970; Stalheim, 1971) and between 'virulent' and 'avirulent' strains of parasitic leptospires (Stalheim, 1971). However, the correlation between *in vitro* and *in vivo* activities is uncertain and the virulence of a leptospiral organism does not appear to be related to any single biochemical reaction and must first rely upon the organism's ability to survive amid bacteriocidal factors *in vivo* and then to grow and multiply (Stalheim, 1971). Although considerable overlap may occur, the major factors determining the presence or absence of clinical disease may be categorized conveniently under three major headings.

1. Host factors
2. Leptospiral factors
3. Host-leptospire interaction

Table 1-1 Summary of the major references pertaining to the pathology and pathogenesis of experimental and natural leptospiral infections of man and domestic animals.

Animal species	Leptospiral serovar	Reference: Experimental Infections	Authors & Date Natural infections
Cats	*ballum*	Fessler & Morter, 1964 Shophet, 1979	
	gryppotyphosa	Van der Hoeden,1953	
	pomona	Fessler & Morter,1964 Shophet,1979	
Cattle	*balcanica*	Mackintosh *et al*,1981	
	bratislava	Fennestad *et al*, 1967	
	canicola	Imbabi *et al*, 1967 Van der Hoeden, 1955	Turner *et al*, 1958
	gryppotyphosa	Van der Hoeden, 1953	
	icterohaemorrhagiae AB		Dodd & Brakenridge,1960
	pomona	Hambdy & Ferguson,1957 Morter *et al*, 1958 Reinhard & Hadlow,1954 Sleight *et al*, 1964 Spradbrow & Seawright,1963 Ferguson *et al*,1957	Hadlow & Stoenner,1955
	szwajizak	Nervig *et al*, 1978	
	unknown	Cordy & Jasper,1952 Reinhard, 1951	
Dogs	*bataviae*	Keenan *et al*, 1978	
	canicola	Finco & Low, 1968 Monlux, 1948 a,b Taylor *et al*, 1970	Bloom, 1941 McIntyre & Montgomery,1952 Morrison & Wright,1976
	gryppotyphosa	Van der Hoeden,1953	
	icterohaemorrhagiae	Gleiser, 1957 Low *et al*, 1956 Monlux, 1948 a,b	Bloom, 1941
Goats	*gryppotyphosa*	Van der Doeden,1953	Van der Hoeden,1953
	pomona	Morse & Langham,1958	

Table 1-1 cont'd

Animal	Leptospiral serovar	Reference: Authors & Date Experimental Infections	Natural Infections
Grivet monkeys	*balcanica* *tarassovi*	Marshall *et al*, 1980	
Guinea pig	*gryppotyphosa*	Van der Hoeden,1953	
	icterohaemorr-hagiae	Arean, 1962b, Arean & Henry,1964 Arean *et al*, 1964 De Brito *et al*,1979 De Brito *et al*,1966 Higgins & Cousineau,1977a,b Higgins *et al*,1979	
Hamsters	*canicola*	Sapp *et al*, 1980	
	gryppotyphosa	Van der Hoeden,1953	
	icterohaemorr-hagiae	Cox & Twigg, 1981 Miller *et al*, 1974	
	hardjo	Adler & Bragger,1979	
	pomona	Abdu & Sleight, 1965 Hambdy & Ferguson,1957 Miller & Wilson,1962 Miller & Wilson,1966 Miller & Wilson,1967 Sanger *et al*, 1961	
Horses	*canicola*	Bryans, 1955	Bryans, 1955
	icterohaemorr-hagiae		Bryans, 1955
	pomona	Bryans, 1955 Morter *et al*,1969	Bryans, 1955
Humans	*bataviae*		Sitprija *et al*,1980
	gryppotyphosa		Breitenfeld & Gug ic,1977
	icterohaemorr-hagiae		Arean, 1962a De Brito *et al*,1965 De Brito *et al*,1967
	bataviae *autumnali* *australis* *akiyami*		Jaroonvesama *et al*,1975

Table 1-1 cont'd

Animal	Leptospiral serovar	Reference: Authors & Date Experimental Infections	Natural Infections
Mice	*pomona*	Marshall, 1973; 1976	
Pigs	*icterohaemorrhagiae*		Nisbet, 1951
	pomona	Langham *et al*, 1958 Morse *et al*, 1958 Sleight *et al*, 1960	Bryan, 1954 Ferguson *et al*, 1956
Sheep	*gryppotyphosa*	Van der Hoeden, 1953	
	pomona	Bauer *et al*, 1961 Decker *et al*, 1970 Hodges, 1974 Hodges *et al*, 1976 Lindqvist *et al*, 1958 Marshall, 1973; 1974 Millar *et al*, 1977 Morse *et al*, 1957 Morter, 1961	

1.1 HOST FACTORS

1.1.1 Species of animal

Certain species of animal are more susceptible to severe clinical disease caused by leptospiral infections than others. Cattle, sheep, pigs and dogs are readily infected both naturally and experimentally. There have been no records of natural leptospiral infections of hamsters and guinea pigs although both may be readily infected experimentally, usually with a fatal result. These species have been widely used in studies of pathogenesis and pathological lesions.

Serological evidence is present for the natural infection of cats (Shophet, 1979) and horses (Bryans, 1955). There is little sign of clinical disease in cats (Fessler & Morter, 1964; Shophet, 1979). Horses exhibit only a slight pyrexia and leucocytosis after infection although periodic opthalmia has been suggested as being caused by the infection (Bryans, 1955; Morter *et al*, 1969).

The presence or absence of haemolysis depends upon the species of animal and the infecting serovar or strain of serovar. It has been shown *in vitro* that parasitic leptospires including *pomona* and *icterohaemorrhagiae* haemolyse ruminant RBC's faster than non-ruminant RBC's (Kasarov, 1970). This is thought to be due to the fact that the membrane of ruminant RBC's contain large amounts of sphingomyelin which is degraded more rapidly than other membrane phospholipids (Kasarov, 1970).

1.1.2 Age of the animal

Young animals are usually more susceptible to severe clinical disease caused by leptospiral infection than adults. Susceptible calves infected with *pomona* develop severe fatal disease (Kemenes, 1974; Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963) more often than adult cattle (Ferguson *et al*, 1957; Hadlow & Stoenner, 1955; Hambdy & Ferguson, 1957; Morter *et al*, 1958; Reinhard & Hadlow, 1954; Sleight *et al*, 1964). Lambs infected with *pomona*

also show more severe disease (Hodges, 1974; Millar *et al*, 1977; Morse *et al*, 1957) than older sheep which show no or only mild symptoms (Lindqvist *et al*, 1958).

Severe disease has been reported in sows and their litters infected with *pomona*, while 'feeder' age pigs show only a very mild form of the disease (Morse *et al*, 1958; Sleight *et al*, 1960). The severe manifestations of the disease included anaemia, icterus and haemoglobinuria (Bryan , 1954; Ferguson *et al*, 1956). This reported occurrence of severe disease in this species is of interest because pigs are usually considered to be maintenance hosts for *pomona* (Hathaway, 1981), and show few clinical signs of infection. However, these observations were on field outbreaks and it is possible that the leptospirosis was coincidental with another condition which was responsible for the haemoglobinuria.

1.1.3 Non-specific antibacterial substances

Normal serum has an inhibitory effect on the survival of saprophytic leptospires and no effect on *Leptospira interrogans*. This activity is mediated via the complement system (Johnson & Muschel, 1965) in conjunction with a β macroglobulin and lysozyme (Faine & Carter, 1968).

Normal non-immune bovine serum has been shown to have an inhibitory effect upon haemolysis induced by *pomona* culture supernatant. This effect increases with the age of the non-immune cattle (Kemenes, 1974).

1.1.4 Specific immunity

The presence of specific antibody in the plasma of animals inhibits the growth of parasitic leptospires (Alexander *et al*, 1971; Faine *et al*, 1974; Johnson & Muschel, 1966) and cross protection occurs only with antigenically similar leptospiral serovars (Alexander *et al*, 1971).

1.1.5 Haemoglobin types

Hodges *et al* (1976) suggest that sheep with haemoglobin type AB are more susceptible to leptospiral haemolysis than haemoglobin types A and B. These results require further confirmation as only nine sheep in total, three of each haemoglobin type were used in the experiment on which the observations were made.

1.2 LEPTOSPIRAL FACTORS

1.2.1 Leptospiral serovar

Different serovars of leptospire induce different disease syndromes and therefore presumably have different pathogenic mechanisms. For instance, *icterohaemorrhagiae* infections of cattle (Dodd & Brakenridge, 1960), guinea pigs (Arean, 1962b; De Brito *et al*, 1966;1979; Higgins & Cousineau, 1977a) and dogs (Low *et al*, 1956; Monlux, 1948b) appear to be more haemorrhagic than *pomona* infections of cattle (Ferguson *et al*, 1957; Spradbrow & Seawright, 1963).

Haematuria rather than haemoglobinuria was reported in *bratislava* infections of cattle (Fennestad *et al*, 1967). *Canicola* infections of cattle varied in the presence (Turner *et al*, 1958) or absence (Imbabi *et al*, 1967) of haemoglobinuria. Although *pomona* infections of cattle and *ballum* infections of hamsters both show similar manifestations of disease, hamsters infected with *pomona* show no signs of RBC destruction (Abdu & Sleight, 1965; Sanger *et al*, 1961).

1.2.2 Strain of serovar

Differences in virulence have been demonstrated in different isolates or strains of *pomona* (Abdu & Sleight, 1965; Bauer & Morse, 1958). Serial passage of *pomona* (Bauer & Morse, 1958; Hambdy & Ferguson, 1957) and *icterohaemorrhagiae* (Cox & Twigg, 1981) have been shown to alter the virulence of the organism.

Stalheim (1971) found that 'avirulent' strains of *pomona* are more susceptible to lysis by fresh rabbit serum than 'virulent' strains.

1.2.3 Leptospiral antigens

Leptospiral organisms contain antigens associated with the axial filament, cell wall and cytoplasm which are composed of polysaccharide, lipid and protein and may be classified as agglutinating, haemagglutinating, complement fixing or precipitating depending on their seroreaction (Faine, 1974). Faine & Carter (1968) postulated that there is a 'z' antigen which is more accessible to the host defences in 'avirulent' forms and suggest that the change from 'virulence' to 'avirulence' is caused by the'uncovering' of the antigen making the organism more susceptible to the host defence mechanisms.

1.2.4 Biochemical activities of leptospires

Leptospires produce 'toxins' or cytotoxic factors (Arean *et al*, 1964; Chaperon *et al*, 1979; Finco & Low, 1967; Higgins *et al*, 1979; Knight *et al*, 1973) and haemolysins (Kasarov, 1970; Stalheim, 1971) which have been associated with pathogenicity of the organism.

The haemolysins from the serovars of leptospires which have haemolytic activity are postulated to consist of a number of different phospholipases (Kasarov, 1970). Haemolytic activity against sheep RBC's has been shown to be present in both the cells and the culture supernatant of a variety of parasitic leptospires including *pomona*, *icterohaemorrhagiae* and *canicola* (Imamura *et al*, 1957). Valentine *et al* (1964) also found that both the *pomona* cells and *pomona* culture supernatant have haemolytic activity and concluded that the leptospires produce the 'toxin' as they grow and release it into the culture supernatant. *In vitro* haemolytic activity has been found to vary with the species of animal from which the RBC's are derived. Against sheep RBC's, *pomona* has the most haemolytic activity, *icterohaemorrhagiae* has less activity and *canicola* very little (Imamura *et al*, 1957). Hathaway (1978) found that *pomona* is haemolytic, *balcanica* less haemolytic and *hardjo* and *ballum* non-haemolytic when cultured with human, cattle, sheep, possum and hamster RBC's. Differences in enzymatic activity for lipids is also seen between 'virulent' and 'avirulent' strains of *pomona*.

'Avirulent' leptospires have less phospholipase C activity against lecithin and lysolecithin and no activity against sphingomyelin (Stalheim, 1971).

The *in vitro* activity of the *pomona* haemolysins can be increased by increasing the amount of haemolysin, increasing the pH, increasing the magnesium ion concentration, decreasing the calcium ion concentration and having an optimal temperature of 37°C for the reaction (Chorvath 1975). The phospholipase has been shown to react similarly to phospholipase A, snake venom phospholipases and other bacterial phospholipases in the breakdown of lecithin to lysolecithin and fatty acids (Chorvath, 1974). *Pomona* culture supernatant produces a similar 'membrane wrinkling' to phospholipase A on bovine lipids and lipoids extracted from the bovine RBC membrane (Kemenes, 1974). This phenomenon is not seen with porcine lipid and RBC lipoids. The addition of crude phospholipids to the incubated cultures of washed sheep RBC's and *pomona* culture supernatant decreases haemolysis of the RBC's (Chorvath, 1975).

Bauer & Morse (1958) demonstrated no positive correlation between *in vivo* and *in vitro* haemolytic activity of *pomona* in hamsters. This was confirmed by Stalheim (1971) who showed that the loss of 'virulence' of *pomona* strains is not characterized by a loss of haemolytic, esterase, lipase, aminopeptidase or phospholipase C activity.

The inability of saprophytic leptospires to haemolyse sheep RBC's is thought to be due to their inability to degrade the large quantities of sphingomyelin in the sheep RBC membrane (Kasarov, 1970). It has been found that sphingomyelin is degraded before other membrane phospholipids (Stalheim, 1971).

1.3 LEPTOSPIRAL-HOST INTERACTION

1.3.1 Specific antibody-mediated immunity

Johnson & Muschel (1966) showed that specific antibody must

be added to complement to inhibit the growth of parasitic leptospires. Antiserum alone does not kill 'virulent' leptospires, it causes the leptospires to clump together, these clumps containing intact and viable leptospires (Johnson & Harris, 1967). It is believed that agglutination assists removal by the macrophage system. Thus, if sufficient antibody is present early in the disease then sufficient numbers of leptospires may be agglutinated and removed, otherwise leptospiral multiplication exceeds removal by macrophages (Johnson & Harris, 1967). The presence of circulating specific antibody has been shown to provide protection against parasitic leptospires such as *pomona* (Alexander *et al* , 1971; Kemenes, 1974) and *icterohaemorrhagiae* (Faine *et al*, 1964) with cross protection occurring between antigenically similar organisms only (Alexander *et al*, 1971).

Contrary to the work discussed in the previous paragraph where antibody was considered protective against leptospiral infections, some evidence suggests that when antibody lyses leptospires, this releases haemolysin which in turn lyses the RBC's. Thus, Morter (1961) reported that in sheep, an initial intravenous injection of killed *pomona* suspension produced an antibody titre but not haemoglobinuria. However following a second intravenous injection, haemoglobinaemia occurred. An alternative explanation is that leptospiral extracts may sensitize the sheep RBC's to the action of leptospiral antiserum and complement and so cause haemolysis (Cox, 1955). These results conflict with those of Bauer *et al* (1961) who showed that the presence of *pomona* antibody protected lambs against the administration of large amounts of *pomona* haemolysin.

Further work to determine a relationship between the occurrence of anaemia and the presence of leptospiral agglutinins in sheep infected with *pomona* failed to produce conclusive evidence and autoimmune antibody against RBC's was not detected (Decker *et al*, 1970).

Ringen (1966) claimed that in bulls and goats infected with *pomona* and showing no clinical signs of infection, there is a decrease in the susceptibility of the RBC's to *pomona* haemolysin 8-13 days after infection. He did not include RBC's from non-infected controls to detect daily fluctuations in haemolysin activity and in only one group of goats was preinoculation haemolysis measured. The reading in one of the preinoculation tested goats was as high as some at postinoculation. No statistical analysis was or could be performed on his figures. The conclusions cannot therefore be considered reliable.

1.3.2 Leptospiral phagocytosis

Leptospiral phagocytosis has been observed *in vivo* in both immune and non-immune mice with greater invasion of the tissues of the non-immune mice (Faine, 1964). In non-immune mice organisms are seen between or within parenchymal cells as well as within Kupffer cells,while in immune mice organisms are seen only within Kupffer cells. Faine (1964) also observed that there is no histologic evidence of tissue reaction to the leptospires within 30 minutes of infection and that the lungs of immune mice 'accumulate' leptospires more rapidly than the lungs of non-immune mice. This function of the lungs has not been described by other workers. Specific antibody in immune mice produced by specific resistence may enhance phagocytosis (Faine *et al*, 1964).

2.0 THE CLINICAL SIGNS AND PATHOLOGY OF LEPTOSPIRAL INFECTIONS WITH PARTICULAR REFERENCE TO RED BLOOD CELL LOSS AND DESTRUCTION

Following the infection of susceptible animals, there is a bacteraemic phase during which the leptospires multiply and spread through the blood stream. At this time, leptospires may be isolated from most body tissues (Sanger *et al*, 1961). If the animal survives, the leptospires disappear from the tissues and blood stream and remain only in the proximal convoluted tubules of the kidneys (Faine, 1964; Marshall, 1973; 1976; Miller & Wilson, 1967; Sanger *et al*, 1961).

2.1 RED BLOOD CELL LOSS AND DESTRUCTION

In any infection, anaemia may result from either increased removal or breakdown of RBC's, haemorrhage, or decreased formation (Benjamin, 1979; Duncan & Prasse, 1978).

Anaemia is often present in calves (Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963) and lambs (Hodges, 1974; Hodges *et al*, 1976; Millar *et al*, 1977; Morse *et al*, 1957) infected with *pomona* and calves infected with *canicola* (Turner *et al*, 1958) as a result of RBC destruction and haemoglobinaemia. The extent of the anaemia depends upon the degree of RBC destruction which is reflected by a decrease in PCV and haemoglobin.

While RBC destruction resulting in haemoglobinaemia has often been noted (Hodges, 1974; Hodges *et al*, 1976; Millar *et al*, 1977; Morse *et al*, 1957; Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963; Turner *et al*, 1958) causal lesions, either biochemical or morphological have not. General studies on RBC's have shown that mature RBC's from most domestic animals are biconcave disks (Bessis, 1973; Clarke & Salisbury, 1967; Jain & Kono, 1972; Petz & Garratty, 1980) while immature RBC's or reticulocytes are larger, rounded cells with large surface irregularities caused by amoeboid movement of the cell and small surface pits due to collapsed pinocytic vesicles (Bessis, 1973).

The biconcave shape is necessary for the normal function and lifespan of the RBC. Flexibility of the membrane and deformability of the cell are necessary for the survival of the cell *in vivo* as extreme shape changes occur when RBC's enter the microcirculation (Bessis, 1973; Jandl, 1965; 1966; La Celle, 1970; Nathan, 1969). The maintenance of shape is not thought to be due to any definite structural factors but rather is due to the combination and balance of physical and metabolic features of the cell contents and the membrane (Grimes, 1980). *In vitro* studies have shown that the cell will regain its normal shape once free of the deforming factors, even if these factors result in the loss of some membrane (La Celle 1970; Weed & La Celle, 1969).

Shape changes will occur with alteration in the transport of sodium (Na^+) and potassium (K^+) across the membrane (Tosteson, 1969; Tosteson & Hoffman, 1960; Zarkowsky *et al*, 1968), depletion of sources of ATP (Bessis, 1973; Brecher & Bessis, 1972; Palek *et al*, 1974; Palek *et al*, 1978), alteration in the membrane cholesterol content (Cooper, 1969; Murphy, 1965; Weed & Reed, 1966) and alteration of cellular magnesium (Mg^{2+}) and calcium (Ca^{2+}) content (LaCelle, 1970; Palek *et al*, 1974; Weed & La Celle, 1969; Weed *et al*, 1969).

The mechanisms for leptospiral destruction of RBC's have not been described but on theoretical grounds it is appropriate to consider that RBC destruction may occur through several mechanisms.

i) Osmotic haemolysis

A balance in Na^+ and K^+ is required for the osmotic regulation of the RBC. This balance requires energy in the form of ATP (Firkin & Wiley, 1966; Jandl, 1965; Jandl & Aster, 1967; Murphy, 1963; Tosteson, 1969).

Osmotic haemolysis is caused by a breakdown in osmotic regulation and volume control which may be the result of either diminished cation transport or increased membrane permeability. The end result of either defect is the accumulation of Na^+ and water although the initiating factor may be quite different (Jandl, 1965). Haemolysis occurs when a 60-70% increase over the normal RBC volume is reached (Jandl, 1965) unless membrane loss has occurred and the haemolytic volume is in fact reduced (Jandl, 1965; Jandl & Aster, 1967; Weed & Reed, 1966). During hypotonic haemolysis unstable holes are formed (Seeman, 1967) which allow the escape of haemoglobin.

ii) Structural damage to the RBC membrane by 'toxins'

This may result from a variety of agents. Saponin (Dourmashkin & Rosse, 1966; Seeman, 1967) and lysolecithin (Brecher & Bessis, 1972; Seeman, 1967) disrupt the bimolecular phospholipid layer of the RBC membrane to form stable holes from which the haemoglobin escapes (Seeman, 1967).

Bacterial 'toxins' (Bernheimer, 1974; Chorvath, 1974; Dourmashkin & Rosse, 1966; Kemenes, 1974) often have phospholipase activity. Streptolysin O produced membrane lesions of 50nm (Bernheimer, 1974) which are large enough to permit the escape of haemoglobin molecules (Seeman, 1967). Most work on leptospiral 'toxins' has been done in order to demonstrate phospholipase activity (Chorvath, 1974; 1975; Kasarov, 1970; Kemenes, 1974; Stalheim, 1971), based upon the assumption that the loss of haemoglobin must result from lesions in the membrane which are large enough to permit the escape of haemoglobin. The majority of work has been done on *pomona*, the 'toxin' from which has been shown to have phospholipase-like activity (Chorvath, 1974; Kasarov, 1970). The greater degree of haemolysis of ruminant RBC's by leptospiral 'toxins' has been explained on the basis that ruminant RBC's have a larger quantity of the membrane phospholipid sphingomyelin (Kasarov, 1970; Kemenes, 1974). The 'toxins' degrade sphingomyelin more rapidly than other membrane phospholipids such as lecithin which are found in higher concentrations in non-ruminant RBC's.

There is, however, a danger in assuming that the above *in vitro* investigations may apply directly *in vivo*. No work has been done to discover alternative *in vivo* mechanisms for RBC destruction.

iii) Immune Complexes

Immune complexes have been shown to produce transmembranous channels by an orderly sequence of complement mediated enzyme reactions (Borsos *et al*, 1964; Dourmashkin & Rosse, 1966). Complement lesions were found to be 10nm in diameter, too small to permit the escape of haemoglobin but large enough to cause increased cation permeability and therefore water uptake (Dourmashkin & Rosse, 1966). The resultant osmotic haemolysis can be inhibited by environment macromolecules (Frank *et al*, 1965; Sears *et al*, 1964). Antibody-complement mediated haemolysis has been suggested to occur in leptospiral

infections (Cox, 1955). He suggested that leptospiral extracts sensitize sheep RBC's to the action of antibody and complement with resultant haemolysis. This aspect was discussed previously in section 1.3.1.

A small degree of haemorrhage is seen in most infections with some infections notably those by *icterohaemorrhagiae* tending to be more haemorrhagic. *Icterohaemorrhagiae* infections of guinea pigs (Arean *et al*, 1964; De Brito *et al*, 1979, Higgins & Cousineau, 1977a,b) hamsters (Miller *et al*, 1974) and cattle (Dodd & Brakenridge, 1960); *bratislava* (Fennestad *et al*, 1967) and *szwajizak* (Nervig *et al*, 1978) infections of cattle; *bataviae* (Keenan *et al*, 1978) and *icterohaemorrhagiae* and *canicola* (Bloom, 1941; Finco & Low, 1968; Low *et al*, 1956; Monlux, 1948b) infections of dogs are all characterized by petechial haemorrhages of the carcase. It is postulated that the haemorrhagic lesions in guinea pigs (Arean *et al*, 1964; De Brito *et al*, 1979; De Brito *et al*, 1966; Higgins & Cousineau, 1977, a,b) and hamsters (Miller *et al*, 1974) caused by *icterohaemorrhagiae* are caused by damage to the endothelial cells of blood vessels (De Brito *et al*, 1966; De Brito *et al*, 1979; Miller *et al*, 1974). Haemorrhagic lesions caused by vascular damage and/or thrombocytopaenia are thought to be associated with the production of a cytoxic factor or an endotoxin (Arean *et al*, 1964; Chaperon *et al*, 1979; De Brito *et al*, 1979; Higgins *et al*, 1979; Miller *et al*, 1974).

It is postulated that haemorrhage may also result from altered clotting factors. While extensive studies of clotting factors have not been done in most leptospiral infections, the known alterations are summarized in Table 1-2.

A cytotoxic factor derived from *canicola* organisms (Chaperon *et al*, 1979; Finco & Low, 1967) was found to produce platelet aggregation and sequestration in the spleen and the associated thrombocytopaenia was thought to be partly responsible for the haemorrhages which were observed in hamsters which were injected with this factor (Chaperon *et al* , 1979).

Table 1-2: Alterations in blood clotting factors during leptospirosis

Animal species	Leptospiral serovar	Clotting Factor Alteration	Author
Dog	*bataviae*	Thrombocytopaenia present; Normal clotting time	Keenan *et al*, 1978
	canicola & *ictero-haemorrhagiae*	Thrombocytopaenia present; Normal clotting time	Monlux, 1948a.
Guinea pig	*ictero-haemorrhagiae*	Thrombocytopaenia present; Decreased clotting factors VIII, IX, X, XI, XIII; Fibrinogen breakdown products present	
		Increased prothrombin, thrombin and partial thromboplastin times	Higgins & Cousineau, 1977a.
Hamster	*canicola* 'toxin'	Thrombocytopaenia	Chaperon *et al*, 1979
Human	*bataviae*	Thromboplastin and Prothrombin times normal; Factors VIII and V normal; Fibrinogen levels normal; Slight thrombocytopaenia	Sitprija *et al*, 1980
	bataviae, *australis* *autumnalis*, *akiyami*	Partial thromboplastin and prothrombin times increased; Factors V and X decreased; Factor VIII normal; Slight thrombocytopaenia; Clotting times normal	Jaroonvesama *et al*, 1975
Rabbits	*canicola* 'toxin'	Thrombocytopaenia	Finco & Low, 1967

A mild to moderate anaemia is associated with haemorrhagic canine *canicola* and *icterohaemorrhagiae* infections, *bratislava* infections of cattle (Fennestad *et al*, 1967) and *icterohaemorrhagiae* infections of guinea pigs (Higgins & Cousineau, 1977b). In these cases the severity of the anaemia was determined by the degree of haemorrhage.

Anaemias resulting from depression of the bone marrow have not been reported during leptospirosis.

2.2 SPLENIC AND OTHER LYMPHOID TISSUE LESIONS

The spleens from acutely ill hamsters (Abdu & Sleight, 1965) and cattle (Cordy & Jasper, 1952; Reinhard, 1951; Spradbrow & Seawright, 1963) infected with *pomona*, and dogs infected with *canicola* and *icterohaemorrhagiae* (Gleiser, 1957; Low *et al*, 1956; Monlux, 1948b) are enlarged, swollen and friable.

Histologically splenic congestion is a constant feature of acute, severe leptospirosis, particularly when the animal is haemoglobinaemic (Arean, 1962b; Arean *et al*, 1964; Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963). Haemosiderosis is particularly obvious in those animals which are haemoglobinaemic as seen in *pomona* infections of cattle (Hadlow & Stoenner, 1955; Reinhard, 1951; Reinhard & Hadlow, 1954) but is also seen in dogs infected with *canicola* and *icterohaemorrhagiae* in which haemoglobinaemia is not a feature (Bloom, 1941).

Focal necrosis and PMN infiltration of the sinusoids of the red pulp is common (Bloom, 1941; Cordy & Jasper, 1952; Spradbrow & Seawright, 1963). Extramedullary haemopoiesis (Arean, 1962b; Arean *et al*, 1964; Bloom, 1941; Monlux, 1948b; Reinhard & Hadlow, 1954) and lymphoid depletion of the lymphoid follicles (Reinhard & Hadlow, 1954) have also been reported.

Few histological descriptions of lymph nodes are present in the literature. Oedema, hyperaemia and enlargement of the

suprascapular, femoral, popliteal and mesenteric lymph node have been reported in *canicola* infected calves (Imbabi *et al*, 1967). In dogs infected with *canicola* and *icterohaemorrhagiae*, the caeco-colic lymph nodes showed severe distension of the medullary sinuses with blood and erythrophagocytic macrophages (Monlux, 1948b).

Blood lymphocyte numbers are given in some references, and show variation as summarized in Table 1-3.

Thus, although the presence of congestion and haemosiderosis has been noted in the spleens of haemoglobinaemic animals only the occasional mention of erythrophagocytosis such as that by Cordy & Jasper (1952) is made. The mechanism for the removal of RBC's has not been discussed in any detail in the literature.

The functions of the spleen are thought to rely upon the complex structure of sinusoids, reticular cords and spaces between the cords and the numerous macrophages and reticular cells (Dellman & Brown, 1976; Galindo & Freeman, 1963; Weiss, 1962a). Fenestrations in the capillary endothelial linings lead to the skimming off of plasma and haemoconcentration (Jandl & Aster, 1967; Weiss, 1962a). RBC's may pass through the spleen by either of two routes (Galindo & Freeman, 1963; Dellman & Brown, 1976; Jandl & Aster, 1967). The first a relatively direct transit through the sinusoids and the second a slow transit through the tortuous and divided spaces between reticular cords. The slow route forces the RBC's to pass through small endothelial openings thereby testing the cells deformability, membrane flexibility and cell filterability. A decrease in filterability for any reason leads to RBC sequestration (Crosby, 1977; Jandl, 1966; Jandl *et al*, 1961; Rifkind, 1965; 1966; Weiss, 1962b). Abnormal RBC's such as those containing Howell-Jolly bodies, Heinz bodies, Cabot Rings and parasites are sequestered and either these abnormal structures removed from the RBC or the RBC removed from circulation (Natham, 1969).

In some cases, splenic sequestration may induce abnormal shape

Table 1-3: Circulating leucocytes in leptospirosis

Animal species	Leptospiral serovar	Leucocyte Alteration	Author
Cattle	*canicola*	Leucocytosis (neutrophilia) followed by a leucopaenia (neutropaenia & lymphopaenia)	Imbabi *et al*, 1967
	pomona	Leucopaenia (neutropaenia and lymphopaenia)	Reinhard & Hadlow, 1954
		Variation in WBC levels	Reinhard & Hadlow, 1954 Spradbrow & Seawright, 1963
	unknown	Leucopaenia (neutropaenia and lymphopaenia)	Reinhard, 1951
Dogs	*canicola* *ictero-haemorrhagiae*	Leucocytosis (neutrophilia and lymphopaenia)	Bloom, 1941 Monlux, 1948a
	bataviae		Keenan *et al*, 1978
Guinea	*ictero-haemorrhagiae*	WBC levels normal	Higgins & Cousineau, 1977b.
Hamsters	*pomona*	Leucocytosis (neutrophiiia and lymphopaenia)	Abdu & Sleight, 1965
Horses	*canicola* & *pomona*	Leucocytosis (neutrophilia and lymphopaenia)	Bryans, 1955
Humans	*ictero-haemorrhagiae*	Leucocytosis or leucopaenia	Arean, 1962a
	gryppotyphosa	Leucocytosis and lymphopaenia	Breitenfeld & Gugic, 1977
	bataviae *australis* *autumnalis* *akiyami*	Leucocytosis Leucotytosis Leucotytosis Leucotytosis	Jaroonvesama *et al*, 1975
Pigs	*pomona*	Leucocytosis followed by leucopaenia	Sleight *et al*, 1960
Sheep	*pomona*	Neutropaenia and lymphocytosis	Marshall, 1973

(Grimes, 1980; Jandl & Aster, 1967). During sequestration, the cells are exposed to lowering glucose and ATP levels and increasing levels of metabolites (Jandl & Aster, 1967) and as has been reported in hereditary spherocytosis, increased fragility and deformation occurs in older cells after passage through the spleen (Griggs *et al*, 1960; Grimes, 1980).

The splenic circulation also exposes the RBC to severe mechanical trauma (Weiss, 1962b) and the more severely damaged RBC's may undergo intravenous lysis while within the splenic pulp (Rifkind, 1965).

Once sequestered in the spleen, the RBC's are rapidly haemolysed and the slight increase in plasma haemoglobin that occurs suggests that some haemoglobin may be released back into circulation (Jandl *et al*, 1957). Following phagocytosis of the entire RBC, the haemoglobin is rapidly removed and insoluble membranes and Heinz bodies are more slowly degraded (Rifkind, 1965).

2.3 HEPATIC LESIONS

Livers from acutely ill animals are enlarged, swollen and friable (Abdu & Sleight, 1965; Arean, 1962a,b; Cordy & Jasper, 1952; Gleiser, 1957; Low *et al*, 1956; Monlux, 1948b; Reinhard, 1951; Spradbrow & Seawright, 1963). Jaundice of the carcase is often seen in cattle (Fennestad *et al*, 1967; Imbabi *et al*, 1967; Nervig *et al*, 1978; Reinhard, 1951; Reinhard & Hadlow, 1954), hamsters (Sanger *et al*, 1961) and dogs (Bloom, 1941; Keenan *et al*, 1978; Low *et al*, 1956; Monlux, 1948b) although some experimental infections of cattle (Spradbrow & Seawright, 1963), hamsters (Abdu & Sleight, 1965) and dogs (Bloom, 1941) have hepatic lesions not associated with jaundice.

Histological examination of all cases in which the animals died from, or were euthanased in the late stages of acute, severe leptospirosis showed severe hepatic congestion (Arean, 1962a,b; Bloom, 1941; Cordy & Jasper, 1952; Higgins & Cousineau, 1977a,b),

hepatocyte vacuolation and necrosis (Abdu & Sleight, 1965; Arean, 1962b; Bloom, 1941; Cordy & Jasper, 1952; Dodd & Brakenridge, 1960; Ferguson *et al*, 1957; De Brito *et al*, 1966; Reinhard & Hadlow, 1954; Sanger *et al*, 1961; Spradbrow & Seawright, 1963), and disruption of hepatic cords (Arean, 1962a,b; Bloom, 1941; De Brito *et al*, 1966; Gleiser, 1957; Dodd & Brakenridge, 1960; Higgins & Cousineau, 1977a,b; Monlux, 1948b). Mononuclear cell infiltrations particularly around portal areas were prominent (Abdu & Sleight, 1965; De Brito *et al*, 1966; Dodd & Brakenridge, 1960; Ferguson *et al*, 1957; Hadlow & Stoenner, 1955; Imbabi *et al*, 1967; Monlux, 1948b; Nervig *et al*, 1978; Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963) and were often accompanied by desquamation of the bile duct epithelium (Reinhard & Hadlow, 1954) and bile stasis (Cordy & Jasper, 1952).

Haemosiderin has been found within the Kupffer cells of calves infected with *pomona* which died within seven days following the onset of haemoglobinaemia associated with RBC destruction (Reinhard & Hadlow, 1954). Haemosiderin has also been seen in the liver of some dogs infected with *canicola* and *icterohaemorrhagiae* (Bloom, 1941; Gleiser, 1957; Monlux, 1948b). The Kupffer cells in *icterohaemorrhagiae* infections show erythrophagocytic activity (Arean 1962a; Gleiser, 1957) and hyperplasia (Arean, 1962a; De Brito *et al*, 1966). Erythrophagocytosis in the Kupffer cells during *icterohaemorrhagiae* infections is suggested as resulting from increased RBC destruction (Gleiser, 1957) even though haemoglobinaemia is absent. In general the liver is thought to be less sensitive than the spleen to minimally damaged RBC's but will detect and sequester severely damaged RBC's (Jandl & Kaplan, 1960; Rifkind, 1965; 1966; Wagner *et al*, 1962; Weiss, 1962b) and is an important site of erythrophagocytosis because of its large blood supply.

Electron microscopic studies of *icterohaemorrhagiae* infections of guinea pigs (De Brito *et al*, 1966) and *pomona* infections of hamsters (Miller & Wilson, 1966) showed enlarged Kupffer cells, damaged bile ducts and hepatocytes had swollen mitochondria and endoplasmic reticulum, with loss of glycogen.

Leptospires are seen between hepatocytes, in the space of Disse and occasionally intracellularly in hamsters infected with *pomona* (Miller & Wilson, 1966).

Histochemical studies of hepatocytes show decreases in the intracellular enzymes isocitrate dehydrogenase, glutamic dehydrogenase and succinic dehydrogenase in *icterohaemorrhagiae* infected guinea pigs (Arean & Henry, 1964). The enzyme depletion in hepatocytes is more severe than indicated by concentrations of the enzymes in serum indicating that serum enzyme levels may not be a particularly sensitive indicator of liver disease (Arean & Henry, 1964).

In animals completely recovered from severe leptospiral infections or those which had only a mild or subclinical infection, no significant hepatic lesions are present (Morter *et al*, 1958; Sleight *et al*, 1964).

Alterations in serum enzymes affected by hepatic disease are summarized in Table 1-4 and, when measured, were usually raised.

While detailed histological and histochemical descriptions of liver lesions have been made, the effects of liver disease upon RBC morphology have not been discussed. Only occasional reference is made to erythrophagocytosis (Arean, 1962a; Gleiser, 1957).

The liver produces lecithin-cholesterol acyl transferase (LCAT) which maintains plasma levels of free and esterified cholesterol (Raz *et al*, 1969). Depletion of LCAT by liver disease may result in the presence of acanthocytic forms of RBC's (Bessis, 1973; Douglass *et al*, 1968; Grimes, 1980). Reduction of LCAT results either directly from reduced hepatocyte production or inhibition of enzyme activity by increased levels of bile salts (Gjone & Norum, 1970). The amount of membrane cholesterol affects RBC shape (Cooper, 1969; Murphy, 1962a,b).

Table 1-4: Biochemical changes in the blood during leptospirosis

Animal species	Leptospiral serovar	Biochemical Alteration	Author
Cattle	*bratislava*	Blood urea nitrogen (BUN) elevation	Fennestad *et al*, 1967
	pomona	Alkaline phosphatase (AP) elevation BUN elevation	Spradbrow & Seawright, 1963
Dogs	*bataviae*	AP elevation Bilirubin elevation	Keenan *et al*, 1978
	canicola & ictero-haemorrhagiae	BUN elevation	Bloom, 1941 Low *et al*, 1956 McIntyre & Montgomery, 1952 Monlux, 1948a
		Bilirubin elevation	Bloom, 1941
Guinea pigs	*ictero-haemorrhagiae*	Aspartate amino transferase (AAT) elevation AP elevation Glutamic pyruvic transaminase (GPT) levels normal BUN elevation	Arean & Henry, 1964
		AAT elevation AP elevation GPT levels normal BUN elevation	Higgins & Cousineau, 1977b
		Bilirubin elevation	Arean, 1962b
Hamsters	*pomona*	BUN elevation	Abdu & Sleight, 1965
Humans	*gryppotyphosa*	AAT elevation AP elevation GPT levels normal Bilirubin elevation	Breitenfeld & Gugic, 1977
	ictero-haemorrhagiae	BUN elevation Bilirubin elevation AP levels normal	Arean, 1962a
	bataviae *autumnalis* *australis* *akiyami*	BUN elevation Bilirubin elevation	Jaroonvesama *et al*, 1975
Horses	*canicola & pomona*	Bilirubin elevation	Bryans, 1955
Sheep	*pomona*	Arginase elevation AAT elevation GPT elevation AP levels normal	Millar *et al*, 1977

Plasma cholesterol and membrane cholesterol are freely exchangeable (Cooper, 1969; Gjone & Norum, 1970; Murphy, 1962a,b; Ways *et al*, 1963; Weed & Reed, 1966). Hence, liver disease may affect RBC shape via plasma cholesterol levels. The only workers to have measured plasma cholesterol in leptospirosis found no alteration in levels in sheep infected with *pomona* (Millar *et al*, 1977). Had alterations in the RBC shape occurred as a result of changes in membrane cholesterol concentration, then detection and sequestration of RBC's may have occurred in the spleen and if severe enough, in the liver (Crosby, 1977; Jandl *et al*, 1965; Jandl & Kaplan, 1960).

Alterations in clotting factors have been summarized in Table 1-2. The liver is the source of clotting factors I, II, V, VII, IX, X, XI, XIII (Benjamin, 1979) and while the haemorrhages in such leptospiral infections as *icterohaemorrhagiae* in guinea pigs was attributed predominantly to vascular damage (De Brito *et al*, 1966; De Brito *et al*, 1979; Miller *et al*, 1974) they may also have resulted from altered clotting factors caused by the liver damage which was also present.

2.4 RENAL LESIONS

The kidney has been the most studied of all organs in leptospirosis because it may show both gross and histological lesions in animals which have recovered from the infection and animals which showed no clinical signs following infection (Gleiser, 1957; Hadlow & Stoenner, 1955; Langham *et al*, 1958; Low *et al*, 1956; Morse *et al*, 1958; Reinhard & Hadlow, 1954; Sleight *et al*, 1960; Spradbrow & Seawright, 1963).

In acute cases of the disease, the kidneys are enlarged, swollen and friable (Abdu & Sleight, 1965; Arean, 1962a,b; Cordy & Jasper, 1952; Gleiser, 1957; Low *et al*, 1956; Monlux, 1948b; Reinhard, 1951; Spradbrow & Seawright, 1963). In those animals which either show no clinical signs or recover from acute infections, the kidneys are either normal in shape or

or slightly contracted. Pale radial or focal cortical lesions were seen in cattle (Hadlow & Stoenner, 1955; Reinhard, 1951; Sleight *et al*, 1964; Spradbrow & Seawright, 1963), cats (Fessler & Morter, 1964; Shophet, 1979), goats (Morse & Langham, 1958), sheep (Lindqvist *et al*, 1958; Marshall, 1973) and pigs (Langham *et al*, 1958; Sleight *et al*, 1960).

Histologically there is much variation in the renal lesions of those animals dying from acute severe leptospiral infections, those euthanased after survival of a severe infection and those which were mildly or subclinically infected. The major cellular changes are summarized in Table 1-6. In cattle (Reinhard, 1951; Spradbrow & Seawright, 1963), dogs (McIntyre & Montgomery, 1952; Monlux, 1948b) and hamsters (Miller & Wilson, 1967) large differences in the renal pathology have been described between animals infected with the same serovar.

Histochemical studies show large decreases in the isocitric, succinic, glutamic, glucose-6-phosphate dehydrogenases and to a lesser extent, lactic and malic dehydrogenases (Arean, 1962b; Arean & Henry, 1964). This was most pronounced in the renal pelvis but occurred to a lesser extent in the renal cortex. These decreases in the absence of histological lesions suggested functional defects (Arean & Henry, 1964) possibly due to renal hypoxia or a 'toxin'. Renal hypoxia has been suggested to be the result of hypovolaemia in humans (Sitprija *et al*, 1980). A leptospiral toxin was implicated in *pomona* infected hamsters (Miller & Wilson, 1967) and *icterohaemorrhagaie* infected guinea pigs (De Brito *et al*, 1966).

Changes in urine associated with leptospirosis are summarized in Table 1-5 and in severe cases are usually large.

Infected animals which were haemoglobinaemic were also haemoglobinuric (Hodges, 1974; Hodges *et al*, 1976; Millar *et al*, 1977; Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963). Haematuria was seen in *bratislava* infections of cattle (Fennestad

Table 1-5: Urinary changes during leptospirosis

Animal species	Leptospiral serovar	Urinary change	Author
Cattle	*bratislava*	Haematuria Proteinuria Urinary casts	Fennestad *et al*, 1967
	canicola	Haemoglobinuria	Turner *et al*, 1958
	pomona	Haemoglobinuria Proteinuria Haemoglobinuria	Reinhard & Hadlow, 1954 Ferguson *et al*, 1957
	unknown	Haemoglobinuria Proteinuria Alteration in specific gravity	Reinhard, 1951
Dogs	*bataviae*	Proteinuria	Keenan *et al*, 1978
	canicola & *ictero-haemorrhagiae*	Proteinuria	Bloom, 1941
		Decreased specific gravity Urinary casts present	McIntyre & Montgomery,1952 Monlux 1948a
		Decreased specific gravity	Taylor *et al*, 1970
Horses	*canicola* & *ictero-haemorrhagiae*	Proteinuria	Bryans, 1955
Humans	*ictero-haemorrhagiae*	Proteinuria Urinary casts present	Arean, 1962a
Sheep	*pomona*	Haemoglobinuria	Hodges, 1974 Hodges *et al*, 1976 Millar *et al*, 1977 Morse *et al*, 1957
Pigs	*pomona*	Haemoglobinuria	Bryan , 1954 Ferguson *et al*, 1956

Table 1-6: Cellular pathology of the kidney during leptospirosis

Renal pathological finding	Animal species	Leptospiral serovar	Author
Swelling of the glomerulus in animals dead or dying from early, severe leptospirosis	Cattle	*pomona*	Spradbrow & Seawright,1963
		szwajizak	Nervig *et al*, 1978
Haemorrhage into the Bowman's Capsule	Dogs	*ictero-haemorrhagiae*	Gleiser, 1957
Thickening of the Bowman's Capsule following severe leptospirosis infections	Cattle	*canicola*	Imbabi *et al*, 1967
		pomona	Ferguson *et al*, 1957
	Dogs	*canicola*	McIntyre & Montgomery,1952 Taylor *et al*, 1970
Shrinkage of the glomerulus as a result of severe leptospiral infections	Cattle	*canicola*	Imbabi *et al*, 1967
		ictero-haemorrhagiae	Dodd & Brakenridge,1960
		pomona	Ferguson *et al*, 1957
Thickening of glomerular basement membrane, fusion of foot processes of glomeruli and occasional endothelial cell damage in the glomerular capillaries	Hamsters	*pomona*	Miller & Wilson, 1967
	Humans	*ictero-haemorrhagiae*	De Brito *et al*, 1965 De Brito *et al*, 1967
	Guinea pigs	*ictero-haemorrhagiae*	De Brito *et al*, 1966 De Brito *et al*, 1979
	Sheep	*pomona*	Marshall, 1973
Vacuolation, loss of microvilli, swelling of the epithelial cells of the proximal convoluted tubules in animals dead or dying from acute, severe leptospirosis	Cattle	*bratislava*	Fennestad *et al*, 1967
		canicola	Imbabi *et al*, 1967
		ictero-haemorrhagiae	Dodd & Brakenridge, 1960
		pomona	Ferguson *et al*, 1957 Reinhard & Hadlow, 1954 Spradbrow & Seawright,1963
		unknown	Cordy & Jasper, 1952
	Dogs	*canicola*	McIntyre & Montgomery,1952 Taylor *et al*, 1970
		ictero-haemorrhagiae	Gleiser, 1957
		canicola & *ictero-haemorrhagiae*	Bloom, 1941 Monlux, 1948b
	Guinea pigs	*ictero-haemorrhagiae*	De Brito *et al*, 1966
	Hamsters	*pomona*	Abdu & Sleight, 1965 Sanger *et al*, 1961 Miller & Wilson, 1967

Table 1-6 continued

Renal pathological finding	Animal species	Leptospiral serovar	Author
	Humans	*icterohaemorrhagiae*	De Brito *et al*, 1965
	Pigs	*icterohaemorrhagiae*	Nisbett, 1951
Damage to the basement membrane of the tubules leading to the regeneration of the epithelial cells often as 'bizarre' cellular forms	Cattle	*bratislava*	Fennestad *et al*, 1967 Spradbrow & Seawright, 1963 Nervig *et al*, 1978
	Dogs		McIntyre & Montgomery, 1952
Mitochondrial changes and increased cellular inclusions and lysosomes during acute leptospirosis	Guinea pigs	*icterohaemorrhagiae*	De Brito *et al*, 1966
	Humans	*icterohaemorrhagiae*	De Brito *et al*, 1965 De Brito *et al*, 1967
Replacement fibrosis in the tubules of those animals surviving acute severe infections	Cattle	*bratislava* *pomona* *szwajizak*	Fennestad *et al*, 1967 Spradbrow & Seawright, 1963 Nervig *et al*, 1978
	Dogs	*canicola*	McIntyre & Montgomery, 1952 Taylor *et al*, 1970
		canicola & *icterohaemorrhagiae*	Bloom, 1941
Large interstitial accumulations of mononuclear cells in those animals surviving acute severe infections and smaller interstitial accumulations of mononuclear cells in sub-clinically or mildly affected animals	Cattle	*bratislava* *canicola* *pomona*	Fennestad *et al*, 1967 Imbabi *et al*, 1967 Ferguson *et al*, 1957 Hadlow & Stoenner, 1955 Reinhard & Hadlow, 1954 Spradbrow & Seawright, 1963
		unknown	Cordy & Jasper, 1952 Reinhard, 1951
	Dogs	*canicola*	McIntyre & Montgomery, 1952 Taylor *et al*, 1970
		icterohaemorrhagiae	Gleiser, 1957
		canicola & *icterohaemorrhagiae*	Bloom, 1941 Monlux, 1948b
	Goats	*pomona*	Morse & Langham, 1958
	Guinea pigs	*icterohaemorrhagiae*	De Brito et al, 1966
	Hamsters	*pomona*	Abdu & Sleight, 1965 Sanger *et al*, 1961
	Horses	*pomona*, *canicola* & *icterohaemorrhagiae*	Bryans, 1955

Table 1-6: continued

Renal pathological finding	Animal species	Leptospiral serovar	Author
	Sheep	*pomona*	Hodges, 1964 Lindqvist *et al*, 1958 Marshall, 1973
	Pigs	*pomona*	Langham *et al*, 1958 Sleight *et al*, 1960
Presence of leptospires either in the interstitium and/or renal tubules in acute, severe leptospiral infections	Cattle unknown	*pomona*	Hambdy & Ferguson, 1957 Spradbrow & Seawright, 1963 Cordy & Jasper, 1952
	Dogs	*canicola*	McIntyre & Montgomery, 1952 Taylor *et al*, 1970
	Hamsters	*pomona*	Abdu & Sleight, 1965 Miller & Wilson, 1967 Sanger *et al*, 1961
	Humans	*icterohaemorrhagiae*	Arean, 1962a
	Mice	*pomona*	Marshall, 1976
	Pigs	*icterohaemorrhagiae*	Nisbet, 1951
	Sheep	*pomona*	Marshall, 1973
Presence of leptospires in proximal convoluted tubules of animals surviving an acute, severe infection, or a mild or subclinical infection	Cattle	*pomona*	Hambdy & Ferguson, 1957 Hadlow & Stoenner, 1955 Reinhard & Hadlow, 1954 Spradbrow & Seawright, 1963
	Dogs	*canicola*	McIntyre & Montgomery, 1952 Morrison & Wright, 1976 Taylor *et al*, 1970
	Sheep	*pomona*	Marshall, 1973 Marshall, 1974
	Goats	*pomona*	Morse & Langham, 1958
	Hamsters	*pomona*	Miller & Wilson, 1967
	Pigs	*pomona*	Langham *et al*, 1958
	Grivet monkeys	*balcanica* & *tarassovi*	Marshall *et al*, 1980

et al, 1967) but was not noted in most other infections characterized by haemorrhage such as *canicola* and *icterohaemorrhagiae* infections of dogs (Gleiser, 1957; Low *et al*, 1956; Monlux, 1948b), *szwajizak* infections of cattle (Nervig *et al*, 1978) and *icterohaemorrhagiae* infections of guinea pigs (Higgins & Cousineau, 1977a,b). Red urine was seen in one *icterohaemorrhagiae* infected calf (Dodd & Brakenridge, 1960) and was probably due to haematuria because *icterohaemorrhagiae* does not usually cause haemoglobinaemia in any species. These workers did not attempt to differentiate between the two conditions.

Chronic renal diseases of causes other than leptospirosis have been associated with the presence of acanthocytes (Benjamin, 1979; Duncan & Prasse, 1978). Chronic renal disease has been associated with decreased RBC glutathione levels and decreased glutathione stability (Theil *et al*, 1961). The significance of this is unknown but glutathione is necessary to prevent oxidation of sulphydryl groups (Grimes, 1980; Weed *et al*, 1962; Weed & Reed, 1966) and to maintain reduced haemoglobin (Kaneko, 1974). Chronic renal disease also results in reduced erythropoietin and hence a non-regenerative anaemia (Benjamin, 1979; Duncan & Prasse, 1978).

2.5 BONE MARROW LESIONS

No marrow cytology and only a few histological studies have been made on the marrow of leptospiral infected animals.

In haemoglobinaemic cattle infected with *pomona* there was depletion of all cellular elements (Reinhard & Hadlow, 1954). In dogs infected with *canicola* and *icterohaemorrhagiae* evidence of granulopoiesis was seen (Monlux, 1948b). The depletion of the erythroid precursors (Reinhard & Hadlow, 1954) and extramedullary haemopoiesis in the spleen (Arean, 1962b; Arean *et al*, 1964; Bloom, 1941; Monlux, 1948b; Reinhard & Hadlow, 1954) reflected the severity of the anaemia.

2.6 BRAIN LESIONS

Nervous signs without corresponding histological lesions were reported in *pomona* infected hamsters (Abdu & Sleight, 1965). Humans infected with *ballum* and *tarassovi* have also been reported as having hallucinations and confused mental states (Marshall & Scrimgeour, 1978). Grivet monkeys *(Cercopithecus aethiops)* infected with *balcanica* and *tarassovi* (Marshall *et al*, 1980) and goats (Morse & Langham, 1958) and pigs (Sleight *et al*, 1960) infected with *pomona* were reported to have minor histological lesions consisting of perivascular cuffing of meningeal blood vessels by mononuclear cells, without observable nervous signs. Small, predominantly meningeal haemorrhages were reported in dogs infected with *canicola* and *icterohaemorrhagiae* (Monlux, 1948b). The nervous signs in hamsters were attributed to electrolyte imbalances rather than neurone damage (Abdu & Sleight, 1965). However, a toxic factor produced by *pomona* and *icterohaemorrhagiae* produced motor instability and muscular spasms shortly before death when injected intracerebrally into mice (Knight *et al*, 1973).

Few examinations of cerebrospinal fluid (CSF) have been made. Jaundiced dogs infected with *canicola* and *icterohaemorrhagiae* have been reported as having increased levels of bilirubin blood and lymphocytes within the CFS (Monlux, 1948b). *Icterohaemorrhagiae* infected humans occasionally are said to have increased protein and RBC's within the CSF (Arean, 1962a).

2.6 PULMONARY LESIONS

Varying degrees of congestion and haemorrhage have been reported in *icterohaemorrhagiae* infected humans (Arean, 1962a), *pomona* infected cattle (Hambdy & Ferguson, 1957) and *canicola* and *icterohaemorrhagiae* infected dogs (Bloom 1941; Monlux, 1948b).

Some dogs had mild bronchopneumonia (Bloom, 1941) while interstitial foci of inflammation composed of lymphocytes, plasma

cells and the occasional eosinophil have been observed in humans (Arean, 1962a). Faine (1964) is the only worker to describe the 'trapping' of leptospires within the lungs.

CHAPTER 2

MATERIALS AND METHODS

1.0 LEPTOSPIRAE

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1.1.2 *Balcanica*

1.2 LEPTOSPIRAL 'TOXIN' PREPARATION

1.3 EVALUATION OF INOCULUM VIRULENCE

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2.2.1 Experiment V: Haemolytic *pomona* infections

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CHAPTER 2

MATERIALS AND METHODS

In this chapter, details of the materials and methods used in several subsequent chapters of this thesis are described. Materials and methods specific to each chapter are included in the appropriate chapter.

1.0 LEPTOSPIRAE

1.1 PREPARATION OF LIVE LEPTOSPIRAL INOCULA

The leptospiral organisms used in the present studies, their origin and method of typing, are detailed in Table 2-1. The strain numbers are those used in the Leptospirosis Research Laboratory of the Department of Veterinary Pathology and Public Health, Massey University. Long term maintenance of leptospiral cultures was performed in semisolid media (Johnson & Seiter, 1977) at 27°C. Before inoculation of animals, the leptospire cultures were subcultured weekly for four weeks in EMJH media[1] (Johnson & Seiter, 1977) at 27°C to stimulate rapid growth and high concentrations of organisms within the culture. Replicate subcultures were made to allow for wastage due to poor growth or contamination which were evaluated weekly by dark field microscopy (dfm) examination[2].

1.1.1 *Ballum* and *pomona*

All hamster and calf inoculations were made using seven day old cultures of at least 10^7 organisms/ml in EMJH media. The volume of the inoculum used and the route of infection is detailed in sections 2.1 and 2.2.1.

1.1.2 *Balcanica*

Two inocula originating from *balcanica* strain E32 were used. The first inoculum was a seven day culture containing 1.5×10^8 organisms/ml in EMJH media. The second inoculum

1. DIFCO, Detroit 1, Michigan, U S A
2. Leitz Wetzlar, Ernst Leitz GMBH Weizlar,Germany 156xmag

Table 2-1 Strains of leptospire, their field source and method of typing.

Leptospiral serovar	Strain	Source	Typing method
ballum	1045	Brown rat (*Rattus norvegicus*) kidney	Restriction-endonuclease analysis (BRENDA)* Massey University (Marshall *et al* 1981).
	M4/9	Mouse (*Mus musculus*) kidney	Cross absorption agglutination: WHO Lab, Brisbane
	964	Brown rat (*Rattus norvegicus*) kidney	BRENDA: Massey University (Marshall *et al*, 1981)
	924	Brown rat (*Rattus norvegicus*) kidney	BRENDA: Massey University (Marshall *et al*, 1981)
	'Cat' 1	Domestic cat kidney. A reisolate of 1045 following an experimental infection	BRENDA: Massey University (Marshall *et al*, 1981)
balcanica	E32	Possum (*Trichosurus vulpecula*) kidney	Cross absorption agglutination: CDC**
pomona	T116	Pig urine	Cross absorption agglutination: CDC**
	790001	Kidney from a calf which died suddenly	Serotyping: Massey University (Alexander 1974).
	'Scamp'	Dog urine	BRENDA: Massey University (Marshall *et al*, 1981)
	MP3	Aqueous humour from the eye of a still-born piglet	BRENDA: Massey University (Marshall *et al*, 1981)
	13231	Aqueous humour from the eye of a calf found dead	Serotyping: Massey University (Alexander, 1974).

* All strains typed by BRENDA were also serotyped (Alexander, 1974) at Massey University

** CDC: Center for Disease Control, Atlanta, Georgia, U S A

consisted of the livers and kidneys from ten hamsters removed seven days post-infection with *balcanica* strain E32. A homogenate was made of 50 gms of liver and kidney in 500 mls of Stuart's media. Cattle were infected as described in Section 2.2.2.

1.2 LEPTOSPIRAL 'TOXIN' PREPARATION

A seven day-old culture of *pomona* strain 790001 was passed twice through a French Press[3]. Complete disruption of the organisms was confirmed by dfm examination and the disrupted culture, henceforth referred to as the 'toxin', was stored in sealed glass vials at 4°C until used as described in Table 2-3.

1.3 EVALUATION OF INOCULUM VIRULENCE

The virulence and haemolytic ability of the strains of *pomona* and *ballum* organisms for hamster experiments were tested by hamster inoculation. The choice of haemolytic organisms for the calf experiments was also based on the results of hamster experiments because of the unavailability of sufficient calves to enable virulence testing in this species.

In order to test the *balcanica* culture and to confirm the presence of leptospires within the homogenate, direct cultures were made of the inocula and two weanling hamsters were infected intraperitoneally (IP), euthanased after 21 days, and their kidneys were cultured. Leptospires were observed both in the direct cultures and in cultures made from hamster kidneys.

1.4 EVALUATION OF MEDIUM TOXICITY

To test for toxicity associated with EMJH medium, two groups of 12 hamsters each were inoculated intraperitoneally with 0.5 mls of EMJH medium and 0.3 mls of live *ballum* culture (strain 1045) respectively. Liver, lung, spleen and kidney were examined from six of the media inoculated hamsters when hamsters from leptospire inoculated group died and from the remaining six after three weeks during which none had shown adverse clinical signs. No lesions were observed in the medium inoculated group.

3. Wabash Hydraulic Div, Wabash, Indiana, USA

2.0 EXPERIMENTAL ANIMALS AND PROCEDURES

2.1 HAMSTERS

Weaned golden hamsters *(Mesocricetus auratus)* 19-42 days of age were obtained from the National Health Institute, Wellington, N.Z. in four groups for four separate experiments. They were kept on sterilized sawdust in stainless steel cages and fed on hamster nuts[4], supplemented with sunflower seeds, fresh fruit and vegetables. Water was freely available.

Experimental infection was carried out by an intraperitoneal injection of 0.25 - 0.30 mls of seven day old culture in EMJH medium. The animals were examined twice daily (9.00 am and 3.00 pm) and any moribund hamsters were euthanased and samples collected as described in Section 3.1. Hamsters were used in the four major experiments which are detailed below.

2.1.1 Experiment I: Haemolytic *ballum* infections

Hamsters infected with *ballum* strain 1045 were divided into experimental groups. In part 1, ten uninfected controls and 44 infected hamsters were euthanased sequentially as detailed in Table 2-2. In part 2 ten hamsters were infected. On day five post-infection, four hamsters were found dead and the six others with clinical signs of leptospirosis were killed.

The samples taken, their preparation and the results of their examination are described in Section 3.1 and Chapters 3, 4 and 5.

2.1.2 Experiment II: Comparison of the virulence of different strains of *ballum*.

Cultures of five strains of *ballum* (1045, M4/9, 964, 924 and 'cat' 1) were used to infect seven groups of six to eight hamsters, as described in Table 6-3.

4. Wairarapa Stock Foods Ltd, Carterton, N Z

Table 2-2 Dates of death of control and *ballum* infected hamsters from Experiment I part I.

Hamster	Day of death	Time of death
Controls		
0-1 to 0-5	Euthanased on day 0, the day of infection	am *
Ballum infected		
1-1 to 1-6	Euthanased on day 1 post infection	am
2-1 to 2-6	Euthanased on day 2 post infection	am
3-1 to 3-6	Euthanased on day 3 post infection	am
4-1 to 4-6	Euthanased on day 4 post infection	am
4-7	Found dead on day 4 post infection	am
5-1 to 5-6	Euthanased on day 4 post infection	pm **
5-7	Found dead on day 4 post infection	pm
6-1 to 6-7	Euthanased on day 5 post infection	am
7-1 to 7-5	Found dead on day 5 post infection	am
Controls		
8-1 to 8-5	Euthanased on day 6 post infection	am

* am: 9.00

** pm: 3.00

The preparation of the samples and results of examination are described in Section 3.1 and Chapters 4 and 6.

2.1.3 Experiment III: Comparison of the virulence of different strains of *pomona*

Three strains of *pomona* (T116, 790001 and 'Scamp') were used to infect three groups of six hamsters. The samples taken, their preparation and results of examination are described in Section 3.1 and Chapters 4 and 6.

2.1.4 Experiment IV: Reticulocyte production

Six weanling hamsters were bled daily for three days. On the first two days blood was removed by periorbital bleeding following ether[5] anaesthesia of the hamsters and on the third day blood was removed by snipping off the end of the tail. The animals were euthanased on day four. The results of examination, specimens taken and their preparation are described in Chapters 4 and 5.

2.2 CATTLE

All cattle used in this study were tested prior to experiment and found seronegative to the leptospiral serovars *pomona, ballum, balcanica, hardjo, tarassovi* and *copenhageni* using the method described by Cole *et al*,(1973). The cattle were used in two separate groups for the two experiments detailed below.

2.2.1 Experiment V: Haemolytic *pomona* infection

Nine female and one male Jersey calves designated 11 - 20 were obtained from a local commercial dairy farm on which no animals had shown serological evidence of *pomona* infection for three successive years. The farm was therefore considered to be *pomona* free. The calves were fed twice daily at 8.00 am and 4.30 pm with a milk substitute[6] from buckets for a week prior

5. May & Baker, Dagenham, England
6. Denkovite: W. & R. Fletcher, N.Z. Ltd

to and during the experiment. Calves 11-17 and 18-20 were managed together in two isolated groups respectively. Rectal temperatures were recorded and clinical examinations made at feeding. Blood and urine samples were collected each morning.

The experimental procedures carried out upon each calf are detailed in Table 2-3. The strain of *pomona* used for both the live leptospiral injections and 'toxin' formation was 790001. The intramuscular (IM) injections of live leptospires were made in the neck. Those calves given intravenous 'toxin' were also given 5 mls of streptopen[7] IM to prevent cross-infection from the calves injected with live leptospires with which they were in contact. The male calf, number 20, and hereafter referred to as the bled calf, was bled to mimic acute blood loss. One third of the total blood volume of this calf was calculated (Schalm *et al*, 1975, p.4) and removed by jugular puncture over 15 minutes.

The dates at which samples were taken are summarized in Table 2-3, the method of removal of samples is described in Section 3-2 and specific examinations and results are described in Chapters 3, 4 and 5.

2.2.2 Experiment VI: Non-haemolytic *balcanica* infection of yearling heifers

Twelve yearling Jersey and Jersey-cross heifers were run on grassed paddocks which had not been stocked for the previous six weeks. Four of the heifers were given 5 mls IM and 5 mls IP of culture of *balcanica* isolate E32 containing 1.5 x 10^8 organisms/ml. Another four heifers were given 5 mls IM and 5 mls IP of the homogenate of the liver and kidney described in Section 1.1.2. Two control heifers were inoculated with 10 mls of media or 10 mls of uninfected hamster liver and kidney respectively by the same IM and IP techniques used for the infected group. Two heifers were run as experimental controls.

7. Glaxo RD (250 mg procaine penicillin/ml and 250 mg dihydrostreptomycin sulphate /ml)

Table 2-3 Treatment of calves in Experiment V, an experimental *pomona* infection

Calf	Day of treatment	Treatment	Day of death
11	Day 0 pm	1.5 mls of live *pomona* culture culture intramuscularly (IM)	Euthanased on day 15, am.
12	Day 0 pm Day 1 am Day 1 pm	20 mls of 'toxin' + 5 mls Streptopen 15 mls of 'toxin' 20 mls of 'toxin' + 5 mls Streptopen	Euthanased on day 2, am.
13	Day 0 pm Day 1 am Day 1 pm	20 mls of 'toxin' + 5 mls Streptopen 15 mls of 'toxin' 20 mls of 'toxin' + 5 mls Streptopen	Euthanased on day 2, am.
14	Day 0, pm	1.5 mls of live *pomona* culture IM	Found dead on day 5, am.
15	Day 0, pm	1.5 mls of live *pomona* culture IM	Moribund on day 6, pm. Euthanased.
16	Day 0, pm	1.5 mls of live *pomona* culture IM	Euthanased on day 16, am.
17	Day 0, pm	1.5 mls of live *pomona* culture IM	Found dead on day 5, am.
18	Control	No treatment	Euthanased on day 16. am.
19	Control	No Treatment	Euthanased on day 15, am.
20	Day 11 pm	600 mls of blood removed from the jugular vein	Euthanased on day 12, pm.

The removal of samples is described in Section 3-2 and the specific examinations and results are described in Chapters 4 and 6.

2.3 EXPERIMENT VII: AN ACCIDENTAL HUMAN *BALLUM* INFECTION

A laboratory worker in the Leptospirosis Research Laboratory Massey University was accidentally infected with *ballum* strain M4/9 through a wound in a finger and developed leptospirosis. RBC samples were taken for electron microscopy as described in Section 3-3 and Chapter 4.

3.0 COLLECTION OF SAMPLES

3.1 HAMSTERS

Samples from all hamsters were collected at the time of euthanasia. Under ether anaesthesia, the jugular veins were severed with a scalpel blade and blood was drawn into heparinized capillary tubes[8]. Samples were collected for the differentiation of bone marrow smears, ultrastructural pathology, and histopathology. The detailed descriptions of the preparation procedures for the various examinations are included in the appropriate chapers.

Urinary changes were noted from visual evaluation only.

3.2 CATTLE

In all cattle experiments, samples for haematology and electron microscopy (EM) of RBC's were collected into EDTA containing vacutainers[9], samples for blood biochemistry and serology into plain vacutainers[9], and blood samples for leptospiral culture into heparinized vacutainers[9].

8. Catalogue No. 2501, Chase Instrument Corp., Poultney VT 05764
9. Venoject: Terumo

A midstream sample of urine was collected into sterile universal bottles. Urination was stimulated by stroking of the perineum. Calves were euthanased by the injection of 20 mls of euthatal[10] into the jugular vein.

In the yearling heifer experiment (2.2.2) blood samples for haematology, serology, and leptospire culture were collected daily for 15 days. RBC's for EM were processed on days 0, 5 and 7. On day 60 post infection the animals were euthanased.

All samples collected from the cattle and the examinations performed are discussed in the appropriate chapters.

3.3 HUMAN

Blood was drawn into a plain vacutainer[9] and two to three drops of blood were immediately transferred into fixative for subsequent processing and examination as described in Chapter 4.

10. May & Baker: Pentobarbitone Sodium 200 mg/ml

CHAPTER 3

CLINICAL SIGNS AND CLINICAL PATHOLOGY OF *POMONA* INFECTED CALVES AND *BALLUM* INFECTED HAMSTERS

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CHAPTER III

CLINICAL SIGNS AND CLINICAL PATHOLOGY OF CALVES INFECTED WITH *POMONA* AND HAMSTERS INFECTED WITH *BALLUM*

1.0 INTRODUCTION

Haemoglobinaemia and haemoglobinuria resulting from RBC destruction have been reported in *pomona* infections of calves (Reinhard, 1951; Spradbrow & Seawright, 1963) but older cattle appear to show few clinical signs apart from abortion (Morter *et al*, 1958; Sleight *et al*, 1964; Ferguson *et al*, 1957). Infections of hamsters with *pomona* are non-haemolytic (Abdu & Sleight, 1965; Miller & Wilson, 1966; 1967; Sanger *et al*, 1961), but a very brief description by Frenkel (1972) of *ballum* infections of hamsters reports that they cause RBC destruction.

This chapter compares the clinical signs, haematology and clinical biochemistry of calves infected with *pomona* and weanling hamsters infected with *ballum*.

2.0 MATERIALS AND METHODS

2.1 LEPTOSPIRES

Calves were infected with *pomona* strain 790001 and hamsters infected with *ballum* strain 1045 as described in Chapter 2. Methods of culture preparation, 'toxin' preparation and animal inoculation are detailed in Chapter 2, Sections 1.1, 1.2, 2.1.1 and 2.2.1 respectively.

2.2 ANIMALS

Calves and hamsters were obtained and maintained as described in Chapter 2, Sections 2.2.1 and 2.1 respectively.

2.2.1 Treatment groups

The treatment of calves (Experiment V) and hamsters (Experiment I, part 1) is detailed in Tables 2-3 and 2-2 respectively. Samples were taken daily from the calves and hamsters were euthanased daily to examine sequential changes in clinical pathology.

Clinical signs of the calves and hamsters were recorded as detailed in Chapter 2.

2.2.2 Controls

A total of ten hamsters were euthanased at the start and the end of the experiment (Table 2-2) and their haematological values used as controls (Table 3-1).

The haematological values of twelve normal 10-21 day old calves taken on two days, three days apart, are tabulated in Table 3-2 and used as population controls. The experimental controls are untreated calves 18 and 19 and bled calf 20.

2.3 LABORATORY PROCEDURES

(Abbreviations used in this chapter are standard and summarized in Appendix VI.)

2.3.1 Haematology

2.3.1.1 Total haemoglobin estimation

Total haemoglobin (hb) was measured by the cyanmethaemoglobin method as described by Schalm *et al* (1975; pp.54-55) using commercially prepared Drabkin's Reagent and standard solutions[1].

2.3.1.2 Packed cell volume

The PCV was measured using the microhaematocrit method (Schalm *et al*, 1975; pp45-47). Blood was centrifuged for five minutes at 13,000g.

1. Boehringer Mannheim GmbH Diagnostica, Catalogue No. 12479

Table 3-1 Normal haematological values for ten hamsters aged 19-42 days used in Experiment I, part 1.

	Mean Value ± S.D.
Hb (g/dℓ)	16.4 ± 1.2
PCV	0.50 ± 0.01
MCHC (g/dℓ)	32.5 ± 2.3
WBC ($x10^9/ℓ$)	4.7 ± 2.2
Lcyte ($x10^9/ℓ$)	4.2 ± 2.1
PMN ($x10^9/ℓ$)*	0.5 ± 0.37
IMM ($x10^9/ℓ$)	0
Mcyte ($x10^9/ℓ$)	0.09 ± 0.08
Eosin ($x10^9/ℓ$)	0.05 ± 0.04
Bas ($x10^9/ℓ$)	0
II**	0
TP (g/ℓ)	51.5 ± 1.1
M:E Ratio	2.8 ± 0.28

* Cells with the nucleus in a ring form are frequently seen

** Hamster plasma is clear to slightly cloudy due to lipaemia

Polychromatic cells were occasionally seen

Table 3-2 Normal haematological values for 12 calves 10-21 days of age. The data is based on two samples being taken from each calf on two days with a three day interval

	Mean value ± S.D.
Hb (g/dℓ)	14.8 ± 4.6
PCV	0.44 ± 0.13
MCHC (g/dℓ)	33.4 ± 1.2
WBC ($x10^9/\ell$)	7.1 ± 2.1
Lcyte ($x10^9/\ell$)	4.2 ± 1.3
PMN ($x10^9/\ell$)	2.7 ± 1.2
IMM ($x10^3/\ell$)	0
Mcyte ($x10^9/\ell$)	0.27 ± 0.2
Eosin ($x10^9/\ell$)	0.02 ± 0.05
Bas ($x10^9/\ell$)	0.03 ± 0.05
II	0
TP (g/ℓ)*	
Fib (g/ℓ)	5.4 ± 2.1

* There is considerable variation due to age in young calves. For more complete data see Thompson & Pauli (1981) (Appendix I)

2.3.1.3 Blood smears

Blood smears were prepared according to standard practices (Schalm *et al*, 1975;pp25-26). The smears were fixed using McNeal's tetrachrome stain[2] for three minutes than stained for six minutes using a phosphate buffer at pH 6.8.

2.3.1.4 Mean cell haemoglobin concentration

The MCHC was calculated according to the formula

$$\text{MCHC} = \frac{\text{Total haemoglobin} \times 100}{\text{PCV}}$$ (Schalm *et al*, 1975; p66).

2.3.1.5 Total white cell count

The total white cell estimations were made using standard dilution techniques of blood in acetic acid (Schalm *et al*, 1975; pp55-58) and counted in a standard haemocytometer[3].

2.3.1.6 Icterus index

The icterus index (II) was estimated using potassium dichromate standards as described by Schalm *et al*(1975; pp44-45).

2.3.1.7 Reticulocyte examination

Examination for reticulocytes was carried out on air dried smears of cattle blood prepared after the incubation of equal volumes of blood and new methylene blue[4] for 15 minutes at room temperature.

2.3.1.8 Bone marrow examination

Air dried smears of bone marrow were prepared from femoral marrow within 15 minutes of death of the animal. Hamster marrow was fixed for 15 minutes then stained for 15 minutes using McNeal's tetrachrome stain and a phosphate buffer at pH 6.8. Calf bone marrow was fixed for 20 minutes then stained for 25 minutes using McNeal's tetrachrome stain and the phosphate buffer.

2. Searle Diagnostic, High Wycombe, Bucks, England
3. Improved Nauerbauer, Hawksley Equipment, Lancing, Essex England
4. Searle Diagnostic, High Wycombe, Bucks, England

Differential counts of bone marrow cells were made on a minimum of 500 cells using suitable areas for counting on two slides of bone marrow smears. Because there is variation in the terminology used by various authors to describe the stages of erythroid cell maturation, the termination adopted in this thesis is defined and briefly described below. In this system, differentiation of cell type is gauged by cytological features rather than cell size *per se*.

ERYTHROBLAST

This is the most immature cell type recognised in the erythroid series. The cell has a large nucleus and small amount of deeply basophilic cytoplasm. The nucleus contains finely stranded chromatin and one or two prominent nucleoli. The cell is the largest in the erythroid series.

EARLY NORMOBLAST

This cell is similar in size and cytoplasmic characteristics to the erythroblast but the nucleus shows loss of the nucleoli and a small degree of condensation of nuclear chromatin.

NORMOBLAST

Normoblasts are smaller than the preceding cells with a relatively smaller nucleus and larger amount of cytoplasm. The nuclear chromatin shows obvious condensation and may have a 'cartwheel' appearance. The decreased cytoplasmic basophilia is due to haemoglobin formation.

LATE NORMOBLAST

This is the last stage of development before extrusion of the nucleus. The nucleus is small, pyknotic and often eccentrically placed in the cell. The amount of cytoplasm is large in relation to cell size and may have a pinkish tinge due to the presence of haemoglobin. Size is much reduced.

Loss of the nucleus results in the presence of free nuclei or haematogenes and reticulocytes or polychromatic cells. The haematogenes are phagocytosed by the macrophages of the marrow. These cellular elements are not included in the differential counts but alteration in the frequency of their occurrence is recorded.

The classification of the other cellular elements of bone marrow is as described by Schalm *et al* (1975; pp317-319).

2.3.2 Clinical biochemistry

Unless stated otherwise, the following tests were carried out on cattle samples only. The small volume of blood obtainable from young hamsters limited the number of tests performed on each sample.

With the exception of total plasma protein and fibrinogen all tests carried out on serum or plasma samples of cattle were on material stored at -4°C within two to four hours of collection and thawed immediately before use in the week following the death of the last calf. This time delay in measuring the biochemical parameters was necessitated by the time required to perform other experimental procedures requiring immediate attention.

2.3.2.1 Total plasma protein

Total plasma protein (TP) was measured on fresh plasma from calves and hamsters using a protein refractometer[5]. Plasma was obtained by the centrifugation of whole blood at 13,000g for five minutes in a sealed microhaematocrit tube (Schalm *et al*, 1975; pp47-50).

2.3.2.2 Fibrinogen

Fresh plasma from calves and hamsters prepared as above was heated to 56°C for three to four minutes to precipitate the fibrinogen. The microhaematocrit tube was then recentrifuged for two to three minutes to pellet the precipitated fibrinogen, and the remaining protein level in the plasma samples measured by the

5. Bellingham & Stanley Ltd

protein refractometer[5]. The fibrinogen level was calculated by the differences between protein recorded in the heated and non-heated plasma samples (Schalm *et al*, 1975; pp50-52).

2.3.2.3 Serum protein electrophoresis

Electrophoretic separation of calf serum proteins was performed within four hours of thawing after storage at -4°C using commercial equipment and reagents[6]. The serum sample was applied to an acetate strip prebuffered at pH 8.6 in barbitol buffer.

The separation was effected by a direct current gradient of 100 volts applied for 15 minutes. The strips were stained in Ponceau red dye for five minutes and the background colour removed by washing in 5% acetic acid. Following dehydration and clearing as described in the Helena method of protein electrophoresis[6] the strips were scanned in a Quickscan Flur Vis densitometer[6] at a wavelength of 525 nm. The levels of albumin and α, β and γ globulins were calculated from a graduated printout (Schalm *et al*, 1975; pp611-616).

2.3.2.4 Serum liver enzymes

The serum enzymes gamma glutamyl transpeptidase (GGT; EC 2322)[7], aspartate amino transferase (AAT; EC 2611)[8] and alkaline phosphatase (AP; EC 3131)[9] were measured on an autoanalyser 100[10] at a temperature of 30°C for GGT and AAT and a temperature of 37°C for AP. All measurements were carried out in duplicate.

2.3.2.5 Bilirubin levels

Total and direct bilirubin[11] levels of fresh hamster sera and stored frozen calf sera were measured using a spectrophotometer[12].

6. Helena Laboratories, Beaumont, Texas, USA
7. Diagnostica Merck No. 14302
8. Diagnostica Merck No. 3342
9. Diagnostica Merck No. 3314
10. Abbott ABA 100, Abbott Diagnostic Instrument Division, USA
11. Diagnostica Merck No. 3358
12. Bausch & Lomb, Spectronic 20, Watson Victor Ltd, Australia, N 3

2.3.2.6 Blood urea nitrogen

The urea nitrogen (BUN) of stored calf sera was measured using a semi-quantitative chromatographic method[13].

2.3.3 Examination of urine

Urine of hamsters was either absent, or present in such small quantities in the bladder that urinary examination was restricted to visual evaluation.

The specific gravity of calf urine was measured with a refractometer[14]. Haemoglobin, protein, blood, bilirubin and glucose content and the pH were measured using dipsticks[15]. The urinary sediment was examined using light microscopy following centrifugation within 20 minutes of collection.

2.3.4 Examination for leptospires

Plasma and urine from calves and plasma from hamsters was examined by dark field microscopy (dfm). The preparation of the plasma sample followed the method described by Mackintosh and Thompson (1979) (Appendix II).

The presence of leptospires in the calf blood was confirmed by culture following the methods described by Johnson & Seiter (1977).

Microscopic agglutination titres (MAT's) were measured on cattle sera stored at -4°C, following the methods described by Cole *et al* (1973) at a minimum dilution of 1:24.

13. 'Urastrats': Warner-Chilcott Laboratories, Morris Plains, New Jersey, 07950
14. Atago SPR-N, Atago Optical Works Co. Ltd., Japan
15. Combur 8 Test, Mannheim, Boehringer

3.0 RESULTS

3.1 CLINICAL SIGNS

3.1.1 Calves

Three of the five calves (numbers 14,15 & 17) infected with live leptospiral culture became haemoglobinuric on days five, six and five respectively post-infection. Calves 11 and 16 showed no signs of haemoglobinuria. The calves were slightly depressed and anorexic the day before haemoglobinuria occurred. After the onset of haemoglobinuria the calves quickly became moribund and calves 14 and 17 died. Calf 15 was euthanased when haemoglobinuric.

The daily temperature readings from the ten calves was tabulated in Appendices XVI to XXV. No calf had a sustained pyrexia and with the following exceptions, temperatures were diurnal and similar in all calves. Calves 14 and 15 had temperatures of 40.5°C and 40.0°C respectively on day five and calf 17 had a temperature of 40.0°C on day three.

Calf 17 showed tenesmus and had hard, dry faeces on day four. Calf 11 was diarrhoeic on day four and on day nine, both calves 11 and 16 had diarrhoea. The colour of faeces from calf 16 was normal. Those from calf 11 on day nine were of a greyish colour becoming creamy white on day 10. The intestinal disturbances at this time coincided with loss of condition, with calf 16 showing a slightly stunted appearance and calf 11 an obviously stunted appearance when compared to the control calves. By day 15, faeces from calf 16 were normal in appearance while those from calf 11 were almost normal in colour but with a jelly-like texture due to the presence of large quantities of mucus.

Urine from calf 16 remained normal in appearance while that from calf 11 appeared very yellow from days 12 to 14.

Immediately following the removal of one third of its blood volume on day ten, calf 20, the experimental control, was reluctant to stand and unsteady upon its feet but two to three hours later

appeared normal. Calves 12 and 13 showed no abnormal clinical signs.

3.1.2 Hamsters

Clinical signs, if seen, consisted of depression, anorexia, huddling in a corner of the cage and were followed rapidly by collapse and death. Occasionally, hamsters appeared to show irritation about the head region by the frequent rubbing in the region of the cheek and ear with the front paws. Some of these animals appeared to be photophobic with tightly shut eyelids and occasionally a yellow ocular discharge. Animals with clinical signs were obviously jaundiced with a yellow tinge to all mucous membranes and hairless areas of skin. Dehydration, shown by the loss of skin elasticity, was observed in moribund animals.

3.2 HAEMATOLOGY

3.2.1 Red blood cells

3.2.1.1 Calves

Measurements of parameters relating to RBC's are detailed in full in Appendices XVI to XXV. Alterations in the PCV and haemoglobin levels are demonstrated graphically in Fig. 3-I(i) & (ii). RBC measurements for the population controls are detailed in Table 3-2.

From days one to four the PCV and haemoglobin levels showed no change in the live leptospiral infected calves apart from minor day to day variation which also occurred in the control calves. On day five, calves 14 and 17 were haemoglobinaemic and had PCV's of 0.22 and 0.23 and haemoglobin levels of 6.8 and 7.3 g/dℓ respectively. Although calf 15 was not yet haemoglobinaemic, the PCV of 0.26 and haemoglobin level of 8.0 g/dℓ were below the levels of the control calves. The appearance of haemoglobinaemia coincided with an increase in the icterus index although accurate estimations of the icterus index could not be made due to the interference by the free plasma haemoglobin. By day six, calf 15

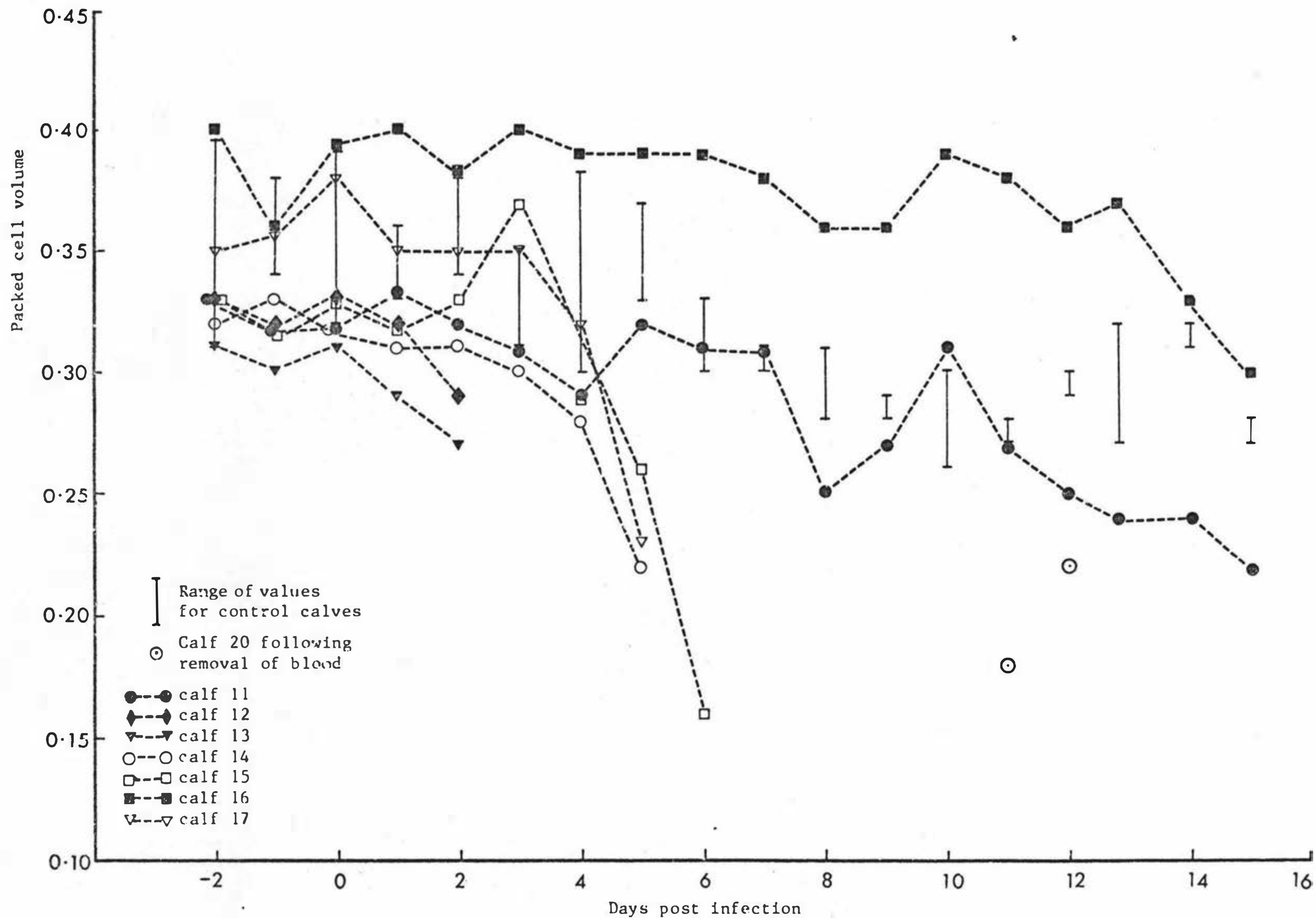


Fig. 3-1(i) Alteration in packed cell volume in control, infected and 'toxin' injected calves

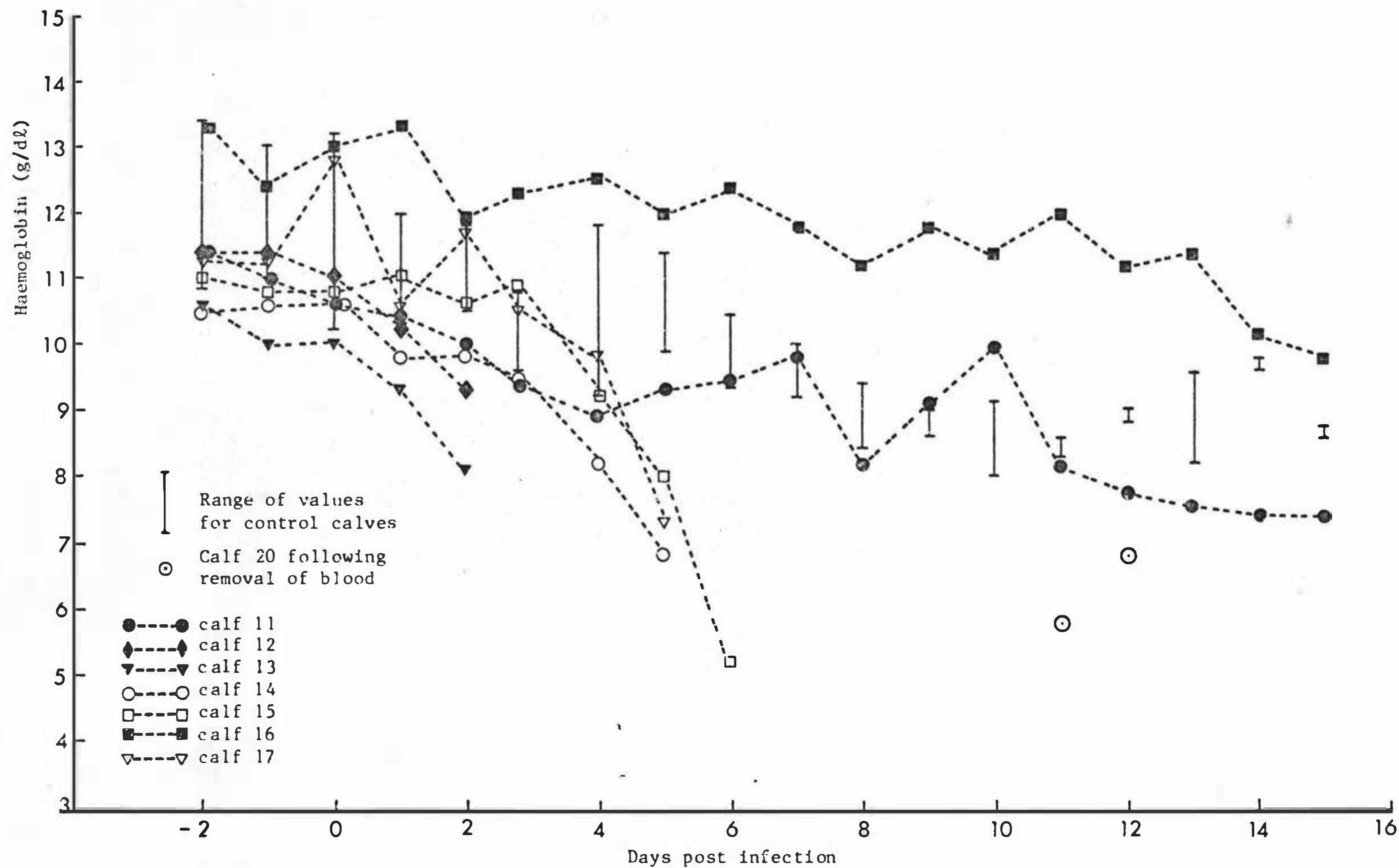


Fig. 3-1(ii) Alteration in haemoglobin concentration in control, infected and 'toxin' injected calves

was also severely haemoglobinaemic with a PCV of 0.16 and haemoglobin level of 5.2 g/dℓ. The PCV and haemoglobin readings for calf 16 remained higher than those of the control calves throughout the duration of the experiment (Fig. 3-I(i) & (ii)). The values for calf 11 dropped below those of the control calves from day 11 post infection (Fig. 3-I(i) & (ii)).

The MCHC values for all calves are tabulated in Appendices XVI to XXV. All calves showed a gradual overall decrease.

Cytological examination of the blood smears made prior to the development of haemoglobinaemia in calves 14, 15 and 17 revealed a slight increase in the numbers of crenated cells. In the haemoglobinaemic animals there was a large degree of anisocytosis and poikilocytosis. Late normoblasts, reticulocytes and erythrophagocytic neutrophils and monocytes were rarely seen even in those animals in which haemoglobinaemia was severe. There was little polychromasia. Rouleaux formation was seen in the RBC's of haemoglobinaemic calves. The bled calf also failed to show increased nucleated RBC's, polychromasia or a reticulocytosis.

Changes in the 'toxin' injected calves consisted of a slight lowering of PCV and haemoglobin levels on day two (Fig. 3-I(i) & (ii)). These values were slightly lower than those of all other control calves on that or previous days. No cytological alterations were observed in RBC's from non-haemoglobinaemic and 'toxin' injected calves.

3.2.1.2 Hamsters

The PCV, haemoglobin and MCHC values are detailed in full in Appendices VII to XIV. Data from selected hamsters is tabulated in Table 3-3 to emphasise the alteration in the PCV and haemoglobin levels in relation to the MCHC values.

There was little change in PCV or haemoglobin levels in hamsters euthanased on days one and two. On day three hamster (3-5) was haemoglobinaemic with a PCV of 0.42 and haemoglobin level of 14.2 g/dℓ.

TABLE 3-3 Data from selected* hamsters demonstrating the decrease in PCV and haemoglobin levels, the increase in the MCHC and alteration in icterus index, total protein and white blood cells.

Hamster	PCV	Hb (g/dℓ)	MCHC (g/dℓ)	II	TP (g/ℓ)	WBC (X10^9/ℓ)	PMN (x10^9/ℓ)	IMM (x10^9/ℓ)	Eosin (x10^9/ℓ)	Bas (x10^9/ℓ)	Lycte (x10^9/ℓ)	Mcyte (x10^9/ℓ)
2-1	0.52	16.2	31.2	0	54	5.4	0.8	0	0	0	4.6	0.1
2-2	0.50	16.2	32.4	0	54	1.2	0.3	0	0	0	0.9	0
3-2	0.44	14.4	32.7	0	58	4.5	1.6	0	0	0	2.8	0.1
3-5	0.42	14.2	33.8	15-20§	58	13.7	5.0	0.6	0.1	0	7.8	0.1
4-4	0.43	13.2	30.7	20+§	56	12.0	7.0	1.0	0.1	0	3.7	0.2
5-5	0.33	12.0	36.4	20+§	74	18.8	10.2	1.3	0.6	0	1.9	8.6
4-1	0.31	11.0	35.5	¶	80	14.5	7.7	1.3	0	0	5.5	0.6
6-5	0.28	12.6	45.0	¶	64	54.0	36.7	5.4	1.1	0	3.2	7.6
4-3	0.18	9.6	53.3	¶	96	17.5	9.2	2.8	0.2	0	5.3	0.4
5-1	0.12	8.6	71.7	¶	90	15.1	6.3	2.3	0.2	0	5.1	1.2
6-4	0.085	12.0	141.2	¶	82	34.3	14.7	4.1	1.0	0	9.9	4.5
6-1	0.035	5.8	165.7	¶	98	8.7	3.8	2.1	0.4	0	2.3	0.3

§ haemoglobinaemia

¶ severe jaundice and haemoglobinaemia

* complete data in Appendices VII to XIV

The plasma was yellow. Haemoglobinaemia was not seen in other animals on day three although one hamster (3-2) had a lower PCV and haemoglobin level.

On day four, three out of the six hamsters euthanased am were haemoglobinaemic and all six hamsters euthanased pm were haemoglobinaemic. On day five am when the remaining surviving hamsters were euthanased, six of the seven were haemoglobinaemic.

The severity of haemoglobinaemia increased with the decrease in the PCV and haemoglobin levels (Table 3-3). The increase in free plasma haemoglobin masked the increase in the icterus index but yellow discolouration of the plasma could still be distinguished (Fig.3-2).

Very low PCV and haemoglobin levels were accompanied by high MCHC values. Thus hamster (6-1) which had a PCV of 0.035 had an MCHC of 165.7 g/dℓ and hamster (6-4) with a PCV of 0.085 had an MCHC of 141.2 g/dℓ (Table 3-3).

Cytological examination of RBC's in blood smears from animals in which the PCV was slightly lower than normal and which were not yet haemoglobinaemic showed a slight increase in crenated cells. Crenated cells were also seen in the control animals. In those animals which were haemoglobinaemic, the RBC's showed a large degree of anisocytosis, poikilocytosis and spherocytosis. The degree of these cytological abnormalities increased with the decrease in the PCV, as did the degree of polychromasia. Few late normoblasts or normoblasts were seen.

Erythrophagocytosis by neutrophils and monocytes was common in those animals which were severely haemoglobinaemic (Fig. 3-3). Those hamsters which were bled to mimic RBC loss showed polychromasia and increased numbers of nucleated RBC's.

3.2.2 White blood cells

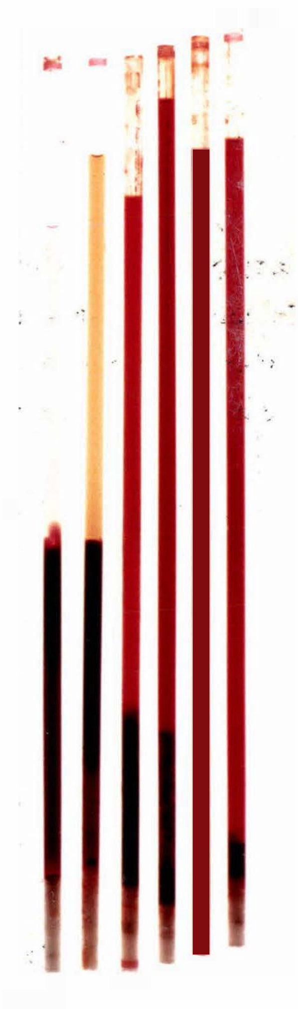
3.2.2.1 Calves

The total and differential WBC counts are detailed in Appendices XVI to XXV. All calves showed day to day variation in

Fig 3-2

Centrifuged capillary tubes of blood from a control hamster on the extreme left and, to the right, blood from five hamsters infected with *ballum* and showing increasing degrees of jaundice and haemoglobinaemia with a decrease in PCV.

PCV's from left to right: 0.50, 0.44, 0.25, 0.18, 0.12, 0.035.



total WBC numbers but significant differences in total counts between control and treated calves were not observed.

The highest WBC counts in the live leptospiral injected and haemoglobinaemic animals were $12.1 \times 10^9/\ell$ in calf 17 on day five, 11.0×10^9 cells/ℓ in calf 14 on day five and 11.7×10^9 cells/ℓ in calf 15 on day six. These counts were no higher than some obtained from the untreated control calves and slightly higher than those of the population controls (Table 3-2). The highest WBC counts for the non-haemoglobinaemic calves were on day ten on which calf 11 had a count of 13.7×10^9 cells/ℓ and calf 16 had a count of 12.9×10^9 cells/ℓ.

Although differences in total WBC counts were minor, the differential cell counts and cytological examination showed abnormalities in the infected calves. In the last blood samples taken before their deaths, calves 14,15 and 17 had increased numbers of band cells, with both mature neutrophils and band cells showing evidence of toxic granulation and vacuolation of the cytoplasm. Calf 14 had a transient neutrophilia of 5.4×10^9 cells/ℓ on day three and calf 17 had a transient neutrophilia of 5.3×10^9 cells/ℓ on day five. Calves 11 and 16 showed no alteration in neutrophil morphology but on day 11, calf 11 had a transient neutrophilia of 5.3×10^9 cells/ℓ. On day ten, calves 11 and 16 each had a lymphocytosis of 9.2×10^9 cells/ℓ and 8.4×10^9 cells/ℓ respectively.

The monocytes showed the greatest changes in numbers. Of the three calves which became haemoglobinaemic two had a monocytosis in the last blood samples taken before their deaths. Thus calf 14 had a count of 2.3×10^9 cells/ℓ on day five and calf 15 had a monocyte count of 2.7×10^9 cells/ℓ on day six. While calves 11 and 16 did not have monocyte counts as high as those from calves 14 and 15, a monocytosis was seen in calf 11 blood smears on days five and ten and in blood smears from calf 16 on days two, seven and ten.

3.2.2.2 Hamsters

The total and differential WBC counts are tabulated in full in Appendices VII-XIV. Data from selected hamsters is presented in Table 3-3 to illustrate particular points.

In contrast to the calves the hamsters showed very marked changes in the leucogram. The total and differential white cell counts could vary greatly depending on the animal and the stage of infection. The earliest changes were seen on day two in which hamster (2-2) had a profound leucopaenia and lymphopaenia and hamster (2-4) had a leucocytosis consisting of a slight neutrophilia and a large lymphocytosis (Table 3-3).

More severe changes occurred in subsequent groups of euthanased animals. For example, extremely high leucocytoses of up to 34.3×10^9 cells/ℓ and 54.0×10^9 cells/ℓ were seen in hamsters (6-4) and (6-5) respectively and comprised a neutrophilia and a left shift (Table 3-3). In all those animals in which haemoglobinaemia was present, regardless of the size of the neutrophilia there was a left shift with both mature and immature neutrophils showing toxic granulation, blue-grey colouration and vacuolation of the cytoplasm (Fig 3-3). Lymphocytoses were less evident but a monocytosis was often present in haemoglobinaemic animals. The largest monocytosis was that of hamster (5-5) (Table 3-3) in which 8.6×10^9 cells/ℓ were seen. In all haemoglobinaemic hamsters the monocytes were large, often clumped, contained dark granular inclusions, many vacuoles within the cytoplasm, and resembled macrophages. Intravascular erythrophagocytosis by neutrophils and monocytes (Fig. 3-3) was more frequent in hamsters than in calves.

3.2.3 Bone marrow

3.2.3.1 Calves

The results of the calf bone marrow differentials are summarized in Appendix XXVI. The myeloid:erythroid (M:E) ratios of untreated control calves 18 and 19 were 0.8 and 3.0 respectively.

The M:E ratios of the 'toxin' injected calves 12 and 13 were 0.8 and 0.4 respectively with 0.8 being normal and 0.4 below normal.

Differential counts of haemoglobinaemic calves were restricted to calf 15 only as the calves 14 and 17 which were found dead were not suitable for bone marrow differential counts due to autolysis of the marrow cells. The M:E ratio of 0.6 in calf 15 was slightly below the normal range of the control calves. Calves 11 and 16 which did not become haemoglobinaemic had M:E ratios of 0.3 and 0.4 respectively which were lower than those of the control calves. Calf 20 which was bled had an M:E ratio of 1.3 which was within the range of the untreated control calves.

The stages in the myeloid cell maturation were in the same proportion in 'toxin' injected calves 12 and 13, and bled calf 20 as in the control calves 18 and 19. There were increased numbers of myeloblasts in the infected calves 11, 15 and 16 with the number of mature neutrophils reduced in calf 11 and almost absent in calf 15. Cells of the myeloid series showed vacuolation of the cytoplasm and toxic granulation with some asynchrony of development between the cytoplasm and nucleus.

Proportions of cells in the various stages of erythroid development did not vary greatly between the control calves and treated calves. The only major cytological abnormalities noted occurred in calf 15 in which late normoblasts contained abnormally shaped nuclei or nuclear fragments (Fig. 3-5) and increased number of haematogenes were present.

3.2.3.2 Hamsters

There was considerable variation in the M:E ratio of control and infected hamsters (Appendices VII to XIV). Up to 30% of the cells in normal hamster bone marrow smears were small to medium sized lymphocytes.

The bone marrow from severely haemoglobinaemic hamsters grossly appeared to be more 'watery' than normal and upon

microscopic examination was hypocellular. Increased numbers of erythroid cell mitoses were seen in bone marrow of some leptospiral infected hamsters early in the disease. The occasional hamster had increased numbers of erythroblasts, as was illustrated by hamster (3-5). The most notable feature in the severely haemoglobinaemic hamsters was the presence of many late normoblasts containing abnormally shaped nuclei or nuclear fragments. In addition many haematogenes were observed and occasionally erythrophagocytosis by macrophages was seen.

In those hamsters which were severely haemoglobinaemic there was an almost complete absence of mature and band neutrophils (Fig. 3-4). Those cells of the myeloid series present showed toxic granulation and vacuolation of the cytoplasm and asynchrony between the nuclear and cytoplasmic development. Cells appeared larger than normal and the occasional binucleated cell was present. Degeneration of cells in all stages of myeloid development and phagocytosis of cellular debris was seen. Mitotic figures in cells of both the myeloid and erythroid series were commonly observed.

Megakaryocyte and lymphocyte morphology appeared normal. Some severely haemoglobinaemic hamsters appeared to have a relative increase in the numbers of marrow lymphocytes.

Although differential cell counts were not made on marrow from those hamsters which were found dead, evaluation of the cell density in smears showed the marrow to be hypocellular.

3.3 BLOOD BIOCHEMISTRY

3.3.1 Protein levels

Calf and hamster protein levels are tabulated in full in Appendices XVI to XXV and VII to XIV respectively.

3.3.1.1 Total plasma protein

The normal variation of total plasma protein levels of young calves are described by Thompson & Pauli (1981; Appendix I).

Fig 3-3

Blood smear from a haemoglobinaemic hamster infected with *ballum*. A monocyte (MO) with a vacuole containing a phagocytosed RBC (R) and a metamyelocyte (MM) with toxic granulation and blue-grey colouration of the cytoplasm are present.

McNeal's 1040x

Fig 3-4

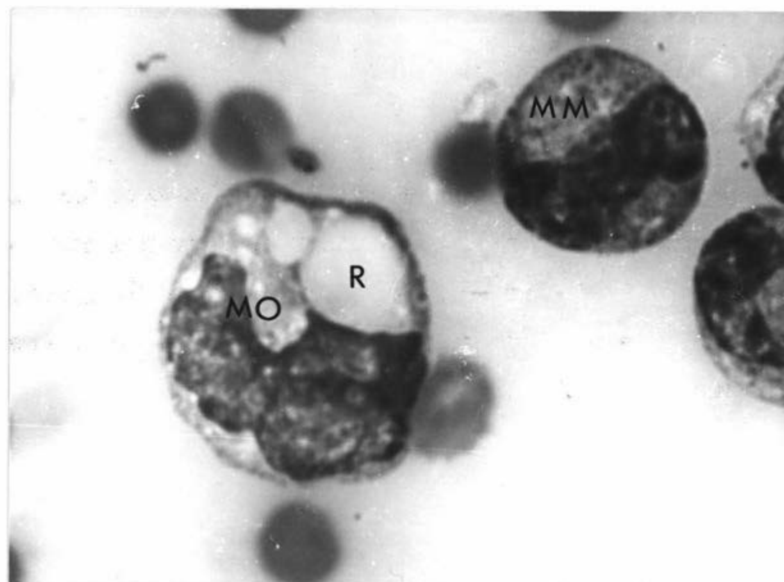
Bone marrow smear from a haemoglobinaemic hamster infected with *ballum*. There are increased numbers of early erythroid (e) and myeloid (m) cells in addition to large numbers of metamyelocytes (large arrows) and normoblasts (small arrows). Few band cells (b) and PMN's (p) are present.

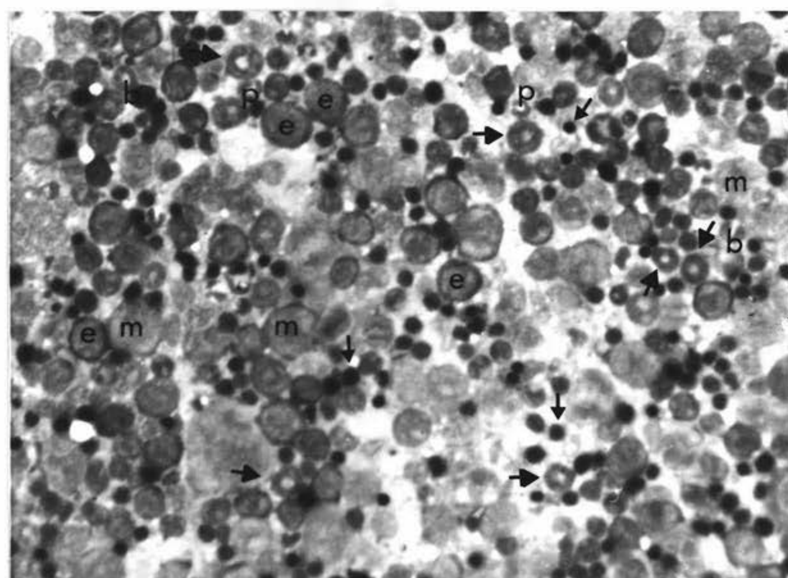
McNeal's 200x

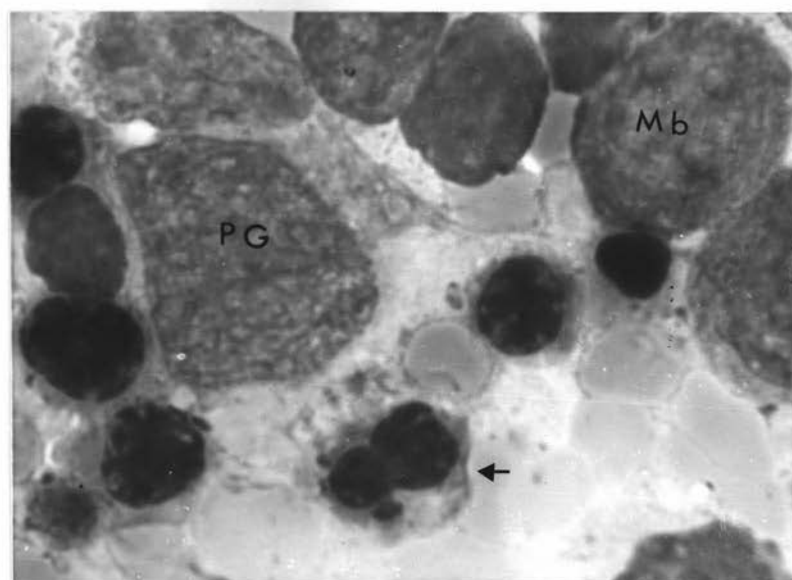
Fig 3-5

Bone marrow smear from haemoglobinaemic calf 15 infected with *pomona*. A normoblast (arrow) with an abnormally shaped nucleus and nuclear fragments, a progranulocyte (PG) and a myeloblast (Mb) are present.

McNeal's 1040x


MM
R
MO


R
e
e
p
m
b
e
e
m
m


Mb
PG

Young calves attain high levels of protein following ingestion of colostrum soon after birth and these levels gradually decline. The untreated calves 18, 19 and 20, and the 'toxin' injected calves 12 and 13 showed this decline.

Calves 14, 15 and 17 which became haemoglobinaemic showed small variation in the total protein levels with calf 15 showing a slight increase in total protein on day six. Total protein levels for calves 11 and 16 remained slightly higher than those of the control calves throughout the experiment but showed the characteristic overall decline in values with some day to day variation.

The total protein levels of selected hamsters are summarized in Table 3-3. Very high levels of total protein were seen in moribund hamsters with values as high as 96 g/ℓ and 98 g/ℓ in hamsters (4-3) and (6 -1) respectively.

3.3.1.2 Fibrinogen

All fibrinogen levels were normal except in calf 17 on day five where a level of 12 g/ℓ was measured.

3.3.1.3 Protein electrophoresis

The decline in total plasma protein levels in the calves was due to a decrease in γ globulin and to a lesser extent β globulin. While the albumen and α globulins showed some day to day variation, their levels did not show an overall decrease.

Those calves which became haemoglobinaemic showed more variation in the protein fractions. On day five calves 14 and 17 had a large increase in α globulin. On day six calf 15 had small increase in α globulin, which, while within the range of the control calves, was slightly higher than the previous readings for that particular calf.

From day nine post infection, values of α globulin for calves 11 and 16 were slightly higher than those of the control calves with calf 11 showing the greater increase.

3.3.2 Liver enzymes

The results are tabulated in full in Appendices XVI to XXV and results of GGT and AAT are demonstrated graphically in Fig. 3-6(i) & (ii) . Duplicate enzyme results were so close in value that the second result only is tabulated in the appendices.

Values for normal calves from birth to six weeks have been described by Thompson & Pauli (1981; Appendix I) and Bouda *et al* (1980). The levels of GGT and AP in all calves in this experiment followed the pattern of change described by them. AAT levels remained low in all control calves.

GGT levels of the calves 14, 15 and 17 decreased as expected until the last sample taken before death where values of 102 IU/ℓ, 5 IU/ℓ and 90 IU/ℓ respectively were obtained (Fig. 3-6(i)). The figure of 5 IU/ℓ was low but repeatable. All figures are probably inaccurate due to the free plasma haemoglobin.

AAT was raised in calves 14, 15 and 17 only when the animals were haemoglobinaemic with values of 77 IU/ℓ, 163 IU/ℓ and 82 IU/ℓ respectively. The value for calf 15 was the only value to alter to well above the normal range (Fig. 3-6(ii)).

Calf 16 showed no abnormality in liver enzyme levels. Alterations in liver enzyme levels of calf 11 occurred from day eight when serum GGT levels rose from 165 IU/ℓ to 179 IU/ℓ to reach a peak of 307 IU/ℓ on day 12. The levels of AAT rose to 78 IU/ℓ which was slightly above the normal values for cattle (Thompson & Pauli, 1981; Appendix I) and higher than values previously obtained for this calf. AP levels rose from 411 IU/ℓ on day eight to a peak of 843 IU/ℓ on day 12 then gradually declined. This peak of 843 IU/ℓ was higher than initial values of AP for this calf.

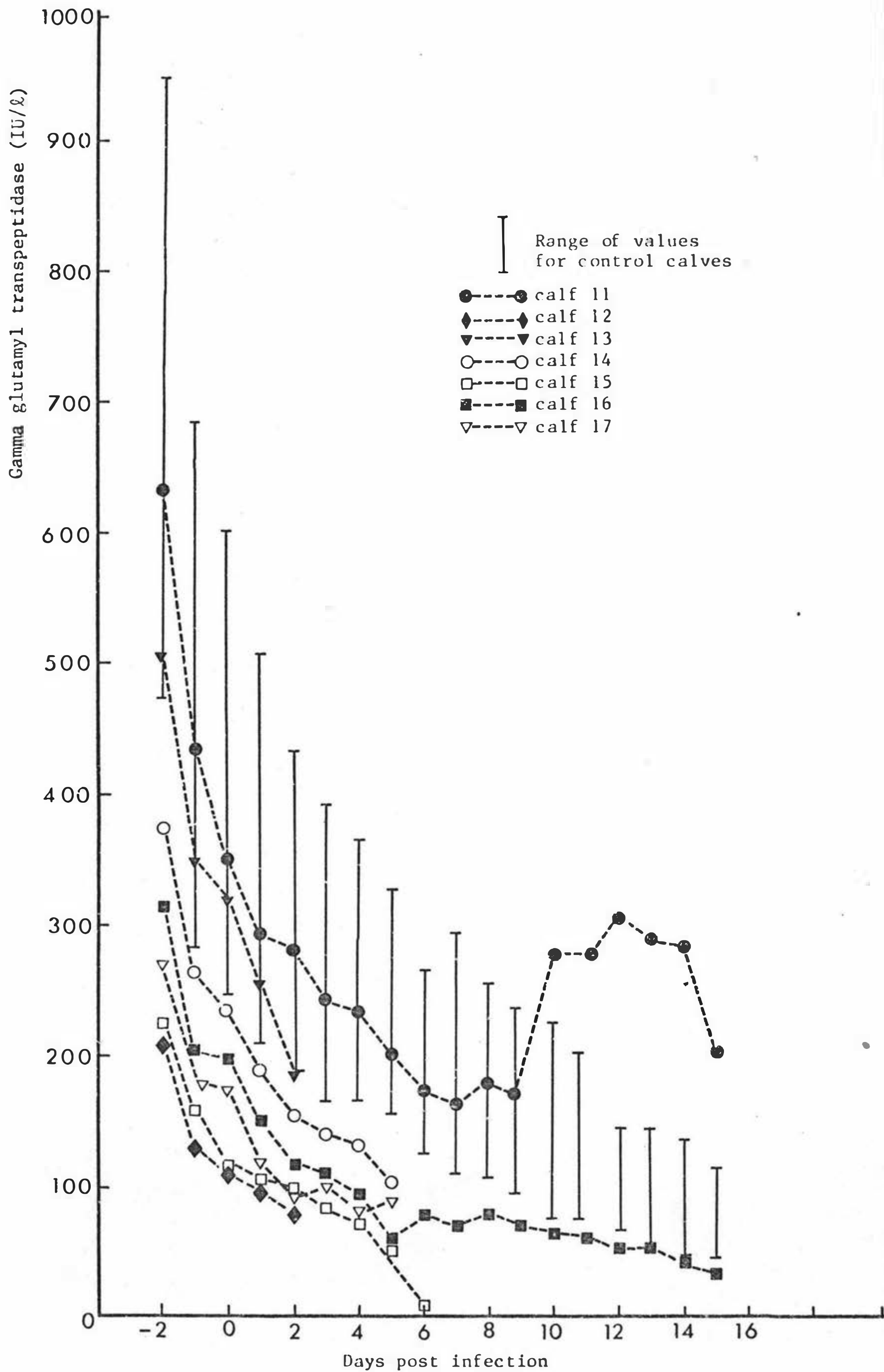


Fig. 3-6(i) Alteration in gamma glutamyl transpeptidase in control, infected and 'toxin' injected calves

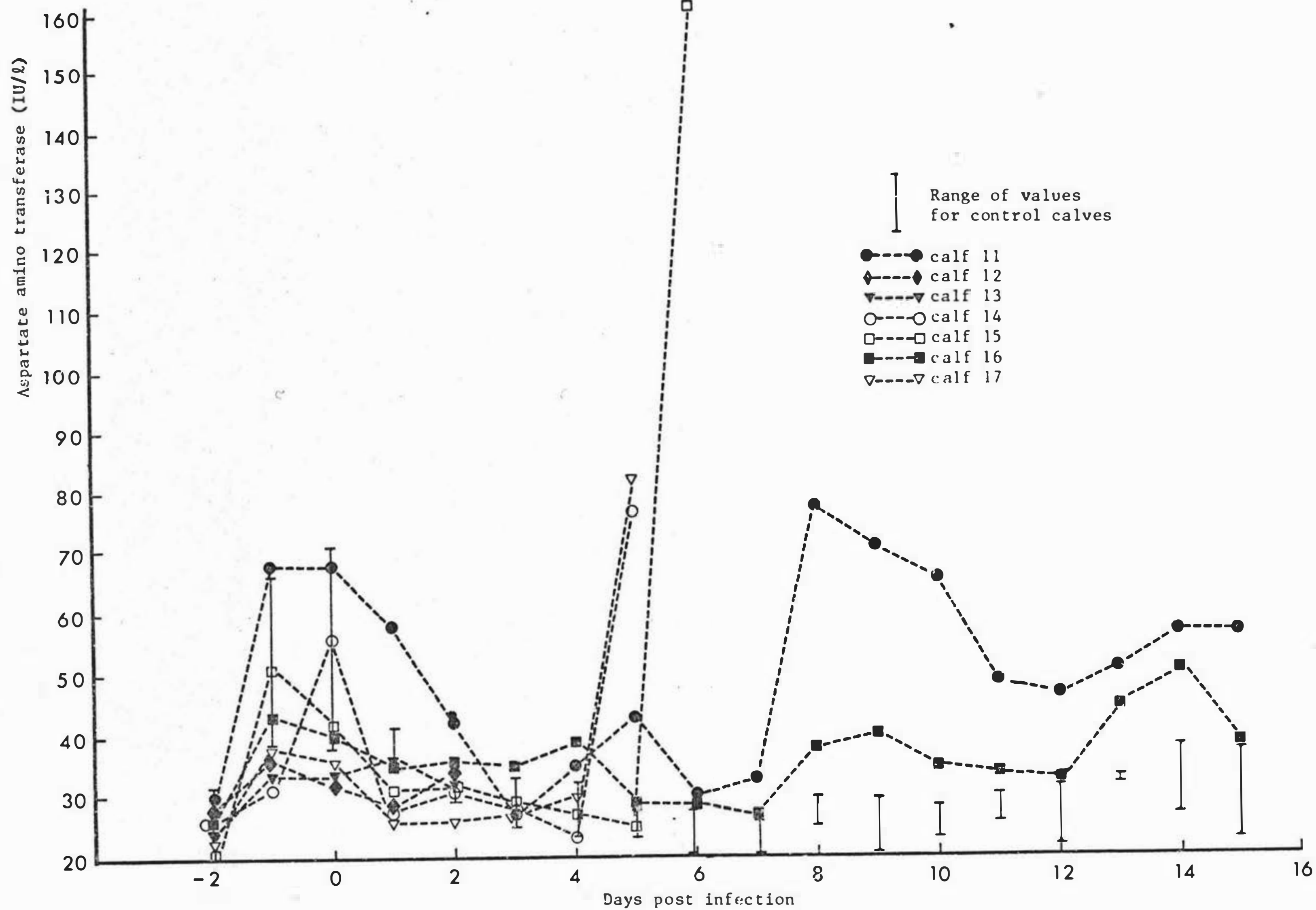


Fig. 3-6(ii) Alteration in aspartate amino transferase in control, infected and 'toxin' injected calves

3.3.3 Bilirubin

The calf bilirubin levels are detailed in full in the Appendices XVI to XXV.

Total bilirubin levels remained low in the non-infected calves 18, 19 and 20 and the 'toxin' injected calves 12 and 13. Higher levels of total bilirubin were measured in serum samples from calves 14, 15 and 17 taken the day before death. This increase in total bilirubin consisted of an increase in indirect or unconjungated bilirubin. Increases in direct or conjugated bilirubin were very small.

Bilirubin levels in calf 11 serum samples were raised from days 9 to 14, this increase consisting of both increased conjugated and unconjugated bilirubin. There were no increases in total bilirubin levels of serum from calf 16.

Attempts were made to measure bilirubin levels in hamsters up to day four. It was found that although increase in bilirubin levels occurred early in the disease, once the haemoglobinaemia became severe, the levels of bilirubin decreased. The bilirubin measurements were discontinued after day four as the large amount of haemoglobin interfered with the readings.

3.3.4 Blood urea nitrogen

The BUN measurements obtained from the calves are tabulated in the Appendices XVI to XXV.

There was no increase in the BUN levels of control calves 18, 19 and 20, the 'toxin' injected calves 12 and 13 and infected calf 16. They remained between 4.5 - 7.1 mmol/ℓ, below the upper limit of normal for cattle which at this laboratory is 9 mmol/ℓ. Calves 14, 15 and 17 had slightly increased BUN levels of 10.7, 12.5 and 11.6 mmol/ℓ in the serum samples taken the day before death occurred, when the animals were haemoglobinaemic. In calf 11 the BUN increased to 9.8 and 13.4 mmol/ℓ on days three and ten respectively.

3.4 EXAMINATION OF URINE

No significant findings were seen in the urinary sediment of calves except for the presence of RBC's in those calves which were haemoglobinuric. All calves showed variation in the pH and specific gravity measurements (Appendices XVI to XXV).

On the day before death calves 14, 15 and 17 were haemoglobinuric and proteinuric and urinary bilirubin was absent.

Calf 16 showed minor urinary changes. On day 13, a trace of protein was present. Calf 11 had greater urinary changes. On day ten traces of protein, glucose and bilirubin were present. By day 12, the urine was strongly positive for protein and glucose and moderately so for bilirubin. The urine was yellow. By day 15, protein, glucose and bilirubin were present only as traces and the colour of the urine was similar to that of the controls.

Visual examination of the hamster urine revealed that all haemoglobinaemic animals were also haemoglobinuric. Normal urine of hamsters is opaque and viscous. Those hamsters in which the PCV was starting to fall yet were not haemoglobinaemic had urine which was clearer than normal.

3.5 EXAMINATION FOR LEPTOSPIRES

Blood from calves 14, 16 and 17 was culturally positive for leptospires from day one while blood from calves 11 and 15 was culturally positive from day two. Positive cultures were obtained daily from calves 14, 15 and 17 until death. Calves 11 and 16 were culturally negative for leptospires from day 11.

Plasma samples on day five from calves 14 and 17 contained the occasional organism /hpf. That from calf 17 contained several organisms/hpf on day six. On day seven plasma samples from calf 11 contained the occasional organism/hpf with many organisms/hpf seen on day nine. In contrast, calf 16 had

only the occasional organism/hpf on days eight and nine. On day ten, calves 11 and 16 were no longer leptospiraemic by dfm.

Direct examination of urine from calves 14, 15 and 17 showed several organisms/hpf on the last samples obtained before death of the calves. Direct examination of urine from calf 11 revealed several organisms/hpf on days nine and ten increasing to many organisms/hpf on the day of euthanasia. No organisms were seen in the urine from calf 16.

The MAT's are summarized in Appendices XVI to XXV. No titres were found in calves 14, 15 and 17 on any day. Titres started to develop in calf 11 from day 11 and calf 16 from day nine.

The plasma from blood samples of haemoglobinaemic hamsters was positive for leptospires by dfm. Very few or no leptospires were seen in non-haemoglobinaemic hamsters.

4.0 DISCUSSION

Anaemia and haemoglobinuria resulting from RBC destruction were the most prominent clinical and clinical-pathological features of *pomona* infection of calves and *ballum* infection of hamsters. Although the RBC destruction when judged from PCV and haemoglobin levels appeared to be more severe in hamsters than in calves, it is likely that had blood samples been taken from the calves shortly before they died naturally from the disease, levels similar to those obtained from hamsters may have been seen. Death in both species appeared to be as a result of RBC destruction and certainly in the hamsters, with the extremely low PCV's of 0.035 and 0.085, tissue hypoxia could be expected to be of considerable importance in the development of lesions (Chapter 5) and clinical signs (Benjamin, 1979). The importance of RBC destruction in the death of hamsters has been demonstrated by experiments in which *ballum* infected hamsters were treated with sulfadiazine which prevented RBC destruction presumably by decreasing the numbers of leptospires in circulation and death occurred two to three weeks later due

to chronic renal failure (Frenkel, 1972). It was observed in the present experiments that severe RBC destruction commenced within five to six days in both species and death rapidly followed.

Although the present experiments show that calf deaths occur shortly after the onset of haemoglobinaemia and haemoglobinuria other work has shown that death following RBC destruction is not inevitable (Reinhard, 1951; Spradbrow & Seawright, 1963). Leptospiral infections of calves are more likely to be fatal or show clinical signs of infection than adult cattle (Fennestad *et al*, 1967; Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963; Turner *et al*, 1958). Although serial daily blood samples could not be obtained from hamsters, death appeared to occur 12-24 hours from the onset of haemoglobinaemia and it is probable that the onset of the clinical signs of anorexia and depression coincided with the onset of RBC destruction because all hamsters showing clinical signs were also haemoglobinaemic.

Those animals with the extremely low PCV's also had elevated total protein and clinical signs of dehydration. It therefore seems likely that the PCV levels were in reality lower than the levels recorded because dehydration leads to high PCV and total protein levels caused by reduction of the total plasma volume (Benjamin, 1979; Duncan & Prasse, 1978; Schalm *et al*, 1975).

The extremely high levels of MCHC in severely haemoglobinaemic hamsters is artefactual, resulting from total haemoglobin readings which include free plasma haemoglobin as well as the haemoglobin contained in intact RBC's. There is no way in which the MCHC may become increased above the upper limit of 36 g/dℓ as the RBC can not be supersaturated with haemoglobin (Benjamin, 1979). The raised MCHC's in these hamsters may have resulted from free plasma haemoglobin being removed more slowly than the haemolysed cells. Hamsters showed greater evidence than calves of intravascular removal of RBC's by monocytes and PMN's. The decreasing PCV, haemoglobin and MCHC values seen in the control calves is a normal phenomenon in neonatal calves (Benjamin, 1979).

The crenated RBC's observed in the blood smears may have been artefactual as they also occurred in some smears from control animals. The longer time taken for thicker smears to dry and the prolonged standing of blood before making the smear will cause such changes (Benjamin, 1979; Schalm *et al*, 1975). The spherocytes and poikilocytes seen in smears from haemoglobinaemic animals appear to correspond to the abnormally shaped RBC's described in Chapter 4.

The wide variations in the M:E ratios of normal animals seen in the present experiments has been reported by other workers (Benjamin, 1979; Schalm *et al*, 1975). In the present experiments, the severity of the infection in the haemoglobinaemic animals was reflected in the bone marrow as well as the peripheral blood. The M:E ratios alone were not particularly informative and must be viewed in conjunction with marrow cell cytology and the peripheral blood picture to provide accurate information on haemopoiesis (Schalm *et al*, 1975). In any situation if the demand for cells is such that cells of both erythroid and myeloid series are removed in approximately similar proportions the M:E ratio may remain within the normal range. If, as occurred in moribund hamsters, sufficient cells from both myeloid and erythroid cell lines are removed then the marrow will appear hypocellular while still maintaining normal M:E ratios. Increased numbers of mitotic figures and blast cells from both series indicated increased haemopoiesis. Abnormally nucleated late normoblasts in the severely haemoglobinaemic animals result from either the stress placed upon the marrow to produce RBC's or 'toxic' effects from the organisms upon the marrow cells. Such abnormally nucleated RBC's are frequently seen in myeloproliferative disorders involving the erythroid cell series (Thompson & Johnstone, In press: 1983; Schalm, 1980). Cytological changes in marrow smears from calf 15 were similar to those of the haemoglobinaemic hamsters which also had decreased M:E ratios. Loss of myeloid cells was also seen. While haemoglobinaemia did not occur in the 'toxin' injected calves, the lower M:E ratios in one calf suggested increased erythroid cell

production and, as will be discussed in Chapter 4, RBC's from the 'toxin' injected calves were abnormal in morphology and probably recognised by the spleen and removed (Crosby, 1977; Griggs *et al*, 1960; Rifkind, 1965; Wagner *et al*, 1962; Weiss, 1962a) with this net loss of cells stimulating erythropoiesis. The two calves which did not become haemoglobinaemic also showed increased erythroid cell production which was indicated by decreased M:E ratios and increased number of erythroblasts. As only two control calves were used in this experiment, the M:E ratios obtained can not be considered representative of normal animals.

Neutrophil abnormalities occurred in the marrow and the peripheral blood of calves and hamsters. The lack of a sustained neutrophilia in the calves was not unexpected as cattle have only limited neutrophil reserves and a leucopaenia is common in acute, severe infections (Benjamin , 1979; Schalm *et al*, 1975) and has been reported in *canicola* infections of cattle (Imbabi *et al*, 1967). The abnormal cytological features seen in neutrophils in both the peripheral blood and bone marrow, and the hypocellularity of the marrow reflect the greatly increased requirements for neutrophils and the inability of the marrow to cope with these demands. Both of these result from toxaemia. Little is known of the neutrophil reserve of hamsters, but the present results indicate that they must be sizeable and/or the bone marrow has the ability to respond rapidly. That species variation occurs, is illustrated by the work of Ludwig & Kohn (1964) who have shown that Chinese hamster marrow has a greater ability than that of the mouse for proliferation.

Monocytosis, while usually associated with inflammatory conditions (Schalm *et al*, 1975) has also been reported to occur with internal haemorrhage and haemolytic disease (Benjamin, 1979; Duncan & Prasse, 1978; Schalm, 1980; Zinkl, 1981). These disorders require phagocytosis to remove macromolecular particles. While monocytes with the characteristics of macrophages have occasionally been reported in peripheral blood, little is known of their significance (Zinkl, 1981). It seems likely that in the present

experiments that the monocytoses were due to an increased demand for phagocytes caused by cellular necrosis and RBC destruction.

The profound blood and bone marrow abnormalities recorded in the haemoglobinaemic animals is in contrast to the minor changes in the nonhaemoglobinaemic calves and the 'toxin' injected calves. In order to reproduce the clinical disease and haematological changes noted in the haemoglobinaemic animals it is probable that larger quantities of 'toxin' would have to be continually infused. In the natural disease it is probable that large quantities of 'toxin' are released from the large numbers of organisms in the bloodstream and tissues. While the deaths of the haemoglobinaemic animals appeared to result primarily from hypoxia following RBC destruction, the effects of 'toxins' produced by the leptospires acting upon other body organs must also be considered. The effects of leptospiral 'toxins' in the absence of haemoglobinaemia was shown by the clinical pathological results of calf 11. That this calf was susceptible to infection was seen by the development of clinical signs and a leptospiraemia which was larger than that of the haemoglobinaemic calves. Some strains of *ballum* as will be demonstrated later produce death without haemoglobinaemia by kidney and liver insufficiency. Also as discussed earlier, sulphadiazine treatment of hamsters infected with a haemolytic strain of *ballum* prevented RBC destruction and the animals died later from nephritis (Frenkel, 1972).

The increased total bilirubin in haemoglobinaemic animals was not an indication of hepatic dysfunction as it consisted mostly of increased unconjugated bilirubin and coincided with the onset of haemoglobinaemia. This reflected the large scale RBC destruction and the inability of the liver to cope with the sudden production of a large quantity of unconjugated bilirubin (Benjamin, 1979; Duncan & Prasse, 1978; Schalm *et al*, 1975). This contrasted with calf 11 in which liver dysfunction was shown by the presence of large quantities of unconjugated and conjugated bilirubin which indicated that the liver could still conjugate the bilirubin but was unable to excrete it into the bile (Benjamin, 1979; Duncan &

Prasse, 1978). While jaundice is sometimes present (Ferguson *et al*, 1957) some workers comment on its absence even though RBC destruction has occurred (Spradbrow & Seawright, 1963). Jaundice in sheep with haemolytic *pomona* infections has been reported as being due to increased unconjugated bilirubin. This indicates that the increases are due to RBC destruction rather than the inability of the liver to conjugate and excrete the bilirubin (Hodges, 1974; Millar *et al*, 1977).

The enzyme levels from the haemoglobinaemic calves are considered inaccurate because plasma haemoglobin interferes with readings. As alterations in enzyme levels were not recorded until the calves became haemoglobinaemic, it is apparent that liver lesions detectable by serum enzymes did not occur until late in the course of the disease. Nevertheless raised levels of AAT are suggestive of hepatocyte necrosis and the presence of this was confirmed histologically (Chapter 5).

As calves 11 and 16 did not become haemoglobinaemic, levels of their serum enzymes may be considered accurate. Hepatic dysfunction as indicated by AP, AAT and GPT was not detected in calf 16 but was seen in calf 11. The rise in AAT was indicative of hepatocyte necrosis as this enzyme is intracellular and released following hepatocyte destruction (Benjamin, 1979; Duncan & Prasse, 1978). AAT may also be released from damaged muscles and may be differentiated from hepatic AAT by isoenzyme studies (Benjamin, 1979). Isoenzyme studies were not done but it is considered that the raised AAT in calf 11 was of hepatic origin as the calf did not show any evidence of muscle disease or spend long periods recumbent and other signs of liver disease were present. Raised GGT levels have been shown to be indicative of bile duct disease (Blackshaw, 1978). Cholestasis was indicated by the presence of raised serum conjugated bilirubin and raised AP. Both AP and conjugated bilirubin are excreted into the bile and levels are raised as a result of cholestasis (Benjamin, 1979). Pale, pasty coloured faeces result from a lack of faecal stercobilin which results from intestinal breakdown of bilirubin. Benjamin (1979) considered that pale,

pasty coloured faeces are characteristic and may be considered diagnostic of cholestasis.

Although haemoglobinaemia did not occur in calf 11 it appears that there was increased removal of RBC's from circulation. Abnormal RBC's were seen at the time of maximum leptospiraemia (Chapter 4) and this coincided with the release of liver enzymes and the decrease in the PCV and haemoglobin levels. RBC morphology is influenced by such factors as plasma cholesterol and fatty acids (Gjone & Norum, 1970; Murphy, 1962a,b; Ways *et al*, 1963), the former factor controlled by the esterification activity of the enzyme lecithin-cholesterol acyltransferase (LCAT) (Raz *et al*, 1969). The activity of LCAT is depressed in patients with obstructive jaundice (Gjone & Norum, 1970) and it is possible that the RBC abnormalities noted in this calf were the result of a similar process. Such an alteration in the RBC membrane would be detected by the spleen and the subsequent removal of RBC's result in anaemia and increased erythropoiesis.

In the haemoglobinuric calves, the raised BUN and proteinuria coincided with the onset of haemoglobinuria. Haemoglobinuria has been described as a cause of tubular nephrosis (Flink, 1947; Jaenike & Schneeberger, 1966) and haemoglobinuria rather than the presence of organisms is considered to be the most likely explanation of the renal changes observed. The abnormal urine in calf 11 coincided with the peak of leptospiraemia and larger numbers of organisms were seen in its urine when compared to the haemoglobinuric calves. The pathogenesis of the renal dysfunction of calf 11 was probably related to damage caused by the presence of leptospires and/or their 'toxins' in the renal tubules. Marshall (1973) reported endothelial cell damage caused by migrating organisms and ultra-structural lesions of glomeruli and tubules in animals surviving 30 days post infection. The cause of the glycosuria is not known but it occurred at the peak of leptospiraemia and was possibly due to tubular dysfunction and inability of the epithelial cells to resorb the glucose from the urinary filtrate. Blood glucose levels were not measured so it is not known if the glycosuria resulted from hyperglycaemia.

In calf 11, biochemical changes indicative of hepatic and renal lesions coincided with the peak of leptospiraemia and resolution occurred after the development of antibody. The development of antibody resulted in a decrease in plasma leptospires followed by the gradual resolution of the hepatic and renal lesions which produced the biochemical changes. The appearance of antibody was associated with decrease in plasma leptospires in other work (Hambdy & Ferguson, 1957; Sleight *et al*, 1964). MAT's developed late in the infection and the haemoglobinuric calves did not survive long enough to develop titres.

Loss of condition of surviving animals as occurred in calf 11 has been noted in other *pomona* infections of cattle (Spradbrow & Seawright, 1963) and those by an unknown serovar (Reinhard, 1951). The survival of calves 11 and 16 appeared to be related to the fact that haemoglobinaemia and RBC destruction did not occur.

The reason for the diarrhoea is not known. Possibly it may be due to digestive disturbances in calf 11 where cholestasis prevented sufficient bile from reaching the intestines. As will be commented on in Chapter 5, no histopathological lesions were present to explain the diarrhoea. Intestinal dysfunction resulting in diarrhoea (Fennestad *et al*, 1967; Imbabi *et al*, 1967; Reinhard, 1951) and constipation (Fennestad *et al*, 1967) have been reported in other bovine leptospiral infections.

Increased α globulin levels are non-specific for leptospirosis and RBC destruction, and may be found in a variety of inflammatory conditions and tissue destruction as well as acute and chronic renal disease (Benjamin, 1979; Schalm, *et al*, 1975).

5.0 CONCLUSIONS AND SUMMARY

1. Haemoglobinaemia occurred in calves and hamsters about five to six days post infection with *pomona* strain 790001 and *ballum* strain 1045 respectively. Death followed 12-24 hours after the onset of haemoglobinaemia and haemoglobinuria.

2. In the present experiments, while the animals could be severely leptospiraemic as was seen in calf 11 and to a lesser extent calf 16, death was not inevitable. RBC destruction appeared to be the cause of death in the haemoglobinaemic animals.

3. The most severe stages of the infection were reflected by severe changes in peripheral blood and bone marrow. The M:E ratios taken in conjunction with the cytological examination of the bone marrow and peripheral blood haematological results showed increased demand for RBC's and WBC's.

4. Bilirubin increases in haemoglobinaemic animals resulted from RBC destruction rather than liver dysfunction as the increased total bilirubin comprised mostly unconjugated bilirubin. In one infected, non-haemoglobinaemic calf an increased total bilirubin level consisting of raised conjugated and unconjugated bilirubin indicated liver dysfunction.

5. Raised hepatic enzymes indicative of liver disease were seen as a terminal condition in haemoglobinaemic calves. Liver enzymes were also raised in one infected non-haemoglobinaemic calf at about the time of the peak of leptospiraemia.

6. Renal dysfunction coincided with haemoglobinuria in the haemoglobinaemic calves and the peak of leptospiraemia in the non-haemoglobinaemic calf. The haemoglobinuric nephrosis was not considered severe enough to have caused death at that time as the signs of renal dysfunction were more severe in the non-haemoglobinuric calf which survived.

7. The leptospiraemia in the surviving calves was terminated at the same time at which their MAT's developed. These calves remained leptospiruric. The haemoglobinaemic calves died before they had time to develop an MAT.

CHAPTER 4

MORPHOLOGICAL CHANGES IN RED BLOOD CELLS IN LEPTOSPIROSIS

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CHAPTER 4

MORPHOLOGICAL CHANGES IN RED BLOOD CELLS IN LEPTOSPIROSIS

1.0 INTRODUCTION

Haemolysis is the early removal of RBC's from circulation (Petz & Garratty, 1980). This may be as a result of intravascular destruction of severely damaged RBC's, the alterations in which are detected by the spleen and the liver (Jandl, 1966; Rifkind, 1966; Weed & Reed, 1966).

Preliminary morphological studies of RBC's from leptospiral infected calves and hamsters (Thompson, 1982; Appendix III) showed ultrastructural changes which were different to those reported in other haemolytic anaemias such as those caused by *Bartonella bacilliformis* (Cuadra & Takano, 1969) and *Plasmodium* species (Balcerzak *et al*, 1973; Bodammer & Bahr, 1973) infections in man.

In this chapter, the ultrastructural changes in RBC's from hamsters, calves and a human infected with leptospirosis are described.

The infections in these experiments are divided into the two main categories of haemolytic and non-haemolytic on the basis of the presence (predicted and proven) or absence of haemoglobinaemia. Clinical pathological and histopathological evidence for increased RBC removal and destruction is discussed in Chapters 3, 5 and 6.

2.0 MATERIALS AND METHODS

2.1 SOURCE OF RBC's FOR ELECTRON MICROSCOPY

2.1.1 Haemolytic leptospiral infections

2.1.1.1. Cattle

Blood samples from calves infected with *pomona* strain 790001 and 'toxin' injected calves in Experiment V were taken from

all living animals on days 0, 2,5,8,9 and 12 post infection for scanning and transmission electron microscopy (SEM and TEM). Blood from the non-infected calves 18 and 19 was processed at the same time as that of the infected calves.

2.1.1.2 Hamsters

Samples were obtained from hamsters infected with *ballum* strain 1045 in Experiment I parts 1 and 2 as described in Chapter 2. In part 1 of the experiment RBC's were taken from control and infected hamsters as described in Table 2-2 and Section 2.1.1, Chapter 2. Part 2 involved processing of RBC's from two control hamsters and six hamsters infected with *ballum* strain 1045 showing signs of varying degrees of severity of disease (Chapter 2, Section 2.1.1, part 2).

2.1.2 Non-haemolytic leptospiral infections

2.1.2.1 Cattle

Blood was taken on days 0, 5 and 7 post-infection from cattle infected with *balcanica* as described in Experiment VI, Chapter 2. Further samples were not taken as the animals showed no clinical signs of infection.

2.1.2.2 Hamsters

Blood was taken from three moribund hamsters infected with *pomona* strain 'Scamp' as described in Experiment III, Chapter 2, Section 2.1.3 and three moribund hamsters infected with *ballum* strain 1045 as described in Experiment II, Chapter 2, Section 2.1.2. None of these animals were showing signs of haemoglobinaemia.

2.1.3 Reticulocyte production

Hamster reticulocyte production was stimulated by bleeding (Experiment IV, Chapter 2, Section 2.1.4). Reticulocyte production by bleeding of calf 20 was unsuccessful (Experiment V, Chapter 2, Section 2.2.1).

2.1.4 Human *ballum* infection

Blood was taken from a human female patient 14 days after an accidental *ballum* strain M4/9 infection and seven days after

the onset of clinical signs of leptospirosis. RBC's were prepared for SEM and TEM examination. Clinical signs consisted of pyrexia, headaches, nausea, muscle pains and inappetance. At seven days the blood was leptospiraemic by dfm. Blood was taken for SEM and TEM preparations of RBC's five months after recovery, seven months after the initial infection. RBC's from a normal woman were also processed as controls.

2.2 FIXATION OF RBC's

RBC's were fixed within 30 minutes of collection by suspension of two to three drops of blood in 10 mls of 0.1M phosphate buffered glutaraldehyde at pH 7.4 (Appendix V) for one to two hours at 4°C.

2.3 PREPARATION FOR SCANNING ELECTRON MICROSCOPY

The SEM preparation method is that of Jain & Kono (1972). Following fixation, the RBC's were washed three times in 0.1 M phosphate buffer (Appendix V) at pH 7.4 and a temperature of 4°C. The supernatant was removed between washings following centrifugation at 4356 g for five minutes. The RBC's were then dehydrated by serial passage through an ascending series of 25% 50% and 75% acetone and water with two final passages through 100% acetone. The RBC's remained in each concentration of acetone for 30-45 minutes at room temperature. Following each dehydration step, the solution containing the RBC's was centrifuged at 4356 g for five minutes, the supernatant removed and the cells resuspended in acetone at a higher concentration. Following the 2nd passage through 100% acetone, the RBC's were centrifuged, the supernatant removed and the cells resuspended in 1-2 mls of fresh 100% acetone. Two to three drops of this suspension were run from a Pasteur pipette down a cover slip held at an angle of 45° from vertical. After evaporation of the acetone, the coverslips were stored in a bell jar containing silica-gel[1] as a moisture absorbent until final preparation for the SEM.

1. BDH, Parkstone Poole, England

Following fracturing, 1-2 mm pieces of coverslip containing RBC's in a range of thicknesses were attached to an aluminium stub with silver conductive glue[2], coated with elemental gold from a sputter coater[3] and examined under a Cwickscan microscope.[4]

2.4 PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY

Fixed and sedimented RBC's were collected by decanting the excess fixative leaving 1-2 mls of fixative. The RBC's were then drawn into a microhaematocrit tube, one end plugged,and the cells pelleted by centrifugation at 13,000g for four to five minutes in a microhaematocrit. The end of the tube containing the pelleted cells was snapped off as close to the cell/fluid interface as possible and the plugged end also removed. While hamster and human RBC's remained pelleted following gentle extrusion from the capillary tube with a blunt probe, the cattle RBC's tended to disperse. For this reason, post fixation and dehydration of cattle blood to 100% alcohol was carried out in the capillary tube, while the same process on hamster and human blood was carried out on extruded cells as detailed in Appendix V.

Following extrusion from the capillary tube the pelleted cells were cut into 1 mm cubes, with one block only being taken from either end of the pellet of cattle RBC's.

Infiltration and embedding of RBC's in resin[5] was routine (Appendix V). Sections of silver-gold thickness were cut by a diamond knife onto distilled water using a ultramicrotome[6],

2. G.C. Electronics, Rockford, Illinois, USA, 61101
3. Polaron E5 100,Selby Wiltons
4. Cwickscan 100 Field Emission SEM, Coates Welter Instrument Company, U S A
5. Durcupan ACM Fluka, Switzerland
6. LKB 8800A Ultrotome III, LKB Produkter AB, S-161 25 Bromma 1, Sweden

collected onto unsupported grids[7], stained using lead citrate and uranyl acetate (Appendix V) and examined in a Philips EM 200 electron microscope[8].

3.0 RESULTS

The clinical parameters such as the presence or absence of haemoglobinaemia and jaundice which have been used to estimate the degree of severity of the infection have been obtained from Chapter 3.

3.1 SEM OF RBC'S

3.1.1 Controls

Control calf and hamster RBC's are of a uniform shape and size with occasional pits, protruberances and surface irregularities (Figs. 4-1, 4-6). Morphology of RBC's from the control calves remained normal throughout the experiment.

3.1.2 Haemolytic infections of calves and hamsters

3.1.2.1 'Toxin' injected calves

Increased numbers of RBC's showed crenation and surface irregularities on day two (Fig. 4-8). The abnormal RBC's were similar in morphology to echinocytes as described by Bessis (1973) and Brecher & Bessis (1972).

3.1.2.2 Animals inoculated with live leptospires

The RBC's from the infected calves 11, 14, 15, 16 and 17 were normal in appearance on day two. On day five, when calves 14, 15 and 17 were haemoglobinaemic the RBC's from these calves were spherical with many surface irregularities (Fig. 4-7).

The RBC's from calf 16 showed no alterations throughout the duration of the experiment. RBC's from calf 11 remained normal until day nine, when there was a small increase in the number of

7. Type G400 (3.05 mm), Polaron Equipment Ltd. Watford, England
8. Philips Electrical Industries of NZ Ltd, Wellington, N Z

irregularly shaped cells, some of which contained the occasional pit (Fig. 4-9).

RBC's from infected hamsters in which the plasma was showing a normal or slightly increased icterus index and was not yet severely haemoglobinaemic were in the form of echinocytes (Fig. 4-2). After the development of haemoglobinaemia the echinocytes became less obvious as spherical transformation occurred (Fig. 4-3). As the animals became increasingly haemoglobinaemic, the degree of spherocytosis increased and deep pits and surface irregularities became increasingly obvious (Figs. 4-4, 4-5).

Leptospires were not seen in SEM preparations from either calves or hamsters.

3.2 TEM OF RBC's

3.2.1 Controls

The RBC's from control calves and hamsters are of a relatively uniform shape and size with occasional vacuoles and protruberances (Fig. 4-14).

3.2.2 Haemolytic infections of calves and hamsters

3.2.2.1 'Toxin' injected calves

The 'toxin' injected calves 12 and 13 showed an increased irregularity in cell shape with an increased number of cells containing vacuoles, some of which contained a small quantity of a fine granular material (Fig. 4-16). In some RBC's, small portions of cytoplasm of normal or slightly reduced electron density appeared to have become separated from the remainder of the cytoplasm (Fig. 4-15).

3.2.2.2 Animals inoculated with live leptospires

The RBC's from calves 14, 15 and 17 contained many vacuoles of irregular shape, size and number on day five (Fig. 4-19). Some of these vacuoles appeared to have tracts communicating with the

cell exterior and some vacuoles and tracts appeared to contain a small amount of a fine granular material. In some RBC's the tracts appeared relatively long and to run under and parallel to the RBC membrane as seen in hamster 2 (Fig. 4-17). Some RBC's also appeared to have segments of the cytoplasm separated from the remainder of the cytoplasm by either a dark membrane or a clear region of cytoplasm similar to that noted in the 'toxin' injected calves.

Haemoglobin loss was recognised by the decrease in staining density of the cells when compared to their neighbours (Figs. 4-19, 4-20). While the majority of RBC's from calves 14 and 15 appeared fully haemoglobinated, many of the RBC's from calf 17 appeared to be only partially haemoglobinated and non-haemoglobinated (Figs. 4-19, 4-20). These partially haemoglobinated RBC's contained fewer empty vacuoles and many vacuoles were seen to contain dark inclusions. The vacuoles in the partially haemoglobinated cells were membrane bound (Figs. 4-19, 4-20, 4-21), but vacuolar membranes would not be distinguished in fully haemoglobinated cells. Many of the partially haemoglobinated RBC's and RBC ghosts had large protruberances (Fig. 4-20). Occasional discontinuities in the cell membrane and the escape of haemoglobin were seen in RBC's from calf 17 (Fig. 4-22). However in most cells which appeared to have lost the majority of their haemoglobin the cell membrane appeared normal (Figs. 4-19, 4-20).

The RBC's from calf 16 remained normal throughout the experiment while those from calf 11 contained a small increase in the number of abnormal RBC's on day nine. The abnormal cells had irregular outlines and increased numbers of vacuoles.

In hamsters before haemoglobinaemia occurred, the RBC's were of an irregular shape and size with the occasional vacuole. The RBC's from haemoglobinaemic hamsters showed features similar to those of the haemoglobinaemic calves 14 and 15 with fewer partially haemoglobinated RBC's present (Figs 4-17,

4-18). The presence of lines forming straight lines and ovoid or circular shapes in the cytoplasm of the fully haemoglobinated vacuolated cells from haemoglobinaemic animals was interpreted as an artefact (Fig. 4-23).

Leptospires were seen close to but not in contact with the RBC's in two infected haemoglobinaemic hamsters (Fig. 4-18). No intracellular leptospires were seen in RBC's from either species.

3.3 NON-HAEMOLYTIC INFECTIONS OF CATTLE AND HAMSTERS

No significant alterations in RBC morphology were noted.

3.4 RETICULOCYTE MORPHOLOGY

SEM examination showed reticulocytes to be larger than normal mature RBC's, rounded with large projecting folds (Fig. 4-10). Pits, smaller than those seen in the RBC's from infected animals were seen on the surface of the cells (Fig. 4-10, 4-11). In addition, biconcave cells of normal size and large flat cells with small shallow pits were also seen (Fig. 4-11).

On TEM examination the reticulocytes were variably shaped cells with the remains of mitochondria, ferritin accumulations, small pinocytic vesicles and larger vacuoles often containing cellular organelles which appeared to be undergoing autophagocytosis (Fig. 4-24). The cytoplasm was less densely staining when compared to that of the mature normal RBC's (Fig. 4-14).

3.5 HUMAN *BALLUM* INFECTION

Increased numbers of knizocytes with no signs of pitting or spherocytosis were seen seven days after the onset of clinical signs (Fig. 4-13). TEM examination showed an increase in cellular irregularity and occasional vacuoles. There was no evidence of haemoglobin loss.

The RBC's processed after recovery from the disease and those from the control donor were normal (Fig. 4-12).

Fig 4-1

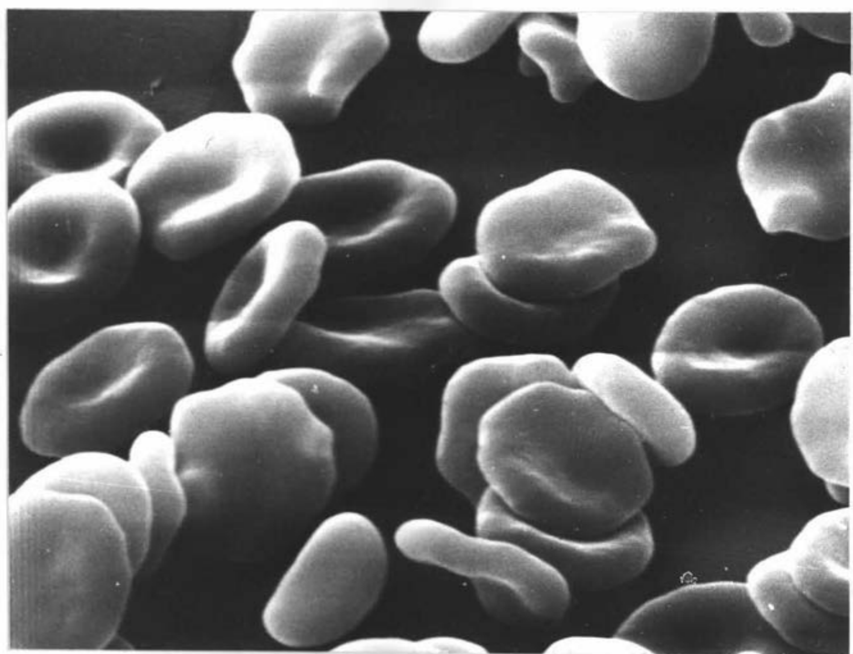
RBC's from a control hamster showing the normal biconcave discoid appearance and minor surface irregularities.
Hamster (8-1) PCV: 0.50 SEM 4,800x

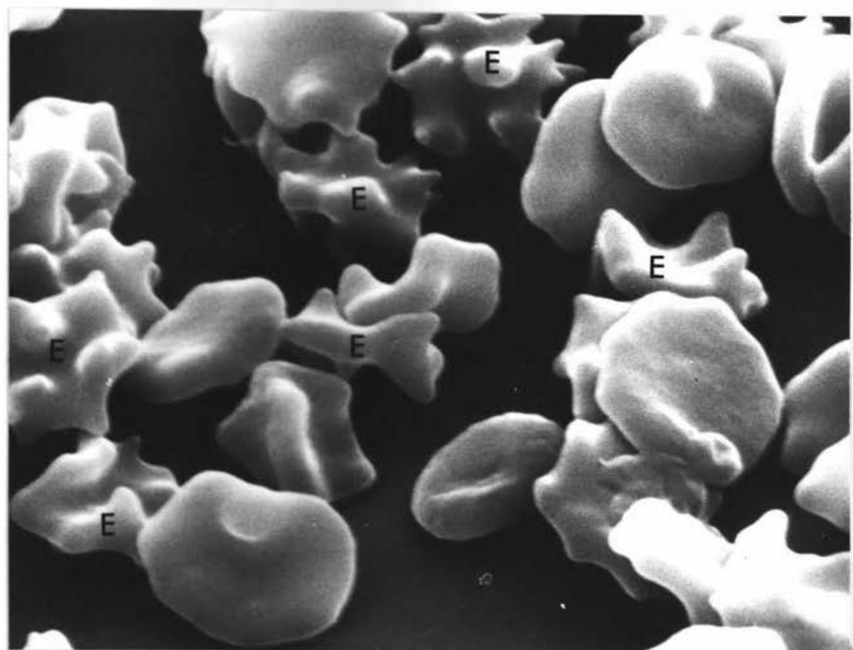
Fig 4-2

RBC's from a non-jaundiced, non haemoglobinaemic hamster three days after infection with *ballum*. Echinocytes (E) are present.
Hamster (3-1) PCV: 0.49 SEM 4,800x

Fig 4-3

RBC's from a jaundiced and haemoglobinaemic hamster three days after infection with *ballum*. Spherocytes (S) with surface pitting (arrows) are present at an earlier stage of development than those in Fig 4-4.
Hamster (3-5) PCV: 0.42 SEM 4,800 x




E
E
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E

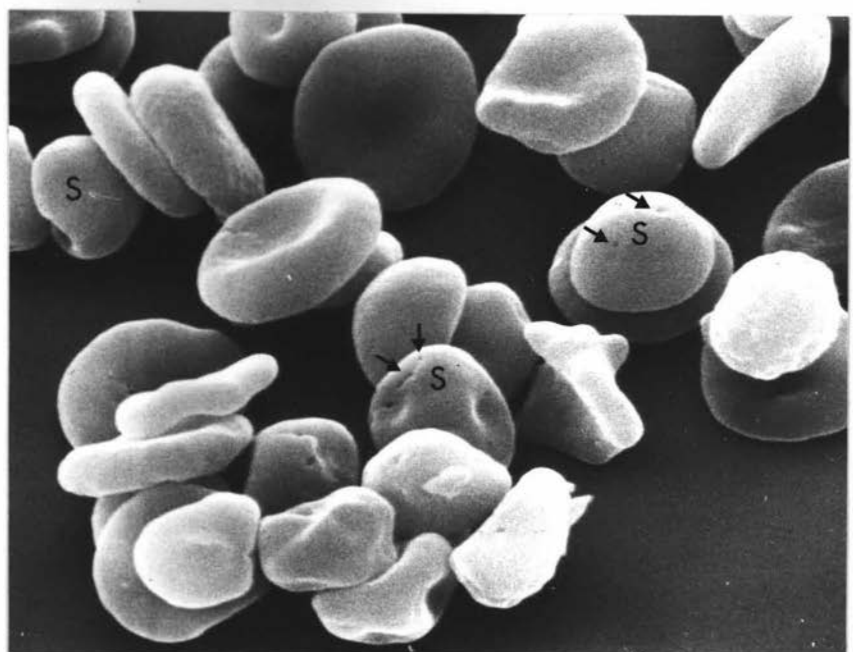

S
S
S

Fig 4-4

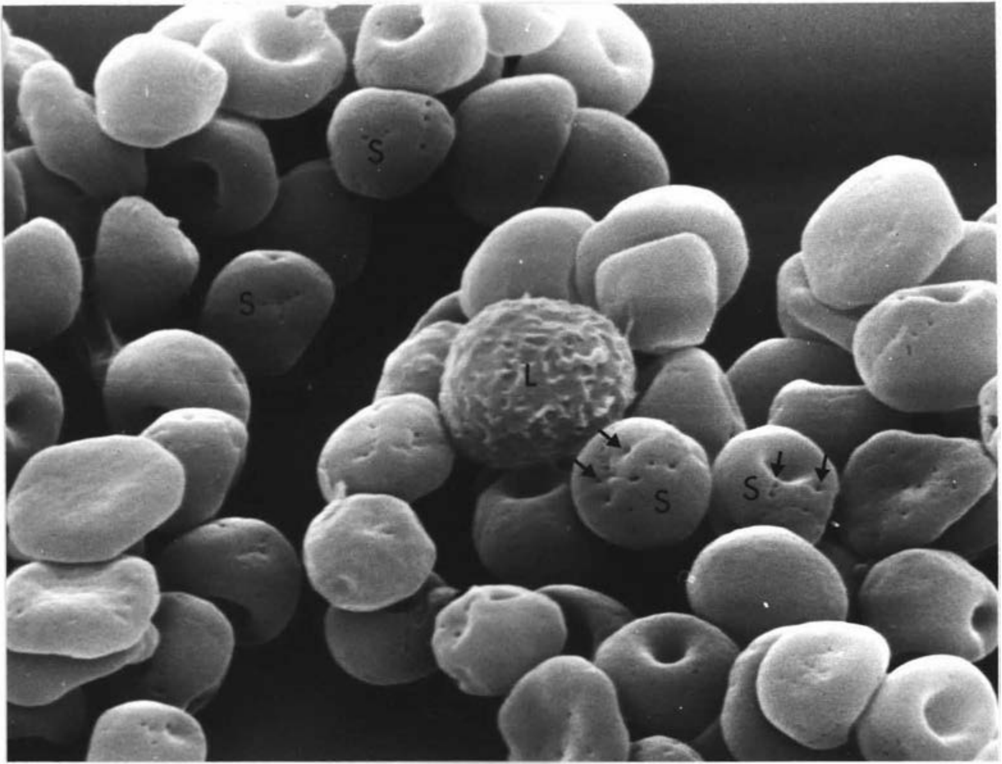
RBC's from a jaundiced and haemoglobinaemic hamster five days after infection with *ballum*. Spherocytes (S) with surface pitting (arrows) are present at a more advanced stage of development than in Fig 4-3. Larger numbers of spherocytes are present, the spherical transformation is more severe, and there are greater numbers of surface pits. A lymphocyte (L) is present.

Hamster 2 (Table 5-1) PCV: 0.10 SEM 4,800x

Fig 4-5

RBC's from a jaundiced and haemoglobinaemic hamster four days after infection with *ballum*. Spherocytosis and pitting are obvious.

Hamster (5-2) PCV: 0.13 SEM 9,600x


S
S
L
S
S

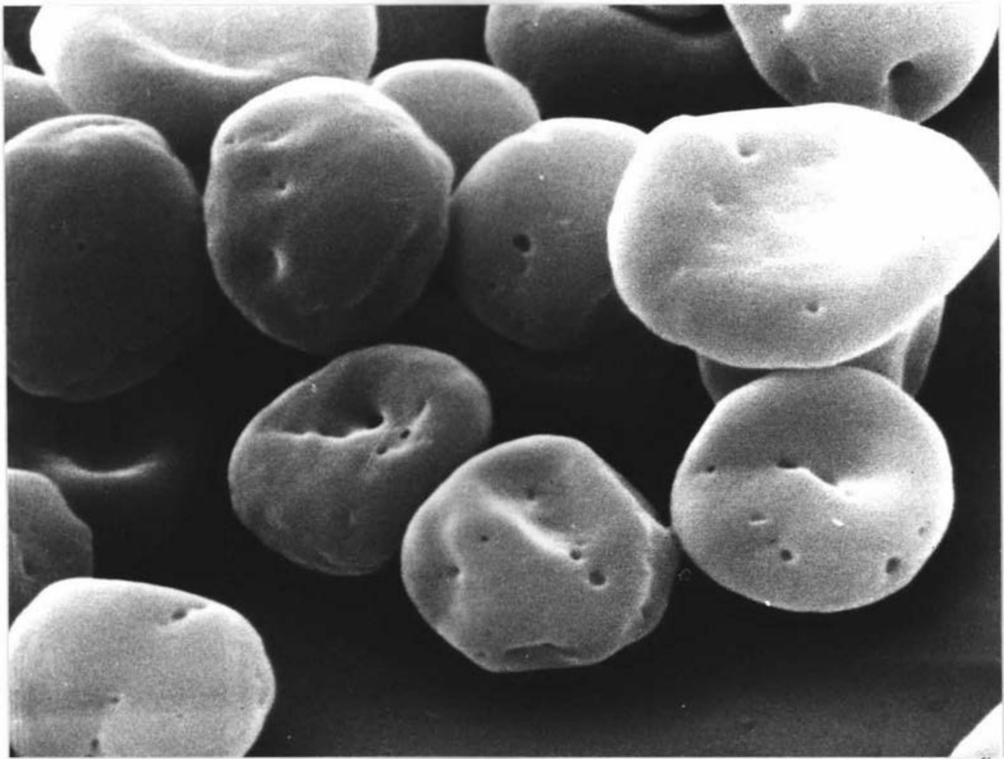


Fig 4-6

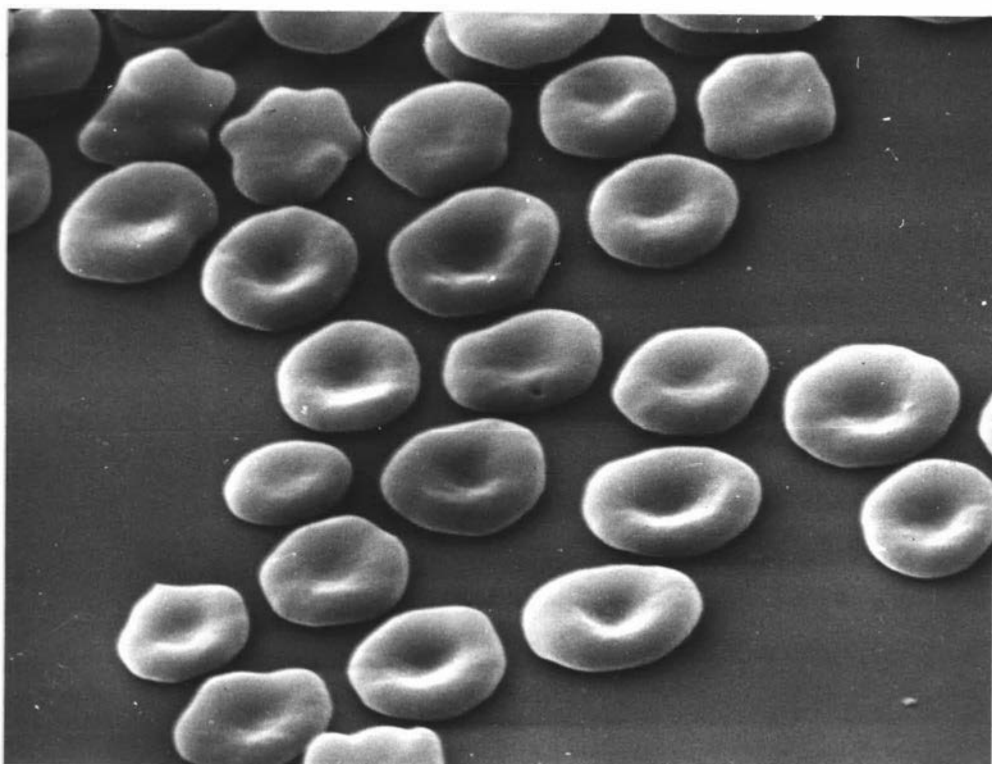
RBC's from a control calf showing the normal biconcave discoid shape with the occasional pit and surface irregularity.

Calf 20 PCV: 0.32 SEM 4,800x

Fig 4-7

RBC's from a jaundiced and haemoglobinaemic calf five days after infection with *pomona*. Spherocytes (S) and other abnormally shaped RBC's (R') showing surface pitting are present.

Calf 17 PCV: 0.23 SEM 7,200x



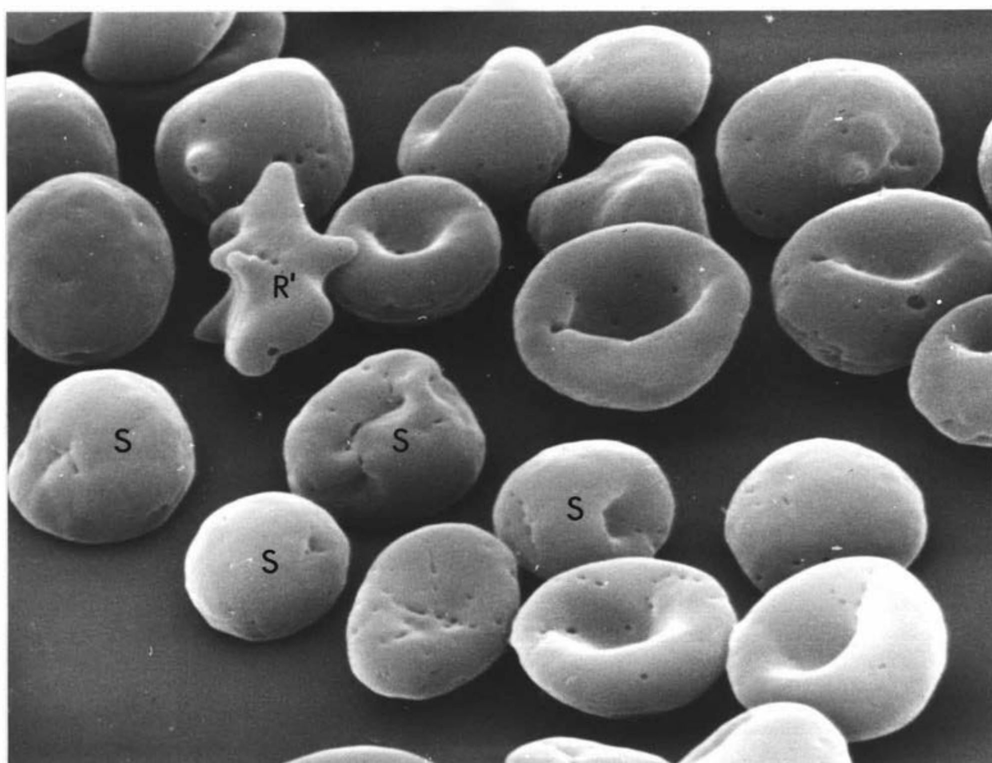

R'
S
S
S
S

Fig 4-8

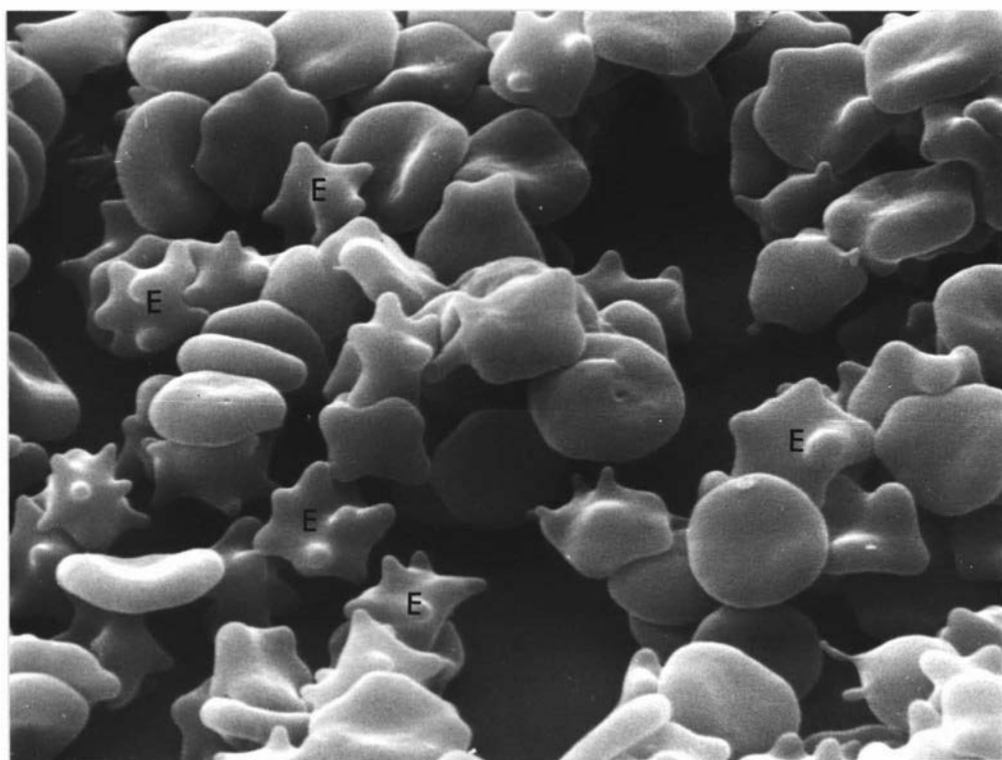
RBC's from a calf two days following injection with a *pomona* 'toxin'. Echinocytes (E) are present.

Calf 13 PCV:0.27 SEM 4,800x

Fig 4-9

RBC's from a jaundiced and non-haemoglobinaemic calf nine days following infection with *pomona*. The occasional RBC (R) with small surface pits and abnormally shaped RBC (R') are present.

Calf 11 PCV: 0.27 SEM 9,600x


E
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E

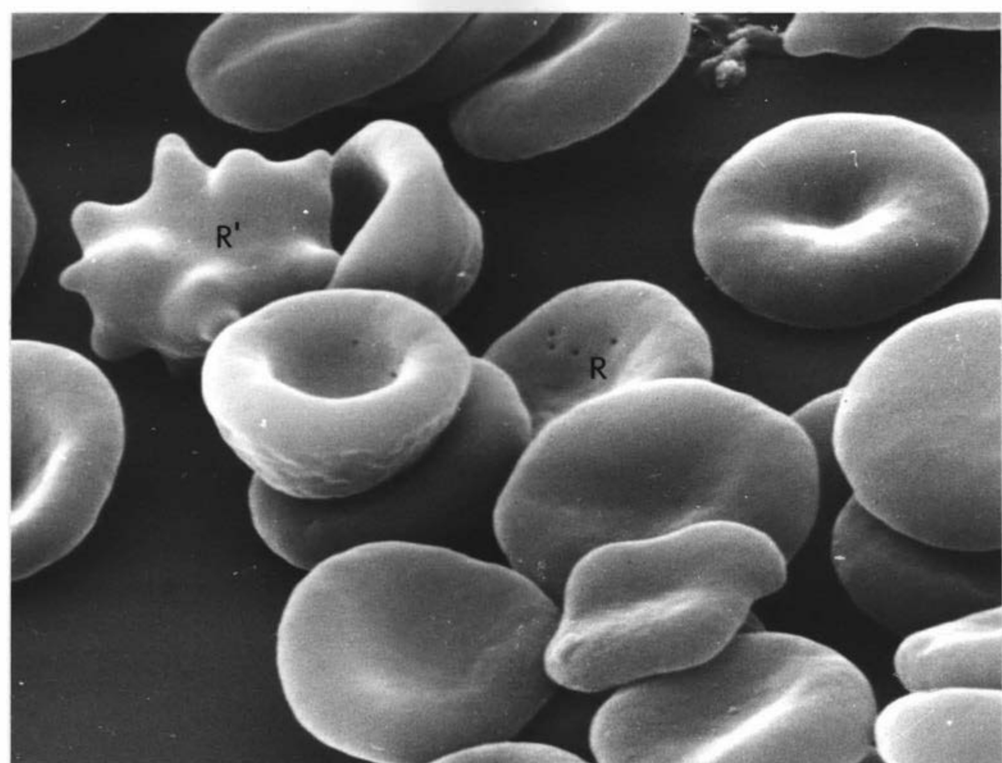

R'
R

Fig 4-10

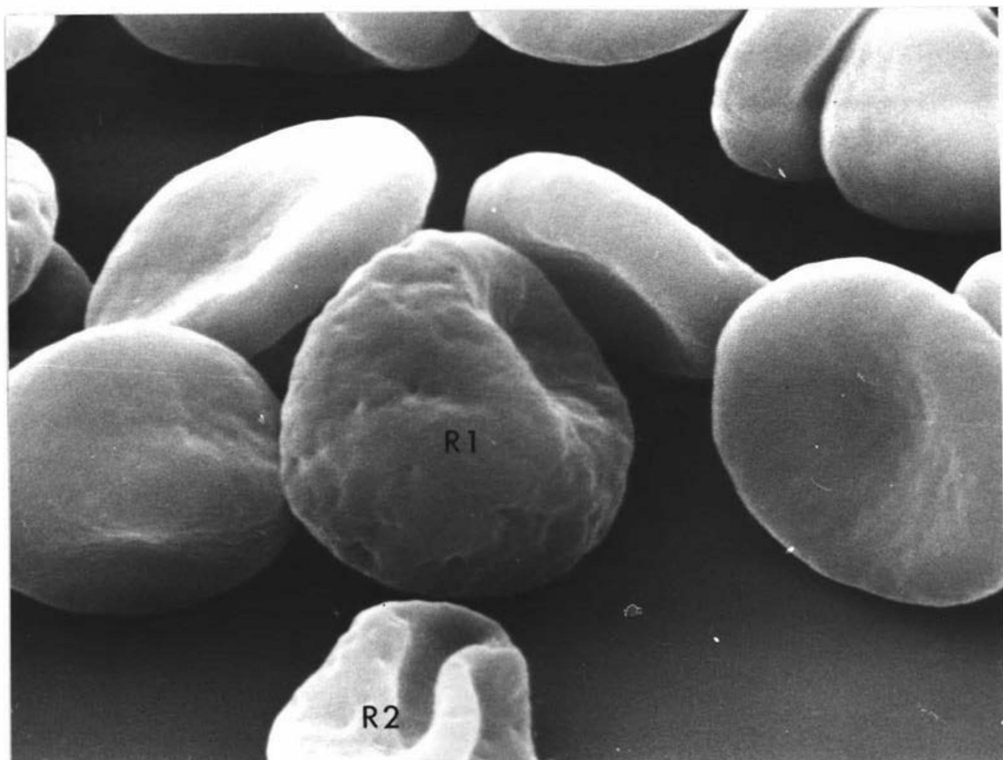
RBC's from a bled hamster. Two cells (R1 and R2) show the typical reticulocyte morphology of small surface pits, membrane folds, round shape and a larger size than is seen in discocytes.

SEM 9,600x

Fig 4-11

RBC's from a bled hamster. The large flat cells (R1) and biconcave discoid cells (R2) which contain small surface pits are considered to be maturing RBC's.

SEM 7,200x


R1
R2

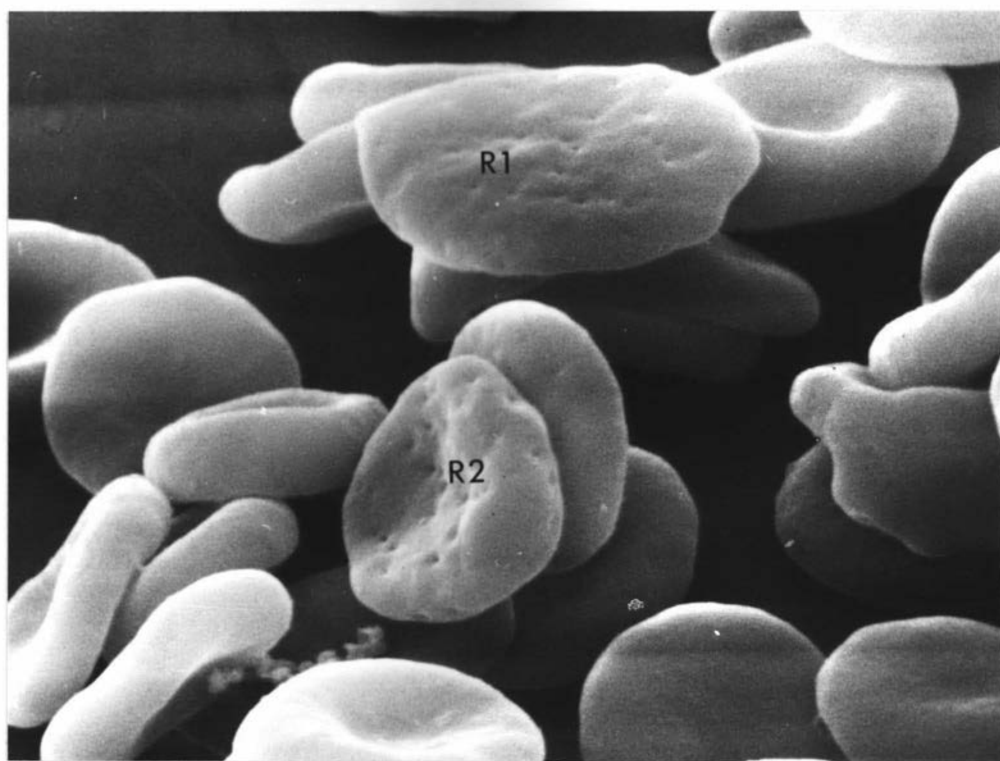

R1
R2

Fig 4-12

Human RBC's demonstrating the normal biconcave discoid shape.

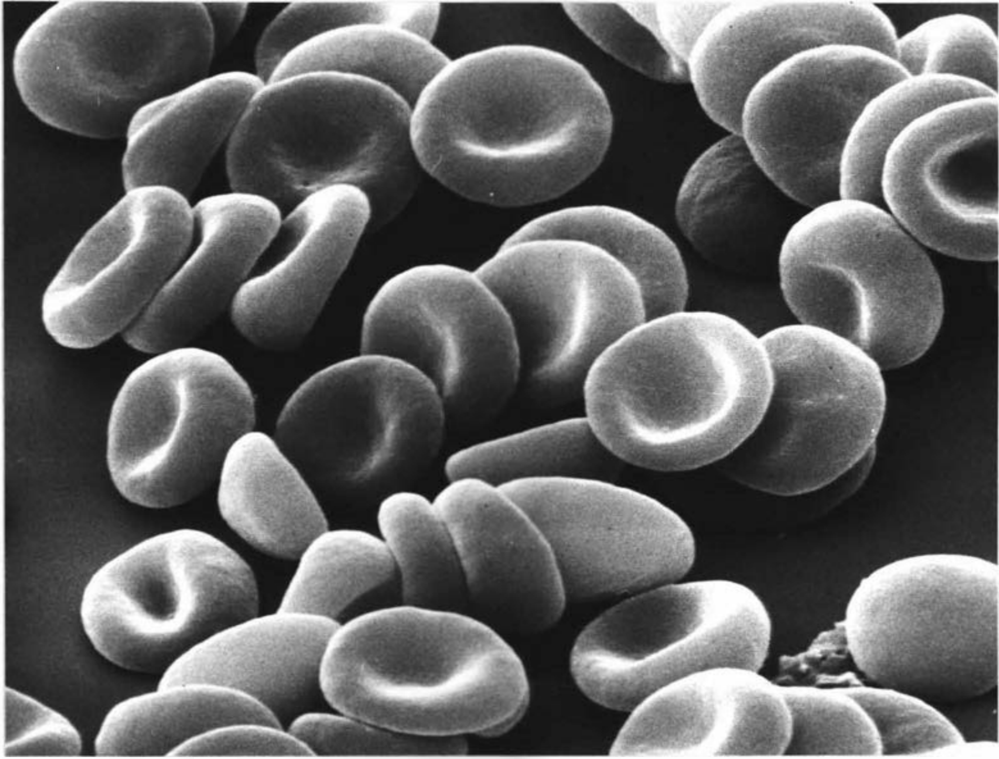
SEM 4,800x

Fig 4-13

RBC's from a leptospiraemic human patient.

Knizocytes (K) are present.

SEM 4,800x



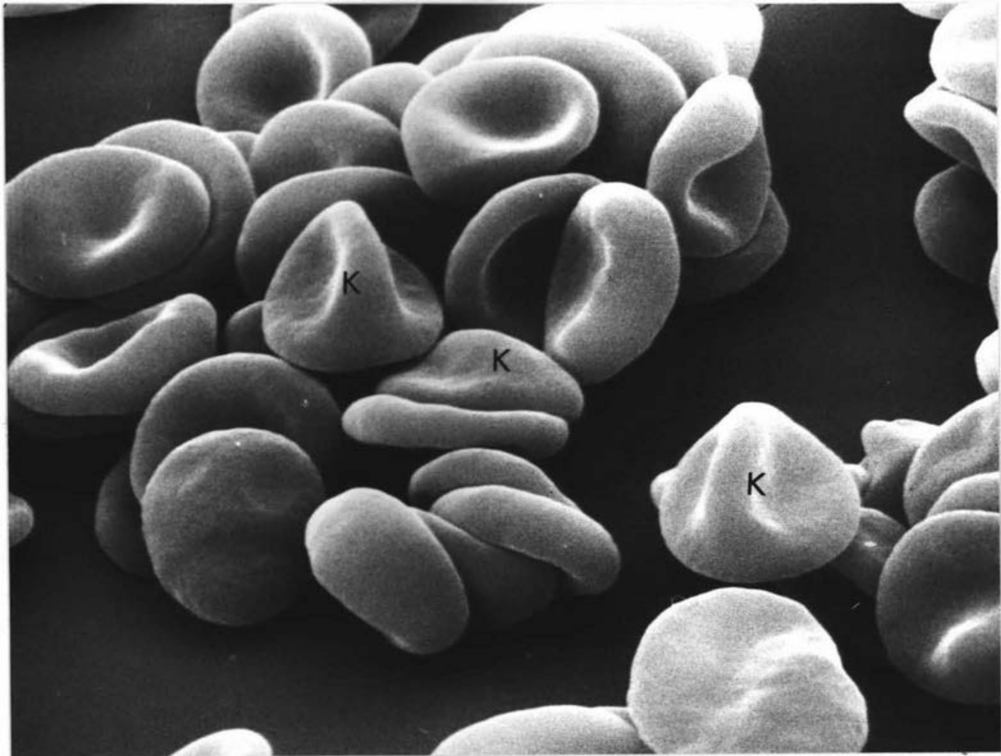

K
K
K

Fig 4-14

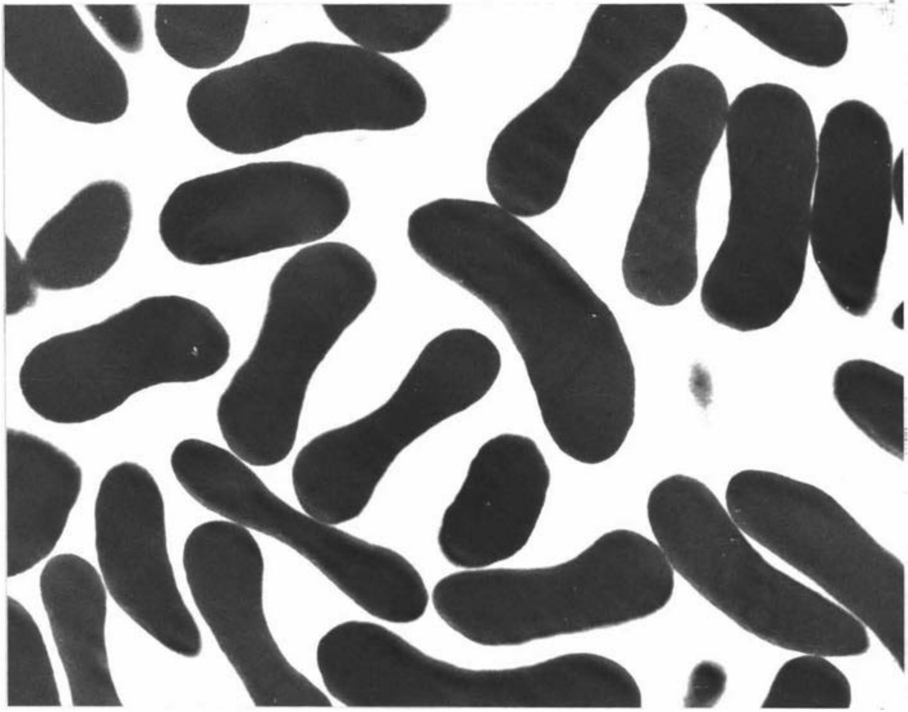
RBC's in cross-section demonstrating the normal biconcave shape.

Calf 18 TEM 7,700x

Fig 4-15

RBC demonstrating lesions typical of calves injected with a *pomona* 'toxin' and non-haemoglobinaemic hamsters infected with *ballum*. Portions of cytoplasm of the same density (arrow) and lesser density (C) separated from the cytoplasm are present. A vacuole (V) is also present.

Calf 12 TEM 27,500x



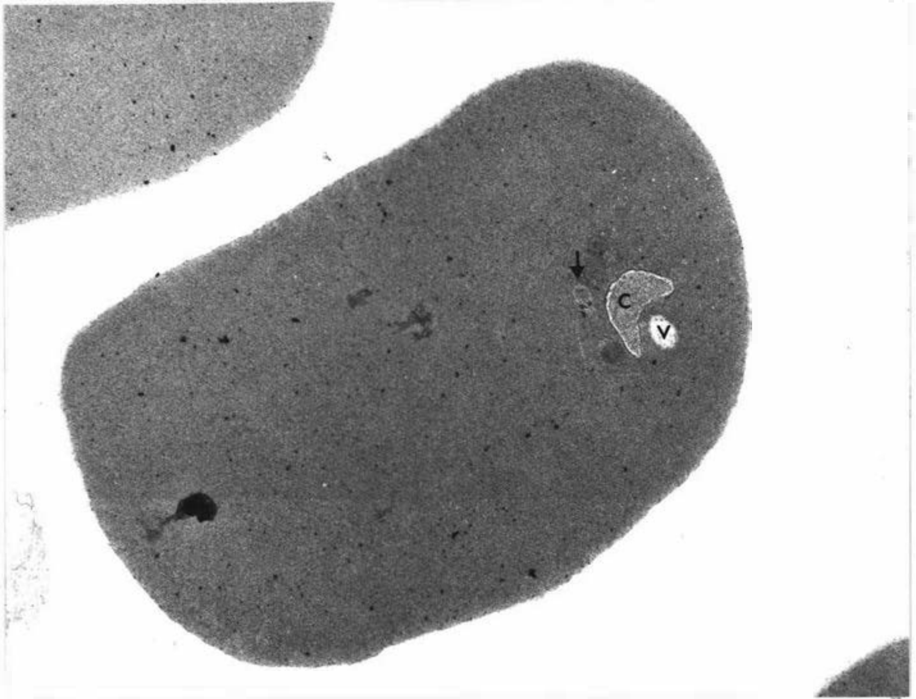

c
v

Fig 4-16

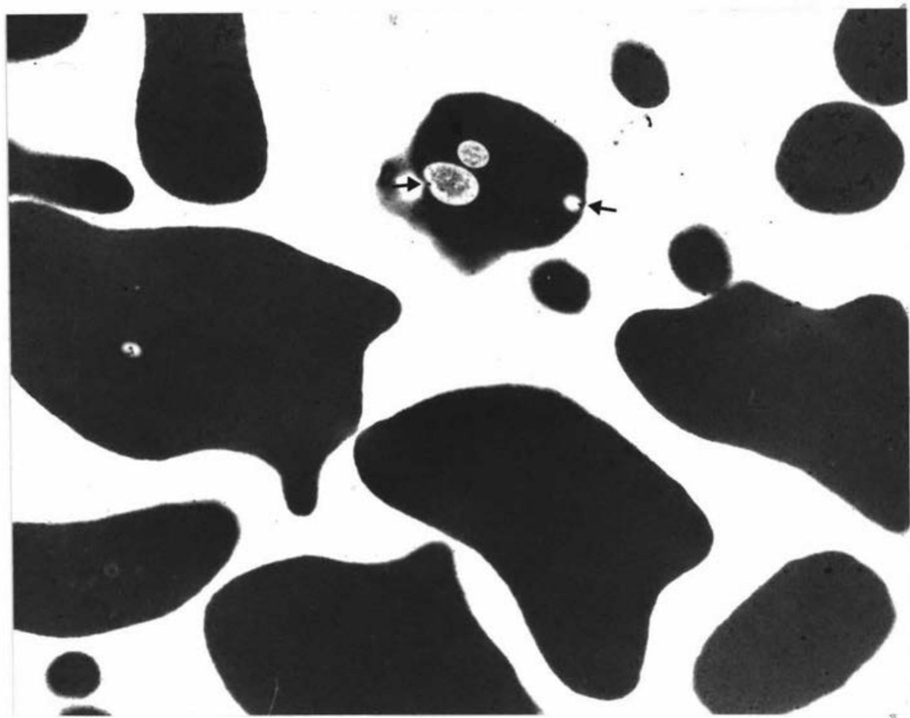
RBC's demonstrating changes typical of calves injected with a *pomona* 'toxin' and non-haemoglobinaemic hamsters infected with *ballum*. There is increased irregularity in shape and the presence of cytoplasmic vacuoles (arrows) often containing a small amount of a granular material.

Calf 12 TEM 12,000x

Fig 4-17

Fully haemoglobinated RBC from a haemoglobinaemic hamster infected with *ballum*. Vacuoles (V) and tracts (T), one of which opens to the cell exterior (arrow), are seen below the cell surface.

Hamster 2 TEM 20,300x



V
T
T

Fig 4-18

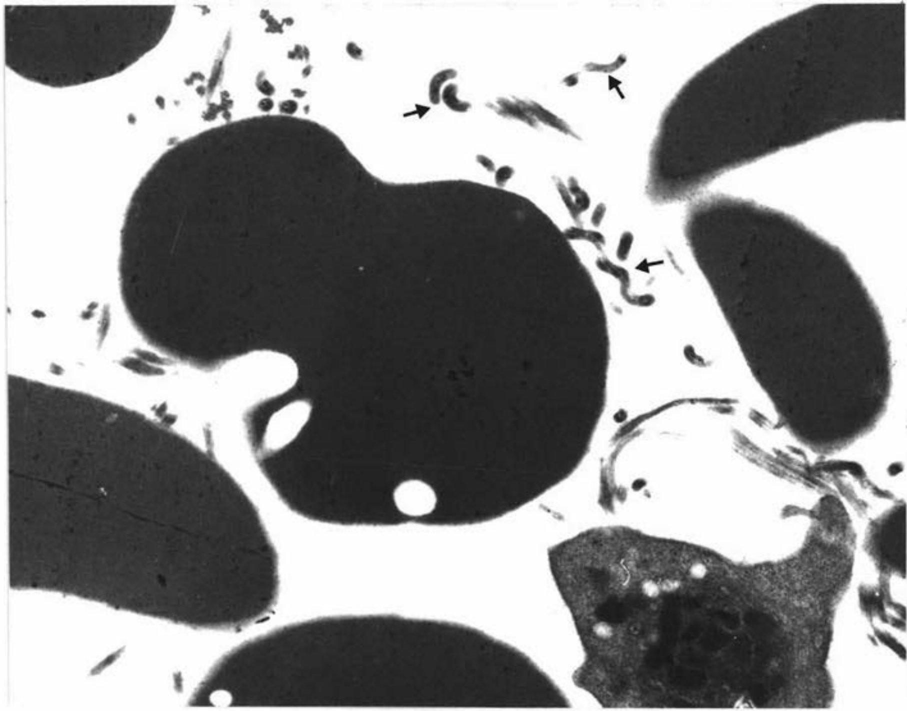
Fully haemoglobinated vacuolated RBC's from a haemoglobinaemic hamster infected with *ballum*. Leptospires (arrows) are seen close to but not in contact with the RBC's.

Hamster 4 TEM 14,600x

Fig 4-19

RBC's from a haemoglobinaemic calf infected with *pomona*. Differences in the degree of haemoglobination are obvious. Fully haemoglobinated RBC's have vacuoles which often contain a granular material (arrows). Partially haemoglobinated RBC's have dark inclusions (I), often surrounded by a distinct membrane.

Calf 17 TEM 20,300x



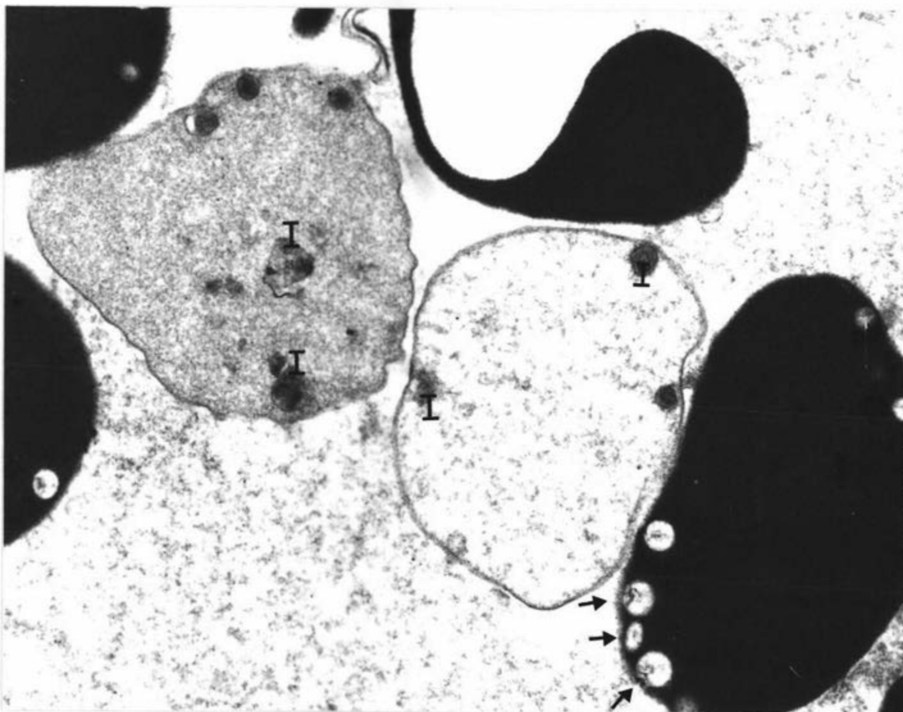


Fig 4-20

RBC's from a haemoglobinaemic calf infected with *pomona*. Large numbers of partially haemoglobinated RBC's (R'), fewer numbers of fully haemoglobinated RBC's (R) and platelets (P) are present. Partially haemoglobinated RBC's are variably shaped with protruberances (arrows). A granular material (G) considered to be haemoglobin because of an appearance similar to that of the material in partially haemoglobinated RBC's is seen in the background.

Calf 17 TEM 9,000x

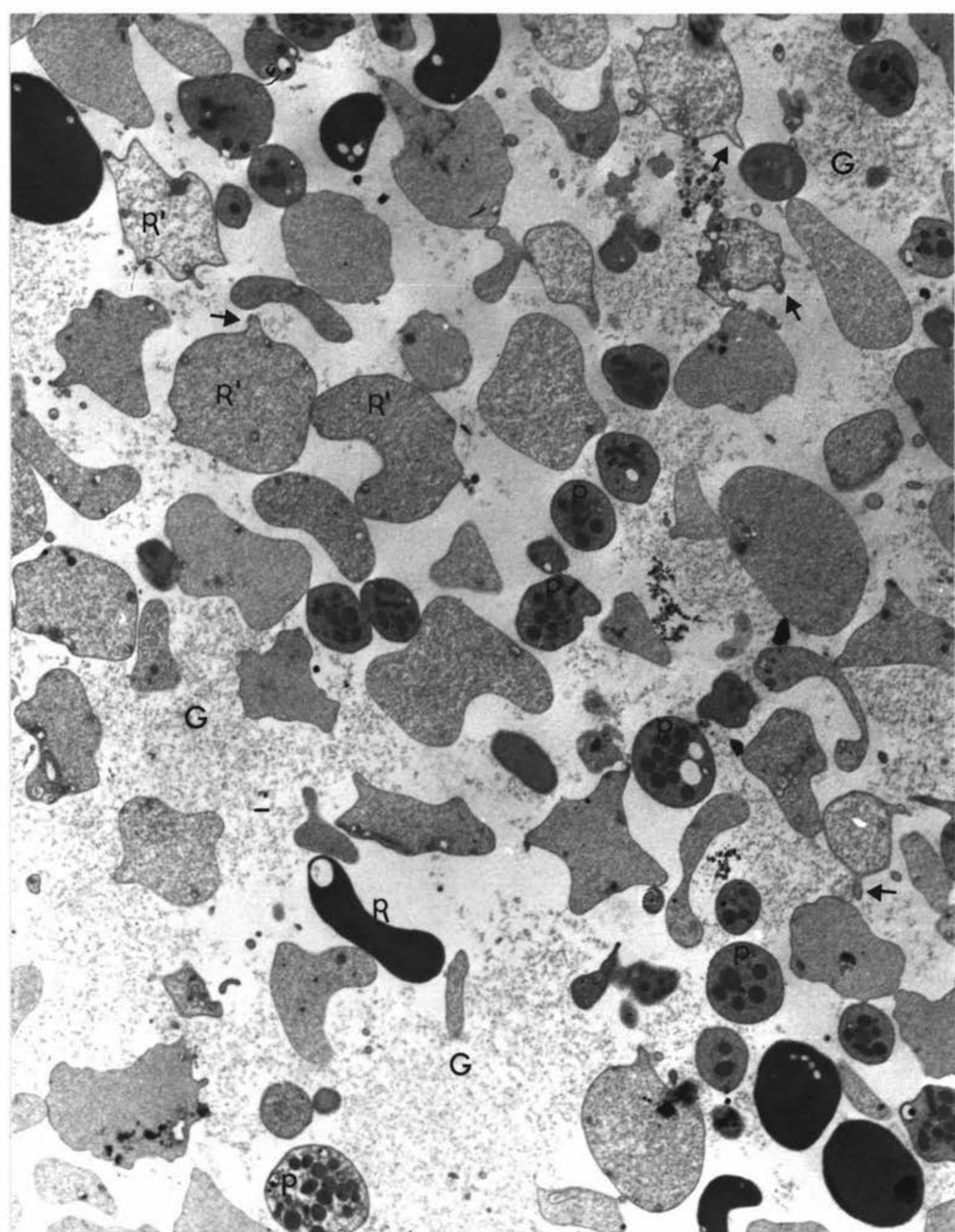
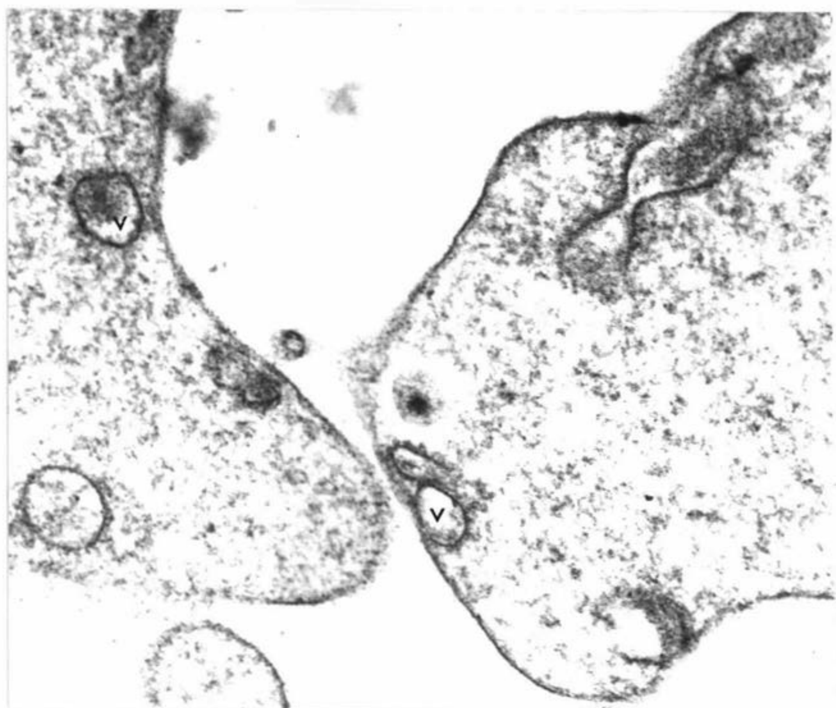

G
R'
R'
R'
P
P
G
P
R
P
G
P

Fig 4-21

High power electron micrographs of RBC's from Calf 17 showing the membrane bound vacuoles (V) and dark granular cytoplasmic inclusions (I).

TEM 41,000x


V
V

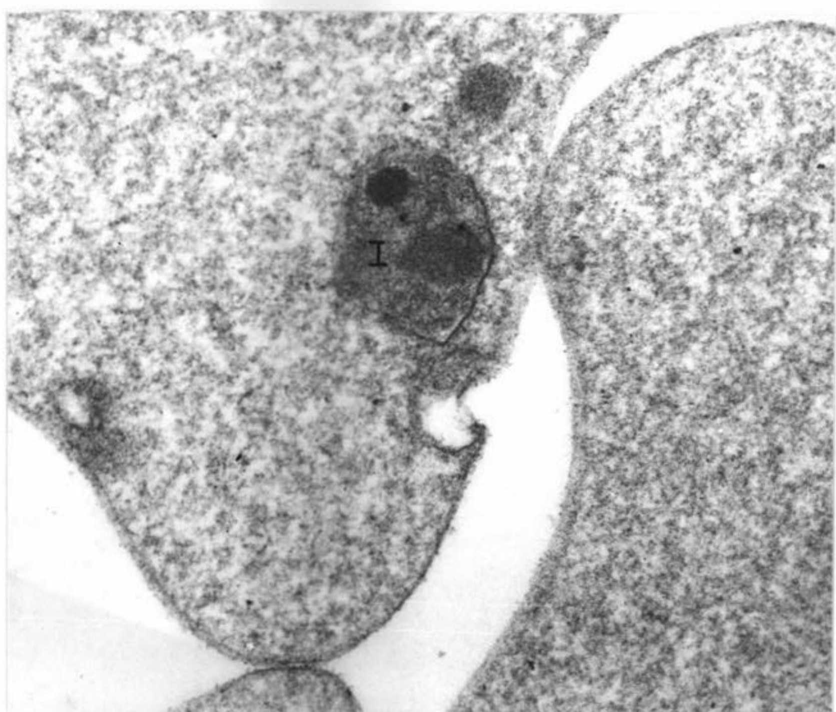

I

Fig 4-22

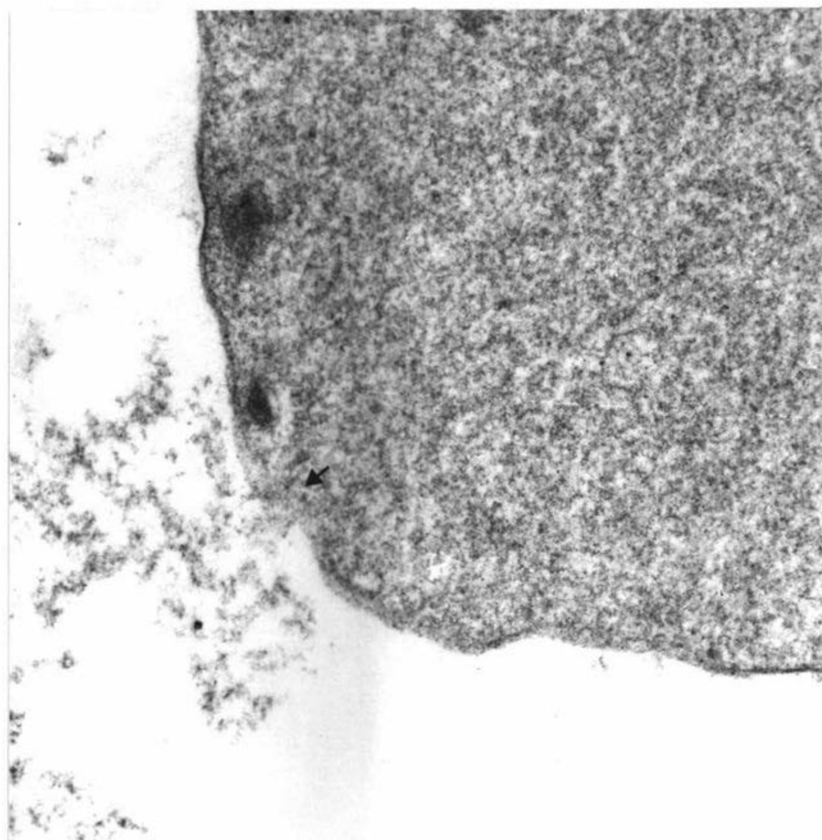
RBC from calf 17 showing a gap in the membrane (arrow) and the apparent escape of haemoglobin.

TEM 41,000x

Fig 4-23

Fully haemoglobinated RBC from a hamster infected with *ballum*. Artefactual dark lines, circles and ovoids are seen arising and terminating at random, within the cytoplasm, at vacuoles, or at the cell membrane.

TEM 24,000x



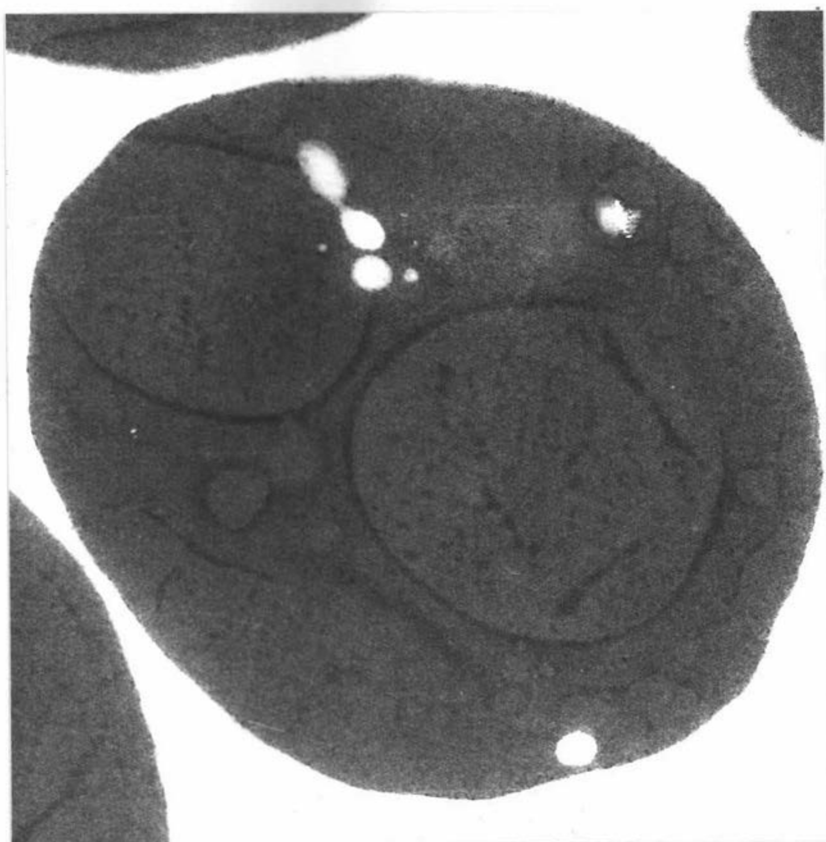
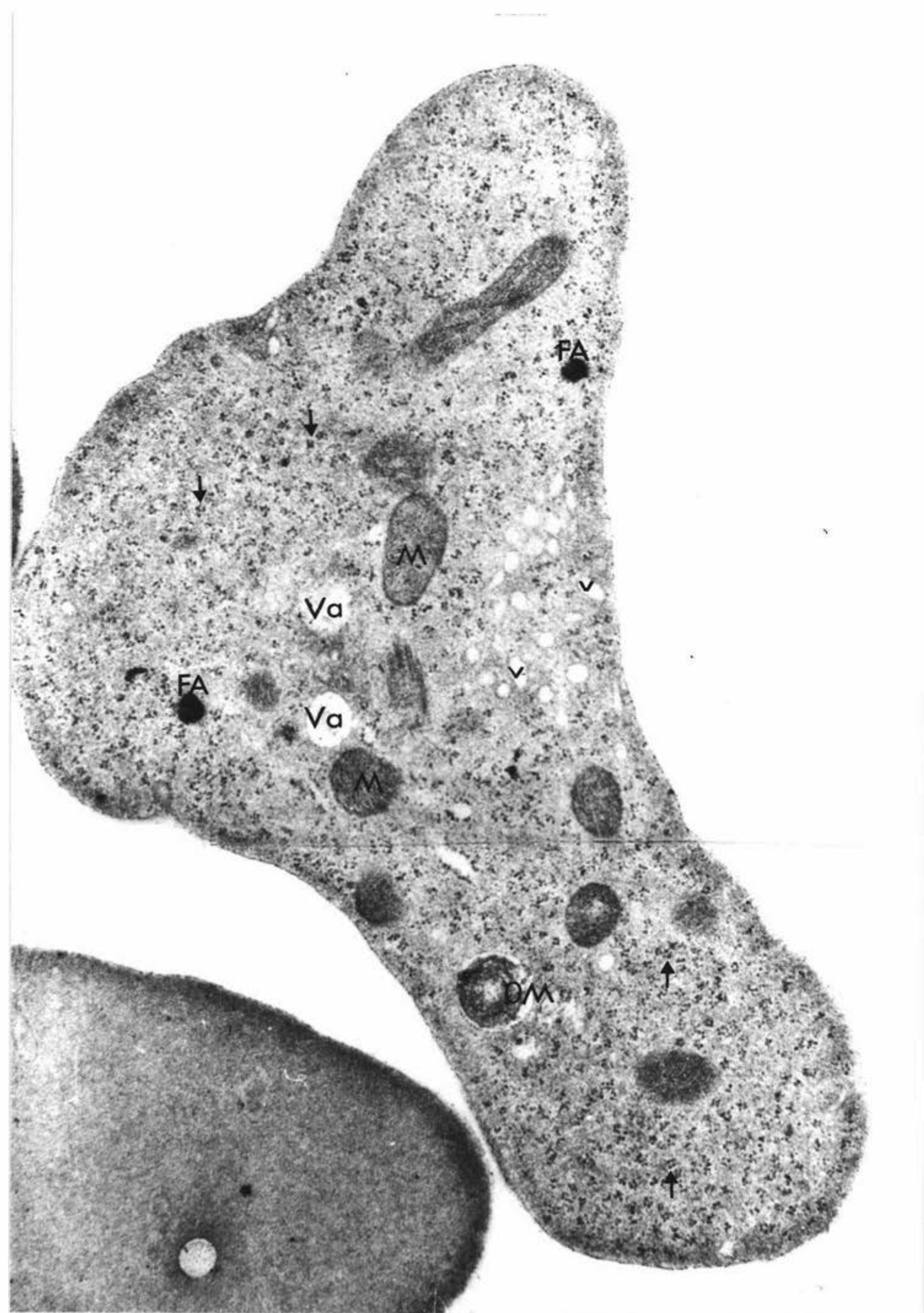


Fig 4-24

Reticulocyte from a bled hamster. Vacuoles (Va), small pinocytic vesicles (V) mitochrondria (M) and a degenerating mitochondrion (DM) within an autophagocytic vacuole are seen. Ribosomes (arrows) and ferruginous accumulations (FA) are also present.

TEM 25,000x


FA
M
Va
FA
Va
M
DM

4.0 DISCUSSION

Sequential morphological changes of increasing severity were seen in hamsters infected with *ballum*. The discocyte-echinocyte transformation noted in the 'toxin' injected calves is probably due to the same factor producing the discocyte-echinocyte transformation in the prehaemoglobinaemic hamsters. Progressive swelling of the echinocytes resulted in the formation of spherocytes and the presence of pits and vacuoles were noted. The severity of the haemoglobinaemia was directly proportional to the degree of swelling and pitting of the RBC's.

It is probable that the pits seen by SEM represent vesicles or vacuoles below the surface of the RBC membrane which have collapsed during preparation. These correspond to structures noted by TEM examination and are in accord with the findings of other workers (Bessis, 1973).

The preliminary change in the formation of vacuoles appears to be segregation of a portion of the cellular cytoplasm and has been described in RBC's from healthy splenectomized humans (Holroyde & Gardner, 1970; Kent *et al*, 1966; Schaeffer *et al*, 1970). The sequestered portion of cytoplasm then undergoes progressive degradation resulting in vacuoles containing dark granular inclusions or a small amount of granular material.

The presence of vacuoles was most obvious in the fully haemoglobinated RBC's while partially haemoglobinated RBC's contained larger numbers of dark granular bodies. The larger numbers of dense inclusions in more severely affected RBC's may be a sign of 'exhaustion' of the RBC's capability to digest and expel defective portions of the cytoplasm. This may indicate that material has been accumulated in a form in which it can not be expelled, or the spleen is unable to carry out its normal function of removal of intracellular debris (Nathan, 1969).

Histochemical evidence for the presence of autophagocytic vacuoles in mature RBC's and reticulocytes has shown that acid phosphatase activity appears to be localized around the regions of the vacuoles or inclusions (Kent *et al*, 1966; Schaeffer *et al*, 1970; Tooze & Davies, 1965). Other workers have shown RBC's appearing to discharge material to the outside of the cell (Kent *et al*, 1966) or from one vacuole into another (Holroyde & Gardner, 1970) and this combined with the actions of the spleen (Holroyde & Gardner, 1970; Kent *et al*, 1966; Nathan, 1969) is probably the method of disposing of unwanted cellular material. It would appear likely that the vacuoles observed in the present experiments are autophagocytic.

The straight lines, ovoid or circular shapes are considered artefactual and a possible result from stress during sectioning as they arise from or terminate in vacuoles, the cell membrane or occur randomly in the cytoplasm.

While the passage of haemoglobin through discontinuous RBC membranes was occasionally seen, very few of the partially haemoglobinated RBC's had membrane discontinuities in the plane of sectioning and membrane damage did not appear to be the major reason for haemoglobin escape. Seeman (1967) described transient 'holes' caused by hypotonic haemolysis and stable 'holes' caused by saponin and lysolecithin in experimentally induced haemolysis which indicated membrane damage was a major cause for haemoglobin loss. However in these present experiments alteration of the cell cytoplasm appears to be the preliminary change. Other workers have compared leptospiral 'toxins' to phospholipases (Chorvath, 1974; Kasarov, 1970; Kemenes, 1974; Stalheim,1971. However while there seems to be some similarities in action between the 'toxins' of leptospires and phospholipases it is not known exactly how an 'enzyme' or 'toxin' acting upon the membrane can cause cytoplasmic damage resulting in autophagocytosis as described in the present work except perhaps by the alteration of the cellular metabolism and the ability of the RBC to maintain its cytoplasmic constituents of haemoglobin and enzymes in a functional state.

The vacuoles do not appear to be directly associated with leptospiral organisms but unless the organisms were firmly attached to the RBC it is to be expected that with the large number of preparatory washes involved in EM preparation that the organisms would be removed. Leptospires (Miller & Wilson, 1962; 1967) and other microorganisms (Balcerzak *et al*, 1972, Bodammer & Bahr, 1973; Cuadra & Takano, 1969) are readily identifiable from intracellular debris and are unlikely to have been overlooked in the present studies.

Because of the lack of close association between RBC and leptospire and echinocyte formation in 'toxin' injected calves it is most likely that the damage is induced by a toxin. That the RBC damage induced by 'toxin' injection did not progress beyond the echinocytic stage is not surprising because in order to truly mimic the natural disease where there are extremely large numbers of organisms present, large amounts of 'toxin' would have to be continually infused for several days.

A biochemical 'lesion', if present in the RBC's could not be detected by the morphological studies described above. However, a biochemical abnormality may exist because discocyte-echinocyte transformations have been reported as a result of ATP or glucose deficiency in which the RBC can not maintain its shape or enzymes in a functional state (Bessis, 1973; Brecher & Bessis, 1972) and following incubation of the RBC with oleic acid and lysolecithin during which the structure of the membrane is altered (Brecher & Bessis, 1972).

It is considered unlikely that haemoglobin loss was induced by complement and antibody damage to the RBC membrane as described by Borsos *et al* (1964) and Dourmashkin & Rosse (1966) because those calves which died following haemoglobinaemia did so before antibody levels were detected and the two calves which developed MAT's did not become haemoglobinaemic.

While the spherical cells containing vacuoles may have a superficial resemblance to reticulocytes, close examination of SEM micrographs of both types of cell showed large differences in the type of pits; those from the reticulocytes being much smaller and more shallow. In addition the reticulocytes had large folds and projections.

The reason for the non-susceptibility to lysis of the RBC's by *pomona* in the two non-haemoglobinaemic calves can not be deduced from the present studies but the lesions described in the RBC's from calf 11 may be explained on the basis of liver disease as discussed in Chapter 3.

The cause of the knizocyte formation in the human infection is unknown as such cells are rare but may be seen in certain haemolytic conditions such as hereditary spherocytosis (Bessis, 1973). However, the presence of knizocytes despite the lack of pits and vacuoles is an interesting observation because human infections with *ballum* are not characterized by haemoglobinaemia (Marshall, pers. comm.) and this patient did not show evidence of jaundice at any stage.

5.0 CONCLUSIONS AND SUMMARY

1. RBC's from leptospiraemic and haemoglobinaemic calves and hamsters were spherical with cytoplasmic inclusions, and vacuoles which by SEM appeared as surface pits.

2. Although one calf had large numbers of partly haemoglobinated cells, the majority of RBC's within circulation of leptospiraemic calves were fully haemoglobinated. Those showing haemoglobin loss contained fewer empty vacuoles and larger numbers of granular inclusions.

3. The earliest change recognised was discocyte-echinocyte transformation. This occurred in hamsters before haemoglobinaemia developed and in those calves given intravenous 'toxin' injections. Echinocytic cells showed spherical transformation as haemoglobinaemia developed.

4. The morphological changes do not result from direct parasitism of the cell by leptospiral organisms and are most likely to be caused by leptospiral toxins.

5. Immune haemolysis was not thought to occur because those calves which became haemoglobinaemic did not concurrently develop MAT's. Those which did not become haemoglobinaemic survived to develop MAT's.

6. The resemblance of the spherical cells to reticulocytes was superficial only.

7. The significance of the knizocytes in the human *ballum* infection is not known as they were not seen in calves and hamsters.

CHAPTER 5

PATHOLOGY OF HAEMOLYTIC LEPTOSPIRAL INFECTIONS OF CALVES AND HAMSTERS

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4.0 DISCUSSION

5.0 CONCLUSIONS AND SUMMARY

CHAPTER 5

PATHOLOGY OF HAEMOLYTIC LEPTOSPIRAL INFECTIONS OF CALVES AND HAMSTERS

1.0 INTRODUCTION

While the presence of haemoglobinaemia and description of other pathological changes have been reported in cattle (Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963) and sheep (Hodges, 1974) infected with *pomona* and hamsters infected with *ballum* (Frenkel, 1972), the major detailed studies of the pathogenesis of leptospiral infections have been confined to *icterohaemorrhagiae* infected guinea pigs (Arean *et al*, 1964; De Brito *et al*, 1979; De Brito *et al*, 1966) and hamsters (Miller *et al*, 1974) in which haemoglobinaemia is not a feature. The published reports on *pomona* in cattle (Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963) and sheep (Hodges, 1974) and *ballum* in hamsters (Frenkel, 1972) have not attempted to examine or discuss the changes in the various organs prior to the onset and during the early stages of haemoglobinaemia.

In this chapter the gross, histological and ultrastructural lesions of the spleen, liver and kidney and histological lesions of the bone marrow are described and correlated with the changes of the RBC's described in Chapters 3 and 4 as haemoglobinaemia develops. Hamsters were used to show the sequential tissue changes.

Because tissue hypoxia also produces histological changes of the liver (Jubb & Kennedy, 1963), the organs from bled hamsters and a bled calf were examined. They were bled to mimic the loss of RBC's without the complicating presence of leptospires and their 'toxins' to ascertain, if possible, what lesions were due to leptospires and what lesions were due to hypoxia.

2.0 MATERIALS AND METHODS

2.1 LEPTOSPIRES

The methods of culture and maintenance of *pomona* strain 790001 used to infect the calves and *ballum* strain 1045 used to infect the hamsters are described in Chapter 2, Section 1.0.

2.2 ANIMALS

The calves and hamsters used in this chapter are those from Experiments V and I (parts 1 & 2) respectively as described in Chapter 2, Sections 2.2.1 and 2.1.1.

The treatment groups for the calves and hamsters of Experiment V and Experiment I part 1 are detailed in Chapter 2, Tables 2-3 and 2-2 respectively.

2.3 LABORATORY PROCEDURES

2.3.1 Tissue weight measurements

The hamsters from Experiment I part 1 were weighed following euthanasia and collection of blood as described in Chapter 2, Section 3.1. Bone marrow smears were made (Chapter 3, Section 2.3.1.8) then the liver, spleen and kidneys were removed, stripped of all fat and weighed. The weight of each of the organs was expressed as a proportion of total body weight.

2.3.2 Histopathology

Tissues were collected from all calves in Experiment V and hamsters from Experiment I (parts 1 & 2) as summarized in Chapter 2. Liver, spleen, kidney, lung, cardiac muscle and one entire femur were taken from hamsters. In part I of the experiment these were fixed immediately following weighing of the organs and in Part 2 immediately following the removal of tissue for ultrastructural examination.

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Fixation of the calf tissues for histopathology was completed after tissue was first removed for ultrastructural examination, bone marrow smear preparation and photography of relevant gross lesions. Blocks of tissue selected for histological examination included the following: liver, spleen, kidney, red marrow from the head of the femur, lung, brain (cerebral cortex, cerebellum, thalamus, medulla oblongata), prescapular lymph node, thymus, cardiac muscle, adrenal gland, thyroid gland, pancreas, eyes, jejunum, ileum and colon. All tissues except for the eyes were fixed in phosphate buffered 10% formalin (Culling, 1974; p45). The tissues were then processed by routine methods and embedded in paraffin wax (Culling, 1974; p73).

Sections approximately five microns thick were cut on a Leitz microtome[1] and mounted and stained on glass slides. All sections were stained with haematoxylin and eosin (H & E) (Culling, 1974; p211) and using Perl's prussian blue reaction for iron (Culling, 1974; p378) and Warthin-Starry silver stain (W/S) for leptospires (Young, 1969). In addition, liver and bone marrow sections were stained by the Periodic Acid Schiff (PAS) technique (Culling, 1974; p267) and a liver section was treated with diastase then stained by the PAS method to demonstrate glycogen (Culling, 1974; p299).

The eyes from the calves were stripped of adherent fat, muscle and connective tissue, fixed for 48 hours in Bouin's fluid (Culling, 1974; p49) then stored in 70% alcohol until further processing. The eye was bisected in the plane of the optic nerve and the lens was removed. Care was taken to minimise retinal detachment and distortion of the ciliary apparatus. The orb and a longitudinal section of the lens were then processed and stained.

2.3.3 Electron microscopy

One mm cubed pieces of liver, spleen and kidney from calves and hamsters and bone marrow from calves were removed and fixed at 4°C in Karnovsky's solution (Appendix V) within five minutes of death. Tissues were post fixed in osmium tetroxide solution,

1. Ernst Leitz, GMBH, Wetzlar, Germany

dehydrated and infiltrated with resin[2] as detailed in Appendix V.

One micron thick sections were cut on an LKB III ultramicrotome[3] and stained with toluidine blue (Appendix V). The sections were examined by a light microscope and selected areas of the blocks trimmed for EM. Silver-gold coloured sections were cut onto distilled water using a glass knife and collected onto grids[4] previously coated with cellotape cement (Appendix V). They were stained with uranyl acetate and lead citrate (Appendix V) immediately before examination in a Philips EM 200 electron microscope[5].

Tissues from calves 14 and 17 were considered unsuitable for ultrastructural examination as an unknown length of time had elapsed between the time of death and post mortem examination.

The infected animals were divided into two categories depending on the presence or absence of haemoglobinaemia or evidence that haemoglobinaemia would have developed had the animal not been euthanased. Evidence for the eventual development of haemoglobinaemia as described in Chapters 3 and 4 was seen in all hamsters and, as such, all hamsters were considered to be haemoglobinaemic along with calves 14, 15 and 17. Calves 11 and 16 were non-haemoglobinaemic.

2. Durcupan ACM Fluka, Switzerland
3. LKB 8800A Ultrotome III: LKB Produkter AB, S-161 25 Bromma 1, Sweden
4. Type G400 (3.05 mm), Polaron Equipment Ltd, Watford, England
5. Philips Electrical Industries of NZ Ltd., Wellington, N Z

3.0 RESULTS

3.1 GROSS PATHOLOGY

3.1.1 Control animals

The only gross lesion in the control calves was a small patch of dull red hepatization in the ventral portion of the right cardiac lobe of calf 19.

Control hamsters had occasional small randomly distributed red patches in the lungs.

3.1.2 Infections associated with haemoglobinaemia

3.1.2.1 Calves

All mucous membranes and body fat deposits of calves 14, 15 and 17 were jaundiced and the bladder contained dark red urine (Fig. 5-1).

The liver, spleen and kidneys were enlarged, reddened and friable. Small dark red foci of about 1 mm in diameter were seen on the capsular surface of the kidneys and extended radially down to the medulla. The cut surface of the kidney was redder than normal and the medulla dark red.

The pleural, peritoneal and pericardial cavities of the calves contained a small amount of a red stained fluid. Calf 17 and to a lesser extent, calves 14 and 15 had marked oedema and red discolouration of the perirenal tissue (Fig. 5-1). The lungs of calf 15 showed diffuse patchy congestion of most lobes.

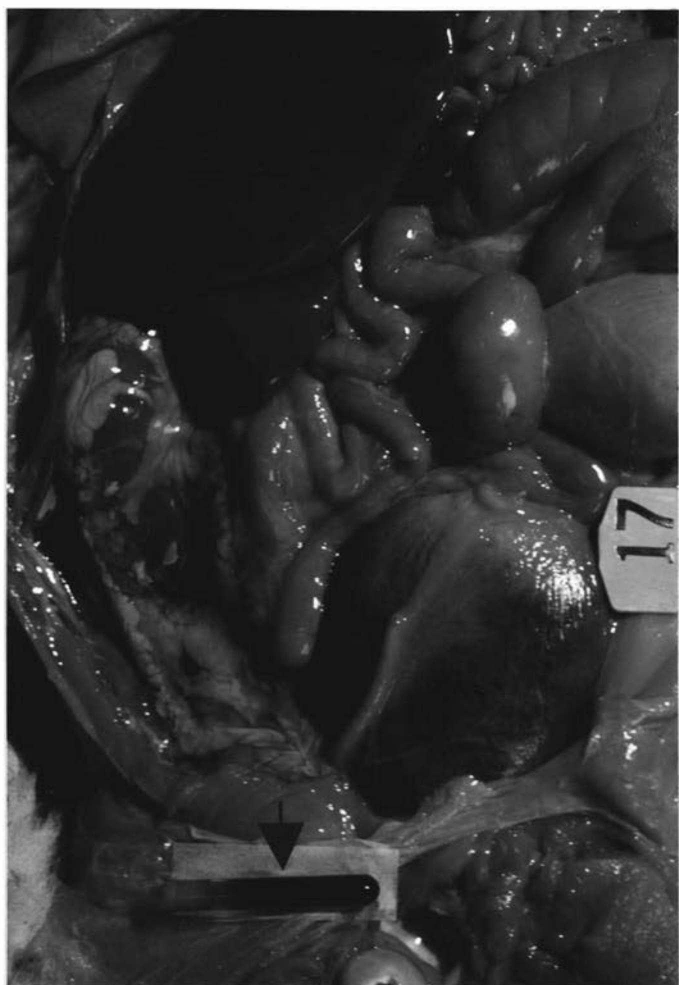
The thymus from calf 15 contained multiple petechial haemorrhages. The prescapular lymph nodes from all calves appeared slightly oedematous. The mesenteric and thoracic lymph nodes were normal.

3.1.2.2 Hamsters

Hamsters killed sequentially showed no gross post mortem changes until day three following infection when two hamsters

Fig 5-1

Post mortem appearance of calf 17. There is intense jaundice of the carcase, perirenal oedema, rounded margins to the dark red congested liver and a dark appearance to the bladder due to the presence of haemoglobinuria. A sample of urine is within the test tube (arrow).


17

showed abnormalities. On following days the progression of the disease followed a relatively consistent pattern, although small variations in the time of onset and the stage to which lesions had developed at defined times varied slightly from hamster to hamster.

The earliest changes were found in the spleen which showed congestion and enlargement. Liver congestion occurred next while alterations in the kidney were seen relatively late in the course of the disease when haemoglobinaemia and haemoglobinuria were severe.

Musculature, body fat and mucous membranes of moribund and dead hamsters were intensely jaundiced. The liver, spleen and kidney were reddened, enlarged and friable. Small red capsular foci less than 1 mm in diameter extended to the medulla. The cut surface of the kidney was more red than normal and the medulla dark red.

Hamsters found dead had hypostatic congestion of the dependent lung and patchy congestion of the other. Those which were found moribund usually had small patches of congestion of both lungs. Dead and moribund animals had a small amount of red stained fluid in the pleural, peritoneal and pericardial cavities.

3.1.3 Infections not associated with haemoglobinaemia

Grossly, calves 11 and 16 were stunted with little body fat present, particularly in calf 11.

The capsular surface of the kidneys of calf 16 contained multiple diffuse pale areas of approximately 0.5 cm diameter which extended 2.5 to 0.5 cm into the cortex. No other lesions were noted in the carcase.

The kidneys from calf 11 contained multiple 1 mm diameter white foci which extended radially from the capsule to the cortico-medullary junction. The liver was enlarged, and bronzed with rounded margins and friable in texture. The bile ducts and wall

of the gall bladder were slightly thickened and the gall bladder was empty.

3.1.4 'Toxin' injected calves

No gross lesions were seen.

3.1.5 Bled animals

Bled animals had no gross lesions.

3.2 HAMSTER ORGAN WEIGHT RATIOS

The hamster organ weight ratios are shown in Appendices VII to XIV, the means and standard deviations of control animals are tabulated in Appendix XV.

The first alteration in organ weight ratios occurred on day two when Hamster (2-3) had an increased spleen to body weight ratio and the liver to body weight ratio was just outside the normal range. Hamsters (2-2), (2-4) and (2-5) had spleen to body weight ratios outside the normal range but liver to body weight ratios were normal.

On subsequent days most hamsters with no other gross lesions had increased spleen to body weight ratios. Increased liver to body weight ratios were always present with increased spleen to body weight ratios. Increased kidney to body weight ratios were present only after haemoglobinuria occurred and were always accompanied by increased spleen and liver to body weight ratios.

3.3. CELLULAR PATHOLOGY

The results of Experiment I, part 2 are summarized in Table 5-1. The haematological results indicated the severity of the stage of infection in that particular animal. Information on the progression of lesions is taken from Experiment I, part 1 in which hamsters were sequentially euthanased and part 2 in which hamsters in different stages of the disease were euthanased on the same day.

Table 5.1 Summary of clinical pathological results from *ballum* infected hamsters in Experiment I part 2

Hamster	Clinical signs immediately prior to euthanasia	PCV	II	Blood smear characteristics	Leptospiraemia	Post mortem examination results	RBC morphology by SEM & TEM
1	Depressed, dehydrated, jaundiced	0.10	¶	Polychromasia, neutrophilia, monocytosis, erythrophagocytosis	<1/hpf	Jaundiced carcase, haemoglobinuria Liver, spleen, kidneys: grossly enlarged, congested, dark red in colour and friable	Spherocytosis, pits and vacuoles
2	Depressed, dehydrated, jaundiced	0.10	¶	Polychromasia, neutrophilia, monocytosis, erythrophagocytosis	3/hpf	Jaundiced carcase, haemoglobinuria. Liver, spleen, kidneys: grossly enlarged, congested and dark red in colour and friable	Spherocytosis, pits and vacuoles
3	Depressed, dehydrated, jaundiced	0.25	20+§	Neutrophilia, monocytosis	3-4/hpf	Jaundiced carcase, haemoglobinuria. Liver and spleen: grossly enlarged, congested, dark red in colour and friable. Kidneys slightly red and enlarged	Spherocytosis, pits and vacuoles. Leptospires seen by TEM
4.	Depressed.	0.35	10+§	Neutrophilia, monocytosis	40-50/hpf	Spleen enlarged, liver slightly enlarged. Kidneys grossly normal.	Spherocytosis, pits and vacuoles. Leptospires seen by TEM
5.	Normal	0.42	0	Normal	1/hpf	Spleen slightly enlarged.	Discocyte-echinocyte transformation
6	Normal	0.45	0	Normal	1/hpf	Normal	Discocyte-echinocyte transformation
C1	Control	0.49	0	Normal	None	Normal	Discocytes
C2	Control	0.48	0	Normal	None	Normal	Discocytes

¶ Severe jaundice and haemoglobinaemia

§ Haemoglobinaemia

3.3.1 Liver

3.3.1.1 Control animals

a) Histopathology

Normal calf hepatocytes are similar to those of cattle described elsewhere (Dellman & Brown, 1976). Normal hamster hepatocytes are shown in Fig. 5-2 and have a foamy appearance due to cytoplasmic vacuolation.

Normal calf and hamster livers contained large amounts of glycogen in most hepatocytes. In hamsters, the normal foamy vacuolated portion of the cell as seen by H & E was still obvious in cells in the PAS stained sections. The glycogen was seen as PAS positive granules within the cytoplasm either distributed through the cytoplasm or localized towards the periphery of the cell and around the nucleus.

Haemosiderin was rarely seen in hepatocytes and Kupffer cells from control calf and hamster livers. In some of the older control hamsters euthanased at the end of Experiment I part 1, intensely staining haemosiderin granules were seen within some hepatocytes of the portal regions.

b) Ultrastructural pathology

There were consistent differences between the hepatocyte ultrastructure of hamsters (Fig. 5-8) and calves which reflected the differences seen in light microscopy. In hamsters, the cellular organelles and endoplasmic reticulum tended to aggregate around the nucleus and the periphery of the cells (Fig. 5-8). Most of the cytoplasm appeared empty and there were no distinct margins between these regions and those where the cellular organelles were located. The hamster mitochondria, endoplasmic reticulum and bile canniculi are similar in structure to those of the calves. The ultrastructure of bovine hepatocytes was similar to that of human hepatocytes described by Laguens & Dumm (1969) and Matthews & Martin (1973).

3.3.1.2 Infections associated with haemoglobinaemia

a) Histopathology

A definite sequence was seen in the development of histological lesions in sequentially euthanased hamsters, although some euthanased on the same day showed lesions of different severity. The early lesions in the sequence occurred before haemoglobinaemia developed but these animals are still considered haemoglobinaemic because according to the results of morphological studies of RBC's (Chapter 4) there were abnormal RBC's in circulation showing changes which would ultimately result in haemoglobin loss.

Early histopathological changes consisted of loss of the foamy appearance to the cytoplasm occurring first around the portal triads. There was swelling of the hepatocytes and a light mononuclear cell infiltration in this region. The size and intensity of the cellular infiltrations and the extent and degree of hepatocyte degeneration increased with time and the increasing severity of haemoglobinaemia. Although the initial degenerative lesions of hepatocytes were observed around the portal triads, with the progression of the disease hepatocyte changes were noted throughout the entire hepatic lobule. As animals became haemoglobinaemic, glycogen depletion occurred. Haemosiderin accumulations were not seen in the early stages of the disease and leptospiral organisms were rare.

The livers of all severely haemoglobinaemic hamsters (Fig. 5-3) and calves showed a marked loss of cellular detail characterized by a loss of distinctness to the cell boundaries and disruption of the hepatic cords. Swollen vacuolated hepatocytes occluded some sinusoids while other sinusoids were dilated. Individual cells showed advanced degenerative changes while neighbouring cells appeared less affected. The degeneration of the hepatocytes was more severe around the central vein and had frequently progressed to coagulative necrosis. The latter change was more marked in hamster livers (Fig. 5-3).

The mononuclear infiltrations observed around the portal triads (Fig. 5-3) from haemoglobinaemic animals were often very extensive and comprised lymphocytes and macrophages with the occasional PMN. Smaller groups of mononuclear and PMN cells were seen throughout the parenchyma often coinciding with areas of coagulative necrosis. These were most frequently seen in hamsters.

The hepatic blood vessels and sinusoids of haemoglobinaemic animals contained large numbers of spherocytes which appeared as intensely staining RBC's of a small diameter without the central pallor. Large numbers of WBC's were seen in the blood vessels and sinusoids. Kupffer cells often contained phagocytosed RBC's.

The haemoglobinaemic animals occasionally contained a pale staining form of haemosiderin within Kupffer cells and occasionally in hepatocytes. In calf 17 and those hamsters which were euthanased last (Table 2-2) these cells had darker staining haemosiderin globules. The haemosiderin was evenly distributed through the parenchyma and did not have a portal distribution.

Glycogen was absent in the severely haemoglobinaemic animals.

Large numbers of leptospires were demonstrated by W/S stain between the hepatocytes of calves 14 and 17 with the occasional organism appearing to be intracellular. Fewer leptospires were seen in liver sections from calf 15. The numbers of leptospires in sections of hamster livers (Fig. 5-5) generally exceeded that of the calf livers. Although some leptospires were seen within hepatic blood vessels, most leptospires were found within the parenchyma.

b) Ultrastructural pathology

In hamsters, the initial changes in hepatocyte ultrastructure consisted of loss of the pale empty portion of the cytoplasm as was seen in hamsters 5 and 6. There was

swelling and disorganisation of the endoplasmic reticulum (Fig. 5-10) which increased as the infection progressed and the animals became haemoglobinaemic. This change varied in degree from cell to cell. In general, there was also congestion of the sinusoids by abnormally shaped and vacuolated RBC's as shown in hamsters 1,2,3 and 4 related to the onset, and increasing in degree proportionally with the severity of haemoglobinaemia (Table 5-1).

Erythrophagocytosis was common with some macrophages containing large numbers of RBC's which were fully haemoglobinated and a few partially haemoglobinated RBC's (Figs. 5-11, 5-12). In addition, phagocytosis of debris of cells other than RBC's was observed (Fig. 5-12).

Leptospires were frequently seen migrating between hepatocytes with little apparent effect on the adjacent cells in calf 15 and hamsters 1, 2, 3, and 4. Intracellular leptospires (Fig. 5-13) were usually degenerate and showed loss of the normal granular structure of the cytoplasm, loss of the axiostyle, and stained more densely than extracellular organisms.

3.3.1.3 Infections not associated with haemoglobinaemia

a) Histopathology

Liver sections from calf 11 showed disorganisation of the hepatic cords, vacuolation of the hepatocytes, and randomly distributed focal accumulations of mononuclear and PMN cells. Kupffer cells appeared larger than those of control calves.

In calf 16, there was the occasional vacuolated hepatocyte. Small numbers of PMN's had infiltrated through the parenchyma in an apparently random distribution.

Haemosiderin was rarely seen in the liver sections from calf 11 while dark staining granules of haemosiderin of a portal distribution were present in calf 16.

Glycogen was present in normal quantities and distribution.

No leptospires were seen in W/S stained liver sections.

b) Ultrastructural pathology

Hepatocytes of calf 16 showed minimal swelling of endoplasmic reticulum, few vacuoles containing cellular debris and the occasional bile plug in the bile canaliculi.

Hepatocytes from calf 11 showed similar but more severe changes. Myelin forms of various sizes and degrees of density (Fig. 5-15) were seen and the cristae of some mitochondria were vacuolated. The space of Disse was frequently swollen and contained cell debris and membranes (Fig. 5-16). No abnormally shaped RBC's were seen in the sinusoids or macrophages of the livers from calves 11 and 16.

No leptospires were seen either intracellularly or extracellularly in calves 11 and 16.

3.3.1.4 'Toxin' injected calves

Histologically, the hepatocytes from calves 12 and 13 were swollen and vacuolated throughout most of the lobule. Those hepatocytes with a normal appearance were centrilobular in distribution. Glycogen levels appeared normal and there were no accumulations of haemosiderin.

Ultrastructually there was a small amount of vacuolation of the cytoplasm of some hepatocytes, swelling and disorganisation of the endoplasmic reticulum, aggregation of the mitochondria and endoplasmic reticulum,and the occasional myelin form (Fig. 5-14).

3.3.1.5 Bled animals

Histological changes in the hamsters consisted of a slight loss to the foamy appearance of the cytoplasm of the hepatocytes. Hepatocytes from the bled calf showed mild hydropic degeneration.

Ultrastructurally this calf showed swelling of the endoplasmic reticulum and an increased volume of pale 'empty' cytoplasm in which the cellular organelles were aggregated around the nucleus and the periphery of the hepatocytes (Fig. 5-9). The sinusoids were occluded by the swollen hepatocytes.

3.3.2 Spleen

3.3.2.1 Control animals

a) Histopathology

The calves and younger hamsters had small foci of erythropoiesis within the sinusoids and spaces between reticular cords of the red pulp. In the lymphoid follicles of the hamsters lymphoblasts were the predominant cell type with few mature lymphocytes present. Very little haemosiderin was present in either group of animals.

b) Ultrastructural pathology

Splenic ultrastructure of calves and hamsters was similar in most respects to that of normal humans (Laguens & Dumm, 1969; Matthews & Martin, 1973; Olah *et al*, 1975). Calf spleens contained more fibrous trabecular material than the hamster spleens. Plasma cells, erythrophagocytic macrophages, erythropoietic cells, reticulocytes and megakaryocytes were seen in normal calves and hamsters. The RBC's in the sinusoids were abnormally shaped due to the close packing of cells. A few vacuolated cells were present. Those RBC's undergoing erythrophagocytosis were frequently vacuolated and showed evidence of intracellular digestion by the patchy loss of haemoglobin from the RBC cytoplasm (Fig. 5-17).

3.3.2.2 Infections associated with haemoglobinaemia

a) Histopathology

Initial changes in spleens from hamsters which showed no clinical or gross pathological signs consisted of lymphoid hyperplasia of the Malpighian follicles and distension of the sinusoids and spaces between reticular cords of the red pulp by RBC's. As haemoglobinaemia developed and became more severe, lymphoid depletion occurred. There were few mature lymphocytes present, the lymphoid cells comprised mainly lymphoblasts. Within the germinal centres these were admixed with pyknotic nuclei of unidentifiable cells. The pathological changes although similar in both species, tended to the more pronounced in the hamsters.

Severely haemoglobinaemic animals showed extremely congested reticular cord spaces and sinusoids in which spherocytes were present. Erythrophagocytosis and the presence of a homogeneous eosinophilic material within large macrophages was common, particularly in hamsters.

Infiltration of the red pulp sinusoids by PMN's occurred in the hamsters and calves 14 and 17. Congestion without PMN infiltration, areas of necrosis and pyknotic nuclei were noted in calf 15. Areas of necrosis and pyknotic nuclei were also seen in haemoglobinaemic hamsters. Macrophages containing cell and nuclear debris from cells other than RBC's were found within the red pulp and lymphoid follicles. Haemosiderin in the form of diffuse and pale staining globules within macrophages was distributed through the red pulp of the spleens of severely haemoglobinaemic animals.

Leptospires were abundant in sections from the most severely haemoglobinaemic hamsters and calves 14 and 17. Few organisms were seen in the sections before the animals became severely haemoglobinaemic and few were seen in sections from calf 15.

b) Ultrastructural pathology

Swelling of the reticular cells of the red pulp was seen in hamsters 5 and 6 without evidence of haemoglobinaemia (Table 5-1). There were large numbers of vacuolated RBC's within the sinusoids and macrophages. Swelling of the reticular cells was shown by the formation of 'blebs' of cytoplasm (Figs 5-19, 5-20).

The most striking feature of the severely haemoglobinaemic animals was the presence of large numbers of abnormally shaped and vacuolated RBC's within the sinusoids (Fig. 5-19). The majority of these cells were fully haemoglobinated. Large numbers of vacuolated and fully haemoglobinated RBC's were also found within macrophages and fewer numbers within PMN's. Occasionally mononuclear cells were seen making close contact with RBC's (Fig. 5-18). Intracellular digestion of RBC's was

seen. Macrophages also contained cellular debris from cells other than RBC's. Dark tubular or round inclusions were observed within some cells (Fig. 5-21). These structures had indistinct boundaries and membranes were difficult to distinguish. These tubular structures were distinct from the leptospires (Figs. 5-19, 5-20). Large areas of cellular degeneration were seen particularly within the severely haemoglobinaemic *ballum* infected hamsters.

Degeneration of leptospires within the macrophages was similar to that which occurred in *pomona* infected hamsters (Chapter 6). All intracellular leptospires were found in membrane-bound vacuoles and were in varying stages of degeneration.

3.3.2.3 Infections not associated with haemoglobinaemia

The spleens of calves 11 and 16 were histologically normal and no leptospires were seen.

Electron microscopy of splenic sections from calf 16 showed no abnormalities. There was an increase in erythrophagocytosis of vacuolated RBC's in the splenic sections from calf 11. Increased numbers of vacuolated RBC's were seen both intracellularly and extracellularly but not in such large numbers as in the haemoglobinaemic calves. The occasional necrotic cell was seen surrounded by apparently normal reticular cells in the red pulp. Phagocytosis of cellular debris was occasionally observed.

3.3.2.4 'Toxin' injected calves

No histological lesions were seen but ultrastructurally there were increased numbers of vacuolated RBC's both intracellularly and extracellularly when compared to the control animals. There were no other significant features.

3.3.2.5 Bled animals

No histological or ultrastructural lesions were seen.

3.3.3 Kidney

3.3.3.1 Control Animals

a) Histopathology

Renal structure was normal in all control animals. Only the occasional granule of haemosiderin was present in epithelial cells of tubules.

b) Ultrastructural pathology

The renal ultrastructure of calves and hamsters was similar to that described in mice (Ericsson, 1964) and humans (Laguens & Dumm, 1969; Matthews & Martin, 1973).

3.3.3.2 Infections associated with haemoglobinaemia

a) Histopathology

In sequentially euthanased hamsters, severe renal lesions were seen only in haemoglobinaemic animals. Initial changes occurred in animals after lesions in the spleen and liver were well established, but before haemoglobinuria occurred, and consisted of the slight dilatation of the Bowman's Capsule by an almost transparent faintly eosinophilic fluid. The onset of severe lesions coincided with the presence of a dark pink proteinaceous fluid in the tubules (Fig. 5-4). This fluid is thought to represent haemoglobin and will be referred to as such in the following results.

In all haemoglobinaemic animals haemoglobin was present in many tubules extending from the Bowman's Capsule to the renal pelvis. The degree of eosinophilia of the haemoglobin containing fluid increased and a granularity became apparent with distal progression. In some cases RBC's, WBC's and epithelial cells were incorporated in the haemoglobin casts. Advanced degenerative changes consisting of loss of the brush border of the proximal tubular epithelial cells, and vacuolation and disintegration of epithelial cells were present in tubules containing haemoglobin. In some cases the epithelial cells had desquamated. In hamsters the corticomedullary junction was often the area most severely affected. In the cortex collapse of the stroma due to loss of tubular epithelial cells was sometimes seen.

The hamster kidneys contained large accumulations of mononuclear cells in the interstitium of tubules and around large blood vessels. Some PMN accumulations were also seen in the interstitium. Mononuclear and PMN accumulations were also seen in the renal pelvis of some hamster kidneys. In calves, cellular infiltrates of a similar cell type were present although of lower density and size.

Glomerular changes were minor in calves and hamsters and consisted of dilatation of Bowman's Capsule with a urinary filtrate, usually darkly eosinophilic but occasionally almost transparent.

Haemosiderin was not found in any of the kidneys.

In moribund or dead hamsters, very large numbers of leptospires were seen singly or in large groups in blood vessels, and in the interstitium, between tubules and between epithelial cells (Fig.5-6). Large groups were seen particularly at the corticomedullary junction. The occasional leptospire was seen in tubular lumina in the cortex and medulla. Numbers of leptospires observed were fewer in calves 14 and 17 and infrequent in calf 15. Large groups of organisms were not observed in calf kidneys.

b) Ultrastructural pathology

Before haemoglobinaemia occurred in hamsters, few changes were seen. Those changes which were present consisted of slightly increased vacuolation of the epithelial cells of proximal convoluted tubules and oedema of the glomerulus with damage to the endothelial cells of the glomerulus. No leptospires were observed in sections from the non-haemoglobinaemic hamsters 5 and 6. All pathological changes were minor until haemoglobinuria was present.

In kidney sections from all haemoglobinaemic and haemoglobinuric animals, a densely staining substance (Fig.5-23) which is considered to be haemoglobin was seen in tubular lumina. In some tubules this substance had a more condensed, granular appearance (Fig. 5-24).

The epithelial cells showed varying degrees of degeneration with those epithelial cells associated with the condensed, granular haemoglobin appearing more degenerate (Fig. 5-24). Large vacuoles and some myelin forms were seen within most epithelial cells (Fig. 5-23). The epithelial cells were often swollen and occluded the tubular lumen, but were sometimes shrunken (Fig. 5-24). In the latter case little cell cytoplasm was seen between the tubular lumen and basement membrane of the proximal tubules. The least affected epithelial cells of proximal convoluted tubules had normal microvilli (Fig. 5-23) which was progressively lost as the cells degenerated (Fig. 5-24). There was an increase in the number of apical dense tubules and vesicles.

Larger quantities of haemoglobin were seen within the epithelial cells of the proximal convoluted tubules of calf 15 (Fig. 5-22) than was seen within those of the haemoglobinaemic hamsters. Few changes were seen in the distal convoluted tubules and collecting ducts apart from the presence of haemoglobin which was often granular.

Abnormally shaped RBC's with vacuoles were seen within the blood vessels and glomerular capillaries of the haemoglobinaemic animals (Figs. 5-25, 5-26). Glomerular changes consisted of oedema, damage to the blood vessel endothelial cells and the presence of small pieces of cell debris in the lumen of the capillaries (Figs.5-25, 5-26).

Few organisms were observed in the calf kidney sections and no organisms seen within the tubule lumina of either calf or hamster kidneys. Leptospires were more frequently seen in the severely haemoglobinaemic hamsters and in all animals were seen migrating between epithelial cells or epithelial cells and the basement membrane (Fig. 5-27) where they were often associated with a small quantity of oedema. The organisms were not directly associated with necrotic cells.

3.3.3.3 Infections not associated with haemoglobinaemia

a) Histopathology

Calf 16 showed only minor kidney changes consisting of vacuolation of the epithelial cells of the proximal convoluted tubules and the presence of mononuclear infiltrations of small numbers of lymphocytes, macrophages and plasma cells.

There were more severe changes in the kidneys from calf 11. There were larger accumulations of mononuclear cells in the interstitium, and tubular casts of PMN's. A pink proteinaceous material was seen in some tubules with cellular debris in some of the collecting duct lumina. No haemosiderin was seen in kidneys of either calf 11 or 16.

Few leptospires were seen in kidneys of calf 16. Colonies of leptospires were seen in the proximal convoluted tubules of calf 11, but none observed in the interstitium.

b) Ultrastructural pathology

Epithelial cells of proximal convoluted tubules from calf 16 occasionally had increased numbers of vacuoles. No leptospires were seen in kidney sections from calf 16.

Changes were most severe in kidney sections from calf 11. The number of vacuoles was increased in many epithelial cells from the proximal convoluted tubules.

Leptospires were seen within the lumina of the proximal convoluted tubules, in some cases associated with normal microvilli (Fig. 5-29) and in others with degenerate microvilli and cellular debris (Fig. 5-28). Degeneration of the mitochondria of the epithelial cells of the proximal convoluted tubules in the form of vacuolated cristae was frequently observed.
Leptospires were seen migrating between apparently normal epithelial cells.

Other regions of the nephron appeared normal, as did the RBC's in blood vessels.

3.3.3.4 'Toxin' injected calves

Renal tissue from the 'toxin' injected calves was histologically and ultrastructurally normal.

3.3.3.5 Bled animals

No histological or ultrastructural lesions were observed in bled animals.

Fig 5-2

Liver from a control hamster showing the normal large 'vacuoles' within the hepatocytes which give a 'foamy' appearance to the cytoplasm.

Hamster C1 H&E 100x

Fig 5-3

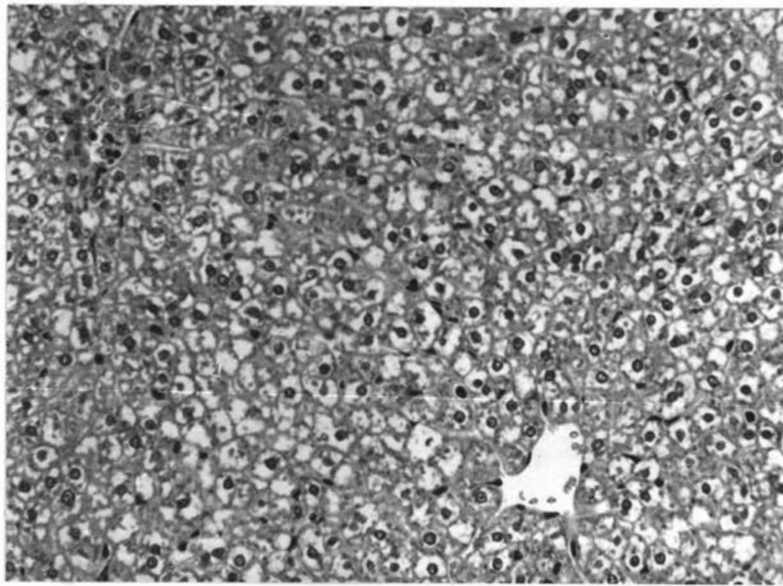
Liver from a moribund haemoglobinaemic hamster infected with *ballum*. There is loss of the characteristics of the normal hepatocyte cytoplasm, dilatation of the sinusoids (arrows), portal infiltration with mononuclear cells (I) and centrilobular coagulative necrosis (CN).

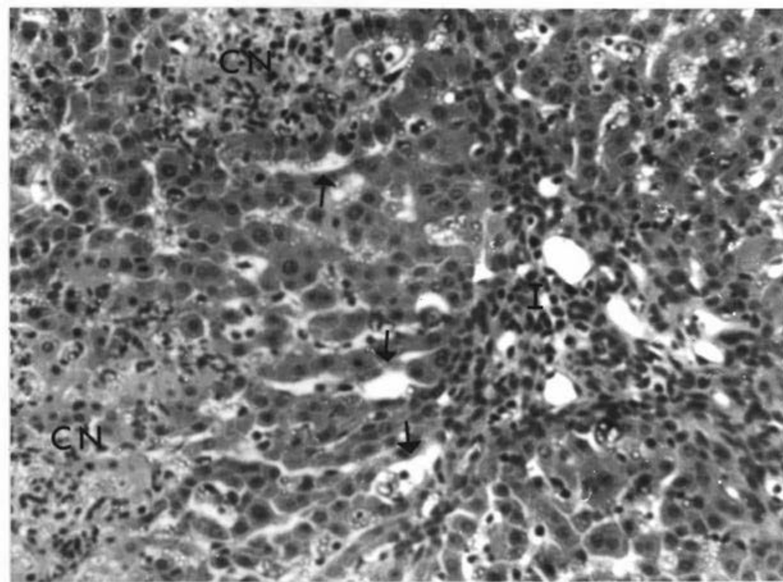
Hamster 1 H&E 100x

Fig 5-4

Kidney from a moribund haemoglobinaemic hamster infected with *ballum*. Most haemoglobin casts have a homogeneous appearance (H) while some appear more granular (G).

Hamster 2 H&E 100x




CN
I
CN

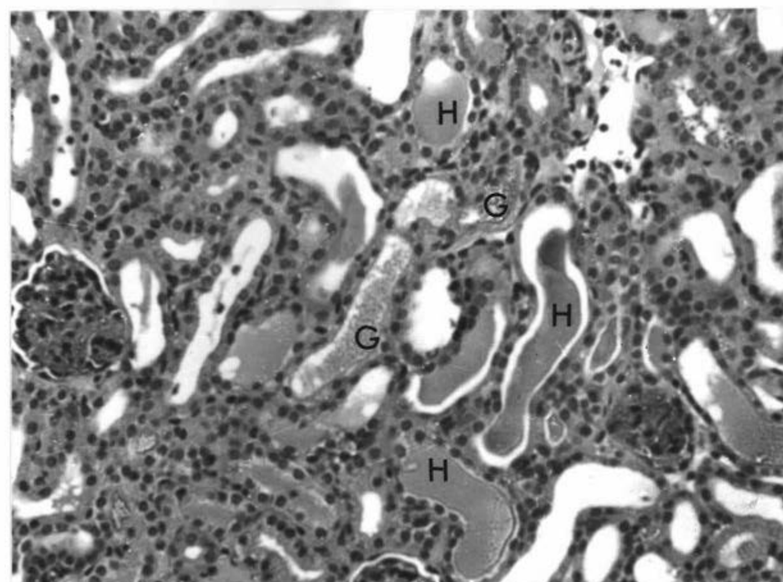

H
G
G
H
H

Fig 5-5

Liver from a moribund haemoglobinaemic hamster. Large numbers of leptospires (arrows) are present extracellularly, between hepatocytes and within sinusoids.

Hamster 2 W/S 200x

Fig 5-6

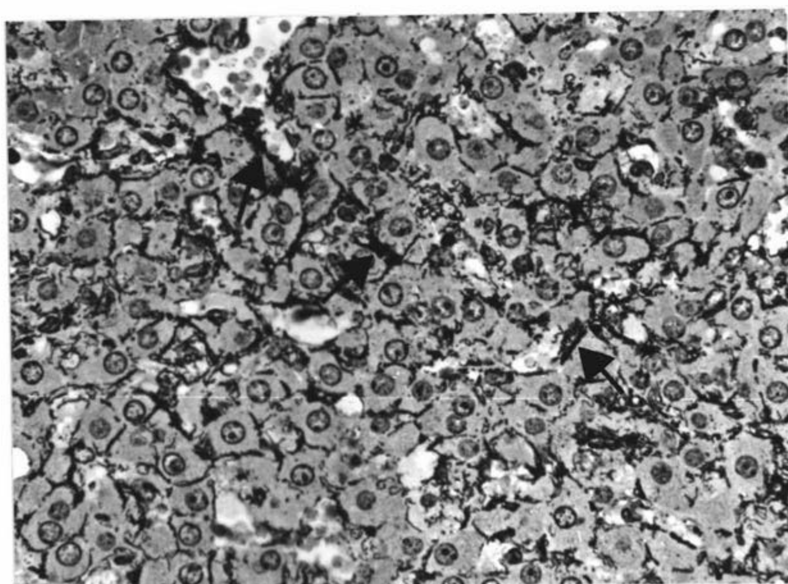
Kidney from a moribund haemoglobinaemic hamster. Large numbers of leptospires (arrows) forming colonies are present within blood vessels and in the interstitium between the tubules. Organisms are rarely seen within tubular lumina.

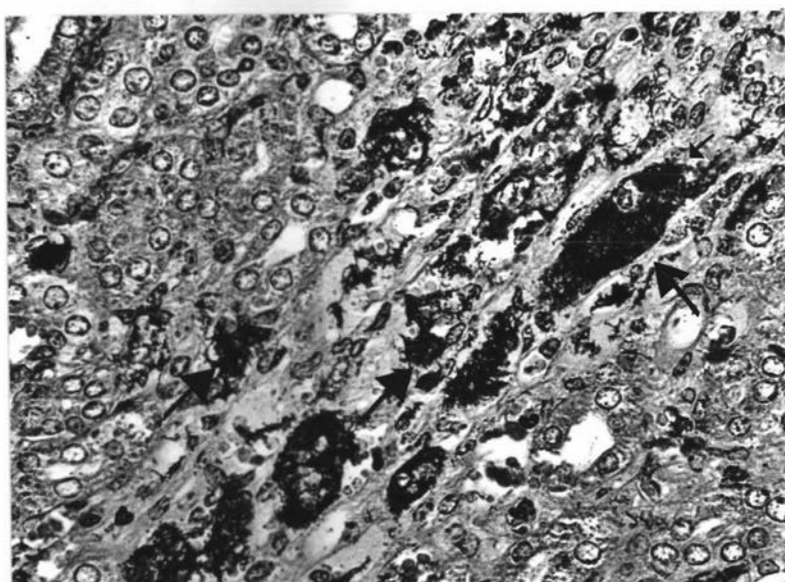
Hamster 2 W/S 200x

Fig 5-7

Lens from an untreated calf. Large vacuoles (V) and smaller vacuoles (arrows) are present within the lens fibres.

Calf 18 H&E 100x





v
v

Fig 5-8

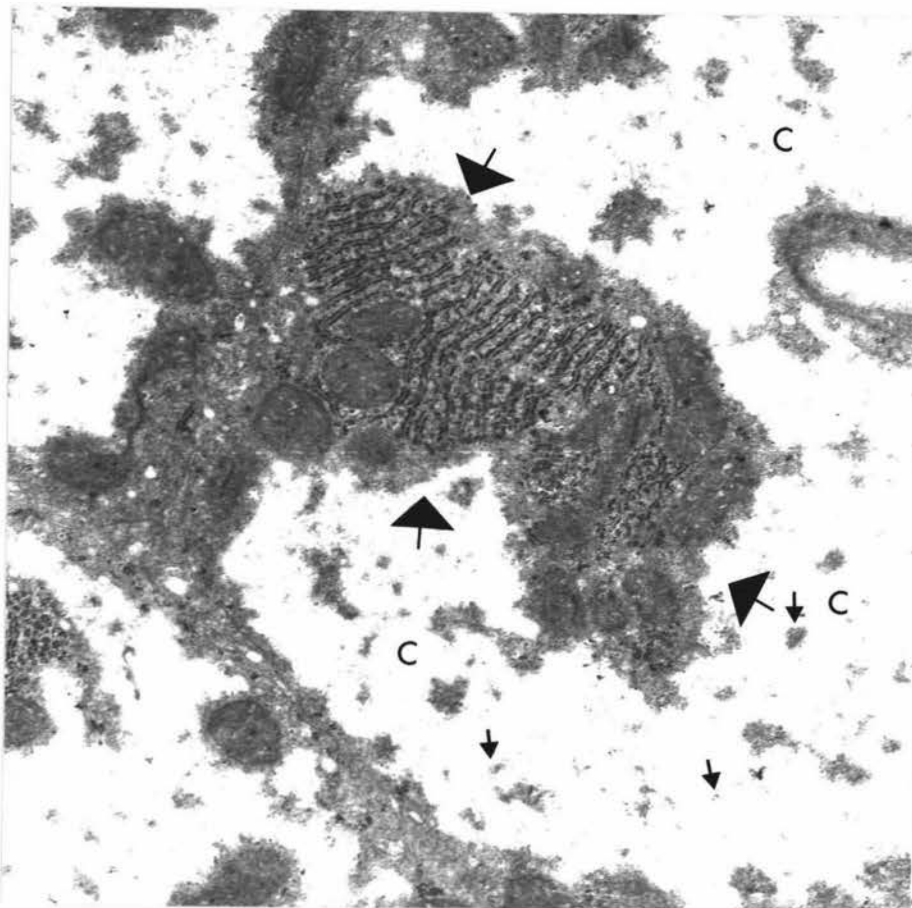
A normal hamster hepatocyte showing aggregated mitochondria and endoplasmic reticulum (large arrows) in a predominantly structureless, empty, cytoplasm (C) which contains small aggregations of a dark material (small arrows).

Hamster C1 TEM 10,500x

Fig 5-9

Hepatocyte cytoplasm of the bled calf demonstrating dilated endoplasmic reticulum (arrows) devoid of ribosomes and terminating in structureless cytoplasm (C).

Calf 20 TEM 17,500x


C
C
C

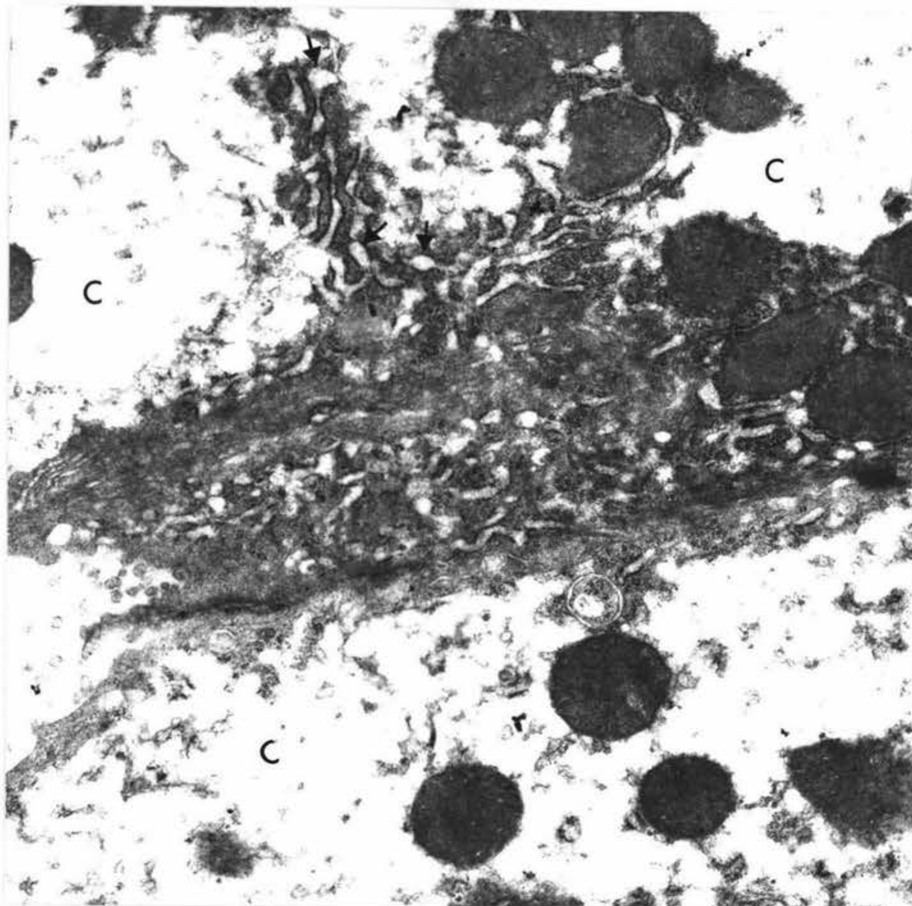

C
C
C

Fig 5-10

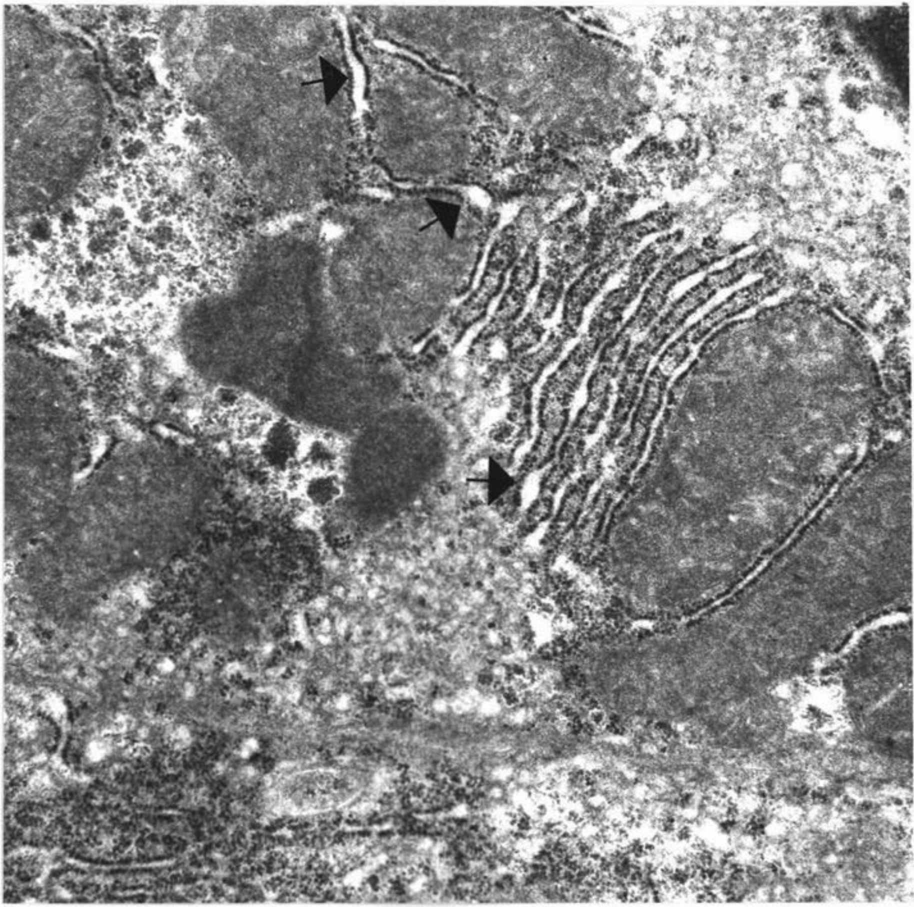
Details of a hepatocyte from a hamster infected with *ballum*. Dilated endoplasmic reticulum (arrows) and a decrease in the amount of empty cytoplasm are seen.

Hamster 5 TEM 25,000x

Fig 5-11

Region of a hepatic macrophage from a haemoglobinaemic hamster infected with *ballum*. Large numbers of RBC's, many fully haemoglobinated (R) and some showing haemoglobin loss (R') are present. Haemoglobinated RBC's are vacuolated and ovoid or circular in shape. A leptospire (arrow) is also seen.

Hamster 2 TEM 6,400x



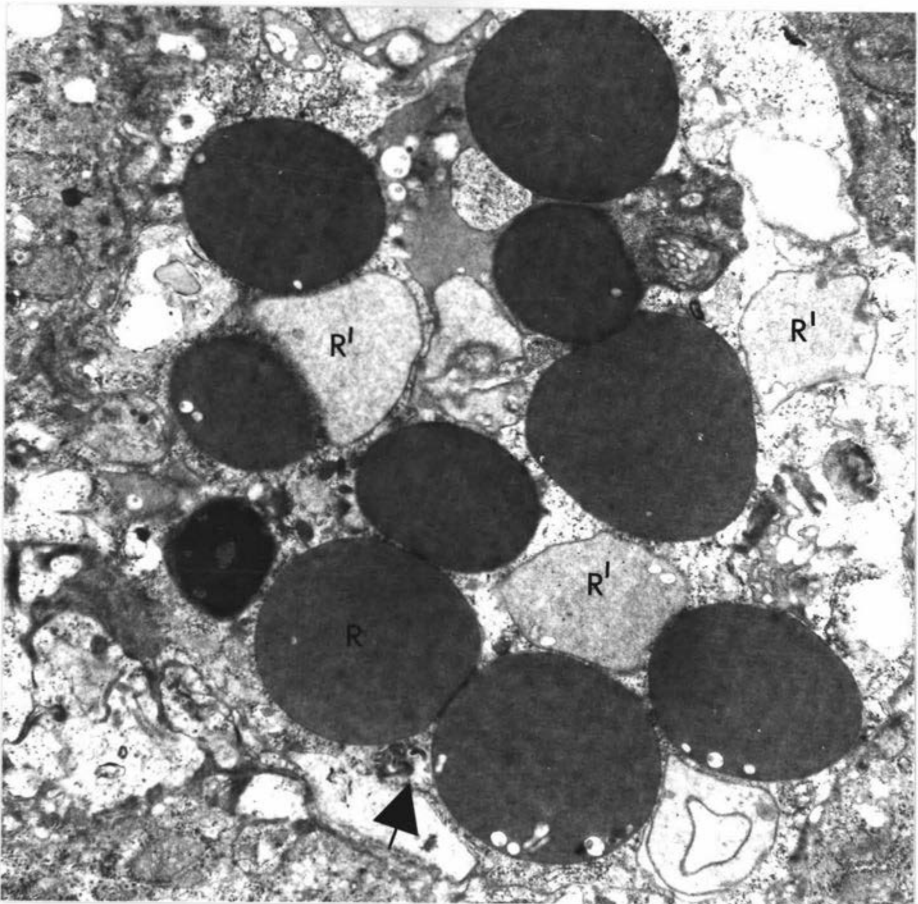

R'
R'
R'
R

Fig 5-12

Hepatic macrophage from a haemoglobinaemic calf infected with *pomona*. The phagocytosis of a RBC (arrow), other phagocytosed RBC's (R), and phagocytosed debris (D) is seen.

Calf 15 TEM 10,500x

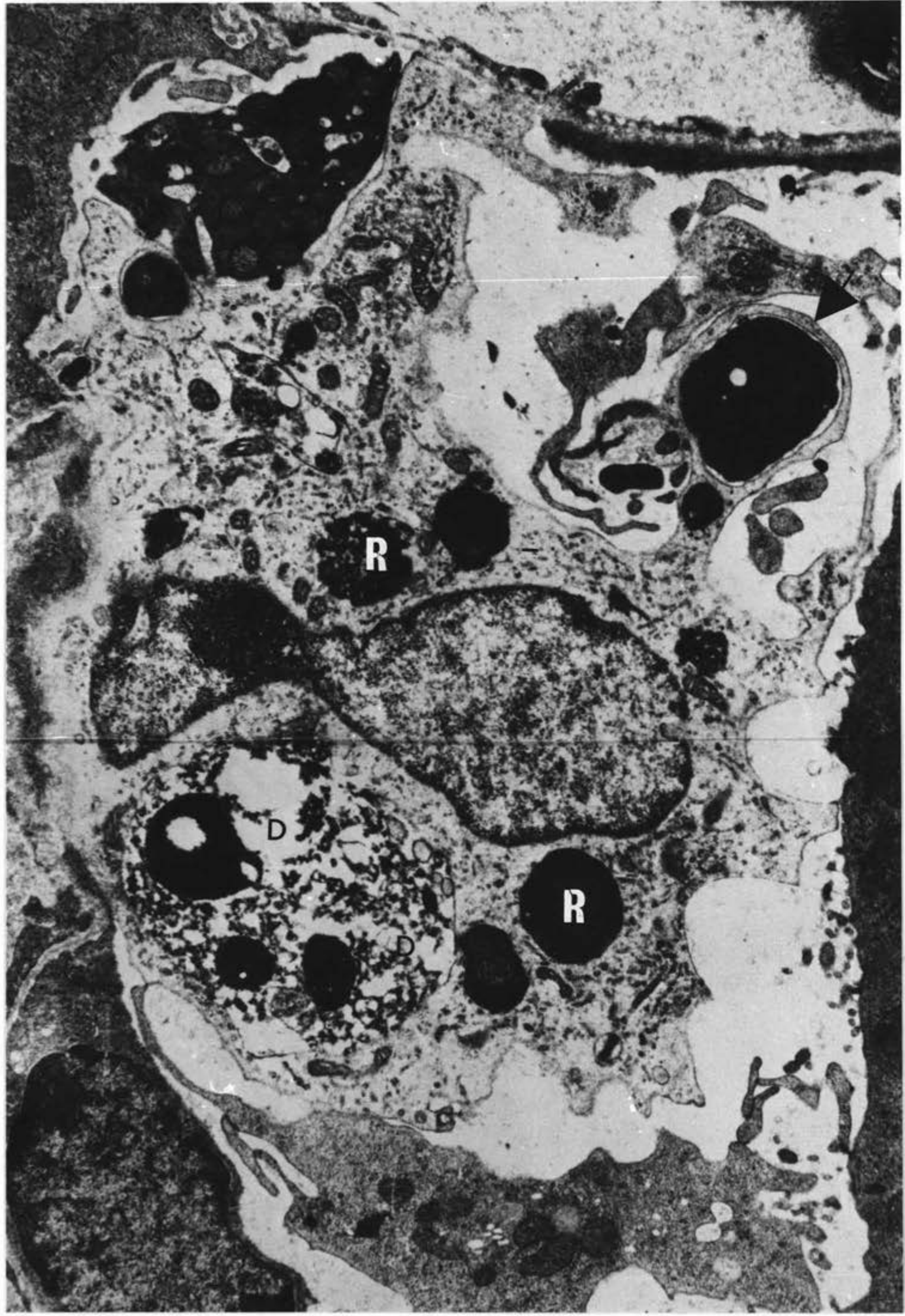

R
R
D
D

Fig 5-13

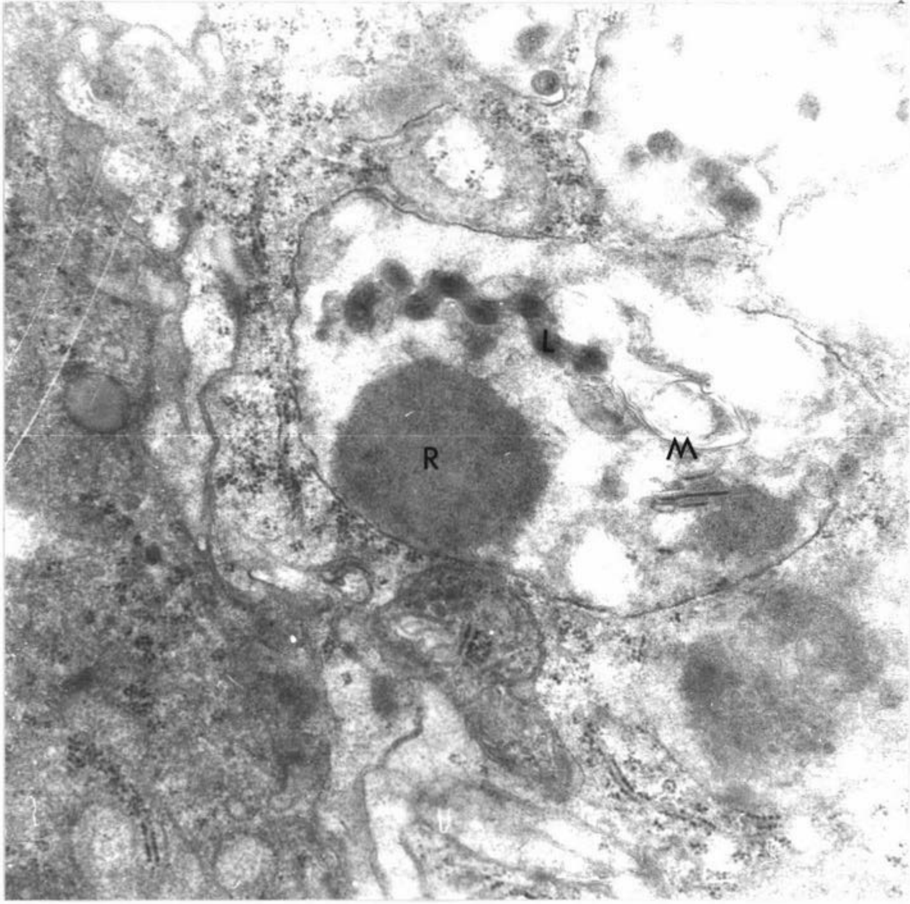
A membrane lined vacuole within a hepatic macrophage from a hamster infected with *ballum*. A degenerate leptospire (L), a haemoglobinated RBC (R), myelin forms (M) and other debris are seen.

Hamster 2 TEM 25,000x

Fig 5-14

Hepatocytes from a calf injected with *pomona* 'toxin' showing dilatation of the endoplasmic reticulum (arrows), loss of ribosomes and presence of intracellular vacuoles (V).

Calf 12 TEM 17,500x


L
R
M

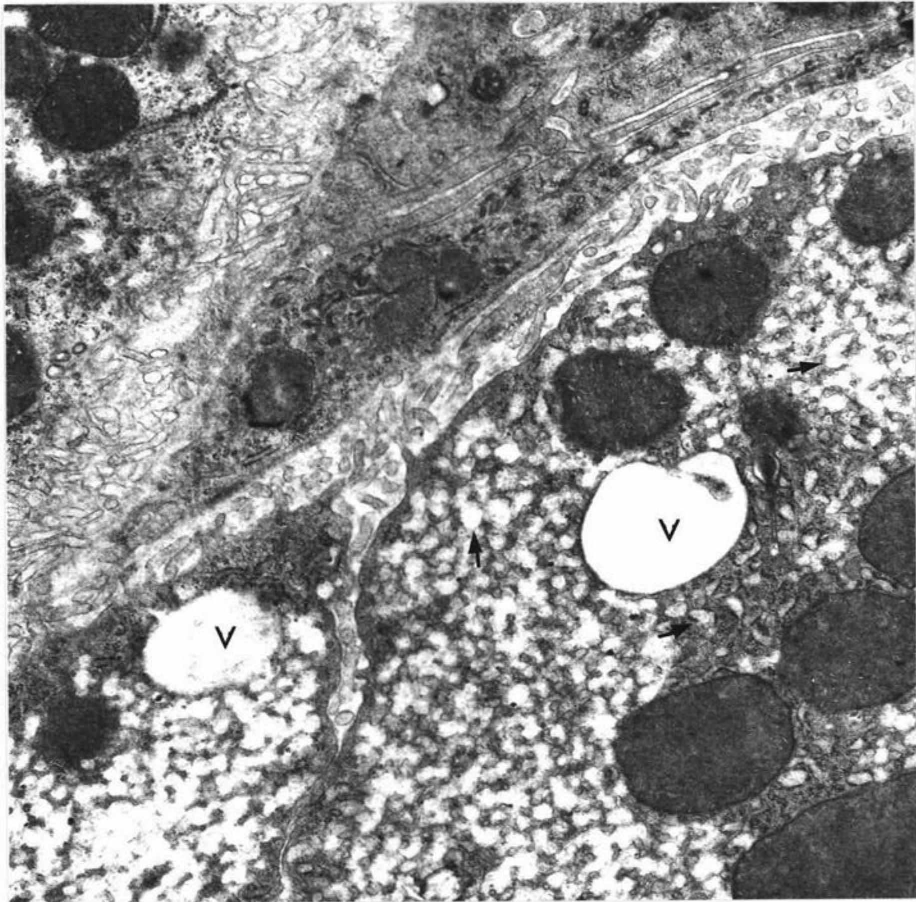

V
V

Fig 5-15

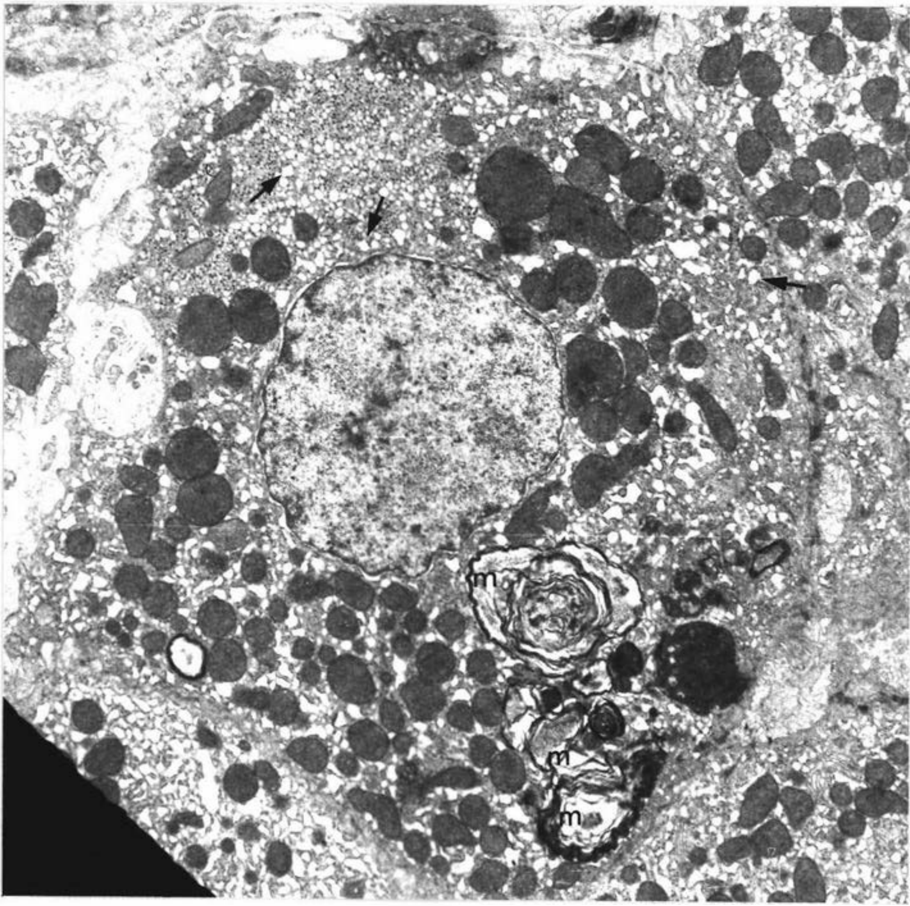
Hepatocyte from a non-haemoglobinaemic calf infected with *pomona*. Myelin forms (M) and dilated endoplasmic reticulum (arrow) are present.

Calf 11 TEM 6,400x

Fig 5-16

Liver from a non-haemoglobinaemic calf infected with *pomona*. There is dilatation of the space of Disse which contains fragments or extrusions of cells (C). The adjacent hepatocytes have slightly dilated and irregular endoplasmic reticulum (E) and loss of ribosomes. The occasional vacuole (arrow) is seen within mitochondria.

Calf 11 TEM 10,500x


m
m
m

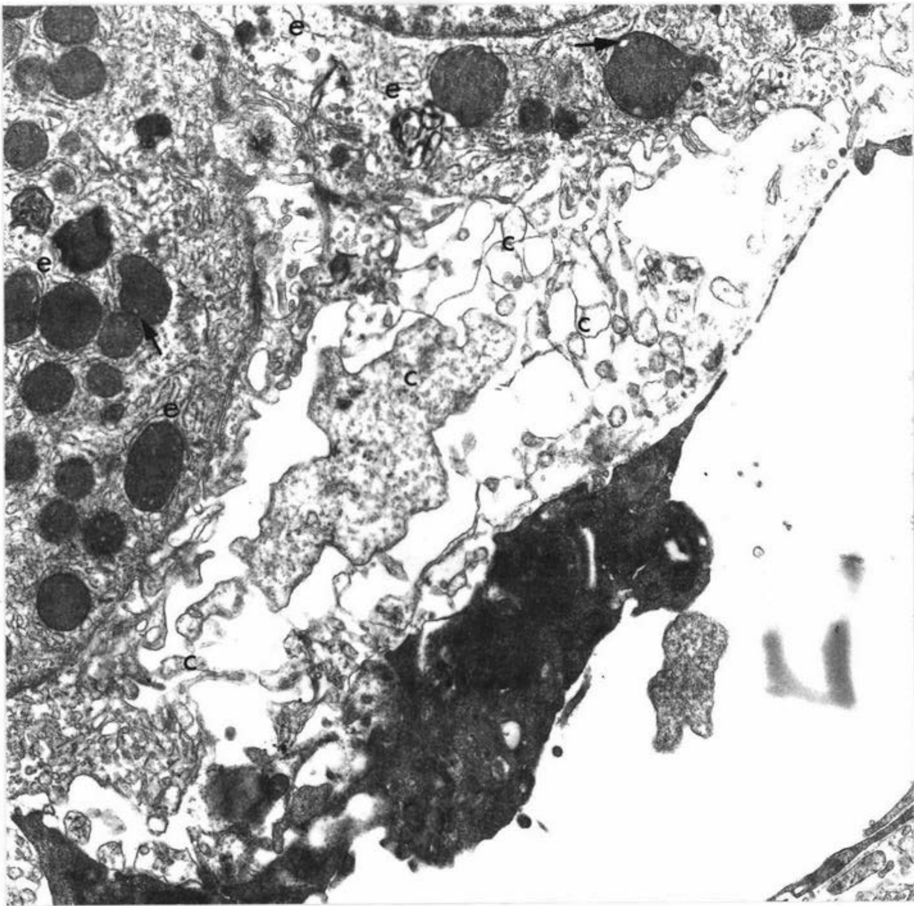

e
e
e
e
c
c
c
c

Fig. 5-17

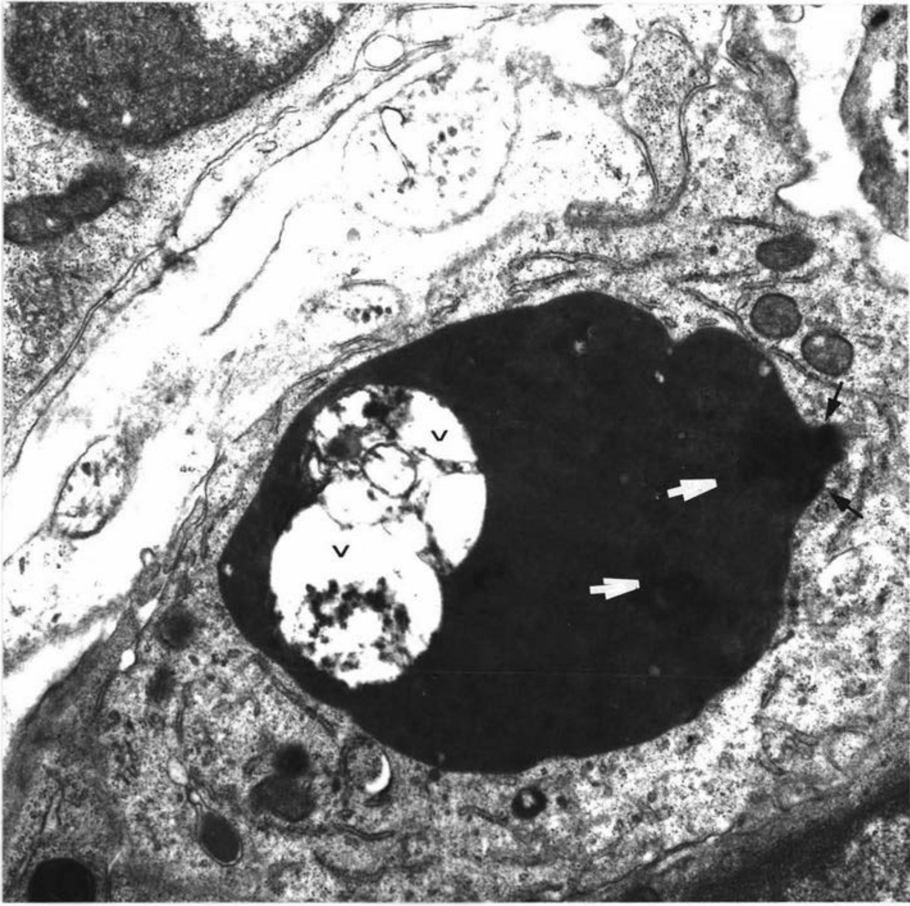
Erythrophagocytosis in a normal hamster spleen. Condensation of haemoglobin (arrow) and vacuolation (V) of the RBC cytoplasm are seen.

Hamster C1 TEM 17,500x

Fig 5-18

Mononuclear cell appearing to contact a RBC (arrow) within the spleen of a hamster infected with *ballum*.

Hamster 4 TEM 13,500x


v
v

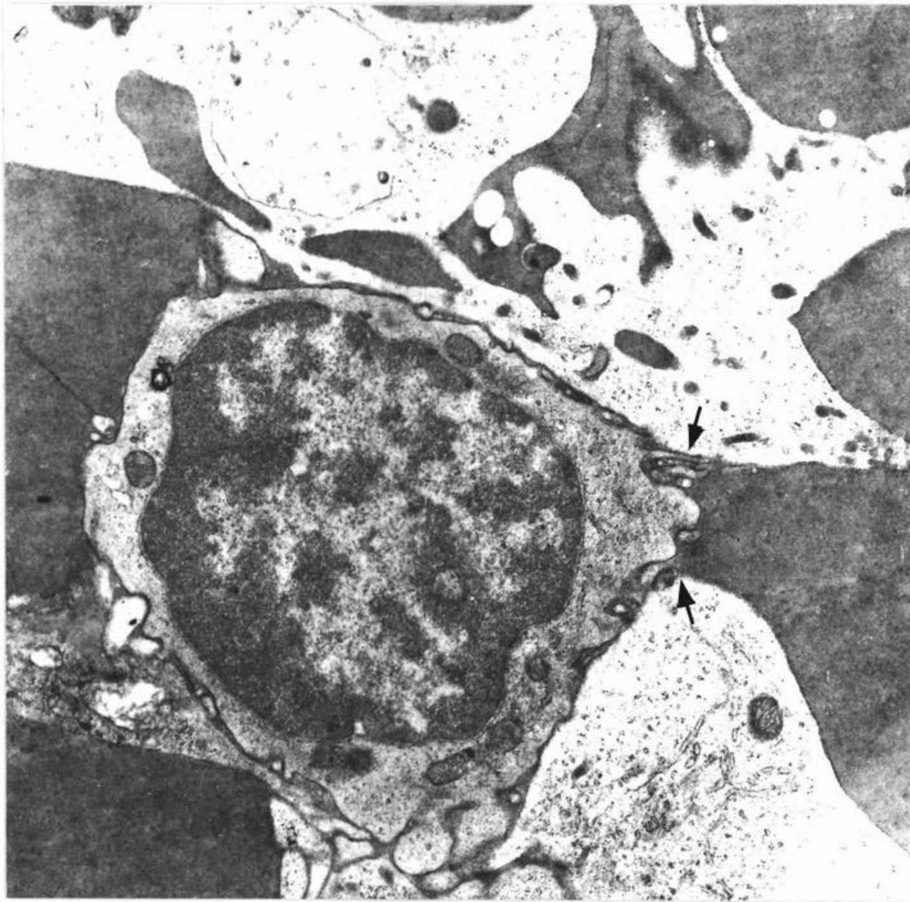


Fig 5-19

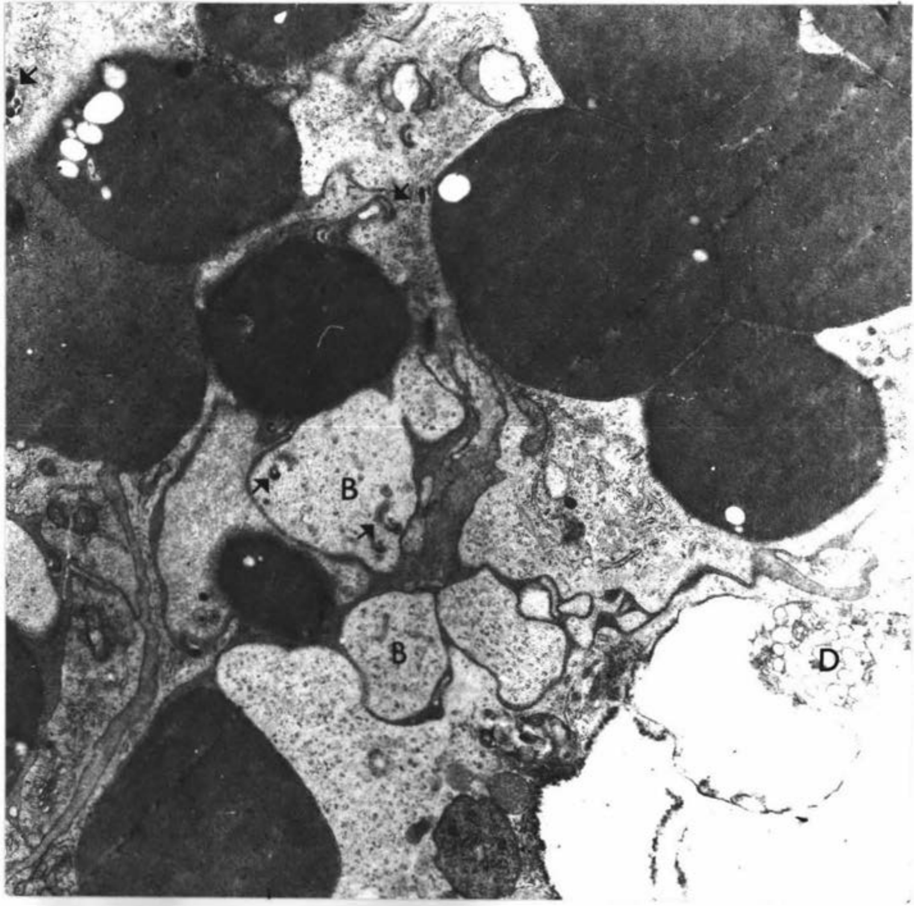
Sequestration of vacuolated, circular RBC's in the spleen of haemoglobinaemic hamster infected with *ballum*. 'Blebs' of swollen reticular cell cytoplasm (B), cell debris (D) and intracellular leptospires (arrows) are present.

Hamster 3 TEM 10,500x

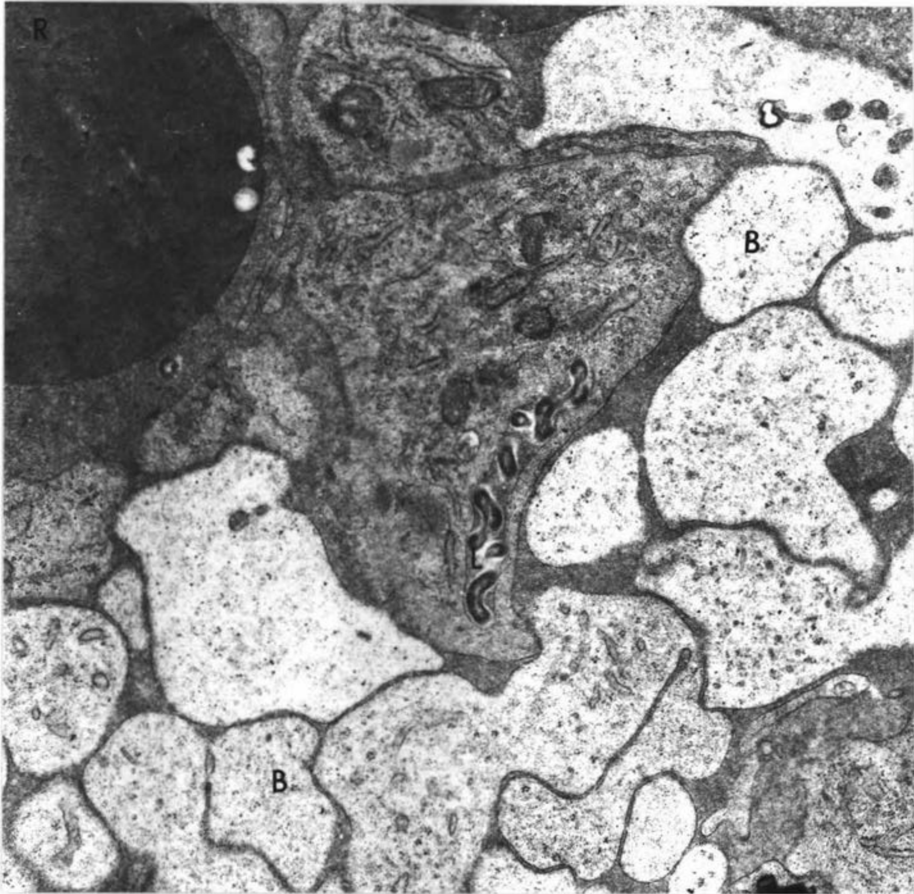
Fig 5-20

Splenic macrophage from a hamster infected with *ballum*. A leptospire (L) is seen within a membrane-bound vacuole. 'Blebs' of reticular cell cytoplasm (B) and a vacuolated RBC (R) are also seen.

Hamster 3 TEM 17,500x



B
B
D



R
B
L
B

Fig 5-21

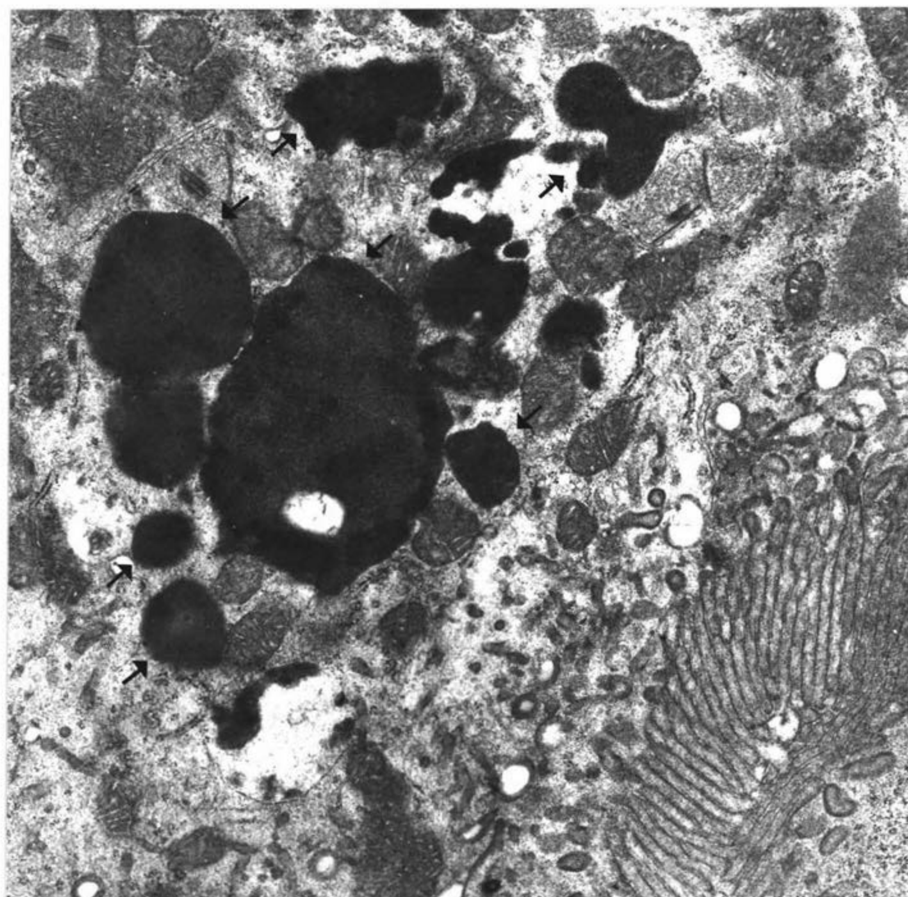
Tubular and round structures (arrows) within the cytoplasm of a reticular cell from the spleen of a hamster infected with *ballum*.

Hamster 6 TEM 17,500x

Fig 5-22

Kidney of a calf infected with *pomona*. An epithelial cell from a proximal convoluted tubule containing intracellular haemoglobin (arrows) is present.

Calf 15 TEM 17,500x



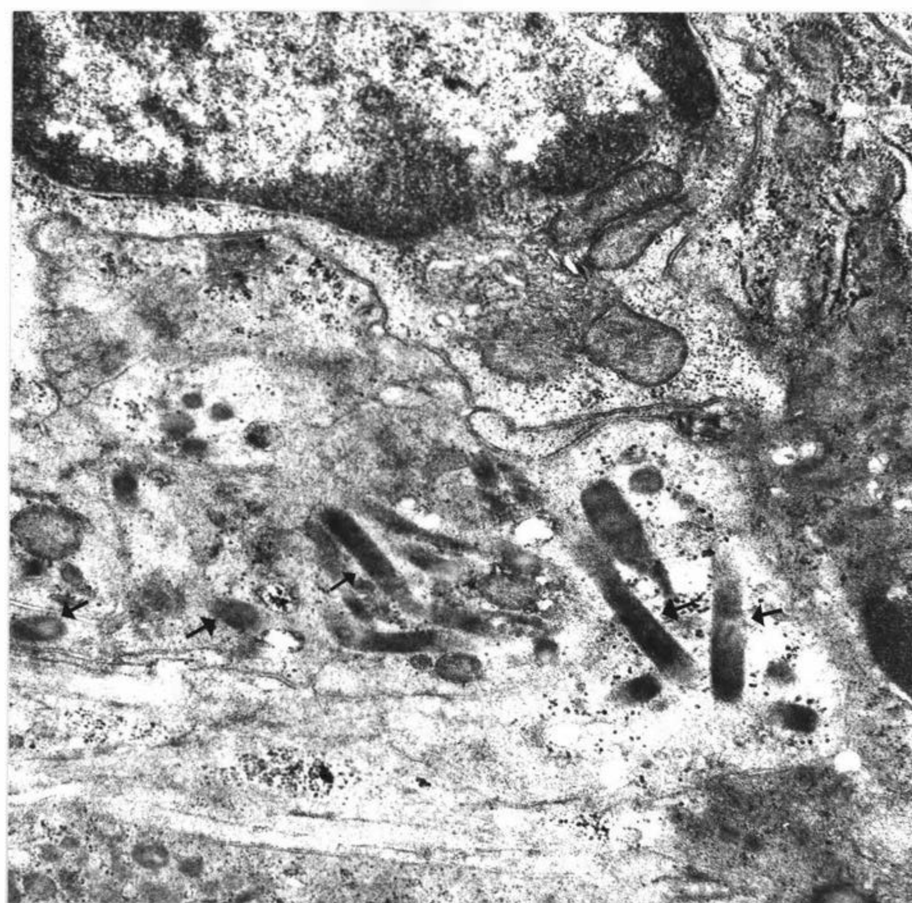


Fig 5-23

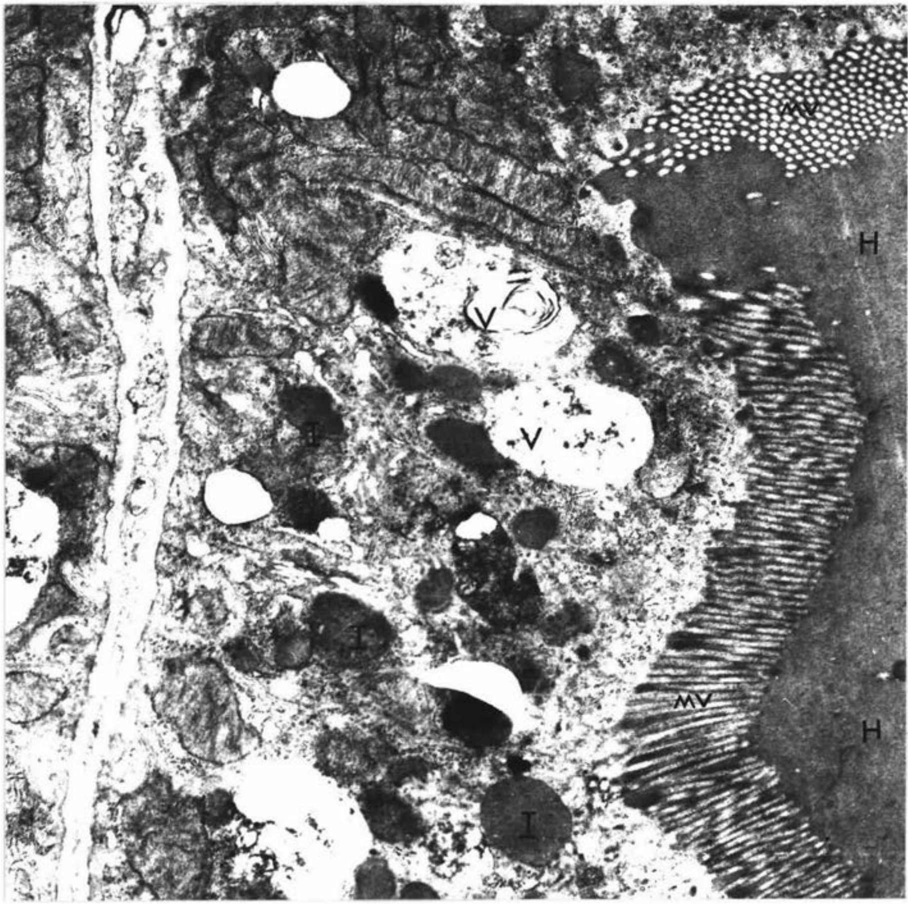
Epithelial cells from a proximal convoluted tubule of a haemoglobinaemic hamster infected with *ballum*. Large numbers of vacuoles (V) and inclusions (I) are seen. The microvilli (MV) appear intact and the haemoglobin (H) within the tubular lumen has a homogeneous appearance.

Hamster 1 TEM 10,500x

Fig 5-24

Epithelial cells from a haemoglobinated hamster infected with *ballum* showing greater degenerative changes when compared to the above figure. The lumenal haemoglobin shows some condensation and has a less homogeneous appearance. The associated cells show shrinkage and loss of microvilli (MV). One cell (C) appears shrunken. Cell debris (D) is present in the tubular lumen.

Hamster 1 TEM 10,500x


mv
H
V
V
I
I
mv
H
I

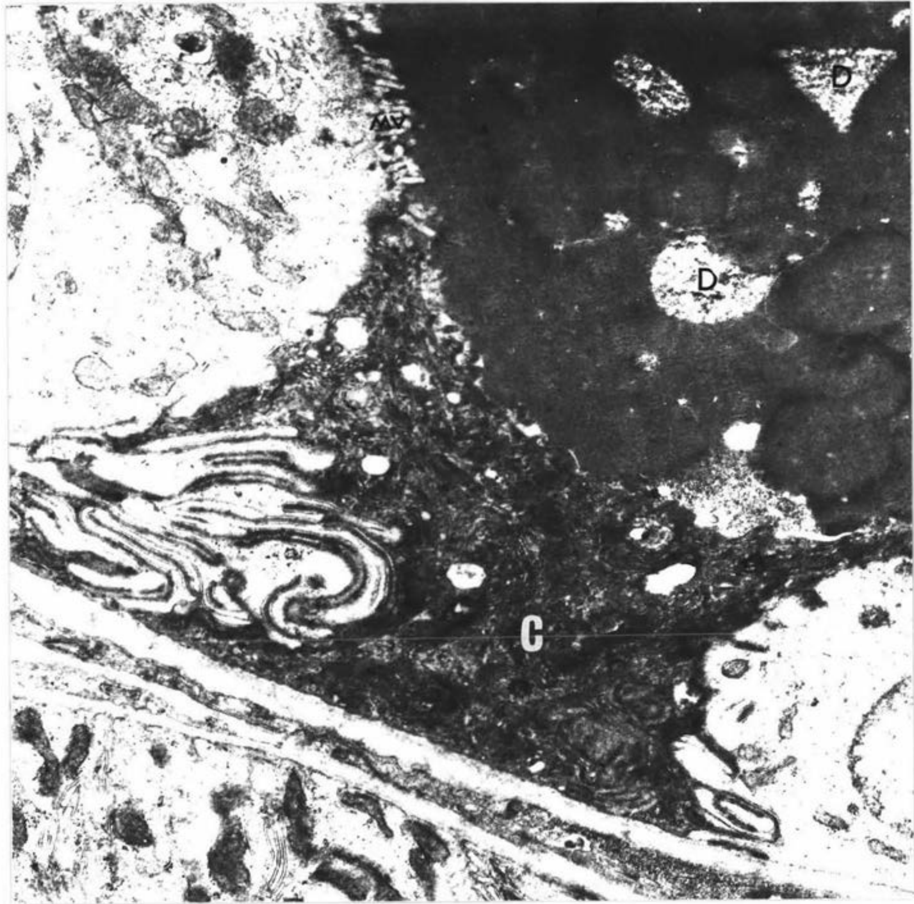

mv
D
D
C

Fig 5-25

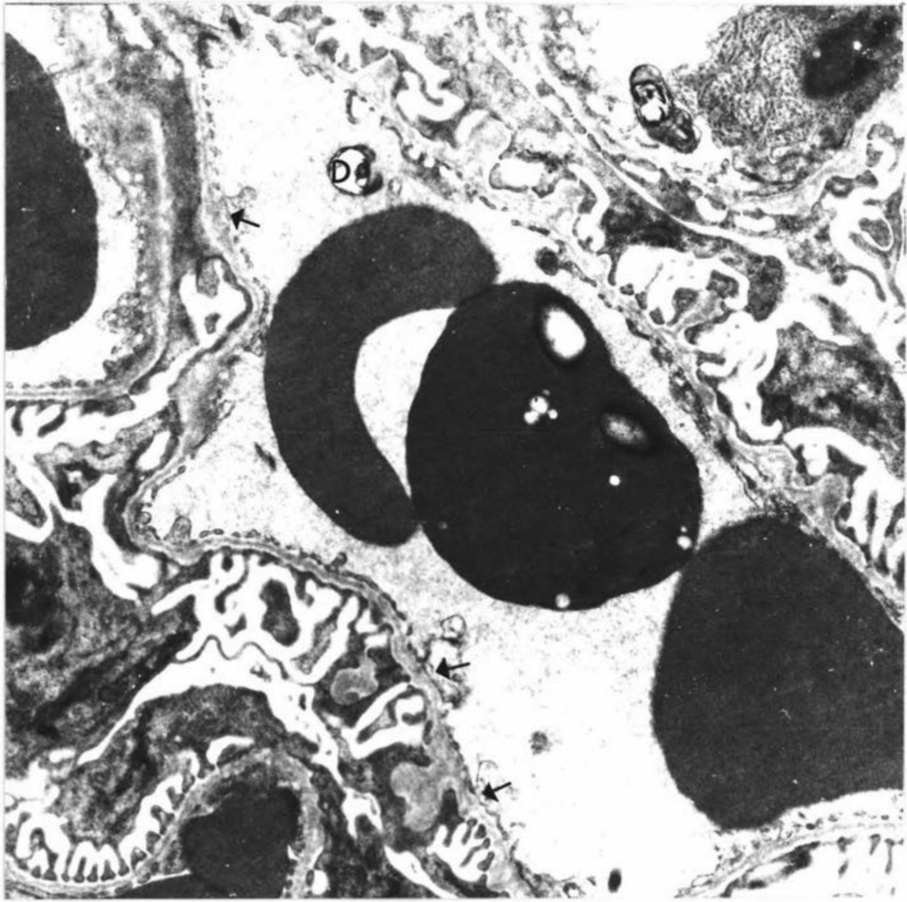
Glomerular capillary from a haemoglobinaemic hamster infected with *ballum*. Vacuolated RBC's and cellular debris (D) are present within the capillary lumen. Portions of the capillary endothelium (arrow) appear to be lifting off the basement membrane.

Hamster 4 TEM 10,500x

Fig 5-26

Glomerular capillary from a non-haemoglobinaemic hamster infected with *ballum*. A circular, vacuolated RBC is seen and also oedema (Oe) between the basement membrane and endothelial cell.

Hamster 5 TEM 10,500x


D

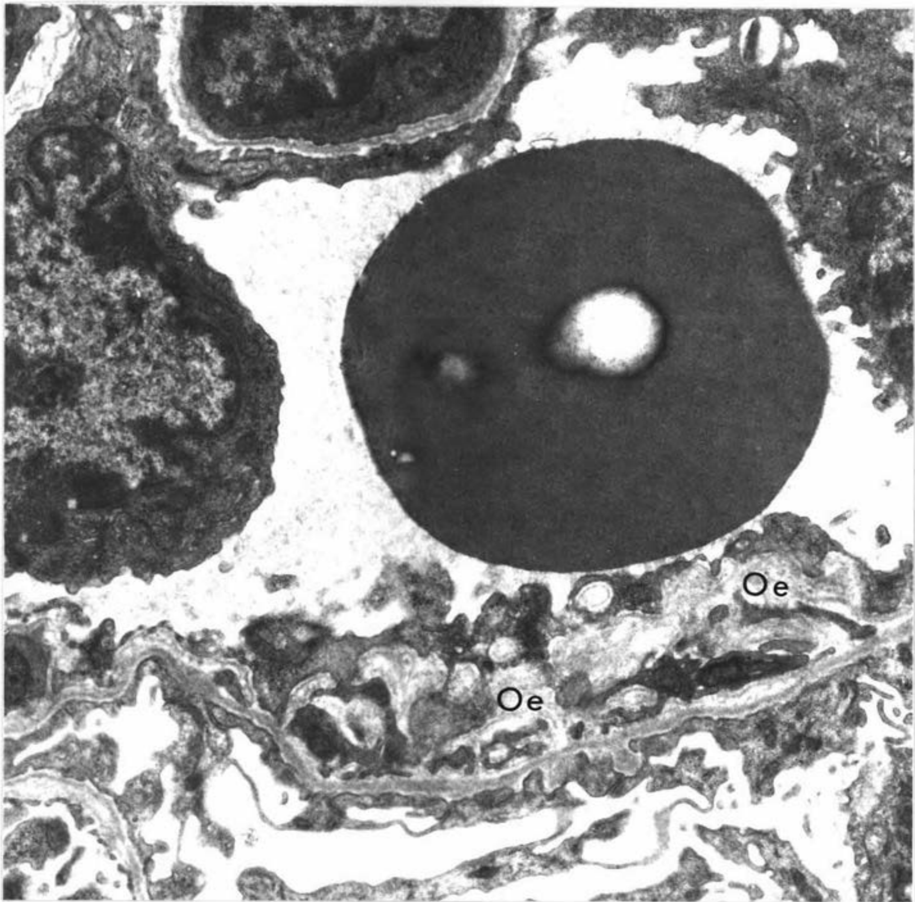

Oe
Oe

Fig 5-27

Leptospire migrating in a pocket of oedema (Oe) between the basement membranes of tubules from a hamster infected with *ballum*.

Hamster 4 TEM 25,000x

Fig 5-28

Leptospires (L) within a proximal convoluted tubule of a calf 14 days after infection with *pomona*. Numbers of microvilli appear reduced and some cell debris (D) is present within the lumen.

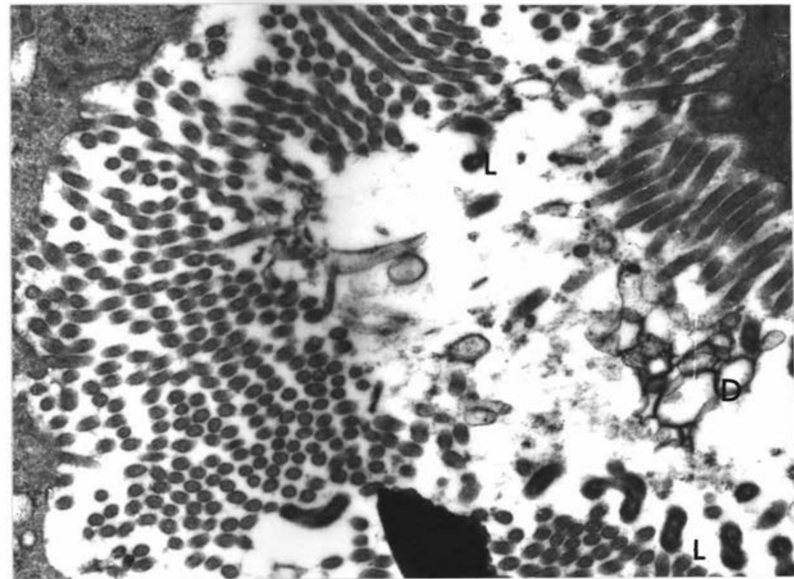
Calf 11 TEM 22,500x

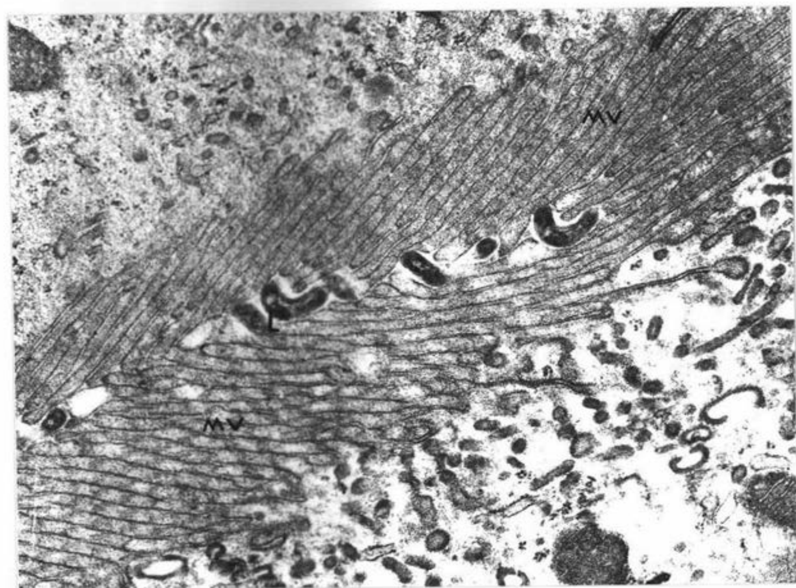
Fig 5-29

Leptospires (L) within the proximal convoluted tubule of a calf 14 days after infection with *pomona*. Microvilli (MV) appear normal.

Calf 11 TEM 22,500x

Oe
Oe


L
D
L


MV
L
MV

3.3.4 Bone marrow

3.3.4.1 Control animals

Bone marrow from control animals was normal histologically and no haemosiderin was seen. Ultrastructural examination showed maturation stages for all haemopoietic cell lines as described by Bessis (1973).

3.3.4.2 Infections associated with haemoglobinaemia

Changes in hamster marrow histology were difficult to distinguish until RBC destruction was well advanced and the animals were severely haemoglobinaemic.

Marrow from haemoglobinaemic calves and hamsters was hypocellular for both erythroid and myeloid cells lines. Blast erythroid and myeloid cells were seen with an almost complete lack of mature cells.

Erythrophagocytosis was frequent in all severely haemoglobinaemic animals. In hamsters, the macrophages were sometimes extremely large and filled with an eosinophilic homogeneous material similar to that seen in splenic macrophages. Haemosiderin was absent in haemoglobinaemic animals.

Large numbers of leptospires were seen in the marrow sinusoids of the severely haemoglobinaemic hamsters.

Ultrastructurally, macrophages and erythrophagocytosis were commonly seen in the haemoglobinaemic calf. The RBC degradation and morphology was similar to that in the splenic and hepatic macrophages. Late normoblasts with abnormal nuclei were seen in marrow from calf 15.

3.3.4.3 Infections not associated with haemoglobinaemia

Marrow from calves 11 and 16 appeared histologically normal with neither haemosiderin or leptospires present.

3.3.4.4 'Toxin' injected calves

No histological and ultrastructural abnormalities or haemosiderin was seen.

3.3.4.5 Bled animals

Marrow from the bled hamsters was normal apart from increased numbers of erythropoietic cells.

3.3.5 Lymph node

Lymph nodes from the haemoglobinaemic calves 14, 15 and 17 showed severe depletion of mature lymphocytes from the lymphoid follicles. Pyknotic nuclei from lymphocytes and the occasional PMN were seen in the medulla. The occasional leptospire was seen in the medulla. Haemorrhage was seen in the medullary sinuses of calf 15.

Lymph nodes from infected calves 11 and 16 and 'toxin' injected calves 12 and 13 were normal except for small infiltrations of PMN's in medullary sinuses. The latter were seen in all calves, including the control animals.

3.3.6 Thymus

In the haemoglobinaemic calves 14, 15 and 17 there was severe depletion of the cortical lymphoid tissue. Petechial haemorrhages were common in the medullary regions of calves 14 and 15 and infrequent in calf 17. No lesions were observed in the other calves.

3.3.7 Lung

In haemoglobinaemic calves 14, 15, 17 and hamsters there were some areas of collapse, emphysema and congestion. Euthanased hamsters often had intra-alveolar RBC's. In those hamsters found dead, blood vessels often contained large numbers of WBC's. Phagocytosis of RBC's and haemosiderin was seen in alveolar macrophages of severely haemoglobinaemic hamsters.

Few leptospires were seen in lung sections from hamsters and calves 14, 15 and 17. No leptospires were seen in calves 11 and 16.

Lungs from all control hamsters showed areas of collapse, emphysema and intra-alveolar RBC's. Intra-alveolar RBC's resulted from the inhalation of RBC's following severance of the jugular veins.

The focal area of dull red hepatization in the ventral portion of the right cardiac lobe noted at post-mortem examination of control calf 19 was an area of subacute pneumonia. No lesions were seen in the lungs of control, 'toxin' injected or 'bled' calves, and infected calves 11 and 16.

3.3.8 Brain

No lesions of significance were noted in any calves. Leptospires were not seen within the brain tissue although some organisms were found within blood vessels of calves 14,15 and 17.

3.3.9 Eye

Vacuolation of lens fibres was seen in all calves (Fig. 5-7). No lesions were seen in other ocular tissues. No leptospires were seen in tissue or in the aqueous or vitrous humours.

3.3.10 Cardiac muscle

With the exception of some lightly staining bundles of cardiac muscle fibres in severely haemoglobinaemic calves and some hamsters, no lesions were present.

Leptospires were present in blood in the lumen of blood vessels and heart chambers of haemoglobinaemic animals.

3.3.11 Other tissues

No significant histological lesions and only the occasional leptospire was seen in the calf adrenal glands, pancreas , thyroid gland or intestines.

4.0 DISCUSSION

The haemolytic infections of the *pomona* infected calves and *ballum* infected hamsters could be divided into two categories:

a) animals with haemoglobinaemia plus those animals that would be expected to become haemoglobinaemic had they not been euthanased early in the course of the disease.

or b) animals without haemoglobinaemia but with evidence of increased RBC destruction and production.

On the basis of the clinical pathological results and RBC ultrastructural studies (Chapters 3 and 4) all infected hamsters and infected calves 14, 15 and 17 could be placed in the first category. The 'toxin' injected calves 12 and 13 showed ultrastructural changes similar to those of pre-haemoglobinaemic hamsters and were also placed in the first category.

Non-haemoglobinaemic calves 11 and 16 showed no signs of becoming haemoglobinaemic and were therefore placed in the latter category on the evidence of increased RBC destruction and production. Calf 11 had altered RBC morphology and a decreased M:E ratio and PCV and although calf 16 showed normal RBC morphology, the M:E ratio was lower than those of the control calves.

The severity of lesions in calves and hamsters appeared to be directly related to the degree of RBC destruction and a definite pattern of lesion development in the various organs was apparent. The earliest noted change, that of increase in the weight of the spleen was shown by light and electron microscopy to be due to RBC sequestration which occurred before clinical signs appeared and before haemoglobinaemia was overt. The spleen is a sensitive detector of altered and damaged RBC's, (Crosby, 1977; Jandl & Aster, 1967; Jandl *et al*, 1965; Rifkind, 1965; Wagner *et al*, 1962; Weiss, 1962a) and it is therefore likely that the echinocytic cells in circulation (Chapter 4) were being detected and removed at an early stage in the disease. This increased rate of RBC destruction

was countered by increased RBC formation shown in the bone marrow response described in Chapter 3. While minor changes in RBC's are detected by the spleen through its complex sinusoidal and reticular cord system which enables RBC's to come into close contact with fixed macrophage cells (Weiss, 1962a) the liver is only able to detect the more gross changes in RBC morphology (Jandl & Aster, 1967; Jandl *et al*, 1965). RBC sequestration therefore occurred later in the liver than in the spleen and hence the increase in liver body weight ratios occurred later than the increase in the spleen: body weight ratio.

In the present experiments the calves which developed haemoglobinaemia showed essentially similar gross and histopathological changes in the spleen, liver and kidney to those reported in haemolytic *pomona* infections of cattle (Cordy & Jasper, 1952; Ferguson *et al*, 1957; Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963) and some haemolytic *canicola* infections of calves (Imbabi *et al*, 1967; Van der Hoeden, 1955).

The gross and histological lesions of hamsters infected with *ballum* have not been described in detail in earlier work but the present experiments were similar to those briefly described by Frenkel (1972).

The pathological findings from the haemoglobinaemic calves and hamsters while being essentially similar, were of a greater severity in hamsters. This observation confirmed the earlier clinical pathological results.

Liver lesions appeared early in the disease before leptospiral organisms were visible by W/S stain. The initially periportal distribution of hepatocyte degeneration suggests the action of a noxious agent infused via the portal vessels. It seems likely that this agent is a 'toxin' released by leptospires multiplying in blood. The leptospires, while shown culturally to be present in calves, would have been very few in number as it was not until the latter stages of disease in the haemoglobinaemic animals that they were visible by dfm.

The degenerative changes shown in the hepatocytes of the 'toxin' injected calves provides further support to the hypothesis that initial hepatocyte degeneration is due to leptospiral 'toxin'. The later centrilobular lesions occurring when anaemia was present are consistent with those in hypoxia (Jubb & Kennedy, 1963). The most severe degenerative changes occurred late in the disease in the haemoglobinaemic animals and it could be argued that to reproduce this stage in the 'toxin' injected calves that much more 'toxin' would have to be infused intravenously in order to cause RBC destruction and haemoglobinaemia resulting in hypoxic lesions of the liver.

Miller & Wilson (1966) also demonstrated that liver lesions occurred before large numbers of organisms were seen in the liver and did not appear directly associated with tissue damage. 'Toxins' prepared from *pomona, canicola* and *icterohaemorrhagiae* have been demonstrated to be cytotoxic to cells in tissue culture and to kill mice (Knight *et al*, 1973). Other workers have noted that intracellular changes in hepatocyte enzymes occur before histopathological lesions become severe suggesting an initial functional defect of enzyme systems (Arean, 1962b; Arean & Henry, 1964) but they did not comment upon a differential distribution of liver lesions. Hepatocytes are very active metabolically and therefore probably very susceptible to factors such as hypoxia. Light microscopy of hamster and calf liver from bled animals and ultra-structural examination of the bled calf showed that loss of RBC's did appear to have some effect but only one calf and six hamsters were examined. Larger numbers of animals would have to be examined to conclusively establish the pathological effects of hypoxia and to differentiate these effects from those of leptospiral 'toxins'.

The ultrastructural changes in hepatocytes of swollen endoplasmic reticulum and degeneration of the mitochondria are similar to those recorded in *pomona* (Miller & Wilson, 1966) and *canicola* (Sapp *et al*, 1980) infections of hamsters in which the severe cellular necrosis was attributed to hypoxia.

The 'cytoplasmic blebs' seen within the spleen before large numbers of organisms were seen resemble those seen in endothelial cells of hamsters and guinea pigs infected with *icterohaemorrhagiae* (De Brito *et al* 1979; Miller *et al*, 1974) and in the glomerulus of sheep infected with *pomona* (Marshall, 1973). It seems likely that this change is a result of leptospiral 'toxin' as leptospires were rarely seen close to the affected cells in any of the reported work.

In those animals which were haemoglobinaemic or in which haemoglobinaemia was likely to occur had the animal not been euthanased, the most noticeable changes seen histologically and by EM were those of RBC sequestration and erythrophagocytosis. RBC's were engulfed whole by splenic and hepatic phagocytes. It has been suggested that the damaged RBC's are detected by alterations in the mechanical properties (La Celle, 1970), metabolic functions (Kaneko, 1974) or surface characteristics (Seaman *et al*, 1977) of the cell.

A notable feature was that although haemoglobinaemia was very severe in some cases, very few partly haemoglobinated RBC's were seen either in circulation and only slightly larger numbers in macrophages. There are two possible explanations for this observation. It seems likely that either partially haemoglobinated RBC's may be removed from circulation and degraded so rapidly that little evidence of them remains, or alternatively damaged fully haemoglobinated RBC's are recognised and removed by the spleen before intravascular haemolysis occurs and then haemoglobin is subsequently released back into circulation. This latter mechanism has been suggested to occur during immune haemolysis (Jandl & Aster, 1967).

The presence of pale staining haemosiderin within the liver and spleen reflects the suddenness of onset of the RBC destruction as dark granular deposits such as those seen in the control hamsters euthanased last in Experiment I, part 1 are probably formed over a long period of time and result from concentration of the pale

form of haemosiderin. The generalized, diffuse distribution of the haemosiderin in macrophages throughout the hepatic lobule and splenic red pulp reflects the generalized uptake by many cells of haemoglobin from the plasma and/or erythrophagocytosis of the sequestered RBC's.

The dark tubular inclusions seen in some splenic cells resemble those seen in the endothelial cells of ocular (Matsuda & Sugiura, 1970) and other blood vessels (Sengel & Stoebner, 1970). They may be the source of thromboplastic substances which are thought to be released by blood vessel walls (Shimamoto & Ishioka, 1963) and may be important in determining clotting time. Increased numbers of these inclusions may be an indication of a slightly increased need for clotting factor production as a result of minor haemorrhages although overall the infection may not appear haemorrhagic.

Previous work on experimentally induced haemoglobinaemia resulted in renal nephrosis (Flink, 1947; Jaenike & Schneeberger, 1966). This was postulated to arise from renal ischaemia caused by the intravascular aggregation of haemoglobin obstructing the passage of metabolites from blood to the tubular epithelial walls and hence a functional defect of the cells (Jaenike & Schneeberger, 1966). Leptospiral induced nephritis has been attributed to hypovolaemia caused by endothelial cell damage and fluid leakage and haemorrhage (Sitprija *et al*, 1980), 'toxin' damage (Arean, 1962b; Miller & Wilson, 1967; Sitprija *et al*, 1980) and mechanical damage to endothelial cells by migrating leptospires (Marshall, 1973).

In the present work, haemoglobinaemia and the presence of large numbers of organisms coincided and occurred at about the time of death. While haemoglobinaemic nephrosis is considered likely, it is difficult to separate these effects from those of the actual organism and its 'toxins'. In addition, the pathogenicity of haemoglobin for renal tubules is further complicated by the fact that haemoglobin free of RBC stroma does not produce nephrosis (Dudziak & Bonhard, 1980; Relihan & Litwan, 1972). Dehydration

and therefore hypovolaemia was particularly noticeable in the hamsters (Chapter 3). No evidence for mechanical damage caused by migrating leptospires was seen and the organisms always appeared to migrate between cells. Severe renal lesions were seen in other experimental animals (Chapter 6) in which haemoglobinaemia was not a feature. In the haemoglobinaemic animals the causes of tubular damage are probably multifactorial arising from hypoxia due to hypovolaemia and RBC loss, leptospiral 'toxins' and the presence of free plasma haemoglobin.

The loss of microvilli from epithelial cells of the proximal convoluted tubules may result from the mechanisms postulated by Ericsson (1964) in work on experimentally induced haemoglobinaemia in rats. The absorption of proteins from the tubular lumen occurs at the base of the microvilli in the tubules which are pinched off at the tips to form vesicles which later coalesce resulting in vacuole formation. The intracellular vesicles are formed using membrane from the microvilli and where absorption is excessive the microvilli shrink. Vacuoles seen by EM were not as large as those reported in sucrose nephrosis which are formed by the enlargement and coalescence of cytosomes (Maunsbach, *et al*, 1962; Trump & Janigan, 1962) and therefore seem more likely to be due to other causes such as proteinuria which was shown to be present in the calves (Chapter 3). Previous work has shown that vacuolation of tubules may be associated with proteinuria (Oliver *et al*, 1954).

The glomerular changes of oedema and degeneration of the endothelial cells were minor. Reports of endothelial cell damage varied in other work. De Brito *et al* (1965) found no evidence for endothelial cell damage but later work (Marshall, 1973; De Brito *et al*, 1967) described endothelial cell damage.

The fusion of the glomerular foot processes described by these workers (De Brito *et al*, 1965, 1967; Marshall, 1973) was not seen in the present work. Marshall (1973) also described

podocyte and mesangial cell changes but his studies were on sheep which survivied for 30 days.

Necrosis, whether induced by leptospiral 'toxins' or hypoxia is probably the major stimulus for the accumulations of macrophages and in some cases PMN's. Lymphocyte accumulation and proliferation locally was most likely in response to antigenic stimulus from leptospires. The depletion of lymphocytes from lymphoid tissue could be as a result of either recruitment of lymphocytes to the blood or site of local reaction, or a lymphotoxic action. While lymphocyte counts in the calves showed some variation, hamsters could have very high lymphocytoses or profound lymphopaenias representing either increased formation and release, increased tissue usage, or decreased production. The hypocellularity of the bone marrow is probably caused by the combined effects of increased demand for all haemopoietic lines and 'toxic' depression of the marrow.

The PMN infiltration of the prescapular lymph nodes of all calves was thought to be due to a local reaction to the products of traumatised tissue resulting from daily venepuncture. Because precautions were taken to clean the site of injection and there was an absence of inflammatory lesions around the jugular vein and microorganisms within the lymph node, the PMN's are unlikely to have resulted from infection.

Although some hamsters showed what could be considered as neurological signs, the calves showed no signs. The absence of brain lesions in calves reflected other work in which *pomona* infected hamsters, while showing neurological signs, had no brain lesions (Abdu & Sleight, 1965). These authors attributed neurological signs to uraemia and electrolyte imbalance. Clinical pathology examinations to test for uraemia in calves (Chapter 3) showed uraemia was not present as BUN levels were only slightly elevated. These tests were not done on hamsters. Other species such as pigs (Sleight *et al*, 1960) and goats (Morse &

Langham, 1958) have had light perivascular cuffing of the cerebellum and meninges following *pomona* infections with no related CNS signs. Similarly, with an absence of neurological signs, Marshall *et al* (1980) observed perivascular cuffing by mononuclear cells in the meninges of grivet monkeys infected with *balcanica* and *tarassovi*.

The lesions of the lens fibres resembled those of early cataracts (Jubb & Kennedy, 1963) and are considered a coincidental finding because they occurred in control as well as infected calves.

It has been suggested that leptospires may enter and remain within cells for prolonged periods (Miller & Wilson, 1966) and Miller & Wilson (1962, 1967) described intraerythrocytic leptospires, suggesting this as a means for dissemination throughout the body. Evidence from the present study suggests that intracellular leptospires are rapidly destroyed. The morphologically normal leptospires noted within cells have probably been recently phagocytosed or are within membrane infoldings which if cut on a different plane may appear as intracellular but in fact are not so. The evidence for intraerythrocytic leptospires (Miller & Wilson, 1962; 1967) was based upon the examination of few animals, and no evidence in support of intraerythrocytic leptospires was found in this present study. It therefore seems unlikely that dissemination of leptospires throughout the body occurs by means of the RBC's especially as leptospires survive well in and can be readily cultured from plasma or serum.

Leptospires are frequently observed in intercellular spaces and pass between adjacent cells. It is presumed that they bypass tight junctions because they lack 'toxins' with lecithinase and hyaluronidase activity (Marshall, 1973) and it has been argued that they achieve this passage by intracellular movement. No evidence for this was seen in the present work as most intracellular leptospires were degenerate.

Colonization of the kidney has been reported frequently in chronic leptospirosis (Hambdy & Ferguson, 1957; Marshall, 1973) and occurred in the two surviving calves in this series but is

unlikely to be due to a particular trophism for this tissue. Leptospires multiply and migrate through the body tissue, some enter the kidney tubules but as a result of antibody formation the organisms are cleared from all sites except the renal tubules where they appear to be protected (Marshall, 1973). Prior to antibody formation, subjectively the site of greatest numbers of organisms appeared to be the liver. This observation was also made by Faine (1964) and Miller *et al*, (1974).

Pathogenicity of leptospires is considered to be related to numerous factors (Stalheim, 1971) and the calves in this experiment illustrate the importance of individual animal variation in susceptibility to leptospirosis. Although all calves became leptospiraemic the degree of leptospiral multiplication and hence presumably the quantity of 'toxin' elaborated also varied. Individual variations in the response to 'toxin' was shown by the presence or absence of RBC destruction and hepatocyte toxicity. An alternative explanation is that the levels of 'toxin' elaborated *in vivo* showed variation from calf to calf. The hamsters showed greater uniformity than calves in their response to infection which was most likely due to their genetic uniformity.

5.0 CONCLUSIONS AND SUMMARY

1. The development of lesions in spleen and liver in hamsters infected with *ballum* strain 1045 followed an orderly sequence of events with some variation in the time of onset. These lesions developed before the appearance of large numbers of organisms.

2. Initial splenic lesions in hamsters consisted of the sequestration of RBC's and the swelling of reticular cells. Early sequestration probably resulted from splenic detection of echinocytic RBC's as at this stage the animal was not yet haemoglobinaemic and corresponded with the increased spleen:body weight ratio. Later in the disease sequestered spherocytes were obvious in conjunction with haemoglobinaemia. Ultrastructural evidence for erythrophagocytosis was also seen in the 'toxin' injected calves. In all cases, the majority of RBC's seen within the splenic circulation were fully haemoglobinated although morphological changes of spheroidicity and vacuolation were obvious. The haemoglobinaemic calves showed histological changes similar to those of the moribund hamsters although slightly less severe.

Erythrophagocytosis was increased above that of the controls in the calf which did not become haemoglobinaemic yet showed signs of liver disease.

3. Initial hepatic lesions in hamsters comprised periportal degeneration of hepatocytes and the beginnings of a portal infiltration of mononuclear cells. The degenerative changes extended to include the centrilobular hepatocytes. In moribund and dead hamsters and the haemoglobinaemic calves, there was severe centrilobular degeneration and coagulative necrosis which were related to hypoxia. Increased liver:body weight ratios occurred after the increase in the spleen:body weight ratio. 'Toxin' injected calves also showed minor degenerative lesions of the hepatocytes. Hepatocyte vacuolation was seen in the non-haemoglobinaemic calves.

4. The renal histopathological lesions and changes in the kidney: body weight ratio coincided with the presence of haemoglobinuria and were consistent histologically with changes due to haemoglobinuric nephrosis. Renal lesions were absent in the 'toxin' injected calves and minor in those calves which did not become haemoglobinaemic although at the time of euthanasia decreases in the raised GGT, AAT, bilirubin and BUN (Chapter 3) indicated recovery of the animal. Gross and histological appearances of the kidneys of this last group were characteristic of chronic leptospirosis.

5. Lesions of other tissues were minor and consisted of cellular depletion of the bone marrow and lymphoid depletion of calf thymuses with petechial haemorrhages. Oedema and congestion were seen in the lungs.

6. Leptospires were readily seen in moribund and dead hamsters and calves. The liver appeared to be the preferred site for multiplication of organisms because numbers here exceeded those in other sites, including the kidney. Leptospires were seen in the kidneys only of the non-haemoglobinaemic calves. Leptospires were recovered culturally from all calves from day one or two post-infection up to the time of death in those which developed haemoglobinaemia or up to the time of the development of MAT's in the non-haemoglobinaemic calves.

7. Colonization in the renal tubules appeared to result from random colonization rather than tissue predilection. Once in this position, the organisms are able to survive and multiply while at other sites, the organisms are killed and removed by host defence mechanisms.

CHAPTER 6

PATHOLOGY OF NON-HAEMOLYTIC LEPTOSPIRAL INFECTIONS OF CATTLE AND HAMSTERS

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3.3.2.2 Spleen
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5.0 CONCLUSIONS AND SUMMARY

CHAPTER 6

PATHOLOGY OF NON-HAEMOLYTIC LEPTOSPIRAL INFECTIONS OF CATTLE AND HAMSTERS

1.0 INTRODUCTION

A comparison of the pathology of different strains of the same serovar was considered important because it has been found by other workers (Cox & Twigg, 1981; Hambdy & Ferguson, 1957) and in this laboratory by Marshall (1973) that repetitive subculturing over a period of time can result in a loss of virulence of a leptospiral isolate. It was noticed with the *ballum* strain 1045 isolate which was used in earlier experiments for work reported in this thesis that the virulence for hamsters changed over a period of about a year. The strain was subcultured at four to six week intervals and checked periodically for virulence by hamster inoculation. The length of time between infection and death of hamsters increased and the number of animals dying from acute RBC destruction decreased. Attempts to recover a virulent form of the 1045 *ballum* organism by infecting hamsters and culturing the kidneys failed. The *ballum* strain 'cat 1' is a reisolate of the *ballum* strain 1045 following a feline infection (Shophet, 1979).

Likewise, it was noticed that recent *pomona* isolates such as 'Scamp' appeared more virulent than isolates such as T116 and 790001 which had been subcultured in the laboratory for a number of years.

The differences between strains of *ballum* which produce haemoglobinaemia and those which do not were considered of importance in order to ascertain which aspects of the disease are associated with the organism *per se* and which are due to hypoxia resulting from RBC destruction.

Of interest also was the comparison of *pomona* and *ballum* in hamsters because *pomona*, while causing haemoglobinaemia in cattle (Reinhard & Hadlow, 1954; Spradbrow & Seawright, 1963) and sheep (Hodges, 1974; Hodges *et al*, 1976; Millar *et al*, 1977) has never been associated with haemoglobinaemia in hamsters (Abdu & Sleight, 1965; Miller & Wilson, 1966; 1967; Sanger *et al*, 1961) while *ballum* is considered haemolytic in hamsters (Frenkel, 1972). The opportunity was also taken to make a study of experimental infection of cattle with serovar *balcanica* because it provided a useful comparative model of a non-haemolytic leptospiral infection in that species.

An accidental *ballum* infection with strain M4/9 produced clinical symptoms of dizziness, severe headaches, nausea, and pain around the ear and cheek regions of a human. The pathogenesis of these symptoms was unknown but may have resulted from brain or ear lesions. Examination of brains from several species infected with *pomona* produced no (Abdu & Sleight, 1965) or only minor histological lesions (Morse & Langham, 1958; Sleight *et al*, 1960). Brains from *pomona* and *ballum* infected hamsters were examined for the presence of lesions. No work has been reported as to the effects of leptospires on the ear canals and inner ear of any species. Therefore aural tissue from hamsters infected with *ballum* was also examined in this chapter. The examination of aural tissue was considered important because it was necessary to determine whether or not organisms survived in the inner ear as they do in the lumen of the proximal convoluted tubules of kidneys (Faine, 1964). This persistence of leptospires in protected sites has been considered as one of the explanations for periodic opthalmia reported in association with equine *pomona* infections (Bryans, 1955; Morter *et al*, 1969).

No attempts were made to trace lesion development in the infections described in this chapter. The aim was to compare the pathological findings resulting from infections of hamsters by a number of strains of *ballum* and *pomona* and the infection of cattle with *balcanica* as well as to examine hamster brains and ears for lesions.

2.0 MATERIALS AND METHODS

2.1 LEPTOSPIRES

The source of the leptospires and methods of identification and culture are described in Chapter 2, Section 1.1 and Table 2-1. The strains of leptospire used in Experiment II, the comparison of different strains of *ballum*, are tabulated in Table 6-3. The second subculture of *ballum* strains 964 and M4/9 was used to infect hamsters two weeks after infection of the first group of hamsters in order to establish the culture's ability for the production of haemoglobinaemia in preparation for Experiment VIII. The strains of leptospire in Experiment III, the comparative *pomona* infections are tabulated in Table 6-1. The strain of *balcanica* used in the cattle infection is described in Chapter 2, Section 1.1.2.

2.2 ANIMALS

The source, maintenance, methods of inoculation and collection of hamster samples are described in Chapter 2, Sections 2.1 and 3.1. The methods of inoculation and collection of samples of the *balcanica* infected cattle are described in Experiment VI, Chapter 2, Section 2.2.2.

2.3 LABORATORY PROCEDURES

2.3.1 Clinical pathology

2.3.1.1 Cattle

Blood was collected for clinical pathology as described in Chapter 2. The clinical pathological examinations carried out were total haemoglobin, PCV, total white blood cell count, total plasma protein, examination of the icterus index and urinanalysis.

The detailed description of these procedures are described in Chapter 3, Section 2.3.

2.3.1.2 Hamsters

After infection the majority of the hamsters were found dead. Those which were moribund were euthanased for humane reasons

and limited tests carried out to determine the presence or absence of haemoglobinaemia. These tests were PCV estimation, examination of the plasma to estimate the icterus index and determine the presence or absence of free plasma haemoglobin, examination of the blood smear for cell cytology, and total plasma protein.

Bone marrow smears were made from the femoral marrow of euthanased hamsters and the cell cytology examined as described in Chapter 3, Section 2.3.1.8.

2.3.2 Histopathology

All tissues for histological examination were fixed in phosphate buffered 10% formalin and routinely processed as described in Chapter 5, Section 2.3.2. In hamsters liver, spleen, kidney, one femur, lung, heart and brain were examined. Ear tissue was also processed from the *ballum* infected hamsters in the following manner.

The hamster heads were disarticulated at the occipitoatlantal articulation, skinned and placed in phosphate buffered 10% formalin for two weeks to allow adequate fixation. After removal, the brain was cut into two by a vertical, longitudinal, paramedian incision and processed routinely. In the *ballum* infected hamsters, the remainder of the head was decalcified in RDO[1] reagent until the bones were soft enough to be cut with a scalpel. Following bisection of the decalcified head, two incisions were made on either side and parallel to the ear canals and the middle and inner ear. The block of tissue between these two incisions contained the majority of the middle and inner ear intact. A block of tissue was taken on either side of the first block to ensure complete removal of the middle and inner ear. The blocks were routinely processed for histological examination as before.

Liver and kidney only were collected from the *balcanica* infected cattle. Preparation for histological examination was as previously described.

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2.3.3 Ultrastructural Pathology

Samples of liver, spleen and kidney were collected and fixed as described in Chapter 5, Section 2.3.3 for ultrastructural examination by TEM. The samples were taken from the first three hamsters to become moribund from a *pomona* infection. This strain of *pomona* was the 'Scamp' isolate. RBC's were also collected from these hamsters for SEM and TEM examination as described in Chapter 4, Sections 2.2, 2.3, and 2.4. Samples from two uninfected control hamsters were processed at the same time.

3.0 RESULTS

3.1 CLINICAL SIGNS AND CLINICAL PATHOLOGY

3.1.1 Cattle

The cattle showed no clinical signs of infection except in two animals which showed a slight transient pyrexia (Appendix XXX a,c). All clinical pathological parameters remained normal throughout the experiment. The results are detailed in Appendices XXVII to XXX.

3.1.2 Hamsters

Clinical signs of hamsters dying up to six days post infection consisted of depression, anorexia, dehydration and huddling in the corner of the cage. Often no clinical signs were seen before death. Those hamsters dying 7 to 14 days post infection had clinical signs of depression, anorexia and dehydration frequently combined with emaciation. Nervous signs consisted of the rubbing of the head and sitting on the haunches with some moribund animals almost toppling backwards. Those animals euthanased on day 14 were apparently healthy with no clinical signs of infection.

The PCV's of those hamsters dying from acute *pomona* infections within six days of infection were normal or increased, as was

the total plasma protein. The plasma was yellow and not haemoglobinaemic (Table 6-1 & 6-2).

Ballum infected hamsters dying within six days of infection developed jaundice and had haemoglobinaemic plasma while those dying 7 to 14 days post infection had normal or slightly low PCV's with no jaundice or free plasma haemoglobin. Strain 964 appeared to lose while M4/9 retained the ability to produce haemoglobinaemia following a further subculture (Table 6-3).

Pomona and *ballum* infected hamsters euthanased 14 days post-infection had normal PCV's and plasma characteristics.

All hamsters dying of non-haemolytic leptospirosis had increased numbers of myeloid cells with depletion of mature and band myeloid cells in the bone marrow.

Those hamsters euthanased 14 days post infection had normal marrow cytology.

3.2 GROSS PATHOLOGY

3.2.1 Cattle

No gross lesions were observed on post mortem examination.

3.2.2 Hamsters

On the basis of lesions noted at post mortem examination, *ballum* infected hamsters may be divided into three groups; namely those dying within six days, 7 to 13 days, and those euthanased on the 14th day. The *pomona* infected hamsters could be divided into two groups, those dying of acute infection within six days and those euthanased at 14 days showing no clinical signs.

The lesions in hamsters dying within six days of *ballum* infections are described in Chapter 5, Section 3.1.2.2.

Those hamsters dying within six days of acute *pomona* infections were less jaundiced than those dying within six days of *ballum*

Table 6-1 Clinical pathological data from euthanased hamsters infected with *pomona* in Experiment III

Hamster	*Pomona* strain	PCV	II	TP (g/ℓ)
1	Control	0.46	0	56
2	Control	0.46	0	60
1	'Scamp'	0.58	50	76
2	'Scamp'	0.59	50	86
3	'Scamp'	0.53	25-50	66
1	790001	0.64	50	50
2	790001	0.62	25	48
3	790001	0.45	10	52
1	T116	0.46	0	60
2	T116	0.45	0	62
3	T116	0.41	0	58
4	T116	0.44	0	61
5	T116	0.49	0	60
6	T116	0.50	0	58

Table 6-2 Summary of clinical data from the hamsters infected with *pomona* : Experiment III, a comparison of three strains.

Strain of *pomona*	'Scamp'	790001	T116
No. of hamsters infected	6	6	6
No. of hamsters found dead	3	3	0
Date of death	Day 3	Day 4	-
No. of hamsters euthanased due to display of clinical signs	3	3	-
Date of euthanasia	Day 4	Day 5	Day 14
Presence of jaundice at euthanasia	6	6	0
Presence of haemaglobinaemia at euthanasia	none	none	none
No. of hamsters surviving to 14 days showing no clinical signs and euthanased	0	0	6

Table 6-3 Summary of clinical data from the hamsters infected with *ballum*: Experiment II, a comparison of five strains.

Ballum isolate	964 *1	964 **2	1045	M4/9 *1	M4/9 **2	924	'Cat 1'
Total no. of infected hamsters	7	7	7	6	8	7	7
No. of hamsters found dead	6	5	7	3	6	4	0
No. of dead hamsters showing haemolytic lesions	5	1	1	3	5	2	0
No. of dead hamsters not showing haemolytic lesions	1	4	6	0	1	2	0
No. of hamsters euthanased due to display of clinical signs	1	1	0	3	2	2	0
No. of hamsters euthanased and showing haemoglobinaemia	0	0	0	3	2	0	0
No. of hamsters surviving to 14 days with no clinical signs	0	1	0	0	0	1	7
Total no. of hamsters with signs of haemolysis	5	1	1	6	7	2	0

* Subculture 1

** Subculture 2

infections. The livers were either red, or pale with red mottling, and were slightly enlarged. The spleens although slightly reddened, were of a normal size. The kidneys were reddened, enlarged and friable with small red foci. There was a small amount of fluid in the body cavities and the occasional petechial haemorrhages in the peritoneal and thoracic serosal surface.

Hamsters dying of *ballum* 7 to 13 days after infection were non-jaundiced, emaciated, had darkened musculature with occasional petechial haemorrhages and body fat had been resorbed. The liver appeared grossly normal while the spleen was often pale and atrophied. The kidneys were pale and misshapen but of approximately normal size.

The only gross lesions seen in the hamsters which survived to 14 days following infection with *ballum* and *pomona* were pale and misshapen kidneys.

Table 6-3 summarizes the results from hamsters infected with five strains of *ballum* and Table 6-2 summarizes the results from hamsters infected with three strains of *pomona*. Those infected with strain 'Scamp' died earlier than those with 790001 and T116 infections.

3.3. CELLULAR PATHOLOGY

3.3.1 <u>Histopathology</u>

3.3.1.1 Liver

a) Cattle

Small infiltrations of mononuclear cells were seen around some portal areas. Very little haemosiderin and no leptospires were seen.

b) Hamsters

In hamsters which died within six days of infection by *pomona*, the hepatocytes were swollen with complete loss of the normal foamy appearance to the cytoplasm (Fig. 6-1). The cell boundaries were indistinct with hepatocyte dissociation in some

regions leading to disruption of the hepatocyte cords. There was obliteration of the sinusoids by swollen hepatocytes in some regions and widening of the sinusoids in others. In moribund animals there was extensive swelling and hydropic degeneration in centrilobular hepatocytes which resulted in the cytoplasm having a lacey appearance (Fig. 6-2). In others, the cytoplasm had a more granular appearance and Councilman body-like structures were seen in hepatocytes in some sections.

Infiltration of portal areas by mononuclear cells and coagulative necrosis of hepatocytes occurred but was not as extensive as in livers from hamsters infected with *ballum* in which haemoglobinaemia was severe.

Glycogen was absent in hepatocytes and haemosiderin deposits were not present in Kupffer cells.

Many leptospires were seen between hepatocytes, some within blood vessels and the occasional leptospire was intracellular in hepatocytes.

Those hamsters dying of *ballum* infection within six days had histological lesions similar to those described in Chapter 5, Section 3.3.1.2 (a).

The histopathological lesions of the hamsters dying of *ballum* infections 7 to 13 days post infection were similar to the lesions of *pomona* infected hamsters dying within six days of infection. In many of these animals, particularly those which died towards 14 days post-infection, there was more haemosiderin than normal within hepatocytes and Kupffer cells. This haemosiderin stained more darkly and was more granular than that in livers from haemoglobinaemic hamsters. There was a complete lack of glycogen in the hepatocytes. Livers from animals which died seven to ten days post-infection contained leptospires and it was noted that those hamsters which lived longer had fewest organisms. Those dying 11 to 13 days post-infection contained very few or no organisms.

The *pomona* and *ballum* infected hamsters euthanased 14 days post-infection had only minor hepatic lesions. These included the slight loss of the foamy appearance to the cytoplasm, random focal areas of PMN infiltration and small mononuclear infiltrations in portal regions. Macrophages, some lymphocytes, and PMN's were also found scattered through the parenchyma. Glycogen and haemosiderin concentrations were normal and no leptospires were seen.

3.3.1.2 Spleen

In hamsters dying within six days of infection by *pomona* strain 'Scamp' there was hyperplasia of the lymphoid follicles. Increased numbers of small lymphocytes were present around the periphery of the lymphoid follicles and within the sinusoids of the red pulp. Aggregations of PMN's were observed within the spaces between the reticular cords of the red pulp. The reticular cells forming the cords of the red pulp were swollen. The degree of extramedullary erythropoiesis was normal.

In animals infected with *pomona* strain 790001 death occurred later than the 'Scamp' strain (Table 6-2). There was a greater degree of infiltration of the sinusoids of the red pulp by PMN's and macrophages. The macrophages were large and the cytoplasm contained cellular debris and pyknotic nuclei. The erythrophagocytosis characteristic of spleens from haemoglobinaemic animals was not seen. There was depletion of lymphocytes from the lymphoid follicles.

Spleens from hamsters infected with *ballum* and dying within six days were similar histologically to those described in Chapter 5, Section 3.3.2.2 (a).

In those hamsters dying of *ballum* infections 7 to 13 post-infection there was widespread necrosis of reticular cells of the red pulp and in these areas, cell debris, and numerous macrophages often containing cell debris, PMN's and pyknotic nuclei were present. The lymphoid follicles were depleted of lymphoid cells. Although overt RBC destruction as judged by the absence of haemoglobinaemia

was absent, there was increased erythrophagocytosis. Some spleens, particularly of those hamsters infected with strain 1045, contained more haemosiderin than normal in macrophages of the lymphoid follicles and red pulp. The haemosiderin in macrophages of the lymphoid follicles and red pulp was darker staining and more granular than haemosiderin seen in spleens from haemoglobinaemic animals. The lymphoid atrophy and atrophy and necrosis of the red pulp was more extensive in those dying ten days or more post-infection.

In the hamsters infected with *ballum* and *pomona* and surviving to 14 days post-infection with no clinical signs of illness (Tables 6-2,6-3) haemosiderin content of the splenic macrophages was normal.

Many leptospires were seen in all spleens from *pomona* and *ballum* infected hamsters up to ten days post-infection, fewer 11 to 13 days post-infection and none in those animals which were euthanased at 14 days with no clinical signs of disease.

The hamsters infected with *ballum* and *pomona* and euthanased 14 days post-infection showed only minor splenic changes which consisted of the occasional areas with necrotic cells, pyknotic nuclei and macrophages containing cell debris. Haemosiderin content was normal and no leptospires visible.

3.3.1.3 Kidney

a) Cattle

Small infiltrations of mononuclear cells were seen in the interstitium. No leptospires were seen.

b) Hamsters

The renal proximal tubules of hamsters dying from *pomona* infections within six days of infection showed degenerative changes of the epithelial cells consisting of loss of the brush border, vacuolation and in some cases disintegration and desquamation of the epithelial cells (Fig 6-3). A pale eosinophilic proteinaceous material was seen in many tubules and Bowman's capsule, the intensity of colour increasing distally in the nephron. Adjacent

tubules could show widely differing degrees of degenerative changes in the epithelial cells, ranging from slight swelling and vacuolation to necrosis and desquamation off the basement membrane. Vacuoles in epithelial cells of some proximal convoluted tubules often contained protein (Fig. 6-3). In some cases the desquamated cells formed casts within the tubule lumina. Glomerular changes were minor and consisted of distention of the Bowman's capsule with an eosinophilic fluid. Infiltrations of varying density by mononuclear and PMN cells were seen in the interstitium. Large numbers of leptospires were seen in the interstitium and between epithelial cells of tubules with few organisms within tubule lumina. The numbers of leptospires did not appear to be as large as within liver sections. Leptospires did not form the large colonies which were seen at the corticomedullary junction of *ballum* infected hamsters.

The histopathological lesions of hamsters infected with *ballum* and dying within six days are described in Chapter 5, Section 3.3.3.2 (a).

The *ballum* infected hamsters dying 7 to 13 days post infection showed severe renal changes. A homogeneous pale eosinophilic material often containing RBC's was seen within the urinary spaces of some Bowman's capsules and tubules, similar in appearance to that of *pomona* infected hamsters dying within six days of infection. Accumulations of mononuclear cells were seen around glomeruli, between tubules and around blood vessels with some extremely large mononuclear and PMN inflitrations in the pelvis. Haemorrhage into some glomeruli and tubules was occasionally observed. *Ballum* strain 1045 showed more severe tissue destruction and haemorrhage within the interstitium of the kidney. In two animals infected with strain 964 and euthanased on day 11 and four animals infected with strain 'cat'1 and euthanased on day 14 there was calcification of cellular debris. Leptospires were seen within the interstitium, migrating between epithelial cells and within the lumina of tubules. Larger colonies of organisms were found at the corticomedullary junction.

The hamsters infected with *ballum* and *pomona* and euthanased at 14 days had renal lesions of varying severity. Some animals had only small infiltrations of mononuclear cells in the interstitium while others had very large infiltrations in the interstitium and around blood vessels. In the more severely affected kidneys there was calcification of necrotic debris. No haemosiderin was seen and leptospires were observed only within the lumina of the proximal convoluted tubules.

3.3.1.4 Bone marrow

All animals dying as a result of leptospirosis had hypocellular marrows. There was an almost complete lack of mature PMN and band neutrophils with an increase in myeloblasts. Some leptospires were seen within sinusoids of the marrow. Haemosiderin was usually absent.

Marrow from all animals euthanased at 14 days appeared normal.

3.3.1.5 Lung

Lungs from hamsters dying from *ballum* and *pomona* infections were congested, had a homogeneous eosinophilic proteinaceous fluid in the alveoli and some contained large numbers of WBC's within the blood vessels. Intra-alveolar RBC's were seen in large numbers in all hamsters which had been euthanased. The intra-alveolar RBC's resulted from inhalation of blood following euthanasia.

Lungs from hamsters euthanased at 14 days were normal apart from intra-alveolar RBC's.

The occasional leptospire was seen in the lungs of hamsters dying within six days and organisms were seen in the blood vessels.

3.3.1.6 Brain

One *ballum* infected hamster euthanased seven days post-infection had perivascular cuffing of some meningeal blood vessels by mononuclear cells. No organisms were seen within brain tissue but occasional organisms were seen within blood vessels of hamsters less than six days post-infection.

3.3.1.7 Ear

No histopathological lesions were seen within the middle or inner ear. Leptospires were occasionally seen in blood vessels and in large numbers in the sinuses of the petrous temporal bone of hamsters less than six days after infection with fewer seven to ten days post-infection.

3.3.1.8 Cardiac muscle

Apart from some pale staining bundles of muscle fibres, the cardiac muscle was histologically normal. Leptospires were not seen within the muscle tissue but were occasionally observed in blood vessels and the heart chambers.

3.3.2 Ultrastructural pathology

Description of liver, spleen and kidneys from control hamsters are given in Chapter 5, Sections 3.3.1.1 (b), 3.3.2.1(b) and 3.3.3.1(b).

3.3.2.1 Liver

There was swelling and disorganisation of the endoplasmic reticulum with loss of ribosomes and the formation of vacuoles (Figs.6-4, 6-5) in the less severely affected hamsters 2 and 3. In all hamsters there was swelling of mitochondria with the cristae coming to lie parallel to the outer mitochondrial membranes (Figs. 6-4 , 6-5) Vacuolation also occurred within the mitochondria (Fig. 6-5).

The dilatation of the endoplasmic reticulum in the more severely affected hepatocytes of hamster 1 produced a lacey appearance (Fig. 6-6). There was dilatation of the nuclear membrane (Fig. 6-6). Progressive mitochondrial degenerative changes were often present in the same cell (Figs. 6-5, 6-6) and were recognised as swelling, vacuolation, and finally disintegration or the formation of dense bodies (Figs. 6-5, 6-6, 6-8). Tubular or round structures (Fig. 6-7) seen within single membrane lined vacuoles and some of the myelin forms were considered to be mitochondrial remains. In some cells vacuoles appeared to bulge into neighbouring vacuoles as if about to empty into them (Fig. 6-7).

Leptospires were often seen between normal hepatocytes and were noted in infoldings of the basement membrane (Fig. 6-9). Intracellular leptospires were uncommon, were present in intracellular vacuoles and were usually degenerate. Extracellular and intracellular organisms were similar in appearance to those described in Chapter 5, Section 3.3.1.2 (b).

3.3.2.2 Spleen

While there was some erythrophagocytosis, the amount was not increased above that of the control animals. RBC's within the sinusoids and blood vessels had normal morphology.

Swelling of the reticular cells produced 'blebs' similar to those described in Chapter 5, Section 3.3.2.2 (b), was seen in all animals, and was most severe in hamster 1. Necrosis of the red pulp and presence of cellular debris was not as marked as in the haemoglobinaemic animals.

Leptospires were frequently seen within macrophages (Figs. 6-10, 6-12, 6-13). The most frequent type of leptospiral inclusion consisted of the organism within a membrane-bound vacuole. Many such vacuoles were observed within some macrophages with the leptospires in different stages of degeneration. In most cases the organism was tightly coiled (Figs. 6-10, 6-11, 6-12, 6-13) and there was progressive loss of the normal structure. Membranous formations orientated parallel to the organisms were present and increased in number in proportion to the degree of degeneration of the organism. The final product of leptospiral digestion consisted of a vacuole containing electron dense linear profiles (Fig. 6-11). Degenerate leptospires were occasionally seen in unlined vacuolar spaces containing the organism and unidentified cell debris (Fig. 6-14).

The macrophages which contained large numbers of organisms also contained many abnormally shaped mitochondria (Fig. 6-12). Some of these mitochondria were circular, others horseshoe shaped, and many contained dilated cristae. The mitochondria enclosed an

area of cytoplasm which stained less densely than the rest of the cytoplasm (Fig. 6-12). Some macrophages also contained vacuoles, bound by a double membrane with discrete membrane bound bodies and vacuoles in a more densely staining cytoplasm (Fig. 6-13).

3.3.2.3 Kidney

Ultrastructural lesions were mostly confined to the epithelial cells of the proximal convoluted tubules. Degenerative changes of swollen cells with the formation of 'blebs' occluding the tubular lumen (Fig. 6-17) and loss of microvilli from the epithelial cells (Fig. 6-15) were common. Shrinkage of the epithelial cells associated with widening of the tubular lumen was occasionally seen. Dilatation of apical tubules and vesicles, mitochondrial degeneration as shown by the loss of the internal mitochondrial structure (Fig. 6-16) and swelling of the endoplasmic reticulum, the latter often in a circular form (Fig. 6-16) were also features of degenerate cells. Cellular debris was often present in the tubular lumen (Fig. 6-15).

Leptospiral organisms and their migration were similar to that described for haemoglobinaemic animals in Chapter 5, Section 3.3.3.2(b).

Glomerular oedema and endothelial cell damage was minor and similar to that seen in hamsters infected with *ballum* which are described in Chapter 5.

Ultrastructural changes to the distal convoluted tubule were less severe than those in the proximal convoluted tubules and consisted of the formation of vacuoles and the presence of cell processes or fragments in the tubular lumina.

All RBC's seen in the blood vessels of hamsters infected with *pomona* were of normal morphology.

The following figures illustrate the histological and ultrastructural lesions in hamsters infected with the 'Scamp' strain of *pomona* (Experiment III). The differences in acute fatal leptospiral infections of hamsters which are not associated with RBC destruction and haemoglobinaemia and acute fatal leptospiral infections of hamsters and calves which are associated with RBC destruction may be seen by the comparison of the following figures with those of Chapter 5.

Fig 6-1

Hepatocyte dissociation with vacuolation and loss of the normal 'foamy' appearance to the hepatocyte cytoplasm in the least affected hamster.

Hamster 3 H&E 100x

Fig 6-2

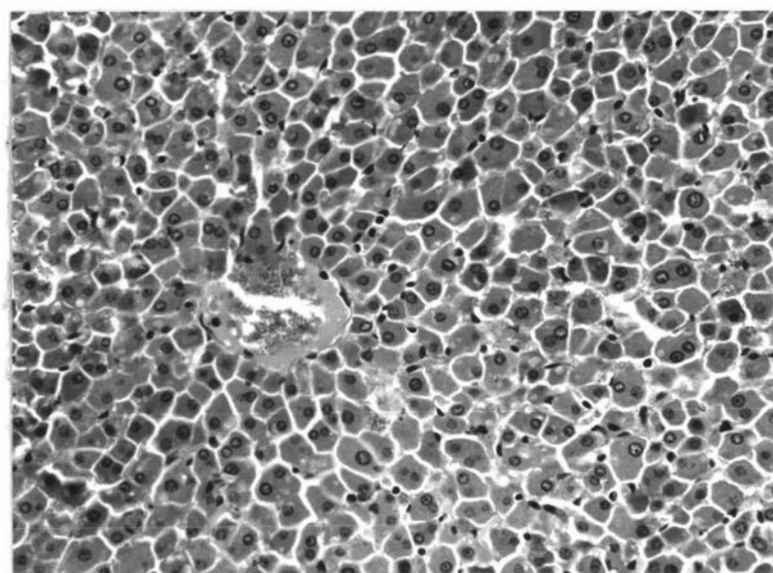
Vacuolation of hepatocyte cytoplasm resulting in a 'lacey' appearance to the cells in the most severely affected hamster.

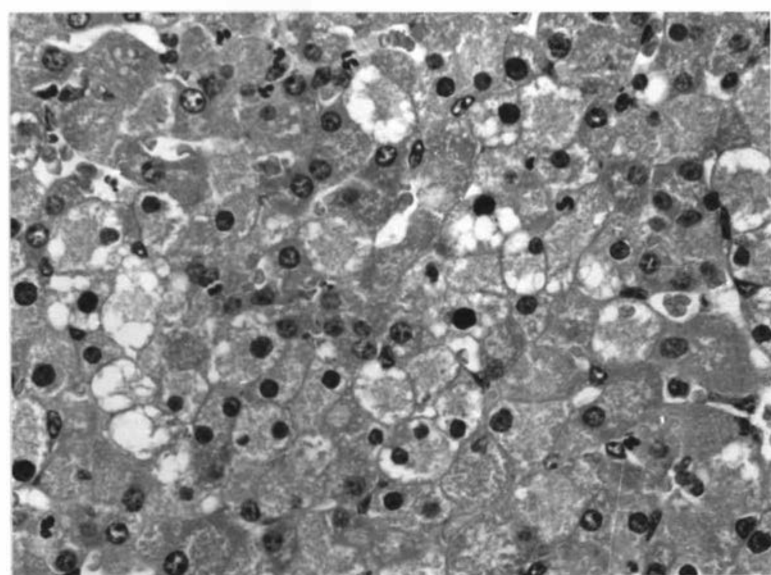
Hamster 1 H&E 200x

Fig 6-3

Renal cortex showing degeneration of the epithelial cells of the proximal convoluted tubules. Homogeneous inclusions (arrows) are seen within epithelial cells and a homogenous material (M) is present within the tubular lumina.

Hamster 1 H&E 200x





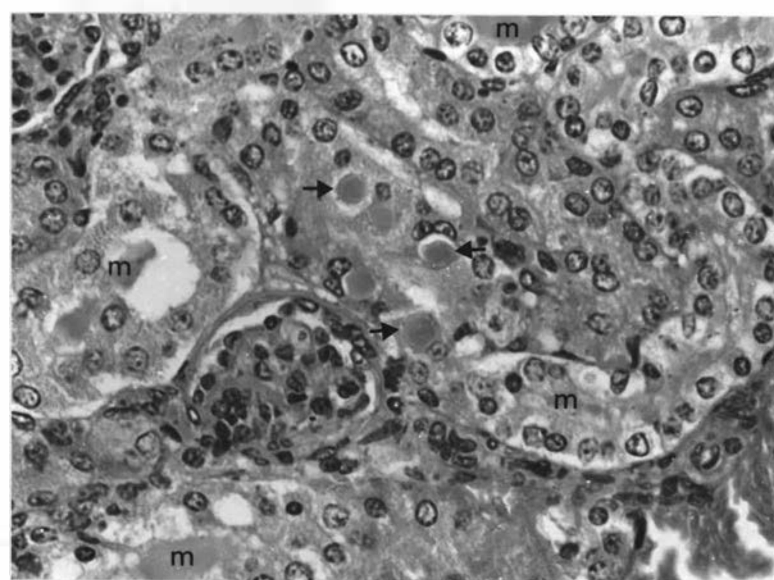

m
m
m
m

Fig 6-4

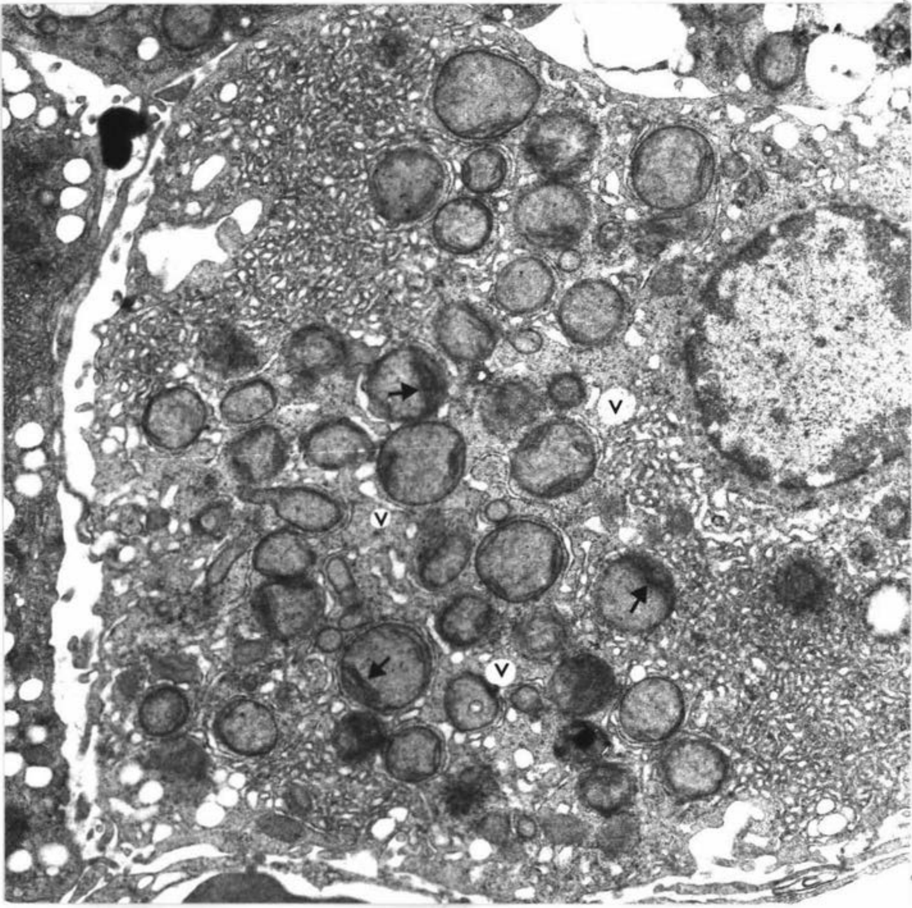
Hepatocyte from a hamster demonstrating swelling of mitochondria with margination of cristae (arrows). Dilatation of the endoplasmic reticulum and vacuole (V) formation are also present.

Hamster 2 TEM 10,500x

Fig 6-5

Hepatocyte demonstrating swollen and distorted mitochondria. Cristae are displaced to the periphery of the mitochondria. Mitochondrial vacuoles (arrows) are obvious and in some cases mitochondrial shrinkage (M) is present.

Hamster 1 TEM 17,500x


V
V
V

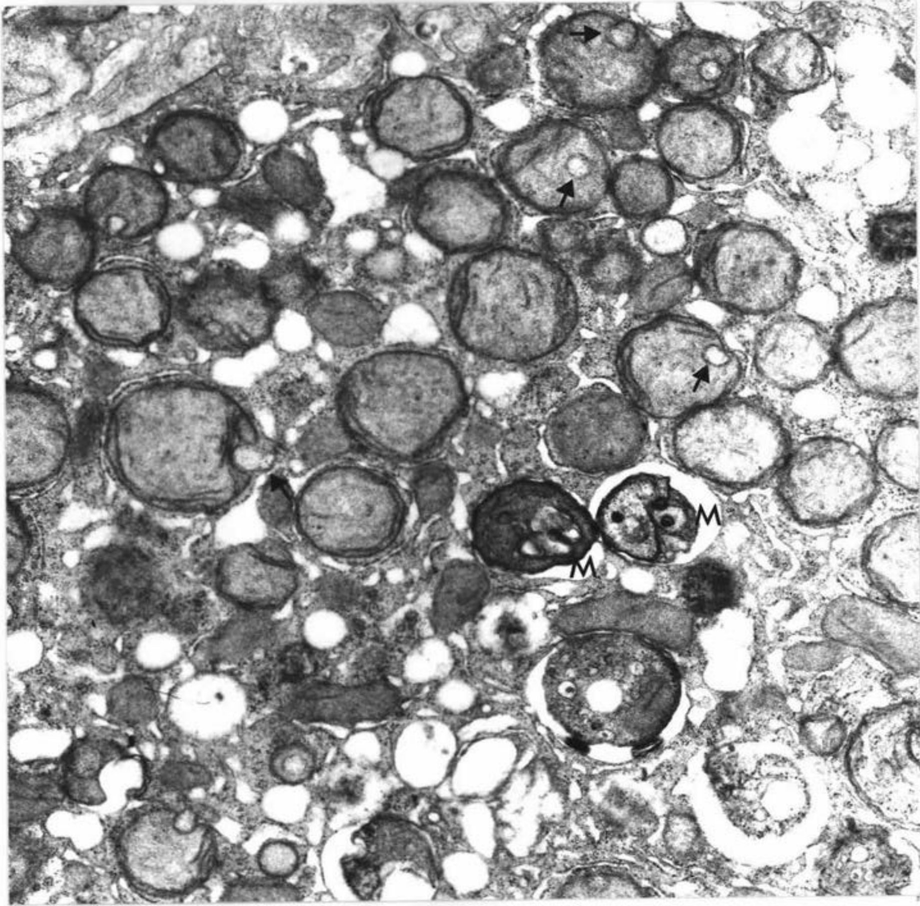

M
M

Fig 6-6

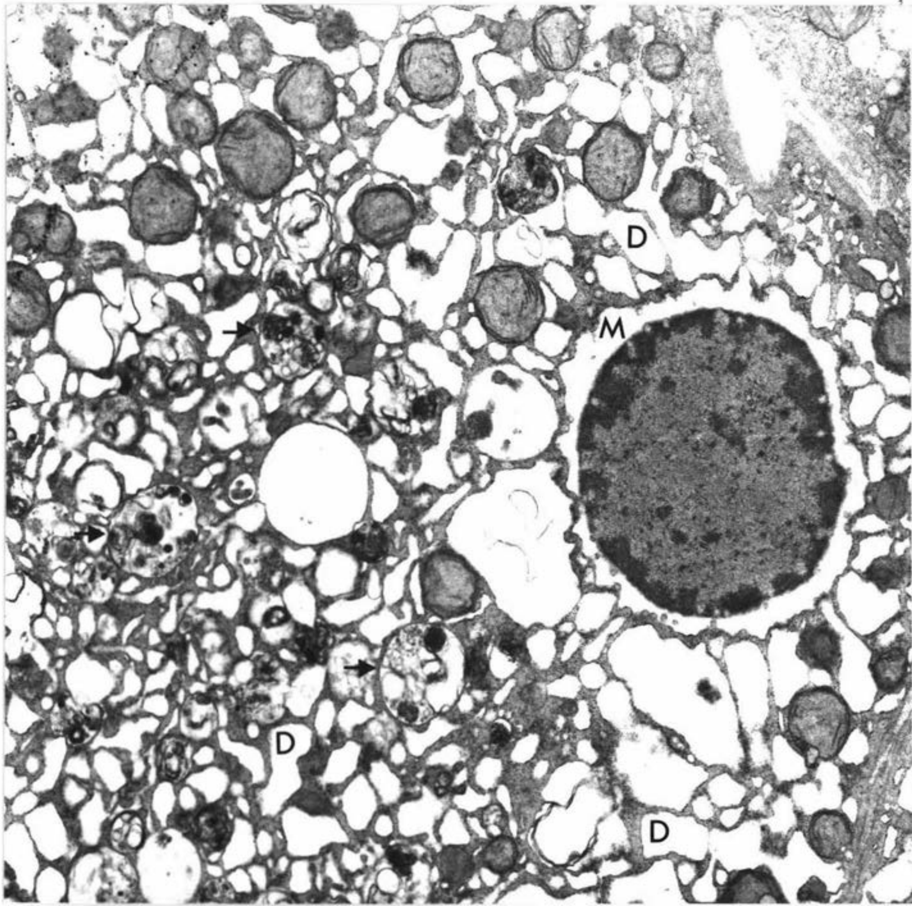
Extreme dilatation of the endoplasmic reticulum (D) and swelling of the space in the perinuclear membrane (M) are seen. All mitchondria are degenerate with some appearing fragmented (arrows).

Hamster 1 TEM 10,500x

Fig 6-7

Vacuoles in a severely affected hepatocyte. One vacuole appears to be emptying into another (arrow). Tubular and round structures (S) are seen within vacuoles and may be remnants of mitochondria or other organelles.

Hamster 1 TEM 22,500x


D
M
D
D

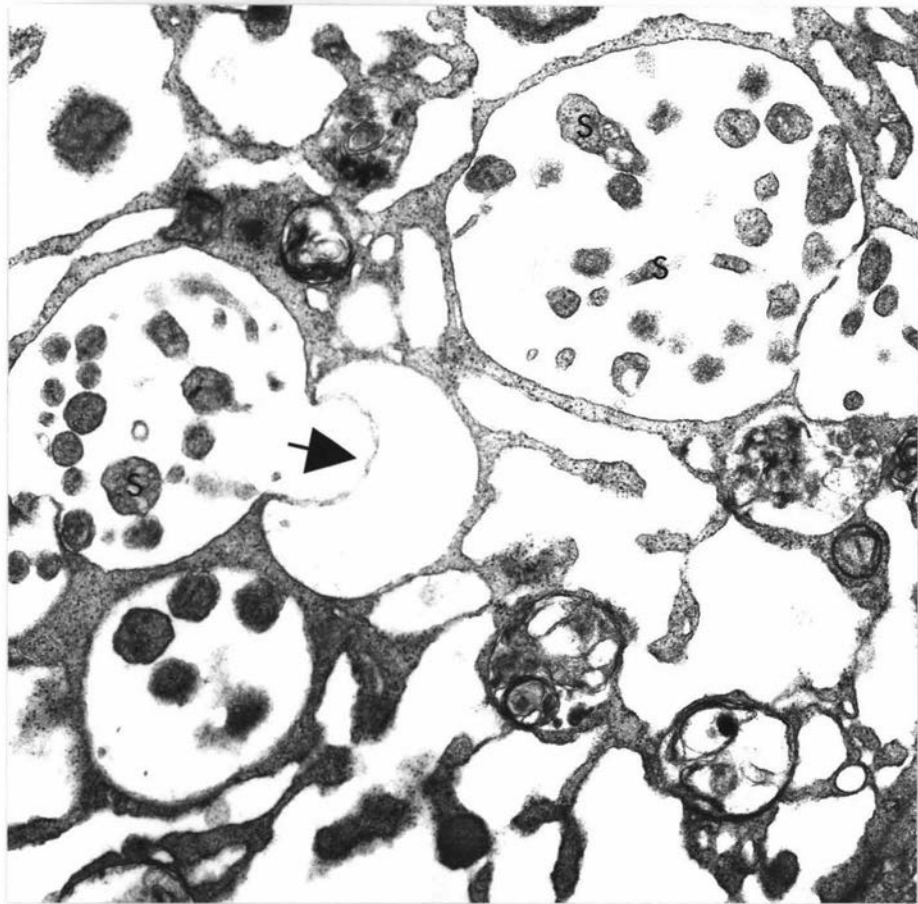

S
S
S

Fig 6-8

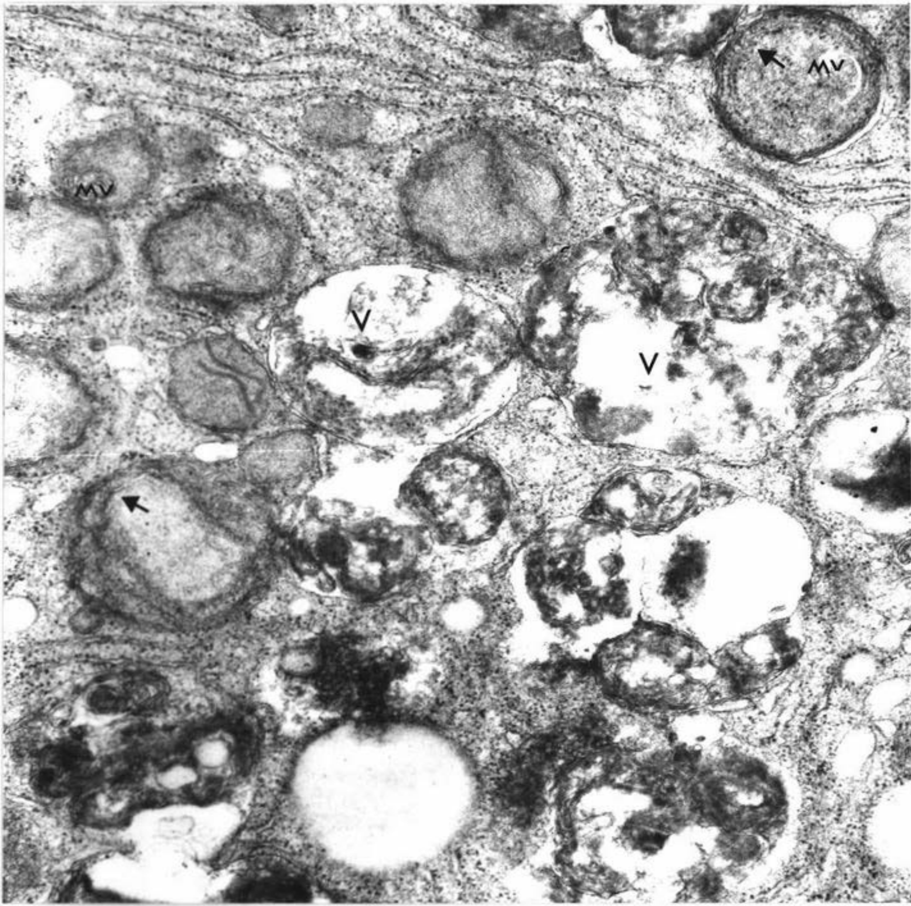
Disintegration of hepatocyte mitochondria producing intracellular debris within a vacuole (V). Margination of mitochondrial cristae (arrows) and mitochondrial vacuoles (MV) are present.

Hamster 1 TEM 25,000x

Fig 6-9

Viable extracellular leptospire (L) within an infolding of the hepatocyte membrane (arrow). If sectioned in a different plane, this organism may appear to be intracellular.

Hamster 2 TEM 46,000x


MV
MV
V
V

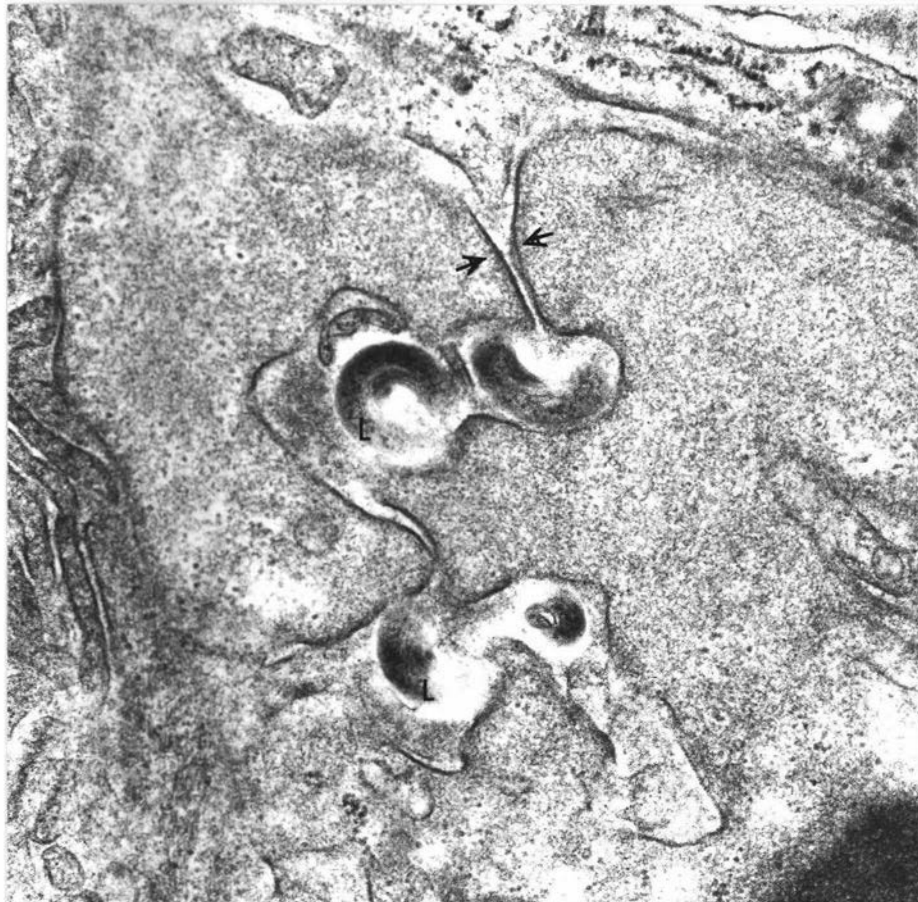

L
L

Fig 6-10

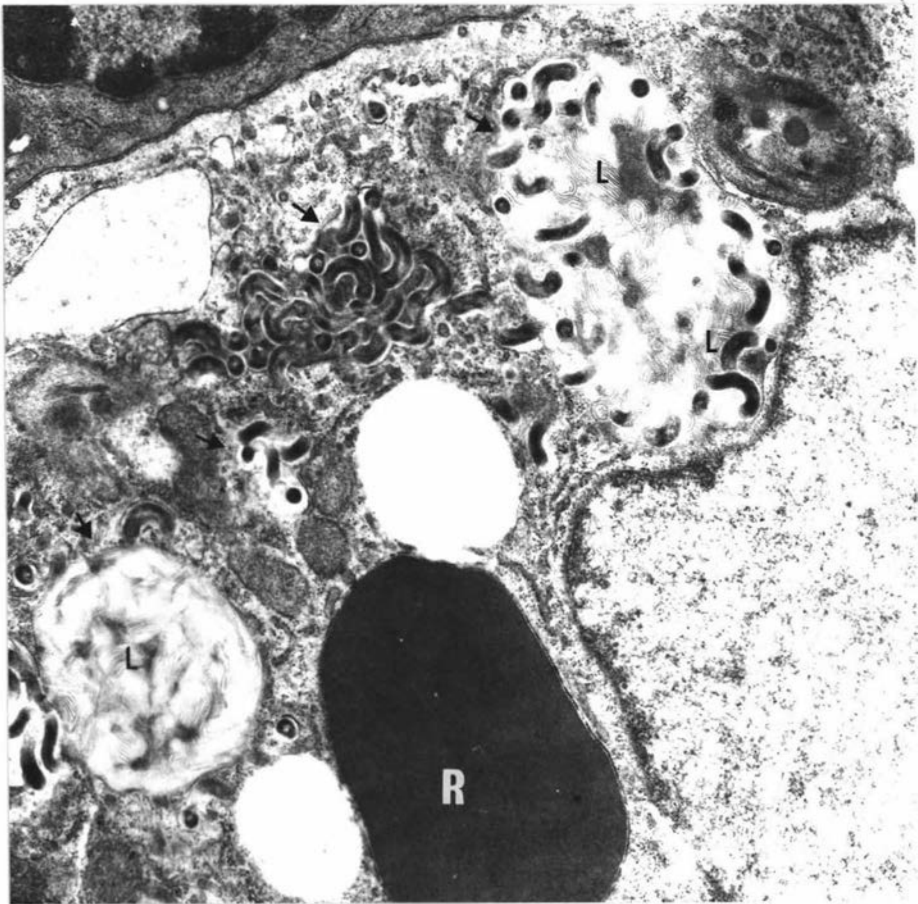
Splenic macrophage containing a number of leptospiral organisms in varying stages of degradation within membrane-bound vacuoles (arrows). As degradation proceeds the leptospires become more dense with the loss of the granular cytoplasmic structure. Electron dense linear profiles (L) are formed as an end result. An intracellular RBC (R) is also seen.

Hamster 1 TEM 17,500x

Fig 6-11

High power area from above electron-micrograph demonstrating differences in degradation of leptospires in two adjacent vacuoles. In the more advanced stage of degradation, electron dense linear profiles (arrows) appear to outline the original position of the leptospire.

Hamster 1 TEM 46,000x


L
L
L
R

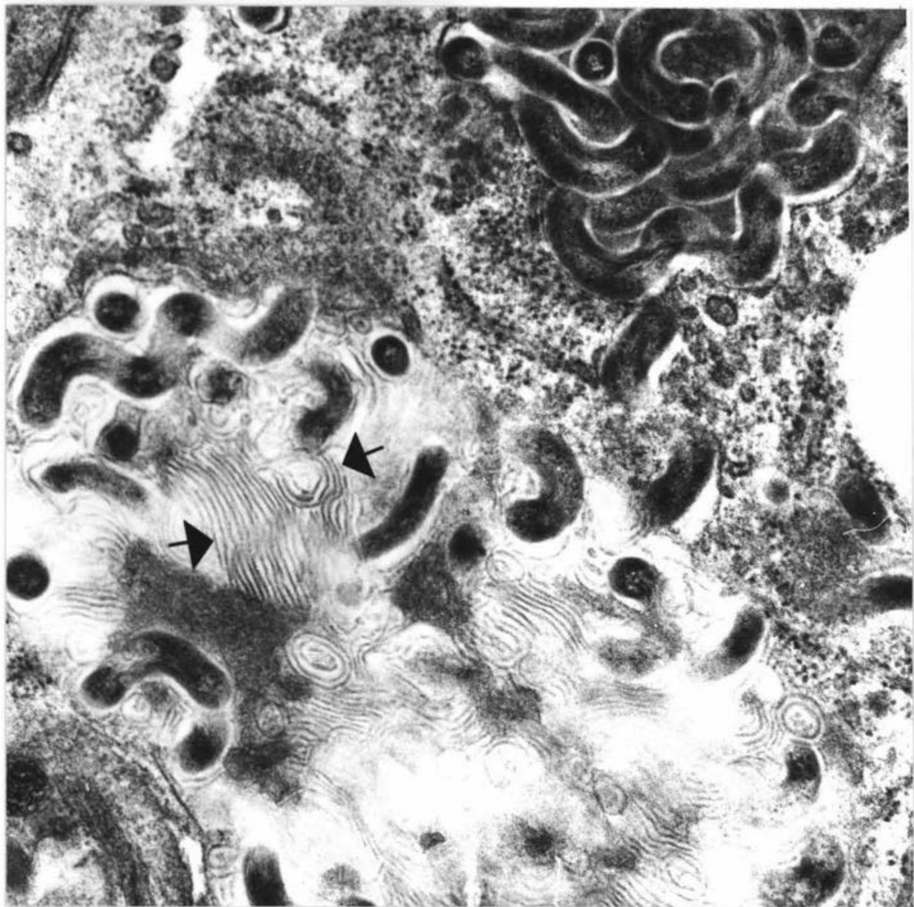


Fig 6-12

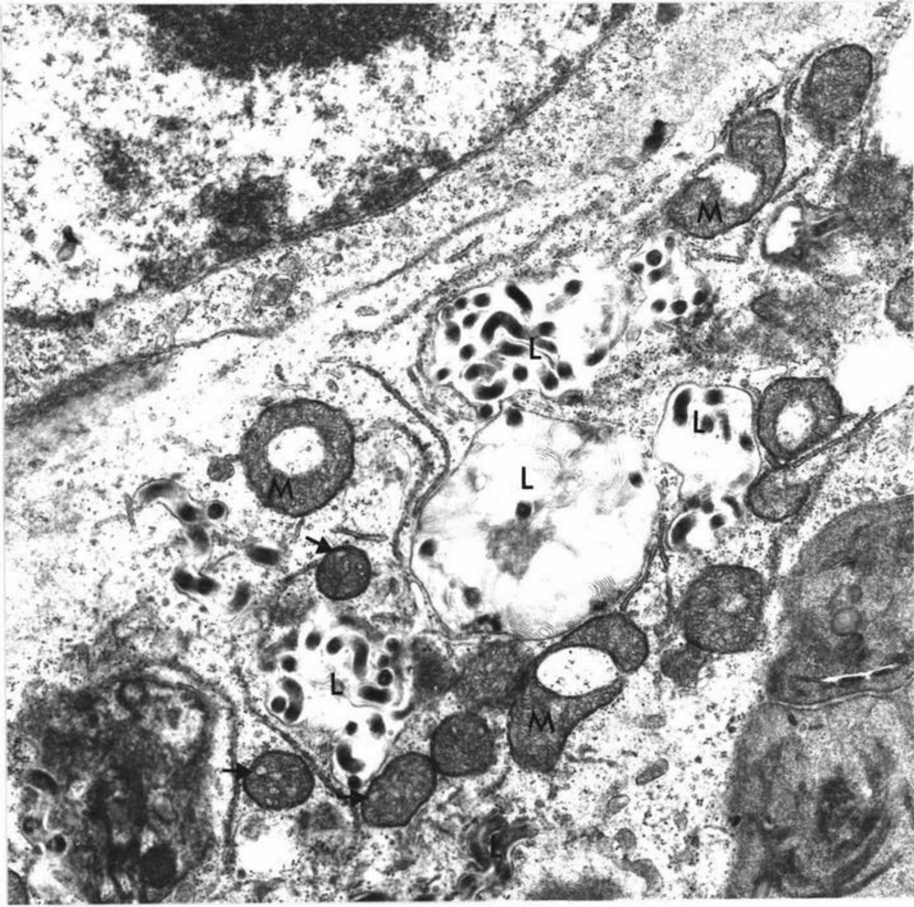
Splenic macrophage demonstrating circular or horseshoe shaped mitochondria (M). The concave surface of the mitochondria encloses a more electron-translucent portion of cytoplasm. Dilatations of mitochondrial cristae (arrows) are frequent. A number of leptospires (L) in varying stages of degeneration are seen within membrane-bound vacuoles.

Hamster 1 TEM 17,500x

Fig 6-13

Splenic macrophage containing a discrete body (B) bound by a double membrane which encloses cytoplasm of greater electron density and smaller inclusions (I) of differing electron densities. A RBC (R), a membrane-bound easily recognisable leptospire (L) and a membrane-bound inclusion (M) containing electron dense linear profiles which are considered to be a completely degraded leptospire are also seen.

Hamster 2 TEM 25,000x


M
L
L
L
M
L
M

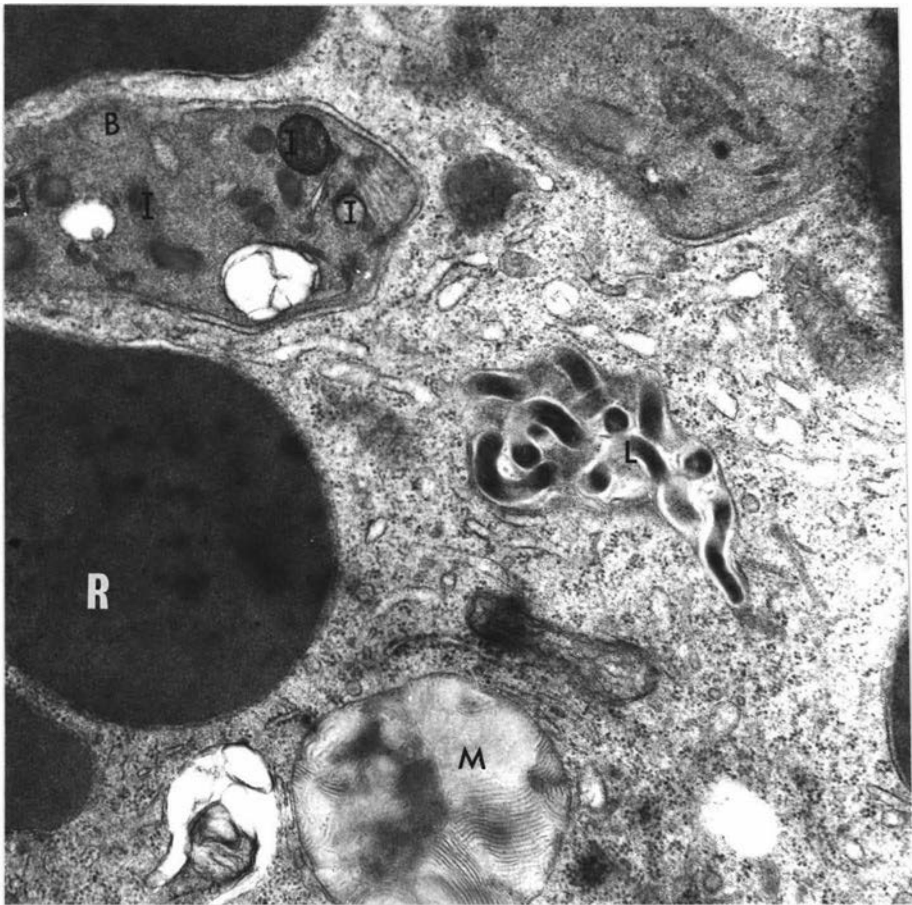

B
I
I
I
L
R
M

Fig 6-14

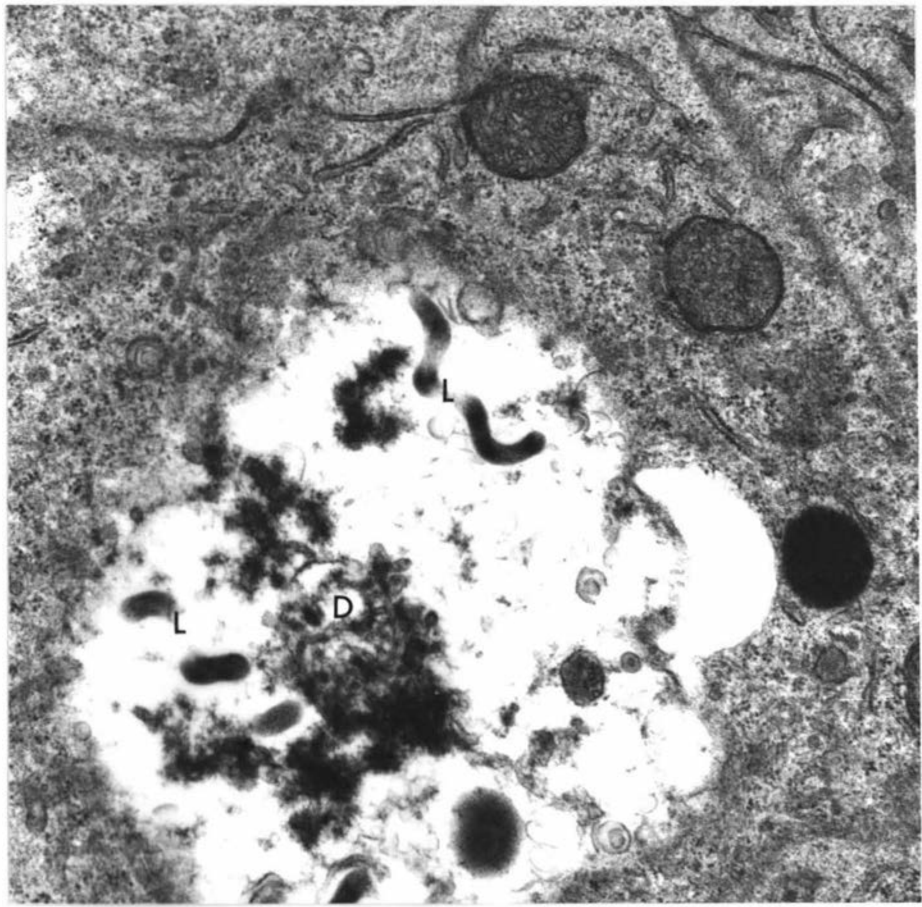
Splenic macrophage containing unrecognisable cellular debris (D) and a degenerate leptospire(s) (L) within a non-membrane bound vacuole.

Hamster 1 TEM 25,000x

Fig 6-15

Apical region of an epithelial cell from a proximal convoluted tubule showing disintegration and loss of microvilli (arrows) and the presence of cellular debris (D) within the tubular lumen.

Hamster 2 TEM 17,500x


L
D
L

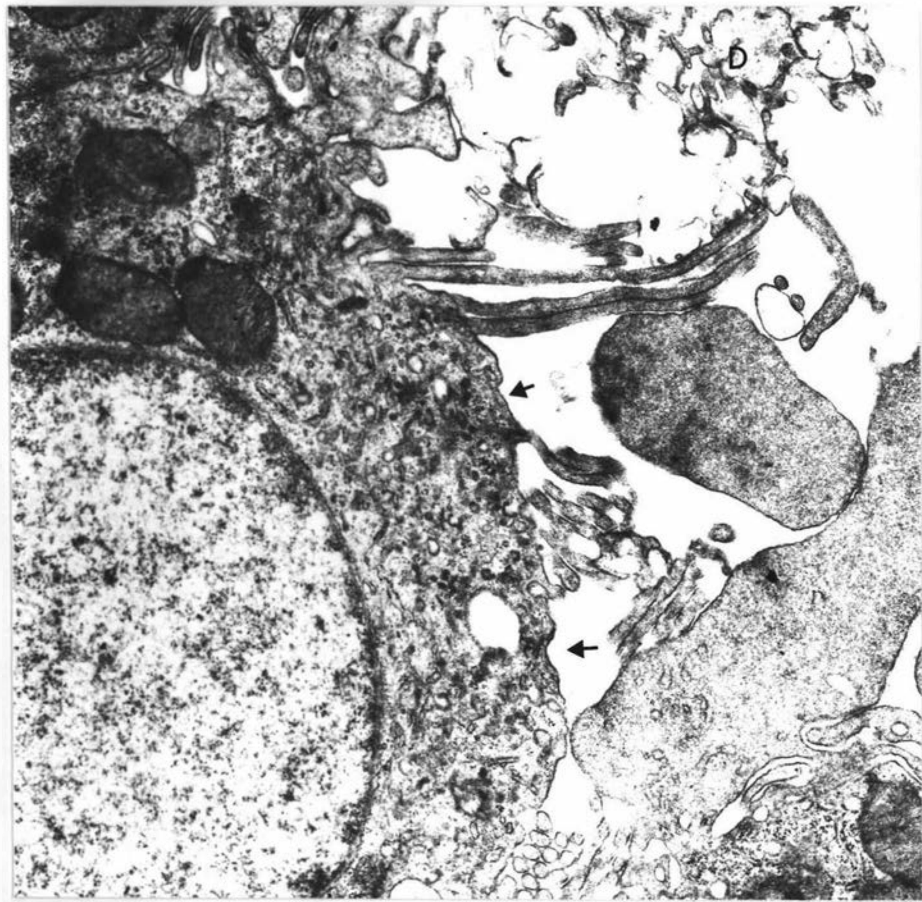

D

Fig 6-16

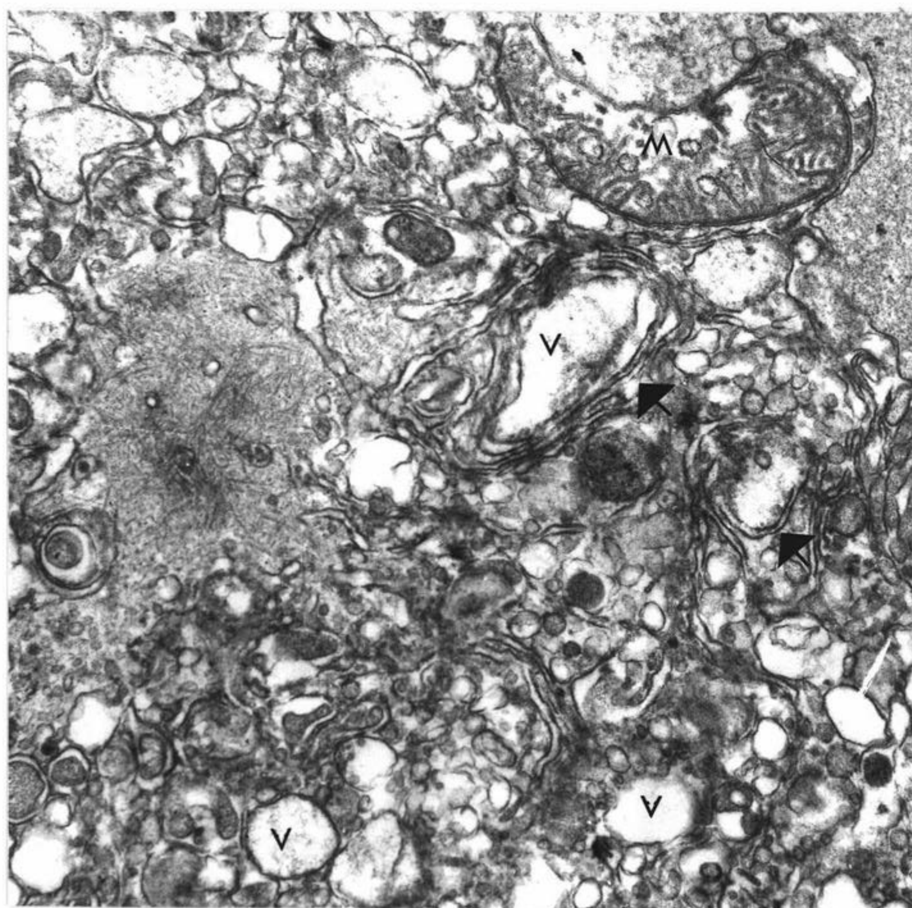
Apical region of an epithelial cell from a proximal convoluted tubule. A disintegrating mitochondrion (M) shows loss of cristae. Smooth endoplasmic reticulum appears to form circular forms (arrows). Numerous vacuoles (V), are seen possibly resulting from swollen endoplasmic reticulum or degenerate mitochondria.

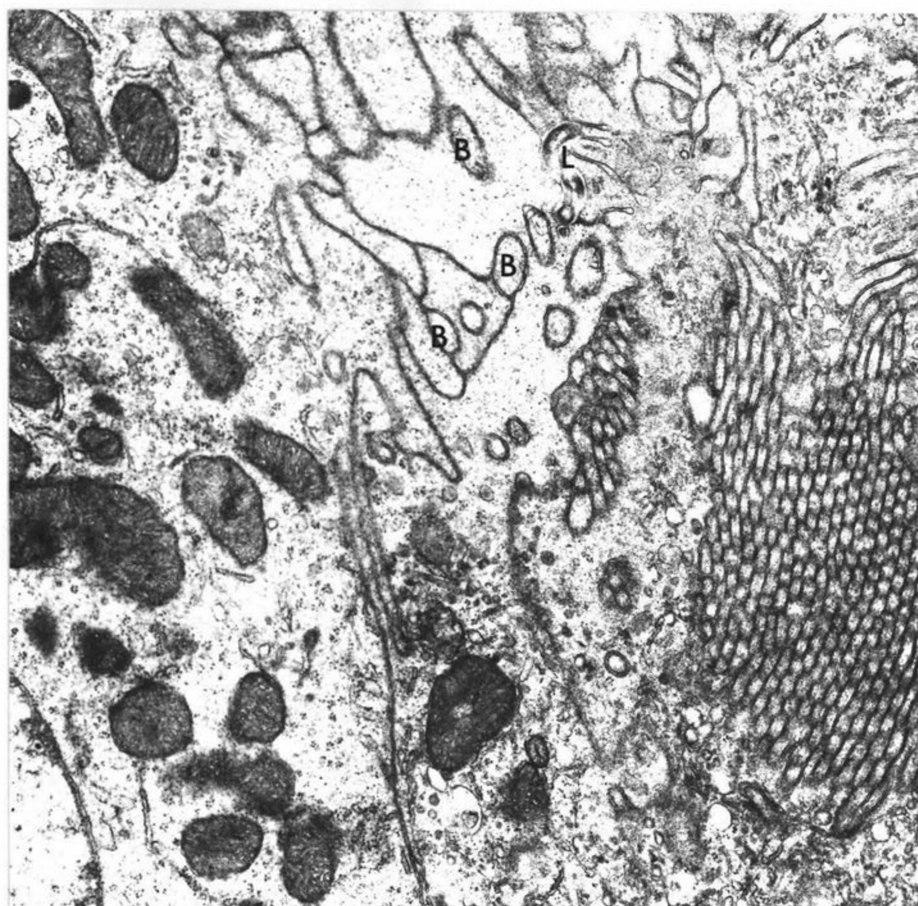
Hamster 2 TEM 25,000x

Fig 6-17

Leptospire (L) migrating between two epithelial cells of a proximal convoluted tubule. Cytoplasmic 'blebs' (B) are present.

Hamster 1 TEM 17,500x


M
V
V
V


B
L
B
B

4.0 DISCUSSION

Following infection, leptospires multiply within the blood stream then localize in the tissues. Whether the animals live or die and the time after infection at which they die depends upon the resistance of the animal and the strain of the infective organism. The clinical and pathological aspects of infection by strains of leptospire producing haemoglobinaemia have been discussed in previous chapters. Death as a result of RBC destruction occurs within five to six days of infection in both calves and hamsters but if the RBC's of hamsters are insusceptible to the leptospiral 'toxins' or the hamsters are protected from the multiplying organisms by administration of sulphadiazine (Frenkel 1972), then death may occur later from kidney and liver disease. While the RBC's of the hamster appear to be insusceptible to the *pomona* 'toxins' which cause haemolysis of calf RBC's, the hamsters may be quite susceptible to infection, dying within a week with acute hepatitis and nephritis. These and other less severe manifestations of leptospirosis, not associated with haemoglobinaemia, occurred in the present experiments.

Previous descriptions of the histological (Abdu & Sleight, 1965; Sanger *et al*, 1961) and ultrastructural lesions (Miller & Wilson, 1966; 1967) seen in *pomona* infections of hamsters are similar to those described in these studies, while descriptions of *ballum* infections of hamsters are scarce and involve only strains of the organism which produced a haemolytic disease (Frenkel, 1972) with no mention of strains which did not have this characteristic.

The difference in severe liver lesions between hamsters infected with *pomona* and *ballum* appears to be related to the presence or absence of hypoxia caused by loss of RBC's. In order to study the effects of leptospiral 'toxins' upon tissues other than RBC's there appeared to be advantages in using strains of organism which do not produce haemoglobinaemia because hypoxia is not under these circumstances likely to be a complicating factor.

Following initial multiplication, there is localization of the organisms in the various organs with the liver appearing to be the preferred site for multiplication as judged by the number of leptospires seen in the sections. This is also reported by other workers (Faine, 1964; Miller *et al*, 1974). Faine (1964) suggested that the leptospires were incapable of initiating a tissue response to their presence resulting in inadequate phagocytosis of leptospires which would otherwise prevent spread of the 'virulent' organism. The present results do not fully support these claims as hepatic phagocytosis of leptospires did occur. However, in comparison with phagocytosis of leptospires in spleen, that noted within the liver was less frequent. Other hepatic factors may be important and Miller & Wilson (1966) postulated that bile released from damaged bile canniculi may be responsible for decreasing leptospire numbers within the liver. Phagocytosis of 'virulent' leptospires has been described by other workers (Faine, 1964; Faine *et al*, 1964). The circular mitochondria noted in the splenic macrophages have been hypothesized as resulting from increased macrophage activity, the larger surface area of the mitochondria resulting in increased metabolite exchange (Stephen & Bils, 1965).

Several histochemical studies have shown that alterations of normal hepatocyte metabolism occur before histological lesions can be observed or leptospires seen within the liver. Such findings indicate that the changes are functional disturbances to the cells (Arean, 1962b; Arean & Henry, 1964) and may involve interference with such enzyme systems as the pentose phosphate pathway (Arean & Henry, 1964). They add further support to the hypothesis that leptospiral 'toxins' rather than the actual organism induce the lesions (Miller & Wilson, 1966). Further support for the presence of a 'toxin' has been provided by the observation of Knight *et al*, (1973) that whole blood and plasma from animals infected with 'virulent' *pomona* and *icterohaemorrhagiae* organisms contain a factor which has a cytopathic effect on cell cultures and kills

mice following intracerebral inoculation while inoculations of cell cultures with plasma from hamsters infected with 'avirulent' *pomona, biflexa* and *P. multocida* does not have a cytopathic effect.

While hepatic lesions were seen only in those animals which died from leptospirosis, renal lesions were seen in most animals. Varying degrees of interstitial nephritis were seen in cattle and hamsters which survived the disease. Hamsters showed the most severe changes. However, even in the most severely affected animals sufficient functional renal tissue remained for survival of the animal at that time. The survival of hamsters following *pomona* infections has also been reported by Miller & Wilson, (1966; 1967).

In dead and moribund hamsters, large numbers of organisms were seen in the kidney sections, the numbers being fewer than in the livers from the same animals. A possible explanation was that the kidney was a less ideal place for leptospiral multiplication. Movement of leptospires into the tubules appeared to be random because the organisms were found initially in collecting ducts, capillaries and the interstitium as well as in proximal and distal convoluted tubules. However in those animals euthanased at 14 days or later, organisms were found only in the proximal convoluted tubules. Leptospiraemia was probably terminated by the development of serum antibody (Taylor *et al*, 1970) and leptospires remained in the renal tubules because in this position they are thought to be in some degree protected from host defence mechanisms (Faine, 1964; Marshall, 1973) and thus could survive and multiply. It is presumed that the proximal convoluted tubules provide a better site for leptospiral survival because in those animals which were long term carriers, the organisms were located in the proximal convoluted tubules although the factors influencing survival are undefined (Marshall, 1973; 1974; 1976). As in the liver, the earliest renal changes were thought to result from alterations in enzyme function (Arean, 1962b; Arean & Henry, 1964) brought about by 'toxins' or non-specific effects resulting from hypovolaemia and renal

hypoxia (Sitprija *et al*, 1980). Hypovolaemia was thought to result from the leakage of fluid and blood following vascular damage (De Brito *et al*, 1979; Sitprija *et al*, 1980). Vascular damage recognised by ultrastructural lesions of the glomerular endothelial cells was seen in both *pomona* and *ballum* (Chapter 5) infections of hamsters. The histological lesions of dilatation of the Bowman's capsule by a pale eosinophilic material was evidence of increased filtration of protein. Haemorrhage in the kidney was also seen. Degeneration of the mitochondria was very obvious and has been noted under hypoxic conditions in the liver by other workers (Glinsmann & Ericsson, 1966). In this latter work, degenerate mitochondria were associated with curved endoplasmic reticulum. In the present studies of proximal convoluted tubular epithelium, curving of the endoplasmic reticulum was also seen and although it could not be directly associated with a degenerate mitochondrion, degenerate mitochondria were seen in the same cell. Although disintegration and loss of the microvilli was seen in the acute *pomona* infections and *ballum* infections, (Chapter 5) of hamsters and *pomona* infections of calves (Chapter 5) the microvilli with bulbous tips described by Marshall (1973;1974) were not seen.

The function of the mononuclear infiltrates of both the liver and kidney is not known. There is no evidence to support the observation that the nephritis is associated with an autoimmune reaction (Sitprija *et al*, 1980). Immunological studies have demonstrated discrete organisms within tubules, as granular deposits within macrophages and as extracellular debris associated with cellular infiltrates (Morrison & Wright, 1976; Taylor *et al*, 1970). It would appear therefore that the function of the cellular infiltrate is phagocytosis or antibody production and cell mediated immunity. Infiltrates resulting from immune reactions are more likely to be represented in the chronically affected animals because in those dying less than a week post-infection there is probably insufficient time for any noteworthy response to develop.

Haemorrhage in the kidneys was noticed more particularly in *ballum* infections,with strain 1045 showing most extensive haemorrhage. Haemorrhage has been reported in *pomona* infected hamsters (Sanger *et al*, 1961), guinea pigs (De Brito *et al*, 1966; 1979; Higgins & Cousineau, 1977a); in cattle (Dodd & Brakenridge, 1960) and dogs infected with *icterohaemorrhagiae* (Bloom, 1941; Low *et al*, 1956; Monlux, 1948b); and cattle infected with *bratislavia* (Fennestad *et al*, 1967) and *szwajizak* (Nervig *et al*, 1978). The erythrophagocytosis and excess haemosiderin observed in the spleens of animals infected with strains of *ballum* causing no overt haemoglobinaemia were considered to be most likely the result of extravasation of RBC's from damaged endothelium rather than the removal of 'toxin' damaged or lysed RBC's in circulation because the RBC lesions seen in haemolytic infections and described in earlier chapters were not noted. However, it is possible that changes may have occurred within the RBC which were not detected by electron microscopy but which nevertheless could be detected by the spleen. An example of morphological changes of RBC's which did not result in haemoglobinaemia was seen in the human case of *ballum* (Chapter 4). Erythrophagocytosis of extravascular RBC's has been described in other haemorrhagic infections such as *icterohaemorrhagiae* infections in guinea pigs (Higgins & Cousineau, 1977b). The mild to moderate anaemia reported in dogs infected with *canicola* and *icterohaemorrhagiae* (Bloom, 1941; Taylor *et al*, 1970) was considered to be the result of extravasation of RBC's from damaged endothelium rather than toxic destruction and removal of damaged RBC's from circulation. Disseminated intravascular coagulation initiated by vascular damage has been reported in *icterohaemorrhagiae* infections of guinea pigs (Higgins & Cousineau, 1977a). The atrophy of the spleen in the *ballum* infections after seven or more days was a likely result of removal of necrotic reticular cells. Although haemorrhage was seen within the kidneys of hamsters infected with *ballum* strain 1045,the progression of lesions could not be determined because sequential euthanasia of hamsters following infection was not done.

The significance of the absence of brain lesions was discussed in Chapter 5. Abdu & Sleight (1965) also considered the absence of lesions possibly due to insufficient time having elapsed for the lesions to develop but those hamsters which survived to 14 days as described in the present experiments did not either show nervous signs or histological lesions in the brain. Jaundice was not thought to be a factor in the production of nervous signs because Knight *et al*, (1973) has shown that intracerebral inoculation of bilirubin does not produce clinical signs. The large numbers of organisms seen in the marrow cavity of the petrous temporal bone may have resulted in pain or discomfort which was responsible for some of the apparent nervous signs.

The loss of 'virulence' or ability to cause haemoglobinaemia following subculture noted previously in the *ballum* strain 1045 and commented upon by other workers (Marshall, 1973) was demonstrated by the changes which occurred in the *ballum* strain 964 and is possibly due to selection of organisms better adapted to survival *in vitro* rather than *in vivo*.

5.0 CONCLUSIONS AND SUMMARY

1. Fatal leptospiral infections can occur in the absence of haemoglobinaemia and haemoglobinuria in *ballum* and *pomona* infected hamsters.

2. Moribund and dead hamsters which were non-haemoglobinaemic showed different hepatic, splenic and renal lesions to those animals which were haemoglobinaemic. The differences are believed to have resulted from the presence of severe hypoxia in the haemoglobinaemic animals.

3. The absence of RBC destruction in some leptospiral infections suggests either an absence of a particular 'toxin' or 'factor' causing RBC destruction or a lack of susceptibility of the infected animal to the 'toxin' which causes destruction of RBC's in other leptospiral-host associations.

4. There was active phagocytosis of leptospires particularly by splenic macrophages in which all stages of leptospiral degradation would be found. In susceptible animals and before antibody formation would be expected it appeared that leptospiral multiplication exceeded leptospiral removal thereby allowing the disease to progress, histopathological lesions to develop, and if the condition was severe enough, death to occur. In those animals in which no antibody was present yet clinical lesions did not develop, the non-specific defence mechanisms or undetectable early antibody development may have limited leptospiral multiplication.

5. *Ballum* infections of hamsters in which overt haemoglobinaemia was not a feature showed increased haemosiderin deposits and splenic necrosis. This was not seen in hamster *pomona* infections. The pathogenesis of the lesions is not known but RBC's may be phagocytosed due to either:

i) Extravasation from normal circulation

or ii) Alterations in the RBC's which may or may not be obvious by light and electron microscopy.

6. Haemorrhagic lesions were seen in particular in the kidneys of the *ballum* infected hamsters.

7. No lesions could be found in the brain, middle or inner ears and the cause of apparent nervous signs seen in some moribund hamsters remains speculative.

CHAPTER 7

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2.1.3 <u>Laboratory procedures</u>

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CHAPTER 7

IN VITRO HAEMOLYTIC STUDIES

1.0 INTRODUCTION

Experimental work involving the pathogenetic mechanisms of haemolysis requires work to be carried out *in vivo* as well as *in vitro* in order to examine certain aspects in isolation.

During RBC destruction *in vivo*, there are problems due to individual animal variation, the decision as to when exactly to take samples in order to demonstrate certain points, and the problem of separating the various facets of the disease and establishing whether the pathogenesis of each facet is due to the same or separate mechanisms. Work done *in vitro* may eliminate some of these problems but create additional ones. The RBC's are not then in their natural environment and environmental homeostasis such as the removal of metabolic wastes and the maintenance of pH may not be possible. Therefore, while work done *in vitro* is important, it may not truly reflect the conditions which exist *in vivo*.

Haemolysis *in vitro* of the RBC's from various animal species has been described by a number of authors (Hathaway, 1978; Hathaway & Marshall, 1980; Imamura *et al*, 1957; Russell, 1956; Stamm & Charon, 1979; Valentine *et al*, 1964). However, there are discrepancies between the presence of haemolysis *in vitro* and *in vivo*. Hathaway (1978) and Hathaway & Marshall (1980) state that *ballum* is always non-haemolytic *in vitro* with RBC's from several animal species including hamsters and *pomona* is always haemolytic for RBC's from several species, also including hamsters. This is difficult to reconcile with results presented from *in vivo* studies reported in earlier chapters of this thesis,and with reports by other workers which state that *ballum* usually causes haemoglobinaemia in hamsters and *pomona* does not.

The experiments in this chapter attempt to examine *in vitro* the effects of some known differences in RBC's on haemolysis and to verify the findings of other workers.

Experiment A was designed to examine changes in the degree of *in vitro* haemolysis of RBC's from calves over the first few weeks of life. The decrease in foetal haemoglobin was also examined over this time because this is one major change which occurs in calf RBC's over the first few weeks of life (Kitchen, 1969; Schalm *et al*, 1975). The susceptibility of young calves to *pomona* infection has never been discussed in relation to foetal or adult haemoglobin types although adult haemoglobin types have been suggested to affect the susceptibility of sheep to RBC destruction following infection by *pomona* (Hodges *et al*, 1976). RBC's from two calves were examined by SEM to compare morphological changes occurring *in vitro* following incubation with *pomona* culture with those changes occurring *in vivo* following *pomona* infection. *Hardjo* and *balcanica* were also incubated with calf RBC's in order to verify the findings of Hathaway (1978) and Hathaway & Marshall (1980) that *balcanica* is haemolytic and *hardjo* is non-haemolytic for bovine RBC's.

Experiment B was a comparison of the degree of haemolysis by two strains of *pomona* and two strains of *ballum* when incubated with RBC's from young calves, adult cattle, humans and hamsters. *Pomona* strains T116 and 790001 were chosen because they most consistently produced dense cultures. *Ballum* strain M4/9 was selected because it produced destruction of hamster RBC's as described in Chapter 6. *Ballum* strain 1045 was selected because it initially produced destruction of hamster RBC's as described in Chapters 3, 4 and 5 then appeared to lose this characteristic as described in Chapter 6.

Experiment C was designed to test the findings of Ringen (1966). Ringen found that RBC's taken from cattle and goats 8 to 13 days following infection with *pomona* showed decreased susceptibility to *pomona* 'toxin' although the infection itself was asymptomatic.

Experiment D examined the effects of incubating washed RBC's from normal calves in leptospiraemic plasma from *pomona* infected calves in which overt RBC destruction was not a feature. RBC's from the calves of Experiment V, Chapter 2 were used in this experiment.

2.0 MATERIALS AND METHODS

2.1 EXPERIMENT A

2.1.1 Leptospires

Pomona strains T116, 790001, MP3 and 'Scamp', *balcanica* strain E32 and *hardjo* strain 'prajitna' were isolated, cultured and maintained as described in Chapter 2, Section 1.0. While replicate cultures were grown in an attempt to obtain dense cultures for incubation with the RBC's, it was found that culture density varied after seven days of growth and throughout the duration of the experiment. The *pomona* strains MP3 and 'Scamp' were consistently less dense in culture than strains T116 and 790001.

2.1.2 Animals

Eleven Jersey-Freisian cross calves were run at pasture, fed on bulked colostrum and bled on the days noted in Appendix XXX1 into two EDTA containing vacutainers from soon after birth to 47 days of age. RBC's from these calves were added to leptospiral cultures and incubated as described in the following sections.

Five calves, numbers 63, 67, 70, 72 and 76 developed coccidiosis and were treated systemically with antibiotics. They were excluded during the course of the experiment from the tube and plate testing described below, but were typed for haemoglobin.

2.1.3 Laboratory procedures

2.1.3.1 Tube haemolysis

The method of tube haemolysis was based upon that described by Hathaway (1978) and Hathaway & Marshall (1980).

RBC's were washed three times using sterile 0.85% saline at room temperature. While the RBC's in one vacutainer were being washed, the RBC's in the second vacutainer were allowed to stand at room temperature. Following washing and centrifugation, the RBC's were resuspended in 0.85% saline to the original plasma volume of the blood sample.

One ml of leptospiral culture was incubated with 1 ml of resuspended washed RBC's from each animal and 1 ml of unwashed RBC's suspended in plasma (whole blood). In addition two osmotic haemolysates consisting of 1 ml each of washed and unwashed RBC's in 1 ml of sterile distilled water and two negative controls, one each of washed and unwashed RBC's in 1 ml of culture medium were prepared.

Following 18 hours of incubation at 27°C, the cultures were diluted to 10 mls with 0.85% saline and centrifuged at 4,356 g for ten minutes to remove the non-haemolysed RBC's. The culture supernatant was then further diluted 1:10 with 0.85% saline and the optical density read at 540 nm on a SP6-550 UV/VIS spectrophotometer.[1] The reading was expressed as a percentage of the osmotic haemolysate.

The supernatant was examined by dfm for the presence of leptospires.

2.1.3.2 Plate haemolysis

Blood agar plates were prepared as described by Hathaway (1978) and Hathaway & Marshall (1980) using whole blood or washed RBC's resuspended in 0.85% saline as described for the tube haemolysis. Culture was streaked over the plate using a flamed microbiological metal loop. Haemolysis was observed visually following incubation at 30°C for 72 hours.

1. Pye Unicam Ltd., Cambridge, England

In addition, on day 32 plates were prepared using osmotically haemolysed RBC's in which the washed RBC's were resuspended to the original plasma volume in sterile distilled water, mixed, and allowed to stand for ten minutes. Haemolysis was observed as before.

2.1.3.3 Electron microscopy

RBC's from all calves were prepared for tube haemolysis on day 40 using *pomona* strain 790001 as this strain had produced the most dense culture on this day. RBC's from calves 66, 68, 69 and 73 were prepared for SEM with those from calves 69 and 73 being lost during processing.

Washed RBC's suspended in saline, culture medium and leptospiral culture and unwashed RBC's suspended in plasma, culture medium and leptospiral culture respectively were fixed in 1% glutaldehyde at 0, ½, 6 and 18 hours of incubation and prepared for SEM as described in Chapter 4, Section 2.3.

2.1.3.4 Haemoglobin typing

RBC's were taken from the 11 calves from birth to 52 days of age as shown in Fig. 7-6 and washed three times in 0.85% saline. A small volume of centrifuged RBC's was haemolysed in six times their volume of distilled water, the osmotic haemolysate being allowed to stand for ten minutes to allow complete rupture of all RBC's. The haemolysate was then centrifuged at 4356 g for 20 minutes to remove the cell stroma. A sample of supernatant was applied to an acetate strip and the haemoglobins separated by electrophoresis using 100 volts for 30 minutes. The acetate strip was then stained and processed as described for protein electrophoresis in Chapter 3, Section 2.3.2.3.

Haemoglobin samples from adult cattle were also prepared to obtain control samples of haemoglobins A and B for comparison with the calf haemoglobins.

2.2 EXPERIMENT B

2.2.1 Leptospires

Pomona strains T116, 790001 and *ballum* strains 1045 and M4/9 were cultured and maintained as described in Chapter 2, Section 1.0.

2.2.2 Animals

2.2.2.1 Hamsters

Six weanling golden hamsters 19-21 days of age were anaesthetized with ether and bled by cardiac puncture into a 3 ml syringe containing 0.3 mls of EDTA which had been rinsed through the needle and barrel of the syringe. Blood from the hamsters was pooled and thoroughly mixed with the EDTA.

2.2.2.2 Cattle

Two adult Jersey cows and two newborn Jersey calves were bled from the jugular vein into three 10 ml EDTA containing vacutainers.

2.2.2.3 Humans

Blood samples from four human donors were each collected into 10 ml EDTA containing vacutainers. Donor number 1 had been infected with *ballum* six months previously (Chapter 4, Section 2.1.4).

2.2.3 Laboratory procedures

Washed and unwashed RBC's were incubated as described in Section 2.1.3.1 with two *pomona* and two *ballum* strains. Due to the small volume of blood obtainable from the hamsters quantities of blood and culture used for incubation were halved.

In addition, RBC's from one vacutainer of calf and adult cattle blood were washed three times in 0.85% saline and then the centrifuged, washed RBC's were resuspended in their own stored plasma. These RBC's were then cultured with the two *pomona* strains as previously described.

The supernatant from all cultures was examined by dfm for leptospires.

The optical density of all incubated cultures was read at 540 nm on a SP6-550 UV/VIS spectrophotometer.

MAT's to *pomona* and *ballum* were measured using the methods of Cole *et al* (1973) and a minimum dilution of 1:24.

2.3 EXPERIMENT C

2.3.1 Leptospires

Pomona strain 13231 was cultured in preparation for animal inoculation as described in Chapter 2, Section 1.0. *Pomona* strains T116 and 790001 were cultured for tube haemolysis as previously described.

2.3.2 Animals

Six Jersey-Freisian cross six to nine month-old heifers (I1, I19, I23, I26, I39, and I41) were run on grassed pastures which had not been stocked for the previous month, and supplemented with hay. Two control heifers (C1 and C2) were run separately. The cattle were presumed leptospiral-free after negative MAT's to *pomona, ballum, hardjo, balcanica, tarassovi* and *copenhageni* were obtained using techniques described by Cole *et al*, (1973).

2.3.3 Laboratory procedures

The cattle were injected IM in the neck with 5.0 mls of *pomona* strain 13231 culture. Blood was collected from the jugular vein into an anti-coagulant free and two EDTA containing vacutainers.

The PCV and WBC levels were measured as described in Chapter 3, Sections 2.3.1.2 and 2.3.1.5 respectively. Serum AAT was measured as described in Chapter 3, Section 2.3.2.4 and MAT's performed following the methods of Cole *et al* (1973). Blood was collected as described in Chapter 2, Section 3.2 on days 0, 1, 5, 7, 11 and 16.

RBC's collected into EDTA containing vacutainers were incubated with *pomona* strains T116 and 790001 as previously

described for the tube haemolysis. The amount of haemolysis was measured and the culture supernatants examined for leptospires by dfm.

The cattle were haemoglobin-typed on days 0 and 16 as previously described in Section 2.1.3.4.

The presence of antibody on RBC's from cattle on all days was examined for by the incubation of washed RBC's with rabbit antibovine globulin conjugate[2] as described by Hudson & Hay (1980). The RBC's were then washed and examined as a smear on a glass slide using long wavelength UV light and viewing through a UV filter on a fluorescent microscope[3].

2.4 EXPERIMENT D

2.4.1 Leptospires

Pomona strain 790001 was used to infect week-old calves as described in Chapter 2, Section 2.2.1.

2.4.2 Animals

Ten week-old calves were obtained and maintained as described in Chapter 2, Section 2.2.1. Five calves were infected with a virulent strain of *pomona*, two were injected IV with a 'toxin' and three were maintained as controls (Experiment V).

2.4.3 Laboratory procedures

The clinical pathological examinations performed are described in Chapter 3, the morphological examinations of RBC's carried out *in vivo* are described in Chapter 4 and the gross and histopathological tissue examinations are described in Chapter 5.

2. Grand Island Biological Company, Grand Island, NY. 14072 USA
3. Reichert, Austria: Supplied by G. Wilton & Co. Ltd

Three calves (14, 15 and 17) died as a result of acute RBC destruction. Two calves (11 and 16) survived without haemoglobinaemia despite a more marked leptospiraemia than the former calves. On day 11, the day of the greatest leptospiraemia, blood was collected in EDTA containing vacutainers from infected calves 11 and 16 and control calves 18, 19 and 20. The blood was centrifuged and the plasma from each calf retained in a sterile test tube. RBC's from each calf were washed three times in sterile 0.85% saline. Following the final centrifugation, approximately 0.5 mls of RBC's from calves 18, 19 and 20 were resuspended in 1.5 to 2.0 mls of plasma from calves 11 and 16, their own plasma and 0.85% saline respectively. Washed RBC's from calves 11 and 16 were resuspended in plasma from calf 20, their own plasma and 0.85% saline respectively. RBC's were fixed and prepared as in Chapter 4, Sections 2.2 and 2.3 at 0, ½, 1, 2 and 6 hours following incubation at 37°C. The temperature of 37°C was chosen as being similar to body temperature and therefore being more likely to mimic the natural course of the disease.

3.0 RESULTS

3.1 EXPERIMENT A

The MAT's to *pomona* and *hardjo* are summarized in Appendix XXXI. Calf 69 had MAT's to *pomona* and calves 65, 66, 68, 70, 73 and 78 had MAT's to *hardjo* throughout the sampling period. The densities of the cultures used for incubation are noted in Appendix XXXI.

3.1.1 Tube haemolysis

The percentage of haemolysed washed and unwashed RBC's are tabulated in Appendix XXXI. Washed RBC's showed varying degrees of haemolysis when incubated with all *pomona* cultures on different days. No unwashed RBC's showed any signs of haemolysis (Fig.7-1(i)).

It was found that although the *pomona* strains MP3 and 'Scamp' consistently produced cultures which were less dense than strains T116 and 790001, the degree of haemolysis of washed RBC's was similar or sometimes greater than that produced by T116 and 790001. Slight variation in the degree of haemolysis occurred from day to day and between calves with no obvious decline in the degree of haemolysis with increasing age.

Hardjo was non-haemolytic and *balcanica* weakly haemolytic (Fig.7-1 (ii)) (Appendix XXXI) as previously reported by Hathaway (1978) and Hathaway & Marshall (1980).

Leptospires were seen in the supernatant of both washed and unwashed RBC's incubated with leptospiral culture. The numbers of organisms were diminished in the supernatant of unwashed RBC's plus culture where homologous antibody to that particular serovar was present when compared to the numbers in the supernatant of unwashed RBC's and culture where homologous antibody was not present. Numbers of organisms although more difficult to estimate in haemolysed samples in which RBC ghosts interfered with observation, appeared similar in all incubated cultures of washed RBC's.

3.1.2 Plate haemolysis

Haemolysis, as indicated by clear areas following the streaked cultures, was present on plates containing both washed and unwashed RBC's. The size of the clear zone varied from calf to calf and day to day. Measurements were not made as there was no accurate method for determining the quantity of culture applied by the loop or the density by which the culture was applied during streaking over the agar plate. By visual appraisal, the plates containing washed RBC's sometimes appeared to have larger clear zones but often little difference was seen.

As described by Hathaway (1978) and Hathaway & Marshall (1980) *hardjo* was non-haemolytic and *balcanica* haemolytic for washed cattle RBC's contained in blood agar plates. The present work also showed haemolysis of unwashed RBC's by *balcanica*.

No clear zones were observed on plates incorporating osmotically haemolysed washed or unwashed RBC's.

3.1.3 Electron microscopy

All washed, and unwashed RBC's whether in plasma, saline, culture medium or leptospiral culture were spherical at the beginning of the period of incubation (Fig. 7-3). The control RBC's and the unwashed RBC's incubated with leptospiral culture remained spherical with only minor changes which consisted of the presence of the occasional unusual shaped RBC and slight alteration in the size of some RBC's after 18 hours incubation.

Following six hours of incubation with leptospiral culture, many of the washed RBC's while showing no haemolysis grossly, were shrunken and flattened (Fig. 7-4). After 18 hours of incubation, haemolysis had occurred and grossly the supernatant was stained red. The majority of the RBC's were shrunken and had pits and the occasional protruberance (Fig. 7-5) similar to those seen on the RBC's from the haemoglobinaemic animals described in Chapter 4.

3.1.4 Haemoglobin typing

The means and standard deviations of the haemoglobin types of nine of the calves in which foetal haemoglobin was replaced by haemoglobin type A are demonstrated in Fig. 7-6. Calves 67 and 76 developed haemoglobin type AB but died before detailed examination of their haemoglobin changes could be made. No calves developed haemoglobin type B.

3.2 EXPERIMENT B

The results of incubation of washed and unwashed RBC's with leptospiral culture in tube haemolysis are described in Table 7-1.

No unwashed RBC's showed any signs of haemolysis with either of the *pomona* or *ballum* cultures (Fig. 7-2(i)).

Table 7-1 Results of Experiment B: Percentage of washed and unwashed hamster, cattle and human RBC's haemolysed by two strains of *pomona* and two strains of *ballum*

Animal	Leptospire strain	*pomona* T116	*pomona* 790001	*ballum* M4/9	*ballum* 1045	MAT* *pomona*	MAT* *ballum*
pooled hamster RBC's	washed	11%	21.8%	31.5%	56.9%	-ve	-ve
	unwashed	0%	0%	0%	0%		
cow 1	washed	83.6%	81.6%	27.9%	86.8%	-ve	-ve
	unwashed	0%	0%	0%	0%		
cow 2	washed	77.5%	77.8%	14.6%	73.5%	-ve	-ve
	unwashed	0%	0%	0%	0%		
calf 1	washed	77.9%	58.0%	4.0%	20.6%	-ve	-ve
	unwashed	0%	0%	0%	0%		
calf 2	washed	98.6%	92.9%	91.0%	98.0%	-ve	-ve
	unwashed	0%	0%	0%	0%		
human 1	washed	5.2%	18.0%	2.3%	3.2%	-ve	1:384
	unwashed	0%	0%	0%	0%		
human 2	washed	8.3%	38.0%	2.2%	15.4%	-ve	-ve
	unwashed	0%	0%	0%	0%		
human 3	washed	14.5%	42.5%	2.4%	20.9%	-ve	-ve
	unwashed	0%	0%	0%	0%		
human 4	washed	2.7%	20.5%	1.6%	32.5%	-ve	-ve
	unwashed	0%	0%	0%	0%		
Culture density		Dense	Dense	Dense	Dense		

* Negative to minimum dilution of 1:24

Fig 7-1(i)

Test tubes containing centrifuged unwashed calf RBC's following 18 hours of incubation with two strains of *pomona* (T116, 790001), one strain of *balcanica* (bal) and one strain of *hardjo*. Haemolysis has occurred only within the test tube containing distilled water (DW).

Fig 7-1(ii)

Test tubes containing centrifuged washed calf RBC's following 18 hours of incubation with two strains of *pomona* (T116, 790001), one strain of *balcanica* (bal) and one strain of *hardjo*. The test tube in which RBC's were incubated with culture medium (CM) was the only one in which haemolysis did not occur.

Fig 7-2(i)

Test tubes containing centrifuged unwashed calf RBC's following 18 hours of incubation with two strains of *pomona* (T116, 790001) and two strains of *ballum* (M4/9, 1045). Haemolysis has occurred only within the test tube containing distilled water (DW).

Fig 7-2(ii)

Test tubes containing centrifuged washed calf RBC's following 18 hours of incubation with two strains of *pomona* (T116, 790001) and two strains of *ballum* (M4/9, 1045). The test tube in which RBC's were incubated with culture medium (CM) was the only one in which haemolysis did not occur.

66 ww DW
66 ww CM
66 TMG ww

66 w DW
66 w CM
66 w HARJ
66 w BUI
66 w TMG
66 w 79001

calf1 ww DW
calf1 ww CM
POM calf1 ww T116
POM calf1 ww 79001
BALL calf1 ww M4/9
BALL calf1 ww 1045

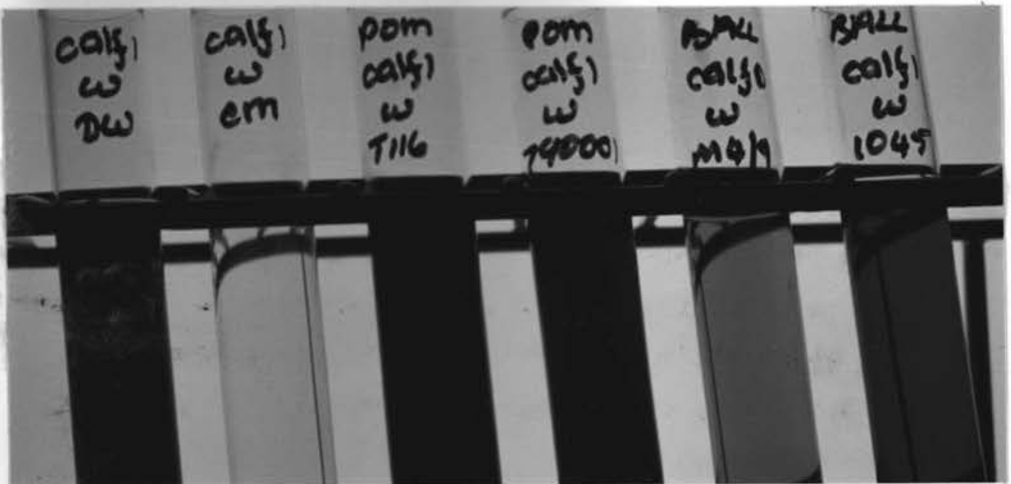

calf1 w DW
calf1 w CM
POM calf1 w T116
POM calf1 w 79001
BALL calf1 w M4/9
BALL calf1 w 1045

Fig 7-3

Cattle RBC's following washing with saline and immediately after combination with a *pomona* culture. All RBC's are spherical.

SEM 4,000x

Fig 7-4

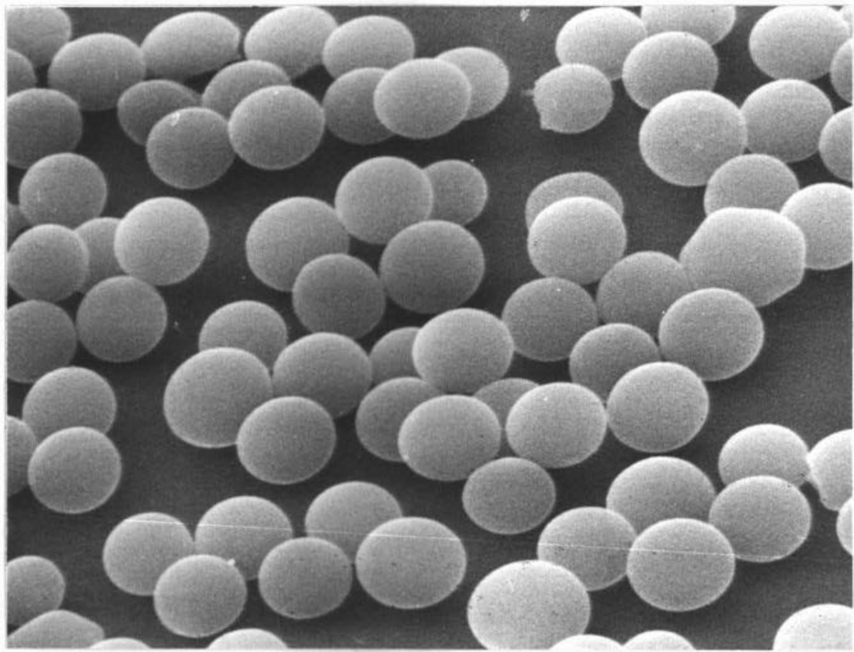
Cattle RBC's after six hours of incubation with *pomona*. Some RBC's remain spherical (s) but the majority have a decreased volume and are flattened (arrows).

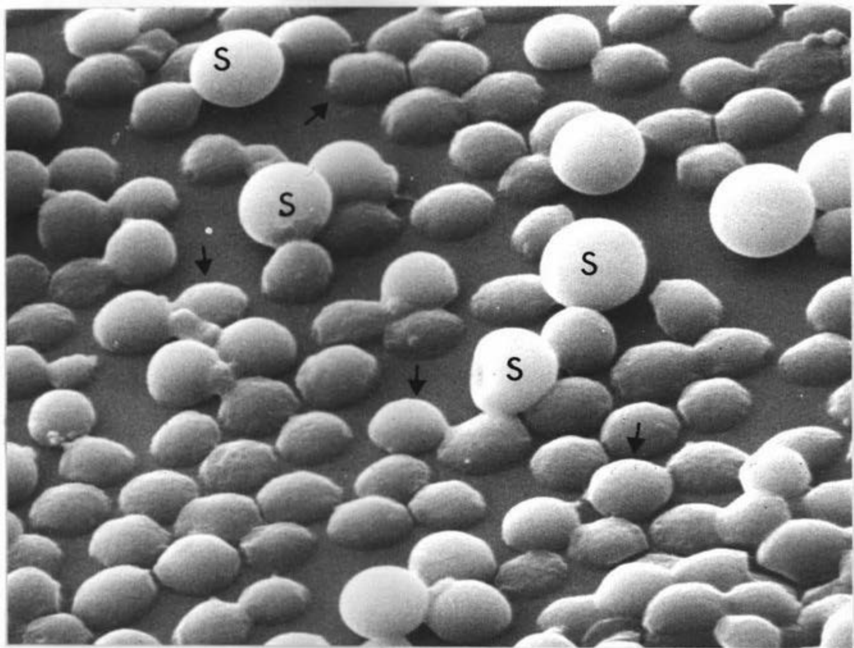
SEM 4,000x

Fig 7-5

Cattle RBC's after 18 hours of incubation with *pomona*. Many of the RBC's have surface pits (arrows) and projections (P).

SEM 10,000x

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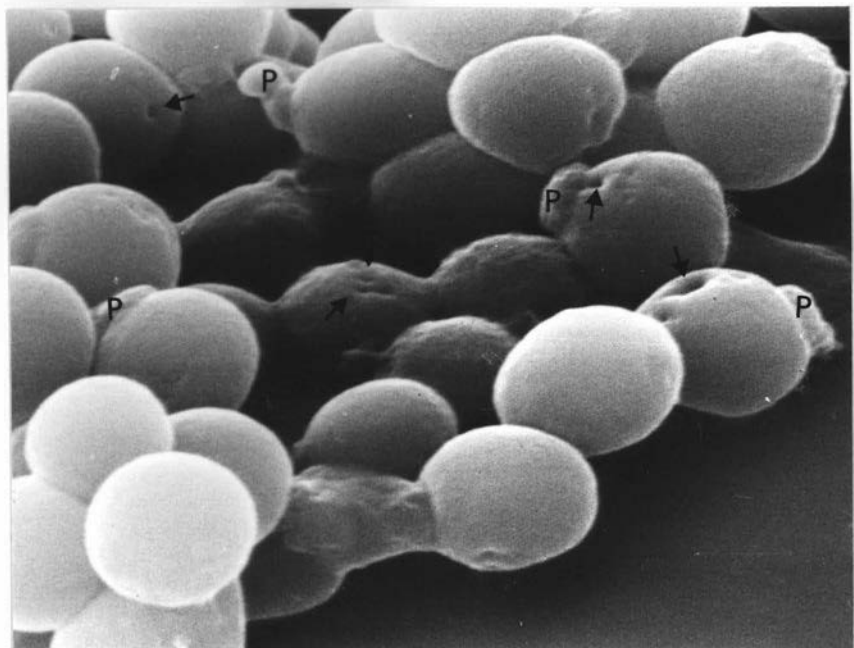
P
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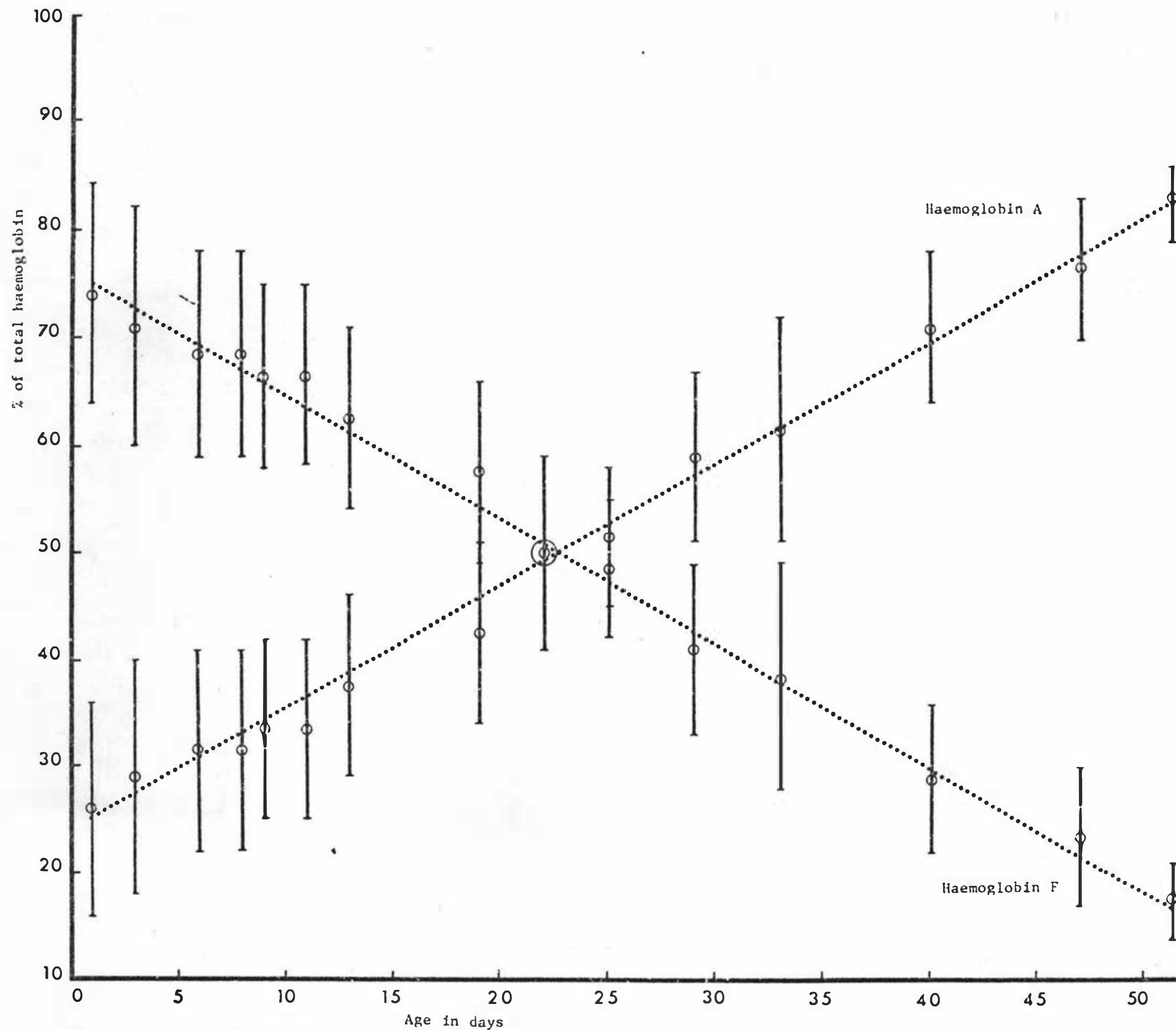

Fig 7-6 Means ± standard deviation for the haemoglobin types of nine calves from birth to 52 days of age.

Pomona was more haemolytic for cattle and calf RBC's than for human and hamster RBC's. There was variation in human RBC susceptibility to the *pomona* strains with 790001 appearing more haemolytic than T116.

Ballum strain 1045 appeared more haemolytic than *ballum* strain M4/9. *Ballum* was more haemolytic than *pomona* for hamster RBC's. The degree of haemolysis of *ballum* for hamsters was less than the degree of haemolysis of *pomona* for cattle RBC's.

In general *ballum* was less haemolytic than *pomona* for all RBC's (Fig. 7-2(ii)).

No relationship between the age of the animals and the degree of haemolysis was seen as both adult cattle and calves showed similar variation in the degree of haemolysis (Table 7-1). Calf 2 showed the greatest degree of haemolysis with both *pomona* and *ballum*.

The only serum to contain specific antibody was that of human 1 in which an MAT of 1:384 to *ballum* was obtained (Table 7-1). Leptospires were found within the supernatant of all incubated cultures of washed and unwashed RBC's with *ballum* and *pomona* cultures. Diminished numbers of *ballum* organisms were found within the supernatant of the incubated culture of unwashed RBC's from human 1 and the *ballum* cultures 1045 and M4/9 when compared to the number of *ballum* organisms in the supernatant of incubated cultures and unwashed human RBC's in plasma not containing homologous antibody.

Washed cattle RBC's replaced in their own plasma then incubated with *pomona* showed no signs of haemolysis. Leptospires were present within the supernatant of the incubated cultures.

3.3. EXPERIMENT C

The infected cattle showed no signs of infection. No alterations in PCV, WBC and AAT occurred. The rise in MAT's

Table 7-2 Results of Experiment C: Percentages of washed and unwashed RBC's haemolysed by two strains of *pomona* up to 14 days post infection

Animal	*pomona* strain	Day 0 T116	Day 0 790001	Day 1 T116	Day 1 790001	Day 5 T116	Day 5 790001	Day 7 T116	Day 7 790001	Day 11 T116	Day 11 790001	Day 16 T116	Day 16 790001
C1	washed	88%	80%	93%	90%	90%	93%	96%	86%	98%	83%	98%	94%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	MAT	-		-		-		-		-		-	
C2	washed	97%	65%	90%	76%	98%	81%	100%	81%	100%	79%	99%	85%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	MAT	-		-		-		-		-		-	
I1	washed	97%	52%	99%	67%	99%	86%	98%	87%	94%	77%	98%	86%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	MAT	-		-		1:24		1:96		1:768		1:384	
I19	washed	93%	72%	99%	83%	93%	89%	99%	87%	100%	89%	99%	tube broken
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	MAT	-		-		tr		1:192		1:384		1:192	
I23	washed	91%	72%	94%	83%	93%	97%	87%	87%	100%	87%	94%	93%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	MAT	-		-		-		1:48		1:192		1:48	
I26	washed	93%	53%	75%	78%	89%	94%	82%	92%	94%	84%	87%	91%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	MAT	-		-		-		1:192		1:384		1:384	
I39	washed	90%	64%	95%	74%	95%	83%	92%	85%	98%	tube broken	96%	91%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	MAT	-		-		-		1:192		1:178		1:384	
I41	washed	93%	76%	80%	tube broken	93%	88%	99%	83%	100%	86%	100%	90%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	MAT	-		-		-		1:96		1:768		1:192	
Culture density		Dense	Dense	Dense	Dense	Dense	Dense	Dense	Dense	Dense	Dense	Dense	Dense

are tabulated in Table 7-2. There was no alteration in the degree of haemolysis of washed RBC's (Table 7-2). No unwashed RBC's were haemolysed but the numbers of organisms in the supernatant of incubated cultures of unwashed RBC's and *pomona* diminished when MAT's were detected. The numbers of organisms did not diminish in the control animals.

No fluorescent antibody was detected on the surface of the RBC's.

All infected cattle were of haemoglobin type A. One control animal had haemoglobin type A and the other haemoglobin type B.

3.4 EXPERIMENT D

No alteration in morphology was seen in the RBC's of calves 18, 19 and 20 as controls or when incubated with the leptospiraemic plasma of calves 11 and 16 apart from similar artefactual changes to those described in Section 3.1.3. Likewise, no significant changes were seen in RBC's from calves 11 and 16.

4.0 DISCUSSION

Results from Experiments A, B and C consistently showed that unwashed RBC's from cattle, hamsters and humans cultured in tubes with *pomona* were never haemolysed while washed RBC's from these species cultured with *pomona* were always haemolysed to a varying extent. The absence of haemolysis of the unwashed RBC's occurred despite the presence of large numbers of leptospires and therefore presumably 'toxin' within the supernatant. The organisms within the plasma were active and motile and the numbers decreased only if homologous antibody was present. This was well demonstrated by calf 69 in Experiment A in which *pomona* organisms were always diminished in numbers when compared with other calves of Experiment A and in those *pomona* infected cattle following the development of MAT's. *Hardjo* and *ballum* numbers were also decreased in the presence of homologous antibody.

There is no reason to suspect that leptospires cultured with plasma and unwashed RBC's do not produce 'toxins' capable of haemolysing RBC's. It would also seem likely that plasma with a greater availability of nutrients would in fact be a better culture medium than saline for leptospiral multiplication. It therefore appears that plasma does have a protective function against RBC haemolysis as has been described by Kemenes, (1974). Kemenes (1974) found that normal calf serum was less protective against haemolysis than normal adult cattle serum which in turn was less protective than serum containing protective antibody.

The present work also demonstrated the protective function of homologous antibody in that the plasma which contained homologous antibody against *pomona, ballum* and *hardjo* had diminished numbers of organisms of the particular serovar when compared to incubated cultures in which homologous antibody was absent. That other serovars were not affected by the antibody adds weight to the observation that cross-protection between organisms from a serovar and antibody to a different serovar does not occur (Alexander *et al*, 1971). Other workers, while showing that non-immune serum will protect against saprophytic leptospires (Johnson & Muschel, 1965; 1966) due to the presence of a β macroglobulin (Faine & Carter, 1968) have also demonstrated that specific antibody is necessary to impair the growth of parasitic leptospires (Johnson & Harris, 1967; Johnson & Muschel, 1966).

The fact that animals do develop haemoglobinaemia and haemoglobinuria as discussed in Chapters 3, 4 and 5 appears to conflict with the *in vitro* findings that plasma has a protective function as demonstrated by the lack of haemolysis of unwashed RBC's. While adult cattle are known to be more resistent to *pomona* infections, it seems unlikely from results of previous experiments (Chapter 5) that had all 11 newborn calves been infected with *pomona* that none would have become haemoglobinaemic as indicated by the lack of haemolysis of any unwashed RBC's *in vitro*. The present results do not show any alteration in resistance of

RBC's to haemolysis as the calves age and it therefore seems unlikely that foetal haemoglobin is a factor. No calves developed haemoglobin B and no infected cattle had haemoglobin type B so a comparison of adult haemoglobin types was not possible.

The protective function of plasma may also be illustrated by the absence of lesions in the RBC's from calves 18, 19 and 20 which were incubated with leptospiraemic plasma. However, small numbers of animals were used in this experiment and it should be repeated using larger numbers of animals and comparing plasma from calves which become haemoglobinaemic with plasma from calves which do not become haemoglobinaemic. It may therefore be hypothesized that an *in vivo* mechanism within the animal may in fact be responsible for the RBC destruction. To a certain extent, the nature of the spontaneous disease was reflected by the *in vitro* haemolysis. *Pomona* may produce haemoglobinaemia in cattle but not in humans and hamsters and the *in vitro* tube haemolysis showed a greater degree of haemolysis with cattle RBC's than those of humans and hamsters. Likewise *ballum* infections of hamsters but not humans may produce haemoglobinaemia and the *in vitro* tube haemolytic test showed a greater degree of haemolysis with hamsters rather than humans.

The difference in susceptibility of washed and unwashed RBC's to *pomona, ballum* and *balcanica* organisms may be explained in part by the fact that *in vitro* test tube haemolysis can never truly reflect the *in vivo* situation. Plasma provides such factors as a nonspecific buffering system against environmental changes such as pH alterations which may be brought about merely by metabolism of RBC's and possibly the metabolism of leptospires. Such plasma factors may in some way neutralize the leptospiral 'toxins' up to a certain point above which haemolysis occurs.

The lesions seen in RBC's from haemoglobinaemic animals were similar to those seen in the *in vitro* experiments although in the latter case some artefactual changes resulted from washing and standing at room temperatures.

As stated by Stalheim (1971) the term'virulence factor' for parasitic leptospires should be replaced by the term 'survival factor' as it was shown that all the serovars examined had the ability to survive in plasma although numbers were diminished in the presence of homologous antibody.

The results of these experiments do not always correspond to previously reported work. Hathaway (1978) and Hathaway & Marshall (1980) used *pomona* as a non-haemolytic control and they do not mention the consistent differences in haemolysis between washed and unwashed RBC's in the tube test. Their work concentrates more on the plate haemolytic test which cannot be considered an accurate representation of *in vivo* haemolysis. The results obtained by examination of the blood agar plates were different from that in tube cultures in that both washed and unwashed RBC's were 'haemolysed'. While tube haemolysis consisted of the release of haemoglobin into the supernatant resulting in red discolouration, 'haemolysis' of blood agar plates resulted in the formation of clear zones immediately around the leptospiral colonies (Hathaway, 1978; Hathaway & Marshall, 1980; Stamm & Charon, 1979). It would appear that haemoglobin is not only released into the environment in the blood agar plates but is also digested or degraded resulting in the clear zone.

The RBC's trapped in the solid agar of plates are in an abnormal environment which may be a factor in making even unwashed RBC's susceptible to haemolysis. In addition the RBC's may be damaged in the making of the plates where they are mixed with hot agar which also may make them more susceptible to leptospiral 'toxins'. The value of the plate haemolytic test is therefore as a test for differentiation of certain serovars such as *balcanica* and *hardjo* (Hathaway, 1978; Hathaway & Marshall, 1980) rather than to demonstrate mechanisms of pathogenesis.

The cause of the absence of 'haemolysis' or clear zones on the plates prepared with deliberately lysed RBC's is not known

but is possibly due to the fact that once out of the RBC without enzyme systems to maintain its normal configuration, substances such as methaemoglobin are formed which may inhibit leptospiral growth.

The results of Experiment C do not support the observation of Ringen (1966) who claimed that there was an increased resistance of RBC's to *pomona* 'toxin' for a short time following infection of cattle and goats by *pomona* which did not produce clinical signs of infection. His work did not include non-infected controls and some animals showed considerable variation in readings on days early in the infective period before the time during which he claimed that increased resistance occurred.

5.0 CONCLUSIONS AND SUMMARY

1. An unknown plasma factor prevented haemolysis of unwashed RBC's during incubation of cattle, human and hamster RBC's with *pomona* and *ballum* serovars.

2. This absence of haemolysis of unwashed RBC's occurred despite the presence of large numbers of leptospires. The presence of specific antibody decreased the numbers of leptospires visible by dfm.

3. The absence of haemolysis in unwashed calf RBC's despite the presence of large numbers of leptospires in the supernatant suggested that an *in vivo* mechanism may be responsible for the haemolysis.

4. Washed RBC's were haemolysed to varying extents with *pomona* being most haemolytic for cattle RBC's and *ballum* being most haemolytic for hamster RBC's. This followed the natural diseases in which *pomona* infections of cattle and *ballum* infections of hamsters may result in haemoglobinaemia.

5. Washed cattle RBC's incubated with *pomona* showed pitting similar to that of haemoglobinaemic animals.

6. Alteration in the foetal haemoglobin content of calf RBC's did not appear to affect the degree of haemolysis in calf RBC's incubated with *pomona*.

7. Antibody was not detected upon the surface of washed RBC's from *pomona* infected cattle.

8. That no cross protection occurred *in vitro* between unrelated leptospiral serovars and antibody was shown by the fact that numbers of organisms in incubated plasma remained undiminished.

9. 'Haemolysis' occurred in plates containing both washed and unwashed RBC's. The plate haemolysis appears to result from a different process to that in the test tube incubation experiments.

CHAPTER 8

INTRAVASCULAR HAEMOLYSIS IN GAMMA-IRRADIATED, *BALLUM* INFECTED HAMSTERS

1.0 INTRODUCTION

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2.2 EXPERIMENT VIII: PART II

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3.1.3 Gross pathology

3.1.4 Histopathology

3.1.4.1 Liver
3.1.4.2 Spleen
3.1.4.3 Kidney
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3.2 PART II

3.2.1 Clinical signs

3.2.2 Clinical pathology

3.2.2.1 Haematology

3.2.2.2 Bone marrow differentials and cytology

a) Irradiated controls
b) Infected hamsters
c) Irradiated-infected hamsters

3.2.2.3 RBC ultrastructural morphology

a) Irradiated controls
b) Infected hamsters
c) Irradiated-infected hamsters

3.2.2.4 Gross pathology

a) Irradiated controls
b) Infected hamsters
c) Irradiated-infected hamsters

3.2.2.5 Histopathology

3.2.2.5.1 *Liver*

a) Irradiated controls
b) Infected hamsters
c) Irradiated-infected hamsters

3.2.2.5.2 *Spleen*

a) Irradiated controls
b) Infected hamsters
c) Irradiated-infected hamsters

3.2.2.5.3 *Kidney*

a) Irradiated controls
b) Infected hamsters
c) Irradiated-infected hamsters

3.2.2.5.4 *Bone marrow*

a) Irradiated controls
b) Infected hamsters
c) Irradiated-infected hamsters

3.2.2.5.5 *Lung*
a) Irradiated controls
b) Infected and irradiated-infected hamsters
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a) Irradiated controls
b) Infected and irradiated-infected hamsters

4.0 DISCUSSION

5.0 CONCLUSIONS AND SUMMARY

CHAPTER 8

INTRAVASCULAR HAEMOLYSIS IN GAMMA-IRRADIATED, *BALLUM* INFECTED HAMSTERS

1.0 INTRODUCTION

Studies on the ultrastructure of RBC's in blood (Chapter 4) and other organs (Chapter 5) from hamsters infected with *ballum* showed that partially haemoglobinated RBC's were rarely seen in blood vessels, suggesting that either they were rapidly removed from circulation or that haemoglobin loss rarely occurred in the general circulation. If the latter was the case, damaged RBC's must have been removed from circulation possibly by detention in the spleen prior to haemoglobin loss and free haemoglobin released back into circulation as previously reported by Jandl *et al* (1957).

Irradiation of guinea pigs infected with *icterohaemorrhagiae* resulted in the animals dying at the same time as non-irradiated infected animals with none of the irradiated animals showing the characteristic jaundice (Higgins & Cousineau, 1977b). The absence of jaundice was considered to result from an absence of phagocytosis of extravasated RBC's. It was suggested that irradiation impaired the function of the reticulomacrophage system and the jaundice was entirely caused by RBC haemolysis rather than liver impairment.

To test whether a similar function of the reticulomacrophage system was in operation in the present studies which had resulted in some animals becoming haemoglobinaemic, it was decided to impair the reticulomacrophage system of *ballum* infected hamsters in order to determine the effects on the degree of haemoglobinaemia and jaundice.

In view of the results of Higgins & Cousineau (1977b), it was decided that irradiation was the preferable method of impairing the reticulomacrophage system, because although carrageenin and silica destroy macrophages *in vitro* (Allison *et al*, 1966; Catanzaro *et al*, 1971) injections of these substances *in vivo* caused liver necrosis and fibrosis (Paronetto *et al*, 1962). As *ballum* also affects the liver, it was thought that these substances may complicate the pathogenesis of the disease.

The degree of depletion of haemopoietic and lymphoid cells from the marrow and spleen was considered to be the best indication of the degree of impairment of the reticulomacrophage system because the haemopoietic and lymphoid systems are the most susceptible of body systems to radiation, due to their high rate of mitosis (Bond *et al*, 1965; Eddy & Casarett, 1972).

Tissue macrophages are derived from blood monocytes released from the bone marrow and are relatively long lived (Benjamin, 1979; Bond *et al*, 1965; Schalm *et al*, 1975). They therefore are less likely to be affected by irradiation although there is work in mice (Bond *et al*, 1965) which suggests that phagocytic function is impaired. However, irradiation would inhibit an increase in the numbers of fixed macrophages and stop the replacement of those which were dying, and would inhibit the production of other phagocytic cells such as PMN's. RBC's are relatively long lived when compared to granulocytes and lymphocytes and anaemia would not become obvious for some time (Bond *et al*, 1965; Eddy & Casarett, 1972) and should therefore not interfere with the pathogenesis of the infection.

Experiment VIII described in this chapter is divided into two parts. Part I of the experiment was conducted to determine the most suitable level of irradiation on the hamsters for the purpose outlined above. Three different levels were used to determine the level at which the haemopoietic and lymphoid systems were most impaired while producing the least damage to other organs such as

liver, kidney and lungs. It was considered desirable to minimise the amount of radiation because of the possibility of producing thrombocytopaenia and vascular damage leading to fluid leakage and haemorrhage (Eddy & Casarett, 1972). Part 2 of the experiment examined the combined effects of *ballum* strain M4/9 infection of hamsters with the level of irradiation judged most satisfactory from the results of Part I.

2.0 MATERIALS AND METHODS

The hamsters used in the following experiments were obtained and maintained as described in Chapter 2, Section 2.1.

2.1 EXPERIMENT VIII: PART I

Twenty-six 19-42 day old hamsters were divided into four groups and were subjected to a single dose of γ radiation from a cobalt source[1] as follows:

i) 6 untreated controls
ii) 6 hamsters exposed to 600 RADS
iii) 6 hamsters exposed to 800 RADS
iv) 6 hamsters exposed to 1000 RADS

Half of the animals from each group were euthanased three days post-irradiation and the remainder euthanased seven days post-irradiation. Euthanasia and the collection of blood and bone marrow was performed in the manner described in Chapter 2, Section 3.1 and the haematological examinations done as described in Chapter 3, Sections 2.3.1.1 to 2.3.1.8. Reticulocyte examinations were not carried out.

Liver, spleen, kidney, bone marrow and lung were collected and prepared for histological examination as described in Chapter 5, Section 2.3.2.

1. Theratron 80: Atomic Energy of Canada, Ltd., Ottawa, Canada

2.2 EXPERIMENT VIII: PART II

2.2.1 Leptospires

Ballum strain M4/9 was obtained, typed and cultured as described in Chapter 2, Section 1.0 and Table 2-1.

2.2.2 Hamsters

Fifty-two 19-26 day old hamsters of mixed sexes were divided into the following four treatment groups of hamsters designated as shown in brackets:

i) 12 untreated controls (C 1 - C 12)
ii) 12 irradiated controls (Irr 13 - Irr 24)
iii) 14 infected animals (Inf 25 - Inf 38)
iv) 14 irradiated and infected animals (II 39 - II 52)

All irradiated animals were given 800 RADS as a single exposure of radiation from a cobalt source[1]. Groups (iii) and (iv) were infected with *ballum* as described in Chapter 2, Section 2.1 within 90 minutes of irradiation of groups (ii) and (iv). During irradiation, the hamsters were confined in an aluminium cage 28 cm x 28 cm with individual cubicles of 14 cm x 6.5 cm for each animal to ensure that each obtained a similar dose of radiation.

2.2.3 Experimental procedures

Following infection, the hamsters were examined three times daily at 8.00 am, 12 noon and 4.30 pm. Hamsters were euthanased following the methods described in Chapter 2, Section 3.1 and blood and bone marrow collected. Days of death are detailed in Appendix XXXIII. Control animals were euthanased when infected animals showed clinical signs or died, and on the day on which the last of the respective infective groups were found dead or moribund.

Blood was collected as described in Chapter 2, Section 3.1 from all euthanased animals. The PCV and icterus index were estimated as described in Chapter 3, Sections 2.3.1.2 and 2.3.1.6

respectively. Blood smears were made for cytological examination of cells as described in Chapter 3, Section 2.3.1.3 and plasma visually examined for the presence of free haemoglobin. Bone marrow smears were made and differential counts performed as described in Chapter 3, Section 2.3.1.8.

Liver, spleen, kidney, bone marrow, lung and cardiac muscle were collected from all animals for histological preparation as described in Chapter 5, Section 2.3.2.

RBC's were collected from the hamsters C 2, 4, 6, 7; Irr 14, 16, 18, 23, 27, 28; Inf 27, 28, 29, 30, 36, 37 and II 41, 45, 46, 47, 52 and prepared for SEM examination as described in Chapter 4, Sections 2.2 and 2.3.

3.0 RESULTS

3.1 PART I

3.1.1 Clinical signs

No abnormal clinical signs were seen in any irradiated hamsters.

3.1.2 Clinical pathology

Haematological and bone marrow differential data are tabulated in Appendix XXXII. Differential counts were not performed on blood smears because of the very low WBC counts in the irradiated hamsters. The majority of WBC's seen in irradiated hamsters were hypersegmented neutrophils.

RBC's were normal in morphology apart from some crenation, and the presence of the occasional elliptocyte and target cell. There was a decrease in the PCV and haemoglobin levels by the seventh day. The MCHC, icterus index and total protein remained normal.

Although the optimum number of cells to be counted for bone marrow differentials is considered to be at least 500

(Schalm *et al*, 1975), the hypocellularity of the marrow from irradiated hamsters with the exception of those exposed to 600 RADS and euthanased on days three and seven did not permit differentiation of this number. The number of cells differentiated on marrow from those hamsters exposed to 800 RADS was between 300-500 with hamster 8 having so few cells on day seven that no differential was performed. On day three, 100-200 cells were differentiated on marrow from those hamsters exposed to 1000 RADS. Only hamster 11 had sufficient intact and recognisable cells for differentiation on day seven. Marrow cells from hamsters 10, 12 and 13 were mostly comprised of reticulum cells, bare nuclei of unknown origin and degenerate cells of unknown origin. There was the occasional, usually hypersegmented PMN, and late normoblast. Cellular fragments were common.

In general, in hamsters euthanased three days post-irradiation, erythropoiesis was inhibited to a greater degree than granulopoiesis as demonstrated by increased M:E ratios. There was a complete absence of mitotic figures in all irradiated marrows (Appendix XXXII).

3.1.3 Gross pathology

On autopsy the carcases had an overall pale appearance and spleens were pale and much thinner than normal. The femur marrow, particularly on day seven, had a 'watery' consistency and appeared to be mostly blood.

3.1.4 Histopathology

3.1.4.1 Liver

After three days the livers showed a slight loss of the normal foamy appearance of the hepatocyte cytoplasm, a variable decrease in the amount of glycogen and accumulation of pale staining haemosiderin within hepatocytes, with dilatation of the sinusoids. There was some variation between hamsters

exposed to the same radiation levels but in general those exposed to 600 RADS were least affected and those exposed to 1000 RADS were most severely affected.

Severe liver changes were seen after seven days in those hamsters exposed to 1000 RADS. The hepatocytes had lost most of the foamy appearance to the cytoplasm and there was very little glycogen present with the exception of those hepatocytes around the central vein in which larger amounts were retained. Sinusoids were widely dilated. Randomly distributed areas of focal necrosis were most pronounced in hamster 12, but were present in all hamsters. Increased amounts of dark staining haemosiderin were seen within hepatocytes.

The liver changes were less severe in those hamsters exposed to 600 and 800 RADS seven days post-irradiation when compared to the 1000 RAD group. Loss of the normal foamy appearance of the cytoplasm of hepatocytes was observed, and also a decrease in glycogen. There was an increase in dark staining granules of haemosiderin within the hepatocytes of portal areas.

The livers in control hamsters showed no abnormalities.

3.1.4.2 Spleen

Lymphocyte depletion of the lymphoid follicles at three days post-irradiation was most severe in those animals exposed to 1000 RADS and least in those exposed to 600 RADS. The small quantities of dark staining haemosiderin seen in macrophages associated with the depleted lymphoid follicles were similar to the quantities of haemosiderin seen in the lymphoid follicles of the controls.

After seven days there was a slight increase in the number of lymphoblasts in the lymphoid follicles of those animals exposed to 600 RADS. Lymphoid follicles of those animals exposed to higher doses of radiation remained depleted. In all

animals the quantities of haemosiderin were similar to that at three days.

The spleens of non-irradiated hamsters showed no lesions.

3.1.4.3 Kidney

No significant lesions were observed in any kidneys.

3.1.4.4 Bone marrow

All marrows were hypocellular three days post irradiation due to loss of nucleated cells. Those hamsters exposed to 1000 RADS showed the most severe changes with the marrow containing predominantly endothelial cells, reticulum cells, and blood vessels and sinusoids filled with RBC's. There was the occasional hypersegmented PMN, late normoblast and megakaryocyte. Active haemopoiesis in the form of groups of undifferentiated blast cells and immature erythroid and granulocytic cells was seen only in those hamsters exposed to 600 RADS. Hypersegmented PMN's were frequently seen.

After seven days, marrow from the hamsters exposed to 1000 RADS was almost acellular with no signs of regenerating nucleated cells. Marrow from those hamsters exposed to 800 RADS was less cellular than at three days but the occasional group of large undifferentiated nucleated cells was seen. Those animals exposed to 600 RADS had a more cellular marrow than either of the other groups.

No haemosiderin was seen in marrow of any irradiated hamster.

Bone marrow in control hamsters was normal.

3.1.4.5 Lungs

Some areas of alveolar collapse, vesicular emphysema, and intra-alveolar RBC's resulting from inhalation of blood following severance of blood vessels were seen.

3.2 PART II

3.2.1 <u>Clinical signs</u>

Irradiated control hamsters euthanased five days post-

irradiation showed no abnormal clinical signs. Irradiated control hamsters euthanased 11 days post-irradiation showed varying degrees of emaciation despite what appeared to be normal appetites. Hamster Irr 19 was the most severely affected and had a bilateral conjunctivitus with a yellow discharge.

The *ballum* infected and the irradiated *ballum* infected hamsters showed clinical signs of illness similar to those described for hamsters infected with *ballum* strain 1045 in Chapter 3, Section 3.1.2. The clinical signs of the irradiated and infected hamsters were noted earlier and persisted for longer before the animals became moribund than those of the infected only hamsters. Deaths also occurred earlier in the infected-irradiated group. Only one hamster (Inf 38) survived the infection with no clinical signs and was euthanased on day 14.

3.2.2 Clinical pathology

3.2.2.1 Haematology

The PCV, icterus index, and present or absence of haemoglobinaemia for all animals are tabulated in Appendix XXXIII.

Irradiated control animals euthanased 11 days post-irradiation had PCV levels lower than the untreated control animals. The plasma of irradiated hamsters remained normal. No haemoglobinaemia was observed.

Infected animals showing clinical signs had decreased PCV levels, those animals with the lowest PCV's having the higher icterus index levels and a more severe haemoglobinaemia. The irradiated-infected hamsters had a greater decrease in the PCV than the infected, non-irradiated hamsters before an increased icterus index and haemoglobinaemia became obvious (Appendix XXXIII). Thus hamsters Inf 29 and Inf 33 had PCV's of 0.19 and 0.23 respectively which were accompanied by a raised icterus index and severe haemoglobinaemia while hamsters II 45 and II 46 which had the respective PCV's of 0.19 and 0.23 had

only a slightly raised icterus index with slight haemoglobinaemia and a normal plasma respectively. Hamster Inf 38 which survived the infection has a normal PCV and plasma.

The RBC cytology of the irradiated controls was normal except for the presence of slight crenation which was also seen in the untreated controls, and some elliptocytes and target cells which were not seen in the untreated controls. Very few WBC's were seen in the blood smears from irradiated hamsters, but when present were usually hypersegmented PMN's. Hamsters Irr 19 to Irr 24 had some band cells and the total numbers of WBC's were increased in these six animals.

RBC and WBC morphology of the infected hamsters was similar to that described in Chapter 3, Sections 3.2.1.2 and 3.2.2.2 respectively. Cells from hamster Inf 38 were normal.

RBC morphology of the irradiated-infected hamsters showed anisocytosis, poikilocytosis and spherocytosis similar to that in the infected hamsters. PMN's were rare and those found in the smears of hamsters dying five to six days after infection were hypersegmented and showed 'toxic' changes. Band cells were more frequently seen in hamsters 7 to 11 days post infection. The total WBC count, as judged by smear examination, appeared larger in those dying at this later date after infection and irradiation.

3.2.2.2 Bone marrow differentials and cytology

Bone marrow cell differential data are presented in Appendix XXXIV. Differentials were not performed on those animals which were found dead. In some irradiated and irradiated-infected hamsters, the marrow was too hypocellular to allow accurate differential counts to be made. In these cases only the cell cytology was described.

a) Irradiated controls

Marrow from the irradiated control hamsters Irr 13 to Irr 18 was hypocellular and contained mostly blood, reticulum

cells and bare nuclei of unknown origin. There was the occasional hypersegmented PMN and late normoblast.

Marrow from irradiated control hamsters Irr 19 to Irr 24 showed increased haemopoiesis. There was asynchrony in development between the nucleus and cytoplasm of granulocyte precursors. This was shown by a more extensive and basophilic cytoplasm than normal, along with the persistence of azurophilic granules, clumped nuclear protein and persistent nucleoli. Some binucleated PMN's were seen, and some PMN's had fragmented nuclei. Some granulocytic cells appeared to become degenerate while undergoing mitosis. Phagocytosis of cells of the granulocytic series was occasionally seen.

Erythroid cells also showed developmental abnormalities in the form of binucleated early normoblasts and normoblasts, with some normoblasts containing abnormally shaped nuclei. Occasional erythroid precursors also appeared to degenerate while undergoing mitosis. Unclassified blast cells with intensely blue cytoplasm, large nuclei and several nucleoli were also seen.

There was a wide variation in the M:E ratios of irradiated hamsters with much variation in the number of mitoses, the proportions of cell types within a cell line and the number of disintegrating cells (Appendix XXXIV).

b) Infected hamsters

There was wide variation in the M:E ratio (Appendix XXXIV) which ranged from 11.2:1 in hamster Inf 36 to 0.4:1 in hamster Inf 27. Cell cytology was similar to that described for *ballum* 1045 infections described in Chapter 3, Section 3.2.3.2. Late normoblasts with abnormally shaped nuclei, and phagocytosis of cellular debris were frequently seen. Marrow from hamster Inf 36 was morphologically normal.

c) Irradiated-infected hamsters

No differentials were performed on hamsters in this group due to the large numbers of bare nuclei and disintegrating cells of unknown origin.

Hamsters which became moribund soon after infection had an almost acellular marrow. The occasional late normoblast and degenerate PMN was seen. Reticulum cells and bare nuclei were seen.

Hamsters II 41 and II 48 showed erythroid and granulocyte precursors indicative of haemopoiesis. PMN's often had fragmented nuclei and were often seen to degenerate under mitosis. The presence of many granulopoietic cells at all stages of development was noted in hamster II 47. Asynchrony of development between the nucleus and cytoplasm was seen. The granulocyte precursors were larger than normal.

Marrow from hamsters II 50 and II 52 was the most cellular and contained many immature erythroid and granulocytic cells, bare nuclei and macrophages containing cell debris. Larger numbers of megakaryocytes were also seen in these hamsters than in animals which died earlier. Large monocyte-like cells were frequently seen.

3.2.2.3 RBC Ultrastructural morphology

RBC's from untreated controls (Fig. 8-1) were morphologically similar to those described in Chapter 4, Section 3.1.1.

a) Irradiated controls

Echinocytes were frequently seen in irradiated controls (Fig. 8-2) and were similar in appearance to those in early *ballum* 1045 infections of hamsters and after *pomona* 'toxin' injections of calves as described in Chapter 4, Section 3.1.2.

b) Infected hamsters

RBC's were examined from hamster Inf 36 which had normal plasma and hamsters Inf 27, Inf 28, Inf 29, Inf 31 and Inf 37 which were haemoglobinaemic. RBC's from hamster Inf 36 were echinocytic while those from the other hamsters were pitted and spherical as described in Chapter 4, Section 3.1.2.2. In addition, some RBC's appeared to have small spherical cellular fragments attached to the surface of the membrane similar to those in hamsters which were both infected and irradiated (Fig. 8-3).

Fig. 8-1

RBC's from a control hamster demonstrating the normal biconcave discoid shape with the occasional surface irregularity.

SEM 4,000x

Fig 8-2

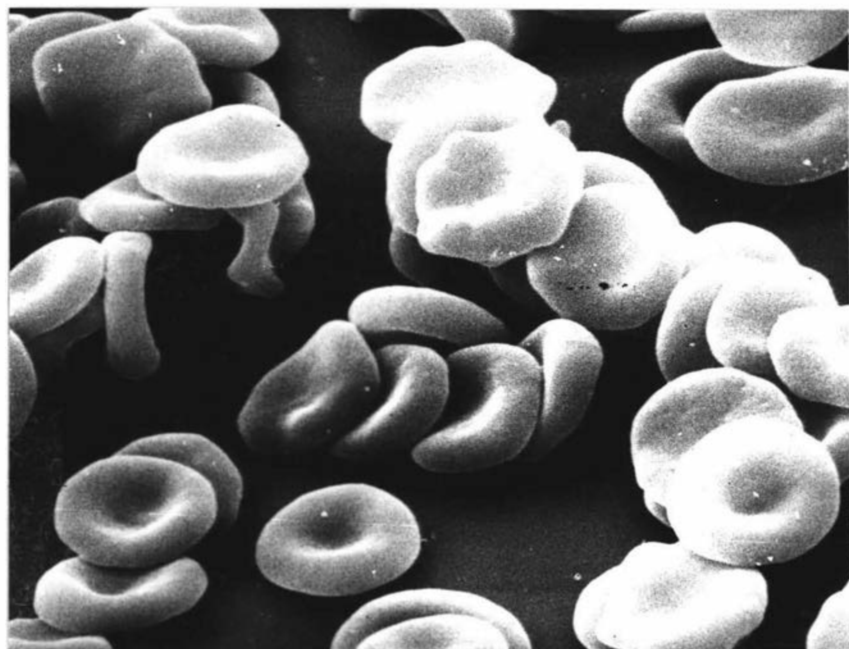
RBC's from an irradiated hamster five days post-irradiation demonstrating echinocytes (E) and the occasional discocyte (D).

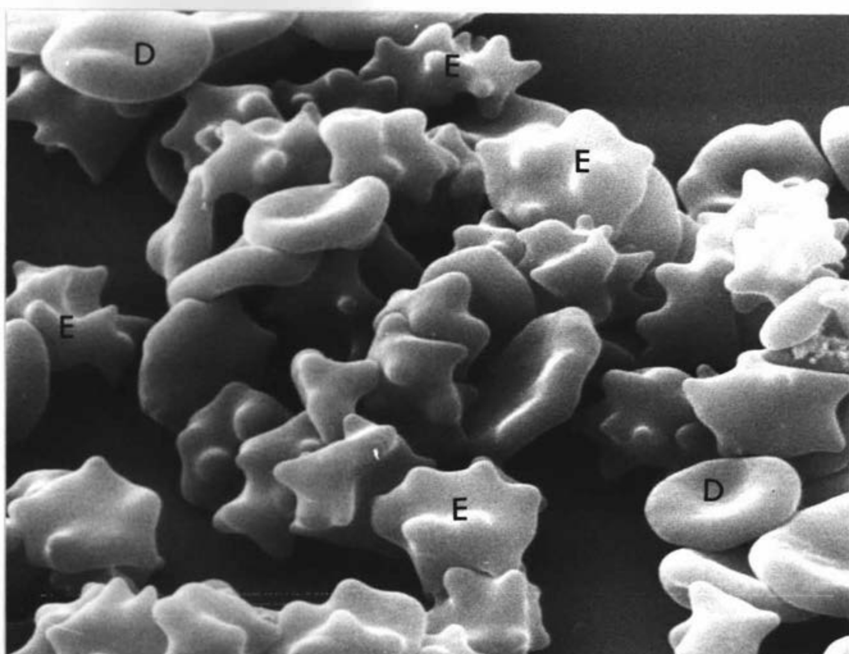
SEM 4,000x

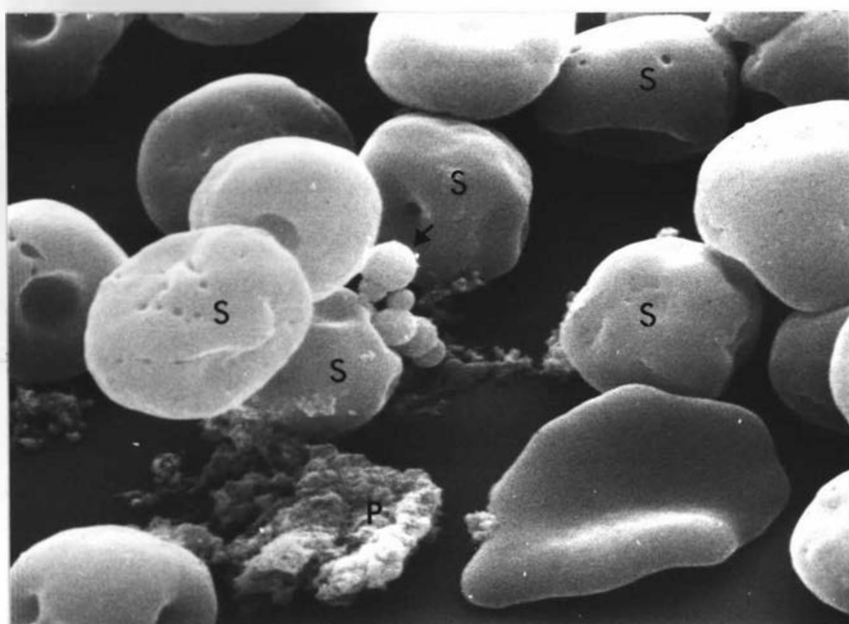
Fig 8-3

RBC's from a haemoglobinaemic hamster euthanased six days following irradiation and infection with *ballum*. Pitted spherocytes (S), microspherule (arrow) formation and platelet debris (P) are present.

SEM 8,000x




D
E
E
E
E
D


S
S
S
S
S
P

c) Irradiated-infected hamsters

RBC's from the anaemic, non-haemoglobinaemic hamster II 41 were echinocytic, similar to those in Fig. 8-2 and described in Chapter 4, Section 3.1.2. RBC's from the anaemic, non-haemoglobinaemic hamster II 46 which had normal plasma and the anaemic hamster II 45 which had an icterus index of 7.5 and slight haemoglobinaemia were spherical and pitted as were RBC's from the anaemic, severely haemoglobinaemic, jaundiced hamsters II 47 and II 52. The severely haemoglobinaemic hamsters often had small spherical cellular fragments attached to the surface of the membrane (Fig. 8-3).

3.2.2.4 Gross pathology

a) Irradiated controls

The gross autopsy findings were similar to those described in Section 3.1.3. In addition those euthanased at 11 days post-irradiation were emaciated. The gross pathological changes are summarized in Appendix XXXIII.

b) Infected hamsters

The pathological features are similar to those described in Chapter 5, Section 3.1.2.2, and summarized in Appendix XXXIII.

c) Irradiated-infected hamsters

The major gross pathological features are summarized in Appendix XXXIII. In general, the carcases were less jaundiced than those of the infected group and the spleens, although red, congested and friable were very much smaller than those from infected animals. Urine was often dark red in the absence of haemoglobinaemia. The kidneys were sometimes pale and enlarged with red urine within the pelvis. Hamster II 46 had a blood clot distending the bladder four to five times its normal size. Hamsters II 49, II 51 and II 52 contained blood in the large intestine, and the body cavities contained larger amounts of blood stained fluid.

3.2.2.5 Histopathology

The histology of the livers, spleens, kidneys, marrow, lung and cardiac muscle of untreated controls was similar to

that described in Chapter 5, Sections 3.3.1.1, 3.3.2.1, 3.3.3.1, 3.3.4.1, 3.3.7 and 3.3.10 respectively.

3.2.2.5.1 *Liver*

a) Irradiated controls

In hamsters Irr 13 to Irr 18, changes were similar to those recorded in hamsters euthanased seven days post-irradiation (Section 3.1.4.1).

Hamsters Irr 19 to Irr 24 had changes similar to those in irradiated controls euthanased earlier. In addition there was extramedullary haemopoiesis and the infiltration of a small number of macrophages and PMN's within the sinusoids. Haemosiderin deposition was more marked in these hamsters than those euthanased five days post-irradiation.

b) Infected hamsters

The histological findings were similar to those described from *ballum* infections in Chapter 5, Section 3.3.1.2.

Hamster Inf 38 showed only minor lesions consisting of a slight loss of the normal foamy cytoplasmic appearance to the hepatocytes and the accumulation of small numbers of mononuclear cells with a portal distribution. No leptospires and little haemosiderin was seen.

c) Irradiated-infected hamsters

There was loss of the normal foamy appearance to the hepatocyte cytoplasm accompanied by cytoplasmic vacuolation. Dilatation of the sinusoids occurred particularly around the central veins. Degeneration of cells was more obvious around the central veins although coagulative necrosis was not as extensive as in the infected hamsters. Mononuclear cell infiltrations were very small or absent. Erythrophagocytosis was frequently seen with some macrophages containing large numbers of RBC's or large quantities of a pink proteinaceous material considered to be haemoglobin. Small round intensely staining RBC's (spherocytes) were seen within the sinusoids and blood vessels.

Pale staining and some dark granular haemosiderin was seen within hepatocytes, Kupffer cells and some macrophages. The quantities of haemosiderin were larger in those hamsters which survived for longer post irradiation and infection.

Leptospires were seen in larger numbers than in the infected non-irradiated hamsters and were located in the sinusoids and between the hepatocytes.

Hamster II 52, moribund and euthanased 11 days post-infection had more coagulative necrosis and PMN infiltration than those which died earlier. Mononuclear cell infiltration of portal areas was also more obvious. Spherocytes were observed within blood vessels and sinusoids. Glycogen was completely absent from the hepatocytes. Both the pale staining and dark granular haemosiderin were more frequently seen in the hepatocytes of this hamster when compared to those hamsters dying earlier following infection. Many leptospires were also seen.

3.2.2.5.2 *Spleen*

a) Irradiated controls

Histological findings of hamsters Irr 13 to Irr 18 euthanased five days post-irradiation were similar to those of the hamsters exposed to 800 RADS in Part I, Section 3.1.4.2. Occasional foci of cellular necrosis were randomly distributed through the red pulp. This was most obvious in Irr 17. These hamsters had larger accumulations of haemosiderin within macrophages and reticular cells of the red pulp and dark staining granules within macrophages and reticular cells of the depleted lymphoid follicles than the control hamsters.

Hamsters Irr 19 to Irr 24 euthanased 11 days post-irradiation showed evidence of lymphoid regeneration by increased numbers of lymphoblasts and some mature lymphocytes. Extramedullary haemopoiesis was evident by the presence of groups of undifferentiated blast cells, granulocytic and erythroid precursors and megakaryocytes within and between the reticular cords of the red

pulp, immediately below the fibrous outer capsule of the spleen and along the fibrous trabeculae (Fig. 8-4). Large amounts of dark granular haemosiderin were seen within macrophages in the lymphoid follicles and large amounts of pale, diffuse haemosiderin within macrophages and reticular cells of the red pulp.

b) Infected animals

Histological lesions were similar to those described in Chapter 5, Section 3.3.2.2.

Hamster Inf 38 showed no signs of splenic congestion and erythrophagocytosis was normal. There was proliferation of lymphocytes with cells extending beyond the marginal zone of the lymphoid follicle. PMN's were seen in the reticular cords of the red pulp. Haemosiderin quantities and distribution was normal. No leptospires were seen.

c) Irradiated-infected animals

Spleens from those animals found dead showed congestion of the sinusoids and spaces between the reticular cords by large numbers of spherocytes. There was much evidence of erythrophagocytosis with macrophages containing either large numbers of RBC's or a homogeneous pink proteinaceous material considered to be haemoglobin. Lymphoid depletion of the lymphoid follicles was greater than in the infected hamsters and larger quantities of pale diffuse and dark granular haemosiderin were seen within macrophages and reticular cells.

Spleens from hamsters II 41, II 46, and II 50 which were not yet haemoglobinaemic showed congestion of the sinusoids and spaces between the reticular cords by RBC's to a lesser extent than was found in haemoglobinaemic animals. Erythrophagocytosis was still very obvious as was lymphoid depletion of the lymphoid follicles.

Animals such as II 52 which survived for longer had more dark granular haemosiderin within the cells associated with the lymphoid follicles and pale diffuse haemosiderin within macrophages

and reticular cells of the red pulp than those animals which died sooner from the infection.

In all animals many leptospires were seen within sinusoids and the congested spaces between the reticular cords. Fewer organisms were seen in the lymphoid follicles.

3.2.2.5.3 *Kidney*

a) Irradiated controls

Kidneys from hamsters Irr 13 to Irr 18 had no significant lesions. Those from hamsters Irr 19 to Irr 24 showed no or only minor changes consisting of vacuolation and loss of microvilli of epithelial cells from the occasional proximal convoluted tubule. There was loss of epithelial cells from the renal papilla in some hamsters. No haemosiderin was seen.

b) Infected hamsters

Histological lesions in these hamsters were similar to those described in Chapter 5, Section 3.3.3.2. Haemorrhage into the Bowman's capsules and tubules appeared more severe in the M4/9 infection of the present experiment when compared to the 1045 infection described in Chapter 5. Areas of necrosis were seen at the corticomedullary junction. Mononuclear cell infiltrations,some very large, were found around blood vessels in the medulla and pelvic areas.

Large colonies of leptospires were observed within blood vessels and between the tubules of the corticomedullary junction, the medulla and renal papilla. Smaller numbers of organisms were seen in the cortex. There were single or small groups of organisms migrating between tubular epithelial cells and within tubules of the cortex and medulla.

Hamster Inf 38 showed few renal lesions. There was some vacuolation of epithelial cells, the occasional cellular cast in tubules and small perivascular infiltrations of mononuclear cells. Few leptospires were seen and those present were in the lumina of the proximal convoluted tubules.

c) Irradiated-infected hamsters

All hamsters showed the same changes described for the infected hamsters with the absence of the mononuclear cell infiltrations. In addition, with the exception of hamsters II 47, II 48 and II 49, there was renal papillary necrosis. Renal papillary necrosis was associated with haemorrhage within the medulla, immediately above the papillary epithelium, and into the urinary space of the pelvis. These pathological changes were most severe in hamster II 46 giving rise to the blood clot within the bladder. Granular deposits of haemosiderin were often found within the tubules. Hamster II 48 while showing no haemorrhage associated with renal papillary necrosis had haemorrhage within the fat deposits around the renal capsule.

The distribution of leptospires was similar to that of the infected hamsters with larger numbers of organisms present.

3.2.2.5.4 *Bone marrow*

a) Irradiated controls

Marrow from hamsters Irr 13 to Irr 18 five days post-irradiation was almost acellular, the remaining nucleated cells were mainly the endothelial and reticulum cells lining blood vessels and sinusoids. Occasional small groups of unclassifiable blast cells (Fig. 8-5), late normoblasts and hypersegmented PMN's were found, mainly in trabeculae at the proximal end of the femur.

Marrow from hamsters Irr 19 to Irr 24 11 days post-irradiation showed considerable regeneration within the marrow cavity. Groups of haemopoietic precursor cells were found, with granulopoiesis occurring more to the periphery of the femur and within the trabeculae at the proximal end and erythropoiesis occurring towards the centre of the marrow cavity. Giant granulopoietic cells, many with nuclei in a large ring form, and increased numbers of megakaryocytes were seen. Little or no haemosiderin was present.

b) Infected hamsters

Marrow from all hamsters except Inf 38 was hypocellular and similar to that described in Chapter 5, Section 3.3.4.2. Marrow from Inf 38 was normal.

Fig 8-4

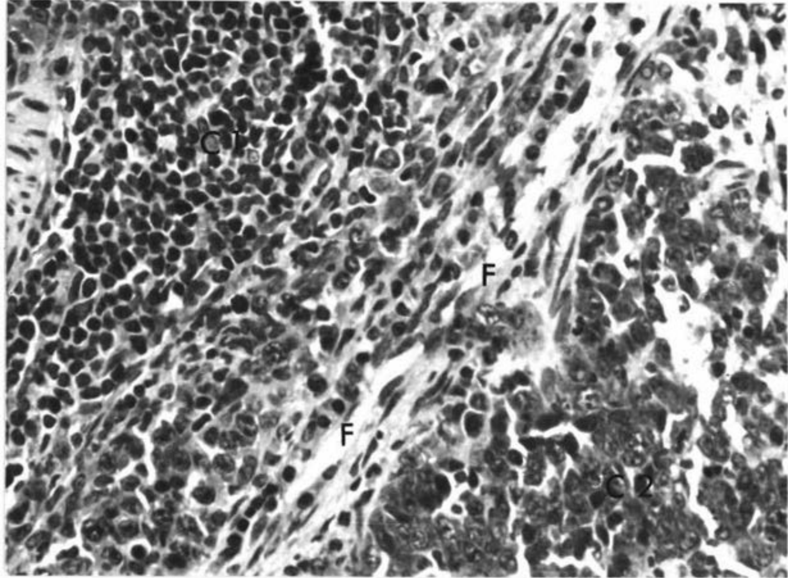
Spleen from a hamster euthanased 11 days post-irradiation. Two colonies (C1 & C2) of regenerating cells with different cytoplasmic and nuclear characteristics are seen on either side of the fibrous trabecula (F).

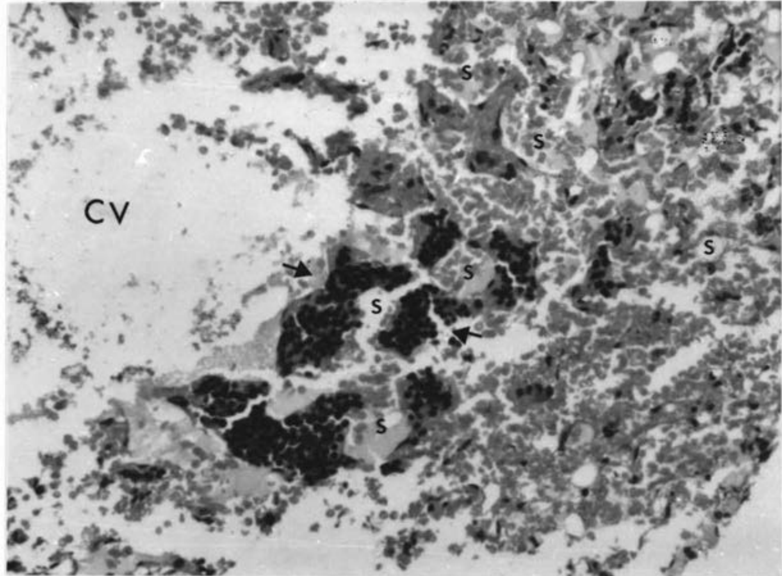
H&E 200x

Fig 8-5

Bone marrow from a hamster euthanased five days post-irradiation. Groups of unclassifiable blast cells (arrows) are present amid the RBC's. Sinusoids (S) and the central vein (CV) may be seen.

H&E 100x


C1
C1
F
F
C2
C2


CV
s
s
s
s
s
s

Large numbers of leptospires were seen in all hamsters except hamster Inf 38 in which no organisms were detected.

c) Irradiated-infected hamsters

Marrow from all hamsters dying five to six days post-infection was acellular except for the presence of endothelial and reticulum cells and the occasional group of unclassifiable blast cells. Erythrophagocytosis was frequent. No haemosiderin was present.

In hamsters dying at a later date there was recolonization which appeared to commence within the trabeculae at the proximal head of the femur. The amount of regeneration varied between hamsters with hamsters II 47 and II 48 showing a slight increase in erythroid and granulocytic cells and hamsters II 49 and II 52 showing greater numbers. The patterns of regeneration and cell cytology was similar to that of the irradiated controls.

Individual, small groups and large colonies of leptospires were seen more frequently than in the infected, non-irradiated group of animals.

3.2.2.5.5 *Lung*

a) Irradiated controls

Lung sections from all hamsters showed areas of alveolar collapse and emphysema with intra-alveolar RBC's. Hamster Irr 13 contained some pink proteinaceous fluid within the bronchioles and alveoli.

b) Infected and irradiated-infected hamsters

Lungs from all infected and irradiated-infected hamsters which were found dead had severe congestion and large quantities of a homogenous pink proteinaceous fluid present in the alveoli, alveolar ducts and bronchioles. Larger blood vessels often contained large numbers of WBC's. Euthanased hamsters usually showed less pulmonary congestion and exudation than those found dead. Collapse, emphysema and intra-alveolar RBC's were similar to that of the controls.

Small numbers of leptospires were present in the alveolar septae and larger numbers were frequently found in blood vessels. No organisms were seen in hamster Inf 38.

Haemosiderin was present in many alveolar macrophages except those of hamster Inf 38.

3.2.2.5.6 *Cardiac muscle*

a) Irradiated controls

No lesions were seen.

b) Infected and irradiated-infected hamsters

Cardiac muscle was histologically normal in all infected hamsters apart from the presence of the occasional bundle of muscle fibres showing a slight pallor. This pallor was also noted in the irradiated-infected hamsters. In addition, hamsters II 43 and II 44 had some haemorrhage between muscle fibres, and vacuolation and degeneration of muscle fibres.

Leptospires were not seen within the cardiac muscle of either group of hamsters but were seen singly, in small groups or large colonies within the coronary blood vessels and heart chambers. Larger numbers were seen in the irradiated-infected group.

4.0 DISCUSSION

The present experiments were performed to determine the effects of suppression of the reticulomacrophage system on the pathogenesis of the disease and in particular, the rate of RBC destruction and the degree of haemoglobinaemia.

The results of the present experiment were inconclusive due to several complicating factors which arose throughout the course of the experiment. By comparison the effects of irradiation upon *icterohaemorrhagiae* infected guinea pigs reported by Higgins & Cousineau, (1977b) gave clear cut results which, according to the authors, were directly attributable to suppression of the reticulomacrophage system. The most important factor in the present

study was the failure of irradiation to completely suppress the activity of fixed macrophages. Although enlargement of the spleen of irradiated *ballum* infected hamsters did not occur to the same extent as in the infected only group, those macrophages already present showed much activity and erythrophagocytosis was obvious. In the irradiated-infected group, there was histological evidence for sequestration of RBC's and erythrophagocytosis in those animals which were not yet haemoglobinaemic indicating that as in the infected only group, abnormal RBC's were being detected and removed.

The radiation level of 800 RADS was chosen for use in Part II of the experiment because the level of 600 RADS appeared too low to deplete the spleen and bone marrow of lymphoid and haemopoietic tissues while those hamsters exposed to 1000 RADS showed more severe tissue damage in the form of coagulative necrosis of hepatocytes. That more tissue damage could be expected in the 1000 RAD group was also shown by previous workers who demonstrated that following exposure to 1,000 to 10,000 RADS, deaths in hamsters were associated with the gastrointestinal syndrome and exposures higher than 8,000 to 10,000 RADS deaths were associated with a central nervous system syndrome (Eddy & Casarett, 1972). As *ballum* also causes hepatic lesions, it was considered that the liver lesions induced by 1,000 RADS would interfere with the interpretation of leptospiral induced disease. Because of the reported prominent reduction in bone marrow cellularity with 825 RADS manifested by the severe reduction in the number of cells and degeneration of the blood cell precursors at five days (Eddy & Casarett, 1972), it was decided that the 800 RAD level would be the most appropriate for use in the present experiments. Higgins & Cousineau (1977b) do not discuss their rationale for their selection of 500 RADS for irradiation of the guinea pigs.

The second factor complicating the results was the rapid recovery of the lymphoid and haemopoietic systems following irradiation. Recovery of haemopoiesis has been reported in mice and Chinese hamsters after six days and to be almost complete in

some animals after ten days (Ludwig & Kohn, 1964). Active regeneration of haemopoiesis has been reported in golden hamsters after eight days (Eddy & Casarett, 1972). From earlier experiments it was estimated that the weanling hamsters used in the present experiments should have died five to six days post-infection as a result of RBC destruction which would have been within the shorter regeneration time of six days reported for Chinese hamsters. It has been suggested that Chinese and golden hamsters have equivalent regenerative ability (Eddy & Casarett, 1972).

Irradiated control and infected hamsters euthanased 11 days post-irradiation had groups of haemopoietic cell precursors in the spleen, bone marrow and liver as a result of the survival of stem cells (Bond *et al*, 1965; Eddy & Casarett, 1972; Quesenberry & Levitt, 1969) which are capable of multiplication and differentiation to the various cell lines (Kretchman & Conover, 1970; Quesenberry & Levitt, 1979). It seems probable that the irradiated and infected animals would have a greater stimulus for haemopoiesis than irradiated animals as the presence of bacterial endotoxins (Quesenberry *et al*, 1978) and anaemia (Necas & Neuwirt, 1970) are capable of stimulating differentiation to and increasing production of granulocytic and erythroid cells respectively. The differential positions of groups of haemopoietic cells depends upon the position of the stem cell within the spleen or bone marrow (Quesenberry & Levitt 1979) which determines the 'haemopoietic inductive microenvironment'. In mice this has been demonstrated by the fact that under haemopoietic stress the spleen becomes an erythropoietic organ while the bone marrow becomes granulopoietic (Quesenberry & Levitt, 1979). The groups of granulopoietic cells which were found within the boney trabeculae of the head of the femur in the present work may have resulted from factors influencing the preferential production of granulocytes which, in mice, appear to be associated with the endosteum and boney trabeculae (Schwartz & Greenberg, 1981). This preferential production of

granulocytes results in the bone marrow becoming granulopoietic rather than erythropoietic (Quesenberry & Levitt, 1979). The description of the irradiation and infection of guinea pigs by Higgins & Cousineau (1977b) did not include mention of active lymphoid and haemopoietic regeneration as interfering with their results.

The increased M:E ratios seen in irradiated animals euthanased three days post-irradiation resulted from the fact that the erythroid precursors are more sensitive to radiation and therefore more effectively destroyed (Eddy & Casarett, 1972).

Another complicating factor was the haemorrhage which resulted from vascular damage and renal papillary necrosis and obscured the degree of anaemia produced by infection and RBC destruction. Irradiation produces fluid loss and haemorrhage as a result of vascular damage and thrombocytopaenia (Eddy & Casarett, 1972). The extent of the anaemia in hamsters as a result of haemorrhage was well demonstrated by hamster II 46 which, while not haemoglobinaemic, had a PCV of 0.23 and a large blood clot within the bladder. The pathogenesis of the renal lesions was discussed in Chapters 5 and 6, as was the significance of endothelial cell damage in those hamsters which were infected but not irradiated. Although *ballum* has not been reported as inducing more haemorrhagic disease than *icterohaemorrhagiae* there is histological evidence of haemorrhage into the Bowman's Capsules and ultrastructural evidence of damage to the glomerular endothelial cells (Chapters 5 and 6). It is not surprising therefore that a combination of irradiation and infection should produce more haemorrhage than would normally be seen in infected only hamsters. It is surprising however, that a similar phenomenon was not observed in the *icterohaemorrhagiae* infected guinea pigs described by Higgins & Cousineau (1977b) particularly as *icterohaemorrhagiae* is reported as producing extensive petechial haemorrhages (Arean, 1962b; Arean *et al*, 1964; De Brito *et al*, 1966; Higgins & Cousineau, 1977a). Although platelet counts and clotting factor examinations were not done in

the present experiments, had a thrombocytopaenia been present as has been reported in infections with *canicola* (Chaperon *et al*, 1979; Finco & Low, 1967) and *icterohaemohaemorrhagiae* (Higgins & Cousineau, 1977a) then this combined with the thrombocytopaenia and vascular damage of irradiation (Eddy & Casarett, 1972) should have produced greater haemorrhage than in the infected, non-irradiated group.

Although the numbers of animals examined by SEM were small it appears that impairment of the reticulomacrophage system did have some effect as pitting and spherocytosis of RBC's was seen in one irradiated-infected animal before haemoglobinaemia was present. This differed from the situation in non-irradiated infected hamsters in both 1045 and M4/9 infections in which pitting and spherocytosis were always accompanied by haemoglobin-aemia, suggesting that these cells were undergoing intravascular haemolysis. The presence of pitted, spherical RBC's in the absence of haemoglobinaemic demonstrated that these cells, in the early stages of the disease at least, were not necessarily undergoing intravascular haemolysis but were being sequestered and phagocytosed. In the later stages of the disease where haemoglobinaemia was present in the irradiated-infected hamsters intravascular haemolysis may be present and the small spherical bodies attached to RBC surfaces may be RBC fragments. Red cell fragmentation is described as one method of haemolysis and may be seen in a variety of anaemias such as Coomb's positive haemolytic anaemia (Weed & Reed, 1966). Further experiments need to be conducted in order to determine the extent of splenic and hepatic sequestration of RBC's and to verify whether haemoglobin is in fact released back into general circulation. The degree of haemoglobinaemia in infected, surgically splenectomised animals would be of interest although, as also discussed in Chapter 5 erythrophagocytosis is seen in both liver and bone marrow.

The presence of echinocytes following irradiation has not been previously recorded. Damage to sulphydryl groups follows

irradiation (Sutherland & Pihl, 1967) and membrane damage by organic mercurials (Sutherland *et al*, 1967; Weed *et al*,1962). The latter agents produce altered Na^+ and K^+ permeability and inhibition of glucose uptake by the RBC's As both glucose deficiency (Bessis, 1973; Brecher & Bessis, 1972) and Na^+ and K^+ imbalance (Jandl, 1965; Kaneko, 1974; Valentine, 1979) cause echinocyte formation, it seems a likely hypothesis that irradiation damaged the RBC's sulphydryl groups resulting in altered glucose uptake and/or Na^+ and K^+ permeability and resulted in echinocyte formation.

While bilirubin levels were not quantitated, it was noticed on visual inspection that the carcases of the irradiated infected hamsters were less jaundiced than the infected hamsters. This difference is most likely due to the difference in the total amount of erythrophagocytosis. Haemorrhage would have resulted in the loss of many RBC's into the renal pelvis and bladder. Hyperplasia of the macrophage system was inhibited. If bilirubin accumulation was due to leptospiral induced liver disfunction then jaundice should have been similar in both groups of animals, maybe with more in the irradiated-infected group as radiation was shown to produce hepatocyte degeneration.

The accumulation of iron in the form of haemosiderin was due to the breakdown of RBC's and haemoglobin. In the infected group of animals, this breakdown occurred over a short period of time and the iron was not immediately re-used in erythropoiesis. Dark granular deposits were seen in those animals which survived for longer allowing concentration of the haemosiderin deposits. Erythropoiesis was completely inhibited for some days in the irradiated animals allowing accumulation of iron or haemosiderin which would normally have been utilized in RBC production. The effects of increased RBC breakdown and decreased erythropoiesis were combined in the irradiated *ballum* infected hamsters causing increased quantities of haemosiderin to accumulate.

This experiment was stimulated by the work of Higgins & Cousineau (1977b) who reported no complications in the interaction

of irradiation and leptospiral infections of guinea pigs and reported very clear cut results in that only the *icterohaemorrhagiae* infected-nonirradiated guinea pigs were jaundiced. There are definite species differences both in actual sensitivity to radiation and the types of lesions resulting from radiation. Species such as guinea pigs, man and dogs show a large degree of haemorrhage as a result of thrombocytopaenia, while the rabbit, rat and mouse show little haemorrhage (Bond *et al*, 1965). Higgins & Cousineau (1977b) used guinea pigs for their radiation studies. That the combination of irradiation and *icterohaemorrhagiae* did not produce more haemorrhage in combination is surprising because Higgins & Cousineau (1977a) themselves state that *icterohaemorrhagiae* infections of guinea pigs produce a thrombocytopaenia and severe endothelial cell damage to blood vessels.

On the basis of the hamster experiments reported in this thesis there is a significant interaction between radiation and leptospiral infection and it seems that some of Higgins & Cousineau's (1977b) work may be rather simplified particularly with respect to the brevity of their histological findings. Indeed, the fact that both groups of animals were reported to die at exactly the same time i.e. five days, is surprising because irradiation would be expected to destroy the nonspecific cellular immune system comprising the PMN's and monocytes and has been shown in mice to impair the fixed macrophage's ability to phagocytise and destroy microorganisms (Bond *et al*, 1965). In mice there is a direct correlation between the number of circulating PMN's and the susceptibility to microorganisms. The numbers of PMN's fall immediately following irradiation reaching the lowest point after three days (Bond *et al*, 1965). It seems likely, as was shown in the hamster experiment reported here that increased numbers of organisms and therefore presumably 'toxin' should be found in infected and irradiated animals when compared to infected and non-irradiated animals. In the current experiments *ballum* infected and irradiated animals started dying earlier than non-irradiated hamsters and the last deaths occurred sooner than

in the infected-only group. Indeed in the infected group one hamster appeared to survive the infection altogether. Increased susceptibility of animals to leptospiral infections has also been shown by chemical immunosuppressants in which hamsters became susceptible to *hardjo* following cyclophosphamide treatment (Adler & Bragger, 1979) and haemorrhage and haematuria were seen.

In the present studies the major problem in studying the cause of haemoglobinaemia was to separate the erythrophagocytic function of macrophages from their protective function of leptospiral phagocytosis. Complete destruction of the reticulomacrophage system, while preventing removal of damaged RBC's also permits unhindered multiplication of the organisms. Any experimental methods used to modify the pathogenesis of the infection in order to study the disease must produce readily explainable results which can be separated from the results of the infection.

5.0 CONCLUSIONS AND SUMMARY

1. Both infected and irradiated-infected hamsters became haemoglobinaemic with spherical and pitted RBC's seen in both groups.

2. All infected haemoglobinaemic hamsters had circulating spherical and pitted RBC's suggesting that such cells were actually undergoing haemolysis although TEM examination showed few partially haemoglobinated RBC's. In the irradiated-infected group, one hamster was present in which spherical, pitted RBC's were not associated with haemoglobinaemia.

3. Erythrophagocytosis was frequent in both infected and irradiated-infected groups of hamsters although the splenic enlargement of the infected hamsters was not seen in the irradiated-infected animals to such a great extent.

4. Complications resulting from the irradiation affected the outcome of the infection. These comprised:

   i) A large amount of fixed macrophage activity remained although proliferation of the macrophages was inhibited.

   ii) Rapid recovery of the haemopoietic system coincided with the onset of severe clinical disease.

   iii) Severe anaemia resulted from haemorrhage in association with renal papillary necrosis.

5. A radiation level of 800 RADS was not sufficient to suppress the reticulomacrophage system.

6. Less jaundice was seen in the irradiated-infected hamsters compared to the infected hamsters and this may be explained as being due to a reduced amount of erythrophagocytosis.

CHAPTER 9

GENERAL DISCUSSION

When this study was commenced, it was often postulated that the different serovars of leptospire which produce different manifestations of the disease do so by qualitative and quantitative differences in pathogenetic mechanisms. This was particularly so in regard to RBC destruction and its associated haemoglobinaemia. This was believed to result from specific leptospiral phospholipase activity on the RBC membrane but there were no satisfactory explanations as to why RBC destruction should vary according to both species and age of the infected host. The only hypothesis advanced was that it could be explained in part by variations in the phospholipid content of RBC membrane in different species (Kasarov, 1970). This however, did not explain satisfactorily the variation in susceptibility between adults and neonates and the occasionally observed differences in response between animals of similar age within the same species.

The ultrastructural lesions within endothelial, hepatic and renal cells of hamsters (Miller *et al*, 1974) and guinea pigs (De Brito *et al*, 1979; De Brito *et al*, 1966) infected with *icterohaemorrhagiae* and hamsters infected with *pomona* (Miller & Wilson, 1967) and histochemical changes within the livers and kidneys of guinea pigs infected with *icterohaemorrhagiae* (Arean, 1962b; Arean & Henry, 1964) were considered to be caused by a leptospiral 'toxin' by the respective workers. The most recent paper published since the completion of the present study (Arriaga *et al*, 1982) has merely produced conclusions similar to those of earlier published work and makes no further progress in establishing the initial cause of lesions. Tissue hypoxia as a result of hypovolaemia is considered important (Arean & Henry, 1964; De Brito *et al*, 1979; Sitprija *et al*, 1980) but the cause of the initial damage to the endothelial cells which leads to the fluid loss and hypovolaemia while being

attributed to a 'leptotoxin' remains unknown. Arean & Henry (1964) suggested that there may be interference to the cellular enzyme systems resulting in the early histochemical changes followed by the histological lesions. In murine hepatitis, large alterations in several intracellular enzymes have been noted before the presence of histological lesions which indicate, in that disease at least, the possibility of a block in the normal glycolytic pathway and an increase in the utilization of the alternative pentose phosphate pathway (Jones & Cohen, 1962).

To become established in the host, the leptospires must be capable of surviving the initial bacteriocidal defence mechanisms of the body (Stalheim, 1971). The present work showed that leptospires are capable of surviving and multiplying within a number of host tissues and as previously reported (Faine, 1964; Miller *et al*, 1974) the largest numbers are most consistently observed within the liver. Fewer numbers are seen initially within the kidney and as discussed in earlier chapters, colonization of the proximal convoluted tubules is most likely as a result of random movement before the development of specific resistance to the organisms. The renal lesions described in the present studies are similar to those of Arriaga *et al* (1982). These workers describe the presence of severe lesions at the corticomedullary junction in guinea pigs infected with *icterohaemorrhagiae*, a feature observed in the present work but not commented upon in earlier papers. These workers do not, however, discuss whether or not the corticomedullary lesions are associated with large numbers of organisms as noted in the experiments reported here.

The present work supports other reports (Arean, 1962b; Arean & Henry, 1964; Arean *et al*, 1964; De Brito *et al*, 1966; Knight *et al*, 1973; Marshall, 1973; Miller *et al*, 1974) that 'toxins' rather than mechanical damage are responsible for the tissue damage. Evidence for this was provided by the fact that lesions were observed before large numbers of organisms were present and most organisms which were intracellular were degenerate. It was further substantiated by the fact that RBC and hepatic lesions were induced in two calves

given only intravenous *pomona* 'toxin'. The histological and ultrastructural lesions described by Arriaga *et al* (1982) were attributed to damage by 'toxins' and were similar to those seen in the present work. The hepatic and renal lesions in infected guinea pigs are associated with alteration to the cellular enzymes (Arean, 1962b; Arean & Henry, 1964). From the present and previous work, a general diagrammatic scheme for the progression of leptospiral infections may be drawn (Fig. 9-1). All serovars of *L. interrogans* in the appropriate host environment are probably capable of multiplying and producing 'toxins' at a sufficient level to induce most manifestations of the disease. The exact lesions depend upon the host susceptibility and the infective serovar. This host susceptibility may depend upon the host's ability to prevent multiplication of organisms and thus to control levels of 'toxin' elaboration, or upon the host's response to the 'toxin'.

RBC destruction is only one manifestation of toxic damage and occurs in some species of animals infected with certain serovars. While the initiating cause is unknown, it appears that RBC damage may result from interference with an endogenous enzyme or enzyme system instead of, or in conjunction with the previously proposed phospholipase which acts directly upon the RBC membrane (Chorvath 1974; 1975; Kemenes, 1974; Stalheim, 1971).

The interspecies and age variation in regard to the presence or absence of RBC destruction may result from basic differences in RBC's in these animals. There are quantitative differences in the cellular enzymes, and metabolic intermediates, substrates and end products as well as in permeabilities to hexose sugars in RBC's from different species (Kaneko, 1974). For example pig RBC's are incapable of taking up glucose (Kim & McManus, 1971) and should a putative leptospiral 'toxin' interfere with the mechanisms for glucose uptake by cells it would then be expected that pig RBC's would be resistent. Such differences have been found between animals of different ages. Differing permeabilities to hexose sugars have been found between adult and foetal RBC's from guinea pigs (Widdas, 1955) and cattle (Johnson & Stewart, 1967; Kaneko, 1974) and

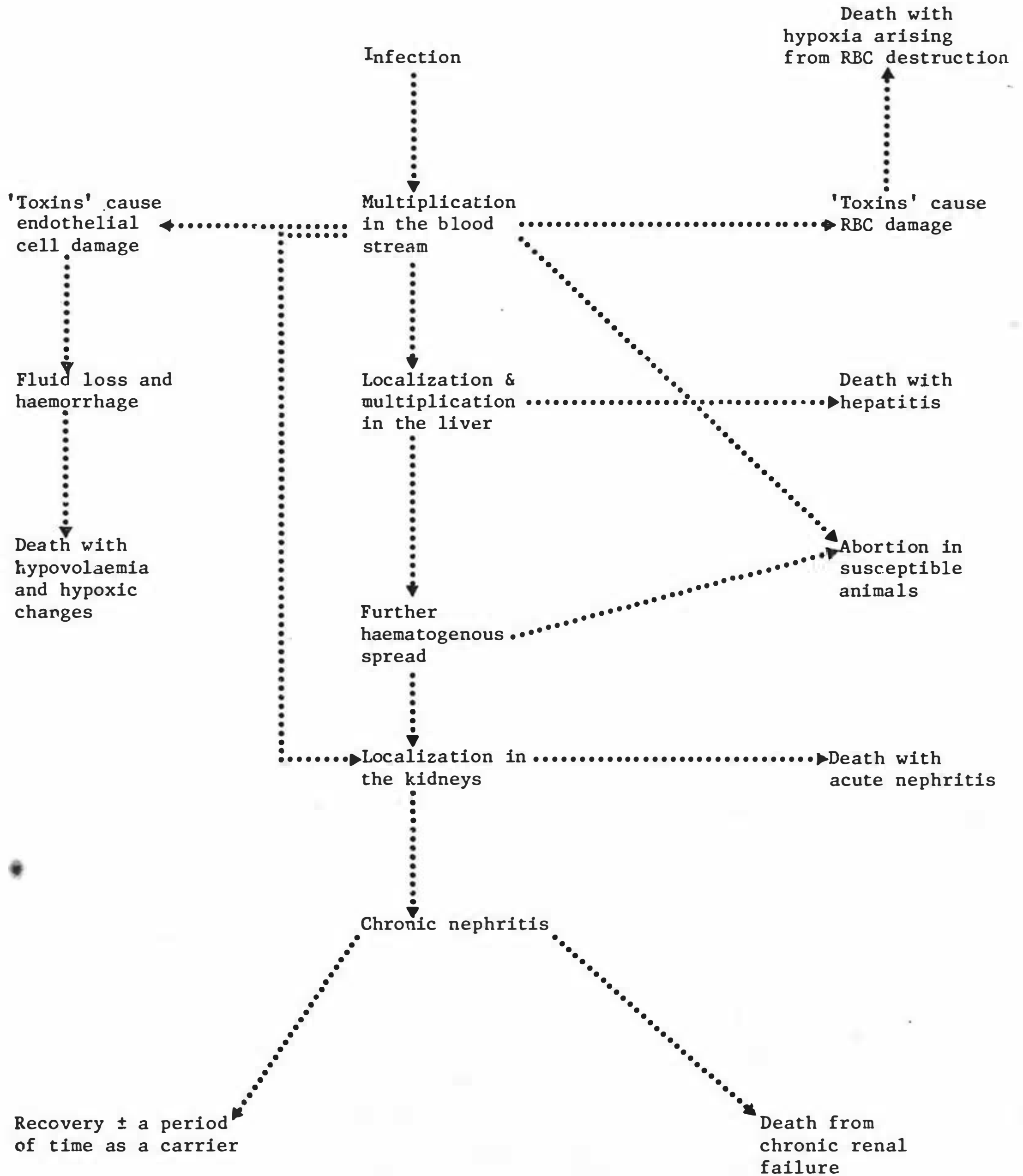


Figure: 9-1 Progression of infection during leptospirosis

differing quantities of 2, 3-diphosphoglycerate have been found in foetal, neonatal and adult cattle (Zinkl & Kaneko, 1973b). Differences in metabolism may be seen between animals of the same age within a species (Kaneko, 1974). RBC's from individual sheep (Tosteson, 1969; Tosteson & Hoffman, 1960) and humans (Zarkowsky *et al*, 1968) may have differing permeabilities to sodium and potassium. In humans, sodium and potassium permeabilities which differ from normal have been associated with a haemolytic anaemia (Zarkowsky *et al* , 1968). In cattle the haemolytic anaemia occurring in erythropoietic porphyria has been associated with alterations in the cellular enzymes and glycolytic intermediates (Zinkl & Kaneko, 1973a). An example of an enzyme defect leading to RBC removal is found in congenital pyruvic kinase deficiency of humans (Valentine, 1979) and dogs (Benjamin, 1979; Duncan & Prasse, 1978) and is characterised by a haemolytic anaemia.

As discussed earlier, foetal haemoglobin content was not found to be important to the *in vitro* susceptibility of RBC's to leptospiral cultures. Abnormalities of haemoglobin can not be excluded. The maintenance of haemoglobin in a functional state requires normal function of the enzyme systems of the RBC (Kaneko, 1974; Kitchen, 1969). Oxidation of haemoglobin leads to Heinz body formation (Rifkind & Danon, 1965), the Heinz bodies being seen as dark inclusions within the RBC cytoplasm (Bessis, 1973; Rifkind, 1965; Rifkind & Danon, 1965). The end stage Heinz bodies described by Rifkind & Danon (1965) are structurally different to the inclusions of the leptospiral affected RBC's and vacuoles have not been described in association with the former. Heinz bodies are not membrane-bound and are inclusions of a greater electron density than the surrounding haemoglobin.

Renal disease may affect haemoglobin through its association with lowered RBC glutathione levels and lowered glutathione stability (Theil *et al*, 1961). Reduced glutathione is necessary to maintain reduced haemoglobin (Kaneko, 1974; Valentine, 1979). *Eperythrozoon ovis* infections of sheep are also associated with interference to the maintenance of reduced glutathione causing the

RBC's to be more susceptible to oxidizing agents (Sutton, 1979).

The destruction of RBC's during leptospirosis appears to be more complex than simply as a result of a 'toxin' acting upon the cell and causing lysis. *In vitro*, plasma appears to be protective with only washed cells being susceptible to lysis by the leptospiral culture. This may be a reflection of the *in vivo* situation whereby early in the development of the disease, RBC sequestration occurs before the development of haemoglobinaemia. Even during haemoglobinaemia very few partially haemoglobinated RBC's can be seen in circulation. Lesions in RBC's were observed in prehaemoglobinaemic animals but the plasma *in vivo* (as also observed *in vitro*) may have helped to prevent haemoglobin loss from RBC's in which a 'toxin' induced lesion had already developed. How plasma exerts its protective effect is not known. It may be as simple as acting as a buffering system against pH changes which have been shown to change RBC morphology (Bessis, 1973; Brecher & Bessis, 1972) or it may act as a source of glucose for RBC metabolism. Depletion of glucose results in discocyte-echinocyte transformation (Brecher & Bessis, 1972). The ability to metabolize may assist the RBC in withstanding the 'toxic' effects of leptospires. It has been shown that sulphydryl groups are destroyed during irradiation (Sutherland & Pihl, 1967) and exposure to organic mercurial compounds (Benesch & Benesch, 1954; Sutherland *et al*, 1967; Weed *et al*, 1962). However, if the RBC's are allowed to metabolize then repair to the sulphydryl groups may occur (Sutherland & Pihl, 1967). Such repair would not be possible in RBC's suspended in saline.

The resistance of the RBC's to lysis when suspended in plasma indicates the possibility of another mechanism *in vivo*. The increased sequestration of RBC's which occurred in most animals suggested that the reticulomacrophage system may be important in the development of haemoglobinaemia. Unfortunately, complications resulting from irradiation prevented definite conclusions from being made during the experiment designed to observe the effects of impairment of the reticulo-macrophage system on the development of haemoglobinaemia.

While it is known that the spleen detects and sequesters abnormal RBC's (Griggs *et al*, 1960; Jandl, 1966; Jandl & Aster, 1967; Jandl *et al*, 1965; Rifkind, 1965; Wagner *et al*, 1962; Weiss, 1962b), exposure to the conditions within the spleen may in fact result in the development of abnormalities, particularly in RBC's in which some abnormality already exists (Griggs *et al*, 1960). Passage of RBC's through the spleen probably exposes the RBC's to lowered glucose levels through competition with splenic cells for glucose and increased accumulations of metabolites (Griggs *et al*, 1960; Jandl & Aster, 1967). These would be of importance to abnormal RBC's as they are more likely to be detained in the spleen for longer periods of time due to the pre-existing defect. Any abnormalities leading to alteration in membrane deformability will result in the cell's inability to conform to the microcirculation and hence sequestration within the spleen (La Celle, 1970) as has been seen in conditions such as Heinz body anaemias (Nathan, 1969; Rifkind, 1965). Damaged RBC's may in fact lyse within the splenic circulation (Rifkind, 1965; 1966). It therefore seems likely that RBC's damaged by leptospiral 'toxins' are detected and sequestered by the spleen where they are either further damaged or lysed and removed from circulation.

Increased haemosiderin deposits within the spleens of non-haemoglobinaemic hamsters infected with *ballum* were possibly due to increased removal of minimally damaged RBC's, the damage in this case not being severe enough to result in overt haemoglobinaemia. Although RBC morphology has not been described in other work, increased erythrophagocytosis has been seen in *icterohaemorrhagiae* infections of dogs (Gleiser, 1957) and humans (Arean 1962a) and Kupffer cell hyperplasia has been noted in *icterohaemorrhagiae* infections of guinea pigs (De Brito *et al*, 1966) and humans (Arean, 1962a). Minimal RBC lesions may have been present in these infections. Had more detailed studies been possible in the human case of *ballum* infection reported in Chapter 4 then increased removal of the RBC's may have been seen.

Except in the case of one calf, morphological changes in RBC's resulting from hepatic and renal malfunction were not thought to occur, probably because death rapidly followed the onset of haemoglobinaemia in those calves which died.

Further work is necessary to elucidate the mechanisms of RBC destruction. Histochemical techniques could be used to examine the presence or absence of interference to the RBC enzymes with care to take into account the enzyme alterations which occur as the cell ages (Kaneko, 1974).

Fractionation of the plasma and both intracellular leptospiral contents and culture supernatant should be carried out and the various fractions incubated together in different combinations with RBC's in order to determine which of the plasma constituents are protective and which constituents of the leptospire are harmful. Tissue cultures of liver and kidney may also be used with combinations of leptospiral 'toxin' and plasma. Cell cultures may also be useful in examining the differences between serovars and determining why serovars such as *pomona, ballum* and *icterohaemorrhagiae* cause serious disease within animals such as cattle, hamsters and guinea pigs while serovars such as *hardjo* and *balcanica* cause little serious disease in animals such as cattle and sheep. Histochemical techniques and ultrastructural examinations may also be useful in examining leptospiral effects on tissue cultures.

In conclusion, while this study examined the morphological changes associated with leptospiral induced RBC destruction and enzyme examination was beyond the scope of the present work, it appears that the initial lesion is likely to be precisely biochemical. It seems reasonable to hypothesise that the basic mechanism for RBC destruction is similar to that which causes the lesions in the other tissues and that the same enzyme or enzyme system is affected in all tissues. Sufficient biochemical differences exist between RBC's from different species of animal and from animals of the same or different ages within the same species to explain the variation in susceptibility to RBC destruction.

If a discreet biochemical lesion can be defined then an alternative therapeutic regime may be revealed. The search for more effective bacteriocidal agents may be better replaced by treatment aimed at alleviating or overcoming the effects of 'toxin' upon the cellular metabolism. Simultaneous destruction of large numbers of organisms could theoretically exacerbate the situation by releasing large quantities of 'toxin' into a host environment already containing large quantities of 'toxin'. Treatment aimed at alleviating the 'toxic' effects of leptospires is already foreshadowed by the demonstrated *in vitro* protective effects of normal plasma. If a therapeutic regime can be developed upon these lines then a significant advance in preventing the more severe clinical effects of the disease could be in prospect.

APPENDIX III: New Zealand Journal of Science, 1982, Vol.25
New Zealand Society for Electron Microscopy
Conference abstract

MORPHOLOGICAL CHANGES IN RED BLOOD CELLS OF CALVES AND HAMSTERS WITH LEPTOSPIROSIS

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Red blood cells (RBC's) in calves and hamsters infected with *Leptospira interrogans* serovar *pomona* and *Leptospira interrogans* serovar *ballum* respectively undergo intravascular destruction resulting in haemoglobinaemia and haemoglobinuria.

Calves and hamsters were experimentally infected with the respective serovars of *Leptospira interrogans* and observations made at the haemoglobinaemic stage of the disease. RBC's were taken from normal control and infected animals and processed for examination by scanning electron microscope (SEM) and transmission electron microscope (TEM). Under SEM examination, normal calf and hamster RBC's were evenly sized, regularly shaped biconcave disks with occasional surface irregularities. By TEM the RBC's were also seen to have occasional small vacuoles randomly distributed in their cytoplasm.

Pathological changes seen in RBC's from haemoglobinaemic animals were similar for both calves and hamsters. The RBC's were swollen and irregular in shape and size. SEM examination revealed many irregular surface pits which it is believed were represented by vacuoles of variable shape and size in TEM preparations. Some of these vacuoles were seen to communicate with the cell exterior and some contained a fine granular material. Leptospires were seen in one TEM preparation of infected hamster RBC's. They were close to, but not in contact with, the RBC's. In TEM preparations from all haemoglobinaemic animals, except one calf, the majority of the RBC's appeared fully haemoglobinated. One calf showed a large number of partially haemoglobinated RBC's. These RBC's also contained vacuoles, though fewer in number than the fully haemoglobinated cells, dark granular inclusions and cytoplasmic protruberances. Apparent gaps in the cell membrane with the escape of haemoglobin were also seen.

APPENDIX V

Preparation of tissue for electron microscopic examination

1. FIXATION

i) Tissues are cut into 1-2 mm cubes, immersed in modified Karnovsky's fixative at 4°C for at least 2 hours or overnight.

Modified Karnovsky's Fixative

2% Formaldehyde
3% Gluteraldehyde
in 0.1M phosphate buffer

To make 100 mls.

a) Heat 2 gms paraformaldehyde in 80 mls of distilled water to 60-70°C
b) Slowly add IN NaOH dropwise until the milky solution clears
c) Add buffer salts 2.51 gms Na_2HPO_4. 12 H_2O.
0.41 gms KH_2 PO_4
d) Add 12 mls of 25% gluteraldehyde
e) Make up to 100 mls. Store in refrigerator.

ii) The tissue cubes are then washed in 0.1M phosphate buffer, pH 7.2 at 4°C for approximately 2 hours, changing the solution 3 times.

Preparation of Phosphate Buffer

1) Preparation of 0.2M solution of dibasic sodium phosphate

Na_2H PO_4	28.39 gms
Na_2H PO_4. $2H_2O$	35.61 gms
Na_2H PO_4. $7H_2O$	53.65 gms
Na_2H PO_4.$12H_2O$	71.64 gms

make up to 1000 mls with distilled water

2) Preparation of 0.2M solution of monobasic sodium phosphate

Na H_2 PO_4. H_2O	27.6 gms
Na H_2 PO_4.$2H_2O$	31.21 gms

make up to 1000 mls with distilled water

Preparation of 0.1M phosphate buffer by mixing the two in proportions given below, and diluting to 100 mls with distilled water.

pH at 25°C	Na_2H PO_4	Na H_2PO_4
7.2	36.0	14.0

(iii) Tissues are then post-fixed in a 1% solution of osmium tetroxide in 0.1M phosphate buffer at 4°C for one hour.

(iv) Following post fixation tissues are washed in phosphate buffer by a brief immersion followed by a 2nd immersion for ½ hour at room temperature.

2. DEHYDRATION

Fixed tissued are dehydrated in capped bottles by continuous stirring in a graded alcohol series at room temperature, 25%, 50%, 70%, 90%, 100% x 2 at 20 minutes for each grade. Tissues may remain in 70% alcohol overnight if necessary.

3. INFILTRATION AND EMBEDDING

Alcohol is removed from the tissues by immersion (2 x 20 minutes periods) and stirring in propylene oxide. The tissues are then immersed in a mixture of 25% resin * and 75% propylene oxide and the bottles left for 12 hours with the tops off at room temperature, on the stirrer. The propylene oxide evaporates leaving the tissues in approximately 100% resin. Fresh 100% resin is added for 6 - 8 hours and tissues are then embedded in fresh resin in trays or capsules and cured for 48-72 hours at 60°C.

4. SECTIONING AND STAINING

Sections of 1μ cut on an ultramicrotome** and stained with 1% toluidine blue in 0.1 M phosphate buffer for 1 minute at 80°C are examined by light microscopy to identify suitable areas for electron microscopy.

Thin sections of silver/gold colour in reflected light are picked up on grids *** which had previously been dipped in a solution containing 40 cm of 12.5 mm cellotape in 100 mls of chloroform.

Immediately before examination using a Philips EM 200 electron microscope ****, thin sections are stained with uranyl acetate for 6 minutes and lead citrate for 6 minutes.

* Durcupan ACM Fluka
** LKB 8800A Ultrotome III: LKB ProdukterAB, S-161 25 Bromma 1, Sweden
*** Type G400 (3.05 mm), Polaron Equipment Ltd., Watford, England
**** Philips Electrical Industries of N.Z. Ltd., Wellington, N Z

Uranyl Acetate

Uranyl acetate is added to 50% ethanol until it no longer dissolves. The solution is centrifuged and the supernatant stored in a brown glass bottle.

Lead Citrate

0.025 g lead citrate

10 mls distilled water

0.1 mls 10 N NaOH

All ingredients are completely dissolved.

APPENDIX VI

Summary of abbreviations for clinical pathological data and bone marrow differentials.

i) Clinical pathological data

PCV	packed cell volume
Hb	haemoglobin
MCHC	mean cell haemoglobin concentration
II	icterus index
WBC	white blood cell
PMN	polymorphneutrophil
IMM	immature neutrophil
Lcyte	lymphocyte
Mcyte	monocyte
Eosin	eosinophil
Bas	basophil
Retic	reticulocyte
GGT	gamma glutamyl transpeptidase
AAT	aspartate amino transferase
AP	alkaline phosphatase
BUN	blood urea nitrogen
Tot.Bil	total bilirubin
Conj. Bil	conjugated bilirubin
TP	total protein
Fib	fibrinogen
glob	globulin
MAT	microscopic agglutination titre
SG	specific gravity
Prot	protein
Bil	bilirubin

ii) Bone marrow differentials

Mblast	myeloblast
Pgran	progranulocyte
m (n)	neutrophilic myelocyte
m (e)	eosinophilic myclocyte
m (b)	basophilic myelocyte
mm (n)	neutrophilic metamyelocyte
mm (e)	eosinophilic metamyelocyte
band (n)	neutrophilic band cell
band (e)	eosinophilic band cell
PMN	polymorphneutrophil
Eosin	eosinophil
Bas	basophil
Eblast	erythroblast
E.Norm	early normoblast
Norm	normoblast
L.Norm	late normoblast
Pl.cell	plasma cell
Lcyte	lymphocyte
Mcyte	monocyte
Mega	megakaryocyte
Retic	reticulum cell
Unclass	unclassifiable cell
Disint	disintegrated cell
M:E ratio	myeloid: erythroid ratio

APPENDIX VII (a) Clinical pathological data from control hamsters of Experiment I, Part I, euthanased on day 0.

Hamster	0 - 1	0 - 2	0 - 3	0 - 4	0 - 5
PCV	0.48	0.49	0.50	0.50	0.53
Hb (g/dℓ)	16.4	14.6	15.6	14.8	16.0
MCHC (g/dℓ)	34.2	29.8	31.2	29.0	32.0
II *	0	0	0	0	0
TP (g/ℓ)	48	48	53	54	52
WBC ($x10^9$/ℓ)	4.6	6.6	3.6	3.3	3.6
PMN ($x10^9$/ℓ)	0.3	0.3	0.3	0.2	0.4
IMM ($x10^9$/ℓ)	0	0	0	0	0
Eosin (x 10^9/ℓ)	0	0.1	0	0	0
Bas ($x10^9$/ℓ)	0	0	0	0	0
Lcyte ($x10^9$/ℓ)	4.2	6.2	3.2	3.0	3.1
Mcyte ($x10^9$/ℓ)	0.1	0	0.1	0.1	0.1
$\frac{\text{Organ Wgt (g)}}{\text{Body Wgt (g)}}$ Ratio					
$\frac{\text{Liver Wgt}}{\text{Body Wgt}}$	0.0616	0.0568	0.0548	0.0555	0.0486
$\frac{\text{Spleen Wgt}}{\text{Body Wgt}}$	0.0012	0.0012	0.0010	0.0009	0.0009
$\frac{\text{Kidney Wgt}}{\text{Body Wgt}}$	0.0101	0.0096	0.0081	0.0078	0.0072

* Normal hamster plasma is clear or slightly lipaemic

APPENDIX VII (b) Bone marrow differentials of control hamsters from Experiment I, Part I euthanased on day 0

Hamster	0 - 1		0 - 2		0 - 3		0 - 4		0 - 5	
	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series
Mblast	0	0	0	0	0	0	0.1	0.2	0.2	0.3
Pgran	2.4	4.2	0.7	1.6	0.4	0.7	0.7	1.2	0.8	1.4
m(n)	24.7	43.7	20.5	43.3	15.8	28.0	19.8	35.0	19.1	33.9
m(e)	0.3	0.5	0.2	0.5	1.0	1.8	0.4	0.7	0.4	0.7
m(b)	0	0	0.2	0.5	0.2	0.4	0	0	0.2	0.3
mm(n)	3.9	6.9	2.0	4.2	7.3	12.7	6.3	11.1	5.6	9.9
mm(e)	0	0	0.1	0.3	0	0	0	0	0.4	0.7
band(n)	11.7	20.6	11.8	24.5	12.4	21.8	8.5	15.1	12.2	21.6
band(e)	0.3	0.5	0	0	0.4	0.7	0	0	0	0
PMN	13.2	23.3	11.7	24.7	19.3	33.8	20.5	36.4	17.6	31.2
Eosin	0	0	0	0	0	0	0	0	0	0
Bas	0	0	0	0	0	0	0	0	0	0
Mitoses	0	0	0	0	0	0	0.1	0.1	0	0
Total	56.1%	100%	47.3%	100%	57.1%	100%	56.4%	100%	56.4%	100%
Eblast	0	0	0	0	0	0	0	0	0.4	2.0
Enorm	0.1	0.8	0.7	3.5	1.2	6.5	0.4	2.0	1.2	6.0
Norm	12.0	61.1	13.2	62.0	13.5	69.9	16.0	81.9	13.5	70.0
L.norm	7.5	38.2	7.3	34.5	4.4	22.6	2.9	14.8	4.1	21.0
Mitoses	0	0	0	0	0.2	1.1	0.3	1.3	0.2	1.0
Total	19.6%	100%	21.2%	100%	19.3%	100%	19.5%	100%	19.3%	100%
Pl.cell	0		0		0		0		0	
Lcyte	22.7		28.9		18.5		17.9		19.7	
Mcyte	0		0		0		0		0	
Mega	0.8		0.2		0.2		0.8		0.6	
Retic	0.4		2.0		0.6		0		0.6	
Unclass	0.4		0.2		0.8		2.6		1.7	
Disint	0		0.1		3.5		2.7		1.7	
M:E ratio	2.9:1		2.3:1		3.0:1		2.9:1		2.9:1	

APPENDIX VIII (a) Clinical pathological data from *ballum* infected hamsters of Experiment I, Part I euthanased on Day 1, am.

Hamster	1 - 1	1 - 2	1 - 3	1 - 4	1 - 5	1 - 6
PCV	0.51	0.49	0.50	0.49	0.48	0.45
Hb (g/dℓ)	16.6	15.0	16.8	16.4	16.0	14.6
MCHC (g/dℓ)	32.5	30.6	33.6	33.5	33.3	3.24
II	0	0	0	0	0	0
TP (g/ℓ)	56	55	54	56	56	50
WBC ($x10^9/ℓ$)	13.3	4.5	3.3	5.1	6.4	4.7
PMN ($x10^9/ℓ$)	1.1	0.8	0.4	0.8	0.8	0.9
IMM ($x10^9/ℓ$)	0	0	0	0	0	0
Eosin ($x10^9/ℓ$)	0	0	0	0.2	0.1	0.1
Bas ($x10^9/ℓ$)	0	0	0	0	0	0
Lcyte ($x10^9/ℓ$)	12.1	3.7	2.9	4.0	5.4	3.8
Mcyte ($x10^9/ℓ$)	0.1	0	0	0.1	0	0
Organ Wgt(g) / Body Wgt(g) Ratio						
Liver Wgt / Body Wgt	0.0456	0.0604	0.0510	0.0605	0.0513	0.0542
Spleen Wgt / Body Wgt	0.0011	0.0018	0.0012	0.0014	0.0013	0.0013
Kidney Wgt / Body Wgt	0.0079	0.0094	0.0074	0.0093	0.0083	0.0080

APPENDIX VIII (b) Bone marrow differentials of *ballum* infected hamsters from Experiment I, Part I, euthanased on Day 1, am.

Hamster	1 - 1		1 - 2		1 - 3		1 - 4		1 - 5		1 - 6	
	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series
Mblast	0	0	0	0	0	0	0	0	0	0	0	0
Pgran	1.2	2.0	1.2	2.8	0.8	1.5	0.2	0.5	0.4	0.9	0.3	0.6
m(n)	24.2	41.3	22.4	52.8	18.2	32.8	15.8	37.3	15.8	37.3	17.6	40.4
m(e)	0.2	0.4	0.3	0.6	0.2	0.4	0.8	1.9	0.2	0.6	0.3	0.6
m(b)	0	0	0.3	0.6	0.1	0.2	0.2	0.5	0.8	1.8	0.4	0.9
mm(n)	0.9	1.6	2.1	5.0	6.0	10.9	0.6	1.4	6.4	14.7	3.9	8.0
mm(e)	0	0	0.1	0.3	0.2	0.4	0.4	1.0	0	0	0.3	0.6
band(n)	9.9	16.9	7.7	18.2	10.5	18.9	4.4	10.5	6.2	14.2	7.2	14.8
band(e)	0	0	0.3	0.6	0.1	0.2	0.4	1.0	0.2	0.6	0.1	0.3
PMN	22.1	37.8	7.9	18.6	19.3	34.7	19.2	45.5	11.6	26.6	22.1	45.3
Eosin	0	0	0.1	0.3	0	0	0.2	0.5	0	0	0	0
Bas	0	0	0	0	0	0	0	0	0	0	0	0
Mitoses	0	0	0	0	0	0	0	0	0.2	0.6	0	0
Total	58.5%	100%	42.3%	100%	55.6%	100%	42.2	100%	43.5%	100%	48.9%	100%
Eblast	0	0	0	0	0.1	0.6	0.2	0.6	0.2	0.6	0	0
Enorm	0	0	0	0	1.3	6.7	0.6	1.8	2.8	8.8	1.4	6.3
Norm	0.7	97.0	16.8	77.8	11.8	60.6	17.2	51.5	22.4	70.0	13.0	56.6
L.norm	0.2	3.0	4.7	21.6	6.0	30.9	15.4	46.1	6.4	20.0	8.5	37.1
Mitoses	0	0	0.1	0.6	0.2	1.2	0	0	0.2	0.6	0	0
Total	7.8%	100%	21.6%	100%	19.5%	100%	33.3%	100%	31.9%	100%	23.0%	100%
Pl.cell	0		0		0		0		0		0.1	
Lcyte	29.0		35.2		19.6		19.8		18.8		18.4	
Mcyte	0		0		0		0.2		0		0.3	
Mega	0.2		0.4		0.2		0		0.2		0	
Retic	1.2		0.3		0.5		1.6		0.4		2.0	
Unclass	2.8		0.3		0		1.0		0.8		1.9	
Disint	0.5		0		3.3		0.2		4.4		5.4	
M:E ratio	7.5:1		2.0:1		2.8:1		1.3:1		1.4:1		2.1:1	

APPENDIX IX(a) Clinical pathological data from *ballum* infected hamsters of Experiment I, Part I euthanased on day 2, am.

Hamster	2 - 1	2 - 2	2 - 3	2 - 4	2 - 5	2 - 6
PCV	0.52	0.50	0.44	0.49	0.50	0.52
Hb (g/dℓ)	16.2	16.2	13.0	16.4	16.6	16.6
MCHC (g/dℓ)	31.2	32.4	29.5	33.5	33.2	31.9
II	0	0	0	0	0	0
TP (g/ℓ)	54	54	50	56	54	52
WBC ($x10^9$/ℓ)	5.4	1.2	4.5	12.7	6.3	4.8
PMN ($x10^9$/ℓ)	0.8	0.3	0.3	1.4	0.6	0.7
IMM ($x10^9$/ℓ)	0	0	0	0	0	0
Eosin ($x10^9$/ℓ)	0	0	0.1	0	0	0
Bas ($x10^9$/ℓ)	0	0	0	0	0	0
Lcyte ($x10^9$/ℓ)	4.6	0.9	4.1	11.0	5.7	4.1
Mcyte ($x10^9$/ℓ)	0.1	0	0	0.3	0.1	0
Organ Wgt(g) / Body Wgt(g) Ratio						
Liver Wgt / Body Wgt	0.0500	0.0426	0.0627	0.0503	0.0504	0.0485
Spleen Wgt / Body Wgt	0.0010	0.0013	0.0016	0.0013	0.0015	0.0010
Kidney Wgt / Body Wgt	0.0086	0.0097	0.0116	0.0098	0.0099	0.0079

APPENDIX IX (b) Bone marrow differentials of *ballum* infected hamsters from Experiment I, Part I euthanased on day 2, am.

Hamster	2 - 1		2 - 2		2 - 3		2 - 4		2 - 5		2 - 6	
	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series
Mblast	0	0	0	0	0	0	0	0	0	0	0	0
Pgran	1.3	2.8	0.8	1.9	1.0	2.2	0.2	0.5	0.6	1.1	0.7	1.3
m(n)	18.4	39.1	24.3	57.1	19.7	44.9	17.2	38.2	17.3	34.1	16.3	32.3
m(e)	0.4	0.8	0.7	1.6	0.5	1.1	0.2	0.5	0.9	1.9	0.7	1.3
m(b)	0.1	0.3	0.3	0.8	0.2	0.4	0.2	0.5	0.9	1.9	0.4	0.8
mm(n)	1.8	3.8	1.3	3.2	4.6	10.5	1.1	2.4	4.7	9.3	1.6	3.5
mm(e)	0.2	0.5	0.3	0.8	0.2	0.4	0.4	0.9	0.4	0.7	0.4	0.8
band(n)	7.8	16.5	4.4	10.3	7.6	17.2	2.5	5.7	9.9	19.6	5.3	10.4
band(e)	0.2	0.5	0.3	0.8	1.2	2.6	0.2	0.5	0.6	1.1	0.3	0.5
PMN	16.7	35.6	9.8	23.0	9.0	20.6	22.7	50.5	15.6	31.5	25.1	49.6
Eosin	0	0	0	0	0	0	0.2	0.5	0	0	0	0
Bas	0	0	0.2	0.8	0	0	0	0	0	0	0	0
Mitoses	0.1	0.3	0	0	0	0	0	0	0	0	0	0
Total	47.0%	100%	42.5%	100%	43.9%	100%	44.9%	100%	50.7%	100%	50.5%	100%
Eblast	0	0	0.2	0.7	0	0	0.4	2.8	0.4	1.5	0.3	1.6
Enorm	0.4	2.7	0.2	0.7	1.3	6.0	1.1	7.0	0.9	3.7	0.9	5.4
Norm	10.1	76.1	14.0	58.5	11.3	51.5	8.1	53.5	15.6	61.9	6.7	38.8
L.norm	2.7	20.4	9.4	39.4	8.9	40.3	5.5	36.6	8.1	32.1	9.0	51.9
Mitoses	0.1	0.9	0.2	0.7	0.5	2.2	0	0	0.2	0.7	0.4	2.3
Total	13.3%	100%	23.9%	100%	22.0%	100%	15.0%	100%	25.1%	100%	17.4%	100%
Pl.cell	0		0		0		0		0		0	
Lcyte	36.3		30.2		29.1		34.7		28.5		31.5	
Mcyte	0.1		0		0		0		0		0	
Mega	0.2		0		0		0.2		0.2		0.3	
Retic	1.5		2.5		1.2		3.4		0		0.3	
Unclass	1.5		0.7		1.2		1.7		0.6		0	
Disint	0		0.2		2.6		0		2.1		0	
M:E ratio	3.5:1		1.1:1		1.0:1		3.0:1		2.0:1		2.9:1	

APPENDIX X (a) Clinical pathological data from *ballum* infected hamsters of Experiment I, Part I euthanased on Day 3, am.

Hamster	3 - 1	3 - 2	3 - 3	3 - 4	3 - 5	3 - 6*
PCV	0.49	0.44	0.48	0.49	0.42	
Hb (g/dℓ)	16.8	14.4	15.4	16.2	14.2	
MCHC (g/dℓ)	34.3	32.7	32.1	33.1	33.8	
II	0	0	0	0	15-20 §	
TP (g/ℓ)	60	58	55	53	58	
WBC ($x10^9/ℓ$)	8.1	4.5	4.6	1.8	13.7	
PMN ($x10^9/ℓ$)	1.2	1.6	0.5	0.5	5.0	
IMM ($x10^9/ℓ$)	0	0	0	0	0.6	
Eosin ($x10^9/ℓ$)	0	0	0.1	0.2	0.1	
Bas ($x10^9/ℓ$)	0	0	0	0	0	
Lcyte ($x10^9/ℓ$)	6.9	2.8	3.9	1.3	7.8	
Mcyte ($x10^9/ℓ$)	0	0.1	0.2	0.1	0.1	
Organ Wgt(g) / Body Wgt(g) Ratio						
Liver Wgt / Body Wgt	0.0541	0.0651	0.0586	0.0494	0.0629	
Spleen Wgt / Body Wgt	0.0012	0.0022	0.0014	0.0015	0.0043	
Kidney Wgt / Body Wgt	0.0097	0.0117	0.0097	0.0093	0.0109	

* Found dead, no sample available

§ haemoglobinaemia

APPENDIX X (b) Bone marrow differentials of *ballum* infected hamsters from Experiment I, Part I euthanased on day 3, am.

Hamster	3 - 1		3 - 2		3 - 3		3 - 4		3 - 5	
	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series
Mblast	0	0	0	0	0.4	0.8	0	0	0	0
Pgran	0.5	0.9	0.7	1.9	0.6	1.4	0.3	0.9	0.6	1.9
m(n)	18.9	42.0	18.4	49.6	19.8	43.0	14.7	42.9	10.3	34.2
m(e)	0.4	0.8	1.1	3.0	0.5	1.1	0.2	0.6	0.6	1.9
m(b)	0.3	0.6	1.0	2.6	0.8	1.6	0.2	0.6	0.6	1.9
mm(n)	4.0	8.8	1.5	4.1	6.3	13.7	1.0	2.8	4.0	13.2
mm(e)	0.7	1.3	0.3	0.7	0	0	0	0	0.1	0.3
band(n)	7.7	17.1	5.8	15.6	7.6	16.4	5.1	14.9	12.1	39.9
band(e)	0.1	0.2	0.3	0.7	0.1	0.3	0.2	0.6	0.1	0.3
PMN	12.6	28.1	7.7	20.7	9.3	20.3	12.5	36.3	1.8	6.1
Eosin	0	0	0.1	0.4	0.1	0.3	0.1	0.3	0	0
Bas	0	0	0.1	0.4	0	0	0	0	0	0
Mitoses	0	0	0.1	0.4	0.5	1.1	0	0	0.1	0.3
Total	44.9%	100%	37.0%	100%	46.0%	100%	34.3%	100%	30.3%	100%
Eblast	0	0	0	0	0.1	0.4	0	0	0.6	1.6
Enorm	0.3	0.8	1.1	2.9	0.5	1.7	0.2	2.3	1.2	3.2
Norm	14.8	48.6	13.2	35.3	17.9	61.5	5.9	62.5	16.8	46.8
L.norm	15.2	49.7	22.1	59.2	10.3	35.5	3.3	35.2	16.8	46.5
Mitoses	0.3	0.8	1.0	2.6	0.3	0.9	0	0	0.7	1.9
Total	30.5%	100%	37.3%	100%	29.1%	100%	9.4%	100%	36.0%	100%
Pl.cell	0		0		0		0		0	
Lcyte	22.5		24.7		23.4		53.9		31.0	
Mcyte	0		0		0		0		0	
Mega	0.2		0.7		0.3		0.3		0.1	
Retic	0.3		0.3		0.5		1.3		0.2	
Unclass	0.6		0		0.5		0.9		1.9	
Disint	1.2		0		0.3		0		0.5	
M:E ratio	1.5:1		1:1		1.6:1		3.7:1		0.8:1	

Hamster 3-6 was heavily pregnant therefore a bone marrow differential was not made

APPENDIX XI (a)Clinical pathological data from *ballum* infected hamsters of Experiment I, Part I euthanased or found dead on day 4, am

Hamster	4 - 1	4 - 2	4 - 3	4 - 4	4 - 5	4 - 6	4 - 7**
PCV	0.31	0.52	0.18	0.43	0.48	0.50	
Hb (g/dℓ)	11.0	16.6	9.6	13.2	15.6	16.6	
MCHC (g/dℓ)	35.5	32.0	53.8	30.7	32.5	33.2	
II	¶	0	¶	20+§	0	0	
TP (g/ℓ)	80	62	96	56	62	62	
WBC ($x10^9/ℓ$)	14.5	14.7	17.5	12.0	5.2	13.2	
PMN ($x10^9/ℓ$)	7.7	6.5	9.2	7.0	2.0	4.0	
IMM ($x10^9/ℓ$)	1.3	0.4	2.8	1.0	0.1	0.1	
Eosin ($x10^9/ℓ$)	0	0	0.2	0.1	0.2	0.1	
Bas ($x10^9/ℓ$)	0	0	0	0	0	0	
Lcyte ($x10^9/ℓ$)	5.5	7.1	5.3	3.7	2.8	8.8	
Mcyte ($x10^9/ℓ$)	0.6	0.7	0.4	0.2	0.2	0.1	
Tot.Bil µmol/ℓ	0*	0	0*	56.86	0	0	
Conj.Bil µmol/ℓ	0	0	0	35.91	0	0	
Organ Wgt(g) / Body Wgt(g) ratio							
Liver Wgt / Body Wgt	0.0705	0.0436	0.0663	0.0490	0.0498	0.0612	0.0711
Spleen Wgt / Body Wgt	0.0044	0.0019	0.0033	0.0036	0.0020	0.0021	0.0031
Kidney Wgt / Body Wgt	0.0139	0.0079	0.0122	0.0091	0.0097	0.0107	0.0148

** Found dead, no sample available

* Free haemoglobin interferred with bilirubin reading as plasma was obviously jaundiced.

¶ Severe jaundice & haemoglobinaemia

§ Haemoglobinaemia

APPENDIX XI (b) Bone marrow differentials of *ballum* infected hamsters from Experiment I, Part I, euthanased on day 4 am.

Hamster	4 - 1		4 - 2		4 - 3		4 - 4		4 - 5		4 - 6	
	% Total	Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series
Mblast	0	0	0	0	0	0	0	0	0	0	0	0
Pgran	0.5	2.2	1.3	2.5	0.4	1.0	0.3	0.4	0.3	0.6	0.1	0.3
m(n)	18.2	76.4	18.1	36.1	24.6	65.2	20.5	58.8	26.6	64.4	19.4	53.2
m(e)	0.3	1.3	0.5	1.0	0.1	0.3	1.5	4.4	0	0	0.5	1.4
m(b)	0.1	0.4	1.1	2.3	1.3	3.4	1.4	4.0	0	0	0.2	0.6
mm(n)	1.6	6.7	5.7	11.4	6.8	18.1	2.7	7.6	6.1	14.9	1.2	3.3
mm(e)	0.2	0.9	0.4	3.1	0.4	1.0	0.8	2.4	0.1	0.3	0.2	0.6
band(n)	2.1	8.4	12.0	24.0	2.7	7.2	4.9	14.0	6.3	15.2	3.4	9.4
band(e)	0.5	2.2	0.5	1.0	0.1	0.3	0.8	2.4	0	0	0.3	0.8
PMN	0.2	0.9	10.2	20.5	0.8	2.0	1.4	4.0	1.9	4.5	10.8	29.6
Eosin	0	0	0	0	0	0	0.1	0.4	0	0	0.2	0.6
Bas	0	0	0.1	0.3	0.5	1.4	0	0	0	0	0	0
Mitoses	0.1	0.4	0.1	0.3	0	0	0.4	1.2	0	0	0.1	0.3
Total	24.6%	100%	50.0%	100%	37.8%	100%	34.9%	100%	41.3%	100%	36.5%	100%
Eblast	0	0	0	0	0	0	0	0	0	0	0.1	0.8
Enorm	0.5	1.3	0.5	2.5	0.4	1.1	1.1	4.3	0.7	4.0	0.1	0.8
Norm	24.0	57.4	8.8	44.3	14.8	43.9	10.1	39.1	8.4	50.8	5.6	45.9
L.norm	17.2	41.0	10.6	53.2	18.3	54.2	14.1	54.9	7.2	43.5	6.4	52.5
Mitoses	0.1	0.3	0	0	0.3	0.8	0.4	1.6	0.3	1.6	0	0
Total	41.9%	100%	19.9%	100%	33.8%	100%	25.7%	100%	16.6%	100%	12.2%	100%
Pl.cell	0		0		0		0		0		0.2	
Lcyte	31.6		26.9		26.3		37.3		36.9		49.1	
Mcyte	0		0		0		0.1		0		0	
Mega	0.2		0.8		0.5		0.4		1.1		0.2	
Retic	0.2		1.1		0.6		1.5		0.7		0.8	
Unclass	0.6		0.8		0.3		0		1.6		0.4	
Disint	0.8		0.5		0.6		0		1.9		0.4	
M:E ratio	0.6:1		2.5:1		1.1:1		1.4:1		2.5:1		3.0:1	

APPENDIX XII(a) Clinical pathological data from *ballum* infected hamsters of experiment I, Part I euthanased or found dead on day 4, pm.

Hamster	5 - 1	5 - 2	5 - 3	5 - 4	5 - 5	5 - 6	5 - 7*
PCV	0.12	0.13	0.25	0.38	0.33	0.44	
Hb (g/dℓ)	8.6	9.2	11.0	13.0	12.0	14.0	
MCHC (g/dℓ)	71.7	70.8	44.0	34.2	36.4	31.8	
II	¶	¶	¶	¶	20+ §	15	
TP (g/ℓ)	90	80	75	72	74	60	
WBC ($x10^9/\ell$)	15.1	29.1	11.7	15.9	18.8	12.8	
PMN ($x10^9/\ell$)	6.3	13.4	3.4	8.9	10.2	6.9	
IMM ($x10^9/\ell$)	2.3	2.0	2.8	2.5	1.3	1.3	
Eosin ($x10^9/\ell$)	0.2	0.6	0.5	0	0.6	0.3	
Bas ($x10^9/\ell$)	0	0	0	0	0	0	
Lcyte ($x10^9/\ell$)	5.1	4.9	4.0	4.3	1.9	2.7	
Mcyte ($x10^9/\ell$)	1.2	2.3	1.1	0.2	8.6	0.5	
Organ Wgt(g) / Body Wgt(g) Ratio							
Liver Wgt / Body Wgt	0.0607	0.0692	0.0688	0.0623	0.0602	0.0595	0.0708
Spleen Wgt / Body Wgt	0.0034	0.0038	0.0051	0.0049	0.0042	0.0045	0.0043
Kidney Wgt / Body Wgt	0.0110	0.0129	0.0131	0.0128	0.0125	0.0106	0.0152

* Found dead, no sample available

¶ Severe jaundice & haemoglobinaemia

§ Haemoglobinaemia

APPENDIX XII(b) Bone marrow differentials of *ballum* infected hamsters from Experiment I, Part I, euthanased on day 4, pm.

Hamster	5 - 3		5 - 4		5 - 6	
	% Total	% Series	% Total	% Series	% Total	% Series
Mblast	0	0	0	0	0	0
Pgran	1.3	5.8	1.1	3.2	1.2	3.3
m(n)	14.2	65.4	20.4	62.3	19.8	56.3
m(e)	0	0	0.5	1.0	0.5	1.3
m(b)	0.8	3.8	0.3	1.0	1.2	3.3
mm(n)	2.5	11.5	3.7	11.4	5.3	15.3
mm(e)	0	0	0.4	1.0	1.6	4.6
band(n)	1.7	7.7	5.1	15.6	4.4	12.6
band(e)	1.3	5.8	0.2	0.6	0.2	0.7
PMN	0	0	0.7	2.3	0.5	1.3
Eosin	0	0	0.1	0.3	0	0
Bas	0	0	0.1	0.3	0.5	1.3
Mitoses	0	0	0	0	0	0
Total	21.8%	100%	32.7%	100%	35.1%	100%
Eblast	0	0	0	0	0	0
Enorm	0	0	0.6	2.5	0.2	0.7
Norm	7.1	51.5	10.7	41.6	51.1	45.1
L.norm	6.7	48.5	14.0	54.3	18.1	54.2
Mitoses	0	0	0.4	1.6	0	0
Total	13.8%	100%	25.8%	100%	33.5%	100%
Pl.cell	0		0		0	
Lcyte	55.6		38.9		29.5	
Mcyte	0		0		0	
Mega	0.4		0.3		0.2	
Retic	2.5		0.8		0.2	
Unclass	0.2		0.3		0.5	
Disint	5.0		1.2		0.9	
M:E ratio	1.6:1		1.8:1		1.0:1	

Marrow smears from hamsters 5-1, 5-2, 5-5 were unsuitable for differentials

APPENDIX XIII (a) Clinical pathological data from *ballum* infected hamsters of Experiment I, Part I euthanased on day 5, am.

Hamster	6 - 1	6 - 2	6 - 3	6 - 4	6 - 5	6 - 6*	6 - 7
PCV	0.035	0.12	0.075	0.085	0.28		0.49
Hb (g/dℓ)	5.8	8.6	6.2	12.0	12.6		14.8
MCHC (g/dℓ)	165.7	71.7	82.7	141.2	45.0		30.2
II	¶	¶	¶	¶	¶	20+§	0
TP (g/ℓ)	98	96	92	82	64		58
WBC ($x10^9/ℓ$)	8.7	8.7	4.1	34.3	54.0		3.0
PMN ($x10^9/ℓ$)	3.8	4.5	2.0	14.7	36.7		0.7
IMM ($x10^9/ℓ$)	2.1	1.8	0.8	4.1	5.4		0
Eosin ($x10^9/ℓ$)	0.4	0.1	0	1.0	1.1		0.1
Bas ($x10^9/ℓ$)	0	0	0.1	0	0		0
Lcyte ($x10^9/ℓ$)	2.3	1.2	1.0	9.9	3.2		2.2
Mcyte ($x10^9/ℓ$)	0.3	1.0	0.2	4.5	7.6		0.1
Organ Wgt(g) / Body Wgt(g) Ratio							
Liver Wgt / Body Wgt	0.0615	0.0584	0.0572	0.0560	0.0611	0.0504	0.0563
Spleen Wgt / Body Wgt	0.0054	0.0023	0.0053	0.0025	0.0033	0.0038	0.0020
Kidney Wgt / Body Wgt	0.0019	0.0138	0.0106	0.0126	0.0130	0.0111	0.0094

* Sample clotted

¶ Severe jaundice & haemoglobinaemia

§ Haemoglobinaemia

APPENDIX XIII (b) Bone marrow differentials of *ballum* infected hamsters from Experiment I, Part I euthanased on day 5, am.

Hamster	6 - 1		6 - 2		6 - 3		6- 4		6 - 5		6 - 6		6 - 7	
	% Total	Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	Series
Mblast	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pgran	0.5	1.3	2.2	5.6	0.3	0.9	3.1	5.4	1.9	2.7	0.4	0.8	1.0	2.3
m(n)	26.0	67.8	20.7	53.1	22.2	64.2	28.2	48.9	31.5	45.6	37.3	75.6	22.9	52.3
m(e)	0.3	0.8	0.7	1.7	0.8	2.3	0.2	0.4	0.2	0.3	0.2	0.4	0.4	0.9
m(b)	0	0	0.3	0.7	0	0	0.2	0.4	0.2	0.3	0	0	0.3	0.6
mm(n)	6.3	16.3	6.3	16.1	5.9	17.0	7.1	12.3	12.6	18.2	2.9	5.6	3.4	7.7
mm(e)	0.2	0.4	0.3	0.7	0.3	0.9	0	0	0.2	0.3	0	0	0.1	0.3
band(n)	4.5	11.7	4.8	12.2	3.5	10.1	9.4	16.3	18.2	26.4	7.3	14.5	10.3	23.4
band(e)	0.3	0.8	2.0	5.2	1.0	2.8	0.4	0.7	0.2	0.3	0.2	0.4	0.8	2.0
PMN	0.2	0.4	1.4	3.5	0.5	1.4	8.4	14.5	1.6	2.4	2.3	4.4	3.6	8.3
Eosin	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bas	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mitoses	0.2	0.4	0.4	1.0	0.2	0.5	0.6	1.1	2.3	3.4	0.6	1.2	1.0	2.3
Total	38.4%	100%	38.9%	100%	34.6%	100%	57.7%	100%	69.0%	100%	51.3%	100%	43.8%	100%
Eblast	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enorm	0.2	0.8	0.3	1.0	0.2	1.1	0	0	0.2	5.0	0	0	0.8	3.1
Norm	14.8	71.8	16.0	61.8	20.5	69.0	10.0	67.6	2.1	45.0	9.7	73.4	16.4	67.5
L.norm	5.6	27.3	9.4	36.1	8.9	29.9	4.6	31.0	2.3	50.0	3.5	26.6	6.8	27.8
Mitoses	0	0	0.3	1.0	0	0	0.2	1.4	0	0	0	0	0.4	1.5
Total	20.6%	100%	26.0%	100%	29.7%	100%	14.9%	100%	4.7%	100%	13.3%	100%	24.3%	100%
Pl.cell	0		0.1		0		0		0.5		0		0.1	
Lcyte	37.1		30.8		30.6		25.3		20.3		31.7		30.4	
Mcyte	0		0		0		0		0		0		0	
Mega	0.3		0.5		0.5		0.4		0.5		0		0.3	
Retic	1.6		1.1		2.9		0.4		2.3		2.3		1.0	
Unclass	1.6		2.0		1.4		0.8		1.9		1.0		0.3	
Disint	0.3		0.5		0.2		0.4		0.9		0.4		0.3	
M:E ratio	1.9:1		1.5:1		1.2:1		3.9:1		14:1		3.9:1		1.8:1	

APPENDIX XIII(c) Organ weight/body weight ratios from *ballum* infected hamsters of Experiment I, Part I found dead on day 5, am.

Hamster	7 - 1	7 - 2	7 - 3	7 - 4	7 - 5
Liver Wgt / Body Wgt	0.0808	0.0700	0.0672	0.0786	0.0642
Spleen Wgt / Body Wgt	0.0050	0.0038	0.0029	0.0057	0.0033
Kidney Wgt / Body Wgt	0.0149	0.0139	0.0121	0.0145	0.0117

APPENDIX XIV (a) Clinical pathological data from control hamsters of experiment I, Part I euthanased on day 6.

Hamster	8 - 1	8 - 2	8 - 3	8 - 4	8 - 5
PCV	0.50	0.50	0.50	0.51	0.53
Hb (g/dℓ)	16.2	16.8	16.8	16.8	17.8
MCHC (g/dℓ)	32.4	33.6	33.6	32.9	33.6
II	0	0	0	0	0
TP (g/ℓ)	52	52	54	50	52
WBC ($x10^9$/ℓ)	1.7	3.2	9.0	4.1	7.3
PMN ($x10^9$/ℓ)	0.5	0.5	0.7	1.8	1.4
IMM ($x10^9$/ℓ)	0	0	0	0	0
Eosin ($x10^9$/ℓ)	0	0	0.2	0.1	0.1
Bas ($x10^9$/ℓ)	0	0	0	0	0
Lcyte ($x10^9$/ℓ)	1.0	2.7	7.8	2.2	5.8
Mcyte ($x10^9$/ℓ)	0	0	0.3	0.1	0
Organ Wgt(g) / Body Wgt(g) Ratio					
Liver Wgt / Body Wgt	0.0438	0.0425	0.0466	0.0492	0.0448
Spleen Wgt / Body Wgt	0.0012	0.0011	0.0011	0.0011	0.0009
Kidney Wgt / Body Wgt	0.0086	0.0072	0.0074	0.0078	0.0074

APPENDIX XIV(b) Bone marrow differentials of control hamsters from Experiment I, Part I euthanased on day 6.

Hamster	8 - 1		8 - 2		8 - 3		8 - 4		8 - 5	
	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series
Mblast	0.6	1.2	0.5	1.1	0	0	0	0	0	0
Pgran	0.2	0.4	0.6	1.3	2.3	4.8	1.8	2.8	2.5	4.5
m(n)	16.2	30.0	15.1	31.5	13.4	27.1	21.2	32.5	22.2	40.5
m(e)	0.6	1.2	0.7	1.4	0.5	1.0	0.3	0.5	0.4	0.7
m(b)	0.4	0.8	0.4	0.9	0.4	0.8	0.3	0.5	0.6	1.1
mm(n)	3.6	6.8	5.1	10.6	4.5	9.1	7.5	11.4	3.9	7.2
mm(e)	0	0	0.2	0.4	0.6	1.2	0.3	0.5	0.3	0.5
band(n)	9.8	18.2	4.7	9.9	8.4	17.0	8.4	12.9	9.2	16.7
band(e)	0.4	0.8	0.7	1.4	0.2	0.4	0.2	0.2	0.2	0.4
PMN	21.6	39.9	19.7	41.2	18.9	38.1	25.2	38.6	15.1	27.5
Eosin	0	0	0.1	0.2	0	0	0	0	0	0
Bas	0	0	0	0	0	0	0	0	0	0
Mitoses	0.4	0.8	0.1	0.2	0.3	0.6	0.2	0.2	0.5	0.9
Total	54.1%	100%	47.9%	100%	49.5%	100%	65.3%	100%	54.7%	100%
Eblast	0.6	2.4	0	0	0	0	0	0	0.3	1.0
Enorm	2.1	8.0	1.4	6.1	1.9	6.4	0.6	5.0	0.9	3.0
Norm	16.7	62.4	14.7	65.8	19.3	66.3	8.5	69.1	16.8	58.1
L.norm	7.3	27.2	6.1	27.3	7.3	25.3	2.9	23.5	10.0	34.6
Mitoses	0	0	0.2	0.8	0.5	2.0	0.3	2.5	1.0	3.3
Total	26.7%	100%	22.4%	100%	29.1%	100%	12.4%	100%	28.9%	100%
Pl.cell	0		0		0		0		0.1	
Lcyte	19.2		29.4		21.3		21.7		16.1	
Mcyte	0		0		0		0		0.1	
Mega	0		0.3		0.1		0.6		0.1	
Retic	0.1		0		0		0.1		0	
Unclass	0		0.1		0		0		0	
Disint	0		0		0.1		0		0	
M:E ratio	2.0:1		2.1:1		1.3:1		5.6:1		1.9:1	

APPENDIX XV: Means, standard deviations and ranges of organ weight: body weight ratios of 10 untreated control hamsters from Experiment I, Part I.

	$\bar{x}$	SD	Range
$\frac{\text{Liver wgt(g)}}{\text{body wgt(g)}}$	0.0504	±0.00065	0.0425 - 0.0616
$\frac{\text{Spleen wgt(g)}}{\text{body wgt(g)}}$	0.0011	±0.00032	0.0009 - 0.0012
$\frac{\text{Kidney wgt(g)}}{\text{body wgt(g)}}$	0.0081	±0.0010	0.0072 - 0.0101

APPENDIX XVI: Clinical pathological data from *pomona* infected calf 11

Day P.I.	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Temp am °C	38.0	38.7	38.0	38.4	38.0	38.6	38.0	38.0	37.4	37.5	37.7	37.9	38.0	38.6	39.1	38.1	38.4	39.4
Temp pm °C	ND	ND	39.2	38.8	38.8	38.4	39.1	39.1	39.2	38.5	39.2	39.0	38.2	39.1	39.2	39.1	38.6	Euth
Haematology																		
PCV	0.33	0.32	0.32	0.33	0.32	0.31	0.29	0.32	0.31	0.31	0.25	0.27	0.31	0.27	0.25	0.24	0.24	0.22
Hb g/dℓ	11.4	11.0	10.6	10.3	10.0	9.4	8.9	9.3	9.4	9.8	8.2	9.0	10.0	8.2	7.8	7.6	7.4	7.4
MCHC g/dℓ	34.5	34.4	33.1	31.2	31.3	30.3	30.7	29.1	30.3	31.6	32.8	33.3	32.3	30.4	31.2	31.7	30.8	33.6
II	2	2	2	2	2	2	2	2	2	2	2	10+	15+	50	50	25	10-15	10-15
WBC x 10^9/ℓ	8.3	5.3	4.8	5.3	5.9	7.0	7.0	7.4	6.2	6.1	7.7	8.0	13.7	12.9	9.7	9.6	11.3	7.5
PMN x 10^9/ℓ	4.1	1.5	1.4	2.0	1.5	1.2	2.8	2.8	2.4	2.2	3.3	2.8	3.0	5.3	3.2	3.5	3.2	2.3
IMM x 10^9/ℓ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lcyte x 10^9/ℓ	3.5	3.3	3.0	2.9	4.0	4.8	3.7	3.0	3.3	3.0	4.1	4.3	9.2	6.2	5.9	5.4	7.7	4.7
Mcyte x 10^9/ℓ	0.7	0.5	0.3	0.3	0.4	1.1	0.5	1.4	0.5	0.9	0.3	0.9	1.5	1.2	0.6	0.6	0.3	0.5
Eosin x 10^9/ℓ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0.1	0
Bas x 10^9/ℓ	0.1	0	0.1	0.1	0	0	0	0.1	0	0.1	0	0	0	0.3	0	0.1	0	0
Retic %	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Blood Biochemistry																		
GGT IU/ℓ	632	434	351	295	283	245	236	202	177	165	179	172	278	271	307	288	286	203
AAT IU/ℓ	30	68	68	58	42	27	35	43	31	33	78	71	66	49	47	51	57	57
AP IU/ ℓ	384	405	386	314	458	499	406	444	337	449	411	519	750	843	810	766	799	792
BUN mmol/ℓ	5.4	6.2	7.1	8.9	8.9	9.8	7.1	5.4	8.0	8.0	8.9	8.0	13.4	8.9	7.1	8.0	5.4	4.5
Tot.Bil μmol/ℓ	4.2	4.2	4.2	4.2	2.1	2.1	6.3	2.1	2.1	4.2	4.2	18.9	75.6	58.8	56.7	42.0	25.2	10.5
Conj.Bil μmol/ℓ	0	1.05	0	1.05	0	0	1.05	1.05	0	0	1.05	10.5	33.6	29.4	29.4	18.9	10.5	4.2
TP g/ℓ	86	80	76	74	80	74	74	76	74	72	74	76	82	76	72	74	74	76
Fib g/ℓ	8	4	8	8	4	10	4	6	6	4	2	8	8	6	4	8	4	10
Albumin g/ℓ	28.7	29.0	26.3	23.0	29.3	25.9	26.7	29.7	29.2	26.1	31.2	25.9	28.6	28.4	26.4	27.1	28.4	27.5
α glob g/ℓ	6.9	8.7	7.4	9.6	9.5	7.6	8.6	8.5	7.1	8.0	6.5	9.1	11.7	11.8	10.8	9.8	11.5	10.0
β glob g/ℓ	11.9	15.1	10.1	12.1	14.6	12.6	14.7	13.0	12.3	16.1	16.5	14.1	14.7	13.9	16.9	14.1	15.5	14.9
γ glob g/ℓ	30.6	23.3	24.2	21.4	22.7	18.0	19.9	18.8	19.5	17.8	17.9	18.8	19.0	15.9	13.9	15.1	14.6	13.7
Leptospire detection																		
Leptospiraemia (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	-ve	-ve	+	+	+++	-ve	-ve	-ve	-ve	-ve	-ve
Leptospiraemia (culture)	ND	ND	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
MAT (*pomona*)	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	trace	1:48	1:48	1:48	1:96
Leptospiruria (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+	+	+	++	++	+++	+++
Urinanalysis																		
SG	1.015	1.005	1.009	1.015	1.015	1.015	1.009	1.015	1.012	1.009	1.010	1.009	1.007	1.012	1.016	1.011	1.011	1.018
pH	7	8	7	6	6	7	6	7	7	6	6	7	7	7	6	7	7	6
Prot	-ve	-ve	-ve	-ve	-ve	-ve	-ve	trace	-ve	-ve	-ve	-ve	trace	++	+++	++	+	trace
Blood	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Bil	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	trace	++	+++	++	+	trace
Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	trace	+	+++	+++	++	trace

ND : Not done
\+ : Occasional leptospire seen or Small quantities of substance present in urine
++ : Several leptospires seen or Moderate quantities of substance present in urine
+++ : Many leptospires seen or Large quantities of substance present in urine

APPENDIX XVII: Clinical pathological data from toxin injected calf 12

Day P.I.	-2	-1	0	1	2
Temp am °C	38.8	39.8	38.6	38.6	38.7
Temp pm °C	ND	ND	38.8	39.6	38.2
<u>Haematology</u>					
PCV	0.33	0.32	0.33	0.32	0.29
Hb g/dℓ	11.4	11.4	11.0	10.2	9.3
MCHC g/dℓ	34.6	35.6	33.3	31.9	32.1
II	2	2	2	2	2
WBC x 10^9/ℓ	6.8	9.1	6.5	4.9	6.1
PMN x 10^9/ℓ	1.4	3.2	1.4	1.4	0.7
IMM x 10^9/ℓ	0	0	0	0	0
Lcyte x 10^9/ℓ	4.9	5.4	4.9	3.3	4.8
Mcyte x 10^9/ℓ	0.4	0.3	0.1	0.2	0.6
Eosin x 10^9/ℓ	0	0	0.1	0	0
Bas x 10^9/ℓ	0.1	0.3	0.1	0	0
Retic %	<1	<1	<1	<1	<1
<u>Blood Biochemistry</u>					
GGT IU/ℓ	208	132	113	99	82
AAT IU/ℓ	28	36	32	29	35
AP IU/ℓ	634	563	518	443	563
BUN mmol/ℓ	4.5	5.4	5.4	7.1	6.2
Tot.Bil μmol/ℓ	8.4	6.3	6.3	4.2	4.2
Conj.Bil μmol/ℓ	2.1	1.05	0	0	0
TP g/ℓ	74	70	70	70	66
Fib g/ℓ	6	8	6	4	2
Albumin g/ℓ	28.0	27.7	27.6	28.5	27.1
α glob g/ℓ	6.7	4.2	6.3	8.1	8.0
β glob g/ℓ	14.7	11.7	12.4	13.5	13.6
γ glob g/ℓ	18.7	18.5	17.7	16.0	15.4
<u>Leptospire detection</u>					
Leptospiraemia (dfm)	Plasma not examined for Leptospires by dfm.				
Leptospiraemia (culture)	Plasma not cultured				
MAT (*pomona*)	-ve	-ve	-ve	-ve	-ve
Leptospiruria (dfm)	Urine not examined for Leptospires by dfm				
<u>Urinanalysis</u>					
SG	1.015	1.005	1.003	1.015	1.010
pH	6	8	7	7	6
Prot	-ve	-ve	-ve	-ve	-ve
Blood	-ve	-ve	-ve	-ve	-ve
Bil	-ve	-ve	-ve	-ve	-ve
Glucose	-ve	-ve	-ve	-ve	-ve

APPENDIX XVIII: Clinical pathological data from toxin injected calf 13

Day P.I.	-2	-1	0	1	2
Temp am °C	37.8	38.5	38.4	38.6	38.8
Temp pm °C	ND	ND	38.8	39.2	38.4
<u>Haematology</u>					
PCV	0.31	0.30	0.31	0.29	0.27
Hb g/dℓ	10.6	10.0	10.0	9.3	8.3
MCHC g/dℓ	34.2	33.3	32.3	32.1	30.7
II	2	2	2	2	2
WBC x 10^9/ℓ	7.1	5.9	9.3	8.7	8.4
PMN x 10^9/ℓ	3.1	1.3	1.7	5.2	3.6
IMM x 10^9/ℓ	0	0	0	0	0
Lcyte x 10^9/ℓ	3.4	4.0	7.0	2.9	4.5
Mcyte x 10^9/ℓ	0.5	0.6	0.5	0.6	0.3
Eosin x 10^9/ℓ	0	0	0	0	0
Bas x 10^9/ℓ	0.1	0	0.2	0	0
Retic %	<1	<1	<1	<1	<1
<u>Blood Biochemistry</u>					
GGT IU/ℓ	503	349	323	254	186
AAT IU/ℓ	24	34	34	35	33
AP IU/ℓ	421	399	381	206	214
BUN mmol/ℓ	5.4	6.2	5.4	6.2	5.4
Tot.Bil μmol/ℓ	6.3	4.2	8.4	8.4	8.4
Conj.Bil μmol/ℓ	2.1	1.05	1.05	0	0
TP g/ℓ	76	78	76	68	70
Fib g/ℓ	6	8	6	2	6
Albumin g/ℓ	27.2	24.6	28.7	26.4	25.2
α glob g/ℓ	7.2	4.8	7.0	7.9	8.3
β glob g/ℓ	13.7	14.9	12.3	12.9	16.3
γ glob g/ℓ	22.0	25.7	22.0	18.9	14.1
<u>Leptospire detection</u>					
Leptospiraemia (dfm)	Plasma not examined for Leptospires by dfm				
Leptospiraemia (culture)	Plasma not cultured				
MAT (*pomona*)	-ve	-ve	-ve	-ve	-ve
Leptospiruria (dfm)	Urine not examined for Leptospires by dfm				
<u>Urinanalysis</u>					
SG	1.015	1.002	1.003	1.021	1.000
pH	6	7	7	7	6
Prot	-ve	-ve	-ve	-ve	-ve
Blood	-ve	-ve	-ve	-ve	-ve
Bil	-ve	-ve	-ve	-ve	-ve
Glucose	-ve	-ve	-ve	-ve	-ve

APPENDIX XIX: Clinical pathological data from *pomona* infected calf 14

Day PI	-2	-1	0	1	2	3	4	5
Temp am °C	38.7	39.4	38.6	38.6	38.0	38.4	38.6	38.0
Temp pm °C	ND	ND	38.6	39.6	39.0	39.8	39.4	39.1
<u>Haematology</u>								
PCV	0.32	0.33	0.32	0.31	0.31	0.30	0.28	0.22
Hb g/dℓ	10.5	10.6	10.6	9.8	9.8	9.4	8.2	6.8
MCHC g/dℓ	32.8	32.1	33.1	31.6	31.6	31.3	29.3	30.9
II	2	2	2	2	2	2	2	5-10*
WBC x 10^9/ℓ	6.3	10.5	9.8	6.4	7.5	10.1	5.8	11.0
PMN x 10^9/ℓ	2.1	2.8	4.2	3.1	2.9	5.4	2.0	2.0
IMM x 10^9/ℓ	0	0	0	0	0	0	0	1.3
Lcyte x 10^9/ℓ	3.3	6.6	5.0	2.6	3.9	4.5	3.4	5.4
Mcyte x 10^9/ℓ	0.9	1.1	0.6	0.5	0.7	0.1	0.4	2.3
Eosin x 10^9/ℓ	0	0	0	0	0	0	0	0
Bas x 10^9/ℓ	0	0	0	0	0.1	0.1	0	0
Retic %	<1	<1	<1	<1	<1	<1	<1	<1
<u>Blood Biochemistry</u>								
GGT IU/ℓ	373	267	236	191	156	144	134	102
AAT IU/ℓ	27	31	56	29	31	27	23	77
AP IU/ℓ	700	697	599	673	519	525	593	630
BUN mmol/ℓ	4.5	5.4	7.1	7.1	7.1	5.4	7.1	10.7
Tot.Bil μmol/ℓ	6.3	6.3	8.4	6.3	4.2	4.2	16.8	35.7
Conj.Bil μmol/ℓ	1.05	2.1	1.05	1.05	1.05	0	1.05	8.4
TP g/ℓ	74	76	72	74	74	68	70	70
Fib g/ℓ	4	8	4	6	4	2	4	6
Albumin g/ℓ	27.4	29.1	28.4	28.8	28.6	29.6	35.2	25.7
α glob g/ℓ	5.2	6.8	5.6	7.2	7.8	6.2	1.5	9.4
β glob g/ℓ	14.8	14.6	15.0	13.1	14.6	13.0	15.3	13.5
γ glob g/ℓ	22.7	17.6	19.0	19.0	19.0	17.2	14.1	15.4
<u>Leptospire detection</u>								
Leptospiraemia (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	+
Leptospiraemia (culture)	ND	ND	-ve	+ve	+ve	+ve	+ve	+ve
MAT (*pomona*)	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Leptospiruria (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	++
<u>Urinanalysis</u>								
SG	1.005	1.005	1.006	1.015	1.015	1.006	1.009	1.016
pH	6	6	6	5	6	7	5	6
Prot	-ve	-ve	-ve	-ve	-ve	-ve	-ve	trace
Blood	-ve	-ve	-ve	-ve	-ve	-ve	-ve	trace
Bil	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

* : Slight haemoglobinaemia
ND : Not done
+ : Occasional leptospire seen
++ : Several leptospires seen

APPENDIX XX : Clinical pathological data from *pomona* infected calf 15

Day PI	-2	-1	0	1	2	3	4	5	6
Temp am °C	39.2	38.8	38.2	38.3	38.8	39.1	38.8	38.1	39.1
Temp pm °C	ND	ND	38.4	38.8	38.3	39.3	39.5	40.0	39.7
<u>Haematology</u>									
PCV	0.33	0.32	0.33	0.32	0.33	0.36	0.29	0.26	0.16
Hb g/dℓ	11.0	10.8	10.8	11.0	10.6	10.9	9.2	8.0	5.2
MCHC g/dℓ	33.3	33.8	32.7	34.4	32.1	30.3	31.7	30.8	32.5
II	2	2	2	2	2	2	2	2	25+
WBC x 10^9/ℓ	9.6	5.4	7.5	5.5	8.0	5.5	5.0	4.6	11.7
PMN x 10^9/ℓ	5.5	1.4	1.2	1.3	0.8	1.2	1.0	1.0	3.0
IMM x 10^9/ℓ	0	0	0	0	0	0	0	0	1.1
Lcyte x 10^9/ℓ	3.8	3.4	5.2	3.6	6.5	4.1	3.5	3.3	4.9
Mcyte x 10^9/ℓ	0.3	0.5	1.1	0.6	0.6	0.3	0.6	0.3	2.7
Eosin x 10^9/ℓ	0	0	0	0	0	0	0	0	0
Bas x 10^9/ℓ	0	0.1	0	0	0.1	0	0	0	0
Retic %	<1	<1	<1	<1	<1	<1	<1	<1	<1
<u>Blood Biochemistry</u>									
GGT IU/ℓ	227	158	116	103	101	87	71	61	5
AAT IU/ℓ	20	51	41	31	32	29	27	25	163
AP IU/ ℓ	374	403	398	340	316	337	461	311	413
BUN mmol/ℓ	4.5	6.2	4.5	4.5	4.5	4.5	5.4	7.1	12.5
Tot.Bil μmol/ℓ	12.6	4.2	6.3	6.3	8.4	9.45	12.6	6.3	50.4
Conj. Bil μmol/ℓ	4.2	2.1	0	1.05	2.1	1.05	1.05	2.1	2.1
TP g/ℓ	82	86	82	80	82	58	80	74	88
Fib g/ℓ	2	4	6	2	2	2	4	6	8
Albumin g/ℓ	31.6	28.7	31.9	29.9	35.1	24.4	31.5	28.6	28.2
α glob g/ℓ	7.4	7.5	6.1	7.6	7.8	4.1	8.1	8.5	15.4
β glob g/ℓ	17.2	16.1	15.2	15.8	17.9	16.5	16.0	13.4	16.6
γ glob g/ℓ	23.8	29.9	26.7	24.8	19.1	15.9	20.4	21.5	19.7
<u>Leptospire detection</u>									
Leptospiraemia (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	-ve	++
Leptospiraemia (culture)	ND	ND	-ve	-ve	+ve	+ve	+ve	+ve	+ve
MAT (*pomona*)	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Leptospiruria (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	-ve	++
<u>Urinanalysis</u>									
SG	1.007	1.003	1.010	1.006	1.005	1.005	1.005	1.014	1.015
pH	5	4	5	5	6	6	5	5	7
Prot	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+++
Blood	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+++
Bil	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+
Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

* : Severe haemoglobinaemia
ND : Not done
+ : Small quantities of substance present
+++ : Large quantities of substance present
++ : Several leptospires seen

APPENDIX XXI: Clinical pathological data from *pomona* infected calf 16

Day PI	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Temp am °C	38.2	39.4	38.2	38.0	38.0	38.2	38.0	38.1	38.0	38.5	37.7	38.2	37.8	37.2	39.1	38.6	38.8	39.6
Temp pm °C	ND	ND	38.2	38.5	38.9	39.0	39.2	38.8	38.0	38.4	39.3	38.8	38.8	39.1	39.0	39.4	39.1	Euth
<u>Haematology</u>																		
PCV	0.40	0.36	0.39	0.40	0.38	0.40	0.39	0.39	0.38	0.36	0.36	0.36	0.39	0.38	0.36	0.37	0.33	0.30
Hb g/dℓ	13.4	12.4	13.0	13.4	11.9	12.3	12.5	12.0	12.4	11.8	11.2	11.8	11.4	12.0	11.2	11.4	10.2	9.8
MCHC g/dℓ	33.5	34.4	33.3	33.5	31.3	30.8	32.1	30.8	31.8	31.1	31.1	32.8	29.2	31.6	31.1	30.8	30.9	32.7
II	2	2	2	2	2	2	2	2	2	2	2	5	2	2	2	2	2	2
WBC x 10^9/ℓ	8.2	6.9	8.6	6.8	7.0	6.7	7.1	7.4	9.1	8.7	11.4	9.4	12.9	9.6	8.6	11.4	9.5	10.2
PMN x 10^9/ℓ	2.7	1.4	1.9	1.2	1.2	1.9	2.0	1.6	2.1	1.8	3.1	1.4	2.7	2.6	3.3	3.8	2.8	2.0
IMM x 10^9/ℓ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lcyte x 10^9/ℓ	4.7	5.1	5.7	4.6	4.6	3.9	4.3	4.6	5.8	5.4	7.3	7.1	8.4	6.0	4.6	6.7	6.2	8.1
Mcyte x 10^9/ℓ	0.7	0.3	0.9	1.0	1.3	0.9	0.8	1.1	1.2	1.5	0.9	0.8	1.5	0.8	0.7	0.9	0.4	0.1
Eosin x 10^9/ℓ	0.1	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0.1	0.1	0
Bas x 10^9/ℓ	0.1	0.1	0	0.1	0	0	0.1	0	0	0	0.1	0	0.3	0.2	0	0	0	0
Retic %	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
<u>Blood Biochemistry</u>																		
GGT IU/ℓ	314	205	198	151	118	113	97	61	80	73	78	73	66	61	54	54	42	35
AAT IU/ℓ	26	43	40	35	36	35	39	29	29	27	38	40	35	34	33	45	51	39
AP IU/ℓ	267	378	357	278	258	313	327	289	276	260	416	279	282	272	305	328	322	284
BUN mmol/ℓ	4.5	5.4	5.4	7.1	5.4	5.4	4.5	5.4	7.1	6.2	5.4	5.4	5.4	5.4	5.4	4.5	6.2	4.5
Tot. Bil µmol/ℓ	8.4	4.2	8.4	4.2	6.3	4.2	8.4	2.1	4.2	2.1	4.2	6.3	4.2	4.2	2.1	2.1	4.2	2.1
Conj. Bil µmol/ℓ	2.1	2.1	2.1	0	2.1	1.05	2.1	0	0	0	0	2.1	1.05	0	1.05	0	1.05	0
TP g/ℓ	84	80	78	80	78	74	78	76	76	80	74	78	80	76	72	72	68	62
Fib g/ℓ	4	4	2	4	2	2	4	6	6	8	6	6	6	6	6	6	4	4
Albumin g/ℓ	31.6	31.5	32.2	32.0	31.0	32.3	31.4	29.3	31.9	30.3	28.6	31.5	32.1	31.8	29.6	31.8	32.3	28.6
α glob g/ℓ	6.2	6.8	6.2	8.4	8.1	5.6	8.7	8.3	7.5	8.6	8.1	9.9	8.9	10.1	8.8	9.3	7.7	6.2
β glob g/ℓ	19.1	14.2	16.4	15.7	16.3	15.5	16.3	12.0	15.0	19.6	14.7	14.2	17.5	13.1	14.3	14.1	12.9	14.0
γ glob g/ℓ	23.0	23.2	21.2	20.0	20.7	18.7	17.7	20.4	15.6	13.5	14.7	16.3	13.5	13.1	13.3	10.9	11.1	9.2
<u>Leptospire detection</u>																		
Leptospiraemia (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+	+	-ve	-ve	-ve	-ve	-ve	-ve
Leptospiraemia (culture)	ND	ND	-ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve	-ve	-ve	-ve
MAT *(pomona)*	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	trace	1:96	1:96	1:384	1:192	1:96	1:48
Leptospiruria (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
<u>Urinanalysis</u>																		
SG	1.007	1.004	1.011	1.006	1.001	1.002	1.001	1.005	1.006	1.008	1.001	1.019	1.010	1.006	1.010	1.020	1.020	1.006
pH	6	7	6	8	6	7	6	5	6	5	5	6	6	6	6	6	6	6
Prot	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	trace	-ve	-ve
Blood	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Bil	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

ND: Not done

\+ Occasional leptospire seen

APPENDIX XXII: Clinical pathological data from *pomona* infected calf 17

Day PI	-2	-1	0	1	2	3	4	5
Temp am °C	38.4	39.2	38.8	38.6	38.6	38.7	39.1	39.4
Temp pm °C	ND	ND	38.4	39.2	39.4	40.0	39.4	39.9
<u>Haematology</u>								
PCV	0.35	0.36	0.38	0.35	0.36	0.35	0.32	0.23
Hb g/dℓ	11.4	11.4	12.8	10.5	11.8	10.5	9.8	7.3
MCHC g/dℓ	32.6	31.7	33.7	30.0	32.8	30.0	30.6	31.7
II	2	2	2	2	2	2	2	25+ *
WBC x 10^9/ℓ	8.7	7.7	8.0	11.5	10.6	9.8	5.8	12.1
PMN x 10^9/ℓ	2.0	1.7	1.2	2.9	2.4	3.7	1.8	4.3
IMM x 10^9/ℓ	0	0	0	0	0	0	0	1.0
Lcyte x 10^9/ℓ	6.5	5.7	6.2	7.4	7.1	5.5	3.5	5.8
Mcyte x 10^9/ℓ	0.2	0.3	0.6	1.3	1.1	0.5	0.5	1.0
Eosin x 10^9/ℓ	0	0	0	0	0	0.1	0	0
Bas x 10^9/ℓ	0	0	0	0.1	0	0	0	0
Retic %	<1	<1	<1	<1	<1	<1	<1	<1
<u>Blood Biochemistry</u>								
GGT IU/ℓ	271	181	175	122	99	101	82	90
AAT IU/ℓ	22	39	36	27	26	27	30	82
AP IU/ℓ	540	677	476	336	318	356	470	279
BUN mmol/ℓ	5.4	6.2	5.4	7.1	7.1	5.4	7.1	11.6
Tot. Bil μmol/ℓ	6.3	6.3	8.4	4.2	4.2	4.2	4.2	29.4
Conj. Bil μmol/ℓ	1.05	0	0	1.05	1.05	0	0	2.1
TP g/ℓ	76	74	72	74	76	70	68	82
Fib g/ℓ	6	6	4	8	4	4	4	12
Albumin g/ℓ	27.7	29.2	28.7	26.8	29.7	29.3	26.7	24.6
α glob g/ℓ	5.8	6.7	5.3	7.1	8.4	6.5	8.5	22.2
β glob g/ℓ	13.0	14.4	14.2	11.8	15.7	14.5	16.3	8.9
γ glob g/ℓ	23.6	17.8	19.8	20.5	18.1	15.8	12.5	14.0
<u>Leptospire detection</u>								
Leptospiraemia (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	+
Leptospiraemia (culture)	ND	ND	-ve	+ve	+ve	+ve	+ve	+ve
MAT *(pomona)*	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Leptospiruria (dfm)	ND	ND	ND	-ve	-ve	-ve	-ve	++
<u>Urinanalysis</u>								
SG	1.007	1.004	1.011	1.009	1.004	1.005	1.001	1.010
pH	5	6	6	7	6	6	6	5
Prot	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+++
Blood	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+++
Bil	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

* : Severe haemoglobinaemia
ND : Not done
+ : Occasional leptospire seen
++ : Several leptospires seen
+++ : Large quantities of substance present

APPENDIX XXIII: Clinical pathological data from control calf 18

DAY PI	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Temp am °C	38.4	38.6	38.4	38.0	38.4	38.5	38.7	38.4	38.3	38.3	38.5	39.3	38.2	39.0	38.0	38.0	38.9	39.0
Temp pm °C	ND	ND	38.5	38.8	38.9	38.0	38.8	39.1	39.3	39.2	38.6	39.3	39.2	39.2	39.0	39.8	39.3	Euth
Haematology																		
PCV	0.32	0.36	0.34	0.34	0.37	0.35	0.30	0.33	0.30	0.31	0.31	0.28	0.30	0.28	0.29	0.32	0.31	0.27
Hb g/dℓ	10.8	11.6	11.4	10.6	11.8	10.8	9.2	9.9	9.3	9.4	9.4	8.8	9.1	8.6	8.8	9.6	9.6	8.4
MCHC g/dℓ	33.8	32.2	33.5	31.2	31.9	30.9	30.7	30.0	31.0	30.3	30.3	31.4	30.3	30.7	30.3	30.0	31.0	31.1
II	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
WBC x 10^9/ℓ	11.8	10.6	9.5	6.0	10.5	8.4	8.8	9.6	10.6	10.6	8.4	9.8	8.3	9.1	9.2	9.5	10.2	9.5
PMN x 10^9/ℓ	3.9	3.5	2.9	1.1	3.6	1.6	2.1	3.1	2.9	3.9	1.3	2.3	2.7	2.5	2.1	2.0	2.7	2.9
IMM x 10^9/ℓ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lcyte x 10^9/ℓ	7.4	5.8	6.0	4.7	6.1	6.2	6.2	5.7	5.8	6.5	6.4	7.2	5.1	6.3	6.6	6.3	7.0	6.4
Mcyte x 10^9/ℓ	0.5	1.0	0.7	0.1	0.7	0.6	0.4	0.9	1.2	0.2	0.7	0.4	0.5	0.3	0.5	1.2	0.4	0.2
Eosin x 10^9/ℓ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bas x 10^9/ℓ	0	0.1	0	0.1	0.1	0	0	0	0	0	0	0	0	0	0	0	0	0
Retic %	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Blood Biochemistry																		
GGT IU/ℓ	948	684	601	505	441	391	356	328	269	297	257	236	224	201	146	146	138	111
AAT IU/ℓ	24	41	38	39	30	25	23	23	20	19	25	20	26	30	22	32	27	23
AP IU/ ℓ	503	563	645	459	456	492	570	499	510	499	622	518	553	650	574	587	485	580
BUN mmol/ℓ	7.1	5.4	5.4	7.1	5.4	5.4	6.2	5.4	7.1	7.1	5.4	6.2	5.4	6.2	5.4	6.2	5.4	6.2
Tot. Bil μmol/ℓ	4.2	4.2	6.3	4.2	4.2	2.1	2.1	6.3	2.1	4.2	2.1	2.1	4.2	2.1	1.05	2.1	4.2	2.1
Conj. Bil μmol/ℓ	0	0	0	0	0	0	0	0	0	0	0	0	1.05	0	0	0	1.05	0
TP g/ℓ	78	78	74	60	80	70	70	74	66	66	68	68	66	62	60	66	66	66
Fib g/ℓ	6	6	8	4	4	2	4	6	6	4	2	6	4	4	4	6	2	4
Albumin g/ℓ	24.9	28.6	27.8	30.8	32.8	30.7	27.2	28.5	28.0	27.0	29.1	28.4	29.1	27.0	26.5	31.4	33.9	35.2
α glob g/ℓ	6.7	5.3	5.7	5.0	7.8	5.3	6.8	7.1	5.0	6.5	6.5	5.8	6.5	7.5	6.6	6.4	6.5	4.3
β glob g/ℓ	13.3	14.1	12.6	10.8	14.1	12.0	13.6	12.3	11.2	12.0	13.7	10.7	11.7	10.3	11.0	11.1	12.4	11.0
γ glob g/ℓ	27.1	24.0	19.9	19.4	21.4	20.0	18.5	18.2	15.9	16.6	16.7	17.1	14.8	13.2	11.9	11.1	11.2	11.5
Leptospire detection																		
Leptospiraemia (dfm)	Plasma not examined for Leptospires by dfm																	
Leptospiraemia (culture)	Plasma not cultured																	
MAT (*pomona*)	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Leptospiruria (dfm)	Urine not examined for Leptospires by dfm																	
Urinanalysis																		
SG	1.005	1.010	1.005	1.010	1.010	1.005	1.001	1.020	1.008	1.010	1.006	1.005	1.011	1.010	1.009	1.006	1.009	1.009
pH	5	6	7	7	6	7	5	5	6	5	5	5	5	6	6	6	6	6
Prot	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Blood	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Bil	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

APPENDIX XXIV: Clinical pathological data from control calf 19

Day PI	-2	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Temp am °C	38.2	38.6	38.5	38.0	38.4	38.2	38.1	38.0	38.5	39.0	39.0	39.5	38.8	38.6	38.6	39.6	39.0	39.0
Temp pm °C	ND	ND	38.8	38.8	39.0	38.2	38.9	39.3	39.4	39.0	39.3	39.1	39.3	38.4	38.8	39.8	39.6	Euth
Haematology																		
PCV	0.39	0.38	0.39	0.36	0.38	0.31	0.38	0.37	0.33	0.30	0.28	0.28	0.26	0.27	0.30	0.27	0.32	0.28
Hb g/dl	13.4	13.0	13.2	12.0	11.8	9.8	11.8	11.4	10.4	9.2	8.4	9.0	8.0	8.3	9.0	8.2	9.8	8.6
MCHC g/dl	34.4	34.2	33.8	33.3	31.1	31.6	31.1	30.8	31.5	30.7	30.0	32.1	30.8	30.7	30.0	30.4	30.6	30.7
II	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
WBC x 10^9/l	7.2	8.3	9.2	10.0	9.6	7.2	10.0	5.6	11.6	11.8	9.8	7.0	8.6	10.8	8.9	8.8	9.9	12.9
PMN x 10^9/l	2.5	3.5	4.2	4.3	2.4	1.4	3.0	1.8	3.8	4.5	3.9	2.5	2.5	4.8	1.1	1.8	2.8	6.1
IMM x 10^9/l	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lcyte x 10^9/l	4.5	4.6	4.6	5.5	6.7	5.5	5.7	3.0	7.2	7.1	5.3	4.2	5.6	5.2	6.9	6.4	6.6	6.7
Mcyte x 10^9/l	0.2	0.2	0.4	0.2	0.4	0.2	0.9	0.6	0.6	0.1	0.6	0.3	0.5	0.8	0.8	0.4	0.3	0.1
Eosin x 10^9/l	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0.2	0
Bas x 10^9/l	0	0.1	0	0	0.1	0.1	0.4	0	0	0.1	0	0	0	0.1	0.1	0	0	0
Retic %	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Blood Biochemistry																		
GGT IU/l	476	283	255	212	188	165	162	156	125	110	104	92	78	76	68	57	47	45
AAT IU/l	31	39	28	36	30	33	30	27	21	20	28	23	23	29	32	33	38	38
AP IU/ l	374	428	382	344	362	333	358	254	292	318	392	373	436	483	488	490	564	509
BUN mmol/l	4.5	5.4	4.5	7.1	7.1	5.4	5.4	5.4	6.2	6.2	5.4	5.4	5.4	5.4	5.4	5.4	6.2	5.4
Tot. Bil µmol/l	4.2	4.2	6.3	4.2	4.2	4.2	8.4	0.2	2.1	4.2	3.3	3.3	2.1	3.3	2.1	4.2	4.2	4.2
Conj. Bil µmol/l	2.1	1.05	0	0	0	0	2.1	0	0	0	1.05	1.05	0	0	0	1.05	1.05	1.05
TP g/l	66	60	60	74	62	80	62	64	60	62	60	60	56	56	60	56	58	56
Fib g/l	4	6	4	4	2	6	2	6	6	6	4	6	4	2	4	6	2	6
Albumin g/l	26.9	22.6	24.5	31.6	28.0	34.2	27.1	25.2	26.7	27.7	26.5	25.0	26.5	27.7	28.3	26.8	30.6	28.6
α glob g/l	7.1	4.8	7.5	8.3	7.3	8.0	8.2	7.7	7.1	7.2	6.6	7.7	6.7	7.5	8.1	7.0	7.1	4.7
β glob g/l	13.7	10.4	11.3	16.0	13.1	15.0	13.3	10.0	10.6	12.2	14.1	13.0	11.6	10.9	13.1	10.3	12.0	11.6
γ glob g/l	14.3	16.3	12.7	14.0	11.6	13.8	11.5	11.1	9.6	8.9	8.9	8.3	7.2	8.0	6.7	6.0	6.3	5.2
Leptospire detection																		
Leptospiraemia (dfm)	Plasma not examined for Leptospires by dfm																	
Leptospiraemia (culture)	Plasma not cultured																	
MAT (*pomona*)	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Leptospiruria (dfm)	Urine not examined for Leptospires by dfm																	
Urinanalysis																		
SG	1.004	1.002	1.002	1.001	1.005	1.001	1.001	1.011	1.005	1.006	1.001	1.004	1.004	1.005	1.006	1.011	1.009	1.005
pH	5	6	7	8	5	5	7	5	7	5	5	5	5	6	5	6	6	6
Prot	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Blood	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Bil	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

APPENDIX XXV: Clinical pathological data from the bled calf 20

Day PI	-2	-1	0	1	2	3	4	5	6	7	8	9	10*	11	12
Temp am °C	38.0	38.0	38.2	38.0	38.0	38.0	38.0	37.5	37.6	37.8	38.4	38.5	39.0	38.1	38.3
Temp pm °C	ND	ND	38.8	38.4	38.0	38.5	38.7	39.0	38.8	39.2	39.0	38.8	39.3	39.6	Euth
<u>Haematology</u>															
PCV	0.33	0.34	0.32	0.33	0.34	0.32	0.32	0.35	0.31	0.31	0.30	0.29	0.28	0.18	0.22
Hb g/dℓ	11.0	11.0	10.2	10.3	10.5	10.8	9.7	11.0	9.4	10.0	9.0	8.7	8.4	5.8	6.8
MCHC g/dℓ	33.3	32.4	31.9	31.2	30.9	33.8	30.3	31.4	30.3	32.3	30.0	30.0	30.0	32.2	30.9
II	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
WBC x 10^9/ℓ	9.0	8.0	12.0	10.0	10.9	13.4	10.6	6.8	11.9	11.7	9.6	9.4	7.9	7.9	9.6
PMN x 10^9/ℓ	3.3	2.5	4.8	4.3	2.6	2.3	2.7	1.5	4.0	4.2	2.6	3.0	1.3	2.1	2.6
IMM x 10^9/ℓ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lcyte x 10^9/ℓ	5.5	5.4	7.1	5.5	7.6	9.8	7.1	5.0	7.5	6.7	6.6	5.8	6.2	4.9	6.5
Mcyte x 10^9/ℓ	0.2	0.1	0.1	0.2	0.4	1.2	0.8	0.3	0.4	0.8	0.4	0.5	0.4	0.7	0.4
Eosin x 10^9/ℓ	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0.1	0
Bas x 10^9/ℓ	0	0	0	0	0	0.1	0	0	0	0	0	0.1	0	0.1	0.2
Retic %	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
<u>Blood Biochemistry</u>															
GGT IU/ℓ	628	437	375	332	309	267	252	238	205	184	184	184	151	113	90
AAT IU/ℓ	29	66	71	41	37	33	32	25	28	27	30	29	28	26	23
AP IU/ℓ	597	714	572	477	530	507	501	567	448	437	691	588	507	680	663
BUN mmol/ℓ	7.1	5.4	5.4	7.1	5.4	5.4	6.2	7.1	5.4	5.4	5.4	5.4	5.4	5.4	4.5
Tot. Bil μmol/ℓ	4.2	6.3	6.3	6.3	4.2	4.2	4.2	2.1	2.1	2.1	2.1	2.1	4.2	2.1	2.1
Conj. Bil μmol/ℓ	0	0	0	0	1.05	0	0	0	0	0	0	0	0	0	0
TP g/ℓ	74	70	68	74	74	68	70	72	66	68	66	66	66	56	62
Fib g/ℓ	8	4	4	8	2	4	4	6	6	6	4	6	4	2	4
Albumin g/ℓ	25.5	25.7	24.3	27.8	29.2	28.7	27.1	27.9	26.3	27.5	27.7	26.8	27.7	25.9	26.6
α glob g/ℓ	5.7	6.5	7.6	6.9	7.7	5.4	7.5	7.8	5.9	7.0	6.1	6.5	7.4	6.2	6.6
β glob g/ℓ	14.1	13.9	12.9	14.4	15.8	12.6	13.3	12.3	11.7	12.5	12.9	13.1	12.9	10.2	13.2
γ glob g/ℓ	20.7	19.9	19.3	17.0	19.3	17.3	17.9	18.2	16.1	14.9	15.4	13.7	14.1	11.8	11.7
<u>Leptospire detection</u>															
Leptospiraemia (dfm)	Plasma not examined for Leptospires by dfm														
Leptospiraemia (culture)	Plasma not cultured														
MAT (*pomona*)	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Leptospiruria (dfm)	Urine not examined for Leptospires by dfm														
<u>Urinanalysis</u>															
SG	1.004	1.010	1.002	1.010	1.005	1.005	1.001	1.010	1.008	1.006	1.001	1.005	1.004	1.010	1.009
pH	5	6	7	7	5	7	7	5	6	5	5	5	5	6	5
Prot	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Blood	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Bil	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Glucose	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

* Calf bled pm

APPENDIX XXVI: Bone marrow differentials from control and *pomona* infected calves

	Calf 18 (control)		Calf 19 (control)		Calf 20 (Bled control)		Calf 12		Calf 13		Calf 11		Calf 16		Calf 15	
	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series	% Total	% Series
Mblast	0.6	1.3	1.1	1.7	0.4	0.8	0.1	0.3	0	0	0.9	4.1	1.0	3.9	2.1	6.0
Pgran	1.1	2.5	3.4	5.1	1.9	3.7	1.9	4.5	0.5	1.8	0.8	3.6	1.0	3.9	1.9	5.4
m (n)	9.8	21.7	26.6	39.8	13.8	26.1	14.2	33.3	8.3	27.8	6.1	27.9	14.5	54.9	9.8	27.2
m (e)	1.5	3.3	1.7	2.5	0.4	0.7	0.8	1.8	1.8	6.8	1.7	7.7	0.3	1.0	3.3	9.3
m (b)	0.2	0.4	0.6	0.8	0	0	0	0	0	0	0.1	0.5	0	0	0	0
mm (n)	4.6	10.2	6.8	10.2	4.9	9.2	5.9	13.7	3.8	12.6	2.9	13.5	2.3	8.8	11.4	32.2
mm (e)	1.1	2.5	1.1	1.7	0.8	1.5	0.4	0.9	0.7	2.4	0.6	2.7	0	0	0.6	1.6
band (n)	11.8	26.2	10.2	15.3	13.2	25.0	9.5	22.3	10.4	34.9	6.5	29.7	2.8	10.8	5.4	15.3
band (e)	1.1	2.5	1.1	1.7	0	0	0.3	0.6	0.5	1.8	0.5	2.3	0.5	2.0	0.3	0.8
PMN	13.1	29.1	13.0	19.5	16.9	32.3	9.3	21.7	3.2	10.8	1.5	6.8	3.9	14.7	0.7	1.9
Eosin	0	0	0	0	0.4	0.7	0.1	0.3	0.4	1.2	0	0	0	0	0	0
Bas	0	0	0	0	0.2	0.4	0.1	0.3	0	0	0.2	0.9	0	0	0.1	0.3
Mitoses	0.2	0.4	1.1	1.7	0	0	0.1	0.3	0.2	0.6	0.1	0.5	0	0	0	0
Total	44.9%	100%	66.7%	100%	52.9%	100%	42.7%	100%	29.9%	100%	21.7%	100%	26.4%	100%	35.5%	100%
Eblast	0.2	0.4	0	0	0.2	0.5	0.1	0.3	0.2	0.3	0	0	0.8	1.1	0.5	0.8
E. norm	2.8	5.6	1.7	7.7	1.4	3.4	1.1	2.3	1.4	2.2	2.5	3.5	5.4	7.9	1.5	2.4
Norm	17.7	35.7	10.2	46.2	16.7	41.5	18.2	35.9	24.8	37.1	30.4	41.6	24.4	35.5	26.2	42.6
L. norm	28.9	58.4	9.6	43.6	20.8	51.7	31.2	61.6	40.1	59.9	39.9	54.5	38.1	55.5	32.5	52.8
Mitoses	0.2	0.4	0.6	2.6	1.2	2.9	0	0	0.4	0.5	0.3	0.4	0	0	0.9	1.4
Total	49.5%	100%	22.0%	100%	40.3%	100%	50.6%	100%	66.9%	100%	73.1%	100%	68.7%	100%	61.6%	100%
Pl.cell	0		0		0		0		0		0		0		0	
Lcyte	1.1		4.0		2.5		2.7		0.7		3.6		1.6		0.6	
Mcyte	0		0		0.2		0.5		0		0		0		0.4	
Mega	0.4		0.6		0.2		0.1		0.4		0.2		0.3		0.1	
Retic	2.9		2.3		2.3		1.3		1.4		0.4		1.0		0.5	
Unclass	0		2.3		1.2		1.0		0		0.2		0		0.6	
Disint	1.1		2.3		0.4		1.0		0.7		0.8		2.1		0.8	
	100%		100%		100%		100%		100%		100%		100%		100%	
M:E Ratio	0.9		3.0		1.3		0.8		0.4		0.3		0.4		0.6	

APPENDIX XXVII: Haematological and urinary results from untreated control heifers

a) Untreated control heifer: number 19

Day P.I.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16
Temp. °C	39.2	38.5	38.6	38.3	38.3	38.3	38.4	38.2	38.3	38.4	38.0	38.0	38.6	38.5	38.8	38.5
PCV	0.36	0.35	0.36	0.36	0.35	0.34	0.36	0.34	0.34	0.34	0.34	0.36	0.33	0.32	0.32	0.32
Hb g/dℓ	12.0	11.8	ND	ND	10.8	11.0	ND	11.4	ND	ND	10.8	ND	ND	10.2	ND	10.4
MCHC g/dℓ	33.3	33.7	ND	ND	30.9	32.4	ND	33.5	ND	ND	31.8	ND	ND	31.9	ND	32.5
II	5	5	5	5	5	5	5	5	5	5	7.5	7.5	7.5	5	7.5	5
TP g/ℓ	66	66	66	62	64	66	66	66	76	64	68	70	64	66	66	66
WBC x 10^9/ℓ	7.0	8.0	6.3	8.4	7.7	7.6	7.7	7.5	6.7	6.7	6.2	5.8	6.7	7.3	7.1	6.6
Urinary pH	7.0	8.0	8.5	8.0	8.0	7.5	8.0	7.5	8.5	9.0	9.0	8.0	8.0	7.5	8.0	8.5
Urinary SG	1.031	1.031	1.038	1.042	1.041	1.031	1.042	1.031	1.007	1.030	1.029	1.036	1.037	1.037	1.038	1.025

b) Untreated control heifer: number 67

Day P.I.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16
Temp. °C	38.9	38.5	39.1	38.7	38.9	38.8	38.2	38.6	38.0	38.7	39.0	37.8	39.0	38.9	38.7	38.4
PCV	0.35	0.34	0.35	0.33	0.33	0.35	0.33	0.32	0.36	0.36	0.33	0.35	0.34	0.31	0.35	0.33
Hb g/dℓ	11.6	10.6	ND	ND	10.6	10.6	ND	10.4	ND	ND	10.0	ND	ND	9.4	ND	10.0
MCHC g/dℓ	33.1	31.2	ND	ND	32.1	30.3	ND	32.5	ND	ND	30.3	ND	ND	30.3	ND	30.3
II	5	5	5	5	7.5	5	5	5	7.5	5	5	5	5	5	5	5
TP g/ℓ	70	66	66	62	66	62	62	86	66	72	66	70	66	66	75	64
WBC x 10^9/ℓ	4.2	8.3	4.7	8.6	7.6	8.8	6.1	6.7	7.8	8.2	7.7	7.8	9.9	7.8	7.8	7.7
Urinary pH	8.0	9.0	8.0	8.0	8.0	8.0	8.0	8.0	8.5	9.0	8.5	9.0	8.5	7.0	8.5	7.0
Urinary SG	1.023	1.023	1.039	1.031	1.030	1.023	1.043	1.032	1.037	1.038	1.021	1.023	1.026	1.028	1.031	1.025

MAT's from both heifers remained negative

ND : Not done

Urine was negative for protein, blood, bilirubin and glucose on all days

APPENDIX XXVIII: Haematological and urinary results from negative control heifers

a) Heifer inoculated with non-infected media : Number 44

Day P.I.	0	1	2	3	4	5	6	7	8	9	10	11	12	14	16
Temp °C	38.7	38.2	39.2	39.2	38.8	39.0	38.9	38.9	38.4	39.2	38.8	38.1	38.5	38.7	38.8
PCV	0.38	0.37	0.40	0.37	0.34	0.35	0.34	0.38	0.35	0.34	0.33	0.35	0.32	0.31	0.32
Hb g/dℓ	12.4	12.0	ND	ND	10.6	11.0	ND	11.6	ND	ND	11.0	ND	ND	ND	10.2
MCHC g/dℓ	32.6	32.4	ND	ND	31.2	31.4	ND	30.5	ND	ND	33.3	ND	ND	ND	31.9
II	5	5	5	5	5	5	5	7.5	5	7.5	10	10	10	7.5	7.5
TP g/ℓ	72	70	70	70	66	70	70	82	72	72	72	70	66	68	72
WBC x 10^9/ℓ	8.9	11.5	6.8	9.6	7.5	7.2	6.7	9.3	8.2	7.5	7.7	8.4	8.7	7.7	6.8
Urinary pH	9.0	9.0	9.0	ND	8.5	9.0	ND	9.0	8.5	9.0	7.5	8.5	8.5	8.5	8.5
Urinary SG	1.077	1.077	1.043	ND	1.037	1.040	ND	1.028	1.016	1.035	1.048	1.029	1.022	1.032	1.036

b) Heifer inoculated with non-infected liver and kidney homogenate : Number 61

Day P.I.	0	1	2	3	4	5	6	7	8	9	10	11	12	14	16
Temp.°C	38.8	38.8	38.9	39.1	38.6	38.7	38.3	38.1	38.5	38.2	38.7	38.6	38.6	38.8	38.8
PCV	0.35	0.34	0.36	0.34	0.36	0.33	0.32	0.31	0.33	0.30	0.31	0.34	0.32	0.32	0.30
Hb g/dℓ	11.8	11.4	ND	ND	12.0	10.2	ND	10.0	ND	ND	9.8	ND	ND	ND	10.0
MCHC g/dℓ	33.7	33.5	ND	ND	33.3	30.9	ND	32.3	ND	ND	31.6	ND	ND	ND	33.3
II	10	7.5	5	7.5	10	10	10	10	15	15	5	10	10	5	10
TP g/ℓ	72	70	70	66	66	70	66	70	72	70	70	74	74	82	76
WBC x 10^9/ℓ	6.8	9.5	9.0	9.8	11.1	10.7	9.0	6.5	11.8	9.1	8.2	8.3	10.9	9.8	9.9
Urinary pH	8.0	8.0	7.0	9.0	9.0	8.0	8.8	8.0	8.0	9.0	8.5	7.5	8.0	9.0	8.0
Urinary SG	1.025	1.025	1.033	1.025	1.028	1.015	1.026	1.020	1.003	1.030	1.026	1.027	1.026	1.023	1.021

MAT's from both heifers remained negative
ND: Not done
Urine was negative for protein, blood, bilirubin, and glucose on all days

APPENDIX XXIX: Haematological and urinary results from heifers infected using *balcan.ica* infected hamster livers and kidneys

a) *Balancica* infected heifer: number 1

Day P.I	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16
Temp. °C	38.8	38.7	38.8	38.8	38.6	38.4	38.4	38.9	38.5	38.5	39.8	37.8	38.8	38.5	38.3	38.6
PCV	0.36	0.38	0.38	0.40	0.36	0.34	0.35	0.33	0.38	0.34	0.35	0.35	0.32	0.33	0.31	0.30
Hb g/ℓ	12.4	12.6	ND	ND	11.2	10.6	ND	10.2	ND	ND	11.0	ND	ND	10.0	ND	9.0
MCHC g/dℓ	34.4	33.2	ND	ND	31.1	31.2	ND	30.9	ND	ND	31.4	ND	ND	30.3	ND	30.0
II	5	5	5	5	5	5	5	5	7.5	7.5	5	10	7.5	7.5	7.5	7.5
TP g/ℓ	70	66	66	78	68	64	66	66	68	68	68	66	64	66	68	64
WBC x 10^9/ℓ	6.3	9.2	9.1	11.3	9.4	11.7	9.0	11.2	9.8	10.4	9.7	9.9	9.2	8.8	10.5	8.8
MAT	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	1:24	1:96	1:384	1:768	1:768	1:384
Urinary pH	9.0	9.0	8.0	8.0	8.5	8.5	8.0	8.0	8.0	9.0	8.0	8.0	9.0	7.5	8.5	8.5
Urinary SG	1.030	1.035	1.039	1.035	1.028	1.031	1.028	1.025	1.037	1.040	1.031	1.032	1.033	1.028	1.033	1.026

b) *Balcanica* infected heifer: number 53

Day P.I	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16
Temp. °C	38.3	38.5	38.2	38.6	38.8	38.4	38.3	38.3	38.7	38.6	38.0	38.1	38.7	38.3	38.3	38.6
PCV	0.38	0.39	0.36	0.34	0.34	0.35	0.34	0.33	0.36	0.35	0.33	0.35	0.31	0.35	0.31	0.32
Hb g/dℓ	12.6	12.2	ND	ND	10.6	11.0	ND	10.6	ND	ND	10.6	ND	ND	11.1	ND	9.8
MCHC g/dℓ	33.2	31.3	ND	ND	31.2	31.4	ND	32.1	ND	ND	32.1	ND	ND	31.7	ND	30.6
II	5	5	5	5	5	5	5	5	5	5	5	7.5	5	5	7.5	5
TP g/ℓ	72	74	74	60	66	70	68	66	72	72	72	72	68	74	68	72
WBC x 10^9/ℓ	4.4	8.3	7.2	8.4	7.5	7.9	7.4	7.4	6.7	6.3	6.1	6.0	7.9	6.4	7.2	7.2
MAT	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	1:96	1:192	1:384	1:768	1:768	1:768	1:384
Urinary pH	6.0	6.5	8.0	8.0	8.0	8.0	8.5	8.0	7.5	8.5	9.0	8.0	9.0	8.0	8.0	8.0
Urinary SG	1.030	1.039	1.039	1.048	1.033	1.036	1.037	1.019	1.020	1.035	1.024	1.033	1.022	1.028	1.025	1.028

ND Not done

Urine remained negative for protein, blood, bilirubin and glucose on all days

APPENDIX XXIX: cont. Haematological and urinary results from heifers infected using *balcanica* infected livers and kidneys.

c) *Balcanica* infected heifer: number 59

	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16
Temp. °C	38.7	38.4	39.1	38.3	39.0	38.9	38.8	38.0	38.3	39.9	38.2	37.5	38.8	38.9	38.7	38.5
PCV	0.35	0.36	0.34	0.37	0.31	0.31	0.31	0.34	0.34	0.32	0.33	0.33	0.30	0.34	0.28	0.27
Hb g/dℓ	11.2	11.2	ND	ND	9.4	9.8	ND	10.6	ND	ND	10.6	ND	ND	11.2	ND	8.4
MCHC g/dℓ	32.0	31.1	ND	ND	30.3	31.6	ND	31.2	ND	ND	32.1	ND	ND	32.9	ND	31.1
II	5	5	5	5	5	5	5	5	5	7.5	5	5	7.5	5	5	5
TP g/ℓ	66	64	64	70	60	60	60	62	62	64	64	66	60	62	60	60
WBC x $10^9/\ell$	8.3	9.3	7.7	8.2	8.3	9.5	10.1	9.3	8.2	10.5	10.7	8.3	8.9	10.9	8.3	85
MAT	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	1:24	1:96	1:192	1:192	1:384
Urinary pH	8.0	8.5	9.0	8.5	8.5	9.0	9.0	8.0	8.5	9.0	9.0	9.0	9.0	8.5	9.0	8.5
Urinary SG	1.020	1.029	1.031	1.030	1.025	1.023	1.025	1.030	1.026	1.032	1.024	1.035	1.023	1.018	1.020	1.024

d) *Balcanica* infected heifer: number 64

Temp. °C	38.8	38.3	38.8	38.3	38.4	38.6	38.2	38.2	39.2	37.8	38.4	37.9	38.5	38.6	38.6	38.3
PCV	0.40	0.39	0.38	0.37	0.38	0.39	0.39	0.37	0.37	0.39	0.36	0.39	0.36	0.32	0.31	0.32
Hb g/dℓ	13.2	12.8	ND	ND	12.8	11.8	ND	11.8	ND	ND	10.8	ND	ND	9.8	ND	9.6
MCHC g/dℓ	33.0	32.8	ND	ND	33.6	30.3	ND	31.9	ND	ND	30.0	ND	ND	30.6	ND	30.0
II	15	10	5	5	5	10	10	10	10	10	5	10	15	10	10	15
TP g/ℓ	68	72	72	60	68	66	68	70	76	70	68	70	66	68	66	66
WBC x $10^9/\ell$	8.5	6.6	5.9	8.2	8.4	9.3	8.4	9.6	9.2	8.7	6.7	7.8	7.7	10.5	8.5	7.7
MAT	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	1:24	1:96	1:192	1:384	1:768	1:768
Urinary pH	8.0	8.5	8.0	8.5	8.0	9.0	8.0	8.5	7.5	9.0	9.0	8.0	8.0	8.5	8.5	8.5
Urinary SG	1.020	1.025	1.038	1.031	1.027	1.031	1.032	1.027	1.003	1.028	1.026	1.031	1.026	1.018	1.031	1.028

ND: Not done
Urine remained negative for protein, blood, bilirubin and glucose on all days

APPENDIX XXX: Haematological and urinary results from heifers infected using *balanica* culture

a) *Balcanica* infected heifer:number 27

Day P.I.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16
Temp. °C	38.3	37.5	39.1	40.8	38.9	38.8	38.6	38.2	38.5	38.7	39.0	38.6	38.8	30.0	38.7	38.6
PCV	0.35	0.35	0.38	0.32	0.32	0.33	0.33	0.31	0.34	0.35	0.34	0.35	0.32	0.30	0.30	0.30
Hb g/dℓ	11.6	11.4	ND	ND	10.0	10.8	ND	10.0	ND	ND	10.6	ND	ND	9.4	ND	9.4
MCHC g/dℓ	33.1	32.6	ND	ND	31.3	32.7	ND	32.3	ND	ND	31.2	ND	ND	31.3	ND	31.3
II	5	5	5	5	5	5	5	5	5	5	7.5	5	5	5	5	5
TP g/ℓ	66	66	66	66	68	70	68	70	68	70	68	66	66	66	66	66
WBC x 10^9/ℓ	4.9	7.1	6.9	8.4	6.1	7.9	6.7	6.9	6.4	7.5	7.1	8.6	8.9	7.5	8.4	7.5
MAT	-ve	-ve	-ve	-ve	1:96	1:384	1:384	1:768	1.768	1:768	1:768	1:1536	1:1536	1:1536	1:1536	1:384
Urinary pH	8.0	8.5	8.0	8.5	8.5	9.0	8.0	7.5	8.0	9.0	8.5	8.0	8.5	8.0	7.5	8.5
Urinary SG	1.040	1.041	1.043	1.043	1.031	1.030	1.041	1.041	1.010	1.029	1.034	1.028	1.025	1.023	1.037	1.030

b) *Balcanica* infected heifer:number 39

Temp. °C	39.3	38.1	38.3	38.9	39.3	38.8	38.5	38.2	38.4	38.3	38.7	38.5	38.6	38.5	38.2	39.1
PCV	0.39	0.36	0.38	0.38	0.37	0.39	0.36	0.37	0.38	0.34	0.35	0.37	0.34	0.30	0.33	0.32
Hb g/dℓ	12.6	11.2	ND	ND	11.4	12.2	ND	11.2	ND	ND	10.8	ND	ND	9.6	ND	9.8
MCHC g/dℓ	32.3	31.1	ND	ND	30.8	31.3	ND	30.3	ND	ND	30.9	ND	ND	32.0	ND	30.6
II	5	5	5	5	5	5	5	5	5	5	5	5	5	5	7.5	5
TP g/ℓ	58	60	60	54	54	62	54	62	60	56	58	60	56	56	58	60
WBC x 10^9/ℓ	9.9	11.0	6.2	9.8	10.5	11.1	6.7	7.6	7.8	7.9	7.9	8.0	9.8	9.6	10.0	9.4
MAT	-ve	-ve	-ve	-ve	-ve	-ve	1:24	1:48	1:96	1:384	1:768	1:768	1:768	1:768	1:768	1:384
Urinary pH	7.0	7.5	8.0	6.5	8.0	7.5	7.0	7.0	6.0	8.0	7.0	8.0	8.5	7.5	7.5	7.5
Urinary SG	1.030	1.034	1.039	1.050	1.032	1.032	1.043	1.043	1.015	1.045	1.022	1.023	1.027	1.033	1.036	1.028

ND: Not done

Urine was negative for protein, blood, bilirubin and glucose on all days.

APPENDIX XXX cont.: Haematolcgical and urinary results from heifers infected using *balcanica* culture

c) Infected heifer: number 41

Day P.I.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16
Temp.°C	38.9	38.6	38.5	40.3	39.0	38.4	38.5	38.2	38.2	38.8	37.8	38.0	38.8	38.9	38.3	38.7
PCV	0.32	0.34	0.34	0.31	0.30	0.31	0.32	0.30	0.32	0.28	0.29	0.30	0.29	0.36	0.28	0.24
Hb g/dℓ	10.6	10.8	ND	ND	9.6	9.4	ND	9.2	ND	ND	9.8	ND	ND	11.0	ND	7.8
MCHC g/dℓ	33.1	31.8	ND	ND	32.0	30.3	ND	30.7	ND	ND	33.8	ND	ND	30.6	ND	32.5
II	7.5	7.5	5	10	10	5	7.5	7.5	5	10	10	5	10	10	5	5
TP g/ℓ	66	72	72	70	70	72	72	72	76	70	68	72	68	70	68	68
WBC x 10^9/ℓ	8.6	9.2	7.8	10.6	8.3	10.4	6.7	6.4	6.8	7.1	6.3	6.8	7.8	6.8	7.3	7.1
MAT	-ve	-ve	-ve	-ve	1:24	1:96	1:192	1:384	1:768	1:768	1:1536	1:1536	1:1536	1:1536	1:536	1:768
Urinary pH	7.0	7.0	9.0	8.5	8.5	9.0	8.5	8.0	9.0	8.5	8.0	7.5	8.5	8.5	9.0	8.0
Urinary SG	1.020	1.026	1.043	1.047	1.028	1.032	1.031	1.025	1.022	1.035	1.024	1.028	1.026	1.027	1.026	1.022

d) Infected heifer: number 62

Day P.I.	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	16
Temp °C	39.2	39.2	38.8	38.8	38.8	38.8	38.7	38.3	38.3	38.6	38.5	38.1	39.0	38.7	38.8	38.3
PCV	0.37	0.35	0.34	0.34	0.36	0.34	0.34	0.33	0.33	0.31	0.30	0.30	0.30	0.29	0.32	0.27
Hb g/dℓ	11.8	11.6	ND	ND	11.4	11.0	ND	10.0	ND	ND	9.8	ND	ND	8.8	ND	8.8
MCHC g/dℓ	31.9	33.1	ND	ND	31.7	32.4	ND	30.3	ND	ND	32.7	ND	ND	30.3	ND	32.6
II	7.5	5	5	7.5	7.5	5	5	5	10	5	5	7.5	7.5	7	7.5	5
TP g/ℓ	72	74	74	66	72	70	72	66	72	72	68	66	72	66	76	68
WBC x 10^9/ℓ	11.8	11.3	6.3	10.5	15.0	9.6	9.7	9.6	9.5	8.7	10.5	10.3	9.3	11.2	11.8	9.7
MAT	-ve	-ve	-ve	-ve	-ve	1:24	1:48	1:192	1:384	1:768	1:768	1:768	1:768	1:1536	1:768	1:768
Urinary pH	8.0	8.0	8.0	9.0	8.5	9.0	8.0	8.0	8.0	8.5	8.0	8.0	8.5	8.0	9.0	9.0
Urinary SG	1.020	1.025	1.041	1.031	1.034	1.030	1.028	1.017	1.003	1.022	1.030	1.027	1.024	1.028	1.032	1.021

ND: Not done

APPENDIX XXX1: Results of *in vitro* Experiment A: Tube haemolysis of washed and unwashed RBC's taken from calves soon after birth to 47 days of age

Calf		Day 0				Day 6				Day 11				Day 18				Day 25			
		T116	790001	Scamp	MP3	T116	790001	Scamp	MP3	T116	790001	Scamp	MP3	T116	790001	Scamp	MP3	T116	790001	Scamp	MP3
63	washed		unborn			53%	80%	77%	91%	49%	ND	ND	35%	60%	79%	81%	84%	Died			
	unwashed					0%	0%	0%	0%	0%			0%	0%	0%	0%	0%				
65	washed						unborn			62%	ND	ND	46%	79%	90%	88%	86%	tube broken			
	unwashed									0%			0%	0%	0%	0%	0%				
66	washed	47%	66%	51%	69%	66%	79%	84%	86%	66%	ND	ND	57%	89%	89%	93%	94%	78%	69%	73%	74%
	unwashed	0%	0%	0%		0%	0%	0%	0%	0%			0%	0%	0%	0%	0%	0%	0%	0%	0%
67	washed						unborn			98%	ND	ND	89%	97%	81%	87%	84%	Died			
	unwashed									0%			0%	0%	0%	0%	0%				
68	washed	83%	98%	70%	97%	69%	76%	83%	84%	92%	ND	ND	72%	88%	84%	80%	80%	72%	59%	55%	55%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%	0%			0%	0%	0%	0%	0%	0%	0%	0%	0%
69	washed		unborn			46%	81%	76%	79%	57%	ND	ND	41%	68%	93%	95%	91%	46%	70%	74%	82%
	unwashed					0%	0%	0%	0%	0%			0%	0%	0%	0%	0%	0%	0%	0%	0%
70¶	washed		unborn			77%	89%	100%	89%	ND	66%	66%	ND								
	unwashed					0%	0%	0%	0%		0%	0%									
72¶	washed	57%	98%	95%	97%	tube broken				ND	70%	100%	ND								
	unwashed	0%	0%	0%	0%						0%	0%									
73	washed	80%	96%	97%	95%	85%	98%	100%	96%	ND	94%	95%	ND					78%	60%	60%	67%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%		0%	0%						0%	0%	0%	0%
76	washed	58%	88%	100%	89%	50%	79%	84%	85%	ND	43%	64%	ND		Died						
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%		0%	0%									
78	washed	94%	97%	95%	98%	79%	91%	100%	92%	ND	81%	89%	ND	tube broken				68%	60%	64%	57%
	unwashed	0%	0%	0%	0%	0%	0%	0%	0%		0%	0%						0%	0%	0%	0%
	Growth of organisms	Dense	Dense	Light	Light	Dense	Moderate	Light	Light	Dense	Moderate	Dense	Light	Dense	Dense	Dense	Moderate	Dense	Dense	Moderate	Dense

ND: Not done

* Contaminated culture therefore not incubated with RBC's
§ RBC's prepared for SEM
¶ Treated with systemic antibiotics containing streptopen: incubation discontinued

APPENDIX XXXI continued:

Calf		Day 32				Day 40	Day 47				MAT	
		T116	790001	*hardjo*	*balcanica*	790001§	T116	790001	Scamp	*MP3	*pomona*	*hardjo*
63	washed					haemolysis present					-ve	-ve
	unwashed					no haemolysis present						
65	washed	47%	53%	2%	5%	haemolysis present	57%	80%	55%	ND	-ve	+ve
	unwashed	0%	0%	0%	0%	no haemolysis present	0%	0%	0%			
66	washed	34%	67%	1%	4%	haemolysis present	68%	59%	71%	ND	-ve	+ve
	unwashed	0%	0%	0%	0%	no haemolysis present	0%	0%	0%			
67	washed										-ve	-ve
	unwashed											
68	washed	65%	59%	2%	5%	haemolysis present	81%	83%	71%	ND	-ve	+ve
	unwashed	0%	0%	0%	0%	no haemolysis present	0%	0%	0%			
69	washed	30%	55%	1%	4%	haemolysis present	44%	64%	83%	ND	+ve	-ve
	unwashed	0%	0%	0%	0%	no haemolysis present	0%	0%	0%			
70¶	washed										-ve	+ve
	unwashed											
72¶	washed										-ve	+ve
	unwashed											
73	washed	34%	68%	1%	7%	haemolysis present	82%	95%	93%	ND	-ve	+ve
	unwashed	0%	0%	0%	0%	no haemolysis present	0%	0%	0%			
76	washed										-ve	+ve
	unwashed											
78	washed	54%	73%	1%	7%	haemolysis present	91%	93%	91%	ND	-ve	+ve
Growth of organisms		Dense	Dense	Dense	Light	Dense	Dense	Dense	Moderate			

ND: Not done

* Contaminated culture therefore not incubated with RBC's
§ RBC's prepared for SEM
¶ Treated with systemic antibiotics containing streptopen: incubation discontinued

APPENDIX XXXII Haematological and Bone Marrow Data of Control and Irradiated Hamsters from Experiment VIII, part I

Hamsters Euthanased 3 days Post Irradiation

Hamster	1	2	3	4	5	6	7	8	9	10	11	12	13
Treatment	Control	Control	Control	600R	600R	600R	800R	800R	800R	1000R	1000R	1000R	1000R
PCV	0.47	0.46	0.49	0.47	0.47	0.45	0.49	0.47	0.46	0.48	0.41	0.43	0.46
Hb (g/dℓ)	15.8	15.2	16.2	15.0	15.6	15.4	16.6	15.8	15.0	15.6	13.8	14.4	15.4
MCHC (g/dℓ)	33.6	33.0	33.1	31.9	33.2	34.2	33.9	33.6	32.6	32.5	33.7	33.5	33.5
II	0	0	0	0	0	0	0	0	0	0	0	0	0
WBC($x10^9$/ℓ)	14.6	11.6	14.4	3.6	3.1	1.8	1.3	1.5	1.5	1.4	0.7	1.1	1.0
TP	60	58	62	56	54	57	55	55	54	62	54	58	58
M blast	0	0	0	0	0.2	0	0	0.2	0.8	0	0	0	0
P gran	1.1	0	0.8	2.0	0.7	0.5	0.6	0.2	2.1	0	0	0	0
m (n)	27.3	22.4	15.6	8.0	17.8	20.1	17.6	17.9	12.6	38.7	28.7	31.3	31.0
m (e)	0.9	0.7	0.4	0.2	0.4	0.3	0	0.2	1.3	0	0	0	1.0
m (b)	0	0	0	0	0	0	0.3	0	0.5	0	0	0	0
mm (n)	8.5	6.4	4.1	9.5	10.4	4.7	7.3	8.6	4.4	8.6	4.1	4.2	2.0
mm (e)	0.2	0	0	0.2	0.2	0.3	0	0	0	0	0	0	0
band (n)	10.8	9.9	6.6	18.0	17.0	12.3	24.3	19.3	12.6	10.2	8.2	9.4	9.0
band (e)	0.5	1.6	1.0	0.2	0.2	0	0.6	0.2	0.3	1.1	0	0.5	0
PMN	10.9	14.4	7.4	27.7	18.6	26.9	33.1	40.1	56.7	29.6	46.2	43.8	48.0
Eosin	0	0.5	0	0.2	0	0	0.3	0	0	0	0	0	0
Bas	0	0	0	0	0	0	0	0	0	0	0	0	0
Mitosis	0.2	0	0	0	0	0	0	0	0	0	0	0	0
Total	52.3%	55.9%	35.9%	65.9%	65.5%	65.0%	84.2%	86.9%	91.2%	28.2%	87.0%	89.1%	82.0%
E blast	0	0	0.2	0.9	0	0	0	0	0	0	0	0	0
E.Norm	0.5	0.3	1.0	4.4	0.6	6.8	0	0	0.5	0	0	0	0
Norm	16.2	16.3	24.4	9.5	11.9	11.5	1.2	0.5	1.6	0	0	0	0
L Norm	7.5	7.6	8.0	5.5	13.0	4.2	5.5	4.7	2.8	1.6	4.1	0.5	1.0
Mitosis	0.3	0	0.4	0.2	0	0	0	0	0	0	0	0	0
Total	24.8%	24.3%	34.0%	20.4%	25.4%	22.5%	6.7%	5.1%	4.9%	1.6%	4.1%	0.5%	1.0%
Pl.cell	0	0.2	0.2	0.2	0.2	0.3	0.6	0.5	0	0	0	0	1.0
L cyte	17.2	18.9	25.6	9.7	7.4	10.7	6.4	5.6	3.1	1.1	4.7	5.8	6.0
M cyte	0	0	0	0.4	0	0	0	0	0	0	0	0	0
Mega	0.3	0.3	0.4	0.9	0.2	0.3	0.3	0.2	0.3	0.5	0.6	0.5	0
Retic	2.3	0	0.6	0.6	0.6	0.8	1.2	0.9	0.5	2.2	0.6	1.6	4.0
Unclass	2.3	0.3	2.7	1.3	0.4	0.5	0.3	0.7	0	3.2	2.3	2.1	3.0
Disint	0.5	0	0.6	0.4	0.4	0	0.3	0	0	3.2	0.6	0.5	3.0
M:E	2.1:1	2.3:1	1.1:1	3.2:1	2.6:1	2.9:1	12.6:1	17:1	18.6:1	55:1	21:1	171:1	82:1

APPENDIX XXXII continued

Hamsters Euthanased 7 days Post Irradiation

Hamster	1	2	3	4	5	6	7	8	9	10	11	12	13
Treatment	Control	Control	Control	600R	600R	600R	800R	800R	800R	1000R	1000R	1000R	1000R
PCV	0.46	0.48	0.48	0.44	0.44	0.44	0.46	0.46	0.46	0.40	0.43	0.38	0.40
Hb (g/dℓ)	14.6	14.6	16.0	14.8	14.6	15.0	14.8	15.2	15.0	13.4	13.6	12.8	13.6
MCHC (g/dℓ)	31.7	30.4	33.3	33.6	33.2	34.1	32.2	33.0	32.6	33.5	31.6	33.7	34.0
II	0	0	0	0	0	0	0	0	0	0	0	0	0
WBC($x10^9/ℓ$)	7.6	6.6	10.7	2.5	1.5	1.3	0.9	1.2	0.7	0.6	0.1	0.2	0.2
TP	54	56	62	54	52	50	56	58	56	66	56	62	58
M blast	0.1	0	0	0	0.1	0.1	0.2	ND	0	ND	0.7	ND	ND
P gran	0.8	0	0.4	0.4	0.7	0.4	0.2		0.3		2.9		
m (n)	17.6	27.0	24.9	22.3	42.7	36.3	54.8		29.1		56.0		
m (e)	0.1	0	0.4	0	0.1	0.1	0.2		0		0		
m (b)	0	0	0	0	0	0	0,2		0		0.7		
mm (n)	4.5	2.9	2.9	3.5	2.2	3.7	2.6		0.3		2.5		
mm (e)	0	0	0	0.4	0	0.3	0.2		0.6		0		
band (n)	8.9	10.7	8.2	8.2	6.0	6.6	3.8		1.8		0		
band (e)	0.1	0	0.2	1.0	0.6	0.6	0.3		0		0		
PMN	18.0	16.9	11.2	39.4	13.9	18.2	13.3		11.4		0.4		
Eosin	0	0	0	0	0	0.6	0		0		0		
Bas	0	0	0	0	0	0	0		0		0		
Mitosis	0	0	0	0	0	0	0		0		0		
Total	50.2%	57.5%	48.1%	80.2%	66.4%	66.9%	75.6%		43.8%		64.7%		
E blast	0	0.2	0	0.2	0	0	0		0		1.8		
E.Norm	1.4	0.6	0.8	1.2	0.3	2.1	0.5		5.1		8.0		
Norm	13.7	13.0	18.4	1.4	6.7	11.3	7.5		9.0		2.9		
L. Norm	9.1	3.1	4.5	2.4	1.8	5.1	5.8		28.8		5.5		
Mitosis	0.3	0	0.2	0	0	0	0		0		0		
Total	24.4%	16.9%	23.9%	5.3%	8.8%	18.5%	13.8%		42.9%		18.2%		
Pl.cell	0	0	0	0.6	0.1	0.3	0		0		0		
L cyte	23.0	23.4	26.0	7.8	17.7	12.3	9.4		8.7		5.1		
M cyte	0	0	0.4	0.2	0.1	0	0		0.3		0.4		
Mega	0.4	0.2	0.4	0.2	0.1	0	0		0		0		
Retic	1.1	0.6	0.4	3.3	3.9	1.1	0.7		2.4		8.4		
Unclass	0.9	1.0	0.4	1.8	1.9	0.7	0.5		0.6		2.6		
Disint	0	0.2	0.4	0.6	0.9	0.3	0		0.4		0.7		
M:E	2.1:1	3.4:1	2.0:1	15.1:1	7.6:1	3.7:1	5.5:1	ND	1.1	ND	3.6:1	ND	ND

ND: Not done

APPENDIX XXXIII Summary of clinical and pathological data from Experiment VIII, part II.

Hamsters	Day of death	Number found dead	Range of PCV	Range of II	Haemoglobin-aemia	Carcase appearance	Haemoglobin-uria	Pathological Lesions of the Organs: Spleen	Liver	Kidney
C1-C6	5	0	0.42-0.52	0	None	Normal	None	Normal	Normal	Normal
C7	11	0	0.47	0	None	Normal	None	Normal	Normal	Normal
C8-C12	14	0	0.45-0.51	0	None	Normal	None	Normal	Normal	Normal
Irr13-Irr18	5	0	0.35-0.43	0	None	Pale	None	Thin, pale	Slightly pale	Normal
Irr19-Irr24	11	0	0.29-0.36	0	None	Pale emaciated	None	Thin, pale	Slightly pale	Normal
Inf25-Inf31	6	2	0.17-0.25	Yellow	Severe	Moderate to severe jaundice	Present	Red, friable enlarged	Red, friable enlarged	Red, friable enlarged
Inf32-Inf33	7	0	0.23-0.44	Yellow slight	Severe - none	Severe jaundice normal	Present - none	Red, friable enlarged	Red, friable enlarged	Red, friable enlarged
Inf34-Inf35	9	2	ND	ND	ND	Severe jaundice	Present	Red, friable enlarged	Red, friable enlarged	Red, friable enlarged
Inf36-Inf37	10	0	0.26-0.030	Yellow	Moderate - slight	Moderate - slight jaundice	Present - none	Red, Friable enlarged	Red, Friable enlarged	Red, friable enlarged
Inf 38	14	0	0.50	0	None	Normal	None	Normal	Normal	Normal
II 39-II40	5	0	0.06*-0.23	Yellow - normal	Severe - none	Slight jaundice - none	Present	Thin, red friable	Red, friable slightly enlarged	Enlarged, friable, either pale or red, with dark red streaks in the cortex plus red urine within the pelvis of most animals
II41-II48	6	3	0.07*-0.36	2Yellow 3normal	2Severe 2none	5Slight jaundice- 3none	Present	Thin, red friable	Red, Friable slightly enlarged	
II49-II50	7	1	0.36	0	None	Pale or slight jaundice	Present	Thin, red friable	Red, friable slightly enlarged	
II51	10	1	ND	ND	ND	Slight jaundice	Present	Thin, red friable	Red, friable slightly enlarged	
II52	11	0	0.07	Yellow	Severe	Slight	Present	Thin, red friable	Red friable slightly enlarged	

ND: Not done (animals found dead)

* Those animals with PCV's 0.23 and above had an II of 0, and no signs of jaundice.

APPENDIX XXXIV Bone Marrow Differentials of Hamsters from Experiment VIII Part II

Hamster	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	IRR20	IRR21	IRR22	IRR23	IRR24
M blast	0.3	0.1	0.4	0	0.2	0	0.1	0.5	0	0.1	0	0.1	0	0.2	0.6	0.5	0.2
P Gran	0.9	1.5	0.6	1.2	1.5	0.6	1.6	2.1	1.7	1.3	1.4	1.3	1.5	2.4	6.5	3.4	2.0
m (m)	7.9	23.0	15.1	9.5	9.9	11.2	10.4	19.0	16.7	13.9	17.8	17.4	13.5	10.6	16.5	14.7	13.6
m (e)	0.1	0.7	0.4	0.9	1.0	1.8	0.9	1.2	1.2	0.4	1.1	0.4	0	0	0	0	0
m (b)	0.4	0.1	0.1	0.2	0.1	0	0.4	0.2	0.9	0.2	0	0.1	0	0.2	0	0.1	0
mm (m)	2.5	2.7	1.9	3.3	3.8	2.0	2.5	3.4	3.7	3.4	3.1	2.6	11.2	9.3	6.7	5.0	3.1
mm (e)	0.2	0.3	0.1	0.5	0	0.4	0.3	0.2	0.1	0.1	0.3	0	0	0	0	0	0
band (n)	8.1	7.2	8.0	8.7	4.1	7.0	8.4	8.7	8.9	7.1	8.0	8.8	20.9	20.5	11.1	7.8	3.9
band (e)	0.4	0.3	0.6	0.5	0.6	0.8	1.4	0.2	0.3	0.1	0.3	0.3	0.3	0	0	0.1	0
PMN	6.4	9.8	12.2	15.1	5.6	10.4	11.3	14.6	15.1	15.8	19.3	12.0	9.4	11.5	13.6	6.3	3.3
Eosin	0.2	0.5	0.5	0.8	0.4	0.6	0.4	0.7	0.5	0.1	0.1	0.3	0	0	0	0	0
Bas	0	0	0	0.1	0	0	0	0	0.3	0	0	0	0	0	0	0	0
Mitosis	0.2	0.1	0.4	0.4	0.7	0	0	0.2	0.4	0.1	0.1	0.1	0.3	0.2	0.3	0	0
Total	27.8%	46.2%	40.2%	41.1%	27.4%	34.7%	37.8%	50.8%	49.8%	42.5%	51.7%	43.8%	57.0%	54.9%	55.3%	37.9%	26.1%
E blast	0.1	0	0.1	0.1	0.3	0	0	0.2	0	0	0	0	0	0.4	0.3	0.4	0.4
E.Norm	1.5	0.9	0.7	1.2	1.2	0.6	0.9	1.2	0.9	0.7	0.9	0.8	0.3	1.4	2.9	0.9	1.1
Norm	16.9	18.7	18.5	11.5	16.8	18.9	16.2	15.8	8.5	12.2	11.0	13.2	8.4	13.8	11.1	17.6	23.3
L.Norm	36.0	26.1	23.1	17.4	23.7	24.1	28.0	17.5	18.5	12.7	13.3	17.5	10.4	14.8	13.5	21.2	23.8
Mitosis	0.7	0.5	0.8	1.0	0.8	0.6	1.4	0.9	0.2	0.4	0.3	0.4	0.3	1.0	0.3	0.8	1.5
Total	55.3%	46.1%	43.2%	31.1%	42.9%	44.2%	46.5%	35.5%	28.2%	25.9%	25.5%	31.8%	19.3%	31.5%	28.2%	41.0%	50.1%
Pl. cell	0	0	0	0.1	0	0	0	0	0	0	0	0	0	0	0.1	0	0
L cyte	13.5	6.7	14.5	27.0	29.4	20.7	13.2	11.3	21.2	31.0	22.8	24.1	8.9	9.1	15.0	20.7	22.7
M cyte	0.1	0.1	0	0	0.2	0	0	0	0	0	0	0	0	0.2	0	0	0
Mega	0.2	0.2	0.2	0.3	0.1	0.4	0	0.3	0.2	0.1	0	0.3	0.5	0.2	0	0.4	0.2
Retic	1.2	0.7	0.6	0.3	0	0	1.0	2.1	0.5	0.3	0	0	7.6	0	0.3	0	0.4
Unclass	1.0	0	0.5	0.1	0	0	0.3	0	0	0.2	0	0	0.5	3.0	0	0	0.1
Disint	1.0	0	0.7	0	0	0	1.2	0	0	0	0	0	6.1	1.0	0	0.1	0.3
M:E Ratio	0.50:1	1:1	0.9:1	1.3:1	0.6:1	0.8:1	0.8:1	1.4:1	1.8:1	1.6:1	2.0:1	1.4:1	3.0:1	1.7:1	2.0:1	0.9:1	0.5:1

Appendix XXXIV continued

Hamster	Inf27	Inf28	Inf29	Inf30	Inf31	Inf32	Inf33	Inf36	Inf37	Inf38
M blast	0.2	0.3	0.2	0.2	0.4	0	0.2	0.8	0.2	0
P Gran	2.2	3.2	2.7	1.4	1.5	1.5	1.7	7.4	2.7	1.3
m (m)	13.2	19.6	17.4	19.1	12.4	21.0	19.7	30.9	13.9	13.9
m (e)	0.2	0.9	1.3	1.1	1.1	0.7	1.6	0	1.2	1.0
m (b)	0	0.1	0	0.1	0.2	0.1	0.1	0.5	0.3	0.1
mm (m)	2.1	5.2	4.7	4.7	3.9	5.5	3.0	6.0	2.1	3.3
mm (e)	0.4	0.4	0.2	0.1	0.3	0.1	0.3	0	0	0.4
band (n)	1.9	4.0	2.5	7.2	4.2	4.4	1.1	6.1	2.1	9.5
band (e)	1.1	1.0	0.6	0.5	0.8	0.4	1.2	0	0.5	0.4
PMN	0.2	1.4	1.2	1.6	0.6	2.1	3.4	26.7	0.3	17.1
Eosin	0.7	0.5	0.6	0.7	0.8	0.6	0.8	0.9	0.4	0.1
Bas	0.3	0.3	0	0	0.4	0	0.9	0.9	0	0.2
Mitosis	0.8	1.1	1.2	0.8	0.1	0.9	0.5	1.1	1.1	0
Total	23.4%	38.0%	32.6%	37.6%	26.8%	37.2%	34.3%	78.5%	24.8%	47.2%
E blast	0.1	0.4	0	0.1	0	0	0.1	0	0	0.1
E.Norm	0.8	1.1	1.0	1.1	0.6	0.7	0.9	0.9	0.3	0.8
Norm	11.4	13.9	12.8	14.7	18.9	11.4	4.8	4.4	4.0	11.6
L. Norm	33.1	22.0	25.0	16.0	30.0	13.8	32.4	1.8	39.6	13.4
Mitosis	0.6	0.8	1.4	0.8	0.3	0.5	0.4	0	1.3	0
Total	46.0%	38.2%	40.3%	32.8%	49.8%	26.4%	38.6%	7.0%	45.2%	25.8%
Pl cell	0	0	0	0	0	0	0	0	0.2	0
L cyte	30.6	23.6	26.7	29.6	23.4	36.1	26.6	12.8	29.6	26.7
M cyte	0	0	0	0	0	0	0	0	0	0
Mega	0.1	0.1	0.4	0.1	0	0.3	0.2	0.6	0.1	0.3
Retic	0	0	0	0	0	0	0	1.1	0	0
Unclass	0	0.1	0	0	0	0	0.3	0	0	0
Disint	0	0	0	0	0	0	0	0	0	0
M:E Ratio	0.5:1	1:1	0.8:1	1.2:1	0.5:1	1.4:1	0.9:1	11.2:1	0.6:1	1.8:1

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