MECHANISMS OF PATHOGENESIS IN EXPERIMENTAL BABESIOSIS

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CONTENTS

SUMMARY

The pathophysiology of Babesia rodhaini infection in intact rats and B.divergens infection in splenectomized calves has been examined. In both infections the anaemia and the pathological changes recorded were closely related to the parasite development and once the parasitaemia declined recovery was apparent.

The roles of the spleen and the reticulo-endothelial system as possible mechanisms of erythrocyte loss in B.rodhaini infections were examined and while this organ and system were responsible for some loss of erythrocytes their contribution to the anaemia was considered to be small.

In both infections the osmotic fragility of the erythrocytes was significantly increased. Studies of B.rodhaini and B.divergens in living blood preparations showed very different modes of parasite activity within erythrocytes but both parasites appeared to maintain a close association with the erythrocyte membrane. The scanning electron microscope revealed considerable morphological alterations on the surface of B.rodhaini-infected erythrocytes and during severe babesiosis in rats the morphological characteristics of the entire erythrocyte population were greatly changed. It was concluded that the anaemia of babesiosis was due not only to the destruction of erythrocytes but probably also to a loss in efficiency of surviving cells.

The serum biochemical and histopathological changes found in both infections were first recorded after the appearance of parasites in the peripheral circulation. The disease in rats was consistently severe while the disease in calves varied in severity. In general the

changes recorded were non-specific and common to other disease states.

A coagulation disturbance leading to thrombosis and frequent splenic infarction was found in rats during B.rodhaini infection. Thrombocytopenia, hyperfibrinogemaemia and raised serum levels of fibrinogen or fibrin degradation products were present from early in infection and these changes were considered to be manifestations of a disseminated intravascular coagulation syndrome. In calves a thrombocytopenia, minor changes in plasma fibrinogen levels and positive protamine paracoagulation tests were found during the course of B.divergens infection. From this evidence, and from reviewing the literature on the pathology of babesiosis, it was concluded that disseminated intravascular coagulation could be an important mechanism in the pathogenesis of babesiosis.

CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

The babesias are non-pigmented amoeboid protozoan parasites found in the blood of many wild and domesticated animals throughout the world. They are transmitted by ixodid ticks and their life cycle involves development within the erythrocytes of the vertebrate host and within the tissues of the tick. They cause disease in domesticated animals with considerable economic loss.

The organisms were first described by Babes in 1888 from the blood of a cow in Rumania. He later described similar organisms from sheep (Babes, 1892). Their role in disease was recognized by Smith and Kilborne (1893) when they showed that a piroplasm, the general name given to the group, was the cause of Texas fever in cattle. They called this organism Pyrosoma bigeminum, and they described the pathology of the disease and the tick transmission of the parasite. Piana and Galli-Valerio (1895) described piroplasms in the blood of dogs with malignant jaundice and Guglielmi (1899) observed parasites in equine blood which Laveran (1901) identified as piroplasms. This early history of the disease has been thoroughly reviewed by Wenyon (1926).

Babes first called the organism he observed a Gonococcus and later an Haematococcus. As the names Haematococcus and Pyrosoma used by Smith and Kilborne (1893) were already applied to other organisms, the generic name Babesia, given by Starcovici (1893) to Babes original two parasites, Babesia bovis and Babesia ovis, is the accepted name for the genus.

The position of the piroplasms within the phylum Protozoa is unsettled. Traditionally they were placed in the Sporozoa Leukart 1879. Wenyon (1926) classified them in the class Sporozoa, order Coccidiida, sub-order Piroplasmidea with two families, the Babesiidae Poche 1913 and the Theileriidae du Toit 1918. The Sporozoa was regarded by Ball (1960) as an unsatisfactory class with poorly defined characteristics and only a parasitic mode of life in common. Levine (1961) placed the piroplasms in an appendage to the Protozoa, class Piroplasmea Wenyon 1926. In this system they were moved away from the malarial parasites, and although the many similarities between these two groups were recognized, the absence of sexual reproduction in the piroplasms was a major factor in their separation. An international committee of protozoologists (Honigberg et al, 1964) placed them in superclass Sarcodina Hertwig and Lesser 1874. This position was accepted by Chessin (1965) because of the mode of reproduction and movement of the organisms. Riek (1968) returned them to the Sporozoa arguing that the matter of sexual reproduction had not been resolved and the similarities of the piroplasms to the malarial parasites justified a closer positioning. Levine (1969), who as one of the international committee (Honigberg et al, 1964) had removed them from the Sporozoa, now returned them to this class in a new subphylum, Apicomplexa. The evidence presented from electronmicroscopic studies had demonstrated broad similarities in the apical regions of many organisms that justified this new grouping and the return to the Sporozoa (Levine, 1971). Honigberg (1974) argued that this new classification was not justified on these grounds alone and the 1964 classification (Honigberg et al, 1964) is accepted in this work.

From the early studies of the piroplasms it became evident that there was more than one genus. Bettencourt et al.(1907) created a new genus Theileria for those organisms with schizogony in their development. Du Toit (1918) placed them in a separate family Theileriidae du Toit 1918. Subsequently, the taxonomy of the piroplasms has been reviewed by many authors including Donatien and Lestoquard (1930), Thompson and Hall (1933) and Neitz (1956). In the early studies there was a great deal of division into genera and subgenera within the Babesiidae which was greatly simplified by Wenyon (1926) who accepted only one genus. Neitz (1956) also accepted one genus and on consideration of the synonymy concluded that there were 17 valid species. This number increased to 71 in the following 15 years (Levine, 1971). The clear distinction between the two families in the Piroplasmida seems to be bridged by a babesia-like parasite in the gerbil (Tsur et al., 1960). This parasite, Nuttallia (Babesia) danii, develops both in the erythrocyte and in the tissues of the host. Ultrastructural studies have shown similarities between these families (Jarrett and Brocklesby, 1966) and to malarial parasites (Rudzinska and Trager, 1962). The classification is likely to remain unsettled until more is known about the life cycle of babesias and parameters other than their size and morphology are used in distinguishing between the species.

The life cycle of babesias in the tick vector has been recently reviewed by Riek (1968). Their transmission by ticks was first recorded by Smith and Kilborne (1893). Their development in the tick was studied by Koch (1906) using B.bigemina in a number of ticks. Developmental forms were seen in both the gut and the ova. Rosenbusch (1927)

clarified the development of B.bigemina in Boophilus microplus. Sexual development was suggested by Dennis (1932) for B.bigemina in Boophilus annulatus. But Regendanz and Reichenow (1933) studying B.bigemina in Boophilus microplus found no evidence of sexual forms. Petrov (1941) claimed that sexual stages did occur in B.bovis development in Ixodes ricinus but this was disputed by Poljansky and Cheissin (1959). Riek (1968) concluded that "the early development in the tick is uncertain, but various bodies have been detected which could be sexual stages".

The babesias are transmitted during tick feeding and pass into the blood stream (Hoyte, 1961). There they enter the erythrocytes and the intraerythrocytic location has been confirmed by the early electron microscopic studies (Flewett and Fulton, 1959; Bayer and Dennig, 1961). Krylov (1964) described exoerythrocytic forms of B.bigemina in the lumina of capillaries in many tissues but these were not found by Hoyte (1965). Levine (1973) reviewing Krylov's paper thought he was describing free parasites that had been pushed together. Matson (1964) has seen similar aggregates of B.rodhaini free from erythrocytes which he considered to be 'clumped' free parasites. The majority of authors describe only erythrocytic forms.

Once within the cell the parasite must feed, grow and reproduce. Reproduction is by a process of budding or binary fission in most babesias (Nuttall and Graham-Smith, 1908; Rudzinska and Trager, 1962) but a few species divide into four (Nuttall and Strickland, 1912; Van den Berghe et al, 1950). This latter form of division, described as quarternary fission or limited schizogony, encourages some authors (Franca, 1909; Aeschlimann and Suter, 1965; Nowell, 1968) to place these babesias in a separate genus, Nuttallia. However, the majority

opinion is against any new genera at least until more is known about the babesias.

Following division the parasites leave the cells by lysis but Holbrook et al (1968), while studying living whole blood preparations in vitro, have observed B.equi and B.caballi leaving the erythrocyte without lysis. The rate at which babesias multiply is difficult to calculate as they are asynchronous. Babesia rodhaini, which multiplies fairly constantly early in infection has been calculated to double in number every nine to ten hours by Bungener (1967) and every $14\frac{1}{2}$ hours by Overdulve and Antonisse (1970).

The erythrocyte preferences of the babesias have not been investigated very thoroughly. Eaton (1934) reported a preference for reticulocytes by B.canis but Simons (1939) found no evidence of young cell preference by B.canis, B.gibsoni, B.argentina or B.bigemina. However, both of these investigations are open to criticism. Eaton had examined only one dog suffering from a severe intercurrent Dirofilaria immitis infection. Simons examined one dog with each canine babesia infection and a bull with a mixed infection of B.argentina and B.bigemina which eventually died from secondary pericarditis due to an unrecognised foreign body. Landsberg and Eskridge (1940) investigated the cell preferences of B.canis in eight splenectomized and four intact dogs. They found that despite high reticulocyte counts there was no predeliction for these cells by the parasite, but parasitized mature cells were larger than non-parasitized cells. Rodhain (1950) found a mature cell preference by B.rodhaini but Nowell (1968) reported a reticulocyte preference by this parasite. Nowell had examined only three rats and his finding has since been disputed by McHardy (1973). Recently

Wright and Kerr (1974), using cell size as the criterion of age, found that B.bigemina parasitized larger (i.e. younger) cells from early in infection. However, another possible explanation for their observation is that parasitization itself may have induced an increase in cell size, as could also have been the case in Landsberg and Eskridge's (1940) findings.

The early investigations into the nature and control of babesiosis met with such success that it might seriously have hindered later research. The description of the pathology and epidemiology of bovine babesiosis by Smith and Kilborne (1893) was quickly followed by the application of a vaccine (Hunt, 1897). In 1909 Nuttall and Hadwen demonstrated the usefulness of trypan blue in the treatment of canine babesiosis. In 1935 acaprin, a synthetic quinilyl, was used to treat canine babesiosis (Kikuth, 1935; Carmichael, 1935) and Kikuth (1938) showed that in low doses it allowed survival of sufficient parasites to stimulate a natural resistance. Legg (1939) found that B.argentina alone, rather than in a mixed vaccine with B.bigemina, provided adequate protection in Australian cattle. In the early 1940s bovine babesiosis was successfully eliminated from the United States by vector control, and large animal babesiosis was not seen again until equine babesiosis was introduced from Cuba (Sippel et al, 1962; Maurer, 1962). With vector control and vaccination as effective control measures for range cattle and the sporadic nature of outbreaks and the usually recognizable clinical signs coupled with efficient therapy in more intensive farming systems, the threat of the disease was greatly reduced. It is only in recent years that interest has revived in babesiosis, and particularly in its immunology (Mahoney, 1972). The

search for better vaccination and reliable serodiagnostic tests have prompted this interest along with the usefulness of babesia models in malarial research and the occurrence of clinical babesiosis in man.

"The destruction of red corpuscles by the micro-parasites within them is the main fact in the pathology of Texas fever" (Smith and Kilborne, 1893). The anaemia they described was progressive and dependent upon the degree of parasitaemia. A regenerative response was seen in all except the most rapidly developing and overwhelming infections. They considered that erythrocyte destruction, congestion of internal organs and the packing of small blood vessels by altered red cells and debris to be responsible for the degeneration of the host tissues. The liver was most profoundly affected, with centrilobular necrosis in severely affected animals. Kidney degeneration was less marked, and haemoglobinuria was present in all but very mild cases. Upon these observations the basic concept of the pathology of babesiosis was laid.

In contrast to rapidly developing disease with high parasitaemia, Nocard and Motas (1902) recorded difficulty in finding parasites in chronic babesiosis in dogs - commonly called malignant jaundice. Apart from this observation and descriptions of less typical manifestations of the disease, summarized by Malherbe and Parkin (1951), nothing was added to the general concept of the disease until investigations into the pathophysiological changes occurring during infection were undertaken in the 1950s.

Malherbe (1956) described the manifestations of canine babesiosis and was struck by the close similarities with malaria in a variety of its disease patterns. Maegraith et al, (1957) brought their

accumulated knowledge of the malarial pathogenesis to the study of babesiosis. Working with B.canis, they emphasized the many similarities between the diseases and the non-specific nature of many of the changes. They endeavoured to relate the progress of the disease to parasite development and the changes in the blood to tissue changes. They showed that the oxygen-carrying capacity of the blood was adequate until erythrocytes fell to a very low level. However, as the disease advanced the plasma became increasingly more lytic for erythrocytes, bilirubin and urea levels increased and, while plasma glycogen remained within the normal limits, liver glycogen stores were seriously depleted. Liver changes were brought about in part by circulatory changes and possibly also by direct 'toxic' action of substances of parasite or host-parasite origin. Shock was an important outcome in some cases and responded to the intravenous injection of nor-adrenaline. Tella and Maegraith (1961a,b) later demonstrated the pathophysiological similarities between acute experimental malarial infections and B.canis infection. Malherbe (1965a,b,c; 1966) applied a range of clinico-pathological tests to clinical cases of babesiosis in dogs and his findings closely paralleled those of Maegraith et al.(1957) in experimental disease.

Goodwin and Richards (1960) described the presence of pharmacologically active substances in the blood and urine of mice with B.rodhaini infection. At the same time they described similar substances during the course of malaria, trypanosome, virus and rickettsia infections. In the following years the role of these substances in malaria and trypanosomiasis was closely examined (Tella and Maegraith, 1962; Boreham, 1968; Boreham and Goodwin, 1969;

Onabanjo and Maegraith, 1969). They were identified as kinins, a group of extremely active substances derived from the kininogen precursors in the plasma by the enzymatic action of kininogenases (e.g. kallikrein) and rapidly destroyed by kininases (Goodwin, 1970). Their action on the vasculature produces increased permeability and is probably responsible for the oedema of chronic inflammation. Wright (1973) found elevation of kallikrein levels in B.argentina infections which eventually fell to subnormal levels. Further investigations (Wright and Mahoney, 1974) found that kallikrein activation occurred prior to the appearance of parasites and remained high for 11 to 12 days, until kallikrein reserves were depleted or liver synthesis failed due to degeneration. Both Goodwin and Richards (1960) and Wright and Mahoney (1974) found that the injection of lysed blood did not activate this system. It was postulated that (i) the parasite or its products might activate Hageman Factor which in turn would activate kallikrein, or (ii) that parasite-induced tissue damage caused the release of products which would directly activate kallikrein. Boreham and Goodwin (1969) have postulated that, in human trypanosomiasis, antigen-antibody complexes can absorb Hageman Factor which then activates kallikrein. The demonstration of these substances and their activity offered very strong support for the shock syndrome in dogs described by Maegraith et al (1957) and since observed by Wright (1973) in cattle with babesiosis.

The concept of an immune mechanism in the pathogenesis of the anaemia of babesiosis was first given experimental support by Schroeder et al (1966). They demonstrated agglutinins for trypsinized erythrocytes, spherocytosis and phagocytosis of apparently normal erythrocytes during

the course of infections in rats that were more closely related to the anaemia than was the degree of parasitaemia. These findings were similar to those reported from studies on malaria infections (McGhee and Corwin, 1964; Cox et al., 1966) and Anaplasma marginale infections (Ristic, 1961; Mann and Ristic, 1963; Kreier et al., 1964; Schroeder and Ristic, 1965). The observation of an anaemia apparently in excess of the degree of parasitaemia in babesiosis was well known in cattle and dogs (Nissle, 1907; Neitz, 1938). Maegraith et al. (1957) found, in addition to disproportionate anaemia in B.canis infection, the phagocytosis of apparently normal erythrocytes. Sibinovic et al.(1965) demonstrated antigens in the sera of horses, dogs and rats with babesiosis which became apparent during the parasitaemia and disappeared with the onset of latency. The inoculation of dog or rat antigens into either dogs or rats was capable of producing an anaemia (Sibinovic et al., 1967a,b). These antigens were elaborated by infected erythrocytes and were bound onto erythrocytes during the development of acute parasitaemia. They were directed against erythrocytes rather than parasites. The actual role of the antigen on normal erythrocytes was not clarified (Sibinovic et al., 1969). Ristic et al.(1972) characterized the antibodies involved in the excessive anaemia of Anaplasma marginale infections in calves. They included haemagglutinins directed against components within the erythrocyte membrane and opsonins sensitizing erythrocytes to phagocytosis. Maximum activity occurred during the anaemic crisis. Rogers (1974) found similar opsonic activity in the serum of B.rodhaini infected rats using an in vitro peritoneal macrophage phagocytosis test. However, the opsonins were only capable of sensitizing

infected erythrocytes. These findings were said to satisfy the requirements of an auto-immune mechanism of anaemia, particularly the demonstration of what appeared to be erythrocyte autoantibodies. However, auto-immune-like phenomena have been demonstrated by the repeated bleeding of rats (Cox et al., 1966) and by the injection of Freund's adjuvant in calves (Mahoney, 1972). Calves treated in this way produced antigens to trypsinized erythrocytes but showed no anaemia and no interference with subsequent B.argentina infection. While Maegraith et al. (1957) described excessive anaemia in B.canis infection they were unable to demonstrate an increased rate of destruction of P_{32} or Fe_{59} labelled erythrocytes. Mahoney (1972) using repeated inoculations of plasma antigen from B.argentina infections failed to induce Cr_{51} labelled erythrocyte destruction in normal calves although the antigens produced an immune response that protected against later B.argentina infection. Goodger (1970, 1971) demonstrated antigens to normal haptoglobin-haemoglobin complexes and normal erythrocyte protein extracts in B.argentina infections but they were not directed against intact erythrocytes.

Other mechanisms of excessive destruction of erythrocytes proposed for malarial infections include toxins, lysins of parasite or host-parasite origin and reticuloendothelial hyperactivity (Zuckermann, 1964, 1966; Dixon, 1966; Zuckermann et al, 1969). It seems very likely that similar mechanisms, in particular reticuloendothelial hyperactivity, are responsible for haemolysis in babesiosis. Mahoney (1972) emphasizes the parallel development of anaemia and parasitaemia in B.argentina and B.bigemina infections but points out the persistence of anaemia in some B.canis infections after the decline in parasitaemia.

The anaemia of B.divergens infection is also closely related to parasitaemia (Davies et al, 1958) but more cells appear to be destroyed than are parasitized (Joyner, 1966).

A different type of immune reaction was proposed by Holbrook (1965) in the pathogenesis of B.equi and B.caballi infections. After the appearance of antibodies in both infections a shock syndrome appeared. He argued that the combination of antigen and antibody produced an anaphylactic-type reaction caused by these circulating complexes. No further investigation of this mechanism has been carried out and shock in babesiosis is generally regarded as physiological (Maegraith et al, 1957; Goodwin and Richards, 1960).

Iturri and Cox (1969) described a correlation between antibodies for trypsinized erythrocytes and kidney damage in B.rodhaini infected rats. They contended that the components of immunological anaemia were also affecting the vascular endothelium of the kidneys. The high titres for agglutinins coincided with the most dramatic glomerular change and swelling of the tubular epithelium. However, their rat stock was infected with Haemobartonella muris and control animals developed an anaemia during the experimental period. Their results must therefore be regarded with caution. The concept of an immune-based renal pathology has been substantiated by Annable and Ward (1974) in the same host-parasite system. These workers demonstrated an immune complex glomerulo-nephritis of a mild transient nature. Using immunofluorescent staining, glomerular deposits of both Ig G and complement (C_3) were identified with an accompanying hypocomplementaemia. The Ig G was eluted and reacted with babesial antigen. The mechanism was thought to be the combination of complement fixing antibodies with

soluble antigens of the parasite and subsequent bombardment of the glomerulus as reported in many other conditions including malaria (Dixon et al, 1971). Babesia models in both these studies were used with the primary aim of investigating malarial pathogenesis.

Cerebral babesiosis is one of the more frequent atypical manifestations of canine and bovine babesiosis. Parant (1905) reported severe nervous signs in dogs with B.canis infection, an observation subsequently reported many times (Basson and Pienaar, 1965). Clark (1918) and Zlotnik (1953) described nervous signs in B.bigemina infections in cattle. Rees (1934) described the aggregation of B.argentina-infected erythrocytes in the vessels of internal organs, particularly in the brain. The clinical signs and histopathological changes in experimental B.argentina infection with cerebral involvement were described by Callow and McGavin (1963).

Wright (1972b) studied the ultrastructural changes in the brain during B.argentina infections with clinical nervous signs. Congestion was present early in infection and local parasitaemia developed very rapidly to about 90% in the capillaries. As the disease progressed the parasites became greatly enlarged, many cells lysed and the shape of parasitized cells changed from round to stellate. These stellate cells were shown to have fine strands attached to the endothelial cells of the small vessels. Non-parasitized cells appeared normal. This type of parasite change and aggregation was not observed in the kidney (Wright, 1972b). Light microscopic examination of the brain showed dilatation of perivascular spaces and some interstitial oedema with occasional perivascular haemorrhages (Callow and McGavin, 1963; Rogers, 1971). The change in size in B.argentina has prompted

Wright (1972b) to suggest that both Clark (1918) and Zlotnik (1953) might have mistaken the enlarged B.argentina for B.bigemina. Also, while congestion is a feature of both infections, high local parasitaemia does not occur in B.bigemina (Callow and McGavin, 1963; Wright, 1972a). Uilenberg (1965) considered that intercurrent viral or rickettsial infection might have provided an environment which permitted B.bigemina to multiply rapidly in the brain.

In B.canis infection, layering of parasitized cells along the capillaries of the brain has been described (Maegraith et al, 1957), a process not seen in other Babesia infections. Ludford (1969) demonstrated a similar type of fluorescence about the periphery of both B.argentina and B.canis infected erythrocytes that was not shown for other Babesia infected cells. It could be that both parasites induce alterations in the erythrocyte surface with consequent disturbances of blood flow similar to that described in Plasmodium knowlesi infection (Knisely et al, 1964). Rogers (1971) has suggested that perivascular dilatation, reflecting plasma fluid accumulations about the vessels, was the result of increased permeability induced by vaso-active substances as earlier described. The local haemoconcentration may have added to the disturbed blood flow. Finally it has been suggested that the utilization of fibrinogen in acute P.falciparum malaria may cause clumping of erythrocytes (Devakul et al, 1966). Maegraith et al.(1957) have described minor fibrin formation without thrombosis in cerebral vessels of dogs, and Mahoney and Goodger (1969) observed a fibrinogen-like substance in the serum of B.argentina infected calves that might have resulted from fibrin or fibrinogen breakdown. The investigation of coagulation disturbances in Babesia

infections forms an important part of the research undertaken in this thesis.

A new species of piroplasm was described from the blood of English cattle by M'Fadyean and Stockman in 1911. The parasite was small, frequently placed at the periphery of the erythrocyte and the paired organisms were widely divergent. They named the parasite Piroplasm divergens and established its separate identity from B.bigemina (P.bigeminum), the cause of Texas fever. They implied that the larger parasite existed at that time in British cattle. Nocard (1901) had first described piroplasms in Ireland and since that first report many observations were made in Britain (Stockman, 1908). Montgomery (1904) had described the causal organism of 'red water' in the west of England. He considered that the parasite he described, the original organism described by Babes (1888) and the cause of Texas fever were all the same parasite, B.bovis (P.bovis). From the time of the description of B.divergens by M'Fadyean and Stockman until Brocklesby and Barnett (1970) described a larger Babesia, identified as B.major (Brocklesby et al, 1971), no reports of any other species were recorded. Matson (1964) noted the close similarity between the B.divergens he was working with and the causal agent of redwater in Devon and Cornwall, described by Montgomery (1904). As yet no description of a larger species has been reported from that part of England and so it is possible that Montgomery's was the first accurate description of B.divergens.

B.divergens was considered by Wenyon (1926), Donatien and Lestoquard (1930) and Neitz (1956) to be a synonym for B.bovis, although du Toit (1918) considered it a valid species. Davies et al

(1958) reviewed the literature and compared B.divergens with a Yugoslavian strain of B.bovis and concluded that on morphological grounds they were different species. Levine (1971, 1973) accepted B.divergens as a separate species, regarding it as the common Babesia in Western and Central Europe. The difficulty of parasite identification on morphological features alone is emphasized in the case of B.divergens by the observations of Danilov et al.(1965). These workers defined four separate epidemiological areas, in each of which the parasite had a different appearance. In an area defined as immune the parasite was very atypical morphologically.

The babesias were generally held to be host specific, but this concept has been gradually eroded by the demonstration of various Babesia species in unusual hosts. B.divergens was the cause of death in a human infection described by Skrabalo and Deanovic (1957) and a number of similar infections have been reported since. Garnham and Bray (1959) infected splenectomized monkeys and chimpanzees, and Enigk and Friedhoff (1962) successfully infected with B.divergens wild sheep and fallow, roe and red deer, all of which had been splenectomized. The fact that infections can be established in unusual hosts is probably, as suggested by Healy and Gleason (1973), because the host is unusually susceptible. This state of susceptibility is most often produced by splenectomy.

There are many reports of the clinical disease caused by B.divergens but relatively few reports on the pathology of the disease. This present investigation follows the course of B.divergens infection in splenectomized calves.

B.rodhaini was first described by Van den Berghe et al. (1950) from the blood of the Congo tree rat (Thamnomys surdaster surdaster). The parasite was inoculated into mice and produced mild infections with low parasitaemias. With passage the pathogenicity increased, producing very high parasitaemia, haemoglobinuria and death (Rodhain, 1950; Rodhain and Demuylder, 1951; Colas-Belcour and Vervent, 1953). Rodhain (1950) produced mild infections in splenectomized cotton rats and intact Syrian hamsters, but squirrels were refractory. Beveridge (1953) successfully adapted the parasite to rats. Phillips (1968) infected sucking guinea pigs but they eliminated the parasite within 12 days while one splenectomized rabbit was resistant to infection. The potential of B.rodhaini as a screening organism for anti-babesial drugs was recognized by Rodhain (1951). Beveridge (1953, 1956) reported the similarity in chemical structure of drugs effective against B.rodhaini and trypanasomes. A range of drugs, which later proved effective in the treatment of babesiosis in domesticated animals, was tested against this parasite.

B.rodhaini has also proved useful in investigation of problems of a general biological nature in babesiosis. As has been already described, Goodwin and Richards (1960) used this model to examine the pharmacological nature of the shock syndrome postulated by Maegraith et al. (1957). Schroeder et al. (1966) investigated autoimmune haemolytic anaemia in babesiosis, and Iturri and Cox (1969) and Annable and Ward (1974) examined the components of immune damage to the kidney - an aspect of the disease which has yet to be examined in economically important babesiosis. Phillips (1968, 1971) examined methods of improving vaccination and reported the changing antigenic

nature of relapse parasitaemias. Bishop and Adams (1974) have since applied irradiated B.bigemina vaccines with some success in calves.

The usefulness of B.rodhaini in rat and mice models for the investigation of many aspects of babesiosis in economically important animals has been clearly shown. Its advantages in this present examination of the pathogenesis of babesiosis are that it allows large numbers of a relatively standard experimental animal to be subjected to constant conditions, and that it provides a system whereby severe and reproducible disease can be set up without recourse to splenectomy. This allows a valid exploration of some disease mechanisms which are not feasible in available alternative systems. The combination of this model with the splenectomized bovine system provides a complementary pair of babesiosis infections from which to explore the pathology of the disease.

This general introduction to the pathology and pathogenesis of babesiosis is followed by a general chapter on materials and methods. Then each group of experiments is presented with an introduction, experimental details, results and discussion. In the introduction to each group of experiments the relevant literature is reviewed and the discussions from all groups of experiments are brought together in a concluding chapter.

CHAPTER 2

GENERAL MATERIALS AND METHODS

(1) The Parasites

The Babesia rodhaini used in these experiments was obtained from Mrs K.M.G. Adam of the Zoology Department, Edinburgh University. It was a mouse passage strain, the earlier history of which had been lost. In this laboratory it was maintained through 23 blood passages in mice (TREU 812) then frozen down in 7.5% glycerol (Analar. BDH, Poole, Dorset) as cryoprotectant and stored at -79°C in solid carbon dioxide. Following storage for 19 months it was thawed and inoculated into mice. When a parasitaemia developed blood was passaged into rats and it proved infective. It was maintained by twice weekly blood passage from a rising parasitaemia. The first six passages were of 0.5 ml of whole blood in heparinized ABPS (Appendix (1)a); Lumsden et al., 1965). For the following forty passages a calculated dose of 10^8 infected erythrocytes was used. Thereafter 0.3 ml of whole blood in heparinized ABPS was used as the standard inoculum for passage. When the parasite had been through 52 passages, 25 ml of pooled infected blood was frozen down in 2M dimethyl sulphoxide (BDH) as cryoprotectant. This was stored at -79°C as a reference stock and insurance against any eventualities resulting in loss in passage.

The Babesia divergens was obtained from Dr L.P. Joyner of the Central Veterinary Laboratory, New Haw, Weybridge. A fresh blood sample with 7% parasitaemia was received on ice. On arrival it was inoculated (20 ml) into a splenectomized calf and when the parasitaemia reached 30% blood was taken into 3.8% sodium citrate and frozen down using 7.5% glycerol as cryoprotectant and stored at -79°C.

(2) Animals

Rats. Wistar female rats, obtained from the Edinburgh University Centre for Laboratory Animals (EUCLA), were used in the majority of the experiments. They were an outbred strain that had been established specific pathogen free (SPF) at the Medical Research Council's Carshalton Laboratory, Surrey. They were purchased at 140 to 160 g bodyweight and introduced into experiments when between 160 and 180 g. According to data supplied by EUCLA they were approximately 60 days old at this weight. They were allowed at least five days to adjust to their new environment prior to use in any experiment.

The rats were healthy, very occasionally showing a slight nasal discharge but no lung lesions were observed. Some rats had a clear or slightly cloudy vaginal discharge usually accompanied by a distended uterus. Bacteriological culture and stained smears from this discharge failed to show any pathogenic organisms. Three of the rats had cystic ovaries. Two rats also had unilateral hydronephrosis, only detected post-mortem. Both of these animals had calculi in the bladder, ureter and kidney. One adult female and her litter of 14 from this same stock were also obtained.

A separate non-SPF strain of Wistar rat was obtained from the Department of Pharmacology, Edinburgh University. This was maintained in isolation and was used in a single experiment in the investigation of coagulation disturbances. A strain of SPF Hooded rats, obtained from EUCLA was also used in experiments related to coagulation studies.

Rabbits. Adult male New Zealand white rabbits were obtained from EUCLA.

Calves. Calves were bought locally from tick free areas. They were identified by ear tags and used in three experiments.

(i) No. B10, an Ayrshire cross male calf bought at three weeks of age from the Veterinary Field Station Farm, Edinburgh, was used in a pilot experiment.

(ii) Nos. 212, 273, 274, 275, EC251, L1160, and A89 were all Ayrshire cross male calves purchased at the Lanark market south of Edinburgh. These calves, four weeks old on arrival, constituted a fairly evenly matched group and were used together in one main experiment.

(iii) No. 296 was a Jersey cross male calf bought from a farm south of Edinburgh. This calf was infected by Mr A.J. Trees for an experiment not related to the work reported here. The calf died during an acute anaemic crisis following infection with B.divergens and material was taken for histopathological examination. The haematological data were kindly supplied by Mr Trees.

(3) Feeding and Management of Animals

Rats. Rats were kept in groups of not more than six in plastic cages, 18 x 13 x 16 inches with wire tops. They were bedded on a mixture of wood shavings and absorbent bedding. Their diet was of a proprietary rat cake (McGregor and Co. Ltd., Edinburgh) and water ad libidum. Rats were maintained separately from other animals at 70°C. Air circulation was by roof fan extraction.

Rabbits. Rabbits were kept in individual cages, 22 x 12 x 18 inches, on slats over drop trays filled with peat moss. They were fed on an Oxoid SG-1 diet and water ad libidum. Room temperature was maintained at 60°C, with air circulation by extraction fan.

Calves. Calves were housed indoors on straw on a concrete floor. They were fed on Nutristart Calf Cubes (Scottish Agricultural Industries, Edinburgh), meadow mixture, hay and water.

On arrival calves were kept in isolation for two weeks, during which time they were observed and examined for evidence of disease. An outbreak of ringworm was treated with Fulcin Feed additive (Griseofulvin 10%, ICI).

(4) Splenectomy

Rats. The rats were starved overnight and anaesthetized by intraperitoneal injection with Nembutal (pentobarbitone sodium) at 10 to 15 mg/100 g bodyweight. The spleen was removed through an incision in the left flank. The vessels were ligated and the wound closed with 2/0 chromic catgut. Recovery was uneventful.

Cattle. The calves were starved overnight and anaesthetized by inhalation through a face mask of an Halothane - Nitrous oxide - Oxygen mixture. Once unconscious they were intubated and maintained by a lighter concentration of the same gaseous mixture. Being small calves the spleens were removed through an incision in the left flank, immediately behind the last rib; a technique demonstrated by Dr J.K.H. Wilde. The vessels were tied off and the muscle layers sutured with 0/2 chromic catgut and the skin closed with Size 2 monofilament nylon. The calves were disbudded while still unconscious. They made an uneventful recovery, some calves had a slight swelling at and below the wound site for three to four days. The sutures were removed after ten days.

One week before being splenectomized all calves were treated

with 10 ml Spirotrypan forte (Hoechst). No Eperythrozoon sp. or any other blood parasites were observed during the period of observation.

(5) Collection of Blood Samples

Rats. Rats were anaesthetized by ether inhalation and the chest wall was opened along the sternum. Blood was then withdrawn from the right ventricle, using a 16 gauge $1\frac{1}{2}$ inch needle into a 5 ml sterile disposable syringe.

Rabbits. Blood was taken from the large ear vein along the lower edge of the medial aspect of the ear. The ear was washed and shaved then swabbed with xylene and when the vessels engorged, the vein towards the tip of the ear was nicked along its course with a sterile scalpel blade and the blood, which ran freely, was collected. The wound was compressed to stop bleeding and the xylene was washed from the ear tip.

Cattle. The calves were bled from the external jugular vein. The skin along the jugular furrow was clipped prior to the experiment. The skin over the vein was swabbed with alcohol and blood was withdrawn through a 20 gauge $1\frac{1}{2}$ inch needle into an evacuated glass tube (Vacutainers, Becton-Dickinson, Ireland). These vacutainers were used for all samples except citrated blood, when an 18 gauge $1\frac{1}{2}$ inch needle was used attached to a 5 ml syringe.

Serum Samples

Rats. Samples were drawn into a 2 or 5 ml syringe and transferred to a sterile test tube. When clotted the samples were freed from the wall of the tube, incubated at 37°C for 30 minutes then left for at least one hour, or overnight, in the refrigerator at 4°C.

Rabbits. The blood was allowed to flow from the ear vein into sterile universal bottles until an adequate sample had been obtained. Two rabbits were anaesthetized with Nembutal and bled from the heart into 50 ml disposable syringes through an 18 gauge $1\frac{1}{2}$ inch needle. Following incubation for 30 minutes these samples were left overnight in the refrigerator.

Cattle. Samples were taken into 10 ml vacutainer tubes and treated in the same manner as for rat samples.

Following clot retraction, in the refrigerator, the serum of all animals was taken off and centrifuged at 2500G for 20 minutes in a Mistral Centrifuge (Measuring and Scientific Equipment, London) at $4^{\circ}C$ to remove any remaining cells. If the serum was not used immediately it was stored at $-20^{\circ}C$.

Plasma Samples

Anti-coagulated blood was spun at 2500 rpm for 10 minutes and the plasma harvested. If not used immediately, it was stored at $-20^{\circ}C$.

Anti-coagulants

Prepared anti-coagulated 5 ml evacuated glass tubes containing either 7 mg dried disodium ethylenediamine tetra acetic acid (EDTA) or 143 USP units of sodium heparin were used for cattle blood. For rats the EDTA from one of the above tubes was tapped into a 5 ml disposable syringe, the blood drawn into the syringe and then returned to the original EDTA container. The sodium heparin used for rat blood was diluted in a balanced salt solution at pH 8.0 (ABP8, Appendix (1)a) to give a final concentration of 30 iu/ml blood.

Citrated blood samples were obtained by withdrawing blood into 3.8% sodium citrate in distilled water, to a final dilution of 1:9.

(6) Parasitaemia

The parasitaemia was estimated from thin blood films stained with Giemsa stain. A minimum of 500 erythrocytes was counted and parasitized cells were expressed as a percentage of that total. When very low numbers of parasites were present 50 microscope fields (15,000 erythrocytes approx.) were examined and the count was expressed as parasitized cells for 50 fields. A total of 70 fields was examined before an animal was recorded as negative. Counts were made using a Wild M20 microscope (Wild, Heerbrugg, Switzerland) with a x 10 eyepiece and fitted Whipple Graticule (Graticules Ltd., Tonbridge, Kent) and x 100 oil immersion lens.

Blood films were made from anticoagulated blood or from the tail tip of rats when parasite development was being followed.

(7) Staining

The blood films were spread on glass slides, 76mm x 25mm previously cleaned in chromic acid. The films were rapidly air dried and fixed in methanol for at least one minute. They were then placed face downwards on a flat tray with each end on a ridge of about 0.1 inch high. The area between the slide and the tray was flooded with 15% Giemsa in phosphate buffer pH7.2 (Appendix (1)b)). After 30 minutes the slide was rinsed in the same buffer and air dried.

Impression smears were stained in the same way, except that drying took longer and they were fixed for three minutes.

(8) Haematology

The blood parameters of erythrocyte count (RBC), white blood cell count (WBC), mean corpuscular volume (MCV) and packed cell volume

(PCV) were estimated using a Coulter* electronic particle counter, Model FN, with an attached MCV and PCV computer. Haemoglobin (Hb) levels were measured using a Coulter Haemoglobinometer. The mean corpuscular haemoglobin concentration (MCHC) was calculated from the above parameters using the formula MCHC = $\frac{\text{Hb gm\%}}{\text{PCV\%}}$ x 100. Dilutions for these estimations were made using the Coulter Dual Diluter (20 ml) from well mixed anticogulated blood in Isoton (Coulter), a normal saline solution with sodium azide as preservative. White blood cell dilutions of 1:500 were made and mixed, and from these 1:50,000 dilutions for RBC counts were prepared. For WBC counts the erythrocytes were lysed with Zap-o-globin (Coulter).

The counter was calibrated daily using Coulter standard blood, 4C, or a standard sample from the Royal Infirmary Edinburgh. Readings were made in duplicate at the following settings:

	RBC	WBC
Threshold	5	15
Attenuation	1	0.707
Aperture	8	8

Correction for counts above 10,000 were made routinely from the Coulter Coincidence Correction Chart.

Normoblasts were not lysed by Zap-o-globin and correction was made for this by estimating the number of normoblasts per 500 erythrocytes in thin blood films. When normoblasts were in very high numbers (which only occurred in rats) counts for total WBC estimation, for those samples, was done according to the method of Dacie and Lewis (1968). Whole blood was diluted 1:20 in 2% acetic acid, with a little gentian violet added, and counted in a Neubauer chamber.

* Coulter Electronics Ltd., Dunstable.

(9) Differential White Blood Cell Counts

Differential WBC counts were carried out on thin blood films fixed in methanol and stained with Giemsa stain (15% for 30 minutes). Films were prepared from EDTA anti-coagulated blood. A battlement procedure was followed where three fields (x 10 x 100) were counted along the edge of the film, then two in, two along, then two out to the edge again and so on. The counts were recorded on a Clay - Adams Multiple counter (Becton-Dickinson) and 200 cells were counted. The cells were differentiated into lymphocytes, neutrophils, eosinophils and monocytes. The differentiation was according to Schalm (1965) and Schermer (1967) for rats and Schalm (1965) for cattle, and immature cells were classified with the mature types with occasional reference to Diggs et al. (1956). As very few cells were so immature as not to be classified these immature cells were not included and smudge cells were ignored if not clearly identifiable.

(10) Thrombocyte Counts

Thrombocyte counts were estimated either directly by dilution in formal citrate or indirectly from thin blood films. The indirect method consisted of counting 500 erythrocytes from anticoagulated blood, as for parasitaemia, using the Whipple graticule to facilitate counting. The number of thrombocytes present among the 500 cells was expressed as a percentage. The actual number was then calculated from the daily erythrocyte count per mm^3. Before counting the blood film was screened for any aggregations or gross irregularity in distribution of platelets. If either of these occurred the count was not carried out.

Direct Thrombocyte Count. Blood taken into citrate without frothing was diluted with formal-citrate diluent (1% formalin in 31.3g/l trisodium citrate and filtered twice). A 1 in 100 dilution was made of blood in diluent, mixed gently for at least two minutes and a Neubauer chamber filled with the solution. The counting chamber was then put into a moist chamber and left to settle for at least 20 minutes. The thrombocytes were counted using a x4 objective with a x10 eyepiece on a Leitz phase contrast microscope. The method was that of Dacie and Lewis (1968) and Brecher and Cronkite (1950).

(11) Osmotic Fragility

The osmotic fragility of erythrocytes was assessed according to the method of Dacie and Lewis (1968). A stock solution of buffered sodium chloride, osmotically equivalent to 10% NaCl was prepared (Appendix (1)c) and stored in a stoppered bottle. From this stock a 1% solution in deionized distilled water was prepared and the following series of dilutions was made; 0.9, 0.85, 0.8, 0.75, 0.7, 0.65, 0.6, 0.55, 0.5, 0.45, 0.4, 0.35, 0.3, 0.2, 0.1%. Each was made up to a final volume of 100 ml and stored at 4^{o}C.

Before samples were tested the salt solutions were allowed to reach room temperature. Then 5.0 ml of each saline concentration was pipetted into a clean, marked test tube and 0.05 ml of well mixed heparinized whole blood was added and mixed. The tubes were allowed to stand at room temperature for 30 minutes then mixed again. They were then centrifuged at 1200G for five minutes. The amount of haemolysis in each tube was compared with 100% lysis, using the 0.9% tube as blank in an SP1800 Spectrophotometer (Pye Unicam, Cambridge,

England) at 540 nm. The results were transferred to graphs in which the axes were % lysis and concentration of saline. The distribution was represented by a sigmoid curve. The 50% lysis point was found and recorded as median corpuscular fragility (MCF) for each sample.

(12) Reticulocyte Counts

One volume of filtered 1% brilliant cresyl blue in citrate saline (Dacie and Lewis, 1968) was mixed with an equal volume of anticoagulated blood and incubated for 20 minutes at 30°C. The cells were resuspended by mixing and a thin blood film made and air dried. When reticulocytes were in very low numbers 50 fields were examined and when in higher numbers 500 cells were counted and, as for parasitaemia, the reticulocytes were expressed as a percentage of this total.

(13) Antiglobulin Tests

Coombs Test. The direct test was used to detect incomplete antibodies on rat erythrocytes. Test animal erythrocytes were washed four times in a large volume of phosphate buffered saline at pH 7.0 (see Appendix (1)c). The saline was warmed before use to 37°C, to avoid any confusion with cold haemagglutinins (Dacie and Lewis, 1968). To one volume of a 20% suspension of washed cells in PBS an equal volume then $^{1}/_{10}$, $^{1}/_{20}$, $^{1}/_{40}$ dilutions of anti-rat-globulin anti serum (Difco Laboratories, Detroit, Michigan, U.S.A.) were added. These were mixed on a glass tile at room temperature, left for ten minutes, then examined under low power on a microscope for agglutination and recorded.

Papainized Red Cell Test. This test was used to detect incomplete antibodies in the serum of both cattle and rats. The test was based

on the method of Dacie and Lewis (1968) from the original enzyme treated red cell test of Morton and Pickles (1947). Normal bovine or rat blood was taken in acid citrate dextrose (ACD Appendix (2)) and washed in PBS as above, prewarmed to 37°C. The cells were spun at 2,000 rpm for ten minutes and mixed with an equal volume of 1% papain diluted 1:9 in Sorensen phosphate buffer pH 7.0 (Appendix (1)d). The resuspended cells were incubated at 37°C for 30 minutes, then washed twice in PBS and resuspended to 5% in PBS. An equal volume of cells was added to undiluted and, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$ dilutions of test animal serum, in WHO plates and left for 30 minutes, then read. Control antiserum was a known anti-rhesus incomplete serum received from the Royal Infirmary, Edinburgh. This was diluted to $\frac{1}{16}$, as was the test serum. A saline control was included to test for non-specific agglutination.

(14) Erythrophagocytosis

Erythrophagocytosis was studied by the examination of spleen and liver impression smears and bone marrow scrapings stained with Giemsa stain. The spleen was cut across its lower third and an impression made by applying the cut surface to a clean glass slide. The liver was cut across the lower third of the large lobe to the right of the midline. The cut surface was touched on absorbent paper to remove excess blood and an impression made. The bone marrow scraping was made from the lower third of the right femur, the pointed end of scissors was introduced into the broken bone and the marrow spread thinly on a slide. These preparations were air dried, then fixed in methanol for three minutes and stained with Giemsa stain at pH 7.2

(Appendix (1)b) for 30 minutes. Erythrophagocytosis was estimated by the examination of 50 thin fields in each preparation that contained at least one phagocytic-type cell.

(15) Serum and Plasma Biochemistry

(i) Sorbitol Dehydrogenase (SDH) was measured using the Boehringer Test Combination (15960). Readings were made at a wavelength of 366 nm in a Pye Unicam SP1800 spectrophotometer. When haemolysis was present it was necessary to dilute the sample, as the Nicotinamide adenine dinucleotide, reduced form (NADH), was not in excess due to its utilization by other substrates released by haemolysis. Dilutions were made to $\frac{1}{10}$ and very rarely to $\frac{1}{20}$.

(ii) Urea was measured using the Boehringer Test Combination (15954). Test standard used was Seronorm (Nyegaard & Co., Oslo) Batch 122. (This standard was also run with both SDH and Bilirubin Tests to provide a standard control). Readings were made at a wavelength of 546 nm.

(iii) Bilirubin was measured using the Boehringer Test Combination (15944). Total bilirubin was read at a wavelength of 578 nm.

(iv) Potassium was estimated using plasma from heparinized blood. The method used was that in the Pye Unicam Manual and standards were prepared from potassium chloride (Analar B.D.H. Poole, Dorset) and sodium chloride (P.V.S. Reagent, Hopkins and Williams, Essex). Readings were taken using a Pye Unicam* atomic absorption spectrophotometer SP90 at a wavelength of 766.5 nm using an acetylene emission head. A potassium calibration graph was drawn from the standards read each day and the test sample values calculated. The calibration was checked after every five samples and a mid range standard between

* Pye Unicam Ltd., Cambridge.

each sample.

(v) Sodium was also calculated from a heparinized plasma sample according to the method in the Pye Unicam Manual. The standard sodium was prepared from sodium chloride. Readings were again taken on the SP90 spectrophotometer at a wavelength of 589.0 nm using an acetylene emission head. A calibration graph was prepared as above from standards and values for the test sample estimated. The same procedure was used to check the calibration.

Standards were prepared in de-ionized distilled water and the spectrophotometer was aspirated with water between samples.

(vi) Total Proteins were measured by the Biuret Reaction according to the method of Henry et al (1957). Samples were measured in duplicate in a Pye Unicam SP1800 spectrophotometer at a wavelength of 540 nm. A standard reading was again made in duplicate from Seronorm. The total protein value of each sample was then calculated from the formula

$$\text{Total Protein} = \frac{\text{Test Sample}}{\text{Standard Reading}} \times \text{Protein conc. of Standard in g.}$$

(16) Histopathology

Tissues for histopathological examination were taken immediately after killing the experimental animal, or as soon as possible from animals dying during the course of the infections. Blocks of 3 to 5mm in thickness were cut from the tissue and put into 10% formalin buffered to pH 7.0. Tissues were left in fixative for at least 48 hours, then they were transferred to 70% alcohol and processed automatically in a Shandon histokinette. The tissues were processed according to the routine of Carleton (1967) and embedded in paraffin wax. Blocks

were trimmed and 5μ sections cut on a rotary microtone and stained with haematoxylin and eosin (HE), Perl's prussian blue (PRB) and Martius scarlet blue (MSB) for fibrin.

Tissue of rats. Blocks were cut from the following sites.

Liver: Lower third of the large lobe immediately to the right of the mid-line.

Kidney: Posterior third of the right kidney.

Spleen: First part of the lower third, irrespective of size.

Lung: Middle third of the right cardiac lobe.

Cerebral hemisphere: Lateral half from the right side.

Small intestine: Anterior end.

Adrenal gland: Lateral half, left side.

Submaxillary lymph node: Anterior half, left side.

Mesentric lymph node: Middle of the small intestine.

Tissues of cattle. Blocks were cut from the following tissues, liver, kidney, heart, lung, cerebral hemispheres, submaxillary and mesentric lymph nodes. From these blocks six sections were cut from two separate areas and stained with HE, MSB and PRB. Control tissues were from a normal calf slaughtered at Gorgie Slaughterhouse, Edinburgh and whose carcase was passed as fit for human consumption.

CHAPTER 3

BABESIA RODHAINI AND BABESIA DIVERGENS IN THEIR EXPERIMENTAL HOSTS

I. Introduction

Babesiosis is a disease usually characterized by fever, progressive anaemia, haemoglobinuria and a variable mortality rate. Following recovery the parasites persist for some time in a latent state after their disappearance from the peripheral circulation. The B.rodhaini strain adapted from mice was an unknown pathogen in rats. In order to assess its general behaviour in rats and to discover its potential as a pathogen, a group of experiments was set up. The development of parasitaemia, the effects of infection on body weight gains and the production of haemoglobinuria and death were first examined. The effect of passage on the virulence of the parasite was then assessed. Finally the disease produced in young rats and the persistence of parasites after recovery were examined.

B.divergens was known to cause a severe disease in splenectomized calves (Davies et al., 1958). As a preliminary examination of the disease it produced and to establish a large volume of infected blood from which later experiments could be set up, a single calf was infected. The disease produced by B.divergens was followed by examination of the parasitaemia, rectal temperature and haematological changes during infection.

II. Materials and Methods

Experiment 3.1 : B.rodhaini in Wistar Rats

Fifteen rats were inoculated with 10^8 B.rodhaini infected erythrocytes and ten control rats were inoculated with normal erythrocytes

equal in number of those in the infective dose. The infected blood was from the 11th parasite passage, (P_{11}), following adaptation from mice and the five passage inocula prior to P_{11} were of a calculated 10^8 infected erythrocytes. The parasitaemia was estimated daily from tail blood films. The Urine was examined by applying digital pressure to the lower abdomen and a drop of urine, expressed in this way, was placed on an absorbent pad and tested for haemoglobinuria, using the Perheme 40 Kit (B.D.H.). The rats were weighed each day before sampling. These parameters were measured each morning between 9.00 a.m. and 9.30 a.m., animals dying after 9.30 a.m. were recorded on the following day.

Experiment 3.2 : The Effect of Passage on Parasite Virulence

The aim of this experiment was to test for any change in the virulence of the parasite with increasing numbers of passages. This was done by comparing the mortality rate at P_{41} with the mortality rate at P_{11}, observed in Exp. 3.1. For all passages from P_{11} to P_{41} a constant inoculum dose of 10^8 B.rodhaini-infected erythrocytes was used. At P_{41} a sample of 24 rats was infected, again using a dose of 10^8 infected erythrocytes.

Experiment 3.3 : The Parasitaemia and Diet in Young Rats

An adult female rat and her litter of 14 were obtained when the litter was five days old. They were allowed to suckle the dam for another seven days, then half the litter was taken at random, placed in a separate cage and fed on normal laboratory diet. They appeared little disturbed by this change. The seven baby rats left with the dam continued to suckle. At 14 days of age the two groups of baby rats were inoculated with 10^7 infected erythrocytes and the parasitaemia

was followed by tail blood films prepared every second day. One month later to test the susceptibility of the dam and to investigate the possibility of any maternal protection for the baby rats the dam and two control rats were inoculated with 10^7 infected erythrocytes and parasitaemia followed as above.

Experiment 3.4 : The Persistence of Parasites Following Recovery

This experiment was designed to test for the persistence of parasites following recovery from acute B.rodhaini infections. Fourteen rats, ten recovered and four controls were splenectomized and examined for parasites for 40 days. Daily tail blood films were examined for the first 20 days then the rats were screened every second day. Of the ten test rats four had shown parasitaemias of 25-55% and the other six had parasitaemias of 55% or more. Five of these rats had last shown patent parasitaemias between two weeks and one month before splenectomy, the remaining five had last shown parasites between one and three months prior to splenectomy.

Experiment 3.5 : B.divergens in a Splenectomized Calf

Calf B10 was splenectomized when approximately three months old and one month later was inoculated with 1.4 ml of infected whole blood (30% parasitaemia) in 7.5% glycerol. This blood had been stored for two years at -79°C, it was thawed in running water and inoculated intravenously. The following parameters were used to follow the course of infection: Rectal temperature, RBC, PCV, MCV, WBC, Hb, MCHC, indirect thrombocyte count and parasitaemia. These parameters were measured on the day prior to infection, the day of infection, then days 1 to 3 and days 6 to 17 after infection.

III. Results

Experiment 3.1 : B.rodhaini in Wistar Rats

The B.rodhaini parasites were present in low numbers on day 1 and parasitaemia developed rapidly during the following four days (Figure 3.1, Table 3.1). Between days 2 and 3 the mean percentage parasitaemia of the group doubled twice but this rate slowed to doubling once between days 4 and 5. A mean peak parasitaemia of 56.8 ± 6.5% was reached on day 5 and by day 7 the parasitaemia had fallen to 3.3 ± 2.2%. Twelve rats had haemoglobinuria on day 4, when the mean parasitaemia was 24.5 ± 6.0% and all rats were showing haemoglobinuria on day 5. Deaths were first recorded on day 6, when five rats died; the relationship between parasitaemia, haemoglobinuria and death is shown in Table 3.1.

Table 3.1

Mean parasitaemia, number with haemoglobinuria and mortality following B.rodhaini infection

Day	1	2	3	4	5	6	7	8	9	10
Parasitaemia	17/50	1.8	7.2	24.5	56.8	52.5	3.3	+	0	0
Standard error		±0.2	±0.6	±6.0	±6.5	±3.2	±2.2			
Haemoglobinuria	0	0	0	12	15	8	3	0	0	0
Mortality	0	0	0	0	0	5	7	0	1	0

By day 9 13 rats had died leaving two survivors which recovered rapidly. No parasites and no haemoglobinuria were seen, and no deaths occurred, in the control group.

The mean bodyweight (BW) in the infected group was seriously affected by the infection and its relationship to the parasitaemia and

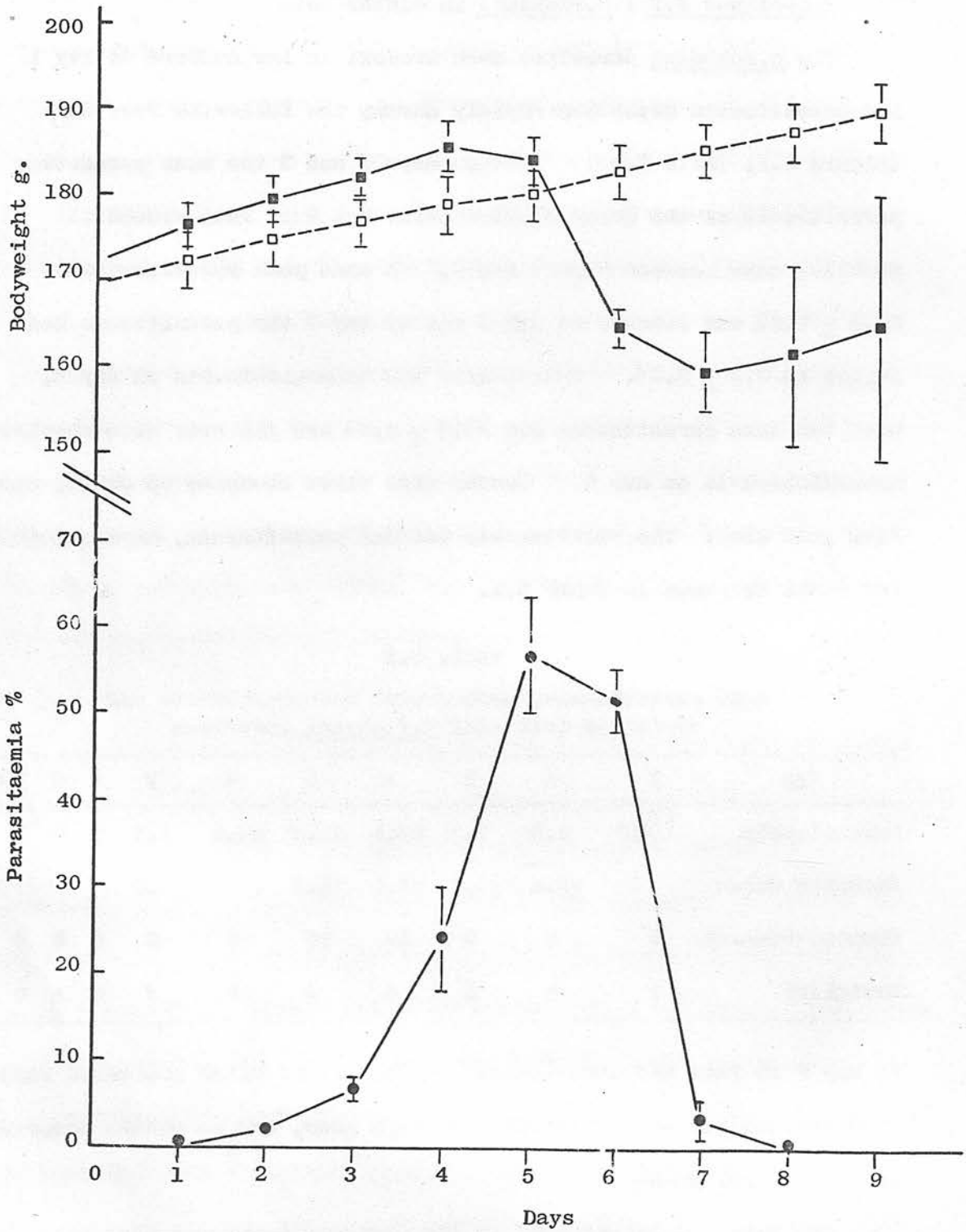


Fig. 3.1. Mean daily parasitaemia (●) and bodyweight of 15 B.rodhaini-infected (■) and ten control rats (□).

to BW in the control group is shown in Figure 3.1. Both groups gained weight steadily for four days following inoculation. From this point the infected group lost weight, while the control group continued to gain at a steady rate to the end of the observation period. On day 5 the test group mean BW had fallen 1.6 g and on day 6 the surviving ten rats had lost another 19.1 g. This drastic weight loss was checked a little and the surviving three rats lost 5.3 g on day 7, after which BW began to recover.

Experiment 3.2 : The Effect of Passage on Parasite Virulence

All rats in the group had parasites in low numbers on day 1. Death was first recorded on day 5 when one rat died, 16 were dead the following day and the remaining seven were dead on day 7. The mortality rate is shown in Table 3.2 and compared with the mortality rate from Expt. 3.1.

Table 3.2
Mortality rates

Day	4	5	6	7	8	9	10	Total mortality
Expt. 3.1	0	0	5	7	0	1	0	$^{13}/_{15}$
Expt. 3.2	0	1	16	7	0	0	0	$^{25}/_{25}$

Experiment 3.3 : The Parasitaemia and Diet in Young Rats

The parasitaemia was examined every second day and its development is shown in Table 3.3. Rat 6 in the weaned group was misinoculated and is excluded from the calculations. This rat eventually died on day 11 having had a 68% parasitaemia on day 10.

Table 3.3

The parasitaemia development in milk fed and weaned rats

	Milk Fed								Weaned							
Day	1	2	3	4	5	6	7	$\bar{x}\pm$ s.e.	1	2	3	4	5	6	7	$\bar{x}\pm$ s.e.
0	-	-	-	-	-	-	-		-	-	-	-	-	-	-	
2	$^{6}/_{50}$	1.2	1.0	2.9	$^{3}/_{50}$	$^{5}/_{50}$	1.0		1.5	3.9	1.5	1.4	$^{2}/_{50}$	0	1.0	
4	19.5	51.2	11.2	64.5	5.6	17.4	24.8	27.7±8.2	44.5	36.3	32.5	14.1	6.3	0	14.5	24.7±6.2
6	45.3	14.7	59.8	18.3	68.5	63.5	34.4	43.5±8.2	20.2	25.0	26.0	71.3	74.3	$^{3}/_{50}$	53.6	45.1±10.0
8	0	0	0	0	0	$^{1}/_{50}$	0		0	0	0	$^{1}/_{50}$	$^{5}/_{50}$	18.2	0	
10	0	0	0	0	0	0	0		0	0	0	0	0	68.0	0	
														D		
Mean max. parasitaemia						55.3±4.6								52.1±7.2		
Day of mean max parasitaemia						5.4±0.4								5.0±0.5		

The two groups developed an almost identical parasitaemia. The mean maximum parasitaemia for the milk fed group was 55.3 ± 4.6% and occurred 5.4 ± 0.4 days after infection, the comparable figures for the weaned group were 52.1 ± 7.2% after 5.0 ± 0.5 days. The mean peak parasitaemias were 43.5 ± 8.2% and 45.1 ± 10.0% on day 6. By day 8 only three rats had parasites and these were in very low numbers. Despite the high individual parasitaemias, the mortality rate was nil excluding the misinoculated rat.

The dam was a fully mature female and weighed 240 g when infected, the two control rats were (i) 174 g and (ii) 165 g. The three rats developed parasitaemias, the dam reaching 9.6% on day 6 and recovering rapidly. Of the two controls (i) died on day 7 having shown a parasitaemia of 65% on day 6 and (ii) survived following a peak parasitaemia of 55% on day 6.

Experiment 3.4 : The Persistence of Parasites Following Recovery

None of the splenectomized rats showed any parasites during the screening period.

Experiment 3.5 : *B.divergens* in a Splenectomized Calf

Parasites were first seen on day 6 and the parasitaemia rose steadily to 9.6% on day 10, on day 11 it dropped to 3.1% and parasites were not seen beyond day 12. The rectal temperature followed the parasite development, it rose to 40.6°C on day 8 and reached a maximum of 41.1°C on day 10 then fell gradually to 39.7°C on day 13. The relationships between the parameters are shown in Table 3.4. The RBC count fell slightly on day 7 then rapidly from day 10 to reach 2.70×10^6 per mm^3 on day 13. The PCV and Hb values fell in parallel with the RBC count. The Hb value began to recover on day 13, a day

Table 3.4

The haematological parameters of calf B10 following B.divergens infection

Day	Temp. °C	RBC x10^6/mm^3	PCV %	MCV µm^3	WBC /mm^3	HB g/100ml	MCHC %	Platelets x10^3/mm^3	Parasites %
-1	39.0	8.79	36.6	37.0	12,000	11.3	30.9	1,616	-
0	38.9	8.92	36.8	43.0	12900	11.0	29.9	1.596	-
1	38.6	10.20	37.8	39.0	12200	11.2	29.6	1,689	-
2	39.2	8.30	33.8	42.0	12400	10.9	32.2	1,243	-
3	39.5	8.70	35.1	42.0	12500	10.7	30.5	1,547	-
6	39.5	8.40	29.6	37.0	11500	11.4	38.5	1,268	+
7	39.7	7.60	29.6	40.0	7200	11.2	37.8	1,075	1.8
8	40.6	7.53	25.6	36.0	4100	9.9	38.4	1,157	4.1
9	40.7	7.60	24.5	36.0	6500	9.9	40.4	894	8.8
10	41.1	5.70	20.0	36.0	9100	8.1	40.5	479	9.6
11	40.0	3.80	12.7	34.0	12500	5.1	40.2	364	3.1
12	40.7	3.10	12.6	41.0	16000	2.7	21.4	506	0.4
13	39.7	2.70	9.8	37.0	33000	3.8	38.8	583	0
14	39.7	2.98	13.7	46.0	16400	4.0	29.2	517	0
15	39.5	3.11	15.9	53.0	15100	4.9	30.8	842	0
16	39.7	3.61	17.6	52.0	6900	5.1	29.0	1152	0
17	39.5	3.94	16.1	52.0	6000	5.0	31.1	1103	0

earlier than the onset of RBC and PCV recoveries. The MCV was unchanged until RBC recovery began then it increased from approximately 40 μm^3 to over 50 μm^3. The MCHC was not greatly changed during the course of infection.

The WBC count showed a dramatic fall between days 6 and 8 to about one third its pre-infection and early infection values. It then rose sharply to reach a peak of 33,000 per mm^3 on day 13 after which it again plunged to 6000 per mm^3 on day 17.

The thrombocytes began to fall steadily after the appearance of parasites reaching a nadir of 346,000 per mm^3 the day after peak parasitaemia. From day 12 the thrombocytes began a steady recovery.

IV. Discussion

The B.rodhaini strain adapted from mice and used in these experiments proved to be highly pathogenic for rats. Infection was followed by a rapidly developing parasitaemia, haemoglobinuria and death. The pathogenic behaviour of B.rodhaini resembled that reported by many workers (Matson, 1964; Philips, 1968; Nowell, 1968). Direct comparisons, however, are not possible as rats of different strains, age and sex were used, with a variety of parasite inocula. Philips (1971) has demonstrated the antigenic variability of B.rodhaini which might further complicate comparisons between experimental situations. Todorovic et al. (1967) have found, on the other hand, that even with splenectomy their rats survived infection; but their rats were fully grown and the parasite dose they used was smaller than that used in these experiments.

Bodyweight losses thought to be due to anorexia have been reported from B.rodhaini infections (Canache-Mata, 1959). The other symptoms of

the disease he noted were pallor and haemoglobinuria. The rats became rough coated with eyes a dull brown. At the crisis of the disease the rats were cold and curled up. This description of the disease agrees with Matson's (1964) findings and those observed during the course of B.rodhaini infection in this study. In addition jaundice was observed in this study and in Matson's (1964) work. Rats dying during Matson's study either died quietly following a period of coma or showed some violent excitement at the point of death. Rats dying in the present experiments lay absolutely still, hunched on their abdomen. They were cold and in a state of complete collapse. If pushed onto their side they showed feeble paddling movements but once returned to their abdomen they became still again. Breathing was laboured for the last two or three hours before death and they died without any sign of excitement. This collapsed state may resemble the shock state reported in dogs with B.canis infection (Maegraith et al., 1957) and cattle with B.argentina infection (Wright, 1973).

Rapid passage of some babesias has been shown to increase the virulence of the organism (Ryley, 1957; Brocklesby et al., 1973). With passage B.rodhaini in these experiments seemed to increase in virulence. However, the total mortality rate was not significantly different between the two groups, as only two rats survived in one experiment compared with none in the other. It is also felt that too much emphasis should not be placed on the number of rats dying on any one day. As deaths were recorded at 9.30 a.m., a matter of a few minutes between the deaths of two rats might have resulted in their deaths being recorded on different days. Further, throughout the course of this study some rats receiving a similar infective dose

of B.rodhaini to that used in Exp. 3.1 and 3.2 survived the infection. For these reasons it is felt that the results in Exp. 3.2 could have been an overestimate of the parasite virulence.

Rodhain (1953) has observed that milk diet suppressed the development of Plasmodium berghei, but not B.rodhaini, in mice. The finding of an almost identical development of B.rodhaini in both milk fed and laboratory diet fed rats would support this observation. More interesting however is the survival of the very young rats following a large inoculum of a virulent B.rodhaini. It was believed of babesiosis, that young animals had a particular 'reversed age resistance' to the disease. This fact was based on the observation, especially in cattle, that very young animals in endemic areas never, or very rarely, showed clinical disease. When this phenomenon was examined experimentally it was found that calves were surviving because of the transfer of maternal antibody (Hall, 1960) and that age conferred no particular resistance (Hall, 1960; Lohr, 1969; Brocklesby et al., 1971). Pipano (1969), however, reported a conflicting finding in that apparently fully susceptible calves showed a resistance to B.berbera infection. Riek (1963) considers that calves between the ages of four and seven months have a heightened resistance to Babesia infection that disappears at nine to 12 months under natural conditions. His opinion is founded upon observations, over many years, of babesiosis in natural and experimental situations. In B.rodhaini infections in the rat, on the other hand, increasing age is associated with an increasing resistance (Philips, 1968). The development of parasitaemia in the dam demonstrated her susceptibility to infection while the relatively low parasitaemia was probably a manifestation of age related

immunity. The virulence of the infecting dose was confirmed by the severe reaction of the control rats. There was no likelihood of the dam having had previous infection with B.rodhaini and consequently providing maternal protection for her litter.

The disappearance of parasites as judged by splenectomy in Exp. 3.4 was more rapid than reported from other work. However, A.J. Trees (personal communication), using B.rodhaini from the same source as that used in this study but maintained in separate passage in the same strain of rats, has found that the parasites disappeared as early as 12 days after infection. Phillips (1969) examined the persistence of parasites following recovery from primary B.rodhaini infections in 100 rats. Failure of splenectomy to provoke a relapse was first seen five to seven weeks after recovery and then in only four of 12 rats. At three months following infection 65% did not show parasitaemia, while at a year a few rats still had parasites. In cattle persistence of parasites with relapses is common (Smith and Kilbourne, 1893; Legg, 1933; Davies et al., 1958) and in intact cattle splenectomy will usually provoke a relapse to B.bigemina but not B.argentina (Legg, 1935; Barnett, 1965). The nature or efficiency of the immunity in the splenectomized rats that had earlier cleared their parasite burden was not examined.

Splenectomy has been widely used in experimental protozoan infections to induce a measurable disease response to needle passaged parasites. In unsplenectomized calves B.divergens produces only 'slight parasitaemia with few or no clinical symptoms' while splenectomized calves respond with a disease similar to that seen in natural conditions (Davies et al., 1958). Barnett (1965) has justified the

use of splenectomy for chemotherapeutic trials in the treatment of babesiosis and his arguments are accepted to apply to wider investigations (Brocklesby et al., 1973). The removal of the spleen deprives the animal of a large haemopoietic organ, its most concentrated source of phagocytic cells and the major source of its initial antibody production to injected antigen (Taliaferro, 1956). In haemoprotozoan infections one of the earliest and most pronounced changes following infection is splenic enlargement, due mainly to hyperplasia of the organ. Thus the splenectomized animal loses not only the potential of the pre-infection spleen but the extra potential of the reactive spleen early in infection, before alternative sources can provide protection. The needle passaged parasite, probably altered by its laboratory life cycle (Brocklesby et al., 1971), is then able to establish itself because of the increased susceptibility of the host.

The disease produced in calf B 10 by B.divergens was very similar to that reported by other workers (Davies et al., 1958; Brocklesby et al., 1971) in terms of the parasite development, the erythrocytic changes and the temperature response. Few studies exist of the leukocyte response of cattle during babesiosis infections and the leucopenia followed by a leucocytosis is an interesting finding that will be discussed fully in Ch. 4. The thrombocytopenia, that developed with the onset of measurable parasitaemia and which began to diminish as parasitaemia regressed, has not previously been reported from studies of babesiosis. It was measured at this stage as a sighting observation for studies to be undertaken on the coagulation mechanism in Ch. 7, where its relevance will be discussed in detail.

Both experimental infections assessed in this chapter produced

measurable disease responses, essential requirements for the investigation of pathological change. Furthermore they resembled similar experimental disease systems and many aspects of the naturally occurring disease. The apparent resistance of the very young rats and the rapid elimination of parasites are interesting findings and while repetition of these experiments and examination of the mechanisms involved would be worthwhile investigations they are beyond the scope of this work.

CHAPTER 4

THE HAEMATOLOGY

I. Introduction

The anaemia of babesiosis is due primarily to the destruction of erythrocytes by the emerging parasites. However, anaemias are frequently reported to be disproportionate to the degree of parasitaemia, although usually parallel to parasite development, except in chronic cases of B.canis infection (Joyner, 1966; Mahoney, 1972). The mechanisms of this additional anaemia have not been clarified. Schroeder et al. (1966) found that the anaemia of B.rodhaini infections was more closely related to developing splenomegaly, erythrocytosis and haemagglutinins than to parasitaemia. They also found that non-parasitized cells were phagocytosed and spherocytosis was present. These findings were similar to reports from Plasmodium and Anaplasma infections where the underlying mechanism was thought to be an autoimmunization. By strict definition autoimmunity is the production of antibody against the host's own unmodified native components or against a foreign antigen whose antibody cross-reacts with a native self antigen (Brent and Medawar, 1959). In none of these diseases has a specific anti-erythrocyte antibody been found. Sibinovic et al. (1969) have shown that a Babesia parasite antigen is released into the plasma and is rapidly coated onto erythrocytes. Ludford (1969) has demonstrated the distribution of antigenic material within infected cells. In both these situations it is reasonable to assume that antibody directed against these antigens could be found on erythrocytes. Cox (1973) who originally supported the autoimmune concept of erythrocyte destruction

(Cox et al., 1966) seems to have modified his view. He argues that soluble antigen might form complexes with antibody, the complexes then become bound onto erythrocytes and act as opsonins in their phagocytosis. Rogers (1974) has described opsonins apparently directed specifically at parasitized erythrocytes, clearly different from the non-specific opsonins described by Cox (1973).

Other possible mechanisms of erythrocyte loss described during the course of protozoan infections, but examined most fully in malaria, are the function of the spleen in relation to altered erythrocytes (Crosby, 1957; 1959) and reticuloendothelial hyperactivity (Doan, 1949; Dameshek, 1955; Jandl and Aster, 1967). The splenic function of pitting rigid bodies or inclusions such as siderocytes or Heinz bodies has also been shown to remove malaria parasites effectively (Schnitzer et al., 1972). These erythrocytes are then returned to the circulation but with a changed shape and a shortened life span. The hyperactive spleen also prolongs the sequestration of erythrocytes. During the unduly long sequestration the limited metabolic capacity of the erythrocyte is reduced further and the cell life span is shortened (see Jandl and Aster, 1967). These two mechanisms are thought to be the main causes of the anaemia in some malaria infections (George et al., 1966; Conrad, 1969).

Phagocytosis has been observed to occur in most Babesia infections removing parasitized erythrocytes, non-parasitized erythrocytes, free parasites and debris. The importance of this process in the response to infections, especially in malaria, has been reviewed and emphasized by Taliaferro (1956). Belding (1965) regarded it as the predominant mode of defense against malaria. More recently it has been looked upon

as a contributing factor in the anaemia of some protozoal infections. Phagocytosis of erythrocytes was observed by Graham-Smith (1905) in the dog. Neitz (1938) described the phagocytosis of free parasites by neutrophils and of parasitized erythrocytes by monocytes in the peripheral circulation. Simpson (1974) found that neutrophils from the buffy coat phagocytosed parasitized cells which were present in large numbers at the top of the red cell column following centrifugation of B.canis infected blood. Maegraith et al. (1957) observed phagocytosis of both infected and non-infected erythrocytes by neutrophils and monocytes in the peripheral circulation and by mononuclear histiocytes in the tissues. Phagocytosis has also been observed during B.rodhaini infections in mice (Paget et al., 1962) and rats (Schroeder et al., 1966; Todorovic et al., 1967) and B.argentina infections in cattle (Rogers, 1971). Kyurtov (1970) found no evidence of phagocytosis in the peripheral circulation of sheep with B.ovis infection but the phagocytic index was increased following primary infection and reinfection.

The white blood cell changes observed during the course of Babesia infections have been reported mainly from B.canis infections. The total cell count was elevated in most of these reports but the response of the individual cell types followed no common pattern. A variable response, with the majority of dogs showing a leucocytosis and a few a leucopenia, has been reported by Wright (1905) and Maegraith et al. (1957). Alperin and Bevins (1963) found a lymphocytosis while others found a neutrophilia (Sanders, 1937; Hindaway, 1951; Brodey and Prier, 1962). Dorner (1967) found that the total count was within the normal range but neutrophils were increased in

number. In mice with B.rodhaini infection the total count was slightly higher in acute fatal infections but neutrophils increased significantly (Gamble, 1974). Cattle responded with a lymphocytosis (Karput, 1966; Suteu and Giurgea-Iacob, 1971); this lymphocytosis was accompanied by a monocytosis, basophilia, and neutropenia which persisted into recovery when an eosinophilia also became apparent.

The aim of the experiments to be reported here was to investigate the effects of the two parasites on the haematological parameters of their respective hosts and to explore possible mechanisms of additional anaemia that might exist.

II. Materials and Methods

Experiment 4.1 : The Development of Parasitaemia and the Haematological Parameters in B.rodhaini infection

(i) This experiment was set up to examine the haematological changes occurring during the course of acute, severe B.rodhaini infections. Forty five rats were inoculated intraperitoneally with 10^8 B.rodhaini infected erythrocytes and 45 control rats were inoculated with an equal number of normal erythrocytes to that contained in the infective dose. (Infected rats were screened for parasites on day 1 in this and all other experiments and rats showing no parasites were rejected). Five test and five control rats were anaesthetized and bled each day. The following parameters were measured: RBC, PCV, MCHC, Hb, parasitaemia, reticulocytes, erythrophagocytosis in spleen, liver and bone marrow and total and differential WBC. A serum sample was also taken and stored for antiglobulin testing and the spleen was weighed.

Table 4.1

The parasitaemia and erythrocyte parameters during *B.rodhaini* infection (Exp. 4.1(i))

	Test						Control					
	1	2	3	4	5	6	1	2	3	4	5	6
RBC $x10^6/mm^3$	6.56 ±0.10	6.30 ±0.16	5.57 ±0.45	5.36 ±0.14	2.65 ±0.38	1.24 ±0.23	7.24 ±0.10	7.07 ±0.14	6.99 ±0.07	6.86 ±0.15	6.48 ±0.13	6.71 ±0.15
PCV %	42.0 ±0.6	38.7 ±0.6	38.0 ±1.7	33.8 ±0.9	17.4 ±2.5	8.9 ±1.4	46.2 ±1.1	44.5 ±2.2	47.7 ±1.2	46.8 ±3.9	43.4 ±1.3	43.3 ±4.4
MCV μm^3	66 ±1	65 ±1	65 ±2	65 ±1	68 ±1	76 ±3	64 ±1	65 ±3	70 ±1	70 ±5	67 ±2	66 ±6
Hb g/100ml	13.9 ±0.4	14.1 ±0.9	12.7 ±0.4	10.9 ±0.4	5.9 ±0.8	2.9 ±0.2	15.2 ±0.2	14.5 ±0.2	15.3 ±0.2	14.6 ±0.2	14.8 ±0.2	14.9 ±0.2
MCHC %	33.1 ±0.9	36.4 ±0.5	33.5 ±0.8	32.3 ±1.0	34.6 ±0.7	30.2 ±2.7	32.8 ±0.6	33.1 ±2.1	32.0 ±0.8	32.0 ±2.5	34.2 ±1.1	35.2 ±3.0
Parasitaemia %	+	2.2 ±0.4	8.8 ±.9	33.6 ±2.9	61.3 ±5.5	47.1 ±0.5	0	0	0	0	0	0
Reticulocytes %	-	2.4 ±0.5	2.2 ±0.7	4.0 ±1.0	12.3 ±3.5	36.0 ±6.8	-	1.0 ±0.3	0.6 ±0.1	0.8 ±0.2	0.5 ±0.1	1.0 ±0.1

Table 4.2

The parasitaemia and erythrocyte parameters during *B.rodhaini* infection (Exp. 4.1(ii))

	Test				Control			
	6	7	8	9	6	7	8	9
RBC $x10^6/mm^3$	1.41 ±0.11	1.39 ±0.04	2.42 ±0.34	3.44 ±0.15	7.01 ±0.14	6.82 ±0.15	7.08 ±0.24	7.23 ±0.13
PCV %	10.4 ±0.7	12.8 ±0.8	22.6 ±2.1	30.2 ±1.6	42.8 ±1.3	44.0 ±1.6	43.5 ±1.6	46.4 ±0.9
MCV μm^3	76 ±1	92 ±4	99 ±6	90 ±3	63 ±1	67 ±3	63 ±2	66 ±2
Hb g/100ml	3.7 ±0.2	4.2 ±0.2	7.2 ±0.7	9.6 ±0.4	15.3 ±0.2	15.3 ±0.2	14.8 ±0.5	15.1 ±0.2
MCHC %	35.2 ±0.3	32.6 ±0.8	31.9 ±0.5	31.9 ±0.9	35.8 ±1.2	35.1 ±1.6	34.2 ±1.3	32.5 ±0.7
Parasitaemia %	54.8 ±5.1	7.1 ±3.5	1.0 ±0.3	0	0	0	0	0
Reticulocytes %	40.9 ±1.9	43.1 ±9.2	-	-	0.8 ±0.2	0.9 ±0.3	-	-

(ii) As all rats infected in Exp. 4.1(i) died, no parameters were measured beyond day 6. In order to examine the disease through the crisis of anaemia and into recovery a further 30 rats were infected. In anticipation of mortalities only four infected rats were examined each day from day 6 to day 9 and the infective dose was reduced to 10^7 infected erythrocytes. Controls were inoculated with normal blood as above.

Experiment 4.2 : The Development of Parasitaemia and the Haematological Parameters in B.divergens infection

In this experiment seven calves, splenectomized a month earlier, were infected with approximately 5.4×10^8 B.divergens-infected erythrocytes. Clinical examination and sampling were carried out each morning at 9.00 a.m. The following parameters were examined, (a) Clinical: Rectal temperature, appetite, mucous membranes, faeces and urine. (b) Haematological: RBC, PCV, MCV, Hb, MCHC, reticulocytes, total differential WBC and a serum sample was taken and stored for antiglobulin testing. Plasma samples were taken for the measurement of other parameters reported in later chapters. Control values were established for these calves in the two weeks prior to infection.

III. Results

Experiment 4.1(i) and (ii) : The Development of Parasitaemia and the Haematological Parameters in B.rodhaini Infection

In Tables 4.1 and 4.2 and Fig. 4.1 the development of parasitaemia and the changes in RBC, PCV, MCV, Hb, MCHC and reticulocyte count are presented. The values represent the mean $\pm$ s.e. of the parameters from five test and five control rats on each day in Exp. 4.1(i) and

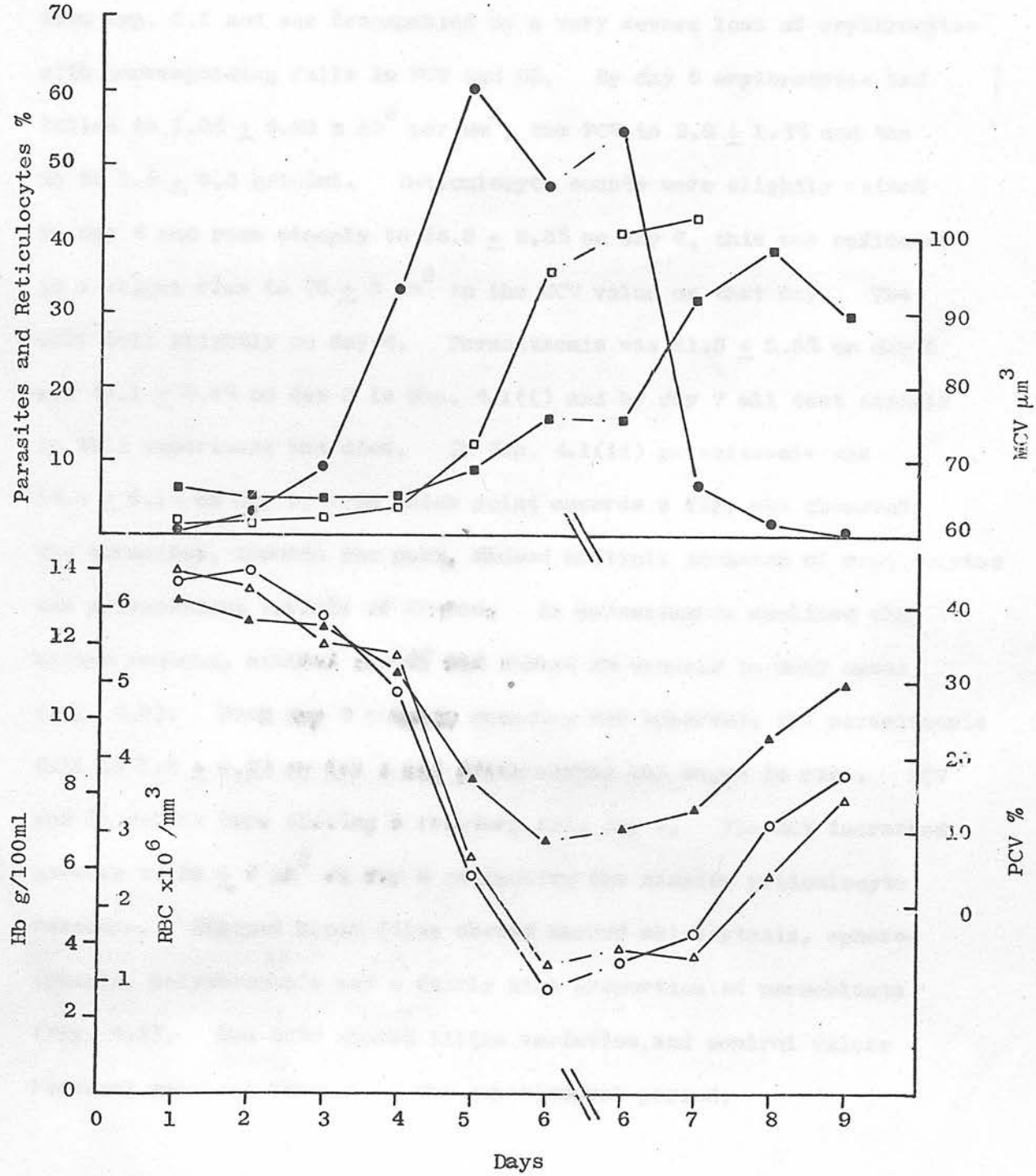


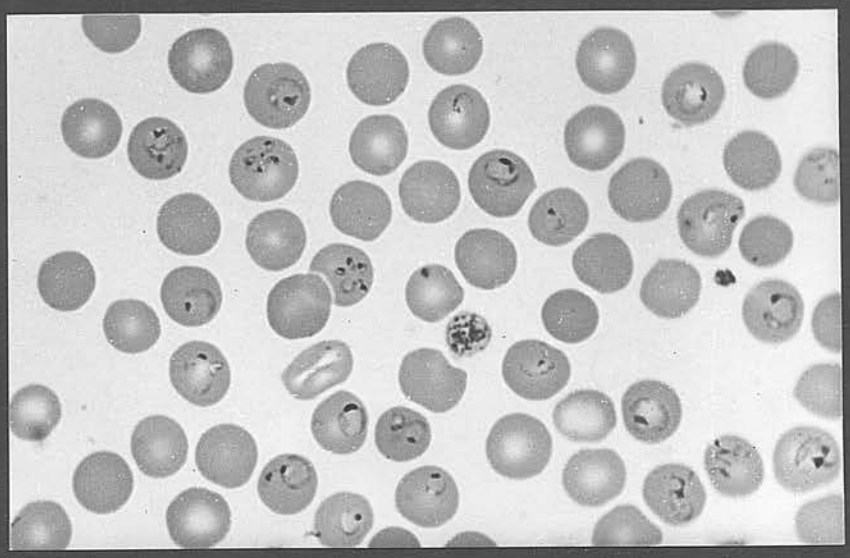
Fig. 4.1. Mean daily parasitaemia (●), RBC (△), PCV (▲), Hv (○), MCV (■), and reticulocyte (□) levels during B.rodhaini infection in rats (Exp. 4.1(i) and (ii)).

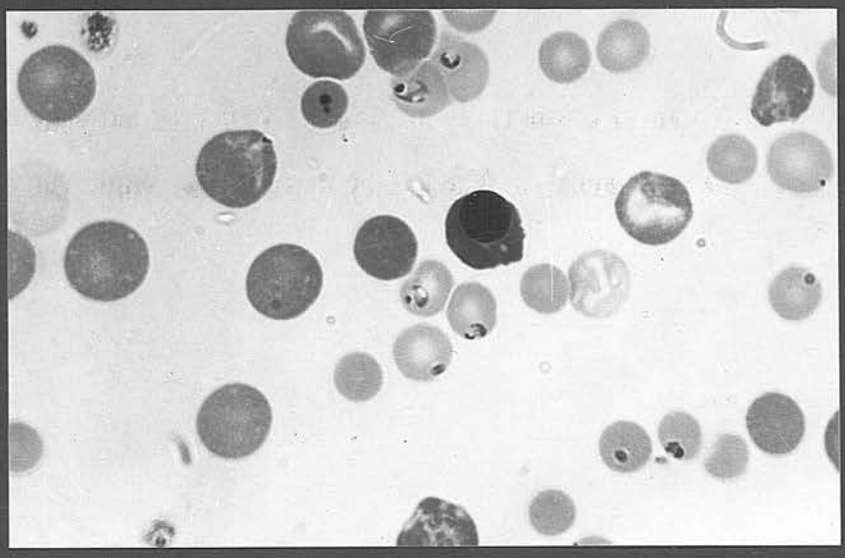
four test and four control rats on each day in Exp. 4.1(ii).

Parasitaemia development was almost identical with that reported from Exp. 3.1 and was accompanied by a very severe loss of erythrocytes with corresponding falls in PCV and Hb. By day 6 erythrocytes had fallen to $1.24 \pm 0.23 \times 10^6$ per mm^3, the PCV to $8.9 \pm 1.4\%$ and the Hb to 2.9 ± 0.2 g/100ml. Reticulocyte counts were slightly raised on day 4 and rose steeply to $36.0 \pm 6.8\%$ on day 6, this was reflected in a slight rise to 76 ± 3 μm^3 in the MCV value on that day. The MCHC fell slightly on day 6. Parasitaemia was $61.3 \pm 5.5\%$ on day 5 and $47.1 \pm 0.5\%$ on day 6 in Exp. 4.1(i) and by day 7 all test animals in this experiment had died. In Exp. 4.1(ii) parasitaemia was $54.8 \pm 5.1\%$ on day 6, from which point onwards a fall was observed. The parasites, towards the peak, showed multiple invasion of erythrocytes and a tremendous variety of shapes. As parasitaemia declined they became rounded, stained poorly and showed no vacuole in many cases (Fig. 4.2). From day 7 onwards recovery was apparent, the parasitaemia fell to $1.0 \pm 0.3\%$ on day 8 and erythrocytes had begun to rise. PCV and Hb values were showing a recovery from day 7. The MCV increased greatly to 99 ± 6 μm^3 on day 8 reflecting the massive reticulocyte response. Stained blood films showed marked anisocytosis, spherocytosis, polychromasia and a fairly high proportion of normoblasts (Fig. 4.2). The MCHC showed little variation, and control values remained constant throughout the experimental period.

Fig. 4.2. B.rodhaini-infected rat blood. Typical appearance of the parasite during rising parasitaemia showing annular, amoeboid and dividing forms (upper) and the appearance of the parasite during recovery showing shrunken forms and loss of the parasite vacuole (lower). The presence of anisocytosis with normoblasts and polychromatophils can be seen in the blood during recovery (x1100. Giemsa).

Fig. 4.3. B.divergens infected calf blood. Typical appearance of the parasite showing its close relationship with the membrane at the periphery of the cell (x1100. Giemsa).





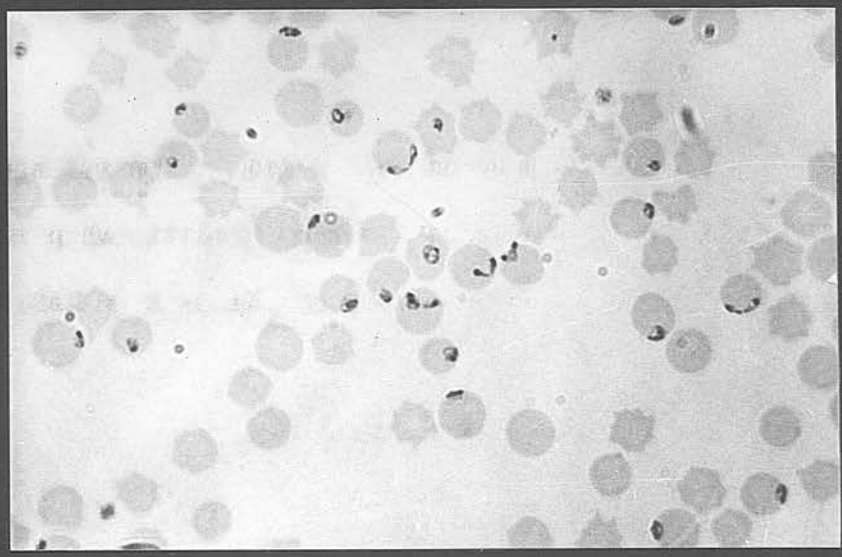


Table 4.3

The (Coombs) antiglobulin test during *B.rodhaini* infection

	Day	3	4	5	6	7	8	9
Animal Test	1	-	-	1/10	1/20	1/10	1/10	-
	2	-	1/10	1/10	1/20	1/10	1/10	-
	3	-	-	1/20	1/20	1/10	-	-
	4	-	-	1/10	1/20	1/10	1/10	-
Mean % Parasitaemia		7.5	47.0	55.6	42.0	6.0	+	-
± s.e.		±3.0	±6.1	±3.1	±5.2	±2.6		-

Antiglobulin test in *B.rodhaini* infections

The papainized red cell test was the test of choice for the identification of globulins on erythrocytes, but the stored sera from experiments Exp. 4.1(i) and (ii) proved to be negative. To test whether this was a true result, sera from five rats showing a high parasitaemia and two normal control rats were examined fresh and following storage at -20°C for three days. The fresh samples were also tested by a direct Coombs test. The fresh test samples were positive to both papainized red cell and Coomb's tests while the control samples were negative. The stored samples were all negative. Because of the loss of activity on storage of sera another experiment was set up in which 28 rats were inoculated with 10^7 *B.rodhaini*-infected erythrocytes and four test animals along with four controls were killed and samples examined by the Coombs test on days 3 to 9. The haematological response and parasitaemia development were very similar to Expt. 4.1, and the Coombs test results are presented in Table 4.3. One rat was positive on day 4 and all were positive on days 5, 6 and 7. Three were positive on day 8 and none on day 9. The control rats were negative. The test became positive with high parasitaemia and persisted through the crisis, becoming negative as the rats recovered and parasites disappeared.

Experiment 4.2 : The Development of Parasitaemia and the Haematological Parameters in *B.divergens* Infection

The calves infected with *B.divergens* showed disease responses of varying severity, animals with higher parasitaemias developing more

profound anaemias. Because of this variation, parameters in most instances are presented from individual calves rather than as means for the group. However, despite the variation the patterns of change were very similar in all calves and to save needless repetition typical results will be described. The complete results for all parameters are presented in Appendices (3) to (11).

The clinical signs of infection

The clinical signs of the disease are summarized in Table 4.4 where rectal temperature, appetite, appearance of mucous membranes, presence or absence of haemoglobinuria and condition of faeces are recorded. All calves developed an elevated temperature after the appearance of parasites in the peripheral circulation and these temperatures reached a peak on or within a day of peak parasitaemia in all cases. The highest peak temperature was 41.0°C shown by calf 275 and the lowest peak was 39.7°C shown by calf 274; both these peaks coincided with the peak of parasitaemia. As the parasitaemia declined the temperatures returned to normal.

Calf A89 died early on the morning of day 13 having shown a temperature of 40.5°C on day 10 which fell to 38.6°C on day 12. Haemoglobinuria was present on days 11 and 12. Appetite was lost on day 12 and the mucous membranes were jaundiced. Despite these findings, on day 12 the calf was fairly bright and its death was unexpected. It was the most advanced calf of the group and unlike the others the erythrocytes tended to form rouleaux. Anticoagulated (EDTA) blood samples allowed to stand on the bench were observed to settle and examination of wet blood films showed the rouleau formation. The calf died apparently without struggling as the bedding about it

Table 4.4

The clinical parameters during *B. divergens* infection

Animal	212					275					L1160					A89				
Day	RT	MM	Ap	U	F	RT	MM	Ap	U	F	RT	MM	Ap	U	F	RT	MM	Ap	U	F
0	38.9					39.1					38.9					39.0				
7	-					39.2					-					39.0				
8	38.8					38.6					-					37.3				
9	38.9					39.4					38.2					39.5				
10	39.4					39.7					38.3					40.5				
11	40.1					40.5			+		38.8					40.1*			+++	
12	40.4*			++		41.0*			+		38.9					38.6	J	A	+++	
13	40.5	P	R	+++		40.6	P		+		40.4*			++						
14	38.0	P	A	++	C	40.4	P	R	++	C	40.1									
15	37.0	P	A		C	39.1	P				39.9									
16	38.7	J	R		C	39.4					38.8	P	R		C					
17	38.4					39.1					39.2		R							
18	38.9					38.4					38.4									

Animal	273	274	251	
0	38.4	38.9	38.8	RT = Rectal temperature °C
7	38.8	-	38.9	MM = Mucous membranes
8	37.3	38.6	39.3	P = Pale
9	38.0	39.4	39.9	J = Jaundice
10	38.6	39.4	39.8	Ap = Appetite
11	39.4	39.7*	40.3	R = Reduced
12	39.5*	39.2	40.2*	A = Absent
13	38.5	38.8	40.0	U = Urine
14	39.3	39.0	40.1	+ = Haemoglobinuria
15	39.4	38.6	40.1 P	F = Faeces
16	39.6	38.9	39.7	C = Constipation
17	39.1	39.4	39.2	* = Peak Parasitaemia
18	38.4	38.4	38.7	

was undisturbed.

Calf 212 suffered a very severe disease episode and became moribund. Its temperature was elevated from day 10 to 13 then fell below normal for two days. (Normal was taken as being within the range of the group 38.4 to 39.1°C on the day of infection). It had severe haemoglobinuria from day 12 to 14 and appetite was very poor or absent from day 13 to 16. Its mucous membranes were pale and showed jaundice on day 16. The faeces were very firm, flecked with fresh blood and coated with mucus. Auscultation of the heart and lungs indicated a progressive weakening of the heart (which ultimately showed incompetence) and a developing pulmonary oedema. This became apparent on day 16 when the parasitaemia had declined and the erythrocyte parameters were showing recovery. It was also noted that from day 13/14 a neutrophila had begun to develop. With the exception of calf A89 none of the other calves showed this change. The likelihood of secondary infection rendering this calf valueless either living or dead was very high and it was decided to institute specific therapy. The calf was treated with Terramycin (Pfizer), Millophyline (Dales Pharmaceuticals) and Vetidrex (Ciba-Geigy) on day 17 and 18 and recovered rapidly. However, it needed intensive physiotherapy and encouragement before it stood and walked unaided.

Haemoglobinuria was shown by calves A89, 212, 275, L1160 and to a very slight degree by calf 251. The first four of these calves were those that developed the highest parasitaemias and the most severe anaemias. Three calves showed constipation and A89 had very firm faeces in the rectum on post mortem examination. There was no evidence of diarrhoea in any of the calves.

The parasitaemia and erythrocyte parameters

Parasites became apparent in thin blood films between days 7 and 9, reaching peak levels between days 11 and 13. The erythrocyte parameters of RBC, PCV and Hb did not begin to fall until the parasitaemias were well established in all calves and then they fell rapidly, continuing to fall for some days after peak parasitaemia. The day of appearance of parasites, the day and degree of peak parasitaemia and the day and value of erythrocyte nadir are shown in Table 4.5.

Table 4.5

The parasite peak and erythrocyte nadir in B.divergens infection

Calf no.	212	275	L1160	A89	273	274	251
Appearance of Parasites	8	7	9	7	7	8	7
Peak % Parasitaemia	33.0	28.6	20.6	28.2	13.5	11.5	10.9
Day of Peak	12	12	13	11	12	11	12
RBC Low $x10^6/mm^3$	1.99	1.98	1.98	D	3.77	3.75	3.41
Day of RBC Low	15	15	16	-	15	15	16

For convenience the four animals 212, 275, L1160 and A89 that developed the highest parasitaemias and most pronounced anaemias are grouped together and the more mildly affected are similarly grouped. Calves 275 and L1160, while showing RBC, PCV and Hb falls similar to calf 212 had a less severe clinical disease. Erythrocyte values began a slow and continuous recovery from the crisis of anaemia but had not attained normal values when observations ceased on day 35. The MCHC values dropped slightly at the crisis of anaemia but were little disturbed except in calf 212 in which they remained low for about 14

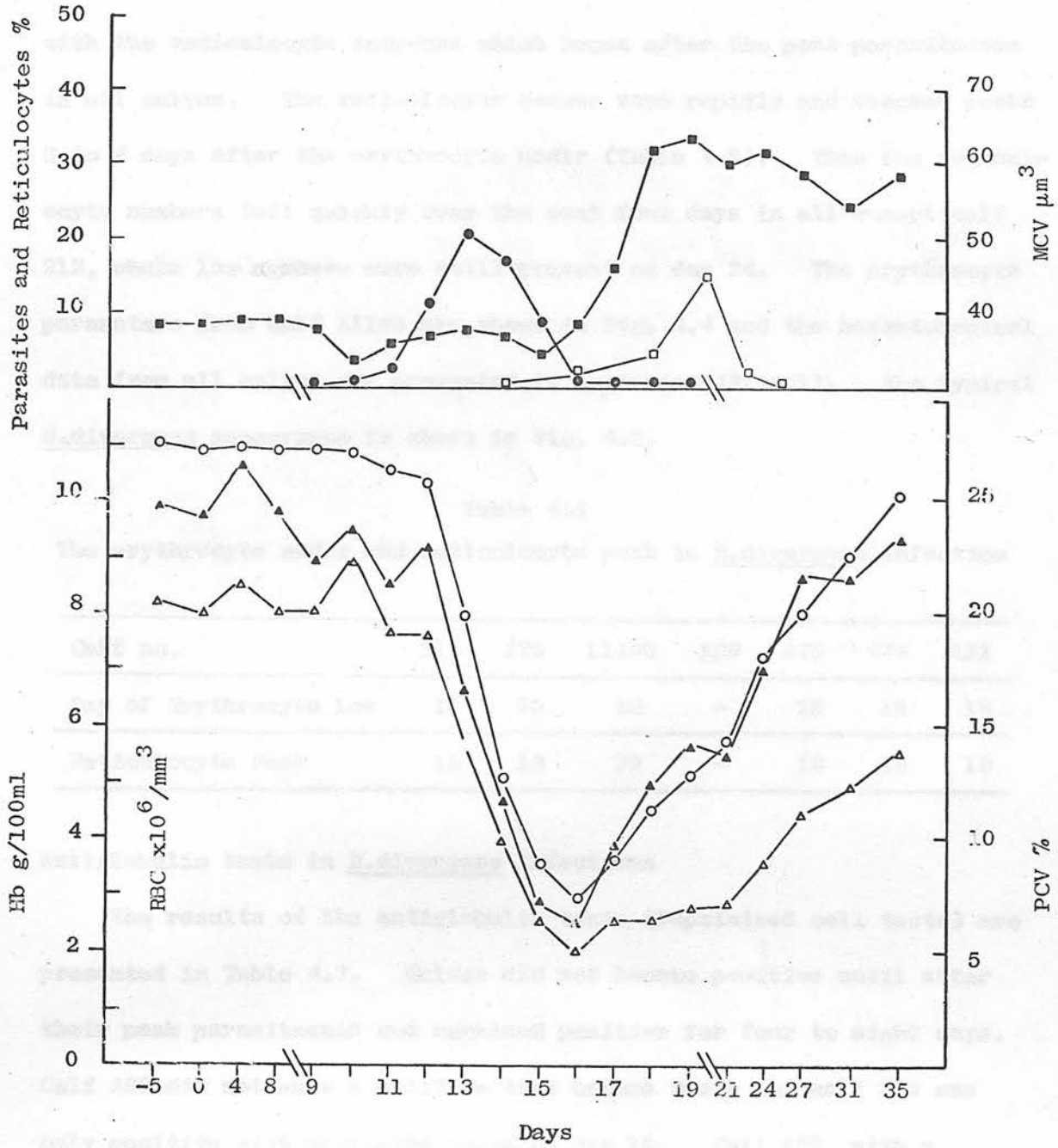


Fig. 4.4. Daily parasitaemia (●), RBC (△) PCV (▲), Hb (○), MCV (■) and reticulocyte (□) levels during B.divergens infection in calf LL160.

days during recovery. The MCV also fell slightly at the crisis but became greatly increased early in recovery and gradually reduced but was still above normal at the end of observations. This was associated with the reticulocyte response which began after the peak parasitaemia in all calves. The reticulocyte counts rose rapidly and reached peaks 3 to 4 days after the erythrocyte nadir (Table 4.6). Then the reticulocyte numbers fell quickly over the next four days in all except calf 212, where low numbers were still present on day 24. The erythrocyte parameters from calf L1160 are shown in Fig. 4.4 and the haematological data from all calves are presented in Appendices (3) to (11). The typical B.divergens appearance is shown in Fig. 4.3.

Table 4.6

The erythrocyte nadir and reticulocyte peak in B.divergens infection

Calf no.	212	275	L1160	A89	273	274	251
Day of Erythrocyte Low	15	15	16	-	15	15	16
Reticulocyte Peak	18	18	20	-	18	18	18

Antiglobulin tests in B.divergens infections

The results of the antiglobulin tests (Papainized cell tests) are presented in Table 4.7. Calves did not become positive until after their peak parasitaemia and remained positive for four to eight days. Calf A89 did not show a positive test before death and calf 212 was only positive with undiluted serum on day 15. Calf 273, with a mild disease response, showed the highest titre and the most prolonged positive reading. The known positive anti incomplete Rh serum was positive at $\frac{1}{8}$ each time a batch of samples was tested and the saline

Table 4.7

Antiglobulin test in B.divergens infection

	Day	6-12	13	14	15	16	17	18	19	20	21	35
Calf no.	212	-	-	-	1/1	-	-	-	-	-	-	-
	275	-	-	1/2	1/2	1/2	1/1	1/1	1/2	1/2	-	-
	L1160	-	-	1/1	1/1	1/2	1/1	1/1	-	-	-	-
	A89	-	D									
	273	-	-	1/4	1/2	1/4	1/2	1/4	1/4	1/8	1/4	-
	274	-	-	-	1/1	1/1	1/1	1/1	-	-	-	-
	251	-	-	-	1/1	1/1	1/1	1/1	-	-	-	-
+ Control		1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8	1/8
- Control			-	-	-	-	-	-	-	-	-	-

Table 4.8

The total and differential WBC counts recorded in Exp. 4.1(i) and (ii)

Test	Total WBC	Lymphocytes	Neutrophils	Monocytes
Day 1	2480 ±274	1738 ±252	608 ±66	129 ±28
2	4220 ±1280	2754 ±956	1339 ±304	121 ±33
3	4180 ±610	2308 ±330	1543 ±287	298 ±102
4	6200 ±550	4263 ±333	1870 ±194	491 ±107
5	8320 ±620	5964 ±563	1635 ±155	671 ±197
6	8360 ±1890	5897 ±1750	1623 ±579	811 ±106
6	15860 ±3020	11378 ±2070	2900 ±663	1505 ±501
7	23900 ±7631	15328 ±4144	6890 ±2804	1620 ±713
8	21800 ±5300	18475 ±4620	3490 ±1021	483 ±199
9	10100 ±2460	9857 ±2053	1653 ±326	352 ±115

Control	Total WBC	Lymphocytes	Neutrophils	Monocytes
Day 1	3640 ±1054	2264 ±475	1200 ±605	159 ±45
2	4420 ±1195	3372 ±941	757 ±293	365 ±278
3	2920 ±420	2110 ±252	651 ±175	158 ±49
4	3660 ±920	2905 ±742	492 ±138	235 ±57
5	4040 ±260	2853 ±249	910 ±49	215 ±36
6	3440 ±270	2505 ±250	604 ±86	199 ±43
6	4660 ±850	3770 ±693	729 ±203	172 ±41
7	4480 ±620	3523 ±514	753 ±85	200 ±42
8	5000 ±1180	3804 ±685	899 ±250	255 ±79
9	4340 ±540	3091 ±251	702 ±134	140 ±27

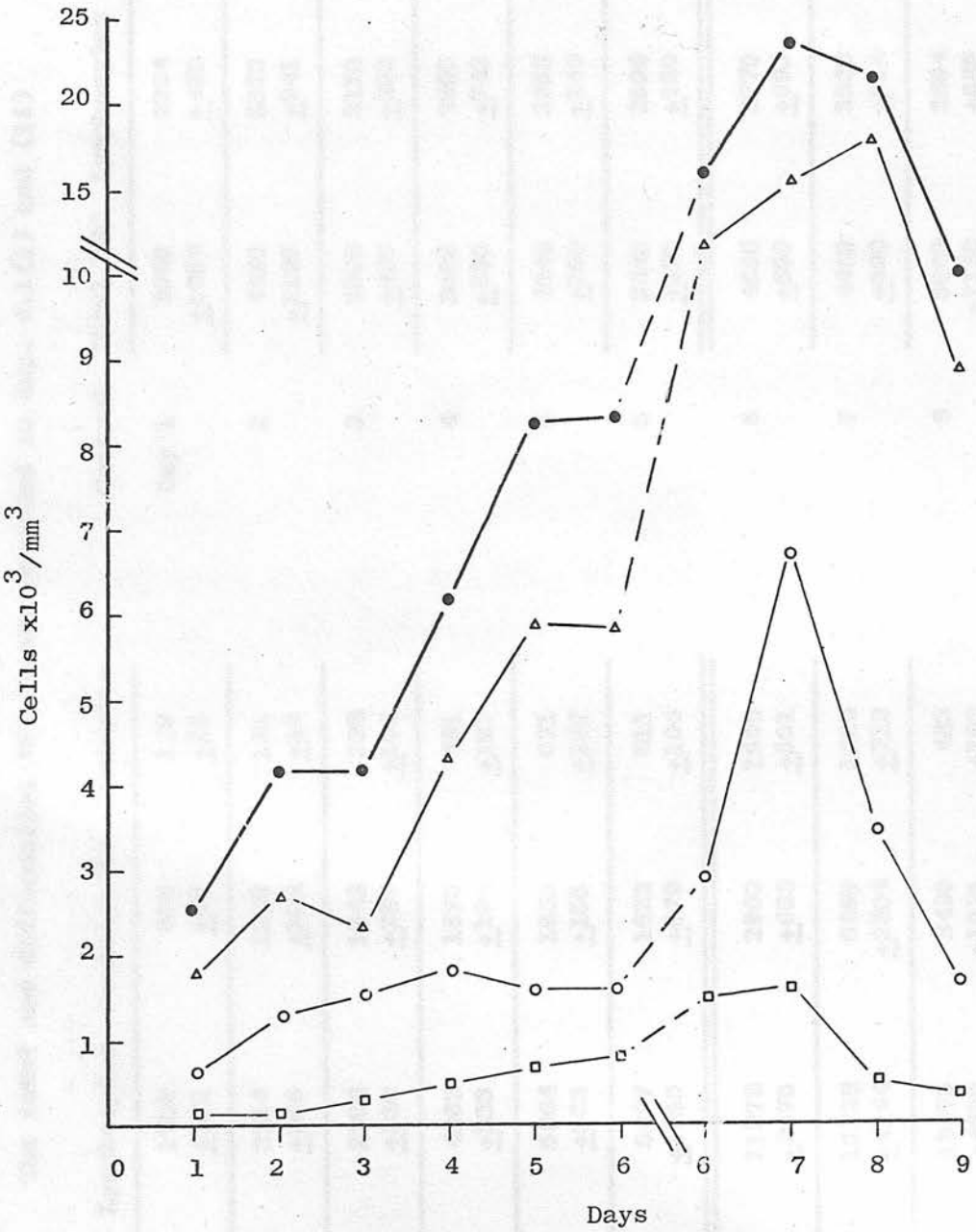


Fig. 4.5. Mean daily total white blood cell (●), lymphocyte (△) neutrophil (○), and monocyte (□) counts during B.rodhaini infection in rats (Exp. 4.1(i) and (ii)).

control was negative.

The leucocyte response

B.rodhaini infection in rats provoked a very marked leucocytosis. The results of total and differential leucocyte counts from Exp. 4.1(i) and (ii) are presented in Table 4.8, and Fig. 4.5. The results of the two experiments are combined for ease of description. No change from control values was observed until day 4 when the total count for the infected rats had risen to 6,2000 ± 550. This continued to rise until day 7, the day after peak parasitaemia, when it reached 23,900 ± 7,631. This count fell slightly on day 8 and was down to 10,100 ± 2,460 on day 9. The response to infection was mainly lymphocytic but as the lymphocytes rose a gradual increase in neutrophils and monocytes was apparent. On day 7 a sudden rise in neutrophils to 6,980 ± 2,890 was recorded which reduced to 3,490 ± 1,021 on day 8. The gradual rise in monocytes reached 1,620 ± 713 on day 7 then fell on day 8. Lymphocytes reached 15,328 ± 4,144 on day 7 and a peak of 18,475 ± 4,620 on day 8 before falling to half that value on day 9.

Following B.divergens infection all the calves developed a leucopenia which began either before parasites became apparent or very early in the parasitaemic development. This leucopenia reached its lowest level and had begun to recover prior to peak parasitaemia. All calves, except 273, which had a mild response, then showed a leucocytosis which quickly reached a peak and fell again. The large variation between calves in pre-infection values together with the variation in time of occurrence of both lowest and highest counts renders the data unsuitable for statistical analysis. Taking a mean over all animals for each day tends to obscure the decreases and

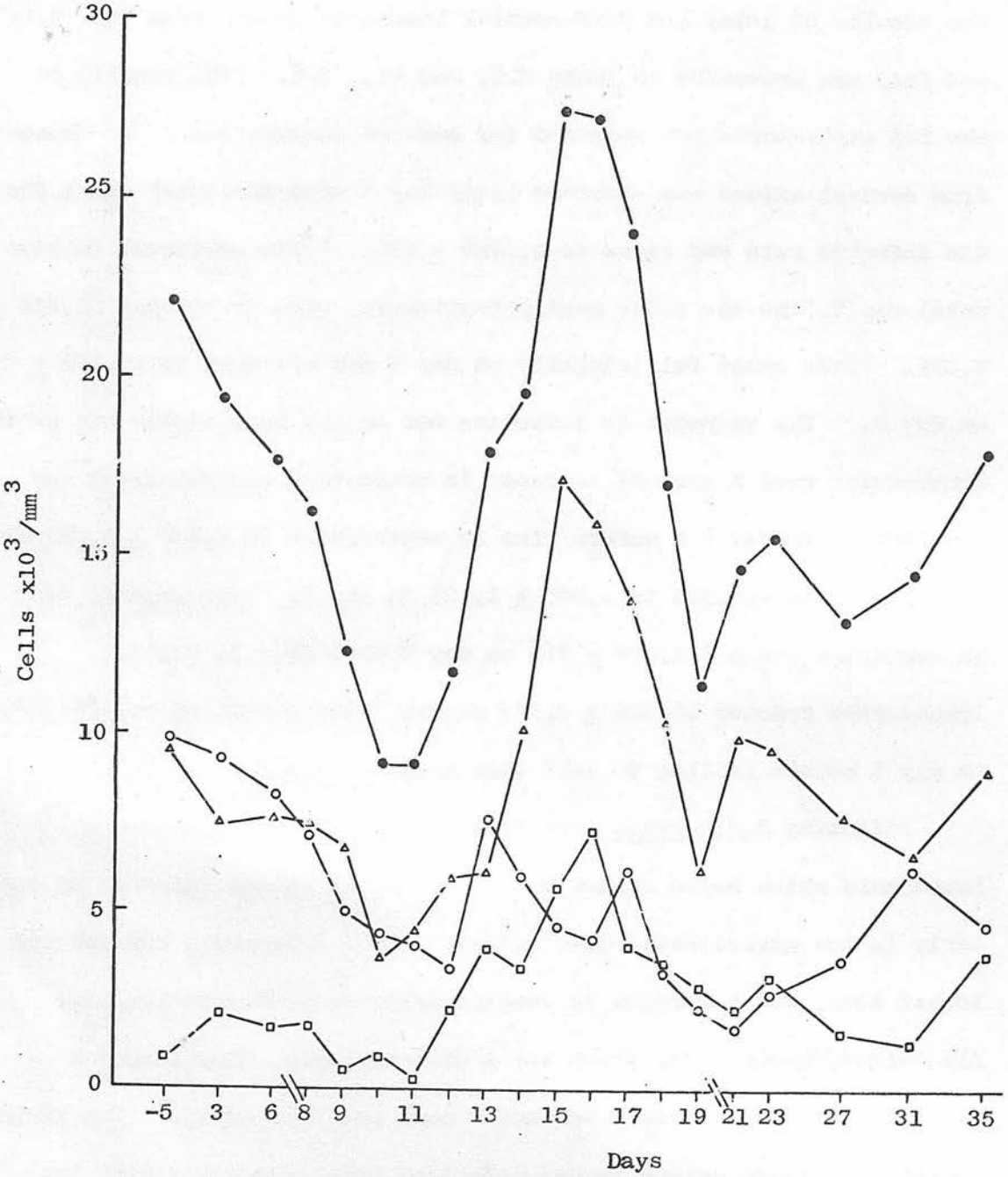


Fig. 4.6. Daily total white blood cell (●), lymphocyte (△), neutrophil (○) and monocyte (□) counts during B.divergens infection in calf L1160.

increases that occurred. The summary of results presented in Table 4.9 was found to provide the best illustration of the changes and the complete results are presented in Appendix (11).

Table 4.9

The WBC low and high in B.divergens infection

Calf no.	212	275	L1160	A89	273	274	251
Pre-infection	19340	10820	22180	10840	16140	10780	7880
Low (Day 8-10)	10400	6100	9000	7300	7000	5300	4900
High (Day 12-16)	52000	12400	27500	25900	14200	24600	17000

During the leucopenia, lymphocytes and neutrophils fell in all calves while monocytes were depressed in calves 273, 274, L1160 and 212. Eosinophils, which were apparent in low numbers in some of the calves before infection, were sporadically present during the parasitaemia and were recognized again late in the observation period in all calves. As the animals began to recover from the leucopenia a monocytosis was observed in all cases. Lymphocytes rapidly rose to near pre-infection values and calves 212, L1160, 274 and 251 developed a lymphocytosis. Neutrophils remained depressed until late in the observation period in all except calves 212 and A89, the two most severely affected. Both of these calves had a pronounced neutrophilia. The responses of calf L1160 is presented in Fig. 4.6 as an example of the response and the leucocyte values for all calves are presented in full in Appendix (11).

Spleen size and phagocytic response

The spleens of infected rats were noticeably enlarged two days after infection and continued to increase in size during the development of parasitaemia. Once parasitaemia had reached a peak and begun to

wane the spleen size began to diminish (Fig. 4.7). Weight was used as the indicator of change in the organ and it can be seen from Table 4.10 that while control groups showed almost no change the infected spleens increased in weight progressively. On day 8 the

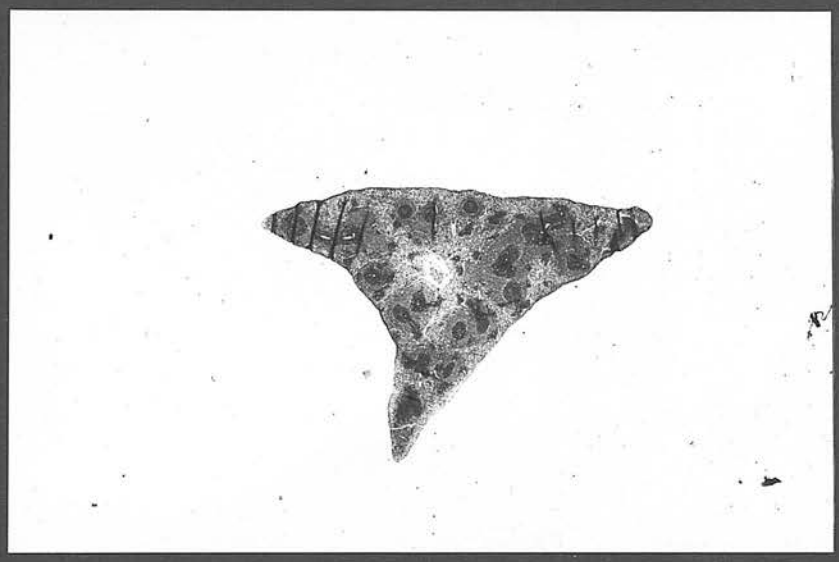
Table 4.10

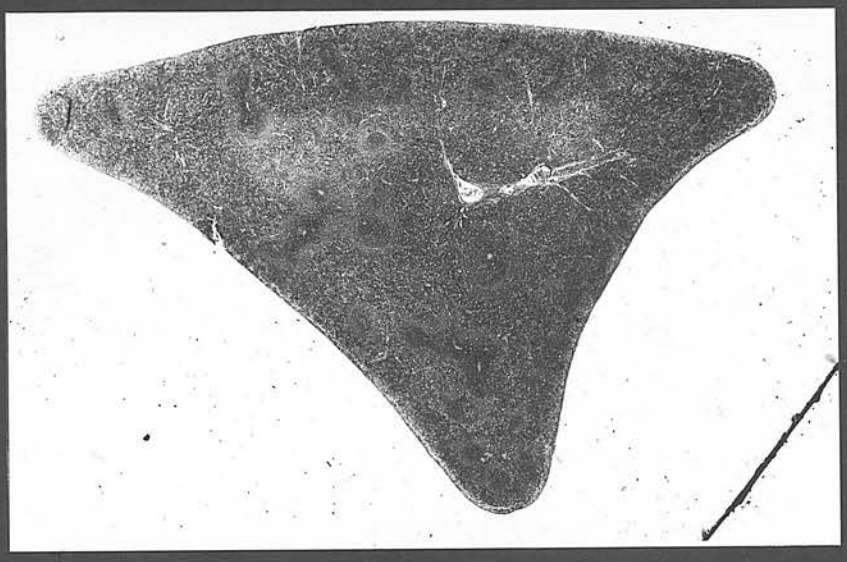
Spleen weight changes during B.rodhaini infection

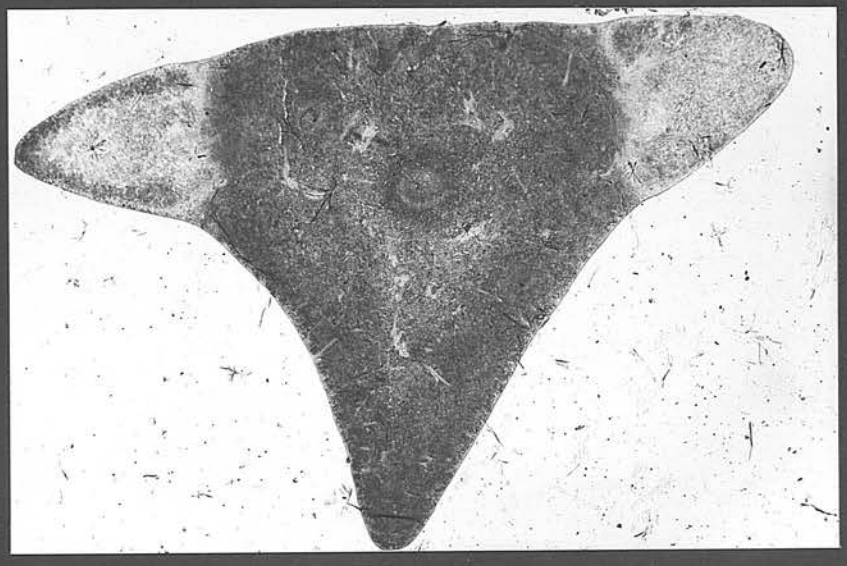
Day	1	2	3	4	5	6	6	7	8	9
Test	0.51	0.77	1.54	1.76	3.05	2.96	3.24	3.90	4.67	2.89
	±0.02	±0.03	±0.05	±0.10	±0.18	±0.29	±0.06	±0.16	±0.52	±0.30
Control	0.41	-	0.47	0.47	0.45	0.55	0.50	0.54	-	0.47
	±0.01		±0.02	±0.03	±0.02	±0.04	±0.04	±0.04		±0.03

test spleens were more than eight times the weight of control spleens and the highest individual weight increase was more than 12 times the control value. On day 9 they had begun to reduce in weight. The weight increase was due to a cellular proliferation and not to congestive splenomegaly. The cut surface of the spleen did not bleed and histopathological examination of the organ carried out later in this study showed the following cellular changes. Early in infection proliferation of the reticulum and phagocytic cells was observed and as the disease progressed intense lymphoid hyperplasia developed. These cell types continued to proliferate with the lymphoid element predominating. The erythroid and myeloid components virtually disappeared although occasional erythroid foci were seen. Megakaryocytes continued to develop during the course of infection. Erythrocytes were present in the sinusoids but congestion was not apparent.

Fig. 4.7. **Spleens of rats. A cross-section taken from the same region from a non-infected rat (upper), a rat six days after infection (middle) and nine days after infection (lower), the great increase in size and lymphoid tissue can be seen following infection. The developing infarct can be seen from the day 6 spleen and the mature lesion from the day 9 spleen (x11.HE).**







Of the eight infected spleens examined on days 8 and 9 in Exp. 4.1(ii) four were seen to have pale round or oval areas at the thin border of the organ. In two of the spleens these lesions were raised giving a beaded appearance to the organ. In one spleen there were six of these lesions and in another four. Histological examination showed these to be areas of necrosis and their position and appearance resembled infarcts; their possible origin and significance will be discussed in Ch. 7.

The erythrophagocytic response during the course of infection is summarized in Table 4.11. The total observations made for each site in the four animals killed on that day are presented, along with the number of parasitized and non-parasitized erythrocytes. The spleen

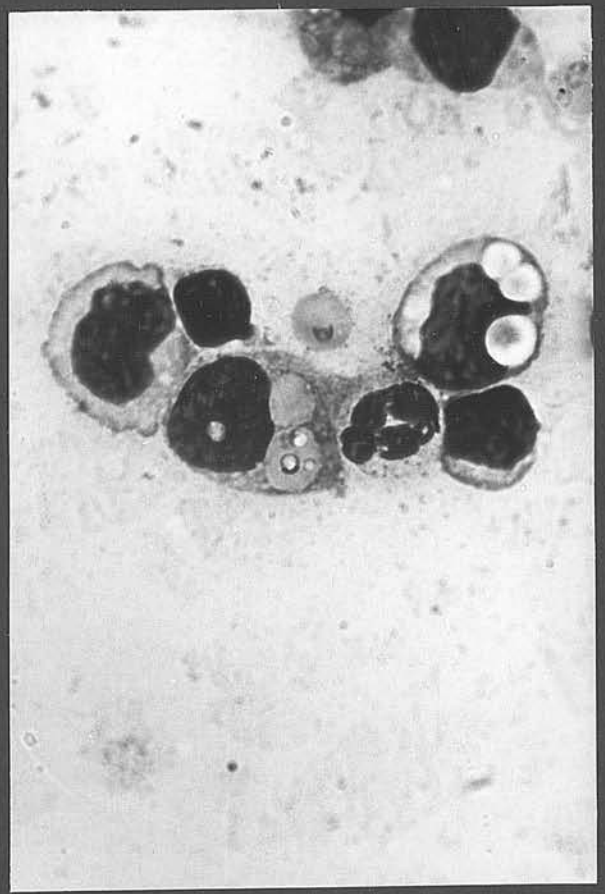
Table 4.11

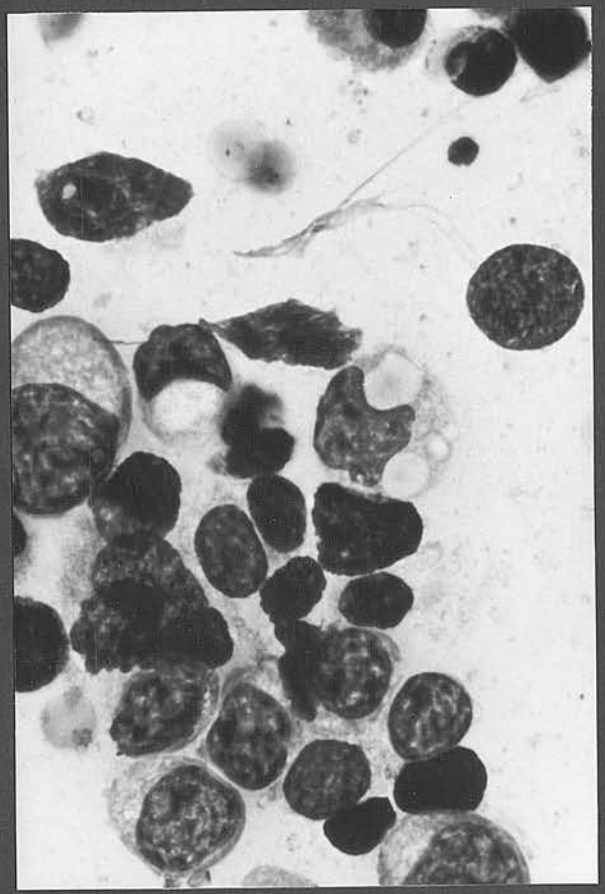
Erythrophagocytosis observed during B.rodhaini infections

Day	1	2	3	4	5	6	6	7	8	9
Spleen	0	0	4	5	8	7	3	5	0	1
Liver	0	0	1	4	15	34	10	8	0	1
Bone Marrow	0	0	0	0	2	2	3	0	0	0
Erythrocytes										
Parasitized	0	0	0	0	11	15	8	3	-	0
Non-parasitized	0	0	5	9	33	52	20	11	-	2

was the earliest site of active phagocytosis which later became apparent in the liver and bone marrow (Fig. 4.8). As the disease progressed the liver apparently became the most avid site of phagocytosis while the bone marrow remained the minor site. It is possible that the phagocytic capability of the liver was exaggerated as the impression

Fig. 4.8. Erythrophagocytosis in B.rodhaini-infected rats showing the presence of both parasitized and non-parasitized erythrocytes in phagocytic cells of the spleen (upper) the liver (middle) and the bone marrow (lower) (x1100. Giemsa).





smears from that organ provided very easy identification of phagocytic cells while the bone marrow scrapings and the spleen impression smears were very much more crowded preparations. On only one occasion was phagocytosis observed in the control rats and that was of one erythrocyte in the spleen. Two infected rats showed phagocytosis in the peripheral circulation, three parasitized erythrocytes were phagocytosed, two by monocytes and one by a lymphocyte. The types of cells involved in phagocytosis in the liver, spleen and bone marrow were mononuclear cells ranging from mainly typical monocytes to the occasional transitional lymphocytes (see Zuckerman and Weiss, 1973).

The predominant phagocytosis of non-parasitized cells might be exaggerated because wherever any doubt existed about the presence or absence of a parasite the cell was recorded as non-parasitized. Pale erythrocytes within phagocytes obviously undergoing digestion were commonly found. It might well be that these contained parasites which had been digested earlier. Free parasites were not identified within phagocytes despite their frequent occurrence in the blood, especially in higher parasitaemias. This might indicate their rapid digestion. Early in infection phagocytosis was of single erythrocytes while later, many ingested erythrocytes were seen in some phagocytes. Phagocytosis appeared to be that of entire erythrocytes, any debris being of erythrocytes undergoing digestion.

IV. Discussion

The development of B.divergens parasitaemia and the rate and degree of erythrocyte destruction with corresponding falls in packed cell volume and haemoglobin values were similar to reports from other studies of the same experimental system. (Davies et al., 1958;

Brocklesby et al., 1971). B.rodhaini, while showing similar parasitaemic characteristics to those described from other work (Matson, 1964; Nowell, 1969), tended to produce a more severe anaemia and a greater mortality. This was probably due to strain of parasite, the size of inoculum and the age and strain of rat used as discussed in Ch. 3.

Following intraperitoneal inoculation of infected blood, intact erythrocytes, both parasitized and non-parasitized, are transferred across the peritoneum to the circulation, while free parasites are destroyed (Rickard, 1970a). Intravenous inoculation introduces the infected blood directly into the parasite's normal vertebrate environment. Once inoculated into a susceptible host there is a variable period of time before parasites become apparent in the peripheral and general circulation. This is due to dilution, as the parasites have no exoerythrocytic development but need a certain time to achieve sufficient numbers for them to be recognized. They are then observed to multiply rapidly. This continues for a time then begins to slow because there is a limited number of suitable cells (which the parasites are destroying) and the innate and later acquired immune mechanisms of the host act to limit their development. With increasing cell destruction and tissue damage to the host the plasma becomes unsuitable or damaging to the parasite and the high parasitaemia and multiple invasion of erythrocytes, as seen in B.rodhaini infection, may lead to competition for nutrients (Nowell, 1969).

In the rats the parasites had reached a peak and begun to decline one to two days before the erythrocyte nadir. In the calves three to four days elapsed between parasite peak and erythrocyte recovery.

These were predictable intervals because parasites continue to destroy erythrocytes on emergence even as the parasitaemia falls. The longer interval shown by the calves reflects their slower response to an erythrocytic stimulus (Valli et al., 1971). If an appreciable number of surviving cells is damaged, perhaps because of the deterioration of the plasma or due to defective production as occurs under stress (Berlin, 1964), then these cells with a shortened life span will contribute to the interval before recovery of erythrocyte numbers becomes apparent.

The reticulocyte response in rats became apparent on day 4 and rose sharply. In calves this response was not observed until the day after peak parasitaemia and then reticulocytes increased rapidly. The delay in the calves' response was presumably due to a slower erythropoietic response. There was no evidence of any interference with erythropoiesis in either host.

Positive antiglobulin tests were recorded in B.rodhaini infections when the parasitaemia was fairly high and persisted into early recovery. B.divergens infections also induced a positive antiglobulin test which became apparent after peak parasitaemia. It persisted for a few days during parasite decline and then disappeared. Antiglobulin tests need to be interpreted with caution. It has been found that repeated bleeding, or anaemia induced by phenylhydrazine treatment, brings about positive tests in rats (Zuckerman and Spira, 1963; Zuckerman, 1963). Mahoney (1972) provoked a positive test in calves by the injection of Freund's adjuvant. Also, reticulocytes are agglutinated by potent antiglobulin serum because of a transferrin type substance on the stromata of the cells (Jandl, 1960). However, Zuckerman (1963) has

pointed out that true positive tests can be found concurrently with false positive reactions. In both infections reported here reticulocytes were present in high numbers during recovery. The test applied in rats became negative whilst the reticulocytes remained in high numbers, indicating that the reticulocytes were not giving a false positive test. This did not arise in calves as the serum and not the erythrocytes was tested. Tests in both experimental systems became negative when parasites fell to low numbers. Sibinovic et al. (1967b, 1969) have shown that during the course of patent B.rodhaini and B.canis infections parasite antigens were released and became bound onto erythrocytes. When these antigens were isolated and injected into the experimental hosts they provoked transient anaemias. Annable and Ward (1974) have also identified soluble antigen on erythrocytes during the course of B.rodhaini infection and Rogers (1974) has detected opsonins directed against parasitized cells in the same infection. Mahoney (1972) found that the plasma antigens of B.argentina were bound to proteins and, while they were capable of provoking an antibody response that gave specific protection against B.argentina, they did not induce anaemia. It is therefore apparent that parasite antigens capable of provoking antibody responses are present in the plasma and on erythrocytes during Babesia infections. It is most likely that some of these antibody types were recognized by the antiglobulin tests that gave positive readings during these studies. The finding of positive tests only while parasites were still present would further emphasize their parasite association. Schroeder et al. (1966), who first described positive antiglobulin tests during babesiosis infections, found that the haemagglutinins, along with splenomegaly, and

erythrophagocytosis were more closely associated with the anaemia than was the parasitaemia. They examined a B.rodhaini infection in rats that was mild and prolonged and in ways resembled the more chronic forms of B.canis infection where anaemias are frequently unrelated to the degree of parasitaemia (Neitz, 1938; Maegraith et al., 1957 and others). In Babesia infections, where chronic anaemia with low parasitaemia is found, an immune based-mechanism in the pathogenesis of the anaemia is an attractive hypothesis. The evidence for the presence of parasite antigens on erythrocytes is so strong that to propose an autoimmune mechanism as suggested by Schroeder et al. (1966) cannot be justified. The antigens on erythrocytes lead to erythrocyte destruction, either directly by lytic antibodies or following opsonization by the RES. In acute disease, such as the experimental systems examined in these studies, the anaemia closely parallels the parasitaemia and antibodies are only detected for a short time while parasites are present in reasonable numbers. This mechanism is unlikely to contribute very greatly to the anaemia. When, however, definite evidence for the existence of this immune loss of erythrocytes has been sought by the inoculation of radioactive labelled erythrocytes, the rate of destruction of these labelled cells has been found to be no greater in the infected than in the normal animal (Maegraith et al., 1957; Mahoney, 1972). Despite these findings much evidence still exists to support the immune hypothesis, not only the presence in plasma and on erythrocytes of parasite antigens and the positive antiglobulin tests but also the frequent observation of phagocytosis of non-parasitized erythrocytes (Maegraith et al., 1957; Schroeder et al., 1966; Rogers, 1971).

The white blood cell response of calves to B.divergens infection was initially a leucopenia and then a leucocytosis. The leucocytosis was sharp, short-lived and in a few cases the counts then fell to below preinfection values, as in calf B10 (Exp. 3.5). It was composed of a lymphocytosis and a monocytosis in all animals while the two most severely affected also showed a neutrophilia. Neutrophils in the other five calves were depressed and did not recover until late in infection. In the rats with B.rodhaini infection a leucocytosis was also found, composed of a marked lymphocytosis, a mild monocytosis and a neutrophilia at the crisis of the disease; all cell types then returned towards normal.

The leucocyte counts of the calves prior to infection ranged from 7800 to 22,100 per mm^3. Following splenectomy such a variation is predictable as a leucocytosis usually occurs, with a variable time, from weeks to months, before returning to the normal range (de Gruchy, 1970). The leucopenia was probably also associated with the absence of a spleen. As the parasitaemia increased to patent levels the calves, in responding to the presence of the parasite, were hampered by their lack of the initial source of antibody production and the phagocytic and productive capacity of the spleen (Taliaferro, 1956). The delay before alternative sites of production could provide the response was manifest as a leucopenia. The fact that the calves had been splenectomized must comprise to some extent the results obtained. However, once over the leucopenia, they resembled the few reports from bovine babesiosis that exist. Karput (1966) described a lymphocytosis which was also found by Jajicek and Hybasek (1971) but did not go outside the normal range. Suteu and Giurgea-Iacob (1971) found a lymphocytosis,

a monocytosis and a neutropenia.

From the many reports of white blood cell studies during canine infections no typical response is recorded although a leucocytosis is most common (Wright, 1905; Maegraith et al., 1957), while the role played by the different cell types in the total varies greatly (Sanders, 1937; Alperin and Bevins, 1963; Dorner, 1967). Gamble (1974) found a marked neutrophilia without a rise in total count in mice. The rats in this study also showed a neutrophilia but it followed an earlier lymphocyte and monocyte response. Maegraith et al. (1957), whose study of the pathological processes in B.canis infections is the most exhaustive work on the disease, were content to describe the white blood cell changes, remark upon the variability of reports from other studies on white blood cells in babesiosis and refrain from any interpretation. This appears a prudent decision as an interpretation must be deeply interwoven with the immunology of the disease.

Splenic enlargement is a consistent finding in babesiosis and other haemoprotozoan infections. The increases in spleen size found in this study were very similar to those found by Schroeder et al. (1966) in the same host-parasite system. The enlargement was due to cellular proliferation and not to congestive splenomegaly. The proliferation was of reticular, phagocytic and lymphoid cell types. Paget et al. (1962) found when examining the spleens of mice infected with B.rodhaini that proliferation of reticular, phagocytic and megakaryocyte cell types occurred early in infection, while later in infection both erythroid and myeloid development occurred. Studies of mice infected with P.berghei showed splenic congestion and proliferation of cell types for three to four days but then the organ became predominantly erythrogenic

(Singer, 1954). Rats on the other hand, with the same infection showed proliferation of reticular and phagocytic cells but lymphoid production was at the expense of erythroid elements (Zuckerman et al., 1973). From the observations of splenic change during B.rodhaini infection in this study it would appear that the rat spleen response is similar to that of rats with P.berghei infection and different from the mouse spleen response to either infection. Where splenic changes in cattle with Babesia infections have been studied, enlargement and congestion were reported (Smith and Kilborne, 1893; Rogers, 1971). Rogers (1971), describing the histopathology of the organ, reported the proliferation of reticular and phagocytic cells and the production of erythroid elements at the expense of the lymphoid tissue. It appears that while animals infected with Babesia spp. or Plasmodium spp. undergo proliferation of splenic reticular and phagocytic cell types the predominance of erythrogenesis or lymphogenesis in the organ during the infection is dependent upon the host species.

The role of the spleen in response to B.rodhaini infections has been subject to many investigations (Todorovic et al., 1967; Philips, 1969; Roberts et al., 1972; Irvin et al., 1973). The present study was concerned with examining the aspects of the reactive spleen which might contribute to erythrocyte loss during infection rather than the innate or specific immune responses which were the concern of other studies. The spleen is a most complex organ of which neither the anatomy nor the functions are fully understood (Bjorkman, 1947; Ham, 1969). More recent electron microscopic studies have increased the knowledge of the vascular structure of the organ while labelled erythrocyte studies have explained some of the haemodynamics

(see Jandl and Aster, 1967). The enlarged and hyperactive spleen is known by the term hypersplenism, irrespective of the aetiology and the effects of this state have been fully reviewed (Doan, 1949; Dameshek, 1955; Jandl and Aster, 1967; Jacob, 1974). The normal splenic function of removal of damaged or slightly altered cells is greatly increased in hypersplenic states, while the rate of blood flow through some compartments of the organ is greatly reduced. The splenic sinusoid has running parallel to it a splenic cord. These two compartments are separated by the sinusoidal endothelial wall which has no preformed openings but a series of intraendothelial slits through which circulating cells must squeeze (Chen and Weiss, 1973). It is in passing through this wall that such cell inclusions as Heinz bodies or malaria parasites have been observed to be pitted from erythrocytes (Chen and Weiss, 1973; Schnitzer et al., 1972). It is also this region of the hyperactive spleen that bears upon the anaemia of babesiosis. In passing through the sinusoidal wall the parasitized cell will be trapped and the cell may lyse, the parasite may be pitted from the cell or the erythrocyte may be removed. The pitted cell will continue to circulate as a smaller, rounded cell with a shortened life span.

The other mechanism of erythrocyte destruction embodied in the hyperactive spleen resides in the sequestration or pooling of blood in the cords. Here erythrocytes are trapped and concentrated in an almost plasma-free state for variable periods of time. This situation can be tolerated by the immature erythrocyte but the mature cell is dependent upon plasma sources of glycogen and phosphate and it rapidly utilizes its own very limited supply. Once used up the cell begins

to deteriorate both in its internal metabolic capability and its membrane structure (Doan, 1947; Jacob and Jandl, 1964). Adding this mechanism to an existing haemolytic disease may considerably increase the cell loss. Further if membrane damage already exists this process will rapidly undermine the cell integrity.

The phagocytic cells of the spleen increase greatly in number and efficiency during malaria infection (Talliaferro, 1956; Zuckerman et al., 1973) and a similar response is thought to occur during babesiosis (Schroeder et al., 1966; Mahoney, 1972). Mahoney (1972) considers that the initial response to babesia infection is non-specific, being the general increase in activity of the hyperactive spleen, while later this response is more specific. This would be manifest by the indiscriminate removal of erythrocytes before more specific mechanisms, such as opsonization, could occur. Schroeder et al. (1966) correlated the onset of erythrophagocytosis with positive antiglobulin tests, a finding confirmed in this present study which also showed a decline in measurable erythrophagocytosis with disappearance of antiglobulins and reduction of parasitaemia. The exact nature of these antibodies has not been determined but Schroeder (1966) has detected opsonins for apparently normal erythrocytes while Rogers (1974) has demonstrated opsonins directed at parasitized erythrocytes. The former would account for an additional loss by the removal of non-parasitized cells. Quantification of the splenic response to malaria infection has been carried out and while the splenic volume increased 20 times the erythrophagocytosis was increased by 200 times (Zuckerman et al., 1973). While similar investigations have not been reported for babesiosis the increases recorded in this study and the cell changes

observed greatly resemble those of Zuckerman et al. (1973).

Erythrophagocytosis occurred, not only within the spleen, but throughout the entire phagocytic system. The initial appearance of active erythrophagocytosis in the spleen and later at other sites has previously been observed (Todorovic et al., 1967). The liver which has a large phagocytic capacity, became very active, while the bone marrow did not show very intense phagocytosis. This latter tissue was, however, very active in the production of erythroid, myeloid and megakaryocyte cell types. The parasite presence produced a marked leucocyte response in the host and here too phagocytosis was observed.

The peak and fall of parasitaemia was the first parasite indication of acquired immunity although the antiglobulin tests probably indicated the presence of antibodies some days earlier. There was considerable variation in the time of peak parasitaemia. Thus when mean parasitaemia is considered the fall in parasite numbers does not appear as dramatic as in the case of individual animals, where marked falls, occasionally as sharp as from 60% to 5% in 24 hours, were recorded. The parasites during this phase were greatly changed, usually rounded, stained badly or showed no vacuole. Whether these parasites were actively extruded from the cell or pitted by the RES is not known. It is likely that these cells were not destroyed at this stage because the rats, with an approximate erythrocyte count of 2.0×10^6 per mm^3, could not withstand the loss of so many erythrocytes in such a short period of time, even allowing for the active erythrocyte regeneration.

The rapid reduction in measurable erythrophagocytosis once parasitaemia fell would indicate that in the absence of parasites no antigen was being coated onto erythrocytes. Thus fewer cells were being

opsonized either by the direct coating of erythrocytes with antigen that attracted opsonic antibody (Mahoney, 1972) or the non-specific antigen- antibody complex opsonin suggested by Cox (1973).

The anaemia of the B.rodhaini and B.divergens infections examined was acute and parasite-related. The hyperactive spleen, phagocytosis and the presence of antiglobulins played additional, but probably minor roles in the erythrocyte loss in B.rodhaini infections. There was no interference with erythrocyte regeneration and both infections provoked a marked leucocyte response.

CHAPTER 5

THE PARASITE AND THE ERYTHROCYTE

I. Introduction

It is essential for the survival of the parasite once within the vertebrate host that it gains entry to a suitable cell. It is not known whether babesias have any special mechanism of attraction to or for the erythrocyte or rely upon chance collision in the circulation. Aikawa and Seed (1973) have found that Plasmodium berghei lacks charged sialic acid residues on the parasite membrane surface. The parasite is thus left "with an absence of net negative charge and so acts as a charged positive body in relation to the erythrocyte". It is possible that a similar mechanism might be active for Babesia, providing a surface attractant between parasite and cell after which the mechanism of penetration could take over.

Protozoa can enter the host cell by either of two processes; those organisms possessing a conoid puncture the membrane and enter while those that do not possess a conoid invaginate the membrane to gain entry (Vickerman, 1972; Aikawa, 1974). Protozoans such as Babesia and Plasmodium are thought to enter by invagination. Observations of the process of Babesia entry into erythrocytes have been described from living preparations (Nuttall and Graham-Smith, 1908; Holbrook et al., 1968), fixed stained blood films (Nowell, 1968) and from electron microscopic studies (Simpson et al., 1967). Recently, studies have attempted to examine the mechanism of penetration. Simpson (1973), using the electron microscope and examining B.canis infected blood, described the parasite as invading the cell by pinocytosis. The membrane invaginated and the parasite was then found in a cell membrane pocket within the erythrocyte. Rudzinska and Trager (1973) could not

identify the process in hamster blood following the inoculation of large volumes of B.microti, freed from erythrocytes. Thus, while the process of entry has been observed and is presumed to be invagination, the mechanisms, such as parasite orientation and changes in the cell membrane as detailed in malarial parasite penetration (Ladda et al., 1969), are as yet not fully known.

This lack of knowledge of the process of penetration of the erythrocytes adds to the difficulty in understanding the relationship between the parasite and the host erythrocyte. The erythrocytic forms of malarial parasites have two membranes and lie in immediate contact with the host erythrocyte cytoplasm (Rudzinska and Trager, 1959; Rudzinska et al., 1965). The outermost membrane however is thought to come from the host cell while the innermost membrane is the parasite limiting membrane (Aikawa, 1966; Hepler et al., 1966). A single membrane has been described in B.rodhaini (Rudzinska and Trager, 1962) and B.microti (McMillan and Brocklesby, 1971; Rudzinska and Trager, 1973). Molyneux and Bafort (1970) have found, in contrast to the other studies of B.microti, that the organism has two membranes. When freed from erythrocytes it still retains two membranes which they concluded were membranes derived solely from the parasite rather than of host and parasite origin. Two membranes have also been described for B.canis (Simpson et al., 1963), B.caballi (Simpson et al., 1963, 1967), B.bigemina (Hoyte, 1966) and B.equi (Simpson et al., 1967). Abramov et al. (1973) have described a three layered cytoplasmic membrane for B.ovis in which the inner layer was discontinuous. This membrane structure possibly resembled the partial three layered membrane sometimes seen in B.caballi (Simpson et al., 1967).

If babesias enter the erythrocyte in the same way as do malarial parasites, by invagination, then a second host cell membrane should be present. In the babesias that do not have a double membrane it is postulated that they might have lost one of them shortly after entry or the parasite might have lysed the erythrocyte membrane at the site of entry (Rudzinska and Trager, 1973).

Once within the erythrocyte, the parasite has been observed to feed by a process of intracellular phagotrophy (Rudzinska and Trager, 1962; Hoyte, 1966). The ingested erythrocyte cytoplasm was digested completely in the food vacuole as no pigment was found in the erythrocyte or parasite. Again, Molyneux and Bafort (1970) presented a contradictory finding where pigment grains similar to those found in malarial parasites were observed in B.microti and these were discharged from the parasite vesicle into the plasma. More recently Rudzinska and Trager (1973) postulated a different process of parasite nutrition. With B.microti they found that, despite the presence of host cell cytoplasm accumulated within B.microti, they could not demonstrate a food vacuole in the parasite when freed from the erythrocyte. Because of this they postulated that the parasite was nourished by diffusion of nutrients across the membrane and this process was facilitated by the single limiting membrane and the amoeboid activity of long and numerous pseudopodia. The presence of a cytostome in some babesias (Molyneux and Bafort, 1970; Frerichs, 1970) provides another possible mechanism of parasite feeding but it is clear that much more investigation is required before these mechanisms are understood.

Biochemical studies of the babesias are very few but offer some insight into the effect of parasitism on the normal function of the

erythrocyte. The respiration of B.canis, both in infected canine blood and freed from erythrocytes by lysis, has shown that the parasites are metabolically active but cause little change in the aerobic respiration of the erythrocytes (Maegraith et al., 1957). Rickard (1969, 1970a,b) carried out a series of experiments on the carbohydrate metabolism of B.rodhaini. Both oxygen uptake and glycolysis were greatly increased in infected erythrocytes. While the parasites possessed their own glycolytic enzymes they stored no endogenous substrate. He concluded that the Babesia might regulate the metabolism of the host erythrocyte through its own maleate dehydrogenase in a similar manner to malarial parasites, as shown by Maegraith and Fletcher (1962) and Sherman (1966). Acid phosphatase activity has been demonstrated throughout intra-erythrocytic B.divergens and exo-erythrocytic B.ovis in the tick haemolymph (Weber and Friedhoff, 1973). Wright and Goodger (1973) have demonstrated non-specific esterase activity capable of haemoglobin hydrolysis in some of the antigens of B.argentina and B.bigemina. Godfrey (1957) has found that diets rich in cod liver oil suppress B.rodhaini in mice while inositol and para-aminobenzoic acid enhance its development. From these studies it appears that the Babesia parasitism at least interferes with the erythrocyte metabolism by a redirection of glucose metabolism; the other factors, if any, affecting the erythrocyte biochemistry remain unstudied.

Measurements of the osmotic fragility of erythrocytes during the course of Babesia infections have yielded no consistent pattern and some confusion. Yakimoff and Weizekhowsky (1926) found that the osmotic fragility of erythrocytes increased during B.bovis infection.

Shirlaw (1939) found a similar increase during B.canis infection but not in B.gibsoni infection, while no increase was found by Maegraith et al. (1957) in B.canis infection. These workers, however, did find that the mechanical fragility of erythrocytes increased. Dorner (1967) found a decrease in osmotic fragility during B.canis infection. Mahoney (1967) found that cells parasitized with B.argentina were more resistant to lysis and could be concentrated because of this resistance. This finding was confirmed by Wright (1974a) who also found an increased fragility of non-parasitized erythrocytes late in infection. B.bigemina induced an increased fragility which was greatest in parasitized cells (Wright, 1974a). Other indications of change in erythrocytes during Babesia infections include a tendency for B.bigemina and B.canis-infected cells to be distributed towards the edge and tail of blood films (Barnett, 1965; Hirsh et al., 1969), while B.caballi and B.canis parasitized erythrocytes tended to concentrate at the top of the red cell column following centrifugation of parasitized blood (Watkins, 1962; Hirsh et al., 1969; Simpson, 1974). Saal (1964), using Giemsa staining, described a darkened rim to erythrocytes parasitized by B.argentina and B.bigemina and Ludford (1962) has shown specific immunofluorescence about the periphery of B.argentina and B.canis infected erythrocytes which could indicate surface changes.

Recent studies of Plasmodium infected erythrocytes have shown profound morphological changes of the erythrocyte surface of both parasitized and non-parasitized cells (Arnold et al., 1969; Balcerzak et al., 1972; Kreier et al., 1972), although conflict exists as to the relationship between the parasite and some of the observed defects on the membrane. Weidekamm et al. (1973) have correlated surface

alterations during P.berghei infections in mice with breakdown of membrane proteins. Bodammer and Bahr (1973), from studies of the same host-parasite system, have postulated that the parasite induces specific changes in the membrane permeability which allows certain nutrients to pass from the plasma to the parasite. The changes observed by Wright (1972b) in B.argentina-parasitized erythrocytes do not resemble these specific parasite related changes but would seem to be a complex relationship between the parasite, the erythrocyte and the physiological changes in the circulation of the capillaries of the brain.

The following experiments were carried out in an effort to understand better some of the effects of parasitism and the possible mechanisms of erythrocyte damage by Babesia organisms.

II. Materials and Methods

Experiment 5.1 : The Osmotic Fragility during B.rodhaini and B.divergens Infections

During the course of Exp. 4.1 and 4.2, in which rats were infected with B.rodhaini, and Exp. 4.3, in which calves were infected with B.divergens, the erythrocyte osmotic fragility was measured by the method previously described (Ch. 2 (11)). The osmotic fragility was recorded as median corpuscular fragility (MCF) and is presented with the results in this chapter.

Experiment 5.2 : Scanning Electron Microscopic (SEM) Examination of Normal and B.rodhaini-infected Rat Blood

Blood was drawn into EDTA, thin blood films, stained with Giemsa stain, were prepared for parasitaemia estimation and an RBC count was carried out. One volume of well mixed blood was added to ten volumes

of 1% glutaraldehyde (Taab Laboratories) in 0.17M phosphate buffered saline at pH 7.4. Fixation was continued for one hour at room temperature on a Matburn blood mixer.* The preparation was centrifuged at 1,500 rpm for three minutes, the supernatant fluid discarded and the cells resuspended to the original volume in 10% alcohol and mixed as above for 30 minutes. The sample was again centrifuged at 1,500 rpm, resuspended in absolute alcohol and mixed for 30 minutes. The sample was finally washed three times in 20 volumes of deionized distilled water, resuspended in distilled water and dropped onto an 18 mm coverslip at an angle of 120° and allowed to dry in a dust free airstream. Once dry, they were placed in marked slide cards and stored dust free. The coverslips were mounted with conducting glue (Evostick 6503) on a large stub and coated with gold and palladium in an AEI shadow caster. They were examined in a 5,600 scanning electron microscope (Cambridge Scientific Instrument Company) at accelerating voltages of 1.5K to 25K.

Seventeen infected and seven normal control samples were examined. The infected samples were chosen to represent different stages of the infection and repeated to confirm earlier observations. Samples were prepared from rats in groups of three or four and each group included one control sample. Infections were established by the inoculation of 10^7 B.rodhaini-infected erythrocytes from rats with rising parasitaemias. The parasitaemia ranges and erythrocyte counts of the samples examined are shown in Table 5.1.

* Matburn Ltd., London.

Table 5.1

Parasitaemia and RBC counts of blood samples examined by SEM

Parasitaemia Group	No. of Samples	%	RBC	%	RBC	%	RBC	%	RBC	%	RBC
0 - 20	3	4	7.87	19	6.21	19	5.28	-	-	-	-
20 - 40	3	21	7.72	24	6.32	33	7.00	-	-	-	-
40 - 60	4	47	4.32	55	4.78	55	4.46	58	1.99	-	-
60 - 70	5	61	2.93	61	3.87	64	1.05	66	1.67	70	1.44
Recovery	2	(70)	3.21	33	5.61	-	-			-	

Experiment 5.3 : Living Parasite Preparations

Blood for observations of the living parasites was taken into EDTA in a pre-warmed syringe and the blood was examined immediately on a heated microscope stage (Wild Heerbrugg, Switzerland). Cleaned glass slides, 16 mm diameter coverslips, clear nail varnish, immersion oil and pasteur pipettes were all warmed in an incubator to 37°C. A drop of fresh blood was placed on a slide and a coverslip gently dropped on. This was quickly sealed with nail varnish, a drop of immersion oil put on the centre of the coverslip and the slide introduced into the heated stage for examination. The drop of blood was judged to spread out as a single layer of cells below the coverslip before being sealed.

Observations were made by light field microscopy (x100 x15) on a Wild M20 microscope, phase contrast microscopy (x100 x10) on a Leitz Orthoplan microscope and by Nomarski interference phase (Leitz) microscopy.

(i) Living preparations of B.rodhaini-infected blood were examined from rats with various levels of parasitaemia. The duration of observation on any sample was dependent upon the quality of the sample and varied from one or two minutes to four hours. On a few occasions

samples of very high parasitaemias and early recovery samples were examined but the majority of time was spent on blood samples of 20 to 40% parasitaemia.

(ii) Living preparations of B.divergens - infected cattle blood were examined from three calves, A89, 274 and 296. Samples were examined on eight occasions from blood samples of 10 to 20% parasitaemia.

Experiment 5.4 : Separation of B.rodhaini-infected Erythrocytes

(i) The distribution of parasitized cells in thin blood films stained with Giemsa stain was examined by estimating the parasitaemia (%) from the middle, the edge and the tip of the blood film. Eight blood films were examined in this way.

(ii) Using a sucrose gradient ten samples of varying levels of parasitaemia were examined for their distribution in the different layers of the gradient following centrifugation.

Gradients were prepared from 0.4 to 0.7M sucrose using a standard laboratory gradient marker. The quality of the gradient was checked by layering on a solution of Acridine Orange and examining its distribution in the gradient by spectrophometry. Blood samples, taken in EDTA and well mixed, were layered onto the top of the gradient and centrifuged at 1000 rpm for five minutes. After centrifugation a 16 gauge needle was introduced into the base of the cellulose nitrate tube, in which the gradient had been prepared, and the various layers collected separately. Some preliminary experiments had established that gradients of 0.4 to 0.7M were suitable for this study.

(iii) Two methods of ultracentrifugation were also used and samples taken from different levels of the packed cells were examined for parasites.

(a) The ten samples used in the sucrose gradient separation attempts were examined following centrifugation in capillary tubes in a Hawksley haematocrit centrifuge. The estimated force in this machine is about 13,000 G. Following centrifugation for five minutes the tube was broken at different levels of the red cell column. The cells were resuspended in their own plasma and thin blood films were prepared, stained and examined.

(b) Five blood samples of about 30% B.rodhaini parasitaemia were centrifuged at 12,500 G in a Beckman ultracentrifuge at 4°C for 20 minutes. The blood was centrifuged in cellulose nitrate tubes and following centrifugation the red cells from different levels of the packed cells were collected by introducing a 16 gauge needle into cells and withdrawing some from different levels. The cells were then resuspended in their own plasma and thin blood films prepared and examined.

III. Results

Experiment 5.1 : Osmotic Fragility during B.rodhaini and B.divergens Infections

The osmotic fragility of erythrocytes during the course of B.rodhaini infection was measured in Exp. 5.1 and is presented in Table 5.2. The infection produced a highly significant increase in fragility, using the median corpuscular fragility (MCF) as the indicator of fragility. The mean values for the five test and the five control animals are presented. The difference between the test and the control groups ($\bar{X}_T - \bar{X}_C$) on days 5 and 6 is highly significant ($p < 0.001$). A similar examination of the osmotic fragility during recovery, where four test and four control animals were used, is

Table 5.2

Osmotic fragility during B.rodhaini infection

Day	Test ($\bar{X}_T$)	Control ($\bar{X}_C$)	Difference ($\bar{X}_T - \bar{X}_C$)	Standard error	t_8	P
3	5442	5387	58	119	0.48	>0.50
4	5525	5362	163	105	1.55	>0.20
5	5847	5075	775	60	12.87	<0.001
6	5739	4973	766	91	8.41	<0.001

Table 5.3

Osmotic fragility during recovery

Day	Test ($\bar{X}_T$)	Control ($\bar{X}_C$)	Difference ($\bar{X}_T - \bar{X}_C$)	Standard error	t_6	P
6	6142	5190	952	213	4.47	<0.005
7	4094	5339	- 1245	159	7.81	<0.001
8	4400	5150	- 750	273	2.75	<0.05
9	4183	5262	- 1079	137	7.89	<0.001

Table 5.4

Mean MCF and parasitaemia during *B.divergens* infection

Day	n	Parasites %	Mean MCF	Day	n	Parasites %	Mean MCF	Day	n	Parasites %	Mean MCF
5	7	0	.5268	10	7	7.6±2.9	.5629	15	6	2.2±1.5	.5558
3	7	0	.5337	11	7	11.9±3.2	.5621	16	6	+	.5583
6	7	0	.5253	12	7	17.8±3.7	.6029	17	3	+	.5592
8	7	+	.5407	13	6	11.2±3.5	.5833	20	6	0	.5442
9	7	2.5±0.9	.5464	14	6	7.1±3.0	.5667	35	5	0	.5245

Table 5.4

Analysis of variance of mean MCF during *B.divergens* infection

Source	df	M.S.	F	p
MCF between days	14	31.4318	7.1	< 0.001
MCF within days	79	4.3721		

presented in Table 5.3. Here the fragility was significantly increased on day 6 and on days 7, 8 and 9 there was a significant decrease in fragility.

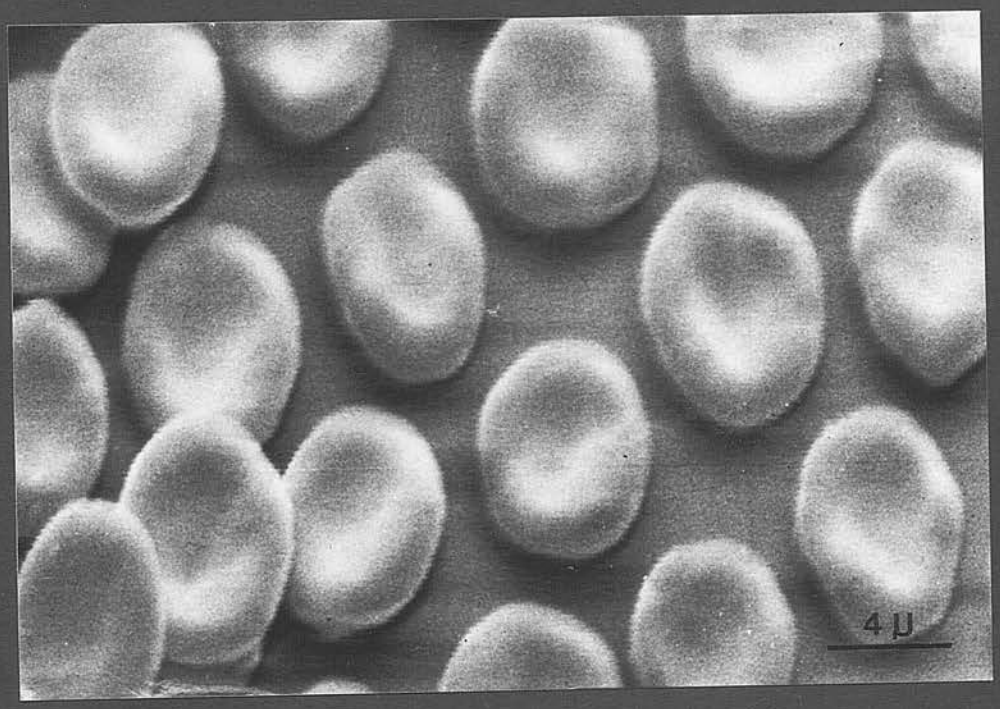
The daily mean MCF values for the infected calves are presented in Table 5.4. together with the percentage parasitaemia for the group and the number of animals whose fragility was tested on that day. The MCF increased from day 8 onwards reaching a maximum on day 12, the day of peak parasitaemia, after which it gradually declined, resuming normal values by the end of the experiment. In order to ascertain if, and when, this increase in mean MCF was significant a single classification analysis of variance, presented in Table 5.5, was performed. There is a significant difference in the MCF values recorded on different days ($F^{14}_{79} = 7.1$; $p < 0.001$). The least significant difference (LSD) was calculated taking the number of observations per day = 6.0 (the harmonic mean). With a significance level of 5% the LSD was 0.0242 indicating that mean MCF on days 10 to 14 inclusive was significantly higher (i) than on the first three days post infection and (ii) than the final level on day 35.

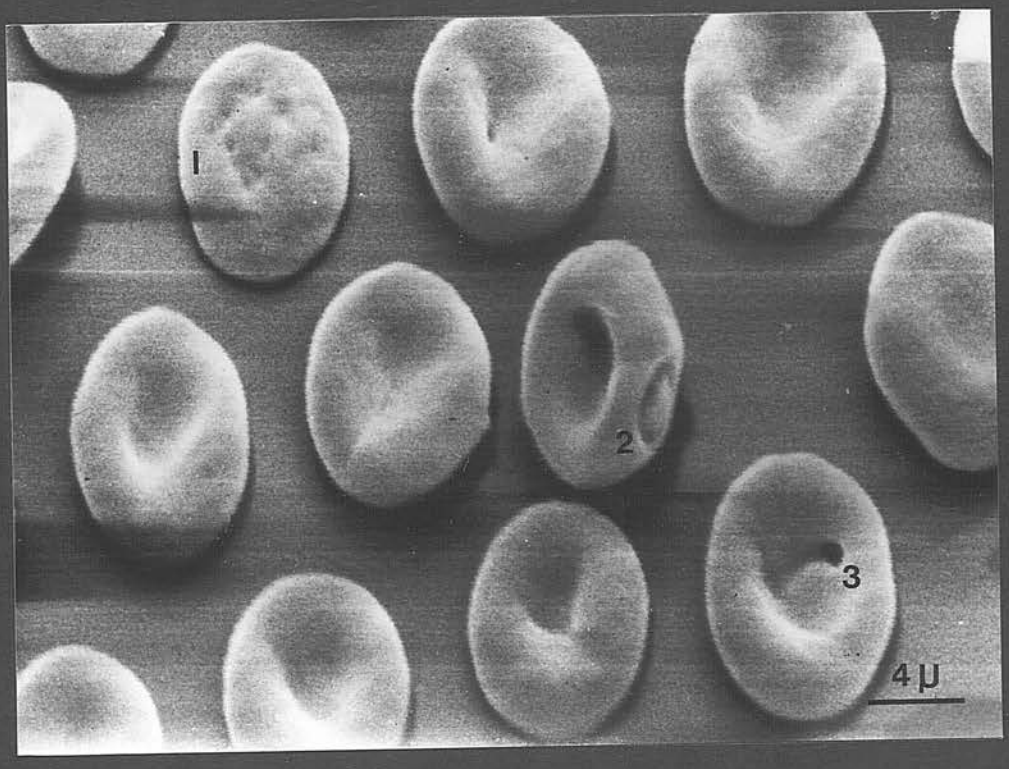
Experiment 5.2 : Scanning Electron Microscopic Examination of Normal and *B.rodhaini*-infected Rat Blood

These studies revealed considerable changes in the normal erythrocyte morphology (Fig. 5.1) which became progressively more pronounced and varied with increasing anaemia. The morphology remained greatly changed into recovery. From early in parasite development three main types of defect were seen (Fig. 5.2). These consisted of an irregular distribution of small undulations over a varying area of membrane (Type 1), a more local defect usually with a pronounced folding of the membrane

Fig. 5.1. **Scanning electron micrograph of normal rat erythrocytes**

Fig. 5.2. **Scanning electron micrograph of B.rodhaini-infected rat blood showing defects designated Type 1, 2, and 3 (55% parasitaemia).**


4 µ


1
2
3
4 µ

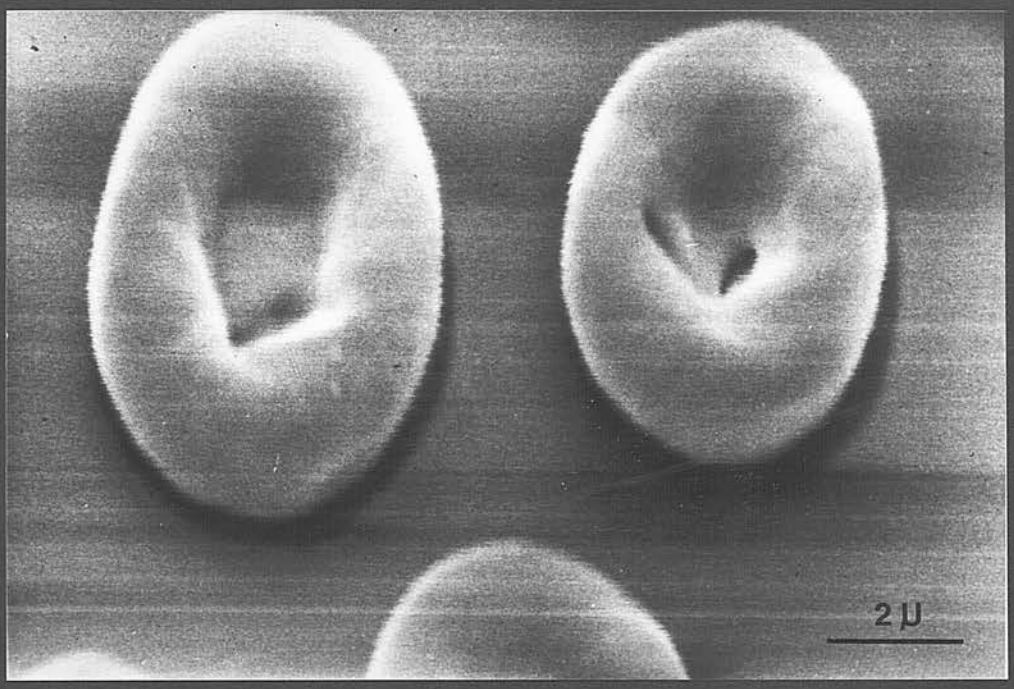
(Type 2) and a sharply defined defect often rounded and apparently penetrating the depth of the surface membrane (Type 3). The base of this latter defect was fairly smooth and appeared capable of maintaining the integrity of the erythrocyte (Fig. 5.3). As the anaemia increased the frequency of these defects increased, Type 3 defects becoming more common. As the erythrocyte count fell and haemoglobinaemia and haemoglobinuria became apparent a general loss of the smooth biconcavity of erythrocytes was seen, some cells showing a more sharply defined biconcavity while others were more spherical (Fig. 5.4).

Type 1 defects affected varying areas of surface membrane and caused a reduction or loss of the biconcavity of the erythrocyte (Fig. 5.2). Type 2 defects, when towards the periphery of the erythrocyte, caused only a local deformation (Fig. 5.5), but when on the centre of the cell they also disturbed the biconcavity (Fig. 5.6). Both these defects could be correlated with the presence of parasites within the cell by light microscopic examination (Fig. 5.7). It is most likely that these defects, while appearing somewhat different on the surface of the cell are essentially the same defect, but reflect a different degree of parasite-membrane contact. The third type of defect was not readily associated with the parasite, for it was recognized on both parasitized and non-parasitized erythrocytes.

The blood from animals with very high parasitaemia and marked anaemia had a grossly mis-shapen erythrocyte population composed, not only of cells with the three types of defect outlined, but of many bizarre shapes and considerable anisocytosis (Fig. 5.8). The response of these rats to infection resulted in the release of a large number of normoblasts and reticulocytes which added to the morphological

Fig. 5.3. Scanning electron micrograph of B.rodhaini-infected rat blood showing one erythrocyte with a marked increase in concavity (left) and another with Type 3 defects (64% parasitaemia).

Fig. 5.4. Scanning electron micrograph of B.rodhaini-infected rat blood showing anisocytosis, spherocytosis, and small Type 3 defects (58% parasitaemia).


2 µ

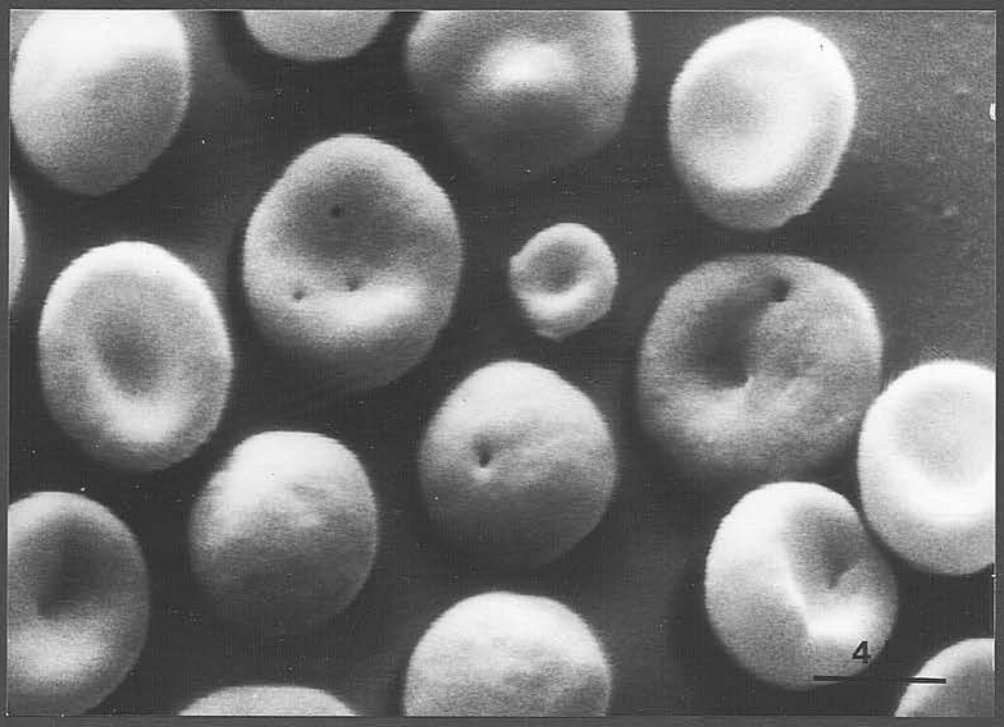
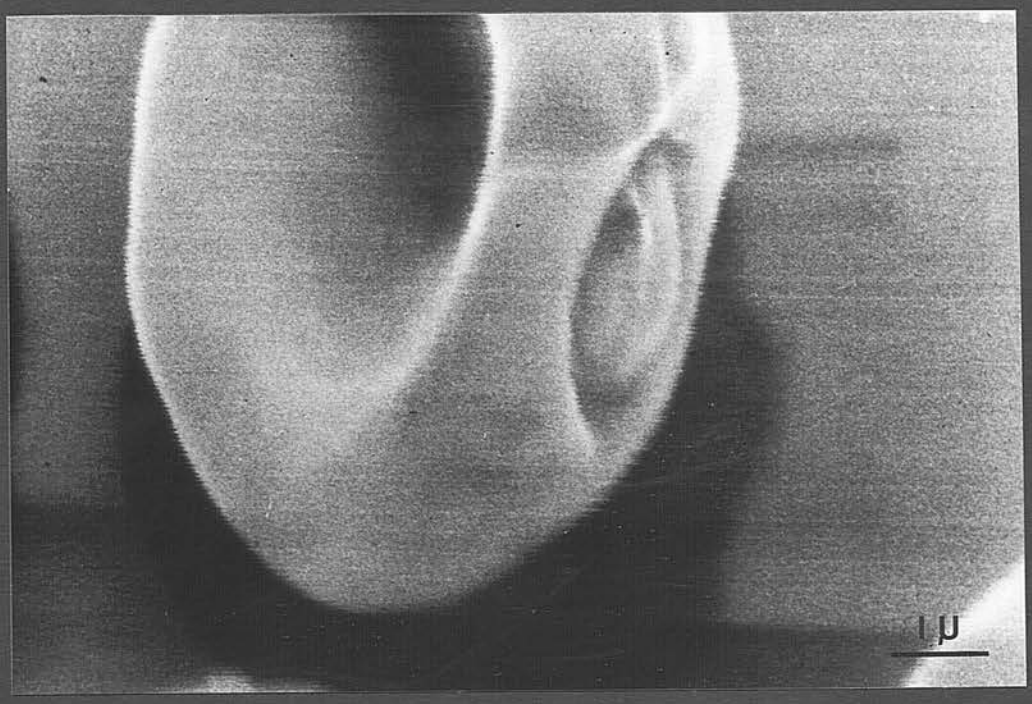

4

Fig. 5.5. Scanning electron micrograph of an erythrocyte showing a Type 2 defect at the periphery of the cell (from Fig. 5.2).

Fig. 5.6. Scanning electron micrograph of an erythrocyte showing a Type 2 defect disturbing the biconcavity of the cell (21% *B.rodhaini* parasitaemia).

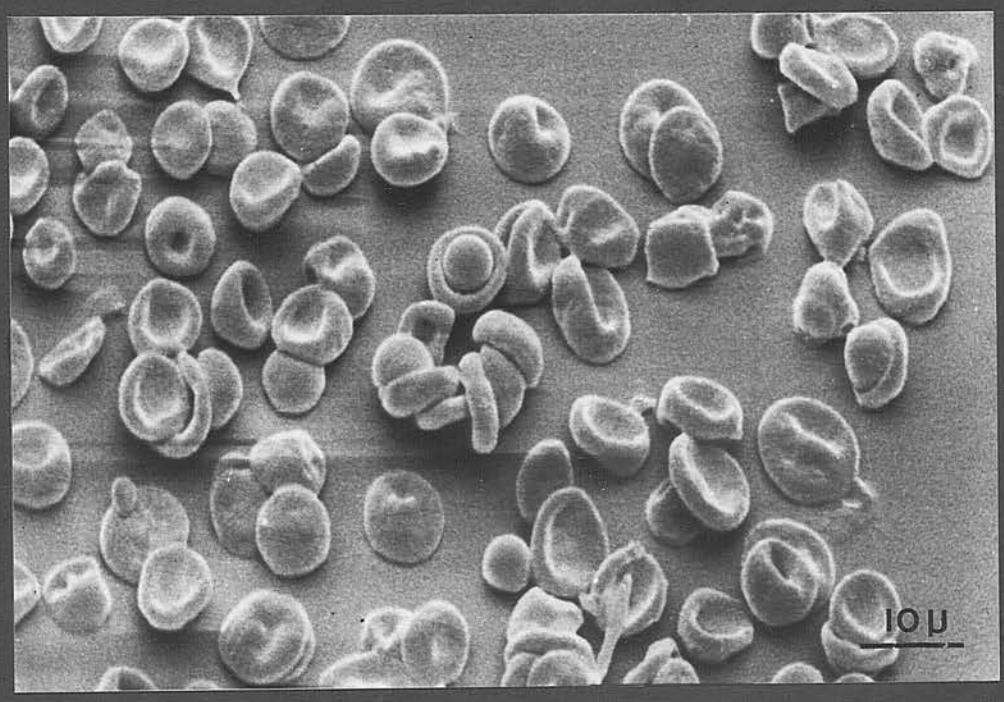

1 μ


1 μ

Fig. 5.7. Light and scanning electron micrographs of the same field of B.rodhaini-infected blood fixed in glutaraldehyde, dehydrated and then stained with Giemsa's stain and examined by light microscopy. The blood preparation was then coated with gold and palladium and examined by SEM. The arrows indicate cells in which B.rodhaini parasites were identified in the light microscope and which show Type 2 defects in the SEM.

10μ

Fig. 5.8. Scanning electron micrograph of rat blood during recovery from B.rodhaini infection showing the variety of mis-shapen cells.


10 µ

variety. Blood examined in early recovery showed many of these morphological changes including cup-shaped cells, microspherocytes, anisocytosis and general loss of the biconcave shape.

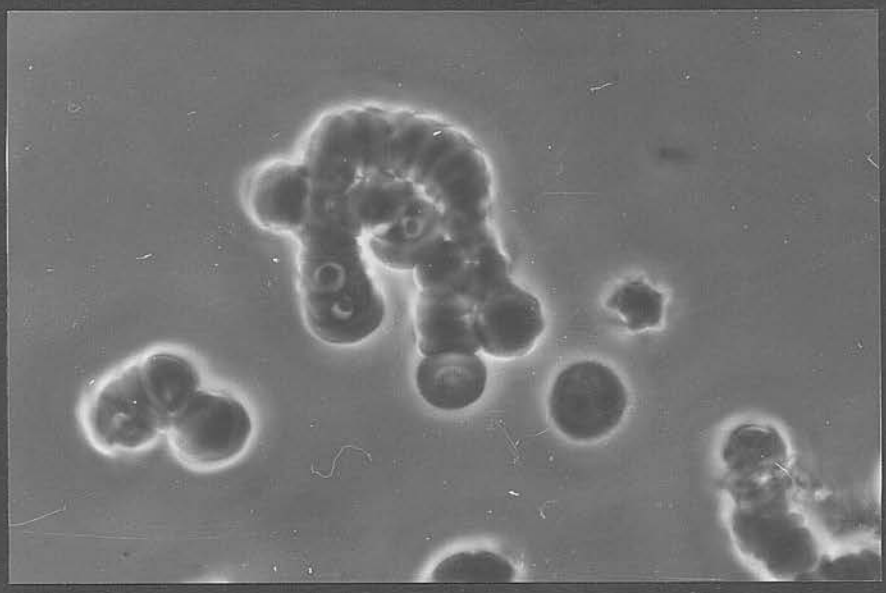
Experiment 5.3 : Living Parasite Preparations

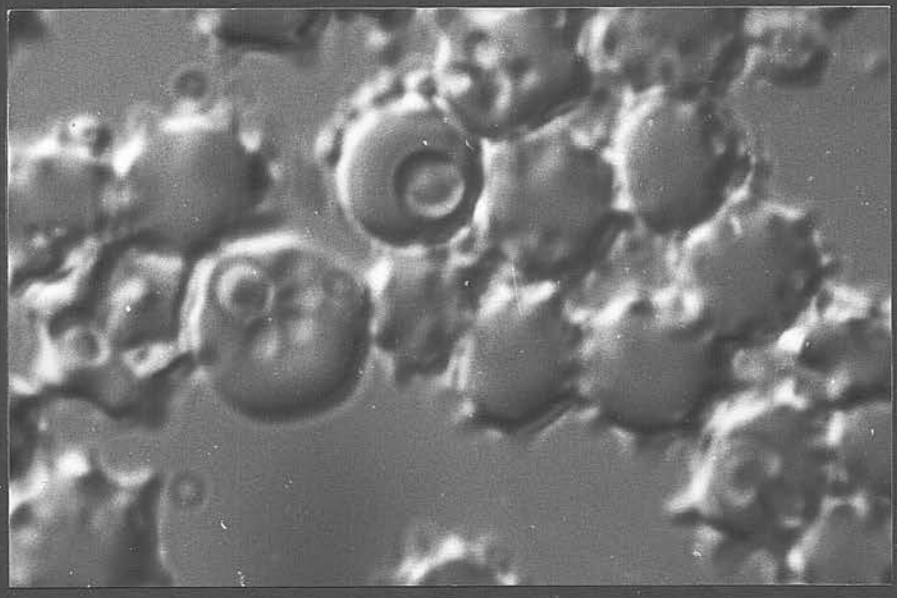
(i) B.rodhaini in living preparations. The parasite was readily recognizable within erythrocytes because of its size and vigorous activity. It did not show any preference for a particular location in the cell but appeared to maintain its basic position once located. With higher parasitaemias it was common to find more than one active parasite within a cell. Rouleaux formations were a feature of rat erythrocytes and parasitized cells were frequently part of these formations (Fig. 5.9). Because of the side-on view of cells in rouleau little of the parasite activity in these particular cells could be observed. However, parasitized erythrocytes free from rouleau were very common, usually lying flat and moving gently as the blood channelled on the slide. This was probably due to changing temperatures in the preparation.

The outstanding features of B.rodhaini were the vigour of its movement and the apparent limit to two or four pseudopodia (Fig. 5.10). The parasite continuously extended and retracted long pseudopodia that could reach across the diameter of the cell or, when near the periphery of the cell, travel down the inside of the membrane and appear again extending below the parasite. Occasionally a parasite showing this activity would apparently rotate within the cell, but this could have been due to the pseudopodia being extended from further around the parasite body each time, rather than true rotation. When no pseudopodia were extended the parasite had a rounded appearance or consisted of a

Fig. 5.9. Phase contrast micrograph of B.rodhaini-infected rat blood showing parasitized erythrocytes in rouleau (x1100).

Fig. 5.10. Nomarski interference phase micrograph of B.rodhaini-infected rat blood. The parasites appear to be in a depression in the erythrocytes, and the parasitized cell to the left of centre shows four parasite pseudopodia (x1500)





squared shape composed of four blunt pseudopodia. During all this activity there always appeared to be a parasite body, however small, from which the pseudopodia were extended. When a very long pseudopodium was seen, it seemed to be at the expense of the other pseudopodia on that parasite at that time.

Although extension of either two or four pseudopodia was the usual pattern shown by these parasites, very occasionally five or six were seen. It was possible that other pseudopodia were extended below the parasite and were not observed, but this seems unlikely. The pseudopodia did not appear to engulf portions of host cell cytoplasm but simply extended and retracted.

There was a tendency for infected cells to lyse; the former full and rounded appearance of the cell was lost and the membrane became slack and wrinkled, although the cell retained a rounded outline. Parasites within such cells remained active and did not leave these cells.

On one occasion a small condensed body, probably the earliest form of the parasite, was observed. This body was less than 1 μ in diameter and corresponded to a dense red body with a very slight blue halo of cytoplasm seen in films stained with Giemsa stain. This 'anaplasmoid' form of the parasite was observed to enlarge and assume an annular appearance and it eventually showed slight pseudopodial activity. This entire process occurred over a period of 45 minutes.

Phase contrast observation permitted recognition of the nuclear material of the parasite. It could be seen to flow within the parasite body and extend into the pseudopodia. But despite many hours spent in observation no reproductive activity was clearly identified,

nor were parasites seen to enter or leave the erythrocytes although occasional free parasites were observed.

The Nomarski interference phase system presents an apparently transparent three dimensional view of the blood preparation. From this it appeared that the parasite caused the surface membrane to deform in the region of the parasite (Fig. 5.10). Apart from this point it yielded no further information than the conventional phase system.

There was considerable variation between preparations despite the standard method of preparation. Some preparations crenated almost immediately while others remained normal in appearance and showed parasite activity for 90 minutes or more. In good preparations parasite activity became reduced after one hour and the parasite rounded up. The range of activities of parasites observed in living preparations was not fully represented in stained films. The very long pseudopodia were not well demonstrated for it would appear that the spreading and drying of the thin blood film causes some retraction of the pseudopodia.

(ii) B.divergens in living preparations. In contrast to B.rodhaini, B.divergens was small, usually rounded and almost invariably located against the erythrocyte membrane at the periphery of the cell. There it constantly pushed at the membrane, forcing it to bulge outwards and then return to its former round outline only to be pushed outwards again. The bulging surface was considerable and large enough to present almost the total parasite body outside the normal erythrocyte outline. That portion of the parasite facing inwards, towards the cytoplasm of the erythrocyte, was composed of numerous tiny pseudopodia

that moved continuously. There were no long pseudopodia and the parasite seemed to stay in the location in which it was first seen.

Blood from calf A89 showed very marked rouleauxformation unlike the samples examined from the other calves. The parasite activity was similar in all calves but in the blood of A89 parasitized erythrocytes did not appear to form part of the rouleaux. Occasionally, parasitized cells were seen in small groups free from non-parasitized cells. Parasite reproduction or entry and exodus from erythrocytes was not observed. In general cattle blood was less susceptible to crenation during preparation and B.divergens tended to retain its activity longer than B.rodhaini.

Experiment 5.4 : Separation of B.rodhaini-infected Erythrocytes

Efforts to separate B.rodhaini infected cells by the methods adopted were uniformly unsuccessful.

(i) In Table 5.6 the parasitaemias estimated from the edge, middle and tip of the thin blood films are compared. A tendency for the

Table 5.6

Parasitaemia in different areas of thin blood films

Rat	Mid-film	Edge	Tip
1	6.4	6.9	8.5
2	6.1	7.5	7.9
3	5.2	5.4	5.7
4	3.9	2.8	5.5
5	2.9	4.1	3.7
6	1.5	1.0	1.3
7	1.9	2.2	1.3
8	6.9	7.4	7.3

parasitaemia to be higher at the edge and tip was noted in some films but the erythrocytes at the edge or tip of the film were often pushed together and sometimes damaged making counting difficult. Low parasitaemias were used as it was found that with high parasitaemia the cells were more fragile and counting with accuracy became impossible. It is clear from the table that there was no consistent separation of parasitized cells by this method.

(ii) In the sucrose gradient blood was separated into four main bands. The top 10% of the gradient showed haemolysis with little cellular debris. About half way down the gradient there was a thick band of cells which was followed by a diffuse band and finally a solid pellet of cells at the bottom of the tube. Damage to erythrocytes was considerable; it was possible to recognize lysed cells, free parasites and many crenated cells. Cells in the diffuse band in the lower half of the gradient were in the best condition. The damage to cells made parasitaemia estimation difficult, however, parasitized cells were present in all but the top 10%, the layer of haemolysis. The parasitaemia levels from two samples in which all layers were counted are shown in Table 5.7.

Table 5.7
Distribution of parasitaemia in sucrose gradients

Position in Gradient	Parasitaemia %	
	Sample 1	Sample 2
Thick Band	47.0	46.0
Diffuse Band	54.0	46.0
Pellet	49.0	45.0

(iii) Ultracentrifugation failed to show any separation of parasitized erythrocytes. Thin blood films made from several different levels of the packed red cell columns all contained parasites and parasitized cells in similar numbers.

IV. Discussion

The erythrocyte is a biconcave disc in most mammals and its major role is the transport of oxygen to the tissues of the body. This main function is accomplished by the avid but weak union formed between the haemoglobin molecule of the erythrocyte cytoplasm and oxygen. The characteristic shape of the erythrocyte is maintained by the molecular configuration of haemoglobin and the unique structure of the cell membrane (Ham, 1969; Bull, 1973; Bull and Brailsford, 1973). This shape provides for a large surface to volume ratio, which, together with the short distance from the surface to any point within the cell, allows for the rapid exchange of gases. The absence of a nucleus increases the carrying capacity of the cell per unit volume. The rounded edges protect the cell from injury in the circulation and the membrane, which is highly flexible but essentially inelastic (Schalm, 1965; Bull, 1973), along with the shape of the cell and the gel-like nature of the cytoplasm, permit the deformation necessary for the cell to pass through the microcirculation. The normal function of tissue oxygenation by the blood is dependent upon an adequate number of erythrocytes and their functional integrity.

The erythrocyte population during both infections showed a significant increase in osmotic fragility which corresponded with maximum parasitaemia. The increase was similar to that found during

B.bovis (Yakimoff and Weizekhowsky, 1926), B.bigemina (Wright, 1974a) and B.canis (Shirlaw, 1939) infections although conflicting findings have been reported from studies of B.canis (Maegraith et al., 1957; Dorner, 1967). Normal erythrocytes exposed to increasing concentrations of hypotonic saline take in water and swell. However, there is a critical limit beyond which they rupture (Ponder, 1961). The swelling of erythrocytes is at the expense of their biconcavity and, in general, cells with a greater biconcavity have a greater resistance to lysis. An increase in osmotic fragility then is associated with change in shape, the cells being more spherical (Castle and Daland, 1937). The factors that affect the fragility of erythrocytes include age, size, volume and form, membrane thickness, haemoglobin type and content, differences in viscoelastic properties of the membrane and the membrane structure and chemical composition (Perk et al., 1964). It has been postulated by Wright (1974a) that changes in the lipoproteins of the membrane may result in the increase in fragility of bovine erythrocytes during Babesia infection but this has not been tested experimentally. It was observed by Holbrook et al. (1968) that in living preparations, parasitized cells tended to lyse easily and a similar observation was made in these studies of B.rodhaini. Kreier et al. (1972), commenting upon the large membrane defects during malaria infections, thought that such defects would increase the fragility of erythrocytes. The findings in this study of similar membrane alterations and an increased fragility would support their suggestion.

During recovery the osmotic fragility of the cattle blood returned to within the normal range while the rat blood showed a marked decrease in fragility. This probably reflects a basic difference in kinetics

of haemopoiesis between the two species. The rat has an erythrocyte life span of around 60 days (Schalm, 1965) and its bone marrow is in near maximum production at all times because of the erythrocytic turnover. Cattle, on the other hand, have a very long erythrocyte life span of about 150 days and they are slow to respond to demand for new cells both in stem cell proliferation and in switching from myelopoiesis to erythropoiesis (Valli et al., 1971). Furthermore young cells are more resistant to lysis in hypotonic saline (Prankerd, 1961; Perk et al., 1964). Thus the rat with a massive and quick release of young cells would have a totally changed population of cells within a few days, which would also be reflected in morphological changes, while the calf, with a slower response and with a longer erythrocyte life span would not show the same change. This was borne out by the reticulocyte responses and the values recorded for mean cell volume in both infections reported in Ch. 4.

Alterations in the surface morphology of erythrocytes observed with the scanning electron microscope (SEM) became progressively more frequent during the course of B.rodhaini infections. Many of these defects resembled defects observed during the course of malaria infections (Arnold et al., 1969; Kreier et al., 1972; Balcerzak et al., 1972). Defects of the types designated (1) and (2) were present when examined by light microscopy on cells that contained parasites. Studies of living B.rodhaini preparations showed that despite the vigour of its activity the parasite tended to occupy a fixed location within the cell from which pseudopodia were extended, as if the parasite was anchored in some way. Later studies with the transmission electron microscope (TEM) (Dolan and Carr, 1974) showed

that the parasite within the cell was located below, and in close contact with, the area of membrane defect (Fig. 5.11).

Living preparations of B.divergens in cattle blood demonstrated the unusual activity of the parasite pushing restlessly against the membrane of the cell from within. Fixed stained films, when examined closely, showed that the parasite, most often placed at the periphery of the cell, caused the membrane to bulge outwards. Observations by Friedhoff (1974) from both SEM and TEM studies of this parasite, unfortunately without illustrations, described a roughened membrane protrusion on the surface of the cell, with the parasite lying immediately beneath the membrane in a pocket-like bulge. This description would agree with the observations on living preparations reported in this study and a small study by TEM (Dolan and Carr, unpublished) which demonstrated the peripheral position of the parasite and its many pseudopodia (Fig. 5.12).

The finding of an intimate relationship between both these parasites and their host cell membranes would suggest that there was a purpose in this relationship. A similarly close membrane relationship has been observed for P.berghei in mouse erythrocytes (Bodammer and Bahr, 1973). These authors have proposed a most interesting hypothesis for the relationship. It is based upon observations of Gutteridge and Trigg (1970) and others that malarial parasites have nutritional requirements during development which are not normally available within the erythrocyte. Bodammer and Bahr (1973) suggest that the parasite induces a change in membrane permeability of the erythrocyte at the site of 'contact' with the parasite. This change allows the plasma substances to cross the membrane barrier and become available

Fig. 5.11. **Transmission electron micrograph of B.rodhaini-infected rat blood showing the frequent close relationship between the parasite and the erythrocyte membrane, and the large accumulations of erythrocytic material within some parasites (Dolan and Carr, 1974).**

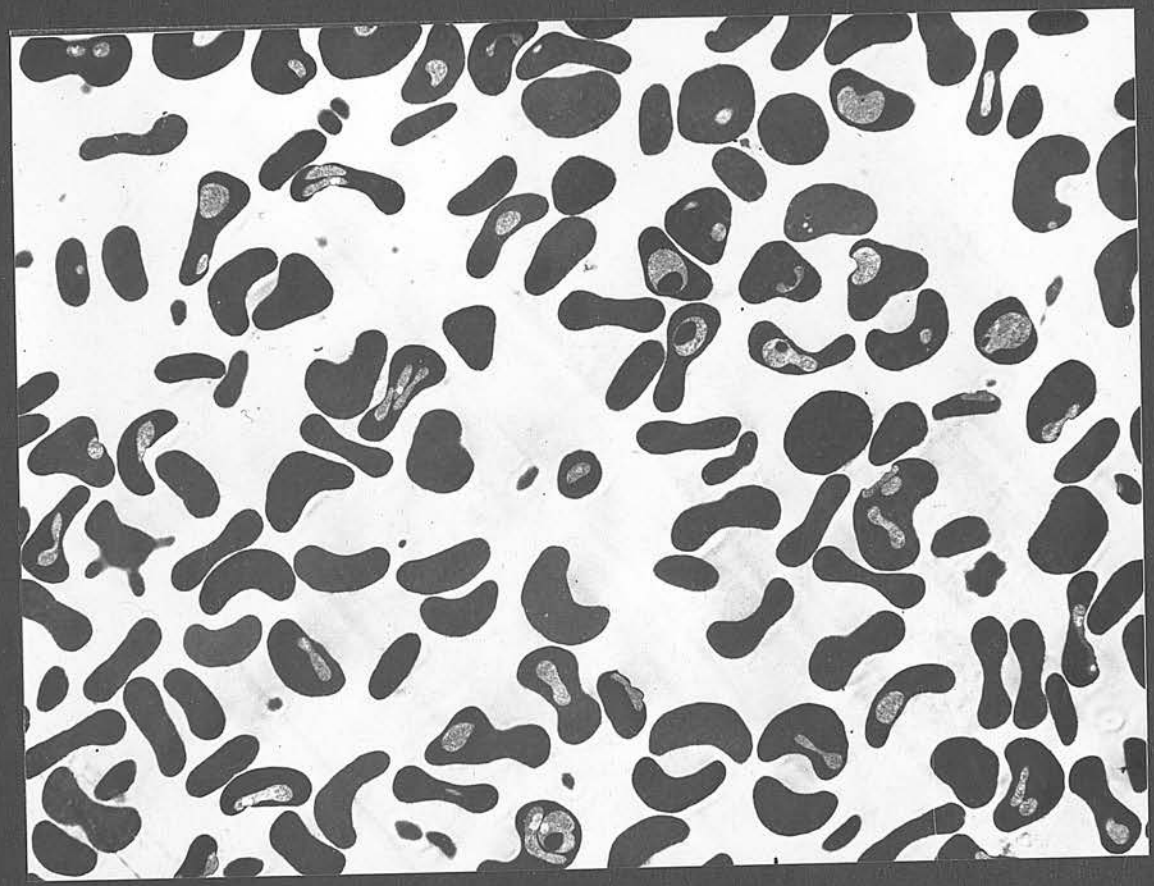
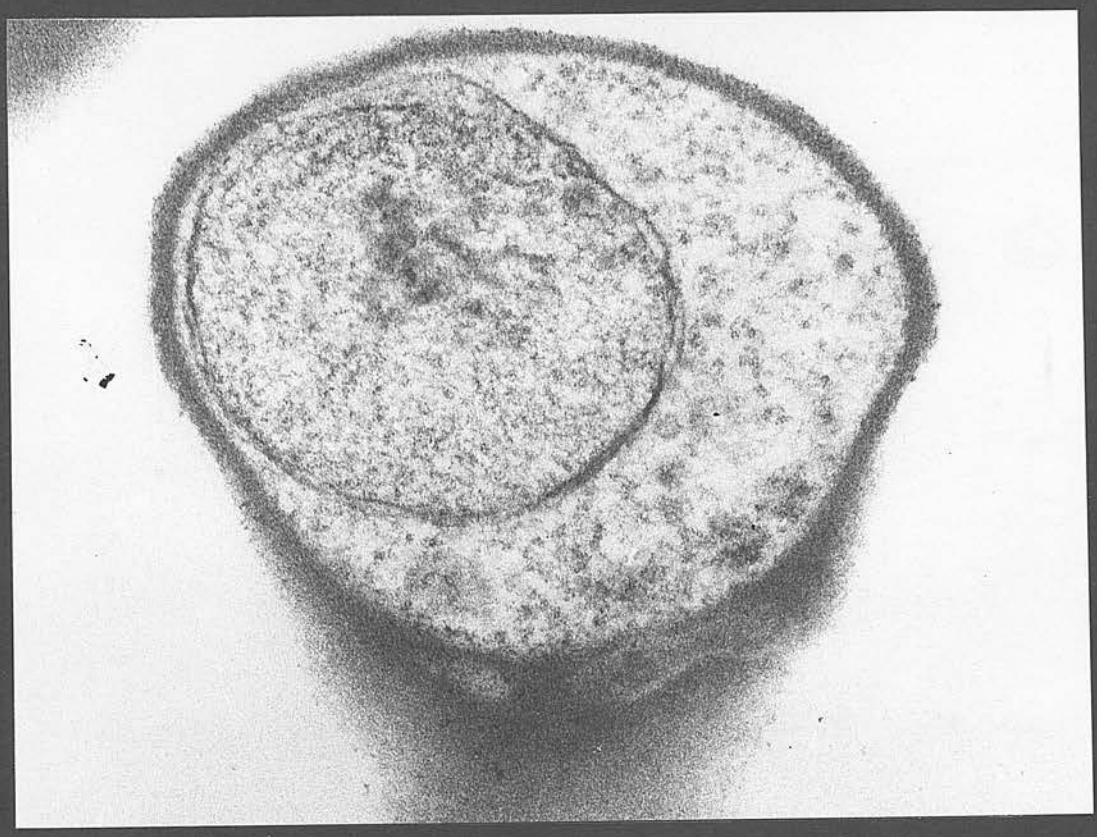
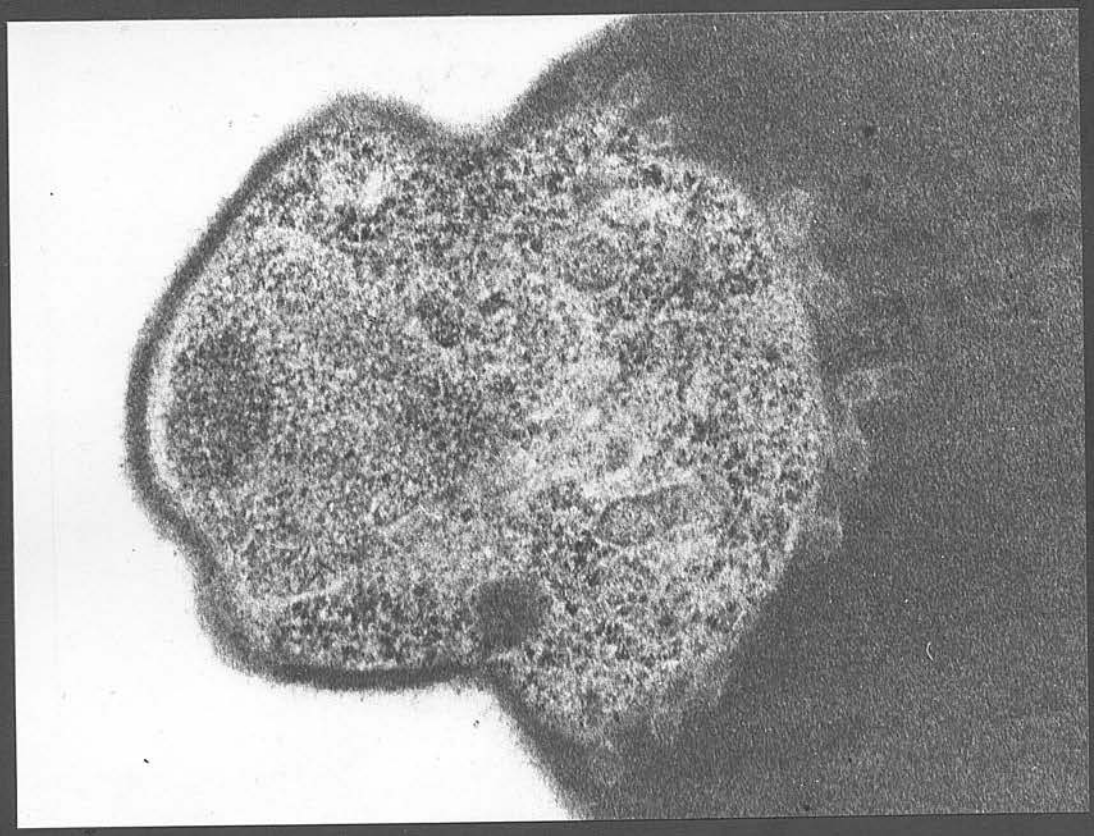


Fig. 5.12. Transmission electron micrographs of B.divergens-infected erythrocytes showing the very close relationship between the parasite and the erythrocyte membrane and the many small pseudopodia facing into the erythrocyte cytoplasm (Dolan and Carr, unpublished).





to the parasite. They also suggest that the parasite could dispose of metabolites in the same way. Further support for this hypothesis would seem to come from the work of Weidekamm et al. (1973) who have correlated surface morphological changes, again during P.berghei infection, with changes in the membrane proteins. The normal morphology of the erythrocyte membrane is dependent upon the integrity of the component proteins and when these are altered the surface morphology is altered. No biochemical studies have been carried out that might support this hypothesis for Babesia organisms. However, the physical relationships of the parasites and the host cell membrane are very similar, as are the membrane defects. It has been reported by Rickard (1970a) that the membrane of B.rodhaini is much more permeable to many carbohydrates than the erythrocyte membrane but similar studies of proteins have not been undertaken.

The defects designated Type 3 were found on both parasitized and non-parasitized cells. This type of defect was quite different from other observed changes in that the membrane surface was penetrated or so drastically altered that it appeared to be penetrated. Yet the cells with this defect remained intact. The base of this defect seemed smooth and did not show the textured base which Arnold et al. (1969) thought to be exteriorized malarial parasites. Nor did it show the protrusions, also observed in malarial studies, which Bodammer and Bahr (1973) thought to be excretory products of the parasite. It could be argued that such defects represent an area of poorly fixed membrane surface that had lifted off during processing but such defects were never seen on normal erythrocytes fixed by the same technique at the same time. It has also been demonstrated in

malarial studies that the erythrocyte is antigenically intact over similar defects (Lo Buglio et al., 1972).

It is possible that these Type 3 defects could represent the scar formed following pitting of the parasite from the cell by the reticulo-endothelial system (RES). Such a mechanism has been proposed by Arnold et al. (1969). However, normal erythrocytes are nucleated during their early development and the nucleus is lost as the cell matures, usually by splenic pitting. In a similar way inclusions, such as siderocytes, are removed from erythrocytes and the cells returned to the circulation (Crosby, 1957, 1959). Yet these cells are smoothly biconcave. If the theory of membrane alteration is valid then the damaged membrane might not be capable of forming the same smooth union seen following pitting from an erythrocyte with a normal membrane.

Another possible source of this type of defect could be that the parasite leaves the erythrocyte without lysis; as suggested for B.equi and B.caballi (Holbrook et al., 1968) and for B.canis (Simpson, 1973). It has been found that when splenectomy has been performed (Schnitzer et al., 1973), or when a hyposplenic state exists as in the newborn (Preston and Shanani, 1970), pits, craters and other defects are found on erythrocytes examined by TEM or SEM. These occur because the erythrocytes extrude inclusions, such as siderocytes or Heinz bodies, and a defect is left, whereas with normal splenic function these bodies are removed and the surface is smoothly closed. The parasite exodus from the cell would resemble extrusion of erythrocytic inclusions in hyposplenic or splenectomized states, and so possibly cause such defects.

The effects of malaria parasite penetration on the erythrocyte membrane has led to a fundamental difference of opinion between Arnold et al. (1973), who hold that the parasite leaves a permanent defect, and Kreier et al. (1972) who hold that it does not. In support of their opinion Kreier et al. (1972) cite the TEM studies of Ladda et al. (1969) which showed the complete closure of the erythrocyte membrane following entry. Simpson (1973) reported the entry of B.canis into erythrocytes and his findings would seem to resemble those of Ladda et al. (1969). From the present study the frequency of Type 1 and 2 defects would support the suggestion of complete closure of the penetration site with no permanent defect.

The three types of defect discussed so far can be related directly to the parasite. Parasitized cells will lose an area of functional surface membrane and the living parasite feeding on the host cell cytoplasm will reduce the available haemoglobin. These erythrocytes will be less efficient because of reduced oxygen carrying capacity and loss of functional membrane surface for gaseous exchange. The parasite or damaged surface membrane may be pitted from the cell by the RES reducing the size of the cell and changing its shape. The defects described probably render the cell less deformable, consequently individual cells will pass with difficulty through the microcirculation. This, however, will be less important as the anaemia increases because the falling packed cell volume will decrease the viscosity of the blood (Weed, 1970). If the theory of altered membrane permeability is valid, and exchange between parasite and plasma occurs across the membrane, this will provide a site for host antibody to act and for parasite antigen to be released (Bodammer and Bahr, 1973). The parasitized

cell could be opsonized for phagocytosis, the antibody might directly attack the parasite, or the released antigen might coat normal erythrocytes which could then be opsonized for phagocytosis.

The morphology of the erythrocyte is very susceptible to change in its environment (Bessis and Weed, 1972; Bessis et al., 1973; Murphy, 1973). During the course of B.rodhaini infection the plasma environment undergoes considerable change. Initially, these changes result from lysis of erythrocytes and the release of parasite products into the circulation. Later as the anaemia becomes more severe and the liver and kidney begin to show signs of failure there is a build up of many products leading to a serious deterioration of the plasma. Thus erythrocytes which are not already showing direct parasite damage begin to show changes resulting from their changed environment.

Finally, the release of many young and immature erythrocytes will add to the changing morphology of the erythrocyte population. For young erythrocytes are not only larger than mature cells but reticulocytes and normoblasts have a roughened surface appearance (Preston and Shahani, 1970). Cells produced under stress are frequently defective (Berlin, 1964) and it is most likely that this would add to the variety of changes observed.

Studies of living preparations demonstrated considerable differences between B.rodhaini and B.divergens. B.rodhaini was large, with actively extended long pseudopodia and was found anywhere in the cell but once located did not appear to change position. B.divergens on the other hand was small, with a frill of tiny pseudopodia extending into the cytoplasm of the erythrocyte (Fig. 5.12) while the other side of the parasite was pressed against the membrane and repeatedly forced

it to bulge outwards. Neither of the parasites was seen to engulf host cell cytoplasm but TEM studies of B.rodhaini have shown large accumulations of what is presumed to be erythrocyte cytoplasm within the parasite (Fig. 5.11) (Rudzinska and Trager, 1962; Dolan and Carr, 1974). This is borne out in fixed stained films where free annular forms of the parasite contain a large central vacuole which stains identically with erythrocytes. Free B.divergens parasites are very rare in thin blood films and accumulations of erythrocyte material was not observed within them in this study. However, TEM observations do show small accumulations of what looks like erythrocyte cytoplasm throughout the parasite cytoplasm (Dolan and Carr, unpublished). It was not possible to decide whether the feeding process was by engulfing portions of erythrocyte cytoplasm or by assimilation, but this latter process appeared more likely for B.divergens. The relationship of both parasites to the membrane of the erythrocyte could well represent a requirement for plasma nutrients but this point can only be clarified by extensive biochemical studies.

The physical activity of the parasites, apart from either their physical or chemical distortion of the cell membrane, is unlikely to interfere with the erythrocyte except where their bulk does not permit them to pass through the narrow slits of the splenic sinusoidal walls. The movement of the pseudopodia will not damage the cell because it has no architectural structure but is in nature a gel and undergoes far greater disturbances in its passage through the micro-circulation.

The fact that the parasite remained within the lysed erythrocyte without showing any urgency about leaving the cell might be a

reflection of the lack of blood flow. In the circulation the lysed cell could be presumed to disintegrate and free the parasite. The parasite could then find another host cell but whether the parasite can invade an erythrocyte at all stages of its development is not known.

The anaplasmoid form of the parasite recognized in both living and fixed stained preparations is similar to that described by Holbrook et al. (1968) for B.caballi and B.equi, for B.gibsoni (Fowler et al., 1970) and presumed by Mahoney (1972) to be an essential Babesia form.

The appearance of B.rodhaini, apparently in a depression in infected erythrocytes, when examined by the Nomarski interference system, is inconsistent with the other findings in this investigation and is probably an artefact. Structures with a different optical density, such as the parasite and the erythrocyte appear in a different observational plane with this system, so the parasite's apparent position in a depression was an optical effect and not a true representation of the host-parasite relationship. A similar effect was observed when malarial parasites were examined by this system (Kreier et al., 1972).

The observation by Holbrook et al. (1968) that erythrocytes parasitized by B.equi and B.caballi were isolated from rouleau and seemed attracted to other parasitized erythrocytes could indicate a change in cell charge. A similar isolation was observed of B.divergens parasitized erythrocytes but not of B.rodhaini-parasitized cells. If this isolation is due to changes in the surface charge of infected erythrocytes then ion-exchange chromatography should separate

these cells. This would confirm the suggestion of change of cell charge and provide a method of cell separation that could be useful for antigen preparation.

Attempts to separate B.rodhaini infected erythrocytes from non-parasitized cells by methods relying upon density changes failed. This is a common finding for Babesia infected cells (Mahoney, 1972), although B.caballi and B.canis-infected erythrocytes can be separated by centrifugation (Watkins, 1962; Hirsh et al., 1969). The distribution of B.canis and B.bigemina infected erythrocytes towards the edge and tail of the thin blood films (Hirsh et al., 1969; Barnett, 1965) was not found to occur for B.rodhaini. Dacie and Lewis (1968), in discussing the distribution of white blood cells in thin blood films, attributed their positions to factors such as stickiness, size or specific gravity. Whether these are the factors that account for the distribution of B.canis or B.bigemina is not known but this parameter at least indicates, crudely, alterations of erythrocytes parasitized by some babesias.

During the course of B.rodhaini and B.divergens infections the erythrocytes not destroyed by emerging parasites undergo changes that seriously compromise their efficiency. The parasites cause morphological alterations in the surface of erythrocytes that are related either directly to the parasite or the response of the RES to the presence of the parasite. These membrane defects will predispose to lysis of the cell either mechanically in the circulation or by sequestration in the spleen. They may also mark the cell for removal by the RES. The cells are likely to be less deformable, they also lose an area of membrane surface and the parasite digests the

haemoglobin essential in oxygen transport. As the disease progresses a general deterioration of erythrocytes is manifest by the increasing osmotic fragility and the grossly changed morphology of the cells. Damaged cells and new cells produced under stress will have a shortened life span. Thus the anaemia of babesiosis must be measured not only by the loss of erythrocytes and the falling PCV and haemoglobin values, but also by the reduced efficiency of the surviving erythrocytes and their shortened life span.

CHAPTER 6

THE SERUM BIOCHEMISTRY AND HISTOPATHOLOGY

I. Introduction

The serum biochemical changes and the histopathology of many naturally occurring and experimentally induced *Babesia* infections of domestic animals have been recorded (Smith and Kilborne, 1893; Shirlaw, 1939; Maegraith *et al.*, 1957; Malherbe, 1966; Wright, 1972a; Fowler *et al.*, 1972). The pathology of *B.rodhaini* infection in mice has also been reported (Paget *et al.*, 1962), while pathological changes in rats with the same *Babesia* infection have been recorded during investigations of other aspects of the disease (Matson, 1964; Nowell, 1968). The disease caused by *B.divergens* has been the subject of few investigations and knowledge of its pathology is poor.

Generalizations about the anaemia of babesiosis and the consequent tissue changes might be justified. Nevertheless, it is clear that some manifestations of the disease, such as symptoms of cerebral babesiosis occurring during *B.argentina* infections (Wright, 1972b), have underlying mechanisms not found in all *Babesia* infections. Similarly, the variety of atypical symptoms observed in *B.canis* infections (Malherbe and Parkin, 1961) can hardly be explained by erythrocyte destruction alone. The possibility that immune-based glomerulonephritis, described in *B.rodhaini* infections (Iturri and Cox, 1969; Annable and Ward, 1974), occurs in babesiosis of domestic animals has yet to be explored. It might cause a similar form of tissue damage in these infections or a comparable immune complex pathogenesis might underlie many of the unusual symptoms of *B.canis* infections. While it is certain that the

Babesia parasite initiates the disease process and that anaemia is the cardinal sign of the disease, different host-parasite systems of babesiosis have different pathogenic mechanisms and to define the disease simply as a haemolytic anaemia might be a serious underestimation of its complexity.

The histopathology of the disease can be briefly summarized in the following way. When Smith and Kilborne (1893) examined the pathology of B.bigemina infections and reviewed earlier reports, they concluded that "of all the organs the liver was the most seriously involved". This has been a consistent finding in all Babesia infections since then. The kidney also showed changes but was not as severely damaged as the liver. Cerebral complications have been reported from a number of different Babesia infections (Parant, 1905; Clark, 1918; Callow and McGavin, 1963; Collins et al., 1970). However, it would seem reasonable to accept as valid only those diagnoses where the nervous signs have been related to identifiable histological change. If this criterion is applied, then B.argentina and possibly B.canis are the only two species so far associated with such changes (Wright, 1972b; Reusse, 1954; Maegraith et al., 1957). Clinical reports of diarrhoea accompanying Babesia infections are frequent, but so too are reports of constipation. Dorner (1967) examined the intestines of dogs with B.canis infection and found some haemorrhages and enteritis. Pulmonary oedema has been reported to develop during equine babesiosis (Sippel et al., 1962) and this has also been found in dogs where it occasionally progresses to broncho-pneumonia (Maegraith et al., 1957). Enlargement of the spleen occurs in all Babesia infections while hyperplasia of the reticular cells of the lymph nodes has been reported from

cattle with B.argentina (Rogers, 1971) and hyperplasia of both reticular and lymphoid cells was found in B.canis infections (Dorner, 1969). The adrenal glands were found to show only mild congestion in B.canis infection (Maegraith et al., 1957).

Investigations of the serum biochemistry of animals with babesiosis have yielded somewhat inconsistent results. Total serum proteins are generally little changed while the albumin/globulin ratio is reversed with persistence of raised globulins observed during recovery. In cattle, however, Collins et al. (1970) found total proteins levels raised while Suteu and Giurgea-Iacob (1971) found that they were depressed. Both these studies were based on natural infections. Serum electrolyte levels remained within the normal range during canine babesiosis, but Jerichow and Jungmann (1969) found potassium levels elevated in B.divergens infections while Wright (1972a) found potassium levels reduced in B.argentina and B.bigemina infections. Tests applied to examine the functional state of the liver and kidney have in general given a reliable indication of damage to these organs. But too much emphasis should not be placed upon non-specific tests such as estimations of transaminase levels, where changes in many tissues occur. Bilirubin levels are elevated due to erythrocyte damage and, less frequently, to liver damage.

In this chapter a group of experiments were set up to examine the serum biochemical and histopathological changes of two experimental babesiosis systems and to explore the pathophysiological mechanisms possibly underlying these changes.

II. Materials and Methods

Experiment 6.1 : The Plasma Sodium and Potassium Levels during *B.rodhaini* Infection

This experiment was set up to examine the sodium and potassium levels in the plasma of rats during the acute stages of *B.rodhaini* infection. Twenty four animals were inoculated with 10^7 *B.rodhaini*-infected erythrocytes and 24 normal rats were used as uninfected controls. On days 5, 6, 7 and 9, four test and four uninfected control animals were killed and erythrocyte counts, parasitaemia and plasma sodium and potassium levels estimated.

Experiment 6.2 : The Plasma Proteins during *B.rodhaini* Infection

In this experiment the effects of infection on the total protein and the albumin/globulin ratio of rats were examined. Thirty five rats were inoculated with 10^7 *B.rodhaini* infected erythrocytes and five infected and two uninfected control animals were killed on days 3, 5, 6, 7 and 9. The erythrocyte count, parasitaemia, total serum protein and albumin levels were estimated. As there was little variation between days, or between animals, in the control values, single overall means were estimated for each parameter and used in comparisons with the test results obtained for each day.

Experiment 6.3 : The Plasma Sorbitol Dehydrogenase (SDH) Bilirubin and Urea Levels during *B.rodhaini* Infection

In this experiment the effects of infection upon the plasma levels of urea, SDH and bilirubin were examined. Thirty six rats were infected with 10^7 *B.rodhaini*-infected erythrocytes and on days 2, 4, 5, 6, 7 and 9 four test and two uninfected control animals were killed and the above parameters were measured, together with the erythrocyte

count and the parasitaemia. As before, the test readings for each day were compared with mean control values.

Experiment 6.4 : The Urine of B.rodhaini-infected Rats

The urine from five rats dying from acute B.rodhaini infection was examined for abnormal substances. Following withdrawal from the distended bladder into a sterile syringe, the urine was centrifuged and the supernatant fluid was examined by spectrophotometry (Pye Unicam SP1800 with an attached recorder) from wavelengths of 350 to 800nm. The deposit was spread on a glass slide, stained with Giemsa and examined for erythrocytes, protein casts and cellular debris.

Control urine was obtained by applying digital pressure to the abdomen of eight normal rats and collecting the urine in a sterile syringe. The urine was pooled and examined as above.

Experiment 6.5 : The Plasma Sodium and Potassium Levels during B.divergens Infection

During the course of B.divergens infections in seven splenectomized calves, described in Chapter 4, the sodium and potassium levels were measured on days 3, 6, 8 to 17, 20 and 35. Pre-infection values were established on day -5.

Experiment 6.6 : The Plasma Proteins during B.divergens Infection

From the above seven calves, stored serum from days -5, 4, 7, 9, 11, 13, 15, 17, 24 and 31 was examined for total protein value and the albumin percentage.

Experiment 6.7 : The Plasma SDH, Bilirubin and Urea Levels during B.divergens Infection

From these calves stored plasma was examined for urea, SDH and bilirubin levels. Samples were examined for days -5, 3, 6, 9, 11 to

18 and 35 and additional samples were examined from calf 212 on days 22 and 29.

Experiment 6.8 : Sites of Distribution of the Parasite

The degree of parasitaemia at different sites in the body was examined by the preparation and staining (Giemsa) of thin blood films or tissue smears. The preparations were made from eight rats killed or dying from B.rodhaini infections and from one calf (AS9) dying from B.divergens infection.

Experiment 6.9 : The Histopathology of B.rodhaini-infected Rats

During the course of the experiments reported in this chapter the histopathology of infected rats was examined. Tissues were examined from one test and one control animal on day 3 and from two test and one control animal on days 4 to 7 and day 9. Tissues were taken and processed as described in Ch. 2 (16) from the liver, kidney, spleen, cerebellum, cerebral hemisphere and lung. From blocks, of 3 to 5 mm in thickness, a total of nine 5μ sections were cut, three from near either edge and three from the middle of the block. One of each set of three sections was stained with HE, one with PRB and one with MSB. On examination the degree of tissue degeneration or PRB reaction was scored by a 4+ system and the presence of fibrin or thrombosis recorded. The results of these examinations are presented in Appendix (13). In addition to these tissues, the intestine, adrenal gland and the submaxillary and mesenteric lymph nodes were examined from five infected rats with high parasitaemias and two control rats. From these observations a picture of the histological progress of the disease was built.

Experiment 6.10 : The Histopathology of B.divergens-infected Calves

The tissues of two splenectomized calves dying from acute B.divergens infections were examined. Calf A89 died during the experiment and details of the infection and pathological changes observed in this calf are presented here and in Ch. 4.

Calf 296 was infected by Mr A.J. Trees in the course of a babesiosis study unrelated to this work. The haematological data from this calf were collected by Mr Trees and are presented in Appendix (14). The calf was infected from the same stored sample (from calf B10, Ch. 3) used to infect the other calves in this study and the disease was allowed to follow its course. The calf developed a similar disease pattern to that of calf A89 reported in this study. Parasites became apparent on day 6, reached a peak of 30% on day 10 and the calf died on the morning of day 13. Control tissues were obtained from a normal animal at slaughter.

III. Results

Experiment 6.1 : The Plasma Sodium and Potassium Levels during B.rodhaini Infection

From the table of means and standard errors (Table 6.1) it can be seen that the development of parasitaemia and the fall in erythrocyte numbers were very similar to the pattern described in earlier experiments. No changes in either sodium or potassium levels were recorded.

Experiment 6.2 : The Plasma Proteins during B.rodhaini Infection

The mean parasitaemia was lower than in other experiments but the erythrocyte numbers fell to levels similar to those recorded in earlier experiments (Table 6.2). The total protein and albumin levels recorded

Table 6.1

Mean plasma sodium and potassium levels in mEq/l during *B.rodhaini* infection

Test	Day 5	Day 6	Day 7	Day 8
Parasitaemia %	14.3±1.2	65.4±2.8	44.3±3.8	3.5±2.1
RBC	6.85±0.07	3.10±0.31	2.03±0.16	4.49±0.38
Plasma Na	150.6±3.1	151.4±3.4	146.8±3.2	150.7±3.6
Plasma K	4.8±0.1	5.1±0.2	5.1±0.2	5.0±0.2
Control				
RBC	7.72±0.21	7.41±0.11	7.25±0.11	7.37±0.22
Plasma Na	152.8±1.1	153.6±1.2	151.2±1.5	149.0±2.6
Plasma K	4.9±0.1	5.1±0.2	5.1±0.3	5.1±0.2

Table 6.2

Mean total protein and albumin percentage during *B.rodhaini* infection

Day	RBC x10^6/cmm	Parasitaemia %	Protein g/100ml	Albumin %
3	6.29±0.24	1.0±0.2	5.62±0.22	50±2
5	6.03±0.16	8.7±2.0	6.10±0.07	43±2
6	3.84±0.67	29.7±6.0	5.94±0.21	42±2
7	1.82±0.24	3.8±0.4	6.14±0.12	37±1
9	3.98±0.13	0	7.00±0.10	38±1
Control	6.87±0.11	0	6.19±0.14	52±1

from control rats were in agreement with levels quoted elsewhere (Long, 1961). No change from the normal range was recorded for total protein values until day 9 when a significant increase occurred ($t_{14} = 4.56$; $p < 0.01$). The albumin level began to fall on day 5 and this fall continued until day 9 when, because of the rise in total protein value, the real albumin value began to recover.

Experiment 6.3 : The Plasma SDH, Bilirubin and Urea Levels during B.rodhaini Infection

The erythrocyte loss and parasitaemia were similar to other experiments. The SDH value was slightly raised on day 5, then rose very sharply to reach 63.36 ± 21.06 mμ/ml on day 7 and then fell on day 9 to 4.19 ± mμ/ml.

The bilirubin values were also raised and reached a peak of 0.74 ± 0.22 mg/100ml on day 7 and fell to 0.26 ± 0.03 mg/100ml on day 9.

The value recorded for plasma urea in the control rats of 57.6 ± 1.4 mg/100ml was higher than that reported from rats by Long (1961) of 33.6 mg/100ml. On day 2 (Table 6.3) a low level was recorded but the levels increased over the next four days. On day 7 the raised level was significantly different from the control level ($t_{14} = 2.19$; $p < 0.05$). On day 9, the urea level was still high but it was not significant.

Experiment 6.4 : The Urine of B.rodhaini-infected Rats

The urine of all infected rats examined was a deep red colour. Absorption peaks were found in all infected urine samples between wavelengths of 390 and 430 nm, at 530 nm and at 560 nm. These peaks corresponded with the absorption spectra of haemoglobin and some of its derivatives (Varley, 1967). The deposit from all infected urine

Table 6.3

Mean plasma urea, SDH, and bilirubin levels during *B.rodhaini* infection

Day	RBC $x10^6$/cmm	Parasitaemia %	Urea mg/100ml	SDH mμ/ml	Bilirubin mg/100ml
2	6.75±0.12	+	47.2±0.7	3.15±0.58	0.16±0.01
4	6.63±0.11	6.0±0.7	52.5±2.3	2.17±0.20	0.20±0.02
5	6.17±0.46	17.1±2.3	59.1±4.6	4.53±0.24	0.26±0.04
6	3.68±0.59	37.4±10.4	64.8±3.3	27.09±3.16	0.50±0.08
7	1.52±0.15	16.7±9.7	68.3±4.7	63.36±21.06	0.74±0.22
9	4.54±0.72	+	68.5±5.7	4.19±1.29	0.26±0.03
Control	6.97±0.1	0	57.6±1.4	2.05±0.15	0.17±0.02

samples showed a pink proteinaceous background with cellular debris and casts. In two samples intact erythrocytes were found, some of which were parasitized. The pooled control sample showed no absorption activity and only a very slight deposit in which no cells or casts were observed.

Experiment 6.5 : The Plasma and Sodium Levels during B.divergens Infection

The levels of these parameters fluctuated slightly throughout the experimental period but no major change occurred and there was no trend (Appendix (12)).

Experiment 6.6 : The Plasma Proteins during B.divergens Infection

The mean pre-infection value for total protein of 6.4 ± 0.1 g/100ml, obtained from the seven calves, was similar to the values presented in other work (Long, 1961) of 6.2 ± 0.3 g/100ml. During the course of infection a reduction in total protein was observed in all calves (Table 6.4). In analysing the results calf A89 was omitted because of its incomplete record. The remaining data constituted a balanced analysis with recordings on six animals over ten days. A cross classification analysis of variance (Table 6.4) was performed in which the data were classified according to days and according to animals. Both sources of variation were found to be significant at the 1% level. However, of major concern here was the significant difference in total protein recorded on different days. In order to ascertain which days during the course of the infection gave values significantly lower than the pre-infection value, the least significant difference was calculated. This showed that differences greater than 0.59 g/100ml were significant at the 5% level. So on days 13 and 15, at the time

Table 6.4

Total protein levels (g/100ml) during B.divergens infections

Day	Pre-infection	4	7	9	11	13	15	17	24	31	Mean
Calf no.											
212	6.7	7.0	6.5	7.1	7.0	6.6	6.4	6.5	6.5	6.6	6.7
275	6.6	6.3	5.9	6.6	6.5	5.9	5.4	7.8	6.7	6.6	6.4
L1160	6.3	6.1	6.2	6.6	6.6	5.9	5.3	8.4	7.3	6.3	6.5
A89	6.4	5.9	5.7	6.3	6.2	D	-	-	-	-	-
273	6.2	6.4	6.2	6.6	6.4	5.2	6.0	6.6	6.4	5.7	6.2
274	6.7	6.6	6.3	7.0	6.1	5.7	5.8	5.9	6.1	6.2	6.2
251	6.0	5.6	6.3	6.0	5.8	5.0	5.6	5.6	6.2	6.3	5.8
Mean	6.4	6.3	6.2	6.6	6.4	5.7	5.8	6.8	6.5	6.3	-
Parasitaemia %	0	0	+	2.5 ±1.0	11.9 ±3.2	11.8 ±3.5	2.3 ±1.5	+	0	0	

Analysis of Variance

Source	df	M.S.	F	p
Days	9	0.732	3.73	<0.001
Animals	5	0.881	4.49	<0.001
Error	45	0.196		

Table 6.5

Total protein and albumin levels during *B.divergens* infections

Day	Pre-infection	4	7	9	11	13	15	17	24	31
Total Protein	6.4	6.3	6.2	6.6	6.4	5.7	5.8	6.8	6.5	6.3
±s.e. g/100ml	±0.1	±0.2	±0.1	±0.1	±0.2	±0.2	±0.2	±0.4	±0.2	±0.1
Albumin as %	41.4	42.8	45.5	40.9	40.6	44.2	47.8	50.8	51.0	43.8
of total ±s.e.	±0.6	±0.9	±0.6	±0.9	±1.1	±1.7	±0.9	±0.8	±1.2	±2.2

Table 6.6

SDH (mμ/ml) and bilirubin (mg/100ml) levels during *B.divergens* infections

Calf no.	212		275		L1160		A89		273		274		251	
Day	SDH	BLR	SDH	BLR	SDH	BLR	SDH	BLR	SDH	BLR	SDH	BLR	SDH	BLR
-5	3.82	0.10	2.19	0.10	3.29	0.10	2.19	0.15	2.73	0.10	2.19	0.05	3.29	0.05
3	3.29	0.20	1.10	0.20	2.19	0.25	2.19	0.20	2.19	0.20	2.73	0.25	2.73	0.25
6	3.26	0.10	1.10	0.16	1.10	0.10	1.10	0.20	-	0.06	2.19	0.10	1.63	0.16
9	-	0.30	2.74	0.16	2.74	0.10	2.74	0.20	1.37	0.10	4.11	0.38	-	-
11	4.11	0.30	4.11	0.65	5.47	0.20	4.11	0.80	2.74	0.40	5.47	0.96	2.74	0.36
12	5.47	0.46	2.74	1.30	5.47	0.60	65.60	5.39	5.47	0.70	5.47	0.70	2.74	1.10
13	8.20	1.70	16.41	1.90	13.66	0.46	D		5.47	0.70	10.93	0.65	-	1.20
14	43.73	3.60	10.94	1.80	5.47	2.20			2.19	1.36	5.47	0.54	2.73	0.20
15	39.36	0.54	10.94	1.15	4.37	1.35			1.64	0.70	4.37	0.30	2.73	0.26
16	37.17	2.25	5.47	0.10	3.82	-			2.19	0.20	5.47	0.10	2.73	0.26
17	28.43	3.90	4.37	0.16	4.92	0.30			2.19	0.06	4.37	0.10	2.73	0.06
18	69.97	4.90	2.73	-	3.27	0.10			2.73	0.10	2.19	-	2.73	0.10
35	3.83	0.16	1.10	0.10	3.47	0.10			1.10	0.10	1.10	0.10	2.19	0.10

of crisis in the disease, the total protein was significantly lower than the pre-infection level.

The percentage albumin recorded prior to infection was close to the value of 43.1% recorded by Irfan (1967). The albumin/globulin ratio remained normal as the parasitaemia developed (Table 6.5) but its absolute values were depressed with the fall in total protein. From day 15, however, albumin began to rise and on day 17 and 24 a reversal of the albumin/globulin ratio was recorded. The ratio returned to normal by day 31.

Experiment 6.7 : The Plasma SDH, Bilirubin and Urea Levels during B.divergens Infection

Taking a SDH value of 4.0 mμ/ml, the upper limit of the pre-infection range recorded in the seven calves, as the normal maximum, a slight SDH increase was first seen in calf 274 on day 9 (Table 6.6) and on day 11 five calves were showing increased levels. During the infection all calves except 251 showed some increase in this parameter and those calves undergoing the most severe reactions showed the greatest increases in plasma levels. Peak activity was recorded close to parasitaemia peak and some days before erythrocyte nadir (Table 6.7) except in calf 212. This calf was moribund for some days and the persistence of raised SDH levels would possibly indicate a continued malfunctioning of the liver. Readings taken on day 22 and 29, as the animals recovered, showed levels of 13.12 and 6.02 mμ/ml respectively. The level in calf A89 rose from 4.11 to 65.00 mμ/ml on day 11/12, indicating very severe liver damage, and the calf died on day 13. The levels in the other calves returned to normal within three to five days.

Table 6.8

Urea levels (mg/100ml) during *B.divergens* infections

Calf No.	212	275	L1160	A89	273	274	251
Day							
-5	18.83	20.27	23.17	18.83	14.47	15.93	21.72
3	17.37	23.17	20.27	18.83	20.27	20.27	23.17
6	19.03	22.19	26.96	19.03	20.61	-	23.69
9	21.72	30.27	30.27	31.80	25.73	27.24	-
11	27.34	36.33	-	40.87	21.19	36.33	30.27
12	44.39	62.44	23.59	52.71	45.79	24.97	30.18
13	69.39	41.63	34.70	D	59.67	27.74	37.46
14	73.53	33.33	45.79		27.75	24.98	16.65
15	123.49	30.53	43.02		45.79	16.65	22.20
16	174.83	15.32	41.63		33.33	19.43	20.81
17	63.84	13.88	30.53		27.20	15.32	18.04
18	45.79	11.09	15.26		22.22	13.88	16.65
35	13.88	16.65	19.43		21.19	12.49	22.22

Table 6.7

The relationship between parasitaemia, erythrocyte nadir and peak levels of SDH, bilirubin, and urea in calves

Calf no.	212	275	L1160	A89	273	274	251
Peak Parasitaemia	12	12	13	11	12	11	12
RBC Nadir	15	15	16	-	15	15	16
Peak Bilirubin	17	13	14	-	14	11	13
Peak SDH	18	13	13	-	12/13	13	-
Peak Urea	16	12	14	-	13	11	13

The plasma bilirubin levels recorded during the infection are presented in Table 6.6. By day 10 all calves, except L1160, were showing raised levels. Levels were raised in all calves reaching a peak slightly later than the SDH levels (Table 6.6). Calf A89 showed a massive rise to 5.39 mg/100ml on the day before death. Calf 212 showed a peak activity of 3.60 mg/100ml on day 14, the level then fell for a few days but remained above normal and peaked again on day 18 at 4.90 mg/100ml. On day 22 its bilirubin level had fallen to 1.00 mg/100ml. The other calves were within the normal range by day 17.

Urea levels were raised on day 11 (Table 6.8) and continued to rise with the increase in parasitaemia. Once the parasitaemia had reached a peak and declined these levels returned to normal. Calf A89 showed a level of 31.80 mg/100ml on day 9, which was above the pre-infection range of these calves, and its level continued to rise reaching 52.71 mg/100ml on day 12. Calf 212 showed a persistently raised level

Table 6.9

Distribution of parasites from different sites of the body

Animal	Tail	Heart	Lung	Liver	Kidney	Spleen	Brain	Heart Blood	Leg Vein	Haemoglobinuria
Rat 1	16/50	12/50	12/50	14/50	10/50	3/50	6/50	12/50	-	0
2	26/50	30/50	32/50	32/50	32/50	26/50	20/50	36/50	-	0
3	2.4%	1.4	1.8	1.5	1.7	1.7	-	1.0	-	0
4	7.2	6.6	6.5	9.2	7.4	6.2	-	7.0	-	0
5	14.0	13.6	11.7	15.7	12.7	14.3	-	11.0	-	0
6	40.2	38.3	37.6	49.2	46.7	40.0	38.9	-	-	++
7	43.6	33.0	38.0	35.1	35.5	33.9	29.1	34.6	-	++
8	58.6	49.8	46.5	48.5	47.3	50.6	-	51.0	-	+++
Bovine A89	=	19.0	-	31.0	12.0	-	22.5	-	21.0	++

of urea and a very marked ureamia, of 172.53 mg/100ml, was recorded on day 16 after which the level declined reaching 26.06 mg/100ml on day 22.

Experiment 6.8 : Sites of Distribution of the Parasite

The results of the examination of blood films made from different sites of animals killed or dying from B.rodhaini or B.divergens infections are presented in Table 6.9. There was some variation between sites in the rats but there was not a consistent preference for any one site. There would appear to have been a higher parasitaemia in the liver and a lower parasitaemia in the kidney of calf A89. But this animal was dead for some hours before sampling and the impression from the examination of sections did not suggest a site preference. It was not possible to repeat this observation.

Experiment 6.9 : The Histopathology of B.rodhaini-infected Rats

Gross pathology. From rats dying or killed at various stages of B.rodhaini infection the following pattern of gross pathological change was observed. The earliest change was of splenic enlargement, first observed on day 2 and by day 6 and 7 weights of as much as 12 times the control weight were recorded (Ch. 4). Haemoglobinuria was observed from day 3 or 4 and a distended bladder was present in all severely affected animals on days 5 to 7. The eyes lost colour from day 3 becoming brown, then very pale by day 6. By day 8 the eyes had regained much of their red colour. The carcase was jaundiced from day 5 or 6 but this was not apparent by day 9. The liver became paler as the infection progressed and thin watery blood ran from the cut surface from day 5. The liver size was not changed but the lobular outline was apparent from days 5 to 7 and the bile was thickened.

The kidney also became pale but no other changes were seen macroscopically. The heart became pale and at the crisis of the disease it was difficult to obtain 5ml of blood from the ventricle and it had to be drawn with great care. The meninges were frequently congested. The rectum was full of hard dry faecal pellets from day 5 and in very sick animals these were coated with mucus. It appeared that the rectum or bladder were not emptied during the disease crisis. Once recovery became apparent organ colour returned rapidly but splenic size was still increased and about 25% of recovering animals showed one or more splenic infarcts.

Histopathology. Liver. On day 4 a mild vacuolar degeneration of the hepatic cells in the centrilobular area was found and its distribution was widespread. On day 5 the hepatic cell degeneration was more marked and the Kupffer cells were enlarged and showed a PAB reaction as fine blue grains. The reticular cells in the portal area showed a slight increase in number and stained more darkly. On day 6 the vacuolar degeneration of hepatic cells was present throughout the entire lobule, with some necrosis of these cells in the central areas. Staining characteristics were poor, pyknotic nuclei were occasionally found and the sinusoids were dilated. There was no congestion but occasionally discrete foci of five or eight blast type cells were found in the sinusoids (Fig. 6.1). The increase in reticular cells in the portal areas continued. On day 7 similar but more severe changes were observed. Some necrosis was present in the central area of almost all the lobules, but only occasionally was the architecture of the organ disturbed. In some areas there was a distinct outline to the hepatic cells which was shown well by MSB

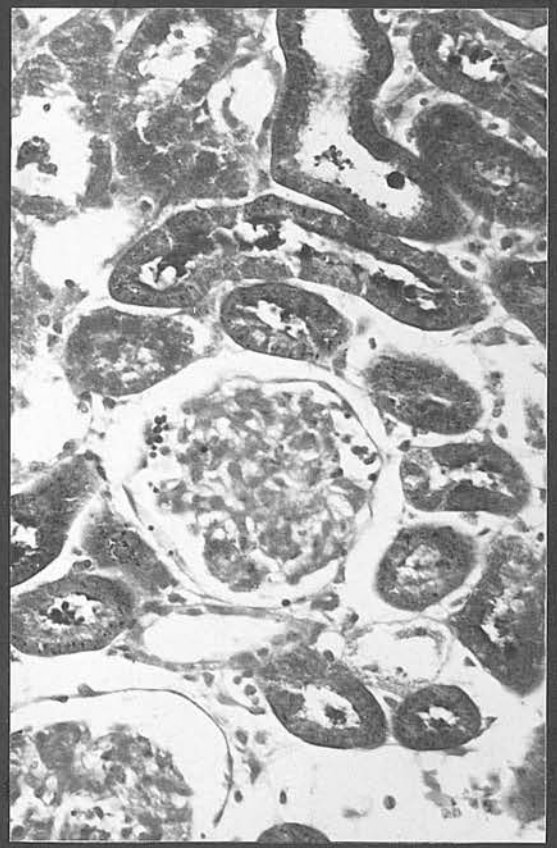
staining and might represent some distention of the bile ducts. The blast cell foci had increased in size. The Kupffer cells were large and projected into the sinusoids. A thrombus was found in the sinusoid of one rat (Fig. 6.2). On day 9 the liver was substantially intact with only mild vacuolar degeneration and a few areas of collapse of the tissue structure. The liver looked very healthy considering the degree of damage observed two days earlier in the infection. The only remaining changes were the enlarged Kupffer cells which continued to stain PRB positive, the scattered blast cell foci and the enlarged portal areas. Congestion was not present and there was no infiltration by inflammatory cells.

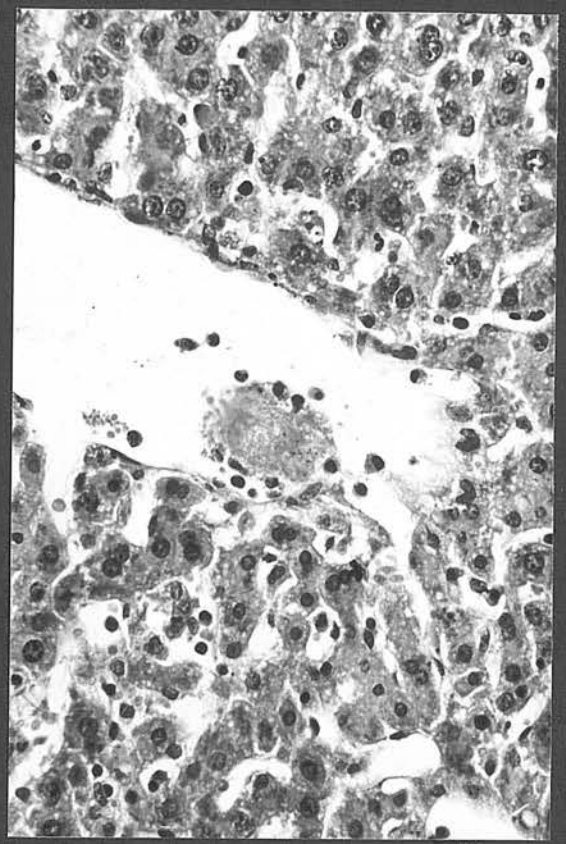
Kidney. Changes were first seen on day 4 when eonsinophilic globules were found in the cytoplasm of the convoluted tubular epithelial cells, and these cells showed a positive PRB reaction. On day 5 the globules were more pronounced and stained reddish with MSB stain. Mild vascular degeneration of tubular cells was occasionally found but it was not widespread. As rat kidney tissue was difficult to fix, these very early vacuolar changes were not easily identified. Small accumulations of debris within some of the lumina of the convoluted tubules were also found on day 5 and few erythrocytes were seen in the glomeruli. On day 6 the epithelium of the convoluted tubules showed a very marked PRB reaction and the granules in the cytoplasm were very noticeable with both HE and MSB staining. Desquamation of some of these cells was observed but was not common and pyknotic nuclei were occasionally seen. The debris within the tubular lumina had increased greatly and was staining similarly to the cytoplasm of the epithelial cells. Very occasionally a small amount of condensed material, staining

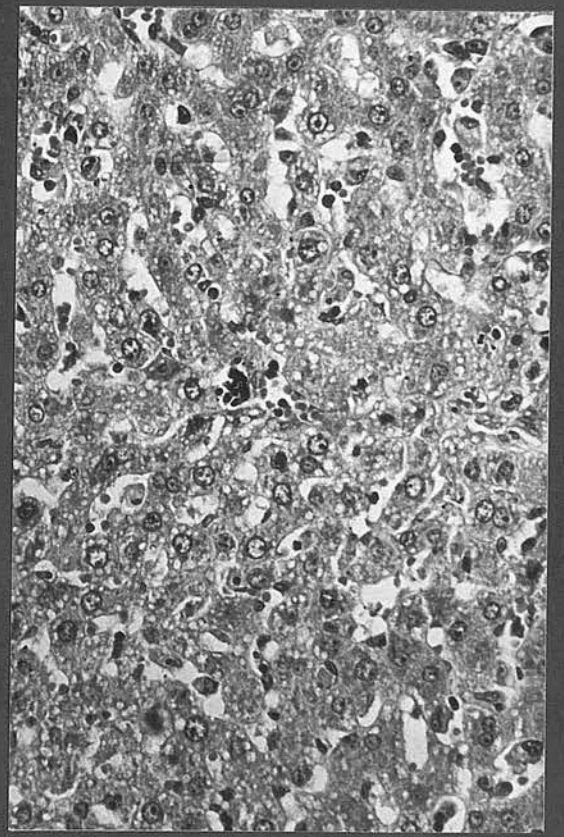
Fig. 6.1. **Liver of rat showing marked vacuolar degeneration and a focus of blast cells (Day 7. x270.HE).**

Fig. 6.2. **Liver of rat showing a thrombus within a large vein (Day 6. x270.HE).**

Fig. 6.3. **Kidney of rat showing the distribution of the PRB reaction (Day 7. x270.PRB).**







pink with HE and light blue with PRB was present in Bowman's space. The glomeruli had few erythrocytes within the capillaries and a few glomeruli were shruken. On day 7 a similar appearance was presented, with the architecture of the organ remaining intact. Considerable quantities of debris remained within the lumina of the convoluted tubules together with a very marked PRB reaction in the epethelium of these tubules (Fig. 6.3). The glomerular basement membrane was slightly thickened. There was mild congestion in the cortico-medullary region and very occasionally in the medulla, otherwise this area of the renal tissue was virtually unchanged. On day 9 the PRB reaction was still observed in the epithelial cells of the convoluted tubules but there was no debris in the lumina. The basement membrane of many glomeruli still showed some thickening but the capillaries of the glomeruli contained a 'normal' amount of blood. The tissue looked healthy.

In Table 6.10 the relationship between histopathological changes found in the liver and kidney are shown, together with the plasma SDH, bilirubin and urea levels, for four infected and four control rats. A very close correspondence was found between the histological and the biochemical changes.

Spleen. Some splenic changes have already been described in Ch. 4. Enlargement and proliferation of phagocytic, reticular and lymphoid cells was found on day 2. Erythrocytes were present in the sinusoids but there was no congestion. By day 6 the lymph follicles were greatly enlarged and plasma cells were present in large numbers in the red pulp about the follicles. On day 9 the follicular activity had changed, the centres of the follicles were much less crowded and the

Table 6.10

The relationship between SDH, bilirubin and urea and histological changes in the liver and kidney

Day	Rat	Parasites	RBC	Liver	SDH	B/R	Kidney	Urea
		%	$x10^6/mm^3$		mμ/ml	mg/100ml		mg/100ml
2	Control	0	7.19		0.49	0.18		6.38
2	Test	1.9	6.68		1.97	0.14		5.65
4	Control	0	7.11		1.97	0.09		5.50
4	Test	52.2	4.51	+	4.93	0.50	+	5.83
5	Control	0	7.19		0.99	0.14		5.25
5	Test	74.14	1.62	++	34.48	0.80	+	7.37
6	Control	0	7.16		3.94	0.18		4.83
6	Test	56.3	1.08	+++	300.43	1.40	+++	7.57

staining less intense.

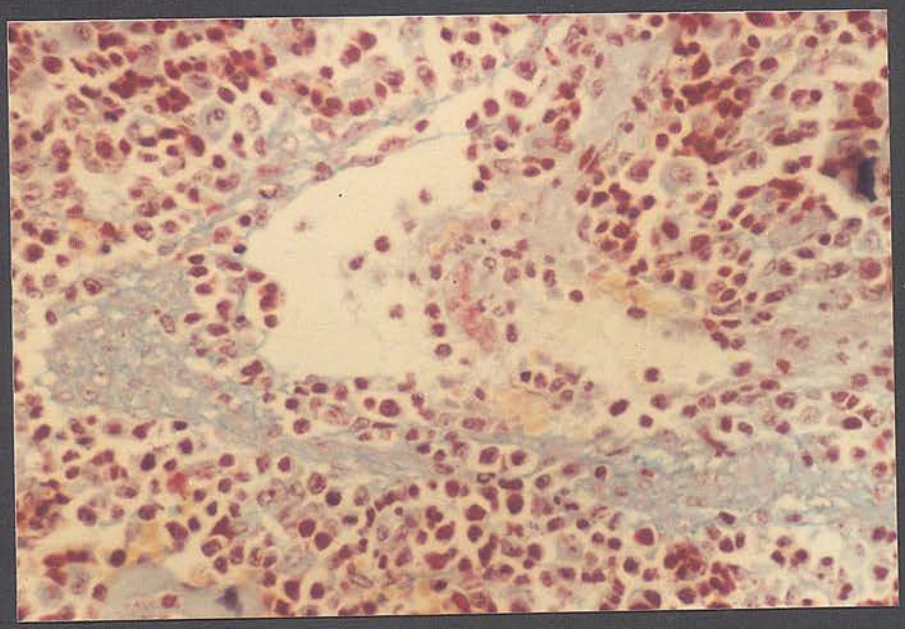
In control rats a mild PRB reaction was found but in infected animals a much more intense reaction was present from day 5. Two rats were observed to have pale areas in the spleen on days 5 and 6 (Fig. 4.7). Examination of these areas showed a considerable depletion of cells but the reticular network of the tissue was intact. These were believed to be the earliest manifestation of developing infarcts, although thrombosis was not identified in these particular spleens. These early lesions were usually about 1 to 2 mm in diameter and placed close to the thin edge of the spleen. On the surface of the organ they appeared as a pale yellow area. The mature infarctive lesion was pale, raised, rounded and about 5 mm in diameter and penetrated a similar distance into the spleen (Fig. 4.7). One or two such lesions could be found in the spleen but occasionally as many as ten were observed around the edge of the organ giving it a regular beaded appearance. The splenic capsule remained intact over these lesions as if nourished by a separate blood supply. Within the lesions few cells survived, occasional erythrocytes were seen and some surviving connective tissue. The interface between healthy and necrotic tissue showed congestion. The fate of these lesions was not studied beyond day 9. Thrombosis, a possible cause of such lesions, was found in four different animals during this investigation (Figs. 6.4 and 6.5).

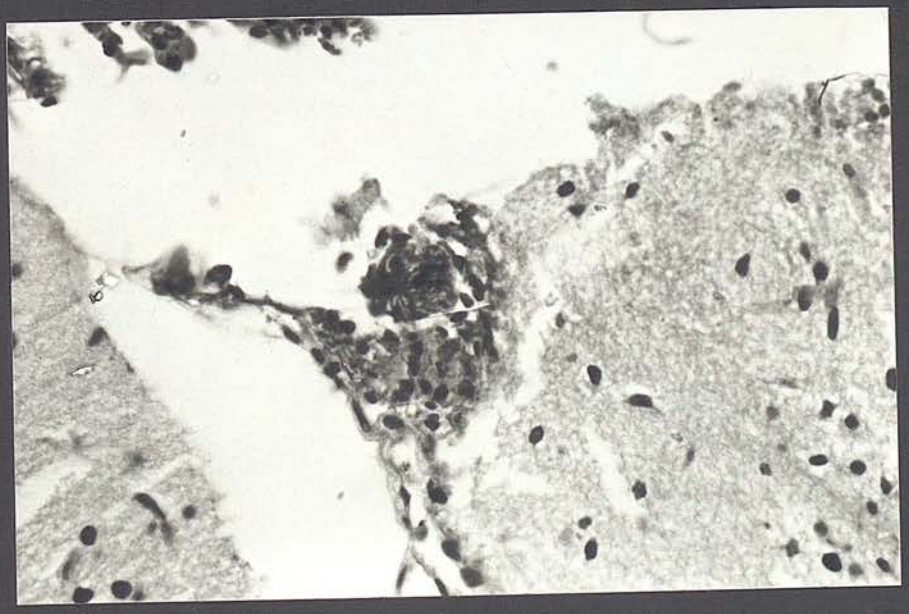
Brain. In both the cerebellum and cerebral hemispheres congestion of small vessels was found on days 6 and 7. On day 6 a small fibrin thrombus was found in a meningeal vessel of the cerebellum of one rat (Fig. 6.6). There were no other changes observed in infected rats.

Fig. 6.4. Spleen of rat showing a large thrombus (Day 6. x270.HE).

Fig. 6.5. Spleen of rat. A section from the same spleen shown in Figure 6.4 cut close to the section above and showing the presence of fibrin within the blood vessel (Day 6 x270.MSB).

Fig. 6.6. Cerebellum of rat showing a thrombus in a broken meningeal vessel (Day 6. x440.MSB).





Lungs. On days 5, 6 and 7 a PRB reaction was found in the alveoli, otherwise the tissue was unchanged.

Other tissues. The intestine and the adrenal glands showed no change. An increase in lymphoid and reticular cells was found in the lymph nodes during the course of infection.

Experiment 6.10 : The Histopathology of B.divergens-infected Calves

Gross Pathology. Both calves were found dead on the morning of day 13 following infection. They were in rigor and calf A89 was still warm on the underside. Neither calf showed any sign of struggling. The mucous membranes were pale and slightly jaundiced and the eyes were sunken. The preputial hairs were damp and stained with reddish urine. The subcutaneous tissues were slightly jaundiced. The lungs appeared normal while the heart was pale with some petechiae on the coronory band and a slight increase in pericardial fluid. The liver was of normal size but pale and fatty, the tissue was friable and the bile thick. The kidneys were pale but otherwise unchanged and there was a small quantity of reddish urine in the bladder. The bowel was normal but the rectum contained very firm mucous coated faeces. The meninges and brain showed no changes.

Histopathology. Liver. This organ was profoundly damaged. Centrilobular necrosis was found in more than half of the lobules in any section examined from calf A89 and an even more widespread necrosis was found in calf 296 (Fig. 6.7). Occasionally, adjacent lobules were completely necrotic so that islands of ill-defined pink staining material with scattered pyknotic nuclei were found. Most surviving hepatic cells showed some degree of vacuolar degeneration. The Kupffer cells were greatly enlarged, contained erythrocyte material and

stained markedly positive for haemosiderin. There was no congestion but unlike the finding in rats there was some infiltration of inflammatory cells. Occasionally bile canaliculi were distended (Fig. 6.8). Fibrin formation was observed in some sinusoids, especially those adjacent to necrotic areas (Fig. 6.9).

Kidney. The kidneys showed considerable change but the architecture of the organ remained intact. Within the cytoplasm of the epithelium of the convoluted tubules there was widespread vacuolar degeneration and positive PRB material. Necrosis was not commonly seen but cells were shed into the lumen of the tubules. There were large accumulations of debris in most of the convoluted tubules and this material was present in 60 to 70% of the glomeruli (Fig. 6.10). The debris had similar staining characteristics to the cytoplasm of the convoluted tubular epithelium. Within Bowman's space the debris was usually found against the parietal membrane, and only rarely within the glomerular tufts. There was some thickening of the glomerular basement membrane. The medulla was unchanged except for the presence of debris occasionally seen in the tubules.

Brain. No changes were found in the brain of calf A89 but four small haemorrhages were present in calf 296 (Fig. 6.11). There was no congestion.

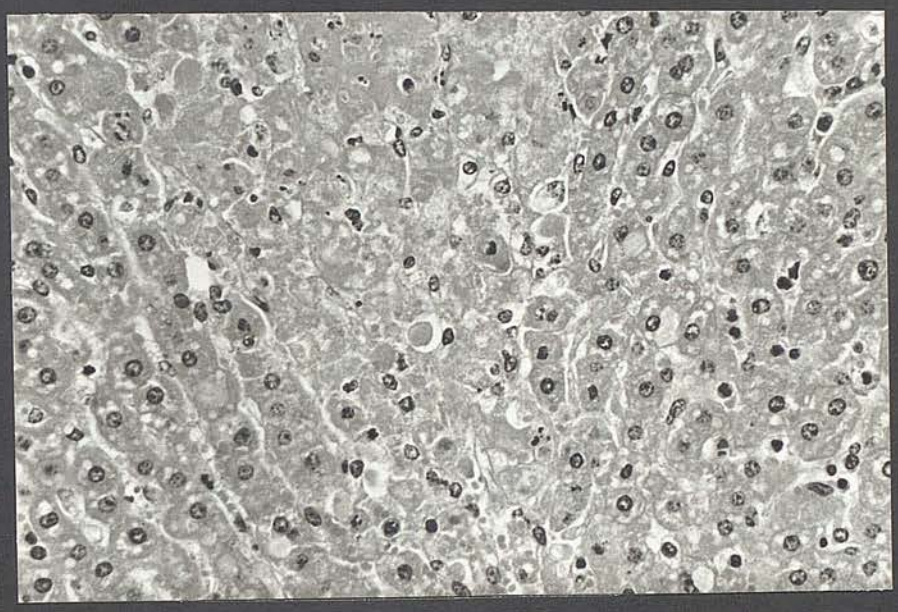
Lungs. Some cells in the alveoli showed a PRB reaction and a similar reaction was seen in large phagocytic cells within the larger veins. A frothy material was present in many alveleoli and bronchioles.

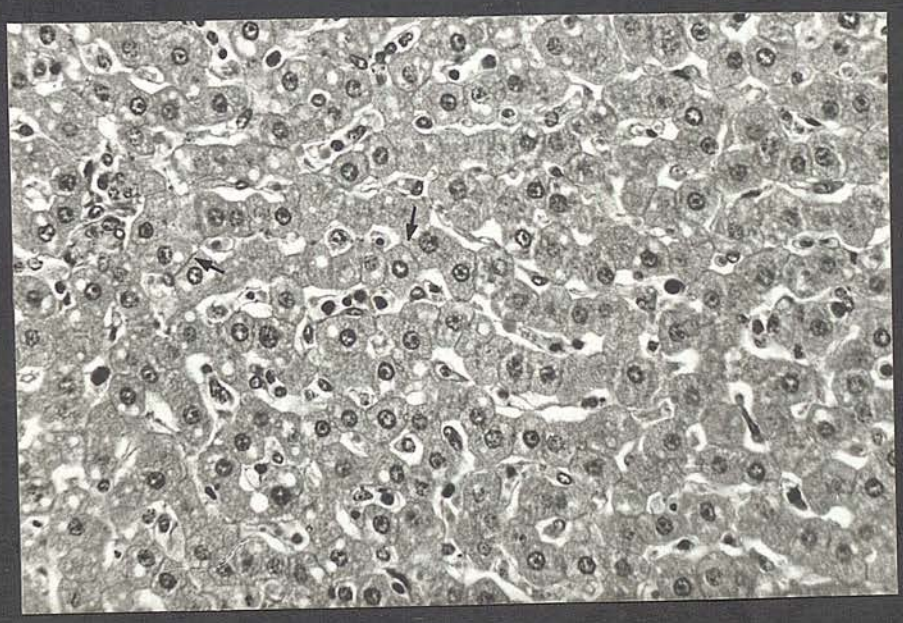
Heart. In calf 296 there was a heavy infection with Sarcocystis fusiformis (Fig. 6.12), otherwise no abnormality was observed.

Fig. 6.7. Liver of calf 296 showing necrosis with marked vacuolar degeneration in surviving hepatic cells (x270.HE).

Fig. 6.8. Liver of calf 296 showing distended bile ducts (arrows), vacuolar degeneration of hepatic cells and enlarged Kupffer cells (x270.MSB).

Fig. 6.9. Liver of calf A89 showing fibrin strands within a sinusoid and hepatic cell necrosis (x590.MSB).





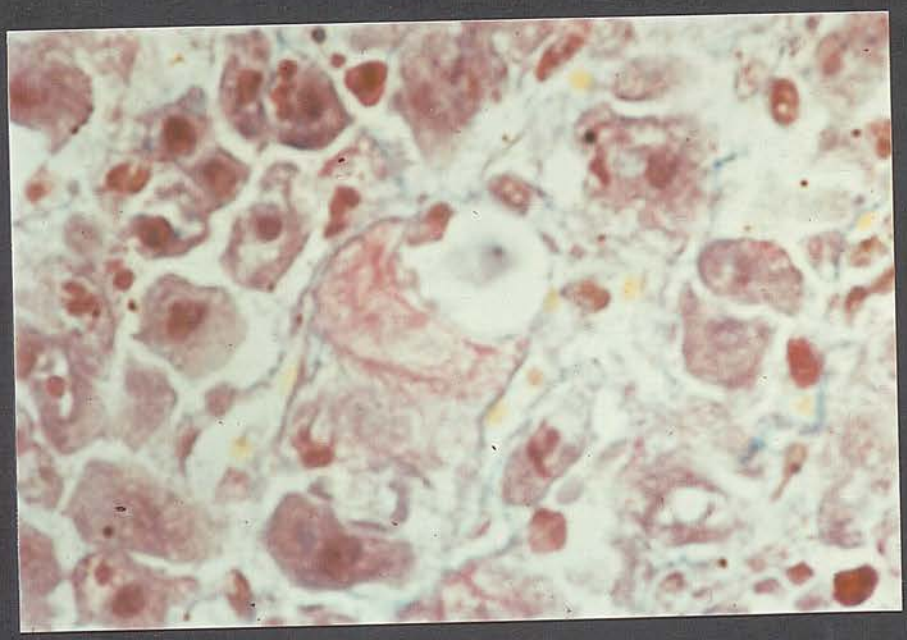
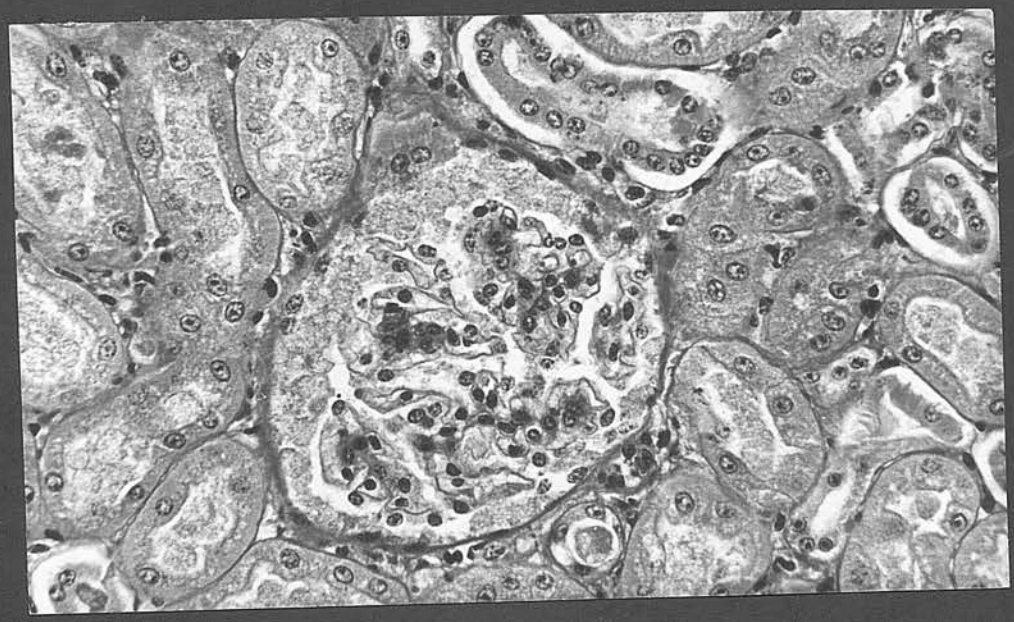
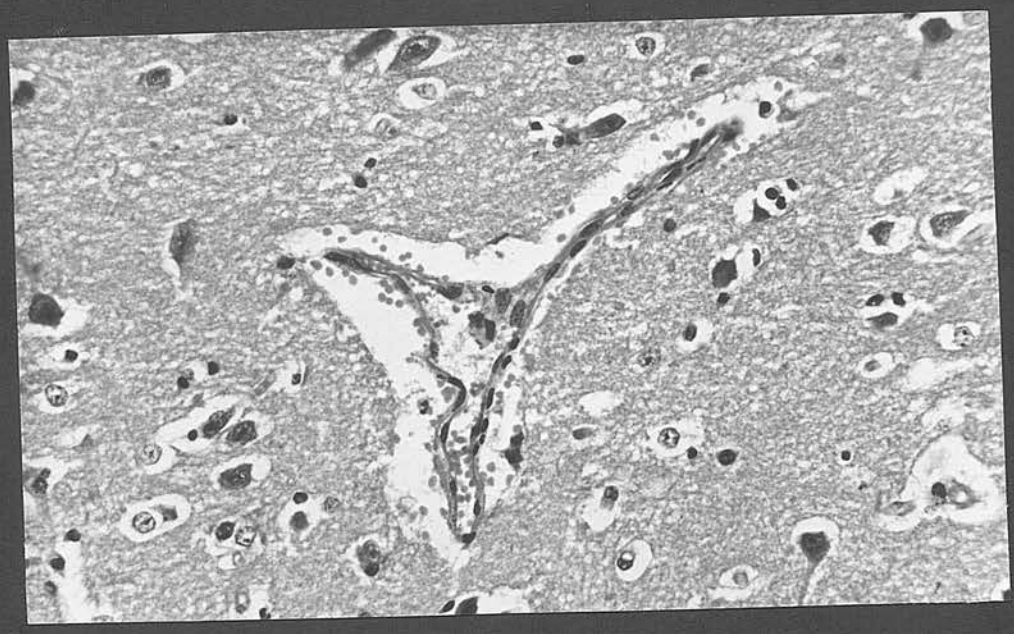


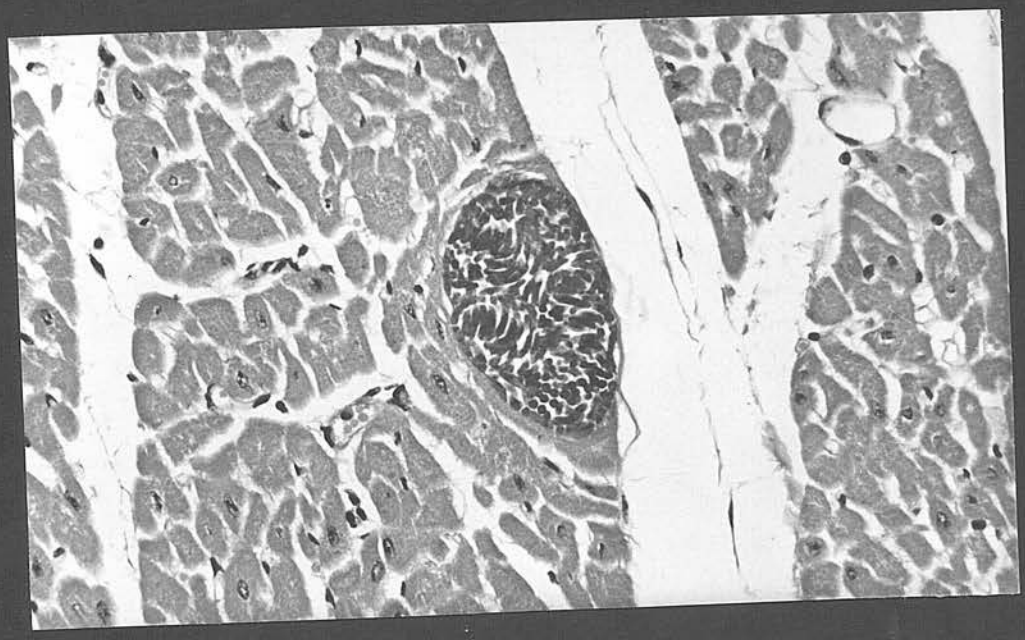
Fig. 6.10. Kidney of calf A89 showing accumulations of debris within the tubules and Bowman's space (x440.MSB).

Fig. 6.11. Cerebellum of calf 296 showing haemorrhage about a small blood vessel (x440.MSB).

Fig. 6.12. Heart of calf 296 showing a Sarcocystis fusiformis cyst (x270.HE).







Lymph nodes. There was some lymphoid proliferation in the submaxillary nodes but the mesenteric nodes seemed less active and were oedematous.

IV. Discussion

The serum biochemical and histopathological changes found during this investigation of experimental B.rodhaini and B.divergens infections were similar to changes reported from other studies of babesiosis. However, the majority of these studies have simply recorded the changes observed, neglecting to relate them to the underlying pathogenic mechanisms. As the mechanisms of pathogenesis are the primary concern of the present study the findings reported will be discussed in relation to the possible underlying mechanisms.

The plasma levels of sodium and potassium were unchanged in this investigation as in the findings in B.canis and B.gibsoni infections (Maegraith et al., 1957; Fowler et al., 1972). But Jerichow and Jungmann (1969) found plasma potassium levels significantly elevated in naturally occurring B.divergens infections. They did not measure plasma sodium levels. Wright (1972a) found plasma potassium levels depressed in both B.argentina and B.bigemina infections while sodium levels remained unchanged. He also found that urinary potassium levels were increased. Overman (1948) found that in malaria infections erythrocyte sodium was reduced and potassium increased. Subsequently, it was shown that, due to a disturbance in the active transport mechanism of the erythrocytes in malaria, these ionic levels were altered and a reciprocal change occurred in the plasma (Dunn, 1969; Seed and Kreier, 1972). In attempting to identify erythrocyte or other cellular ionic

disturbances by an indirect plasma measurement, two factors can confuse the result. Firstly, the destruction of erythrocytes on any scale might release sufficient sodium and potassium into the plasma to mask any changes resulting from internal cellular disturbances (Maegraith et al., 1957). Secondly the maintenance of electrolyte homeostasis is dependent upon the functional integrity of both the liver and kidney (Maher and Nolph, 1972). Thus Wright's (1972a) finding of depressed potassium without plasma sodium alteration could have reflected a degree of kidney damage, as he found evidence of kidney dysfunction very early in infection, before appreciable erythrocyte destruction. The finding of no changes in this study where considerable haemolysis occurred means that no conclusion can be drawn about the erythrocyte sodium/potassium balance but it does indicate that the kidney remained capable of maintaining normal plasma levels of these ions despite their increased release by haemolysis. It would appear that a reliable indication of erythrocyte ionic balance can only be obtained by the direct measurement of erythrocyte sodium and potassium.

SDH is localized mainly in the liver and meets the requirements of an organ specific enzyme (Hess, 1962; Gerber, 1968). It has been widely applied in human clinical medicine as an indicator of liver cell damage, but has not had a wide application in veterinary investigations. Ford (1967) examined its distribution in the tissues of calves and sheep while Gerber (1968) examined its distribution in the horse and both authors emphasized its potential usefulness. Hammond (1971) found that serum SDH levels were elevated during Fasciola infections in calves and that the activity of the enzyme was not lost

on storage. Shlosberg et al. (1973) found SDH levels elevated during the febrile response to Theileria annulata infections in calves. The bromsulphthalein (BSP) clearance rate is a specific indicator of liver function and has been found to be reduced (indicating damage) during chronic B.canis infections (Maegraith et al., 1957) and severe B.argentina infections (Wright, 1972a). However, the application of the test demands frequent handling and sampling of the animal and the dye competes with bilirubin for uptake by hepatic cells. Thus its value in the investigation of liver damage in babesiosis is reduced, while more simple, but specific, indicators of damage, such as SDH measurement would seem to have much merit, although where concurrent kidney damage occurs this will contribute a little to the total SDH found. The close correspondence between the degree of damage found histologically and the plasma SDH levels in rats confirmed its usefulness and the test probably reflected accurately the degree of liver damage in the infected calves.

The measurement of total bilirubin levels does not indicate whether the origin of the pigment is due to haemolysis or hepatocellular damage. The raised bilirubin levels found in both infections in this study were observed after the onset of erythrocyte loss and returned towards normal in early recovery from the disease. Thus they were probably a reflection of haemolysis and the fact that they did not persist above normal levels in the recovery phase indicated the functional normality of the liver. The infection in calf 212 provided an exception to this pattern showing a second and higher peak of both bilirubin and SDH activity on day 18. These peaks occurred despite the uninterrupted recovery of the haematological parameters. As there was no haemolysis

observed at that time in the infection the elevation of bilirubin levels was probably due to a second, but unexplained, liver crisis.

The liver showed a progressive degeneration during both B.rodhaini and B.divergens infections. The degree of liver damage was more severe in the two calves examined than in the rats. The lesions of centrilobular necrosis were typical of the liver pathology reported from all studies of babesiosis and were similar to the changes recorded in malaria. A number of mechanisms have been proposed to explain the centrilobular degeneration. The blood supply to the liver lobule arrives via the hepatic artery and the portal vein and flows into the larger sinusoids at the periphery of the lobule. The blood then percolates through the sinusoids to the central vein (Andrews et al., 1949). It is argued that in the anaemic state the tissues at the periphery of the lobule utilize the available oxygen and the more central tissues are deprived and degenerate. However, as degeneration of the centrilobular areas is observed in malaria and babesiosis before the oxygen carrying capacity of the blood has fallen very low some other mechanism was sought. Skirrow et al (1964) demonstrated that the vascular tree in the liver was greatly constricted in malaria and they showed that this change was due to a stimulation of the sympathetic nervous system. The origin of the stimulus was somewhere in the host-parasite interrelationship. This mechanism produced a constriction of the portal vein and its tributaries with a reduction in blood flow through the sinusoids and a consequent reduction of tissue oxygenation. A similar mechanism has been demonstrated in the pathogenesis of liver damage in carbon tetrachloride poisoning (Calvert and Brody, 1960).

Polani (1954) found that in producing a comparable degree of anoxia in rats by means of haemolytic serum, phenylhydrazine treatment and low oxygen tension, only the haemolytic serum caused hepatic cell damage. He concluded that the antibody must have had a direct effect upon the hepatic cell. Paget et al. (1962) adapted Polani's findings in attempting to explain the centrilobular liver degeneration in mice with B.rodhaini infection. They postulated that this change would occur only with an abnormality of the reticulo-endothelial system. Kupffer cell enlargement is a consistent finding in babesiosis but in neither babesiosis nor in other conditions with a similar hyperplasia has an obstruction to sinusoidal blood flow been found (Maegraith et al., 1957; Knisely et al., 1945). It seems even more unlikely that the enlarged Kupffer cells would interfere with hepatic cell oxygenation. Polani's (1954) original suggestion of damage being due to a secondary effect of anti-erythrocyte antibody has not been investigated and in babesiosis and malaria no specific anti-erythrocyte antibodies have been identified.

It would seem that the initial degeneration of the liver in these conditions results from an alteration in lobular blood flow leading to oxygen deprivation in the centrilobular area and degeneration. Later in the infection with marked anaemia this degeneration will be compounded by anaemic anoxia. But a number of other mechanisms will also damage the liver as the disease progresses. It has been postulated that a state of histotoxic anoxia might exist whereby the hepatic cells would be unable to utilize the available oxygen (Maegraith, 1966). This mechanism is dependent upon the presence of circulating 'toxin' or 'toxins' elaborated either by the parasite or as a result of host-parasite interaction. The existence of this latter type of non-specific

toxin has already been implied when discussing plasma alterations (in earlier chapters). During overt malaria infections a substance was found in the plasma which was capable of causing damage to the mitochondria of hepatic cells. Wright (1972a) considers this to be a most likely mechanism but no electron microscopic studies of early liver damage in babesiosis are reported that might confirm this possibility. The non-specific nature of this 'toxin' is shown by the finding of a similar but somewhat less severe type of mitochondrial damage induced by starvation (Riley and Maegraith, 1962; Maegraith et al., 1962).

Another mechanism that might contribute to the liver pathology is the circulatory disturbance caused by stasis or sludging. Stasis may be due to nervous stimulation of the vasculature of the organ while sludging is a phenomenon in which some conversion of fibrinogen to fibrin occurs. Both can produce a state of stagnant anoxia and have been found in malaria, babesiosis and, as a terminal phenomenon, in many acute infections (Knisely et al., 1941; Wright, 1972a). No evidence of either disturbance was found in the present study, although Wright (1972a) found stasis in B.argentina but not in B.bigemina infections. Despite the presence of stasis in one infection and not in the other he did not find an appreciable difference in the degree of liver damage, possibly because it was a terminal phenomenon.

The liver glycogen levels have been found to be greatly reduced in B.canis and B.rodhaini infections (Gilles et al., 1953; Maegraith et al., 1957; Paget et al., 1962) while the plasma levels remain within normal limits in dogs infected with B.canis or B.gibsoni

(Gilles et al., 1953; Fowler et al., 1972). Wright (1972a) considered that these changes were a result of liver cell damage and increased glycogen catabolism due to pyrexia. However, in malaria and carbon tetrachloride poisoning gross changes in circulating corticosteroids have been recorded and in carbon tetrachloride poisoning the adrenal glands were found to show histopathological change (Mercado and von Brand, 1957; Calvert and Brody, 1960). In both of these conditions glycogen changes similar to those found in babesiosis occur and the hormonal disturbances might provide a more satisfactory explanation of the glycogen changes in babesiosis than the explanation offered by Wright (1972a). The fact that no changes were observed in the adrenal glands of rats in this study probably excludes major hormonal disturbances of glycogen and lipid metabolism in the disease pathogenesis but it provides no information on possible physiologically altered corticosteroid levels that could lead to considerable liver changes.

The small foci of blast cells found in the liver sinusoids during B.rodhaini infection were not clearly identified. They were possibly lymphoid cells but sufficient numbers of lymphocytes or plasma cells were not observed in intimate contact with the foci to conclusively establish their nature. Rogers (1971) has observed focal accumulations of lymphoid cells in the kidney and an infiltration of these cells into the portal triads in the liver of cattle with B.argentina infections. As the cells were not identified in the present study no conclusion could be drawn as regards their role.

Total plasma protein values remained unchanged in B.rodhaini infected rats until day 9 when they showed a significant increase.

The albumin/globulin ratio was reversed from day 5, although, because of the rise in total protein value on day 9, the albumin levels were returning towards normal. A different plasma protein response was found in B.divergens infections of splenectomized calves. Total protein values were significantly reduced at the crisis of the disease and then returned to normal. During recovery albumin levels were elevated. During severe babesiosis, protein is lost both by plasma haemoglobin exceeding the renal threshold for its retention with consequent haemoglobinuria, and by protein leakage resulting from kidney damage (Malherbe, 1966). The ability of the animal to compensate for this loss might be hindered seriously by concurrent liver damage. The normal control of blood volume is a complex homeostatic mechanism dependent upon the intravascular colloid osmotic pressure (Crane et al., 1974). The maintenance of this pressure is controlled by finely regulated protein synthesis (Rothschild et al., 1972). The response of the host to a sudden protein loss will be to return the protein level to normal, irrespective of the protein type. It would appear that in rats this was achieved by raised globulin levels while in calves albumin synthesis effected the return to normal. Clearly the rat was more efficient in maintaining normal levels of protein and showed a reactive hyperproteinaemia in recovery. The response of most animals during recovery from an infectious disease is by a reversal of the albumin/globulin ratio with raised immunoglobulin levels. This was most likely to have been the response in rats in this study, although the globulin fractions were not examined. Nowell (1968) found a similar albumin depression in B.rodhaini-infected rats while globulin levels were raised, with an increase in immunoglobulins.

The calves did not show raised globulins, but this does not exclude the possibility of immune globulin increases at the expense of some other globulin or globulins. Suteu and Giurea-Iacob (1971) also found a hypoproteinaemia in infected cattle, but globulins were raised during recovery and immune globulins were still raised three weeks after recovery. The observation by Collins et al. (1970) of elevated total protein levels in natural cases of babesiosis must be interpreted with caution as the protein measurement was by a 'dip stick' technique and the samples might have aged.

The damage to the kidney found in this study in both infections was less severe than that in the liver but was still considerable. Necrosis of tubular cells was found, but the architecture of the organs was undisturbed. The damage was concentrated mainly in the cortical tubules with virtually no changes in the medulla. There was some thickening of the basement membrane of the glomeruli and in rats glomerular ischaemia was common. Within Bowman's space in the calves, accumulations of material were found which had similar staining characteristics to the debris in the convoluted tubules and the cytoplasm of tubular cells. In rats it was rare to find material in Bowman's space, and when it was found, it was in small quantities and very condensed. Maegraith et al. (1957) have observed similar accumulations of material in the glomeruli of dogs. The most likely source of this material was the proximal convoluted tubule. Possibly because of tubular obstruction and reduction, or shut down in glomerular filtration, the debris moved back into the glomerular space.

The plasma urea level is widely accepted as being a reliable indicator of kidney function. In the calves examined in the present

study the pre-infection values were close to other quoted values (Long, 1961). Urea levels began to rise once parasitaemia became apparent and erythrocyte destruction began. In the rats studied in the present investigation this did not prove to be a reliable parameter. The control values were very high and, despite the histological observation of kidney damage, the expected uraemia did not occur. The plasma levels of urea are influenced by dietary factors (Osbaldiston and Moore, 1971) and the rats were fed ad libidum on a high protein diet. This was presumed to be the reason for the high 'normal' urea levels. During the infection, urea levels were raised on days 6, 7 and 9, but the increase was only found to be significant on day 7. As the disease progressed and the animals lost their appetites the dietary urea levels were probably reduced while the pathological levels were raised. Thus the raised levels recorded on days 6, 7 and 9 probably indicated kidney dysfunction, but because of the high control values and the fact that the origin of the urea was not identified, the parameter failed to provide the information for which it was employed.

In calf A89, urea levels showed a progressive rise before death. Calf 212 also showed a gradual rise, but this continued to reach 174.8 mg/100ml on day 16. The SDH and bilirubin values had reached peaks on day 14 and then began to recover. Judging by these parameters, the degree of organ damage in this animal must have been very severe, and the uraemia probably reflected almost total kidney failure. The prompt recovery following treatment was probably greatly assisted by the fact that liver function and erythrocyte numbers were recovering at the time. The degree of kidney damage was not so severe in the other calves.

During babesiosis the kidney is subject to the same general damaging forces as is the liver. But in disease states where either intravascular haemolysis or breakdown of muscle tissue with release of myoglobin occur, a specific kidney damage is found. Two theories are proposed to explain this damage. In one, an old theory, the pigments are thought to be precipitated within the tubules leading to vascular changes with gross intrarenal shunts, hypoxia or ischaemia. Goldberg (1962) revived this theory because of his finding of normal glomerular filtration rates during the establishment of tubular damage. Once the tubular damage was established vascular changes of hypoxia or ischaemia occurred. The other theory places these events in the reverse order. The haemodynamics of the kidney are altered and the tubular changes are secondary to hypoxia or ischaemia. This latter theory is favoured by Maegraith et al. (1957) and Malherbe (1966) in the pathogenesis of canine babesiosis. It is also favoured by Wright (1972a) in B.argentina and B.bigemina infections, in which he found that kidney damage preceded liver damage by one to two days, the kidney damage occurring before much erythrocyte destruction had occurred. In the present study, liver and kidney damage, observed histologically or detected by plasma indicators, began about the same time. However erythrocyte destruction had been observed before any signs of such organ damage. The general belief of Maegraith and his coworkers in the non-specific nature of the changes in malaria and babesiosis, in which widespread vascular changes occur, could justify their adoption of the second theory. Wright and Mahoney (1974) recently found that vaso-active substances were released before B.argentina parasitaemias became patent. These substances could

initiate vascular changes in the kidney before haemoglobin-induced changes take place. If this is the initiating mechanism of kidney damage, it does not resolve the question of whether vascular changes precede or follow tubular degeneration in purely haemolytic states. However the kinin-induced vascular changes might render the kidney more susceptible to haemoglobin damage once haemolysis of any consequence occurs. It has not been established if this very early kinin release occurs in conditions other than B.argentina infection. The fact that kidney damage was not found in the present study until erythrocyte destruction had occurred possibly indicates that the haemolysis was the more important factor in initiating kidney damage in B.rodhaini and B.divergens infections.

Both the observations of basement membrane thickening and glomerular shrinkage are subjective, as no quantitative measurements were undertaken. Annable and Ward (1974) have established beyond dispute the development of changes in the basement membrane of B.rodhaini infected rats caused by immune complex bombardment. This might well have been the mechanism of the changes observed in this study, but immunofluorescent studies were not carried out. The shrinkage of the glomeruli, which might also give an impression of basement membrane thickening, has been described from B.canis and B.argentina infections (Maegraith et al., 1957; Wright, 1972a). Maegraith associated glomerular shrinkage with anuria. In this study urinary retention was a consistent finding in rats and they might have been anuric.

The urine of calves was examined for the presence of haemoglobin, but a more complete examination of rat urine was undertaken. Severely ill rats showed a considerable deposit of proteinaceous material, casts

and intact erythrocytes indicating severe kidney damage. Spectrophotometric examination showed peaks of activity at wavelengths of between 390-430 nm and at 530 nm and 560 nm corresponding with the absorption spectra of haemoglobin and some of its derivatives (Varley, 1967). No attempt to determine whether these derivatives arose before or after leaving the kidney was made.

The splenic changes found in the rats are discussed in Ch. 4 and Ch. 7. The other tissues examined showed very little change. Haemosiderin was found in phagocytic cells throughout the spleen, liver and lung of rats and liver and lung of calves. In the kidney it was present in the cytoplasm of the epithelial cells of the convoluted tubules, in the debris in the tubules and in the debris within Bowman's space of the calves. The material in the space in rats was condensed and, while staining a light translucent blue it did not show the typical PRB reaction. Occasionally the spleen of control rats showed a mild PRB reaction and this would reflect normal splenic iron metabolism in an animal with a rapid erythrocyte turnover.

The lungs showed no histological change apart from increased phagocytic activity. However, calf 212 did show, on clinical examination, a developing pulmonary oedema. This type of change has been recorded in dogs with B.canis infection (Maegraith et al., 1957; Basson and Peinaar, 1965). The origin of this change was probably complex, being due to profound anaemia, severe kidney dysfunction and cardiac incompetence. Following therapy to support the heart and to increase urinary flow, the animal recovered quickly, but the return of the blood parameters towards pre-infection levels was slower than in the other calves.

Some small haemorrhages were found in the brain of calf 296, but no clinical nervous signs were observed. Whether these haemorrhages were a terminal phenomenon or were due to some other mechanism is not known. However, this type of change can result from a disturbance of the coagulation mechanism and this will be more fully discussed in Ch. 7. A similar mechanism could explain the thrombosis found in the meninges, liver and spleen of rats and the pathological consequences of these events could be very severe. The changes in cerebral vessels, with congestion, high local parasitaemia and perivascular fluid infiltration found during B.argentina infections were not found in infections with either B.rodhaini or B.divergens examined in this study. These parasites showed no tendency to concentrate in any organ or site, unlike other Babesia spp. Collins et al (1970) reported nervous signs during natural B.divergens infections, but from clinical experience of such infections, a more simple explanation might be offered. When a severely anaemic animal is forced to move or is startled into sudden movement it might suffer an acute cerebral anoxia because of the diversion of blood to the musculature. This might cause staggering, bellowing, collapse and, in rare cases, even death. It is unlikely that histological change would be observed in such animals.

Vascular changes leading to varying degrees of congestion throughout the body are reported from most studies of babesiosis. These were not commonly seen in the present investigation although some congestion of the meninges and the renal medulla was found in rats. As the majority of rats examined were exsanguinated, the blood distribution in the tissues might have been disturbed. Also, only two

calves were examined and such a small number might not be representative. Indeed Maegraith et al. (1957) found that of 36 dogs dying from B.canis infections, six showed no kidney changes whatsoever. Thus, while common patterns of pathological change may be formulated, individual animals might show wide variation in their disease responses. Different host species might show different susceptibilities to certain types of damage and different Babesia Spp. can provoke a different pathological response in the same host species.

CHAPTER 7

THE COAGULATION MECHANISM

I. Introduction

Disseminated intravascular coagulation (DIC) is a pathological process initiated in many disease states by a triggering of the coagulation mechanism resulting in fibrin formation. The fibrin formed may be rapidly degraded by the fibrinolytic system (Alkjaersig et al., 1959) or may lead to thrombosis. In the first instance, if the triggering stimulus is low grade and of short duration the fibrin is broken down and the degradation products (FDPs) are removed from the circulation by neutrophils and reticulo-endothelial cells (Barnhart, 1967). However, if the triggering episode is prolonged, it can lead to depletion of the coagulation factors and consequent haemorrhagic diathesis (Verstaete et al., 1965) which can be exacerbated by the potent anticoagulant action of certain FDPs (Fletcher et al., 1962; Kowalski, 1968). These events are believed to form the basis of abnormal bleeding tendencies sometimes found in the DIC syndrome. Alternatively, the stimulus to the coagulation mechanism may lead to thrombosis, either in the microcirculation or in larger vessels. Again, dissolution of the thrombi may occur (Merskey, 1973) or they may be very small and missed in histopathological examinations (Robboy et al., 1972). However, where appreciable thrombosis does occur, the consequences can be very severe, as in pulmonary embolism and glomerulo-nephritis found in infantile diarrhoea due to Escherichia coli infection (McKay, 1965). Copley (1954) believes that fibrin formation is a normal process in the maintenance

of the integrity of the vascular system and Roos (1957) has also suggested that intravascular fibrin formation and dissolution is a dynamic and continuous process. The syndrome of DIC then represents an accelerated rate of coagulation above the normal physiological rate caused by some factors arising from the primary disease.

DIC occurs widely in disease states of many different aetiologies (McKay, 1965), but it is of particular interest in the present study because it is often associated with diseases in which there is haemolysis, anoxia or anoxaemia, tissue damage and circulating antigen-antibody complexes. Mahoney and Goodger (1969) found a substance in sera from calves infected with B.argentina which was immunologically identical with fibrinogen. Some of the breakdown products of fibrinogen and fibrin are immunologically similar to the parent fibrinogen (Nussenzweig et al., 1961), and they postulated that a coagulation disturbance might occur in babesiosis giving rise to these products. In examining the literature there are indeed reports that might support this hypothesis. Smith and Kilborne (1893) found thrombosis in the liver of an animal infected with B.bigemina, Malherbe and Parkin (1951) described purpuric lesions in dogs with B.canis infection, and purpuric lesions are also commonly found in equine babesiosis (Sippel et al., 1962; Maurer, 1962). Increased megakaryocyte activity has also been observed during Babesia infections (Paget et al., 1962; Dorner, 1969), but thrombocyte turnover has not been measured. Possibly the most convincing evidence that the DIC syndrome exists in babesiosis is that presented by Basson and Pienaar (1965). They examined three cases of cerebral babesiosis in dogs and found that one dog had haemorrhagic lesions in the epicardium, endocardium, throughout the musculature and in the brain, together with

splenic infarcts while another dog had haemorrhagic lesions in the brain and bilateral thrombosis of the opthalmic veins.

Merskey (1973) has questioned the accuracy of the term DIC which has also been named consumptive coagulopathy or defibrination, because, in the wide spectrum of diseases within which it occurs and because of its varying manifestations, it may be neither disseminated, intravascular or coagulation. However, the term DIC is broadly accepted as descriptive of a disease syndrome in which the coagulation mechanism is triggered, coagulation factors are consumed, thrombocytes are reduced, the fibrinolytic system is activated and sometimes thrombosis or a haemorrhagic diathesis occur. The following experiments were carried out to test the hypothesis that a coagulation disturbance might contribute to the pathogenesis of babesiosis.

II. Materials and Methods

Experiment 7.1 : The RBC, Parasitaemia, Thrombocyte and Fibrinogen Levels during *B.rodhaini* Infection in Rats

This experiment was carried out to investigate the effects of *B.rodhaini* infection on the thrombocyte and fibrinogen levels of rats.

(i) Forty rats were inoculated with 10^7 *B.rodhaini*-infected erythrocytes and ten control rats were inoculated with normal erythrocytes equal in number to the total number of erythrocytes contained in the infective dose. Five test rats and two control rats were killed on day 3, 5, 6, 7, 8 and 9 and the parameters listed above were measured. Thrombocytes were estimated by the indirect method (Ch. 2 (10)). Fibrinogen was estimated by the method of Burmester *et al.* (1970) using 'Thrombin Topical' (Parke-Davis) to clot fibrinogen

and optical density readings of test solutions were recorded following thrombin action for ten minutes. Recordings were made at a fixed wavelength of 300 nm using a Pye Unicam SP1800 spectrophotometer.

(ii) As raised fibrinogen levels and thrombocytopenia were found this experiment was repeated using further strains of rats to determine whether the raised fibrinogen levels were related to the basal disease status of the host or a strain effect. Twelve rats of each strain (non-SPF Wistar and SPF Hooded) were used; six were infected and six treated as normal controls. Two infected SPF Wistars were added to each group. Three infected and three normal control rats of each strain, together with two SPF Wistars, were killed on days 3 and 5 and the same parameters were measured.

Experiment 7.2 : The Detection of FDPs in Rats Infected with *B.rodhaini*

FDP levels in the plasma are raised during DIC because the fibrinolytic system is activated in the syndrome and digests both fibrin and fibrinogen. A series of experiments was carried out in an attempt to detect and measure these products in rats. The techniques used were based mainly upon a FDP test assay kit designed to detect FDPs in human serum.

(i) Using the Wellcome FDP assay kit (Wellcome Reagents Ltd.)

FDPs and fibrinogen are assayed in a haemagglutination-inhibition system by a modification of the method using formalin-treated sheep red cells (Merskey *et al.*, 1966). A suspension of these fibrinogen coated cells is a very sensitive indicator of the presence of antibodies to fibrinogen, showing macroscopic agglutination in the presence of such antibodies under suitable conditions. Aliquots of a specific

anti-fibrinogen serum diluted to a concentration just high enough to agglutinate the sensitized cells are added to serial dilutions of the sample to be assayed. If sufficient fibrinogen is present in the sample it will combine with the anti-fibrinogen serum and sensitized red cells added subsequently will fail to agglutinate. The row of serial dilutions should thus show one or more unagglutinated patterns, a short intermediate zone and finally a number of agglutinated patterns. The last completely unagglutinated well is taken as the end-point and the amount of fibrinogen or FDPs in the sample can be assessed by comparison of the results obtained with the sample and with a known fibrinogen standard. The units of FDPs or fibrinogen measured are expressed in μg/ml.

Reagents : Fibrinogen sensitized sheep red cells

Anti-human fibrinogen serum

Fibrinogen standard (human)

Citrate buffer

Serum collection tubes containing both thrombin to clot the sample and an enzyme inhibitor to stop plasmin degradation of fibrin and fibrinogen

Sheep cells for absorption of heterologous species agglutinins.

The test was carried out using 0.025 ml volumes in the Flow Microtitre system.

The technique was used as in the booklet 'Detection of fibrinogen degradation products and fibrinogen' (Wellcome Reagents Ltd.). The test included the following controls : a reagent control of antiserum, buffer and sensitized cells, a buffer control of buffer and sensitized

cells and a sample control of sample, buffer and sensitized cells. Results were calculated from the sensitivity of the assay (the concentration of fibrinogen at the end-point) multiplied by the dilution of the sample end-point to give the concentration of FDPs in μg/ml from undiluted serum.

(ii) Using a FDP assay system prepared for experimental use.

An assay system identical in design to the Wellcome FDP kit already used but based on material from rats was set up according to the methods of Hoq and Das (1970) and Das (1970a,b).

Preparation of Fibrinogen. SPF Hooded rats were used as donors as it was found in preliminary experiments that SPF Wistar rats gave poor fibrinogen yields. Blood was drawn into 3.8% sodium citrate (10 volumes to 1 volume) and the plasma separated. To 10 ml of the plasma, 2 ml of 6M β-alanine (BDH) was added slowly and stirred gently, this gave a 1M β-alanine concentration. This was placed on an ice bath for 30 minutes and then centrifuged at 2000G for 30 minutes at 4°C. The slight precipitate was discarded. To the supernatant, 3 ml of 6M β-alanine was added slowly to give a final 2M β-alanine concentration. It was then left overnight at 4°C after which it was centrifuged at 9000G for 20 minutes at 4°C and the supernatant was discarded. The fibrinogen was dissolved in citrate saline to the original volume of 10 ml. The precipitation was repeated adding 5 ml of 6M β-alanine. This was placed on an ice bath for 30 minutes and then centrifuged at 2000G for 30 minutes at 4°C. This was repeated twice, finally adding citrate saline to give a final volume of 3.3 ml which was dialysed overnight against citrate saline. The purity of the fibrinogen sample was determined by measuring

the total protein and the fibrinogen level of both the original plasma sample and the final fibrinogen sample. Total protein was estimated by the Biuret method and fibrinogen estimated according to the method of Burmester et al. (1970). This yielded a fibrinogen sample in solution containing 149.3 mg/100ml of clottable protein. This was 75% pure fibrinogen and 25% unidentified protein.

The preparation of anti-rat fibrinogen serum. Anti-rat fibrinogen serum was prepared in two adult male New Zealand white rabbits. A serum sample was obtained from the rabbits as a pre-inoculation control. The antiserum was raised by the following regimen using the rat fibrinogen solution (149.3 mg/100ml). Rabbits were injected intramuscularly with 0.5 ml of fibrinogen solution on day 0 and then intravenously with 0.2, 0.4 and 0.8 ml fibrinogen solution on days 7, 17 and 21 respectively. A serum sample from each animal was collected on day 31 and tested for anti-fibrinogen activity and the rabbits were anaesthetized and bled for the main sample of serum on day 33. Both samples showed anti-fibrinogen activity on day 31 and the samples taken on day 33 were pooled and tested in the following way.

The antiserum was tested for anti-fibrinogen activity according to the routine methods of immunodiffusion and immunoelectrophoresis in Williams and Chase (1967). Immunodiffusion was carried out in 1% Ionagar No. 2 (Oxoid) in barbitone buffer, pH 8.6. The pre-inoculation serum, the day 33 anti-fibrinogen serum and a sample of absorbed day 33 antiserum were tested against normal rat serum, plasma and fibrinogen solution as antigens. The antiserum was absorbed by the incubation of ten volumes of antiserum with one of normal rat serum at 37°C for 30 minutes. The immunodiffusion plates were put in a

moist chamber at 4°C for 24 hours and then photographed.

Immunoelectrophoresis was also carried out in 1% Ionagar No. 2 (Oxoid) in a barbitone buffer pH 8.6 by applying a constant 150 volts using a Shandon Vokam 2541 power source. Electrophoresis was carried out for 70 minutes then the antisera added. The same antigens and antisera were tested as for immunodiffusion. The plates were incubated at 4°C for 24 hours then photographed.

In Fig. 7.1 the precipitation pattern in the immunodiffusion plates is shown. No precipitation was seen in the sample taken before priming of the rabbits with fibrinogen solution. Both the antisera showed common lines of identity with fibrinogen and plasma but no identity with serum. The strength of the line was similar in both antisera.

In Fig. 7.2 the immunoelectrophoresis patterns are shown. A single arc was present close to the plasma and fibrinogen wells following incubation with the day 33 absorbed and unabsorbed antisera, but no precipitation was present close to the serum antigen well. The pre-inoculation serum showed no precipitation with any of the antigens.

The finding of a single clear line of identity common to both the fibrinogen solution and the plasma and no reaction with the serum antigen, indicated that the antiserum raised against rat fibrinogen was of a specific anti-fibrinogen activity and the absorbed day 33 anti-rat fibrinogen serum was used in the experiments to detect FDPs.

Preparation of fibrinogen sensitized rat erythrocytes. Blood was drawn into ACD (Ch. 2 (12)) four volumes to one and the blood was allowed to settle for three days at 4°C. The plasma was removed

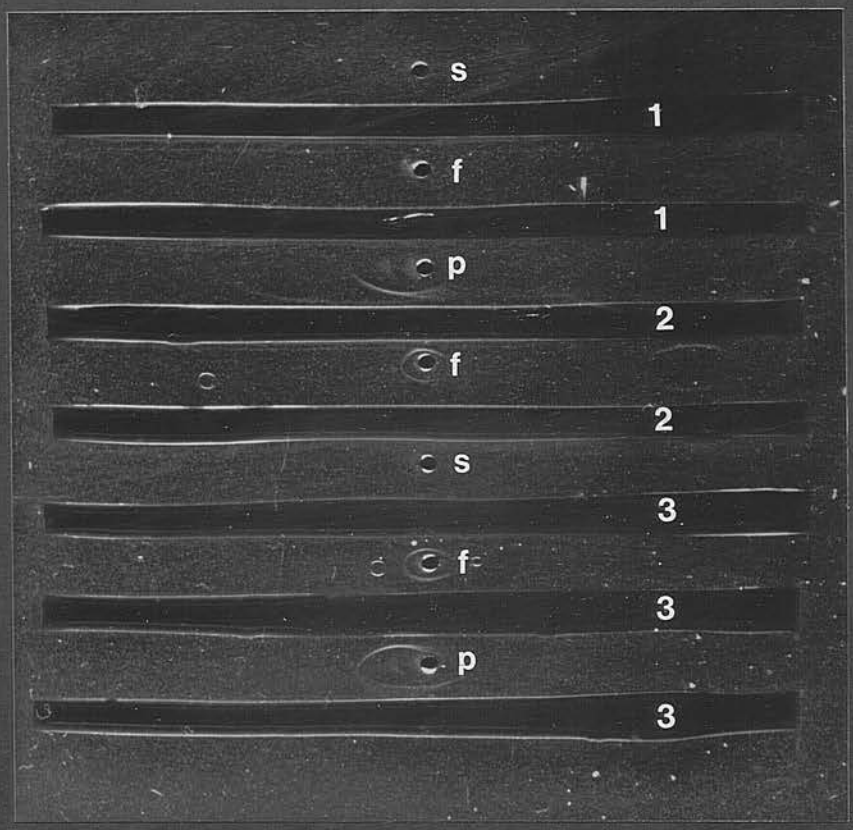
Fig. 7.1. The immunodiffusion plates showing the precipitation bands between the pre-inoculation serum (1) the day 33 anti-fibrinogen serum (2) and the day 33 absorbed anti-fibrinogen serum (3) and the antigens, normal rat serum (S), plasma (P) and fibrinogen (F) after 24 hours incubation.

S
P
1
F

S
P
2
F

S
P
3
F

Fig. 7.2. The immunoelectrophoresis plates showing the precipitation between the pre-inoculation serum (1) the day 33 anti-fibrinogen serum (2) and the day 33 absorbed anti-fibrinogen serum and the antigens, normal rat serum (s), plasma (p) and fibrinogen (f).


s
1
f
1
p
2
f
2
s
3
f
3
p
3

and the cells were washed in saline. The cells were then formalized and tanned according to the methods of Hoq and Das (1970) and Das (1970a). The cells were sensitized by incubation with fibrinogen at 37°C for 30 minutes. One volume of a 4% suspension of cells in buffer was incubated with one volume of fibrinogen solution (10 µg/ml of clottable protein) prepared from the fibrinogen sample containing 149.3 mg/ml of fibrinogen. The cells were then washed three times in buffer and stored at 4°C as a 10% suspension. These cells were used along with the rat fibrinogen antiserum in an assay system to detect FDPs according to the method of Das (1970b). The protocols of the test were identical with the Wellcome FDP assay kit. For the test, blood was drawn into Trasylol (Bayer) to a concentration of 80 iu/ml of blood. This blood was incubated at 37°C for four hours then centrifuged and 10 NIH units of thrombin were added to the serum. It was incubated again for one hour at 37°C then the sample was centrifuged and the serum, if not tested immediately, was stored at -20°C.

To determine the value of this experimental assay system in rats, eight rats were inoculated with 10^7 B.rodhaini-infected erythrocytes and six rats were used as untreated controls. On days 4 and 7 four test and three control rats were killed and RBC, parasitaemia and thrombocyte levels estimated and serum samples were collected as above for examination for FDPs.

(iii) The Plasma Protamine Paracoagulation Test

The addition of protamine sulphate to plasma containing soluble complexes of fibrin monomer with fibrinogen degradation products permits the release of the fibrin monomer molecules which polymerize and appear

as insoluble visible fibrin fibres. Normal plasmas do not contain these soluble complexes. The plasma protamine paracoagulation test was carried out according to the method of Seaman (1970). Blood was drawn into 3.8% sodium citrate (10 volumes to 1 volume) and the plasma separated. One ml of citrated plasma was warmed to 37°C, and then 0.1 ml of 1% protamine sulphate (B.D.H.) was added. The contents of the tube were mixed by tilting and returned to the water bath at 37°C for 15 minutes. The tube was tilted and examined and if fibrin threads were present the test was positive; opalescence was regarded as negative.

This test was applied to plasma from 14 rats. Eight rats were inoculated with 10^7 B.rodhaini infected erythrocytes and six rats were used as uninfected controls. On days 3 and 6, four test and three control rats were killed and sampled and RBCs, parasitaemia and thrombocytes were measured and samples of plasma were examined for fibrin monomers by the paracoagulation test.

(iv) Using a modified Wellcome FDP assay kit

In this experiment the Wellcome FDP assay kit was used, but the anti-human fibrinogen antiserum was replaced by the antirat fibrinogen serum raised in rabbits. Otherwise the protocols of the test were the same.

Blood was collected into Trasylol at 80 iu/ml, incubated at 37°C for four hours, then introduced into thrombin, 10 NIH units/ml for one hour at 37°C. The serum was absorbed with an equal volume of formalized sheep red cells overnight at 4°C then centrifuged at 1500G for 20 minutes and the serum harvested and stored.

(a) This system was tested using seven rats infected with 10^7 B.rodhaini-infected erythrocytes and two normal controls. The RBCs, parasitaemia, thrombocytes and serum FDPs were estimated on day 6.

(b) This experiment was repeated using 12 infected rats inoculated with 10^7 B.rodhaini-infected erythrocytes and six controls inoculated with normal erythrocytes equal in number to the total erythrocyte number contained in the infective dose. Four test and two control rats were killed on days 3, 5 and 7, and RBCs, parasitaemia and serum FDPs measured.

Experiment 7.3 : The Thrombocyte and Fibrinogen Levels, and the Protamine Paracoagulation Test, during B.divergens Infection in Calves

Haematological data obtained during the course of B.divergens infections in seven splenectomized calves have been presented in Ch. 4. In the present experiment thrombocytes were estimated in fresh citrated whole blood from these animals, the protamine paracoagulation test was applied to fresh citrated plasma and the fibrinogen levels were estimated in stored citrated plasma. Thrombocytes were estimated by the direct method (Ch. 2 (10)) and control values were established for the group on day 5 before infection. Fibrinogen was estimated by the method of Burmester et al.(1970) and a preinfection control mean value for each animal was established in samples taken on three separate days prior to infection. To check that the technique was showing a consistent reading and that fibrinogen activity was not lost with freezing and thawing a citrated plasma sample from a normal calf was used as a system control. The sample was divided into aliquots and any one aliquot was used four times and discarded. A control sample

Table 7.1

RBC, parasitaemia, thrombocyte and fibrinogen levels during *B.rodhaini* infection

	Control	Day 3	5	6	7	8	9
RBC $x10^6/_{mm}3$	6.8±0.1	6.3±0.2	6.0±0.2	3.8±0.7	1.82±0.2	3.9±0.2	4.6±0.7
Parasitaemia %	0	1.0±0.2	8.7±2.0	24.8±8.6	3.8±0.5	0	0
Thrombocytes/$_{mm}3$	1,159,300	842,900	262,300	100,400	207,900	1,072,300	1,613,300
	±44,000	±67,900	±16,100	±15,700	±33,800	±155,400	±79,400
Fibrinogen mg/100ml	194.7±6.6	259.0±11.7	225.0±117	251.6±13.8	282.3±9.0	212.8±13.3	177.8±4.3

was estimated with each batch of test samples.

III. Results

Experiment 7.1 : The RBC, Parasitaemia, Thrombocyte and Fibrinogen Levels during the Course of *B.rodhaini* Infection in Rats

(i) The parasite development and erythrocyte loss followed a similar pattern to that found in earlier experiments, but the parasitaemia was somewhat lower (Table 7.1). The parasitaemia declined rapidly from day 6 and erythrocyte numbers had begun to recover on day 8. Thrombocyte numbers had fallen on day 3 and this fall continued to reach a nadir on day 6, the day of peak parasitaemia. Thrombocytes had begun to recover in number on day 7 as the parasitaemia declined. On day 8 they had almost recovered to the control value and on day 9 there was a marked thrombocytosis, on days 7 to 9 many large and bizarre shaped thrombocytes were observed. Fibrinogen values showed a very pronounced rise from a control value of 194.7 $\pm$ 6.6 mg/100ml to 249.0 $\pm$ 11.7 mg/100ml on day 3. The fibrinogen level then remained above normal until parasites disappeared on day 8. On day 9 the level had fallen to slightly below the control value.

(ii) Both the Hooded and non-SPF Wistar rats developed parasitaemia and anaemia. Thrombocytopenias and elevated fibrinogen levels were found in these small groups of rats like those found in SPF Wistar rats in Exp. 7.1(i). The results are presented in Table 7.2 and 7.3 and it can be seen that changes were apparent while the parasitaemia was low.

Table 7.2

RBC, parasitaemia, thrombocyte and fibrinogen levels during B. rodhaini infection in non-SPF Wistar rats

Day	Animals	RBC $x10^6/mm^3$	Parasites %	Thrombocytes $/mm^3$	Fibrinogen mg/100ml
3	Test	6.99±0.1	4.8±0.1	530,000±19,000	267.9±18.9
	Normal	7.23±0.2	0	935,000±36,000	199.4±15.0
	Wistar Control	6.03	18.3	380,000	271.6
5	Test	3.64±0.5	55.2±5.9	125,000±15,000	264.1±2.4
	Normal	7.16±0.2	0	1,301,000±14,000	181.3±8.5
	Wistar Control	2.90	61.7	171,000	274.1

Table 7.3

RBC, parasitaemia, thrombocyte and fibrinogen levels during B. rodhaini infection in Hooded rats

Day	Animals	RBC $x10^6/mm^3$	Parasites %	Thrombocytes $/mm^3$	Fibrinogen mg/100ml
3	Test	6.52±0.2	1.57±0.4	748,000±20,000	238.2±11.9
	Normal	6.60±0.2	0	845,000±53,000	200.7±2.4
	Wistar Control	5.91	10.3	471,000	251.0
5	Test	6.09±1.0	16.4±8.3	278,000±13,000	250.0±11.9
	Normal	6.66±0.2	0	1,119,000±47,000	205.9±7.3
	Wistar Control	3.10	48.3	209,000	284.5

Experiment 7.2 : The Detection of FDPs in Rats Infected with B.rodhaini

(i) Using the Wellcome FDP assay kit.

The parasite development and erythrocyte loss in the rats were similar to other experiments and a thrombocytopenia was apparent (Table 7.4). The FDP test showed no inhibition of haemagglutination. The controls included in the test indicated that the components of the test were functioning normally and there was no evidence of species agglutination.

(ii) Using an FDP assay system prepared for experimental use

Again anaemia developed with the rise in parasite numbers and the thrombocytes were reduced (Table 7.5). The pattern of haemagglutination in the test wells was irregular, although the control wells indicated that the components of the test were functioning. The anti-rat fibrinogen serum gave a fibrinogen sensitivity reading with the human fibrinogen standard, comparable to that in the previous experiment, indicating that the anti-rat fibrinogen serum was reacting satisfactorily with the human fibrinogen. Because of the irregular haemagglutination found no FDP values were recorded.

(iii) The Plasma Protamine Paracoagulation test in rats

Again the patterns of parasite development, anaemia and thrombocytopenia were similar to those found in earlier experiments, but the rats showed no positive paracoagulation tests (Table 7.6).

(iv) Using a modified Wellcome FDP assay kit

(a) All the infected rats showed a very high parasitaemia and a marked anaemia. Thrombocyte numbers were also greatly reduced. There were no changes in the normal rats. The assay detected FDPs in both

Table 7.4

RBC, parasitaemia, thrombocyte and FDP (Wellcome kit) levels during *B. rodhaini* infection

Day	Animals	RBC $x10^6/mm^3$	Parasites %	Thrombocytes $/mm^3$	FDPS µg/ml
3	T_1	6.47	2.5	380,000	-
	T_2	5.93	8.2	275,000	-
	T_3	6.19	4.8	763,000	-
	T_4	6.38	6.1	417,000	-
	C_1	6.72	0	1,090,000	-
	C_2	6.50	0	996,000	-
	C_3	7.40	0	1,120,000	-
6	T_1	2.91	56.2	109,000	-
	T_2	5.35	27.5	280,000	-
	T_3	1.07	71.3	201,000	-
	TT_4	1.66	68.5	77,000	-
	C_1	6.85	0	1,212,000	-
	C_2	6.63	0	1,036,000	-
	C_3	6.70	0	1,001,000	-

Table 7.5

RBC, parasitaemia, thrombocytes and FDP (rat system) levels during *B.rodhaini* infection

Day	Animal	RBC $x10^6/mm^3$	Parasites %	Thrombocytes $/mm^3$	FDPS µg/ml
3	T_1	6.30	9.1	330,000	-
	T_2	6.09	2.1	530,000	-
	T_3	5.63	2.4	743,000	-
	T_4	6.10	7.4	294,000	-
	C_1	7.10	0	840,000	-
	C_2	6.72	0	925,000	-
	C_3	6.83	0	1,082,000	-
6	T_1	4.45	20.0	75,000	-
	T_2	4.21	30.8	71,000	-
	T_3	3.41	52.1	46,000	-
	T_4	2.98	33.2	31,000	-
	C_1	6.54	0	981,000	-
	C_2	7.11	0	1,402,000	-
	C_3	7.16	0	1,195,700	-

Table 7.6

RBC, parasitaemia and thrombocyte levels and protamine paracoagulation tests during B.rodhaini infection

Day	Animal	RBC	Parasites	Thrombocytes	FDPs
3	T_1	6.26	6.4	480,000	-
	T_2	5.85	6.1	401,000	-
	T_3	5.80	32.1	110,000	-
	T_4	5.60	27.5	198,000	-
	C_1	6.54	0	1,281,000	-
	C_2	6.69	0	1,403,000	-
	C_3	6.90	0	1,003,000	-
6	T_1	2.32	74.8	192,000	-
	T_2	3.34	75.6	97,000	-
	T_3	3.69	69.8	161,000	-
	T_4	2.74	64.3	107,000	-
	C_1	7.03	0	1,310,000	-
	C_2	6.44	0	920,000	-
	C_3	6.93	0	1,121,000	-

Table 7.7

RBC, parasitaemia, thrombocyte and FDP (modified Wellcome Kit) levels during B.rodhaini infection (Exp. 7.2(iv)a)

Day	Animal	RBC	Parasites	Thrombocytes	FDPs
6	T_1	2.35	76.6	133,000	2.56
	T_2	1.70	85.3	162,000	1.28
	T_3	3.22	66.5	184,000	2.56
	T_4	1.97	77.6	115,000	2.56
	T_5	1.80	79.6	123,000	2.56
	T_6	1.84	81.2	130,000	2.56
	T_7	2.74	69.3	193,000	5.12
	C_1	7.50	0	1,301,000	1.28
	C_2	6.94	0	1,271,000	0.64

Table 7.8

RBC, parasitaemia, thrombocyte and FDP (modified Wellcome kit) levels during B.rodhaini infection (Exp. 7.2(iv)b)

Day	Animal	RBC	Parasites	Thrombocytes	FDPs
3	T_1	6.14	7.5	501,000	5.12
	T_2	6.47	9.2	380,000	5.12
	T_3	5.13	13.7	112,000	2.56
	T_4	6.71	3.0	759,000	2.56
	C_1	7.23	0	1,157,000	1.28
	C_2	6.61	0	1,093,000	1.28
5	T_1	3.41	31.0	53,000	2.56
	T_2	3.47	47.5	101,000	2.56
	T_3	3.68	49.5	52,000	2.56
	T_4	4.01	63.1	39,000	2.56
	C_1	7.65	0	994,000	1.28
	C_2	6.80	0	1,021,000	1.28
7	T_1	1.72	3.2	543,000	2.56
	T_2	3.12	0.6	674,000	1.28
	T_3	1.34	15.0	331,000	2.56
	T_4	1.83	4.4	184,000	1.28
	C_1	7.15	0	1,177,000	1.28
	C_2	6.94	0	991,000	0.04

Table 7.9

RBC, parasitaemia and thrombocyte levels during B.divergens infection

	Pre-infection	Day 7	8	9	10	11
RBC $x10^6/_{mm}^3$	9.86±0.44	9.19±0.43	8.92±0.43	9.13±0.43	8.50±0.54	7.22±0.77
Parasitaemia %	0	+(0.2)	+(0.7)	2.5±1.0	7.6±2.9	11.9±3.2
Thrombocytes/$_{mm}^3$	601,400	551,100	560,600	478,000	406,300	400,900
	±40,200	±21,200	±18,900	±28,300	±21,400	±21,900

12	13	14	15	16	20
5.35±0.68	4.27±0.57	3.20±0.43	2.82±0.43	3.03±0.32	4.05±0.43
17.8±3.7	11.8±3.5	7.1±3.0	2.3±1.5	+(0.2)	0
390,600	376,000	381,300	527,000	533,300	808,000
±25,100	±23,000	±38,900	±42,100	±80,600	±55,200

test and control animals (Table 7.7). In all samples except one, the serum FDP values in test rats were higher than the values in untreated animals.

(b) The infected rats developed parasitaemia, anaemia and thrombocytopenia as in earlier experiments, while the values in control rats remained unchanged (Table 7.8). On day 7 the parasitaemia had fallen and thrombocytes were showing a recovery in number. FDPs were detected in control animals to a maximum of 1.28 μg/ml. On days 3 and 5 the values in the test animals were either two or four times higher than the upper control level, while on day 7, two test animals had FDP values 1.28 μg/ml and the other two animals had twice this amount.

Experiment 7.3 : The Thrombocyte and Fibrinogen Levels and the Protamine Paracoagulation Test during B.divergens Infection in Calves

The mean values and standard errors for erythrocytes, parasites and thrombocytes of the seven splenectomized calves are presented in Table 7.9. Parasites were present in low numbers on day 7 and rose to a peak on day 12 from which they gradually declined. Erythrocyte numbers had fallen slightly to $7.22 \pm 0.77 \times 10^6$ per mm^3 on day 11 and reached a nadir of $2.82 \pm 0.43 \times 10^6$ per mm^3 on day 15. Thrombocyte numbers remained close to their pre-infection value until day 9 when they showed a slight fall. This fall continued until day 13, the day after peak parasitaemia, but then the number of thrombocytes began to recover and by day 20 there was a marked thrombocytosis. When thrombocyte numbers began to recover many large and bizarre thrombocytes were observed. The relationship between parasitaemia, RBC, thrombocyte and fibrinogen levels from calf 251 are shown in Fig. 7.3.

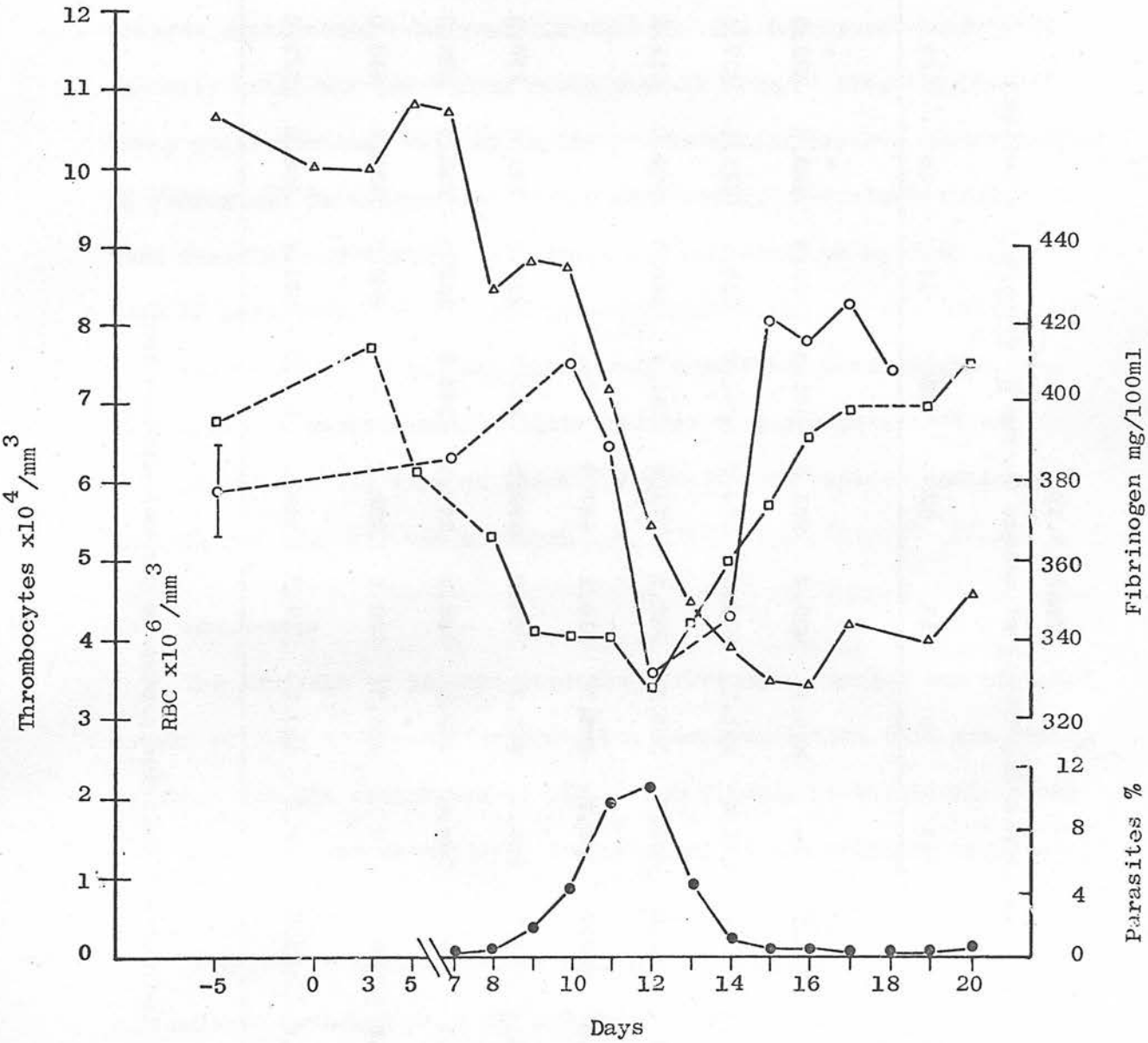


Fig. 7.3. Daily parasitaemia (●), RBC (△), Thrombocyte (□) and fibrinogen (○) levels during B.divergens infection in calf 251.

Table 7.10

Fibrinogen levels (mg/100ml) of calves during *B. divergens* infection

Day	Pre-Infection	7	9	10	11	12	13	14	15	16	17	18
Calf No.												
212	436.6±10.8	342.7	-	369.9	429.4	371.6	400.5	429.4	483.6*	433.0*	498.0*	487.2
275	345.1±17.8	331.9	313.8	321.1	378.8	324.7	303.0	324.7	411.3	317.5	425.8	331.9
L1160	352.3±11.9	443.8	400.5	386.1	343.5	371.6	360.8	360.8	360.8	317.5	407.7	393.3
A89	394.2±7.3	422.0	465.5	483.6*	516.1	487.2	D					
273	330±24.1	281.3	266.9*	281.3	303.0	295.8	303.0	252.5	321.0	270.5	259.7	252.2
274	375.6±6.1	-	360.8	313.8	389.9	277.7	245.2	223.6	288.6	238.0	274.1	292.2
251	376.4±7.3	386.1	-	411.3*	389.9	328.6	-	346.3	422.2	415.0	425.8	407.7
Control Sample	509.9±2.5	512.5	505.2	523.3	519.7	516.1	494.2	501.6	508.8	523.0	512.5	501.6

* Indicates a positive protamine paracoagulation test

In Table 7.10 the fibrinogen values for the seven calves are presented and the days on which a positive paracoagulation test was recorded are marked with an asterisk. The pre-infection samples for each animal showed some variation. Following infection the fibrinogen levels continued to vary, but there was a general trend towards depression. However, in Calf A89 the fibrinogen level rose steadily until the day before death then it dropped slightly, but its level still remained well above its pre-infection value. Estimations of fibrinogen in the control sample were fairly consistent indicating that freezing and thawing or storage did not cause an appreciable loss of activity.

Calves A89, 273 and 251 all showed a positive paracoagulation test early in parasitaemia. Calf 212 showed a positive test on days 15, 16 and 17, days on which its thrombocyte numbers continued to fall and the calf was moribund (Ch. 4).

IV. Discussion

The findings of thrombocytopenia, fibrinogen changes and elevated levels of FDPs or a positive protamine paracoagulation test are strong evidence for the occurrence of DIC. The finding of thrombosis along with these changes establishes the presence of the syndrome beyond doubt.

Attempts to detect FDPs in rats proved difficult despite the accumulated evidence of a DIC syndrome in which FDPs are said to be invariably present (McKay, 1969; Deykin, 1970). The initial attempt to measure FDPs employed a commercial assay kit prepared for use in man in which rabbit anit-human fibrinogen serum and sheep erythrocytes

coated with human fibrinogen were used in testing for rat FDPs. The rat serum was preabsorbed with sheep cells to remove any heterologous species agglutinins and the use of anti-human fibrinogen serum to test for rat FDPs caused no complications as the fibrinogen molecules from different species are very similar (Barnhart, 1967) and yet no inhibition of agglutination was found. In the second attempt to assay FDPs an all rat system was used, avoiding the need to preabsorb the test serum. Difficulties were encountered with this experimental system. The haemagglutination patterns were very irregular and the problem was thought to be the impure (75% fibrinogen and 25% unidentified protein) fibrinogen coated onto the erythrocytes, although Das (1972) argues that pure anti-fibrinogen serum and impure fibrinogen antigen or vice versa should work adequately in this test.

A protamine paracoagulation test was then used in the search for FDPs but it gave no positive readings. This test identifies circulating fibrinogen monomers produced during fibrinogen proteolysis by thrombin and is regarded as diagnostic of intravascular coagulation (Seaman, 1970; O'Leary et al., 1972). The failure of this test, despite the occurrence of DIC, was possibly due to the breakdown products of fibrinogen or fibrin being present in very small amounts in the circulation, or possibly as a result of the hyperactivity of the reticulo-endothelial system already identified during this Babesia infection. Finally, using anti-rat fibrinogen serum in the Wellcome assay kit in place of anti-human fibrinogen serum, it was possible to detect FDPs in both normal and infected rats. While the infected animals showed FDP levels two or four times those of control animals, much

higher FDP levels have been recorded in other DIC syndromes. This finding may reflect the hyperactivity of the reticulo-endothelial system in maintaining low levels of FDP as suggested, but it may also be due to the insensitivity of the test. Since the completion of these experiments, Boreham and Facer (1974) have reported the successful application of the Wellcome assay kit system to the measurement of FDPs in rabbits with a DIC syndrome associated with Trypanosoma brucei infection. They found that the test would only work after prior absorption of the rabbit serum with either fibrin or fibrinogen-coated erythrocytes. The complicating factor, on which further work is being carried out, was thought to be an antibody against some component of the fibrinogen molecule or its derivatives. It is possible that a similar factor might exist in the rat serum, but this possibility was not investigated.

The application of the protamine paracoagulation test to test for evidence of fibrinogen breakdown during B.divergens infection proved much more successful. Positive results were obtained with plasma from three calves prior to peak parasitaemia, while samples from calf 212 were positive on days 15, 16 and 17, when its thrombocytes were also low and the animal hovered near death.

The coagulation mechanism of the body is counterbalanced by a fibrinolytic system otherwise once coagulation is initiated it would lead to vascular occlusion. Haemostasis is essential in the maintenance of life, but it is equally important that the vasculature remain patent. While the actual mechanisms of coagulation and fibrinolysis are not the primary concern of this study, the effects of the fibrinolytic system on formed fibrin and fibrinogen can be

important in disease pathogenesis once intravascular coagulation occurs.

The action of the fibrinolytic system on fibrin or fibrinogen produces seven breakdown fragments (Marder et al., 1967), four of which are recognized by anti-fibrinogen serum, and can be identified by the haemagglutination inhibition test in FDP assays. These breakdown products have biological properties that may effect the character of disease changes. Their anticoagulant effect that may potentiate haemorrhagic disorders has already been mentioned. They are capable of greatly increasing the vascular permeability and of increasing the resistance of erythrocytes to hypotonic saline (Triantaphyllopoulos and Triantaphyllopoulos, 1970). FDPs also directly effect the liver synthesis and release of fibrinogen (Barnhart et al., 1970). The effect of FDPs on vascular permeability might contribute to the vaso-active effects of kinins which are also known to be greatly increased in babesiosis (Goodwin and Richards, 1960; Wright, 1973). Whatever effects FDPs might have had upon the hypotonic saline fragility of erythrocytes, in both of the infections examined in this study the factors acting to increase erythrocyte fragility were much more powerful.

In both the rats and the cattle infected in this study a pronounced fall in thrombocytes was recorded which began with the rise in para-sitaemia but once the numbers of parasites had reached a peak and begun to decline thrombocyte numbers recovered. In the early recovery from the disease there was a noticeable thrombocytopenia in both species. The primary function of thrombocytes is in haemostasis and the major cause of the thrombocytopenias almost certainly lies in a disturbance of the coagulation mechanism but other factors may have

contributed to the thrombocytopenia.

Thrombocytes are complex cells and recent research has provided much new information on their function. They are actively phagocytic, a function first established by Govaerts (1921) and confirmed by electron microscopic studies (Movat et al., 1965). They have been shown to remove malaria parasites from the blood (Thompson and Huff, 1944; Fajardo, 1973) and to engulf other particulate matter such as viruses, bacteria and antigen-antibody complexes. In this scavenging role they provide an early protective action for the host (Danon et al., 1959) and this probably accounts for the transient fall in thrombocyte numbers recorded in many infectious conditions.

In conditions in which splenomegaly occurs a thrombocytopenia is almost invariably recorded. This is due to the sequestration of the thrombocytes in the hyperactive spleen by a mechanism similar to that already discussed for erythrocyte sequestration. At any given time the spleen contains a thrombocyte pool estimated to be 30% of the total circulating thrombocyte mass. The thrombocytes of this pool are in constant dynamic exchange with those in the circulation and an increase in splenic size and activity results in a corresponding increase in splenic pool size and a considerable reduction in the circulating thrombocyte number (Penny et al., 1966). In the present study, enlarged and hyperactive spleens probably added to the thrombocytopenia of the rats, but could not have been a factor in the thrombocytopenia found in calves.

In both trypanosome infections and malaria coagulation disturbances have been recorded and thrombosis has been found in Trypanosoma congolense infections in cattle (Fiennes, 1970) and in P.falciparum infections in man (Spitz, 1946). However, in recent studies of infections with

these parasites thrombocytopenias have been found without any changes in coagulation factors or thrombosis. Neva et al. (1970) demonstrated that in some P.falciparum infections in man an immunological mechanism was damaging the thrombocytes, while Davis et al. (1974) found that some product of T.rhodesiense was damaging thrombocytes in experimental infections in rats. In the present investigation mechanisms of selective thrombocyte damage were not thought to be acting as other evidence pointed to coagulation disturbances.

The level of thrombocyte numbers in the circulation is under humoral control and a fall below threshold levels results in the release of thrombopoietin (de Gabrielle and Pennington, 1957a,b). This substance acts by stimulating an increase in megakaryocyte production from precursor cells and by increasing the amount of thrombocyte-producing cytoplasm of these cells. Both Paget et al. (1962) and Dorner (1969) found increased numbers of megakaryocytes in their studies of B.rodhaini and B.canis infections, which probably indicated a response to thrombocytopenia. During the course of this study no detailed measurements of megakaryocyte numbers were undertaken, but they were present in at least comparable numbers to those of control animals and their appearance was normal in the bone marrow and spleen of rats. However, large and bizarre thrombocytes were seen in rats from early in infection, and similar forms appeared somewhat later in calves. According to Kraytman (1973) these are an indication of megakaryocyte response to a thrombocytopenic stimulus, and also suggest that the thrombocytopenias observed were not due to bone marrow damage or inhibition.

Thrombocytes are a potent source of chemically active substances,

containing most of the body serotonin and a proportion of its histamine and adrenaline (Zucker, 1962). In their involvement in either phagocytosis or aggregation they undergo degranulation and release of these substances. It has already been noted that FDPs have an effect on the vasculature, and now another possible source of vaso-active substances must be considered in assessing the changes that could occur during babesiosis in which a DIC syndrome occurs.

With a triggering of the coagulation mechanism and fibrin formation a fall in circulating coagulation factors would be expected. However, in rats fibrinogen levels were elevated very early in infection and fibrinogen remained high until the numbers of parasites had begun to decline. This increase in fibrinogen, along with a thrombocytopenia, was also found in both a non-SPF Wistar and a Hooded strain of rats. This finding is not without precedent for in malaria of monkeys and in trypanosomiasis of rabbits, raised fibrinogen levels have also been found as components of a DIC syndrome accompanying the primary disease (Dennis et al., 1966; Boreham and Facer, 1974). One of the effects of FDPs is to stimulate hepatic fibrinogen synthesis and release (Barnhart et al., 1970). The response to this stimulus probably depends upon the species involved, the level of FDP stimulus and the state of health of the liver. Host species which show elevated fibrinogen levels presumably exhibit a sensitive response to this stimulus.

In calves, the fibrinogen levels did not show a consistent pattern of change. Calf A89 showed a gradual rise prior to death while the other calves showed an irregular depression of this parameter. The fibrinogen level of cattle is normally very high and in disease states

the plasma levels are very variable (Schalm, 1970). In general, fibrinogen levels of cattle are elevated during infectious disease, although in anaplasmosis they stay within the normal range (Schalm, 1970). The finding in this investigation of an irregular depression might have some significance, but as very little is known about changes in the fibrinogen parameters in cattle disease, no definite conclusions could be drawn from the observation.

In diseases in which splenomegaly occurs vascular accidents within the spleen are frequent and splenic vein thrombosis is not uncommon (de Gruchy, 1970; Jacob, 1974). Jacob considers that these vascular accidents might be either due to too much blood flow to the organ or because the blood supply might not be adequate to nourish the hyperactive tissue. The explanation of the splenic lesions found in rats in the present study is more likely to be a generalized coagulation disturbance causing thrombosis, evidence of which was found in the spleen, liver and meninges. In DIC syndromes, fibrin thrombi begin to form as fibrin aggregates in the systemic circulation and are carried into smaller vessels where they become tightly packed (McKay, 1969). These thrombi tend to be trapped in the very small vessels usually of the blood filtering organs and if they are not digested they can lead to tissue damage, especially in organs with an end-arterial blood supply. However these thrombi are often very difficult to find (Rabiner and Friedman, 1968; Robboy et al., 1972). Robboy et al. (1972) have demonstrated that specific organs are more likely to be targets for the accumulation of thrombi. For example thrombosis is common in the liver of still born infants as this organ receives a large blood supply from the placenta, likewise, the kidney is a common target as

it receives a considerable proportion of the cardiac output. Also, in sickle cell anaemia, in which haemolysis, splenomegaly and DIC occur, splenic infarcts are frequently found as well as pulmonary and cerebral thrombosis (Lau, 1959; McKay, 1965). The enlarged spleens found in rats infected with B.rodhaini in the present study were probably more susceptible to thrombotic damage in the DIC syndrome because of their increased blood supply, their blood filtering role and their restricted collateral circulation. A similar explanation might be offered for the splenic infarcts found by Basson and Pienaar (1965) in B.canis infection. However, in the calves with B.divergens infection thrombosis was not found but fibrin strands were present in the sinusoids of the liver and minor fibrin formation has been reported in the cerebral vessels of dogs with B.canis infection (Maegraith et al., 1957). As this type of change can be found during routine post mortem examinations following disease in which DIC has not been a component it cannot be accepted as evidence of DIC (Robboy et al., 1972). The possible triggering mechanisms of the coagulation disturbance found in the present study were not investigated. However, haemolysis, tissue damage, anoxia and circulating antigen-antibody complexes are capable of triggering DIC and occur in babesiosis.

The suggestion by Mahoney and Goodger (1969) that DIC might occur in babesiosis has been substantiated in the present experimental study. No investigations into the occurrence of the DIC syndrome in natural Babesia infections have been undertaken but there is a body of evidence to suggest that it occurs and that it is manifest both by thrombosis and haemorrhagic diathesis.

CHAPTER 8

GENERAL DISCUSSION

In both the B.rodhaini and B.divergens infections examined in this study, the development of anaemia and the other pathological changes were very closely related to the development of parasitaemia. Once the parasitaemia declined recovery was apparent. The experimental systems were deliberately chosen to provide models of severe babesiosis in which the development of infection and consequent pathological changes would outstrip the innate and specific immune responses of the host in the early stages of infection.

In both the discussion and interpretation of many of the changes observed in babesiosis frequent reference has been made to analogous findings reported in studies of malaria. Attempts to draw analogies between one disease and another are not always justified but these two diseases have much in common, although for various reasons the pathogenesis of malaria infections has been much more thoroughly examined. While the life cycles of Babesia and malaria parasites are different, both parasites have a phase where they are free in the plasma before they invade the erythrocyte. The development of malaria parasites within erythrocytes and their biochemical requirements are better known than in the case of Babesia Spp. but this study and others (Rudzinska and Trager, 1962; Dolan and Carr, 1974) have shown similarities in their physical relationships with the host erythrocytes. Many of the experimental examinations of both infections have been undertaken in the same host species and some of the changes recorded have been similar. Furthermore, B.rodhaini infections in the rat were

recently considered a suitable model for investigations of immuno-pathological phenomena of malaria (Iturri and Cox, 1969; Annable and Ward, 1974).

Maegraith et al. (1957) regarded the pathological changes found in B.canis infections as non-specific and similar to those of malaria and other acute disease states. These changes result from the infection of the host by the parasite and the responses of the host to the parasite. The parasite in the erythrocyte initiates the disease process and then the metabolizing organs become involved and disturbances in the biochemical and physiological balance of the host occur. Thus a series of local and general changes are brought about and, as the disease progresses, a complex of inter-dependent physiological mechanisms are disturbed (Maegraith, 1966). Circulatory changes are fundamental in the pathogenesis of malaria and Babesia infections (Goble, 1966). Garnham (1966) has summarized the origin of these changes in malaria as resulting from stimulation of the vasomotor centres of the brain and the sympathetic nerve supply of the organs by non-specific substances of parasite or host-parasite origin. These stimuli lead to local or general vascular changes, and evidence of this type of change in babesiosis frequently takes the form of organ congestion or renal ischaemia. Maegraith et al. (1957) also demonstrated a shock syndrome in B.canis infection and the finding of kinin-like substances during B.rodhaini infection (Goodwin and Richards, 1960) provides a reasonable explanation for this type of shock. More recently Wright (1973) has demonstrated similar substances (e.g. kallikrein) in B.argentina infections and these have been shown to be present from an early stage of infection (Wright and Mahoney, 1974).

Wright (1974b) regards vaso-dilatory shock as one of the earliest pathogenic mechanisms of B.argentina infection but it is not known if kallikrein activation occurs as early or at all in other Babesia infections. It can be postulated that the finding of a coagulation disturbance in this investigation, which began early in infection and which would release vasoactive substances by thrombocyte degranulation and fibrinolysis might add to such vascular changes. It could also be, that the initiation of coagulation via the intrinsic pathway (Rowsell, 1968) could provide the activated Hageman factor which might activate the kallikrein system.

The circulatory changes of congestion, stasis, sludging and thrombosis have all been reported in babesiosis. Both congestion and stasis may be due to the stimulation of the nerve supply to the vascular system, but sludging, in which erythrocytes are contained in a "glassy precipitate" of fibrin (Garnham, 1966), and thrombosis, involve the conversion of fibrinogen to fibrin. The stage at which sludging becomes thrombosis depends upon the balance between coagulation and fibrinolysis. In B.rodhaini infections in this study, where thrombosis and tissue damage were found, the stimulus to coagulation was profound and the fibrinolytic system was probably unable to arrest thrombosis, and this condition persisted long enough for ischaemic necrosis to occur.

The occurrence or importance of DIC as a pathogenic mechanism in natural babesiosis has not been investigated. However, there are reports from studies of almost all forms of babesiosis which indicate that it probably does occur. The potential of this syndrome to compound an already severe condition resulting either from thrombotic

damage or haemorrhage cannot be overlooked. Either of these sequelae of DIC could well explain many of the less typical manifestations of the disease. They might explain, for example true syndromes of cerebral babesiosis that have their origin in a cause other than the probably unique changes induced by B.argentina. The meningeal thrombus found in the rat and the small haemorrhages found in the brain of one calf, if occurring at a vital site, could conceivably cause nervous signs and recognisable histological change.

The clinical disease pattern shown by calf 212 is of interest and may reflect a disease complication resulting from a coagulation disturbance, although the evidence for the presence of DIC gathered from B.divergens infected calves was not as complete as that in the rodent infection. The parasitaemia in this calf had fallen considerably by day 15, erythrocyte recovery was apparent by day 16, and the leucocyte count had reached a peak on day 16 (Appendix (3)) yet this animal hovered near death. The uraemia had reduced considerably on day 17, but both bilirubin and SDH values, which were returning towards normal from a peak on day 14, showed a second crisis on day 18 without any further erythrocyte loss. The only indicators of a disturbed physiological mechanism at this stage were the continued fall in thrombocyte numbers, until day 17, and positive protamine paracoagulation tests on days 15 to 17. It could be that the liver had been damaged by the coagulation disturbance. This disease pattern resembles some field cases of bovine babesiosis, in which despite successful elimination of the parasite, the animals fail to show the expected recovery. If a coagulation disturbance is part of the disease complex that interferes with recovery following specific anti-babesial treatment

then supportive therapy with anti-coagulants, could benefit such animals and hasten recovery.

In considering the potential causes of erythrocyte loss or reduction in efficiency in rats the roles of the spleen and the RES were emphasized (Ch. 4). It was concluded that in B.rodhaini infections the contribution of this organ and system to the anaemia was not very great. It was not, however, intended to place an undue emphasis upon the possibility of the host destroying itself, as the examination or discussion did not include the total role of the spleen in the disease. The protective, rather than the destructive, function of the spleen was more than adequately demonstrated by the need to splenectomize calves before a measurable pathological effect could be induced by B.divergens. Splenic and RES mechanisms, however, probably contribute substantially to the serious loss of erythrocytes in chronic B.canis infections and to the predominantly extravascular haemolysis associated with B.gibsoni infection in dogs (Shirlaw, 1938; Seneveritna, 1965). However the relative importance of the immune response, the RES and the spleen in these diseases has not been determined.

Further mechanisms of erythrocyte loss might be encountered associated with the coagulation disturbance found in the experimental systems of babesiosis examined, although their contribution to the anaemia would be small. Some erythrocytes might be lost by being trapped in the fibrin mesh of thrombi while other erythrocytes may be trapped by the thrombus in nec rotic tissue following infarction. The fine fibrin strands formed during the coagulation episode provide another hazzard for circulating erythrocytes. These cells strike

the fibrin strands while moving rapidly in the circulation and are fragmented (Brain et al., 1967). In this way erythrocytes are destroyed but the fragments continue to circulate for some time and can presumably, carry out a limited oxygen transport function. These fragments or schistocytes have a mis-shapen appearance (Bull and Kuhn, 1970) and would contribute to the variety of abnormally shaped erythrocytes observed by SEM in Ch. 5. If the coagulation disturbance was manifest as a haemorrhagic diathesis then this would also contribute to the anaemia.

The use of SEM (Ch. 5) to visualize alterations of the surface of erythrocytes induced by B.rodhaini has illustrated many changes and has led to much speculation as to how they came about. It has already been suggested that some surface defects may lead to a loss of cell deformability. It has been found in Plasmodium infections that parasitized cells lose their flexibility (Miller et al., 1971, 1972). The loss of flexibility can lead to a blockage of small blood vessels. This might be the initiating mechanism in the aggregation of erythrocytes found in B.argentina infection following which changes in the parasite, erythrocyte and vascular endothelium occur. Wright (1972b) does not consider this a likely mechanism, arguing that B.bigemina, a much larger parasite than B.argentina would be much more likely to cause mechanical blockage but it does not do so. Smith and Kilborne (1893) originally made this suggestion of mechanical blockage of small blood vessels by B.bigemina when it reached its mature size within the erythrocyte. What has been overlooked in these arguments, however, is that firstly, Babesia parasites are amoeboid and therefore deformable and consequently unlikely to be

trapped, except by very fine passages such as those in the splenic sinusoidal wall (Ch. 4): secondly, if the parasite induces biochemical changes of the erythrocyte membrane, it would be the degree of loss of flexibility of the membrane that would lead to trapping of the erythrocyte and not the presence of the parasite. However, this too is speculation and until more is learned about the parasites' biochemical requirements and the nature of erythrocyte changes they induce, discussion must remain speculative.

The possibility that toxic substances play a role in the pathogenesis of babesiosis and malaria is unresolved. Smith (1973) in reviewing toxins in microbial disease regarded gross toxic effects of a non-specific nature as part of the pathogenesis of malaria. A similar non-specific toxic effect might also be presumed to occur in babesiosis, the origin of the 'toxin' being in the host-parasite interaction. The effects of this 'toxin' are possibly indicated by the common liver changes found in babesiosis and malaria (Ch. 6), the increasing 'lytic' effects of plasma in B.canis infection (Maegraith et al., 1957) and the increase in osmotic fragility of erythrocytes found in most Babesia infections.

In this investigation of pathological changes in babesiosis many organs and systems have been examined. The examination of an organ or system in isolation in the search for changes is useful, but in the disease process, especially in the case of a generalized disease, probably no organ or system will be affected alone. A system not directly affected by the parasite may be affected by changes in another system. The liver undergoing degeneration places a greater burden upon the kidney. The kidney, itself suffering damage, fails to perform

its normal functions and so throws a further stress back onto the liver. The homeostatic mechanisms thus will have difficulty in maintaining balance because of the failure of metabolizing organs. A consideration of the possible origin and effect of the fever found in B.divergens infections, and which is a consistent finding in the naturally occurring disease, can be used to illustrate the interactions between disturbed physiological mechanisms in the disease. The infection results in the release of pyrogens, possibly of parasite origin, but almost certainly endogenous pyrogens released following phagocytosis by neutrophils, monocytes or cells of the RES (Page, 1972). The presence of these substances in sufficient concentration alters the balance of amines in the hypothalamus and leads to elevation of the body temperature set-point (Bligh, 1966). The resulting pyrexia will lead to an alteration in the water and salt balance of the body by cellular retention of chlorides and alteration in protein metabolism by increasing nitrogen excretion (Best and Taylor, 1961). It is also interesting that the amines primarily responsible for body temperature control are serotonin and adrenaline. Both of these amines are present in considerable quantities in thrombocytes and it has already been described (Ch. 7) how they are released from the thrombocyte during phagocytosis of particulate matter and during aggregation in haemostasis or coagulation. Their possible effects upon the vasculature have been considered but it is possible that they also exert an influence upon the temperature control mechanism.

The examination of disease as a process, by exploring the patho-physiological mechanism underlying the pathology, has greatly increased the understanding of disease and led to more rational forms of therapy

(Frohlich, 1972). Maegraith and his co-workers adopted this approach to the study of malaria and babesiosis and it has also been followed in this investigation. Although the essentially pathological observations of parasite-induced erythrocyte changes demonstrated by SEM really only emphasize the need for further investigation into the host-parasite relationship and biochemistry of the parasite, the finding of a severe DIC syndrome has added considerably to the knowledge of the babesiosis disease complex.

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Appendix (1) Buffers

a. Phosphate Buffer for ABP8 (Lumsden et al., 1965)

Stock solutions 1 24.02 g/l sodium dihydrogen orthophosphate

2 36.89 g/l disodium hydrogen orthophosphate

pH 8.0 3.6 ml 1 to 96.4 ml 2

b. Giemsa Buffer, pH 7.2, prepared using buffer tablets supplied by Hopkins and Williams, Essex.

c. Buffered Sodium Chloride for osmotic fragility estimations (10% Na Cl)

90 g sodium chloride

13.65 g disodium hydrogen orthophosphate

2.43 sodium dihydrogen orthophosphate

disolved in distilled water and made up to 1 .

d. Phosphate Buffer (Sorensen's) used in antiglobulin tests.

Stock solutions 1 9.1 g/l potassium dihydrogen orthophosphate

2 11.9 g/l disodium hydrogen orthophosphate

pH 7.0 38.9 ml 1 to 61.1 ml 2

e. Phosphate Buffer (iso-osmotic) 0.15M used for SEM fixation

Stock solutions 1 23.4 g/l sodium dihydrogen orthophosphate

2 21.3 g/l disodium hydrogen orthophosphate

pH 7.4 18.0 ml 1 to 82.0 ml 2

Appendix (1) contd.

f. Barbital Buffer, pH 8.6, used for Millipore electrophoresis

0.331 g diethyl barbituric acid

1.848 g sodium diethyl barbiturate

120 ml distilled water

g. Barbitone Saline Buffer used in fibrinogen estimation

2.95 g sodium chloride

5.71 g sodium diethyl barbiturate

dissolved in 900 ml of distilled water and pH adjusted to 7.2 using 1.0 N hydrochloric acid and the volume made up to 1 .

h. Barbitone Acetate Buffer, pH 8.6, used in immunoelectrophoresis

6.5 g sodium acetate

8.87 g sodium barbitone

1.13 barbitone

dissolved in distilled water to final volume of 1 .

Appendix (2)

Acid Citrate Dextrose (ACD) Solution

3.0 g dextrose

2.0 g disodium hydrogen citrate

120 ml distilled water

Appendix (3) Calf 212 Haematology

Day	Temp.	RBC	PCV	MCV	MCHC	Hb	MCF	WBC	Thrombo-cytes	Para-sites
	°C	$x10^6_3$/mm	%	μm^3	%	g/100ml	Sal. Conc.	/mm^3	$x10^3_3$/mm	%
-22	-	10.47	34.2	34	-	14.5	-	19,900	-	0
-18	-	10.96	31.1	35	37.3	11.6	-	10,500	-	0
-15	-	10.39	34.5	35	38.9	13.2	-	20,800	-	0
-13	-	10.89	35.1	34	39.6	13.9	-	21,900	-	0
-11	-	9.94	33.2	35	38.6	12.8	-	17,000	-	0
- 5	39.6	9.40	39.4	44	31.7	12.5	.5575	18,000	332	0
0	38.9	9.40	33.8	38	36.1	12.2	-	19,000	550	0
1	38.7	10.20	36.6	38	35.8	13.1	-	19,100	-	0
2	38.8	10.71	39.2	39	34.9	13.7	-	19,300	-	0
3	38.4	10.73	37.8	37	37.0	14.0	.5450	18,100	584	0
4	38.5	10.37	36.0	37	36.7	13.2	-	17,800	-	0
5	39.2	10.05	38.2	38	33.8	12.9	-	17,900	-	0
6	38.6	10.84	36.8	36	38.6	14.2	.5400	18,000	694	0
7	39.1	9.70	31.3	34	40.6	12.7	-	18,200	-	0
8	38.8	9.97	35.9	37	36.5	13.1	.5250	18,700	552	4/50
9	38.9	10.42	35.1	36	39.0	13.7	.5575	14,800	460	1.0
10	39.4	10.32	36.8	38	36.7	13.5	.5700	10,400	446	2.6
11	40.1	7.48	27.9	35	31.1	11.8	.5650	13,100	438	15.5
12	40.4	5.97	20.9	39	38.3	8.0	.6125	17,100	458	33.0
13	40.5	3.16	12.1	40	35.5	4.3	.6200	17,800	410	23.9
14	38.0	2.33	8.3	37	36.1	3.0	.6175	19,200	392	13.7
15	37.0	1.99	7.6	39	35.5	2.7	.5650	38,000	362	3.7
16	38.7	2.10	12.0	56	30.8	3.7	.5250	57,000	224	4.5
17	38.4	2.30	13.4	60	30.6	4.1	.5625	36,000	120	1.3
18	38.9	2.90	18.5	66	29.2	5.4	-	27,000	164	1.0
19	38.5	2.90	19.0	68	30.5	5.8	-	27,800	176	0.5
20	38.4	3.20	22.7	72	27.3	6.2	.5400	18,200	158	4/50
21	38.9	3.25	23.6	69	29.7	7.0	-	18,300	162	0
22	38.0	3.58	25.7	74	29.2	7.5	-	13,000	302	0
23	38.7	3.62	25.5	72	29.8	7.6	-	13,000	458	0
24	38.4	4.81	32.0	68	27.8	8.9	-	14,400	728	0
27	38.7	5.46	34.8	66	32.5	11.3	-	17,700	-	0
29	38.9	5.23	32.6	64	35.9	11.7	-	14,400	1,148	0
31	39.0	6.06	36.0	61	33.6	12.1	-	11,700	1,656	0
34	-	6.72	39.2	60	34.2	13.4	-	14,300	-	0
35	38.2	6.42	35.9	58	34.0	12.2	.5225	11,000	1,150	0

Appendix (4) Calf 275 Haematology

Day	Temp.	RBC	PCV	MCV	MCHC	Hb	MCF	WBC	Thrombo-cytes	Para-sites
	°C	$x10^6_3$/mm	%	μm^3	%	g/100ml	Sal. Conc.	/mm^3	$x10^3_3$/mm	%
-22	-	9.14	28.6	39	43.4	12.4	-	9,900	-	0
-18	-	7.58		38	42.2	9.8	-	8,500	-	0
-15	-	8.63	26.7	32	40.1	10.7	-	11,000	-	0
-13	-	9.00	30.5	36	36.4	11.1	-	9,400	-	0
-11	-	8.29	29.3	35	35.5	10.4	-	10,300	-	0
- 5	39.8	8.34	29.3	37	38.6	11.3	.5525	13,700	598	0
0	39.1	8.75	32.2	39	34.2	11.0	-	9,700	-	0
1	38.8	8.07	28.7	37	36.6	10.5	-	9,500	-	0
2	39.2	8.13	26.9	35	39.0	10.5	-	11,800	-	0
3	38.8	8.55	29.3	36	36.5	10.7	.5525	8,900	626	0
4	38.5	8.48	30.1	37	35.8	10.8	-	9,380	-	0
5	38.8	8.60	31.9	39	33.5	10.7	-	8,600	-	0
6	38.1	8.91	31.2	37	36.2	11.3	.5050	10,400	578	0
7	39.2	8.58	30.1	36	36.9	11.1	-	9,000	-	1/50
8	38.2	7.92	26.9	36	39.4	10.6	.5375	7,700	566	1.0
9	39.4	8.00	28.0	37	37.1	10.4	.5625	6,500	576	2.1
10	39.7	7.80	27.1	36	37.3	10.1	.5625	6,100	502	4.1
11	40.5	6.68	23.7	37	36.7	8.7	.5625	8,300	486	11.1
12	41.0	4.76	19.4	41	30.9	6.0	.6025	9,400	398	28.6
13	40.6	2.67	9.9	39	36.4	3.6	.5725	12,400	444	10.6
14	40.4	2.01	6.9	35	39.1	2.7	.5600	11,700	486	1.0
15	39.1	1.98	6.9	39	39.1	2.7	.5500	7,800	592	3/50
16	39.4	2.35	10.4	45	31.7	3.3	.5500	10,600	718	1/50
17	39.1	2.77	14.4	54	30.6	4.4	-	10,500	956	7/50
18	38.4	2.70	14.3	56	30.8	4.4	-	7,000	858	-
19	38.5	2.90	15.3	54	33.3	5.1	-	6,500	-	6/50
20	38.3	3.37	16.5	50	33.3	5.5	.5300	6,700	912	0
21	38.3	3.27	16.6	52	33.7	5.6	-	8,100	-	0
22	39.4	4.02	19.6	50	34.2	6.7	-	10,300	-	0
23	38.7	4.27	21.4	52	33.2	7.1	-	8,900	-	0
24	38.2	4.25	22.5	55	31.6	7.1	-	9,100	-	0
27	38.0	4.65	20.8	46	38.0	7.9	-	9,300	-	0
29	39.0	5.05	23.7	49	34.8	8.1	-	8,100	-	0
31	38.5	5.15	23.1	47	36.4	8.4	-	9,900	-	0
34	-	5.71	27.0	49	33.3	9.0	-	10,600	-	0
35	38.8	5.26	24.5	48	34.7	8.5	-	9,400	744	0

Appendix (5) Calf L1160 Haematology

Day	Temp.	RBC	PCV	MCV	MCHC	Hb	MCF	WBC	Thrombo-cytes	Para-sites
	°C	$x10^6_3$/mm	%	μm^3	%	g/100ml	Sal. Conc.	/mm^3	$x10^3_3$/mm	%
-22	-	8.53	28.0	34	36.3	11.4	-	12,200	-	0
-18	-	8.84	28.3	34	35.0	11.1	-	20,700	-	0
-15	-	8.13	28.4	37	34.0	11.3	-	21,300	-	0
-13	-	10.52	33.0	33	39.5	11.5	-	22,500	-	0
-11	-	8.34	30.7	38	40.7	11.2	-	23,000	-	0
- 5	39.0	8.20	29.8	38	39.2	11.0	.5550	20,800	-	0
0	38.9	8.03	29.2	38	37.3	10.9	-	23,300	552	0
1	38.4	8.10	28.6	37	38.5	11.0	-	19,100	-	0
2	38.4	8.30	29.2	37	34.3	10.0	-	17,900	-	0
3	38.6	8.27	29.2	37	37.7	10.9	.5575	19,400	542	0
4	38.7	8.66	32.2	39	33.9	11.0	-	17,000	-	0
5	38.8	8,00	28.6	37	39.5	11.3	-	19,000	-	0
6	37.6	8.30	29.5	37	37.3	11.0	.5450	17,600	492	0
7	38.5	9.00	32.6	38	35.6	11.6	-	18,100	-	0
8	38.1	8.00	29.5	39	36.6	10.8	.5450	16,200	578	0
9	38.2	8.02	26.6	38	41.0	10.9	.5450	12,300	590	7/50
10	38.3	9.12	28.6	33	37.8	10.8	.5550	9,000	438	0.7
11	38.8	7.62	25.9	36	40.5	10.5	.5350	9,000	408	1.9
12	39.6	7.65	27.7	37	37.6	10.4	.5500	11,700	368	11.1
13	40.4	5.71	20.1	38	39.8	8.0	.5625	17,900	302	20.6
14	40.1	3.94	13.9	37	36.7	5.1	.5725	19,600	268	17.7
15	39.9	2.50	8.5	35	41.2	3.5	.5700	27,500	538	9.1
16	38.8	1.98	7.4	38	37.8	2.8	.5725	27,300	372	0.5
17	39.2	2.59	11.5	46	30.4	3.5	.5700	24,000	460	0.5
18	38.4	2.50	14.8	62	29.7	4.4	-	17,000	584	0.5
19	38.4	2.80	17.0	64	30.0	5.1	-	11,200	622	0.1
20	38.1	2.74	17.3	62	30.0	5.2	.5400	11,900	624	0.1
21	38.3	2.85	16.6	60	33.7	5.6	-	14,600	-	0
22	38.5	3.16	18.8	61	30.9	5.8	-	14,000	-	0
23	38.7	3.38	20.6	63	33.0	6.8	-	15,500	-	0
24	39.0	3.59	21.5	62	33.5	7.2	-	15,600	-	1/50
27	38.2	4.53	26.0	59	30.4	7.9	-	13,100	-	0
29	38.5	4.54	24.0	54	35.4	8.5	-	12,100	-	0
31	38.4	4.89	25.7	54	35.0	9.0	-	14,400	-	0
34	-	5.91	30.5	53	34.4	10.5	-	14,600	-	0
35	37.8	5.52	28.2	-	35.8	10.1	.5000	17,900	-	0

Appendix (6) Calf A89 Haematology

Day	Temp.	RBC	PCV	MCV	MCHC	Hb	MCF	WBC	Thrombo-cytes	Para-sites
	°C	$X10^6_3$/mm	%	μm^3	%	g/100ml	Sal. Conc.	/mm^3	$x10^6_3$/mm	%
-22	-	9.86	32.8	35	41.8	13.7	-	9,900	-	0
-18	-	8.81	31.1	36	37.3	11.6	-	10,500	-	0
-15	-	9.36	34.8	39	35.6	12.4	-	9,600	-	0
-13	-	9.96	37.2	39	35.0	13.0	-	9,600	-	0
-11	-	8.92	32.4	38	36.7	11.9	-	11,900	-	0
- 5	38.4	9.39	34.2	38	38.0	13.0	-	12,500	-	0
0	39.0	8.07	29.2	38	35.6	10.4	.4850	10,600	702	0
1	38.9	10.09	34.9	36	30.1	10.5	-	8,800	-	0
2	38.7	8.08	30.1	39	36.2	10.9	-	8,700	-	0
3	38.5	8.30	31.5	40	35.6	11.2	.5075	8,500	604	0
4	38.5	8.07	27.4	36	38.3	10.5	-	8,190	-	0
5	38.7	9.70	34.5	37	32.2	11.1	-	8,700	-	0
6	38.0	7.88	30.0	40	36.0	10.8	.5125	9,500	484	0
7	39.0	7.75	29.3	40	36.5	10.7	-	10,900	-	2/50
8	37.3	7.73	28.5	38	38.3	10.9	.5400	7,300	490	
9	39.5	7.79	27.2	37	39.7	10.8	.5525	7,300	424	4.5
10	40.5	6.79	25.8	40	34.5	8.9	.5525	16,800	362	24.5
11	40.1	3.99	15.5	41	35.5	5.5	.5800	18,000	374	28.2
12	38.6	2.07	7.7	40	39.0	3.0	.6425	25,900	502	20.7
13	DEAD									

Appendix (7) Calf 273 Haematology

Day	Temp.	RBC	PCV	MCV	MCHC	Hb	MCF	WBC	Thrombo-cytes	Para-sites
	°C	$x10^6_3$/mm		μm^3		g/100 ml	Sal. Conc.	/mm^3	$x10^3_3$/mm	%
-22	-	11.56	36.2	31	36.5	13.2	-	22,500	-	0
-18	-	11.20	33.1	30	-	14.4	-	17,100	-	0
-15	-	10.64	32.4	32	40.7	13.2	-	16,300	-	0
-13	-	11.10	39.6	34	34.3	13.6	-	17,100	-	0
-11	-	10.94	30.4	30	-	13.3	-	16,200	-	0
- 5	38.6	11.20	39.5	35	35.2	13.9	.4725	14,300	598	0
0	38.4	10.73	34.9	35	38.1	13.3	-	16,800	-	0
1	39.2	10.26	35.9	35	34.5	12.4	-	13,900	-	0
2	39.0	10.67	34.4	34	38.4	13.2	-	12,600	-	0
3	38.3	10.94	37.8	34	37.9	14.2	.5225	13,600	626	0
4	38.1	-	35.4	33	36.7	13.4	-	13,900	-	0
5	39.5	12.0	40.9	37	32.2	13.2	.5325	11,700	506	0
6	37.9	11.64	39.5	37	35.2	13.9	-	12,800	-	0
7	38.8	10.40	31.4	32	40.0	12.6	-	10,700	-	1/50
8	37.3	10.29	33.8	35	37.3	12.6	.5675	8,700	550	5/50
9	38.0	10.80	40.0	38	32.5	13.0	.5575	7,000	422	1.0
10	38.6	10.56	40.4	39	31.7	12.8	.5625	7,300	326	2.9
11	39.4	10.90	36.0	35	34.2	12.3	.5725	9,300	338	5.8
12	39.5	8.15	27.4	38	36.9	10.1	.5925	10,000	342	13.5
13	38.5	6.63	22.7	36	34.4	7.8	.5925	10,500	328	8.8
14	39.3	4.57	14.6	33	34.9	5.1	.5525	12,500	330	8.7
15	39.4	3.77	11.4	31	40.4	4.6	.5375	14,200	454	1.0
16	39.6	3.89	11.4	30	37.7	4.3	.5625	9,000	542	5/50
17	39.1	4.18	13.9	34	35.3	4.9	-	9,400	652	2/50
18	38.4	3.90	13.9	37	3244	4.5	-	7,000	698	0
19	38.0	4.10	14.5	36	37.2	5.4	-	7,500	-	2/50
20	37.9	4.53	16.0	37	36.3	5.8	.5400	6,500	918	1/50
21	38.4	4.48	17.2	40	36.1	6.2	-	7,500	948	0
22	38.4	4.56	16.8	38	35.1	6.0	-	6,700	924	0
23	38.1	5.14	20.0	41	33.5	6.7	-	7,700	832	0
24	38.7	5.37	19,3	40	36.3	7.0	-	9,700	1,000	0
27	39.0	5.10	18.5	38	37.8	7.0	-	12,800	-	0
29	39.1	5.87	23.1	41	32.9	7.6	-	10,900	824	0
31	39.1	5.54	21.0	40	36.2	7.6	-	11,900	988	0
34	-	5.76	22.4	41	34.4	7.7	-	10,700	-	0
35	37.8	6.40	25.0	41	33.6	8.4	.5150	11,500	992	0

Appendix (8) Calf 274 Haematology

Day	Temp.	RBC	PCV	MCV	MCHC	Hb	MCF	WBC	Thrombo-cytes	Para-sites
	°C	$x10^6_3$/mm	%	μm^3	%	g/100ml	Sal. Conc.	/mm^3	$x10^3_3$/mm	%
-22	-	11.66	36.8	38	-	14.2	-	9,000	-	0
-18	-	10.89	36.4	35	36.5	13.3	-	9,500	-	0
-15	-	10.33	34.4	35	35.2	12.1	-	9,700	-	0
-13	-	10.63	34.2	34	38.3	13.1	-	11,000	-	0
-11	-	10.07	34.3	36	38.2	13.1	-	11,900	-	0
- 5	38.2	10.53	37.3	37	36.5	13.6	.5550	10,400	694	0
0	38.9	9.50	34.6	38	35.6	12.3	-	10,900	-	0
1	39.1	9.61	34.2	37	37.7	12.9	-	9,800	-	0
2	38.9	9.18	33.5	38	36.1	12.1	-	8,900	-	0
3	38.7	9.87	36.3	39	35.3	12.8	.5450	10,800	672	0
4	37.8	10.06	36.9	39	34.2	12.6	-	11,100	-	0
5	39.0	10.20	37.5	39	33.1	12.4	-	10,500	-	0
6	38.4	9.44	34.0	38	37.4	12.7	.5450	9,900	574	0
7	38.9	9.11	33.9	39	37.8	12.8	-	8,700	-	0
8	38.6	10.12	32.6	38	34.7	11.3	.5475	5,300	654	1.5
9	39.4	8.99	33.5	36	37.0	12.4	.5550	7,700	466	7.4
10	39.4	7.70	25.8	35	39.5	10.2	.5725	11,200	398	10.5
11	39.5	6.70	21.0	35	39.5	8.3	.5575	13,200	326	11.5
12	39.2	5.32	19.7	39	33.0	6.5	.6050	16,900	332	6.8
13	38.8	4.77	17.8	39	33.2	5.9	.5450	24,600	354	2.6
14	39.0	3.94	13.6	36	38.2	5.2	.5150	14,700	312	0.5
15	39.6	3.75	12.3	34	40.7	5.0	.5575	8,900	648	1/50
16	38.9	3.87	14.8	40	35.8	5.3	.5750	7,400	686	4/50
17	39.4	4.33	17.1	41	35.1	6.0	.5450	8,900	754	4/50
18	38.7	4.20	18.9	46	34.4	6.5	-	10,800	698	2/50
19	38.3	4.40	19.2	45	36.5	7.0	-	8,900	-	0
20	38.1	5.05	22.5	46	34.7	7.8	.5550	10,700	836	4/50
21	38.5	4.53	20.3	46	36.0	7.3	-	10,500	-	0
22	39.3	5.26	23.8	47	34.5	8.2	-	11,100	-	0
23	38.6	5.02	23.4	49	32.8	7.8	-	7,700	-	0
24	38.5	5.53	25.4	47	35.0	8.9	-	9,500	-	0
27	38.5	5.93	25.5	45	38.0	9.7	-	12,400	-	0
29	38.5	6.03	26.1	45	37.9	9.9	-	8,100	-	0
31	38.5	6.21	26.8	45	39.9	10.7	-	9,700	-	0
34	-	6.76	31.2	48	34.3	10.9	-	12,400	-	0
35	38.1	6.58	29.2	46	37.0	10.8	.5225	12,300	688	0

Appendix (9) Calf 251 Haematology

Day	Temp.	RBC	PCV	MCV	MCHC	Hb	MCF	WBC	Thrombo-cytes	Para-sites
	°C	$x10^6_3$/mm	%	μm^3	%	g/100ml	Sal. Conc.	/mm^3	$x10^3_3$/mm	%
-22	-	10.98	32.6	31	42.3	13.8	-	5,600	-	0
-18	-	11.61	33.3	31	38.4	12.8	-	6,900	-	0
-15	-	10.24	32.1	33	40.8	13.1	-	7,200	-	0
-13	-	10.63	33.1	33	42.0	13.9	-	6,300	-	0
-11	-	10.42	34.4	35	38.1	13.1	-	10,700	-	0
- 5	38.6	10.69	35.2	35	37.0	13.0	.5450	7,700	680	0
0	38.3	10.02	33.6	35	38.7	13.0	-	7,500	-	0
1	38.8	8.05	-	36	-	12.8	-	8,000	-	0
2	39.2	10.18	34.0	35	35.6	12.1	-	7,900	-	0
3	38.4	10.00	34.0	36	36.6	12.6	.5060	7,200	770	0
4	38.1	10.27	34.8	36	36.2	12.6	-	6,900	-	0
5	38.9	10.90	38.4	37	33.3	12.8	-	7,100	-	0
6	37.8	9.84	32.8	35	38.7	12.7	.4975	6,800	620	0
7	38.9	10.78	37.5	37	32.5	12.2	-	6,200	-	0.1
8	39.3	8.41	30.4	38	41.5	12.6	.5225	4,900	534	0.3
9	39.9	8.82	26.4	34	42.4	11.2	.5650	5,600	408	1.8
10	39.8	8.71	30.0	36	36.0	10.8	.5700	6,300	402	4.4
11	40.3	7.15	24.3	36	37.5	9.1	.5675	8,300	402	9.8
12	40.2	5.46	18.3	35	36.6	6.7	.6150	9,500	334	10.9
13	40.0	4.46	14.4	36	38.9	5.6	.6075	17,100	418	4.4
14	40.1	3.84	12.4	33	38.7	4.8	.5825	14,100	500	-
15	40.1	3.43	11.3	34	38.9	4.4	.5550	9,100	568	4/50
16	39.7	3.41	12.0	36	36.7	4.4	.5575	6,600	660	3/50
17	39.2	4.20	16.0	39	32.5	5.2	-	6,600	696	0
18	38.7	-	-	-	-	-	-	-	-	-
19	39.4	3.90	17.0	44	34.7	5.9	-	6,000	692	2/50
20	38.4	4.55	19.1	43	35.1	6.7	.5625	6,700	752	0.1
21	38.9	4.59	19.5	44	34.4	6.7	-	7,300	-	0
22	38.5	4.78	20.0	43	35.5	7.1	-	7,100	-	0
23	38.7	4.93	21.8	46	34.4	7.5	-	6,500	-	0
24	39.1	5.35	21.3	45	38.0	8.1	-	7,400	-	0
27	39.0	5.52	22.0	41	39.1	8.6	-	8,400	-	0
29	38.0	5.48	23.4	44	37.6	8.8	-	6,100	-	0
31	38.7	6.18	27.7	47	36.8	10.2	-	11,000	-	0
34	39.1	7.05	30.7	45	33.2	10.2	-	10,700	-	0
35	39.1	6.68	28.6	45	34.6	9.9	.5100	9,000	-	0

Appendix (10) Reticulocyte counts during *B. divergens* infection

Calf No.	212	275	L1160	A89	273	274	251
Day -5	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0
13	1/70	2/70	0	D	2/70	2/70	1/70
14	1.0	6/70	2/70		3/70	3/70	1/70
16	2.5	0.5	0.5		5/70	0.5	0.5
17	17.5	18.0	4.4		1.7	3.1	7.5
20	9.1	1.0	15.0		1.6	2/70	1.0
22	2.3	3/70	1.0		1/70	0/70	0/70
24	6/70	0/70	0/70		0/70	0	0
29	0	0	0		0	0	0

Appendix (II) Total and Differential white blood cell counts during B.divergens infections.

DAY		-5	3	6	8	9	10	11	12	13	14
Calf No.											
212	WBC	18000	18100	18000	18700	14800	10400	13100	17100	17800	19200
Lymphocyte		12330	13575	11070	13931	9028	5512	7140	8550	9523	8640
Neutrophil		4050	2534	5310	3834	4736	3588	5109	5387	3382	6816
Monocyte		1170	1448	1620	748	740	1300	917	2823	4806	3744
Eosinophil		450	543	0	187	296	0	0	0	89	0
275	T	13700	8900	10400	7700	6500	6100	8300	9400	12400	11700
	L	9110	6363	7072	4851	4160	3446	5395	5499	6200	6961
	N	3494	1379	1976	1963	1267	1251	1784	1645	2108	1287
	M	1096	1068	1352	847	1073	1342	1121	2256	4092	3452
	E	137	180	0	39	0	61	0	0	0	0
L1160	T	20800	19400	17600	16200	12300	9000	9000	11700	17900	19600
	L	9880	7372	7656	7290	6765	3690	4230	5967	6086	10192
	N	9880	9312	8272	7047	4981	4365	4005	3393	7786	5978
	M	728	2134	1672	1701	492	900	360	2280	4028	3430
	E	312	582	0	162	62	45	45	0	0	0
A89	T	12500	8500	9500	7300	7300	16800	18000	25900	DEAD	
	L	8375	6332	6412	4234	3030	5628	8370	7252		
	N	3500	1360	2090	1606	3321	8064	6120	8417		
	M	625	723	998	1387	839	3108	3510	10101		
	E	0	85	0	73	109	0	0	0		
273	T	14300	13600	12800	8700	7000	7300	9300	10000	10500	12500
	L	10153	7072	7104	4437	3710	3431	5115	5400	6090	7312
	N	3217	2040	4393	3523	2625	2920	2883	2100	1890	1563
	M	930	5	1403	609	630	875	1302	2500	2467	3625
	E	0	0	0	131	35	74	0	0	53	0
274	T	10900	10800	9900	5300	7700	11200	13200	16900	24600	14700
	L	6485	6426	6336	3207	3888	5320	6996	10731	15621	8526
	N	3325	2970	1930	1908	2849	3584	2640	1268	2091	735
	M	926	1134	1436	185	963	56	3564	4901	6888	5439
	E	164	270	198	20	0		0	0	0	0
251	T	7700	7200	6800	4900	5600	6300	8300	9500	17100	14100
	L	5390	3456	3944	2793	2296	2520	3859	4227	8464	8178
	N	1617	2772	1462	1127	2184	1575	1660	1425	513	1353
	M	733	1260	1360	931	1120	2079	2781	3468	8123	5569
	E	0	0	34	49	0	126	0	0	0	0

15	16	17	18	19	21	23	27	31	35
38000	57000	36000	27000	27800	18300	13000	17700	11700	11000
12920	19665	20880	11610	8340	8418	6695	7877	7196	7040
20520	31065	9360	11475	13761	8601	5655	8407	3334	2860
4560	5985	5580	3915	5699	1281	650	1416	526	440
	285	180	0	0	0	0	0	644	660
7800	10600	10500	7000	6500	8100	8900	9300	9900	9400
4875	6996	7087	5040	4680	5872	6764	6975	6782	5922
1599	2385	1417	700	682	770	579	1348	1881	1128
1131	1060	1943	1260	1040	1458	1557	977	1039	2303
0	159	53	0	98	0	0	0	99	47
27500	27300	24000	17000	11200	14600	15500	13100	14400	17900
17187	15834	13680	10370	6048	10658	9610	7794	6552	9039
4538	4095	6240	3230	2184	1752	2713	3603	6336	4654
5775	7371	4080	3400	2968	2190	3177	1637	1296	3849
0	0	0	0	0	0	0	66	216	358
14200	9000	9400	7000	7500	7500	7700	12800	11900	11500
10792	5805	5687	4235	5850	5175	6122	7744	8092	6957
923	1125	940	630	600	900	885	3328	2796	2473
2485	2070	2726	2135	975	1425	693	1664	1369	2070
0	45	47	0	0	0	0	64	238	0
8900	7400	8900	10800	8900	105000	7700	12400	9700	12300
5785	4958	3604	6048	5607	7140	5852	7006	5238	6519
445	592	1825	2592	623	1260	924	3410	2813	3259
2314	1776	3471	2160	2581	2046	924	1736	1164	1784
0	37	0	0	89	54	0	248	485	798
9100	6600	6600	-	6000	7300	6500	8400	11000	9000
5733	4818	3894		3060	5110	5200	5880	7755	5490
1137	660	1386		840	803	748	1554	1320	1530
2230	1122	1320		2040	1350	552	882	1870	1980
0	0	0		60	37	0	0	55	0

Appendix (12) Plasma Na and K values (m Eq/l) during B.divergens infection

Calf No.	212		275		L1160		A89		273		274		251	
Day	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K	Na	K
-5	156	4.4	143	3.9	159	4.0	145	3.9	147	4.0	145	3.8	143	3.8
3	156	4.2	157	3.8	172	4.2	161	4.0	159	4.3	165	4.0	170	4.0
6	146	4.1	156	4.3	159	4.1	149	3.9	137	4.3	159	3.9	146	4.2
8	147	4.4	138	4.3	142	4.2	145	4.1	136	4.3	139	4.3	133	4.1
9	143	4.4	141	4.6	137	4.6	145	4.1	158	4.4	139	4.5	137	4.3
10	152	4.2	143	4.3	143	-	143	4.2	141	4.7	146	4.3	144	4.6
11	157	4.9	144	5.1	156	4.4	131	4.2	139	4.1	138	4.0	163	4.7
12	179	4.6	143	4.4	148	4.4	153	4.1	160	3.8	145	3.7	141	4.0
13	153	4.3	151	4.0	138	4.1	D		141	4.0	141	3.6	146	3.8
14	138	3.5	131	3.6	150	4.3			140	3.6	141	3.7	141	4.1
15	151	4.5	146	4.0	136	4.1			144	3.8	142	3.7	140	3.9
16	150	3.3	143	3.5	145	3.8			137	3.8	137	3.9	155	4.0
17	143	2.8	135	3.7	140	3.9			149	3.8	145	3.8	-	4.0
20	150	3.2	154	4.0	136	3.9			140	3.9	146	3.8	146	3.9
35	157	5.1	156	3.7	144	3.9			151	3.9	161	3.6	154	3.7

Appendix (13) Histopathology during B.rodhaini infection

Day 3	T_2			C_1		
Organ	HE	PRB	MSB	HE	PRB	MSB
Liver	-	-	-	-	-	-
Kidney	-	-	-	-	-	-
Spleen	-	3+	-	-	3+	-
Cb. Hemis.	-	-	-	-	-	-
Cerebellum	-	-	-	-	-	-
Lung	-	-	-	-	-	-

Day 4	T_3			T_4			C_2		
Organ	HE	PRB	MSB	HE	PRB	MSB	HE	PRB	MSB
Liver	4+	3+	-	3+	3+	-	-	-	-
Kidney	2+	2+	-	3+	3+	-	-	-	-
Spleen	-	3+	-	E	3+	-	-	2+	-
Cb. Hemis.	-	-	-	-	-	-	-	-	-
Cerebellum	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-	-

Day 5	T_2			T_3			C_1		
Organ	HE	PRB	MSB	HE	PRB	MSB	HE	PRB	MSB
Liver	3+	3+	-	3+	3+	-	-	-	-
Kidney	2+	3+	-	-	3+	-	-	-	-
Spleen	-	3+	-	1+	3+	-	-	3+	-
Cb. Hemis.	-	-	-	-	-	-	-	-	-
Cerebellum	-	-	-	-	-	-	-	-	-
Lung	-	3+	-	-	2+	-	-	-	-

Day 6	T_3			T_4			C_1		
Organ	HE	PRB	MSB	HE	PRB	MSB	HE	PRB	MSB
Liver	6+	3+	1+	6+	3+	-	-	-	-
Kidney	6+	6+	-	2+	3+	-	-	-	-
Spleen	1+	6+	-	-	6+	-	-	3+	-
Cb. Hemis.	-	-	-	-	-	-	-	-	-
Cerebellum	-	-	1+	-	-	-	-	-	-
Lung	-	3+	-	-	3+	-	-	-	-

Day 7	T_1			T_2			C_3		
Organ	HE	PRB	MSB	HE	PRB	MSB	HE	PRB	MSB
Liver	9+	3+	-	6+	6+	-	-	-	-
Kidney	6+	9+	-	3+	6+	-	-	-	-
Spleen	2+	6+	-	-	6+	-	-	3+	-
Cb. Hemis.	-	-	-	-	-	-	-	-	-
Cerebellum	-	-	-	-	-	-	-	-	-
Lung	-	3+	-	-	3+	-	-	-	-

Day 9	T_1			T_3			C_1		
Organ	HE	PRB	MSB	HE	PRB	MSB	HE	PRB	MSB
Liver	4+	6+	-	3+	6+	-	-	-	-
Kidney	-	6+	-	-	6+	-	-	-	-
Spleen	-	6+	-	-	6+	-	-	3+	-
Cb. Hemis	-	-	-	-	-	-	-	-	-
Cerebellum	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-	-

Appendix (14) Calf 296 Haematology

Day	Temp.	RBC	PCV	MCV	WBC	Hb	Parasites
	^{o}C	$x10^{6}_{3}$/mm	%	μm^{3}	$/mm^{3}$	g/100/ml	%
-9	38.2	8.1	42.9	55	12,300	14.4	0
-7	38.6	7.9	41.2	54	11,300	14.5	0
-1	38.8	7.6	39.5	54	12,300	14.8	0
0	38.4	7.5	39.9	55	11,100	13.8	0
1	38.4	7.9	42.9	56	9,500	15.5	0
2	38.2	-	-	-	-	-	-
3	38.5	8.3	44.2	55	9,400	14.6	0
4	38.8	7.5	39.8	55	9,400	13.6	0
5	39.0	8.0	43.2	56	8,900	14.1	0
6	38.4	8.7	43.4	52	8,300	14.0	+
7	39.4	9.0	44.4	57	6,000	15.8	0.5
8	39.1	8.4	44.8	56	5,200	15.3	2.6
9	40.8	7.3	40.0	57	6,300	13.3	7.4
10	40.2	5.3	28.0	55	8,300	8.7	30.0
11	40.0	3.1	16.9	56	13,600	5.3	19.0
12	36.4	2.1	13.2	65	28,600	3.7	9.2
13	DEAD						