STUDIES ON *EPERYTHROZOON OVIS* INFECTION IN SHEEP

A thesis presented in partial fulfilment of the
requirements for the degree of Doctor of Philosophy
in Veterinary Clinical Pathology
at Massey University

Richard Hugh Sutton

1974

ACKNOWLDGEMENTS

I would like to thank Dr. R. D. Jolly, my Chief Supervisor, for his continual guidance, advice and encouragement throughout this study. Special thanks are also due to my other supervisor, Dr. W. A. G. Charleston, particularly for his guidance and assistance in the preparation of this manuscript. Professor B. W. Manktelow and Dr. R. M. Greenway gave helpful advice during the writing of this thesis, and Professor R. E. Munford gave advice and assistance in the statistical analysis of the data.

I am grateful for technical assistance provided at various times by Mrs. S. Cordery, B.H.Sc., Miss C. M. MacDonald, Mrs. D. Lovelock, Miss B. Hobman, Mrs. C. Tse, and Mr. A. T. deCleene.

I am indebted to Mr. J. Austin for the preparation of the photographs, to Mr. D. Ward, and Mrs. J. Laing for the preparation of histopathological tissues, and to the staff of the Massey University Printery for the reproduction of the figures presented in this thesis.

The co-operation of many people in providing assistance and the use of facilities is very much appreciated. In particular I would like to thank Mr. P. H. Whitehead, the Massey Sheep Farm Supervisor, for the provision of grazing land, Dr, A. N. Bruere, Department of Veterinary Clinical Sciences, for providing indoor housing facilities, Dr. B. E. Goulden, Department of Veterinary Clinical Sciences, for surgical assistance. The staff of the Massey University Library were very helpful, particularly in obtaining material from outside sources.

The use of equipment outside the Veterinary Faculty was only possible through the co-operation of Dr. R. M. Greenway, Department of Chemistry, Biochemistry and Biophysics, Massey University, Dr. D. Newstead, Dairy Research Institute,

Palmerston North, and Dr. A. A. Corbett of the Palmerston North Hospital.

Special thanks are due to Mrs. G. Percy for typing the final copy of this manuscript.

I would like to sincerely thank my wife Tina for typing the draft copies of this thesis. Without the patience, understanding and sacrifices made by her and children, Peter and Susan, this work would never have been completed.

This investigation was supported by a research grant from the Vernon Willey Trust, to whom I am most grateful.

STUDIES ON EPERYTHROZOON OVIS INFECTION IN SHEEP

ABSTRACT

This thesis concerns the effect of Eperythrozoon ovis on its host, with particular emphasis on some of the haematological, pathological and biochemical changes which occur.

Haematological studies on experimentally infected sheep showed that both the maximum degree of anaemia and maximum degree of parasitaemia were similar in all groups and independent of the size of inoculating dose. The smaller the dose rate, however, the longer the prepatent period, so that at the lowest dose rate used (1×10^{-6} ml of infected blood intravenously injected) the peak of parasitaemia and peak of anaemia were synchronized. The time taken for anaemia to develop following inoculation was similar in all experimentally infected groups and independent of the time taken for parasitaemia development. It is suggested from these observations that the anaemia is at least partially associated with a host immune response against the parasite and involving the erythrocyte. In natural infection the severity of anaemia and parasitaemia was variable. It is postulated that in such infection the host immune response may be expressed before infection is fully developed. It was also shown that there was a significant ($p < 0.01$) correlation between the maximum degree of anaemia and maximum degree of parasitaemia for all infected (experimental and natural) sheep. For this reason the possibility of a direct effect by the parasite on the erythrocyte, contributing to the anaemia cannot be discounted.

All infected groups of sheep showed a slower rate of weight gain as compared with controls. In one experiment the difference between groups was significant ($p < 0.01$).

The main pathological changes in E. ovis infection were splenic enlargement and haemosiderosis of the liver and kidney cortex. These latter findings indicated that intravascular haemolysis is probably the predominant mode of erythrocyte destruction. Infection is accompanied by an increased intravascular clearance rate of carbon by the reticuloendothelial system. The possible role of the reticuloendothelial system in the host immune response and in erythrocyte destruction, along with the role of complement and the parasite in the pathogenesis of anaemia, is discussed.

Comparative glycolytic studies between E. ovis infected and control erythrocytes showed that infected erythrocytes utilized approximately 24 times as much glucose and produced 18 times as much lactic acid as the controls. Other findings included an egress of oxygen from infected erythrocytes, thought to result from a drop in intracellular pH and a reduction in the amount of glucose which could be accounted for as lactic acid, pyruvic acid and oxygen uptake. This latter finding was thought to be due to utilization of glucose by the parasite for synthetic purposes.

Changes in infected sheep which were considered to result from the increased glycolytic activity of erythrocytes were a fall in venous blood glucose levels (in some cases to negligible values) and a large increase in blood lactic acid levels. Acid-base studies showed that these changes were accompanied by significant ($p < 0.05$) falls in venous pH and standard bicarbonate. The fall in standard bicarbonate would be expected to result from the neutralization of lactic acid, but there was no apparent explanation for the increase in pCO_2 which also occurred. Such an increase is normally indicative of a respiratory acidosis. Apart from a period of inappetance during heavy parasitaemia, which may affect weight gain, no clinical effect due to the acidosis was noted.

The reductive potential, i.e., the ability of an erythrocyte to withstand oxidative damage was assessed

during an infection cycle by measuring erythrocyte reduced glutathione levels and blood methaemoglobin levels before and after incubation with acetylphenylhydrazine. It was concluded that an infected erythrocyte's ability to withstand oxidative challenge was severely affected as compared with control erythrocytes, and that haemolysis could be a likely result. Without such a challenge, however, an infected erythrocyte's reductive potential is not greatly affected. It is probable that such metabolic changes occurring in infected erythrocytes do not play a role in the pathogenesis of anaemia.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

REVIEW OF THE LITERATURE

INTRODUCTION

Eperythrozoon ovis is an epi-erythrocytic blood parasite of sheep which can cause anaemia. There is circumstantial evidence that it may be a cause of unthriftiness in sheep (Sheriff et al., 1966).

Eperythrozoon ovis was first reported from South Africa by Neitz et al. in 1934. In the course of studies on the transmission of Rickettsia ruminantium, involving a splenectomized sheep, ring-shaped bodies of approximately 0.5 to 1.0 μ diameter were seen in blood smears. On the basis of morphology, staining reaction with Giemsa stain, and the location of the parasite on the erythrocytes, it was considered that this was a previously undescribed species of the genus Eperythrozoon, and the name Eperythrozoon ovis proposed. Although Neitz et al. suggested that Eperythrozoon be included within the family Anaplasmidae, then considered protozoal organisms, Peters and Wigand (1955) considered that Eperythrozoon could be set aside from protozoa on account of its small size and lack of cellular structure. At present Eperythrozoon is included in the family Bartonellaceae which are blood parasites, in the order Rickettsiales (Weinman, 1957). However, results of recent structural studies have suggested that Eperythrozoon should be included in the Mycoplasmatales and not the Rickettsiales (Avakian et al., 1973).

Since the original discovery, E. ovis has been reported from Algeria (Donatien and Lestoquard, 1935); France (Lafenetre, 1936); Iran (Delpy, 1936); U.S.A. (Jensen, 1943); Norway (Øverås, 1959); Australia (Littlejohns, 1960); Scotland (Foggie, 1961); India and Pakistan (Sarwar et al., 1962); Yugoslavia (Begovic et al., 1963); Russia (D'yakonov, 1964); England (Rouse and Johnson, 1966); Kenya (Ohder, 1967); New Zealand (Jolly, 1967); Spain (Rodriguez, 1968); Germany (Friedhoff

et al., 1971); Bulgaria (Kyurtov, 1971) and Poland (Ramisz et al., 1972).

INFECTIVITY

Transmission

Experimentally, transmission has been effected by inoculation of susceptible animals with infected blood by intravenous, subcutaneous, intramuscular, intraperitoneal (Kreier and Ristic, 1968), and oral routes (Øverås, 1962, 1969).

The natural mode of transmission has not been established, though it is probable that an insect vector is involved. Øverås (1962) produced circumstantial evidence suggesting that the biting fly Stomoxys calcitrans could be a vector. Subsequently he reported that he was able to transmit E. ovis by subcutaneous injection of macerated Stomoxys, but an attempt to transmit infection by inducing flies to feed on infected and then uninfected sheep failed (Øverås, 1969). Similar results were obtained with the sheep ked (Melophagus ovinus). Previous attempts by other workers to transmit E. ovis by infecting with suspensions of ground up keds (Neitz, 1937; Rouse and Johnson, 1966) and by transference of live keds (Foggie and Nisbet, 1964) had failed. Foggie and Nisbet (1964) produced a mild infection in a sheep inoculated with a saline suspension of ground up lice from an infected sheep but failed to reproduce the disease when they transferred sucking lice (Linognathus ovillus) from naturally infected sheep to susceptible sheep.

Nikol'skii and Slipchenko (1969) reported the experimental transmission of E. ovis by the ticks Hyalomma plumbeum and Rhipicephalus bursa, and they were able to demonstrate transovarian transmission between tick generations.

Circumstantial evidence also supports the hypothesis that infection is spread by insects. Foggie and Nisbet (1964) observed that all diagnosed spontaneous cases of E. ovis infection occurred in summer and autumn, and suggested a biting

fly vector. Sheriff et al. (1966) found that infection occurred in higher rainfall areas at a time of the year favourable to insect vectors. Both these groups also noted that, in the course of experimental work, infections occurred in control sheep which were not in direct contact with artificially infected sheep.

Host Specificity

Eperythrozoon ovis is relatively host specific. Rabbits, guinea pigs, splenectomized and intact mice, and a splenectomized dog have proved refractory to infection (Neitz, 1937; Foggie and Nisbet, 1964). Reports of possible transmission to calves by Neitz (1937, 1940) and Ohder (1967) are not substantiated by the data presented. Hoyte (1971) was able to show that E. ovis survived in a calf for at least 9 days, although no parasitaemia was demonstrated.

Successful transmissions to a splenectomized goat and a splenectomized deer (Dama virginiana) have been reported (Kreier and Ristic, 1963). An antelope (blesbuck) (Damaliscus albifrons), injected with blood from an infected sheep appeared to develop a latent infection as the organism was later recovered by inoculation of the antelope's blood into another susceptible sheep (Neitz, 1939).

In Iran, E. ovis has been incriminated as a cause of goat mortality (Rafyi and Maghami, 1966).

MICROBIOLOGY

Cultivation and Preservation

Attempts to culture E. ovis on common bacteriological media under different atmospheric conditions have failed (Neitz, 1937; Rouse and Johnson, 1966). Similarly, attempted culture on sheep kidney monolayers (Foggie and Nisbet, 1964) and a "mixed hamster kidney - bovine lymphatic tissue culture" (Ohder, 1967)

have also failed. Seamer (1959), using embryonated hen eggs, successfully passaged the rodent specific organism E. coccoides 14 times by yolk sac inoculation, and 16 times by intravenous inoculation of the chick embryo. In the only recorded attempt to repeat this work with E. ovis, Ohder (1967) found no sign of infection in eggs and failed to recover the organism by inoculation of sheep with allantoic or yolk fluid and embryo tissue. However, he was able to transmit infection to susceptible sheep with infected blood stored for 5 weeks at -20°C with and without 30% glycerol, but was unable to transmit infection with blood stored for a longer period, or with freeze dried blood.

Jensen (1943) found that E. ovis was killed when infected blood was heated for 10 minutes at 48°C to 50°C.

Morphology

Most descriptions of E. ovis have been made from observations on smears of infected blood, stained with one of the modified Romanowsky stains. It is generally agreed that E. ovis is pleomorphic. The most characteristic form described is a small purple-staining ring approximately 0.4 to 0.5 μ in diameter with a pale or clear centre (Neitz, 1937), this being the first form to appear in a parasitaemia (Foggie and Nisbet, 1964; Sheriff et al., 1966). Neitz (1937) noticed that some ring forms had up to 3 darker staining points which he suggested may have some relation to multiplication. Variations from the ring form have also been described; Littlejohns (1960) noted a more bacillary form which showed bipolar staining and resembled diminutive pasteurellae; Sheriff et al. (1966) noted that some rings had short solid extensions on one side or at opposite poles, and Øverås (1969) noted rings of irregular thickness and some which appeared spheroidal with only a slight central pallor. Sheriff et al. (1966) also noted that, as parasitaemia developed, the ring forms tended to disappear and a variety of forms, including long chains resembling minute streptococci, "wispy" filaments resembling leptospirae, and many combinations of these forms appeared. Later the "wispy" forms predominated although

some short rods were noted. Foggie and Nisbet (1964) described these rod forms as curved to fit the margin of the erythrocytes. Other morphological types have been described as ovoid, comma, dumb-bell and tennis racket shaped, as triangles with rounded angles (Neitz, 1937), and large pale-staining irregular rings up to 2μ in diameter (Littlejohns, 1960). These large rings were observed during the phase of decreasing parasitaemia. Øverås (1969) described the appearance of threadlike structures projecting out from the erythrocyte and in some cases he also observed large numbers of minute coccoid bodies, mainly in those sheep which had been infected orally.

So numerous are the morphological variations described that it is difficult to ignore the possibility that some at least are artefact and attributable perhaps to variations in the techniques of preparation, staining and examination of blood smears (see Øveras for example). Kreier and Ristic (1963) studied the organism's morphology using indirect fluoresence, phase contrast and bright field microscopy, and concluded that the most typical form is a rod or sphere on the periphery of the erythrocyte. The ring forms which were seen in dry film preparations under bright field microscopy, appeared as dark spheres under phase contrast microscopy. Peters and Wigand (1955), working with E. coccoides, suggested that such ring forms probably arise during the process of air drying and that the organism in circulation was more likely to be in a coccoid or vesicular form.

Fine Structure

Observations on the ultrastructure of E. ovis have been recorded by Kreier and Ristic (1963). The organism is round or oval with a peripheral dense area which is thought to be a membrane. The inner structure consisted of central and peripheral aggregates of finely granular material within an electron-lucid substance. At its point of contact with an erythrocyte, the organism appears to have partly penetrated or eroded the erythrocyte membrane. A definite single unit membrane was described by McKee et al. (1973) but in contrast to Kreier and

Ristic, they considered the parasite to be situated only on the surface of the erythrocyte.

Relation of E. ovis to Erythrocyte

In blood smears most organisms are seen on the surface of erythrocytes, some apparently free in the plasma; mechanical separation of the organisms from the erythrocytes in the process of making the smear is considered the probable explanation for these "free" organisms (Neitz, 1937; Littlejohns, 1960; Foggie and Nisbet, 1964; Sheriff et al., 1966). The bond between organism and cell does not appear to be strong; Neitz (1937) found that centrifugation of blood at 3,000 rpm for 1 hour detached most of the organisms, which were packed just below the layer of leucocytes.

PATHOGENESIS AND PATHOLOGY OF INFECTION

Incubation Period and Development of Parasitaemia

Following inoculation of susceptible sheep by the intravenous or subcutaneous routes, Neitz (1937) recorded that the incubation (or prepatent) period - i.e. the period between inoculation and first appearance of the organisms in blood smears - ranged from 2 to 26 days, but was commonly between 5 and 7 days. Subsequent observations have confirmed this (Jensen, 1943; Øverås, 1969).

Although no critical work has been reported on the relationship between size of infecting dose and prepatent period with E. ovis, it has been found with E. coccoides that the prepatent period is inversely related to the size of the infecting dose (Schindler and Krampitz, 1964). Foggie and Nisbet (1964) noted that the prepatent period following inoculation with heavily parasitized blood was shorter than when using blood from convalescent sheep, in which parasites were no longer detectable in smears.

There is little information on the prepatent period in

natural cases where the infecting dose would be expected to be relatively small. Ohder (1967) noted that infection was apparent in 3 control sheep 8 to 21 days after being transferred to a stall that held experimentally infected sheep.

Neitz (1937) recorded that, after organisms were first noted in sequential blood smears, they increased rapidly and within 5 to 10 days became 25 to 100 times as numerous as erythrocytes. Neitz also noted that this phase of active multiplication appeared to continue up to about the time that anaemia first appeared. A sudden decrease in numbers occurred at this stage, so that when the anaemia was most marked there were comparatively few or no organisms to be seen. The parasite was demonstrable microscopically in the peripheral blood for a period of 6 to 42 days, with an average of 14 days. This relationship of parasitaemia to anaemia was also noted by Littlejohns (1960) and Sheriff et al. (1966). However, other workers have observed longer parasitaemic episodes with concurrent anaemia (Foggie and Nisbet, 1964; Øverås, 1962, 1969; Rouse and Johnson, 1966). In experimental infection Øverås (1969) found that the average duration of parasitaemia for the 23 sheep used was 97 days, whereas the duration in naturally infected sheep was shorter. In these longer parasitaemic episodes, the haemoglobin values returned to normal as the parasitaemia diminished.

In parasitaemias of longer duration, Neitz (1937) found that there was considerable fluctuation in the numbers of circulating parasites. Reappearance of the parasites in blood smears after being apparently free from infection, has also been noted (Neitz, 1937; Sheriff et al., 1966; Øverås, 1969). These relapses of parasitaemia occurred at intervals ranging from a few days to a few months, although Weinman (1944) noted that there was apparently a tendency for a progressive increase in the "parasite-free" period between successive relapses.

There are considerable difficulties in reliably detecting very small numbers of organisms in blood smears. Blood from animals with no detectable parasitaemia may still be infective

for other animals. It is, therefore, difficult to pinpoint precisely the beginning and end of parasitaemia and, since techniques for staining and examination of smears vary, difficulty in comparing the results of different workers occurs. It is clear, however, that both the prepatent period and the duration of parasitaemia may vary considerably, as may the incidence and extent of relapses.

Clinical Signs of Infection

Foggie and Nisbet (1964) found that in spontaneously infected sheep, with only slight parasitaemia, there were no significant clinical effects, but that anaemia and transient jaundice occurred in animals that had received an intravenous inoculation of heavily infected blood. Neitz (1937) considered that the severity of anaemia was related directly to the intensity of parasitaemia.

Neitz (1937) found in experimentally infected sheep that one of the first clinical signs of infection was a rise in body temperature. These febrile reactions, sometimes as high as 41.5°C, were intermittent or continuous for 3 to 4 days with remissions at intervals of a week or more. Intermittent high temperatures have also been described by Jensen (1943); Øverås (1959, 1969) and Ohder (1967).

Icterus (Neitz, 1937; Foggie and Nisbet, 1964; Øverås, 1969) and haemoglobinuria (Neitz, 1937; Øverås, 1962, 1969) have been observed, these signs being indicative of rapid erythrocyte destruction.

Other clinical signs observed are mainly referable to the severity of the anaemia, namely dullness, rapid weak pulse and accelerated respiration (Neitz, 1937). A disinclination to eat accompanied by a loss of weight has been noted (Neitz, 1937; Sheriff *et al.*, 1966). The latter authors found that, in lambs, this loss of body weight began about 1 to 3 weeks after the organisms were no longer demonstrable in blood smears. Øverås (1969) was unable to draw any definite conclusion

regarding the effect of E. ovis on weight gain in lambs, although he did note that infected animals tended to gain less than the controls, and that the retardation of weight gain was more apparent in individuals heavily infected for a considerable time. Foggie and Nisbet (1964), also working with lambs, found that, apart from a slight check in growth about 4 to 6 weeks after the parasites first appeared there was a "reasonable weight gain".

Mortality following experimental infection is not common. Neitz (1937) reported one death in his experimental sheep and Sheriff et al. (1966) destroyed an infected sheep in extremis.

The variation in pathogenicity observed with this organism led Foggie and Nisbet (1964, 1966) and Harbutt (1969a) to suggest the possibility that different strains occur. It is of interest to note that two species have been identified in pigs (Splitter, 1950). One, E. suis, is considered to cause anaemia and icterus with mortality, and the other, E. parvum, is considered to be relatively non-pathogenic. Three separate species have been identified in cattle. The first described species, E. wenyoni, parasitizes the erythrocytes. Hoyte (1962) identified parasites which appeared free in the plasma and which he considered to be a separate species and named them E. teganodes. Uilenberg (1965, 1967) observed organisms associated with bovine platelets for which he proposed the name E. tuomii. Evidence establishing the validity of this latter species was later presented by Zwart et al. (1969).

Haematological Changes and Blood Chemistry

Morphologically, the anaemia is most commonly macrocytic and normochromic (Kreier and Ristic, 1963; Sheriff et al., 1966; Rouse and Johnson, 1966; Harbutt, 1969b; Campbell et al., 1971) although, in less severe cases, it may be normocytic and normochromic (Sheriff et al., 1966; Rouse and Johnson, 1966; Harbutt, 1969b). Øverås (1969) observed hypochromia in sheep with a pronounced anaemia. He, as well as Neitz (1937) and Littlejohns (1960), recorded profound anaemia

with reduction in haemoglobin levels to about 40% of normal. The anaemia is followed by the appearance in the peripheral circulation of immature cell types indicative of a stimulated erythroid response in the bone marrow (Neitz, 1937; Kreier and Ristic, 1963; Ohder, 1967; Øverås, 1969). This has been confirmed by study of serial bone marrow biopsies taken during the course of infection (Kreier et al., 1964), which showed that the erythroid-myeloid ratio shifted from 1:1 to 2:1 at the height of anaemia. A similar observation was made by Øverås (1969).

Recovery of erythroid values is relatively slow. Neitz (1937) noted that, 3 weeks after reaching the minimum level, the haemoglobin concentration was still only 60% of the pre-infection level, and Sheriff et al. (1966) found that recovery took 4-8 weeks and even then haemoglobin levels tended to be lower than before infection. Relapse of infection often occurred at this stage of partial recovery.

It has been demonstrated that infection may be accompanied by a drop in total serum protein levels (Neitz, 1937; Sheriff et al., 1966). However, Øverås (1969) found that E. ovis did not usually produce a marked fall in serum protein level. Neitz (1937) also noted a slight increase in serum non-protein nitrogen levels due mainly to increased urea nitrogen. An increase in serum bilirubin level was recorded by Neitz (1937) and Littlejohns (1960).

No work on the metabolism of the organism or its effect on erythrocyte metabolism has been reported.

Post-Mortem Findings

The post-mortem appearance of infected sheep varies, depending on the stage and severity of the infection. Neitz (1937) found icterus in some cases. Other features recorded include thin watery blood, pallor of various muscles, excess pericardial fluid, enlarged spleen, emaciation, excess peritoneal fluid, and brownish discolouration of the kidneys (Neitz,

1937; Littlejohns, 1960; Foggie and Nisbet, 1964; Sheriff et al., 1966; Jolly, 1967; Øverås, 1969).

Although the kidneys often appear grossly normal they may, on occasions, be visibly discoloured by ferric iron; this can be readily demonstrated by the Prussian blue reaction (Foggie and Nisbet, 1964). Histologically the iron is to be found as "vacuoles" (Foggie and Nisbet, 1964) or granules in the cells lining the convoluted tubules (Foggie and Nisbet, 1964; Sheriff et al., 1966; Jolly, 1967; Øverås, 1969). In some circumstances a positive Prussian blue reaction may extend to the kidney medulla also (Rouse and Johnson, 1966). It has been suggested that this is due to haemosiderin free in the collecting tubules (Øverås, 1969). In some animals, particularly those which have had long periods of haemoglobinuria, subacute and chronic lesions of the glomeruli and interstitial tissues of the kidney have been observed (Øverås, 1969).

Splenic enlargement is a common though not invariable finding at post-mortem (Foggie and Nisbet, 1964). This enlargement may be first observed during parasitaemia and may persist for up to a year afterward. The Malpighian corpuscles are more prominent than normal due to lymphoid hyperplasia. In some animals iron can be demonstrated in phagocytic cells of the splenic red pulp (Foggie and Nisbet, 1964).

Pathogenesis of Anaemia

The anaemia is haemolytic in nature (Weinman, 1944; Kreier and Ristic, 1968) but the pathogenesis of erythrocyte destruction is not known. Littlejohns (1960), who observed large pale erythrocytes in blood smears from infected sheep, suggested that exogenous enzymes of the parasites may have digested the erythrocyte contents. This hypothesis was put forward by Thurston (1954) with regard to E. coccoides infection of mice. However observations from other studies on E. ovis as well as on other blood parasite infections do not support this view.

On evidence available, it would seem that intravascular

haemolysis is the predominant form of erythrocyte loss. In severe infections this is evident from the occurrence of haemoglobinuria (Neitz, 1937; Øverås, 1962, 1969), and the presence of renal haemosiderin at necropsy (Foggie and Nisbet, 1964; Rouse and Johnson, 1966; Jolly, 1967). Intravascular haemolysis is the cause of anaemia in other blood parasite infections such as malaria and babesiosis (Manwell, 1968; Riek, 1968), and involves uninfected erythrocytes (Riek, 1968). Removal of uninfected erythrocytes also occurs in anaplasmosis (Ristic, 1968) although in this disease anaemia is caused by increased erythrophagocytosis, and intravascular haemolysis does not occur.

In all these blood parasite infections the mechanism by which the erythrocyte's susceptibility, either to haemolysis or removal from the circulation is poorly understood. A possibility in E. ovis infection is a host antibody response directed against the parasite, and/or involving the erythrocyte; it has been shown that the antiglobulin (Coombs) test is positive during an infection cycle (Sheriff, 1967; Sheriff and Geering, 1969), (see under DIAGNOSIS).

Association with Ill-Thrift

In many cases the organism has been recovered from individual sheep showing definite clinical symptoms, or from flocks with an ill-thrift problem. Proof of E. ovis being the sole causative agent of these effects has been lacking. Rouse and Johnson (1966) recovered the organism from sheep that were unsaleable because of poor weight gain, but no relative weight loss was recorded in animals which were experimentally infected with the organism. Jolly (1967) recovered the organism from a 4 month old lamb, submitted for post-mortem examination because of flock ill-thrift, but no direct evidence of the organism's association with the ill-thrift in the rest of the flock was obtained.

In a study of ill-thrift in lambs and hoggets in South Australia, Pulsford et al. (1966) found that in many instances

there was a depression of growth which could not be attributed to commonly recognized causes, such as cobalt deficiency, selenium-responsive disease and helminth infection. Further investigations to assess the significance of E. ovis in relation to this unthriftiness resulted in the isolation of the organism from the majority of the flocks (Sheriff et al., 1966). Haematological studies on these flocks showed changes in blood picture similar to those found in sheep artifically infected with E. ovis. A close correlation was evident between haemoglobin values and the physical condition of individuals. In two flocks the course of the disease was followed in detail and found to resemble closely that of experimental E. ovis infection. These workers considered that the majority of ill-thrift problems in South Australia were caused primarily by E. ovis. In Victoria, however, Harbutt (1969a) found that, although E. ovis was widespread, there was no consistent relationship between E. ovis infection and ill-thrift in any age of sheep. Helminthosis, fascioliasis, septicaemia and low copper status were considered to be more important factors. Observations on four lamb flocks (Harbutt, 1969b) showed that uncomplicated infection caused a subclinical disease only, with no significant depression of growth rate. In a later report from Victoria (Campbell et al., 1971) mortalities in unweaned lamb flocks were associated with the presence of E. ovis, although in two of the three flocks investigated the evidence that E. ovis was the direct cause of the mortality was only circumstantial. In Western Australia, E. ovis was observed in flocks with anaemic lambs and a 2 to 6% mortality (Maxwell, 1969). These outbreaks were similar to that previously described in New South Wales (Littlejohns, 1960). However, Littlejohns considered that some other condition, "either nutritive, immunological or otherwise", may have increased susceptibility of the lambs to E. ovis. The fact that he recovered the organism by splenectomizing apparently normal sheep from his experimental flock led him to believe that the organism was common. This could also be concluded from the observations of Neitz (1937) who found that 15 to 20% of his experimental sheep harboured a latent infection, and of Jensen (1943) who isolated the organism at his first attempt, by inoculation of blood from ten apparently healthy ewes into

splenectomized sheep.

It has been suggested that sheep, infected when young, become latent carriers of infection and then serve as a source of infection for subsequent generations of young animals (Littlejohns, 1960; Sheriff et al., 1966). This hypothesis has been tested by splenectomy of groups of young and old sheep; parasitaemia was induced in some of the older sheep but not in the younger ones (Littlejohns, 1960).

Interaction of E. ovis with Other Agents

In view of the many reports of the interaction of E. coccoides with other infectious agents in mice, it is possible that a similar situation could occur in sheep. Although no direct experimental work on this has been reported, E. ovis has often been discovered incidentally during the course of investigations into other diseases (Neitz et al., 1934; Øverås, 1959; Foggie, 1961; Ohder, 1967). In the course of experimental work on bovine petechial fever in sheep, Ohder (1967) found that latent E. ovis infection appeared to be activated by the concurrent bovine petechial fever infection. E. ovis then appeared to suppress bovine petechial fever. However Foggie and Nisbet (1964) found that the onset of tick-borne fever in sheep coincided with the complete disappearance of E. ovis from the circulating blood.

In mice it has been shown that a concomitant or prior infection with E. coccoides greatly enhances the pathogenic action of mouse hepatitis virus (Niven et al., 1952) and lymphocytic choriomeningitis virus (Seamer et al., 1961). Observations have suggested that these viruses initially undergo a cycle of growth within cells of the reticuloendothelial system (Gledhill et al., 1965), and the view has been advanced that E. coccoides increases the susceptibility of the reticuloendothelial cells to these viruses, thereby increasing their pathogenicity (Gledhill et al., 1965). Glasgow et al. (1971) suggested the possibility of increased pathogenicity for mice of viruses such as Newcastle disease virus, Chikungunya virus and poly I:C

virus, which are involved with the reticulo-endothelial system, because of suppression of interferon production by E. coccoides. This suppression of interferon was most striking during the acute phase of E. coccoides infection. Work with Semliki Forest virus, which is not primarily associated with the reticulo-endothelial system, has shown that a prior infection of E. coccoides protects mice from the lethal effects of this virus (Voller and Bidwell, 1968). A synergistic interaction between E. coccoides and a lactose dehydrogenase-elevating virus has also been reported (Riley, 1964); in this case, the effects of both virus and E. coccoides were potentiated and a metabolic interaction was suggested.

Gledhill and Niven (1957) found that in mice Gram-negative bacterial filtrates prepared by inactivating the bacteria with formalin, centrifuging and filtering the supernatant, were toxic for mice with E. coccoides infection. The toxic principle which was believed to be lipopolysaccharide, had little or no effect in normal mice.

Other observations with E. coccoides in mice have shown that the infection suppressed the protozoal organisms Plasmodium berghei, Babesia rodhaini (Peters, 1965), and Plasmodium chabaudi (Ott and Stauber, 1967; Ott et al., 1967).

Because of these interactions, it has been suggested that Eperythrozoon infection of laboratory animals can cause some subtle complications in biomedical research (Baker et al., 1971). The host's response in experiments involving studies of reticulo-endothelial function, radiation injury, antibody production, experimental viral and protozoal infections, interferon induction and tumour transplantation could be affected by the presence of these organisms.

IMMUNITY

The immunological state produced is thought to be one of premunition, an immunity which depends on a persistent latent infection (Neitz, 1937). The duration and strength of this

immunity appear to be variable.

It is evident that the spleen plays an important role in this host-parasite relationship, as splenectomy of latent carriers causes a relapse with parasitaemia and anaemia (Neitz, 1937; Littlejohns, 1960). The anaemia produced may be very severe and result in haemoglobinuria (Neitz, 1937; Rouse and Johnson, 1966). In contrast the severity of infection caused by inoculation of splenectomized sheep may not differ significantly from that of non-splenectomized sheep. Splenectomized sheep recover from the relapse and subsequently become latent carriers (Neitz, 1937).

The duration of immunity varies considerably. The earliest successful reinfection by inoculation recorded is at 6 weeks after the first inoculation (Ohder, 1967) though some sheep were still refractory after 5 months. Using a fluorescent antibody technique, Ohder (1967) found that circulating antibodies were first detectable when the parasitaemia had decreased considerably or had disappeared completely. He found that the presence of these antibodies appeared to inhibit reinfection and that animals lacking antibody were susceptible to reinfection.

Kreier and Ristic (1963), using fluorescent antibody, demonstrated that E. ovis has antigens in common with E. wenyoni, an organism which infects cattle.

DIAGNOSIS

Diagnosis of infection is usually dependent on demonstration of the organism in blood smears, or inoculation of known susceptible animals with suspect blood (Littlejohns, 1960). As the organism may not be demonstrable at the peak of anaemia when clinical effects are most evident (Neitz, 1937; Littlejohns, 1960; Sheriff et al., 1966), smears from a considerable number of clinically normal in-contact sheep may have to be examined before infection is detected in a flock. Splenectomy may reveal latent carriers.

Serological studies have been limited. Kreier and Ristic

(1963), using Anaplasma marginale complement-fixation antigen produced from cattle, obtained consistently positive complement-fixation reactions in acute infections. However, this may have been due to the presence of E. wenyoni antigen in the A. marginale antigen preparation. Sheriff (1967) and Sheriff and Geering (1969), working in South Australia, used a modification of the antiglobulin test of Coombs et al. (1945) and obtained a positive agglutination of cells from animals in which infection was present, or had recently been active. The test generally became positive 7 to 14 days after parasites were first seen in smears, with the titre continuing to rise after the parasites had begun to decrease in number or were no longer detectable. This rise in titre often coincided with the fall in haemoglobin values. Although Sheriff (1967) found, in a limited number of observations, that the test remained negative during and after parasitaemic episodes to the first, later work (Sheriff and Geering, 1969) on naturally infected flocks, showed that the test was invariably positive at every parasitaemic cycle. In these cases, the titre and persistence of the antibody tended to diminish in the later cycles. Generally, results were negative in uninfected sheep, in the incubative and early parasitaemic period, and in the latent period of infection. It was found that, in flocks in which E. ovis was absent or inactive, 90% or more of antiglobulin tests were negative and, in flocks with active infection, the proportion of positive results varied with the incidence of detectable infection and ranged up to 40%. Sheriff (1967) has pointed out that in areas other than South Australia the test may not be specific for E. ovis, particularly if other blood parasites are present.

For diagnosis on a flock basis, Sheriff (1972) has recommended the examination of from 6 to 10 blood samples collected from a cross-section of the flock, and that diagnosis of the presence of E. ovis can be made from the following three examinations; the haemoglobin concentration, erythrocyte morphology, and more particularly, the antiglobulin test. This latter test has been found to be a valuable diagnostic aid in South Australia as it may remain positive after parasites are no longer detectable in blood smears.

TREATMENT

In earlier studies on treatment, Neitz (1937) found that use of the arsenical compound neoarsphenamine at a dose of 5.0 to 7.5 mg/Kg had a temporary parasitacidal action, but a relapse invariably occurred. A dose of 45 mg/Kg did not give complete eradication. Similar results with the same drug were obtained by Littlejohns (1960).

Tetracyclines have been found to be effective in the treatment of other species of Eperythrozoon (Splitter and Castro, 1957; Thurston, 1953) but it was found that treatment of lambs clinically affected by E. ovis with oxytetracycline had no apparent effect (Campbell et al., 1971). However intravenous injection of Spirotrypan forte into sheep has proved effective in eliminating E. ovis infection (Sheriff, 1973), though treatment of flock outbreaks in such a manner is not considered economically feasible.

SUMMARY

Eperythrozoon ovis is an organism, classified in the order Rickettsiales, which parasitises the erythrocytes of sheep and, possibly, goats. It is world wide in distribution and is probably transmitted by biting insects. The organism is pleomorphic, but is most commonly seen as small ring-shaped, coccoid or rod-shaped bodies on the surface of erythrocytes. The principal effect of the organism is to produce a haemolytic anaemia. The mechanism by which this anaemia is caused is not clear. The evidence indicates that the organism is common, and that most infections are not detected. Severe, clinically obvious anaemia is sometimes produced and there is some evidence that the infection may cause,,or contribute to, unthriftiness of young sheep. Infections are rarely fatal and animals usually recover without treatment. Recovered animals are potentially latent carriers of infection and resistant to superinfection for a variable period. Resistance appears to depend on a state of premunity, Animals with latent infections may suffer relapses and parasitaemia can also be induced by splenectomy. Work on *Eperythrozoon* infections in mice and, to a lesser extent in sheep, suggests that sheep could be predisposed to *E. ovis* infections by other diseases, and that *E. ovis* infections could alter the susceptibility of sheep to some infectious diseases. Diagnosis of *E. ovis* infection is usually based on the detection of organisms in Romanowsky stained blood smears.

CHAPTER II

MATERIALS AND METHODS

EXPERIMENTAL APPROACH AND GENERAL MATERIALS AND METHODS

It is clear from the literature that knowledge of Eperythrozoon ovis is far from complete. Even in those areas where experimental work has been concentrated there are considerable areas of disagreement. The effect of the infection on the productivity of sheep under field conditions is disputed. The haematological changes induced by E. ovis have been studied by a number of workers with sometimes contradictory results. Scarcely any work has been done on the pathogenesis of the anaemia and none at all on the effect of the organism on erythrocyte metabolism. The experimental studies described herein were planned with these deficiencies in mind.

This study of Eperythrozoon ovis infection in sheep comprises seven major experiments (Table I). The majority of observations were made on experimentally infected sheep. Although each experiment was designed to yield information on some specific aspect of E. ovis infection (see Table I), data relevant to other aspects were also collected. For this reason it is necessary to describe the techniques used in a single chapter to avoid repetition.

The Parasite

The strain of Eperythrozoon ovis used was isolated at Massey University from an unthrifty lamb with anaemia (Jolly, 1967). The organism was subsequently maintained by serial passage at intervals of 2 to 4 weeks by intravenous inoculation of young susceptible sheep. Infected blood from these sheep was used for infecting experimental animals.

Experimental Animals

In all experiments 6 to 15 month females or castrated males

TABLE I

MAJOR EXPERIMENTS IN THE INVESTIGATION OF EPERYTHROZOON OVIS INFECTION

Experiment Number	Primary Purpose of Experiment
I	To examine the influence of the size of infecting dose on the course of E. ovis infection.
II	To examine the effect of E. ovis infection on the haemogram and body weight of young grazing sheep.
III and IV	To examine the effect of E. ovis on the reticulo-endothelial system.
V	To investigate the effect of E. ovis on the carbohydrate metabolism of erythrocytes.
VI	To examine the effect on sheep of the increased carbohydrate metabolism in E. ovis infected erythrocytes.
VII	To examine the effect of E. ovis on some factors controlling the structure and function of the erythrocyte.

of Romney and Perendale breeds were used.

Bleeding of Experimental Animals

Experimental sheep were bled from the jugular vein. Unless otherwise stated, sodium ethylenediaminetetra-acetic acid (EDTA) was the anticoagulant.

Haematological Estimations

In all experiments the following haematological parameters were measured:

(i) Haemoglobin (Hb) concentration.
(ii) Packed cell volume (PCV).
(iii) The degree of parasitaemia.

Blood smears from control sheep were also examined for the presence of E. ovis to check for the possibility of cross-infection.

Other haematological parameters measured in some of the experiments were:

(i) Total erythrocyte count.
(ii) Reticulocyte percentage.
(iii) Mean corpuscular haemoglobin concentration (MCHC).
(iv) Mean corpuscular volume. (MCV)
(v) Mean corpuscular haemoglobin (MCH).
(vi) Total leucocyte count.
(vii) Differential leucocyte count.
(viii) Plasma haemoglobin concentration.
(ix) Osmotic fragility of erythrocytes.
(x) Haemoglobin (Hb) type.
(xi) Plasma and erythrocyte potassium concentration.

The experiments in which the various parameters were measured are listed in Table II.

Haemoglobin concentrations were estimated by the cyanmethaemoglobin method described by Dacie and Lewis (1968). Commercially prepared Drabkin's reagent and standard solutions were used.(1)

---

(1) Manufactured by Diagnostic Reagents Limited, Thame, Oxon, England.

TABLE II

General haematological, biochemical and organ and tissue data obtained in each experiment.

Exp. number	Number of infected groups	Number of control groups	Haematological data												Biochemical data				Organ and tissue data							
	1					2			2	2	2	2			2											5
I	Four (5)	One (5)	Hb	PCV	BS	RBC		MCHC	MCV	MCH	WBC	DWC	HT	RK	OF			TP		LW	DW	SPL			EP	KUP
II	One (32)	One (32)	Hb	PCV	BS			MCHC												LW						
III	One (9)	Two (5) (4)	Hb	PCV	BS			MCHC	MCV	MCH						BUN		SGOT	TP	LW	DW	SPL	LIV	6 HLN	EP	7 KUP
IV	Three (5)	Three (5)	Hb	PCV	BS			MCHC					HT						TP FP	LW	DW	SPL	LIV	LNG	HLN	HP	5 KUP
V	One (6)	Two (6) (8)[3]	Hb	PCV	BS		R[4]	MCHC					HT	RK												
VI	One (7)	One (7)	Hb	PCV	BS			MCHC																		
VII	Two (7)	One (7)	Hb.	PCV	BS		R	MCHC					HT		PH		BU	BIL	TP	LW						

1. Number of sheep in each group
2. Estimations on control group and one infected group only.
3. This group of 8 was subdivided into two groups of 4. One sub group was bled to simulate the severity of anaemia produced by *T.ovis* only.
4. On smears of washed red cells only.
5. Counts at post-mortem only.
6. Haemolymph node size graded on 1 to 5 basis (see text).
7. Counts before infection and at post mortem.

Key:

Hb = Haemoglobin.
PCV = Packed cell volume.
BS = Blood smear examination for parasites.
RBC = Red blood cell (erythrocyte count)
R = Reticulocyte count
MCHC = Mean corpuscular haemoglobin concentration.
MCV = Mean corpuscular volume.
MCH = Mean corpuscular haemoglobin.
WBC = White blood cell (Leucocyte count).
DWC = Differential white cell (leucocyte) count.
HT = Haemoglobin type.
RK = Red cell potassium.
PH = Plasma haemoglobin.
OF = Osmotic fragility.
BUN = Blood urea nitrogen.
BU = Blood urea.
BIL = Bilirubin.
SGOT = Serum glutamic oxalacetic transaminase.
TP = Total protein.
FP = Fractionated proteins.
LW = Live weight.
DW = Dressed weight.
SPL = Spleen weight.
LIV = Liver weight.
LNG = Lung weight.
HLN = Haemolymph node size measured.
HP = Histopathology.
KUP = Kupffer cell counts.

Packed cell volumes were measured by the microhaematocrit method. Blood was centrifuged at 13,000 g for 5 min.

Blood smears were stained with Tetrachrome (MacNeal) stain at pH 6.8 for 6 minutes after a prior fixing period of 3 minutes.

Estimation of the degree of parasitaemia: In the field trial experiment (Experiment II) parasitaemia was estimated subjectively on peripheral blood smears by the method of Littlejohns (1960). The scale used by Littlejohns is as follows:

Grade 1. Present, but required searching of the smear to be found.
Grade 2. Found readily in small numbers in all fields.
Grade 3. Most cells parasitized with 3 to 4 parasites per cell common.
Grade 4. Heavy infection. Practically all cells affected by several parasites.
Grade 5. Extremely heavy parasitaemia.

In the other experiments a more accurate estimation of the degree of parasitaemia was made by counting the number of parasites (N) attached to, or overlying, 1,000 erythrocytes. Counts were performed in an area of the blood smear where there was no overlapping of the erythrocytes. Parasites lying free of the erythrocytes were not counted. The degree of parasitaemia is expressed as N/1000, the mean number of organisms/erythrocyte.

Erythrocytes and leucocytes were counted by standard dilution methods (Dacie and Lewis, 1968; pp 21 and 61) using formol citrate and acetic acid diluents respectively. Duplicate counts were made and, if differing by less than 10%, means calculated. Where counts differed by more than 10% fresh dilutions were made and counts repeated. Results are expressed in cells per mm^3.

The indices (MCHC, MCV, MCH) were calculated as described by Dacie and Lewis (1968, p. 78).

Reticulocyte counts were made on air dried smears prepared after

equal volumes of blood and new methylene blue stain[2] had been incubated at room temperature for 10 to 15 min. The percentage of reticulocytes in a minimum of one thousand erythrocytes was estimated by using a Miller ocular eyepiece[3] as described by Dacie and Lewis (1968, p. 32).

Plasma haemoglobin was estimated by the method of Harboe (1959).

Osmotic fragility of erythrocytes was determined using dilutions of buffered saline containing the equivalent of 0.85, 0.80, 0.75, 0.70, 0.65, 0.60, 0.55, 0.50, 0.45, 0.40, 0.30, and 0.10% NaCl: 0.025 ml samples of blood were added to 5 ml of each dilution. Haemolysis was judged after 30 min. at room temperature (20°-24°C) as described by Dacie and Lewis (1968, p. 167). All stock and working solutions were stored at 4°C.

Haemoglobin types were determined by electrophoresis of a prepared haemolysate. Erythrocytes were washed 3 times in normal saline and then mixed with 5 times their packed volume of distilled water and allowed to lyse over a period of 10 min. at room temperature. To remove the stromal material, 1.0 ml of toluene was mixed with the haemolysate which was then centrifuged at 2,000 g for 15 min.

Electrophoretic separation was performed on cellulose acetate (Kohn, 1969) using the PhoroSlide system[4]. The acetate strips were pre-buffered in TEB buffer[4] and placed in the electrophoresis cell containing a barbital buffer solution at pH 8.6[4]. After application of 0.3 ul of the haemolysate to the strip an operating voltage of 100 volts (direct current) (D.C.) was applied for 30 min. The slide was then stained and fixed in a Ponceau-S concentrate for 10 min and rinsed in 5% acetic acid until the background was free of colour.

---

(2) New Methylene Blue (Basic Blue 24). Matheson Coleman and Bell. East Rutherford. New Jersey. U.S.A.

(3) Graticules Limited. 57/60 Holborn Viaduct. London. E.C.1.

(4) Millipore Corporation. Bedford. Mass. U.S.A. Buffer formulae contained in booklet "PhoroSlide Electrophoresis System - Principles and Operating Instructions".

Erythrocyte potassium concentrations were calculated from whole blood, plasma and PCV data (Evans et al., 1963). Concentrations (m Eq/litre) in the samples were measured with a Gallenkamp[5] flame photometer.

Biochemical Methods

The experiments in which biochemical measurements were performed are indicated in Table II. Unless otherwise stated colorimetric measurements were made with a "Spectronic 20"[6] spectrophotometer.

Serum glutamic oxalacetic transaminase (SGOT) was measured by a Sigma[7] modification of the method described by Reitman and Frankel (1957) and using Sigma reagents. The results were expressed in Sigma Frankel Units (SFU) per ml.

Blood urea was estimated in the form of blood urea nitrogen (BUN) in Experiment I by a semi-quantitative chromatographic technique[8]. Because of the unreliability of this method, blood urea was measured in Experiment VII by the Diacetyl method (Wootton, 1964).

Bilirubin in serum was determined by the method of Malloy and Evelyn (1937). Both free and conjugated forms were estimated.

Total plasma protein was measured by two methods. In the initial experiments, the Biuret method (Wootton, 1964) was used. In Experiment VII estimations were made with a protein refractometer[9] which gave comparable results to the Biuret method and was much simpler to use.

---

(5) Gallenkamp, Christopher Street, London, E.C.2, England.

(6) Bausch and Lomb, Rochester, New York, U.S.A.

(7) Sigma Technical Bulletin, No. 505, Sigma Chemical Company, 3500 De Kalb St., St. Louis, Missouri, U.S.A.

(8) "Urastrats", Manufactured by Warner-Chilcott Laboratories.

(9) Hitachi Perkin-Elmer. (Model PRP-B).

Plasma proteins were fractionated by electrophoresis on cellulose acetate using PhoroSlides. The acetate strips were pre-buffered in barbital buffer at pH 8.6. The same buffer was used as the cell medium, and after application of the plasma sample to the strip within the cell, the separation was run for twenty minutes at 100 volts (D.C.). The strips were stained as described for haemoglobin separation, and scanned in a Canalco Model G Densitometer.(10)

Gross and Microscopic Examinations of Animals

The data recorded in various experiments are shown in Table II.

Body weights: Animals were weighed in a commercially available sheep weighing crate which employs a spring scale. Weights were read to the nearest 250 gm.

Dressed weight and body organ weights: Sheep were killed by severance of the major vessels of the neck and then the cervical spinal cord. The carcases were skinned and dressed according to normal slaughterhouse practice. Each dressed carcase was weighed on a spring balance to the nearest 250 gm and the organs were weighed on a pan balance, to the nearest gm.

Haemolymph node size was subjectively graded in Experiment III using a scale ranging from 1 to 5 as follows:

Grade 1	Estimated 1.0-2.0 mm diameter
Grade 2	Estimated 2.0-3.0 mm diameter
Grade 3	Estimated 3.0-4.0 mm diameter
Grade 4	Estimated 4.0-5.0 mm diameter
Grade 5	> 5.0 mm diameter.

In Experiment IV more accurate measurements were made using callipers. Measurements of diameter were made of 5 to 10 sublumbar haemolymph nodes in each sheep and the mean value of the readings calculated.

---

(10) Canal Co., Rockville, Maryland, U.S.A.

Histopathological examination of various tissues was performed on paraffin sections of material which had been fixed in 10% formol saline. Sections were stained either with haemotoxylin and eosin (H and E) or Perl's Prussian Blue stain (Culling, 1963).

Kupffer cells were counted in H and E stained paraffin sections. Blocks were taken from two approximately standard positions in each liver; one at the edge of the ventral lobe and the other deep in the liver parenchyma adjacent to the vena cava. Cells were counted at 250X magnification using a calibrated eyepiece graticule. Ten fields, confined to mid-acinar regions and each of 0.09 mm^2 area, were examined in each slide. Total counts were converted to cells per 0.1 mm^2. Cells designated as Kupffer cells were those with prominent nuclei lining the sinusoids. Some cells included in the counts may have been in the transition stage between the flat endothelial cells and true Kupffer (phagocytic) cells, (Popper and Schaffner, 1957).

EXPERIMENTAL DETAILS AND SPECIFIC MATERIALS AND METHODS

Experiment I - To Examine the Influence of the Size of Infecting Dose on the Course of E. ovis Infection

Twenty-five castrated male, 12 to 14 month old Romney sheep were divided randomly into 5 equal groups which were housed in separate but adjacent indoor pens. Feed, in the form of commercial sheep nuts and lucerne hay, was provided ad lib. Various dilutions of infected blood (approximately 8 parasites per erythrocyte) were made in non-infected ovine plasma at room temperature; the plasma had been filtered under pressure through a 0.22 µ pore-size cellulose acetate membrane filter, and heated to 56°C for 30 minutes on the previous day and stored over night in a refrigerator at 4°C. Groups were inoculated intravenously as follows:

Group I	1 ml plasma used as diluent (control group).
Group II	1 ml infected blood not diluted.
Group III	1 ml plasma, containing 1 X 10^{-2} ml of infected blood.

Group IV — 1 ml plasma, containing 1 X 10^{-4} ml of infected blood.

Group V — 1 ml plasma, containing 1 X 10^{-6} ml of infected blood.

All sheep were bled twice weekly and haematological and biochemical data as indicated in Table II were obtained. Sheep were weighed on the day of inoculation and at the termination of the experiment; organ and tissue data were also obtained (see Table II).

Experiment II - To Examine the Effect of E. ovis Infection on the Haemogram and Body Weight of Young Grazing Sheep

Sixty-four shorn hill-country 5 to 6 month old Perendale mixed castrated male and female lambs were randomly divided into 2 groups. The groups were grazed together, at approximately 50 to the hectare, for the duration of the experiment, commencing in February and terminating in April. No supplementary feed was supplied. At the start of the experiment the sheep were identified by eartags, weighed and bled. Blood smears from all animals were examined for the presence of E. ovis. Sheep in one group were inoculated intravenously with 1.0 ml of heavily infected blood. Thereafter all sheep were bled and weighed once weekly until the termination of the experiment. Haematological data as indicated in Table II were obtained.

Experiment III - To Examine the Effect of E. ovis on the Reticulo-endothelial System

Eighteen, 8 to 10 month old Perendale castrated male sheep were divided randomly into 2 groups of 9, and individually identified with ear tags. Liver biopsies were taken from the 9 sheep destined to become the infected group, and from 5 sheep of the control group. Two days after the liver biopsies, sheep in the test group were infected by intravenous inoculation of 1.0 ml of blood heavily infected with E. ovis. The 2 biopsied groups (9 and 5 sheep respectively) were housed in separate indoor pens in different sections of the building. The remaining 4 control sheep were housed with the infected group as

"in-contact" controls. All sheep were bled at 3 day intervals, and weighed on the day of inoculation and at the end of the experiment. Haematological, biochemical and organ and tissue data were obtained as indicated in Table II.

Liver biopsy technique: Sheep were anaesthetized with thiopentone sodium. An incision was made in the right sublumbar fossa, and a portion of liver approximately 1 cm cube was removed from the lateral border of the ventral lobe. This was immediately fixed in 10% formol saline. A similar sample of liver was obtained at the end of the experiment when the sheep were killed and necropsied.

Experiment IV - To Examine the Effect of E. ovis on the Phagocytic Activity of the Reticuloendothelial System

Thirty mixed Perendale and Romney castrated male, 10 to 12 month old sheep were used. These were randomly divided into 6 equal groups, 3 of which were inoculated with 1.0 ml of blood heavily infected with E. ovis, and the remaining groups kept as controls. All sheep were run together on pasture at approximately 25 to the hectare. They were bled at intervals of 3 or 4 days, and haematological and biochemical data as shown in Table II were obtained.

Carbon clearance studies were performed on control and infected groups as shown in Table III. The day following carbon clearance studies, sheep were killed and organ and tissue data obtained (Table II). Representative portions of various body organs were taken for carbon analysis.

Carbon clearance technique: A suspension of carbon (C 11/1431a)[11] which had been manufactured for reticuloendothelial studies, was used. The carbon particles are suspended in a solution of fish glue with a small amount of phenol as preservative. The suspension was centrifuged at 2,500 g for 15 min. to remove any large particles present. The supernatant contains approximately

---

(11) Supplied by Gunther Wagner, Hanover, Germany.

TABLE III

CARBON CLEARANCE TESTS IN SHEEP IN RELATION TO STAGE OF INFECTION WITH *E. OVIS*

Days after inoculation of *E. ovis* when carbon clearance tests performed	Groups of sheep tested and degree of *E. ovis* infection of red cells
5	A - early light infection
	B - non-infected controls
12	C - heavy infection at peak parasitaemia
	D - non-infected controls
26	E - light infection at recovery stage
	F - non-infected controls

90 mg carbon per ml (Biozzi et al., 1953) with particle size of approximately 25 nm diameter. For in vivo clearance studies, the method as described by Biozzi et al. (1953), was used with some modifications. The carbon suspension was diluted 1:1 with 0.85% NaCl to give a final carbon concentration of approximately 45 mg/ml of suspension. This was injected intravenously at a dose rate of 8 mg/100 gm body weight. At regular intervals after administration of the carbon, 0.02 ml of blood was drawn into a Sahli pipette from an ear vein puncture and lysed in 3.0 ml of 0.1% Na_2CO_3. The concentration of carbon was calculated from a pre-calibrated graph after determination of the optical density (O.D.) in a Unicam SP 500 Spectrophotometer at a wavelength of 675 nm. From these readings, the phagocytic index (k), and the corrected phagocytic index (α) were calculated by the following formulae: $k = (\text{Log } C1 - \text{Log } C2)/(T2 - T1)$ and $\alpha = (W/Wls)\sqrt[3]{k}$ (Biozzi et al., 1953). (C1 and C2 are the concentrations of carbon at times T1 and T2. W = body weight of the animal, Wls = combined weight of liver and spleen).

Carbon analysis of body organs: Preliminary experiments showed that there were no gross variations in the distribution of carbon within organs. Portions of spleen, liver, lung, lymph node, kidney and haemolymph node were taken at necropsy for digestion and estimation of carbon particle content by a modification of a method described by Fisher et al. (1968). Weighed samples of the order of magnitude of 0.5 gm were digested in 2.0 ml of 2% gum acacia and 1.0 ml of 70% ethanol containing 10% KOH. The digest was incubated in a water bath at 56°C for 36 hours at which time the volume was adjusted to 10 ml with distilled water. After light centrifugation (800 g for 5 min.) to remove large tissue particles, the digest O.D. was determined at 800 nm against a blank of gum acacia and alcoholic KOH. The concentration of carbon in tissues was estimated by comparison of the O.D. with a standard line.

Experiment V - To Investigate the Effect of E. ovis on the Carbohydrate Metabolism of Erythrocytes

The glucose utilization, lactic and pyruvic acid production,

oxygen uptake and Glucose-6-Phosphate Dehydrogenase (G6PD) activity of infected and non-infected erythrocytes were studied *in vitro* (Part 1). The same parameters were compared in non-infected erythrocytes from anaemic sheep and non-infected erythrocytes from normal sheep (Part 2).

Part 1. Twelve Romney castrated male and female sheep 12 to 15 months old were divided randomly into 2 equal groups. Because of logistical problems, the experiment was performed 3 times, using 2 infected and 2 control sheep each time, but all infected sheep were examined at equivalent stages and degree of parasitaemia. The results from the pairs were combined.

Part 2. Eight sheep, similar to those used in Part 1 were divided into 2 groups of 4. The experiment was performed twice using 2 test and 2 control sheep each time. The 2 test sheep were bled 3 times from the jugular vein at daily intervals so that a total of 1.2-1.5 litres of blood was removed. This resulted in a degree of anaemia similar to that of the *E. ovis* infected sheep in Part 1.

In both experimental series sheep were bled every 2 or 3 days for haematological (Table II) and biochemical studies.

Preparation of cell suspensions: Ten ml of blood were collected aseptically into ice cold test tubes containing 0.05 ml of 1% heparin. After mixing, the blood samples were cooled in ice prior to centrifugation at 1,500 g for 15 min. at 4°C. The plasma, buffy coat and approximately 0.75 ml of the uppermost packed erythrocytes were removed and discarded. The remaining erythrocytes were washed twice in cold (4°C) isotonic saline. After the last centrifugation, 1.0 ml of packed erythrocytes was removed from the bottom of the tube with a pasteur pipette and transferred to a graduated centrifuge tube for G6PD estimation. The remainder of the packed erythrocytes was resuspended in an incubation medium of Krebs-Ringer phosphate ($KRPO_4$) buffer solution, pH 7.4 (Umbreit *et al.*, 1964). A sample was then removed for estimation of PCV, reticulocyte percentage and to check for the presence of leucocytes.

Incubation of erythrocytes was carried out in a Braun Warburg apparatus[12] using 3.0 ml capacity single side arm manometer flasks. Stock solutions of components of $KRPO_4$ were stored at 4^oC and fresh buffer was made up immediately before each set of incubations. Solutions of glucose in $KRPO_4$ were prepared immediately before use and 0.1 ml was added via the side arm of each manometer flask. The concentration of the glucose solution was such that the final concentration in the flask was 90 mg/100 ml of medium. The erythrocytes were incubated aerobically for 2 hours at 37^oC, with the flasks shaking at 100 oscillations per minute. The CO_2 was removed by means of 0.2 ml of 40% KOH in the centre well of each flask.

After incubation, the contents of each flask were transferred into 3.0 ml of cold 10% trichloracetic acid (TCA); each flask was then washed twice with 1.0 ml of distilled water and the washings added to the TCA, mixed, placed in an ice bath for 20 minutes and centrifuged at 2,600 g for 45 minutes (4^oC). Glucose, lactic acid and pyruvic acid estimations were performed on the supernatant.

Biochemical methods: Glucose-6-Phosphate Dehydrogenase (G6PD) was estimated by the method of Kornberg and Horecker (1955), after preparation of the haemolysate as described by Loder and de Gruchy (1965). The assay system contained tris-HCl buffer, pH 7.4, 250 mM, nicotinamide adenine dinucleotide phosphate (NADP)[13] 0.5 mg, $MgCl_2$ 20 mM, haemolysate 0.05 ml and glucose-6-phosphate 2.0 mM.

Glucose was measured using glucose oxidase and peroxidase[13] as a coupled enzymatic reaction using O-dianisidine as the colour reagent. The method is a Sigma[14] modification of the procedure described by Raabo and Terkildsen (1960).

Lactic acid determinations on the supernatant were made using the method of Barker and Summerson (1941), and pyruvic

---

(12) Warburg Apparatus Model V 85 B. Braun. Apparatebau. Melsungen. West Germany.

(13) Sigma Chemicals. St. Louis, Missouri, U.S.A.

(14) "Sigma" Tentative Technical Bulletin. No. 510, January 1967.

acid was determined by the method of Koepsell and Sharpe (1952).

Experiment VI - To Examine the Effect on Sheep of the Increased Carbohydrate Metabolism in E. ovis Infected Erythrocytes

Fourteen mixed female and castrated male 9 to 10 month old Perendale sheep were divided randomly into 2 equalgroups and identified with ear tags. One group was inoculated with 1.0 ml of blood heavily infected with E. ovis. Both groups were run together on pasture at approximately 20 to the hectare.

At 2 or 3 day intervals 2 blood samples from each sheep were collected in Vacutainers and stored in ice until estimations were performed. For haematological (Table II) and glucose, lactic and pyruvic acid estimations, EDTA was used as anticoagulant. For acid-base determinations heparin was used.

Plasma glucose concentrations were measured by the method previously described (P 34).

Lactic acid and pyruvic acid levels in whole blood were determined by an ultra-violet method (340 nm) using Boehringer kits.(15)

The estimation of lactic acid is based on the conversion of lactic acid to pyruvic acid by lactic acid dehydrogenase (LDH) with concomitant reduction of nicotinamide adenine dinucleotide (NAD) to reduced nicotinamide adenine dinucleotide (NADH).

$$\text{Lactic Acid} + \text{NAD} \xrightleftharpoons{\text{LDH}} \text{Pyruvic Acid} + \text{NADH}$$

The amount of NAD reduced, which is dependant on the amount of lactic acid in the sample, is determined from the increase in O.D. at 340 nm.

The estimation of pyruvic acid is based on the reverse reaction, where the amount of NADH oxidized, which is dependant on the amount of pyruvic acid in the sample, is determined from the decrease in O.D. at 340 nm.

---

(15) The Boehringer Corp. Ealing. London. W.5. England.

The acid-base status of blood was determined with an Astrup Micro-Apparatus.(16) The determinations were started within 1 hour of blood collection and completed within 3 hours. The apparatus uses the pH microelectrode and microtonometry systems of Astrup et al. (1960), and the results were calculated by the use of the modified Siggaard-Andersen Curve Nomogram (Siggaard-Andersen, 1962).

Parameters measured on anaerobically drawn venous blood were:

(i) Actual pH.

(ii) Actual pCO_2 (mm Hg) (The partial pressure of CO_2 in anaerobically drawn blood).

(iii) Actual bicarbonate (m Eq/litre) (The bicarbonate concentration in plasma of anaerobically drawn blood.

(iv) Total CO_2 (m Eq/litre) (The CO_2 derived from carbonic acid and bicarbonate in plasma from anaerobically drawn blood).

(v) Standard Bicarbonate (m Eq/litre) (The bicarbonate concentration in the plasma of blood equilibrated at a pCO_2 of 40 mm Hg and with oxygen at full saturation level of haemoglobin).

(vi) Buffer Base (m Eq/litre) (The sum of buffer anions, which are mainly bicarbonate and proteinate ions.

(vii) Base Excess (m Eq/litre) (Defined as zero for blood with pH 7.40 at pCO_2 of 40 mm Hg.) Positive values indicate an excess of base or deficit of fixed acid. Negative values indicate a deficit of base or excess of fixed acid.

Experiment VII - To Examine the Effect of E. ovis on some Factors Controlling the Structure and Function of the Erythrocyte

Twenty-one mixed Perendale and Romney, castrated males and females 9 to 10 months old, were divided randomly into 3 equal groups and identified with ear tags. One group (A) was a control group and the other two groups (B and C) were inoculated with 1.0 ml of E. ovis infected blood. All sheep were run together

(16) Type AME 1. Manufactured by Radiometer. Copenhagen. Denmark

on pasture outdoors at approximately 30 to the hectare. Sheep were bled at 3 or 4 day intervals throughout the experiment. Group C sheep were withheld from feed and water for 24 hours prior to bleeding.

Venous blood was collected into ice-cold Vacutainer tubes containing heparin. Haematological and biochemical data (as indicated in Table II) were collected.

Reduced glutathione (GSH) and methaemoglobin levels in blood were determined both prior to and after incubation for 2 hours at 37°C. Separate samples of blood were similarly incubated with acetylphenylhydrazine after which GSH and methaemoglobin levels were measured. Examinations for Heinz bodies in blood were performed on representative samples from all treatments.

Blood GSH levels were determined by the method of Beutler et al. (1963). Methaemoglobin was measured by the method of Evelyn and Malloy (1938) modified by Henry (1964). The stability test for GSH using acetylphenylhydrazine was by the method of Beutler (1957). For the examination of Heinz bodies erythrocytes were stained supravitally with methyl violet (Dacie and Lewis, 1968).

CHAPTER III

HAEMATOLOGICAL CHANGES IN SHEEP INFECTED WITH EPERYTHROZOON OVIS

INTRODUCTION

Most reports on Eperythrozoon ovis infection include data on the more common haematological parameters, though there are considerable variations in the results reported, particularly with regard to the severity of, and cellular response to, the anaemia (Neitz, 1937; Littlejohns, 1960; Harbutt, 1969b; Øverås, 1969). A difference in severity of disease has been noted between natural and experimental infections (Foggie and Nisbet, 1964) probably reflecting differences in infecting dose rates and routes of administration. In this chapter these aspects are investigated, the common haematological parameters re-examined and further observations made on the blood changes during infection. These include the extent of damage to circulating erythrocytes as measured by plasma haemoglobin levels, osmotic fragility tests of erythrocytes, and the relationship between severity of disease, haemoglobin type and erythrocyte potassium level.

In normal (non-anaemic) adult sheep there are two different haemoglobins, Hb-A and Hb-B (Evans et al., 1956) which are genetically determined by two co-dominant autosomal alleles; these produce three phenotypes, namely A, AB and B (Evans et al., 1956; Huisman et al., 1958). In sheep with Hb type A, or AB, the production of another haemoglobin (type C) occurs under anaemic conditions (see Tucker, 1971). In such conditions production of Hb-A is reduced, or, if the anaemia is severe, it ceases altogether. Because there is evidence to suggest that Hb-A type sheep are less susceptible to some anaemias (Evans et al., 1963; Jilek and Bradley, 1969; Neethling et al., 1969), the effect of different haemoglobin type on E. ovis infection required investigation. Similarly, it has been shown that sheep can be classified on the basis of erythrocyte potassium concentration (Evans, 1954), namely HK-type (80-90 m Eq/litre erythrocytes) and LK-type (20-30 m Eq/litre erythrocytes). Although many studies have been made to define some physiological

or adaptive advantage of either type (see Tucker, 1971), the evidence presented has been inconclusive; but because of the close relationship of E. ovis to the erythrocyte, the concentration of potassium could perhaps influence the course of infection.

MATERIALS AND METHODS

The haematological data recorded in this chapter were obtained from various experiments. Experiment I was designed to investigate the relationship between infecting dose and the severity of the anaemia induced. Haematological data obtained in Experiments II to VII (see Table II) are also recorded in this section. The methods are described in Chapter II. The decline in haemoglobin values in individual animals were calculated as the percentage decrease in Hb level from the last estimate prior to infection. Group means were calculated from these data.

RESULTS

The Effect of Size of Infecting Dose on the Course of E. ovis Infection - Experiment I

The relationships between size of infection dose and the rate of development of parasitaemia and anaemia are shown in Figure 3.1. Times after infection at which parasitaemia was first discernible (prepatent period), of peak parasitaemia and of maximum anaemia for each group are summarized in Table IV. From this data the following observations were made:

(i) The mean prepatent period was inversely proportional to the size of infecting dose.

(ii) The time for peak parasitaemia to be reached was similarly, inversely proportional to the infecting dose.

(iii) In contrast, there was no evidence that the size of infecting dose affected the maximum level of parasitaemia reached.

(iv) The haemoglobin concentrations for each group began

TABLE IV

Experimental infection. The effect of dose rate on various time intervals during infection and the maximum percentage fall in haemoglobin (Experiment I - Group mean values).

Group No.	Dose of infected blood (mls)	Time intervals - days. Inoculation to discernible parasitaemia (pre-patent)	Inoculation to peak parasitaemia	Inoculation to maximum degree of anaemia	Onset to peak parasitaemia	Onset of parasitaemia to maximum degree of anaemia	Peak of parasitaemia to maximum degree of anaemia	Maximum** percentage fall in Hb.	Mean number of parasites per erythrocyte (Maximum)
II	1.0	4	14	25	10	21	11	46.32±5.07*	8.43
III	1.0×10^{-2}	7	14	21	7	14	7	45.79±2.76	10.02
IV	1.0×10^{-4}	11	21	25	10	14	4	54.26±2.71	9.35
V	1.0×10^{-6}	14	25	25	11	11	0	41.61±3.65	8.98

Number of sheep in each group = 5.

*Standard error.

**Analysis of variance between group means for this parameter.

F = 1.66

NS = Not significant (>0.05).

to fall as peak parasitaemia approached and, with the exception of Group V, where peak anaemia coincided with peak parasitaemia, haemoglobin concentrations continued to fall as parasitaemia declined.

(v) There was no significant difference in the maximum degree of anaemia developed between the infected groups (Table IV).

(vi) The time taken to reach the maximum degree of anaemia after inoculation was not apparently influenced by the size of the infecting dose.

(vii) The time taken to reach the maximum degree of anaemia after either the onset of discernible parasitaemia or the peak of parasitaemia decreased with decreasing dose.

Haemoglobin Values in Sheep Experimentally Infected with E. ovis - Experiments I - VII inclusive

For assessment of the degree of anaemia, haemoglobin concentrations were used, because they are more directly related to the oxygen carrying capacity of the blood. Packed cell volume percentages and erythrocyte totals followed a pattern generally similar to haemoglobin concentrations in all experiments. All three parameters were incorporated in the calculation of erythrocyte indices. Information on the degree of anaemia relative to various stages of infection was obtained from those sheep infected with a minimum of 1.0 ml of heavily infected blood. The time relationships between the degree of parasitaemia and haemoglobin values for all the experiments are shown in Figure 3.2. The time relationships between the degree of parasitaemia and haemoglobin values were similar in all experiments; the haemoglobin concentration for each group began to fall as peak parasitaemia approached and continued to fall as parasitaemia declined. The means of the various time relationships obtained are summarized in Table V. There was very little variation in timing between individuals within group means. With the exception of the infected group of Experiment III, the maximum percentage fall of Hb in all groups were not significantly different (Table VI).

TABLE V

Experimental infection. The various time intervals of infection following inoculation with a minimum of 1.0 ml of heavily infected blood. (All experiments - group mean values.)

Exp. No.	Group No	Number of sheep	Time intervals - days. Inoculation to discernible parasitaemia (Pre-patent)	Inoculation to peak parasitaemia	Inoculation to maximum degree of anaemia	From onset to peak parasitaemia	Onset of parasitaemia to maximum degree of anaemia	Peak of parasitaemia to maximum degree of anaemia
I	II	5	4	14	25	10	21	11
II	-	32	7	14	21	7	14	7
III	II	9	6	12	31	6	25	19
IV	-	5	7	14	18	7	11	4
V	-	6	3	12	22	9	19	10
VI	-	7	3	14	24	11	21	10
VII	B	7	3	17	24	14	21	7
VII	C	7	3	14	21	11	18	7

TABLE VI

EXPERIMENTAL INFECTION. MAXIMUM PERCENTAGE FALL OF HAEMOGLOBIN AND THE MAXIMUM DEGREE OF PARASITAEMIA RECORDED. (ALL EXPERIMENTS - GROUP MEAN VALUES)

Exp. No.	Group No.	No. of Sheep	Percent fall in Hb [xxx]	Mean number of parasites per erythrocyte
I	II	5	46.34±5.07[x]	8.43
II	-	32	44.22±1.34	-[xx]
III	II	9	26.11±2.54[xxxx]	4.73
IV	-	5	44.43±1.52	7.86
V	-	6	42.49±3.81	5.92
VI	-	7	35.55±7.04	6.70
VII	B	7	51.38±2.57[+]	8.22
VII	C	7	44.96±5.77[+]	7.79

x = Standard Error

xx = Graded on scale of Littlejohns (1960) and not comparable with other means (Value = 3.44)

xxx = Analysis of Variance between group means for this parameter F = 4.41, P < 0.01

xxxx = This mean significantly (P < 0.05) lower than all means except Exp. VI (t-test analysis)

+ t-test analysis between Groups B and C of Exp. VII - t = 1.02, P = Not Significant (P > 0.05)

The Effect of Withholding Food and Water for 24 Hour Periods on the Haemoglobin level in E. ovis Infection - Experiment VII

Food and water were withheld from Group C sheep in Experiment VII for 24 hours prior to bleeding, a procedure which commonly occurs prior to shearing or during transportation. The sheep appeared unaffected by this treatment and there were no significant differences in the maximum decline of Hb values between the 2 groups of infected sheep (Table VI).

Haemoglobin Values in Sheep Naturally Infected with E. ovis

In Experiments II, III and IV cross-infection of control sheep occurred. The time relationships between experimental and natural E. ovis infection in terms of the onset and degree of parasitaemia and haemoglobin levels are shown in Figure 3.3. The various time relationships in each of the naturally infected groups as well as degree of parasitaemia, and fall in haemoglobin level are summarized in Table VII. In Experiments II and III the study of natural infection was carried through until after the peak of anaemia; in Experiment IV the study was terminated in the early parasitaemic phase. From the data (Tables V, VI and VII) it can be seen that:

(i) The group mean percentage fall in haemoglobin was smaller in the naturally infected groups than in the inoculated groups.

(ii) In the naturally infected group of Experiment II the mean times from onset of parasitaemia to peak parasitaemia, and the maximum degree of anaemia were longer than in the artificially infected groups.

(iii) In contrast, the time relationships in the naturally infected group of Experiment III were similar to those of Group V (low dose rate) in Experiment I (Compare Tables IV and VII).

(iv) The time taken for parasitaemia to commence in the contact group after parasite appearance in the experimentally infected group was shorter in Experiment II, which was conducted in late summer, than in the other 2 experiments, which were held in winter and spring.

TABLE VII

Natural infection. The maximum percentage fall of haemoglobin, the maximum degree of parasitaemia and the various time intervals of infection. (Experiments II, III, IV - group mean values.)

Exp No	Number of sheep in group	Number of sheep infected	Time intervals - days: From inoculation of experimentally infected sheep to discernible parasitaemia	From parasitaemia in experimentally infected sheep to discernible parasitaemia	From onset of parasitaemia to peak (Natural).	From onset of parasitaemia to maximum degree of anaemia (Natural).	From peak of parasitaemia to maximum anaemia (Natural).	Maximum percentage fall in Hb	Mean number of parasites per erythrocyte (Maximum).	Time of year
II	32	31	14	7	21	35	14	25.90 ±2.05*	1.97**	February-April
III	4	4	24	18	9	9	0	8.66 ±4.14	1.69	June-July
IV	5	4	21	14	Not determined	Not determined	Not determined	Not determined	Not determined	September-October

*Standard error.

**Graded on scale of Littlejohns (1960) and not comparable with the other mean.

Relationship Between Degree of Parasitaemia and Severity of Anaemia

In the infected sheep there was a significant ($p < 0.01$) positive correlation between the highest degree of parasitaemia and the maximum percentage fall in haemoglobin (Figure 3.4).

Comparison of Severity of Anaemia between Experimentally and Naturally Infected Sheep - Experiment II

Cross-infection of all but one of the control sheep occurred in Experiment II. Data on the highest degree of parasitaemia and severity of anaemia, from the sheep in both the experimentally and naturally infected groups of Experiment II, are shown in Table VIII. In general, naturally infected sheep did not become as severely parasitised as experimentally infected sheep, nor did they develop as severe an anaemia. However some individual animals did develop parasitaemia and anaemia of similar degree to experimentally inoculated sheep.

Reticulocytes and Mean Corpuscular Haemoglobin Concentration (MCHC)

Reticulocyte percentages were estimated on whole blood in Experiments III and VII, and are shown in relation to the degree of parasitaemia and haemoglobin level of experimentally inoculated sheep in Figure 3.5. Reticulocytes first appeared shortly after the initial fall in haemoglobin when parasitaemia was at its peak. In Experiment VII the mean reticulocyte response in both infected groups reached a peak of 5% and in Experiment III where the anaemia was not as severe (Table VI) the mean maximum response was 2.5%. In the naturally infected sheep of Experiment III, where the anaemia was less severe (Table VII) reticulocytes were only occasionally present.

The relationships between the haemoglobin level and mean corpuscular haemoglobin concentration (MCHC) levels of experimentally inoculated sheep in all experiments (with the exception of Experiment II) are shown in Figure 3.6. Compared with control groups significant ($p < 0.05$) falls in MCHC occurred in infected groups.

TABLE VIII

COMPARISON BETWEEN EXPERIMENTAL AND NATURAL INFECTION OF THE MAXIMUM PERCENTAGE FALL IN HAEMOGLOBIN LEVELS, LOWEST HAEMOGLOBIN LEVELS, AND MAXIMUM DEGREE OF PARASITAEMIA (EXPERIMENT II)

	Experimentally infected sheep	Naturally infected sheep		P
Maximum degree of parasitaemia (Littlejohns, 1960)	3.44±0.12[x]	2.71±0.19		<0.01[xx]
Lowest mean Hb value at peak of anaemia (gms/100 ml)	5.72±0.13	7.25±0.25	t=5.38	<0.001
Range of Hb values at peak of anaemia (gms/100 ml)	4.4-7.3	4.7-10.8		
Mean maximum percentage fall in Hb level (%)	44.22±1.34	32.94±1.95	t=4.76	<0.001
Range of percentage falls in Hb level (%)	27.45-59.09	9.82-52.53		

x = Standard Error

xx = Comparison between these two means by the Rank-Sum test.

In naturally infected sheep significant ($p < 0.01$) falls of MCHC occurred in Experiment II (Table IX) but no fall occurred in the sheep of Experiment III.

In experimentally infected groups the magnitude of the MCHC fall was significantly correlated with the percentage fall in haemoglobin level (group mean fall in MCHC versus group mean percentage fall in haemoglobin, $r = 0.64$, $n = 11$, $p < 0.05$); and with the percentage of reticulocytes present (individual maximum fall in MCHC versus individual maximum reticulocyte percentage, $r = 0.58$, $n = 23$, $p < 0.01$).

Mean Corpuscular Volume (MCV) and Mean Corpuscular Haemoglobin (MCH) - Experiments I and III

Changes in MCV and MCH in relation to the degree of parasitaemia are shown in Figures 3.7 and 3.8 respectively. Significant ($p < 0.05$) increases in both these indices occurred in infected groups compared with control groups. These followed a pattern similar to the MCHC.

The Total and Differential Leucocyte Count in E. ovis Infection - Experiment I

No significant differences in the total leucocyte count between the control and infected groups were recorded during *E. ovis* infection. The relationships between the degree of parasitaemia, and the mean neutrophil and lymphocyte totals for each group during *E. ovis* infection are shown in Figure 3.9. The relationship between degree of parasitaemia and the mean monocyte totals is shown in Figure 3.10. It can be seen that:

(i) There was no apparent effect on the neutrophil and lymphocyte counts resulting from *E. ovis* infection. The significant ($p < 0.05$) differences which were recorded between the control and infected groups, indicated in Figure 3.9, were not related to parasitaemia; similar levels of significant difference (not indicated in the figure) were recorded between infected groups, during the course of infection.

TABLE IX

MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATIONS (MCHC's) OF CONTROL AND EXPERIMENTALLY INFECTED SHEEP - EXPERIMENT II

Week	Control	Inoculated	t	P
0	32.1 ± 0.21^{x}	31.8 ± 0.25^{x}	0.93	NS
1	31.4 ± 0.25	30.9 ± 0.25	1.32	NS
2	32.2 ± 0.49	28.3 ± 0.51	5.51	<0.001
3	30.6 ± 0.27^{xx}	25.8 ± 0.42	9.26	<0.001
4	30.4 ± 0.36^{xx}	28.2 ± 0.25	4.92	<0.001
5	28.9 ± 0.40^{xx}	28.5 ± 0.28	0.80	NS
6	30.0 ± 0.37^{xx}	30.7 ± 0.33	1.33	NS
7	29.2 ± 0.46^{xx}	30.1 ± 0.38	1.44	NS
8	32.5 ± 0.35	33.4 ± 0.36	1.75	NS
9	29.4 ± 0.34^{xx}	30.2 ± 0.32	1.72	NS

Number of sheep in each group = 32

x = Standard error

NS = Not significant ($p > 0.05$)

xx = Significantly ($p < 0.001$) lower by t-test analysis than the control value for week 0.

(ii) The monocyte count in the infected groups showed small but significant ($p < 0.05$) elevations after the peak of parasitaemia (Figure 3.10).

Eosinophils and basophils were occasionally seen, but showed no change in number in relation to E. ovis infection.

Plasma Haemoglobin Levels During E. ovis Infection - Experiment VII

The overall mean plasma haemoglobin level in the control group of Experiment VII was 9.29 ± 0.78 mg/100 ml. The relationships between the degree of parasitaemia and the plasma haemoglobin levels for both the infected groups of Experiment VII are shown in Figure 3.11. In both of these groups increases in plasma haemoglobin levels over those of the control group did occur and were occasionally significant ($p < 0.05$). However, although the group mean correlations between the degree of parasitaemia and the plasma haemoglobin levels were positive, they were not significant (Group B. $r = 0.47$, $n = 14$, $p > 0.05$; Group C. $r = 0.19$, $p > 0.05$).

The Effect of E. ovis on the Osmotic Fragility of Erythrocytes - Experiment I

The relationship between the Median Corpuscular Fragility (MCF)[17] and development of parasitaemia in the infected group is shown in Figure 3.12. Control group values are also shown. The erythrocytes became more fragile as infection progressed and the correlation between the degree of parasitaemia and MCF was significant ($r = 0.80$, $n = 8$, $p < 0.01$). However the peak of osmotic fragility of infected erythrocytes did not occur until the degree of parasitaemia was in decline (Figure 3.12).

The Effect of Different Haemoglobin Type on E. ovis Infection - Experiments I, IV, V and VII

For evaluation of the effect of haemoglobin type in E. ovis infection, the results from Experiments I, IV, V and VII were

---

(17) MCF: Defined as the concentration of buffered saline at which 50% of erythrocytes are haemolysed (Dacie and Lewis, 1968).

pooled.

The frequency of each haemoglobin type and the overall mean haemoglobin values for each type in the control sheep are shown in Table X. The frequency of each haemoglobin type in the infected sheep, the mean minimum haemoglobin level recorded, and the mean maximum percentage fall in haemoglobin level are shown in Table XI.

From the data it is apparent that:

(i) There were no significant differences in control haemoglobin levels between types,

(ii) There was no evidence that Hb type affected the decline in Hb levels in E. ovis infection.

During the course of infection, there was some conversion of Hb-A to Hb-C in both Hb-A and Hb-AB type sheep (Figure 3.13).

In Part II of Experiment V, in which sheep were made anaemic by bleeding, a comparison between different haemoglobin type sheep was not possible because all but 2 of the sheep were of Hb-AB type.

The Effect of E. ovis Infection on Plasma and Erythrocyte Potassium Levels - Experiments I and V

Plasma potassium levels were not affected by E. ovis infection or by anaemia induced by phlebotomy (Table XII).

All but 2 of the sheep (both controls in Experiment V, Part I) had erythrocyte potassium levels of type HK: data from these 2 HK sheep have been excluded.

The relationships between the degree of parasitaemia, reticulocyte percentage and erythrocyte potassium levels for both parts of Experiment V are shown in Figure 3.14. A similar relationship, not shown in the figure, was obtained in Experiment I.

TABLE X

FREQUENCY OF EACH HAEMOGLOBIN TYPE AND THE MEAN HAEMOGLOBIN VALUE FOR EACH HAEMOGLOBIN TYPE IN CONTROL SHEEP

Haemoglobin Type	A	AB	B
Number of sheep	8	16	9
Mean Hb value (gms/100 ml)	10.60 ± 0.14^{x}	10.95 ± 0.09	10.76 ± 0.12

Analysis of variance between the means

F = 0.38

Probability = Not Significant ($p > 0.05$)

x = Standard error

TABLE XI

THE EFFECT OF DIFFERENT HAEMOGLOBIN TYPE ON *E. OVIS* INFECTION

Haemoglobin type	A	B	AB
Number of sheep	16	9	20
Pre-infection mean level of haemoglobin (gms/100 ml)	12.05±0.30[x]	11.28±1.12	11.41±1.14
Mean lowest haemoglobin level during infection (gms/100 ml)	5.81±0.21	5.62±0.20	5.91±0.26
Maximum mean[xx] percentage fall in haemoglobin	51.19±2.30	50.15±1.81	47.90±2.28

x = Standard error

xx = Analysis of variance between these means

F = 0.57

P = NS (>0.05)

TABLE XII

OVERALL MEAN PLASMA POTASSIUM LEVELS IN *E. OVIS* INFECTED SHEEP (EXPERIMENTS I AND V - PART I) AND IN ANAEMIC NON INFECTED SHEEP (EXPERIMENT V - PART II)

EXPERIMENT I (n = 80)

	Control	Infected				F	P
Group Number	I	II	III	IV	V		
Plasma K (m.Eq/litre)	4.77± 0.13 x	4.42± 0.11	4.18± 0.13	4.49± 0.16	4.65± 0.18	0.28	NS

EXPERIMENT V - Part I (n = 78)

	Control	Infected	F	P
Plasma K (m.Eq/litre)	5.26± 0.09	5.33± 0.11	0.39	NS

Part II (n = 40)

	Control	Anaemic non infected	F	P
Plasma K (m.Eq/litre)	5.22± 0.27	5.34± 0.27	1.05	NS

x = Standard Error

F = F ratio by analysis of variance

NS = Not significant ($p > 0.05$)

The correlations of erythrocyte potassium level with reticulocyte percentage and degree of parasitaemia are given in Table XIII. The data show that:

(i) Erythrocyte potassium levels were significantly ($p < 0.05$) increased in both *E. ovis* infected and anaemic non-infected sheep.

(ii) The increase in erythrocyte potassium level in *E. ovis* infected sheep was not significantly correlated with the degree of parasitaemia.

(iii) There was a highly significant ($p < 0.01$) correlation between erythrocyte potassium level and reticulocyte percentage in both *E. ovis* infected sheep and anaemic non-infected sheep.

DISCUSSION

If conclusions drawn from experimental infections with *E. ovis* are to be related to events in natural infections, it is clearly necessary to establish whether or not the course of infection is affected by the size of the infecting dose. The results of the experiments described in this chapter show that, whilst some aspects of the infection are affected by dose size, others are not.

The observation that increasing the size of the infecting dose shortens the time lapse before parasitaemia becomes detectable and reaches a peak agrees with the observations of others on *E. coccoides* (Schindler and Krampitz, 1964) and *E. ovis* (Foggie and Nisbet, 1964). The finding that experimental dose size has no effect on the level of parasitaemia finally reached, the duration of parasitaemia, the level of anaemia produced or the time after infection at which anaemia is most severe, also confirms Schindler and Krampitz's report.

The effect of dose on the development of parasitaemia is to be expected if it is assumed that during the development phase the multiplication of the organism is unrestricted. The absence of a dose-effect on later events, however, raises a number of questions as to the cause of the decline in

TABLE XIII

THE CORRELATION BETWEEN ERYTHROCYTE POTASSIUM CONCENTRATION AND THE DEGREE OF PARASITAEMIA (EXPS. I AND V) AND THE CORRELATION BETWEEN ERYTHROCYTE POTASSIUM LEVEL AND RETICULOCYTE PERCENTAGE IN *E. OVIS* INFECTED (EXP. V - Part I) AND IN ANAEMIC NON INFECTED SHEEP (EXP. V - Part II)

Between erythrocyte potassium level and degree of parasitaemia

EXPERIMENT I (n = 15)

	r	p
Group II	-0.31	NS
III	-0.05	NS
IV	0.29	NS
V	0.32	NS

EXPERIMENT V - Part I (n = 13)

r	p
0.54	NS

Between erythrocyte potassium level and reticulocyte percentage

EXPERIMENT V - Part I (n = 13)

r	p
0.98	< 0.01

Part II (n = 10)

0.84	< 0.01

r = correlation coefficient

NS = not significant ($p > 0.05$)

parasitaemia and the precise mechanism by which anaemia is brought about; it is possible that these two events are linked together.

Although the timing of the onset of anaemia appeared to be affected to some extent by the dose-size, the time after infection at which anaemia was greatest was similar in all groups (21 to 25 days, Table IV). It seems likely from this that the development of anaemia is primarly governed by factors, associated with the host animal, which require a relatively constant period of exposure to infection for their full expression. The anaemia must develop either through intravascular haemolysis or the removal of parasitised and damaged cells from circulation. In either case, it is not inconceivable that immune mechanisms of the host are involved and their activation could well be independent of the size of the initial infecting dose. The fact that E. ovis infected sheep develop a positive Coombs test (Sheriff, 1967; Sheriff and Geering, 1969) suggests that there is an immune response against the parasite, indirectly involving the erythrocyte, or against the erythrocyte itself. In either case intravascular lysis or removal of erythrocytes from circulation would be the likely result. Experimental evidence relating to these phenomena will be discussed later (p 59).

The decline in parasitaemia is likely to be governed by the host's response to infection. This decline may result from the selective removal of parasitised cells or from some effect on the multiplication or survival of the organisms or a combination of the two. Since recovered animals are refractory to further infection, at least for a period (Neitz, 1937; Ohder, 1967), there is clearly some kind of immune response to the parasite itself.

The fact that the inoculation of a large infecting dose reduced the prepatent period to a minimum but did not otherwise affect the course of infection led to the adoption of a standard dose of 1.0 ml of heavily infected blood for subsequent experiments. An additional advantage of minimising the prepatent period was that where cross-infection to control animals was a

possibility, the infection would be well developed in the inoculated group by the time parasitaemia commenced in the controls.

In 3 experiments where control animals were maintained in contact with infected ones, cross-infection did in fact occur. Although there were individual exceptions, the degree of parasitaemia and anaemia developed in these naturally infected animals was less than in inoculated sheep. This observation is in agreement with those of Neitz (1937) and Foggie and Nisbet (1964) who also concluded that naturally infected animals were less severely affected than those experimentally infected. At first sight these results appear in conflict with those derived from sheep experimentally infected with graded doses. There are, however, a number of possible explanations for this apparent anomaly. In the first place it is not known with what number of organisms the in-contact animals were infected. If the host response is primarily time-based and the infecting dose considerably lower than the 130,000 organisms estimated for the minimum dose in Experiment I, the parasitaemia could be terminated before it had time to reach the levels attained in experimental infections. There is clear evidence that the degree of anaemia is related to the severity of parasitaemia (Figure 3.4).

A further consideration relates to the route of infection. Although there is no direct proof, it has been postulated that the infection is spread by blood-sucking insects (Foggie and Nisbet, 1964; Sheriff et al., 1966; Ohder, 1967; Øverås, 1969). If it is assumed that in these experiments Simulud flies or mosquitoes were responsible for transmission of infection it is still not certain whether the organisms would be injected directly into blood vessels or into cutaneous or subcutaneous tissues. The observed greater degree of variability in the course of infection in the naturally infected animals could in part be due to variations in the size of inoculating dose and/or the route of administration. Part of this apparent variability is, however, associated with the lack of synchrony of infection in the group.

The similarity in the course of infection between groups was

one of the features of experimental E. ovis infection. From the evidence obtained it was clear that the magnitude of anaemia was not influenced by Hb type (Table XII). The formation of some Hb-C by sheep with Hb-A is a usual response to such an anaemia (Tucker, 1971) (Figure 3.13). This change of Hb type is probably due to the fact that Hb-C has a higher oxygen affinity which may be advantageous to anaemic animals in transporting oxygen to their tissues (Huisman and Kitchens, 1968). It is worth noting though that the similarity in haemoglobin levels between control sheep of different Hb type (Table XI) is at variance with the findings of Dawson and Evans (1965) and van der Helm et al. (1957), who found that Hb-A and Hb-AB sheep had higher haemoglobin values and packed cell volumes than Hb-B sheep. The number of sheep used in this investigation may have been too small to reach any definite conclusion in this regard. Likewise, no conclusion was obtained on the possible influences of different erythrocyte potassium levels on the course of E. ovis infection. Further investigation on this aspect is required. Increases in erythrocyte potassium level during E. ovis infection, shown to be strongly correlated with the reticulocyte percentage (Figure 3.14), are consistent with the findings of Ellory and Tucker (1970) who found that reticulocytes were responsible for the high potassium values found in anaemic blood.

The development of anaemia, whether it is due to an immune response by the host as mentioned previously (p 57), or to direct damage to the erythrocyte by the parasite, is mediated either by intravascular haemolysis or by an increased rate of removal of affected cells from the circulation. Infected erythrocytes were more susceptible to osmotic lysis than control cells, but whether or not this is sufficient to bring about lysis of the cell in circulation is not clear. The factors that control a cell's osmotic fragility are complex and any alterations in this fragility are usually non-specific. Increased fragility does occur in haemolytic anaemias particularly when there is accompanying spherocytosis (Øverås, 1969; Dacie and Lewis, 1968) which may result from an autoimmune process (Schalm, 1972). Dacie and Lewis (1968) considered that the osmotic fragility test was a more objective measurement of spherocytosis than can be obtained

by inspection of stained blood films. The presence of spherocytes in smears of E. ovis infected blood was noted by Øverås (1969). However the sheep erythrocyte, being naturally small (Schalm, 1965), stains evenly with MacNeal stain without central pallor, and in this study, no definite conclusion could be reached as to whether spherocytes were present in infected blood smears.

Evidence has been produced to show that Eperythrozoon spp. cause some erosion of the erythrocytic membrane (Tanaka et al., 1965; Kreier and Ristic, 1963). The fact that there was a time lag between peak parasitaemia and peak osmotic fragility of infected erythrocytes (Figure 3.12), is more consistent with an immune response by the host than with a direct mechanical effect by the parasite. Attempts were made to measure the mechanical fragility of erythrocytes during E. ovis infection by the method described by Dacie and Lewis (1968) (p 177), but the results obtained were inconsistent. The possibilities exist that the process of collecting blood was sufficient to disrupt mechanically fragile cells thereby increasing plasma haemoglobin levels (Figure 3.11) or that infected cells are not mechanically fragile. These points and the possibility that the parasite directly affects osmotic fragility require clarification.

It is pertinent to note that the time interval between peak parasitaemia and peak plasma haemoglobin levels was similar to that between the peaks of parasitaemia and osmotic fragility. Whilst the collection of the blood samples could cause lysis of abnormally fragile cells, there is histological evidence (see Chapter V) that intravascular haemolysis does occur in infected sheep.

The possibility of an increased rate of removal of affected erythrocytes must also be considered, if the increase in circulating monocytes recorded in E. ovis infected sheep is a result of increased reticuloendothelial activity (Neitz, 1937). This aspect is examined in Chapter VI.

It is apparent that, apart from an increased monocyte count,

consistent changes in the total and differential leucocyte counts did not occur in infected sheep, and were, therefore, of no diagnostic significance.

The observations made, along with the data collected, indicate that the general erythrocyte morphology during the response of the bone marrow to the anaemia produced, is macrocytic and hypochromic. It is therefore surprising, in view of the results obtained in this study, that normochromic cells rather than hypochromic cells have been considered part of the typical cellular change in the bone marrow response to the anaemia of E. ovis infection (Kreier and Ristic, 1963, 1968; Sheriff et al., 1966; Rouse and Johnson, 1966; Harbutt, 1969b; Campbell et al., 1971). It must be conceded that the mean amount of haemoglobin in each erythrocyte (MCH) increased in infected blood, but this was more than offset by the increase in size of the cell (MCV), with a resultant fall in the concentration of haemoglobin within the cell (MCHC). Increases in MCH are usually found in anaemias of sudden onset, with large erythrocytes arising directly from normoblasts without passing through the late normoblast stage (Schalm, 1965). The magnitude of the hypochromic response as measured by the fall in MCHC level was dependent on the severity of anaemia, with no evidence of bone marrow suppression having occurred. This finding is in accord with those of Kreier et al. (1964), and Øverås (1969) who described erythroid hyperplasia of the bone marrow in E. ovis infected sheep. The erythroid hyperplasia was considered by Øverås to resemble that described by Winter (1967) in post-haemorrhagic anaemia of sheep. Prolongation of the bone marrow response to anaemia with a delay longer than in bled sheep for haemoglobin levels to return to normal was noted by Sheriff et al. (1966). No objective comparison to confirm this point was made in this study, but in view of the apparently normal response of the bone marrow to the anaemia of E. ovis infection, any delay in the return of haemoglobin levels to normal is most likely due to a continuing excessive rate of erythrocyte removal from circulation.

SUMMARY

Groups of sheep were experimentally infected with Eperythrozoon ovis by intravenous injection. Doses of variable size were used. The following observations were made:

1. The prepatent period, and the time for peak parasitaemia to be reached following inoculation were inversely proportional to the size of the inoculating dose.
2. The time taken from inoculation until the peak of anaemia was similar, irrespective of inoculating dose size.
3. The time relationship between degree of parasitaemia and fall in haemoglobin concentration varied according to the size of the inoculating dose, with, at the low dose rate (1×10^{-6} ml of heavily infected blood), peak parasitaemia and peak anaemia being synchronised.
4. The maximum degree of parasitaemia and the maximum percentage fall in haemoglobin concentration was similar in all groups.

It is concluded from these observations that the onset and degree of anaemia, being unrelated to inoculating dose size, is dependent upon an immune response by the host. Further circumstantial evidence to support this is provided by the fact that the peak of increase in erythrocyte osmotic fragilities and peak plasma haemoglobin levels during infection did not coincide with the peak of parasitaemia.

Observations were also made on naturally infected sheep, which in contrast to experimentally infected sheep showed variable but generally less severe degrees of anaemia and parasitaemia. Although this appears to conflict with the conclusions made from experimental infection, it is possible that, if the host response is time based, it may have reached full expression before infection had time to develop. Factors contributing to this may be:

(1) The route of administration, which, if mediated by blood insects, could mean that organisms are injected into cutaneous or subcutaneous tissues, and
(2) The size of infecting dose which could be lower than the

smallest dose given experimentally (130,000 organisms).

The maximum percentage fall in haemoglobin level, and the maximum degree of parasitaemia recorded in individual sheep were significantly ($p < 0.01$) correlated. This was once again consistent with a host immune response against the parasite which also involves the erythrocyte. The possibility of a direct mechanical effect by the parasite, facilitating the erythrocyte's removal from circulation cannot be discounted.

The morphology of erythrocytes during the response of the bone marrow to the anaemia is macrocytic and hypochromic, which is at variance with most reports in the literature. There appeared to be no evidence of bone marrow suppression. The anaemia of E. ovis infection was not altered by different Hb types in sheep. Infection did not appear to affect erythrocyte potassium levels; increases recorded were probably due to reticulocytosis.

Total and differential leucocyte counts were performed during an infection cycle. A significant ($p < 0.05$) increase in monocytes was recorded in infected sheep at the peak of parasitaemia, but all levels were still within the accepted normal range. Changes in the total count and in the differential count for the other cell types were not consistent with E. ovis infection and were therefore of no diagnostic significance.

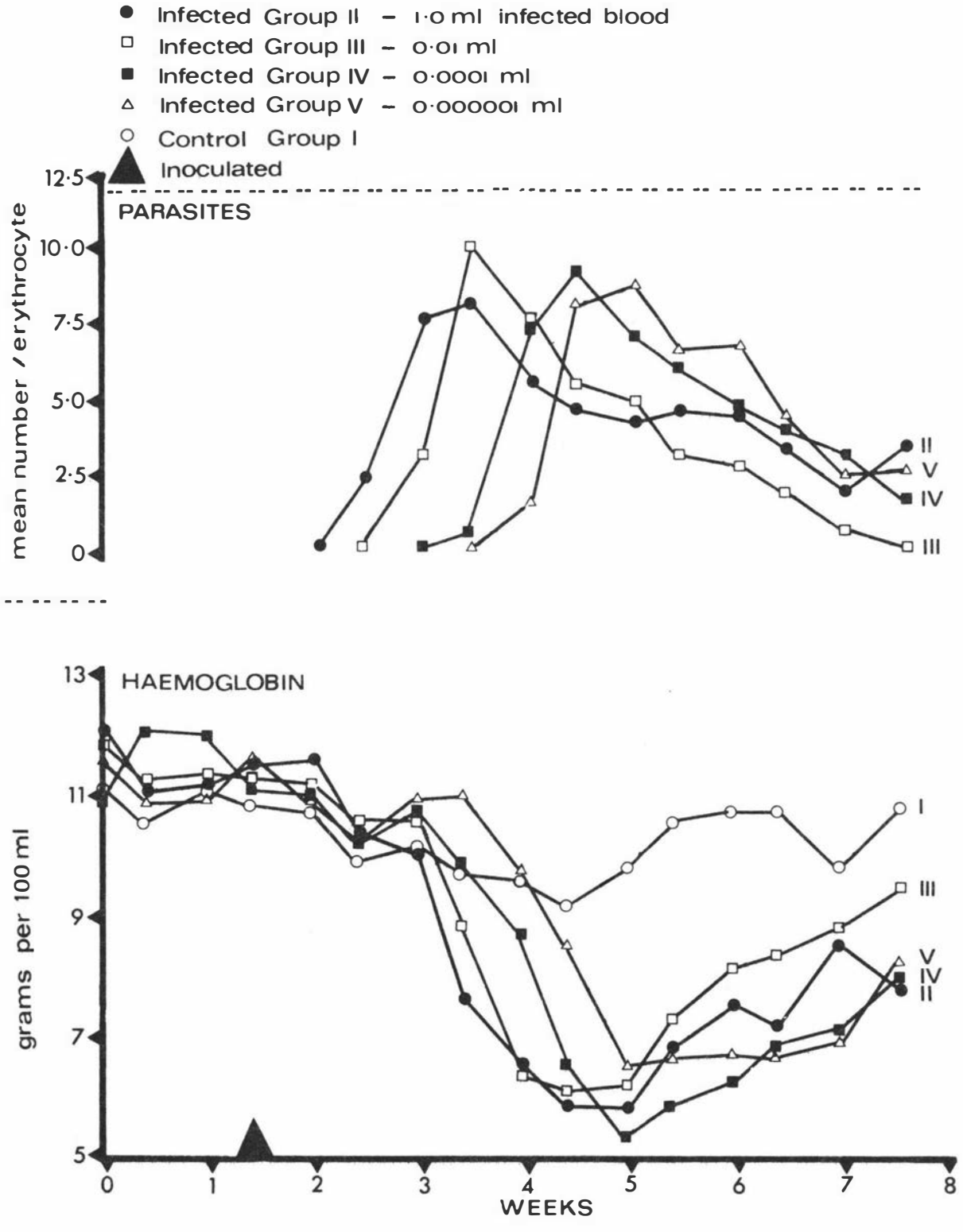


Figure 3.1. The time relationships between the size of infecting dose and consequent degrees of parasitaemia and anaemia. (Exp. I. — Group mean values)

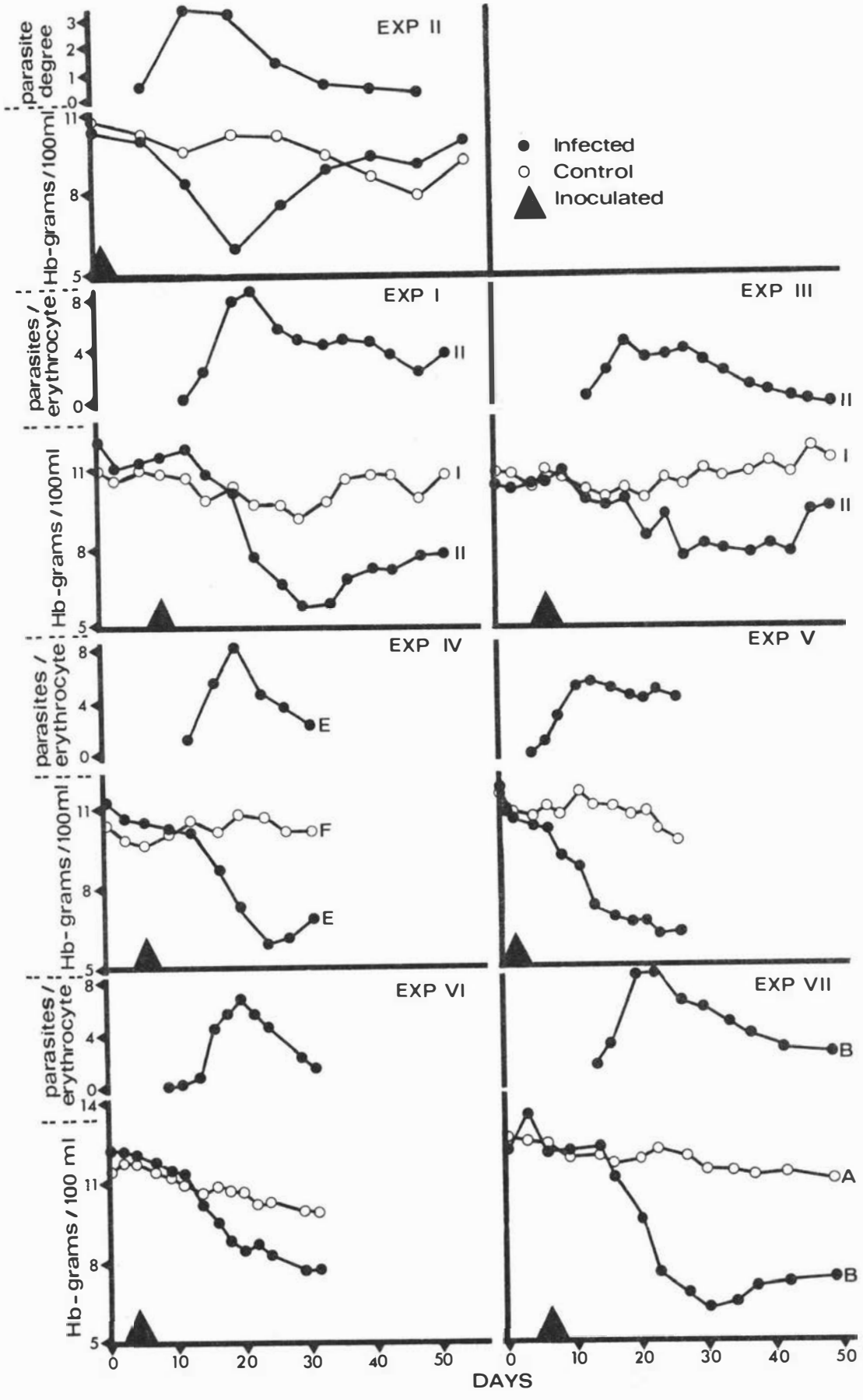


Figure 3.2. The time relationship between the degree of parasitaemia and haemoglobin concentration. (All experiments. — Group mean values)

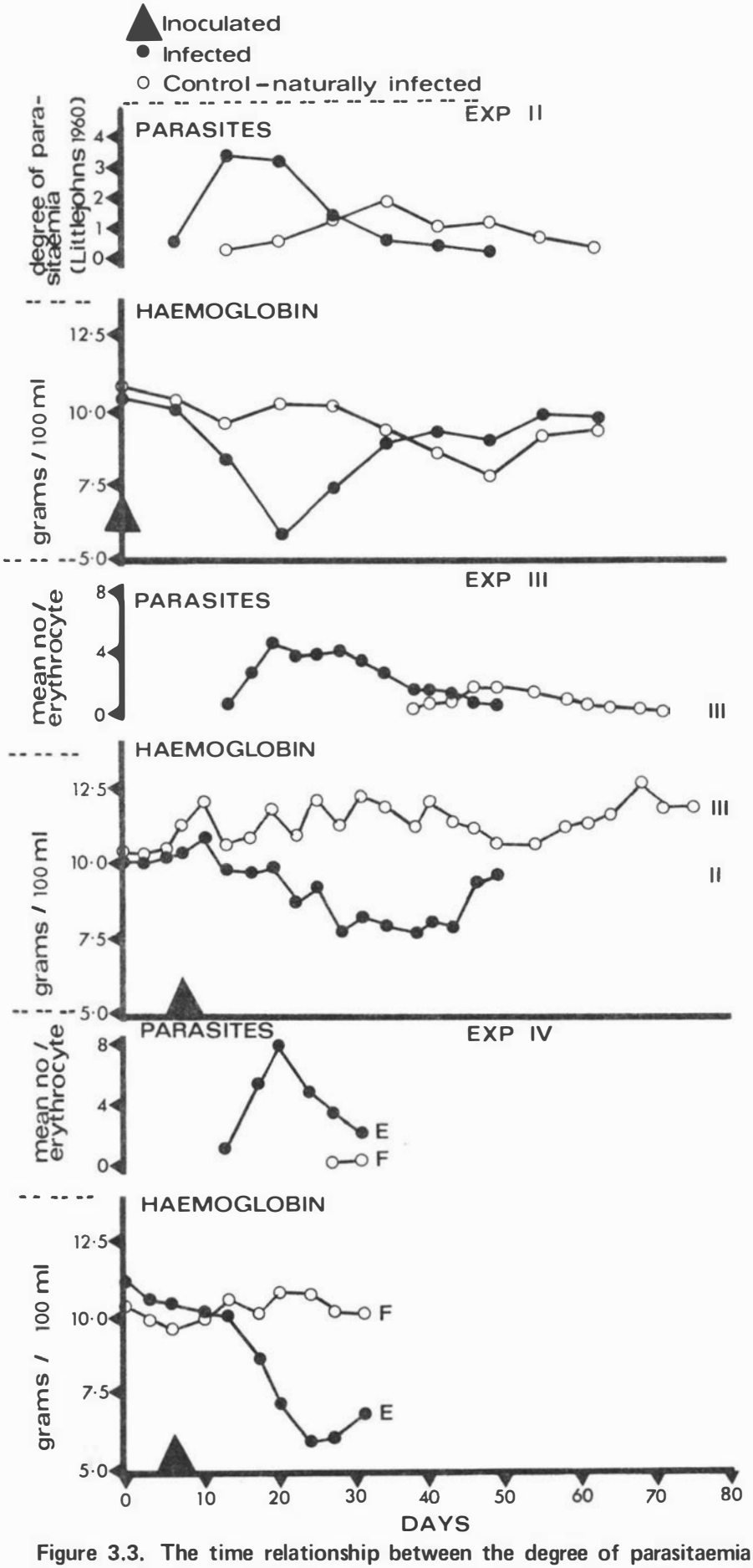


Figure 3.3. The time relationship between the degree of parasitaemia and haemoglobin concentration in experimentally and naturally infected sheep (Exps. II, III and IV – Group mean values)

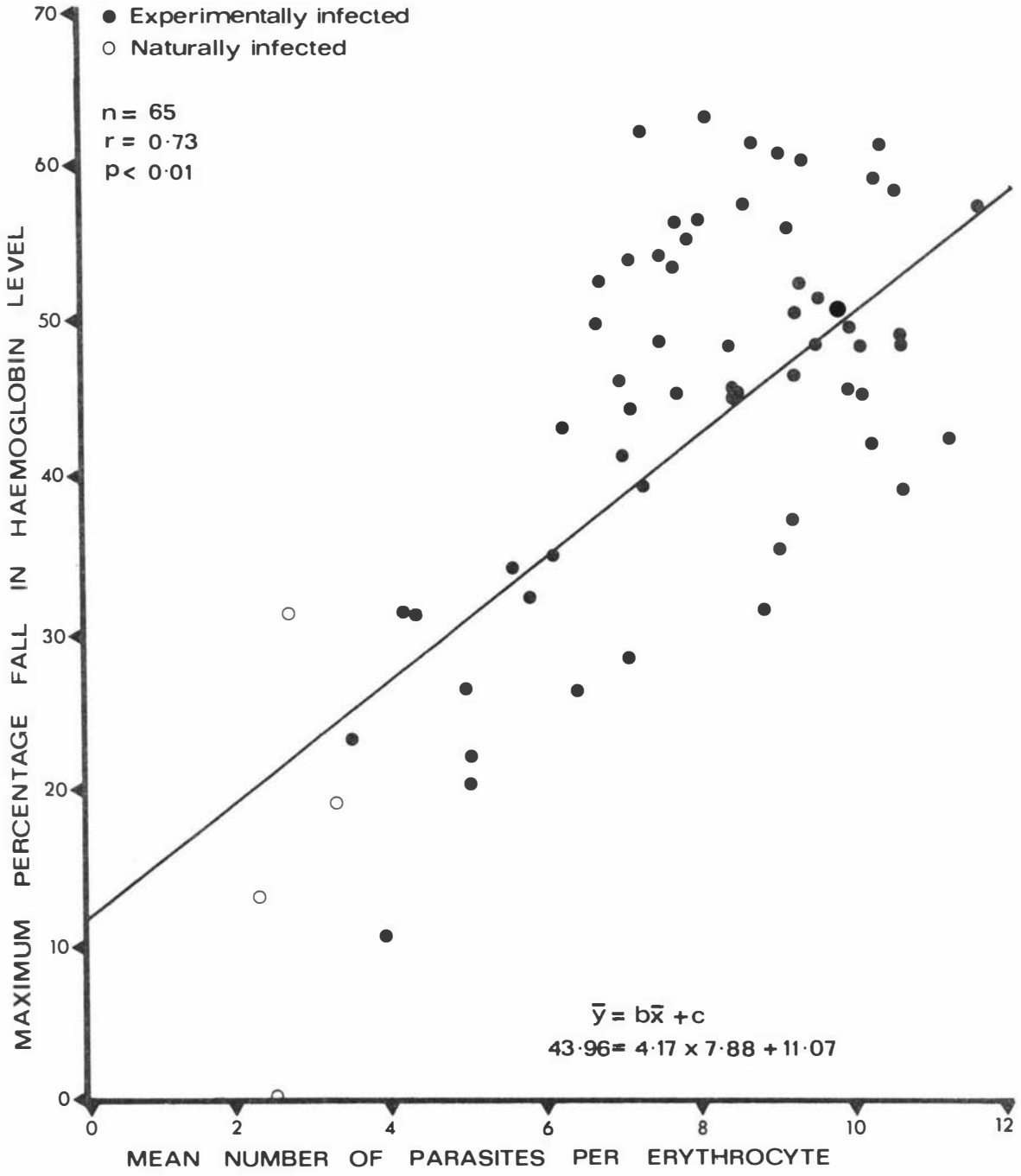


Figure 3.4. The correlation between the maximum degree of parasitaemia and maximum percentage fall in haemoglobin concentration in *E. ovis* infected sheep. (Individual sheep values from all experiments except Exp. II)

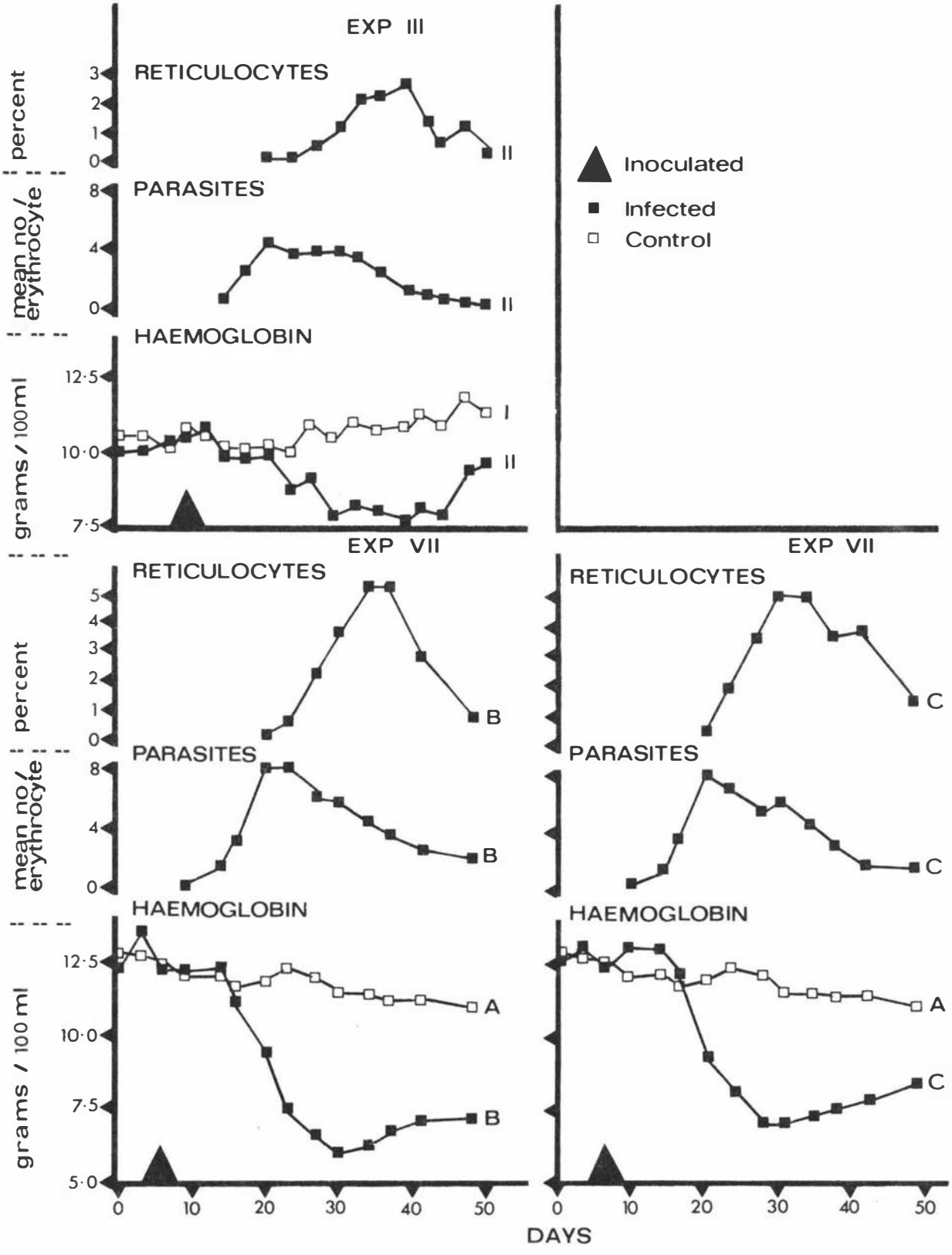


Figure 3.5. The time relationships between the degree of parasitaemia, haemoglobin concentration and reticulocyte percentage in experimentally infected sheep. (Exps. III and VII — Group mean values)

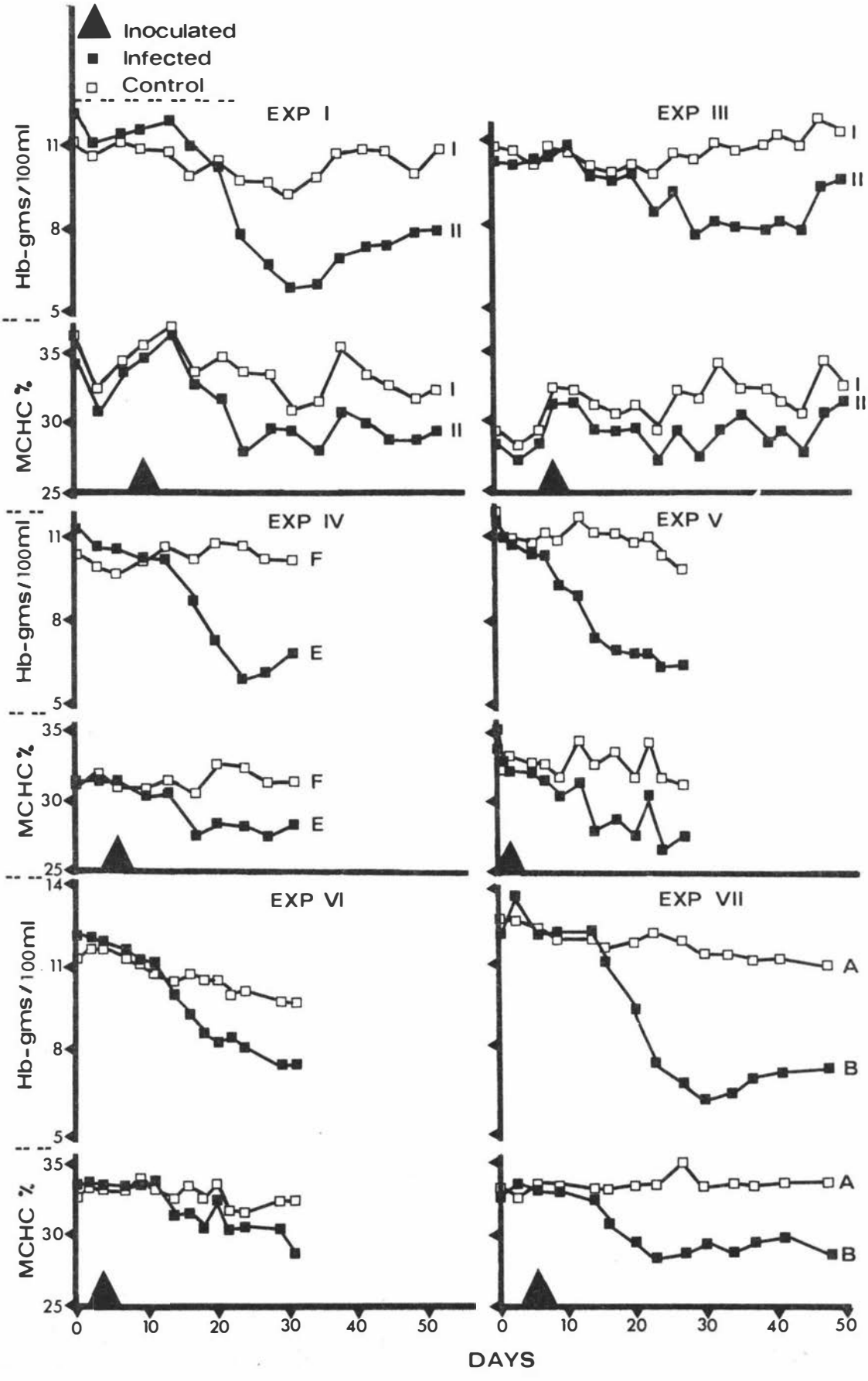


Figure 3.6. The time relationship between haemoglobin concentration and the mean corpuscular haemoglobin concentration (MCHC) in experimentally infected sheep. (Group mean values from all experiments except Exp. II)

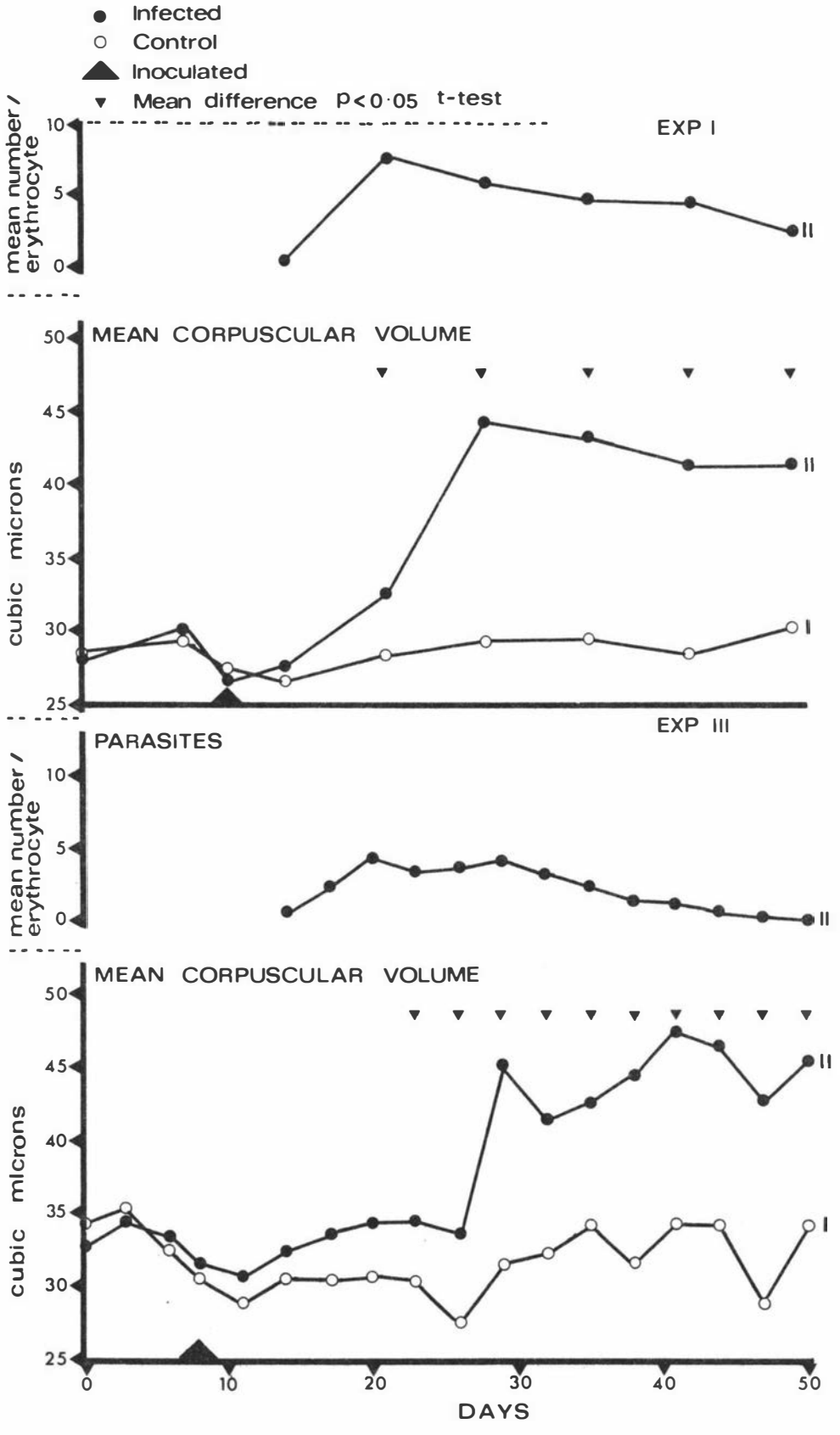


Figure 3.7. The time relationship between the degree of parasitaemia and the mean corpuscular volume (MCV) in experimentally infected sheep. (Exps. I and III — Group mean values)

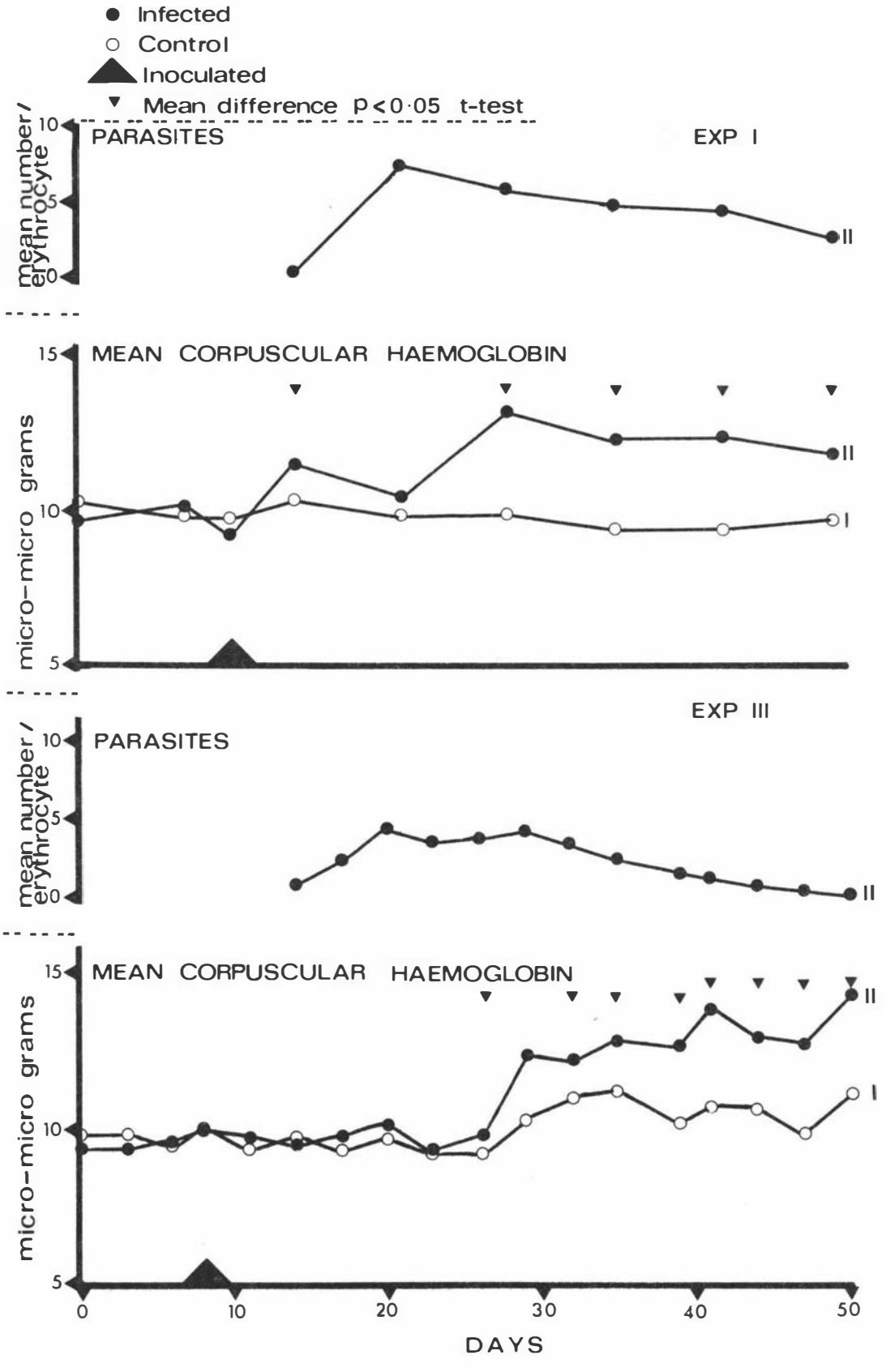


Figure 3.8. The time relationship between the degree of parasitaemia and the mean corpuscular haemoglobin (MCH) in experimentally infected sheep. (Exps. I and III – Group mean values)

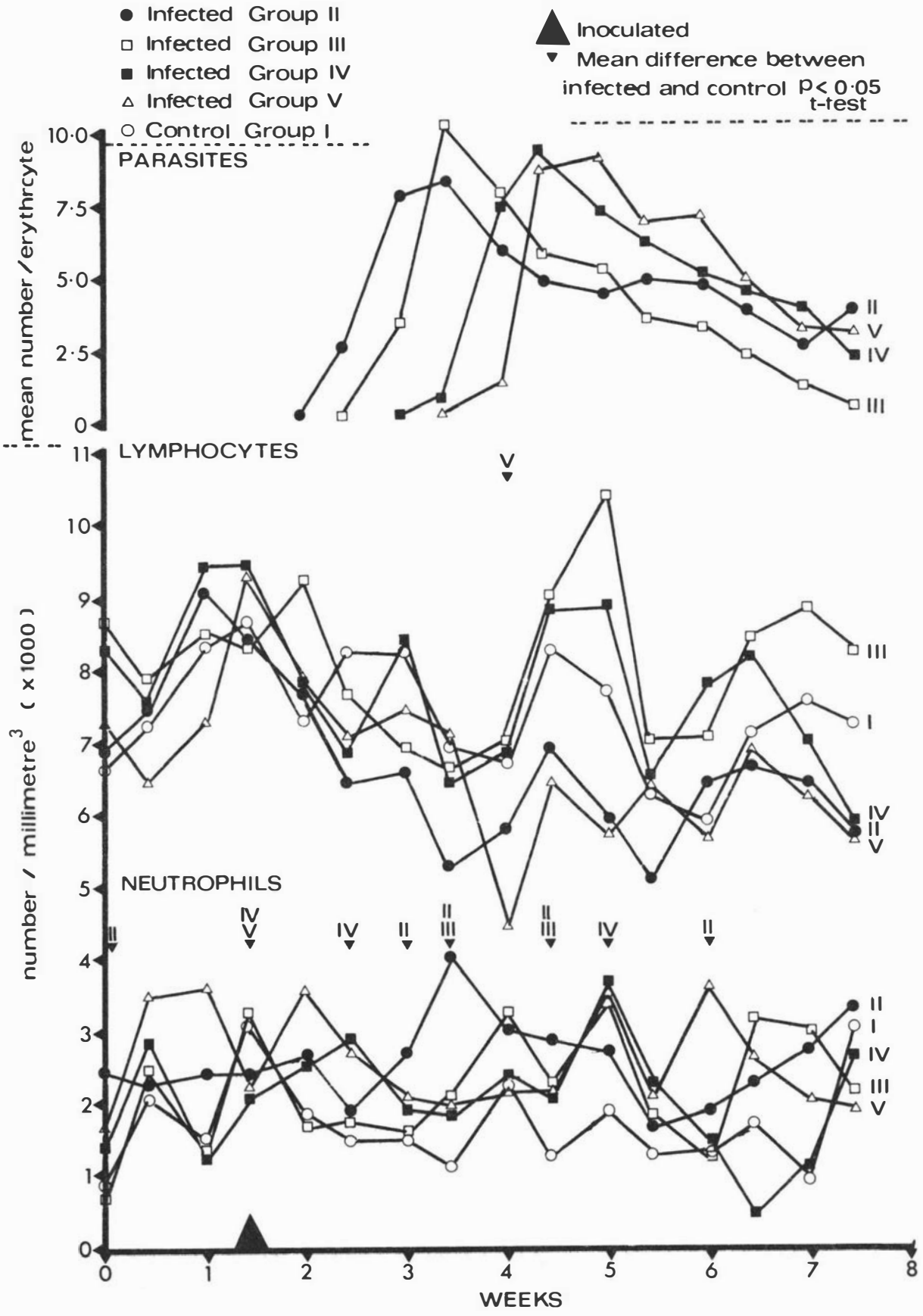


Figure 3.9. The relationship between the degree of parasitaemia and absolute lymphocyte and neutrophil counts (Exp. I — Group mean values)

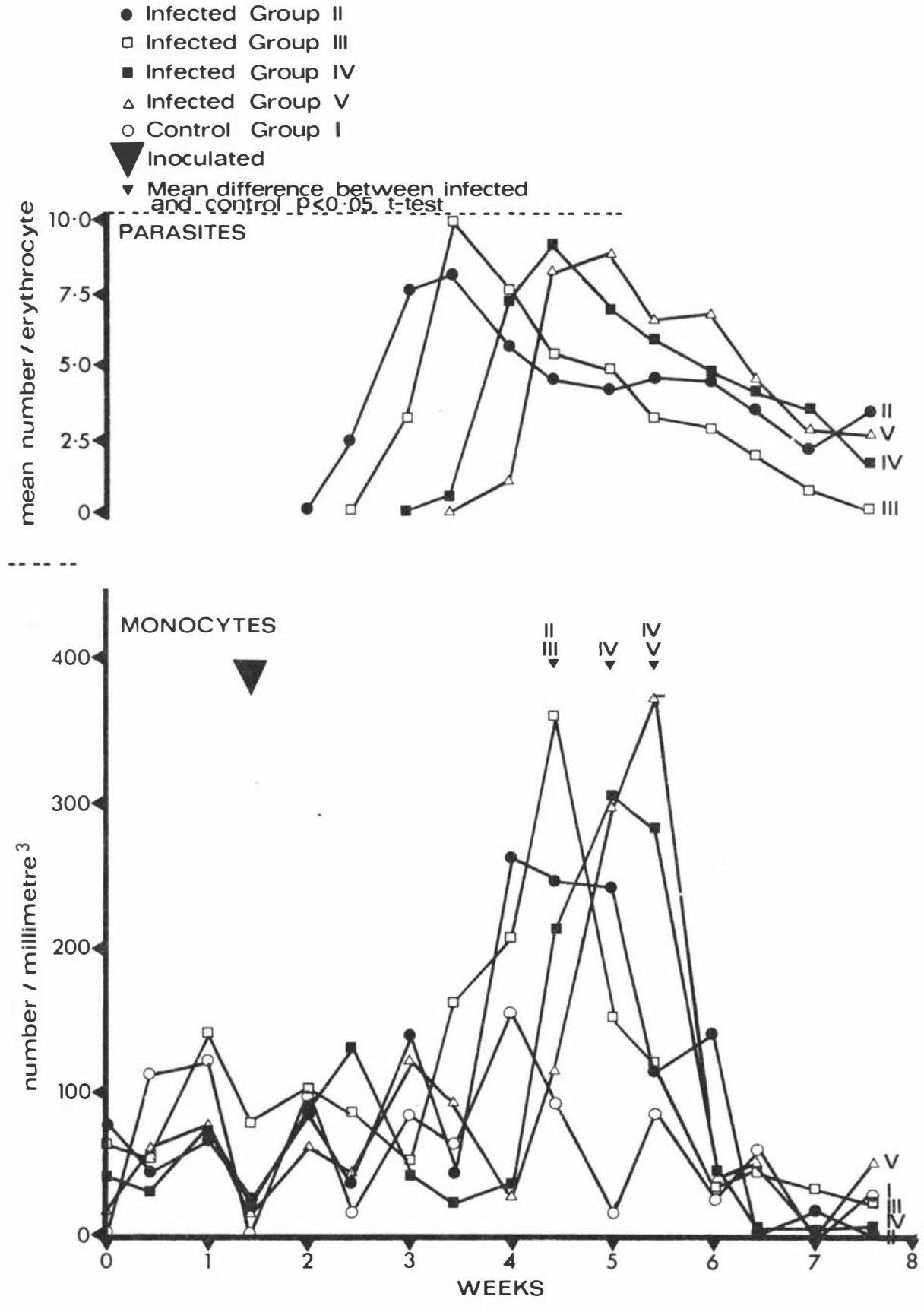


Figure 3.10. The relationship between the degree of parasitaemia and absolute monocyte counts — Exp. I (Group mean values)

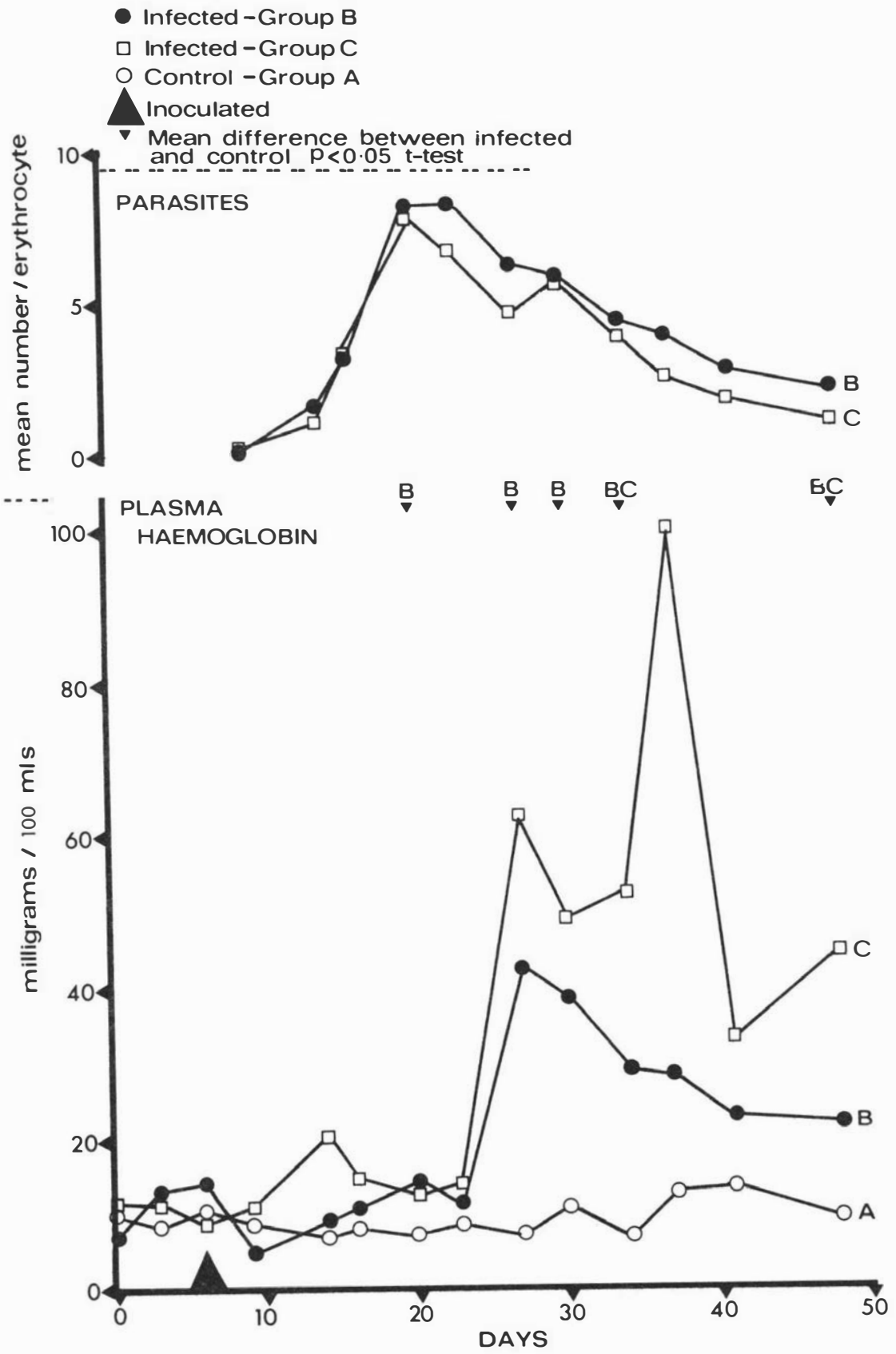


Figure 3.11. The time relationship between the degree of parasitaemia and plasma haemoglobin concentration. (Exp. VII — Group mean values)

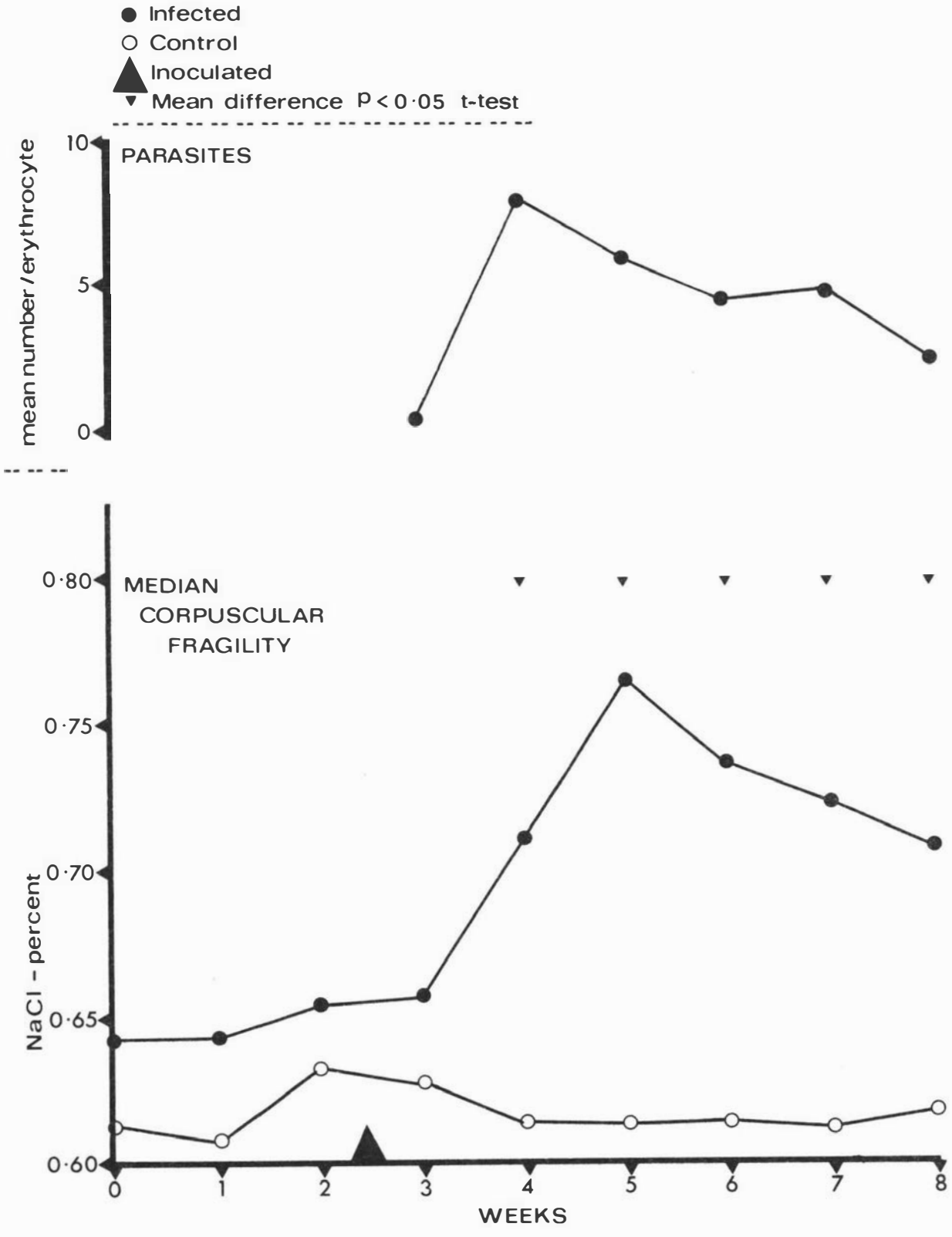


Figure 3.12. The time relationship between the degree of parasitaemia and the median corpuscular fragility (MCF) (Exp. I — Mean values of control group I and infected group II)

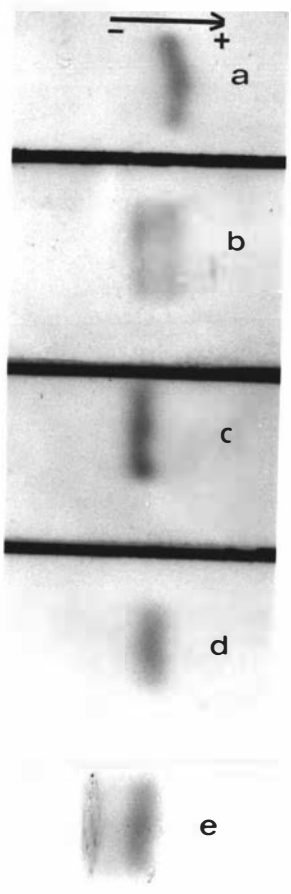


Figure 3.13: Haemoglobin types A, AB, and B as shown by electrophoresis on cellulose acetate (Phoro Slide) pH 8.6. Partial conversion of Hb-A to Hb-C with developing anaemia in infected sheep 37 (Experiment I) is also shown.

(a) Hb type A
(b) Hb type AB
(c) Hb type B
(d) Experimental sheep No. 37
Hb type A. 11.9 gm/100 ml
(e) Sheep 37
Hb type AC. 7.6 gm/100 ml

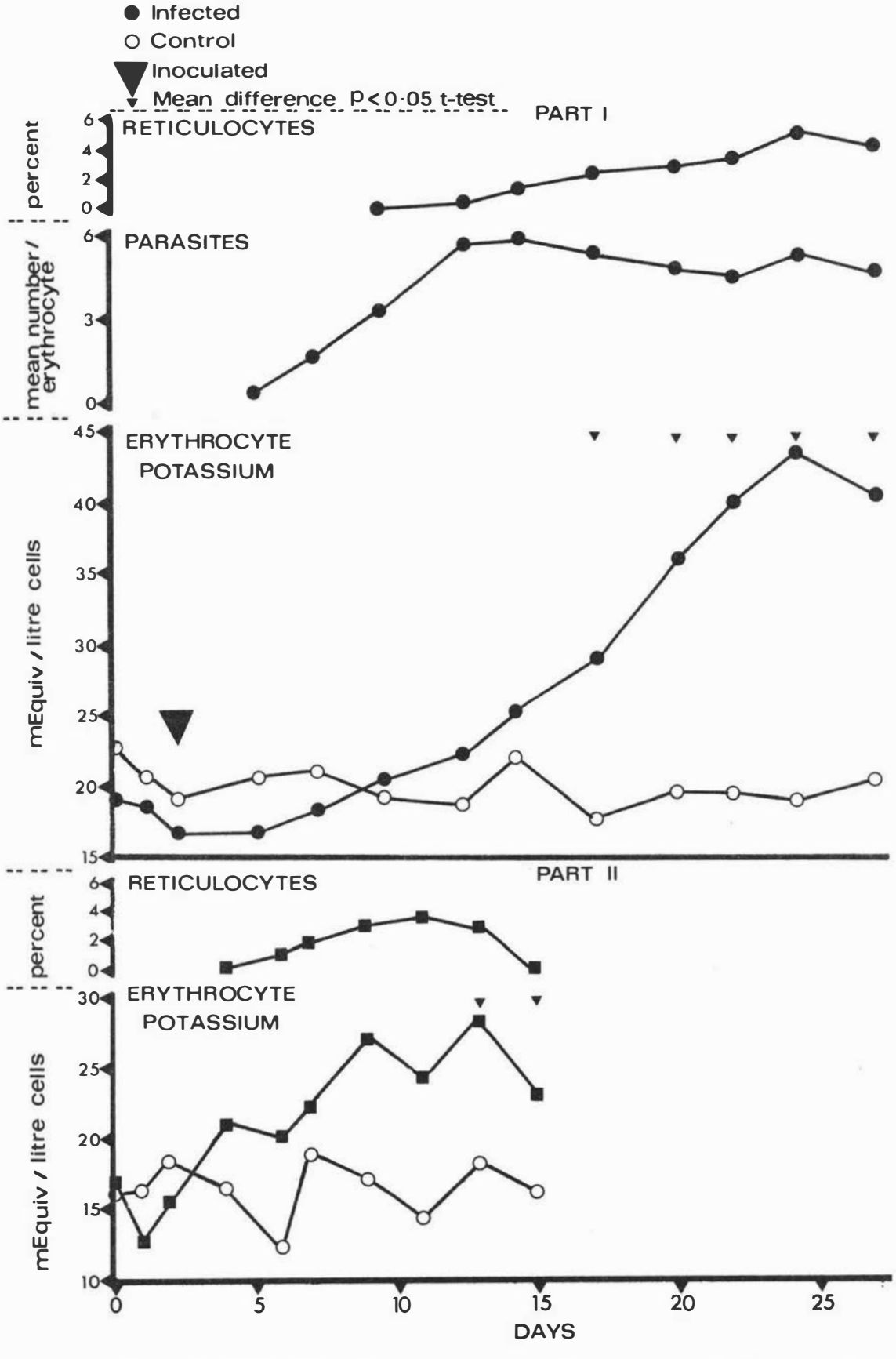


Figure 3.14. The relationship between erythrocyte potassium concentration and reticulocyte percentage in infected (Part I) and non infected (Part II) blood. (Exp. V – Group mean values)

CHAPTER IV

THE EFFECT OF EPERYTHROZOON OVIS INFECTION ON SOME BLOOD BIOCHEMICAL PARAMETERS

INTRODUCTION

There are few reports in the literature on the effect of Eperythrozoon ovis infection on the routinely measured biochemical parameters in blood. Increases in blood urea nitrogen and serum bilirubin levels have been noted (Neitz, 1937; Littlejohns, 1960) but no attempt has been made to relate this to the stage of the infection cycle. Investigations on total and fractional serum protein components have yielded equivocal results. While Neitz (1937), and Sheriff et al. (1966) recorded falls in total protein levels, Øverås (1969) found no change in total protein, and no significant changes in the different serum protein fractions.

During the various experiments, opportunity was taken to measure both blood urea and blood urea nitrogen, serum bilirubin (free and conjugated) and total plasma proteins. In Experiment IV the proteins were fractionated by electrophoresis. Although reports in the literature on E. ovis infection do not suggest any likely tissue or organ damage which might cause an increase in serum glutamic oxalacetic transaminase (SGOT) levels, these were measured in Experiment III, mainly to monitor the extent of liver and tissue damage, which may have resulted from the biopsy operation. The SGOT levels of the biopsied control and infected groups, and the control group that was not biopsied, were also estimated.

MATERIALS AND METHODS

The various experiments in which the biochemical examinations were performed, as well as the experimental methods are outlined in Chapter II. In Experiment VII, one of the infected groups (Group C) was housed without food and water 24 hours prior to bleeding for reasons given previously (Chapter III).

RESULTS

The Effect of E. ovis Infection on Blood Urea Nitrogen (BUN) (Experiment III) and Blood Urea (Experiment VII)

The blood urea and blood urea nitrogen results of infected sheep were all within normal range throughout the experiments, and did not differ significantly from the control values. These latter are given in Table XIV, and are comparable with values recorded in the literature (see Doxey, 1971).

The Effect of E. ovis Infection on Bilirubin (Experiment VII)

The mean values for total free and conjugated bilirubin in the control sheep are given in Table XIV. Increases in total bilirubin levels were recorded in both the infected groups during parasitaemia. This was due to an increase in the free bilirubin fraction; conjugated bilirubin levels were not affected. The time relationship between parasitaemia and the mean levels of free bilirubin in all groups at each reading are shown in Figure 4.1. The increases which were recorded in the infected groups were variable in magnitude, and only occasionally significantly ($p < 0.05$) different from controls.

The Effect of E. ovis Infection on Serum Glutamic Oxalacetic Transaminase (SGOT) - Experiment III

SGOT levels in all sheep remained consistently within the normal range as cited in the literature (Done *et al.*, 1958; Buck *et al.*, 1961) but there were small differences between groups. The mean levels of SGOT obtained from both the control groups of sheep are given in Table XIV. The mean in the biopsied control sheep was consistently higher throughout the experiment than in the group of intact control sheep, but in only two of the seven estimates was the difference between the two groups significant ($p < 0.05$). The level in the biopsied infected group (65.39 ± 5.43 SFU/ml) was intermediate between the two control groups and was not apparently influenced by *E. ovis* infection.

TABLE XIV

MEAN LEVELS FOR SOME BIOCHEMICAL PARAMETERS IN CONTROL SHEEP

Biochemical Parameter	Experiment No.	No. of Sheep	No. of Readings for each Sheep	Mean ± S.E.
Blood Urea Nitrogen (mgs/100 ml)	III	5 (biopsied)	5	21.47±1.32
		4 (intact)	5	22.49±0.81
Blood Urea (mgs/100 ml)	VII	7	6	49.70±4.18[x]
Bilirubin (mgs/100 ml)	VII			
Total		7	10	0.057±0.005
Free		7	10	0.030±0.006
Conjugated		7	10	0.026±0.003
SGOT (Sigma Frankel Units/ml)	III	5 (liver biopsied)	7	81.75±6.97
		4 (intact)	7	57.09±4.10
Total Plasma Protein (gms/100 ml)				
	I	5	16	7.31±0.21
	III	5 (biopsied)	7	7.10±0.16
		4 (intact)	7	6.98±0.06
	IV	5	10	7.13±0.05
	VII	7	14	6.70±0.06 xx

x = BUN. equivalent is 23.22 mgs/100 ml

xx = Estimated by refractometer. Other total protein levels estimated by the Biuret Method (see Chapter II)

The Effect of E. ovis Infection on Total and Fractionated Protein Levels

Total plasma protein levels were measured in Experiments I, III, IV and VII, and values obtained in the control group of sheep are given in Table XIV.

No alteration in the level of plasma protein of the infected groups of Experiments I and III was noted during the course of the experiments.

In Experiment IV, protein estimations were done on infected group E and control group F. The relationship between the mean degree of parasitaemia and the total plasma protein level of both groups (F and E) are shown in Figure 4.2. Significant ($p < 0.05$) increases in the level in infected animals over the controls were recorded as parasitaemia was declining.

The relationship between the mean degree of parasitaemia and total protein levels for the control (A) and infected groups (B and C) in Experiment VII are shown in Figure 4.3. As can be seen in the figure, the level of plasma protein in the 2 infected groups was higher than the control level both prior to, and after the onset of parasitaemia. Significant ($p < 0.05$) differences were recorded between the infected (particularly group C) and control groups as parasitaemia declined.

The relationship of the degree of parasitaemia with total albumin, globulin and the albumin-globulin ratio (A/G) in Experiment IV is shown in Figure 4.4. The increase in the total protein level of the infected group as parasitaemia declined (Figure 4.2) was mainly due to an increase in the globulin fraction. The increase in globulin occurred in only some of the infected sheep. The overall control mean for the A/G ratio was 0.86 ± 0.05. The A/G ratio was consistently higher in the control group during parasitaemia, but except for one reading the difference between the 2 groups was not significant.

Typical patterns of separated plasma proteins from control animals and infected animals at peak parasitaemia and as it declined are shown in Figure 4.5. No consistent changes in the pattern were noted during the infection cycle.

DISCUSSION

The Effect of E. ovis Infection on Blood Urea Concentrations (Blood Urea and Blood Urea Nitrogen)

Blood urea concentrations provide one evaluation of renal function. It has been shown in the dog that urea accumulation only occurs when 75% of functional kidney has been destroyed (see Kronfeld and Medway, 1969). Relatively little work has been done in the sheep but a similar situation is probable. Blood urea elevation may also occur in shock, dehydration and following a high protein meal (Wilkinson, 1969).

The values obtained in the control sheep of Experiments III and VII are similar to those recorded in the literature (see Coles, 1967; Doxey, 1971). In contrast to the findings of Neitz (1937) the E. ovis infections described here were not accompanied by any increase in the blood urea levels. Considerable variations in the degree of renal pathology in E. ovis infection are reported and this may account for the differences in blood urea levels observed (see Chapter V).

The Effect of E. ovis Infection on Serum Glutamic Oxalacetic Transaminase (SGOT) Levels

Because of its wide tissue distribution, alterations in the activity of SGOT can be associated with necrosis or damage to many tissues. The highest enzyme activity is found in the liver, cardiac and skeletal muscle, and the intestines (Doxey, 1971). Activity of one isozyme has been noted in mature erythrocytes, and increased activity, as well as an additional isozyme, has been found in reticulocytes (Nisselbaum and Bodansky, 1965). The group SGOT levels showed the same relationship to each other before and after biopsy. It appeared,

therefore, that the surgical interference was without significant effect on these levels. Furthermore, there was no evidence that E. ovis infection affected SGOT levels. Release of the enzyme from erythrocytes may have been expected to cause some alteration in blood levels during infection but these were not detected.

The Effect of E. ovis Infection on Bilirubin Levels

The total and conjugated bilirubin values obtained from control sheep were similar to those reported in the literature (see Cornelius, 1970). In the infected group some significant ($p < 0.05$) increases were recorded in the level of free bilirubin, but these were variable and not large. The highest level recorded was still within normal limits.

Increase in free bilirubin is indicative of a haemolytic anaemia as a result of increased erythrophagocytosis and/or intravascular haemolysis (Cornelius, 1970). Jaundice associated with E. ovis infection has been reported by both Neitz (1937) and Øverås (1969), though only in the more heavily infected sheep. None of the infected sheep in this investigation showed jaundice and the highest level of free bilirubin recorded was lower than that usually associated with icteric changes (Doxey, 1971).

The Effect of E. ovis Infection on Plasma Proteins

Previous studies of plasma or serum proteins during Eperythrozoon infection have yielded varying results. The fall in total proteins recorded by Neitz (1937) and Sheriff et al. (1966) may have been the result of a hypoglobulinaemia which has been found in natural Eperythrozoon infection of cattle (Dimopollous et al., 1959). However, in the only substantial study of blood proteins during E. ovis infection Øverås (1969) found no change in the total protein level, and the only alteration in the electrophoretic pattern was a loss of the distinct boundary between the β and γ globulins. Øverås (1969) concluded that E. ovis infection did not induce any rise in γ globulin.

The findings from all four experiments in this study, together with those of others (Neitz, 1937; Sheriff et al., 1966; Øverås, 1969), illustrate the absence of a consistent effect of E. ovis on plasma proteins. Dimopoullos (1970), in a review on plasma proteins, emphasized a number of factors which influence the plasma protein profile. These include the methods of total and fractional analysis, the nutritional and hormonal status of the animal, and its age and sex. None of these factors, except perhaps the nutritional status, would be expected to alter the protein patterns within an experiment; but they would most certainly be a consideration when comparing the results of two separate investigations.

The most common alteration found in plasma protein profiles in bacterial, mycotic and helminth infection is a lowering of the A/G ratio. This is mainly a result either of increased antibody production leading to increased globulin, especially γ globulin, levels and/or a decrease in the albumin fraction. In viral diseases on the other hand, there is little or no change in the plasma protein picture even despite extremely high antibody titres (Dimopoullos, 1970).

Although antibody has been demonstrated in E. ovis infection (Kreier and Ristic, 1963) and an increase in immunoglobulin G (IG_G) has been found in E. coccoides infection (Baker et al., 1971), the findings in this study suggest a picture similar to that of viral infections, where the increase in globulin is small and in some cases inapparent. In Experiment IV (outdoors) concurrent increases in both albumin and globulin contributed to the significant ($p < 0.05$) total protein increase in the infected group over the controls. Increases in both albumin and globulin most commonly occur with dehydration leading to haemoconcentration (Dimopoullos, 1970; Schalm, 1970). Whilst there was no other evidence to suggest dehydration of these animals, it is possible that this accounted for the protein rise. It is, further, difficult to see why this should have occurred unless the infection had some influence on the animals' inclination to seek water.

In the other outdoor experiment, in which plasma proteins were measured (Experiment VII) a general tendency for a decrease of the protein level occurred in all groups, particularly in the latter stages of the experiment. This was probably due to poor nutrition as the live weight of these sheep followed a similar pattern (Chapter V). The possibility of a dehydration effect causing the small relative increases in total protein of infected group (C) as compared with the control group (Figure 4.3), must be considered as the infected group was housed without food and water for 24 hours. Whatever the cause of the increased total proteins in the two outdoor experiments, its effect was not apparent in the two experiments which were run indoors (Experiments I and III).

It would appear that estimation of total and fractionated plasma proteins during E. ovis infection has little or no diagnostic value. Determination of the distribution of antibodies, and their effect on the plasma protein profile, requires more sensitive and critical fractionation methods than used in this study.

SUMMARY

Estimation of blood urea, serum bilirubin, serum glutamic oxalacetic transaminase (SGOT), total and fractional plasma protein levels were performed during E. ovis infection.

Results showed that E. ovis infection had no effect on blood urea or SGOT levels but small rises in free bilirubin, probably as a result of excessive erythrocyte removal or destruction, were recorded. However the magnitude of the rise was not sufficient to cause clinical icterus.

Changes in total and fractional protein levels were inconsistent, and it is concluded that in E. ovis infection such estimations are of little or no diagnostic value.

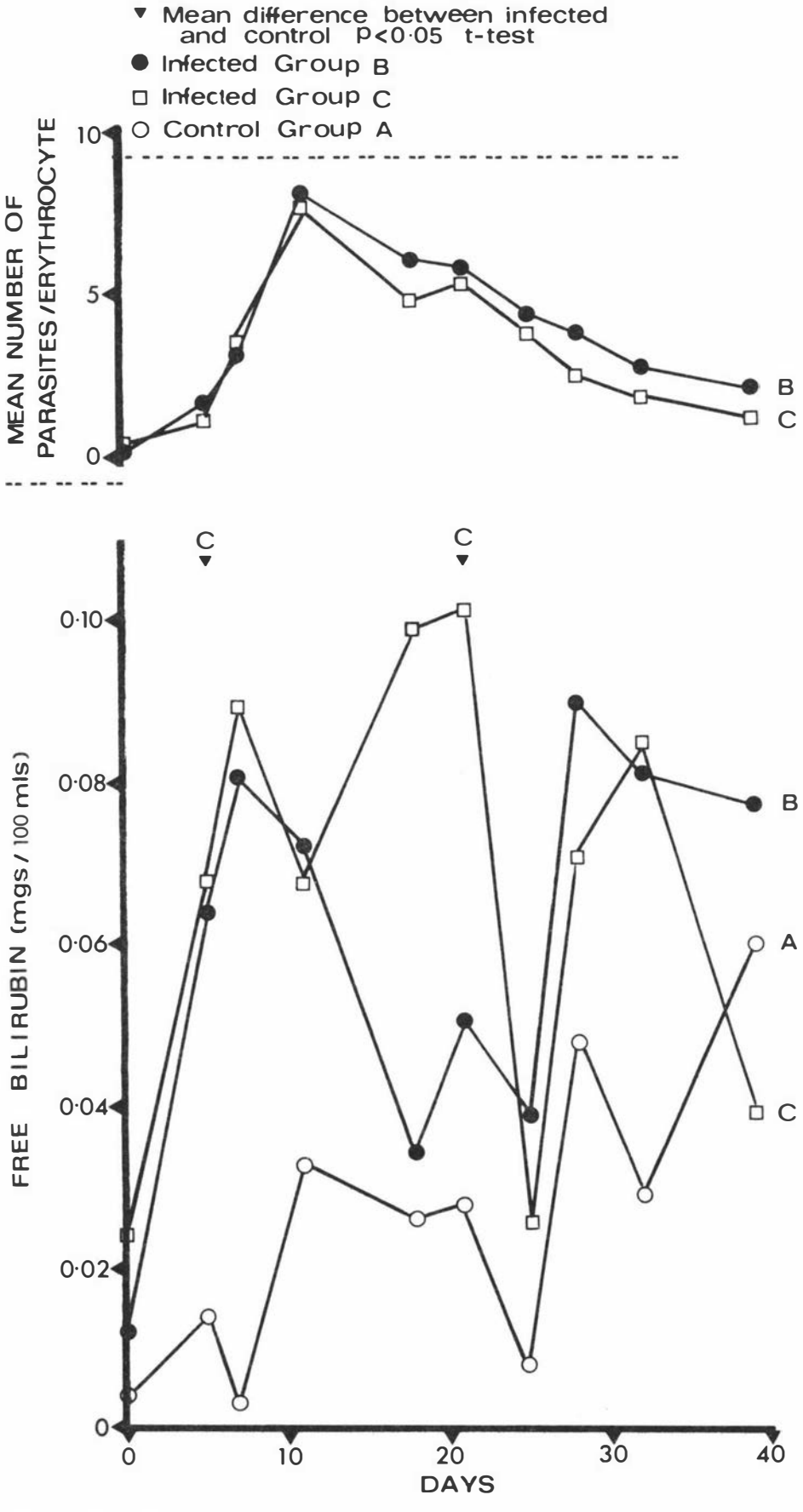


Figure 4.1. The time relationship between the degree of parasitaemia and the concentration of plasma free bilirubin (Exp. VII – Group mean values)

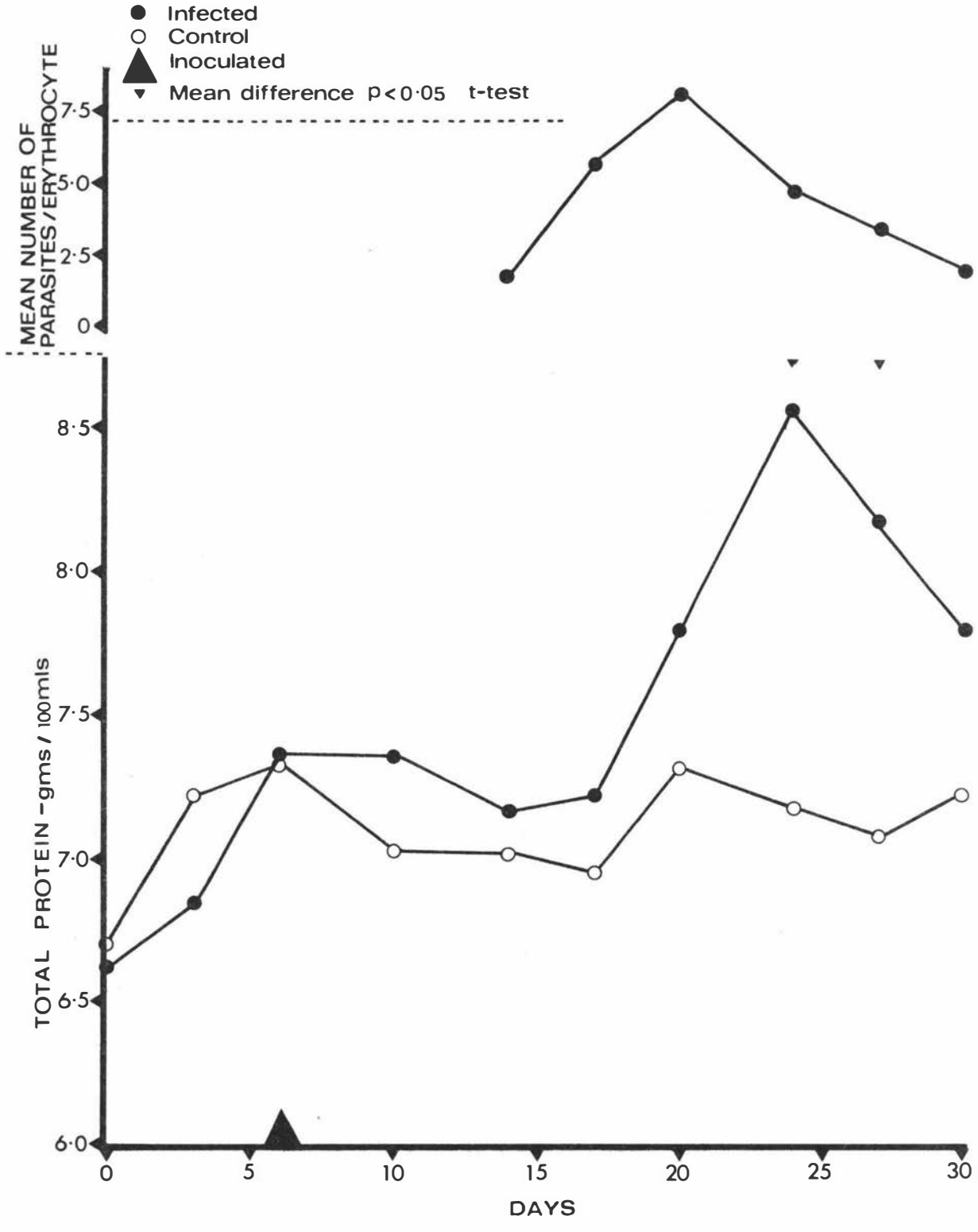


Figure 4.2. The time relationship between the degree of parasitaemia and total plasma protein level (Exp. IV – Mean values of control group F and infected group E)

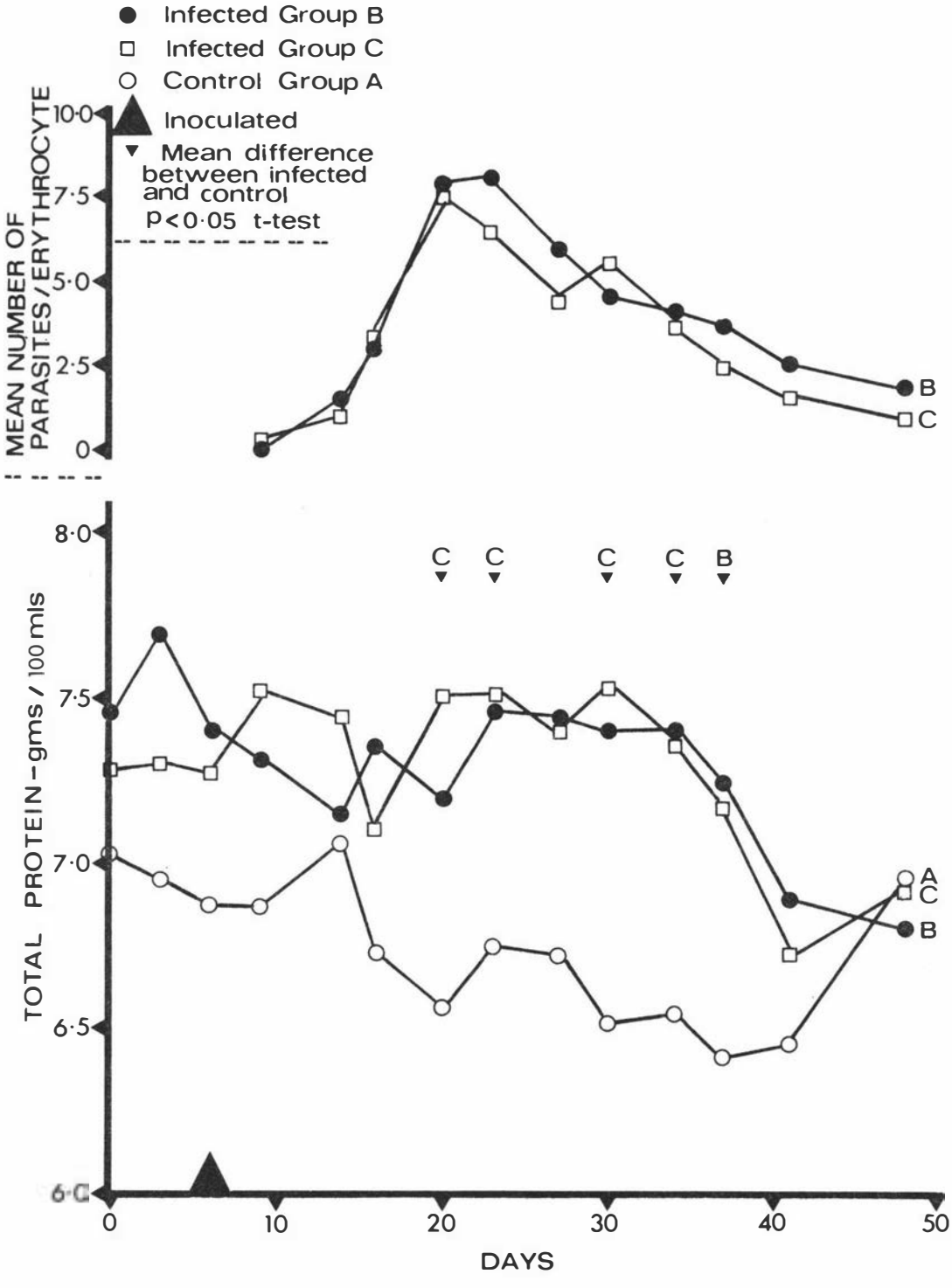


Figure 4.3. The time relationship between the degree of parasitaemia and total plasma protein level (Exp. VII — Group mean values)

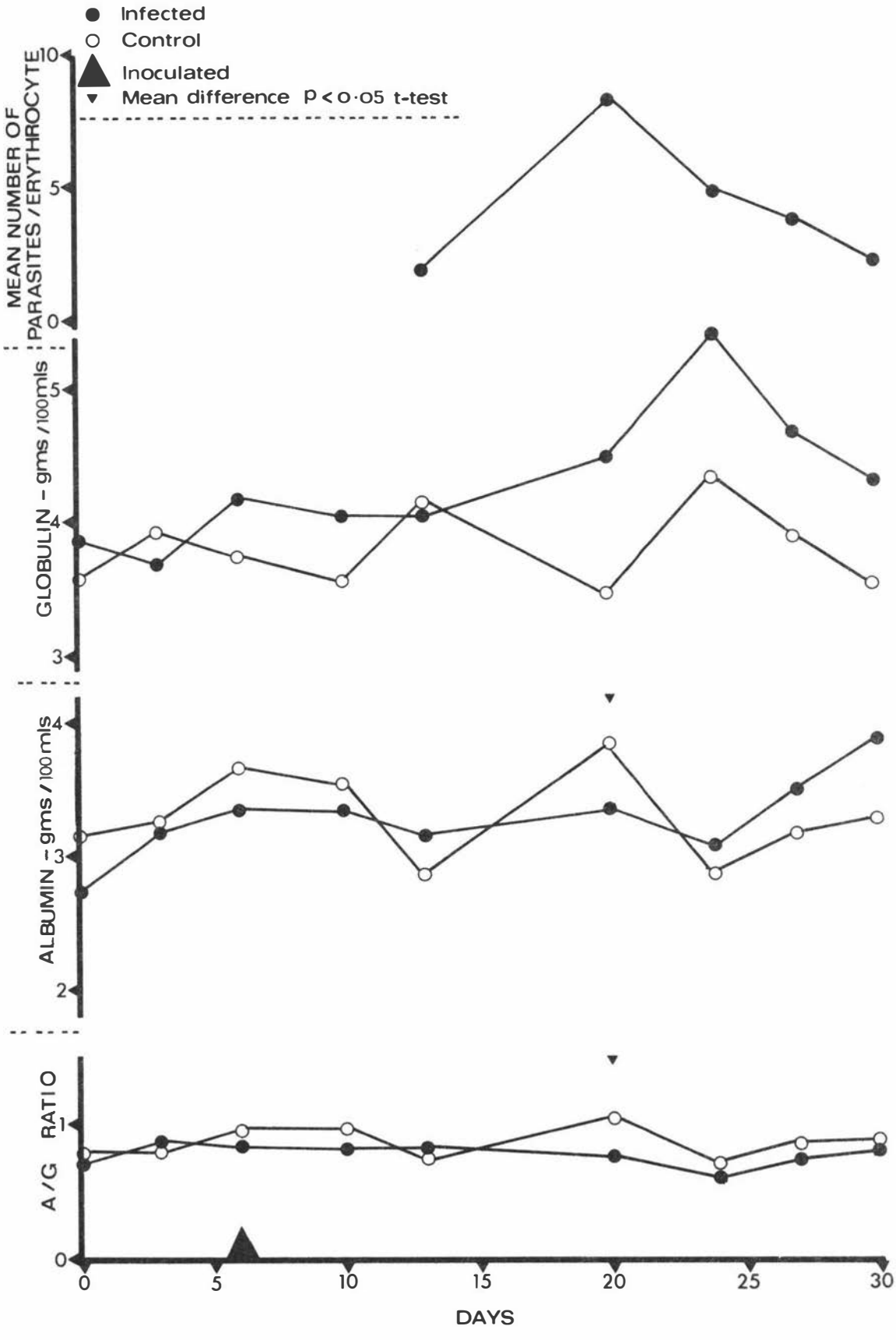


Figure 4.4. The time relationship between the degree of parasitaemia, total plasma albumin and globulin concentrations, and the albumin : globulin ratio. (Exp. IV — Mean values of control group F and infected group E)

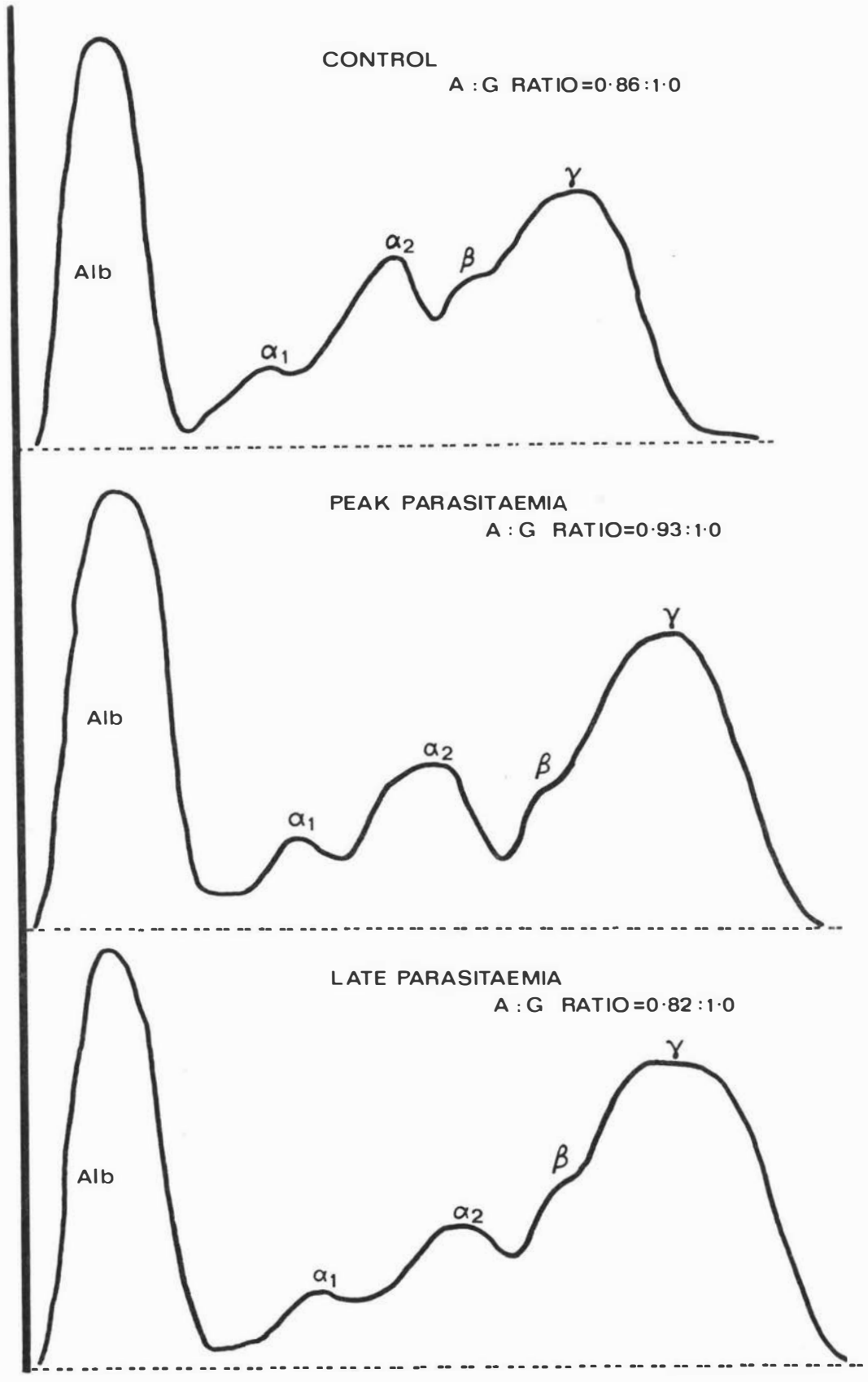


Figure 4.5. Plasma protein electrophoretic patterns for control sheep and for sheep in the peak and late stages of parasitaemia. (Exp. IV — Representative examples)

CHAPTER V

MORPHOLOGICAL CHANGES IN EPERYTHROZOON OVIS INFECTION OF SHEEP

INTRODUCTION

Eperythrozoon ovis infection has been incriminated as a cause of illthrift in sheep (Sheriff et al., 1966), but whether or not it is a disease of economic importance remains uncertain. In observations on experimental infection Foggie and Nisbet (1964) found no significant difference in weight gain between infected and control sheep. Similar observations in naturally infected sheep were recorded by Øverås (1969) and Harbutt (1969b). In this investigation of E. ovis infection, weight measurements were taken in three outdoor experiments and two indoor experiments.

Certain aspects of the pathology of E. ovis infection have been described and are consistent with haemolytic anaemia (Neitz, 1937; Foggie and Nisbet, 1964). Lesions include an enlarged spleen, occasional jaundice, pale musculature and pale mucous membranes. Examination of organ and tissue changes, as well as live and certain organ weight measurements were included in the present study of E. ovis infection.

MATERIALS AND METHODS

To Examine the Effect of E. ovis Infection on Body Weight

In 2 of the indoor experiments (Experiments I and III) sheep were weighed prior to infection and at the end of the experiment. In 3 of the outdoor experiments (Experiments II, IV and VII), sheep were weighed at each bleeding. These experiments were run at different times of the year with sheep of different ages as indicated in Table XV.

Stocking rates, inoculation and weighing procedures, and bleeding intervals were as described in Chapter II.

TABLE XV

THE AGE OF SHEEP AND THE TIME OF YEAR IN WHICH BODY WEIGHT MEASUREMENTS WERE TAKEN FROM SHEEP GRAZED ON PASTURE

Experiment	Age of Sheep	Time of Year	Season
II	6-8 months	February to April	Late Summer to Autumn
IV	10-12 months	September to October	Spring
VII	9-10 months	June to July	Winter

Statistical Methods

Differences in the body weight growth curves between the groups of sheep in the outdoor experiments were examined in 2 ways by analyses of covariance. In both cases the response analysed was the gain in body weight at the end of each interval between weighings. In the first series of analyses the independent variable was the initial body weight, and the significance of the cumulative difference in body weight between the groups was tested, after correction for group differences in initial body weight. In the second series of analyses the independent variable was the preceding weight measurement. This series tested the significance of the differences in body weight at each reading after correcting for differences in body weight at the preceding weight measurement.

Necropsy Procedure and Body Organ Weight Measurements

The necropsy procedure was described in Chapter II. Organs that were weighed are indicated in Table II.

RESULTS

The Effect of E. ovis Infection on the Weight Gain of Sheep Maintained on Pasture

Experiment II: The time relationships between mean degree of parasitaemia, mean Hb level, and mean weight of lambs is shown in Figure 5.1. Cross-infection of the control lambs occurred, and by the fourth week they were as severely affected as the experimentally infected group, which was now in the convalescent stage. The actual cumulative and interval weight differences are recorded in Table XVI, together with levels of significance. The results indicate that infection with E. ovis caused a significant ($p < 0.01$) reduction in weight gain which was most pronounced 5 weeks after infection. Thereafter this became less pronounced probably due to convalescence of the inoculated group and cross-infection of the controls. It is noteworthy that significant ($p < 0.01$) reduction of weight gain occurred relatively

TABLE XVI

CUMULATIVE AND INTERVAL MEAN WEIGHT DIFFERENCES BETWEEN INFECTED AND CONTROL SHEEP - EXPERIMENT II

Week	Weekly Difference Weight (Kgs)	P	Cumulative Difference Weight (Kgs)	P
1	^{x}+0.17	NS	+0.17	NS
2	+0.44	<0.01	+0.61	< 0.01
3	+0.64	<0.01	+1.25	< 0.01
4	+0.04	NS	+1.29	< 0.01
5	+0.69	<0.01	+1.98	< 0.01
6	^{xx}-0.67	<0.05	+1.31	< 0.01
7	+0.03	NS	+1.34	< 0.01
8	-0.16	NS	+1.18	< 0.01
9	+0.09	NS	+1.27	< 0.01

Number of sheep in each group = 32

^{x}+ = Gain of control over infected sheep

^{xx}- = Gain of infected over control sheep

NS = Not Significant (P> 0.05)

early in the disease before the anaemia was severe and continued into the convalescent period.

Experiment IV: In this experiment groups of infected and control animals were killed 6, 13, and 27 days after inoculation. The cumulative and interval mean weight differences between the 3 sets of control and infected groups are shown in Table XVII. Eperythrozoon ovis infection had no significant effect on the final cumulative weight gain of infected sheep compared with the controls. The time relationships between the mean degree of parasitaemia, mean Hb level, and the mean weight gains of control group F and infected group E is shown in Figure 5.2. Both groups showed a steady rate of weight gain throughout the experiment.

Experiment VII: The time relationships between the mean degree of parasitaemia, mean Hb level and the mean weight gains of the control and 2 infected groups is shown in Figure 5.3. All groups showed a loss in weight by the end of the experiment. The actual cumulative and interval weight differences between the control group (A) and the two infected groups (B and C) and between the two infected groups together with levels of significance are shown in Table XVIII. The results indicate no significant differences in weight between the control group (A) and the infected group (B). However there was in the initial stages of infection some significant ($p < 0.05$) gain in weight of the control group over infected group C, reaching a peak of 1.55 kilograms at day 11, but this difference decreased to non-significant levels as the infection continued (Table XVIII). Infected group B also showed significant ($p < 0.0$ gain in weight over infected group C reaching a peak of 1.32 kilograms at day 15, but this difference also decreased to non-significant levels as the infection continued (Table XVIII).

The Effect of E. ovis Infection on the Weight Gain of Sheep Maintained Indoors - Experiments I and III

Animals were weighed prior to inoculation and at the conclusion of the experiment only. In both Experiments I and III, the control sheep gained more weight than the sheep infected with E. ovis (Tables XIX and XX), but only in Experiment III was this weight gain difference significant ($p < 0.05$) (Table XX).

TABLE XVII

CUMULATIVE AND INTERVAL MEAN WEIGHT DIFFERENCES BETWEEN INFECTED AND CONTROL SHEEP - EXPERIMENT IV

Days (After Inoculation)	Interval Difference Weight (Kgs)	P	Cumulative Difference Weight (Kgs)	P
	(Group A - infected and Group B - control)			
4	xx-1.45	<0.01	-1.45	<0.01
	(Group C - infected and Group D - control)			
4	-0.73	NS	-0.73	NS
7	x+0.77	NS	+0.04	NS
11	-0.09	NS	-0.05	NS
	(Group E - infected and Group F - control)			
4	-1.36	< 0.05	-1.36	<0.05
7	+0.59	NS	-0.77	NS
11	+0.55	< 0.05	-0.22	NS
14	+1.14	< 0.01	+0.92	NS
18	-0.92	< 0.05	0	NS
21	+1.18	< 0.05	+1.18	NS
25	-0.77	NS	+0.41	NS

Number of sheep in each group = 5

x = + Gain of control sheep over infected sheep

xx = - Gain of infected sheep over control sheep

NS = Not significant ($p > 0.05$)

TABLE XVIII

Cumulative and interval mean weight differences between infected and control sheep and between infected group B and infected group C. (Experiment VII.)

Days (Post inoculation)	Control group A and infected group B				Control group A and infected group C			
	Interval weight gain (kgs)	P	Cumulative weight gain (kgs)	P	Interval weight gain (kgs)	P	Cumulative weight gain (kgs)	P
5	**-0.36	NS	-0.36	NS	+0.23	NS	+0.23	NS
7	*+1.05	NS	+0.69	NS	+1.05	<0.05	+1.28	<0.01
11	-0.32	NS	+0.37	NS	+0.27	NS	+1.55	<0.01
15	-0.32	NS	+0.05	NS	-0.18	NS	+1.37	0.05
19	-0.05	NS	0	NS	-0.64	<0.05	+0.73	NS
22	+0.09	NS	+0.09	NS	+0.50	NS	+1.23	NS
26	+0.18	NS	+0.27	NS	0	NS	+1.23	<0.05
29	-0.05	NS	+0.22	NS	-0.32	NS	+0.91	NS
33	-0.09	NS	+0.12	NS	+0.36	NS	+1.27	NS
40	+0.32	NS	+0.44	NS	-0.64	<0.05	+0.63	NS

*+ = Gain of control over infected.

**- = Gain of infected over control.

	Infected group B and infected group C.			
	Interval weight gain (kgs)	P	Cumulative weight gain (kgs)	P
5	*+0.59	NS	+0.59	NS
7	0	NS	+0.59	NS
11	+0.59	NS	+1.18	<0.01
15	+0.14	NS	+1.32	<0.05
19	**-0.59	NS	+0.73	NS
22	+0.41	NS	+1.14	<0.05
26	-0.18	NS	+0.96	NS
29	-0.27	NS	+0.69	NS
33	+0.45	NS	+1.14	NS
40	-0.95	<0.05	+0.19	NSZ

*+ = Gain of infected group B over infected group C

**- = Gain of infected group C over infected group B.

Number of sheep in each group = 7.

NS = Not significant (P >0.05).

TABLE XIX

THE MEAN WEIGHT GAINS OF EACH GROUP FOLLOWING INOCULATION WITH VARIOUS DILUTIONS OF INFECTED BLOOD - EXPERIMENT I

Group No.	Amount of Infected Blood	Mean wt. gain (kgs)
I	Nil (Control)	7.0 ± 0.6^{x}
II	1.0 ml	6.1 ± 0.3
III	1.0×10^{-2} ml	5.9 ± 0.5
IV	1.0×10^{-4} ml	5.6 ± 0.7
V	1.0×10^{-6} ml	6.5 ± 0.9

No. of sheep in each group = 5

x = Standard error

Analysis of Variance: F = 0.83

P = NS (> 0.05)

TABLE XX

THE MEAN WEIGHT GAIN OF EACH GROUP IN EXPERIMENT III

Group No.		No. of Sheep	Weight gain (Kilograms)
I	Control Group "Biopsied"	5	4.1 ± 0.42^{x}
II	Infected Group "Biopsied"	9	2.1 ± 0.38
III	Control Group "In Contact" Unbiopsied	4	3.8 ± 0.31

x = Standard Error

		P
Analysis of Variance between all groups.	F = 6.42	< 0.01
Analysis of Variance between Group I & II.	F = 8.90	< 0.05
Analysis of Variance between Group II & III.	F = 6.58	< 0.05
Analysis of Variance between Group I & III.	F = 0.15	NS

NS = Not significant ($p > 0.05$)

The Effect of E. ovis Infection on the Body Weight - Dressed Weight Ratio - Experiments I, III and IV

The ratio of body weight to dressed weight was higher in the infected groups than in the control groups in all experiments (Table XXI). This was significant ($p < 0.02$) in Experiment III only.

The Effect of E. ovis Infection on the Weight of Spleen, Liver and Lung - Experiments I, III and IV

In Experiments I and III the spleens were weighed 42 days post-inoculation. In both experiments the spleen weights were higher in the experimentally infected sheep than in the controls, but only the weight difference in Experiment III was significant (Table XXII). The liver weights of the experimentally infected sheep in Experiment III were higher than the controls but not significantly so (Table XXIII). Livers were not weighed in Experiment I.

In Experiment IV, spleen, liver and lung weights were measured at early (6 days post-inoculation), peak (13 days) and late parasitaemia (27 days). The following observations were made:

1. Experimental E. ovis infection induced significant ($p < 0.05$) spleen and liver weight increases as early as 6 days post-inoculation (Figure 5.4, Tables XXII and XXIII).
2. The liver and spleen weight increases paralleled, and were significantly ($p < 0.05$) correlated with, the degree of parasitaemia until the peak of parasitaemia 13 days post-inoculation (Figure 5.5).
3. From 13 to 27 days during which parasitaemia, decreased, the liver weights still increased although at a slower rate, whereas the spleen weights remained unchanged (Figure 5.4).
4. Control animals (Group F) killed on day 27 had become naturally infected and showed increases in liver and spleen weight similar to those of experimental infection

TABLE XXI

Mean body weight: Dress weight ratios (Experiments I, III and IV.)

	Control	Infected						
						Number of sheep in each group	F	P
EXPERIMENT I								
Group No	I	II	III	IV	V			
	2.47	2.56	2.65	2.78	2.65	5	2.73	NS.
	±0.05*	±0.06	±0.04	±0.07	±0.08			
EXPERIMENT III								
Group No	I	II						
	1.97	2.16				5(I)	8.77	<0.02
	±0.04	±0.04				9(II)		
EXPERIMENT IV								
Group No. (Early parasitaemia)	B	A	F**					
	2.17	2.33	2.42			5	2.55	NS.
	±0.03	±0.02	±0.03					
Group No. (Peak parasitaemia)	D		C					
	2.35		2.43			5	1.97	NS.
	±0.02		±0.05					
Group No. (Late parasitaemia	F		E			5	-	-
	(See Above)		2.46					
			±0.02					

*Standard error

**Early natural infection of control group

F = F ratio by analysis of variance

NS = Not significant (P >0.05).

TABLE XXII

Mean spleen weights (grams) in Experiments I, III and IV.

	Control	Infected				Number of sheep in each group	F	P
EXPERIMENT I (42 days past inoculation)								
Group No.	I	II	III	IV	V			
	73.4	97.2	99.4	90.6	88.6	5	0.73	NS
	±11.03*	±5.48	±17.46	±4.82	±9.29			
EXPERIMENT III (42 days post inoculation)								
Group No.	I	II						
	46.8	86.2				5(I)	11.52	<0.01
	±2.41	±7.90				9(II)		
EXPERIMENT IV								
(Early parasitaemia) - (6 days post inoculation)								
Group No.	B	A	F**					
	42.4	66.0	75.0			5	5.94	<0.05
	±2.53	±4.24	±9.47					
Peak parasitaemia - (13 days post inoculation).								
Group No.	D	C						
	44.4	110.4				5	84.55	<0.001
	±2.98	±5.67						
Late parasitaemia (27 days post inoculation)								
Group No	F	E						
	(see above)**	110.0				5	-	-
		±9.32						

*Standard error.

**Early natural infection of control group.

F = F ratio calculated by analysis of variance.

NS = Not significant (P >0.05).

TABLE XXIII

Mean liver weights (grams) in Experiments III and IV.

	Control	Infected		Number of sheep in each group	F	P
EXPERIMENT III (42 days post inoculation)						
Group No	I	II				
	481.0	520.7		5(I)	2.47	NS
	±19.11*	±13.83		9(II)		
EXPERIMENT IV						
Early parasitaemia (6 days post inoculation)						
Group No	B	A	F**			
	528.8	624.4	700.4	5	10.72	<0.01
	±12.54	±25.79	±28.75			
Peak parasitaemia (13 days post inoculation)						
Group No.	D	C				
	551.4	752.2		5	34.59	<0.01
	±20.22	±22.81				
Late parasitaemia (27 days post inoculation)						
Group No.	F	E		5	-	-
	(see above)**	798.2				
		±13.89				

*Standard error.

**Early natural infection of control group.

F = F ratio calculated by analysis of variance.

NS = Not significant (P >0.05).

(Figure 5.4, Tables XXII and XXIII).

5. Lung weights were not affected by E. ovis infection (Figure 5.4).

The Effect of E. ovis Infection on Haemolymph Node Size

Experiment III: The results of the grading of haemolymph node size on a visual basis is shown in Table XXIV. By this grading the infected sheep had significantly ($p < 0.001$) larger nodes at the late convalescent stage, i.e. 42 days post-inoculation.

Experiment IV: The mean diameters at the early, peak and late parasitaemia stages are also shown in Table XXIV. Significant ($p < 0.05$) increase in haemolymph node size was not recorded until the late parasitaemic stage, i.e. 27 days post-inoculation.

The Pathology of E. ovis Infection

Macroscopic Findings: The general body condition of E. ovis infected sheep was similar to the control sheep. The most notable lesion in the infected sheep necropsied at the peak and late stages of parasitaemia was enlargement of the spleen, in which large Malpighian corpuscles were visible on the cut surface. The haemolymph nodes of these sheep were also enlarged, but the liver, lungs, kidneys, lymph nodes and urine all appeared normal.

Microscopic Findings: In both normal and infected sheep kidneys a homogeneous eosinophilic protein-like substance was often found in the space of Bowman's capsule and within the lumina of the proximal convoluted tubules. With Perl's iron stain there was no evidence of iron containing pigment (haemosiderin) in the control sheep kidneys (Figure 5.6). All E. ovis infected sheep, necropsied at the late parasitaemic stage showed varying amounts of haemosiderin in the kidney cortex. Two distinct staining reactions were obtained with Perl's stain. The first appeared as a blue homogeneous substance

TABLE XXIV

THE EFFECT OF E. OVIS INFECTION ON HAEMOLYMPH NODE SIZE

Experiment III

Late Parasitaemia

	Control	Infected	No. of Sheep in each group	F	P
Mean Grade Size	1.20±0.18[x]	4.22±0.26	5 (control) 9 (infected)	55.44	< 0.001

Experiment IV

Early Parasitaemia

Mean diameter (mm)	3.1±0.36	3.6±0.52 2.6±0.26xxx	5	1.29	NS

Peak Parasitaemia

	2.8±0.18	3.4±0.61	5	0.72	NS

Late Parasitaemia

	Became infected	5.2±0.44xx	5	-	-

x = Standard Error

xx = This value significantly higher ($p < 0.05$) than the controls in early and peak parasitaemia

xxx = Control group for late parasitaemia stage with early natural infection

P = Probability. NS = $P > 0.05$

F = F ratio by Analysis of Variance

which appeared to correspond with the protein-like material found in the Bowman's space and lumina of the proximal convoluted tubules. The other appeared as deep blue staining granules found within the cytoplasm and close to the nucleus of the epithelial cells of the proximal convoluted tubules (Figure 5.7). The kidneys of 5 sheep necropsied before peak parasitaemia of Experiment IV showed no evidence of haemosiderin. Only small amounts were seen in the kidneys of 5 sheep necropsied at peak parasitaemia.

The livers of E. ovis infected sheep appeared normal with H and E stain, with the exception that there was probably some increase in the number of Kupffer cells in those livers from sheep necropsied at the late parasitaemic stage (Chapter VI). With the Perl's iron stain most of the infected sheep, particularly in the late parasitaemic stage showed varying amounts of haemosiderin granules within the hepatic parenchymal cells adjacent to the portal vessels (Figure 5.8). A small number of Kupffer cells contained large haemosiderin granules. This was seen particularly when the amount of haemosiderin in the parenchymal cells was small (Figure 5.9).

The macroscopically visible enlargement of the Malpighian corpuscles of the spleen in infected sheep was due to lymphoid hyperplasia. The spleens of both infected and control sheep showed haemosiderin deposits particularly in the sinusoidal lining cells and substance of the red pulp. The 2 distinct staining reactions with Perl's stain observed in the kidney, were again apparent (Figure 5.10). In general the infected sheep had greater amounts of haemosiderin in the spleen than controls although there was considerable variation between sheep.

The haemolymph nodes of infected sheep showed some lymphoid hyperplasia, although this did not appear to be as severe as that seen in the spleen.

The lungs and lymph nodes of all sheep examined appeared

microscopically normal.

DISCUSSION

The Effect of E. ovis Infection on the Weight Gain of Sheep

In all experiments in which sheep were weighed, the control sheep showed greater weight gains than E. ovis infected sheep, but the magnitude of the weight gains varied. In the outdoor experiments, only the control sheep of Experiment II showed significant ($p < 0.01$) gains in weight over experimentally infected sheep (Table XVI). The sheep in this experiment were younger than in other experiments and therefore would be expected to have a higher rate of weight gain than older sheep. As is shown in Figure 5.1 and Table XVI, the effect on weight gain was noted before peak parasitaemia was reached and continued on into the period of convalescence. It could not be determined how long the rate of weight gain was affected, since by 6 weeks post-inoculation, the majority of control group lambs were also infected and showing loss of weight relative to the inoculated group. However the weight gain was not as adversely affected in the control group as in the experimentally infected group. This was probably due, not only to variable stages of E. ovis infection occurring within the control group at any one time but, if it is assumed that changes in haemoglobin concentration are a measure of infection severity, it could have been also due to a generally less severe infection in the naturally infected group (see Chapter III).

Although E. ovis infection had a significant ($p < 0.01$) depressive effect on the weight gain of sheep in Experiment II, results from the other 2 outdoor experiments (IV and VII) were equivocable. As can be seen in Tables XVII and XVIII and Figures 5.2 and 5.3, there were inconsistencies in the relative weight gains between infected and control groups. It is apparent from the findings in Experiments IV and VII, that factors other than E. ovis infection were influencing the weight response obtained. These could include the age

of sheep, the time of the year in which the experiment was run, and the severity of the gastro-intestinal parasite burden.

It is worth noting that, by the termination of Experiment VII, all sheep had lost weight. In this experiment, run in the middle of winter, there was little pasture growth and food was scarce. In contrast all sheep in Experiment IV showed weight gain, and these were on pasture in the flush of spring growth. The sheep in these 2 experiments, run on extremes of pasture conditions, at similar stocking rates did not appear to differ in their response to E. ovis. Group C sheep of Experiment VII were held without food and water for 24 hours prior to bleeding. As can be seen in Figure 5.3, a relative loss of weight of sheep in this group did occur, which was most likely the result of fluid and intestinal contents loss over the 24 hour period.

The weight measurements in the 2 indoor experiments (I and III) showed relative weight gains of control sheep over infected sheep (Tables XIX and XX). Sheep in these 2 experiments were of similar age to the sheep in Experiments IV and VII. It is apparent from the results of the experiments in which live weights were measured that under certain conditions E. ovis has a deleterious effect on the weight gain of sheep. However it is also apparent that other influencing factors do exist either in conjunction with, or unrelated to E. ovis which may decrease the effect of E. ovis on weight gain. Further work with replicate experiments is required to define these factors.

It was thought prior to this study that the main effect of E. ovis was mediated by the severity of the anaemia occurring. However as can be seen in the results from Experiment II (Table XV and Figure 5.1) and to a lesser extent in Experiment VII (Table XVII), the cumulative weight gain of control sheep was higher than infected sheep before anaemia had developed. In addition, it was noted in the indoor experiments (I and III) that infected sheep were inappetent

for a short period in the early stages of the disease. These observations suggest that the effect of E. ovis infection may be mediated by factors other than anaemia. This is discussed further in Chapter VIII.

The Pathology of E. ovis Infection

The most notable finding in E. ovis infected sheep at necropsy was an enlarged spleen, which at peak parasitaemia was approximately 2½ times as heavy as that of control sheep.

The role of the spleen in Eperythrozoon infection has been the subject of study. Stansly et al. (1962) found a 3 to 5 fold increase in spleen weight of mice bearing transplantable tumours and reported that this lesion was caused by an agent, which was later shown to be E. coccoides (Stansly and Neilson, 1965).

The observation of lymphoid hyperplasia which accompanied increases in spleen weight in infected sheep is in accord with the findings of Neitz (1937) and Foggie and Nisbet (1964). In both mice with E. coccoides infection, and sheep with E. ovis, the spleen clearly plays an important role in the limitation of infection. In mice extreme hyperplasia occurs in the spleen with loss of definition of the Malpighian corpuscles (Baker et al., 1971). It is probable that this reaction is associated with antibody production by the lymphoid tissues; and this is suggested by observations that plasma of infected mice contains protective elements which can be diluted out (Stansly and Neilson, 1966). Whether or not lymphoid hyperplasia in sheep with E. ovis is associated with an antibody response has not been determined. Certainly, splenectomy of previously infected sheep is usually followed by a resurgence of parasitaemia (Neitz, 1937; Littlejohns, 1960), though the severity of infection is similar to that of non-splenectomized sheep. Splenectomy of mice with E. coccoides produces a severe prolonged infection with severe anaemia (Thurston, 1954). In intact animals, E. coccoides infection is less severe in mice than E. ovis is in sheep (Kreier and Ristic,

1968). These results suggest the spleen is more important in controlling infection in mice than in sheep.

The haemolymph node has usually been considered to play a role comparable to that of the spleen, but this has not been confirmed. Some structural features are similar to the spleen but it has been established that it is a distinct lymphoid organ, of unknown precise function (Folse et al., 1971). Although lymphoid hyperplasia did not appear to be a prominent feature of the haemolymph nodes early in the parasitaemia cycle (Table XXIV) it is probable that changes found following peak parasitaemia were ancillary to those changes observed in the spleen.

In infected sheep significant ($p < 0.01$) increases in liver weight as compared to control sheep were recorded. Although liver dimensions were not specifically measured, increases in liver size were not obvious at necropsy. Microscopically some increase in numbers of Kupffer cells was seen, though not until the late parasitaemic stage (see Chapter VI).

The higher body weight to dressed weight ratio in the infected sheep (Table XXI) was most probably due to the increased weight of the liver and spleen. The original purpose of taking this measurement was to try to determine any difference in food intake between infected and control sheep but, because of the alteration in liver and spleen weight, this point was impossible to ascertain.

Haemosiderosis in E. ovis Infection

Haemosiderin is a water insoluble iron storage compound usually found in granular form (Sturgeon and Shoden, 1969). It is thought that it is formed in tissues when the concentration of another iron storage compound, ferritin, has reached a critical level (Shoden and Sturgeon, 1962). Haemosiderin stains readily with Perl's Prussian blue stain, whereas ferritin, a water soluble compound, is dispersed in

tissues and does not.

One of the main histopathological features of experimental E. ovis infection in this study was the presence of haemosiderin in the kidneys. This was a constant feature of sheep kidneys examined after peak parasitaemia. Renal haemosiderosis with E. ovis infection has previously been described by Foggie and Nisbet (1964), Rouse and Johnson (1966), Jolly (1967) and Øverås (1969). The presence of haemosiderin in epithelial cells of the proximal convoluted tubules is indicative of prolonged intravascular haemolysis (Leonardi and Ruol, 1960; Roberts, 1966; Liddy and Roberts, 1970). Low concentrations of haemoglobin released into plasma are bound to haptoglobin (Lathem, 1959). The binding capacity of the haptoglobin in humans is of the order of 100 to 140 mg/100 ml (Liddy and Roberts, 1970), and when the level of free haemoglobin exceeds this binding capacity it is filtered through the renal glomeruli (Lathem, 1959). Prolonged intravascular haemolysis, however, depletes the plasma haptoglobin and the renal threshold falls accordingly (Venziale et al., 1966). The haemoglobin is reabsorbed by and deposited in the proximal tubules. Acute intravascular haemolysis on the other hand may cause glomerular filtration of haemoglobin with resultant haemoglobinuria. Haemosiderin in these cases is present only in the tubular lumina and Bowman's spaces (Roberts and Morrow, 1966).

Although haemoglobinuria has been observed in E. ovis infected sheep (Neitz, 1937; Øverås, 1969), it was not observed macroscopically in this study. It is reasonable to assume that the haemosiderin deposits in the renal proximal convoluted tubules of infected sheep is the result of a prolonged intravascular haemolysis. The 2 distinct positive staining reactions obtained with Perl's Prussian blue, were also noted in E. ovis infection by Foggie and Nisbet (1964). These 2 forms are apparently similar to those described by Shoden and Sturgeon (1962) and Sturgeon and Shoden (1969), in relation to hepatic haemosiderosis. The blue-staining homogeneous material was considered by these investigators

to be an intermediate storage form of iron between ferritin and haemosiderin. They concluded that once the storage of ferritin had reached a certain level, the deposition of haemosiderin commenced with the appearance of the homogeneous material immediately preceding that of intra-parenchymal granules. Whether this is similar to the stainable iron (grade 1) described in the kidney tubule lumina by Roberts (1966) in cases of intravascular haemolysis, is not known but it does appear that the homogeneous staining positive iron material found in the infected kidneys of this study is probably an intermediate form between ferritin and haemosiderin.

The clinical question as to whether haemosiderin affects renal function is a problem about which very little is known. It is generally believed from studies on man that renal haemosiderosis does not cause significant tubular impairment (Crosby and Damshek, 1953). Leonardi and Ruol (1960) found that pronounced haemosiderin infiltration of the proximal convoluted tubules of human kidneys was not accompanied by impairment of renal function, and urine specimens were always normal.

Reports on the presence of renal lesions in E. ovis infection are variable. Øverås (1969) observed lesions which were described as a subacute to chronic nephrosis, with, in extreme cases, glomerular sclerosis. Some interstitial inflammatory change was also present. Damage was most pronounced in animals showing prolonged haemoglobinuria. Haemosiderin deposition was extensive, and included the epithelial cells, the tubular lumina, and the connective tissue between the proximal convoluted tubules. Extensive lesions were not described by the other authors who have noted renal haemosiderosis with E. ovis infection (Foggie and Nisbet, 1964; Rouse and Johnson, 1966; Jolly, 1967), but it is worthy of note that Neitz (1937) who described haemoglobinuria, also recorded an increase in blood urea levels (see Chapter IV). He did not however examine the kidneys. It has been suggested that the presence of high

levels of free haemoglobin in plasma as indicated by haemoglobinuria may be associated with renal vasoconstriction, leading to extensive damage (Øverås, 1969). The fact that haemosiderosis does not appear to damage renal tissue would suggest that a critical level of free haemoglobin is required before renal damage occurs. The observations in this study on E. ovis infection namely, unaffected blood urea levels (Chapter IV), the absence of renal lesions despite extensive haemosiderosis, as well as no observation of haemoglobinuria would support this conclusion.

The increased amounts of haemosiderin deposits in the spleen, and the variable amounts of haemosiderin within the Kupffer and parenchymal cells of the liver, situated near and adjacent to the portal triads from E. ovis infected sheep, necropsied at late parasitaemia is consistent with increased erythrocyte destruction (MacDonald, 1969). Knowledge on the mechanism of iron uptake by these organs is incomplete, but there is evidence to suggest that the liver and spleen may function in different ways (Iyengar and Chandra, 1972). While it is assumed that there is an intravascular haemolysis in E. ovis infection, with release of free haemoglobin into the plasma, it has recently been shown that free haemoglobin is not processed by the reticulo-endothelial cells of the spleen. One function of the spleen appears to be the removal of injured erythrocytes with comparatively minor abnormalities (Ultmann and Gordon, 1965). The fact that the amount of haemosiderin in the spleens of E. ovis infected sheep appears to be greater than in the control sheep may indicate that there is erythrocyte destruction by increased erythrophagocytosis. This would therefore mean that the anaemia is produced in 2 ways; intravascular haemolysis and increased erythrophagocytosis.

The ability of the liver, in haemolytic disorders, to recognise and remove injured erythrocytes is less than that of the spleen, and intrahepatic erythrophagocytosis is indicative of pronounced erythrocytic defects (Ultmann and Gordon, 1965). Histologically there was no evidence of

increased erythrophagocytosis by the liver in E. ovis infection.

It has been shown in man that in intravascular haemolytic conditions the liver is the main site of free haemoglobin uptake, accounting for more than 70% of the haptoglobin-bound haemoglobin (Keene and Jandl, 1965; Pimstone, 1972). If the Kupffer cells removed this haemoglobin in E. ovis infection it appears to have been transferred quickly to the parenchymal cells, as haemosiderin deposits in the Kupffer cells were infrequent, particularly when haemosiderin levels in the parenchymal cells were high. This apparent inverse relationship between the Kupffer cells and parenchymal cells in regard to the amount of haemosiderin contained in them (see Figures 5.8 and 5.9) may be the result of different stages of haemoglobin uptake. In the early stages most haemosiderin would be in the Kupffer cells, with little being deposited in the parenchymal cells, and in the late stages most iron taken up by the Kupffer cells would have been deposited in the parenchymal cells, and uptake activity would have fallen off. Whether the parenchymal cells take up iron directly, as postulated by MacDonald (1969), or not, is of little importance in this study, as the amount of haemosiderin deposited depends on the iron level already in storage and on the amount phagocytosed.

Iron in excess of that needed to maintain a functional "iron balance" is found in several organs, but most abundantly in the liver, spleen and bone marrow (Shoden and Sturgeon, 1962; Sturgeon and Shoden, 1969). It is of interest to note that hepatic haemosiderosis was not a feature of E. ovis infection in the studies of Foggie and Nisbet (1964) and in 2 of the 12 spleens examined by them there was no evidence of haemosiderin. In this study, however, haemosiderin deposits in the spleen were a normal feature, not only in the infected sheep, but in the control sheep as well. While it must be conceded that the anaemia from E. ovis infection appeared to be more severe in this study than in that of Foggie and Nisbet, it is possible that the sheep used in this

investigation, because of dietary or other factors, had high levels of iron in storage, thereby requiring less phagocytosed iron from erythrocyte breakdown to be stored as haemosiderin.

SUMMARY

It has been shown that under certain conditions E. ovis infection causes a retardation of growth rate. The main pathological feature of infection is enlargement of the spleen due mainly to hyperplasia of the Malpighian corpuscles. This is believed associated with the development of an immune response to infection.

Other pathological features included haemosiderin deposits in the proximal tubules of the kidney, in Kupffer cells and in parenchymal cells of the liver. There was also increased amounts of haemosiderin in the red pulp of the spleens of infected animals.

These findings support the conclusion that haemolytic anaemia in E. ovis infection is induced by both intravascular haemolysis and by increased erythrophagocytosis.

No renal lesions which were referable to the presence of haemosiderin were discernible with light microscope examination, but it is suggested that renal damage could occur in severe cases of infection when haemoglobinuria may be a feature of the disease.

Weight measurements of control and infected sheep revealed that E. ovis had a depressive effect on weight gain, but in only one experiment (Experiment II) was this effect significant. The effect was noticeable before anaemia had developed in infected sheep and some factors which may cause this, are referred to in Chapter VIII.

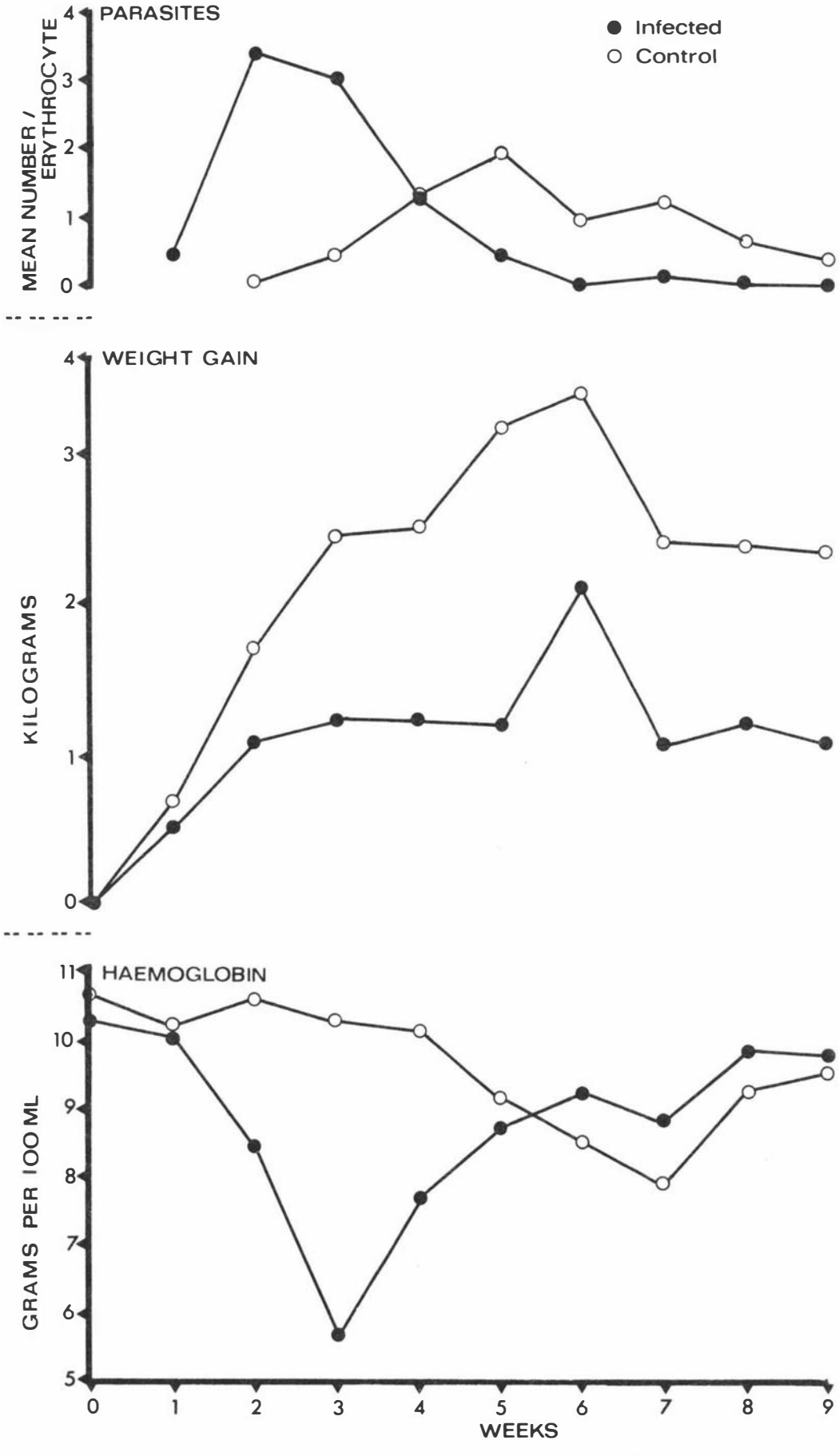


Figure 5.1. The time relationships between the degree of parasitaemia, haemoglobin concentration and weight gain of sheep. (Exp. II – Group mean values)

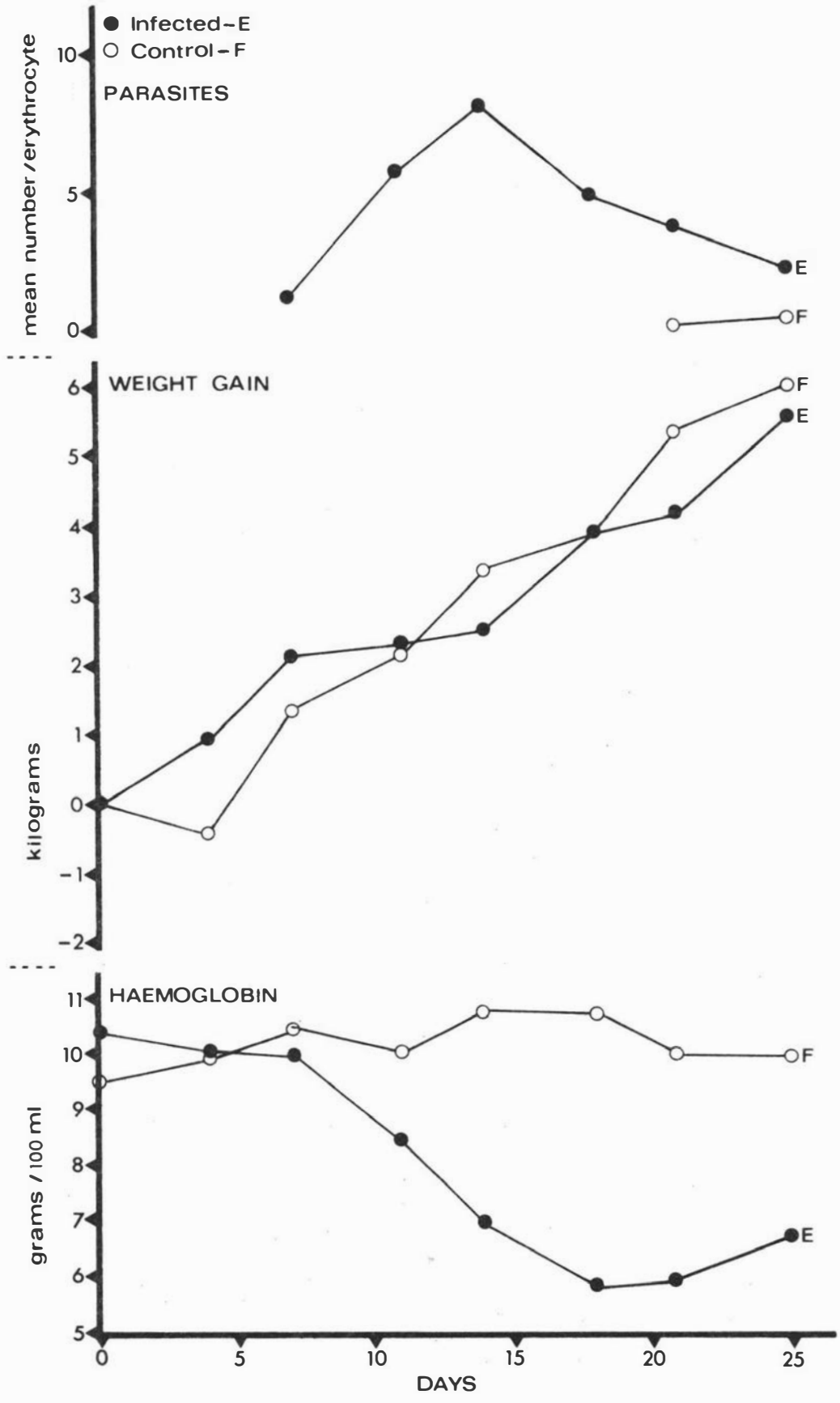


Figure 5.2. The time relationships between the degree of parasitaemia, haemoglobin concentration and weight gain of sheep. (Exp. IV – Mean values of control group F and infected group E)

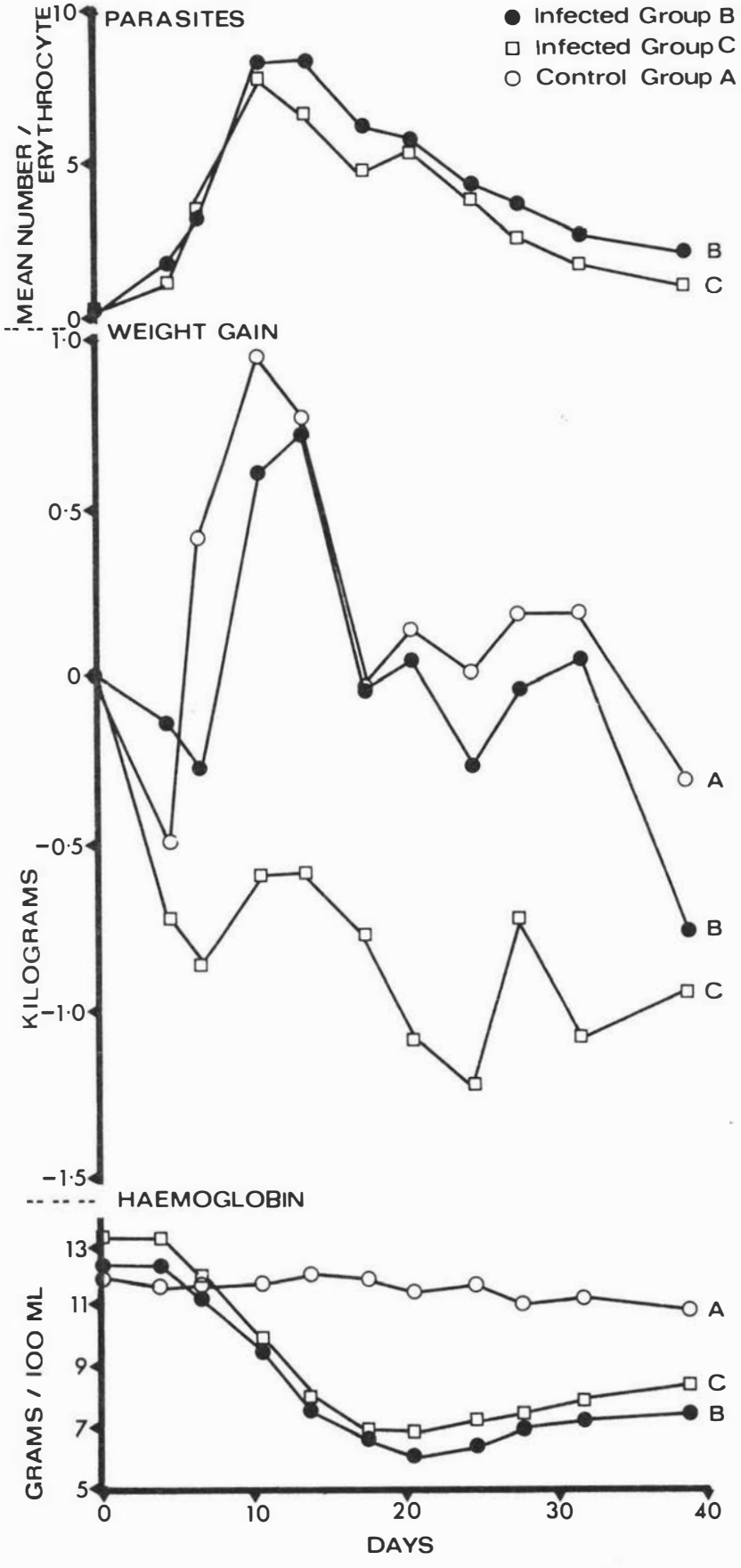


Figure 5.3. The time relationships between the degree of parasitaemia, haemoglobin concentration and weight gain of sheep. (Exp. VII – Group mean values)

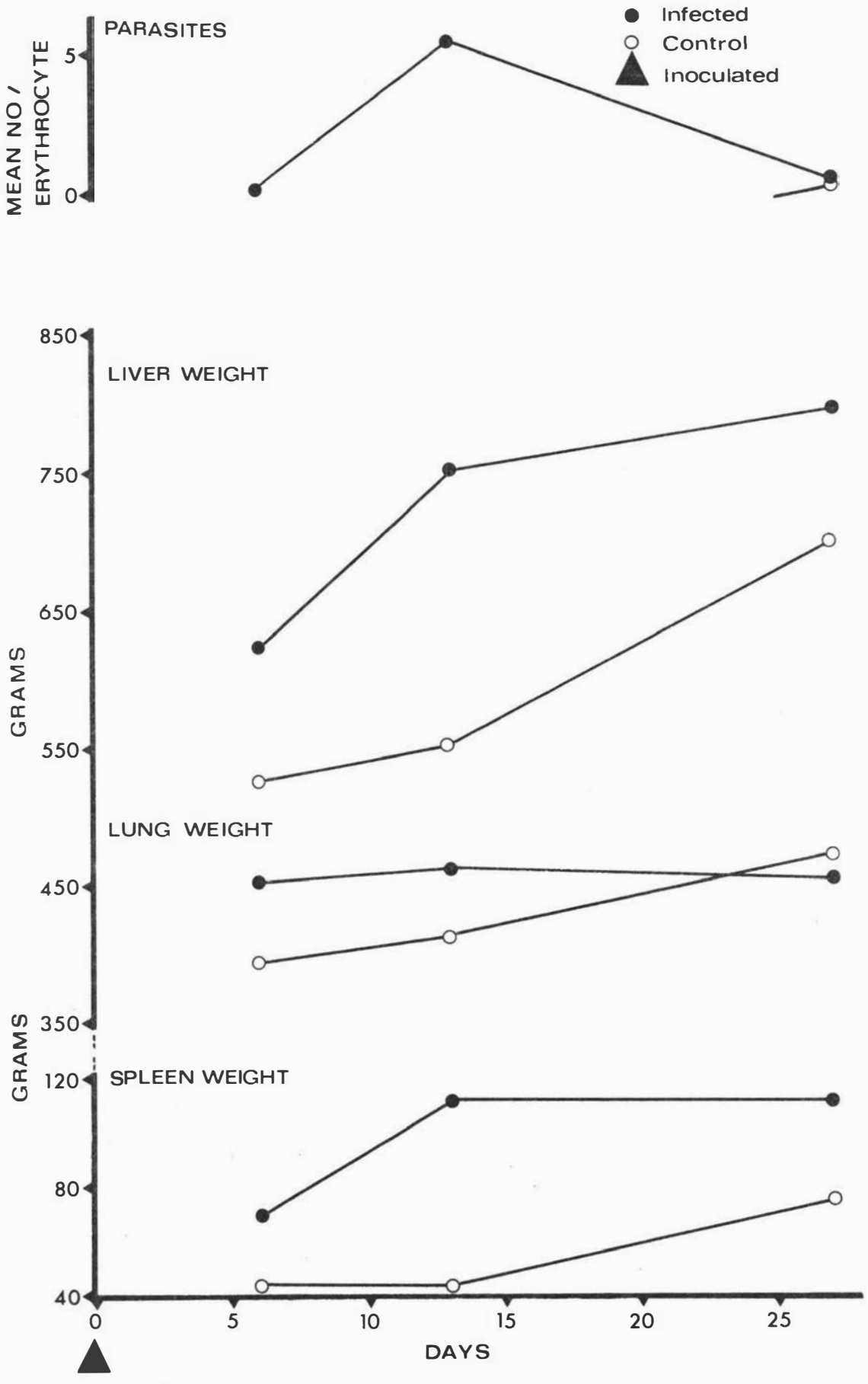


Figure 5.4. Liver, lung and spleen weights of sheep during early, peak and late parasitaemia (Exp. IV – Group mean values)

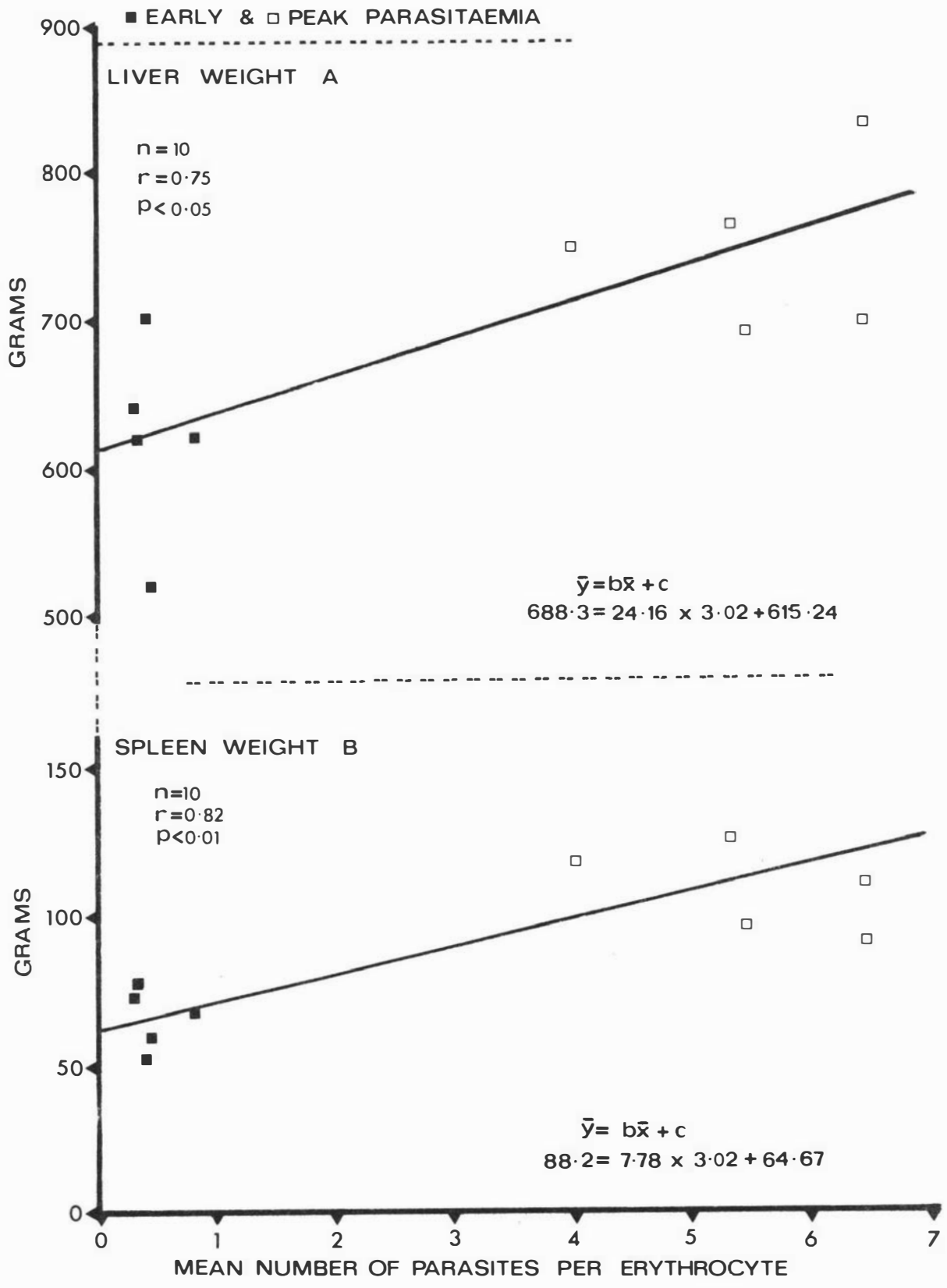


Figure 5.5. The correlation of liver weight (A) and spleen weight (B) with early and peak stages of parasitaemia (Exp. IV — Individual sheep values)

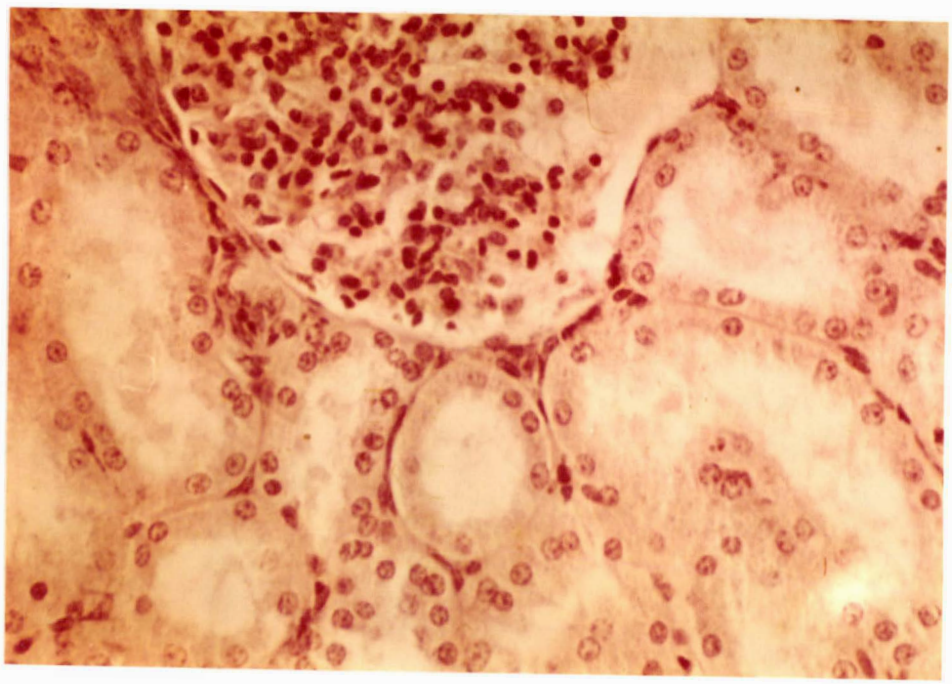


Figure 5.6: The glomerulus and convoluted tubules of a control sheep kidney showing no evidence of iron containing pigment. (Perl's Prussian Blue x 250).

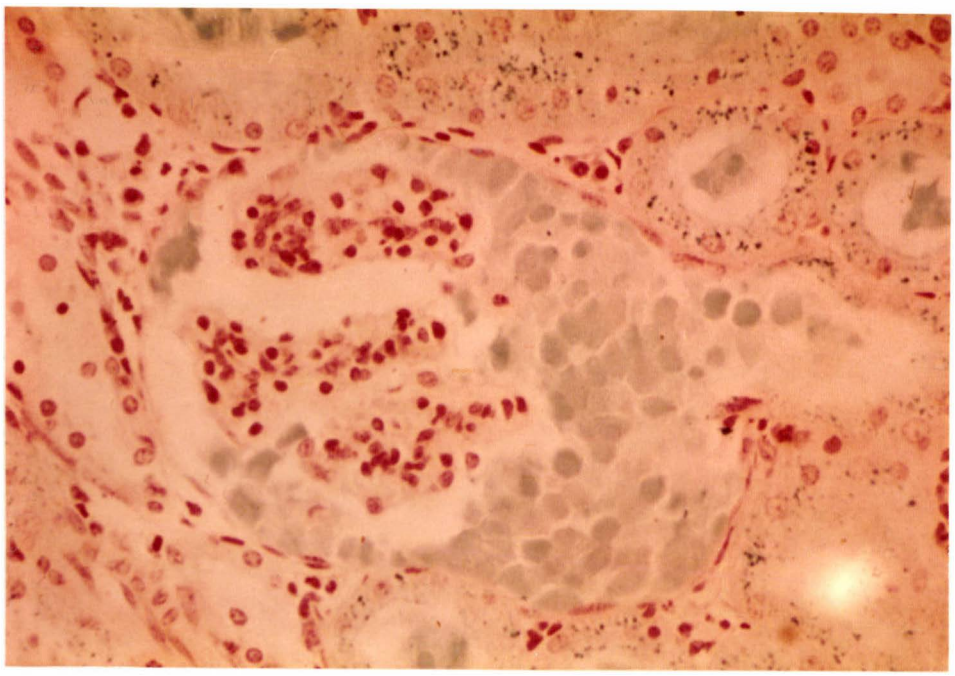


Figure 5.7: The glomerulus and convoluted tubules of a sheep kidney at the late parasitaemic stage of E. ovis infection. Two distinct positive staining reactions for iron are present. In the tubular cells are distinct blue granules. These contrast with a more diffuse staining of glomerular filtrate in Bowman's space and in the lumina of the proximal tubules. (Perl's Prussian Blue x 250).

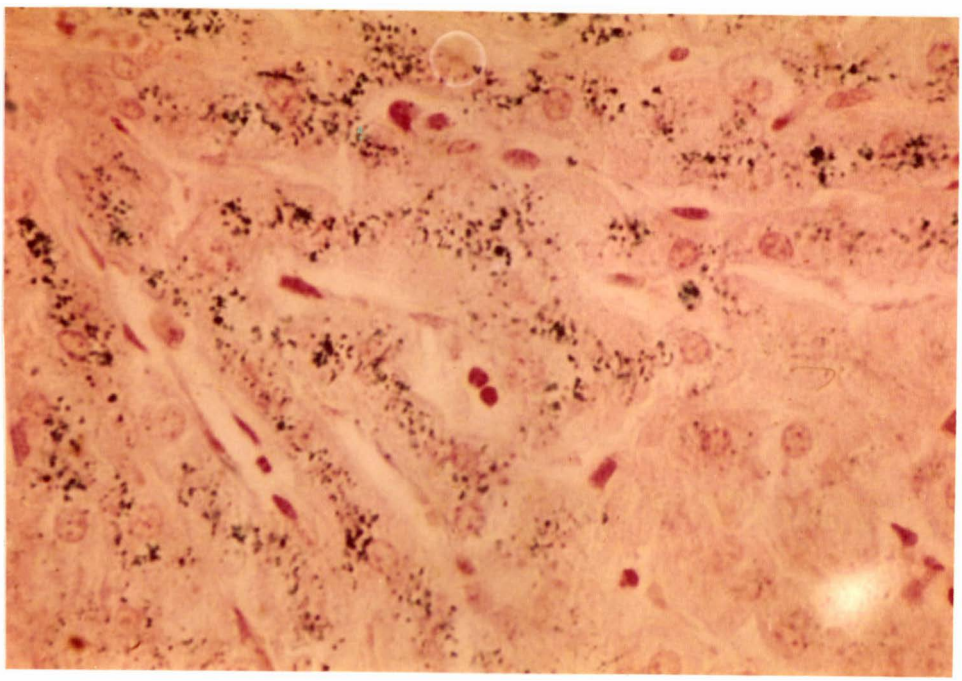


Figure 5.8: Area of liver in periportal area from an E. ovis infected sheep in the late parasitaemic stage of infection and showing many iron containing granules (haemosiderin) within the parenchymal cells. (Perl's Prussian Blue x 400).

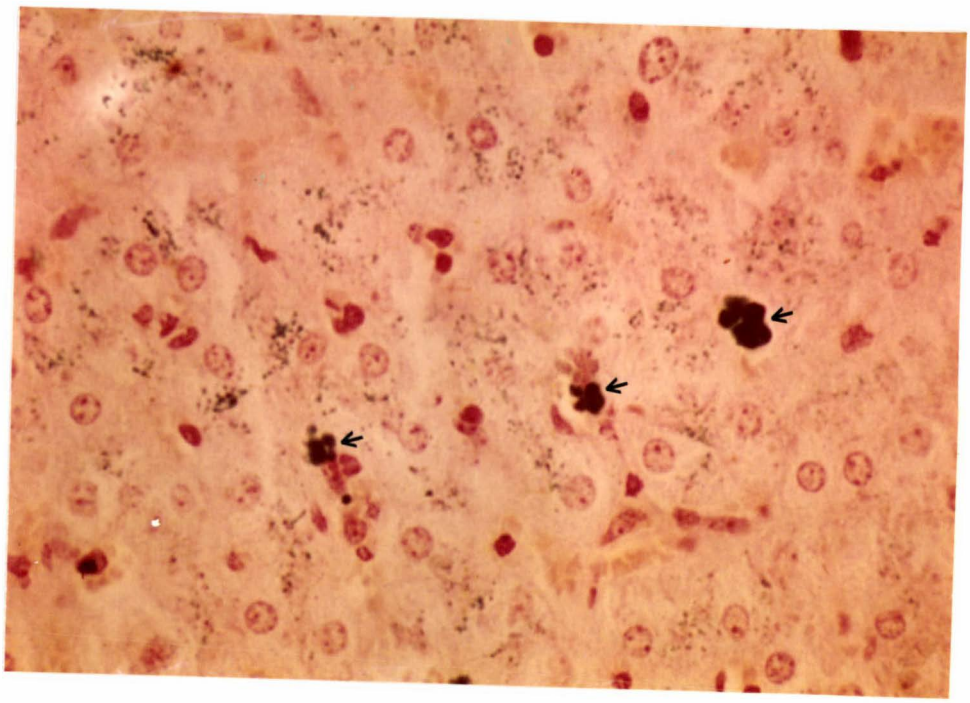


Figure 5.9: Area of liver in periportal area from an *E. ovis* infected sheep in the late parasitaemic stage of infection and showing a moderate amount of iron containing granules (haemosiderin) within the parenchymal cells. Some of the Kupffer cells (↑) contain large accumulations of haemosiderin. (Perl's Prussian Blue x 400).

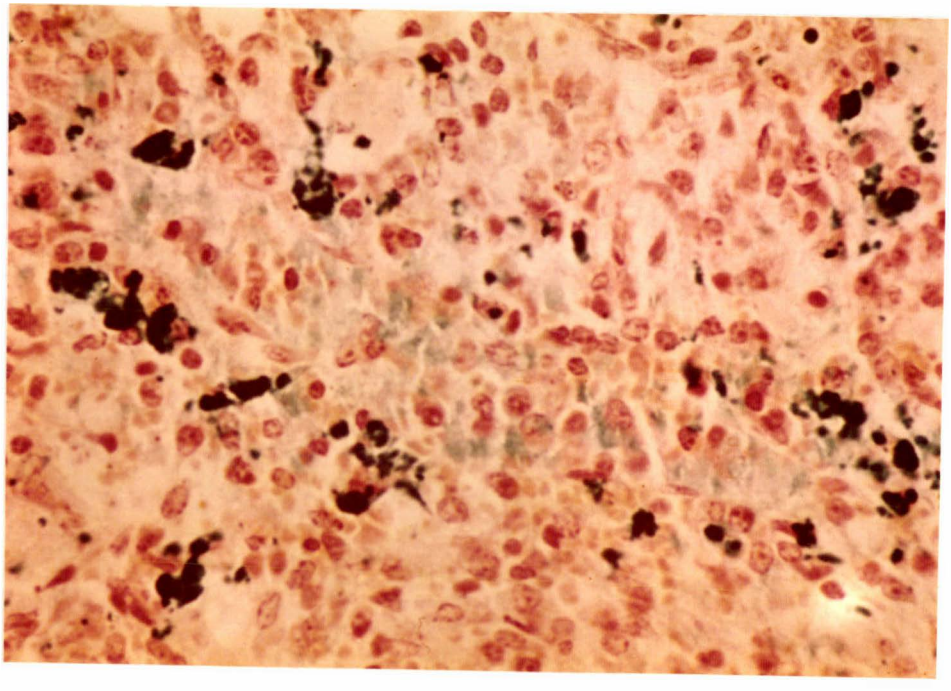


Figure 5.10: Red pulp of the spleen from an E. ovis infected sheep showing two staining reactions for iron. In the reticuloendothelial cells of the sinusoids there are distinct granules and inclusions of iron staining material. In contrast there is a more diffuse staining of material possibly within the sinusoids (Perl's Prussian Blue x 400).

CHAPTER VI

THE EFFECT OF EPERYTHROZOON OVIS INFECTION ON THE RETICULO-ENDOTHELIAL SYSTEM

INTRODUCTION

Historical - General Definition and Description

It was realised in the 19th century that a variety of morphologically diverse cells widely distributed in the vertebrate body were intensely phagocytic. Experiments showed that these cells comprised a system capable of ingesting colloidal dyes and other particulate materials. Although many names, such as spleen apparatus or "Milzapparat" and endothelial metabolism apparatus or endothelium "Stoffwechselapparat" were given to this system of cells (Landau and McNee, 1914), it was Aschoff (1924) who first used the descriptive term "Reticuloendothelial System". Although Aschoff defined the term as applying to cells with the property of phagocytosis, he was aware of the morphological diversity of the system, and there are still differences of opinion as to which cells contribute to the Reticuloendothelial System.

According to Stuart (1970) the Reticuloendothelial System is made up of three components:

1. Reticulum cells representing the argyrophilic structural component.
2. Phagocytic endothelial cells which are found lining the blood capillaries of the liver, the sinusoids of the spleen, the lymph nodes and the bone marrow. This can also include the endothelial cells lining the capillaries of the adrenal and pituitary gland.
3. The macrophages which are abundant in lymph nodes, tonsil and spleen.

Method for Investigation of Reticulo-endothelial Activity

Inorganic particles were first used about a century ago for histological demonstration of phagocytosis in vertebrate animals. Various authors have described methods based on the injection of vital dyes, or suspensions of particles such as colloidal silver, manganese, India ink and thorium (Cappell, 1929; Nagao, 1920; Wislocki, 1924; Brickner, 1927; Gordon and Katsh, 1949), with subsequent histological examination of the tissues. Work on the rate of clearance of particles from the blood was first attempted by Saxl and Donath (1924) who measured the rate of disappearance of fat particles injected intravenously into mammals. Since then a number of similar techniques using a variety of materials, including colloid particles which have been labelled with radioactive isotopes, have been used (Sheppard et al., 1951; Zilversmit et al., 1952).

One of the most common methods for measuring reticulo-endothelial activity was developed by Halpern et al. (1953) and Biozzi et al. (1953). They perfected a technique for the estimation of blood clearance rate and distribution of phagocytic activity in organs using a shellac-free colloidal suspension of carbon in fish glue.

With this technique mathematical formulae were developed which expressed the relationship between the speed of phagocytic activity, the dose of carbon administered and the weight of the organs (liver and spleen) principally responsible for the phagocytic activity (Biozzi et al., 1953).

It is well recognized that in blood parasite infections such as malaria, the phagocytic activity of the reticulo-endothelial system plays a prominent role in host defence (Cantrell and Elko, 1964; Belding, 1965; Cantrell et al., 1970). Accordingly, the technique of Biozzi et al. (1953) was used to ascertain the effect of E. ovis infection on the phagocytic activity of the reticuloendothelial system of sheep. The amount of carbon taken up by the various body

organs was also measured. Because phagocytic activity is mainly dependent on the Kupffer cells of the liver (Biozzi et al., 1953), the numbers of these cells present during E. ovis infection were also estimated.

MATERIALS AND METHODS

The experimental procedures, including the descriptions of the carbon clearance technique, calculation of phagocytic index (k), organ digestion for carbon recovery, and method of Kupffer cell counting are described in Chapter II. For the purposes of correlation estimations, and the calculation of the corrected phagocytic index (α), liver and spleen weight data, recorded in Chapter V, were used.

The final techniques used were developed after a series of preliminary experiments. A major problem centred around the constitution of the diluent in which the carbon was suspended. It became clear that the distribution of carbon following injection was affected by an interaction of the suspension with the animals' clotting mechanisms. Accordingly a small experiment was carried out to investigate this,

Two sheep (A and B) were injected with colloidal carbon, prepared as described in Chapter II. Blood samples were collected into EDTA "Vacutainers" at regular intervals over the succeeding 30 min. for the following estimations:

(i) Plasma fibrinogen levels by the method of Kaneko and Smith (1967).
(ii) Platelet counts by the Rees Ecker method as described by Schalm (1965).
(iii) Leucocyte counts as described in Chapter II.

RESULTS

The effect of E. ovis infection on the number of Kupffer cells per 0.1 mm^2 of liver tissue - Experiments I, III and IV

The mean numbers of Kupffer cells per 0.1 mm^2 of liver tissue from both control and infected groups in Experiments I,

III and IV are summarized in Table XXV. The counts were made on samples taken at post-mortem and were restricted to the mid-acinar region.

The data in Table XXV show that:

1. Variable counts were obtained within and between control and infected groups of sheep.
2. At both early (Experiment IV) and late (Experiments I and III) stages of parasitaemia there were no significant differences in Kupffer cell numbers between control and experimentally infected sheep, although numbers in the infected groups tended to be higher late in infection.
3. At peak parasitaemia (Experiment IV) there was a significant ($p < 0.05$) difference between control and infected sheep, due mainly to a low number of Kupffer cells per 0.1 mm^2 in the infected group, but also partly to a high mean count in the control group.

The comparison of Kupffer cell numbers between pre-infection biopsy samples and post-mortem samples at a late stage of parasitaemia for both control and infected groups of Experiment III is given in Table XXVI. The infected group showed a significant ($p < 0.05$) increase in Kupffer cell numbers at post-mortem compared with the pre-infection level.

Phagocytic activity during E. ovis infection - Experiment IV

Figure 6.1 summarises the disappearance of carbon particles as measured by the phagocytic index (k) - (Chapter II, p. 32) from control and infected sheep during early, peak and late parasitaemia. The disappearance rate in the first two minutes after injection was much more rapid than indicated by the overall slope of the regression line. This was mainly due to equilibration of the carbon with the vascular pool during this time.

From the figure it can be seen:

TABLE XXV

Kupffer cell counts, expressed as the number of cells per 0.1 mm^2 of liver tissue - Experiments I, III and IV (group mean values).

	Control	Infected						
EXPERIMENT I						Number of sheep in each group	F	P
Group No	I	II	III	IV	V			
	94.74 ±8.90*	96.72 ±5.24	90.88 ±3.42	103.96 ±5.17	107.36 ±8.11	5	0.87	NS
EXPERIMENT III								
Group No	I	II						
	98.64 ±6.82	113.91 ±9.32				9(II) 5(I)	1.10	NS
EXPERIMENT IV								
Group No	B	A	F**			5	0.89	NS
(Early parasitaemia)	94.10 ±3.17	95.52 ±2.80	100.66 ±3.79					
Group No	D	C						
(Peak parasitaemia)	109.70 ±7.47	78.78 ±7.12				5	7.16	<0.05
Group No	F	E						
(Late parasitaemia)	Became infected (see above)	99.94 ±3.97				5	-	-

*Standard error.

**Control group for late parasitaemia in early stage of natural infection.

F = F ratio as calculated by analysis of variance

NS = Not significant ($p > 0.05$)

TABLE XXVI

KUPFFER CELL COUNTS FROM SHEEP LIVERS BEFORE INFECTION (BIOPSIED TISSUE) AND AT THE LATE PARASITAEMIA STAGE OF INFECTION - EXPERIMENT III (GROUP MEAN VALUE)

	Before Infection cells per 0.1 mm^2	Late Infection	No. of sheep in each group	t	P
Infected Group	88.62 ± 1.74^{x}	113.91 ± 3.11	9	2.37	<0.05
Control Group	99.20 ± 5.20	98.64 ± 2.27	5	0.04	NS

x = Standard Error

NS = Not Significant (P >0.05)

1. Relative to the controls, the value of k increased with developing parasitaemia reaching a peak concurrently with parasitaemia, and falling off with the decrease in parasitaemia. The correlation between k and the degree of parasitaemia was significant ($p < 0.01$) (Figure 6.2).
2. The value of k also increased with spleen and liver weight and the correlation of k with these 2 body organ weights was again significant ($p < 0.01$) (Figure 6.2).
3. The control group in the experiment for the late stage of infection became infected and developed a low degree of parasitaemia (mean 0.78 parasites per erythrocyte) and consequently, a higher value for k was recorded than in the other control groups at the previous time intervals (Figure 6.1).

The results for the corrected phagocytic index (α) which is a measure of the phagocytic activity per unit weight of tissue (Chapter II, p. 32) are summarised in Table XXVII. The phagocytic activity showed some increase per unit weight of tissue at peak of infection but this was not significant.

The distribution of carbon in body organs during E. ovis infection

Sheep were necropsied 24 hours after carbon injection. The liver, lungs and spleen were visibly blackened due to the presence of carbon.

In the liver, carbon was present in association with the Kupffer cells lining the sinusoids. It was not possible to be invariably certain of the location of such deposits in relation to cell structure since the density of the carbon obscured underlying detail (Figure 6.3). The carbon in general was more dense in the periportal area (Figure 6.4). Some carbon was also observed in the centri-lobular veins (Figure 6.5). In late parasitaemia the Kupffer cells appeared to be more numerous, a finding which is supported by the Kupffer cell counts (Table XXV). Not all Kupffer cells

TABLE XXVII

Values for the phagocytic activity per unit of reticuloendothelial tissue (corrected phagocytic index α) during early, peak and late parasitaemia. (Exp.IV - group mean values.)

	Control	Infected		Number of sheep in each group	F	P
	Early parasitaemia (5 days post inoculation)					
Group No	B	A	F**			
	16.86	16.72	15.95	5	0.20	NS
	±1.00*	±0.20	±1.35			
	Peak parasitaemia (12 days post inoculation)					
Group No	D	C		5	2.13	NS
	17.42	20.09				
	±0.99	±1.54				
	Late parasitaemia (26 days post inoculation)					
Group No	F	E		5	-	-
	Became infected (see Early parasitaemia above).	16.40 ±1.14				

*Standard error

**Control group for late parasitaemia in early stage of natural infection

F = F ratio as calculated by analysis of variance

NS = Not significant ($p > 0.05$).

contained carbon.

In the spleen there were some carbon particles in the red pulp, but most was within macrophages surrounding lymphoid follicles (Figure 6.6). This was in contrast to the distribution of haemosiderin which was confined to the red pulp area (Figure 6.7). There was no carbon within the lymphoid follicles. Except for the greater size of lymphoid follicles in the infected sheep, there appeared to be no difference between groups in carbon distribution in this organ.

Carbon particles were infrequent in both lymph nodes and haemolymph nodes, but it was not possible in paraffin sections to determine their precise localisation in regard to structures or cells. In the kidneys, occasional carbon particles were lodged in the glomerular capillaries and the capillaries of the interstitial tissue.

The lungs were congested and large deposits of carbon were seen in the interstitial tissue probably within capillaries. The relationship of the carbon to structure however, was difficult to determine by light microscopy (Figure 6.8).

Carbon Concentration in Body Organs During E. ovis Infection Experiment IV

The concentrations of carbon in samples of tissue taken from kidney, lymph node, haemolymph node, spleen, liver and lung the day following administration, in relation to the degree of parasitaemia, are given in Table XXVIII. The data showed that:

1. Significant differences ($p < 0.05$) between control and experimentally infected sheep only occurred at day 13 post inoculation when the carbon concentration was lower in the liver, lung and lymph node of the infected group (Table XXVIII). A similar tendency occurred with splenic concentrations but this was not significant ($p < 0.10$).

TABLE XXVIII

Concentration of carbon (mgs per gram of body tissue) in various body tissues at early, peak and late stages of parasitaemia - Experiment IV (Group mean values).

Group No.	Early					Peak				Late
	Control	Infected		F	P	Control	Infected	F	P	Infected
	B	A	F**			D	C			E
Lung	2.75 ±0.65*	2.49 ±0.76	2.18 ±0.33	0.23	NS.	3.12 ±0.20	2.27 ±0.21	8.53	<0.05	2.50 ±0.23
Liver	2.01 ±0.21	1.59 ±0.29	1.32 ±0.15	1.89	NS.	2.02 ±0.23	1.28 ±0.08	9.49	<0.05	1.33 ±0.19
Spleen	1.45 ±0.02	0.92 ±0.15	1.05 ±0.15	2.24	NS.	1.75 ±0.17	1.10 ±0.19	6.25	NS.	0.94 ±0.13
Haemolymph node	0.59 ±0.44	0.92 ±1.07	0.26 ±0.20	0.25	NS.	1.57 ±0.52	1.38 ±0.91	0.03	NS.	0.58 ±0.65
Lymph node	0.45 ±0.10	0.38 ±0.14	0.18 ±0.02	0.69	NS.	0.48 ±0.07	0.19 ±0.03	13.84	<0.02	0.36 ±0.09
Kidney	0.37 ±0.17	0.22 ±0.03	0.18 ±0.04	2.64	NS.	0.29 ±0.01	0.35 ±0.02	5.38	NS.	0.22 ±0.06

*Standard error

**Control group for late parasitaemia in early stage of natural infection.

NS = Not significant p(>0.05)

F = F ratio by analysis of variance.

2. The carbon concentration in the liver was significantly correlated with the Kupffer cell count ($r = 0.37$, $n = 30$, $p < 0.05$) and negatively, but not significantly correlated with the rate of phagocytosis (k) ($r = -0.32$, $n = 30$, p = NS).

Total Carbon in Organs During E. ovis Infection - Experiment IV

The total amount of carbon in the liver, lung and spleen was calculated from the concentration per gm of tissue and the total organ weight, and is shown in Figure 6.9. This amount was compared with the total amount of carbon injected. From the figure it can be seen that:

1. Nearly all the carbon injected into the control sheep was recovered in the lungs, spleen and liver.
2. Both experimentally and naturally infected groups had less carbon than control groups in liver, spleen and lungs. The differences between infected and control groups, however, were not significant because of wide variations within groups.

Plasma Fibrinogen Levels, Total Leucocytes and Total Platelets During Carbon Clearance

In the pilot study designed to follow plasma fibrinogen levels during carbon clearance, the sample collected from Sheep A, 1.5 min. after carbon inoculation, clotted, despite mixing with EDTA. The clot, when removed from the sample was black with carbon, and centrifugation of the remainder of the sample revealed a carbon-free serum. The corresponding sample in Sheep B did not clot. Centrifugation of the remaining sequential blood samples showed the plasma to be markedly discoloured with carbon; the carbon concentration decreased over the 30 minute period. Because of this, protein estimation and therefore fibrinogen estimation by refractometry was not possible. However, heating of the plasma at 56°C for 3 minutes and centrifugation to precipitate and pack the fibrinogen (Kaneko and Smith, 1967) produced a carbon-free plasma (Figure 6.10).

The sequential leucocyte and platelet counts after carbon injection in the 2 sheep are shown in Figure 6.11. The depletion of leucocytes was due to an almost complete neutropaenia which slowly recovered over the 30 minute period. Although no platelet count was performed on Sheep A, smear examination revealed a depletion of platelets which appeared to follow a similar pattern to the platelet count of Sheep B. In Sheep B, the correlation between the platelet and leucocyte count was highly significant ($r = 0.90$, $n = 7$, $p < 0.01$).

DISCUSSION

The Kinetics of Carbon Clearance and Uptake by Body Organs in the Sheep

The use of colloidal carbon as a means of investigating the function of the reticuloendothelial system has been confined to laboratory mammals (Biozzi et al., 1953; Stiffel, 1958), the fowl, chick embryo and frog (Kent, 1966). The comparison between sheep and the other species for the phagocytic index (k), the corrected phagocytic index (α), and the ratio of body weight to the weight of the liver and spleen (W/Wls) is shown in Table XXIX. The overall results for the 3 parameters in the sheep most resemble those of the fowl. The α value, which is a measure of the phagocytic activity per unit weight of organ tissue (liver and spleen), is considerably higher than for the other mammals. The high α value for the frog and fowl was considered by Kent (1966) to be associated with low relative weight of the liver and spleen, which therefore suggested that the phagocytic efficiency of these 2 organs was very high. Even allowing for possible variation in the W/Wls ratio and the α index due to the effect of fleece weight on body weight, the data in Table XXIX would suggest that the phagocytic efficiency of the sheep liver and spleen was also very high.

In laboratory animals, fowl and frog, it has also been shown that vascular clearance is primarily a measure of

TABLE XXIX

THE RATE OF CARBON CLEARANCE (PHAGOCYTIC INDEX k) AND THE PHAGOCYTIC ACTIVITY PER UNIT OF RETICULOENDOTHELIAL TISSUE (CORRECTED PHAGOCYTIC INDEX α) IN VARIOUS ANIMALS FOLLOWING INTRAVENOUS INJECTION OF 8 MGS CARBON PER 100 GMS BODY WEIGHT. THE W/WLS RATIO IS ALSO SHOWN WHERE W IS THE LIVE BODY WEIGHT, AND WLS IS THE WEIGHT OF THE LIVER AND SPLEEN

Animal	k	α	W/WLS	Reference
Fowl	0.0562	17.1	43.0	Kent (1966)
Frog	0.0143	12.5	51.5	Kent (1966)
Rat	0.026	6.0	20.0	Biozzi et al. (1953)
Mouse	0.047	5.7	16.0	Stiffel (1958)
Guinea Pig	0.023	6.9	25.0	Stiffel (1958)
Rabbit	0.008	5.4	27.0	Stiffel (1958)
Sheep	0.05	17.1	46.5	(This Study)

hepatic reticuloendothelial cell clearance (Biozzi et al., 1953; Odeblad et al., 1955; Zilversmit et al., 1952; Di Luzio and Riggi, 1964; Munson et al., 1970) with 90% of injected carbon being recovered in the rat liver (Biozzi et al., 1953; Halpern et al., 1957), 75% in the fowl liver and 70 - 85% in the frog liver (Kent, 1966). However in normal sheep in this study only about 45% of the carbon recovered was in the liver, with a high percentage also being recovered in the lung (Figure 6.9). It is clear that the interpretation of carbon clearance rates is not entirely straightforward. In the initial work on laboratory animals using carbon particles as a measure of reticuloendothelial activity, a considerable amount of carbon was retained in the lungs, kidneys and other organs, particularly where a dose rate greater than 16 mg/100 gm body weight was used (Biozzi et al., 1951; Halpern et al., 1953). This was accompanied by a depletion of plasma fibrinogen (Biozzi et al., 1951), the liberation of a large quantity of thromboplastin (Halpern et al., 1953) and was associated with flocculation of carbon, which then lodged in the capillaries of the lungs, kidneys and other organs. The carbon was trapped in fibrin deposits (Halpern et al., 1953). The shellac preservative in the ink was found to be the cause of this phenomenon, and development of a shellac-free carbon suspension (C 11/1431a), with a fish glue stabilizer and 1 or 2% neutralized gelatin as a diluent was claimed to overcome the problem of flocculation (Biozzi et al., 1953; Halpern et al., 1953). The role of gelatin has been the subject of study since it has been shown that serum opsonins play an important role in facilitating the phagocytosis of inert particles such as carbon (Jenkin and Rowley, 1961; Murray, 1963; Normann and Benditt, 1965a, b; Saba and Di Luzio, 1969; Jeunet and Good, 1967; Pisano et al., 1968). Gelatin can affect phagocytic rates (Murray, 1963; Normann and Benditt, 1965a; Kampschmidt et al., 1965; Koenig et al., 1965) by interaction with the plasma opsonin system (Filkins and Di Luzio, 1966a). At low concentrations of gelatin an increased removal rate has been found, suggesting that gelatin bound to carbon has a high affinity for the plasma opsonins while

at high gelatin concentrations, carbon removal was inhibited probably due to the competition of free gelatin for plasma opsonin (Filkins and Di Luzio, 1966b). In spite of earlier claims (see above), gelatin has also been shown to affect the stability of carbon in circulation, causing some aggregation accompanied by a transient fall in plasma fibrinogen level (Murray, 1963; Gabrielli et al., 1967).

In preliminary experiments on sheep, using gelatin diluent, the carbon clearance rate was extremely rapid, the animals showed respiratory distress and in some cases died. At necropsy the lungs were grossly blackened with carbon. It was concluded that these events were associated with the gelatin content of the diluent, and, thereafter, physiological saline was used. Although the clinical effect was less severe than with gelatin, large amounts of carbon still lodged in the lung capillaries (Figures 6.8 and 6.9). It has been found that "in vitro" experiments that, with saline diluted carbon, aggregation of carbon particles occurs, accompanied by a fall in fibrinogen level (Gabrielli et al., 1967). The results of the pilot experiment (Figure 6.10) carried out suggest that this could occur in the sheep though the matter needs further investigation. The transient fall in platelet and leucocyte counts which was observed (Figure 6.11) is of interest. Although other workers have not commented on leucopenia associated with carbon clearance experiments, falls in platelet counts have been recorded. There is, to make matters more complicated, evidence that the attachment of carbon particles to platelets may be a major factor in determining clearance rates (Van Aken et al., 1968; Donald and Pound, 1971; Donald, 1972a, b).

The concentration of carbon in the lymph nodes, haemolymph nodes and kidneys was variable but low (Table XXVIII). In the kidney particularly, the histopathology indicated that carbon is lodged in capillaries, rather than ingested by, or attached to cells. It appears therefore, that under the conditions of this experiment the kinetics of carbon clearance from the circulation depended not only on

reticuloendothelial clearance activity but on plasma constituent-particle interaction with the deposition of aggregates in small blood vessels.

The Effect of E. ovis on Carbon Clearance by the Reticuloendothelial System

Increased reticuloendothelial activity as measured by carbon clearance occurred in E. ovis infected sheep. This increase, as compared with controls, commenced early in infection (5 days post-inoculation - Figure 6.1), and was significant ($p < 0.01$) at peak parasitaemia. In the late parasitaemic stage (26 days) the clearance rate of infected sheep had nearly returned to normal. The enhanced phagocytic activity which was similar to that found in mice infected with E. coccoides (Gledhill et al., 1965) was accompanied by an increase in both spleen and liver weight; the weight of these organs remained high at late parasitaemia, while phagocytic activity declined.

At peak parasitaemia, carbon concentration in both organs and the Kupffer cell counts in the liver had decreased. Proportionately the spleen increased more in weight than the liver; this increase appeared due principally to lymphoid hyperplasia and since no carbon was found in the lymphoid nodules a decrease in overall carbon concentration would be expected and, in fact, occurred. In spite of an increase in liver weight in infected animals relative to controls of about 35% there were no obvious differences in liver dimensions. Although these were not measured they would, on theoretical grounds, have been relatively small. From the decrease in Kupffer cell counts and carbon concentration, it seems reasonable to conclude that the increase in weight was due to an increase in parenchymal tissue or fluid content. This could not be assessed in paraffin sections. It would also appear that little or no proliferation of Kupffer cells had occurred at peak parasitaemia (day 12) and that the increase in clearance rate was probably attributable to increased phagocytosis by existing cells.

At late parasitaemia (day 26) however there was evidence suggestive of a modest proliferation of Kupffer cells; liver weights remained high whilst cell counts rose to, or exceeded, control levels.

In relating these changes in the reticuloendothelial system to E. ovis infection, it must first of all be assumed that the increase in carbon clearance rate noted in infected sheep was the result of increased phagocytosis. The possibility of other causes associated with changes in plasma constitution, which leads to particle-constituent interaction unrelated to phagocytic activity cannot be entirely ignored. Increased carbon clearance usually results from an increase in non-specific serum factors or opsonins (Jenkin and Rowley, 1961). While reflecting an increase in reticuloendothelial activity, it gives no indication as to the stimulating mechanisms which bring this about. Discussion on these mechanisms can therefore only be of a speculative nature. The increased phagocytic activity of the reticuloendothelial system to carbon during E. ovis infection is also likely to be directed against E. ovis itself and parasitized erythrocytes. This hypothesis is supported by the observation that activity was greatest at peak parasitaemia and as such could be associated with the subsequent decline in the degree of parasitaemia. Although increased phagocytosis to carbon is clearly a non-specific phenomenon it could be augmented in E. ovis clearance by specific opsonizing antibodies against the organism or even the erythrocyte. On theoretical grounds a micro-organism such as E. ovis would be expected to stimulate an immune response. The reaction of antibody with the organism is likely to make it more susceptible to phagocytosis and possibly to lysis. The close association of E. ovis to erythrocytes may also lead to involvement of the erythrocyte in this immune reaction although an immune reaction developed against a damaged erythrocyte cannot be ruled out. A positive Coombs test has been demonstrated in E. ovis infection (Sheriff, 1967; Sheriff and Geering, 1969) and the pattern of anaemia development in experimental infection is suggestive of an immune reaction which involves

the erythrocyte (see Chapter III). Histological evidence available would suggest that the majority of erythrocytes prematurely removed are lysed in circulation (Chapter V). It is likely therefore that complement may play an important role in haemolysis following the antibody-antigen reaction. Complement fixing antibodies have been demonstrated in acute infection (Kreier and Ristic, 1963) but because of possible contamination of the antigen preparation, some doubt as to the validity of this result remains. Whether or not the postulated immune reaction associated with haemolysis plays any direct or indirect part in immunity to infection is also a matter for speculation. It is quite possible that the same antibodies associated with haemolysis may also be specific opsonizing antibodies. It is also possible that the products of haemolysis may stimulate the mechanisms responsible for increased clearance of carbon.

Non-specific stimulation of the reticuloendothelial system occurs with a number of agents such as oestrogen, glucan, zymosan, and tubercle lipid (Kelly et al., 1962; Fred et al., 1970; Kelly and Dobson, 1971; Donald, 1972c; Donald and Pound, 1973). Stimulation in these cases is usually accompanied by proliferation of reticuloendothelial cells, whereas bacterial endotoxins, for example, only activate existing cells (Howard, 1959). Reticuloendothelial stimulation also occurs in malaria (Lucia and Nussenzweig, 1969; Elko and Cantrell, 1970; Cantrell et al., 1970; Sheagren et al., 1970), accompanied by an initial phase of Kupffer cell hypertrophy followed by hyperplasia (McCallum, 1969). It has also been found, however, that intravascular haemolysis, apart from stimulating the reticuloendothelial system, can cause cellular proliferation; the degree of proliferation being dependent on the severity of the haemolysis and the amount of cell debris (Jandl et al., 1965). The apparent difference in the degree of Kupffer cell proliferation between E. ovis and malaria may be a result of this. In malaria a large amount of cell debris and other material is formed as a result of the interaction between host and parasite (Sheagren et al., 1970) whereas

in E. ovis the haemolysis, although prolonged, does not appear on histological examination to generate much debris at all. In cases where large amounts of haemolysate are released into circulation, phagocytic function eventually becomes depressed (Okuyama and Ito, 1973); this is most likely due to a depletion of serum opsonins. The decrease in phagocytic activity at late parasitaemia could be a result of this, but it may also be due to a fall in the amount of cell debris and particulate antigen needing to be removed from circulation. This may be accompanied by a fall in non-specific opsonins.

Although the preceeding discussion is largely speculative, there is some support for the ideas put forward, from the literature on other blood parasites. In anaplasmosis (Mann and Ristic, 1963), malaria (Zuckerman, 1969) and babesiosis (Schroeder and Ristic, 1968a) immune mechanisms involving the erythrocyte have been reported, but the nature of the antibodies involved and the importance of them in the development of anaemia is unclear. However, opsonizing antibodies have been reported to be present in Plasmodium berghei infected rats (Cox et al., 1966) and opsonin titres have been recorded in anaplasmosis (Schroeder and Ristic, 1968b). In the latter report it was considered that opsonization of uninfected erythrocytes facilitated their removal from circulation and was responsible for the severe anaemia in anaplasma infection, even though the degree of parasitaemia was very small.

Arising out of the activation of the reticuloendothelial system during E. ovis infection is the possibility that the host response to other concurrent infections could be altered. There is evidence that mice infected with E. coccoides are more resistant to spp of Plasmodium (Peters, 1965; Ott et al., 1967; Voller and Bidwell, 1968; Cox, 1966; Seamer, 1967) and show an increased interferon-like response to some viruses (Suntharasamai and Rytel, 1973), and endotoxin (Glasgow et al., 1971). On the other hand, specific interferon production to some viruses is suppressed

(Glasgow et al., 1971) and infected animals may be more susceptible to certain viruses which multiply in reticuloendothelial cells (Niven et al., 1952; Gledhill et al., 1955; Seamer et al., 1961).

One of the disappointing aspects of this study on reticuloendothelial activity during E. ovis infection was the occurrence of carbon aggregations which were then deposited at such non-reticuloendothelial sites as the lung capillaries. It is therefore clear that this problem must be overcome, either by changing the test substance, or the diluent and stabilizers of the carbon particles used before further study of reticuloendothelial function in the sheep can be carried out.

SUMMARY

The phagocytic activity of the reticuloendothelial system was estimated in E. ovis infected and control sheep by measuring the intravascular clearance (k) of intravenously injected carbon. Considerable problems were encountered with this technique in sheep, where carbon aggregation occurred, leading to deposition of large amounts of carbon in non-reticuloendothelial sites, particularly the lung capillaries. Evidence is provided which suggests that there is an interaction between the carbon and some plasma substances the most notable of which is fibrinogen, leading to this aggregation. This phenomenon does not appear to occur to the same extent in laboratory animals using similar dose rates of carbon with similar diluents.

E. ovis infected sheep showed increased carbon clearance rates as compared with control sheep. This was small and non-significant at early parasitaemia (5 days post-inoculation), but was highly significant ($p < 0.01$) at peak parasitaemia (12 days). The clearance rate had nearly returned to normal at day 26. The increased clearance rate during parasitaemia was accompanied by increased liver and spleen weight.

It is postulated that the increased reticuloendothelial phagocytic activity, as measured by carbon clearance, may also be directed against E. ovis itself or parasitized erythrocytes. The increase in non-specific opsonins, which reflect increased carbon clearance may be augmented by specific opsonizing antibodies against the organism and/or the erythrocyte. In the latter instance mechanical damage to the erythrocyte by the parasite may possibly lead to antibodies directed against the erythrocyte. Intravascular haemolysis, which is a feature of E. ovis infection, may result from the participation of complement in the antibody-antigen reaction.

Non-specific stimulation of the reticuloendothelial

system could also have resulted from the haemolysis of erythrocytes, and it is suggested that the small proliferation of Kupffer cells which was noted at late parasitaemia, resulted from this.

Further studies of this nature in sheep should only be made when the problems encountered with carbon aggregation are overcome, either by using a different stabilizer and/or diluent, or by changing the test substance.

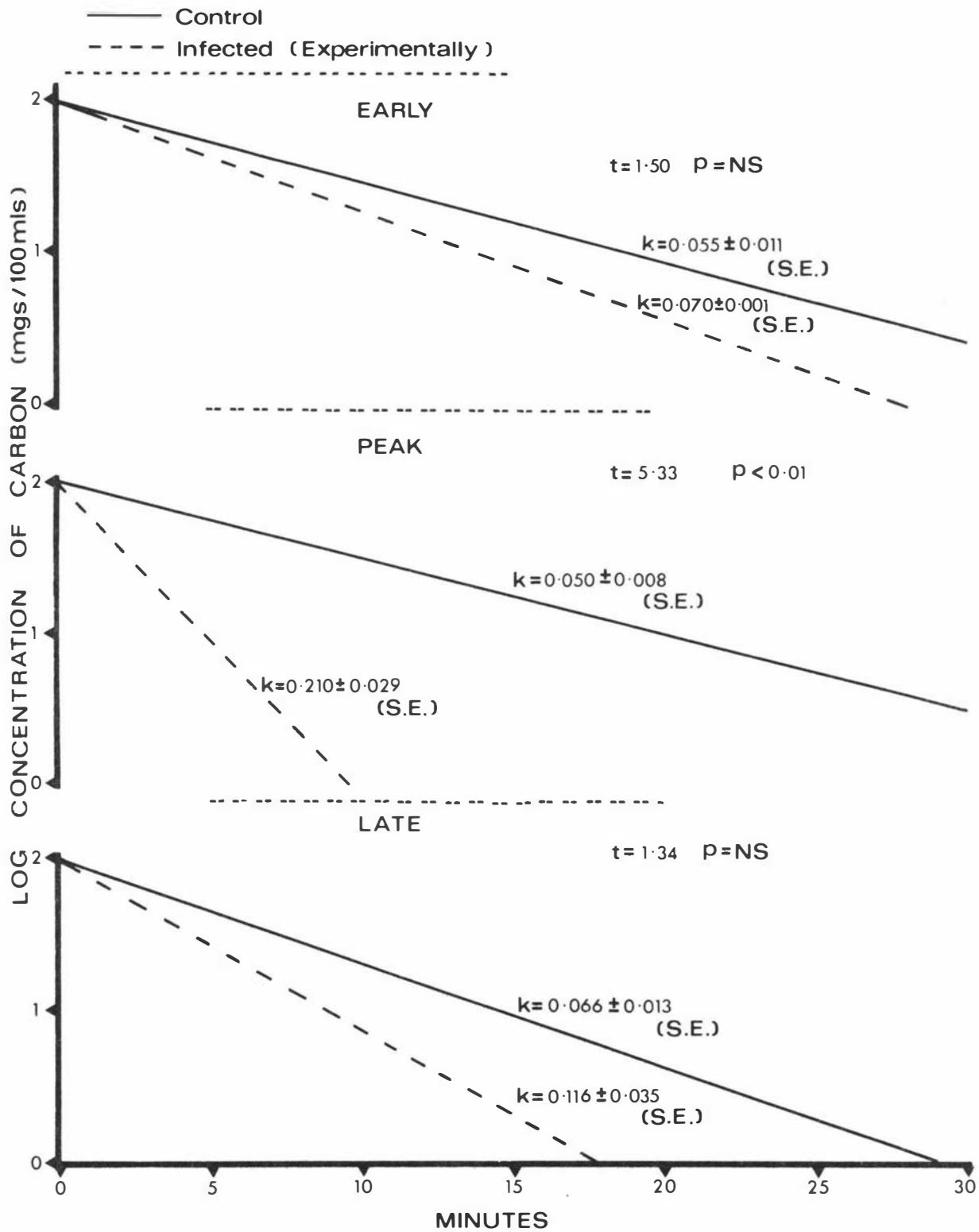


Figure 6.1. Phagocytic index (k) values of infected and control sheep during early, peak and late parasitaemia (Exp. IV — Group mean values)

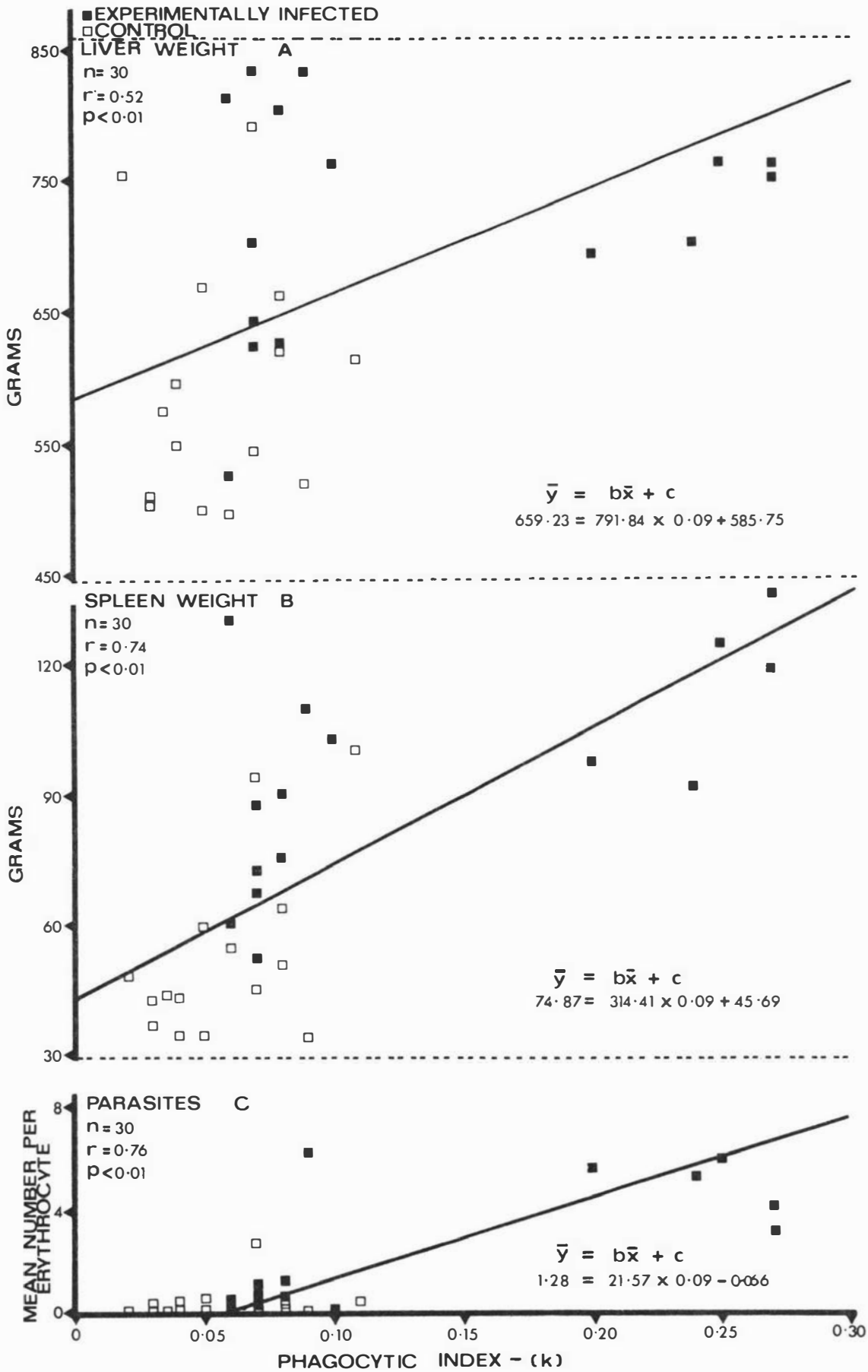


Figure 6.2. The correlation of phagocytic index in control and infected sheep with liver weight (A), spleen weight (B) and the degree of parasitaemia (C). (Exp. IV — Individual sheep values)

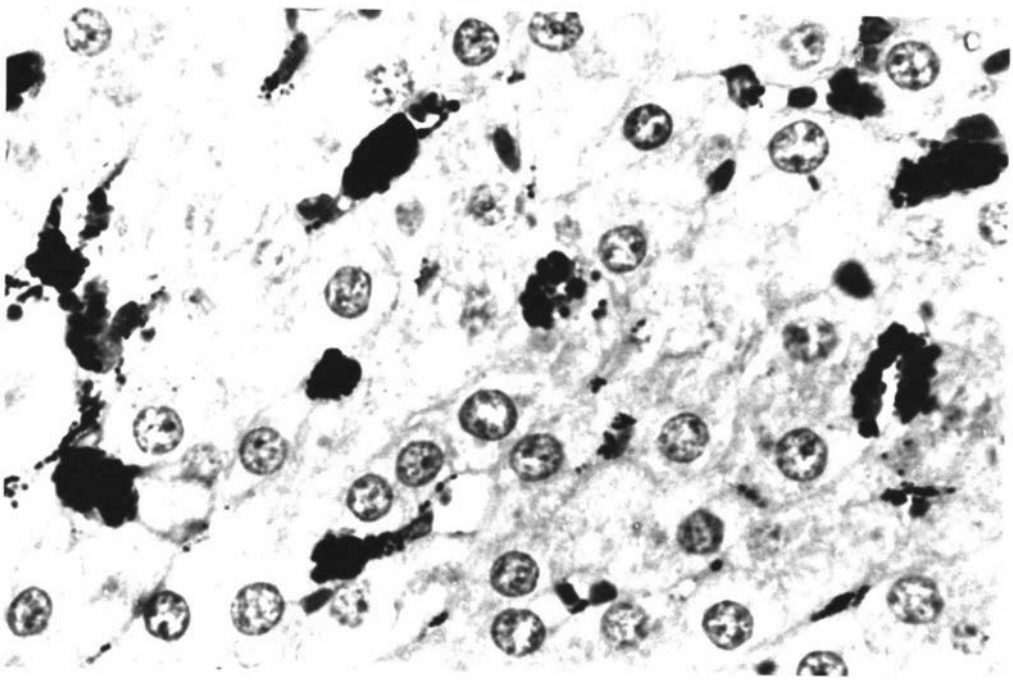


Figure 6.3: An area of sheep liver, 24 hours after intravenous administration of carbon and showing the relationship of carbon to Kupffer cells. (H & E x 400).

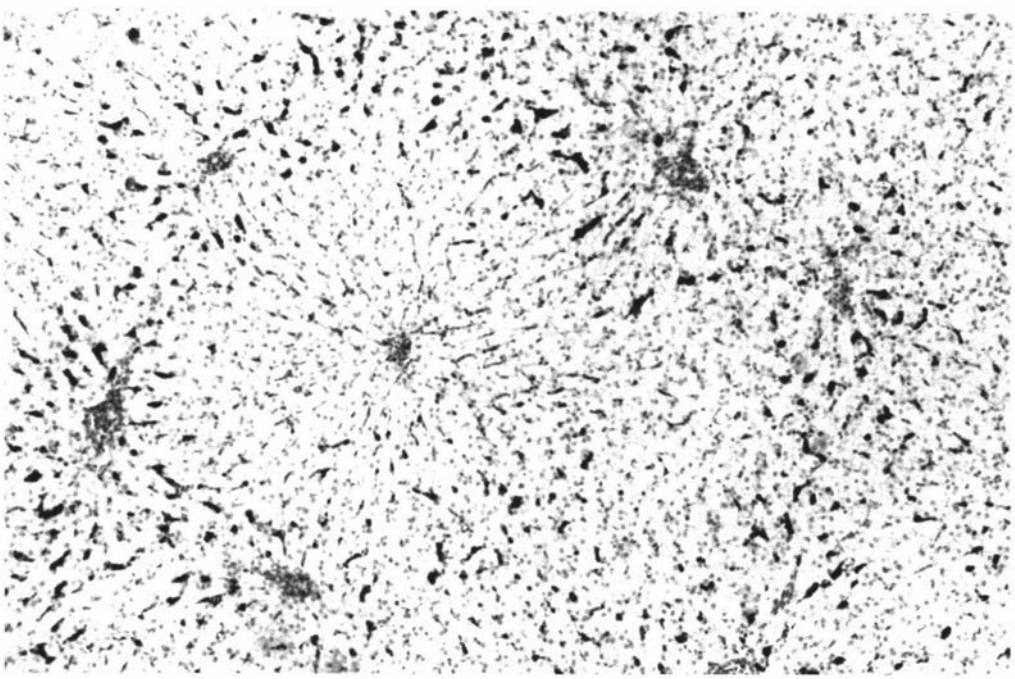


Figure 6.4: An area of sheep liver 24 hours after intravenous administration of carbon. The distribution of carbon particles is most dense in the periportal regions. (H & E x 100).

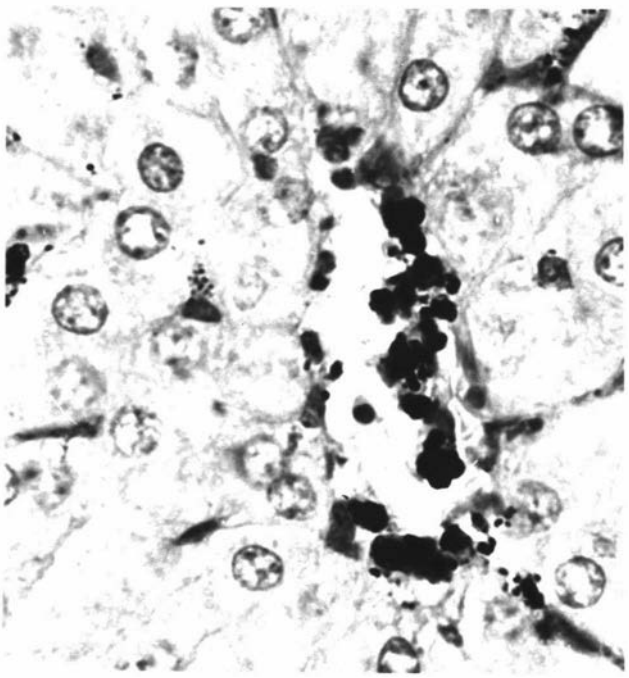


Figure 6.5: The centrilobular vein of a sheep liver, 24 hours after intravenous administration of carbon showing accumulations of carbon particles within the vein. (H & E x 400).

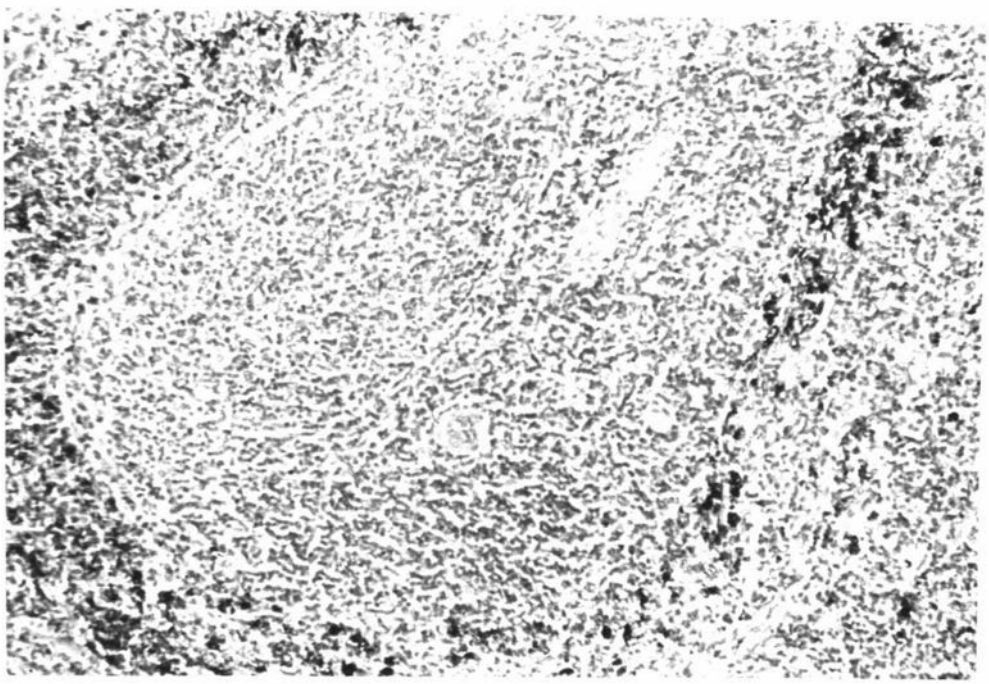


Figure 6.6: The distribution of carbon in the spleen of a sheep 24 hours after the intravenous administration of the carbon, Some carbon is in the red pulp but most is associated with macrophages which surround the lymphoid follicle. (H & E x 100).

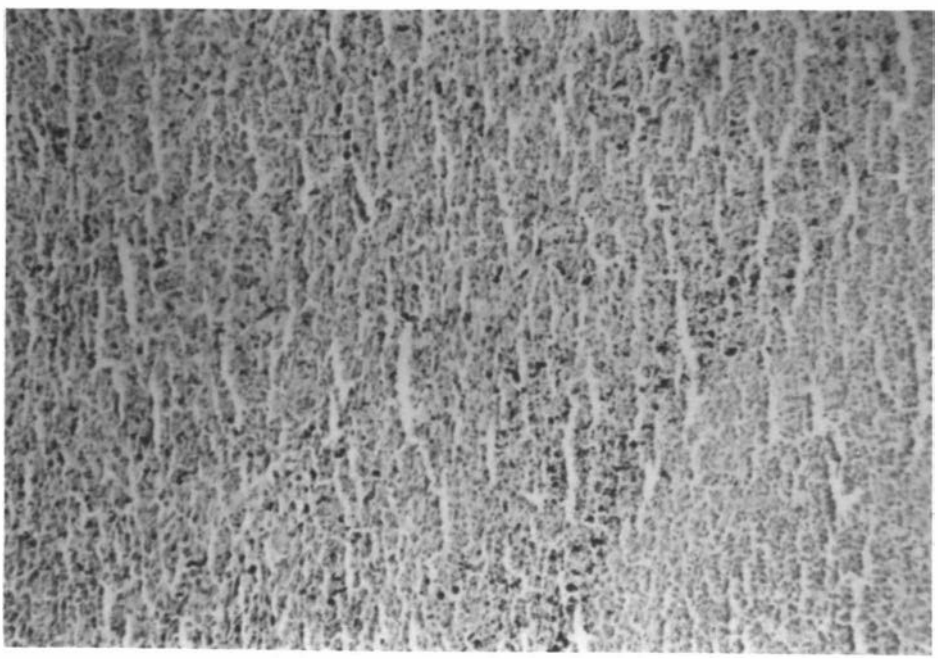


Figure 6.7: Comparison of distribution of haemosiderin and intravenously administered carbon 24 hours after carbon injection. The blue staining haemosiderin is confined to the red pulp area, whereas carbon is more evenly distributed with a large amount adjacent to the lymphoid follicle. (Perl's Prussian Blue x 100).

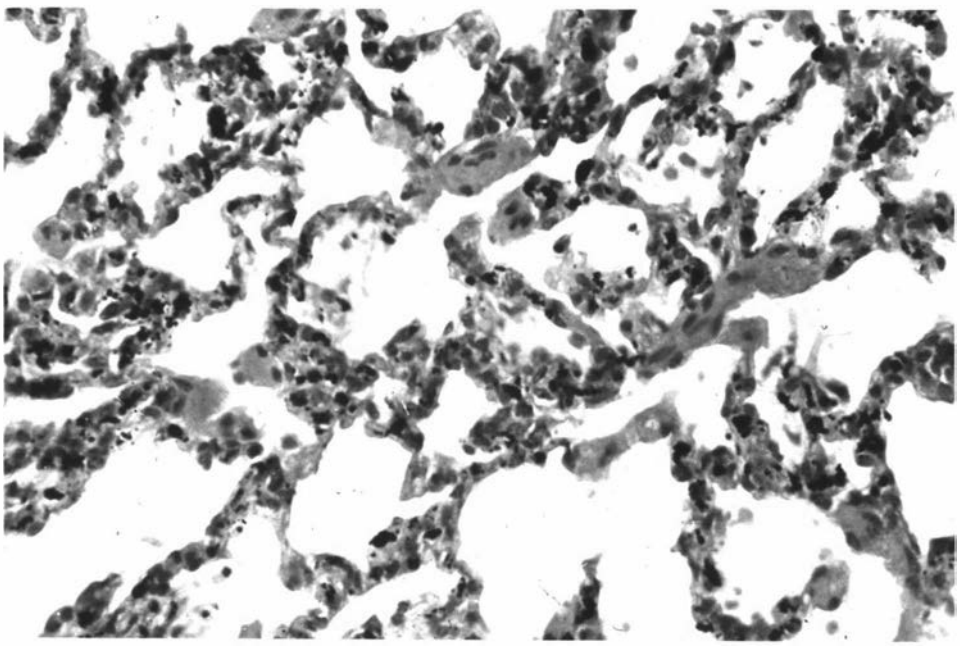


Figure 6.8: Lung of a sheep, 24 hours after the intravenous administration of carbon. Carbon is deposited in the interstitial tissue probably within capillaries but the relationship between carbon and lung structure is difficult to ascertain. (H & E x 250).

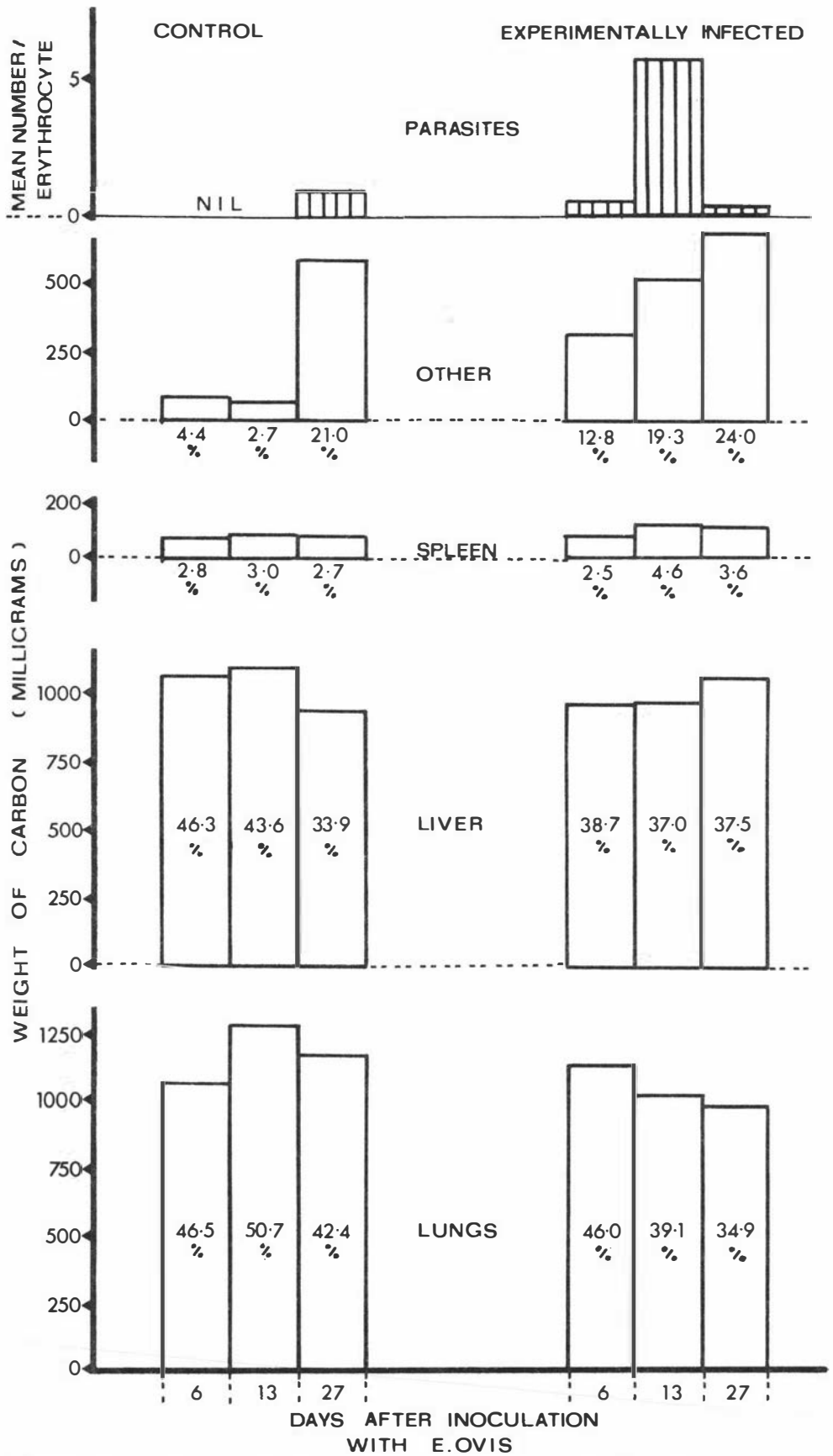


Figure 6.9. The weight of carbon recovered from the spleen, liver, lungs, and other tissues of infected and control sheep, following carbon injection at early peak and late stages of parasitaemia. Percentages of the total amount of carbon injected, recovered in each body tissue are indicated. (Exp. IV — Group mean values)

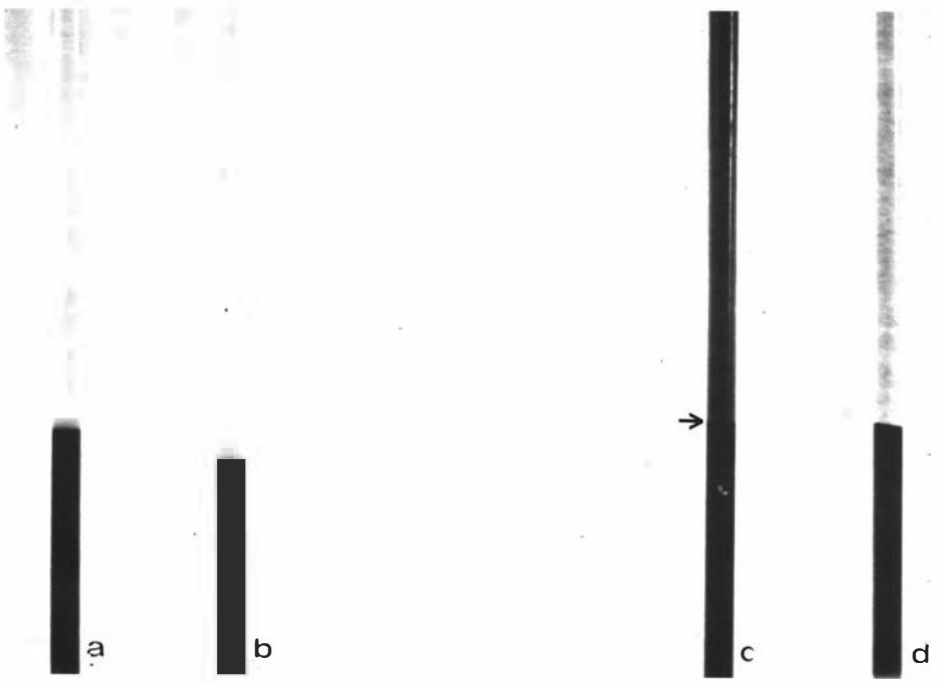


Figure 6.10: The interaction between carbon and fibrinogen in plasma.

(a) Packed erythrocytes with buffy coat in normal sheep blood.

(b) Packed fibrinogen, incorporated with the buffy coat after heating the plasma column for 3 min at 56°C and recentrifuging. (Normal sheep blood).

(c) Plasma containing a large amount of carbon following centrifugation of blood taken from a sheep 5 min after intravenous administration of carbon. The plasma and packed cell interphase is shown by (→).

(d) Sample (c) recentrifuged after heating of the plasma column for 3 min at 56°C. Most of the carbon has packed with the precipitated fibrinogen.

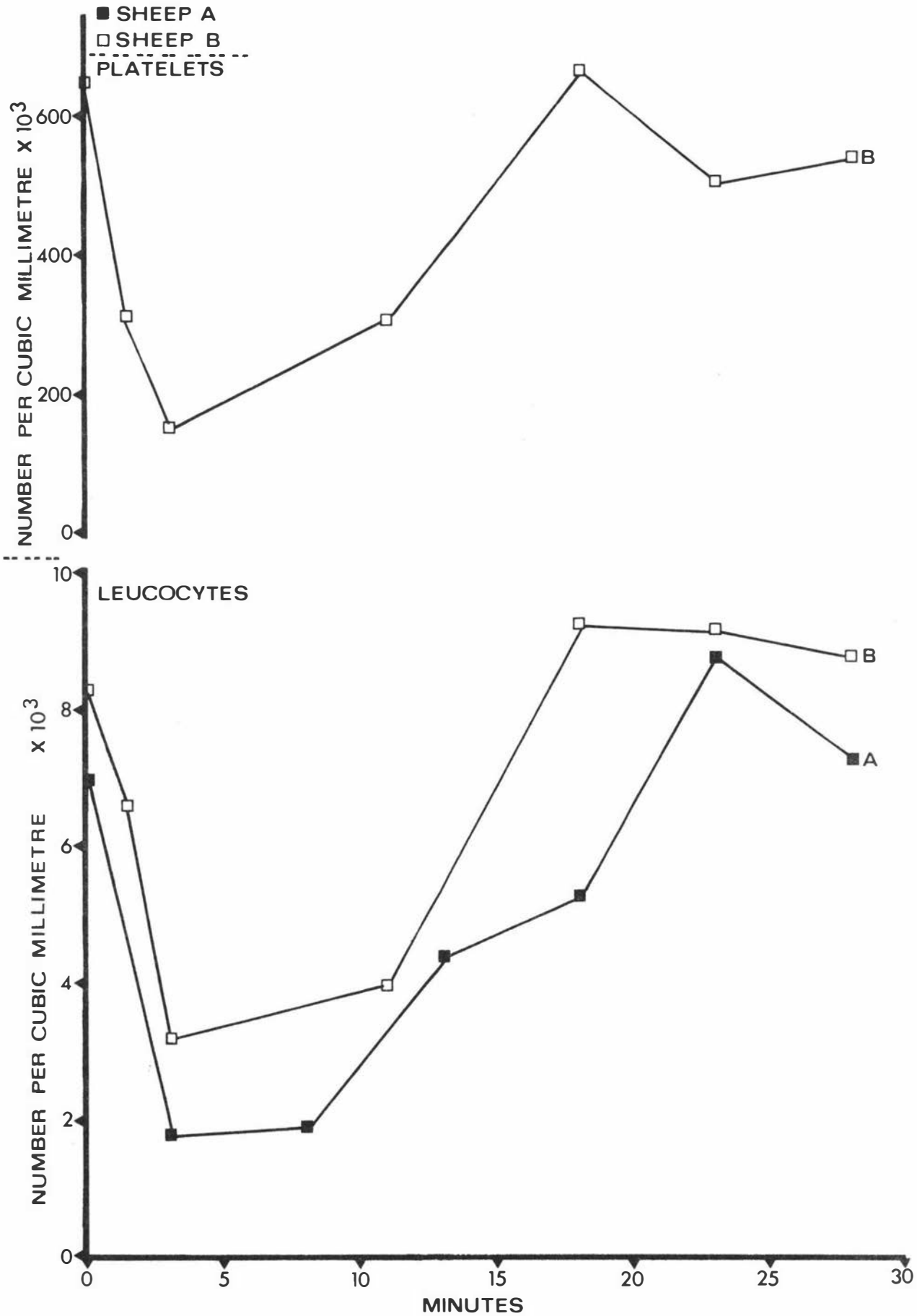


Figure 6.11. Total leucocyte and platelet counts of two sheep (A and B) following the intravenous injection of 8 mgs of carbon particles per 100 grams body weight

CHAPTER VII

THE EFFECT OF EPERYTHROZOON OVIS ON THE CARBOHYDRATE METABOLISM OF ERYTHROCYTES

INTRODUCTION

Since the first observation of Eperythrozoa (Schilling, 1928), reports on the biochemistry of the organism have been confined to histochemical studies (Kreier and Ristic, 1968). There have been no reports on the organism's metabolism, growth or energy requirements. Study of the metabolism of blood parasites has been mainly restricted to the Protozoa of the genera Plasmodium (Moulder, 1962) and Babesia (Rickard, 1969, 1970a, b); and the Rickettsiae which cause typhus fevers, spotted fevers, scrub typhus and Q fever (Moulder, 1962).

All of the aforementioned organisms are intracellular parasites and metabolic studies have been approached either by comparing the metabolism of non-infected and infected erythrocytes or by isolation of the parasite from the erythrocyte and studying its activity in artificial media. It has been frequently found that the freed Plasmodium and Babesia organisms are permeable to substances which are not able to enter intact erythrocytes and therefore are not available to the intracellular organism (Moulder, 1962; Rickard, 1970a). As a result it is possible that an organism isolated from its host cell could show an altered metabolism because it may not have the advantages of host cell metabolism, or because of damage in the isolation process. Although the Eperythrozoa are epicellular, they are attached to the erythrocyte and cause erosion of the erythrocyte surface (Kreier and Ristic, 1963) and, therefore, it might be expected that they depend on the erythrocyte for all or part of their energy and synthetic requirements. For these reasons, metabolic studies were made without attempting to separate the organism from the host erythrocyte.

The metabolic characteristics of the erythrocyte at various stages of maturation are listed in Table XXX. The 2 glycolytic pathways of carbohydrate metabolism, namely the anaerobic (Embden-Myerhoff) pathway and the aerobic (Pentose-Phosphate) pathway (Figure 7.1) form the main basis of mature erythrocyte metabolism. Most work on this aspect of erythrocyte metabolism has been confined to human cells and general reviews have been published (Prankerd, 1961; Bishop, 1964; Rapoport, 1968; Fornaini and Bossu, 1969; Harris and Kellermeyer, 1970). Glucose is the main monosaccharide utilized by erythrocytes with approximately 90% metabolized via the anaerobic pathway to form lactic acid as the major end product and 10% metabolized via the aerobic pathway (Murphy, 1960) (Figure 7.1). This glycolytic activity ensures the continued availability of energy and reductive potential necessary for maintaining the structure and function of the cell (Fornaini and Bossu, 1969).

Because glycolysis is the most important metabolic activity of the erythrocyte, a comparative study of the glycolytic activity of E. ovis infected and non-infected sheep erythrocytes was made (Part I). As reticulocytes have additional metabolic pathways (Table XXX) and are present in the later stages of E. ovis infection, a comparative study was also made of the glycolytic activity of non-infected erythrocytes and erythrocytes from non-infected anaemic sheep with a reticulocyte percentage equivalent to that found with E. ovis infection (Part II).

MATERIALS AND METHODS

The experimental procedures and biochemical assays were as described for Experiment V in Chapter II.

RESULTS

Glucose Utilization

The overall mean glucose utilization by the erythrocytes

TABLE XXX

METABOLIC CHARACTERISTICS OF THE ERYTHROCYTE AT VARIOUS STAGES OF ITS DEVELOPMENT (FROM HARRIS & KELLERMEYER, 1970)

	Nucleated cell	Reticulocyte	Adult cell
Replication	+	0	0
DNA Synthesis	+	0	0
RNA Synthesis	+	0	0
RNA Present	+	+	0
Lipid Synthesis	+	+	0
Heme Synthesis	+	+	0
Protein Synthesis	+	+	0
Cytochrome and Electron Transfer System	+	+	0
Carbohydrate Metabolism			
Tricarboxylic Acid (TCA) Cycle	+	+	0
Embden-Meyerhof Pathway	+	+	+
Pentose Phosphate Pathway	+	+	+
Maturation and/or Senescence	+	+	+

from the control sheep was 0.63±0.004 μ moles per ml of cells per hour. The time relationships between the glucose utilization, degree of parasitaemia and reticulocyte percentage for infected erythrocytes (Part I) and a similar relationship for non-infected erythrocytes between glucose utilization and reticulocyte percentage (Part II) are shown in Figure 7.2. The maximum mean glucose utilization by the erythrocytes from the infected and non-infected groups of anaemic sheep with the corresponding control values are shown in Tables XXXI and XXXII respectively. The results show that:

1. Glucose utilization by infected erythrocytes increased rapidly with the onset of parasitaemia and significant ($p < 0.05$) increases in utilization over that of the control cells occurred when the parasitaemia reached a mean level of 0.32 parasites per erythrocyte.
2. Maximum glucose utilization by infected erythrocytes corresponded with peak parasitaemia, and was approximately 24 times that of control erythrocytes (Figure 7.2 and Table XXXI).
3. Glucose utilization by non-infected erythrocytes from anaemic sheep was similar to that of control erythrocytes.

The increase in glucose utilization by infected erythrocytes was correlated significantly ($p < 0.01$) with both the degree of parasitaemia and reticulocyte percentage (Table XXXIII). However because of the relationship between parasitaemia and reticulocyte percentages ($n = 13$, $r = 0.65$, $p < 0.05$) and the relationship between glucose utilization and reticulocyte percentage in Part II (Figure 7.2 and Tables XXXII and XXXIII), it was evident that the increase in glucose utilization was due to the parasite, and not influenced by reticulocytosis.

Lactic Acid Production

The mean lactic acid production by erythrocytes from control sheep was 1.18±0.01 μ moles per ml of erythrocytes per hour. The relationships between the lactic acid production, degree of parasitaemia and reticulocyte percentage

TABLE XXXI

The metabolism of control and E.ovis infected erythrocytes at the time of maximum difference between readings. (Exp.V, Part I = Group mean values.)

	Control	Infected	t	P	Reticulocyte percentage	No. of parasites/ erythrocyte	Days after parasitaemia onset
Glucose utilization (u moles/ml erythrocytes/hour)	0.64 ±0.01*	15.52 ±0.88	16.99	<0.001	2.59	5.42	12
Lactic acid production (u moles/ml erythrocytes/hour)	1.19 ±0.06	21.91 ±5.69	3.65	<0.02	0.27	5.85	7
Glycolytic ration (percent)	91.94 ±3.09	61.88 ±5.05	5.10	<0.01	0.002	3.34	4
Pyruvic acid production (u moles/ml erythrocytes/hour)	0.16 ±0.01	0.80 ±0.05	11.46	<0.001	2.59	5.42	12
Pyruvic acid as a percentage of lactic acid	11.01 ±0.98	2.08 ±0.20	8.91	<0.01	5.17	5.20	19
Percent glucose accounted for as oxygen uptake, lactic and pyruvic acid	112.29 ±3.79	64.09 ±5.17	7.55	<0.001	0.002	3.34	4
Oxygen uptake (u litres/ml erythrocytes/hour)	7.39 ±0.83	20.86 ±1.50	7.90	<0.001	5.17	5.20	19
Glucose-6-phosphate dehydrogenase (u moles NADP reduced/min/ 3.2 gms Hb	0.82 ±0.06	1.82 ±0.18	5.27	<0.01	4.53	4.68	22

Number of sheep in each group = 6

*Standard error.

TABLE XXXII

THE METABOLISM OF CONTROL ERYTHROCYTES AND ERYTHROCYTES FROM ANAEMIC NON-INFECTED SHEEP AT THE TIME OF MAXIMUM DIFFERENCE BETWEEN READINGS (EXP V PART II - GROUP MEAN VALUES)

	Control	Anaemic	t	p	Reticulocyte Percentage in anaemic blood
Glucose Utilization (μ moles/ml erythrocytes/hour)	0.59±0.02[x]	0.71±0.03	3.66	<0.05	1.50
Lactic Acid Production (μ moles/ml erythrocytes/hour)	1.06±0.02	1.29±0.06	3.39	<0.05	1.50
Glycolytic Ratio (Percent)	94.06±4.53	82.72±4.90	1.70	NS	0.13
Pyruvic Acid Production (μ moles/ml erythrocytes/hour)	0.13±0.01	0.18±0.01	3.06	NS	0.98
Pyruvic Acid as a Percentage of Lactic Acid	14.68±0.60	10.98±0.47	4.83	<0.02	1.50
Percent glucose accounted for as Oxygen Uptake, Lactic and Pyruvic Acid	111.50±1.67	138.39±2.19	9.91	<0.001	3.53
Oxygen Uptake (μ litres/ml erythrocytes/hr)	8.88±0.77	24.00±2.28	6.30	<0.01	3.53
Glucose-6-Phosphate Dehydrogenase (μ moles NADP reduced/min/ 3.2 gms Hb)	0.88±0.11	1.89±0.15	5.33	<0.05	0.98

Number of sheep in each group = 4
x = Standard Error
NS = Not Significant (P > 0.05)

TABLE XXXIII

THE CORRELATIONS BETWEEN ERYTHROCYTE METABOLISM AND THE DEGREE OF PARASITAEMIA AND RETICULOCYTE PERCENTAGE (PART I) AND IN UNINFECTED ERYTHROCYTES BETWEEN ERYTHROCYTE METABOLISM AND RETICULOCYTE PERCENTAGE ONLY (PART II) - EXPERIMENT V

PART I - Number of Readings = 13

	Parasitaemia		Reticulocyte Percentage	
	r	p	r	p
Glucose Utilization	0.99	< 0.01	0.69	< 0.01
Lactic Acid Production	0.99	< 0.01	0.71	< 0.01
Glycolytic Ratio	-0.56	< 0.05	-0.21	NS
Pyruvic Acid Production	0.89	< 0.01	0.58	< 0.05
Oxygen Uptake	0.74	< 0.01	0.95	< 0.01
Percent Glucose as O_2 Lactic & Pyruvic Acid	-0.69	< 0.01	-0.35	NS
Pyruvic Acid : Lactic Acid	-0.85	< 0.01	-0.59	< 0.05
Glucose-6-Phosphate Dehydrogenase	0.76	< 0.01	0.87	< 0.01

Correlation between Parasitaemia and Reticulocyte Percentage $r = 0.65$ $p < 0.05$

PART II - Number of Readings = 10

	Reticulocyte Percentage	
	r	p
Glucose Utilization	0.45	NS
Lactic Acid Production	0.57	NS
Glycolytic Ratio	0.41	NS
Pyruvic Acid Production	0.19	NS
Oxygen Uptake	0.90	< 0.01
Percent Glucose as O_2 Lactic & Pyruvic Acid	0.73	< 0.05
Pyruvic Acid : Lactic Acid	-0.41	NS
Glucose-6-Phosphate Dehydrogenase	0.21	NS

r = correlation coefficient

NS = Not significant ($p > 0.05$)

(Part II) are shown in Figure 7.3. The maximum mean values obtained from the erythrocytes of the infected and non-infected groups of anaemic sheep with the corresponding control values are shown in Tables XXXI and XXXII respectively. From the figure and tables it can be seen that the pattern of lactic acid production in infected and non-infected erythrocytes was similar to that of the glucose utilization. Peak lactic acid production by infected erythrocytes occurred at the peak of parasitaemia and was approximately 18 times that of control erythrocytes. Similarly, it was also apparent that the increase in lactic acid production was due to the presence of the parasite rather than the presence of reticulocytes (Figure 7.3 and Tables XXXII and XXXIII).

Glycolytic Ratio (Percentage Lactic Acid/Glucose)(1)

The mean glycolytic ratio, i.e. the percentage of glucose utilization accounted for as lactic acid, for the erythrocytes of control sheep was 92.96±0.68%. The time relationships as described for glucose utilization and lactic acid production are shown in Figure 7.4 and the values at maximum group mean difference in Parts I and II are shown in Tables XXXI and XXXII respectively.

The glycolytic ratio for infected erythrocytes fell with the onset of parasitaemia to a minimum level of 61.88% (Table XXXI). This fall was due to the presence of parasites rather than reticulocytes (Figure 7.4, Tables XXXII and XXXIII).

Pyruvic Acid Production

The mean pyruvic acid production by erythrocytes from control sheep was 0.145±0.003 μ moles per ml of erythrocytes per hour. As for lactic acid production, the level of pyruvic acid produced by infected erythrocytes (Figure 7.5),

(1) Calculated on the basis that if 2 moles of lactic acid are produced from 1 mole of glucose, the glycolytic ratio is 100%.

increased with parasitaemia reaching a maximum group mean level at the peak of parasitaemia. This was approximately 5 times the amount of pyruvic acid produced by the control group erythrocytes. This large increase did not occur with the erythrocytes from the anaemic non-infected sheep (Figure 7.5).

Pyruvic Acid : Lactic Acid Ratio

The mean percentage ratio of pyruvic acid to lactic acid for the erythrocytes of the control sheep was 12.52±0.24. With the onset of parasitaemia the infected erythrocytes showed a marked fall in this ratio (Figure 7.6) to a level of approximately one-fifth that of the controls (Table XXXI). As can be seen in Figure 7.6, this low level persisted during the course of parasitaemia. As with the other biochemical parameters previously described, the level was not influenced to any degree by the percentage of reticulocytes present (Figure 7.6, Tables XXXII and XXXIII).

Oxygen Uptake

The mean rate of oxygen uptake by erythrocytes from control sheep during the 2 hour incubation period was 8.16±0.19 μ litres per ml of erythrocytes per hour. Although there was a steady rate of uptake over the 2 hour incubation period, less oxygen was utilized in the second hour (Table XXXIV). The time relationships as described for the biochemical parameters are shown in Figure 7.7 and the maximum levels in Part I and II are shown in Tables XXXI and XXXII respectively. The data show that:

1. Oxygen uptake increased in both *E. ovis* infected erythrocytes and in erythrocytes from anaemic non-infected sheep.
2. The increase in the infected erythrocytes reached a maximum mean level, approximately 2.5 times the control mean level, and in the non-infected erythrocytes from anaemic sheep a maximum mean level approximately 3 times the control mean level (Tables XXXI and XXXII).

TABLE XXXIV

COMPARISON BETWEEN THE FIRST HOUR AND THE SECOND HOUR IN THE OXYGEN UPTAKE OF CONTROL ERYTHROCYTES FOR A TWO HOUR INCUBATION PERIOD (EXP' V PART I - GROUP MEAN VALUES)

	Oxygen Uptake			
Day	Hour 1	Hour 2	t	P
0	8.41±1.96[x]	4.58±1.94	1.39	NS
1	10.22±1.41	5.27±0.97	2.91	<0.05
2	8.82±1.93	8.20±1.52	0.26	NS
5	10.17±1.32	4.07±0.61	4.22	<0.01
7	11.30±2.26	7.08±1.51	1.56	NS
9	11.13±1.17	4.04±1.23	4.18	<0.01
12	10.91±0.82	6.31±1.02	3.52	<0.02
14	10.58±1.14	7.04±0.60	2.75	<0.05
17	7.48±1.18	6.99±1.19	0.29	NS
20	11.23±1.59	5.63±1.01	2.97	<0.05
22	11.32±0.62	3.83±0.62	8.53	<0.001
24	12.25±1.73	2.75±0.89	4.90	<0.01
27	12.14±1.98	6.03±1.65	2.38	NS

Number of Sheep in each group = 6

x = Standard Error

NS = Not significant ($p > 0.05$)

3. The increase was correlated significantly with both reticulocyte percentage and parasitaemia (Table XXXIII), but it is apparent that the increase is almost entirely due to the presence of reticulocytes (Figure 7.7, Table XXXII).

The mean rates of oxygen uptake measured at 10 minute intervals over the 2 hour incubation period at 3 different stages of E. ovis infection are shown in Figure 7.8. Erythrocytes taken from animals as peak parasitaemia was reached, and before the reticulocyte response was marked, released gas (presumably oxygen) over the first hour's incubation. This was reversed during the second hour. At the later stage of parasitaemia, when the reticulocyte percentage was high, egress of oxygen from the infected erythrocytes was not apparent, and oxygen uptake proceeded at a steady, though higher than normal, rate.

Percentage of Glucose Accounted for as Oxygen, Lactic Acid and Pyruvic Acid

The total amount of glucose utilized, that could be accounted for as oxygen uptake, lactic acid and pyruvic acid production in the control erythrocytes was 113.98±0.81%. With the onset of infection (Part I) the percentage of glucose utilized in this manner decreased (Figure 7.9), reaching a minimum level in the early parasitaemic stage of approximately half that of the control level (Table XXXI). In Part II the percentage of glucose utilized increased (Figure 7.9) because of the higher oxygen uptake, while the lactic and pyruvic acid level remained virtually unchanged (Figures 7.3 and 7.5). The peak percentage obtained for Part II was nearly 25% higher than the controls (Table XXXII).

Glucose-6-Phosphate Dehydrogenase (G6PD)

The mean G6PD level for the erythrocytes of the control sheep was 0.883±0.029 μ moles NADP reduced per minute per 3.2 grams Hb. The time relationships as for the other

erythrocyte biochemical parameters are shown in Figure 7.10. In Part I significant increases of G6PD in the infected erythrocytes over the controls only occurred towards the end of the experiment at the late parasitaemic stage. The maximum level was approximately 2.5 times that of the control level (Table XXXI) and although this increase was significantly correlated with both the degree of parasitaemia and reticulocyte percentage (Table XXXIII) it appeared to be more closely associated with the latter.

In Part II the level of G6PD for the erythrocytes from the non-infected sheep increased irregularly (Figure 7.10). This increase did not correlate significantly with reticulocyte percentage (Table XXXIII) although the maximum level finally reached was approximately 2.25 times the value obtained for the control erythrocytes.

DISCUSSION

Carbohydrate Metabolism of Normal Sheep Erythrocytes

The results obtained in this study on the glycolytic activity of normal sheep erythrocytes are similar to those obtained by Leng and Annison (1962) (Table XXXV). In comparison with other mammalian species Somogyi (1933) found that the glycolytic activity of sheep blood was the lowest of those examined (Table XXXVI). One of the problems in examining such activity in erythrocytes is the high metabolic activity of leucocytes which must be taken into account if they are present (Bishop, 1964). Because Leng and Annison (1962) had difficulty in preparing sheep erythrocyte suspensions free of leucocytes, their results were based on whole blood readings rather than erythrocytes alone. In this study a similar difficulty was initially experienced but, by carefully removing the upper layers of erythrocytes, the leucocyte count was reduced to less than 500 cells per cubic mm. No correction was made for the leucocytes present, and as the counts were similar in both control and treated incubates, any differences in the biochemical parameters measured

TABLE XXXV

RESULTS OF GLYCOLYTIC ACTIVITY OF SHEEP BLOOD OBTAINED IN THIS INVESTIGATION AND BY LENG AND ANNISON (1962) (MEAN VALUES)

	This Study (Erythrocytes only)	Leng and Annison (Whole Blood)
Glucose Utilization μ moles/ml cells/hour	0.63	0.69
Lactic Acid Production μ moles/ml cells/hour	1.18	1.62
Oxygen Uptake μ litres/ml cells/hour	8.16	10.9

TABLE XXXVI

RELATIVE GLYCOLYTIC RATES OF MAMMALIAN ERYTHROCYTES (FROM SOMOGYI, 1933)

Species	Hourly Rate of Glycolysis mgs/100 ml cells
Rabbit	28
Guinea Pig	23
Dog	22
Cat	21
Monkey	17
Man	16-18
Calf	4.5
Ox	4.5
Pig	3.5
Sheep	2.5

were considered not to be due to leucocytes.

Under aerobic conditions, lactic acid was the main product of glucose metabolism, and the glycolytic ratio was close to 100% (Figure 7.4). This indicates that glucose utilization is predominantly anaerobic and proceeds via the Embden-Myerhoff glycolytic pathway (Prankerd, 1961). This is similar to the situation in erythrocytes of other mammals (Barron and Harrop, 1928; Bird, 1947; Bartlett and Marlow, 1953; Bernstein, 1959; Rivkin and Simon, 1965; Rickard, 1969).

The percentage of glucose accounted for as oxygen uptake and lactic and pyruvic acid production was greater than 100% under aerobic conditions (Figure 7.9). This was similar to the results obtained by Rickard (1969) with rat erythrocytes. The sheep erythrocyte contains little or no stored glucose (Somogyi, 1933; Reid, 1953; Leng and Annison, 1962), so it follows from the results that there must be some additional source of lactic acid and/or pyruvic acid, other than the glucose added to the incubation medium. One possibility is the depletion of glycolytic pathway intermediates, particularly 2-3, diphosphoglycerate (2-3 DPG) present in the erythrocyte. It is generally accepted that 2-3 DPG is an important regulator of oxygen dissociation at constant pH and temperature (Rørth, 1970). A depletion of erythrocyte 2-3 DPG, while not likely to occur as a result of insufficient glucose to meet the demands of erythrocyte metabolism, may result from a lowering of intracellular pH due to the *in vitro* glycolytic activity of the cell (Astrup, 1970). It has been shown that other mammalian erythrocytes incubated without added glucose continue to take up oxygen and produce lactic acid and pyruvic acid for some hours (Nossal, 1948; Sherwood-Jones *et al.*, 1953; Brandt and Rapoport, 1959; Rickard, 1969). It was suggested by Brandt and Rapoport (1959) that endogenous glycine and possibly other amino-acids provide part of the endogenous respiration of erythrocytes, and it has been shown that purine ribosides such as inosine are converted to lactic acid by erythrocytes

(Gabrio et al., 1956; Rubinstein and Denstedt, 1956; Jaffe et al., 1957; Lowy et al., 1958; Jaffe, 1959).

It is therefore apparent from the percentage of glucose accounted for, that glucose is little used for synthetic purposes in normal erythrocytes (Leng and Annison, 1962), but mainly for the production of energy, which is the principal requirement of the erythrocyte (Prankerd, 1961; Bishop, 1964; Fornaini and Bossu, 1969; Beutler, 1969).

The pyruvic acid to lactic acid ratio of 12.52 obtained for normal sheep erythrocytes was much higher than normally found in human erythrocytes (Kosower et al., 1963) but similar to that found in rat erythrocytes by Rickard (1969). Rickard suggested that the high ratio in rat erythrocytes was due to a high normal activity of NADH-dependent methaemoglobin diaphorase. Of the 2 enzymes concerned in methaemoglobin reduction (NADH-dependent diaphorase, and NADPH-dependent reductase), the diaphorase is the more important physiologically (Gibson, 1948; Scott et al., 1965; Tonz, 1968). In vitro, this can be maintained by dehydrogenation of lactic acid, mediated by lactic dehydrogenase (Tonz, 1968). Because it appears that increased susceptibility to methaemoglobinaemia in ruminants is offset by an increased ability to reduce methaemoglobin (Smith and Beutler, 1966), it is conceivable that in sheep a high normal level of NADH is required to maintain haemoglobin in the reduced state.

The oxygen utilization of 8.16 μ litres per ml erythrocytes per hour was consistent with the observations of Leng and Annison (1962) who estimated that about 15% of the glucose metabolism by sheep erythrocytes enters the aerobic pentose phosphate pathway. The smaller amount of oxygen taken up in the second hour as compared with the first hour was possibly due to an accumulation of an energy reserve during the initial collection and preparation period when the cells were kept at 4^{o}C (Sirs, 1963). Cell metabolism is slower at that temperature (Prankerd, 1961), and it is assumed that the low temperature prevents the utilization of energy, and for

a time ATP and other energy sources are accumulated (Sirs, 1963). As can be seen in Figure 7.8, the higher rate of oxygen uptake in the first hour occurred mainly in the first 10 minutes, despite time being allowed beforehand for equilibration (Umbreit et al., 1964). It is possible that had the erythrocytes been stored overnight at 4°C, the higher rate of oxygen uptake may have lasted for an hour (Davson and Danielli, 1952; Sirs, 1963).

Some workers have failed to detect G6PD activity in sheep erythrocytes (Baxi et al., 1963; Budtz-Olsen et al., 1963; Wagner and Brown, 1966). However some activity has been found by others (Salvidio et al., 1963; Koch, 1963a, b; Kaneko and Smith, 1964; Thompson and Todd, 1964; Smith et al., 1965; Cheun, 1966; Smith, 1968; Maronpot, 1972), but this is very low and approximately 5% of the level in human erythrocytes (Koch, 1963b; Smith, 1968). Although each author has reported his results in a different format, making comparison between each result difficult, the level of G6PD obtained for normal erythrocytes in this study is similar to that obtained by Smith et al. (1965).

Despite the fact that the level of G6PD activity in sheep erythrocytes is very low, the proportion of glucose metabolism which is directed through the pentose phosphate pathway (15%) is similar to that of human erythrocytes (Leng and Annison, 1962; Murphy, 1960).

The Metabolism of Reticulocytes

The presence of reticulocytes caused a marked increase in the oxygen uptake by sheep erythrocytes. This is consistent with the findings within human erythrocytes (Harrop, 1919; Nossal, 1948; Sherwood-Jones et al., 1953). In contrast, glucose utilization and lactic acid production were not altered to any extent by the presence of reticulocytes, This observation is in agreement with that of Leng and Annison (1962). It has been shown that human reticulocytes have increased glycolytic rates compared with mature

erythrocytes (Bernstein, 1959; Grimes, 1963), possess a complete tricarboxylic acid (TCA) cycle and have cytochrome activity (Rubinstein et al., 1956; Dajani and Orten, 1958; Bernstein et al., 1965).

In the study on infected erythrocytes (Part I) a significant increase in G6PD level was obtained (Figure 7.10) which was significantly ($p < 0.05$) correlated with both degree of parasitaemia and reticulocyte percentage (Table XXXIII). It is possible that either one or both of these factors cause an increase in G6PD. It has been shown that young erythrocytes have a higher G6PD activity than nature erythrocytes (Lohr and Waller, 1961; Marks et al., 1958; Maronport, 1972). Todd and Ross (1968), working with sheep, obtained a 3-fold increase in the level of erythrocyte G6PD following phlebotomy which they attributed to higher activity in younger erythrocytes. They did not, however, correlate their findings with reticulocyte percentage. In Part II of this study, however, the G6PD level was not invariably increased in proportion to the reticulocyte percentage (Figure 7.10) and the correlation between the two, although positive, was not significant (Table XXXIII). A study of Figure 7.10 does reveal an apparent rise in G6PD level, corresponding with reticulocyte percentage, but with a sudden drop in the penultimate set of readings when the reticulocyte percentage was high, followed by a high level of G6PD in the final reading when the reticulocyte percentage was low. Although the drop in the penultimate set of readings is inexplicable, the last reading could be explained by the fact that reticulocyte percentage does not necessarily indicate the total proportion of young erythrocytes present. Considering the findings of other workers, and the higher correlation of G6PD with reticulocyte percentages than with parasitaemia in Part I, it does seem likely that the presence of reticulocytes and a higher than normal proportion of young non-reticular erythrocytes was responsible for the majority of the increase in G6PD level.

As the increase of G6PD level, and hence pentose

phosphate pathway activity, is very small with virtually no alteration in the glycolytic ratio, it must be assumed that the activity of the TCA cycle or some other oxidative pathway in reticulocytes is the cause of the major increase in oxygen uptake. This greater activity also led to the higher ratio of total metabolic products expressed as a percentage of glucose utilized in the samples containing reticulocytes (Table XXXII).

Carbohydrate Metabolism of E. ovis Infected Erythrocytes

The glucose utilization and lactic acid production of E. ovis infected erythrocytes was markedly increased (Figures 7.2 and 7.3), and was similar to that reported in malarial infections (Christophers and Fulton, 1938; Fulton, 1939; Wendel, 1943; Silverman et al., 1944; McKee et al., 1946; Moulder, 1962; Sherman et al., 1969; Schiebel and Pflaum, 1970) and in Babesia infection (Rickard, 1969).

One of the interesting points emerging from this study was the significant lowering of both the glycolytic ratio (Figure 7.4) and the percentage of glucose accounted for as oxygen uptake, lactic acid and pyruvic acid in the infected cells (Figure 7.9). This was, once again, similar to the findings of Silverman et al. (1944) with Plasmodium gallinaceum, and Rickard (1969) with Babesia rodhaini. What happens to the unaccounted for glucose in erythrocytes infected with parasites has been the subject of much study. It has been suggested that it is used by the parasite for synthesis and energy production (Rickard, 1969) and in malaria-infected erythrocytes it has been shown that glucose serves as a precursor for amino-acids (Bryant et al., 1964; Garnham, 1966; Sherman et al., 1969); but whether these are formed by the TCA cycle (Bryant et al., 1964; Garnham, 1966) or by the fixation of CO_2 (Sherman and Tung, 1966, 1968; Siu, 1967; Sherman et al., 1969) is still a matter for conjecture.

Although the ratio of pyruvic acid to lactic acid

remained constant in the control erythrocytes, there was a significant fall in the E. ovis infected cells (Figure 7.6). One possible reason for this fall is the incorporation of pyruvic acid into the TCA cycle by the parasite. The existence of a TCA cycle in the malarial parasites has been suggested (Speck et al., 1946; Bovarinck et al., 1946; Moulder, 1962; Garnham, 1966), but not substantiated (Bowman, et al., 1959, 1960, 1961; Bryant and Voller, 1961; Bryant et al., 1964; Schiebel and Pflaum, 1970). Similarly Rickard (1970b) considered the existence of a functional TCA cycle in Babesia rodhaini to be questionable. Whether any parallel can be drawn with E. ovis is not clear, although the apparent lack of increase in oxygen uptake by infected erythrocytes would suggest some utilization of pyruvic acid other than by the oxidative TCA cycle. Schiebel and Pflaum (1970), for example, were able to show that accumulation of large amounts of neutral and volatile acids resulted from carbohydrate metabolism of P. knowlesi. One of the most significant products was acetate, which was thought to be formed from pyruvate, without the liberation of significant quantities of CO_2.

The results of this study also suggest that pentose phosphate pathway activity, as measured by the level of G6PD is not altered by E. ovis infection. It has been shown with malaria that this pathway does not exist in the parasite (Fletcher and Maegraith, 1962) but it has been suggested that the organism may have some stimulatory effect on the pathway of the host erythrocyte (Fletcher and Maegraith, 1962; Barnes and Polet, 1969). This pathway was not, however, considered to be an important factor in the energy metabolism of the parasite (Schiebel and Miller, 1969).

The egress of oxygen from the infected erythrocytes at the peak of parasitaemia (Figure 7.8) has not been reported with other blood parasite infections. Egress of oxygen has been reported in sheep erythrocytes following dissociation of oxyhaemoglobin after mixing the erythrocytes with a solution containing $Na_2S_2O_4$ (Legge and Roughton, 1950). Sirs

(1966) also found that the rate of this oxygen egress was determined by the rate of chemical dissociation of oxyhaemoglobin and that the intact erythrocyte membrane offered no effective resistance. The oxygen tension of venous blood is low, but further release of oxygen can occur by lowering the pH or by increasing the temperature of the reaction (Harris and Kellermeyer, 1970). Although the incubation temperature was 37°C and a phosphate buffer was included in the medium, the high lactic acid production by the infected erythrocytes may have caused some lowering of intracellular and extracellular pH. As a result of this increase in glycolysis, deoxygenation of haemoglobin may have occurred by mediation of the Bohr effect, whereby oxyhaemoglobin takes up a proton when an oxygen molecule is released; this causing an increase in the intracellular pH (Williamson, 1970). The oxygen release during heavy infection was compensated over the 2 hour incubation period, probably by the oxidative metabolism of the erythrocyte. This resulted in a final positive oxygen uptake. It is worthy of note that the oxygen uptake of cells from the anaemic non-infected sheep was higher than E. ovis infected cells, even though the reticulocyte count was lower (Figure 7.7, Tables XXXI and XXXII).

Although this investigation does not elucidate the metabolism of E. ovis or any of its biochemical pathways, it does reveal that either as a result of parasite metabolism, or enhanced erythrocyte metabolism caused by the parasite, there is a very high utilization of glucose with a concurrent high output of lactic acid.

The effect of these factors on blood glucose levels and on the acid-base status of the host animal is studied in a later chapter.

SUMMARY

The glucose utilization, lactic acid production and oxygen uptake of aerobically incubated normal sheep erythrocytes were comparable to values reported in the literature. The main features observed during incubation of E. ovis infected sheep erythrocytes were considerable increases in glucose utilization and lactic acid production. This was accompanied by a fall in both the glycolytic ratio, and the percentage of glucose accounted for as lactic acid, pyruvic acid and oxygen uptake. The fate of the unaccounted for glucose is not known, but it is suggested that it was used for synthetic and energy purposes by E. ovis.

The level of pyruvic acid produced increased with aerobic incubation of E. ovis infected erythrocytes, but the pyruvic acid : lactic acid ratio was lower in the infected erythrocytes than in the normal control sheep erythrocytes. This was suggestive of some utilization of pyruvic acid by the parasite.

Uninfected erythrocytes which incorporated a percentage of reticulocytes comparable to that found in E. ovis infection showed no apparent difference in glucose utilization and lactic acid production from the normal control sheep erythrocytes. However considerable increases in the oxygen uptake were recorded, which is consistent with findings reported in the literature for human erythrocytes. Erythrocytes heavily infected with E. ovis had net oxygen uptakes for the 2 hour incubation period similar to the normal control erythrocytes. In contrast to normal erythrocytes, the initial stages of this incubation period with heavily infected erythrocytes was characterized by negative oxygen uptake readings. The cause of this apparent dissociation and release of oxygen from the haemoglobin molecule was most probably due to a decreased intracellular pH, as a result of the increased glycolytic activity. This phenomenon was not apparent in the later stages of infection, when reticulocytes were present.

The activity of glucose-6-phosphate dehydrogenase (G6PD), an enzyme of the aerobic pentose phosphate pathway, in normal control erythrocytes was similar to values reported in the literature. E. ovis infection did not affect the activity of this enzyme. Small increases in activity were attributed to the presence of reticulocytes.

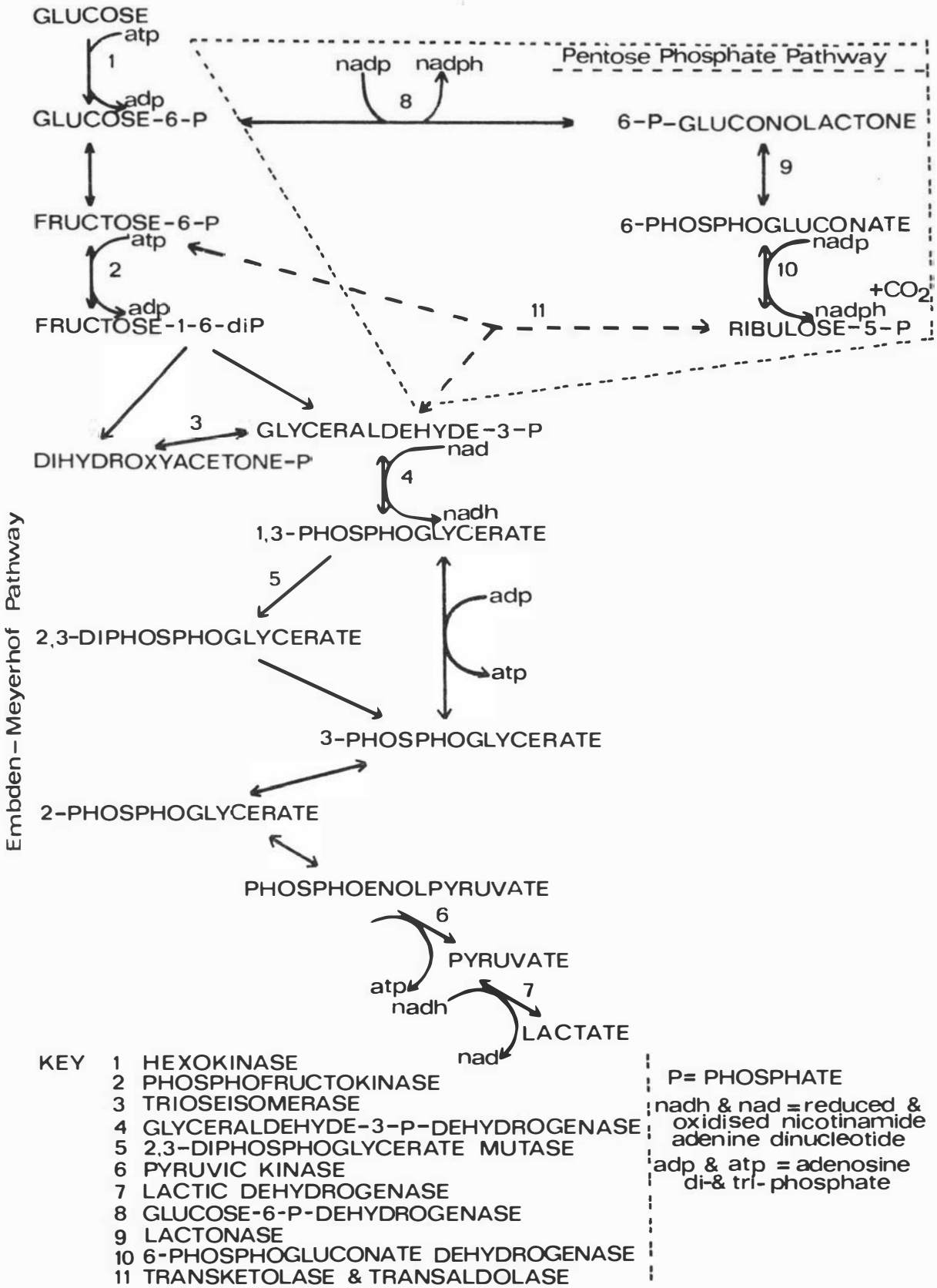


Figure 7.1. Glucose catabolism in human erythrocytes. (From Harris and Kellermeyer, 1970)

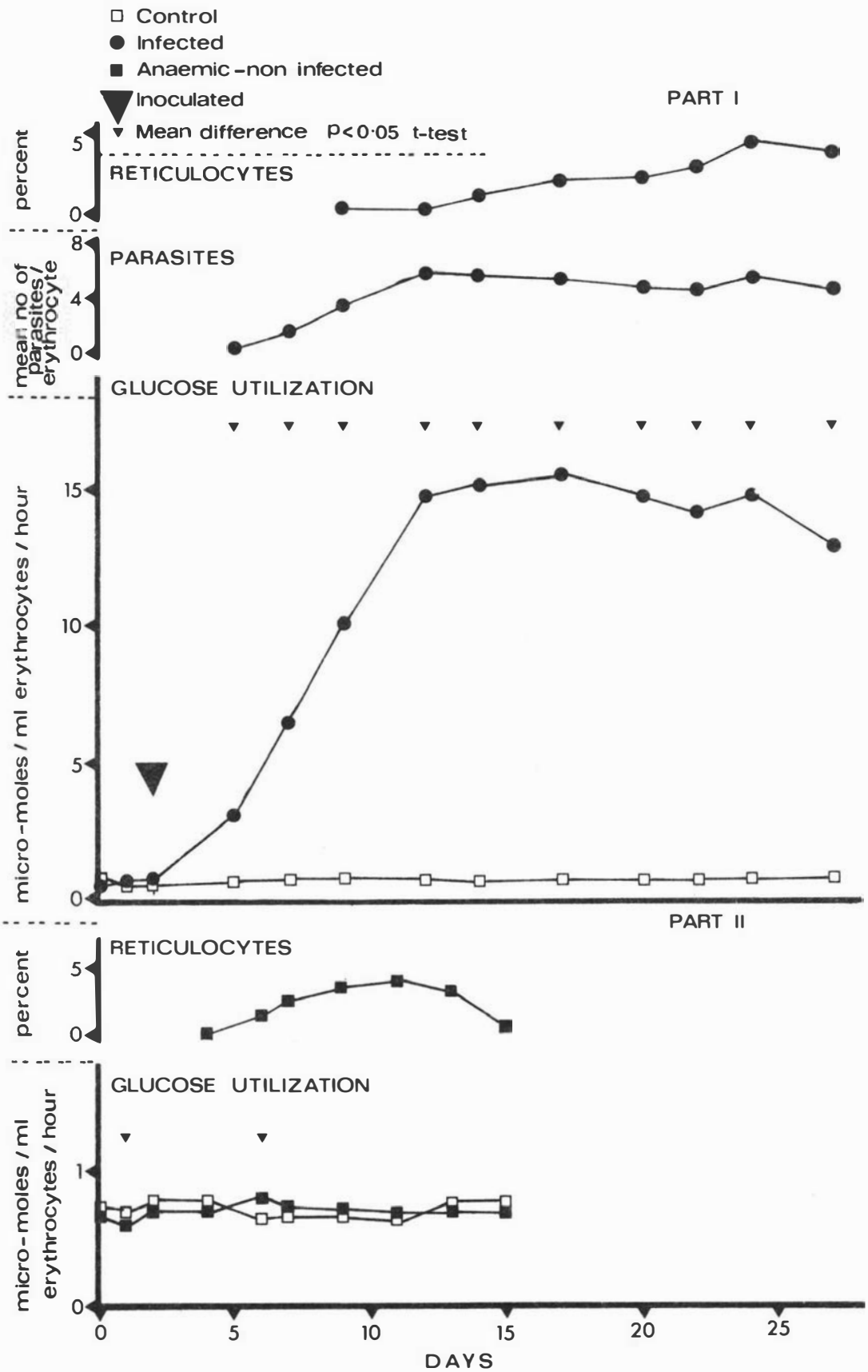


Figure 7.2. The relationship between glucose utilization and reticulocyte percentage in erythrocytes from infected (Part I) and anaemic non infected (Part II) sheep (Exp. V — Group mean values)

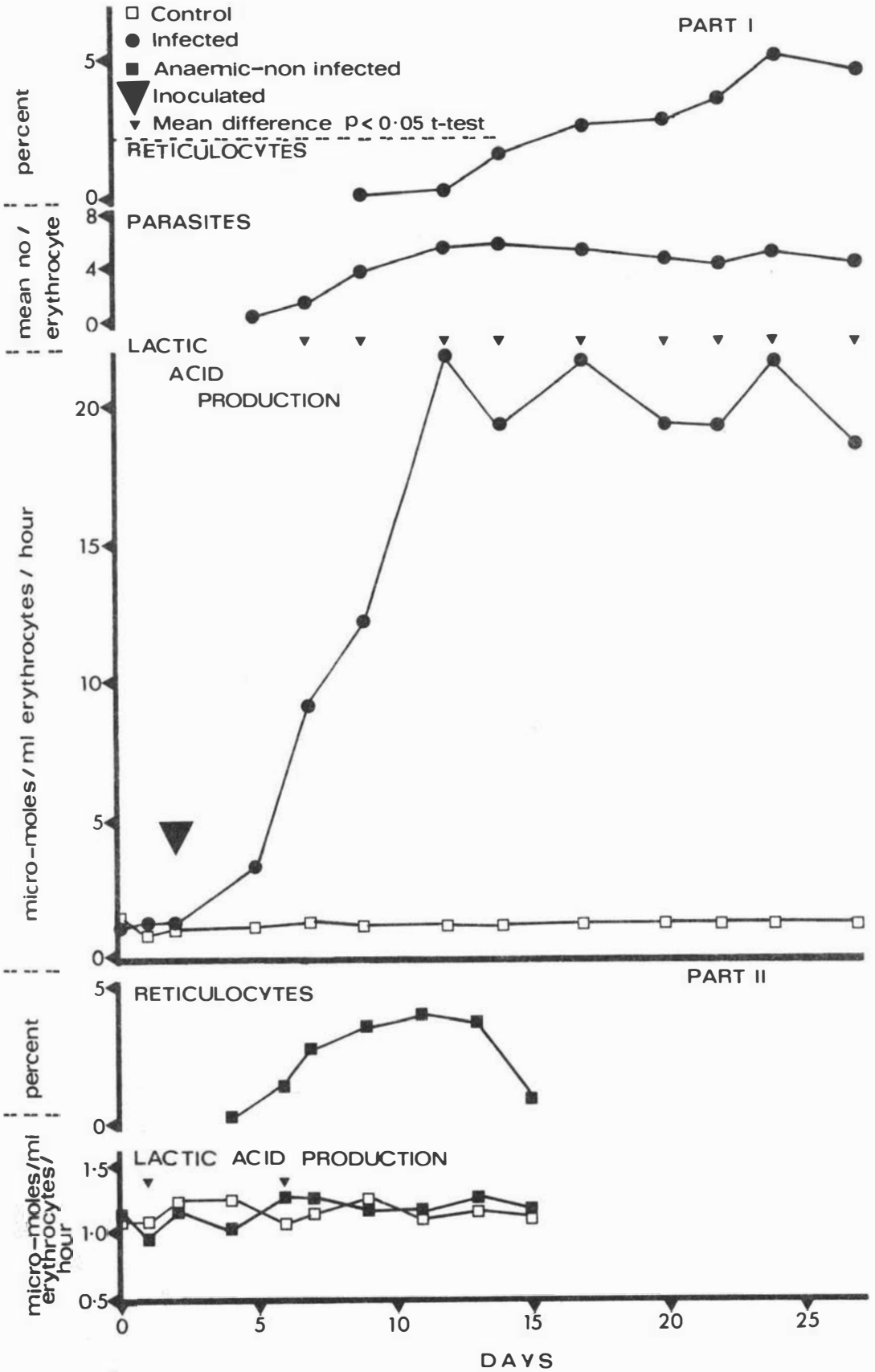


Figure 7.3. The relationship between lactic acid production and reticulocyte percentage in erythrocytes from infected (Part I) and anaemic non infected (Part II) sheep (Exp. V – Group mean values)

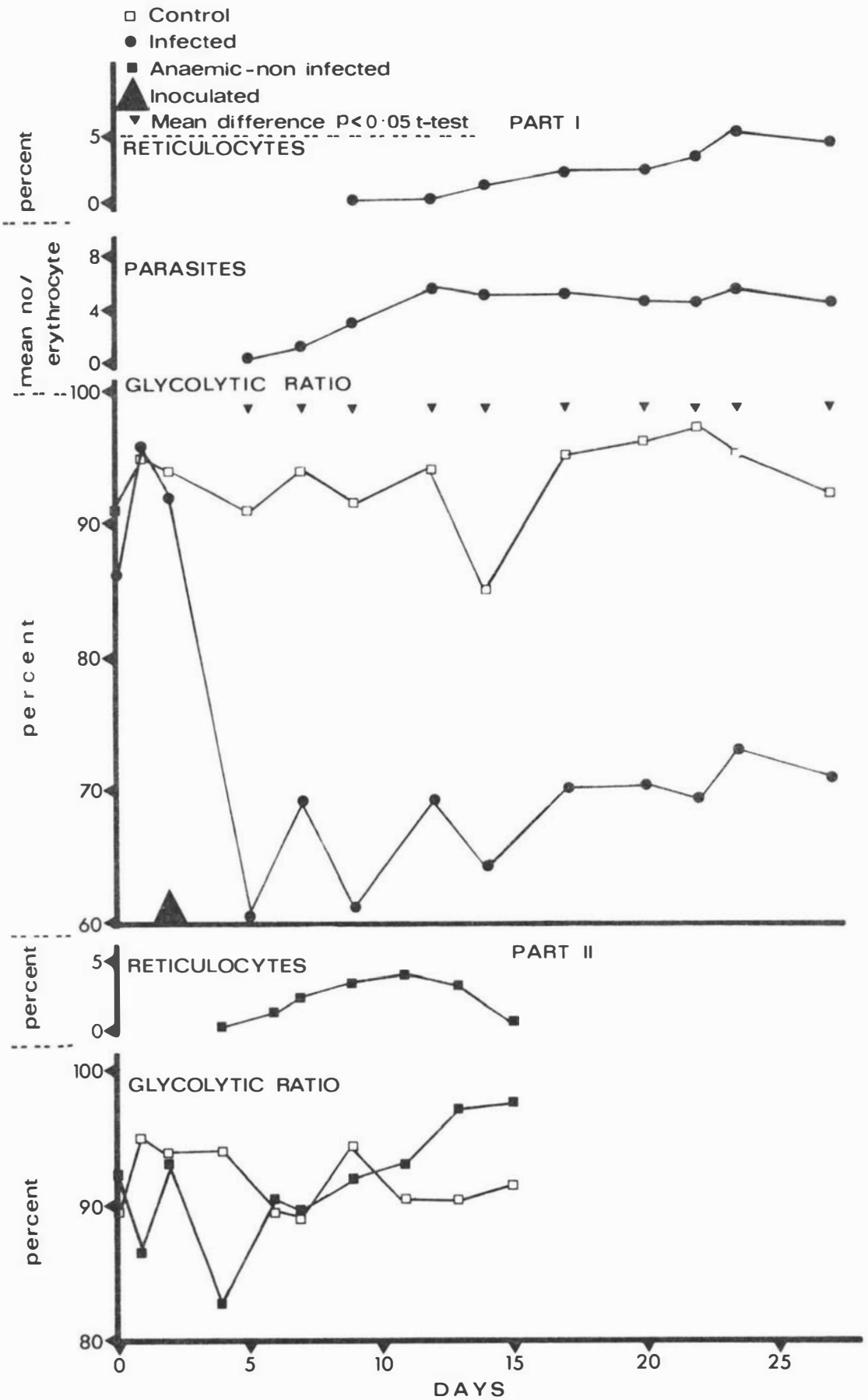


Figure 7.4. The relationship between the glycolytic ratio percentage and reticulocyte percentage in erythrocytes from infected (Part I) and anaemic non infected (Part II) sheep. (Exp. V – Group mean values)

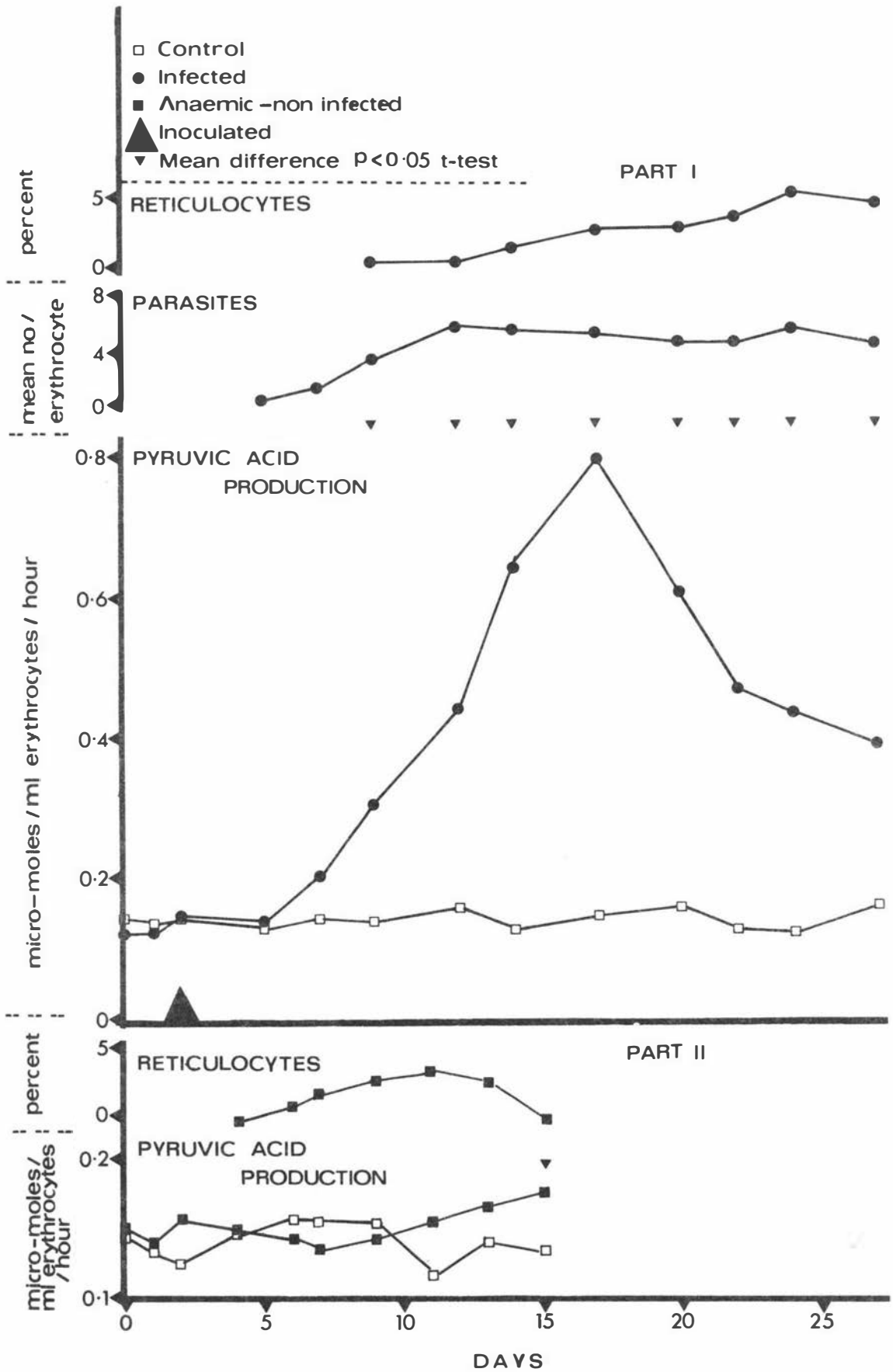


Figure 7.5. The relationship between pyruvic acid production and reticulocyte percentage in erythrocytes from infected (Part I) and anaemic non infected (Part II) sheep. (Exp. V — Group mean values)

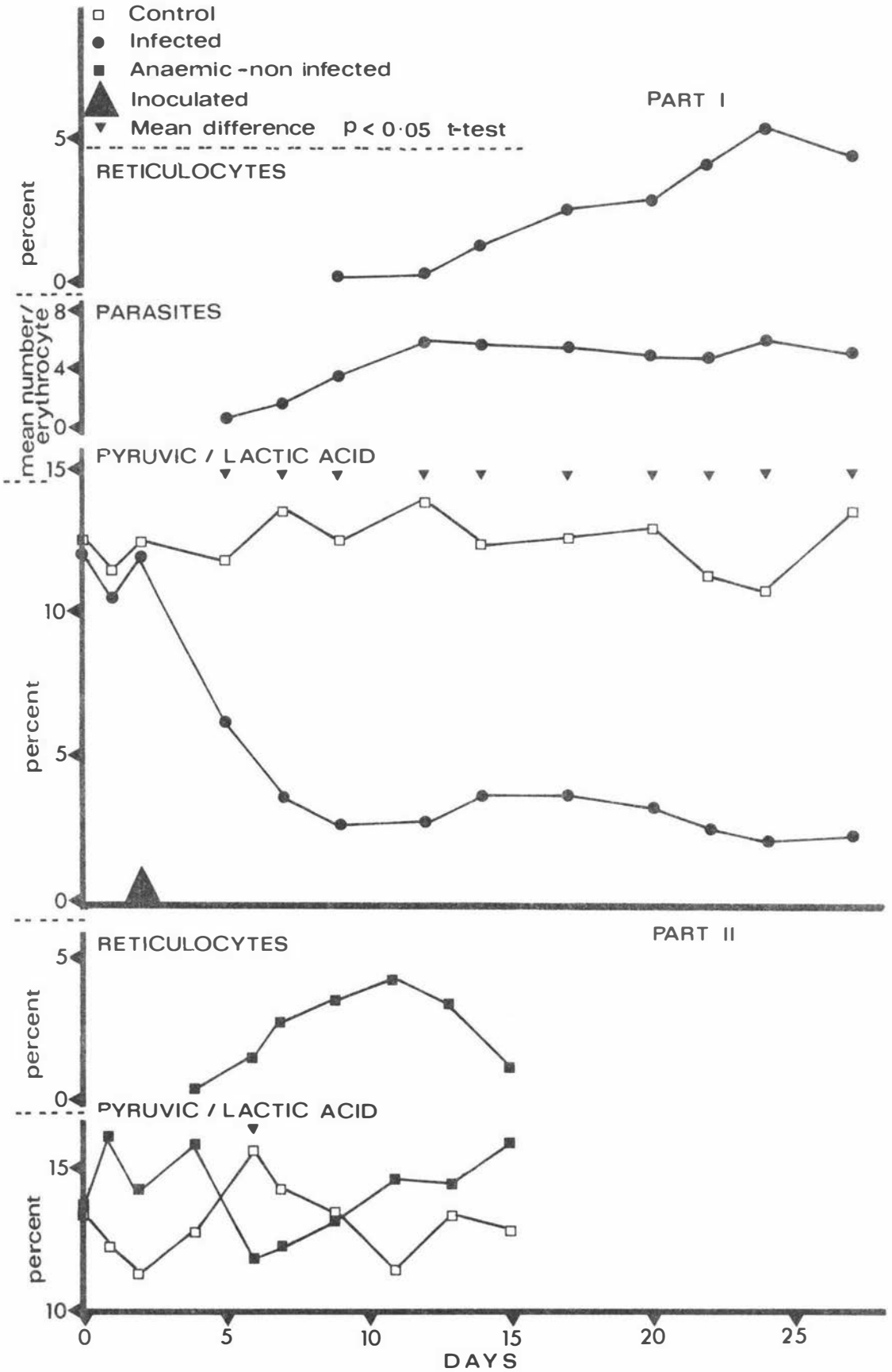


Figure 7.6. The relationship between the percentage ratio of pyruvic acid to lactic acid production and reticulocyte percentage in erythrocytes from infected (Part I) and anaemic non infected (Part II) sheep. (Exp. V — Group mean values)

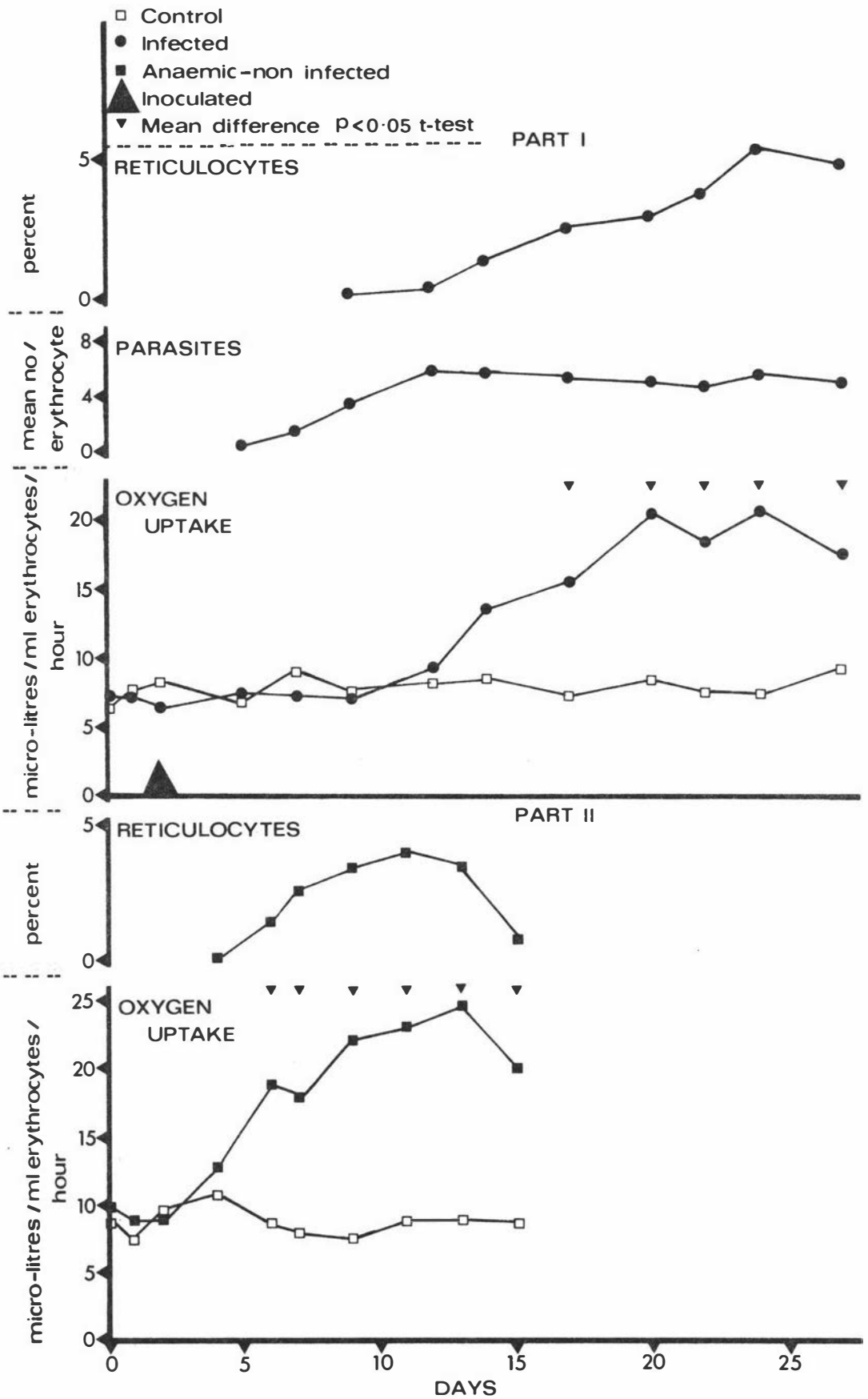


Figure 7.7. The relationship between oxygen uptake and reticulocyte percentage in erythrocytes from infected (Part I) and anaemic non infected (Part II) sheep. (Exp. V — Group mean values)

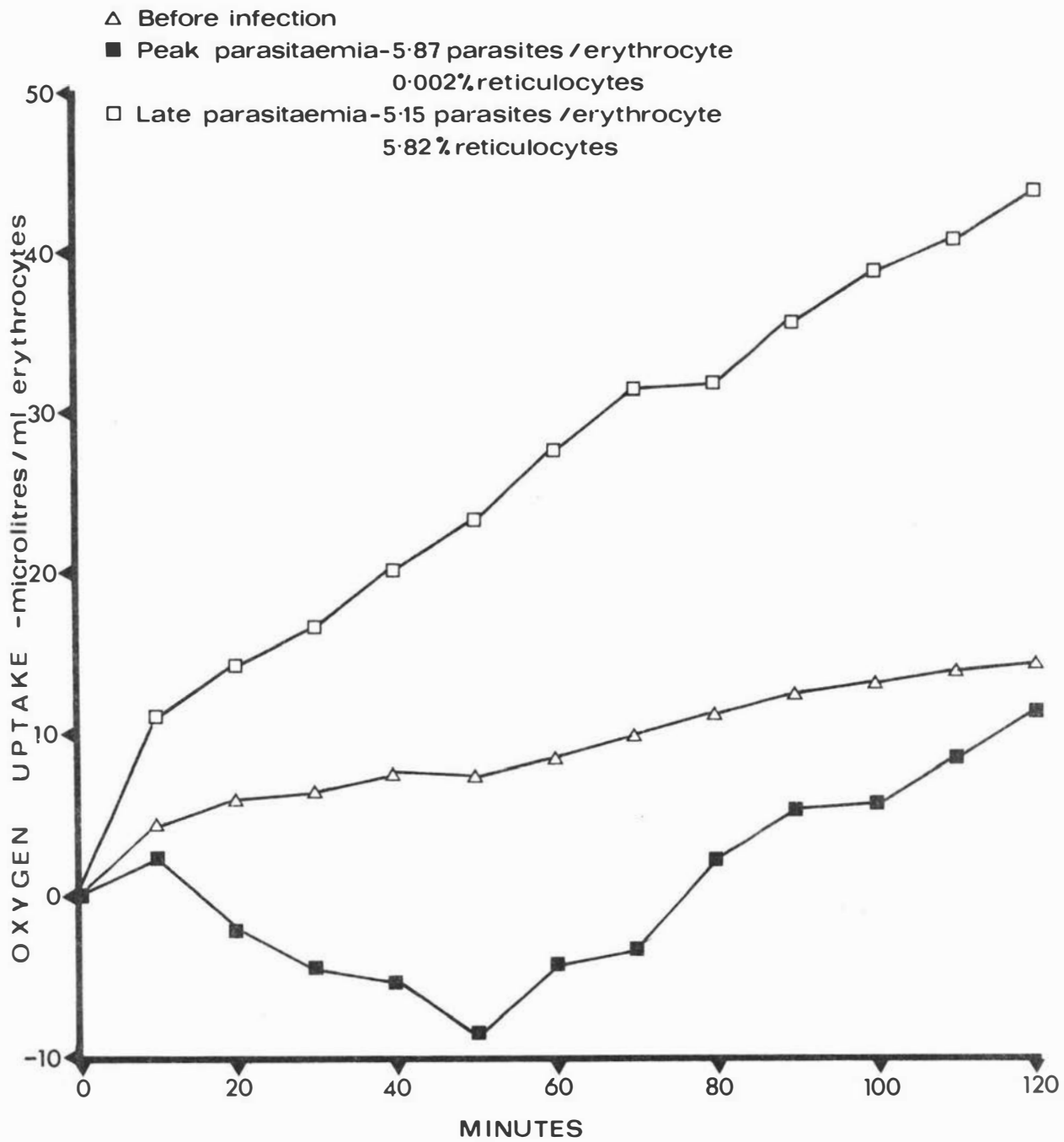


Figure 7.8. The rate of oxygen uptake over a two hour period at three different stages of *E. ovis* infection. (Exp. V — Group mean values)

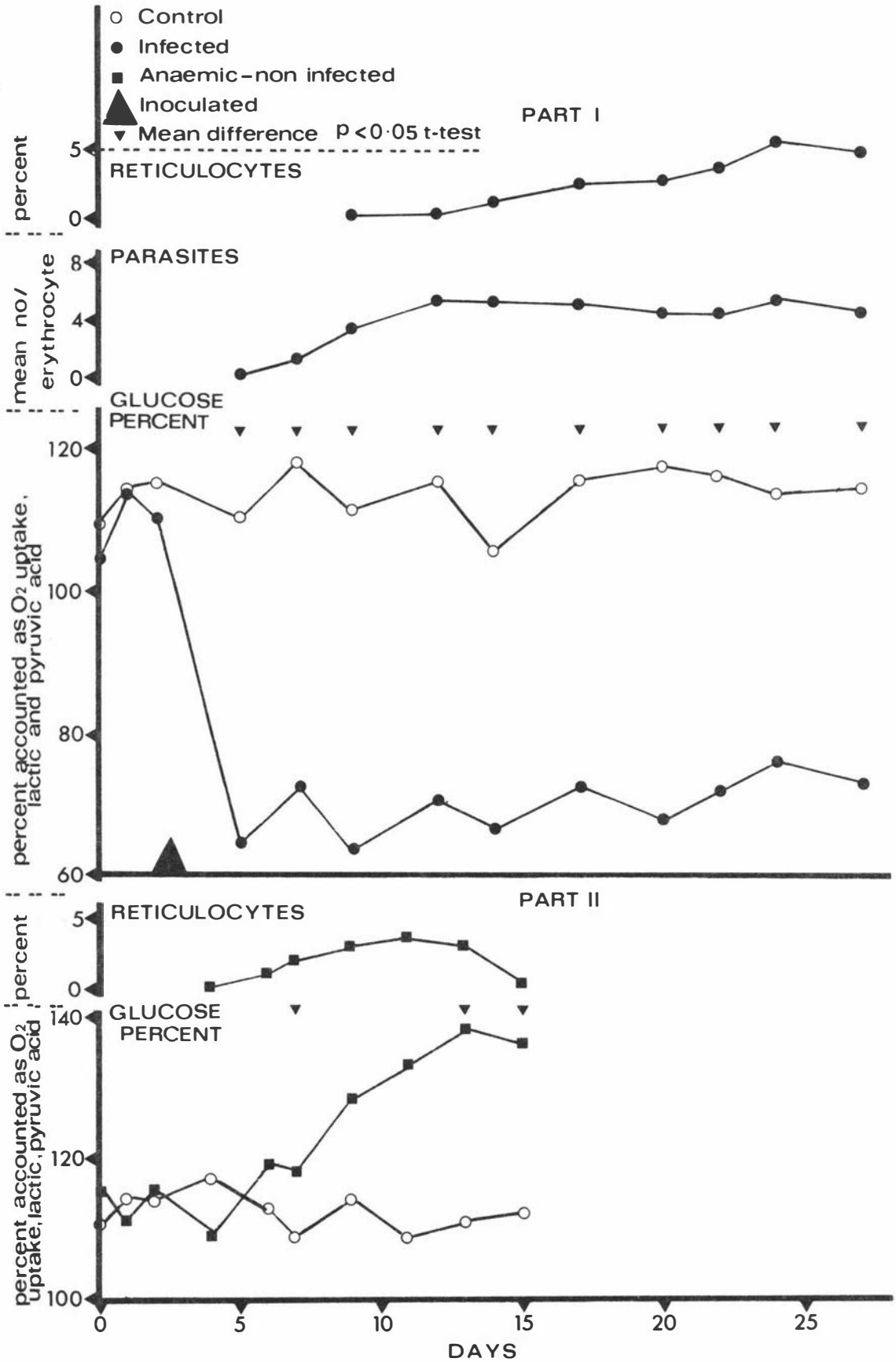


Figure 7.9. The relationship between the percentage of glucose utilized and accounted for as oxygen uptake, lactic and pyruvic acid production, and reticulocyte percentage in erythrocytes from infected (Part I) and anaemic non infected (Part II) sheep. (Exp. V — Group mean values)

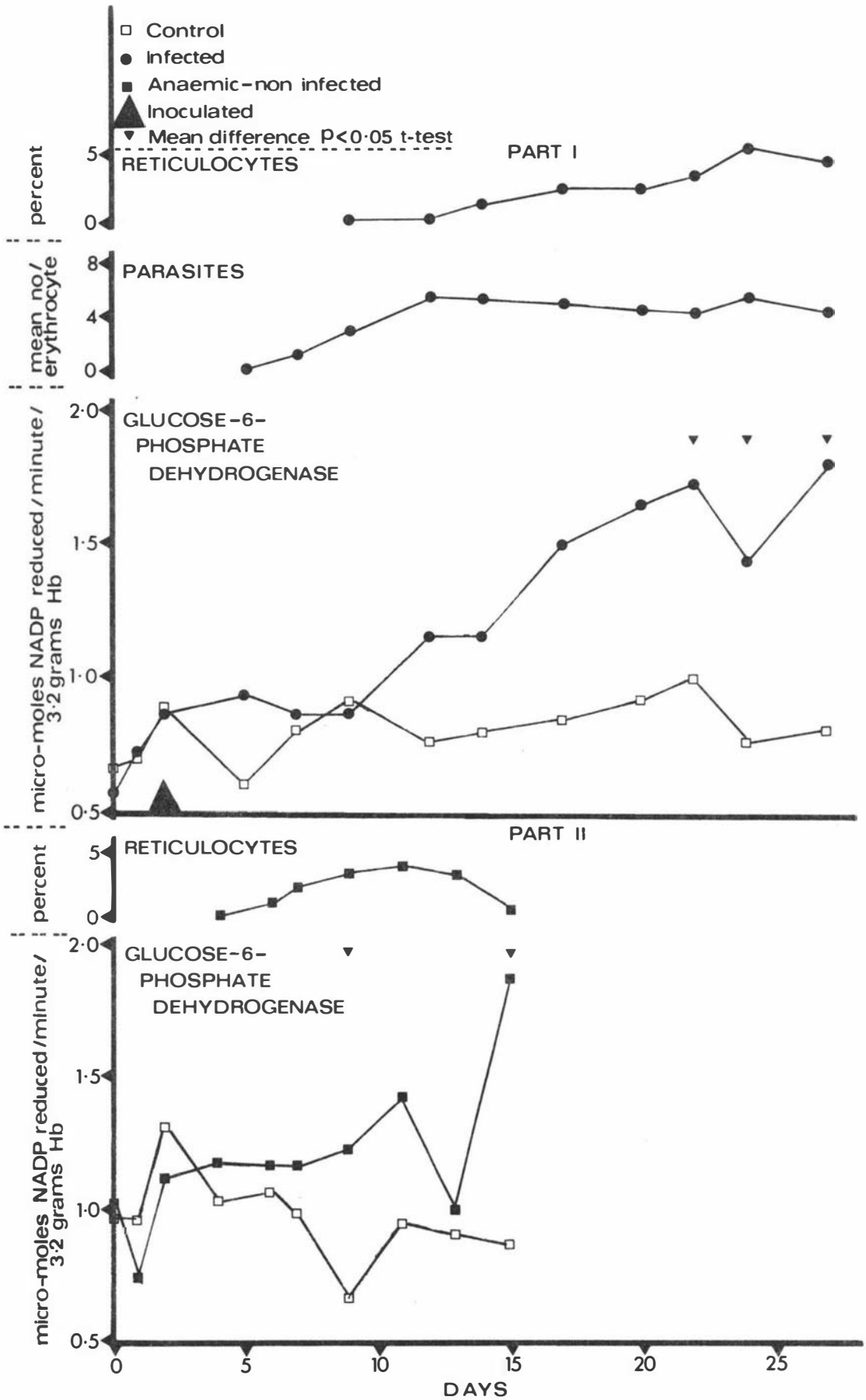


Figure 7.10. The relationship between erythrocyte Glucose - 6 - phosphate dehydrogenase concentration and reticulocyte percentage in blood from infected (Part I) and anaemic non infected (Part II) sheep. (Exp.V — Group mean values)

CHAPTER VIII

THE EFFECT ON SHEEP OF THE INCREASED CARBOHYDRATE METABOLISM IN EPERYTHROZOON OVIS INFECTED ERYTHROCYTES

INTRODUCTION

In vitro studies on the metabolism of erythrocytes infected with *E. ovis* showed that either the erythrocytes or the organisms utilize large amounts of glucose and produce much lactic acid (see Chapter VII). Experiments were carried out to see if these events could be detected in the live animal, and if the lactic acid production affected the animals' acid-base balance.

An acid-base nomogram for the calculation of acid-base values of human blood was described by Siggaard-Andersen and Engel (1960), and later revised (Siggaard-Andersen, 1962). The acid-base parameters which can be measured on anaerobically drawn blood using the pH microelectrode and microtonometry systems of Astrup *et al*. (1960), and calculated from the above nomogram are listed and defined in Chapter II.

These parameters, together with blood glucose, lactic and pyruvic acid levels were measured on sheep experimentally infected with *E. ovis*.

MATERIALS AND METHODS

The techniques and materials used were as described for Experiment VI in Chapter II. Routine haematological data was also collected and has been described in Chapter III.

RESULTS

The Effect of E. ovis Infection on Blood Glucose, Lactic and Pyruvic Acid Levels

The overall mean venous blood levels for glucose, lactic

and pyruvic acid in the control sheep are shown in Table XXXVII. The changes in blood glucose and lactic acid with parasitaemia and relative to control sheep are shown in Figure 8.1. In Table XXXVIII, the maximum differences noted for these parameters between the 2 groups are given.

The onset of parasitaemia led to a considerable fall in blood glucose to a minimum mean level approximately a quarter that of control sheep (Table XXXVIII). There was a corresponding increase in lactic acid concentration to a maximum mean level approximately 3.5 times that of the control sheep. Some individual sheep in the infected group reached a minimum glucose level of 2 to 3 mg/100 ml and maximum lactic acid levels of 80 to 85 mg/100 ml. The blood levels of both glucose and lactic acid were significantly ($p < 0.01$) correlated with the degree of parasitaemia (Table XXXIX).

There were no significant differences in the pyruvic acid level between the control and infected sheep.

The Effect of E. ovis Infection on Acid-Base Parameters Measured in Venous Blood

The overall mean venous blood levels for the acid-base parameters in the control sheep are listed in Table XXXVII. The changes in the acid-base parameters relative to parasitaemia and control levels are shown in Figures 8.2 and 8.3. In Table XXXVIII the maximum mean differences noted for these parameters between the two groups are listed.

The onset of parasitaemia was accompanied by a fall in both the standard bicarbonate and pH; differences between control and infected groups were significant at the higher levels of parasitaemia (Figure 8.2). Both these parameters showed a significant ($p < 0.01$) negative correlation with the degree of parasitaemia (Table XXXIX).

In contrast, the pCO_2 levels in the infected group showed sporadic and variable increases with the onset of

TABLE XXXVII

MEAN CONTROL VALUES FOR BLOOD GLUCOSE, LACTIC ACID, PYRUVIC ACID AND VARIOUS ACID BASE PARAMETERS
(EXPERIMENT VI - VENOUS BLOOD)

Glucose (mgs/100 ml)	50.41 ± 1.19^{x}
Lactic Acid (mgs/100 ml)	16.68 ± 0.92
Pyruvic Acid (mgs/100 ml)	0.176 ± 0.004
pH	7.392 ± 0.004
pCO_2 (mm Hg)	40.28 ± 0.46
Total CO_2 (m Eq/litre)	25.06 ± 0.23
Standard Bicarbonate (m Eq/litre)	23.86 ± 0.17
Actual Bicarbonate (m Eq/litre)	23.85 ± 0.22
Buffer Base (m Eq/litre)	45.69 ± 0.25
Base Excess (m Eq/litre)	-0.61 ± 0.20

x = Standard Error
Number of Sheep = 7
Number of Readings from each sheep = 14

TABLE XXXVIII

THE MAXIMUM DIFFERENCES IN VENOUS BLOOD GLUCOSE, LACTIC ACID AND PYRUVIC ACID CONCENTRATIONS AND ACID-BASE PARAMETERS BETWEEN SAMPLES TAKEN FROM *E. OVIS* INFECTED AND CONTROL SHEEP (EXP. VI - GROUP MEAN VALUES)

	Control	Infected	Mean No. of Parasites/Erythrocyte	t	P
pH	7.42±0.007[x]	7.28±0.019	5.58	7.54	<0.001
Glucose (mgs/100 ml)	48.77±4.71	11.48±4.34	6.70	5.84	<0.001
Lactic Acid (mgs/100 ml)	15.62±2.76	54.59±8.18	5.63	4.52	<0.01
Pyruvic Acid (mgs/100 ml)	0.14±0.016	0.21±0.050	4.47	1.26	NS
Actual HCO_3 (m Eq/litre)	25.07±0.74	21.79±0.55	5.63	3.56	<0.01
Std. HCO_3 (m Eq/litre)	25.39±0.47	21.39±0.54	5.58	5.59	<0.001
pCO_2 (mm Hg)	40.57±2.34	51.93±2.01	5.58	3.69	<0.01
Total CO_2 (m Eq/litre)	25.83±0.62	22.03±0.62	2.40	4.34	<0.01
Buffer Base (m Eq/litre)	46.57±0.61	41.14±0.67	5.58	6.00	<0.001
Base Excess (m Eq/litre)	1.14±0.39	-3.39±0.62	5.58	5.56	<0.001

x = Standard Error

P = Probability

NS = Not Significant (P >0.05)

Number of sheep in each group = 7

TABLE XXXIX

THE CORRELATION BETWEEN THE DEGREE OF PARASITAEMIA AND VENOUS ACID-BASE PARAMETERS IN *E. OVIS* INFECTED SHEEP (EXP. VI)

	r	p
pH	-0.72	< 0.01
Glucose	-0.88	< 0.01
Lactic Acid	+0.93	< 0.01
p CO_2	+0.57	< 0.05
Total CO_2	-0.48	NS
Std. HCO_3	-0.84	< 0.01
Actual HCO_3	-0.56	< 0.05
Buffer Base	-0.71	< 0.01
Base Excess	-0.82	< 0.01

r = Correlation Coefficient
P = Probability
NS = Not Significant ($p > 0.05$)
Number of Readings = 14

parasitaemia. Significant ($p < 0.05$) differences between control and infected sheep did occur but the correlation with the degree of parasitaemia, although positive and significant ($p < 0.05$), was not as high as the correlation between parasitaemia and standard bicarbonate (Table XXXIX).

With the onset of parasitaemia, the fall in the actual bicarbonate and total CO_2 levels in the infected group was variable and only occasionally significant (Figure 8.3). The correlations of these 2 parameters with the degree of parasitaemia were the lowest of the acid-base parameters measured, and in the case of total CO_2, not significant (Table XXXIX).

The onset of parasitaemia produced significant falls in the buffer base level of the infected sheep with a concurrent negative base excess. Both of these parameters showed significant differences between the 2 groups of sheep at the higher levels of parasitaemia, and both showed a significant negative correlation with the degree of parasitaemia (Table XXXIX).

DISCUSSION

The method used for the measurement and calculation of the acid-base parameters (Astrup et al., 1960; Siggaard-Andersen, 1962) was derived from studies on human blood. Phillips (1970) conducted a series of experiments to ascertain whether the Siggaard-Andersen nomogram was suitable for calculating acid-base values for sheep and cattle blood. He concluded that the use of the nomogram will allow estimates of base excess and buffer base with an accuracy of about $\pm$ 1.0 m Eq/litre. Standard bicarbonate estimations were found to be accurate to $\pm$1.0 m Eq/litre at high values and to less than $\pm$ 0.5 m Eq/litre at low values.

There is some controversy as to whether acid-base measurements should be made on venous or on arterial blood.

Some workers consider that venous samples yield as reliable results as do arterial samples (Zahn and Weil, 1966; Carter and Brobst, 1969). The latter authors point out that pH measurements made on arterial blood are less repeatable since they are liable to comparatively rapid fluctuation in response to changes in respiratory pattern. The buffering effect of the whole acid-base system prevents such short-term fluctuations in venous blood, which should therefore reflect more accurately changes in acid-base balance. From comparisons made it is claimed that only in respect of pCO_2 and pO_2 estimations do arterial and venous samples yield significantly different results. Others have also shown that arterial blood yields more reliable estimates of these 2 parameters (Sutton et al., 1967; Tennant et al., 1969).

On the other hand, it has been suggested that venous samples may not allow accurate assessment of more subtle changes in acid-base balance (Sutton et al., 1967; Donawich and Baue, 1969). It was also considered that local or regional variations in metabolism and circulation would alter pCO_2 and pH levels to such an extent that the respiratory contribution to an alteration in acid-base balance could not be accurately assessed.

In a preliminary experiment an attempt was made to obtain arterial blood samples using an indwelling catheter. This was found to be unsatisfactory. It proved difficult to keep the catheter in place and to prevent it from becoming blocked by coagulated blood. Since it was necessary to use relatively large numbers of experimental animals over a comparatively long period (approximately 30 days), it was clearly more convenient to use venous blood for the estimations. These may not provide for the most sensitive estimation of all the acid-base measurements but since the study was essentially a comparison of infected and control animals, it was considered that the data yielded by venous samples should be satisfactory for this purpose.

All the parameters measured indicate to a variable

extent the severity of an acid-base disorder, but some give a better indication than others of the type of disturbance (Astrup et al., 1960; Bach, 1963). The pH value reflects the effect of respiratory and/or metabolic disturbances, and the total CO_2 and actual bicarbonate, although indicative of metabolic disturbance, can also be influenced by respiratory disturbances (Astrup et al., 1960). A respiratory acid-base disturbance is normally reflected by alteration in the pCO_2, which is elevated in respiratory acidosis and lowered in respiratory alkalosis. Standard bicarbonate is an indicator of the non-respiratory component of acid-base metabolism, It is calculated as under standard conditions, so that the direct influence of respiration is eliminated and any deviation from normal reflects either a primary non-respiratory or compensated respiratory acid-base disturbance. However, the standard bicarbonate estimation has the disadvantage that it does not show directly the amount in m Eq/litre of blood, of fixed acid or base causing a change in the base content of a blood sample (Astrup et al., 1960). While a change in the buffer base level in m Eq/litre directly expresses the amount of acid or base which causes the change; it is influenced by a number of different buffer systems. It has been estimated, for instance, that buffer base values change by 0.36 m Eq/litre for each change of 1.0 gram Hb per 100 ml (Singer and Hastings, 1948; Phillips, 1970). On the other hand the base excess measurement appears to be independent of haemoglobin content and is indicative of surplus fixed acid or base in plasma (Astrup et al., 1960; Phillips, 1970).

It has generally been considered that most vertebrates give similar values for these acid-base parameters (Rossdale and Mullen, 1970). Wide ranges have been reported in the literature, generally from methods other than that of Astrup et al. (1960). Data from normal sheep is limited, but the results for the control sheep in this investigation are similar to those obtained by Annison et al. (1959). The levels of blood glucose and lactic acid obtained were

similar to the range reported by Spector (1956), while that of pyruvic acid tended to be lower.

Eperythrozoon ovis infection caused a severe depletion of blood glucose, with a concurrent elevation of blood lactic acid. Consistent with this was a lowering of the venous pH with falls in standard bicarbonate (Figure 8.2) and base excess (Figure 8.3). Similar findings have been obtained in calves infected with E. wenyoni (Schotman, 1970; Zwart et al., 1970), and a hypoglycaemia has been reported with Haemobartonella bovis infection in splenectomized calves (Love and McEwan, 1972). Pyruvic acid levels in blood were not altered in the blood of infected sheep even though considerable increases did occur with in vitro incubation of infected erythrocytes (Chapter VII). Zwart et al. (1970) found some increase in blood pyruvic acid level in Eperythrozoon wenyoni infected calves but glucose levels were only slightly reduced.

This variation in blood glucose and pyruvic acid levels between the 2 Eperythrozoon species could be a reflection of differences, not only of infection severity (Kreier and Ristic, 1968), but also in the metabolism of the 2 organisms. It is also possible that, as a result of the considerable fall in blood glucose during E. ovis infection, the excess pyruvic acid formed (Chapter VII) is oxidized, in vivo, via the tricarboxylic acid cycle, in order to maintain energy requirements.

One unusual finding in this investigation was an increased pCO_2 level in the infected sheep, which was indicative of a respiratory acidosis. This was again similar to the findings with E. wenyoni infection (Schotman, 1970; Zwart et al., 1970).

These findings suggest that, as a result of increased glucose utilization and lactic acid production by infected erythrocytes, an acidosis results which, by the definition of Astrup et al. (1960), is due to both respiratory and

metabolic disturbances in acid-base balance. A metabolic acidosis, as indicated by the fall in standard bicarbonate due to the neutralization of the lactic acid, was expected. However a resultant respiratory compensation which is normally indicated by a lowering of the pCO_2 did not occur.

The reason for the increased pCO_2 and apparent loss of the ability to compensate is unexplained. The infected sheep showed no clinical evidence of respiratory malfunction preventing hyperventilation, and the necropsy examination of infected sheep in other experiments revealed no respiratory abnormality (Chapter V).

The possible effect of the acidosis on other aspects of the infected sheep's physiology is uncertain but could vary with circumstances. It is possible that the fall in pH and base excess observed during parasitaemia could be a cause of the inappetance which occurs at this time. In some circumstances animals may suffer a metabolic acidosis from dietary causes (Annison et al., 1959; Phillips and Knox, 1969; Rossdale and Mullen, 1970) and should this coincide in sheep with E. ovis infection, the acidosis could well be aggravated; in such a case the combined effects of anaemia and acidosis could produce a significant effect on animal production.

SUMMARY

Eperythrozoon ovis infected sheep have low venous blood glucose levels and correspondingly increased blood lactic acid levels as compared with control sheep. Pyruvic acid levels are unchanged. Acid-base studies showed that these changes were accompanied by significant ($p < 0.05$) falls in venous pH, standard bicarbonate and buffer base levels and a negative base excess. All these changes were considered to result from the increased glycolytic activity of infected erythrocytes (Chapter VII).

While a fall in standard bicarbonate due to neutralization of lactic acid might be expected to occur in infected sheep, an increase in the pCO_2 level which is indicative of a respiratory acidosis was not. There is no apparent explanation for this latter finding.

The acidosis which occurs may be a cause of inappetance observed during the parasitaemic phase of the infection cycle.

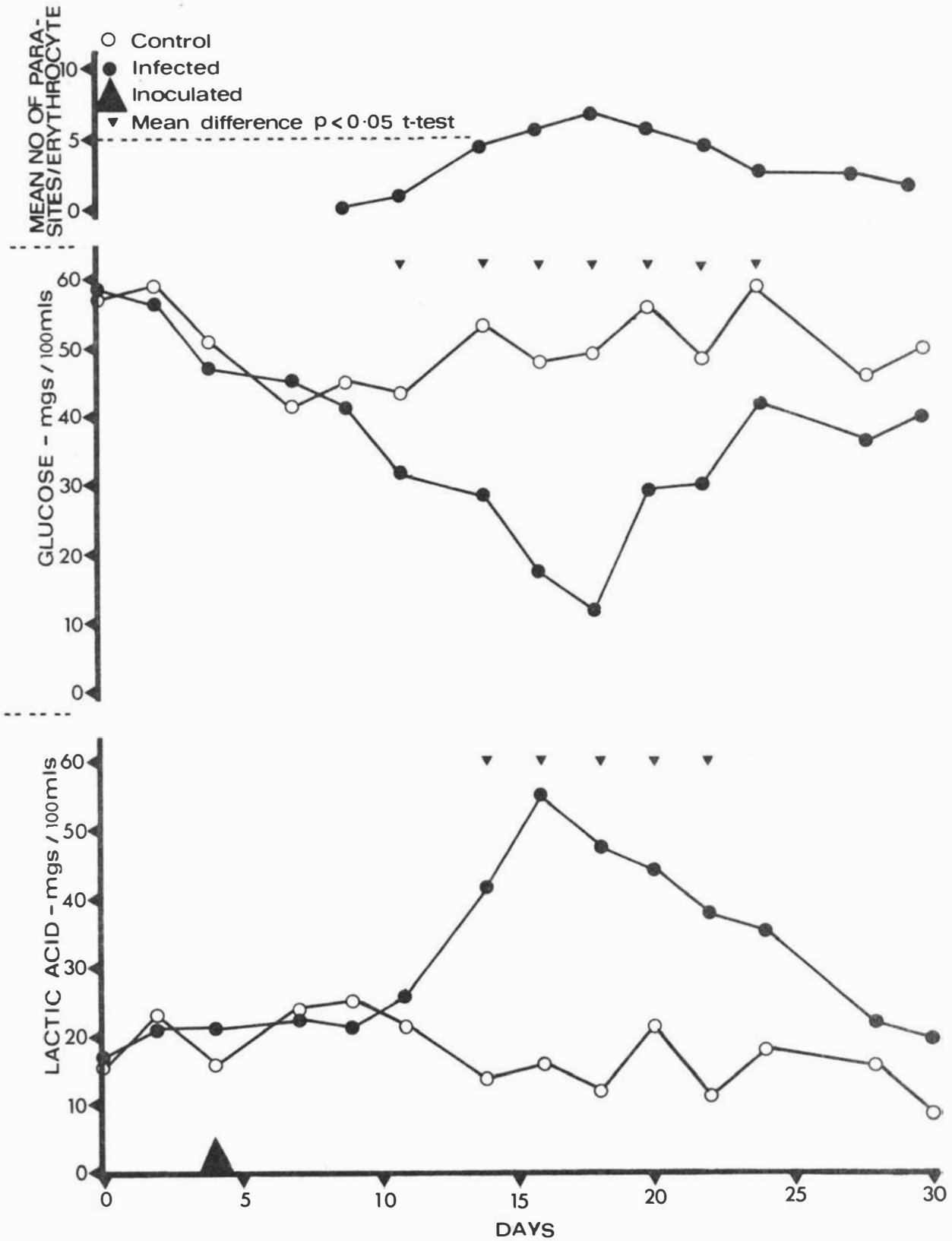


Figure 8.1. The time relationships between the degree of parasitaemia, and the blood concentrations of glucose and lactic acid. (Exp. VI — Group mean values)

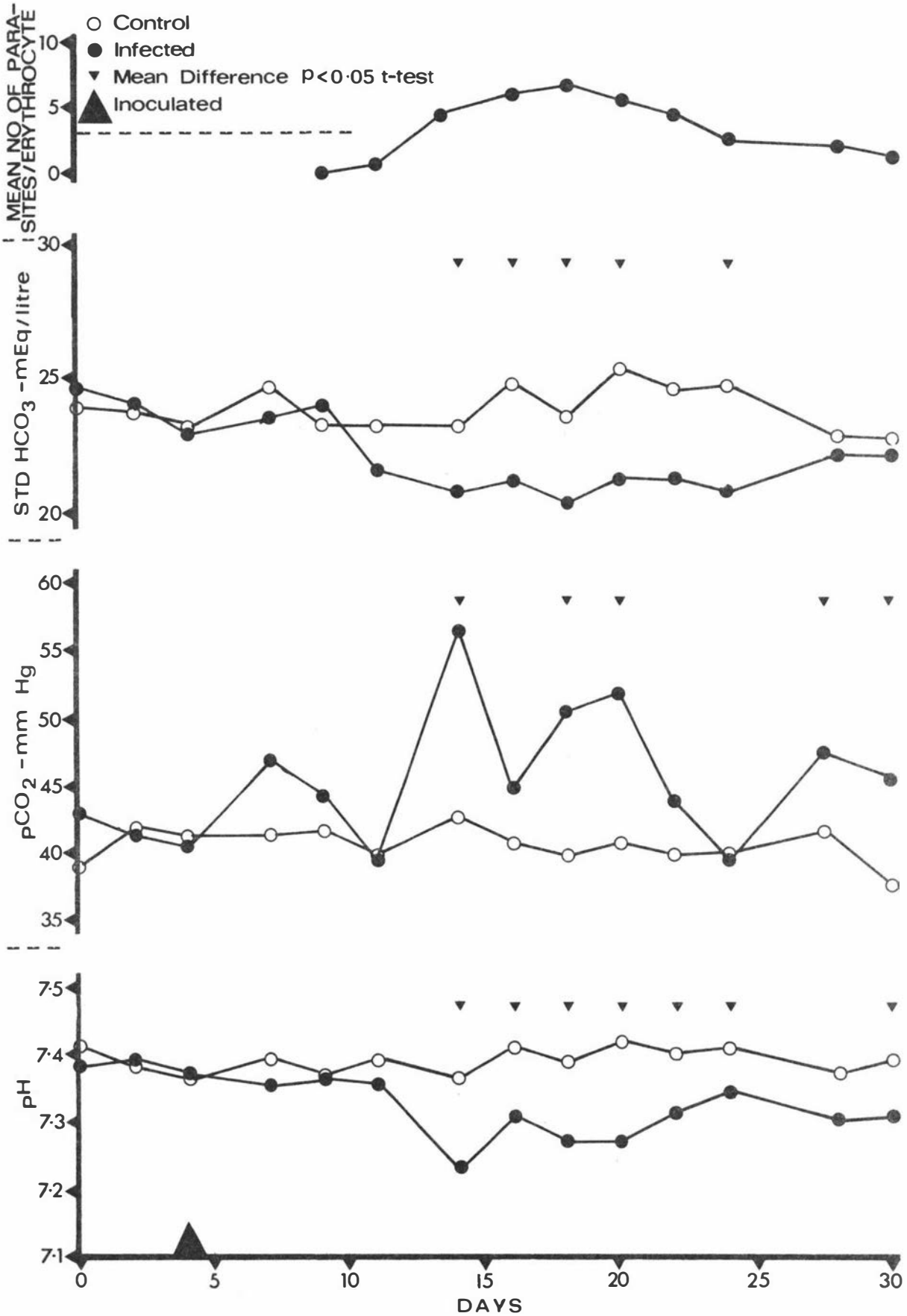


Figure 8.2. The time relationships between the degree of parasitaemia and blood pH, pCO_2 and standard bicarbonate. (Exp VI — Group mean values)

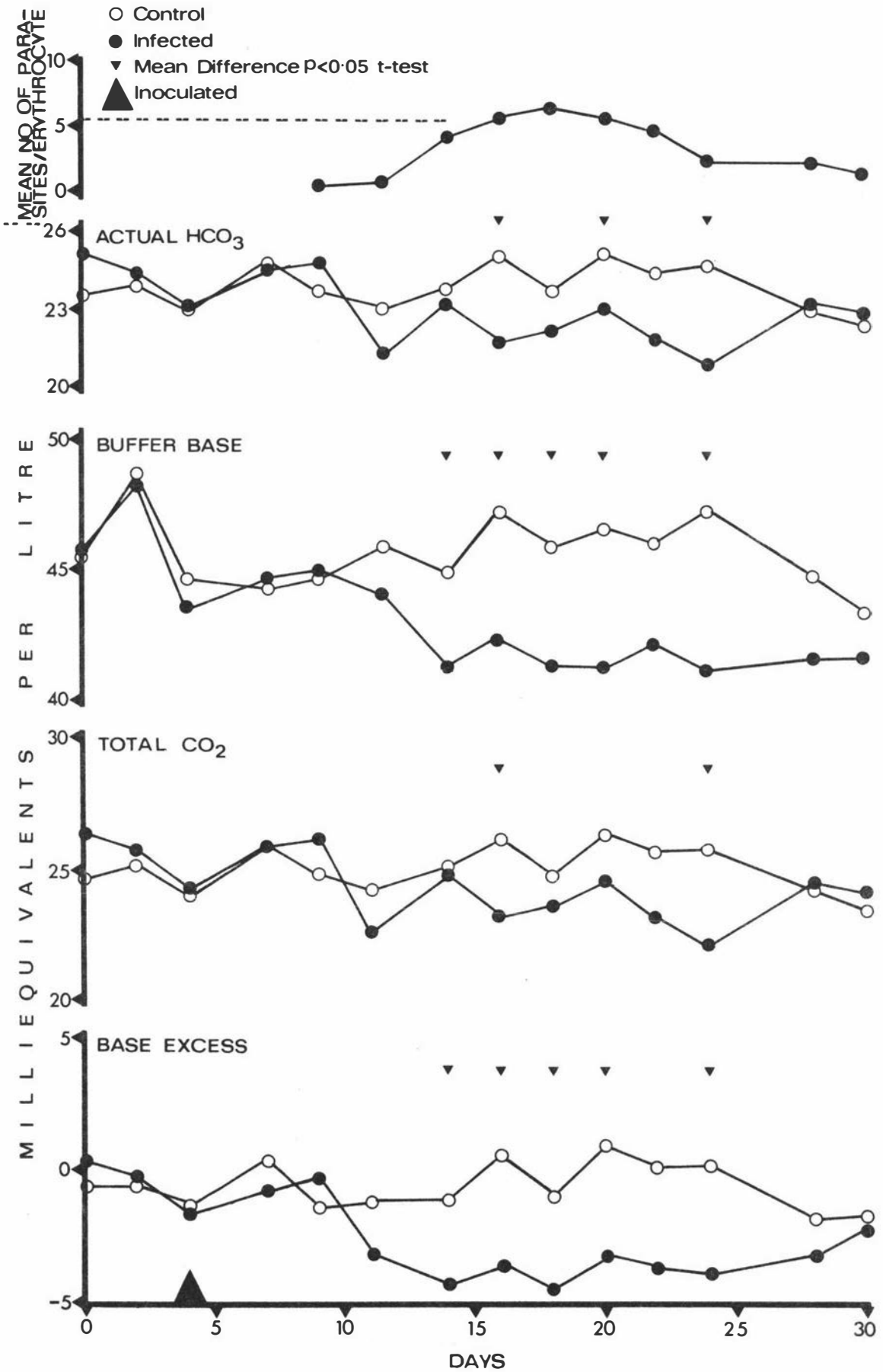


Figure 8.3. The time relationships between the degree of parasitaemia and the actual bicarbonate, buffer base, total CO_2 and base excess levels in blood. (Exp. VI – Group mean values)

CHAPTER IX

THE EFFECT OF *EPERYTHROZOON OVIS* INFECTION ON SOME BIOCHEMICAL FACTORS WHICH INFLUENCE THE FUNCTIONAL AND STRUCTURAL INTEGRITY OF THE ERYTHROCYTE

INTRODUCTION

The glycolytic pathways are the most important energy producing processes in the erythrocyte (Fornaini and Bossu, 1969). It has already been shown that *E. ovis* infected erythrocytes show increased glycolytic activity, but whether this is due directly to parasite activity, or to altered erythrocyte activity as a result of the infection, is not yet known (Chapter VII).

Allied to the glycolytic activity of the erythrocyte is a "reductive potential" which balances the oxidative processes which arise in a natural way from soluble components of the blood. In the absence of a cytochrome system (Rubinstein *et al.*, 1956) the reduction of oxidative processes by the erythrocyte depends on the reduced nicotinamide adenine dinucleotides (NADPH and NADH). The interrelationships of these dinucleotides with the glycolytic pathways in the erythrocyte are shown in Figure 9.1.

The reduction of NAD is coupled to the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase and lactic dehydrogenase. The main function of NADH in the Embden-Myerhoff pathway (Figure 9.1) appears to be the maintenance of haemoglobin in the reduced state, through methaemoglobin diaphorase. *In vitro* this function has been estimated to be responsible for about 67% of the methaemoglobin reducing role (Scott *et al.*, 1965), but under normal physiological conditions *in vivo*, it is believed to be the only active mechanism for methaemoglobin reduction (Tonz, 1968).

The reduction of NADP is limited to the pentose phosphate pathway. This pathway, which utilizes approximately 10% of

the erythrocyte's glucose requirements (Murphy, 1960; Leng and Annison, 1962), contains 2 enzymes, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), which are coupled with the reduction of NADP to NADPH (Figure 9.1). The most important function of NADPH is its utilization as a hydrogen donor by the enzyme glutathione reductase, for the reduction of oxidized glutathione (GSSG). The maintenance of glutathione, which contains a sulphydryl group, in the reduced state (GSH) is necessary for the preservation of normal function and structural integrity of the erythrocyte (Kosower and Kosower, 1969), particularly in maintaining membrane sulphydryl groups (Weed and Reed, 1966) and haemoglobin sulphydryl groups (Tarlov et al., 1962) in the reduced form.

Although the physiological significance of the GSH-GSSG oxidation-reduction system in erythrocytes has been in question (Mills and Randall, 1958; Szeinberg and Marks, 1961; Oort et al., 1961; Prins et al., 1966), it has been shown to be extremely important in protecting the erythrocyte membrane and the haemoglobin molecule from damage when certain oxidizing drugs are administered. If all GSH is oxidized, either in the presence of such chemicals as acetylphenylhydrazine, or as a result of the erythrocyte's failure to maintain glutathione in the reduced form, damage occurs to the cell. Alterations of shape, permeability, osmotic resistance, haemoglobin metabolism and structural and enzymatic protein, including the formation of Heinz bodies, may result (Jones and McCange, 1949; Jensen, 1959; Jacob and Karnowsky, 1967; Jandl et al., 1960; Allen and Jandl, 1961; Beutler, 1962; Robinson, 1966). These alterations have been defined by Jandl et al. (1960) as being part of the process of "oxidative haemolysis".

Results of earlier studies in this investigation and by other workers (Sheriff, 1967; Sheriff and Geering, 1969) on E. ovis infection have shown that the anaemia which develops could be induced by 2 possible mechanisms:

1. By an immune response against either the parasite, erythrocyte or both.

2. By a direct effect of the parasite on the erythrocyte.

Although it was suggested that the direct effect could be a result of mechanical damage to the cell (Chapter III), the high glycolytic activity of the organism and/or the infected erythrocyte could interfere with the oxidation-reduction mechanisms of the host cell, particularly if the high glycolytic activity is confined to the organism and at the expense of the cell.

In this chapter an experiment is described in which reduced glutathione (GSH) and methaemoglobin levels in E. ovis infected and control blood, were measured during a cycle of E. ovis infection. The effect on these 2 parameters of incubating blood, with and without acetylphenylhydrazine, was also studied in an endeavour to measure the effect of E. ovis on the biochemical integrity of the erythrocyte. Representative samples of blood were examined following incubation, for the presence of Heinz bodies.

MATERIALS AND METHODS

The procedures and materials used are as described for Experiment VII in Chapter II. One of the infected groups (Group C) was housed prior to each bleeding to study the effect of such treatment on the haematology (Chapter III) and weight gain (Chapter V).

RESULTS

The Effect of E. ovis Infection on the Reduced Glutathione (GSH) Level in Erythrocytes

Non-incubated blood: The overall mean level of GSH in the blood of the 7 control sheep was 95.75 ± 1.85 mg per 100 ml of erythrocytes. The time relationships between the mean degree of parasitaemia, reticulocyte percentage and the GSH levels in the 2 E. ovis infected groups (B and C) and the level in the control group (A) are shown in Figure 9.2

Examination of data revealed that:

1. Significant ($p < 0.05$) differences in the GSH level between the infected and control groups occurred in the latter half of the experiment, due partly to a small increase in the control level, but mainly due to a sharp decrease in the infected group level between days 21 and 28 post-inoculation.
2. The GSH levels in the infected groups were not correlated significantly with either the reticulocyte percentage (Table XL) or the degree of parasitaemia (Table XLI).

Because of changes in GSH level within the control group during the experiment, the mean differences at each reading between the infected and control groups were calculated. The correlations of these mean differences with the reticulocyte percentage were negative and significant in both infected groups ($p < 0.05$) (Table XL), whereas the correlations with the degree of parasitaemia were very small in both groups and not significant (Table XLI).

Incubated blood - without acetylphenylhydrazine: Reduced glutathione (GSH) levels in all groups after incubation of blood for 2 hours at 37°C, were lower than pre-incubation values. The overall mean level for the blood of the control sheep was 90.86 ± 2.02 mg per 100 ml erythrocytes. The time relationships between mean degree of parasitaemia, reticulocyte percentage and the GSH levels after the incubation of blood are shown in Figure 9.3.

It was apparent that:

1. Significant ($p < 0.05$) differences in the GSH level between the control and infected groups occurred both at the peak of parasitaemia and as parasitaemia declined. The pattern of increase in the control group, and decrease in the infected group, was similar to the pre-incubation pattern (compare Figures 9.2 and 9.3).
2. The GSH levels in the infected groups following incubation of blood were not correlated significantly

TABLE XL

CORRELATIONS BETWEEN RETICULOCYTE PERCENTAGE AND SOME REDUCED GLUTATHIONE (GSH) PARAMETERS IN E. OVIS INFECTED SHEEP

EXPERIMENT VII

GSH (mgs/100 ml Erythrocytes)	Degrees of Freedom	Group B r	Group B p	Group C r	Group C p
Total GSH	11	-0.50	NS	-0.16	NS
Mean difference between control and infected groups	11	-0.77	< 0.01	-0.62	<0.05
Total GSH following incubation without Acetylphenylhydrazine	8	-0.51	NS	-0.50	NS
Fall after incubation without Acetylphenylhydrazine	8	0.19	NS	0.34	NS
Mean difference after incubation without Acetylphenylhydrazine	8	0.06	NS	0.33	NS
Total GSH following incubation with Acetylphenylhydrazine	10	-0.37	NS	-0.61	<0.05
Fall aften incubation with Acetylphenylhydrazine	10	0.30	NS	0.51	NS
Mean difference after incubation with Acetylphenylhydrazine	10	0.24	NS	0.10	NS

r = Correlation Coefficient P = Probability NS = Not Significant (P`>0.05)

TABLE XLI

CORRELATIONS BETWEEN DEGREE OF PARASITAEMIA AND SOME REDUCED GLUTATHIONE (GSH) PARAMETERS IN *E. OVIS* INFECTED SHEEP - EXPERIMENT VII

GSH (mgs/100 ml Erythrocytes)	Degrees of Freedom	Group B r	Group B p	Group C r	Group C p
Total GSH	11	0.05	NS	0.18	NS
Mean difference between control and infected groups	11	0.17	NS	0.08	NS
Total GSH after incubation without Acetylphenylhydrazine	8	-0.11	NS	0.15	NS
Fall after incubation without Acetylphenylhydrazine	8	0.40	NS	0.52	NS
Mean difference after incubation without Acetylphenylhydrazine	8	0.59	NS	0.83	< 0.01
Total GSH after incubation with Acetylphenylhydrazine	10	-0.84	< 0.01	-0.73	< 0.01
Fall after incubation with Acetylphenylhydrazine	10	0.76	< 0.01	0.77	< 0.01
Mean difference after incubation with Acetylphenylhydrazine	10	0.70	< 0.05	0.82	< 0.01

r = Correlation Coefficient P = Probability NS = Not Significant (P > 0.05)

with either reticulocyte percentage (Table XL) or the degree of parasitaemia (Table XLI). The mean fall in GSH (mg per 100 ml erythrocytes) from the pre-incubation level in control and infected groups relative to the mean degree of parasitaemia and reticulocyte percentage is shown in Figure 9.4. Although the infected groups tended to show a larger fall in GSH than the control group, only one reading was significantly ($p < 0.05$) different; the correlation of this fall with the degree of parasitaemia, although positive, was not significant (Table XLI).

The correlations of the mean difference in GSH level between the control and infected groups, with the degree of parasitaemia were also positive and in group C was highly significant ($p < 0.01$) (Table XLI). However correlations of fall in GSH level, and the mean differences between the infected and control groups in GSH values, with the reticulocyte percentage were small and not significant (Table XL).

Incubated blood - with acetylphenylhydrazine: Incubation of blood with acetylphenylhydrazine for 2 hours at 37°C caused a large fall in the GSH level of all groups. The overall mean control level was 42.96 ± 2.05 mg per 100 ml erythrocytes, which was less than half the pre-incubation level. The time relationships between the mean degree of parasitaemia, reticulocyte percentage, and the GSH level in the infected groups, and the GSH level in the control group are shown in Figure 9.5. From the figure it is apparent that:

1. The level of GSH in the infected groups fell almost to zero.
2. The correlations between the degree of parasitaemia and the GSH level in the infected groups were negative and significant ($p < 0.01$) (Table XLI).
3. The correlations between the reticulocyte percentage and the GSH level in the infected groups were also negative and, in the case of Group C, was significant ($p < 0.05$) (Table XL).

The size of the fall in GSH in mg per 100 ml of erythrocytes for all groups, after incubation of the blood with acetylphenylhydrazine, is shown in Figure 9.6. Although the size of the fall was variable it was significantly ($p < 0.05$) higher in the infected groups at the peak parasitaemic stage. This fall and the mean difference between the infected and control groups at each reading were significantly ($p < 0.05$) correlated with the degree of parasitaemia (Table XLI) but not with reticulocyte percentage (Table XL).

The Effect of E. ovis Infection on Blood Methaemoglobin Levels

Non-incubated blood: The overall mean level of methaemoglobin in the blood of the 7 control sheep was 0.45 ± 0.04 gm per 100 ml. This was $3.85 \pm 0.33\%$ of the total Hb level. The time relationships between the mean degree of parasitaemia, reticulocyte percentage and methaemoglobin level in the 2 *E. ovis* infected groups (B and C) and the methaemoglobin level in the control group (A) are shown in Figure 9.7.

It can be seen that:

1. The methaemoglobin levels varied with time and a considerable increase occurred in all groups from day 36 of the experiment.
2. In the infected groups the level of methaemoglobin was significantly ($p < 0.05$) correlated with reticulocyte percentage (Table XLII) but not with the degree of parasitaemia (Table XLIII).

Because of variation recorded in the control methaemoglobin levels during the experiment (Figure 9.7) the mean differences between the infected groups and the control group were calculated at each reading. These are plotted relative to the control values (Figure 9.7). The infected groups had higher levels of methaemoglobin than the control group during parasitaemia but these were only occasionally significant ($p < 0.05$) (Figure 9.7). However the correlation of this mean difference with the degree of parasitaemia was

TABLE XLII

CORRELATIONS BETWEEN RETICULOCYTE PERCENTAGE AND SOME METHAEMOGLOBIN PARAMETERS IN E. OVIS INFECTED SHEEP - EXPERIMENT VII

	Degrees of Freedom	Group B		Group C	
		r	p	r	p
Methaemoglobin (gms/100 ml)	12	0.62	< 0.05	0.62	<0.05
Mean difference between control and infected groups (Methaemoglobin)	12	0.09	NS	0.20	NS
Methaemoglobin (% of total Hb)	12	0.64	< 0.05	0.81	<0.01
Mean difference between control and infected groups (% total Hb)	12	0.77	< 0.01	0.69	<0.01
Methaemoglobin (gms/100 ml) after incubation for 2 hours at 37°C	8	0.14	NS	0.48	NS
Mean difference between control and infected groups (Methaemoglobin) after incubation for 2 hours at 37°C	8	0.25	NS	0.10	NS
Methaemoglobin (gms/100 ml) after incubation with Acetylphenylhydrazine	8	-0.43	NS	-0.39	NS
Mean difference between control and infected groups (Acetylphenylhydrazine)	8	0.39	NS	0.51	NS
Mean difference between control and infected groups (%) Acetylphenyl-hydrazine)	8	0.05	NS	0.05	NS
Methaemoglobin (% of total Hb) after incubation with Acetylphenylhydrazine	8	-0.05	NS	0.07	NS

r = Correlation Coefficient P = Probability NS = Not Significant (p > 0.05)

TABLE XLIII

CORRELATIONS BETWEEN DEGREE OF PARASITAEMIA AND SOME METHAEMOGLOBIN PARAMETERS IN E. OVIS INFECTED SHEEP - EXPERIMENT VII

	Degrees of Freedom	Group B r	Group B P	Group C r	Group C P
Methaemoglobin (gms/100 ml)	12	0.0018	NS	0.22	NS
Mean difference between control and infected groups (Methaemoglobin)	12	0.21	NS	0.54	< 0.05
Methaemoglobin (% of total Hb)	12	0.25	NS	0.45	NS
Mean difference between control and infected groups (% total Hb)	12	0.71	< 0.01	0.77	< 0.01
Methaemoglobin (gms/100 ml) after incubation for 2 hours at 37°C	8	0.53	NS	0.17	NS
Mean difference between control and infected groups (Methaemoglobin) after incubation for 2 hours at 37°C	8	0.64	< 0.05	0.62	NS
Methaemoglobin (gms/100 ml) after incubation with Acetylphenylhydrazine	8	-0.45	NS	-0.59	NS
Mean difference between control and infected groups (Acetylphenylhydrazine)	8	0.64	< 0.05	0.78	< 0.01
Mean difference between control and infected groups (%) Acetylphenylhydrazine	8	0.65	< 0.05	0.77	< 0.01
Methaemoglobin (% of total Hb) after incubation with Acetylphenylhydrazine	8	-0.38	NS	-0.48	NS
Reticulocyte Percentage	12	0.34	NS	0.42	NS

r = Correlation Coefficient P = Probability NS = Not Significant (P > 0.05)

significant ($p < 0.05$) in Group C (Table XLIII) and higher in both infected groups than the correlation with reticulocyte percentage (Tables XLII and XLIII).

The level of methaemoglobin as a percentage of total haemoglobin was calculated and is shown together with the degree of parasitaemia and reticulocyte percentage in Figure 9.8. The mean differences between the infected and control groups at each reading in methaemoglobin percentage are shown relative to the control values in the figure. The data show that:

1. The percentage level of methaemoglobin followed a pattern similar to total methaemoglobin with increases in all groups occurring in the latter half of the experiment.
2. The increase in the infected groups was correlated significantly ($p < 0.05$) with the reticulocyte percentage (Table XLII) but not with the degree of parasitaemia (Table XLIII).
3. With adjustment of the methaemoglobin data, for the degree of anaemia, i.e. by expressing the levels as a percentage of total haemoglobin, the mean difference calculations revealed a significantly ($p < 0.05$) higher percentage of methaemoglobin in these infected groups.
4. The mean differences between the infected and control groups were significantly ($p < 0.01$) correlated with both the degree of parasitaemia (Table XLIII) and the reticulocyte percentage (Table XLII).

Incubated blood - without acetylphenylhydrazine: The overall mean methaemoglobin level in the blood of the control sheep, following incubation for 2 hours at 37°C was 0.24 ± 0.05 gm per 100 ml. The time relationships between mean reticulocyte percentage, degree of parasitaemia and methaemoglobin levels following incubation in this manner are shown in Figure 9.9. The mean differences between the infected and control groups at each reading, in methaemoglobin levels are also shown relative to the control values in the figure. The results showed that:

1. The methaemoglobin levels in all groups were, with few exceptions, less than the pre-incubation value.
2. There were some significant ($p < 0.05$) increases in levels in infected groups which coincided with the peak of parasitaemia.
3. The methaemoglobin levels in the infected groups did not however correlate significantly with either the degree of parasitaemia (Table XLIII) or reticulocyte percentage (Table XLII).
4. The mean difference readings between the infected groups and the control group were not significantly correlated with the reticulocyte percentage (Table XLII), but were significantly ($p < 0.05$) correlated in Group B and nearly significantly correlated (p approx. 0.06) in Group C with the degree of parasitaemia (Table XLIII).

Incubated blood - with acetylphenylhydrazine: The overall mean methaemoglobin level in the control sheep following incubation with acetylphenylhydrazine for 2 hours at 37°C was 3.49±0.12 gm per 100 ml. This was 30.54±0.98% of total Hb, and represented a 7.5 fold increase over the pre-incubation level.

The time relationships between the mean degree of parasitaemia, reticulocyte percentage and methaemoglobin level following incubation with acetylphenylhydrazine are shown in Figure 9.10. The mean differences in the methaemoglobin level between the infected and control groups, at each reading are also shown relative to the control values in the figure. The results show that:

1. The level of methaemoglobin in all the groups fluctuated, but in contrast to pre-incubation patterns, the level was consistently higher in the control group than in the 2 infected groups.
2. In the 2 infected groups correlations of the methaemoglobin level with the reticulocyte percentage and the degree of parasitaemia were negative but not significant (Tables XLII and XLIII).

3. The mean differences calculations revealed significantly ($p < 0.05$) lower levels of methaemoglobin in the infected groups.
4. The mean differences were correlated significantly ($p < 0.05$) with the degree of parasitaemia (Table XLIII) but not with the reticulocyte percentage (Table XLII).

The time relationships between the mean degree of parasitaemia, reticulocyte percentage and the methaemoglobin level as a percentage of total Hb following incubation with acetylphenylhydrazine are shown in Figure 9.11. The mean difference calculations between the infected and control groups for methaemoglobin percent are also shown relative to the control values in the figure. From Figure 9.11 it can be seen that:

1. Although the E. ovis infected sheep were anaemic and methaemoglobin levels increased on incubation with acetylphenylhydrazine, the percentage of haemoglobin in the form of methaemoglobin was significantly ($p < 0.05$) less in the infected groups than in the control group whilst parasitaemia was at peak levels.
2. The mean difference readings between the infected and control groups for methaemoglobin percentage were significantly ($p < 0.05$) correlated with the degree of parasitaemia (Table XLIII) but not with the reticulocyte percentage (Table XLII).

The Effect of Incubation for 2 Hours at 37°C of Control and E. ovis Infected Blood with and without Acetylphenylhydrazine on the Formation of Heinz Bodies

Following incubation for 2 hours at 37°C, without acetylphenylhydrazine, the blood from both infected and control sheep was found to contain small numbers of erythrocytes with, usually single, Heinz bodies. The percentage of erythrocytes affected was variable but there was a tendency for a higher frequency of infected erythrocytes to be involved (Figure 9.12, a and b).

Following incubation with acetylphenylhydrazine most erythrocytes of both infected and non-infected blood contained variable numbers of Heinz bodies (Figure 9.12, c and d). No apparent difference in the frequency and size of these bodies was apparent between the infected and non-infected blood. Some of these acetylphenylhydrazine incubated erythrocytes stained diffusely with the methyl violet (see Figure 9.12, c and d). This phenomenon was not apparent in the erythrocytes incubated without acetylphenylhydrazine, and occurred with similar frequency in both E. ovis infected and control blood.

The Relationship Between Methaemoglobin and Reduced Glutathione (GSH) in Normal and E. ovis Infected Blood Before and After Incubation, With and Without Acetylphenylhydrazine

The correlation between GSH and methaemoglobin levels for the control and infected groups before and after incubation, with and without acetylphenylhydrazine are shown in Table XLIV. In control blood there was no correlation between the 2 parameters, but in the infected blood, there was a negative correlation recorded for both groups, which was significant ($p < 0.01$) in Group B.

Incubation without acetylphenylhydrazine caused some fall in both parameters (Figures 9.3 and 9.9). All groups showed a positive correlation between the 2 parameters, but only the coefficient for the control group approached significance (Table XLIV).

Incubation with acetylphenylhydrazine caused a marked fall in GSH and a marked increase in methaemoglobin. All groups showed a significant ($p < 0.05$) positive correlation between the 2 parameters.

TABLE XLIV

CORRELATION BETWEEN METHAEMOGLOBIN AND REDUCED GLUTATHIONE (GSH) BEFORE AND AFTER INCUBATION WITH AND WITHOUT ACETYLPHENYLHYDRAZINE

EXPERIMENT VII

Treatment	Degrees of Freedom	Control A		Infected B		Infected C	
		r	p	r	p	r	p
Before Incubation	11	0.07	NS	-0.73	<0.01	-0.51	NS
Incubation with Acetylphenylhydrazine	8	0.88	<0.01	0.87	<0.01	0.71	<0.05
Incubation without Acetylphenylhydrazine	8	0.630[x]	NS	0.23	NS	0.39	NS

r = Correlation Coefficient

P = Probability

NS = Not Significant (P >0.05)

x P <0.05 when r = 0.632

DISCUSSION

Methaemoglobin and Reduced Glutathione (GSH) Levels in Non-Incubated Blood from Normal and E. ovis Infected Sheep

Control values: Values for the GSH level in sheep erythrocytes have been reported by a number of investigators (Smith and Osburn, 1967; Kaneko and Smith, 1964; Tucker and Kilgour, 1970; Tucker, 1971; Agar et al., 1972) and are summarised in Table XLV. The discovery by Smith and Osburn (1967) that some sheep had GSH levels which were less than 20% of the mean level in other sheep, was later shown to be an inherited autosomal recessive disorder (Tucker and Kilgour, 1970). This has led to a classification of normal (GSH^H) and glutathione deficient (GSH^h) sheep (Table XLV).

In this investigation, which was on sheep of the Romney and Perendale breeds, no GSH^h sheep were detected, and no apparent difference in GSH values between the 2 breeds was noted. The mean value obtained for the control group was similar to that obtained by other investigators with other breeds of sheep. Although some fluctuation in the control values was recorded during the experiment, there was a tendency for the levels to rise as the experiment continued (Figure 9.2). It is probable that the removal of 20 ml of blood at each bleeding, while not producing a reticulocyte response, would lead to some decrease in the average age of the erythrocyte population. Although Prankerd (1958) observed no variation in the GSH content of human erythrocytes with age, Sass et al. (1965) found a decrease with increasing age of erythrocyte. In sheep erythrocytes there is a distinct increase in GSH content when erythrogenesis is most active (Todd and Ross, 1968). A similar situation has been described in cattle erythrocytes (Steensgaard and Møller, 1970).

The level of methaemoglobin in control blood recorded in the early stages of the experiment was similar to that recorded for human blood (Henry, 1964; Tonz, 1968). The

TABLE XLV

Reduced glutathione (GSH) levels of sheep.
GSH^H - sheep with normal levels of reduced glutathione.
GSH^h - sheep deficient in reduced glutathione.

Author	Breed	Value (mgs/100 ml of red cells.)	Method
Kaneko and Smith (1963)***	Suffolk	49.0 ± 3.1*	Beutler (1957)
	GSH^H Values		
Tucker and Kilgour (1970)	Finnish Landrace	96.65 ± 2.09	Beutler et al. (1963)
	Clun Forest	89.85 ± 1.10	
	Soay	85.82 ± 1.06	
	Merino Cross	89.25 ± 2.72	
	Welsh Mountain	83.82 ± 3.78	
	Shetland	89.01 ± 5.74	
Agar et al. (1972)	Border Leicester	88.3 ± 1.95	Roberts and Agar (1971)**
	Corriedale	112.4 ± 2.93	
	Dorset Horn	108.9 ± 1.57	
	Poll Dorset	109.9 ± 1.46	
	Merino	108.3 ± 1.56	
Smith and Osburn (1967)	-	104.0 ± 2.72	Beutler et al. (1963)
	GSH^h Values		
Tucker and Kilgour (1970)	Finnish Landrace	31.07 ± 1.70	
	Soay	48.17	
	Merino Cross	19.46	
Agar et al. (1972)	Corriedale	33.4	
	Merino	42.8 ± 1.60	
Smith and Osburn (1967)	-	11.4	Beutler et al. (1963)
Sutton (EXP 7)	Romney	95.75 ± 1.85	Beutler et al.(1963)

* Standard error.

** This is an automated version of the manual method of Beutler et al (1963) which gives results approximately 20% higher.

*** These results are similar to those of GSH^h sheep.

reason for the increase which occurred in both the infected and control groups during the latter half of the experiment (Figure 9.7) is obscure. All groups lost weight at this time, presumably because of a reduced standard of nutrition and this may account for the observed increase (Chapter V). It has been shown that the degree of methaemoglobinaemia may vary with the quality of the diet. For example, experimental nitrite poisoning with accompanying methaemoglobinaemia has been recorded in animals fed an inadequate ration after dosing with nitrite at a level which is innocuous to animals fed on good ration (Gwatkin and Plummer, 1946). The possibility cannot be discounted that a low level diet could facilitate the absorption of such chemicals as hydroxylamine which is a catabolic product of nitrite (Nicholas, 1959) normally found in the rumen of sheep (Holtenius, 1957; Jamieson, 1958; Winter, 1962) thereby leading to a higher level of methaemoglobin.

Resulting from this increase in blood methaemoglobin was a significant correlation ($p < 0.05$) in the infected groups between methaemoglobin levels and the reticulocyte percentage (Table XLII), although correlations with the degree of parasitaemia were not significant (Table XLIII). However in view of the fact that the increase in control methaemoglobin levels was similar to that in the infected groups, and that reticulocytes are relatively more resistant to methaemoglobin formation (Matthies, 1956; Bethlenfalvay, 1971), it seems likely that the correlation between methaemoglobin and reticulocyte percentage is coincidental.

Values in infected blood: In infected blood the GSH levels were slightly lower than the control values during the development of, and at peak, parasitaemia. With decreasing parasitaemia in the latter half of the experiment, infected values showed a significant fall (Figure 9.2) relative to control values. Although the difference between the infected and control groups was significantly ($p < 0.05$) correlated with reticulocyte percentage (Table XL), 2 contradictory points arise. The first is that the difference

between the infected and control groups is due, in part, to an increase in control levels, which could result from the presence of young erythrocytes. However, the decline in the infected groups occurred in spite of the presence of larger numbers of immature cells.

As the glycolytic metabolism of erythrocytes infected with E. ovis appears to be similar to that of erythrocytes infected with plasmodia (Chapter VII), it is possible that the nutritional demands are similar in spite of their different morphological relationship with the erythrocyte. It has been shown that P. knowlesi utilizes GSH for some of its protein requirements (Fulton and Grant, 1956). In the earlier stages of malarial infection there is a tendency for GSH levels to remain normal or even increase slightly (Fulton and Grant, 1956; Sherman, 1965; George and Pollack, 1966; Fletcher and Maegraith, 1970). By analogy this would suggest that early in E. ovis infection the erythrocyte's capacity for regenerating GSH is able to meet any nutritive demand by the parasite. In the later stages of parasitaemia, where it might be expected that the nutritive demands of the parasite are much less, it is possible that the erythrocyte may have difficulty maintaining adequate GSH levels, either as a result of GSH and oxidized glutathione (GSSG) store depletion by the parasite, or of a diminished capacity for GSH regeneration caused by the parasite. The fact that G6PD activity is not lowered during parasitaemia (Chapter VII) indicates that pentose phosphate pathway activity and hence NADPH regeneration is probably not affected. The possibility of some interference, either directly or indirectly on NADPH utilization, by glutathione reductase must be considered.

The presence of considerable numbers of reticulocytes, and other young erythrocytes, which under normal circumstances would tend to increase the mean erythrocyte GSH level in the infected blood (Todd and Ross, 1968; Sass et al., 1965), indicates that some erythrocytes may be more heavily depleted in GSH than the mean values would suggest.

Absolute methaemoglobin values were slightly higher in E. ovis infected blood than in control blood, but only occasional significant differences were recorded (Figure 9.7). As a percentage of total haemoglobin, the levels of methaemoglobin in infected blood were considerably higher than control levels, but did not reach a level of clinical significance (Prewitt and Derilan, 1958). It has been calculated that, because of the normally low concentration of methaemoglobin, the reductive systems are at least 250 times as active as the oxidative ones (Fornaini and Bossu, 1969). The 4 systems which are known to contribute to the in vitro reduction of methaemoglobin utilize GSH, NADH-dependent diaphorase, ascorbic acid and NAPDH dependent methaemoglobin reductase (Scott et al., 1965). From the evidence available, the 2 systems that may be affected during E. ovis infection are the ones that utilize GSH and methaemoglobin diaphorase. Although there is a significant negative correlation between absolute methaemoglobin level and GSH level in infected blood (Table XLIV), which suggests that a fall in GSH may cause methaemoglobin levels to rise, the NADH-dependent diaphorase is probably the only methaemoglobin reducing system effective in vivo (Tonz, 1968). It seems therefore that the correlation between GSH and methaemoglobin is a result of parasitic activity on each parameter individually, rather than a direct effect between the two. Regeneration of NADH is dependent mainly on the activity of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase.

Because of the apparently high level of anaerobic glycolysis in infected blood (Chapter VII), the possibility of glycolytic substrate depletion by the parasite with a resultant decrease in the efficiency of the NADH diaphorase system of the erythrocyte must be considered a possible cause of the slight increase in the methaemoglobin level of infected blood. The other reducing systems are believed to only play a role in vivo, when there is an excess of methaemoglobin, or in the presence of some specific oxidizing chemicals (Tonz, 1968).

The Effect of Incubation of Blood from E. ovis Infected and Normal Sheep on Reduced Glutathione (GSH) and Methaemoglobin Levels

Incubation without acetylphenylhydrazine: The effect of 2 hours incubation at 37°C on the level of GSH in both E. ovis infected and control bloods was to produce a fall in the level of GSH comparable to that shown by Allen and Jandl (1961). At the peak of parasitaemia this fall was higher in the infected groups with a significant ($p < 0.05$) difference between the infected and control groups being recorded (Figure 9.4).

It is well established that at a physiological pH, spontaneous oxidation of sulphydryl group compounds such as GSH does occur, and is influenced by pH change as well as oxidant drugs (Allen and Jandl, 1961). At an acid pH, which might be expected in the infected blood as a result of the increased glycolytic activity, the compounds are more stable. However, the possibility of increased GSH utilization for nutritional and synthetic purposes by the parasite in the infected blood may more than offset the slower oxidation which could be expected to occur with the lower pH.

Jandl et al. (1960) studied the effect of prolonged incubation of blood at 37°C on haemoglobin and found a very slow conversion to methaemoglobin during the first 2 days which subsequently became more rapid. At the end of a week about 70% of the haemoglobin had been converted to methaemoglobin. In this study, the level of methaemoglobin in the control group was less than the pre-incubation level (Figures 9.7 and 9.9). There was, however, an increase of methaemoglobin in the blood of the infected animals, which correlated with the degree of parasitaemia (Table XLIII). The erythrocyte in circulation is subject to many oxidative processes which probably arise in a natural way from soluble components of the blood (Kosower and Kosower, 1969). These processes may not be as evident in vitro. What role other reducing agents play in such circumstances is uncertain, but

results from *in vitro* studies have shown some contribution to methaemoglobin reduction by factors other than the NADH-dependent diaphorase (Scott *et al.*, 1965). Any reduction in methaemoglobin level which does occur can only be considered to be transitory as depletion of glucose due to continuing glycolytic activity will lead to a slow and steady methaemoglobin formation as described by Allen and Jandl (1961). The higher methaemoglobin levels recorded in the *E. ovis* infected blood after incubation are probably the result of circumstances previously described for non-incubated blood.

Incubation with acetylphenylhydrazine: It has been known for some time that certain drugs such as 8 amino quinoline, primaquine, acetanilid, sulphanilamide, and nitrofurantoin will, under some circumstances, induce in man a haemolytic anaemia (Cordes, 1926; Hochwald *et al.*, 1952; Dern *et al.*, 1954; Beutler *et al.*, 1955a, b; Beutler, 1957, 1959). It was demonstrated that the GSH content of sensitive human erythrocytes tended to be below normal (Beutler *et al.*, 1955b) and fell markedly when cells were incubated with acetylphenylhydrazine (Beutler, 1957).

Although as yet not properly defined, it is believed that oxidation mechanisms in the normal erythrocyte are mediated through the formation of H_2O_2 and free radicals (Fornaini and Bossu, 1969). These are reduced through the action of the enzymes catalase and glutathione peroxidase. The oxidized glutathione (GSSG), which is formed as a result of the peroxidative destruction of hydrogen peroxide, is in turn reduced through the mediation of glutathione reductase, with consequent oxidation of NADPH (Figure 9.1). The instability of GSH level in the drug sensitive haemolytic anaemias was shown by Cohen and Hochstein (1961) to be a result of low glucose-6-phosphate dehydrogenase (G6PD): this deficiency was first described in drug sensitive erythrocytes by Carson *et al.* (1956). It is believed that G6PD deficiency results in a failure to reduce NADP with a consequent fall in GSH an important source of reductive potential in the erythrocyte (Szeinberg and Marks, 1961).

The action of acetylphenylhydrazine and oxidant compounds on the sulphydryl groups of the erythrocytes was shown by Allen and Jandl (1961) to proceed in order of decreasing reaction rate as follows:

1. The reaction of oxygen with the compounds (acetylphenylhydrazine) to form oxidized derivatives.
2. The reaction of the oxidized derivatives with glutathione (GSH).
3. The reaction of the oxidized derivatives with the ferrous hemes to form methaemoglobin.
4. The reaction of the oxidized derivatives with the haemoglobin sulphydryl groups to form Heinz bodies (Figure 9.13).

The exact mode of action of acetylphenylhydrazine on GSH is uncertain. While Beutler et al. (1957) considered that acetylphenylhydrazine did not destroy GSH and other sulphydryl group compounds, but produced an altered form of haemoglobin which oxidizes GSH, Allen and Jandl (1961) found that acetylphenylhydrazine alone oxidizes GSH once it has been converted in the presence of oxygen to an active form; this conversion is catalysed either by haemoglobin or trace metals. Cohen and Hochstein (1963) who showed that under normal physiologic conditions glutathione peroxidase was the main pathway of H_2O_2 reduction, later found (Cohen and Hochstein, 1964) that phenylhydrazine and therefore, presumably, acetylphenylhydrazine interacted with H_2O_2 in 2 ways. The first was by direct action with molecular oxygen to generate H_2O_2 and the second was by reaction with oxyhaemoglobin to form free O_2 and/or a haematin-H_2O_2 complex. The H_2O_2 then brings about a depletion of GSH by oxidation in G6PD deficient erythrocytes.

In this study on sheep erythrocytes, both normal and E. ovis infected cells showed a large fall in GSH level on incubation with acetylphenylhydrazine (Figure 9.5). This fall was significantly ($p < 0.05$) higher in the infected erythrocytes (Figure 9.6) and correlated significantly ($p < 0.01$) with the degree of parasitaemia, but not with

reticulocyte percentage. The fall in the normal sheep erythrocytes was similar to that described by Kaneko and Smith (1964), and intermediate between normal human erythrocytes and those which are G6PD deficient (Beutler et al., 1955b; Beutler, 1957). Although the level of the enzyme G6PD in sheep erythrocytes is similar to that of a G6PD deficient human erythrocyte, and therefore a likely explanation for the apparent GSH instability of the normal sheep erythrocyte, the following points must be considered:

1. Sheep erythrocytes do not undergo haemolysis in vivo when large amounts of primaquine are administered (Carson, 1960; Smith, 1968; Maronpot, 1972), where as G6PD deficient human erythrocytes do.
2. Cattle erythrocytes show a similar GSH instability, although their G6PD level is similar to the normal human level (Kaneko and Mills, 1969; Steensgaard and Møller, 1970).
3. Using the methaemoglobin reduction test of Brewer et al. (1960) to measure the ability of sheep erythrocytes to generate NADPH and hence indirectly their G6PD activity, Brown (1963) and Kaneko and Smith (1964) found levels similar to those of G6PD deficient human erythrocytes. In contrast Salividio et al. (1963) and Smith and Beutler (1966) obtained a higher level of methaemoglobin reduction intermediate between the G6PD deficient and normal human erythrocyte.
4. In the incubation of normal human erythrocytes a lack of glucose leads to GSH instability (Beutler et al., 1957; Cohen and Hochstein, 1963; Davies and Gower, 1964).

From these points it is apparent that despite a normal G6PD level in the sheep erythrocyte which is similar to a G6PD deficient human erythrocyte, the sheep cells can use the pentose phosphate pathway at a higher rate than the deficient human cell and maintain a GSH level comparable to that of other species (Table XLVI). In a study of the kinetic characteristics of G6PD in the sheep erythrocyte Smith and Holdridge (1967) were, however, unable to provide

TABLE XLVI

REDUCED GLUTATHIONE (GSH) LEVELS IN SPECIES OTHER THAN SHEEP

Author	Species	Value mgs/100 ml Erythrocytes	Method
Beutler (1957)	Human	66.3 ± 9.6^{x}	Beutler (1957)
Beutler et al. (1963)	Human	58.55 ± 0.24^{xx} 66.69 ± 0.94 68.89 ± 0.95 62.74 ± 0.68	Stevenson et al. (1960)
		59.53 ± 0.45 67.76 ± 0.43 69.01 ± 0.88 63.04 ± 0.70	Beutler et al. (1963)
Kaneko and Mills (1969)	Bovine	89 ± 14.2^{x}	Beutler (1957)
Sherman (1965)	Duck	145.0	Beutler (1957)
Fletcher and Maegraith (1970)	Mice	65.0 ± 15.1^{x}	Kay and Murfitt (1960)
	Rhesus Monkey	125.0 ± 30.0^{x}	

x = Standard Deviation

xx = 2 X Standard Error

an explanation for the apparent ability of the cell to utilize the pentose-phosphate pathway. The possibility (Salvidio et al., 1963) that the erythrocytes of some animal species may have different metabolic pathways associated with the protection of erythrocytes, but dissociated from the NADPH linked pentose phosphate pathway cannot be discounted in the sheep.

Kaneko and Mills (1969) considered that the comparative GSH instability of normal sheep erythrocytes was due to a low normal level of blood glucose, which may lead to a lack of necessary substrate in the GSH maintaining pathways. It was shown earlier (Chapter VII) that infected erythrocytes had a very high consumption of glucose, and as no glucose was added to the blood incubated with acetylphenylhydrazine, the bigger fall in GSH level which occurred in the E. ovis infected erythrocytes at the peak of parasitaemia (Figures 9.5 and 9.6) would be expected.

Other effects of acetylphenylhydrazine incubation on both E. ovis infected and control blood were:

1. The formation of a high level of methaemoglobin Figures 9.10 and 9.11).
2. A diffuse staining reaction similar to that of the coccoid (Heinz) bodies present in some erythrocytes of both infected and control blood (Figure 9.12).
3. A large number of coccoid bodies, which were presumably Heinz bodies, formed in most erythrocytes (Figure 9.12).

The higher level of methaemoglobin formed in the control blood compared with infected blood was probably due to differences in the relative advancement of haemoglobin oxidation within the erythrocyte (Figure 9.13). The depletion of GSH with acetylphenylhydrazine incubation in infected blood would lead to the oxidation process advancing further beyond the methaemoglobin stage.

The diffuse staining reaction occurring in some

erythrocytes of both infected and non-infected blood as distinct from the definite coccoid (Heinz) bodies formed (Figure 9.12 c, d) may be due to the presence of denatured haemoglobin products other than true methaemoglobin (Mills, 1959; Jandl et al., 1960). These denatured products which cause spectroscopic interference with methaemoglobin, gradually supplant methaemoglobin at a rate dependent on the concentration of the oxidizing drug. Included in this group are the pyridine haemochromagens (sulph-haemoglobins) which are soluble, and the "green haemoglobins" which may not contain intact porphyrin rings and are relatively insoluble.

Heinz body formation is the last stage of the oxidative haemolytic process in the erythrocyte (Figure 9.13). It was not possible however, because of variability in size and number, to determine the relative degree of Heinz body formation between the E. ovis infected and control groups (Figure 9.12). The importance of Heinz bodies in the pathogenesis of haemolytic anaemias has been the subject of some study. The spleen is believed to play an active role in the removal of affected cells (Rothberg et al., 1959; Rifkind and Danon, 1965; Weed and Reed, 1965), and phagocytosis by neutrophils in the peripheral circulation is thought to be another means of removal (Simpson, 1971). However Dacie (1967) suggested that associated factors must be considered in the haemolytic process. These include the overwhelming of the mechanisms that maintain GSH and as a consequence an inability to generate GSH. As a result of this the erythrocyte membrane is affected, leading to alteration in morphology, osmotic fragility and permeability, and eventual haemolysis.

The Possible Clinical Effect Arising from Alterations in Erythrocyte GSH and Methaemoglobin Levels during E. ovis Infection

Eperythrozoon ovis infection causes some fall in erythrocyte GSH level, and some increase in methaemoglobin level, but neither alteration is great, and in the case of

methaemoglobin does not reach a level of clinical significance. It has been shown that very low levels of GSH as such do not cause erythrocyte destruction (Smith and Osburn, 1967; Kosower and Kosower, 1969; Tucker and Kilgour, 1970; Lo et al., 1971). However it is worthy of note that the GSH level in infected blood does not fall until after anaemia has started to develop; where it may be expected that some rise in GSH would occur, due to the presence of reticulocytes. It appears that parasitized erythrocytes at this latter stage of infection may have a diminished capacity for GSH regeneration, and therefore, because of continual chemical challenges to which they may be subjected by soluble components in the blood (Kosower and Kosower, 1969) may not survive as long as normal erythrocytes. The fall in erythrocyte GSH levels may in itself be a reason for the decreasing parasitaemia as it has been shown that humans with G6PD deficient erythrocytes have a selective advantage against the malarial parasites (Luzzatto et al., 1969) where it is thought that the parasites are inhibited by the build up of oxidized glutathione levels (Kosower and Kosower, 1970).

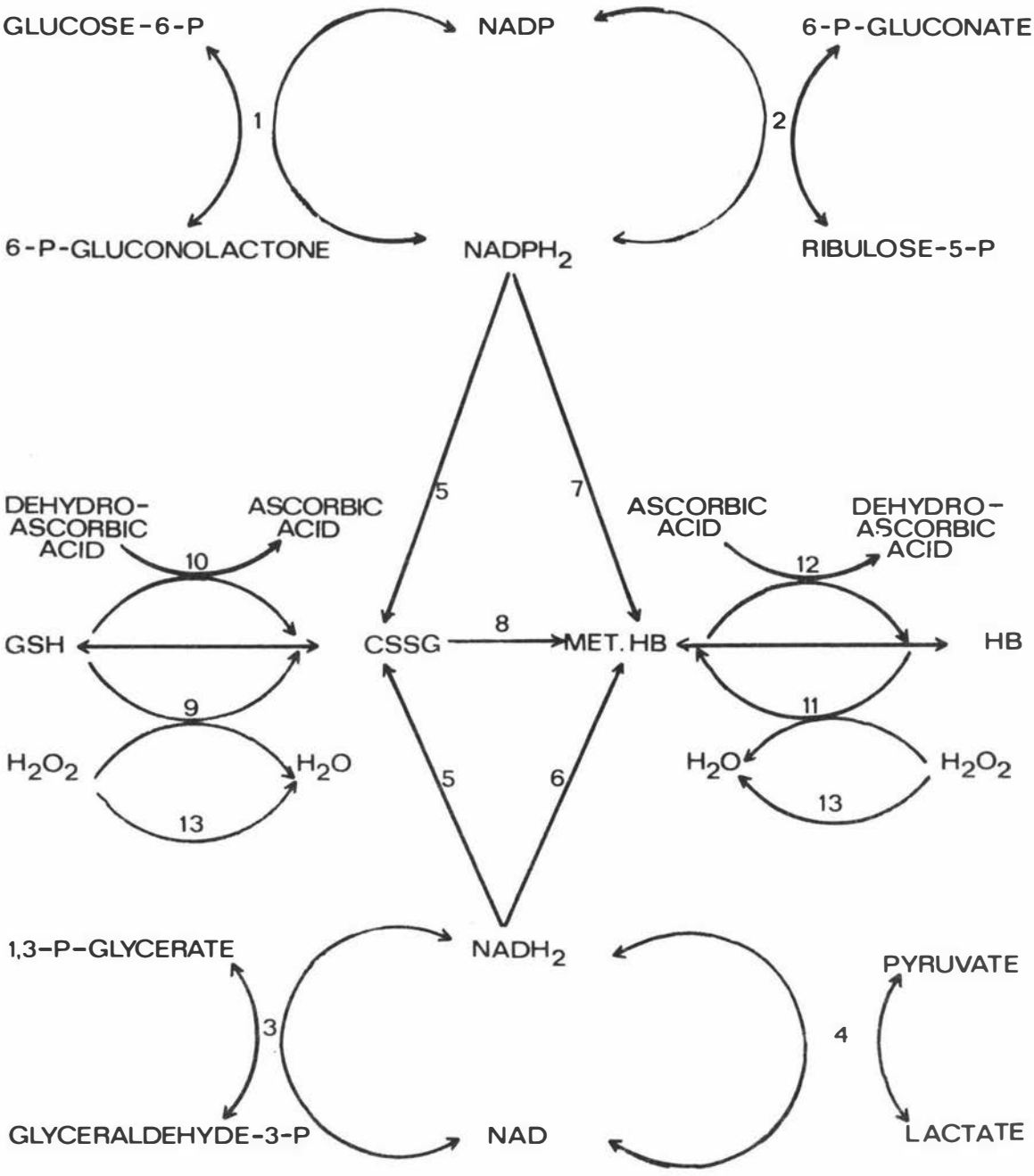
Although it has been shown that low GSH activity in erythrocytes does not cause haemolysis, it does make the erythrocyte more prone to oxidative destruction (Kosower and Kosower, 1969; Tucker and Kilgour, 1970; Lo et al., 1971). This has been shown both in the G6PD deficient human erythrocyte where oxidative challenge by drugs such as primaquine lead to a haemolytic anaemia due to a failure of GSH regeneration (Beutler, 1959), and in the sheep erythrocyte where the haemolytic crisis of copper poisoning is associated not only with a fall in GSH, but with some rise in blood methaemoglobin level (Todd and Thompson, 1963, 1964). The fall of GSH levels to negligible value in infected erythrocytes under the influence of acetylphenylhydrazine, compared with the higher level in control erythrocytes, would suggest that while E. ovis infection does not decrease GSH sufficiently to induce haemolysis by breakdown of erythrocyte membrane integrity, it does increase the susceptibility of the erythrocyte to oxidative haemolysis.

SUMMARY

Reduced glutathione (GSH) and methaemoglobin, 2 parameters which were considered important indicators of an erythrocyte's "reductive potential" and therefore its ability to withstand oxidative damage, were measured in E. ovis infected and control blood during an infection cycle. The effect on these 2 parameters of incubating blood with and without acetylphenylhydrazine was also measured.

The results showed that E. ovis infection had no appreciable effect on GSH and methaemoglobin levels, either before or after incubation of the blood for 2 hours at 37°C. Following incubation with acetylphenylhydrazine, methaemoglobin levels in both infected and control blood were markedly increased. Reduced glutathione levels in infected erythrocytes fell to negligible values, whereas control levels fell approximately 50%.

It was concluded that an infected erythrocyte's ability to withstand challenge from an oxidizing chemical was severely affected. In the absence of such a challenge however, E. ovis infection does not appear to affect the reductive potential of the erythrocyte. It is therefore probable that biochemical changes occurring in infected erythrocytes do not play a role in the pathogenesis of anaemia.



KEY 1 GLUCOSE-6-P-DEHYDROGENASE
2 PHOSPHOGLUCONATE DEHYDROGENASE
3 GLYCERALDEHYDE-3-P-DEHYDROGENASE
4 LACTATE DEHYDROGENASE
5 GLUTATHIONE REDUCTASE
6 METHAEMOGLOBIN DIAPHORASE
7 METHAEMOGLOBIN REDUCTASE
8 GLUTATHIONE-METHAEMOGLOBIN TRANSHYDROGENASE
9 GLUTATHIONE PEROXIDASE
10 GLUTATHIONE-ASCORBIC ACID REDUCTASE
11 HAEMOGLOBIN (PROTEIN) OXIDASE
12 METHAEMOGLOBIN-ASCORBIC ACID REDUCTASE
13 CATALASE

Figure 9.1. The oxidation - reduction systems in human erythrocytes. (From Fornaini and Bossu, 1969)

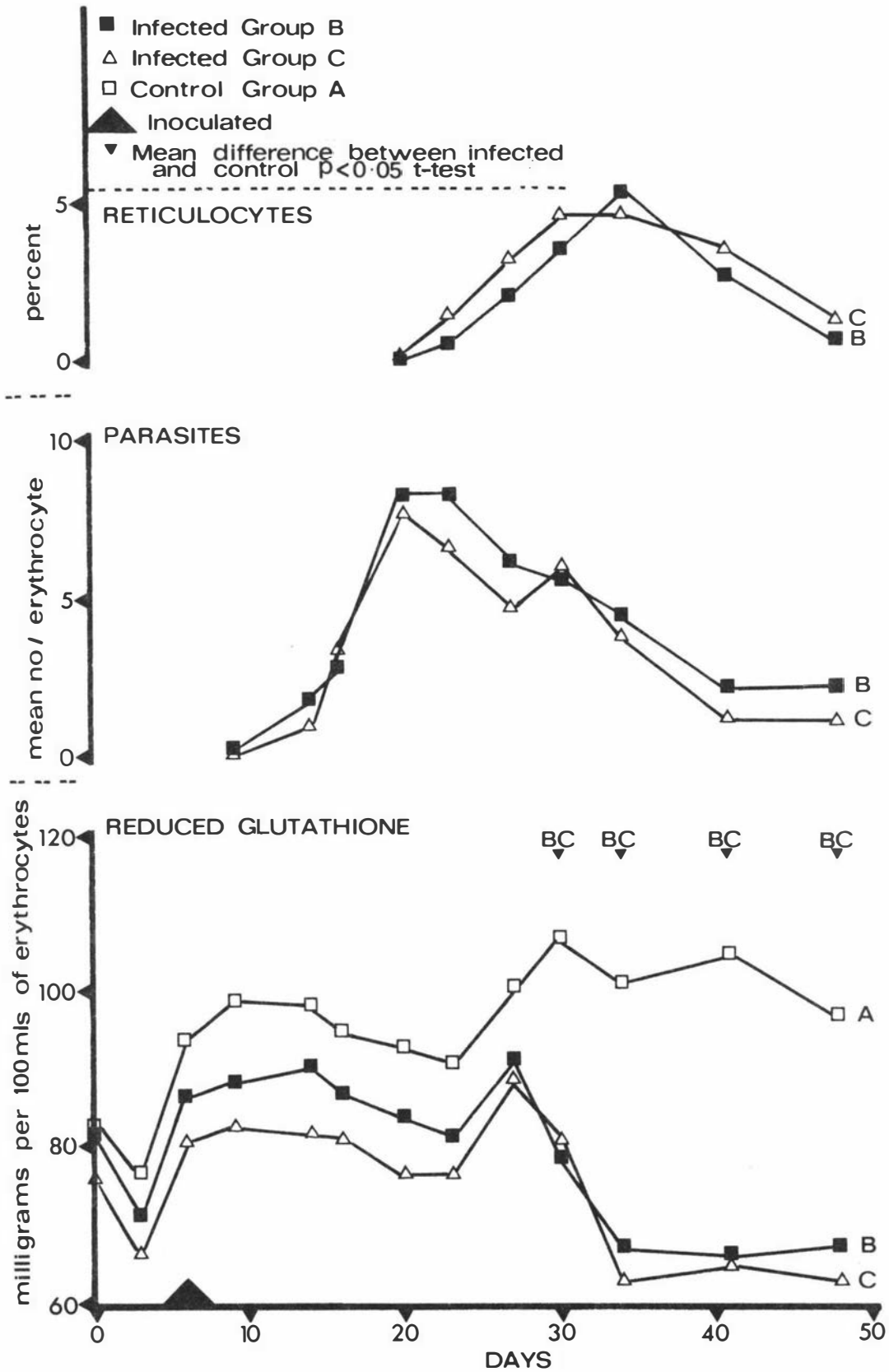


Figure 9.2. The relationships between the degree of parasitaemia, reticulocyte percentage, and reduced glutathione levels of erythrocytes (Exp. VII — Group mean values)

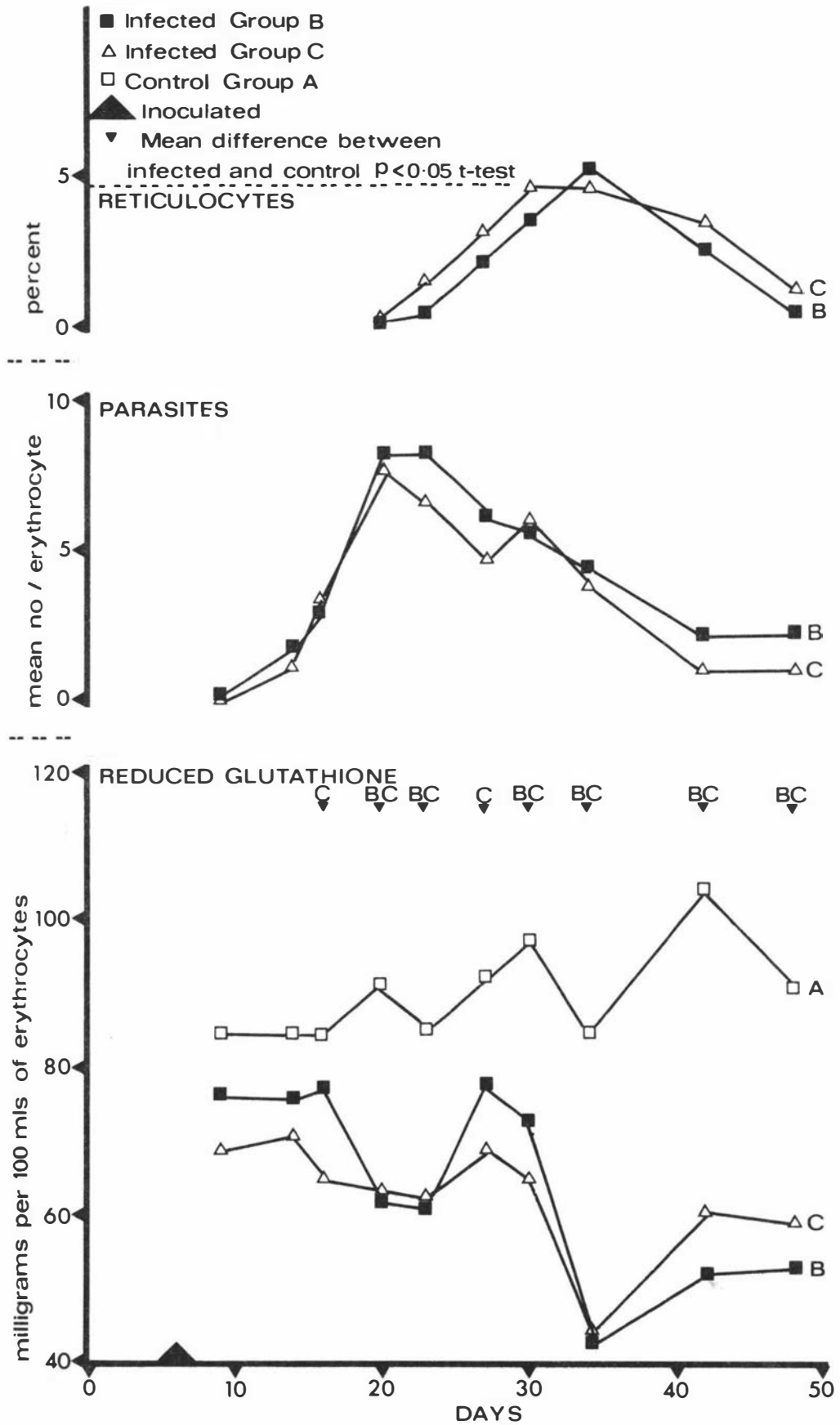


Figure 9.3. The relationships between the degree of parasitaemia, reticulocyte percentage and reduced glutathione levels of erythrocytes following incubation of blood for two hours at 37°C. (Exp. VII – Group mean values)

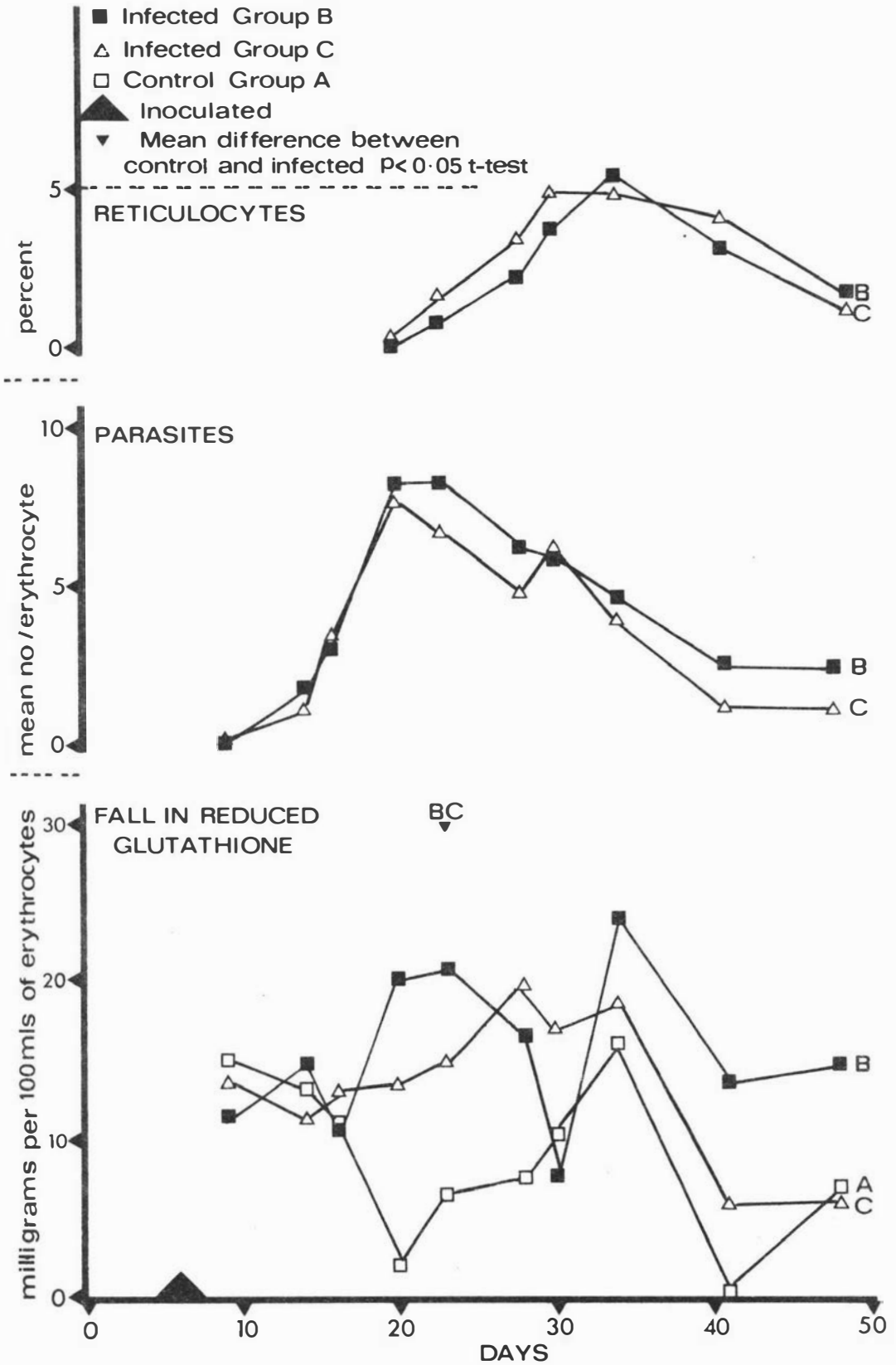


Figure 9.4. The fall in reduced glutathione, related to the degree of parasitaemia and reticulocyte percentage, resulting from incubation of blood for two hours at 37°C (Exp. VII – Group mean values)

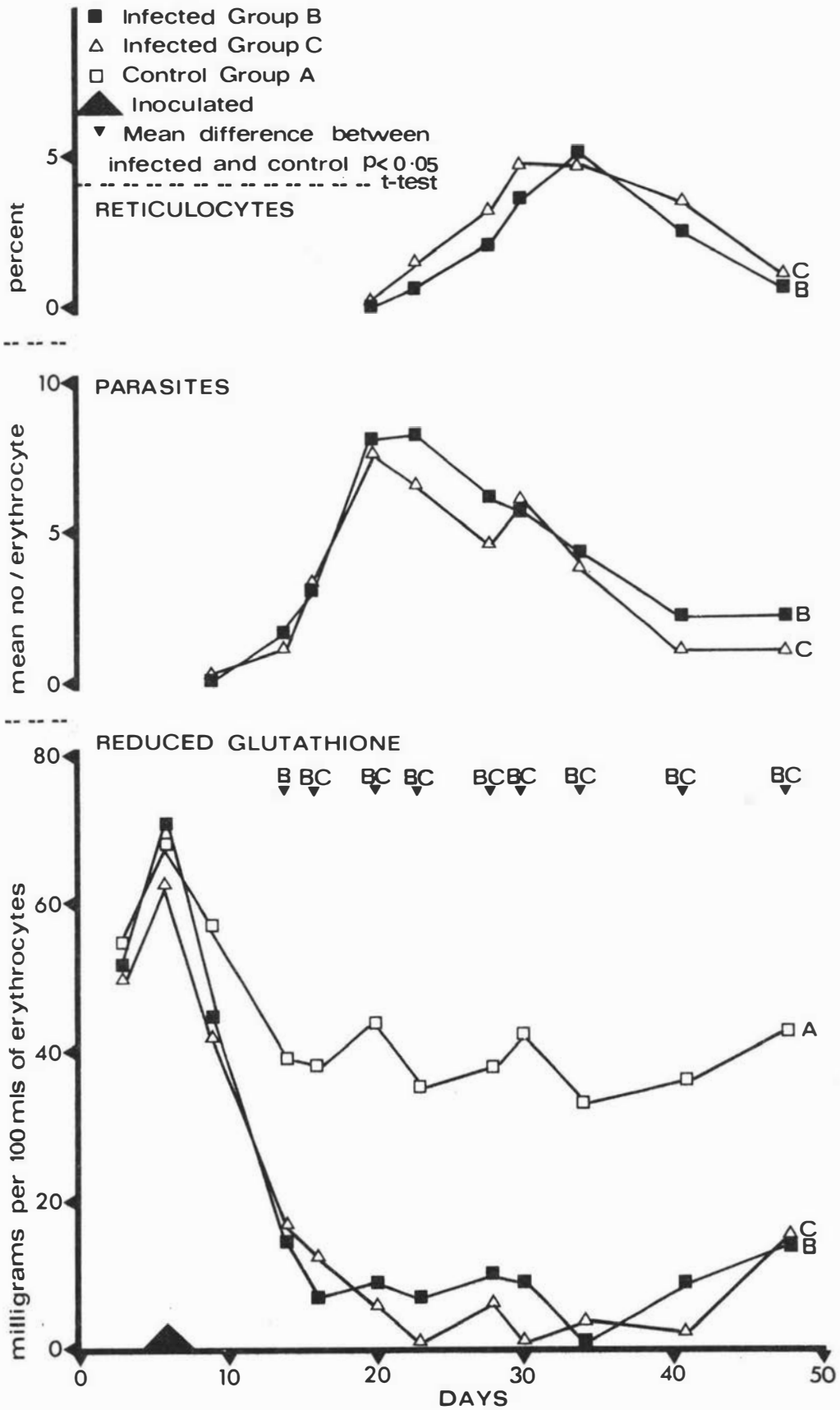


Figure 9.5. The relationships between the degree of parasitaemia, reticulocyte percentage and reduced glutathione levels of erythrocytes following incubation of blood with acetylphenylhydrazine for two hours at 37°C. (Exp. VII — Group mean values)

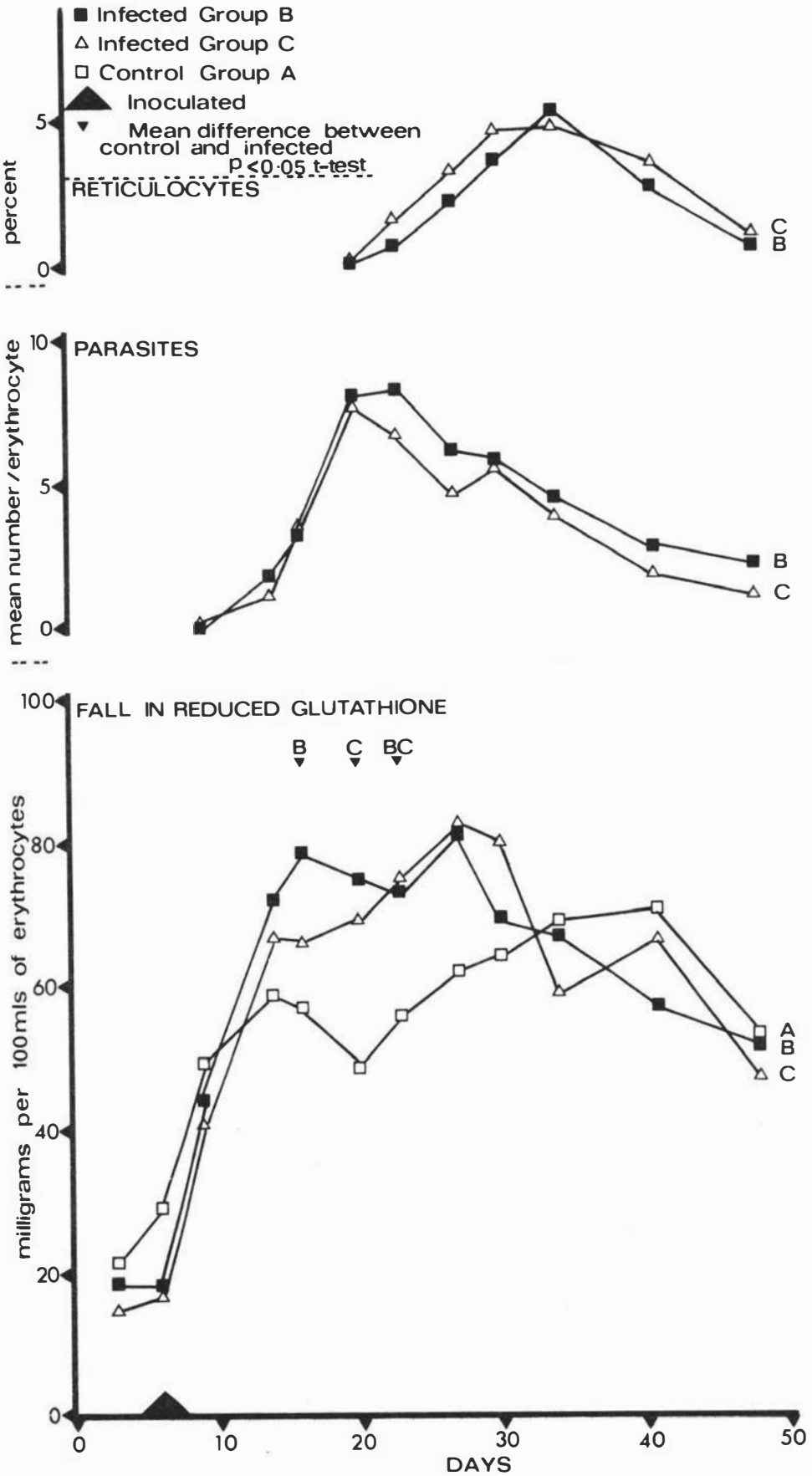


Figure 9.6. The fall in reduced glutathione, related to the degree of parasitaemia and reticulocyte percentage resulting from incubation of blood with acetylphenylhydrazine for two hours at 37°C (Exp. VII — Group mean values)

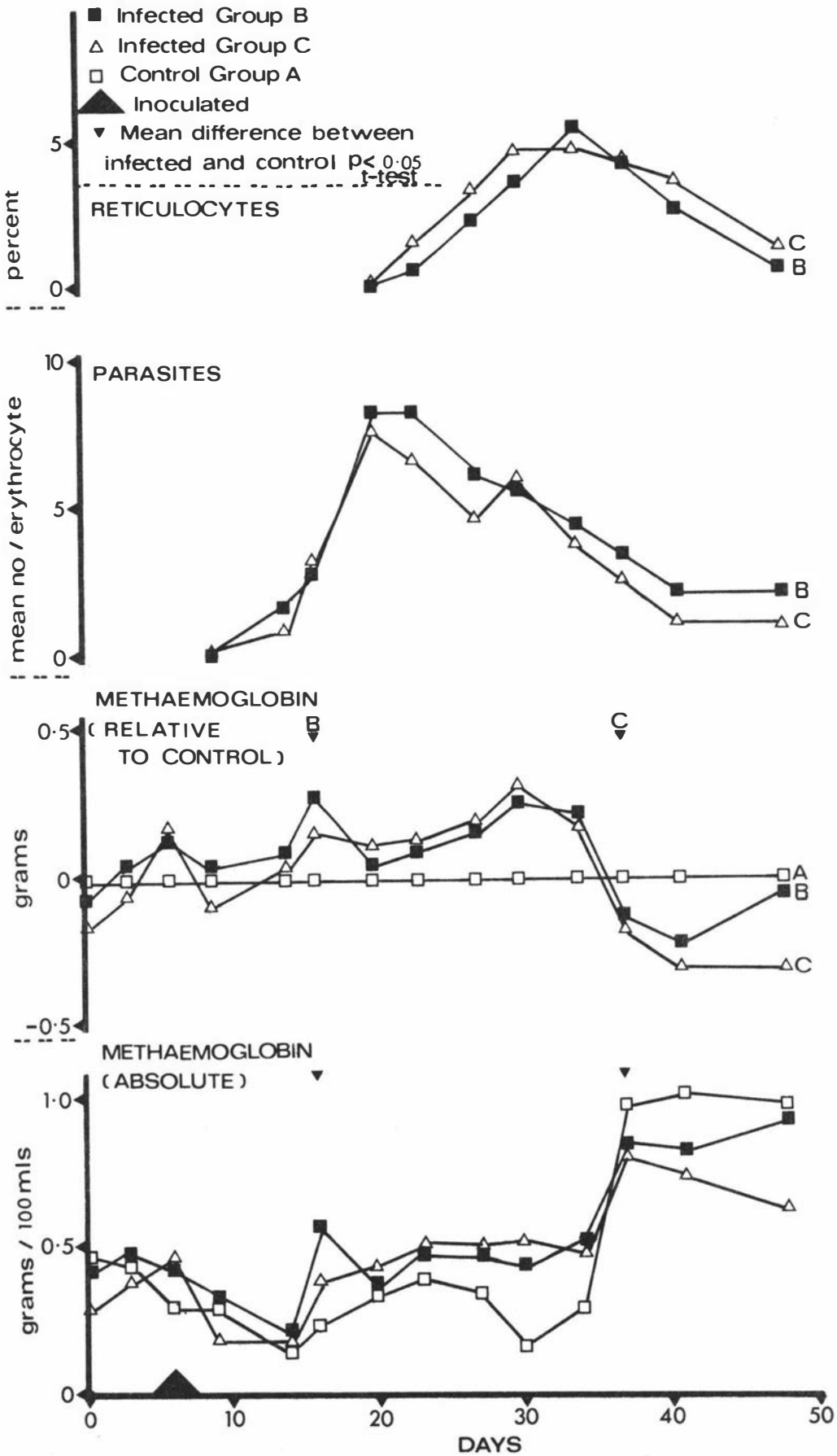


Figure 9.7. The relationship between the degree of parasitaemia, reticulocyte percentage and the methaemoglobin concentration of blood. The levels of methaemoglobin of infected groups are also plotted relative to the control level. (Exp. VII – Group mean values)

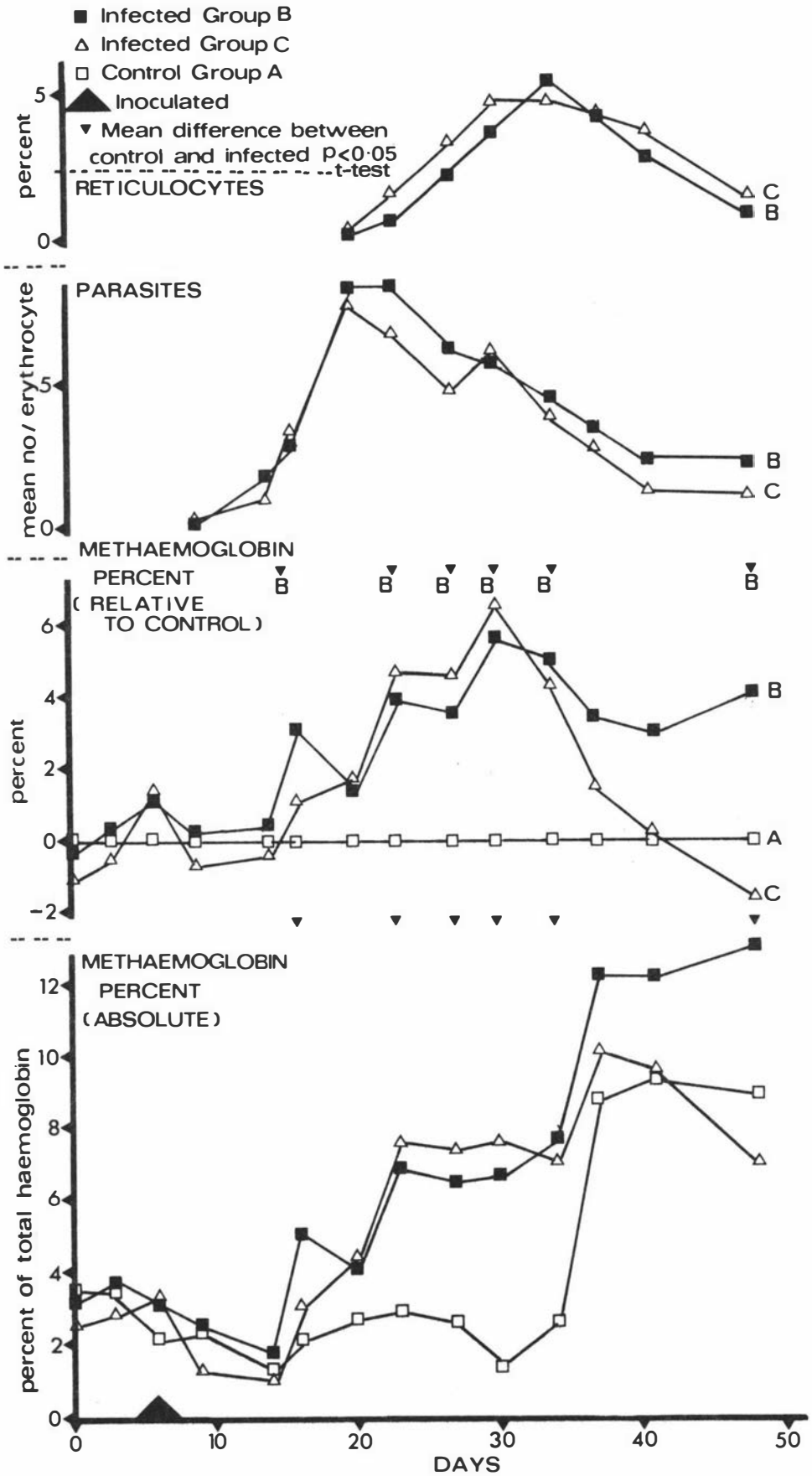


Figure 9.8. The relationships between the degree of parasitaemia, reticulocyte percentage and the methaemoglobin concentration of blood expressed as a percentage of total haemoglobin. The levels of the methaemoglobin percentage of infected groups are also plotted relative to the control level. (Exp. VII – Group mean values)

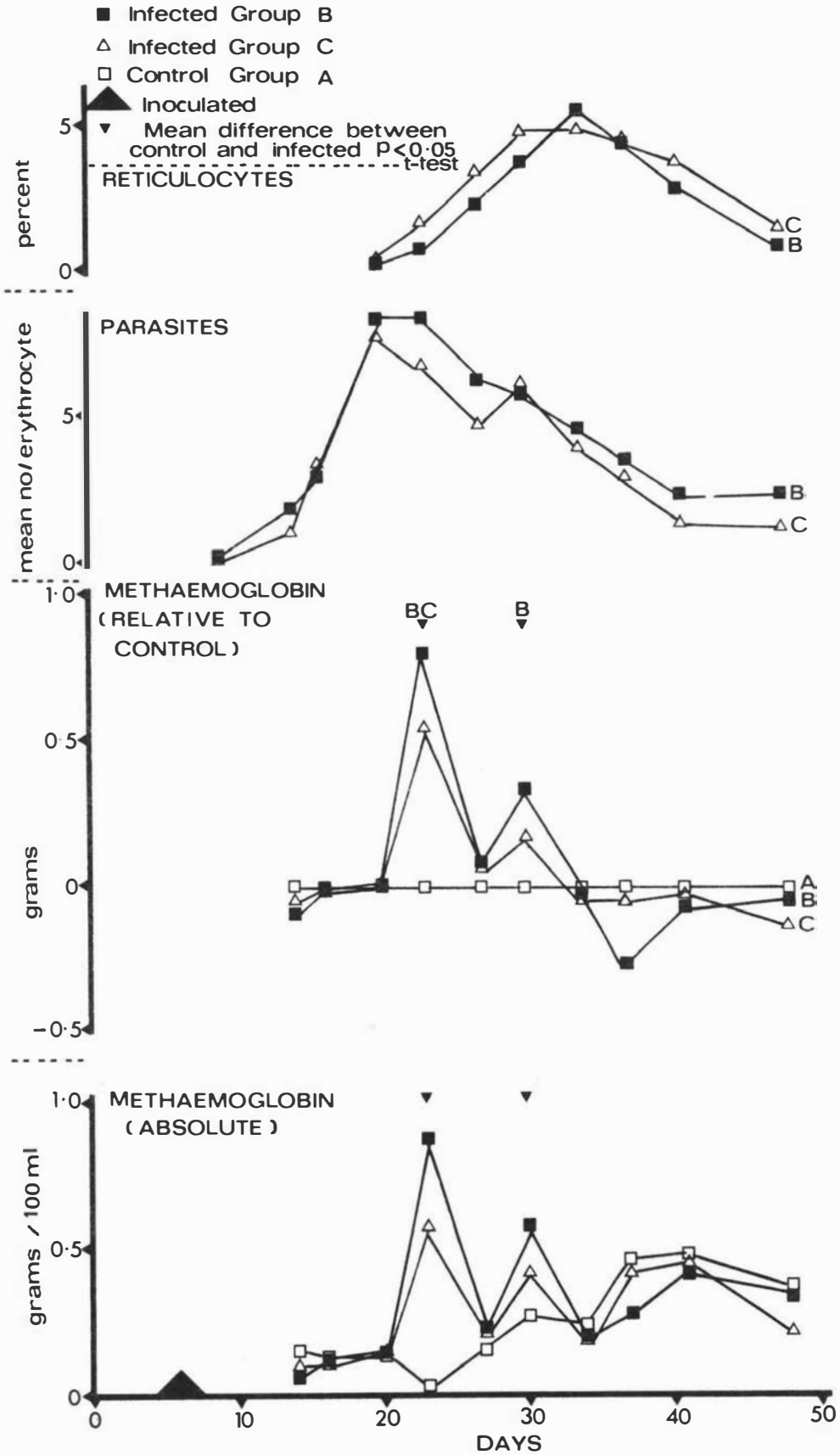


Figure 9.9. The relationships between the degree of parasitaemia, reticulocyte percentage, and the methaemoglobin concentration of blood, following incubation for two hours at 37°C. The levels of methaemoglobin of infected groups are also plotted relative to the control level. (Exp. VII — Group mean values).

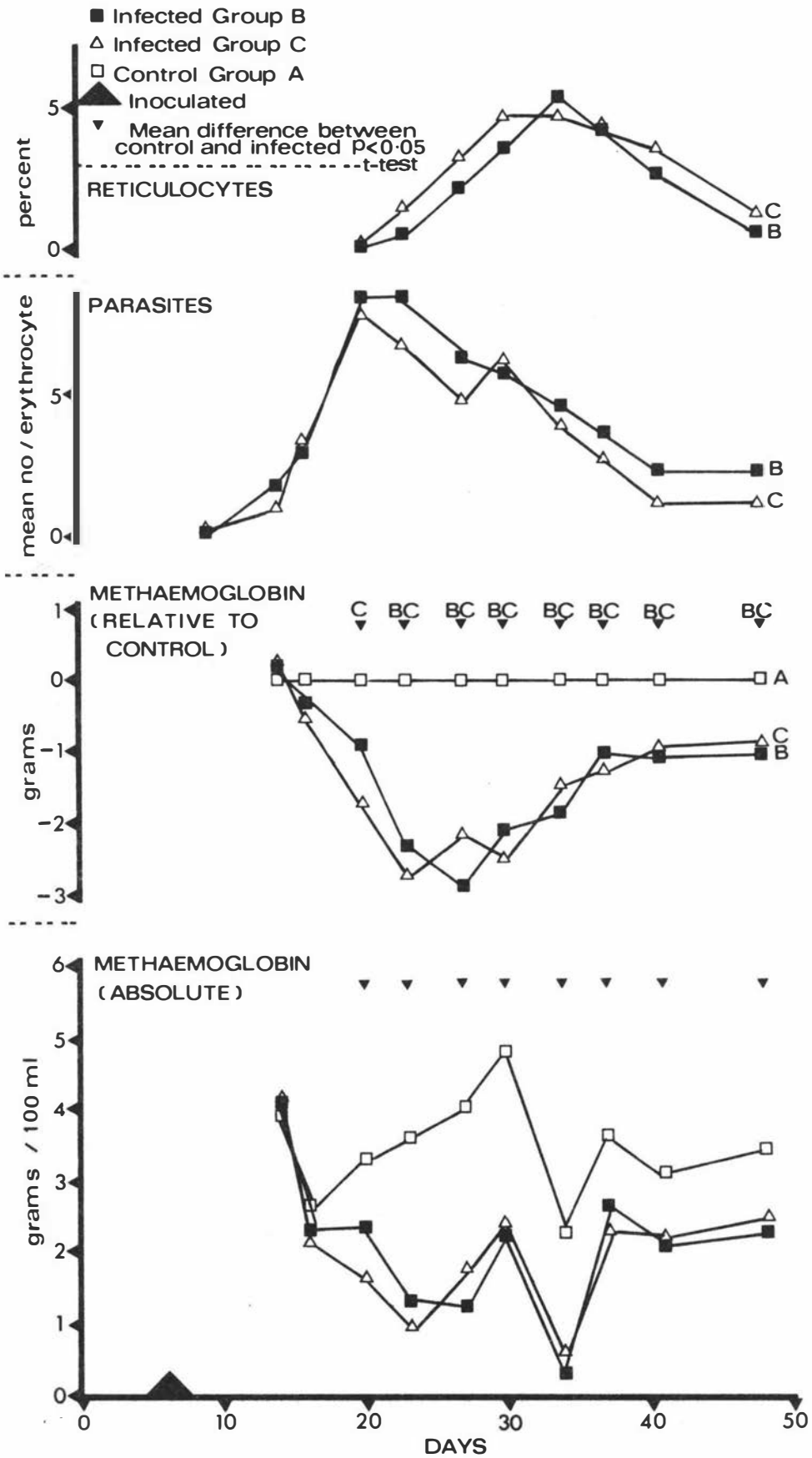


Figure 9.10. The relationships between the degree of parasitaemia, reticulocyte percentage and the methaemoglobin concentration of blood, following incubation with acetylphenylhydrazine for two hours at 37°C. The levels of methaemoglobin of infected groups are also plotted relative to the control level. (Exp. VII – Group mean values)

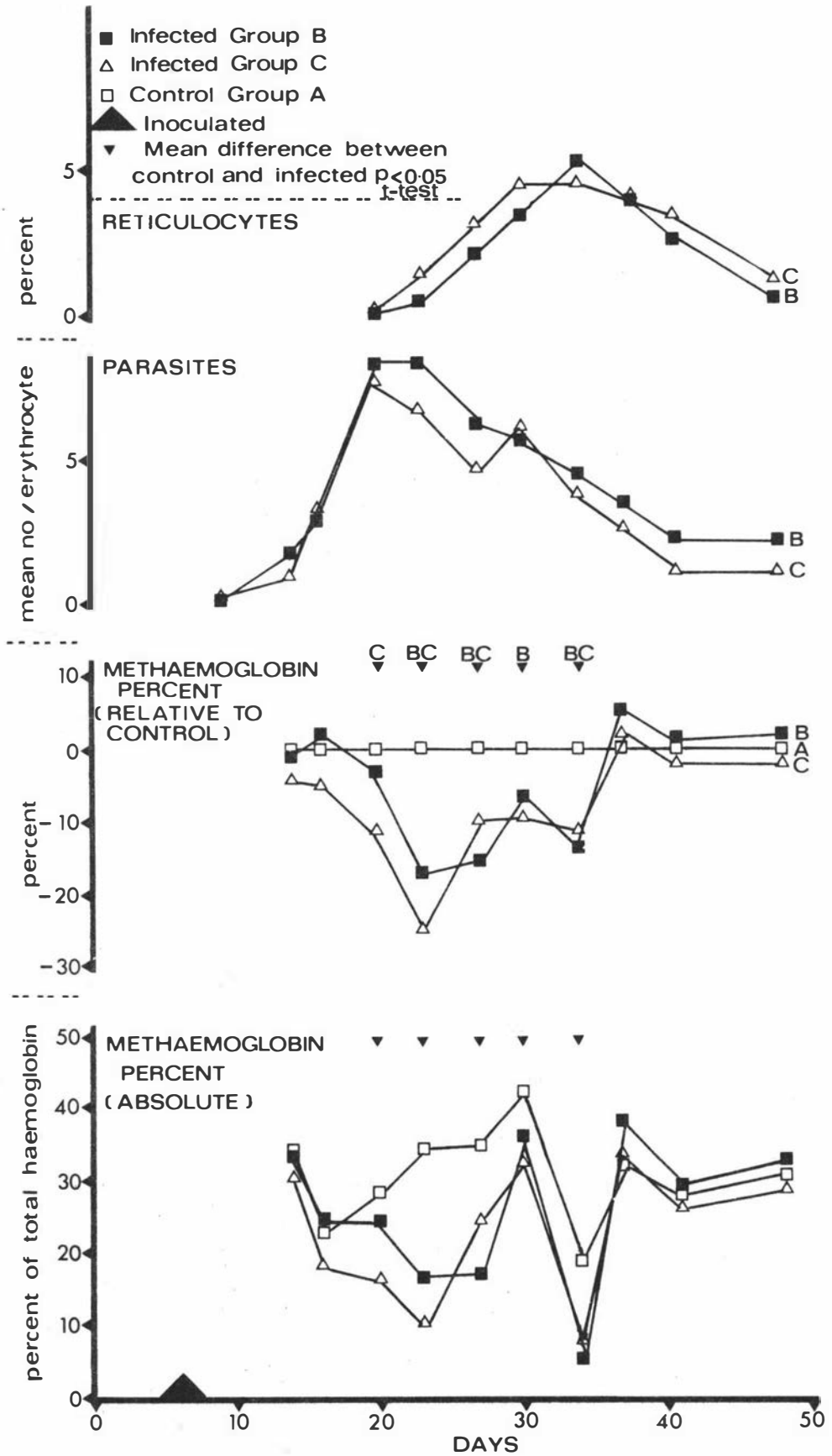


Figure 9.11. The relationships between the degree of parasitaemia, reticulocyte percentage and the methaemoglobin concentration of blood expressed as a percentage of total haemoglobin, following incubation with acetylphenylhydrazine for two hours at 37°C. The levels of the methaemoglobin percentage of infected groups are also plotted relative to the control level. (Exp. VII. — Group mean values)

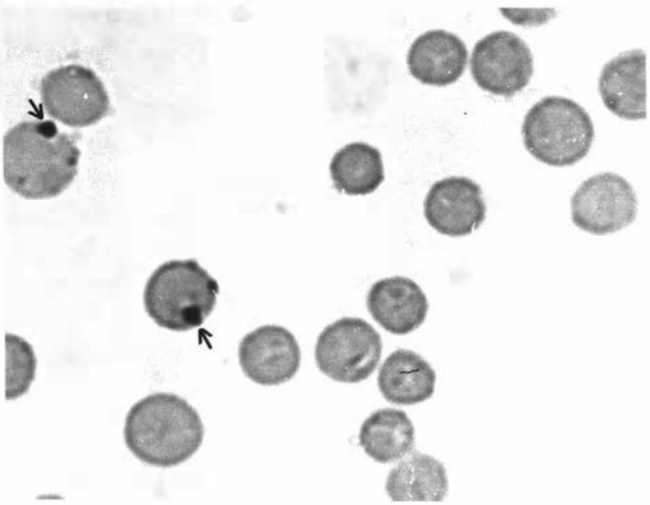


Figure 9.12a: Heinz bodies (↑) in erythrocytes of control sheep following incubation of blood for 2 hours at 37°C. Compare the frequency of Heinz bodies with that shown in Figure 9.12b. (Methyl Violet Supravital Stain x 1000).

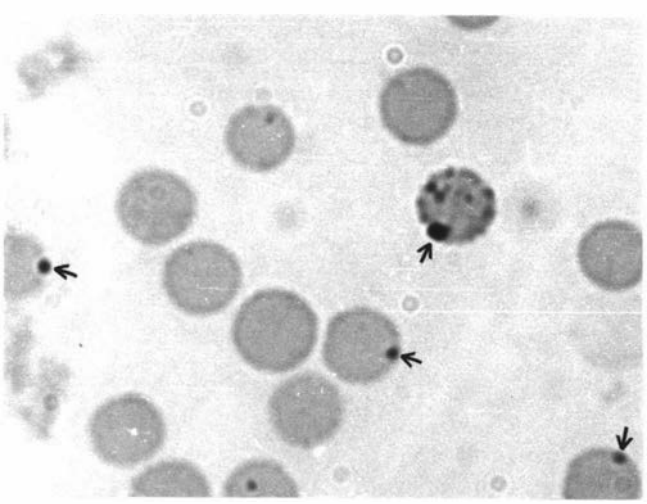


Figure 9.12 b: Heinz bodies (↑) in erythrocytes of *E. ovis* infected sheep following incubation of blood for 2 hours at 37°C. The frequency of Heinz bodies present is higher than that shown in Figure 9.12a. (Methyl Violet Supravital Stain x 1000).

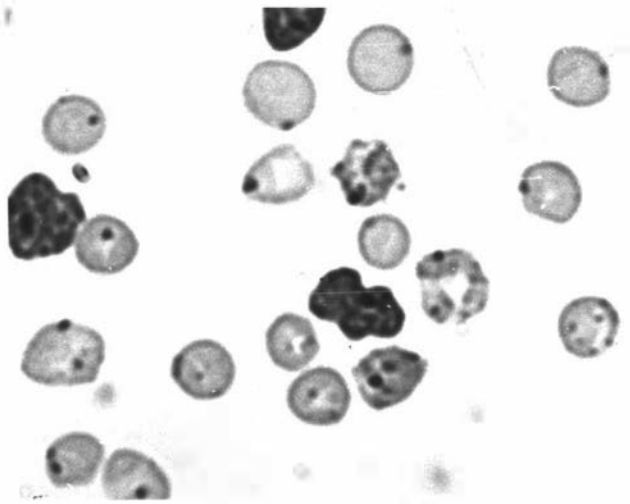


Figure 9.12c: Heinz bodies in erythrocytes of control sheep following incubation of blood with acetylphenylhydrazine for 2 hours at 37°C. The frequency of Heinz bodies is similar to that shown in Figure 9.12d. Note some cells with a diffuse dark staining reaction (see text p234).
(Methyl Violet Supravital Stain x 1000).

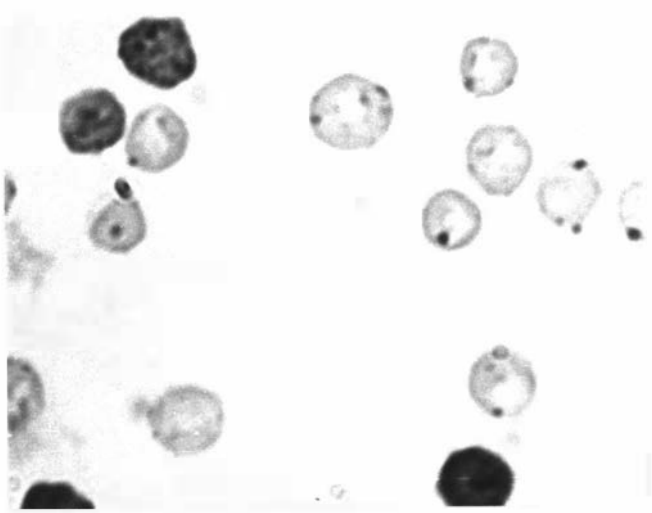


Figure 9.12d: Heinz bodies in erythrocytes of E. ovis infected sheep following incubation of blood with acetylphenylhydrazine for 2 hours at 37°C. The frequency of Heinz bodies is similar to that shown in Figure 9.12c. Note some cells with a diffuse dark staining reaction (see text p 234). (Methyl Violet Supravital Stain x 1000).

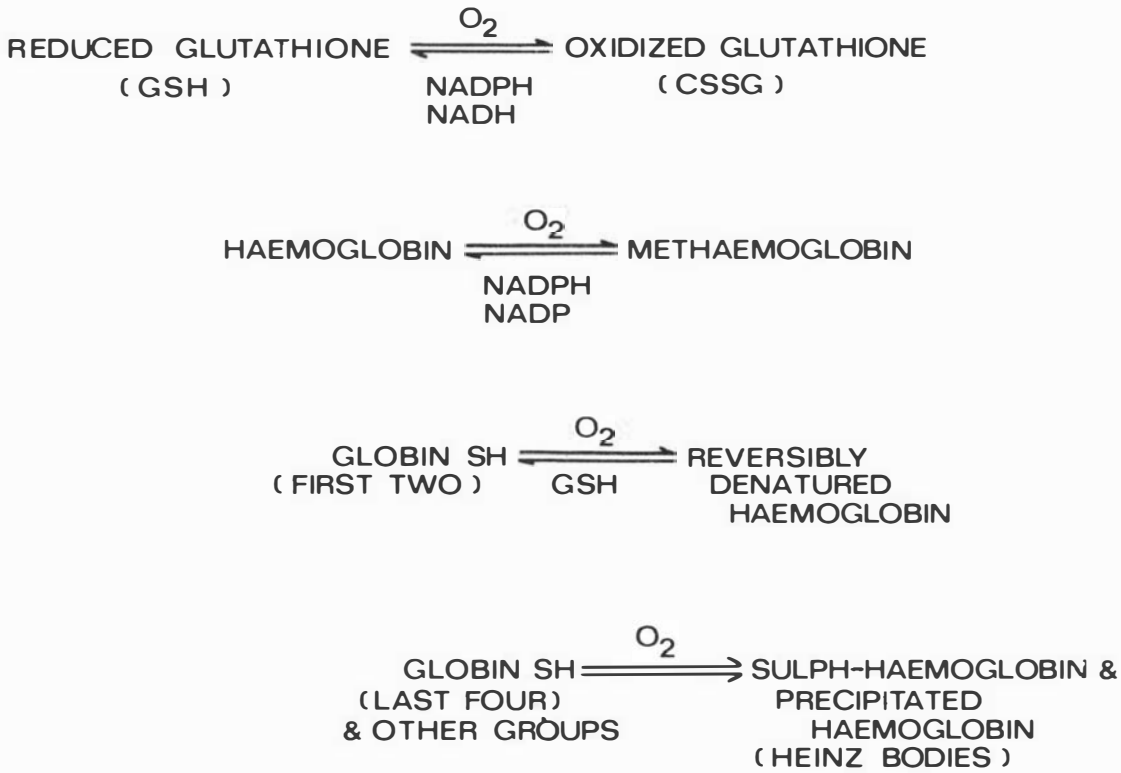


Figure 9.13. Scheme of biochemical sequence during oxidative precipitation of haemoglobin and Heinz body formation (From Allen and Jandl, 1961)

CHAPTER X

GENERAL DISCUSSION AND CONCLUSIONS

This study of Eperythrozoon ovis infection in sheep comprised 7 experiments, each designed to obtain information on some specific aspect of the host-parasite relationship (Table I). The number of sheep in each experiment was, with 2 exceptions, a minimum of 5 per group. This was considered the smallest number from which a statistically valid result was likely to be obtained, and yet not too large for the number of tests to be done. In Experiment III a group of 4 unbiopsied (liver) sheep was housed with biopsied infected sheep; the main purpose of this former group being to check for cross-infection. This in fact occurred with all 4 sheep. In the study of erythrocyte carbohydrate metabolism in anaemic non-infected sheep (Experiment V, Part II, Chapter VII) for logistical reasons only 4 sheep were used in each group. Although this may have been undesirable from the statistical viewpoint, the results obtained were clear cut and presented no statistical problems.

With only a limited number of sheep available it was necessary to make the most efficient use of each experiment. This resulted in the collection of a considerable amount of data which, whilst not directly relevant to the specific aspect under study, was nonetheless useful. Data of this type has its limitations and ideally it would be desirable to carry out experiments specifically designed to investigate all aspects of this infection. This was impractical. Because of the specificity of the parasite to the host it was not possible to adapt the organism to a laboratory animal. If this had been possible some experiments would have been designed differently and in some cases extended by taking advantage of large numbers of animals. For example, the dose rate experiment described (Chapter III) could have been more elaborate, using smaller dose rates and different routes of administration.

Perhaps the biggest advantage in working with sheep is that repeated collection of large (10 to 20 ml) blood samples can be made, and a cycle of infection followed in the same animal. The size of the sample which can be collected enables a greater range of tests to be done; the main restriction on the number performed in this investigation was the logistical problem of handling and testing within a limited period of time. One problem which can arise from repetitive blood sampling is the inducement of reticulocytosis, which could have some effect on the results from control sheep, particularly when erythrocyte biochemical factors are under study. Reticulocytosis in control sheep did not in fact occur, but in one experiment (Exp. VII, Chapter IX) an increase in erythrocyte reduced glutathione (GSH) levels in control sheep occurred, which was attributed to a lessening in the average age of the erythrocytes. In this case about 50 to 60 ml of blood were being removed per week, which was approximately 3 to 4% of the total blood volume of the animals concerned.

The main results obtained and the conclusions reached are summarized at the end of each of the preceding chapters and, therefore, it is unnecessary to consider each individual result in turn. However the overall pattern which has emerged, with some emphasis on the main findings, will be discussed further.

One aspect of E. ovis infection which is of economic interest is the clinical effect of the parasite on the host animal, the sheep. In New Zealand unthriftiness in young sheep occurs commonly from a variety of causes. However the incidence and distribution of E. ovis in New Zealand, and its possible contribution to this, is not known. The only report concerns its initial discovery in an unthrifty lamb (Jolly, 1967). It was therefore not possible to study field outbreaks of E. ovis infection to ascertain its effect in infected flocks. Consequently one experiment (Exp. II) was designed to measure the effect of E. ovis on the weight gain of young sheep. The infected and control groups of sheep

were kept in the same paddock to equalise environmental factors. There was a significant ($p < 0.01$) depression in weight gain of infected sheep but the experiment was complicated by cross-infection of control sheep. However a decrease in the rate of weight gain also occurred in the latter group of sheep after infection. One point that was noted and will be referred to later in this discussion, was that retardation in the rate of weight gain in infected sheep was evident before anaemia developed. It has often been assumed that any effect E. ovis had on the host animal is associated with the severity of the anaemia. In other experiments the weight gain of infected sheep was less than that of controls, but results were not statistically significant. The groups in these experiments were smaller than in Experiment II. It appears clear that at least under certain conditions E. ovis infection may cause a decrease in weight gain but the economic importance of this has not been defined. For further clarification an extensive study would be required, involving a large number of infected and control groups of sheep subjected to varying pasture and disease conditions. The effect of E. ovis in combination with gastro-intestinal parasites on sheep is certainly worthy of investigation.

Eperythrozoon ovis infection causes a haemolytic anaemia in sheep but, as mentioned in the introduction to Chapter III, the reports in the literature on the severity of anaemia are contradictory. During the course of this investigation considerable haematological data were collected and Experiment I was specifically designed to measure the effect of a graded dose rate on the development and severity of anaemia in relation to the development and maximum degree of parasitaemia. The most important facts which arose out of the haematological studies on experimental infection were that the severity of anaemia, as measured by the fall in haemoglobin concentration, and the maximum number of parasites per erythrocyte were similar in all groups of sheep irrespective of the size of inoculating dose. The decline in haemoglobin concentration was remarkably well synchronized

in all groups whereas the onset and development of parasitaemia was not. Time of onset and development of parasitaemia was however inversely proportional to the size of inoculating dose. The consistent degree of anaemia obtained in experimental infection was not expected, and at first it was difficult to offer an explanation for this. The haematological data obtained from naturally cross-infected sheep has however enabled a possible explanation to be provided. Although Experiment II was specifically run as a weight gain trial, it was set up at a time of the year when it was thought possible that cross-infection would occur. No positive proof has been presented to incriminate a specific insect vector in the transmission of infection, but circumstantial evidence supporting blood sucking insects as vectors has been mentioned (see Chapter I).

In the event cross-infection did occur and from the haematological data obtained, along with the results from experimental infection, an explanation for this apparent conflict in literature reports is provided. In brief, it is postulated that the development of anaemia is dependent upon a time-based host immune response against the parasite and which involves the erythrocyte. Thus in experimental infection, even at the lowest dose rate used (1×10^{-6} ml of heavily infected blood), parasitaemia was fully developed by the time the immune response reached full expression. The results obtained from natural infection were more variable; some sheep had a degree of infection comparable to experimental infection, while others had a very mild infection. It is possible, therefore, that the host immune response had reached its peak before the parasitaemia had developed to its full extent. This could happen if the dose rate was lower than that used experimentally, and if the route of administration was other than directly into blood vessels, as might be expected to occur with insect vectors. It would require a large number of animals to confirm this postulate.

Further evidence supporting the above hypothesis is provided by the observation that development of maximum

plasma haemoglobin levels and osmotic fragility did not correspond with the time of peak parasitaemia but in fact followed it. In addition, a positive antiglobulin (Coombs) test has been obtained during infection (Sheriff, 1967; Sheriff and Geering, 1969). In other blood parasite infections the development of anaemia involving an immune response against the erythrocyte has been questioned. The occurrence of autoantibodies has been reported in malaria, babesiosis and anaplasmosis (Mann and Ristic, 1963; Schroeder and Ristic, 1968b; Zuckerman, 1969), but it is not known whether or not this is a non-specific phenomenon. In Plasmodium berghei infection George et al. (1966) considered that a positive Coombs reaction resulted from the non-specific adherence of transferrins to immature erythrocytes. However in Plasmodium vivax infection of humans a positive Coombs test has been obtained in the absence of reticulocytosis (see Zuckerman, 1969). The fact that reticulocytosis does develop in E. ovis infection and in view of the findings of George et al., there must be some doubt as to the precise role of an immune response by the host in the development of anaemia.

There must also be considered in the development of anaemia the possibility of a direct effect of the parasite on the erythrocyte. A significant ($p < 0.01$) correlation was obtained between the maximum degree of parasitaemia and the maximum degree of anaemia in all infected sheep (see Chapter III). This could be consistent with an immune reaction against the parasite which also involves the erythrocyte, but a direct effect by the parasite on the erythrocyte must also be considered. Arising from this is the question of how an infected erythrocyte is removed from circulation. Intravascular haemolysis does occur and in some circumstances may lead to haemoglobinuria. Haemosiderin was found in the proximal tubules of the kidney and considerable amounts were found in the parenchymal cells of the liver (Chapter V). Evidence for the occurrence of an increased rate of erythrophagocytosis by the reticuloendothelial system was not so conclusive. The amount of haemosiderin present in

spleens (the organ most likely to remove infected erythrocytes) of infected sheep appeared to be higher than in those from control sheep, but there was considerable variation in amount within groups.

It is generally recognised that the role of the reticuloendothelial system is allied to host immune mechanisms. Stimulation of the system to remove inert particulate matter is mediated by serum protein factors known as opsonins (Jenkin and Rowley, 1961). Development of techniques in which the phagocytic activity of the system is measured have mainly depended upon the measurement of the intravascular clearance rate of an inert colloid. The colloid which has been most commonly used for this purpose is the carbon particle. Ever since the early reports on the use of carbon, which showed that there was an interaction between carbon and plasma clotting agents (Halpern et al., 1953; Biozzi et al., 1953) many modifications in the preparation of carbon have been made. These have included the development of carbon with a fish glue stabilizer (C11/1431a - Gunther Wagner, Hanover) especially for reticuloendothelial studies, and the use of gelatin as a diluent. No reports in the literature were found of carbon clearance techniques being used in sheep, so the method used was an adaptation of that used in laboratory mammals. Some problems were encountered with this technique. It was found in a preliminary study, for example, that the use of gelatin as a carbon diluent caused respiratory distress and death in some animals. The use of saline as a diluent overcame this problem, but it was shown that interaction between the carbon particles and some plasma constituents (probably fibrinogen) resulted in aggregation of the carbon and lodgement in non-reticuloendothelial sites; particularly in the lung capillaries. In retrospect it would probably have been valuable to repeat the experiment using another test substance, but this was not practicable within the time limits available for the present study. Despite carbon aggregations in blood vessels of lungs and other organs, interpretation of carbon clearance studies was possible as the aggregation appeared to be

non-specific.

Increased phagocytosis of carbon by reticuloendothelial cells did occur in E. ovis infection; increases at 5 days post-inoculation were non-significant but at day 12 they were highly significant ($p < 0.01$). Increase in carbon clearance usually results from an increase in non-specific serum factors, i.e. : opsonins. It is possible that such an increase in reticuloendothelial activity is also directed against E. ovis itself. It is probable that E. ovis does stimulate a host immune response. Although carbon clearance increase is non-specific it could be augmented in E. ovis infection by specific opsonizing antibodies against the organism or even the erythrocyte. The balance between the number of infected erythrocytes which are prematurely phagocytosed, and the number which are lysed in circulation is, from the evidence available, heavily in favour of the latter. Opsonization of an infected erythrocyte by specific antibody would facilitate an erythrocyte's removal from circulation, but it is probable that antigen-antibody alone would not cause intravascular lysis; this could result either from the participation of complement following the antigen-antibody reaction, or from a direct mechanical effect of the parasite on the erythrocyte. In the latter case as a result of damage, exposure of normally covert erythrocyte antigens may occur, leading to a direct auto-immune response by the host against the erythrocyte. The role of a mechanical effect by the parasite as being a direct or indirect cause of lysis cannot be discounted when one considers other blood parasite infection. In anaplasmosis, for example, erythrophagocytosis is the only way erythrocytes are prematurely removed from circulation; this presumably follows opsonization of the cell (Schroeder and Ristic, 1968b). The number of organisms present and the percentage of erythrocytes infected in anaplasmosis is very small compared with E. ovis infection, and therefore less likely to cause mechanical damage to the erythrocyte. In E. ovis infection, on the other hand, up to ten or more parasites can be attached to one erythrocyte and 100% of

erythrocytes infected. It seems, therefore, that the compounding effect of opsonization and the attachment of a large number of organisms to the erythrocyte membrane with consequent erosion (Kreier and Ristic, 1963) and/or the possible participation of complement could be sufficient to lyse the erythrocyte.

In summary, therefore, it appears that in E. ovis infection intravascular haemolysis is the predominant form of erythrocyte removal from circulation and that this could be mediated by an immune mechanism against the parasite which also involves the erythrocyte; perhaps with the participation of complement, and/or by a mechanical effect against the erythrocyte. This latter effect may either cause direct lysis or lead to the exposure of erythrocyte antigens leading to an autoimmune haemolytic process.

Perhaps the most interesting area of this investigation was the comparative study of the carbohydrate metabolism between infected and non-infected erythrocytes. It was shown that infected erythrocytes, at the peak of parasitaemia, utilized approximately 24 times as much glucose and produced approximately 18 times as much lactic acid as the control erythrocytes (Chapter VII). Accompanying these changes was a fall in the percentage of glucose which was recovered as lactic acid, and a fall in the percentage of glucose which could be accounted for as lactic acid, pyruvic acid and oxygen uptake. There was also an egress of oxygen in the early stage of incubation at peak parasitaemia from infected erythrocytes; this is thought to result from a drop in intracellular pH. An increase in oxygen uptake at the late parasitaemic stage was attributed to the presence of reticulocytes.

This experiment did not elucidate whether the increased glycolytic activity was due to the parasite or an effect on the erythrocyte by the parasite, nor did it define the biochemical pathways of the parasite. It was considered that these aspects could only be studied with isolated

parasites. One of the most important problems to be overcome, if such a study is to be made, is the effect which isolation procedures may have on the parasite (see Chapter VII).

Arising out of this work, which showed that there was increased glycolytic activity by infected erythrocytes, were two very interesting questions. What was the effect of this activity on (1) the host animal, and, (2) the host erythrocyte? As the results of Experiment VI show (see Chapter VIII), a metabolic acidosis due to high blood levels of lactic acid does occur in infected sheep. One remarkable finding was the severe lowering of blood glucose levels, in some cases as low as 2 to 3 mg/100 ml. At about the time this work was in progress, similar findings were published in relation to calves infected with Eperythrozoon wenyoni (Schotman, 1970; Zwart et al., 1970). These workers measured acid-base parameters including lactic acid levels, after they noticed that infected calves were hyperventilating. In contrast to what was expected they found an elevated pCO_2. This could not, however, be reconciled with any respiratory abnormality. An elevated pCO_2 was also obtained with E. ovis infection, and at present there is no apparent explanation for this.

The effect of the metabolic acidosis on the host animal is difficult to assess. As was noted earlier, infected sheep showed a retarded rate of weight gain before anaemia had developed and housed sheep were noted to be inappetant at a similar stage of infection. Although no other clinical effects were noted, the possibility of these factors being due to the acidosis must be considered. Another consideration is the possible potentiating effect of E. ovis on an acidosis arising from other pathological or physiological changes. An illustration of this is provided by the study of the metabolic status of sheep previously fed on a supplemented diet (hay and concentrates) and then transferred to lush spring pasture (Annison et al., 1959). A metabolic

acidosis, similar in degree to that obtained with E. ovis, occurred. Many E. ovis outbreaks, reported in the literature, have occurred in the spring. Serious consequences of the acidosis in E. ovis may not be expected if the changes recorded are no more severe than in this study, but if other factors are present, such as a dietary change just described, then acid-base changes may prove of great importance. The validity of this suggestion needs investigating.

The effect of increased glycolytic activity on the ability of the erythrocyte to maintain "reductive potential" and therefore retain normal structure and function, was investigated (Chapter IX). The parameters studied, notably erythrocyte reduced glutathione and blood methaemoglobin levels, were considered the most likely to provide information on the ability of the erythrocyte to withstand biochemical challenge from oxidative processes. Although the maintenance of the erythrocyte's reductive potential is dependent on the glycolytic activity of the cell (see introduction to Chapter IX) it was possible that the increased activity in infected cells was mainly due to the parasite, the cell itself having reduced activity. If glycolytic activity of the erythrocyte is impaired, then "oxidative haemolysis" could be the eventual result (Allen and Jandl, 1961). There was, however, nothing in the results to suggest that E. ovis infection caused an oxidative haemolysis. Small increases occurred in blood methaemoglobin concentration and small decreases occurred in erythrocyte reduced glutathione, but these alterations did not approach critical levels. On the other hand, exposure of infected erythrocytes to acetylphenylhydrazine, a chemical which tests the ability of a cell to withstand oxidative challenge, caused reduced glutathione levels to fall to negligible levels, whereas control erythrocytes still maintained adequate levels. Methaemoglobin levels showed a large increase in both infected and control blood. It is probable, therefore, that an E. ovis infected sheep, if treated with an oxidizing chemical, could suffer a severe oxidative haemolytic anaemia, whereas uninfected sheep would be resistant. Without such a

challenge, however, it is unlikely that oxidative haemolysis would occur in *E. ovis* infected sheep. It seems, therefore, that *E. ovis* does not affect an erythrocyte's glycolytic metabolism sufficiently to initiate its removal from circulation by the above means.

The relationship between the parasite and the erythrocyte is complex and probably involves immunological, mechanical and biochemical factors. An attempt has been made in this work to explain the relative importance of these factors in the pathogenesis of anaemia, and the effect that interactions involving these factors, between the parasite and the erythrocyte, have on the host animal. Further work is necessary before a complete understanding on the pathogenesis of *E. ovis* infection can be reached.

BIBLIOGRAPHY

Agar, N.S.; Roberts, J.; Evans, J.V. (1972): Erythrocyte glutathione polymorphism in sheep. Aust. J. biol. Sci., 25 : 619-26

Allen, D.W.; Jandl, J.H. (1961): Oxidative haemolysis and precipitation of haemoglobin. II. Role of thiols in oxidant drug action. J. clin. Invest., 40 : 454-75.

Annison, E.F.; Lewis, D.; Lindsay, D.B. (1959): The metabolic changes which occur in sheep transferred to lush spring pastures. II. Changes in the acid-base balance of the whole animal. J. agric. Sci., Camb., 53 : 42-5

Aschoff, L. (1924): Das reticulo-endotheliale system. Ergebn. inn. Med. Kinderheilk., 26 : 1-118. (Cited by Stuart, 1970).

Astrup, P. (1970): Dependence of oxyhaemoglobin dissociation and intraerythrocytic 2,3-DPG on acid-base status of blood. II. Clinical and experimental studies. In Red Cell Metabolism and Function. (G.J. Brewer, ed.), P. 67-79. Plenum Press. New York - London.

Astrup, P.; Jørgensen, K.; Siggaard-Andersen, O.; Engel, K. (1960): The acid-base metabolism. A new approach. Lancet, i : 1035-9.

Avakian, A.A.; D'yakonov, L.P.; Nadtochei, G.A. (1973): (Ultrastructure and nature of representatives of the genus Eperythrozoon Schilling, 1928). Izvestiya Akademii Navk SSR, Seriya Biologicheskaya. No 1 : 135-7. (Abstr. Vet. Bull., 43 : 2026. 1973).

Bach, J.R. (1963): The Astrup method. A micromethod for determination of pH and other acid-base values in blood. Radiometer Bulletin. Radiometer, 72 Emdrupvej, Copenhagen NV, Denmark.

Baker, H.J.; Cassell, Gail H.; Lindsey, J.R. (1971): Research complications due to Haemobartonella and Eperythrozoon infections in experimental animals. Am. J. Path., 64 : 625-50.

Barker, S.B.; Summerson, W.H. (1941): The colorimetric determination of lactic acid in biological material. J. biol. Chem., 138 : 535-54.

Barnes, M.G.; Polet, H. (1969): The influence of methylene blue on the pentose phosphate pathway in erythrocytes of monkeys infected with Plasmodium knowlesi. J. Lab. clin. Med., 74 : 1-11.

Barron, E.S.G.; Harrop, G.A. (1928): Studies on blood cell metabolism. II. The effect of methylene blue and other dyes upon the glycolysis and lactic acid formation of mammalian and avian erythrocytes. J. biol. Chem., 79 : 65-87.

Bartlett, G.R.; Marlow, A.A. (1953): Erythrocyte carbohydrate metabolism. I. The flow of C^{14} glucose carbon into lactic acid, carbon dioxide, cell polymers and carbohydrate intermediate pool. J. Lab. clin. Med., 42 : 178-87.

Baxi, A.J.; Nail, S.N.; Bhatiz, H.M. (1963): Erythrocytic glucose-6-phosphate dehydrogenase in various animal species. Curr. Sci., 32 : 405.

Begovic, S.; Delic, S.; Rukavina, J. (1963): (First finding of Eperythrozoon ovis in Yugoslavia). Veterinaria, Sarajevo. 12 : 177-85. (Abstr. Vet. Bull., 34 : No 100. (1964)).

Belding, D.L. (1965): Textbook of Parasitology. 3rd ed. Appleton-Century-Crofts. New York.

Bernstein, R.E. (1959): Alterations in metabolic energetics and cation transport during ageing of red cells. J. clin. Invest., 38 : 1572-86.

Bernstein, R.E.; Troughton, V.A.; Donly, D.L. (1965): Citric acid cycle in the maturing rabbit reticulocyte on incubation in vitro. Biochem. J., 94 : 14P.

Bethlenfalvay, N.C. (1971): Resistance of reticulocytes to oxidation by nitrite: A cytologic demonstration. J. Lab. clin. med., 77 : 361-5.

Beutler, E. (1957): The glutathione instability of drug-sensitive red cells. A new method for the in vitro detection of drug sensitivity. J. Lab. clin. Med., 49 : 84-95.

Beutler, E. (1959): The haemolytic effect of primaquine and related compounds. A review. Blood, 14 : 103-39.

Beutler, E. (1962): Drug induced haemolytic anaemia and the mechanism and significance of Heinz body formation in red blood cells. Nature, Lond., 196 : 1095-6.

Beutler, E. (1969): Biochemistry of the erythrocyte. Experientia, 25 : 470-73.

Beutler, E.; Dern, R.J.; Alving, A.S. (1955a): The hemolytic effect of primaquine. VI. An in vitro test for sensitivity of erythrocytes to primaquine. J. Lab. clin. Med., 45 : 40-50.

Beutler, E.; Dern, R.J.; Flanagan, C.L.; Alving, A.S. (1955b): The hemolytic effect of primaquine. VII. Biochemical studies of drug-sensitive erythrocytes. J. Lab. clin. Med., 45 : 286-95.

Beutler, E.; Robson, Melba; Buttenwieser, Ellen (1957): The mechanism of glutathione destruction and protection in drug-sensitive and non-sensitive erythrocytes. In vivo studies. J. clin. Invest., 36 : 617-28.

Beutler, E.; Duron, Olga; Kelly, Barbara M. (1963): Improved method for the determination of blood glutathione. J. Lab. clin. Med., 61 : 882-888.

Biozzi, G.; Benacerraf, B.; Mene, G.; Halpern, B.N. (1951): Etude quantitative de l'activite granulopexique du systeme reticuloendothelial par l'injection intraveineuse d'encre de chine chez les diverses especes animales; relations entre les modifications de la coagulation du sang in vitro sous l'effet de l'injection intraveineuse de doses croissantes d'encre de chine et sa repartition dans l'organisme. Ann. Inst. Pasteur, 81 : 164-72. (Cited by Halpern et al., 1953).

Biozzi, G.; Benacerraf, B.; Halpern, B.N. (1953): Quantitative study of the granulopectic activity of the reticuloendothelial system. II. A study of the kinetics of the granulopectic activity of the RES in relation to the dose of carbon injected. Relationship between the weight of the organs and their activity. Br. J. exp. Path., 34 : 441-57.

Bird, R.M. (1947): Glycolysis in human blood. J. biol. Chem., 169 : 493-501.

Bishop, C. (1964): Overall red cell metabolism. Chap. 4 of The Red Blood Cell. (Bishop, C.; Surgenor, D.M.; eds.), Academic Press. New York and London.

Bovarinck, Marianna, R.; Lindsay, Ann; Hellerman, L. (1946): Metabolism of the malarial parasite, with reference particularly to the action of antimalarial agents. II. Atabrine (quinacrine) inhibition of glucose oxidation in parasites initially depleted of substrate. Reversal by adenylic acid. J. biol. Chem., 163 : 535-51.

Bowman, I.B.R.; Grant, P.T.; Kermack, W.O.; Ogston, D. (1959): An inhibitory effect of mepacrine on the catabolism of glucose by Plasmodium berghei. Biochem. J., 73 : 42P-43P.

Bowman, I.B.R.; Grant, P.T.; Kermack, W.O. (1960): The metabolism of Plasmodium berghei, the malaria parasite of rodents. I. The separation of the erythrocytic form of P. berghei separated from the host cell. Expl. Parasit., 9 : 131-36.

Bowman, I.B.R.; Grant, P.T.; Kermack, W.O.; Ogston, D. (1961): The metabolism of Plasmodium berghei, the malaria parasite of rodents. II. An effect of mepacrine on the metabolism of glucose by the parasite separated from its host cell. Biochem. J., 78 : 472-8.

Brandt, H.; Rapoport, S. (1959): Zur frage des endogenen substrats und der glukose-hemmung de atmung der retikulozyten und ascitestumorzellen. Acta biologica et medica germanica, 2 : 290-97. (Cited by Rickard, 1969).

Brewer, G.J.; Tarlov, A.R.; Alving, A.S. (1960): Methaemoglobin reduction test. A new simple in vitro test for identifying primaquine sensitivity. Bull. Wld. Hlth. Org., 22 : 633-40.

Brickner, R.M. (1927): Role of capillaries and their endothelium in distribution of colloidal carbon by blood stream. Johns Hopkins Hosp. Bull., 40 : 90-109.

Brown, J.M.M. (1963): Biochemical lesions in the pathogenesis of Geeldikkop (Tribulosis ovis) and enzootic icterus in sheep in South Africa. Ann. N. Y. Acad. Sci., 104 : 504-38.

Bryant, C.; Voller, A. (1961): Preliminary studies on carbon utilization patterns of Plasmodium vinckei. Trans. R. Soc. trop. Med. Hyg., 55 : 305.

Bryant, C.; Voller, A.; Smith, M.J.H. (1964): The incorporation of radioactivity from (C^{14}) glucose into the soluble metabolic intermediates of malaria parasites. Am. J. trop. med. Hyg., 13 : 515-19.

Buck, W.B.; James, L.F.; Binns, W. (1961): Serum transaminase activities associated with plant and mineral toxicity in sheep and cattle. Cornell Vet., 51 : 568-85.

Budtz-Olsen, O.E.; Axten, B.; Haigh, S. (1963): Glucose-6-phosphate dehydrogenase deficiency in erythrocytes of sheep and goats. Nature, Lond., 198 : 1101-2.

Campbell, R.W.; Sloan, C.A.; Harbutt, Patricia R. (1971): Observations on mortality in lambs in Victoria associated with Eperythrozoon ovis. Aust. vet. J., 47 : 538-41.

Cantrell, W.; Elko, E.E. (1964): Reticulo-endothelial function in rats infected with malaria. Fedn. Proc., 23 : 139.

Cantrell, W.; Elko, E.E.; Hopff, Barbara M. (1970): Plasmodium berghei: Phagocytic hyperactivity of infected rats. Expl. Parasit., 28 : 291-7.

Cappell, D.F. (1929): Intravitam and supravital staining. J. Path. Bact., 32 : 595-708.

Carson, P.E. (1960): Glucose-6-phosphate dehydrogenase deficiency in hemolytic anaemia. Fedn. Proc., 19 : 995-1006.

Carson, P.E.; Flanagan, C.L.; Ickes, C.E.; Alving, A.S. (1956): Enzymatic deficiency in primaquine sensitive erythrocytes. Science, 124 : 484-85.

Carter, J.M.; Brobst, D. (1969): A comparison of the pH of canine capillary, arterial and venous blood. J. comp. lab. Med., 3 : 19-24.

Cheun, L.H. (1966): Glucose-6-phosphate dehydrogenase activity in erythrocytes of experimental animals. J. clin. Path., 19 : 614-16.

Christophers, S.R.; Fulton, J.D. (1938): Observations on the respiratory metabolism of malaria parasites and trypanosomes. Ann. trop. Med. Parasit., 32 : 43-75.

Cohen, G.; Hochstein, P. (1961): Glucose-6-phosphate dehydrogenase and detoxification of hydrogen peroxide in human erythrocytes. Science, 134 : 1756-57.

Cohen, G.; Hochstein, P. (1963): Glutathione peroxidase: The primary agent for the elimination of hydrogen peroxide in erythrocytes. Biochem., Easton, 2 : 1420-28.

Cohen, G.; Hochstein, P. (1964): Generation of hydrogen peroxide in erythrocytes by hemolytic agents. Biochem, Easton, 3 : 895-900.

Coles, E.H. (1967): Veterinary Clinical Pathology. W.B. Saunders Co. Philadelphia and London.

Coombs, R.R.A.; Mourant, A.E.; Race, R.R. (1945): Detection of weak and incomplete Rh agglutinins: A new test. Lancet, ii : 15-16.

Cordes, W. (1926): Experiences with plasmochin in malaria (preliminary reports). 15th Annual Report, United Fruit Co. (Med. Dept.), p. 66-71. (Cited by Beutler, 1959).

Cornelius, C.E. (1970): Liver function. In Clinical Biochemistry of Domestic Animals. 2nd Ed. Vol. I. Chap. 5. (Kaneko, J.J.; Cornelius, C.E. eds.) Academic Press. New York and London.

Cox, F.E.G. (1966): Acquired immunity to Plasmodium vinckei in mice. Parasitology, 56 : 719-32.

Cox, H.W.; Schroeder, W.F.; Ristic, M. (1966): Hemagglutination and erythrophagocytosis associated with the anaemia of Plasmodium berghei infection of rats. J. Protozool., 13 : 327-32.

Crosby, W.H.; Dameshek, W. (1953): Paroxymal nocturnal hemoglobinuria. Relation of the clinical manifestations to underlying pathogenic mechanisms. Blood, 8 : 769-812.

Culling, C.F.A. (1963): Handbook of Histopathological Techniques. 2nd ed. Butterworths. London.

Dacie, J.V. (1967): The Haemolytic Anaemias. Part IV. Drug induced haemolytic anaemias etc. 2nd ed. pp 1008-1030. J. and A. Churchill Ltd. London.

Dacie, J.V.; Lewis, S.M. (1968): Practical Haematology. 4th ed. J. and A. Churchill Ltd. London.

Dajani, R.M.; Orten, J.M. (1958): A study of the citric acid cycle in erythrocytes. J. biol. Chem., 231 : 913-24.

Davies, J.P.H.; Gower, D.B. (1964): Conditions affecting the glucose-6-phosphate dehydrogenase activity and glutathione stability of human erythrocytes. Nature, Lond., 203 : 310-11.

Davson, H.; Danielli, J.F. (1952): The Permeability of Natural Membranes. 2nd ed. P.150. Cambridge University Press.

Dawson, T.J.; Evans, J.V. (1965): Effect of hemoglobin type on the cardiorespiratory system of sheep. Am. J. Physiol., 209 : 593-98.

Delpy, L. (1936): Agents pathogenes observes en Iran dans le sang des animaux domestiques. (Note preliminaire). Bull. Soc. Path. exot., 29 : 157-61.

Dern, R.J.; Beutler, E.; Alving, A.S. (1954): The hemolytic effect of primaquine. II. The natural course of the hemolytic anemia and the mechanism of its self-limited character. J. Lab. clin. Med., 44 : 171-76.

Di Luzio, N.R.; Riggi, S.J. (1964): The development of a lipid emulsion for the measurement of reticuloendothelial function. J. Reticuloendothel. Soc., 1 : 136-49.

Dimopoullos, G.T.; Schrader, G.T.; Foote, L.E. (1959): Electrophoretic studies of bovine serum. II. Concurrent hypoglobulinemia and natural infections of eperythrozoonosis. Proc. Soc. exp. Biol. Med., 100 : 55-58.

Dimopoullos, G.T. (1970): Plasma proteins. In Clinical Biochemistry of Domestic Animals, 2nd ed. Vol. I. Chap. 3. (Kaneko, J.J.; Cornelius, C.E., eds). Academic Press. New York and London.

Donald, K.J. (1972a): The role of platelets in the clearance of colloidal carbon from blood in rabbits: A light and electron microscope study. Pathology, 4 : 295-301.

Donald, K.J. (1972b): The mechanism of enhanced clearance of colloidal carbon from the blood of rabbits stimulated by a tubercle bacillary lipid. J. Path., 108 : 97-104.

Donald, K.J. (1972c): The kinetics of carbon clearance in rabbits stimulated by a tubercle bacillary lipid - A statistical model for carbon clearance studies. Br. J. exp. Path., 53 ; 560-59.

Donald, K.J.; Pound, A.W. (1971): The effect of a tubercle bacillary lipid on the clearance rate of colloidal carbon from the blood of rabbits. Br. J. exp. Path., 52 : 256-63.

Donald, K.J.; Pound, A.W. (1973): Proliferation and migration of reticuloendothelial cells following injection of a tubercle bacillary lipid. Br. J. exp. Path., 54 : 79-89.

Donatien, A.; Lestoquard, F. (1935): Existence d'Eperythrozoon ovis en Algerie. Bull. Soc. Path. exot., 28 : 423-26.

Donawick, W.J.; Baue, A.E. (1968): Blood gases, acid-base balance and alveolar-arterial oxygen gradient in calves. Am. J. vet. Res., 29 : 561-67.

Done, J.; Mortimer, P.H.; Taylor, A. (1958): Some observations on field cases of facial eczema; liver pathology and determination of serum bilirubin, cholesterol, transaminase and alkaline phosphatase. Res. vet. Sci., 1 : 76-83.

Doxey, D.L. (1971): Veterinary Clinical Pathology. Bailliere Tindall. London.

D'yakanov, L.D. (1964): (The finding of Eperythrozoon ovis). Veterinariya, Moscow, 41 : 62-63. (Abstr. Vet. Bull., 34 : No. 2490, 1964).

Ellory, J.C.; Tucker, Elizabeth M. (1970): Active potassium transport in the immature red cells of anaemic sheep. J. Physiol., Lond., 208 : 18P-19P.

Evans, J.V. (1954): Electrolyte concentrations in red blood cells of British breeds of sheep. Nature, Lond., 174 : 931-32.

Evans, J.V.; King, J.W.B.; Cohen, B.L.; Harris, H.; Warren, F.L. (1956): Genetics of haemoglobin and blood potassium differences in sheep. Nature, Lond., 178 : 849-50.

Evans, J.V.; Blunt, M.H.; Southcott, W.H. (1963): The effects of infection with Haemonchus contortus on the sodium and potassium concentrations in the erythrocytes and plasma, in sheep of different haemoglobin types. Aust. J. agric. Res., 14 : 549-58.

Evelyn, K.A.; Malloy, Helga T. (1938): Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. biol. Chem., 126 : 655-62.

Filkins, J.P.; DiLuzio, N.R. (1966a): Mechanism of gelatin inhibition of reticuloendothelial function. Proc. Soc. exp. Biol. Med., 122 : 177-81.

Filkins, J.P.; DiLuzio, N.R. (1966b): Effects of gelatin and heparin on intravascular phagocytosis. J. Reticuloendothel. Soc., 3 : 398-414.

Fisher, S.; Riley, Wendy B.; Shorley, C.D. (1968): Studies on the mechanism of increased splenic particle uptake in mice after the injection of some finely divided agents. J. Path. Bact., 96 : 463-71.

Fletcher, K.A.; Maegraith, B.G. (1962): Glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities in erythrocytes of monkeys infected with Plasmodium knowlesi. Nature, Lond., 196 : 1316-18.

Fletcher, K.A.; Maegraith, B.G. (1970): Erythrocyte reduced glutathione in malaria (Plasmodium berghei and P. knowlesi). Ann. trop. Med. Parasit., 64 : 481-86.

Foggie, A. (1961): Eperythrozoon infection in sheep in Scotland. Vet. Rec., 73 : 453-54.

Foggie, A.; Nisbet, D.I. (1964): Studies on Eperythrozoon infection in sheep. J. comp. Path., 74 : 45-61.

Foggie, A.; Nisbet, D.I. (1966): Eperythrozoon ovis. Vet. Rec., 79 : 297-98.

Folse, D.S.; Beathard, G.A.; Marshall, R.B.; Fish, J.C.; Sarles, H.E.; Remmers, A.R. Jnr.; Ritzmann, S.E. (1971): Characterization of the bovine hemal node. J. Reticulo-endothel. Soc., 10 : 461-81.

Fornaini, G.; Bossu, M. (1969): The inherited defects of erythrocyte metabolism. Ital. J. Biochem., 18 : 185-326.

Fred, R.K.; Dobson, E.L.; Kelly, Lola S.; Shore, M.L. (1970): The proliferative and phagocytic capacity of the reticulo-endothelial system of sublethally irradiated mice. J. Reticuloendothel. Soc., 7 : 453-70.

Friedhoff, K.; Drommer, W.; Wolfhagen, M. (1971): (Eperythro-zoon ovis infections in sheep in North Germany). Infektioner mit Eperythrozoon ovis bei schafen in Nord-Deutschland. Berliner und Munchener Tierarytliche Wochenschrift, 84 : 361-68. (Abstr. Vet. Bull., 42 : No. 1789, 1972).

Fulton, J.D. (1939): Experiments on the utilization of sugars by malaria parasites (Plasmodium knowlesi). Ann. trop. Med. Parasit., 33 : 217-27.

Fulton, J.D.; Grant, P.J. (1956): The sulphur requirements of the erythrocytic form of Plasmodium knowlesi. Biochem. J., 63 : 274-82.

Gabrieli, E.R.; Pyzikiewicz, T.; Mlodozeniec, P. (1967): Comments on the india ink clearance as a reticulo-endothelial test. J. Reticuloendothel. Soc., 4 : 223-27.

Gabrio, B.W.; Finch, C.A.; Heunnekens, F.M. (1956): Erythrocyte preservation: A topic in molecular biochemistry. Blood, 11 : 103-115.

Garnham, P.C.C. (1966): Malaria Parasites and other Haemosporidia. Blackwell. Oxford.

George, C.N.J.; Pollack, S. (1966): Glucose-6-phosphate dehydrogenase deficiency and malaria. II. The mechanism of oxidative drug action in Plasmodium berghei infections. Proc. 1st int. Cong. Parasit., 1 : 252.

George, J.N.; Stokes, E.F.; Wicker, D.J.; Conrad, M.E. (1966): Studies on the mechanism of hemolysis in experimental malaria. Milit. Med., 131 : 1217-24.

Gibson, Q.H. (1948): The reduction of methaemoglobin in red blood cells and studies on the cause of idiopathic methaemoglobinaemia. Biochem. J., 42 : 13-23.

Glasgow, L.A.; Odugbemi, T.; Dwyer, P.; Ritterson, A.L. (1971): Eperythrozoon coccoides. I. Effect on the interferon response in mice. Infec. Immunity, 4 : 425-30.

Gledhill, A.W.; Dick, G.W.A.; Niven, Janet S.F. (1955): Mouse hepatitis virus and its pathogenic action. J. Path. Bact., 69 : 299-309.

Gledhill, A.W.; Bilbey, D.L.J.; Niven, Janet S.F. (1965): Effect of certain murine pathogens on phagocytic activity. Br. J. exp. Path., 46 : 433-42.

Gledhill, A.W.; Niven, Janet S.F. (1957): The toxicity of some bacterial filtrates for mice pre-infected with Eperythrozoon coccoides. Br. J. exp. Path., 38 : 284-90.

Gordon, A.S.; Katsh, G.F. (1949): Regulation of adrenal cortex to structure and phagocytic activity of macrophagic system. Ann. N. Y. Acad. Sci., 52 : 1-30.

Grimes, A.J. (1963): Glycolysis in young and mature normal human erythrocytes. Nature, Lond., 198 : 1312-13.

Gwatkin, R.; Plummer, P.J.G. (1946): Toxicity of certain salts of sodium and potassium for swine. Can. J. comp. Med., 10 : 183-90.

Halpern, B.N.; Benacerraf, B.; Biozzi, G. (1953): Quantitative study of the granulopectic activity of the reticuloendothelial system. I. The effect of the ingredients present in india ink and of substances affecting blood clotting in vivo on the fate of carbon particles administered intravenously in rats, mice and rabbits. Br. J. exp. Path., 34 : 426-40.

Halpern, B.N.; Biozzi, G.; Nicol, T.; Bilbey, D.L.J. (1957): Effect of experimental biliary obstruction on the phagocytic activity of the reticuloendothelial system. Nature, Lond., 180 : 503-4.

Harboe, M. (1959): A method for determination of hemoglobin in plasma by near-ultraviolet spectrophotometry. Scand. J. clin. lab. Invest., 11 : 66-70.

Harbutt, Patricia R. (1969a): The incidence and clinical significance of Eperythrozoon ovis infections of sheep in Victoria. Aust. vet. J., 45 : 493-99.

Harbutt, Patricia R. (1969b): The effect of Eperythrozoon ovis infection on body weight gain and haematology of lambs in Victoria. Aust. vet. J., 45 : 500-4

Harris, J.W.; Kellermeyer, R.W. (1970): The Red Cell. Production, Metabolism, Destruction: Normal and Abnormal. Revised ed. Harvard University Press. Cambridge. Massachusetts

Harrop, G.A. (1919): The oxygen consumption of human erythrocytes. Arch. Intern. Med., 23 : 745-52.

Henry, R.J. (1964): Clinical Chemistry (1st reprint ed. 1966). Harper and Row, New York, Evanston and London. John Weatherill Inc. Tokyo.

Hockwald, R.S.; Arnold, J.; Clayman, C.B.; Alving, A.S. (1952): Status of primaquine. IV. Toxicity of primaquine in Negroes. J. Am. Med. Ass., 149 : 1568-70,

Holtenius, P. (1957): Nitrite poisoning in sheep, with special reference to the detoxification of nitrite in the rumen. Acta agric. Scand., 7 : 113-63.

Howard, J.G. (1959): Activation of the reticuloendothelial cells of the mouse liver by bacterial lipopolysaccharide. J. Path. Bact., 78 : 465-70.

Hoyte, H.M.D. (1962): Eperythrozoon teganodes sp. nov. (Rickettsiales) parasitic in cattle. Parasitology, 52 : 527-32.

Hoyte, H.M.D. (1971): The infectivity of Theileria mutans, Eperythrozoon wenyoni and E. teganodes to sheep and of Eperythrozoon ovis to cattle. Br. vet. J., 127 : lv-lvii.

Huisman, T.H.J.; Kitchens, T. (1968): Oxygen equilibria studies of the hemoglobins from normal and anaemic sheep and goats. Am. J. Physiol., 215 : 140-146

Huisman, T.H.J.; van Vliet, G.; Sebens, T. (1958): Sheep haemoglobins. Some genetic and physiological aspects of two different adult haemoglobins in sheep. Nature, Lond., 182 : 171-72.

Iyengar, B.; Chandra, K. (1972): Iron release in the reticulo-endothelial cells of the spleen after red cell damage. Acta. haemat., 48 : 354-59.

Jacob, H.S.; Karnowsky, M.L. (1967): Concomitant alterations of sodium flux and membrane phospholipid metabolism in red blood cells: Studies in hereditary spherocytosis. J. clin. Invest., 46 : 173-85.

Jaffe, E.R. (1959): The reduction of methemoglobin in human erythrocytes incubated with purine nucleosides. J. clin. Invest., 38 : 1555-63.

Jaffe, E.R.; Vanderhoff, Grace A.; Lowy, B.A.; London, I.M. (1957): Effect of nucleosides on osmotic resistance of mammalian erythrocytes in relation to the age of cells. J. clin. Invest., 36 : 903.

Jamieson, N.D. (1958): Adverse effect of nitrate metabolic products on sheep growth. Nature, Lond., 181 : 1601-2.

Jandl, J.H.; Engle, L.K.; Allen, D.W. (1960): Oxidative hemolysis and precipitation of the hemoglobin. I. Heinz body anaemias as an acceleration of red cell aging. J. clin. Invest., 39 : 1818-36.

Jandl, J.H.; Files, N.M.; Barnett, S.B.; MacDonald, R.A. (1965): Proliferative response of the spleen and liver to hemolysis. J. exp. Med., 122 : 299-326.

Jenkin, C.R.; Rowley, D. (1961): The role of opsonins in the clearance of living and inert particles by cells of the reticuloendothelial system. J. exp. Med., 114 : 363-74.

Jensen, E.V. (1959): Sulphydryl-disulphide interchange. Science, 130 : 1319-23.

Jensen, R. (1943): Eperythrozoonosis in cattle and sheep of Louisiana, preliminary report. Bull. La. agric. exp. Stat., No 366 : 2-8

Jeunet, F.S.; Good, R.A. (1967): Reticuloendothelial function in the isolated perfused liver. Study of rates of clearance, role of a plasma factor, and the nature of RE blockade. J. Reticuloendothel. Soc., 4 : 351-69.

Jilek, A.J.; Bradley, R.E. (1969): Hemoglobin types and resistance to Haemonchus contortus in sheep. Am. J. vet. Res., 30 : 1773-78.

Jolly, R.D. (1967); Eperythrozoon ovis infection in a lamb. N. Z. vet. J., 15 : 47-48.

Jones, P.E.H.; McCange, R.A. (1949): Enzyme activities in the blood of infants and adults. Biochem. J., 45 : 464-67.

Kampschmidt, R.F.; Upchurch, H.F.; Park, A. (1965): Factors in the suspending media which alter the carbon clearance rate. J. Reticuloendothel. Soc., 3 : 214-22.

Kaneko, J.J.; Mills, R. (1969): Erythrocytic enzyme activity, ion concentrations, osmotic fragility, and glutathione stability in bovine erythropoietic porphyria and its carrier state. Am. J. vet. Res., 30 : 1805-10.

Kaneko, J.J.; Smith, J.E. (1964): Glucose-6-phosphate dehydrogenase activity, glutathione stability and the methemoglobin-reducing ability of erythrocytes of sheep acclimatized to high altitude. Am. J. vet. Res., 25 : 846-48.

Kaneko, J.J.; Smith, Rachel (1967): The estimation of plasma fibrinogen and its clinical significance in the dog. Calif. Vet., 21 : 21-24.

Kay, W.W.; Murfitt, K.C. (1960): The determination of blood glutathione. Biochem. J., 74 : 203-8.

Keene, W.R.; Jandl, J.H. (1965): The sites of hemoglobin catabolism. Blood, 26 : 705-19.

Kelly, Lola S.; Brown, B.A.; Dobson, E.L. (1962): Cell division and phagocytic activity in liver reticuloendothelial cells. Proc. Soc. exp. Biol. Med., 110 : 555-59.

Kelly, Lola S.; Dobson, E.L. (1971): Evidence concerning the origin of liver macrophages. Br. J. exp. Path., 52 : 88-99.

Kent, Rosalind (1966): Uptake of carbon particles by the RES of the fowl, the frog, and the chick embryo. J. Reticulo-endothel. Soc., 3 : 271-93.

Koch, J.H. (1963a): The toxic action of phenothiazine and some disturbances of intermediary metabolism in under-nourished sheep. Aust. J. agric. Res., 14 : 529-39.

Koch, J.H. (1963b): Glucose-6-phosphate dehydrogenase and potassium concentration in sheep erythrocytes. Nature, Lond., 200 : 1334-35.

Koenig, M.G.; Heyssel, R.M.; Melly, M.A.; Rogers, D.E. (1965): The dynamics of reticuloendothelial blockade. J. exp. Med., 122 : 117-42.

Koepsell, H.J.; Sharpe, E.S. (1952): Microdetermination of pyruvic and α keto-glutaric acids. Arch. Biochem. Biophys., 38 : 443-54.

Kohn, J. (1969): Separation of haemoglobins on cellulose acetate. J. clin. Path., 22 : 109-11.

Kornberg, A.; Horecker, B.L. (1955): Glucose-6-phosphate dehydrogenase. In Methods in Enzymology. (Colowick, S.P.; Kaplan, N.O. eds). 1 : 306. Academic Press. New York.

Kosower, E.M.; Kosower, Nechama S. (1969): Lest I forget thee, glutathione. Nature, Lond., 224 : 117-20.

Kosower, Nechama S.; Kosower, E.M. (1970): Molecular basis for selective advantage of glucose-6-phosphate dehydro-genase deficient individuals exposed to malaria. Lancet, ii : 1343-45.

Kosower, Nechama S.; Vanderhoff, G.A.; Jaffe, E.R. (1963): Metabolic changes in normal and glucose-6-phosphate dehydrogenase deficient erythrocytes induced by acetylphenylhydrazine. J. clin. Invest., 42 : 1025-30.

Kreier, J.P.; Ristic, M. (1963): Morphologic antigenic and pathogenic characteristics of Eperythrozoon ovis and Eperythrozoon wenyoni. Am. J. vet. Res., 24 : 488-500.

Kreier, J.P.; Ristic, M.; Schroeder, W. (1964): Anaplasmosis. XVI. The pathogenesis of anaemia produced by infection with Anaplasma. Am. J. vet. Res., 25 : 343-52.

Kreier, J.P.; Ristic, M. (1968): Haemobartonellosis and related diseases. In Infectious Blood Diseases of Man

and Animals. Diseases caused by Protista. Vol. II. The Pathogens, the Infections and the Consequences. (Weinman, D.; Ristic, M. eds.) Chap. 22. Academic Press. New York and London.

Kronfeld, D.S.; Medway, W. (1969): A Textbook of Veterinary Clinical Pathology. Chap. 2. (Medway, W.; Prier, J.E.; Wilkinson, J.S. eds). The Williams and Wilkins Co., Baltimore.

Kyurtov, N. (1971): (Eperythrozoon ovis in sheep in Bulgaria). Veterinarna Shirka (Sofia), 68 : 19-20.

Lafenetre, H. (1936): Existence d'Eperythrozoon ovis en France. Rev. vet. Toulouse, 88 : 200-1. (Cited by Weinman, 1944).

Landau, M.; McNee, J.W. (1914): Zur physiologie des chold-sterinstoffwechsels. Beitr. path. Anat., 58 : 667-93. (Cited by Stuart, 1970).

Lathem, W. (1959): The renal excretion of hemoglobin. Regulatory mechanisms and the differential excretion of free and protein-bound hemoglobin. J. clin. Invest., 38 : 652-58.

Legge, J.W.; Roughton, F.J.W. (1950): Some observations on the kinetics of haemoglobin in solution and in the red blood corpuscle. Biochem. J., 47 : 43-52.

Leng, R.A.; Annison, E.F. (1962): Metabolic activities of sheep erythrocytes. I. Glycolytic activities. Aust. J. agric. Res., 13 : 31-44.

Leonardi, P.; Ruol, A. (1960): Renal hemosiderosis in the hemolytic anemias: Diagnosis by means of a needle biopsy. Blood, 16 : 1029-38.

Liddy, T.J.; Roberts, W.C. (1970): Chronic intravascular hemolysis (renal hemosiderosis) after incomplete prosthetic closure of a ventricular septal defect and noncalcific aortic regurgitation. Am. J. clin. Path., 53 : 839-42.

Littlejohns, I.R. (1960): Eperythrozoonosis in sheep. Aust. vet. J., 36 : 260-65.

Lo, S.S.; Marti, H.R.; Hitzig, W.H. (1971): Hemolytic anemia associated with decreased concentration of reduced glutathione in red cells. Acta haemat., 46 : 14-23.

Loder, P. Bronwen; de Gruchy, G.C. (1965): Red-cell enzymes and co-enzymes in non-spherocytic congenital haemolytic anaemias. Br. J. Haemat., 11 : 21-31.

Lohr, G.W.; Waller, H.D. (1961): Zur biochemie der erythrozytenalterung. Folia. Haemat., 78 : 385. (Cited by Tonz, 1968).

Love, J.N.; McEwan, E.G. (1972): Hypoglycemia associated with Haemobartonella-like infection in splenectomized calves. Am. J. vet. Res., 33 : 2087-89.

Lowy, B.A.; Jaffe, E.R.; Vanderhoff, G.A.; Crook, L.; London, I.M. (1958): The metabolism of purine nucleosides by the human erythrocyte in vitro. J. biol. Chem., 230 : 409-19.

Luzzatto, L.; Usanga, E.A.; Reddy, S. (1969): Glucose-6-phosphate dehydrogenase deficient red cells: Resistance to infection by malarial parasites. Science, 164 : 839-42.

MacCallum, D.K. (1969): Time sequence study on the hepatic system of macrophages in malaria-infected hamsters. J. Reticuloendothel. Soc., 6 : 232-52.

MacDonald, R.A. (1969): Human and experimental hemochromatosis and hemosiderosis. In Pigments in Pathology. (Wolman, M. ed.) Chap. 5. Academic Press. New York and London.

McKee, A.E.; Ziegler, R.F.; Giles, R.C. (1973): Scanning and transmission electron microscopy of Haemobartonella canis and Eperythrozoon ovis. Am. J. vet. Res., 34 : 1196-201.

McKee, R.W.; Ormsbee, R.A.; Anfinsen, C.B.; Geiman, Q.M.; Ball, E.G. (1946): Studies on malarial parasites. VI. The chemistry and metabolism of normal and parasitized (P. knowlesi) blood. J. exp. Med., 84 : 569-621.

Malloy, Helga T.; Evelyn, K.A. (1937): The determination of bilirubin with the photoelectric colorimeter. J. biol. Chem., 119 : 481-90.

Mann, D.K.; Ristic, M. (1963): Anaplasmosis. XIII. Studies concerning the nature of autoimmunity. Am. J. vet. Res., 24 : 703-8.

Manwell, R.D. (1968): Malaria. In Infectious Blood Diseases of Man and Animals. Diseases caused by Protista. Vol. II. The Pathogens, the Infections and the Consequences. (Weinman, D.; Ristic, M. eds). Chap. 16. Academic Press. New York and London.

Marks, P.A.; Johnson, A.B.; Hirschberg, E. (1958): Effect of age on the enzyme activity in erythrocytes. Proc. Nat. Acad. Sci. U. S. A., 44 : 529-36.

Maronpot, R.R. (1972): Erythrocyte glucose-6-phosphate dehydrogenase and glutathione deficiency in sheep. Can. J. comp. Med., 36 : 55-60.

Matthies, H. (1956): Methaemoglobinruckbiddung in reticulocyten. Arch. exp. Path. Pharmakol., 229 : 331. (Cited by Tonz, 1968).

Maxwell, J.A.L. (1969): Eperythrozoon ovis infection in Western Australia. Aust. vet. J., 45 : 436.

Mills, G.C. (1959): The purification and properties of glutathione peroxidase of erythrocytes. J. biol. Chem., 234 : 502-6.

Mills, G.C.; Randall, H.P. (1958): Hemoglobin catabolism. II. The protection of hemoglobin from oxidative breakdown in the intact erythrocyte. J. biol. Chem., 232 : 589-98.

Moulder, J. (1962): The Biochemistry of Intracellular Parasitism. University of Chicago Press. Chicago. Illinois.

Munson, A.E.; Regelson, W.; Wooles, W.R. (1970): Tissue localization studies in evaluating the functional role of the RES. J. Reticuloendothel. Soc., 7 : 366-74.

Murphy, J.R. (1960): Erythrocyte metabolism. II. Glucose metabolism and pathways. J. Lab. clin. Med., 55 : 286-302.

Murray, I.M. (1963): The mechanism of blockade of the reticuloendothelial system. J. exp. Med., 117 : 139-47.

Nagao, K. (1920): The fate of india ink injected into the blood. I. General observations. J. infect. Dis., 27 : 527-38.

Neethling, L.P.; Brown, J.M.M.; Osterhoff, D.R.; de Wet, P.J.; Ward-Cox, I.S. (1969): The functional advantage of haemoglobin Type A in haemolytic syndromes in sheep. J. S. Afr. vet. med. Ass., 40 : 121-28.

Neitz, W.O.; Alexander, R.A.; du Toit, P.J. (1934): Eperythrozoon ovis (sp. nov.) infection in sheep. Ondersterpoort J. vet. Res., 3 : 263-71.

Neitz, W.O. (1937): Eperythrozoonosis in sheep. Onderster-poort J. vet. Res., 9 : 9-30.

Neitz, W.O. (1939): Ovine anaplasmosis: The transmission of Anaplasma ovis and Eperythrozoon ovis to the blesbuck (Damaliscus albifrons). Ondersterpoort J. vet. Res., 13 : 9-16.

Neitz, W.O. (1940): Eperythroonosis in cattle. Onderster-poort J. vet. Res., 14 : 9-28.

Nicholas, D.J.D. (1959): Cambridge Review Papers. Soc. exp. Biol. Symposia. XIII : pp 1-23.

Nikol'skii, S.N.; Slipchenko, S.N. (1969): (Experimental transmission of Eperythrozoon ovis by the ticks Hyalomma plumbeum and Rhipicephalus bursa). Veterinariya, Moscow, 5 : 46. (Abstr. Vet. Bull., 40 : No. 52, 1970).

Nisselbaum, J.S.; Bodansky, O. (1965): Glutamic-oxalacetic transaminase in reticulocytes and erythrocytes. Science, 149 : 195-97.

Niven, Janet S.F.; Gledhill, A.W.; Dick, G.W.A.; Andrewes, C.H. (1952): Further light on mouse hepatitis. Lancet, ii : 1061.

Normann, S.J.; Benditt, E.P. (1965a): Function of the reticuloendothelial system. I. A study on the phenomenon of carbon clearance inhibition. J. exp. Med., 122 : 693-707.

Normann, S.J.; Benditt, E.P. (1965b): Function of the reticuloendothelial system. II. Participation of a serum factor in carbon clearance. J. exp. Med., 122 : 709-19.

Nossal, P.M. (1948): The metabolism of erythrocytes. I. Respiration in the absence and presence of methylene blue. Aust. J. exp. Biol. Med. Sci., 26 : 123-38.

Odeblad, E.; Dobson, E.L.; Odeblad, A.M.; Jones, H.B. (1955): Autoradiographic study of the distribution of radioactive particulate chromic phosphate in liver, spleen and lung of the mouse. Am. J. Physiol., 181 : 210-14.

Ohder, H. (1967): Some observations on Eperythrozoon infection in non-splenectomized sheep and the detection of the parasites and their antibodies by immunofluores-cence. Zentbl. Bakt. Parasitkde, I (Orig)., 203 : 391-401.

Okuyama, S.; Ito, Y. (1973): Reticuloendothelial uptake of hemoglobin assessed with radiocolloid: An experimental approach to intravascular hemolytic sequelae. J. Reticuloendothel. Soc., 14 : 68-78.

Oort, M.; Loos, J.A.; Prins, H.K. (1961): Hereditary absence of reduced glutathione in the erythrocytes, a new clinical and biochemical entity? (Preliminary communication). Vox Sang, 6 : 370-73. (Cited by Tonz, 1968).

Ott, Karen J.; Astin, J.F.; Stauber, L.A. (1967): Eperythrozoon coccoides and rodent malaria: Plasmodium chabaudi and Plasmodium berghei. Expl. Parasit., 21 : 68-77.

Ott, Karen J.; Stauber, L.A. (1967): Eperythrozoon coccoides: Influence on course of infection of Plasmodium chabaudi in mouse. Science, 155 : 1546-48.

Øverås, J. (1959): Eperythrozoon ovis en ny blodparasitt hos sau i Norge. Nord. Vet. Med., 11 : 791-800.

Øverås, J. (1962): Medl. blad. for Den. Norske Veter., 7 : 187. (Cited by Foggie and Misbet, 1964).

Øverås, J. (1969): Studies on Eperythrozoon ovis infection in sheep. Acta vet. Scand., Supplement 28.

Peters, W. (1965): Competitive relationship between Eperythrozoon coccoides and Plasmodium berghei in the mouse. Expl. Parasit., 16 : 158-66.

Peters, D.; Wigand, R. (1955): Bartonellaceae. Bact. Rev., 19 : 150-55.

Phillips, G.D. (1970): The assessment of blood acid-base parameters in ruminants. Br. vet. J., 126 : 325-32.

Phillips, R.W.; Knox, K.L. (1969): Diarrheic acidosis in calves. J. Comp. lab. Med., 3 : 1-3.

Pimstone, N.R. (1972): Renal degradation of hemoglobin. Semin. Haemat., 9 : 31-42.

Pisano, J.C.; Patterson, J.T.; DiLuzio, N.R. (1968): Reticuloendothelial blockade: Effect of puromycin on opsonin-dependent recovery. Science, 162 : 565-67.

Popper, H.; Schaffner, F. (1957): Liver: Structure and Function. (p96) The Blakiston Division. McGraw-Hill Book Co. Inc. New York. Toronto. London.

Prankerd, T.A.J. (1958): The ageing of red cells. J. Physiol., 143 : 325-31.

Prankerd, T.A.J. (1961): The Red Cell. Blackwell Scientific Publications. Oxford.

Prewitt, R.D.; Merilan, C.P. (1958): Effect of potassium nitrate intake on dairy calves. J. Dairy Sci., 41 : 807-11.

Prins, H.K.; Oort, M.; Loos, J.A.; Zurcher, C.; Beckers, T. (1966): Congenital nonspherocytic hemolytic anemia, associated with glutathione deficiency of the erythrocytes. Blood, 27 : 145-66.

Pulsford, M.F.; Rac, R.; Irving, Elizabeth A. (1966): Unthriftiness of lambs and weaners in the spring and early summer in South Australia. Aust. vet. J., 42 : 165-68.

Raabo, E.; Terkildsen, T.C. (1960): On the enzymatic determination of blood glucose. Scand. J. clin. lab. Invest., 12 : 402-7.

Rafyi, A.; Maghami, S. (1966): (On some blood parasites of sheep and goats in Iran and neighbouring countries). Bull. Off. int. Epizoot., 65 : 1769-83. (Abstr. Vet. Bull., 37 : No 3111, 1967).

Ramisz, A.; Szankowska, ?; Lubieniecki, B. (1972): (The first outbreak of eperythrozoonosis in sheep in South Poland). Medycyna Weterynaryjna, 28 : 143-45.

Rapoport, S. (1968): The regulation of glycolysis in mammalian erythrocytes. Essays. Biochem., 4 : 69-103.

Reid, R.L. (1953): Studies on the carbohydrate metabolism of sheep. VI. Interrelationships between changes in the distribution and levels of glucose and in the levels of volatile fatty acid in the blood of lambs. Aust. J. agric. Res., 4 : 213-23.

Reitman, S.; Frankel, S. (1957): A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am. J. clin. Path., 28 : 56-63.

Rickard, M.D. (1969): Carbohydrate metabolism in Babesia rodhaini: Differences in the metabolism of normal and infected rat erythrocytes. Expl. Parasit., 25 : 16-31.

Rickard, M.D. (1970a): Babesia rodhaini: Carbohydrate metabolism and infectivity for rats of cells freed from host erythrocytes. Expl. Parasit., 27 : 136-42.

Rickard, M.D. (1970b): Carbohydrate metabolism in Babesia rodhaini: Investigations with ^{14}C-labelled substrates and enzyme assays. Expl. Parasit., 28 : 512-20.

Riek, R.F. (1968): Babesiosis. In Infectious Blood Diseases of Man and Animals. Diseases caused by Protista. Vol II. The Pathogens, the Infections and the Consequences. (Weinman, D.; Ristic, M. eds). Chap. 19. Academic Press. New York and London.

Rifkind, R.A.; Danon, D. (1965): Heinz body anemia - An ultrastructural study. I. Heinz body formation. Blood, 25 : 885-96.

Riley, V. (1964): Synergism between a lactate dehydrogenase-elevating virus and Eperythrozoon coccoides. Science, 146 : 921-23.

Ristic, M. (1968): Anaplasmosis. In Infectious Blood Diseases of Man and Animals. Diseases caused by Protista. Vol II. The Pathogens, the Infections and the Consequences. (Weinman, D.; Ristic, M. eds). Chap. 23. Academic Press. New York and London.

Rivkin, S.E.; Simon, E.R. (1965): Comparative carbohydrate catabolism and methemoglobin reduction in pig and human erythrocytes. J. Cell. comp. Physiol., 66 : 49-56.

Roberts, J.; Agar, N.S. (1971): An improved method for the automated analysis of erythrocyte reduced glutathione. Clinica. Chim. Acta., 34 : 475-480.

Roberts, W.C. (1966): Renal hemosiderosis (blue kidney) in patients with valvular heart disease. Am. J. Path., 40 : 671-79.

Roberts, W.C.; Morrow, A.G. (1966): Renal hemosiderosis in patients with prosthetic aortic valves. Circulation, 33 : 390-98.

Robinson, J.D. (1966): Interaction between protein sulphydryl groups and lipid double bonds in biological membranes. Nature, Lond., 212 : 199-200.

Rodriguez, I.G. (1968): (Ovine eperythrozoonosis recorded for the first time in Spain). Revta. Patron. Biol. anim., 12 : 23-32. (Abstr. Vet. Bull., 40 : No 1027, 1970).

Rørth, M. (1970): Dependence of oxyhemoglobin dissociation and intra-erythrocytic 2.3-DPG on acid-base status of blood. I. In vitro studies on reduced and oxygenated blood. In Red Cell Metabolism and Function. (Brewer, G.J. ed). P57-65. Plenum Press. New York - London.

Rossdale, P.D.; Mullen, P.A. (1970): Alterations to whole blood pH, pCO_2 and plasma bicarbonate index values during a metabolic acidosis occasioned by neonatal diarrhoea in thoroughbred foals. Br. vet. J., 126 : 82-88.

Rothberg, H.; Corallo, L.A.; Crosby, W.H. (1959): Observations on Heinz bodies in normal and splenectomized rabbits. Blood, 14 : 1180-86.

Rouse, B.T.; Johnson, R.H. (1966): Eperythrozoon ovis. Vet. Rec., 79 : 223-24.

Rubinstein, D.; Denstedt, O.F. (1956): Metabolism of the erythrocyte. XIV. Metabolism of nucleosides by the erythrocyte. Can. J. Biochem., 34 : 927-37.

Rubinstein, D.; Ottolenghi, P.; Denstedt, O.F. (1956): The metabolism of the erythrocyte. XIII. Enzyme activity in the reticulocyte. Can. J. Biochem., 34 : 222-35.

Saba, T.M.; DiLuzio, N.R. (1969): Reticuloendothelial blockade and recovery as a function of opsonic activity. Am. J. Physiol., 216 : 199-205.

Salvidio, E.; Pannacciulli, T.; Tizianello, A. (1963): Glucose-6-phosphate and 6-phosphogluconic dehydrogenase activities in the red blood cells of several animal species. Nature, Lond., 200 : 372-73.

Sarwar, M.M.; Akram, S.M.; Qureshi, M.A. (1962): Occurrence of Eperythrozoon ovis in sheep and goats in Indo-Pakistan. Pakistan J. anim. Sci., 1 : 23-28.

Sass, M.D.; Caruso, C.J.; O'Connell, D.J. (1965): Decreased glutathione in aging red cells. Clinica. Chim. Acta, 11 : 334-40.

Saxl, P.; Donath, F. (1924): Wasserhaushalt und reticulo-endotheliales system. Klin. Wochschr. 3 : 1397-99. (Cited by Kent, 1966).

Schalm, O.W. (1965): Veterinary Hematology. 2nd ed. Lea and Febiger. Philadelphia.

Schalm, O.W. (1970): Clinical significance of plasma protein concentration. J. Am. vet. med. Ass., 157 : 1672-75.

Schalm, O.W. (1972): Proceedings No. 17. Blood Disorders. P. 109. The Post-Graduate Committee in Veterinary Science. The University of Sydney.

Scheibel, L.W.; Miller, J. (1969): Glycolytic and cytochrome oxidase activity in Plasmodia. Milit. Med., 134 : 1074-80.

Scheibel, L.W.; Pflaum, W.K. (1970): Carbohydrate metabolism in Plasmodium knowlesi. Comp. Biochem. Physiol., 37 : 543-53.

Schilling, V. (1928): Eperythrozoon coccoides, eine neue durch splenektomie aktivierbare daverinfektion der weissen mavs. Klin Wchnschr., 7 : 1853-55. (Cited by Weinman, 1944).

Schindler, R.; Krampitz, H.E. (1964): Quantitative prufungen des wirtswer haltens eines "Spontanstammes" von Eperythrozoon coccoides Schilling 1928. Z. Tropenmed. Parasit., 15 : 18-29. (Cited by Kreier and Ristic, 1968).

Schotman, A.J.H. (1970): De biochemische aspecten bij de Eperythrozoon infektie. Tijdschr. Diergeneesk., 95 : 142-4.

Schroeder, W.F.; Ristic, M. (1968a): Autoimmune response and pathogenesis of blood parasite disease. In Infectious Blood Diseases of Man and Animals. Vol. I. Disease caused by Protista. (Weinman, D.; Ristic, M. eds.) Chap. 4. Academic Press. New York and London.

Schroeder, W.F.; Ristic, M. (1968b): Blood serum factors associated with erythrophagocytosis in calves with anaplasmosis. Am. J. vet. Res., 29 : 1991-95.

Scott, E.M.; Duncan, Irma W.; Ekstrand, Virginia V. (1965): The reduced nucleotide dehydrogenases of human erythrocytes. J. biol. Chem., 240 : 481-85.

Seamer, J. (1959): The propagation and preservation of Eperythrozoon coccoides. J. gen. Microbiol., 21 : 344-51.

Seamer, J. (1967): In Pathology of Laboratory Rats and Mice. (Cotchin, E. and Roe, F.J.C., eds.) P. 537. Blackwell. Oxford.

Seamer, J.; Gledhill, A.W.; Barlow, J.L.; Hotchin, J. (1961): Effect of Eperythrozoon coccoides upon lymphocytic choriomeningitis in mice. J. Immun., 86 : 512-15.

Sheagren, J.N.; Tobie, J.E.; Fox, L.M.; Wolff, S.M. (1970): Reticuloendothelial system phagocytic function in naturally acquired human malaria. J. Lab. clin. Med., 75 : 481-87.

Sheppard, C.W.; Jordan, G.; Hahn, P.F. (1951): Disappearance of isotopically labelled gold colloids from the circulation of the dog. Am. J. Physiol., 164 : 345-50.

Sheriff, D. (1967): Eperythrozoon ovis and the antiglobulin test. Nature, Lond., 215 : 101-2.

Sheriff, D. (1972): Diagnosis of Eperythrozoon ovis infection. Aust. vet. J., 48 : 128.

Sheriff, D. (1973): The effect of spirotrypan on Eperythrozoon ovis in sheep. Vet. Rec., 93 : 288-89.

Sheriff, D.; Clapp, K.H.; Reid, Marjorie, A. (1966): Eperythrozoon ovis infection in South Australia. Aust. vet. J., 42 : 169-76.

Sheriff, D.; Geering, M.C. (1969): The antiglobulin (Coombs) test in Eperythrozoon ovis infection in sheep. Aust. vet. J., 45 : 505-7.

Sherman, I.W. (1965): Glucose-6-phosphate dehydrogenase and reduced glutathione in malaria-infected erythrocytes (Plasmodium lophurae and P. berghei). J. Protozool., 12 : 394-96.

Sherman, I.W.; Ting, I.P. (1966): Carbon dioxide fixation in malaria (Plasmodium lophurae). Nature, Lond., 212 : 1387-89.

Sherman, I.W.; Ting, I.P. (1968): Carbon dioxide fixation in malaria. II. Plasmodium knowlesi (monkey malaria). Comp. Biochem. Physiol., 24 : 639-42.

Sherman, I.W.; Ruble, Judith A.; Ting, I.P. (1969): Plasmodium lophurae: (U-^{14}C)-glucose catabolism by free plasmodia and duckling host erythrocytes. Expl. Parasit., 25 : 181-92.

Sherwood-Jones, E.; Maegraith, B.G.; Gibson, Q.H. (1953): Pathological processes in disease. IV. Oxidations in the rat reticulocyte, a host cell of Plasmodium berghei. Ann. trop. Med. Parasit., 47 : 431-37.

Shoden, A.; Sturgeon, P. (1962): Iron storage. IV. Cellular distribution of excess liver iron. Am. J. Path., 40 : 671-79.

Siggaard-Andersen, O. (1962): The pH-log pCO_2 blood acid-base nomogram revised. Scand. J. clin. lab. Invest., 14 : 598-604.

Siggaard-Andersen, O.; Engel, K. (1960): A new acid-base nomogram. An improved method for the calculation of the relevant blood acid-base data. Scand. J. clin. lab. Invest., 12 : 177-86.

Silverman, M.; Ceithaml, J.; Taliaferro, L.G.; Evans, E.A. Jnr. (1944): The in vitro metabolism of Plasmodium gallinaceum. J. infect. Dis., 75 : 212-30.

Simpson, C.F. (1971): The ultrastructure of Heinz bodies in horse, dog, and turkey erythrocytes. Cornell vet., 61 : 228-38.

Singer, R.B.; Hastings, A.B. (1948): An improved clinical method for the estimation of disturbances of the acid-base balance of human blood. Medicine, Baltimore., 27 : 223-42.

Sirs, J.A. (1963): Metabolic aspects of the uptake of oxygen by haemoglobin in erythrocytes. Biochem. biophys. Acta, 66 : 378-85.

Sirs, J.A. (1966): The egress of oxygen from sheep erythrocytes. Biochim. biophys. Acta, 112 : 538-49.

Siu, P.M.L. (1967): Carbon dioxide fixation in plasmodia and the effect of some antimalarial drugs on the enzyme. Comp. Biochem. Physiol., 23 : 785-95.

Smith, J.E. (1968): Low erythrocyte glucose-6-phosphate dehydrogenase activity and primaquine insensitivity in sheep. J. Lab. clin. Med., 71 : 826-33.

Smith, J.E.; Barnes, J.M.; Kaneko, J.J.; Freedland, R.A. (1965): Erythrocytic enzymes of various animal species. Nature, Lond., 205 : 298-99.

Smith, J.E.; Beutler, E. (1966): Methemoglobin formation and reduction in man and various animal species. Am. J. Physiol., 210 : 347-50.

Smith, J.E.; Holdridge, Barbara A. (1967): Comparison of erythrocyte glucose-6-phosphate dehydrogenases of man and various ungulates. Comp. Biochem. Physiol., 22 : 737-43.

Smith, J.E.; Osburn, B.E. (1967): Glutathione deficiency in sheep erythrocytes. Science, 158 : 374-75.

Somogyi, M. (1933): The distribution of sugar and rate of glycolysis in the blood of some mammals. J. biol. Chem., 103 : 665-70

Speck, J.F.; Moulder, J.W.; Evans, E.A. Jnr. (1946): The biochemistry of the malaria parasite. V. Mechanism of pyruvate oxidation in the malaria parasite. J. biol. Chem., 164 : 119-44.

Spector, W.S. (1956): Handbook of Biological Data. W.B. Saunders Company. Philadelphia and London.

Splitter, E.J. (1950): Eperythrozoon suis n. sp. and Eperythrozoon parvum n. sp., two new blood parasites of swine. Science, 111 : 513-14.

Splitter, E.J.; Castro, E.R. (1957): Antibiotic therapy in acute eperythrozoonosis in swine. J. Am. vet. med. Ass., 131 : 293-94.

Stansly, P.G.; Neilson, C.F. (1965): Relationship between spleen weight increase factor (SWIF) of mice and E. coccoides. Proc. Soc. exp. Biol. Med., 119 : 1059-63.

Stansly, P.G.; Neilson, C.F. (1966): Distinctive behaviour of E. coccoides in plasma and spleen of infected mice. Nature, Lond., 211 : 1203-4.

Stansly, P.G.; Ramsey, D.S.; Neilson, C.F. (1962): A transmissable spleen weight increase factor (SWIF) of mice. Proc. Soc. exp. Biol. Med., 109 : 264-67.

Steensgaard, J.; Møller, N.P.H. (1970): Glucose-6-phosphate dehydrogenase activity and glutathione stability in fetal and adult bovine erythrocytes. Acta. vet. Scand., 11 : 322-23.

Stevenson, T.D.; McDonald, Berdenia L.; Roston, S. (1960): Colorimetric method for determination of erythrocyte glutathione. J. Lab. clin. Med., 56 : 157-60.

Stiffel, C. (1958): Etude de la fonction phagocytaire du systeme reticulo-endothelial. J. Physiol. (Paris), 50 : 1087-1106. (Cited by Kent, 1966).

Stuart, A.E. (1970): The Reticulo-endothelial System. E. and S. Livingstone, Edinburgh and London.

Sturgeon, P.; Shoden, A. (1969): Hemosiderin and ferritin. In Pigments in Pathology. (Wolman, M. ed.) Chap. 4. Academic Press. New York and London.

Sunthrasamai, P.; Rytel, M.W. (1973): Interferon response and interferon action in E. coccoides infection of mice. Proc. Soc. exp. Biol. Med., 142 : 811-16.

Sutton, R.N.; Wilson, R.F.; Watt, A.J. (1967): Differences in acid-base levels and oxygen-saturation between central venous and arterial blood. Lancet, ii : 748-51.

Szeinberg, A.; Marks, P.A. (1961): Substances stimulating glucose catabolism by the oxidative reactions of the pentose phosphate pathway in human erythrocytes. J. clin. Invest., 40 : 914-24.

Tanaka, H.; Hall, W.T.; Sheffield, J.B.; Moore, D.H. (1965): Fine structure of Haemobartonella muris as compared with Eperythrozoon coccoides and Mycoplasma pulmonis. J. Bact., 90 : 1735-49.

Tarlov, A.R.; Brewer, G.J.; Carson, P.E.; Alving, A.S. (1962): Primaquine sensitivity. Glucose-6-phosphate dehydrogenase deficiency: An indoor error of metabolism of medical and biological significance. Arch. Intern. Med., 109 : 209-34.

Tennant, B.; Harrold, D.; Reina-Guerra, M.; Laben, R.C. (1969): Arterial pH, pCO_2 and pO_2 of calves with familial bovine polycythemia. Cornell vet., 59 : 594-601.

Thompson, R.H.; Todd, J.R. (1964): Estimation of glucose-6-phosphate dehydrogenase in sheep erythrocytes. Nature, Lond., 201 : 718.

Thurston, June P. (1953): The chemotherapy of Eperythrozoon coccoides (Schilling, 1928). Parasitology, 43 : 170-74.

Thurston, June P. (1954): Anaemia in mice caused by Eperythrozoon coccoides (Schilling, 1928). Parasitology, 44 : 81-98.

Todd, J.R.; Ross, J.G. (1968): Biochemical and haematological changes in the blood of normal sheep following repeated phlebotomy. Br. vet. J., 124 : 353-61.

Todd, J.R.; Thompson, R.H. (1963): Studies on chronic copper poisoning: II. Biochemical studies on the blood of sheep during the haemolytic crisis. Br. vet. J., 119 : 161-73.

Todd, J.R.; Thompson, R.H. (1964): Studies on chronic copper poisoning: III. Effects of copper acetate injected into the blood stream of sheep. J. comp. Path., 74 : 542-51.

Tonz, O. (1968): The Congenital Methemoglobinemias. S. Karger. Basel (Switzerland). New York.

Tucker, Elizabeth M. (1971): Genetic variation in the sheep red blood cell. Biol. Rev., 46 : 341-86.

Tucker, Elizabeth M.; Kilgour, L. (1970): An inherited glutathione deficiency and a concomitant reduction in potassium concentration in sheep red cells. Experientia, 26 : 203-4.

Uilenberg, G. (1965): Notes on Eperythrozoon in cattle in Madagascar. Rev. Elev. med. vet. Pays. trop., 18 : 73-81. (Cited by Zwart et al., 1969).

Uilenberg, G. (1967): Rev. Elev. med. vet. Pays. trop., 20 : 563. (Cited by Zwart et al., 1969).

Ultman, J.E.; Gordon, C.S. (1965): The removal of in vitro damaged erythrocytes from the circulation of normal and splenectomized rats. Blood, 26 : 49-62.

Umbreit, W.W.; Burris, R.H.; Stauffer, J.F. (1964): Manometric Techniques. 4th ed. Burgess, Minneapolis, Minnesota.

van Aken, W.G.; Goote, T.M.; Vreeken, J. (1968): Platelet aggregation: An intermediary mechanism in carbon clearance. Scand. J. Haemat., 5 : 333-38.

van der Helm, H.J.; van Vliet, G.; Huisman, T.H.J. (1957): Investigations on two different hemoglobins of the sheep. Arch. Biochem. Biophys., 72 : 331-39.

Veneziale, C.M.; McGuckin, W.F.; Hermans, P.E.; Mankin, H.T. (1966): Hypohaptoglobinemia and valvular heart disease: Association with hemolysis after insertion of valvular prostheses and in cases in which operation had not been performed. Mayo Clin. Proc., 41 : 657-62.

Voller, A.; Bidwell, D.E. (1968): The effect of Eperythrozoon coccoides infection in mice on superimposed Plasmodium chabaudi and Semliki Forest Virus infections. Ann. trop. med. Parasit., 62 : 342-48.

Wagner, Adriana M.; Brown, J.M.M. (1966): Contributions to the study of blood constituents in domestic animals in South Africa. 3. Normal values for the activity of glyceraldehyde-phosphate dehydrogenase and glucose-6-phosphate dehydrogenase in the erythrocytes of the sheep. Onderstepoort J. vet. Res., 33 : 337-42.

Weed, R.I.; Reed, C.F. (1966): Membrane alterations leading to red cell destruction. Am. J. Med., 41 : 681-98.

Weinman, D. (1944): Infectious anaemias due to Bartonella and related red cell parasites. Trans. Amer. phil. Soc., 33 : 243-338.

Weinman, D. (1957): Bartonellaceae. Bergey's Manual of Determinative Bacteriology. 7th ed. Williams and Wilkins. Baltimore.

Wendel, W.B. (1943): Respiratory and carbohydrate metabolism of malaria parasites (Plasmodium knowlesi). J. biol. Chem., 148 ; 21-34.

Wilkinson, J.S. (1969): Kidney disease and urine analysis. In A Textbook of Veterinary Clinical Pathology. Chap. 5. (Medway, W.; Prier, J.E.; Wilkinson, J.S., eds.) The Williams and Wilkins Co. Baltimore.

Williamson, J.R. (1970): General features of metabolic control as applied to the erythrocyte. In Red Cell Metabolism and Function. (Brewer, G.J., ed.) P. 117-36. Plenum Press. New York and London.

Winter, A.J. (1962): Studies on nitrate metabolism in cattle. Am. J. vet. Res., 23 : 500-5.

Winter, H. (1967): Myelogram of sheep in post-haemorrhagic anaemia. Am. J. vet. Res., 28 : 1389-95.

Wislocki, G.B. (1924): On the fate of carbon particles injected into the circulation with especial reference to the lungs. Am. J. Anat., 32 : 423-45.

Wootton, I.D.P. (1964): Micro-Analysis in Medical Biochemistry. 4th ed. J. and A, Churchill Ltd. London.

Zahn, R.L.; Weil, M.H. (1966): Central venous blood for monitoring pH and pCO_2 in the critically ill patient. J. Thorac. Cardiovasc. Surg., 52 : 105-11.

Zilversmit, D.B.; Boyd, G.A.; Bauer, M. (1952): The effect of particle size on blood clearance and tissue distribution of radioactive gold colloids. J. Lab. clin. Med., 40 : 255-60.

Zuckerman, A. (1969): Current status of the immunology of malaria and of the antigenic analysis of plasmodia. Bull. Wld. Hlth. Org., 40 : 55-66.

Zwart, D.; Leeflang, P.; van Vorten-Bosch, C.J.A.H.V. (1969): Studies on an Eperythrozoon associated with bovine thrombocytes. Zentbl. Bakt. Parasitkde., I (Orig.), 210 : 82-102.

Zwart, D.; Schotman, A.J.H.; Straver, A.J.M. (1970): Metabolic changes during Eperythrozoon infection in cattle. Res. vet. Sci., 11 : 105-11.