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AFRICAN TRYPANOSOMIASIS: ITS EFFECT ON PLATELET MORPHOLOGY, FUNCTION AND SURVIVAL

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Frontispiece

CALF PLATELETS AND TRYPANOSOMES IN A T VIVAX INFECTION

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Abstract

AFRICAN TRYPANOSOMIASIS: ITS EFFECT ON PLATELET MORPHOLOGY, FUNCTION AND SURVIVAL.

Denise Syndercombe Court

Thrombocytopenia, occurring sometimes in conjunction with disseminated intravascular coagulation, has been reported sporadically in both human and animal trypanosomiases.

The extent of the thrombocytopenia and its relationship to the anaemia and parasitaemia has been investigated in experimental infections of Trypanosoma brucei brucei and Trypanosoma congolense in rabbits, and of Trypanosoma vivax in calves. Studies of platelet size, structure, function and survival have been undertaken in an attempt to ellucidate the mechanism for the thrombocytopenia.

A significant decrease in platelet number is associated with the early parasitaemia of the infection. A parasite-mediated mechanism, either immune or toxic, leads to in vivo platelet clumping, marked platelet ultrastructural changes and functional inhibition of the circulating platelets. The potential ischaemic action of the resultant microthrombi and the haemostatic problems of poorly functioning platelets may contribute significantly to the pathology of the disease.

Platelet destruction, precipitated by the parasite, occurs in the spleen resulting in a reduced platelet lifespan. Experiments in asplenic animals show that other organs can take over the splenic role in this respect.

Thrombocytopenic stimulation of platelet production from the increased megakaryocytic mass in the bone marrow only partially compensates for the platelet loss which continues late in the infection by a mechanism unrelated to parasite number. Evidence is provided suggesting that the expanded mononuclear phagocytic ability of the reticular endothelial system actively or passively continues the platelet destruction at this time. Support for two different mechanisms of platelet destruction, active at different stages of the disease, is also provided by experiments involving different strains of trypanosome and limited immunity experiments.

The pattern of platelet destruction is similar to that suggested for red cells of an early haemolytic anaemia with a non-haemolytic anaemia active in the later stages.

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I. INTRODUCTION

1.1 AFRICAN TRYPANOSOMIASIS - HISTORICAL CONSIDERATIONS AND THE IMPORTANCE OF THE DISEASE TODAY

Trypanosomiasis remains today one of the most serious threats to the health of man, not only directly, but also through the serious obstacle to the development of agricultural industry in tropical Africa. The disease is caused by parasitic protozoa, trypanosomes, which attack men, domesticated and wild animals. The form that the disease takes depends not only upon the host animal species but also on the particular species of infecting trypanosome and the particular strain of trypanosome involved(1). The parasites are transmitted between hosts by different species of the tsetse fly, genus Glossina. Once a fly carries the trypanosome it continues to do so for the rest of its life. Glossina palpalis is found principally in the tropical rain forests whereas the habitat of G morsitans is mainly the dry savannah land. This difference too affects the distribution and forms of the disease seen in different geographical areas of Africa.

There is evidence that African trypanosomiasis has been found in the region of the upper Niger since the 14th century and it is thought that the King of Mali, Mansa

Djata, died of the disease in 1374. Reports of the disease came from the Guinea coast in 1721 and Sierra Leone in 1803. It is thought that the disease spread along the Congo basin from West Africa in the late 19th century reaching the Nile in Uganda and southern Sudan at the beginning of the 20th century. The caravan trading route taken over by the French in 1901 between Ubangui and Chad was probably the cause of the epidemic in 1915. Attempts to flee from infected villages caused a spread of the disease and in some areas more than half of the population was infected - it was common to see 30% of the population die during an epidemic. While the disease was endemic in West Africa and possibly also East Africa before the 19th century, colonisation changed all this(2). Increased contact between people and greater use of rivers under the European influence has brought about the recurrent epidemics that we have seen throughout this century(3).

Trypanosomiasis is prevalant between the latitudes $15^{o}N$ and $21^{o}S$ involving 35 million people and 10 million km^2 of land, areas where there can be no livestock development and where one can use susceptible animals only with curative and prophylactic trypanocides. Whereas the direct result of the disease is mainly the animal death and the finance needed for control, the indirect influences are enormous. Human

health is affected through lack of meat and milk resulting in protein deficiencies; better breeds of livestock can't be used; in tsetse-free areas there is overstocking which causes the land to deteriorate; agricultural production suffers through a lack of manure; rural land cannot be used efficiently because of seasonal restrictions; the national economy suffers through its need to import meat and dairy products(4).

Advances in diminishing the disease have been negligible despite the rapid development of medicine this century. The reasons are many: the remote geographical locations of disease foci; the political situation within the area; the relatively small number of human cases; the cost of control or eradication; the potential non-profitability for drug firms investing research money(5).

It is said that "trypanosomiasis is probably the only disease that has profoundly affected the settlement and economic development of a major part of a continent"(4) and in Resolution XI of the 1974 World Food Conference of the United Nations the FAO was given the mandate "to launch, as a matter of urgency, a long term programme for the control of African animal trypanosomiasis as a project of high priority".

1.2 AFRICAN TRYPANSOMES

Five different species of trypanosomes are of major importance in tropical Africa and can be divided into two groups according to their distribution within the body of the host.

Trypanosomes of the Trypanosoma brucei group (T brucei brucei, T brucei rhodesiense and T brucei gambiense) can penetrate vessel wall endothelium and multiply outside the blood vascular system. They can be found distributed widely throughout the body and have a preference for connective tissue and intracellular tissue fluids[6]. Thus the numbers of these parasites found in the blood are no guide to the parasitaemia[7].

In contrast, T congolense and T vivax are found mainly in plasma. T congolense has a preference for the microcirculation while T vivax is more evenly distributed with some tissue invasion, probably passive[6].

The trypanosomes infecting horses (T equiperdum) and camels (T evansi) are biologically quite different from the above and are not transmitted by the tsetse fly. Diseases caused by these trypanosomes are not

considered.

1.3 CLINICAL FEATURES OF AFRICAN TRYPANOSOMIASIS

Two of the T brucei species (T b gambiense and T b rhodesiense) cause disease in humans. Three phases are recognised in Gambian sleeping sickness: the development of a chancre at the infecting site; a systemic infection with fever, lymphadenopathy and splenomegaly; a neurological infection with invasion of the central nervous system bringing features of meningitis and encephalitis. Death may follow, often due to secondary infections[8]. The progression of the disease may be very slow and in consequence mortality is also low.

T b rhodesiense infections are similar but more rapid bringing about an acute disease, often with an overwhelming parasitaemia, and early death. Myocarditis may be prominent and death may follow cardiac failure. Anaemia and disseminated intravascular coagulation (DIC) may be clinically important[8].

T b brucei infects animals, the disease in cattle being particularly mild. In rabbits, often used as a laboratory model of T.brucei infections, there are signs of the tissue involvement of the disease with scabby lesions, tissue oedema and muscle wastage[7].

T vivax and T congolense are the most important causes of bovine trypanosomiasis in West and East Africa respectively, although both forms are found throughout tropical Africa. The disease may be acute or chronic depending on the host genetic constitution, exposure and virulence of the parasite[10]. The main feature is one of anaemia, reflecting the circulatory preferences of the parasites, with splenomegaly and stunted growth[11] followed by recovery or death due to a variety of causes such as cardiovascular collapse, acute thrombosis, septicaemia or pneumonia[7].

1.4 PATHOLOGY OF AFRICAN TRYPANOSOMIASIS

Several reviews have been published on the pathology of trypanosomiasis[1,7,8,10,12-14]. Although the features of the disease are well recognised, the pathological mechanisms and their relative importance remain unclear. The main pathogenic features are those involving tissue and organ damage and immunological and haematological changes. A short review of the changes seen in these systems follows before the evidence for specific involvement of platelets is considered.

1.4.1 Tissue lesions

1.4.1.1 Effects on major organs

Both humans and animals dying of trypanosomiasis show a remarkable lack of gross lesions apart from enlargement of the spleen, liver and lymph nodes[1,12,13,15]. Microscopically there are many lesions which individually are not specific but collectively produce a spectrum of change not seen in any other disease.

Hyperplastic changes are seen in the organs of the reticulo-endothelial system[14-18] but in prolonged infections the organs may become cellularly depleted and atrophy[14,16,19]. Gross alteration and disruption of lymph node structure have been seen in T brucei infections[20] and atrophy of the thymic dependent areas has been reported[16].

Renal lesions of glomerular nephritis and tubular atrophy have been widely reported as well as the presence of haemosiderin deposits[15,16,18,21,22]. The lungs and brain show oedema, vessel dilatation and inflammatory cell deposits, often also with trypanosome presence [14,16,18,22].

The organ most severely effected is the heart,

especially in T congolense infections. The histopathology is mainly one of a myocardial mononuclear cell infiltrate[16,19] leading to a generalised myocarditis[10,20].

Bone marrow is uniformly affected with hyperplasia of the erythropoietic system being prominent[15-18,23]. Megakaryocytes are also increased[15,23]. Terminally the marrow may show depression of erythropoiesis[11,24].

1.4.1.2 Microvascular lesions

Extensive endothelial damage leading to increased vascular permeability has been reported by several workers[1,12,25,26]. Microthrombi, the development of which may be increased by hyperviscosity[27], have been found in blood vessels in acute infections[18] and may also result in DIC[28-32]. These lesions will produce local anoxia and necrosis.

Lesions seen in tissues may be due to immunological mechanisms, release of toxins or the anaemia.

1.4.2 Immunological lesions

When trypanosomes invade the host body there is a marked immune response to the different variable antigen types[19] bringing about a massive expansion of the

lymphoproliferative system. Much of the production from the expanded system is non-specific IgM. The reason for this non-specificity may be caused by trypanosomes breaking the T cell - B cell link, the T cells preventing B cells from transforming into specific antibody-producing cells. The B cells make IgM autoantibodies instead[17].

The massive immune reaction may have adverse effects when stimulated[20], caused by the formation of antigen-antibody complexes. Deposition of these complexes has been implicated in tissue damage[7,19] and there is a suggestion that they may, in combination with Hageman factor, bring about release of kinins which may also damage tissues[7]. The haemolytic anaemia seen in trypanosome infection may too be due to immunological mechanisms[19] since antigen-antibody complexes are found on red cells[33-35] and these sensitised cells may be lysed by complement or agglutinated and removed by the spleen[36]. Further evidence of this mechanism comes from Facer et al[37] who found that IgG and IgM with and without C_3 on red cells in bovine trypanosomiasis was associated with a lower haematocrit, which recovered slightly when the direct anti-globulin test became negative with removal of the sensitised red cells over the next 2 to 4 days. As early as 1917, Rieckenberg[38] reported that

platelets became coated with T b brucei antibody complexes dependent on a thermolabile substance, probably complement. Slots et al[39] showed that, in vitro, complexes of T vivax and its antibody could bring about platelet release; each component alone had no effect. Antigen-antibody complexes may also be involved in the development of glomerulo-nephritis[15] and myocarditis[40]. Trypanosomes can activate complement alone and the resultant decrease in available complement may compromise the immunological destruction of trypanosomes[20]. Greenwood et al[8] criticise the suggestion that the pathology of human sleeping sickness is due to its immunopathology as being 'fashionable', saying that, although the immune reactions are obviously important, there is little evidence for the supposition in man in contrast with the evidence from experimental animals.

T b brucei produces severe immune depression in mice and rabbits and a similar, but less extreme, suppression is seen in cattle with T vivax or T congolense infections[41]. Immunosuppression involves both antibody mediated and cell mediated immune systems and may be the most important pathogenic mechanism by its exposure of infected individuals to unsuppressed secondary infection[9]. The possible mechanisms have been reviewed by Urquhart[40] and it is interesting

that despite immunodepression the body continues to respond to new trypanosome variants[19].

1.4.3 Role of toxins

The first evidence for toxin production by trypanosomes came in 1902 by Laveran and Mesnil[42] when they described the death of a rat after injection of frozen and thawed T b brucei. There were however many failures to demonstrate trypanotoxins after this time and the idea fell into disrepute.

Toxins may be produced from two sources - the trypanosome itself or the host as a result of reactions to the infection. Tizard has reviewed production from the former[9]. As well as release of antigenic coats, which may contribute to immune complex formation, the live trypanosome can also synthesise potentially toxic indole and catechol metabolites; it is not known whether these latter substances are in sufficient concentration to have any effect. Dead trypanosomes on the other hand make available a variety of substances which may be important, although their action may be through induction of immunopathological mechanisms[8]. Enzymes such as proteases and phospholipases are produced. Proteases such as cathepsin D can activate plasminogen and kininogen to form plasmin and kinin respectively[43]. Kinins have been reported in T b

<u>brucei</u> and <u>T b rhodesiense</u> infections although their production may also be due to immune complexes and Hageman factor activating the kallikrein-kinin system[44]. Phospholipase A_1 is only found in significant amounts in pathogenic trypanosomes and may have direct cytotoxic effects by destruction of membrane phospholipids[43] or contribute to respiratory distress by degradation of lung surfactant[45]; it also releases free fatty acids (FFA) from the tissue. On disruption of trypanosomes lipids are also released; lipopolysaccharides activate complement[9], and FFA are both haemolytic and cytotoxic[43]. Linoleic acid has been shown to be primarily responsible for the haemolytic properties of <u>T congolense</u> <u>in vitro</u> [43] but <u>in vivo</u> it will be bound by albumin and only if there is high parasite death will albumin be saturated allowing red cell destruction[9]. FFA have other effects though and can induce thrombosis, myocardial lesions, thrombocytopenia and immunosuppression[9]. Tai et al[46] have shown however that the FFA, linoleic and arachidonic acids, are reduced in <u>T.b.brucei</u> S42 infections in mice and so are unlikely to contribute to immunodepressive effects although their possible conversion into PGE_1 and PGE_2 respectively may be the immunodepressive mechanism involved. Haemolysis, mitogens and a vascular permeability factor may also be produced by trypanosome lysis[9].

The release of other pharmacologically active substances such as bradykinin, serotonin, and histamine by the host have been reported by several other workers[31,44,47-49]. Activation of Hageman factor alone may initiate the coagulation, fibrinolytic and complement systems[44]. Thus the release of toxins from or by trypanosomes can account for the major pathological changes of immunosuppression, hypocomplementaemia, microvascular damage and red cell damage.

1.4.4 Anaemia

There have been many reports on the effect of African trypanosomiasis on the haemopoietic system. The importance of the anaemia varies with the species of organism and the infected host. Reports originating in the 1950's often suggested that the anaemia in cattle infections was due to inhibition of haemopoiesis, based on the absence of reticulocytes in the peripheral blood[50], and Boycott and Price-Jones[51] suggested that marrow response in T b brucei infections of rabbits was defective based on their marrow differential counts, despite their evidence that the long bones contained red marrow. Since then there have been many reports in T b brucei infections in rodents and rabbits[52-54], in T vivax infections of sheep, goats and cattle[14,33,37,55-58], in T congolense infections or rabbits and cattle[19,33,41,52,57,59,60-62] and T

<u>rhodesiense</u> infections in monkeys[15] and man[8] of a normoblastic macrocytic haemolytic anaemia associated with a decreased red cell life span[37,53,59,62,63] and erythroid hyperplasia[52,61,64-66]. Possible mechanisms of the anaemia include red cell sensitisation (see 1.4.2) with extravascular haemolysis, direct trauma to the red cells by trypanosomes or a microangiopathic element[64], non specific removal of red cells due to extended splenic vasculature[54], red cell pooling (probably a minor component only)[67] and haemodilution. There has been some discussion over the latter mechanism but more rigorous studies have suggested that plasma volume increases to compensate for a decrease in red cell volume with the blood volume remaining the same[56,68], ie: no evidence for haemodilution. Most people favour the immunological mechanism.

In more chronic infections the aetiology of the anaemia may be different[41]. Early the onset of anaemia correlates with the parasitaemia but later the anaemia may become normocytic or microcytic with a pattern associated with the anaemia of chronic disorders - continued red cell destruction not influenced by parasites, defective iron utilisation and dyshaemopoiesis[19,50,59,62,64].

1.4.5 Coagulation defects

Where coagulation defects occur in trypanosomiasis they can prove life-threatening or fatal and are often associated with the development of disseminated intravascular coagulation (DIC). Such reports are relatively rare. On the other hand, a mild coagulopathy may exist in the infection and contribute to the pathology without giving any obvious signs or causing death.

There have been several reports of a haemostatic defect in trypanosomiasis[69-71] and Boulton et al[72] reported increases in clotting factors VIII and XII, without a shortened partial thromboplastin time, occurring late in a T b brucei infection in rabbits. They suggested that their results implied an increased fibrinogen turnover and DIC but did not measure platelet levels or fibrinogen-degradation products (FDP). In another experimental infection involving T.b.brucei FDP have been demonstrated[73], evidence for increased fibrinolysis, again suggesting the possibility of DIC. Another feature of DIC is thrombocytopenia and this has also been reported in T b brucei infections[39,74] but it is the association with fibrinogen consumption that provides a more convincing thesis of DIC. Barrett-Connor et al[28] described a case of T rhodesiense in a man with severe thrombocytopenia, increased

prothrombin and partial thromboplastin times and increased FDPs and concluded that he was suffering from DIC. Similar reports of DIC in T rhodesiense infections have come from Greenwood and Whittle in man[18], Robbins-Browne et al in man[30] and Rickman and Cox in rats[30]. There are also reports of DIC in T simiae infections of pigs[75], T congolense infections of dogs[76] and T vivax infections of cattle[31,77]. Tizard[43] has suggested that local platelet aggregates, seen often in trypanosome infections[47,78], may initiate the DIC but Evensen has shown that platelets contain little procoagulant material which will anyway be rapidly cleared by the reticulo-endothelial system, although a moderate DIC may develop if the reticulo-endothelial system is sufficiently depressed[79]. There are other possible mechanisms for the DIC: release of procoagulant material from red cell membranes as a result of an intravascular haemolysis[80], kinin activation and antigen-antibody complex formation[34]. all of which occur in trypanosomiasis.

1.4.6 Influence of the disease on platelets

A platelet defect, whether of number or function, will result in abnormal haemostasis although in man the platelet count needs to be less than 20 x 10^9/litre for spontaneous haemorrhage to occur[81]. Petechial

haemorrhages and purpura have been reported quite frequently however[15,28.77,82].

Severe thrombocytopenia has been reported in naturally acquired T rhodesiense infections of man[28.30,82] whereas the reduction in platelet number seen in T gambiense infections is much less severe [8.29] and different mechanisms have been suggested. In experimental infections thrombocytopenia has also been described in T rhodesiense infections of rats[34,83] and monkeys[15], T b brucei infections of rabbits[74] and T congolense infections of dogs[76]. Thrombocytopenia is also a commonly reported feature of T congolense infections of cattle[57.84-86] and T vivax infections of cattle[31,77] and goats[39,47,48].

A particular feature of the fall in platelet number in some cases is its correlation with trypanosome number, at least initially[83,86,87], or with a decrease in complement and increase in immune complex formation[8,28.34]. These have been reported in T rhodesiense infections and suggest the possibility of different mechanisms for the low platelet number.

Possible causes for the thrombocytopenia are: increased platelet destruction, dysthrombopoiesis, excess pooling

or a dilutional loss.

1.4.6.1 Increased platelet destruction

Increased destruction of platelets may be a primary event, such as direct damage by the trypanosomes, or secondary to DIC, increased reticulo-endothelial activity or development of immune complexes and antiplatelet antibodies.

Davis et al[83] have shown a strong inverse correlation between platelet and trypanosome number and have suggested that a direct injury was likely. Their experiments with T rhodesiense lysates in vitro causing platelet aggregation lend force to this argument and they were able to extract a heat labile substance which was not blocked by ADP, kinin or complement inhibitors and not extracted with petroleum ether suggesting an enzyme or toxin. Such a substance has not been found using T gambiense[29], T vivax or T brucei[39]. DIC has already been referred to as an important cause of thrombocytopenia (see 1.4.5) and thrombocytopenia is probably the most important feature of the acute disease in association with increased FDP and DIC a significant cause of mortality. It is not present in all forms of the disease however. In the more chronic T gambiense infections thrombocytopenia is mild and FDPs are not significantly raised[29], suggesting that other

mechanisms are involved. Davis et al[83] have demonstrated a marked thrombocytopenia in rats with T rhodesiense which occurred without fibrinogen consumption or FDP release, again suggesting that the fall in platelet number is not secondary to DIC. Forsberg et al[84] have remarked that because of the sudden death that DIC causes and its relative rarity, the demonstration and reporting of some cases will not be made.

Non-specific removal of platelets by an expanded reticulo-endothelial system is a possible cause of the thrombocytopenia and advocated by Greenwood and Whittle to explain their studies with T gambiense patients[29]. Both Davis[83] and Preston[85] have reported thrombocytopenia in splenectomised animals. Neither looked at the contribution to platelet destruction that the liver or bone marrow may have made.

As has been suggested for red cells in bovine trypanosomiasis[37] and by Shulman[88] a decreased platelet lifespan may also be mediated by complement fixing immune complexes. This mechanism is supported by the association with decreased complement and increased immune complexes previously referred to[8,28,34], and the occurrence of a severe thrombocytopenia just after the parasitaemic peak[31,48,57], although Davis et

al[83] suggest that, in rats at least, as the thrombocytopenia was marked before possible development of an immune response a direct mechanism is more likely.

Autoantibodies to platelets may be produced but one study has not shown their presence[39].

Where there is platelet destruction, for whatever reason, this will involve changes in platelet kinetics(see 1.4.6.2).

1.4.6.2 Dysthrombopoiesis

Increased megakaryocyte numbers in the bone marrow are a well reported feature of trypanosomiasis (see 1.4.1.1) and Valli and Forsberg[22] confirmed this finding but remarked that, although the megakaryocytes were also increased in size, this size increase was due to the nuclear size caused by an asynchrony, indicative of dysthrombopoiesis. Confirmation of their thesis came from the finding of a normal platelet lifespan in T congolense infected calves using a ^{35}S-methionine in vitro cohort label and they suggested that ineffective thrombopoiesis was the cause of the thrombocytopenia and a reflection of general bone marrow stress. Robbins-Browne et al[30] however reported a reduced ^{51}Cr-labelled platelet lifespan in patients with T rhodesiense and Preston et al[85] also in ^{51}Cr-

labelled platelet lifespan of cattle with T congolense, supporting the previous theory of increased platelet destruction. ^{51}Cr is used as a random label and has certain advantages over cohort labelling which will be mentioned later(89).

1.4.6.3 Excess platelet pooling

The recovery of labelled platelets varies inversely with the size of the splenic pool and Robbins-Browne et al(30) found that pooling was a significant feature in their patients with T rhodesiense. It was however unlikely to be the only mechanism for the thrombocytopenia since platelet lifespan is usually normal or near normal in such conditions(90) and, as referred to previously, thrombocytopenia is found in splenectomised animals.

1.4.6.4 Dilutional loss

A significant fall in platelet number could occur by simple dilution in the presence of an increased plasma volume. This increase would need to be accompanied also by an increased blood volume and not just be compensatory for the loss in red cell volume. As discussed previously (1.4.4) the evidence is in favour of blood volume remaining normal.

2. AIMS

Many studies have been carried out to investigate the aetiology of the anaemia in trypanosomiasis. Thrombocytopenia, occurring sometimes in conjunction with disseminated intravascular coagulation, has been reported sporadically both in human and animals infections. Generally, however, the influence of the disease on platelets has been neglected.

The aim of the work therefore was to investigate the influence that trypanosome infections have on platelets in terms of their number, structure and function.

Two different organisms, T b brucei and T congolense, both of which cause widespread infection of livestock particularly in central Africa, have been studied in a laboratory animal, the rabbit, and a further organism, T vivax, has been studied in its more usual animal host, the calf.

3. METHODS

3.1 ANIMALS

3.1.1 Mice

Eight week old male and female Balb/C mice were obtained from Bantin and Kingman Ltd. Grimston, Aldbrough, Near Hull. Yorkshire.

3.1.2 Rabbits

Sandy full-lop and albino half-lop male and female rabbits weighing 3-3.5kg were obtained from Ranch Rabbits, Crawley Down. Sussex.

3.1.3 Calves

Friesian bull calves, weighing between 53 and 63 kg. were obtained from Leahurst Farm and housed at the Liverpool School of Tropical Medicine.

3.2 TRYPANOSOMES

3.2.1 T b brucei

This organism was obtained from The London School of Hygiene and Tropical Medicine and stored under liquid nitrogen. The strain S42 was used. This strain had originally been obtained from an old female warthog in Tanzania on March 12th 1966 and subsequently passaged in

mice.

3.2.2 T congolense

Two strains of T congolense were provided by The London School of Hygiene and Tropical Medicine and maintained in this laboratory by syringe passage of infected blood through mice. Both strains were isolated in 1977: LEU TSW 99/77 (TSW) from a pig in Liberia. the enzyme patterns of this isolate showing a
typical forest or riverine pattern: GAMB 19 (GAMB) from cattle in the Gambia, here the enzymes showing a typical savannah pattern. Both strains showed differences in drug susceptibility.

3.2.3 T vivax

T vivax (WA 64/23) was obtained and used at The Liverpool School of Tropical Medicine. Originally isolated from a sheep in Nigeria in 1964. it had been maintained in Liverpool under liquid nitrogen and passaged by fly and syringe.

3.3 INFECTION OF ANIMALS

3.3.1 Mice

Mice were infected by intraperitoneal injections of trypanosome stabilates which had been stored in glass capillaries under liquid nitrogen. The thawed organisms

were diluted with 1ml quantities of phosphate buffered glucose-saline (PSG) - (Appendix I.1) and 0.2ml volume injected.

The infection was monitored daily by wet-preparation examination using a microscope for observation of trypanosomes in blood obtained from tail clippings. Once the infection was established (usually within three or four days) it was either passaged to another mouse. using blood obtained from tail clippings mixed with PSG, or used for rabbit infections. using the infected blood from a freshly killed mouse.

3.3.2 Rabbits

Trypanosomes obtained from infected mouse blood. were counted in an Improved Neubauer counting chamber and further diluted with PSG to obtain a count of 1×10^6 trypanosomes within a suitable volume of the diluent. Approximately 0.4ml to 1 ml volumes of the mouse blood/PSG mixture, each containing 1×10^6 trypanosomes, were injected into the marginal ear vein of the rabbit. For the T.congolense experiment, 2.5×10^6 trypanosomes were injected.

Throughout the experimental period, 46 rabbits were infected with T b brucei S42, 4 rabbits with T congolense (TSW 99/77) and 4 rabbits with T congolense

(GAMB 19).

3.3.3 Calves

Forty-nine different stabilates of T vivax (WA 64/23) were thawed, mixed and injected subcutaneously in the neck and around the ribs (Table 3.1). The infections were performed by John Crosskey at the Liverpool School of Tropical Medicine. Four calves were infected in this way.

3.4 SPLENECTOMY OPERATION

A total of eight rabbits involved in three separate experiments, were splenectomised. This operation was performed, under halothane-induced anaesthetic, either by myself (four operations) or by Mr AJ Murday (three operations). A three-inch incision was made in the left upper abdominal quandrant below the rib cage and the spleen exposed. Each blood vessel supplying the spleen was ligated individually and two haemostat clamps placed on the splenic pedicle, both on the splenic side of the ligatures. The pedicle was divided between the two clamps, and the spleen with one clamp attached was removed. The remaining clamp was released to check for signs of bleeding from ligated vessels and when the field was dry the clamp was removed, ligated vessels divided and the peritoneum closed using continuous

T.VIVAX (WA 64/23) STABILATES GIVEN TO CALVES

NO.	CALF	STABILATE	SURVIVAL	NO.	CALF	STABILATE	SURVIVAL
1	175	T5	Poor	26	1943	T130	90%
2	175	T9	Poor	27	1943	T136	90%
3	175	T13	Bad	28	1943	T149	1%
4	176	T47	Very good	29	1943	T155	90%
5	175	T50	Good	30	1025	T5	90%
6	176	T51	Good	31	1025	T9	90%
7	176	T58	Poor	32	695	T48	0%
8	175	T59	Very poor	33	1025	T50	90%
9	175	T78	Good	34	695	T59	90%
10	176	T79	Very good	35	1025	T61	90%
11	175	T83	Very good	36	1025	T66	80%
12	1942	T8	Very good	37	695	T70	50%
13	1943	T8	Good	38	1025	T74	80%
14	1942	T12	Good	39	1025	T83	80%
15	1943	T12	Very poor	40	1025	T87	80%
16	1942	T16	Very poor	41	695	T95	90%
17	1943	T16	Poor	42	1025	T101	90%
18	1942	T19	80%	43	1025	T109	80%
19	1943	T23	20%	44	695	T111	90%
20	1943	T42	90%	45	1025	T115	75%
21	1943	T53	90%	46	695	T117	75%
22	1943	T75	90%	47	104	T7	Good
23	1943	T95	90%	48	105	T7	Good
24	1943	T102	90%	49	106	T6	Poor
25	1943	T118	90%				

subcuticular stitching for the skin.

The animals were allowed to recover and maintained on a normal laboratory diet and water for at least one month before being infected. Two rabbits were splenectomised during infections.

3.5 PARASITOLOGICAL METHODS

All infections were followed by wet preparation or buffy coat examination of blood from the animals or examination of stained blood films. Typically trypanosomes in twenty low power (x40) fields were counted. Where quantitation was particularly important, trypanosomes were counted in a haemocytometer after a suitable dilution of blood in ammonium oxalate solution.

In the calf experiments, the parasitaemia was assessed by examination of buffy coat preparations[91]. counting the number of parasites in low power (x40) fields. The buffy coat quantitations were all made by John Crosskey at the Liverpool School of Tropical Medicine.

3.6 HAEMATOLOGICAL METHODS

Blood counts, from EDTA anticoagulated blood samples.

were obtained throughout the infections using a Coulter Model S (Coulter Electronics Ltd. Luton. Bedfordshire) or Coulter Model S Plus IV. Although counts of white cell, red cells. amounts of haemoglobin and various red cell parameters were all obtained in this way, the haematocrit (Hct) was used in particular to monitor the anaemia and will be the index presented.

3.7 PLATELET METHODS

Various methods have been used throughout the experiments to assess the effect that trypanosome infections have on the platelets in particular, both in terms of morphology and function.

3.7.1 Platelet counting

Various established techniques have been employed to make assessments of platelet numbers throughout the series of rabbit infections; the problems involved will be discussed further. Calf platelets were counted using one technique only. Counts were made. from venous or arterial blood samples taken into potassium ethylene diamine tetra-acetic acid (EDTA) (1.5mg/ml blood) within six hours of sampling in the case of rabbit counts and within 24 hrs in the case of calf samples. The validity of all counts was assessed by examination of stained blood films.

3.7.1.1 Counting-chamber methods for rabbit and calf platelets[92].

20ul of well mixed blood was added to 0.38ml ammonium oxalate (10mg/L) to make a 1 in 20 dilution and the suspension roller-mixed for five minutes. An improved Neubauer counting chamber was filled and allowed to stand in a damp atmosphere for 20 minutes[93]. Platelet counts were made over a $1mm^2$ area (0.1µl volume) of the chamber under phase contrast microscopy.

3.7.1.2 Electronic counting using the Coulter ZF for rabbit platelets.

For many of the experiments a Coulter Counter Model ZF was used. The principles of the method are those given by Bull et al[94] but the threshold settings, aperture currents and attenuation settings were recalibrated for particular use with rabbit platelets which have smaller mean platelet volume than human platelets (4.52fl compared with 6.42fl)[95].

A 50µm orifice was used for counting a sample volume of 0.1ml with threshold settings at 8 and 100, aperture current at 16 and attenuation at 0.500. These settings represented a volume range of 1.5 to 19fl. A Coulter Platelet Kit was used for separation of platelet-rich plasma and counts corrected for coincidence and haematocrit using formulae and tables provided by

Coulter Electronics Ltd.

3.7.1.3 Electronic counting using the Coulter Model S plus IV for rabbit platelets.

With the development of cell counting machines over the years, a model was produced by Coulter that also counted platelets over the range 2-20fl, which would be applicable to rabbit platelets as well as human platelets for which it was designed. 0.1ml volumes of whole blood were counted using this method.

3.7.1.4 Electronic counting using the Coulter Model ZF for Calf Platelets

Bovine blood has an exceptionally low erythrocyte sedimentation rate and so the method described in 3.7.1.2 could not be used for counting platelets. Instead a method described by Maxie[96] which correlates well with visual counting was used.

100µl whole blood was diluted in 10ml Isoton (Coulter Electronics Ltd) in plastic Universal bottles (Sterilin Ltd, Islington, Middlesex), recapped and allowed to stand for three hours. After sedimentation, 100µl of the supernatant was further diluted in 10ml Isoton and platelets counted using a 70µm orifice with a 0.1ml sample volume, threshold settings of 9.6 and 96, aperture current of 16 and attenuation of 0.500. These

settings represented a volume range of 1.8 - 18fl. Counts were corrected for background, coincidence and dilution.

3.7.2 Blood films for platelet count estimation.

Blood films were made using a spun-film technique with an ADC 500 spinner (Abbott Laboratories Ltd. Dallas, Texas, USA) and stained using a May Grunwald-Giemsa automated technique (Searle Diagnostic, High Wycombe, Buckinghamshire) in a Shandon Elliott Automatic Stainer (Shandon Scientific Co Ltd. London, NW10) (Appendix I.2).

All films were examined for estimation of platelet count and for consideration of possible changes in platelet morphology.

3.7.3 Platelet size

Assessment of platelet volume (MPV) and platelet distribution width (PDW) was made in two experiments involving six uninfected rabbits and twelve rabbits infected with T b brucei S42. MVP and PDW were both measured on a Coulter S Plus IV (Coulter Electronics Ltd), being derived from the platelet distribution histogram, and followed throughout the infection using the same EDTA samples taken for measurement of platelet count during these experiments.

3.7.4 Platelet aggregate formation

Most studies of platelet aggregate formation examine platelets *in vitro* but in 1974 Wu and Hoak[97] described a technique designed to quantify the circulating platelet aggregates. The method is based on the differential enumeration of platelets in blood drawn into a buffered EDTA-formalin solution, which fixes the aggregates, and blood drawn into a buffered EDTA alone solution, which breaks down the aggregates.

This experiment was done in a group of six rabbits infected with *T.b.brucei* S42 and aggregates were monitored throughout the infection.

Using a 23-gauge Butterfly needle (Abbott Laboratories Ltd, Queenborough, Kent) free flowing blood was taken from the central ear artery of each rabbit. The first few drops of blood were allowed to drip from the Luer fixing of the Butterfly set to ensure free flow and a 2ml syringe attached containing 1ml of EDTA-formalin buffered solution (Appendix I.3). 1ml of blood was taken into this syringe, air introduced, the sample mixed. A second 2ml syringe was attached containing 1ml of buffered EDTA solution (Appendix I.3) and a further 1ml blood sample taken in a similar fashion. The contents of each syringe were delivered into separate polypropylene tubes and kept at room temperature for 15

minutes. Both samples were centrifuged at 200g for 8 minutes and the platelets counted in the resultant platelet-rich material using a Coulter Model S Plus IV (Coulter Electronics Ltd). This method was a slight modification of that suggested by Wu in 1977[98] and all counts were performed within one hour of blood sampling[99]. The results were expressed as a ratio of counts in EDTA-formalin suspension to counts in EDTA suspension.

3.7.5 Electronmicrograph studies on platelets

Electronmicrographs were examined in two series of experiments, one involving infection of six rabbits with T b brucei S42 and the other involving infection of four calves with T vivax. In both cases the method of preparation of platelets for these studies was the same.

4.5ml blood was taken from the central ear artery of the rabbit into a syringe containing 0.5ml 0.106M trisodium citrate (9ml from the jugular vein of the calf into 1ml trisodium citrate) and transferred to a sterile round bottomed 10ml tube (142 AS tubes, Sterilin Ltd). After centrifugation at 200g for 15 minutes, the platelet-rich plasma was removed and an equal volume of 0.2% glutaraldehyde buffer (Appendix I.4) added and the sample mixed. The sample was recentrifuged at 200g for 5 minutes, the platelet-poor plasma discarded and the

resultant platelet button covered with 1-2ml 3% glutaraldehyde in buffer (Appendix I.4). The platelet button was left to fix at 4°C for 2-4 hours and then washed twice with 0.2M cacodylate buffer, and stored under cacodylate buffer at 4°C until despatched to the electron microscopy unit for post fixation with 1% osmium tetroxide and processed into cylinders of Araldite containing the platelet button. 60-100nm sections were cut using a glass knife, stained with lead citrate and photographs made of several areas of each section.

A qualitative examination was made of platelet ultrastructure throughout the infection and several quantitative measurements were also made. All photographs were coded according to their source by another person and the code was not broken until examination and measurements were computed.

Measurements were made of the short (L short) and long (L long) axes of all platelets lying completely within the area of the photographs obtained from each individual animal on a particular day. The derived mean measured axial ratio (AR) was used to characterise shape. Platelet volume (PV) and platelet surface area (PSA) were calculated based on the theoretical volume of a rotational ellipsoid with the short axis as rotation

axis$^{(100)}$. These formulae, given below, do not take any account of the presence of pseudopodia, and therefore may give rise to an underestimate: this emphasises the importance of the associated qualitative examination.

$$PV = (4/3)\pi\ (L\ long/2)^2\ (L\ short/2)$$
$$= (\pi/6)\ (L\ long^2)(L\ short)$$

$$PSA = (\pi/2)\ (L\ long^2) + (\pi/4)(L\ short^2)x$$
$$[(L\ long + L\ short)/(L\ long - L\ short)]$$
$$x\ log\ (L\ long/L\ short)$$

Counts were also made of platelet granules (alpha and dense bodies) and vacuoles within the measured platelets.

3.7.6 Platelet aggregometry

The ability of platelets to aggregate, in response to various *in vitro* stimuli, was examined within two separate experiments. Platelets from four cattle, infected with *T vivax*, were examined using an Accutech Aggregraph (Accutech Ltd, Littleborough, Lancashire), the use of which was kindly allowed by the Department of Haematology, Liverpool Royal Infirmary. Platelets from six rabbits, infected with *T b brucei*, were examined using a Payton 1010 aggregation module (Centronic Sales

Ltd, New Addington, Croydon).

Blood samples (4.5ml) were taken from the central ear artery (rabbit) or jugular vein (calf) into 5ml syringes containing 0.5ml of 0.106M trisodium citrate using a 23 gauge Butterfly needle (rabbit) or 21 gauge needle (calf). Because of the particular importance of achieving clean samples with good flow in work with platelets, only samples that were achieved within one minute and without any evidence of bleeding out within the tissue were accepted. Bleeding was only ever a problem with the rabbits and repeat samplings that were sometimes necessary were always made from the opposite ear vessel or at least 3cm proximal to the previous puncture site. Blood was mixed within the syringe during sampling and transferred to a 10ml polypropylene tube, sealed and centrifuged at 200g for 8 minutes to obtain platelet-rich plasma. A polypropylene Pasteur pipette was used to transfer the platelet-rich plasma to a second polypropylene tube and the original sample then centrifuged at 1000g for a further 8 minutes to obtain platelet-poor plasma. Platelet counts of the platelet-rich plasma were made using the techniques described in 3.7.1.1 (for calf platelets) and 3.7.1.2 (for rabbit platelets) and individual counts were then standardised to 400 x 10^9/L by adding platelet-poor plasma obtained from the second centrifugation. Excess platelet-poor

plasma was used to provide a blank for each individual sample during aggregometry.

Various agents were used to stimulate aggregation:

(a) adenosine diphosphate (ADP) (Diamed Diagnostics Ltd. Liverpool) was used at various final concentrations of between 0.625-10µg/ml.

(b) collagen (Diamed Diagnostics Ltd, Liverpool) was used at various final concentrations of between 10-20µl/ml, (4-8 µg/ml).

(c) sodium arachidonate (Sigma London Chemical Co. Poole, Dorset) was made up to a 20mM concentration and used at final concentrations of 0.125-1mM. This particular aggregation inducer was only used in the rabbit experiments.

Investigation was also made of the effects of trypanosomes and infected platelet-poor plasma on normal platelets in the aggregometer.

Occasionally it was not possible to obtain final platelet counts of 400 x 10^9/L in the platelet-rich plasma. On these occasions platelets from uninfected animals were also diluted to achieve the same platelet count as that from the infected animals. The resultant aggregation responses were then compared.

3.7.7 Platelet malondialdehyde formation

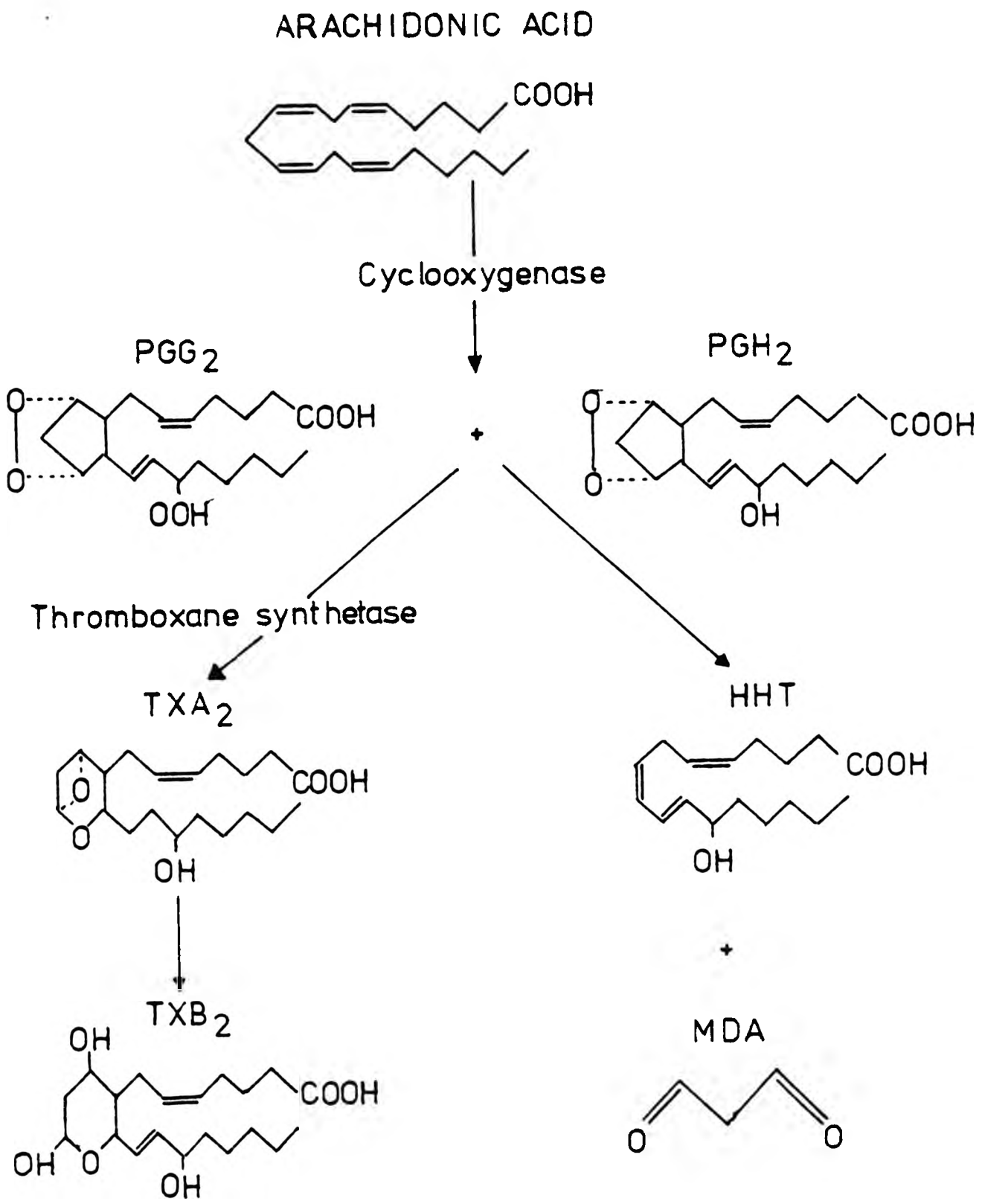
A different assessment of platelet function during an infection was made by exposing platelets to N-ethylmaleimide (NEM) which leads to platelet aggregation and release. This reaction is accompanied by production, from arachidonic acid within the platelets, of cyclic endoperoxides and thromboxane A_2. These substances decay to non-prostaglandin products, over 50% of which is found as a C17 hydroxy fatty acid 12L-hydroxy-5.8.10-hepta-decatrienoic acid (HHT) formed by cleavage of the cyclopentane ring yielding also a 3 carbon fragment, malondialdehyde(MDA)[101](fig.3.1).

The MDA generated is reacted with thiobarbituric acid to produce a red colour which has an absorption maximum at 532nm in a spectrophotometer[102,103]. However MacFarlane et al[102] have described a technique which concentrates the coloured product and allows measurements down to 0.02μM MDA and it is a modification of this method that has been used.

Blood samples, 4.5ml into trisodium citrate as previously described (3.7.6), were taken from 6 rabbits, four being infected with T.b.brucei and two remaining uninfected as control animals. The samples were centrifuged at 150g for 15 minutes and the platelet-rich plasma removed and counted (as in 3.7.1.1).

Figure 3.1

PRODUCTION OF MALONDIALDEHYDE (MDA) IN PLATELETS



To a 2ml aliquot of platelet-rich plasma was added 0.2ml of 1.25mg/ml NEM providing a final concentration of 1mM and the mixture incubated at 37°C for one hour. MDA was extracted by adding 0.95ml of 40% trichloracetic acid in 1M HCl, mixing and centrifuging at 1000g to remove the protein. Samples were then heated at 70°C for 30 minutes after addition of 400µl of 0.12M thiobarbituric acid in 0.26M 2-amino-2(hydroxy-methyl)-1,3-propanediol adjusted to pH 7.0.

After cooling, the mixture was pipetted on to a column prepared in a Pasteur pipette containing an 8% slurry of DEAE cellulose (Whatman DE 52) and supported on a glass fibre plug and 7mm disc punched from Whatman GFA glass fibre paper. A second disc was placed at the top of the column before addition of the coloured reaction mixture. The coloured product remained within the cellulose and the column was washed with 2ml water and eluted with 1.2ml 6M KOH, collecting the final 1ml.

Using a 6M KOH blank the samples were read in a spectrophotometer at 518nm, 548nm and 578nm within 5 minutes and the final adjusted absorbance (A) calculated from the formula:

$$A = OD \text{ at } 548nm - \frac{(OD \text{ at } 518nm + OD \text{ at } 578nm)}{2}$$

where OD = optical density.

548nm is the absorption maximum of the coloured substance after treatment with KOH and adjustments made using readings obtained at 518 and 578nm are to correct for optical effects on absorbance caused by any turbidity in the sample[104].

The concentration of MDA in the sample is calculated from the formula:

$$\mu M \text{ MDA}/10^9 = \frac{Vf \times A}{Vo \times 0.152 \times P}$$

where A - corrected absorbance

Vf = final volume

Vo = original volume

P = no. of platelets ($x10^9$)/ml

and the molar absorbance coefficient of malondialdehyde is 1.52×10^5 at 548nm[105].

3.7.8 Platelet lifespan

Platelets, when labelled with a suitable radioactive isotope, have been shown to provide important information on platelet kinetics both in the normal and the diseased state. ^{111}Indium (In) oxine was first used as a platelet label in 1976[106] because of its suitable physical characteristics. It has a half life of 67.2 hours which is long enough to monitor a platelet

lifespan of up to about 10 days.

Oxine is a bidentate ligand and provides a covalent bond via an hydroxyl group and a donor bond via the nitrogen atom to form a neutrally charged complex with ^{111}In at pH 5-6[106]. The complex, suspended in a non-plasma salt-balanced medium, diffuses passively through the cell membrane; ^{111}In associates with cytoplasmic components and oxine elutes from the cells. Only small amounts of ^{111}In taken up by the platelets are lost as a result of platelet damage or induction of the platelet release reaction[106].

Rabbit platelets have been labelled successfully by several workers[107-110] and although different methods have been used, the <u>in vivo</u> survival has been similar to that reported by other workers using different labels.

For both rabbit and calf platelet labelling, we have used the method of Hawker et al[111], which involves preparation of the ^{111}In oxine complex, and labelling of platelets in calcium free Tyrode's buffer containing prostaglandin E_1, to prevent activation of platelets during centrifugation procedures. The method is described fully in Appendix I.5 and has been modified only slightly. In order to facilitate the taking of a

large volume of blood from the rabbit ear without problems, the animals were sometimes anaesthetised with 50mg/kg ketamine prior to the procedure.

Platelet lifespan has been measured in eight rabbits before and during a T b brucei infection and in four calves before, during and after recovery from a T vivax infection For estimation of lifespan the maximum likelihood estimate of the integral ordered gamma function, based on a multiple-hit model, has been used as one recommended by the International Committee for Standardisation in Haematology[89], using a computer program prepared by David R. Bolling (Division of Medical Genetics, Department of Medicine, John Hopkins University School of Medicine, Baltimore, Maryland 21205, USA).

3.8 EXPERIMENTAL DESIGN

3.8.1 T b brucei S42 experiments (Table 3.2)

The major series of experiments have involved infecting rabbits with the T.b.brucei organism. The strain S42 used produces an acute infection in rabbits lasting about one month[53].

Experiment I

This was a pilot infection of three rabbits followed

Table 3.2

T b brucei infections

EXPERIMENT	NO. RABBITS INFECTED	CONTROLS	PARAMETERS STUDIED
I	3	1	Trypanosome count Haematocrit Platelet count (3.7.1.1)
II	4 non-splenectomised 3 splenectomised	-	Trypanosome count Haematocrit Platelet count (3.7.1.1)
III	4	2	Trypanosome count Haematocrit Platelet count (3.7.1.2)
IV	3	3	Trypanosome count Haematocrit Platelet count (3.7.1.2)
V	2 No treatment 2 given ASA daily	1 1	Trypanosome count Haematocrit Platelet count (3.7.1.2) Platelet ultrastructure
VI	4	2	Trypanosome count Platelet count (3.7.1.2) Malondialdehyde production
VII	3 non-splenectomised 3 splenectomised	3 3	Trypanosome count Haematocrit Platelet count (3.7.1.3) Platelet indices
VIII	6	6	Trypanosome count Haematocrit Platelet count (3.7.1.3) Platelet indices Platelet aggregate formation
IX	6	-	Trypanosome count Platelet function
X	8	13	Platelet lifespan

over 27 days with one further rabbit as control, done to assess the effect of the organism on the platelet count and to establish experimental methods.

Experiment II

Making use of the methods in Experiment I, a further infection in four animals was followed over a similar period of time and compared with an infection in a group of three animals that had previously had their spleens removed.

Experiments III and IV

Because of problems encountered in the first two experiments a further set of two experiments, each using six animals, was done using a different method of assessing the platelet count.

Experiment V

This experiment was used to assess the effect of aspirin (ASA) ingestion on the infection. Two groups of three animals were used, each group containing a control animal. This set of animals was also used to make a study of platelet ultrastructure and changes observed during the infection.

Experiment VI

An assessment of platelet biochemistry during an

infection was made by a measurement of platelet malondialdehyde production. Four rabbits were infected and two control animals were also studied.

Experiment VII

Measurement of the platelet volume (MPV) and platelet distribution width (PDW) was made in this experiment. Six rabbits (three splenectomised) were followed for 10 days and for up to 28 days after infection.

Experiment VIII

An infection in six animals was used to assess *in vivo* platelet aggregate formation along side other measures used previously.

Experiment IX

Platelet function before and during infection was assessed in a group of six animals using *in vitro* responses of platelet rich plasma to various aggregating agents.

Experiment X

Platelet lifespan, before infection and at different times during the infection, was measured in a total of 13 animals of which 8 were infected.

3.8.2 *T congolense* infection in rabbits

T congolense infections in cattle and sheep have been reported to cause a rapid acute anaemia later becoming chronic [17,122]. Two strains. GAMB 19 and TSW 99/77. isolated in geographically different regions and showing distinct isoenzyme patterns. have been used to infect a group of eight rabbits, four being infected with each strain. Three further rabbits were studied alongside this group as controls and the infection was studied over 225 days.

3.8.3 *T vivax* infection in cattle

This experiment was set up at The Liverpool School of Tropical Medicine principally to study the development of immunity to a series of multistabilate induced infections in four calves. We used the opportunity to make a study of platelet number, structure, function and lifespan during these series of infections which are summarised in Table 3.3.

3.9 STATISTICAL METHODS

Because of individual differences in baseline levels of many of the parameters measured. most of them have been converted to the percentage of their value before infection; this also helps when assessing the change in a parameter over the period of the infection.

Table 3.3

T.vivax infections

CALF

DAY	Blue	Boris	George	Tog
0	Control	Control	1st infection	1st infection
13	All received Berenil 28mg.kg			
55	Control	1st infection	Post-infection control	2nd infection
87	All received Berenil 15mg/kg			
102	All received Berenil 15mg/kg			
136	1st infection	2nd infection	2nd infection	3rd infection
198	Experiment ended			

Groups of animals within experiments have generally been compared using a paired t-test. where it was possible to pair across the days, or an unpaired t-test where this was not possible. Where the data departed significantly from normal, a non-parametric method was used (Kendall's S test).

Spearman's rank correlation coefficient was used to compare trypanosome counts and malondialdehyde levels in experiment VI. The data on platelet counts and haematocrits from the calf experiments was not analysed statistically because only four calves were involved and each calf underwent a different number of infections for variable periods of time.

4. RESULTS

4.1 PARASITAEMIA

4.1.1 *T b brucei* infections

T b brucei is principally an infection of extravascular tissues with particular affinities for connective tissue[7]. Although the trypanosomes are found in the circulation, their numbers are not always a good guide to the parasitaemia or the prospects for the animal survival[113]; often the numbers are much lower than seen in infections with other trypanosome species and sometimes no trypanosomes can be found in the blood[6]. The organism is however very pathogenic producing severe inflammatory reactions[44] and widespread tissue changes[19,20].

In the series of infections in rabbits reported here, the debilitating effects of the disease were obvious within the first week of the infection, the rabbits showing signs of lassitude. In the second week of infection, the ears in particular showed gross thickening and by the third week skin lesions, thickening and inflammation of tissues around the eyes and often a nasal discharge were also present. Sometimes testicular and rectal bleeding were seen towards the end of the fourth week by which time the

animals were severely debilitated and had suffered a considerable loss of weight. Animals that reached this distressing state were killed.

Parasites were first observed in the peripheral blood, usually between the third and seventh day of the infection although the numbers were normally very low. typically 1-2 per 20 low power fields. Jenkins et al[53] in their series of infections of rabbits with T.b.brucei S42 did not detect parasites in wet films until day 14 although buffy coat preparations revealed parasites from day 3. The numbers of parasites seen in the initial parasitaemia in the splenectomised animals of experiment VII were higher than the control infected animals but this phenomenon was not seen in experiment II until the later stages of infection. The higher parasitaemia in splenectomised animals has been previously reported in T b brucei infections of rabbits[67] and in T simiae infections of rabbits[114]. In all animals the parasites disappeared from the circulation within one or two days, as detected by wet preparation examination of blood. although more sensitive methods showed the continued presence of parasites in very low numbers. Parasites again became apparent in the peripheral blood between days 11 and 17 (mean 14) and the numbers then rapidly increased reaching high levels (typically around 100-

2000/20 low power fields) within four or five days. The rabbits normally had to be killed or died around this time, most animals surviving from between 28 and 32 days after infection.

Five rabbits died early:

JOB (Experiment II) died on day 8 with a high parasitaemia and a fall in haematocrit to 42% of initial values. Thrombocytopenia, although present, was not marked.

LUP (Experiment IV) died on day 7 with a relatively high and accelerating parasitaemia and significant fall in haematocrit (to 68% of pre-infection values) and in platelet number (to 52% of pre-infection numbers).

JAS (Experiment IV) died on day 8 with no detectable parasitaemia or thrombocytopenia but a fall in haematocrit to 58% of pre-infection values.

GRE (Experiment VI) died on day 12 with a high parasitaemia after a marked thrombocytopenia (6% on day 7) although platelet numbers recovered after day 10.

CHE (Experiment VII) was asplenic and died on day 7 with a low parasitaemia and no significant fall in

haematocrit. The animal had experienced a marked thrombocytopenia on days 4-7.

In summary, the infection caused by T b brucei was mainly characterised by its debilitating effects and inflammatory tissue reactions. Two peaks of parasitaemia were observed, the first towards the end of the first week, usually low in intensity and of short duration, and the second beginning in the third week and increasing without check. Deaths which occurred early in the infection were associated with either higher parasitaemias and more rapid falls in haematocrit or with periods of marked thrombocytopenia not seen in surviving animals at this time.

4.1.2 T congolense infection

T congolense parasites, in contrast to T b brucei, are found mainly in plasma with a preference for the microcirculation[7], although tissue foci at the site of injection may provide an important source of parasites later in the infection[115]. Most reports of this infection have been made in their natural hosts, cattle, goats and sheep. Generally the reports have been of an early acute phase with high parasitaemia[60,113] becoming sub-acute or chronic. Naylor[61] however reported that he found the disease in cattle to be chronic with few trypanosomes and 8/10

showed a spontaneous recovery.

In our experiments four rabbits were infected with one strain of T congolense - GAMB 19 - and a further four rabbits with a different strain - TSW 99/77 - with the aim of assessing any differences between the infections which might be characteristic of the strain used. None of the animals showed the severe tissue inflammation seen in the T.b.brucei infections and appeared reasonably healthy throughout.

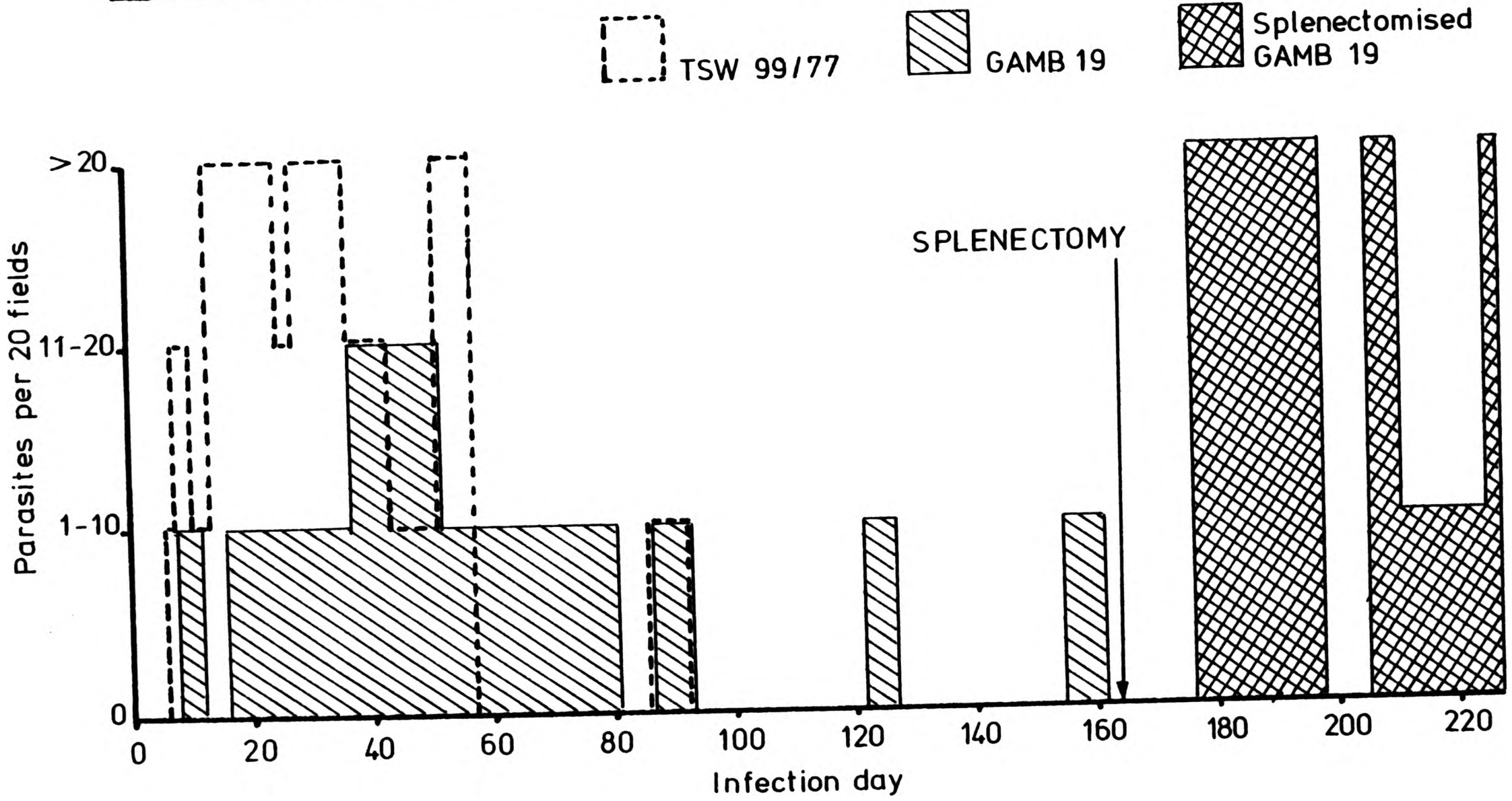
Trypanosomes appeared on day 7 in one animal and were present on day 8 in all eight animals.

Levels of parasites in the GAMB 19 infection (fig 4.1) peaked on day 37, although numbers were low (mean 5.0+/- 3.8 per 20 low power fields) and remained detectable up to day 72. Between days 72 and 225 when the experiment ended, parasites were seen only sporadically. One animal (HAN) underwent splenectomy on day 162 and after that, trypanosome levels increased to 85 per 20 low power fields on day 205. Two animals died after 184 days of infection with no detectable parasitaemia.

In the TSW 99/77 infection, considerably higher levels of parasites were seen with a major peak on day 21 (mean 70.7 +/- 40.1 per 20 low power fields), falling away to

Fig 4.1

PARASITE LEVELS IN T. CONGOLENSE INFECTIONS (mean range)



nothing on day 58. From then no parasites were detected within the remaining experimental period of 167 days. One rabbit (NIE) died on day 9 with no detectable parasitaemia but with a marked thrombocytopenia. A second rabbit (PUC) died on day 155, while undergoing splenectomy.

In all the infected rabbits the spleen was found to be enlarged when examined at splenectomy or post-mortem and with the rise in trypanosome numbers after splenectomy, the importance of this organ for continued removal of trypanosomes in this apparently chronic infection is emphasised. In 60 day T congolense infections of cattle, Losos et al[116] have reported increased spleen, liver, heart and kidney weights.

4.1.3 T vivax infections

T vivax parasites are found mainly in blood in infected animals although there is some tissue infiltration[6] and is most virulent in ruminants[24]. In goats there is a prepatent phase of about one week with a rapid early parasitaemia producing an acute phase or subacute phase, which later becomes a chronic infection[18,113,117]. Usually the highest parasitaemia is found earliest in the infection[6]. In cattle a similar prepatent phase of one week is found with the first parasitaemia peak showing the highest

number of trypanosomes and thereafter cyclic fluctuations[31,37,66].

In our series of infection in four calves parasites were observed on days 3 or 4 in the first infection (four animals), on days 4 and 7 in the second infection (two animals) and on day 9 in the third infection(one animal) showing an apparent delay in the development of parasitaemia due to previous exposure to the disease. In the first group of infections in two calves (GEO & TOG), terminated after 13 days with Berenil, parasites were detected on all days thereafter peaking on day 7 and day 12. The early pattern of the first infection for the other two calves (BOR & BLU) was similar. BOR's first infection was terminated after 31 days and the pattern of parasitaemia after the 13th day showed large numbers of parasites on 7/9 of the days that they were looked for, being negative on the other two days. BLU's first and only infection was followed for 62 days and parasites were observed on 28/34 days that preparations were examined, the first negative result being found on day 42.

As well as the apparent delay in onset of parasitaemia in the second and third infections the early parasitaemia showed lower numbers of parasites and this was especially so in TOG's third infection. With time,

however, the high parasitaemias were again reached so any apparent immunity was still overcome.

Parasitaemias (shown in Fig.4.2) were compared using Kendall's S test (Table 4.1) but TOG was the only calf to show a significant decrease in parasite number from the first infection to the second ($p<0.05$) and from the second infection to the third ($p<0.01$).

All four calves survived to day 62 of the final infection and none showed a loss of weight over this time.

4.2 ANAEMIA (APPENDIX II)

4.2.1 T b brucei infections

T b brucei has varied host virulence and although infections with this organism in cattle are common, the infection is usually subpatent[118] and only produces a mild anaemia with low mortality[119]. In natural and experimental infections of goats, sheep[113] and lions[120] a progressive significant anaemia is seen early in the infection although the parasitaemia may appear very low. In rodents there is a significant fall in haematocrit, by about 20%, in the first few days of infection[54] and in splenectomised mice, the fall is much greater[121]. The spleen is, however, in mice,

PARASITE LEVELS IN T. VIVAX INFECTIONS

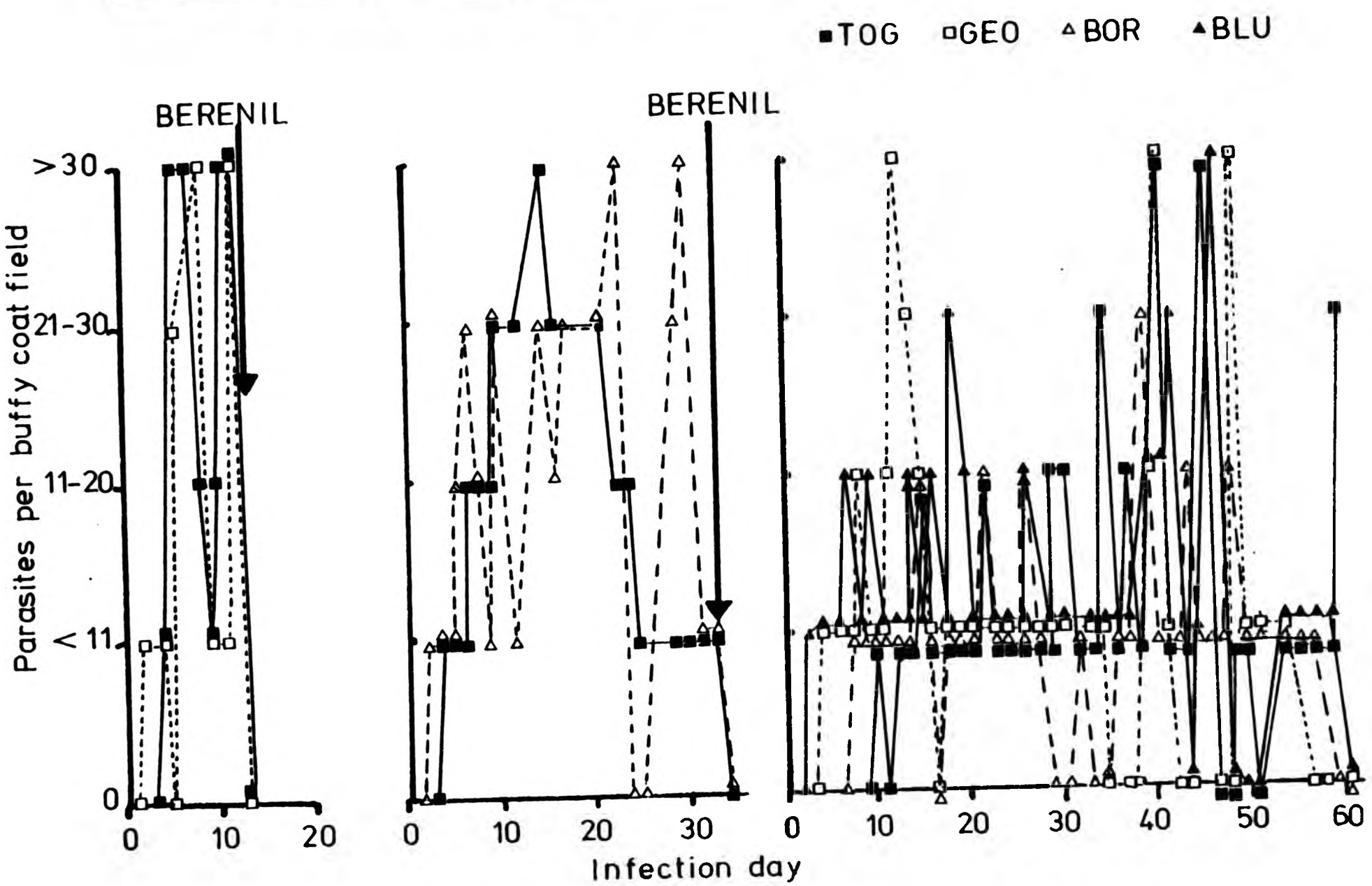


Fig 4.2

Table 4.1

COMPARISON OF PARASITE LEVELS IN T.VIVAX EXPERIMENTS

KENDALL'S S TEST (Z LEVELS)

1st INFECTION	BLU	BOR	GEO	TOG
BLU	-	0.83	1.04	1.95
BOR		-	0.76	1.40
GEO			-	1.40
TOG				-

All are non-significant

2nd INFECTION	BOR	GEO	TOG
BOR	-	1.59	1.33
GEO		-	0.08
TOG			-

All are non-significant

1st INFECTION v 2nd INFECTION	BOR	GEO	TOG
BOR	1.79	-	-
GEO	-	0.67	-
TOG	-	-	2.00*

* significant ($p<0.05$)

2md INFECTION v 3rd INFECTION	TOG
	2.58**

** significant ($p<0.01$)

significantly involved in erythropoiesis[67]. Rabbits typically show a progressive fall in haematocrit during the first two weeks of infection which then remains stable[51,52].

In our experiments I, II, IV, VII and VIII, we also observed a rapid fall in haematocrit to levels of 61%, 56%, 65%, 77% and 78% of pre-infection levels respectively by day 7. These values then normally stabilised out at around 65-75% until the experiment was ended (Figs.4.3, 4.4, 4.5, 4.6 and 4.7). Only one animal showed a marked fall in haematocrit later in the infection resulting in a premature death (EST experiment I, 45%, day 18).

In two experiments, III and V, the haematocrit had only fallen to 93% and 92% respectively by day 7 but continued to fall in both cases reaching a means of 67% and 65% respectively by about day 24 (Figs. 4.8 and 4.9). In experiment III one of the three animals showed the expected fall in haematocrit by day 7 but in experiment V, the pattern was similar for all the infected animals. The reason for the difference in response was not obvious. All animals had the same number of trypanosomes injected but it was possible that their viability within the rabbits varied according to when the organisms were obtained from the infected mice.

EXPERIMENT I - HAEMATOCRIT IN T B BRUCEI INFECTION

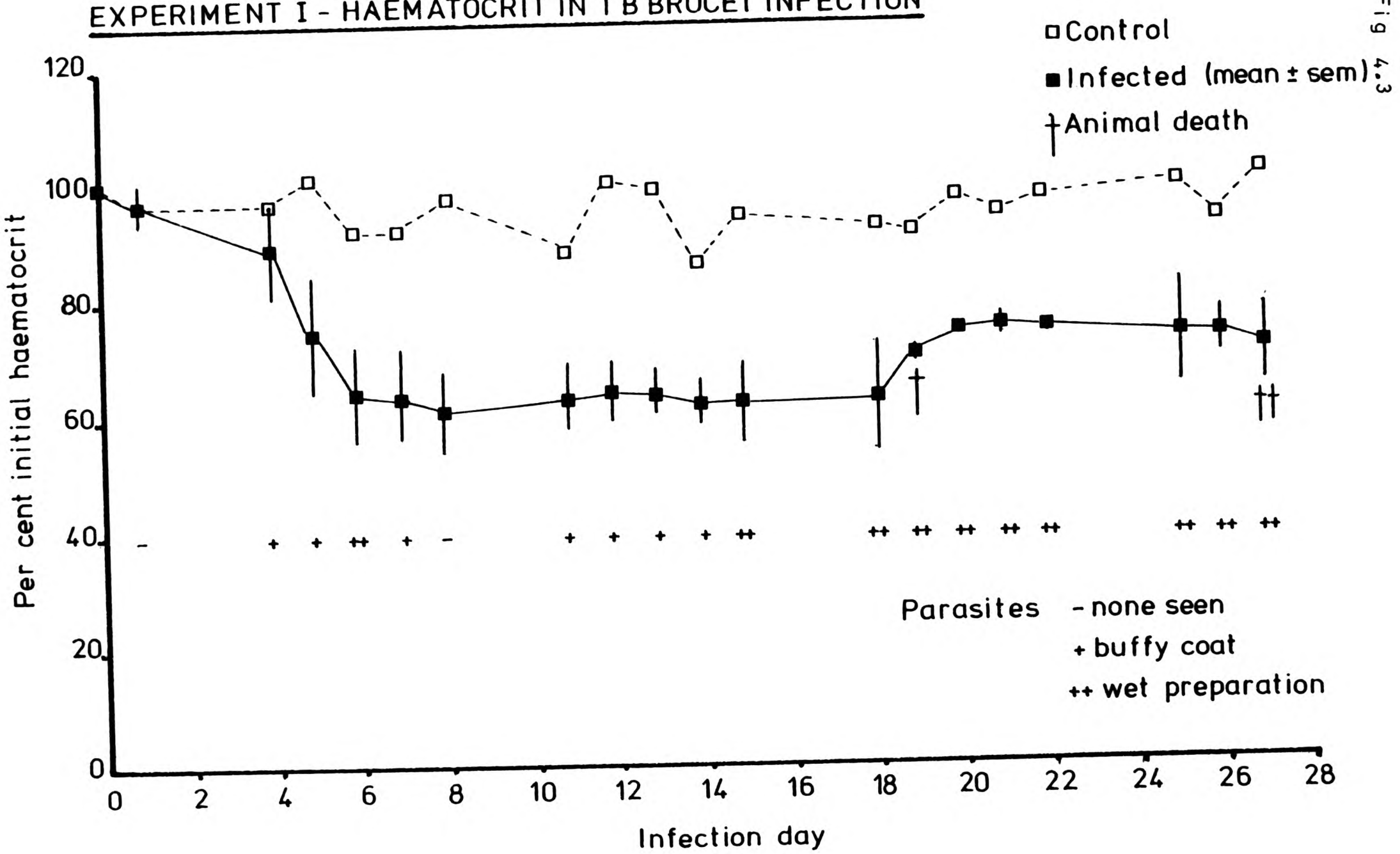
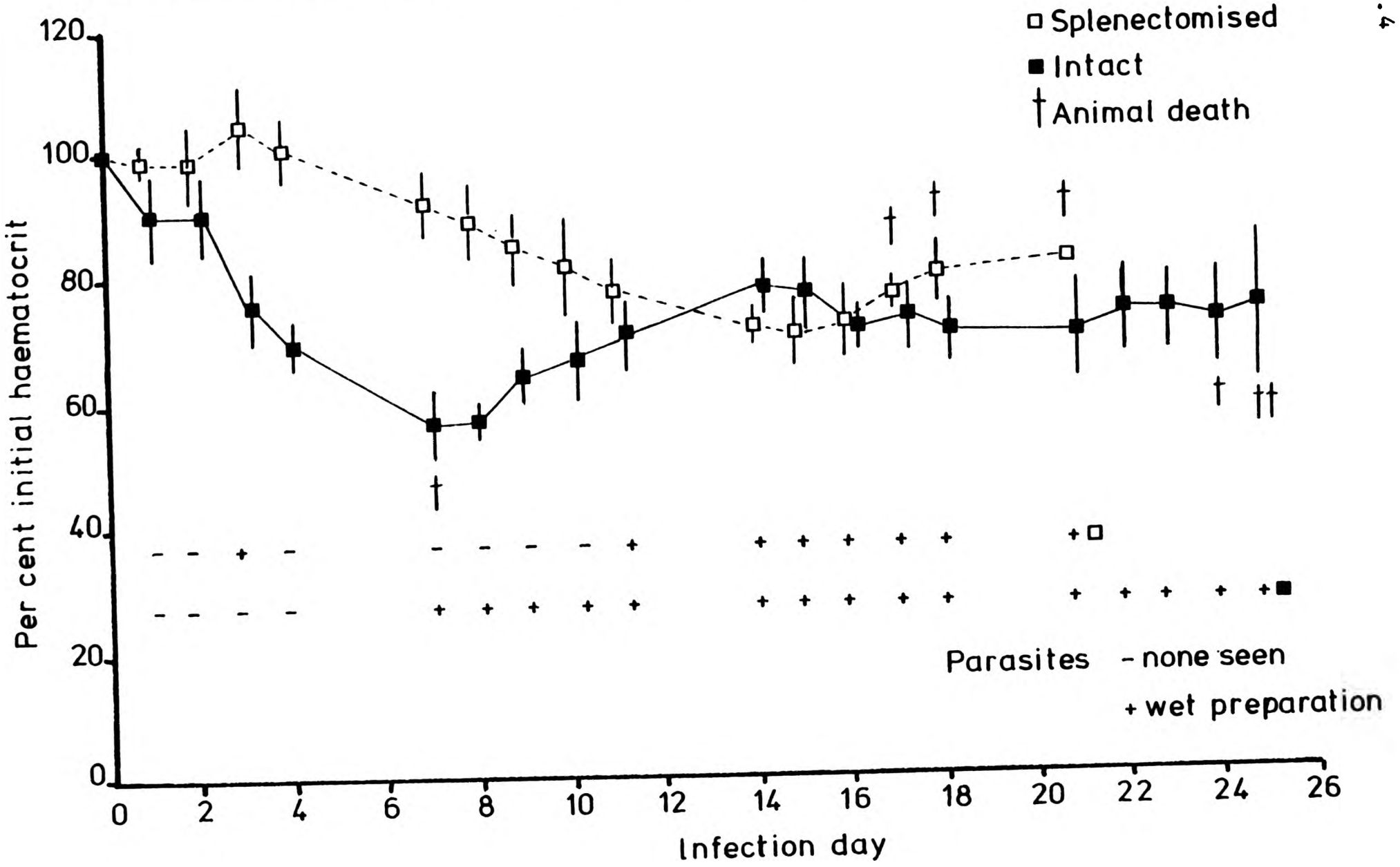


Fig 4.3

Fig 4.4

EXPERIMENT II - HAEMATOCRIT IN T B BRUCEI INFECTIONS (mean ± sem)



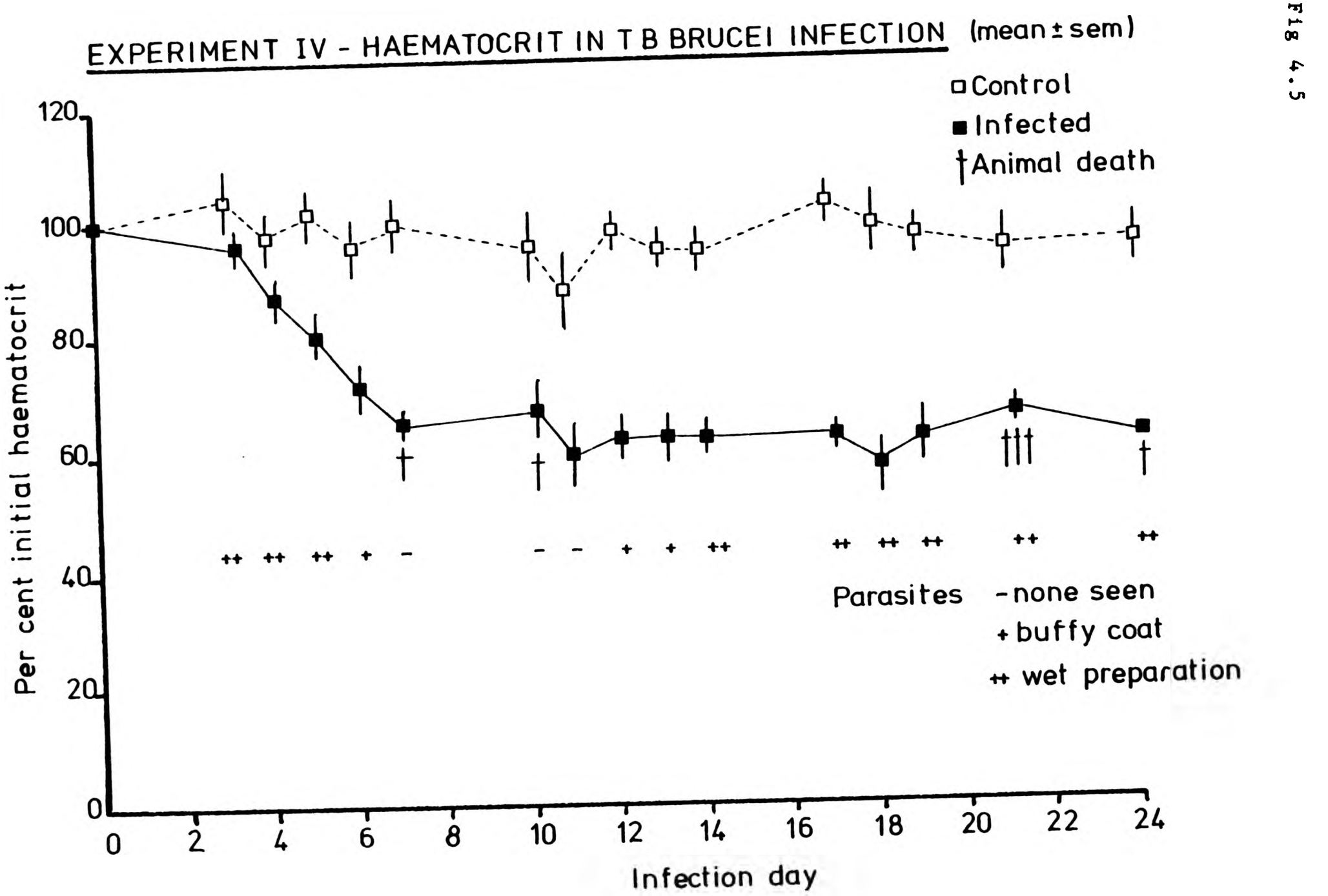

EXPERIMENT IV - HAEMATOCRIT IN T B BRUCEI INFECTION (mean ± sem)
□ Control
■ Infected
† Animal death
Per cent initial haematocrit
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Infection day
Parasites - none seen
+ buffy coat
++ wet preparation

Fig 4.5

Fig 4.6

EXPERIMENT VII - HAEMATOCRIT IN T B BRUCEI INFECTIONS (mean ± sem)

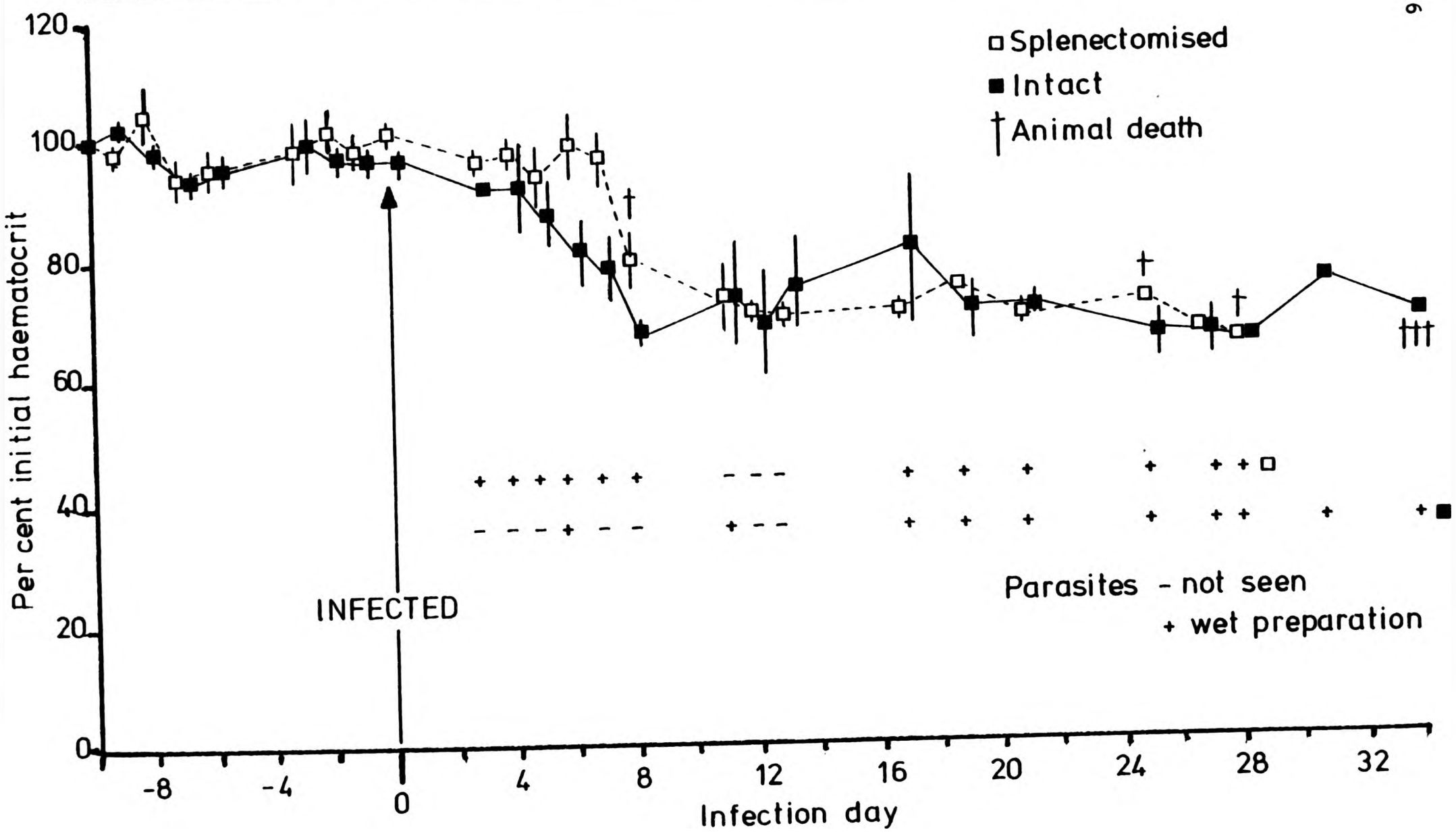
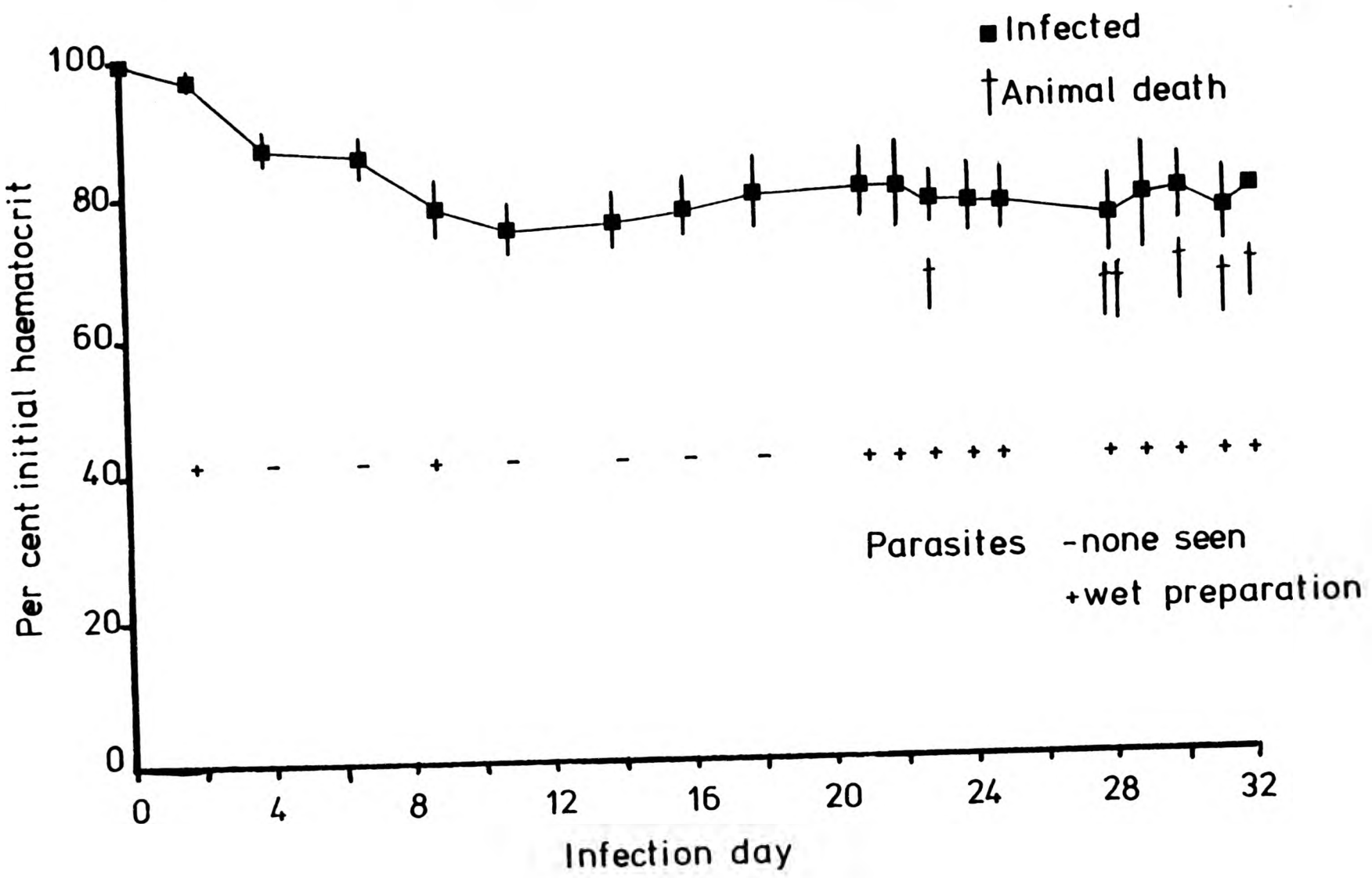


Fig 4.7

EXPERIMENT VIII - HAEMATOCRIT IN T B BRUCEI INFECTION (mean ± sem)



EXPERIMENT III - HAEMATOCRIT IN T B BRUCEI INFECTION (mean ± sem)

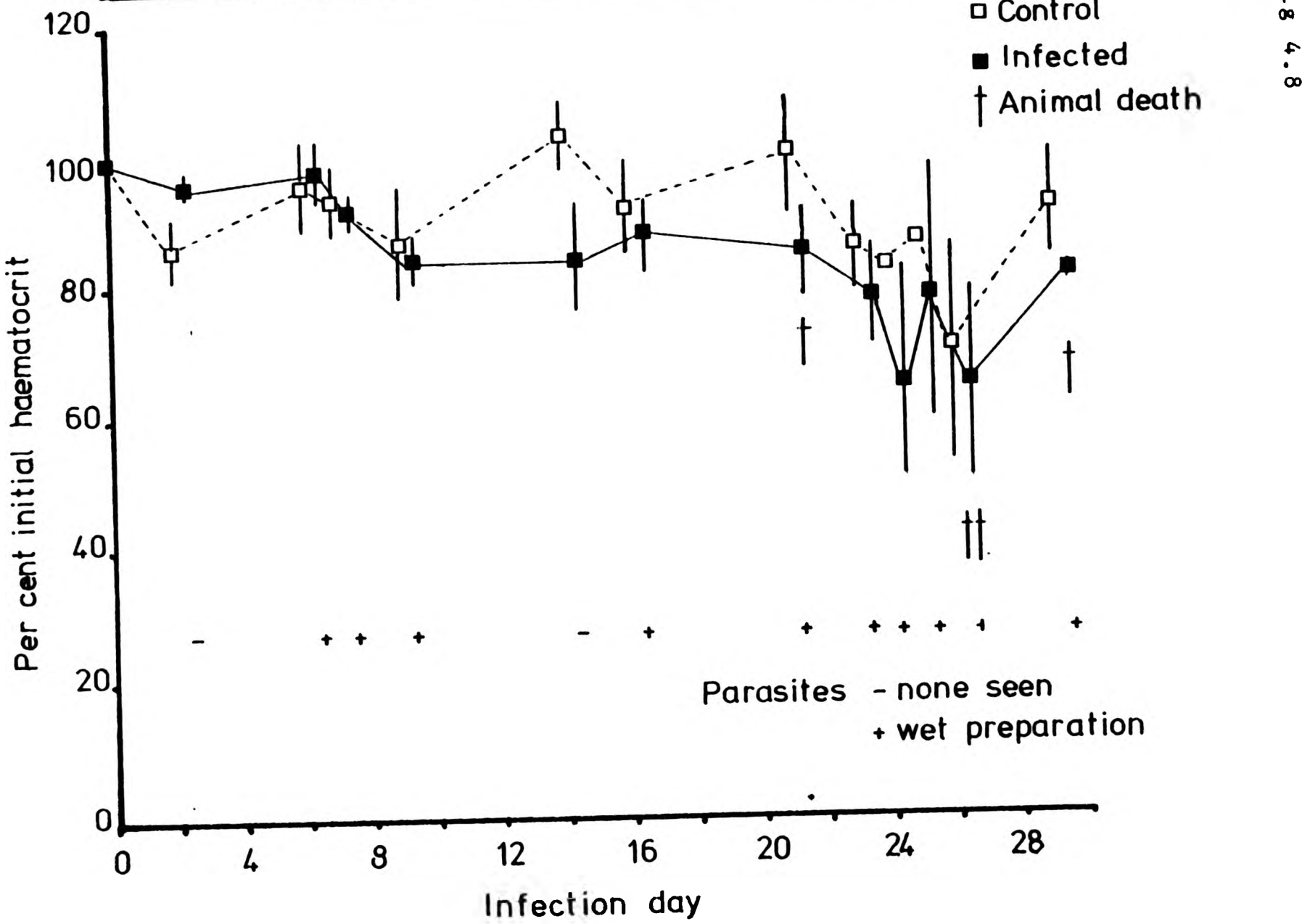


FIG 4.8

EXPERIMENT V - HAEMATOCRIT IN T B BRUCEI INFECTIONS (mean±sem)

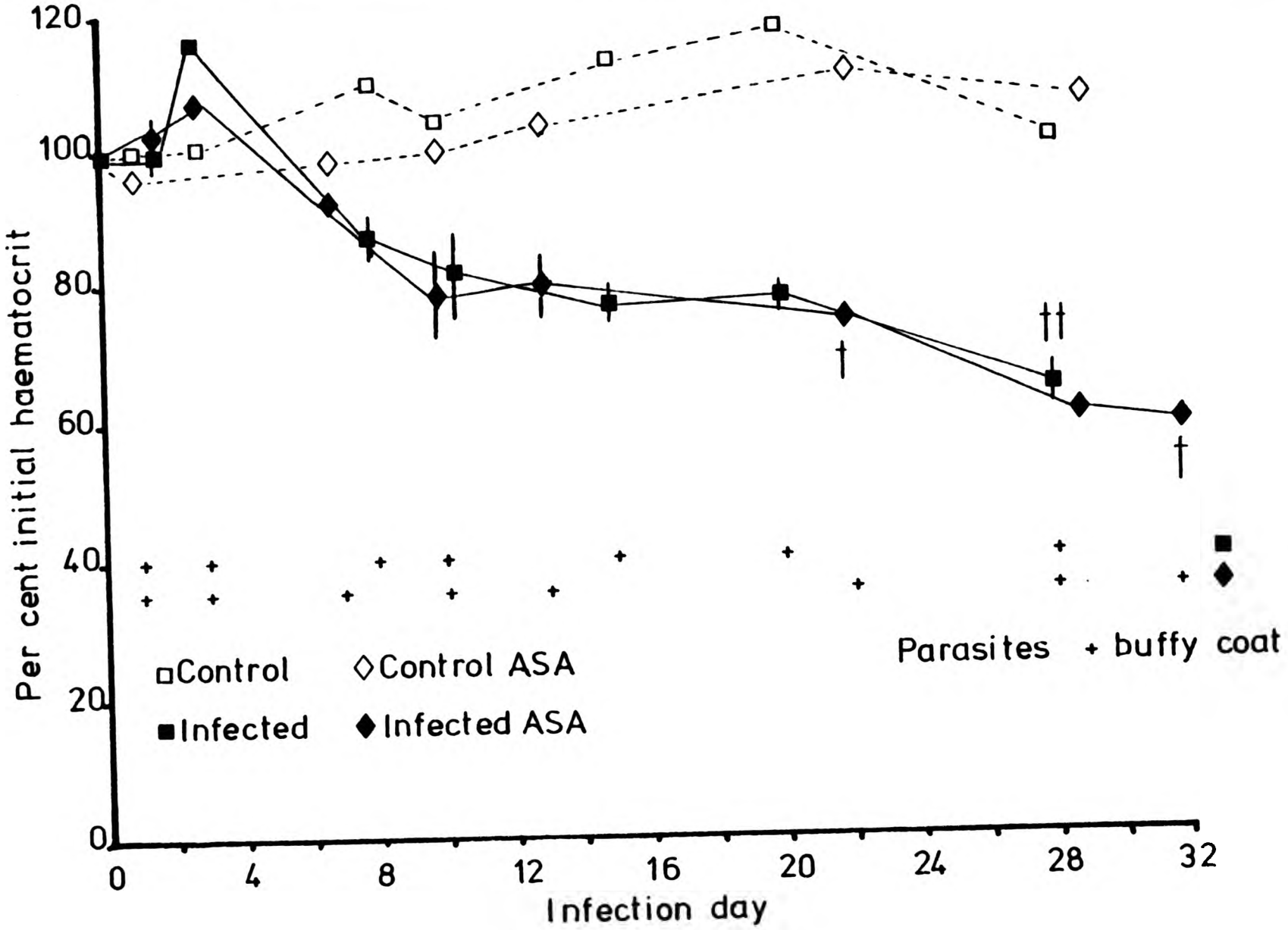


Fig 4.9

This would not, however, explain the variance seen in experiment III.

In all experiments the fall in haematocrit during the infection was significant when compared to control values (Table 4.2). In experiment V, where half the animals received ASA treatment this had no effect on the haematocrit levels either between controls (unpaired $t=1.04$, $df=13$, $p>0.05$) or between the infected animals (unpaired $t=0.25$, $df=15$, $p>0.05$) although there was a significant fall in haematocrit in both the ASA treated and untreated infected animals when compared to controls (Table 4.2).

Experiments II and VII each involved a group of splenectomised animals. In experiment II the haematocrit had only fallen to 91% by day 7 in the splenectomised animals (compared with 56% in the intact group) although by day 14 the levels were 70% and 76% respectively and the splenectomised animals did not survive more than a few days after that. Over the two groups, however, the haematocrit was found to be significantly lower in the intact animals compared with the splenectomised animals (mean difference 12.4%, paired-$t=3.69$, $df=15$, $p<0.01$). In experiment VII a similar pattern was seen with levels in the splenectomised group falling to 92% on day 7 (compared

Table 4.2

COMPARISON OF HAEMATOCRIT LEVELS IN T.B.BRUCEI INFECTED RABBITS AND CONTROL RABBITS

EXPERIMENT	TEST	MEAN DIFFERENCE	t	df	p
I	Paired-t	23.7%	10.17	19	<0.001
III	Paired-t	5.3%	2.12	12	<0.05
IV	Paired-t	27.9%	9.20	15	<0.001
V					
(untreated)	Unpaired-t	-	3.40	14	<0.01
(ASA treated)	Unpaired-t	-	2.41	14	<0.05

with 77% in the intact gtoup) and both groups falling to around 70% by day 21 with earlier deaths in the splenectomised group. Again the splenectomised group showed a higher overall haematocrit (mean difference 4.0%, paired $t=2.17$, $df=15$, $p<0.05$) but both groups showed significant falls in haematocrit during the infection when compared to controls (Kendall's S test: intact - $z = 3.31$, $p<0.001$; splenectomised - $z = 2.49$, $p<0.02$).

In summary, the falls in haematocrit that we observed were similar to those reported by other workers and is in the later stages unrelated to the appearance or level of parasites in the peripheral blood. The differences in the splenectomised rabbits were similar to those observed by McCrorie et al[67].

4.2.2 *T congolense* infections

T congolense, unlike *T b brucei*, is found mainly in plasma with a preference for the microcirculation and anaemia is the main pathogenic factor[7]. In infections of sheep and goats with this organism, the red cell count fell rapidly to one-third of its original value after the initial high parasitaemia[113] but MacKenzie et al[17] reported an acute episode in only 3/9 infected sheep, the rest showing a gradual fall in haemoglobin. Cattle with *T congolense* infections may

die suddenly from an acute episode but many show a prolonged chronic disease[84]. Generally the severity of the anaemia correlates with the onset and degree of parasitaemia[41] but is also influenced by the weight and breed of the animal[19]. In Zebu cattle the haematocrit falls by about 41% during weeks one to six of infection whereas the more trypanotolerant N'dama cattle show a fall of only 25% in this time[65]. Significant falls in haematocrit after a pre-patent period have been reported by many workers[10,25, 33,60,66,122]. The report by Valli[22] is typical of these, showing a prepatent period of three days with a fall in haematocrit from 32% to 22% on day 16 rising gradually thereafter and stabilising out at 28% over the 127 days of infection.

Holmes and Jennings[52] have reported the effect of T congolense infection in rabbits and showed a fall in haematocrit over the first two weeks of infection which was associated with the first detection of parasites.

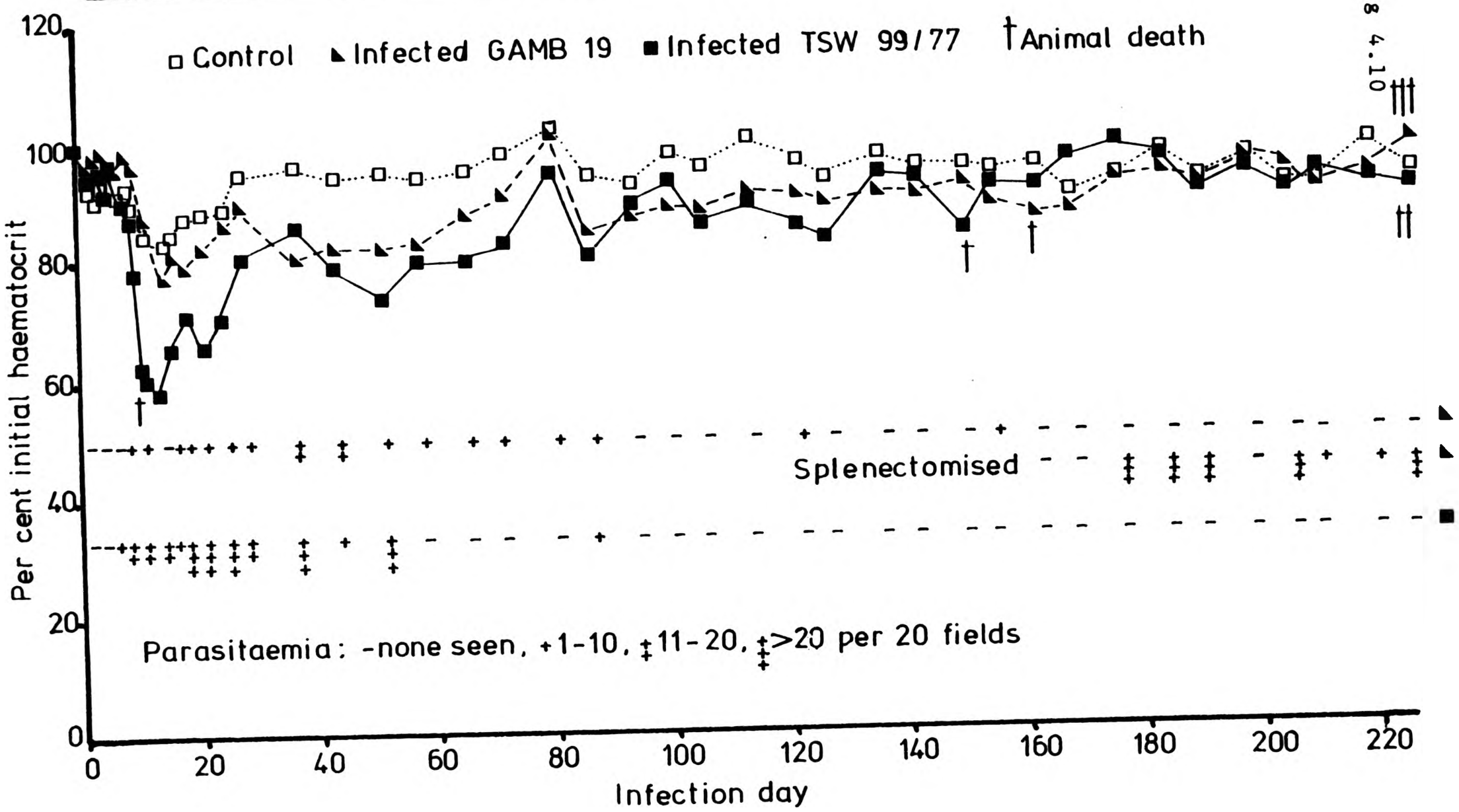
Our experiment involved infection of rabbits with two different strains of T congolense (GAMB 19 and TSW 99/77). In the GAMB 19 infection the haematocrit began to decline from day 10 (with parasites becoming evident first on day 8) but falling to a level of only 78% of starting values on day 14. After that the values rose

gradually over the 225 days of infection reaching levels that were virtually normal (Fig.4.10). The mean level was, however, significantly lower than mean control level (mean difference 3.2%, paired $t=3.42$, $df=43$, $p<0.001$). In the TSW 99/77 infection there was a much greater fall in haematocrit from day 8 to 59% of starting values on day 14 and this fall was associated with the higher initial parasitaemia seen in this group, compared with the GAMB 19 strain, from day 8. These levels too rose gradually and were similar to those in the GAMB 19 group from about day 81 although overall the level in the TSW 99/77 group was significantly lower (mean difference 4.2%, paired $t=3.20$, $df=43$, $p<0.01$) (Fig.4.10). Like the GAMB 19 group the TSW 99/77 group was also significantly lower than controls (mean difference 5.4%, paired $t=2.05$, $df=43$, $p<0.05$).

In summary, we have shown falls in haematocrit in T congolense infections associated with the first parasitaemia and a gradual recovery to normal levels as others have reported. It is obvious that the strain of organism is important too in the initial fall although after that it does not appear to be relevant. This emphasises the probable different mechanisms involved in the anaemia in the initial stage compared with the more chronic stage where the expanded mononuclear phagocytic system continues to remove red cells at a faster rate

HAEMATOCRIT IN T CONGOLENSE INFECTIONS

Fig 4.10



than normal, irrespective of parasite level, the earlier difference being caused by the greater ability of the TSW 99/77 strain to divide or avoid immune mechanisms, allowing larger numbers of trypanosomes to proliferate and resulting in a more rapid breakdown of red cells.

4.2.3 *T vivax* infections

T vivax is a common infection of livestock, particularly in West Africa, and is most virulent in ruminants[24]. Like *T congolense*, *T vivax* is mainly found in plasma but is evenly distributed throughout the circulation and produces cyclical waves of parasitaemia[7]. Of all the different species *T vivax* probably produces the highest parasitaemia[6] in livestock.

There are many reports of *T.vivax* infection in sheep, goats and cattle all of which show a significant fall in haematocrit associated with the onset of parasitaemia, although severity is not related to the parasitaemia.

In sheep the anaemia is relatively mild with the haematocrit falling by about 23-30%[55,56]. In goats three different patterns of infection are seen: acute, subacute or chronic[63], but all show a fall in haematocrit of around 50%, the time after infection that it takes to reach this level being dependent on which course the infection is following[123].

In cattle also the fall in haematocrit is associated with development of the parasitaemia within the first week after infection[58]. The haematocrit is significantly lower after day 12 and falls by about 30% in total[57]. Facer et al[37] have shown that the haematocrit level may be partly associated with immunological events; the detection of red cells sensitised with IgG or IgM is associated with lower packed cell volumes which recover slightly when the direct anti-globulin test becomes negative.

The T vivax infection carried out by us was in cattle also, haematocrit levels falling by between 32 and 47% over the first 12 days and rising gradually by a few percent after that. Initiation of the fall was associated with detection of parasites in the peripheral blood. In the second infection (undergone by three animals) the haematocrit fell by between 9 and 27% over the first 12 days although 2/3 began this infection about 25% down on their original pre-first infection levels. Only one animal underwent a third infection, again starting about 25% down on pre-infection levels. No fall in haematocrit was seen over the first 12 days although as soon as significant numbers of parasites were detected (day 12) the fall was precipitated and continued to show a reduction with an eventual drop of 37% by day 62 of this infection, similar to the greatest

drops shown over shorter periods of time in the second infection. The individual results are shown in figure 4.11.

The fall in haematocrit associated with parasitaemia reported here was thus similar to that reported by others. The previous exposure to infection in 3/4 animals appeared only to delay the onset of parasitaemia and thus the amount of fall in haematocrit in a similar period of time after infection. The haematocrit in all calves, however, fell eventually by similar amounts suggesting that the initial stage in the anaemia was related to the parasitaemia, which may have been moderated initially by previous exposure and that later the mechanism might have become independent of parasite presence. It was not possible to tell whether this was the case in these animals because sporadic parasitaemia was seen throughout and the animal undergoing his third infection also showed the highest parasitaemia throughout even though the onset was delayed.

4.3 PLATELET COUNTS (Appendix II)

4.3.1 T b brucei infections

Thrombocytopenia has been reported in infections with many different trypanosome species and T b brucei is no exception. An experimental infection in rats[87]

HAEMATOCRIT IN T VIVAX EXPERIMENTS

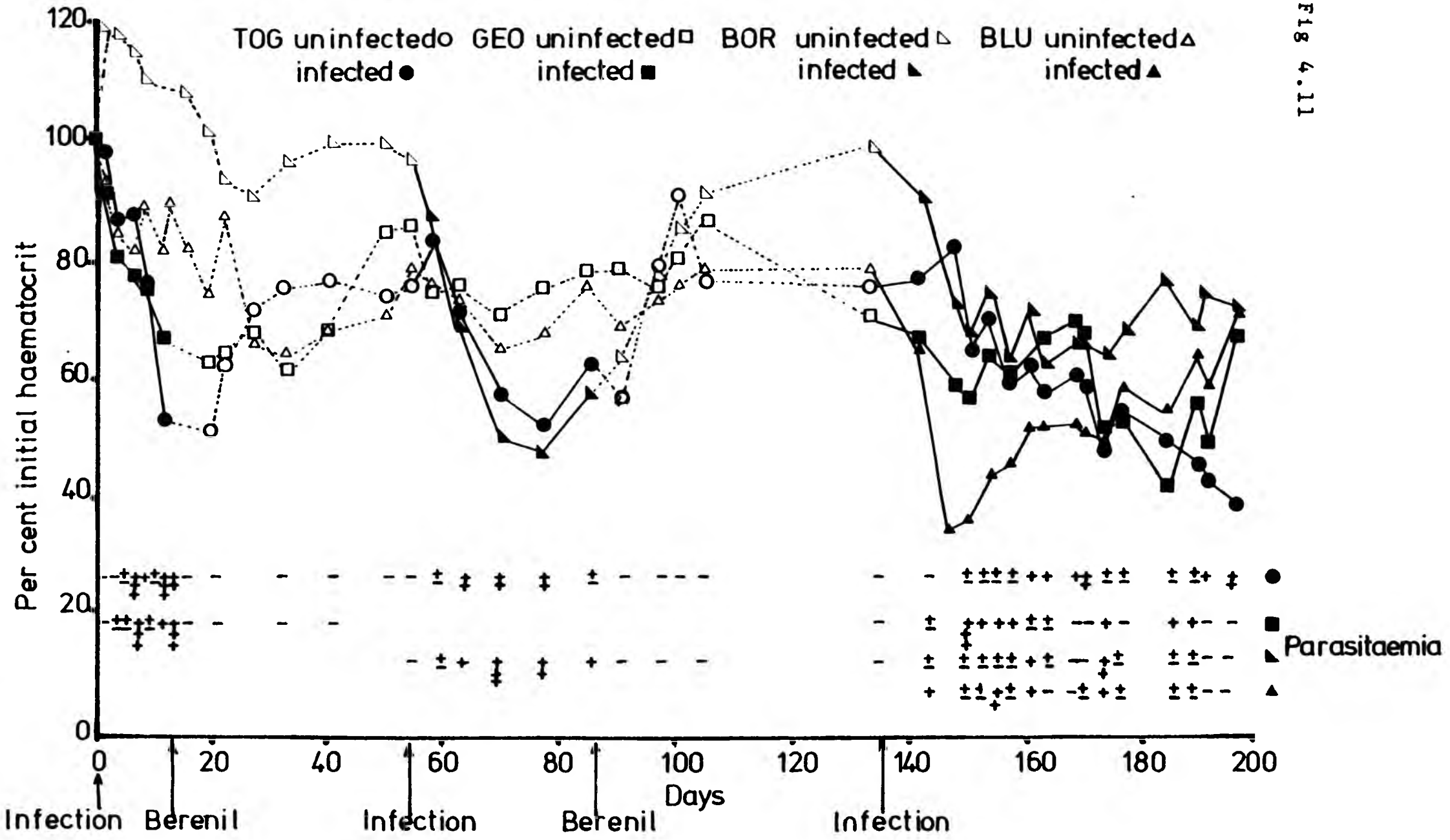


Fig 4.11

showed a 78% reduction in platelet number at the first peak of parasitaemia, the relative severity being related to the height of parasitaemia within individuals. Davis[87] also showed a 69% reduction in goats and sheep. Essien[124] however reported an increase in platelet numbers during infection of 2 horses and 2 goats. However, since all three animals appeared to be thrombocytopenic before infection having counts of 23, 40, 87 and 106 x 10^9/L, there may be some doubt as to the importance of this observation. Davis[87] has also shown a 79% reduction in platelet number in cattle infected with this organism. Rabbits became thrombocytopenic[74].

The series of experiments described here involving T b brucei infections of rabbits began using conventional phase contrast chamber counting of platelet number (see Methods 3.7.1.1). Experiment I showed a significant reduction in number throughout the infection reaching a mean level of 19% on day 27 (mean difference 54.9%, paired $t=11.34$, $df=19$, $p<0.001$), compared with an uninfected control animal whose platelet count varied between 92% and 138% of his original starting value (Fig.4.12). The platelet count in 2/3 animals appeared to have fallen substantially on day 1 of the infection. Experiment II, which compared splenectomised and intact rabbits, showed a similar marked reduction in platelet

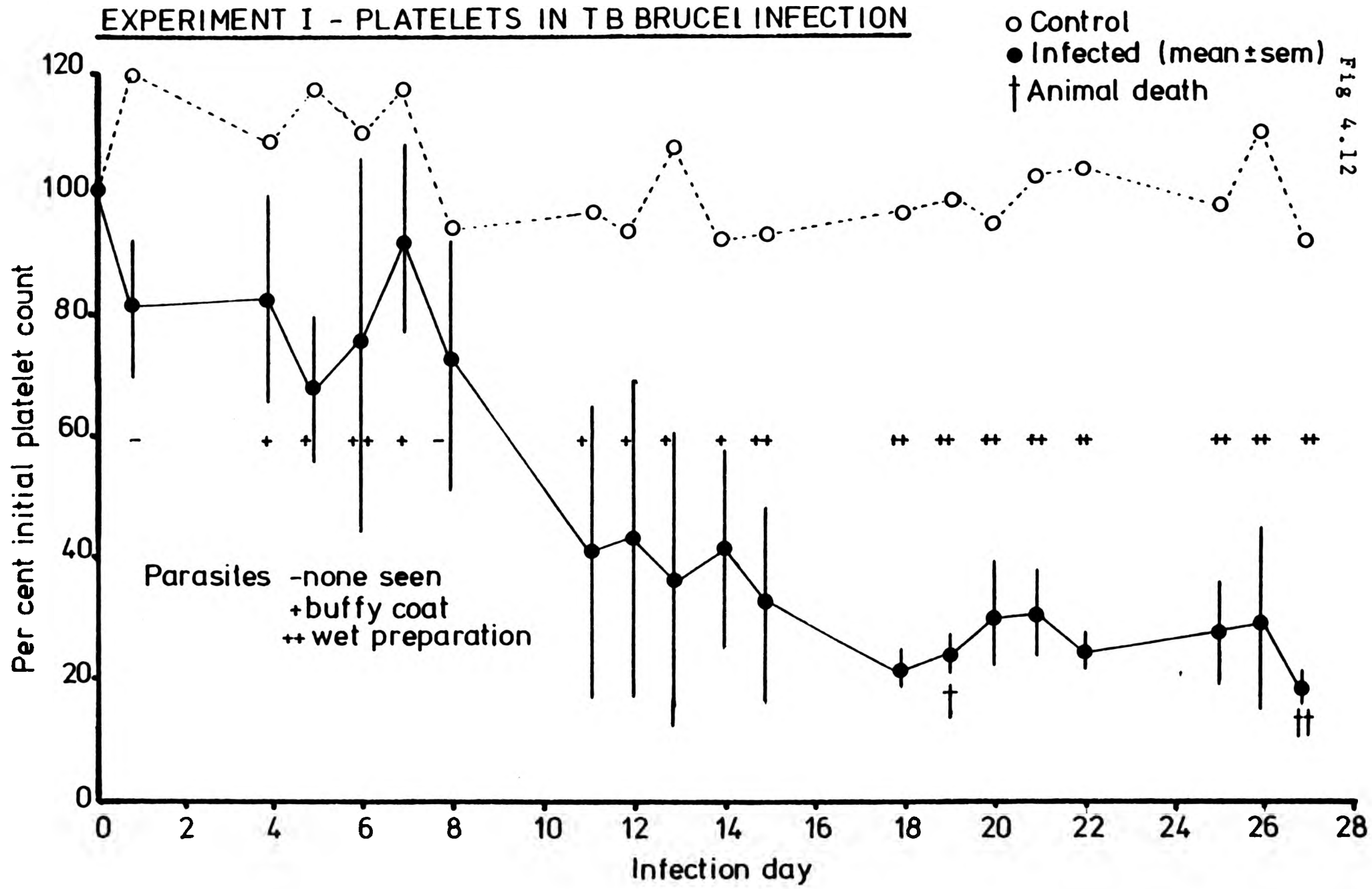

EXPERIMENT I - PLATELETS IN T B BRUCEI INFECTION
o Control
● Infected (mean ± sem)
† Animal death
Per cent initial platelet count
120
100
80
60
40
20
0
Parasites -none seen
+buffy coat
++wet preparation
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Infection day

Fig 4.12

number with levels falling immediately and well before demonstration of parasites. There was no difference between the intact and splenectomised group (paired t=0.06, df=15, p>0.05) (Fig.4.13).

A main complication of visual counting in these two experiments was the presence of clumps of platelets in the counting chamber. This has been reported also by Davis in his series of infections[87]. The size of clumps varied enormously and it was never possible to accurately determine the number of platelets within each clump and estimates only could be obtained. The question arose as to whether the numbers of cells within the clumps should be counted as contributing to the platelet count since they might have formed in vitro, whether each clump should be counted as a single platelet, or whether they should be ignored in the count, with the assumption that they were haemostatically inactive. The latter method was chosen for these counts but a decision was made to investigate this clumping further and another method of platelet counting investigated (Method 3.7.1.2). By using electronic counting it was hoped that precision would be improved and that while some small clumps might be counted as individual platelets, the larger clumps would be excluded at a specific size. It is of course possible that clumps of two or three platelets were

EXPERIMENT II - PLATELETS IN T B BRUCEI INFECTIONS (mean ± sem)

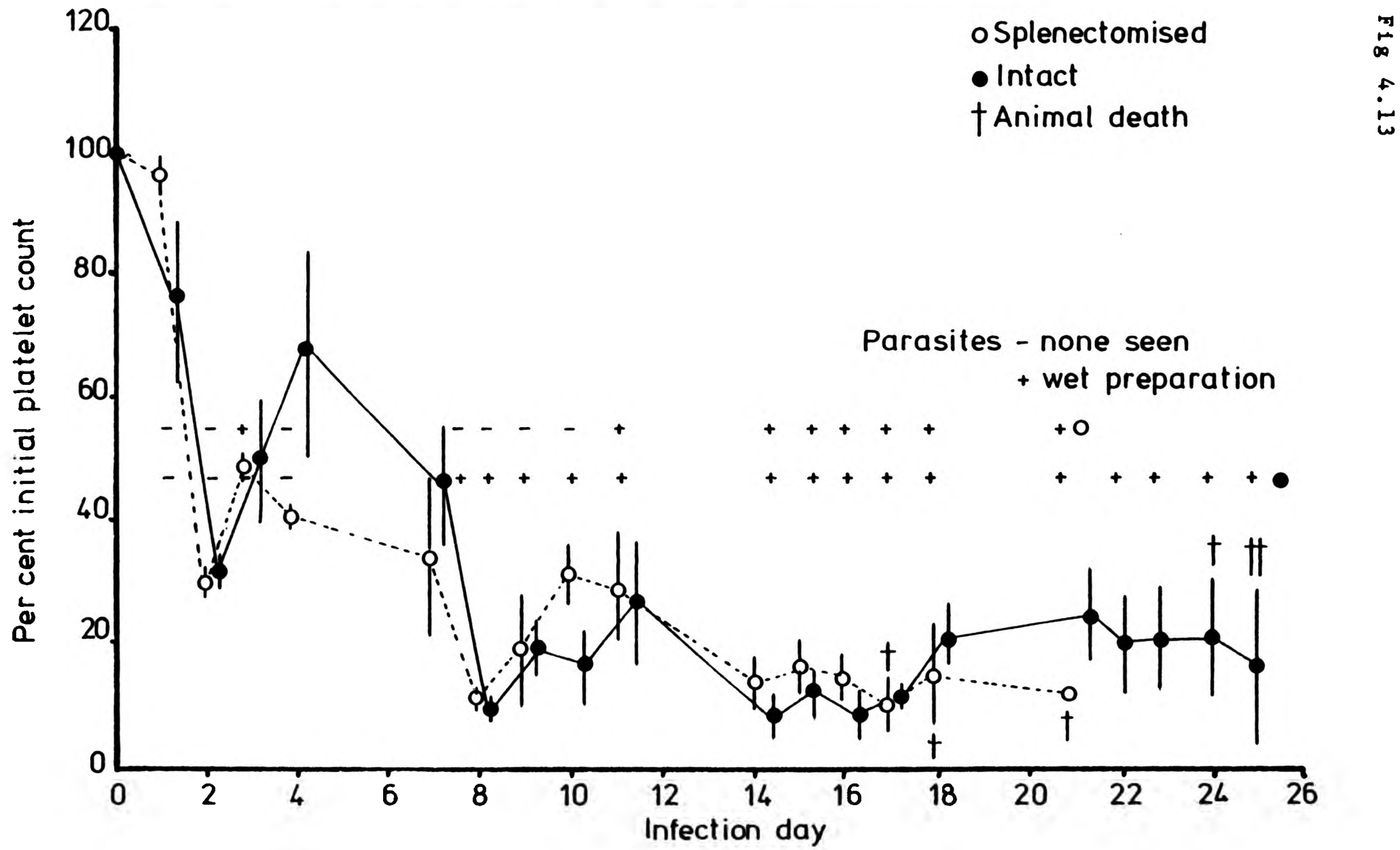


Fig 4.13

composed of only temporarily adherent elements or that they were haemostatically quite active and it removed the difficult decisions about what one should do when the number of small clumps were great compared with the individual platelet number. The clumping was not seen in the control animal of experiment I, nor in a series of uninfected rabbits examined in relation to this problem and it is not clear what others have done in these circumstances. It did appear though that electronic counting produced slightly higher platelet counts as we would have expected. Forsberg et al[84], although not mentioning any problems with clumping, reported 20% lower cattle platelet counts electronically compared with visual counts, but she offered no explanation as to the possible cause. They chose to use electronic counting for greater convenience.

Experiment III was therefore performed using electronic counting methods. The reduction in number was again immediate, although an earlier parasitaemia was seen here but not as marked as the previous two experiments. A low level of 17% was reached on one day late in the infection and one animal appeared to die early (URI, day 16) from a marked thrombocytopenia (11%) with little fall in haematocrit and no detectable parasitaemia. The infected group platelet counts were significantly lower than those from the control group (mean difference

29.7%, paired t=4.15, df=10. p<0.001) (Fig.4.14).

A more extensive study (Experiment IV) was done involving three control and six infected animals; two of the latter died early, one (LUP, day 7) with a marked thrombocytopenia after a massive parasitaemia. Although the platelet counts in the surviving animals fell within the first week to around 50%, associated with the first parasitaemia peak, they rose again to levels higher than the controls and remained normal for the remainder of the infection despite further significant parasitaemias. The picture was confused by a reduction in platelet number seen also in the control group in the middle section of the experiment and so overall there was no significant difference shown between the groups (mean difference 0.6%, t=0.09, df=15, p>0.05) although the suggestion of an early thrombocytopenia remains (Fig.4.15).

Animals with trypanosomiasis suffer from periodic fevers associated with their parasitaemias and there has been some suggestion that fever is associated with the pathogenesis of the anaemia by causing increased red cell fragility[10]. Since a fall in serotonin level is also associated with the fever (and parasitaemia)[48] the latter too may be involved in the platelet pathogenesis. Goodwin stated in 1974[7]:

EXPERIMENT III - PLATELETS IN T B BRUCEI INFECTION (mean±sem)

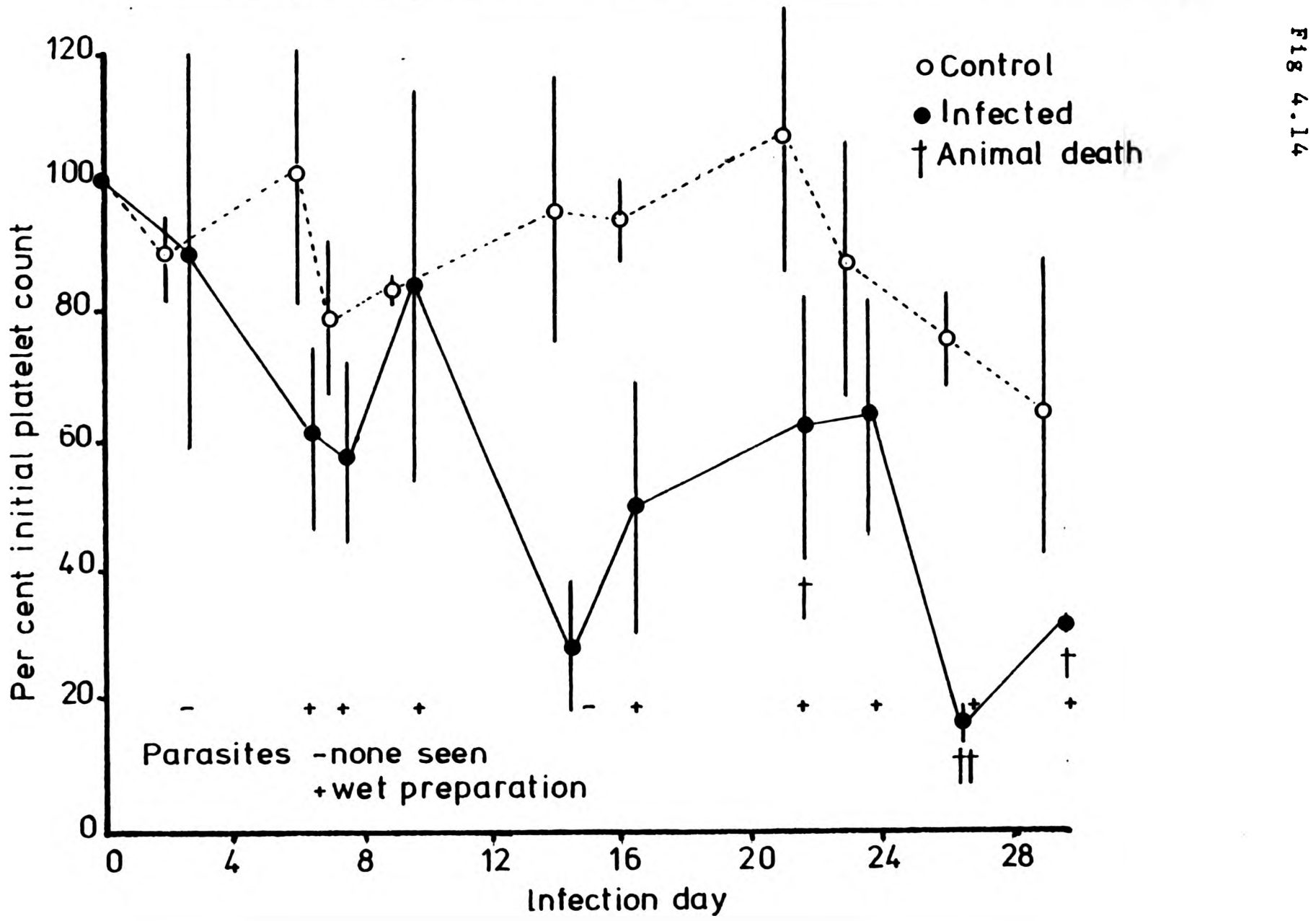


Fig 4.14

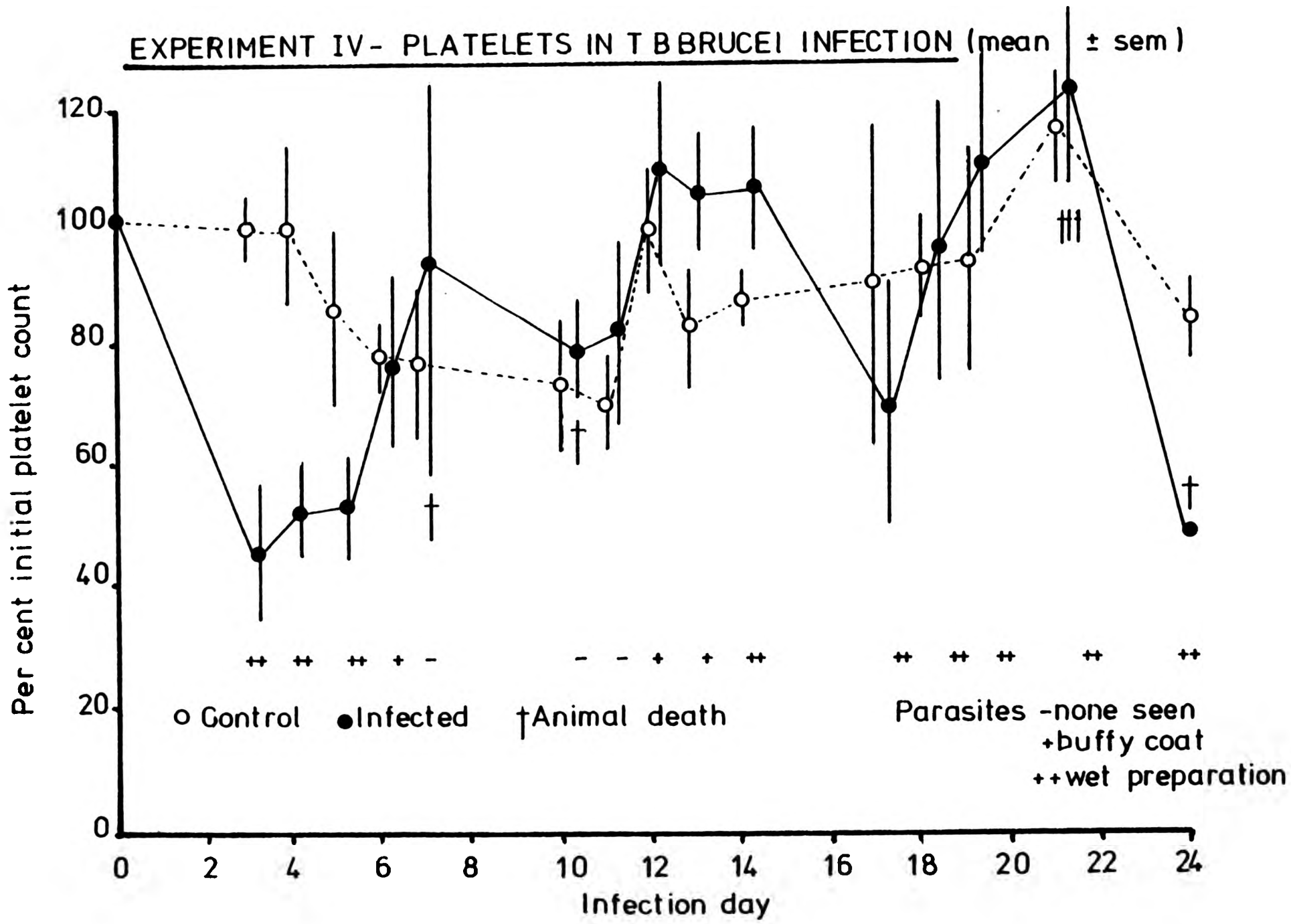

EXPERIMENT IV- PLATELETS IN T B BRUCEI INFECTION (mean ± sem)
Per cent initial platelet count
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20 22 24
Infection day
o Control ●Infected †Animal death
Parasites -none seen
+buffy coat
++wet preparation

Fig 4.15

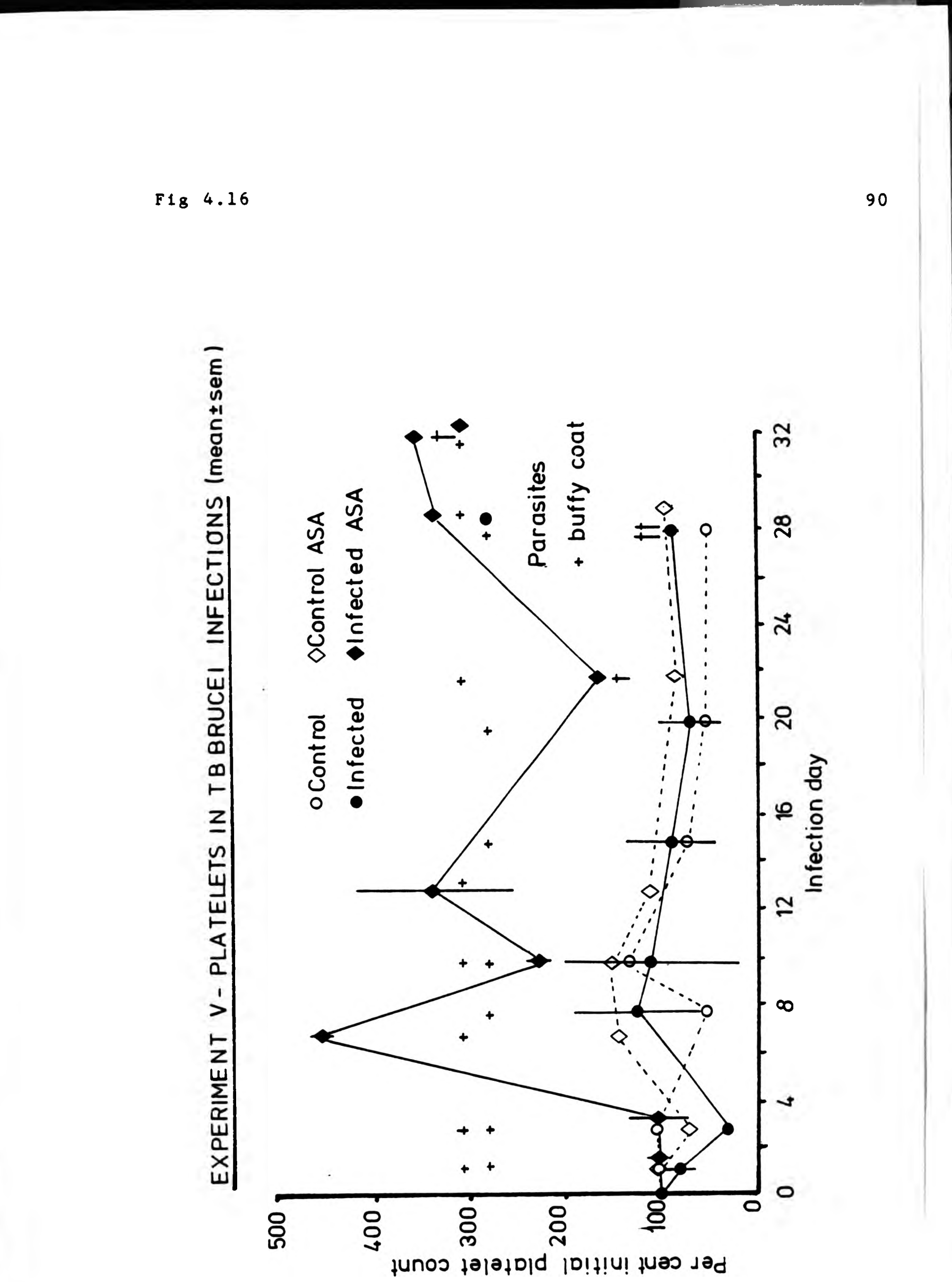
"Anti-inflamatory drugs, given with the purpose of damping down the catastrophic effects of successive allergic reactions, would be expected to benefit the patient" and would of course reduce the fever. The non-steroidal anti-inflamatory drugs have potent effects on platelets also, irreversibly blocking the cyclo-oxygenase enzyme which converts arachidonic acid to the potent aggregating prostaglandin derivative thromboxane A_2[125]. While other mechanisms exist which can bring about platelet activation (and possible aggregation) *in vivo* , it was thought useful to attempt to block prostaglandin synthesis in the platelets of a group of animals with aspirin to assess its effect. A dose of 300mg. was given orally once a day. This dose has been shown to completely inhibit *in vitro* platelet aggregation induced by 1mM sodium arachidonate in rabbits[126]. There was no significant difference in platelet count between the treated and untreated control animals (unpaired $t=1.66$, $df=14$, $p>0.05$) although there was great variability within the animals. This variability was also seen in the untreated infected rabbits and only one showed a significant thrombocytopenia. Thus overall the untreated infected group were not significantly different from their control group (unpaired $t=0.52$, $df=14$, $p>0.05$). In contrast with other infections the aspirin treated group showed a marked increase in platelet count during the

infection which was both significantly higher from its control (unpaired $t=2.78$, $df=15$, $p<0.01$) and from the untreated infected group (unpaired $t=3.17$, $df=15$, $p<0.01$) (Fig.4.16). Visual examination of blood films from these infected animals showed the clumping typical of all infections which did not appear decreased despite the increased platelet counts. Although the thrombocytopenia had apparently been prevented by aspirin treatment the course of the disease remained similar in both groups with deaths on days 21 and 32 compared with days 28 and 34 in the untreated group.

In experiment VI platelet counts were again compared in infected and control animals and trypanosomes quantitated. An early thrombocytopenia, although only to 60% on day 4, was followed by recovery and a terminal thrombocytopenia to 41% (Fig.4.17). Marked falls in platelet number in individual animals appeared to coincide with higher parasite numbers and one animal (GRE) died on day 12 after a persistently high parasitaemia and thrombocytopenia, platelet counts falling as low as 6%. The platelet count was significantly reduced in the infected group (Kendall's S test, $z=3.40$, $p<0.001$).

Experiments VII and VIII used an automated method of counting platelets in whole blood (Methods 3.7.1.3). It

Fig 4.16


EXPERIMENT V - PLATELETS IN T B BRUCEI INFECTIONS (mean±sem)
○Control ◇Control ASA
●Infected ◆Infected ASA
Parasites
+ buffy coat
Per cent initial platelet count
500
400
300
200
100
0
0
4
8
12
16
20
24
28
32
Infection day

EXPERIMENT VI- PLATELETS AND TRYPANOSOMES IN T B BRUCEI INFECTION

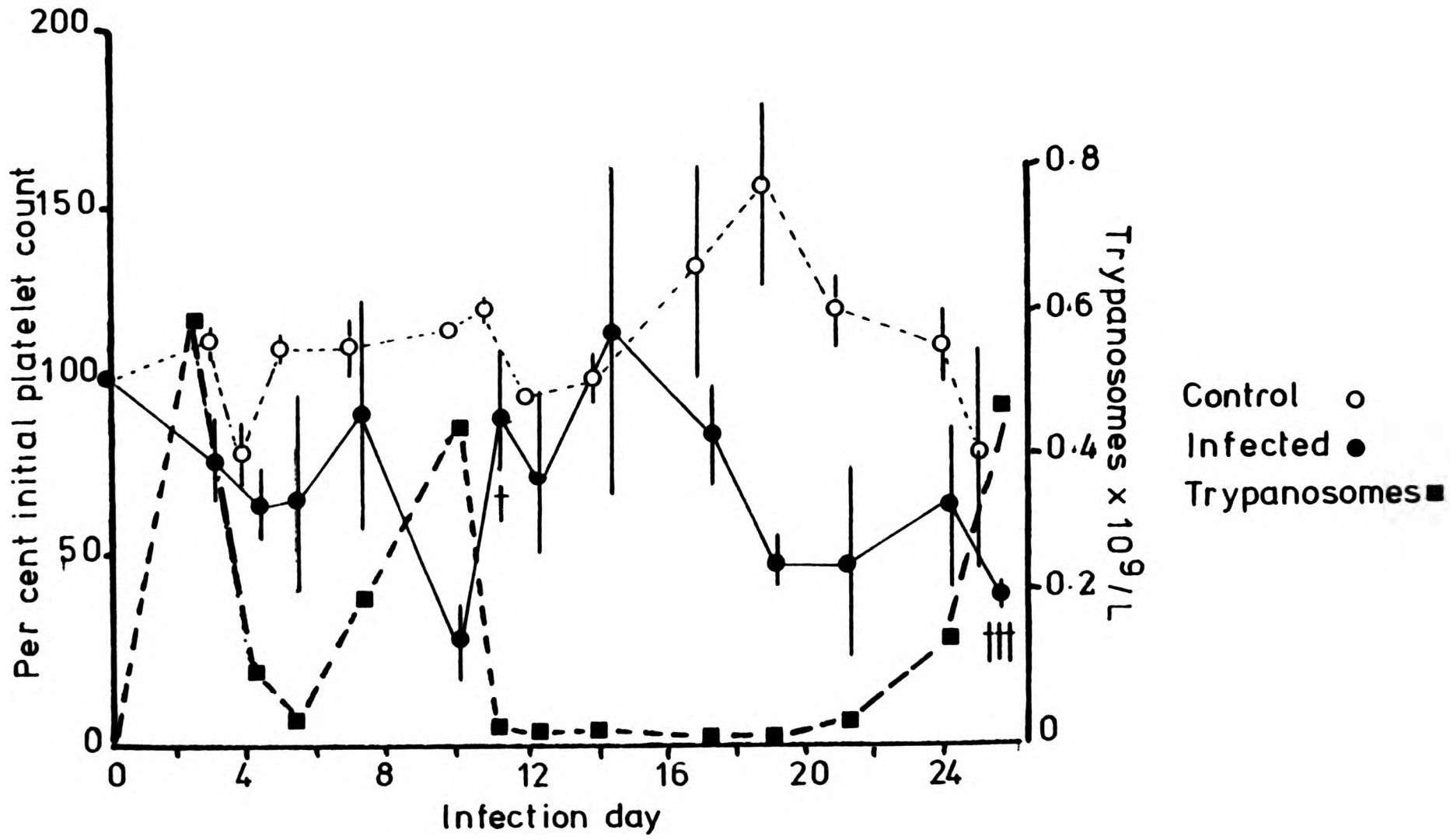


Fig 4.17

was hoped that variability observed in platelet counts of control animals, which may have been related to preparation of dilutions in the previous method, would thus be reduced. Experiment VII looked at counts in groups of intact and splenectomised animals before and during an infection (Fig.4.18). Both groups showed significant falls in platelet number when compared with the same animals followed for 10 days before infection (Kendall's S test: intact - $z=2.87$, $p<0.01$; splenectomised - $z=2.46$, $p<0.02$). There was no significant difference between the intact and splenectomised groups during infection (mean difference=17.8%, paired $t=1.23$, $df=15$, $p>0.05$) although there was a considerable variance in counts within each group on any one day. The splenectomised animals all died earlier or had to be killed sooner than the non-splenectomised animals. CHE, a splenectomised animal, died on day 8 of infection after a marked thrombocytopenia with platelet levels falling as low as 5% on day 5. Experiment VIII showed a marked reduction in platelet count with levels of 40% and 16% on days 2 and 9 respectively associated with the demonstration of parasites in the peripheral blood (fig 4.19). Platelet levels recovered in 5/6 animals despite continued trypanosome presence, two animals having particularly high platelet counts (although these two had lower trypanosome levels in the terminal stages). The other

EXPERIMENT VII - PLATELETS IN T B BRUCEI INFECTIONS (mean ± sem)

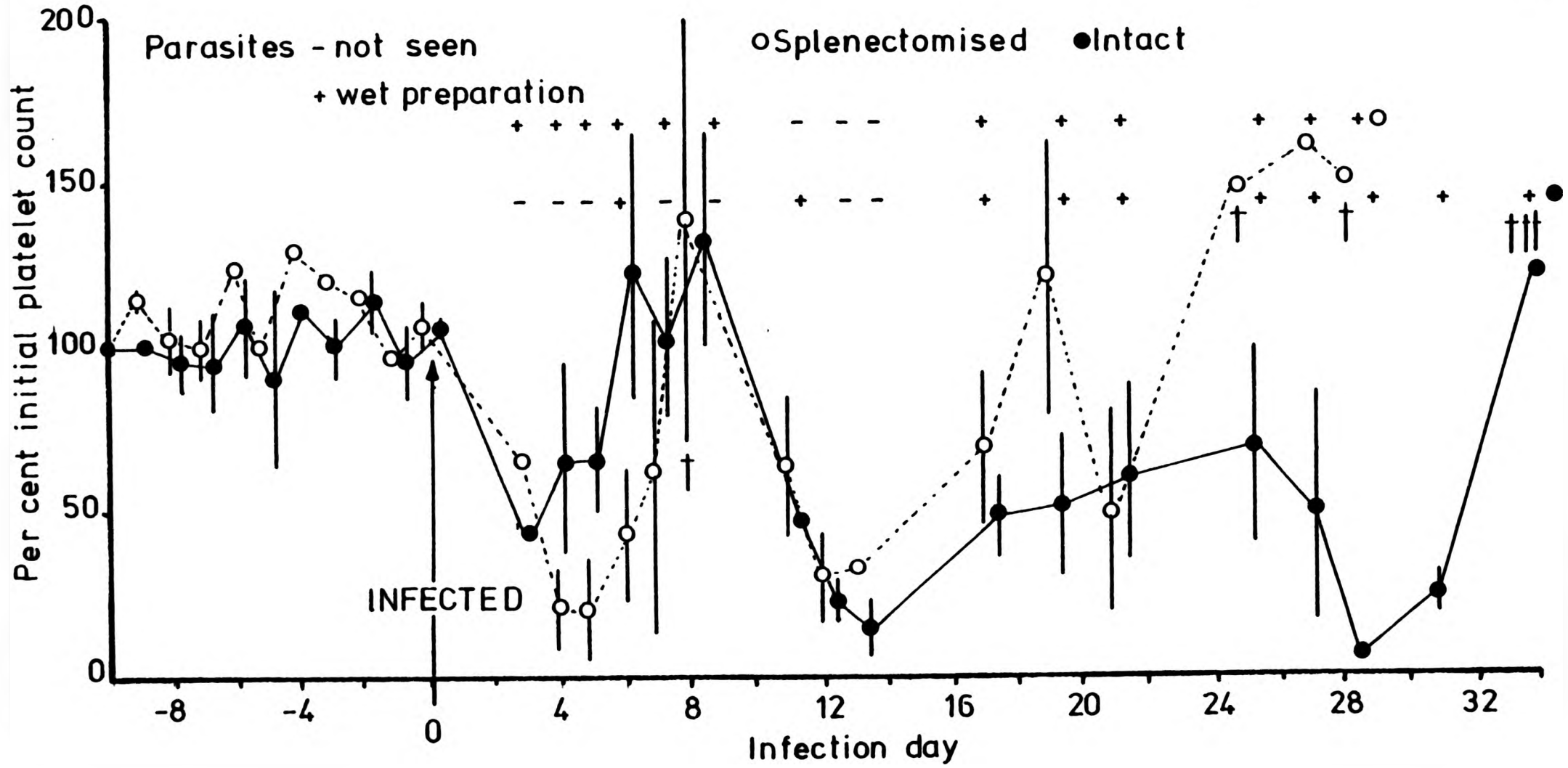


Fig 4.18

EXPERIMENT VIII - PLATELETS IN T B BRUCEI INFECTION (mean± sem)

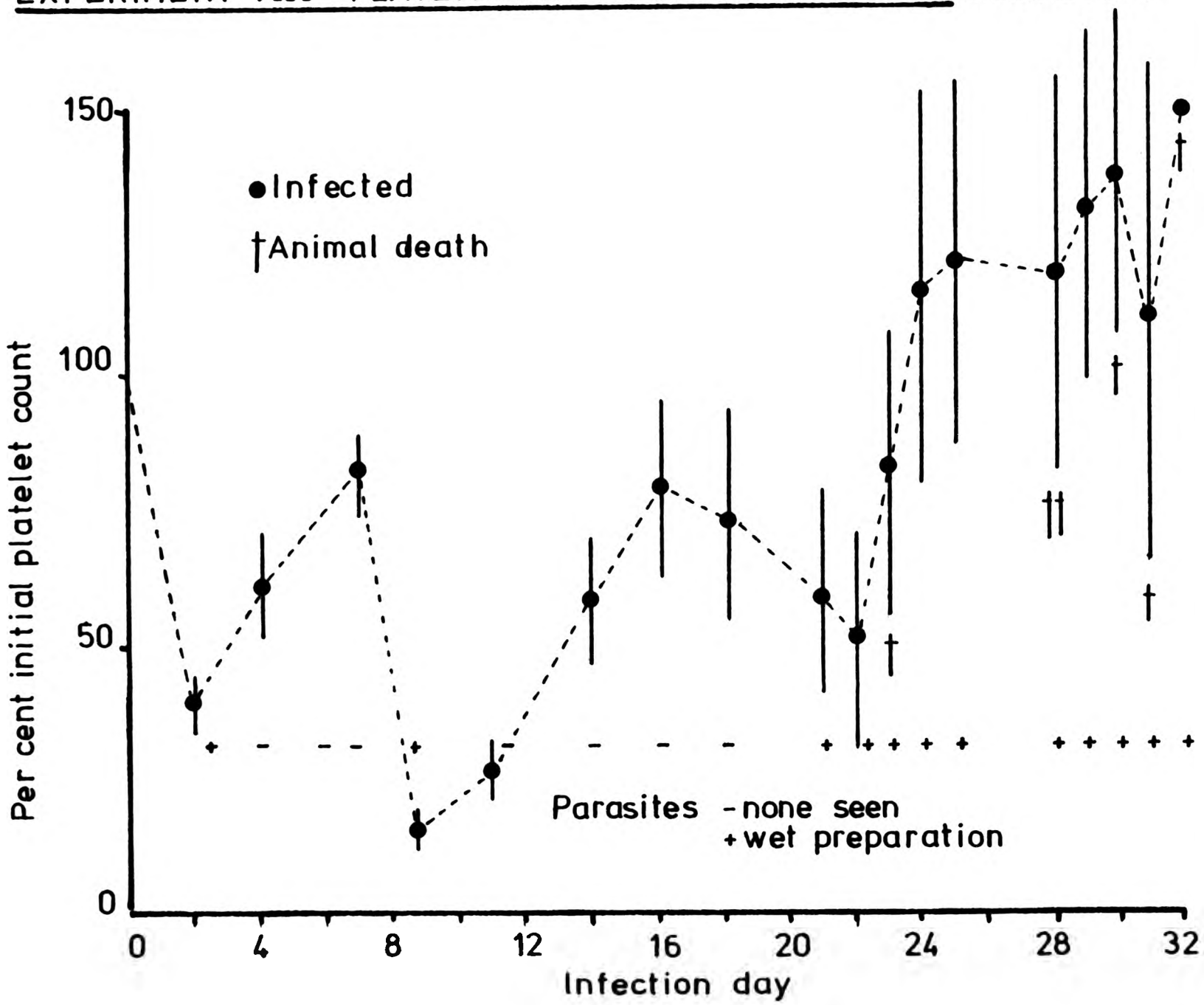


Fig 4.19

surviving to day 28 had been severely thrombocytopenic and parasitaemic from day 21.

4.3.2 T congolense infection

Thrombocytopenia has been reported in several studies involving infection with T congolense. Davis[87] has shown reductions in platelet number of between 70 and 86% in experimental infections in rats, sheep and cattle. Maxie et al[57] reported a reduction of platelets by 60% within one week of infection in calves, levels remaining low for the remainder of the infection. A 50% reduction was reported by Forsberg et al[84] in calves. Wellde and co-workers[86] found a thrombocytopenia in cattle when parasites first appeared which was most severe with high parasitaemia whereas Preston et al[85] later reported an evident thrombocytopenia before trypanosomes were detected. A severe thrombocytopenia with a rapidly fatal clinical course has also been seen in an experimental infection of dogs[76].

Rabbits infected with T congolense GAMB 19 showed a significant fall by about 20% in platelet numbers from day 3 before parasites were detected on day 8 and numbers continued to fall to around 35% on day 25. These levels were maintained with parasitic presence and rose marginally from day 90 when parasites were detected

only sporadically and three animals ended their infections with percentage reductions of 75%, 60% and 18% respectively. The fourth animal (HAN) which was moderately thrombocytopenic at the time, was splenectomised on day 162 of the infection. Over the next 63 days there followed a marked parasitaemia with no change in haematocrit but a rapid return of platelet count to normal. This is in contrast to other workers who have always reported a high association between parasite and platelet numbers. The mean reduction in count was significant when compared with controls (mean difference=42.9%, $t=13.3$, $df=43$, $p<0.001$) (Fig. 4.20).

In the TSW 99/77 infection the thrombocytopenia was more marked and associated with a higher parasitaemia in these animals (Fig.4.20). One animal (NIE) died on day 9 with a rapid fall from day 2 in platelet numbers to 59% of starting values. No parasites had been detected in this animal. The remainder of counts fell to a level of 10% on day 21 with a gradual rise over the remainder of the infection to a mean of 80% of starting levels. This does not mean to say that the situation in this infection was significantly better than in the GAMB 19 infection at the end of the experiment - the mean of 80% was composed of only two animals, one of which had attained normal platelet levels but the other of which remained thrombocytopenic at a level similar to the GAMB

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PLATELETS IN T CONGOLENSE INFECTIONS

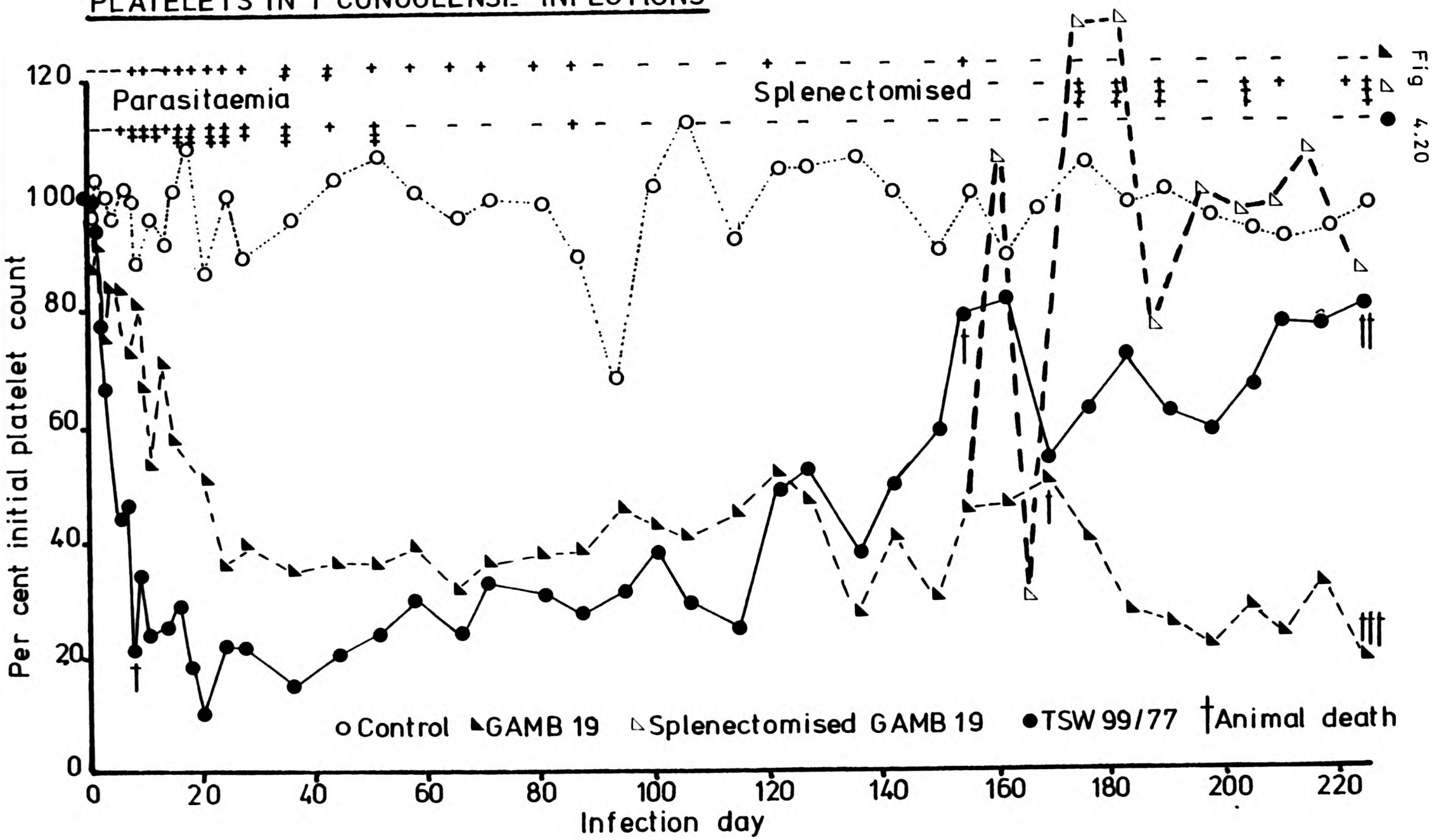


Fig 4.20

19 infection.

One animal (PUC), as in the GAMB 19 infection, was splenectomised but died during the operation. The spleens were considerably enlarged in both the animals and probably death was due to hypovolaemic shock on splenic removal. Because only two animals were left in this series, this animal was not replaced.

In summary, like others have found, a thrombocytopenia is seen in experimental T congolense infections and in the early stages the severity appears to be directly related to the height of parasitaemia (except for NIE). Later though the presence of large numbers of parasites in the peripheral blood of the splenectomised animal apparently had little effect on platelet number suggesting a different mechanism for the continuing thrombocytopenia of the chronic stages.

4.3.3 T vivax infections

Marked thrombocytopenia has been reported in infections of rats[87], goats[18,48] and cattle[31,66,77,87], the reports associating development of the thrombocytopenia with parasitaemia. This was also the case in our series of infection in four calves (Fig.4.21).

The first infections of all calves showed a rapid

PLATELETS IN T VIVAX EXPERIMENTS

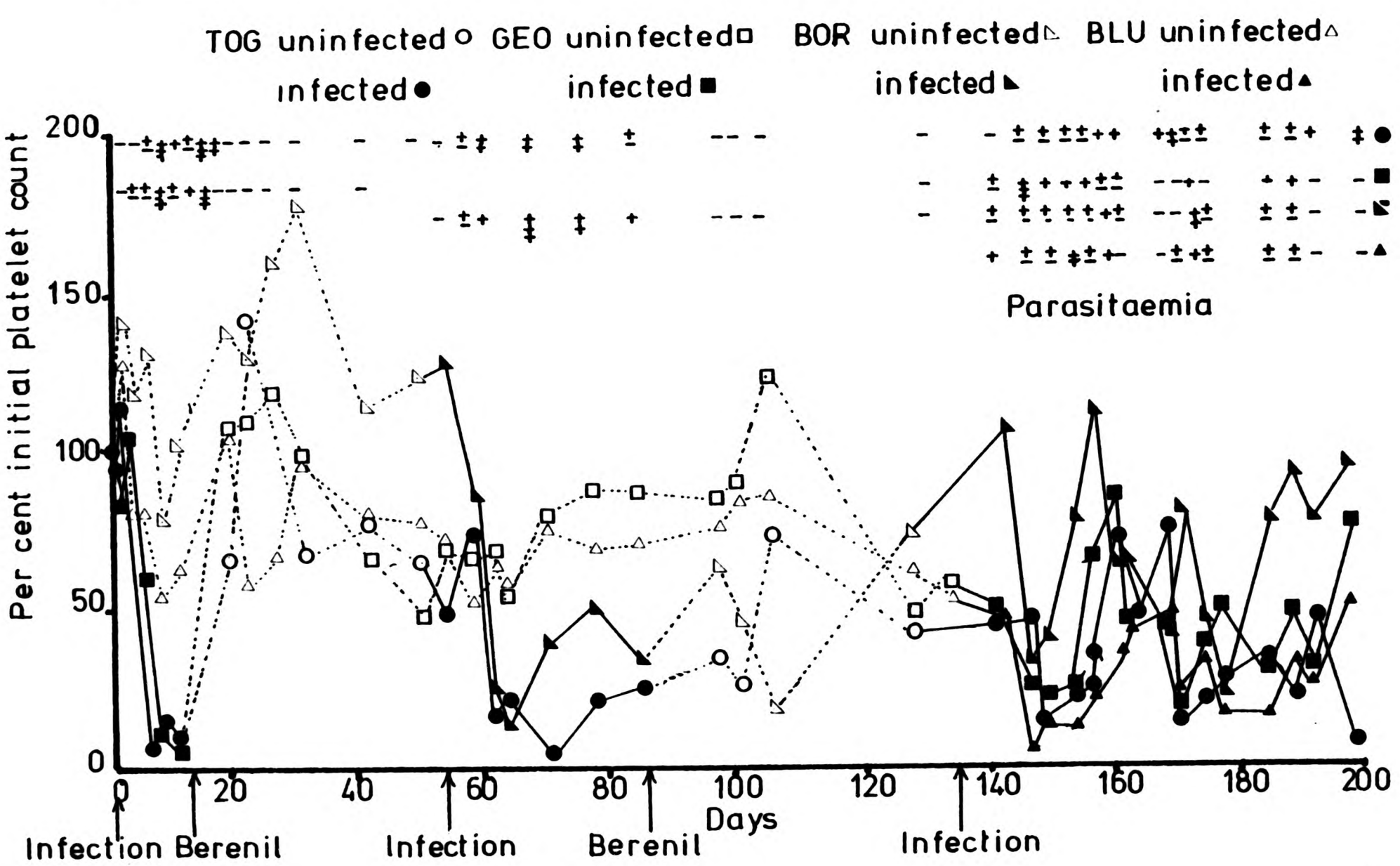


Fig 4.21

thrombocytopenia with levels of 6%, 6%, 7% and 17% on days 12, 12, 12 and 9 respectively, the levels not falling until after parasites had been detected. Two infections (GEO, TOG) were terminated at this time with Berenil, platelet numbers returning to normal or near normal. One infection, (BOR), allowed to continue showed a rise in platelet number up to 50% over 31 days which then rose to normal on Berenil treatment. The fourth (BLU) was followed for 62 days and platelets rose to pre-infection levels by this time.

In the second infection, one count (TOG) dropped to 5% from pre-second infection levels by day 16, rising again to 50% levels over 31 days and returning to 70% after Berenil - a similar course to the first infection. The other two calves showed less marked thrombocytopenia in their second infection (low levels of 33% and 42% on day 12) with levels fluctuating enormously over the rest of the infection, counts sometimes being relatively low and at other times greater than pre-second infection levels but never reaching the early levels. TOG was the only animal to undergo a third infection and there had been no fall in platelet count by day 12 although a 26% pre-third infection level was reached by day 15 after development of the parasitaemia. Again there was a fluctuating platelet count through the rest of the infection with a fluctuating parasitaemia.

Like other workers we have found a thrombocytopenia associated with development of the parasitaemia. Further challenges with the organism appeared to ammeliorate the thrombocytopenia although not to the extent reported by Wellde et al[127] in his series of infections with T congolense in calves, in which 'immunes' showed a fall in platelet number to 89% compared with a fall to 23% in the control infection animals.

4.4 PLATELET SIZE (Appendix II)

One of the main impressions on examination of stained blood films to look at platelet morphology during the infection was the appearance of giant platelets. Giant platelets are well recognised as a sign of underlying stress in platelet production. Although there is much confusion about whether 'larger' platelets are intrinsically 'young' platelets[128,129] it appears to be accepted that thrombocytopenic stress results in the production of megakaryocytes with increased cytoplasmic volume[130,131] and the normal production of platelets from megakaryocytes by physical fragmentation mechanisms will produce cells with a larger mean volume[93]. The number of megakaryocytes also correlates with the percentage production of large platelets and will increase in disorders of increased peripheral

destruction, including hypersplenism[132].

Bone marrow hyperplasia has been reported in cattle in both T congolense[61] and T vivax[31] and the hyperplasia reported by MacKenzie[17] in sheep with T congolense was said to involve 'all forms'. An increase in megakaryocytes in particular has been reported in T vivax in goats[123] and T congolense infections in calves[84]. In the latter infection the megakaryocytes were said to be larger than normal in both nuclear and cytoplasmic size but only the nuclear increase was significant[23]. Jenkins[64] reported an increase in megakaryocyte number rising to a maximum at 18 days in T brucei infections of rabbits.

Two methods of looking at platelet size have been used. First an electronic measurement of mean platelet volume (MPV) and platelet distribution width (PDW) in two series of T.b.brucei infection in rabbits and second a derived measurement from electromicrographs has been made in rabbit T b brucei infections and calf T vivax infections (Section 4.6).

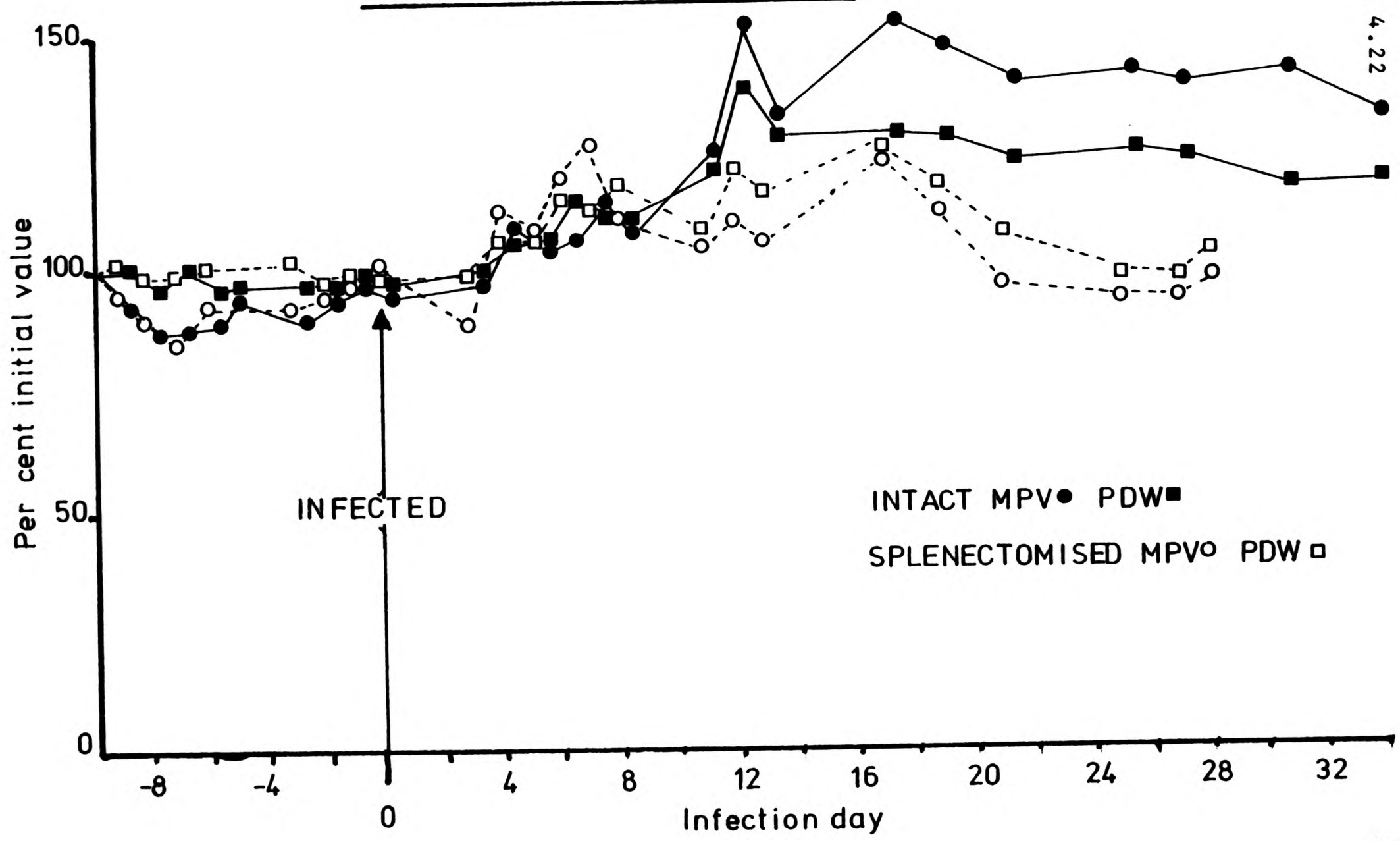
Clearly the size measured by electronic methods will be influenced by any platelet clumps present in the sample although if above 20fl they will be disregarded. The MPV in a group of six uninfected intact rabbits was 5.11 +/- 0.13 (sem) fl. This compares reasonably with a

theoretical volume of 4.87fl in rabbits reported by Trowbridge et al[95]. Human platelets are much larger. There was however a significant difference in MPV and PDW between the intact and splenectomised group (MPV: unpaired $t=2.36$, $df=4$, $p<0.05$; PDW: unpaired $t=3.40$, $df=4$, $p<0.05$) with the asplenic animals having larger and more heterologous platelets (means:- intact MPV=4.92fl, PDW=14.66fl; splenectomised MPV=5.94fl, PDW=15.68fl). This possibly reflects the normal removal by the spleen of platelets above a certain size although why this mechanism should be operative at such a small size is not clear. More probably the apparent increase in size is a reflection of in vivo platelet clumps which would normally be removed in the splenic circulation, the tail end measurement of which will be included in the electronic counting. To account for this difference the measurements have all been normalised to 100%.

Experiment VII showed that both intact and splenectomised animals increased their MPV and PDW within a day of thrombocytopenia developing to maximum values of 157% and 137% respectively for the intact animals and 122% and 125% respectively for the splenectomised animals (Fig.4.22). These increases were significant (Table 4.3). There were differences too when these measurements were compared in the two infected groups, even though there had been no

EXPERIMENT VII - MEAN PLATELET VOLUME AND PLATELET DISTRIBUTION WIDTH IN T B BRUCEI INFECTION

Fig 4.22



EXPERIMENT VII

A. DIFFERENCES IN MPV AND PDW (control v.infected)

Kendall's S test	z	p	
INTACT	MPV	4.07	<0.001
	PDW	4.01	<0.001
SPLENECTOMISED	MPV	3.45	<0.001
	PDW	3.48	<0.001

B. DIFFERENCES IN INFECTED MPV AND PDW (intact v.splenectomised)

Paired-t test	mean diff. %	t	df	p
MPV	12.70	1.80	15	<0.05
PDW	6.68	2.53	15	<0.05

differences seen in platelet number (Section 4.3.1), with the MPV and PDW being significantly less in the splenectomised group (Table 4.3). This was surprising as there did not appear to have been any significant differences in the thrombopoietic stress between the two groups (if anything the splenectomised group showed the greatest fall in platelet number in the first half of the infection) and one would have expected the spleen to act by removing the larger platelets or clumps. This phenomenon was seen only in the second half of the infection however (Fig.4.22).

In experiment VIII, which involved intact animals only, a similar pattern was seen of increasing MPV and PDW just after development of the thrombocytopenia (Fig.4.23), followed by a decrease when the thrombopoietic stress was not as great.

Overall there was an apparent increase in platelet volume and distribution of platelet size associated with, but occurring after, the development of thrombocytopenia in infected animals. The differences in the splenectomised and intact animals will be discussed further.

EXPERIMENT VIII - MEAN PLATELET VOLUME AND PLATELET DISTRIBUTION WIDTH IN T B BRUCEI INFECTION (mean ± sem)

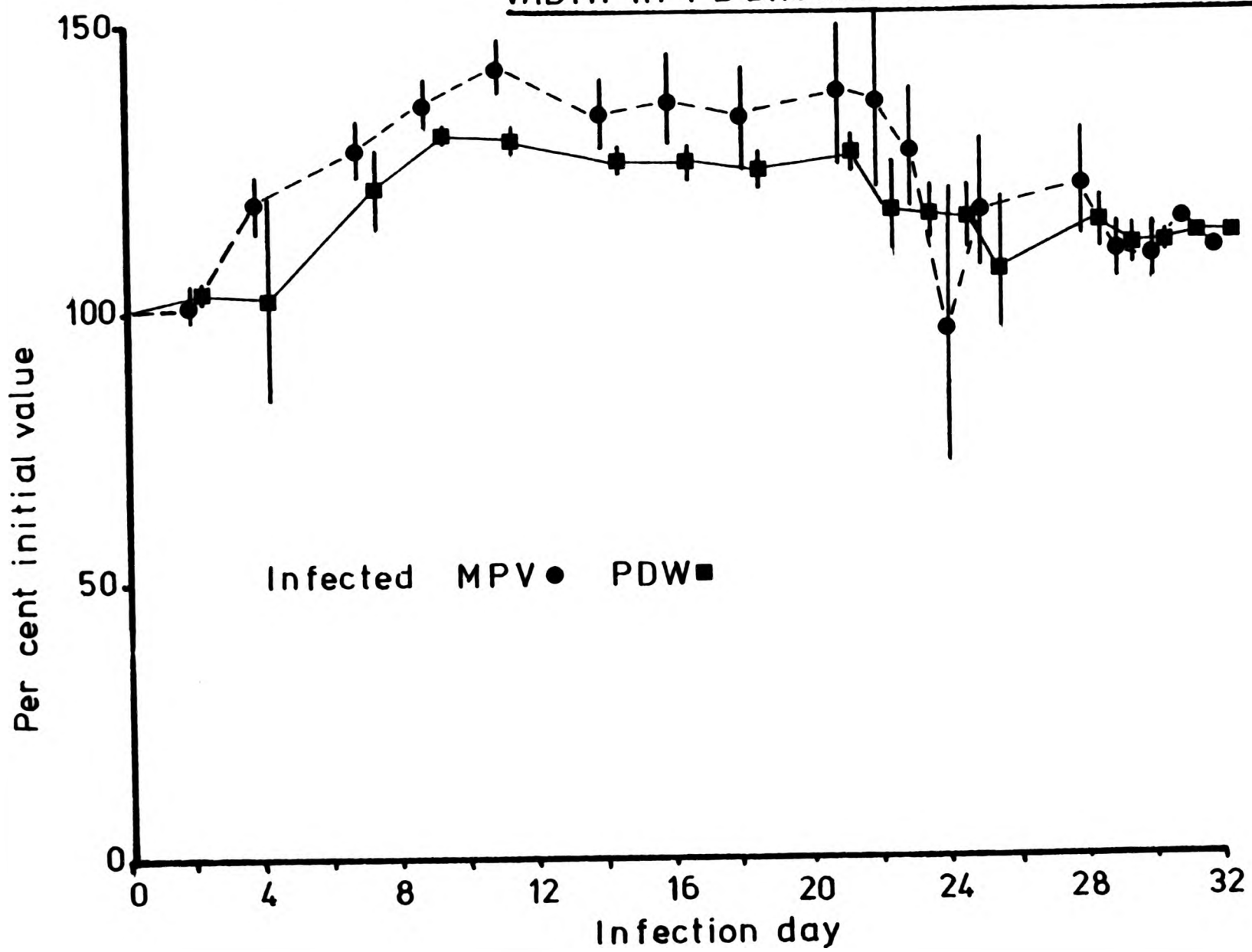


Fig 4.23

4.5 PLATELET AGGREGATE FORMATION (Appendix II)

Platelet aggregates play a key role in the pathogenesis of thromboembolism and small aggregates may occlude small branches of arteries producing local areas of ischaemia[133]. Microthrombi and thromboembolism are pathological conditions seen in trypanosomiasis and microthrombi have been reported in acute T b brucei infections in dogs[20], acute T vivax infections in goats[18,48] and cattle[31].

Small platelet aggregates, which might contribute to microthrombi formation, have been seen consistently by us and others[87] in platelet counting chambers and stained blood films. In order to decide whether these clumps were an in vivo or an in vitro occurrence a method[97] was used, said to measure preformed circulating platelet aggregates[134].

Measures of circulating aggregates (reported as F/E ratios) were made over 10 days in 6 uninfected rabbits and the measurement continued throughout an experimental T.b.brucei infection. The animals involved were those in experiment VIII and the mean ratios are shown in Fig. 4.24. The F/E ratio, although very variable, fell immediately in association with the parasitaemia and thrombo-cytopenia, indicating the presence of

EXPERIMENT VIII - PLATELET AGGREGATES IN T B BRUCEI INFECTION (mean±sem)

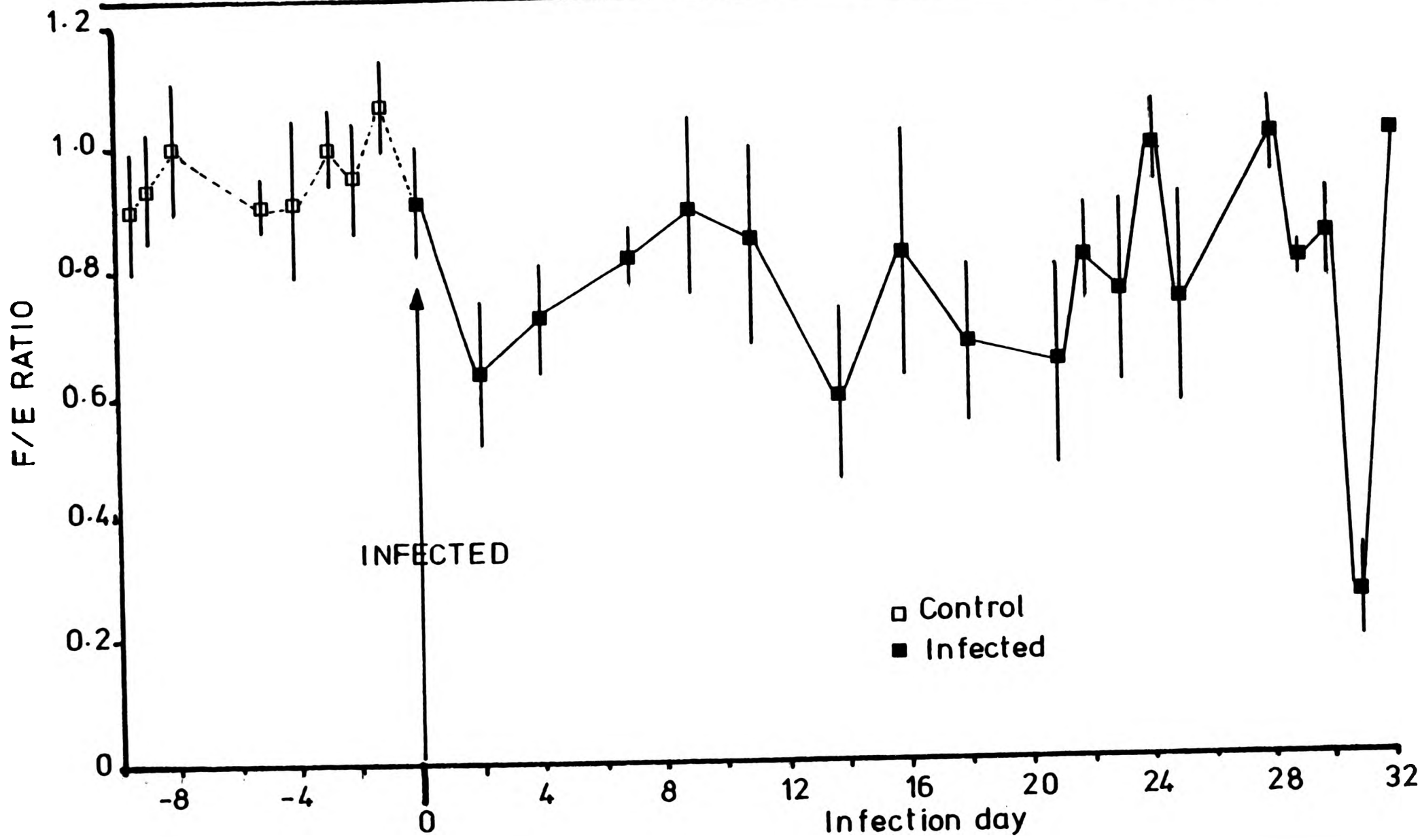


Fig 4.24

circulating platelet aggregates. Before infection the ratio showed a mean level of 0.95 +/- 0.02(sem) and after infection it had fallen to a mean of 0.79 +/- 0.04(sem). An unpaired t test comparing the control and infected groups showed that this difference was significant ($t=2.08$, $df=26$, $p<0.05$).

4.6 PLATELET ULTRASTRUCTURE

Alfred Donne has been given the credit for the first observation of platelets in 1842 but two English practitioners, George Gulliver and William Addison independently described them in 1841. The role of platelets in thrombosis was demonstrated by Bizzozero in 1881 and in haemostasis by Hayem in 1882[135]. Electron microscope studies of platelets were first made by Wolpers and Ruska in 1939[136].

The use of glutaraldehyde allows rapid fixation of platelets which are very labile and may be influenced by handling, producing ultrastructural changes. Platelets are normally discoid in shape and morphologically can be divided into: a peripheral zone for acception of stimuli; a sol-gel zone containing supporting fibres and contractile mechanisms; an organelle zone consisting of granules, dense bodies and mitochondria for storage and metabolic processes; a membrane zone consisting of the

dense tubular system and surface canalicular system[137]. The shape is maintained by microtubules, sub-membrane filaments, cyclic nucleotides and maintenance of high levels of ATP[138].

Platelets that have been exposed to activating substances, which may result in their eventual aggregation, show a shape change becoming roughly spherical with pseudopod formation and centralisation or organelles[138,139]. The centripetal movement of organelles is a manifestation of the contractile process and platelets may show degranulation and vacuolisation[140] or may recover their discoid shape even after secretion[138]. Not all platelets are uniformly affected on exposure to aggregating agents; Hovig[140] exposed rabbit platelets to thrombin and found some to be entirely normal while others were degranulated.

Rabbit platelets were examined by Elchaninova[141] and compared with human platelets; the electron micrograph (EM) structure of each was said to be similar. Dense bodies in rabbit platelets are said to occupy 3-4% of the total area and alpha granules about 10%[142].

Cattle platelets have been described by Kimeto[143] as being round with a regular outline and of similar size.

The granules were round or oval and of different sizes but equally distributed. Cattle with East Coast Fever, caused by a tick-transmitted protozoon, Theleria parva, suffer from thrombocytopenia, petechial haemorrhages and pulmonary oedema. During infection the platelets show indentations, pseudopodia, peripheral granules, vacuoles of different size and shape and degranulation.

Davis et al[83] examined rat platelets in aggregates formed after exposure to T rhodesiense parasites for 30 minutes and showed typical changes of aggregation - degranulation, centralisation of granules, presence of micelles and reduction in cytoplasmic density.

4.6.1 T b brucei infection

Platelet ultrastructure was examined in the group of six rabbits involved in Experiment V. Platelets were prepared from these rabbits on days 7, 10, 17, 27 and 31 of the infection and compared with two control animal preparations made on three of the days. On seven occasions the material was unsuitable for EM preparations.

None of this group of animals became particularly thrombocytopenic during the infection yet significant changes were seen in platelet morphology. Mean measurements of platelet volume, platelet surface area,

alpha and dense granule numbers, made from the electronmicrographs, were not significantly different in infected and control animals overall (Table 4.4). There were generally only three electronmicrographs per animal and so it was unlikely that we would have found any significant differences. However, there were associations between measurements and particular characteristics seen in the EM photographs from individual animals.

Platelets from the uninfected animals were typically elongated and ovoid in shape (Figs.4.25 and 4.26). Alpha granules, dense bodies, vacuoles and microtubules could all be easily distinguished and this general appearance did not differ on any other days that the platelets were examined.

On day 7 of the infection one animal in particular (YEW) showed considerable rounding of the platelets (Fig.4.27) and this was noted in several other animals although not so marked. Generally on day 7 the infected animals had a higher than normal platelet volume and surface area, reflecting the difference in shape, and probably slightly greater numbers of granules, reflecting the increase in platelet size (Table 4.4). Pseudopodia were prominent in some of the animals and there appeared to be increasing vacuolisation.

MEASUREMENTS MADE FROM EM PHOTOGRAPHS OF RABBITS IN EXPERIMENT V (means)

A. PLATELET VOLUME (μm^3)

	CONTROL			INFECTED		
	POP	XAN	YEW	VIO	ULE	RHO
Day						
7	1.36	1.30	2.75	2.61	1.80	1.18
10	1.66	-	1.09	2.32	0.85	-
17	1.99	1.16	1.04	1.99	-	1.98
27	-	-	1.01	-	3.18	-
31	-	1.94	1.36	-	-	1.78

B. PLATELET SURFACE AREA (μm^2)

Day						
7	6.09	5.15	7.91	7.68	7.53	5.08
10	7.09	-	5.32	7.23	4.90	-
17	7.04	6.13	4.92	6.82	-	6.24
27	-	-	4.08	-	10.16	-
31	-	7.48	4.91	-	-	6.98

C. ALPHA GRANULES NUMBER

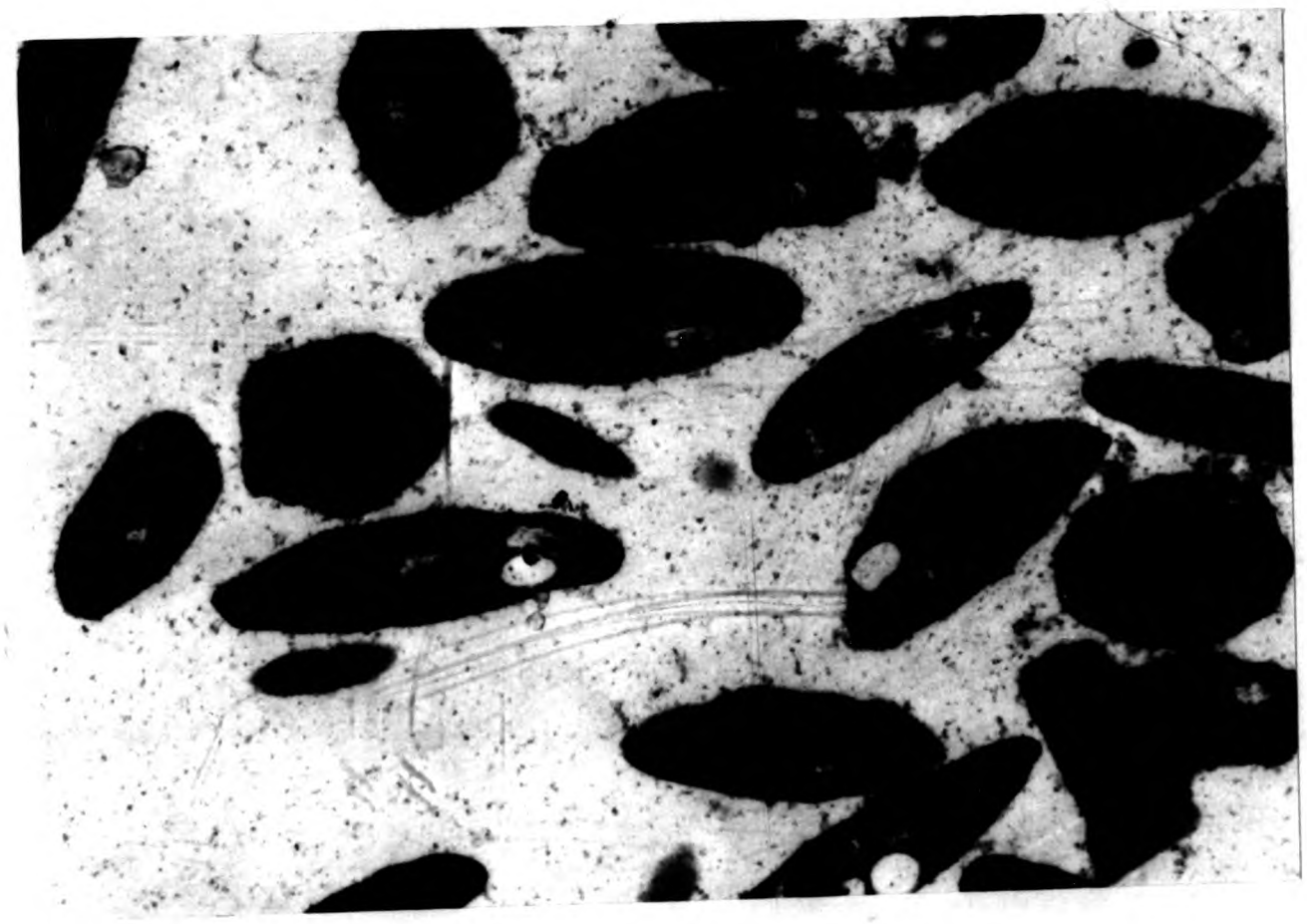
Day						
7	2.9	3.7	3.0	3.0	3.0	1.5
10	2.9	-	2.4	6.0	1.6	-
17	2.0	3.2	1.3	1.6	-	1.4
27	-	-	1.4	-	4.0	-
31	-	2.6	3.4	-	-	1.4

D. DENSE GRANULE NUMBER

Day						
7	1.0	1.3	1.5	1.3	1.3	0.4
10	1.3	-	0.5	0.9	0.9	-
17	1.1	0.8	0.4	0.3	-	0.5
27	-	-	0.4	-	0.8	-
31	-	1.2	0.3	-	-	0.5

ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

UNINFECTED



ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

UNINFECTED

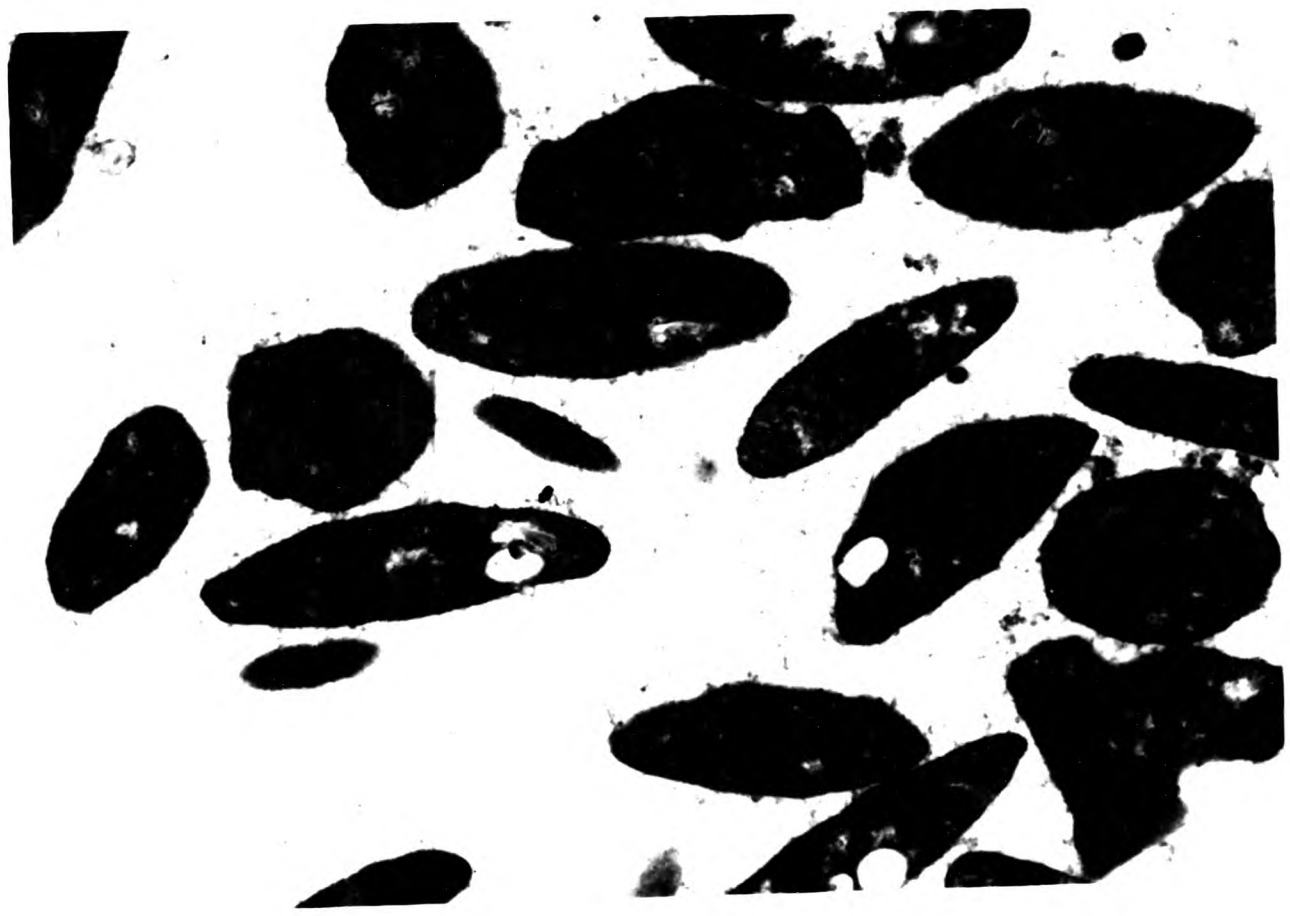


Fig 4.26

ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

UNINFECTED

ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

UNINFECTED

ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

T B BRUCEI INFECTION DAY 7

ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

T B BRUCEI INFECTION DAY 7

On day 10 both vacuolisation and pseudopodia formation were marked in all 3 infected animals examined but the platelets appeared to have returned to a more elongated shape in 2/3. The rabbit whose platelets remained round in appearance was the only one to continue to show the increased platelet volume, surface area and granule number at this time (Table 4.4). The cells in general appeared less uniform and some centralisation of granules and degranulation was seen (Fig. 4.28).

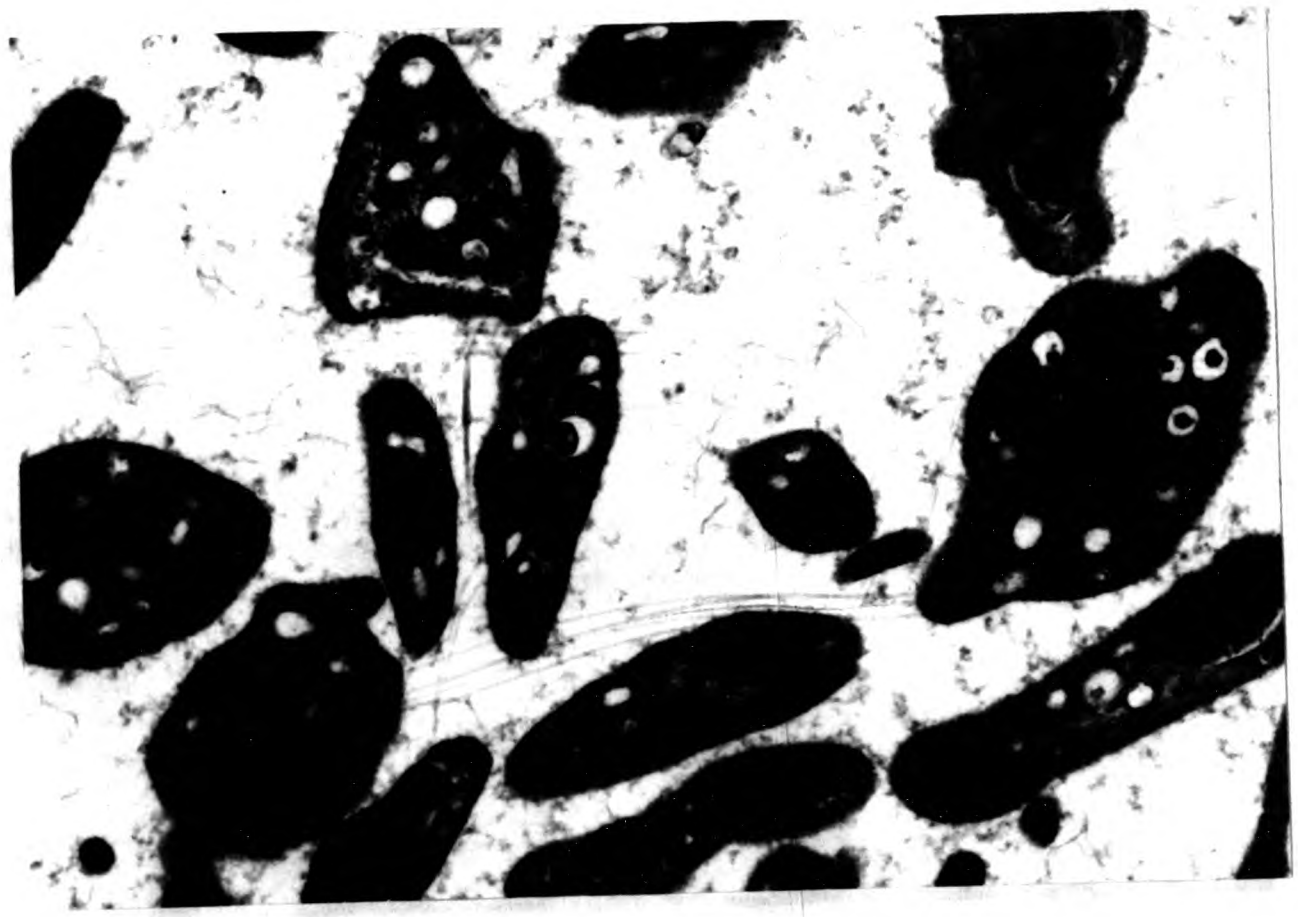
Day 17 of the infection showed similar changes in platelet morphology with the cells appearing very ragged because of the marked pseudopodia formation. Degranulation was more marked and this has perhaps resulted in the granule numbers now appearing lower than in the control animals (Table 4.4).

On days 27 and 31 the changes were more marked still. Pseudopodia were more obvious and the cells appeared very distorted. Degranulated platelets (Fig.4.29), centralisation and partial disintigration of organelles with their contracted band of microtubules (Fig.4.30) were commonly seen.

Thus, even where platelet levels were maintained, the effect of the infection on platelet morphology, and thus probably function, was significant.

ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

T B BRUCEI INFECTION DAY 10



ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

T B BRUCEI INFECTION DAY 10

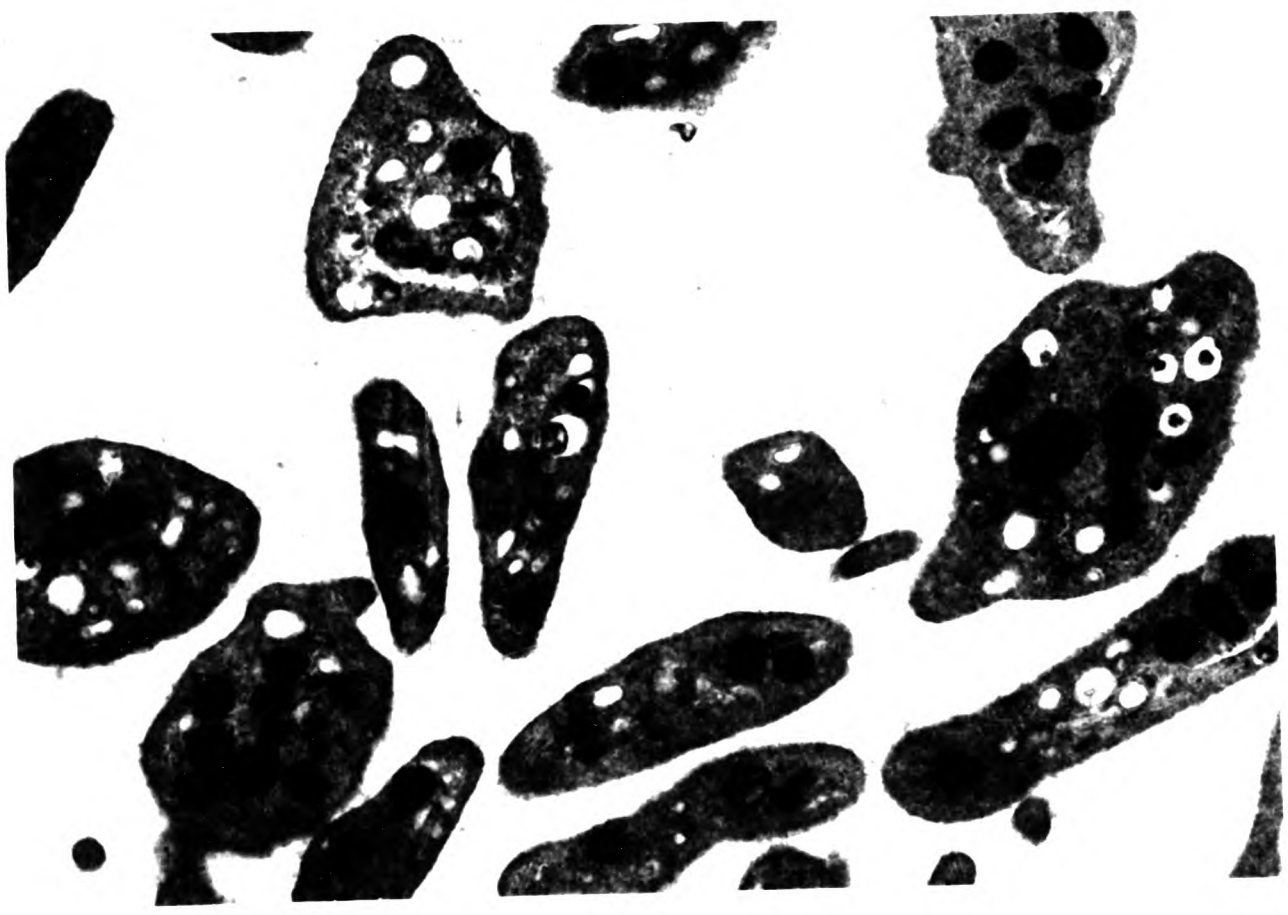


Fig 4.29

ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

T B BRUCEI INFECTION DAY 27

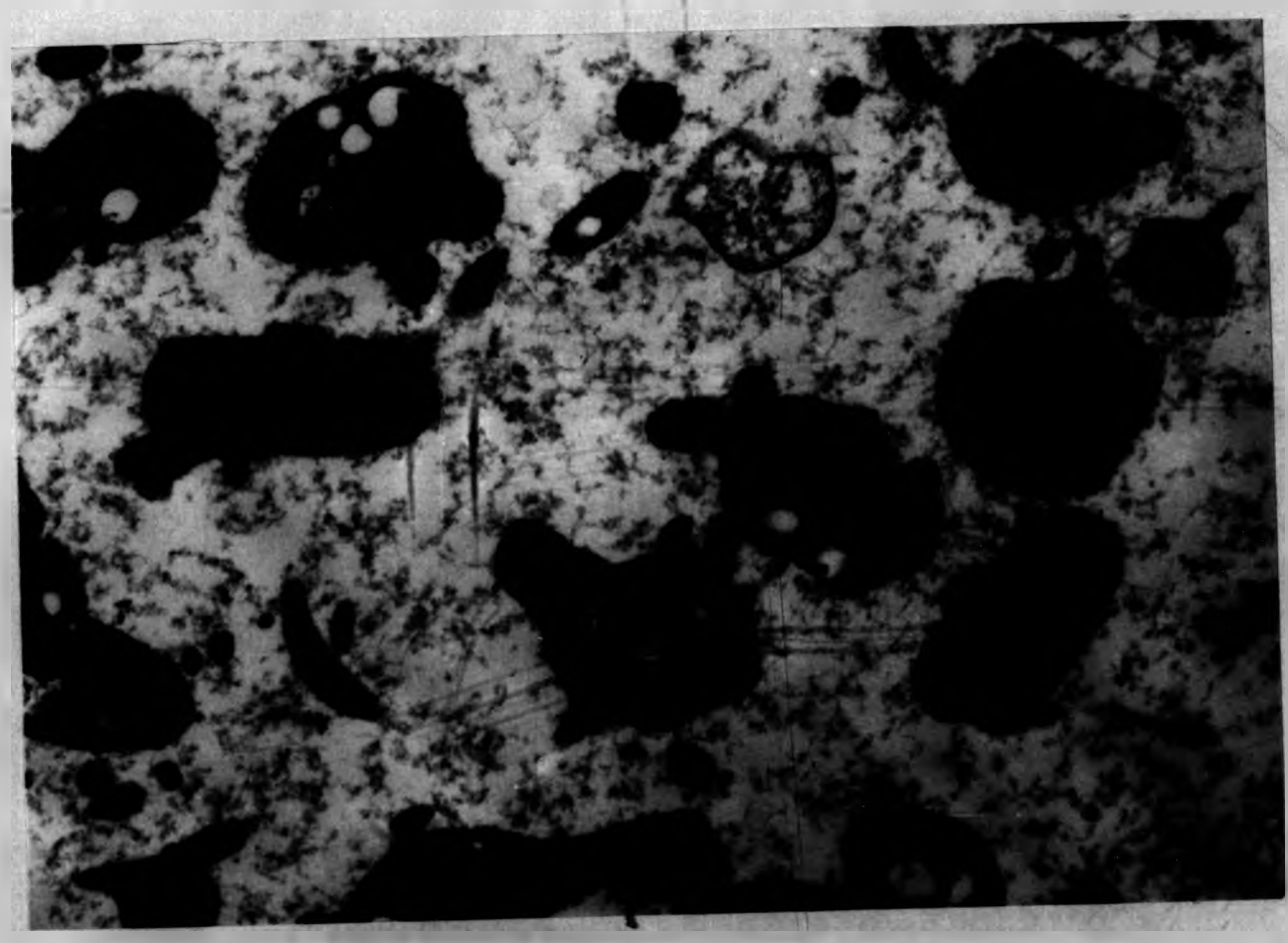


Fig 4.29

ELECTRON MICROGRAPH RABBIT PLATELETS (x22,000)

T B BRUCEI INFECTION DAY 27

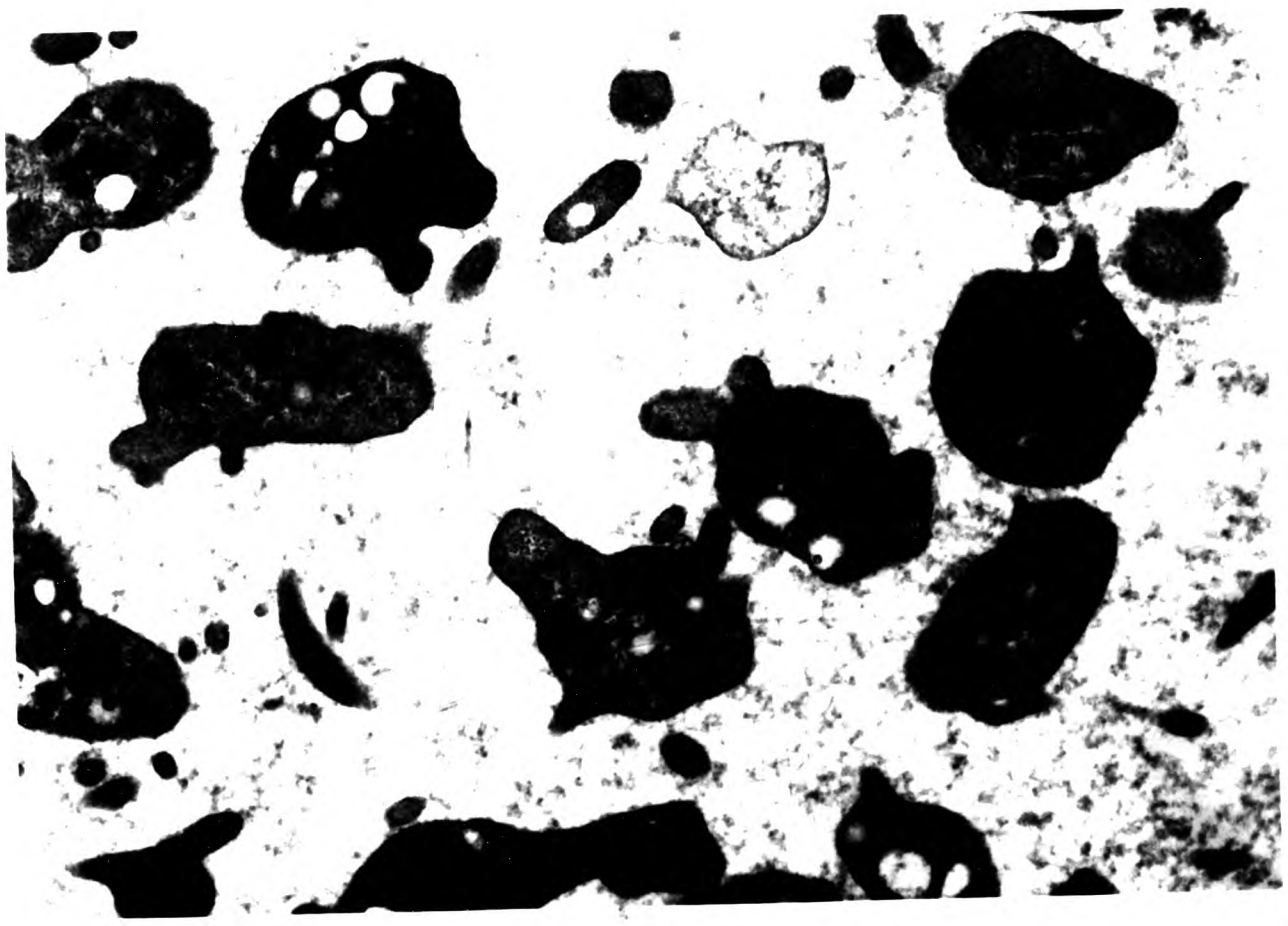


Fig 4.30

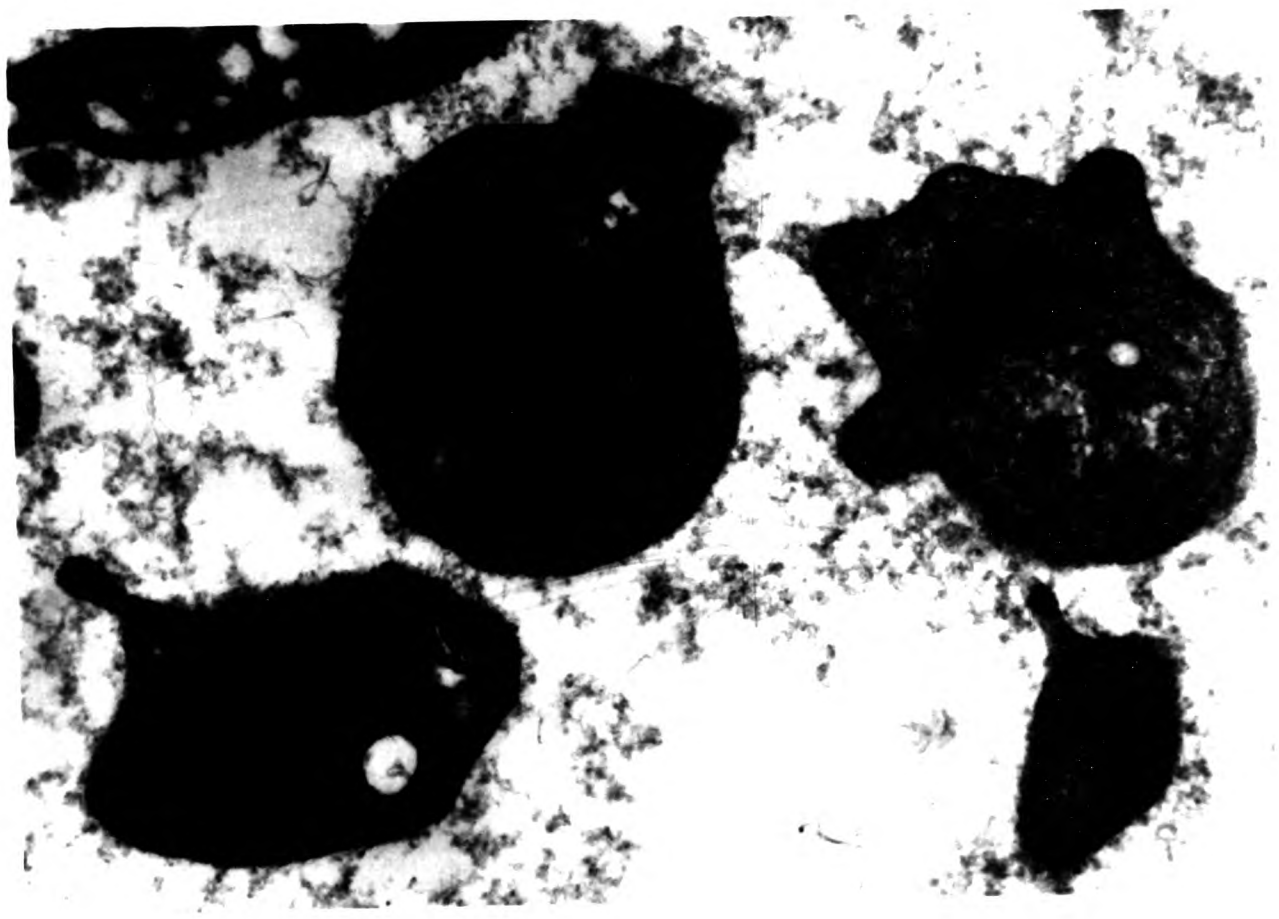
ELECTRON MICROGRAPH RABBIT PLATELETS (x30,000).

T B BRUCEI INFECTION DAY 27

Fig 4.30

ELECTRON MICROGRAPH RABBIT PLATELETS (x30,000)

T B BRUCEI INFECTION DAY 27



4.6.2 T vivax infection

Calf platelets appear more ovoid in shape than rabbit platelets and they seem to have, on average, more alpha granules and fewer dense bodies. Some of the granules are stick-like. Vacuoles are few and glycogen presence is obvious (Fig.4.31).

By day 7 of the first infection there is a marked thrombocytopenia and many trypanosomes are seen in the plasma. The platelets appear much rounder, some with obvious pseudopodia and this shape change is reflected in the increases seen in platelet volume and platelet surface area at this time (Fig.4.32). Degranulation or centralisation of granules with their associated bundle of microtubules is seen in many of the cells (Figs.4.33, 4.34 and 4.35). Mean granule number did not appear to alter significantly during the infection (Fig.4.36); possibly degranulation of some cells was balanced by production of larger platelets with slightly larger numbers of granules on average.

Later in the first infection the platelets appeared more oval again with an apparent reduction in volume and surface area. Trypanosome numbers were decreased on this occasion but the cells continued to show differences in size, pseudopodia and appeared generally distorted in shape. There was still some

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Fig 4.31

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

UNINFECTED

Fig 4.31

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

UNINFECTED

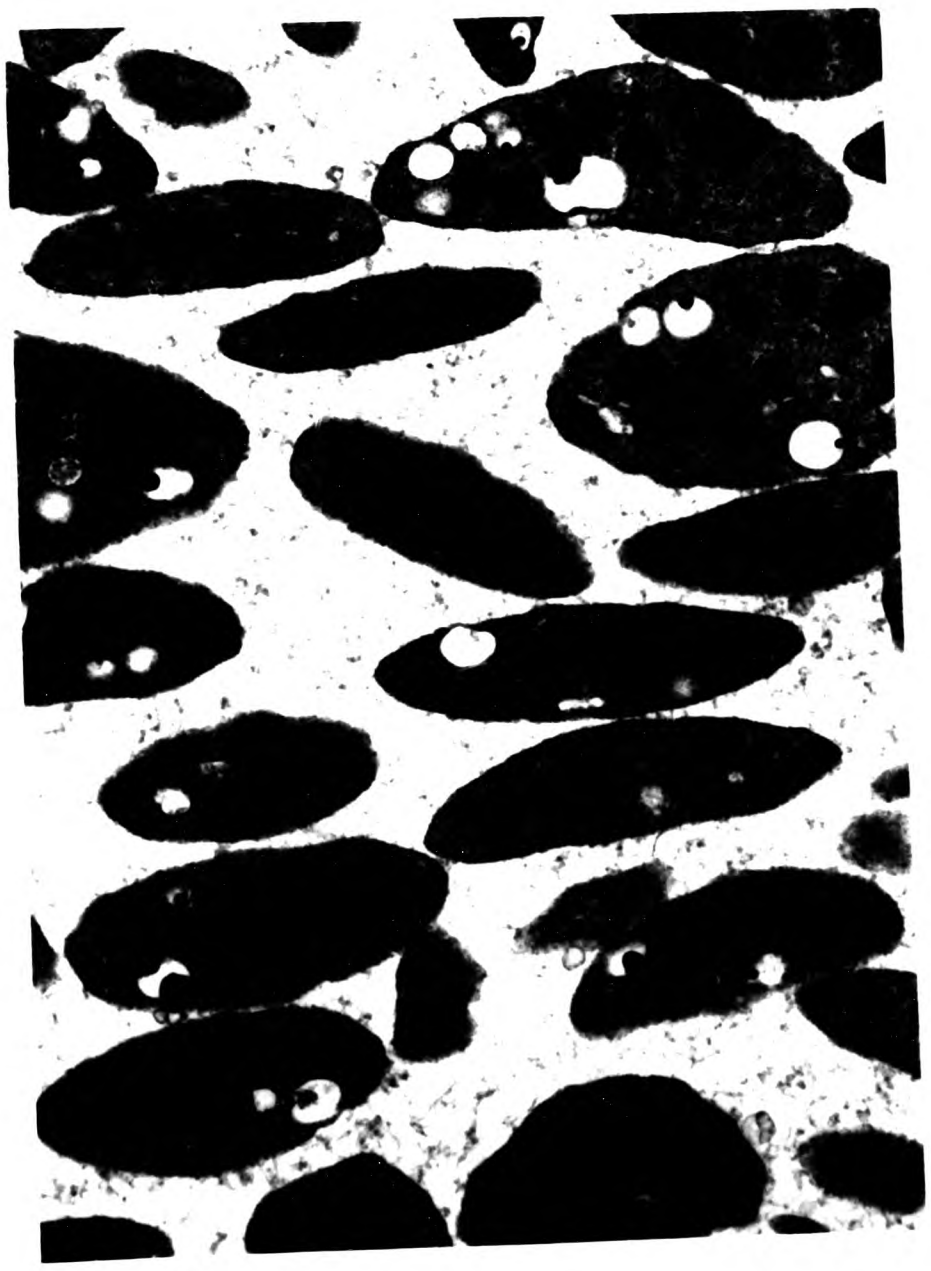


Fig 4.32

PLATELET VOLUME AND SURFACE AREA IN T VIVAX INFECTIONS

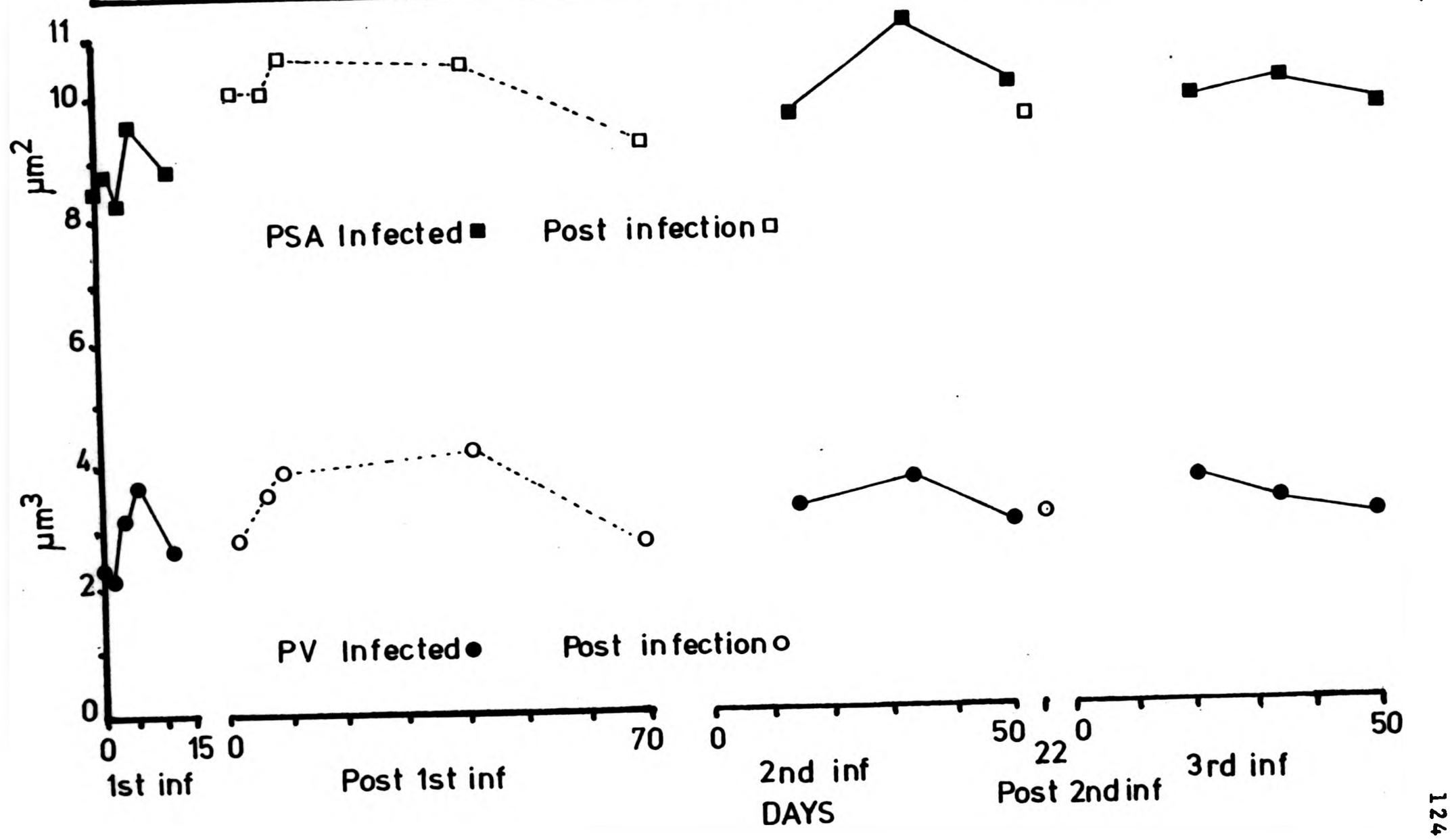


Fig 4.33

ELECTRON MICROGRAPH CALF PLATELETS (x9,000)

T VIVAX INFECTION DAY 7

Fig 4.33

ELECTRON MICROGRAPH CALF PLATELETS (x9,000)

T VIVAX INFECTION DAY 7

Fig 4.34

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION DAY 7

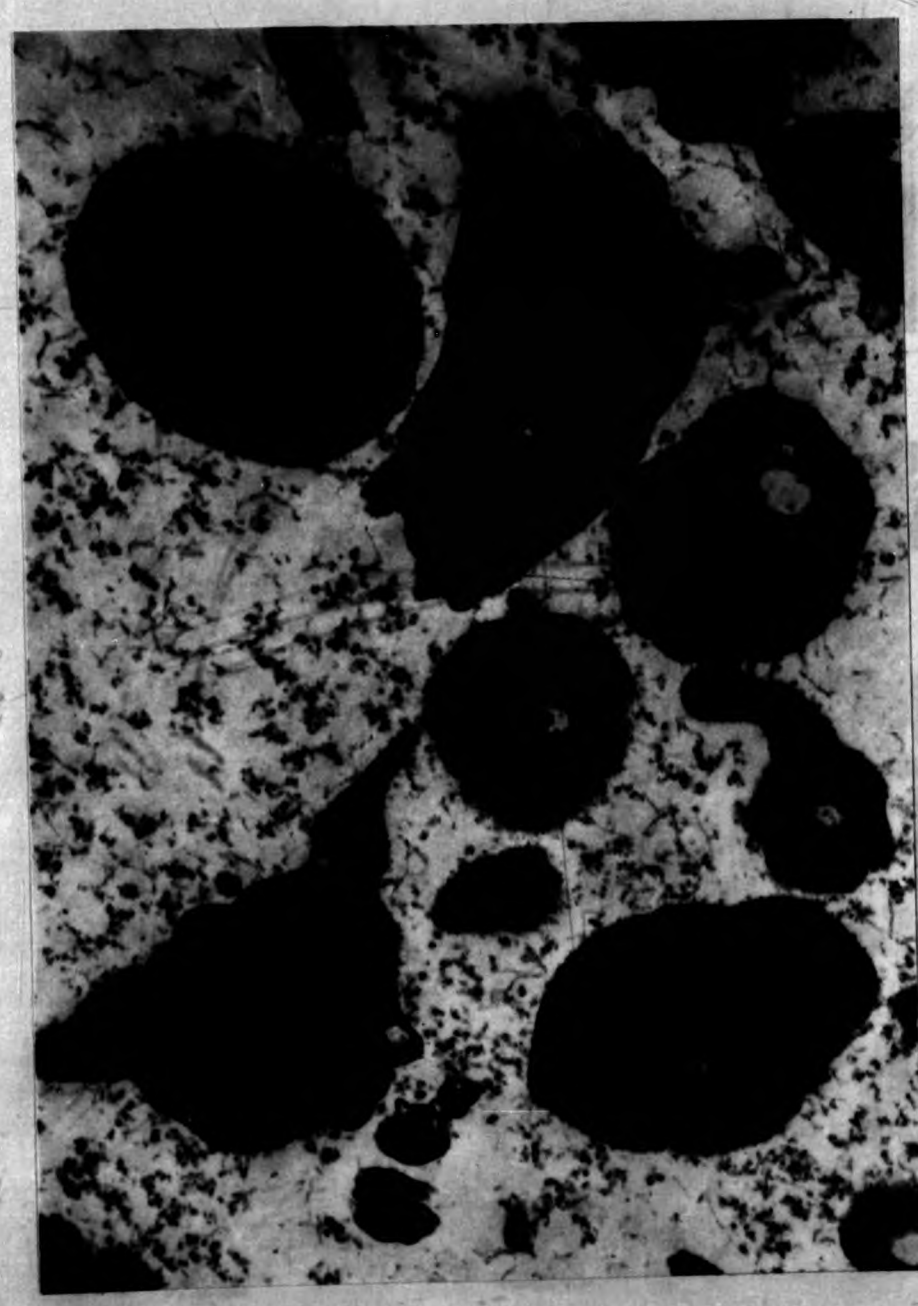


Fig 4.34

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION DAY 7

Fig 4.35

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION DAY 7

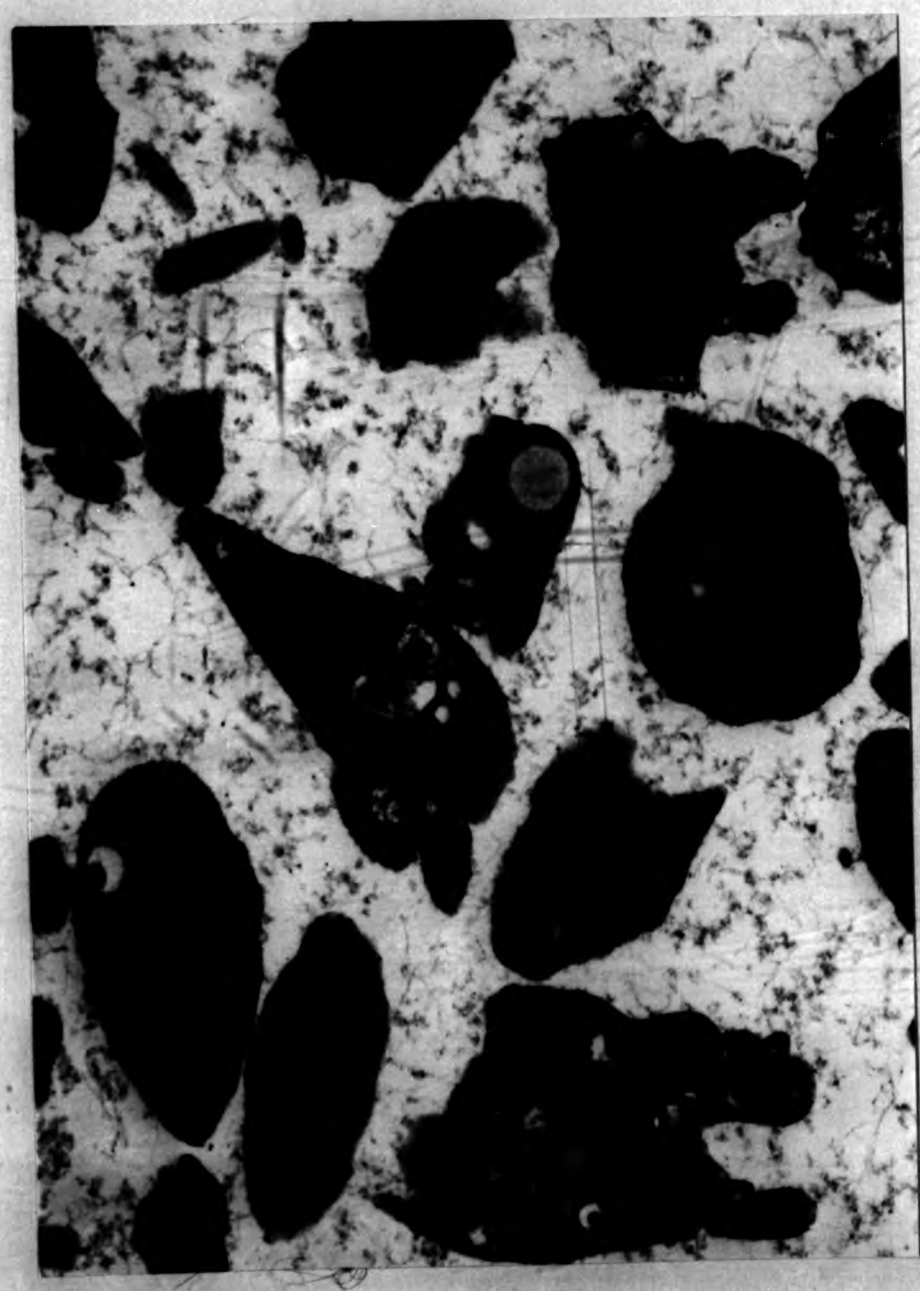


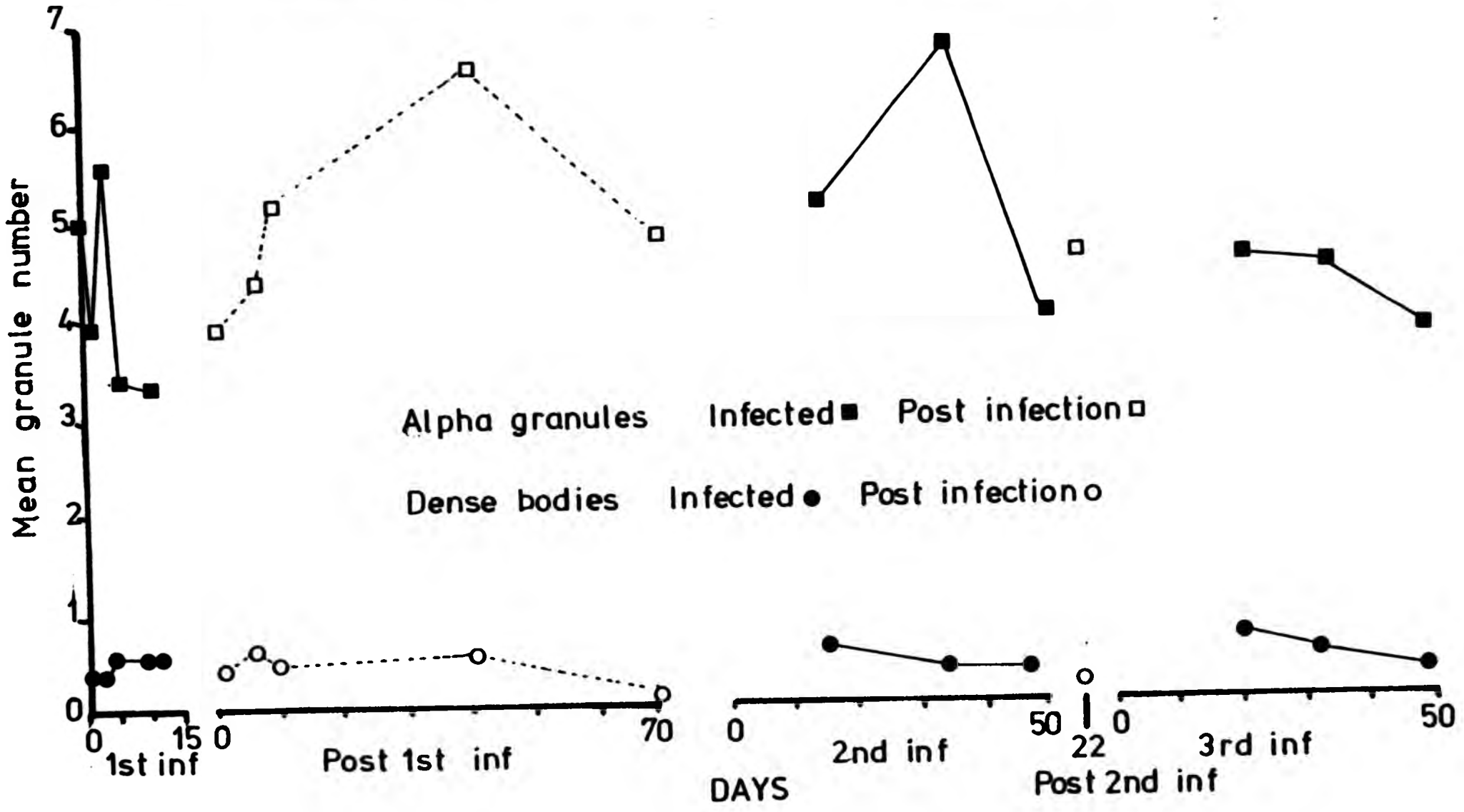
Fig 4.35

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION DAY 7

Fig 4.36

PLATELET ALPHA AND DENSE GRANULES IN T VIVAX INFECTIONS



degranulation seen (Fig.4.37).

After treatment with Berenil the parasites disappeared within 24 hours and there followed a rapid rise in platelet number. 11 days after treatment morphological changes remained. Some degranulated cells could still be demonstrated (Fig.4.38) and even 52 days after infection the cell morphology was not entirely normal (Fig.4.39). These cells were quite round and there was a greater variation in size than normal, both changes reflected in the larger platelet volume, surface area and alpha granule content during this period. The platelet membrane appeared much more regular however and granules were evenly distributed throughout the cytoplasm, as in the pre-infection platelets.

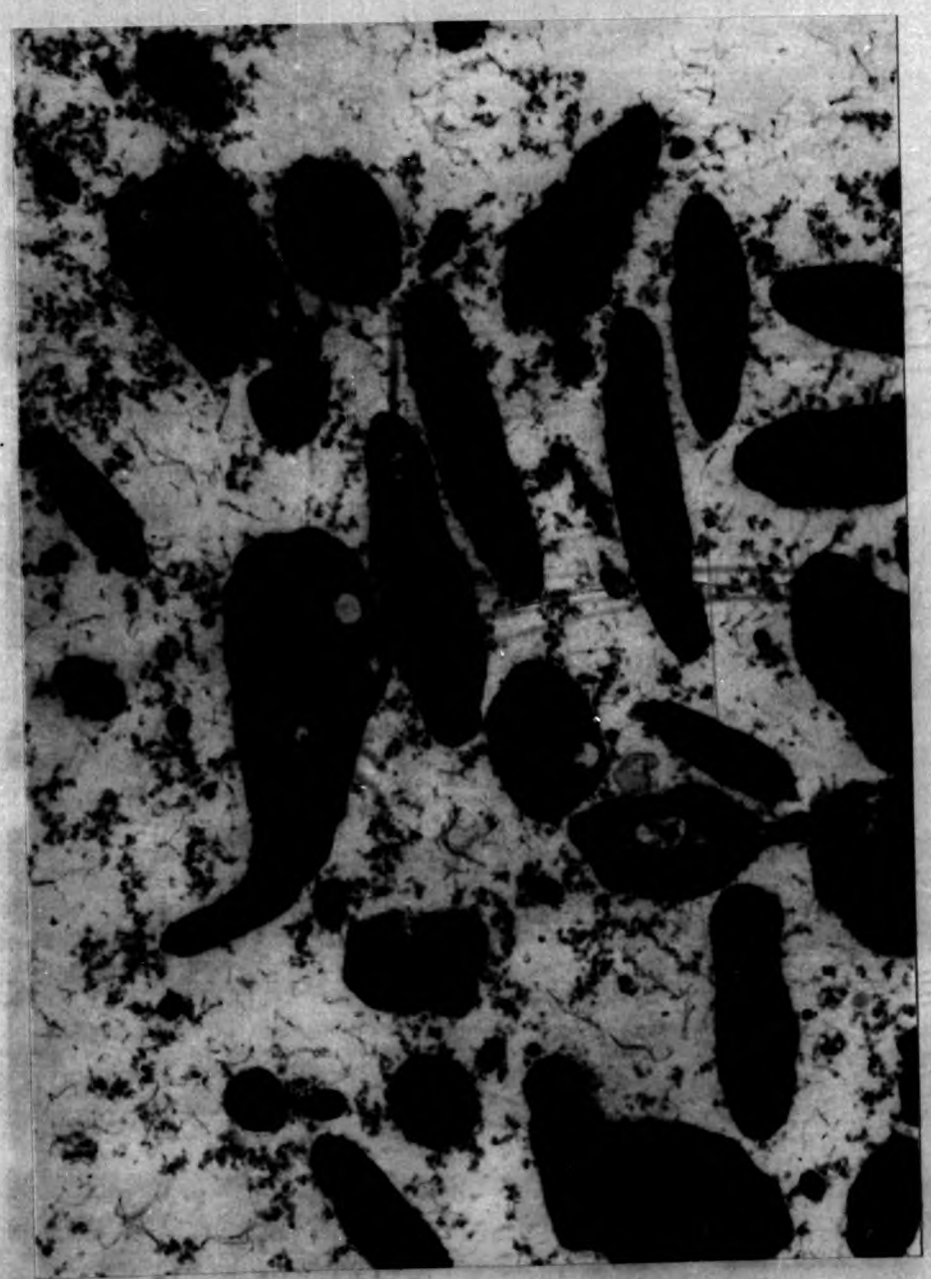
Repeated infections did not result in prevention of platelet ultrastructure changes although the numbers of platelets were higher and trypanosome numbers not as great. Those cells continued to show the distortion, pseudopodia and size change typical of the previous infections and the centralisation of granules, indicating platelet activation, was also seen (Figs.4.40 & 4.41).

The most significant changes in morphology were undoubtedly seen early in the first infection, when the

Fig 4.37

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION DAY 12

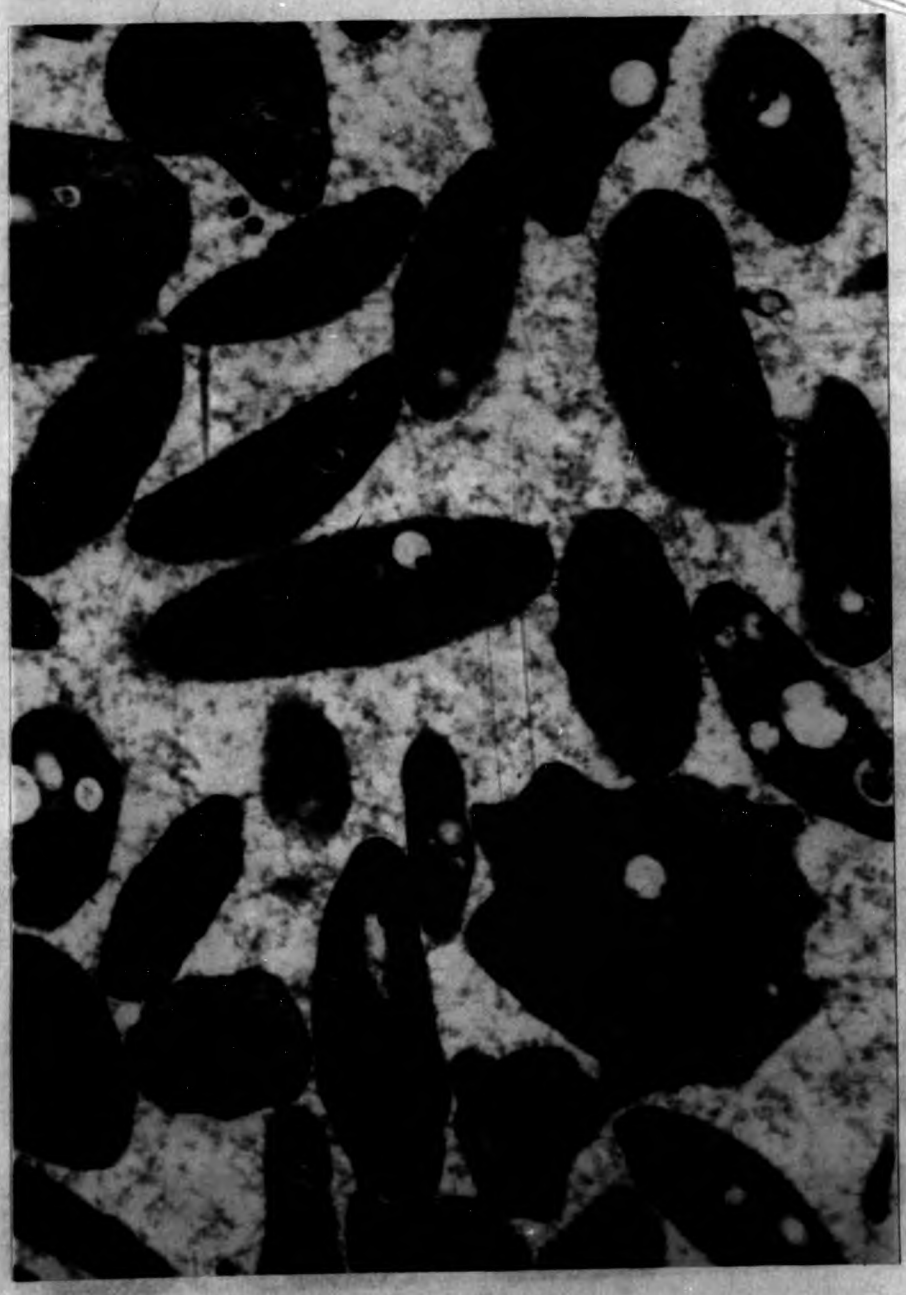


ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION DAY 12

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION 11 DAYS POST BERENIL



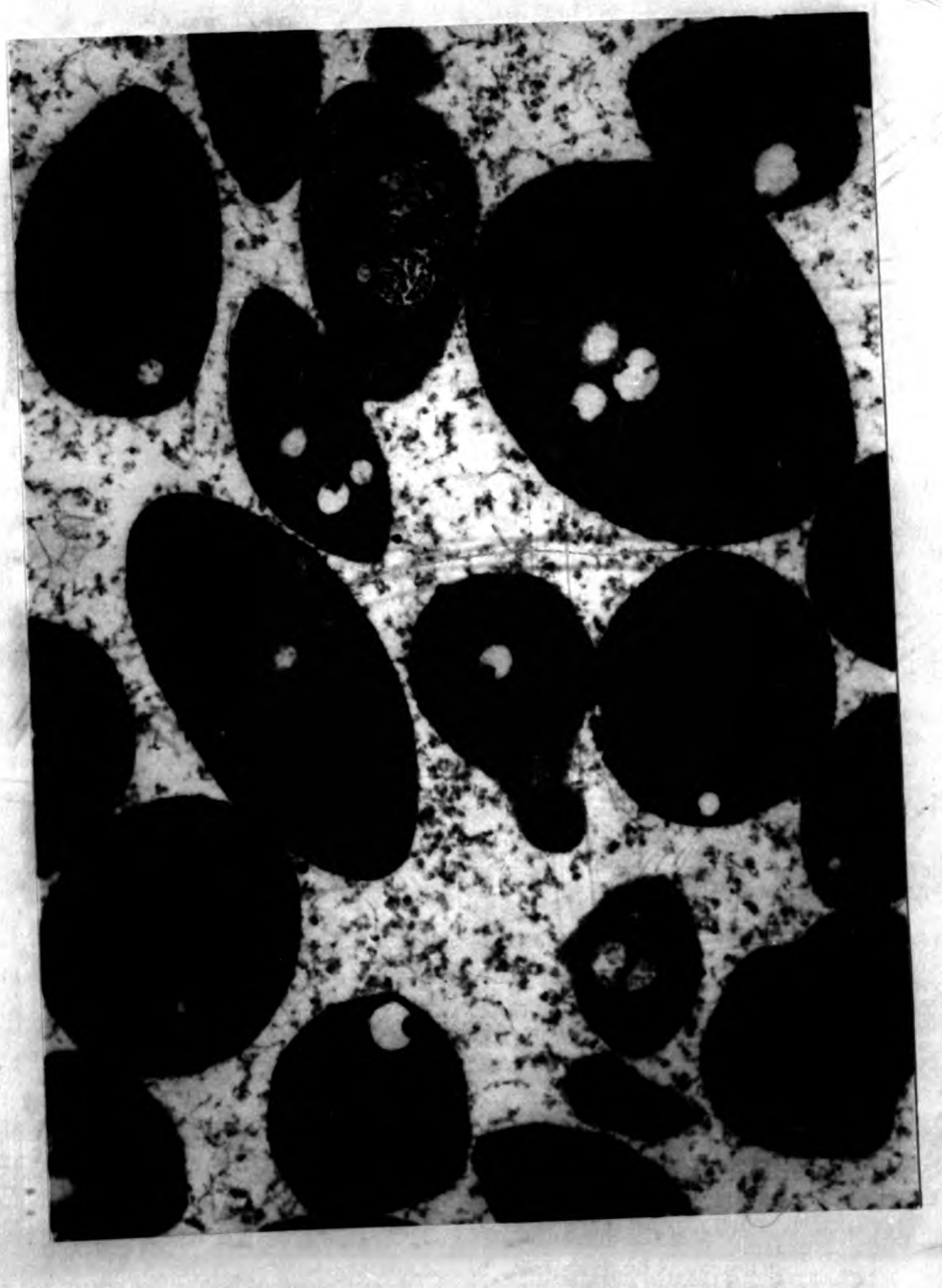
ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION 11 DAYS POST BERENIL

Fig 4.39

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION 52 DAYS POST BERENIL



ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION 52 DAYS POST BERENIL

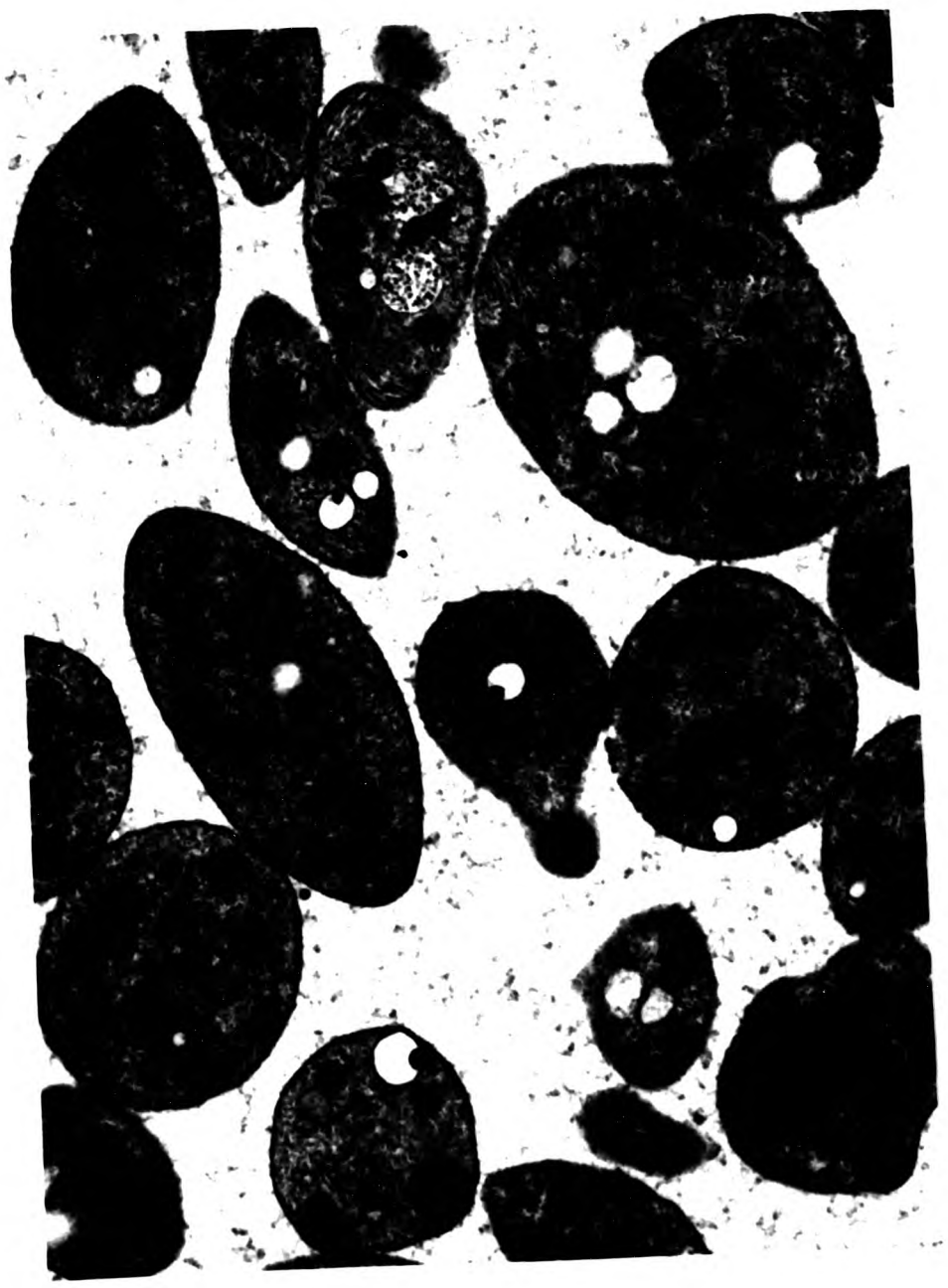


Fig 4.40

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION DAY 22 3RD INFECTION

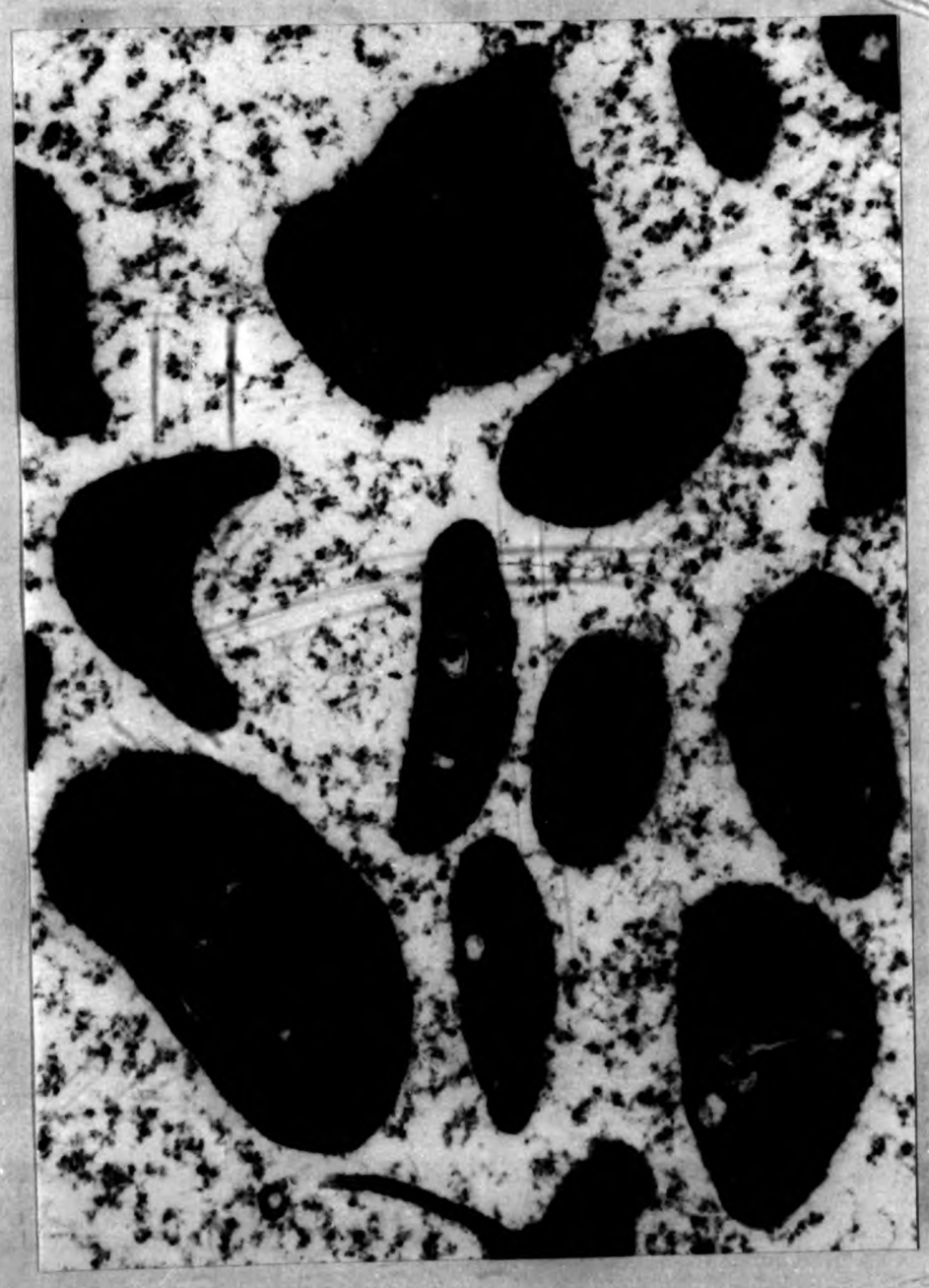


Fig 4.40

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION DAY 22 3RD INFECTION

Fig 4.41

ELECTRON MICROGRAPH CALF PLATELETS (x20,000)

T VIVAX INFECTION DAY 54 3RD INFECTION

Fig 4.41

ELECTRON MICROGRAPH CALF PLATELETS (x20,C00)

T VIVAX INFECTION DAY 54 3RD INFECTION

parasitaemia was high, but it was interesting that the platelet morphology did not appear to return entirely to normal after Berenil treament.

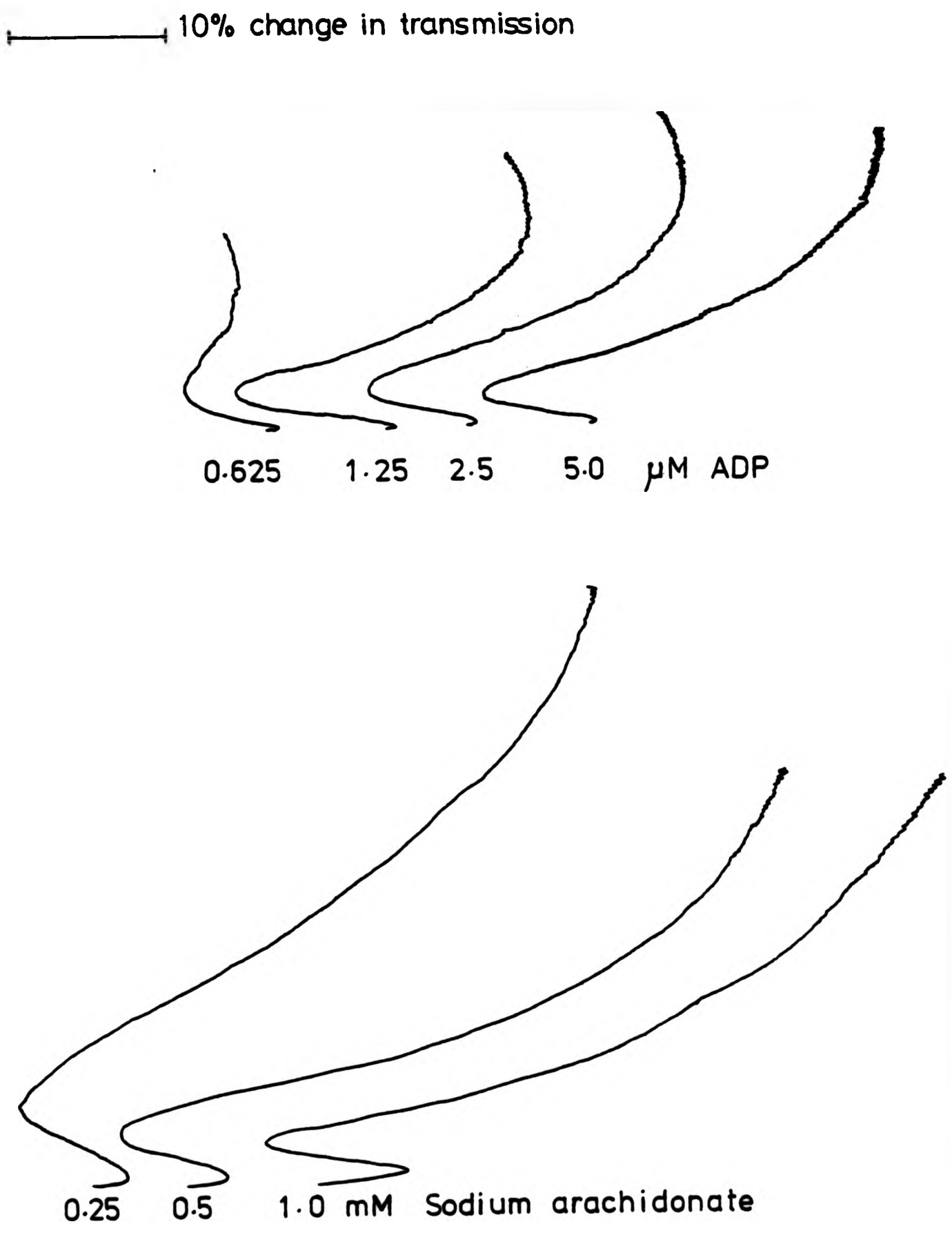
4.7 PLATELET AGGREGATION

The reports in the literature concerning platelet aggregation in the presence of trypanosomes show conflicting results. Davis et al[83] infected rats with the blood from a patient suffering with a T rhodesiense infection, producing a severe thrombocytopenia in the rats. No platelet aggregates were seen when performing platelet counts. When they added trypanosomes to normal rat, rabbit or human blood in vitro, platelet aggregates formed although the platelet count was not significantly reduced until 15 minutes after mixing. By 30 minutes aggregation was virtually complete and involved most of the platelets. Greenwood and Whittle[29] repeated this experiment with exposure of viable T gambiense trypanosomes and trypanosome extracts to human blood and rat blood and found no significant reduction in platelet counts after a one hour incubation period. They speculated that the difference was due to T gambiense causing a more chronic disease than that found in Rhodesian trypanosomiasis. Slots and van Miert[39] investigated addition of whole and homogenised T vivax and T brucei to goat platelet-

rich plasma. No aggregation was seen but mixtures of platelets, T vivax and plasma containing T vivax antibodies showed release of ^{14}C-serotonin from platelets after 15 minutes in vitro incubation. Release of serotonin from platelets was also evident after a second injection, but not after the first injection, of dead trypanosomes to rabbits suggesting that both the organism and antibodies are required to stimulate the release reaction in platelets.

4.7.1 T b brucei infections (Experiment IX)

We have examined the platelets from rabbits infected with T b brucei. Normal rabbit platelets show slight differences from human platelets in aggregation - they do not aggregate with adrenaline and show only a single reversible phase of aggregation with ADP. The initial shape change seen on addition of aggregating agents is more marked and slower that that found in human platelets. Typical responses to various concentrations of the aggregating agents ADP, collagen and sodium arachidonate are shown in Fig.4.42. In several experiments the supernatant from repeatedly frozen and thawed T b brucei suspensions was added to normal rabbit platelet-rich plasma in an aggregometer. At no time did aggregates form although 15 minutes was the longest time of incubation used; slight fluctuations in the trace were similar to those produced on addition of adrenaline



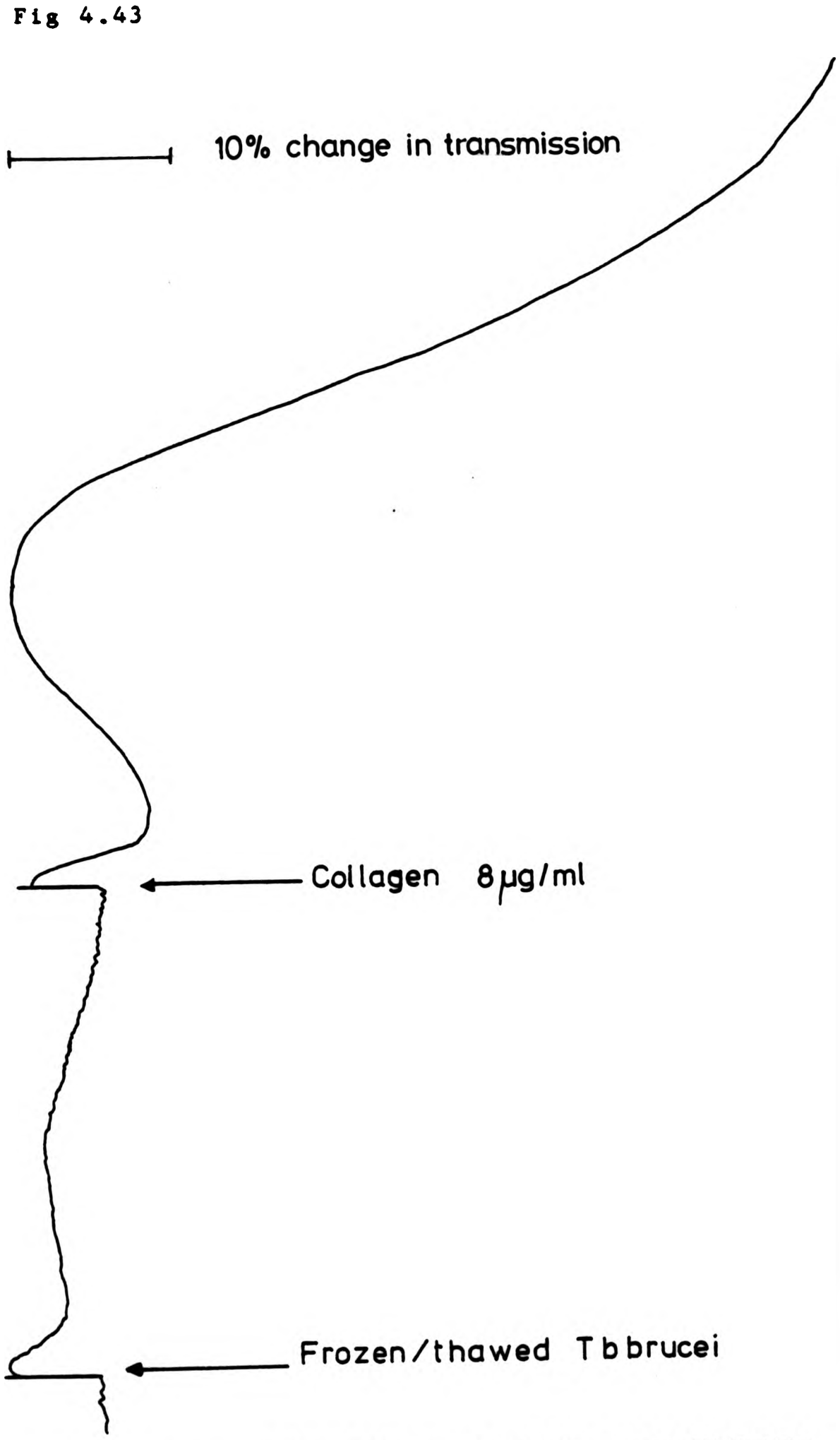
AGGREGATION RESPONSES - RABBIT UNINFECTED

(no aggregation) and were probably due to disturbances caused by addition of material and dilution effects rather than the nature of the material. A typical trace is illustrated in Fig.4.43. Addition of collagen to the platelets after the 15 minutes incubation gave apparently normal aggregation responses.

In order to quantify changes in aggregation throughout the infection, different concentrations of ADP and sodium arachidonate were used to find the lowest concentration producing an aggregation response. Collagen was used at a single concentration only and responses classified as good (+ve), poor (+/-) or no response (-ve). The results are shown in Tables 4.5, 4.6 and 4.7. Responses to collagen and sodium arachidonate (Tables 4.6 & 4.7) only began to show significant changes at the beginning of the third week and these were marked towards the end of the infection, associated with the presence of trypanosomes. Responses to ADP (Table 4.5) began to change noticeably in the second week of infection. This may reflect the increased sensitivity of ADP over other aggregating agents to detect changes.

Fig.4.44 shows typical aggregation responses that were obtained towards the end of the infection; alcngside each curve is the response produced by the same

Fig 4.43



AGGREGATION RESPONSES - RABBIT UNINFECTED

THRESHOLD LEVELS OF AGGREGATION RESPONSES TO ADP - FINAL CONCENTRATION IN ug/ml

RABBIT	GRE	HUR	MCN	SHA	WIL	WOR
Pre-infection	0.625	1.25	0.625	0.625	0.625	0.625
Infection day						
3	0.625(0)	1.25(0)	1.25(0)	0.625(0)	1.25(0)	1.25(0)
6	0.625(0)	0.625(1)	1.25(0)	-	-	-
7	-	-	-	***	1.25(0)	1.25(0)
8	1.25(1)	1.25(0)	2.5(0)	-	-	-
12	-	-	-	>10.0(0)	5.0(3)	***
15	5.0(7)	5.0(2)	5.0(2)	*	-	-
22	10.0(36)	>10.0(45)	*	-	-	*
Control	5.0	5.0				
27	>10.0(144)	*	*	-	10.0(162)	
Control	2.5				2.5	

NOTE: The threshold levels for response of an uninfected control rabbit at the same platelet count as the test animal are given when baseline platelet counts of 400 x 10^9/L could not be reached in platelet-rich platelet.

(-) number in brackets represents number of trypanosomes in 30 low power fields of whole blood

* animal dead

** platelet count too low for study

*** sample clotted

Table 4.6

AGGREGATION RESPONSES TO COLLAGEN

RABBIT	GRE	HUR	MCN	SHA	WIL	WOR
Pre-infection	+ve	+ve	+ve	+ve	+ve	+ve
Infection day						
3	+ve(0)	+ve(0)	+ve(0)	+ve(0)	+ve(0)	+ve(0)
6	+ve(0)	+ve(1)	+ve(0)	-	-	-
7	-	-	-	***	+ve(0)	+ve(0)
8	+ve(1)	+ve(0)	+ve(0)	-	-	-
12	-	-	-	-ve(0)	+/-(3)	***
15	+/-(7)	+/-(2)	+/-(2)	*	-	-
22	+ve(36)	+/-(45)	**		-	*
Control	+ve	+ve				
27	+/-(144)	*	*		+/-(162)	
Control	+ve				+ve	

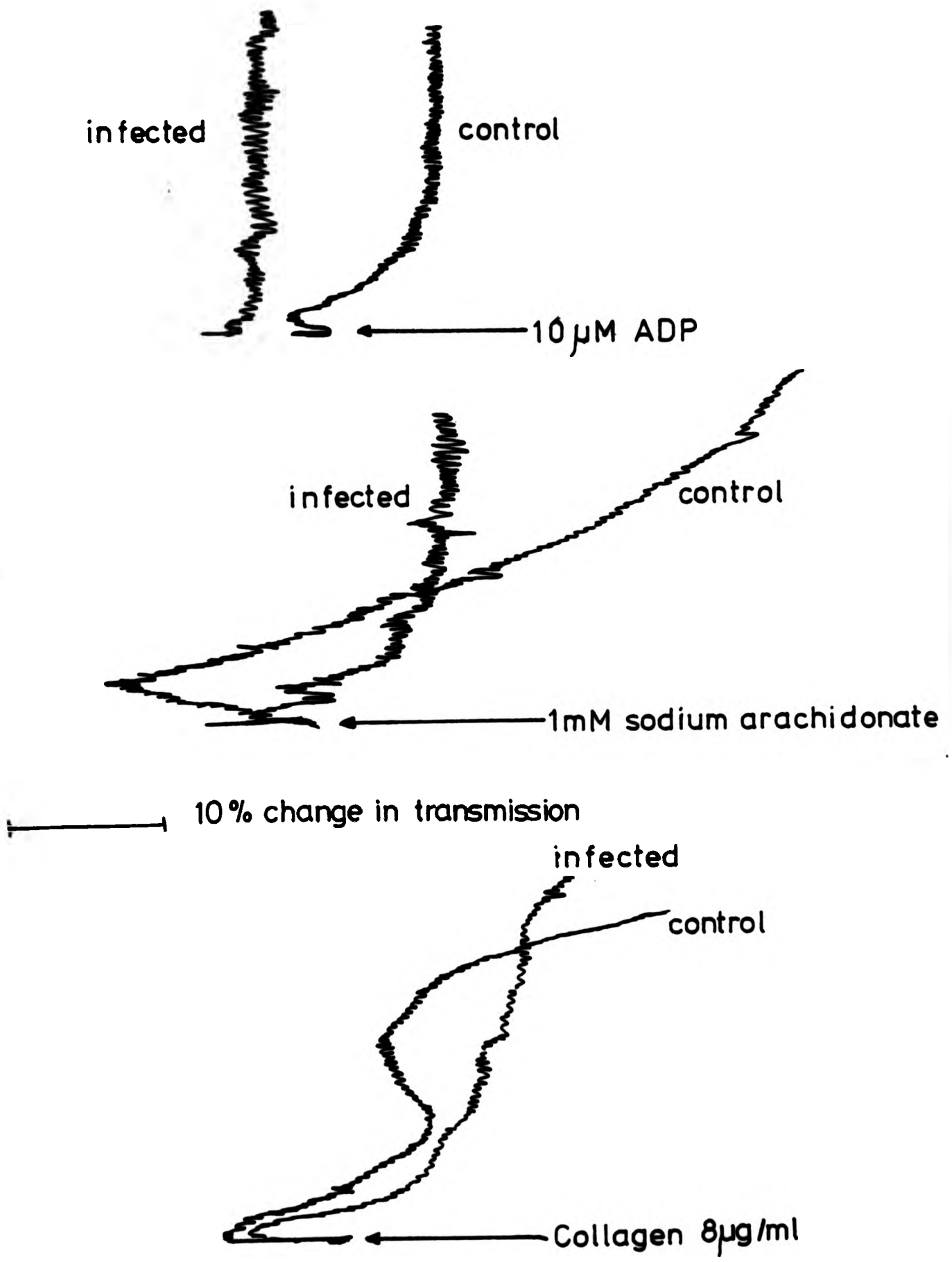
For key see Table 4.5

THRESHOLD LEVELS OF AGGREGATION RESPONSES TO SODIUM ARACHIDONATE - FINAL CONCENTRATIONS IN mM

RABBIT	GRE	HUR	MCN	SHA	WIL	WOR
Pre-infection	0.25	0.5	0.5	0.25	0.5	0.5
Infection day						
3	0.25(0)	0.5(0)	0.5(0)	0.25(0)	0.5(0)	0.25(0)
6	0.25(0)	0.25(1)	0.25(0)	-	-	-
7	-	-	-	***	0.5(0)	0.5(0)
8	0.125(1)	0.25(0)	0.5(0)	-	-	-
12	-	-	-	>1.0(0)	1.0(3)	***
15	1.0(7)	0.5(2)	0.5(2)	*	-	-
22	1.0(36)	1.0(45)	**		-	*
Control	0.5	0.5				
27	1.0(144)	*	*			1.0(162)
Control	0.25					0.25

For key see Table 4.5

Fig 4.44



AGGREGATION RESPONSES - RABBIT INFECTED T B BRUCEI (DAY 27) PLUS CONTROL

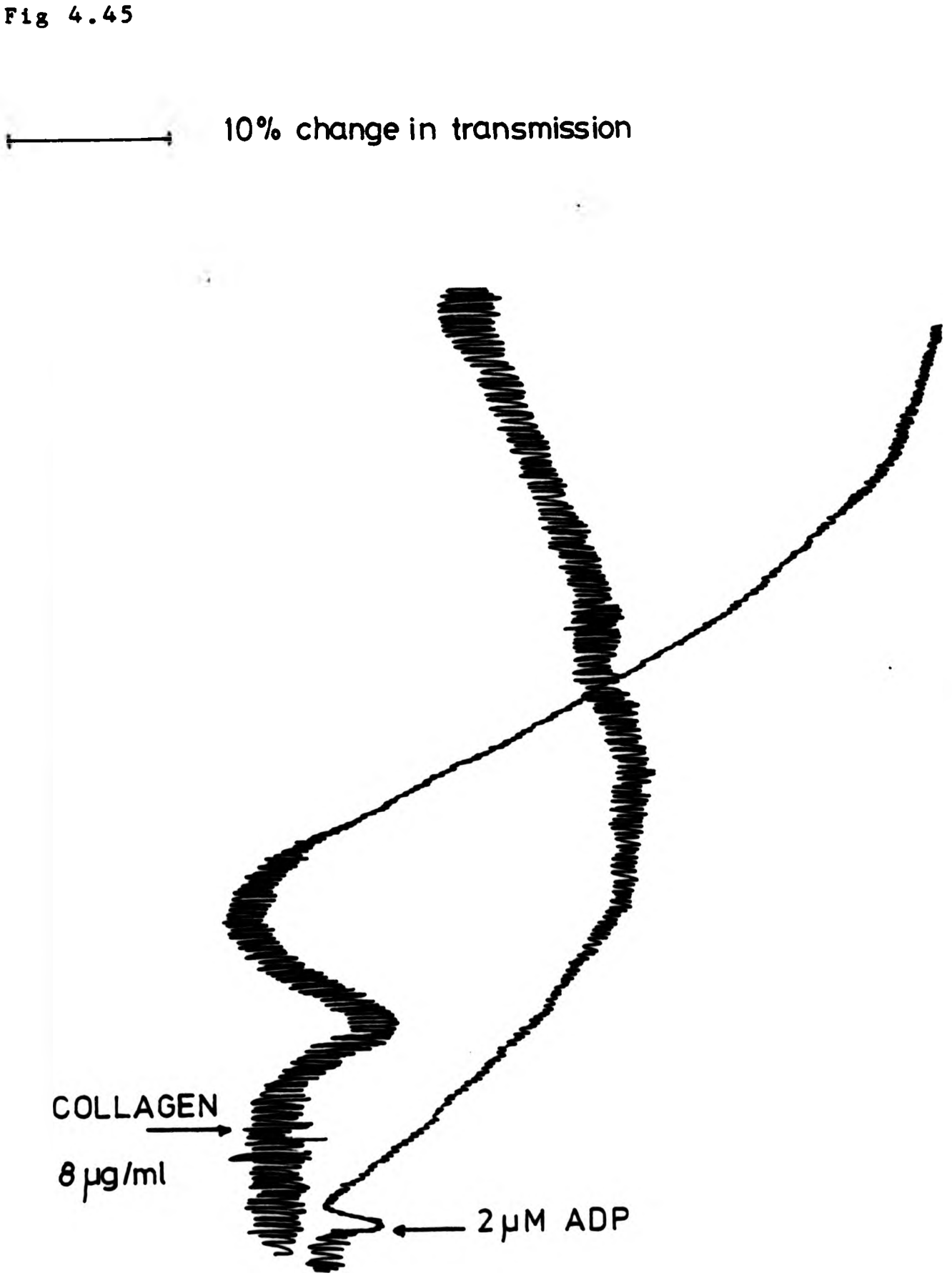
concentration of aggregating agent in an uninfected control sample at the same platelet count as the test.

In summary, ex vivo platelets from infected animals showed a deterioration in response to aggregating agents in vivo in the second week of infection. Normal platelets did not appear to be aggregated by trypanosome extracts in vitro and these platelets aggregated normally when exposed to collagen.

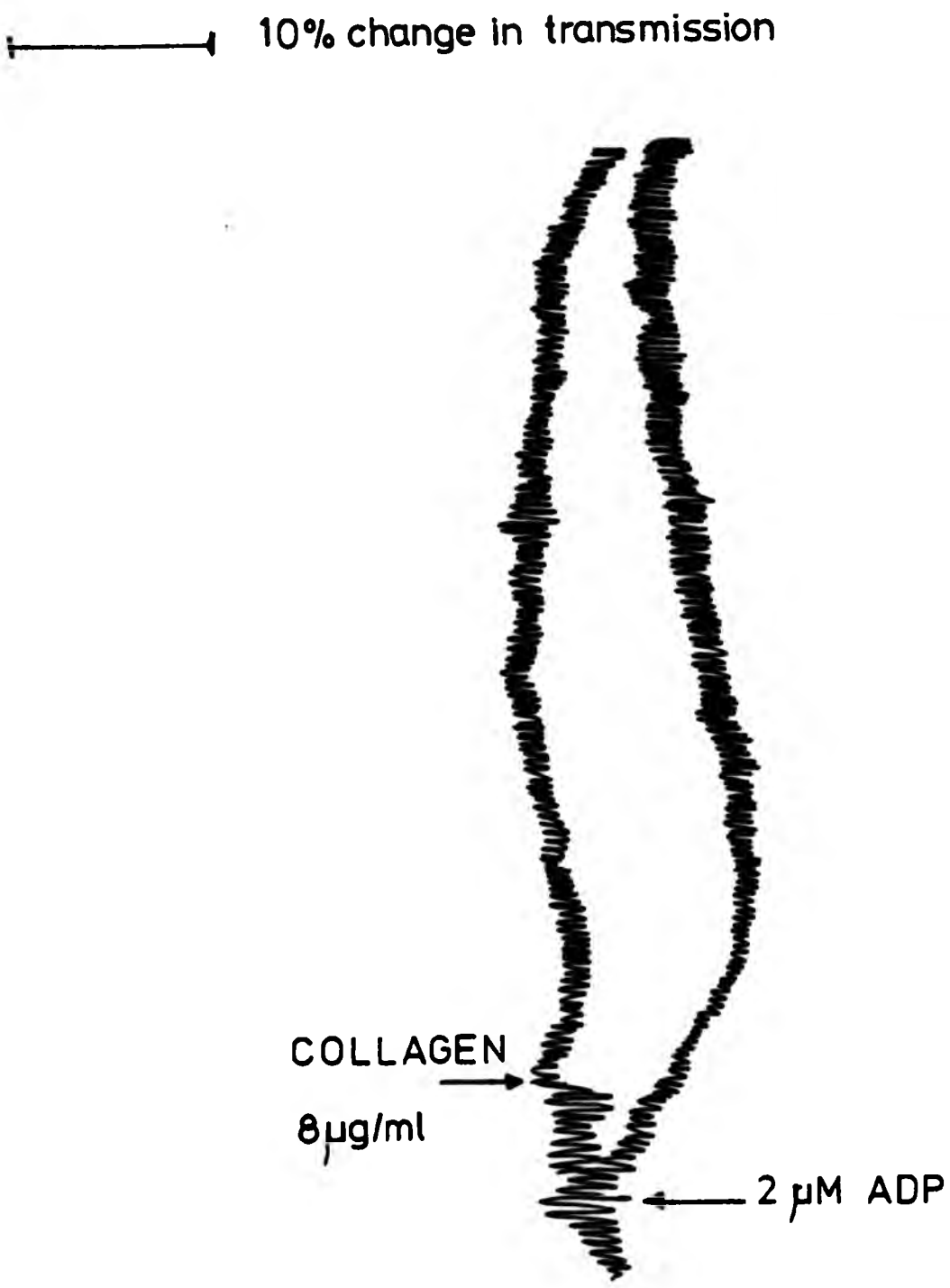
4.7.2 T vivax infections

Because of the volume of platelet-rich plasma required by the aggregometer used in these experiments, qualitative responses only to aggregating agents were examined. ADP was used at a final concentration of 2µg/ml and collagen used at a 1/50 dilution. A typical response to ADP and collagen pre-infection is shown in Fig.4.45.

Calves examained on day 1 of the infection gave normal aggregation responses. By day 7 aggregation was poor and associated with high trypanosome counts (Fig.4.46). From day 10 no aggregation responses were seen although a response could sometimes be elicited if extra quantities of aggregating agents were added. Generally, at this time, trypanosomes were absent. From day 17 until any infection was terminated, the response was



AGGREGATION RESPONSES - CALF UNINFECTED



AGGREGATION RESPONSES - CALF INFECTED T VIVAX
(DAY 7)

either poor or negative and was not dependent on the presence or absence of trypanosomes as judged by a buffy coat detection method.

One calf was examined about 36 hours after Berenil treatment; the platelets aggregated normally as did other calves' platelets during other between infection periods.

A mixing experiment was performed on day 12 of an infection when trypanosome numbers were very high using various 50% incubation mixtures of infected and uninfected platelet-rich and platelet-poor plasma. The results are shown in Table 4.8. Platelets from the infected animal gave aggregation responses only when mixed with control platelets and control platelets were not prevented from aggregation when mixed with infected platelet-poor plasma. If trypanosomes were actively producing substances inhibiting platelet aggregation, one might expect it to be present in the platelet-poor plasma - it appears that this is not the case, and that there is a need for the platelets to acquire their damage in vivo or that a more prolonged exposure to trypanosomes or their toxins is required.

Thus, throughout the infection aggregation responses are considerably diminished. The rapidity with which these

Table 4.8

AGGREGATION RESPONSES IN T.VIVAX INFECTION

MIXING EXPERIMENT

	Infected		Control	
	PRP	PPP	PRP	PPP
PRP	-ve	-ve	+ve	-ve
Infected PPP	-	-	+ve	-
PRP	-	-	+ve	+ve
Control PPP	-	-	-	-

PRP = platelet-rich plasma

PPP = platelet-poor plasma

responses return to normal after treatment with Berenil is of interest.

4.8 PLATELET MALONDIALDEHYDE PRODUCTION

Several workers[44,49] have suggested that early release of pharmacologically active substances such as plasmin and kinins are involved in the pathogenic mechanisms in trypanosomiasis. Goodwin[7] has speculated that prostaglandins may be involved and Tizard[43] has shown that trypanosomes produce phospholipase A, which acts on all membranes and surface active molecules such as free fatty acids. It is phospholipase A_2 that releases arachidonic acid from platelet membranes to take part in prostaglandin production.

MDA levels in platelets were measured during a *T b brucei* infection (Experiment VI) in order to assess the role that prostaglandins might have during the disease. Levels of MDA fluctuated enormously in both control and infected animals but higher levels were seen in the infected group in the latter part of the experiment (Fig.4.47). Kendall's S test confirmed that production of MDA was higher in the infected animals than in the controls ($z=3.256$, $p<0.01$). There appeared to be little association between the amount of MDA produced and the

Fig 4.47

EXPERIMENT VI - MDA IN T B BRUCEI INFECTION

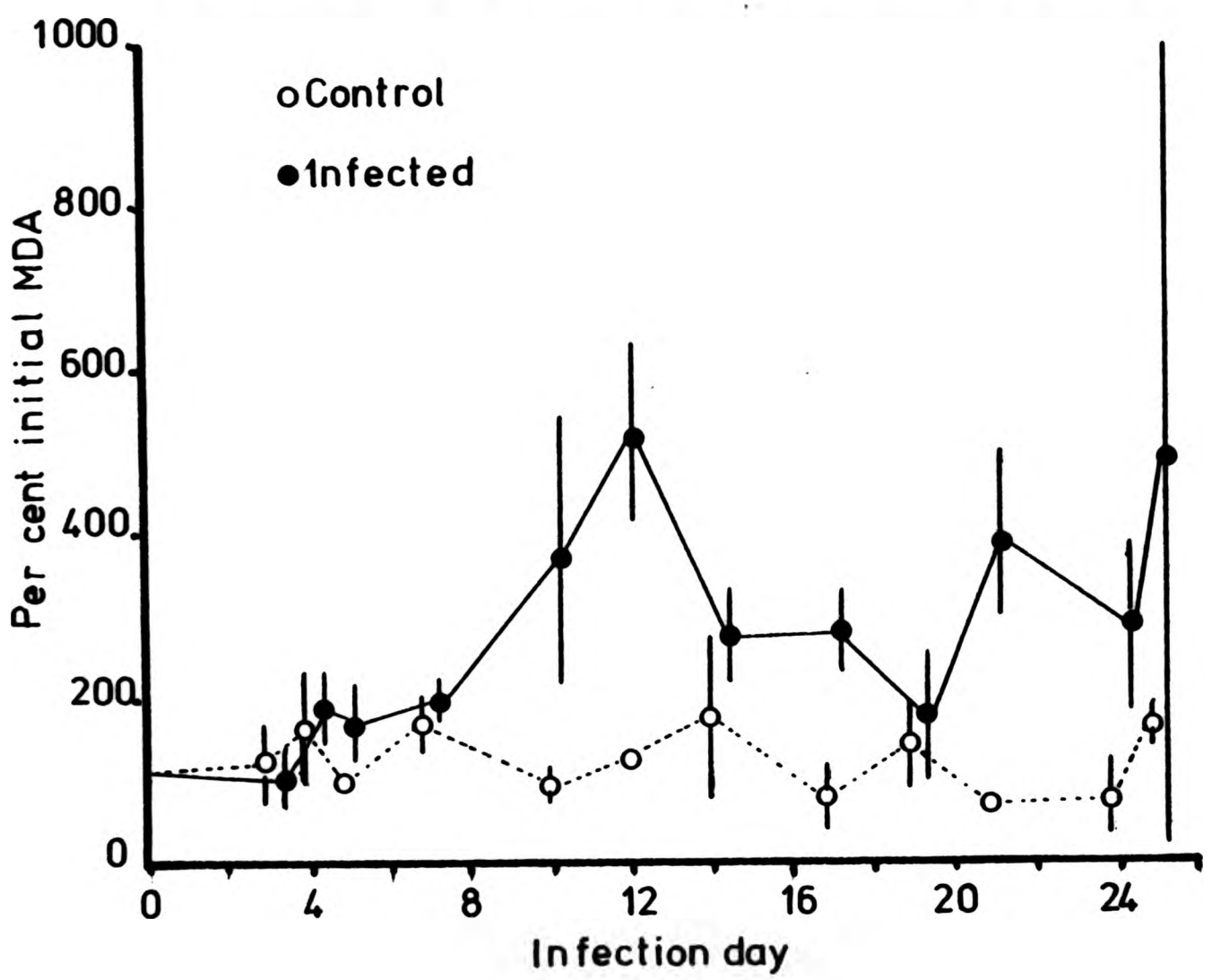
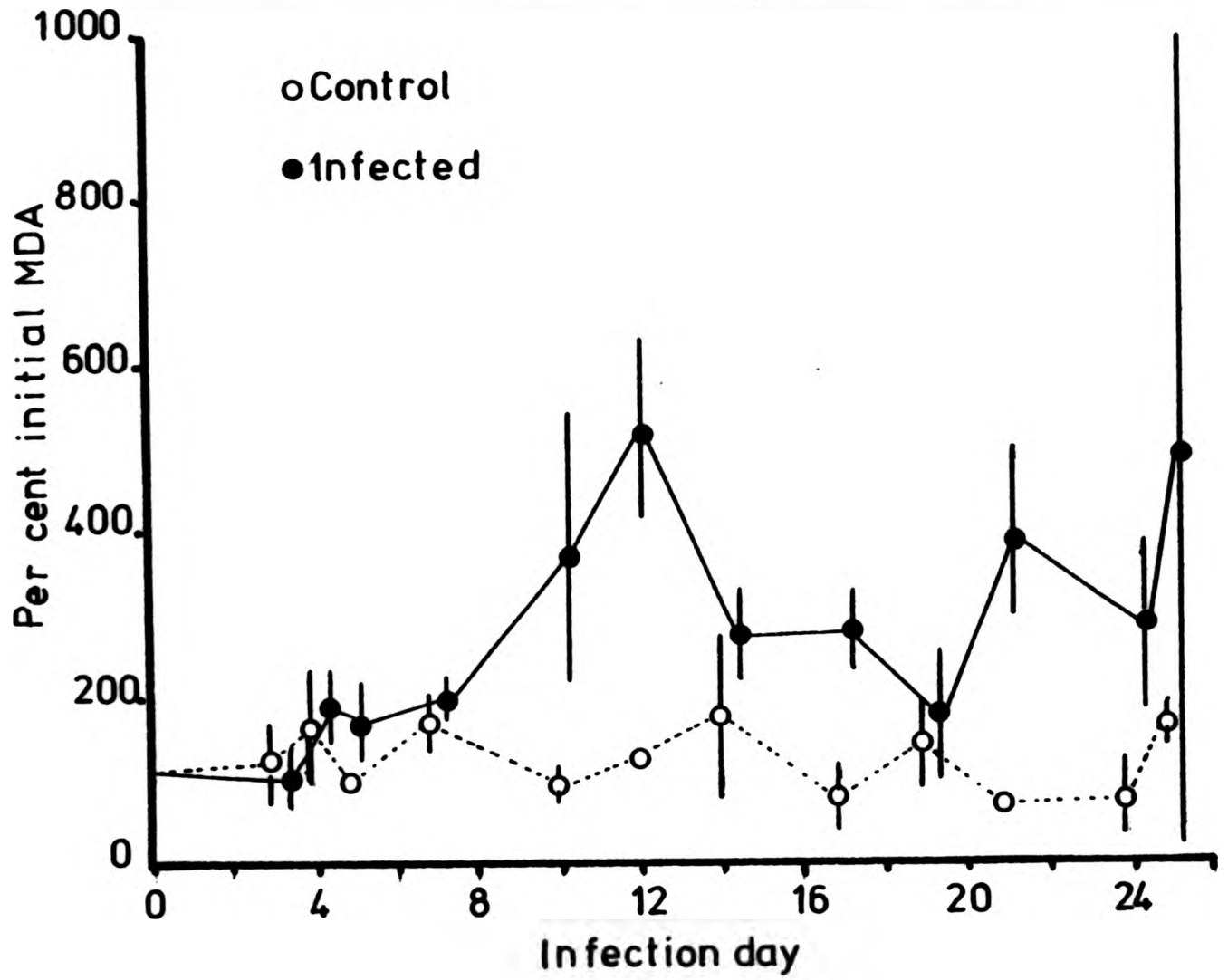


Fig 4.47

EXPERIMENT VI - MDA IN T B BRUCEI INFECTION



trypanosome concentration (Spearman's rank correlation coefficient = 0.437).

Thus, far from finding that platelets had reduced amounts of MDA caused by early release of pharmacologically active mechanisms, increased levels were found in the infected platelets. This will be discussed further.

4.9 PLATELET LIFESPAN (Experiment X)

In an attempt to measure platelet lifespan under normal and pathophysiological conditions many different radio-isotopic labels have been used, some being more appropriate than others[144]. Not only does careful choice have to be made of label but the method of analysis is also important[89,145]. Different methods of calculating survival produce quite different results and cannot be compared with each other directly.

Several people have measured platelet lifespan in the rabbit; all used ^{111}Indium oxine as label. Wistow et al[107] found that survival followed an exponential function and gave a mean survival of 1.3 days. Schmidt and Rasmussen[110] measured survival in 5 rabbits by a linear and an exponential method giving mean results of 4.1 days and 1.4 days respectively. Trowbridge and

Martin[146] have used the Dornhurst model, one recommended by the International Committee for Standardisation in Haematology[89], which gave a 2.53 day mean lifespan in rabbits. Scheffel and others[109] calculated rabbit platelet survival as 5.12 days (linear model), 1.22 days (exponential model) and 2.84 days (gamma function model).

Reports on platelet survival in trypanosomiasis are few. Robbins-Browne et al[30] used ^{51}Cr labelled allogenic platelets in three patients with T rhodesiense and measured lifespan with a linear model. They found mean survival of 13 hours, 4.7 days and 1.9 days in their patients which contrast with a normal survival of 7-10 days in normals using this method. Reduced platelet lifespans have also been reported in T congolense infections of steers using ^{51}Cr labelled heterologous platelets and regression analysis[85]; infected cattle showed lifespans of 0.8-2.1 days compared with 2.9-4.5 days in controls. In contrast, Forsberg et al[84] using ^{35}S-methionine as a cohort label of platelets in calves infected with T congolense, found no evidence of a reduced platelet lifespan.

Platelet survival has been measured in our experiments using $^{111}Indium$ oxine labelled platelets and a gamma function model as recommended by the ICSH[89].

Comparisons were made with linear and exponential models at the same time but because the residual sum of squares was considerably smaller in the gamma function model in all cases the latter model has been used throughout. Labelling efficiency was around 75%.

4.9.1 T b brucei infections

Platelet lifespan has been measured in two series of T.b.brucei infections of rabbits. In the first (A) measurements were made in seven control rabbits. Three of these rabbits were infected, platelets labelled on days 2 and 22 of the infection and measurements of lifespan made over the next 8 days. The results are shown in Table 4.9. Although there was an apparent reduction in lifespan in the early part of the infection only three animals were involved and the difference was not significant (unpaired $t=1.14$, $df=7$, $p>0.05$). The reduction in lifespan was marked towards the end of the infection and highly significant (unpaired $t=5.02$, $df=7$, $p<0.001$). In the second infection (B) six rabbits (three splenectomised) were measured before and from day 5 of an infection. The results are given in Table 4.9. Platelet lifespan was significantly reduced (paired $t=2.81$, $df=4$, $p<0.05$) and there were no apparent differences between splenectomised and intact animals.

PLATELET LIFESPAN IN T.B.BRUCEI INFECTION (days)

A.

RABBIT	PRE-INFECTION	DAY 2	DAY 22
IVE	2.40	1.98	1.25
URI	1.68	1.65	*
RAW	2.40	2.14	0.97
TIP	2.18		
POP	2.48		
VIV	2.01		
STR	2.14		
mean	2.18	1.92	1.11
Sem	0.10	0.15	0.14

B.

RABBIT	PRE-INFECTION	DAY 5
APP**	1.53	0.63
BLA**	1.79	1.69
CHE**	2.38	1.71
DAM	1.88	*
ELD	1.57	1.47
FIG	2.59	2.17
mean	1.96	1.53
sem	0.18	0.25

* rabbit died

** splenectomised rabbit

4.9.2 T vivax infections

Because of the timing of infections only two calves had their platelet lifespan measured before infection. Platelets were labelled on day 0 and lifespan measured over 10 days of the infection; there was a significant reduction (unpaired $t=2.24$, $df=4$, $p<0.05$). Lifespan measurements were also made at two different times after treatment with Berenil and appeared to be considerably prolonged. The difference was however just not significant when compared with pre-infection values (unpaired $t= -2.02$, $df=3$, $p=0.068$) but was highly significant compared to infection values (unpaired $t= -4.04$, $df=5$, $p<0.01$). Results are given in Table 4.10. The possible statistical significance of these results were obviously impaired by the inherent restrictions in the study of animal number and timing of infections but a clear reduction in lifespan was demonstrated.

Table 4.10

PLATELET LIFESPAN IN T.VIVAX INFECTIONS (days)

CALF	PRE-INFECTION	DAY 0	POST-BERENIL(days)
BLU	3.02	1.08	-
BOR	2.92	1.92	4.83(4)
GEO	-	2.61	4.16(22)
TOG	-	2.03	7.23(4)
mean	2.97	1.91	
sem	0.05	0.31	

5. DISCUSSION

5.1 THE INFECTION

The infection observed in rabbits with T.b.brucei was typical of that described by others[26,53]. Five rabbits underwent a very acute course with death around 8 days into the infection. Two of these deaths appeared to be due to a massive haemolytic anaemia and related to the very high parasitaemia also seen. Although the anaemia may have been caused by a massive immune sensitisation of the red cells, it is possible that death was precipitated by a massive immune trypanolysis causing a shock syndrome and haemolytic crisis, similar to that sometimes seen after administration of trypanocidal drugs with a high parasitaemia[9]. The high concentrations of various toxins produced in these circumstances could easily bring about death. A further two rabbits had experienced a rapid and marked thrombocytopenia and it is likely that DIC was the cause of death. Since neither rabbit had experienced a massive intravascular tryopanolysis it is likely that the mechanism was due to an initiation of DIC through antigen-antibody complex formation.

The high parasitaemias seen here and by others[67] in splenectomised animals are likely to be due to the

absence of an organ which can rapidly sequester the infecting organisms. Desowitz et al[114] suggest that splenectomy diminishes the production of antibody and while this may be the case the differences between the two groups were right at the beginning of the infection, probably before a sufficient antibody response had been mounted.

The role of the spleen for the continuing removal of parasites was emphasised in the T congolense infections, which had been particularly long lasting chronic infections with rarely detectable parasites. Removal of the spleen brought about a rapid increase in parasite number in the days following.

Reports of T congolense infections have described some acute, some subacute and some chronic infections and certainly we have seen significant differences even between the two strains used to infect our rabbits which may account for the different courses. There was also a death in this infection, probably again due to DIC.

The T vivax infections in calves were similar to those described by others, the first parasitaemia peak showing the highest number of trypanosomes with cyclic fluctuations but the pre-patent period was only a few days in the first infections. There appeared to be some

conferment of immunity by repeated infection but it was not long lasting. Wellde et al[127] also showed immunity in T congolense infected calves which was apparently complete on a third challenge. He points out though that the degree of resistance also correlates with the duration of infection and in our experiment these times were particularly short (13 and 31 days respectively).

5.2 THE ANAEMIA

As others have observed T b brucei causes a significant anaemia in rabbits related to the developing parasitaemia suggesting an early haemolytic mechanism with the red cells being destroyed in the spleen. We found quite a variability in the severity of the initial fall in haematocrit between experiments although later the severity of the anaemia was similar across the experiments. The same number of organisms were injected in each case and it is possible that the differences were related to the passaged material being in different stages when the mice were killed resulting in an initial variability in immune response within the new host.

The splenectomised animals did not become anaemic as rapidly as the intact animals, presumably because the early sensitised red cells are removed by this organ.

As it appears that the spleen also has a role in removing circulating parasites the early advantage is diminished and the resulting higher parasitaemia may be contributing to the early death seen by us and others[67].

McCrorie et al[67] have shown that the red cell life span is near normal in splenectomised animals; if the role of the spleen had been taken over by the liver or bone marrow this would have also been reflected in the survival curves but as we and they have shown, the anaemia continues to develop in these animals and suggests that another mechanism is involved. Haemodilution is an unlikely possibility, especially as splenectomy often alleviates increased plasma volume in patients with splenomegaly[147] and a more likely hypothesis is that the later anaemia is due to development of an anaemia of chronic disorders[64].

The anaemia in the T congolense infections was relatively mild compared with reports of infections in more usual hosts. The differences seen in the two strains have already been discussed.

In the T vivax infections the only remarkable event in terms of the anaemia was the apparent development of an early immunity with repeated infections which along

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In the T vivax infections the only remarkable event in terms of the anaemia was the apparent development of an early immunity with repeated infections which along

with, and probably because of, the later parasitaemia, delayed the onset of the anaemia.

5.3 THE PLATELETS

5.3.1 Platelet number

There seems little doubt that platelet numbers were reduced by T b brucei infections in rabbits. The early experiments produced a rather confusing picture and it was not clear whether the platelet number reported were accurate in any way. The platelet counting method used from experiment III onwards, while not necessarily improving the accuracy of the counts, was likely to improve the precision and made assessments of the situation easier.

In both experiment I and II the thrombocytopenia was immediately marked, before the development of a significant parasitaemia and certainly before an immune response could be mounted. Production of a toxin released from the multiplying living trypanosomes[9] seems to be the only possible mechanism at such an early time although whether sufficiently high concentrations could be reached so early is unknown. A more likely explanation is the chance variability due to the inherent difficulties in performing these manual platelet counts. Early thrombocytopenias are found in

the later experiments also but these were associated with early parasitaemias.

The changing pattern of platelet number seen in most of the experiments was a marked thrombocytopenia over the early part of the infection associated with the parasitaemia, an increase in number towards the middle of the infection with a reduction in parasite number and a later milder thrombocytopenia, again apparently associated with the increased parasitaemia often seen towards the end of the experiment.

Experiment VIIIwas slightly different showing the early changes but then platelet numbers remained high throughout the rest of the experimental period despite the high parasite numbers at this time, suggesting that, like that proposed for the anaemia, two different mechanisms are involved in the aetiology of the thrombocytopenia. Probably the initial reduction is related to the parasite presence with the resultant formation of complement-fixing immune complexes causing platelet destruction. As a result of the increased platelet destruction, the bone marrow is stimulated to produce more platelets by increasing its megakaryocyte number to a maximum around day 18 of the infection declining thereafter[64]. When the immune stimulus decreases in intensity with the reduction in parasite

number so will the platelet destruction. The marrow cannot respond as rapidly and will continue to produce greater numbers of platelets resulting in a reverse thrombocytosis which, depending on the remaining influence of the immune removal, may result in platelet counts much higher than normal. Essien and Ikede[124] have reported a thrombocytosis also, in goats infected with T b brucei. Because of the massive red cell destruction there has by now been an enormous expansion of the reticulo-endothelial system and it may be that this system, either because of its extended size or because it is in some way primed, takes over the removal of platelets which continues at a slower rate irrespective of parasite numbers.

The pattern of rapidly decreasing platelet number followed by a partial recovery was repeated in the T congolense infections. Here the platelet count appeared to fall before the parasites appeared but only wet preparations had been examined for trypanosomes and there may well have been significant numbers (in terms of their effect on platelets) earlier. The splenectomised animal again highlighted the probability of later destructive mechanisms being different - a rapid return of platelet count to normal despite the increasing parasitaemia. It seems likely that the spleen was playing an important part in keeping the

platelet number reduced in the chronic stage of the infection. When splenectomies were done before the T b brucei infections (experiment II and VII) there were no differences in the platelet counts. It seems likely that another part of the reticulo-endothelial system had expanded in the early stages of infection because of the lack of a spleen and assumed the splenic role in the later stages.

There was nothing unusual about the thrombocytopenia in the cattle infections - like the anaemia its onset was delayed with repeated infections, but not its extent, again supporting the idea of different mechanisms.

The experiment (V) with aspirin was interesting although the thrombocytopenia seen in the untreated infected animals was not very great. The treated animals however showed massive increases in platelet number in the infection. Van Miert et al[47] had done a similar experiment in goats infected with T vivax in an attempt to prevent the platelet aggregation and serotonin release he had found associated with the parasitaemia. He gave flurbiprofen, like aspirin also a potent non-steroidal anti-inflamatory agent blocking the cyclo-oxygenase enzyme, but found not only that platelets continued to release serotonin, and form aggregates as before but that blocking the febrile response produced

a massive parasitaemia culminating in the animals' early death. He suggested that the drug was inhibiting the host defence mechanism and that a fulminating disease with DIC resulted. The same pattern was not observed in our animals, parasite numbers remaining similar, and that may simply be a reflection of the different tissue locations of these parasites. We too though continued to see the platelet clumping in the treated animals. The arachidonic acid pathway is only one of several mechanisms through which platelets are activated and aggregate and it may be that one of the other paths are active here. Van Miert[47] did show some effect of drug treatment - the microthrombi found at post-mortem tended to be fibrinous rather than composed wholly of platelets.

The increased platelet number in our aspirin treated animals was more of a mystery - with no thrombocytopenic stimulus to the bone marrow, there would be no reason for the increased production - but perhaps the stimulus comes instead from the trypanosome and aspirin here is indeed having a beneficial effect on preventing activation, aggregation and removal thus causing the build up. Another possible explanation would be that the aspirin is prolonging platelet lifespan producing a temporary build up in these animals. This seems unlikely as Stuart et al[148] have shown that platelet

lifespan, measured using an aspirin technique, is no different from the lifespan obtained with ^{51}Cr techniques. Prevention of the thrombocytopenia has not helped in the final outcome of the disease.

5.3.2 Platelet size

The giant platelets seen in blood films in both the T b brucei and T vivax experiments are evidence for increased megakaryocyto-poiesis and consequent increased platelet production. Both the electronic measurements (in T b brucei infections) and measurements made from the electronmicrographs (in T b brucei and T vivax infections) of platelet volume reflected the stained blood film picture and the shape change seen in the electronmicrographs. The increase in size was noted within a day of the thrombocytopenia being recognised in the counts. Ebbe et al[149] have showed that rat platelet production from megakaryocytes begins two days after induction of a thrombocytopenia and the megakaryocytes reach a maximum size within 10 days. The one day lag phase in our findings is probably compatible with these results and if megakaryocyte size can be used as a prediction of platelet size[95] then the increasing platelet volume seen over the first ten days or so will be simply a reflection of the bone marrow changes in megakaryocyte volume.

Removal of parasites with Berenil in the T vivax infections did not result in a return in calf platelet size to normal, even after a long period of time. The animals remained relatively thrombocytopenic so presumably the stimulus for giant cell production remained.

The difference in platelet size between splenectomised and intact animals in the second half of a T b brucei infection was interesting and raises more questions than it answers. The splenectomised rabbits' platelets appeared smaller. It is possible that the spleen was, in the intact animals, actively and differentially removing the smaller (older) platelets although we would expect the platelet distribution width to have shifted rather than increase. Another possibility is that the higher MPV and PDW measurements reflect increased clumping as well as larger platelets and that if the spleen is actively sequestering platelets it may also be damaging platelets in such a way to cause greater clumping as some of the platelets continue to circulate. One might ask if the trypanosomes being destroyed in the spleen are providing an unhealthy environment for platelets that continue to circulate and they are being activated by trypanosome toxins produced locally.

5.3.3 Platelet aggregates

The finding that platelets were circulating as preformed aggregates during the infection was a significant one in terms of the pathogenesis of the disease. Originally it was thought that the clumps, commonly seen in wet preparations and blood films, might have been due to cold acting autoantibodies active *in vitro*, although warming the wet preparations did not decrease the clumping. The method used is designed to rapidly fix preformed aggregates as soon as they leave the blood vessel, thus reflecting as near as possible the *in vivo* situation; the authors have used the method finding decreased ratios in patients with arterial insufficiency[97].

Platelet aggregates interfere with the vascular supply and cause impairment of organ function; thus their presence may contribute to the pathology of trypanosomiasis. Micro-thrombi have been reported in the small vessels of infected animals post-mortem [18,20,31,34,48,48,78]. Maxie and Valli[57] considered that formation of platelet aggregates was not life threatening unless DIC occurred. There have however been several reports of microaggregates without thrombus formation (and without DIC) associated with sudden death[150-152] and the possibility of this phenomenon also causing death in trypanosomiasis cannot

be disregarded.

The possible cause of the platelet clumping may be platelet activation by a trypanosome-release toxin, immune complex formation or possibly a reduction in surface charge on the platelet.

5.3.4 Platelet ultrastructure

Davis et al[83] are the only people to have examined platelets in the disease by electronmicroscopy. They were looking at preformed aggregates produced by parasites in vitro so whatever the mechanism of aggregate formation was, one would have expected to see marked morphological changes.

In our experiments platelets from infected T b brucei and T vivax animals have been fixed within a few minutes of their removal from the body. Thus we hoped to attain a true picture of the circulating platelet morphology in the infection. The changes were marked, especially in the early stages of infection when parasites were present. Not all the platelets showed changes by any means but the most common observation was probably the change in shape seen in both infections. The centralisation of organelles with the movement inwards of microtubules and degranulation indicative of platelet activation were also prominent features.

It is difficult to escape the feeling that these changes have not been brought about by an in vivo pharmacological stimulation. Tizard et al[9] have reviewed the numerous different substances that are produced from living and dead trypanosomes, among them phospholipases and free fatty acids. Phospholipase A_2 mobilises the fatty acid, arachidonic acid, from platelet membranes to participate in the pathway forming the pro-aggregatory thromboxane A_2, and free arachidonic acid also stimulates thromboxane A_2 production. While Tizard[153] has not shown arachidonic acid to be produced in large amounts, Tai et al[146] have shown that arachidonic acid is produced in T b brucei S42 infections of mice. The levels of this and other polyunsaturated fatty acids reduce during the infection, probably because they are metabolised to other substances such as prostaglandins and thromboxanes. The combination of trypanosomes and their antibodies activating platelets as suggested by Slots' experiments[39] cannot be ignored as a mechanism.

Although platelet number and morphology improved in the T vivax infections after Berenil treatment the morphology did not return completely to normal. The platelets appeared relatively normal apart from their more rounded shape and more variable size. It is possible that the changes seen relatively late after the

Berenil 'cure' were due only to the altered megakaryocyte mass continuing to produce larger platelets and that this would normalise in time.

5.3.5 Platelet aggregation

We could not repeat the in vitro mixing experiments of Davis et al[83] and Slots and van Miert[39]. We did not measure serotonin release but platelet aggregation to collagen remained normal after incubation with the trypanosomes. Anti-trypanosome antibodies were likely to be present in the platelet rich plasma of the infected source of our trypanosomes in these experiments but whether they were directed against the current variable antigen type is not known.

The ex vivo quantitative experiments in T b brucei rabbit infections showed that platelets were functionally as well as morphologically abnormal, being less responsive to aggregating agents. The lesion in calf platelets appeared to be more severe with little or no aggregation response to the aggregating agents. The defect was most marked shortly after the first parasitaemia when the levels of trypanosome antigen-antibody complexes or trypanosome toxins would be expected to be at their highest. Circulating trypanosome numbers are higher in T vivax infections than T b brucei which might have caused the more severe

defect in the former infections.

There was thus a marked contrast between the ex vivo and in vitro experiments. Uninfected control platelets used in these experiments have not been exposed to the possible inhibiting or aggregating agents either for long periods of time or within particular micro-environments in the body which are likely to be quite different from the environment in a plastic tube.

Whether the apparent depression of platelet function was due to some form of inhibitory action to the platelets or whether the platelets were 'spent' of their enzymes by granule release from previous encounters is not clear. It appears that platelets 'activated' by exposure to pro-aggregatory environments in the body are hypersensitive to aggregating agents rather than less sensitive[154]. The numbers of degranulated platelets in the electronmicrographs was not sufficiently large to account for such a gross response in the T vivax infections. Johnson[155] has shown that after thrombin addition to platelet rich plasma the granules disintegrate into a collection of vesicles which may be mistaken for storage granules so it is possible that we too had not recognised this change in the platelets. An inhibitory action seems more likely although the mechanism is not obvious. If this is the case then the

platelet clumps found are unlikely to have been produced by a platelet aggregatory mechanism. It would not explain how platelets seen in the electronmicrographs had been 'activated'.

Whatever the mechanism of decreased platelet aggregation in the infection there is a rapid return to normal aggregation after treatment with Berenil and it is tempting to speculate that an inhibiting factor provided by the trypanosomes might have been removed allowing the presently circulating platelets to return to normal. New platelet production can be very rapid at this time and without the activating mechanism will aggregate normally.

An inhibition of platelet function during the infection may put the host at greater risk haemostatically. Severe haemorrhages are associated with T vivax infections but DIC is thought to be the mechanism here[77].

5.3.6 Malondialdehyde production

Production of malondialdehyde from platelets is a measure of prostaglandin synthetase activity. Stuart[156] has used the production as a measure of hypersensitivity. Our findings of higher MDA levels in the infected animals supported the theory of inhibition

of platelet aggregation rather than most platelets having gone through aggregation and release. There was obviously plenty of potential for MDA production.

Not only was MDA not reduced but it was higher than in the control animals and not apparently derived from trypanosomes. There is plenty of evidence to suggest that larger platelets are more active - because of their size they will contain more ATP, BTG and enzymes[130,157-159]. Platelets produced with a larger mean platelet volume under thrombocytopenic stress also produce more thromboxane B_2 per unit volume, that is, two platelets of the same size will produce different amounts of TXB_2 if each is produced under different circumstances - normal and thrombocytopenic[131]. This would explain the higher MDA levels found in the infection.

5.3.7 Platelet lifespan

Significant reductions in platelet lifespan, using the cohort label ^{111}Indium oxine, were found in both the T b brucei and T vivax infection and supports the theory for increased platelet destruction being the cause of the thrombocytopenia. Robins-Browne et al[30] and Preston et al[85] have also shown reduced lifespans in T rhodesiense and T congolense infections respectively using ^{51}Cr as a cohort label.

In contrast Forsberg and her co-workers reported a normal platelet lifespan in T congolense infections in calves[84]. They suggested that thrombocytopenia was due to ineffective thrombopoiesis and backed up their hypothesis with histological examination of the megakaryocytes[23]. Their study can be criticised however by their choice of ^{35}S-methionine as platelet label. The International Committee for Standardisation in Haematology (ICSH) in their recommended methods for readioisotope platelet survival studies[89] have said that cohort labelling using ^{35}S-methionine is not a satisfactory method as the compound remains available for labelling megakaryocytes and platelets for a long time compared with the platelet survival time; thus a true shortened lifespan may appear to be normal. The ICSH have recommended ^{51}Cr as the most useful label but since their report the use of ^{111}Indium oxine has been shown to produce similar results to ^{51}Cr and to have other advantages over the latter label[106].

Berenil treatment in the T vivax experiments produced platelet lifespans that were much longer than normal. With removal of the probable parasite-related stimulus for platelet destruction relatively early in the infection large numbers of platelets would continue to be released from the expanded megakaryocyte mass - a rebound thrombocytosis. The combination of large

numbers of young platelets and the fact that platelets which are larger in size also have an intrinsically longer survival[157] is likely to be the cause of the prolonged survival reported in this study.

We saw no difference in platelet lifespan in splenectomised and intact animals. We might have seen differences if the spleen had been removed late in the infection when this organ was probably involved in the continuing removal of platelets, but in animals asplenic before infection the sequestration role of the spleen was probably taken over by other areas of the expanding reticulo-endothelial system. We have evidence from in vivo studies (not presented in this thesis) that both the liver and bone marrow are important in the sequestration of platelets in this situation.

In view of the reduced lifespan in both normal and asplenic animals, pooling, in the spleen or another organ, seems an unlikely mechanism for the thrombocytopenia by itself.

5.4 CONCLUSIONS

1. T b brucei, T congolense and T vivax all produce an anaemia and thrombocytopenia which may, although rarely, result in death.

2. Both the anaemia and the thrombocytopenia are mediated through different mechanisms at different stages of the disease. The early stage is parasite-related, probably through an immune complex mechanism, and the later more chronic stage mediated by the expanded reticulo-endothelial system.

3. Measurements of platelet size show an increase during the infection. The increase appears to be a reflection of both true size increase (giant platelet formation) and increased platelet clumping.

4. Circulating platelet aggregates are present which might contribute to the pathology of the disease by causing death or local tissue and organ ischaemia.

5. Platelets show ultrastructural changes of activation and granule release which are more marked early in the infection when parasites are prominent.

6. Normal aggregation responses in the infection are reduced, especially in T vivax infections, putting animals at risk haemostatically.

7. The high potential for prostaglandin synthesis in the platelets from infected animals suggests that their reduced aggregation response ex vivo results from an

inhibition received <u>in vivo</u> rather than an exhaustion of the platelets through aggregatory mechanisms.

8. Platelet survival is reduced in the infection. Treatment with Berenil results in prolonged survival and suggests that there is increased platelet production with even greater destruction. Organs other than the spleen may be involved in the destructive process.

MATERIALS

1. PHOSPHATE BUFFERED GLUCOSE-SALINE (PSG)

Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$)	0.468g
Di-sodium hydrogen orthophosphate (Na_2HPO_4)	8.094g
Sodium chloride (NaCl)	2.55g
Glucose ($C_6H_{12}O_6$)	15.0g

Make up with distilled water to 1 litre and adjust pH to 8.0.

2. MAY GRUNWALD-GIEMSA AUTOMATED STAINING METHOD

Solutions:

1. May Grunwald stain - diluted equal parts with buffered water pH 6.8
2. Giemsa stain 'R.66' - diluted 1/10 with buffered water pH 6.8
3. Methanol
4. Buffered distilled water, pH 6.8

Method:

STATION	REAGENT	TIME
1	Methanol	5 mins
2	May Grunwald (as above)	8 mins
3	Giemsa (as above)	15 mins
4	Buffered water pH 6.8	1 min
5	Buffered water pH 6.8	1 min
6	Buffered water pH 6.8	1 min
7	Buffered water pH 6.8	1 min

3. REAGENTS FOR IN VIVO AGGREGATION METHOD OF WU AND HOAK

1. Formalin: 10 ml of 40% formalin made up to 100ml with distilled water
2. 0.077M EDTA: 28.66g disodium EDTA ($Na_2C_{10}H_{14}O_8N_2.2H_2O$) added to approximately 750ml distilled water. pH was adjusted to 7.4 and diluted to 1000ml with distilled water.
3. Concentrated phosphate buffered saline (PBS): 2g potassium chloride (KCl), 2g monopotassium phosphate (KH_2PO_4), 80g sodium chloride (NaCl) and 11.5g disodium hydrogen phosphate ($Na_2HPO_4/2H_2O$) added to 1000ml distilled

water.
4. Test solutions (made up daily for use):
(a) buffered EDTA-formalin solution: 3ml 0.077M EDTA, 5ml 4% formalin and 2ml concentrated PBS added to 10ml distilled water.
(b) buffered EDTA solution: 3ml 0.077M EDTA and 5ml concentrated PBS added to 12ml distilled water.

4. REAGENTS FOR FIXATION OF PLATELETS FOR ELECTRON MICROSCOPY

1. 0.4M sodium cacodylate: 21.4g sodium cacodylate ($Na(CH_3)_2AsO_2.3H_2O$) made up to 250ml with distilled water.
2. 0.2M cacodylate buffer: 50ml 0.4M sodium cacodylate and 8ml 0.2M HCl made up to 100ml with distilled water. The pH was adjusted to 7.3.
3. 0.2% glutaraldehyde buffer: 0.08ml in 10ml 0.2M cacodylate buffer.
4. 3.0% glutaraldehyde buffer: 1.2ml in 8.8ml 0.2M cacodylate buffer.

5. LABELLING OF PLATELETS USING ^{111}INDIUM OXINE

A. PREPARATION ^{111}INDIUM OXINE

(1) To ^{111}In, as supplied by the Radiochemical Centre (Amersham, Bucks) is added 200ul 0.2M NaOH and 400ul 0.3M acetic acid/sodium acetate buffer.
(2) 100ul of a 1mg/1ml solution of 8-hydroxy-quinoline in ethanol is added, mixed and allowed to stand for 5 minutes.
(3) The mixture is extracted three times in glass with 1.5ml dichloromethane and the dichloromethane evaporated to dryness.
(4) The residue is dissolved in 100ul redistilled ethanol and provides a sample with 90% extraction efficiency.

B. PLATELET LABELLING

(1) Acid citrate is prepared from solution of 2.5g trisodium citrate dihydrate and 1.49g citric acid monohydrate in 100ml distilled water, passed through a 0.2um filter.
(2) Calcium-free Tyrodes solution is prepared from solution of 8g NaCl, 0.2g KCl, 1g sodium bicarbonate, 50mg sodium dihydrogen-orthophosphate dihydrate, 406.6mg $MgCl_2$ hexahydrate, 1g D-

glucose and 25,000 units heparin in 1L distilled water. The pH is adjusted to 6.5 with 1M HCl, and prostaglandin E1, added at a concentration of 300ug/ml. The buffer is passed through a 0.2um filter and stored in 30ml aliquots at -25°C.
(3) Using a 21 gauge Butterfly, 17ml blood is taken into a 20ml syringe containing 3ml acid citrate and a further 4.5ml taken into a 5ml syringe containing 0.5ml 0.133M trisodium citrate.
(4) The samples, placed in sterilin 10ml plastic tubes, are centrifuged at 200g for 16 minutes.
(5) The platelet-rich plasma from the acid citrate tube is removed to another tube, made up to 10ml with warmed (37°C) calcium free Tyrodes buffer and re-centrifuged at 640g for 10 minutes to produce a platelet button.
(6) The supernatant is decanted and saved and the platelet button washed gently with 20ml of the warm calcium free Tyrodes buffer, discarding the washings.
(7) 2.5ml warm buffer is added to the button and the platelets resuspended by gentle mixing and rotation, maintaining the suspension at 37°C.
(8) 200uCi ^{111}In oxine are added to the platelets at 37°C and after two minutes the supernatant from (6) is added and after gentle mixing the suspension is recentrifuged at 640g for 10 minutes.
(9) The red cells remaining from step (5) are washed in sterile saline four times and resuspended in 10ml saline for reinjection.
(10) A portion of the platelet-rich citrated plasma prepared in (4) is removed for aggregation studies and the remainder centrifuged at 1000g to provide citrated platelet-poor plasma. A portion of this is used as an aggregation blank.
(11) The supernatant from the platelet button is drained and used to measure labelling efficiency and the platelets resuspended in the remainder of the citrated platelet poor plasma.
(12) A portion of the platelet suspension is placed at 37°C for 15 minutes to elute off PGE_1 and this and the sample from (10) are used in aggregation studies using ADP to assess the effect of the labelling procedure on platelet function.
(13) The labelled platelets and washed red cells are returned to the animal via the marginal ear vein in the rabbit or jugular vein in the calf, using a 21 gauge Butterfly needle.
(14) 2ml blood samples are taken daily for 9 days for determination of platelet lifespan, exactly 1ml delivered by slow release from Grade 'A' glass pipette being counted for ^{111}Indium.

RESULTS

1. EXPERIMENT I (T.B.BRUCEI S42)

	CONTROL				INFECTED			
RABBIT	DOR		EST		ONE		ZAR	
	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.320	320	.403	430	.390	300	.340	331
DAY 1	.310	400	.416	445	.355	228	.330	223
DAY 4	.310	350	.392	420	.280	143	.330	335
DAY 5	.323	375	.300	196	.226	293	.270	145
DAY 6	.293	352	.251	654	.200	93	.270	145
DAY 7	.295	440	.247	490	.200	181	.270	333
DAY 8	.314	300	.224	355	.205	96	.250	340
DAY 11	.283	311	.222	385	.235	33	.250	63
DAY 12	.321	300	.228	398	.235	40	.250	63
DAY 13	.317	345	.237	362	.240	32	.240	46
DAY 14	.275	295	.219	320	.250	93	.230	74
DAY 15	.300	298	.196	281	.263	40	.240	31
DAY 18	.294	310	.183	115	.292	55	.230	40
DAY 19	.290	318	-	-	.275	81	.235	53
DAY 20	.309	304	-	-	.284	121	.250	104
DAY 21	.301	328	-	-	.283	114	.260	131
DAY 22	.311	332	-	-	.285	83	.250	91
DAY 23	.318	-	-	-	-	-	-	-
DAY 24	.309	-	-	-	-	-	-	-
DAY 25	.316	315	-	-	.250	113	.280	63
DAY 26	.297	351	-	-	.271	45	.263	150
DAY 27	.323	293	-	-	.251	49	.261	72

RESULTS

1. EXPERIMENT I (T.B.BRUCEI S42)

	CONTROL				INFECTED			
RABBIT	DOR		EST		ONE		ZAR	
	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.320	320	.403	430	.390	300	.340	331
DAY 1	.310	400	.416	445	.355	228	.330	223
DAY 4	.310	350	.392	420	.280	143	.330	335
DAY 5	.323	375	.300	196	.226	293	.270	145
DAY 6	.293	352	.251	654	.200	93	.270	145
DAY 7	.295	440	.247	490	.200	181	.270	333
DAY 8	.314	300	.224	355	.205	96	.250	340
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DAY 22	.311	332	-	-	.285	83	.250	91
DAY 23	.318	-	-	-	-	-	-	-
DAY 24	.309	-	-	-	-	-	-	-
DAY 25	.316	315	-	-	.250	113	.280	63
DAY 26	.297	351	-	-	.271	45	.263	150
DAY 27	.323	293	-	-	.251	49	.261	72

2. EXPERIMENT II (T.B.BRUCEI S42)

INTACT

RABBIT	HEB		MIC		PSA		JOB	
	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.330	488	.375	428	.370	434	.320	682
DAY 1	.325	446	.350	346	.260	165	.310	653
DAY 2	.320	130	.355	118	.260	138	.305	278
DAY 3	.275	228	.280	199	.230	335	.255	192
DAY 4	.250	235	.240	350	.230	<492	.235	295
DAY 7	.210	070	.220	142	.210	121	.135	405
DAY 8	.200	023	.195	049	.210	053	-	-
DAY 9	.255	044	.250	108	.205	099	-	-
DAY 10	.250	019	.255	096	.200	092	-	-
DAY 11	.255	028	.270	164	.230	152	-	-
DAY 14	.275	015	.300	022	.260	078	-	-
DAY 15	.285	013	.270	067	.260	072	-	-
DAY 16	.265	<10	.255	100	.245	064	-	-
DAY 17	.270	031	.265	066	.240	054	-	-
DAY 18	.255	062	.260	138	.235	082	-	-
DAY 21	.275	041	.280	152	.220	132	-	-
DAY 22	.275	024	.280	143	.275	196	-	-
DAY 23	.275	027	.240	099	.225	182	-	-
DAY 24	.270	025	.280	072	.220	172	-	-
DAY 25	.282	020	-	-	.230	122	-	-

SPLENECTOMISED

RABBIT	GEN		ISI		KIN	
	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.340	515	.335	452	.370	505
DAY 1	.325	552	.340	413	.375	476
DAY 2	.340	172	.290	120	.395	165
DAY 3	.385	260	.360	228	.340	245
DAY 4	.350	294	.355	134	.335	244
DAY 7	.280	058	.335	143	.340	291
DAY 8	.255	014	.320	68	.340	047
DAY 9	.255	206	.310	27	.305	065
DAY 10	.235	225	.300	120	.300	145
DAY 11	.235	245	.280	54	.290	152
DAY 14	.230	047	.245	74	.270	091
DAY 15	.210	130	.260	73	.260	042
DAY 16	.210	082	.260	64	.280	025
DAY 17	-	-	.280	21	.275	074
DAY 18	-	-	.280	16	.275	130

3. EXPERIMENT III (T.B.BRUCEI S42)

CONTROL

RABBIT	POP		STR	
	Hct	Plt	Hct	Plt
DAY -1	.345	-	.375	-
DAY 0	.330	396	.360	381
DAY 2	.330	324	.300	434
DAY 6	.370	293	.335	583
DAY 7	.355	267	.320	410
DAY 9	.345	333	.295	371
DAY 14	.390	464	.365	327
DAY 16	.350	348	.320	457
DAY 21	.390	326	.340	584
DAY 23	.330	246	.300	496
DAY 24	-	-	.310	-
DAY 25	-	-	.325	-
DAY 26	.290	271	.320	375
DAY 29	.360	141	.315	405

INFECTED

RABBIT	IVE		URI		RAW		VIV	
	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt
DAY -1	.330	-	.360	-	.310	-	.290	-
DAY 0	.290	330	.380	465	.315	409	.275	189
DAY 2	.330	523	.350	207	.305	207	.295	122
DAY 6	.365	204	.340	110	.290	225	.320	162
DAY 7	.330	250	.330	176	.295	114	.270	134
DAY 9	.320	310	.330	71	.240	159	.250	312
DAY 14	.340	78	.340	52	.180	325	.270	48
DAY 16	.335	70	.335	310	.235	382	.275	26
DAY 21	.310	237			.220	80	.275	149
DAY 23	.310	256			.200	107	.255	137
DAY 24	.290	-			.155	-	-	-
DAY 25	.345	-			.180	-	-	-
DAY 26	.299	65			.150	57	.295	32
DAY 27	.305	146					.220	32

4. EXPERIMENT IV(T.B.BRUCEI S42)

CONTROL

RABBIT	DOR		FLO		POP	
	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.330	304	.263	272	.355	310
DAY 3	.362	310	.247	290	.370	277
DAY 4	.297	228	.272	350	.348	286
DAY 5	.349	214	.271	315	.329	210
DAY 6	.332	208	.264	232	.301	251
DAY 7	.339	161	.266	232	.331	288
DAY 10	.334	189	.267	251	.292	203
DAY 11	.324	184	.207	16	.294	242
DAY 12	.311	183	.268	322	.344	268
DAY 13	.290	231	.254	284	.348	215
DAY 14	.323	271	.228	221	.354	281
DAY 17	.250	193	.277	59	.342	364
DAY 18	.336	246	.276	300	.310	267
DAY 19	.308	162	.267	285	.326	377
DAY 21	.336	294	.243	349	.312	375
DAY 24	.220	185	.259	236	.325	231

INFECTED

RABBIT	JAS		KIG		LUP		MAR		NAS		ORL	
	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.399	390	.360	362	.429	113	.420	240	.395	356	.451	451
DAY 3	.358	357	.375	108	.360	70	.427	66	.370	186	.458	74
DAY 4	.342	164	.350	201	.340	39	.342	164	.340	175	.374	121
DAY 5	.307	376	.345	185	.328	37	.343	145	.325	138	.303	205
DAY 6	.232	556	.323	168	.291	59	.290	245	.295	218	.299	291
DAY 7	.231	439	.238	239			.270	675	.265	353	.317	192
DAY 10			.232	227			.221	244	.290	232	.343	409
DAY 11			.196	189			.235	286	265	226	.227	450
DAY 12			.202	253			.245	327	.275	456	.286	480
DAY 13			.202	272			.249	314	.270	397	.278	455
DAY 14			.221	356			.240	233	.265	492	.279	422
DAY 17			.218	144			.235	280	.265	345	.277	133
DAY 18			.192	244			.191	486	.270	338	.255	143
DAY 19			.193	371			.246	514	.280	343	.286	244
DAY 21			.244	477			.280	283	.240	555	.305	320
DAY 24											.279	255

5. EXPERIMENT V (T.B.BRUCEI S42)

UNTREATED

	CONTROL		INFECTED			
RABBIT	POP		YEW		VIO	
	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.356	363	.383	432	.363	403
DAY 3	.361	376			.388	188
DAY 8	.391	175	.321	835	.323	259
DAY 10	.371	493	.290	887	.319	86
DAY 15	.401	253	.310	563	.273	154
DAY 20	.420	176	.295	439	.284	173
DAY 28	.358	193	.236	405	.245	356

ASA TREATED

	CONTROL		INFECTED			
RABBIT	XAN		RHO		ULE	
	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.383	238	0391	153	.315	186
DAY 3	-	-	.452	98	.365	244
DAY 7	.381	334	.361	684	.291	860
DAY 10	.383	349	.287	341	.271	429
DAY 13	.398	268	.295	676	.264	424
DAY 22	.424	204	.292	255		
DAY 28	-	-	.238	506		
DAY 29	.413	225	-	-		
DAY 32			.229	533		

6. EXPERIMENT VI (T.B.BRUCEI S42)

RABBIT	CONTROLS IVY		POP	
	Plt	MDA	Plt	MDA
DAY 0	264	.089	337	.066
DAY 3	305	.168	363	.057
DAY 4	231	.086	244	.164
DAY 5	299	.080	355	.070
DAY 7	312	.013	352	.135
DAY 10	303	.075	385	.066
DAY 11	334	-	382	-
DAY 12	254	.108	317	.089
DAY 14	296	.063	309	.192
DAY 17	270	.040	552	.069
DAY 19	319	.166	635	.058
DAY 21	294	.051	446	-
DAY 24	319	.030	337	.083
DAY 25	296	.174	148	.104

NOTE: Levels of MDA are in μM per 10^9 platelets

INFECTED ANIMALS

RABBIT	FER			GER		
	T	Plt	MDA	T	Plt	MDA
DAY 0	-	446	.034	-	262	0.97
DAY 3	0.22	238	-	0.33	247	.118
DAY 4	0.02	298	.096	0.02	241	.098
DAY 5	0.04	660	.057	0.04	41	.122
DAY 7	0.01	561	.051	0.75	17	.170
DAY 10	0.02	210	.300	0.66	28	.089
DAY 11	-	262	-	-	305	-
DAY 12	0.02	147	.213		*	
DAY 14	0.02	204	.083			
DAY 17	0.02	335	.120			
DAY 19	-	178	.089			
DAY 21	0.02	113	.090			
DAY 24	0.15	183	.151			
DAY 25	0.64	169	.776			

T= tryanosomes x 10^9/Litre

* animal died

EXPERIMENT VI (ctd)

	INFECTED ANIMALS					
RABBIT	HOP			XAN		
	T	Plt	MDA	T	Plt	MDA
DAY 0	-	314	.042	-	361	.078
DAY 3	0.44	390	.070	1.50	206	.052
DAY 4	0.02	141	.111	0.23	209	.153
DAY 5	0.02	212	.132	0.02	120	.076
DAY 7	0.02	321	.112	0.02	529	0157
DAY 10	0.04	236	.184	0.02	435	-
DAY 14	0.02	133	.103	0.02	365	.266
DAY 17	0.02	200	.119	0	425	.157
DAY 19	0	221	-	0	178	.081
DAY 21	0	325	.246	0.07	64	.254
DAY 24	0.15	287	.090		*	
DAY 25	0.33	276	.301			

7. EXPERIMENT VII (T.B.BRUCEI S42)

SPLENECTOMISED

RABBIT	APP		BLA		CHE	
	Hct	Plt	Hct	Plt	Hct	Plt
DAY -11	-	-	.322	644	.372	415
DAY -10	.412	379	.310	682	.359	493
DAY -9	.403	396	.333	522	.356	439
DAY -8	.456	396	.289	656	.354	497
DAY -7	.379	311	.285	724	.361	564
DAY -6	.396	456	-	-	-	-
DAY -5	.375	386	-	-	-	-
DAY -4	.359	494	.331	749	.380	470
DAY -3	.356	482	.315	671	.383	511
DAY -2	.408	454	.328	607	.360	393
DAY -1	.382	357	.326	616	.374	491
DAY 0	.416	297	.401	283	.410	412
DAY 3	.422	165	.357	224	-	-
DAY 4	.413	116	.384	79	.381	240
DAY 5	.377	156	.35	146	.397	28
DAY 6	.403	24	.356	236	.436	158
DAY 7	.360	34	-	-	.421	471
DAY 8	.337	183	.301	609		
DAY 11	.326	135	.265	239		
DAY 12	.294	127	.282	45		
DAY 13	.291	102	.274	99		
DAY 17	.292	284	.283	134		
DAY 19	.303	486	.291	239		
DAY 21	.290	252	.270	52		
DAY 25	.295	446				
DAY 27	.276	481				
DAY 28	.269	445				

7. EXPERIMENT VII (ctd)

	SPLENECTOMISED					
RABBIT	APP		BLA		CHE	
	MPV	PDW	MPV	PDW	MPV	PDW
DAY -11	-	-	6.6	16.1	5.2	15.2
DAY -10	7.3	15.5	6.4	15.7	5.5	15.5
DAY -9	5.9	16.6	6.2	15.7	4.9	14.9
DAY -8	6.3	16.0	5.8	16.3	49	15.1
DAY -7	5.7	15.5	6.1	16.2	5.1	15.5
DAY -6	6.8	15.5	-	-	-	-
DAY -5	5.9	15.9	-	-	-	-
DAY -4	6.1	16.1	6.4	15.3	4.8	14.9
DAY -3	6.1	17.5	6.7	15.5	5.1	15.1
DAY -2	6.1	15.3	6.7	15.6	5.1	15.1
DAY -1	6.5	15.7	6.8	15.6	5.3	15.4
DAY 0	6.6	15.8	7.4	15.2	5.5	14.8
DAY 3	5.7	14.8	6.7	16.3	-	-
DAY 4	8.3	17.2	8.4	16.6	5.5	14.8
DAY 5	7.6	16.7	8.6	17.0	5.0	15.5
DAY 6	7.9	18.2	8.3	17.2	7.0	17.1
DAY 7	9.4	18.9	-	-	6.3	15.6
DAY 8	7.5	18.0	8.1	18.7		
DAY 11	7.3	17.8	7.4	16.6		
DAY 12	8.5	19.3	6.7	18.4		
DAY 13	7.2	17.8	7.6	18.2		
DAY 17	8.5	19.6	8.5	19.1		
DAY 19	6.5	16.8	9.4	19.5		
DAY 21	6.2	15.7	7.4	17.6		
DAY 25	6.2	15.6				
DAY 27	6.2	16.4				

7. EXPERIMENT VII (ctd)

INTACT

RABBIT	DAM		ELD		FIG	
	Hct	Plt	Hct	Plt	Hct	Plt
DAY -12	.384	524	-	-	-	-
DAY -11	.384	568	.417	354	-	-
DAY -10	.370	603	.417	392	.371	361
DAY -9	.351	568	.400	345	.378	304
DAY -8	.365	713	.378	404	.364	298
DAY -7	.359	649	.380	377	.355	242
DAY -6	.345	583	-	-	.359	295
DAY -5	.344	436	-	-	.363	204
DAY -4	-	-	.395	421	-	-
DAY -3	-	-	.393	442	-	-
DAY -2	.373	569	.388	295	.359	395
DAY -1	.353	603	.388	380	.358	387
DAY 0	.438	352	.411	352	.375	354
DAY 3	.371	147	-	-	-	-
DAY 4	.339	48	.369	399	.387	240
DAY 5	.333	333	.350	201	.366	137
DAY 6	.330	643	.315	240	.333	410
DAY 7	.309	241	.292	307	.336	523
DAY 8	-	-	.290	346	.245	600
DAY 9	-	-	.278	247	.338	592
DAY 10	-	-	.216	135	-	-
DAY 11	.316	157	.216	135	.304	60
DAY 12	.305	41	-	-	-	-
DAY 13	.290	73	-	-	.304	19
DAY 17	.318	-	.263	123	.393	210
DAY 19	.341	71	.262	190	.255	292
DAY 21	.321	53	.279	193	.262	392
DAY 25	.282	129	.246	98	.270	517
DAY 27	.303	66	-	-	-	-
DAY 28	-	-	.267	47	.278	430
DAY 29	-	-	.239	9	-	-
DAY 31	.322	41			-	-
DAY 32					.279	74
DAY 36					.254	442

7. **EXPERIMENT VII (ctd)**

INTACT

RABBIT	DAM		ELD		FIG	
	MPV	PDW	MPV	PDW	MPV	PDW
DAY -12	5.6	15.2	-	-	-	-
DAY -11	4.6	14.4	5.4	15.1	-	-
DAY -10	4.6	14.3	5.4	16.3	5.2	14.8
DAY -9	4.5	14.4	5.1	15.0	4.9	14.7
DAY -8	4.8	14.7	4.9	15.7	4.4	14.1
DAY -7	4.4	14.3	4.8	14.7	4.7	14.5
DAY -6	4.5	14.3	-	-	4.7	14.0
DAY -5	4.7	14.8	-	-	5.7	14.9
DAY -4	-	-	4.8	14.6	-	-
DAY -3	-	-	4.9	14.5	5.1	14.2
DAY -2	4.5	14.4	4.7	14.5	5.0	14.2
DAY -1	4.98	14.5	4.8	14.4	5.7	15.0
DAY 0	5.2	14.3	4.6	12.9	4.9	14.1
DAY 3	5.1	14.3	-	-	-	-
DAY 4	6.3	16.3	4.5	13.6	5.0	14.2
DAY 5	5.4	14.4	4.5	14.0	5.3	15.1
DAY 6	4.9	14.1	4.9	14.4	5.7	15.0
DAY 7	6.0	17.1	5.5	15.4	5.3	14.6
DAY 8	-	-	5.3	15.2	-	-
DAY 9	-	-	5.0	14.6	5.0	14.3
DAY 11	6.5	17.2	7.0	17.2	8.8	20.9
DAY 12	7.3	18.6	-	-	-	-
DAY 13	6.9	18.4	-	-	6.4	17.7
DAY 17	-	-	8.2	17.8	6.7	16.7
DAY 19	8.6	19.2	6.2	16.0	6.9	17.8
DAY 21	7.4	18.1	6.6	16.0	6.4	16.2
DAY 25	7.1	17.5	6.9	17.1	6.7	16.8
DAY 27	6.3	16.4	-	-	-	-
DAY 28	-	-	6.8	17.0	6.7	17.0
DAY 29	-	-	5.2	14.5	-	-
DAY 31	6.4	15.8			-	-
DAY 32					7.7	17.4
DAY 36					6.4	16.5

8. EXPERIMENT VIII (T.B.BRUCEI S42)

INFECTED

RABBIT	ALW		BUT		CRU	
	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.345	594	.336	390	.269	548
DAY 2	.344	297	.320	193	.266	83
DAY 4	.323	228	.275	105	.251	486
DAY 7	.300	472	.262	459	.274	424
DAY 9	.286	166	.253	91	.253	57
DAY 11	.269	197	.231	81	.241	104
DAY 14	.276	190	.220	212	.255	535
DAY 16	.274	289	.219	183	.262	799
DAY 18	.284	273	.209	123	.267	895
DAY 21	.286	106	.211	103	.264	775
DAY 22	.316	73	.247	295	-	-
DAY 23	.260	49	.247	295	.262	1080
DAY 24	.239	31			.258	1166
DAY 25	.233	36			.257	1197
DAY 28	.198	19			.256	1192
DAY 29					.249	1042
DAY 30					.248	1057
	MPV	PDW	MPV	PDW	MPV	PDW
DAY 0	5.1	14.4	4.9	13.9	5.4	14.4
DAY 2	5.0	14.1	5.0	14.0	4.9	13.9
DAY 4	6.1	17.9	6.4	17.1	5.6	15.3
DAY 7	6.3	16.4	7.0	18.5	5.9	15.9
DAY 9	7.5	18.6	6.4	18.3	7.0	18.5
DAY 11	7.4	18.1	6.7	18.3	8.0	19.3
DAY 14	7.5	19.2	6.4	17.6	7.2	17.9
DAY 16	8.0	18.9	8.1	19.3	6.4	16.9
DAY 18	7.4	18.0	8.2	20.0	6.1	16.2
DAY 21	9.0	19.0	8.8	19.9	6.2	6.6
DAY 22	8.3	17.9	7.1	17.1	-	-
DAY 23	8.5	17.6	7.0	17.1	5.8	15.6
DAY 24	7.1	18.1			5.7	15.6
DAY 25	8.0	19.0			5.3	15.1
DAY 28	7.4	16.8			5.4	15.2

8. EXPERIMENT VIII (ctd)

F/E RATIO

RABBIT	ALW		BUT		CRU	
	Cont	Inf	Cont	Inf	Cont	Inf
DAY 0	-	0.94	0.75	1.07	1.39	1.32
DAY 1	0.76	-	1.36	-	0.92	-
DAY 2	1.37	1.15	0.99	0.68	0.99	0.22
DAY 4	-	0.96	-	0.80	-	0.73
DAY 5	0.80	-	1.01	-	0.92	-
DAY 6	1.22	-	1.32	-	0.98	-
DAY 7	1.02	0.72	1.16	0.98	1.02	0.94
DAY 8	1.35	-	0.90	-	1.06	-
DAY 9	1.17	0.91	1.29	1.28	1.15	0.76
DAY 10	0.31	-	0.95	-	1.12	-
DAY 11	-	1.15	-	1.15	-	0.75
DAY 14	-	1.10	-	0.46	-	0.73
DAY 16	-	1.26	-	1.06	-	0.29
DAY 18	-	1.06	-	0.49	-	0.95
DAY 21	-	1.02	-	0.23	-	0.99
DAY 22	-	0.97	-	0.80	-	-
DAY 23	-	1.22	-	0.80	-	1.03
DAY 24	-	1.14	-	-	-	1.15
DAY 25	-	0.97	-	-	-	1.08
DAY 28	-	1.23	-	-	-	0.93
DAY 29					-	0.84
DAY 30					-	0.98

8. EXPERIMENT VIII (ctd)

INFECTED

RABBIT	DEL		ELG		FIN	
	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.303	561	.312	485	.342	718
DAY 2	.287	269	.309	172	.333	300
DAY 4	.263	373	.255	345	.283	538
DAY 7	.248	382	.270	344	.270	584
DAY 9	.230	49	-	50	.241	115
DAY 11	.223	44	.234	293	.227	174
DAY 14	.229	139	-	50	.241	115
DAY 16	.247	185	.234	293	.227	174
DAY 18	.249	148	.254	484	.258	531
DAY 21	.262	138	.245	334	.257	590
DAY 23	.250	114	.230	435	.251	731
DAY 24	.249	385	.232	621	3242	1017
DAY 25	.237	512	.230	657	.261	1131
DAY 28	.274	612	.229	637	.247	979
DAY 29			.217	571	.256	885
DAY 30			.231	495	.268	1015
DAY 31			.224	308	.280	1130
DAY 32					.274	1070
	MPV	PDW	MPV	PDW	MPV	PDW
DAY 0	5.5	14.5	5.6	14.7	4.8	13.7
DAY 2	6.0	15.4	5.5	16.6	4.7	13.7
DAY 4	7.3	19.0	5.8	15.4	5.6	15.4
DAY 7	7.5	19.3	7.1	17.5	6.5	17.6
DAY 9	7.3	19.0	7.0	18.0	7.2	18.9
DAY 11	7.6	18.9	7.4	17.8	7.4	17.7
DAY 14	8.7	18.8	6.6	17.1	6.1	16.7
DAY 16	7.3	18.3	6.7	17.2	5.9	16.2
DAY 18	7.5	19.2	6.4	16.7	5.9	15.9
DAY 21	7.5	18.0	5.8	18.2	5.9	15.8
DAY 22	-	-	5.7	15.5	-	-
DAY 23	6.8	17.4	5.5	15.4	5.8	16.0
DAY 24	7.5	18.5	5.4	15.2	5.2	14.9
DAY 25	6.7	18.2	5.4	15.2	5.3	15.1
DAY 28	7.8	17.9	5.8	15.5	5.4	15.6
DAY 29			5.7	15.6	5.6	15.7
DAY 30			5.6	17.6	5.5	15.5
DAY 31			6.4	16.2	5.4	15.3
DAY 32					5.2	15.2

8. EXPERIMENT VIII (ctd)

F/E RATIOS

RABBIT	DEL		ELG		FIN	
	Cont	Inf	Cont	Fin	Cont	Fin
DAY 0	0.92	0.79	0.78	0.81	0.23	0.60
DAY 1	0.87	-	1.04	-	0.68	-
DAY 2	0.94	0.70	0.57	0.63	1.51	0.41
DAY 4	-	0.26	-	0.85	-	0.76
DAY 5	1.03	-	0.85	-	0.84	-
DAY 6	0.98	-	.066	-	.05	-
DAY 7	0.77	1.00	0.70	0.80	1.13	0.75
DAY 8	0.84	-	0.69	-	0.86	-
DAY 9	1.02	0.57	1.08	0.54	0.69	1.34
DAY 10	0.89	-	1.13	-	0.79	-
DAY 11	-	0.89	-	0.16	-	0.95
DAY 14	-	0.76	-	0.11	-	0.39
DAY 16	-	0.91	-	0.12	-	1026
DAY 18	-	0.51	-	.026	-	0.81
DAY 21	-	.061	-	-	-	0.35
DAY 23	-	0.45	-	0.75	-	0.37
DAY 24	-	0.95	-	0.69	-	0.99
DAY 25	-	0.40	-	1.00	-	0.26
DAY 28	-	0.87	-	0.98	-	1.03
DAY 29			-	0.82	-	0.74
DAY 30			-	0.82	-	0.72
DAY 31			-	0.34	-	0.18
DAY 32					-	1.01

9. EXPERIMENT - T.CONGOLENSE

CONTROL

RABBIT	KOR		LIS		QUI	
	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.404	399	.383	388	.480	307
DAY 1	.386	348	.383	414	.386	286
DAY 2	.386	410	.381	405	.401	310
DAY 3	.383	399	.366	369	.389	323
DAY 4	.392	330	.382	423	.393	297
DAY 7	.384	446	.398	393	.387	294
DAY 8	.387	398	.384	377	.392	310
DAY 9	.389	284	.375	377	.366	294
DAY 10	.337	349	.360	419	.385	301
DAY 11	.372	343	.352	414	.328	288
DAY 14	.371	316	.355	427	.311	268
DAY 16	.385	379	.356	400	.324	321
DAY 18	.370	436	.378	453	.351	303
DAY 21	.374	362	.376	343	.367	251
DAY 25	.381	401	.364	411	.370	288
DAY 28	.400	286	.385	406	.412	276
DAY 37	.395	321	.401	395	.413	322
DAY 44	.382	376	.387	411	.414	311
DAY 52	.393	435	.323	440	.414	302
DAY 58	.393	376	.373	416	.427	310
DAY 66	.387	350	.375	422	.439	279
DAY 72	.403	401	.391	408	.440	275
DAY 81	.419	370	.406	399	.465	298
DAY 87	.397	303	.358	408	.436	261
DAY 94	.413	326	.313	340	.429	104
DAY 100	.423	387	.374	400	.440	320
DAY 106	.400	386	.359	563	.439	290
DAY 114	.430	308	.367	377	.473	310
DAY 122	.407	403	.365	454	.443	289
DAY 127	.404	328	.335	416	.438	381
DAY 136	.419	307	.350	552	.455	300
DAY 142	.403	416	.350	348	.451	325
DAY 150	.420	348	.347	381	.449	264
DAY 155	.393	351	.346	414	.456	322
DAY 162	.422	295	.332	426	.452	267
DAY 168	.398	335	.313	425	.429	305
DAY 176	.397	412	.330	457	.451	290
DAY 184	.146	381			.431	306
DAY 190	.379	382			.432	317
DAY 198	.398	372			.448	303
DAY 205	.372	392			.434	270
DAY 210	.363	417			.441	319
DAY 219	.403	378			.457	281
DAY 225	.377	398			.456	295

9. EXPERIMENT - T.CONGOLENSE(ctd)

GAMB 19

RABBIT	GRA		HAN		IVE		JOS	
	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.358	355	.383	350	.395	574	.437	356
DAY 1	.366	302	.377	297	.382	536	.389	226
DAY 2	.366	333	.398	344	.385	518	.386	221
DAY 3	.363	201	.401	294	.388	467	.393	186
DAY 4	.353	279	.435	260	.386	487	.375	254
DAY 7	.355	240	.394	284	.375	486	.380	254
DAY 8	.373	219	.387	333	.400	311	.397	205
DAY 9	.371	278	.388	392	.372	261	.387	231
DAY 10	.340	184	.355	329	.373	265	.369	198
DAY 11	.336	100	.355	348	.365	271	.309	107
DAY 14	.264	258	.354	287	.282	325	.328	178
DAY 16	.302	204	.341	234	.313	382	.336	63
DAY 18	.316	148	.330	226	.294	227	.306	154
DAY 21	.340	108	.320	244	.324	272	.303	144
DAY 25	.325	157	.346	168	.331	236	.365	25
DAY 28	.330	104	.359	123	.339	263	.375	119
DAY 37	.300	208	.314	152	.293	85	.348	71
DAY 44	.328	152	.345	171	.304	172	.299	55
DAY 52	.348	182	.335	121	.268	157	.328	65
DAY 58	.322	166	.340	62	.317	110	.322	183
DAY 66	.303	185	.357	71	.367	62	.342	92
DAY 72	.325	284	.369	39	.378	24	.325	125
DAY 81	.330	186	.326	104	.349	162	.351	94
DAY 87	.321	184	.355	137	.321	182	.333	73
DAY 94	.342	190	.319	167	.375	115	.326	162
DAY 100	.347	204	.317	165	.377	160	.341	106
DAY 106	.347	229	.309	121	.346	167	.385	73
DAY 114	.332	182	.317	114	.389	111	.394	185
DAY 122	.321	195	.353	119	.368	239	.376	197
DAY 127	.335	138	.351	201	.350	147	.358	175
DAY 136	.356	120	.357	81	.350	95	.358	88
DAY 142	.343	167	.356	182	.339	153	.372	132
DAY 150	.365	23	.350	44	.366	141	.369	83
DAY 155	.363	171	.300	155	.371	169	.362	159
DAY 162	.362	191	.256	400	.394	104	.333	166
DAY 168	.327	202	.309	100	.389	191	.356	154
DAY 176	.362	212	.319	483	.387	250	.387	191
DAY 184			.332	488	.399	157		
DAY 190			.352	265	.369	150		
DAY 198			.359	348	.386	121		
DAY 205			.347	337	.376	168		
DAY 210			.336	340	.367	133		
DAY 219			.344	370	.380	192		
DAY 225			.372	297	.395	105		

9. EXPERIMENT - T.CONGOLENSE(ctd)

GAMB 19

RABBIT	GRA		HAN		IVE		JOS	
	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.358	355	.383	350	.395	574	.437	356
DAY 1	.366	302	.377	297	.382	536	.389	226
DAY 2	.366	333	.398	344	.385	518	.386	221
DAY 3	.363	201	.401	294	.388	467	.393	186
DAY 4	.353	279	.435	260	.386	487	.375	254
DAY 7	.355	240	.394	284	.375	486	.380	254
DAY 8	.373	219	.387	333	.400	311	.397	205
DAY 9	.371	278	.388	392	.372	261	.387	231
DAY 10	.340	184	.355	329	.373	265	.369	198
DAY 11	.336	100	.355	348	.365	271	.309	107
DAY 14	.264	258	.354	287	.282	325	.328	178
DAY 16	.302	204	.341	234	.313	382	.336	63
DAY 18	.316	148	.330	226	.294	227	.306	154
DAY 21	.340	108	.320	244	.324	272	.303	144
DAY 25	.325	157	.346	168	.331	236	.365	25
DAY 28	.330	104	.359	123	.339	263	.375	119
DAY 37	.300	208	.314	152	.293	85	.348	71
DAY 44	.328	152	.345	171	.304	172	.299	55
DAY 52	.348	182	.335	121	.268	157	.328	65
DAY 58	.322	166	.340	62	.317	110	.322	183
DAY 66	.303	185	.357	71	.367	62	.342	92
DAY 72	.325	284	.369	39	.378	24	.325	125
DAY 81	.330	186	.326	104	.349	162	.351	94
DAY 87	.321	184	.355	137	.321	182	.333	73
DAY 94	.342	190	.319	167	.375	115	.326	162
DAY 100	.347	204	.317	165	.377	160	.341	106
DAY 106	.347	229	.309	121	.346	167	.385	73
DAY 114	.332	182	.317	114	.389	111	.394	185
DAY 122	.321	195	.353	119	.368	239	.376	197
DAY 127	.335	138	.351	201	.350	147	.358	175
DAY 136	.356	120	.357	81	.350	95	.358	88
DAY 142	.343	167	.356	182	.339	153	.372	132
DAY 150	.365	23	.350	44	.366	141	.369	83
DAY 155	.363	171	.300	155	.371	169	.362	159
DAY 162	.362	191	.256	400	.394	104	.333	166
DAY 168	.327	202	.309	100	.389	191	.356	154
DAY 176	.362	212	.319	483	.387	250	.387	191
DAY 184			.332	488	.399	157		
DAY 190			.352	265	.369	150		
DAY 198			.359	348	.386	121		
DAY 205			.347	337	.376	168		
DAY 210			.336	340	.367	133		
DAY 219			.344	370	.380	192		
DAY 225			.372	297	.395	105		

9. EXPERIMENT (ctd)

TSW 99/77

RABBIT	MOZ		NIE		ORF		PUC	
	Hct	Plt	Hct	Plt	Hct	Plt	Hct	Plt
DAY 0	.392	456	.487	460	.431	404	.430	410
DAY 1	.392	426	.416	403	.428	442	.408	439
DAY 2	.390	421	.422	417	.436	402	.412	397
DAY 3	.378	386	.413	251	.410	365	.408	302
DAY 4	.397	254	.475	122	.417	391	.395	342
DAY 7	.379	254	.443	70	.365	194	.382	215
DAY 8	.367	405	.476	23	.343	189	.347	188
DAY 9	.354	131			.306	15	.321	118
DAY 10	.270	198			.242	93	.267	144
DAY 11	.271	107			.254	44	.238	142
DAY 14	.281	78			.241	137	.211	93
DAY 16	.313	163			.260	77	.253	126
DAY 18	.322	154			.289	<10	.292	87
DAY 21	.310	44			.266	<10	.283	73
DAY 25	.321	125			.289	88	.272	64
DAY 28	.352	119			.330	56	.325	90
DAY 37	.336	71			.358	108	.340	40
DAY 44	.312	55			.371	55	.305	130
DAY 52	.318	165			.306	54	.304	93
DAY 58	.352	183			.352	194	.301	40
DAY 66	.348	125			.338	73	.317	97
DAY 72	.354	192			.368	125	.316	90
DAY 81	.380	173			.404	106	.396	106
DAY 87	.359	262			.344	63	.307	105
DAY 94	.370	354			.384	42	.346	15
DAY 100	.367	144			.429	191	.368	139
DAY 106	.358	125			.350	108	.368	130
DAY 114	.345	119			.380	81	.402	104
DAY 122	.348	371			.385	110	.334	150
DAY 127	.383	355			.409	175	.376	150
DAY 136	.395	265			.388	163	.375	71
DAY 142	.437	283			.375	165	.375	193
DAY 150	.357	425			.398	161	.294	171
DAY 155	.365	502			.393	195		
DAY 162	.357	492			.391	210		
DAY 168	.381	473			.412	40		
DAY 176	.398	385			.412	153		
DAY 184	.407	397			.382	231		
DAY 190	.369	375			.381	165		
DAY 198	.379	388			.389	131		
DAY 205	.363	432			.384	153		
DAY 210	.368	483			.386	201		
DAY 219	.367	459			.368	215		
DAY 225	.349	466			.394	281		

10. EXPERIMENTS - T.VIVAX

CALF	BLU		BOR		GEO		TOG	
	Inf. Hct	Plt	Inf Hct	Plt	Inf Hct	Plt	Inf. Hct	Plt
DAY 0	.340	658	.320	532	0 .340	911	0 .380	932
DAY 1	.320	841	.400	756	1 .320	832	1 .375	876
DAY 4	.290	545	.380	628	4 .280	965	4 .330	1065
DAY 7	.280	528	.370	694	7 .290	529	7 .340	73
DAY 9	.305	354	.355	416	9 .260	92	9 .290	135
DAY 12	.280	413	.350	539	12 .230	56	12 .200	56
	B E R E N I L 2 8 m g / k g							
DAY 20	.255	719	.325	735	.210	943	.195	620
DAY 23	.265	381	.360	685	.220	975	.245	1315
DAY 28	.225	444	.290	848	-	-	-	-
DAY 34	.220	628	.310	941	.210	1073	.275	632
DAY 42	.235	527	.320	596	.235	877	.290	730
DAY 51	.240	515	.320	655	.290	585	.280	606
DAY 55	.270	475	0 .310	664	.260	423	0 .290	411
DAY 59	.260	347	4 .280	460	.255	625	4 .320	200
DAY 63	.240	412	8 .210	142	.260	602	8 .280	140
DAY 64	.250	384	9 .220	93	.240	497	9 .270	203
DAY 71	.220	506	16 .160	220	.240	707	16 .220	50
DAY 78	.230	446	23 .150	264	.260	796	23 .200	200
DAY 86	.260	469	31 .185	174	.270	778	37 .240	227
	B E R E N I L 1 5 m g / k g							
DAY 91	.235	-	.205	-	.270	-	.215	-
DAY 98	.250	502	.250	336	.260	756	.300	323
DAY 101	.260	537	.275	250	.295	798	.345	247
	B E R E N I L 1 5 m g / k g							
DAY 106	.270	558	.295	100	.295	1100	.295	665
DAY 129	-	408	-	392	-	437	-	387
DAY 135	.270	350	.320	-	.240	541	.290	-
DAY 143 7	.220	325	7 .290	570	7 .225	430	7 .295	405
DAY 148 12	.120	25	12 .235	178	12 .205	215	12 .300	421
DAY 151 15	.125	97	15 .220	213	15 .195	195	15 .250	105
DAY 155 19	.150	75	19 .240	419	19 .220	339	19 .270	218
DAY 158 22	.155	163	22 .205	590	22 .210	612	22 .230	33
DAY 162 26	.180	251	26 .230	343	26 .230	797	26 .240	656
DAY 164 28	.180	284	28 .200	338	28 .230	371	28 .220	400
DAY 170 34	.180	326	34 .210	230	34 .240	344	34 .230	704
DAY 171 35	.175	180	35 .210	438	35 .220	384	35 .225	148
DAY 175 39	.170	226	39 .205	251	39 .175	368	39 .190	176
DAY 178 42	.200	97	42 .220	117	42 .180	164	42 .210	222
DAY 185 49	.190	113	49 .250	140	49 .145	338	49 .190	294
DAY 190 54	.220	228	54 .225	486	54 .195	435	54 .175	192
DAY 192 56	.200	188	56 .240	410	56 .165	263	56 .165	434
DAY 198 62	.240	347	62 .260	494	62 .230	679	62 .150	74

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