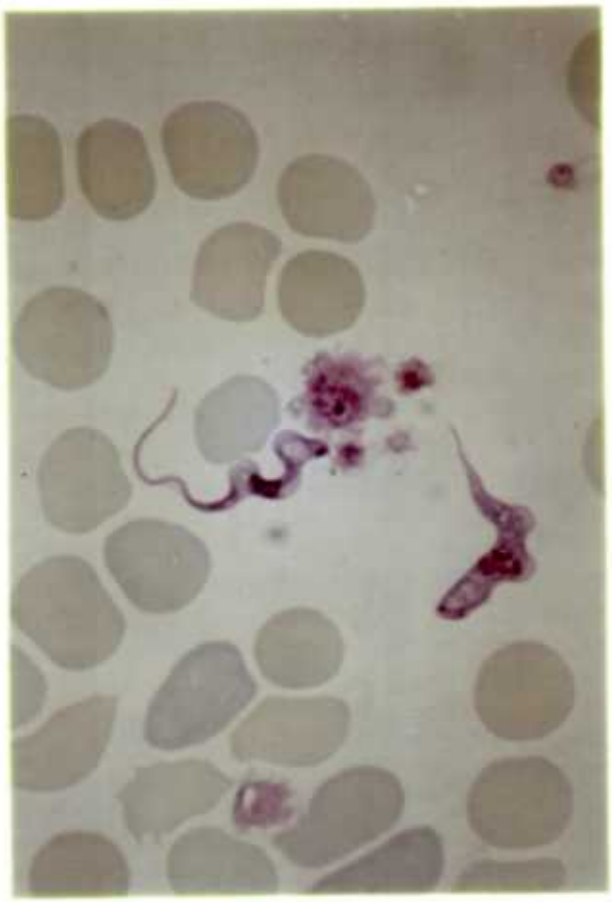


Slender and stumpy forms of Trypanosoma (Trypanozoon) brucei 427 in the blood of a rabbit 12 days after infection.

PHYSIOPATHOLOGICAL CHANGES IN THE BLOOD OF ANIMALS INFECTED WITH AFRICAN TRYPANOSOMES

by

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ABSTRACT

Investigations into physiological changes in the blood of rabbits experimentally infected with Trypanosoma (Trypanozoon) brucei were made in an attempt to elucidate some of the pathogenic mechanisms of this disease.

Studies on the fibrinolytic system showed important alterations in infected animals with significant increases in serum fibrinogen/fibrin degradation products (FDP). Measurement of serum plasminogen concentrations and experimental inhibition of plasmin by specific drugs, suggested that plasminogen was being activated during the infection, giving rise to FDP. Experiments are described to elucidate the mechanism of activation. Trypanocidal therapy did not cause a rapid change in FDP levels unless a high parasitaemia was evident at the time of drug cure, when a sudden increase in serum FDP occurred shortly after treatment. This suggested fibrinolytic activity was associated with the destruction of trypanosomes. In vitro experiments utilising trypanosome extracts and purified fibrinogen confirmed this suggestion. Large increases in plasma fibrinogen concentration also occurred early on in the infection. These findings give a picture very similar to that of diffuse intravascular coagulation with secondary fibrinolysis. Sera from patients with sleeping sickness were also

shown to have high FDP and fibrinogen concentrations.

An increase in an auto-antibody to one or more components of the fibrinogen/fibrin system (anti-F) was found to be elevated during infection. This auto-antibody was found to react with homologous and heterologous fibrinogen and shown to be Immunoglobulin M (IgM) at all stages of the infection. There was no evidence to suggest that the anti-F auto-antibody causes any direct pathological effects and in fact it might have a beneficial effect in enhancing phagocytosis of intra-capillary fibrin. High titres of serum anti-F were found to be present in both human and cattle infections.

Rheological studies on infected rabbit blood showed significant elevations in the viscosity of whole blood, plasma and serum due to the effect of raised plasma fibrinogen, globulin and lipoprotein levels. These proteins were also shown to be the cause of a non-immunological reversible erythrocyte agglutination and adhesiveness resulting in an elevated erythrocyte sedimentation rate.

The above findings can be considered to cause microcirculatory insufficiency in trypanosome infections thus forming an important contribution towards the pathogenesis of the disease.

PART 1 INTRODUCTION

1.1. Object of Study

The purpose of the study was to investigate, during trypanosome infections of rabbits, any abnormalities of fibrinolysis and blood viscosity and attempt to ascertain their aetiology and significance in the pathology of the disease. It is intended as a contribution towards a more complete understanding of the disease state in African trypanosomiasis with the hope that it may lead to more effective alleviation of human suffering and possible reversion of certain pathological processes.

1.2. Trypanosomes and Trypanosomiasis

1) Importance of trypanosomiasis

The importance of the trypanosomiasis of man and animals in tropical Africa was put into perspective by a recent statement by Kershaw (1973) - "the World Health Organization in determining the ten major health problems facing mankind places trypanosomiasis of man and his domestic animals high on the list along with malaria, cancer and heart diseases".

Focal resurgence of trypanosomiasis of man causes much ill-health. In addition nagana, or trypanosomiasis of livestock, results in losses of food production and consequently contributes to the widespread malnutrition. In Africa, in 1960, when it was estimated that trypan-

osomiasis of cattle was costing £1,250 million a year, about ten million square kilometres of infested land south of the Sahara existed which could otherwise carry about 125 million cattle (Goodwin 1973).

The problem today has changed very little as regards eradicating the disease. In fact, the impact of trypanosomiasis retarding the development of Africa increases rather than diminishes.

However, as knowledge on aspects of the epidemiology, pathology and immunology of the disease accumulate one would hope that in the near future there will be more effective and economical prophylactic measures together with better methods of treatment.

ii) Taxonomy of Trypanosomes

The nomenclature of trypanosomes will follow the classification given by Hoare (1966) where trypanosomes of the former brucei group (brucei and evansi sub-groups) are considered members of the subgenus Trypanozoon. Although this classification is not universally accepted (Ormerod, 1967) it is proposed to refer to three species of the subgenus Trypanozoon viz. Trypanosoma (Trypanozoon) brucei, Plimmer and Bradford 1899, T. (T.) gambiense, Dutton 1902, and T. (T.) rhodesiense, Stephens and Fantham, 1910.

1.3. Immunity to Trypanosome Infections

There is no evidence showing unequivocally an acquired protection after recovery from the African trypanosomes, either in man or animals, indicating an ineffective or defective immune response to trypanosomal antigens. To elucidate why the immune response appears to be ineffective has been the interest of immunologists for many years and, only recently with increasing knowledge concerning immunological phenomena, has advance been made in this field.

In trypanosomiasis there exists the paradoxical situation of trypanosomes living in an apparently hostile environment. Despite the large amounts of immunoglobulin produced the parasite survives and the host frequently succumbs to the infection. The success on behalf of the parasite lies in its ability to evade the host immune response by antigenic variation - a phenomenon first described by Franke in 1905. Almost all investigations into antigenic variation have employed, for various technical reasons, trypanosomes of the *brucei* sub-group, (Inoki *et al*, 1952; Soltys, 1957; Cunningham and Vickerman, 1962), although recently workers are now beginning to show interest in the important parasites of domestic livestock, *T. (D.) vivax* and *T. (N.) congolense* (Jones, 1973; Uilenberg and Giret, 1973). The subject of antigenic variation has been reviewed in

detail by de Raadt (1974).

The antigens of trypanosomes are also of interest as an important potential means of controlling trypanosomiasis although, to date, there has been no immunoprophylaxis achieved by experimental methods against the pathogenic trypanosomes which would be of practical value. However experimental animals have been successfully immunised by injections of trypanosome suspensions, with and without adjuvants (Soltys, 1973) and by the use of irradiated parasites (Duxbury and Sadun, 1969; Duxbury et al, 1972; Wellde et al, 1973). In addition, Soltys (1965) successfully attempted immunoprophylaxis in mice by immunisation with trypanosomes inactivated by β-propiolactone.

No one has yet isolated a protective antigen with anything more than limited variant specifity, and inducing protection against a wide range of trypanosome types is still a major problem.

1.3.1. The Host Response

Alterations in the serum proteins of hosts infected with a variety of trypanosome species have been the subject of much study. The general picture is one of a polyclonal gammopathy, the γ-globulin fraction following paper electrophoresis showing a typical serrated hetergeneous / "saw-tooth"-like configuration. This condition is usually associated with a hyper-

proteinaemia (Burtin, 1964). Electrophoretic analysis of serum from trypanosome infections of man and experimental animals all showed a characteristic γ-globulin elevation (Trincao et al 1953; Petard and Ridet, 1954; Olberg, 1955; Seneca et al 1958; Pautrizel et al, 1959). In addition Gall (1956) described an extra component which appeared as a shoulder on the γ peak between the β and γ globulins using serum from humans and monkeys infected with T. (T.) gambiense. Similarly, Desowitz (1956) by means of micro-Tizelius electrophoresis, showed an extra peak on the γ-globulin component in sera from N'Dama cattle that were hyper-immune to T. (Dutonella) vivax.

In bovines and sheep infected with T. (D.)vivax, γ-globulin shows a significant increase whereas African antelopes, one of the reservoir hosts of trypanosomes, exhibit in addition, a large increase in the β-globulin fraction. It is suggested that perhaps the β-globulin may play a role in maintaining the host-parasite relationship (Desowitz, 1960).

Recent interest has been centered on the elevation of immunoglobulin M (IgM), a characteristic feature of the humoral response in trypanosomiasis. This was first reported by Mattern (1961; 1963) and suggested to be a response to the rapid antigenic variation of the parasite (Lumsden, 1972).

In sleeping sickness, the serum IgM level has been shown to rise four times the normal level (Mattern, 1964) although the IgG component did not rise significantly. The high IgM levels revert to normal following chemotherapy and significant reductions occur 18-30 days following the beginning of specific treatment (Marcario and Bentz, 1963; Courtois and Bideau, 1966; de Raadt et al, 1966; Onyango et al, 1972).

Determination of IgM levels in plasma is used as a valuable screening test for suspected trypanosomiasis. However, major increases in IgM are not specific for trypanosomiasis and have been found in high levels in apparently uninfected Africans, particularly in some forms of liver disease (Hollins and Lewis-Fanning, 1947) It has subsequently been shown that measurement of IgM levels in cerebrospinal fluid is a more specific diagnostic test (Mattern, 1969).

Elevations of serum IgM also occur in other infectious diseases e.g. malaria, kala-azar, leprosy and syphilis (McKelvey and Fahey, 1965). However, here the ratio of IgG:IgM is usually high whereas in human trypanosomiasis the ratio is characteristically 1:3.

In the animal trypanosomiasis serum IgM increases have been quantitatively measured usually by the single radial diffusion method of Mancini et al (1965). Rees

(1969) studied both IgM and IgG production in sheep in response to a rat-adapted strain of T. (D.) vivax. IgM levels increased more than two fold over the first 20-30 days during the period of maximum parasitaemia, and then decreased slowly. Houba et al (1969), studying T. (T.) brucei, T. (T.) gambiense and T. (T.) rhodesiense infections in rhesus monkeys found large increases in serum IgM but no significant rises in IgG or in IgA.

Hyper γ-M-globulinaemia has also been demonstrated in bovine trypanosomiasis where IgM showed a 10 fold increase within 2 weeks and persisted for several months. The IgG in comparison showed only a 2 fold increase (Luckins 1972). Clarkson and Penhale (1972) showed that calves infected with 4 different strains of T. (D.) vivax showed marked increases in IgM commencing at a much later time after infection - 45 days. Again there was a 10 fold increase.

In experimental infections of rabbits with the brucei group trypanosomes, notable IgM increases occur. The early production of IgM in T. (T.) gambiense infections was demonstrated by Mattern (1963), who showed that after 5 days of infection with this parasite a considerable increase occurred and that by the 8th day this increase had reached its maximum. Likewise, Seed et al (1969) showed comparable elevations of IgM

in T. (T.) gambiense infections of rabbits, where 4 to 8 fold increases were shown as early as 8 days after infection rising to a 16 fold increase approximately 16 days later and thereafter forming a plateau. This condition remained for as long as 118 days.

Not only quantitative, but also qualitative changes have been shown in the IgM produced. Thus the occurrence of low molecular weight (7S) IgM immunoglobulin has been shown to occur in rabbits infected with T. (T.) gambiense by Frommel et al (1970). IgM levels were found to increase rapidly after infection doubling within 10 days and rising to 10 times the normal value within three weeks of infections. Following elution of infected serum on agarose columns, a 7S IgM appeared on the ascending IgG peak. This low molecular weight IgM was detectable late in the infection and did not exceed 5% of the total IgM. The extra peak between the β and γ-globulins discovered after two-dimensional electrophoresis of infected cattle sera as previously described, probably consists of a similar low molecular weight IgM (Desowitz, 1956). However, the recent studies of Clarkson and Penhale (1972) on cattle infected with T. (D.) vivax showed that in this infection there was no change in the molecular weight of IgM.

A criticism of how I9S IgM is measured was made by Klein et al (1970). Using T. (T.) equiperdum infections of rabbits the results of IgM determinations by ultracentrifugation were compared by those obtained by agar diffusion and immune precipitation. Their results showed that the measurement of I9S IgM was overestimated by agar diffusion since 7S IgM diffused more easily into the agar and, as it also shares antigenic determinants common with I9S IgM, it was detected as the latter.

The macroglobulinaemia of sleeping sickness has been likened to the pathogenic condition of Waldenstrom's disease (Waldenstrom, 1968). However, care must be taken in equating the two conditions since the terminal amino acids of IgM in trypanosomiasis are glutamic and aspartic acids, whereas the IgM of Waldenstrom's disease has glutamic acid only (Josselin et al, 1964). In addition, the macroglobulinaemia of Waldenstrom is often accompanied by the presence of 22S components (Franklin and Kunkel, 1957) as yet not demonstrated in trypanosome infections.

Non-trypanosome specific immunoglobulin

Much of the vast amounts of immunoglobulin produced during trypanosome infections is not specific for the antigens of the parasite (Neal et al, 1969).

Freeman et al (1970) have shown that less than 5% of the IgG produced in T. (T.) brucei infections of

rhesus monkeys is specific for the parasite as judged by the catabolic rates of immune and normal IgG preparations. This situation should not be regarded as a peculiar feature of parastic infections since the earlier work of Askonas and Humphrey (1958) demonstrated that following experimental immunization to single antigens not all the γ- globulin produced is specific for that antigen.

In addition to anti-trypanosomal antibodies, the IgM fraction has been shown to contain heterophile and anti-immune complex antibodies like the rheumatoid factor (Houba and Allison, 1966; Houba et al, 1969; Klein and Mattern, 1965; Klein et al, 1971). Klein et al (1970) stressed the close analogy between the occurrence of rheumatoid factor (RF) reported in human T. (T.) gambiense infections (Klein and Mattern, 1965) and its presence in T. (T.) equiperdum infections of rabbits. The agglutinating substance in infected sera was IgM in nature and had a predilliction for a heterologous substrate (rabbit IgG in the case of humans, human IgG in rabbits). Monkeys have also been shown to produce RF, but here IgG was the agglutinating factor (Klein et al, 1970,b; Klein et al, 1971). The host-parasite relationship appears to be very critical as regards appearance of RF since negative results were obtained from mice infected with T. (T.) equiperdum (Klein et al, 1970, a) and monkeys infected with

T. (T.) gambiense, T. (T.) rhodesiense and T. (T.) brucei (Houba et al, 1969) - the latter a system which should represent a close analogy to the situation in human infections. In addition no RF was produced in T. (T.) gambiense infections of mice nor in rats carrying on infection with the non-pathogenic T. (H.)lewisi (Klein et al, 1971).

The raised non-specific immunoglobulin component of IgM in trypanosomiasis not only includes heterophile antibodies and rheumatoid factor but also anti-tissue autoantibodies (Mackenzie and Boreham, 1974). Anti-tissue autoantibodies have been found in low levels in normal animals. Adult rabbits were shown by Kidd and Friedewald (1942) to possess a serum factor which fixed complement (C') in the presence of saline extracts of normal rabbit liver and shared many of the general properties of the two "natural" antibodies found in rabbits viz. Wassermann antibody and sheep red blood cell haemolysin. This work was later supported by Dumonde (1966) who showed that rabbit "natural" anti-bodies occur in varying titres in most adult rabbits.

Studies by Gajdusek (1957, 1958) showed that tissue antibodies were relatively common in man, where 75% of patients with macroglobulinaemia were positive and 82% of patients with systemic lupus erythematosus (SLE) gave positive results with autopsy tissue. More recently anti-liver autoantibody has also been demon-

strated in other diseases e.g. in rabbits infected with Eimeria stiedae (Asherson and Rose, 1963) and in schistosomiasis (Bassily et al, 1973).

Seed and Gam (1967) were the first to describe increases in an anti-liver autoantibody in trypanosomiasis using experimental T. (T.) gambiense infections of rabbits. Later Mansfield and Krier (1972) using both precipitin and complement fixation tests (CFT) demonstrated autoantibodies to kidney, liver, brain and heart in rabbits infected to T. (N.) congolense. More recent studies by Mackenzie and Boreham (1974) have demonstrated by means of CFT, autoantibodies to liver and Wassermann antigens in T. (T.) brucei infections of rabbits. These autoantibodies were shown to be IgM at all times during the infection. They differ from those experimentally produced by injecting rat liver in Freunds Complete Adjuvant which are heat stable at 65°C for 30 min and represent IgG autoantibodies. The anti-liver autoantibody described by Mackenzie and Boreham (1974) also cross reacts with other various rabbit tissues particularly kidney, since absorption with this tissue removed anti-liver activity. Absorption with trypanosomes had no effect on the autoantibody titre indicating that there are no shared antigens between rabbit tissue and trypanosomes which might explain the autoantibody increase during infection. Since high levels of circulating anti-liver autoantibody

were present yet no apparent liver damage occurred (this assumption based on the low level of liver transaminase in the serum), it was concluded that African trypanosomiasis in rabbits had no autoimmune aetiology.

In South American trypanosomiasis (Chagas' disease) autoantibodies to heart tissue have been shown to occur by Kozma (1962).

Other autoimmune phenomena have been described in trypanosomiasis. Immunoconglutinin (Ik), the antibody to hidden determinants of complement components C_3 and C_4 revealed following the fixation of complement, have been described in T. (T.) brucei infections of laboratory animals (Ingram and Soltys, 1960). Coombs considered the presence of Ik to be "one of the best examples of autoimmunisation" (Coombs and Coombs, 1953). It is of interest to note that Ik has also been described in human malaria (Topley et al, 1973).

The enhanced erythrophagocytosis which occurs in trypanosomiasis has been suggested by Zuckermann (1964) to arise from autoimmunity to host erythrocytes, a situation well documented in malarial infections Zuckermann (1970). However there is a lack of clear and reproducible evidence for the presence of auto-antibodies to red blood cells both in experimental and natural infections with African trypanosomes. The demonstration of a positive direct or indirect anti-

globulin (Coomb's) test indicative of red blood cell sensitisation *in vivo* with incomplete (non-agglutinating) antibody was first reported by Zoutendyk and Gear (1951) in a single sleeping sickness case. However, the blood for the Coomb's test was taken after chemotherapeutic treatment and it remains uncertain as to whether sensitisation of red blood cells resulted from the infection or from its treatment. More recently, a single case of Coomb's positive haemolytic anaemia has been described by Barrett-Connor (1973). In addition, Mackenzie and Boreham (inpress) found that out of 9 cases of active sleeping sickness in African patients, 8 showed Coomb's positivity. The test repeated on *T. (T.) brucei* infections in rabbits suggested that it was improbable that incomplete antibodies were produced in this infection. However it was proposed that the test warranted further investigation since it was considered possible that the Coomb's reagent used (an anti-rabbit serum) was not sensitive enough. The reason given for this suggestion was that incomplete antibodies, like other autoantibodies found in trypanosome infections, may be of the IgM class. A specific anti-rabbit IgM antiserum as the Coomb's reagent might provide the answer.

Coomb's positivity is often found accompanied by an accelerated erythrocyte sedimentation rate (ESR) and red blood cell autoagglutination or clumping. However Coomb's tests, performed when red cell clumping was

observed in cattle infected with T. (N.) congolense and T. (T.) brucei were negative (Mackenzie, 1973). Barrett-Connor (1973) also found an inconsistent relationship between Coomb's positivity in sleeping sickness and the ESR and autoagglutination of red cells.

1.3.2. Immunosuppression

In recent years it has been shown that certain infections appear to exert an immunosuppressive effect on the response of the host to other antigens. Such a situation has been shown to exist in certain malarial infections. (McGregor and Barr, 1962; Greenwood, 1968; reviewed by McGregor, 1972) and more recently in the field of trypanosomiasis. Goodwin and his colleagues (1970; 1972) showed that in chronic T. (T.) brucei infections of mice the immune response to sheep erythrocytes was diminished. In addition Alt et al (1971) reported a significant proportion of rabbits with T. (T.) brucei infections failed to develop experimentally induced allergic neuritis. These results prompted Urquhart et al (1973) to study the immune response of trypanosome infected rats to a primary infection with the nematode Nippostrongylus braziliensis. Results showed that rats with a chronic infection were unable to mount a proper immune response to the nematode and the expulsion of adult worms which usually occurs in normal rats was delayed. Production of protective antibody and reaginic antibody was also suppressed.

Mice infected with T. (T.) brucei have been shown to mount a poor response to challenge with sheep red blood cells as judged by the haemolytic plaque assay (Freeman et al, 1974). Within 10 days of infection IgG and IgM producing cells of the spleen were reduced to below 2% of control values.

Numerous examples are given in the literature describing secondary infections as a common cause of death in trypanosomiasis. Thus lobar pneumonia has been described in sleeping sickness cases (Low and Castellani, 1903; Goodwin, 1970b), together with depressed immunologic reactions to Candida and streptococal antigens (Greenwood et al, 1973). Pneumonia has also been described in rats infected with T. (T.) brucei (Jennings et al, 1974a). Streptococcal infections were found to be of common occurrence in dogs infected with T. (T.) brucei (Rodet and Valet, 1908) and in T. (N.) congolense infections of ruminants (Fiennes, 1954; Kramer, 1966; Krampitz, 1970).

An attempt to explain the apparent paradox of heightened immunological activity (i.e. raised IgG and IgM levels) accompained by immunosuppression has been made by Terry et al (1973). They tried to link T-dependent immunosuppression with the high levels of T-independent IgM characteristics of trypanosome infections. They suggested that defective T-cells would not co-operate in thymus dependent antibody responses

and, at the same time, fail in their normal inhibitory control of certain B lymphocyte clones particularly those producing IgM autoantibodies.

1.3.3. Cell mediated immunity

Few references to cell mediated responses in trypanosomiasis are to be found in the literature (Tizard and Soltys, 1971; Aiyedun and Amodu, 1973; Viens et al, 1974). The significance of these responses needs elucidating since they may explain the persistance of trypanosomes in the host.

1.3.4. Immunopathology

Immune phenomena have been shown to play an important role in the pathology of trypanosomiasis.

Hypersensitivity reactions against trypanosomal antigens in cattle infected with T. (N.) congolense were considered by Fiennes (1950a) to be associated with the anaemia, liver degeneration and lung oedema seen in cattle. However there is no detailed evidence at present that homocytotropic antibody is formed in trypanosomiasis, although Ormerod (1970) and Fiennes (1970) support the view that anaphylaxis is important in human and cattle infections respectively. In experimental infections of guinea-pigs with T. (T.) brucei contraction of infected guinea-pig ileum in an in vitro system was brought about following the addition of the sensitising antigen, here a T. (T.) brucei suspension (Boreham and Kimber, 1970). However, if

anaphylaxis does occur, its importance must vary greatly between species.

Activation and release of kinins in the plasma and urine occurs in trypanosome infected mice, rabbits, cattle and man (Goodwin and Richards, 1960; Boreham, 1968, a, b; 1970). Although kinins are rapidly destroyed by plasma kininase, they could be the cause of changes in vascular permeability leading to oedema, haemostasis and tissue anoxia. The activation of the kinin system is probably brought about by the absorption of Hageman factor onto the surface of antigen-antibody complexes (Boreham and Goodwin, 1969, 1970). Immune complexes have been demonstrated on the endothelium of renal blood vessels in T. (T.) brucei infections of rabbits (Boreham and Kimber, 1970). These may play a part in mediating the renal insufficiency and proteinuria that develops during the same infection (Goodwin and Guy, 1973; Itazi and Enyaru, 1973). Glomerular deposits of antigen-antibody complexes have been shown to occur in T. (T.) brucei infections of mice (K. M. Hudson, personal communication) and more recently in T. (T.) rhodesiense infections of rhesus monkeys (Nagle et al, 1974). Here the exudate and proliferative glomerulonephritis was associated with deposits of IgM and properdin.

The occurrence of antigen-antibody reactions in trypanosomiasis has been likened to the Arthus reaction

(Goodwin and Hook, 1968) in rabbits. Here there is an inflammatory invasion of blood vessels, consequent upon the deposition of antigen-antibody complexes in their walls. How these antigen-antibody complexes have their effect is not known although it is probably via release of pharmacologically active amines, although complement (C') may be of importance.

An 'agglutin' in the plasma of sleeping sickness patients was first described by Yorke (1911) who showed that red blood cell autoagglutination was due to a cold reactive protein, the action of which was inhibited at 37°C. However, it was later to be shown that the increases in ESR could not be fully explained by sensitisation of erythrocytes with antibody (cold agglutinin) - instead by a "property of the plasma" (Gall, 1956; Gall et al, 1957).

Pronounced increases in ESR and autogglutination have been recorded by other workers in sleeping sickness (Hollins and Lewis-Fanning, 1947; Deegan et al, 1956; de Raadt et al, 1965; Taylor, 1971) and in trypanosome infections of guinea-pigs (Nicolle and Simons, 1939), in rats and camels with T. evansi (Jatkar and Purohit, 1971), in rats with T. (T.) brucei and T. equiperdum (Ikejiani, 1946), and in cattle infections with T. (D.) vivax (Vohradsky, 1971). Similarly, with T. (N.) congolense and T. (T.) brucei in cattle (Edwards et al, 1957). Explanations for the increase in sedi-

mentation are not given by these authors. It is certain that an autoimmune reaction to erythrocytes occurs in Rhodesiense sleeping sickness as indicated by the presence of Coomb's positivity, but this may not be the only explanation for autoagglutination and subsequent erythrophagocytosis.

Other alternate immunological mechanisms for sequestration and erythrophagocytosis by the reticulo-endothelial organs have been suggested (Zuckermann, 1964). These range from the coating of erythrocytes with parasitic antigen or the sharing of antigens between host red cell membrane and trypanosomes. Modification and alteration of host erythrocytes so as to become auto-autogenic has been suggested by the same author. Alternatively, it can be envisaged that antigen-antibody complexes and C' may coat the red cell membrane.

1.4. Pathological mechanisms in trypanosomiasis

Recently there have been excellent reviews published on the pathology of trypanosomiasis (Fiennes, 1970; Ormerod, 1970; Losos and Ikede, 1972; Goodwin, 1974). Each author has stressed the paucity of knowledge of the mechanisms responsible for the production of clinical disease in trypanosomiasis which has subsequently led to the inadequate description of physiopathological events occurring in the infected host. In fact, it could be said that up until a few years ago, very little advance had been made in this field since the pioneer works of Mott, Laveran and Peruzzi.

The review following deals only with pathological events occurring in infections with African pathogenic trypanosomes, particularly infections in the rabbit. Occasional references to Chagas' disease will be made where similarities to the African trypanosomiasis have been recognised. Involvement of the coagulation and fibrinolytic systems during trypanosome infections is discussed separately under Section 1.6.

1.4.1. Clinical signs of infection

Losos and Ikede (1972) divide the pathogenic trypanosomes into two groups according to their location within the host. The haematic group, comprising T. (N.)congolense and T. (D.) vivax remain essentially blood parasites confined within the plasma of the blood

vessels. In contrast, those of the humoral group, made of T. (T.) brucei, T. (T.) rhodesiense and T. (T.) gambiense have a predilection for the connective tissues and intracellular tissue fluids in a variety of hosts in addition to infecting the plasma. Because of these differences in location, lesions produced during the disease in the two groups tends to differ. Thus anaemia plays an important part in the pathology of the haematic group whereas extensive degenerative inflammatory and necrotic changes of various organs are important in the brucei sub-group trypanosomes.

The clinical signs of infection in rabbits with T. (T.) brucei are considered to be typical of infections with trypanosomes of the brucei sub-group (Brumpt, 1905; Yorke, 1911; Tobie and Highman, 1956; Goble, et al, 1959) and the rabbit is usually used as a laboratory model for human and cattle trypanosomiasis. However, there is a limitation of this model with regard to validity of conclusions since in rabbit trypanosomiasis there are important differences such as the apparent non-existence of CNS involvement. However there are certain common signs of infection, the first being an oedema and erythema of the ears, eyelids, external nares and scrotum (Durme, 1905) followed by exudative erosive dermatitis (Fig. 1). Parasitaemia is relatively low and the course of infection in rabbits is from 3 to 9 weeks or longer. Enlargement of the

lymph nodes (Mackenzie, personal communication) occurs - a feature common in sleeping sickness (Ormerod, 1970) and in cattle with both T. (D.) vivax (Hudson, 1944), T. (N.) congolense and T. (T.) brucei (Ikede and Losos, 1972; Losos and Ikede, 1972). Along with hyperplasia of the lymphatic system, splenomegaly, with prominent Malpighian corpuscles, is a constant feature both in rabbits (Losos and Ikede, 1970), in cattle with T. (N.) congolense (Naylor, 1971) or T. (D.) vivax (Hudson, 1944) and in sleeping sickness (Woodruff et al, 1973). The splenomegaly is probably due to a multitude of causes such as an increase in phagocytic activity, immunoglobulin synthesis and extra-medullary erythropoesis.

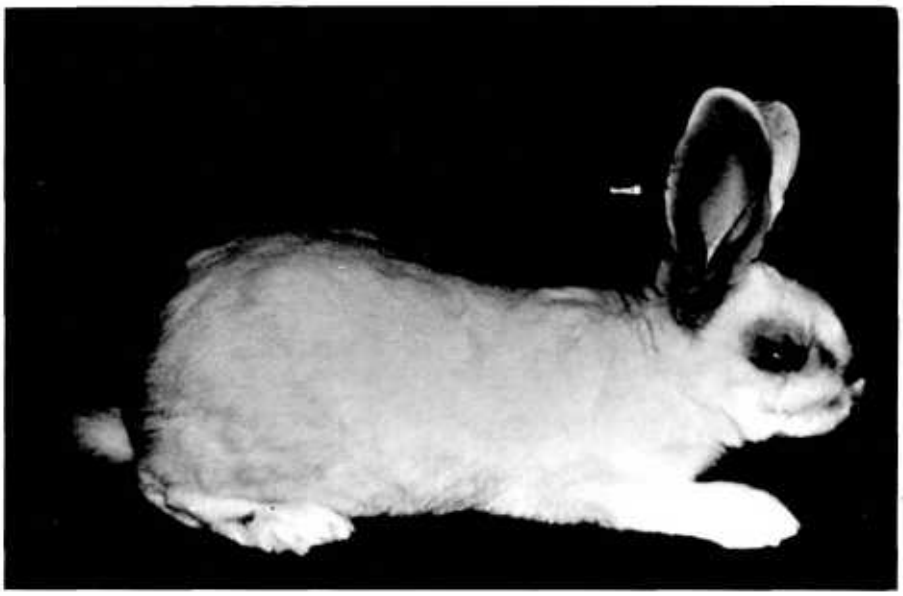
1.4.2. Central nervous system involvement

In sleeping sickness, the most important histo-pathological lesion is meningoencephalitis (Ormerod, 1970) following infection with T. (T.) rhodesiense.

Cerebral involvement becomes manifest between the 3rd and 12th weeks after infection. In contrast, sleeping sickness due to T. (T.) gambiense, occurring mainly in West and Central Africa, gives a more chronic infection with symptoms less severe and cerebral changes may or may not develop.

Post mortem examination of human T. (T.) rhodesiense trypanosomiasis patients indicated that all had central nervous system involvement (de Raadt et al, 1965).

(a)



(b)



Fig. 1

Fig. 1.

(a) Rabbit 35 days after infection with *T. (T.) brucei* 427. Note the development of oedema.

(b) Rabbit 28 days after infection showing lesions developing around eyes, nares and on the limbs.

However a remarkable observation was the varying degree of severity of the punctate haemorrhages and cerebral oedema. Both features were definately more pronounced in that patients having received anti-trypanosomal treatment - suggesting that a Herxheimer-like reaction was possibly more important than previously considered in the causation of cerebral complications.

Ikede and Losos (1972) investigated a dog, naturally infected with T. (T.) brucei that developed incoordination and blindness where there as a diffuse meninogoencephalitis characterised by perivascular accummulations of large lymphocytes, plasma cells and occasionally cells of Mott. Again, in T. (T.) brucei infections of sheep and goats necrosis of parenchymal cells in the pars anterior of the pituitary was attributed to invasion of the tissues by trypanosomes. These lesions were not observed in experimental infections of rats and rabbits with the same trypanosome species (Losos and Ikede, 1970). A mild encephalitis appeared to occur with natural T. (T.) brucei infections of game animals (Losos and Gwamaka, 1973).

Cases of CNS involvement in cattle with the same parasite are described by Gray (1964) and Mott (1906).

The physiological changes occuring within the CNS during infection is certainly a field of work which requires further detailing, possibly with comparisons

to the similar conditions known to occur in cerebral malaria.

1.4.3. Cardiovascular changes

While tissue damage and cellular infiltration occurs in most organs during trypanosomiasis, especially in the vicinity of the trypanosomes, the organ most severely affected is the heart. Rhodesian sleeping sickness patients often exhibit vascular changes such as diminished and labile pulse pressures, and electrocardiographic (ECG) abnormalities particularly in the T wave (Bertrand et al, 1965). Macroscopically the heart may be normal or enlarged - the enlargement caused more by dilation than hypertrophy. Hisopathological lesions in T. (N.) congolense infections of cattle are mainly inflammatory changes consisting of oedema and infiltration with lymphocytes, macrophages and plasma cells - symptoms very similar to those of Chagas' cardiomyopathy (Ormerod, 1970).

Trypanosomes have been observed intravascularly in the capillaries of the myocardium of cattle infected with T.(N.) congolense (Fiennes, 1950) and T. (D.) vivax (Lester, 1932) where they are considered to produce the severe myocardial lesions. More recently this situation in cattle has been likened to the chronic experimental infections of rats with infections of T. (T.) brucei (Murray et al, 1974). Severe myocarditis developed in many rats as demonstrated histologically

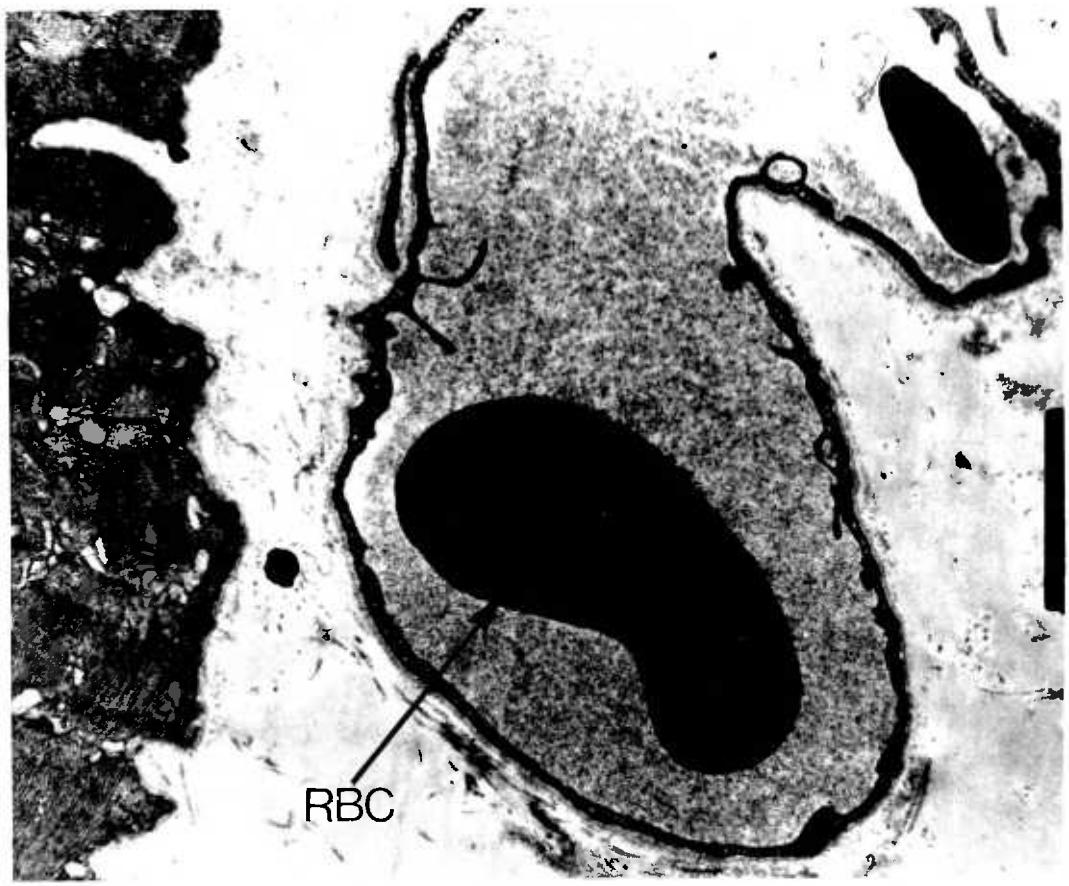
and by ECG. It was considered by the authors that a large proportion of the animals died in heart failure as a result.

1.4.4. Vascular lesions in trypanosomiasis

Advancement in this field of pathology was made by Goodwin and his colleagues. Using X-ray angiography they showed that the main arteries in the ears of rabbits experimentally infected with T. (T.) brucei became irregularly constricted during the disease (Goodwin and Hook, 1968; Goodwin, 1971). Evidence was produced that this feature was mediated through the autonomic nervous system. Constriction of the vessels resulted in areas of anoxia causing tissue damage. In addition they showed congestion of the venous drainage from the muscles and viscera.

Later studies involving ear chamber experiments investigated changes in regenerative tissue and the constituents of tissue fluid (Goodwin, 1971; Goodwin et al, 1973). A constant feature occuring around the 14th day of infection was the adherence of leucocytes and macrophages to the vascular endothelium, probably as a result of injury to the vascular bed by trypanosomes (Fig. 2). Adherent cells and platelets soon were shown to form raised obstructions to blood flow and gave a characteristically "serrated" appearance to the venous flow. Occasionally blood flow ceased followed by vascular disintegration due to anoxia of the tissues. Extravascular tissue was seen to be

(a)



(b)

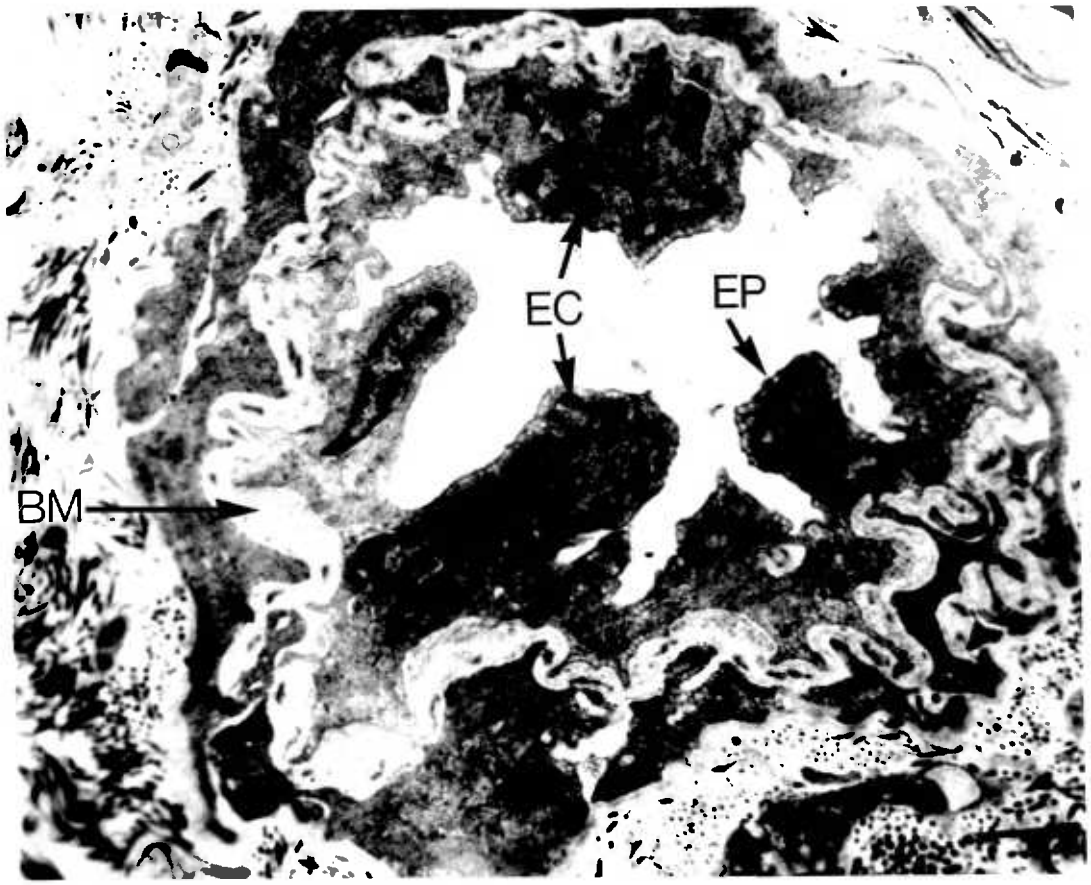


Fig. 2.

Fig. 2.

(a) Electromicrographs through a capillary in the eye muscle of:-

(a) non-infected rabbit x 7,500 mag.

(b) rabbit infected with T. (T.) brucei x 7,500 mag. This capillary is almost occluded by swollen nucleated endothelial cells (EC) and basement membrane (BM).

EP = epithelium

RBC = erythrocyte

Both photographs reproduced by kind permission of Dr. P. Boreham.

heavily infiltrated with mononuclear cells although very few polymorphs were present. What initiates these changes however has yet to be explained and their importance ascertained.

1.4.5. Anaemia in trypanosomiasis

The anaemia in trypanosomiasis is an area of the pathology of this disease which has received most interest and attention. However, the importance of anaemia in experimental trypanosome infections of the brucei sub-group is still a matter of conjecture. The available evidence certainly suggests it as being a contributory facter towards the death of the host. Not only is its importance open to question, but also its aetiology. In discussing the anaemia, it is important to consider the two groups of African pathogenic trypanosomes separately.

The cause of the anaemia in cattle infected with T. (N.) congolense and T. (D.) vivax has been attributed to the inhibition of haemopoeisis by various workers (French and Hornby, 1934, 1935, 1938; Parkin, 1935; Fiennes, 1950; Hornby, 1952). This conclusion was based on the absence of immature red blood cells in the circulating blood and lack of evidence of stimulation of long bone erythropoeisis. In contrast Naylor (1971, a, b, c) found, on examination of bone marrow smears from cattle infected with T. (N.) congolense, that hyperplasia developed early on in the disease and

persisted up to 16-20 weeks after infection when it became normoplastic. However, he also found no obvious extensions of red bone marrow down the long bones in agreement with the findings of French and Hornby (1934) but contrary to those of Fiennes et al (1946). Naylor also detailed a normochromic macrocytic anaemia accompanied by a leucopenia - lymphocyte neutrophil, and eosinophil levels all being depressed. Regenerative forms of erythrocytes (reticulocytes included) were uncommon in the peripheral blood at all times, and anisocytosis was the only common morphological change observed. There is no detailed description of haematological changes in T. (T.) brucei infections of cattle - the only report is a decrease in eosinophils (Naylor, 1967).

Bone marrow in T. (T.) evansi infection has been shown to be depressed by Richardson and Kendall (1963), and Ershov (1956) suggested that during infection some "toxic" material was present which depressed the bone marrow. Contrary to these findings Jatkar and Purohit (1971) showed that rats and cattle infected with T. (T.) evansi suffer from a macrocytic anaemia which was not due to bone marrow depression since the marrow was shown to be hyperplastic (as in T. (N.) congolense infections of cattle) during the anaemic phase.

Increased serum bilirubin has been described in T. (N.) congolense infected cattle and, since liver

lesions were not severe, its presence was taken to indicate a haemolytic condition although haemoglobinaemia was never seen (Naylor, 1971). However Fiennes (1954) considers the anaemia to be non-haemolytic in the same host-parasite system.

Anaemia of unknown aetiology also occurs in the brucei sub-group trypanosomes and is considered by some workers to be only of secondary importance in the pathogenesis of the disease (Losos & Ikede, 1972). However a situation similar to that of T. (N.) congolense was described in rabbits infected with T. (T.) brucei (Boycott and Price-Jones, 1912). These authors showed a depression of bone marrow activity and storage of iron pigments in the spleen, kidney and liver.

Polychromasia, anisocytosis and normoblast responses have been reported in rabbit infections of T. (T.) brucei, and, in contrast to the above findings, a reticulocytosis was apparent (Boreham, 1968a). This situation is identical to that occurring in rats infected with the same parasite, findings recently reported by Jennings et al (1974). In addition these results have received supportive evidence from Jenkins and his colleagues (Jenkins et al, 1974) who found that in rabbit infections reticulocytes increase, erythrocytes and haemoglobin decrease accompanied by a lymphocytosis macrocytosis and a thrombocytopenia. Morphological examination of erythrocytes showed an interesting

variety of abnormal forms, including spherocytes. Total plasma bilirubin increased as previously shown by Naylor (1971) in cattle trypanosomiasis. Bone marrow hyperplasia was evident. The authors concluded from these findings together with results from coagulation studies, that the anaemia associated with T. (T.) brucei infections partly involves a microangiopathic haemolytic mechanism.

A haemolytic anaemia has been shown to occur in T. (T.) brucei infections of both mice and rats, the animals often succumbing at an acute haemolytic crisis (Jennings, et al, 1972, 1974). Circulating half-life values of red cells was reduced in infected rats and the label excreted in the urine. Fe^{59} was rapidly removed from the serum of infected mice and incorporated into the circulating red cells. There was no increased loss of iron from the body. These results, together with histological findings of haemosiderosis and marked splenic erythrophagocytosis pointed to the anaemia as being haemolytic in origin.

A similar decrease in red cell half-life occurs in rabbits infected with a different strain of the same parasite (Jenkins et al, 1974).

It is possible that, since the red cell morphology is altered, a weakening of the red cell membrane might also occur and be the cause of the observed haemolysis.

However, this theory is discounted since the osmotic fragility of infected rabbit erythrocytes to hypertonic solutions is unaltered (Mackenzie and Boreham, in press).

Reports of increased phagocytosis of apparently normal red blood cells suggests a mechanism perhaps similar to that occurring in malarial infections. An extravascular haemolysis associated with erythrophagocytosis within the spleen has recently been described in sleeping sickness (Woodruff et al, 1973) - demonstrated by surface counting in patients whose erythrocytes had been labelled with Cr^{51}. Woodruff suggested that, if erythrophagocytosis is explained by an immunological mechanism, then C' coating of red cells is likely to occur. Jenkins (personal communication) suggests a similar mechanism is in operation in trypanosome infections of rabbits. Alternative immunological mechanisms which could explain the anaemia (e.g. red cell auto-sensitisation) have been previously discussed under Section 1.3.4.

The anaemia in trypanosome infections is often accompanied by an increase in plasma volume e.g. T. (N.) congolense in cattle (Fiennes, 1970; Naylor, 1971); T. (T.) brucei in rabbits (Boreham, 1968) and T. vivax in sheep (Clarkson et al, 1966; Clarkson and Awan, 1969) - a feature almost certainly contributing to the anaemia.

The cause of the anaemia in trypanosomiasis thus

remains uncertain, although it is becoming evident that not only one mechanism is in operation, and immunological mechanisms such as coating of the red cells with antigen-antibody complexes and C', may play an important part depending on the host-parasite system investigated.

1.4.6. Serological alterations

The alteration in numbers of cellular components of the blood and haemodilution is also accompained by changes in the proportion of the various plasma proteins. As previously discussed under Section 1.3 there are marked elevations of both IgG and IgM. In conjunction with this there is often a drop in serum albumin described in T. (T.) gambiense infections of monkeys (Smithers and Terry, 1959) and in experimental T. (T.) brucei infections of lions (Mortelimans and Kageruka, 1971), rats (Ikejiani, 1946) and rabbits (Goodwin and Guy, 1973). Goodwin and Guy developed a technique for collecting tissue fluids for biochemical analysis, by implanting perforated plastic hair curlers subcutaneously into rabbits. They showed that the tissue fluid also showed a decrease in albumin content along with an increase in globulin content reflecting the intra-vascular situation. The aetiology of the decreased albumin and the physiological effects it may have in the blood have not been considered.

C-reactive protein (CRP) a globulin of γ_1 electrophoretic mobility not normally present in the serum but

a common feature of many inflammatory processes, has recently been demonstrated to occur in high titres around the third week of infection in rabbits infected with T. (N.) congolense (Thomasson et al, 1973). An interesting feature was that CRP levels rose before any external signs of disease occurred and then dropped as tissue necrosis became progressively more severe.

Serum lipid alterations have received little attention. Roberts (1974) described a decrease in all serum lipid components during T. (N.)congolense and T. (D.) vivax infections of cattle and suggested an interference by the trypanosomes of the host's lipid metabolism.

Goodwin and Guy (1973) have shown that as infections with T. (T.) brucei in rabbits progresses the pyruvate concentration in the blood and tissue fluids rises to about five times normal and appears to be associated with changes in the structure of connective tissue. Fibroblasts formed during infection became filled with lipid droplets and gradually ceased to produce collagen fibres. It is suggested by the authors that the high concentrations of pyruvate provide a substrate for the accummulation of lipid in fibroblasts. In this way the parasite metabolism initiates the degenerative connective tissue changes.

Increases in phosphate and decreases in the amount of bicarbonate in serum and tissue fluids of rabbits

carrying T. (T.) brucei (Goodwin and Guy, 1973) are explained by the renal insufficiency that develops. The same explanation was applied to the decrease in calcium and increases in urea and creatinine in the same fluids.

1.4.7. The cause of death in trypanosomiasis

Various theories have been proposed to explain the cause of death of animals infected with trypanosomes.

A trypanosome "toxin" was first described by Laveran and Pettit (1911) but was never demonstrated. Seed (1969) also suggested the presence of a trypanosome "toxin" and showed that injection of soluble T. (T.) gambiense extracts into the skin of rabbits produced inflammation at the site of injection. It was considered possible that the "toxin" might be a hyaluronidase or protease.

Hypoglycaemia has been implicated to be the cause of death in acute trypanosome infections of rats and mice with T. (T.) brucei (Schern, 1925). Here, the high parisitaemia (10^8 to 10^9 trypanosomes per ml of blood) exhausts the blood sugar and carbohydrate reserves. In addition Kli gler et al (1929) considered this feature to be complicated by asphyxiation of the host due to utlization of available oxygen. However, since more recent studies have shown that the blood stream forms of trypanosomes of the brucei sub-group do not metabolise carbohydrate beyond pyruvate (Vickerman,

1965) this theory is unacceptable.

The cause of death in many sleeping sickness patients is most frequently due to secondary infections usually resulting in broncho-pneumonia (de Raadt et al, 1965).

Rabbits and rats infected with T. (T.) brucei are considered to die because of renal failure (Goodwin and Guy, 1973) and heart failure (Murray et al, 1974) respectively. However, the underlying cause leading up to these conditions i.e what initiates these changes, still remains obscure. From the evidence available immune complexes are likely candidates.

Obviously the pathological mechanisms operating in both the human and animal trypanosomiasis still require further investigation - particularly into circulatory and CNS changes together with the relationship and importance of immunopathology in the disease.

1.5. Coagulation and fibrinolysis

According to current knowledge, it appears that both the blood coagulation system and fibrinolytic system are in a state of dynamic equilibrium. If this equilibrium is upset by changes in either system, then haemostasis is not maintained and pathological symptoms ensue. Since both coagulation and fibrinolysis have been well documented (Astrup, 1956; Sherry et al, 1959; Biggs and MacFarlane, 1962; Jorpes, 1964; Ambrus, 1966; Laki, 1968), emphasis in this review will be placed on recent advancements particularly in the field of fibrinolysis. To avoid repetition a list of coagulation factors and their terminology is given in Table 1.

1.5.1. Brief review of the Coagulation System

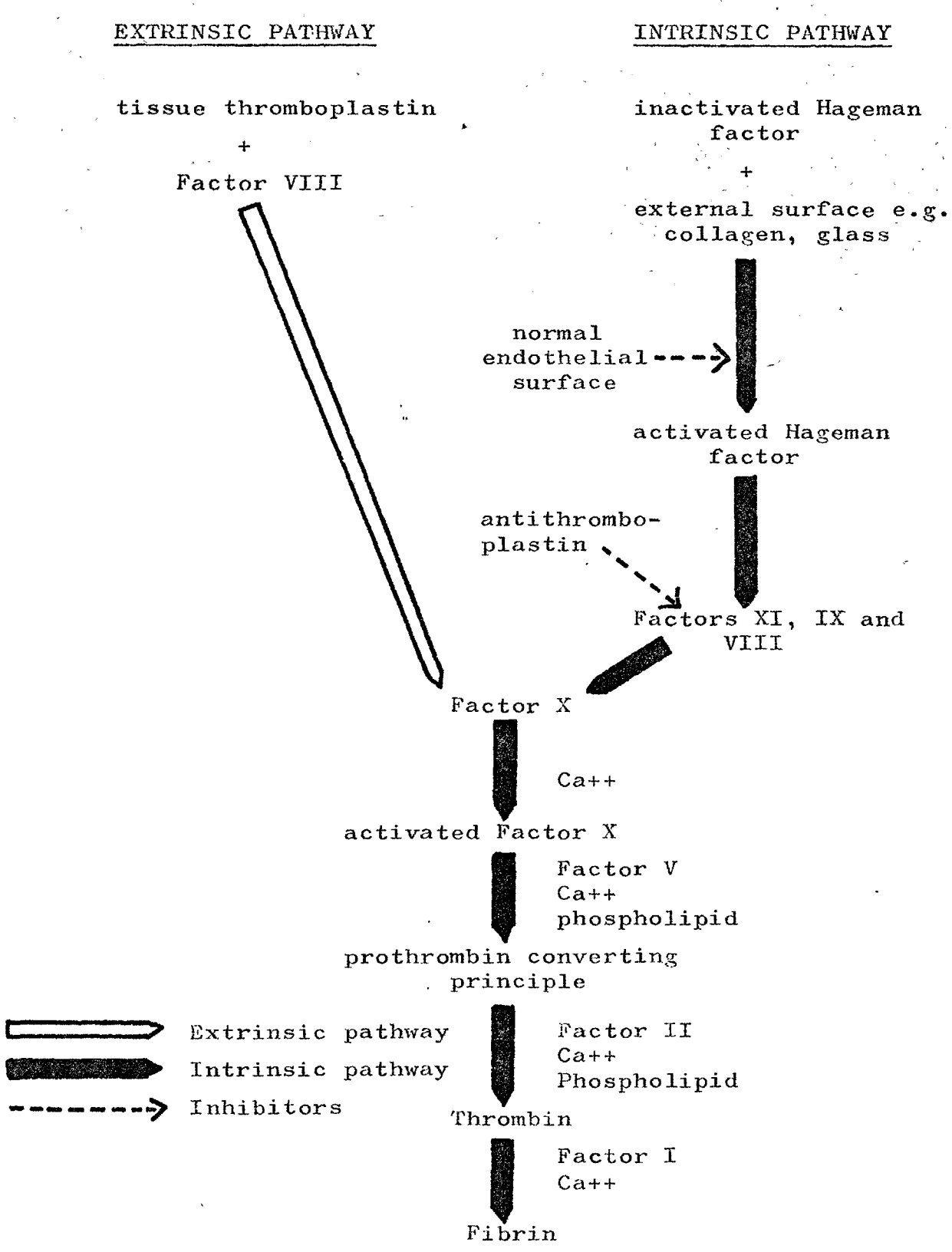
Blood clotting reflects the conversion of plasma fibrinogen to an insoluble network of fibres - the fibrin clot. Fibrinogen is one of the larger plasma proteins with a molecular weight of 340,000 for all species except the horse and donkey, for which the molecular weight is about 500,000 (Blomback, 1964). The molecule consists of 3 pairs of polypeptide chains α, β and γ (Mckee et al, 1966) linked together by clusters of disulphide bonds (Blomback, 1969). Electron microscope studies by Hall and Slayter (1959) have suggested that fibrinogen has a trilobular structure, each globule being interconnected with a strand-like structure. Fibrinogen constitutes about 4% of the total

Clotting factor and alternate name		Found in	
		plasma	serum
I	Fibrinogen	Y *	N *
II	Prothrombin	Y	N
III	Thromboplastin	N	-
IV	Calcium	Y	Y
V/VI	Ac-globulin labile factor	Y	N
VII	serum prothrombin conversion accelerator (SPCA) autoprothrombin I	Y	Y
VIII	Antihaemophilic factor (AHF) haemophilic factor A platelet cofactor I	Y	N
IX	Christmas factor haemophilic factor B autoprothrombin II platelet cofactor II	Y	Y
X	Stuart-Prower factor autoprothrombin C	Y	Y
XI	Plasma thrombin antecedent (PTA) haemophilic factor C	Y	Y
XII	Hageman factor	Y	D *
XIII	Fibrin stabilising factor (FSF)	Y	D

Table 1 Nomenclature of the Clotting Factors

* Y, yes N, no D, decreased

Figure 3. Diagrammatic representation of the extrinsic and intrinsic clotting mechanisms



plasma proteins and is synthesised and stored in the liver parenchymal cells (Barnhart and Forman, 1963; Barnhart, et al, 1970). The protein synthesis is rapid and is readily released into the circulation, the thoracic duct lymph being the major route for delivery (Barnhart and Cress, 1968). Radioactive labelling experiments have shown that, in rabbits, 80% of the total fibrinogen is confined to the plasma, and the remaining 20% is extravascular (McFarlane, 1963).

The coagulation system centres around the activation of prothrombin to thrombin, a process which has two known pathways. The intrinsic pathway requires only a foreign surface to trigger the series of events in which platelets and the haemophilic factors play an important role. The extrinsic pathway requires the addition of tissue fluid to rapidly activate the clotting system by circumventing the chain of events of the intrinsic pathway. Fig. 3 summarises both these pathways. The whole clotting process has been likened to an enzyme cascade in which proenzymes are converted to enzymes at each stage (MacFarlane, 1964).

Apart from the mechanisms shown in Fig. 3, other stimuli may bring about clotting. Reptilase, a proteolytic enzyme from the venom of Bothrops atrox leads to clot formation. Recently Boulton et al (1974) while investigating the coagulation system of rabbits found that, unlike human plasma, rabbit plasma could

not be coagulated by Reptilase in the absence of Ca^{++}.

Fibrinopeptides

The action of thrombin on fibrinogen results in the release of two acidic polypeptides - fibrinopeptides A and B (only A with Reptilase). The remaining fibrin monomers polymerise to form an insoluble fibrin clot (Blomack, 1958; Stocker and Straub, 1970). The release of both fibrinopeptides is mandatory prior toclot formation and visible clotting does not take place if fibrinopeptide B is cleaved selectively. In vivo and in vitro studies by Gladner and Murtaugh (1968) have shown that these peptides have potent biological activities in stimulation of smooth muscle contraction using oestrus rat uterus and bradykinin as a contracting agent.

Pathological variations in the clotting mechanism

Various clinical entities have been associated with the intravascular activation of the clotting mechanism. Since blood stasis is known to play an important role in pathological clotting, intravascular coagulation may be seen in certain vascular malformations as well. A classification of the clinical entities into four groups has recently been proposed by Rodriguez-Erdmann (1973). The classification is as follows:-

Group 1. Shwartzman - type reactions following liberation of endotoxin into the circulation.

Group 2. Entities in which thromboplastic material enters into the circulation e.g. "auto-infusion" of tissue thromboplastin into the blood following trauma, increased vascular permeability, certain obstetric complications and thromboplastic material from an exogenous source e.g. viper bites.

Group 3 Giant vascular formations e.g. haemangiomas and aneurysms. In addition stasis of blood is important in this group of diseases.

Group 4 Mixed clinical conditions of poorly understood aetiology and pathogenesis e.g. shock, trauma, infection, haemolytic crisis in Plasmodium falciparum malaria, rickettsial infections.

The tendency for abnormal intravascular coagulation to occur may take place in the presence of increased clotting factors. For example, there are numerous clinical entities in which fibrinogen synthesis is increased resulting in a hyperfibrinogenaemia with an associated tendency towards intravascular clotting e.g. in acute and chronic bacterial infections (McFarlane,

1964), postoperative states (Izak et al, 1967; Regoeczi, 1970), nephrotic syndrome (Takeda and Chen, 1967), rheumatoid arthritis (Takeda, 1967) and pregnancy (Regoeczi and Hobbs, 1969).

1.5.2. Primary and secondary fibrinolysis

In the normal process of haemostasis and repair of injury, coagulated blood after acting as a haemostatic barrier and possibly a supporting framework for the growth of fibroblasts and new capillaries, gradually dissolves - a process known as secondary fibrinolysis. A scheme of the fibrinolytic mechanism describes a system of activators that act on plasminogen (profibrinolysin) an inert plasma proenzyme, resulting in the formation of an active proteolytic enzyme plasmin (fibrinolysin). For convenience, the activators are classified as exogenous (e.g. streptokinase) and endogenous (e.g. urokinase). All the steps participating in the activation of plasminogen, in addition to the enzyme itself, are blocked by inhibitors. The total net balance of this continuous activation and inhibition results in slight fibrinolytic activity under normal conditions (Fearnley, 1961). Primary fibrinolysis or fibrinogenolysis does not occur under normal conditions and usually follows acute episodes of secondary fibrinolysis (Bowman, 1958).

In the review following, components of the fibrinolytic system, its activators and inhibitors are discussed.

This is followed by a consideration of abnormal primary and secondary fibrinolysis and the pathological effects of the end products of these processes.

Plasminogen and plasmin

Plasminogen, although abundant in the plasma where its concentration in man has been estimated at 0·1 to 0·2 mg/ml and it's activity at 2·5 ± 1·0 caseinolytic units/ml (Marder and Sherry, 1972), is also present in smaller amounts in all body fluids and secretions including urine. It has a high affinity for fibrin and significant amounts are incorporated into the fibrin meshwork to mediate its subsequent lysis when plasminogen activator diffuses into the clot from the circulating plasma or surrounding endothelium. In various exudates and transudates a correlation exists between plasminogen and fibrinogen levels: where fibrinogen is low or absent, plasminogen is reduced or absent, and where fibrinogen is abundant plasminogen is present in high concentrations (Christensen, 1954). The precise origin of plasminogen has not yet been elucidated and its synthesis in the bone marrow eosinophil (Barnhart and Riddle, 1963) in the liver (Pechet, 1965) and more recently in the kidney (Highsmith and Kline, 1971) has been demonstrated or suggested.

Activation of plasminogen leads to its conversion to plasmin by the enzymatic splitting of a single arginine-valine bond resulting in two peptide chains

held together by a single disulphide bond. Plasmin itself is capable of hydrolysing susceptible arginine and lysine bonds in a variety of naturally occurring proteins at a neutral pH (Johnson et al, 1952; Coon and Duff, 1958; Mirsky et al, 1959). It is also known to inactivate complement (C') by hydrolysing certain of the components of the C' system. Clotting factors are also susceptible to hydrolysis by plasmin (Donaldson, 1960).

Activation of fibrinolysis

Four groups of naturally occurring (endogenous) activators of plasminogen are known to occur (Sherry et al, 1959). These activators are also proteolytic enzymes highly specific for the plasminogen substrate and include tissue activators (tissue kinases), an activator in plasma (plasma kinase), a urinary activator (urokinase) and trypsin. Exogenous activators are also known to occur and include streptokinase, staphylokinase, chloroform and peptone - these however will not be discussed here and are reviewed in detail by Sherry et al (1959). The following discussion is concerned only with endogenous activation.

The presence of an activator in a variety of animal tissues, originally demonstrated by Astrup and Permin (1947), has been investigated and confirmed by others (Lewis and Furguson, 1950; Tagnon and Palade, 1950; Todd, 1964; Kwaan, 1966). It is likely that the tissue

activators are concentrated in the cellular lysosomal granules in the vascular endothelial cells of most organs (Astrup, 1966). The type derived from the vascular cells is soluble and presumably diffuses into the circulation in response to vasoactive stimuli (Astrup, 1966). The amount of activator extractable from different tissues varies widely and a physiological basis for this distribution has been suggested (Astrup, 1956, 1966, 1968). Astrup observed that organs with low fibrinolytic activity in man (e.g. liver) were classical sites of fibrosis following injury. Assays of animal tissues revealed that they were less active than human tissues - rabbit tissues were found to be particularly low in activity.

Little is known about the plasma activator or "proactivator" and its existence is even questioned by some authors (Marder & Sherry, 1972). It was originally invoked to explain the two-step activation of plasminogen to plasmin by streptokinase. Because streptokinase is less effective in activating plasminogen of some animal species than that of man (Wulf and Mertz, 1964) and, in particular, completely ineffective with ox blood, it was postulated that these species have little or no proactivator. The finding that an addition of a small amount of human serum to the streptokinase/bovine plasma (or plasminogen) is followed by a prompt activation, seemed to lend further support to the proactivator

theory. However, the purification of such an entity has never been accomplished and Kline and Fishman (1961) demonstrated that 'proactivator' was in fact human plasminogen itself which has the particular property to become activated by streptokinase.

Plasminogen activator can also be found in normal urine and was first demonstrated independently by Williams (1951) in rabbits and later by Astrup and Sterndorff (1952) and Sobel et al (1952). It was termed urokinase by the latter group of workers. This plasminogen activator has subsequently been purified and crystalized (Sgouris et al, 1960; Lesuk et al, 1965) and measurements indicate that it is a single polypeptide chain with an active enzyme centre capable of hydrolysing arginine and lysine bonds (Lesuk et al, 1965). Human urokinase can activate plasminogen from bovine and other species of plasminogen (Mohler et al, 1958). In contrast to plasmin, its activity is not blocked by soybean trypsin inhibitor (Lorand and Mozen, 1964). Although the precise origin of this urinary enzyme is unknown, it is thought to be produced by the kidneys independent of circulating plasma activator levels and excreted through the urinary tract. Its function is presumably to keep the urinary tract free of fibrin deposits (Astrup and Sterndorff, 1952).

The activation of plasminogen by trypsin (Jacobsson, 1953; Jackson and Mertz, 1954; Alkjaersig, et al 1958)

is relatively slow compared to the specific activators. It is not inhibited by EACA (Sherry et al, 1959).

Besides blood and tissue activators, other sources capable of playing a part in the activation of plasminogen are certain cellular components of the blood. Platelets known to contain anti-plasmin, have also been shown to contain activator (Holemans and Gross, 1961). Leucocytes, with the exception of eosinophils, are known to contain in dogs and rabbits, proteases that manifest activator as well as fibrinolytic activity (Gans, 1964). A parellelism between fibrinolytic, proteolytic and esterolytic activity of 7 difference leucocyte proteases in fibrinolysis was supported by Gottschall, (1965). However, unlike that of plasmin the fibrinolytic activity of leucocyte extracts is not inhibited by either EACA or antiplasmin (Caviezel et al, 1964).

Although haemolysed red cells might be devoid of direct activator potential (Holmans and Gross, 1961) urokinase and urokinase-treated plasminogen were found more effective in lysing clots when the perfusion medium contained red cells (Back et al, 1964). This could be due to an indirect effect by the action of urokinase, or to a fibrinolysis potentiating agent contained by the red cells. It is interesting to note in this context, that urokinase has been shown to be absorbed onto the red cell membrane (Kolman et al, 1959).

Inhibitors of fibrinolysis

The inhibitors of plasminogen system have been divided into two categories (Sherry et al, 1959) - those acting upon the activation process (inhibitors of action) and those acting upon plasmin (antiplasmins). Moreover certain synthetic compounds such as BAMe (penzoyl-arginyl-methyl ester), LEe (lysine-ethyl ester) and EACA are also capable of inhibiting plasminogen activation competively (Barnhart and Riddle, 1963) and are used in the management of clinical conditions.

Whereas the nature of the inhibitors of plasminogen activation are well documented, the mechanisms of their action are still poorly understood. Two antiplasmins have been isolated from blood and characterised - one an α_1, macroglobulin and the other an α_2 macroglobulin (Taylor et al, 1964). Diminished levels of inhibitors are invariably seen in pathologic fibrinolysis.

Lipaemia is believed to inhibit the in vivo fibrinolytic system and also induce changes in the coagulation mechanism favouring accelerated clotting. Clots induced in dogs during alimentary lipaemia were markedly resistant to fibrinolysis (Bang and Clifton, 1960). In rats given a diet high in saturated fatty acids, thrombi and infarcts, prolonged lysis time and increased coagulability were found to develop (Naimi et al, 1962). Alimentary lipaemia was also found to have an inhibitory effect on the fibrinolytic system of man (Konttinen, 1962). Conversly, no changes were found in

either coagulation or fibrinolysis in patients with hyper-cholesterolaemia and idiopathic hyperlipaemia (Peyman et al, 1960). Lipids may have an indirect effect by activating Hageman Factor (Lasch, 1966) which itself is a plasminogen activator. The importance of serum lipids in initiating coagulation has received more recent attention by several workers (Cullen and Swank, 1954; Dennis et al, 1966; Rodriguez-Erdmann, 1965; Zbinden, 1967; Kwaan and Suwanwela, 1969; Newall et al, 1973).

Two major drugs used in fibrinolytic therapy are EACA and aprotonin ('Trasylol'). EACA is a potent competitive inhibitor of plasminogen activation and a relatively weak inhibitor of plasmin and trypsin (Ablondi et al, 1959). In small concentrations, EACA may actually enhance the action of plasmin on different substrates (Alkjaersig, et al, 1958). The other blocking agent, 'Trasylol', is a kallikrein inhibitor isolated from bovine parotid gland. This agent not only blocks plasmin but also inhibits the activation of plasminogen. It seems to be more effective than EACA in preventing fibrinolysis (Beck et al, 1963).

Abnormal states of fibrinolysis

A shifting of the 'balance' between coagulation and fibrinolysis can lead to pathological conditions. A vast literature, much of it of uncertain quality, covers the field of fibrinolysis in disease states.

In this review only key references will be cited where pathological fibrinolysis in disease states illustrates principles of general interest.

MacFarlane and Biggs (1948) first suggested the relationship between stress and the development of fibrinolytic activity. They observed increased fibrinolytic activity in man following adrenalin injections. In immune reactions e.g. anaphylactic shock and peptone shock, fibrinolytic activity is produced (Rocha e Silva and Teixeira, 1946; Ungar, 1953). More recently Barnhart et al (1970) have confirmed the early work of Macfarlane and Biggs (1948) and shown alterations of fibrinogen metabolism and fibrinolysis in stress states in the dog.

Three mechanisms exist for the production of pathological fibrinolysis. First inordinate amounts of plasminogen activator may be released into the circulation from neoplastic tissue, e.g. metastic prostrate carcinoma (Swan et al, 1957) or from the activator rich vascular endothelium, after trauma, anoxia or shock (Sherry et al, 1959). Impaired clearance of plasminogen activator produces fibrinogenolysis e.g. in hepatic cirrhosis (Frick, 1956). Lastly, proteolytic enzymes other than plasmin capable of degrading fibrinogen may be liberated from leukaemic cells or damaged leucocytes (Cooperberg and Neiman, 1955; Gottschall, 1965). The spontaneous occurrence of

pathological fibrinogenolysis, severe enough to cause hypofibrinogenoemia, is rare and usually clinical symptoms represent the condition of diffuse intravascular coagulation (DIC) - a condition also referred to as defibrination syndrome or consumption coagulopathy (Bachman, 1971). This syndrome can be initiated by many diseases and represents a haemostatic breakdown, where thromboplastin-like substances are released into the blood leading to extensive deposition of fibrin in the microcirculation of many organs. Anoxia followed by necrosis may occur as a result of thrombi in the brain, lungs, kidneys and skin (Marder & Sherry, 1972). A further complication during episodes of DIC is a haemolytic anaemia typified by the appearance of red cell fragmentation. This anaemia has subsequently been termed microangiopathic haemolytic anaemia by Brain et al (1962). Brain and his colleagues (review by Brain, 1970) demonstrated in vivo the aetiology of the anaemia. They showed that red cells became caught up on intravascular fibrin strands and were damaged in the process resulting in the appearance of the abnormally shaped (schistocytes) erythrocytes in the peripheral blood. Concurrent haemolysis is also a common finding in DIC (Klein et al, 1973).

Following DIC, there appears rapid local fibrinolysis of microthrombi resulting in the formation of breakdown products of fibrin. These degradation products

(described in detail under a later heading) accummulate and produce derangements of coagulation and also interfere with other physiologic functions (Bachmann, 1971; Fletcher and Alkjaersig, 1973).

In brief, the classical laboratory picture of DIC syndrome is a deficiency of coagulation factors (e.g. platelets, fibrinogen, Factors V and VIII), the consumption of fibrinolytic components (e.g. plasminogen) and the disruption of normal clotting by circulating fibrin degradation products. Circulating fibrin monomer is often found together with a fibrinogen that exhibits a tendency to clot at 4°C (cryofibrinogen). In addition other 'abnormal' fibrinogen components may be found (Cotton and Craven, 1970) such as the heparin precipitable fraction of human plasma described by Smith and von Korff (1957) and further demonstrated following major surgical operations by Godal (1962). 'Fibrinogen B' originally described in 1948 by Cummine and Lyons and later by Smith et al, (1951) and Godal (1962) is assumed to be an intermediate between fibrinogen and fibrin (but not fibrin monomer) which gells on addition of a napthoquinone complex, and is often found in thromboembolic states.

1.5.3. Fibrin and fibrinogen degradation products (FDP)

Enzymatic digestion by plasmin of fibrin clots or circulating fibrinogen results in the appearance, in the systemic circulation, of soluble polypeptide

fragments referred to as fibrin degradation products (fibrin DP) and fibrinogen degradation products (fibrinogen DP) respectively, or collectively as FDP. These substances have also been referred to as fibrinogen/fibrin related antigens (or FR-antigens) (Wood et al, 1972) and fibrinogen/fibrin split products (or FSP, Merskey, et al, 1966). In vitro studies, where plasmin and other proteolytic enzymes such as trypsin are incubated with fibrinogen or fibrin, have shown that an enzymatic breakdown of these proteins occurs in a defined sequence of stages - resulting in the formation of FDP of varying molecular weights divided into "early" and "late" FDP (Fig. 4). This enzymatic hydrolysis has been extensively studied by means of analytical ultracentrifugal, gel electrophoretic and biochemical techniques with subsequent characterisation of fragments (Nussenzweig and Seligmann, 1960; Fletcher, 1965; Nilehn, 1967; Marder et al, 1969). From Fig. 4 it can be seen that 2 Fragment D and one Fragment E molecules are derived from each parent fibrinogen molecule (Marder et al, 1969).

The above widely accepted concept of fibrinogen degradation described by Marder may be slightly oversimplified. Fletcher's group (Fletcher, 1970) suggests the existence of several intermediates (MW 250,000 - 100,000) before the final plasmin resistant D and E fragments are formed. In addition

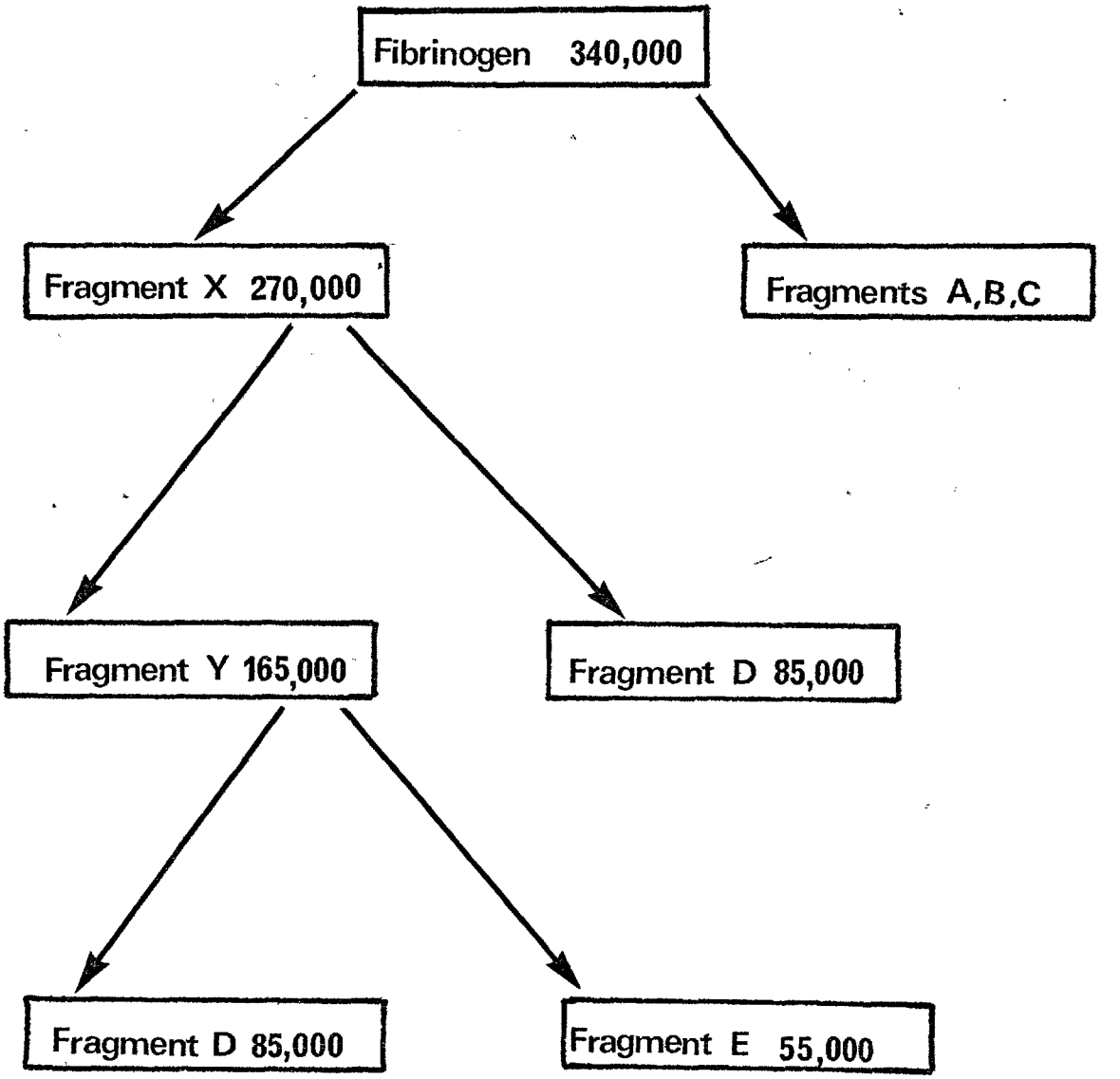


Fig. 4: Degradation pathway of fibrinogen by plasmin

The molecular weight of each fragment formed is indicated.

Fragments X and Y = "early" FDP

Fragments D and E = "late" FDP

Only Fragment X is clotted by thrombin.

the degradation scheme of McKee's group (Pizzo et al, 1972) fits better in accordance with the demonstration of 4 bands in the 'X' area in polyacrylamide electrophoresis, and of D and E fragments of different mobility in agarose (Gormsen et al, 1972).

It has recently been shown that there are two types of X fragment, X^{fp} bound to the fibrinopeptides and Fragment X^{o} free of fibrinopeptides (Schorn, 1973). The fibrin moroners are prevented from spontaneous polymerisation within the circulation by forming soluble complexes with fibrinogen or fibrinogen-DP (Shainoff and Page, 1962). Fragment X^{fp} formed by the limited action of plasmin on fibrinogen have been shown to retain their fibrinopeptide chain which prevents their polymerisation in the circulation. When plasmin acts on fibrin to form fibrin-DP the Fragment X^{o} is also prevented from polymerisation by forming soluble complexes with fibrinogen or Fragments Y and D (Niewiarowski et al, 1970). These reactions, shown in Fig. 5 are part of the normal process to prevent polymerisation in the circulation and so keep the blood free of precipitates - the "final anti-clotting defense mechanism" (Gurewich and Hutchinson, 1971).

Immunochemical properties

It has been suggested by Nussenweig et al (1961) that fibrinogen contains four antigenic determinants. "Early" and "late" FDP have antigen determinants common

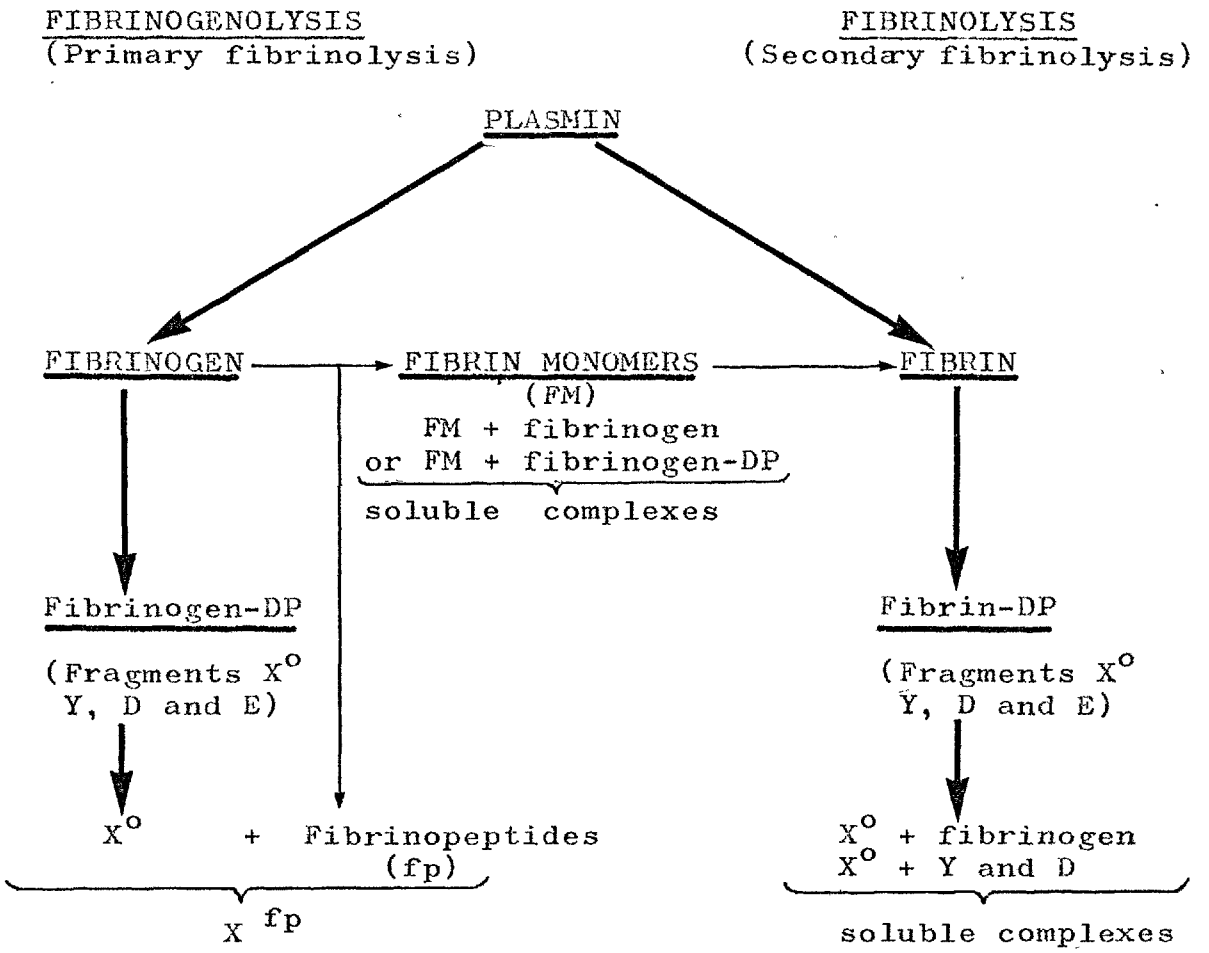


Fig. 5 Diagram Showing the Formation of FDP from Primary and Secondary Fibrinolysis with Plasmin

→ Clotting process

⟶ fibrinolysis

to those of fibrinogen and fibrin (Salmon, 1959; Nussenweig and Seligmann, 1960; Niljehn, 1967) thus making the immunological determination of these products possible using either an anti-fibrinogen or anti-fibrin sera. The 'minor' fragments A, B and C form a group of separate non-antigenic polypeptides (Gormsen et al, 1973). Although Fragments X and Y are immunologically identical and share the multiple antigenic determinants of fibrinogen, the situation with Fragments D and E is rather different. Both Fragments D and E have each been shown to contain only one antigenic determinant of the multiple determinants of the fibrinogen molecule, the D antigenic site being immunologically distinct from that of E. Recently Plow and Edgington (1973) have demonstrated a neo-antigen expressed on Fragments X, Y and D but absent on Fragment E and on the native fibrinogen molecule. Thus hidden amino-acid sequences on the fibrinogen molecule are exposed during its catabolism.

The most widely used techniques for the detection and characterisation of FDP are those of immunoelectrophoresis and immunodiffusion in agar gel. It has been shown that when specific antiserum is used to precipitate FDP and fibrinogen following electrophoresis, the pattern for each fragment is quite distinctive (Barnhart et al, 1967; Fisher et al, 1967; Marder et al, 1967, 1969; Cortes et al, 1973; Rayner et al, 1969; Plow et al,

1973; Gaffney et al, 1974).

Measurement of FDP

The occurrence of fibrin or fibrinogen degradation products in certain disease situations with their potential physiopathologic significance, have focused attention on the development of clinical tests for the quantification of FDP in serum and urine. Current methods for demonstrating FDP have been extensively reviewed (Merskey et al, 1967; Marder et al, 1971; Thuot and Larrieu, 1971; Donati et al, 1973). For the purpose of the following brief summary these methods can be sub-divided into two categories, immunologic and non-immunologic.

The common basis for all immunologic techniques is the ability of an antiserum to either fibrinogen a FDP or to detect non-coagulable FDP in sera samples. The FDP antigens may be demonstrated by precipitin techniques (Thuot and Larrieu, 1971) by the tanned red cell haemagglutination inhibition immunoassay (Merskey et al, 1966), or by a similar assay utilising antibody sensitised latex particles (Melliger, 1970; Allington, 1971; Donati et al, 1973). The more recent ratio immunoassay methods are supposedly more sensitive (Catt et al, 1968).

The non-immunologic techniques depend on the ability of certain substances when added to plasma or serum, to aggregate and precipitate FDP. Such assays include the

protamine sulphate and the ethanol gelation tests. The protamine sulphate test (Lipinski and Worowski, 1968; Latello et al, 1970; Gurewich and Hutchinson, 1971; Niewiarowski and Gurevich, 1971; Gurewich, 1973) when used at low concentrations of protamine sulphate detects only fibrin monomer and fibrin-DP. It is sensitive to low levels of each but is insensitive to fibrinogen-DP. It can thus distinguish between fibrinolysis and fibrinogenolysis. Similar to protamine sulphate, the addition of ethanol to plasma evokes precipitation of fibrin monomer and fibrin-DP (Godal and Abildgaard, 1966: Breen and Tullis, 1968). Comparison of this ethanol gelation technique with the protamine sulphate method has yeilded variable results, and it has been suggested (Sanfelippo et al, 1971) that ethanol gelation is less sensitive and specific.

A staphylococcal clumping test has been described, the basis of which is the ability of fibrinogen, fibrin monomer and their degradation products to aggregate a strain of coagulase negative staphylococci (Allington, 1967; Hawiger et al, 1970). The test appears to detect all cleavage products but to a variable degree, and sensitivity to D and E fragments is quite poor (Thomas et al, 1970).

Biological properties of FDP

The anticoagulant effects of plasmin-digest fibrinogen-DP have been recognised since 1957 (Niew-

iarowski and Kowalski, 1957). The 'earlier' (large) degradation products (X and Y) have antithrombin activity and the 'late' (small) fragments (D and E) have the property of interfering with polymerisation (Triantaphyllopoulos and Triantaphyllopoulos, 1970). Thus in patients with fibrinolytic syndromes, the bleeding associated with the disorders is usually attributed to the elevated levels of FDP found in the serum of the patients. Non-clottable complexes formed between fibrin monomer, Fragment X and other degradation products also contribute to the anti-coagulant effect, interfering with thromboplastin generation (Triantaphyllopoulos and Triantaphyllopoulos, 1970) and platelet function (Barnhart et al, 1967) and producing unstable clots with faulty microscopic structure (Bang et al, 1962).

Other physiological effects attributed to FDP include potentiation of biogenic amines (Barnhart, 1969), increasing vascular permeability (Sack and Nefa, 1970) and the resistance of erythrocytes to hypertonic solutions (Triantaphyllopoulos and Triantaphyllopoulos, 1970), and regulation of fibrinogen synthesis (Barnhart et al, 1970).

Significance of FDP in serum and urine

The detection of FDP in the urine and elevated concentrations in the circulation indicates either pathologic activation of the coagulation system or pathologic fibrinogenolysis.

It has been well documented that in DIC, FDP are markedly elevated e.g. metastic carcinomas, leukaemia, sepsis, meningococcal meningitis and obstetrical complications (Bachmann, 1971). In certain parasitic protozoal infections, the elevation of FDP in the serum has been taken as indicative of DIC e.g. in malaria of man (Naroonvesama, 1972; Reid and Nkrumah, 1972) and of rhesus monkeys (Dennis et al, 1966), and possibly in calves infected with the piroplasm Babesia argentina (Mahoney and Goodger, 1969).

Treatment with tetracycline of patients with louse-borne relapsing fever resulted in fibrinolysis and subsequent elevation of FDP in the serum (Perine et al, 1971). It is possible here that chemotherapy triggered off DIC.

Elevations of FDP may also occur in localised thrombotic conditions in addition to DIC. Merskey et al (1966) were the first to observe increases in FDP in the sera of patients with pulmonary embolism. This finding was subsequently confirmed by Cash et al (1969) and Ruckley et al (1970) who also noted increased FDP in patients with venous thrombosis and myocardial infection.

In certain nephrotic diseases and in renal homotransplantation, FDP have also been detected in the urine (Clarkson et al, 1970, 1971; Wardle et al, 1970;

Cortes et al, 1973; Whitworth et al, 1973). Their presence in the urine has been explained by lysis of intraglomerular fibrin (fibrinolysis) in both minimal lesion and membranous glomerulonephritis (Clarkson et al, 1971). In addition urinary FDP may occur as a result of proteolysis in the upper renal tract of fibrinogen (fibrinogenolysis) filtered through an abnormally permeable basement membrane (Clarkson et al, 1971). However, other non-renal conditions such as deep vein thrombosis and DIC are associated with a rise in urinary FDP (Ruckley et al, 1970; Merskey et al, 1967) suggesting that serum FDP may be filtered through the glomerulus and into the urine.

1.6. Abnormalities in Coagulation and Fibrinolysis in trypanosome infections

These two very important systems have not, until recently, been investigated in trypanosomiasis. Early observations on cattle infected with T. (N.) congolense (Fiennes, 1950 (a), (b), 1954, 1970) describe thrombus formation in smaller arteries and veins, the aetiology of which was ascribed to agglutinated trypanosomes. A similar situation has been described in T. (D.) vivax infections of goats (Losos and Ikede, 1972), T. (T.) brucei in horses and rabbits (Helm, 1929), and dogs (Rodet and Valet, 1907). More recently in T. (T.) rhodesiense infections of rats the choroid plexus was found blocked with trypanosomes with a white thrombus formation (Ormerod and Venkatesan, 1971).

Only very recently has any detailed examination of the coagulation system been made. Christine Hawkey (personal communication) first showed changes in certain coagulation factors which were suggestive of DIC in rabbits infected with T. (T.) brucei. This evidence has received support from Bolton et al (1974) using the same host-parasite system, and by Barrett-Connor et al, (1973) on examination of a sleeping sickness patient. All three authors show a general increase in most coagulation factors (Table 1A).

To date, no detailed study has been carried out on alterations in the fibrinolytic system in trypanosome infections (either natural or experimental) - an essential adjunct to coagulation studies.

Observation	(1) *T.(T.) brucei* in rabbits	(2) *T.(T.) brucei* in rabbits	(3) *T.(T.)rhodesiense* in man
Partial thromboplastin time	Increased	N/T	Increased
Prothrombin time	No change	N/T	Increased
Thrombin time	No change	N/T	N/T
Stypven time	No change	N/T	N/T
Re-calcification time	Increased	N/T	N/T
Cephalin time	Increased	Increased	N/T
Factor VIII	Increased	Increased	N/T
Factor IX	N/T	Increased	N/T
Factor XI	N/T	Increased	N/T
Factor XII	N/T	Increased (6x)	N/T
Platelets	N/T	Decreased	Decreased

N/T = not tested.

Table 1 a: Summary of observations on blood coagulation in trypanosomiasis.

1. Hawkey (unpublished observations)
2. Bolton *et al* (1974)
3. Barrett-Connor *et al* (1973)

1.7. Rheology of the Blood

The discipline of rheology is an important aspect in the understanding of haematologic phenomena in the microcirculation, and forms a close link with disorders of coagulation such as found in thrombo-occlusive disease states (Eisenberg, 1966). The rheology of blood or its flow properties include the intrinsic properties of blood and the influence of these properties by the system in which the flow is concerned - reviewed by Wells (1962, 1963 a, b); Whitmore (1968); Merrill, (1969); Chien (1969); Cokelet (1972). Although most experimental work has been attached to studies on human blood, the rheology of blood from different mammals is remarkably similar and it might be

expected that, from the rheological point of view, they can be treated as one system (Whitmore, 1968).

The viscosity of blood denotes the resistance to blood flow. It is defined as the ratio of shear stress (force imposed per unit area of fluid plane) to shear rate (difference in the velocity of 2 planes or centimetres per second divided by the distance between them in centimetres - hence the dimension 1 per sec or sec $^{-1}$). Newton assumed that for a given material this ratio was constant and therefore its viscosity was constant. In other words, to move a fluid twice as fast, twice as much force would be required.

However, blood is not a Newtonian fluid, a property that has been well recognised for many years (Bayliss, 1952; Merrill et al, 1963, a, b, c, 1965). Only fairly recently have the rheological properties of blood been studied under shear rates of near zero flow (Dintenfass, 1962; Wells, 1962). These studies have shown that as the force moving the blood (i.e. shear stress) declines, so the decrease in the velocity of the blood flow (i.e. shear rate) gets proportionately less. Thus the viscosity of whole blood rises as the shear rate approaches zero. This creates the apparent paradox that when the blood moves slowly it needs proportionately more driving force than when it moves rapidly.

Rheology of Normal Whole Blood

As previously mentioned, it is generally accepted that human blood is a non-Newtonian fluid. Earlier measurements of whole blood viscosity were made on capillary tube instruments, where the diameter of the tube may have had some influence on the result obtained (Whitmore, 1968). Recent workers have used cone and plate viscometers (Wells et al, 1961; Charm et al, 1964; Dormandy, 1970, 1971) and rotating cylinder viscometers (Cokelet et al, 1962) where the measured viscosity is substantially independent of red blood cell concentration changes occurring during shear and have thus given generally confirmatory results.

A number of factors have been defined for blood which contribute to its non-Newtonian behaviour. These include properties of the red cells and cell - cell interations. The concentration of red cells (haematocrit) in normal plasma has a profound influence on the viscosity of blood (Meiselman and Merrill, 1966; Wells, 1962, 1967; Chien et al, 1967; Kemble and Hickman, 1972). Doubling the haematocrit of blood has been shown to result in more than a two-fold increase in the viscosity. The relationship of viscosity to haematocrit is exponential as it exceeds 50% (Nygaard et al, 1935). However at haematocrits below 50% there is a linear relationship if the logarithm of the viscosity is plotted against the haematocrit (Dormandy, 1970).

Cell-cell interations are altered not only by changes in haematocrit but also by changes in protein concentration of the suspending medium (Merrill et al, 1963a; Wells, 1963a, 1967; Wells et al, 1964; Chien et al, 1967; Harkness and Whittington, 1967; Rosenblum, 1969). Aggregation and rouleaux formation with the dysproteinaemias or increased fibrinogen are both greater with a higher concentration of cells (Wells et al, 1964).

The forces of fluid shear are primarily a function of vessel diameter and pressure gradient (Whitmore, 1968). The higher pressures and smaller size vessels in the arterial system are associated with greater shear rates and less aggregation of cells. Thus aggregation is more likely to occur within the venous system (Murphy, 1973).

Another factor contributing to the non-Newtonian viscosity of blood concerns the rheologic properties of the individual red cells (Erslev and Atwater, 1963; Gregerson et al, 1963; Chien et al, 1967; Rosenblum, 1969). The red cell undergoes change in shape both in the large vessels because of shearing forces, and in capillaries because of restrictions of vessel size. The deformability of the red cell is also related to its internal viscosity and to changes in the ratio of surface area to volume (Rosenblum, 1969).

Apart from the pathologically altered red cells, which will be discussed under a subsequent heading,

change in shape of red cells has been shown to occur on exposure of blood to certain anticoagulants, and thus presents a problem when determining blood viscosity. Merrill and Wells (1961) and Rosenblum (1969) have shown that erythrocyte shrinkage induced by sodium citrate and oxalate (5 mg/ml) caused an increase in blood viscosity as measured in a capillary viscometer - even though the haematocrit was reduced and the plasma viscosity remained the same. EDTA (1 mg/ml) on the other hand, did not alter cell size or shape and thus failed to elevate the apparent viscosity.

Temperature changes also effect whole blood viscosity but in a less complex fashion than changes in haematocrit or shear rate (Pirofsky, 1953; Merrill et al, 1963b; Rand et al, 1964). When viscosity is plotted against temperature (in a region between 27°C and 37°C) there is a linear rise at each shear rate regardless of haematocrit.

Rheology of normal plasma and serum

Human plasma has been shown to be slightly non-Newtonian (Lawrence, 1950; Wells et al, 1961) the apparent viscosity falling with increasing rate of shear to an asymptotic constant value. This observation is perhaps not surprising in view of the large protein molecules it contains. The addition of anticoagulants sometimes influences the rheological behaviour so that heparin reduces slightly the general level of viscosity

of the plasma and subsequently whole blood (Copely, 1960; Cerney et al, 1962; Charm and Kurland, 1962). EDTA has no effect on altering plasma viscosity (Rosenblum, 1969).

At high shear rates (100 to 120$^{sec\ -1}$), Merrill and Wells (1961) have shown that human plasma behaves as a Newtonian fluid.

Serum is also non-Newtonian and to approximately the same degree as plasma (Whitmore, 1968). For serum, a shear rate of around 250^{sec-1} must be exceeded in order to obtain Newtonian behaviour. Serum viscosities are greatly effected by changes in the globulin content particularly large molecular weight globulins.

Abnormalities of blood viscosity

An abnormality in the microcirculation due to increased plasma viscosity is usually indicated by observations on red cell agglutination and a high ESR. Fahraeus (1929) first described the clinical significance of cell aggregation in vivo demonstrating its correlation with that observed in vitro by the ESR. Knisely and his colleagues (Knisely et al, 1947) studied the micro-circulation by photomicrographic techniques in a variety of illness noting the major role that cell aggregation played in blood flow cessation. From their studies the now widely used term "blood sludging" arose to describe cell aggregation of any form or cause. Similar studies relating sludging of blood to elevations of ESR

were shown by Thorsen and Hint (1951); by Bicher et al (1966) as significant in the pathology of ischaemic myocardial damage; by Wells et al (1964) who showed rates of ESR correlated well with viscosity and the presence of yeild stress. Likewise, Eastham (1954) showed a direct logarithmic correlation between ESR and plasma viscosity. Sludged blood and raised ESR has also been described in multiple sclerosis (Roizin et al, 1953) and in cases of multiple myeloma (Marmont et al, (1957). The lipaemias (including alimentary lipaemia) also cause blood 'sludging' (Elliot, 1973).

The use of syndromes in describe complex lists of symptoms and signs represents a convenient economy of scientific expression. The state of serum (or plasma) hyperviscosity - which involves spontaneous bleeding, with neurologic and occular disorders - has been called the hyperviscosity syndrome (Fahey et al, 1965). Hyperviscosity of blood may be due not only to elevated serum or plasma viscosity with subsequent blood 'sludging', but also to increased numbers of cells (polycythaemia or leukaemia) or increased resistance of cells to change in shape (sickle-cell anaemia or spherocytosis) as described by Wells (1964). These three conditions will be briefly considered below.

i) Syndrome of serum hyperviscosity

The macroglobulinaemia of Waldenstrom (1944) represents the classic disease process in which the

hyperviscosity syndrome occurs. In this particular disease the abnormal protein is IgM macroglobulin. Some of the IgM globulins have properties of cryoglobulins in that they form a precipitate or gel below temperatures of 37°C. Further studies on Waldenstroms disease show that the increase in serum IgM is in excess of 5 times the normal and produces marked elevations in serum viscosity as determined using an Ostwald (capillary) viscometer (Ritzman et al, 1960; Fahey et al, 1965).

In most cases of multiple myeloma the increase in globulins, primarily the lower molecular weight proteins IgG and IgA, cause a slight increase in serum viscosity without the clinical manifestations of hyperviscosity (Reisman, 1932; Albers, 1937). However extremely high concentrations of IgG or IgA globulin can occur with sufficient increase in serum viscosity to cause the hyperviscosity syndrome (Murphy, 1973), but there have been some patients described with a hyperviscous state without the very high concentrations of myeloma proteins. Here the IgG or IgA proteins which cause the hyperviscous state at relatively low concentrations have been shown to either form molecular aggregates or to have an unusual molecular shape (Smith et al, 1964, 1965; Mackenzie et al, 1970).

Alterations in the concentration of the globulin

fraction in syphilis, leprosy and kala-azar (visceral leishmaniasis) were considered responsible for elevations in the relative viscosity of infected serum by Chopra and Choudhury (1928). The highest values in this survey were found in the patients with leishmaniasis.

Increases whole blood viscosity due to a relative increase in the fibrinogen content of blood is a common finding following surgical procedures (Litwin, 1968; Kemble and Hickman, 1972). Dormandy (1970) showed an increased viscosity in those patients where vascular reconstruction had failed.

All the above described conditions of hyperviscosity are usually treated by plasmapheresis, a valuable form of therapy which preferentially removes more of the heavier molecular weight proteins than it does albumin, so that serum and whole blood viscosity are reduced, (Solomon and Fahey, 1963; Fahey, et al, 1965).

ii) Syndrome of polycythaemic hyperviscosity

Hyperviscosity due to increased numbers of red cells is the most common of all hyperviscosity syndromes. Secondary polycythaemia is primarily related to hypoxia resulting from chronic lung disease, congenital heart disease and altitude (Murphy, 1973). The observations of Lertzman et al (1964) suggest that there are changes in cardiopulmonary function in polycythaemic vera.

iii) Syndrome of sclerocythaemic hyperviscosity

There are a number of haematologic disorders in which the red cell undergoes loss of ability to change shape. The most apparent of these is the genetically inherited sickle-cell disease where sickling results in doubling of viscosity at a haematocrit of 20% (Murphy, 1972).

Other examples are spherocytosis, pyruvate kinase deficiency, burr-cell formation and certain haemoglobinopathies (Murphy, 1973).

Decreased cell deformability has also been described in Plasmodium knowlesi infected erythrocytes. Suspensions of infected rhesus monkey red cells compared with normal red cells at the same haematocrit showed a significant increase is viscosity (Miller et al, 1971).

Physiopathological effects of raised blood viscosity

Recent attention has focused on the viscosity of blood in thrombo-occlusive disease. Wells and Merrill (1961) and Burch and de Pasquale (1962) have suggested that an increase in blood viscosity might play a key role in precipitating thrombosis. A similar conclusion was reached by Eisenberg (1966) who showed an increased fibrinogen concentration following cerebral infarction, which caused an increase in blood viscosity. The changes in viscosity were of sufficient magnitude to be considered significant in causing a reduction in blood flow with the possibility of thrombus formation.

Comparative analysis of the viscous properties of blood is thus of obvious physiological and clinical interest. Conditions associated with DIC, plasma protein alterations, or abnormal red cell morphology demand further study. One of the purposes of the following studies is to examine the existence of these three conditions in experimental African trypanosomiasis and relate them to changes in blood viscosity.

Part 2. MATERIALS AND METHODS

2.1. Animals

Male New Zealand white rabbits weighing 2·5-3 kg. obtained from A. E. Moss, White Cloud Farm, Tring, Hertfordshire, were used. They were fed standard laboratory diet, water being supplied ad libitum.

Outbred mice, of either sex, weighing 18-20 g, were obtained from Animal Supplies Ltd., Hertfordshire.

Outbred male rats, weighing approximately 200 g, were again obtained from Animal Supplies Ltd.

The Zebu cattle were bred at the East African Trypanosomiasis Research Organization (EATRO) farm at Tororo, Uganda. Material for experimental work was collected by A. R. MacKenzie, who kindly made its availability at Silwood Park.

Human blood samples, obtained by venepuncture from patients attending the EATRO hospital, were also made available by A. R. MacKenzie.

2.2. Trypanosomes

a) Trypanosoma (Trypanozoan) brucei, Strain S42 and 427

Two strains of Trypanosoma (Trypanozoan) brucei, S42 and 427, were obtained from the Lister Institute of Preventive Medicine.

Strain S42 was isolated from an old female warthog in Tanzania on March 12th, 1966 and has since been maintained in mice.

Strain 427 was isolated from a sheep in South East Uganda in 1960 and subsequently passaged in mice.

Maintenance in this laboratory was by syringe passage of infected blood through mice every two to three days.

b) Trypanosoma (Trypanozoan) brucei, EATRO Strain 1386

T. (T.) brucei EATRO 1386 was isolated from a male Glossina fuscipes (L-334) caught at Lugala, South Busoga, Uganda in 1969. Innoculation of the salivary glands into a single mouse was followed by blood passage three days later into clean mice. The strain was frozen after the seventh passage and stored in liquid nitrogen.

c) Trypanosoma (Nanomonas) congolense, EATRO 505

This strain of T. (N.) congolense was isolated from a cow at Mtakara, Uganda in 1960 by M. P. Cunningham.

2.3. Infection of Animals

a) Rabbits

Rabbits were infected with either T. (T.) brucei S42 or 427 by subcutaneous innoculation with between 10^6-10^{10} trypanosomes (previously separated from infected rat blood as described under section 2.4.2.). Strain S42 usually kills rabbits within 3-4 weeks, whereas strain 427 causes a more chronic infection, usually killing between 5-8 weeks. Occasionally rabbits infected with strain 427 became asymptomatic and aparasitaemic.

b) Cattle

Infection of cattle was achieved by intravenous injection of diluted whole stabilate. Cattle infected with EATRO 505 received an inoculation of approximately $4{\cdot}8 \times 10^4$ trypanosomes, and those infected with EATRO 1386 received $1{\cdot}5 \times 10^6$ trypanosomes.

2.4. Parasitological Methods

2.4.1. Parasitaemia

Parasitaemia was determined microscopically in wet film preparations of blood, by counting the numbers of trypanosomes seen in 30 randomly selected fields under high power magnification (x40 objective).

2.4.2. Separation of trypanosomes

Trypanosomes were separated from heavily infected rat or mouse blood by the DEAE-cellulose method of Lanham (1968). The eluant used was phosphate buffered glucose (PSG, pH 8.0). Separations were most successful when the majority of rodent erythrocytes had been separated from the trypanosomes by centrifugation (1,600 g for 10 min.) prior to the application of the trypanosomes onto the cellulose column. To achieve a fast rate of flow it was found satisfactory to contain the cellulose in a glass Buchner funnel of porosity 1 (Anderman and Co. Ltd., London). Before use trypanosomes were washed five times in PSG.

2.4.3. Trypanosome counts

Separated and washed trypanosomes were counted on an Improved Neubauer haemocytometer, employing the same counting procedure as used for erythrocytes (Dacie and Lewis, 1963)

2.5. Haematological Methods

2.5.1. Packed cell volume (PCV) or haematocrit

Venous blood was collected in ethylenediaminetetra-acetic acid dipotassium salt (EDTA, 2mg/ml) and the PCV determined by a microhaematocrit method using an MSE microhaematocrit head on an MSE bench centrifuge (MSE Ltd., London).

2.5.2. Erythrocyte sedimentation rate (ESR)

The ESR was measured in standard haematocrit capillary tubes. The procedure was to collect whole blood in EDTA (2 mg/ml), which was then allowed to sediment in the capillary tubes at either room temperature or at 37° for 1 or 2 hours. The degree of sedimentation was expressed as mm/mm/h.

2.6. Biochemical techniques

2.6.1. Preparation and measurement of fibrinogen

i) Preparation

Rabbit venous blood was collected in one-tenth its volume of a 3·8% trisodium citrate solution and centrifuged to remove cellular components. The supernatant plasma was then centrifuged at 1,000 g for 20-30 mins. to remove platelets, and diluted with two volumes of 0·85% sodium chloride. Fibrinogen was then prepared according to the method of McFarlane (1963) where reprecipitation of fibrinogen was carried out at 23% saturation ammonium sulphate, followed by precipitation at 21% saturation. The precipitate was dissolved in 10mM trisodium citrate in 8mM phosphate buffer, pH 7·5 and dialysed against two changes of this buffer for 36 hours. ϵ-aminocaproic acid (EACA), 50 mg/ml, was added to the buffer to prevent fibrinogenolysis during dialysis. The dialysate was centrifuged at 36,000 g for 30 mins. and the supernatant containing the partially purified fibrinogen stored at -20°C in aliquots of 1ml.

ii) Quantitative fibrinogen determination

The method for determining the concentration of fibrinogen in normal and infected plasma was based on the estimation of fibrin obtained after inducing clotting with thrombin (Ratnoff and Menzie, 1951). In this procedure, plasma collected in EDTA, was first diluted with saline containing EACA (50 mg/100 ml). Thrombin

(Leo Pharmaceutical Co. Ltd.) at 50u/ml in 0·5M calcium chloride was added and the plasma left for 30 mins. at room temperature to form a clot. The fibrin clot was then washed thoroughly with saline/ EACA to remove any contaminating plasma proteins, dissolved in 1% sodium hydroxide, and the total protein content determined by the Biuret method. All solutions were read at 550 mμ on a Beckman DB spectrophotometer against a reagent blank. Determinations were performed in duplicate.

iii) Qualitative fibrinogen determinations

a) Heparin precipitable fraction (HPF)

The HPF of fibrinogen in normal and infected rabbit plasma was detected following the technique of Smith and von Korff (1957). The method was slightly modified by adding 4·5 ml of blood to 0·5 ml of saline solution containing 1 mg heparin (Cotton and Craven, 1970). The heparinised plasma was left at $4^{o}C$ for 24 hours and any precipitate formed graded visually from 1+ (light precipitate) to 4+ (heavy precipitate).

b) Cryofibrinogen

The method of Kalbfleisch and Bird (1960) was

used to detect the presence of cryofibrinogen. A volume, 0.5 ml, of citrated plasma was chilled at 4°C for 24 hours and the presence or absence of any precipitate or gel formation noted. The precipitates were graded as described above for HPF.

Since cryoglobulins will also precipitate at this temperature, a serum control was included for every rabbit plasma sample tested.

c) Fibrinogen - B

Fibrinogen-B was determined according to the method of Lyons (Cummine and Lyons, 1948) and the results graded as proposed by Smith et al (1951). Infected and normal citrated plasma was brought to room temperature (20-25°C) and 0·5 ml added to 0·15 ml of reagent. The reagent was prepared by dissolving 2 g of β-naphthol in 100 ml of 50% ethyl-alcohol (Cummine and Lyons, 1948). As a control 1 ml of plasma was added to 0·1 ml of ethyl-alcohol.

After adding the reagent, the tubes were quickly shaken and left to stand for 10 mins. Solutions that were still cloudy at the end of 10 mins. and contained no grossly visible particulate matter were considered negative. Solutions containing heavy particulate matter were graded as 1+, small threads of fibrin as 2+ and those containing clumps of fibrin without coagulation

of the entire solution graded as 3+. Solutions that clotted or gelled were called 4+.

2.6.2. Fibrin plates

i) Plasminogen rich plates

For the estimation of fibrinolytic activity in rabbit tissues and trypanosome extracts, fibrin plates were prepared according to directions given by Astrup (1966). Purified, lyophilised human fibrinogen (Grade L, Kabi Pharmaceuticals) 96% clottable fraction, was dissolved in phosphate buffered saline (PBS), pH 7·0, to a final concentration of 200 mg/100 ml. 10 ml aliquots of this solution were pipetted into standard petri dishes and clotted with 5 IU thrombin for 30 mins. at room temperatures.

The plasminogen content of the fibrinogen preparation as detailed by Kabi Pharmaceuticals Ltd., was 0·26 casein units per mg of fibrinogen as estimated according to Nissen (1964), corresponding to 0·026 CTA units as estimated by means of the standard preparation of Johnson et al (1969).

To each of the plates was added 0·5 ml of test solution in 3 test areas. The plates were then incubated at 37°C for 24 hours and examined for areas of lysis of fibrin. The areas of any lysed zones were recorded as the product in mm^2 of two perpendicular diameters and used as an index of fibrinolytic activity. For permanent preparations the fibrin was dialysed against distilled

water, dried, and stained with the Martius-Scarlet-Blue method for fibrin (for details of method see under section 2.9).

ii) Plasminogen inactivated plates

The above Astrup plate was heated at 80°C for 30 min. to inactivate plasminogen (Lassen, 1952).

iii) EACA plates

EACA was added to the PBS before diluting the fibrinogen, in a concentration of 0·4 g per 50 ml buffer, in order to inactivate plasminogen and plasmin present in the fibrin.

iv 'Trasylol' plates

Transexamic acid ('Trasylol', FBA Pharmaceuticals Ltd.) was added to the PBS prior to diluting the fibrinogen, to prevent enzyme activity of test solutions. The amount added was 20,000 KIU per 50 ml of buffer.

v) Preparation of homogenates

Normal rabbit organ tissues were either perfused or washed well in cold isotonic saline to remove blood and frozen at -20°C. Using a ground glass homogeniser, 1g (wet weight) of tissue was homogenised in 4 ml PBS. The homogenate was then centrifuged at 32,000 g for 15 mins. at 4°C, and the supernatant retained for estimation of fibrinolytic activity.

Trypanosomes, $1{\cdot}9 \times 10^{10}$/ml, separated and washed, were disintegrated by repeated freezing and thawing at -20°C. Centrifugation was at 38,000 g for 15 mins. The supernatant was used immediately for detection of any enzyme activity.

2.6.3. Plasminogen assay

Plasminogen concentrations were determined in pre-infection sera and infected sera by Dr. Christine Hawkey at the Nuffield Institute for Comparative Medicine, using the method described by Remert and Cohen (1949) as modified by Alkjaersig et al (1959) and using human urokinase as activator. In principle this method consists of the conversion of all the plasminogen in plasma to plasmin, which is then measured by caseinolysis.

2.6.4. Protamine sulphate test

A modification of the serial protamine sulphate test described by Gurewich and Hutchinson (1971) and later by Schorn (1973) was used on citrate plasma samples (3.8% sodium citrate) taken from non-infected rabbits and rabbits infected with trypanosomes.

A 1% solution of protamine sulphate (Salmine sulphate; BDH Chemicals Ltd.) was mixed with 0·05 M tris-buffered saline, pH 6·5, in dilutions of 1:5, 1:10; 1:20; 1:40. To 0·2 ml of each dilution of protamine sulphate was added 0·2 ml of plasma sample in glass test tubes. (The plasma had first been freed of platelets by centrifugation at 1000 g for 30 mins).

The concentrations of protamine sulphate were thus 1,000, 500, 250 and 125 µg/ml (Gurewich and Hutchinson, 1971). The contents of the tube were mixed gently, left to stand at room temperature and then examined after 30 mins. and again at 24 hours.

The reactions were recorded as follows:-
G = gel; FS = fibrin strands; FY = feathery precipitates (graded 1+ to 4+; - = amorphous granular precipitates. The test was considered positive if gel, fibrin strands or feathery precipitates resulted.

The fibrinolytic inhibitor, Trasylol, (80 IU/ml) was added to the plasma samples to prevent lysis of any precipitates or fibrin strands which might develop during the 24 hours incubation period.

2.6.5. Total protein determinations

Determinations of protein concentrations in serum throughout the infection were made using the Bio-Dynamics Unitest for total protein (M.C.P. Pharmaceuticals Ltd.). The procedure utilises the Biuret reaction. Protein levels are expressed as g/100 ml serum.

2.6.6. Globulin determinations

Serum globulin levels were determined using the Bio-Dynamics Unitest for globulin, which is based on a factor relating tryptophan to total serum globulins. The Unitest procedure determined tryptophan directly by its reaction with dimethyaminokenzaldehyde and sodium nitrite in HCL. Globulin is expressed as g/100 ml serum.

2.6.7 Albumin calculation

Albumin levels in g/100 ml were calculated by subtracting the serum globulin value from the total serum protein value.

2.6.8. Determination of lipid levels

Rabbits were starved overnight before collecting sera for lipid determinations which were made using the Boehringer Test Combination Kits (Boehringer Corporation, London).

Neutral fats (triglycerides) were calculated by subtracting the total lipid value from the combined values of phospholipid and cholesterol.

All lipid levels are expressed as mg/100 ml serum.

2.7. Immunological techniques

2.7.1. Collection of serum samples

Samples of blood were collected from the marginal ear vein of rabbits at intervals during the infection. The blood was allowed to clot at 37°C for 4 hours and the serum removed by centrifugation. When sera was required for the assay of FDP blood was collected in glass tubes containing 'Trasylol' (100 IU/ml) to prevent any *in vitro* digestion of fibrin. The serum was then clotted with thrombin (10 IU/ml; Thrombin Topical, Parke-Davis and Co.), to clot any residual fibrinogen or high molecular weight FDP present. All sera were kept at -20°C and tested within 3 weeks of storage.

2.7.2. Collection of urine samples

In order to obtain urine samples free from contaminating faecal material, rabbits were housed in metabolism cages to which were attached glass urine/faeces separators. The volume of 24 hour urine collected in 'Trasylol' (100 IU/ml) was measured, and freeze-dried. Reconstitution in distilled water to 1/10th the original sample volume was followed by dialysis against tap water over a period of 8 hours.

2.7.3. Immuno-assay of FDP

The assay of serum and urinary FDP was made following the tanned red cell haemagglutination inhibition immunoassay (TRCHII) of Merskey *et al* (1966), and using

the Wellcome FDP assay kit (Wellcome Reagents Ltd.). A diagrammatic representation of the principle of the test is shown in Fig. 6.

The experimental procedure can be divided into 4 sections:-

i) Absorption of anti-species agglutinins

Anti-species agglutinins were removed from the serum prior to FDP assay by two absorptions with equal volumes of washed packed sheep red blood cells. The absorptions were carried out at room temperature for 30 mins.

ii) Absorption with human fibrinogen sensitised sheep red blood cells (HF-SRBC)

Following absorption with SRBC, all sera were absorbed with an equal volume of HF-SRBC to remove a factor present in normal and infected sera which caused agglutination of the commercially prepared sensitised cell suspension. This factor is explained in greater detail under the experimental section.

iii) Inhibition test

Doubling dilutions of absorbed sera or concentrated urine were prepared using the microtitre system (Flow Laboratories Ltd.). An equal volume of antiserum diluted 1 in 10,000 in citrate buffer pH 8·0, was added to each well. After standing at 4°C for 1 hour, one volume of a 2·5. suspension of human fibrinogen sensitised sheep red cells was added to each well. The

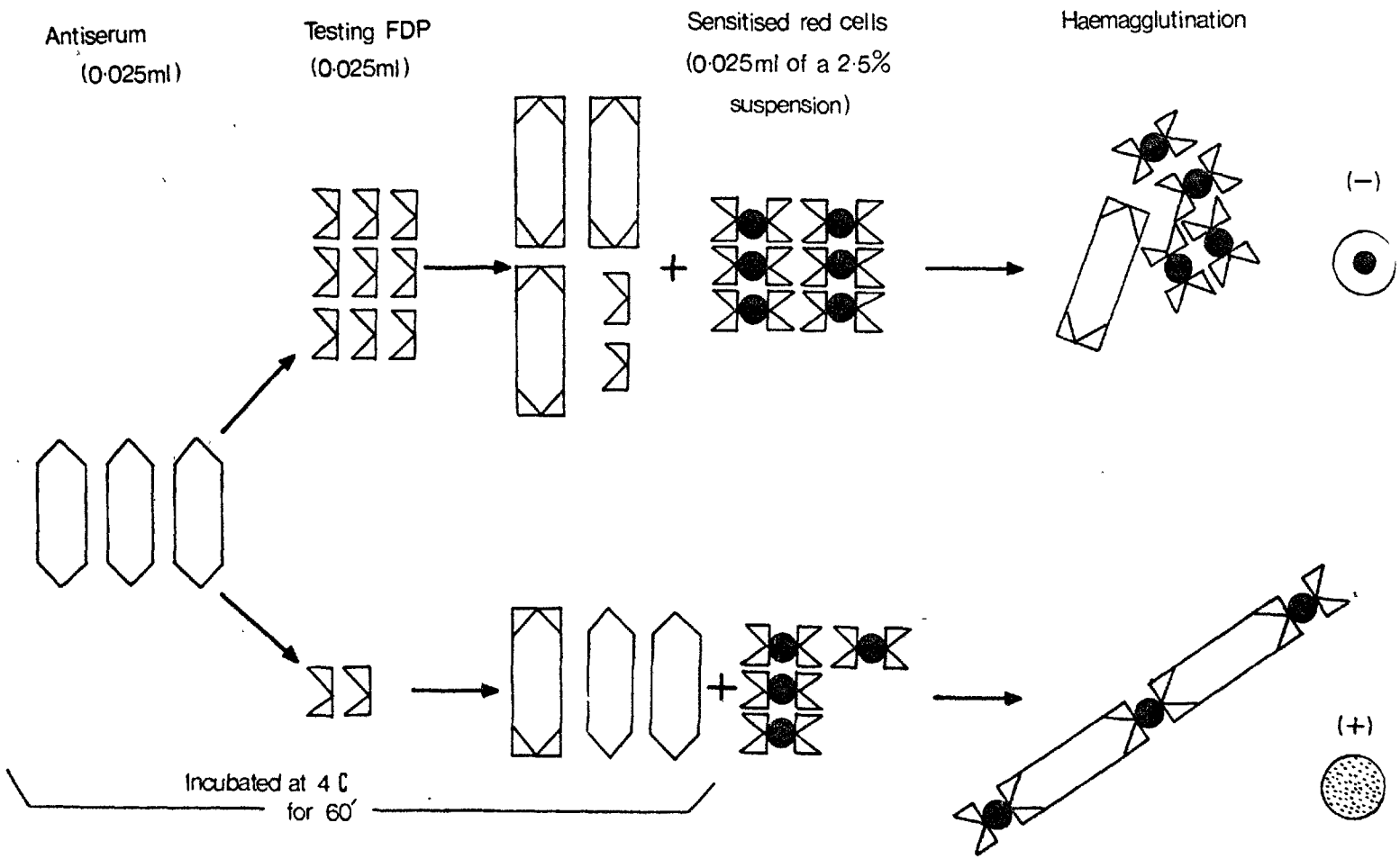


Fig. 6. Diagrammatic representation of the tanned red cell haemagglutination inhibition immunoassay used for determination of serum and urine FDP

plates were shaken and left to stand overnight at 4°C. The end point was taken as the last dilution that showed no trace of agglutination. The concentration of FDP in the samples tested was subsequently assessed by comparison of the results obtained with a known fibrinogen standard. Suitable controls were included in each assay, these being dilutions of normal (non-infected) serum, buffer and reagent controls.

iv) Reactivity of antiserum to human fibrinogen with rabbit fibrinogen

The specificity of the rabbit anti-human fibrinogen antiserum for rabbit fibrinogen was tested by first coating tanned sheep red cells with rabbit fibrinogen (Das, 1970) and then carrying out direct agglutinations with the antiserum.

2.7.4. Anti-F autoantibody titration

i) Preparation of sensitised cells

a) Fibrinogen

Lyophilised bovine fibrinogen, 95% clottable, was obtained from BDH Chemicals, Poole, Dorset. Human fibrinogen was obtained as previously described. Both bovine and human fibrinogen were reconstituted with distilled water to give a concentration of 10 mg/ml and stored at -20°C until required for sensitisation.

b) Sheep red blood cells (SRBC)

SRBC, preserved in Alsevers solution were obtained from Wellcome Reagents Ltd., Beckenham, Kent. They were

stored at 4°C until used.

c) Formalinisation of SRBC

Modifications of the method described by Das (1970) were used for the formalinisation, tanning and subsequent sensitisation of sheep erythrocytes. Red cells were washed five times in cold physiological saline and packed by centrifugation after the final wash. 25 ml of the packed cells were then re-suspended in 200 ml of PBS pH 7·2. Dialysis tubing filled with commercial formalin was submerged in the red cell suspension and after 3 hours the swollen sac was punctured and the formalin allowed to escape. Gentle mixing was then continued overnight by means of a magnetic stirrer. Formalinisation by this method prevented spontaneous haemolysis of the red cells. The cells, now dark brown in colour, were washed 3 times in 50 volumes of distilled water. Finally a 10% (v/v) suspension was made in distilled water containing sodium azide (1 mg/ml) as preservative and stored at 4°C. The cells were normally tanned and coated within two weeks of formalinisation.

d) Tanning of cells

The standard method was to take 1 volume of formalinised sheep red cells and mix them with one volume of freshly prepared tannic acid stock solution (10 mg/ml) in PBS (Boyden, 1951). This was followed by incubation of the cells in a water bath at 56°C,

stirring at 10 min. intervals to keep the cells in suspension.

e) Coating of the cells

After tanning the cells were washed three times with 50 volumes of PBS and finally suspended (2·8% suspension) in 0·15 m citrate buffer, pH 6·4, and mixed with an equal volume of fibrinogen solution to a final concentration of 2 µg/ml of fibrinogen. It was found that a fibrinogen concentration in excess of this level did not improve the sensitivity. By this method cells were coated with either rabbit, human or bovine fibrinogen or with whole rabbit plasma. After sensitisation the cells were washed 4 times in 50 volumes of citrate buffer, suspended as a 2·5% (v/v) solution in the same buffer containing 0·1% bovine serum albumin (BSA) and 1 mg/ml sodium azide, and stored at 4°C.

ii) The agglutination test

Any serum sample to be tested for agglutinating activity against fibrinogen sensitised red cells was first absorbed with SRBC to remove any residual anti-species agglutinins. The procedure was to add an equal volume of washed packed SRBC to the sample for 30 mins. at room temperature. Two absorptions were carried out before using the serum in the agglutination test.

Serial dilutions of infected or normal sera were made in 0·1 m citrate buffer (pH 6·4) in the wells of

microtitre plates (V-plates, Coke Engineering Co., U.S.A.) commencing at a dilution of 1 in 2. To one volume of test serum, one volume of citrate buffer and one volume of a 2·5% suspension of fibrinogen sensitised red cells was added. Controls, including 2 volumes of diluting fluid and 1 volume of sensitised cells were incorporated into each plate. The plates were then shaken and placed overnight at 4°C.

The end point was taken to be the highest dilution of serum causing complete agglutination of the cells, and the titre of the serum expressed as the recipricol of that dilution.

iii) Preparation of rabbit fibrin suspension

Normal citrated rabbit plasma was re-calcified and clotted with thrombin (IU/ml). The fibrin clot was squeezed between filter paper and washed 3 times in isotonic saline to remove any contaminating plasma proteins. 60 mg dried weight of fibrin was added to 1 ml of saline containing EACA (50 mg/100 ml). The fibrin was then subjected to ultrasonic disintegration at a frequency of 20,000 c/s for 15 mins. at 4°C.

iv) Absorptions

Titres of control and infected sera were determined before and after absorption using the following procedure:

a) Human fibrinogen sensitised sheep red blood cells (HF-SRBC)

Sera were absorbed twice with an equal volume of

packed HF-SRBC. Absorption times were 30 mins. at room temperature.

b) Bovine fibrinogen sensitised sheep red blood cells (BF-SRBC)

Sera were absorbed with an equal volume of packed BF-SRBC using the method described above.

c) Rabbit fibrinogen sensitised sheep red blood cells (RF-SRBC)

Sera to be absorbed were treated with an equal volume of RF-SRBC as described above.

d) Rabbit fibrin

Sonicated rabbit fibrin suspension (60 mg/ml) was centrifuged at 38,000 g for 20 mins. and the sediment added to 0·5 ml of test serum. Two absorptions were carried out at room temperature for 30 mins.

2.7.5. Inactivation of IgM

i) Heat inactivation

Sera from rabbits infected with trypanosomes and pre-infection sera were heated in a water bath at 65°C for 30 mins.

ii) 2-mercaptoethanol (2-ME) inactivation

Normal and infected rabbit sera were inactivated with 0·2 M 2-ME solution as described by MacKenzie and Boreham (in press). For each sample, an alkylation control was included in which buffer was substituted for 2-ME.

2.7.6. Gel filtration of plasma proteins

Sephadex G200 chromatography was used to separate plasma proteins at weekly intervals during the infection. 2 ml samples were applied to the column. The eluting buffer consisted of 0·1 M Tris-HCl in 1 M NaCl, pH 8·0, with the addition of 0·2 M EACA. The void volume was determined by elution of blue dextran 2000 (Pharmacia Ltd.). The protein concentration of 3 ml fractions was measured on a Beckman DB spectrophotometer at 280 nm. Fractions were then pooled according to the major peaks and dialysed overnight at 4°C in 0·15 M phosphate buffer pH 7·2. The pooled fractions were then freeze-dried, re-constituted to the original sample volume, and dialysed in PBS for 3 hours.

2.7.7. Immunoelectrophoresis

Immunoelectrophoretic studies on plasma fractions and urine were carried out using a Shandon Universal electrophoresis tank (Shandon Scientific Co. Ltd., London). The supporting medium was a 1·3% agar (Difco Laboratories Ltd.) on microscope slides. The buffer used was a barbitone buffer pH 8·6, with an ionic strength of 0·06 M. Sample size was standardised at 0·05 ml.

After applying the sample to the agar wells, electrophoresis was carried out for 4 h with the current adjusted to 0·4-0·5 mA/cm width (160-200v). This was followed by application of the appropriate antiserum to the troughs, and the slides left for 48 hours in a humidity cabinet for precipitin arcs to develop. The slides

were then examined and lines of precipitation noted. For permanent preparations, the slides were washed overnight in 0·15 M NaCl to remove unprecipitated protein followed by a final rinse in distilled water. The agar was then dried onto the slides at 37°C. The slides were then stained for 10 mins. in a 0·2% w/v solution of napthalene black in 90% v/v methanol with 10% v/v acetic acid. Faint precipitin lines became more distinct following staining.

Two antisera were used, rabbit anti-human fibrinogen (Wellcome Reagents Ltd.) and goat anti-rabbit fibrinogen (Sera Service Ltd.). Both were used undiluted.

2.7.8. Immunofluorescence

The direct fluorescent antibody technique (IFT) was used throughout. Each test was carried out in duplicate and for each run of the test suitable controls were included such as a saline control, un-conjugated antiserum, and absorbed conjugate.

For antigen slides, thin blood smears were made from blood taken throughout infection. These were quickly dried in air and stored in a dessicator for 3 hours. They were then wrapped in paper and kept at -20°C in sealed polythene bags. Before using them in the test, they were brought to room temperature in a dessicator.

Lyophilised goat anti-rabbit fibrinogen conjugated with fluoroscein isothiocyanate was obtained from Sera Service Ltd. After rehydration it was dispensed in 0·1 ml aliquots and stored at -20°C until required. Before use, it was necessary to titrate the conjugate to find its optimal dilution - the dilution which minimises the cross-reaction fluorescence and non-specific background fluorescence. It was determined using serial two-fold dilutions of the conjugate in 0·15 M PBS (pH 7·2) beginning at a dilution of 1 in 2. The optimal dilution was taken as one dilution higher than the last dilution showing a very strong fluorescence. The batch of conjugate used had an optimal dilution of 1 in 10.

The procedure of the test was standard as outlined

in Nairn (1969).

All examinations were made with a Zeiss microscope using bright field illumination which required the use of exciter filter No. 2 and barrier filter nos. 44 and 50. The degree of fluorescence was recorded for each slide from a 1+ grading (faint fluorescence) to 4+ (strongly fluorescent).

The conjugate control consisted of absorbing the conjugate with an equal volume of RF-SRBC. This procedure was carried out at 30 min. intervals at room temperature.

2.8. Histochemical staining for fibrin

Small pieces (4 mm cubes) of normal and infected rabbit tissues were quenched in an isopentane bath in liquid nitrogen, and then stored at -40°C. Sections (10μ in thickness) of specimens of heart, liver, kidney, spleen, brain and lung were cut in a Cryostat (Slee Medical Equipment Ltd.) at -15°C, and left to dry on cover slips at room temperature.

Dried preparations were fixed in neutral formalin (Lillie, 1954) and stained for fibrin with the Martius-Scarlet-Blue method of Lendrum et al (1962) and mounted in DPX prior to microscopical examination.

2.9. Rheological techniques

2.9.1. Collection of blood samples

Blood samples were taken from the marginal ear vein of rabbits at weekly intervals during the infection. The anticoagulant used was EDTA at a concentration of 2 mg per ml of blood. The viscosity of each blood sample was tested within 4 hours of collection since it has been shown that alterations in the rheological properties of whole blood occurs if it is left standing for longer periods of time (Dormandy, personal communication).

PCV's for each blood sample were determined prior to rheological measurement.

Blood was also collected for plasma and serum samples for the determination of the viscosity of plasma and serum respectively. Plasma was centrifuged at 1000 g for 30 mins. to remove all cellular components of the blood, including platelets.

2.9.2. Viscometry

Viscometric studies were carried out in the Department of Physiology, Royal College of Surgeons, by the kind permission of Professor Slome and Mr. J. A. Dormandy.

Two viscometers were used in the present studies:-

a) Wells-Brookfield microviscometer

This cone-plate viscometer is an adaptation of an industrial viscometer originally described by Wells et al (1961). The procedure was first to check the

accuracy of the viscometer. 1 ml of calibration fluid of 4·0 centipoise (cP) was added to the rhodium cup in which a conical disc was rotated through a beryllium copper spring connection. The drag on the rotating cone, due to the viscosity of the calibration fluid sample, was then measured by the torque on the spring suspension.

Throughout the procedure the cup was kept at 37°C $\pm$1°C by means of a heated water jacket.

After calibration of the viscometer, 1 ml of either blood, plasma or serum sample was added. Readings were noted at given shear rates after 3 to 4 mins. to ensure equilibration of the rotating spring mechanism and steady state in the sample. All measurements were taken in duplicate at two shear rates, 23 and 230 inverse seconds (sec^{-1}).

b) Low Shear Rheometer (LS100)

Viscosity was also measured on a new model viscometer, the LS100 (Contraves, A. G., Zurich) which was designed to measure small samples at low shear stresses and low viscosities. The machine, an improvement on the Brookfield viscometer, consisted of a thermostatically controlled measuring cup, driven by a Rheomat-15 unit with a speed range from 0·056 to 3·52 rpm divided into fifteen steps. The measuring bob was immersed into the sample (0·7 ml) and fastened to an electro-optical rotating system. The cup's rotation caused a torque to

be exerted on the bob through the sample; the torque was then measured electro-optically and displayed on an indicating instrument as shear stress value. The indicating instrument had 3 sensitivity degrees which were changed in accordance with changes in viscosity at different shear rates.

Shear rates on this viscometer were usually taken at three shear rates $4{\cdot}616^{sec-1}$, $0{\cdot}581^{sec-1}$ and $0{\cdot}0734^{sec-1}$.

2.9.3. Calculation of viscosity

a) using the Brookfield viscometer

Resistance to rotation of the cone, due to placing of the sample between the cone and plate, was measured by the degrees of deflection indicated on the vision dial. This reading was then converted to absolute centipoise units from pre-calculated range charts (Brookfield Engineering Labs. Inc. U.S.A.).

Occasionally, if the calibration fluid did not give the standard centipoise reading at any shear rate, it was necessary to correct the sample reading. The correction factors calculated for the Brookfield viscometer used are given in Appendix I.

b) using the LS100 viscometer

Absolute viscosities were calculated on the LS100 by multiplying the reading taken within 1 minute at 37°C by the sensitivity degree value for that particular shear rate.

PART 3 EXPERIMENTAL

3.1. Studies on the Fibrinolytic System

3.1.1. Serum FDP concentrations in rabbits

a) Reactivity of rabbit anti-human fibrinogen with rabbit fibrinogen

Since the FDP kit used in these studies was designed for the quantitative assay of human FDP, it was necessary before measuring rabbit FDP, to test the reactivity of the anti-human fibrinogen serum (AHFS) with rabbit fibrinogen.

Similar haemagglutination titres were obtained when the assay was carried out using AHFS and SRBC sensitised with either human or rabbit fibrinogen (Table 1 b). It is therefore possible to assay rabbit FDP using the commercially available products intended for clinical use.

b) FDP concentration as measured by the TRCH11

Fig. 7 shows the increase in serum FDP concentration of six rabbits infected with T. (T.) brucei 427 and four rabbits infected with T. (T.) brucei S42 over a period of four weeks. The complete data for individual rabbits is given in Appendix 1.

The concentration of serum FDP in normal non-infected rabbits was between 20-40 µg/ml and this rose following infection to about 160 µg/ml. Occasionally higher levels up to 322 µg/ml were found. There was a peak in FDP concentration which varied between individual rabbits, but usually occurred between 14 and 21 days after infection. No significant differences were found either in the

DAYS AFTER INFECTION / CELL SUSPENSION	SERUM FDP CONCENTRATION µg/ml		
	D.0	D.10	D.20
2.5% HF-SRBC	20	40	180
2.5% RF-SRBC	20	60	180

TABLE I.b Comparison of FDP concentrations as measured by the TRCHII in rabbit S198 serum when titrated with SRBC sensitised with either human fibrinogen (HF-SRBC) or rabbit fibrinogen (RF-SRBC). Serum was taken prior to infection then 10 and 20 days following infection with *T. (T.) brucei* 427.

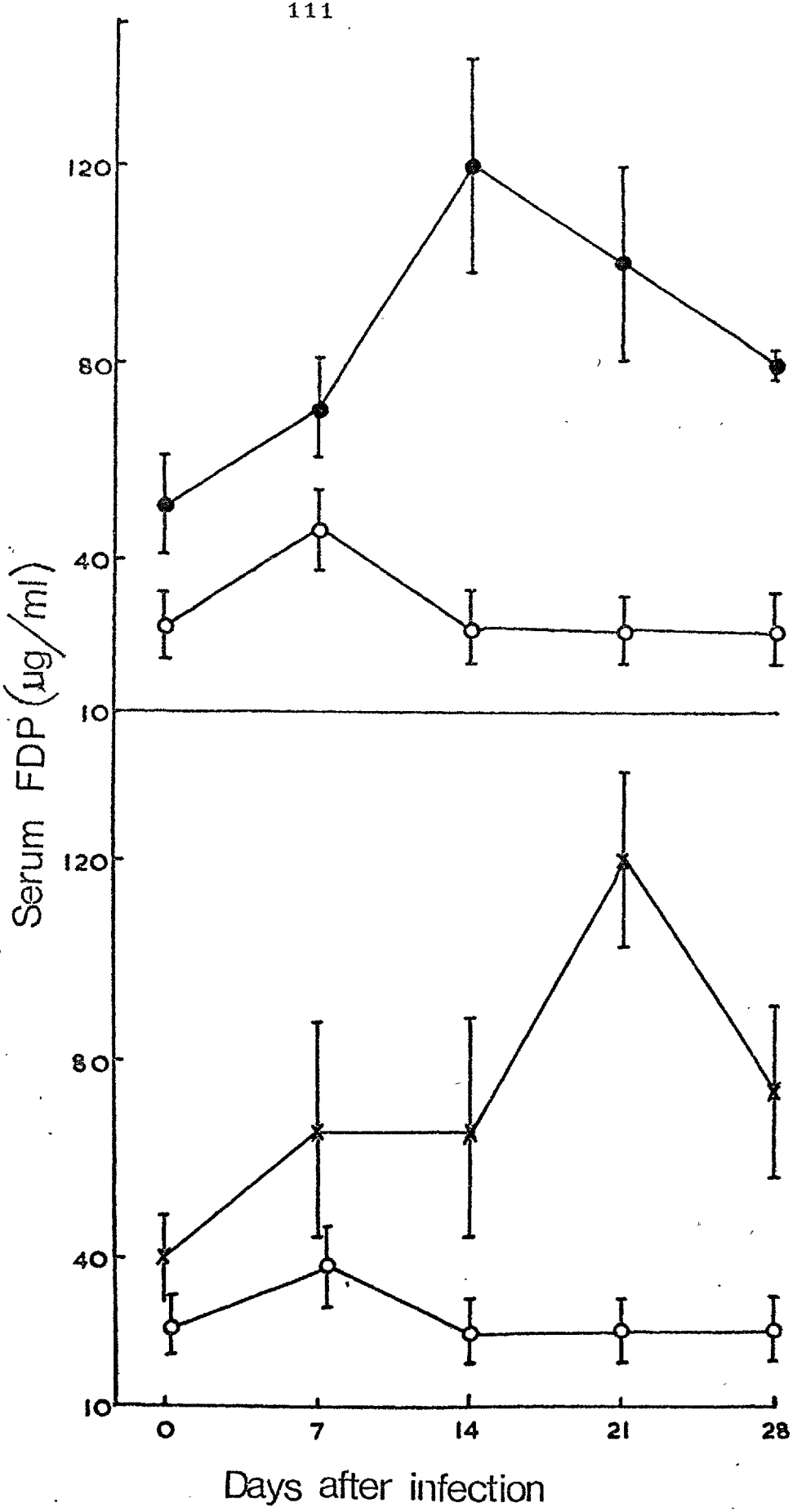

120
80
40
10
Serum FDP (μg/ml)
120
80
40
10
0
7
14
21
28
Days after infection

Fig. 7.

Fig. 7. FDP concentrations in the serum of rabbits infected with T. (T.) brucei

●———● strain 427 mean (± S.E.) plotted for six rabbits.

X———X strain S42 mean (± S.E.) for four rabbits.

O———O non-infected controls mean (± S.E.) for three rabbits.

concentration of serum FDP, or the time when maximum concentrations were found, for the two strains of trypanosome.

A typical test plate is shown in Fig. 8.

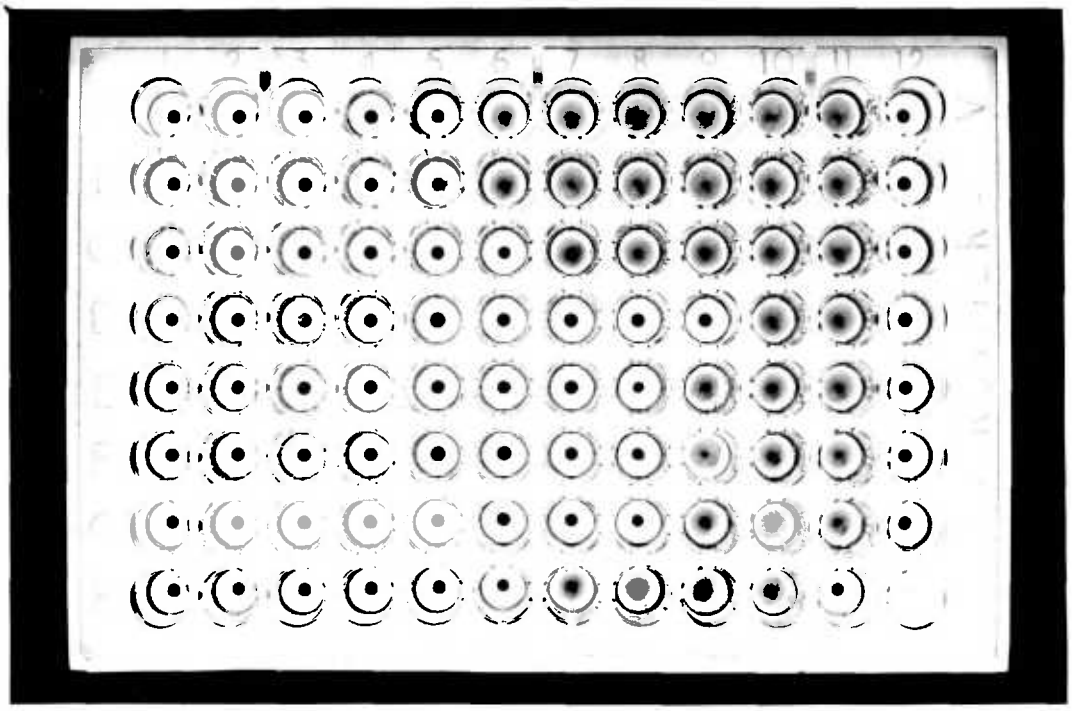


Fig. 8. TRCHII for FDP in a "Microtitre" plate using rabbit S262 sera. Rows A-G represents doubling dilutions of sera collected at days 0, 7, 14, 21, 28, 35 and 42 of an infection with T. (T.) brucei 427. Neat serum in well 1 of each row.

Well 11, Row A : reagent control.

Well 12, Row A : buffer control.

Well 12, Rows B-H : sample control.

Row H: Fibrinogen standard, starting concentration 10 µg/ml. Sensitivity of assay 0.63 µg/ml.

3.1.2. Characteristics of serum FDP

In order to obtain a clearer picture of what FDP fragments were being measured the following tests were carried out.

a) Thrombin treatment of serum

Although Fragment X is considered clottable by thrombin, it is in fact only 85% clottable and then clots very slowly (Marder, 1970). Since blood was allowed to clot for only 4 h and the serum removed it was probable that Fragment X was present in the FDP measured.

Serum samples from two rabbits, S262 and S263 infected with T. (T.) brucei 427, after collection in 'Trasylol', were mixed with thrombin (10 u/ml) and left for 5 h at 37°C to clot any Fragment X present.

Treatment of serum samples with thrombin did not alter the titre of FDP. This suggests that only 'late' FDP and perhaps Fragment Y were present in the samples, although it is possible that the action of thrombin in the original clotting process may have removed some 'early' FDP present.

b) Sephadex G200 gel filtration and immunoelectrophoresis

Four rabbits (S383-S386) were infected with T. (T.) brucei 427 and plasma prepared from blood samples collected in EDTA (2 mg/ml) before infection and at 7, 14, 21 and 28 days after infection.

A mixed plasma sample (for each of these days) was obtained by taking 0.5 ml of plasma from each of the 4 rabbits giving a final volume of 2 ml of mixed plasma.

The five 2 ml plasma samples were then subjected to gel filtration on Sephadex G200.

The elution patterns of the combined weekly plasma samples are seen in Figs. 32 to 36 under the following Section 3.2. The presence of FDP in pooled fractions was detected by immunoelectrophoresis using a specific goat anti-rabbit fibrinogen antiserum (GARF).

The results of immunoelectrophoresis are given as photographs of the stained slides and also as drawings where diffusion patterns are not clearly seen in the photographs due to loss of definition during photographic processing (Fig. 9 a-e).

Since each degradation product and fibrinogen have a distinctive immunologic pattern (Marder, 1968) it is possible to distinguish between the 'early' and 'late' FDP. Plasma taken prior to infection (day 0) yielded only a single arc of precipitation just opposite the origin (Fig. 9 a). This represents native fibrinogen. It was eluted just after the macroglobulin fraction (i.e. precipitin lines were formed only with fractions I and II) - a result to be expected considering the large molecular weight of this protein (340,000). No other precipitin lines were found indicating that FDP were present in only small amounts.

On day 7 although FDP were again absent, fibrinogen was found in the first 4 fractions - possibly indicating an increase in this protein (see Section 3.1.4.) giving rise to a spread of the elution peak (Fig. 9 b).

A new component, in fraction V which migrated towards the cathode, (Fig. 9 c) was found in day 14 plasma. This faint precipitation line probably represents Fragment D. Fibrinogen again is present in fractions II and III.

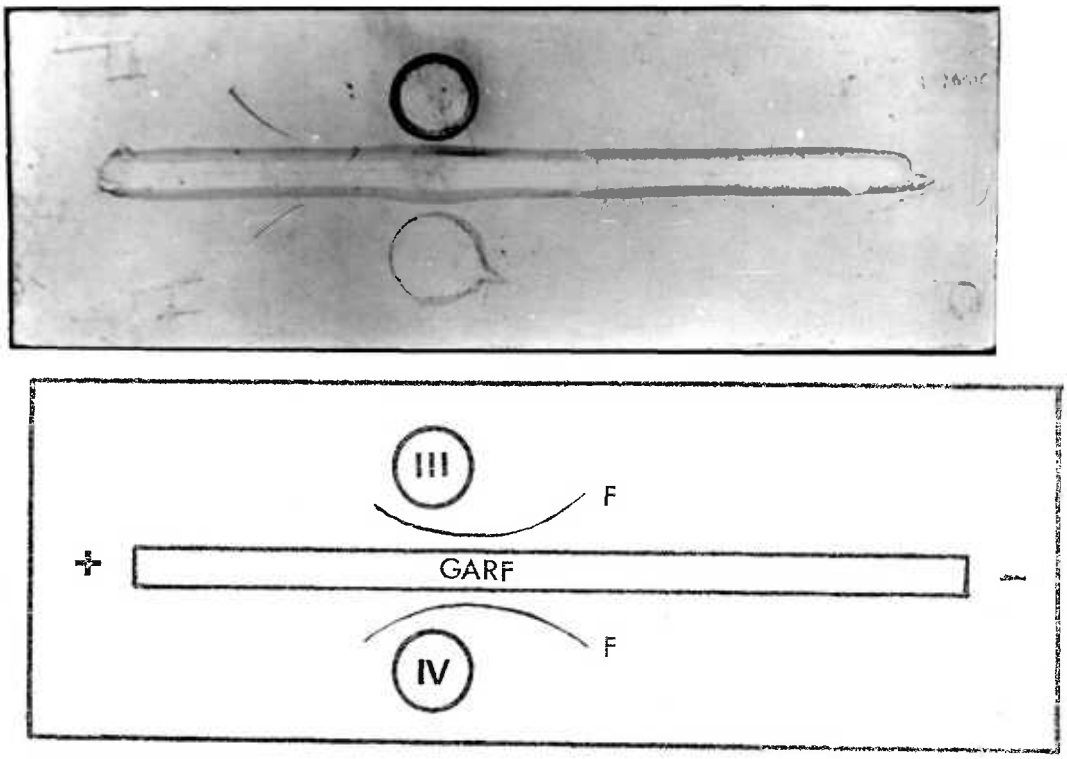
Fraction III of plasma taken three weeks after infection showed a line of precipitation over the origin and extending towards the cathodic end of the slide (Fig. 9 d). This is probably Fragment X or Fragment x and fibrinogen in combination. Fragment X enters the gel more readily than fibrinogen and migrates slowly towards the cathode and thus tends to overlap with fibrinogen.

A further change in the precipitation pattern was seen in plasma collected on day 28 of the infection. In fractions II and III Fragment D is clearly seen as a single precipitin arc either side of the antiserum trough and slowly migrating towards the cathode (Fig. 9 e).

A summary of the above results can be seen in Table II.

Thus immunoelectrophoresis demonstrating 'early' and 'late' degradation products, provides a means for characterisation of the degradation products circulating in the blood of infected rabbits. It is shown here that Fragments X and D are formed in large amounts (to be detected by this method) from the second week of infection onwards. However no Fragment E, which has a distinct anodal mobility, could be demonstrated. It is probable that it was present in too low a concentration to be detected by this method.

Day 0 (a)



Day 7 (b)

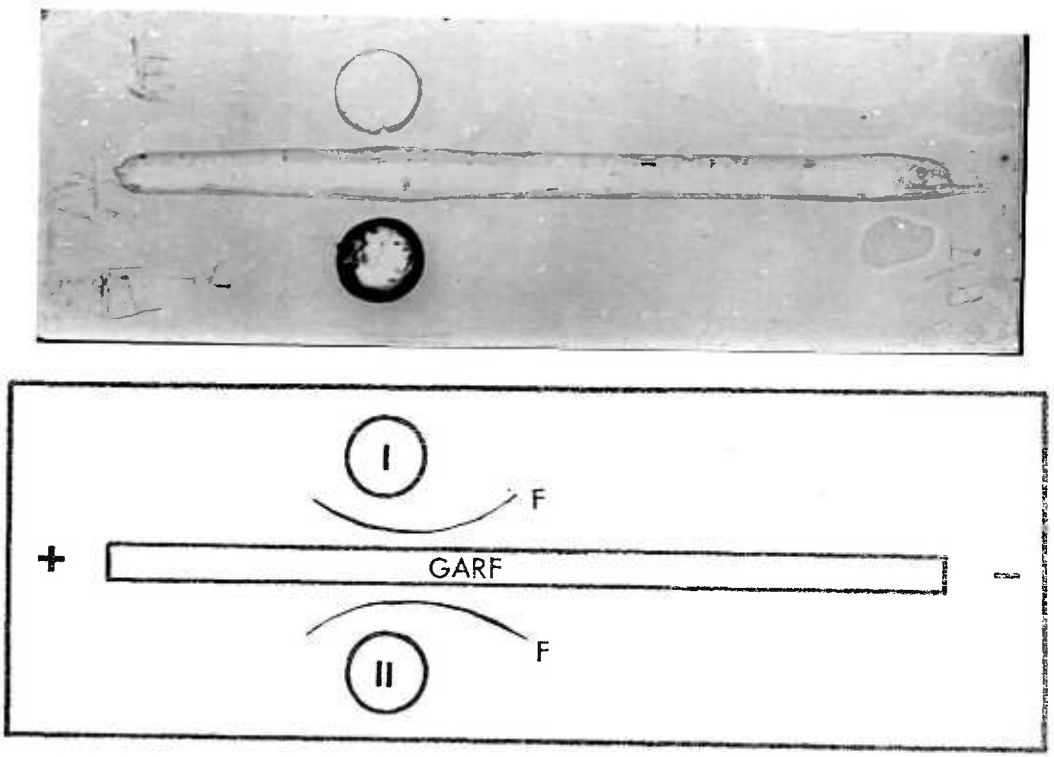
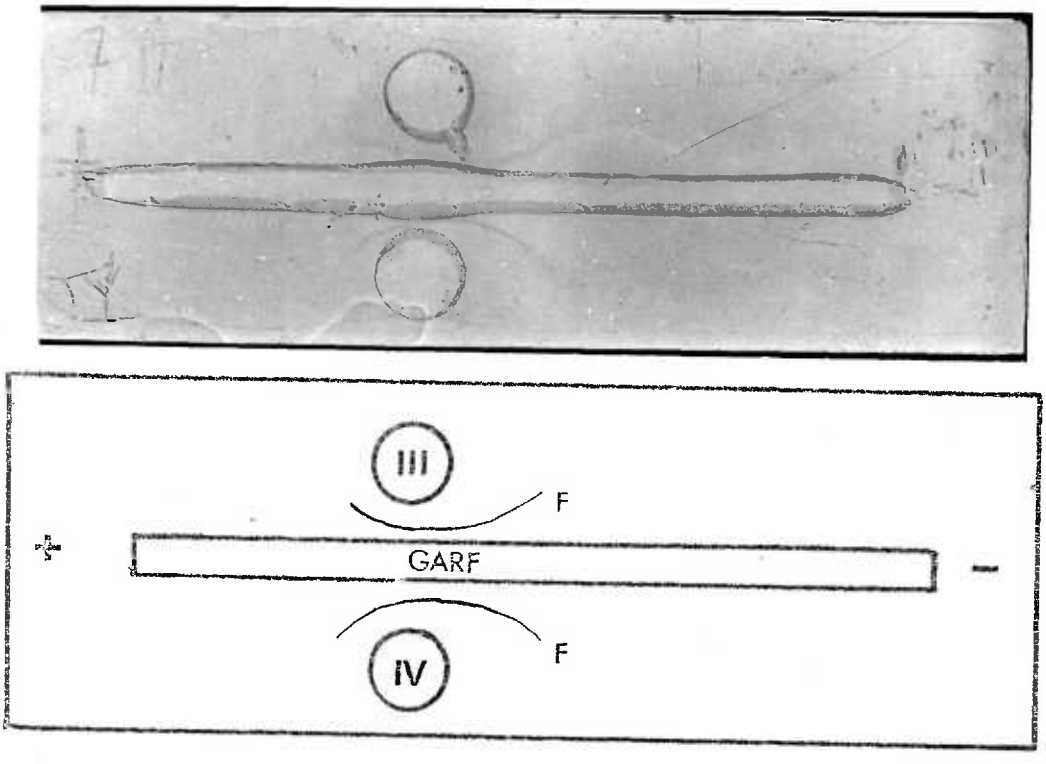
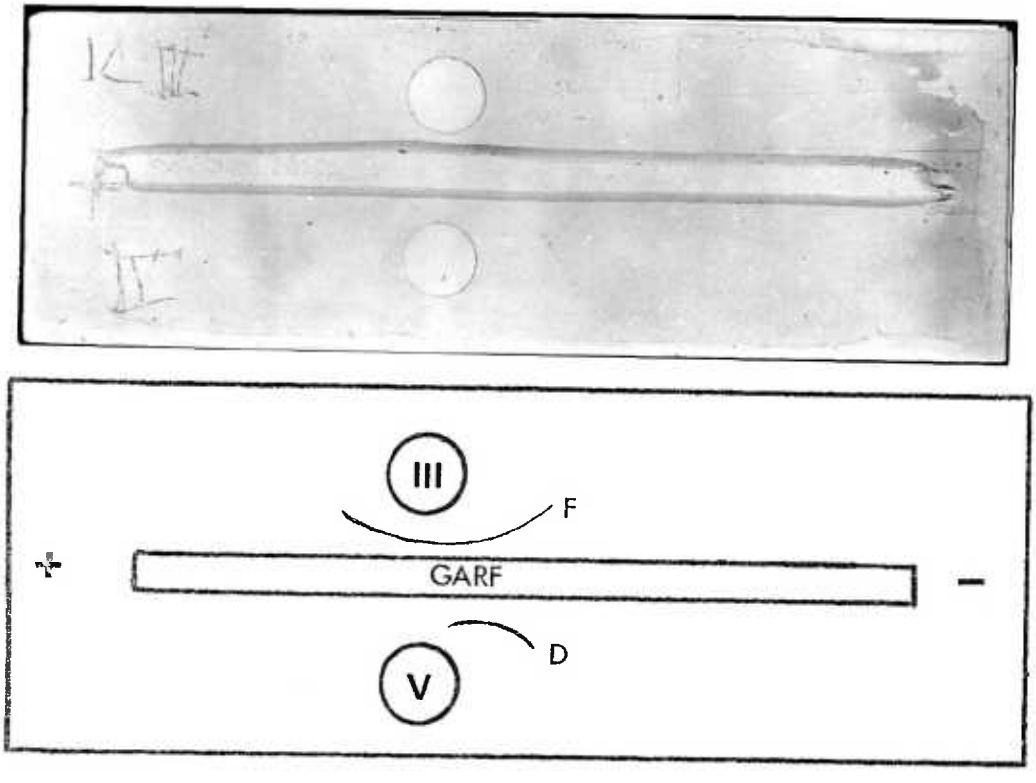


Fig. 9.

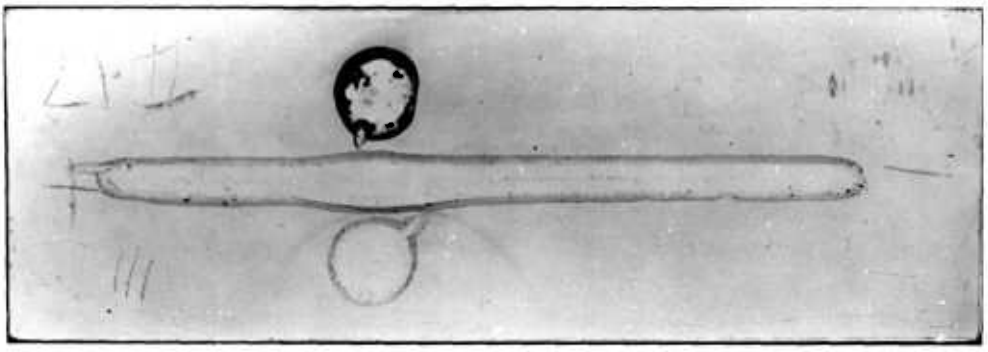
Day 7 (c)

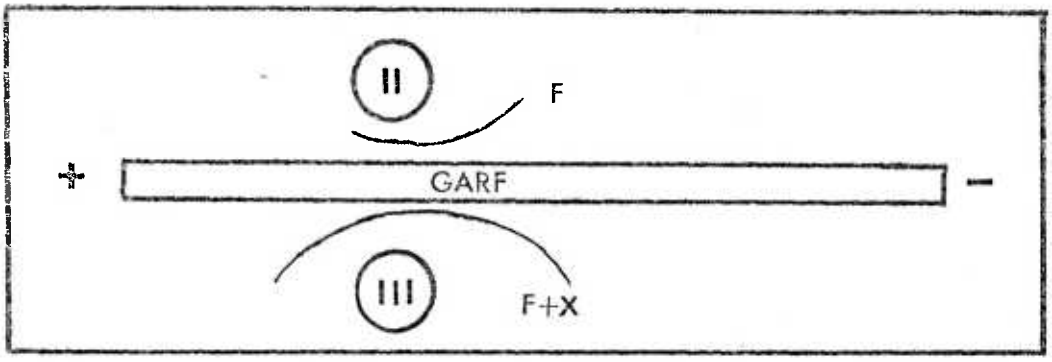


Day 14 (d)

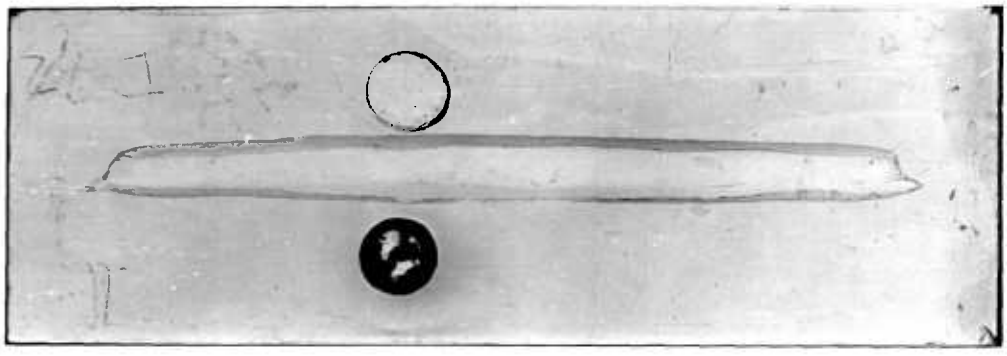


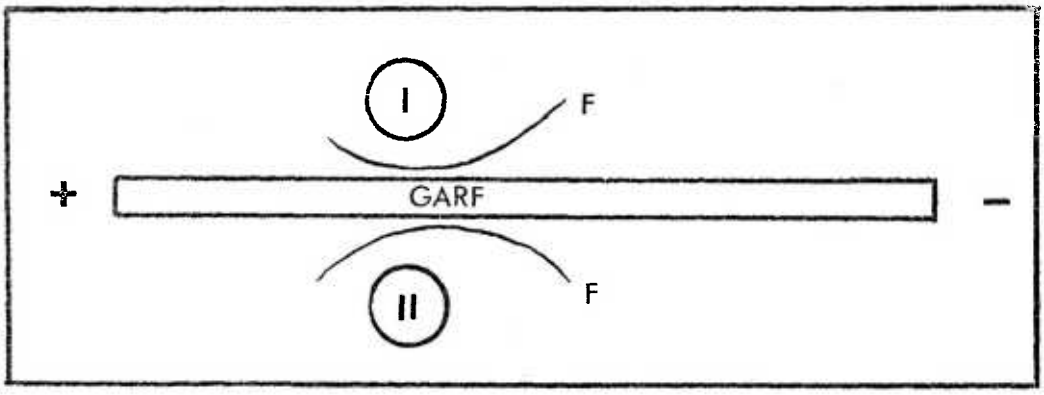
Day 21 (e)





Day 28 (f)





Day 28 (g)

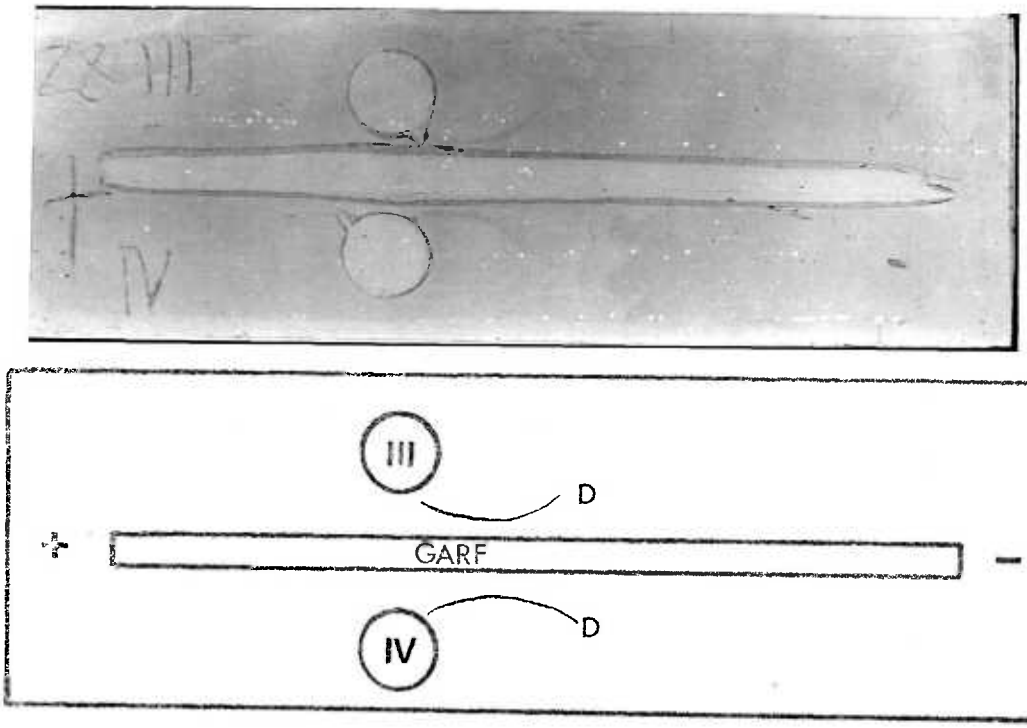


Fig. 9.

Fig. 9. Identification of fibrinogen and degradation products in normal and infected plasma identified by agar gel electrophoresis of fractions following Sephadex G200 column chromatography.

Drawings are given after the originals in addition to photographic representation, since drying of the agar slides caused the troughs to widen in some cases thereby obscuring the precipitin lines.

The fraction samples were placed in the wells. The antiserum in the central trough was a goat anti-rabbit fibrinogen serum (GARF).

Only those samples in which fibrinogen or FDP were identified are shown. Results are also given in Table 2.

a. Pre-infection plasma.

b & c. Day 7 plasma.

d. Day 14 plasma.

e. Day 21 plasma.

f & g. Day 28 plasma.

Fraction number

Days after infection	I	II	III	IV	V	VI	VII
0	-	F	F	-	-	-	-
7	F	F	F	F	-	-	-
14	-	F	F	-	D	-	-
21	N/T	F	F+X	-	-	-	-
28	F	F	D	D	-	-	-

TABLE II. Results of immune precipitation of fibrinogen and degradation products in plasma fractions separated on a Sephadex GL00 column. The plasma samples were mixed plasmas from 4 rabbits (S383 - S386) infected with T. (T.) brucei 427, taken at weekly intervals following infection.

F = fibrinogen
X = Fragment X
D = Fragment D
- = no precipitation lines
N/T = not tested

c) Protamine sulphate test

The protamine sulphate test was applied to non-infected and infected rabbit sera in order to determine whether the breakdown products were derived from fibrinogen or fibrin and whether fibrin monomer was present.

The appearance of a precipitate or gel was similar to that described by Gurevich and Hutchinson (1971). A test was considered positive if a gel, fibrin strands or a feathery precipitate resulted.

It was found essential to carry out the test using citrated plasma (60 mg sodium citrate / 10 ml blood) as detailed by Gurewich (1971, 1973). If heparinised or sequestered plasma was used, a milky precipitate formed immedately the protamine sulphate was added. This occurred in a range of normal as well as infected sera. It is known that protamine sulphate inhibits the effect of heparin (Brown, 1952), but why such a gross amorphous precipitate should form is left open to question.

Six rabbits infected with strain 427 were bled before infection and at seven day intervals thereafter. The results are shown in Table III and in Appendix 1. It can be seen that the four of the rabbits gave negative results prior to infection indicating that fibrin-DP or fibrin monomer were either absent or in too small a concentration to be detected by the test.

However, following infection all rabbits gave a positive protamine sulphate test. Only on the 7th day of infection did 3 of the rabbits, S358, S360 and S361, give

	Days after infection							
Rabbit number	0	7	14	21	28	35	42	49
S358	-VE	G	FY	FY	FY	N/T	FY	FY
S359	-VE	FY	FY	FY	FY	N/T	FY	FY
S360	-VE	G	FY	FY	FY	N/T	FY	FS
S361	-VE	G	FY	FY	FS	N/T	FY	FY
S375	FY	FY	FY	FY	FY	FY	FY	FY
S376	FY	FY	FY	FY	FY	FY	FY	FY

TABLE III. Results of the protamine sulphate test on serum from 6 rabbits taken at weekly intervals during infection with T. (T.) brucei 427.

The reactions are graded as follows in increasing degrees of reactivity:-

-VE = negative
FY = feathery precipitates
FS = fibrin strands
G = gel

Further details for individual rabbits are given in Appendix 1.

strong reactions with the formation of a fibrin gel.

These results indicate that not only fibrinogen-DP, but also fibrin-DP and fibrin monomer are formed in trypanosomiasis of the rabbit.

3.1.3. Mechanism of FDP formation

The significant elevation of serum FDP during trypanosome infections suggests that either increased activation of the plasminogen/plasmin system occurs, or that substances with direct fibrinolytic activity (from trypanosomes or damaged host tissues) are released into the circulation.

a) Plasminogen concentrations

Plasminogen concentrations from two rabbits, S267 and S269, infected with T. (T.) brucei 427 were measured. The results showed that the serum plasminogen concentrations decreased during infection (Table IV). Even at the end of the experiment the plasminogen concentrations were below pre-infection levels. It should be noted that rabbit S267 had a low concentration of plasminogen at the commencement of the experiment. The plasminogen and FDP concentrations showed an inverse relationship (Fig. 10). At peak FDP levels, there was a significant fall in the amount of plasminogen present in the serum suggesting that plasminogen is being activated to form plasmin.

b) Effect of plasmin inhibition in vivo

Five rabbits were used in this experiment. Three (S310-S312) were infected with T. (T.) brucei 427 and two (S295 and S296) were left as controls. Serum samples were assayed at intervals to determine the FDP concentration. When raised FDP levels were found (21 days after infection) two infected and two control rabbits were treated with

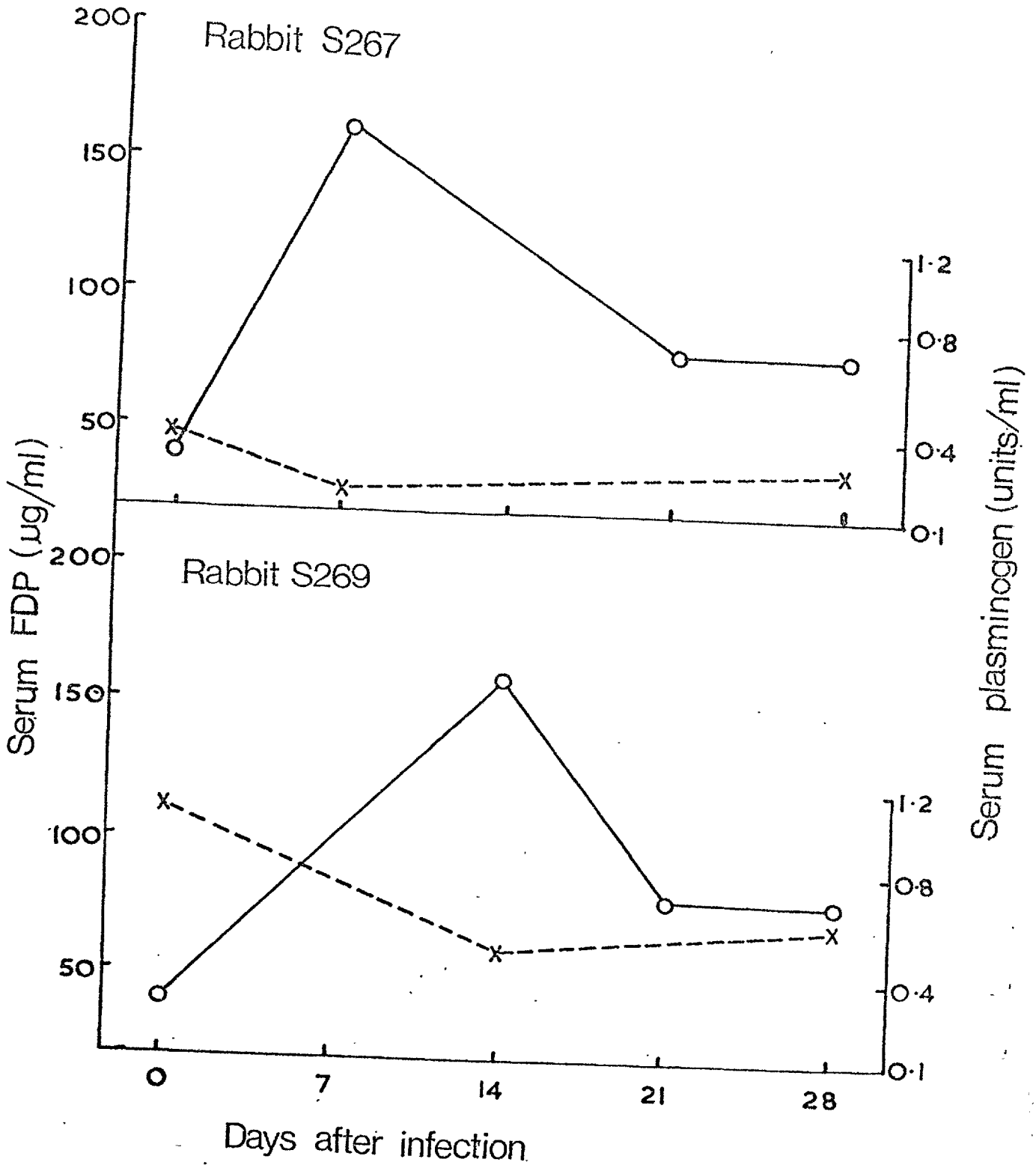


Fig. 10. Serum FDP and plasminogen concentrations in 2 rabbits infected with T. (T.) brucei 427 to show inverse relationship.

O———O FDP concentration.

X———X Plasminogen concentration.

	S267		S269	
Days after infection	FDP (µg/ml)	Plasminogen (IU/ml)	FDP (µg/ml)	Plasminogen (IU/ml)
0	40	0·38	40	1·12
7	161	0·17	N/T	N/T
14	N/T	N/T	161	0·5
28	80	0·29	80	0·6

N/T = Not tested.

TABLE IV. FDP and plasminogen concentrations in two rabbits infected with T. (T.) brucei 427

plasmin/plasminogen inhibitors and the effect on FDP levels noted. Since the half-life of FDP in trypanosome infections was not known, it was decided to treat rabbits with 1 ml EACA (400 mg/ml) and 1 ml of 'Trasylol' (5000 K.I.U./ml). These drugs were administered separately by intravenous injections into the marginal ear vein at 8 h intervals (08.00, 16.00 and 24.00 h) for 3 days. The drugs caused no obvious side effects in the rabbits.

The results of these treatments are shown in Fig. 11 and data for each rabbit can be found in Appendix 1. The effect of inhibiting plasmin in rabbits infected with trypanosomes and showing high FDP concentrations in the serum, was to produce an immediate and dramatic fall in the level of these substances. In rabbit, S309 infected with trypanosomes but not given EACA and 'Trasylol', a fall also occurred but it was not precipitous and did not take place until several days later. This was a similar pattern found in most infected (not drug-treated) rabbits as shown earlier under Section 3.1.1. (b). The effect of both drugs on non-infected rabbits was to reduce the 'normal' concentration of FDP slightly.

It was of interest to note that perhaps only a 24 h period of drug treatment is required to inhibit plasmin/plasminogen effectively, since rabbit S312 showed an immediate drop in serum FDP after this period. The other rabbits of this group were not investigated for changes in FDP at this time.

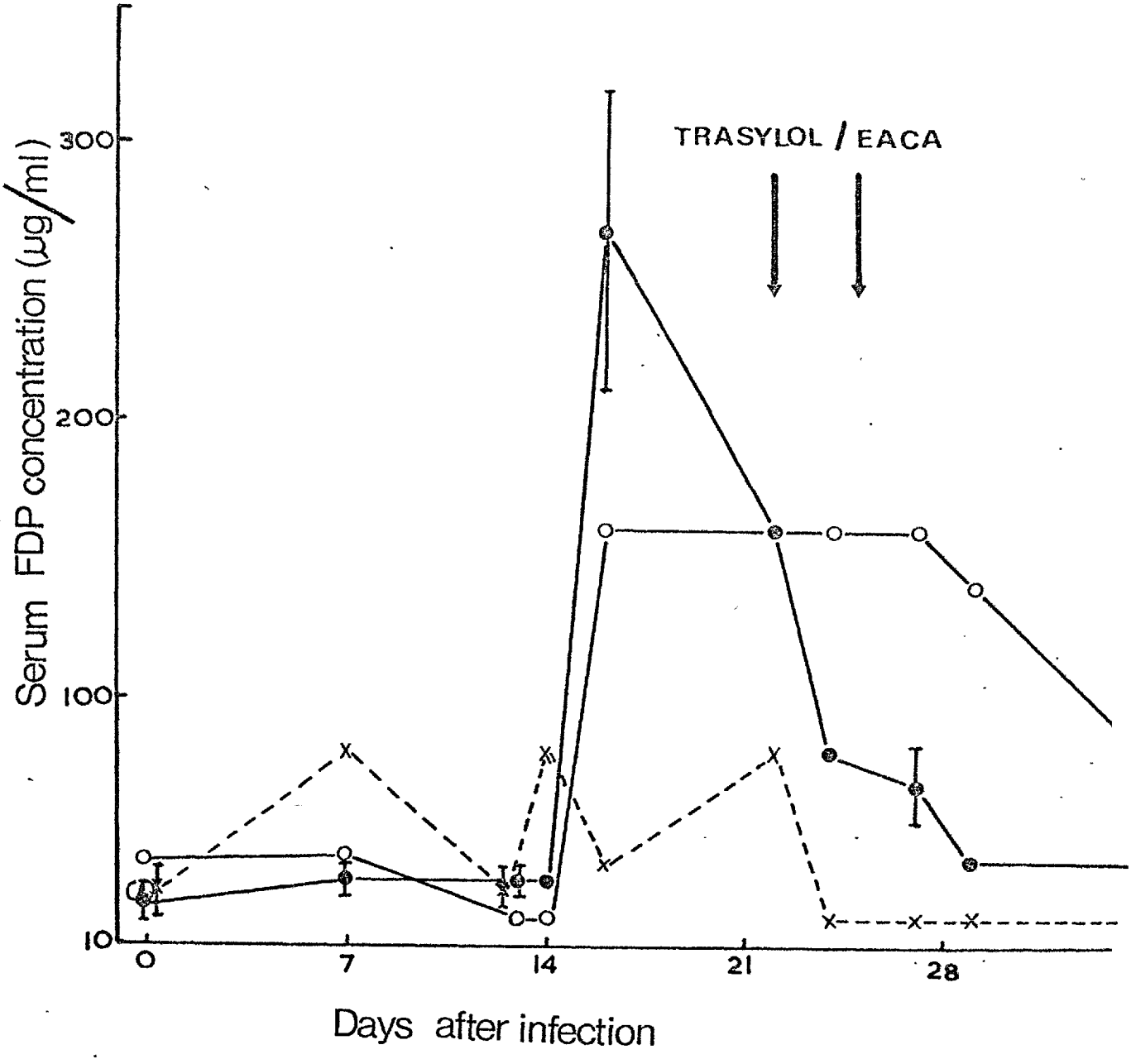

TRASYLOL / EACA
Serum FDP concentration (µg/ml)
300
200
100
10
0
7
14
21
28
Days after infection

Fig. 11.

Fig. 11. The effect on serum FDP concentration of plasminogen inhibition by administration of 'Trasylol' (5000 K.I.U.) and EACA (400 mg) every 8 h for 3 days. The drugs were given on days 22, 23 and 24 of the infection as indicated by the arrows.

●————● Rabbits infected with T. (T.) brucei 427 and treated with 'Trasylol'/EACA. Mean (± S.E.) of 2 rabbits.

O————O Rabbit infected with T. (T.) brucei 427 remaining as untreated control.

X————X Non-infected control rabbits treated with 'Trasylol'/EACA. Mean (± S.E.) of 2 rabbits.

c) Plasminogen activator in rabbit tissues

The presence of plasminogen activator potential in rabbit tissues was examined since tissue damage might provide a mechanism whereby FDP formation could occur.

Saline extracts of normal rabbit tissues viz. lung, kidney, spleen, liver and heart, gave no indication of containing plasminogen activator as judged by the absence of lysed zones of fibrin on Astrup fibrin plates after incubation for 24 h at 37°C. Heat treated fibrin plates in which the plasminogen content was destroyed, were also negative indicating, in addition, the absence of proteolytic activity in the extracts. These results compare favourably with those of Astrup (1966) who also demonstrated that rabbit tissues were very low in plasminogen activator compared with human or porcine tissues.

d) Plasminogen activator in trypanosome extracts

A homogenate of T. (T.) brucei 427 in PBS (pH 7.3) similarly appeared to have no activation potential and also gave negative results on the heated, EACA and 'Trasylol' plates. Trypsin (10 µg/ml; BDH Chemicals Ltd.,) which had been added to the plates as a positive control gave a mean fibrinolytic index of 242 $\pm$ 21.87 mm^3 (on plasminogen rich plates) 237 $\pm$ 7.3 mm^3 (on EACA plates), 158 $\pm$ 8.8 mm^3 (on heat-inactivated plates) and no evidence of fibrinolytic activity on 'Trasylol' plates. Thus 'Trasylol' completely inhibited the proteolytic activity of the enzyme which did not occur however on the EACA plate.

There are 2 possible explanations for the absence of visible fibrinolysis using trypanosome extracts on Astrup plates. To begin with, any plasminogen activator contained within the extracts might be in too low a concentration to be detected by this method. Secondly, the fibrin layer of the plate may have been too thick (1.5 mm) for the low concentration of any activator to have a visible effect.

It was therefore decided to incubate saline extracts of trypanosomes (supernatant fraction of a homogenised preparation, pH 7.3, see Section 2.6. for details) with fibrinogen in solution and detect any fibrinolytic activity by both a decrease in the clottable fibrinogen concentration and immunoelectrophoresis of 'digests' at various periods during the incubation.

The following test solutions were set up:-

1) An incubation control consisting of an equal volume of PBS (pH 7.3) and human fibrinogen (10 mg/ml).

2) Trypanosome extract (protein concentration = 2 mg/ml) and human fibrinogen (10 mg/ml) in equal volumes.

3) Trypanosome extract plus fibrinogen and streptomycin (25 µg/ml, Glaxo Laboratories Ltd.,) to prevent growth of any bacteria contained within the trypanosome extract.

4) Trypanosome extract plus fibrinogen and EACA (8 mg/ml) to inhibit plasminogen activation and plasmin activity.

5) Trypanosome extract plus fibrinogen incubated together in equal volumes as (2). However since it was possible that the trypanosome extract might have anti-thrombin activity, additional thrombin (500 U/ml instead of the normal 50 U/ml) was added during the clotting process for determination of fibrinogen.

Clottable fibrinogen in the samples

Results of the clottable fibrinogen remaining after incubation at various time intervals for the above test solutions is shown in Fig. 12. Fibrinogen concentration is plotted as optical density read at 550 mμ on a Beckman DB spectrophotometer, against time on a $\log_{10}$ scale.

It can be seen that only after a 5 min incubation period of trypanosome extract with fibrinogen, there was a marked drop in the clottable fibrinogen. This decline continued until, at 24 h, no clot was formed after the addition of thrombin (50 U/ml). This decrease in fibrinogen may be due to fibrinolytic activity associated with bacterial growth, fibrinolytic activity of trypanosome extracts or increased anti-thrombin activity of the same extracts.

Addition of streptomycin had no effect on the sharp initial decrease in fibrinogen during the first hour of incubation. However a clot could be obtained following 24 h of incubation, indicating that in samples minus antibiotic, growth of bacteria possessing fibrinolytic activity had occurred resulting in complete breakdown of all fibrinogen contained within the sample.

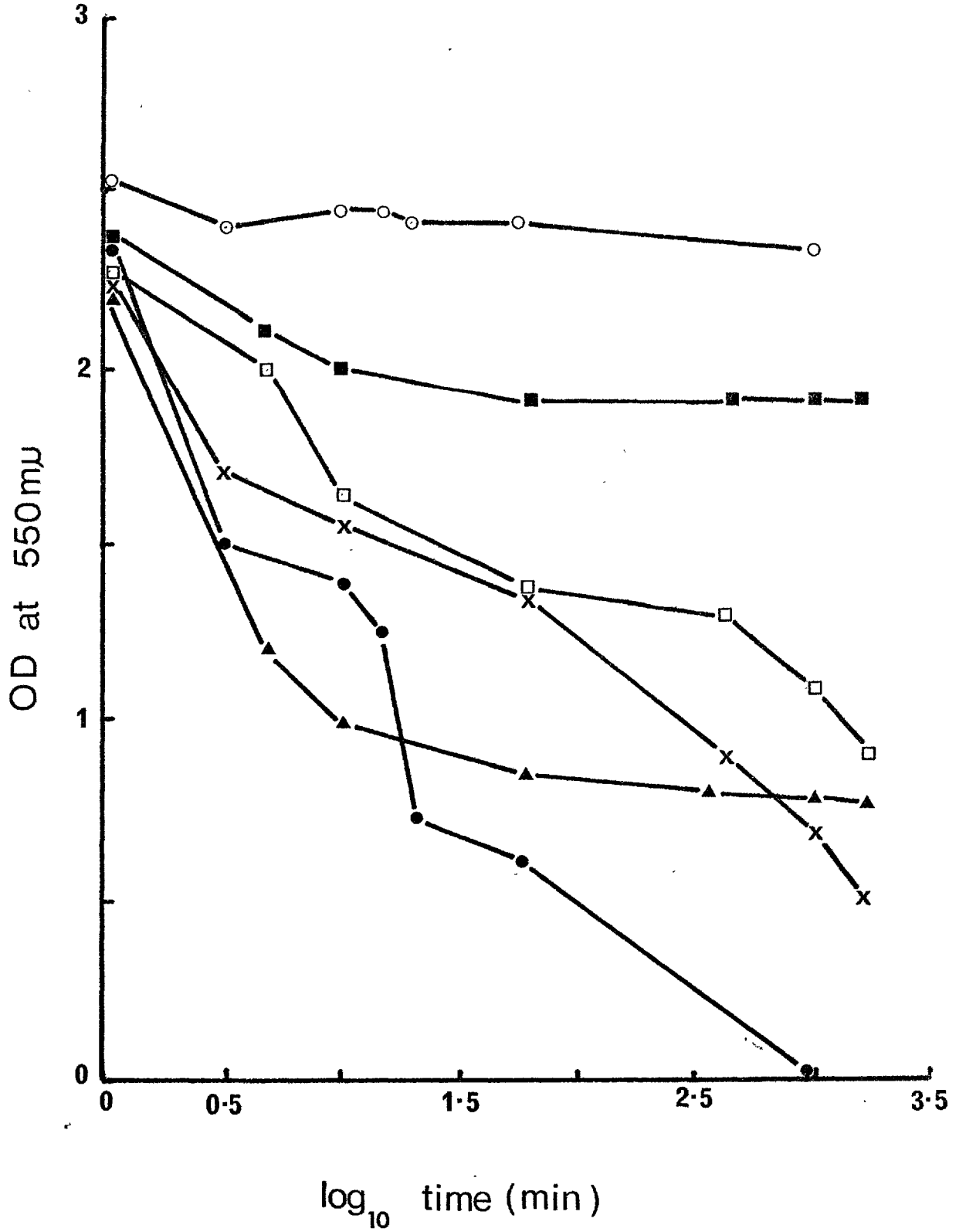


Fig. 12.

Fig. 12. Effect of T. (T.) brucei 427 extract (supernatant fraction of 2.0 x 10^8 trypanosomes/ml) on human fibrinogen incubated at 37°C, at pH 7.4 over a 24 h period. Fibrinogen concentrations plotted as OD read at 550 mμ on a Beckman DB spectrophotometer.

●——● Tryp. extract + fibrinogen.

O——O Incubation control. Buffer + fibrinogen.

■——■ Tryp. extract + fibrinogen + 'Trasylol' (200 K.I.U./ml).

□——□ Tryp. extract + fibrinogen + EACA (8 mg/ml).

▲——▲ Tryp. extract + fibrinogen + streptomycin (25 μg/ml).

X——X Tryp. extract + fibrinogen + additional thrombin (500 U/ml) during the clotting process.

Addition of EACA had a slight effect in inhibiting the fall in the fibrinogen concentration indicating that trypanosome extracts do possess a plasminogen activator.

When extra thrombin was added to overcome any antithrombins in the extracts which might be interfering with the clotting process, a significant decrease in clottable fibrinogen still occurred. Thus if antithrombins are present they only had a minimal effect on the results.

Immunoelectrophoresis of samples

Following electrophoresis of samples taken at 3, 10, 15, 20 and 60 min, only one precipitin line, in the position of fibrinogen, developed. However at 12 h and 24 h degradation of fibrinogen occurred to give 2 precipitin lines crossing one another (Fig. 13 a and b). It is possible that from the positioning of the lines, that they represent Fragments D and E.

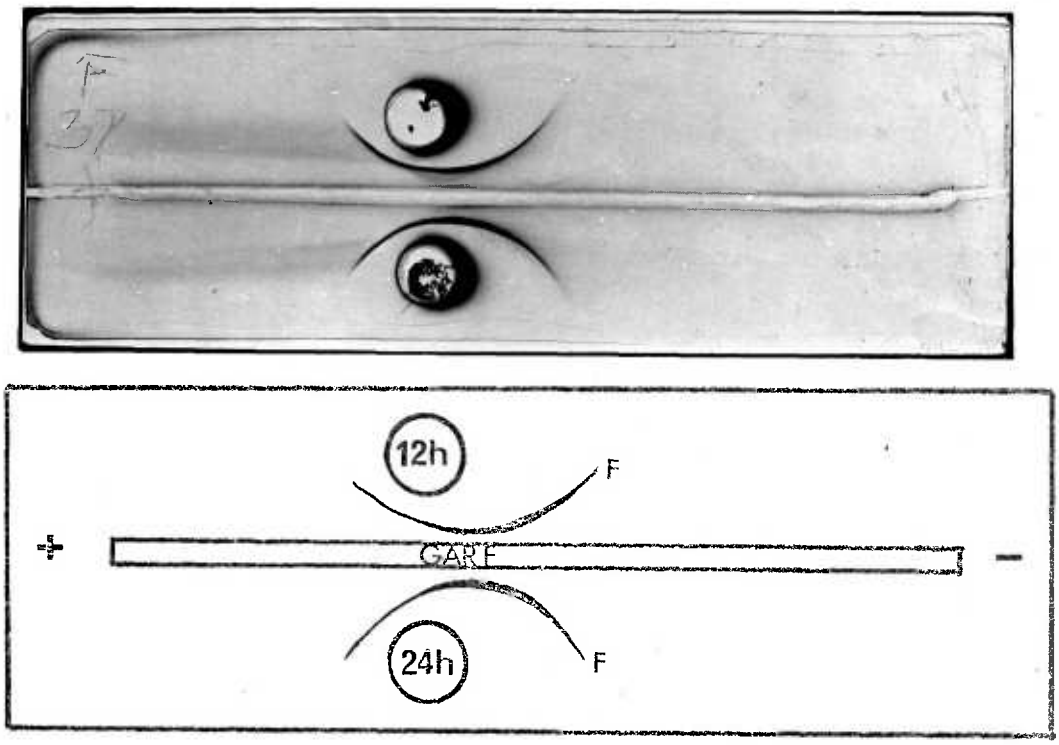
e) Enzymatic activity of trypanosome extracts

It was considered possible that, in addition to activating plasminogen, the trypanosome extracts might contain a protease enzyme capable of degrading fibrinogen directly.

Trypanosome extracts were incubated with fibrinogen and 'Trasylol' (200 K.I.U./ml) to inhibit any proteolytic activity.

It can be seen from Fig. 12 that addition of 'Trasylol' almost completely prevented the fall in fibrinogen concentration. This would indicate that the major pathway for fibrinogen degradation *in vitro* is direct via. a trypanosome protease.

(a)



(b)

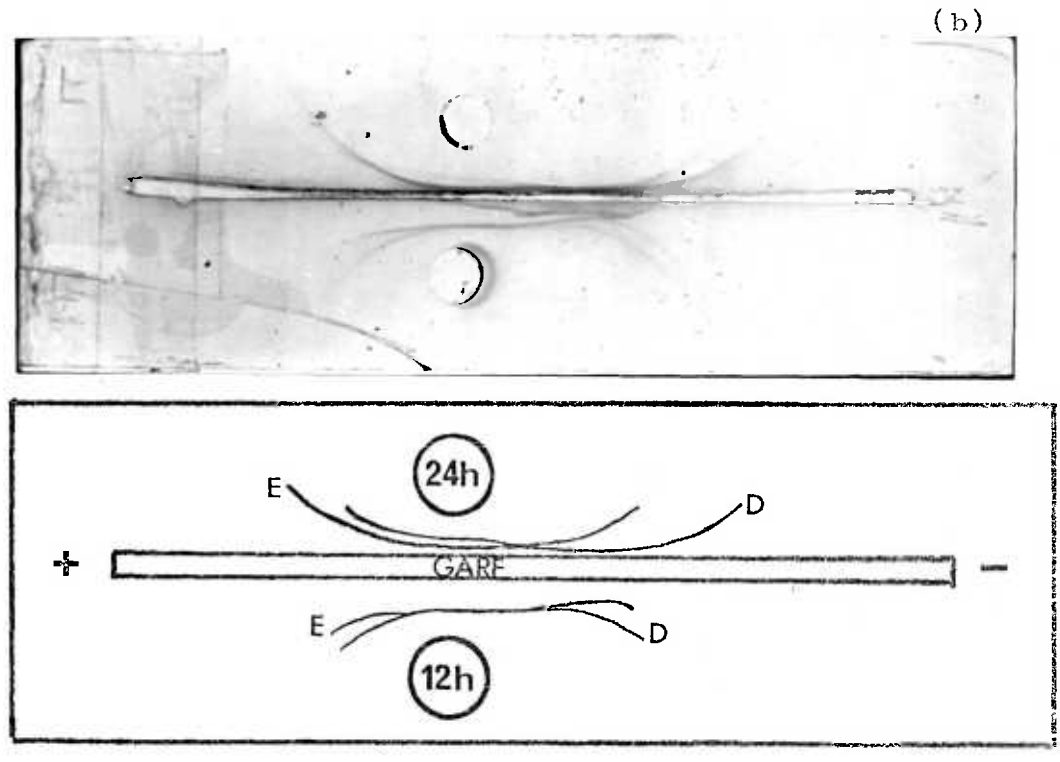


Fig. 13.

(c)

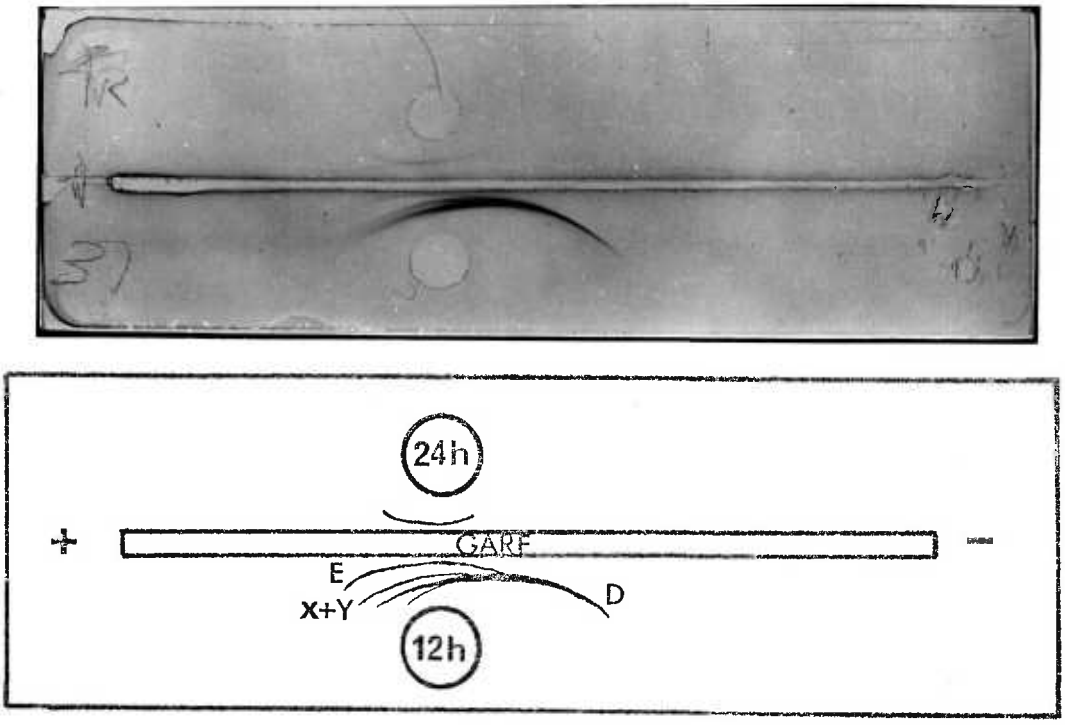


Fig. 13. Immunolectrophoresis of fibrinogen solutions following incubation with a trypanosome extract for 12 h and 24 h at 37°C.

a. Incubation control comprising fibrinogen and buffer alone. Single precipitin line represents fibrinogen.

b. Fragments D and E formed by incubation of fibrinogen with trypanosome extracts.

c. Positive control fragments X, X, D and E formed following incubation of fibrinogen with trypsin (10 µg/ml). At 24 h incubation only a single precipitin line. Note disappearance of antigenicity of fragments following prolonged digestion with this enzyme.

3.1.4. Effect of chemotherapy on FDP levels

Six infected rabbits were divided into 3 groups:-

Group 1: Infected with T. (T.) brucei 427 and drug-treated two weeks after infection.

Group 2: Infected with T. (T.) brucei 427 and treated when serum FDP levels were maximal.

Group 3: Infected with T. (T.) brucei S42 and treated when the parasitaemia was high (> 100 trypanosomes per microscope field)

All rabbits, were given a single intravenous injection of Melarsoprol (Mel-B, May and Baker Ltd.,) 3-6 mg/kg, at the times indicated above.

Blood for serum FDP assay was taken:

- 1 h after treatment
- 24 h after treatment
- at weekly intervals thereafter for the following two weeks.

The results shown in Figs. 14 and 15 indicate the trypanocidal therapy given early in the infection and at peak serum FDP, did not cause a rise in FDP values. FDP either remained constant (Group 1) or fell to normal levels 24 h after treatment (Group 2).

Rabbits given Mel-B when the parasitaemia was high showed an increased FDP concentration 1 h following drug administration (Table V). Because of this rapid onset of

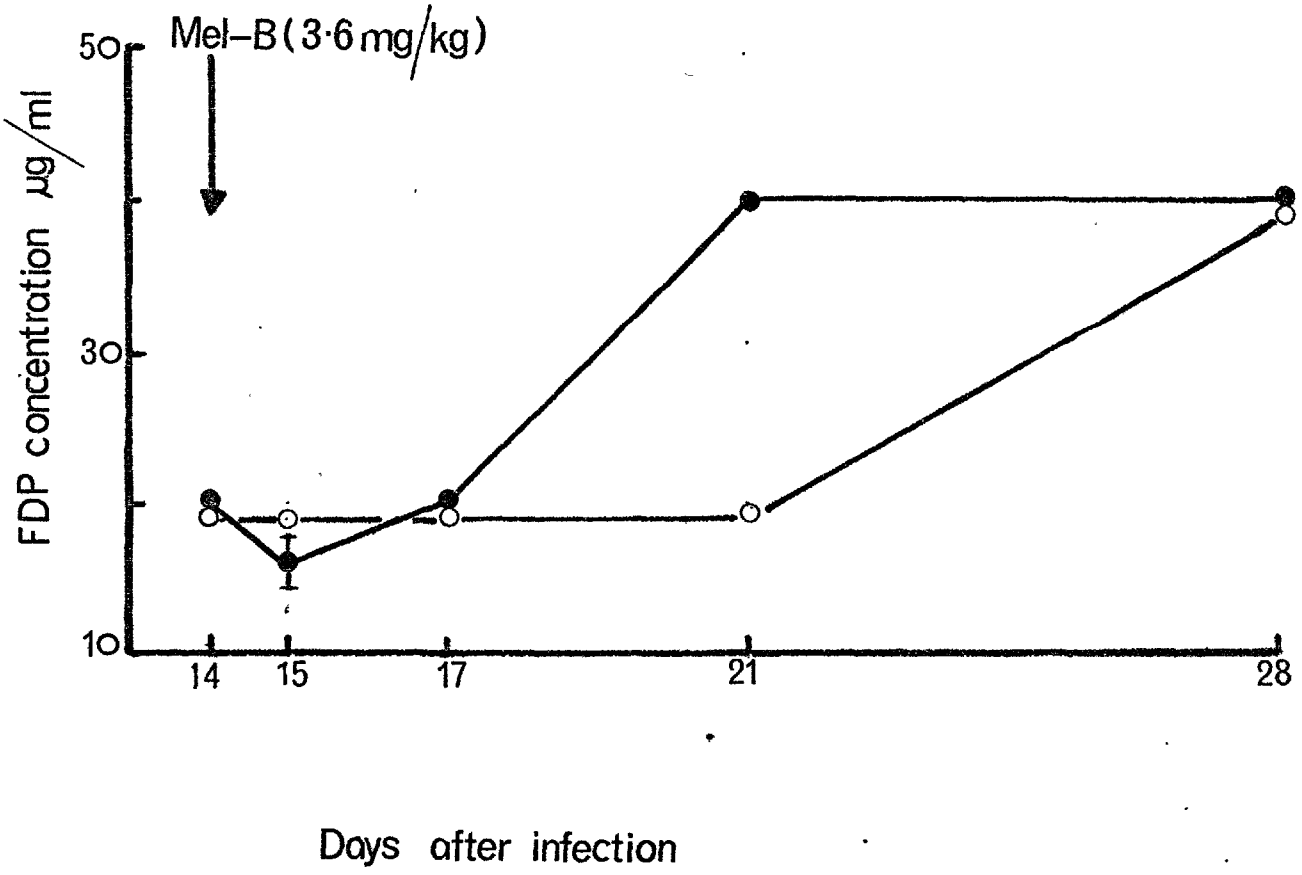


Fig. 14. Effect of chemotherapeutic treatment with Mel-B on serum FDP levels. Drug treatment given early in infection (day 14).

●———● Mean of three rabbits (S318, S319 and S320) infected with T. (T.) brucei 427.

O———O Control non-infected rabbit (S321).

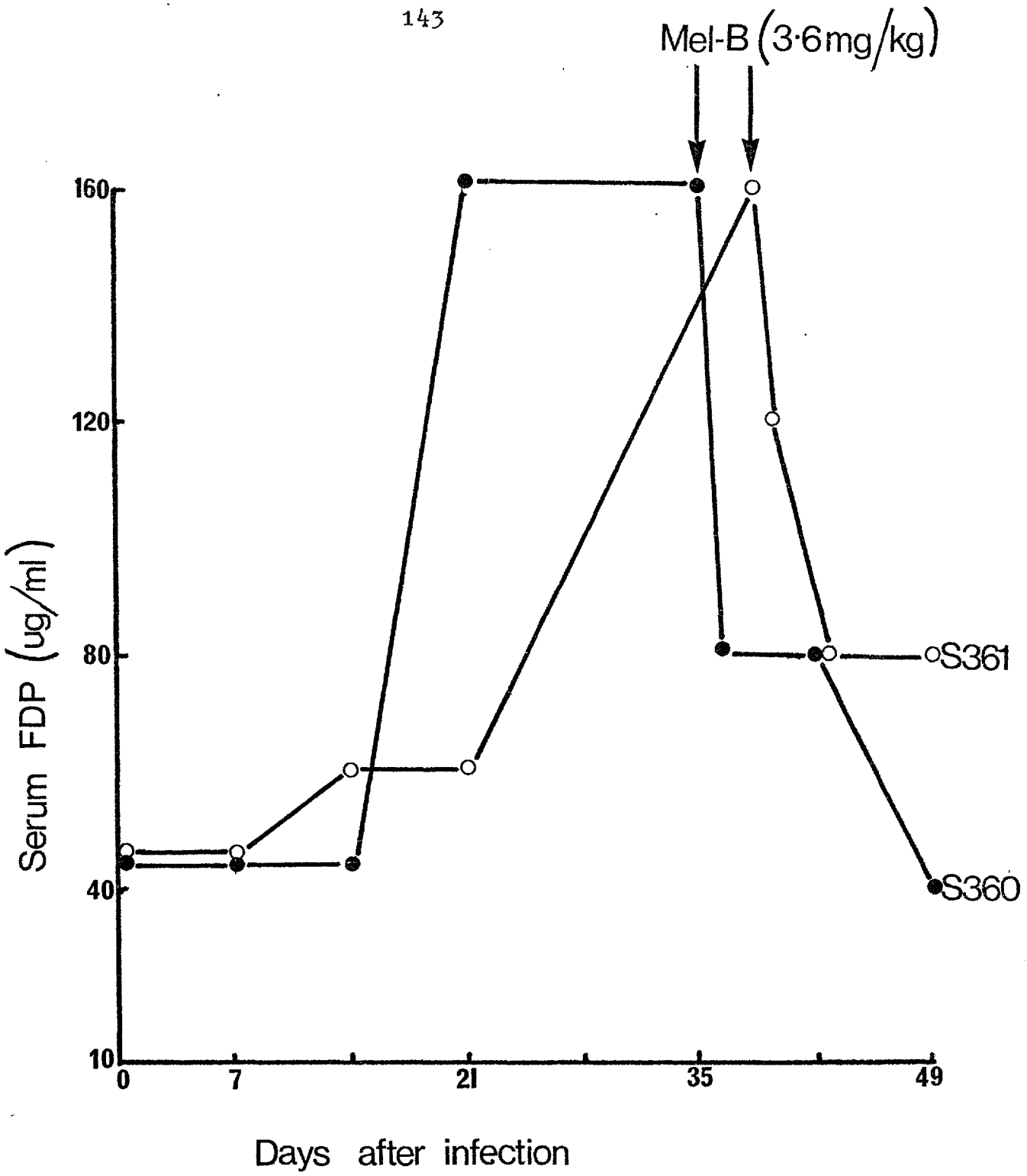


Fig. 15. Effect of chemotherapeutic treatment (Mel-B) on serum FDP levels. Drug treatment given when FDP levels were maximal.

●———● Rabbit S360.

o———o Rabbit S361.

Both rabbits infected with T. (T.) brucei 427

Rabbit S409

Days after infection and time of drug treatment	Parasit-aemia *	FDP µg/ml
10	2	N/T
20	19	40
25 prior to Mel-B	130	80
25 1 h after Mel-B	4	160
26 24h after Mel-B	0	80-160
32 1 wk after Mel-B	0	40

Rabbit S410

10	11	20
20	35	20
26 prior to Mel-B	90	20
26 1 h after Mel-B	6	10
26 approx. 8 h after Mel-B	died	N/T

Rabbit S411

10	27	10
20	8	40
27 prior to Mel-B	390	40
27 1 h after Mel-B	11	80
28 24h after Mel-B	0	40-80
34 1 wk after Mel-B	0	40

Rabbit S412

10	15	20
20	10	80
27 prior to Mel-B	115	80
27 1 h after Mel-B	3	80-160
28 24h after Mel-B	0	80
34 1 wk after Mel-B	0	80

TABLE V. Effect of Melarsoprol-B at a dose of 3·6 mg/kg on the elevation of serum FDP in 4 rabbits (S409, S410, S411, S412) infected with T. (T.) brucei 427

* parasitaemia = number of trypanosomes in 30 microscope fields (magnification x 400).

FDP elevation it is likely that fibrinolysis is associated with release of trypanosomal material or formation of antigen-antibody complexes. Mel-B alone had no effect on the fibrinolytic system of control rabbits. Of interest was the death of one of the rabbits in this group, S410, only a few hours after chemotherapy suggesting that perhaps a Jarish-Herxheimer-like reaction had occurred.

3.1.5. Plasma fibrinogen

Since elevated levels of FDP were found in infected rabbits, it was important to investigate changes (both quantitative and qualitative) of the fibrinogen molecule.

i) Quantitative fibrinogen

a) Levels throughout infection

Six rabbits (S267, S268, S269, S252, S340 and S341) were infected with T. (T.) brucei 427 and the clottable plasma fibrinogen concentration followed during the infection. Three uninfected rabbits (S270, S271 and S337) were kept as controls.

It can be seen from Fig. 16 that little variation in the concentration of fibrinogen in control rabbits occurred over the period of investigation and levels did not exceed 300 mg/100 ml blood. A considerable range was noted in non-infected animals (150 to 300 mg/100 ml). Individual rabbit fibrinogen concentrations are given in Appendix 1.

There was a marked increase in fibrinogen during the first two weeks of infection reaching a peak between the 3rd and 4th week. In most animals this increase was between 100-150%. Occasionally levels up to 800 mg/100 ml were found. In prolonged infections lasting eight weeks or more, the concentration of fibrinogen usually dropped to just below pre-infection levels.

b) Effect of chemotherapy

Since plasma fibrinogen showed a sharp increase

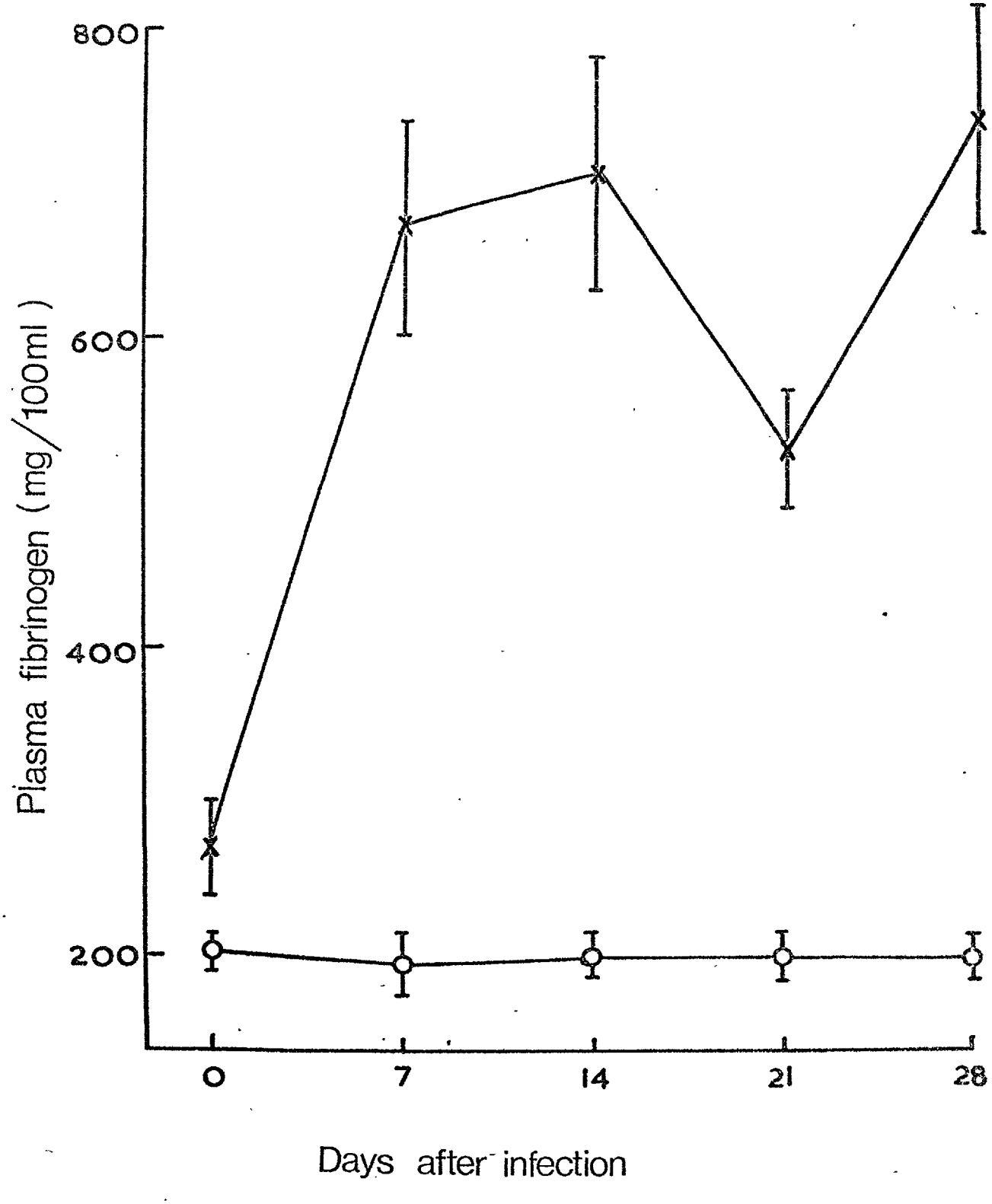


Fig. 16. Fibrinogen concentrations in the plasma of rabbits.

X———X Mean (± S.E.) for 6 rabbits infected with T. (T.) brucei 427

O———O Mean (± S.E.) for 3 non-infected controls.

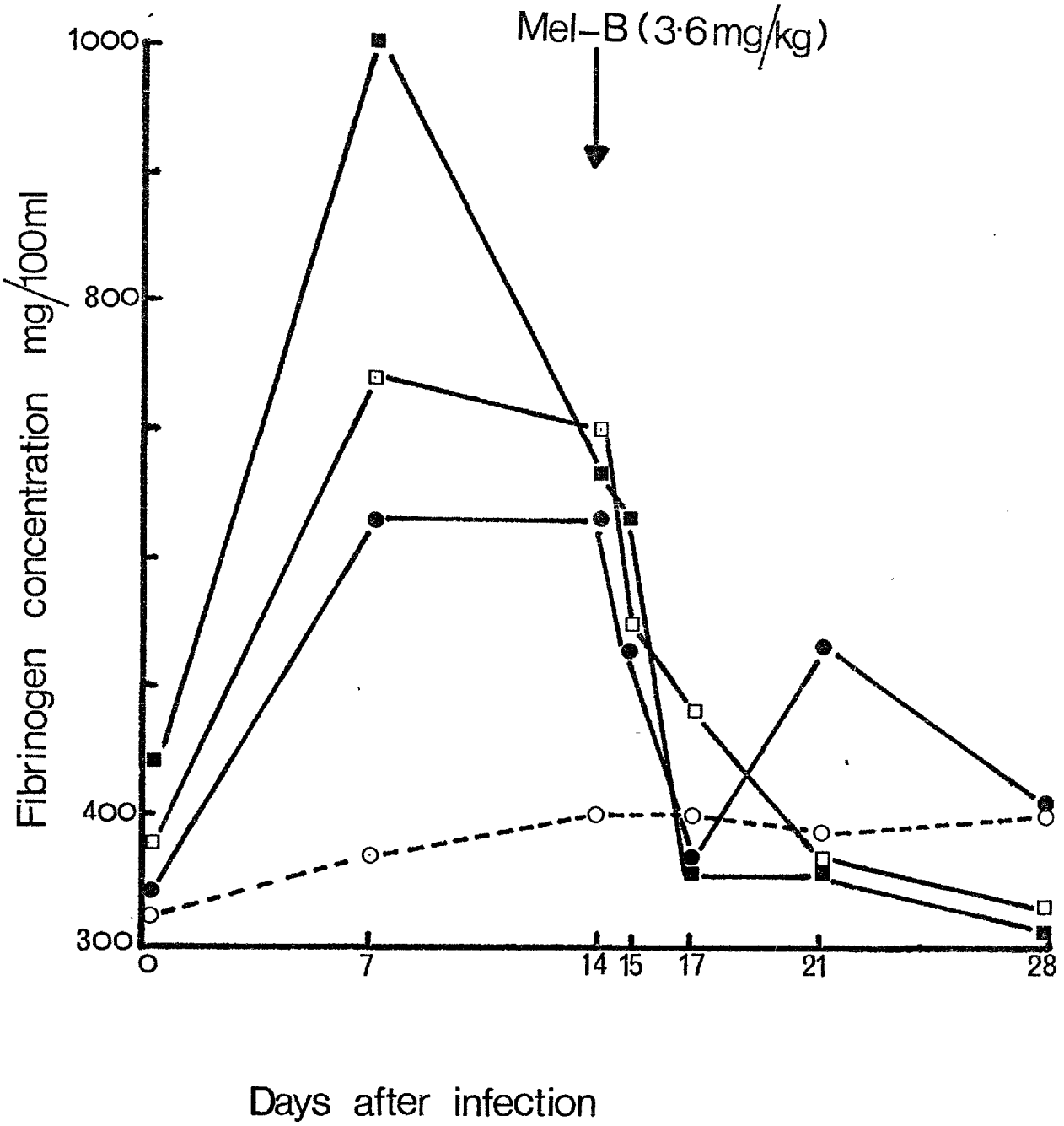

Mel-B (3·6 mg/kg)
1000
800
400
300
Fibrinogen concentration mg/100ml
0
7
14
15
17
21
28
Days after infection

Fig. 17.

Fig. 17. Effect of chemotherapeutic treatment (Mel-B) on the plasma fibrinogen concentration of rabbits infected with T. (T.) brucei 427.

●——● Rabbit S320.

■——■ Rabbit S319.

□——□ Rabbit S318.

O——O Non-infected control rabbit S321.

following infection, it was of interest to investigate whether this was maintained during chemotherapy or whether there was a rapid fall to normal levels.

Blood for plasma was collected from infected rabbits S318, S319, S320 and control rabbit S321.

The effect of chemotherapy with Mel-B (3.6 mg/kg)was was dramatic. There was a sharp decrease to normal pre-infection levels within 3 days after drug administration (Fig. 17) indicating a rapid "switch-off mechanism" of the increased fibrinogen synthesis by the liver.

c) Effect of immunisation with dead trypanosomes

Two rabbits (S422 and S433) were given intramuscular injections into the hind limbs at 3-4 day intervals (for a period of 3 weeks) of 0.5 ml Freunds Complete Adjuvant (FCA) and 0.5 ml of a lysed T. (T.) brucei 427 suspension (2.02×10^9 trypanosomes). Another rabbit (S424) was given 0.5 ml of trypanosome suspension only, at the same intervals. A control rabbit (S425) was injected with 0.5 ml FCA alone. Plasma for fibrinogen determination was collected from each rabbit prior to the first and subsequent innoculations.

Table VI shows the results. Since rabbit S424, immunised with trypanosomes alone, showed no elevation of plasma fibrinogen it suggests that there is no non-specific release or synthesis of fibrinogen due to the presence of dead trypanosome material.

The remaining three rabbits all showed an elevated

Days after immunisation	Rabbit number			
	S422	S423	S424	S425
	plasma fibrinogen mg/100 ml			
0	130	190	211	182
3	150	169	182	133
6	284	252	221	382
10	319	390	252	412
13	487	470	316	539
17	542	505	319	535
20	483	539	340	542
24	459	503	306	522

TABLE VI. Fibrinogen levels in rabbits during the following immunisation procedures:-

Rabbit S422 - 1 ml Freunds Complete Adjuvant + 1 ml *T. (T.) brucei* 427 ($2 \cdot 02 \times 10^9$ trypanosomes) IM.

Rabbit S423 - as for rabbit S422

Rabbit S424 - 1 ml *T. (T.) brucei* 427 ($2 \cdot 02 \times 10^9$ trypanosomes) alone.

Rabbit S425 - 1 ml Freunds Complete Adjuvant alone.

plasma fibrinogen after the fourth injection of either FCA plus trypanosomes or FCA by itself. This increase was not as marked as in a trypanosome infection. At this time, the rabbits had developed abcesses on the limbs at the site of injection. It has been well documented that inflammatory states are associated with increased fibrinogen synthesis (McSherry et al, 1970) and that FCA induces an inflammation (Dumonde and Glynn, 1962). It would appear therefore, that the rabbits in this experiment, immunised with FCA, were experiencing a localised inflammation as indicated by the appearance of the abcesses. The dead mycobacteria may have had some indirect effect (i.e. not via. inflammation), although this is unlikely.

ii) Qualitative fibrinogen

a) Fibrinogen-B

The β-naphthol reaction for 'Fibrinogen-B' in plasma was positive for 4 rabbits (S358-S361) within the first seven days of infection with T. (T.) brucei 427. It's presence in the plasma was also detected during the following 6 weeks of observation. However no plasma sample taken at any time during infection gave a 4 + (gel) reaction (Table VII).

Out of the 4 plasma samples taken from the above rabbits prior to infection with trypanosomes, only 1 rabbit, S358 gave a positive reaction for 'Fibrinogen-B'. Most reactions were complete, i.e. there was no increase in precipitate, after the 3 h incubation period.

Days infected	Rabbit number							
	S358		S359		S360		S361	
	Reaction Time (min.)							
	10'	180'	10'	180'	10'	180'	10'	180'
0	1+	1+	-ve	-ve	-ve	-ve	-ve	-ve
7	-ve	1+	-ve	1+	3+	3+	3+	3+
16	-ve	1+	1+	3+	1+	3+	1+	3+
21	1+	3+	1+	3+	-ve	3+	-ve	3+
28	1+	3+	1+	3+	1+	3+	2+	3+
35	1+	N/T	1+	N/T	-ve	N/T	2+	N/T
42	1+	1+	2+	2+	*-ve	1+	*1+	2+
49	1+	N/T	1+	N/T	2+	N/T	1+	N/T

TABLE VII. The β-naphthol reaction of plasma (graded 1+ to 4+) from 4 rabbits infected with T. (T.) brucei 427.

N/T = not tested.

* Rabbit S360 had received chemotherapeutic treatment (Mel-B , 3·6 mg/kg) on day 35 after infection, and rabbit S361 received the same treatment on day 38 of the infection.

It was of interest to note that two of the rabbits, S360 and S361, having received trypanocidal therapy on days 35 and 38 after infection respectively, still retained a positive reaction 14 and 11 days following this treatment.

Addition of 50% ethanol alone caused no gelation of the plasma samples, indicating that the reaction was specific for β-naphthol and that the precipitate formed did not represent fibrin monomer which is gelled by the addition of alcohol (Cummine and Lyons, 1948).

The presence of 'Fibrinogen-B' in the plasma was considered abnormal by Cummine and Lyons (1948) who reported a positive β-naphthol reaction prior to intravascular thrombosis both in man and rabbits. However Smith et al (1951) criticised Lyons' reports and suggested that the β-naphthol reaction was dependant only on quantitative and not qualitative changes in fibrinogen in a variety of disease states in man.

To examine the occurrence of this situation in trypanosomiasis, four rabbits (S347-S350) were infected with T. (T.) brucei 427 and 2 rabbits (S345 and S346) were left as non-infected controls. Plasma, collected in EDTA, was analysed at weekly intervals following infection for both clottable fibrinogen and the presence of 'Fibrinogen-B'. The previously stored plasma (-20°C) of rabbit S341 (infected with the same strain of trypanosome) was also used in these experiments. The proceedure following

precipitation by β-naphthol was modified slightly by grading reactions according to the dry weight of the precipitate formed. To do this, the plasma/β-naphthol mixtures were centrifuged at 10,000 g for 20 min, the precipitate freeze-dried, and the dry weight noted. The results are represented as a scatter-diagram (Fig. 18) and the weights also shown in Table VIII. There appears to be no direct parallelism between the concentration of clottable fibrinogen and the reaction with β-naphthol. Very high fibrinogen levels (e.g. rabbit S341) were present without the same plasma showing a gross precipitate following mixing with the reagent. It would appear therefore, that β-naphthol is complexing with and precipating a plasma protein (presumably a fibrinogen derivative since no precipitate was formed in serum) which is not measured during clottable fibrinogen determinations. This derivative is unlikely to be FDP since they are only present in microgram amounts and their contributory weight, if precipitated, would be negligable in these results.

The substance precipitated, in addition, is not likely to be fibrin monomer since no precipitate was formed in plasma on the addition of ethanol alone.

It would appear therefore, that during trypanosomiasis of the rabbit, a large propation of the fibrinogen synthesised is 'abnormal' in that it precipitates following mixing with a naphthoquinone complex. The precipitate is not related to the concentrations of clottable fibrinogen in the circulation.

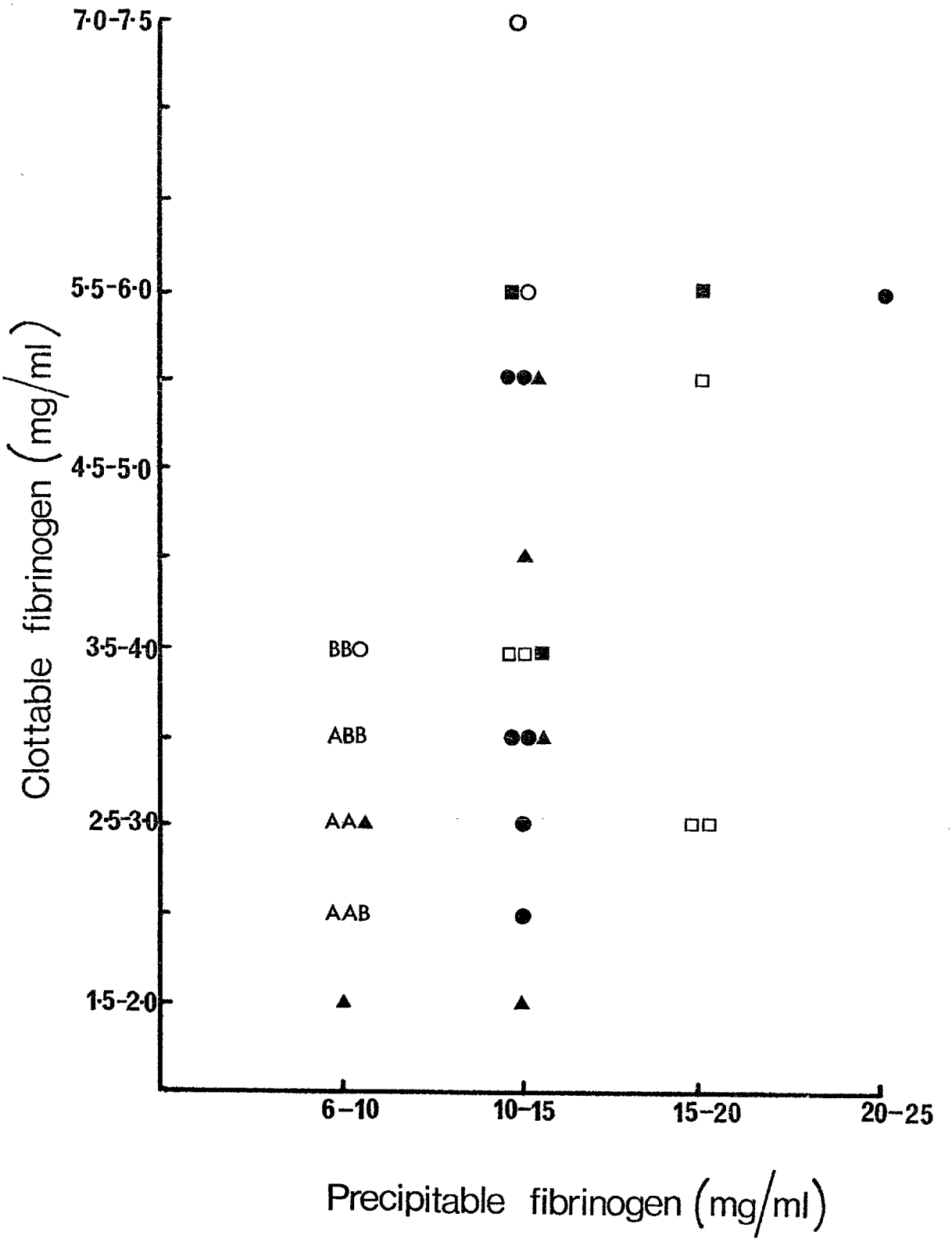

7·0-7·5
5·5-6·0
4·5-5·0
3·5-4·0
2·5-3·0
1·5-2·0
Clottable fibrinogen (mg/ml)
BBO
ABB
AAA
AAB
6–10
10–15
15–20
20–25
Precipitable fibrinogen (mg/ml)

Fig. 18.

Fig. 18. Scatter graph to show the relationship between fibrinogen-B and the clottable fibrinogen concentration in the plasma of rabbits infected with T. (T.) brucei 427.

O = Rabbit S341

● = Rabbit S347

■ = Rabbit S348

X = Rabbit S349

□ = Rabbit S350

A and B = Two control non-infected rabbits S345 and S346.

(a)

Days infected	Rabbit number S345	S346
0	5·5	7·0
7	8·0	N/T
14	8·0	N/T
21	8·0	7·0
28	9·5	6·0
35	9·0	8·5
49	7·0	6·0

(b)

Days infected	Rabbit number S347	S348	S349	S350	S341
0	10·0	9·0	9·5	9·5	N/T
7	22·0	14·0	12·0	N/T	12·0
14	12·0	N/T	N/T	16·0	12·0
21	12·5	N/T	14·5	N/T	10·0
28	12·0	N/T	11·5	16·0	N/T
35	12·5	16·0	10·0	12·5	N/T
49	11·5	12·0	9·5	16·0	N/T

TABLE VIII. The dry weight of precipitate (in mg) formed by the addition of β-naphthol to 0·5 ml of plasma.

a) weights for control non-infected rabbits

b) weights for rabbits infected with T. (T.) brucei 427

N/T = not tested.

The clottable fibrinogen concentration for rabbits S345 - S350 in Appendix 1.

b) Heparin - precipitable fibrinogen (HPF)

The HPF fraction, partly clottable, precipitates from heparinised human and rabbit plasma at a temperature of about 4°C (Thomas et al, 1954; Smith and von Korff, 1957). It has been shown to be markedly increased during a multitude of inflammatory and degenerative diseases (Smith, 1957).

Four rabbits (S358, S359, S375 and S376) showed the presence of HPF in pre-infection plasma as indicated in Fig. 19 a. There was an increase in this fraction around 2 weeks following infection with T. (T.) brucei 427, approaching a peak 5 weeks later.

All precipitates formed at 4°C dissolved on re-heating the plasma to 37°C. No precipitate was formed in citrated plasma or in serum samples.

Since the clottable fibrinogen levels were not measured in this group of rabbits, it was not possible to correlate HPF with clottable fibrinogen. However the previous experiments have shown that the typical pattern following a trypanosome infection is an increase in clottable plasma fibrinogen 2-3 weeks after infection declining towards termination of the infection approximately 8 weeks later. Although HPF would correlate well with clottable fibrinogen within the first few weeks of infection, it is obvious that an inverse relationship is in existence towards the end of the infection.

c) Cryofibrinogen

In the presence of high levels of fibrinogen, a cold precipitable and clottable protein has repeatedly been demonstrated in human plasma (Korst and Kratochvil, 1955; Campbell et al, 1959; Kalbfleisch and Bird, 1960). It was therefore of interest to see whether cold insoluble fibrinogen also occurred in trypanosome infections.

The plasma of 2 rabbits, S375 and S376 infected with T. (T.) brucei 427, was collected at weekly intervals during the infection and examined for the presence of cryofibrinogen.

The results (Table VIII and Fig. 19 b) indicate that following chilling of the plasma at 4°C for 24 h cryofibrinogen was not demonstrated in normal rabbit plasma, but a heavy precipitate was formed in infected plasma collected at 21 days following infection.

Concurrent chilling of serum samples collected at the same weekly intervals, indicated the absence of cryoglobulin in these 2 rabbits.

One problem encountered in detecting cryofibrinogen is that it might be confused with HPF since both are formed at 4°C. However by comparing Figs. 19 a and 19 b it can be seen that the changes in cryofibrinogen and HPF do not parallel each other and are therefore probably not synonomous.

Again there was an increase in cryofibrinogen towards the end of the infection at a time when clottable

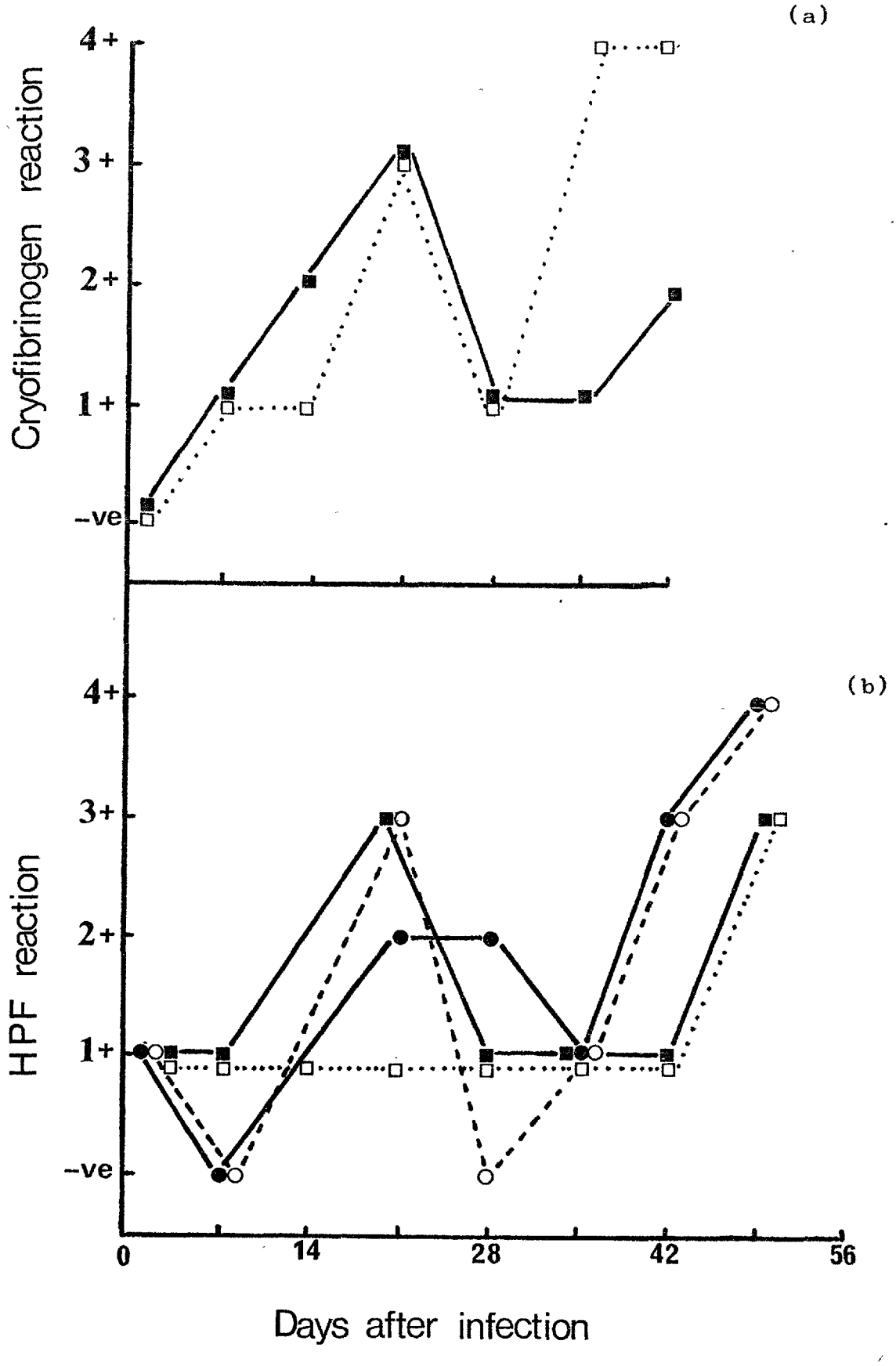

(a)
Cryofibrinogen reaction
4+
3+
2+
1+
-ve
(b)
HPF reaction
4+
3+
2+
1+
-ve
0
14
28
42
56
Days after infection

Fig. 19.

Fig. 19.

a) Cryofibrinogen reaction in the plasma of 2 rabbits infected with T. (T.) brucei 427.

□———□ Rabbit S375

■———■ Rabbit S376

b) Heparin precipitable reaction (HPF) in the plasma of 4 rabbits infected with T. (T.) brucei 427.

●———● Rabbit S358

O———O Rabbit S359

□———□ Rabbit S375

■———■ Rabbit S376

fibrinogen decreases. Only light precipitates were found 2-3 weeks after infection indicating that even high fibrinogen levels do not necessarily render fibrinogen insoluble in the cold. These findings are in agreement with the observations of Godal (1962) who examined the properties of human plasma following surgical operations.

3.1.6. Fibrin deposition in the tissues

Elevated fibrin-DP provides evidence for secondary fibrinolysis following activation of the coagulation system. In this situation one might expect that fibrin could be demonstrated in the circulation, particularly the microcirculation of various organs.

Three rabbits infected with T. (T.) brucei S42 (S219, S251 and S220) and 5 rabbits (S340, S341, S348, S349, S350) infected with strain 427, were killed between 4-5 weeks after infection. Frozen sections of tissue were then stained histochemically for fibrin.

No apparent fibrin deposits were detected in the brain, liver and heart. Small amounts were detected in the spleen although extravascular to the trabecular veins. The most marked deposits were seen in the lungs (Fig. 20) where alveolar cells were swollen and material staining for fibrin was apparent, again extravascularly in the alveolar tissue. This feature suggested a leakage of plasma proteins, including fibrinogen into the tissues where it had clotted.

There was no evidence, using this histochemical staining technique, of microthrombi formation within the blood vessels of any tissue examined.

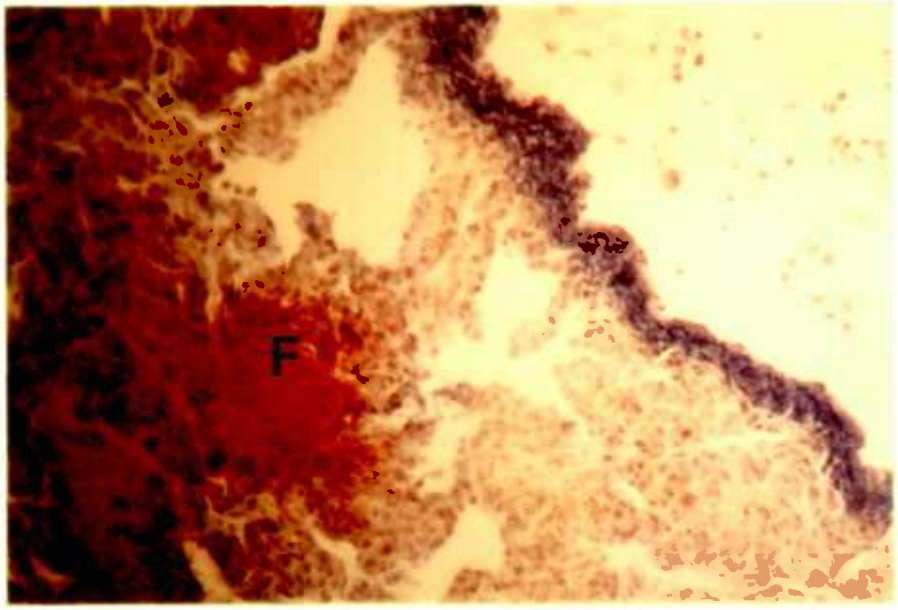


Fig. 20. Extravascular localisation of fibrin (F) in the lung of a rabbit on day 21 of infection with T. (T.) brucei 427. Original mag. 1200. Stained MSB for fibrin.

3.1.7. Urinary fibrinogen and FDP

Since a proteinuria occurs in trypanosome infections of rabbits (Goodwin and Guy, 1973; Itazi and Enyaru, 1973), it was decided to investigate whether fibrinogen and its derivatives could be detected in the urine. Positive results might be indicative of kidney damage occurring during infection.

a) Urinary fibrinogen

At no time during the infection was any clottable fibrinogen detected in the urine as measured by the method of Ratnoff and Menzie (1951).

b) Urinary FDP

Urine was collected the day before infecting with trypanosomes and at weekly intervals after infection. The TRCHII was then applied to urine concentrates. Fig. 21 shows the results for 5 rabbits infected with *T. (T.) brucei* 427 (rabbits S358, S359, S376, S388, S389). The FDP concentrations are expressed as total urinary FDP excreted within each 24 h collection period. Total urine volumes and FDP in µg/ml for each rabbit are given in Appendix 1.

FDP in infected rabbits appeared in the urine only late in the infection i.e. between the 6th and 7th week of the disease. The maximum FDP found to be excreted was 315 µg. In 2 control rabbits, S356 and S357, no FDP was detectable at any time during the 7 week observation period.

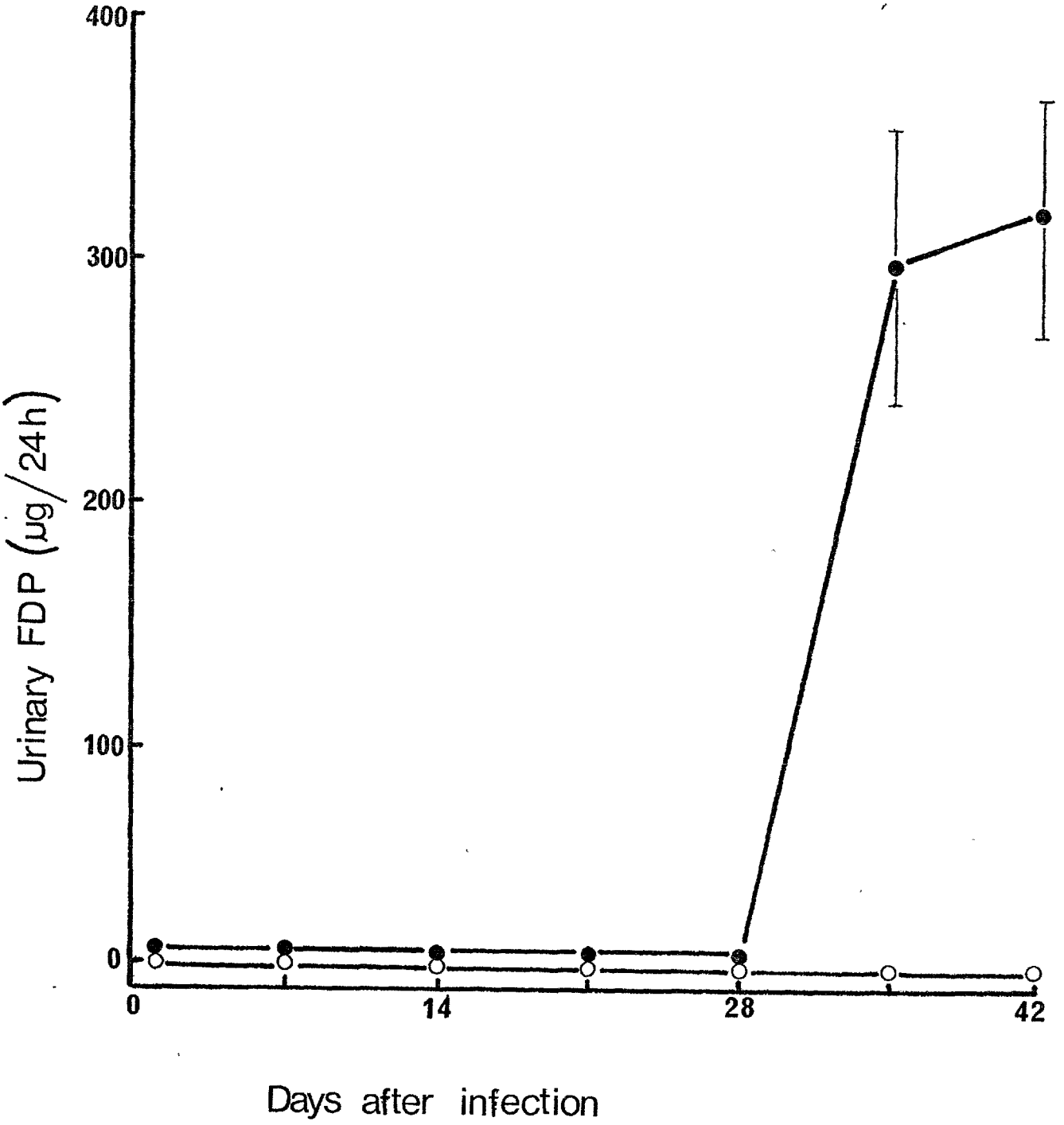


Fig. 21. Concentration of FDP in the urine of rabbits infected with T. (T.) brucei 427.

●———● Mean (± S.E.) for 5 infected rabbits.

O———O Result from 2 control non-infected rabbits.

c) Origin of urinary FDP

The time course of FDP in the urine of infected rabbits did not follow that in the serum. Since FDP appeared only late in the infection, it is possible that at this time glomerular damage allowed the passage of both 'early' and 'late' FDP into the urine.

Urinary FDP might arise from the breakdown of intra-glomerular fibrin. However as shown in the previous section (Section 3.1.5) there was no apparent evidence of fibrin deposition at any location in the kidney.

A third possibility is that FDP may arise from fibrinogen filtering through an abnormally permeable glomerular basement membrane late in infection, and undergoing proteolysis in the renal tract. It was decided to examine this possibility by investigating the fibrinolytic activity of rabbit urine.

Fresh urine was collected from 2 normal rabbits, S389 and S390, and centrifuged at 3,000 g for 10 min. Experimentation was carried out within 1 h of collection.

To 0.2 ml of purified rabbit fibrinogen (5 mg/ml or 2.5 mg/ml) was added 0.2 ml fresh urine. This was followed by incubation for either 1 or 2 h at 37°C. Two controls were included. The first comprised of 0.2 ml of the fibrinogen solution together with PBS (pH 7.3), and the second included the addition of 0.05 ml EACA (500 U/ml) to the fibrinogen/urine mixtures.

Fibrinogenolysis was measured by:-

i) presence or absence of clot formation after the addition of thrombin (5QU/ml)/$CaCl_2$. Protein estimation of any clot formed was made.

ii) characterisation of proteolysis products by means of immunoelectrophoresis.

It can be seen from Table VIX that after 1 h incubation only a small clot was formed from the fibrinogen solution incubated with rabbit S389 urine after the addition of thrombin/$CaCl_2$. This reaction completely disappeared after a further 1 h incubation period. At no time was it possible to obtain a clot from the fibrinogen solution incubated with rabbit S390 urine.

Protein determination of fibrin clots which did form (with rabbit S389 urine at 5.0 mg/ml and 2.5 mg/ml of fibrinogen) showed that in these samples a marked reduction had occurred in the initial fibrinogen level to approximately 22% of the original concentration.

There was no change in the fibrinogen concentration when the protein was incubated with buffer alone over the 1 h and 2 h periods.

Figs. 22 a and b show the immunoelectrophoretic characterisation of fibrinogen proteolysis products formed by the action of the urokinase from both rabbits. Also

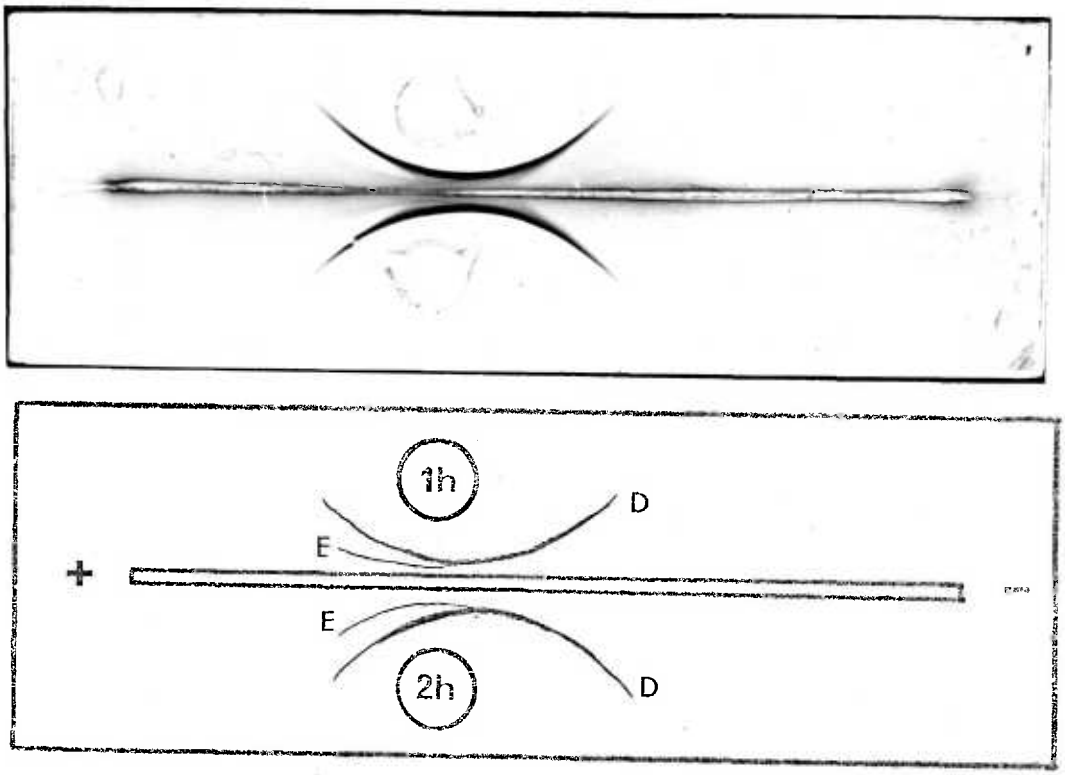
		Rabbit number			
		S389		S390	
Incubation time	Initial fibrinogen$_n$ conc mg/ml	Clot formation	fibrinogen$_n$ conc mg/ml	Clot formation	fibrinogen$_n$ conc mg/ml
1 h	5	small	N/T	-ve	N/M
2 h	5	-ve	N/M	-ve	N/M
1 h	2·5	small	1·39	-ve	N/M
2 h	2·5	-ve	2·23	-ve	N/M

TABLE IX. Effect of normal rabbit urine on homologous fibrinogen. The degree of clot formation and the amount of clottable fibrinogen remaining following incubation at 37°C.

N/T = not tested

N/M = not measured i.e. no clot was formed in these samples making it impossible to measure the clottable fibrinogen concentration remaining.

Rabbit S 390 (a)



Rabbit S 389 (b)

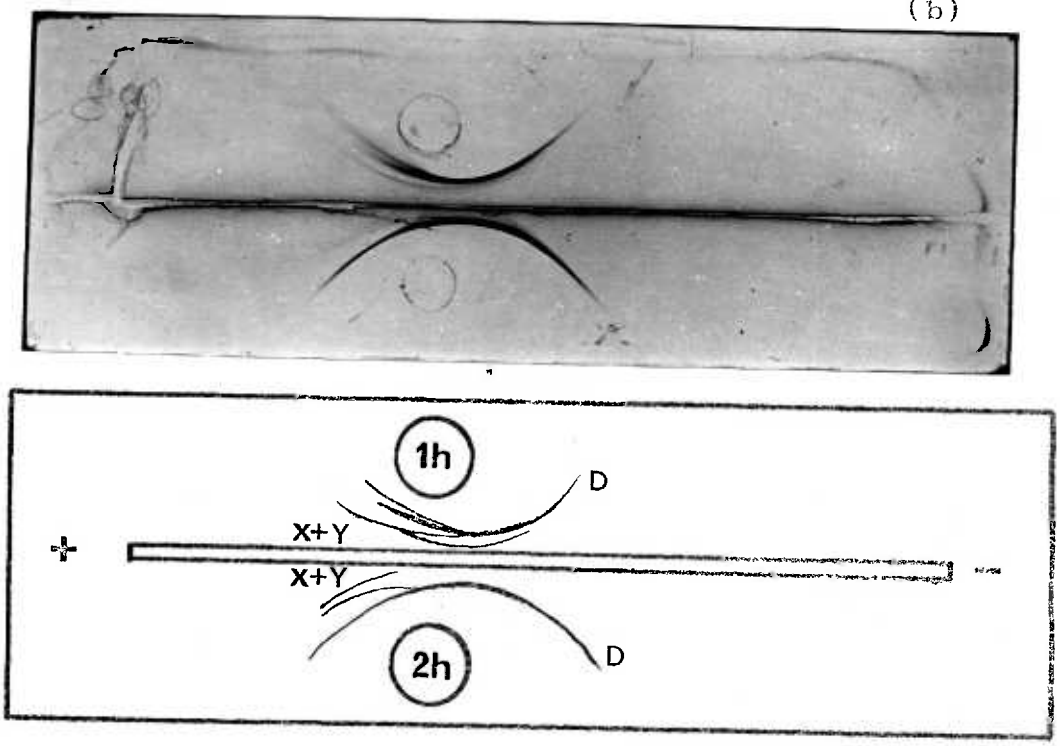


Fig. 22.

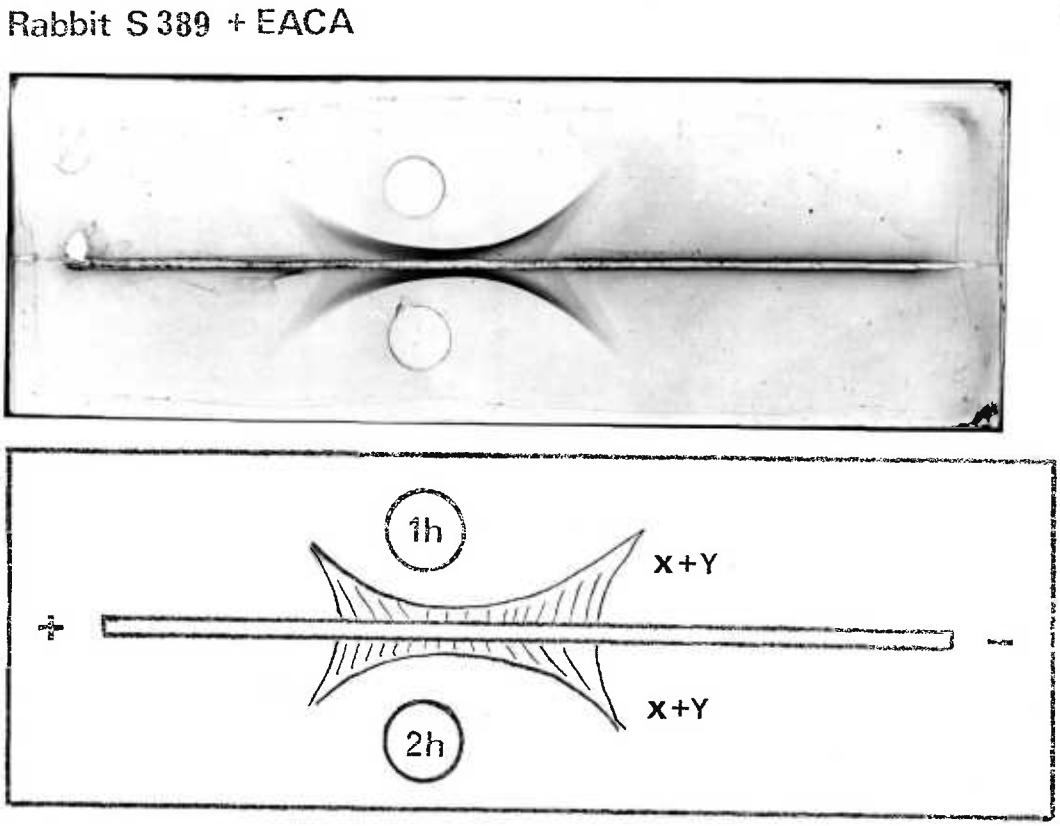


Fig. 23. Immunolectrophoresis of fibrinogen digests following incubation of fibrinogen with normal rabbit (S389) urine plus EACA for 1 h and 2 h at 37°C.

Fig. 23. (a) Immunoelectrophoresis of fibrinogen digests following incubation with normal rabbit (S390) urine for 1 h and 2 h at 37°C.

(b) As for 22 (a) except normal rabbit (S389) urine used.

shown are the results of the incubation with EACA (Fig. 23) and the fibrinogen control (Fig. 24). Immunoelectrophoresis gave 3 arcs of precipitation similar to those of fibrinogen, 'late' and 'early' FDP. Fragment D was formed in large concentrations as indicated by the density of precipitate. Fibrinogenolysis was slower with urine from rabbit S389 since Fragments X and Y appeared in both 1 and 2 h incubation periods. The assymetry of the Fragment D are with S389 urine after 1 h at 37°C probably indicates that a certain amount of fibrinogen was present. This is substantiated by the finding of clottable material within this sample.

An interesting observation was that inhibition of the plasminogen, (contained within the fibrinogen solution) by EACA, gave rise to a single much wider and shallower precipitin are demonstrating only partial proteolysis to Fragments X and Y (Fig. 23). The diffuse nature of this precipitin probably indicates a situation of antigen excess i.e. large amounts of these two fragments were present. These results would suggest that, in addition to plasminogen activation by urokinase, another mechanism for fibrinogenolysis was in operation. This might represent direct proteolytic activity by urokinase or the presence of distinct rabbit proteases in the urine.

3.1.8. FDP and fibrinogen in cattle trypanosomiasis

i) Cattle

Three Zebu cattle aged 1.5-2.0 years were infected with a T. (T.) brucei subgroup stabilate (EATRO 1386). A further three cattle of similar age were infected with a T. (N.) congolense stabilate (EATRO 505). A mixed T. (T.) brucei/T. (N.) congolense infection subsequently developed in one of the cattle, no. 417. It was assumed that the origin of the T. (T.) brucei was due to non-cyclical transmission whilst the animals were in close contact or possibly by accidental cross infection during examination. Blood for plasma and serum samples was collected by syringe from the jugular vein every 2-3 days during the infection. After 5 weeks following innoculation of parasites the T. (N.) congolense and mixed infection cattle were all showing some signs of the disease. However, the T. (T.) brucei infected cattle showed no symptoms during the infection. Full details are given by MacKenzie (1973).

ii) Serum FDP

The same proceedure as used for measuring rabbit FDP (Section 3.1.1.) was followed. It was found however, that all cattle sera tested gave suprisingly low FDP concentrations - less than 10 µg/ml. It was thus decided to test the specificity of the Wellcome anti-human fibrinogen serum for bovine fibrinogen by agar diffusion. The results are indicated in Table X. It can be seen that

Agar precipitation reaction with:	Anti-BF	Anti-HF	Anti-RF
BF	+ve	-ve	-ve
HF	-ve	+ve	+ve
RF	-ve	+ve	+ve

TABLE X. An immunological cross reaction study using a fibrinogen/antifibrinogen system.

Anti-BF = antiserum to bovine fibrinogen

Anti-HF = antiserum to human fibrinogen

Anti-RF = antiserum to rabbit fibrinogen

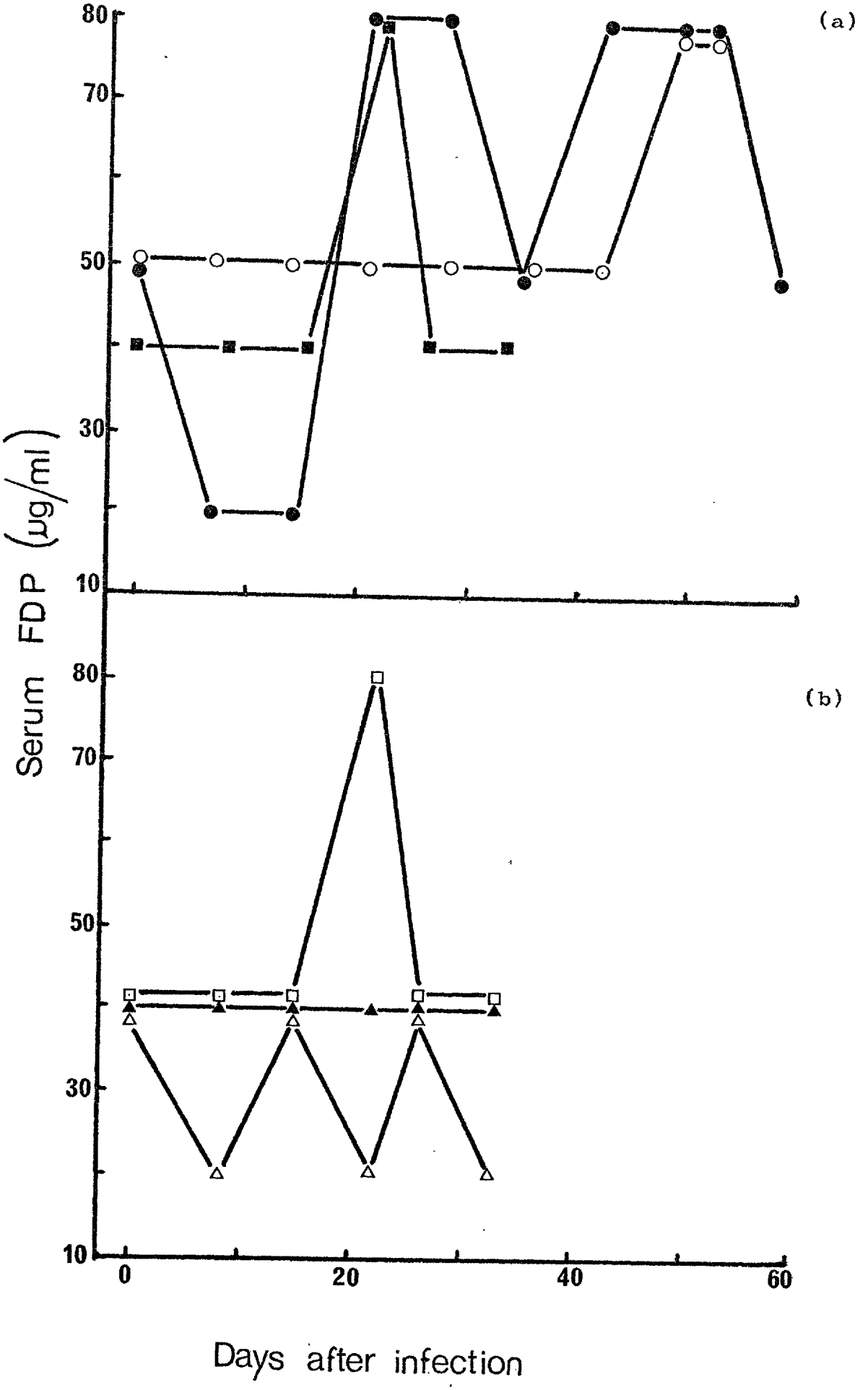


Fig. 25.

Fig. 24. Serum FDP concentrations in

(a) T. (T.) brucei sub-group infected cattle.

●——● 393

o——o 364

■——■ 349

(b) T. (N.) congolense (s88 & 411) and mixed T. brucei/congolense (417) infected cattle.

□——□ 388

▲——▲ 411

△——△ 417

bovine fibrinogen showed no immunological cross-reaction with either an antiserum to human or to rabbit fibrinogen. This explained the reason for the low levels of FDP measured in bovine serum.

The test was modified by using bovine fibrinogen sensitised sheep red cells as the indicator cells, and an antiserum to bovine fibrinogen (Miles - Seravac Ltd.). Before use the antiserum was diluted with citrate buffer (pH 6.4) to a concentration sufficient to agglutinate the sensitised cells (1 : 20,000). The standard used was a bovine fibrinogen solution 10 µg/ml.

Fig. 24 a and b shows serum FDP levels in the infected cattle. FDP levels were never elevated above 80 µg/ml during the infection in either group of cattle. Two animals in the T. (T.) brucei infected group, nos. 393 and 349, showed a peak FDP concentration about 3 weeks after infection which subsequently dropped only to increase again around the 7th week. This result was very similar to that of one of the T. (N.) congolense infected cattle, No. 388. Ox No. 411 showed no change in concentration of circulating FDP at any stage during infection.

iii) Plasma fibrinogen

Measurements of fibrinogen were carried out on plasma samples from two of the cattle, ox Nos. 393 and 364 collected over an 8 week period of a T. (T.) brucei infection. The results are shown in Fig. 25. After two

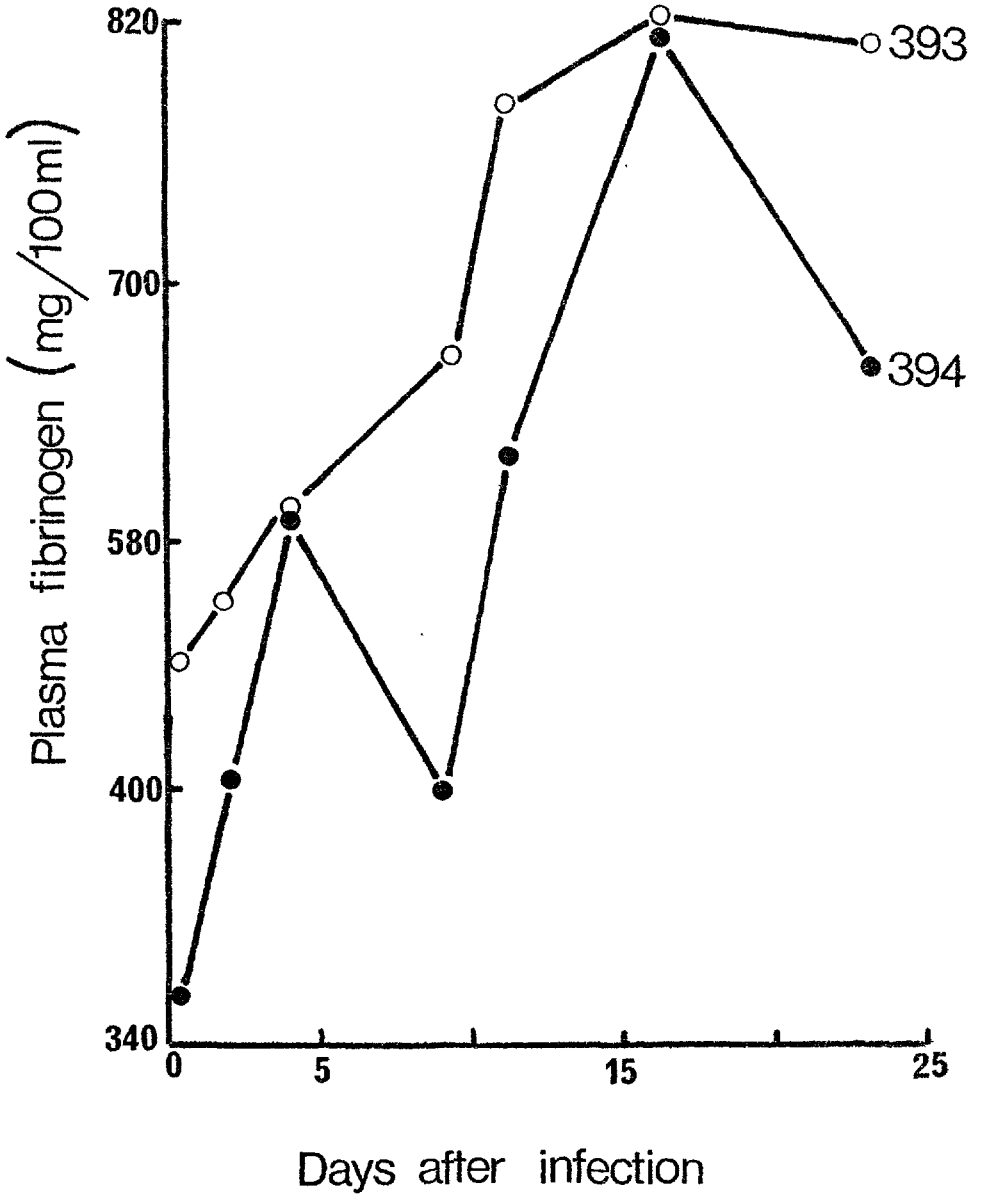


Fig. 25. Plasma fibrinogen concentration in two cattle infected with T. (T.) brucei sub-group trypanosomes.

days of infection there was a marked increase in fibrinogen in both animals and levels reached a peak between the 2nd and 3rd weeks of infection. A decrease in fibrinogen occurred during the following weeks, a result very similar to the picture in rabbits infected with a different strain of the same species.

3.1.9. FDP and fibrinogen in human trypanosomiasis

Sera and plasma were obtained from 13 active sleeping sickness patients and 3 patients undergoing follow-up examination at the EATRO hospital. Sera from 2 control patients with other diseases were also obtained. Case reports of the all above patients, compiled by Dr. A. R. MacKenzie, are given in Appendix 3.

i) Serum FDP concentrations

The results of FDP assays on serum from all patients, including the controls, are given in Table XI. By comparison with the 2 control patients, serum FDP would at first appear not to be elevated except for one patient SS.1026 who had an extremely high concentration of serum FDP (320 µg/ml). However, FDP levels in normal (European) sera have been well documented. Das *et al* (1967) demonstrated serum FDP in 95% of normal subjects, with a mean level of 4.9 ± 2.8 µg/ml. Thus the FDP levels in sleeping sickness patients were elevated, and it is probable that the high levels of FDP in the 2 control patients (M.O. and S.E.) were a result of their complications viz. bruising and tropical ulcer.

ii) Plasma fibrinogen

Only a limited number of plasma samples from infected patients and follow-up cases were available for measurement of the clottable fibrinogen content.

The results (Fig. 26) show that the concentration of fibrinogen appears to be related to the length of infection.

S.S. Patients		Controls	
Number	FDP	Name	FDP
1013	48	M.O.	80
1020	80	S.E.	80
1023	48		
1024	80		
1026	320		
1021	48		
1025	80		
1032	80		
1030	48		
1031	80		
956/FU	80		
944/FU	48		
859/FU	80		

TABLE XI. Serum FDP concentrations (µg/ml) found in active and follow-up (FU) cases of Rhodesian sleeping sickness. Concentrations found in two control patients are also shown. The estimated age of infection (see Appendix 3) increases from the top to the bottom of the table.

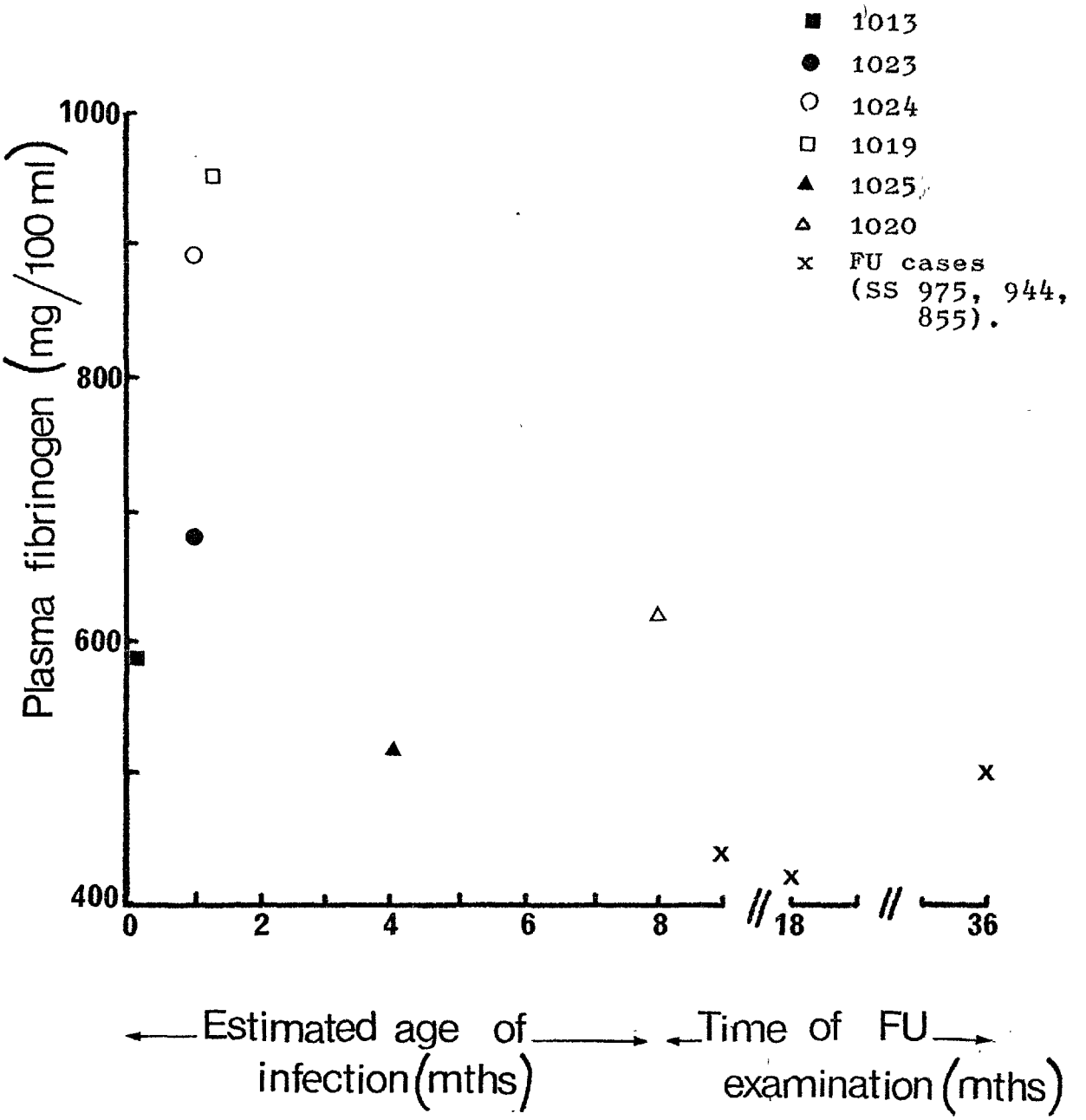


Fig. 26. Plasma fibrinogen concentration in human sleeping sickness patients.

0 - 8 months:- Plasma samples from patients with active infections.

9 - 36 months:- Plasma samples from patients undergoing follow-up (FU) examinations.

Infections estimated as more than 4 months duration were associated with higher fibrinogen levels. Unfortunately no control (noninfected) plasma was available for comparison, but it is widely documented that in the normal adult the fibrinogen concentration ranges from 190-330 mg/100 ml plasma with most values lying between 220-280 mg/100 ml (Petermann, 1960). The increase in fibrinogen in sleeping sickness patients is thus very marked - up to 400% above normal levels e.g. SS.1019. Follow-up patients had much lower levels than the patients with active trypanosomiasis.

3.2. Anti-F autoantibody

During attempts to measure the concentration of serum FDP, it was found that a factor was causing agglutination of the fibrinogen coated sheep red cells thereby interfering with the determination of the end point of the FDP assay. The work reported here is an investigation into the nature of this factor.

3.2.1. Anti-F autoantibody titre

It was found that when SRBC coated with fibrinogen were added to normal or infected rabbit serum from which all anti-species agglutinins had been removed (Section 2.7.4.) agglutination of the sensitised cells occurred. This agglutinating activity will be referred to as anti-F (anti-fibrin/fibrinogen) autoantibody since the component of the fibrinogen/fibrin system against which it is directed is not known.

Blood samples from rabbits infected with both strains of T. (T.) brucei were collected before, and at weekly intervals after infection. The results of anti-F activity for 15 rabbits shown in Fig. 27. A typical plate is shown in Fig. 28. Similar results were seen for 6 of the rabbits infected with strain 427 (S217, S267, S268, S269, S293 and S294) and 5 rabbits infected with strain 42 (S199, S198, S219, S220, S251). Low titres of between $^{1}/_{2}$ and $^{1}/_{4}$ in pre-infection sera rose to $^{1}/_{128}$ - $^{1}/_{256}$ 14-21 days after infection. Titres remained elevated throughout the period of study although slightly lower than maximal levels.

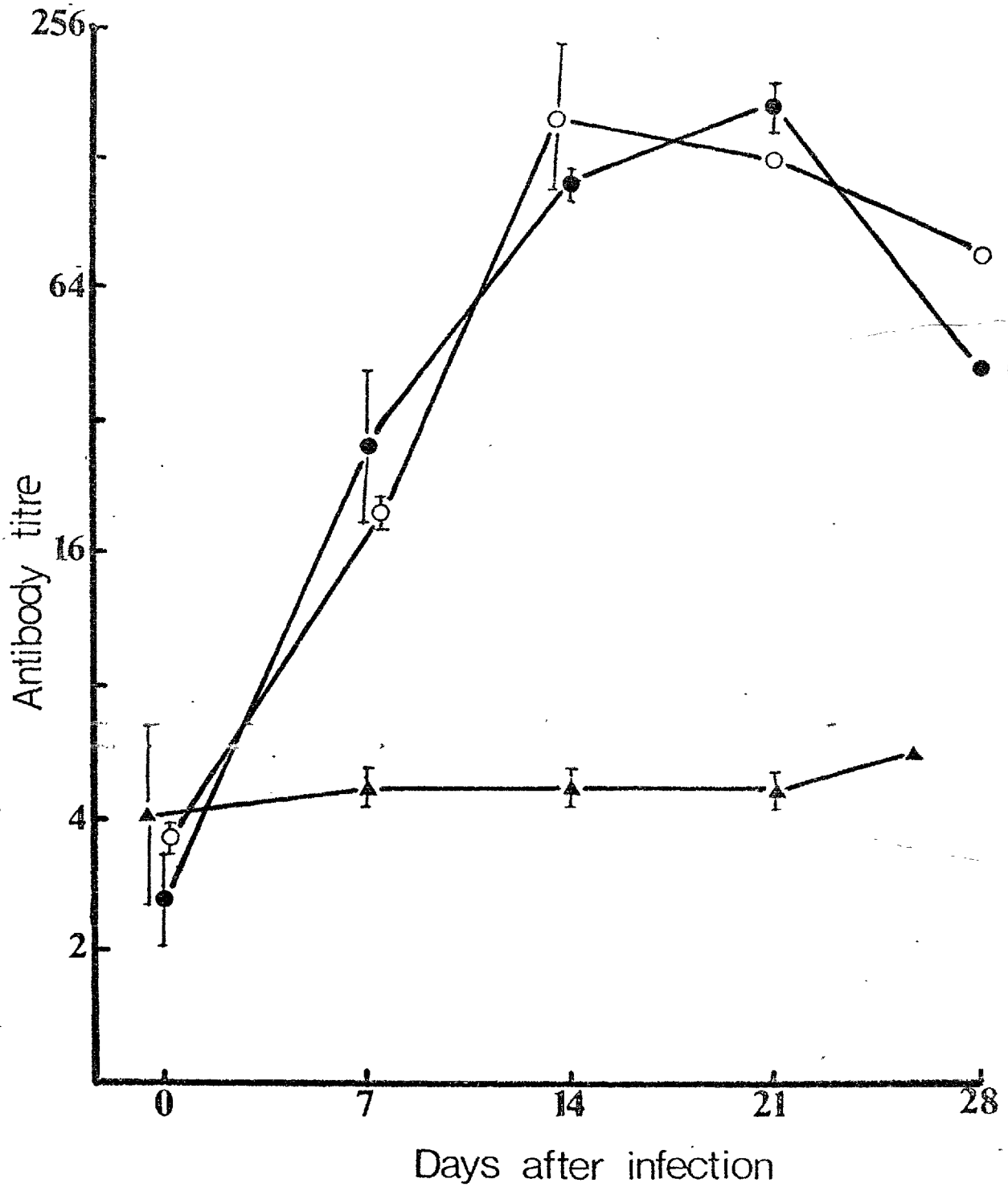


Fig. 27.

Fig. 27. Agglutinating anti-F autoantibody titres in the serum of rabbits infected with T. (T.) brucei.

●——● strain 427, mean (± S.E.) for 5 rabbits.

O——O strain S42, mean (± S.E.) for 6 rabbits.

▲——▲ non-infected controls, mean (± S.E.) for 4 rabbits.

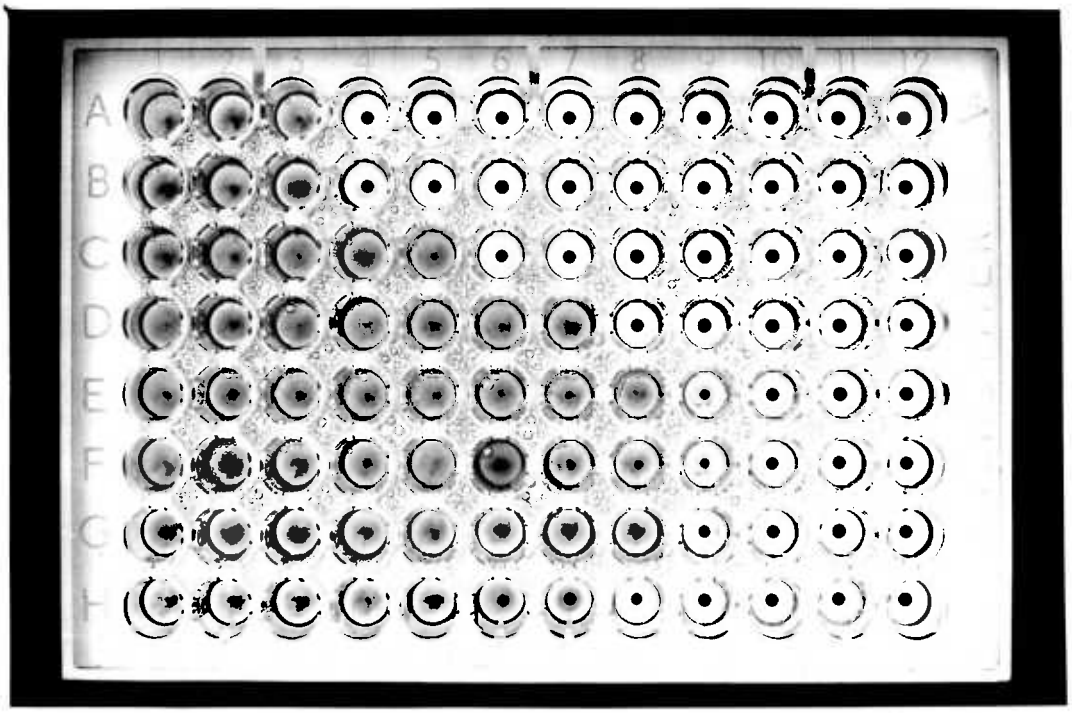


Fig. 28. Anti-F autoantibody titration in a "Microtitre" plate of rabbit S262 sera. Each row represents serial dilutions of sera collected at days 0, 7, 14, 21, 28, 35, 42 and 49 of an infection with T. (T.) brucei 427. Neat serum in well 1 and a $^{1}/_{2048}$ dilution of serum in well 12.

In a group of control rabbits (S218, S270, S295 and S296), the titres did not vary significantly throughout a 4 week period. Titres for individual rabbits are given in Appendix 1.

No natural agglutinins to SRBC were found in the human, bovine or rabbit fibrinogen preparations.

3.2.2. Persistence of anti-F autoantibody following chemotherapy

Two rabbits infected with T. (T.) brucei 427 (S399 and S400) and 2 non-infected control rabbits were given Mel-B (3.6 mg/kg) I.V. on day 14 following infection. Agglutinating anti-F activity in the serum was measured prior to treatment, and weekly for 5 weeks after treatment.

The results, Fig. 29, show a gradual reduction of anti-F autoantibody titre until, on the 25th day after curing, titres were only slightly higher than control levels.

Administration of Mel-B to non-infected rabbits had no effect on the existing autoantibody titres.

The results indicate that Mel-B has no direct effect on the antibody producing cells and that the imbalance of the immunological system caused by the infection, takes over 5 weeks to return to normal.

3.2.3. Autoimmune nature of anti-F antibody

Since the antigen used in the above tests was homologous rabbit fibrinogen, it was necessary to show that

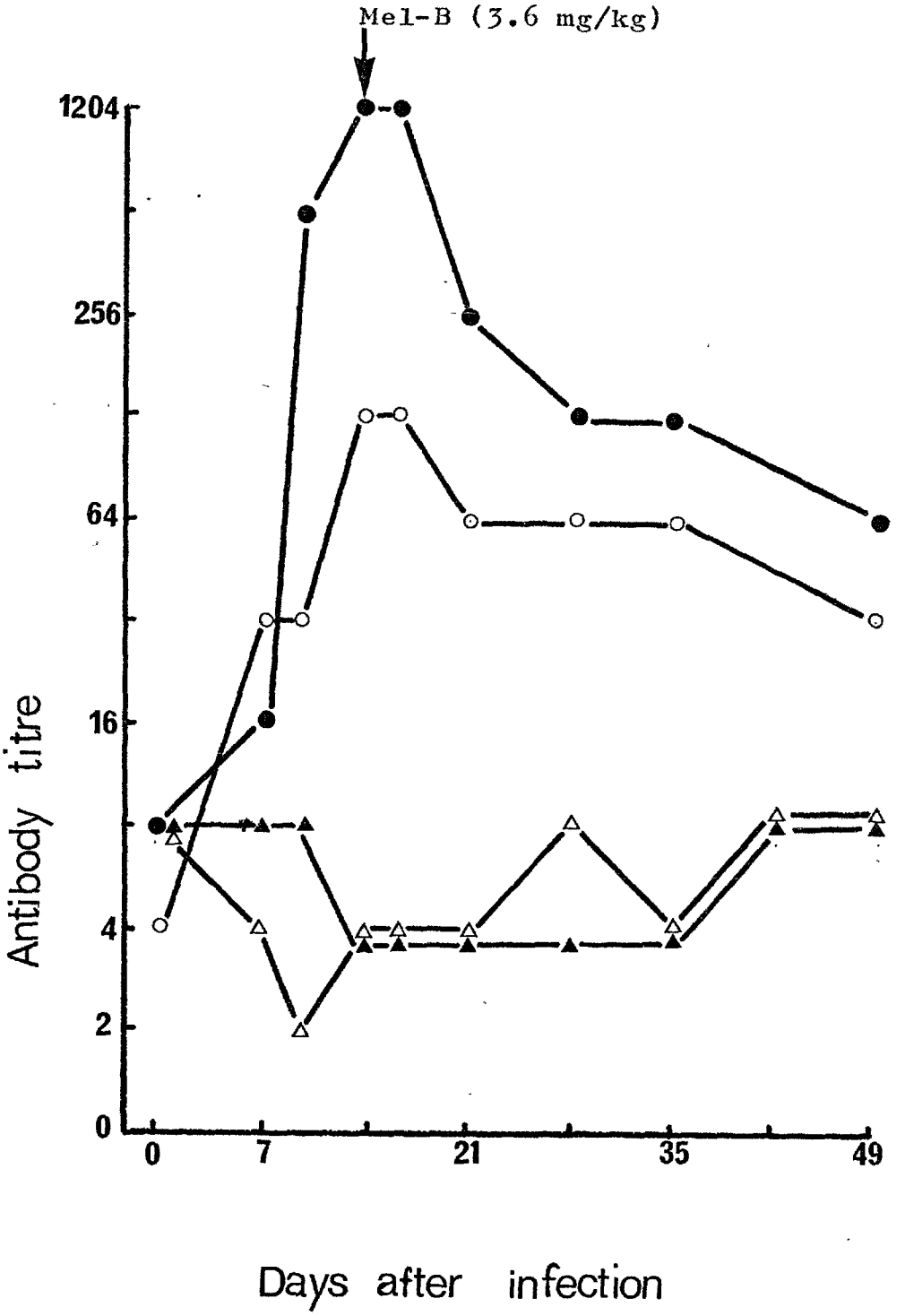


Fig. 29. Effect of chemotherapeutic treatment (Mel-B) on the serum anti-F autoantibody titres.

●———● S400 rabbits infected with
o———o S399 T. (T.) brucei 427

▲———▲ S297 control non-infected
△———△ S298 rabbits.

the antibody was truly auto-directed.

Serum from rabbit S220 was therefore tested against A SRBC suspension comprising cells sensitised with fibrinogen from the same rabbit. Table XII is a comparison of titres obtained with normal heterologous rabbit fibrinogen and both normal and infected autologous fibrinogen. The results show that the antibody reacts equally well with either antigen and therefore a true autoantibody.

3.2.4. Species specificity

The anti-F autoantibody found in two rabbits, rabbit S217 infected with strain 427 and rabbit S251 infected with strain S42, gave similar titres when titrated against either rabbit fibrinogen sensitised SRBC or human fibrinogen sensitised SRBC as shown in Table XIII. This indicates that SRBC sensitised with either rabbit or human fibrinogen can be used to detect and measure antibody activity.

3.2.5. Absorption studies

i) Sheep red blood cells (SRBC)

Absorption with 2 volumes of packed SRBC removed all antispecies agglutinins from the serum even when high titres were present, as was often seen in infected sera. However, the agglutinating titre to cells sensitised with either rabbit or human fibrinogen remained the same.

	Days after infection					
	0	7	14	21	28	35
Homologous F.	2	32	512	256	32	32
Autologous F.	2	32	512	256	32	32

TABLE XII. Comparison of anti-F agglutinating auto-antibody titres using SRBC sensitised with homologous or autologous rabbit fibrinogen. Sera from rabbit S220 taken at weekly intervals during infection with *T. (T.) brucei* 427.

F = fibrinogen

	Days after infection					
Fibrinogen	0	7	14	21	28	35
Human	4	16	64	256	32	32
Rabbit	4	16	64	256	32	32

(a) Rabbit S217 infected with T. (T.) brucei 427

	Days after infection				
Fibrinogen	0	7	14	21	28
Human	8	16	512	512	256
Rabbit	8	16	512	512	256

(b) Rabbit S251 infected with T. (T.) brucei S42

TABLE XIII. Comparison of anti-F agglutinating antibody titres of infected sera using sheep red blood cells sensitised with either rabbit or human fibrinogen as indicator cells.

ii) Absorption with rabbit, human and bovine sensitised SRBC

Cells sensitised with any of the 3 fibrinogen molecules caused a reduction in the anti-F titre of serum from rabbit S220 infected with T. (T.) brucei strain S42 for 20 days. The reductions in titre were from 512 to 32 (Table XIV). These results indicate that the antibody is not specific for rabbit fibrinogen.

iii) Rabbit plasma sensitised red cells (RP-SRBC)

On absorption with RP-SRBC, a 3-fold dilution difference in titre was noted (Table XIV) i.e. the absorptive capacity of the cells was less than above (ii). A possible explanation for this observation is that complete absorption did not occur because of competition with other plasma proteins, during the sensitisation process, for sites normally occupied by fibrinogen. This would result in less than 100% sensitisation of the cells with fibrinogen.

iv) Sonicated rabbit fibrin

Table XV shows the results of absorbing two infected sera with sonicated rabbit fibrin. In all cases there was a reduction of the titre suggesting that the determinant(s) responsible for the absorption was found not only on fibrinogen molecules but also on fibrin.

3.2.6. Macroglobulin nature of the autoantibody

In order to determine whether the anti-F autoantibody was IgM in nature and similar to those described

Day	Original titre	Reciprocal of antibody titre after absorption with sensitised SRBC			
		HF	BF	RF	RP
0	2	0	0	0	0
20	512	32	32	32	64

TABLE XIV. Effect of absorption with fibrinogen sensitised sheep red blood cells on serum anti-F autoantibody titres. Rabbit S220 infected with T. (T.) brucei S42.

HF = Human fibrinogen

BF = Bovid fibrinogen

RF = Rabbit fibrinogen

RP = Rabbit plasma

SERUM	S220/D0	S220/D21	S268/D0	S268/D14
Control	10	80	10	40
Absorbed	0	20	0	0
$\log_2$ Unit Reduction	1	2	1	3

TABLE XV. Effect of absorption of infected sera with sonicated rabbit fibrin, using serum from Rabbits S220 and S268, pre-infection serum (D0) and days 21 and 14 after infection respectively. Doubling dilutions of serum beginning at a dilution of $^1/_{10}$.

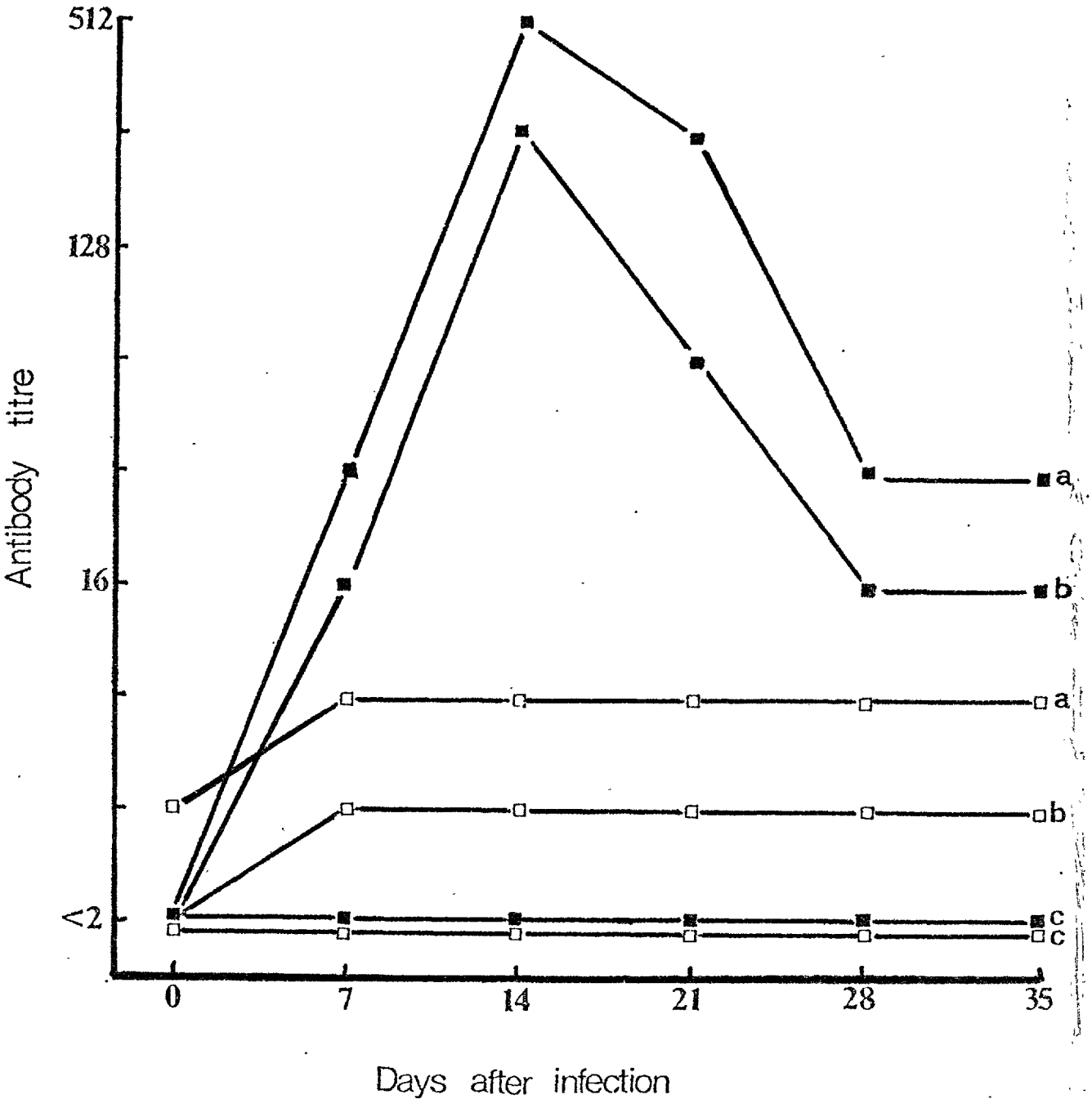


Fig. 30. Effect of 2-mercaptoethanol reduction and alkylation on serum anti-F titres in two rabbits.

■———■ Rabbit S220 infected with T. (T.) brucei 427

□———□ Rabbit S270 non-infected control.

a = untreated
b = alkylation only
c = 2-ME and alkylation

Day	S220	S270	S220	S270
0	<2	<2	<2	2
7	<2	<2	16	4
14	<2	<2	256	4
21	<2	<2	64	4
28	<2	<2	16	4
35	<2	<2	16	4

TABLE XVI. Effect of 2-mercaptoethanol (2ME) reduction and alkylation on the anti-F antibody titres of two rabbits, one S220 infected with *T. (T.) brucei* S42, and S270, a control non-infected rabbit.

by Mackenzie and Boreham (1974), gel filtration, heat lability and 2-ME reduction and alkylation experments were carried out.

i) Heat lability

Heating normal and infected sera to 65°C for 30 min destroyed all anti-F activity.

ii) Sensitivity to reduction by 2-ME

Weekly serum samples were taken from control rabbit S270 and from rabbit S220 infected with T. (T.) brucei S42.

The results of mild reduction and alkylation of the sera of these two rabbits are shown in Fig. 30 and Table XVI.

All anti-F activity was lost by 2-ME treatment, whereas alkylation only caused only a slight reduction of the titre, strongly suggesting that the autoantibody is IgM.

iii) Gel-filtration

Four rabbits (S383-S386) were infected with T. (T.) brucei 427 and plasma samples collected in EDTA before infection and at weekly intervals up to the 4th week of infection. The rabbit plasmas were then bulked (in equal quantities from each rabbit) in order to give a combined plasma sample for each of the days to be tested.

Results of separation of rabbit plasma on Sephadex G200 are shown in Figs. 31 to 35. The figures show the total protein distribution for each serum sample and the

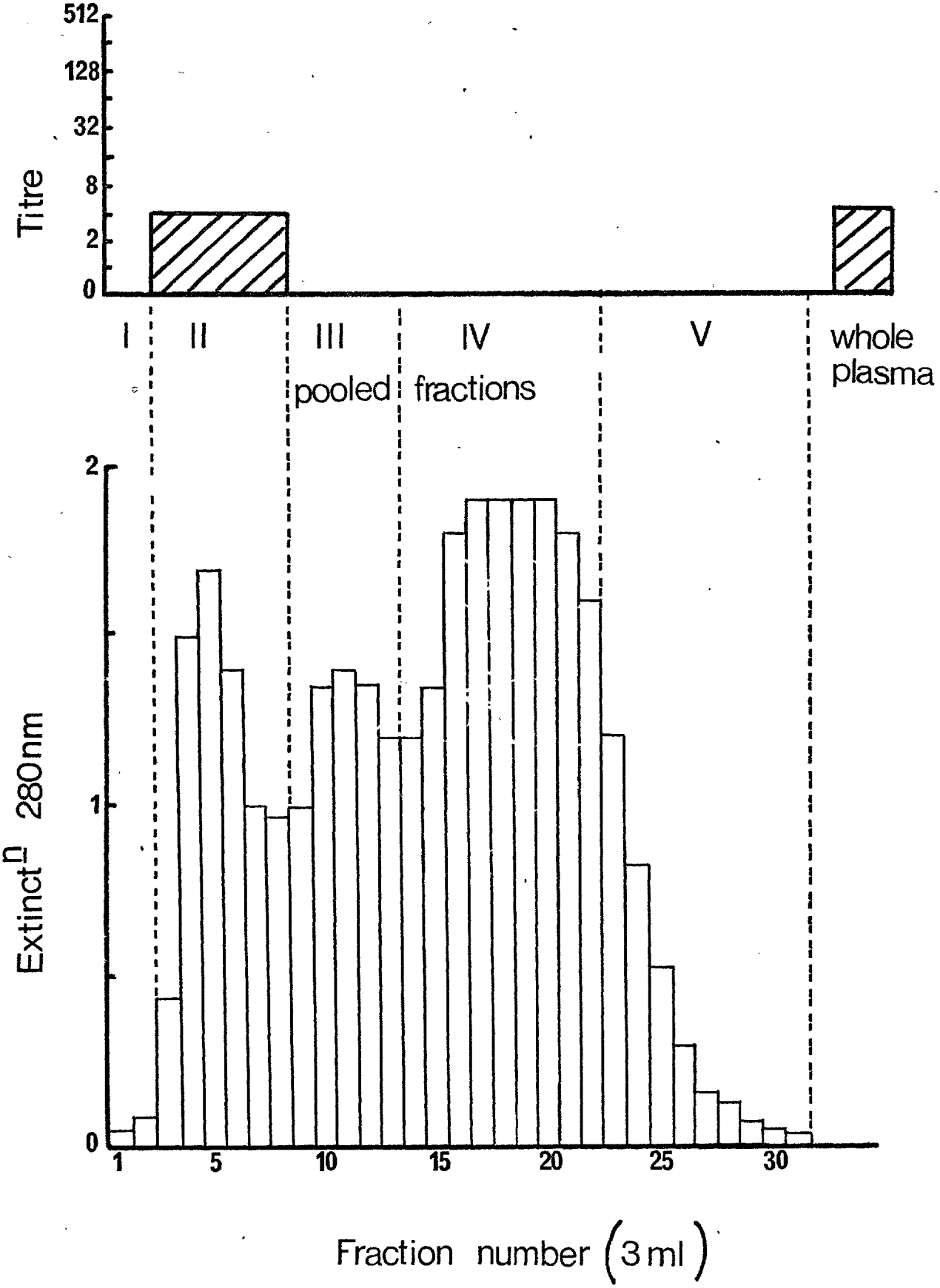

512
128
32
8
2
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Titre
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II
III
IV
V
whole plasma
pooled fractions
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Extinct^{n} 280nm
1
5
10
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20
25
30
Fraction number (3ml)

Fig. 31.

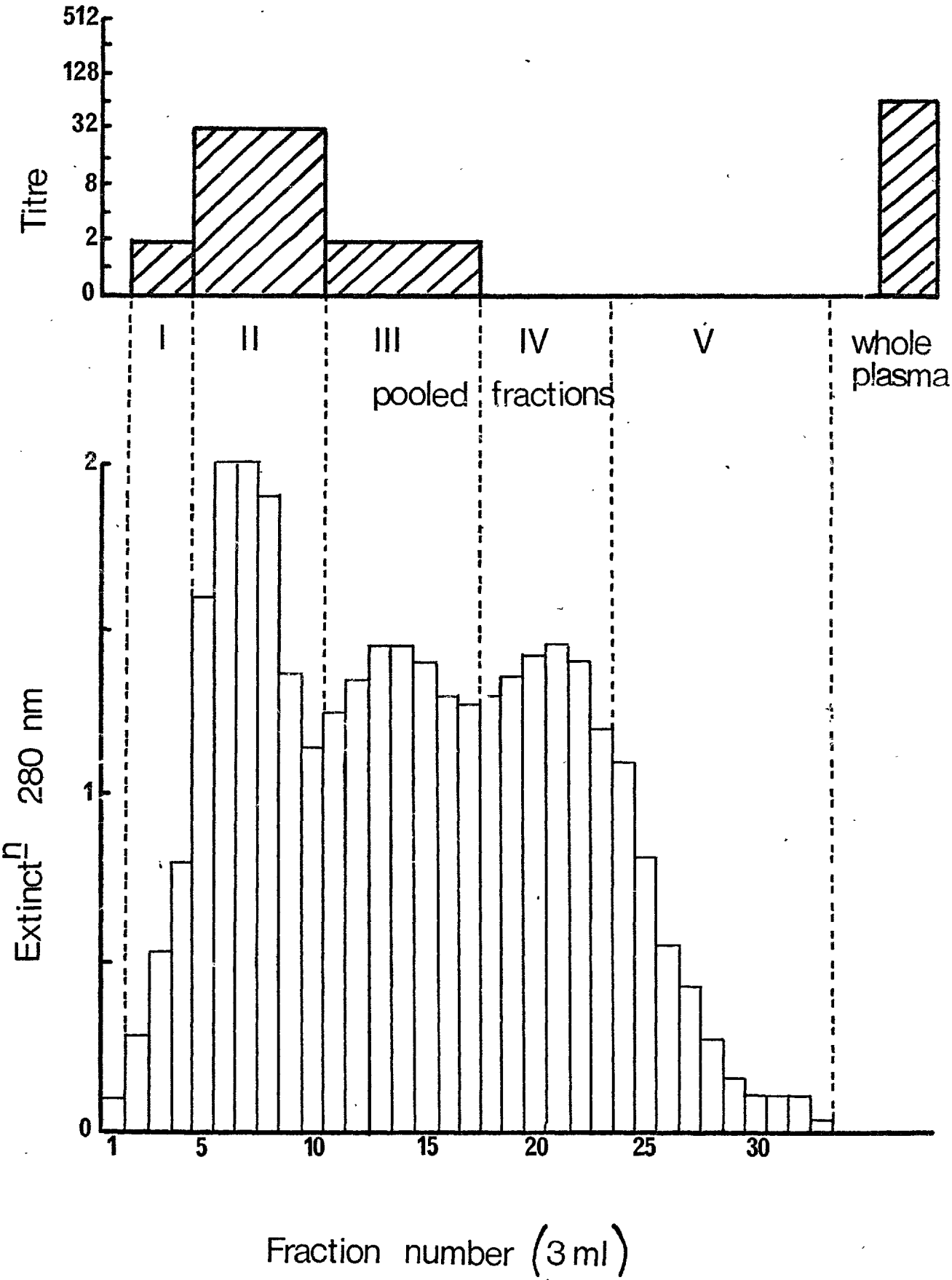

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Fraction number (3 ml)

Fig. 32.

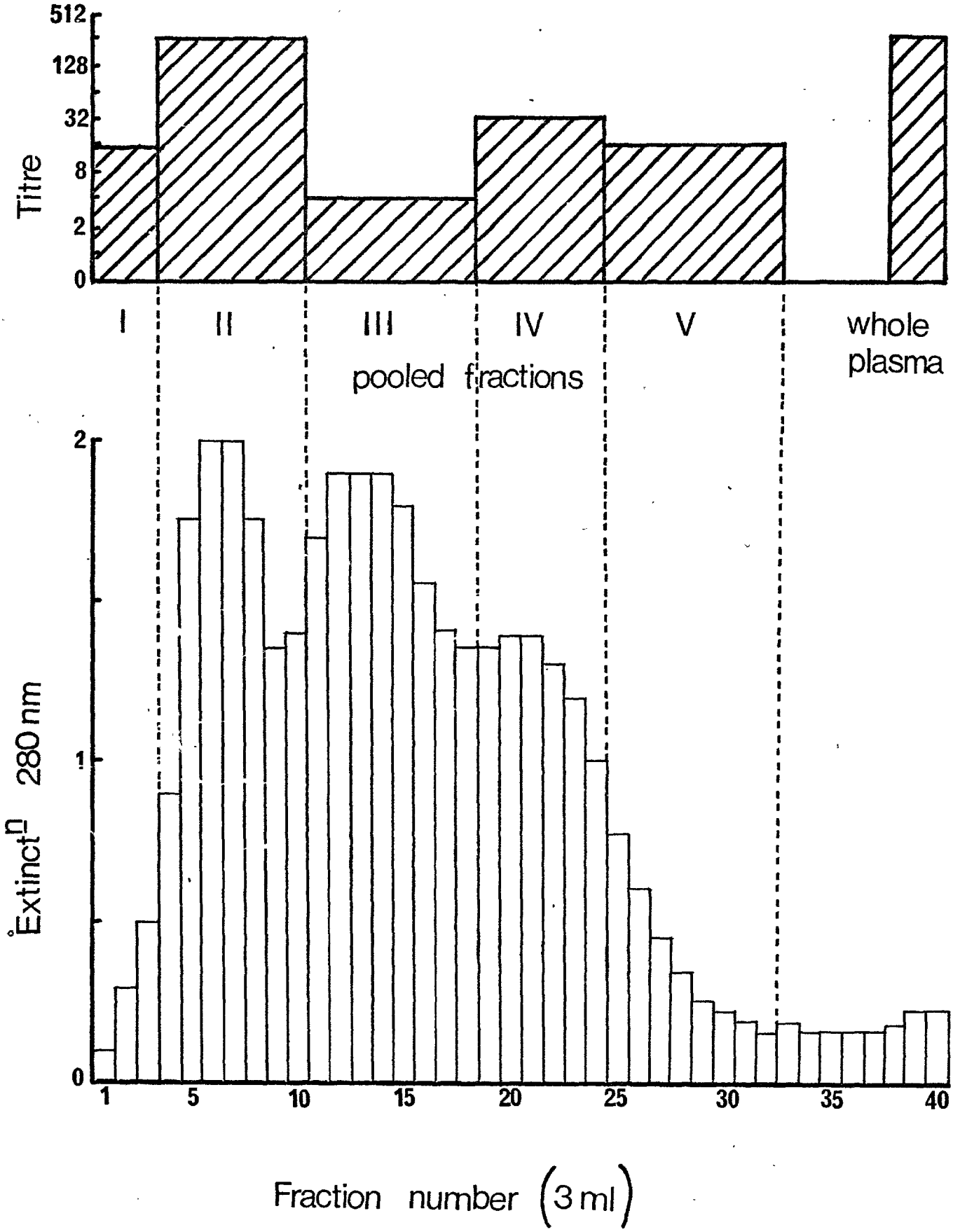

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pooled fractions
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Fraction number (3 ml)

Fig. 33.

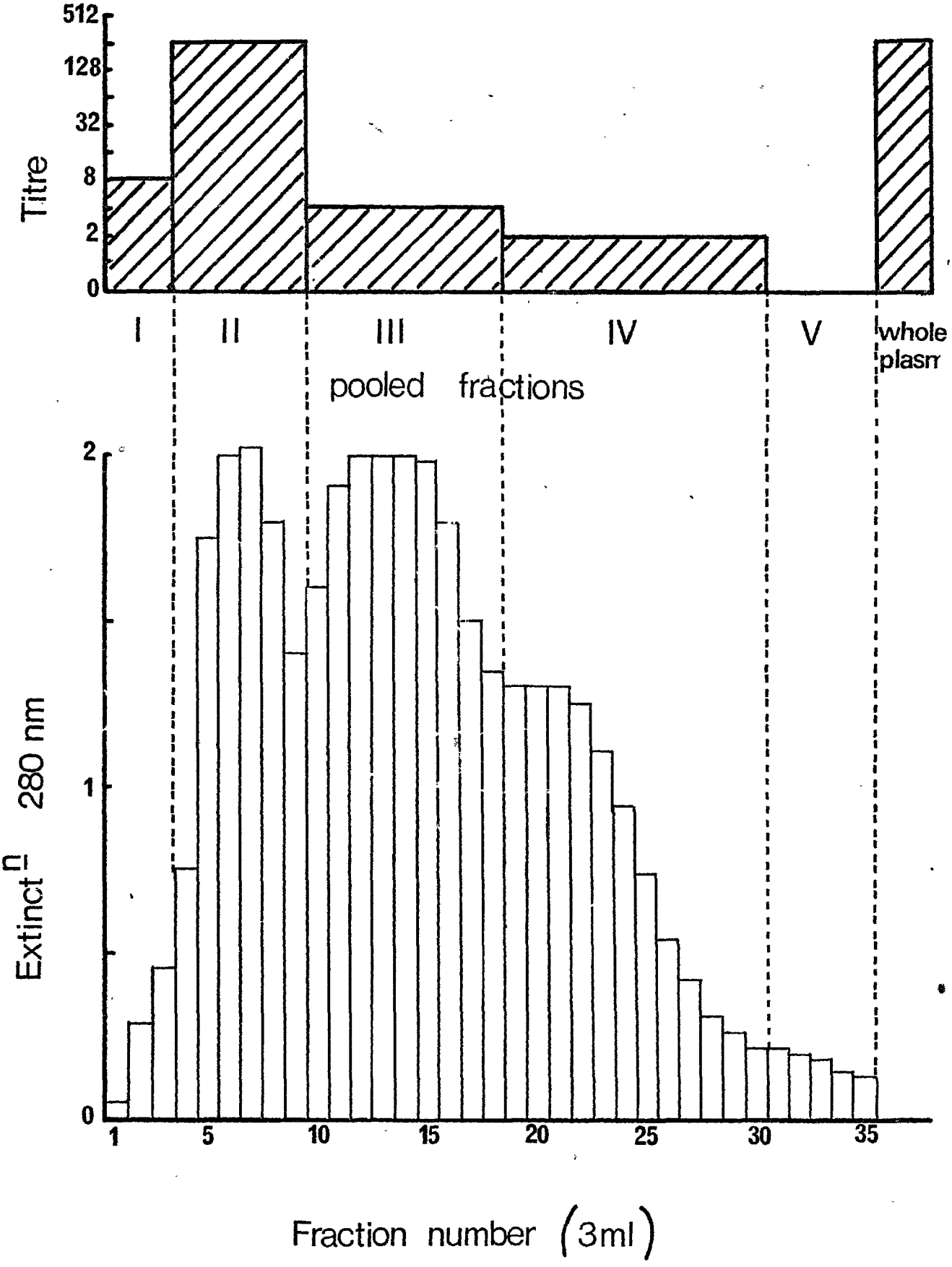

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Fraction number (3ml)

Fig. 34.

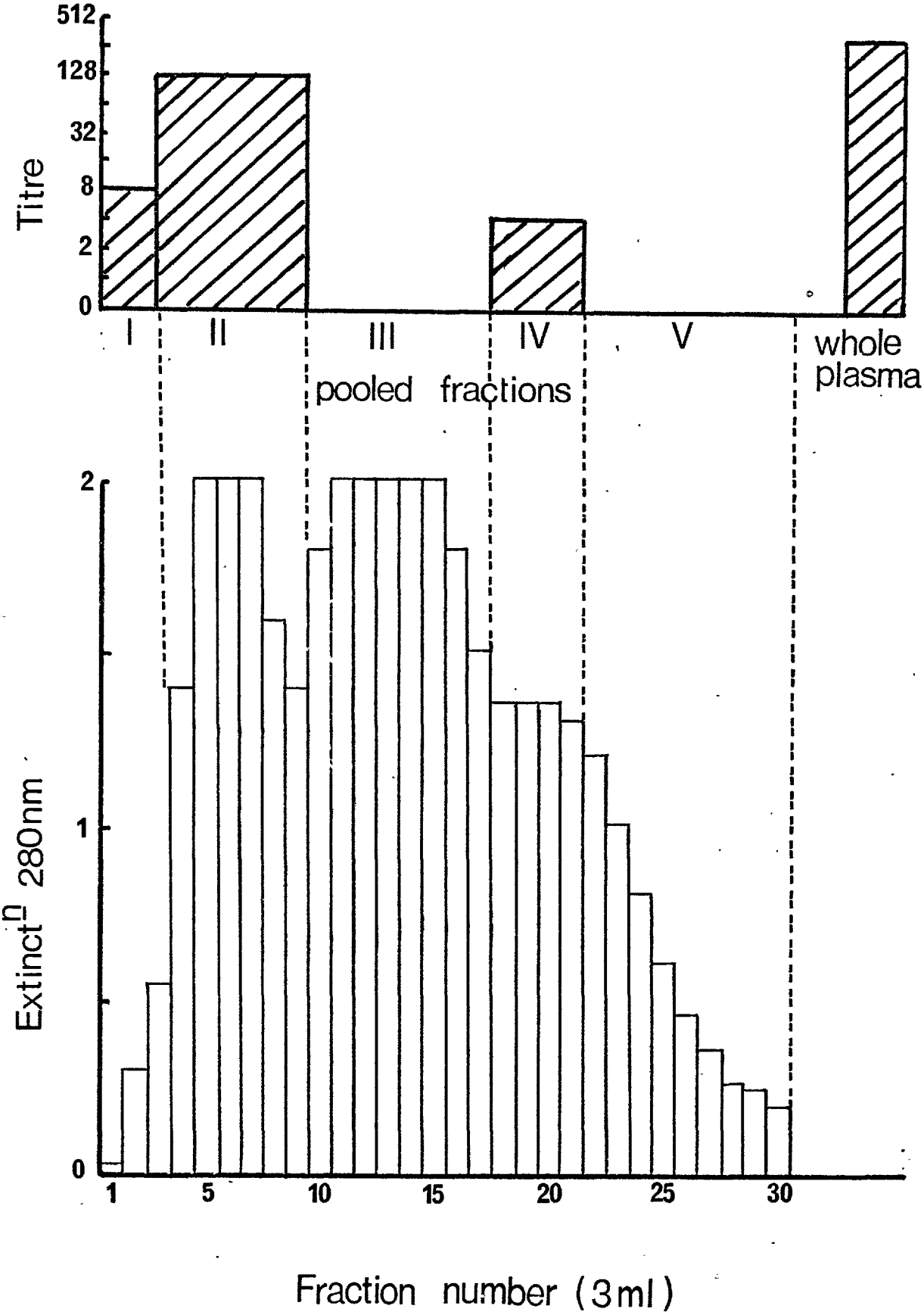

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whole plasma
pooled fractions
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Extinctn 280nm
1
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10
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20
25
30
Fraction number (3ml)

Fig. 35.

Fig. 31 - 35. Separation of anti-F activity in the bulked plasma collected from rabbits on Sephadex G200.

Fig. 31. collected prior to infection with trypanosomes.

Fig. 32. day 7 plasma following infection with T. (T.) brucei 427.

Fig. 33. day 14 plasma.

Fig. 34. day 21 plasma.

Fig. 35. day 28 plasma.

Histograms represent autoantibody titres.

anti-F activity in pooled fractions.

In control plasma (day 0) and the majority of infected plasma, anti-F activity lies in the high molecular weight macroglobulin fraction. However, in addition anti-F activity was also found in fraction III (day 7); fractions III, IV and V (day 14); fractions III and IV (day 21) and fraction IV (day 28). Thus smaller peaks of activity on the low molecular side of the γ-globulin peak and also on the albumin peak. This situation may have several explanations. Fraction III contains IgG activity to trypanosomal antigen. It could also represent 7s IgM with anti-F specificity. In fact, monomeric IgM has been shown to occur in rabbits with trypanosomiasis by Frommel et al (1970) but its specificity has not been studied.

One explanation for the finding of activity in the albumin peak is that 7s IgM might bind with albumin to form a complex and in doing so become in some way retarded in the gel. Complexing of monoclonal IgM with albumin has recently been described by Harboe and Folling (1974).

3.2.7. Anti-F autoantibody in cattle trypanosomiasis

i) Anti-F titres

Sera collected from cattle infected with T. (T.) brucei sub-group trypanosomes or T. (N.) congolense were tested for agglutinating anti-F antibody, using in the test HF-SRBC as the sensitised cell suspension.

The autoimmune nature of cattle anti-F antibody could not be tested due to lack of material. However from the previous studies on rabbit anti-F (loc cit) and from those of Mackenzie and Boreham (1974) it is very likely that cattle anti-F is also an autoantibody. It will therefore be termed an autoantibody in this section and also the following section investigating human anti-F titres in sleeping sickness.

The results (Figs. 36 and 37) for both groups are very similar to those obtained with infected rabbit sera. Pre-infection titres were between $^{1}/8$ and $^{1}/64$. One of the cattle, No. 349, showed an extremely high anti-F titre ($^{1}/356$) before injection of trypanosomes possibly due to an intercurrent infection. Peak titres in both T. (T.) brucei and T. (N.) congolense infected cattle were found to occur 2-3 weeks after infection, with a gradual decline over the following weeks but still remaining elevated above control non-infected titres.

ii) Heat inactivation of sera

To investigate whether the anti-F antibody was an IgM immunoglobulin as in rabbit trypanosomiasis, sera

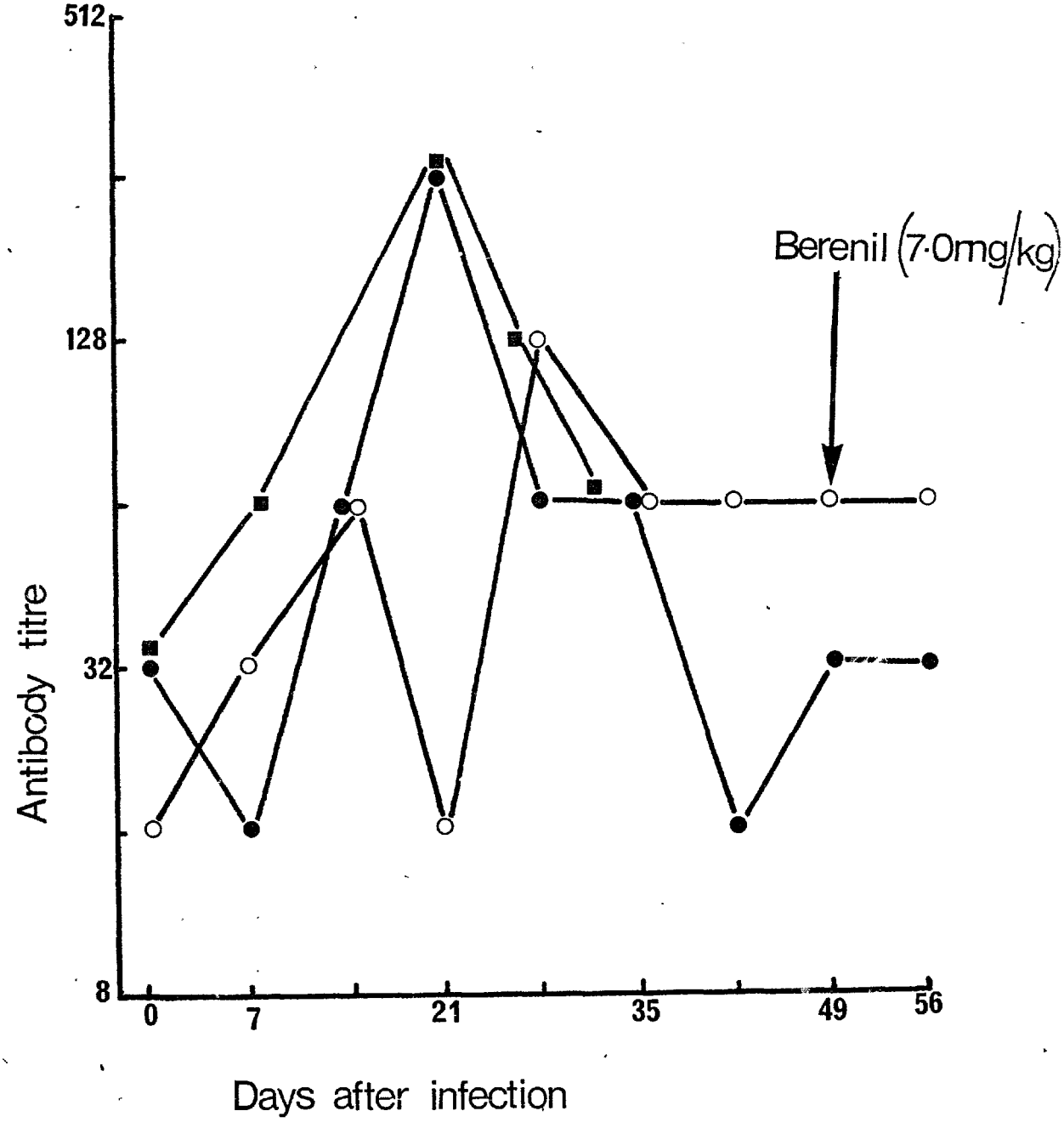


Fig. 36. Anti-F titre in 3 cattle infected with T. (T.) brucei sub-group trypanosomes.

●——● 364

o——o 393

x——x 349

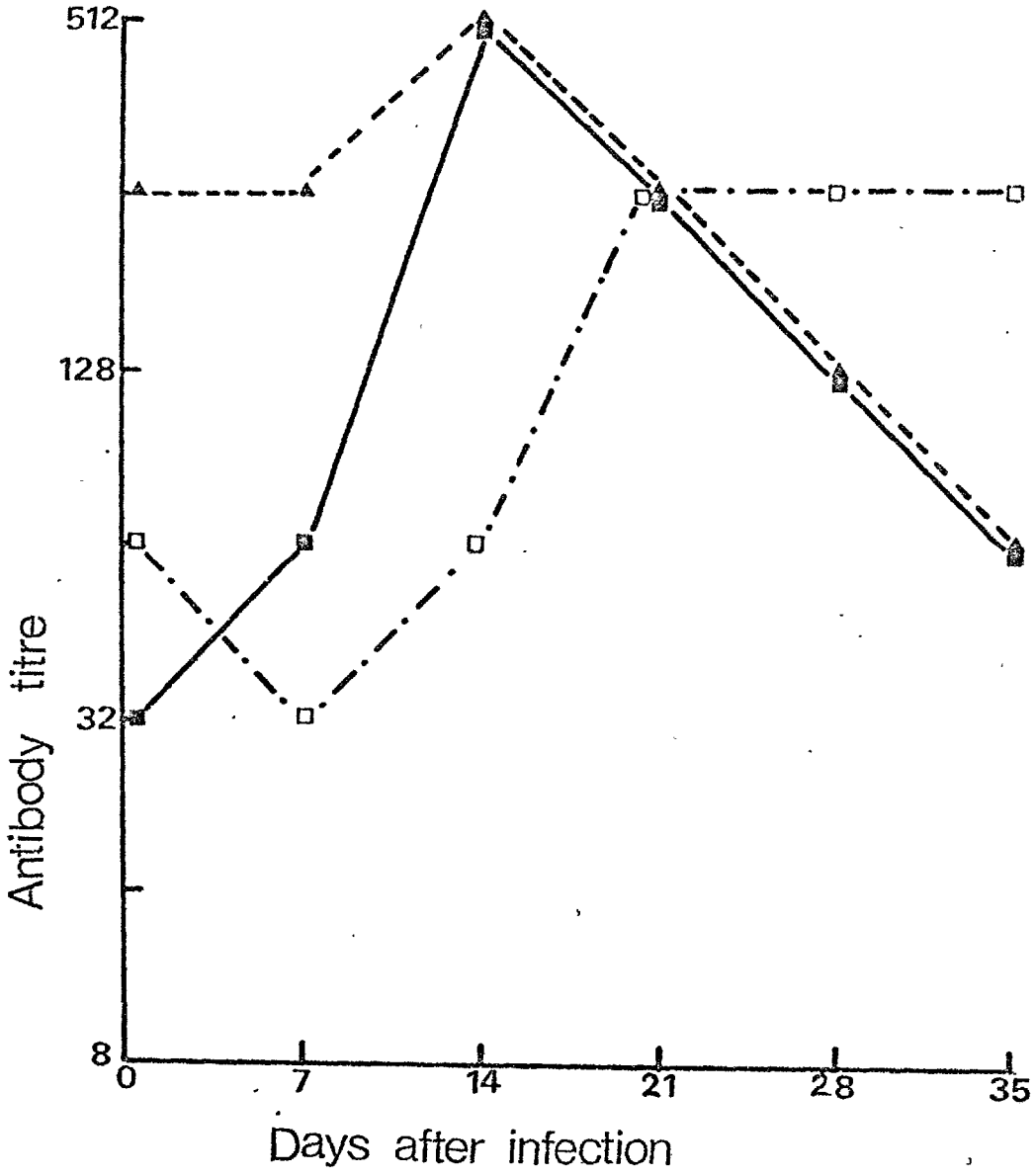


Fig. 37. Anti-F titres in 3 infected cattle:

□——□ 411 infected with T. (N.) congolense.

▲——▲ 388

△——△ 417 infected with T. brucei/congolense.

(a)

	Days after infection			
	0	14	21	35
Unheated	32	64	256	64
Heated	2	8	16	8

(b)

	Days after infection			
	0	15	26	33
Unheated	32	64	256	256
Heated	2	8	32	32

TABLE XVIII. Effect of heating cattle sera at 65°C for 30 min. on the anti-F titre.

(a) Ox no. 364 infected with T. (T.) brucei sub-group.

(b) Ox no. 417 infected with a mixed T. brucei/congolense.

from cattle Nos. 364, 349 and 417 were heated at 65°C for 30 min.

Sera from ox No. 349 gelled on heating probably due to the presence of pyroglobulin and was therefore not suitable for testing.

The results of heating sera from ox Nos. 364 and 417 on anti-F titres is shown in Table XVIII. There was a 3-4 fold decrease in antibody titre indicating its heat lability and suggesting it is possibly IgM in nature.

3.2.8. Anti-F autoantibody in human trypanosomiasis

i) Anti-F titres

Fig. 38 shows that the highest anti-F antibody titres were found in patients with active sleeping sickness.

Only one follow-up (FU) patient (SS.939) had a high anti-F titre of $^{1}/128$. A titre of between $^{1}/4$ to $^{1}/8$ was more usual. Control patients had very low levels of anti-F and none were found to have titres $^{1}/4$.

ii) 2-ME reduction and alkylation

It was found impossible to inactivate and test sera by heating since all samples, apart from the controls and sera from long term FU cases, gelled at 65°C.

Therefore, to examine the nature of anti-F, reduction of the sera was carried out using the 2-ME method.

Table XIX indicates that the anti-F antibody was of the IgM class, since titres were reduced following 2-ME treatment with the exception of SS.1030 where the titre remained the same. Alkylation only, as with rabbit sera, had little effect on the original titre.

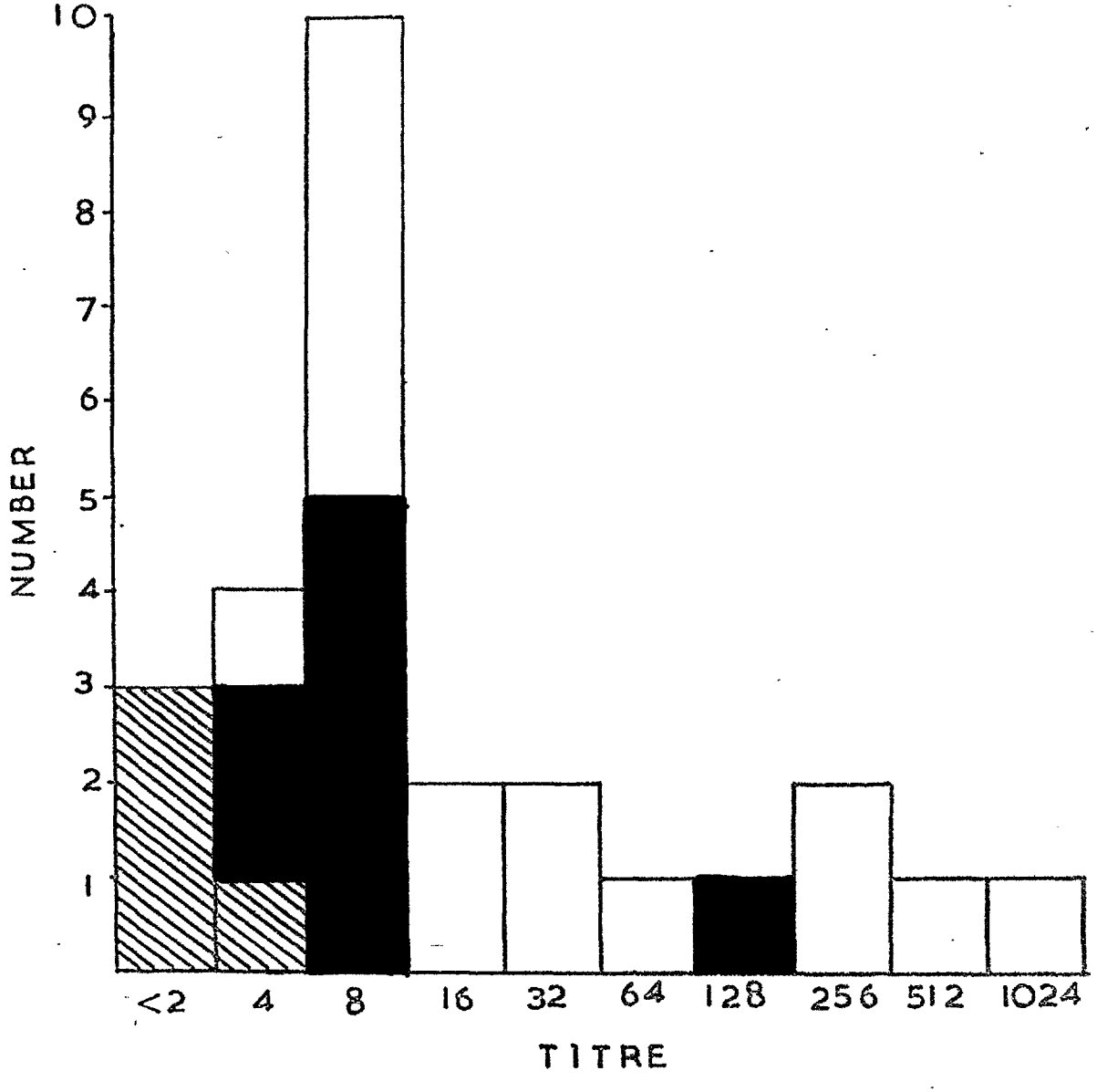


Fig. 38. Anti-F antibody titres in human cases of T. (T.) rhodesiense sleeping sickness.

control patients

active cases

follow-up cases

SS number	Titre	Titre after 2-ME
1020	256	0
1024	16	0
1030	8	8
1032	8	0
944/FU	8	0
1002/FU	4	0
1004/FU	8	0
1005/FU	8	0

SS number	Titre	Titre after alkylation only
944/FU	8	4
1002/FU	4	4
1005/FU	8	8

Control	Titre	Titre after 2-ME
E.F.	4	0
M.W.	8	0

TABLE XIX. Effect of 2-ME reduction and alkylation on the serum anti-F antibody titre in sleeping sickness patients and 2 control patients.

3.3. Changes in other serum proteins

In many infectious diseases, whatever the causative organism, there is a characteristic change in the serum protein composition with a rise in the γ-globulin fraction associated with the acquisition of immunity. Elevated γ-globulin levels in trypanosomiasis have been well documented (Trincao et al, 1953; Pautrizel et al, 1959; Sadun et al, 1973). This feature together with that of high fibrinogen levels and serum macroglobulins described under the previous sections, suggested that hyperproteinaemia may be a feature of rabbit trypanosomiasis. To investigate this situation, a differential serum protein analysis during infections of rabbits with T. (T.) brucei 427 was made.

Serum from 4 infected rabbits (S347-S350) was collected prior to infection and at seven day intervals following innoculation with trypanosomes. Two rabbits (S345 and S346) were reserved as non-infected controls and serum collected at the same time intervals. All results are expressed as geometric means $\pm$ standard errors. Data for individual rabbits can be found in Appendix 1.

3.3.1. Total protein

Fig. 39 a, shows the total protein concentration for infected and normal rabbits. The total protein content for the control rabbits showed very little variation over the 4 week investigation period with values ranging from 6.3 g/100 ml ($\pm$ 0.78 g) to 6.7 g/100 ml ($\pm$ 0.75 g). How-

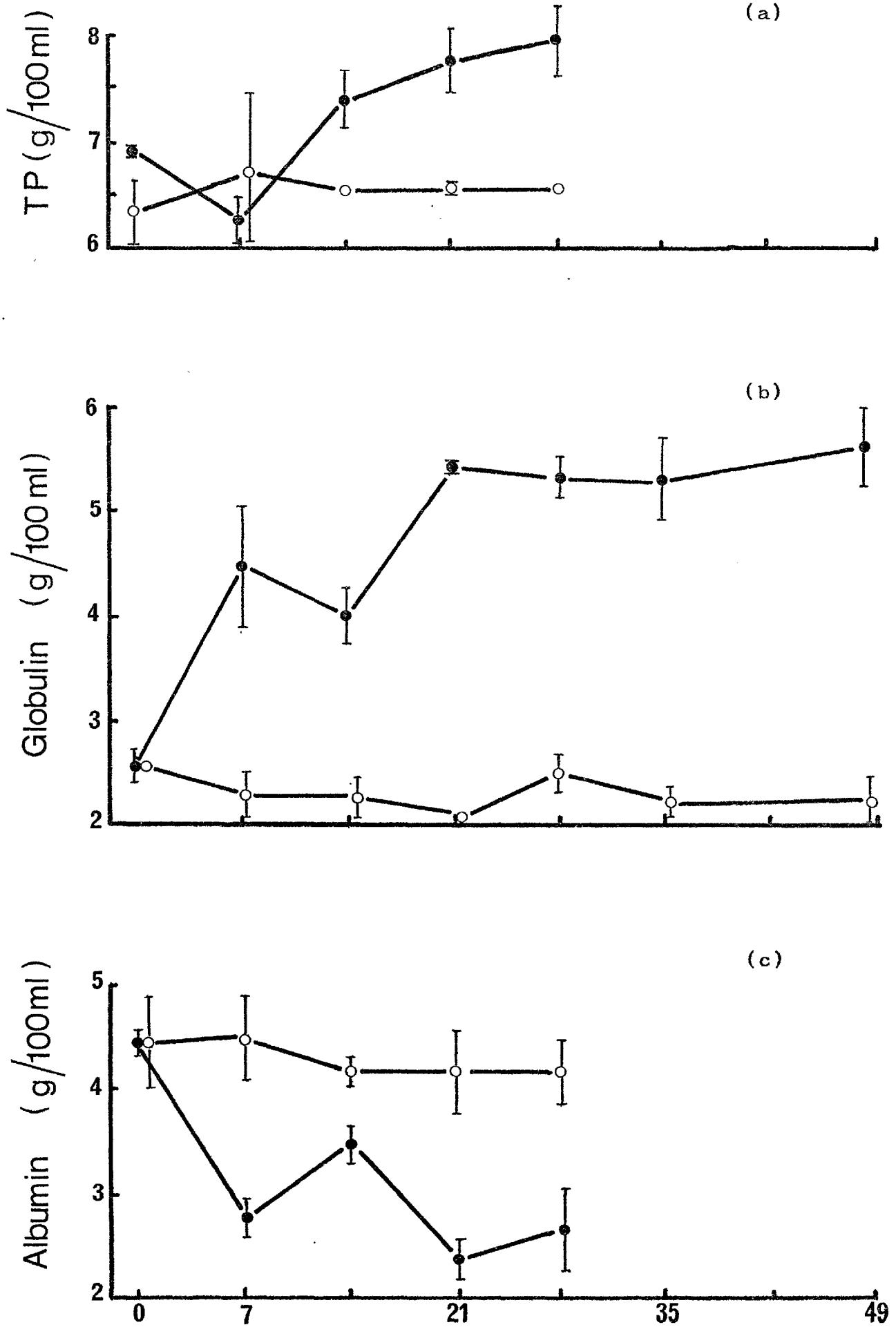

(a)
TP (g/100ml)
8
7
6
(b)
Globulin (g/100 ml)
6
5
4
3
2
(c)
Albumin (g/100ml)
5
4
3
2
0
7
21
35
49
Days after infection

Fig. 39.

Fig. 39. Total protein (a); globulin (b), and albumin (c) in the serum of two groups of rabbits.

●———● mean (± S.E.) for 4 rabbits infected with T. (T.) brucei 427.

O———O mean (± S.E.) for 2 control non-infected rabbits.

ever, in infected rabbits, after an initial decrease during the first week, a gradual increase occurred during the following 3 weeks reaching a mean of 7.95 g/100 ml (± 0.35) - an increase of approximately 33% from pre-infection values.

3.3.2. Globulin

In infected rabbits there was a steep increase in globulin beginning around the 7th day of infection and continuing to rise during the following weeks eventually reaching a 100% increase over normal levels (Fig. 39 b). The two control rabbits had globulin values of 2.08 g/100 ml (± 0.27 g) and 2.45 g/100 ml (± 0.38 g).

The elevation of globulin in infected rabbits is probably determined largely by the high IgM and IgG concentrations as shown in the previous Section 3.2. (Figs. 31 to 35). Chromatography of infected plasma on Sephadex G200 columns showed an increased height and width of the macroglobulin and γ-globulin peaks indicating increased concentrations of these proteins. The peak shown in Fig. 39 b, on the 7th day of infection almost certainly represents the early appearance of 19s IgM. The subsequent rise in globulin values after 21 days of infection probably represent the increased synthesis and accumulation of IgG.

3.3.3. Albumin

In non-infected rabbits, the serum albumin levels

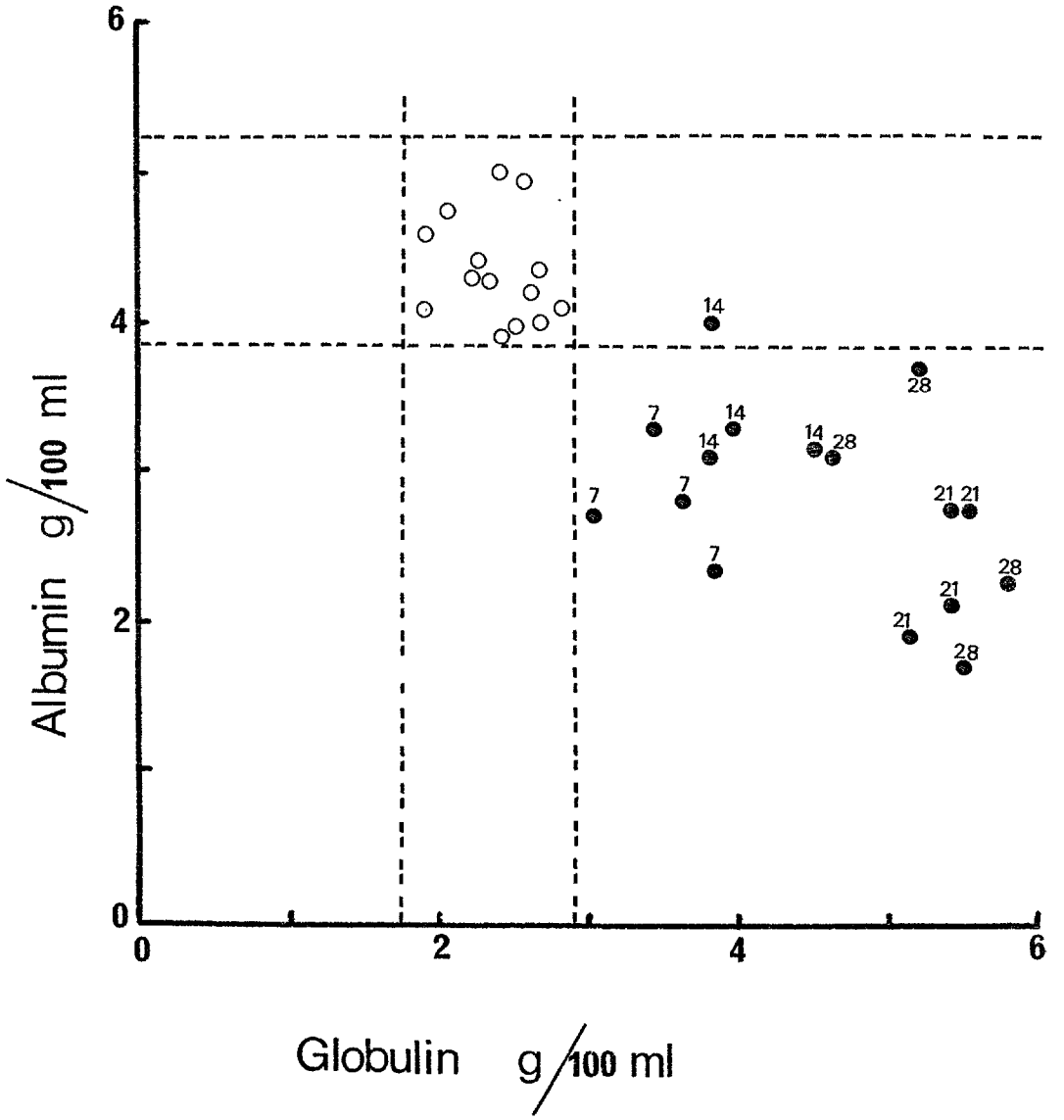

6
4
2
0
0
2
4
6
Albumin g/100 ml
Globulin g/100 ml
14
28
7
14
14
28
7
7
21
21
7
28
21
21
28

Fig. 40.

Fig. 40. Relationship between serum albumin and globulin concentrations in normal rabbits and rabbits infected with T. (T.) brucei 427.

● means for 4 infected rabbits

O means for a group of control non-infected rabbits.

Figures by the points denote days after infection. Note shift to the lower right hand corner of the graph as the infection progresses.

remained almost constant throughout the period of investigation. However, in the trypanosome infected rabbits, concurrent with the 2-fold increase in globulin, was a 2-fold decrease in albumin (Fig. 39 c). The decline in albumin was gradual and at its lowest by the 3rd week of infection (2.36 g/100 ml $\pm$ 0.23 g). Again, this picture can be seen clearly during the separation of albumin from other serum proteins on Sephadex G200 (Figs. 32 to 36) where on day 28 of infection albumin is represented by a small, almost unidentifiable, peak following the extensive γ-globulin peak.

Thus an increase in serum globulin is accompanied by a decrease in the albumin content. This is clearly seen in Fig. 39 b and c, where an inverse relationship between the two exists. A similar situation does not occur between total protein and albumin. By plotting the globulin against albumin values for control and infected rabbits this relationship is made clearer (Fig. 40). The control rabbits form a distinct group with little scatter. However infected animals show a greater scatter of values but a distinct correlation is seen between the two groups of proteins.

3.3.4. Albumin: globulin ratio (A:G ratio)

The A:G ratio was obtained by dividing the albumin value by the globulin value.

In control non-infected rabbits the A:G ratio lies between 1.2 to 2.6.

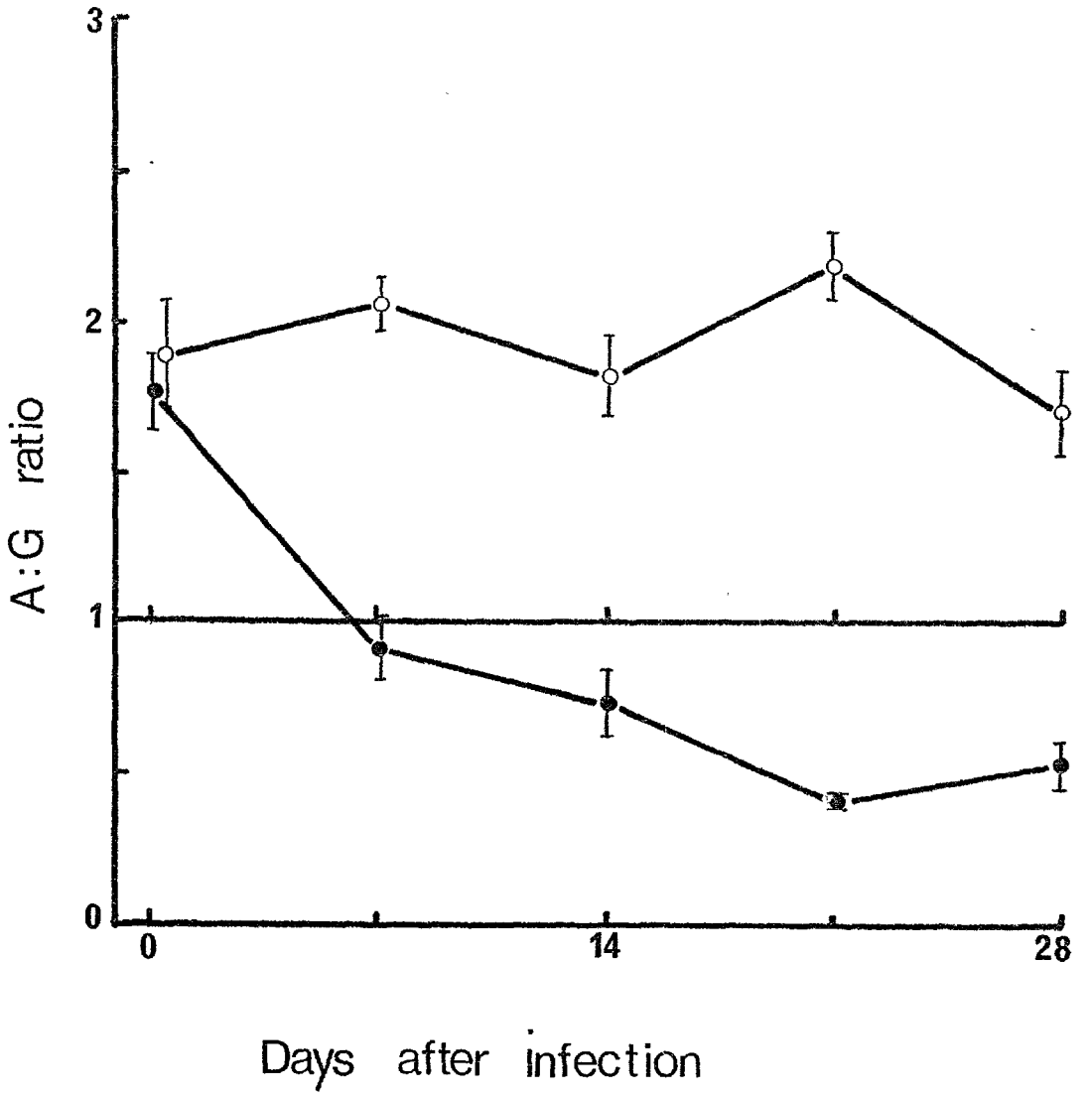


Fig. 41. Serum albumin: globulin ratios in 4 rabbits infected with T. (T.) brucei 427, and 2 non-infected control rabbits.

●———● mean (± S.E.) for infected rabbits.

O———O mean (± S.E.) for control rabbits.

There was a sharp decrease in this ratio during the first week of infection with trypanosomes. The mean ratios ± S.E. for the control and infected rabbits is given in Fig. 41.

The most significant feature is that the A:G ratio is reversed as early as day 7 following infection and remains as such during the following 4 weeks. At this time of infection rabbits are showing external signs of oedema and the two conditions are probably linked as regards plasma osmotic pressure changes. However this is certainly not the only reason for the development of oedema.

3.3.5. Lipoproteins

After 2-3 weeks of infection with either strain of T. (T.) brucei, rabbit plasma and serum was found to be "milky" or opalescent. This condition persisted until the death of the animals when the plasma appeared opaque. Storage of serum or plasma at 4°C for 24 h resulted in the formation of a white supernatant layer and a clearing of the infranatant portion. This situation suggested a lipaemia developed in infected rabbits which, it was considered, might cause direct or indirect pathological effects within the microcirculation.

The serum lipids have been broadly classified under two major headings: the water soluble lipoproteins and the free (or non-esterified) fatty acids. The lipoproteins

represent a whole spectrum of lipid molecules and are composed of the phospholipids, cholesterol (free and esterified) and the tryglycerides. The density of the lipoproteins is largely a function of the proportion of lipid contained within it - the lipoprotein species with the lowest density are the chylomicrons.

Because of the pathological importance of increased lipids in the blood in producing a hypercoagulable state and other microcirculatory disorders (Cullen and Swank, 1954; Kwaan and Suwanwela, 1969), it was decided to investigate the lipids involved in rabbit trypanosomiasis in order to obtain an exact diagnosis of the condition and to ascertain what the pathological consequences of the lipaemia might be.

Five rabbits (S217, S219, S370, S372 and S374) were infected with T. (T.) brucei strain 427, and 3 rabbits (S218, S381 and S382) were kept as non-infected controls. All rabbits were starved overnight on every seventh day and bled the following morning for serum. The clarity of each serum sample was noted. The mean blood total lipid, tryglyceride (neutral fat), phospholipid and total cholesterol levels for infected and control rabbits are shown in Figs. 42 to 45.

The total lipid levels in infected rabbits rose dramatically during the first two weeks of infection and reached extremely high levels during the 7th week of infection - approximately 350% greater than pre-infection

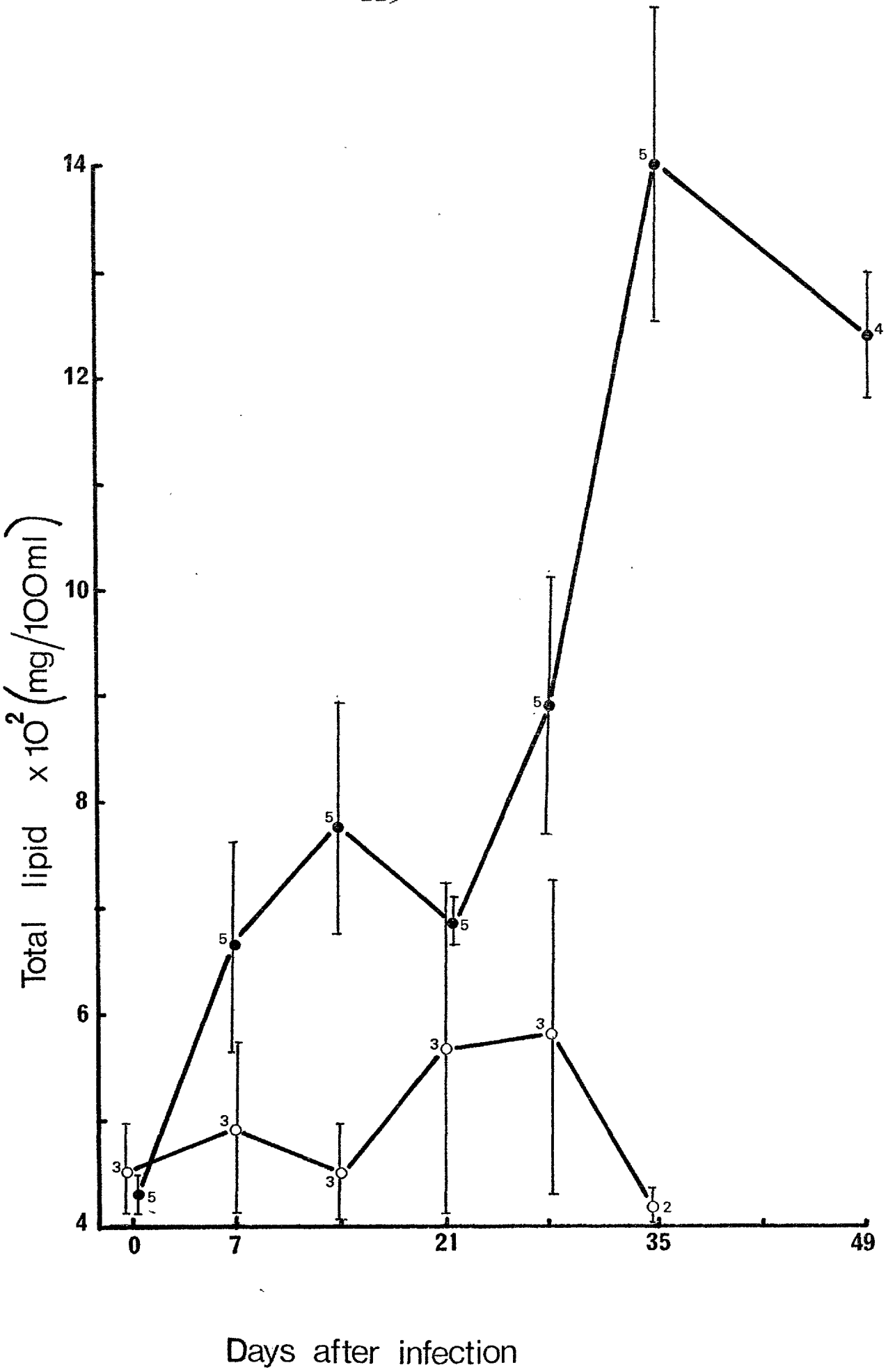

Total lipid x 10² (mg/100ml)
14
12
10
8
6
4
0
7
21
35
49
Days after infection

Fig. 42.

Fig. 42. Serum total lipid levels in control non-infected rabbits and in rabbits infected with T. (T.) brucei 427.

●———● mean (± S.E.) for infected rabbits.

O———O mean (± S.E.) for control rabbits.

Figures beside each point indicate the number of animals used for estimation of means.

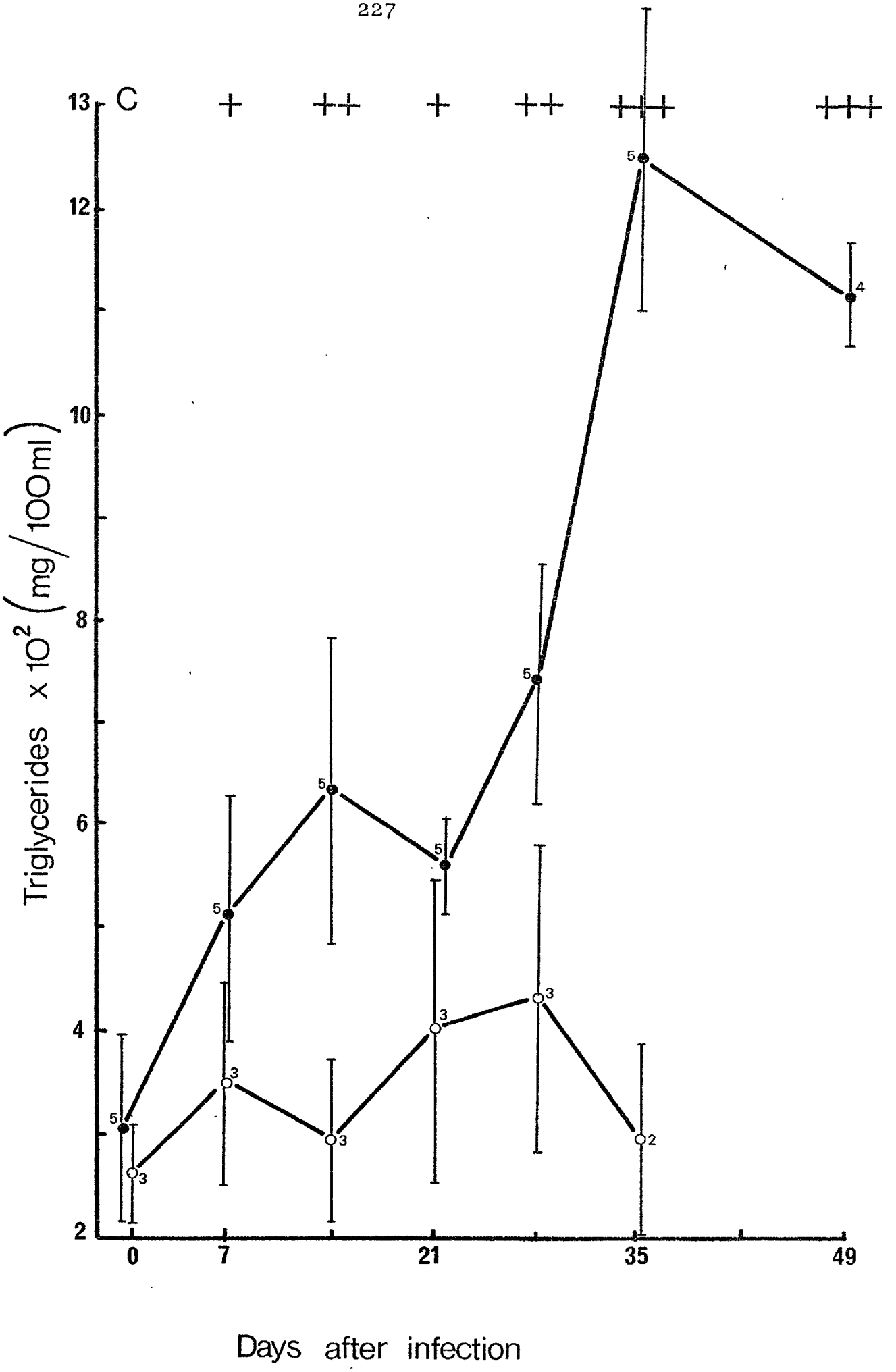

C
+
++
+
++
+++
+++
13
12
10
8
6
4
2
Triglycerides x 10² (mg/100ml)
0
7
21
35
49
Days after infection

Fig. 43.

Fig. 43. Serum triglyceride (neutral fat) concentrations in non-infected control rabbits and rabbits infected with T. (T.) brucei 427.

●————● mean (± S.E.) for infected rabbits.

O————O mean (± S.E.) for control rabbits.

Figures beside each point indicate the number of animals used for estimation of means.

The opacity of the serum is indicated along the top of the graph and is graded as follows:-

C = clear

\+ = slightly cloudy or "milky".

++ = moderately cloudy

+++ = very cloudy

++++ = almost opaque

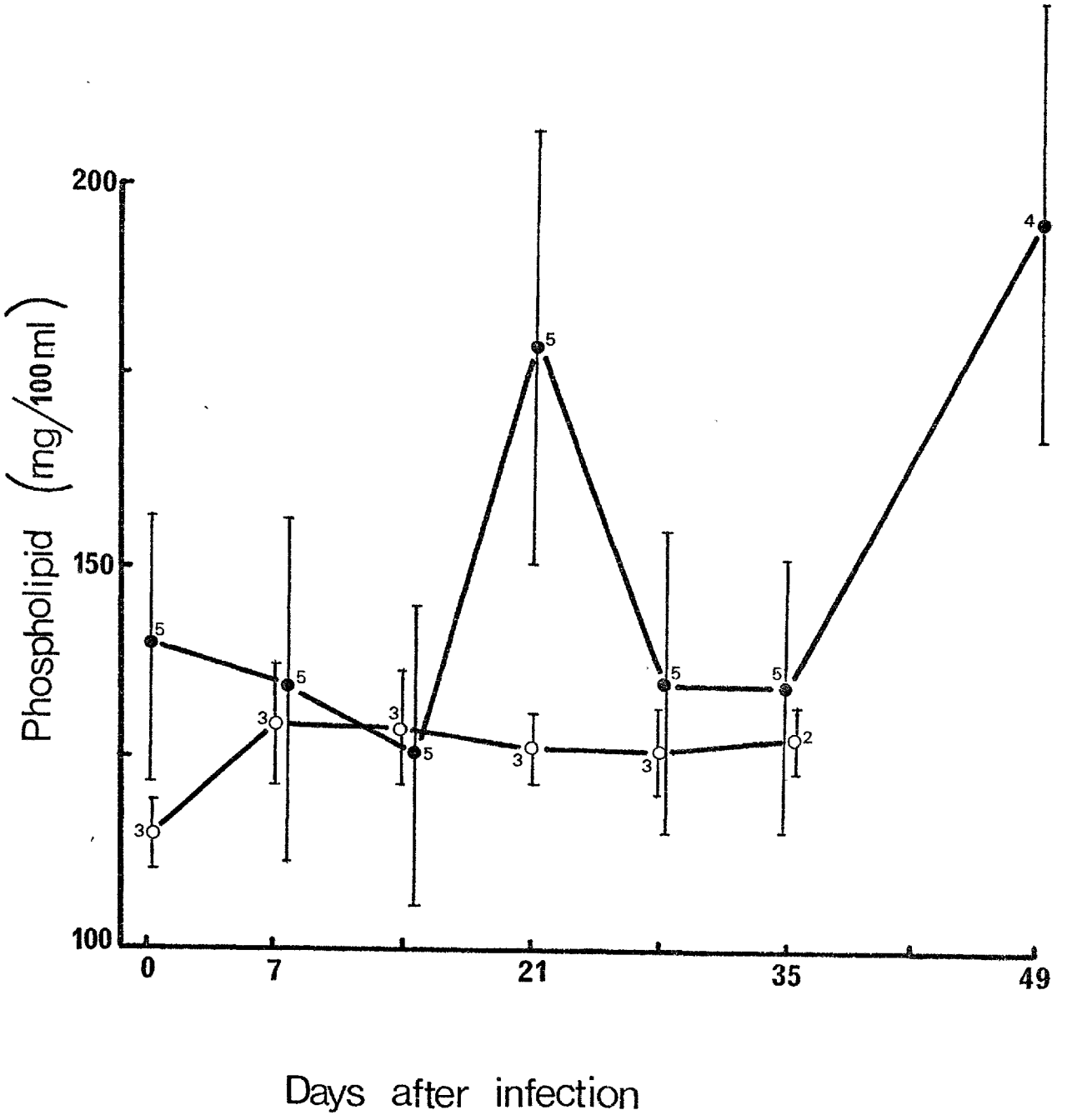


Fig. 44. Serum phospholipid levels in non-infected control rabbits and rabbits infected with T. (T.) brucei 427.

●———● Mean (± S.E.) for infected rabbits

O———O Mean (± S.E.) for control rabbits

Figures beside each point indicate the number of animals used for estimation of means.

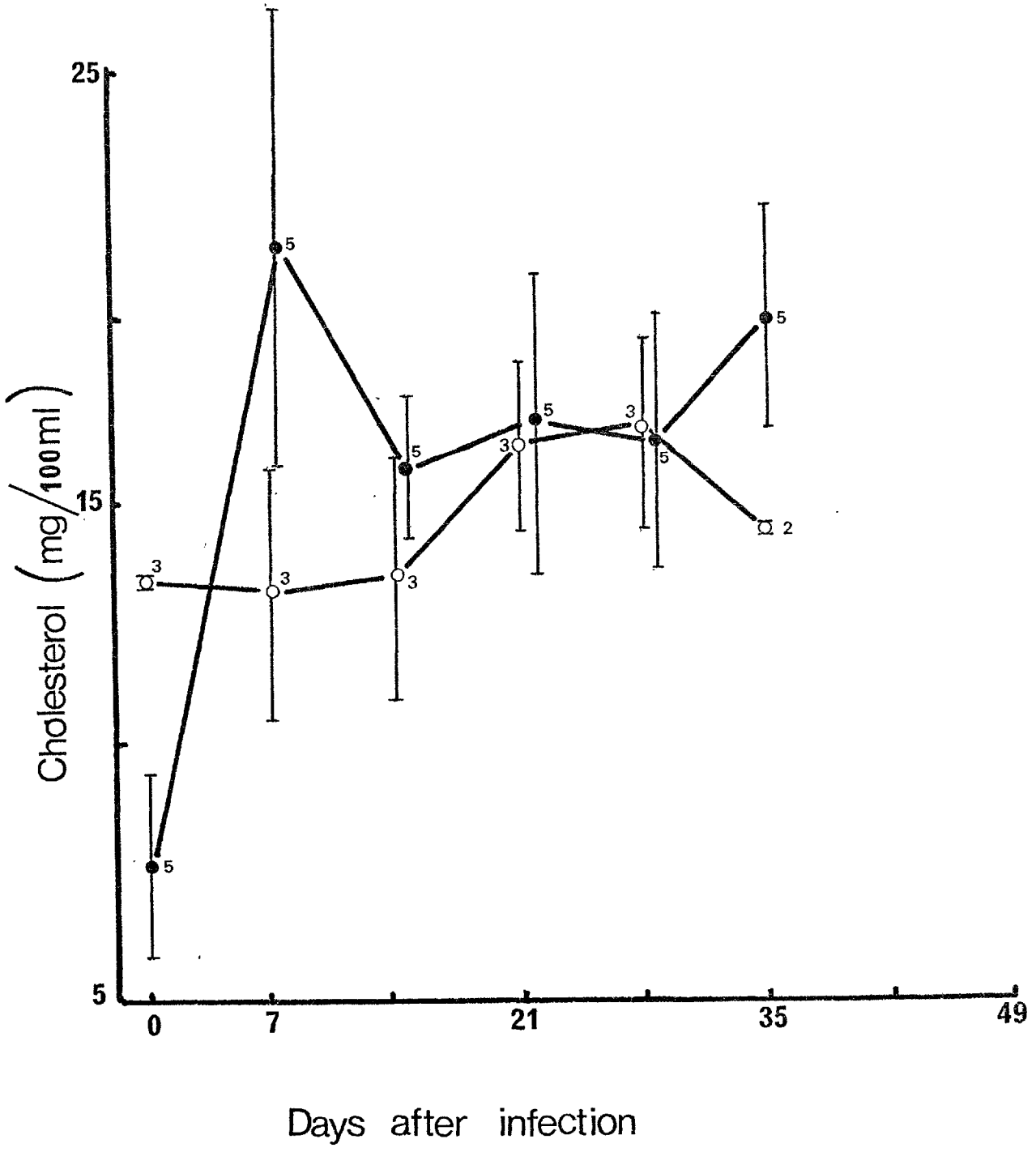


Fig. 45.

Fig. 45. Serum total cholesterol in non-infected control rabbits and rabbits infected with T. (T.) brucei 427.

●———● mean (± S.E.) for infected rabbits.

O———O mean (± S.E.) for control rabbits.

Figures beside each point indicate the number of animals used for estimation of means.

values (Fig. 42). It can be seen from Fig. 43 that the primary lipoprotein constituent of this elevation was a tryglyceride (neutral fat) increase - the pattern of variation during the 7 week infection period being identical to that of the total lipid. Sera which had a high tryglyceride content were noticibly "milky". Only slight fluctuations in the blood composition of total lipid and tryglycerides were seen in control rabbits.

Two peaks of phospholipid (Fig. 44) at days 21 and 49 after infection were found to occur in the trypanosome infected rabbits. However, between these peaks, the phospholipid content of serum remained within the range for control rabbits.

There was no significant difference in cholesterol levels of infected and control groups (Fig. 45). The peak of cholesterol at day 7 in the infected group is perhaps misleading since it was due to the result of rabbit S370, the serum of which was found to contain an abnormally high level of cholesterol (48 mg/100 ml serum).

The variation of both serum cholesterol and phospholipid in most normal rabbits was within $\pm$ 10-15%, and $\pm$ 10-20% in the infected rabbits.

In the 21 samples of normal sera tested, in which the total lipids ranged from 412-582 mg/100 ml, the ratio of phospholipid to total lipid (PL/TL ratio) was found to have a maximum range of 0.26 to 0.33. In Fig. 46 b, the PL/TL ratio for infected and control sera is plotted

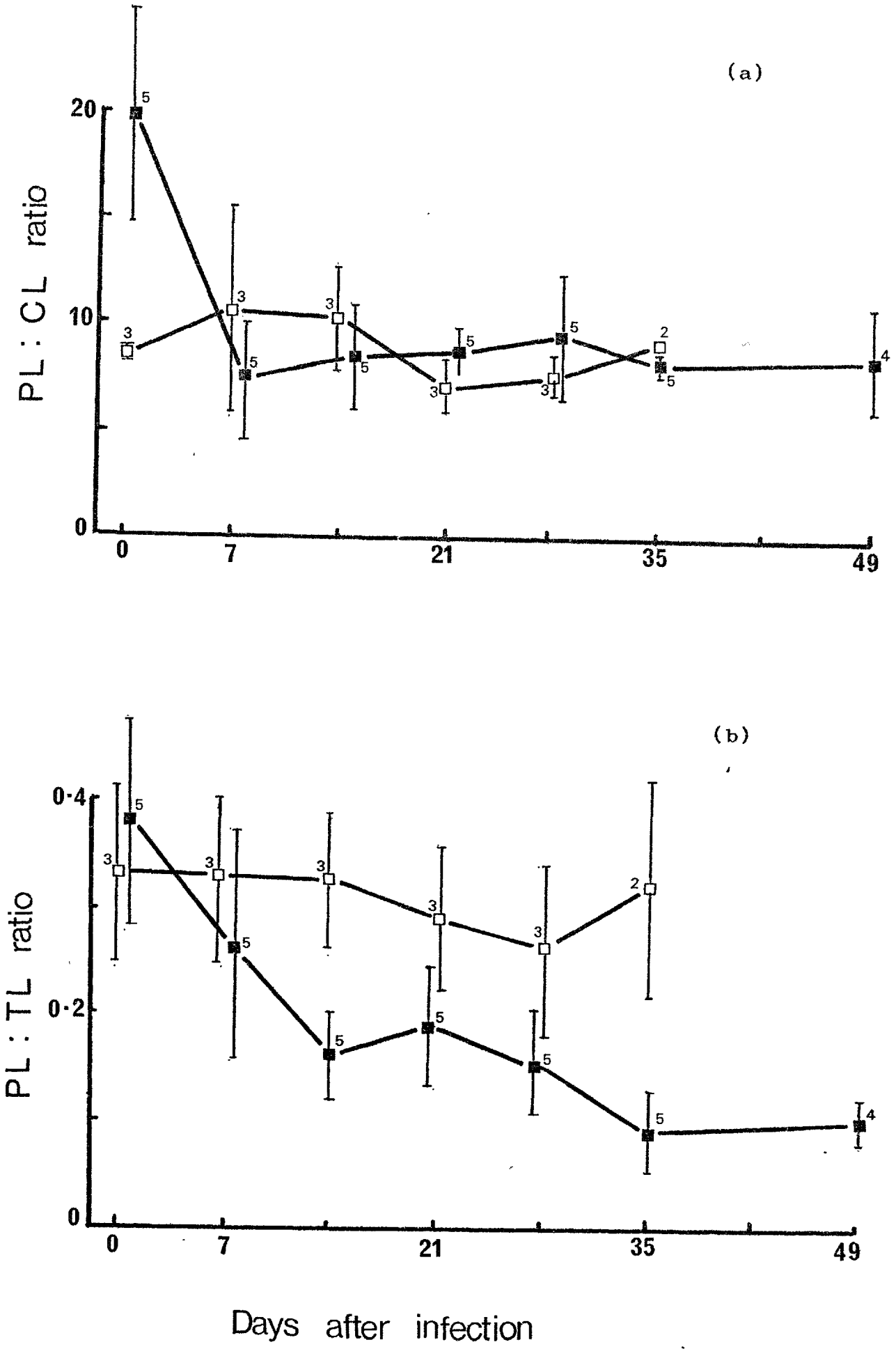

(a)
PL : CL ratio
20
10
0
0
7
21
35
49
(b)
PL : TL ratio
0·4
0·2
0
0
7
21
35
49
Days after infection

Fig. 46.

Fig. 46. Phospholipid: cholesterol ratio (a); and phospholipid: total lipid ratio (b) in control non-infected rabbits and rabbits infected with T. (T.) brucei 427.

■———■ mean (± S.E.) for infected rabbits.

□———□ mean (± S.E.) for control rabbits.

Figures beside each point indicate the number of animals used for estimation of means.

against time. It can be seen that infected rabbits show a lowering of this ratio to a mean ratio of 0.9 (± 0.04 S.E.) 35 days after infection. Ahrens and Kunkel (1949) studying a variety of clinical conditions involving a hyperlipaemia, found that a lowering of the PL/TL ratio, (due to a relatively increased tryglyceride portion of the total lipid), caused the serum of the patients to appear 'milky'. This situation probably occurs in the trypanosome infected rabbits where a lowering of the PL/TL ratio is associated with a gradual increase in the opacity of the serum due to the increased tryglyceride content.

How do tryglycerides effect the opacity of serum? Furman et al (1966) have shown that the lactescence characteristic of hyperlipaemic sera is attributable to the presence of lipoprotein molecules having a diameter of 700-800 Å or more i.e. the tryglycerides particularly chylomicrons. Their presence in the serum causes light scattering and reflectance thereby imparting the opalescence or 'milkiness' characteristic of this type of hyperlipaemia.

The phospholipid: cholesterol ratio (PL/CL ratio) did not vary significantly in either control or infected rabbits (Fig. 46 a).

Thus to summarise, rabbits infected with T. (T.)

brucei exhibit a hyperlipoproteinaemia the main constituent of which is an elevated tryglyceride content. This condition results in a lowering of the PL/TL ratio which was found to be associated with the degree of 'milkiness' of the serum. It is possible that such high lipoprotein levels might cause platelets and other cellular elements of the blood to adhere to each other resulting in a predisposition for the occurrence of thrombotic events (Kwaan and Suwanwela, 1969; Newall *et al*, 1973).

3.4. Mechanisms involved in erythrocyte agglutination and sedimentation

During the estimation of parasitaemia on wet film preparations of blood from infected rabbits, two features were observed.

First although no direct counts were made, the number of erythrocytes per microscope field, progressively diminished. This is explained by the considerable anaemia that develops in this infection (Boreham, 1968; Jenkins et al, 1974) and is confirmed by the packed cell volume measurements shown in Fig. 47 and Appendix 1.

A second feature seen in Fig. 49 b, c, was the tendency for the cellular elements of the blood to either form rouleaux (in the early stages of infection) or to autoagglutinate (from the 2nd week of infection to death of the animal). These stages suggested that different phases of erythrocyte aggregation occur. During auto-agglutination in the later stages of infection the cells take on the macroscopic appearance of an immunologically mediated agglutination forming large distinct clumps (Fig. 49 c). In addition, it was noticed during routine measurements of the erythrocyte sedimentation rate (ESR), that rabbit blood which showed agglutination also gave high ESR readings - suggesting a correlation between the two.

Autoagglutination and high sedimentation of red cells are two phenomena well recognised in trypanosome infections. However, apart from the suggestion of

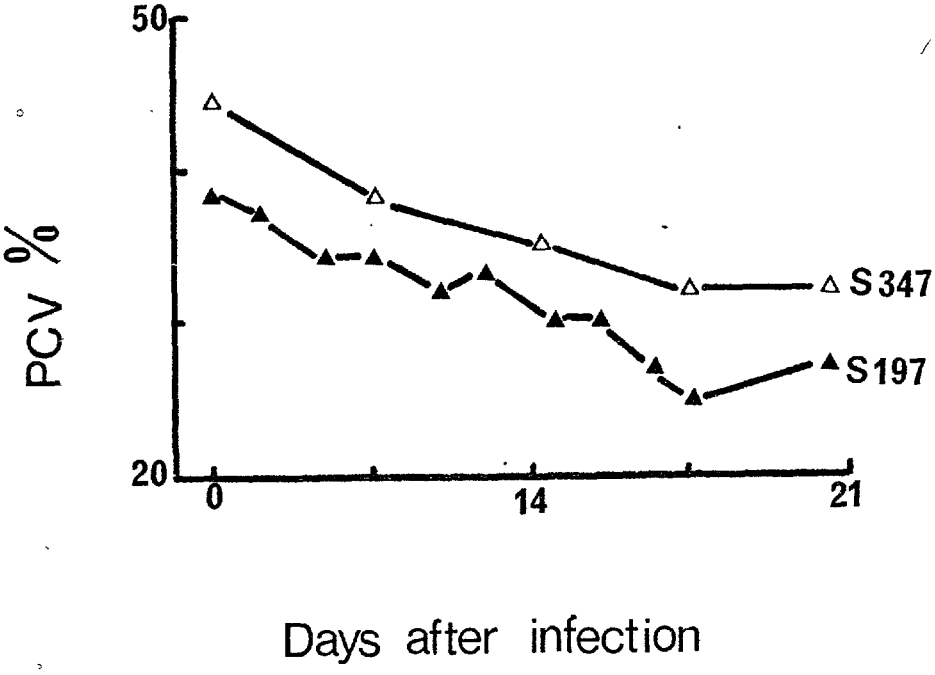

50
PCV %
20
0
14
21
S347
S197
Days after infection

Fig. 47.

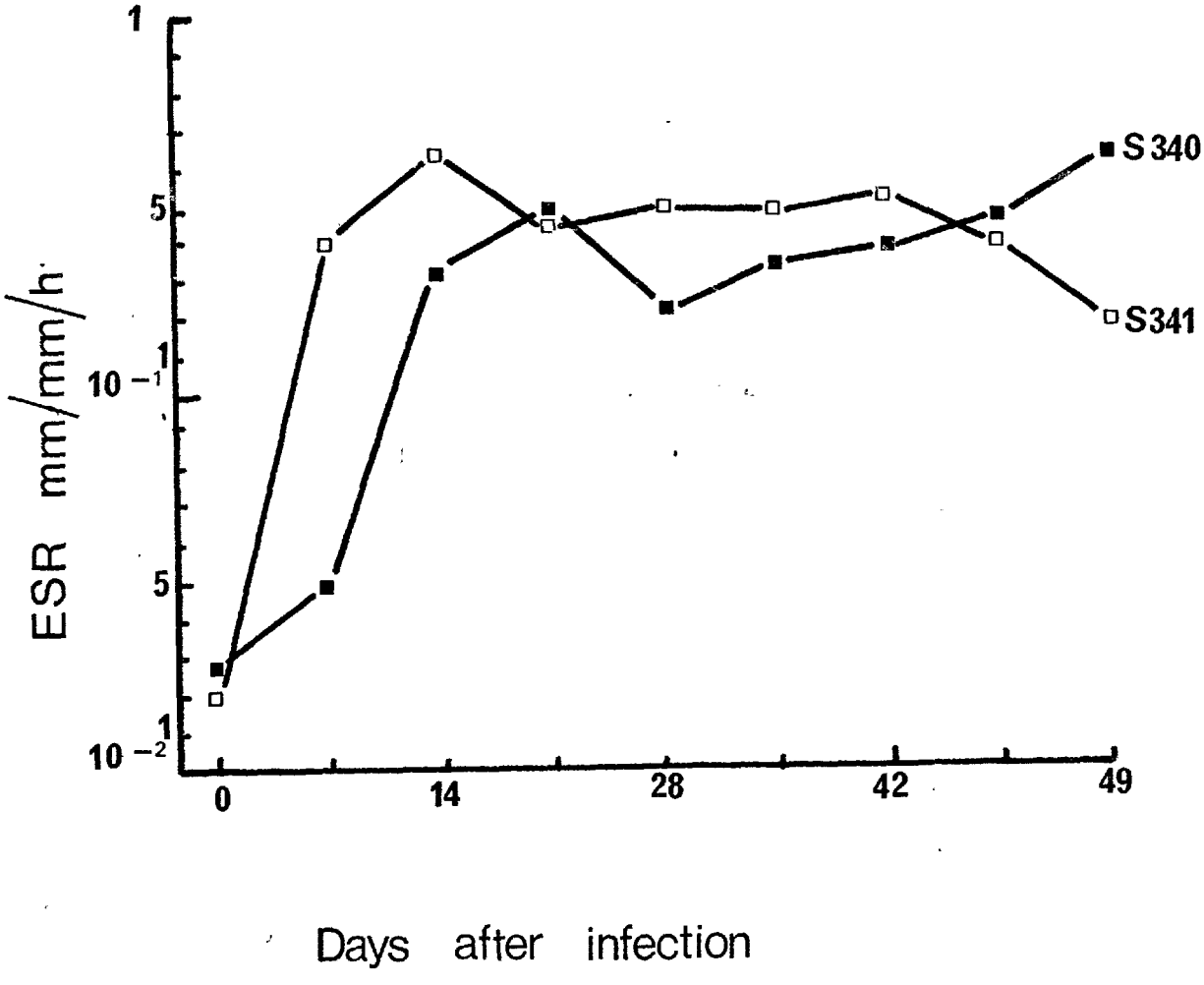

1
5
1
10 −1
5
1
10 −2
ESR mm/mm/h
0
14
28
42
49
S340
S341
Days after infection

Fig. 48.

Fig. 47. Packed cell volume changes in the blood of two rabbits, S198 infected with T. (T.) brucei S42, and S347 infected with T. (T.) brucei 427.

Fig. 48. Erythrocyte sedimentation rate in two rabbits infected with T. (T.) brucei 427. Sedimentation carried out at 37°C for 1 h.

Zuckerman (1964) that both are caused by an immunological reaction similar to that observed in malarial infections, no other suggestions or investigations into the mechanism of autoagglutination in trypanosomiasis have been made.

Since large molecular weight, assymetric proteins such as fibrinogen are known to increase the ESR (Hardwicke et al, 1950) and fibrinogen increases during trypanosomiasis of the rabbit, it was decided to investigate:

i) changes in the ESR during infection.

ii) the effect of red cell concentration on the ESR.

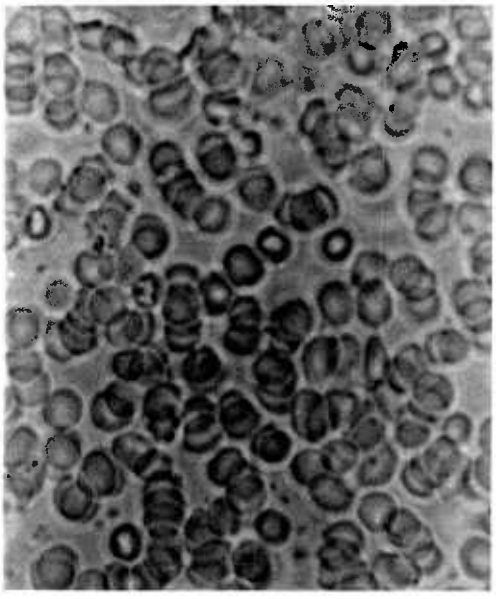
iii) the relationship between individual plasma proteins and the ESR.

iv) why autoagglutination of red blood cells should occur despite evidence (although inconclusive) of the absence of autoantibodies to the red cells in infected serum (Mackenzie and Boreham, in press).

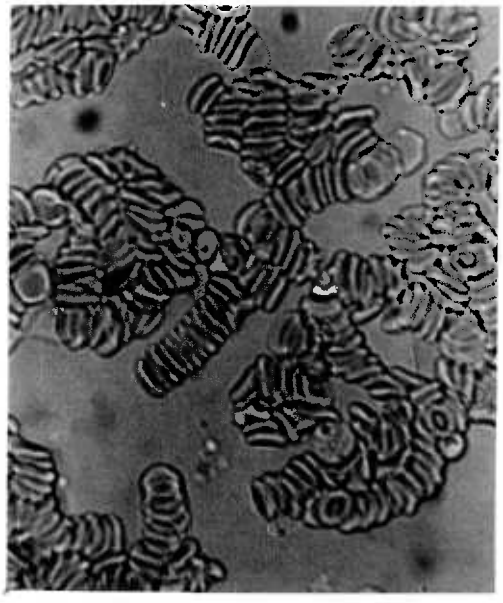
i) Changes in the ESR during infection

The typical change in pattern of the ESR can be seen in Fig. 48 which shows sedimentation rates for 2 rabbits, S340 and S341, infected with T. (T.) brucei strain 427. A marked increase in the ESR occurred with the greatest rise apparent during the first or second week of infection. After this period of time the increase was more gradual. This feature coincided with 3 factors viz. autoagglutination of red cells, the onset of anaemia, and, as

(a)



(b)



(c)

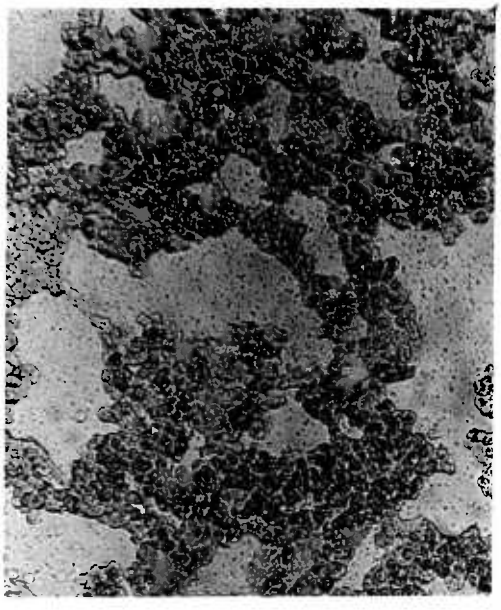


Fig. 49. Wet film preparation of blood taken from one normal non-infected rabbit and two rabbits infected with T. (T.) brucei 427, temperature 30°C.

(a) Normal rabbit blood. x 400 mag.

(b) Rabbit S402. day 4. Rouleaux formation of red cells. x 400 mag.

(c) Rabbit S403. day 21. Strong auto-agglutination of red cells. x 100 mag.

shown in the previous sections, an increase in fibrinogen, globulin (mainly macroglobulin) and lipoproteins in the blood. It was certain that clumping of the red cells resulted in a greater sedimentation rate, but, since the PCV also fell, fewer red cells might have an additive effect.

ii) Influence of the erythrocyte concentration on the ESR

The influence of the concentration of erythrocytes on the sedimentation rate is clearly seen in Fig. 50 where a straight line relationship is demonstrated between the two. Thus in normal rabbit plasma, a reduction of 10% in the volume of red cells from a PCV of 10-40% results in a rise in the ESR of approximately 6 mm/mm/h. However in infected blood this relationship does not exist (Table XX). For example, rabbit S199 showed a 12.5 mm increase in the ESR during the first week of infection and a fall in haematocrit from 39-30%. According to the correlation figure (Fig. 50) for normal rabbit blood, an increase of only 5.5 mm would be expected with a decreased PCV of 9%.

It would appear therefore, that although the anaemia does contribute to the observed high sedimentation rate, other factors in the blood also effect the ESR. That these factors reside in infected plasma is demonstrated by the following studies.

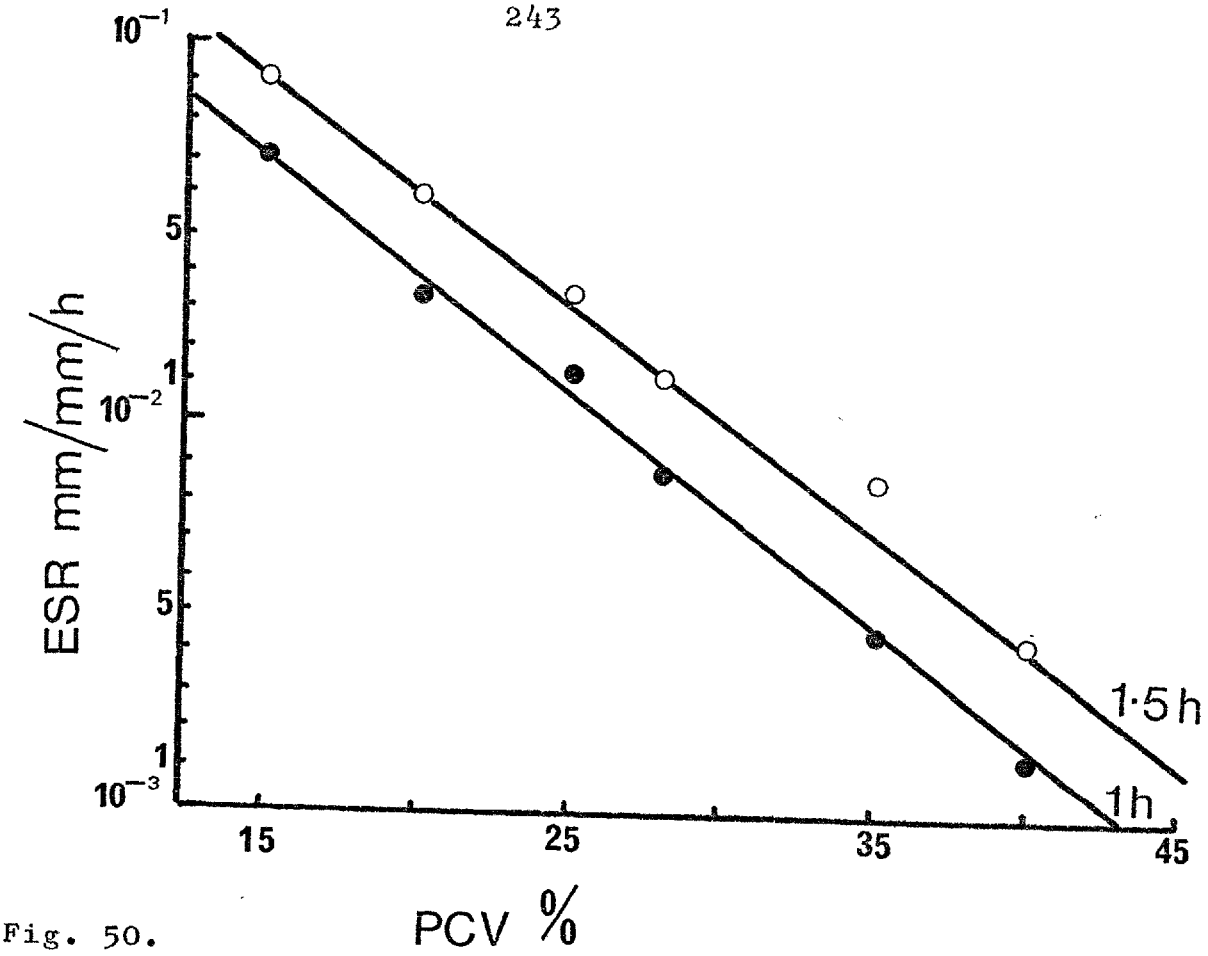

ESR mm/mm/h
10^{-1}
5
1
10^{-2}
5
1
10^{-3}
15
25
35
45
PCV %
1·5h
1h

Fig. 50.

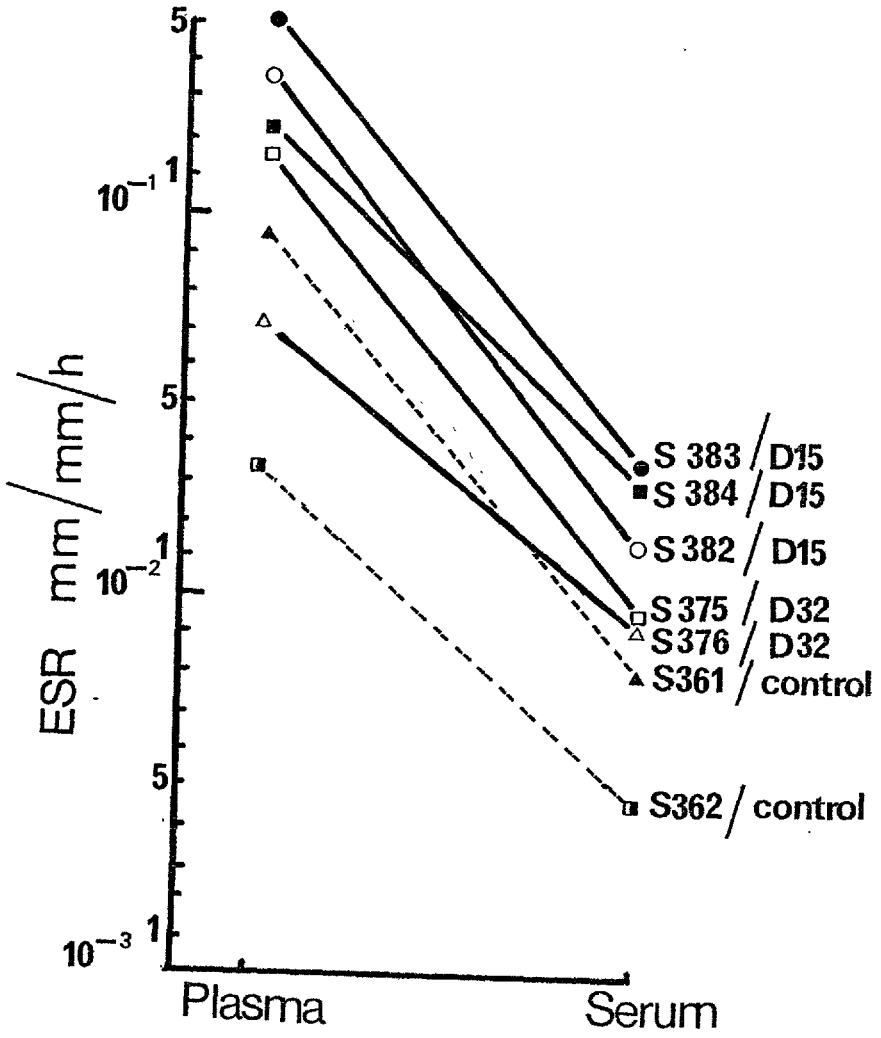

ESR mm/mm/h
5
10^{-1}
1
5
10^{-2}
1
5
10^{-3}
1
Plasma
Serum
S 383 / D15
S 384 / D15
S 382 / D15
S 375 / D32
S 376 / D32
S361 / control
S362 / control

Fig. 51.

Fig. 50. Effect of red cell concentration (PCV%) on the erythrocyte sedimentation rate. Sedimentation carried out at 37°C for 1 h and 1.5 h.

Fig. 51. The difference in sedimentation when red cells are suspended in autologous plasma or serum. Haematocrit was standardised *in vitro* to 45%. ESR values for 5 rabbits infected with *T. (T.) brucei* 427 and 2 non-infected control rabbits.

Rabbit S198

Days after infection	Change in ESR (mm)	Change in PCV (%)
0 - 7	+ 9·5	- 9
7 -14	+ 0·3	+ 1
14 -21	+ 2·3	+ 2
21 -28	+ 2·5	- 4

Rabbit S199

Days after infection	Change in ESR (mm)	Change in PCV (%)
0 - 7	+ 12·5	- 9
7 -14	+ 1·5	- 3
14 -21	+ 1·0	+ 4
21 -28	+ 2·0	- 7

TABLE XX. Change in the ESR and packed cell volume in 2 rabbits infected with T. (T.) brucei 427

iii) Relationship between plasma proteins, auto-agglutination and the ESR

It can be seen from Fig. 51 that the ESR is higher in infected compared with control serum at a standardised haematocrit of 45%. In addition, sedimentation rates were markedly elevated in both control and infected plasma. This suggested that coaggulation factors removed during the clotting process were largely responsible for the increase.

In context with this particular investigation it was also found that when normal washed red cells were mixed with infected plasma the ESR was raised and vice-versa (Table XXI). In addition, clumped erythrocytes in infected plasma reverted to individual cells following re-suspension in saline or normal plasma. This strongly suggests that the autoagglutination is non-immunological in origin.

Washed infected rabbit red cells were suspended in various concentrations of purified rabbit fibrinogen in 0.15 M NaCl. The results shown in Fig. 52 a, indicate a substantial increase in the ESR when the fibrinogen concentration was raised above 200 mg/100 ml. Microscope examination of red cells in the various fibrinogen concentrations showed gross autoagglutination at 1000 mg/100 ml (Fig. 53 a), slightly less clumping at 500 mg/100 ml (Fig. 53 b) and only a few rouleaux formations at 250 mg/100 ml (Fig. 53 c). A majority of the red cells

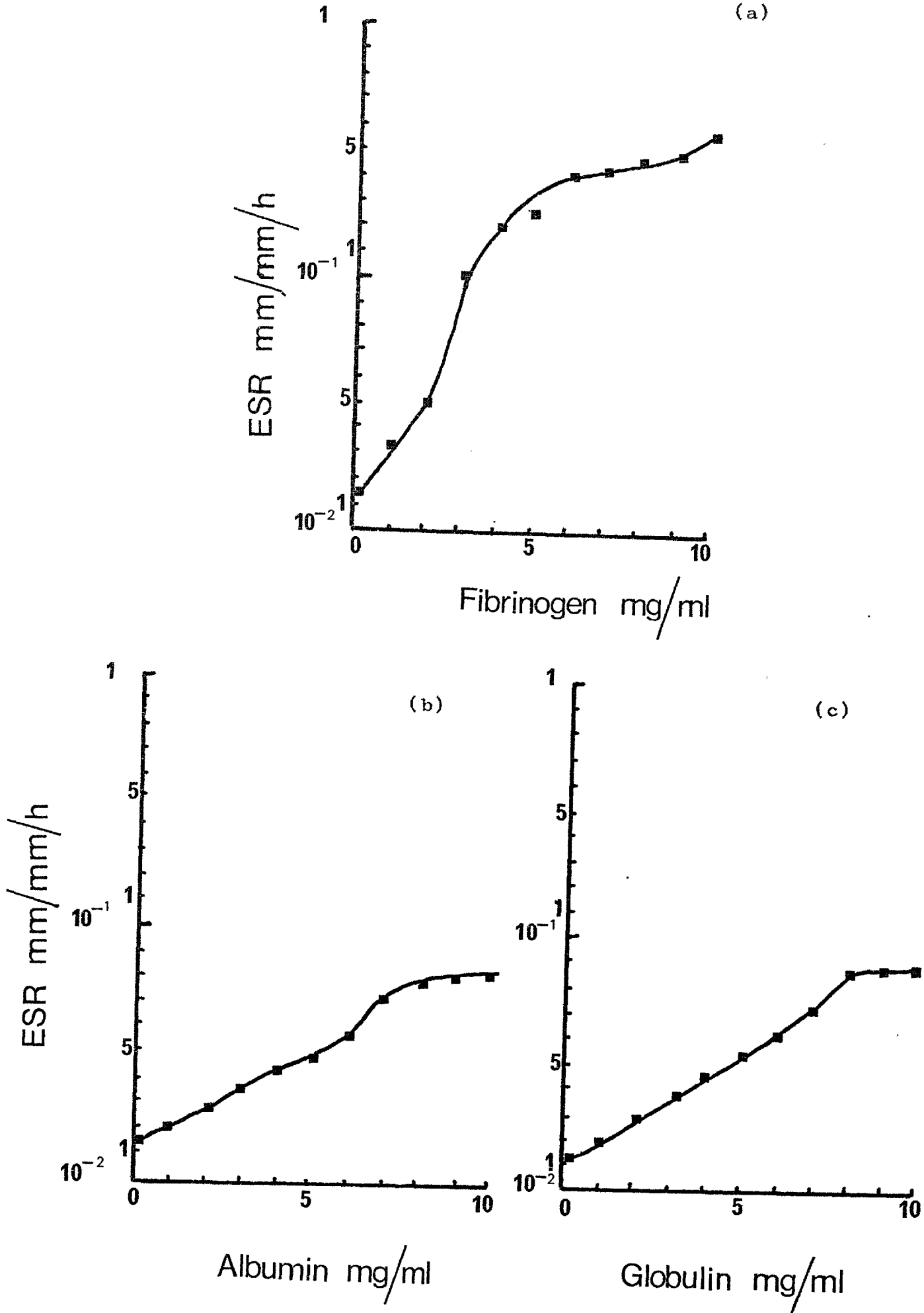

(a)
ESR mm/mm/h
1
5
1
10^{-1}
5
1
10^{-2}
0
5
10
Fibrinogen mg/ml
(b)
ESR mm/mm/h
1
5
1
10^{-1}
5
1
10^{-2}
0
5
10
Albumin mg/ml
(c)
1
5
1
10^{-1}
5
1
10^{-2}
0
5
10
Globulin mg/ml

Fig. 52.

Fig. 52. Effect on the ESR of various concentrations of :

(a) purified rabbit fibrinogen.

(b) bovine serum albumin.

(c) rabbit γ-globulin.

Sedimentation carried out at 37°C for 1 h.

Plasma	Erythrocytes	ESR mm/mm/h
S201 NORMAL WHOLE BLOOD		$6 \cdot 6 \times 10^{-2}$
S198 DAY 22 WHOLE BLOOD / A		$3 \cdot 3 \times 10^{-1}$
S199 DAY 22 WHOLE BLOOD / A		$4 \cdot 1 \times 10^{-1}$
S201 NORMAL	S194 DAY 22 WASHED	$3 \cdot 3 \times 10^{-2}$
S199 DAY 22	S201 NORMAL/A WASHED	$5 \cdot 6 \times 10^{-1}$
S199 DAY 22	S198 DAY 22/A WASHED	$3 \cdot 9 \times 10^{-1}$

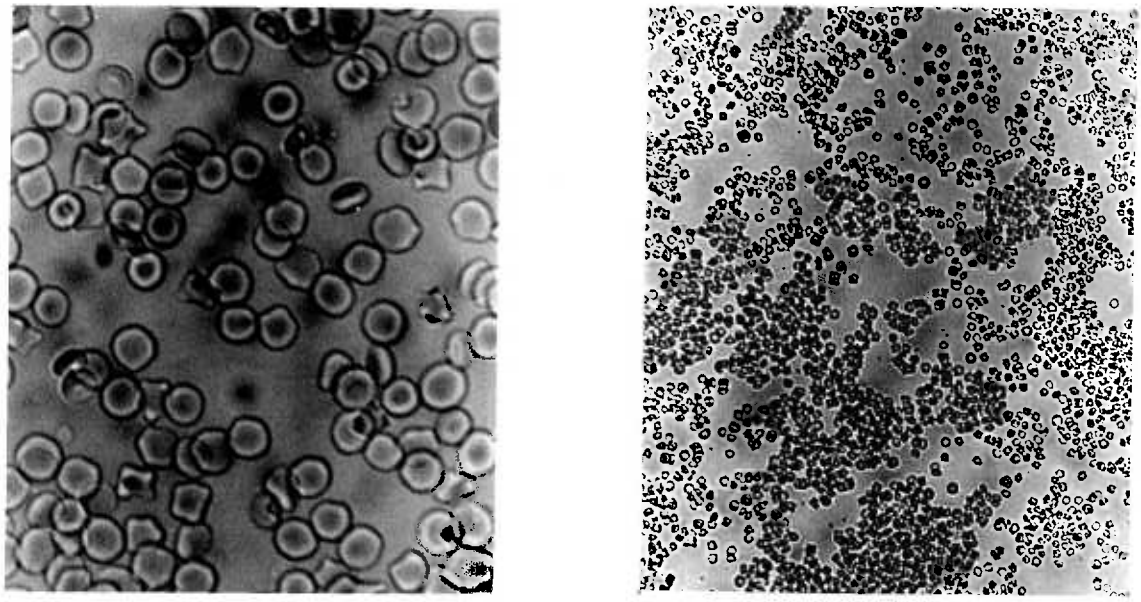
TABLE XXI. Erythrocyte sedimentation rates following mixing of normal plasma with infected erythrocytes and vice-versa.

A = autoagglutination of red cells occurred.

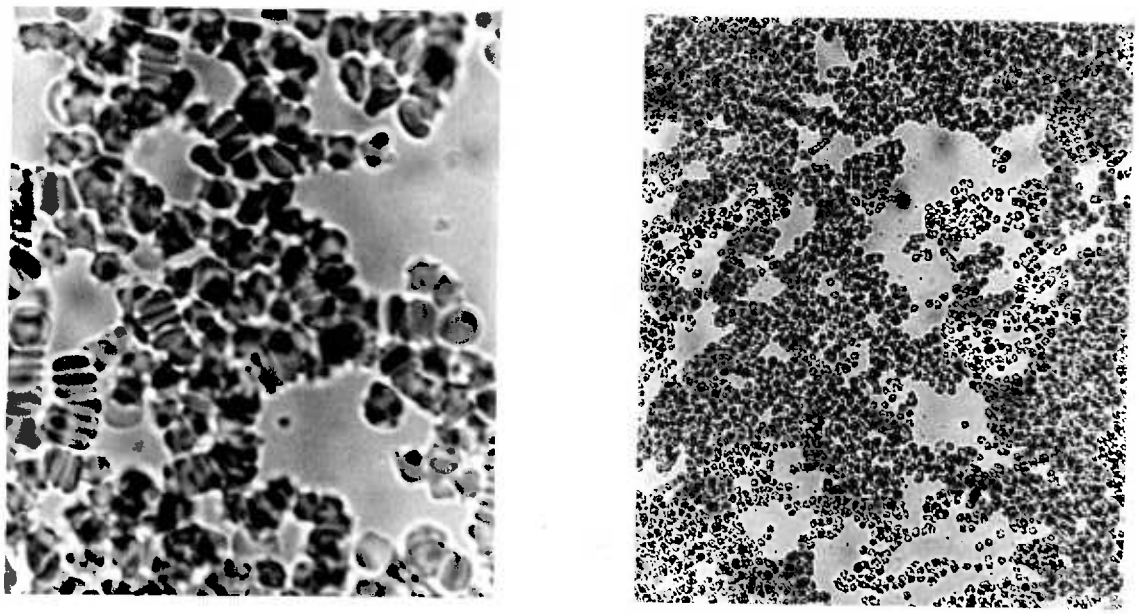
in the high fibrinogen concentrations (levels > 500 mg/100 ml) became microcytic and crenated - similar in appearance to red cells from infected rabbit blood. This crenation was found to be reversible either by diluting the fibrinogen solution with additional saline, or by adding albumin (bovine serum albumin, Armour Pharmaceutricals Ltd.,) to a normal rabbit plasma concentration of 4 g/100 ml. The addition of albumin also caused slight dispersion of erythrocytes in fibrinogen concentrations of 500 mg/100 ml (Fig. 53 d). This feature is interesting as regards the effect of plasma proteins on red cell shape, but its mechanism of operation remains to be explained.

It was not uncommon, in infected blood, to see red cells adhering to each other so that when the cells moved (gentle pressure on the cover slip) their shape became elongated and drawn out into a variety of shapes but maintaining contact with each other via. thin needle-like processes. The same situation was found to occur when normal rabbit red cells were suspended in a high concentration of fibrinogen (Fig. 54 a and b). It can be envisaged that fibrinogen forms a 'sticky' coat around the red cells which then adhere to one another. In this context the plasmanemes of trypanosomes (Wright and Lumsden, 1969) may have a similar explanation.

When red cells were suspended in dilutions of a hyperimmune rabbit anti-human antiserum (globulin content = 4 g/100 ml) containing large amounts of IgG (as



(b)



(c)

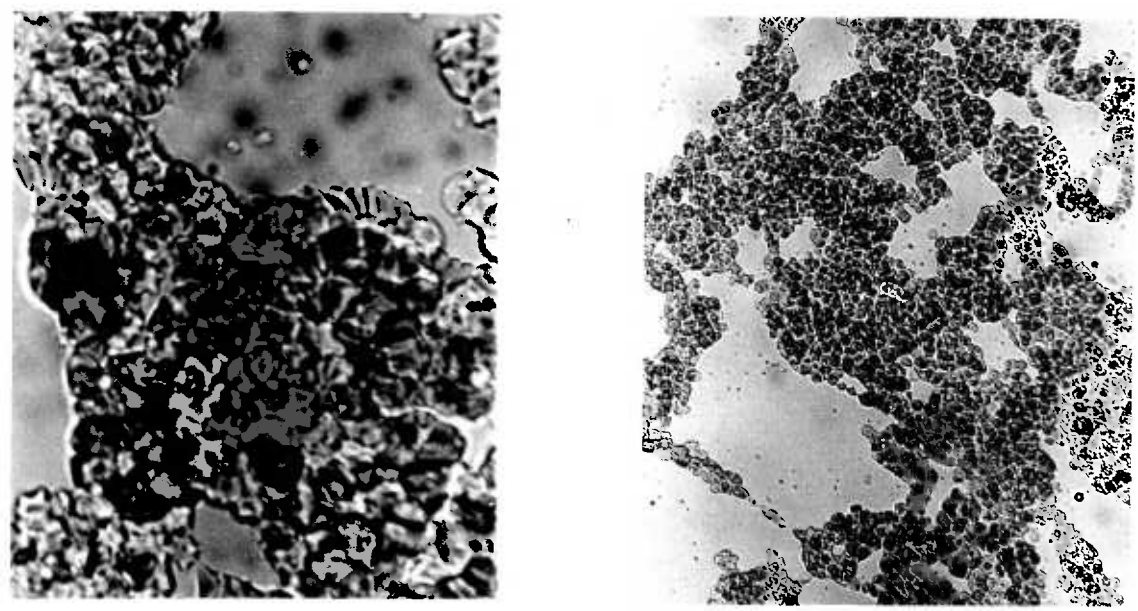


Fig. 53.

(d)

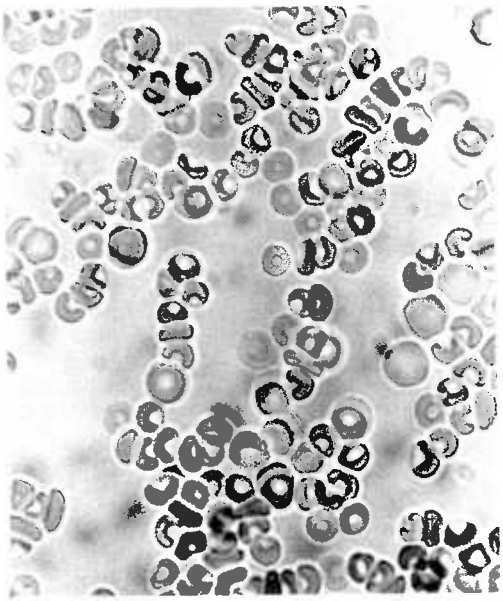


Fig. 53. Autoagglutination of normal rabbit red cells in various concentrations of rabbit fibrinogen x 100 and x 400 mag.

a) 250 mg/100 ml fibrinogen. Monodispersed cells.

b) 500 mg/100 ml fibrinogen.

c) 1000 mg/100 ml fibrinogen.

d) 500 mg/100 ml fibrinogen + 4 g/100 ml albumin x 400 mag. Note segregation of cells which have now become spherocytic.

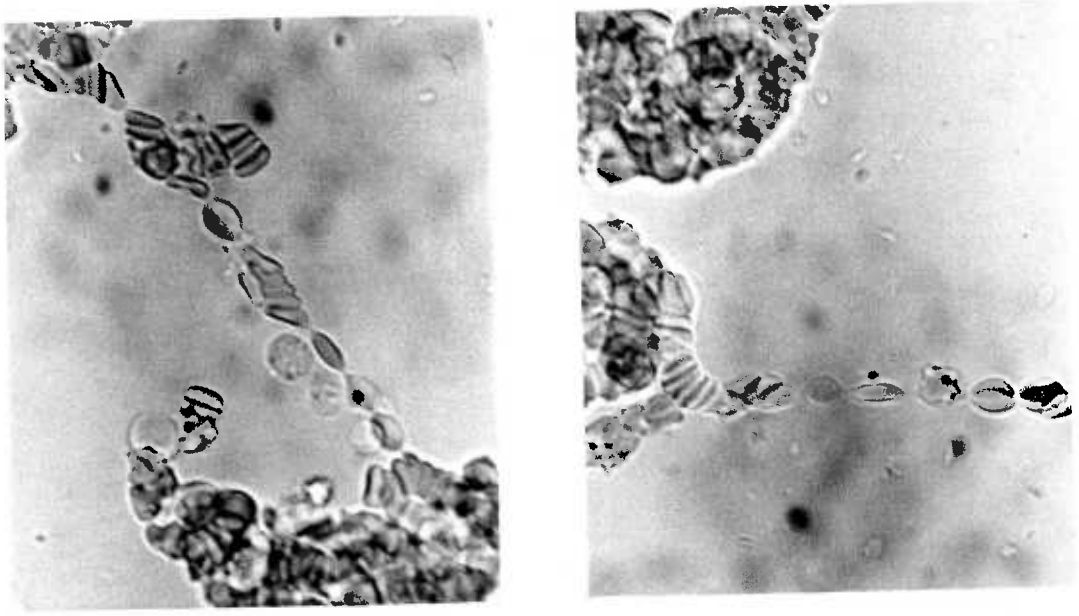


Fig. 54. Normal rabbit erythrocytes in a rabbit fibrinogen concentration of 1000 mg/ml. Red cells within rouleaux formation become greatly elongated and spindle shaped maintaining contact with each other via thin needle-like processes. The long axis of such cells is in the direction of streaming. x 400 mag.

demonstrated by immunoelectrophoresis), the ESR only increased slightly with an increasing γ-globulin concentration (Fig. 52 c). There was no marked clumping of the red cells.

Albumin (BSA) in concentrations ranging from 0-1000 mg/100 ml did not cause aggregation of red cells and consequently little variation in sedimentation (Fig. 52 b).

Removal of lipids from lipaemic serum of infected rabbits by the ether extraction method of Weitz (1952) indicated that lipids have a slight effect in raising the ESR (Table XXII).

From these experiments it would appear that the erythrocyte autoagglutination and sedimentation are largely governed by a non-immunological mechanism i.e. alterations in the colloid composition of plasma during infection. Increases in proteins with a high molecular weight such as fibrinogen, are the main factors responsible for the major increase in the ESR by causing red cell clumping. The lower molecular weight proteins such as γ-globulin and albumin contributing to a small extent. The large amounts of 19s IgM produced during infection will probably have an effect on red cell sedimentation but have not been investigated here.

Day	Lipaemia	Infected serum	Ether extracted infected serum
28	++	$3 \cdot 51 \times 10^{-2}$	$2 \cdot 19 \times 10^{-2}$
35	+++	$5 \cdot 45 \times 10^{-2}$	$2 \cdot 92 \times 10^{-2}$
49	++++	$6 \cdot 12 \times 10^{-2}$	$2 \cdot 92 \times 10^{-2}$

TABLE XXII. Effect of lipid removal from the serum by ether extraction on the ESR of rabbit S370 infected with *T. (T.) brucei* 427

++ = moderately raised lipid level (1000-1500mg/100ml)

+++ = high lipid level (1500-2000mg/100ml)

++++ = very high lipid level (>2000mg/100ml)

The importance of the above findings will be discussed in detail under a later Section (Part 4). However it must be remembered that these *in vitro* findings will reflect the situation *in vivo* only if similar conditions prevail i.e. a temperature of about 37°C and low blood flow or capillary stasis. This latter feature certainly occurs in *T. (T.) brucei* infections of the rabbit (Goodwin, 1970).

Immunofluorescent studies

From the previous studies it can be visualised that fibrinogen coats red cells rendering them adherent to one another. To investigate this situation blood smears were made from four infected rabbits (S384-S386) at weekly intervals during infection and stained with a goat anti-rabbit fibrinogen conjugate. The same proceedure was carried out for two control non-infected rabbits (S381 and S382).

In all blood smears, there was a visible fluorescence around the periphery of the red cells. However this was very faint except for smears taken from 3 of the infected rabbits where a 3+ fluorescence was seen (Table XXIII). The fluorescence occasionally took on a granular appearance on the red cell membrane.

The results therefore indicate that fibrinogen is absorbed from the plasma onto the red cell membrane in both normal and infected rabbits. The amount absorbed (as judged by the intensity of the fluorescence) appears to be dependent on the concentration of fibrinogen in the plasma.

Rabbit Number	Days after infection				
	0	7	14	21	28
S381 CONTROL	1+	2+	1+	1+	±
S382 CONTROL	±	1+	2+	1+	1+
S383	±	3+	2+	2+	2+
S384	±	1+	1+	3+	±
S385	±	2+	2+	2+	±
S386	1+	2+	1+	3+	±

TABLE XXIII. Degree of fluorescence of red cells from normal and trypanosome infected rabbits following staining with GARF conjugate.

3.5. Blood viscosity in trypanosome infections

The marked increase in trypanosome infections of plasma proteins, particularly the larger molecular weight proteins such as fibrinogen and macroglobulins, together with the tendency for red cell autoagglutination leading to a high sedimentation rate, suggested a change might occur in blood viscosity. Any alteration tending towards an increase in blood viscosity would certainly cause pathological changes in microcirculatory function related to blood flow - the degree of pathogenicity being a function of the magnitude of the increase in viscosity.

3.5.1. Blood viscosity measurements in T. (T.) brucei infections of rabbits

The viscosity of whole blood plasma and serum was determined for 6 rabbits, 4 of which were infected with strain 427 trypanosomes (rabbits S347-S350); the other two animals (S345 and S346) kept as non-infected controls. Viscosity, measured at two shear rates 230 and 23^{sec-1}, was determined prior to infection and thereafter at weekly intervals during infection up to the 7th week. In addition, essential data for these studies, such as plasma protein changes (see Section 3.3; and Appendix I) and alterations in the haematocrit (Appendix I) were noted for all rabbits throughout infection.

(i) Whole blood viscosity (η WB)

Fig. 55 shows the mean η WB (mean $\pm$ S.E.) for the four infected rabbits plotted at weekly intervals. The

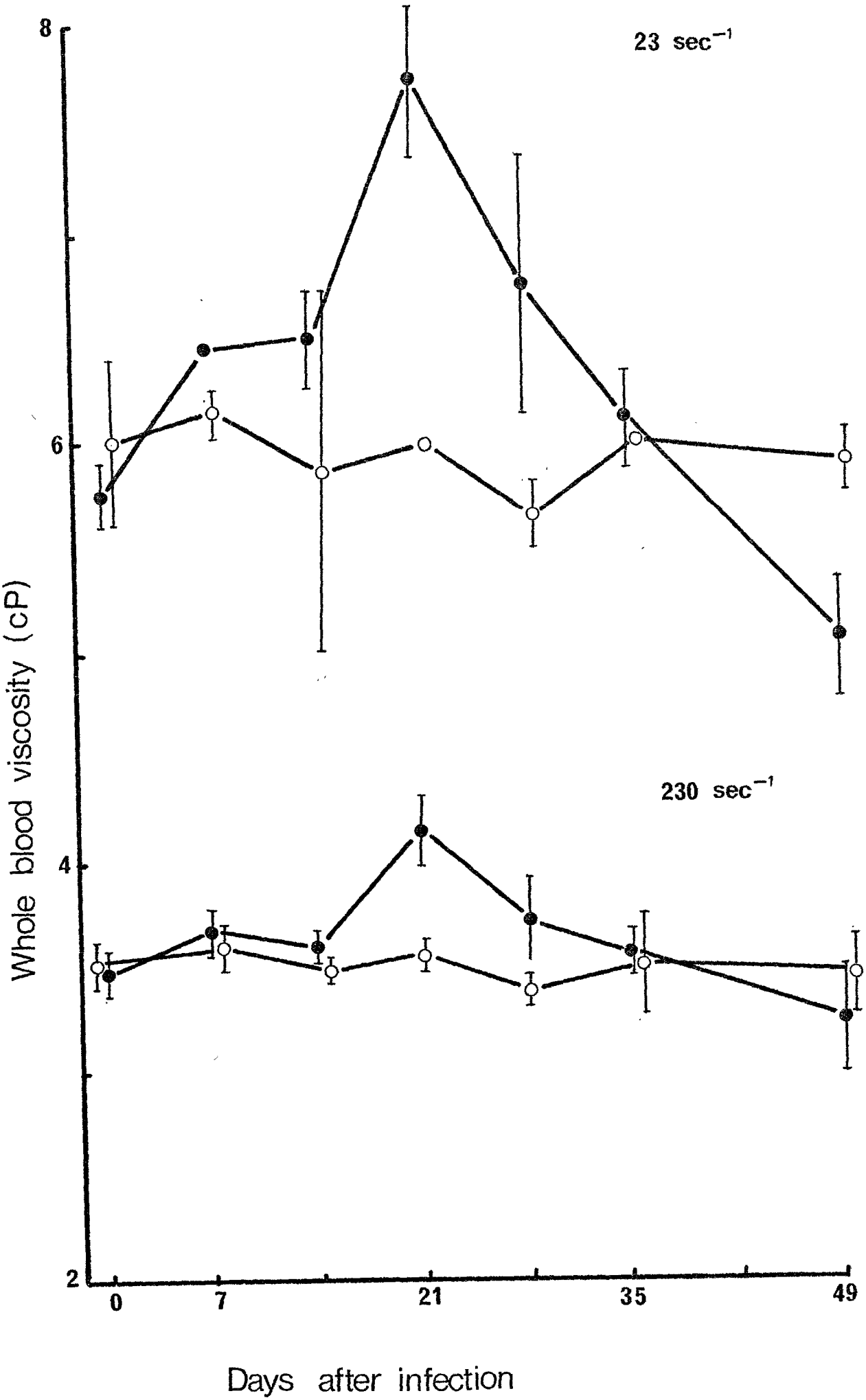

8
6
4
2
23 sec⁻¹
230 sec⁻¹
Whole blood viscosity (cP)
0
7
21
35
49
Days after infection

Fig. 55.

Fig. 55. Viscosity of whole blood in normal non-infected rabbits and rabbits infected with T. (T.) brucei 427 measured at two shear rates (23 sec^{-1} and 230 sec^{-1}).

●———● mean (± S.E.) for 4 infected rabbits

0———0 mean (± S.E.) for 2 control rabbits.

data for the control rabbits (mean ± S.E.) is given for comparison. It can be seen that the non Newtonian characteristic of blood is retained during all stages of the infection since an decrease in shear rate is accompanied by an increase in viscosity.

A rise in η WB was apparent after 7 days following innoculation with trypanosomes. By 21 days this rise had reached a peak increasing 33% at a shear rate of 23 sec^{-1}, and 19% at a shear rate of 230 sec^{-1} above pre-infection values. The η WB of the two control rabbits varied only slightly over the seven week investigation period variations being within a range of 0.5 cP. At the same shear rate (230 sec^{-1}) infected rabbits for the first two weeks of infection showed a range of 0.4 cP i.e. within the normal range for uninfected animals. However during the following weeks, the same rabbits showed a variation of 0.85 cP i.e. an increase of 70% above normal.

Thus the viscosity of blood reaches its highest value between days 21 and 28 of infection. It is probable that at this time the increased viscosity will have pathological effects within the microvasculature such as slowing of blood flow (see Section 4). The elevated η WB is obviously not due to increased haematocrit since at this time infected rabbits had decreased PCV's of between 35 and 31% (Appendix I).

From early studies on the flow of human blood in tubes with diameters < 300μ, Gelin (1961) has shown that

red cells become more concentrated in the faster axial stream with the result that the haematocrit decreases and the η WB is determined more and more by the viscosity of plasma. Thus a study of plasma viscosity is as important as that of the whole blood.

(ii) Plasma viscosity (η P)

The plasma viscosity for both infected and control rabbits, measured at shear rates of 230 sec^{-1} and 23 sec^{-1}, is shown in Fig. 56. Reflecting the picture of η WB, an increase in η P occurs at 21 days following infection, remaining high during the following weeks, then falling but not to pre-infection values as with η WB. The percentage increase in η P was 59% whereas with η WB the increase was only 33% greater than normal. This relatively small increase in η WB however can be ascribed to the anaemia that develops.

Another interesting feature to emerge from these results was the notable shear rate dependence of infected compared with non-infected plasma. The plasma normally only a slightly non-Newtonian fluid became markedly non-Newtonian. The reason for this observation almost certainly lies within changes of the colloid composition of plasma during infection particularly increases in large molecular weight proteins such as fibrinogen and macroglobulin. Statistical correlations between the various plasma proteins changes and viscosity would prove this point. In addition if fibrinogen plays a major

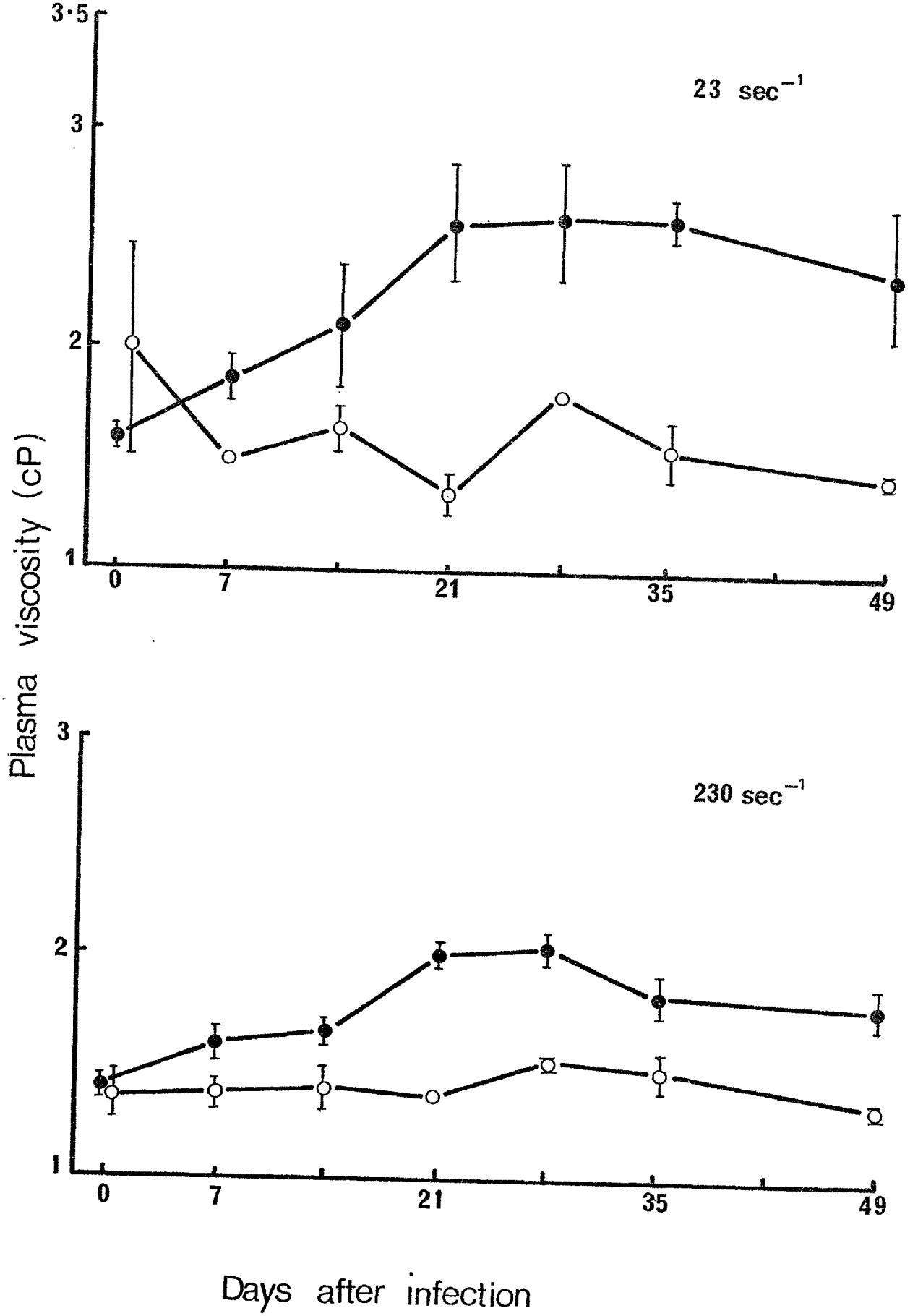

3·5
3
2
1
23 sec⁻¹
0
7
21
35
49
Plasma viscosity (cP)
3
2
1
230 sec⁻¹
0
7
21
35
49
Days after infection

Fig. 56.

Fig. 56. Viscosity of plasma in normal non-infected rabbits and rabbits infected with T. (T.) brucei 427 measured at two shear rates (23 sec^{-1} and 230 sec^{-1}).

●———● mean (± S.E.) for 4 infected rabbits.

O———O mean (± S.E.) for 2 control rabbits.

role in determining ηP then its removal should result in a lower serum viscosity.

(iii) Serum viscosity (ηS)

Fig. 57 indicates that removal of clottable protein results in only a slight reduction in viscosity e.g. only 0.1 centipoise units on day 21 of infection. Nevertheless, ηS increased and reached a peak co-incident with both ηWB and ηP - i.e. around day 21. It can be assumed therefore that at this point serum globulins are probably the most important factors involved in elevating the viscosity of plasma and serum and, because of this, also ηWB. Supportive evidence for this statement comes from the findings of maximal levels of α and β globulins (particularly β_2-macroglobulins) produced by infected rabbits at this time (Fig. 34, Section 3.3.).

3.5.2. Statistical analyses of results

In order to examine in detail the effects of alteration in the plasma protein composition, during infection, on blood viscosity, statistical correlations were made by Dr. G. Murdie between plasma protein constitutents and the viscosity of whole blood, plasma and serum.

Correlation co-efficients (r) calculated between the various protein levels and ηWB, ηP and ηS at a shear rate of 230 sec^{-1} are shown in Table 25. These can be divided into 3 groups depending on the levels of significance found:

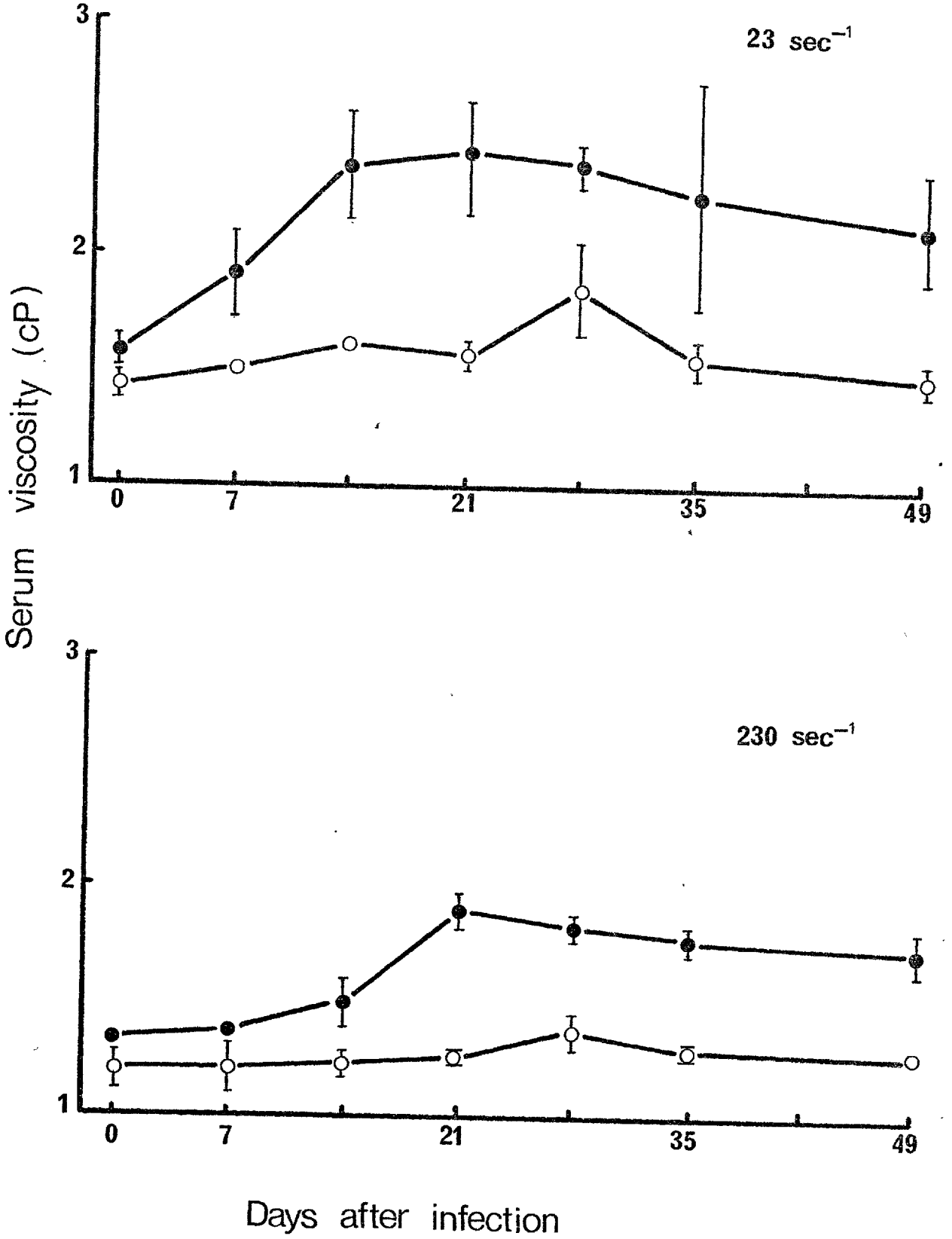

23 sec⁻¹
230 sec⁻¹
Serum viscosity (cP)
3
2
1
0
7
21
35
49
Days after infection

Fig. 57.

Fig. 57. Viscosity of serum in normal non-infected rabbits and rabbits infected with T. (T.) brucei 427 measured at two shear rates (23 sec^{-1} and 230 sec^{-1}).

●———● mean (± S.E.) for 4 infected rabbits.

O———O mean (± S.E.) for 2 control rabbits.

	η_{WB}	η_P	η_S
F	Normal rabbits r =0·61 P =0·1-0·01% 1-2 wks. infection r =0·16 P =<5·0% 3-6 wks. infection r =0·836 P =<0·001%**	r =0·561 P =0·1-0·01%*	r =0·847 P =0·1-0·01%*
G	N/T	r =0·766 P =0·1-0·01%*	r =0·884 P =<0·001%**
TSP	r =0·281 P =>0·5%	r =0·532 P =0·01*	N/T
F+G	1 & 2 wks. infection r =0·28 P =>0·5% 3 - 4 wks. infection r =0·812** P =<0·001%	N/T	N/T

** High significance

* Moderate significance

TABLE XXIV. Correlation co-efficients (r) and probabilities (P) for infected rabbits between changes in whole blood viscosity (η_{WB}); plasma viscosity (η_P); serum viscosity (η_S) and alterations in the concentration of plasma fibrinogen (F); globulin (G) and total protein (TSP).

a) High significance (P = 0.001%)

FIBRINOGEN V. η WB from the second week to termination of the infection (Fig. 58).

FIBRINOGEN + GLOBULIN V. η WB for the same period of time as above.

GLOBULIN V. η S during the whole period of infection (Fig. 59).

b) Moderate significance (P = 0.1 - 0.01%)

FIBRINOGEN V. η WB in a group of control non-infected rabbits.

FIBRINOGEN V. η P for infected rabbits.

FIBRINOGEN V. η S during infection.

GLOBULIN V. η P during infection (Fig. 61).

TOTAL SERUM PROTEIN V. η P throughout infection (Fig. 60).

c) No significance (P = < 0.05%)

FIBRINOGEN V. η WB from the second week of infection onwards.

TOTAL SERUM PROTEIN V. η WB during infection.

FIBRINOGEN + GLOBULIN V. η WB for the initial 14 days of infection.

Thus a number of significant correlations were observed: globulin with serum viscosity; fibrinogen with plasma viscosity. Increases in fibrinogen and globulin were also correlated with η WB - this effect probably due to these proteins causing aggregation of red cells - a feature known to cause a significant rise in η WB at low shear rates (Elliot, 1973).

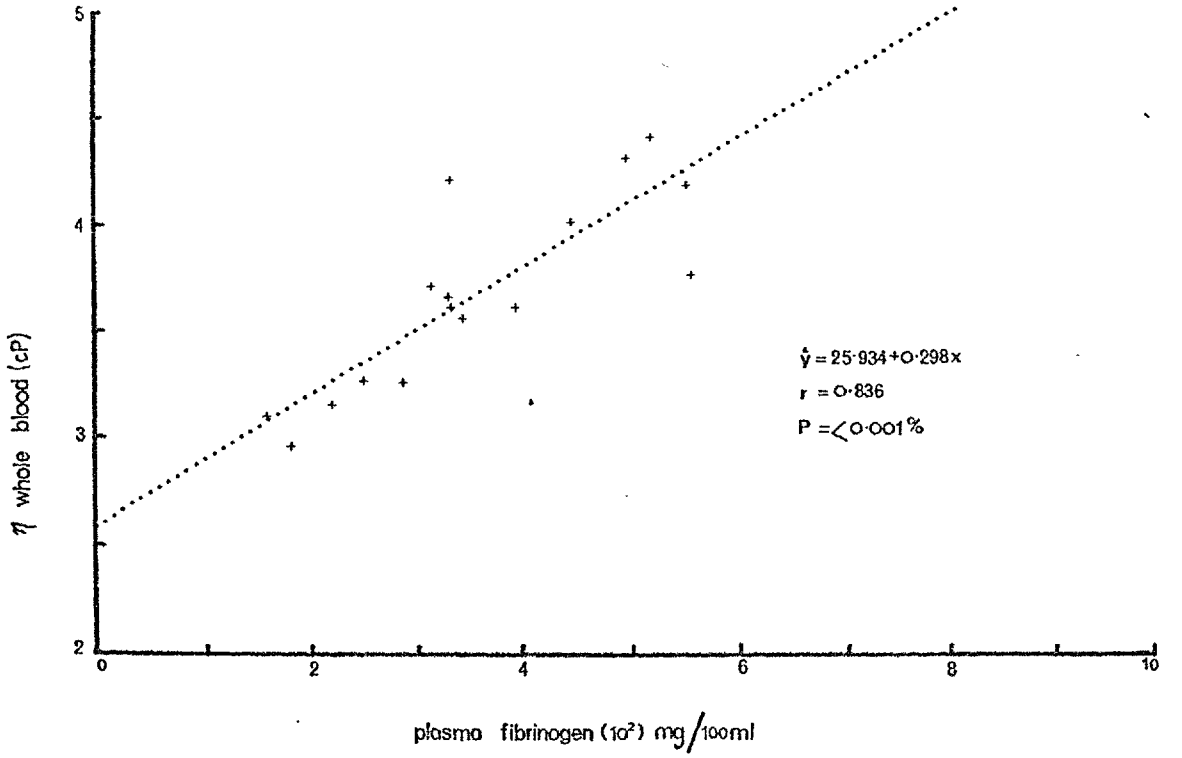

η whole blood (cP)
plasma fibrinogen (10²) mg/100ml
ŷ = 25·934+0·298x
r = 0·836
P =< 0·001%

Fig. 58.

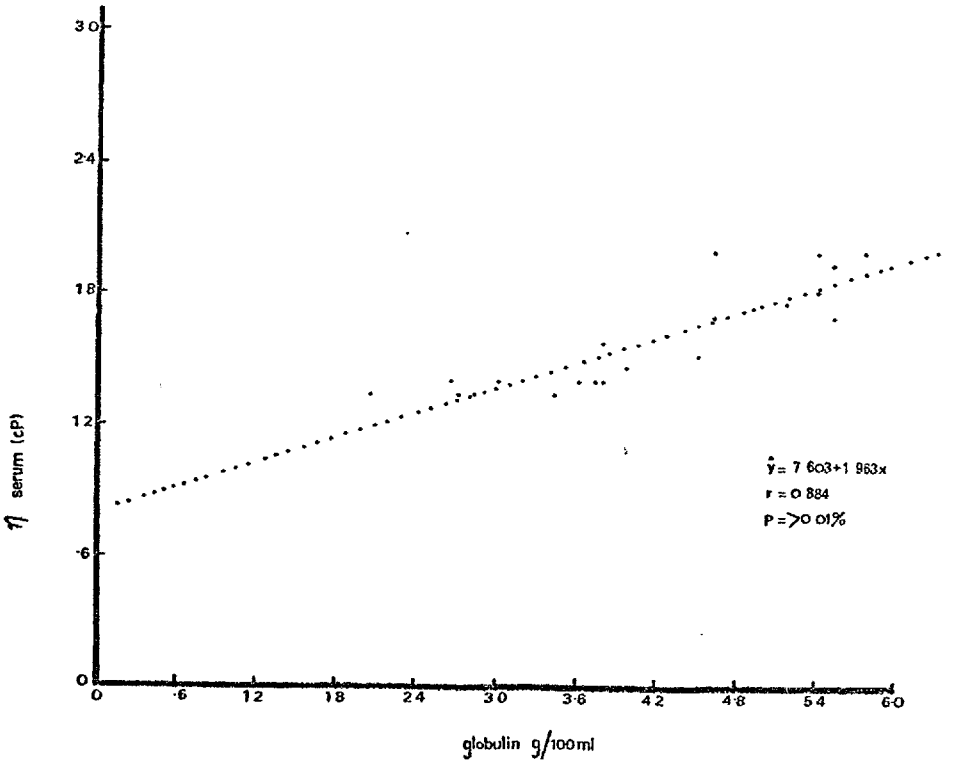

η serum (cP)
globulin g/100ml
ŷ = 7 603+1 963x
r = 0 884
P => 0 01%

Fig. 59.

Fig. 58. The relationship between η WB and plasma fibrinogen concentration in infected rabbits. The line represents the regression equation $y = 4.267 + 1.817\ x$.

Fig. 59. The relationship between η serum and serum globulin concentration in infected rabbit's represents the regression equation $y = 7.603 + 1.963\ x$.

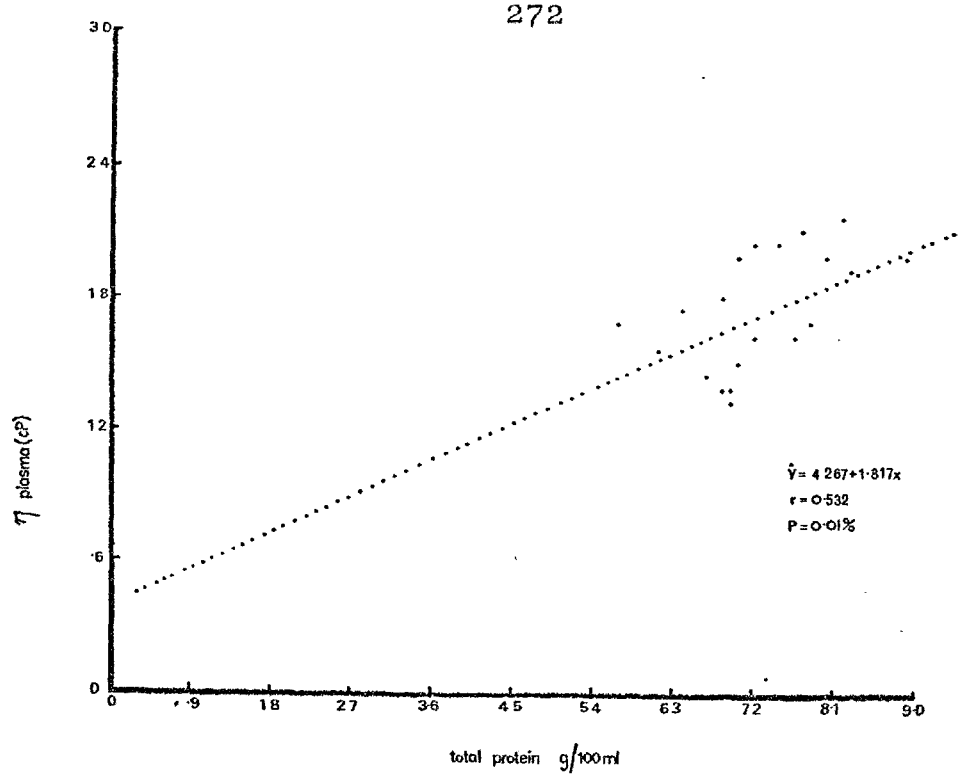

30
24
18
12
6
0
η plasma (cP)
0
·9
18
27
36
45
54
63
72
81
90
total protein g/100ml
Ŷ = 4·267+1·817x
r = 0·532
P = 0·01%

Fig. 60.

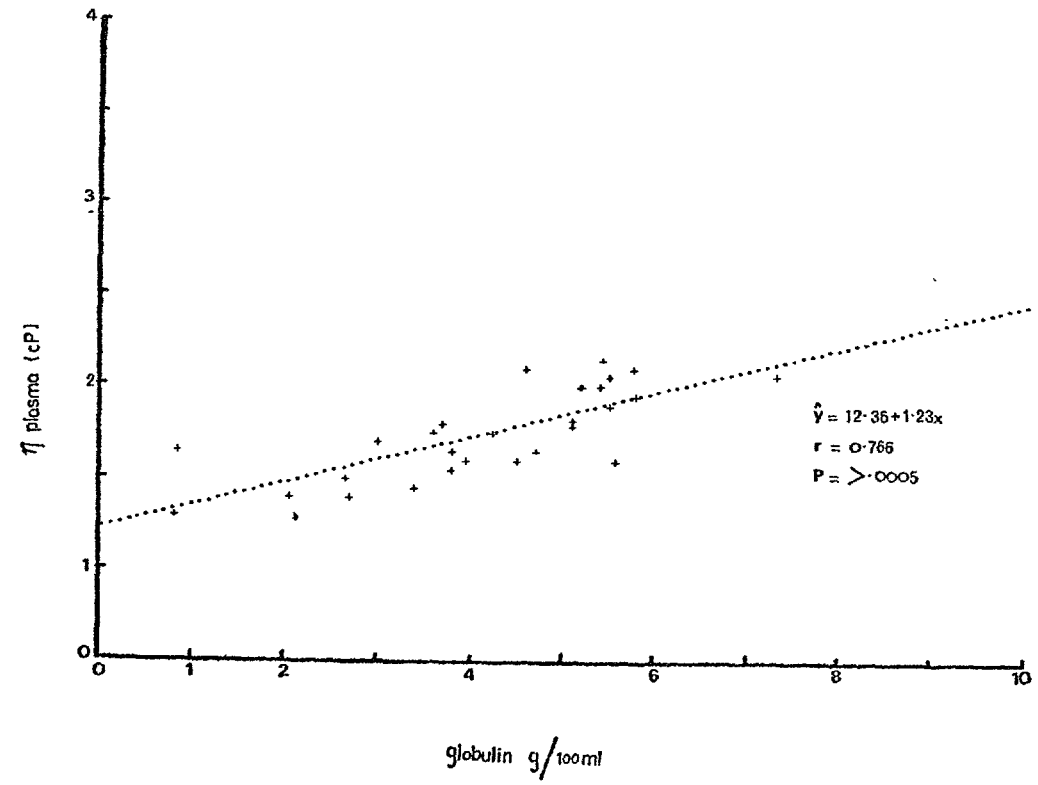

4
3
2
1
0
η plasma (cP)
0
1
2
4
6
8
10
globulin g/100ml
Ŷ = 12·36+1·23x
r = 0·766
P = >·0005

Fig. 61.

Fig. 60. The relationship between η plasma and serum total protein concentration. The dotted line represents regression equation y = 4.261 + 1.817 x.

Fig. 61. The relationship between η plasma and serum globulin concentration. The dotted line represents regression equation y = 12.36 + 1.23 x.

Multiple correlation co-efficients were calculated, and analysis of variance for the multiple linear regressions were made for the correlation between:-

a) fibrinogen, globulin, albumin and η P for the first 4 weeks of infection.

b) fibrinogen, globulin, PCV and η WB during the same time interval.

Regressions were significant for both with multiple correlation co-efficients of 0.87 and 0.71 respectively.

PART 4: DISCUSSION

A series of studies have been carried out in an attempt to demonstrate and elucidate some of the changes that occur within the blood during trypanosome infections.

The first group of studies clearly demonstrates that an increased activity of the fibrinolytic system occurs in both experimental infections of rabbits infected with T. (T.) brucei and in man infected with T. (T.) rhodesiense. The elevated FDP levels in this study were not due to in vitro fibrinolysis since serum for the FDP assay was obtained with the addition of 'Trasylol' a fibrinolytic inhibitor. An increase in serum FDP provides evidence for either primary activation of the fibrinolytic system (i.e. pathological fibrinogenolysis) or secondary activation following intravascular coagulation (Merskey et al 1959).

Zebu cattle infected with either T. (T.) brucei sub-group trypanosomes or T. (N.) congolense did not show a significant increase in serum FDP during a five to six week period of infection. It was considered possible that the results obtained were misleading since immuno-diffusion experiments indicated that an autiserum to either human or rabbit fibrinogen did not cross-react with bovid fibrinogen confirming the findings of Schwick (1964). A modification of the assay using a homologous system indicated relatively high levels of FDP in the cattle before innoculation with parasites. No signi-

ficant elevations occurred during infection. There are two explanations for these findings. First, the processes initiating increased fibrinolysis in rabbits and man do not exist in cattle trypanosomiasis. A second explanation is that cattle FDP have a shorter half-life and, because of this, unless serum samples are taken at shorter time intervals e.g. hourly intervals, during infection, a transient change in FDP levels will be missed. Species differences of the fibrinolytic system have been observed (Niewiarowski & Latallo, 1959).

A comment on the method used to measure FDP must be made. Although the TRCHII is a sensitive and rapid assay method for FDP, it does pose certain limitations. It utilizes an anti-fibrinogen serum and therefore intact fibrinogen will be detected as FDP if not totally removed from the serum. Effort was therefore made to ensure complete removal of all thrombin-clottable material. Unfortunately this had the disadvantage in that not only fibrinogen was removed but also the 'early' clottable FDP (Fragment X) capable of complexing with fibrinogen. Since 'early' and 'late' FDP have different biological properties (Triantaphyllopoulos & Triantaphyllopoulos, 1970) it was necessary not only to quantitate but also characterise the fragments involved. Immunoelectrophoresis of plasma proteins following Sephadex G200 column chromatography indicated that Fragments X and D were present in the circulation of infected rabbits. Fragments Y and E were not demonstrated possibly because

they were present in too low a concentration to be detected by this method.

There are several explanations for the raised FDP found in trypanosome infections:-

1) Endogenous activation of the fibrinolytic system via. tissue or plasma kinase.

2) Exogenous activation via. trypanosome material or antigen-antibody complexes either trypanosomal or non-trypanosomal.

3) Release of protease enzyme activity from degenerating leucocytes (Gans, 1964).

4) Direct proteolytic activity of trypanosomal material.

Evidence that activation of the plasminogen - plasmin system is a major pathway for the observed increased fibrinolysis, comes from the results of serum plasminogen assays. At peaks of FDP, the amount of plasminogen was reduced by half. Experimental inhibition of plasmin and plasminogen by EACA and 'Trasylol' indicated that immediately plasmin was inhibited, FDP levels fell, and the low concentration was maintained for at least two weeks following drug-treatment. This provides additional evidence that FDP are formed by the proteolytic activity of plasmin on either fibrinogen or fibrin. The question remains as to whether the activation is endogenous or exogenous. In fact, an examination of either possibility, it is likely that both mechanisms of activation occur in trypanosomiasis.

Endogenous activation via. plasma kinase, tissue kinase, cathepsins and other proteolytic enzymes from cells (including phagocytes), is most probable. Release of these substances occurs following tissue damage, particularly damage to the endothelium which is rich in tissue activator (Astrup, 1966). It has been previously shown by Goodwin (1971) that a vast amount of connective and vascular tissue damage occurs in T. (T.) brucei infections of rabbits. The pathological processes taking place in the venules and capillaries first occurred between the 12th and 14th day of infection. This period of time co-incides with the observations here of the time of maximum increases in FDP. Goodwin (1971) showed that phagocytes first adhered to the vascular endothelium followed by collections of platelets and circulating leucocytes which eventually caused occlusions and the blood vessels disintegrated. Such a situation would be associated with the local release of plasminogen activator from the endothelium. Damage to other rabbit tissues is unlikely to contribute much more to increased fibrinolysis. No plasminogen activation could be observed using the Astrup plate method when testing a variety of rabbit organ tissues including liver, kidney, heart and brain. This observation is in agreement with that of Astrup and Buluk (1963) who also found that rabbit tissues (apart from the vascular endothelium) were particularly low in fibrinolytic activity. This was believed to be the

reason for the known response of fibrosis after injury in the rabbit.

The possibility that trypanosomes might be causing exogenous activation was considered. The *in vitro* test system utilising a soluble fraction of trypanosome homogenates and a plasminogen rich fibrinogen solution, indicated that trypanosomal protein does activate plasminogen. It was found that complete hydrolysis of fibrinogen occurred over a 24 h period if incubated with the trypanosome homogenate. After approximately 18 h of incubation, an accelerated breakdown was explained by the growth and activity of bacteria since addition of streptomycin to the samples prevented the total degradation. EACA only partially inhibited the reaction indicating that although a certain degree of plasminogen activation is stimulated by trypanosomal material, it cannot explain the overall breakdown of fibrinogen.

This observation led to the suggestion that trypanosomes might possess proteolytic enzymes capable of degrading fibrinogen. This was confirmed by the results obtained when 'Trasylol', a potent inhibitor of a wide range of proteolytic enzymes, was added. Following the addition of 'Trasylol' to the trypanosome/fibrinogen samples, a very marked reduction in the amount of fibrinogen degraded was apparent. Thus an enzyme found in T. (T.) *brucei* extracts, possibly lysosomal in origin (Venkatesan & Bard, 1973), has a prediction for a mammalian substrate (fibrinogen) and can be

inhibited by 'Trasylol' (an inhibitor usually specific for mammalian enzyme systems). Electrophoretic characterisation of the digests indicated two protein components with electrophoretic mobility common to those of Fragments D and E. However, care must be taken in comparing the two enzyme systems. The trypanosome enzyme will not necessarily degrade fibrinogen in the same manner as plasmin. Even other mammalian enzymes e.g. trypsin and chymotrypsin degrade fibrinogen in a similar, but not identical, sequence to plasmin (Gormsen et al, 1973). It is possible that the trypanosome enzyme hydrolyses the same arginine and lysine bonds susceptible to plasmin, but this requires further investigation. It would be of interest to examine the effects of intravenously administered concentrated trypanosome extracts into rabbits. An immediate rise in serum FDP might be expected.

In many respects, the events which occur after treating trypanosomiasis patients with trypanocidal drugs resemble the Jarisch-Herxheimer reaction of syphilis (de Raadt & Onyango, 1965; Boreham, 1968a). Perine et al (1971) showed that treatment of louse-borne relapsing fever with tetracycline caused a sudden rise in the serum concentration of FDP of patients providing evidence for increased fibrinolysis attributed to intravascular clotting in two of the patients. In the present studies, drug treatment of rabbits with Mel-B two weeks after infection when FDP levels were low or at the time of

maximum FDP appearance in the serum, did not increase the level of circulating degradation products further. Since these two experiments were carried out on rabbits infected with T. (T.) brucei 427, only low parasitaemias were apparent at the time of drug cure. In order to achieve a high parasitaemia rabbits were innoculated with T. (T.) brucei S42. Three of the four rabbits used in this experiment which were treated with Mel-B when the parasitaemia was high, showed an increase in serum FDP within 1 h of cure. Since Mel-B alone did not induce fibrinolytic activity in a control animal, the increased fibrinolysis must be associated with either the release of trypanosomal material possessing proteolytic activity (thereby supporting the in vitro studies previously discussed); or the direct or indirect (via. Hagemann factor) of immune complexes on plasminogen. Since Mel-B acts by inhibiting glycolysis of the trypansome thereby causing immobilisation rather than disruption of the parasite (Flynn & Bowman, 1968), it is probable that activation is by immune complexes.

Immune complexes have been shown to affect the microcirculation in two ways. In the presence of reticulo-endothelial 'blockade' they cause intravascular coagulation with obstruction of capillaries (especially in renal glomeruli) by microthrombi (Evensen et al, 1972). When they are injected into unprepared animals, the resultant prolifertive glomerulonephritis is associated with fibrin deposition and reactive fibrin-

olysis (Humair et al, 1969). Immune complexes are formed during trypanosome infections and it is believed that they are responsible for the release of plasma kinins (Boreham, 1968b). It was suggested that immune complexes cause release of kinins by activation of Hageman factor which in turn activates prekallikrein to kallikrein (Boreham & Goodwin, 1970). Kallikrein is a kininogenase enzyme which converts kininogen to kinin, a biologically active peptide. Plasminogen may be activated indirectly through Hageman factor in the presence of immune complexes (Iatridis, 1966). In addition in vitro studies by Ungar and Mist (1949) indicated that immune complexes can also activate plasminogen directly.

Considerable activation of the plasminogen - plasmin system has been demonstrated in rabbits following induction of anaphylactic shock (Gans & Krivit, 1961). Evidence for activation was obtained by observations of a decrease in plasminogen concentration and rise in plasminogen activation activity (PPA). Whether the activation was associated with biologically active amines released during anaphylactic shock, or due to the action of immune complexes formed following administration of the shocking antigen dose, was not considered by the authors.

Simpson (personal communication) found that antigen administration to rabbits sensitised to ovalbumin, or rabbits given pre-formed complexes, showed reactive

fibrinolysis as indicated by a rapid overshoot in PPA. It would be of interest to investigate whether pre-formed trypanosome antigen-antibody complexes *in vitro* or complexes formed *in vivo* have a similar effect.

Stress states such as pyrexia cause activation of fibrinolysis (Sherry *et al*, 1959). Rabbits infected with trypanosomes are continually under a state of stress due to parasitaemic crises and antigenic variation of trypanosomes. Body temperature increases have been described in rabbits infected with *T. (T.) brucei* (Goodwin, 1970), in cattle infected with *T. (D.) vivax* (Unsworth, 1953) and in man infected with *T. (T.) rhodesiense* (Bailey & Boreham, 1969). Stress, therefore, might represent yet another mechanism for fibrinolytic activation.

Several biological properties have been assigned to FDP and include:

1. Direct antithrombin effect.
2. The inhibition of an orderly polymerisation of fibrin monomer to fibrin resulting in faulty clot structure.
3. Formation of unclottable complexes with fibrin monomer.
4. Inhibition of platelet aggregation (Triantaphyllopoulous and Triantaphyllopoulos, 1970).
5. Potentiation of biogenic amines and peptides (Barnhart, 1969).

6. Increased vascular permeability (Triantaphyllopoulos and Triantaphyllopoulos, 1970; Sack & Nefa, 1970).
7. Increased resistance of red cells to hypertonic solutions (Triantaphyllopoulos and Triantaphyllopoulos, 1970).
8. Regulation of plasma fibrinogen concentration by controlling the synthesis and release of hepatic fibrinogen (Barnhart et al, 1970).

The first four properties will have a demonstrable inhibitory effect on the blood coagulation process. This can be beneficial in inhibiting any hypercoagulable state that might have initiated the cycle. It is important to note that the first three mechanisms listed above lead to prolonged thrombin times while the fourth results in prolonged bleeding times and increased capillary permeability. Both latter features occur in trypanosome infected rabbits although prolonged bleeding times are usually only observed late on in the infection (unpublished observations).

During studies on the release of pharmacologically active substances during trypanosome infections, it has been noted that if infected serum is added to isolated guinea-pig ileum, subsequent doses of acetylcholine, 5-hydroxytryptamine, histamine and bradykinin are potentiated (Goodwin & Richards, 1960; Bercham, personal communication). The factor/factors responsible for this potentiation are unknown, but since FDP are present in

infected serum they must certainly be possible candidates. However washed trypanosomes also cause potentiation but do not contain FDP (Boreham, unpublished).

One of the pathological changes in experimental trypanosomiasis which is believed to be important in the events leading to the eventual death of infected animals, is the increase in vascular permeability which has been noted (Boreham & Goodwin, 1967; Goodwin & Hook, 1968). A number of causes of this permeability have been postulated which include the release of pharmacologically active substances, particularly kinins (Boreham, 1968a, 1970), the permeability factor present in trypanosomes (Seed, 1969). and the direct action of immune complexes on the vascular endothelium. The presence of high concentrations of serum FDP in infected rabbits provides yet another mechanism. Barnhart (1969) has shown that Fragment E causes a larger and greater vascular permeability in the intradermal test in rabbits than histamine.

It is not possible to say whether one or more of these factors are responsible for the increased vascular permeability, but almost certainly they will contribute to the observed change.

It is unlikely that FDP have any effect on the membrane stability of erythrocytes and so contribute to the observed anaemia because no change is seen in the fragility of erythrocytes during trypanosome infections of the rabbit (MacKenzie and Boreham, in press).

It should be noted that all the work to date on the biological properties of FDP result from *in vitro* digestion of fibrin and fibrinogen by proteolytic enzymes. There is no evidence to show that FDP formed *in vivo* in trypanosome infections will have the same properties, although there are no reasons to suppose otherwise. In order to settle this point it would be necessary to separate FDP from other serum proteins and test their biological activity.

Activation of the fibrinolytic system not only causes circulatory disturbances due to the formation of FDP, but plasmin in addition to degrading fibrinogen and fibrin, also degrades specific coagulation factors and inactivates complement (C') by hydrolysing C'_1 esterase (Fletcher *et al*, 1962). Thus the activation of plasminogen to plasmin would limit the action of C' on the lysis of trypanosomes unless compensatory production of C'_1 esterase occurred. In addition fibrinogen and its derivatives are powerful anti-complementary substances (Barnhart, 1969) in common with all large molecular weight substances. High levels of these proteins in infected rabbits might explain the increasing anti-complementary activity of rabbit sera observed by Mac-Kenzie (1973).

Since increased FDP are found in trypanosomiasis, one might expect a corresponding decrease in plasma fibrinogen due to the activated fibrinolytic system. However in rabbit, cattle and human infections, the

concentration of fibrinogen actually increases. Since the increase was very marked - up to 800 mg/100 ml plasma in infected rabbits - the only explanation is of an increased synthesis in excess of its catabolism. In normal rabbits it has been shown that the catabolism of fibrinogen proceeds at a remarkably constant rate, the same fraction of the pool being broken down daily, regardless of the size of the pool (Recoeczi et al, 1963). Once a stimulus for fibrinogen production has been applied, the rate of synthesis of this protein is extremely rapid. For example, following defibrination in the dog, the circulating fibrinogen was restored to normal within 1 h (Forman and Barnhart, 1964). A similar situation exists in infected rabbits where marked increases in fibrinogen occurred within the first few days following innoculation with trypanosomes. In addition the "switch-off" mechanism for fibrinogen synthesis is just as rapid since, following chemotherapy, there was a dramatic fall in plasma fibrinogen to almost normal values within 1 h of cure. There is evidence that other proteins are synthesised more rapidly in trypanosomiasis for example α_2 globulin (Boreham, 1968a).

The mechanisms responsible for increased fibrinogen synthesis in disease states is poorly understood. Alterations in fibrinogen metabolism frequently occur in physiologic adaptations to stress and also accompany many pathological states particularly those involving inflammation (Barnhart, 1968; McSherry et al, 1970).

In trypanosomiasis extensive inflammation occurs as a result of a variety of causes (see review by Goodwin, 1974). In the present studies it was found that although trypanosomal material when injected into normal rabbits did not cause a non-specific release or synthesis of fibrinogen, trypanosome material given in combination with Freunds Complete Adjuvant (FCA) or FCA given alone, both caused a slight increase in plasma fibrinogen. FCA is known to cause granuloma formation and ulceration at the site of injection (Humphrey and White, 1971). This condition occurred in the rabbits employed in the present experiments and the severity of inflammation, as judged visually by the size and state of the lesions, correlated well with the degree of plasma fibrinogen elevation.

It has been shown that FDP, when injected intravenously into dogs, causes an increase in plasma fibrinogen (Barnhart et al, 1970; Triantaphyllopoulos and Triantaphyllopoulos, 1970). Later experiments by Simpson et al (1973) showed a similar situation to occur in rabbits. The precise cellular mechanisms responsible for these observations are unknown although the immediate rise in fibrinogen following FDP infusion must reflect the release of pre-formed fibrinogen from tissue stores since synthesis could not take place in such a short time. The sustained rise that followed might be due to the direct action of FDP on hepatocytes or could result from the depletion of fibrinogen from

these cells caused by the earlier release. It is difficult to know how these two mechanisms could be differentiated. Simpson et al (1973) also found that human FDP (fibrinogen - derived) appeared as efficient in stimulating fibrinogen increase as rabbit FDP (fibrin - derived) presumably reflecting their chemical similarity - a suggestion also confirmed in the present studies.

Large pharmacologic doses of corticosteroids have been shown to effect fibrinogen metabolism when injected intramuscularly into rabbits (Seligsohn et al, 1973). Alterations in the endocrine system during traypanosome infections have, as yet, not been fully investigated. However changes have been recently implicated. Murray et al (1974) investigating the pathology of trypanosomiasis of the rat caused by T. (T.) brucei found massive trypanosome invasion of the adrenal cortex and medulla and suggested that this may well have led to serious hormonal imbalance. This is obviously an area in the pathology of trypanosomiasis that deserves further investigation.

The most important conclusion from the findings of raised FDP, increased fibrinogen and decreased plasminogen, is that it is suggestive of the occurrence of low-grade diffuse intravascular coagulation (DIC) in association with T. (T.) brucei infections in rabbit. In addition to these three observations other features are used in the diagnosis of DIC viz. qualitative changes

in plasma fibrinogen, the presence of serum fibrin-DP and fibrin monomer, the demonstration of fibrin deposits in the microvessels and abnormalities in the standard blood coagulation tests (Fletcher & Alkjaersig, 1973).

One assay for distinguishing blood "hypercoagulability" is the demonstration of cryofibrinogen. Cryofibrinogen was found to be present in measurable amounts about three weeks after infection of rabbits with trypanosomes. Thomas et al (1954) found that a typical feature of the Sanarelli - Shwartzman reaction (SSR) in rabbits was the appearance of a cryoderivative of fibrinogen which precipitated only in heparinised plasma (HPF). This same abnormal fibrinogen has been described in a multitude of inflammatory diseases (Smith, 1957). In the present studies HPF was not demonstrated in normal rabbits but appeared in the plasma of rabbits infected with trypanosomes within two weeks of infection and appeared to increase as the infection progressed.

The precipitate, fibrin strands or gel which forms in plasma after the addition of low concentrations of protamine sulphate is indicative of the presence of fibrin-DP or fibrin monomer (Gurevich, 1973; Gurevich et al, 1973; Schorn, 1973). The protamine sulphate test is insensitive to fibrinogen-DP and thus is capable of differentiating between fibrinogenolysis and fibrinolysis. The presence of either fibrin-DP or fibrin monomer indicates secondary fibrinolysis as a

consequence of intravascular coagulation. Since all infected rabbits gave positive reactions in the protamine sulphate test, most only one week after infection, it can be assumed that a proportion of the degradation products measured by the TRCHII originated from the lysis of fibrin.

The presence of another "abnormal" fibrinogen, "Fibrinogen-B", was also found in trypanosome infected rabbits. "Fibrinogen-B" is thought to be an intermediate between fibrinogen and fibrin (but is not fibrin monomer) and has previously been described to appear in the plasma following thromboembolic complications in human patients (Cottan & Craven, 1970). The presence of "Fibrinogen-B" in infected rabbits is thus yet another indication of microthrombi formation.

Additional evidence of DIC in rabbit trypanosomiasis would come from findings of fibrin deposition within the microcirculation. However, no fibrin could be demonstrated intravascularly in any of the rabbit agar tissues examined. Such findings are perhaps not surprising in view of the fact that pathological evidence for clinically documented DIC in humans and animals is frequently difficult to obtain even using sophisticated staining techniques (McKay, 1969; Pitney, 1971). Where DIC is suspected in other infections e.g. Plasmodium falciparum malaria in humans (Reid & Nkrumah, 1972), fibrin has not been demonstrated.

There are three possible explanations for the apparent absence of intravascular fibrin. First, any fibrin formed may be readily phagocytosed by the activated macrophage system in trypanosomiasis. Fibrinogen and its derivatives have been shown to be the most potent chemotactic agents for granulocyte (predominately neutrophil) emigration in inflammation (Barnhart, 1968). Furthermore, Barnhart's (1969) observations have established that formation of antigen-antibody complexes in any medium containing fibrinogen, removes some of it from the solution suggesting a role of fibrinogen in phagocytosis by the coating of complexes.

Another plausible explanation for the lack of fibrin is that the brisk fibrinolysis occurring secondarily to DIC effectively digests the fibrin as it is elaborated and thus presents the development of organised thrombi (Pitney, 1971). Inhibition of fibrinolysis by administration of EACA to rabbits continuously during infection might provide the answer. However one problem here is that fibrinolysis of micro-thrombi may proceed as a consequence of activation of plasminogen already incorporated into the fibrin clot - a situation found to occur during fibrinolytic inhibition in dogs by Barnhart and Cress (1967).

A final, most likely, reason is that the histo-chemical method used for the demonstration of fibrin was perhaps not the most suitable method to use under

the circumstances described. The problem relates to the degree of fibrin polymerisation (Simpson, personal communication). Only fully polymerised fibrin is stained by conventional staining techniques. In DIC fibrin polymerisation is never complete since raised FDP levels prevent this. The problem could be solved by either employing immunofluorescent methods or by electron microscopy since fibrin shows on either regardless of its polymerisation.

There is some evidence in the literature that thrombus formation does occur in trypanosome infections. Cattle infected with T. (N.) congolense showed thrombosis of smaller arteries and veins which was attributed to denudation of the vascular endothelium by the trypanosomes (Fiennes, 1970). The same parasite in dogs, caused thrombosis in the blood vessels of the brain and heart (Hildebrandt et al, 1973). Earlier work by Helm (1929) showed that trypanosomes of the humoral group also produce a similar pathology. Thus horses infected with T. (T.) brucei developed thrombus formation. Ormerod and Venkatesan (1971) have demonstrated that the choroid plexus of rats infected with T. (T.) rhodesiense showed some capillaries completely blocked with amastigote forms together with a substance believed to be a white thrombus. More recently in the same host, T. (T.) brucei infections were associated with fibrin deposition within renal glomeruli (Max Murray, personal communication).

These observations led to detailed examinations of the coagulation system in trypanosomiasis by Hawkey and Bolton *et al* (see Table 1a; Section 1.6). Both groups of workers have shown increases in most clotting factors investigated during *T. (T.) brucei* infections of rabbits which Bolton *et al* suggested was similar to the situation following the SSR reaction. In fact rabbits are the ideal lieges for SSR compared to other species since they are capable of eliciting only a weak focal fibrinolysis (Rodriquez - Erdmann, 1973). Together with the above observations and the demonstration of a red cell anomaly, Jenkins *et al* (1974) suggested that the anaemia which developed in trypanosome infected rabbits may be partly microangiopathic in origin. The syndrome of microangiopathic haemolytic anaemia with fragmentation of red cells in the vascular system developed around the studies of Brain and Hourihane (1967). Visual demonstrations of red cells "hung up" on strands of fibrin with subsequent buffeting by the blood flow around the cells clearly showed the mechanism of damage to red cells in this syndrome.

The question now remains as to what initiates DIC in trypanosomiasis. Hypercoagulability of the blood is the starting point for the chain of events resulting in microthrombi formation. A number of factors, either alone or in combination, can bring about a hypercoagulable state. These aetiological mechanisms have been listed by Fletcher and Alkjaersig (1973) as:-

1. extrinsic release of tissue activators into the circulation.
2. blood stasis.
3. underlying endothelial damage.
4. poorly defined mechanisms which may involve more than one of the above mechanisms.

As previously discussed, a vast amount of tissue damage occurrs in trypanosomiasis which would cause the local release of activator substances into the blood. Other factors might activate the extrinsic coagulation pathway including procoagulants released from lysed red cells during intravascular haemolysis; endothelial injury and antigen-antibody complexes. A haemolytic anaemia does occur in T. (T.) brucei infections of rabbits (Jenkins et al, 1974) possibly with a multiple aetiology; vascular injury is a feature of trypanosomiasis (Goodwin, 1971); and antigen-antibody reactions occur due to antigenic variation of trypanosomes. The importance of blood stasis in initiating blood coagulation will be discussed later on.

Liver functional impairment is the only other pathophysiological process which might explain the multiple coagulation and fibrinolytic abnormalities observed in trypanosomiasis of the rabbit. However the plasma protamine sulphate test is negative in liver disease (Niewiarowski & Gurewich, 1971). In addition enzyme studies by MacKenzie and Boreham (1974) indicated only

minimal liver damage in infected rabbits. Finally, cirrhosis is the only type of liver disease in which increased fibrinolytic activity is usually detectable (Ratnoff, 1949; Kwaan et al, 1956). Pathological examination of livers by the author from rabbits infected with trypanosomes revealed no evidence of cirrhosis.

The findings in this study are similar to those previously described in human P. falciparum malaria and in P. knowlesi malaria of rhesus monkeys by Dennis et al (1967). These studies are also comparable to the observations of Voller et al (1969) who suggested that DIC might account for several coagulation abnormalities observed in one of their owl monkeys infected with P. falciparum. The finding by Devakul et al (1966) of shortened fibrinogen turnover times in human P. falciparum cerebral malarial also supports the belief that DIC does occur at least in some instances of human and simian malaria. Synthesis of plasma fibrinogen has also been investigated in rhesus monkeys with P. coatneyi (Areekul et al, 1971a) or P. knowlesi (Sucharit & Reid, 1973a), and P. falciparum in Aotus monkeys (O'Leary et al, 1972). Areekul and his co-workers found an increased consumption of I^{131} - fibrinogen in human malaria, but little change in experimental monkey malaria. However, in both cases the total amount of plasma fibrinogen increased considerably indicating an increased synthesis of this protein. Contrary to this O'Leary and his colleagues showed that there is a marked decrease in

fibrinogen concentration in the blood of infected Aotus monkeys but an increase in FDP which, they suggested, indicated diffuse intravascular coagulation. Likewise Sucharit and Reid (1973b) concluded that the raised serum FDP and alterations in clotting factors (increased fibrinogen, decreased factors VII and X) during simian P. knowlesi malaria were consistent with the occurrence of intravascular coagulation. A study of fibrinogen turnover in experimental trypanosomiasis would be an important addition to the fibrinolytic studies described here.

Thrombocytopenia is a frequent finding in T. (T.) brucei infections of rabbits (Hawkey, unpublished observations; Bolton et al 1974). The occurrence of DIC in the same infection provides one possible explanation for the development of thrombocytopenia in these animals. It is of interest that antigen-antibody complexes have now been recognised as mediators of both platelet destruction per se (Shulman, 1964) and DIC (Robbins and Stetson, 1959; McKay, 1969). A severe thrombocytopenia has also been shown to occur in acute infections of rats infected with T. (T.) rhodesiense by Davis et al (1974). In vitro experiments by the authors showed that platelet injury was initiated by soluble extracts of disrupted trypanosomes, and since during infection an inverse relationship existed between parasitaemia and platelet counts, they suggested that a similar mechanism might occur in vivo. It is possible

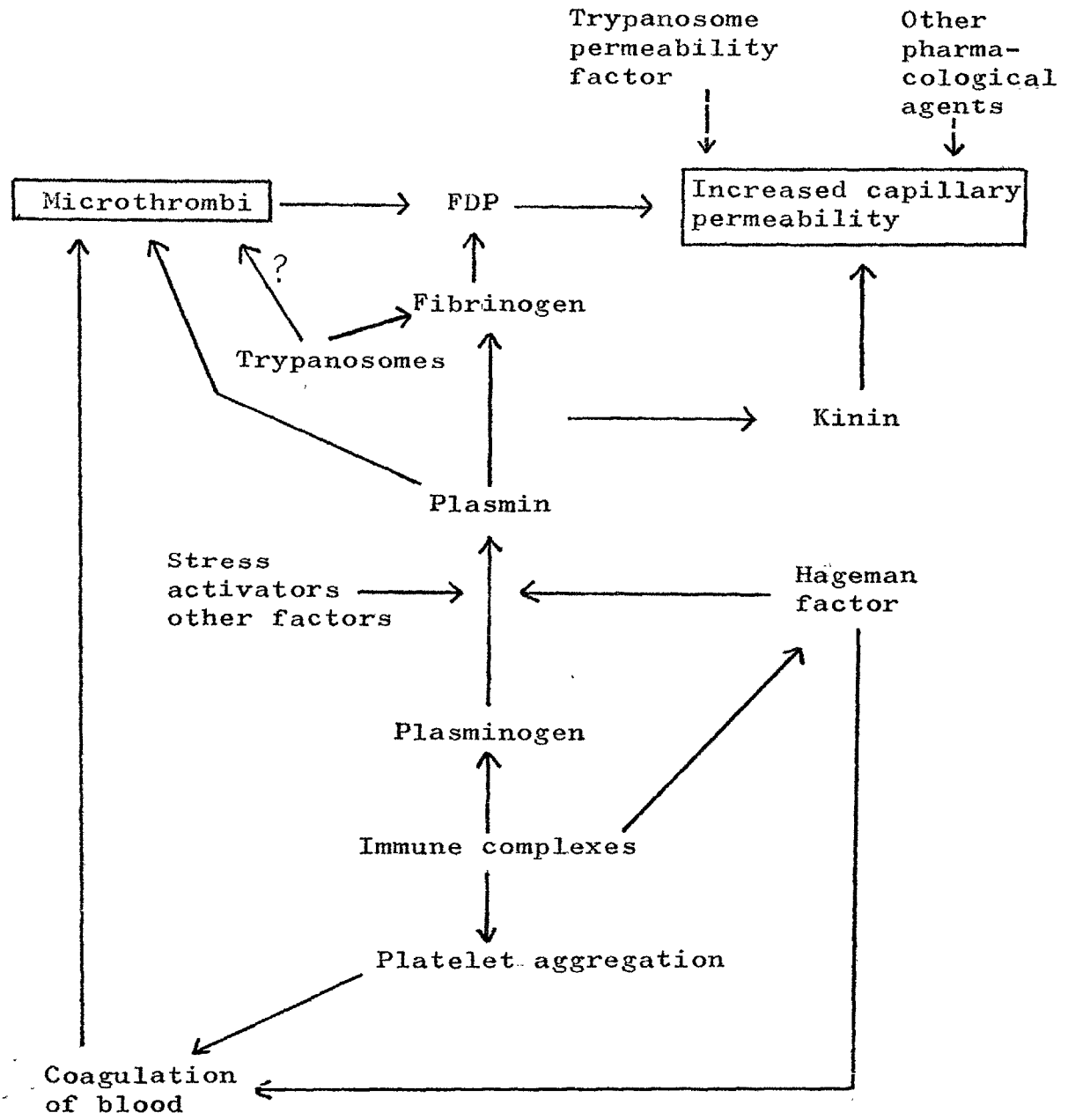


Fig. 62. Diagram to show possible mechanisms whereby increased capillary permeability and microthrombi could occur in experimental trypanosomiasis of the rabbit.

platelet injury in rabbits infected with T. (T.) brucei is brought about in a similar way although in this chronic infection very few parasites are ever seen in the peripheral blood at any time. An attempt has been made in Fig. 62 to demonstrate the interrelationship of various factors which are formed or altered in experimental trypanosomiasis and to show how they might contribute to the pathological changes. Table XXV is a summary of all factors investigated in the present studies and their alteration during infection.

A common finding in infected rabbits was the appearance of FDP in the urine late on in the infection. At no time could clottable fibrinogen be detected. There are three explanations for the appearance of FDP in the urine. First they may reflect a clearance of circulating serum FDP. Late FDP are nearly as small (Fragment D) or smaller (Fragment E) than albumin and thus should pass through the glomeruli (glomerular pores permeable to molecules up to a molecular weight of approximately 70,000) even during highly selective proteinuria. If direct filtration of FDP had occurred then one might expect to find low concentrations of "late" FDP from the second week of infection onwards. However it was interesting to note that there was no direct correlation between the serum and urine FDP

Observation	T. (T.) brucei infected rabbits	T. brucei/ congolense infected cattle	T. (T.) rhodesiense infected humans
Fibrinogen	Increased	Increased	Increased
Cryofibrinogen	Present	N/T	N/T
HPF	Present	N/T	N/T
"Fibrinogen-B"	Present	N/T	N/T
FDP	Increased	Little change	Increased
Fibrinogen-DP	Increased	N/T	N/T
Fibrin-DP	Present	N/T	N/T
Fibrin monomer			
Plasminogen	Decreased	N/T	N/T
Fibrin deposition in tissues	Found extravascularly in lungs		

Table XXV Summary table of changes in factors associated with intravascular clotting in trypanosome infections of rabbits, cattle and man.

N/T = not tested.

values, although it is possible that gross kidney damage occurring late in the infection might allow the passage of serum FDP into the urine at this time.

A second alternative is that urinary FDP are derived from fibrinolysis of intrarenal fibrin. However no extensive fibrin deposits were found in any part of the infected kidneys examined, but since these results proved inconclusive because of the staining method used for fibrin, the possibility of lysis of deposited fibrin must be kept in mind.

A final explanation is that the urinary FDP are derived from local fibrinogenolysis within the urinary tract. This implies that fibrinogen gains entry to the urinary tubules where it is subsequently degraded. In fact a proteinuria does occur in T. (T.) brucei infections of the rabbit (Goodwin & Guy, 1973; Itazi & Enyaru, 1973), and Naish et al (1974) have shown that a positive correlation exists between proteinuria and FDP excretion in patients with a variety of renal diseases even in the absence of glomerular fibrin deposition. The theory that FDP arise from fibrinogenolysis within the urinary tract is supported by the in vitro observations described in the present studies. The degradation of fibrinogen to Fragments D and E by in vitro incubation with unconcentrated samples of normal rabbit urine at 37°C for 1 h and 2 h extended previous demonstrations of the presence of plasminogen activator (urokinase) in normal urine samples by the fibrin plate

technique (Astrup & Sterndorff, 1952). This suggestion of "renal fibrinogenolysis" presupposes that plasminogen also escapes into the urine - a feature almost certain to occur since plasminogen has a high absorptive affinity for fibrinogen and their separation is a difficult process (Hawkey, personal communication). In addition, it has recently been suggested that the kidney is the primary source of plasminogen production in cats (Highsmith & Kline, 1971). If this is also true for rabbits, it is possible renal lesions triggered an excessive production of plasminogen.

These findings indicate that renal failure may be the result of death in infected rabbits in agreement with the suggestion of Goodwin (1974). That fibrinogen with a molecular weight of 340,000 should pass through the glomerular sieve implies gross renal damage with disruption of renal function late on in the infection. It is possible that actual dissolution of basement membrane may occur in segmental areas of a few capillarly loops of diseased glomeruli. Such gaps in the continuity of the basement membrane could be responsible for the passage into the urine of large molecular weight proteins such a fibrinogen.

During attempts to measure the concentration of serum FDP it was found that a factor present in both

normal and infected sera was causing agglutination of the fibrinogen coated red cells and thus interfered with the FDP assay. This factor was present not only in rabbit sera but also normal and infected cattle and human sera. Agglutination did not occur when fresh washed erythrocytes or formalinised tanned SRBC were added to the sera suggesting that this factor must be directed against the fibrinogen attached to the cells. The results of the experiments described here suggest that it is an autoantibody. An autoantibody is defined by Herbert and Wilkinson (1971) as "antibody capable of specific reaction with an antigen that is a normal constituent of the body of the individual by which that antibody was formed". The factor present in the serum of rabbits, cattle and man fits this definition and since it can be separated in the macroglobulin fraction on a Sephadex G200 column, destroyed by heating at 65°C or inactivated by 2-ME reduction, it appears to be an autoantibody of the IgM class.

Since fibrinogen has many derivatives and it is not certain which derivatives the autoantibody is directed against, it has been referred to here as an anti-F autoantibody. The pattern of response of anti-F in rabbits is identical to the anti-liver and anti-Wassermann autoantibodies described recently by Mac-Kenzie and Boreham (1974) measured by precipitation and completment fixation tests.

One question which arises from these studies is:

what component of the fibrinogen system is acting as antigen? The answer to this question must also explain why small concentrations of anti-F can be detected in non-infected sera. The finding of high plasma fibrinogen levels and increased FDP suggests that neither of these substances is acting as antigen since a complex would be formed with the antibody making it impossible to measure them.

One explanation is that the antibody has a very low affinity for the antigen. Such low affinity antibody would be relatively inefficient in eliminating the antigen and, since antigen is continually being released in trypanosomiasis, synthesis of low affinity antibody might result in the formation of soluble antigen-antibody complexes. Low affinity antibody to transferrin has been described in Plasmodium berghei yoelii infections in mice (Steward & Voller, 1973).

Absorption with fibrinogen-sensitised cells removed the antibody and the test system utilised the same cells, indicating that the autoantibody only reacts with fibrinogen after attachment to red cells. One explanation for this is that during attachment to the red cell membrane, the fibrinogen molecule is altered in some way and one or more hidden antigenic determinants are exposed and recognised as "foreign" by the cells of the lymphoid series. One might visualise that fibrinogen in vivo becomes absorbed onto red cells as suggested by Woodruff et al (1972) to occur in leish-

maniasis. In fact this possibility was investigated in the present studies and it was demonstrated that absorption of fibrinogen onto the red cell membrane does occur in both normal and infected rabbits. This phenomenon will be discussed in more detail later on in connection with red cell autoagglutination.

It was also found that absorption of serum with autologous fibrin removed most of the antibody activity, so that possibly, during polymerisation of fibrinogen to fibrin, the same antigenic sites are exposed. The unfolding of the fibrinogen molecule might expose hydrophobic areas, normally concealed in the interior, a feature which is known to occur following denaturation of human serum albumin (DiLuzio & Flemming, 1971).

The possible mechanism of increased synthesis of pre-existing autoantibodies in trypanosomiasis have already been discussed in detail (MacKenzie & Boreham, 1974). Terry et al (1973) suggested that, by one means or another, trypanosome infections break the control link between T and B lymphocytes. This would mean that some B cells would escape from T cell control and would proceed to produce IgM antibodies some of which would be autoantibodies. They also considered other responses which require cellular reactions, would be suppressed. The above mechanism is in contrast to the effect of FCA which appears to act directly through the T cell system (Allison & Davies, 1971). Furthermore Askonas and Humphrey (1958) have shown that when immunisation to a

defined antigen is induced, non-specific antibody elevation occurs. Successive waves of antigenically different variants during trypanosomiasis may have a similar effect.

Homeostatic mechanisms within the normal circulation require that following minor injury to blood vessels, fibrin is laid down as a prerequisite to repair of the vascular endothelium. Under these conditions polymerisation of fibrinogen to fibrin would expose the suggested antigenic sites and so lead to anti-F autoantibody formation. Since it would appear that low-grade DIC occurs in rabbit trypanosomiasis, formation of microthrombi at intervals during the disease may represent constant antigenic stimulation of lymphoid tissue giving rise to the increased levels of anti-F.

Dumonde and Glynn (1962) have shown that an anti-human fibrin antibody produced in rabbits following immunisation with human fibrin, was species specific. The anti-F autoantibody in trypanosomiasis appears to be multispecific in that it reacts with rabbit, human and bovine fibrinogen. It may well be that the antigenic determinant(s) exposed on sensitisation of red cells with fibrinogen molecules from different species, is the same in all cases.

During experiments to determine the role of fibrinogen in inflammation, Barnhart (1968) described a circulating anti-fibrin antibody in a patient suffering from thrombophlebitis migrans. No details of the

method used to detect this antibody is given nor details of its specificity. The appearance and disappearance of the antibody coincided with the presence and absence of an active inflammatory state.

The presence of autoantibodies in the serum will contribute to the raised IgM levels in trypanosomiasis. The contribution that the anti-F autoantibody makes to the pathogenesis of trypanosomiasis remains uncertain. The low level of antibody in normal rabbits appears to do no harm and the high concentrations in infected rabbits do not induce a hypofibrinogenaemia. In fact, if the antibody is, as suggested, directed against one or more hidden determinants on fibrinogen exposed during the clotting process, and a condition of DIC exists, then the antibody may have a beneficial effect in forming complexes with the antigen and thereby stimulating phagocytosis and removal of fibrin strands.

The indirect, non-immunologic, pathological effects of raised IgM concentrations are possibly more important as indicated by additional studies on erythrocyte autoagglutination and blood viscosity. These studies will be discussed in the following paragraphs.

From the observations of increased levels of plasma fibrinogen and macroglobulin, it was not surprising to find that a hyperproteinaemia occurs in rabbit trypan-

osomiasis. Increased total protein levels occurred approximately two to three weeks after infection, the main constituent of which was an increase in serum globulin - almost certainly due to immunoglobulin synthesis. Separation of plasma on Sephadex G200 columns indicated an increase in both the IgG and IgM fractions relative to albumin. It was found that a two-fold increase in globulin was accompanied by a two-fold decrease in albumin - results comparable to those of Goodwin and Guy (1973) who investigated rabbits infected with T. (T.) brucei strain S42. It is well known that in many diseases where globulin levels are increased, the albumin level in plasma is proportionately decreased (Bjorneboe, 1946; Ogryzlo et al, 1959). Furthermore, if a state of hypergamma-globulinaemia is induced by means of hyperimmunisation, the albumin level is also decreased (Bjorneboe & Jarnum, 1961). In addition Bjorneboe (1946) stated that the relationship between the serum globulin and serum albumin could be explained by the assumption of a regulatory mechanism which attempts to maintain the colloid osmotic pressure constant. This is probably the reason for the observed serum protein changes seen here in rabbit trypanosomiasis, although it remains an area which requires further investigation. For example albumin is excreted into the urine in increasing amounts from the first week of infection with trypanosomes (unpublished observations). There are few references in the literature on the path-

ological effects of decreased serum albumin in the presence of elevated levels of other serum proteins. It was observed that the majority of crenated erythrocytes-a typical feature in blood smears taken from rabbits infected with trypanosomes (Boreham, 1967) - reverted to a near normal discoid shape if albumin was added to the blood sample to bring the level of this protein to a normal concentration. This suggests that possibly crenation of red cells observed in infected rabbits is due to a low serum albumin level. Albumin is a large charge-carrying molecule that acts to reduce the gradient in the vicinity of the red cell by shielding it from the field (Evans & LeBlond, 1973). If albumin is reduced then the red cell is effected by factors within that field which might change its shape e.g. fibrinogen (see page 251). Crenated cells are recognised by the reticuloendotheial system as effete cells and as such are readily phagocytosed (Cooper et al, 1974). This could explain at least one mechanism for the increased erythrophagocytosis that occurs in trypanosomiasis (Jenkins et al, 1974; Murray et al, 1974) and the anaemia which subsequently develops.

A possible abnormality in lipid metabolism was indicated by the development of a hyperlipoproteinaemia in infected rabbits associated with an increasing opacity of the serum. The main constituent of the total lipid elevation was a marked increase in the serum triglyceride (neutral fat) content mainly in the form of

chylomicrons. No experiments were carried out to determine the mechanisms responsible for the lipid changes and the reasons remain only speculative. Albumin is an important protein for triglyceride transport in the circulation (Schettler, 1967). A decrease in this protein will thus result in accumulation of triglycerides in the blood. Alternatively, since it is the function of liver parenchymal cells to assimilate and metabolise chylomicron tryglycerides (DiLuzio, 1960), disturbance of liver function in trypanosomiasis might result in elevated serum triglycerides. Loss of albumin into the urine in nephrotic syndromes is usually accompanied by an increased synthetic activity by the liver which is also stimulated to produce low density lipoprotein (Marsh & Drabkin, 1958; Radding & Steinberg, 1960). Since increased liver synthesis of fibrinogen occurs it is also possible that concurrent lipoprotein synthesis is stimulated. Another explanation is that the trypanosome infection causes a reduction in lipoprotein lipase activity in some way, so allowing the large accumulations of lipid to occur (Shapiro, 1967).

It is of interest to note the importance of the host-parasite system as regards these findings. Roberts and Godfrey (1968) and Roberts (1974) investigating changes that occur in the serum lipid levels of cattle infected with T. (N.) congolense and sheep with T. (D.) vivax, showed a marked reduction in both phospholipids and triglycerides. Roberts explanation for his

results was a reduction in lipolytic activity secondary to suppression of lipoprotein formation. Thus alterations in lipid metabolism in rabbit trypanosomiasis would appear the complete opposite to trypanosomiasis of cattle and sheep.

Also of interest are the findings of alterations in lipid metabolism in simian malaria infections (Angus et al, 1971). It has been suggested that an increase in the non-esterified fatty acid fraction of plasma that occurs in infected monkeys could counteract the effect of albumin and lecithin and lead to lysis of non-infected as well as infected erythrocytes (Laser, 1950).

Both DIC (Attar et al, 1969; McKay, 1969) and hyperlipoproteinaemia (Watson, 1970) are well recognised systemic manifestations of shock and injury. Usually these phenomena have been studied separately and there is a paucity of information regarding possible causal relationships between them although the connection between DIC and increased lipid levels is well documented (Lasch et al, 1966; Kwaan & Suwanwela, 1969; Voller et al, 1969; Newall et al, 1973). Elevation of plasma lipids, especially free fatty acids (FFA), may be thrombogenic. FFA infusion in experimental animals produced massive thrombosis through the activation of factors XII and XI (Kwaan & Suwanwela, 1969).

The converse proposition, that an alteration in plasma lipid fractions is a consequence of DIC has received scant attention. However, recently Whitaker

et al (1974) showed that a brief episode of DIC induced by thrombin infusion into rabbits produced a hyperlipoproteinaemia characterized by elevations in FFA, cholesterol and triglycerides. Since the plasma lipid derangements correlated with parameters of haemolysis, they considered the changes might contribute to the genesis of microangiopathic haemolysis. All these features reflect the situation in trypanosomiasis of the rabbit.

Autoimmunity has been implicated in the anaemia of sleeping sickness (Barrett-Connor et al, 1973; MacKenzie & Boreham, in press) by the demonstration of Coombs' positive red cells. Additional studies by MacKenzie & Boreham (in press) however failed to demonstrate a similar condition in T. (T.) brucei infections of rabbits despite observations of red cells adhering to one another and to trypanosomes. The Coombs reagent used in the studies was a goat anti-rabbit serum. Since the autoantibodies so far studied in rabbit trypanosomiasis have been of the IgM class, a specific anti-rabbit IgM serum used, as the Coombs reagent might have provided more conclusive evidence. The possibility that some autoagglutination and mixed autoagglutination are immunologically mediated by other mechanisms other than via. incomplete antibodies should also be considered.

For example, it is possible both may be, in part, mediated by trypanosomal antigen absorbed onto the red cells and that anti-trypanosomal antibody is the agglutinating agent. Herbert and Inglis (1973) have shown that syngeneic red blood cells which were exposed to infected plasma *in vitro* and then washed, produced resistance to challenge if innoculated into clean mice. If this occurs *in vivo* mixed and auto-agglutination may well occur. It is certain that immune adherence cannot be a mechanism for auto-agglutination since the activated C'3 receptors responsible for the phenomenon appear only on primate red cells and not on those of species in other orders.

However the present investigation shows that the increased erythrocyte sedimentation and autoagglutination are largely governed by a non-immunological phenomenon as discussed below.

The blood proteins and their conjugates *in vivo* act as colloids and, as such, significantly influence important biophysical characteristics of the circulating blood e.g. suspension stability, osmotic pressure and viscosity. Changes in these physical properties in turn may affect the rate of flow of blood, the caliber of the small vessels and the permeability through vessel walls. Therefore modification in the plasma protein concentration in trypanosomiasis may have a considerable bearing on the behaviour and function of the peripheral vascular bed in general, as well as on

the insiduous development of degenerative changes in the smaller vessels.

Alteration in the suspension stability of blood from infected rabbits was observed by an autoagglutination (reversible) of red cells accompanied by a markedly elevated ESR. A drop in the ESR occurred *in vitro* when infected plasma was diluted, and infected cells in normal plasma had almost normal ESR's. The marked difference between red cell sedimentation in plasma and serum suggested that fibrinogen might be especially active. *In vitro* investigations into the effect of various concentrations of fibrinogen, γ-globulin, albumin and lipids indicated that fibrinogen was the prime factor determining the degree of red cell autoagglutination and sedimentation. The effect of fibrinogen was most marked at concentrations greater than 600 mg/100 ml. Since fibrinogen levels were normally elevated above this value in trypanosome infections, a similar effect might be expected *in vivo*. These results compare favourably with those of Lipinski *et al* (1969) who found that the ESR was highly correlated with proteins of high molecular weight, derived from fibrinogen and detected by the protamine sulphate test. The ESR is, in fact, a measure of the tendancy of red cells to form rouleaux or aggregates (Ditzel & Moinat, 1959).

Lipids had a slight effect in elevating the ESR confirming the results of Cullen and Swank (1954). In the hamster, alimentary lipaemia has been shown to cause

aggregation of red cells, increased ESR, reduced oxygen uptake by the brain, and changes in the electrocardiogram (Swank & Bartsch, 1971).

Several workers (Thorsen & Hint, 1950; Ditzel, 1959) have presented evidence that red cell aggregation is enhanced by the formation of a viscous and "sticky" coat on the erythrocyte membrane due to adsorption of plasma colloids such as fibrinogen and macroglobulin. In view of these suggestions and the evidence supporting them it is pertinant to recall that during the present investigations, red cells in infected plasma or varying concentrations of purified fibrinogen, needle-like processes were observed to connect adjacent elongated erythrocytes. Similar processes were also observed by Thorsen and Hint (1950) who interpreted them as being formed of attenuated surface film composed of adsorbed colloid and linking adjacent cells. The nature of the plasmanemes (filopodia), long fine processes extending from the trypanosome surface, described by Wright and Lumsden (1969) and Lumsden (1970), has not yet been detailed. It may well be that they represent a similar surface film observed around the red cells. The formation of plasmanemes depends greatly on the medium in which the trypanosomes lie - a fact which supports the view that they are formed from extraneous substances.

Investigations into the nature of this surface film on red cells by immunofluorescence, indicates that it is composed largely of fibrinogen supporting the

previous findings of Traber and Kolmen (1963) who showed that canine fibrinogen is absorbed onto the membrane of canine red cells. In the present studies the amount absorbed onto rabbit erythrocytes was dependent on the concentration of fibrinogen in the surrounding medium suggesting that a dynamic equilibrium exists between fibrinogen in free solution and fibrinogen absorbed onto the red cell membrane. The absorption of fibrinogen followed by aggregation of red cells might be explained by charge differences between the red cell membrane and the fibrinogen molecule. Fibrinogen, a long (axial length-width ratio 15:1), fibrillar and high molecular weight protein is slightly positively charged and will attracted to the negatively charged erythrocytes. In addition, since β_1 IgM and β_2 IgM are both positively charged molecules, the latter having almost the same charge as fibrinogen (Dettelbach & Ritzmann, 1968), it might be expected that both molecules would also attach to the red cell membrane. In contrast, albumin, a small relatively low molecular weight polymer (axial length-width ratio, 3:1) with a strong negative charge would not be adsorbed because of charge similarity with the red cells.

A correlation between erythrocyte aggregation and an increase in the viscosity of whole blood has been

observed in a variety of clinical conditions including macroglobulinaemia (Rosenblum, 1969), multiple myeloma (Whitmore, 1968), rheumatoid arthritis (Crockson & Crockson, 1974) and peripheral vascular disease (Dormandy, 1971). The increase in η WB in rabbits three weeks after infection with trypanosomes is probably attributed to the time of maximum increase in large molecular weight proteins with subsequent erythrocyte aggregation. It is certainly not a result polycythaemia since infected rabbits were anaemic at this stage of infection and had an expanded plasma volume. An increase in the rigidity of the erythrocytes themselves (Miller et al, 1971; Elliot, 1973) might contribute to the syndrome. Abnormally shaped erythrocytes observed in rabbit trypanosomiasis e.g. crenated cells and spherocytes (Jenkins et al, 1974) represent inflexible cells. In fact Gregersen (1967) found that suspensions of crenated cells had a higher viscosity than normal.

A determinant of ηWB is the rate of shear that the blood is subjected to. An increased shear rate tends to disperse red cell aggregates thereby decreasing the overall viscosity (Whitmore, 1968). The mean rates of shear in most blood vessels are high enough, under normal circumstances, to ensure that aggregation makes an insignificant contribution to the viscosity of blood. Since the forces of fluid shear are primarily a function of vessel diameter and pressure gradient, increased shear rates are most marked in the micro-

circulation where they may reach 250^{sec-1} (Dormandy, 1971). However, one condition effecting the flow properties of blood in the microvasculature is the condition of the blood vessels themselves. Both dilation and constriction of arterioles reduces the rate of shear thereby favouring aggregation (Elliot, 1971). In addition vascular damage causes leucocytes and platelets to adhere to the endothelium and both red cells and platelets form into clumps. MacFarlane (1961) found aggregation as "sludging" of red cells occurred after cells had passed through an inflamed vessel. As already mentioned previously, a study of the blood vessels of the ears and cremaster muscles of rabbits by Goodwin and Hook (1968) showed that infection with T. (T.) brucei in rabbits led to chronic vasculitis followed by complete breakdown of some blood vessels. Goodwin (1971) showed injury to the small vascular bed caused leucocytes to adhere to the endothelium. A common site for the leucocytes to stick and aggregate was at a junction of venules. Both features would lead to further slowing of blood flow, increase in viscosity and the initiation of a vicious circle in which blood viscosity becomes a major hoemodynamic variable. As a result, complete cessation of flow might eventually occur. In fact stasis of blood flow is a feature of rabbit trypanosomiasis (Goodwin, 1970). A summary of these events visualised as occurring during infection is shown in Fig. 63.

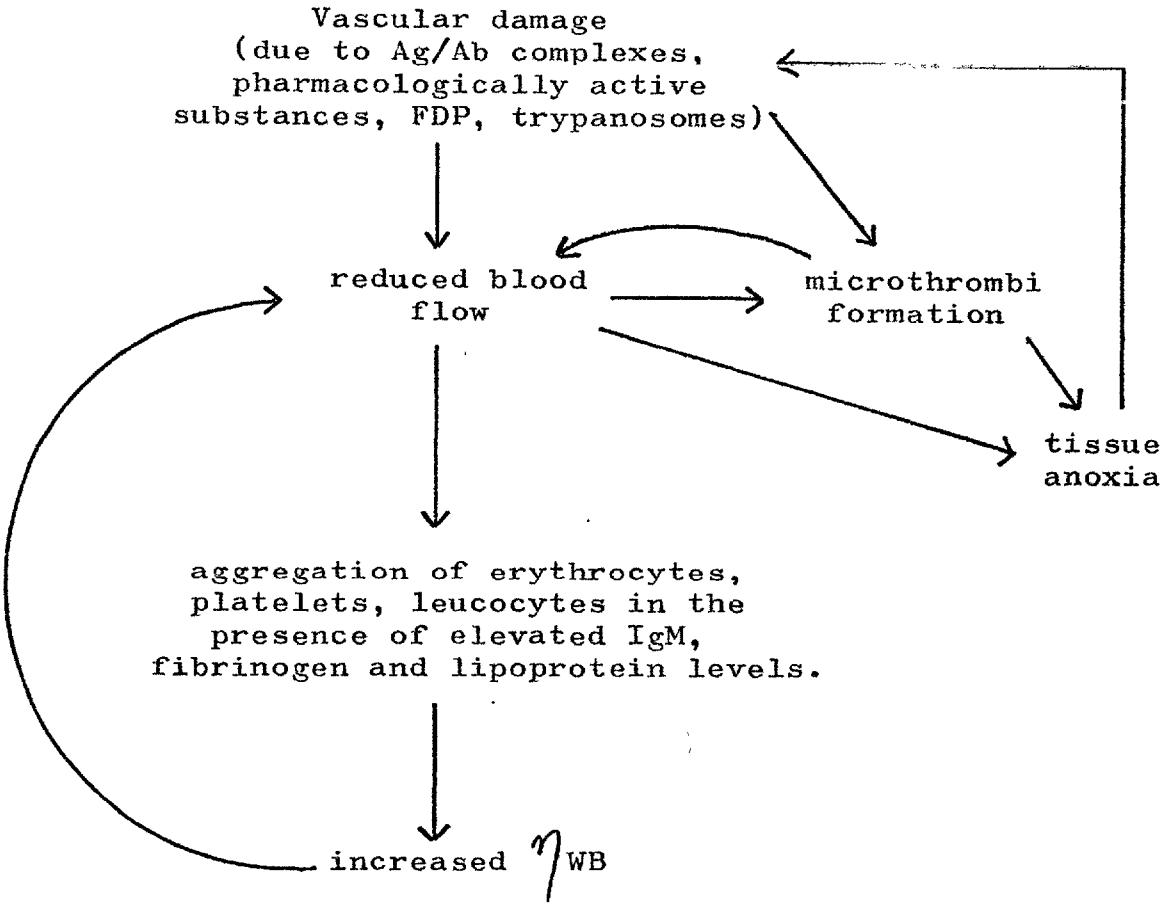


Fig. 63. Schematic representation of a vicious circle of events which occurs following vascular injury in rabbit trypanosomiasis.

As indicated previously, the red cell autoagglutination in infected rabbits is primarily governed by the presence of large molecular weight proteins such as fibrinogen and IgM. Not a surprising finding therefore was an increase in plasma and serum viscosity found to accompany the increase in whole blood viscosity. Statistical analysis of results showed that plasma fibrinogen, globulin and albumin were responsible for 75% of the change in plasma viscosity, with globulin as the major determinant. Globulin was also found to be an important factor in determining ηWB. Non-infected plasma, usually slightly Newtonian in behaviour, became markedly non-Newtonian following infection. The viscosity of plasma is important in these studies - in the smallest vessels of the microcirculation where blood would not retain its homogeneous character because of similarities of red cell size and vessel diameter, the rheological behaviour of the plasma itself becomes a significant factor since the plasma rather than whole blood is being sheared.

In an investigation into the viscosity of patients with peripheral vascular disease, Dormandy (personal communication) has shown that changes in viscosity do have a pathological significance in that alteration in the ηWB produced a two-fold opposite change in blood flow. A similar situation must occur in the damaged blood vessels of infected rabbits particularly in the venous system and in the more sluggish sinusoids of the

spleen and liver where aggregation of red cells would be favoured. The low pH and other unusual features of the microcirculation of the spleen are related to the removal and destruction of red cells recognised as "abnormal". It has been shown that aggregated red cells are recognised as "abnormal" and phagocytosed in the spleen (Murphy, 1973). This condition might therefore represent at least one cause of the anaemia which develops in infected rabbits. The development of anaemia could, in some respects, be considered beneficial to infected animals in that the viscosity of blood would be reduced as a result of a decreased haematocrit. Anaemia is a common finding in other diseases associated with an elevated viscosity e.g. Waldenstrom's macroglobulinaemia (Cline et al, 1963); multiple myeloma (MacKenzie et al, 1970) and more recently rheumatoid arthritis (Crockson & Crockson, 1974).

As a result of reduced or stagnant blood flow, adverse effects on tissue perfusion and oxygenation might be expected (Fig. 63). The influence of intravascular red cell aggregation and consequent changes in blood flow on tissue nutrition were first demonstrated by Lofstrom (1959). He found that oxygen consumption decreased during a period of intravascular aggregation and increased again when this was reversed. Alterations in the gas exchange between blood and tissue during periods of aggregation might be explained as arising from the decreased rate of flow to the tissue, stasis

of cells, and the shunting of blood through arteriolo-venular anastomoses. It is also conceivable that aggregation of red cells diminishes the area available for exchange.

An additional pathological effect of increased blood viscosity and an accompanied decreased rate of blood flow, particularly in the capillaries and venules, is a situation favouring the formation of microthrombi and deposition of carbohydrate-lipid-protein complexes in the walls of blood vessels (Ditzel & Moinat, 1959). As previously discussed, evidence from the initial studies described in the present work suggests a condition of DIC occurs in rabbit trypanosomiasis. It is probable that the increased ηWB and ηP contributes towards this syndrome two to three weeks after infection.

Other manifestations of hyperviscosity seen in patients with macroglobulinaemia are either absent or as yet have not been detailed in trypanosomiasis of the rabbit. These include symptoms related to CNS anoxia (e.g. vertigo and mystagmus), bleeding disorders and opthalmic manifestations (e.g. venous distention and engorgement).

It has been recognised for many years that changes in the health of an animal frequently lead to alterations in the characteristics of the blood and blood

vessel walls. Indeed the alteration in red cell stability is the basis of the sedimentation test which is widely used as a quick check on the health of human patients. The operating mechanism in any disease is usually highly complex which may be specific to the particular disorder. It would appear that trypanosomiasis does not represent a unique pathological condition but represents a combination of many disorders found in a variety of disease states - either other protozoal infections e.g. malaria or completely unassociated human complications such as Waldenstroms macroglobulinaemia.

Unfortunately it is very seldom that a single parameter of the blood alters in disease and trypanosomiasis is no exception. Changes in the constitution of blood are usually symptoms of a wider malaise which effects other bodily functions and organs with inevitable repercussions on the whole of the system. The resulting balance between the various influences depends on the intensity and extent of each. For example, what is important in trypanosomiasis is whether the balance of clotting and fibrinolysis is disturbed. The existence of intravascular coagulation in infection will become significant in the total economy of the host only if compensatory fibrinolysis fails to keep pace. That this balance is upset in favour of increased coagulation is suggested by the decreased suspension stability of the blood and increased viscosity. This alteration in

the balance between coagulation and fibrinolysis probably occurs in the later stages of infection when the above mentioned parameters increased and fibrinolysis decreases (as evidenced by diminished serum FDP levels and increased serum plasminogen).

The question as to whether DIC in rabbit trypanosomiasis is a result or a cause of pathological processes is not clear - in fact it probably represents both. The conditions described in these studies are conducive towards increased clotting. A vicious circle is then set up since microthrombi formation would have serious effects on microcirculatory function by causing tissue anoxia either by either blocking the blood supply to the tissues or by causing a decrease in blood flow. This condition would have the most serious consequences in those organs where microcirculatory function is of greatest importance i.e. lungs, kidney and brain.

It would appear that the anti-F autoantibody is unlikely to be of value in diagnosing human sleeping sickness or Nagana in cattle. Autoantibodies have been described in T. (T.) brucei infections of rabbits, cattle and man (Seed and Gam, 1967; MacKenzie & Boreham, 1974) and in T. (N.) congolense infections of rabbits and cattle (Mansfield & Krier, 1972; MacKenzie, 1973). Tissue autoantibodies have been described in a wide variety of infections diseases including yellow fever (Hughes, 1933), syphilis, malaria, leprosy, infectious

hepatitis (Davies, 1944), coccidiosis (Asherson & Rose, 1963) and schistosomiasis (Bassily et al, 1973).

Instead, the anti-F autoantibody forms part of the total IgM, which itself may have a pathological significance. The increased adhesiveness of red cells caused by high molecular weight substances such as fibrinogen has already been discussed. Circulatory malfunction is likely due to increased viscosity and slowing of blood flow. Since the osmotic pressure of the plasma is likely to increased, a haemodilution will result which in turn contributes towards the anaemia.

Perhaps the question now to be asked is: can any of these complications be reversed or inhibited? Heparin therapy would reduce the tendency towards clotting. Heparin has been found to inhibit tissue damage in hypersensitivity reactions such as the SSR and the Arthus lesion (Halpern et al, 1965). In addition administration of "Trasylol" and EACA during antitrypanosomal therapy would be beneficial in counteracting the effects of antigen-antibody complexes and the release of trypanosomal material in causing fibrinogenolysis. If the rheological picture in human sleeping sickness is similar to that of experimental rabbit trypanosomiasis, then either plasmapheresis or infusion of low molecular weight dextran might temporarily eleviate the macroglobulinaemia and hyperfibrinogenaemia.

The range of physiopathological findings found to occur in trypanosomiasis reflects the complexity of

of this disease. There appears to be little or no establishment of a balance between host and parasite and thus no time for physiological and pathological disturbances to be corrected. Instead the degree of disturbance increases in severity until death of the host ensues. The parasite, by means of antigenic variation, always remains one step ahead of the host's protective responses. The importance of pathological studies cannot be overemphasised if one is to understand the disease processes in operation, and, perhaps by the reversal of certain of these processes, to find a way of eleviating human and animal suffering.

PART 5: CONCLUSIONS

1. Activation of the fibrinolytic system occurs in experimental trypanosomiasis of the rabbit. It is probable that the same sequence of events also occurs in human sleeping sickness.

2. Increased fibrinolysis occurs mainly through the activation of plasminogen. Trypanosome extracts activate plasminogen in addition to possessing direct proteolytic activity on fibrinogen *in vitro*.

3. The combined findings in infected rabbits of increased fibrinogen and FDP, positive protamine paracoagulation tests and the presence of "abnormal" fibrinogen, all constitute findings which are specific for the occurrence of low-grade DIC. It is probable that DIC is a result of tissue injury initiating activation of the coagulation system. The existence of DIC expands a vicious circle of events which, in turn, probably lead to further intravascular coagulation. The occurrence of DIC suggests that the anaemia in rabbit trypanosomiasis may be partly micro-angiopathic in origin. It provides at least

one mechanism for the development of thrombocytopenia in the same species.

4. Autoimmunity develops during trypanosome infections of rabbits, man and cattle as evidenced by elevated titres of an autoantibody directed against one or more components of the fibrinogen system. It is considered likely that this autoantibody causes no direct pathological effect but has important secondary effects in contributing to the macroglobulinaemia of infected hosts.

5. The raised levels of fibrinogen, lipoprotein and IgM are the cause of a non-immunological erythrocyte autoagglutination accompanied by an elevated ESR. Phagocytosis of aggregated cells in the splenic sinusoids may contribute to the anaemia of infected rabbits.

6. Augtoagglutination of the red cells is considered to be the major cause of the raised whole blood viscosity. It is probable that the increased viscosity causes a reduction in blood flow resulting in reduced tissue oxygenation, tissue damage and impaired organ function.

APPENDIX I: RESULTS FROM INDIVIDUAL RABBITS

Serum FDP concentrations (mg/ml) in rabbits infected with T. (T.) brucei

Infected rabbit no.	Days after infection				
T. (T.) brucei S42	0	7	14	21	28
S251	40	80	159	80	80
S219	40	80	161	80	80
S220	80	80	80	80	80
S217	40	40	76	161	76
$\bar{x}$ =	50	70	119	100	79
T. (T.) brucei 427					
S267	40	161	80	80	80
S268	80	80	80	161	80
S269	40	80	161	80	80
S315	20	20	20	163	163
S316	40	20	20	163	40
S517	20	40	40	81	81
$\bar{x}$ =	40	66	66	121	74
Control rabbit no.					
S270	40	40	40	40	40
S321	20	20	20	20	20
S295	20	80	20	20	20
$\bar{x}$	26·6	46	26·6	26·6	26·6

Results of protamine sulphate tests in rabbits infected with T. (T.) brucei 427

FS = fibrin strands
\- = negative

1 + to 4 + = gradation of feathery precipitates
G = gel

	Incubation time (h)									
	0.5					24.0				
Dilution of protamine sulphate	1/5	1/10	1/20	1/40	1/80	1/5	1/10	1/20	1/40	1/80
Rabbit number and days after infection										
S358/ 0	-	-	-	-	-	-	-	-	-	-
7	+	+	G	G	G	+	G	G	G	G
14	+	-	-	-	-	+	+	-	-	-
21	++	-	-	-	-	++	++	-	-	-
28	+	-	-	-	-	+	+	-	-	-
42	+	-	-	-	-	+	+	-	-	-
49	+	-	-	-	-	+	+	-	-	-
S359/ 0	-	-	-	-	-	-	-	-	-	-
7	++	-	-	-	-	++	-	-	-	-
14	++	-	-	-	-	++	-	-	-	-
21	+	-	-	-	-	+	+	-	-	-
28	+	-	-	-	-	+	+	-	-	-
42	+	-	-	-	-	+	-	-	-	-
49	+	-	-	-	-	+	-	-	-	-
S360/ 0	-	-	-	-	-	-	-	-	-	-
7	+	+	+	G	G	+	+	+	G	G
14	++	-	-	-	-	++	++	++	-	-
21	-	-	-	-	-	+	-	-	-	-
28	+	-	-	-	-	+	+	-	-	-
42	+	-	-	-	-	+	-	-	-	-
49	+	-	-	-	-	FS	FS	-	-	-

	Incubation time (h)									
	0.5					24.0				
Dilution of protamine sulphate	1/5	1/10	1/20	1/40	1/80	1/5	1/10	1/20	1/40	1/80
Rabbit number and days after infection										
S361/ 0	-	-	-	-	-	-	-	-	-	-
7	+	+	+	+	-	+	+	+	G	G
14	+	-	-	-	-	+	+	+	-	-
21	+	-	-	-	-	+	-	-	-	-
28	+	+	-	-	-	FS	FS	+	-	-
42	-	-	-	-	-	+	+	+	-	N/T
49	++	++	-	-	-	++	++	-	-	-
S375/ 0	-	-	-	-	-	+	-	-	-	-
7	++	-	-	-	-	++	-	-	-	-
14	++	-	-	-	-	++	-	-	-	-
21	++	-	-	-	-	++	-	-	-	-
28	+	-	-	-	-	++	-	-	-	-
35	+	-	-	-	-	++	-	-	-	-
S376/ 0	-	-	-	-	-	+	-	-	-	-
7	+	-	-	-	-	+	-	-	-	-
14	+	-	-	-	-	+	-	-	-	-
21	+	-	-	-	-	+	-	-	-	-
28	++	-	-	-	-	++	-	-	-	-
35	+	-	-	-	-	+	-	-	-	-

N/T = not tested

Effect of chemotherapy on serum FDP levels

Infected drug	Days after infection				
treated rabbits	14*	15	17	21	28
S318	20	10	20	20	40
S319	20	20	20	20	40
S320	20	20	20	80	40
$\bar{x}$ =	20	16	20	40	40
Non-infected drug treated rabbit					
S321	20	20	20	20	40

* Rabbits given Mel-B (3.6 mg/kg) intravenously.

Effect of plasmin inhibition on serum FDP levels

Infected, drug	Days after infection								
treated rabbits	0	7	13	14	22*	24	27	29	35
S310	40	20	40	20	163	81	40	40	40
S311	20	40	20	40	161	81	81	40	40
S312	20	40	40	40	161	81	81	40	40
$\bar{x}$ =	26	33	33	33	161	81	67	40	40

Non-infected, drug									
treated rabbits	0	7	13	14	22*	24	27	29	35
S295	20	80	20	80	80	20	20	20	20
S296	40	80	40	80	80	20	20	20	20
$\bar{x}$ =	30	80	30	80	80	20	20	20	20

Infected only									
	0	7	13	14	22	24	27	29	35
S309	40	40	20	20	163	163	163	140	80

* Drug treatment (1 ml EACA; 1 ml 'Trasylol') commenced.

Plasma fibrinogen (mg/100 ml) in normal non-infected rabbits and rabbits infected with T. (T.) brucei 427

Infected rabbits		Days after infection				
		0	7	14	21	28
S267		371	577	780	613	705
S268		220	613	543	547	483
S269		241	481	520	608	613
S252		205	345	408	664	713
S340		364	631	780	400	780
S341		223	743	557	353	427
$\bar{x}$	=	270	678	711	530	744
Non-infected rabbits						
S270		240	204	185	204	204
S271		230	230	235	220	230
S337		185	148	185	185	185
$\bar{x}$	=	206	194	201	203	206

Plasma fibrinogen (mg/100 ml) in infected rabbits used for the blood viscosity studies

Infected rabbits

	S347	S348	S349	S350
0	353.1	463.8	408.9	397.7
7	594.3	594.7	546.4	773.2
14	297.9	539.0	483.2	546.4
21	520.4	498.0	323.2	553.8
28	315.9	446.0	167.2	334.5
35	334.5	557.6	289.9	394.0
42	N/T	N/T	N/T	N/T
49	230.0	353.15	185.7	345.7

N/T = not tested

Urinary FDP in rabbits infected with T. (T.) brucei 427

Days after infection	Rabbit number S358 vol. urine (ml)	S358 FDP µg/ml	S358 total FDP	S359 vol. urine (ml)	S359 FDP µg/ml	S359 total FDP	S376 vol. urine (ml)	S376 FDP µg/ml	S376 total FDP
0	142	0	0	128	0	0	115	0	0
7	88	0	0	190	0	0	94	0	0
14	127	0	0	104	0	0	94	0	0
21	84	0	0	86	0	0	78	0	0
28	50	0	0	115	0	0	130	0	0
35	34	2.5	69.7	126	2.5	315	120	2.5	300.0
42	60	5.0	300.0	92	2.5	230	96	0	0

Days after infection	Rabbit number S388 vol. urine (ml)	S388 FDP µg/ml	S388 total FDP	S389 vol. urine (ml)	S389 FDP µg/ml	S389 total FDP
0	120	0	0	136	0	0
7	103	0	0	166	0	0
14	96	0	0	220	0	0
21	115	0	0	150	0	0
28	90	0	0	112	0	0
35	100	1.25	125	75	2.5	187.5
42	15	2.5	37.5	90	2.5	225.0

Anti-F autoantibody titres in control rabbits and rabbits infected with two strains of T. (T.) brucei

(a) Strain S42

Rabbit no.	Days after infection					
	0	7	14	21	28	35
S199	4	64	64	128	32	32
S198	0	16	16	64	64	64
S219	4	8	32	128	32	32
S220	2	32	512	256	32	32
S251	8	64	1024	512	64	64
$\bar{x}$ =	3.6	36.8	329.6	217.6	44.8	44.8

(b) Strain 427

Rabbit no.	0	7	14	21	28	35
S217	4	16	64	256	32	32
S267	4	16	64	128	128	64
S268	2	32	256	128	64	64
S269	2	16	128	64	64	32
S293	4	32	128	64	128	128
S294	8	16	1024	256	128	128
$\bar{x}$ =	4	21.3	244	149.3	90.6	74.3

(c) Control, non-infected rabbits

	0	7	14	21	28	35
S218	1	2	4	4	4	N/T
S270	4	8	8	8	8	N/T
S295	8	8	4	4	4	N/T
S296	8	4	4	4	8	N/T
$\bar{x}$ =	5.2	5.5	5	5	6	

Serum protein changes (g/100 ml) in control non-infected rabbits.

Abbreviations: G = globulin; TP = total protein

	Control rabbit S345			
Day	TP	G	A	A:G
0	7.4	2.4	5.0	2.08
7	6.0	1.9	4.1	2.15
14	6.55	2.2	4.35	1.97
21	6.5	1.89	4.61	2.43
28	6.55	2.25	4.3	1.91
35	N/T	2.05	N/T	N/C
49	N/T	2.1	N/T	N/C

	Control rabbit S346			
Day	TP	G	A	A:G
0	6.3	2.4	3.9	1.62
7	7.5	2.55	4.95	1.94
14	6.55	2.5	4.05	1.62
21	6.6	2.28	4.32	1.89
28	6.65	2.65	4.0	1.5
35	N/T	2.35	N/T	N/C
49	N/T	2.52	N/T	N/C

N/T = not tested

N/C = not calculated

Serum protein changes (g/100 ml) in rabbits infected with T. (T.) brucei 427

Days after infection	Rabbit S347			
	TP	G	A	A:G
0	6.9	2.69	4.21	1.56
7	6.4	3.6	2.8	1.28
14	7.85	3.8	4.05	1.06
21	8.2	5.42	2.78	0.51
28	8.9	5.2	3.7	0.71
35	N/T	4.24	N/T	N/C
49	N/T	4.7	N/T	N/C

Days after infection	Rabbit S348			
	TP	G	A	A:G
0	6.8	2.05	4.75	2.31
7	6.15	3.8	2.35	1.61
14	7.65	4.5	3.15	0.7
21	7.5	5.4	2.1	0.38
28	7.7	4.6	3.1	0.67
35	N/T	5.1	N/T	N/C
49	N/T	5.0	N/T	N/C

N/T = not tested

N/C = not calculated

Serum protein changes (g/100 ml) in rabbits infected with T. (T.) brucei 427

Days after infection	Rabbit S349			
	TP	G	A	A:G
0	7.0	2.65	4.35	1.64
7	6.7	3.4	3.3	1.03
14	7.2	3.95	3.35	1.17
21	8.25	5.5	2.75	0.5
28	8.05	5.78	2.27	0.39
35	N/T	6.0	N/T	N/C
49	N/T	5.56	N/T	N/C

Days after infection	Rabbit S350			
	TP	G	A	A:G
0	6.9	2.8	4.1	1.46
7	5.7	3.0	2.7	1.11
14	6.85	3.7	3.15	0.85
21	7.0	5.18	1.82	0.35
28	7.2	5.5	1.7	0.3
35	N/T	5.76	N/T	N/C
49	N/T	7.3	N/T	N/C

N/T = not tested

N/C = not calculated

Serum total lipid levels (mg/100 ml) in control non-infected rabbits and in rabbits infected with T. (T.) brucei 427

Days after infection	Infected rabbit no: S217	S219	S370	S371	S374
0	560	600	428	219	195
7	660	960	714	285	714
14	1200	800	761	728	380
21	720	730	571	731	690
28	846	1384	690	900	642
35	807	2700	619	2000	904
42	1118	N/T	N/T	N/T	N/T
49	1240	N/T	1142	1428	1285

Day	Control rabbit no: S218	S381	S382
0	620	510	209
7	640	525	310
14	520	495	311
21	884	515	304
28	846	580	320
35	N/T	560	315

Serum phospholipid (mg/100 ml) levels in control non-infected rabbits and in rabbits infected with T. (T.) brucei

	Infected rabbit no:				
Days after infection	S217	S219	S370	S371	S374
0	139	131.2	193	180	75
7	139	131.2	181.2	180	31.2
14	139	128	193	127.2	40.4
21	139	131.2	193	171.2	64.75
28	139	131.2	193	170	43.12
35	139	131.2	193	147.5	53.37
42	139	N/T	N/T	N/T	N/T
49	139	N/T	127.2	180	78.37

	Control rabbit no:		
Day	S218	S381	S382
0	125	115	110
7	131.17	118.5	150
14	131.17	120	148
21	131.17	120	131
28	131.17	112	138
35	131.17	120	140

Serum triglyceride levels (mg/100 ml) in control non-infected rabbits and in rabbits infected with T. (T.) brucei 427

Days after infection	Infected rabbit no: S217	S219	S370	S371	S374
0	413	456	225	104	115
7	511	809	484	91	667
14	1049	653	550	582	327
21	564	585	360	542	607
28	697	1238	479	710	577
35	659	2551	406	1819	833
42	969	N/T	N/T	N/T	N/T
49	1091	N/T	992	1227	1183

Day	Control rabbit no: S218	S381	S382
0	482	380	86
7	489	391	149
14	369	359	154
21	733	311	161
28	694	450	169
35	N/T	425	161

Serum cholesterol (mg/100 ml) in control non-infected rabbits and in rabbits infected with T. (T.) brucei 427

Days after infection	Infected rabbit no: S217	S219	S370	S371	S374
0	7.0	12.0	10.0	5.44	4.5
7	9.5	19.0	48.0	13.62	15.0
14	11.5	15.0	17.26	17.26	16.0
21	16.5	13.0	17.26	17.72	17.2
28	9.5	14.0	17.26	19.09	20.9
35	8.5	17.0	19.54	32.72	17.26
42	9.5	N/T	N/T	N/T	N/T
49	9.0	N/T	21.76	20.9	23.62

Day	Control rabbit no: S218	S380	S381
0	13.0	15.0	13.0
7	19.0	10.0	11.0
14	19.0	13.0	9.0
21	19.0	18.0	12.0
28	20.0	18.0	13.0
35	N/T	15.0	14.0

Phospholipid: total lipid ratio in normal non-infected rabbits and in rabbits infected with T. (T.) brucei 427

Days after infection	Infected rabbit no: S217	S219	S370	S372	S374
0	0.25	0.22	0.45	0.62	0.38
7	0.21	0.14	0.25	0.63	0.04
14	0.12	0.16	0.25	0.17	0.11
21	0.19	0.17	0.33	0.23	0.09
28	0.16	0.09	0.28	0.19	0.06
35	0.17	0.04	0.31	0.07	0.05
42	0.12	N/T	N/T	N/T	N/T
49	0.11	N/T	0.11	0.12	0.06

Day	Control rabbit no: S218	S380	S381
0	0.2	0.22	0.52
7	0.2	0.22	0.48
14	0.25	0.24	0.47
21	0.21	0.23	0.43
28	0.15	0.19	0.43
35	N/T	0.21	0.44

Phospholipid: cholesterol ratio in normal non-infected rabbits and in rabbits infected with T. (T.) brucei 427

Infected rabbit no:

Days after infection	S217	S219	S370	S372	S374
0	19.8	10.9	19.3	33.0	16.5
7	14.6	6.9	3.7	13.2	2.0
14	12.1	8.7	11.2	7.3	2.52
21	8.4	10.0	11.2	9.6	3.65
28	14.6	9.3	11.2	8.9	2.0
35	16.3	7.7	9.8	4.5	3.0
42	14.6	N/T	N/T	N/T	N/T
49	15.4	N/T	5.8	8.6	3.32

Control rabbit no:

Day	S218	S380	S381
0	9.6	7.6	8.4
7	6.9	11.8	13.6
14	6.9	9.2	16.4
21	4.2	6.6	10.9
28	6.5	6.2	10.6
35	N/T	8.0	10.0

Packed cell volumes (PCV%) for control non-infected rabbits and rabbits infected with T. (T.) brucei 427. This data was used in the blood viscosity studies.

Control rabbits

Day	S345	S346
0	41	45
7	37	39
14	37	43
21	39	41
28	39	42
35	39	42
42	N/T	N/T
49	42	42

Infected rabbits

Days after infection	S347	S348	S349	S350
0	44	40	41	38
7	38	35	32	32
14	35	33	34	30
21	34	35	31	33
28	33	33	33	32
35	35	36	31	31
42	N/T	N/T	N/T	N/T
49	33	35	29	30

N/T = not tested

Whole blood viscosity (in centipoise) in control non-infected rabbits and in rabbits infected with T. (T.) brucei 427.

Control rabbits

Day	S345		S346	
	shear rate sec^{-1}			
	230	23	230	23
0	3.65	6.5	3.4	5.5
7	3.65	6.0	3.5	6.3
14	3.7	7.0	3.26	4.7
21	3.5	6.0	3.63	6.0
28	3.45	5.5	3.32	5.8
35	3.6	6.0	3.52	6.0
42	N/T	N/T	N/T	N/T
49	3.4	5.8	3.55	6.0

Infected rabbits

Days after infection	S347		S348		S349		S350	
	shear rate sec^{-1}							
	230	23	230	23	230	23	230	23
0	3.65	5.5	3.3	5.5	3.3	5.6	3.62	6.5
7	3.9	7.0	3.4	6.0	3.6	6.2	3.7	6.7
14	3.7	7.0	3.55	6.5	3.4	6.0	3.63	6.5
21	4.4	8.0	4.3	9.0	3.65	6.5	4.17	7.4
28	3.7	6.5	4.0	7.0	3.1	5.0	4.2	8.5
35	3.6	6.3	3.75	6.4	3.25	5.5	3.6	6.5
42	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T
49	3.15	5.3	3.26	5.0	2.96	4.0	3.55	5.3

N/T = not tested

Plasma viscosity (in centipoise) in control non-infected rabbits and in rabbits infected with T. (T.) brucei 427.

Control rabbits

Day	S345		S346	
	shear rate sec^{-1}			
	230	23	230	23
0	1.25	1.5	1.45	1.6
7	1.3	1.5	1.4	1.5
14	1.3	1.5	1.5	1.8
21	1.3	1.5	1.3	1.2
28	1.51	1.8	1.55	1.8
35	1.6	1.8	1.35	1.3
42	N/T	N/T	N/T	N/T
49	1.3	1.4	1.35	1.5

Infected rabbits

Days after infection	S347		S348		S349		S350	
	shear rate sec^{-1}							
	230	23	230	23	230	23	230	23
0	1.4	1.5	1.4	1.55	1.5	1.7	1.3	1.5
7	1.75	1.9	1.55	1.8	1.45	1.58	1.7	2.1
14	1.65	2.5	1.6	1.82	1.6	1.8	1.8	2.3
21	2.15	3.5	2.0	3.8	1.9	2.0	2.0	2.3
28	2.0	3.9	2.1	2.8	1.95	2.8	2.06	3.0
35	1.75	2.6	1.82	2.5	1.65	2.3	2.1	3.0
42	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T
49	1.65	2.5	1.79	2.1	1.6	1.8	2.07	3.1

N/T = not tested

Serum viscosity (in centipoise) in control non-infected rabbits and in rabbits infected with T. (T.) brucei 427.

Control rabbits

Day	S345		S346	
	shear rate sec^{-1}			
	230	23	230	23
0	1.3	1.5	1.25	1.4
7	1.17	1.5	1.3	1.5
14	1.18	1.6	1.29	1.6
21	1.23	1.6	1.3	1.5
28	1.29	1.6	1.45	2.1
35	1.3	1.6	1.26	1.5
42	N/T	N/T	N/T	N/T
49	1.26	1.4	1.23	1.5

Infected rabbits

Days after infection	S347		S348		S349		S350	
	shear rate sec^{-1}							
	230	23	230	23	230	23	230	23
0	1.31	1.8	1.3	1.5	1.35	1.5	1.3	1.5
7	1.35	1.82	1.4	1.8	1.3	1.5	1.37	2.5
14	1.55	3.0	1.5	2.0	1.45	2.0	1.4	2.5
21	1.95	2.0	1.8	2.3	1.7	2.0	1.75	2.3
28	2.0	4.0	1.65	2.5	1.92	3.0	1.91	3.8
35	1.75	2.6	1.72	2.4	1.65	3.0	1.92	4.6
42	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T
49	1.68	2.6	1.75	2.5	1.5	1.9	1.92	3.8

N/T = not tested

APPENDIX 2

Case Histories

A. Sleeping Sickness Cases.

S.S.No.	Age	Sex	Duration	Blood Group	Parasitaemia Blood	Parasitaemia Glands	IFT Titre
859	30y	F	3y FU	B	-ve	-ve	2
866	47y	M	32M FU	O	-ve	-ve	3
939	36y	F	19M FU	O	-ve	-ve	3
944	25y	F	18M FU	O	-ve	-ve	3
956	43y	F	15M FU	A	-ve	-ve	3
975	20y	M	9M FU	A	-ve	-ve	2
992	20y	M	7M FU	A	-ve	-ve	-
1002	25y	F	3M FU	A	-ve	-ve	2
1004	28y	F	3M FU	O	-ve	-ve	3
1005	45y	F	3M FU	A	-ve	-ve	3
1011	21y	M	Reinf. 2 days	B	+ve	+ve	-
1013	19y	F	5 days	O	+ve	+ve	1
1019	43y	M	5 weeks	B	+ve	+ve	3
1020	17y	F	8M	A	*-ve	-ve	-
1021	20y	M	4M	B	-ve	-ve	3
1023	12y	M	1M	O	+ve	+ve	3
1024	20y	M	1M	O	+ve	+ve	3
1025	55y	M	4M	B	+ve	+ve	3
1026	45y	M	3M	B	+ve	+ve	3
1030	48y	M	7M	O	-ve	+ve	-
1031	28y	M	12M	O	-ve	+ve	-
1032	13y	M	4M	B	+ve	+ve	-

* Positive in CSF

B. Control Patients

Patient	Sex	Diagnosis
S.M.	F	Malaria
A.A.	F	Mumps
E.F.	F	Adenitis
M.W.	F	Conjunctivitis
M.O.	M	Bruising
S.E.	M	Tropical Ulcer

Older Sleeping Sickness Plasma

S.S. 624 Male, age 15 y. Blood smear and gland juice positive. Early stage of FNS involvement. No complications.

S.S. 625 Female, age 45 y. Blood smear and gland juice positive. Early stage. Complicated by hookworm, microfilaria and mild bronchitis and E. coli cystitis.

S.S. 626 Male, age 23 y. Blood smear and gland juice positive. Early stage. Complicated by hookworm, schistosomiasis (S. mansoni) and Ascaris.

Histories of trypanosome stabilates

T. (T.) brucei S42

Isolated from an old ♀ warthog on 12/3/66 in Tanzania. The strain was tested on two volunteers at Tororo in April 1966 and subsequently proven negative - for man. Since that time it has been frozen and passaged in mice, rats and chimpanzees. Recently it has been cyclically transmitted through mice and Glossina mortisans.

T. (T.) brucei 427 (≡ NIMR 15)

Isolated from sheep in S.E. Uganda in 1960 and subsequently passaged in mice. Since then it has been frozen and passaged through rats and mice.

Both strains were obtained from the Lister Institute for Preventive Medicine and maintained by mouse passage at Silwood Park.

APPENDIX 3

Correction factor table for blood viscosity

Calibration fluid η	% correction
3.9	+ 5
3.8	+ 7.5
3.7	+ 11
3.6	+ 14
4.0	no correction
4.1	no correction
4.2	no correction
4.3	- 5
4.4	-77
4.5	- 9
4.6	- 11
4.7	- 13

Measured η	5%	7%	7.5%	9%	11%	13%	14%
3.0	.15	.2	.2	.25	.3	.4	.4
3.5	.2	.25	.25	.3	.4	.45	.5
4.0	.2	.3	.3	.35	.45	.5	.6
4.5	.2	.3	.35	.4	.5	.55	.65
5.0	.25	.35	.4	.45	.55	.6	.7
5.5	.3	.4	.4	.5	.6	.7	.8
6.0	.3	.4	.45	.55	.65	.75	.85
6.5	.3	.45	.5	.6	.7	.8	.9
7.0	.35	.5	.55	.65	.8	.85	1.0
7.5	.4	.5	.6	.7	.85	.9	1.05
8.0	.4	.55	.6	.7	.9	1.0	1.15
8.5	.4	.6	.65	.75	.95	1.05	1.2
9.0	.4	.65	.7	.85	1.05	1.2	1.35
9.5	.45	.7	.7	.85	1.05	1.2	1.35
10.0	.5	.7	.75	.9	1.1	1.25	1.4

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I would like to express my gratitude to the following with whom I have had useful discussions during this work - Prof. R. J. Terry, Mr. K. M. Hudson; Dr. A. R. MacKenzie, Dr. G. C. Jenkins; Dr. L. E. Glynn and Dr. Christine Hawkey.

The thesis was competently typed by Miss Carole Collins to whom I offer my thanks. Thanks are also due to Mr. H. Devitt for photographic work. Finally I would like to record my appreciation to Mr. D. J. Harrison for his continued help in correcting and copy-reading.

Trans. R. Soc. trop. Med. Hyg. 66, 341. 1972

length of time. In macrophages, multiplication was less synchronous than in HeLa cells, and interpretation was complicated by the continued presence of extracellular trypanosomes which presumably continued (actively or passively) to enter cells throughout the period of observation. However, transformation into small trypomastigotes has been seen in macrophages.

In monophasic medium *in vitro,* initial multiplication of *T. dionisii* as epimastigotes culminated in the development of long thin trypomastigotes thought to be the forms which are infective to mammalian cells; some shorter, broader trypomastigotes also appeared.

Preliminary results of attempts to use this system to study cell mediated immunity (BRYCESON et al., 1970) indicate that lymphocytes exposed *in vivo* to *T. dionisii* or *T. vespertilionis* antigens may selectively destroy more target cells infected with *T. dionisii* than do unexposed lymphocytes (see Table). Work is continuing but it has not yet proved possible to reproduce this effect regularly.

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A histochemical investigation of fibrin deposits in the tissues of rabbits infected with *Trypanosoma brucei*

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In many kinds of inflammation the resulting exudates lead to the deposition of material referred to as fibrinoid. These deposits consist mainly of fibrin. It has been suggested that immune complexes may be involved in producing this exudation (VASSALLI and MCCLUSKEY, 1964).

Immune complexes have been detected in the endothelial lining of renal arterioles and venules of rabbits with trypanosomiasis (BOREHAM and KIMBER, 1970). The present study has been undertaken to determine whether immune complexes cause tissue damage leading to fibrinoid deposition.

Rabbits were killed 4–5 weeks after infection with *Trypanosoma brucei* 427. Frozen sections of tissue were stained histochemically for fibrin using the Martius-Scarlet-Blue method.

No fibrin deposits were detected in the brain, liver and heart. Small amounts were detected in the spleen and occasional deposits in the kidney. The most marked lesions were in the lungs, where alveolar cells were swollen and fibrinoid deposition was apparent in the alveolar tissue suggesting a leakage of plasma proteins, including fibrinogen.

The main anaphylactic shock organ in rabbits is the lung. A possible explanation for these results is that the pharmacologically active substances, including kinins, released in trypanosomiasis infections causes increased vascular permeability in the lungs. This would result in a fibrinous exudation and thus affect the transfer of oxygen across the alveolar membranes.

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Investigation of changes in the physiology of the cerebrospinal fluid in rabbits infected with *Trypanosoma brucei*

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LE PORT (1933) suggested that the headache which occurs in the early stages of Rhodesian sleeping sickness might be caused by cerebral hypertension due to swelling of

Trans. R. Soc. trop Med. Hyg. 66, 344-45. 1972

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The trypanolytic factor in normal human serum

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Normal human serum contains a factor which kills certain species of trypanosomes *in vitro* and *in vivo*; it is probable that this factor is largely responsible for the insusceptibility of man to infection with *Trypanosoma brucei brucei*.

Our earlier work, using gel filtration on Sephadex G 200, indicated that this factor was associated with the fraction of human serum which contained high molecular weight proteins. Further studies have now been undertaken, separating the serum proteins by ultracentrifugation in a sucrose density gradient. Initial separations using fresh human serum showed that the active fraction was among the most rapidly sedimenting proteins which comprise the IgM immunoglobulins and the α_2 macroglobulins. As antibody activity is associated with the IgM rather than the α_2 macroglobulins, attempts were made to isolate and assay purified human IgM.

The starting material was a macroglobulinaemic serum containing large amounts of IgM. The IgM fraction was further concentrated by precipitating it with the euglobulin fraction and then subjecting the euglobulins to ultracentrifugation. The resulting IgM rich factor could not be assayed directly because the trypanolytic activity had been lost during the prolonged fractionation procedure. Instead an indirect assay was devised. A rabbit antiserum was raised against the IgM rich fraction and this was shown to inhibit strongly the trypanolytic activity of fresh normal human serum as demonstrated in the mouse protection test. Agar gel diffusion and immunoelectrophoresis showed that the rabbit antiserum was not absolutely monospecific for IgM but showed slight cross reaction with human IgG. Accordingly, the rabbit antiserum was absorbed with purified human IgG, until it was shown, by diffusion tests, to be monospecific for IgM. This anti-IgM serum again strongly inhibited the trypanolytic activity of normal human serum.

It is concluded that this trypanolytic factor is an IgM immunoglobulin; its origin and the nature of the receptor sites on the trypanosomes it attacks are unknown.

Non-trypanosome specific components of the elevated IgM levels in rabbit trypanosomiasis

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Much of the immunoglobulin formed during trypanosome infections is not trypanosome specific. FREEMAN et al. (1970) have shown that in the rhesus monkey the non-trypanosome specific component of IgG may be as much as 95%. Recently we have investigated 3 naturally occurring IgM antibodies in rabbits infected with *Trypanosoma brucei*.

2 of the antibodies, anti-liver autoantibody and anti-Wasserman antibody were measured by the complement fixation test. The third antibody, anti-fibrinogen, was measured by a haemagglutination test using sheep red blood cells sensitized with human fibrinogen. For each antibody the natural preinfection titres of ½ to 1/16 increased to between 1/64 and 1/512. The peak occurred around day 14 of the infection. Each antibody was found to be heat labile (65°C for 30 minutes) and sensitive to reduction by 2-mercaptoethanol indicating that at all stages of the infection the antibodies are of the IgM class.

The anti-liver autoantibody was also detected by the Ouchterlony method in agar gel in which a single line developed close to the serum well again suggesting that the antibody is a macroglobulin.

The possibility that the anti-liver autoantibody is really directed to cardiolipin (Wasserman antigen) is unlikely on the evidence produced by KID and FRIEDEWALD (1942 a, b) who first described the natural anti-tissue antibody. They showed by absorption studies and by ethanol extraction that the natural anti-tissue antigen was not Wasserman antigen.

The anti-fibrinogen antibody has been shown to cross react with rabbit fibrinogen by absorption of the sera with sheep red blood cells, tanned and sensitized with whole rabbit plasma. Work is in progress to determine whether or not autologous fibrinogen cross reacts. It seems probable that the anti-fibrinogen antibody will also cross react with fibrin and possibly fibrinogen degradation products.

Considered together these antibodies represent part of the elevated IgM level found in the trypanosomiasis of the rabbit. All 3 antibodies occur naturally and increase during a trypanosome infection but without the normal IgM/IgG switch occurring. The mechanisms underlying this type of increase are unknown.

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Further studies on the cotton rat, and the action of its serum on *Trypanosoma vivax*

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As has been reported previously, the resistance of the cotton rat to infection with *T. vivax* is mediated by a serum protein.

The studies reported on last year at this seminar indicated a molecular weight for this anti-vivax factor of 550,000 ± 50,000 as determined by gel filtration. As the accuracy of the value obtained by this method depends on the closeness of the molecule to a spherical shape, studies with a high-speed centrifuge were embarked upon in an attempt to gain further information.

High-speed centrifugation of normal cotton rat serum on a sucrose density gradient with radio-labelled marker proteins indicated that the factor sedimented with the high density proteins, the macroglobulins.

Treatment with 2-mercaptoethanol failed, however, to destroy the anti-vivax activity when used at concentrations which reduce IgM to its constituent subunits.

The factor also failed to react in the "immune adherence" reaction although this test was found to work well with IgM complement—fixing, anti-vivax antibodies (carried out by Mrs. P. E. Mackenzie in our laboratory).

The association of the factor with certain of the so-called external secretions i.e., into gut and genital tract, reported previously, failed when we looked at maternal transfer. When newly-born Wistar rats were suckled on nursing cotton rat mothers there was no indication of the factor having passed on to the nurslings in the milk.

Conclusions drawn from this project; it seems that the cotton rat possesses a protein factor which behaves in many ways like a specifically acquired antibody to *T. vivax*. But this factor seems to be natural and not acquired. Further its physico-chemical characteristics and detailed biological behaviour cannot be correlated with any known immunoglobulin. Its origin and true function are unknown.

Partial purification of immunogenic (protective) antigens of *Trypanosoma brucei brucei*

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Although many workers have fractionated trypanosome homogenates in attempts to purify the antigens and determine their properties, little attention has been paid to those antigens which are immunogenic (protective). A contributory factor to this deficiency in our knowledge was undoubtedly the fact that separation of trypanosomes from host components was laborious and gave low yields, until column-separation of trypanosomes (LANHAM, 1968) was shown to be an efficient and rapid method for obtaining large quantities of pure trypanosomes.

Trans. R. Soc. trop. Med. Hyg. 67, 268. 1973

Immunization of mice, against *T. brucei* infection, by the administration of released antigen adsorbed to erythrocytes

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Syngeneic red blood cells were exposed to plasma taken from infected animals, and then repeatedly washed.

Mice given intravenous inoculations of erythrocytes prepared in this way were found to be resistant to challenge by 100 ID_{63} of *T. brucei* antigenic type ETat 2, the homologous organism.

This finding provides another example of the rôle that the erythrocyte may play in the immune response (BUXTON, 1959; HADDOW and HERBERT, in preparation). It may also help to elucidate the mechanism that causes anaemia in trypanosomiasis (JENNINGS et al., 1972).

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The development of immune response during the course of infection with *Trypanosoma cruzi* in rabbits

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Adult rabbits were infected with cultures of *T. cruzi*. The development of immune response was studied by the effect of the growth and morphology of *T. cruzi* in culture of sera from these animals. By these means an increasing trend to immunity was demonstrated.

Autoantibodies in African trypanosomiasis

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We have previously reported the occurrence of several non-trypanosome specific antibodies in rabbits infected with *Trypanosoma brucei* (MACKENZIE, BOREHAM and FACER, 1972). This work has now been extended to other species.

Anti-liver, anti-Wassermann and anti-fibrinogen antibodies have been measured in 6 experimentally infected cattle. 2 were infected with *T. congolense*, 3 with *T. brucei* and 1 a mixed infection of *T. brucei* and *T. congolense*. In addition, anti-liver and anti-Wassermann antibodies have been measured in single samples from 14 other infected cattle.

Raised levels of anti-liver antibodies were found in 50% of the cattle while 75% showed elevated anti-Wassermann titres. Increases of anti-fibrinogen antibodies were seen in 5 of the 6 cattle tested.

Human serum collected from sleeping sickness patients at the E.A.T.R.O. Hospital were mostly found to have raised levels of these antibodies. Anti-liver antibodies were not usually seen during the first 4 weeks of the infection, whereas anti-Wassermann antibodies were only detected between 4 and 5 weeks.

8 lions and 10 hyaenas naturally infected with trypanosomes were also tested for anti-Wassermann and anti-liver antibodies using extracts of cat and hyaena livers as antigens. All sera tested were negative for both antibodies.

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Trans. R. Soc. trop. Med. Hyg. 67, 279. 1973

periods of 30 days. Subsequently, blood samples were taken from 5 cattle and another sheep with patent *T. congolense* parasitaemia, and 2·0 ml. portions were inoculated into groups of 4 rats. Trypanosomes were never detected in 5 groups of rats, but 3 out of 4 rats inoculated with blood from an ox developed patent parasitaemias after long prepatent periods. A third attempt was made to isolate *T. congolense* by inoculating single rats with blood samples taken from 15 cattle known to be infected with the organism though not all showing patent parasitaemia at the time of sampling. *T. congolense* was only detected in the blood of 8 of the 15 rats, and 7 of the infections were very mild, trypanosomes only being found in low numbers on a few occasions.

The lack of infectivity and low pathogenicity of the isolates of *T. congolense* in laboratory rats suggested that they were mostly of the *congolense* type although morphologically they were of the *dimorphon* type. These results indicate that where transmission of *T. congolense* is taking place through wild hosts, the pattern of infectivity for laboratory animals may be very different from patterns described previously, and support the suggestion of WELLS and LUMSDEN (1968) that certain diagnostic methods in current use, particularly animal inoculation, might be inadequate for detection of infection in game animals.

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Fibrinogen degradation products in African trypanosomiasis

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Fibrinogen and fibrin are broken down in the body by proteolytic enzymes, in particular plasmin, to form smaller molecular weight protein units known as Fibrinogen Degradation Products (FDPs). It has recently been realized that some of these substances have biological properties and interfere with several haemostatic mechanisms.

FDPs have been measured in the serum of rabbits and humans infected with trypanosomes, using the tanned red cell haemagglutination inhibition assay (MERSKEY et al., 1966).

Rabbits infected with either *Trypanosoma brucei* S42 or 427 showed marked increases in the concentration of FDPs from control levels of 20–40 μg./ml. to 160 μg./ml. 14–21 days after infection. It has been shown that the degradation products formed are the smaller molecular weight fragments known to exhibit biological activity.

The formation of FDPs appears to be due to the activation of plasminogen during the infection, since plasminogen levels in the plasma are reduced.

Raised FDP levels have also been demonstrated in sera collected from patients in Uganda with active trypanosomiasis. The biological significance of the release of FDPs remains to be investigated.

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Serum lipids of ruminants infected with trypanosomes

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During the first 2 months of infection with pathogenic trypanosomes, the serum phospholipid levels of Zebu cattle decline steadily to less than half of the preinfection levels. The rate and extent of the decline depend on the species of the infecting organism and the severity of the infection, although the reduction is not directly related to the number

group of heterochromatic supernumary B chromosomes (and the Y chromosome in males) do not engage in synthetic activity in these giant nuclei and are eliminated at an early stage of polytene development. A similar situation has been reported for *G. m. morsitans*. Although polytene nuclei are found in a variety of tissues, the most suitable for cytological study are those located in trichogen and tormogen cells associated with thoracic macrochaetae. Development is initiated on about day 12 after larval deposition and the chromosomes grow through endomitotic reduplication reaching their maximum size at day 19–20 in the apical scutella hairs. By this time these two bristles have reached what is probably their maximum size and this is followed shortly after by a rapid disintegration of the chromosomes. Throughout development, bands along the chromosomes display puff patterns which reflect the sequence of DNA transcription.

After mapping these chromosomes it has been found that approximately 72% of the bands of *G. austeni* are represented on the same chromosomes of *G. m. morsitans*. It is evident that during the process of evolution and speciation the positions of many of these sequences have been changed; not apparently *via* translocations or centric fusion or fission, or even as far as we can ascertain through extensive gene duplication, but rather following a complex series of pericentric and paracentric inversions. However, there are small sequences in *G. austeni* which are not found in *G. m. morsitans*. This is probably not surprising as *G. austeni* is an aberrant member of the *morsitans* group and on taxonomic grounds has affinities with both the *fusca* and *palpalis* groups.

Plasma fibrinogen in rabbits infected with *T. (T.) brucei*

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New Zealand white rabbits were infected with 2 strains of *T. (T.) brucei* (427 and S42) and plasma fibrinogen concentrations followed throughout the infection. Similar results were obtained for both strains of trypanosomes. The concentration of fibrinogen was found to rise from preinfection levels of 270 ± 32 mg./100 ml. plasma to peak levels of 711 ± 80 mg./100 ml. 14 days after infection. In prolonged infections of the rabbit, there was a fall in fibrinogen concentration during the 5th and 6th weeks to below preinfection levels.

Since there was also a rise in the breakdown products of fibrin/fibrinogen (Boreham and Facer, 1973) it is suggested that there is an increased turnover of fibrinogen in infected rabbits.

The raised fibrinogen concentration probably contributes to some of the pathological features seen in the disease, such as the raised erythrocyte sedimentation rate. Another likely effect of raised plasma fibrinogen would be changes in the flow properties of the blood, leading to stasis in the capillary beds and possible microthrombus formation.

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Some studies on the coagulation of rabbit blood during infection with *T. brucei* S42

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Some of the rabbits infected in the programme of work carried out at The London Hospital Medical College have had their clotting mechanisms investigated in order to elucidate the possible rôle of "disseminated intravascular coagulation" (DIC) in causing haemolysis and anaemia. Particular attention was paid to the "intrinsic coagulation mechanism" in view of an earlier suggestion that the kaolin-cephalin time (KCT) became prolonged in the late stages of infection, and also of the rôle of factor XII (Hageman factor) in immune responses. Relative to human blood, the KCT of normal rabbit plasma is shorter (about 20 secs. compared with 35 secs.) and the activities of factors VIII, IX, and XII are higher. In the late stages of infection all these factors increase, especially VIII and XII. The KCT's, however, did not shorten; according to some techniques they increased by 5 seconds. This may not be significant. It was apparent that scrupulous attention to sampling and manipulation was required; accordingly all samples were taken by cardiac puncture.

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FIBRINOGEN AND FIBRINOGEN/FIBRIN DEGRADATION PRODUCTS IN EXPERIMENTAL AFRICAN TRYPANOSOMIASIS

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(*Received* 24 *July* 1973)

Abstract—BOREHAM P. F. L. and FACER CHRISTINE A. 1974. Fibrinogen and fibrinogen/fibrin degradation products in experimental African trypanosomiasis. *International Journal for Parasitology* **4**: 143–151 Studies have been carried out on some components of the fibrinolytic system of rabbits infected with two strains of *Trypanosoma* (*Trypanozoon*) *brucei*. Significant increases in fibrinogen/fibrin degradation products (FDP) occur with a peak of activity 14–21 days after infection. Plasma fibrinogen concentrations increase during the infection. Measurement of plasminogen concentrations and experimental inhibition of plasmin by drugs suggests that plasminogen is being activated during the infection giving rise to FDP production. Administration of the trypanocidal drug Mel B to infected rabbits did not cause a rapid change in FDP levels. The results suggest an increased synthesis of fibrinogen. This, together with the increased amount of FDP, may be indicative of an increased fibrinolytic activity suggesting the formation of microthrombi in the circulation.

INDEX KEY WORDS: *Trypanosoma* (*Trypanozoon*) *brucei*; rabbits; fibrinogen; fibrinogen/fibrin degradation products (FDP); fibrinolysis; plasminogen activation; trypanocidal drugs; pathogenesis; capillary permeability; microthrombi.

INTRODUCTION

THE BLOOD coagulation system lays down fibrin to repair any damage that may occur to the vascular endothelium; the fibrinolytic system removes fibrin deposits. Both systems are complicated, involving a large number of different factors in the blood and tissues. The coagulation system essentially consists of the conversion, by prothrombin activator ('thromboplastin'), of prothrombin to thrombin which, in turn, converts fibrinogen to fibrin. In the fibrinolytic system plasminogen, an inactive plasma globulin, is converted by activators to plasmin, a proteolytic enzyme capable of digesting fibrin and fibrinogen. Activators may be endogenous or exogenous. A simplified diagram of the coagulation and fibrinolytic systems to demonstrate their inter-relationship is shown in Fig. 1.

The proteolysis of intravascular fibrin or fibrinogen results in the formation of soluble polypeptide fragments referred to as fibrinogen/fibrin degradation products (FDP). These substances are also known as fibrinogen–fibrin related antigen (F.R. antigen) since they react with antibody (Wood *et al.*, 1972). This enzymic breakdown of fibrinogen and fibrin occurs in stages, causing the formation of FDP of varying molecular weights (Fig. 2). The higher molecular weight fragment (X) is clottable with thrombin while fragments Y, D and E are not clotted by thrombin. Fragments X and Y are referred to as 'early' FDP while the smaller molecular weight fragments (D and E) are known as 'late' FDP. It has recently been shown that the late FDP have important biological properties which may be important in the pathogenesis of disease. (Triantaphyllopoulos & Triantaphyllopoulos, 1970).

Since FDP in serum appear to be relatively persistent in the circulation (Fletcher *et al.*, 1962) they may provide a sensitive indication of increased fibrinolysis subsequent to intravascular fibrin form-

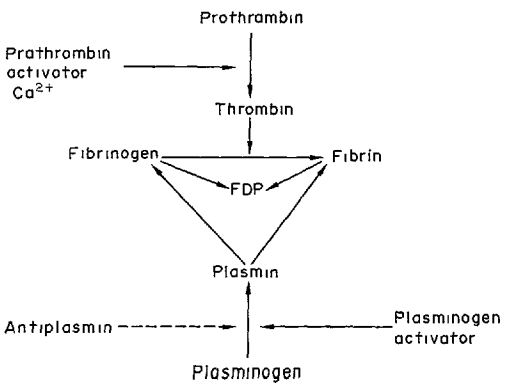


FIG. 1. Diagram of the blood clotting and fibrinolytic systems to show their inter-relationship. The broken line indicates inhibition.

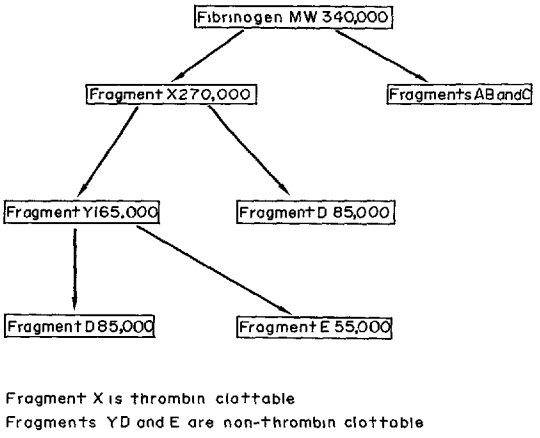


FIG. 2. Formation of FDP from fibrin or fibrinogen by proteolytic enzymes such as plasmin. Fragments D and E show greatest biological activity.

ation. It has been shown that alterations in fibrinogen metabolism and fibrinolysis readily occur under conditions of stress (Barnhart *et al.*, 1970) and since it is known that in experimental trypanosomiasis of the rabbit, such conditions occur (Goodwin, 1970) it was considered of interest to study any changes that might occur in fibrinogen and FDP concentrations.

MATERIALS AND METHODS

1. *Experimental infections*

Adult New Zealand white rabbits of either sex, weighing 2·5–3 kg were infected subcutaneously with one of two strains of *Trypanosoma* (*Trypanozoon*) *brucei*, S42 or 427.

Strain S42 was isolated from an old female warthog, in Tanzania on 12 March, 1966, and since has been maintained in mice. It usually kills rabbits after 2–4 weeks. Strain 427 was isolated from a sheep in South East Uganda in 1960 and also passaged in mice. This strain kills rabbits 4–6 weeks after infection although some animals survive longer and may become aparasitaemic. Both strains were obtained from the Lister Institute of Preventive Medicine.

Trypanosomes were separated from heavily infected mouse blood by the anion exchange column method of Lanham (1968) and 10^8 parasites injected subcutaneously into each rabbit.

2. *Collection of serum and plasma samples*

Three-ml samples of venous blood were taken from the marginal ear vein of rabbits for FDP assay at intervals throughout the infection. The blood was collected in tubes containing 100 K.I.U/ml blood of transexamic acid ('Trasylol' FBA Pharmaceuticals Ltd.), to prevent any *in vitro* digestion of fibrin. The blood was allowed to clot at room temperature and the serum removed by centrifugation. Serum samples were stored at −40°C until required for testing. Blood was also collected in ethylenediaminetetra-acetic acid dipotassium salt (EDTA 2 mg/ml) for plasma samples to determine fibrinogen concentrations.

3. *Thrombin treatment of serum*

A sample of serum collected in 'Trasylol' was mixed with thrombin (10 units/ml Thrombin Topical, Parke Davis & Co.) to clot any high molecular weight FDP present.

4. *Preparation of rabbit fibrinogen for sensitization of sheep red blood cells*

Fibrinogen was prepared according to the method of McFarlane (1963) from citrated rabbit plasma where the re-precipitation of fibrinogen was carried out at 21 and 23 per cent saturation of ammonium sulphate. The precipitate was dissolved in 10 mM trisodium citrate in 8 mM phosphate buffer pH 7·5 and dialysed against two changes of this buffer for 36 h. The dialysate was centrifuged and the supernatant containing the partially purified fibrinogen stored at −20°C.

5. *FDP assay*

The concentration of serum FDP was estimated by the tanned red cell haemagglutination inhibition immunoassay using the Wellcome assay kit (Wellcome Reagents Ltd.). The principle of the test is shown in Fig. 3.

The experimental procedure can be divided into three sections:

(a) *Absorption of anti-species agglutinins.* Anti-species agglutinins were removed from the serum prior to FDP assay by two absorptions with equal volumes of washed packed sheep red cells. The absorptions were carried out at room temeprature for 30 min.

(b) *Inhibition test.* Doubling dilutions of absorbed sera were prepared using the microtitre system (Flow Laboratories Ltd.). An equal volume of antiserum diluted 1 in 10,000 in citrate buffer pH 8·0 was added to each well. After allowing to stand at 4°C for 1 h, 1 vol. of a 2·5 per cent suspension of human fibrinogen sensitized sheep red cells was added to each well. The plate was shaken and left overnight at 4°C. The end point was taken as the last dilution that showed no trace of agglutination. The concentration of FDP in the serum sample was subsequently assessed by comparison of the results obtained with a known fibrinogen standard, since fibrinogen, fibrin and FDP are known to have the same antigenic determinants. Suitable controls were included in each assay, these were dilutions of non-infected serum, buffer and reagent controls.

(c) *Reactivity of antibody to human fibrinogen with rabbit fibrinogen.* The specificity of the rabbit anti-human fibinogen serum for rabbit fibrinogen was tested by first coating tanned red cells with rabbit fibrinogen (Das, 1970), and then carrying out direct agglutinations with the antiserum.

6. *Fibrinogen determination*

The method for determining the concentration of fibrinogen in non-infected and infected plasma was based on the estimation of fibrin obtained after inducing clotting with thrombin (Ratnoff & Menzie, 1951). In this procedure, plasma was first diluted with saline containing ε-aminocaproic acid (EACA) 50 mg/100 ml to prevent fibrinolytic activity during the test. Thrombin (Leo Pharmaceutical Co.) 50 units/ml in 0·5 M calcium chloride was added and the plasma left for 30 min to form a clot. The fibrin clot was then washed with the saline/EACA

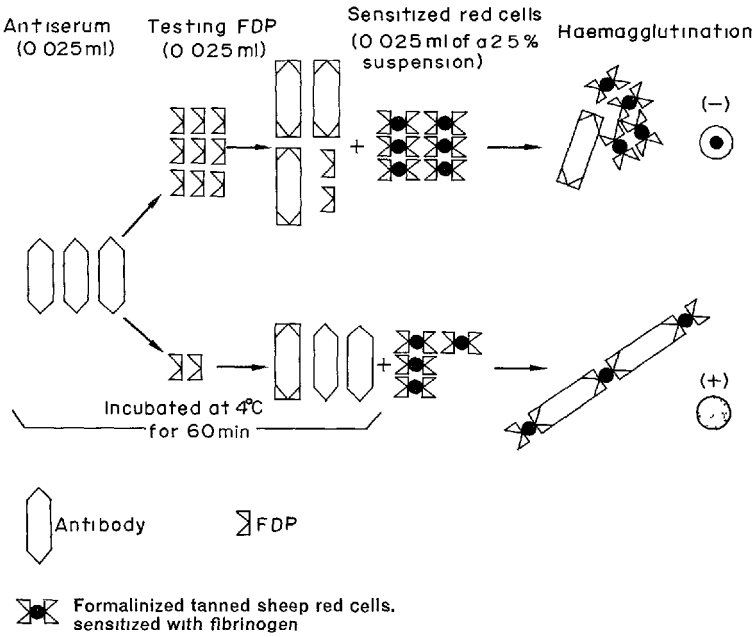


FIG. 3. Diagrammatic representation of the tanned red cell haemagglutination inhibition immunoassay used for determination of serum FDP concentrations.

mixture to remove any contaminating plasma proteins, dissolved in 1% sodium hydroxide solution, and its total protein content determined spectrophometrically by the biuret method. All determinations were performed in duplicate.

7. *Plasminogen assays*

Plasminogen concentrations were determined in pre-infection sera and sera containing high FDP concentrations from two rabbits by Dr. Christine Hawkey, Nuffield Institute of Comparative Medicine, using the method described by Remmert & Cohen (1949) as modified by Alkjaersig *et al.* (1959) and using human urokinase as activator.

RESULTS

1. *Reactivity of rabbit anti-human fibrinogen serum with rabbit fibrinogen*

Similar haemagglutination titres were obtained when direct agglutinations were carried out using an anti-human fibrinogen serum and sheep red blood cells sensitized with either human or rabbit fibrinogen. This indicates that it is possible to assay rabbit FDP using commercially available products intended for clinical use.

2. *FDP concentrations*

Figure 4 shows the increase in FDP concentration of six rabbits infected with *T.* (*T.*) *brucei* 427 and four rabbits infected with *T.* (*T.*) *brucei* S42, over a period of 4 weeks. The concentration of serum FDP in normal rabbits was between

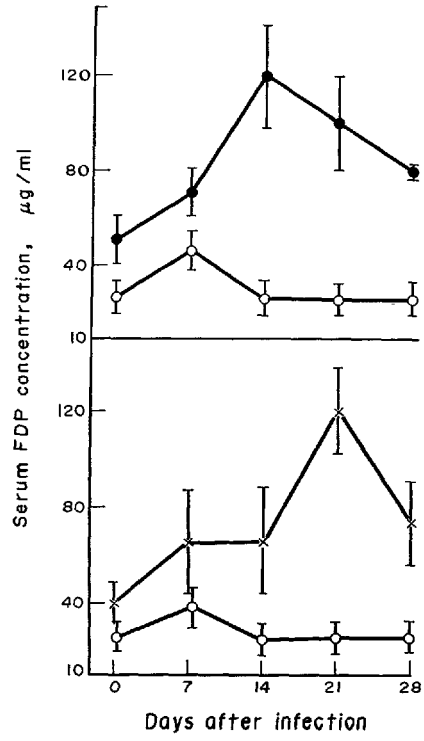


FIG. 4. FDP concentrations in the serum of rabbits infected with *T.* (*T.*) *brucei*. ×———× Strain 427 mean (±S.E.) are plotted for six rabbits; ●———● Strain S42 mean (±S.E.) for four rabbits; ○———○ Non infected controls mean (±S.E.) for three rabbits.

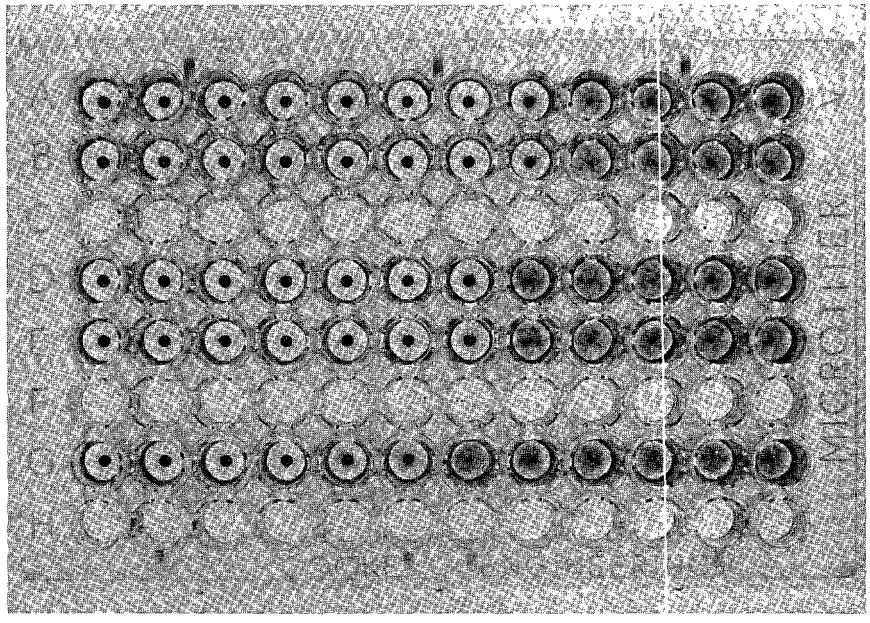


FIG. 5. Assay of serum FDP of two rabbits infected with *T.* (*T.*) *brucei* 427 using the haemagglutination inhibition assay. Doubling dilutions of serum from neat in well 1 to 2048 in well 12. Rows A and B—rabbit S262 day 14: row A serum treated with thrombin; row B non-thrombin treated serum; both giving a titre of 160 μg/ml (well 8). Rows C and D—rabbit S263 day 11: row C serum treated with thrombin; row D non-thrombin treated serum; both giving a titre of 80 μg/ml (well 7). Row G—titration of doubling dilutions of fibrinogen standard 10 μg/ml in row 1. Sensitivity of assay as shown by end point well 4 1·25 μg/ml.

20–40 μg/ml and this rose following infection to about 160 μg/ml. The peak FDP concentration varied with individual rabbits, but usually occurred between 14 and 21 days after infection. No significant differences were found in the concentrations of serum FDP or the time when maximum concentrations were seen for the two strains of trypanosomes.

3. *Thrombin treatment of serum*

Treatment of serum samples with thrombin did not alter the titre of FDP (Fig. 5). This suggests that only 'late' FDP were present in the samples, although it is possible that the action of thrombin in the original clotting process may have removed any early FDP.

4. *Fibrinogen concentrations*

Little variation in the concentration of fibrinogen occurred in non-infected rabbits over the period of the investigation, and the concentration did not exceed 300 mg/100 ml blood. Figure 6 shows the results obtained for fibrinogen concentrations of rabbits infected with *T.* (*T.*) *brucei* 427. Increased concentrations were seen during the 1st week of infection, reaching a peak between the 3rd and 4th week. In most cases, the maximum increase in fibrinogen concentration was about 100 per cent. Again, little difference was seen between infections with the two strains of trypanosome.

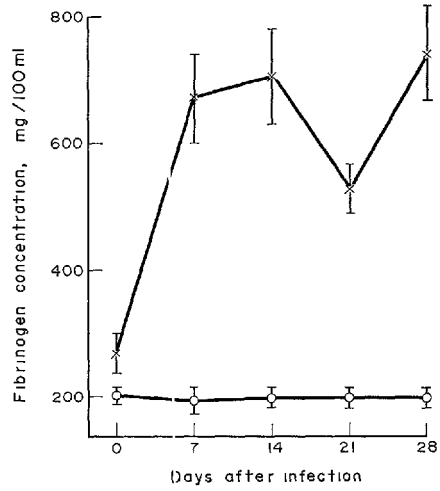


FIG. 6. Fibrinogen concentrations in the plasma of rabbits. ×———× Infected with *T.* (*T.*) *brucei* 427 mean (±S.E.) for six rabbits; ○———○ Non infected controls mean (±S.E.) for three rabbits.

TABLE 1—FDP AND PLASMINOGEN CONCENTRATIONS IN TWO RABBITS INFECTED WITH *T.* (*T.*) *brucei* 427

Days after infection	S267 FDP (μg/ml)	S267 Plasminogen (i.u./ml)	S269 FDP (μg/ml)	S269 Plasminogen (i.u./ml)
0	40	0·38	40	1·12
7	161	0·17	N.T.	N.T.
14	N.T.	N.T.	161	0·5
28	80	0·29	80	0·6

N.T. = Not tested.

5. *Plasminogen concentrations*

Although samples were tested from only two rabbits, both infected with *T.* (*T.*) *brucei* 427, the results showed that the plasminogen concentrations decreased during the infection (Table 1). Even at the end of the experiment, the plasminogen concentrations were below pre-infection levels. It should be noted that rabbit S267 had a low concentration of plasminogen at the commencement of the experiment. The plasminogen and FDP concentrations showed an inverse relationship (Fig. 7). At peaks of FDP concentrations, there was a fall in the amount of plasminogen present in the serum.

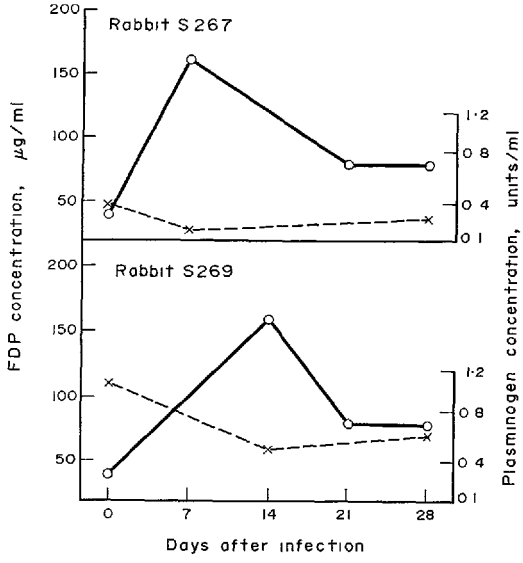


FIG. 7. Serum FDP and plasminogen concentrations in two rabbits infected with *T.* (*T.*) *brucei* 427 to show inverse relationship. ○———○ FDP concentration; ×- - - -× Plasminogen concentration.

6. *Effect of plasmin inhibition* in vivo

Five rabbits were used in this experiment, three were infected with *T.* (*T.*) *brucei* 427 and two were left as controls. Serum samples were assayed at intervals to determine FDP concentration. When raised FDP concentrations were found (21 days after infection), the two non-infected rabbits and two infected rabbits were treated with plasmin inhibitors and the effect on FDP concentrations noted. Since the half-life of the FDP in trypanosome infections was not known, it was decided to treat the rabbits with a mixture of 1 ml EACA 400 mg/ml and 1 ml of 'Trasylol' (5000 K.I.U./ml). These drugs were administered separately by intravenous injections into marginal ear veins at 8 h intervals (08.00, 16.00 and 24.00 h) for 3 days. The results of these treatments are shown in Fig. 8. The effect of inhibiting plasmin in rabbits infected with trypanosomes

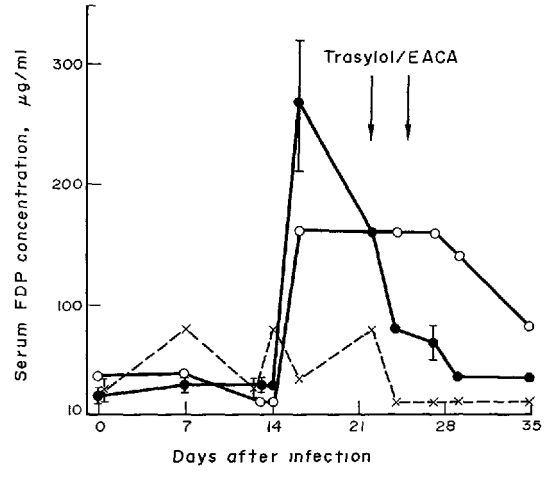


FIG. 8. The effect on FDP concentration of plasminogen inhibition by administration of 'Trasylol' (5000 K.I.U.) and EACA (400 mg) every 8 h for 3 days. The drugs were given on days 22, 23 and 24 of the infection as indicated by the arrows. ●———● Rabbits infected with *T.* (*T.*) *brucei* 427 and treated with 'Trasylol'/EACA. Mean (± S.E.) of 2 rabbits; ○———○ Rabbit infected with *T.* (*T.*) *brucei* 427. Untreated control; ×- - - -× Non infected control rabbits treated with 'Trasylol'/EACA. Mean (±S.E.) of two rabbits.

and showing high FDP concentrations was to produce an immediate and dramatic fall in the levels of these substances. In the control rabbit infected with trypanosomes but not treated with EACA and 'Trasylol' a fall also occurred but it was not precipitous and did not take place until several days after administration of the drugs. This was similar to infected non-drug treated rabbits (Fig. 4). The effect on the non-infected rabbits was to reduce the concentrations of FDP slightly.

7. *Effect of trypanocidal drugs on FDP concentrations*

Three rabbits were infected with *T.* (*T.*) *brucei* 427 and a further rabbit was used as a non-infected control. Fourteen days after infection, all four rabbits were treated with Melarsoprol (Mel B, May & Baker Ltd) 3·6 mg/kg intravenously, before the infected rabbits showed raised FDP. FDP concentrations were measured in all four rabbits before drug treatment, then 3, 7 and 14 days after drug administration. This treatment did not appear to alter the pattern of FDP concentrations in the serum, and they remained below 40 μg/ml throughout the experiment.

DISCUSSION

The presence of FDP in the serum provides evidence of fibrinolysis (Merskey *et al.*, 1966). Elevated levels of FDP in human patients have been reported in a variety of disease states, including myocardial infarction, venous thrombosis, pulmonary embolism, rheumatoid arthritis (Melliger, 1970), post-operative deep vein thrombosis (Wood *et al.*, 1972), and cerebral malaria (Reid & Nkrumah, 1972). In addition, Perine *et al.* (1971) have reported that following treatment of louse-borne relapsing fever with tetracycline (250 mg intravenously) six out of seven patients showed a sudden increase of FDP.

Two major problems were encountered while carrying out the FDP assays. First, as soon as attempts were made to determine the concentrations of FDP in any serum sample it was found that a substance was present which directly agglutinated the sheep red cells sensitized with fibrinogen. This substance thus had to be absorbed out before any measurements of FDP could be made. It was found that by adding fibrinogen coated red cells or fibrin to the serum this substance could be removed. For this reason every serum sample was absorbed before being assayed for FDP. The presence of an antibody directed against one or more components of the fibrinogen molecule and/or its derivatives will form a separate paper to be published shortly.

The second problem was that the tests were being carried out using sheep red cells sensitized with human fibrinogen and that we were measuring breakdown products of rabbit fibrinogen. It is known that human fibrin, fibrinogen and FDP have antigenic determinants in common (Bonnar, 1969) but whether rabbit fibrinogen and FDP have similar determinants to human fibrinogen was not known. For this reason it was necessary to sensitize sheep red cells with rabbit fibrinogen, and use these cells in the test system. The results (Fig. 5) showed that both rabbit and human fibrinogen have antigenic determinants in common and that the sheep cells sensitized with human fibrinogen, commercially available, were satisfactory for this test.

Significant elevations of FDP were noted 7 days after infection of rabbits with *T.* (*T.*) *brucei* with maximum concentrations of 160 μg/ml, 14–21 days after infection. The time of the peak varied slightly for different animals, but the pattern was always similar. Twenty-eight days after infection there was a fall in FDP but concentrations were still raised above control levels.

There are two likely explanations for the raised FDP in trypanosome infections. First, plasminogen might be activated to form the proteolytic enzyme plasmin, which would cause the breakdown of fibrin and fibrinogen. The plasminogen assays would support this view since, at peaks of FDP concentration, the amount of plasminogen in the serum was reduced by half. The second possibility is that trypanosomes might contain an exogenous activator, but to date this has not been investigated.

Experimental inhibition of plasmin by EACA and 'Trasylol' indicated that immediately plasmin was inhibited, no further FDP appeared, and the low concentration was maintained for at least 2 weeks following drug treatment. This also suggests that plasminogen activation occurs during the infection. Stress states such as pyrexia cause activation of fibrinolysis (Sherry *et al.*, 1959) and rabbits infected with trypanosomes are continually under a state of stress, due to parasitaemic crises and antigenic variation of the trypanosomes. It has been shown that the temperature increases in rabbits infected with *T.* (*T.*) *brucei* but that this does not parallel the parasitaemia (Goodwin, 1970). However, in man infected with *T.* (*T.*) *rhodesiense* temperature parallels parasitaemia (Bailey & Boreham, 1969). Plasmin may also be activated indirectly through the activation of Hageman factor in the presence of immune complexes. Immune complexes are formed during trypanosome infections and it is believed that they are responsible for the release of the plasma kinins (Boreham, 1968*a*). There is evidence that these immune complexes cause the activation of Hageman factor which activates prekallikrein to kallikrein (Boreham & Goodwin, 1970). Kallikrein is a kininogenase enzyme which converts kininogen to kinin, a biologically active polypeptide. It is thus possible that this may be the mechanism by which plasmin is activated.

In many respects, the events which can occur after treating trypanosomiasis patients with trypanocidal drugs, resemble the Jarisch–Herxheimer reaction of syphilis (see Boreham, 1968*b* for review). Perine *et*

al. (1971) showed that treatment of louse-borne relapsing fever with tetracycline caused a sudden rise in the serum concentration of FDP in six out of seven patients and, in two patients, it was believed to be due to fibrinolysis secondary to intravascular clotting. In our experiments, there was no increase in FDP after treatment with a trypanocidal drug. However, it must be noted that treatment was only carried out 14 days after infection, and it is possible that, at other times, e.g. at high parasitaemias or late in the infection, different results may have been obtained. It is hoped to extend this work further.

Several biological properties have been assigned to FDP:

1. Inhibition of platelet aggregation
2. Inhibition of thromboplastin formation
3. Inhibition of thrombin (Triantaphyllopoulos & Triantaphyllopoulos, 1970)
4. Potentiation of biogenic amines and peptides (Barnhart 1969)
5. Increased vascular permeability (Triantaphyllopoulos & Triantaphyllopoulos, 1970; Sack & Nefa, 1970)
6. Increased resistance of red blood cells to hypertonic solutions (Triantaphyllopoulos & Triantaphyllopoulos, 1970)
7. Regulation of plasma fibrinogen concentration and synthesis of hepatic fibrinogen (Barnhart *et al.*, 1970).

Since the FDP produced in experimental trypanosomiasis appear to be not clotted by thrombin (fragments Y, D and E) which show biological activity, it is of interest to speculate whether any of these properties are likely to add to the pathogenesis of the disease.

During studies on the release of pharmacologically active susbtances during trypanosome infections, it has been noted that if serum from an infected animal is added to an isolated tissue, such as guinea-pig ileum, subsequent doses of acetylcholine, 5-hydroxytryptamine, histamine and bradykinin are potentiated (Goodwin & Richards, 1960, and Boreham, unpublished observation). The factor/factors responsible for this potentiation are unknown, but since FDP are present in infected serum, they must certainly be possible candidates. However, washed separated trypanosomes will also cause a similar potentiation, although they do not contain any detectable FDP (Boreham unpublished observations).

One of the pathological changes in experimental trypanosomiasis which is believed to be important in the events leading to the eventual death of infected animals, is the increase in vascular permeability which has been noted (Goodwin & Hook, 1968; Boreham & Goodwin, 1967). A number of causes of this permeability might be postulated—the release of pharmacologically active substances, particularly kinins, which are known to occur in trypanosomiasis (Boreham, 1968*a*, 1970) the permeability factor present in trypanosomes (Seed, 1969)—direct action of immune complexes on the vascular endothelium and FDP released in trypanosome infections. FDP fragment E is known to be a potent permeability increasing factor which causes a longer and greater vascular permeability in the intradermal test in rabbits than histamine (Barnhart, 1969).

It is not possible to say whether one or more of these factors are responsible for the increased vascular permeability, but almost certainly they will contribute to the observed change.

It is unlikely that the FDP has any effect on the membrane stability of erythrocytes and so contribute to the observed anaemia because no change is seen in the fragility of erythrocytes during trypanosome infections of the rabbit (Boreham, 1968*b*).

It should be noted that all the work to date on the biological properties of FDP result from *in vitro* digestion of fibrin or fibrinogen by proteolytic enzymes. There is no evidence to show that FDP formed *in vivo* in trypanosome infections will have the same properties, although there are no reasons to suppose otherwise. In order to settle this point it would be necessary to separate the substances and test their biological properties.

Since increased FDP are found in trypanosomiasis, one might expect a corresponding decrease in plasma fibrinogen due to the activated fibrinolytic system. However, our findings show that the opposite occurs and that the concentration of fibrinogen actually increases. One explanation would be that there is an increased synthesis of fibrinogen in excess of its catabolism. In normal animals, the regulation of synthesis and breakdown of proteins is finely balanced so as to maintain relatively constant levels. It is possible that this dynamic balance is upset and the result is an increase in plasma fibrinogen. FDP may play some part in regulating plasma fibrinogen levels and high levels have been shown to cause increased release and synthesis of hepatic fibrinogen (Barnhart *et al.*, 1970). There is evidence that other proteins are synthesized more rapidly in trypanosomiasis for example, kininogen an α_2 globulin (Boreham, 1968*a*).

Plasma fibrinogen has been investigated in human cases of malaria caused by *P. falciparum* (Areekul *et al.*, 1971*b*) and in rhesus monkeys infected with *P. coatneyi* (Areekul *et al.*, 1971*a*) and *P. falciparum* in *Aotus* monkeys (O'Leary *et al.*, 1972).

Areekul and his co-workers found an increased consumption of ^{131}I-fibrinogen in human malaria but little change in experimental monkey malaria. However, in both cases, the total amount of fibrinogen increased considerably indicating an increased synthesis of this protein. Contrary to this, O'Leary and his colleagues showed that there is a marked

decrease in fibrinogen concentration in the blood of *Aotus* monkeys infected with *P. falciparum* but an increase in FDP which they suggested indicated diffuse intravascular coagulation.

The most important conclusion from the finding of raised FDP and increased fibrinogen, is that it is suggestive of intravascular clotting with the formation of microthrombi. There is some evidence that microthrombi are formed in trypanosome infections. Cattle infected with *T.* (*N.*) *congolense*, showed thrombosis of smaller arteries and veins which was attributed to denudation of the vascular endothelium by trypanosomes (Fiennes, 1970).

Ormerod & Venkatesan (1971) have demonstrated that the choroid plexus of rats infected with *T.* (*T.*) *rhodesiense* showed some capillaries completely blocked with amastigote forms, together with a substance they believe to be a white thrombus.

In *T.* (*T.*) *brucei* infection of dogs, extravascular deposition of fibrin particularly in the pericardial sac, has been noted by earlier workers (Rodet & Vallet, 1907). Hawkey (unpublished observation) has shown that the partial thromboplastin time and recalcification time are increased in rabbits infected with *T.* (*T.*) *brucei* but that the prothrombin time was unchanged. The prolonged recalcification time (cephalin time) may be due to lack of platelet factor 3 since a thrombocytopenia is known to occur in rabbits infected with *T.* (*T.*) *brucei*.

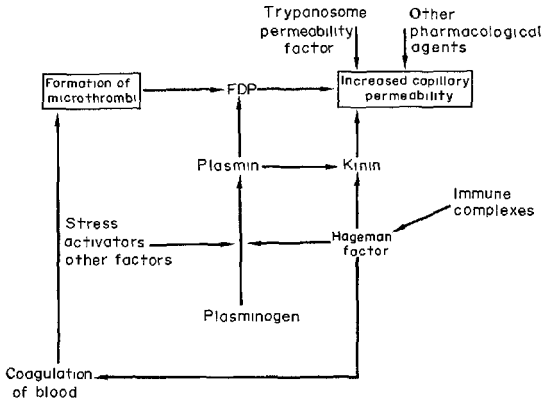


FIG. 9. Diagram to show possible mechanism whereby increased capillary permeability and microthrombi formation could occur in experimental trypanosomiasis of the rabbit.

An attempt has been made in Fig. 9 to demonstrate the interrelationship of various factors which are formed or altered in experimental trypanosomiasis and to show how they might contribute to the pathological changes. The two most important changes in the circulatory system resulting in pathological changes are probably the increased capillary permeability and the deposition of fibrinogen, with the probable formation of microthrombi. It can be seen that several factors may be responsible for the capillary permeability increase but that the exact mechanism of fibrinogen deposition is not yet clear.

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AUTOIMMUNITY IN TRYPANOSOME INFECTIONS—II. ANTI-FIBRIN/FIBRINOGEN (ANTI-F) AUTOANTIBODY IN *TRYPANOSOMA (TRYPANOZOON) BRUCEI* INFECTIONS OF THE RABBIT

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Abstract—BOREHAM P. F. L. & FACER CHRISTINE A. 1974. Autoimmunity in trypanosome infections—II. Anti-fibrin/fibrinogen (anti-F) autoantibody in *Trypanosoma* (*Trypanozoon*) *brucei* infections of the rabbit. *International Journal for Parasitology* **4**: 000–000. Rabbits infected with two strains of *T.* (*T.*) *brucei* showed an increase in the levels of a naturally occurring serum autoantibody directed against a component of the fibrin/fibrinogen system. This anti-F autoantibody is IgM throughout the infection. Absorption studies suggest that it reacts with one or more hidden determinants which are exposed after attachment of several species of fibrinogen molecule to red cell membranes. Absorption with autologous fibrin suggest the presence of the same determinant on the fibrin matrix after polymerisation of fibrinogen by thrombin. The antibody does not appear to react with fibrinogen in the circulation.

There is no evidence that this autoantibody causes any direct pathological change in the rabbit although secondary effects may be important. The antibody may have a beneficial effect by forming complexes with any fibrin deposited in tissues thereby enhancing its phagocytosis and rapid removal from the circulatory system.

INDEX KEY WORDS: *Trypanosoma* (*Trypanozoon*) *brucei;* rabbit; autoantibody; anti-fibrin/fibrinogen (anti-F); IgM; fibrin; fibrinogen; pathogenesis.

INTRODUCTION

Recent studies by several groups of workers have shown that autoantibodies are found in the serum of rabbits infected with trypanosomes. Seed & Gam (1967) described an anti-liver autoantibody in *Trypanosoma* (*Trypanozoon*) *gambiense* infections and Mansfield & Kreier (1972), using both precipitin and complement fixation tests, demonstrated autoantibodies to kidney, liver, brain and heart in rabbits infected with *Trypanosoma* (*Nannomonas*) *congolense* infections. More recent studies by MacKenzie & Boreham (in press) have demonstrated autoantibodies to liver and Wassermann antigens in rabbits infected with two strains of *T.* (*T.*) *brucei.* These antibodies were shown to be of the IgM class and it was suggested that trypanosomes have an adjuvant effect enhancing low natural levels of these antibodies. The mechanism suggested for this process was that depression of certain T-cell functions may release antibody secreting B-cell descendants from T-cell control resulting in an elevation of IgM.

The reason for this interest in autoantibodies in trypanosome infections is that the cause of death in domestic animals and in man is uncertain and such autoantibodies could contribute to the pathogenesis of the disease and give a clue to improved methods of treatment. Goodwin & Hook (1968) have suggested that severe vascular shock may be an important cause of death. The shock could be initiated by immune complexes causing the release of kinins and other pharmacologically active substances (Boreham, 1968). More recent studies by Goodwin & Guy (1973) have shown that chronic renal failure is a contributing factor to death in rabbits infected with *T.* (*T.*) *brucei.* The events leading to these changes still remain obscure.

During attempts to measure the concentration of serum fibrin/fibrinogen degradation products (FDP) it was found that a factor was causing agglutination of the fibrinogen coated sheep red cells and interfered with the FDP assay (Boreham & Facer, in press). The work reported here is an investigation into the nature of this factor and a discussion of its possible role in the pathogenesis of this disease.

MATERIALS AND METHODS

Male New Zealand White Rabbits weighing 2·5–3 kg were obtained from A. E. Moss, White Cloud Farm, Tring, Herts.

Trypanosomes

Two laboratory strains of *T.* (*T.*) *brucei* S42 and 427

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were used in these experiments. Full details of the histories of these strains are given by Boreham & Facer (in press). Strain 427 normally kills rabbits in 4–6 weeks while strain S42 is more acute, killing rabbits in 2–4 weeks. Rabbits were infected by subcutaneous injection of between 2×10^7 and 4×10^8 trypanosomes which had been separated on an anion exchange column from the blood of heavily infected mice or rats (Lanham, 1968).

Sera

Blood samples were collected from rabbits prior to infection then at weekly intervals after infection from the marginal ear vein. Blood was allowed to clot at 37°C for 4 h and the serum removed by centrifugation. Sera were stored at −20°C in 1 ml aliquots until required.

Preparation of rabbit fibrinogen

A modification of the ammonium sulphate precipitation method of preparing fibrinogen from citrated plasma as described by McFarlane (1963) was used (Boreham & Facer, in press). Separated fibrinogen was either freeze-dried or stored in phosphate buffer pH 7·3 at −20°C.

Preparation of bovine fibrinogen

Bovine fibrinogen was prepared in a similar manner to rabbit fibrinogen. It was also obtained commercially from BDH Biochemicals Ltd., Poole, Dorset.

Human fibrinogen

Purified lyophilised human fibrinogen batch 59440 was obtained from Kabi Pharmaceuticals Ltd., Uxbridge, Middlesex. It was reconstituted with distilled water to give a concentration of 10 mg/ml and stored at −20°C until required.

Sheep red blood cells (SRBC)

Sheep red blood cells in Alsevers solution were obtained from Wellcome Reagents Ltd., Beckenham, Kent. They were stored at 4°C until used.

Preparation of fibrinogen coated SRBC

Modifications of the method described by Das (1970) were used for the formalisation, tanning and subsequent sensitisation of sheep erythrocytes. Dialysis tubing filled with formalin was submerged in a 2·5% SRBC suspension and after 3 h the swollen dialysis sac was punctured and the formalin allowed to escape. Formalisation by this method prevented spontaneous haemolysis of the cells. After tanning, the cells were coated with either rabbit, human or bovine fibrinogen or with whole rabbit plasma, at a concentration of 2 µg/ml protein. Sensitisation was not improved if the concentration of fibrinogen was increased above this level. A 2·5% suspension of sensitised washed SRBC was prepared in 0·15 M citrate buffer pH 6·4. The cells were stored at 4°C until required.

Preparation of rabbit fibrin suspension

Normal citrated rabbit plasma was re-calcified and clotted with thrombin (10 units/ml thrombin topical, Parke, Davis & Co., Ltd.). The fibrin clot was squeezed and washed three times in saline to remove any contaminating plasma proteins and then dried on filter paper. 60 mg dried weight of fibrin was added to 1 ml of saline containing ε-amino-caproic acid (EACA) 50 mg/100 ml. The fibrin suspension was subjected to ultrasonic disintegration at a frequency of 20,000 c/s for 15 min at 4°C.

Inactivation of IgM

1. *Heat inactivation.* Sera from rabbits infected with trypansomes and pre-infection sera were heated at 65°C for 30 min.

2. *2-mercaptoethanol (2ME) inactivation.* Normal and infected rabbit sera were inactivated with an 0·2 M 2ME solution as described by MacKenzie & Boreham (in press). For each sample, an alkylation control was included in which buffer was substituted for 2ME

Sephadex G200 separation

Sera from a normal rabbit and rabbit S288 infected with *T.* (*T.*) *brucei* 427 collected 35 days after infection were separated on a Sephadex G200 column using a 0·1 M tris/HCl buffer pH 8·0 with 1·0 M NaCl. Details of the method are given by MacKenzie & Boreham (in press).

The agglutination test

Any serum sample to be tested for agglutinating activity against fibrinogen sensitised SRBC was first absorbed with sheep red blood cells to remove any antispecies agglutinins. The procedure was to add an equal volume of washed, packed SRBC to the sample for 30 min at room temperature. Two absorptions were carried out before the serum was used in the agglutination tests. Doubling dilutions of infected or control sera were prepared in 0·1 M citrate buffer pH 6·4 in the wells of microtitre plates (Cook Engineering Co., U.S.A.) commencing at a dilution of 1 : 2. To one volume of test serum, one volume of citrate buffer and one volume of 2·5% human or rabbit fibrinogen sensitised cell suspension were added. Controls, including two volumes of diluting fluid and one volume of sensitised cells, were incorporated into each plate. The plates were shaken and placed at 4°C overnight. The titre is expressed as a reciprocal of the greatest dilution of serum causing complete agglutination of the cells.

Absorptions

Titres of control and infected sera were determined before and after absorption using the following procedures:

1. *Human fibrinogen sensitised sheep red blood cells (HF-SRBC).* Sera were absorbed twice with an equal volume of packed HF-SRBC. Absorption times were 30 min at room temperature.

2. *Bovine fibrinogen sensitised sheep red blood cells (BF-SRBC).* Sera were absorbed with an equal volume of packed BF-SRBC using the method described above.

3. *Rabbit fibrinogen sensitised sheep red blood cells (RF-SRBC).* Sera to be absorbed were treated with an equal volume of RF-SRBC as described above.

4. *Rabbit fibrin.* Sonicated rabbit fibrin (60 mg/ml) was centrifuged at 38,000 g for 20 min and the sediment added to 0·5 ml of test serum. Two absorptions were carried out at room temperature for 30 min.

RESULTS

When SRBC coated with fibrinogen were added [redacted] to rabbit serum from which antispecies agglutinins had been removed it was found that agglutination occurred. This agglutinating activity will be referred to as anti-F (anti-fibrin/fibrinogen) autoantibody.

Anti-F titres

The results of anti-F activity of 15 rabbits are shown in Fig. 1. Similar results were seen for 6

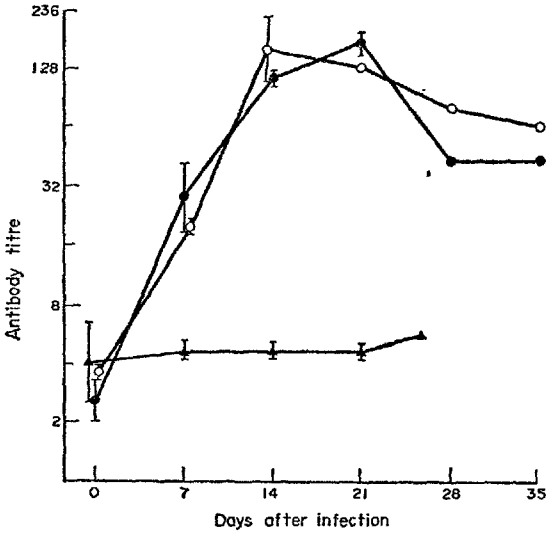


FIG. 1. Agglutinating anti-F autoantibody titres in the serum of rabbits infected with *T. (T.) brucei* ●——● Strain 427 Mean (± S.E.) for five rabbits; ○——○ Strain S42 mean (± S.E.) for six rabbits; ▼——▼ Non-infected control rabbits mean (± S.E.) for four rabbits.

rabbits infected with strain 427 and 5 rabbits infected with strain S42. Low titres of about 2–4 in preinfected sera rose to 128 to 512, 14–21 days after infection. Titres remained elevated throughout the period of study although slightly lower than maximal levels.

In a group of 4 control rabbits, the titres did not vary significantly throughout a 4-week period. No natural agglutinins to SRBC were found in the human, bovine or rabbit fibrinogen preparations.

Species specificity

The anti-F antibody found in 2 rabbits S217 infected with *T. (T.) brucei* 427 and S251 infected with *T. (T.) brucei* S42 gave similar titres when titrated against RF-SRBC and HF-SRBC (Table 1).

TABLE 1.—COMPARISON OF ANTI-F AGGLUTINATING ANTIBODY TITRES OF INFECTED SERA USING SHEEP RED BLOOD CELLS SENSITISED WITH EITHER RABBIT OR HUMAN FIBRINOGEN AS INDICATOR CELLS

Fibrinogen	Days after infection 0	7	14	21	28	35
(a) *Rabbit S217 infected with* T. (T.) brucei 427						
Human	4	16	64	256	32	32
Rabbit	4	16	64	256	32	32
(b) *Rabbit S251 infected with* T. (T.) brucei *S42*						
Human	8	16	512	512	256	—
Rabbit	8	16	512	512	256	—

This indicates that SRBC sensitised with either rabbit fibrinogen or human fibrinogen can be used to measure the antibody activity.

Absorption studies

1. *Sheep red blood cells.* Absorption with two volumes of packed SRBC removes all antispecies agglutinins from serum even when high titres are present as was often seen in infected sera. However, the agglutinating titre to cells sensitised with either rabbit or human fibrinogen remained unchanged.

2. *Absorption with rabbit, human and bovine sensitised sheep red blood cells.* Cells sensitised with any of the three fibrinogens caused a reduction in the anti-F titre of the serum of rabbit S220 infected with *T. (T.) brucei* S42 for 20 days. The reduction in titre was from 512 to 32 (Table 2). These results indicate that the antibody is not specific for rabbit fibrinogen.

TABLE 2.—EFFECT OF ABSORPTION WITH FIBRINOGEN SENSITISED SHEEP RED BLOOD CELLS ON SERUM ANTI-F AUTOANTIBODY TITRES

(Rabbit S220 infected with *T. (T.) brucei* S42)

Day	Reciprocal of antibody titre after absorption with sensitised SRBC: Original titre	HF	BF	RF	RP
0	2	0	0	0	0
20	512	32	32	32	64

HF = Human fibrinogen
BF = Bovid fibrinogen
RF = Rabbit fibrinogen
RP = Rabbit plasma

3. *Rabbit plasma sensitised sheep red blood cells (RP-SRBC).* On absorptions with RP-SRBC a three-fold dilution difference in titre was noted (Table 2). It is possible that complete absorption of the anti-F autoantibody did not occur because of competition of other plasma proteins for sites on the sheep red cell membrane normally occupied by the fibrinogen molecule. This would result in less than 100 per cent sensitisation.

4. *Sonicated rabbit fibrin.* Table 3 shows the results of absorbing two infected sera with sonicated rabbit fibrin. In all cases there was a reduction of the titre suggesting that the determinant responsible for the absorption was found not only on the fibrinogen molecule but also on fibrin.

TABLE 3.—EFFECT OF ABSORPTION OF INFECTED SERA WITH SONICATED RABBIT FIBRIN, USING SERUM FROM RABBITS S220 AND S268, PRE-INFECTION SERUM (DO) AND DAYS 21 AND 14 AFTER INFECTION RESPECTIVELY (doubling dilutions of serum beginning at a dilution of 1/10)

Serum	S220/DO	S220/D21	S268/DO	S268/D14
Control	10	80	10	40
Absorbed	0	20	0	0
$\log_2$ Unit Reduction	1	2	1	3

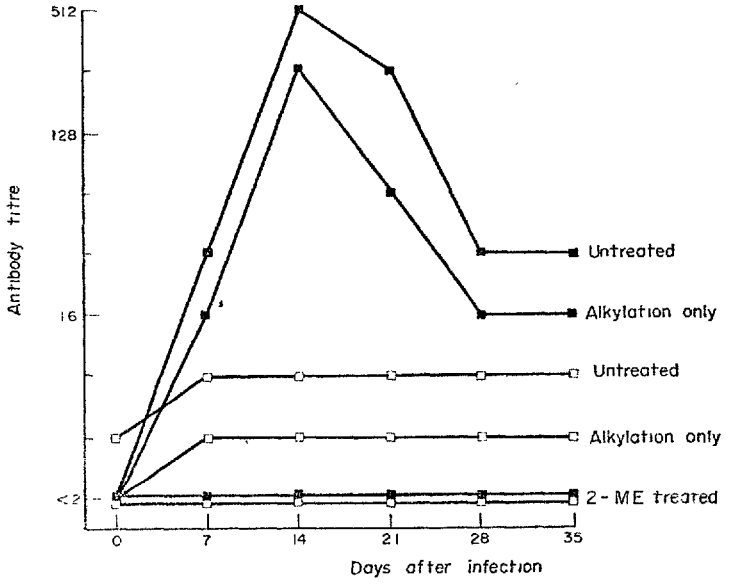


FIG. 2. Effect of 2-mercaptoethanol (2ME) reduction and alkylation on serum anti-F titres in two rabbits. ■——■ Rabbit S220 infected with *T.* (*T.*) *brucei* S42. □——□ Rabbit S270 non-infected control.

Macroglobulin nature of the antibody

In order to determine whether the anti-F antibody was IgM in nature and similar to those autoantibodies described by MacKenzie & Boreham (in press), gel filtration, heat lability and 2ME reduction and alkylation experiments were carried out.

1. *Gel filtration.* Results of separation of infected rabbit serum on Sephadex G200 are shown in Fig. 3. The majority of the anti-F activity was found in the high molecular weight macroglobulin fraction although a small proportion of the activity was found between the γ-globulin and albumin peaks.

2. *Heat lability.* All the anti-F activity in normal and infected sera samples was destroyed by heating to 65°C for 30 min.

3. *2-mercaptoethanol reduction and alkylation.* The results of 2ME treatment of the sera of two rabbits are shown in Fig. 2 and Table 4. Weekly serum samples were taken from control rabbit S270 and from rabbit S220 infected with *T.* (*T.*) *brucei* S42. All anti-F activity was lost by 2ME treatment,

TABLE 4.—EFFECT OF 2-MERCAPTOETHANOL (2ME) REDUCTION AND ALKYLATION ON THE ANTI-F ANTIBODY TITRES OF TWO RABBITS, ONE S220 INFECTED WITH *T.* (*T.*) *brucei* S42 AND S270, A CONTROL NON-INFECTED RABBIT

Day	2ME and alkylation		Alkylation only control	
	S220	S270	S220	S270
0	<2	<2	<2	2
7	<2	<2	16	4
14	<2	<2	256	4
21	<2	<2	64	4
28	<2	<2	16	4
35	<2	<2	16	4

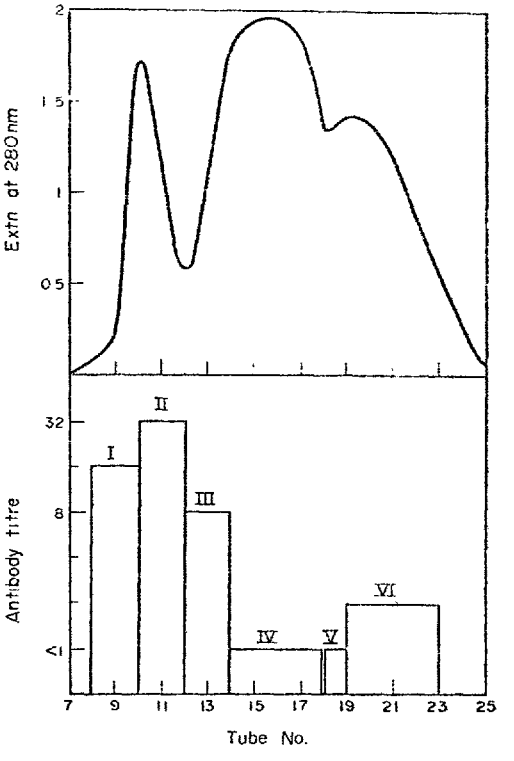


FIG. 3. Separation of anti-F autoantibody on Sephadex G200 from serum of a rabbit taken 5 weeks after infection with *T.* (*T.*) *brucei* 427. The upper curve shows the extinction at 280 nm and the histograms represent the anti-F titre as measured by the agglutination test. 3-ml fractions were collected in each tube.

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whereas alkylation only caused a slight reduction of the titre.

The results of these experiments using gel filtration, heat lability and 2ME reduction and alkylation indicate that the antibody is IgM at all stages throughout the infection. Since a small amount of antibody was found between the β-globulin and albumin peaks it is possible that a small component of the IgM is 7s as described by Frommel *et al.* (1970).

DISCUSSION

While attempting to measure the concentration of fibrin/fibrinogen degradation products (FDP) in the sera of rabbits infected with *T.* (*T.*) *brucei*, it was found that a factor was present in both normal and infected sera which caused the agglutination of fibrinogen sensitised SRBC (Boreham & Facer, in press). Since agglutination did not occur when fresh sheep erythrocytes or formalised tanned SRBC were added to rabbit serum, it was concluded that this factor was directed against the fibrinogen attached to the cells. The results of the experiments described here suggest that it is an autoantibody. An autoantibody is defined by Herbert & Wilkinson (1971) as "antibody capable of specific reaction with an antigen that is a normal constituent of the body of the individual by which that antibody was formed". The factor present in the serum of rabbits fits this definition and since it can be separated in the macroglobulin fraction on Sephadex G200 column, is heat labile at 65°C and its activity is destroyed by 2ME reduction is an IgM autoantibody.

There are several components of the fibrinogen system which the antibody could be directed against, i.e. fibrinogen itself, one or more of its breakdown products, or fibrin. Since we are not certain which of these fractions is involved, we prefer to term this an anti-F autoantibody. The development of the anti-F autoantibody is identical to the anti-liver and anti-Wassermann autoantibodies described recently by MacKenzie & Boreham (in press) and measured by precipitin and complement fixation tests. Maximum levels of these three antibodies are reached in the rabbit 2–3 weeks after infection. Mansfield & Kreier (1972) describe similar autoantibodies in rabbits infected with *T.* (*N.*) *congolense* but in this case maximum anti-liver activity was not reached until 4 weeks after infection.

The immediate question that arises from the finding of this autoantibody is: what exactly is it directed against, i.e. which component of the fibrin-fibrinogen complex is acting as antigen? The answer to this question must also explain why small concentrations of this anti-F autoantibody can be detected in non-infected rabbit sera. Recent work has shown that a situation exists in rabbits infected with *T.* (*T.*) *brucei* where there are high levels of fibrinogen (up to 800 mg/100 ml) and raised FDP concentrations (about 160 μg/ml) (Boreham & Facer, in press).

One would expect that, if any of these substances were acting as antigen, a complex would be formed with the antibody and it would not be possible to measure it by a direct agglutination test. This situation does not exist. Absorption with fibrinogen sensitised cells removes the antibody and the test system used to measure the antibody utilises sensitised cells. It therefore seems probable that the antibody reacts with fibrinogen only after attachment to red blood cells. A possible reason for this is that, during attachment to the red cell membrane, the fibrinogen molecule is altered in some way and one or more hidden antigenic determinants are exposed and recognised as "foreign" by cells of the lymphoid series. One might also suppose that this would imply that *in vivo* fibrinogen is absorbed on to red cells as has been suggested as a possibility in leishmaniasis (Woodruffe *et al.*, 1972).

In these experiments it was also found that absorption of serum with autologous fibrin would remove most of the antibody activity. It is possible therefore that during polymerisation of fibrinogen to fibrin the same antigenic sites are exposed.

An alternative explanation would be that the antibody has a very low affinity for the antigen. Such low affinity antibody would be relatively inefficient at eliminating antigen. Since antigen is continually being released in trypanosomiasis, synthesis of low affinity antibody might result in the formation of soluble antigen-antibody complexes. Low affinity antibodies to transferrin have been described in mice infected with *Plasmodium berghei yoelii* (Steward & Voller, 1973).

The possible mechanism of increased synthesis of pre-existing autoantibodies in trypanosomiasis have already been discussed in detail (MacKenzie & Boreham, in press). Terry *et al.* (1973) attempted to link T-dependent immunosuppression with the high levels of T-independent IgM characteristic of trypanosome infections. They suggested that defective T-cells would not co-operate in thymus dependent antibody responses and at the same time fail in their normal inhibitory control of certain B lymphocyte clones particularly those producing IgM autoantibodies (Allison, 1971). This is in contrast to the effect of Freunds complete adjuvant which appears to act directly through the T-cell system (Allison & Davies, 1971). Askonas & Humphrey (1958) have shown that when immunisation to a defined antigen is induced nonspecific antibody elevation occurs. Successive waves of antigenically different variants during trypanosomiasis may have a similar effect.

Homeostatic mechanisms within the normal circulation require that, after minor injury within the circulation, fibrin is laid down to seal the injured site prior to a permanent repair. Under these conditions, polymerisation of fibrinogen into fibrin would occur exposing the ~~sequestered~~ Suggested antigenic sites

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and so lead to anti-F autoantibody formation. Previous work has suggested that diffuse intravascular coagulation (DIC) occurs in these experimentally infected rabbits (Boreham & Facer, in press). Formation of fibrin at intervals during the disease may represent a constant antigenic stimulation of lymphoid tissue giving rise to increased levels of anti-F autoantibody.

Dumonde & Glynn (1962) showed that an anti-human fibrin antibody was produced in rabbits after immunisation with human fibrin and that it was species specific. This antibody precipitated with human fibrinogen but did not cross react with rabbit fibrinogen. The anti-F autoantibody in trypanosomiasis described here appears to have broad affinity since it reacts with rabbit, human and bovine fibrinogens. It may well be that the antigenic determinant exposed on sensitisation of red cells with various fibrinogen molecules is the same in all cases.

During experiments to determine the role of fibrinogen in inflammation Barnhart (1968) described a circulating anti-fibrin antibody in a human patient suffering from thrombophlebitis migrans. No details of the method used to detect this antibody is given nor details of its specificity. The appearance and disappearance of the antibody coincided with the presence and absence of an active inflammatory site.

It would appear that autoantibodies are unlikely to be of value in diagnosing human sleeping sickness or Nagana. Autoantibodies have now been described in *T. (T.) brucei* subgroup infections of rabbits, cattle and man (Seed & Gam, 1967; MacKenzie & Boreham, in press; MacKenzie *et al.*, 1973) and in *T. (N.) congolense* infections of rabbits and cattle (Mansfield & Kreier, 1972; MacKenzie *et al.*, 1973). Tissue autoantibodies have been described in a wide variety of infectious diseases including yellow fever (Hughes, 1933), syphilis, malaria, leprosy and infectious hepatitis (Davis, 1944) coccidiosis (Asherson & Rose, 1963) and schistosomiasis (Bassily *et al.*, 1973).

The presence of these autoantibodies in the serum will contribute to the raised IgM levels in trypanosomiasis (Mattern, 1968). The contribution that the anti-F autoantibody makes to the pathogenesis of trypanosomiasis remains uncertain. The low level of antibody in normal rabbits appears to have no harmful effect and the high concentrations found in infected rabbit sera do not induce a hypofibrinogenaemia. In fact if the antibody is, as suggested, directed against one or more hidden determinants on fibrinogen exposed during the clotting process and a condition of DIC exists then the antibody may have a beneficial effect in forming complexes with the antigen and thereby stimulating phagocytosis and removal of fibrin strands.

The indirect effects of raised IgM concentrations in the serum of which the anti-F autoantibody forms a part are probably more important. Since they are large molecular weight proteins, they may well upset the osmotic balance within the circulation and also contribute towards the increased blood viscosity which has been noted in infected rabbits (unpublished observations). Other possible effects of raised IgM levels are increased erythrocyte sedimentation rate and a tendency for cellular components of the blood to clump together. Both of these features have been noted in rabbits infected with trypanosomiasis.

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