

KALLIKREIN AND ITS ACTIVATION IN EXPERIMENTAL
Trypanosoma brucei INFECTIONS OF RABBITS

by

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ABSTRACT

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Kallikrein and its activation in experimental Trypanosoma brucei infections of rabbits.

Changes in the levels of plasma kallikrein, during T.brucei infections of rabbits, have been demonstrated using an esterolytic assay employing the hydrolysis of TAME. Initially the working parameters of the assay were established for rabbit plasma kallikrein.

Peak values of free kallikrein were found to occur 9-14 days post infection and this kallikrein activity was inhibited both in vivo, following administration of aprotinin (Trasylol), and in vitro using aprotinin or soy-bean trypsin inhibitor (SBTI).

As the increases in kallikrein occurred at a similar time in the infection to the first variant peak of parasitaemia, trypanosome material was used to establish if this activated the kallikrein-kinin system directly. In vitro studies revealed that activation was only achieved using immune complexes prepared from trypanosomes and immune serum. This was confirmed with in vivo experimental work.

An assay employing radiolabelled (125-I) protein A from Staphylococcus aureus was used to determine the levels of circulating IgG complexes. These were found to increase from day 6 after infection reaching amounts three to four times greater than uninfected control values, between days 16 and 22. SDS-polyacrylamide gel analysis of the complexes suggested that the antigen component was non-trypanosomal in origin. Increases were evident in immunoglobulin concentrations (IgG, IgM) while a decrease in C3 was associated with increasing immune complex levels and circulating parasites.

Anti-inflammatory drugs, corticosteroids and cyclophosphamide administered to rabbits did not inhibit complex formation. A reduction in levels was seen, this being most marked with dexamethasone treatment.

The presence of immune complexes appears to be the major contributing factor to activation of the kallikrein-kinin system, although a second pathway of activation early in the infection, associated with greatly increased parasite numbers in some drug treated animals, may be of importance.

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1 INTRODUCTION

1.1 Object of study

To investigate plasma kallikrein levels in rabbits infected with Trypanosoma (Trypanozoon) brucei brucei and possible activation of the kallikrein-kinin system by factors produced during the disease.

1.2 Trypanosomes and trypanosomiasis

1.2.1 Taxonomy

The species of the genus Trypanosoma used to be divided into groups and subgroups, but these being unsatisfactory in terms of taxonomy, have been replaced by a new classification according to Hoare (1966.1972) and this will be used throughout. The specific characteristics of the organisms within the subgenus Trypanozoon are polymorphism of the blood forms and morphological similarity to Trypanosoma (Trypanozoon) brucei brucei, the type species.

The taxonomic status of T.brucei, T.gambiense and T.rhodesiense has been disputed since their first definition. Classically the gambiense and rhodesiense trypanosomes were regarded as distinct species (reviewed by Baker, 1974). Other suggestions however have been made (Ormerod, 1967) where the brucei, gambiense, rhodesiense complex should be considered as one species. Hoare (1970) and Lumsden (1970) have regarded the three trypanosomes as subspecies of T.brucei and it is intended to follow this compromise. Hoare (1972) also designates both

the trypanosomes pathogenic to man, T.b.gambiense, regarding the Gambian and Rhodesian parasites as nosodemes.

As a consequence of the present classification, the taxonomic standards require a quadrinomial nomenclature plus nosodeme indication each time the gambiense or rhodesiense variety is referred to. The major species of trypanosomes considered in this study, Trypanosoma (Trypanozoon) brucei brucei (Plimmer and Bradford, 1899) (Hoare, 1966, 1972), will be referred to by the simple binomial name Trypanosoma brucei omitting the subgeneric term Trypanozoon, following the convention of WHO (1978).

1.2.2 The importance of trypanosomiasis

The genus Trypanosoma includes organisms parasitic over the entire range of vertebrate classes - amphibia, fish, reptiles, birds and mammals - with the transmission of the parasite occurring mainly via the tsetse (Glossina sp.). In 1898 Bruce discovered trypanosomes to be the causative organisms of nagana, cattle trypanosomiasis, with the first infection in man being discovered in 1902 by Forde in West Africa. At that time no connection was made between human trypanosomiasis and sleeping sickness, which had been known since the fifteenth century, until Castellani (1903) found trypanosomes in the cerebrospinal fluid of a sleeping sickness patient. These findings were later confirmed by Bruce who identified the vector of T.gambiense sleeping sickness as Glossina palpalis. Further work by Kleine (1909) unravelled the cycle of the parasites in the vector. Robertson (1913) described in detail the morphological transformations of the trypanosomes while in the vector; although more recent evidence of Evans and Ellis (1975) has demonstrated that this process is more complicated.

A large number of significant contributions have been made during the present century towards an understanding of trypanosomiasis, yet the present-day knowledge of human and animal pathology and of the various pathological processes themselves remains surprisingly sparse (Losos and Ikede, 1972; Goodwin, 1974). ' Trypanosomiasis with its toll of human and domestic animal life, was (and still is) of particular importance in impeding the progress of African development' (Desowitz, 1970). It has become increasingly evident that the nature of the disease produced in different hosts by different causative organisms can vary considerably (Goodwin, 1970). Nagana is the classical wasting condition found in cattle caused by T.congolense, the most important cause of bovine trypanosomiasis in East Africa, and T.vivax which is considered to be of greater importance in West Africa (Losos and Ikede, 1972). A third important trypanosome to infect cattle is T.brucei but this organism is somewhat less pathogenic (Goodwin, 1970; Killick-Kendrick, 1971). However as these three species are widespread throughout tropical Africa, it is not unusual for cattle to be infected with two or three species of trypanosome simultaneously (Losos and Ikede, 1972).

The African trypanosomes that cause disease in man are T.gambiense and T.rhodesiense which are morphologically indistinguishable from T.brucei. T.rhodesiense is the more virulent giving rise to acute infections of relatively short duration with insignificant neurological signs whereas T.gambiense infections cause a chronic, slowly progressing neurological disease known as sleeping sickness. The mortality from either form of the disease is close to 100% if not treated. With

10,000 new patients per year the disease does not give the impression of being important in terms of morbidity, but its importance as a public health problem lies in its being a continuous threat. The alarming symptoms and the high mortality rate have a dramatic impact on the population at risk. Epidemic outbreaks, if not controlled, rapidly drive more people away from villages and fertile areas (de Raadt and Seed, 1977). Current control measures with continued effort, produce suppression of the disease rather than eradication, but any interruption in these efforts, whether through political or economic circumstances, usually results in a resurgence. Tsetse control has proved difficult, some methods causing secondary problems more devastating than the original but these are being overcome with improved ecologically-balanced measures. The successful establishment of colonies of Glossina (Nash and Jordan, 1970) has greatly encouraged studies on all aspects of the behaviour of the vector. Immunological methods of control have been largely unsuccessful due to the multiplicity of strains and species of trypanosomes. The greatest obstacles however arise from the instability and diversity of the antigens that trypanosomes produce in infected hosts. Some experimental animals have been successfully immunized by injections of trypanosomes, with and without adjuvants (Soltys, 1973) and by the use of inactivated parasites (Duxbury et al., 1972; Welldé et al., 1974). Trypanosomiasis in the African continent is still therefore an important disease, as recognised by its inclusion in WHO's Special Programme for Research and Training in Tropical Diseases. With an increasing understanding of the parasite and the disease processes these factors may lead to a better solution of the problem.

1.2.3 Immunity to trypanosomes

Almost without exception parasitic protozoa involve an anti-body response when they invade the host. Much immunological research on trypanosomiasis has concentrated on serological typing of trypanosomes with a view to studying the feasibility of immunological surveillance and control methods. Study on the immunology of trypanosome infections has been devoted mainly to the humoral response to brucei subgroup organisms and general studies of protection against challenge, although investigations on the immunity to congolense and vivax have been useful (Vickerman, 1974). However much of the current research on immunological aspects of trypanosomiasis centres on antigenic variation as this is pertinent to the problem of vaccination (Desowitz, 1970), although at present an unattainable ideal, in preference to drug prophylaxis with its attendant problems of drug resistance, toxicity and production costs. Other considerations in this area of research are the presence of reservoir infections, natural immunity in wild animal populations and a general inability of man and cattle to mount an effective immune response.

A prerequisite to any study of immunity is the elucidation of the nature of the stimulating agents, the antigens, followed by the host response to them. Advances in biochemistry and immunochemistry have led to a considerable amount of new information on the chemical composition and antigens of the parasite.

It is characteristic of the salivarian trypanosomes that, during the course of an infection, a sequence of populations of different antigenic types, reflected by relapsing parasitaemias, appears at regular intervals (Gray, 1970; Lumsden, 1972). The long persistence of trypanosomal infection in the vertebrate host is due to the ability of the parasite to elude the host's immune response by changing its antigenic structure, a phenomenon first described by Franke (1905) and so playing an essential role in its survival. Comprehensive reviews of antigenic variation have appeared in recent years by Vickerman (1971,1974); Gray and Luckins (1976); Doyle (1977) and Cross(1978) .

Trypanosomes are complex organisms, comprising a large number of diverse antigenic components which can be broadly classified into two groups on the basis of their immunological specificity. Internal bound or common antigens consist mainly of enzymes, structural proteins and nucleoproteins, and are therefore associated particularly with trypanosomal homogenates (Brown and Williamson, 1962; Seed,1963; Bigalke, 1966). They tend to remain common to the same trypanosome species throughout its development (Lumsden, 1972)and remain unchanged (de Raadt, 1974). They are released at the crisis of each parasitaemic wave and are eliminated from the blood stream by antibody action or by catabolism. At least 20 common antigenic components have been observed in different isolates of T.brucei (Le Ray et al., 1973). Although used in immunodiagnosis they seem to have little relevance to protective immunity and have received little attention (Terry, 1976).

The variant antigens mark the identity of different populations of trypanosomes within a given species and are responsible for the differences between serological variants of a strain. They can direct protecting, agglutinating, lysing and precipitating antibodies (Seed, 1963; Miller, 1965; Clarkson and Awan, 1969) and are glycoprotein in nature (Allsop and Njogu, 1974; De Souza, 1975), earlier reports indicating that trypanosomes were almost devoid of any polysaccharide material that could be antigenic (Williamson and Desowitz, 1961; Desowitz, 1970). The variant antigens are considered to be located on the cell surface of the trypanosomes (Vickerman, 1969; Vickerman and Luckins, 1969). The loss of this coat by bloodstream trypanosomes on entering the tsetse fly or culture vessel correlates with the loss of variable antigen under these circumstances (Seed, 1964; Barry, 1977). It is believed that the surface coat material finds its way to the trypanosome surface by a process of secretion (Steiger, 1971) and that this coat can be separated from the plasma membrane in the form of plasmanemes. This is possibly associated with the presence of soluble antigen in the serum of infected animals (Wright et al., 1970). Earlier reports by Weitz (1963) of soluble antigen (exoantigen) detected in infected animals has been shown to be the same as the 4S antigen in trypanosomal homogenates (Brown and Williamson, 1964) by Allsop et al., (1971).

There is still considerable uncertainty about the total number of variant antigens each trypanosome strain can produce. Ritz (1916) believed that the capacity of T.brucei for antigenic variation was inexhaustible, the only criteria however seems to be the length of time that the host survives (Gray, 1965). Up to 23 antigenic types of

T.gambiense have been described by Osaki (1959). Other work has led to the interpretation of antigenic variation being an orderly programmed sequence of variants with antibody acting as the inducer of antigenic change (Vickerman, 1974). However the role of the immune response in the control of antigenic variation is not yet resolved. A number of workers have reported that under conditions of natural or artificially induced immunocompetence, antigenic variation appears to be suppressed (Luckins, 1972a; Hudson et al., 1976; Doyle, 1977). Introduction of techniques enabling identification of variable antigen types (VATs) suggested that antigenic variation, the changes in the nature of the glycoprotein surface coat, may be a spontaneous random process capable of producing a large number of VATs in any given population (van Meirvenne et al., 1977). This would also indicate that the immune response of the host is selective rather than inductive. Marked VAT heterogeneity was found in the first patent trypanosome population following a fly-induced infection (Le Ray et al., 1977), suggesting in these experiments that the diversity of VATs in T.brucei developed quickly, if it was not already present in the injected metacyclic trypanosomes. No experiments have been published which demonstrate unequivocally that variation can arise spontaneously, but with the advent of the culturing of bloodstream trypanosomes (Hirumi et al., 1977) the control of antigenic variation might be understood more fully. 'Elucidation of the mechanisms of antigenic variation will be a key to many of the mysteries of the parasitic relation between trypanosomes and their hosts' (Cross, 1978).

1.2.4 The host response

It has been established that the amounts of serum protéins are increased or decreased in trypanosome infections of man and experimental animals. The striking feature of the humoral response in African trypanosomiasis has been demonstrated by a large increase in γ -globulin levels and a decrease in serum albumin (Trincao et al., 1953; Mattern, 1964). The main area of interest has centred on the elevation of immunoglobulin M (IgM). This was first reported by Mattern (1961,1963) and suggested to be a response to the rapid antigenic variation of the parasite (Lumsden, 1972). The high IgM levels have been found in experimentally or naturally infected animals of several species, including mice (Clarkson, 1975), guinea-pigs (Fink et al., 1971), rabbits (Seed et al., 1969), monkeys (Baker and Taylor, 1971), dogs (van Meirvenne et al., 1972), sheep (Rees, 1969) and cattle (Luckins, 1972b, 1974, 1975; Clarkson and Penhale, 1973; Clarkson et al, 1975). The subject of immunoglobulin M in trypanosomiasis has recently been examined by Clarkson (1976).

Increases in IgM have been reported from 4 times the normal levels in humans (Mattern, 1964) to as much as 24 times normal levels in cattle (Kobayashi and Tizard, 1976), and Nielsen et al. (1978) have related the increases in IgM of calves infected with T.congolense to the parasite burden. The amount of circulating IgM is even higher than that indicated by the concentration, as increase in plasma volume has been reported by Clarkson (1968) and Naylor (1971).

The IgM increase is a typical early feature of brucei sub-group infections but not only quantitative changes have been shown in the IgM produced. Mattern et al. (1967) indicated that a proportion of IgM produced during late infection, especially in human trypanosomiasis, was 7S IgM - approximately 5-10% (Houba et al., 1969) - compared with 19S IgM (Frommel et al., 1970). Free light chain was also shown to be produced in Gambian trypanosomiasis (Greenwood and Whittle, 1975).

The IgM produced during trypanosomiasis appears to be directed against a number of antigens. Although some are specifically anti-trypanosome (Seed et al., 1969) the remainder may be heterophile, antiglobulin (Houba et al., 1969; MacKenzie, 1973) or directed against autoantigens (MacKenzie and Boreham, 1974; Mansfield and Kreier, 1972; Zuckerman, 1964). It may also play a part in B lymphocyte reception and as an effector mechanism in cellular cooperation (Allison, 1974).

In contrast to the large increase in IgM, IgG (7S γ -globulin) concentrations may rise slowly during infection and fluctuate. The ratio of IgG to IgM in human trypanosomiasis has typically been found as 1 : 3 (McKelvey and Fahey, 1965). Houba et al. (1969) were only able to find very slight increases in IgG in T.brucei infections of rhesus monkeys and actual reports on the amount of γ -globulin, especially IgG, present within infections vary considerably (Desowitz, 1970). Increased levels of IgG have been reported in T.gambiense infections of mice (Capbern et al., 1974), in T.equiperdum infection in deer mice (Moulton et al., 1974) and rabbits (Klein et al., 1970), and in rabbits infected with T.gambiense and T.rhodesiense (Seed et al., 1969). Van den Ingh (1976) has also shown a 200-250% increase in IgG in rabbits infected with T.brucei, the levels increasing

rapidly 7-9 days post infection.

In bovine trypanosomiasis a rather inconsistent picture seems to emerge. Nielsen et al. (1978) found little change in the amounts of IgG1 and IgG2 in three calves experimentally infected with T.congolense, and that the half life of all serum immunoglobulin classes were shortened. In a study of 13 calves infected with the same parasite Kobayashi and Tizard (1976) showed that serum IgG1 levels were elevated to 2.5 times the preinfection levels at 7 weeks and the increase in IgG2 levels at this time was minimal. Clarkson et al. (1975) reported no changes in IgG for the first 65 days of infection with T.vivax in some animals. Previous experiments of Luckins (1972b) showed a 1.5-2 fold increase in IgG levels of Zebu cattle 2-4 weeks after exposure to natural infection and Clarkson and Penhale (1973) reported a 2-fold increase 40 days after infection with T.vivax. It would seem in bovine trypanosomiasis at least that serum IgG levels may or may not rise in infected animals and this may depend on the breed of animal used, their state of health through the course of infection and environmental conditions. Serum IgA and IgE levels were found to be severely depressed in cattle infected with T.congolense (Nielsen et al., 1978). They demonstrated that the changes in serum IgM and IgE occurred at approximately the same time as the first parasitaemic peak, while the decrease in IgA preceded any observable blood parasitaemia. The significance in reduction of both IgA and IgE was not clear.

The involvement of complement in the immunological mechanism of the host response to parasitic infections has been suggested by

several workers (reviewed by Santoro et al., 1979). Activation of complement has been reported in trypanosome infections of man (Greenwood and Whittle, 1976), monkey (Nagle et al., 1974), cattle (Kobayashi and Tizard, 1976; Nielsen et al., 1978), and rats (Jarvinen and Dalmaso, 1976). It would appear from the literature cited above that both the classical and alternative pathways of complement activation are involved, with a reduction in C3, C1 and properdin as well as the appearance of high levels of serum immunoconglutinins (Ingram and Soltys, 1960; Kobayashi et al., 1976) and complement-fixing antitrypanosome antibody (Kobayashi and Tizard 1976). Assoku et al. (1977) suggest that the sustained hypocomplementaemia seen in African trypanosomiasis is likely to lead to the suppression of IgG, IgA and IgE and excess biosynthesis of IgM. Deprivation of complement in animals may lead to a failure of the normal IgM to IgG antibody switch mechanism (Nielsen and White, 1974).

The mechanism involved in the consumption of complement however does not seem to be clear. One of the factors appears to be the formation of immune complexes, associated with a reduction in complement levels (Nagle et al., 1974; Lambert and Galvao Castro, 1977), although antibody independent activation of complement has been reported (Kierszenbaum and Weinman, 1977; Nielsen et al., 1978). Direct activation by whole or purified trypanosomal antigens has also been described (Musoke and Barbet, 1977; Nielsen and Sheppard, 1977) and the whole subject of biologically active products from

African trypanosomiasis and their possible actions has been comprehensively reviewed by Tizard et al. (1978). By reducing the haemolytic complement levels, trypanosome infections may predispose the host to secondary infections. In addition, lowered complement levels in experimental animals has been shown to effect lymphoid tissue architecture (White et al., 1975; Pepys et al., 1976), and with the influence on IgM production, these are of significant consequence in trypanosomiasis. More work is required in the area of complement activation with regard to immunological implications and the overall pathology of the disease.

With regard to cell mediated immunity (CMI), Lumsden (1970) considered that there was no evidence for this in salivarian trypanosome infections. Since that time cell mediated responses have been shown to occur as Tizard and Soltys (1971) were able to elicit both delayed-type slow reactions in T.brucei infected rabbits by the administration of trypanosome antigens and CMI in normal rabbits which had received splenic cells. Mansfield and Wallace (1974) undertook various investigations, skin tests, lymphocyte stimulation lymphokine assays for CMI to mycobacterial protein in T.congolense infected rabbits, and they concluded that T-cell functions were probably being depressed during trypanosomiasis.

The role of CMI has been viewed from the evidence of a profound immunosuppression in trypanosomiasis. The immunosuppression is perhaps the most significant of all the immunological lesions which

occur involving both the antibody-mediated and cell-mediated immune systems. At present there is no question that the immune system of trypanosome-infected mammals is suppressed. A number of theories have been advanced recently based upon studies of experimental African trypanosomiasis, which propose to explain the underlying cellular mechanisms responsible and these have been discussed by Mansfield (1978). A number of workers have proposed that unresponsiveness occurs primarily within T-lymphocyte subpopulations of infected animals (Mansfield and Wallace, 1974; Mansfield and Bagasra, 1978; MacKenzie and Boreham, 1974; Terry, 1976). A loss or suppression of helper T-cell function would lead to the inability of B-cells to respond to T dependent antigens such as SRBC or protein molecules and an inability of proliferating B-cell clones to switch from IgM to IgG synthesis would be predicted. In contrast to the theory of T-cell subpopulation suppression it has also been proposed that a non-specific B lymphocyte mitogen is associated with trypanosomiasis (Greenwood, 1974; Urquhart et al., 1973; Corsini et al., 1977; Assoku et al., 1977). It is thought that a trypanosomal mitogen is responsible for the unregulated proliferation and IgM synthesis of B-cell clones, and that clonal exhaustion ultimately results in the observed suppression of B-cell functions. Esuruoso (1976) was the first to demonstrate that homogenised T.brucei brucei suspensions were mitogenic for the spleen cells of both normal and nu/nu mice, and he suggested that some component of these organisms served as a B-cell mitogen. However Mansfield et al. (1976) were unable to

demonstrate any effect on mouse spleen cells, in contrast to Assoku and Tizard (1978), using T.congolense but did show that the material was capable of stimulating rabbit cells.

Eardley and Jayawardena (1977) have demonstrated the presence of suppressor macrophage cells as well as suppressor T-cells in the spleens of T.brucei infected mice in vitro, suggesting the theory that trypanosomiasis generates suppressor cells. These workers hypothesize that trypanosome antigens may directly stimulate T-cells with the concomitant release of factors with affinity for macrophage surfaces, thus becoming suppressive for T- and B-cell responses. Similar findings were reported by Corsini et al. (1977) but they suggested that the immunosuppression occurred due to the combined effects of an undefined trypanosomal mitogen on B-cells as well as a stimulation of suppressor T-cells and macrophages.

Extensive changes in the numbers of T-, B- and null cells in chronic T.congolense infection have been reported (Morrison et al., 1978). Further work by Mayor Withey et al. (1978) with T.brucei in mice has shown an initial transient increase in splenic T-cells, and they suggested that these could be the T-suppressors previously demonstrated (Corsini et al., 1977; Jayawardena and Waksman, 1977). This was followed by dramatic increases in B- and null cells, which correlated with the time of infection and underlined the profound impact of trypanosomes on the cells of spleen and bone marrow. There is no one cell type which is clearly activated before the others and so it is still unclear whether

there is any primary target cell for the immunosuppression. A complete understanding of immunosuppression will come only after identification of target cells, the nature of suppression and the molecular mechanism by which the parasites induce suppression in vitro and in vivo.

1.2.5 Pathology of trypanosome infections

A number of extensive reviews concerning the pathology of African trypanosomiasis have been produced (Ormerod, 1970; Losos and Ikede, 1972; Goodwin, 1974; Boreham, 1979a). Therefore only a brief survey of the general pathology of T.brucei in rabbits will be given here, this subject having been covered in some detail by van den Ingh (1976).

The tsetse-transmitted African pathogenic trypanosomes are thought to be divisible into two groups because of their distribution in the mammalian host and the characteristic lesions they produce during infection (Losos and Ikede, 1972). The haematic group comprising T.congolense and T.vivax remain essentially blood parasites confined within the plasma of the blood vessels, although recently Luckins and Gray (1978) have shown the presence of T.congolense in connective tissue at the reaction site following infection by tsetse. In contrast, those of the humoral group, the brucei-subgroup trypanosomes, have a predilection for the connective tissues where they have been found both intra- and extracellularly (van den Ingh, 1976) and associated with inflammatory reactions in a variety of hosts in addition to being present at intervals in the plasma (Goodwin, 1974). Generally trypanosome distribution depends upon the duration of the disease (Losos and Ikede, 1972). In severe acute infections, as seen in laboratory rats and mice, massive numbers of trypanosomes are seen in the blood with little tissue invasion occurring.

In chronic forms of the disease there is solid tissue invasion and the trypanosomes present in the blood may have little to do with the disease process itself (Goodwin, 1971). The blood parasitaemia of infected rabbits remains low with the infection lasting 4-8 weeks, occasionally longer, with infrequent and late asymptomatic cases occurring (Losos and Ikede, 1972).

The clinical signs of infection in rabbits with T.brucei are considered to be typical of infections with trypanosomes of the brucei subgroup. The rabbit is usually used as a laboratory model for human and cattle trypanosomiasis but there are important differences, such as the apparent non-existence of CNS involvement. The first signs are usually apparent between the second and fourth week, these being oedema and erythema of the ears, eyelids, external nares and scrotum. These are followed by exudative erosive dermatitis and skin lesions, accompanied by micropurulent rhinitis and conjunctivitis, with recurrent keratitis and ophthalmitis being common. The rabbit shows progressive muscular wastage and finally dies (Goodwin and Hook, 1968), although the actual cause of death still remains obscure, as trypanosomes fail to produce gross lesions which could be considered obviously fatal.

The anaemia in trypanosomiasis is an area of the pathology of this disease which has received most interest and attention, and has recently been reviewed by Boreham (1979a). Reticulocytosis, as well as polychromosia and normblast development are typical responses

to infection (Boreham, 1967; Losos and Ikede, 1972). Jenkins et al. (1974) found that in rabbit infections reticulocytes increase, erythrocytes and haemoglobin decrease, accompanied by a lymphocytosis, macrocytosis and a thrombocytosis. They considered that the observed anaemia partially involved a microangiopathic haemolytic mechanism. Contributory factors may be an increase in aspecific phagocytosis of normal erythrocytes by the activated mononuclear phagocyte system (Jennings et al., 1974), especially in the spleen (Holmes and Jennings, 1976; Ikede et al., 1977), the erythrophagocytosis being an immunological phenomenon with the coating of erythrocytes by trypanosomal antigen (Herbert and Inglis, 1973), immunoglobulins (Barrett-Conner et al., 1973; van den Ingh et al., 1976b), or by complement components (Woodruff et al., 1973). It may also be related to a direct effect of the parasite as Huan et al. (1975) reported that a slow-acting haemolytic activity was generated in suspensions of T.brucei, T.gambiense, T.vivax and T.congolense, although Tizard et al. (1978) have questioned whether this is due to living trypanosomes or is an autolytic process. Autoimmune processes may also be involved (MacKenzie, 1973; Facer, 1974).

Changes in the plasma proteins are important in the pathogenesis, with marked increases in immunoglobulins, serum lipids and cholesterol, and a fall in serum albumin (Goodwin and Guy, 1973; Diehl and Risby, 1974; Boreham et al, 1977). An increase in fibrinogen, in addition, alters the flow properties of the blood (Facer, 1976) and with mononuclear cells adhering to the vascular endothelium, often as a result of trypanosome damage (Goodwin, 1971; Losos and Ikede, 1972), microthrombi formation and pooling of the blood in

the microvessels will result, causing tissue anoxia. Microvascular damage may also be mediated directly by phospholipases, proteases or by free fatty acids (Tizard et al., 1978). Thus proteases acting on plasminogen generate plasmin, as indicated by the presence of both plasmin and fibrinogen degradation products (FDP) in T.brucei infected rabbits (Boreham and Facer, 1974), which can also effect haemostatic processes.

The release of pharmacologically active substances (reviewed by Boreham and Wright, 1976a) is important in the alteration of vascular permeability, leading to the typical oedematous symptoms. A number of substances have been implicated, such as plasma kinins (Boreham, 1968, 1970) released as the result of an antigen-antibody reaction (Boreham and Wright, 1976b), FDPs (Boreham and Facer, 1974), the permeability factor of trypanosomes (Seed, 1969), as well as 5-hydroxytryptamine and histamine (Goodwin, 1970, 1976). Goodwin (1971), Goodwin and Hook (1968) and Goodwin et al. (1973) have demonstrated vascular lesions which develop during the disease with the damage occurring shortly after the release of these active substances. The role of the kallikrein-kinin system and immune complexes in trypanosomiasis will be discussed in more detail in sections 1.3.6 and 1.4.3.

1.3 Kallikrein

This section of the introduction deals with kallikrein, although due to the nature of the kallikrein-kinin enzyme system some reference will be made to the kinins and their actions, as well as to the interrelationship of this system with other blood systems.

1.3.1 History of kallikrein

The history of kallikreins can be regarded as having started with the observations of Abelous and Bardier (1909) when the intravenous injection of human urine into an anaesthetized dog caused hypotension. Two decades later studies were begun, which resulted eventually in the isolation and characterization of the material responsible for this property (Frey, 1926; Kraut et al., 1928). These early German workers, seeking the source of the vasodepressor substance in urine, found a similar substance in blood (Frey and Kraut, 1928) and in the pancreas (Kraut et al., 1930). They determined that it was an enzyme and called it kallikrein, a Greek synonym for pancreas, in the mistaken belief that it originated in that organ (Werle, 1937). They also concluded that, unlike urinary kallikrein, the kallikrein in blood and pancreas was present in an inactive form reversibly bound to an inhibitor. These inactive kallikreins, originally termed kallikreinogens but now called prekallikreins, as recommended by the International Union of Biochemistry on Nomenclature (Enzyme Nomenclature, 1965), could be activated by change in pH, by organic solvents (Werle, 1934; 1936) and by enzymes such as pepsin and trypsin (Werle and Urhahn, 1940, Werle et al., 1955).

Subsequent investigations resulted in a modification of earlier views. The kallikreins from different sources have been shown to be different by immunological techniques (Webster et al., 1963), susceptibility to proteolytic inhibitors (Webster and Pierce, 1963), ability to hydrolyze synthetic substrate (Webster and Pierce, 1961) and electrophoretic mobility (Moriya et al., 1963). It is now established that urinary kallikrein originates in and is identical to the enzyme in renal tissue (Nustad, 1970; Nustad, et al., 1975). Kidney kallikrein is considered to be one of many glandular kallikreins with similar properties, which are different from those of plasma kallikrein or its precursor (Nustad et al, 1976).

The nomenclature for the polypeptides that cause a depression in blood pressure became extremely complicated during the early work and so recommendations for standardising the terminology were made (Webster, 1966; Schachter, 1964). These recommendations have been generally accepted and they will be followed in this work.

Kallikrein is therefore the name of a class of proteolytic enzymes that effect the release of vasodepressor peptides or kinins from a plasma α_2 -globulin substrate, termed kininogen. While investigations in Europe began with observations on kallikrein, Rocha e Silva et al., (1949) discovered and began to describe the now well known pharmacology of the potent nonapeptide bradykinin, the product of the action of plasma kallikrein upon the circulating kininogen substrate.

Pierce and Webster (1961) discovered that glandular kallikreins, made specifically in the salivary glands, pancreas and kidney, released another kinin, the decapeptide lysyl-bradykinin (kallidin) from a kininogen. Three naturally occurring kinins have been isolated from plasma and their structure is shown in Fig. 1.1. Other kinins have been found in colostrum (Guth, 1959), urine (Jensen et al., 1965), wasp and hornet venoms (Jacques and Schachter, 1954; Bhoola et al., 1961; Pisano, 1968), skin of amphibia (Anastasi et al., 1966; Erspamer and Anastasi 1966) and the plasma of reptiles and birds (Werle et al., 1966; Erdos et al., 1967; Seki et al., 1973).

Fig 1.1 Amino acid sequence of three naturally occurring plasma kinins

Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Kinin I ¹
Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Kinin II ²
Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Kinin III ³

¹ Bradykinin (Elliot et al., 1961)

² Kallidin (Pierce and Webster, 1961)

³ Met-Lys Bradykinin (Elliott et al., 1963)

The kinins of natural origin have a number of pharmacological properties in common. All are hypotensive and contract most isolated smooth muscle preparations but relax the rat duodenum; they increase capillary permeability (Holdstock et al., 1957; Elliott et al., 1960; Fox et al., 1961) produce pain when applied to a blister or injected intradermally (Armstrong et al., 1957; Mitchell and Krell, 1964). They also cause broncho-

constriction in the guinea-pig (Collier et al., 1960), vasodilation (Holton and Holton, 1952; Allwood and Lewis, 1963) and accumulation and migration of leucocytes (Lewis, 1962).

The quantitative differences in the pharmacological activities of the more commonly occurring kinins have been the subject of intensive study (Rocha e Silva, 1974). This has been demonstrated in the fact that kallidin is only half as effective as bradykinin on isolated guinea-pig ileum, but 2-3 times more effective in lowering the arterial blood pressure of the rabbit or rat (Schachter, 1969).

Kinins have a very short half-life in blood, less than one complete circulation of the blood (Ferreira and Vane, 1967) due to the presence of kininases. Several kininases are known but the most important have been named as kininase I and II (Erdos and Yang, 1970). Kininase I is a carboxypeptidase called carboxypeptidase N or arginine carboxypeptidase (E.C. 3.4.12.7) which generally cleaves the C-terminal lysine. It probably originates from the liver (Oshima et al., 1974, 1975) but can be detected in other organs such as the lung (Petakova et al., 1972) and possibly in the skin (Gecse et al., 1971). Kininase II is a polypeptide hydrolase (E.C. 3.4.15.1) which cleaves Phe-Arg from bradykinin, the vascular endothelium of the lung being a rich source of this membrane bound enzyme (Bakhle, 1968; Ryan and Ryan, 1975).

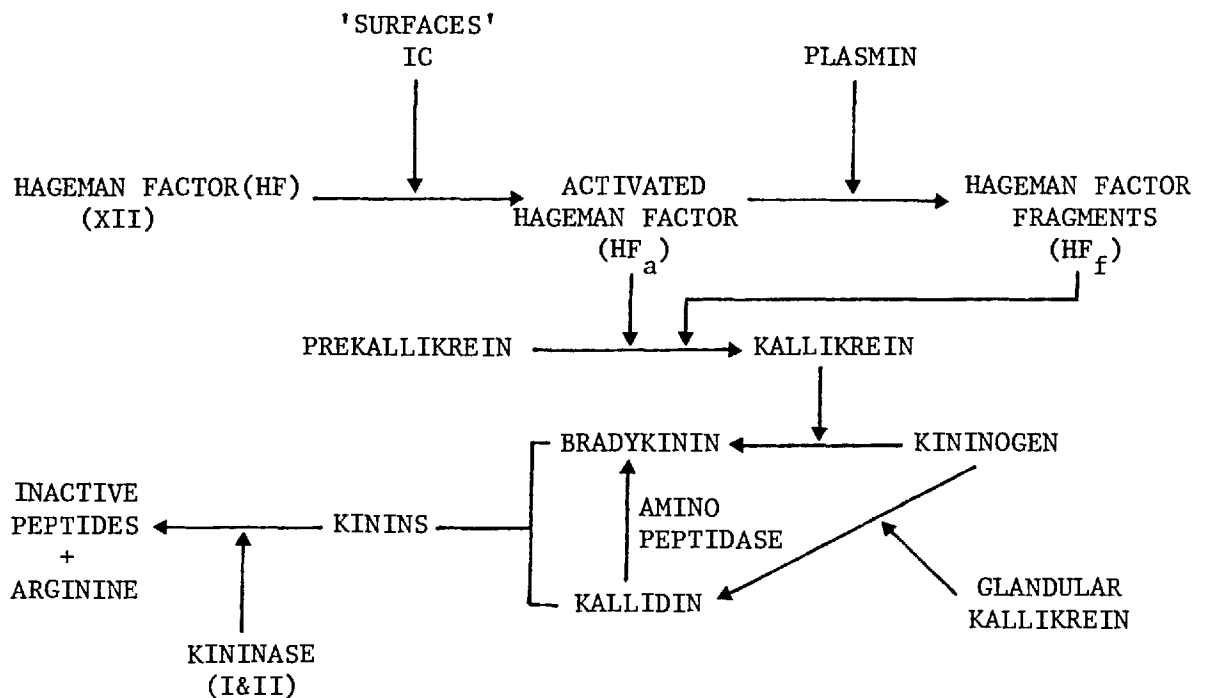
1.3.2 Activity of kallikrein

Many investigators around the world have contributed to our present level of understanding of the biochemical properties, behaviour and pharmacology of components of the plasma or glandular kallikrein-kinin systems (Margolius, 1978). Specific kallikreins have now been shown to occur in mammalian urine, pancreas, submaxillary gland, saliva, tears, sweat, lymph and cerebrospinal fluid (Schachter, 1969; Webster, 1968; Zeitlin, 1970). The main pharmacological actions of the kallikreins in vivo are due to the kinins they release. In addition to these effects there are also direct actions of the kallikreins. Strong contractions of isolated small intestines of the dog and cat have been reported to pancreatic and urinary kallikreins in vitro (Werle, 1936). Vasodilation of coronary vessels of rabbit heart (Felix, 1934), vasodilation and increased capillary permeability in vivo (Frey et al., 1950; Lewis, 1960) and the lowering of blood pressure (Frey et al., 1950) were also noted. Two new functions discovered more recently were the capacity to induce chemotaxis of neutrophils (Kaplan et al., 1973) and the capacity to accelerate blood coagulation in vitro (Wuepper and Cochrane, 1972b).

Kallikrein is formed from the inactive precursor prekallikrein under the influence of the active form of Hageman factor (HF_a) blood coagulation factor XII by rupturing one or two peptide bonds in the proenzyme molecule, without splitting off a fragment (Kaplan et al., 1976) and kallikrein then acts on the kininogen to release kinin (Fig. 1.2). The activation of HF is described in more detail in section 1.3.4.

The picture as represented in Fig. 1.2 is however complicated further with genetic studies revealing that two kininogens are present in plasma (Jacobsen, 1966a, b; Pierce and Webster, 1966). One, the so called high molecular weight (HMW) kininogen (Habal and Movat, 1976), appears to be the preferred substrate of plasma kallikrein (Jacobsen and Kriz, 1967) while the low molecular weight (LMW) kininogen (Spragg and Austen, 1971) is a substrate of glandular (tissue) kallikrein. Extensive studies on the kallikrein-kinin system have been possible with the identification and purification of a large number of the components and with the discovery of a number of families, Fitzgerald, Williams and Flaujeac, who exhibit blood clotting deficiencies in vitro, which relate to the activity of the kinin system.

Fig. 1.2 Simplified diagram of the kallikrein-kinin system



Further complications in the kallikrein-kinin system arise from the presence of kallikrein inhibitors in blood and tissues (Fritz, 1974). It has been recognised that kallikreins are highly substrate specific and that they can also be distinguished by their behaviour towards inhibitors. For example soybean trypsin inhibitor (SBTI) inhibits only plasma kallikrein, whereas aprotinin (Trasylol), an inhibitor derived from animal sources, inhibits both tissue and plasma kallikrein (Colman, 1974). A number of naturally occurring kallikrein inhibitors are also known. Margolis (1958) showed that kallikrein was very rapidly inactivated in plasma and presented data indicating the presence of an inactivator of kallikrein in human, dog and rabbit plasma. Werle and Shmal (1968) later described a kallikrein degrading enzyme in rat plasma which has been shown to be similar to a kallikrein inactivator from human plasma (Henriques et al., 1976).

At least four different plasma proteins have been described that inhibit plasma kallikrein in vitro, however their roles in regulating the activity of this protease in pathophysiologic situations are not fully known. Two of these inhibitors, α_2 -macroglobulin (Harpel, 1970; McConnell, 1972) and the inactivator of the first component of complement (C_1 INH) (Ratnoff et al., 1969; Gigli et al., 1970), react rapidly with human plasma kallikrein, whereas the reaction between α_1 -antitrypsin (Fritz et al., 1972) or antithrombin III (Rosenberg, 1975) and plasma kallikrein appears to be relatively slow and progressive. These studies

have suggested that the α_2 -macroglobulin and $\bar{\text{C}}\bar{\text{I}}$ INH comprise the major circulating kallikrein inhibitors.

The first plasma inhibitor observed to play a role in regulating the kinin forming pathway was $\bar{\text{C}}\bar{\text{I}}$ INH (Kagen, 1964), with its ability to inhibit the coagulant activity of HF_a (Forbes et al., 1970) and HF fragments (Ozge-Anwar et al., 1972) as well as inhibiting the capacity of both these factors to activate prekallikrein to kallikrein (Schreiber et al., 1973a). Subsequent studies have revealed that $\bar{\text{C}}\bar{\text{I}}$ INH is capable of inhibiting the chemotactic activity of kallikrein for the polymorphonuclear leucocytes (Goetzl et al., 1976). α_2 -macroglobulin on the other hand has no effect on activated HF but has been shown, in a highly purified form, to inhibit both the esterase activity and the kinin generating capacity of kallikrein (Harpel, 1970). Dilution affects the potency of both α_2 -macroglobulin and $\bar{\text{C}}\bar{\text{I}}$ INH in different ways (Harpel, loc. cit.) and this finding could be related to that of Schachter (1956) who demonstrated the release of kinins when plasma or serum were diluted in vitro.

The role of α_1 -antitrypsin is at present controversial and is thought to be limited. It has been shown to have no activity in regulating the action of HF_a on prekallikrein and, similarly, at physiologic concentrations the action of kallikrein on kininogen (Schreiber et al., 1973b). Less purified preparations have demonstrated a capacity to inhibit plasma kallikrein (McConnell, 1972) as well as some activity on human kallikrein when long incubation periods were used (Fritz et al., 1972). Vennerod et al. (1976) have recently shown

that purified human antithrombin III inactivates the esterase, kininogenase and plasminogen activator activity of purified plasma kallikrein, these reactions being more rapid in the presence of heparin. They also suggested that the antithrombin III was bound to kallikrein during the inactivation, similar to the situation found with other proteinases (Rosenberg and Damos, 1973; Rosenberg, 1975). As kallikrein plays a role in the inflammatory response, ability to pass through vessel walls should be considered in assessing the relative importance of each inhibitor. As α_2 -antitrypsin and C1 INH are smaller than α_2 -macroglobulin (McConnell, 1972), these would probably be more effective and Ganrot et al. (1970) demonstrated that the ratio of α_1 -antitrypsin to α_2 -macroglobulin in lymph is twice that in plasma.

The role of these inhibitors is obviously not restricted only to the kallikrein-kinin system and indeed, some of their effects on this system may be mediated via components from other interrelating blood enzyme systems. A more detailed account of plasma inhibitors of HF dependent pathways has recently been published by Schreiber (1976). Table 1.1 shows the effect of the plasma inhibitors described, on a number of plasma components.

Table 1.1

Effect of plasma inhibitors upon Hageman factor, the
Hageman factor substrates and plasmin*

	$\bar{C}1$ INH	α_2 -macroglobulin	α_1 -antitrypsin	antithrombin III
HF _a	+	-	-	?
HF _f	+	-	-	?
Activated PTA	+	-	+	+
Kallikrein	+	+	-(+)	+
Plasminogen Activator	-	+	-	?
Plasmin	+	+	+	+

*taken from Kaplan et al. (1976)

HF_a = activated Hageman factor

HF_f = Hageman factor fragments

PTA = plasma thromboplastin antecedent

1.3.3 Measurement of kallikrein

Despite the importance of the plasma prekallikrein-kallikrein enzyme system as a major regulator of kinin production the activity of this system has been inferred only from studies on kininogen depletion or kinin production, or both. Biological assays of kallikrein activity are based on the measurement of kinin released and the activity has been quantitatively determined by means of its hypotensive effect in experimental animals in comparison to a standard preparation (Padutin, Bayer) or by contraction of smooth muscle (Trautschold, 1970; Shroder, 1970). These assays however are time consuming and give poor reproducibility. A more convenient assay of kallikrein activity is the measurement of the esterolytic activity of the enzyme using one of the synthetic amino acid esters. Esterolytic activity has been determined in a variety of ways including titrimetric or spectrophotometric determination of the acid liberated on hydrolysis of the ester, colorimetric measurement of the unhydrolyzed ester or measurement of the liberated methanol or ethanol. Methanol has been assayed by oxidation to formaldehyde (Siegelman et al., 1962) and by the measurement of (^3H) methanol after hydrolysis of an isotopically labelled substrate α -N-p-tosyl-L-arginine-(^3H)methyl ester (^3H TAME) (Beavan et al., 1971), ethanol by a coupled reaction with alcohol dehydrogenase (Trautschold, 1970). Following the purification and characterization of human plasma kallikrein (Colman et al., 1969a) and its identification with the plasma arginine esterase activity activated by exposure to kaolin (Colman et al., 1969b) made possible

the biochemical assay for plasma prekallikrein and kallikrein. Colman et al. (1969c) assayed the enzymatic action of kallikrein on a synthetic substrate TAME rather than its natural substrate kininogen, and they have demonstrated that TAME hydrolysis parallels kinin release at all stages of purification of plasma kallikrein. This method provides a reproducible measure of kallikrein activity unaffected by those variables (Kellermeyer and Graham, 1968) that may influence bioassays, and have been used by other workers (Wright, 1973). A number of immunological assays are available for measurement of components of the human plasma kinin forming systems (Spragg, 1976). Quantitative immunoassays for plasma prekallikrein and kallikrein however have not been reported, even though the preparation of antiserum directed against both reagents has been described (Johnston et al., 1974; Saito et al., 1974).

A number of radioimmunoassays have also been used for bradykinin, that reported by Mashford and Roberts (1971) showed the highest sensitivity, detecting up to 50 pg/tube. A more recent specific radioimmunoassay for urinary kinins used antiserum against synthetic bradykinin in combination with labelled tyr^8 -bradykinin (Shimamoto et al., 1978), the assay detecting as little as 6 pg/tube of bradykinin. These assays are however fairly involved and require the preparation of a number of materials before they can be performed, but the techniques may be applicable to measurement of kallikreins. Amundsen et al. (1976) have used a new chromogenic tripeptide derivative, N^α -benzoyl-L-proline-L-phenylalanine-L-arginine-4-nitroanilide for measurement of plasma kallikrein.

The tripeptide is identical with the amino acid sequence at the C-terminal end of bradykinin and proved to be highly susceptible to the action of plasma kallikrein with virtually no activity with glandular kallikrein. The advantages of the method used were that it was a one step reaction that was rapid, accurate and simple to perform. As more specific substrates are produced, and purification of components continues, more sensitive assays for kallikreins and kinins will no doubt be developed.

1.3.4 Interaction of kallikrein-kinin system with blood enzyme systems

The tendency of workers has been to view blood clotting, clot lysis, kinin generation and the immunological defence network as separate systems, each performing special functions quite independent of the others and of other biologic events. In fact this is not true for any biologic system; it would be physiologically uneconomic. The subject of the interrelationship of the metabolic cycles in blood has been observed for some time and has become a very involved and complicated situation (Ratnoff, 1971; Schreiber and Austen, 1973). 'A complete description of all the interrelating blood systems that either result in the activation and inactivation of the plasma kallikrein-kinin system or are affected by plasma kallikrein could show more intersecting circles than the Olympic emblem' (Erdos, 1976). A brief account of the systems involved and some of the connections will be given here with the emphasis being given to the prekallikrein-kallikrein system and the involvement of kallikrein specifically. A recent review (Murano, 1978) outlines the connections in more detail.

There are four main proteolytic 'cascade' systems in the blood and these are kinin forming, clotting, fibrinolytic and complement systems. All these are multicomponent systems and include a number of specialised proteases, primarily of the trypsinase type. These proteases are activated on the principle of limited proteolysis in a specific sequence in a cascade mechanism (Muller Eberhard, 1975) as a result of which numerous functional links, direct and indirect

are formed within and between each system. The central role among these general components belongs to Hageman factor (HF) itself an enzyme of the trypsinase type which in its active form (HF_a) has three main substrates in plasma: Factor XI (Weupper, 1972,1974), plasminogen proactivator (Kaplan, 1974) and prekallikrein (Weupper, 1973). HF was initially identified as a plasma protein that was bound and activated upon negatively charged surfaces (Ratnoff and Colopy, 1955). It was clear from early experiments of Gjonnoes (Stormorken et al., 1973/1974) that different ways of activation led to different results. There appear to be two ways at least of promoting activation of HF.

In solid phase contact activation, which can be induced by negatively charged particles such as glass, kaolin, uric acid, particles of collagen or particles of the basement membrane of the vessels (Cochrane et al., 1973), conformational changes occur in the molecule which are not accompanied by any change in molecular weight (Cochrane et al., 1974). However this negative surface charge of particles is not the only property of the surface to play a part in the activation, as negatively charged particles such as latex and barium sulphate do not activate HF (Colman, 1974). In fluid phase activation by trypsin or plasminogen on the other hand (Kaplan et al., 1976; Wuepper, 1972) gradual degradation of the HF_a molecule occurs, leading to molecular fragments which are free to diffuse away from the surface. Each form of HF_a is a potent activator of prekallikrein, while only the surface bound (80,000 MW) form of HF_a in the presence of HMW kininogen is a potent activator of factor XI (Meier et al., 1977). The presence of HMW kininogen as a necessary factor for activation of HF was independently ascertained utilizing the plasma of patients named Williams (Colman et al., 1975,

1976a) and Flaujeac (Wuepper et al., 1975) who possessed unique abnormalities of the HF-dependent pathways. The kallikrein formed after activation of prekallikrein serves to convert more HF to the active form. This reaction is also enhanced by HMW kininogen and constitutes a positive feedback mechanism with HF_a as well as its fragments activating more prekallikrein to kallikrein in the presence of HMW kininogen (Liu et al., 1977; Meier et al., 1977). It appears that HMW kininogen is not the only precursor of kinins but is also a linkage for prekallikrein and factor XI to the exposed surface, where they are activated by surface bound HF_a (Wiggins et al., 1977).

Blood coagulation consists basically of the conversion of fibrinogen to fibrin, and their degradation products by thrombin which exists as a zymogen, prothrombin in plasma. A number of zymogens, cofactors, calcium ions and surfaces are involved prior to the formation of an active enzyme in both the intrinsic and extrinsic systems. Stormorken et al. (1973/1974) have established links between the clotting and kinin systems. The description of Fletcher factor deficiency by Hathaway et al. (1965) with the fact that prekallikrein was shown to be identical to Fletcher factor (Wuepper, 1973), being additional to those provided by the cold activation of factor VII and the feedback activation of HF by kallikrein. Simultaneous with the initiation of clot formation HF_a also initiates the steps leading to clot dissolution and kinin generation.

The enzyme responsible for clot lysis is plasmin which has a strong affinity for fibrinogen and fibrin as well as activating other clotting factors including HF_a (Kaplan et al., 1976), components of the complement system (Ratnoff and Naff, 1967) and liberating kinins from kininogen (Habal et al., 1976). As stated earlier the substrate in plasma which is acted upon by HF_a is plasminogen proactivator. This interaction results in plasminogen activator which converts plasminogen to plasmin. Vennerod and Laake. (1976) have demonstrated that plasminogen proactivator is the same as plasma prekallikrein and this implies that kallikrein directly activates plasminogen although this may not be a pathway of major physiologic significance. Yecies et al. (1978) have described the HF dependent fibrinolytic pathway in more detail.

It has been demonstrated in experiments with a highly purified preparation of inactivated human HF and purified preparations of kallikrein, plasmin and factor XIa, that all three of these proteases in low concentrations are capable of activating HF (Kaplan, 1974; Cochrane et al., 1975). The catalytic activity of kallikrein based on the molar concentrations was ten times higher than that of plasmin and XIa. Since kallikrein and factor XIa are direct products and plasmin is an indirect product of the effect of HF_a on their substrates in plasma, the hypothesis was advanced that these three enzymes are capable of catalysing by reciprocal means the reaction in blood plasma of $\text{HF} - \text{HF}_a$ and plasmin is also capable of the further conversion, $\text{HF}_a - \text{HF}_a$ fragments. However, under physiological conditions the reciprocal effect of kallikrein apparently is the most important,

since complete impairment of kinin production and defects in coagulation and fibrinolysis (Cochrane et al., 1973; Weiss et al., 1974) are observed only upon a deficiency of prekallikrein (Hathaway et al., 1965) but not of factor XI. Under such conditions, the activation of kallikrein by HF, and HF by kallikrein is probably a continuous process. The formation in blood plasma of a certain amount of free kallikrein follows from the fact that bradykinin is always present in plasma although in very small amounts (0.07-5.0ng/ml) (Spragg, 1976).

The complement system is a multimolecular, self-assembling system, constituting the primary humoral mediator of inflammation and tissue damage. Comprehensive descriptions of the components of the system, and their means and sequence of activation, have been given by Muller Eberhard (1975) and Frank (1975). The first direct evidence associating clotting and other haemostatic mechanisms with immunological events was derived from the fact that plasmin can initiate the classical pathway of complement activation by activating C1s to C1 esterase (Ratnoff and Naff, 1967) and by damage of C3 (Taylor and Ward, 1967) to yield anaphylatoxin (C3a). Considering the interrelationship of complement further, the immune adherence of the C3b component leads to the release of lysosomal enzymes that can produce C5a from C5 and can fragment basement membranes, thereby possibly contributing to HF activation. This final stage reveals a complete cycle in the interrelationships of the systems outlined. Fig. 1.3 summarises diagrammatically the systems described above.

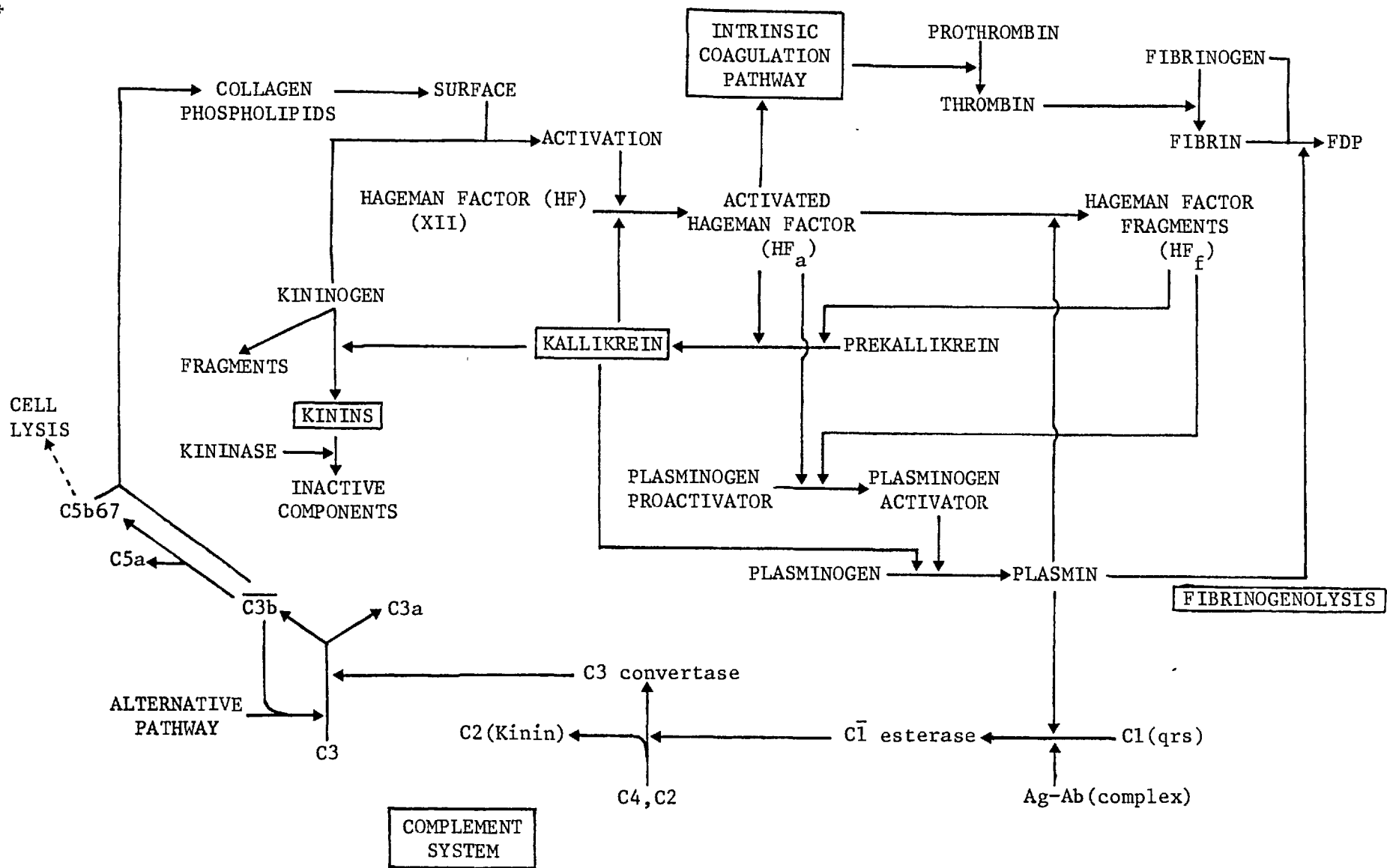


Fig. 1.3 Interrelationship of the four major blood enzyme systems *

1.3.5 Kallikrein-kinin system in disease

Kallikrein, other kininogenases and kinins have been suggested as being involved in a wide variety of pathological conditions. Among these are carcinoid syndrome (Oates et al., 1964), hereditary angioedema (Landerman et al., 1962), dumping syndrome (Zeitlin and Smith, 1966), thermal oedema, clinical allergies, protozoal infections and shock reactions (Schachter, 1969). Some of the evidence however is circumstantial with raised kinin concentrations being found in the vicinity of pathological tissue, and it has still to be determined whether in some instances, kinins induce the reaction or are the result of activation of kallikrein by some means, locally. Some of the properties of kallikreins and kinins are outlined in section 1.3.2

Some years ago, a number of articles discussed the role of the kallikrein-kinin system in human disease (Kellermeyer and Graham, 1968; Miles, 1969; Colman et al., 1969c), and more recently the participation of this system and other Hageman factor dependent pathways has been reported and reviewed (Pisano and Austen, 1976; Colman and Wong, 1977). Before describing some of the diseases and factors involved, it should be noted that there are now three well known deficiencies in which the plasma proteolytic enzyme systems are involved which result in no clinical abnormalities. Hageman trait, a factor XII deficiency (Ratnoff and Colopy, 1955), Fletcher factor trait, a prekallikrein deficiency (Hathaway et al., 1965), and a HMW kininogen deficiency, a common defect recognised by three groups of workers (Colman et al., 1975; Saito et al., 1975; Lacombe et al., 1975), each

using the name of the patient in which the defect was found. Two additional patients have recently been described (Donaldson et al., 1976; Lutchner, 1976). These conditions should prove useful in elucidating the direct involvement of the kallikrein-kinin system in disease states, although at present few studies are available on factor XII or prekallikrein deficient patients suffering from diseases in which the kallikrein system has been implicated. It seems likely that no activation will result unless there is another mode of initiation that is not dependent on factor XII. It has been reported in arthritis however that kinins have been found in the synovial fluid of a patient with severe deficiency of HF, indicating that HF was not required for the induction of inflammation (Donaldson et al., 1972). In the aetiology of rheumatoid arthritis there is some evidence to implicate the formation of kinins in synovial fluid (Melmon et al., 1967; Keele and Eisen, 1970). The relationship between synovial kinin levels and clinical symptoms is however inconsistent (Keele and Eisen, loc. cit.; Webster and Maling, 1970). It has been suggested that the components of the kinin system present in synovial fluid are derived mainly from the plasma (Jasani et al., 1969) and if this is so, changes in systemic kininogen levels, possibly stimulating compensatory synthesis of kininogen, should occur. Levels of plasma kininogen in rheumatoid patients has been found to be twice the mean control value of healthy volunteers (Zeitlin et al., 1976). In effusions from patients with gouty arthritis, kinins have been found (Kellermeyer and Breckenridge, 1965), with only two components appearing to be absolutely necessary, monosodium urate crystals, which are capable of activating HF in vitro and granulocytic leukocytes (Phelps and McCarty,

1966), again however there is no direct evidence to support the role of kinins in the inflammatory process in acute gouty joints.

The demonstration of the lack of an inhibitor of plasma kallikrein (Landerman et al., 1962) and Cl^- (Donaldson and Evans, 1963) in hereditary angioedema, raised the question of whether excess of either enzyme might be responsible for the symptoms of the disease. Injections of either plasma kallikrein (Landerman et al., 1962) or Cl^- (Klemperer et al., 1968) intradermally increased vascular permeability, presumably due to the uninhibited action of these enzymes. The part that both these components play in the production of oedema has been elucidated by studies of Donaldson et al. (1969).

An acquired abnormality involving the kinin-forming system is the malignant carcinoid syndrome, a large amount of evidence having accumulated implicating kinins in the pathogenesis of this disease state (Oates et al., 1964; Melmon et al., 1965; Colman et al., 1969c, 1976b; Oates et al., 1966). At least two mechanisms appear to be responsible for the kinin release, an anionic kallikrein in the carcinoid tumour tissue, and, although less frequent, the release of bradykinin mediated by plasma kallikrein, activated by HF. A third mechanism derived from animal studies showed that sympathomimetic amines (adrenaline and noradrenaline) activated the kallikrein system of rat blood causing up to 50% lowering of kininogen, and transient kinin release (Rothschild et al., 1974a,b, 1976). In normal man however adrenaline infusion in 1-2 μg doses had no effect (Colman et al., 1976b). Similarities between the carcinoid syndrome and post-

gastrectomy dumping syndrome were noted with bradykinin and serotonin being suggested as the humoral mediators of the latter condition (Zeitlin and Smith, 1966). Patients with the dumping syndrome were given hypertonic glucose by mouth and at the peak of the dumping reaction, free plasma kinin as measured by bioassay (Brocklehurst and Zeitlin, 1967) was markedly elevated, with a decrease in kininogen concentrations. These findings have been confirmed in both humans (Cuschieri and Onabanjo, 1971) and in dogs (MacDonald et al., 1969). The release of kinins by plasma or tissue kallikrein activation would account for the vasomotor symptoms of a striking increase in peripheral blood flow, with increased skin temperature.

Because of its ability to lower blood pressure in normal man (Mason and Melmon, 1965) and animals (Rocha e Silva et al., 1949), bradykinin has been implicated in the pathogenesis of several shock syndromes, (i.e. septic, cardiogenic and anaphylactic), the majority of work being concentrated on the changes in the kallikrein-kinin system in septic shock. Studies on unanaesthetized monkeys (Nies et al., 1968) showed an early increase in kinins determined by bioassay that correlated with an initial decrease in peripheral vascular resistance and an increase in cardiac output similar to the changes seen in man. In rats and mice after injection of endotoxin there is a transient appearance of free kallikrein, with a concomitant fall in both pre-kallikrein and kallikrein-inhibitor levels as measured by biochemical assay (Mason et al., 1970). Endotoxin infusion into human subjects leads to a rise in bradykinin and a fall in kininogen (Kimball et al., 1972 ; Hirsch et al., 1974), a similar situation being evident

in lethal endotoxin shock in dogs (Gallimore et al., 1978). The reduced levels of plasma prekallikrein indicating consumption of this protein, reflecting losses due to activation of kallikrein and reduced synthesis by the liver which is damaged in endotoxaemia (De Palma et al., 1967).

Other chronic diseases of the liver lead to a decreased synthesis of many plasma proteins as reflected in low plasma concentrations. A great deal of evidence has shown decreases in plasma prekallikrein in patients with cirrhosis of the liver (Colman et al., 1969c; Wong et al., 1972; Fanciullacci et al., 1976). These workers indicated that the decrease appears to be due to a decreased synthesis as reduced amounts of HF did not account for the low prekallikrein levels. An HF level of 25% or above was adequate for complete conversion of prekallikrein to kallikrein, and kallikrein inhibitor levels were found to be normal, suggesting there was no consumption of prekallikrein.

Changes in kallikrein excretion are usually assumed to be an index of changes in the activity of the kallikrein-kinin system intrarenally, however this assumption does not take into account the potential importance of kininases as regulators of the actions of kinins. It has been shown that there is a direct relationship between glomerular blood flow and urinary kinin and between urinary volume and glomerular filtration rate (Webster and Gilmore, 1964). In both human and rat hypertension, decreased urinary kallikrein have been reported which results in decreased water and electrolyte excretion: this is thought to play a major part in the pathogenicity of the syndrome (Rosas et al., 1977). The case for the participation of the renal kallikrein-kinin

system in disease states has been presented by Margolius et al. (1976). Margolius et al. (1971) rediscovered abnormally low kallikrein excretion in essential hypertension (Elliot and Nuzum, 1934). In addition, a series of studies demonstrated increased kallikrein excretion in primary aldosteronism (Margolius et al., 1971, 1974), reduced excretion in renovascular hypertension (Keiser et al., 1976a) and various abnormalities in kallikrein excretion in hypertensive animal models (Keiser et al., 1976b).

Growing connections between renal kinins and prostaglandins have been established since McGiff et al. (1972) showed that infused bradykinin released prostaglandins from the isolated perfused kidney. Nasjletti and Colina-Choario (1976) extended this observation by showing that aprotinin could decrease urinary prostaglandin excretion in the rat. Halushka et al. (1977) have clearly linked abnormally elevated prostaglandin E excretion with similarly increased kallikrein excretion in children with Bartter's syndrome. Prostaglandin deficiency, secondary to decreased kallikrein release therefore could contribute to elevated blood pressure. Taken together these studies suggest the hypothesis that a deficiency in the renal kallikrein-kinin system could contribute to the development of hypertension by decreasing the production of PGE_2 which normally mediates the anti-hypertensive function of the kidney (Vane and McGiff, 1975).

Plasma kallikrein and bradykinin have also been implicated in the pathogenesis of three acute haemoprotozoan diseases in mammals: Plasmodium knowlesi infections of monkeys (Onabanjo and Maegraith,

1970), Babesia bovis infections of cattle (Wright, 1973) - and chronic Trypanosoma infections of a number of animals and man, which will be discussed in the next section. Tella and Maeagraith (1966) showed a rapid decrease in kininogen due to increased utilization in acute P.knowlesi infections occurring at a time of high parasite multiplication. Although these workers showed no increase in kinin, later work has demonstrated elevated kinin levels (Onabanjo and Maeagraith, 1970; Onabanjo, 1974). Kallikrein is also involved in this infection being demonstrated as a potent and long acting capillary permeability-enhancing chemotactic agent, which also released kinin from kininogen (Onabanjo and Maeagraith, 1970). Broad spectrum esterases have been described in numerous malaria parasites, including P.falciparum and P.knowlesi (Levy et al., 1974) and these may possibly be responsible for the initiation of kallikrein activation in these infections. Wright (1975) has isolated relatively pure esterases from both the cytoplasm of B.bovis infected red cells and from B.bovis itself, which in vitro activate plasma kallikrein probably by a direct action. The enzyme also acts directly on kininogen substrate in vitro producing kinin. In acute terminal B.bovis infection of calves and cattle, a large increase in plasma kallikrein was observed by Wright (1973). Conversely, in acute B.bigemina infection, Wright and Goodger (1977) were unable to demonstrate any activation of the kallikrein or coagulation systems until there was a high parasitaemia (10^{13} parasites) although plasma kallikrein levels did not alter significantly. They suggested that the protease isolated previously had no counterpart in B.bigemina infections and that intravascular haemolysis may be at least partly responsible.

1.3.6 Kallikrein-kinin system in trypanosomiasis

Goodwin and Richards (1960) were the first to describe the presence of pharmacologically active peptides in the urine of mice infected with T.rhodesiense and they suggested that the pharmacological effects of the peptides may be the cause of some of the signs and symptoms of infectious diseases. Following this work, Richards (1965) found that in mice acutely infected with T.brucei plasma kinin levels rose markedly on the fifth day of infection just prior to death. Kinin levels in the ears, skin and feet also rose approximately threefold during this time. Since these initial observations kallikrein and kinins have been demonstrated in the urine of rats and rabbits (Boreham, 1966; Wright and Boreham, 1977) and kinins have been found in the blood of rabbits, cattle and man infected with T.brucei subgroup organisms (Boreham, 1968, 1970). Kinins have also been demonstrated in T.vivax infections of cattle (Van den Ingh et al., 1976b) and T.evansi infected guinea-pigs (Bhattacharya et al., 1965). It has been suggested that kinins are released in all pathogenic infections caused by African trypanosomes (Boreham, 1977), supportive evidence being the observation that no kinin was detected in rats infected with the non-pathogenic organism T.lewisi (Boreham, 1966). The release of pharmacologically active substances in parasitic infections, including trypanosomiasis, has recently been reviewed by Boreham and Wright (1976a), and a subsequent publication (Boreham, 1979b) discussed substances released in T.brucei infections specifically.

It was stated earlier that trypanosomiasis was an inflammatory disease, one of the major pathological changes in experimental T.brucei being an increase in capillary permeability (Boreham and Goodwin, 1967; Goodwin and Hook, 1968; Goodwin, 1970; Boreham, 1974). In the rabbit at least, the similarity of chronic T.brucei infection with the Arthus reaction has been stressed by Goodwin and Hook (1968), while other workers have likened the vascular reaction to the Schwartzman reaction (Boulton et al., 1974). The release of kinin early in infection may be responsible for some of the observed changes although other substances may be involved (Boreham, 1977).

The mechanism of activation of kallikrein with the release of kinins has been studied (Boreham, 1968; Boreham and Goodwin, 1970; Boreham and Wright, 1976a). Boreham (1968) demonstrated a direct relationship between the decreasing parasitaemia, followed by increasing kinin levels, with the appearance of variant-specific antibody in T.brucei infected cattle. Subsequently Boreham and Goodwin (1970) showed with T.brucei that when immune complexes of trypanosomes and immune sera, prepared in vitro, were incubated with fresh rabbit plasma kinin was released, with trypanosomes alone having no effect. This activation by immune complexes was shown to be HF but not complement dependent. Boreham and Wright (1976b) subsequently provided evidence that immune complexes were important in mediating kinin release and causing pathological changes in vivo. Rabbits infected with T.brucei are hypotensive as early as ten days post infection but there was no change in heart rate, and the injection of trypanosomes intravenously

into an infected animal causes a further reduction in blood pressure. Hypotension was also produced in non-infected rabbits infused with immune complexes prepared in vitro, these reactions being prevented by prior administration of aprotinin, a kallikrein inhibitor, suggesting that the reactions were mediated by kallikrein. In T.brucei infections at least, it appears that immune complex activation of HF could be a major cause of kallikrein activation which may influence subsequent coagulation changes. Boreham (1977) has postulated that the immune complex is negatively charged, resulting in partial HF activation, the remainder converted enzymatically by plasma kallikrein through a positive feedback system. Slots et al. (1977) have recently demonstrated that dead T.brucei injected into rabbits did not have any effect on fever, thrombocytopenia or on whole blood serotonin levels, but on subsequent injection 10 and then 20 days later a marked increase in febrile response and a decrease in whole blood serotonin was observed, indicating that immune complex formation caused the release of at least one pharmacologically active substance, serotonin, although they did not measure kinin levels.

In chronic T.brucei infected rabbits elevated urinary kallikrein and kinin levels in association with increased urinary output and tubular damage have been reported (Richards, 1965; Wright and Boreham, 1977). This would directly help to maintain the hypotensive state and suggests that the kidney plays an important role in the maintenance of blood pressure in this disease state. Various mechanisms may be hypothesized to explain the early release of urinary kallikrein as

this occurs within 3-4 days of infection when immune complexes would not be expected to have been formed. Their release could therefore be due to circulating endogenous chemicals, increased kidney blood flow, possibly caused by plasma kinins released locally, and alterations in aldosterone concentrations (Keiser et al., 1976b).

1.4 Immune complexes

'With the many techniques now available for detecting circulating immune complexes (IC), these have been found in a large variety of diseases and even in normal conditions. This high frequency of circulating IC raises questions about their pathogenic roles, their origin, and the possible clinical interest of their determination' (Masson, 1978).

The concept that immunologic injury to specific organs or tissues might occur as a result of antigen-antibody reactions, immunologically unrelated to the structures injured, was first suggested by Von Pirquet (1911) in his classical description of 'serum disease' in both humans and experimental animals. He postulated that the co-existence of foreign serum antigens and their homologous antibodies in the circulation resulted in the formation of toxic components, which were the cause of the rather specific vascular, renal, cardiac, cutaneous and joint lesions developing in tissues without immunologic relationship to the injected antigen. With refinement of techniques and the development of appropriate experimental animal models, the possible pathogenic role of IC in diseases of animals and man, as well as their mode of action have been established (Dixon, 1963).

It can be considered that IC are formed in vivo each time a humoral response to antigen is made when either free antigen molecules are still persisting, or are released from microorganisms, or from host cells into the extracellular fluid (Zubler and Lambert, 1977).

The fate of IC formed in the circulation depends upon a number of variables, including the rate of production, rate of removal and state of the phagocytic system, the degree of lattice formation and the nature of the antigens and antibodies (Mannik et al., 1974). Antigens involved in IC diseases may be of either exogenous or endogenous origin. Exogenous antigens include administered therapeutic agents producing serum sickness, inhaled and ingested antigens, and microbial and parasitic products derived from invading organisms.

1.4.1 Detection of immune complexes

IC may be found in plasma, serum, other body fluids or in tissue at sites of IC deposition. A variety of methods for the detection of complexes has therefore arisen, the main emphasis being placed on methods designed to detect IC in biological fluids (WHO, 1977; Zubler and Lambert, 1977). These methods may be separated into two main groups. Some have been devised in order to detect complexes independently of the nature of the antigen involved in the formation of these IC and represent 'antigen non-specific' methods. All these methods will detect non-specifically aggregated immunoglobulins as well as IC. Others however allow for a selective detection of IC involving one given antigen, through the discrimination between free and antibody bound antigens, and are the 'antigen specific' methods. The techniques are based on the physical properties of IC, their specific recognition by free molecules, such as complement components, rheumatoid factors (RF) and conglutinin, or their interaction with receptors for the F_c part of the immunoglobulins, or for complement on various cells. An evaluation of 18 methods for the detection of IC

in serum covering the properties outlined above has recently been produced (Lambert et al., 1978). A further survey of 16 principal methods have been critically discussed with respect to their specificity, sensitivity and possible interest in classical medicine by Nydegger and Kazatchkina (1979). At least 50 procedures are known at the present time with the continual development of new or modified techniques. However most methods only detect complexes which contain IgG and bind complement, yet other immunoglobulin classes may be involved in IC formation.

The techniques at present used for the detection of IC, including physico-chemical methods such as analytical centrifugation (Kunkel et al, 1961), cryoprecipitation (Meltzer and Franklin, 1966) and binding to anion exchange resins (Fox et al., 1974), as well as biological methods, for example precipitation with Clq (Agnello et al., 1970) or rheumatoid factor (Winchester et al., 1971) and inhibition of antibody dependent cell-mediated cytotoxicity (Jewell and MacLennan, 1973), have not really provided both a sensitive and simple means of detecting circulating immune complexes. One of the main problems associated with many tests is that of interference by endogenous complement or by polyanions (Soothill 1977). More recently the use of solid phase Clq (Hay et al., 1976), polyethylene glycol (PEG) precipitation (Digeon et al., 1977; Harkiss and Brown, 1979), and protein A binding (Hallgren and Wide, 1976; Crawford and Lane, 1977) have made the assays for IC more quantitative, reproducible and easier to perform although those utilizing Clq and protein A do require radioactive substances for their measurements.

One of the most interesting of IC determinations involves protein A. Most of the strains of heat killed Staphylococcus aureus Cowan 1 strain (S.aureus) contain a cell surface component, protein A, that binds to the F_c portion of most subclasses of IgG. In man, IgG1, 2 and 4 bind readily whereas IgG3, IgA and IgM are only adsorbed to a limited extent (Kronvall and Williams, 1969; Harboe and Folling, 1974). It is of value when looking at situations in which IC are found, to be able to isolate the IC and identify its antigenic moiety after dissociation of the antigen-antibody union. This has been achieved satisfactorily using protein A, as Kessler (1975) showed that complexes containing radiolabelled antigens readily bound to S.aureus. These complexes could be released from the bacteria with sodium dodecyl sulphate (SDS) and the antigen and antibody components could be identified by SDS polyacrylamide slab gel electrophoresis (SDS-PAGE) and autoradiography. This method has been refined even further recently by Tucker et al. (1978) utilizing Sephadex G-150 filtration with affinity chromatography on protein A-sepharose. Studies of McDougal et al. (1979) have determined conditions to maximise complexed IgG binding and minimized monomeric IgG binding with complexes of human sera. The assay that they derived from their studies was both quantitative, sensitive and not complement dependent. The isolation and identification of antigens therefore, using these and other methods, will possibly provide the information necessary for the formulation of a rational approach to the early diagnosis of diseases and perhaps their treatment and prevention.

1.4.2 Immune complexes in disease

A wide variety of chronically disabling diseases are thought to be caused by circulating soluble IC. These include collagen vascular diseases (Agnello et al., 1971), rheumatoid arthritis (Zubler et al., 1976), glomerulonephritis (Cochrane and Koffler, 1973) and possibly inflammatory bowel diseases (Jewell and MacLennan, 1973). A number of reviews have been produced that discuss the pathogenic mechanisms by which IC produce disease (Weigle, 1961; Unanue and Dixon, 1967; Germuth and Rodriguez, 1973; Cochrane and Koffler, 1973 ; Cochrane and Dixon, 1976; WHO, 1977), therefore only a brief description of some of the factors and disease states will be given here.

IC mediated reactions have been classified as Type III hypersensitivity reactions by Coombs and Gell (1968) and the simplest complex induced lesion is the Arthus reaction, a localised acute necrotising vasculitis, characterised by an intense infiltration with polymorphonuclear leucocytes. This lesion occurs near the site of antigen entry in relative antibody excess, the complexes being rapidly precipitated. In conditions of antigen excess, such as the abrupt release of antigens brought about by chemotherapy or in parasitic diseases, severe temporary hypersensitivity reactions, originally described as the Herxheimer reaction, are seen.

IC have been implicated in human disease following their identification in the tissues where lesions are found, and by comparison with similar damage produced by serum sickness. However the detection of IC in sera of patients with particular disease states does not necessarily mean that the complexes are the pathogenic cause. Although in many instances good correlation with disease activity is obtained, raised levels of complexes may be secondary events. The major pathological consequence of IC is usually the induction of inflammatory reactions, but the interference of IC with immune mechanisms and the depression of some specific cell functions may represent their most important effect in some diseases (Zubler and Lambert, 1977). The biological properties of IC are those of activation of complement, other plasma enzyme systems, with vasoactive amine release, and the interaction of the complexes with cell-surfaces (Haakenstad and Mannik, 1977; WHO, 1977).

It is becoming clear that IC are heterogeneous not only in the antigen involved but also in immunoglobulin class, and complement binding capacity, as well as size. The rate of formation is also important, as is the capacity of the reticuloendothelial system, especially the macrophages of the liver and spleen, for clearing them from the circulation. The kidney is particularly vulnerable because of its large blood flow and its filtering role, but complement binding complexes may also be preferentially deposited due to the C3 receptors on the glomerular epithelial cells (Gelfand et al., 1975).

As stated, the size of the complexes is an important feature since to be eliminated they must reach a critical size to be picked up by the reticuloendothelial system (Mannik and Arend, 1971). Similarly smaller complexes of bovine serum albumin with antibody have been found to be nephrotoxic in animals, whereas larger ones were not (Germuth et al., 1972). Similar observations in human disease have been noted in that size variation of complexes may account for the different chemical manifestations of systemic lupus erythematosus (SLE), as those patients with renal signs had predominantly medium sized IgG complexes, whereas extrarenal manifestations were associated with very large IgG complexes (Levinsky et al., 1977). Large concentrations of pathogenic complexes in the circulation for brief periods as seen in serum sickness, usually cause exudative polymorphonuclear leucocytic and proliferative endothelial lesions with necrosis of tissue, while lower levels of similar complexes in the circulation for periods of weeks or months cause chronic lesions with hyalin degeneration of vessel walls (Dixon et al., 1961). Once deposited in the tissues, complexes interacting with humoral and cellular elements of the host induce biochemical, pharmacological and morphological events. A sequence of events which are believed to be involved in the induction of vascular injury ('vasculitis') leading to tissue damage by soluble IC is given by Maini (1978).

Localization and concentration of IC, especially those of large size (more than 19S), should generally occur in tissues having F_c or C3 receptors. Except for blood cells, macrophages and lymph-

ocytes the distribution of F_c and C3 receptors is largely unknown. Local increase of vascular permeability as it is caused by IC containing IgE antibodies (Cochrane and Koffler, 1973) also facilitates the deposition of IC. The localising properties of the antigen itself should also be considered. DNA has affinity for collagen and can concentrate on basal membrane where it will react with its antibodies (Izui et al., 1976). It is also possible that collagen has a particular affinity for the F_c portion of aggregated immunoglobulins, as Eeckhout et al. (1976) have observed that collagen from guinea-pig skin was capable of agglutinating human IgG-coated particles. IC interference with the immune response is evident in cancer, where the normal defence mechanism could be hampered by the presence of IC (Sjorgen et al., 1971; Baldwin et al., 1972). Masson et al. (1977) detected factors resembling IC in the sera of pregnant women which could play a role in the tolerance of the foetus, as the sera from these women displayed a higher inhibitory activity than sera from non-pregnant women toward rabbit rheumatoid factor. Experimental studies have shown that IC can modify the receptors and reactions of lymphocytes (WHO, 1977).

IC have been suggested and described in disease states for a long time and their problem can be considered as two-fold : one is connected with the size and type of the immunoglobulin involved, and the other is related to the antigen covered up by the complex. The theory of this antigen/ antibody reaction, the isolation of complexes and the nature of the antigen have recently been considered in detail (Peeters, 1978). The study of IC has proved complicated and is an

expanding field of research and more work will be required to elucidate the many conditions and interactions of IC involvement in disease.

1.4.3 Immune complexes in trypanosomiasis

Some of the lesions of African trypanosomiasis are examples of immunopathology due to IC, their formation being the reaction between antigen and antibody, which must be favoured in trypanosomiasis, as a result of the persistent antigen production and resulting immune stimulation (Boreham and Kimber, 1970; Lambert and Houba, 1974). Occasions of antigen excess continue to alternate with periods of excess of the corresponding antibodies, and this situation is therefore analogous to that of chronic serum sickness. The classical and histopathological features of African trypanosomiasis are in some respects similar to those of generalised IC disease such as SLE.

The evidence for IC involvement in trypanosomiasis has developed from the identification of complexes in tissues, particularly the kidney, and the indirect evidence of soluble circulating complexes by depletion of certain complement components. The importance of specific kidney damage in trypanosome pathology was first suggested by Goodwin and Guy (1973) who presented clinical data suggesting renal failure as a cause of death in experimental T.brucei infections of rabbits. Further ultrastructural immunofluorescent observations have demonstrated the involvement of IC in the pathogenesis of proliferative glomerulonephritis, as suggested by Lambert and Houba (1974)

and Goodwin (1974). Similar results have been obtained in T.equiperdum infection in deer mice (Moulton et al., 1974), T. brucei infections in mice (Murray et al., 1975), T.rhodesiense infection in rhesus monkeys (Nagle et al., 1974), T.vivax infection in goats (van den Ingh et al., 1976a), and in T.brucei infections of rabbits (van den Ingh, 1976b; Facer et al., 1978). These workers have described granular deposits of immunoglobulin and complement along the glomerular capillary wall and mesangium and this correlated with the appearance of sub-epithelial electron-dense deposits in the glomerulus. Both are considered diagnostic of soluble IC glomerulonephritis (Unanue and Dixon, 1967). The presence of antibodies with anti-trypanosomal specificity in eluate of kidney tissue from rabbits chronically infected with T.brucei (Lambert and Houba, 1974) and the identification of trypanosomal antigen in the glomerular IC in T.equiperdum infection of deer mice (Moulton et al., 1974) also supports the idea of IC nephritis initiated by circulating trypanosomal antigen and anti-trypanosomal antibody. Facer et al. (1978) have demonstrated that antibody eluted from nephritic kidney failed to react with normal rabbit kidney, indicating the absence of an autoimmune response. In addition, IC have been identified in the endocardium, epicardium and around blood vessels in the myocardium in T.brucei infected mice, these complexes consisting of variant antigens and their respective antibodies, although the variant types involved were not identical to the predominant population present in the blood when the animal was sacrificed (Lambert and Houba, 1974).

Antigen-antibody complexes can bind to macrophage surface receptor sites (Takayanagi and Nakatake, 1974) and fix complement; these may be ingested, activate alternate receptor sites, or attack particulate antigen. Alternatively, trypanosome antigen-antibody complexes could activate complement in the fluid phase, where, after dissociation, it could bind to both trypanosomes and red cells, causing surface damage, if not complete lysis. This could be part of the immunological mechanism that is the cause of the anaemia seen in trypanosomiasis. Woodruff et al. (1973) have detected C3 on the red cells of humans infected with T.rhodesiense. Woo and Kobayashi (1975) have demonstrated, under in vitro conditions, that T.brucei antigen is absorbed onto normal rabbit red cells, while immunoglobulin has been shown to be present on the erythrocyte surface of cattle infected with T.congolense (Kobayashi et al., 1976). Rickman and Cox (1979) reported that in T.rhodesiense infections of rats, coating of erythrocytes and parasites with fibrin and complement suggested that IC of fibrinogen/fibrin related products, antibody to these products, and complement may have coated both cells and parasites, and that the conglutinating activity of immunoconglutinin caused both to be sequestered and phagocytized in the spleen. High immunoconglutinin titres have been detected in T.brucei infected rabbits, these antibodies being produced against modified complement components bound to antigen-antibody complexes (Ingram and Soltys, 1960).

IC of trypanosomes and antibody have a significantly higher chemotactic index than trypanosomes or immune sera alone, as measured

in vitro, the reaction being complement independent (Cook, 1977). The release of pharmacologically active substances in parasitic infections has recently been reviewed by Boreham and Wright (1976a). The liberation of kinins specifically in trypanosomiasis is known to occur early in the infection, as a result of the antigen-antibody reaction (Boreham and Goodwin, 1970; Boreham and Wright, 1976b). The generation of kinins and other pharmacological mediators by IC would lead to an increase in vascular permeability and correspondingly widen the chemoattractant field, generating a greater influx of activated mononuclear cells. Slots et al. (1977) have demonstrated that mixtures of T.vivax and corresponding antibody containing plasma, induced (^{14}C)serotonin release from prelabelled goat platelets in vitro. Neither the parasite nor the antibody alone had this effect, suggesting the formation of IC as the essential step in affecting platelet behaviour. Previous studies on the pathogenesis of trypanosomiasis in rabbits (van den Ingh et al., 1977), goats (Veenendaal et al., 1976) and cattle (van den Ingh et al., 1976b) suggested a correlation between circulating trypanosomes, platelet aggregation and decrease in blood serotonin levels.

The formation and presence of IC in trypanosome infections therefore appears to be of major significance in the pathology of the disease and it is only recently that circulating complexes have been identified and quantitated. Lambert and Galvao Castro (1977) have demonstrated IC in the blood of mice infected with T.brucei

from the tenth day after infection, by means of the (^{125}I)Clq binding technique. The appearance of the complexes was subsequent to the first parasitaemic peak and coincided with a fall in level of plasma C3. Circulating IC have also been shown in T.gambiense infections in humans and in the rat infected with T.brucei, using the same technique, by Fruit et al. (1977).

2 MATERIALS AND METHODS

2.1 Animals

Male CD-1 mice, weighing 18-25g and male outbred albino CD rats, 225-250g, were obtained from Charles River UK Ltd., Kent.

Male New Zealand white rabbits weighing 2.5-3.0kg were purchased from 'Morton Commercial Rabbits', Parsonage Farm, Essex.

All animals were fed on a standard laboratory diet with water freely available. The rabbits were kept for seven days before use.

2.2 Trypanosomes

Trypanosoma (Trypanozoon) brucei strain 427 was isolated from a sheep in South East Uganda in 1960. The strain used was originally obtained from the Lister Institute of Preventive Medicine, London and stabilates cryopreserved in liquid nitrogen (see section 2.4.5) at Silwood Park. The parasites were maintained by syringe passage of infected blood through albino mice every two or three days.

2.3 Infection of animals

(a) Mice and rats were infected by intraperitoneal injection of whole mouse blood containing trypanosomes. T. brucei strain 427 leads to an acute infection in these animals with death occurring after 2-5 days.

(b) Rabbits were infected by the subcutaneous route, using 1ml of trypanosomes previously separated from infected rat blood (see section 2.4.3) at a concentration of 2.0×10^8 - 9.0×10^9 organisms/ml. Strain 427 causes a chronic infection with death occurring after 5-8 weeks, although some rabbits did occasionally become asymptomatic and aparasitaemic.

2.4 Parasitological techniques

2.4.1 Trypanosome counts

Trypanosomes were counted on an Improved Neubauer haemocytometer, after separating and washing in buffer, in the same way as used for lymphocytes (Hudson and Hay, 1976).

2.4.2 Parasitaemia

Parasitaemia of the mice and rats was determined subjectively by a wet film examination of whole blood. A scale of 0 - 4+ was chosen, as indicated below, using a x40 microscope objective.

0	= no parasites	
1+	= <10 parasites/field	
2+	= 10-50	"
3+	= 50-100	"
4+	= >100	"

Parasitaemia of the rabbits was determined by microscopical examination (x40 objective) of a wet film preparation of whole blood by counting the number of trypanosomes present in 30 randomly selected fields.

2.4.3 Separation of trypanosomes

Mice and rats showing a high parasitaemia, three days after infection, were deeply anaesthetized and blood collected from the heart. The trypanosomes were then separated from the blood components by the DEAE- cellulose method of Lanham (1968). The eluant used was buffered phosphate saline glucose (PSG), pH 8.0, the separations made with the cellulose contained in a glass Buchner funnel with a No.1 sintered glass support. Having lightly centrifuged the blood sample, to partially separate the erythrocytes, the supernatant fraction containing the trypanosomes was layered onto the cellulose surface with a pasteur pipette and the eluate collected in a flask. The trypanosomes were washed three times in PSG and resuspended in the same buffer before use.

2.4.4 Preparation of trypanosome material

Trypanosome antigen, subsequently used in the immune complex preparation and gel separations was prepared in two ways, as indicated in Fig 2.1.

2.4.5 Preparation of stabilates

Stabilates were prepared according to the method of Cunningham et al. (1963). Blood was collected from mice showing a high parasitaemia

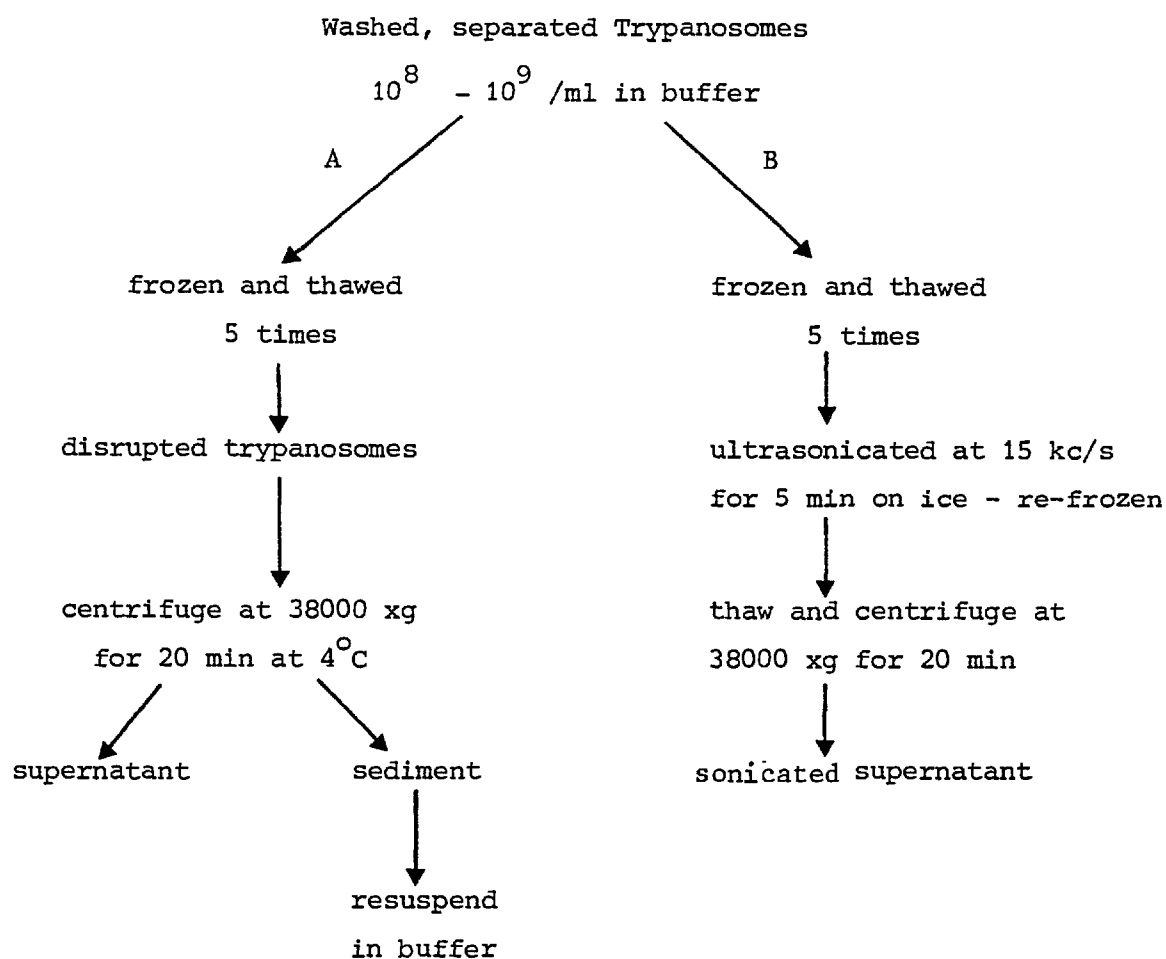


Fig. 2.1 Preparation of trypanosome antigen

and placed in plastic tubes containing lithium heparin, 0.15mg/ml, as an anticoagulant. Glycerol was added to give a final concentration of 7.5% in the blood. Glass capillary tubes, approximately one-third full of this blood, were sealed at each end in a low bunsen flame. These capillaries were then placed in a labelled glass bottle and stored in a liquid nitrogen refrigerator until required. The viability and infectivity of the trypanosomes stored in this way remain for several years.

2.5 Immunological techniques

2.5.1 Collection of serum

Blood samples were taken by cutting the marginal ear vein of rabbits, at intervals throughout the infection (Herbert, 1973) and collecting them in small glass bottles. The blood was allowed to clot at 37°C for 3h in a water bath, the serum removed by centrifugation at 1500xg for 10 min, and small aliquots placed in 2ml plastic tubes. These were stored at 4°C after the addition of 0.01% sodium azide (Digeon et al., 1977) or at -20°C. The sera were used within 6 weeks of collection.

2.5.2 Collection of plasma

Blood samples were initially taken by cutting the marginal ear vein of rabbits and collected into 5ml plastic tubes containing EDTA, 2mg/ml (Greyward). Subsequently samples were taken into a 2ml plastic syringe with the same concentration of EDTA and transferred to 5ml plastic tubes. All samples were centrifuged for 10 min at 1500xg. The plasma was removed using a 1ml plastic syringe, without a needle, and stored in 2ml plastic tubes at -20°C until required.

2.5.3 Immunization of animals

Rabbits were immunized by inoculating them intramuscularly with 2.0ml of an equal volume emulsion of dead disrupted T.brucei 427, at a concentration between 2.0×10^8 and 9.0×10^9 /ml and Freund's Complete Adjuvant (FCA) (Difco). The inoculations were made on days 1, 5, 8 and 20, and the sera collected as described in section 2.5.1, 23 days after the initial inoculation. This hyperimmune sera was tested for precipitating and agglutinating antibodies (section 2.5.6 and 2.5.10) within seven days of preparation.

2.5.4 Gel filtration

Serum samples were separated according to the molecular weight of the components by Sephadex G200 chromatography, at a temperature of 10°C . 2.0ml samples, after the addition of 10% sucrose to increase the density, were carefully layered onto the column and 3ml eluate fractions collected. The eluting buffer used was 0.1M Tris-HCl in 1.0M sodium chloride, pH 8.0. The void volume was determined by elution of blue dextran 2000 (Pharmacia Ltd.). Measurement of the protein concentration of the fractions was carried out on a Beckman DB spectrophotometer at 280nm using the buffer as a blank.

2.5.5 Preparation of immune complexes

(a) Live trypanosomes

Trypanosomes, separated on a DEAE cellulose column, were resuspended to a maximum concentration, after washing in PSG. 0.5ml of this suspension was added to 0.5ml of hyperimmune serum (section 2.5.3). After incubation at 37°C for 1h the suspension was centrifuged at 1500xg for 3 min and this supernatant removed. 0.5ml PSG was added and after agitating thoroughly the suspension was centrifuged as before. The washings were repeated 3 times. The trypanosomes were then resuspended in 0.5ml PSG and their mobility checked by examining a small drop under a microscope before use.

(b) Disrupted trypanosome material

Trypanosomes were disrupted (see section 2.4.4, method A). 1ml of the suspension was incubated at 37°C, with an equal volume of hyperimmune serum for 1h. The material was placed at 4°C for 12h (Crawford and Lane, 1977) centrifuged, the supernatant removed and finally the sediment resuspended in 0.5ml PSG, after repeated washings.

2.5.6 Immuno-electrophoresis

Cross over electrophoresis was carried out on microscope slides or glass plates precoated with a thin layer of 0.5% agar. These were covered with 1.3% agar in barbitone buffer pH 8.6 to an even depth on a level surface. A Shandon electrophoresis cutter was used to stamp out the sample wells. The agar plugs were removed using a modified cutter connected to a vacuum system. This ensured that the wells had vertical sides. The slides were placed in a Shandon immuno-electrophoresis tank and connected to the buffer with filter paper wicks. The voltage was switched on, the samples were applied with drawn out Pasteur pipettes, and electrophoresed at 160-200V, 0.2-0.3 amps for 15 min, topping up the wells as necessary. Initial examination of the slides for precipitation reactions were made using an indirect light source (Shandon). In order to preserve and clarify the results obtained, the slides were washed in three changes of phosphate buffered saline and then wrapped tightly in water saturated filter paper. The wrapped slides were then placed in a glassware drying oven overnight at 60°C before removing the filter paper after re-wetting. This left the dry film of agar firmly attached to the slide, which was stained with brilliant crescein coomassie blue (see Appendix 1). The slides were examined again as an enhancement of sensitivity was achieved with this staining procedure.

2.5.7 S.D.S. polyacrylamide slab gel electrophoresis

Slab gels were poured between cleaned specially cut 14cm x 12cm glass plates held apart by 2mm thick perspex spacers and the edges sealed with agar to prevent leakage. After polymerisation of the running gel, the stacking gel was carefully poured on, and a toothed comb slot former was inserted. When this gel had polymerised the comb was removed and the slots so formed were washed with distilled water. The back surface of the glass plate was cleaned thoroughly before loading the complete gel into a specially designed electrophoresis tank filled with the running buffer. Care was taken to remove any bubbles that formed at the bottom of the gel, these were dispersed using a modified Pasteur pipette. All the samples were made up to 100 μ l with SDS sample buffer, and after the addition of 5 μ l 2- Mercaptoethanol, were boiled for 10 min, before carefully loading them onto the gel using capillary tubes. Electrophoresis was carried out at 50V through the stacking gel and 150V through the running gel. The electrical supply was switched off when the tracking dye had reached the bottom of the gel. The gel plates were then prised apart and the gel stained in the Coomassie blue solution overnight on a rocking platform. Destaining was for 3h and the gel was examined. A photograph was taken as a permanent record. The method described here is a modification of that of Studier (1972) and the solutions used are indicated in Appendix 1.

2.5.8 IgG and IgM levels

IgG. Quantitative determination of rabbit IgG was performed using a standard kit (Miles Biochemicals) based on the principle of single radial immunodiffusion.

A standard curve was prepared in each set of determinations by accurately pipetting 5.0 μ l of each of four reference standards into the wells of the central row of the agar plate using the graduated capillary tubes provided. 5.0 μ l serum samples were applied to the remaining wells with a total of 17 samples determined in one set. A few drops of water were pipetted into the trough bordering the plate, the cover replaced and the plate left undisturbed at room temperature for 18h.

The precipitation rings were directly measured from the immunodiffusion plate and the concentration of IgG present determined from the standard curve.

IgM. The amount of IgM present in the serum was determined by diffusion into agarose containing anti-IgM. The standard used in these measurements was normal rabbit serum. 75 μ l of anti-rabbit IgM (1.9mg antibody/ml) (Miles Biochemicals) was diluted with 1.9ml PBS and warmed to 56°C before adding to 1ml 2% agarose in barbitone buffer at 56°C. This was layered onto a precoated slide on a level surface. After the agar had set, eight wells 2-3mm in diameter were cut with a gel punch. The agar plugs were removed with a Pasteur pipette attached to a water vacuum system.

Each of the wells was then filled quickly with sample, until the meniscus just disappeared, and the slide was placed in a humidity cabinet. After 72h the slide was examined for precipitation rings and their diameter measured. These values were compared directly with the standard on each slide. To improve the resolution of the precipitates formed, the slides were treated and stained, as in section 2.5.6.

2.5.9 C3 determinations

Levels of C3 in serum were measured with a single radial immunodiffusion technique. This was as described in the measurement of IgM (section 2.5.8), with 75 μ l anti-rabbit C3 (Cappel Laboratories, USA) present in the agarose. The measurements of the precipitation rings were made in the same way. A standard of normal rabbit serum was used throughout the determination.

2.5.10 Agglutination test

The method described by Cunningham and Vickerman (1962) was used in these experiments. Serial twofold dilutions of the serum were prepared in phosphate buffered saline pH 7.8, with a starting dilution of 1/10. 0.1ml of each dilution was placed onto a siliconised glass plate, marked out into squared areas. A tube of stabilate (see section 2.4.5) was removed from the liquid nitrogen and both ends broken off. A very small amount of blood containing trypanosomes

was placed on each drop of sera, starting at the greatest dilution, by touching one end of the tube onto the surface of the drop. The plate was then left at room temperature for 1h and examined under a microscope, magnification x40, for agglutination. The dilution of the serum showing any agglutination was taken as the agglutinating titre.

2.6 Biochemical techniques

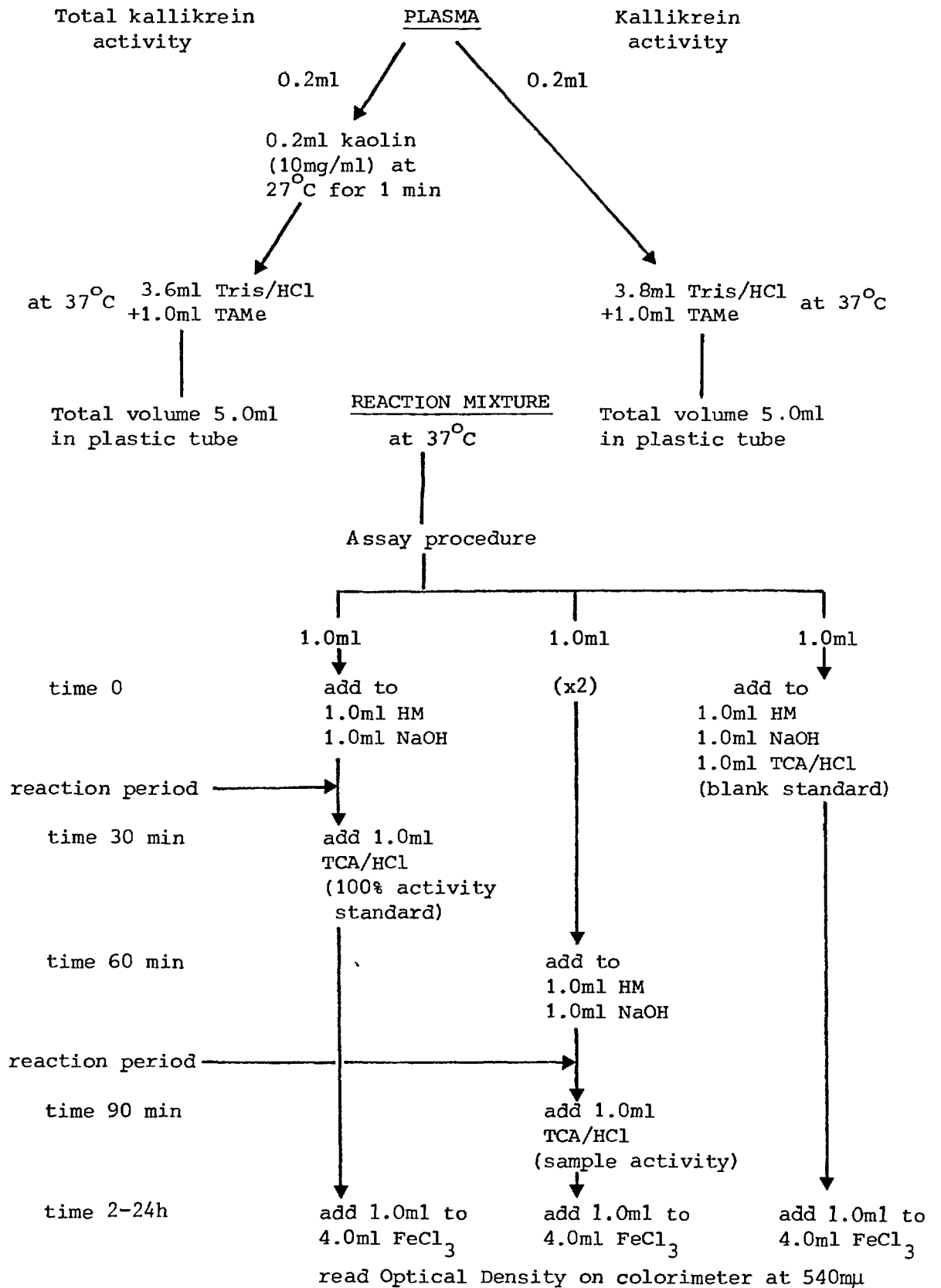
2.6.1 Assay for kallikrein

Kallikrein specifically hydrolyses synthetic arginine methyl esters (Webster and Pierce, 1961). This esterolytic property was used to assay the amount of kallikrein present in the plasma samples. The method followed was adapted from Wright and Mahoney (1974), which is based on the hydrolysis of N- α tosyl- L- arginine methyl ester (TAME), (Roberts, 1958).

Kallikrein exists in plasma in both an active form, measured by the spontaneous hydrolysis of TAME by untreated plasma and an inactive precursor, prekallikrein. The total kallikrein activity (prekallikrein and kallikrein) was measured by TAME hydrolysis after activation of the plasma with kaolin and the level of prekallikrein obtained by subtraction of the two values. The method used is outlined in figure 2.2. Plastic pipettes and tubes were used throughout (Eisen and Vogt, 1970), unless stated otherwise.

0.2ml plasma was added to 3.8ml 0.05M Tris/HCl buffer pH 8.4 and 1.0ml 0.05M TAME in a plastic tube at 37°C. 1.0ml of this reaction mixture was immediately taken (time 0) and added to a glass tube containing 1.0ml 2M hydroxylamine monohydrochloride (HM), 1.0ml 3.5M sodium hydroxide (NaOH) and 1.0ml trichloroacetic acid/hydrochloric acid (1 part concentrated hydrochloric acid diluted with 2 parts distilled water, plus 6g trichloro-

Figure 2.2 Diagrammatic representation of kallikrein measurement



acetic acid per 100ml of acid solution, TCA/HCl). A further 1.0ml of the reaction mixture was added to a second tube with only 1.0ml HM and 1.0ml NaOH. 1.0ml TCA/HCl was added after a 30 min reaction period. These two tubes were the blank and 100% activity standards, respectively. The activity of the sample was determined after incubating the remaining reaction mixture at 37°C for 60 min, with intermittent agitation. At the end of the incubation period 1.0ml aliquots of this reaction mixture were added to two tubes each containing 1.0ml HM and 1.0ml NaOH. 1.0ml TCA/HCl was added after the 30min reaction period. All samples were thoroughly mixed using a vortex mixer (Fisons) during the reaction period, with at least ten separate mixings, to prevent gas bubble formation. All the tubes were then allowed to stand at room temperature for a minimum period of 30 min. If the tubes were left for an extended period, up to 24h, they were covered to prevent evaporation of the sample. The samples were centrifuged at 1500xg for 10 min. 1.0ml of the supernatant was added to 4.0ml 0.11M ferric chloride in 0.04N HCl. The optical density of the sample solution was read against its own blank standard within 3 min at 540mμ using a colorimeter (Gallenkamp).

Plasma samples were activated by incubating equal volumes (0.2ml) of plasma and kaolin (10mg/ml Tris/HCl pH 8.4) in plastic tubes at 27°C for 1 min. The assay was then repeated as described above with 3.6ml Tris/HCl buffer, so that the plasma/kaolin and buffer volume was 4.0ml. This ensured that there was no dilution factor affecting the results, and that the plasma volume remained the same in activated and unactivated samples. The amount of TAME

hydrolysed was determined from the standard curve of TAME dilution (see section 3.1). The amount of prekallikrein or kallikrein present in the sample was represented as μ moles TAME hydrolysed/ml plasma/h after correction for spontaneous hydrolysis. All determinations were performed in duplicate.

2.6.2 Measurement of protein concentration

Lowry et al.(1951) described a method for quantitative protein determination employing the Folin phenol reagent.

The reagents used were:

- A : 2% sodium carbonate in 0.1N sodium hydroxide
- B : 0.5% copper sulphate in 1% sodium tartrate
- C : alkaline copper sulphate prepared before use and discarded after standing for 24h, by adding 1ml B to 50ml A.
- D : dilute Folin reagent kept at 4°C

To 0.4ml of the test sample 2ml reagent C was added, mixed thoroughly and allowed to stand at room temperature for 20 min. 0.2ml reagent D was then added rapidly and shaken immediately. After standing for at least 30 min the optical density of the sample was read on a colorimeter (Gallenkamp) at 670 m μ . Distilled water was used as a reference and treated in the same way as the sample.

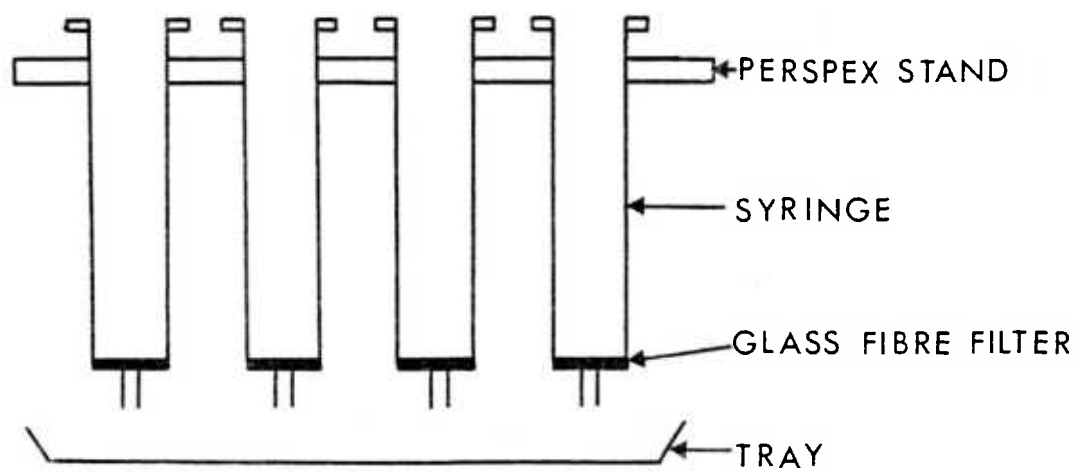
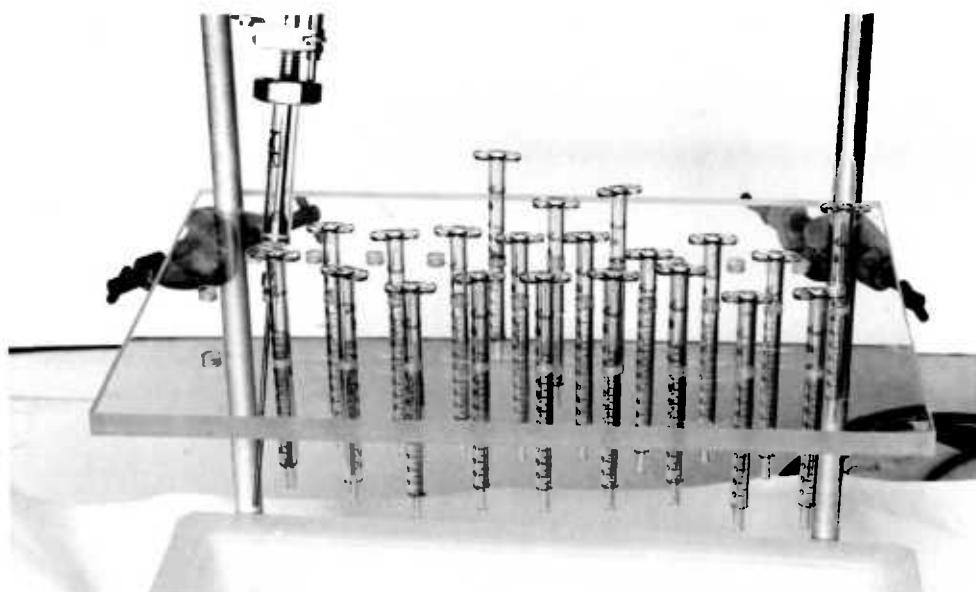
A standard curve (figure 3.25) was constructed for protein levels by determining the optical density of versatol (W.R.Warner and Co.,Hants), a pooled serum standardised for each of 14 constituents, the total protein content being 7.2g/100ml. Doubling dilutions of the versatol were made and treated as for the samples. The amount of protein present in each test sample was read directly from the standard curve.

2.7 Radiochemical methods

2.7.1 Assay for immune complexes

The method used was a modification of an immune complex assay described by Crawford and Lane (1977). 5mm discs of GF/C glass microfibre filter paper (Whatman) were cut with a cork borer and carefully positioned in the base of 1ml disposable plastic syringe barrels (Gillette) held in a perspex stand, as shown in figure 2.3. The filter was then wetted with 0.2ml NET buffer (150mM sodium chloride, 5mM EDTA, 50mM Tris, pH 7.4) containing 0.25% gelatin and 0.05% NP40 (BDH) applied with a long form Pasteur pipette. Dilutions of test and control sera were then made in plastic v-bottomed microtitre trays. 50 μ l of the neat serum was placed in the first well with an adjustable Oxford pipette. 10 μ l of this serum was transferred to a second well containing 40 μ l NET buffer and thoroughly mixed before taking 10 μ l of the diluted sample and adding to a further 40 μ l NET buffer and mixing. 10 μ l of the second dilution was discarded, leaving all the wells with 40 μ l of sample. The neat and second dilution sample were then loaded onto filters, with a Pasteur pipette, and allowed to soak through. The filter was washed twice with 0.2ml of the same buffer before blowing through the excess liquid, using a rubber teat positioned at the top of the syringe. Care was taken not to exert too much pressure or the filter would be displaced from the base of the syringe.

Fig. 2.3 Filter apparatus for immune complex separation



I-125 Protein A in buffer was obtained from the Radiochemical Centre, Amersham with an activity of $10\mu\text{C}/0.1\text{ml}$. $30\mu\text{l}$ of this material was diluted with 1.0ml NET buffer (pH 9.1) containing 0.25% gelatin and stored at a temperature not above 20°C , as the diluted stock. All radioactive material was used within one month of purchase.

$10\mu\text{l}$ of the diluted I-125 Protein A stock, which gave a count of approximately 10^4 cpm, was pipetted directly on to the filter using a multiple dispensing pipette (Jencons Scientific), fitted with a spinal needle adapted to rest just above the filter. This ensured that the active material was only put onto the filter. The amount of activity present on the filter was measured by transferring the syringe to a sodium iodide detector and recording the number of counts over a period of 30 or 40s. The counting system is described in section 2.7.2. The syringes were left at room temperature for 10 min, allowing the Protein A to react with the immune complexes, before repeating the washing procedure. The excess liquid was blown through and the filters counted twice on the detector.

2.7.2 The counting equipment

The counting equipment is shown in figure 2.4a. It consisted of a detector assembly 1653B, a scintillation detector adaptor 2179,

Fig. 2.4a Gamma-ray spectroscopy system

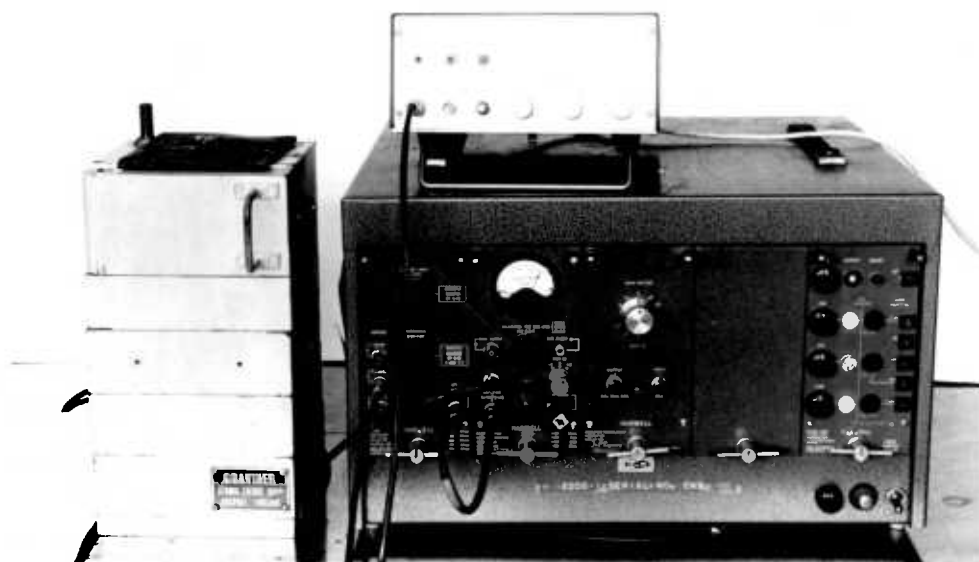
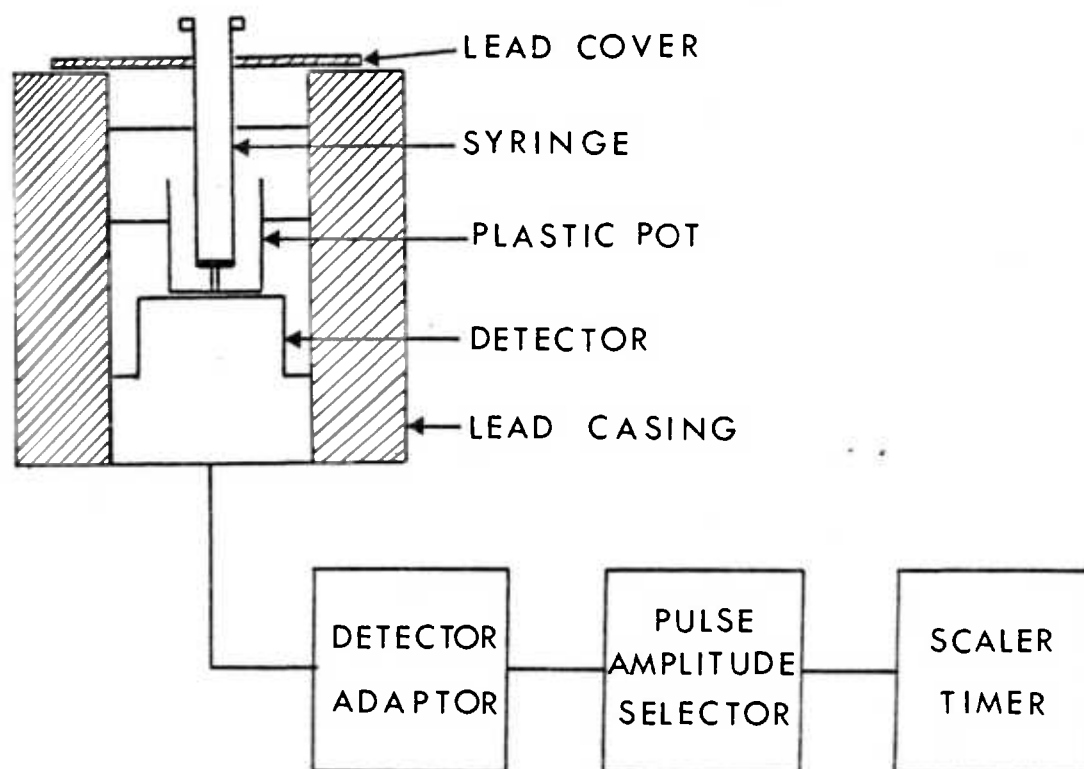


Fig. 2.4b Sectional diagram of detector assembly



a pulse amplitude selector 2216 and a scaler timer. The detector assembly (Fig 2.4b) was made up of a number of parts. The sodium iodide crystal, 1.5 x 1 in., connected to a photomultiplier, surrounded by its own housing, with the entire unit encased in a lead castle. On top of this assembly was a lead plate, approximately 4 in. square with a hole cut centrally, just larger than the syringes used in the assay, enabling the syringes to be positioned accurately above the detector, to improve the reproducibility of the counting geometry. A plastic pot was placed between the detector and the syringe to prevent any radioactive material from contaminating the detector cap (Fig. 2.5). The pots were replaced if any contamination occurred.

Fig. 2.5 Detailed view of detector counting geometry



The scintillation counter adaptor converts the output signal from the photomultiplier tube of the detector to a form suitable for feeding to an analogue or digital recording system. A single lead

providing the e.h.t. supply to the photomultiplier as well as accepting the input signal to the adaptor. The pulse amplitude selector accepts the output signal and with careful adjustment of the threshold and channel width controls, preferentially selects the 37 keV gamma-ray produced by I-125, giving a high count-rate with low background interference. The output then passes into a scaler timer which gives a visual display of the counts over a preset time period.

2.7.3 Clq solid phase radioimmunoassay for immune complexes

A modification of the method of Hay et al. (1976) was used. 1.0ml volumes of human Clq solution of 10µg/ml in PBS/0.2% sodium azide were incubated in polystyrene tubes (LP3 Luckham) for 18h at 4°C. After three washes with cold PBS the tubes were filled with 1.5ml 0.3% gelatin solution in PBS/azide and incubated at room temperature for 2h. After two washings with PBS/azide containing 0.05% tween 20 (PBS/tween) the tubes were used in the assay.

50µl of test serum was mixed with 100µl disodium ethylene diamine tetracetic acid (EDTA), 0.2M (adjusted to pH 7.5 with NaOH), and incubated at 37°C for 30min. The mixture was then transferred to an ice bath (Zubler et al., 1976). Duplicate 50µl samples were placed in the coated plastic tubes together with 950µl PBS/tween and incubated for 60min at 37°C and 30min at 4°C. Coated tubes contain-

ing 1.0ml PBS/tween were used as background controls. The unbound proteins were then removed by washing three times with cold PBS/tween. The washing was performed by tipping out, as described by Hay et al. (1976).

Immune complexes bound to the Clq-coated tubes were detected by incubating the tubes with approximately 25ng of radiolabelled Protein A (Pharmacia) in 1.0ml PBS/tween. On this occasion washing was carried out by aspiration with a pipette attached to a vacuum supply. The tip of the pipette may be touched lightly on the bottom of each tube without impairing duplicate reproducibility. The tubes were then counted on a gamma spectrometer (LKB, Wallac 1280 Ultrogamma), the amount of radioactivity bound being a measure of the immune complexes in the serum.

2.8 Isolation of soluble complexes

200 μ l of serum was added to 500 μ l borate buffer, pH 8.4 and 700 μ l polyethylene glycol (PEG). These were mixed thoroughly and left at 4°C for 2h. The tubes were then centrifuged at 6500xg (Eppendorf centrifuge, 5413) for 4 min and the supernatant removed by aspiration. The sediment was washed once with 3.5% PEG resuspending the pellet with a glass rod and vortex apparatus. The material was centrifuged again, 6500xg for 2 min, and the supernatant removed. 200 μ l NET-NP 40 was then added to the sample tubes and the sediment resuspended by vortex. 1ml PBS was added to one sample tube instead of NET-NP 40 and this was used for an optical density reading.

5.0x10⁹ Staphylococcus aureus (80 μ l stock suspension) were added to the tubes. These were mixed and the tubes fixed to a rocker for 30 min. The tubes were centrifuged at 6500xg for 2 min. The supernatant was removed and retained in separate tubes, to which 10% TCA was added and the tubes left for 1h at 4°C. They were then centrifuged as before for 2 min, the supernatant removed and the sediment washed twice with cold absolute alcohol.

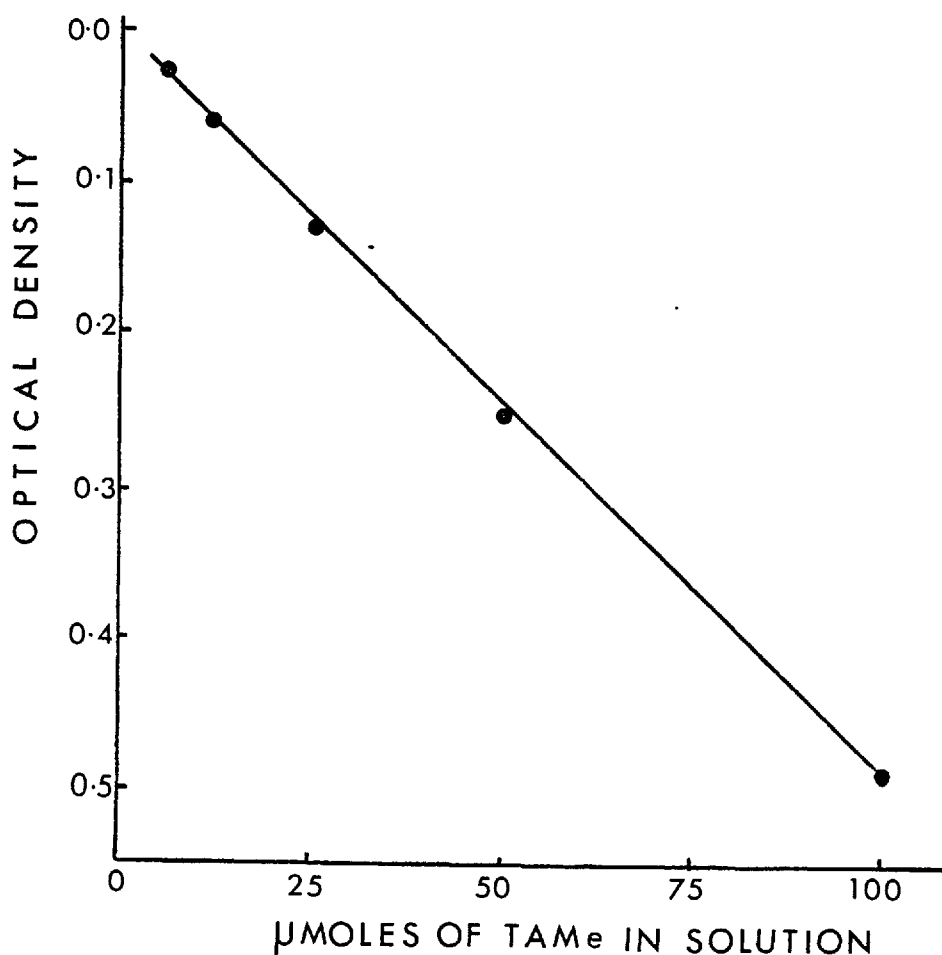
The pellet of Staphylococcus aureus and bound material was washed three times with PBS to remove NP 40 and the sediment resuspended using a vortex apparatus. 150 μ l of SDS was then added to both pellet fractions and the tubes again fixed to a rocker and left at room temperature overnight. 5 μ l 2-Mercaptoethanol was added to 100 μ l of the sample. After boiling for 10 min the sample was applied to an SDS slab gel (see section 2.5.7).

3 EXPERIMENTAL

3.1 Calibration of TAME concentration

In order to represent the amount of TAME hydrolysed in the following experimental procedures a calibration curve was determined for the ferric complex of the hydroxamic acid, produced as a result of the hydrolysis of the ester. Doubling dilutions of TAME were made in 0.05M Tris/HCl with a maximum concentration of 0.1M. 1ml samples of these TAME solutions were then added to tubes containing 1.0ml HM and 1.0ml NaOH. After a 30min reaction period 1.0ml TCA/HCl was added. 1ml of the final solution, combined with 4.0ml FeCl_3/HCl was transferred to a cuvette to measure the optical density, the results are illustrated in Fig. 3.1.

Fig. 3.1 Calibration graph for TAME concentration



3.2 Evaluation of the kallikrein assay

Before experimental data could be accumulated on the changes occurring in the levels of plasma kallikrein in rabbits infected with T.brucei a number of parameters concerning the assay of kallikrein had to be determined, as it is possible that the enzyme may react in a different manner in diverse animal species (Rocha e Silva et al., 1967). Previously, Colman et al. (1969c), using human plasma, and Wright and Mahoney (1974) using cattle plasma, had estimated similar parameters in each case, and these will be referred to briefly in the sections below, where appropriate.

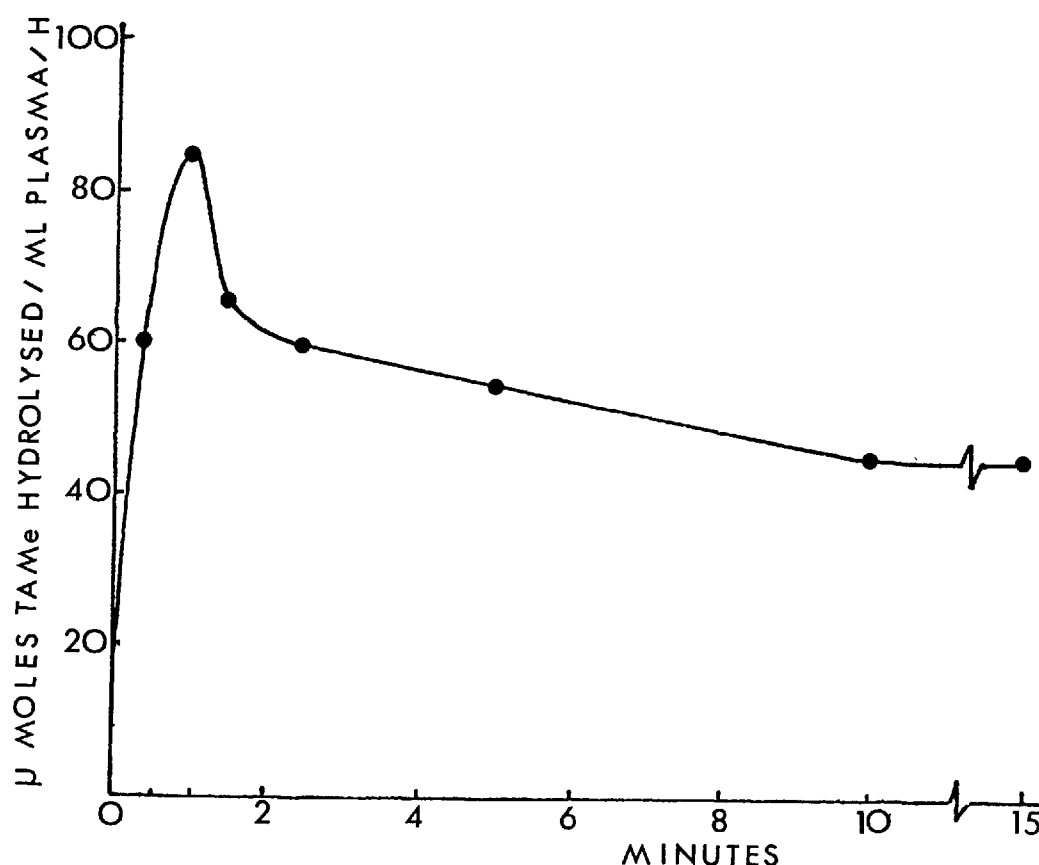
3.2.1 Kaolin activation of plasma kallikrein

The optimum activation period of rabbit plasma kallikrein was determined following previous work demonstrating maximum activation after one and four minutes, using human and cattle plasma, respectively.

Plasma was collected from 4 normal uninfected rabbits. 1.5ml of each plasma sample was mixed with 1.5ml of kaolin (10 mg/ml in 0.05M Tris/HCl) in a plastic tube at 27°C. 0.4ml samples were removed (section 3.2.4) at 30sec, 1, 1.5, 2.5, 5, 10 and 15min and assayed as described in section 2.6.1. 0.2ml of plasma was assayed without addition of kaolin, this sample giving the spontaneous (kallikrein) activity. Fig. 3.2 shows the kallikrein activation curve for normal rabbit plasma.

The mean spontaneous kallikrein activity was low : 15 ± 5 μ moles TAME hydrolysed/ml plasma/h. The maximum total plasma kallikrein, 85 ± 5 μ moles TAME hydrolysed/ml plasma/h, was present after 1min activation with maximum inhibition of about 50% occurring between 5 and 10min. The level remained constant after this time.

Fig. 3.2 Time course of activation of plasma kallikrein by kaolin (10mg/ml)



The difference between the spontaneous activity and the maximum activity obtained at 1min represents the available prekallikrein in the plasma. The decrease after 1min is probably due to the presence

of natural plasma inhibitors. Rocha e Silva et al. (1967) have demonstrated that this decrease does not occur in inhibitor-depleted plasma, neither is it evident in patients with hereditary angioneurotic oedema who lack plasma kallikrein inhibitor (Colman, 1974). The levels of plasma kallikrein inhibitors have not been investigated in the present study. Other workers have estimated the kallikrein inhibition as a percentage of the total activated kallikrein (Wright and Mahoney, 1974) or in arbitrary units (Colman et al., 1969c). The nature of kallikrein inhibition is obviously complicated due to the presence of a number of inhibitors, as outlined in the introduction (1.3.2). Plasma samples were subsequently activated, where appropriate, with kaolin (10mg/ml), for 1min.

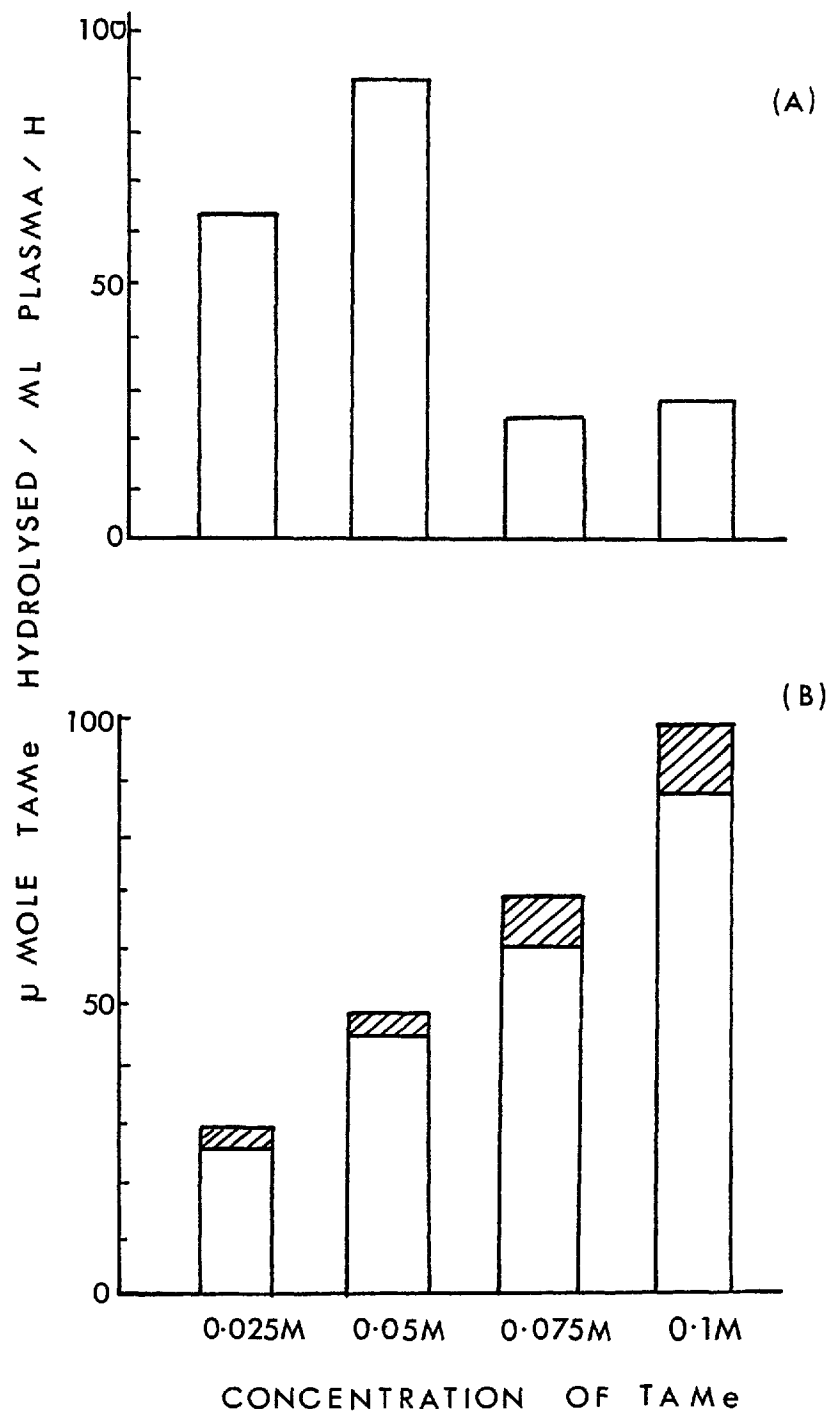
3.2.2 Optimal substrate concentration

Colman et al. (1969c) and Wright and Mahoney (1974) have both used a substrate concentration of 0.05M in their determinations of kallikrein activity. The former authors' investigation of the kinetic constants revealed a K_m of 1.36×10^{-2} M and a V_{max} for the kallikrein-TAME reaction of 0.10M. For many enzymes the rate of catalysis, V , varies with the substrate concentration (S) and the maximal rate, V_{max} , is attained when the enzyme sites are saturated with substrate. K_m is equal to the substrate concentration at which the reaction rate is half of its maximal value, although this depends on the particular substrate and environmental conditions, such as temperature and ionic strength. Section 3.2.3 shows a linear relationship for TAME utilisation

with time for rabbit plasma in the present experiments. Although theoretically it is desirable to measure the enzyme at a concentration greater than its $V_{\max}$, the concentration of the TAME substrate is limited by solubility.

TAME was prepared at concentrations of 0.025, 0.05, 0.075 and 0.1M in 0.05M Tris/HCl. Normal rabbit plasma (0.2ml) was activated with equal volumes of kaolin and assayed for TAME hydrolysis with the various TAME concentrations. It can be seen from Fig. 3.3 (A) that the optimum substrate concentration for activated samples was 0.05M. Although no kinetic studies were undertaken, the result is comparable with those already described. The scale of the colorimeter was logarithmic and therefore the mid-scale reading, corresponding to a 0.05M TAME concentration, gave good sensitivity to change. Despite the fact that the reading for 0.025M was at the lower, and most sensitive, end of the scale, there was not as marked a utilisation of TAME and this is due to the period of sample incubation with the substrate, a longer period of activation being required for the weaker solution. The results also indicate that at the concentrations of 0.075 and 0.1M substrate sufficient TAME is still available, after degradation by kallikrein present in the plasma, to utilise the rest of the HM. This leads to a situation where there appears to be very little TAME degradation and therefore a small amount of kallikrein. There is a surprisingly small difference between 0.075 and 0.1M concentrations. This is probably due to the higher degree of spontaneous hydrolysis in the case of the 0.1M solution, as can be seen in Fig. 3.3 (B),

Fig. 3.3 Utilisation of TAME at different concentrations



(A) activated plasma samples (0.2ml)

(B) spontaneous hydrolysis 

which causes an apparently higher TAME utilisation. Therefore 0.05M TAME was chosen as the substrate concentration for this study.

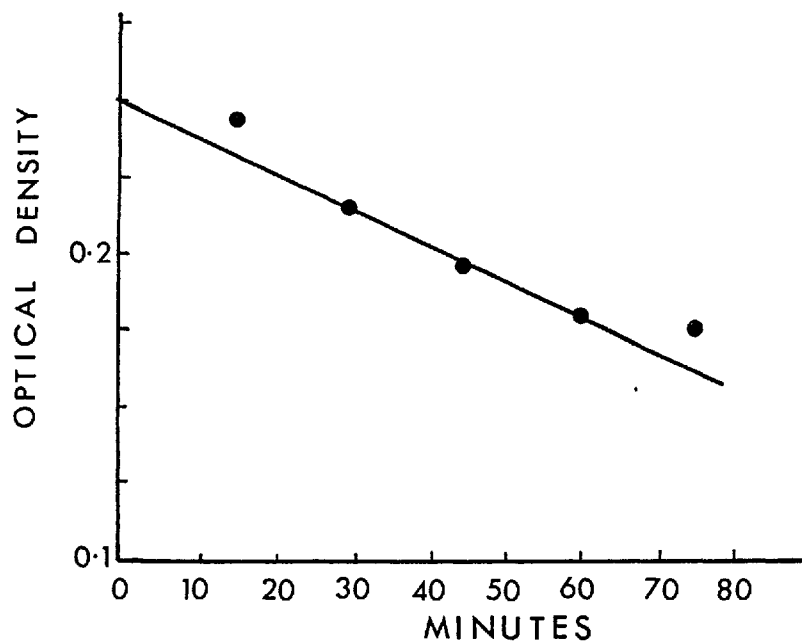
3.2.3 Substrate utilisation

It is of value to determine the rate at which the substrate is utilised during an enzymatic reaction so that the optimum conditions can be achieved. Experiments by both Colman et al.(1969c) and Wright and Mahoney (1974) have indicated that the hydrolysis of ester proceeds as a linear function of time for at least 30min and 60min, respectively.

Plasma was collected from four normal uninfected rabbits. 0.2ml of this plasma was incubated with an equal volume of kaolin at 27°C for 1min before assaying the sample as normal, but incubating the reaction mixture at 37°C for a total period of 10min. This process was repeated with four further 0.2ml samples of the same plasma being incubated with the reaction mixture for periods of 30, 45, 60 and 75min. The amount of hydrolysis in each sample was determined as before.

The results are represented in Fig. 3.4. It can be seen that the substrate is utilised in a linear manner for a period of 60min when the optical density is plotted against time. However the graph shows non-linearity during the first 10min of the reaction, indicating some delay in the reaction process, also after 60min, when the utilisation

Fig. 3.4 TAME utilisation as a function of time



of the substrate was proceeding at a slower rate. The fact that the hydrolysis of the ester is a linear function of time indicates that the presence of the substrate protected against the continuing action of inhibitor. Ideally the reaction should proceed until half the available substrate has been used, but this could not be achieved with the amount of plasma used in the assay (see section 3.2.4). It was decided to incubate the reaction mixture for a period of 60min at 37°C in all subsequent determinations of kallikrein activity, as this gave maximum possible inhibition under the conditions used.

3.2.4 Plasma volume in kallikrein assay

The volume of plasma used is obviously an important factor in the kallikrein assay, with the relationship between enzyme and substrate having to be at an optimum. Colman et al.(1969c) have indicated in their studies, on the quantity of human plasma used, that there was a linear relationship between plasma volume and enzymatic activity in a range from 0.025ml to 0.1ml. At 0.2ml and higher, this relationship no longer applied since the larger concentration of kallikrein inhibitor competed more effectively with the substrate. They subsequently used 0.1ml of plasma for routine assay.

0.1, 0.2, 0.3 and 0.5ml aliquots of the same plasma sample were assayed for kallikrein and total kallikrein activity, by un-activated and activated plasma, respectively. The results, shown in Table 3.1, indicate that the optimum value for both measurements was obtained with a plasma volume of 0.2ml. These values were obtained after correcting for the spontaneous hydrolysis of the substrate and representing them as μ moles of TAME hydrolysed/ml of plasma. A sample calculation of the TAME hydrolysis is described here to indicate how the values in Table 3.1, previous and subsequent studies were obtained:

From the optical density reading the μ moles of TAME present in the solution is found from the calibration graph (Fig. 3.1). This value is deducted from the total μ moles TAME added in the assay, giving the value of μ moles of TAME hydrolysed. The value for the spontaneous hydrolysis is then deducted for each sample, and the final result adjusted to give μ moles TAME hydrolysed/ml plasma/h.

Table 3.1 Variation of TAME hydrolysis with plasma volume

	O.D.	TAME hydrolysed/h (μ moles)	Corrected for spontaneous hydrolysis	Volume of plasma (ml)	TAME hydrolysed/ ml plasma/h (μ moles)
ACTIVATED PLASMA	0.21	8	4	0.1	40
	0.17	16	12	0.2	60
	0.155	19	15	0.3	50
	0.135	23	19	0.5	38
UNACTIVATED PLASMA	0.22	6	2	0.1	20
	0.20	10	6	0.2	30
	0.19	12	8	0.3	27
	0.165	17	13	0.5	26

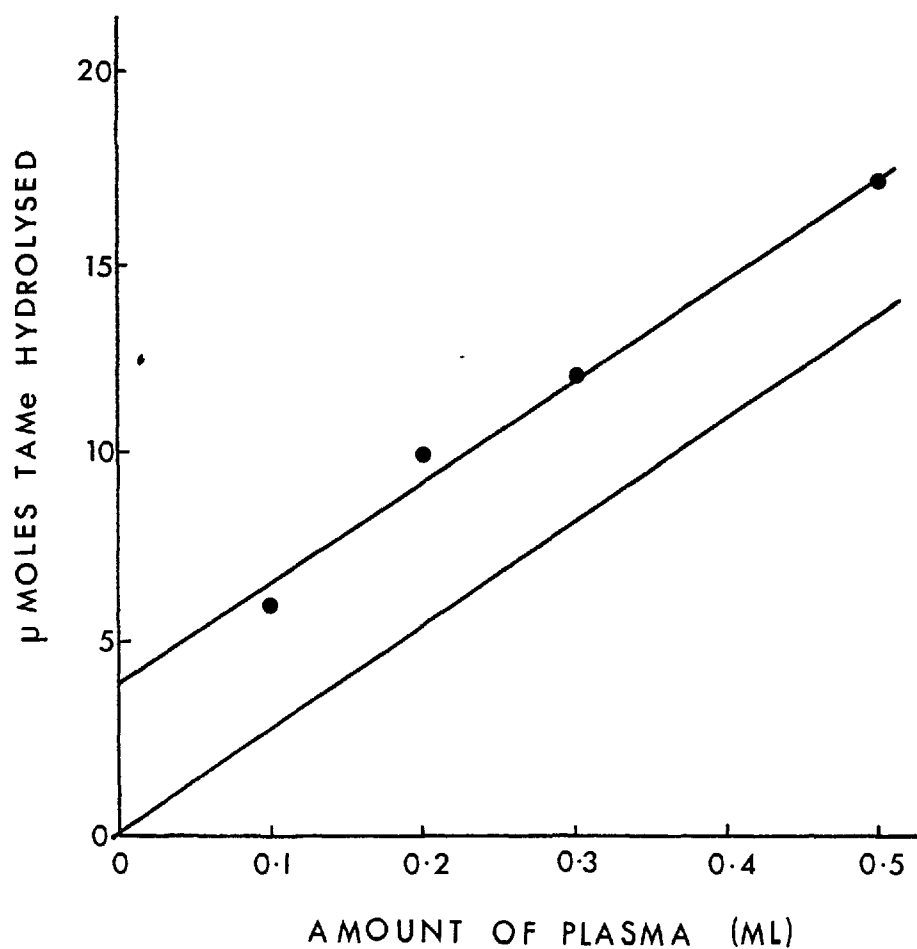


Fig. 3.5 Correction for spontaneous hydrolysis of TAME

The slightly higher (spontaneous) kallikrein levels obtained in this section, using unactivated plasma, are possibly caused by some activation of the kinin system and a consequent effect of inhibitor when collecting such a large amount of plasma for use in these determinations (see section 3.2.6).

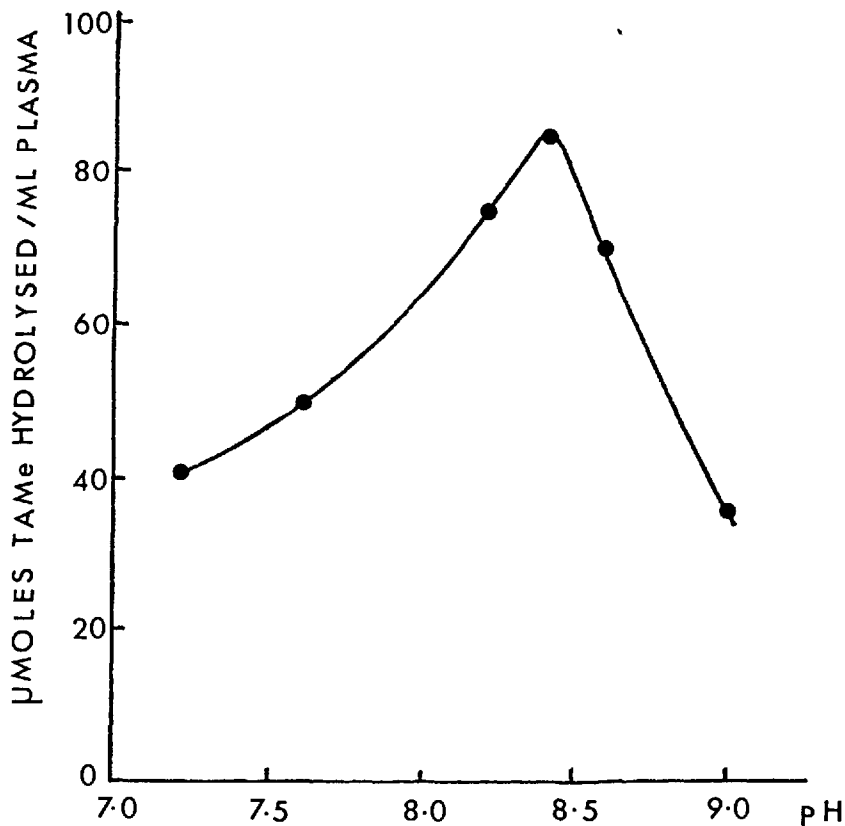
Fig. 3.5 shows the correction for the spontaneous hydrolysis of the substrate (TAMe). With no plasma present, and under optimal conditions, it would be expected that there would be little or no hydrolysis of the substrate. Where the graph intercepts the y-axis gives the experimental value of a case where there is no plasma present and the results obtained are therefore corrected for this apparent hydrolysis. 0.2ml of plasma was used in subsequent kallikrein assays, as this gave satisfactory values without any apparent competition of natural kallikrein inhibitor. It was also a convenient volume to use and measure accurately, from the small volume of rabbit plasma collected at any one time (section 3.2.6).

3.2.5 pH profile

0.2ml plasma samples were activated and assayed as normal, the pH of the Tris/HCl buffer being varied within the range 7.2-9.0. Fig 3.6, a mean of four determinations, indicates that when the μ moles TAMe hydrolysed/ml plasma/h are plotted against the pH, the pH optimum occurs at 8.4. This result is in agreement with Webster and Pierce (1961), who demonstrated an optimum pH of 8.5 for human plasma kallikrein and suggested that TAMe itself undergoes a small digestion at higher

pHs. Wright and Mahoney (1974) used Tris/HCl at pH 8.4 in assaying cattle plasma.

Fig 3.6 Effect of pH on degradation of TAME by rabbit plasma kallikrein



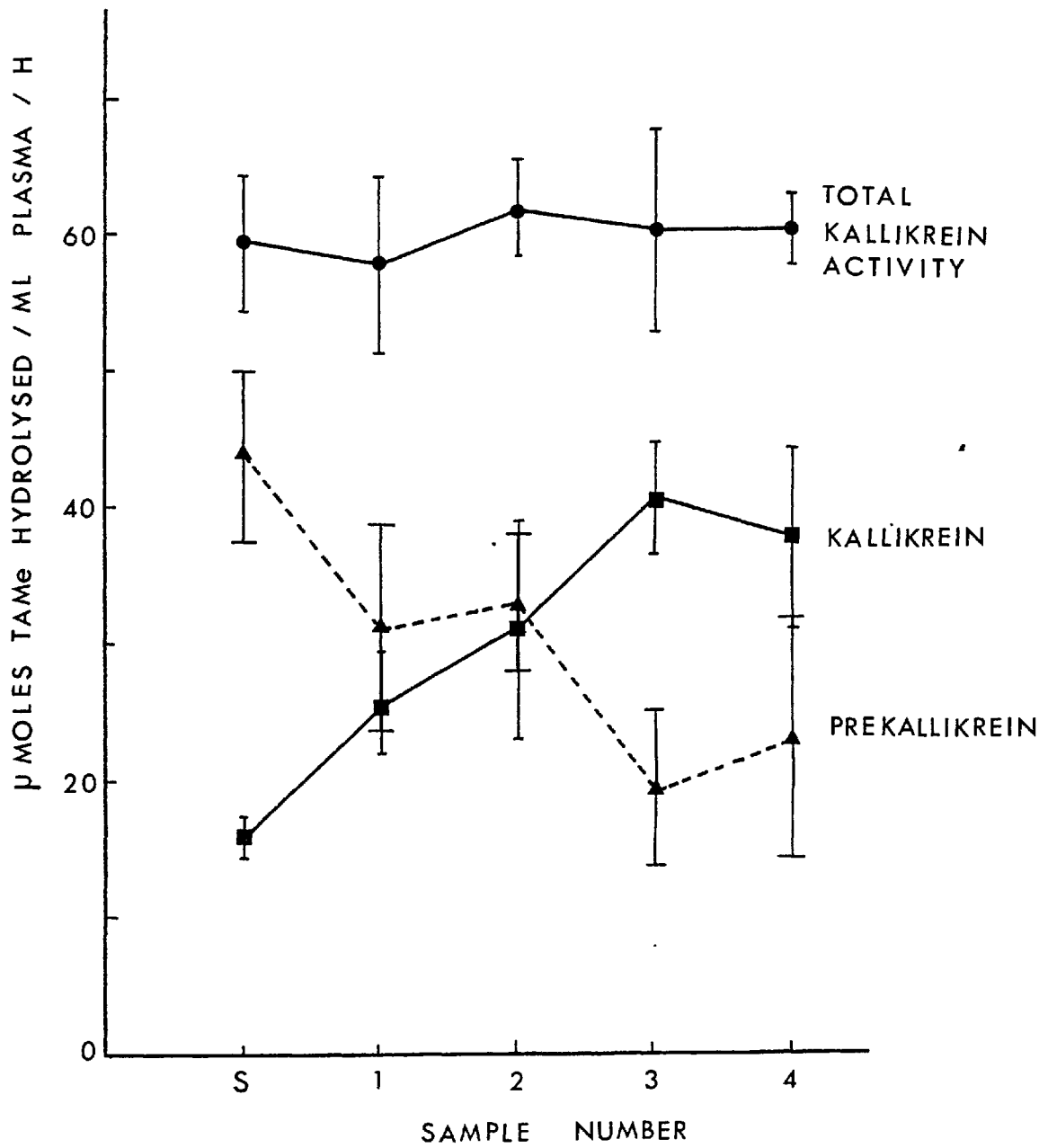
3.2.6 Kallikrein activation with plasma collection

Part of the introduction dealt with the complicated interactions of a number of blood enzyme systems (represented diagrammatically in Fig. 1.3). It can be seen that if there is initiation of the coagulation system, then there will also be activation of the kinin system, leading to changes in levels of plasma prekallikrein and kallikrein. This assumption was of major significance when considering the collection of blood plasma samples as these

were obtained by cutting the marginal ear vein of the rabbits.

Five 1.0ml blood samples were collected consecutively. The first was taken by syringe containing the same amount of EDTA as the tubes which were used for the last four samples. Having transferred the blood from the syringe to a plastic tube, all the samples were centrifuged for 10min at 1500 xg and the plasma assayed for kallikrein activity. Blood samples were collected from three normal rabbits. Fig. 3.7 shows that as the volume of blood collected increased so the level of prekallikrein fell corresponding to a rise in plasma kallikrein indicating activation of the kinin system. The increases in plasma kallikrein were most marked after collection of approximately 3ml of blood but there were changes, although relatively small, after the syringe sample. This change would therefore distort any increase or decrease in plasma kallikrein levels, particularly if these were small themselves, and it was felt that blood samples should be collected by syringe. This however did prove difficult as the disease progressed due to the pathological changes that occurred, with fragility and breakdown of the vessels being most evident. As indicated earlier (section 3.2.4), a small volume of plasma proved adequate for the assay of kallikrein activity and so the relatively small amount of blood collected by syringe from the marginal ear vein was sufficient for the determinations to be made.

Fig. 3.7 Effect of blood sample collection on kallikrein levels



S = syringe sample

3.3 Plasma kallikrein and prekallikrein levels in infected rabbits

Six rabbits, weighing approximately 3kg, were infected subcutaneously with 1.0ml T.brucei 427 at a concentration of 6.8×10^8 organisms/ml. Blood samples were collected three times per week throughout the infection. The plasma obtained from these samples was assayed for kallikrein activity as described (section 2.6.1). The complete data can be found in Table A (Appendix 2). The results for three rabbits are represented graphically in Fig. 3.8.

It is apparent that elevated plasma kallikrein levels occur between days 9 and 14 after infection. In all cases the peak levels show an increase of approximately 100% in this component. Accompanying this increase in kallikrein was a decrease in prekallikrein, which would be expected if activation of the system was occurring in the normal manner, with conversion of the precursor (prekallikrein) to kallikrein, mediated by HF. After the initial rise seen in kallikrein the levels fell, remaining above preinfection values, with smaller peaks, particularly between days 18 and 21, evident as the infection progressed. The data have been presented for individual rabbits as variations occur in the timing of both the maximum and subsequent alterations in levels, and pooling the results would tend to reduce or mask these changes.

Elevated levels seen during the later stages of the infection could be due to activation occurring during the sample collection,

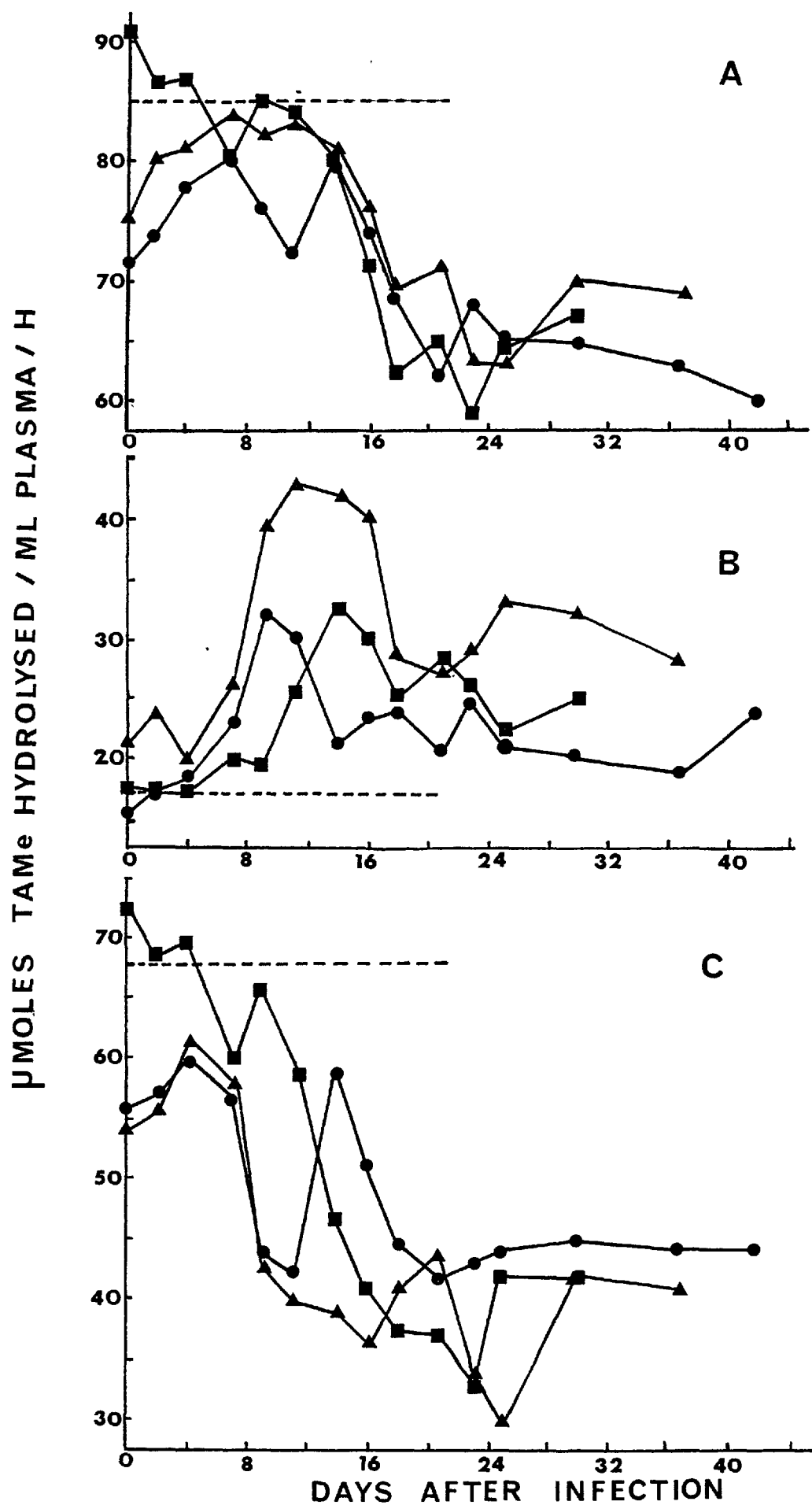
Fig. 3.8 Plasma kallikrein levels in *T.brucei* infected rabbits

Fig. 3.8 Plasma kallikrein levels in T.brucei infected rabbits

(A) Total kallikrein

(B) Kallikrein

(C) Prekallikrein

●—● 550

▲—▲ 553

■—■ 554

----- control levels (mean of 3 uninfected rabbits)

as the vessels were fragile and severe necrosis and oedema of the ears was present, which has been demonstrated in rabbits 3 - 4 weeks after infection with T.brucei (Goodwin, 1970). As the sample collection proved difficult, due to these pathological changes, the results obtained late in the infection cannot be expected to give an accurate assessment of the actual levels in the blood.

Parasitaemia was also monitored during the infection (see Table A, Appendix 2). Parasites were present in the blood of all the rabbits 1-2 days before the peak in kallikrein. In four instances, a small number of parasites were seen on day 2 or 4 and it has been shown that these parasites are similar to those injected into the animals at the day of infection (Boreham, 1968). There was no significant change in the kallikrein level at this time. The parasites observed between days 7 and 11 of infection are the first variant population (Gray, 1965) and it is possible that the presence of these parasites may in some way, either directly or indirectly, be mediating the changes which will lead to alteration in plasma kallikrein levels.

It was decided in the following studies to concentrate on the first 2-3 weeks of the infection, with T.brucei, as the changes in kallikrein during this period were considered important because of their possible implication in the initiation of pathological changes.

3.4 Characterisation of kallikrein

The measurement of spontaneous arginine esterase activity of plasma is a very useful method for detecting increased amounts of circulating kallikrein, but it is known that as well as plasma kallikrein, some other proteolytic enzymes such as plasmin and HF also hydrolyse synthetic arginine methyl esters (Speer et al., 1965). It might therefore be assumed that all three of these components could be involved in the hydrolysis of TAME, leading to artificially increased kallikrein levels during trypanosome infections. However, plasmin and HF hydrolyse synthetic lysine methylester (LMe), but plasma kallikrein does not (Webster and Pierce, 1961). Samples of control and infected plasma were therefore assayed for activity using LMe as the substrate to determine if the hydrolysis of TAME, seen in the previous section, could possibly be attributed to any other proteolytic enzymes in the plasma.

0.2ml samples of unactivated plasma from three rabbits, collected during the first three weeks of infection, were assayed using the same method as that employed for TAME, except that 0.04M LMe was substituted for TAME and 0.2M imidazole buffer, pH 6.5 for Tris/HCl (Troll et al., 1954). A calibration graph for LMe concentration was determined as for TAME and is shown in Fig. 3.9. The reaction period employed in this assay was 5min as Roberts (1958) has demonstrated that the time for complete reaction of LMe with alkaline hydroxylamine (HM) is less than 2min.

Fig. 3.9 Calibration graph for LMe concentration

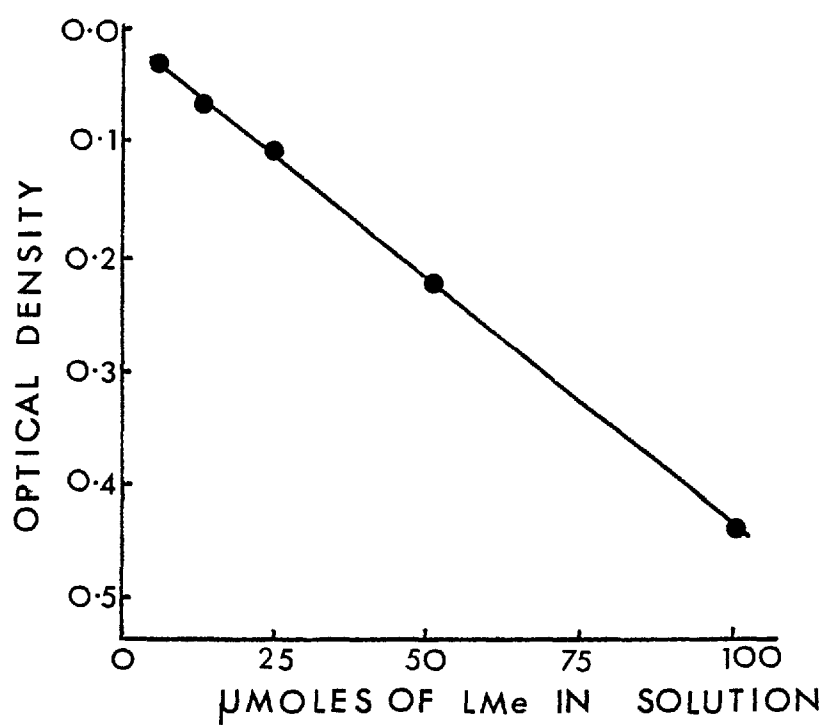


Table 3.2 Hydrolysis of LMe during infection

Rabbit No.	<u>μmoles LMe hydrolysed/ml plasma/h</u>		
	568	569	570
Day 0	3.0	2.1	3.5
7	4.5	2.0	3.0
9	6.0	3.0	4.0
11	4.0	5.0	5.0
14	8.0	5.0	7.5
18	9.5	6.5	6.5
21	8.0	8.5	7.0

The results (Table 3.2) show small fluctuations in amount of hydrolysis of LMe during the first 12 days of the infection. After this time, however, a slight increase is evident. These results would suggest therefore that the major esterolytic component in the plasma was kallikrein and that the increase in hydrolysis of TAME, reaching a maximum between days 9 and 14 of the infection, is due to the elevated levels of kallikrein. The increased hydrolysis of LMe however, after the peak kallikrein values, is interesting as this could be due to an elevation in amounts of plasmin. It may be implied that because HF_a acts upon plasminogen proactivator, which is the same as plasma prekallikrein (Vennerod and Laake, 1976), kallikrein could directly activate plasminogen to release plasmin.

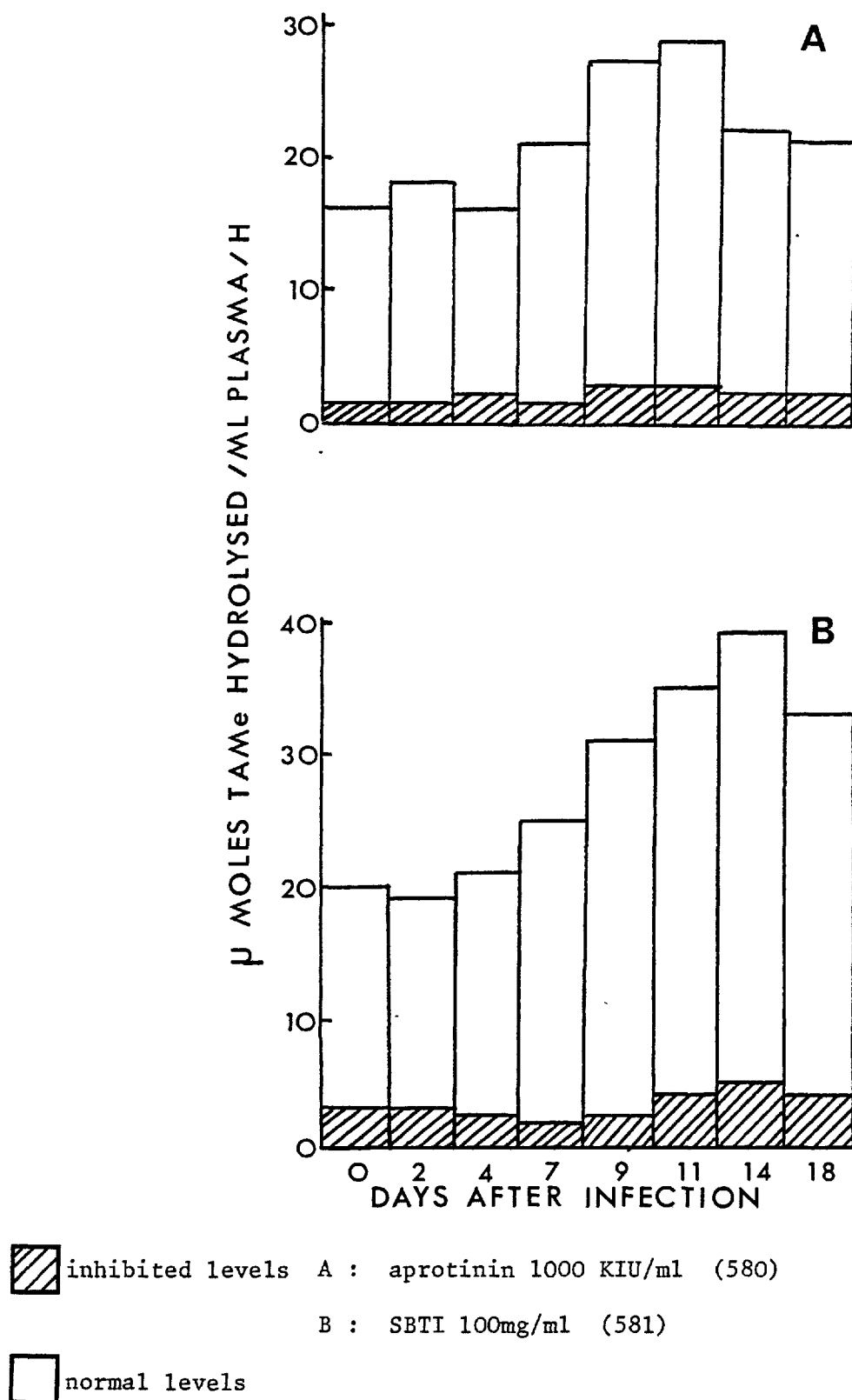
3.5 Inhibition of plasma kallikrein

3.5.1 In vitro inhibition

As outlined in the introduction (1.3.2), a number of inhibitors exist that prevent kallikrein activity. Werle and Maier (1952) have shown that SBTI inhibits plasma but not glandular or urinary kallikreins, whereas aprotinin affects the hypotensive esterase and kinin forming activities of most kallikreins (Vogel and Werle, 1970). The use of inhibitors is an important method of distinguishing the relevant component under study, in this case plasma kallikrein, and for identifying any possible actions which it may possess.

Levels of kallikrein were determined in plasma samples collected regularly during the first three weeks of infection, from four rabbits, after incubation at 37°C with either SBTI (100mg/ml) or aprotinin (1000 kallikrein inhibitor units (KIU)/ml). Fig. 3.10 shows that both SBTI and aprotinin caused a marked inhibition of plasma kallikrein activity, between 80 and 95%, as determined by the esterolytic assay. The complete data appears in Table B, Appendix 2. This shows inhibition of both kallikrein alone and total kallikrein activity, after activation of the plasma samples. The active site of kallikrein is blocked by both inhibitors. A common feature of the inhibition by non-plasma-derived inhibitors is the hydrolysis of a peptide bond (cleavage of a single lysine or arginine residue) located within the disulphide loop of the inhibitor molecule, and this may be essential for the enzyme complex formation and the inhibition (Fritz et al., 1979).

Fig. 3.10 Inhibition of plasma kallikrein



It is known that both aprotinin and SBTI inhibit activated kallikrein but do not affect the conversion of prekallikrein to kallikrein (Ambrus et al., 1968). The experiment, as performed, therefore indicates that there is sufficient inhibitor present to be able to complex with both the kallikrein already present in the unactivated plasma samples, and with the total amount produced after the conversion of prekallikrein following kaolin activation. As the TAME assay only measures the amount of kallikrein present, the inhibited values of μ moles TAME hydrolysed from both the activated and unactivated plasma samples proved to be very similar, for the reason given above.

3.5.2 In vivo inhibition

The previous section demonstrated that kallikrein could be inhibited in vitro, and this inhibition would obviously be of advantage in an in vivo situation. It was decided to use aprotinin for this in vivo work as it is a broad spectrum protease inhibitor and has attained by far the most interest of biochemists, pharmacologists and physicians. It was discovered 50 years ago by Frey, Kraut and Werle as 'kallikrein inhibitor' in bovine lymph nodes, and independently some years later by Kunitz and Northrop as trypsin inhibitor in bovine pancreas, the two now being known to be identical (see Vogel and Werle, 1970). Wright and Kerr (1975) have demonstrated the ability of aprotinin administered intravenously to significantly lower the levels of activated plasma kallikrein during infections of B. argentina in splenectomised calves. Therefore aprotinin was used in order to study the inhibition

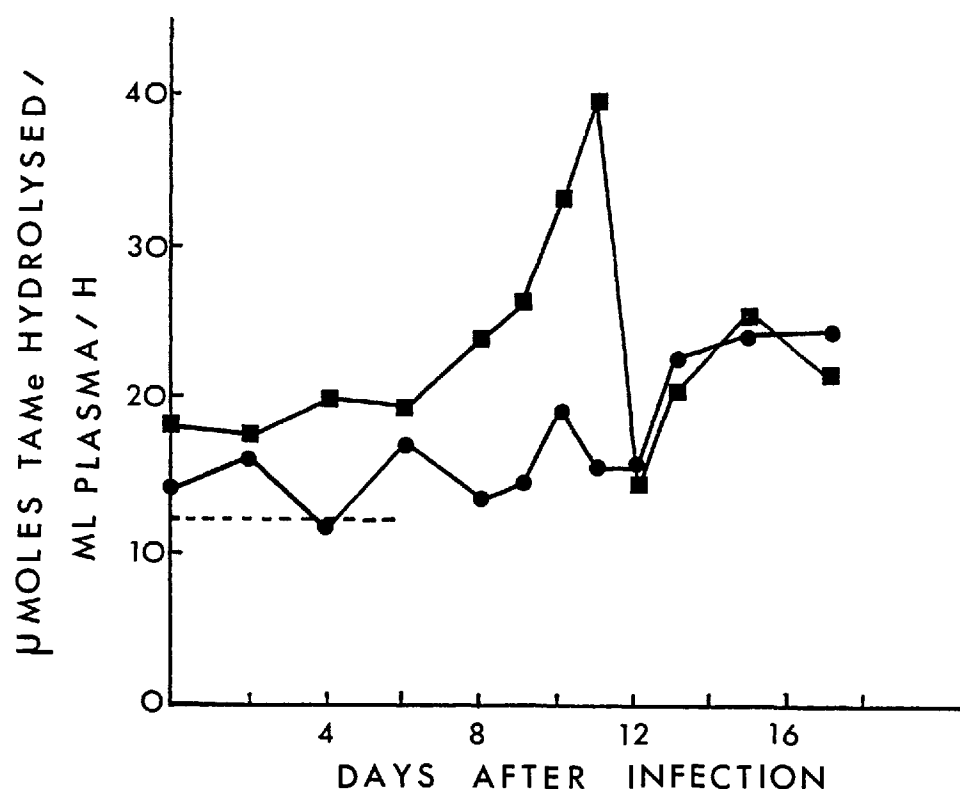
of kallikrein in rabbits at different stages in the infection. Since previous sections (3.3 and 3.5.1) have shown, respectively, the levels of prekallikrein during infection, and the inhibition of kallikrein and total kallikrein, it was considered appropriate to only measure the amount of kallikrein present in unactivated plasma samples in this and following sections.

Six rabbits infected with T.brucei were divided into two groups of three. Aprotinin, 5000 KIU/kg/day, was administered to the first group by intravenous injection, into the marginal ear vein, between days 0 and 12 ('early-treated group'). The second group received the same amount of aprotinin for four days when the level of kallikrein, as determined by esterolytic assay, reached maximum levels ('late-treated group'). Two additional rabbits were used as controls. One infected animal was given an equivalent volume of sterile saline on days 0-12, inclusive, and another, uninfected, was injected with aprotinin (5000 KIU/kg/day) for six days.

The results indicate that in the early-treated group, the rise to peak levels of plasma kallikrein was inhibited. In the late-treated group an immediate fall in levels was produced. Fig. 3.11 represents the data for one animal from each experimental group, and the complete data are given in Table C, Appendix 2. Although complete inhibition of kallikrein was not achieved, significantly lower levels were obtained in both early and late-treated groups, as compared to the untreated infected animal. Small fluctuations were evident in the early-treated

group during the first 12 days of infection and this may possibly be related to low circulating concentrations of the inhibitor. The rise in levels of kallikrein, associated with the removal of aprotinin therapy, is a return to levels which have been demonstrated, at a comparable time, in untreated infected animals, and indicates a fairly short circulating half life of aprotinin (Beller et al., 1966). A similar situation can be seen in the late-treated group, where elevated levels occurred as soon as the inhibitory treatment was stopped.

Fig. 3.11 In vivo inhibition of plasma kallikrein



Aprotinin administered: ●—● days 0-12 (601); ■—■ days 11-14 (604)
 ---- uninfected control (607)

These results would therefore suggest that aprotinin, administered intravenously, is an effective inhibitor of plasma kallikrein in vivo.

3.6 Activation of plasma kallikrein

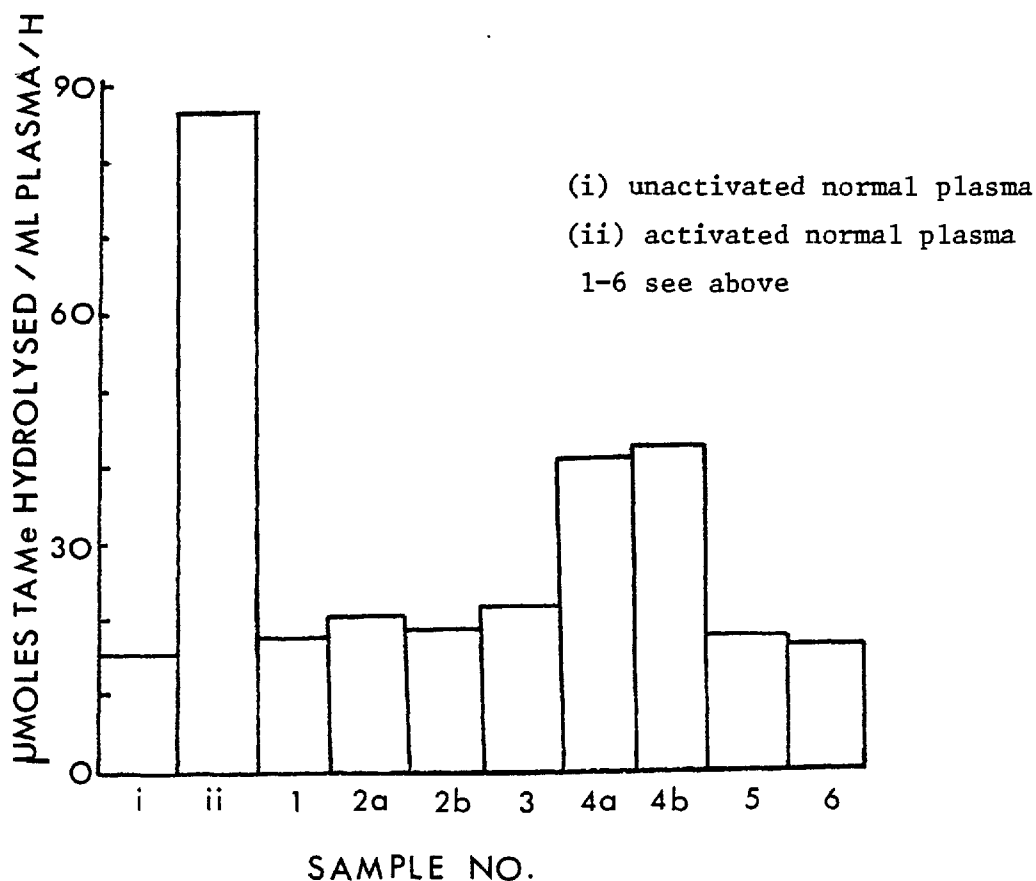
3.6.1 In vitro activation

It has been shown that, following the introduction of T.brucei into rabbits, as the infection progresses a change in the level of plasma kallikrein is apparent, associated with a reduction in prekallikrein. The factors involved in the activation of the kallikrein system are therefore important when considering possible measures, other than direct inhibition, to reduce or eliminate these changes as they occur during this disease. The maximum level of plasma kallikrein was seen 1-2 days after the first variant peak in parasitaemia. The activation of kallikrein could then possibly be caused by direct action of the trypanosomes, their intracellular components following immune destruction or by immune complex formation which is assumed to occur at this time.

Wright (1975) has demonstrated an esterase from B.bovis which is capable of activating the kallikrein system directly, while Boreham and Goodwin (1970) have shown that immune complexes of trypanosomes and immune sera caused the release of kinins from fresh rabbit plasma. No kinin release was observed using trypanosomes alone. A number of factors were therefore considered in relation to plasma kallikrein activation. Small volumes of plasma, collected from an uninfected rabbit over a period of one week, in order to minimise the spontaneous kallikrein activity, were pooled. 0.3ml aliquots of this plasma were then incubated for 1h at 37°C, in plastic tubes, with an equal volume of test material, as listed in Table 3.3. All the trypanosome material

Table 3.3 Test materials for in vitro activation studies

<u>Sample no.</u>	<u>Test material</u>
1	Live trypanosomes in PSG
2	Disrupted trypanosomes
	(a) supernatant } (see Fig.2.1
	(b) sediment } method A)
3	Sonicated trypanosome supernatant. (see Fig.2.1 method B)
4	Immune complexes
	(a) whole trypanosomes (2.5.5a)
	(b) disrupted trypanosomes (2.5.5b)
5	PSG
6	None

Fig. 3.12 In vitro activation with test materials

used was prepared from a suspension of T.brucei at a concentration of 7.6×10^8 /ml. Following the incubation the samples were centrifuged for 5min at 2000xg where necessary, to deposit any particulate matter. 0.4ml of each sample was then assayed for kallikrein activity. In addition, the amount of kallikrein and total kallikrein activity were determined for 0.2ml samples of the pooled plasma, unactivated and kaolin activated, respectively.

The results represented in Fig. 3.12 show that a significant increase in the kallikrein level was only obtained after incubation of the plasma with IC (sample 4a and b). This would suggest that the IC of trypanosomes and immune sera are the only factors which are capable of causing conversion of prekallikrein to kallikrein and this is probably mediated via HF. This suggestion was made by Boreham and Goodwin (1970) when they did not obtain any kinin release after incubating IC with plasma previously heated to 65°C for 15min. A procedure which is known to destroy HF (Nossel, 1964).

To establish that the trypanosomes or their intracellular components were not able to activate kallikrein because they were inhibiting a step in the kinin pathway, both live and sonicated material (0.6ml samples) were incubated for 10min with 0.6ml of kallikrein (Depot Glumorin, 20 biological units). 1.0ml of each sample was then assayed for kallikrein activity, with appropriate adjustment of the buffer volume, after centrifuging as before. The experiment was performed in duplicate. No significant difference in the amount of kallikrein present, calculated as μ moles TAME hydrolysed,

was seen in these samples compared to that of the same volume of kallikrein incubated in the presence or absence of buffer. This would indicate that the trypanosomes did not contain any component which would inhibit kallikrein activity. However, Boreham (1968a) has shown that T.brucei 427 contains low levels of kininase but that the number of trypanosomes present during the infection is unlikely to significantly affect the amount of kinin found in the plasma.

3.6.2 In vivo activation

The previous section indicated that activation of the kallikrein system, as demonstrated by elevated levels of plasma kallikrein, was achieved with the addition of IC to normal plasma. Injection of antigen-antibody aggregates into guinea-pigs has been shown to activate the kinin system (Movat and DiLorenzo, 1968, Movat et al., 1968) and, more recently, Boreham and Wright (1976b) have demonstrated a dramatic change in the blood pressure of rabbits injected with trypanosome antibody complexes. They suggested that the hypotension produced was due to activation of the kallikrein system, since this effect was inhibited with the prior administration of aprotinin (5000 KIU/kg). It was therefore of interest to determine whether activation of kallikrein could be demonstrated in vivo, using immune complexes prepared in vitro and by challenging immunized animals with trypanosomes.

Six rabbits were immunized against T.brucei (see section 2.5.3), Serum collected from each of three rabbits 23 days after the initial inoculation proved to have a high antibody titre, as determined by the

two methods described (2.5.6 and 2.5.10), and so the rabbits were used at this time. Plasma samples were obtained from these rabbits and five uninfected control animals 24h before injection of any test materials. The injected materials, administered intravenously, and levels of plasma kallikrein are shown in Table 3.4.

Table 3.4 Plasma kallikrein levels after in vivo activation

Test material	Rabbit	Plasma kallikrein			
		pre-injection	15min	1h	24h
A	618	18	25	25	20
	619	16	34	23	19
(1) B	620	15	Died	-	-
	621	15	46	28	18
C+B	622	19	12	13	16
	623	18	13	13	20
D	624	16	40	24	20
C+D	625	20	12	14	18
(2) A	626	17	16	18	16
	B	627	18	20	18
	E	628	16	17	15

(1) immunized (2) uninfected controls

(A) 1.0ml live T.brucei (8.4×10^8)

(B) 1.0ml disrupted T.brucei (8.4×10^8) (Fig. 2.1a, fractions not separated by centrifugation)

(C) Aprotinin (5000 KIU/kg) 10min prior to test material

(D) 1.0ml immune complexes (disrupted trypanosomes)

(E) 1.0ml hyperimmune serum

It was intended to collect blood samples from the rabbits 3min, 1 and 24h after injection. However, 2-3min after the injection of live or disrupted trypanosomes into the immunized animals, the marginal ear

veins were found to be markedly constricted and sampling was not possible. These rabbits also seemed to have an increased rate of breathing and one animal died after approximately 10min. These symptoms were also evident in one control rabbit given preformed IC. The first blood sample was therefore taken after 15min.

As can be seen from Table 3.4, plasma kallikrein levels were elevated in all immunized rabbits injected with trypanosome material, with the exception of those previously given aprotinin (5000 KIU/kg). This would indicate that activation of the kallikrein system occurs through the formation of IC in these animals, as IC injected into uninfected control rabbits produced a similar response, again this being inhibited by aprotinin. No change in plasma kallikrein was seen when either live trypanosomes, disrupted trypanosomes or hyperimmune serum was injected into the rabbits. Plasma kallikrein levels were significantly reduced, in those rabbits where changes were noted, after 1h and this is possibly due to the action of natural inhibitors, as well as activation of the available prekallikrein and HF. The levels had fallen to near normal after 24h, the small amount of activity presumably being related to the residual presence of complexed material.

The fact that samples could not be collected from some animals shortly after injection of the test materials, due to constriction of the ear veins, was of interest. This is probably due to the induction of vasoconstriction by bradykinin, the product of the action of plasma kallikrein upon kininogen. Barabé et al. (1979) have shown, in isolated preparations, that bradykinin exerts vasoconstrictor actions in both rabbit ear and kidney, while having vasodilator actions on the heart and

mesentery. Vasodilation is thought to prevail, resulting in a hypotensive effect. This has been observed in vivo by Boreham and Wright (1976b) in T.brucei infected rabbits. The higher level of plasma kallikrein seen in one rabbit (621), and the death of another (620), after injection of disrupted trypanosomes, as compared to live trypanosomes, may be related to the amount of complex produced, as more antigenic material would be present in the former. It may also be due to the type of immunoglobulin involved in the complex formation.

It is evident from the previous sections concerning activation of plasma kallikrein that IC are intimately involved in the process. As increases in levels of plasma kallikrein occurred 1-2 days after the first variant peak of parasitaemia (section 3.3), corresponding to a second exposure of common antigens, it is possible that the IC formed at this time, between the common antigens and antibodies, may be responsible for the kallikrein activation. IgG antibodies evidently have a strong affinity for the common antigens of trypanosomes since Mattern et al. (1967a) found that IgG fractions of serum from 59 sleeping sickness patients reacted with a single strain of T.gambiense. Boreham (1968) has demonstrated the presence of common antibodies, by precipitin tests, in serum from two cows infected with T.brucei E.A.T.R.O. 2/1/4 at a similar time to increased kinin levels. The levels of circulating soluble IgG immune complexes were consequently determined in the serum of rabbits infected with T.brucei.

3.7 Soluble IgG immune complexes

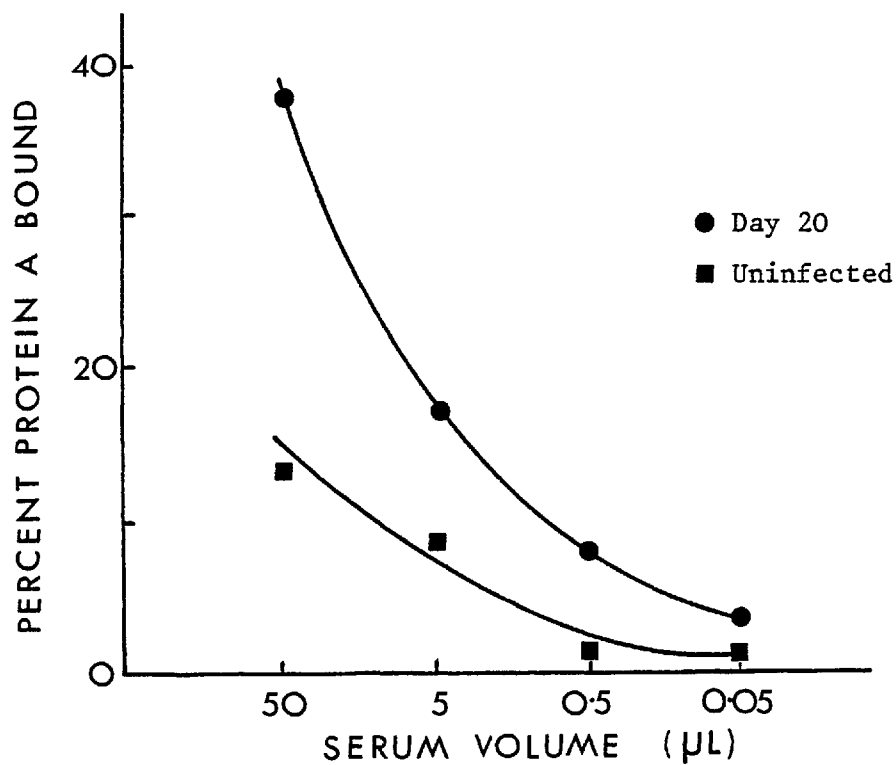
3.7.1 Levels during infection

The method used for measurement of these complexes was originally developed for the detection and quantitation of T antigen, a protein present in cells transformed or infected by simian virus 40 (Crawford and Lane, 1977). The protein A assay makes use of two observations, firstly the trapping of IC on glass fibre filters (Pollard and Waldron, 1966) and secondly the strong affinity of the protein A from Staphylococcus aureus for the F_c region of most classes of IgG (Kronvall et al., 1970). This affinity for IgG has been described using rabbit serum (Forsgren and Sjoquist, 1967). A preliminary study was undertaken to determine if a change in the level of IgG complexes was evident in infected serum using this method. In this initial experiment 50 μ l serum samples were used, and the filters were counted in a Mini-assay type 6-20 gamma counter. Fig. 3.13 illustrates the results obtained for both an uninfected and 20 day infected serum sample, diluted as shown. An increase in the amount of material bound to protein A can be seen at all dilutions and therefore indicates that a marked elevation of IgG complexes has occurred by this time in the infection. The amount of complexes present in a number of infected rabbit serum samples were examined at this time and all demonstrated similar increases to that shown in Fig. 3.13.

Having established that the method was capable of detecting changes in the amount of complexes present in the serum of infected

rabbits, measurements were made throughout the course of the infection. The method and counting equipment used are described in sections 2.7.1 and 2.7.2, respectively.

Fig. 3.13 Elevation of protein A binding in infected serum



In the experimental procedure the neat and second dilution serum samples were used, the latter being an indication that maximum protein A binding was not being reached, with the neat serum samples, as the amount of complexes increased during the infection. It was found that the second dilution was always a constant proportion of the neat serum, at any

stage of infection, and the results were therefore calculated from the number of counts obtained using the neat serum sample. Further preliminary work showed that the assay was extremely reproducible. The assay was repeated five times on a single infected serum sample, using individual 50 μ l volumes, and the results gave values of 9.9% $\pm$ 0.7% and 21.0% $\pm$ 1.2% for the second dilution and neat serum samples, respectively.

Serum samples were collected at regular intervals from three rabbits infected with T.brucei 427, and stored at 4 $^{\circ}$ C (section 2.5.1). The levels of circulating complexes found in these rabbits are shown in Fig. 3.14. The actual data and additional results are in Table D Appendix 2. It can be seen that the levels of complexes started to rise from day6 and reached maximum concentrations 16-22 days post infection. The peak values were approximately three times greater than the day0 samples. This would suggest that as the infection progresses so the amount of IgG produced increases and some of this immunoglobulin forms immune complexes, which can be detected by this method. Repeated sampling of an uninfected rabbit gave results of 8% $\pm$ 2% binding of the added 125-I and blank filters 1.5% $\pm$ 0.5%. The background of the counting equipment was 60-80 counts per minute.

The levels of complexes of all three rabbits followed a similar pattern and in each case the increase correlated well with the parasites seen in the circulation. As noted in these and previous experimental animals (Table A, Appendix 2) a small number of parasites can normally be detected 2-4 days after infection, and these will stimulate the

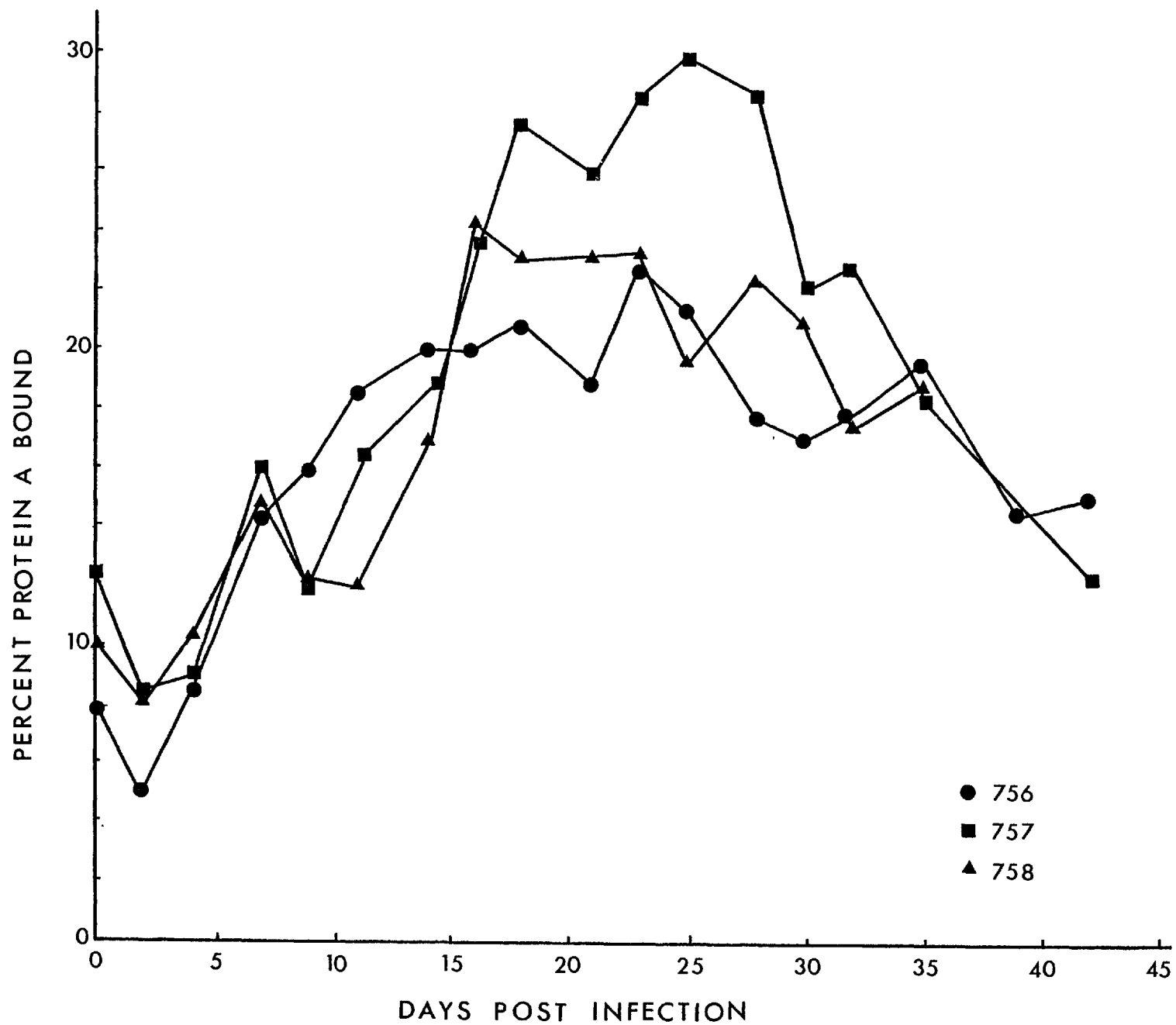


Fig. 3.14 Soluble IgG immune complexes in T. brucei infected rabbits

production of antibodies, mainly IgM but with some IgG (Gray and Luckins, 1976). After a further five or six days of the infection the first variant parasites can be detected and this will lead to an increased production of IgM and particularly IgG, this being the major immunoglobulin synthesised during a secondary response, with complex formation. The data shows that the major rise in complex levels is seen after this second exposure of the immune system to antigen in the form of the first variant parasite. The levels then remained high with slight fluctuations and a marked reduction was seen after 4-5 weeks of infection. Lambert and Galvao Castro (1977) have shown a rise in levels of IgG complexes in infected mice, measured by the 125-IClq binding assay, and correlated these increases with increased parasitaemia. The results found in rabbits appear to be similar to those described by the above workers.

The protein A, obtained already labelled with 125-I, from the Radiochemical Centre, Amersham, proved to be stable, giving reproducible results over a period of 3-4 weeks when stored at a temperature of 15-20°C. After this time comparable data could not be obtained. This is not unexpected as the shelf-life of iodinated protein preparations is generally found in practice to be much shorter than that dictated by the half-life of the radioiodine. Various factors are involved in limiting the useful life of labelled protein-tracers, such as radiation decomposition, loss of iodide, aggregation of labelled protein and general loss of immuno-reactivity of the labelled preparation (Bolton, 1977). The serum samples were stored at 4°C and used after as short a time as possible in order to prevent immunoprecipitation of the

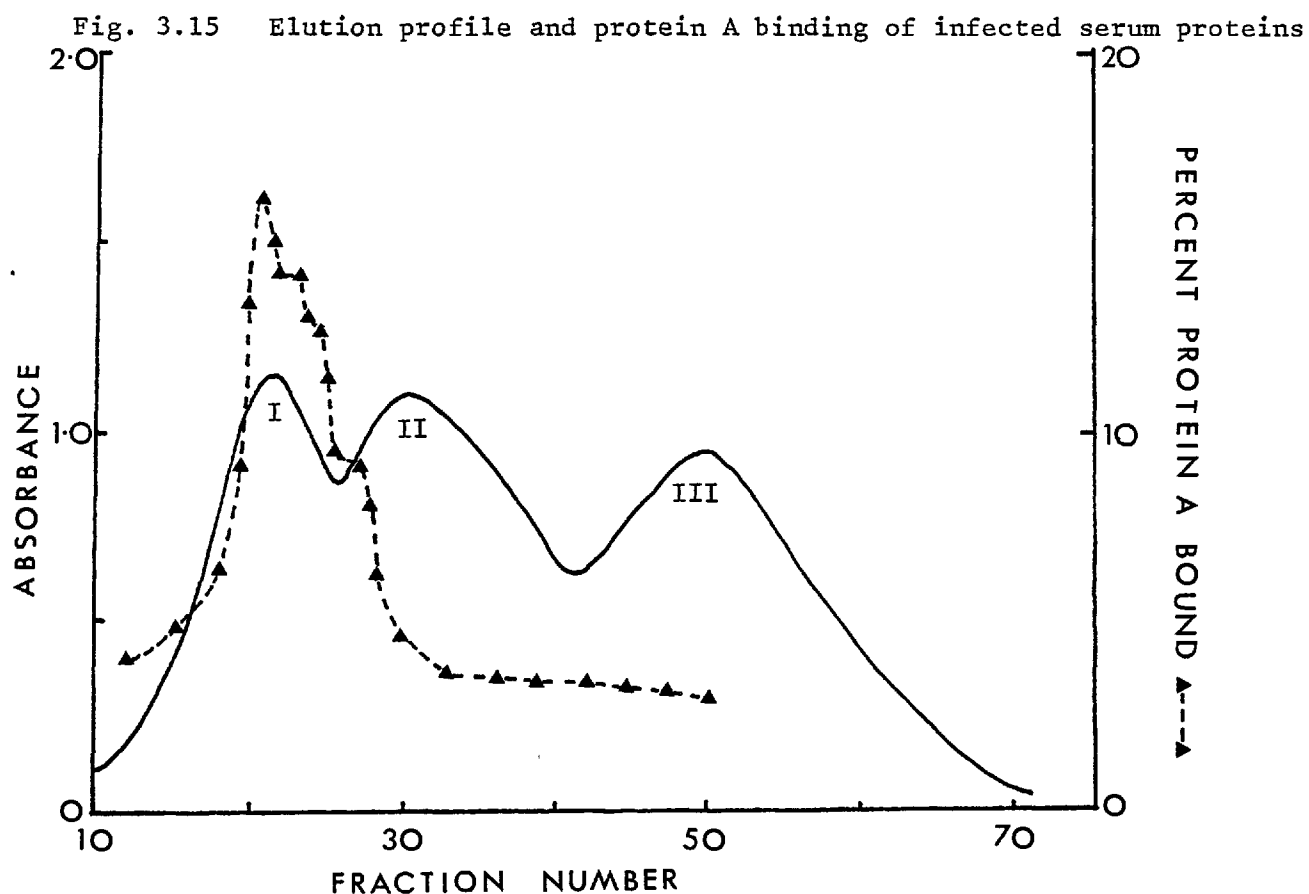
globulins which could block the filter and artificially elevate the results. It has been noted previously that if sera from patients with IC disease are kept at 4°C, many form a precipitate, usually containing IgM and IgG. The IgM is an antiglobulin which is thought to bind to IgG, itself perhaps acting as an antibody complexed to antigen (Roitt, 1977). It was decided however that rabbit serum collected during an experiment should be tested at one time, so that the iodinated protein was of a standard condition and activity.

In the experimental work, the results obtained for % binding of 125-I protein A were slightly lower than those of the preliminary study. This is probably related to a number of factors which would increase the accuracy and sensitivity of the method in the former case. Counting was performed for a longer period, the equipment used for counting was more sensitive, therefore increasing the counts of 125-I and reducing the background interference. The filters were counted twice at the end of the assay and the results averaged, this eliminated to some extent any counting errors.

3.7.2 Gel filtration of infected serum

In order to determine that the increase in complexes, seen in the previous section as determined by the radiochemical method, was not due purely to an increase in the amount of IgG synthesised during the infection, 2.0ml of 26day infected serum was separated into its protein components using G-200 Sephadex (section 2.5.4). The amount of protein A binding, for a number of 2.0ml samples of the separated eluates, was

then determined by passing them through the fibre glass filters and treating as for serum. Fig. 3.15 shows the distribution of binding activity for the separated fractions and indicates that the majority of the material capable of binding to the protein A is found in the macroglobulin fraction (I). A small but not statistically significant amount of binding was seen in the γ -globulin peak (II) and this would suggest that the monomeric IgG is not retained by the filter.



As it has been shown previously that protein A is specific for rabbit IgG (Forsgren and Sjoquist, 1967) the protein A binding evident in the macroglobulin fractions is probably due to IgG complexes of a similar mobility in G-200 Sephadex to IgM. Increasing amounts of IgG synthesised

during trypanosome infections do not therefore contribute to the increase in protein A binding, unless its molecular size is increased in some way. The most probable means being the formation of IC.

3.7.3 Identification of filter binding material

The identification of the components present on the filter is of value in discovering the antigens and antibodies involved in the immune complex formation, during trypanosome infections. From the results described so far, the assay will detect complexes containing immunoglobulin species reactive with protein A, and only complexes above a certain minimum size and configuration will be retained by the filters. The minimum size of complex retained has not been determined, but using GF/F filters it is thought to be less than 1micron (Crawford and Lane, 1977).

The separation of polypeptides by their molecular size can be estimated from the relative electrophoretic mobilities of their SDS complexes on polyacrylamide gels (Shapiro et al., 1967). Thus the size of the polypeptide chains of a given protein can be determined by comparing their electrophoretic mobilities with well-characterised polypeptide chain molecular weights. The method is rapid and versatile and requires very little sample.

0.3ml samples of normal and infected serum were passed through filters supported in syringes and the eluates retained. The filter bound material was then washed with 1.0ml of NET buffer and the material

present on the filter was eluted with 150 μ l of SDS sample buffer. The filter was carefully removed from the syringe and soaked in the SDS eluate for 1h. The samples were then centrifuged at 1000rpm for 2min. Following the preparation of all the samples, 100 μ l of SDS/sample (50 μ l for normal serum) were carefully applied to a prepared SDS polyacrylamide gel. The volumes of materials and samples used are shown in Table 3.5. The marker proteins used in these gels are shown in Table 3.6, with their relative mobilities calculated from four separate gels used in the course of these experiments. Relative mobility was calculated as : $\frac{\text{distance travelled by protein}}{\text{distance travelled by dye}}$.

Fig. 3.16 shows the relative mobility of each polypeptide plotted against the logarithm of its molecular weight, yielding a straight line over the molecular weight range which is characteristic of the gel composition. The inflection in the plot occurs when the molecular weight falls below a critical size (Dunker and Rueckert, 1969).

Fig. 3.17 shows the protein bands obtained after electrophoresis and staining. It would be expected that IgG heavy (H) and light (L) chains would be seen in the gel as some complexed IgG will be retained on the filter, and this has been demonstrated by the fact that protein A binds to the retained material. The H chain of rabbit IgG (γ) usually gives a doublet of bands at approximately 55,000, while the L chain gives a spread of bands from 20-30,000 M.W. on this gel (D. Lane, personal communication) and is confirmed in the present work. A similar rabbit H chain molecular weight was also described by Van Tol et al. (1978). Human IgM H chain (μ) has been shown to be approximately 73-74,000

Table 3.5 Samples applied to SDS polyacrylamide gel (i)

Gel slot	Sample	Volume of sample (μ l)	Volume of SDS (μ l)	Volume of sample/ SDS loaded onto gel (μ l)
1	Markers†	25	-	25
2	NS	1	99	50
3	NS F	60	40	100
4	NS E	1	99	100
5	(1) IS F	100	-	100
6	(2) IS F	100	-	100
7	(3) IS F	100	-	100
8	(3) IS E	1	99	100
9	(4) IS F	100	-	100
10	(4) IS E	1	99	100
11	Tsol	10	90	100
12	Twhole	5	95	100

NS=normal serum; IS=infected serum;

F=filter bound material; E=filtered material;

Tsol=soluble trypanosome fraction, } see Fig. 2.1, method A

Twhole=whole trypanosome fraction

(1) rabbit no. 834* day18

(2) 843 day15, untreated infected serum

(3) 835* } day13

(4) 836* }

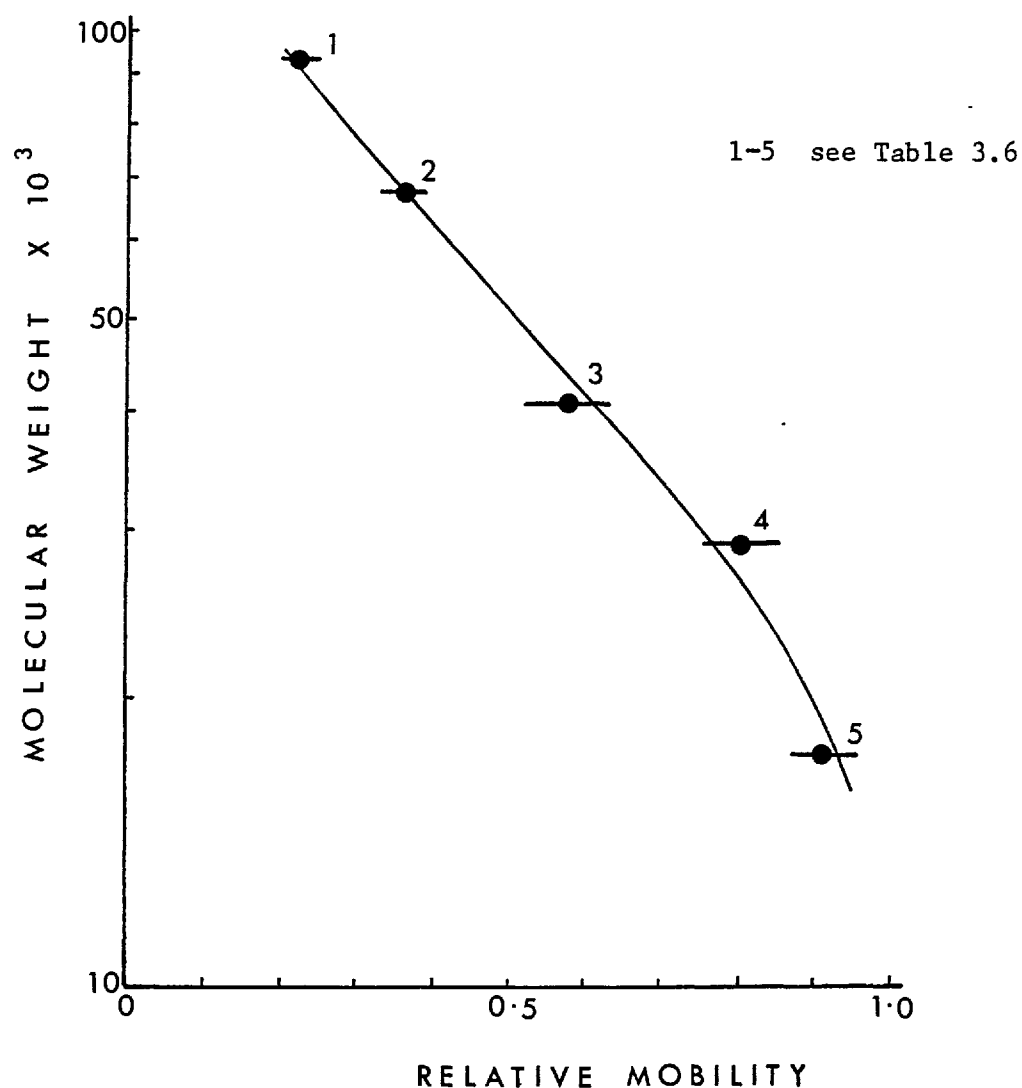
† see Table 3.6

* see section 3.8 for drug treatments

Table 3.6 Range of marker proteins used in SDS polyacrylamide gel electrophoresis

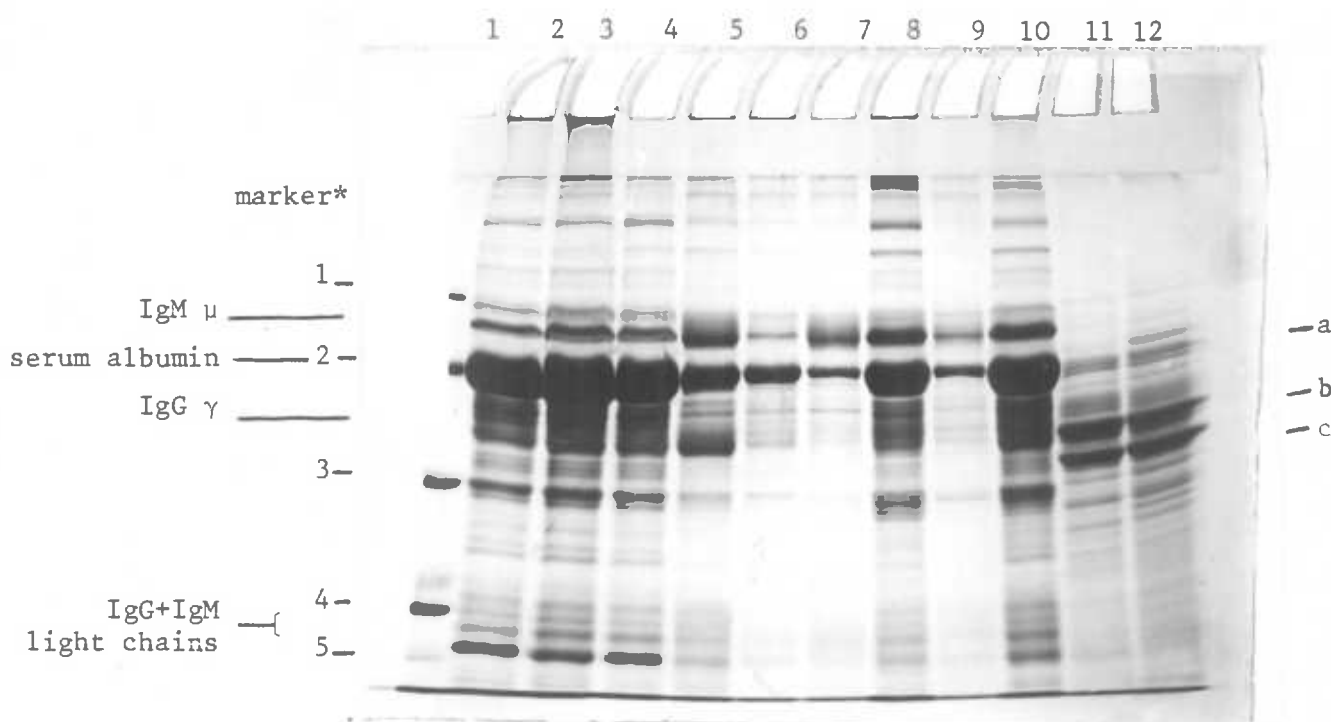
Markers	Molecular Weight	Electrophoretic mobilities
1 phospholipase	94,000	0.22 $\pm$ 0.02
2 BSA	68,000	0.36 $\pm$ 0.02
3 alcohol dehydrogenase	41,000	0.57 $\pm$ 0.04
4 carbonic anhydrase	29,000	0.81 $\pm$ 0.04
5 myoglobin	17,000	0.90 $\pm$ 0.05

Fig. 3.16 Marker protein mobilities in 10% SDS polyacrylamide gel



but in this gel system rabbit IgM H chain appears to have a molecular weight of 82-84,000, this value also being found by Van Tol et al. (loc. cit.). The largest band in the gel which has a molecular weight equivalent to the 68,000 M.W. marker is serum albumin, large amounts of this protein being present in normal serum. As can be seen from Table 3.5, different volumes of samples were used in the separation

Fig. 3.17 Serum protein analysis in SDS polyacrylamide gel electrophoresis(i)



major protein bands of trypanosomes (a) 68,000
 (b) 58,000
 (c) 50,000

*for markers see Table 3.6

and therefore this must be taken into account when making comparisons between the protein bands from different samples. Infected serum was also taken from four rabbits, three of which had been drug-treated as described in section 3.8.

A large number of bands are present in all three normal serum gel slots (2-4) and these include both IgG and IgM heavy and light chains, as well as a band at a similar position to the 41,000 M.W.

marker. It is apparent that a reasonable proportion of the serum albumin, IgM and IgG have passed through the filter, the presence of similar components in both filter bound and eluate material possibly being related to the volume of serum separated by the filter initially. This may have led to some clogging of the filter, particularly by serum albumin. Gel slots 5-7 and 9 can be compared directly, as they represent four infected rabbit filter bound samples, all with the same sample volume. Each one contains some albumin and IgM heavy chain. The amount of albumin is reduced compared to the uninfected serum and this would be expected, as it is known that the serum albumin levels fall during T.brucei infections (Boreham et al., 1977). The amount of heavy chain IgM appears to have increased, as represented by a more diffuse and darker staining band in slots 5,7 and 9, although this is not as evident in slot 6.

IgG heavy chain is increased in slot 5 (rabbit 834) as compared to the untreated infected serum (slot 6), and this correlates well with the measurement of serum IgG in this rabbit (see Table D, Appendix 2). In both dexamethasone treated rabbits (slots 7 and 9) the amount of IgG is reduced and again this is reflected in the serum concentrations. The two eluate fractions of the uninfected rabbit serum both show significant amounts of IgM, albumin, some IgG H and L chains, as well as the 41,000 M.W. component, which have therefore not been retained on the filter and do not contribute to the immune complex. The major trypanosome protein bands at 58 and 50,000 M.W. do not appear to be present in the filter bound or eluate material when compared to the uninfected serum. However a 64,000 M.W. protein is present in both the

filter bound infected serum and trypanosome material although a direct comparison with normal serum and eluate samples is made difficult with the large amounts of albumin present. The other band at 68,000 is a similar M.W. to albumin and this is one M.W. component that is present in the filter bound material.

The method used here, of initial serum filtration followed by elution of the filter bound material and separation by gel electrophoresis, has demonstrated that IgG is present on the filter, that a number of other proteins are also present and therefore possibly associated as a complex with IgG. However this method does not give a satisfactory identification of the IgG complex as some proteins could be present on the filter, and consequently in the gel, having bound to immunoglobulins other than IgG, or are retained due to their molecular size and configuration.

A more specific method is described in section 2.8. This entails a preliminary step of PEG precipitation, which is used to precipitate high molecular weight IC from serum in a manner described by Creighton et al. (1973). IC are precipitated by low concentrations of PEG (2.5-3.5%) while only very small amounts of monomer immunoglobulin come down in the pellet. The optical density readings of the uninfected and infected serum samples, after PEG precipitation and resuspension in PBS, were 0.032 and 2.402 OD units, respectively. For the extinction of IgG, $0.1 \text{ OD unit} \equiv 0.07 \text{ mg/ml}$ and so this represents 0.022 mg/ml IgG in the uninfected serum and 1.681 mg/ml in the infected serum, indicating an increase in complexed IgG. Following this step

S.aureus was added to the precipitate which rapidly binds to the IgG complexes, the non-specific uptake of other material being minimal (Kessler, 1975).

Figs. 3.18 and 3.19 show the protein bands obtained from a number of samples, as indicated in Tables 3.7 and 3.8, respectively, after the procedure described in section 2.8. The infected serum samples eluted from S.aureus in Fig. 3.18 (slots 4 and 11) show major bands at 55,000 and 83,000 M.W. and are therefore IgG and IgM H chains, respectively. Some additional bands are also present in the M.W. range 15-30,000 and are presumably the immunoglobulin L chains. Eluted uninfected serum has a little IgG H and L chain with some IgM evident in the corresponding supernatant (slot 8). However in the infected serum the protein components in the eluted fractions are also present in the supernatant fractions, with the exception of the slot 11 sample. This may indicate that the PEG has precipitated some monomer immunoglobulin or that there is insufficient protein A to bind all the IgG complexes. Fig 3.19 again illustrates the presence of both IgG H and L chains and IgM H chain in slot 5, while comparison of the eluted material in both Figs. 3.18 and 3.19 with the trypanosome fractions (Fig. 3.19, slots 11,12) does not indicate an involvement of any particular component in the IgG immune complex. This finding is not too unexpected as Freeman et al. (1970) have suggested, in rhesus monkeys, that the trypanosome specific component of IgG is only about 5%.

Table 3.7 Samples applied to SDS polyacrylamide gel (ii)

Gel slot	Sample	Volume of sample (μ l)	Volume of SDS (μ l)	Volume of sample/SDS loaded onto gel (μ l)
1	-	-	-	-
2	Markers†	25	-	25
3	NS	1	99	100
4	(1) IS EL	25	7.5	100
5	(1) IS S	50	50	100
6	-	-	-	-
7	NS EL	100	-	100
8	NS S	100	-	100
9	-	-	-	-
10	-	-	-	-
11	(2) IS EL	50	50	100
12	(2) IS S	100	-	100

EL=eluted from protein A; S=TCA precipitated supernatant;
(other abbreviations see Table 3.5)

(1) 843, (2)844, untreated infected rabbits

† see Table 3.6

Fig. 3.18 Serum protein analysis in SDS polyacrylamide gel electrophoresis (ii)

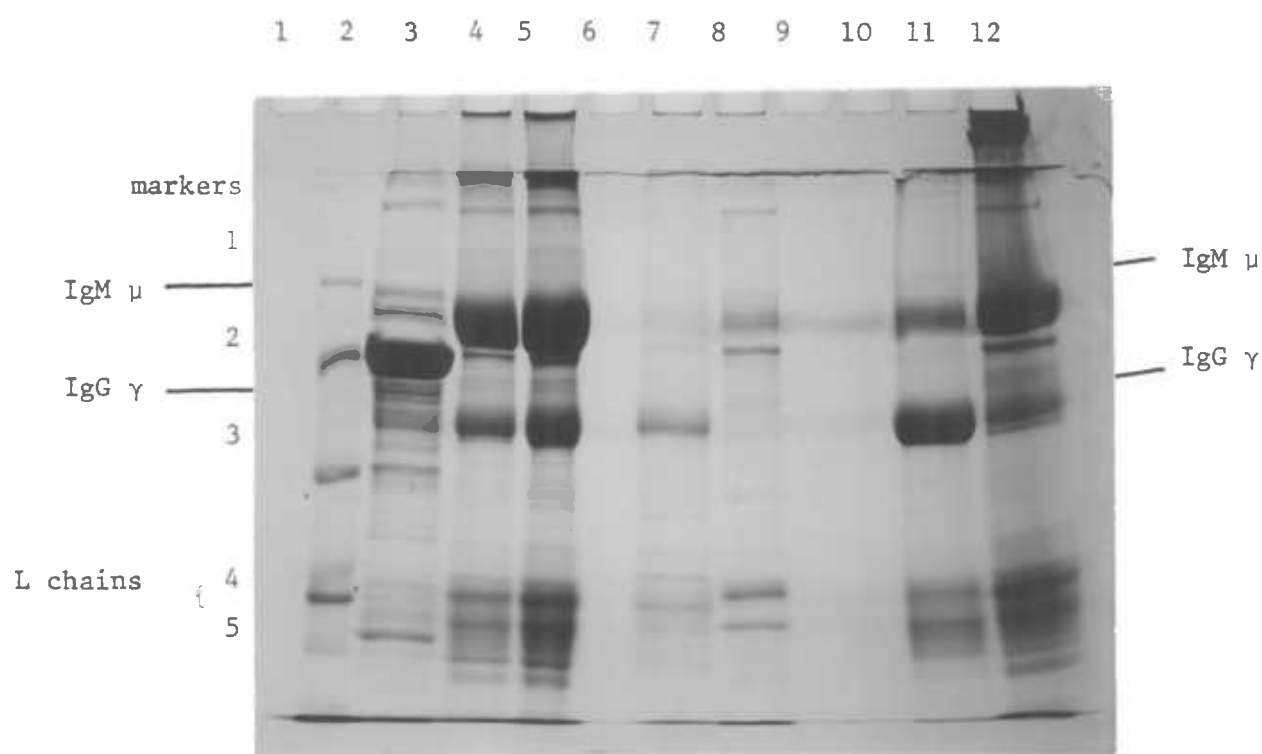


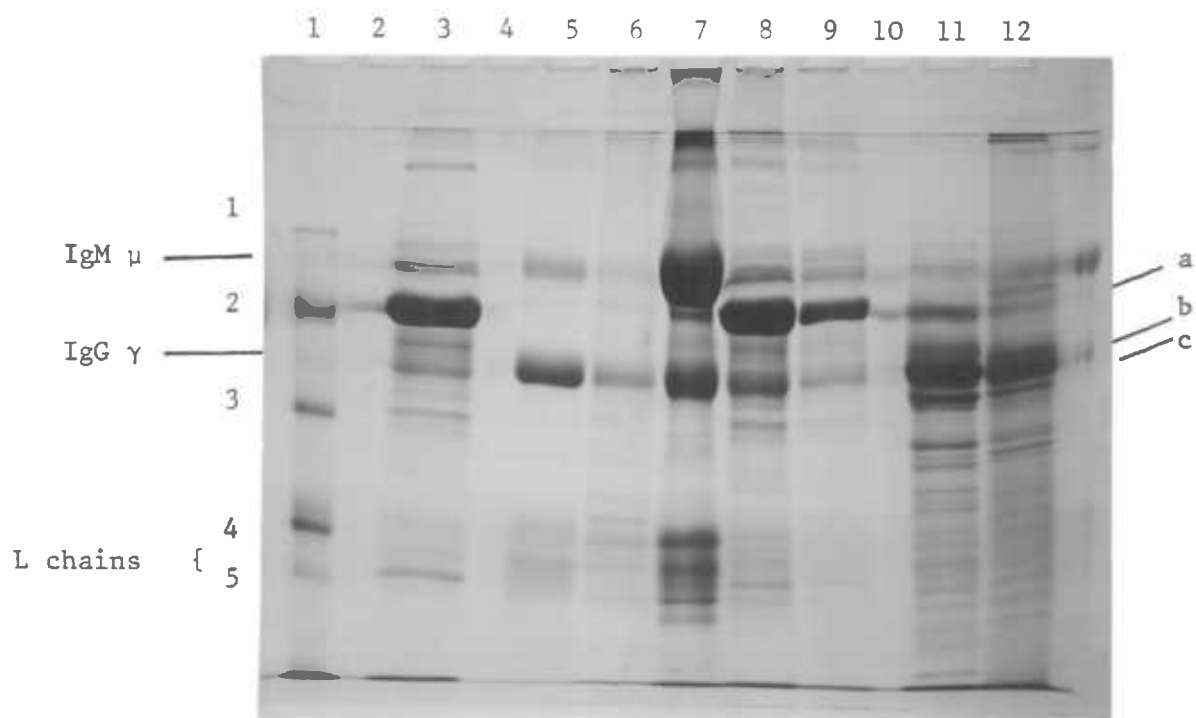
Table 3.8 Samples applied to SDS polyacrylamide gel (iii)

Gel slot	Sample	Volume of sample (μ l)	Volume of SDS (μ l)	Volume of sample/SDS loaded onto gel (μ l)
1	Markers†	25	-	25
2	-	-	-	-
3	NS	1	99	100
4	-	-	-	-
5	(2) IS EL	25	75	100
6	NS EL	100	-	100
7	(2) IS S	50	-	50
8	HS E	1	99	100
9	HS F	100	-	100
10	-	-	-	-
11	Tsol	10	90	100
12	Twhole	5	95	100

HS=hyperimmune serum (other abbreviations see Tables 3.5 & 3.7)

† see Table 3.6

Fig. 3.19 Serum protein analysis in SDS polyacrylamide gel electrophoresis (iii)



The results obtained following the use of different concentrations of PEG and increasing the number of S.aureus to 7.5×10^9 (100 μ l stock suspension) are shown in Fig. 3.20. The samples applied to the gel are given in Table 3.9. An increase in both IgG and IgM with a slight decrease in albumin is apparent in the infected serum when compared to the uninfected serum. No additional bands are evident although they may be obscured by the large amounts of immunoglobulins and proteins present in the sample. All samples eluted from S.aureus have similar M.W. bands consisting of IgG, IgM and a single band at 72,000, the amounts of these components decreasing as the PEG concentration is reduced. Whether the 72,000 M.W. protein constitutes part of the immune complex is uncertain as a similar but slightly lower M.W. (70,000) protein can be seen in the uninfected serum supernatant sample (slot 9), although C3 fractions have been identified in IC/aggregates of rheumatoid synovial fluid with M.W. of 75 and 72,000 (Male et al., 1980).

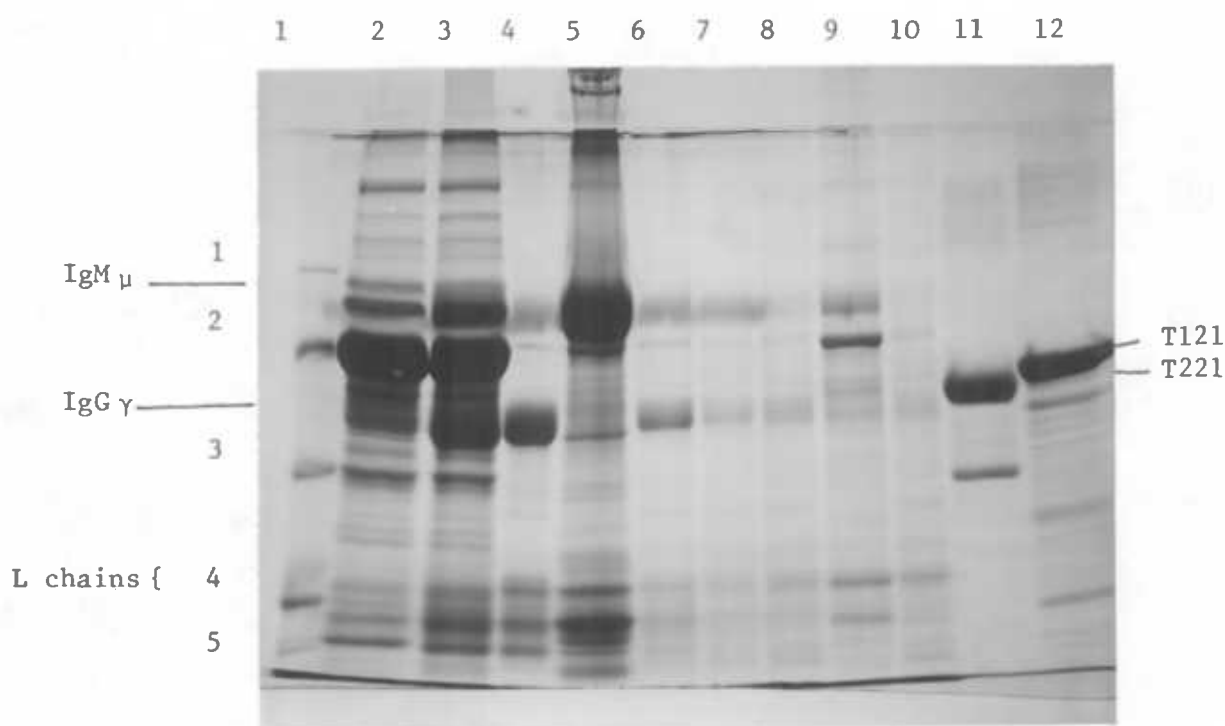
The use of PEG and S.aureus seems therefore to have selectively precipitated the immune complexes and has avoided contamination with other proteins. The complexes found in this study appear to consist of IgG, IgM and possibly some trypanosome components. Further work would be required for more positive identification of any trypanosome component with the use of a greater number of purified antigen preparations. IgM may be present in the separated material through two possible mechanisms. Firstly, it may bind to the protein A of S.aureus. This has been found to occur to a limited extent in man (Brunda et al., 1977), but has not been reported for rabbits. A way of confirming

Table 3.9 Samples applied to SDS polyacrylamide gel (iv)

Gel slot	Sample	Volume of sample (μ l)	Volume of SDS (μ l)	Volume of sample/SDS loaded onto gel (μ l)
1	Markers	25	-	25
2	NS	1	99	100
3	IS	1	99	100
4	IS(a)EL	50	-	50
5	IS(a)S	100	-	100
6	IS(b)EL	50	-	50
7	IS(c)EL	50	-	50
8	NS(a)EL	100	-	100
9	NS(a)S	100	-	100
10	NS(b)EL	100	-	100
11	T221	5	95	100
12	T121	5	95	100

IS=sample from rabbit 844; (a)=3.5%PEG;(b)=2.5%PEG;(c)=1.25%PEG;
T221 & T121 = purified *T.brucei* antigens 221 & 121, respectively
(kindly supplied by Dr M. Turner, Molteno Institute).
(other abbreviations see Tables 3.5 & 3.7)

Fig. 3.20 Serum protein analysis in SDS polyacrylamide gel electrophoresis (iv)



this would be to overlay a separated SDS gel with radiolabelled protein A, and after a suitable incubation period and washing, the radioactivity bound to the proteins could be detected by autoradiography. Secondly, the IgM may be rheumatoid factor-like with specificity for IgG, this type of antibody has been described in trypanosomiasis (Houba et al., 1969; Klein et al., 1970). The IgG seen in the gels has been demonstrated to be present in IC and not as monomer immunoglobulin (section 3.7.2) and preliminary results reported by Harkiss and Brown (1979) also suggest little interference in their IC assay from monomer IgG, even though it is thought to be precipitated by PEG to an extent proportional to its serum concentration (Creighton et al., 1973; Digeon et al., 1977).

3.8 Drug treatment of infected rabbits

Circulating IC have been described in this work, their formation occurring at a similar time to, or shortly after, peak numbers of variant parasites. IC have also been described in T.gambiense infections in humans, T.brucei in rats (Fruit et al., 1977) and T.brucei infections of mice (Lambert and Galvao Castro, 1977). The presence of these circulating complexes is a feature which must be considered in relation to the pathology of the disease, as the work presented in this thesis, and that of Boreham and Goodwin (1970) has indicated that the IC are certainly involved in kallikrein activation and kinin production.

One of the main considerations in this section of experimental work was whether the complexes produced might be acting in a direct manner on the kallikrein-kinin system, leading to the increases in the levels of free plasma kallikrein seen during the infection. The most obvious method to study was the inhibition of IC formation by the administration of a number of drugs, during the infection. This can be approached in two ways: the first would be to immunosuppress the animal, to prevent the production of specific antibodies that would lead to IC formation. Ideal immunosuppressive therapy should selectively suppress either the antibody or the cell-mediated response to a specific antigen, without altering responses to other agents. The second method would be to interfere in some way with the antibody-antigen interaction.

A number of anti-inflammatory and immunosuppressive drugs, as well as aprotinin, were administered to rabbits during infection with T.brucei. The drugs and time periods of administration are outlined, individually, below. Serum and plasma samples were collected at regular intervals throughout infection, as before.

3.8.1 Drug treatment

(1) Aprotinin

Aprotinin is a specific kallikrein inhibitor, its inhibitory action being effected via a reversible linkage to proteinases. The action of aprotinin on plasma kallikrein has already been demonstrated in previous experimental sections (3.5.1 and 3.5.2). Because of this defined inhibitory activity it was included in this section of work and the levels of IC noted during treatment. A total of five rabbits were infected with T.brucei. Two of these animals received 5000 kIU/kg/day aprotinin, intravenously, for six days, from day8 after infection, while the remaining three rabbits received the same dose of aprotinin from day5 to day13 after infection.

(2) Indomethacin

Indomethacin is analgesic, anti-inflammatory and antipyretic, and is used in the treatment of musculoskeletal disorders, such as osteoarthritis, rheumatoid arthritis and ankylosing spondylitis. In humans it is usually administered as capsules at a dosage of 25-50mg, 2-3 times daily with food. Up to 600mg daily has been given but with a higher incidence of side effects.

It has been suggested by Goodwin (1974) that anti-inflammatory drug treatment might be of use in controlling the symptoms of trypanosomiasis. Cook (1979) has shown that rabbits dosed orally with indomethacin (5mg/kg) have reduced levels of serum C-reactive protein (CxRP)

and show a retardation in the development of external inflammatory symptoms. Two infected rabbits were dosed daily, by mouth, with a suspension of 5mg/kg indomethacin, in a small volume of distilled water via a tube inserted into the oesophagus, from day8 to day18, inclusive, after infection. Two further rabbits were dosed in the same way between day4 and day18.

(3) Penicillamine (D-penicillamine hydrochloride, Beechams Pharmaceuticals)

D-penicillamine hydrochloride is an orally administered chelating agent which aids the body's elimination of certain toxic metal ions. The normal human dose is 0.9-1.5g daily, in divided doses. The administration of the drug may occasionally cause toxic effects which include headache, sore throat, proteinaemia and a reversible nephrotic syndrome. L-penicillamine is said to be more toxic than the D-isomer.

Suppression of specific antibody production by penicillamine has been demonstrated by a number of workers (Altman and Tobin, 1965; Hubner and Gengozian, 1965). The chemical use of penicillamine in macroglobulin-associated immune disorders was motivated by its in vitro ability to depolymerize and inactivate proteins through its sulphhydryl radical. That this could be effected in vivo was demonstrated by Bloch et al. (1960) in the serum of a patient with Waldenstrom's disease, and by Jaffe (1962) in the synovial fluid of rheumatoid arthritis patients. More recent work has confirmed the effectiveness of penicillamine in rheumatoid arthritis, leading to clinical improvement and a reduction in disease activity, with suggestions that in this disease there is a reduction in levels of circulating soluble

IC in which IgM rheumatoid factor is a component (Multicentre Trial Group, 1973; Bluestone and Goldberg, 1973; Mohammed et al., 1976). A reduction of IC and immunoglobulins, induced by D-penicillamine, has also recently been reported in primary biliary cirrhosis (Epstein et al., 1979).

Four infected rabbits were dosed daily with D-penicillamine hydrochloride (5mg/kg), the drug being administered orally in solid form, contained in gelatin capsules. Two of these rabbits were dosed from day9 to day24 after infection and the other two from day-4 to day24. Following these dose regimes, a further two infected rabbits were dosed in the same manner from day-4 to day22, using 25mg/kg of the drug.

(4) Corticosteroids

Glucocorticosteroid hormones of the adrenal cortex have striking pharmacologic effects on lymphoid tissues and cells. These effects form part of the widespread use of the hormones in the treatment of a variety of diseases, including immunologic, inflammatory or neoplastic processes. The variety of effects which these compounds have on the immune response was reviewed by Gabrielson and Good (1967) and they have been shown to influence various immunological test systems related to cellular immunity, including adjuvant-induced arthritis and allergic encephalomyelitis (Rosenthale and Nagra, 1967). Their effectiveness has also been demonstrated in delayed type hypersensitivity (Dietrich and Hess, 1970), anaphylaxis (Dietrich et al., 1971), systemic tuber-

culin reactivity (Dietrich, 1970), and on the humoral autoantibody response (Makker, 1978). In trypanosomiasis, several authors (Ashcroft, 1957,1959; Petana, 1964; Luckins, 1972a; Seed et al., 1972; Balber, 1974) have found that corticosteroid treatment elevated the parasitaemia during infection but did not completely eliminate remissions, and Balber (loc. cit.) has suggested that corticosteroids, through their immunosuppressive action, attenuate the anaemia in T.brucei infections of mice. Seed et al. (1972) showed that hydrocortisone suppresses the formation of skin lesions in rabbits infected with T.gambiense.

There are remarkable differences in susceptibility to glucocorticosteroids between various species (Shewell and Long, 1956; Long, 1957). The exact basis of these differences is not known but diurnal species have been divided into steroid-sensitive and steroid-resistant species. The differentiation is usually based on the relative ease of producing lymphoid depletion after a given regimen of systemic glucocorticosteroids. The hamster, mouse, rat and rabbit are recognised as steroid-sensitive, whereas the monkey, guinea-pig and man are resistant (Claman, 1972). Inhibition of circulating antibody production by steroids has been repeatedly shown in steroid-sensitive animals (Germuth, 1956; McMaster and Franzl, 1961; Bjornboe et al., 1951). Two corticosteroids, dexamethasone and hydrocortisone were therefore used in an attempt to inhibit IC formation.

(a) Hydrocortisone acetate

Two rabbits were injected intramuscularly with 2.5mg on alternate days, from the day of infection (day0) until day15. The hydrocortisone acetate was obtained as a sterile suspension 'Hydrocortistab' injection

25mg/ml (Boots Co. Ltd.). The injections were carried out with suitable aseptic precautions, as tissues injected with corticosteroids have an increased susceptibility to infection.

(b) Dexamethasone sodium phosphate

Dexamethasone is a synthetic adrenocortical steroid possessing basic glucocorticoid actions and effects. It is among the most active member of its class and at equipotent anti-inflammatory doses it almost completely lacks the sodium-retaining property of hydrocortisone. The drug was obtained as 'Decadron' injection (Merck, Sharp and Dohme Ltd.), 4mg/ml of dexamethasone sodium phosphate equivalent to 4mg dexamethasone phosphate or 3.33mg dexamethasone. Six rabbits, infected with T.brucei, were injected daily intramuscularly, again using aseptic techniques, with 1mg dexamethasone equivalent, for 13,15 or 18 days.

(c) Cyclophosphamide

Cyclophosphamide (Cy) is an alkylating agent which has similar actions and uses to mustine hydrochloride. It is thought that it is broken down in the liver or blood, liberating an active alkylating agent. It is not a tissue irritant or vesicant and can be given by a number of routes. It has been used as an immunosuppressive agent in prolonging the survival of homografts as in kidney transplantation. Cy has been effective in rheumatoid arthritis (Cooperating Clinics Committee, 1970; Hurd, 1972). Clinical indications are not established however, because the drug has a narrow therapeutic index, serious side effects (Decker, 1973) and guidelines for dosage are poorly defined. Horwitz (1974) revealed several dose-dependent effects of

Cy on blood leucocytes, and that granulocytopenia or marked lymphopenia were not needed for a favourable clinical response. He also demonstrated that arbitrary fixed amounts of Cy may be excessive in some patients and insufficient in others. Neilson (1978) has shown some immunosuppression in hamsters to Dipetalonemia vitae when dosed twice weekly with 150mg/kg Cy. MacKenzie et al. (1978) abolished the development of adjuvant disease in the PVG/c rat using 100mg/kg Cy, given intraperitoneally three days before adjuvant. Turk et al. (1972) has shown that in the guinea-pig this regimen depresses the level of circulating B lymphocytes, but leaves the T lymphocyte numbers largely unaltered. This is reflected in the lymphoid tissues by a marked depletion of B-dependent areas.

Two rabbits were dosed with 150mg/kg Cy (Koch Light Labs.) as a freshly made solution in alcohol, on the same day as infection with T.brucei, intraperitoneally, and one of these rabbits was also given 50mg/kg Cy on day3. A third infected rabbit was dosed once only with 150mg/kg on day3. Following these regimens, two further rabbits were given Cy intraperitoneally at a dose of 100mg/kg on day2, and 50mg/kg on day5 after infection.

For each drug treatment, excluding aprotinin, one uninfected rabbit received a similar dose of the drug as the infected animals, over a comparable time period. The drugs were administered at the same time each day during the treatment. The drug doses used in these experiments were calculated from known therapeutic doses in man and other animal species. An important factor however in determining drug

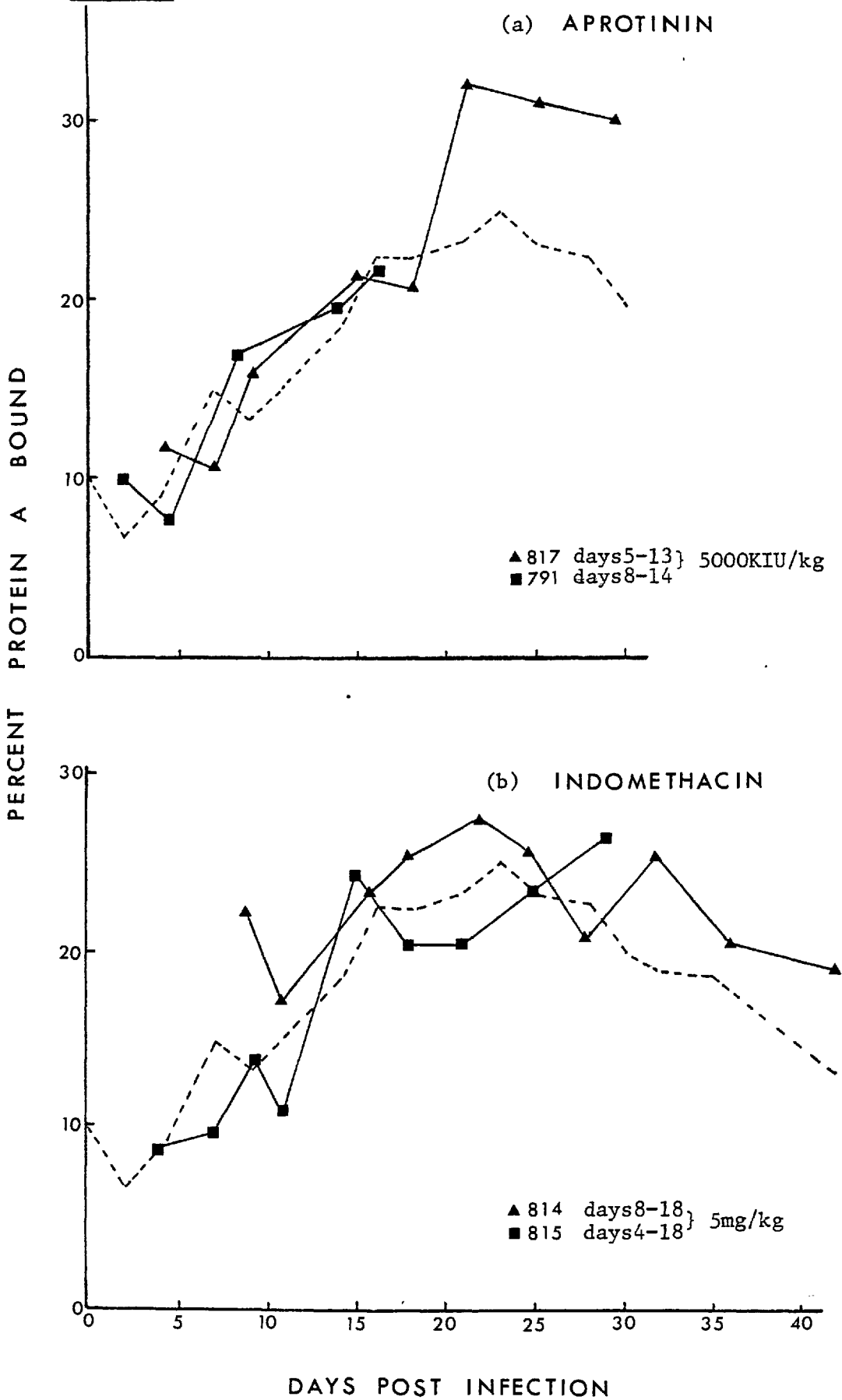
dose equivalents in different species is that degradation and inactivation are proportional to metabolic rate, which in turn is proportional to surface area and not to body weight (Berenbaum, 1975).

3.8.2 Immune complex levels

The levels of IC were determined for the infected rabbits as previously described (section 2.7.1), with the period of sampling restricted, in most cases, to the first 3-4 weeks of infection. The results are represented in Fig. 3.21 (a-f), the IC levels of two rabbits from each drug treatment plotted together with the average values obtained from three untreated infected animals (see section 3.7.1). The complete data can be found in Table E, Appendix 2.

The only drug to have a significant effect in limiting the levels of circulating soluble IgG complexes, at the doses used, was dexamethasone. The low values seen in four of the six rabbits (835,850,851,853) were marked by an initial fall, with some fluctuations occurring throughout the drug treatment. Increases in levels were seen in two of these rabbits (835,853) when the drug treatment ceased. Penicillamine at 5mg/kg (744) and cyclophosphamide (821,834) appeared to enhance the response with maximum levels of complexes significantly higher than the untreated infected animals. A similar increase was also apparent 16-18 days after infection in one of the hydrocortisone (837) and aprotinin (817) treated animals.

Fig. 3.21 Soluble IgG immune complexes in drug-treated rabbits infected with *T.brucei*



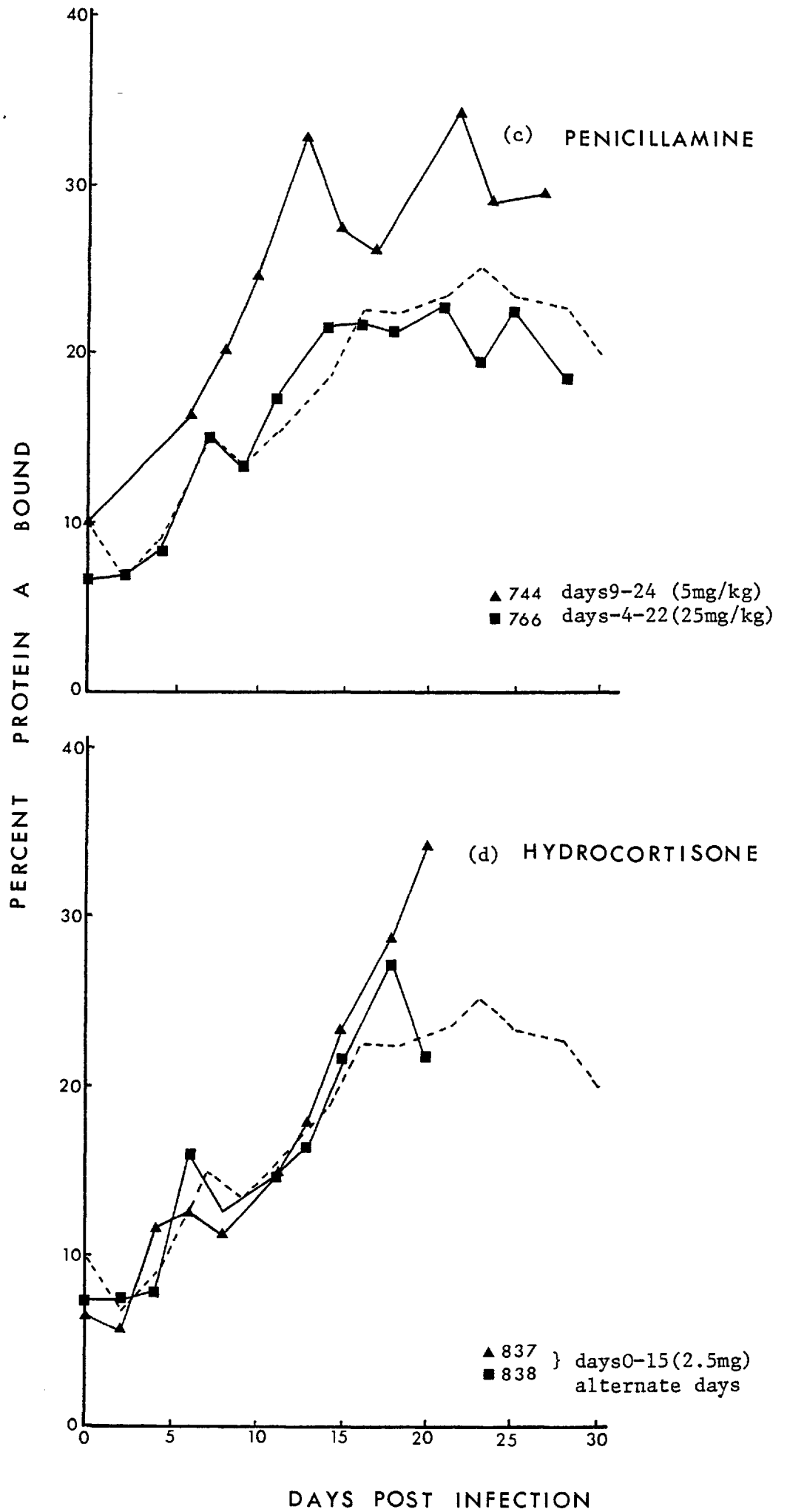
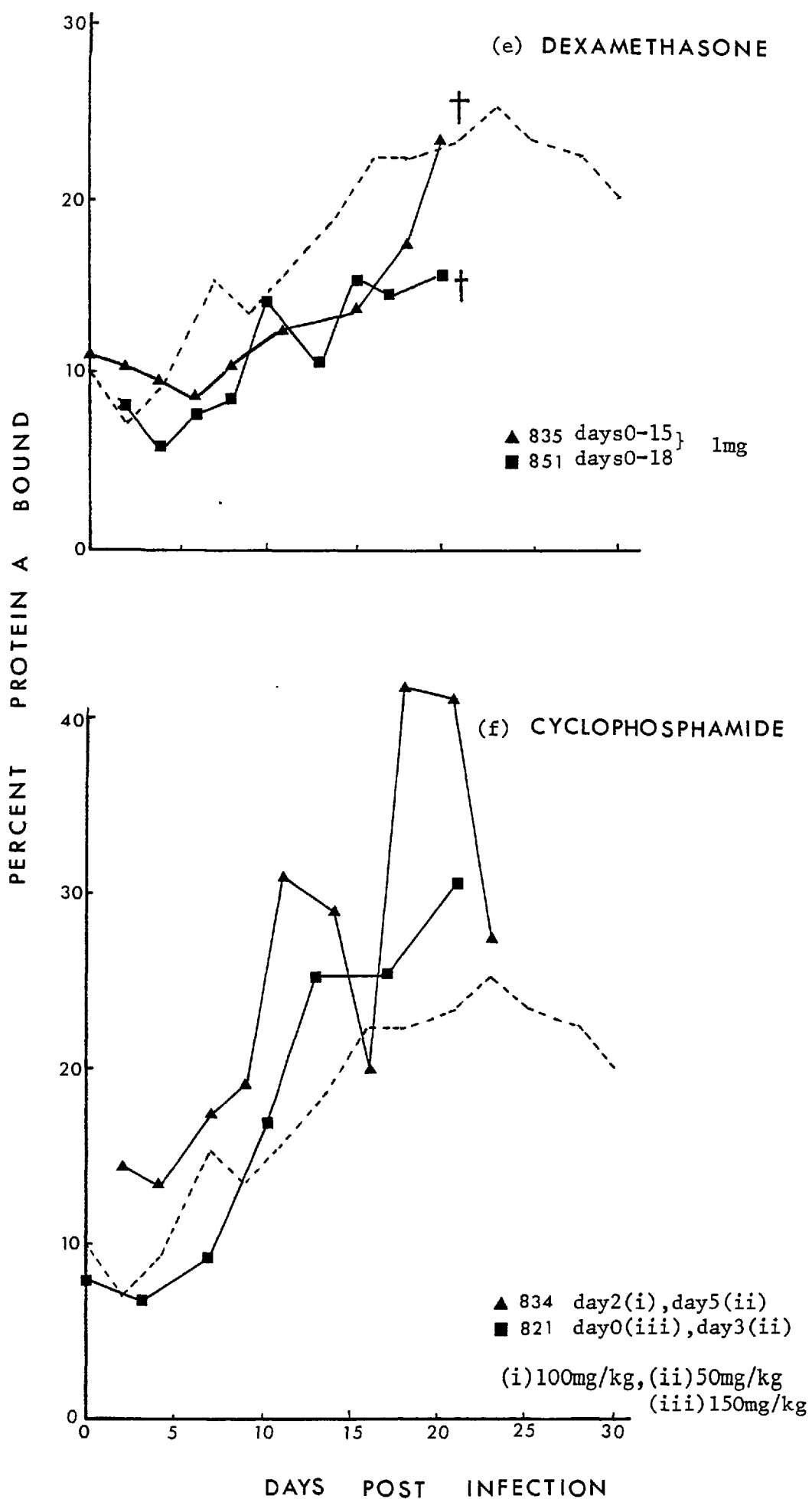


Fig. 3.21 (contd)



3.8.3 Disease symptoms

The physical state of the rabbits, as well as the parasitaemia, was monitored throughout the period of drug treatment, primarily by noting any external symptoms characteristic of the disease. The typical disease symptoms of T.brucei in rabbits are a low blood parasitaemia with some fluctuations during the infection. The first sign of oedema and erythema occurs approximately 15 days after parasitic inoculation and gradually becomes more severe with small skin lesions evident by four weeks. At this stage the eyes are usually swollen and purulent rhinitis is established, with the animals losing weight rapidly in the terminal stage of the disease.

Rabbits dosed with penicillamine (5mg/kg and 25mg/kg) did not demonstrate any significant improvement or deterioration compared to the symptoms described above. A slight delay in the onset of oedema was apparent in the rabbits given aprotinin, this was more marked in the two rabbits dosed with indomethacin from day4 to 18(815,816). The parasitaemia was unaffected and external symptoms appeared after cessation of treatment in all cases.

Cy had a pronounced effect on the rabbits, and the doses used did not appear to be tolerated at all well. Two rabbits (822,823) died on days2 and 7, respectively. Rabbit 821 was very emaciated and had severe necrotic lesions on the face with pronounced swelling round the eyes by day22. Breathing was laboured and the experiment was therefore terminated. Symptoms were evident from day10 in this animal. In a

further two rabbits injected with lower doses of Cy (100mg/kg), rabbit 833 died on day10, being noticeably emaciated by day9 with high blood parasitaemia, the parasites were evident from day7. Rabbit 834 appeared to follow a typical disease course, with regard to visible symptoms, but the time period was reduced considerably. This animal was put down on day24, having severe necrotic lesions of the face and scrotum. Evidence of oedema and reddening round the eyes was noticed at day7 and the parasite numbers were elevated at day14, when loss of fur was also pronounced. At the level used, this drug seemed to be extremely toxic in rabbits, but as described, these levels did appear to be tolerated in other animal species. A reduced amount of Cy (10mg/kg), given intraperitoneally as before on alternate days up to day11 after infection in one additional rabbit (841), led to a marked elevation in the parasitaemia at day9. External lesions were marked by day14 and the animal died on day27.

Hydrocortisone treatment did not cause any significant changes in the external symptoms, but the number of parasites seen in rabbit 838 were much increased at day8 and 11 post infection. A similar finding, although not as pronounced, was evident in rabbit 837. A marked increase in parasite numbers at day4 post infection was seen in four of the six rabbits given dexamethasone (835,850,851,853). Rabbit 850 died on day14 with a very high parasitaemia, loss of weight was seen from day8, the animal being in poor condition with typical lesions on the face at this time. Rabbit 851 followed a normal disease course but died on day21. 852 and 853 were necrotic and oedematous from day16, both animals survived, although in poor condition, to day27 when the experiment was terminated. Rabbits 835 and 836 had elevated parasite numbers, with 835

developing oedema of the face and necrotic lesions round the eyes. Both rabbits lost weight and after cessation of drug treatment their condition deteriorated and they died on day20.

Stabilates were prepared from rabbits 850-853 on days10, 17 and 24, where possible, to see if different populations were apparent as the infection progressed, since parasites were demonstrated more frequently and their numbers much increased during infection in dexamethasone treated animals. 0.2ml of blood, taken from individual rabbits, was injected into clean mice. Within three days the resultant infections were re-passaged and two days later the mice were sacrificed, and stabilates prepared as described (see section 2.4.5). These were then used to determine the agglutinating antibody titre present in serum collected at the same time as the mice were inoculated. The results, shown in Table 3.10, indicate that in the four rabbits tested different and distinct parasite populations did occur as agglutination of trypanosome stabilates was not achieved with any serum collected at the same time or before the stabilate. The increased number of parasites seen at differing intervals during the infection may therefore be due to the immunosuppressive effect of dexamethasone, the division phase of any particular population proceeding for a longer time period before antigenic variation occurs.

The Clq binding assay was also used to determine the levels of circulating IC in a number of drug treated animals and the results can be seen in Table E, Appendix 2. The levels of complexes determined by this method correlated well with those already described using the filter separation and protein A binding, the percentage binding being lower, due to the assay method, but any changes being statistically significant.

Table 3.10 Agglutinating antibody titres of serum from dexamethasone treated rabbits

Autologous serum	Stabilates								
	850	851		852			853		
Days	10	10	17	10	17	24	10	17	24
0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0
17	ND	$>10^4$	0	$>10^4$	0	0	$>10^4$	0	0
24	ND	$>10^4$	10^4	10^4	10^4	0	$>10^4$	$>10^4$	0

NB. Agglutination of basic strain *T.brucei* 427 occurred with the sera collected from all rabbits at day10, 17 and 24. No agglutination was seen with day0 serum.

3.8.4 Immunoglobulins and C3

Levels of IgG, IgM and C3 were measured in serum samples (see sections 2.5.8 and 2.5.9) collected at regular intervals throughout the infection, from both untreated and drug treated rabbits. The concentration of IgM, expressed as a percentage of the day0 value, in three infected rabbits increased rapidly from day5, with increases in IgG evident from day7. Maximum values for both immunoglobulins occurred between day18 and day25 after infection, the levels decreasing slightly in rabbits 756 and 758 after this time. These results are represented in Fig. 3.22 (a,b). Quantitative measurements of IgG, as determined from the standard curve (Fig. 3.23), indicated that pre-infection concentrations were 700 ± 300 mg/100ml serum sample, rising to 2500 ± 200 mg/100ml at peak levels during infection.

Fig. 3.22 Immunoglobulin and C3 concentrations in
T.brucei infected rabbits

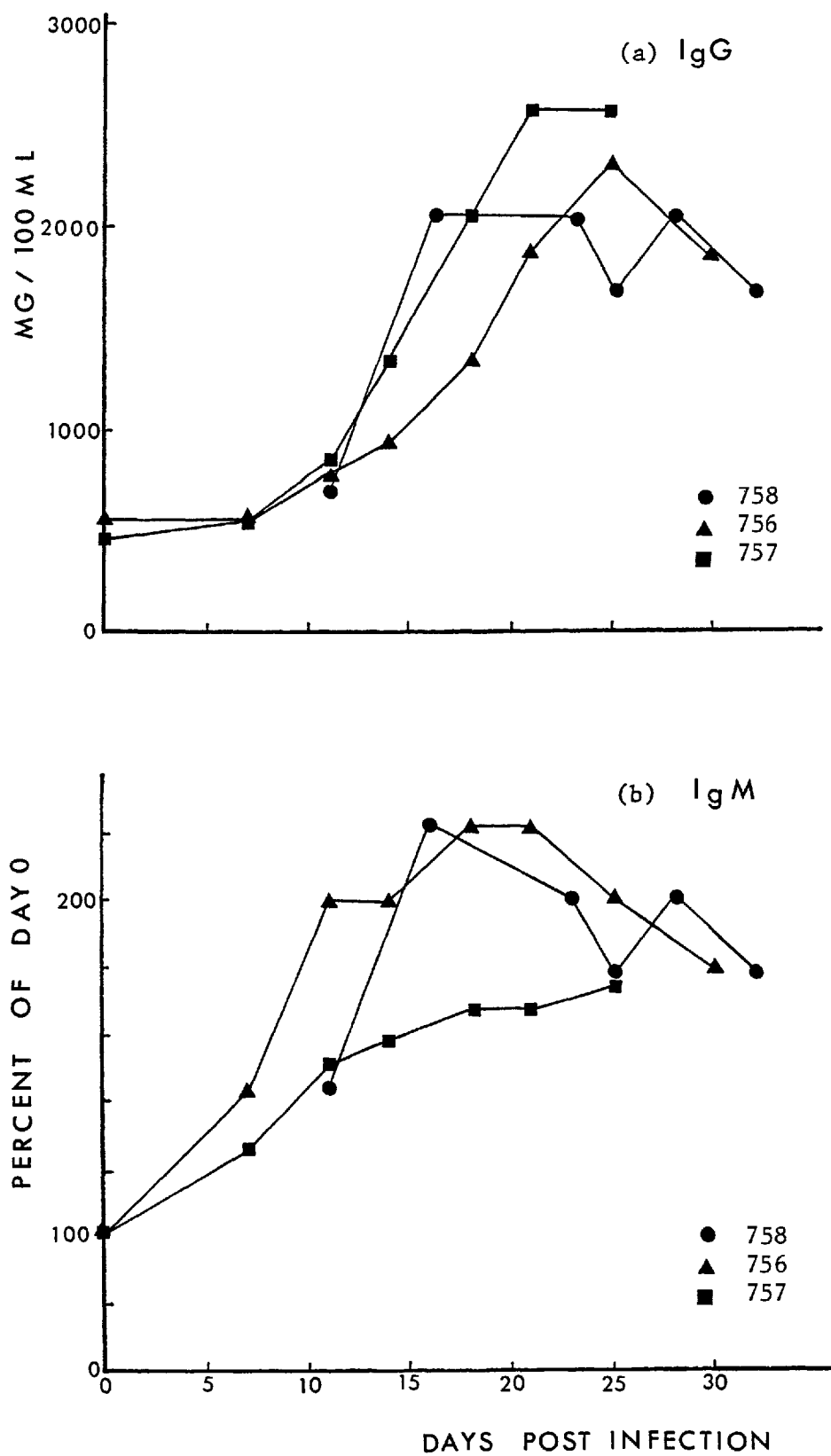
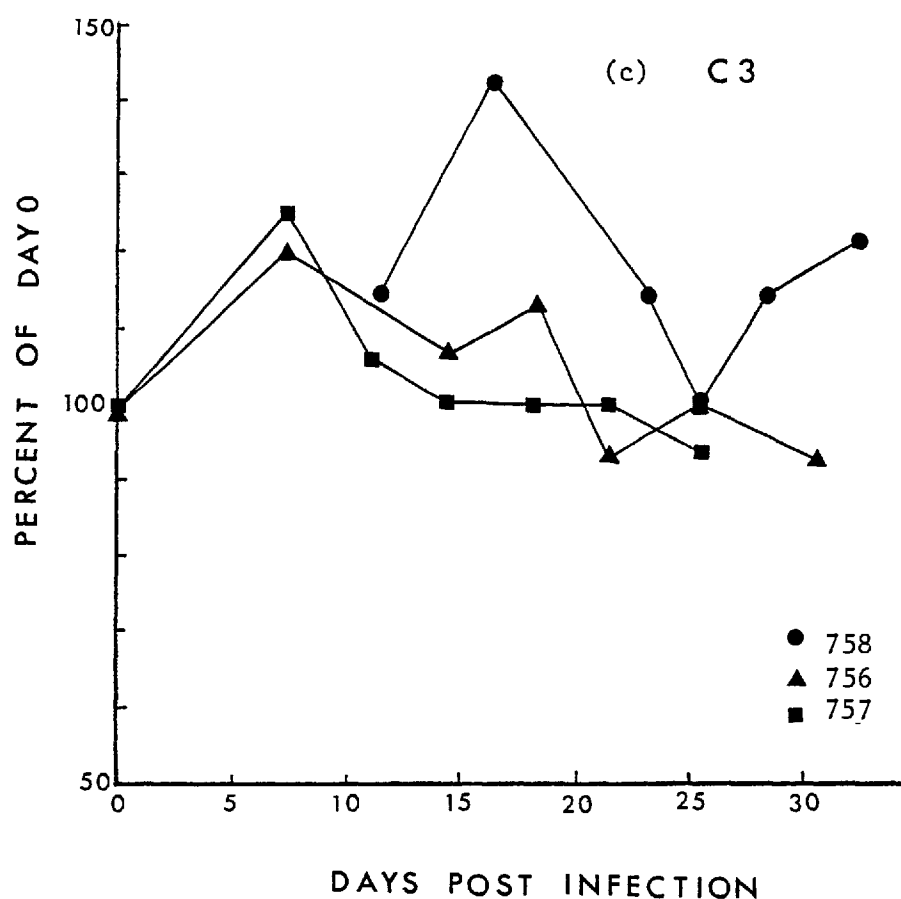
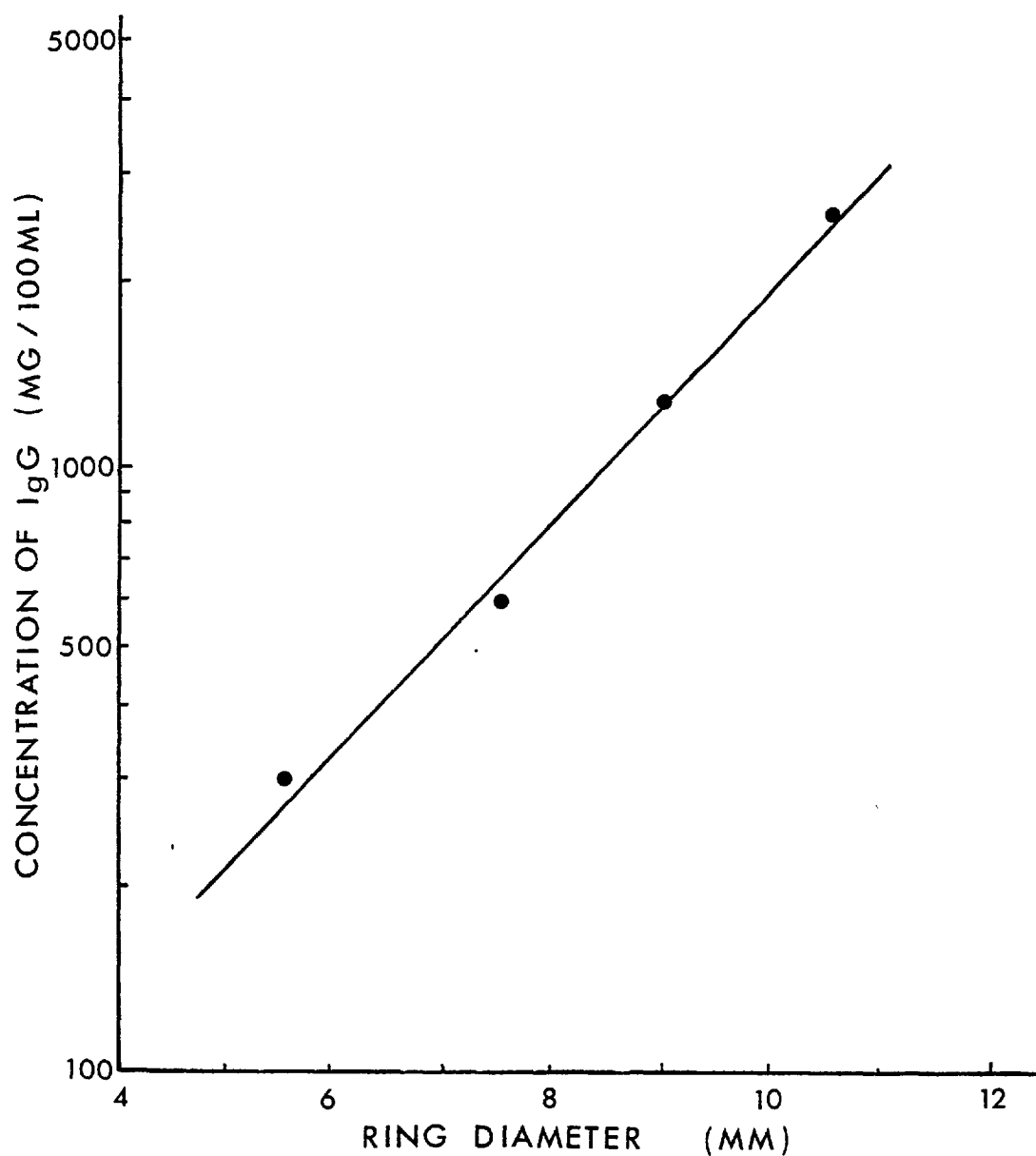


Fig. 3.22 (contd)



C3 concentrations, as shown in Fig. 3.22(c), again expressed as a percentage of the day 0 value, varied between the three infected animals. An initial increase followed by a fall in levels was seen in rabbits 756 and 757, a larger increase however was seen in rabbit 758 at a much later stage in the infection. These results suggest that the complement system was being activated in some way, with consumption of C3. Some synthesis is probably occurring as the concentration increases initially but did not fall significantly below pre-infection levels. The reduction in C3 concentrations seen in these rabbits correlates well with both the increase in IC and the presence of parasites in the

Fig. 3.23 Standard curve for rabbit IgG concentrations



circulation. The data for these parameters can be found in Table D, Appendix 2.

The concentration of IgG in the drug treated rabbits followed a similar course to the levels of IC already described (section 3.8.2).

The only drug therefore to cause a significant depression in serum IgG was dexamethasone (Fig. 3.24(e)). The change in levels was marked by a slight fall compared to day0, with increases above normal only seen when drug administration ceased in four of the six rabbits sampled (835,851,852,853). The results of IgG, IgM and C3 for two rabbits from each treatment group are shown in Fig. 3.24(a-f) plotted together with the average values obtained from three untreated infected rabbits (756-8). The actual data can be found in Table E, Appendix 2. A slight reduction was evident in one hydrocortisone treated animal (838), while another (837) had an increased response early in the infection. Cy resulted in an initial reduction in two rabbits (833,834) but significantly higher levels were seen 13-14 days post infection (834). Increases comparable to untreated infected animals were seen in most rabbits receiving aprotinin, indomethacin or penicillamine (5mg/kg), however the larger dose of penicillamine (25mg/kg) did lead to a reduction in concentration in one animal (766).

IgM concentrations were not significantly affected by aprotinin, penicillamine or indomethacin, although one rabbit (814) treated with indomethacin maintained low levels throughout the sample period. Dexamethasone led to a fall by day4 of infection in four rabbits (835, 836,851,853) but these soon increased to be similar to untreated animals by day10. Cy prevented a rise in levels up to day7 and then, as with IgG concentrations, rose to be greater than those of untreated rabbits and remained elevated - a similar situation recorded with hydrocortisone although one of these rabbits (837) showed a marked increase by day8. This might suggest that IgM production is being enhanced in some way by these agents.

Fig. 3.24 IgG, IgM and C3 in drug treated rabbits infected with T.brucei

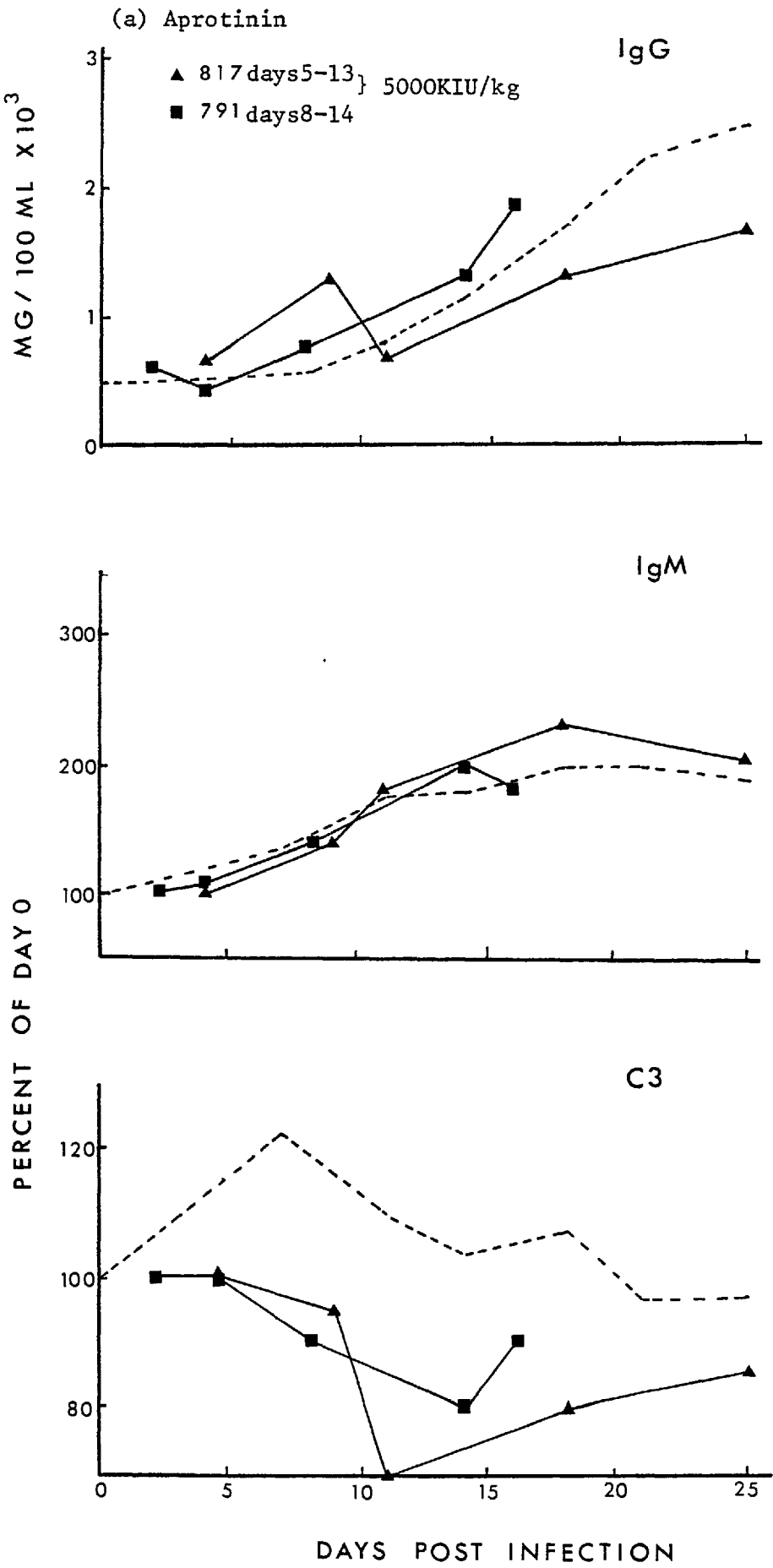


Fig. 3.24 (contd)

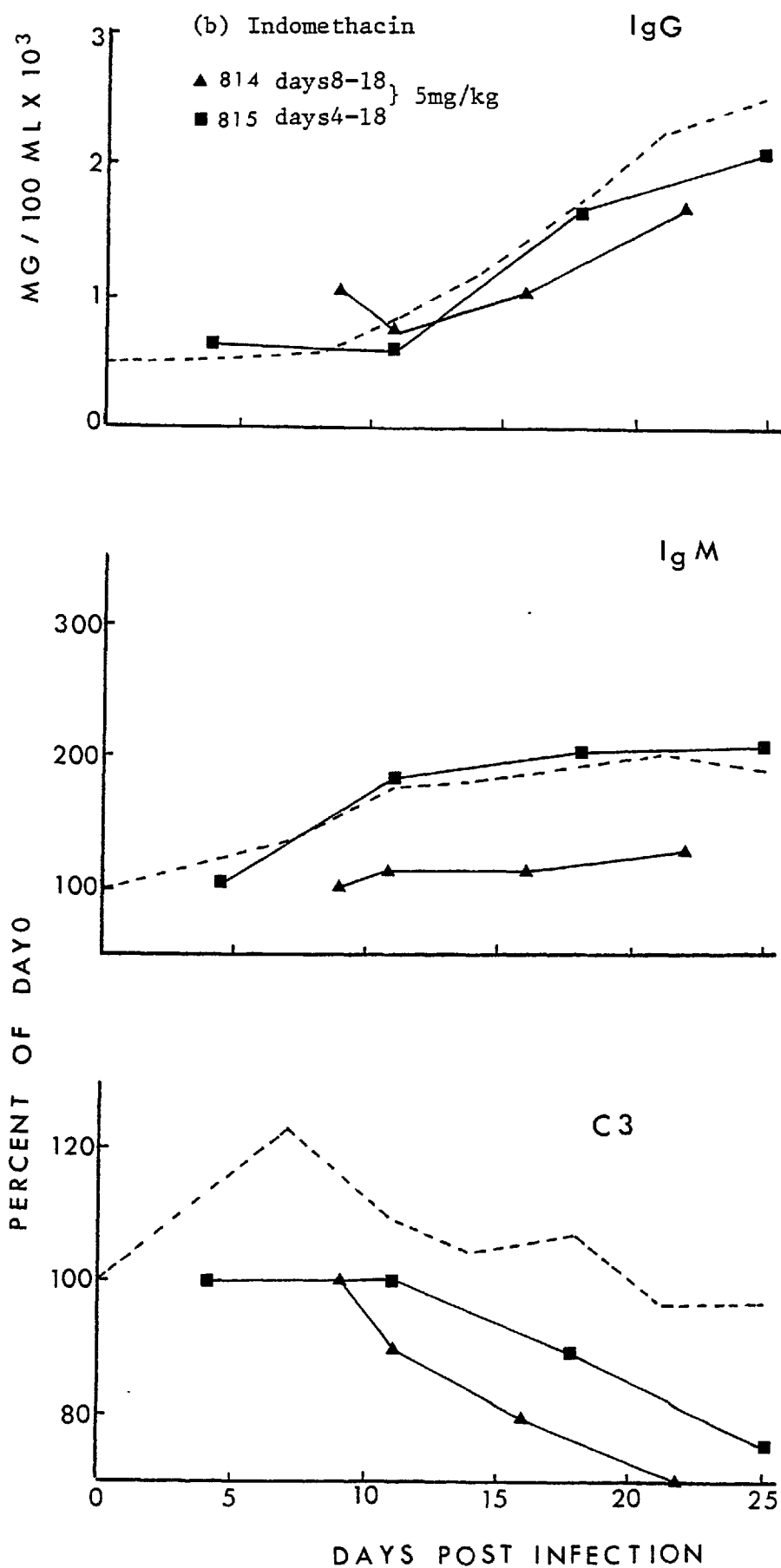


Fig. 3.24 (contd)

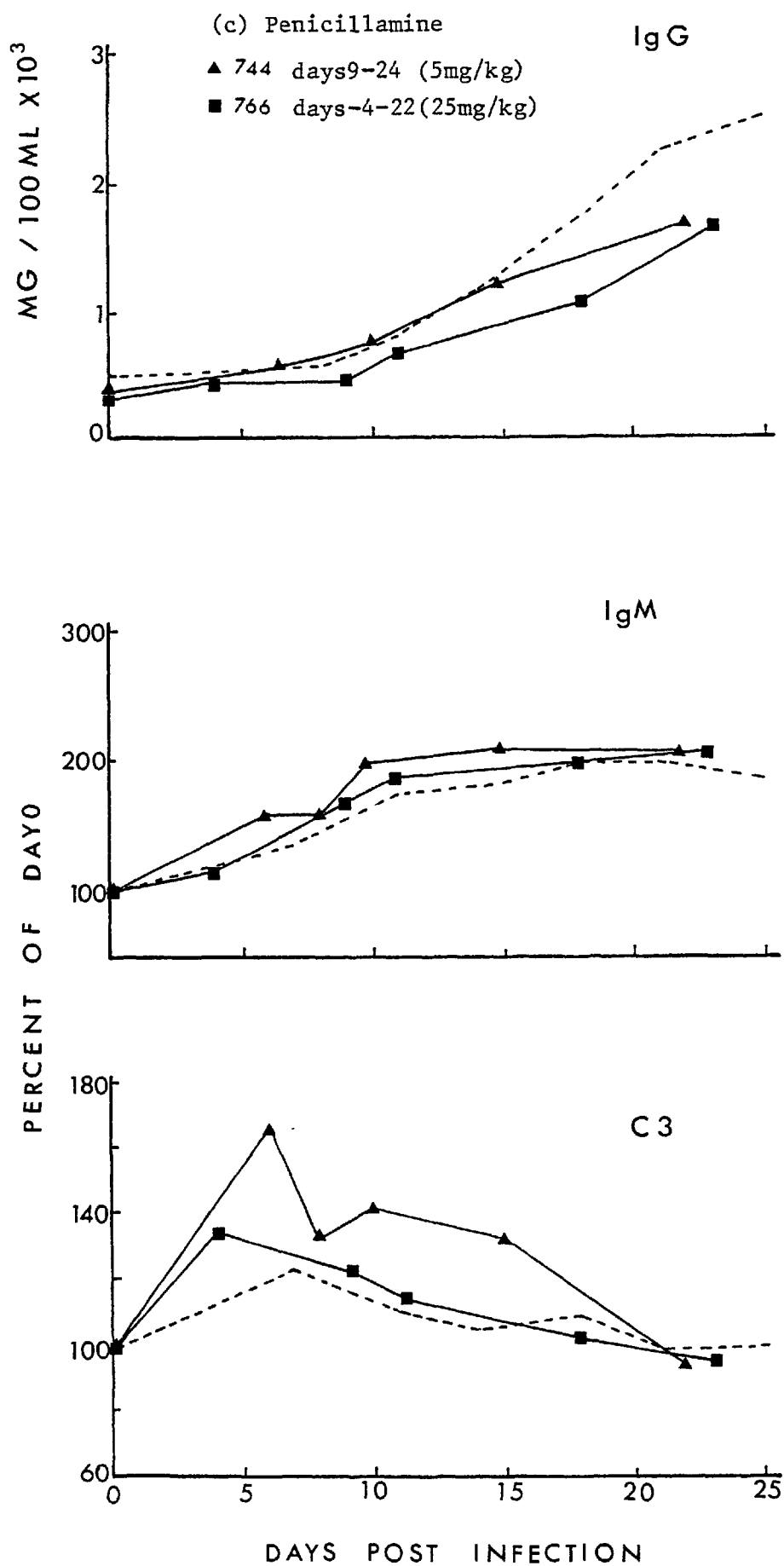


Fig. 3.24 (contd)

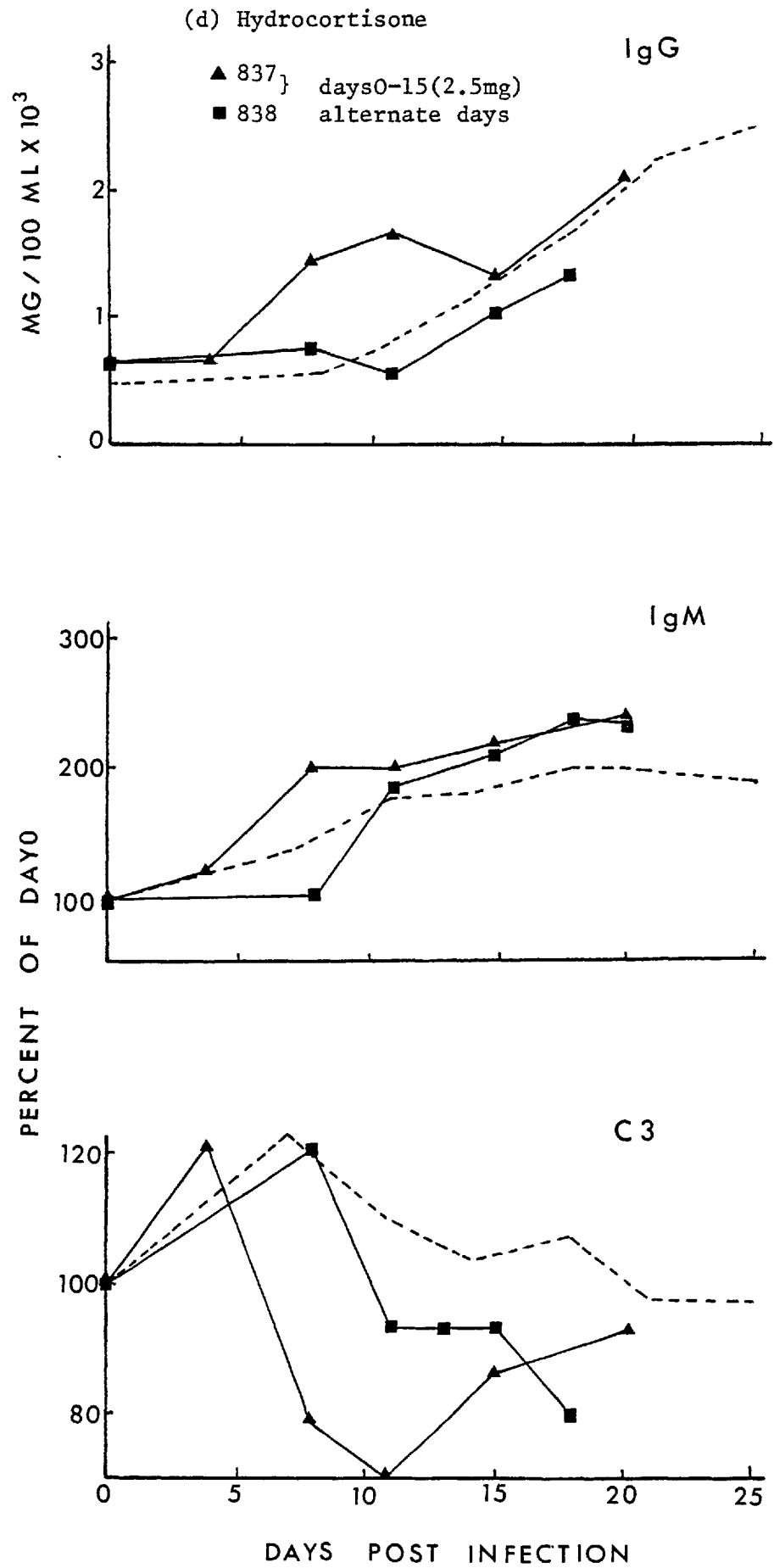


Fig 3.24 (contd)

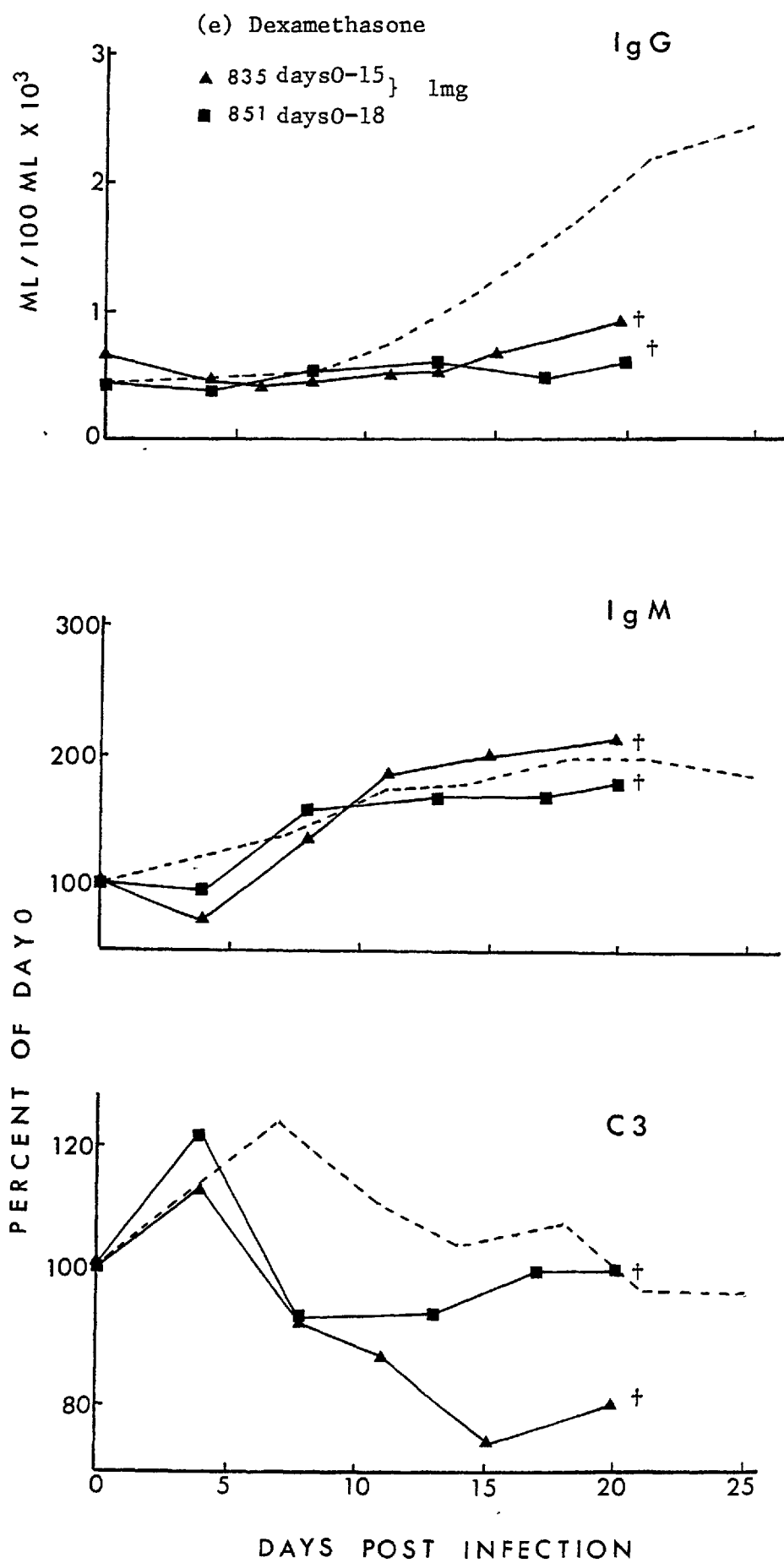
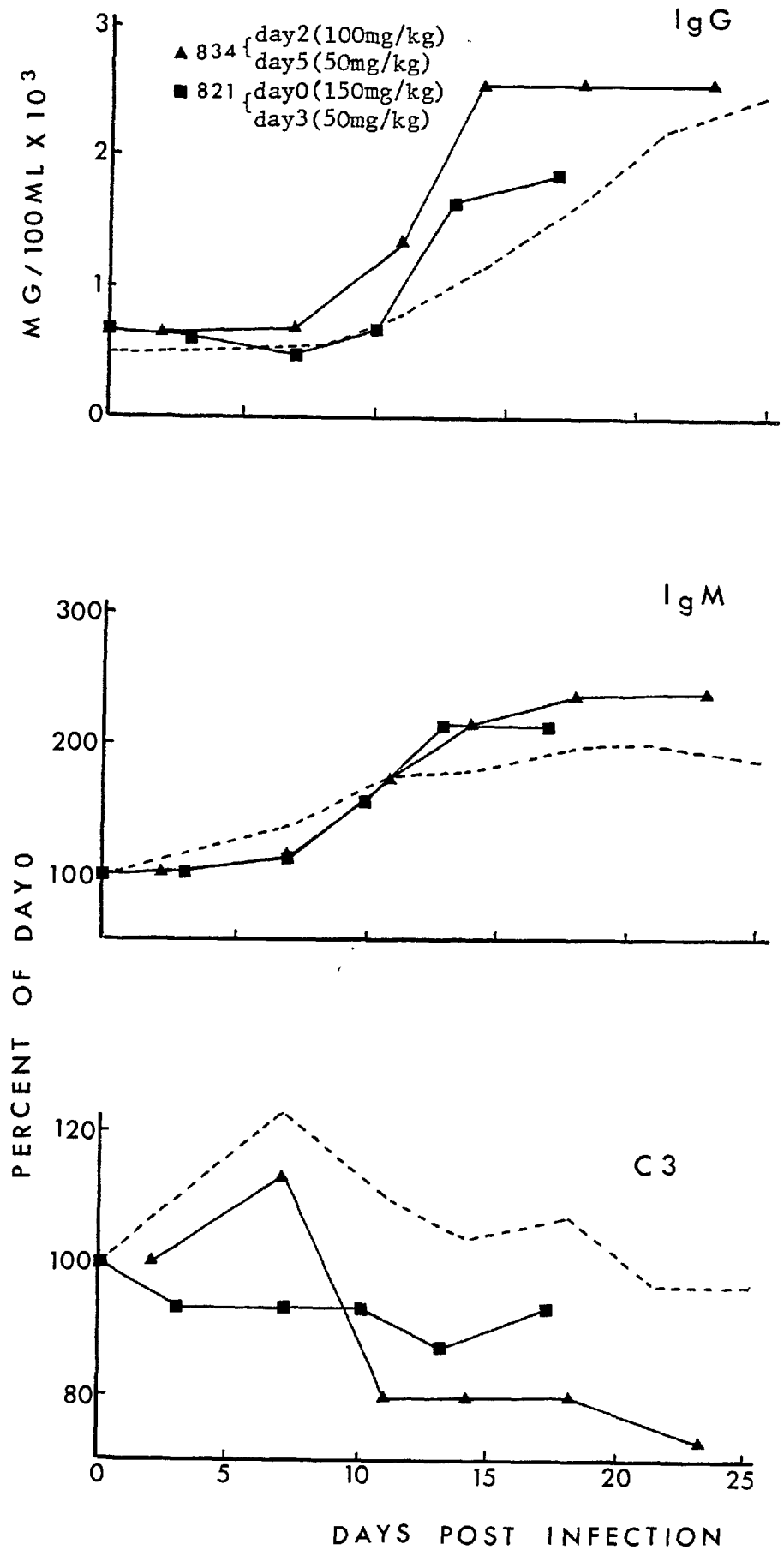


Fig. 3.24 (contd)

(f) Cyclophosphamide



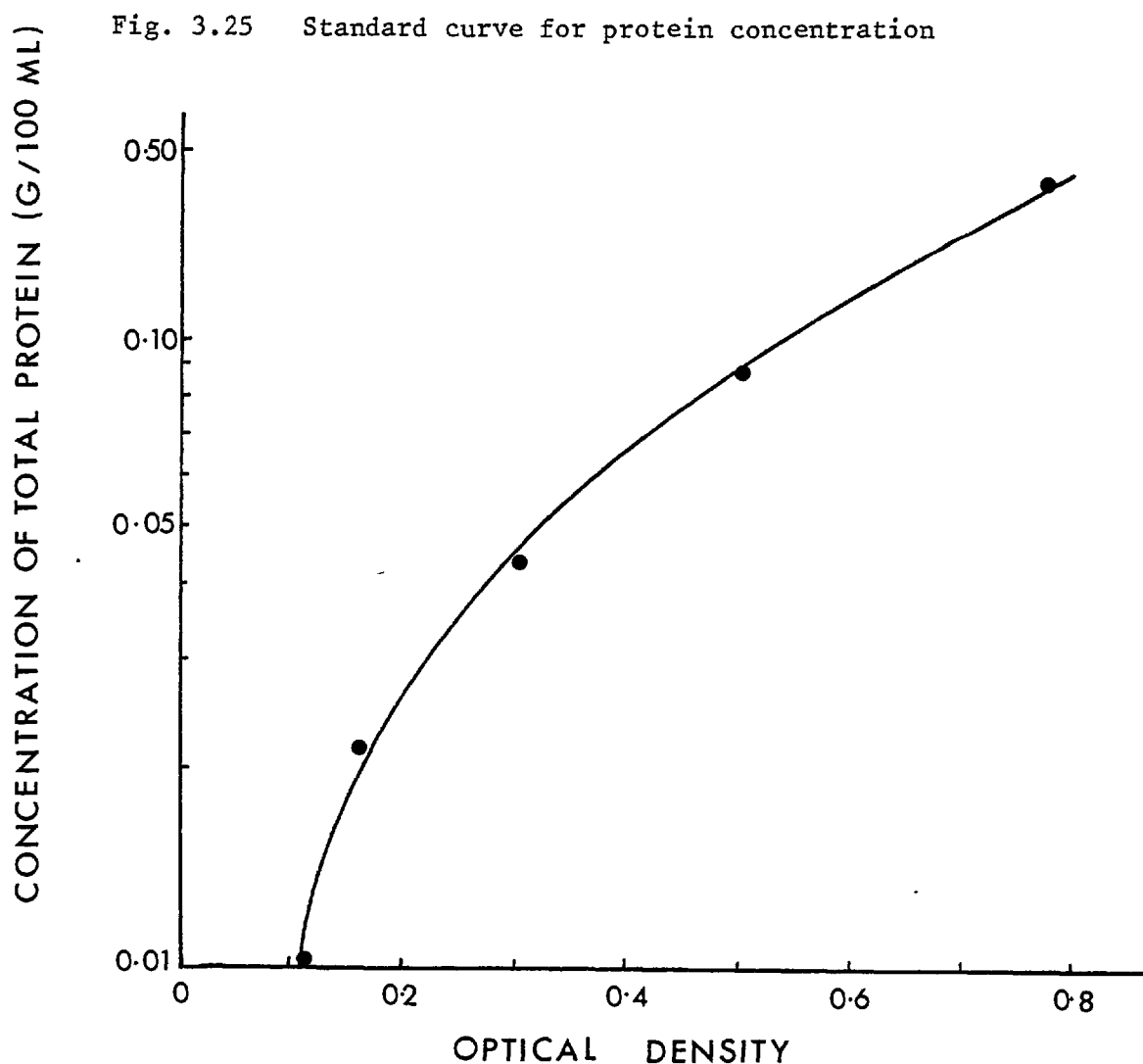
Concentrations of C3 varied from those already described, in every drug treatment. This may have been due to the activity of the drug or possibly to the natural variation between individual rabbits. However a similar trend was established with an initial increase between day 3 and 7, followed by a decrease in levels with some fluctuation later in the infection. This trend was most marked in rabbits treated with dexamethasone and hydrocortisone, although a larger increase was seen in the rabbits dosed with penicillamine (744,766-7).

It is interesting to note that C3 decreased in all drug treatments and that this occurred at a similar time to increases in levels of immunoglobulin and IC. Where these increases were depressed, in dexamethasone treated animals particularly, the decrease in C3 was apparent at the same time as a large number of parasites in the circulation. These results imply that complement is being activated by both the classical and alternative pathways in this infection. A number of authors have reported activation of the complement system in trypanosome infections of a number of animal species (see section 1.2.4) and have indicated that both pathways are involved.

3.8.5 Total protein measurements

It is known that increases in serum globulins and a decrease in albumin occurs during trypanosome infections of rabbits (see Goodwin and Guy, 1973). Fibrinogen and total serum lipid concentrations, as well as Cx-RP are also raised in T.brucei infected rabbits (Boreham and

Facer 1974; Boreham et al., 1977; Cook, 1979). Measurement of the total serum protein can therefore give some indication of gross changes occurring during the infection and it has already been established that both IgM and IgG are markedly increased in T.brucei infection



(section 3.8.4). Boreham et al. (1977) have shown that the increase in blood proteins and lipids will contribute to the pathological changes seen in rabbits. Any change in the amount of serum proteins, due to the drugs used in this experimental work may therefore have some effect on the pathology of the disease.

The protein concentration of serum samples collected at intervals throughout infection, for both untreated and drug treated animals, were determined as described in section 2.6.2. It was found that the serum diluted 1:80 gave suitable mid-scale readings on the colorimeter, and the protein concentrations were calculated from the standard curve (Fig. 3.25). The results (Tables D and E, Appendix 2) show that the protein concentration in untreated rabbits (756,757,758) fell initially, between day 0 and 7, and then gradually increased during the infection. These results are in agreement with those previously described (van den Ingh, 1976; Boreham et al., 1977).

No significant alteration in concentration was seen in animals dosed with aprotinin. Treatment of rabbits early in the infection with indomethacin (815,816) led to a drop in concentrations and although these did increase they did not rise above those found initially, as compared to the rabbit dosed at a later stage in the infection (814). This may be related to a differential reduction and inhibition of acute phase proteins such as Cx-RP. A similar difference in concentrations was noted with penicillamine, the higher doses (25mg/kg) having a more pronounced effect (766,767) with the amount of protein fluctuating slightly during the sampling period. The concentrations in one hydrocortisone treated rabbit (838) only altered to a small degree and this correlated with a lower level of IgG in this particular case. Dexamethasone treatment reduced the concentrations in most cases, with amounts not rising significantly above pre-infection until administration of the drug ceased. This again correlated well with reduced immunoglobulin levels but the anti-inflammatory aspect of dexamethasone also

probably plays some part in lowering the protein concentration. A significant elevation was seen in one rabbit (834) treated with Cy, and again enhanced immunoglobulin synthesis may be responsible.

It is apparent from these observations that protein concentration has been affected in some drug treatments, and that the changes are related to other parameters already considered. A more detailed study would be required in order to establish changes in individual protein components, such as fibrinogen or lipids, and to identify changes in the blood chemistry. This may indicate whether the drugs are having an effect on any tissues or organs leading to an increase or decrease in synthesis of certain serum components.

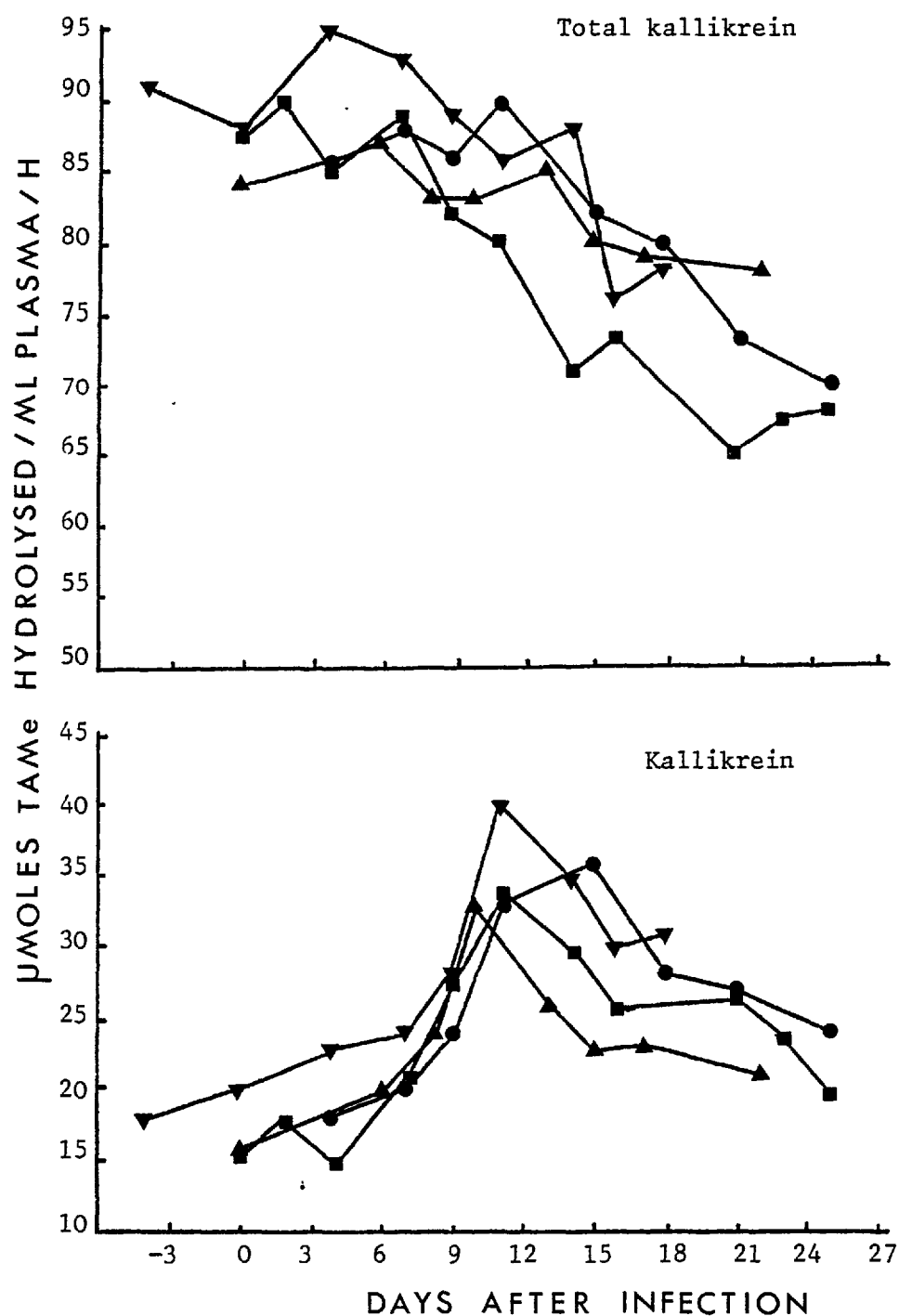
3.8.6 Plasma kallikrein in drug treated rabbits

The object of the drug treatment of infected rabbits was to determine if the formation of IC could be inhibited and whether this would alter the levels of plasma kallikrein. As already described (section 3.8.2), the only drug to have a significant effect on IC levels was dexamethasone. Although it did not completely inhibit IC formation the levels of IC were significantly lower compared to untreated infected animals, at a time (day9-14 after infection) when plasma kallikrein levels were shown to be maximum (section 3.3). Plasma samples collected from rabbits during drug treatment were assayed for kallikrein and total kallikrein, as described in section 2.6.1. Samples of at least two rabbits from each drug used were assayed, with the omission of aprotinin as the in vivo inhibitory activity of this drug has already been demonstrated (section 3.5.2). Particular emphasis was placed on samples from

dexamethasone treated animals. The results of one rabbit from each treatment are shown graphically in Fig. 3.26(a,b), together with those of an untreated animal, the complete data for the samples assayed are given in Table F, Appendix 2.

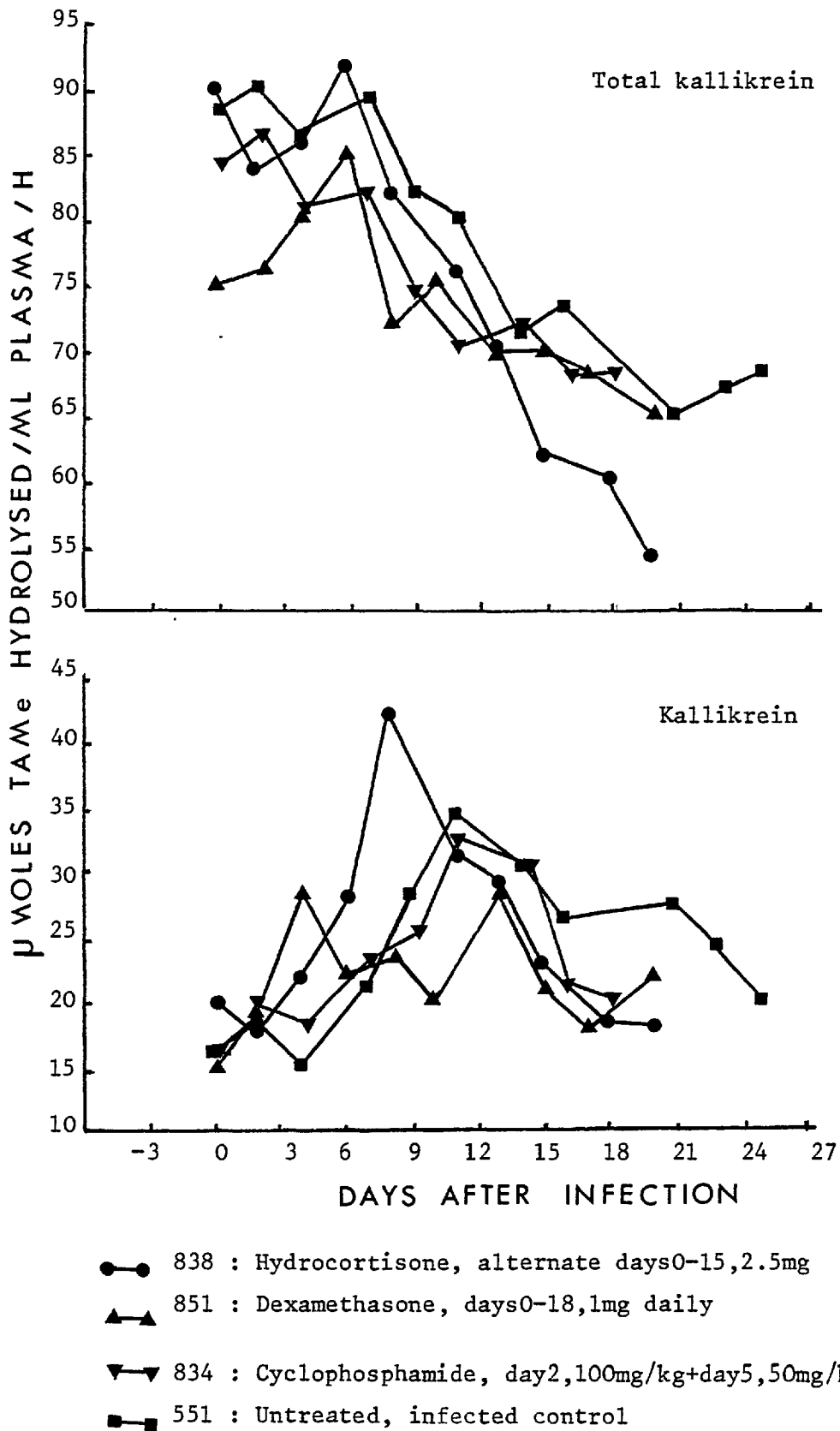
No significant alteration in the levels of total kallikrein or kallikrein were seen in indomethacin or penicillamine treated animals. The rabbits dosed at the higher level of penicillamine (766,767) however did show slightly elevated total kallikrein levels, with little evidence of a drop in one rabbit (766) to values seen in untreated infected animals later in the infection. Hydrocortisone reduced the total kallikrein by 40%, 20 days after infection in one rabbit (838). The peak value for kallikrein was seen at day8 in this animal and although the IC levels were elevated at this time, large numbers of parasites were also noted in the circulation (Table E, Appendix 2). A similar pattern was observed in four dexamethasone treated rabbits (835,836,850,851), significant increases in kallikrein activity present at day4 after infection, following large numbers of circulating parasites noted at day2, in three of these rabbits (835,850,851). Subsequent increases in kallikrein were also seen at a comparable time to an increase in IC. As significant amounts of IC have not been shown as early as day4 of infection, and dexamethasone inhibits IC formation at this time, another mechanism seems to be involved in kallikrein activation. This is probably related to the large numbers of circulating parasites, although a direct action has not been demonstrated either in vitro or in vivo (section 3.6.1, 3.6.2), as a similar early rise in kallikrein was not seen in untreated infected animals with low parasite numbers.

Fig. 3.26(a) Plasma kallikrein levels in drug treated infected rabbits



- 815 : Indomethacin, days 4-18, 5mg/kg
- ▲—▲ 744 : Penicillamine, days 9-24, 5mg/kg
- ▼—▼ 767 : Penicillamine, days -4-22, 25mg/kg
- 551 : Untreated, infected control

Fig. 3.26(b)



Previous work has illustrated an elevated parasitaemia in mice (Balber, 1974), rats (Luckins, 1972a) and rabbits (Seed et al., 1972) treated with corticosteroids. Two rabbits (851, 852) both had slightly lower total kallikrein levels throughout the infection and this may have been due to an inhibitory action of dexamethasone or to the natural variation between rabbits within one treatment group. Some earlier in vitro studies did not show any effect of anti-inflammatory drugs on kallikrein-mediated kinin formation (Davies et al., 1966). However, Cline and Melmon (1966) suggested that certain adrenocorticosteroids exert their anti-inflammatory effect by inhibiting the release of plasma kinins and this may account for their ability to inhibit the early phenomena of the inflammatory process. Some later work in baboons (Herman et al., 1974) has demonstrated a difference in plasma kallikrein response in septicemic shock in animals pretreated with an adrenocorticosteroid (methylprednisolone, 30mg/kg). Their measurements however were made over a short time period (maximum 6h), after a single injection of Escherichia coli, and plasma kallikrein levels were determined indirectly by estimating kinin release. No significant kallikrein inhibition was noted in any of the drug treatments in the present experimental study.

Cy did not significantly alter the total kallikrein or kallikrein levels at any of the doses used. However the severity of the disease appeared to be increased, with a maximum survival time of only 27 days at the lowest dose used. A similar severe reaction was demonstrated with dexamethasone and this may indicate some drug toxicity, particularly as the uninfected control rabbits injected with either Cy (100mg/kg) or dexamethasone (1mg, 15 days) both showed a deterioration in condition.

This was most pronounced with Cy, but both rabbits recovered after termination of their respective drug treatments. None of the other drugs used led to any physical changes in these uninfected animals. IgG, IgM, C3 and protein levels for these drug treated control rabbits are given in Table E, Appendix 2. The most significant changes were seen with dexamethasone, which reduced the IgG concentration by approximately 25%, although the initial level was higher than the other animals studied, and IgM by 15% of the pre-dose levels. Penicillamine (25mg/kg) also reduced the IgG concentration but not the IgM. C3 and total protein fluctuated throughout the dosing period in all the control rabbits. A significant reduction in C3 however was evident in both the dexamethasone and hydrocortisone treated rabbits, this being most pronounced after 14 days of drug dosing. The decrease in total protein following dexamethasone treatment is probably related to the fall in IgG concentration. None of the drugs used significantly altered the total plasma kallikrein and kallikrein levels in these rabbits, with values of 84 ± 6 and 19 ± 4 μ moles TAME hydrolysed/ml plasma/h, respectively. Therefore no inhibition of prekallikrein or kallikrein was apparent with these drugs, at the doses given.

4 DISCUSSION

This work has investigated the changes that occur in the plasma kallikrein-kinin system of rabbits infected with a chronic strain of T.brucei. Emphasis has been placed on the probable activation of this enzyme system by immune complexes (IC).

Plasma kallikrein levels were found to increase to a maximum between 9 and 14 days after infection associated with a decrease in plasma prekallikrein. This suggested that at this time the inactive precursor prekallikrein was being activated in some way leading to the increase in free kallikrein. This would in turn act on kininogen to release kinins and these are rapidly inactivated in plasma by kininase enzymes. The timing of the kallikrein increase is in agreement with earlier results of Boreham (1968), who established a pattern of events in man, cattle and rabbits where at approximately 8-10 days after infection with trypanosomes raised kinin concentrations were found in the blood. This was seen to follow an initial crisis in the infection, marked by the first variant parasitaemic remission and pyrexia, these two factors being associated in man infected with T.rhodesiense (Bailey and Boreham, 1969), although conflicting evidence has been presented for T.brucei infected rabbits (Goodwin, 1970; van den Ingh, 1976). A stress state such as pyrexia however, might well have some bearing during the disease as fibrinolysis has been shown to occur with this condition (Sherry et al., 1959).

The increase in kallikrein levels seen early in the infection could arise in one of several possible ways. The conversion of pre-kallikrein to kallikrein would naturally result in this increase, some of the kallikrein presumably being inhibited by the circulating plasma protein inhibitors, such as α_2 -macroglobulin and C $\bar{1}$ INH. Whether these inhibitors are capable of inhibiting all the kallikrein produced from the available prekallikrein is unknown, but would seem unlikely as it has been demonstrated in this work that a small amount of spontaneous kallikrein activity is present in normal rabbit plasma. A reduction in the natural kallikrein inhibitors or an alteration of the inhibitor profile, due to the disease state, could be responsible for the observed changes in kallikrein levels. Changes in these parameters have been noted previously by Colman et al. (1969c) in a case of E.coli bacteraemia and in B.argentina infections of splenectomised calves by Wright and Mahoney (1974). It would therefore be of interest to study inhibitor levels in T.brucei infections.

Another mechanism which could be envisaged is an increased synthesis of the components of the kallikrein-kinin system. Some rabbits did show elevated values for total kallikrein shortly after infection and this is presumably due to an increase in the level of circulating prekallikrein. Both prekallikrein and kininogen are synthesised in the liver (Fanciullacci et al., 1976) and trauma, as well as local inflammation, leads to an increased synthesis by the liver of certain plasma proteins, the acute phase reactants. Kininogen has been shown to belong to this category (Borges and Gordon, 1976), while studies of Boreham (1968) suggested an increased turnover rate of plasma kininogen in the course of T.brucei infection in cattle.

Following the peak levels of kallikrein a reduction was seen, although the levels remaining were above those found initially, and some fluctuation was apparent as the infection progressed. This reduction would indicate that the available prekallikrein, having been activated, was not being synthesised at a sufficient rate to restore the levels to normal, with continual activation of the kallikrein-kinin system, through a number of mechanisms, being one possible explanation for this situation, during the disease. The experimental work would support this suggestion as the total kallikrein present in the plasma fell, representing a decrease in prekallikrein, from day 14 after infection. The depression of prekallikrein may also be compounded by an impaired synthesis resulting from damage to the liver (Seed and Gam, 1967; van den Ingh, 1976).

The increase in the levels of plasma kallikrein at this time in the infection must be considered an important factor in the initiation of pathological changes in tissues and organs which may eventually contribute to the death of the host. In principle there are no fundamental differences between the effects of plasma kallikrein and the kinins on the cardiovascular system. Kallikrein, as outlined in the introduction, does have some direct actions, the most important of these is in effecting changes in the circulation, through the liberation of kinins.

A number of effects of kallikrein related to the signs and symptoms of inflammation, which is probably the most damaging aspect of trypanosome infection caused by brucei subgroup organisms, can therefore be considered. The inflammatory response is a normal host

reaction following the initial introduction and subsequent establishment of a viable parasite population and when chronic will have severe and far-reaching effects on fundamental tissue haemostasis. The four cardinal signs of inflammation - vasodilation, increased vascular permeability, pain and accumulation of leucocytes - appear after all kinds of tissue injury and this led to the idea that these actions may be mediated by a single chemical substance, such as kinins. Therefore kinins may be largely responsible for the observed oedema and vascular changes as well as the cardiovascular failure, and contribute towards haemostasis and tissue anoxia seen in trypanosome infected animals (Goodwin, 1974). Bradykinin and related peptides exhibit pharmacological actions which qualify them as plausible mediators of inflammation but a relevant criterion in suggesting the action of an endogenous substance concerns the duration of its effects. One objection which has been raised against any role of plasma kinins in inflammatory reactions, however, is that their action is transient whereas the inflammatory response is prolonged. An explanation for this though, is that once the kinin forming system is activated the duration of the response depends not upon the transient actions of the kinins themselves but upon those of the activated kallikreins which results in the continuous formation of kinins. The results already presented have indicated that the kallikrein-kinin system is being continually activated during T.brucei infection, the hypotension seen in infected rabbits, suggested as being mediated by kallikrein or kinin (Boreham and Wright, 1976b), is an indication of this. The onset of all reactions to kallikrein is also somewhat delayed in comparison to that elicited by kinins, while the reactions fade away more slowly.

The initial lesion in most inflammatory reactions is a change in the endothelial wall of the small blood vessels which cause leucocytes to stick, then aggregate, and often completely occlude the vessel (Florey and Grant, 1961). The sequence of early events has been described in detail more recently by Hurley (1978). Mild vascular lesions are present after about 7-10 days in chronic trypanosomiasis and are extremely severe after 3-4 weeks. Goodwin and Hook (1968) demonstrated, in the rabbit infected with T.brucei, that venous drainage of muscles and viscera was congested, the tissues were oedematous and that the walls of the blood vessels were fragile and surrounded by granulomatous tissue. Similar lesions have also been observed in cattle with relapsing T.congolense infections (Fiennes, 1946) and T.vivax infections of cattle, goats and pigs (Losos and Ikede, 1972; van den Ingh et al., 1976a). Recent histopathological studies (Edeghere, 1980) have demonstrated the features of inflammation in T.brucei infected rabbits, manifested by the infiltration of tissues and the lining of venules and capillaries by leucocytes, oedema and tissue necrosis, the changes being evident 14-21 days after infection. The observations correlate well with the timing of increased kallikrein levels in the present study and are consistent with known vascular events in inflammation due to chemical mediators.

One of the most important pathological changes that occurs in T.brucei infections is the increase in vascular permeability (Boreham and Goodwin, 1967; Boreham, 1974). Bradykinin and kallidin are rated among the most effective permeability-increasing factors in most species (Garcia Leme, 1978), although several pharmacologically active substances have been implicated in trypanosomiasis (reviewed by Boreham and

Wright, 1976a). As a direct consequence of their permeability-increasing properties, kinins induce accumulation of fluid in the interstitial spaces, resulting in oedema, and bradykinin is some ten times more potent than histamine in producing swelling (Maling et al., 1974). T.brucei infected rabbits show gross oedema affecting all tissues (Goodwin, 1970) and van den Ingh et al.(1976a) found oedema in three goats killed during the acute phase of T.vivax infection. The increased permeability induced following activation of the kallikrein-kinin system therefore has severe consequences for the host, as plasma and lymph constituents are allowed to escape from the vessels. This could be another explanation for the reduction of prekallikrein in the later stages of the infection and presumably if kininogen also escapes, suitable activating conditions would lead to formation of kinins with consequent localised tissue damage. It is generally recognised that an acidic environment prevails in tissue damage and that increased proteolytic activity occurs (Morsdorf, 1969). Prekallikrein is activated at acid pH (Werle, 1934) and Edery and Lewis (1962) have shown that kininases are inhibited under these conditions. Both factors would promote kallikrein-kinin system activation and accumulation of kinins locally. Damage to blood vessels and exposure of subendothelial connective tissue may also activate the kallikrein-kinin system.

Collagen has been shown to activate kallikrein through an HF-dependent pathway with the enzymes becoming bound to the collagen surface, thereby protecting them to some extent from fluid phase inhibitors (Harpel,1972). More recently Wiggins et al.(1980) have demonstrated that cultured rabbit endothelial cells contain an

enzyme that cleaves and activates HF with subsequent cleavage of both factor XI and prekallikrein. Whether this activity can be released from stimulated or damaged endothelial cells, or remains associated in some way with the injured cell, has not been determined. These factors would seem to suggest a mechanism of continued prekallikrein activation in a self-perpetuating manner, via HF, as the infection progresses.

An integral part of the inflammatory reaction is the migration of cells into the affected area. Graham et al.(1965) have observed sticking and migration of leucocytes in vessels of the rabbit ear, induced by bradykinin, and although this finding is not an indication that the peptide is the cause, it did promote leucocyte accumulation in the mesenteric vessels of the rat (Lewis,1962). It is now clear that chemoattractants are generated by activation of enzyme cascades other than complement in plasma. Kallikrein is one such factor for both neutrophils and human blood monocytes (Kaplan et al., 1972; Gallin and Kaplan, 1974). Chemoattractants for neutrophils are also released during clotting of blood and clot retraction (Stecher et al., 1971),and such activity has been shown in fibrinopeptides (Kay et al., 1973). Chemotactically attracted phagocytes are of importance in tissue histiolysis,the destruction of basement membranes by cathepsins, elastases, proteases and collagenases appears a common feature in all general inflammations (Wilkinson,1974). Epithelial attachment of macrophages could provide an opportunity for the secretion of various enzymes directly onto the surface, and stimulated macrophages are able

to release C3 cleavage enzymes which can in turn react with the macrophages causing the release of lysosomal enzymes into the medium (Schorlemmer and Allison, 1976). Membrane and other tissues are enzymatically disrupted and this, combined with the release of vasoactive substances, enhances vascular permeability.

Platelet clumping and degranulation is often associated with an increased local phagocytosis and platelets adhere to exposed collagen fibres and subendothelial microfibrils when blood vessels are damaged (Zucker, 1974). While it is probably true to say that in general platelets do not often play the central role in inflammatory processes, they probably alter the course of the reaction by acting as yet another source for the release of pharmacologically active substances. A detailed account of platelet aggregation mechanisms, and their implications in haemostasis and inflammatory disease, has been given by Willis (1978). One important factor released by platelets however is prostaglandin E_2 (PGE_2), which not only increases vascular permeability and releases histamine from mast cells (Crunkhorn and Willis, 1971), but also potentiates the pro-inflammatory effects of other mediators such as bradykinin (McGiff et al., 1976). Abundant experimental evidence supports the view that PGs influence the development and modulation of immune and inflammatory reactions (Vane, 1976; Zurier, 1979) and is an area of experimental research which is increasing rapidly. PGE compounds can both suppress and enhance cell-mediated and humoral immunity in vitro and appear to regulate the character and intensity of the immune response in vivo (Zurier, loc. cit.).

Their role in immune/inflammatory reactions is complex and not well understood but they do appear important in regulating cell functions and host defences (reviewed by Goodwin and Webb, 1980).

The interrelationships of the four major blood enzyme systems, kallikrein-kinin, fibrinolytic, coagulation and complement, are important in determining some of the factors which lead to pathological consequences in trypanosomiasis. It is evident that activated HF is an important controlling factor when considering these systems and, as already mentioned, kallikrein plays a significant role in the fluid phase activation of this component. Plasma kallikrein will therefore influence the changes in the other systems when its levels increase through conversion of prekallikrein via HF. Both plasma kallikrein and HMW kininogen are however also involved in the solid phase activation of the intrinsic blood clotting cascade and fibrinogenolysis directly. Evidence of changes in these systems has been demonstrated in trypanosome infections.

The hydrolysis of LMe by rabbit plasma, at a time shortly after maximum kallikrein levels were seen, could indicate an increase in the amount of plasmin present following activation of plasminogen through an HF dependent pathway involving kallikrein as already described. Boreham and Facer (1974) have demonstrated a decrease in plasminogen concentrations in T.brucei infected rabbits at a corresponding time after infection. They also reported an inverse relationship between these concentrations and FDP, the breakdown products of fibrinogen

and fibrin, which would be expected following an increase in amounts of plasmin. Plasmin itself may also play a role in HF activation, since it has been shown to digest HF to its active fragments (Revak et al., 1974). FDP have also been reported in human trypanosomiasis (Greenwood and Whittle, 1976b; Robins-Browne and Schneider, 1977). Alterations in the intrinsic pathway of coagulation are evident in both humans and cattle with acute trypanosomiasis (Robins-Browne and Schneider, loc.cit., Wellde et al., 1978), and in the former study clinical evidence of a bleeding disorder was seen, which was attributed to a marked thrombocytopenia due to platelet pooling and a reduction in platelet half-life. An increase in some of the clotting factors, particularly factors VIII and XII (HF), have also been reported in T.brucei infections of the rabbit (Boulton et al., 1974). On the basis of thrombocytopenia and elevated FDP, investigators have implicated disseminated intravascular coagulation (DIC) as an additional consequence of trypanosome infections (Barrett-Conner et al., 1973; Boreham and Facer, 1974; Wellde et al., 1978).

The increase in kallikrein seen during T.brucei infection in the rabbit occurred at a time when immunoglobulin levels, both IgM and IgG, were increasing and it was associated particularly with the first variant parasitaemic peak. IC proved to be the only factors capable of activating the kallikrein-kinin system both in vitro and in vivo as demonstrated in this experimental work. The soluble IgG complexes were seen to be increasing at a similar time to kallikrein release and were therefore suggested as being responsible for the

initial activation, as IgG has a strong affinity for the common antigens of trypanosomes (Mattern et al., 1963).

It has been proposed for some time that IC are important in the activation of the kinin system in trypanosomiasis. The suggested mechanism of activation was that IC formed during the infection absorb HF onto their surfaces, causing its activation (reviewed by Boreham and Wright, 1976a). Work in related subjects would seem to support this hypothesis. Davies and Lowe (1960) demonstrated a factor (PF/P) which increased capillary permeability in guinea-pigs when a washed precipitate prepared from egg albumin and anti-egg albumin rabbit serum was added to fresh neat or moderately diluted guinea-pig serum. Movat and DiLorenzo (1968) and Movat et al. (1968) have shown that when non-glass contacted guinea-pig or rabbit serum was incubated with washed antigen-antibody precipitates, the serum induced enhancement of vascular permeability upon injection intradermally into blue guinea-pigs or rabbits. Some of this property of activated serum was due to the activation of the kinin system. It seems reasonable to speculate that Ag-Ab aggregates have 'active-sites' which can interact with pro-Hageman factor, leading to activation and release of kinin. The work of Cochrane et al. (1972) however offers no evidence that HF interacts with IC and fails to suggest the possibility that immune aggregates are capable of activating HF. It should be pointed out that in their studies they made use of purified HF and this may have a different activation profile when not present in serum or plasma, although the addition of plasma or serum to immune aggregates did not lead to activation of HF.

In trypanosome infections the most important pieces of evidence to date implicating IC are that complexes cause the release of kinins in vitro from a kininogen substrate and that injection of preformed IC in vivo into rabbits causes a profound hypotension which is inhibited by aprotinin. The intravenous injection of trypanosomal antigen into previously immunized animals also results in hypotension caused by the activation of the kallikrein-kinin system (Boreham and Wright, 1976b). Although blood pressure was not measured in the current experimental work, severe reactions were apparent following injection of trypanosome material into immunized animals, with contraction of blood vessels and death occurring in one case. Barabé et al. (1979) have shown two different types of specific receptor, B_1 and B_2 , in vascular preparations, on which bradykinin exerts direct or indirect actions. Receptors of the B_2 type are widely distributed in peripheral vessels and appear to subserve both vasoconstriction and vasodilation leading to a dual role for bradykinin action.

It has been suggested by Boreham (1969) that the trypanosome complexes may be negatively charged and this may lead to the activation of HF. It is interesting to note that T.brucei bloodstream forms are electrically neutral at physiological pH (Cross, 1978). However, surface charge measurements have never been carried out using antigenically homogeneous populations of trypanosome variants whose isolated surface glycoproteins differ widely in iso-electric point. After the loss of surface coat, cell ghosts carry a strong negative charge. This may have some relevance, possibly by the direct activation of HF. The fact that no significant alteration of kallikrein levels was seen early in the infection (day2-4), when circulating parasites were detected,

and no activation was achieved in vitro or in vivo using trypanosome material, would suggest that the negatively charged surface alone is not contributing to HF activation in this instance. However, Eisen (1969) has shown that precipitated experimental complexes carry a slight negative charge and if the ionic charges on complexes occurring in vivo were similar, these complexes would tend to activate and absorb the intrinsic plasma kinin-forming system, owing to their surface properties. The activation of HF by soluble complexes (Kaplan et al., 1971) may also be explained by the binding of such complexes to Clq, which possesses a chemical composition similar to collagen (Yonemasu et al., 1971). The formation of IC during the disease is clearly an important factor in the pathology. In this present study circulating IgG complexes were present for a long period throughout the disease and this could be a mechanism of continual kallikrein activation. A number of other pathological effects have already been outlined in the introduction but a significant factor in this present context would be the activation of the HF-dependent pathways by collagen or vascular basement membranes as a result of immunologic tissue injury initiated by the complement pathway. Coagulation abnormalities seen during the infection may be due to activation of the intrinsic pathway in the presence of IC (Robbins and Stetson, 1959), whereas indirect action may result from the interaction of complement-fixing IC with cellular elements, such as platelets (WHO, 1977). On the basis of thrombocytopenia and elevated FDP, investigators have implicated disseminated intravascular coagulation (DIC) as an additional consequence of trypanosome infections (Barrett-Conner et al., 1973; Boreham and Facer, 1974; Wellde et al., 1978).

The decrease in levels of C3 seen at a similar time to increase in both IC and parasite numbers in infected animals in the present work is indicative of activation of complement through the classical and alternative pathways. Most IC formed in vivo can activate the complement system, although IC containing IgG₄, IgA, IgD and IgE do not activate C1 (reviewed by Muller-Eberhard, 1975). A fall in serum complement levels would be expected in a type III hypersensitivity reaction in antigen excess, as is found in trypanosome infections with a number of stages being reached when optimum proportions of antigen and antibody will permit complex formation. A decrease in levels of C3 has been reported previously in experimental infections (Nagle et al., 1974; Kobayashi and Tizard, 1976; Lambert and Galvao Castro, 1977) and in man (Greenwood and Whittle, 1976a).

Complement has several roles to play in disease processes, especially in the inflammatory reaction (Nelson, 1974), and these are mainly through the chemotactic and anaphylatoxin activities of C3a and C5a, the adherence reactions of C3b and the neutrophil mobilising factor, which is a cleavage product of C3 distinct from C3a. C3a and C5a have been reported to exhibit little specificity being chemotactic for neutrophils, eosinophils and monocytes (Goetzl and Austen, 1974), while the haemolytically inactive fluid phase complex C5b67 is chemotactic for neutrophils and eosinophils (Ward, 1972). In a number of clinical conditions the activation of complement may be masked by an increased synthesis and therefore the analysis of the complement profile is not conclusive (Ruddy et al., 1972), although the demonstration of some complement components by metabolic studies, or

more practically by demonstration and quantitation of complement breakdown products, is very useful in suggesting the presence of IC. In the present investigation initial increases in C3 levels were suggestive of an increased synthesis. The primary site of synthesis of C3 in vivo is the liver (Alper et al., 1969) and limited data would suggest that the hepatocyte may be its cell of origin (Johnson et al., 1971). However evidence for extrahepatic C3 biosynthesis by cells of the monocyte/macrophage series has also been obtained. (Stecher and Thorbeck, 1967; Einstein et al., 1977). Whether part of the fall in C3 levels could be attributed to a decreased synthesis of this component is not known. It would not be anticipated that the liver would be damaged early in the infection but this may occur at a later stage as already mentioned and changes in the cytopathology of hepatocytes have been noted in T.gambiense infections of guinea-pigs (Lumsden et al., 1972). More recently a direct activation of the complement system by trypanosomes has been demonstrated (Musoke and Barbet, 1977; Nielsen and Sheppard, 1977; Nielsen et al., 1978, 1979). The fact that C3 levels in T.brucei infected rabbits were not depressed to such a marked extent as those described in other trypanosome infections could indicate that not all the mechanisms suggested for complement activation are effective in this case. Complement depletion in vivo with cobra venom factor has been shown to cause a decrease in IgG antibody production (Pepys, 1974) and an elevation in IgM antibody (Nielsen and White, 1974). The present work has demonstrated a marked increase in both IgG as well as IgM concentrations during the infection, which may indicate less disruption of the complement system, or that synthesis of the complement components continues in parallel with activation.

The increase in both IgG and IgM seen during T.brucei infection in rabbits would be expected, as African trypanosomiasis is characterised by waves of parasitaemia which reflect the successive occurrence of different antigenic variants of the parasite surface coat, as well as the release of common antigens on lysis of the parasite. It is known that the levels of both variant and common antibodies rise shortly after infection (Gray, 1962). Since macroglobulins are usually associated with the early immune response, the high levels of IgM could be explained by the sequential production of antibodies against variant surface antigens (Seed, 1972). However, this is not necessarily the explanation because in addition to anti-trypanosomal antibodies, the IgM fraction contains heterophile and anti-IC antibodies very similar to rheumatoid factor (MacKenzie and Boreham, 1974; Boreham, 1974). The IgG on the other hand, which is directed against the common antigens, may be of particular importance in trypanosomiasis since, in man, circulating antigen-IgG complexes occur repeatedly during the infection (de Raadt, 1974).

The immunological effects of the various antibodies produced during infection remains controversial. Takayanagi and Enriquez (1973) have indicated that IgM is more effective in agglutinating trypanosomes and inducing antigenic variation than is IgG. IgG however appears to be the most effective opsonising Ig capable of attaching to specific trypanosome surface receptor sites with its efficiency possibly enhanced due to the powerful agglutinating ability of IgM. Other experimental evidence strongly suggested that IgG molecules, either alone (Tizard and Soltys, 1971), or more effectively, those capable of fixing complement

(Cook, 1977) were of importance in chronic trypanosome infections in rabbits. Opsonic and macrophage cytophilic antibody activity was present in the sera of mice and rabbits infected with T.brucei, the degree of attachment dependent on the presence of specific antibodies, most notably IgG (Cook, 1977). Cellular receptors appear to bind complexed immunoglobulin (IgG) more avidly than they do free immunoglobulin because of the potential for multipoint attachment. In several systems this has been confirmed in thermodynamic terms (Arend and Mannik, 1973; Segal and Hurwitz, 1977), and the importance of IgG subclasses resides in part with their functional properties, usually associated with the Fc portion of the molecules. Receptors for Fc of some IgG subclasses are present on a number of cells including lymphocytes, monocytes - macrophages, platelets, neutrophils and basophils - and mast cells (reviewed by Spiegelberg, 1974). The role of immunoglobulin classes and subclasses and their interrelationships needs further clarification, but the presence of IgG does seem to be important during T.brucei infections as outlined here, and in its ability to form IC with their attendant pathological activity.

The increase in IgM however cannot be ignored, and high levels will presumably affect the host in some way. Increased IgM in conjunction with increased fibrinogen will affect blood viscosity (Boreham and Facer, 1974) and the formation of IC may have some effects. Nagle et al. (1974) have demonstrated the development of glomerulonephritis during the course of T.rhodesiense infection in monkeys, associated with glomerular deposits consisting of C3, properdin and IgM. In terms of activation of the kallikrein-kinin system however, if IC of

IgM and trypanosome antigen are formed early in the infection, as IgM levels were seen to rise before IgG, no increase in kallikrein was evident at this time. Further experimental evidence for involvement of IC containing specific immunoglobulins could be obtained by separation of infected serum, by gel filtration, and preparing complexes with the separated fractions and trypanosome material. IgG complexes prepared in this way have demonstrated a kallikrein activating ability in vivo, but as yet IgM complexes have not been tested due to some IgG contamination (S. Whittle, personal communication).

The fate of circulating IC depends upon their size, composition and on the activity of macrophages and the reticuloendothelial system (RES). The circulating IgG complexes measured in the serum of T.brucei infected rabbits in this work were present for some weeks, whereas it could be expected that larger complexes would be removed from the circulation more rapidly. Circulation of larger complexes however may occur when the RES is saturated and when macrophages and other phagocytic cells fail to remove them (Mannik et al., 1974). Several other factors may play an important role in the persistence of circulating IC, their deposition and perpetuation of the immunopathogenic lesions, such as affinity of the antibodies involved and complement activation. Apart from circulating IC it is likely that a large amount of IC are also formed locally in tissues where trypanosome infiltrates are frequently observed; and T.brucei is a tissue parasite (Goodwin, 1974). Again immunoglobulin size is probably important, with the lower molecular weight IgG more likely to be initially present in tissue fluid than the larger often membrane resistant IgM, although increases

in vascular permeability do occur as already mentioned, and may lead to leakage of IgM (Goodwin and Guy, 1973). A local formation of IC in the heart tissue of mice infected with T.brucei has been shown with the complexes formed mainly of trypanosome antigen and corresponding antibody (Lambert and Houba, 1974).

Identification of the components of the soluble circulating IgG complexes, following PEG precipitation and protein A binding, revealed the presence of IgG, IgM and one other major component of 72,000 M.W. A small number of protein bands in addition to those evident in uninfected serum were seen in the filtered infected serum fractions, and these latter bands may be trypanosome antigen components. In terms of the IgG complexes found during the infection, the lack of trypanosome antigen involved has already been suggested due to the small amount of IgG, purported to be specific for trypanosomes. The method involving PEG precipitation however could also compound these findings as the precipitation of IgG-trypanosome antigen complexes may not occur due to their molecular size or that conformational changes in the combination of the immunoglobulin and antigen plays a significant role in the precipitability.

Two proposals for the involvement of IgM in the complex have already been made. It is possible that free rheumatoid factor-like IgM and monomer IgG may coprecipitate in PEG and form an artificial IC, since reaction of rheumatoid factor (RF) with normal IgG has been reported (Gabriel and Agnello, 1977). Harkiss and Brown (1979) however, found no relationship between the RF and IC levels in their studies and

the proposed reaction probably does not occur to any great extent, due to the small amounts of monomer IgG precipitated at low PEG concentrations. It is also possible that IgG itself could be acting as an antigen, as in rheumatoid patients, immunofluorescent studies on the synovial tissue have shown that 50% or more of the plasma cells are making antibody to IgG (Munthe and Natvig, 1972). The data of Male et al. (1980) is consistent with the hypothesis that IgG is the main, if not the only, antigen responsible for provoking and maintaining the pathological changes in rheumatoid arthritis.

Available evidence would suggest that a C3 fraction (72,000 M.W.), is associated with IgG and IgM, as the levels of this serum component have been shown to decrease both in the present study and as mentioned earlier, at a similar time to increases in the circulating complexes indicating that some of these complexes activate the complement system. Deposits of immunoglobulins, both IgG and IgM, and C3 have also been demonstrated in the kidneys of various animal species in a number of parasitic infections, including trypanosomiasis (see Houba, 1976), and this provides evidence of a circulating IC disease.

It is interesting to note that circulating IC, which are assumed to contain both antigen and antibody, are being reported in an increasing number of seemingly unrelated conditions. In vitro experiments of Soltis and Wilson (1978) suggest the possibility that immunoglobulin aggregation might occur in vivo in pathological conditions associated with hypoalbuminaemia and/or hypergammaglobulinaemia. Alterations in

the concentrations of other serum proteins might also affect the degree of immunoglobulin aggregation. These aggregates, like true IC, could mediate tissue injury by deposition in vessels, activation of the kinin and complement systems in blood, as well as binding to cells possessing Fc receptors. Abnormalities in both IgG and albumin concentrations are seen in trypanosome infections, indicated by changes in serum total protein concentrations. Other experimental evidence indicates a greatly increased turnover of serum proteins in T.congolense infected cattle, the most dramatic changes seen in IgG₁ and IgG₂ (Nielsen et al., 1978a). Similarly the catabolic rate of IgM, IgA and IgE increased. Similar findings have been described for globulins, but not albumin, in T.brucei infected mice, and Freeman et al. (1970) were unable to demonstrate any change in the catabolic rate of serum globulin in T.brucei infected rhesus monkeys. These changes if associated with an impaired clearing by the RES could lead to conditions favouring immunoglobulin aggregation. Further studies are clearly needed to test this hypothesis.

The fact that trypanosomes, and their products on lysis, are present in the circulation at intervals during T.brucei infections must also be considered when looking at pathological mechanisms in this disease. Although several early workers postulated the presence of toxins, injection of large numbers of trypanosomes intravenously or intracranially produced no clinical symptoms (Boreham, 1974). A recent comprehensive review (Tizard et al., 1978) has described a range of factors which can be derived from African trypanosomes that may be capable of inducing all the major lesions common to most forms of these diseases, including

a profound immunosuppression, hypocomplementemia, widespread micro-vascular damage and erythrocyte destruction. More quantitative information will be required before the connections between these factors and the major lesions can be made.

Experimental work described in this thesis indicated that live or disrupted trypanosomes were unable to activate the kallikrein-kinin system both in vitro and in vivo, as determined by plasma kallikrein measurements. These results are in agreement with those of Boreham and Goodwin (1970) who could not obtain any kinin release from fresh plasma when incubated with trypanosomes. However this is not analagous to the situation seen in acute babesiosis and malaria, where it is likely that a common initiating mechanism exists for activation of plasma kallikrein, namely the release of kallikrein-activating enzymes from the parasites (Levy et al., 1974; Wright, 1975). In B.bovis infections these enzymes have also been shown to cleave kinin from its precursor kininogen and to cause activation of the coagulation system (Goodger and Wright, 1977). The lack of any change in plasma kallikrein activity early in trypanosome infections when parasites are first detected is therefore not unexpected. A change in blood bradykinin-like activity however has been reported to occur at this time, associated with the first temperature peak (van den Ingh, 1976), and this could possibly be explained by the release from the trypanosomes of proteases, particularly cathepsin D (Venkatesan et al., 1977). This protease can act on leukokininogen to generate leukokinins, although it is thought to be a system more involved in the chronic phase of the disease as leukokininogen is considered a pathological substrate (Greenbaum, 1975). It is interesting to note that urinary

kallikrein concentrations begin to increase after 2-3 days in rabbits infected with T.brucei (Wright and Boreham, 1977) which would seem to preclude an immunological mechanism. This may relate to the presence of parasite products, with alterations in mineralocorticoid levels in the host being one possible explanation (Boreham and Parry, 1979). The presence of free fatty acids and phospholipases with the ability to release prostaglandins of the E series may also be of some importance.

The use of a number of drugs, in the experimental work, did not prove conclusive in establishing a direct mechanism of kallikrein activation by IC. No significant alteration in kallikrein levels was evident when the complex levels were reduced, indicating that the formation of IC, even in comparatively small amounts may be sufficient to activate the kallikrein-kinin system via HF. An interesting observation in some drug treated rabbits however was an increase in kallikrein levels early in the infection associated with large numbers of circulating parasites. Another mechanism of kallikrein activation would therefore have to be postulated at this time. With little antibody present IC activation of the system would not occur and direct tissue damage may be a contributory factor to solid phase activation of HF and an increase in kallikrein activity. In T.vivax infected goats, Veenendaal et al. (1976) have suggested that bradykinin-like activity found early in the infection may be associated with pyrexia and be similar to endotoxin shock. However, injection of disrupted T.brucei into uninfected rabbits in the current work did not cause any alteration in kallikrein levels. Another possibility is that direct activation of complement, by factors such as the trypanosome surface glycoprotein (Musoke and Barbet, 1977), is involved, with possible adherence of the C3b component leading to lysosomal enzyme release. This can produce C5a from C5 and may fragment basement

membranes and so activate HF. The involvement of platelets in an immune adherence type phenomenon naturally results in thrombocytopenia and, with their disruption, the release of phospholipids, serotonin and ADP all of which serve to accelerate the clotting process and may therefore affect the kallikrein activation. Free fatty acids are capable of inducing thrombocytopenia in experimental animals (Zbinden, 1964) and they have been demonstrated in trypanosome autolysates (see Tizard et al., 1978), although whether sufficient material capable of producing an effect could be generated in vivo is not known. The fact that parasite numbers were markedly different from untreated infected animals could be due to either a depression of the 'natural' immunity of the rabbits, as Petana (1964) suggested that cortisone treatment of T.rhodesiense infected rats may have aided parasite reproduction by reducing the immune response, or to a delay in the induction of the normal immune response, assuming that these factors are instrumental in initiating antigenic variation. In the present study a reduction in both serum IgG and IgM concentrations was apparent in the dexamethasone treated animals when the parasite numbers increased initially. The alteration in the parasitaemia will depend on numerous factors including the drug dose and the time of administration in relation to the course of the infection, as well as the response of the host and the virulence, susceptibility and adaptability of the trypanosomes. A varied response was obtained by Ashcroft (1959) who found that the administration of cortisone to rats infected with T.rhodesiense altered the parasitaemia but some experiments led to an enhancement and others to a reduction. Some of the pathological symptoms evident during trypanosome infections could perhaps be due to the prolonged destruction of trypanosomes in large numbers, as indicated by an initial alteration in the kallikrein activation profile in some drug treated animals.

The fact that the severity of the disease was also enhanced in the corticosteroid and Cy treatments may have been due in part to the increased parasitaemia, although the immunosuppressive effects of these drugs was probably the major contributing factor as immunosuppression may predispose the animal to secondary infections, which could contribute to the severe physical condition and premature death. The possibility that toxicity of these two drugs may have had some effect must also be considered, as uninfected animals did show some deterioration in condition following administration of these agents. The reduction in C3 seen with these drug treatments may also compound the susceptibility to infection. The reduction, as mentioned earlier, was associated with IC formation and circulating parasites, although a depression in the titre of a number of complement components including C3 has been observed following high dose corticosteroid treatment (Atkinson and Frank, 1973). However, these studies were undertaken in the guinea-pig, a steroid sensitive species. The mechanism of action of the drugs used in the current experimental work, on alteration of C3 concentration, is not known. Synthesis may be affected because serum complement levels rise in a number of acute inflammatory states and anti-inflammatory drug activity may therefore reduce the synthesis, leading to lower titres compared to untreated infected animals, when activation of the system occurs. This was true for indomethacin but not for penicillamine treatment, although the view that penicillamine is not an anti-inflammatory drug is supported by its lack of effect on conventional models such as adjuvant arthritis (see Huskisson, 1979). A reduction in C3 in aprotinin treated rabbits may also be related to the 'anti-inflammatory effect' through inhibition of kallikrein and plasmin.

How useful the immunosuppressive drugs are in affecting the course of trypanosome infections is questionable. Kallikrein activity was not altered significantly in these experiments but the humoral antibody response and IC levels were reduced, but this in turn may lead to more severe consequences with a failure of the immune system to respond to other antigenic stimuli. As immunosuppression is a known consequence of trypanosome infections further suppression imposed by drug treatment should possibly be avoided. However a reduction in IC levels could alleviate some of the IC mediated pathological symptoms that occur during the disease. The mode of action of the drugs is complex (see Berenbaum, 1974) with effects on both T and B cell populations as well as alteration of some macrophage functions, such as antigen presentation. Experimental evidence (MacKenzie et al., 1978) has suggested that suppression of adjuvant disease by Cy might involve the depletion of B lymphocytes, although depletion of non-specific inflammatory cells might also account for the observed suppression. In the present work an enhanced antibody response was apparent in some instances, with elevated levels of IgG and IgM, and a number of mechanisms may be involved in this situation. IC can modulate the activation of T and B lymphocytes and a selective inhibition of B cells by the drugs might, by reducing the formation of IC, lead to increased cytotoxic activity of T cells, or conversely, a selective inhibition of suppressor T cells might result in the increased antibody production by B cells. As several types of immune reactions, including immediate hypersensitivity (Tizard and Soltys, 1971), delayed hypersensitivity (Mansfield and Kreier, 1972; Tizard and Soltys, 1971) and auto-antibody production (MacKenzie and Boreham, 1974), have all been considered in relation to the pathology of trypanosomiasis, some form of immunosuppressive therapy may be of value. Corticosteroids have been shown to influence all the reactions outlined above and more recent experimental

evidence (Makker, 1978) may be of some relevance to trypanosome infections, as dexamethasone did not significantly suppress the humoral antibody response of rats to SRBC but it did have a pronounced effect on the humoral autoantibody response, suggesting that the mechanisms of the two responses may be different.

With inflammation being a significant consequence of T.brucei infections, the activity of anti-inflammatory drugs could prove useful in restricting this condition during the disease. As several classes of these drugs are currently believed to exert their anti-inflammatory action, at least in part, by inhibiting immunological processes their activity is therefore obviously varied and has been comprehensively considered recently (Vane and Ferreira, 1979). No direct evidence was obtained for inhibition of the kallikrein system with indomethacin or penicillamine during experimental treatment, but the activity of these drugs, particularly penicillamine, may only be effective when given for prolonged periods. Hunneyball et al. (1978a,b) have demonstrated an effect of D-penicillamine on both humoral and cellular parameters of the immune response in experimental arthritis, with drug treatment daily for periods of up to 410 days.

In considering the pathology of trypanosome infections it is evident that a number of different interacting and sequential events occur, a significant factor being the formation of IC and the activation of the kallikrein-kinin system. It would appear that modification of the immune response, in terms of suppression, is not a satisfactory practical measure for altering some of these symptoms and may in fact compound the situation. The role of non-specific stimulation of the immune system however, although not investigated in depth at present,

may prove more beneficial, as this has been shown to significantly alter the course of trypanosome infections in mice (Murray, 1978). Inhibition of kallikrein activity, on the other hand, may be a short term measure for minimising some of the initial changes, such as vascular permeability and the hypotensive state. The experimental data indicated a marked inhibition of kallikrein activity by aprotinin and the action of this drug was regarded for a long period of time as unspecific because it was effective in a variety of proteolytic systems, including coagulation, lysis, kinin generation, complement activation and the formation of myocardial depressant factor. However these symptoms, as already described, have in common esteroproteinasases including plasma kallikrein, various tissue kallikreins and plasmin, and it is known that aprotinin is specifically an esteroproteinasase inhibitor. This drug would therefore appear ideally suited, in terms of its inhibitory spectrum, for use in restricting the pathological changes in trypanosomiasis. However the reversible nature of the inhibitor and enzyme complex is dependent on the plasma concentration of aprotinin (Haberland, 1973), and recent studies have indicated that it is removed rapidly from the circulation (Philipp, 1977; Kaller et al., 1978). Therefore, in order to maintain a constant tissue and blood level, a continuous supply by drip infusion or repeated injections would be necessary during experimental or clinical therapy. The drug has previously been used in human disease states such as pancreatitis (see Haberland, 1976) but its use in animal trypanosomiasis would not seem to be practical. However it may be of some benefit in human cases, particularly if used with trypanocidal therapy, as a shock syndrome similar to a Jarisch-Herxheimer reaction is a feature of the response of individuals when treated at a time of elevated parasitaemia (Ormerod, 1970). Although severe hypotension occurs in T.brucei (Boreham and Wright,

1976b) it is not commonly found in human trypanosomiasis, but some reports do exist (Buyst, 1975) and aprotinin may be of some benefit in alleviating this condition. Other naturally occurring protease inhibitors, with an inhibitory spectrum comparable to that of aprotinin such as lima bean or soy-bean trypsin inhibitor, are therapeutically effective in animals. In man, however, they are potent antigens and therefore unsuitable as pharmacological agents. With the kallikrein-kinin system and IC playing an important role in the pathology of the disease alternative 'interfering' mechanisms could well be considered. The actions of both kallikrein and kinins could be affected by the use of synthetic analogues, or by alteration of their natural inhibitor profiles. A number of approaches to intervention in the activity of IC have previously been outlined (WHO,1977) but as already intimated, interference in the normal function of the immune system is probably unsatisfactory and the most suitable approach should be to alter the complex's potential for activating effector mechanisms.

Undoubtedly trypanosome infections are complex in terms of their pathogenesis and immunology, with some variation depending on the host and species of parasite. It is clear that further elucidation of both the basic biochemical interrelationships of the enzyme systems and the involvement of the immune response is required, before a more efficient application of therapy can be implemented to afford some relief from this disease.

APPENDIX 1

Buffers and Solutions

(a) Barbitone buffer pH 8.6

Barbital (5.5 Diethylbarbituric acid)	1.84g
Barbital sodium (5.5 Diethylbarbituric acid, Na salt)	10.3g
Distilled water	1.0 l

(b) Borate buffer pH 8.4

Boric acid	6.18g
Sodium tetraborate	9.54g
Sodium chloride	4.38g

Make up to 1.0 l in distilled water

(c) Brilliant croscein, coomassie blue (stain)

Brilliant croscein	250mg
Coomassie blue	15mg

Dissolve the brilliant croscein in 100ml 5% acetic acid and 3% TCA at 60°C. Cool to room temperature and add the coomassie blue.

(d) Phosphate buffered saline pH 7.2

NaCl	8.0g
KCl	0.2g
Na ₂ HPO ₄	1.15g
KH ₂ PO ₄	0.2g

Dissolve in 1 l distilled water.

pH adjusted when necessary by dropwise addition of 1N NaOH.

(e) Phosphate saline glucose (PSG) pH 8.0

0.85% NaCl	300ml
2.5% D-Glucose	400ml
0.2M Na_2HPO_4	285ml
0.2M NaH_2PO_4	15ml

(f) SDS gel electrophoresis

(i) Acrylamide stock

30% Acrylamide (recrystallised) BDH

0.8% Bis acrylamide

Dissolve in distilled water and filter. Protect from light.

(ii) SDS sample buffer - for 50ml volume

Glycerol	5.0ml
SDS (20%)	5.0ml
1M Tris-HCl pH 6.8	4.0ml
0.2% Bromophenol blue	0.5ml
Distilled water	35.5ml

(iii) Running gel (10%)

Acrylamide stock	10ml
1M Tris-HCl pH 8.8	11.2ml
20% SDS	0.15ml
Distilled water	8.7ml

De-gas for 5min then add:

10% Ammonium persulphate (100mg/ml) 100 μ l

TEMED

20 μ l

(f) SDS gel electrophoresis (contd)

(iv) Stacking gel (5%)

Acrylamide stock	1.67ml
1M Tris-HCl pH 6.8	1.25ml
20% SDS	0.05ml
Distilled water	7.03ml
De-gas for 5min then add:	
10% Ammonium persulphate (100mg/ml)	50 μ l
TEMED	10 μ l

(v) Running buffer

Tris base	3.03g
Glycine	14.42g
SDS	1.0g
Make up to 1.0 l in distilled water	

(vi) Staining

Methanol AR	202.5ml
Glacial acetic acid AR	26.5ml
Distilled water	260.0ml
Coomassie blue	62.5mg
Destain, omitting coomassie blue	

(g) (i) Tris-HCl buffer pH 8.0

0.1M Tris-aminomethane 250ml

0.1N HCl 134ml

Make up to 1.0 l in distilled water plus 58.4g NaCl.

(ii) Tri-HCl pH 8.4

Tris-aminomethane 1.514g

HCl (5.0ml 1N + 95.0ml distilled water) 83.0ml

Make up to 1.0 l in distilled water.

Plasma kallikrein levels in T.brucei infected rabbits

	Control*			549				550				551				552				553				554			
Day	K	PK	TK	K	PK	TK	P	K	PK	TK	P	K	PK	TK	P	K	PK	TK	P	K	PK	TK	P	K	PK	TK	P
0	17±3	67	84±6	20	62	82	0	15	56	71	0	16	72	88	0	19	53	72	0	21	54	75	0	18	73	91	0
2				23	61	84	1	17	57	74	0	18	72	90	2	17	63	80	0	24	56	80	0	17	69	86	1
4				24	61	85	0	18	60	78	0	15	71	86	0	18	64	82	0	20	61	81	1	17	70	87	0
7	15±4	68	83±4	22	65	87	2	23	57	80	3	21	68	89	0	23	48	71	8	26	58	84	0	20	60	80	0
9				35	49	84	4	32	44	76	0	28	54	82	5	29	46	75	3	39	43	82	2	19	66	85	6
11				39	40	79	0	30	42	72	1	34	46	80	0	37	32	69	0	43	40	83	2	25	59	84	2
14	16±4	69	85±5	33	42	75	0	21	61	82	0	30	41	71	0	31	30	61	0	42	39	81	0	33	47	80	0
16				28	53	81	1	23	51	74	2	26	47	73	8	31	22	53	0	40	36	76	0	30	41	71	4
18				26	56	82	0	24	45	69	12	ND	ND	ND	ND	33	25	58	4	28	41	69	2	25	37	62	12
21	18±3	66	82±6	25	54	79	0	20	42	62	0	27	38	65	0	26	32	58	0	27	44	71	0	28	37	65	0
23				28	48	76	0	25	43	68	0	24	43	67	1	24	40	64	0	29	34	63	0	26	33	59	0
25				29	46	75	2	21	44	65	0	20	48	68	0	ND	ND	ND	ND	33	30	63	1	22	42	64	0
30				27	45	72	0	20	45	65	3	23	43	66	0	29	31	60	0	32	42	70	0	25	42	67	1
37				29	47	76	ND	19	44	63	ND	26	44	70	ND	23	42	65	ND	28	41	69	ND	(Died Day35)			
42				32	42	74	ND	24	44	68	ND	(Died Day41)			ND	ND	ND	ND	(Died Day42)				(Died Day44)				
				(Died Day44)				(Died Day44)								(Died Day44)											

K = kallikrein PK = prekallikrein TK = total kallikrein (K+PK) P = parasitaemia ND = not determined

* Mean value for three rabbits

Table A

APPENDIX 2

Appendix 2 (contd)

Table B

Inhibition of plasma kallikrein

Day	<u>580</u>						<u>581</u>					
	<u>K</u>			<u>TK</u>			<u>K</u>			<u>TK</u>		
	N	A	B	N	A	B	N	A	B	N	A	B
0	16	1.5	1.5	89	4.5	5.0	20	2.5	3.0	90	5.0	5.5
2	18	1.5	1.5	87	5.0	5.0	19	2.5	3.0	93	4.5	5.5
4	16	2.0	2.5	90	4.5	4.5	21	2.0	2.5	92	6.0	5.0
7	21	1.5	2.0	86	5.0	5.0	25	3.0	2.0	89	5.5	6.0
9	27	2.5	2.0	85	5.0	6.0	31	3.5	2.5	91	6.5	6.0
11	29	2.5	3.0	83	4.5	6.0	35	3.5	4.0	90	6.5	5.5
14	22	2.0	2.5	80	6.0	5.5	39	5.0	5.0	88	7.0	6.5
18	21	2.0	3.0	75	5.5	4.5	33	3.5	4.5	79	7.0	6.5

Day	<u>582</u>						<u>583</u>					
	<u>K</u>			<u>TK</u>			<u>K</u>			<u>TK</u>		
	N	A	B	N	A	B	N	A	B	N	A	B
0	18	2.0	1.5	85	6.0	5.5	18	3.0	5.0	88	6.0	7.0
2	16	2.0	2.5	88	6.0	6.5	18	3.0	5.0	89	5.5	7.0
4	19	3.5	2.5	89	5.0	7.0	20	2.5	3.5	94	7.5	6.5
7	25	3.0	3.0	89	5.0	7.0	21	4.0	3.5	90	8.0	9.0
9	34	3.0	2.5	90	5.5	7.5	25	5.0	4.0	88	7.5	9.0
11	31	2.5	4.0	87	6.5	6.5	30	4.5	4.5	84	7.5	7.5
14	23	2.5	3.5	83	6.5	7.5	40	5.0	5.0	75	6.5	8.0
18	23	3.0	3.5	76	6.0	5.0	33	4.0	4.5	80	7.0	7.5

K = kallikrein TK = total kallikrein N = normal (uninhibited) plasma
A = inhibited with aprotinin B = inhibited with SBTI

Appendix 2 (contd)

Table CIn vivo inhibition of plasma kallikrein

Day	Early-treated group			Late-treated group			Saline
	<u>600</u>	<u>601</u>	<u>602</u>	<u>603</u>	<u>604</u>	<u>605</u>	<u>606</u>
0	12	14	10	15	18	19	18
2	14	16	11	18	17	23	20
4	15	12	11	16	20	21	22
6	12	17	15	20	19	23	25
6	17	18	13	20	24	36	30
9	13	14	15	17	26	39*	38
10	15	19	14	21	33	18	ND
11	17	15	14	28	40*	19	36
12	17	15	18	35*	14	16	ND
13	31	22	20	13	20	28	31
15	24	24	21	15	25	23	26
17	22	24	26	21	21	23	27

* start of aprotinin therapy (4 days)

Rabbit 607 (uninfected) - aprotinin 5000 KIU/kg/day (6 days) :
kallikrein = 12 ± 5 μ moles TAME hydrolysed/ml plasma/h.

Appendix 2 (contd.)

Table D

Parameters determined during T.brucei infections of untreated rabbits

No.	Day	IgG mg/100ml	IgM %	C ₃ %	Complexes	Parasitaemia /30 fields	Total Protein g/100ml
756	0	550	100	100	7.8		6.8
	2				4.8	0	
	4				8.1	0	6.5
	7	550	144	120	14.2	0	
	9				15.8	4	6.9
	11	760	200	113	18.4	0	
	14	950	200	107	19.9	0	7.2
	16				19.8	3	
	18	1320	222	113	20.8	8	8.0
	21	1840	222	93	18.7	0	8.4
	23				23.7	0	
	25	2300	200	100	21.2	0	8.2
	28				17.5	15	
	30	1840	178	93	16.8	0	
	32				17.6	6	
	35				19.5	0	8.0
	39				14.2	6	
	42				14.9	0	
757	0	440	100	100	12.0		7.6
	2				8.3	0	
	4				8.7	2	
	7	550	125	125	15.8	0	6.6
	9				11.8	0	
	11	840	150	106	16.0	13	8.0
	14	1320	158	100	18.5	2	7.6
	16				23.6	22	
	18	2060	167	100	27.5	0	8.9
	21	2560	167	100	25.8	1	8.6
	23				28.3	40	
	25	2560	175	94	29.7	0	9.4
	28				28.4	0	
	30				21.9	1	
	32				22.6	7	
	35				18.1	0	
	42				12.0	2	

Appendix 2 (contd.)

Table D (contd.)

No.	Day	IgG mg/100ml	IgM %	C ₃ %	Complexes	Parasitaemia /30 fields	Total Protein g/100ml
758	0				9.97		5.3
	2				7.95	1	5.4
	4				10.1	0	
	7				14.9	0	5.1
	9				12.2	2	5.6
	11	680	144	114	11.9	0	
	14				16.8	4	5.9
	16	2060	222	142	24.1	23	
	18				22.9	0	6.6
	21				23.0	0	6.1
	23	2060	200	114	23.0	0	
	25	1640	178	100	19.4	0	6.8
	28	2060	200	114	22.2	0	
	30				20.7	2	
	32	1640	178	121	17.2		
	35				18.7		7.2

Appendix 2 (contd.)

Table E

Parameters determined during T.brucei infections of drug treated rabbits
(1) Aprotinin (Trasylol)

No.	Day	IgG mg/100ml	IgM %	C3 %	Complexes	Parasitaemia /30 fields	Total Protein g/100ml
790	2	550	100	100	10.2	0	6.0
	4	550	108	105	13.2	0	5.8
	6	760	120	100	15.4	0	5.8
	8	950	135	95	21.6	1	6.3
	11	1320	160	100	22.3	0	7.4
	14	1180	200	85	28.6	2	6.9
	16	1320	215	90	26.5	0	7.6
	22		224	90	26.0	0	7.6
791	2	610	100	100	10.8	0	6.2
	4	440	110	100	8.0	1	5.1
	8	760	140	90	17.4	5	6.8
	14	1320	200	80	19.4	0	6.1
	16	1840	180	90	22.6	2	7.6
797	2	760	100	100	7.4	0	5.8
	4	1060	105	115	13.5	0	5.8
	7	1060	128	105	17.5	0	6.3
	9	1320	160	90	22.4	4	6.0
	15		220	75	22.8	0	6.8
	17	1640	200	85	19.0	0	7.2
800	2	1060	100	100	8.9	0	6.2
	4	1060	109	117	12.5	2	6.4
	7	1180	136	100	13.9	0	5.9
	9	1060	145	94		0	5.9
	15				22.4	0	
	17	1320	200	94	29.3	1	6.8
817	4	680	100	100	11.9	1	5.7
	7				10.5	0	
	9	1320	140	95	15.9	2	5.4
	11	680	180	70	17.7	6	5.7
	15				21.5	0	
	18	1320	230	80	20.3	1	7.4
	21				32.0	0	
	25	1640			31.1	0	7.5
	29		200	85	29.8	2	

790 } 5000 KIU/kg day8-14
791 }

Drugs administered intravenously

797
800 } 5000 KIU/kg day5-13
817 }

Appendix 2 (contd.)

Table E (contd.)

(2) Indomethacin

No.	Day	IgG mg/100ml	IgM %	C3 %	Complexes	Parasitaemia /30 fields	Total Protein g/100ml
813	2	550	100	100	10.6	0	6.8
	4				10.8	0	6.9
	7	620	160	104	11.5	0	
	9	680	186	100	12.6	0	6.5
	11	1000	200	86	20.5	12	7.1
	14	1320	210	78	24.0	1	6.9
	18				26.8	0	
	22	1640	180	92	20.3	4	7.8
	25				21.4	0	
	35	1020	154	90	16.2	0	7.7
814	9	1000	100	100	21.6	0	6.4
	11	680	113	90	17.0	3	6.1
	16	1000	113	80	23.1		7.2
	18				25.0	1	6.8
	22	1620	125	70	27.4	2	7.6
	25				25.3	0	
	28				20.6	0	
	32	1000	125	80	25.3	1	8.4
	36				20.3	2	
	42				18.9	2	
815	4	620	100	100	9.0	0	7.6
	7				9.8	0	5.7
	9				13.9	0	
	11	680	180	100	10.7	8	4.8
	15				24.4	0	
	18	1620	200	88	20.4	0	5.7
	21				20.2	0	
	25	2090	200	75	23.4	1	6.6
	29	2560	138	81	26.3	0	
816	4	530	100	100	7.5	2	7.0
	9				9.8	0	
	11	680	190	75	13.8	1	5.3
	15				13.2	1	
	18	1320	200	54	11.2	0	7.2
	21	1620	220	57	19.6	0	6.4
	25	2560	220	67	21.7	0	6.8
	29				20.2	0	
	35				14.8	1	

813 }
814 } 5mg/kg day8-18

Drugs administered orally

815 }
816 } 5mg/kg day4-18

Appendix 2 (contd.)

Table E (contd.)

(3) Penicillamine

No.	Day	IgG mg/100ml	IgM %	C3 %	Complexes	Parasitaemia /30 fields	Total Protein g/100ml
741	0	440	100	100	8.5	0	5.8
	3	380	100	110	10.6	2	5.6
	6	520	104	143	16.3	0	
	8	960	137	126	20.5	0	6.2
	10	850	152	108	22.5	8	6.8
	13	1280	200	115	19.1	1	6.3
	15				30.9	0	7.4
	17	1560	187	103	36.4	0	7.5
	22				30.0	2	
	24	1440	164	100	24.1	0	7.1
	27	1320	160	93	26.5	1	7.8
742	-4	530	100	100	10.6		6.6
	0	480	98	96	11.1		6.3
	3	540	102	103	12.4	0	5.8
	6	520	108	115	16.5	0	6.1
	8	680	158	134	18.2	3	
	10	680	183	108	24.3	15	6.4
	13	1320	180	106	25.7	1	6.8
	15	1260	210	84	23.7	0	
	17				19.3	0	6.8
	22	1060	217	101	27.9	2	7.1
	24	1320	189	121	26.8	0	7.3
	27				25.6	0	6.9
	29				18.9	0	
743	0	490	100	100	10.0		6.2
	3	540	105	108	12.5	0	6.4
	6	580	138	96	13.6	0	6.0
	8	670	160	102	10.8	0	6.8
	10	950	200	84	13.3	8	
	13				17.2	2	6.8
	15	1260	235	98	22.6	0	7.1
	17				30.2	3	6.7
	24	1840	210	110	25.4	0	7.3
	27				24.1	0	
	29	1320	185	93		4	7.8

741 } 5mg/kg day-4-24
 742 }

Drug administered orally

743 5mg/kg day9-24

Appendix 2 (contd.)

Table E (contd.)

(3) Penicillamine (contd.)

No.	Day	IgG mg/100ml	IgM %	C ₃ %	Complexes	Parasitaemia /30 fields	Total Protein g/100ml
744	0	400	100	100	10.4		5.4
	6	550	160	167	16.4	0	6.4
	8	680	160	133	20.2	0	5.7
	10	760	200	142	24.7	1	6.4
	13				32.9	0	
	15	1200	210	133	27.4	0	6.8
	17				26.1	0	
	22	1640	210	92	34.4	2	7.2
	24				29.0	0	
	27		180	92	29.4	0	7.8
766	-3				6.5		
	0	360	100	100	6.8		5.4
	2				7.0	0	
	4	440	120	136	8.6	0	6.4
	7				15.1	0	
	9	440	170	121	13.2	0	5.1
	11	680	190	114	17.4	2	6.8
	14				21.4	8	
	16				21.8	0	
	18	1060	200	100	21.2	0	5.6
	21				22.8	1	
	23	1640	210	93	19.3	3	6.5
	25				22.4		
	28				18.5		
767	-3				9.1		6.2
	0	280	100	100	6.7		
	2				8.6	1	6.4
	4				5.7	0	
	7	680	140	114	12.4	0	8.0
	9	1320	176	171	9.4	5	7.6
	11	440	192	129	11.6	20	7.0
	14	1640	210	185	19.5	0	7.8
	16				13.4	3	
	18	440	206	114	12.5	14	
	21				18.6	0	8.0
	23	1060	185	129	17.4	0	8.0
	25				14.6	1	

744 5mg/kg day9-24

Drug administered orally

766 } 25mg/kg day-4-22
767 }

Appendix 2 (contd.)

Table E (contd.)

(4a) Hydrocortisone

No.	Day	IgG mg/100ml	IgM %	C ₃ %	Complexes	Clq %	Parasit- aemia/30 fields	Total Protein g/100ml
837	0	680	100	100	6.3	3.34		6.8
	2				5.7	3.52	5	
	4	680	120	121	11.6	4.02	0	
	6				12.6	4.39	0	6.1
	8	1440	200	79	11.2	4.22	27	6.1
	11	1640	200	71	14.6	4.42	14	8.4
	13				17.9	4.72	3	
	15	1320	220	86	23.2	4.91	3	7.5
	18				28.7	6.17	1	9.4
	20	2060	240	93	34.1	7.65	0	
838	0	680	100	100	7.4	3.16		5.7
	2				7.2	3.12	8	7.6
	4				8.1	3.38	0	5.7
	6				15.9	3.43	0	6.8
	8	760	100	120	12.7	3.44	150	5.9
	11	550	182	93	14.5	6.71	450	6.8
	13			93	16.3	5.80	0	6.8
	15	1060	218	93	21.3	6.49	0	6.2
	18	1320	236	80	27.5	7.56	0	
	20		236		21.5	8.52	15	5.9

837, 838 2.5mg alternate days (day0-15) injected intramuscularly

(4b) Dexamethasone

No.	Day	IgG mg/100ml	IgM %	C ₃ %	Complexes	Clq %	Parasit- aemia/30 fields	Total Protein g/100ml
835	0	680	100	100	11.1	3.57		6.2
	2				10.4	3.47	11	
	4	490	77	113	9.7	3.30	52	
	6	440			8.5	3.30	0	6.2
	8	490	138	93	10.4	3.39	45	
	11	550	184	87	12.7	3.78	12	6.4
	13	550					29	6.1
	15	680	200	73	13.5	4.09	0	5.6
	18				17.3	4.90	0	
	20	840	215	80	23.3	5.17	0	6.4

†

†died

Appendix 2 (contd.)

Table E (contd.)

(4b) Dexamethasone (contd.)

No.	Day	IgG mg/100ml	IgM %	C ₃ %	Complexes	Clq %	Parasit- aemia/30 fields	Total Protein g/100ml
836	0	1060	100	100	10.6	3.37		5.1
	2				10.6	3.33	4	6.1
	4	1060	77	121	9.7	3.21	0	4.8
	6				12.2	3.25	0	5.4
	8	950	123	93	16.9	3.76	14	6.4
	11	1320	108	100	20.5	4.64	32	6.2
	13				24.0		1	6.2
	15	1320	138	100	25.8	5.12	0	6.6
	18				29.3	6.62	0	
	20	2060	154	93			0	
850	0	550	100	100	7.4			4.6
	2				5.3		0	5.7
	4	400	100	121	5.4		62	5.7
	6	490	133	129	8.6		0	5.3
	8	550	200	107	11.9		195	5.9
	10	950	233	107	13.7		>30/field	6.2
	13	1060	288	107	14.6		113 ⁺	5.9
851	0	490	100	100				5.9
	2				7.9		1	4.8
	4	440	92	121	5.8		58	
	6				7.8		0	5.3
	8	550	158	93	8.5		3	5.7
	10				14.2		0	4.3
	13	610	167	93	10.2		30	5.4
	15				15.4		59	5.9
	17	490	167	100	14.4		0	
	20	680	183	100	15.7		1 ⁺	7.2
852	0	950	100	100	7.6			5.6
	2				6.3		2	4.6
	4	680	100	130	10.0		1	
	6				13.4		0	5.1
	8	680	150	96	16.8		16	4.5
	10				21.9		0	4.5
	13	760	183	96	21.0		28	4.8
	15				22.1		2	
	17	680	192	96	22.8		2	5.6
	20	1060	200	89	18.0		2	4.8
	22				22.2		2	
	24				22.5		0	
	27							5.3

†died

Appendix 2 (contd.)

Table E (contd.)

(4b) Dexamethasone (contd.)

No.	Day	IgG mg/100ml	IgM %	C ₃ %	Complexes	Clq %	Parasit- aemia/30 fields	Total Protein g/100ml
853	0	680	100	100	16.1			6.6
	2				13.4		0	4.8
	4	550	86	115	9.0		12	5.7
	6	490	90	121	12.4		0	6.2
	8	610	108	106	10.3		92	
	10	550	136	84	12.2		14	6.6
	13				16.1		24	5.9
	15	610	189	100	13.3		0	6.4
	17	680	215	92	22.2		1	
	20	760	234	86	20.3		75	5.1
	22				14.4		0	
	24	1020	192	94	20.2		0	
	27						2	5.9

835 } 1mg daily day0-15
836 }

Drug injected intramuscularly

850

851 } 1mg daily day0-18
852 }

853

Appendix 2 (contd.)

Table E (contd.)

(5) Cyclophosphamide

No.	Day	IgG mg/100ml	IgM %	C ₃ %	Complexes	Clq %	Parasit- aemia/30 fields	Total Protein g/100ml
821	0	680	100	100	8.1			5.7
	3	610	100	93	6.9		3	5.3
	7	490	111	93	9.3		0	5.3
	10	680	156	93	16.4		15	5.6
	13	1670	211	87	25.3		28	5.6
	17	1840	211	93	25.2		0	6.3
	21				30.6		4	8.4
834	2	680	100	100	14.4	3.06	0	7.1
	4				13.5	3.26	2	
	7	680	118	113	17.3	3.23	0	6.1
	9				19.0	4.38		
	11	1320	172	80	31.0	6.80	1	9.0
	14	2560	218	80	29.4	6.60	34	10.0
	16				20.7	8.08	2	
	18	2560	236	80	42.1		0	11.6
	21				41.4	9.40	2	14.0
	23	2560	236	73	27.5	9.02	8	
833	2	550	100	100	16.1		0	6.4
	4	490	106	104	12.4		0	6.2
	7	680	102	81	8.6		60	6.5
	9	680	143	75	5.6		10-15/field	6.3
	10	840	160	78	17.8		†	6.8
841	2	760	100	100	10.2		6	5.3
	4	680	94	100	9.4		0	5.7
	6	720	106	113	12.3		0	4.6
	9	840	120	90	13.6		105	6.2
	11	860	160	84	16.8		3	4.2
	14						8	
	16	1320	232	86	24.4		0	5.8
	18	2060	214	78	27.3		0	6.6
	23						3	

821 150mg/kg day0 + 50mg/kg day3

822 150mg/kg day0 - died day2

823 150mg/kg day3 - died day7 } results not presented

833 } 100mg/kg day2 + 50mg/kg day5

834 }

Drug administered intraperitoneally

841 10mg/kg on alternate days, day0-11

†died

Appendix 2 (contd.)

Table E (contd.)

(6) Uninfected treated rabbits

Day	IgG mg/100ml					IgM %				
	827	830	831	843	844	827	830	831	843	844
0	550	680	610	950	760	100	100	100	100	100
2	520	720	680	840	680	106	100	91	94	104
4	580	610	600	680	660	110	98	102	85	122
7	600	540	580	720	740	95	106	111	88	125
9	620	560	620	750	830	102	110	104	86	118
11	580	620	540	710	780	111	121	102	91	117
14	680	550	550	690	820	108	114	102	89	109
16	610	600	610	720	820	115	109	118	93	110
18	480	580	610	880	860	120	116	114	110	115
21	540	620	720	1020	780	119	107	118	115	104

Day	C3 %					Total Protein g/100ml				
	827	830	831	843	844	827	830	831	843	844
0	100	100	100	100	100	6.2	5.8	5.3	6.4	6.6
2	90	103	97	102	108	6.4	6.2	5.8	5.8	6.0
4	104	100	93	96	102	6.6	5.8	5.1	5.3	6.8
7	96	107	100	88	110	5.9	5.6	5.7	5.1	7.1
9	98	99	92	98	98	6.1	5.6	5.9	5.4	7.4
11	110	108	89	104	107	5.8	6.1	5.2	5.0	7.0
14	103	106	87	85	111	5.9	6.0	6.1	6.0	6.8
16	101	100	93	89	100	6.3	5.6	6.3	6.2	6.8
18	105	109	100	87	114	6.2	6.0	5.8	5.8	7.0
21	98	107	108	96	101	5.9	6.4	5.8	6.8	6.5

827 indomethacin: 5mg/kg day0-12

830 penicillamine: 25mg/kg day0-20

831 hydrocortisone: 2.5mg day0-15 alternate days

843 dexamethasone: 1mg day0-15

844 cyclophosphamide: 100mg/kg day1

Appendix 2 (contd)

Table F

Plasma kallikrein levels in drug treated infected rabbits

INDOMETHACIN					PENICILLAMINE							
Days	814		815		Days	744		Days	766		767	
	TK	K	TK	K		TK	K		TK	K	TK	K
4	ND	ND	86	18	0	84	16	-4	80	20	91	18
7	ND	ND	88	20	6	87	20	0	82	18	88	20
9	76	30	86	24	8	83	24	4	83	16	95	23
11	74	41	90	33	10	83	33	7	90	22	93	24
15	ND	ND	82	36	13	85	26	9	84	23	89	28
16	80	32	ND	ND	15	80	23	11	92	28	86	40
18	72	29	80	28	17	79	23	14	86	39	88	35
21	ND	ND	73	27	22	78	21	16	86	36	76	30
22	68	26	ND	ND				18	83	28	78	31
25	70	26	70	24								

HYDROCORTISONE					CYCLOPHOSPHAMIDE				
Days	837		838		Days	834		841	
	TK	K	TK	K		TK	K	TK	K
0	85	16	90	20	0	84	16	89	19
2	86	18	84	18	2	86	20	88	16
4	90	20	86	22	4	80	18	92	21
6	82	23	92	28	7	82	23	82	28
8	78	25	82	42	9	74	25	83	39
11	80	33	76	31	11	70	32	79	34
13	72	23	70	29	14	72	30	74	30
15	84	24	62	23	16	68	21	76	18
18	74	29	60	18	18	68	20	73	23
20	68	22	54	18					

(814)days8-18,5mg/kg; (815)days4-18,5mg/kg.

(744)days9-24,5mg/kg; (766,767)days-4-22,25mg/kg

(837,838)alternate days0-15,2.5mg.

(834)day2,100mg/kg+day5,50mg/kg; (841)alternate days0-11,10mg/kg.

TK=total kallikrein, K=kallikrein, ND=not determined

Appendix 2 (contd)

Table F (contd)

DEXAMETHASONE										
Days	835		836		850		851		852	
	TK	K	TK	K	TK	K	TK	K	TK	K
0	90	16	85	18	82	15	75	15	80	21
2	87	20	87	16	86	20	76	19	75	18
4	95	26	92	16	80	34	80	28	76	22
6	94	22	87	23	83	30	85	22	74	24
8	84	20	95	32	76	23	72	23	68	30
10	ND	ND	ND	ND	70	26	75	20	62	40
11	80	24	85	23	ND	ND	ND	ND	ND	ND
13	83	25	88	24	68	25	70	28	63	28
15	81	28	78	20	†	†	70	21	65	26
17	ND	ND	ND	ND			68	18	60	26
18	78	21	79	22			ND	ND	ND	ND
20	74	18	81	24			65	22	61	15

(835,836)days0-15, 1mg daily

(850,851,852)days0-18, 1mg daily

† rabbit died day14

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The Role of Kallikrein in the Pathogenesis of African Trypanosomiasis

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ABSTRACT

Urinary and plasma kallikreins have been shown to be activated during experimental *Trypanosoma brucei* infections of rabbits. Plasma kallikrein is probably released by immune complexes and contributes to the inflammatory reactions and hypotension seen in this disease. Raised urinary kallikrein concentrations are found very early in the infection and it is postulated that changes in mineralocorticoid levels may be responsible. The production of intra-renal kinins would increase renal blood flow which may also cause urinary kallikrein release.

KEYWORDS

Trypanosoma brucei, pathogenesis, urinary kallikrein, plasma kallikrein, immune complexes.

AFRICAN TRYPANOSOMIASIS

The African trypanosomiasis, which cause sleeping sickness in humans and Nagana in cattle, are important economic diseases. Some 35 million people are at risk from sleeping sickness with 10,000 new cases reported annually. In addition approximately 80 million cattle are exposed to the disease (WHO, 1976). These diseases are caused by parasitic protozoans of the genus *Trypanosoma* which live in the blood and tissues of their vertebrate hosts and are transmitted by tsetse flies. The two important trypanosome species which infect man are *T. rhodesiense* and *T. gambiense*, while *T. congolense* and *T. vivax* cause disease in cattle. Another species morphologically identical to the human trypanosomes, *T. brucei*, causes a mild disease in cattle but is not infective to man. It provides a useful laboratory model since it produces chronic infections in rabbits which normally kill the animals in 4-8 weeks. The disease is characterised by waves of parasitaemia every few days, such waves consisting of different antigenic variants of the parasite (Vickerman, 1978). There is a great heterogeneity of antibodies present many of which are not trypanosome specific (Boreham and Mackenzie, 1974).

Trypanosomiasis is basically an inflammatory disease with oedema, meningo-encephalitis and myocarditis being most noticeable. The first signs of the infection in experimental rabbits are oedematous and erythematous involvement of the extremities such as ears, eyelids, external nares and scrotum. This is followed by an exuda-

tive dermatitis and skin lesions. Infected rabbits die in a state of vascular shock and have renal failure which may have an immunological origin (Goodwin, 1970; Goodwin and Guy, 1973). They show evidence of diffuse intravascular coagulation, probably as a result of hypotension (Boreham and Wright, 1976a) coupled with increased concentrations of plasma fibrinogen (Boreham and Facer, 1974) which lead to increases in blood viscosity (Boreham, 1974; Facer, 1976).

It has been well documented that pharmacologically active substances, and in particular the kinins, are released during trypanosomiasis (Boreham, 1968, 1970; Goodwin, 1976). There is now considerable evidence that immune complexes absorb Hageman factor causing its activation and initiating the kinin cascade (Boreham and Wright, 1976b).

The object of this paper is to discuss recent observations on kallikrein in trypanosomiasis and to see how they might help to explain the pathogenesis of this disease.

KALLIKREIN IN TRYPANOSOMIASIS

Urinary Kallikrein

The amount of kallikrein produced in the urine of rabbits infected with *T. brucei* was measured using a modification of the esterolytic assay described by Roberts

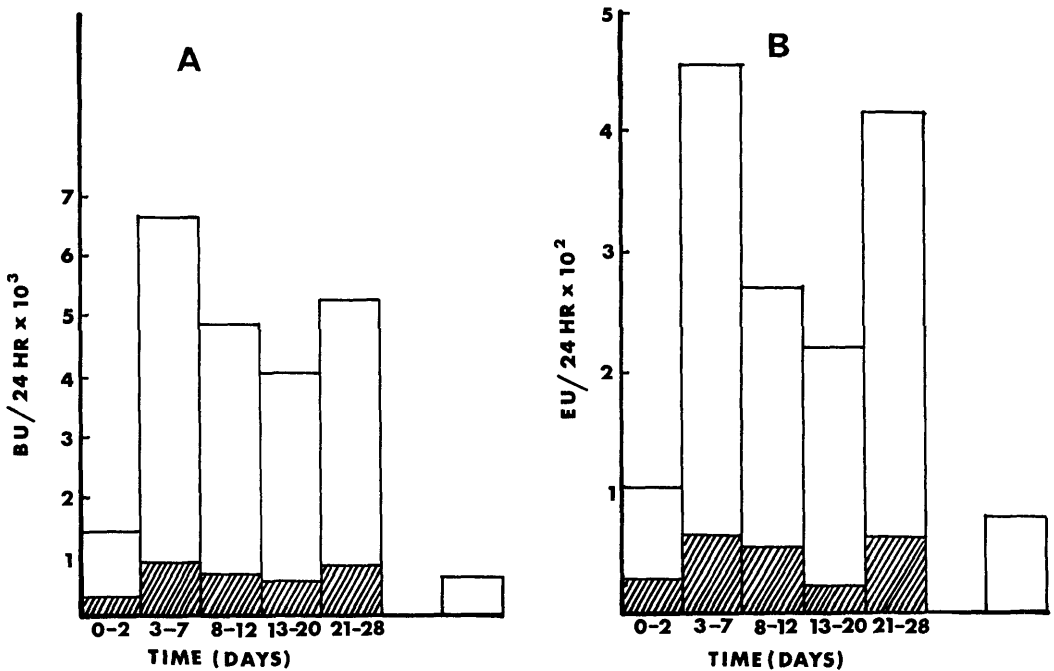


Fig.1. Urinary kallikrein concentration in rabbits infected with *T. brucei* as measured biologically (A) and enzymatically (B). Hatched areas indicate inhibition by aprotinin 5000 IU/ml (A) and 3000 IU/ml (B).

(1958) and by biological assay on the blood pressure of rabbits. One enzyme unit (EU) was defined as the amount of enzyme necessary to hydrolyse 1 μ mol of N-tosyl L-arginine methyl ester (TAME)/min at 37°C and one biological unit (BU) as the volume of urine which caused a fall in blood pressure equivalent to that of one BU of Padutin (kallikrein, Bayer Ltd.) (Wright and Boreham, 1977).

Increased amounts of urinary kallikrein were first detected 2-3 days after infection (Fig. 1). These concentrations increased to 4-8 times normal after 6-10 days. Subsequently fluctuations were seen with additional peaks at intervals which appeared to correlate with changes in parasitaemia. Significant correlation coefficients of 0.97 between BU/24 h and EU/24 h existed. Both biological and enzymic activity were inhibited by aprotinin (Trasylol) but not by soya bean trypsin inhibitor (SBTI).

Plasma Kallikrein

The total kallikrein activity was measured by activating 0.25 ml of plasma with an equal volume of kaolin (10 mg/ml) in distilled water at 27°C for 1 min (Coleman, Mason and Sherry, 1969). This was then added to 4.5 ml of 0.05M TAME in 0.05M Tris/HCl buffer pH 8.4 at 37°C. The amount of hydrolysis was then determined as

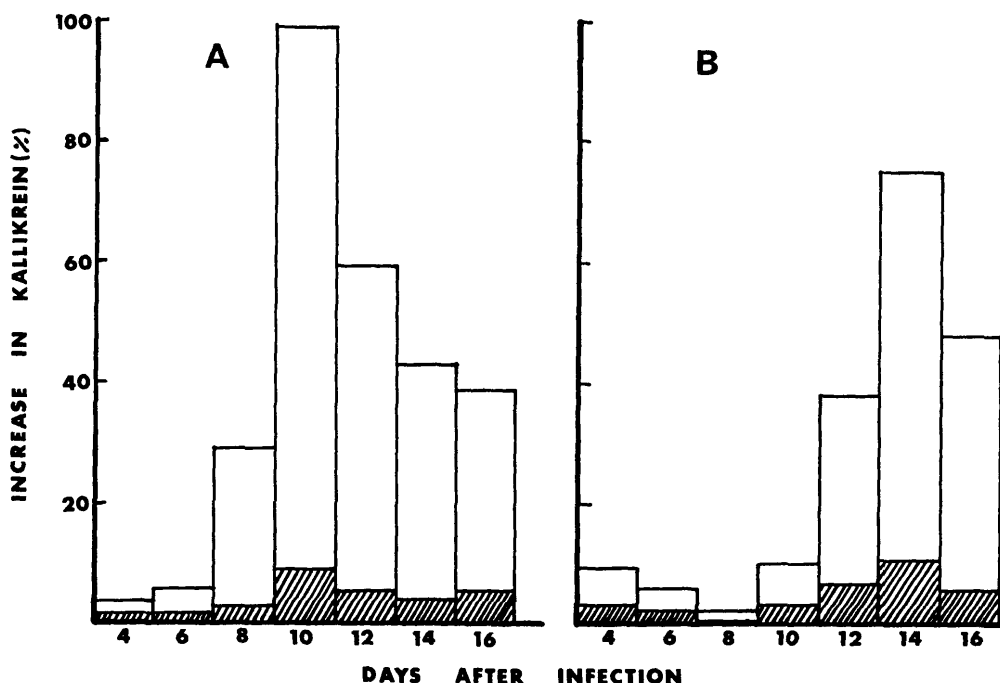


Fig. 2. Percentage increase in plasma kallikrein concentrations in rabbits infected with *T. brucei*. Hatched areas indicate inhibition with aprotinin 1000 IU/ml (A) and SBTI 100 mg/ml (B).

described by Roberts (1958). The free kallikrein was measured in a similar way omitting the kaolin activation. Great care was necessary in taking the blood samples for kallikrein assay, especially late in the infection, when the ear veins were fragile and massive oedema was present. Oedema fluid readily activated the kallikrein system. The results are shown in Fig. 2. An increase in free kallikrein is seen reaching a peak 10-14 days after infection and this is associated with a fall in prekallikrein concentrations. The kallikrein activity can be inhibited *in vitro* by aprotinin (1000 IU/ml) or SBTI (100 mg/ml). *In vivo* daily intravenous injections of aprotinin (5000 IU/day) prevented the increase in kallikrein concentrations and produced an immediate fall in concentrations when introduced at peak levels (Fig. 3).

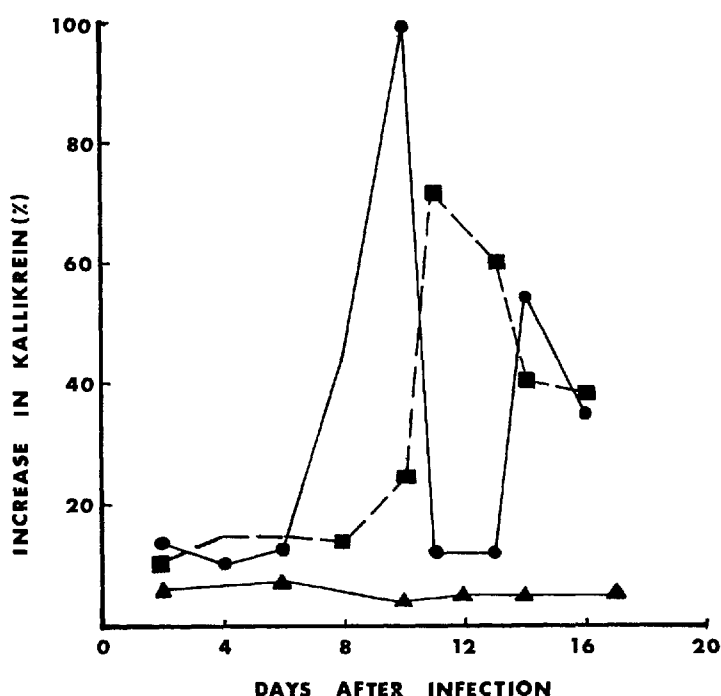


Fig. 3. *In vivo* inhibition of plasma kallikrein by aprotinin 5000 IU/day. Rabbits infected with *T. brucei*. ●—●: treated days 9-13; ■—■: treated days 0-9; ▲—▲: uninfected control.

Immune Complexes

There is evidence from recent work that circulating soluble immune complexes are found in human and experimental trypanosomiasis (Fruit and co-workers, 1977; Lambert and Castro, 1977). In the rabbit infected with *T. brucei* there is a five-fold increase in soluble IgG complexes as measured by the T25-I Iodinated protein A from *Staphylococcus aureus* assay of Crawford and Lane (1977). The maximum concentrations of complexes were detected about day 14 at the same time as maximum kallikrein levels.

DISCUSSION

The recent report by Boreham and Wright (1976a) that injection of immune complexes of trypanosomes and antibody, prepared *in vitro*, into non-infected rabbits produced hypotension, mediated by kallikrein, provides the first real evidence of the involvement of the kinin system in the pathogenesis of this disease. Up until then the evidence had been circumstantial in that free kinin could be detected at the time pathological changes occurred. Hypotension in rabbits infected with *T. brucei* is marked at 10 days when plasma kallikrein concentrations and circulating IgG complexes are high. These observations are compatible with the hypothesis previously presented that immune complexes activate the kinin system causing increases in capillary permeability and hypotension. Increases in plasma proteins, especially globulins and fibrinogen, cause a rise in blood viscosity leading to stasis and micro-thrombi formation. Such stasis will almost certainly cause further activation of the kinin system locally (Boreham, 1976). Another possible role of kallikrein in the inflammatory response of trypanosomiasis is chemoattraction. The conversion of pre-kallikrein to kallikrein results in the formation of cytotoxins for neutrophils and human blood monocytes (Gallin and Kaplan, 1974).

It is intriguing to find that urinary kallikrein concentrations begin to increase very early in the infection, significant rises being detected after only 2-3 days. This almost certainly precludes an immunological mechanism. Urinary kallikrein is produced in the kidney, probably in the juxta-glomerular complex (Nustad, 1970). Keiser and co-workers (1976) have provided evidence that urinary kallikrein is regulated by the levels of sodium retaining steroids such as aldosterone and that it is independent of the rate of excretion of sodium or water. If these conclusions are correct it is necessary to postulate that trypanosome infections in some way are able to modify mineralocorticoid levels in the host.

A second mechanism of control of urinary kallikrein has also been suggested by Keiser and co-workers (1976). When the blood flow to the kidneys of dogs is restricted there is a decrease in urinary kallikrein excretion suggesting that renal blood flow also controls urinary kallikrein release. Therefore an increase in kidney blood flow would also account for our observations. At first sight this is the opposite to expectations since haemostasis and decreased blood flow seem likely. However Nasjletti and Colina-Chourio (1976) have shown that increased kallikrein-kinin activity in the isolated perfused rabbit kidney causes renal vasodilatation and it is probable that the formation of intra-renal kinins is modulated by prostaglandins of the E series (McGiff and Nasjletti, 1976). Therefore in trypanosomiasis urinary kallikrein may be released as a result of changes in mineralocorticoids early in the infection but the production of intra-renal kinins will also contribute to increased blood flow and further urinary kallikrein excretion. Figure 4 summarises the possible effects of plasma kallikrein and urinary kallikrein on the pathogenesis of trypanosomiasis.

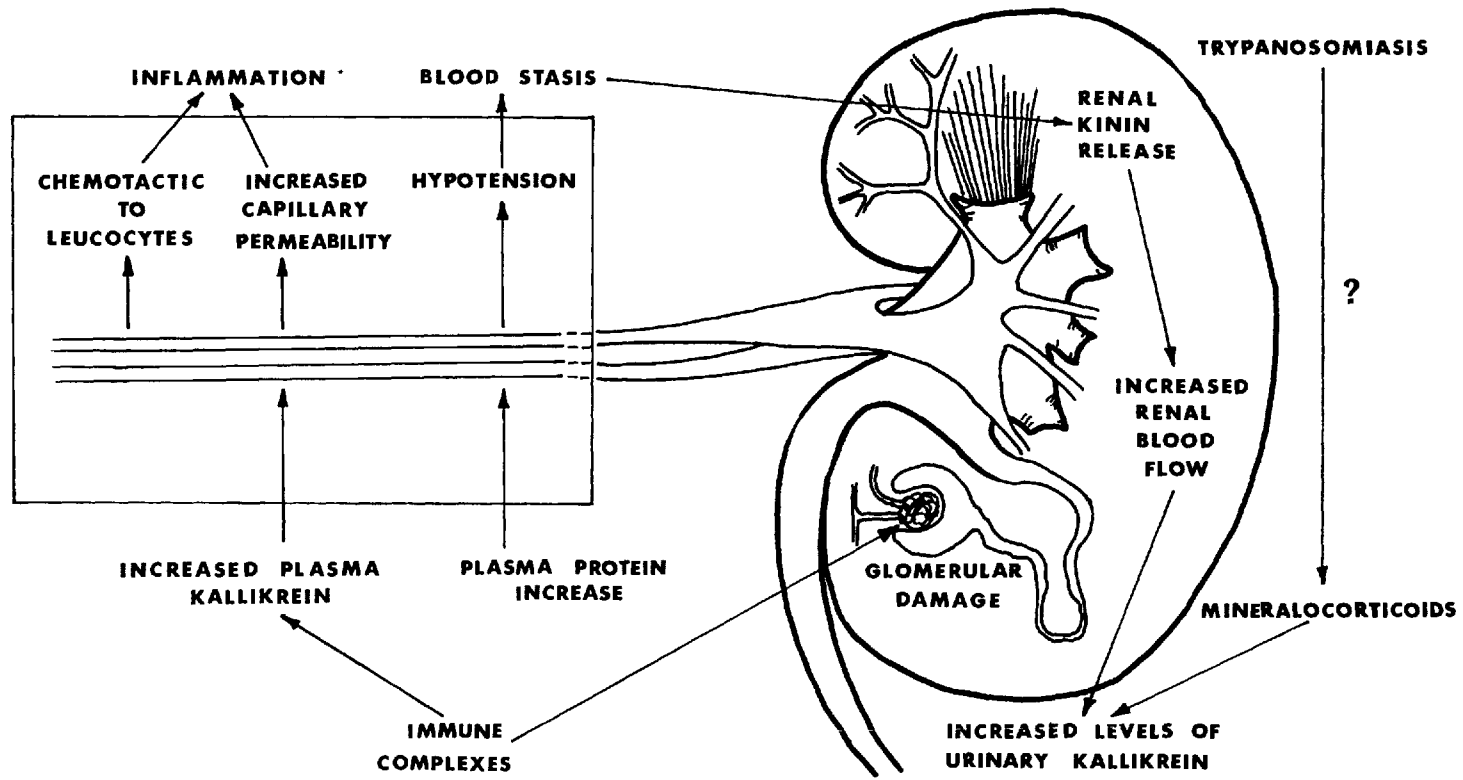


Fig. 4. Kallikrein and Trypanosomiasis

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