

STUDIES ON AFRICAN TRYPANOSOMES.

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SUMMARY.

After introducing the basic problems of trypanosomiasis of domestic stock in tropical Africa, a review is given of the historical development of research, trypanosome nomenclature, some immunological aspects of the disease, the diagnostic techniques in current use, and some of the trypanosome handling methods.

The development of techniques for the serological study of Trypanosoma congolense, and their subsequent use in experiments studying antigenic variation of these trypanosomes and the immune response they induce in infected cattle, is described. A serological examination of populations of T.congolense, before and after cyclical passage through the tsetse fly (Glossina morsitans), is also undertaken using the same techniques. The agglutination test is used to examine the antigenic relationship of geographically distinct isolates of the brucei sub-group trypanosomes. The efficacy of infection with virulent trypanosomes followed by chemotherapy as an immunization procedure, and the value of an indirect fluorescent antibody test as a diagnostic aid, in cattle infected with pathogenic trypanosomes, are subsequently studied.

A discussion follows with particular reference to the advances made in this study, the types of acquired immune mechanism which develop in cattle infected with pathogenic trypanosomes, the feasibility of immunological control of trypanosomiasis, and the use of serological tests in diagnosis.



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1. INTRODUCTION.

Little work has been carried out on the immunology of the disease in animals caused by Trypanosoma congolense. The aims of this study were to develop suitable methods for the serological examination of T. congolense, to obtain information on the mechanism of antigenic variation and on the immune response they induce in cattle and, to study immunization and serodiagnostic techniques.

1.1. Historical.

African pathogenic trypanosomiasis is typically a chronic, wasting and often fatal disease of man and domestic animals. It is caused by a flagellated protozoon, and is transmitted by biting flies of which the most important is the tsetse fly.

It is estimated that over four million square miles of tropical Africa harbour the tsetse fly(128). Throughout this vast area, man and his domestic animals are liable, in varying degree, to infection with trypanosomes, thus limiting stock farming. With vaccines now available for pandemics such as rinderpest and bovine pleuropneumonia, trypanosomiasis remains one of the greatest single impediments to the social and economic advance in many of the countries concerned.

The disease has been known for many centuries(223), but serious research did not commence until about 1890. Much of this early work was concerned with the trypanosome, and although by 1920 many papers had been published, the identity of the various

organisms was confused and no effective chemotherapeutic agents had yet been discovered. However, some fundamental knowledge concerning the disease had been ascertained. The main vector of the parasites was shown to be the tsetse fly(37,38), in which a cyclical transmission took place(217) in the anterior station (103). Trypanosomes had the capacity for antigenic variation (130), which appeared to be unlimited(299,300).

About 1920, studies became concentrated on the tsetse fly due to the introduction by Swynnerton of a 'single Key' approach to control by vector elimination. Two control methods were introduced, namely, bush clearance and game destruction, and throughout the next thirty years many tsetse eradication schemes were conducted using these methods. In most of these schemes, only temporary removal of the fly was achieved, as the cleared areas were populated with people who had little agricultural knowledge. This resulted in a reinfestation by the fly. A change from the 'single key' approach to a 'multiple key' involving antitrypanosome methods was introduced in the mid-1950s for reasons explained by Lumsden(244). By this time, residual insecticides and effective curative and prophylactic drugs had been developed, thus making a dual attack possible.

The discovery of sensitive serological tests and the use of preservation at low temperature then led to more exact and detailed information on the trypanosome.

1.2.Nomenclature.

The genus Trypanosoma was created by Gruby in 1843 for the

parasite in the blood of a frog, now known as T. rotatorium. Members of this genus are characterized by a spindle-shaped body, a single nucleus, a kinetoplast and a flagellum which arises near the last named structure and runs anteriorly adhering to the body to form an undulating membrane. Trypanosomes are protozoa, belonging to the family Trypanosomatidae, Doflein, 1901, and in the most recent classification of the phylum (192) are placed in the order Kinetoplastida and class Zoomastigophorea.

These trypanosomes assumed great importance in Africa on the discovery by Bruce in 1895 of a trypanosome in the blood of horses and cattle suffering from 'nagana' in Zululand. This trypanosome was named T. brucei (285) and was described as a homogeneous mass of protoplasm, of worm-like form, with at one end a thick, stiff extremity, and at the other a long, wavy flagellum. Forde, in Gambia, then described a trypanosome in the blood of a man, which was named T. gambiense (105) ^{by Dutton} and shown to be morphologically similar to T. brucei. The presence of a polymorphic trypanosome in the blood of horses was then reported (106) and called 'the Gambian horse trypanosome', but later called T. dimorphon (229). A small, comma-shaped trypanosome was identified in the blood of cattle and named T. congolense (30). This was followed by the report of a fast moving trypanosome in the blood of cattle in the Cameroons, which was named T. vivax (389).

The classification of these organisms was aided by the discovery that differences occurred in the sites of cyclical development in the tsetse fly. T. vivax developed only in the proboscis (39), T. congolense in the gut and the proboscis (307)

and T. brucei in the gut and salivary glands(44). Wenyon(372) divided trypanosomes into two main groups, those in which the infective forms developed in the posterior station or hindgut of the insect, and those in which the infective forms developed in the anterior station or mouthparts. These two groups have recently been designated stercoraria and salivaria respectively (188). The African pathogenic trypanosomes fell into the last named group and have been divided into three groups on the basis of morphology and site of development in anterior station(185, 186). The groups were called brucei, congolense and vivax. The brucei group was further subdivided into the brucei and evansi sub-groups according to whether they developed in the tsetse fly.

Different morphological forms of the African pathogenic trypanosomes exist, namely, the trypanosome form in which the kinetoplast is behind the nucleus at the posterior end of the body, and the crithidial form in which the kinetoplast is in front of the nucleus in the anterior half of the body. Blood forms refer to the trypanosomes found in the mammal host and insect forms to those found in the insect host. Hoare and Wallace(189) suggested an alternative nomenclature for the morphological forms of trypanosomes employing the term mastigote.

This study is mainly concerned with trypanosomes of the congolense group, which contains three species, T. simiae, T. congolense and T. dimorphon(188). While the characteristics of T. simiae are well defined(41,69,183,184), those of T. congolense

and T.dimorphon are not. After the discovery of T.congolense in 1904, further small trypanosomes, namely, T.nanum(226), T.confusum(257) and T.pecorum(40) were described, which were probably similar to T.congolense in spite of some differences in pathogenicity(372). T.dimorphon was named by Laveran and Mesnil(229) using material from the 'Gambian horse trypanosome', which had been described as being polymorphic and to contain trypanosomes with free flagellae(106) by Dutton and Todd. The work of Laveran and Mesnil differed from that of Dutton and Todd in that the trypanosomes were not polymorphic and no long forms with free flagellae were seen. The discrepancy between these two descriptions may be explained by the fact that T.dimorphon was described from Dutton and Todd's case VI, whereas the 'Gambian horse trypanosome' was described from their case I. There can be no doubt that case I harboured a mixed infection consisting of a congolense group and T.brucei, a view held by other workers at the time(40,231). However, the name T.dimorphon continued to be used to describe mixed infections containing T.congolense (107,219,256,258), and even pure infections of T.brucei(14,252). The resulting taxonomical chaos caused the name T.dimorphon to pass out of circulation. Reports appeared from time to time of trypanosomes, which were considerably longer than the typical T.congolense(193,196,317). This led Hoare(187) to reinvestigate the original type material of T.dimorphon. He concluded that not only does T.dimorphon exist as a separate species but it can be distinguished from T.congolense by mean length measurements, the former being between 15.3 and 17.6 μ and the latter between 12.0

and 14.4 μ . The view that T.congolense and T.dimorphon can be separated so simply is not generally accepted(151). A biometrical and morphological study on five separate isolations of T.congolense, in different species of host, indicated that mean length was not a valid criterion for identification. Godfrey(151) distinguished three forms of T.congolense, which differed morphologically and biometrically. A population of T.congolense could then be typed according to the ratio of these forms in it. This study also showed that mean length of trypanosome varied with the species of host. The method of staining may also affect size(358). Hence the separation of T.congolense and T.dimorphon into two distinct species(187) may not be valid.

In addition to the sites of development in the tsetse fly, the African pathogenic trypanosomes can be distinguished by the morphology of their insect forms(237). T.congolense was found to produce long, trypanosome forms in the stomach of Glossina palpalis which were without free flagellae, and which passed to the hypopharynx via the labial cavity to change into crithidial forms. The crithidials then changed into metacyclics. T.vivax only developed in the proboscis where the forms had free flagellae. T.brucei developed in both the gut and the salivary glands; in all forms a free flagellum was present. The metacyclics are the final developmental stage of the trypanosome in the insect. They are infective to the mammal and are of the trypanosome form. The first population of trypanosomes detected in a mammal infected with metacyclics are termed the first cyclicals(165).

Confusion has sometimes arisen from a lack of clear definition

of general terms used to describe trypanosome populations. The terms strain and isolate were separated by Lumsden(245). An isolate was defined as a section of a wild population of trypanosomes separated off by transference into artificial conditions of maintenance, usually by inoculation into cultures or laboratory rodents. A strain was defined as a population of trypanosomes derived from an isolate by inducing it to reproduce continuously by serial passage in cultures or in laboratory animals. The term stabilate was introduced(248) to describe a population of trypanosomes whose reproduction has been arrested by viable preservation on a unique occasion.

1.3. Immunological aspects of trypanosomiasis.

Most of the work, which has been carried out on the immunology of trypanosomiasis, has involved brucei group antigens. High parasitaemias of these trypanosomes can be obtained in laboratory rodents and they can be separated from blood components relatively easily. Many isolates of T.congolense and all isolates of T.vivax do not infect rodents. Also, T.congolense can be separated from blood components only with great difficulty. Methods, which have been used to obtain pure suspensions of trypanosomes, include the addition of phytohaemagglutinin(235) or anti-erythrocyte serum followed by centrifugation(262,325, 386), sedimentation gradient centrifugation(380) and the passage of infected blood through anion exchange cellulose(224).

a. Antigenic variation.

Trypanosomiasis in mammals, caused by species of the

brucei sub-group, is known to be a relapsing parasitaemia in which the trypanosome numbers(115,306) and their infectivity (80) are continually changing. Since the discovery of antigenic variation in trypanosomes(130), many workers have shown that each relapse population, which developed during the course of an infection, was antigenically different to those which preceded it(234,254,264,265,304). It is widely believed that the capacity of trypanosomes for antigenic variation is very great, and that the only limiting factor is the death of the mammalian host(164,299,300,309,361). Twenty two and twenty four different serotypes of the brucei group have been demonstrated in mice(299 and 278 respectively). Fifteen distinct antigenic variants, appearing one after another, have been isolated from a 52 day infection in a rabbit(164). Antigenic variation has also been demonstrated from infections derived from single trypanosomes (164,205,239).

Methods used to demonstrate antigenic variation can be divided into those used to induce antigenic change and those used to detect this change. The former can be brought about by subcurative drug therapy(299), the use of human plasma(206) and the action of serum antibodies(163), and the latter by the use of certain serological tests, the most important of which will now be examined.

^hErlich and Shiga(111) found that mice infected with trypanosomes, when cured, produced protective antibodies to the homologous strain only. This property was used to distinguish between antigenic variants of a strain. This test, known as Erlich's method, is now rarely used, but forms the basis of the neutralization test.

The property of certain human and primate erythrocytes to adhere to trypanosomes in the presence of complement and specific antiserum was termed the red cell adhesion test(104). The test, which was based on the Rickenburg reaction(294), was subsequently applied to distinguish antigenic variants of T.brucei (31) but is now rarely used as simpler tests are available.

Tests based on the lysis of trypanosomes by antisera, in the presence of complement, have been used since the discovery of trypanolytic antibodies by Schilling in 1902. Two modifications of the test exist, namely the lysin protection test(316) in which the effect of complement and test serum is gauged by subinoculation into mice, and the visual test(238) where the effect is studied microscopically. Both types of the test were used by the quoted workers to detect antigenic change in brucei sub-group trypanosomes. It has been reported that species specific antibodies only were detected by the lysin protection test(56). Lytic tests have also been used in the study of coccidiosis(199).

When specific antiserum and trypanosomes of the brucei group are mixed together agglutination of the trypanosomes results(231). Improved techniques of the test have been described (75,334), and the test has recently been used in detailed studies on antigenic variation of brucei sub-group in infected mammals (82,163-165). The test cannot be used with T.congolense since these trypanosomes autoagglutinate(231,72), and it has only been employed using the rat-adapted strain(95-97) of T.vivax as no other strains infect laboratory rodents(58). Agglutination tests have been used in immunological studies of malaria(108), coccidiosis(266), trichomoniasis(214,284) and toxoplasmosis(138,

139,141).

In the neutralization test, standard numbers of brucei group organisms were incubated in test serum for one hour in vitro and then inoculated into mice(333). If the mice failed to become infected, the trypanosomes were said to have been neutralized. Antigens have also been standardized in terms of the numbers of trypanosomes infective for mice(77). The test has been used to study antigenic variation of the brucei sub-group in cattle(82).

b. Pattern of antigenic variation.

Antigenic variants of the brucei sub-group were formed in a definite order in animals infected with the same or similar antigenic types(163-165). The variants, which developed early in the infections, were antigenically similar; those, which developed later in the infections, were not necessarily the same(164). When variants of a strain were transmitted to new hosts by syringe, similar antigenic types developed early in the resulting infections, and were termed 'predominant strain antigens'(165). This antigen may correspond to the 'parent antigenic type', to which trypanosomes were said to revert, on prolonged syringe passage of a strain in rodents(239). It is not known if, either antigenic variation within strains is limited, or similar antigenic types occur twice during an infection.

c. Mechanism of antigenic variation.

Antigenic variation of trypanosomes is thought to be due to the action of variant specific antibodies, which have been shown to induce antigenic change both in vitro(207) and in vivo(163). The process is thought to be either adaptive(166, 207) or mutative(52,233,322,361). The relapsing nature of the

parasitaemia can be explained by either mechanism.

d. Trypanosomal antigens.

The study of some of the characteristics of trypanosomal antigens became possible with the use of double diffusion techniques(71,161), which were first introduced in the late 1940s for the study of bacterial antigens(110,279,280). The antisera and trypanosomal antigens are placed in wells cut in agar-gel media. After 24-48 hours of reaction, a positive is indicated by lines of precipitated protein which can be detected with or without staining.

Thillet and Chandler(346) immunized rats against T.lewisi using the metabolic products of the trypanosomes. This led to the discovery of a soluble antigen in the serum of rats infected with T.brucei(368,369), which was termed 'exoantigen'. This antigen protected mice from challenge with homologous organisms, played a vital role in the pathogenicity of trypanosomes, and was distinct from many of the antigens within the trypanosome. It was later found to consist of two distinct antigens, designated PR and AG (318,320). The former, which was shown to be protective, could not be detected by precipitin techniques, while the latter could. The protective antigen was subsequently shown to be different to the agglutinogenic antigen(321). Soluble antigens were then demonstrated in the serum of rats infected with T.vivax and T.gambiense, in goats infected with T.vivax and T.brucei(162), in mice infected with T.gambiense, T.congolense and T.equiperdum(101,102), and in rats infected with T.evansi(146). In further work(255), it was suggested that, 'exoantigen contained both precipitinogens and agglutinogens

that antigenic variation of brucei sub-group trypanosomes was caused by specific changes in the soluble antigen complex, and that the immunogenicity of the antigenic components of each variant population decreased as the infection proceeded. Lepage(232) has recently demonstrated that distinct antigenic variants contain some different peptides.

A second group of antigens have also been demonstrated (368,369). These were only detected when the trypanosomes were disrupted and were called 'bound antigens'. Seed(318) considered that these antigens consisted of at least two types, were not involved in the determination of the serotype, and had no biological properties by which they could be characterized. Common 'bound' antigens have been demonstrated in different antigenic variants of a strain(82) and in different species of trypanosome (161,162,370).

The antigens of trypanosomes have been reviewed by Weitz(371)

e. Antigenic relationships of cyclically passaged trypanosomes.

When the first cyclical populations, obtained from G.morsitans infected with antigenic variants of three geographically distinct strains of T.brucei, were compared with each other and with their parents, an antigenic reversion limited to within strains was reported(31). These findings were later confirmed(73, 165), and it was also shown that antigenic variation did not occur in the tsetse fly, and that the antigenic type of the first cyclicals was probably similar to the metacyclics which produced them. The immunological relationships of trypanosomes of the

congolense group, before and after cyclical passage through Glossina has never been studied.

Trypanosomes, which are grown in culture, are thought to be similar to those, which develop in the tsetse fly (159, 291, 348, 364). Culture forms, obtained from five strains of T. rhodesiense, one strain of T. brucei and one strain of T. congolense, were shown to contain common agglutinogens (319), further illustrating the antigenic similarity of insect forms.

f. Resistance of man and animals to trypanosomiasis.

The different types of resistance of man and animals to pathogenic organisms have been defined by Humphrey and White (200). Resistance was divided into non-specific immunity and specific acquired immunity. In[~]ate or natural immunity was defined as the resistance displayed by an animal 'ab initio', by an animal that has never experienced the particular pathogenic organism either as a pathogen or as a related non-pathogenic variant. In[~]ate immunity includes non-specific immunity as well as some degree of passively acquired specific immunity.

The resistance of man and animals to trypanosomiasis varies according to the species and strain of infecting trypanosome, and to the species and physiological state of the mammalian host. The species of tsetse fly, which transmits the disease, may play an indirect role (178). Man is totally resistant to T. vivax. Certain breeds of cattle are thought to be totally (15, 288) or partially (55, 56) resistant to the pathogenic effects of trypanosomiasis, while grade cattle have little or no resistance (251). It has long been known that many species of game are resistant to the disease.

Livingstone(236) noted that game survived in areas where cattle succumbed to trypanosomiasis. Natural resistance is thought to be hereditary. The partial resistance of N'dama cattle was also thought to be hereditary(56), but it was later shown that acquired immunity played an important role(93). The position of game is uncertain. Some species are more susceptible than others to the disease(5,8), and anti-trypanosomal antibodies are present in bushbuck(46) and other species of antelope(94) from which trypanosomes have been isolated. Whether the resistance of game is hereditary or acquired during life is not known.

The host-parasite relationships, which develop during the patent disease, are not understood. In infected cattle, premunition (197,120,281,282), sterile immunity(120,312,315,316) and tolerance (22,56) states have all been described. Tolerance and premunition cannot be distinguished as, in both states, trypanosomes are found in the blood of resistant hosts. That premune mechanisms operate has never been demonstrated, since no methods have been developed to show that the presence of circulating trypanosomes cause the elimination of inoculated organisms.

g. Immune response.

The presence of serum antibodies, in animals infected with trypanosomes, was demonstrated at the beginning of this century(308). However, it has only been with the recent improvement in techniques, that more detailed knowledge of their properties has been obtained. Two distinct types of serum antibody, designated specific and common, can be detected during the disease process.

The serological tests used to study antigenic variation are also used to detect variant specific antibodies. The development, in a rabbit, of different variant specific antibodies to successive

antigenic variants was demonstrated using the agglutination test (163). A similar process was shown to occur in cattle with the use of both the agglutination and neutralization tests(82).

The precipitin, indirect fluorescent antibody and indirect haemagglutination tests have been shown to detect common antibodies in infected animals(161,383, and 147 respectively). Both the last two named tests were initially developed for other protozoal diseases before being applied to trypanosomiasis. The pattern of production of common antibodies in cattle infected with trypanosomes has been studied by Gray(161), Cunningham and Van Hove(82) and Boreham(24). Antibodies were detected by the second week of infection(24,82), they persisted only in the presence of trypanosomes in the host(82), and they disappeared relatively quickly after chemotherapy(161). In these studies, too few animals were used to draw definite conclusions.

The fluorescent antibody method(63) employs immune serum globulin labelled with fluorescent dye to locate the corresponding antigen. Two types of test are used, namely, direct and indirect. The former consists of a single stage antibody-antigen reaction, where, to the fixed antigen homologous conjugated antiglobulin is added. The latter involves two stages; unlabelled antibody and antigen are allowed to react; homologous labelled antiglobulin is then added after washing. The indirect method shows extra sensitivity attributable to additional combining sites made available by the antibody molecules of the middle layer acting as an antigen for the fluorescent antiglobulin. Until 1958, the only labelling substance of practical value was fluorescein isocyanate, FIC(64,65),

chosen because of the brilliant apple-green fluorescence of its protein conjugates. The chemical procedures originally required for the labelling of proteins with FIC were too complicated to appeal to most workers, and a welcome advance was made by the introduction of a simpler conjugation method using fluorescein isothiocyanate, FITC(297,298). FITC has a disadvantage, in that the colour contrast with tissue autofluorescence, may be unsatisfactory. Difficulties in colour contrast were overcome by the use of an orange fluorescent label, lissamine rhodamine B, which was very cheap and easily conjugated(53,54). Immunofluorescent techniques were first used in protozoology to differentiate Entamoeba histolytica and E.coli(155). They have been subsequently used in the study of toxoplasmosis (156,157), trichomoniasis(269), babesiosis(314), malaria(220-222, 349,350,353), and to examine the changes which occur in the surface antigens of Paramecium aurelia(20,21). In trypanosomiasis, the indirect method was first used to detect antibodies to T.cruzi in man(126). Further studies on human trypanosomes(216,242,313,378) established the potential of the test as a serological aid to diagnosis, especially when a simple test, involving the use of blood collected on filter paper(3), was described(10). In animal trypanosomiasis, the test has been used as a general screening method employing blood collected on filter paper and brucei subgroup antigen only(46,147,354). The patterns of common antibody production to all the pathogenic trypanosomes has never been studied.

Some chemical substances will become attached to the surface of erythrocytes, which will then agglutinate, in the presence of antiserum prepared against the attached chemical.

Haemagglutination can be direct, when substances attach directly, or indirect, when pretreatment with chemicals is required before molecular attachment occurs. Only the indirect test is of value in protozoology, as most important antigens are proteins, which will only attach to pretreated erythrocytes. In the initial description of the test(25), proteins were conjugated to fresh, tannic acid treated sheep erythrocytes. Formalinized erythrocytes(127) were then used in the test(60,90,202,270). This resulted in increased standardization of the antigen and a saving of time and labour. Formalinized cells are thought to be less sensitive than fresh cells(180). Although sheep erythrocytes are normally used, human 'O' cells are thought to be more sensitive(342). Bisdiazotised benzidine(BDB) has also been used for coupling proteins onto erythrocytes(290), and then used in the indirect test(61,340,341). The tanned and BDB techniques may not be equally sensitive and non-specific haemagglutination may occur if impure antigens are used (295). In protozoology, the indirect tanned cell test has been used with success in the study of toxoplasmosis(208,250), trichomoniasis (267,268), amoebiasis(213), malaria(98,99,343), and in the detection of antibodies to T.evansi(145,147) and T.cruzi(272). Failures in the application of the test to African pathogenic trypanosomes have been reported(144).

h. Serological typing of trypanosomes.

As an antigenic variant of the brucei sub-group can be identified by its variant specific antibody, the serological typing of the trypanosomes in this group may be feasible. Six different, major antigenic types were identified from ten isolates of brucei

sub-group trypanosomes(75). It was suggested that the species parasitizing man may have distinctive antigens since all the isolates from man contained a different major antigen to the animal isolates. This finding is still to be confirmed. The antigenic relationships of clones, prepared from the 'basic' and 'predominant' antigens of strains of the brucei sub-group, have also been compared with reference to geographical location(167,168). Many strains obtained from one area were antigenically similar, while few similarities existed in strains obtained from different areas. No attempts have been made to examine the antigenic relationships of different isolates of T.congolense or T.vivax.

i. Immunization methods.

Immune serum has been used with success in the treatment and prophylaxis of animal trypanosomiasis(100,311). Some passive immunity may be passed on from the female to her offspring, as young calves are less susceptible to infection than adult animals (118,121,315). As no detailed studies have ever been undertaken, it must be assumed that passive immunity lasts for a short time. It may play a part in subsequent adult immunity(93).

Three techniques have been used to induce active immunity in animals, namely, the inoculation of dead trypanosomes, attempts to produce premunition, and infection with the virulent organisms followed by chemotherapy. The last named approach is thought to give a longer and more durable immunity(35,218,333)

Considerable success has, however, been reported with the use of dead trypanosomes(68,225,336). The efficacy of this method seems to depend on the method of killing the organism,

the frequency of inoculations and the strain of trypanosome used (336). The use of adjuvants may potentiate the antigenic stimulus of dead trypanosomes(211).

No successful method to induce premunition has been reported. A claim that a premune herd had been created by the inoculation of small numbers of trypanosomes(315) was shown to be false when the animals were moved to a different area(197), where the challenge was increased.

The work of ^hErlich and Shiga(111) stimulated interest in the interaction between chemotherapeutic agents and host defence mechanisms. The assumption, that the effect of a drug in animals infected with trypanosomes was due to the combined action of the chemical and the defences of the host(35,209,345), was experimentally confirmed by Soltys(335). A strain of T.brucei, which was gradually exposed to antibodies and so became antibody tolerant, was less sensitive to suramin and antrycide, than a strain which had never been exposed to antibodies. In rodents, a more solid immunity was shown to develop when the strain of trypanosomes used was very pathogenic(36), and when the number of trypanosome challenges was increased(137). The use of drugs was shown to increase the strength of antibody response in mice infected with T.gambiense(278) and in mice infected with T.vivax, Tequiperdum, T&equinum and T.congolense (140). In cattle, the efficiency of the drug-antibody complex was shown to be dependent on the timing of the chemotherapeutic treatment(22), the breed of animal(251) and the strength of trypanosomal challenge(374). The use of large and frequent drug treatments was considered to be not only unnecessary, but also

harmful, as sterilized animals were apparently more susceptible to reinfection(22). When the infection and drug treatment approach was applied in the field, drugs were used whose fate in the body was not accurately known, and the trypanosomal challenge administered was not clearly defined. This led to erroneous results. For example, a resistance to the natural disease, which was produced in two groups of zebu cattle with the use of an antrycide regime and which was thought to be due to the immune response(332), could have been due to the action of the drug alone(327). These difficulties were partially overcome with the use of stabilates as challenge material(89) and berenil, whose fate in the bovine body was accurately known(83). Cattle were made resistant to one stabilate of T.brucei for up to 8 months. It has never been adequately shown that cattle can be made immune against T.congolense.

j. T.congolense.

Little detailed work has been carried out on any immunological aspects of T.congolense, due mainly to their biological properties which make them difficult to handle. Many strains do not infect laboratory rodents and they can be separated from blood components only with great difficulty.

Fiennes(119,120,122) suggested, that in cattle infected with T.congolense, serum antibodies were the first line of defence of the host. These antibodies could only be demonstrated with regularity during the chronic stages of the disease. The antigenicity of four strains of T.congolense was compared; three were thought to be allied, whilst the fourth was thought to be distinct. No detailed results were given in these papers to substantiate these claims. The development of complement fixing antibodies in sheep(331) and lytic

antibodies in N'dama cattle(56) infected with T.congolense has been reported. Laveran(228) claimed that a group of sheep and goats resisted a strain of T.congolense 22 months after the initial infection. It is not known if either antigenic variation occurs in T.congolense, or if two types of antibody response are produced.

1.4. Diagnosis of animal trypanosomiasis.

The diagnosis of trypanosomiasis in animals is dependent on the demonstration of the organisms by the examination of blood films, the inoculation of test blood into laboratory rodents or culture, and the ability of a suspect host to infect a group of uninfected tsetse flies. The detection of serum antibody may aid diagnosis, but can only be regarded as an indicator as to the presence or absence of infection.

a. Trypanosome detection methods.

For the first half of this century, blood films alone (305) were employed to detect trypanosomes in infected mammal hosts. The thick film was thought to be at least 120 times more effective in trypanosome detection than the thin film(125). The introduction of the inoculation of blood or tissue into laboratory rodents as a survey procedure(81,153,154) and a culture method(365) greatly increased the efficiency of detection of trypanosomes in infected hosts. The brucei sub-group were mainly detected by rodent inoculation, the congolense group by both rodent inoculation and blood films and the vivax group by blood films only. Wet film examination was thought to be of moderate value in the detection of T.vivax and of little value in the detection of either

T.brucei or T.congolense(215). Although culture methods are of great benefit in the detection of T.theileri(292), their use in the detection of pathogenic trypanosomes may not be warranted. Culture methods do not seem to detect many more positives than those detected by other methods(337). Also, contamination by T.theileri in cultures may make the identification of the pathogenic trypanosomes more difficult(170). The use of uninfected tsetse flies is of limited value due to the low infection rates and the time required to obtain a result.

b. Serodiagnosis.

Serological tests, which detect common antibodies, give most hope for the development of a suitable serodiagnostic aid. At present there is no test, which is considered suitable to fulfil this vital role.

Studies on the relationship between antibody status and detectable disease have been carried out in cattle using brucei sub-group antigen only(81,167,354). The number of serological positives greatly exceeded the trypanosome positives. The former were not necessarily correlated with the latter. This indicates, either the inadequacy of the trypanosome detection methods used, or that in a large number of the animals examined the infection had been eliminated. A study of the pattern of common antibody production, in relation to the known presence or absence of trypanosomes, in a large group of cattle whose infection history is known, should elucidate this problem.

1.5. Detection of metacyclic trypanosomes.

The detection of metacyclic trypanosomes is necessary to identify trypanosomes, to ascertain the time taken for cyclical development and to study aspects of tsetse fly feeding. Either flies can be dissected and the relevant organs examined microscopically, or fly material is inoculated into laboratory rodents which are subsequently examined for trypanosomes, or hungry flies are induced to probe onto warm microscope slides thereby depositing salivary drops which are then stained and examined.

Animal inoculation techniques are indirect as a fly is said to be ejecting metacyclics only if it produces an infection in a mammal. Either flies are induced to feed directly on the animal (42,43), or to feed through a membrane into a capsule containing blood and the blood inoculated into rodents(59,195,303), or whole flies are triturated in salt solution and aliquots inoculated(109). The membrane capsule has been used to estimate the number of metacyclic trypanosomes of T.rhodesiense and T.congòlense ejected by G.morsitans(76,382).

The fly probing technique(44,47,48) has been used to count the number of metacyclics of T.rhodesiense ejected by G.morsitans.

1.6. Trypanosome counting methods.

Methods used to count trypanosomes are direct and indirect. They have been used to study changes in trypanosome numbers during infection(80,306), to examine the effects of drugs on trypanosomes (83) and in the standardization of antigens for serological tests (77). Direct methods, in which the trypanosomes are visually counted

include the use of the haemocytometer, the number of parasites per 'x40' objective lens field in a wet preparation, the ratio of organisms to red or white blood cells in stained preparations and the number of trypanosomes detected in a constant volume of blood in a stained thick smear. The only indirect method involved the calculation of the number of trypanosomes, infective for mice, by titration(246).

The haemocytometer method consists of counting the number of trypanosomes in a given number of square millimetres. The organisms may be counted alive or dead, stained or unstained. If trypanosomes in blood are to be counted, the blood must be diluted at least fifty times or the erythrocytes will mask the movement of the trypanosomes. If the trypanosomes are scanty, a very large number of squares must be counted to give a reliable result(362). It is thus only practical to use this approach to obtain an exact result in samples containing 10^8 trypanosomes per ml. As in domestic animals the concentration of trypanosomes in the blood rarely exceeds 10^6 per ml., the haemocytometer method is used to estimate numbers in most cases.

By observing a predetermined number of microscopic fields of infected blood, between a slide and a coverslip under a 'x40' objective, some estimate of trypanosome numbers may be obtained.

The advantages of using white or red cells as reference particles are that permanent preparations can be used and light parasitaemias can be detected. However, the random error of counting two types of particle and the lack of uniform distribution of cells and trypanosomes in smears make this method unreliable.

The counting of trypanosomes in a measured quantity of blood

after dehaemoglobinizing and staining(305), is not suitable in heavy infections and some trypanosomes may be lysed in the process.

All the direct methods are inaccurate, as significant differences were found when they were used to count trypanosomes in the same sample(359).

In the indirect method, serial ten-fold dilutions of the test trypanosome suspension are made. 0.1ml aliquots of each dilution are then inoculated into each of six mice. From the number of mice, which become infected, the infectivity of the trypanosome suspension is calculated, and the number of infective trypanosomes is deduced. The technique has been shown to give consistent results with samples of brucei sub-group organisms and differences of $10^{1.2}$ infective trypanosomes or more between titration end points were shown to be significant(246).

1.7. Preservation of trypanosomes at low temperature.

Trypanosomes multiplying in a mammalian host are continually changing. The study of some of these changes is enhanced by the use of stabilates, as it enables definite stages to be examined in a continuous system.

Early work indicated that some trypanosomes were resistant to sudden falls in temperature(143,231). Attention was drawn to the importance of slow cooling when a two-stage freezing process was introduced(367), a finding which was later confirmed(363) when three fast and three slow cooling rates were used to preserve populations of T.brucei. Between -25°C and -35°C appeared to be critical(87). If the cooling rate was slow through this 'danger zone' good survival

was obtained; if it was too fast, survival was poor. After the discovery of glycerol as a freezing protectant(286), it was introduced for the preservation of trypanosomes(287,292). Concentrations of between 5 and 15% increased the number of viable organisms after preservation. Although glycerol is an ideal freezing protectant, in some tissues it permeates slowly. In such circumstances dimethylsulphoxide was suggested as an alternative protectant(240). DMS has been used, with success, in the preservation of T.congolense and is thought to be a better protectant than glycerol in the preservation of Trichomonas vaginalis(393). The volume of blood, which can be efficiently preserved, is closely related to the cooling rate. Very small volumes can sustain fast cooling without the presence of freezing protectant(92,182), while slightly larger volumes cannot (78). The effect of low temperature storage on the infectivity of trypanosomes indicated that organisms could be stored for several years without loss of pathogenicity(79). Some species of trypanosome may preserve better than others(92). The thawing rate of organisms from the temperature of storage does not seem to be critical(88).

Most workers use solid carbon dioxide to maintain trypanosomes at low temperature. The use of liquid nitrogen is becoming more popular as it is cheaper and easier to maintain. Permanent storage units vary from the use of vacuum jars(287) to specially designed structures to hold capillaries in insulated chests(78).

Gordon(158) could not preserve metacyclics by the methods he used. The successful preservation of metacyclics has subsequently been reported(73,76,338).

The mechanism of freezing in living cells has been reviewed by Meryman(253) and Farrant(116). The formation of ice crystals and their subsequent growth are the most important factors. Crystal size is increased by rapid cooling and increased volume but is decreased by the addition of glycerol which is a strong hydrogen bonder. The temperature at which ice crystals of injurious size are formed is known as the eutectic point. In simple solutions this occurs at a definite temperature when the suspension becomes solid. In complex solutions, such as blood, there are many eutectics resulting in a gradual hardening of the suspension(171).

1.8. Trypanosome diluents.

In many chemotherapeutic, immunological and metabolic studies trypanosomes are maintained for short periods in vitro. For this purpose, a diluent is required which will not affect the infectivity of trypanosomes over the period required. The multiplicity of different and often not clearly defined solutions which have been used to suspend trypanosomes(11,12,4,185,287,333) has recently been emphasized(247). Specific information on the maintenance of infectivity of trypanosomes in these various solutions is lacking. It has been claimed, that the incorporation of normal sera into diluents, enhanced the maintenance of trypanosomes at 37°C(387). The precise mode of action of sera is uncertain as, in some circumstances, they are antitrypanosomal(190,206,231,347). For reasons of simplicity, solutions of buffered salts were introduced as diluents for the in vitro study of brucei sub-group trypanosomes (246). Concentrations of metallic and acidic ions are similar to those used in Krebs mammalian ringer solutions(91), and a buffer component added to control the pH. Nutrients are excluded, to and

to minimize metabolism and reproduction the suspensions are kept at 0-2°C.

1.9. T.congolense disease of cattle.

The course and parasitaemic picture of the disease in cattle, caused by T.congolense, has been described as falling into two phases. The first, known as the major crisis, lasts for two to four months following infection and is characterized by the constant presence of trypanosomes in the blood. The second, known as the secondary crisis, is characterized by the scanty appearance of trypanosomes in the blood and may last for many months(121,132). Death may occur after the major crisis, or after prolonged disease during the secondary crisis. Alternatively, a self-cure may result, which may be premunity or sterile immunity. Fiennes (120,122) claimed that in premune animals serum antibody was present, but in sterile immune animals it was absent. He also visualized an antigenic reversion of the trypanosomes in premunity, where the organisms reverted back to an antigenic type to which the animals had already been sensitized.

The plane of nutrition of cattle does not seem to affect the disease process from infection to major crisis, but is very important in the subsequent recovery(131,132).

The habitat of T.congolense in the mammalian host is unknown. It may be entirely in the blood(131), or in the blood and parenchymatous organs(124). Diurnal variations in the numbers of T.congolense occur in the blood of infected hosts(123,198), which appears to be associated with temperature change.

2. MATERIALS AND METHODS.

2.1. Laboratory animal maintenance.

A closed colony of Swiss white mice, brought to East Africa from the USA(329), was maintained at EATRO(the East African trypanosomiasis research organization) in a separate building. They were fed with pellets containing ground nut meal, wheat germ, and dried milk. Experimental mice were obtained from the colony when they were between 28 and 63 days old and they were starved 24 hours prior to use. A closed colony of white rats was maintained in a similar manner.

A stock of East African shorthorned zebu cattle(391) was maintained on the 1,500 acre, Combryetum savannah(352), EATRO farm. The cattle were kept under range conditions and consisted of two herds, one for breeding and the other for experimental purposes. The two herds were kept in separate paddocks, which were divided by a main road, and in which crushes were constructed. No supplementary feeding was necessary as the annual rainfall averaged sixty inches. Dipping was carried out weekly by plunging the animals into a 'Cooper tox' bath dip(Cooper, McDougal & Robertson, Nairobi, Kenya). Yearly vaccinations were carried out against anthrax and blackquarter. Calves were weaned at three months of age and either kept in the breeding herd or transferred to the second herd for experimental work. Each animal was given an EATRO number at birth by means of an ear tag and a record sheet giving the sex, date of birth and history. No tsetse flies were present on the farm although they occurred along the banks of the

Malaba river, which ran along the eastern boundary. This tsetse population was recently sampled for the presence of trypanosomes and found to be negative(177). No cattle were kept in the vicinity of the river.

2.2. Maintenance of and methods used to infect Glossina morsitans.

Pupae of G.morsitans were obtained from Singida, Tanzania and sent to EATRO by post, wrapped in cotton wool. They were placed in sand, inside cages, in a room with a temperature of 22-23°C and relative humidity 75-80%, until hatching took place. Males and females were separated and placed in geigy cages. Twenty four to forty eight hour old flies were given their infecting feed, either by inducing them to feed directly on an infected animal, or by allowing them to feed through a rat-skin membrane into a capsule containing infected blood. The flies were subsequently maintained in geigy cages at a mean temperature of 23-24°C and relative humidity of 60-70%. Uninfected bovine blood, in a capsule, was offered to the flies daily.

2.3. Trypanosome diluents.

a. Phosphate buffered saline(PBS).

PBS is a solution of sodium chloride, buffered to pH 7.2, with a phosphate buffer system. 36.0gm of NaCl, 7.4gm of Na₂HPO₄ and 2.15gm of KH₂PO₄ were dissolved in a litre of distilled water. 100ml aliquots were stored at -26°C in polythene, stoppered bottles. For use, the stock suspension was diluted one in five with distilled water. The pH of the PBS was checked regularly with a pH metre(EIL, Richmond, UK).

b. Buffered salts solutions.

Buffered salt solutions, buffered to pHs varying from 5.8 to 8.0, were prepared from two stock solutions A and B in a similar manner to that described by Humphryes(201). The constituents of solutions A and B are shown in appendix L. Bulk stocks of both solutions were stored at -26°C in glass, stoppered bottles. From the bulk stocks, solutions A and B were dispensed, respectively, to universal and bijou bottles(Unified glass Ltd, Staines,UK), which were then tightly capped and autoclaved at 20lb/sq.in for twenty minutes. The bottles were then stored at approximately 4°C . The complete diluent was made up from them, just prior to use, in a ratio of 9 volumes of A to 1 volume of B. BS-1 is a solution of salts buffered to pH 7.4 with a phosphate buffer system(247).

2.4. Detection of trypanosomes in animals.**a. Salivaria.****i. Wet film.**

A drop of the blood to be examined was placed on a microscope slide(Chance, Smethwick, UK), a coverslip dropped on it, and the resulting wet film was examined for motile trypanosomes under the 'x40' objective of a GFL microscope(Zeiss, Germany). If a trypanosome was seen, the blood was recorded positive; if no trypanosomes were seen in 200 fields, the blood was recorded as negative. In suspect cases, 1,000 fields were examined.

ii. Thick film.

A drop of the blood to be examined was placed in the centre of a microscope slide and made approximately a quarter of an

inch in diameter by gentle stirring with the tip of another slide. The thick film was allowed to dry in air, placed on a staining rack and flooded with giemsa(Gurr,London, UK) stain solution(one part stain to twenty parts distilled water) for 25 minutes. The stain was washed off and the film allowed to dry. The film was examined for the presence of trypanosomes under the 'oil immersion' using the 200 field criterion described for wet films.

iii. Mouse inoculation.

0.5ml of the blood to be examined was inoculated intra-peritoneally into each of two mice. From day 3 after inoculation, each mouse was examined daily for trypanosomes by wet films of tail blood. If trypanosomes were detected the sample was recorded as positive. Examination was continued for 60 days before a sample was recorded as negative.

The three trypanosome detection methods will be referred to as the STD(M the standard trypanosome detection methods).

b. Stercoraria.

T.theileri was detected by a culture method. 24.8gm of Difco nutrient agar(Difco, Detroit, USA) of pH 7.3, and 1.6gm of dextrose were added to 800ml of distilled water. The suspension was autoclaved for 15 minutes at 15lb/sq.in, then cooled to 55°C, and 200ml defibrinated bovine blood, penicillin to a concentration of 625 units/ml, and dihydrostreptomycin to a concentration of 50 µgm/ml were added. 5ml quantities of this medium were added to loz screw capped bottles, which were slanted to set and incubated for 24 hours at 37°C to check sterility. Using aseptic technique, 6ml of the test blood were inoculated into one bottle. A drop of

the mixture in the culture was examined for trypanosomes by wet film 5,10,15,20 and 25 days after inoculation. T.theileri was identified by the length and motility of the trypanosomes seen. If no trypanosomes were observed by day 25, the test blood sample was recorded as negative.

2.5. Identification of trypanosomes.

Trypanosomes were identified by the examination of their morphological characteristics in stained thin films. A drop of the suspension, containing the trypanosomes, was placed on the end of a microscope slide. Using the tip of a second slide, the drop was drawn across the surface so-forming a thin film, which was allowed to dry in air. Methanol(BDH, Poole, UK) was flooded on to the film for two to three minutes followed by washing in distilled water and drying at room temperature. The films were stained with giemsa, as described for thick films. The stained trypanosomes were identified microscopically using the criteria of Hoare(186) for the group, of Godfrey(151) for T.congolense, and of Lloyd and Johnson (237) for insect forms.

2.6. Detection of metacyclic trypanosomes.

a. Dissection.

The proboscis was broken off the fly where it was attached to the head. The labrum and hypopharynx were separated using probes, each placed in a drop of PBS, and mounted with a coverglass. A lateral incision was made along the abdomen of the fly, the contents teased out with a probe, the gut and salivary

glands isolated and both placed in a drop of PBS and mounted. The mounted specimens were examined for trypanosomes under the 'x40' objective. Thin films were prepared from the organs of positive flies for identification.

b. Mouse inoculation.

0.5ml aliquots of the blood remaining in a membrane capsule after feeding(see section 2.2) were inoculated into each of two mice. The mice were examined for trypanosomes as described in section 2.4.a.iii. Thin films were prepared from the tail blood of positive mice for identification and the relevant fly or group of flies were recorded as metacyclic positive.

c. Stained salivary probe.

The flies to be examined were placed, individually, in 1x3" double ended tubes with a cork at one end and a gauze covering at the other. The tubes were marked with the fly number and placed in a room at environmental temperature and humidity. Glass, microscope slides were warmed to body temperature by placing them over a 37°C water bath(Laboratory thermal equipment, Greenford, UK) on a glass plate. The flies were induced to probe by placing the tubes, gauze-end down, on the slides inside a circle made with a wax pencil. A probe was considered to be positive when the fly deposited drops of saliva inside the circle. If a fly failed to probe after ten minutes, it was removed and tried again the following day. Probed slides were marked with the respective fly number and the salivary drops stained with giemsa for the examination and identification of any trypanosomes present. A slide was not considered to be negative until all the stained,

salivary probes had been examined.

When these detection methods were used (see section 3.1), dissection was carried out as the flies died, mouse inoculation from the fifteenth day, and stained probe daily from the twenty first day after the infecting feed.

2.7. Trypanosome counting methods.

a. Haemocytometer.

Facial sebum was thinly smeared on the ridges on either side of the counting chamber of a Neubauer haemocytometer (Hawksley, Lancing, UK), and a coverglass gently pressed onto the ridges until Newton's rings appeared on both sides. A drop of the suspension to be counted was placed under the cover glass, on the edge of both counting chambers, so that there were no air bubbles. The trypanosome suspension was either whole blood, diluted fifty times in BS-1, or stabilate material, when no dilution was necessary. The counting chamber was left in a horizontal position for ten minutes to allow for settling of the trypanosomes. All the organisms, seen in two diagonally opposite millimetre squares of each chamber, were counted. Since approximately 10% of T. congolense adhere to the coverslip (362), two levels were focused, the bottom of the chamber and the coverslip. The number of trypanosomes per ml of the suspension was calculated by multiplying the mean number of organisms per square millimetre by the suspension diluent $\times 10,000$.

b. 'x40' objective lens.

By counting the number of motile trypanosomes in wet films under 'x40' objective lens fields, an approximate estimate of the numbers of trypanosomes can be obtained. A simple scoring system

shown on table 2.1, was used to give the approximate concentration of trypanosomes per ml.

NUMBERS SEEN ON WET FILM EXAMINATION	-	S	+	+	+++	++++
APPROXIMATE Log ₁₀ Nos/ML	7.4	4	5	6	7	8 ⁺

Table 2.1. Approximate relationship between the numbers of trypanosomes seen on wet film examination and counts per ml. - is no trypanosomes seen in a 'x40' objective lens field, S is one, + is between one and five, ++ is between six and ten, +++ is between eleven and twenty, and ++++ is more than twenty.

c. Mouse infectivity titration.

The trypanosome suspension was diluted serially ten-fold by carrying over 0.2ml quantities to 1.8ml of BS-1 in test tubes using teat-operated pasteur pipettes. All suspensions were maintained at 0-2°C by immersion of the test tubes, in iced water, in racks, in photographic trays. The trays were placed in insulating jackets of expanded rubber. The starting dilution of a titration was dependent on the volume of material available. For stabilates in capillaries, it was 1/10; but was undiluted blood, when stabilates in ampoules or unpreserved blood was used. The last dilution of a titration was judged according to the number of trypanosomes present. On completion

of the dilution series, mouse inoculation commenced beginning with the highest dilution. Using a tuberculin syringe (Syringue, industrielles, Clermont-Ferand, France), 0.1ml aliquots of each dilution were inoculated intraperitoneally into each of six mice. The mice were lightly anaesthetized with ether (Hopkin & Williams, Chadwell Heath, UK) to facilitate injection. In the +1 dilution, which was used in some titrations, 1ml aliquots of whole blood were inoculated. Each group of six mice was placed in a separate cage on which the relevant dilution number was marked. Commencing at the lowest dilution, all the mice in the group were examined for trypanosomes, as described in section 2.4.a.iii. If a mouse was positive, examination of the mice in the next dilution began.

The infectivity end point of a titration and significance were calculated, according to the number of mice recorded positive, from tables (246), which are shown in appendix 2. The statistical unit was the mouse ID₆₃, which was taken to be equivalent to one infective trypanosome.

2.8. Preservation methods.

a. Blood forms.

The source of trypanosomes was either blood obtained from the first peak of parasitaemia in infected mice, or blood from infected cattle. The blood was collected by heart puncture of mice using sterile pasteur pipettes, or venepuncture of the jugular vein of cattle using a sterile 20ml syringe. The blood was deposited in bijoux or universal bottles in beakers surrounded by ice, and heparin to a concentration of 4 units per ml was added. The freezing

protectant, glycerol (BDH, Poole, UK), was then added and the blood deposited in preservation receptacles, which were either lymph tubes (Messrs Plowden and Thomson, Stourbridge, UK), one ml ampoules (Baird and Tatlock, Chadwell Heath, UK), or bijou bottles. The lymph tubes and ampoules were sealed in a bunsen burner flame, and the bijou bottles tightly capped. They were then placed in tubes containing methanol (BDH, Poole, UK), and were either wrapped in pieces of one foot square brown paper or deposited in polystyrene holders, and placed in an onazote cabinet filled with solid carbondioxide. Twenty four hours later, the stabilates were transferred to the permanent storage unit, an onazote cabinet of 27 cu ft internal volume which was divided into sections and filled with solid carbondioxide. The stabilates in capillaries were deposited in numbered metal containers similar to those described by Cunningham et al (78). The stabilates in ampoules and bijou bottles were deposited in one of the sections.

b. Metacyclic forms.

Known infective flies were induced to feed through a rat-skin membrane into uninfected, defibrinated bovine blood. After feeding, the blood remaining in the capsule containing any ejected trypanosomes, was transferred to one ml ampoules and preserved by the same technique as that described for blood forms.

c. Stabilate designation.

Every stabilate prepared was designated according to origin and given a specific number. Stabilates prepared from the strains maintained at the Zoology department of Edinburgh university were prefixed by KB, those prepared from EATRO stabilates were given an oblique stroke and a number after the EATRO number, and those

obtained from the Edinburgh veterinary expedition were prefixed by EVE. Record sheets were kept for each stabilate giving the date of preservation, the origin of the stabilate and information regarding identification, the concentration of trypanosomes present and their infectivity.

2.9. Sera.

a. Production and collection.

Antisera were either prepared specifically against a population of trypanosomes in a rat, or collected from known infected animals, or obtained from animals in field surveys when it was not known whether they were infected or not. Specific antiserum was obtained by inoculating 10^6 trypanosomes into a weighed adult rat. Three days after the detection of first parasitaemia, by wet film examination of tail blood, the infection was cured by berenil (Farbwerke Hoechst, Frankfurt, Germany) at a dosage rate of 150µgm per gram body weight. The rat was killed six days later and the serum collected. The method was based on that of Cunningham and Grainge(74).

Collection of sera varied according to the species of animal. In rodents, the animals were killed with chloroform(Hopkin & Williams, Chadwell Heath, UK), pinned to a dissection board ventral side up and the heart exposed by cutting across the rib-cage on both sides. Blood was removed by piercing the left ventricle with a teat-operated pasteur pipette and was deposited in a bijou bottle. In rabbits, the blood was removed from the ear vein. Prior to bleeding, the ear was covered with a thin layer of vaseline and

then sponged with a wad of cotton wool containing xylol. The main ear vein was nicked with a razor blade and 4 to 5ml collected in a bijou bottle. The xylol was removed by washing the ear with methanol immediately after bleeding. In cattle, 20ml of blood was withdrawn by venepuncture of the jugular vein and deposited in a universal bottle. Blood was also collected on Whatman No. 4 filter paper (Balston, London, UK) when the ear vein was punctured with a razor blade, and blood spots were deposited on the paper and allowed to dry. The blood collected from all the animals was allowed to clot over a twenty four hour period at room temperature. The resulting serum was removed and deposited in 0.7ml aliquots in plastic tubes (Xylon products, London, UK).

b. Designation and storage.

Each serum collected was given a number and a record sheet giving the number, the origin and date of collection. The serum tubes were placed in boxes in the deep freeze at -26°C . The dried blood spots on filter paper were numbered, placed in envelopes in groups of ten and stored at -26°C .

2.10. Serological tests.

a. Agglutination test.

Antigen was prepared by the rapid sub-passage of trypanosomes in mice, every 2-3 days, until the concentration of trypanosomes in the blood had been boosted to at least 10^8 per ml. The infected blood was removed by heat stpuncture and preserved in lymph tubes (capillaries). Two modifications of the test were used. In the first (A), drops of 1/10 dilutions of the test sera in PBS were placed on siliconed microscope slides. In the second (B), serial

two-fold dilutions of the test sera were made on siliconed, glass plates using the microtitre technique with BS-1 as diluent. 0.025ml aliquots of diluent were deposited onto a glass plate with the microtitre constant dropping pipette (Cooke engineering, Virginia, USA). Dilutions were made by carrying over 0.025ml amounts of the serum with the microtitre loop. Two controls were included, a BS-1 and a normal serum. A capillary tube of the antigen was removed from storage, the sealed ends broken off and one end made into a fine point by melting it over a bunsen flame. A drop of the antigen was added to each of the test sera dilutions and controls through the fine point. The slides or glass plates were placed in a humidity chamber at room temperature for 30 minutes. The test was read under the 'x40' objective using the plus scoring system of Cunningham and Vickerman (75). The degrees of agglutination ranged from 4 to 1 plus according to the size of clump. The titre of a serum was the highest dilution to give a one plus.

b. Trypanolytic test.

Brucei sub-group antigen was prepared as described for the agglutination test. For T. congolense, ten mice were inoculated with the stabilate to be examined. At the first peak of parasitaemia infected blood was removed, heparinized and centrifuged at 850g for 2 minutes. The supernatant was removed and the trypanosomes therein preserved in capillaries. Sera dilutions were made on glass plates with the microtitre equipment. To each dilution, a drop of a 1/4 solution of guinea pig complement in PBS was added. Three controls were included in each test, a BS-1, a normal serum and a complement inhibitor (EDTA; 0.02M, in 1/4 guinea-pig serum). The antigen was

added as in the agglutination test and the plates placed in a humidity chamber at 37°C for 30 minutes. In the reading of the test a simple scoring system, based on the % lysis of the trypanosomes, was used. Four-plus was 90% lysis, three-plus 50 to 80%, two-plus 20 to 50%, one-plus 10 to 20%, and zero no lysis. The titre of a serum was the highest dilution to give one-plus.

c. Neutralization test.

Preserved antigens were standardized in terms of mouse ID_{63s}. Three modifications of the test were used. In the first (A), approximately 10³ mouse ID_{63s} in 0.9ml were incubated with 0.1ml of the test serum for 30 minutes at 0-2°C. Aliquots containing 10² ID_{63s} were then inoculated into each of six mice. In the second (B) a similar procedure was adopted to A, except that 1/10, 1/20, 1/50, 1/100 and 1/200 dilutions of the test serum were used. In the third (C), mouse infectivity titrations of the test stabilate were carried out using the highest dilution of the test serum, which neutralized the trypanosomes in B, as diluent. Two controls were included in all tests, a BS-1 and a normal serum. All inoculated mice were examined for trypanosomes, daily from the third day until the fortyeth day, when the results were assessed using a simple plus and minus scoring system. Plus(+) indicated neutralization when none of the mice became infected. Minus(-) indicated no neutralization when one or more mice became infected. Neutralization -A indicated whether a serum had neutralising antibodies,; B gave an approximate titre; and C gave a precise titre called the neutralising index(77).

d. Indirect haemagglutination.

A parasitaemia of at least 10^8 trypanosomes per ml was induced in a group of five mice by rapid sub-passage of a population of brucei sub-group. Infected blood was collected by heart puncture, and the trypanosomes removed by centrifugation and deposited in 1ml of BS-1. The organisms were washed three times by centrifuging them at 3,000g with subsequent removal of the supernate and resuspension in BS-1. The trypanosomes, in a final volume of 1ml, were lysed by rapid freezing and thawing. The resulting lysate was centrifuged at 3,000g for 5 minutes. The supernatant was removed, a portion of which was used to estimate the protein concentration while the remainder was stored in 0.1ml aliquots at -26°C . The method of Lowry et al was used to estimate protein(241). Five ml of solution A(100ml, 0.2M NaOH; 100ml, 4% NaCO_3 ; 2% sodium potassium tartrate, 2ml; 2ml, 1% CuSO_4 , added in the order given) was added to 0.5ml of the test sample and to 0, 0.1, 0.2, 0.4 and 0.5ml aliquots of the standard sample(a solution of bovine serum albumin, 400 μgm per ml) made up to 0.5ml distilled water in test tubes. The mixtures were left at 30 minutes at room temperature. 0.5ml of solution B (half dilution of Folin and Ciocalteu's reagent) were added to each tube, the contents mixed well and left in the dark for 30 minutes at room temperature. Each of the standard and test samples were read in a spectrophotometer(Beckman, Glenrothes, UK) at 660 μp against a blank(0.5ml distilled water). The results obtained from the standards were plotted graphically from which a reading of the test sample resulted.

Formalinized sheep erythrocytes were prepared by the method of Czismas(90). One hundred ml of sheep blood was collected

from an abbatoir in a 200ml conical flask and defibrinated using glass beads. One hundred ml of a 10% suspension of erythrocytes were made up in PBS, washed five times and resuspended to the original volume. Twenty five ml of formalin were poured into a cellophane dialysis sac, which was hung in a 200ml beaker by means of a glass rod. The 10% cell suspension was poured into the beaker and gentle agitation of the mixture was carried out for 4 hours by means of a magnetic stirrer. The dialysis sac was then punctured with scissors and the agitation continued for a further 18 hours. After the removal of the surface cell debris, the 125ml formalinized cell suspension was centrifuged at 3,000g for 10 minutes, the supernatant decanted, and the cells washed four times in PBS. The cells were finally resuspended to the original volume, 5ml aliquots deposited in bijou bottles, which were immersed in thermos flasks (Thermos Ltd, Brentwood, UK) containing solid carbondioxide and methanol.

2.5ml of thawed, formalinized erythrocytes were put into two universal bottles and the volume made up to 10ml with PBS(a 2.5% cell suspension). 10ml of a 1/10,000 suspension of tannic acid (Mallinkrodt Chem. Corp, London, UK) made up in PBS, was added to both bottles, which were placed in a 37°C water bath for 15 minutes. Both bottles were centrifuged, the supernatant removed and one bottle was put aside and designated the 'tanned cell only control'. To the other bottle, 10ml of the antigen solution made up in buffered salts solution at pH 6.6 was added and allowed to react with the cells for 15 minutes at room temperature. The cells were centrifuged and the supernatant removed. Both the tanned only and the antigen

coated cells were washed once in 1% normal rabbit serum(NRS) with which they were resuspended to 25ml giving a 1% suspension for the test. The NRS had been inactivated at 56°C and the natural antibodies to sheep erythrocytes removed by adsorption. 0.15ml of the test serum was added to 1.35ml of fresh sheep erythrocytes and the suspension allowed to react for 10 minutes at room temperature to remove natural agglutinins. The cells were centrifuged at 3,000g for 5 minutes and the supernatant, containing the serum at 1/10 dilution, was removed. Two series of serial two-fold dilutions of the test serum were made in 1%NRS from 1/10 to 1/5,120(*) and from 1/10 to 1/640(**) and placed in the holes of a WHO agglutination tray. To series * 0.1ml aliquots of the antigen coated cells were added, and to series ** 0.1ml of the tanned only cells were added. Each hole in the tray contained 0.5ml of the serum dilution and 0.1 ml of cell suspension. To three different 0.5ml aliquots of 1%NRS, 0.1ml amounts of antigen coated, tanned only and fresh cells respectively were added as further controls. The trays were left for 24 hours after which the test was read according to the settling patterns of the erythrocytes. The scoring system of Stavitsky(339), shown on figure 2.1, was used.

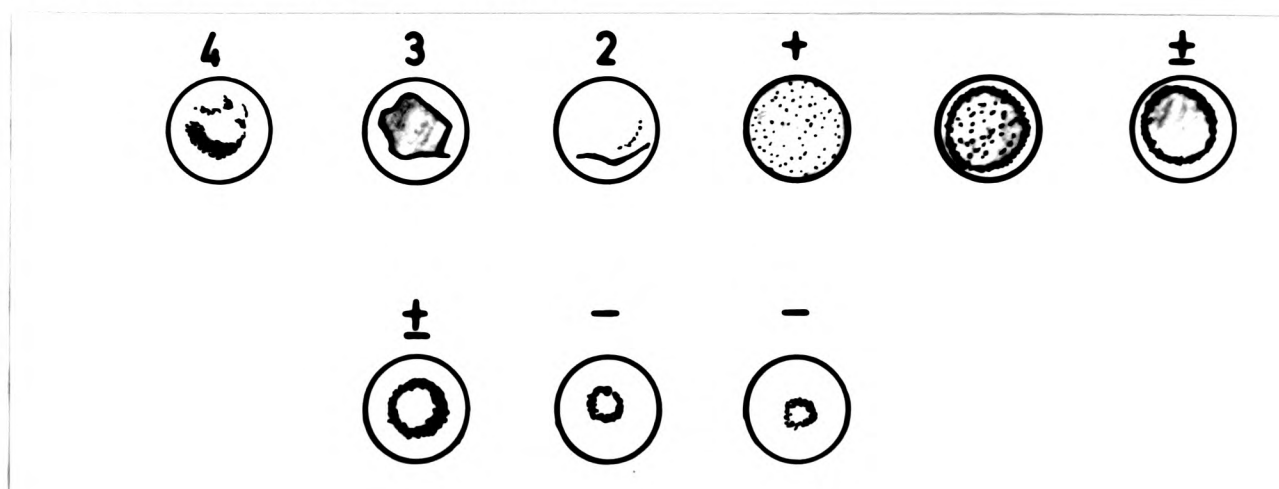


Figure 2.1. Appearance of the patterns of haemagglutination.

4, 3 and 2 are definite positives, + weak positive, ± unknown and - negative.

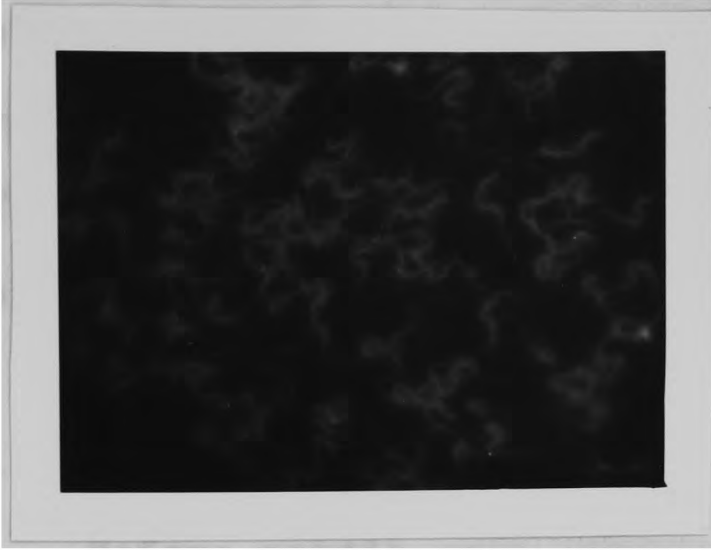
e. Indirect fluorescent antibody test.

Antigens were prepared from T.congolense EATRO-585, brucei sub-group EATRO-3 and T.vivax EATRO-1090 (a derivative of the rat-adapted strain of Desowitz and Watson (95-97)). Adult rats were inoculated with 10^6 organisms of EATRO-3 and EATRO-1090. Infected blood was removed at the first peak of parasitaemia, heparinized, an equal volume of PBS added and the suspension centrifuged for two minutes at 850g. The resulting supernatant, containing approximately 10^8 trypanosomes per ml, was removed and put in a test tube. Thin smears of the trypanosome suspension were made on microscope slides, allowed to dry at room temperature and heat-fixed over a microburner (Baird & Tatlock, Chadwell Heath, UK). The antigen coated slides were placed, in batches of seven, in 3x5" polythene bags (Marks, Nairobi, Kenya) containing a few crystals of silica gel (BDH, Poole, UK). The bags were sealed at both ends with a polysealer (Hulme-Martin, London, UK), wrapped in aluminium foil (Alcan, London, UK) and stored at -26°C . A group of ten mice were inoculated with EATRO-585. Thin films of tail blood were made from those mice in which the trypanosome concentration reached approximately 10^8 per ml at the first peak of parasitaemia. Fixation and storage of this antigen was similar to that described for T.vivax.

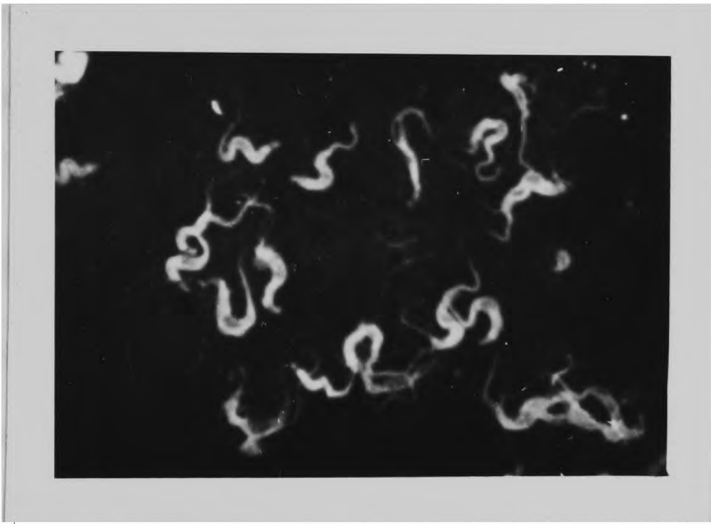
For the test, the required number of antigen slides was removed from the deep freeze, allowed to warm to room temperature and taken out of the polythene bags. Two rows of seven circles were drawn on each slide with red nail varnish (Woltz, Turin, Italy). The slides were numbered with a diamond pencil and placed in a

humidity chamber. Two modifications of the test were used. In the first(IPT-A), serial two-fold dilutions of the test serum, from 1/20 to 1/1,280, were prepared in test tubes. Drops of each dilution were placed in nail varnish circles so as to fill the entire area and the antibody-antigen complex was allowed to react for 40 minutes. In the second(IPT- B), 0.025ml amounts of PBS were placed in the circles and dried blood discs of 3mm diameter were added. The serum eluted from each disc and reacted with the antigen for 60 minutes. In both tests, the sera were washed off the slides with PBS, and the slides were dried with filter paper and replaced in the humidity chamber. The circles were filled with a 1/80 dilution of an anti-bovine gamma globulin FITC conjugate(Difco, Detroit, USA), which had been previously titrated for potency. Forty minutes later, the slides were washed and dried as before. Drops of PBS, conjugate and dilutions of 1/20, 1/40 and 1/80 of normal bovine serum were included in every test as controls. The antigen was mounted by placing drops of buffered glycerol(1 part PBS to 9 parts glycerol) in the circles on which coverslips(Chance No 1, 22x22mm) were placed. The test was read under the 'x40' objective of a conference microscope (Gillett & Sibert, London, UK). The light source was a 100 watt iodine quartz bulb working at a potential of 12 volts. The light was transmitted through two primary filters(Schott BG-12, 4mm thick) and an aplanat condenser onto the object. The light then passed through a yellow secondary filter to a x6 eyepiece lens. A simple scoring system, described by Fife and Muschel(126), was used. A reading of 4 or 3-plus was positive, 2-plus weak positive and 1-plus negative. The titre of a test serum was the highest dilution to give a 2-plus. Photographs 2.1 and 2.2 show the difference between

1-plus and 4-plus fluorescence.



Photograph 2.1. Brucei subgroup showing 1-plus fluorescence.



Photograph 2.2. Brucei subgroup showing 4-plus fluorescence.

Both photographs were taken on Ilford PAN F with 15 minutes exposure and were developed in Microdol X.

2.11. Estimation of trypanosome sensitivity to berenil(diamine-zine acetate).

Sixteen weighed mice were inoculated with the trypanosomes to be tested. When the mice became parasitaemic, 3 groups of 4 mice were inoculated with the maximum tolerated dose(MTD) of berenil (150µgm per gm body weight), half the MTD and a quarter the MTD

respectively. The remaining group of 4 mice was left as the untreated control. The tail blood of all the mice was examined for trypanosomes by wet film for the next 30 days. If a quarter the MTD cured the infection, the trypanosomes were said to be berenil-sensitive.

2.12. Pre-experimental cattle procedures.

Before cattle were used for experimental work, it had to be shown that they were free from trypanosomiasis. Heparinized blood, removed by venepuncture, was examined for the presence of trypanosomes by the STDM daily for the 10 days immediately prior to use. Sera were tested for anti-trypanosomal activity and for the presence of antibodies by using them as diluents in mouse infectivity titrations of a standard stabilate and in the IFT-A respectively. A cow was not used if trypanosomes were detected in the blood, if the serum inhibited infectivity of the stabilate by $10^{1.2}$ mouse ID_{63s} or more, or if the fluorescence titre was 1/40 or above.

2.13. Study locations.

In the work reported in some experiments of section 3 references are made to locations in East Africa. These are shown in figure 2.2.

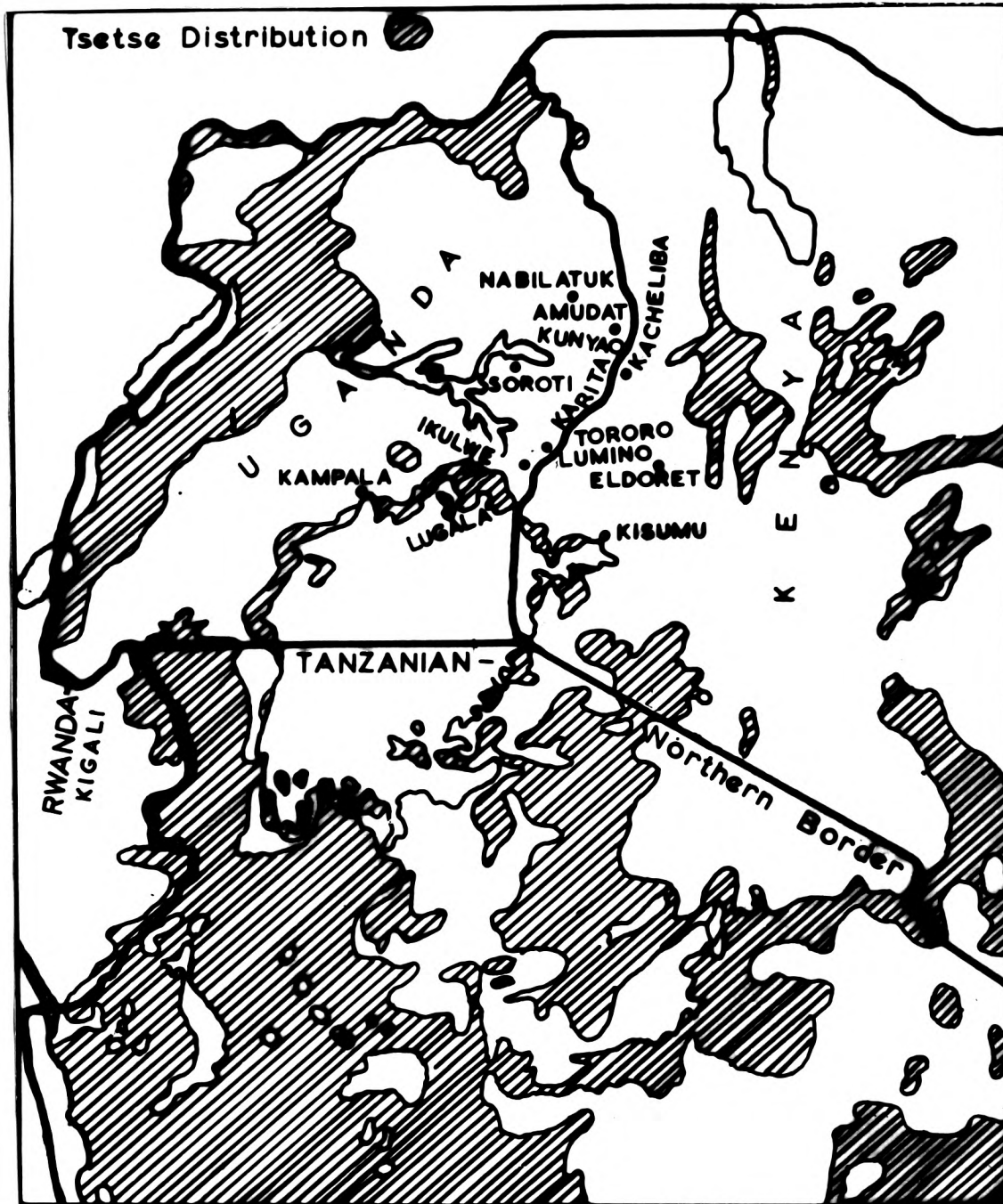


Figure 2.2. Map of East Africa showing locations from which trypanosomes were isolated. Scale: 1.5cm is approximately equivalent to 100 miles.

1 is Mtakara(see section 3.1).

Mavubwe and Bugiri(see section 3.23), and Myarinya (see section 3.1) are near Ikulwe.

EATRO-835(see isolate 6, section 3.23) was

isolated near Kisumu in Nyanza province, Kenya.

3. EXPERIMENTAL.

In this section, investigations were conducted on some immunological aspects of trypanosomiasis in cattle with particular reference to T.congolense. Studies were divided into the following sub-sections:-

- a. the development of suitable methods for the serological study of T.congolense(sections 3.1 to 3.19).
- b. The examination of antigenic changes of T.congolense in cattle and on cyclical passage through G.morsitans, and the immune response of cattle to infection(sections 3.20 to 3.22).
- c. antigenic variation of brucei sub-group with reference to geographical location(section 3.23).
- d. the feasibility of immunizing cattle against a single stabilate of T.congolense and against the natural disease by infection and chemotherapeutic treatment(sections 3.24 to 3.26).
- e. the evaluation of the indirect fluorescent antibody test as a serodiagnostic aid(sections 3.27 and 3.28).

3.1. Taxonomical identification of T.congolense.

Aim.

Before studies on T.congolense commenced, the organisms used had to be identified. Therefore, stabilates obtained from the EATRO trypanosome bank, which had the morphological characteristics of T.congolense and were infective to mice, were passaged through G.morsitans. The sites of development, and the morphology of the insect forms and resulting first cyclicals in mice were examined.

Procedure.

Two stabilates were selected, namely, EATRO-189 and EATRO-585. The former was isolated from a cow at Mtakara, Ankole, Uganda on 29.9.60, after which it was passaged 31 times in mice before preservation. The latter was isolated from a group of G.brevipalpis caught at Myarinya, Busoga, Uganda on 15.7.61, after which it was passaged once in a rat and 10 times in mice before preservation.

Mice infected with EATRO-189, 9 days previously, were used to infect a group of 52 G.morsitans by feeding individual flies on single mice. Blood collected from zebu 495, infected with a first mouse passage of EATRO-585 7 days previously, was used to infect a group of 47 G.morsitans by inducing the flies to feed into the blood through a rat skin membrane. The two groups of flies were maintained in geigy cages as described in section 2.2. On day 21 after the infecting feed, all the flies, which were still alive, were placed individually in double-ended tubes. Infected flies were detected by the methods described in section 2.6. Dissection was carried out as the flies died, mouse inoculation from day 15 and stained probe from day 21. The last two methods were carried out on all the flies, which remained alive, until the end of the experiment. Trypanosomes were identified by the examination of all positive stained probes, all the thin films of the first cyclicals and thin films prepared from gut and proboscis forms of flies found positive by dissection. Thin, stained films of the first cyclicals, obtained from the first infected mouse in each group, were prepared daily from tail blood for 5 days, after the detection of first parasitaemia. The morphological characteristics of 100

trypanosomes on each of the preparations were examined. Colour photographs of blood and insect forms, derived from EATRO-585, were taken with a Zeiss photomicroscope.

Results.

In the group of flies infected with EATRO-189, 26 flies were alive on day 21 and the last fly died on day 72. In the group of flies infected with EATRO-585, 31 flies remained alive on day 21 and the last fly died on day 67.

Tables 3.1 and 3.2 show the % of flies detected trypanosome positive by dissection and stained probe, the time taken for cyclical development to take place as judged by the first appearance of trypanosomes in the trypanosome form detected by the three methods used, and the arrangement of the trypanosomes in the proboscis-infected flies. The % of positive flies detected by dissection was calculated from the total number of flies used, whereas those detected by stained probe were calculated from the number of flies remaining alive on day 21. The length of cyclical development in G.morsitans varied in both stabilates, and with the detection method used. With the EATRO-189 derivatives, it varied from 22 days as judged by dissection to 34 days as judged by mouse inoculation. With the EATRO-585 derivatives, it varied from 17 days as judged by mouse inoculation to 24 days as judged by dissection. The ability of EATRO-189 to infect G.morsitans was much greater than that of EATRO-585; 23.1% by dissection, 30.7% by stained probe as compared with 6.4% and 3.2% respectively.

Figure 3.1 shows the times after the infecting feed when the trypanosomes in the infected flies, which remained alive,

were first detected by mouse inoculation and stained probe. Only one fly infected with EATRO-585 derivatives remained alive. It was detected trypanosome-positive by mouse inoculation on day 17 and by stained probe on day 22. Eight flies infected with EATRO-189 derivatives were detected trypanosome-positive by stained probe between days 28 and 33. Seven of these flies were detected positive by mouse inoculation between days 34 and 38.

The examination of the trypanosomes in all the stained thin films showed organisms without free flagellae and with marginal kinetoplasts. Photographs 3.1 to 3.5 show the different morphological forms of the EATRO-585 derivatives. Three types of blood form were detected, which were differentiated by length, shape of posterior end and prominence of flagellum (photographs 3.1 to 3.3). The insect gut forms were much thicker than the slender proboscis forms (photographs 3.4 and 3.5). Tables 3.3 and 3.4 show the proportion of different types observed in the series of prepared first cyclicals. Those recorded as short are similar to the type shown in photograph 3.1, transitional as in photograph 3.2, and long as in photograph 3.3. In both populations, there was a predominance of transitional forms.

Conclusions.

1. Both stabilates were T.congolense as development in the fly occurred in the gut and proboscis and both the blood and insect forms showed the typical morphological features, and intermediate type as there was a predominance of intermediate forms.

2. The time taken for cyclical development of the

EATRO-585 derivatives in G.morsitans was shorter than that taken by the derivatives of EATRO-189. In the flies infected with EATRO-189, the trypanosomes were present in the proboscis for at least 5 days before they became infective.

3. The ability of the two stabilates of T.congolense to infect G.morsitans varied considerably.

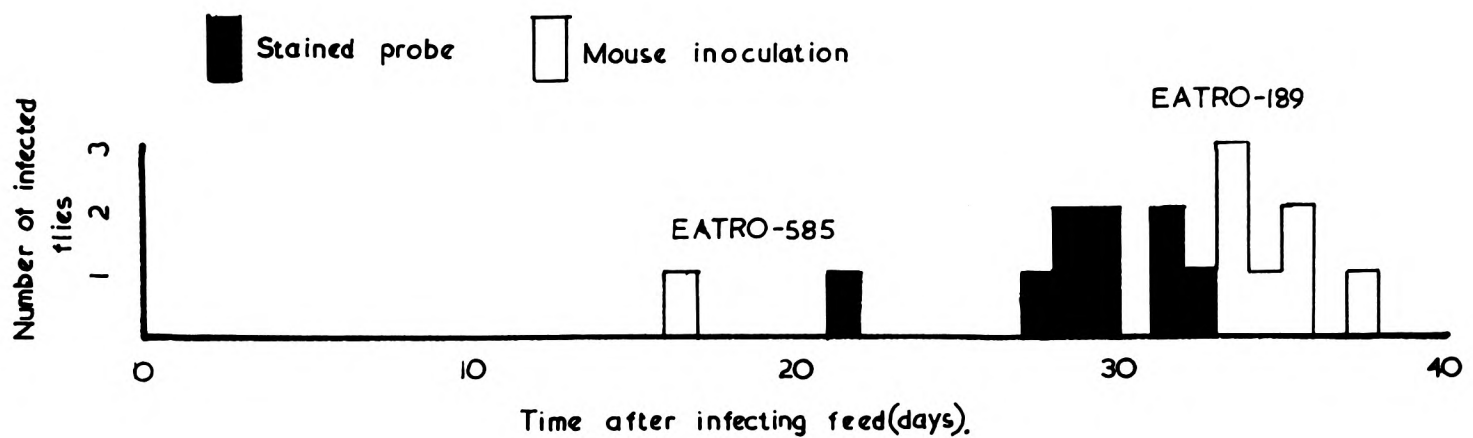


Figure 3.1. The time after the infecting feed that trypanosomes of the trypanosome form were first detected in the proboscis of flies infected with derivatives of stabilates EATRO-189 and EATRO-585.

DETECTION METHOD		DISECTION	STAINED PROBE	MOUSE INOCULATION
FIRST PROBOSCIS INFECTION (days after infecting feed)		22	28	34
% FLIES INFECTED (Proboscis)		23.1	30.7	
LOCATION IN TSETSE %	PROBOSCIS	—		
	PROBOSCIS & GUT	23.1		
	GUT	20.5		
	SALIVARY GLANDS	—		
ARRANGEMENT IN PROBOSCIS		Diffuse		

— NO TRYPANOSOMES DETECTED.

Table 3.1. Passage of EATRO-189 derivatives through G.morsitans-% of flies infected and sites of infection.

FIRST PROBOSCIS INFECTION (days after infecting feed)		24	22	17
% FLIES INFECTED (Proboscis)		6.4	3.2	
LOCATION IN TSETSE %	PROBOSCIS	—		
	PROBOSCIS & GUT	6.4		
	SALIVARY GLAND	—		
	GUT ONLY	4.2		
ARRANGEMENT IN PROBOSCIS		Diffuse		

Table 3.2. Passage of EATRO-585 derivatives through G.morsitans-% of flies infected and sites of infection.

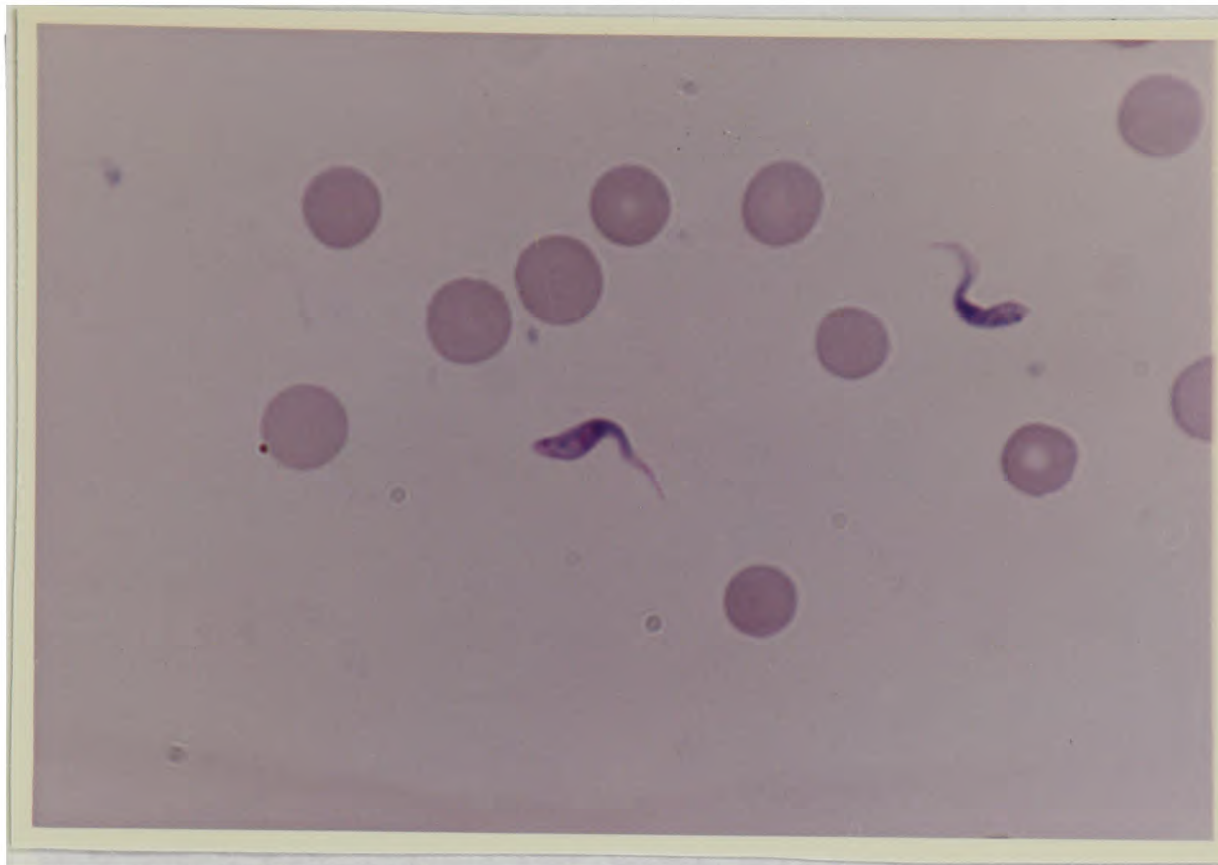
	FORMS		
DAYS	SHORT	TRANSITIONAL	LONG
1	22	68	10
2	17	65	18
3	26	60	14
4	18	64	18
5	25	61	14

Table 3.4. The % of morphological forms of EATRO-
189 derivatives in stained thin films taken
from tail blood of an infected mouse on
five consecutive days.

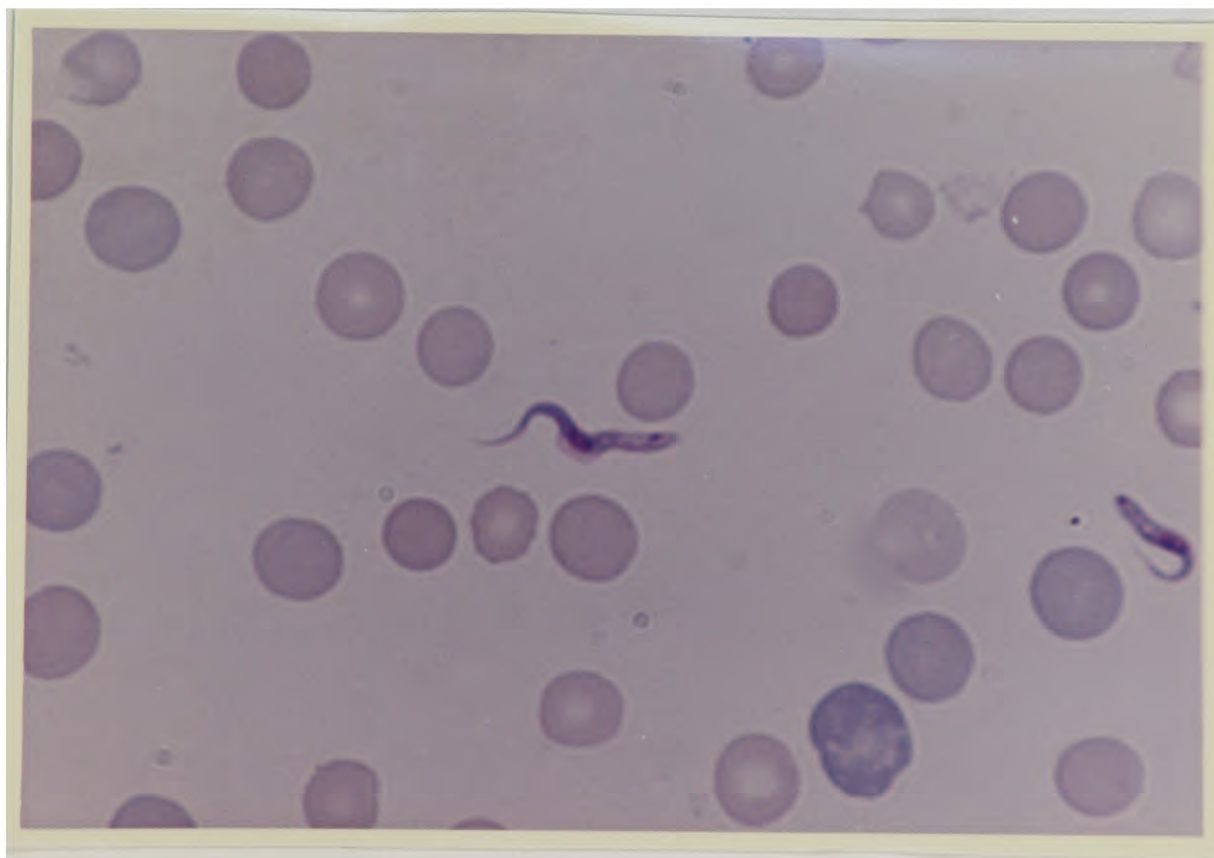
	FORMS		
DAYS	SHORT	TRANSITIONAL	LONG
1	22	50	28
2	20	54	26
3	18	58	24
4	19	57	24
5	18	55	27

Table 3.3. The % of morphological forms of EATRO-
585 derivatives in stained thin films taken
from tail blood of an infected mouse on
five consecutive days.

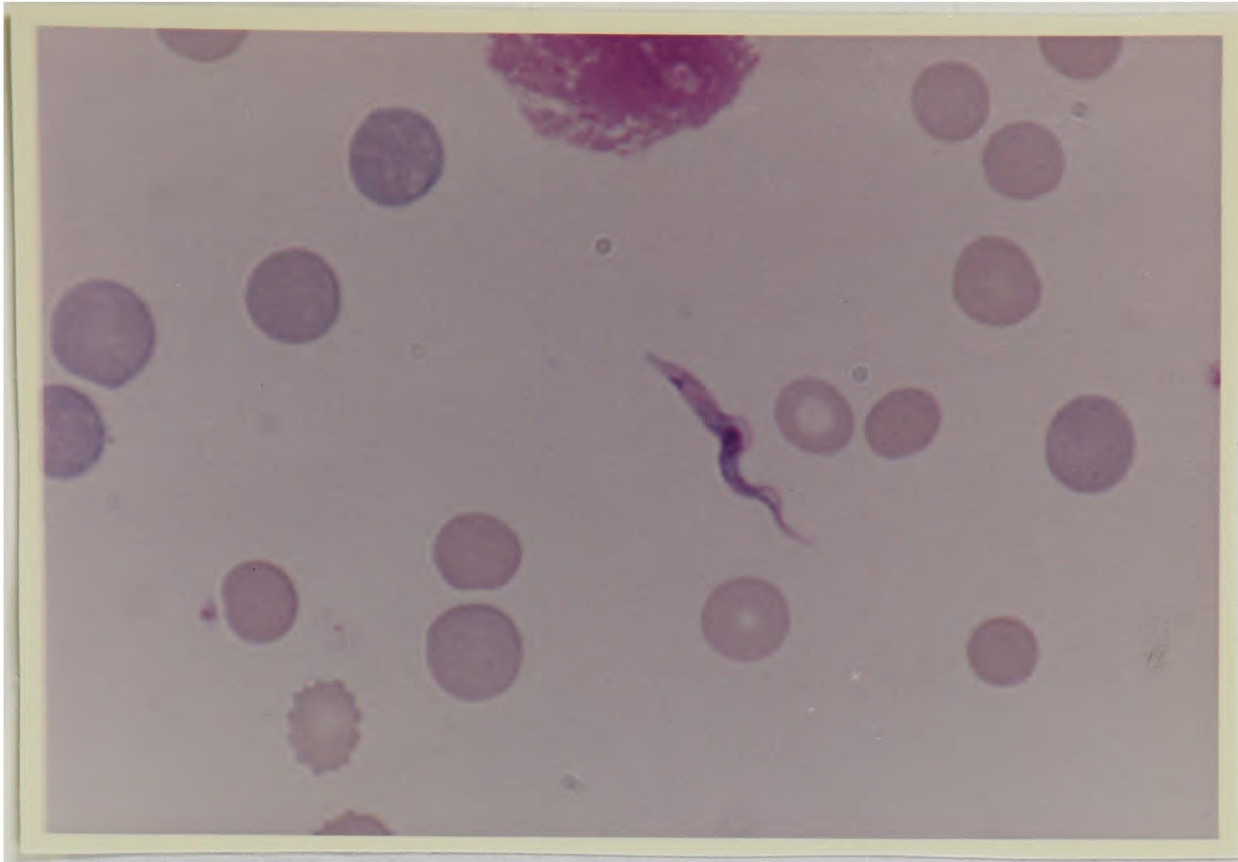
The following photographs, taken on Kodacolor X, show different morphological forms of EATRO-585 derivatives in the blood of mice ,and in the tsetse fly.



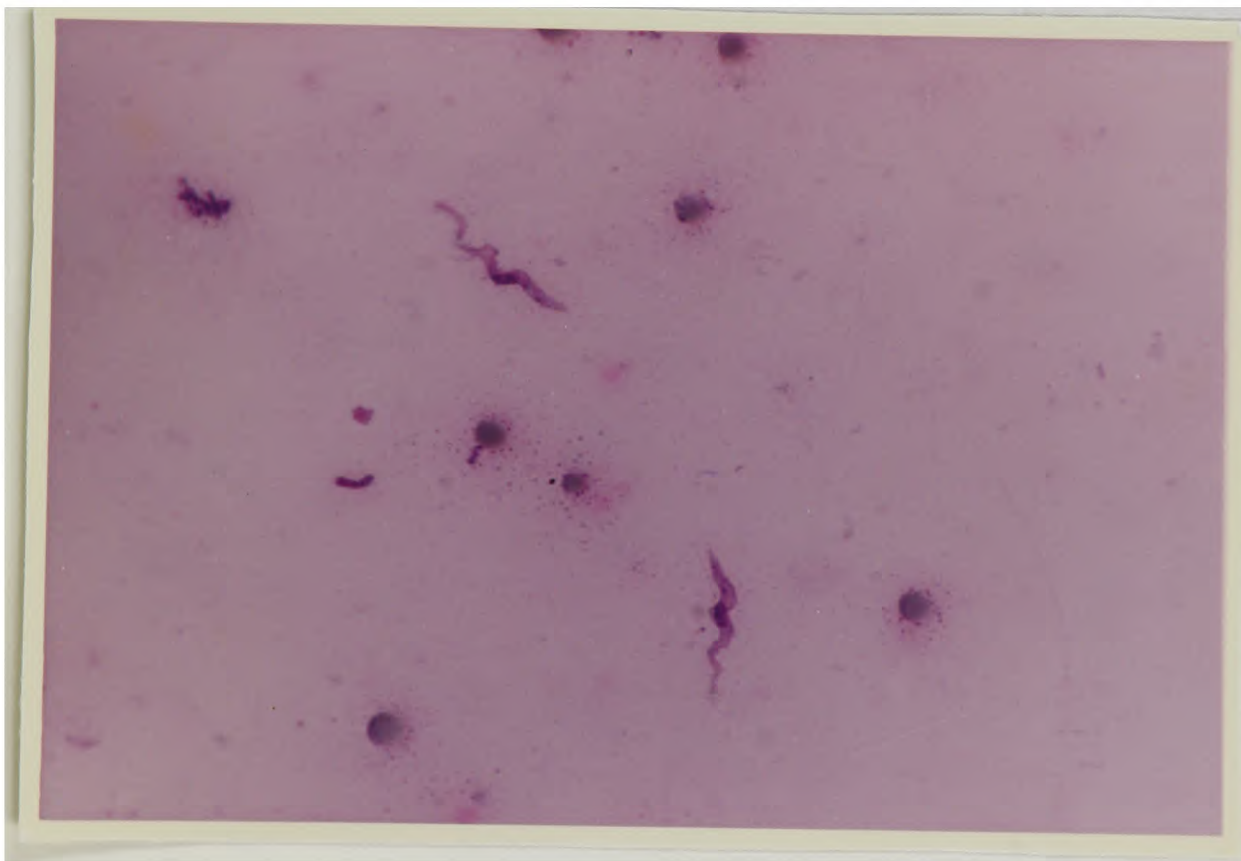
Photograph 3.1. Short, blood forms.



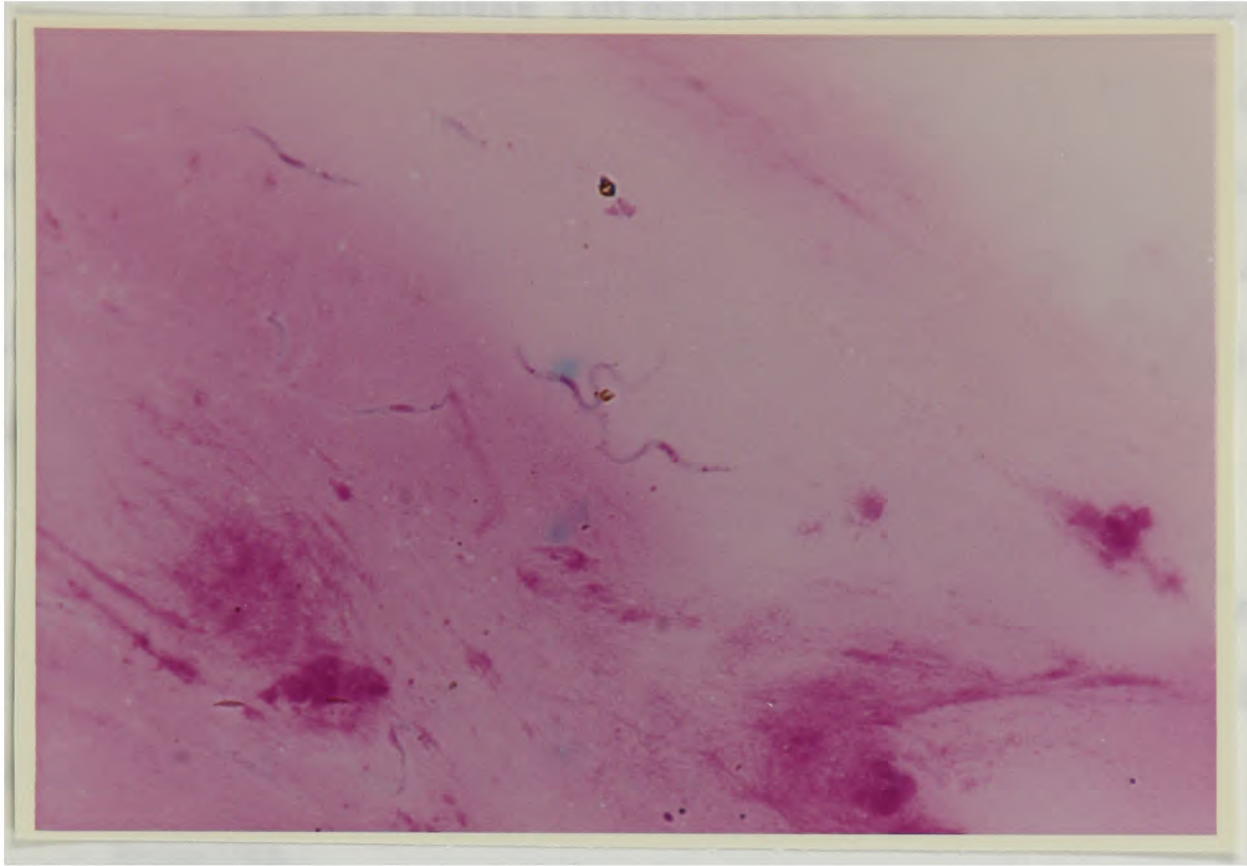
Photograph 3.2. Transitional, blood forms.



Photograph 3.3. Long, blood form.



Photograph 3.4. Insect, gut forms.



Photograph 3.5. Insect, proboscis forms.

3.2. Reproducibility of the mouse infectivity titration technique using T.congolense.

Aim.

As the mouse infectivity titration technique was used to assess the concentration of neutralizing antibody to populations of T.congolense and to study trypanosome infectivity changes during infections in cattle, it was necessary to show that the technique gave reproducible results when samples from the same source were tested.

Procedure.

T.congolense stabilates KB-1, prepared in capillaries, and EATRO-585/4, prepared in ampoules were titrated on five separate occasions in mice.

Results.

The infectivity end points of the titrations and their significance are recorded on table 3.5. The infectivity of the five samples examined from each stabilate was not significantly different.

Conclusion.

The accuracy of the technique, in the titration of infectivity of stabilates containing T.congolense, was demonstrated.

3.3. Relationship between infectivity and numbers of trypanosomes in a rat infected with T.congolense.

Aim.

It was important to establish that, significant changes of infectivity in T.congolense during the course of an infection in an animal, could be detected by the mouse infectivity titration. Therefore, the relationship between the actual numbers and the numbers of infective trypanosomes was explored during the course of an infection in a rat.

Procedure.

The contents of two capillaries of EATRO-585 were inoculated intraperitoneally into an adult white rat. Tail blood was removed daily in heparinized capillaries from days 8 to 22 postinfection. The number of trypanosomes in the blood was estimated by haemocytometer and the number of infective organisms by mouse infectivity titration.

Results.

The tail blood of the rat became trypanosome-positive on day 12. From days 12 to 22, trypanosome numbers remained relatively constant, between an estimated 10^5 and 10^6 per ml as shown on figure 3.2. Significant changes in trypanosome infectivity occurred between days 8 and 22; there were two major troughs on days 9 and 20. Except for day 16, the number of infective trypanosomes was lower than the number found by counting.

Conclusion.

Significant changes of trypanosome infectivity were detected, during the infection in the rat, by the titration

technique. The actual number of trypanosomes was not necessarily the same as the number of infective trypanosomes as on 9 of the 10 days when they were compared the latter were less than the former.

STABILATES	Log ₁₀ No. MOUSE I.D. ₆₃ /ML.				
	KB-1	5.6 ± 0.3	4.9 ± 0.5	5.0 ± 0.3	5.5 ± 0.3
EATRO 585/4	2.8 ± 0.4	2.6 ± 0.3	2.8 ± 0.4	2.4 ± 0.5	2.4 ± 0.5

Table 3.5. Mouse infectivity end points of titrations of stabilates KB-1 and EATRO-585/4 carried out on five different occasions.

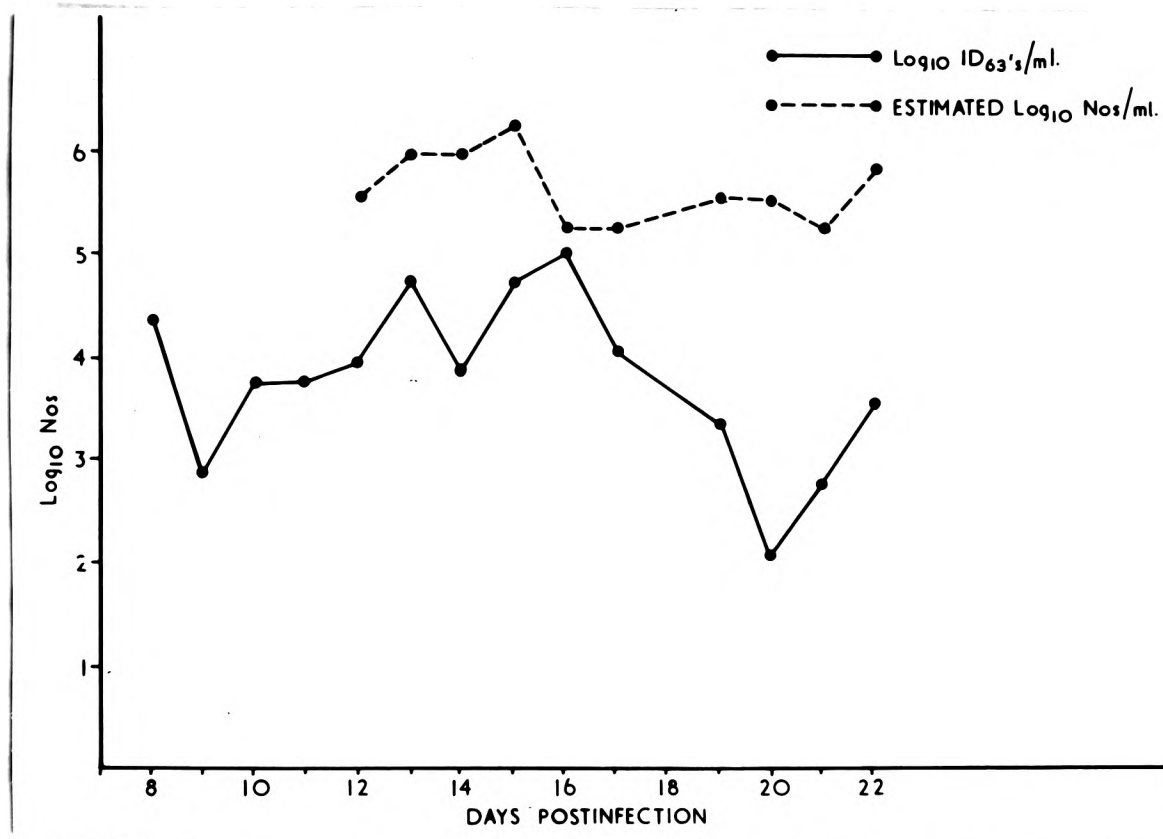


Figure 3.2. Relationship between numbers and infectivity during the early infection in a rat infected with EATRO-585.

Preservation of T.congolense.**3.4. Effect of cooling rate on the preservation of T.congolense.****Aim.**

Since it is known that the rate of cooling is an important factor in the successful preservation of brucei sub-group trypanosomes, the following experiment was designed to determine a safe cooling rate for the preservation of T.congolense.

Procedure.

Different groups of mice were infected with T.congolense stabilates KB-1, EATRO-189 and EATRO-585 respectively. At the first peak of parasitaemia, infected blood was removed by heart puncture. A portion of the blood was titrated for infectivity and to the remainder glycerol was added to a concentration of 7.5%. The suspensions were loaded into capillaries, which were placed in a series of tubes containing methanol in which the trypanosomes were cooled to -79°C at different rates (see figure 3.3). These rates of cooling were obtained by insulating the tubes in polystyrene containers of different thickness, or by wrapping them in layers of brown paper one ft.sq. The temperature of each rate was recorded by means of a thermocouple inserted into each tube. Each treatment was repeated three times to ascertain the reproducibility of the cooling rate. After one week at low temperature, two capillaries of each stabilate were titrated in mice. The infectivity end points so obtained were compared with prefreeze controls. The number of trypanosomes was estimated in the controls and in all the stabilates.

Results.

Table 3.6 shows that all the cooling rates were satisfactory except C,D and E' since no significant reductions in the number or infectivity of the trypanosomes resulted. Rates D and E' were destructive to the trypanosomes. In the population of KB-1, cooled by rate C, there was a $10^{1.3}$ fall in the number of infective trypanosomes and an approximately ten-fold drop in numbers compared to the control.

Conclusion.

For the successful preservation of 25 to 40mg volumes of blood containing T.congolense, the cooling rate should be less than 4°C per minute. Cooling rate C' was chosen as the standard cooling rate.

STABILATE No	COOLING RATE	POST PRESERVATION	
		NUMBERS Log ₁₀ /ml.	INFECTIVITY Log ₁₀ ID ₆₃ 's/ml.
KB-1	CONTROL	6.4	5.6 ± 0.3
	A	5.8	5.2 ± 0.4
	B	6.0	5.4 ± 0.5
	C	5.1	4.3 ± 0.2
	D	—	<2.4
EATRO-189	CONTROL	5.7	4.9 ± 0.5
	A'	5.6	4.9 ± 0.5
	B'	5.4	4.6 ± 0.3
	C'	5.7	4.7 ± 0.3
	D'	5.4	4.4 ± 0.5
	E'	—	<2.4
EATRO-585	CONTROL	6.0	3.8 ± 0.5
	A'	5.6	3.1 ± 0.5
	B'	5.7	3.4 ± 0.5
	C'	5.4	3.6 ± 0.3
	D'	5.7	3.1 ± 0.5
	E'	—	<2.4

—NO TRYPANOSOMES DETECTED

Table 3.6. The effects of different cooling rates from 0°C to -79°C , on the preservation of trypanosome populations derived from KB-1, EATRO-189 and EATRO-585 as judged by numbers and infectivity.

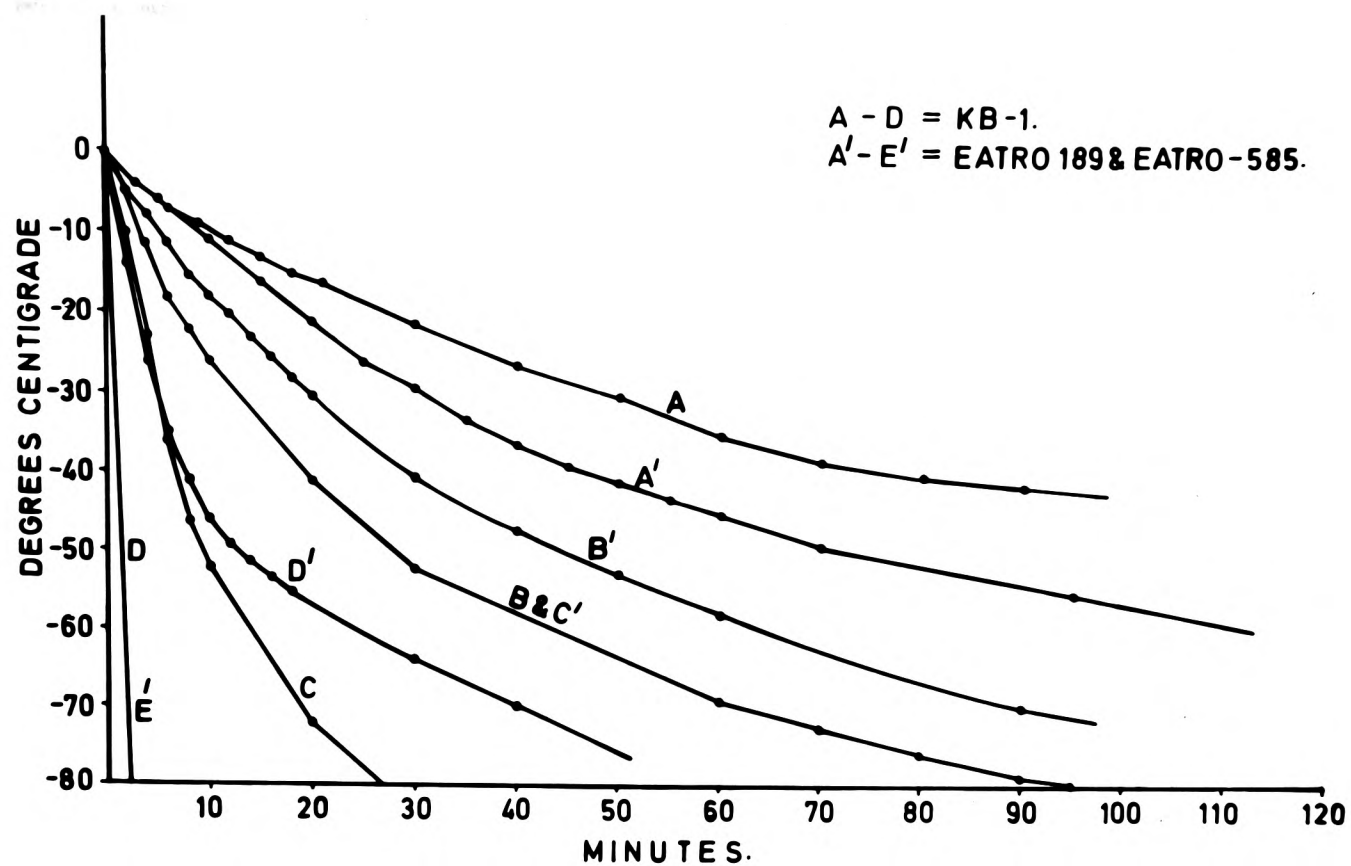


Figure 3.3. Cooling rates through which populations of KB-1, EATRO-189 and EATRO-585 were preserved to -79°C . For cooling rates A, B and C, the methanol tubes were placed in polystyrene containers of 3, 2 and 1" thickness respectively. For rates A', B', C', and D', the tubes were wrapped in 4, 3, 2 and 1 pieces of 1ft.sq. brown paper respectively. For rates D and E', the tubes were immersed directly in solid carbondioxide and methanol.

3.5. Effect of volume of sample and glycerol concentration on the preservation of T.congolense.

Aim.

In addition to the cooling rate, other factors which may influence the preservation of trypanosomes are the volume of the sample and the concentration of the freezing protectant. These factors were investigated as follows.

Procedure.

A group of six mice was inoculated with the stabilate KB-1. Heparinized blood, removed by heart puncture at the first peak of parasitaemia, was divided into four 1ml aliquots. Aliquot 1 was immediately titrated in mice to serve as the control. 2 and 3 were preserved to -79°C in capillaries by cooling rate C' with the addition of 7.5% and 15% glycerol respectively. 4 was preserved in a similar manner without the addition of glycerol. Zebus 495 and 530 were inoculated with stabilates EATRO-585/1 and EATRO-189/1 respectively. At the first peak of parasitaemia, 25ml of blood was removed by venepuncture, heparinized and divided into eight aliquots(1-8). 1 to 4 were treated as described for KB-1. To 5 and 6, glycerol to a final concentration of 7.5% and 15%, was added respectively, and 1ml amounts were preserved in ampoules. 7 and 8 were similarly treated and 5ml amounts were preserved in bijou bottles. All samples were cooled by rate C'. After a week at low temperature, the 17 stabilates were titrated in mice and the number of trypanosomes was counted by haemocytometer.

Results.

Table 3.7 shows the results of these tests. Good

survival of the trypanosomes was noted in the capillaries to which 7.5 and 15% glycerol had been added and in the ampoules containing 15%. In the ampoules containing 7.5% glycerol, trypanosome survival was significantly reduced and was even further reduced in the bijou bottles at both the concentrations tested. In the stabilates, to which no protectant had been added, no infective trypanosomes could be detected.

Conclusion.

When the volume of blood was small (25 to 40mg in capillaries) either 7.5 or 15% glycerol was satisfactory; when the volume was increased (1ml in ampoules) only 15% was effective, as judged by comparisons of numbers and infectivity with the controls. Under the conditions of the experiment, increasing the glycerol concentration to 15% did not improve the viability of trypanosomes preserved in 5ml volumes.

STABILATE No	GLYCEROL CONCENTRATION %	PRESERVATION RECEPTACLES	POST PRESERVATION	
			NUMBERS Log ₁₀ /ml.	INFECTIVITY Log ₁₀ ID ₆₃ 's/ml.
KB-1	CONTROL	CAPILLARIES	5.6	5.4 ± 0.5
	7.5		5.4	5.6 ± 0.3
	15		5.1	5.4 ± 0.5
	NONE		—	—
EATRO - 189	CONTROL	CAPILLARIES	5.7	4.0 ± 0.3
	7.5		5.7	3.4 ± 0.3
	1.5		5.7	3.6 ± 0.3
	NONE		—	—
	7.5	AMPOULES	—	2.8 ± 0.5
	15		5.1	3.4 ± 0.3
	7.5		—	1.4 ± 0.5
	1.5		—	1.8 ± 0.5
EATRO - 585	CONTROL	CAPILLARIES	6.0	4.5 ± 0.3
	7.5		5.7	4.3 ± 0.5
	1.5		6.0	4.1 ± 0.5
	NONE		—	—
	7.5	AMPOULES	—	3.0 ± 0.3
	1.5		5.7	4.0 ± 0.3
	7.5		—	2.1 ± 0.5
	1.5		—	2.4 ± 0.3

— NO TRYPANOSOMES DETECTED.

Table 3.7. The effects of glycerol and volume of sample on the preservation of trypanosomes derived from stabilates KB-1, EATRO-189 and EATRO-585.

T2(R1 and R2).

Figure 3.5 shows the results from zebu 364. A similar relapsing parasitaemia occurred; the number of infective organisms detected was approximately 10^2 less than in zebu 530. There was poor survival of the trypanosomes preserved in capillaries over most of the infection period studied(A1-A3); good survival was recorded only during the rising parasitaemia. Much better survival occurred with the trypanosomes preserved in ampoules as only on day 15, one day prior to the first trough of infectivity(T1), was there a statistical reduction in infectivity. On all the remaining days, there were no differences in infectivity between the ampoule stabilates and the prefreeze controls.

Conclusions.

Trypanosomes were preserved satisfactorily in capillaries only during the rising parasitaemia. In ampoules, however, many populations were successfully preserved also during the falling parasitaemia. The latter method should be used to obtain antigens for the neutralization test.

As a result of the tests, reported in sections 3.4 to 3.6, two methods of preservation were adopted, namely,

- a) capillary, when infected blood was dispensed in capillaries with a glycerol concentration of 7.5%
- b) ampoule, when infected blood was dispensed in 1ml amounts in ampoules with a glycerol concentration of 15%.

The cooling rate in both methods was C'.

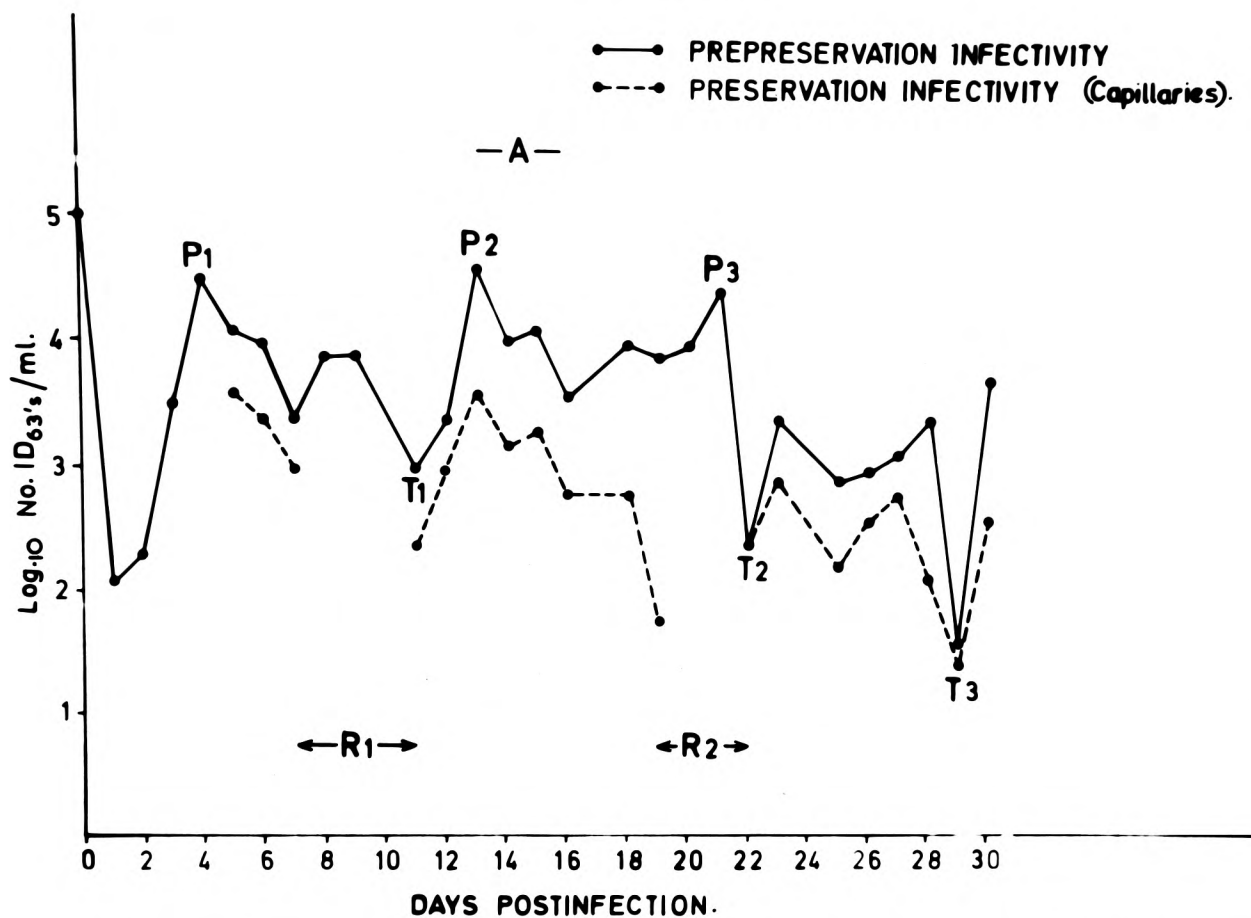


Figure 3.4. Efficiency of the capillary method in the preservation of trypanosomes collected over a 26 day in Zebu 530.

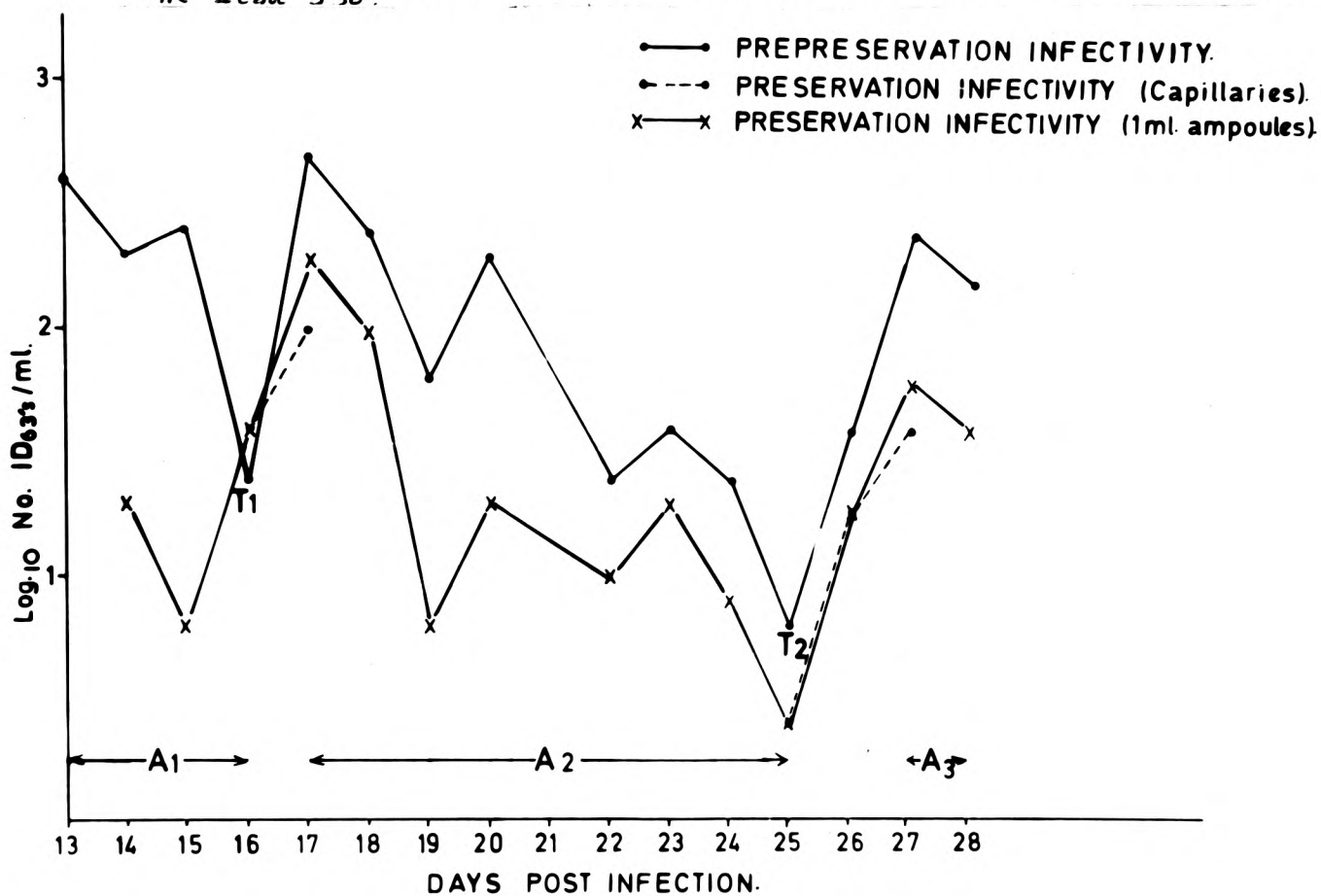


Figure 3.5. Efficiency of the capillary and ampoule methods in the preservation of trypanosomes collected over a 15 day period in zebu 364, as judged by infectivity.

3.7. Effect of length of storage at low temperature on the preservation of T.congolense.

Aim.

Prolonged storage of T.congolense would be necessary in order to challenge animals with the same antigenic material at different times. The effect of low temperature storage on two stabilates of T.congolense was therefore studied.

Procedure.

Stabilates KB-1, preserved in capillaries, and EATRO-585/4, preserved in ampoules, were titrated in mice after varying periods at -79°C .

Results.

Table 3.8 shows that no loss in infectivity of the trypanosomes occurred when they were preserved for at least 329 days in capillaries and for at least 410 days in ampoules.

Conclusion.

Stabilates of T.congolense can be preserved for a year at least without loss of infectivity.

STABILATE No	DAYS IN STORAGE AT -79°C	INFECTIVITY $\text{Log}_{10} \text{ID}_{63}\text{'s/ml.}$
KB-1	CONTROL	5.8 ± 0.3
	16	5.6 ± 0.3
	43	5.0 ± 0.3
	118	5.5 ± 0.3
	329	5.0 ± 0.5
EATRO-585/4	CONTROL	2.8 ± 0.4
	77	2.6 ± 0.3
	154	2.8 ± 0.4
	238	2.4 ± 0.5
	308	2.3 ± 0.3
	410	2.6 ± 0.3

Table 3.8. Length of survival of stabilates KB-1 and EATRO-585/4 at -79°C , as judged by infectivity.

3.8. Preservation of field isolates of T.congolense.

Aim.

In sections 3.4 to 3.7, preservation was studied in only three strains of T.congolense, of which one was totally laboratory adapted. It was of interest to find out whether the blood forms of field isolates could be preserved using the same techniques.

Procedure.

Fifteen isolates of T.congolense, which were identified by morphology, were obtained from mice inoculated with bovine and bushbuck blood by the Edinburgh veterinary expedition of 1966. Blood was removed from the mice at the first peak of parasitaemia, heparinized, a portion immediately titrated in mice, and the remainder preserved in capillaries. After one week at low temperature, 4-5 capillaries of each stabilate were titrated in mice.

Results.

Table 3.9 shows the results of the titrations of each stabilate, before and after preservation. In 11, there was no difference in infectivity. The results of EVE-5 can be discounted since no mice became infected before preservation. In only EVE-16 was there a significant fall in the number of infective trypanosomes after preservation. No conclusions could be drawn from the results of EVE-1 and EVE-23 as their prepreservation infectivities were too low.

Conclusion.

Field isolates of T.congolense can be successfully

preserved by the capillary method.

EVE STABILATE No.	ANIMAL SOURCE	INFECTIVITY Log ₁₀ ID ₆₃ 's/ml.	
		BEFORE PRESERVATION	AFTER PRESERVATION
1	BUSH BUCK	1.9 ± 0.5	<1.4
2	"	2.1 ± 0.3	1.8 ± 0.5
3	BOVINE	2.4 ± 0.5	2.8 ± 0.4
5	"	<1.4	<1.4
9	"	2.3 ± 0.5	2.1 ± 0.3
10	"	2.3 ± 0.5	1.8 ± 0.5
14	"	3.4 ± 0.5	2.8 ± 0.5
15	"	2.6 ± 0.3	2.6 ± 0.3
16	BUSH BUCK	2.7 ± 0.3	<1.4
17	"	2.8 ± 0.3	2.4 ± 0.5
18	"	2.1 ± 0.3	1.8 ± 0.5
19	"	2.3 ± 0.5	2.0 ± 0.3
22	"	2.7 ± 0.3	2.1 ± 0.6
23	"	2.0 ± 0.3	<1.4
26	"	2.3 ± 0.5	1.9 ± 0.5

Table 3.9. Efficiency of the capillary method in the preservation of field isolates of T.congolense, as judged by infectivity.

3.9. Preservation of metacyclic trypanosomes of T.congolense.

Aim.

The successful preservation of the metacyclics of T.congolense has not been reported. It thus seemed important to discover whether the techniques developed for the preservation of blood forms were also applicable for metacyclics.

Procedure.

Two G.morsitans, known to be infected with T.congolense (section 3.1), were induced to feed twice-weekly through a rat-skin membrane into 9ml defibrinated bovine blood, from the 25th day postinfection until death. On each occasion the flies took a full feed as judged by a swollen abdomen. After feeding, a portion of the residual blood in the capsule was titrated in mice and the remainder preserved in ampoules. Stabilates, whose pre-preservation infectivity was more than $10^{0.4}$ ID_{63s} per ml, were titrated in mice after one month at low temperature.

Results.

Table 3.10 show the results of the titrations. Of the 9 metacyclic populations, obtained from the two flies, efficient preservation was achieved in 7, as judged by infectivity. In the samples obtained on day 46 from fly 1 and on day 29 from fly 2, the prepreservation infectivity was too low for a comparison to be made.

Conclusion.

The ampoule method is satisfactory for the preservation of T.congolense.

FLY No.	TIME AFTER INFECTING FEED (days)	INFECTIVITY Log ₁₀ ID ₆₃ 's/ml.	
		BEFORE PRESERVATION	AFTER PRESERVATION
1	25	1.6±0.3	1.4±0.5
	32	1.4±0.5	1.4±0.5
	46	0.6±0.3	<0.4
	49	1.1±0.3	0.8±0.5
	60	1.1±0.3	1.1±0.3
2	29	0.8±0.5	<0.4
	32	0.8±0.5	0.8±0.5
	56	1.4±0.5	0.8±0.5
	62	1.6±0.3	1.2±0.4

Table 3.10. Efficiency of the ampoule method in the preservation of metacyclics of T.congolense, as judged by infectivity.

3.10. Effects of pH and temperature on the maintenance of infectivity of T.congolense in vitro.

Aim.

As both pH and temperature are known to affect the infectivity of the brucei sub-group trypanosomes, it was essential to study their effects on T.congolense so that an adequate maintenance technique could be developed.

Procedure.

To test the effect of pH on survival, the stabilate KB-1 was used. A series of mouse infectivity titrations were carried out in which the pH of the diluent was varied by altering the proportions of the phosphate salts (see section 2.3b and appendix 1). Titrations were carried out immediately and then after 4 and 8 hours.

To test the effect of temperature on survival, the stabilates KB-1 and EATRO-189 were used. The trypanosomes were maintained at 0-2°C and at room temperature (approximately 20°C) for varying periods of time before being titrated in mice. Counts of the trypanosomes were made just prior to inoculation.

Results.

From table 3.11, it can be seen that there was no significant fall in infectivity of the trypanosomes in KB-1 for at least 8 hours at pHs 6.6, 7.4 and 8.0, and for at least 4 hours at pH 5.8. At pH 4.8, there was a significant fall in infectivity after 4 hours in vitro.

Table 3.12 shows the results of the temperature experiment. With KB-1, no significant falls in trypanosome numbers and infectivity occurred after 5 hours at 0-2°C; after 24 hours, no trypanosomes were seen and a fall of 10^3 infective trypanosomes was detected. After 5 hours at room temperature, a significant fall in the number of infective trypanosomes resulted although motile trypanosomes were undiminished. With EATRO-189, no significant falls in trypanosome numbers or infectivity resulted after 6 hours at 0-2°C; after 24 hours, no trypanosomes were seen and the number of infective organisms fell to below $10^{2.4}$ ID_{63s}.

Conclusions.

T. congolense can be maintained for at least 8 hours at 0-2°C at pHs of between 6.6 and 8.0. As there was an apparent loss of infectivity of KB-1 after 5 hours at room temperature, trypanosomes should always be maintained at 0-2°C.

pH	TIME (hours) IN VITRO		
	0	4	8
8.0	5.0 ± 0.3	4.8 ± 0.3	4.4 ± 0.5
7.4	5.0 ± 0.3	4.1 ± 0.5	4.1 ± 0.5
6.6	4.8 ± 0.3	4.1 ± 0.5	3.8 ± 0.3
5.8	5.0 ± 0.3	4.1 ± 0.5	3.6 ± 0.3
4.8	4.6 ± 0.3	2.8 ± 0.3	<2.4

Table 3.11. Effect of hydrogen ion concentration on the infectivity of the trypanosomes in T.congolense stabilate KB-1.

STABILATE No.	CONDITIONS OF MAINTENANCE		TRYPANOSOME	
	TEMPERATURE °C	TIME IN VITRO (hours)	NUMBERS Log ₁₀ /ml	INFECTIVITY Log ₁₀ ID ₆₃ 's/ml.
KB-1	0-2	0	5.7	5.0 ± 0.3
		5	5.1	4.6 ± 0.3
		24	—	2.4 ± 0.5
	20	5	5.0	3.8 ± 0.3
EATRO-189	0-2	0	6.0	3.9 ± 0.5
		3	6.0	3.9 ± 0.5
		6	5.7	3.8 ± 0.3
		24	—	<2.4

— NO TRYPANOSOMES DETECTED.

Table 3.12. Effects of temperature and length of time in vitro on the infectivity and actual numbers of the trypanosomes in T.congolense stabilates KB-1 and EATRO-189.

A number of different techniques have been successfully employed in the detection of antigens and of antibodies in the brucei group. It was not known which of these tests could be applied to T.congolense. The object of the next series of experiments was to find suitable methods for detecting and measuring antibodies in animals infected with T.congolense.

3.11. Isolation of antigenic variants of the brucei sub-group from a rabbit.

Aim.

In the initial study of the trypanolytic and indirect haemagglutination tests (sections 3.12-3.14), brucei sub-group organisms were employed. Antigenic variants of these trypanosomes thus had to be produced.

Procedure.

An adult white rabbit was inoculated with approximately 7×10^6 trypanosomes of stabilate KB-3 T.brucei, a preinoculation serum having been taken. Every 5 days for the 30 days following inoculation, 5ml of blood was removed from the ear vein and a drop was examined for trypanosomes by wet film, 0.5ml inoculated into each of two mice for agglutinogenic antigen production, and the remainder allowed to clot for serum collection. The antigens were preserved in capillaries and designated as in table 3.13. On days 35, 40 and 50 postinfection, blood was examined by wet film and serum collected. Cross agglutination-B tests were carried out using all the sera and antigens collected from the rabbit.

Results.

Agglutinins to KB-3 were detected by the 5th day post-infection, rose to a peak by day 10, and then fell sharply but were still present in detectable quantities on day 50. Using the serial isolates as antigens, agglutinins were not generally found in the sera collected prior to or at the same time as the organisms. The patterns of antibody production were similar to that produced by KB-3, as shown in figure 3.6.

Conclusions.

All the prepared antigens were antigenically distinct. Antigenic variation of the trypanosomes occurred at least once every 5 days, confirming the findings of Gray(163).

KB STABILATE NUMBER	3	4	5	6	7	8	9
DAYS POST- INFECTION	-	5	10	15	20	25	30

Table 3.13. Designations of the agglutinogenic antigens prepared from the rabbit.

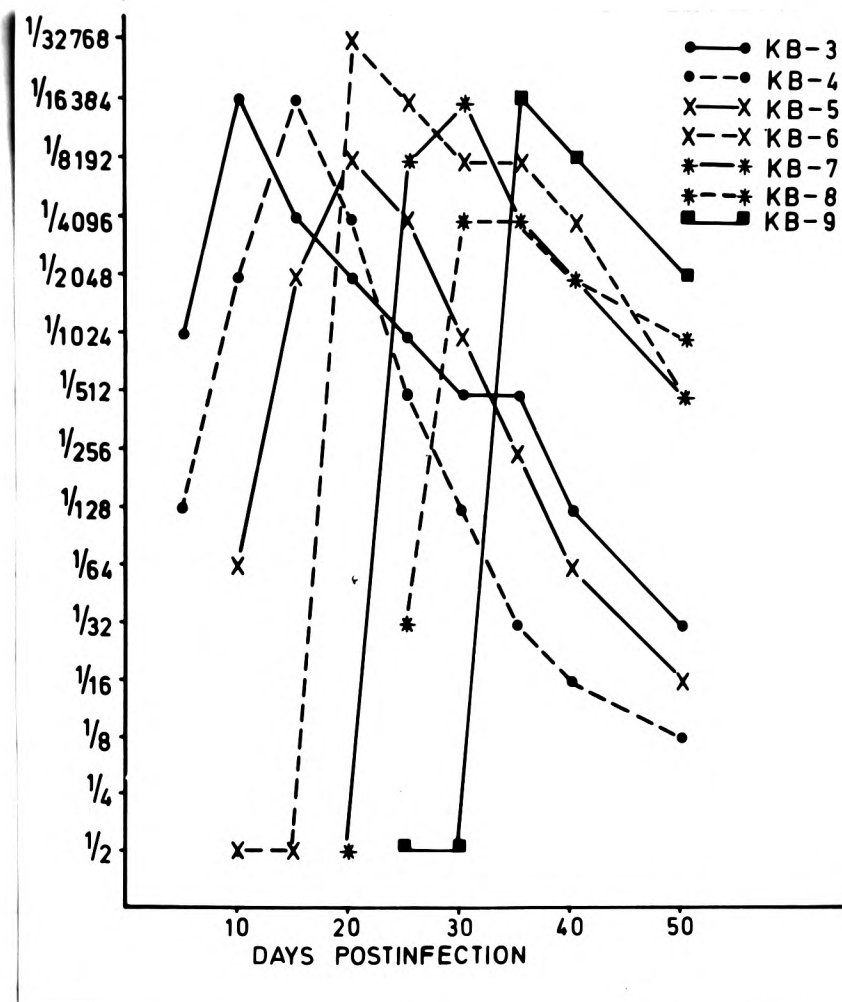


Figure 3.6. Serum agglutinin titres in the rabbit to all the prepared antigens.

3.12. The trypanolytic test.

Aim.

Attempts were made to ascertain whether the trypanolytic test(see section 2.10b) detected different antigenic variants of T.brucei and T.congolense.

Procedure.

Stabilates of T.brucei, KB-3, KB-6 and KB-9, shown to be antigenically distinct by the agglutination test, were used in trypanolytic tests employing the 5 day serum from the rabbit used in section 3.11.

Two stabilates of T.congolense, KB-1 and EATRO-189, were used as antigen in trypanolytic tests employing an anti-KB-1 serum prepared in a rat(see section 2.9a).

Results.

Tables 3.14 and 3.15 show the results of these tests. The 5 day postinfection serum from the rabbit caused lysis of the trypanosomes in KB-3 but not in KB-6 or KB-9. The anti-KB-1 serum lysed the trypanosomes in KB-1 but in EATRO-189.

Conclusion.

The trypanolytic test distinguished antigenic variants of T.brucei and T.congolense.

ANTIGEN	TEST SERUM DILUTIONS											E D T A	NORMAL SERUM
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240		
KB-3	4	4	4	4	4	4	4	3	3	1	—	—	—
KB-6	—	—	—	—	—	—	—	—	—	—	—	—	—
KB-9	—	—	—	—	—	—	—	—	—	—	—	—	—

Table 3.14. Trypanolytic tests using anti-KB-3 serum and antigens prepared from KB-3, KB-6 and KB-9.

ANTIGEN	TEST SERUM DILUTIONS									E D T A	NORMAL SERUM
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560		
KB-1	4	4	4	3	3	2	1	1	—	—	—
EATRO-189	—	—	—	—	—	—	—	—	—	—	—

Table 3.15. Trypanolytic tests using anti-KB-1 serum and antigens prepared from KB-1 and EATRO-189.

3.13. Indirect haemagglutination-use of formalinized cells.

Aim.

The indirect haemagglutination test has been shown to detect small amounts of antibody to many protozoa. Attempts were made to apply the test, in the first instance, to T.brucei. Before coating the prepared formalinized cells with trypanosomal antigens, they were coated with a simple antigen to demonstrate that reproducible results could be obtained by haemagglutination.

Procedure.

Formalinized sheep erythrocytes, prepared as described in section 2.10d, were tanned and coated with purified ovalbumin(2mg/ml). Haemagglutination resulted in the presence of specific antiserum. Four separate titrations were carried out using the same material.

Results.

Table 3.16 shows the results of the titrations. Positive haemagglutination only occurred with the tanned, coated cells and the titres of the test serum in the four titrations were similar.

Conclusion.

As the cells gave positive results with the antigen-coated cells only, and consistent results were obtained in the four titrations, they could then be used in the application of the test to trypanosomiasis.

3.14. Indirect haemagglutination—use of T.brucei antigen.

Procedure.

Haemagglutination tests were carried out using materials collected from a rabbit infected with T.brucei (section 3.11) under the following conditions:-

- a. sheep erythrocytes at concentrations of 0.25, 0.5, 1.0, 2.0 and 5.0%.
- b. trypanosomal protein concentrations of 5.0, 2.0, 0.2 and 0.02 mg/ml.
- c. coating pHs of 5.6, 6.4, 7.2 and 8.0.
- d. concentrations of NRS diluent of 1,2,3,4 and 5%.

Results.

Non-specific haemagglutination occurred in all the tests.

Conclusion.

Until the cause of the non-specific reaction could be ascertained, the test was considered unsuitable for the study of immunological aspects of trypanosomiasis.

SHEEP CELLS	TITRATION NUMBER	ANTISERUM DILUTIONS								1% NRS
		1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	
TANNED COATED	1	3	3	3	3	3	1	—	—	—
	2	3	3	3	3	3	1	1	—	—
	3	3	3	3	3	3	1	—	—	—
	4	3	3	3	3	3	2	—	—	—
TANNED	1	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—
	3	—	—	—	—	—	—	—	—	—
	4	—	—	—	—	—	—	—	—	—
NORMAL	1									—
	2									—
	3									—
	4									—

Table 3.16. Haemagglutination titrations employing the formalinized sheep erythrocytes and a homologous ovalbumin- anti-ovalbumin system.

3.15. Some properties of neutralizing antibodies to T.congolense.

Aim.

It is known that the neutralization test detects variant specific antibodies in animals infected with the brucei subgroup. In order to apply the test to T.congolense, it was necessary to show that neutralising antibodies react with these trypanosomes, and to ascertain which type of antibody the test detected in infected animals.

Procedure.

0.9ml aliquots of EATRO-585/4 were allowed to react with 0.1ml amounts of known positive and negative sera for a series of time intervals at 0-2°C. At the end of each reaction time, 0.15ml aliquots of the suspensions from each sera, containing approximately 10^2 infective trypanosomes, were inoculated into each of six mice and the number of trypanosomes present estimated. The mice were examined as described in section 2.10c.

Zebu 495 and 530 were inoculated with stabilates EATRO-585/1 and EATRO-189/1 respectively, preinoculation sera having been obtained. Sera collected on days 7,14,20 and 29 post-infection from zebu 495 and on days 12,30 and 44 from zebu 530 were used in neutralization-A tests against antigens preserved on days 7,13 and 20 from zebu 495 and on days 8,15 and 27 from zebu 530 respectively.

Results.

Tables 3.17 to 3.19 show the results of the neutralization tests. The trypanosomes were neutralized by the positive serum at all the reaction times in vitro between 5 and 360 minutes. They were not neutralized by the negative serum or by the positive serum after a 24 hour reaction time.

In zebu 495, the 14 days serum neutralized EATRO-585/1 and the 7 day stabilate, but not the 13 or 20 day stabilates which were neutralized by the 20 and 29 day sera. In zebu 530, the 12 day serum neutralized the 8 day stabilate and EATRO-189/1, but not the 15 or 27 day stabilates which were neutralized by the 30 and 44 day sera respectively.

Conclusions.

1. As trypanosomes were neutralized within 5 minutes of contact with a positive serum and remained neutralized for at least 360 minutes, a reaction time of 30 minutes was adopted for all neutralization tests.

2. The neutralization of T.congolense was not associated with a loss of motility.

3. The neutralization test detected antigenically different populations of T.congolense.

REACTION TIME (minutes)	0	5	10	15	20	25	30	40	50	60	120	180	360
POSITIVE SERUM	-	+	+	+	+	+	+	+	+	+	+	+	+
NEGATIVE SERUM	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3.17. Neutralization-A tests using stabilate EATRO-585/4 as antigen and 1/10 dilutions of known positive and negative sera at different reaction times at 0°C. The trypanosomes were not infective after 24 hours in vitro and there was no loss in motility or numbers over the period studied.

STABILATES		ANTISERA (days postinfection)				
DAYS POST- INFECTION	EATRO No.	0	7	14	20	29
0	585/1	-	-	+	+	+
7	585/4	-	-	+	+	+
13	585/10	-	-	-	+	+
20	585/17	-	-	-	-	+

Table 3.18. Cross neutralization-A tests using antigens and sera obtained from zebu 495.

STABILATES		ANTISERA (days postinfection)			
DAYS POST- INFECTION	EATRO No.	0	12	30	44
0	189/1	-	+	+	+
8	189/5	-	+	+	+
15	189/12	-	-	+	+
27	189/24	-	-	-	+

Table 3.19. Cross neutralization-A tests using antigens and sera obtained from zebu 530.

In sections 3.16 to 3.19, the indirect fluorescent antibody tests, described in section 2.10e, were studied using brucei sub-group, T.congolense and T.vivax antigens, to establish their suitability in immunological studies of bovine trypanosomiasis.

3.16. Possible interference of immunofluorescence by T.theileri.

Aim.

It is known that cattle are commonly infected with the non-pathogenic trypanosome T.theileri. The extent of interference by this species, in IF tests for the detection of antibody to the pathogenic trypanosomes, was therefore examined.

Procedure.

Sera were obtained from three groups of cattle shown to be free from pathogenic trypanosomes by the STDM (see section 2.4a). Group 1 consisted of 26 red poll cattle sampled at Tororo prison, group 2 of 52 boran cattle sampled at Moyben, Eldoret, Kenya, and group 3 of 50 zebu cattle from the EATRO breeding herd. Three dilutions of each serum were prepared and tested by the IFT-A test against brucei sub-group, T.congolense and T.vivax antigens. All cattle were examined for the presence or absence of T.theileri by the culture method (section 2.4b).

Results.

Table 3.20 shows the relationship between the known presence of T.theileri in the cattle and the fluorescence reactions of their sera to the pathogenic trypanosomes. T.theileri was present in the cattle of groups 1 and 3, in 58 and 100% of the animals respectively; the trypanosome was not detected in any of the

cattle of group 2. In groups 1 and 3, positive fluorescence was shown by all the sera at the 1/10 dilution, by many of the sera at the 1/20 dilution but by none of the sera at the 1/40 dilution. In group 2, many sera gave positive fluorescence at the 1/10 dilution; however, there were no positives at the 1/20 and 1/40 dilutions.

Conclusions.

1. As the fluorescence reactions of the sera obtained from groups 1 and 3 were stronger than those obtained from group 2, T.theileri may have some common antigens with the pathogenic trypanosomes.

2. When the IFT-A test is employed using unknown sera, the starting dilution should be 1/40.

CATTLE GROUP	REACTION	ANTIGENS								
		T. brucei			T. congolense			T. vivax		
		SERUM DILUTIONS			SERUM DILUTIONS			SERUM DILUTIONS		
		1/10	1/20	1/40	1/10	1/20	1/40	1/10	1/20	1/40
1	+	100	65	—	77	36	—	100	49	—
	W	—	35	73	19	52	43	—	45	37
	—	—	—	27	4	12	57	—	6	63
2	+	38	—	—	19	—	—	40	—	—
	W	39	46	2	27	21	—	37	47	—
	—	23	54	98	54	79	100	23	53	100
3	+	100	44	—	97	13	—	97	33	—
	W	—	53	26	3	67	23	3	50	23
	—	—	3	74	—	20	77	—	17	77

+ POSITIVE.
W WEAK POSITIVE.
— NEGATIVE.

Table 3.20. IFT-A tests using the 1/10, 1/20 and 1/40 dilutions of the sera collected from the cattle against the three antigens. Results are expressed as the % of sera which gave positive reactions.

3.17. Indirect fluorescent antibody-A- Production and storage of antigens.

Aim.

The method used to make T.congolense antigen (section 2.10e) involved growing the trypanosomes in mice until a 4-plus parasitaemia resulted. As this often took as long as 10 days, it was thought possible that the development of antibodies in mice might interfere with the subsequent fluorescence of the trypanosomes when they were used as antigens. This possibility was therefore examined.

If the stored antigens did not deteriorate, it would mean that new antigens would not have to be prepared for each test. Antigens stored over a 6 month period were thus examined for fluorescence activity.

Procedure.

Six mice, inoculated with EATRO-585, were examined for the presence of trypanosomes by wet films of tail blood daily from days 5 to 20 postinfection. Antigens were prepared on the days when the mice showed 4-plus parasitaemias. All the prepared antigen slides were used in IFT-A tests employing the same positive and negative sera.

Antigens of the brucei sub-group, T.congolense and T.vivax, stored at -26°C from periods varying from 1 to 180 days, were examined by the IFT-A test using the same known positive and negative sera.

Results.

All the antigens prepared from the six mice gave the

same fluorescence titre with the positive serum.

Samples of the antigens, removed after different storage times, gave similar titres to the positive serum (table 3.21).

Conclusions.

1. The method of preparation of T.congolense antigen was satisfactory.

2. Antigens, prepared on one occasion and stored at -26°C , do not lose their potency for at least 6 months.

ANTIGEN	DAYS IN STORAGE AT -20°C										
	1	8	15	22	29	36	63	90	115	140	180
T. brucei Sub-group	←				$\frac{1}{80}$						→
T.congolense group	←				$\frac{1}{80}$						→
T.vivax group	$\frac{1}{60}$	←				$\frac{1}{320}$					→

Table 3.21. Titres of IFT-A tests using three antigens stored for different periods and the same known positive serum.

3.18. Detection of antibody by indirect fluorescent antibody-A.

Aim.

Before the general application of the IFT-A test, it was essential to know whether the antibodies detected were common or variant specific. The type of antibody that the test detected was therefore examined.

Procedure.

Antigens, prepared from four different antigenic variants of T.congolense and five of T.brucei, were used in IFT-A tests employing sera obtained from zebus 495 and 480 infected with T.congolense and T.brucei respectively. The T.congolense antigen was prepared from four stabilates, which were shown to be antigenically distinct by the neutralization test. The T.brucei antigen was prepared from the 7,28,56,84 and 140 day isolations of zebu 480, which were shown to be antigenically distinct by the agglutination test.

Results and conclusion.

From tables 3.22 and 3.23, it can be seen that the titres of the sera were similar. Hence it seemed likely that common antibodies were being detected.

SERA (days post- infection)	ANTIGENIC VARIANTS			
	EATRO-585	EATRO-505	EATRO-585 ₁₀	EATRO-649
0	—	—	—	—
29	1/20	1/20	1/20	1/20
104	1/80	1/160	1/160	1/80
122	1/160	1/80	1/80	1/160

Table 3.22. Titres of IFT-A tests using sera obtained from zebu 495 and antigens prepared from distinct antigenic variants of T.congolense.

SERA & STABILATES (days post- infection)	ANTIGENIC VARIANTS				
	7	28	56	84	140
6	—	—	—	—	—
27	1/320	1/160	1/80	1/320	1/160
63	1/320	1/320	1/160	1/320	1/160
84	1/320	1/320	1/320	1/160	1/160
140	1/160	1/160	1/160	1/160	1/160

Table 3.23. Titres of IFT-A tests using sera obtained from zebu 480 and antigens prepared from distinct antigenic variants of T.brucei isolated from the same animal.

3.19. Indirect fluorescent antibody-B.

a. Storage of blood spots.

Aim.

The use of blood spots on filter paper increases the speed of collection of sera from animals and has the advantage that samples can be sent from remote areas by post. This might entail delays of 7 to 14 days before the blood spots could be tested for fluorescence activity. The effect of time and temperature on the potency of sera collected in this manner were therefore examined.

Procedure.

Two blood spots on Whatman No 4 filter paper were collected from each of 10 cattle which had been infected with all three groups of pathogenic trypanosome 21 days previously. Blood discs from the spots were used immediately in IFT-B tests employing brucei sub-group, T.congolense and T.vivax antigens. The spots were subsequently divided into two groups of 10. One group was stored at -26°C , and the other at room temperature. Blood discs, prepared from the spots in each group, were used in IFT-B tests against the same antigens after 7, 21 and 35 days in storage.

Results.

The blood spots stored at -26°C gave positive reactions after storage of 35 days, while those stored at room temperature gave positive reactions after 7 and 21 days only.

Conclusion.

Samples of blood collected on filter paper and then sent to the laboratory by post should be stored at -26°C on

arrival. As the samples stored at room temperature for 21 days were still reactive, blood spots can be collected with safety many miles from the laboratory.

b. Sensitivity.

Aim.

The detection of antibodies in drug-treated animals was studied to establish the sensitivity of the IFT-B test. The relationship between positives by IFT-B and antibody titre was also investigated.

Procedure.

Zebus 496 and 539 were infected with T.brucei. The infections, which became patent on day 4, was cured with berenil (7mg/kg) on day 21. Sera and blood spots were collected from both animals at regular intervals until day 55 postinfection. These materials were used in IFT-B tests initially, and then IFT-A tests.

Results.

Figure 3.7 and table 3.24 show the results of the tests. In both cattle, the common antibody titres reached a peak (1/160 in zebu 496, 1/80 in zebu 539) on day 21 as detected by IFT-A. The titres fell after drug treatment being 1/40 on day 55. Antibodies were detected by IFT-B between 14 and 48 days in zebu 496, but only between days 21 and 29 in zebu 539.

Conclusions.

1. The detection of serological positives by IFT-B was correlated with serum titre. Positives were detected only when the serum titre was 1/80 or above.

2. As a positive at 1/40 is likely to indicate the presence of common antibodies (see section 3.16), a positive reaction by IFT-B is meaningful.

c. Antigen.

Aim.

Blood spots collected from cattle infected with T.congolense only were used in IFT-B tests against brucei sub-group and T.congolense antigens to establish whether the type of antigen used affected positive reactions.

Procedure.

Five zebu cattle were inoculated intravenously with 1ml of EATRO-585/4. The resulting infections were cured with berenil (7mg/kg) on day 21. The cattle were rechallenged on day 77 and as they did not resist this challenge they were cured again on day 98. These animals were part of an immunization experiment (see section 3.25). Blood spots were collected from the five cattle at regular intervals until day 154 and from two controls until death. The spots were used in IFT-B tests employing brucei sub-group and T.congolense antigens.

Table 3.25 shows the results of the tests. Antibodies to the homologous antigen were detected in the two controls by days 35 and 42 respectively and remained in detectable quantities until death. In three of the test animals, two peaks of antibody response were detected in response to the two challenges, in the fourth one peak of antibody was demonstrated, while in the fifth (zebu 384) a poor antibody response was noted. Few positives

were detected when the heterologous brucei sub-group antigen was used.

Conclusinn.

As the heterologous antigen gave little indication as to the presence of antibodies, unknown samples should be tested against all three group antigens by IFT-B.

DAYS POST- INFECTION	BOVID 496	BOVID 539
0	-	-
7	-	-
14	+	-
21	+	+
29	+	-
48	+	-
55	-	-

Table 3.24. Results of IFT-B tests using filter paper discs collected from zebus 496 and 539 and T.brucei antigen.

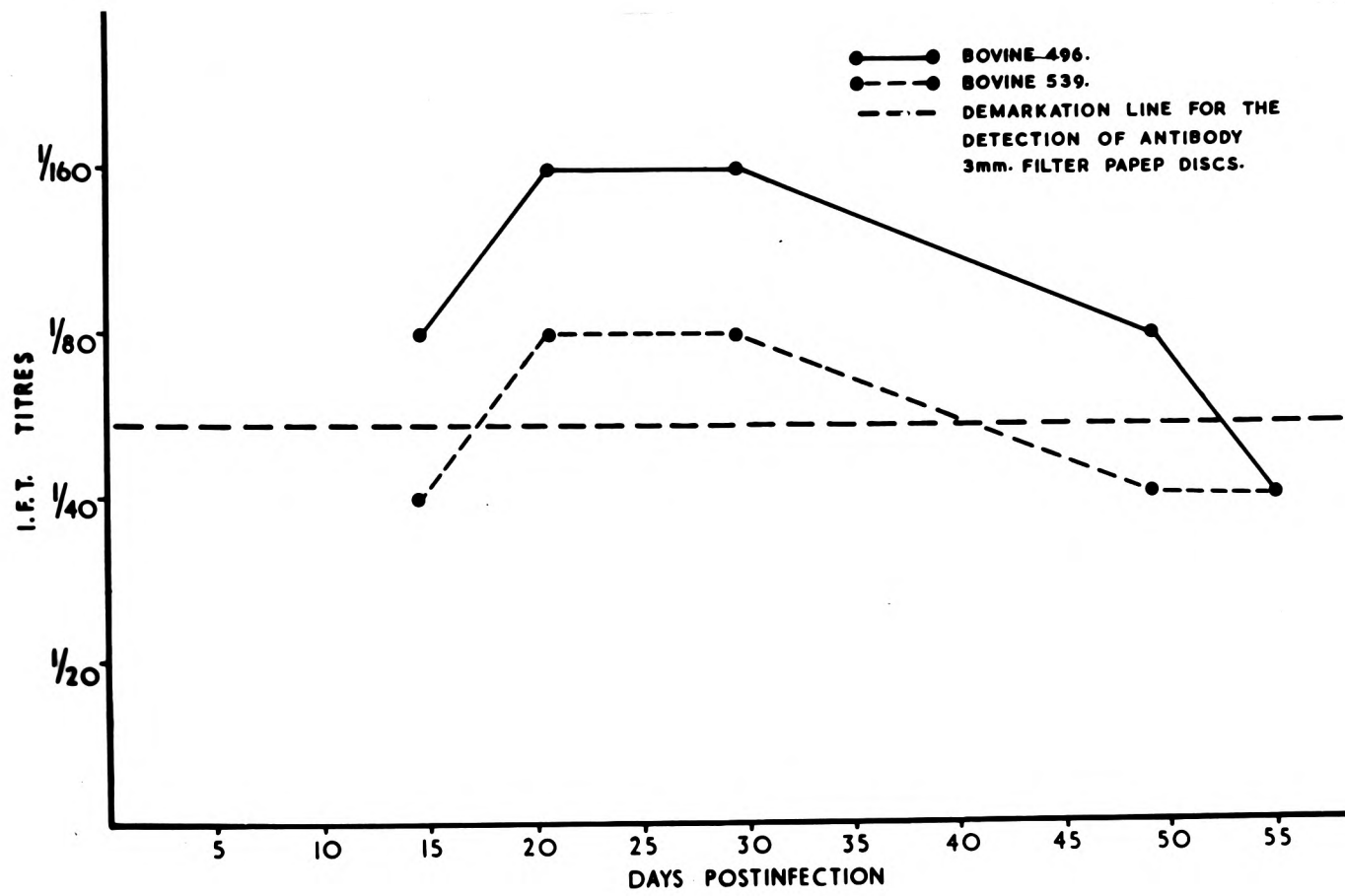


Figure 3.7. Titres of IFT-A tests using sera collected from zebus 496 and 539 and T.brucei antigen.

BOVINE No	DAYS POSTINFECTION																	
	0	7	14	21	28	35	42	49	56	77	84	91	98	105	112	126	135	154
	↑			↑						↑			↑					
TEST	216	-	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+
	379	-	-	-	+	+	+	-	-	-	-	+	+	+	+	+	+	+
	384	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-
	387	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
	397	-	-	-	-	+	+	+	-	-	-	+	+	+	+	+	+	+
CON-TROL	438	-	-	-	-	-	-	+	+	+	+							
	455	-	-	-	-	-	-	+	+	+	+	+	+	+	+			

2) *T. brucei* SUB-GROUP ANTIGEN

BOVINE No	DAYS POSTINFECTION																	
	0	7	14	21	28	35	42	49	56	77	84	91	98	105	112	126	135	154
	↑			↑						↑			↑					
TEST	216	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
	379	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
	384	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	387	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	397	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
CON-TROL	438	-	-	-	-	-	-	-	+	-								
	455	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		

↑ TRYPANOSOMAL CHALLENGE

↑ BERENIL (7mg/Kg)

Table 3.25. Results of IFT-B tests using filter paper discs collected from 7 cattle infected with *T. congolense* and *T. congolense* and *brucei* sub-group antigens.



3.20. T.congolense infection in cattle-antigenic variation of the organisms, immune response of the host, parasitaemia and body weight changes.

Aim.

The infections, in two cattle infected with T.congolense, were studied to examine:-

- i. the variations in the level of parasitaemia
- ii. the variations taking place in the antigenic type of the organisms
- iii. the type, strength and persistence of the antibody response of the host
- iv. the changes in the body weight of the host.

Procedure.

Two animals were studied, zebu 495 and 530, and they were infected with T.congolense stabilates EATRO-585/1 and EATRO-189/1 respectively. Materials from zebu 495 were used in section 3.18, from zebu 530 in section 3.6 and from both animals in section 3.15. Zebu 495 survived until the end of the observation on day 360. Zebu 530 died on day 138. The examinations carried out, summarized on figures 3.8 and 3.9, were as follows.

a) Parasitaemia.

Wet films of jugular blood were examined daily at 0900 hours until day 200 and thence at weekly intervals in zebu 495, and daily throughout the infection in zebu 530. The concentration of trypanosomes in the jugular blood was estimated on days 5 to 13 in zebu 495 and on days 4 to 30 in zebu 530.

b) Infectivity to mice.

Jugular blood, collected at 0900 hours, was titrated in mice on days 5 to 13 and 161 to 207 in zebu 495, and on days

0 to 30, 77 to 100 and 123 to 138 in zebu 530. Also, blood was inoculated into mice at weekly intervals, in zebu 495 only, from days 214 to 360.

c) Preservation.

Stabilates of the jugular trypanosome population were preserved in capillaries, daily, from days 5 to 20 in zebu 495, and until day 30 in zebu 530, and subsequently at weekly intervals in the latter animal only. Each stabilate was titrated for infectivity in mice.

d) Serology.

Serum was collected, daily, for the first 30 days of the infection in both animals and then at approximately weekly intervals until day 360 in zebu 495 and until death in zebu 530. Homologous neutralization-A tests were carried out using sera and stabilates from both animals. Antisera to distinct antigenic variants were thus isolated. The stabilates prepared on days 7 to 12 from zebu 495 and on days 18 to 24 from zebu 530 were used in the test with sera in which neutralizing antibodies to distinct antigenic variants were present. Using the first distinct antigenic variant detected in zebu 495 (EATRO-585/4) as antigen, tests were carried out employing sera from zebu 530 in which antibodies to five distinct antigenic variants were present. Using as antigens the first two distinct variants detected, neutralization-C tests were carried out with sera from zebu 495 up to day 227 and from zebu 530 up to death. Homologous IFT-A tests were conducted with sera from zebu 495 up to day 360 and from zebu 530 up to death.

e) Body weight.

Both cattle were weighed every 3 weeks up to death in zebu 530 and up to day 360 in zebu 495.

Results.

Figures 3.8 and 3.9 show the changes in parasitaemia of the trypanosomes and their infectivity for mice, changes in body weight and the patterns of development of neutralizing and immunofluorescence antibodies in the two cattle.

a) Parasitaemia and infectivity of the trypanosomes.

Trypanosomes were first detected microscopically, in a concentration of 1-plus, on day 5 in zebu 495 and subsequently on days 6 to 126, 135 to 138, 146, 152, 162, 178, 179 and 189. No trypanosomes were detected on the days not mentioned above or over the period 190 to 360. No trypanosomes were detected from the weekly mouse inoculations with blood from days 214 to 360. Figure 3.10 shows the relationship between the numbers of trypanosomes in the jugular blood and their infectivity on days 5 to 13. Numbers remained relatively constant between 10^5 and 10^6 per ml; numbers of infective trypanosomes showed similar concentrations with the exception of day 9 when there was a statistically significant fall in infectivity. The infectivity of the trypanosomes over the period of days 161 to 207 was much lower than that of the trypanosomes over the 5 to 13 day period. On many of the days no infective organisms were detected; on the remainder, infectivity varied from $10^{2.4}$ to $10^{\bar{1}.4}$ (see figure 3.8). The last infective trypanosomes were found on day 193.

Trypanosomes were first detected microscopically,

in a concentration of 1-plus, on day 4 in zebu 530 and subsequently on days 5 to 92, 112 and 120. Figure 3.11 shows the relationship between the number of trypanosomes in the jugular blood and their infectivity on days 4 to 30. Numbers remained relatively constant between 10^5 and 10^6 per ml; numbers of infective trypanosomes were mainly between 10^3 and $10^{4.6}$ but there were statistically significant falls in infectivity on days 11, 22 and 29. The infectivity of the trypanosomes steadily decreased as the infection proceeded (see fig 3.9). No infective organisms were detected on the three days prior to death.

b) Serology.

Tables 3.18 (see section 3.15) and 3.26, and figure 3.12 show the results of the neutralization tests using the materials collected from zebu 495. The 7, 13 and 20 day stabilates were shown to be antigenically different. The infecting and 7 day stabilates were both neutralized by the 14 day serum. The 14 day serum also neutralized the 8 day stabilate but not the trypanosomes preserved on days 9 to 12, which were neutralized by the 29 day serum. Antibodies to the 7 day stabilate (EATRO-585/4) were first detected by neutralization-C on day 9, rose to a peak until day 20 and subsequently fell in titre until between days 112 and 141, when a statistically significant rise was detected on day 134. The titre then fell but antibodies were still present in detectable quantities on day 227. Antibodies to the second antigenic variant (EATRO-585/10) were first detected on day 29, rose to a peak until day 50 and subsequently fell off in titre

but were still detectable on day 227.

Tables 3.27 and 3.28, and figure 3.13 show the results of the neutralization tests using the materials collected from zebu 530. The 8, 27, 49, 71 and 92 day stabilates were shown to be antigenically different. The infecting and the 8 day stabilate were both neutralized by the 12 day serum. The 30 day serum neutralized the 18 and 19 day stabilates but not the 23 and 24 day stabilates, which were neutralized by the 44 day serum. Antibodies to the infecting variant(EATRO-189/1) were first detected by neutralization-C on day 12, rose to a peak until day 25 and subsequently declined but were still present in detectable quantities on day 138 when this animal died. Antibodies to the second antigenic variant(EATRO-189/15) were first detected on day 35, rose to a peak until day 60 and then declined but were still detectable on day 138.

Table 3.29 shows the results of the neutralization-A tests involving EATRO-585/4 as the antigen and sera collected from zebu 530. The 91 day serum from this animal neutralized the antigen.

Common antibodies, as demonstrated by the IFT-A using T.congolense antigen, were first detected in zebu 495 on day 12. From this time until approximately day 200, the titre fluctuated between 1/40 and 1/360 with peaks on days 77, 119, 152, 166 and 186. From days 200 to 360, the titre gradually but steadily decreased from 1/360 to 1/20(see figure 3.8). Common antibodies in zebu 530 were first detected on day 9 but remained

at low levels throughout the remainder of the infection(see figure 3.9).

c) Body weight.

In zebu 495, body weight decreased from 125kg to 85kg from day 0 to day 84. From this time until day 126 it remained at this low level and then began to increase steadily so that by day 360 the original weight had been regained. In zebu 530, body weight decreased steadily throughout the infection from 120kg to 80kg.

Conclusions.

1. The parasitaemic picture of T.congolense infection in the two cattle was divided into three phases. The first was characterized by the regular appearance of the organisms in the venous blood, the second by their intermittent appearance and the third by their total disappearance, as judged by the detection methods used. Phase-1 parasitaemia was characterized by periodic falls in trypanosome infectivity which was not related to falls in trypanosome numbers.

2. The results of the neutralization-A tests demonstrated that, in both cattle, a succession of antigenic variants of T.congolense were formed. In zebu 530, 6 distinct variants were detected during the first 92 days of infection. The statistically significant falls in infectivity, which occurred in the trypanosome populations during phase-1 parasitaemia, were related to antigenic change. The stabilates isolated before the infectivity trough on day 9 in zebu 495 were antigenically distinct from those isolated after(see table 3.26). Similarly, the stabilates

isolated prior to the infectivity trough on day 22 in zebu 530 were antigenically distinct from those isolated after (see table 3.28). The first trypanosome populations isolated from both cattle were of a similar antigenic type to the infecting stabilates. The first antigenic type detected in zebu 495 was similar to the fifth detected in zebu 530.

3. The pattern of development of neutralizing antibodies to antigenic variants of T.congolense in the cattle was similar to that described for the brucei sub-group(82) with the exception of the second rise in antibody titre to the infecting stabilate in zebu 495 on day 134. This second rise may indicate that a reversion to the first antigenic type had taken place and may explain the selfcure of this animal.

4. The results of the IFT-A tests indicated that antibodies were synthesized to antigens common to each variant population. Common antibody titre may be used as an indicator as to the presence or absence of trypanosomes in a host. The titre gradually decreased after day 200 in zebu 495 while trypanosomes were last detected on day 189 by wet film and on day 193 by infectivity titration.

5. There was a relationship between body weight and the course of the disease. A steadily declining body weight, as in zebu 530 indicates a poor prognosis.

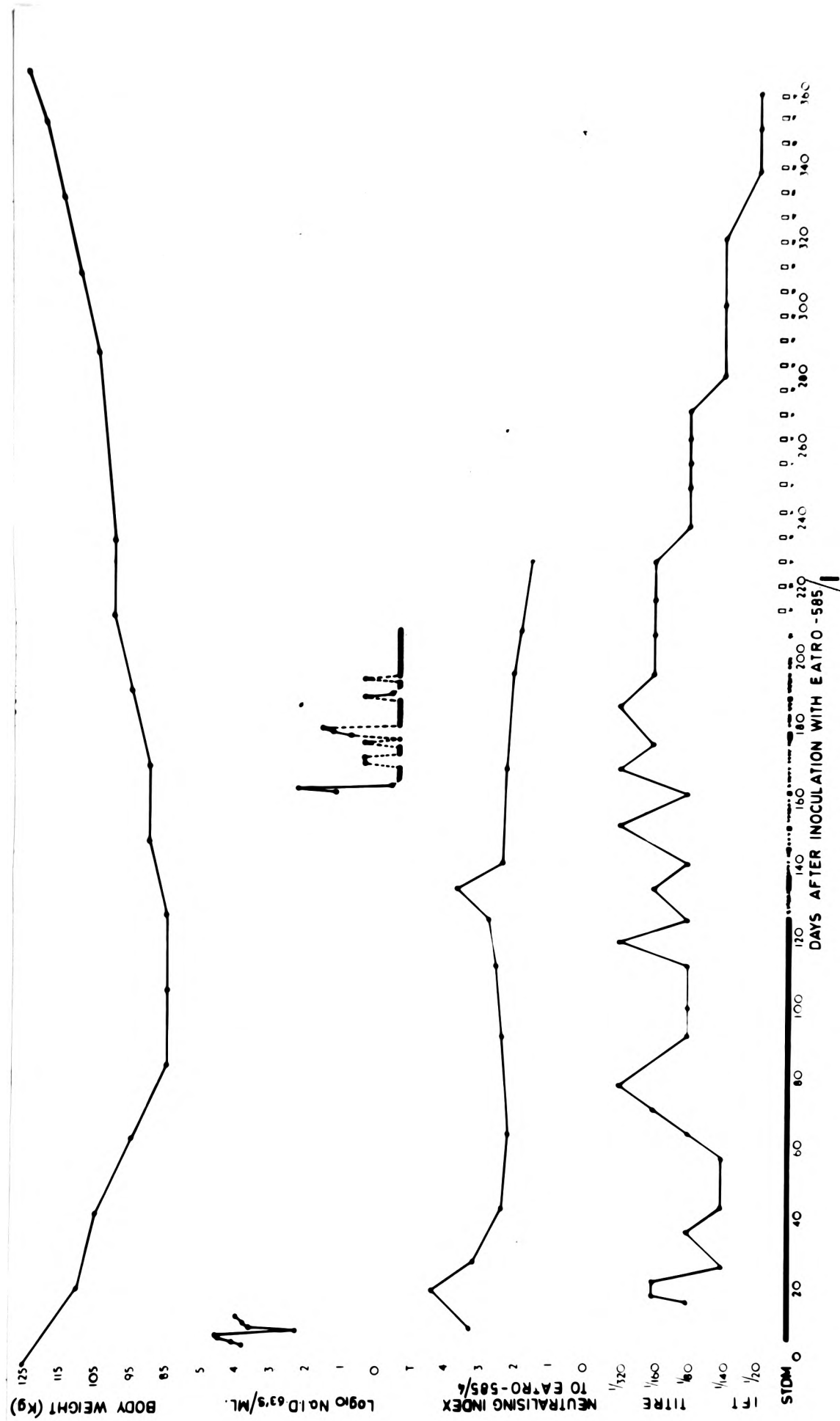


Figure 3.8. Changes in the parasitaemia of the trypanosomes and their infectivity, changes in body weight, and patterns of neutralizing and immunofluorescence antibodies during the infection of zebu 495.

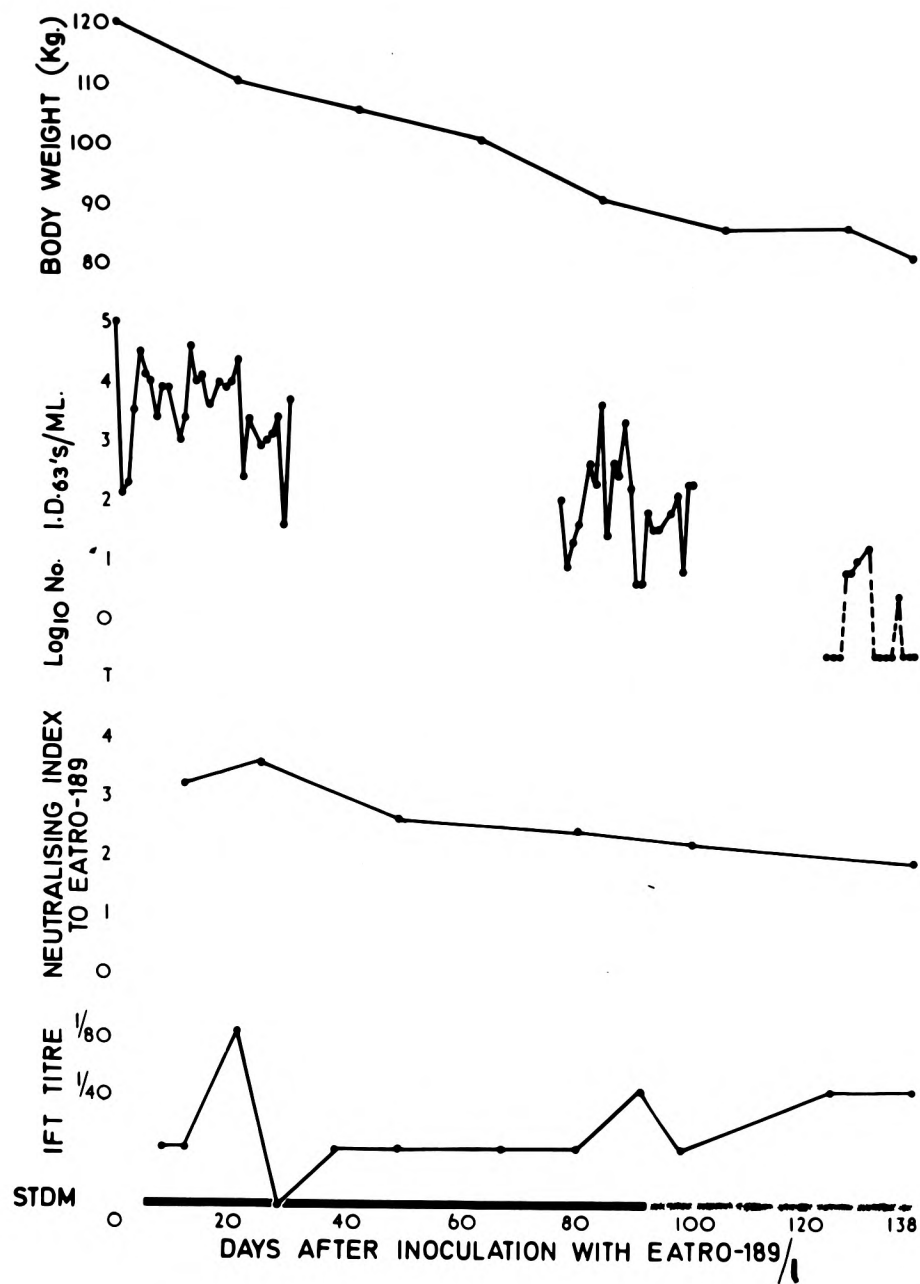


Figure 3.9. Changes in the parasitaemia of the trypanosomes and their infectivity, changes in body weight, and patterns of neutralizing and immunofluorescence antibodies during the infection of zebu 530.

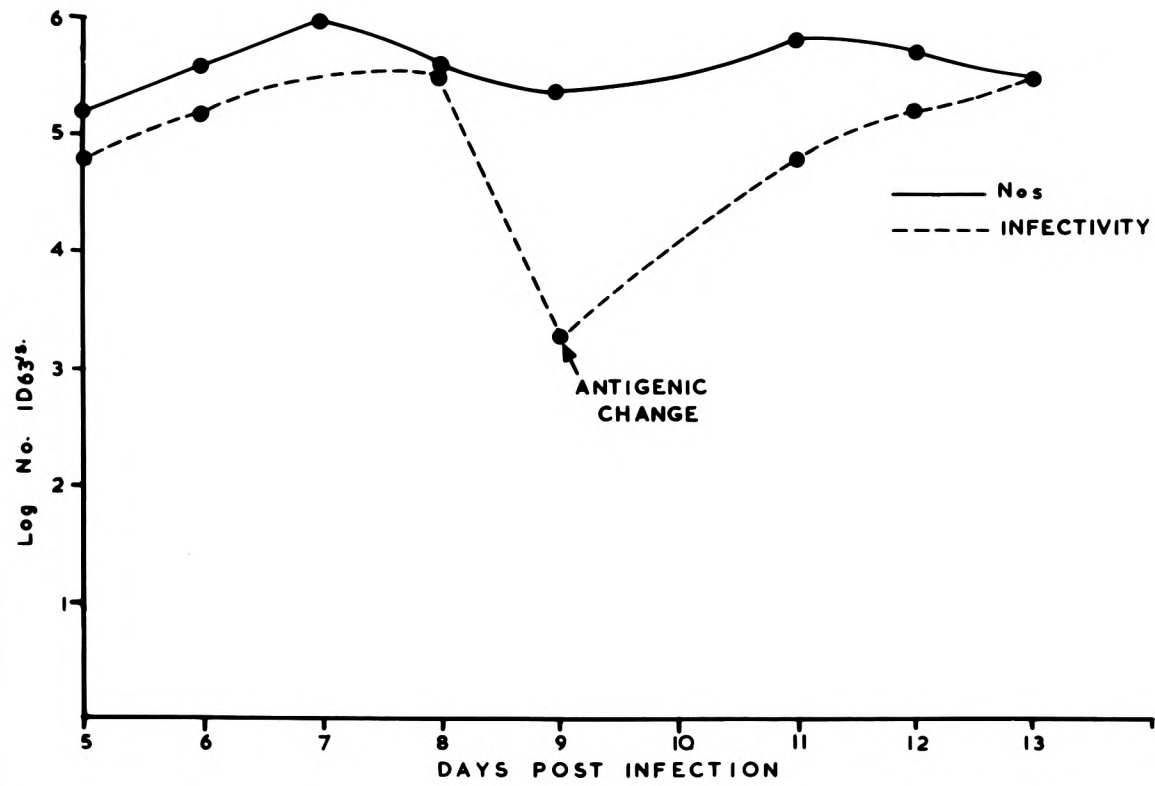


Figure 3.10. The numbers of trypanosomes and their infectivity in the jugular blood of zebu 495 on days 5 to 13 postinfection.

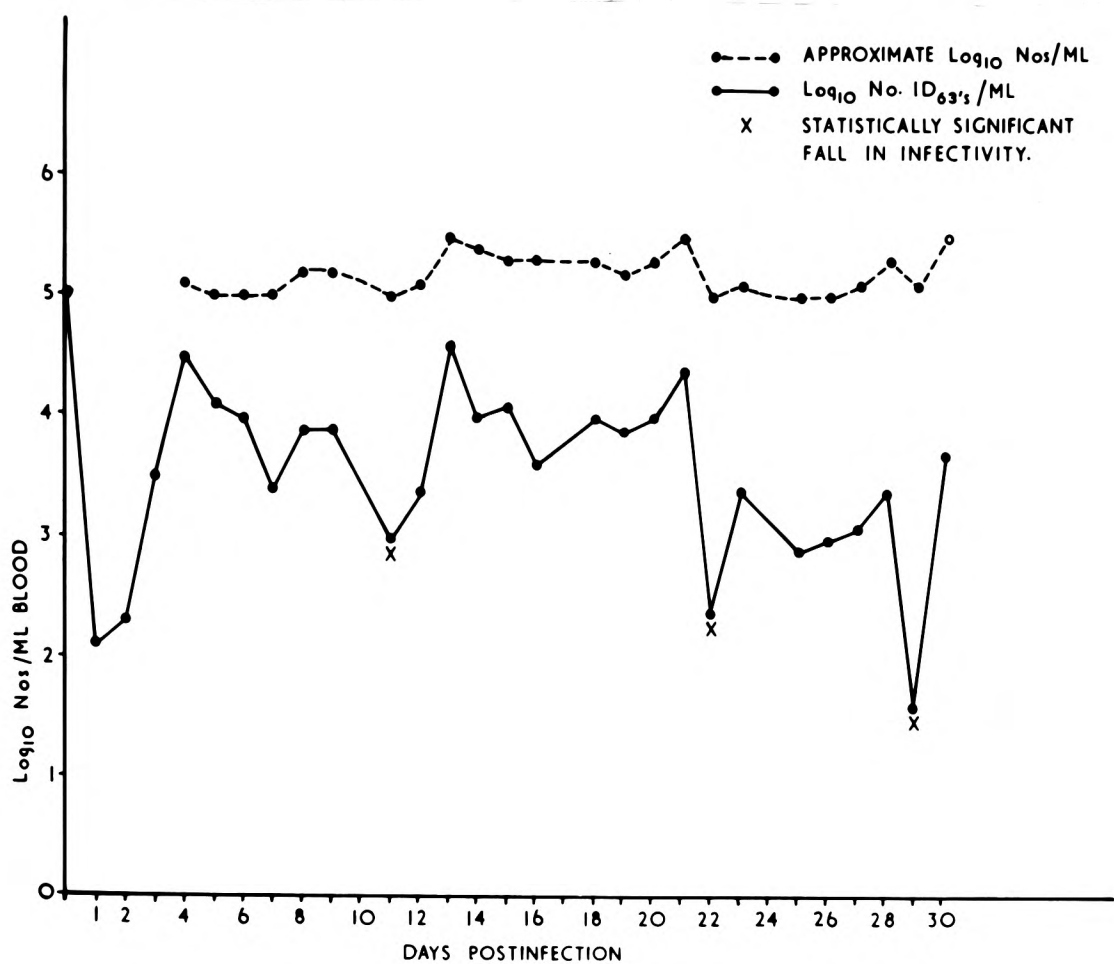


Figure 3.11. The numbers of trypanosomes and their infectivity in the jugular blood of zebu 530 during the first 30 days of infection.

STABILATES		SERA (days postinfection)		
DAYS POST- INFECTION	NUMBER	0	14	29
0	585/1	-	+	+
7	585/4	-	+	+
8	585/5	-	+	+
9	585/6	-	-	+
10	585/7	-	-	+
11	585/8	-	-	+
12	585/9	-	-	+

Table 3.26. Neutralization-A tests using the 14 and 29 days postinfection sera and antigens prepared on days 7-12 postinfection in zebu 495.

STABILATES		SERA (days postinfection)					
DAYS POST- INFECTION.	NUMBER	0	12	44	57	91	117
8	189/5	-	+	+	+	+	+
27	189/24	-	-	+	+	+	+
49	189/29	-	-	-	+	+	+
71	189/32	-	-	-	-	+	+
92	189/35	-	-	-	-	-	+

Table 3.27. Neutralization-A tests using sera and antigens collected at regular intervals throughout the infection from zebu 530

3-64.

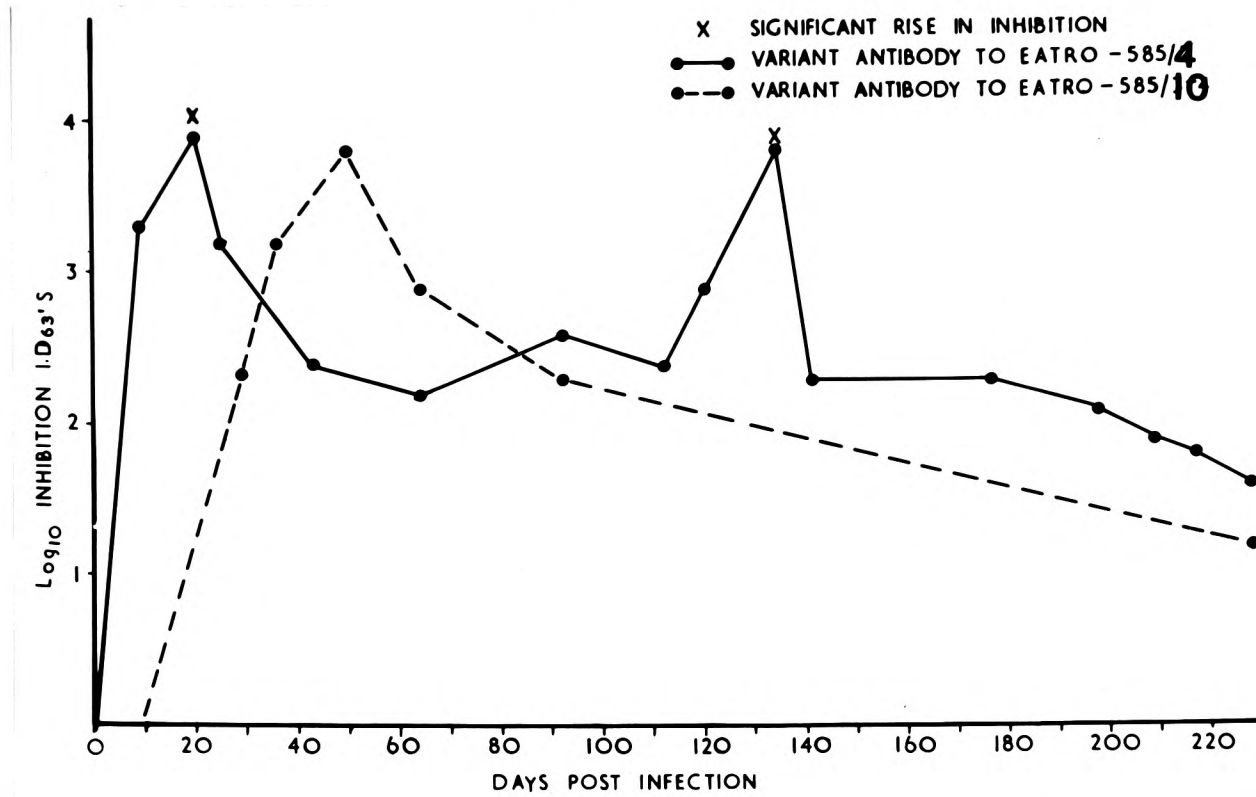


Figure 3.12. Neutralizing indices to antigens EATRO-585/4 and EATRO-585/10 in sera collected from zebu 495 at regular intervals during the first 227 days of infection.

DAYS POST-INFECTION	STABILATE No.	ANTISERA (days post-infection)			
		0	12	30	44
0	189 / 1	-	+	+	+
8	189 / 5	-	+	+	+
18	189/15	-	-	+	+
19	189/16	-	-	+	+
20	189/17	*			
21	189/18	*			
22	189/19	*			
23	189/20	-	-	-	+
24	189/21	-	-	-	+

* INADEQUATE PRESERVATION.

Table 3.28. Neutralization-A tests using the 12,30 and 44 days postinfection sera and antigens prepared during the first 24 days of infection from zebu 530.

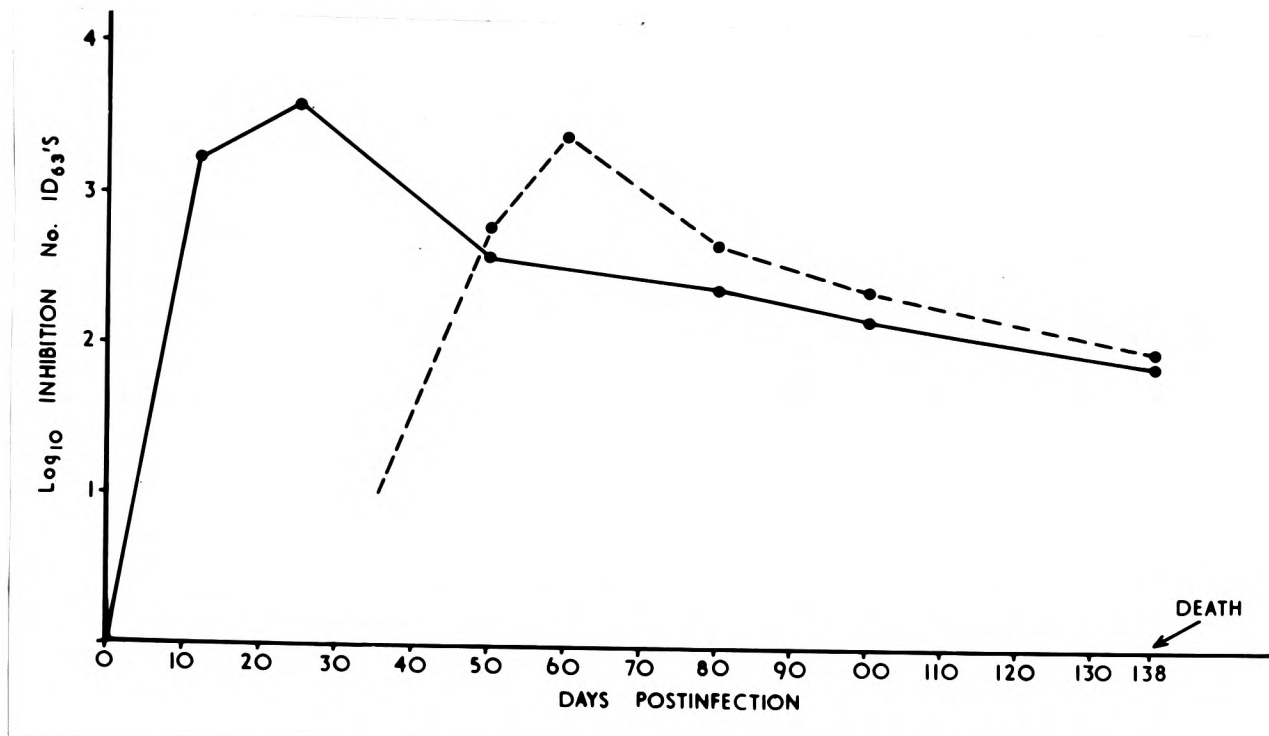


Figure 3.13. Neutralizing indices to antigens EATRO-189/1 and EATRO-189/15 in sera collected from zebu 530 at regular intervals throughout the infection.

7 DAY STABILATE BOVINE 495	ANTISERA FROM BOVINE 530 (days postinfection.)					
	0	12	44	57	91	117
EATRO- 585/4	-	-	-	-	+	-

Table 3.29. Neutralization-A tests using sera collected at regular intervals throughout the infection from zebu 530 and stabilate EATRO-585/4 as antigen

3.21. T.congolense infection in cattle-antigenic variation of the parasites in five animals infected with the same stabilate.

Aim.

A distinct pattern of antigenic variation occurs in animals infected with brucei sub-group organisms(163-165). It seemed important to ascertain whether a similar phenomenon occurred with T.congolense. The antigenic relationships of populations of T.congolense, which were preserved at regular intervals from five cattle inoculated with the same stabilate, were therefore studied.

Procedure.

Five zebus, numbers 353, 383, 492, 498 and 555, were each inoculated intravenously with approximately 10^3 mouse ID_{63s} of EATRO-585/40, preinoculation sera having been taken. EATRO-585/40 was a first cyclical population of EATRO-585 after cyclical passage through G.morsitans and was obtained from an infected fly detected in section 3.1. Venous blood was examined daily by wet film from day 5 until trypanosomes were detected, after which stabilates were prepared weekly by the ampoule method until day 84. Sera were collected from all the animals until day 105. All the stabilates were titrated for infectivity in mice. Stabilates, which contained at least 10^3 infective organisms, were employed in neutralization-A tests using sera collected from the same cow. Antigenic variants and antisera were thus identified. Only the first sera to contain neutralising antibodies to stabilates were recorded with the exception of the

infecting stabilate(see tables 3.30-3.34). Cross-neutralization tests were then conducted using variants isolated from the five cattle and their specific antisera.

Results.

Tables 3.30 to 3.35 show the results of the tests. Thirty antigenic variants, each being given a number, were isolated from the five animals(see tables 3.30 to 3.34). When these variants and their antisera were used in cross-neutralization tests, 12 distinct types were identified(see table 3.35). The first two antigenic types detected in all the cattle were similar, designated A and B. Many of the variants, which developed later in the infections, were dissimilar. However, the 28 and 63 day stabilates(numbers 29 and 30) of zebu 555 were antigenically similar to the stabilates collected on the same days from zebu 498(numbers 22 and 25). They were identified as types E and J respectively. Similarly, the 49 and 84 day stabilates(numbers 5 and 7) of zebu 353 were similar to the stabilates collected on the same days from zebu 383(numbers 12 and 13). They were identified as types H and K respectively.

Conclusion.

Antigenic variants of T.congolense develop in a definite order in cattle infected with the same antigenic type.

Tables 3.30 to 3.34 show the results of neutralization-A tests using sera and antigens collected at regular intervals throughout the infections from zebus 353,383,492,498 and 555 respectively.

STABILATES (days postinfection)	A N T I S E R A (days postinfection).								DISTINCT ANTIGENIC VARIANTS
	0	14	21	35	56	63	77	98	
-	-	+	+	+	+	+	+	+	1
7	-	+							
14	-	-	+						2
21	-	-	-	+					3
28 *									
35 *									
42	-		-		+				4
49	-		-		-	+			5
56 *									
63	-				-	-	+		6
70	-				-	-	+		
77 *									
84	-					-	-	+	7

* INADEQUATE Log₁₀ No. MOUSE ID₆₃'s FOR NEUTRALISATION TEST.

Table 3.30

STABILATES (days postinfection)								DISTINCT ANTIGENIC VARIANTS
	0	14	21	49	56	63	98	
-	-	+	+	+	+	-	-	8
7	-	+						
14	-	-	+					9
21	-	-	-	+				10
28	-	-	-	-	+			11
35 *								
42	-	-	-	-	+			
49	-	-	-	-	-	+		12
56 *								
63 *								
70 *								
77 *								
84	-	-	-	-	-	-	+	13

Table 3.31

STABILATES (days postinfection)								DISTINCT ANTIGENIC VARIANTS
	0	14	21	42	56	77	98	
-	-	+	+	+	+	+	-	14
7	-	+						
14	-	-	+					15
21	-	-	+					
28 *								
35	-	-	-	+				16
42	-	-	-	-	+			17
49 *								
56 *								
63	-	-	-	-	-	+		
70 *								
77 *								
84	-	-	-	-	-	-	+	19

Table 3.32.

STABILATES (days postinfection)	ANTISERA (days postinfection)								DISTINCT ANTIGENIC VARIANTS
	0	14	21	42	56	63	77	98	
-	-	+	+	+	+	+	+	-	20
7	-	+	+						
14	-	-	+		+			+	21
21 *									
28	-		-	+	+			+	22
35	-		-	+	+			+	
42	-		-	-	+	+		+	23
49	-				-	+		+	24
56 *									
63	-					-	+	+	25
70 *									
77 *									
84	-					-	-	+	26

Table 3.33.

STABILATES (days postinfection)						DISTINCT ANTIGENIC VARIANTS
	0	14	21	56	77	
-	-	+	+	+	+	127
7 *						
14	-	-	+			28
21 *						
28	-	-	-	+		29
35 *						
42 *						
49 *						
56 *						
63	-	-	-	-	+	30
70 *						
77 *						
84 *						

Table 3.34.

EXPERIMENTAL VARIANT NUMBER	1 8 14 20 27	2 9 15 21 28	3	10	11 16 22 29	17	4 23	5 12 24	18	6 25 30	7 13	19 26
DISTINCT ANTIGENIC TYPES	A	B	C	D	E	F	G	H	I	J	K	L

Table 3.35. Antigenic variants, isolated from the five bovines, which were serologically similar as judged by neutralization-A.

3.22. Examination of populations of T.congolense before and after cyclical passage through G.morsitans.

Aim.

It is known that an antigenic reversion occurs when antigenic variants of a strain of the brucei sub-group are passaged through G.morsitans(31,73,165). The antigenic relationships of populations of T.congolense, before and after cyclical passage through G.morsitans, were therefore studied to ascertain whether a similar mechanism resulted.

Procedure.

Figure 3.14 shows the protocol of the experiment. Batches of 42 and 38 newly hatched G.morsitans were induced to feed into 4ml defibrinated bovine blood, containing stabilates EATRO-585/8 and EATRO-585/18 respectively. These stabilates were preserved from zebu 495(see section 3.20) and were shown to be antigenically different by the neutralization-A tests. After the infecting feed, the flies were maintained as described in section 2.2. The first fly to become infected with metacyclics, as detected by the methods described in section 2.6, was isolated and placed in a double-ended tube. These two flies were induced to feed into defibrinated bovine blood every 2-3 days. The blood was then inoculated into mice and resulting first cyclicals preserved in capillaries.

Specific antisera to both the infecting stabilates and eight first cyclicals isolated were prepared in rats, as described in section 2.9a. Cross-neutralization-A tests were carried out using the ten stabilates and their specific antisera.

Results.

The two infecting trypanosome populations were antigenically different to all 8 first cyclicals, as shown in table 3.36. Three antigenic types were identified from the first cyclical populations, namely A', which consisted of 1,5 and 7; B', which consisted of 2,3 and 4; and C', which consisted of 6 and 8. The populations of type B' were solely derived from fly 1, those of C' from fly 2, and those of A' from both the flies.

Conclusions.

1. The antigenic type of the first cyclicals was different to that of the trypanosomes prior to cyclical passage.

2. An antigenic reversion, as has been reported for the brucei sub-group, did not occur on cyclical passage. A tendency for such a mechanism maybe indicated by first cyclical type A', which contained derivatives of both infecting variants.

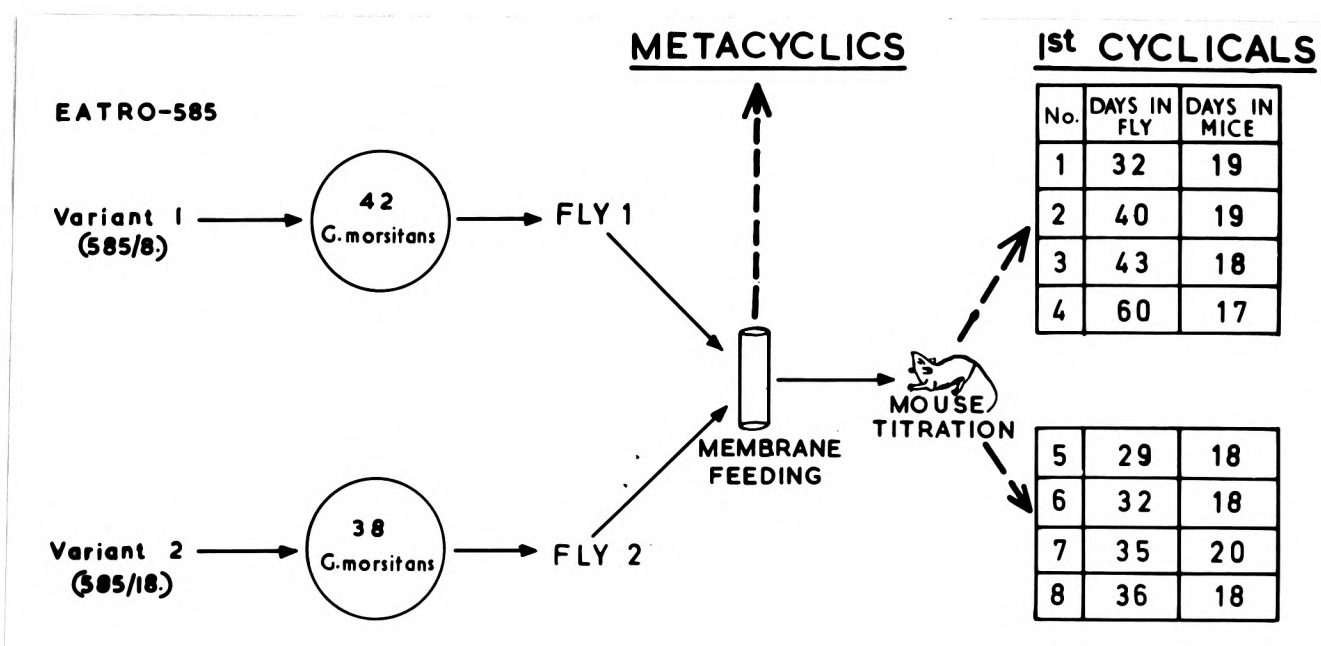


Figure 3.14. Protocol of the experiment showing the numbers of flies used and the histories of the first cyclicals obtained. Metacyclic material from the same flies was used in section 3.9.

ORIGIN		STABILATE NUMBER	A N T I S E R A									
			585/8	585/18	1	2	3	4	5	6	7	8
INFECTING VARIANTS		585/8	+	-	-	-	-	-	-	-	-	-
		585/18	-	+	-	-	-	-	-	-	-	-
1st CYCLICAL POPULATIONS	FLY 1	1	-	-	+	-	-	-	+	-	+	-
		2	-	-	-	+	+	+	-	-	-	-
		3	-	-	-	+	+	+	-	-	-	-
		4	-	-	-	+	+	+	-	-	-	-
	FLY 2	5	-	-	+	-	-	-	+	-	+	-
		6	-	-	-	-	-	-	-	+	-	+
		7	-	-	+	-	-	-	+	-	+	-
		8	-	-	-	-	-	-	-	+	-	+

Table 3.36. Neutralization-A tests using antigens prepared from the infecting variants and the 8 first cyclical populations, and their specific antisera.

3.23. Immunological relationships of isolates of the brucei sub-group obtained from different areas of East Africa.

Aim.

It has been reported that antigenic variation of the brucei sub-group trypanosomes is related to area(167,168). This experiment was carried out to examine these findings.

Procedure.

Agglutinogenic antigens and specific antisera were prepared from each of seven brucei sub-group stabilates, which were prepared from isolates obtained from different areas of Uganda, Kenya, Tanzania and Rwanda. The geographical position of the localities mentioned is shown on the map in section 2.13.

The history of the stabilates used is as follows.

1. EATRO-1137, T.rhodesiense, the 6th mouse passage of trypanosomes isolated from a man at Kigali, Rwanda.
2. EVE-8, brucei sub-group, the first mouse passage of trypanosomes isolated from a cow at Bugiri, Busoga, Uganda.
3. EVE-24, brucei sub-group, the first mouse passage of trypanosomes isolated from a bushbuck at Mavubwe, Busoga, Uganda.
4. EATRO-1135, brucei sub-group, the first mouse passage of trypanosomes isolated from 10 G.fuscipes at Soroti, Uganda.
5. EATRO-2/1, brucei sub-group, the second mouse passage of metacyclics isolated from G.pallidipes at Lugala, Busoga, Uganda.
6. EATRO-964, T.rhodesiense, metacyclics from a single G.morsitans infected with EATRO-835, isolated from a cow in Nyanza, Kenya.
7. Brucei sub-group with no designation, the third mouse passage of trypanosomes isolated from a cow in central Tanzania.

Cross-agglutination-A tests were carried out with all 7 isolates as antigens and their specific antisera.

Agglutination-A tests were then conducted using the 7 antigens and 95 sera obtained from different cattle and bushbuck in the south Busoga area of Uganda.

Results.

Tables 3.37 and 3.38 show the antigenic relationships of the 7 antigens, as judged by agglutination, and the agglutination reactions of these antigens with the 95 animal sera respectively.

Isolates 1, 2 and 7 were immunologically distinct; cross agglutination reactions occurred with the remaining four.

Some of the 95 animal sera gave positive agglutination with all the antigens. The largest number of positives were recorded using isolates 2 and 6, which were isolated from the same tsetse belt as the sera. Isolate 1 gave the lowest number of positive reactions. Isolates 3, 4, 5 and 7 gave intermediate readings.

Conclusion.

The reactivity of the 7 antigens to the 95 sera indicated that a proportion of the animals, from which the sera had been collected, had had immunological experience of brucei sub-group isolates collected many hundreds of miles away. Notably, isolate 7, which was collected over 1,000 miles away in central Tanzania, was agglutinated by 41 of the 95 sera.

TRYPANOSOME ISOLATES		ANTISERA						
LOCATION	DESIGNATION	1	2	3	4	5	6	7
RWANDA	1	+	-	-	-	-	-	-
BUSOGA	2	-	+	-	-	-	-	-
BUSOGA	3	-	-	+	+	-	-	-
SOROTI	4	-	-	+	+	+	-	-
LUGALA	5	-	-	-	-	+	-	-
NYANZA	6	-	-	-	+	-	+	-
TANZANIA	7	-	-	-	-	-	-	+

Table 3.37. Cross agglutination-A tests of the 7 brucei sub-group isolates studied and their specific antisera.

TRYPANOSOME ISOLATE	ANTISERA		
	POSITIVE	WEAK POSITIVE	NEGATIVE
1	6	14	75
2	40	26	29
3	15	41	39
4	29	26	40
5	21	27	47
6	39	38	18
7	18	23	54

Table 3.38. Agglutination-A tests using antigens prepared from the 7 brucei sub-group isolates and 95 animal sera obtained from south Busoga.

Two types of antibody response were detected in cattle infected with T.congolense (see section 3.20). Some of the properties of these antibodies were studied further. Attempts were made to immunize cattle against pathogenic trypanosomes by stimulation of the variant specific antibody response (sections 3.24 to 3.26). The pattern of production of common antibodies in a large group of cattle and the relationship between antibody titre and the presence of trypanosomes were studied. The information so obtained was then used to evaluate the indirect fluorescent antibody tests as diagnostic aids (sections 3.27 and 3.28).

3.24. Infectivity of T.congolense EATRO-585/4 for cattle.

Aim.

In the first immunization experiment, the trypanosomal challenge was standardized in terms of the numbers of infective trypanosomes. Therefore, EATRO-585/4, which had already been titrated for infectivity in mice (see sections 3.2 and 3.7), was also titrated for infectivity in cattle. It could then be determined if there were adequate infective organisms in this stabilate for it to be used as challenge material.

Procedure.

Eight ml of EATRO-585/4 were removed after low temperature storage of 210 days. One ml was titrated for infectivity in mice and the remaining 7ml was titrated for infectivity in cattle, starting at the +1 dilution. The two titrations were carried out within 20 minutes, the technique of the cow titration

being the same as that described for mice (section 2.7c) except that the trypanosomes were inoculated intravenously and the animals were observed over a period of 100 days.

Results.

Table 3.39 shows the results of the titrations. The infectivity end point for cattle was $10^{1.2}$ lower than that for mice. From this single titration comparison, 16 mouse ID_{63s} would be needed to infect a cow.

Conclusion.

The stabilate was considered suitable as challenge material for cattle as it contained 25 bovine ID_{63s} .

TIME IN STORAGE (days)	INFECTIVITY TITRATION END POINTS	
	MICE	CATTLE
210	2.6 ± 0.3	1.4 ± 0.5

Table 3.39. Simultaneous infectivity titrations of stabilate EATRO-585/4 in mice and cattle.

3.25. Immunization of cattle against a single stabilate of T.congolense.

Aim.

In order to examine the practical use of immunization procedures in cattle, it had first to be shown whether animals could be made immune to one stabilate of T.congolense. A regime involving the infection of the animals with living trypanosomes followed by berenil treatment was used. The cattle were treated with the drug 21 days after challenge, as it was at this time that the maximum titre of variant specific antibody to the infecting stabilate occurred (see section 3.20). Animals were rechallenged 18 weeks after drug treatment to eliminate the possibility of protection by the drug.

Procedure.

a) Drug sensitivity.

Tests, as described in section 2.11, were carried out to show that the trypanosomes used, EATRO-585/4, were sensitive to berenil.

b) Challenge procedure.

Sixteen zebu cattle, of 1-2 years old, were divided into two groups, one of 5 designated the 'test' animals, and one of 11 designated the 'controls'. The protocol of the experiment is shown on table 3.40 and figure 3.15. All the 'test' and two of the 'control' animals were each inoculated intravenously with 1ml of EATRO-585/4. After the inoculation, challenge 1, the residual portion of the stabilate was titrated in mice. This was termed the challenge procedure. On day 21, the 'test'

and the 9 remaining 'control' cattle were inoculated intramuscularly with berenil(7mg/kg). On day 77, the challenge procedure was repeated to the 'test' and two new 'control' cattle followed by berenil treatment to the 'test' and the remaining 7 'control' cattle on day 98. On day 154, the 'test' and two new 'controls' were challenged again. Individual 'test' animals were then challenged at 4-weekly intervals. Thus zebu 216 was challenged on day 182, zebu 379 on day 210, zebu 384 on day 238, zebu 387 on day 266, and zebu 397 on day 294. Single clean control cattle were included in each challenge.

c) STDM.

Wet films of jugular blood were examined from all the infected animals, daily on days 5 to 21 after each challenge, and every 3 to 4 days after berenil treatment and after the 21st day following challenges 3 to 8 until death or the termination of the experiment on day 400. Weekly mouse inoculations were carried out using jugular blood from all the infected animals until death or the termination of the experiment.

d) Serology.

Sera, taken at regular intervals from the 'test' animals from day 21 to day 300, were used in neutralization-B tests employing EATRO-585/4 as antigen.

e) Body weight.

Body weight measurements were obtained at approximately weekly intervals throughout the experiment.

Results.

a) Drug sensitivity.

EATRO-585/4 was sensitive to berenil.

b) STDM.

Figure 3.15 shows the STDM results over the period of study. The 'test' and 'control' animals became infected after both challenge 1 and 2. The 'test' animals resisted challenge 3, as judged by the failure to detect trypanosomes, whereas the relevant 'controls' did not. Trypanosomes were not detected in zebus 216 or 387 after challenges 4 and 7 respectively; they were detected in zebus 379, 384 and 397 after challenges 5, 6 and 8 respectively. All the 'control' cattle became infected 7 to 10 days after inoculation.

c) Serology.

Figure 3.16 shows the development of neutralizing antibody to EATRO-585/4 in the 'test' cattle from day 21 to day 300. After challenges 1 and 2, the antibody titres rose; they then fell after the elimination of trypanosomes by berenil. After challenges 2 and 3, falls in titre were detected in zebus 379, 384 and 387, and in zebus 216 and 379 respectively. After the remaining challenges, a fall in titre was noted in zebu 387 after challenge 7 only. The antibody titres, in zebus 379 and 384, rose after challenges 5 and 6 respectively when the trypanosomes reappeared in the blood. There was a marked rise in antibody titre in zebus 216, 379, 384 and 387 after the drug administration on day 21, and in zebu 384 only after the second administration on day 98.

d) Body weight.

Figure 3.17 shows the changes in body weight in the five 'test' animals throughout the experiment. All the animals lost weight after challenge 1. In zebus 216 and 387, body weight remained relatively unchanged throughout the rest of the experiment. In zebu 379, body weight remained at approximately 120 kg. In zebus 384 and 397, body weight remained at steady levels until 40-60 days prior to death when it gradually declined.

e) Clinical condition.

Zebus 384 and 397 died after challenges 6 and 8 respectively. Zebu 379, which became infected after challenge 5, did not die. Six of the eleven 'controls' died; the remaining five overcame the infection. In all the animals, which died, the disease took a chronic course with anaemia and emaciation as the main symptoms.

Conclusions.

1. Zebu cattle were made resistant to one stabilate of T.congolense, for periods of between 8 and 24 weeks, with a regime of infection with the virulent organisms followed by drug treatment. The resistance was characterized by the absence of trypanosomes, maintenance of a steady body weight and a variant specific antibody titre to the infecting stabilate of 10^2 in the animals concerned.

2. The development of neutralizing antibodies to the infecting stabilate in the 'test' cattle was closely related to parasitaemia. Titres strengthened when circulating trypanosomes

were present and fell when they were removed.

3. It was confirmed that body weight measurements were a good indicator for the stage of the disease process.

CHALLENGE NUMBER	1	2	3	4	5	6	7	8
DAYS AFTER CHALLENGE 1	—	77	154	182	210	238	266	294
BOVINES INOCULATED	test 438 435	test 374 375	test 383 410	216 364	379 353	384 575	387 480	397 593
Log ₁₀ MOUSE I.D. 63's	2.8 ±0.4	2.6 ±0.3	2.8 ±0.4	2.4 ±0.5	2.6 ±0.3	2.4 ±0.5	2.8 ±0.4	2.6 ±0.3

Table 3.40. The dose of trypanosomes infective for mice, which the cattle received at each challenge, and the days after challenge 1 on which the remainder of the challenges were administered.

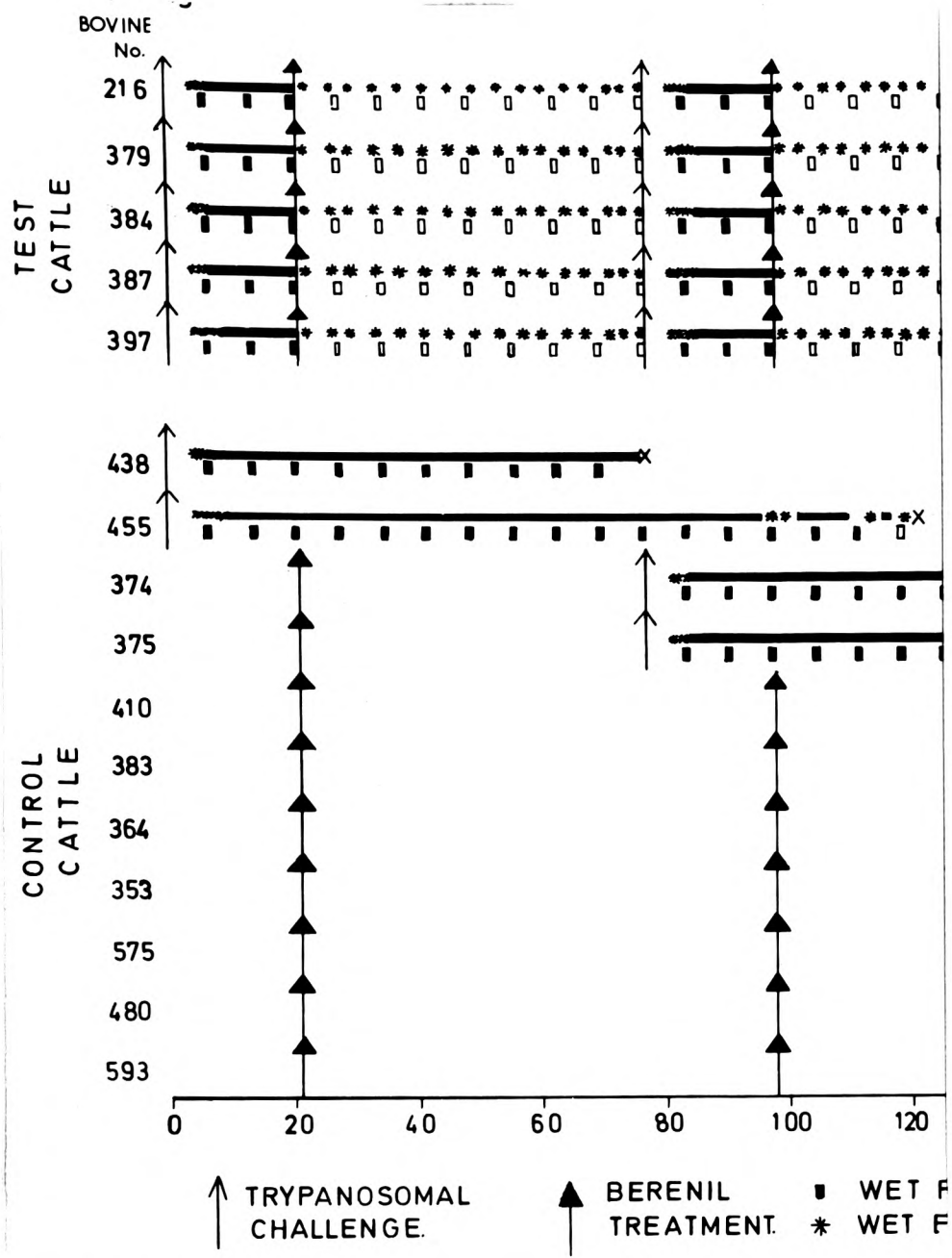


Figure 3.15. The protocol of the experiment, and the STD results from all the infected animals over the period of study.

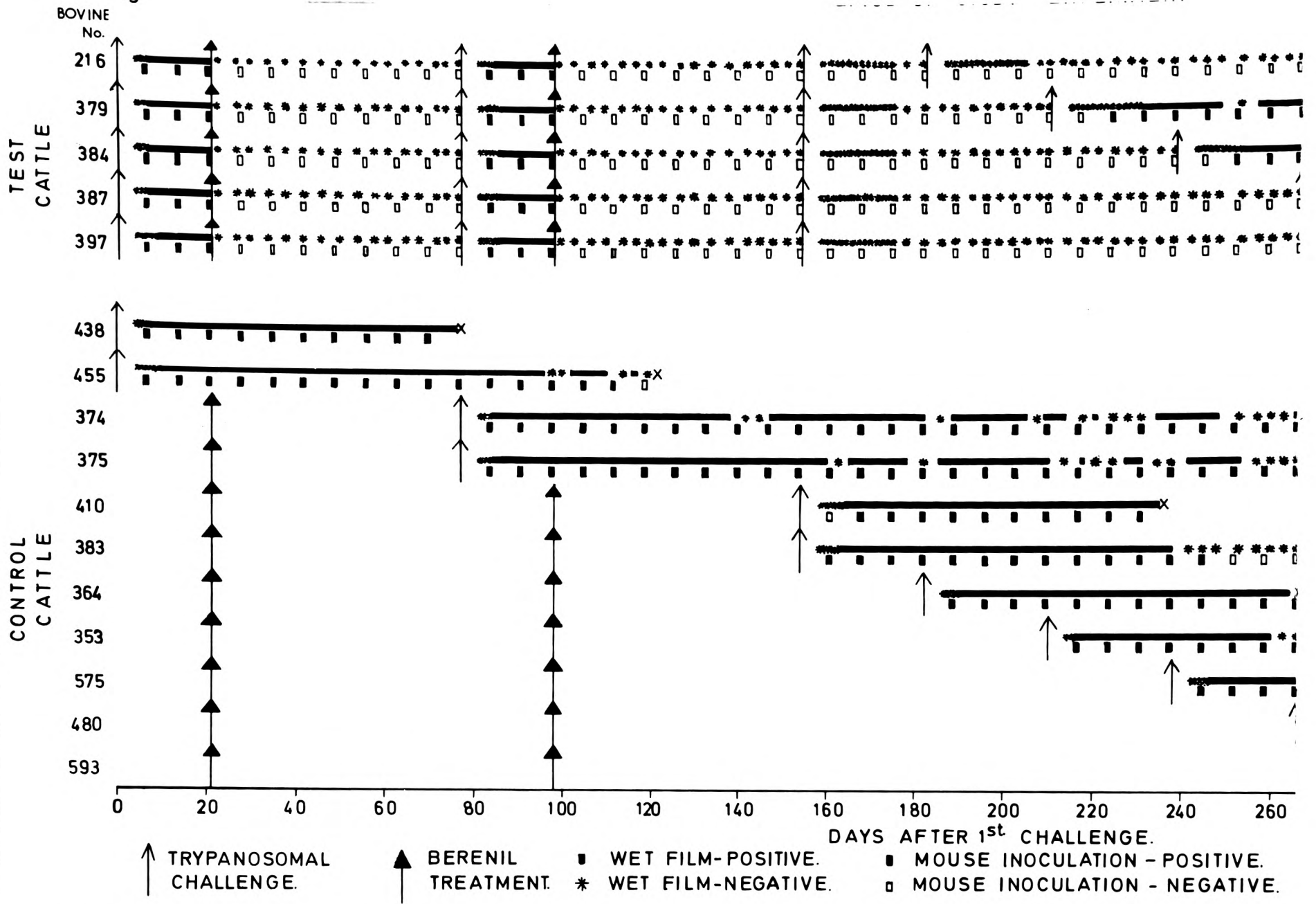
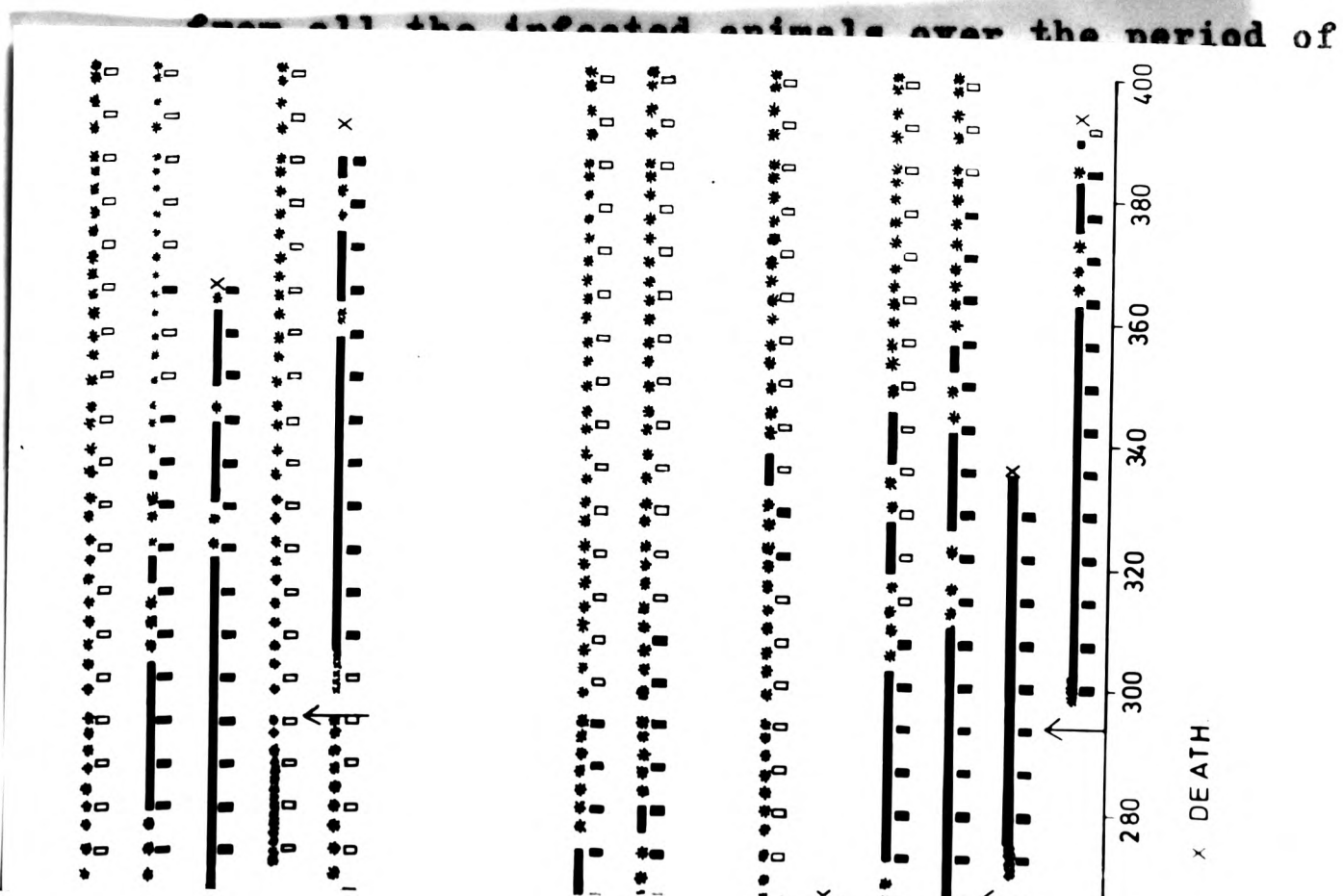


Figure 3.15. The protocol of the experiment, and the STDM results



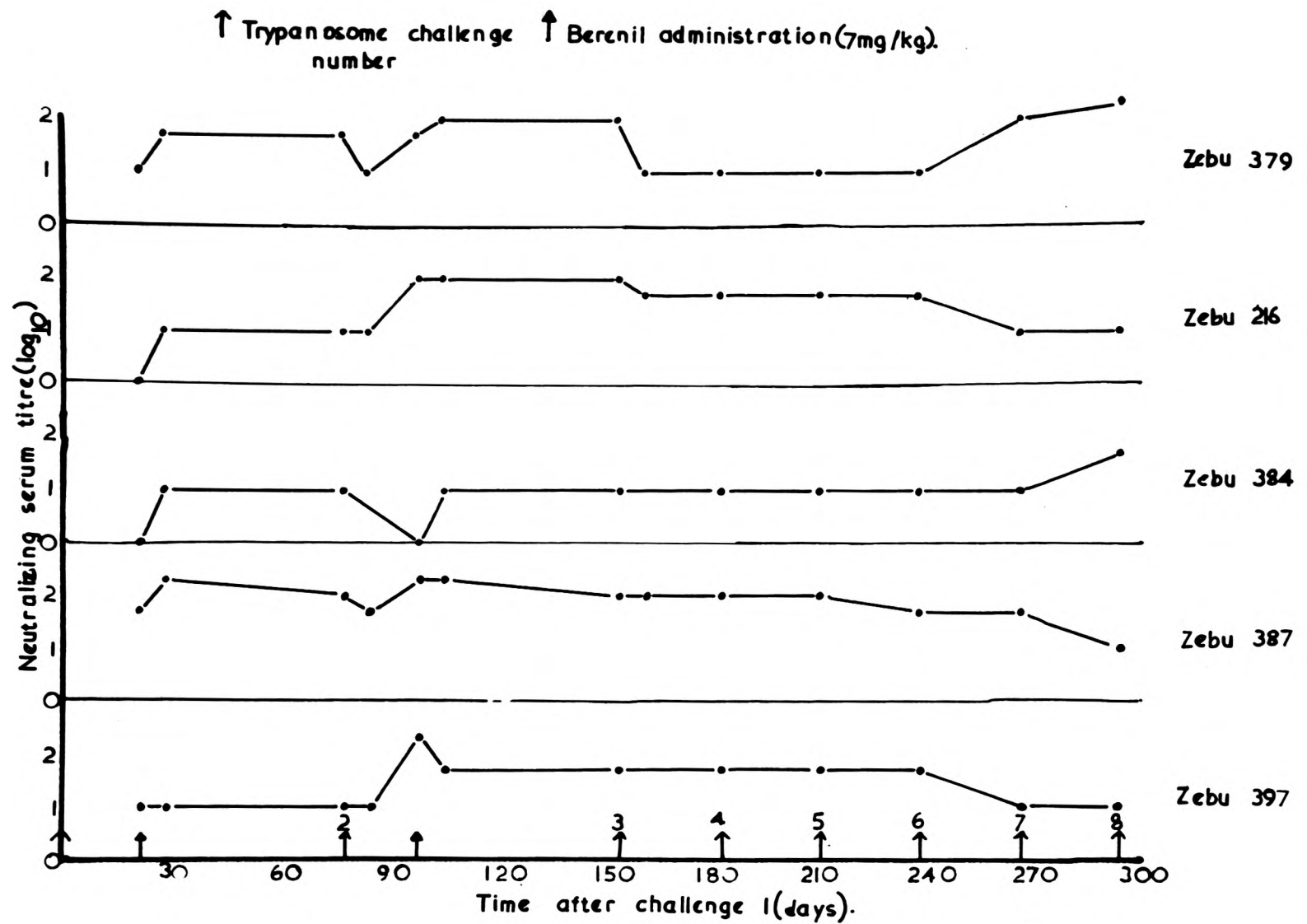


Figure 3.16. Titres of neutralizing antibody, in the 'test' cattle, to EATRO-585/4 antigen, over the experimental period studied.

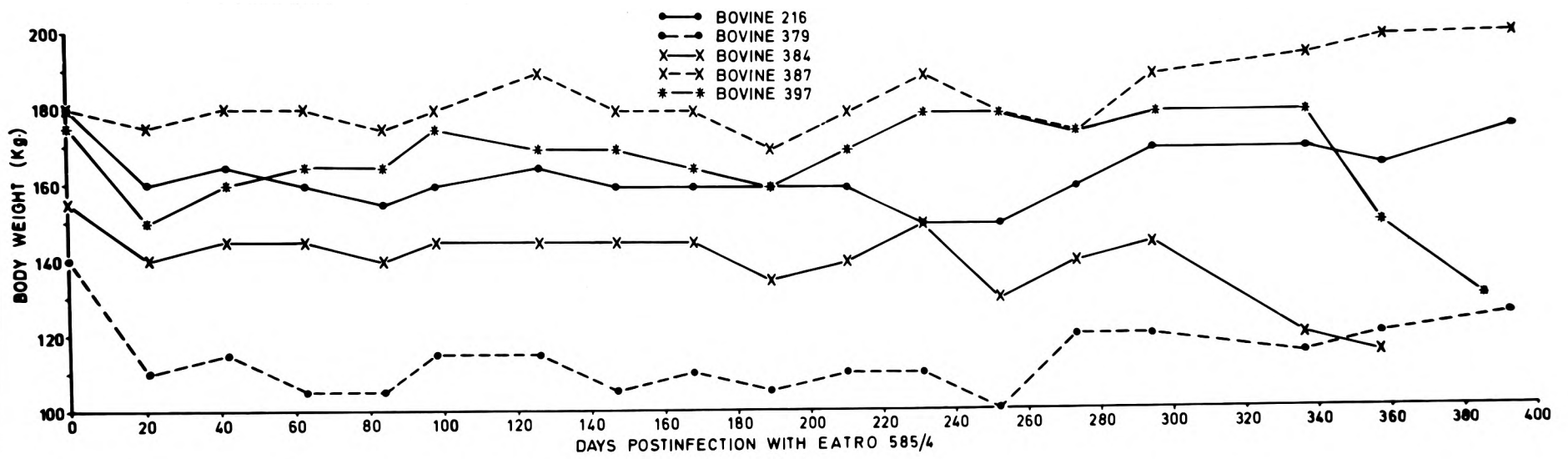


Figure 3.17. Body weight measurements of the 'test' cattle over the period of study.

3.26. An attempt to immunize cattle against the natural disease.

Aim.

In the previous experiment, it was shown that cattle could be made immune to one stabilate of T.congolense for periods of 8 weeks or more. An attempt was thus made to immunize a group of cattle against the natural disease.

Procedure.

Twenty adult zebu cattle were divided into two groups of ten designated 'test' and 'control' respectively. Nine of the 'test' animals had been exposed to the natural disease at Lugala, for between 16 and 36 days followed by berenil treatment, two years previously. The tenth 'test' animal had never been exposed to the disease.

a) Challenge procedure.

The experimental protocol, shown in figure 3.18, was similar to that of section 3.25 except that the infected 'control' cattle were treated with berenil 21 days after each challenge and six challenges were given to the 'test' animals. The challenge material consisted of metacyclic trypanosomes obtained from tsetse flies caught at Lugala. Batches of between six and eight hundred flies, consisting of G.pallidipes, G.fuscipes and G.brevipalpis, were caught off bait animals, brought to EATRO and placed in geigy cages, approximately 100 to a cage. A different batch of flies were caught for each challenge. The flies in each geigy cage were induced to feed into defibrinated bovine blood in a capsule, on three consecutive days. After the flies had finished feeding, the blood from the capsules was

pooled and made up to 45ml by the addition of 2 to 5ml of BS-1. 3ml of the blood was then inoculated intravenously into each of the 'test' animals and two 'controls'. The remaining 9ml was preserved in ampoules. The number of flies used in each of the challenges is shown on table 3.41. The number of flies was less on days 2 and 3 of each challenge due to death at the laboratory. The approximate infection rates of the flies were deduced, by sample dissection, in the flies of challenge 6 only.

The 'test' animals were treated with berenil (7mg/kg) 21 days after challenge 1. Five more challenges were given. Berenil was administered 21 days after challenges 2 to 5 to all the cattle. After challenge 6, five of the 'test' animals (zebus 20, 51, 107, 135 and 921) were given the drug 21 days later, one animal (zebu 263) 26 days later and the remaining four animals were left untreated until the end of the experiment.

The cured, control cattle of challenges 2 to 4 and one uninfected cow were rechallenged with 3ml amounts of the stabilate material prepared from the trypanosomes used to infect them. This challenge was given on three consecutive days at the same time as the 'test' animals received their next challenge.

B) STDM.

The infected animals were examined for trypanosomes at similar times as the 'test' cattle of section 3.25, except that mouse inoculation was carried out at 2-weekly intervals between berenil treatment and the next challenge.

c) Trypanosome identification.

The species of infecting trypanosome were identified from stained thin films of jugular blood from infected cattle and from films of tail from positive mice.

d) Clinical observations.

The clinical condition of each infected animal was noted throughout the experiment.

Results.

a) Trypanosome detection and identification.

Figure 3.18 and table 3.42 show the STDM and stained thin film results respectively. Some of the 'test' animals became infected after each challenge with one or more of the three main groups of African pathogenic trypanosome. Zebus 20, 51, 107, 135, 263 and 921 did not resist any of the challenges; zebu 132 resisted challenge 1 only; zebu 138 resisted challenges 2, 3 and 5; zebu 251 resisted challenges 1, 2, 3 and 5; and zebu 264 resisted challenges 1 and 5. Brucei sub-group was rarely detected. Vivax and congolense groups were much more common and appeared in some of the animals of each challenge. In some challenges, vivax group was more common(challenge 1); in others congolense group was more common(challenge 6). In some animals, brucei, vivax and congolense groups were detected together, while in others they appeared alone when challenged with the same material(challenge 4).

Figure 3.19 shows the results of the rechallenges in the control cattle used in challenges 2 to 4. A sterile protective immunity was produced in four(zebus 360, 572, 573 and 517) of the

six animals, as judged by the failure to detect trypanosomes by STDM.

b) Clinical observations.

The clinical condition of all the infected cattle, 21 days after challenges 1 to 5, was poor. Weakness, extreme emaciation and anaemia were the common symptoms. After challenge 6 however, the two controls, zebus 583 and 475 were moribund after 21 days; zebus 20, 51, 107, 135, 263 and 921 were in poor condition but zebus 132, 138, 251 and 264 were in good condition. The clinical condition of the last named four animals did not deteriorate for the 50 days following challenge 6 although trypanosomes were detected daily in the jugular blood. Photographs 3.6 and 3.7 show the clinical differences between zebus 132, 138, 251 and 264, and the remaining six 'test' cattle on the 400th day of the experiment.

Conclusions.

1. The trypanosomal challenge was considered to be heavy as judged by the poor clinical condition of infected cattle 21 days after each challenge. The irregular appearance of trypanosomes in cattle infected with the same challenge indicated either some parasitic competition mechanism or differing host susceptibilities.

2. Two distinct immune mechanisms were shown to operate. A non-sterile immunity developed in four of the 'test' cattle after challenge 6. A sterile immunity developed in four of the rechallenged control cattle.

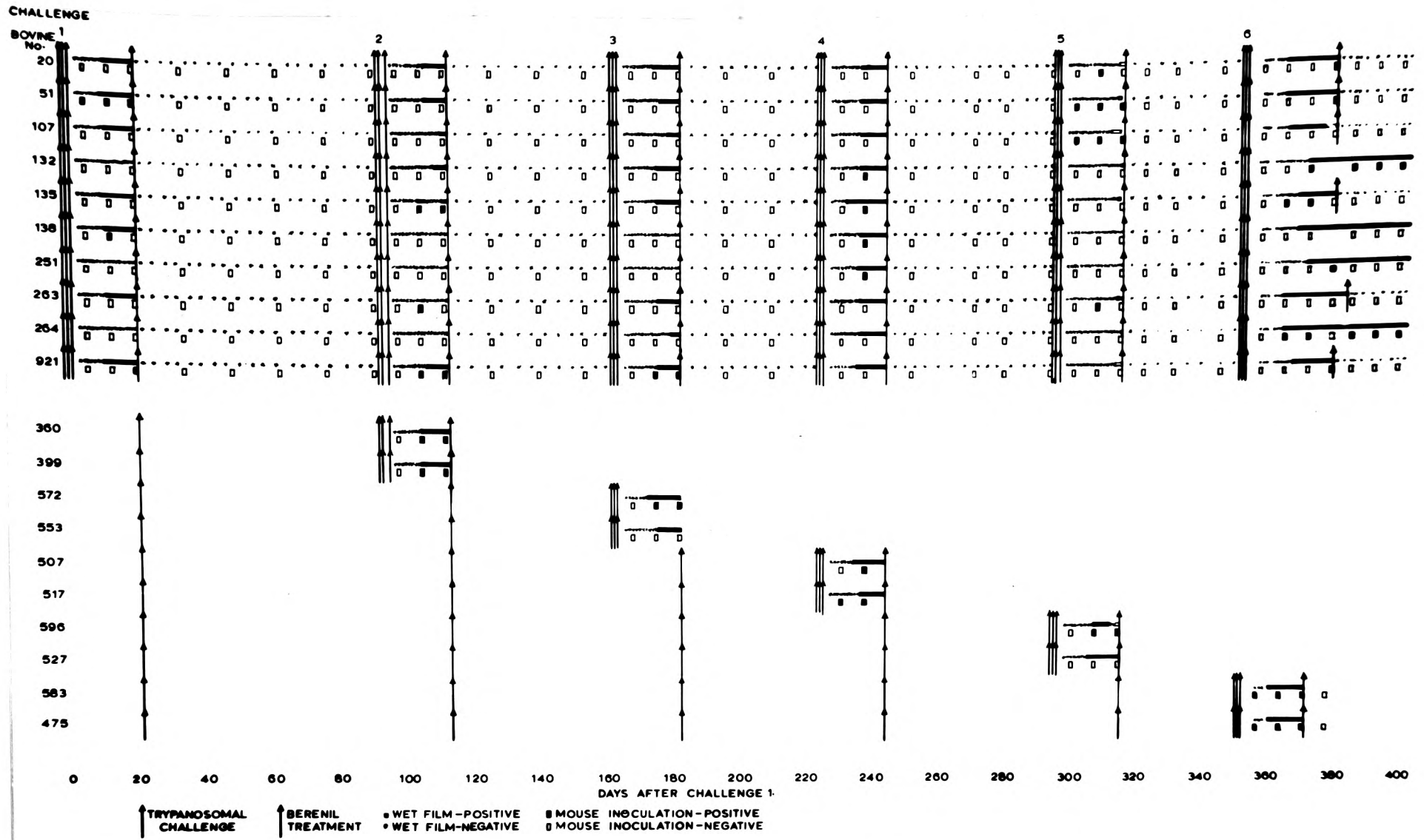


Figure 3.18. The protocol of the experiment and the STDM results of all the infected cattle, until day 400 after challenge 1.

CHALLENGE NUMBER	DAYS OF CHALLENGE			SPECIES OF TSETSE	(%) APPROXIMATE INFECTION RATE	
	1	2	3		♀	♂
1	350	187	157	pallidipes	—	
	432	305	267	fuscipes		
	85	59	30	brevipalpis		
2	300	160	131	pallidipes	—	
	335	246	234	fuscipes		
	—	—	—	brevipalpis		
3	233	203	196	pallidipes	—	
	587	525	513	fuscipes		
	—	—	—	brevipalpis		
4	526	488	434	pallidipes	—	
	369	356	330	fuscipes		
	74	69	56	brevipalpis		
5	476	327	290	pallidipes	—	
	58	52	50	fuscipes		
	81	57	45	brevipalpis		
6	517	472	471	pallidipes	17.5	26.5
	307	302	298	fuscipes	21.1	22.6
	228	204	185	brevipalpis	—	3.3

Table 3.41. Numbers and species of tsetse fly used at each challenge, and approximate infection rates of challenge 6 only.

BOVINE NUMBER	C H A L L E N G E N U M B E R					
	1	2	3	4	5	6
20	vivax	vivax congolense	congolense	vivax congolense	vivax congolense	congolense
51	vivax congolense	vivax	congolense	congolense	brucei vivax	vivax congolense
107	vivax	vivax	vivax congolense	vivax	brucei congolense	congolense
132	—	brucei congolense	vivax	vivax congolense	vivax	vivax congolense
135	vivax	vivax congolense	congolense	brucei vivax congolense	congolense	congolense
138	congolense	—	—	vivax	—	congolense
251	—	—	—	congolense vivax	—	congolense
263	vivax	brucei congolense	vivax congolense	vivax brucei	congolense	vivax
264	—	—	vivax	vivax congolense	—	congolense
921	vivax congolense	brucei vivax congolense	congolense	brucei vivax congolense	vivax	congolense

Table 3.42. The organisms, which were identified,
in all the infected 'test' cattle after
each of the challenges.

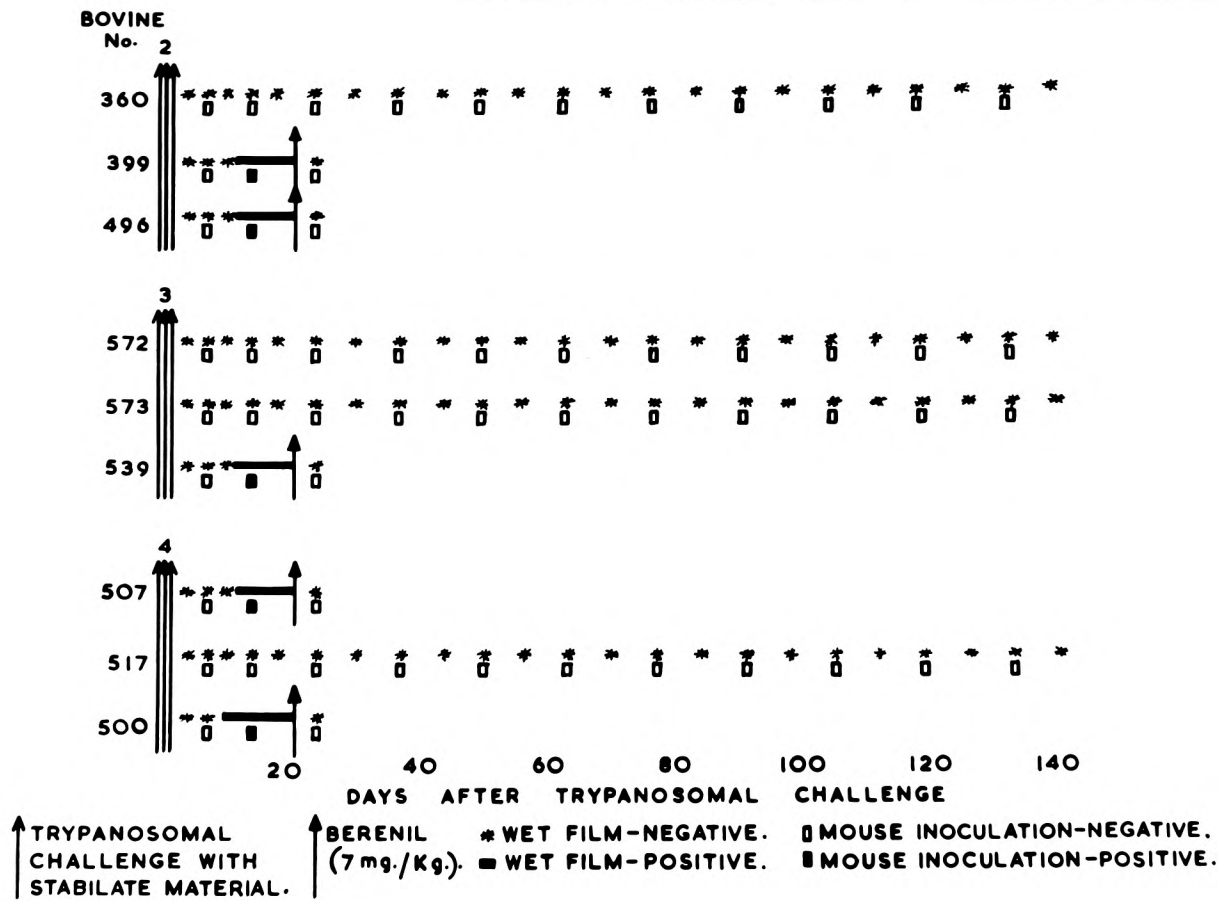


Figure 3.19. The protocol of the regime used to rechallenge the control cattle, and their STD results.

3-96.



Photograph 3.6. Clinical condition of zebus 132, 138,
251 and 264 on day 400 after challenge 1.



Photograph 3.7. Clinical condition of zebus 20, 51,
107, 135 and 263 on day 400 after
challenge 1.

327. Studies on common antibody production in cattle infected with T.congolense, T.vivax and brucei sub-group organisms.

Aim.

Immunofluorescence tests have been used to aid the diagnosis of bovine trypanosomiasis using brucei sub-group antigen only(354). The relationship between the presence of antibody, its titre and the presence or absence of trypanosomes in hosts has not been followed in detail. The following experiments were designed to determine this relationship.

Procedure.

a. Cattle.

Nine groups of cattle, shown in table 3.43, were used. The animals of groups 1 to 3 were also used in sections 3.20, 3.21 and 3.25; those of groups 5 in section 3.25; those of group 8 in section 3.19; and those of group 9 from section 3.26. The history of the animals of groups 6 and 7 was obtained from the EATRO serum bank. The animals of group 4 were infected specifically for this experiment and were treated with berenil(7.5mg/kg) early in the infection. The animals of groups 1 to 5 were infected with T.congolense, groups 6 to 8 with brucei sub-group and group 9 with all three groups of pathogenic trypanosome.

b. STDM.

The findings of STDM carried out on all the animals were obtained from the sections mentioned above with the exception of the animals of group 4. Blood was collected regularly from the animals in this group until day 160 postinfection and examined for the presence of trypanosomes.

c. Serology.

Sera, collected throughout the infection from the animals in all the groups, were used in IFT-A tests. Sera from groups 2 to 5 were tested against T.congolense antigen only, sera from group 8 against a brucei sub-group antigen only and sera from groups 1,6,7 and 9 against T.congolense, T.vivax and brucei sub-group antigens.

Results.

a. Relationship between the pattern of homologous common antibody production and results of the STDM.

Figure 3.20 shows the relationship between homologous common antibody titre and the presence of trypanosomes in zebus 343 and 353(group 2). Similar figures were drawn from the results obtained from all the cattle studied. On many occasions, the antibody titre continued to rise when no trypanosomes could be detected.

The times after infection when common antibodies to the homologous antigen were first detected, at a titre of 1/40, in 37 of the 38 cattle are shown on figure 3.21. In most of the animals, the antibodies were present at this titre between 8 and 21 days postinfection.

The patterns of antibody production in the different groups of animals are shown on figures 3.22 to 3.29. In the infected, untreated animals(groups 1to3,6 and 7), antibody levels to the homologous antigen only increased in the presence of trypanosomes. Although fluctuations in titre occurred, the amount of antibody steadily increased to an upper level of titres of

between 1/80 and 1/1,280. This level, which occurred after approximately 100 days of infection, was maintained until death (groups 1 and 7) and until the end of the experiment in the chronically infected animals (group 2). The level gradually decreased however after the disappearance of the trypanosomes in the animals which cured themselves (groups 3 and 6) and in the animals which were treated with berenil (groups 4, 5, 8 and 9). In two of the animals (zebus 384 and 530), a poor antibody response was noted as their serum antibody titres rarely rose above 1/40 during the patent infection.

The time in days for the titres to fall to 1/40 after the removal of the trypanosomes in the self-cure and drug-treated animals is shown in figure 3.30. These animals were divided into three groups, namely, those treated early in the infection, those known to have been infected two years previously, and the animals which cured themselves late in the infection. The mean times for the titres to fall to the 1/40 level were different in each group being 30.6, 58.1 and 110.0 days respectively.

b. Pattern of heterologous common antibody production.

Figures 3.31 and 3.32 show the results of IFT-A tests using sera, obtained from cattle known to be infected with either T.congolense or brucei sub-group against T.congolense, T.vivax and brucei sub-group antigens. Titres to the homologous antigen were always much higher. Titres to the heterologous antigens rarely exceeded 1/40.

The results of tests using sera collected after

challenges 1,5 and 6, in the test cattle of section 3.26 (this section group 9), against the three antigens are shown on figure 3.29. The group of infecting trypanosome detected in each animal by STDM (see table 3.42 in section 3.26) is also noted in this figure. In most animals, a much stronger homologous antibody titre was detected. Heterologous antibodies either could not be detected or were detected in low titres.

Conclusions.

1. Rising homologous common antibody titres or high titres which were maintained in cattle were associated with patent trypanosomiasis. Steadily falling titres were associated with the removal of trypanosomes from the host by chemotherapy or self-cure.

2. The failure to detect trypanosomes by STDM was insufficient evidence to conclude that animals were not harbouring trypanosomes. On many occasions, in the 38 cattle studied, trypanosomes could not be detected by STDM when the homologous common antibody titre continued to rise.

3. The development of much weaker heterologous common antibody responses confirmed the findings of section 3.19 that all three group antigens must be used when testing unknown samples. By the criterion of serum titre, the infecting group of pathogenic trypanosome was detected, in many instances, in the cattle of group 9. These cattle had received trypanosomal challenge consisting of all three groups of trypanosome (see section 3.26).

GROUP No	No. OF BOVINES USED	BOVINE Nos	TRYPANOSOME INFECTION	CHEMOTHERAPUTIC TREATMENT (if any)	RESULT
1	5	530 435 498 455 446	EATRO - 189/1 EATRO - 585/4	—	Death
2	5	343 353 383 492 555	EATRO - 585/40	—	Chronic infection
3	1	495	EATRO - 585/1	—	Self-cure
4	5	244 507 511 572 573	EATRO - 585/4	Berenil (7mg/Kg) EARLY IN INFECTION	Cure
5	5	216 379 384 387 397	EATRO - 585/4 (2 challenges)	Berenil (7mg/Kg) (2 treatments)	Cure
6	4	4 480 616 840	EATRO - 3	—	Self-cure
7	1	612	EATRO - 3	—	Death
8	2	496 539	EATRO - 2/1/4	Berenil (7mg/Kg) EARLY IN INFECTION	Cure
9	10	20 138 51 251 107 263 132 264 135 921	Natural infection (repeated challenges)	Berenil (7mg/Kg) (repeated treatment)	

Table 3.43. The numbers, type of infection, chemotherapeutic treatment(if any) and results of all the cattle used.

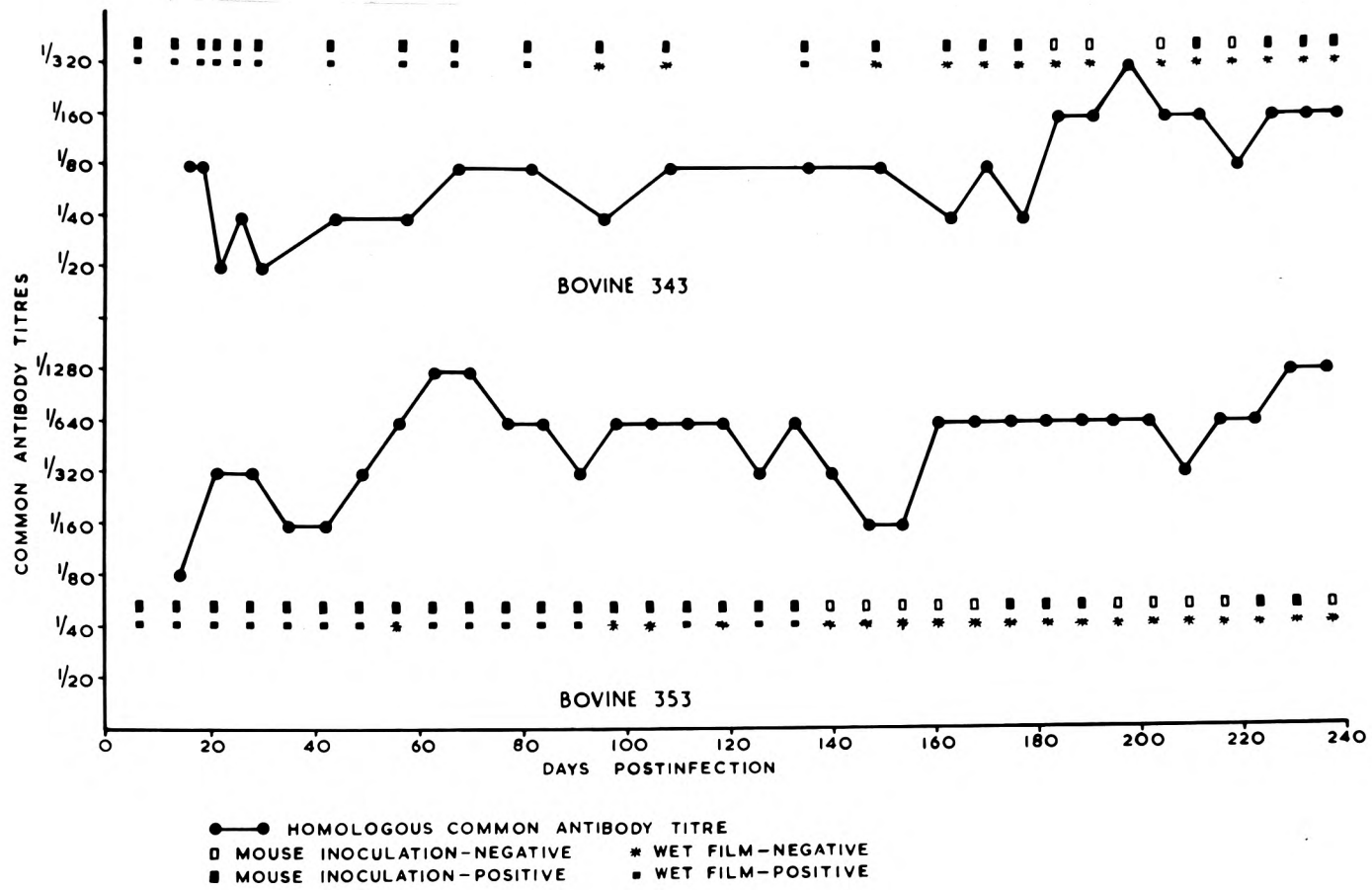


Figure 3.20. Titres of common antibody to the homologous antigen and STD results during the first 240 days of infection in zebus 343 and 353.

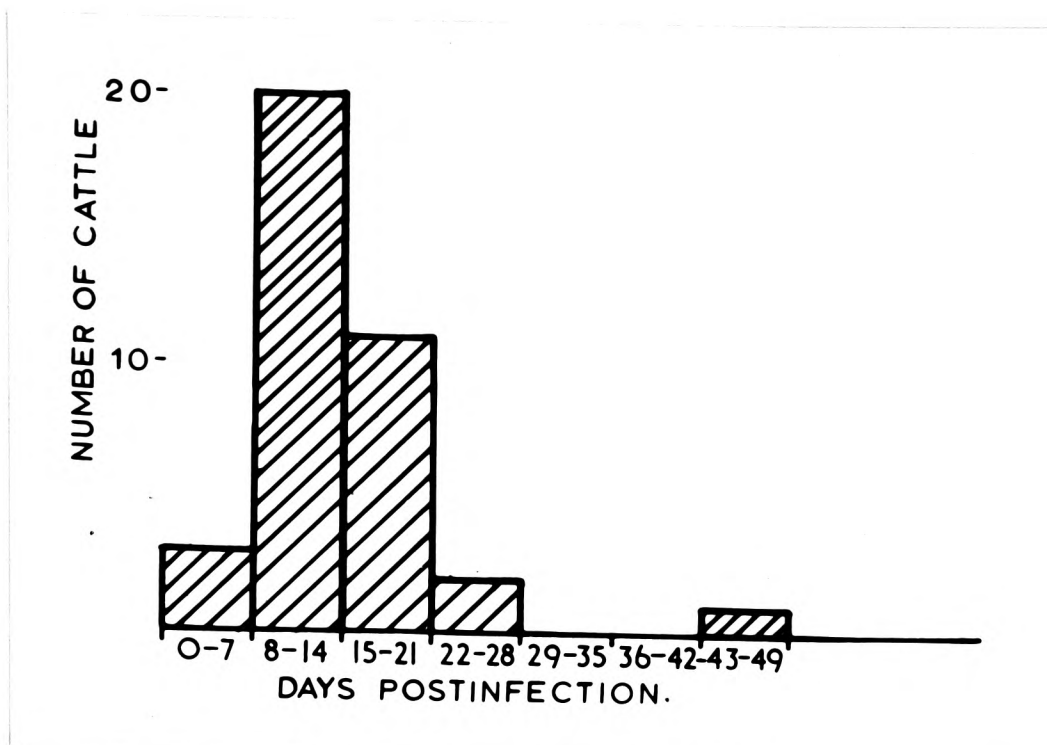


Figure 3.21. Histogram to illustrate when common antibodies to the homologous antigen were first detected, at a titre of 1/40, in 37 of the cattle used.

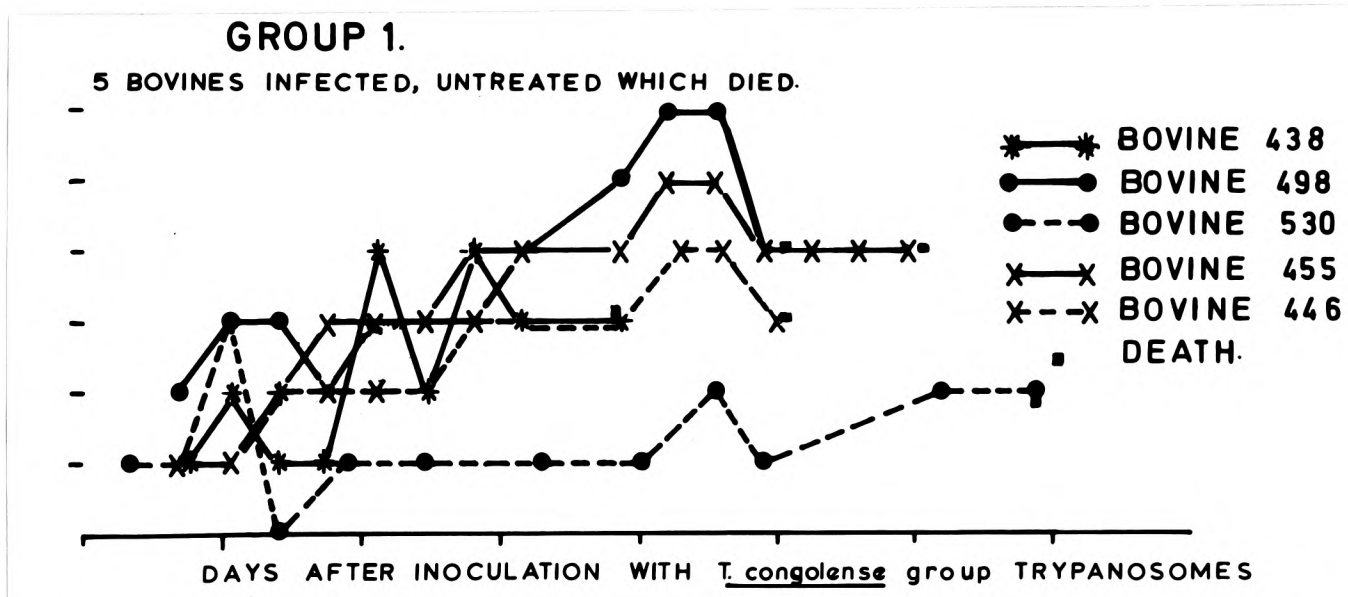


Figure 3.22. Homologous common antibody titres of the sera, obtained at regular intervals throughout the infections, in the cattle of group 1. Each demarcation denotes 20 days postinfection.

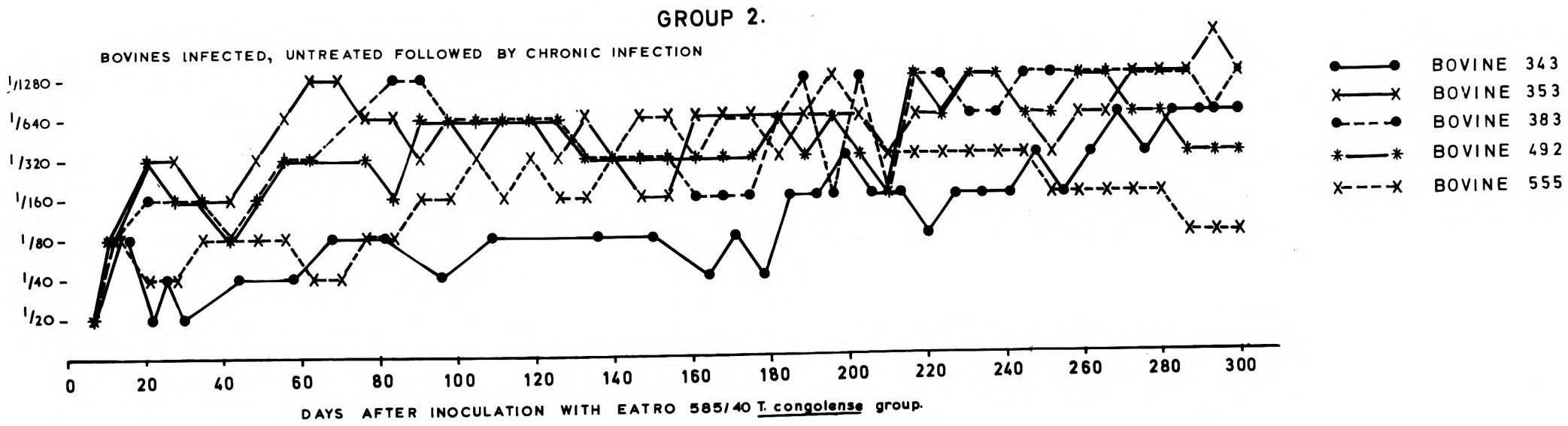


Figure 3.23. Homologous common antibody titres of the sera, obtained at regular intervals until day 300 post-infection, in the cattle of group 2.

GROUP 3.

1 BOVINE INFECTED, UNTREATED FOLLOWED BY SELF-CURE.

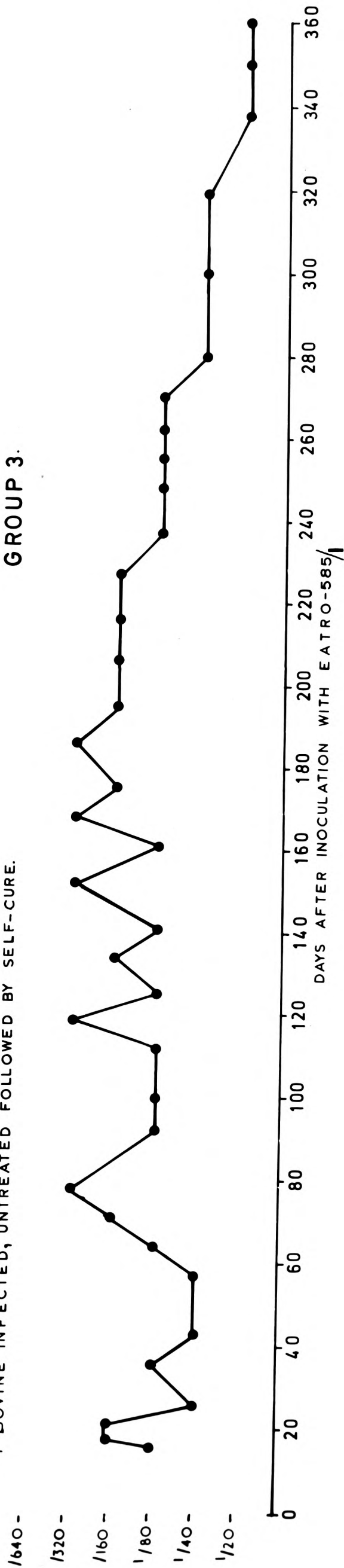


Figure 3.25. Homologous common antibody titres of the sera, obtained at regular intervals throughout the infection, in zebu 495 which cured itself.

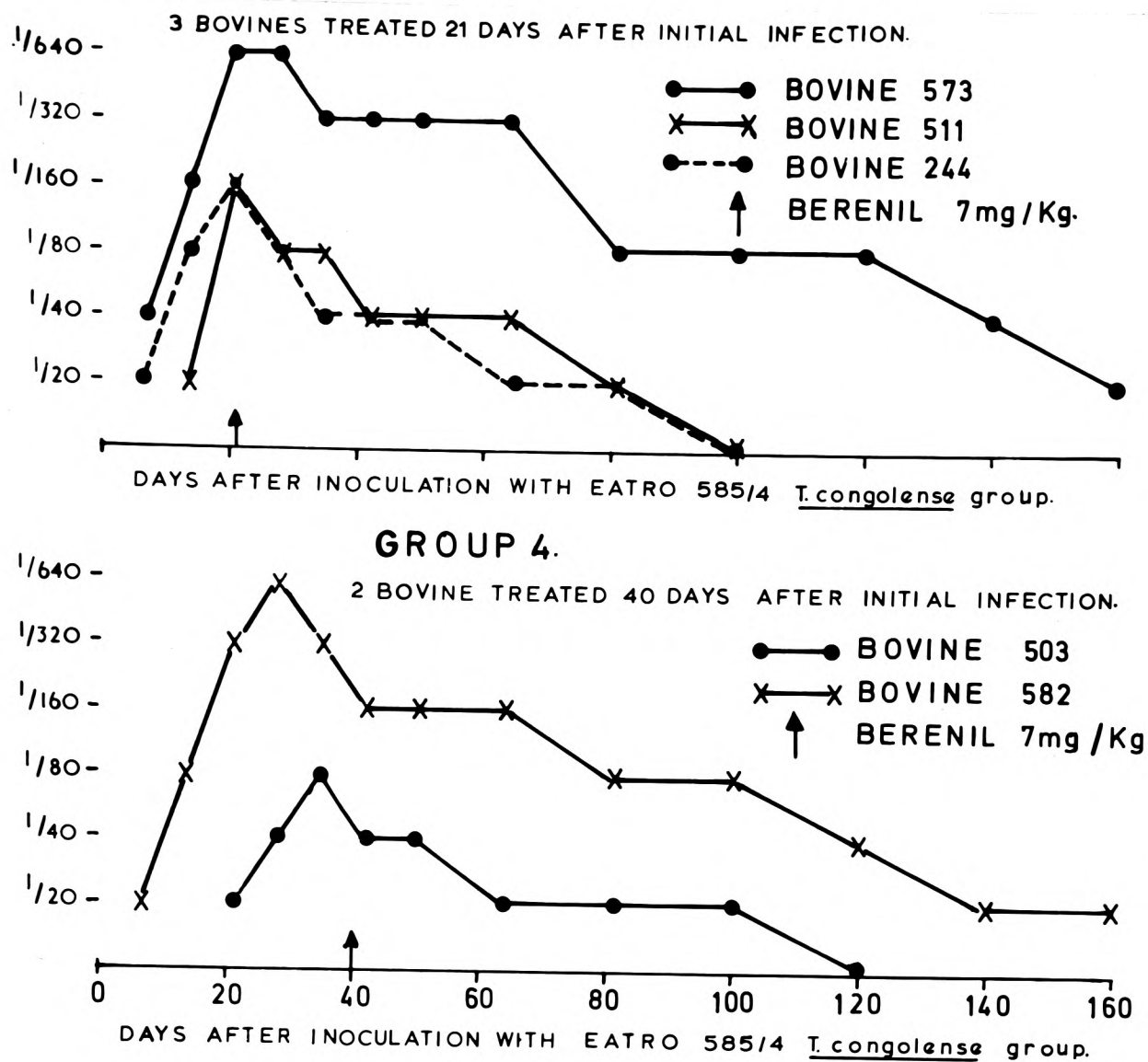


Figure 3.24. Homologous common antibody titres in the sera, obtained at regular intervals until day 160 post-infection, in the cattle of group 4.

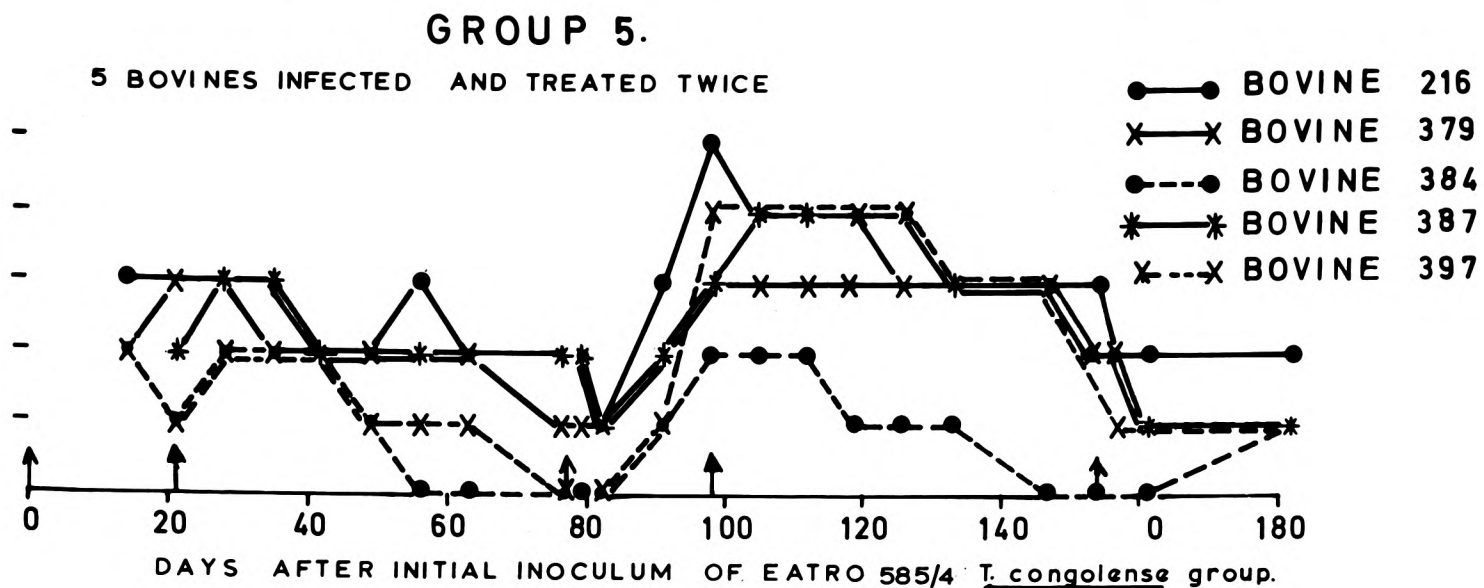


Figure 3.26. Homologous common antibody titres of the sera, obtained at regular intervals until day 180 post-infection, in the cattle of group 5.

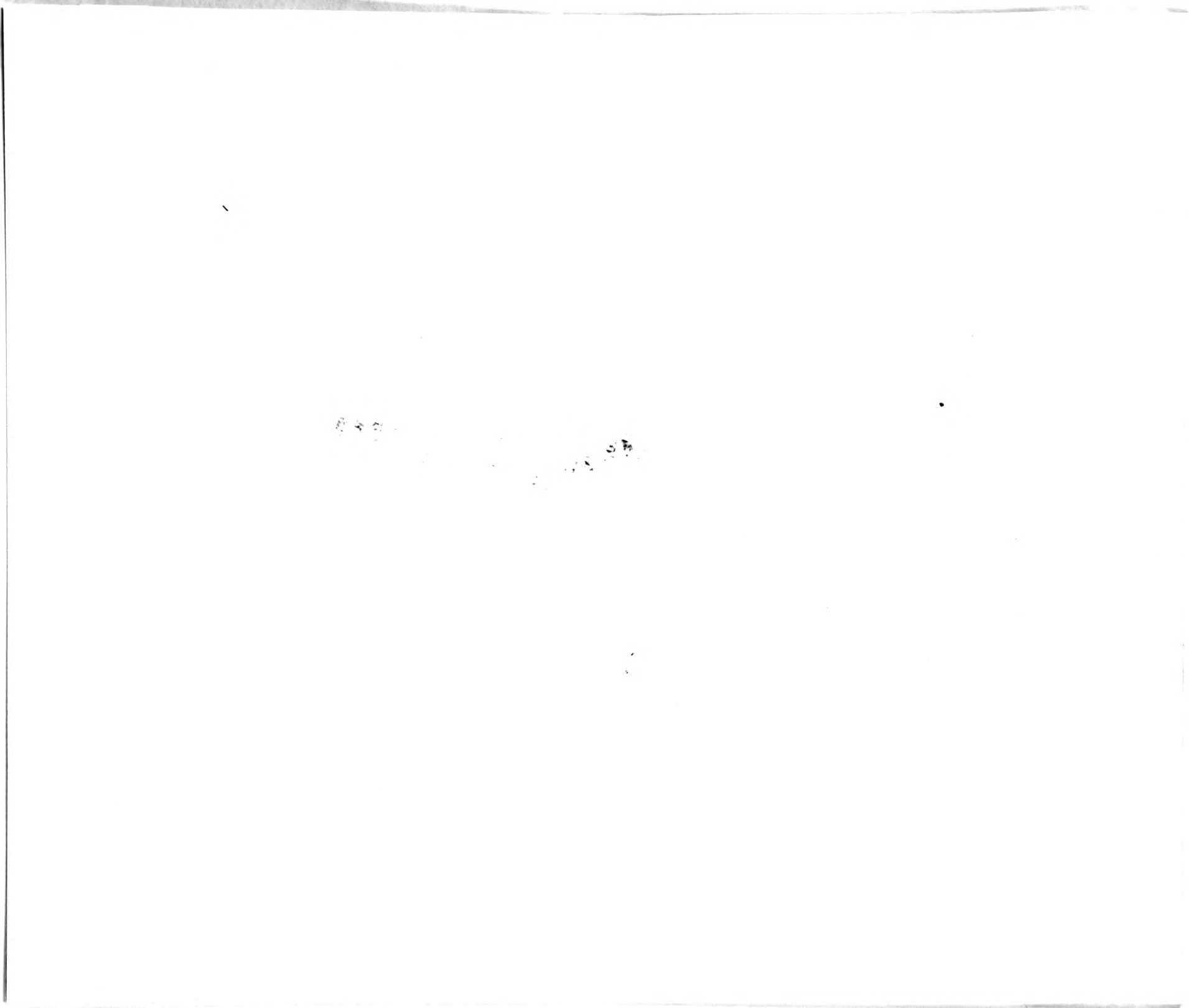
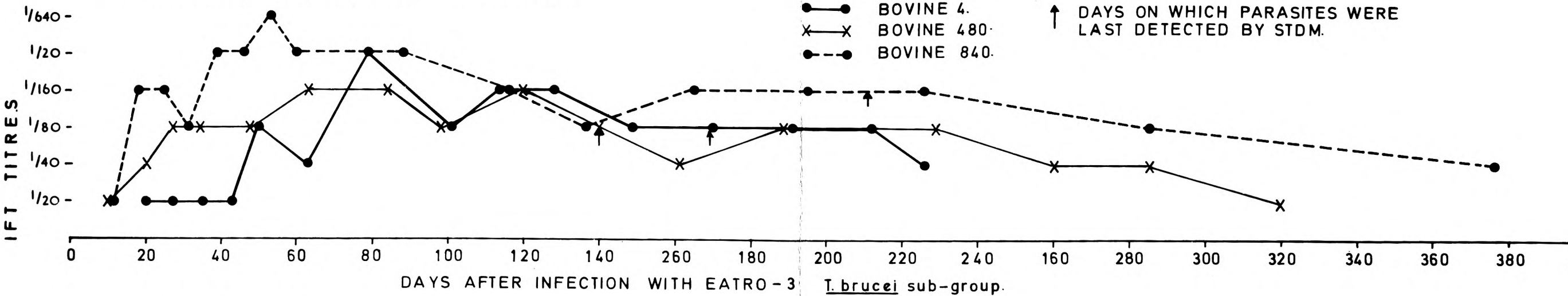


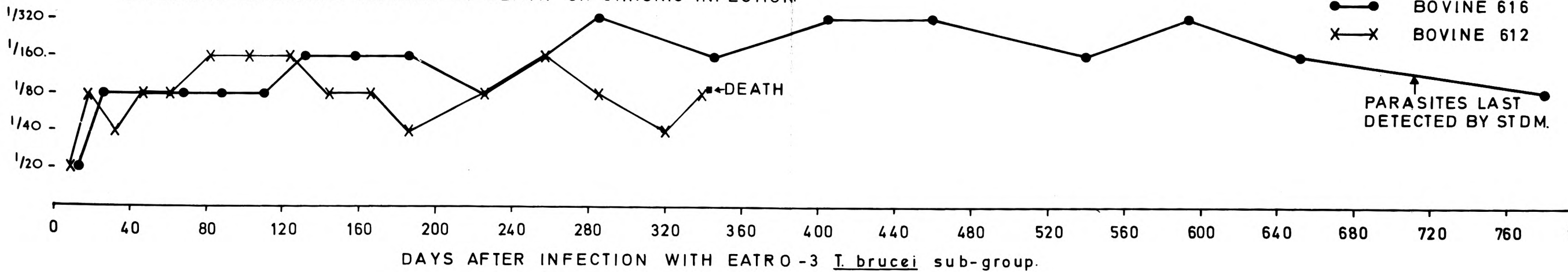
Figure 3.27. Homologous common antibody titres of the sera, obtained at regular intervals throughout the infections, in the cattle of groups 6 and 7.

3 BOVINES INFECTED FOLLOWED BY SELF-CURE

GROUPS 6 and 7



2 BOVINES INFECTED FOLLOWED BY DEATH OR CHRONIC INFECTION.



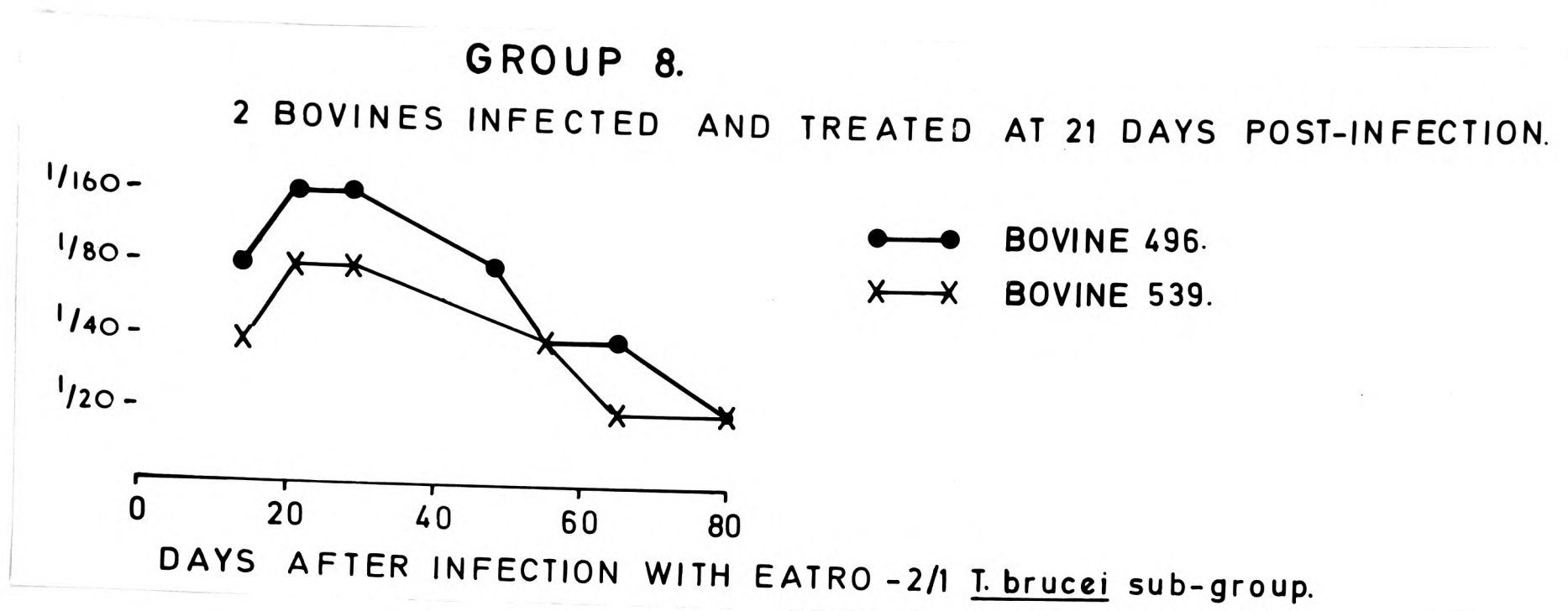


Figure 3.28. Homologous common antibody titres of the sera, obtained at regular intervals until day 80 post-infection, in the cattle of group 8.

●—● ANTIBODIES TO T. VIVAX GROUP
 ●—● ANTIBODIES TO T. CONGOLENSE GROUP
 X—X ANTIBODIES TO T. BRUCEI SUB-GROUP

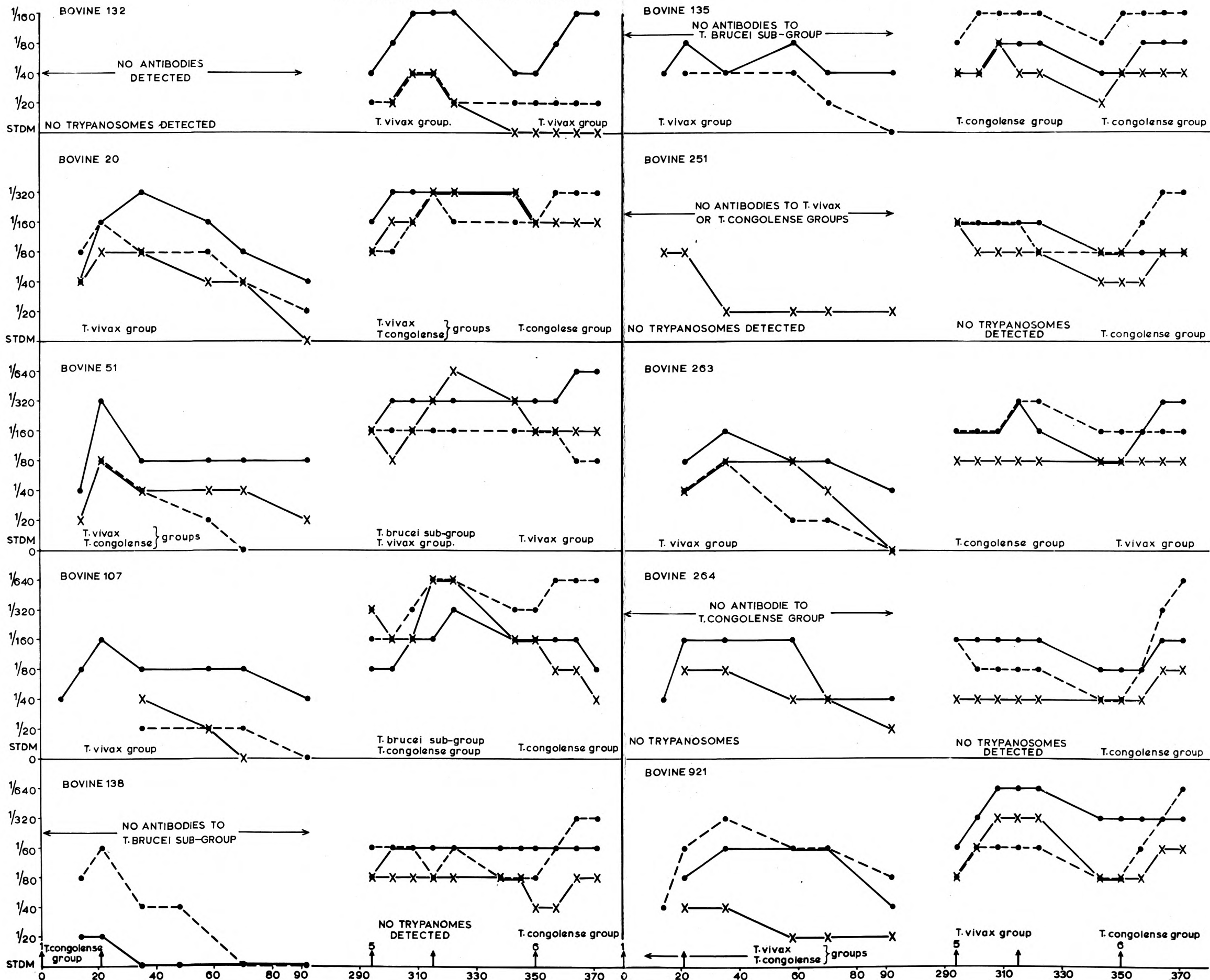


Figure 3.29. Titres of homologous and heterologous common antibodies of the sera collected from the cattle of group 9, and the group of infecting trypanosome in the same animals.

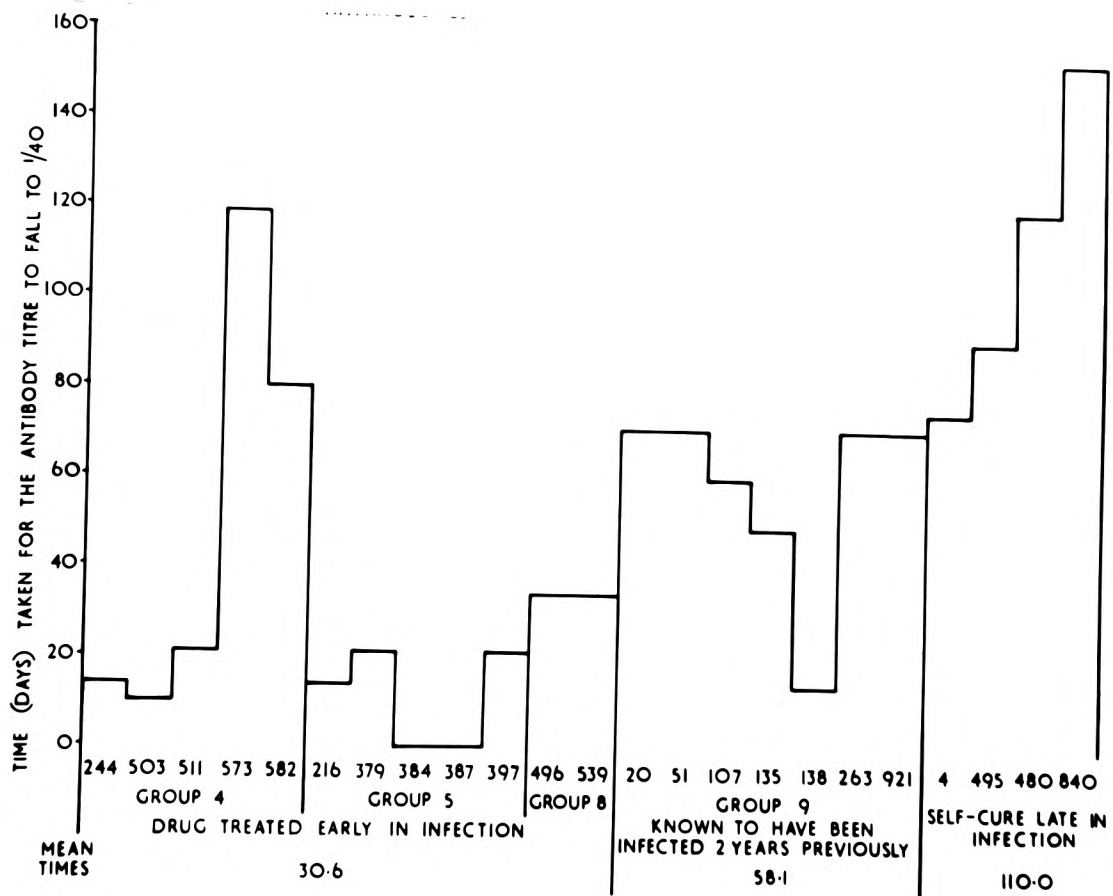


Figure 3.30. The time taken for the homologous common antibody titre to fall, to a titre of 1/40, in the drug-treated and self-cured animals.

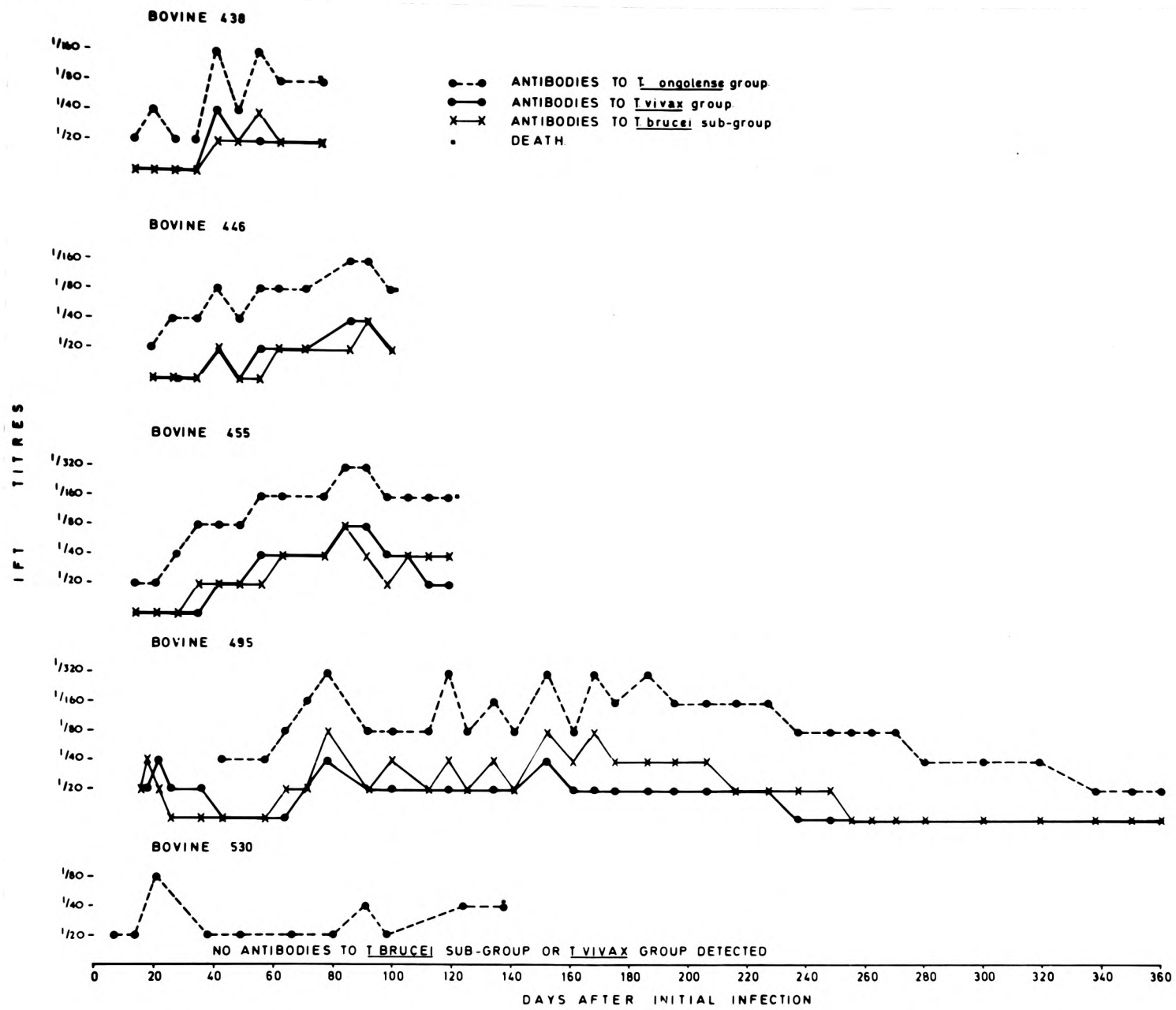


Figure 3.31. Titres of homologous and heterologous common antibody in sera collected throughout the infection from five cattle infected with T. congolense.

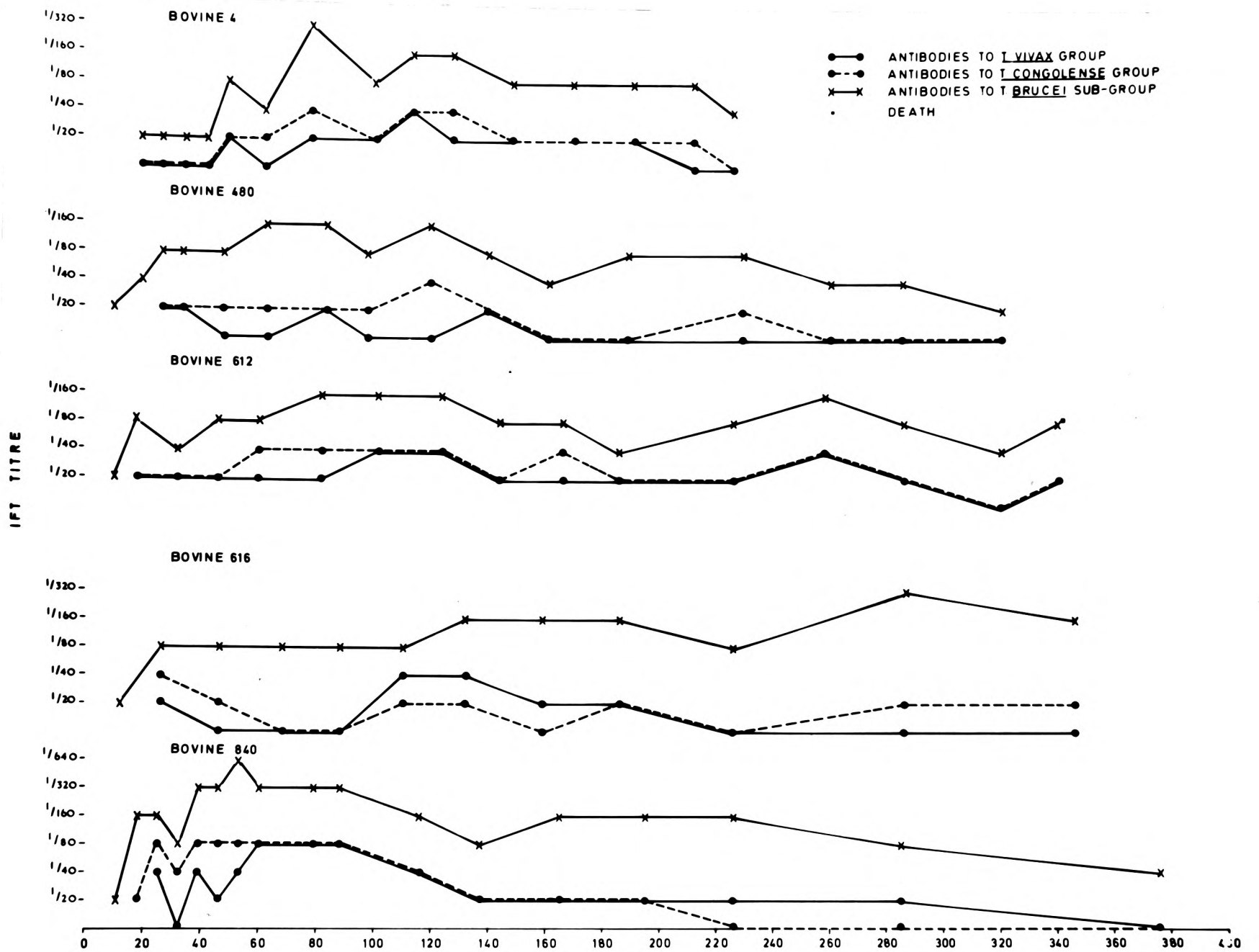


Figure 3.32. Titres of homologous and heterologous common antibody in sera collected throughout the infection from five cattle infected with brucei sub-group.

3.28. Evaluation of the indirect fluorescent antibody and agglutination tests as serological aids for the diagnosis of bovine trypanosomiasis.

Aim.

If the indirect fluorescent antibody tests were to be of value in the serodiagnosis of bovine trypanosomiasis, the findings of section 3.27 should be of use in the analysis of materials obtained from cattle in the field. A study of the relationship between common antibody titre and the detectable presence of trypanosomes in cattle sampled from two areas was thus undertaken. A comparison of the detection of serological positives by the agglutination and indirect fluorescent antibody tests was also carried out to ascertain which was the most efficient.

Procedure.

a) Areas.

Cattle were surveyed for the presence of trypanosomiasis in two distinct areas of eastern Uganda. In the first, in and around Amudat (see map in section 2.13), the disease was present in low levels. Fairclough (114) reported the presence of G. pallidipes in very low numbers at Kunyao and the local veterinary authorities did not consider the disease to be a problem. In the second area, at Lumino, the disease was endemic and considered to be a severe problem.

b) STDM.

Blood was collected from 387 cattle in the Amudat area and 80 cattle at Lumino. Wet and thick films were prepared and blood was inoculated into two mice. The wet films were

examined immediately. The thick films and the tail blood from the inoculated mice were examined in the laboratory.

c) Serology.

Sera and blood spots were collected from each animal. IFT-B tests were carried out using all the blood spots and brucei sub-group, T.congolense and T.vivax antigens. IFT-A tests were then conducted using the sera from animals whose blood spot gave positive fluorescence. Agglutination-A tests were conducted using all the sera collected against an antigen, which contained the six major antigenic types of the brucei sub-group detected by Cunningham and Vickerman(75).

Results.

Table 3.44 shows the percentage of trypanosome and serological positives detected in the animals from the two areas. Many more positives were detected at Lumino(11.25% by STDM, 70% by IFT-B) than at any of the locations in the Amudat area, where the maximum number of positives was detected at Kunyao(1.6% by STDM, 39.7% by IFT-B). The agglutination test only detected positives at Karita and Lumino, 1.2% and 47.5% respectively. Trypanosomes were present in the cattle sampled at Kunyao and Lumino only. In both the last named areas, the number of serological positives greatly exceeded the number of trypanosome positives.

Table 3.45 shows the titres of common antibodies, as detected by IFT-A, in all the cattle shown to be serologically positive by IFT-B or trypanosome positive by STDM. In all the animals detected positive by STDM, with the exception of Kunyao

number 17, the antibody titres to one or more of the antigens were 1/80 or above. In Kunyao number 17, no antibodies were detected. In the remaining animals, which were STDM negative, antibody titres varied from 1/80 to 1/1,280.

Table 3.46 shows the methods which detected the trypanosomes in the 10 animals detected positive by STDM. T.vivax was only detected microscopically, the brucei sub-group regularly by mouse inoculation and T.congolense by all the methods.

Conclusions.

1. The STDM results confirmed earlier findings (81,153, 154) that T.vivax could only be detected microscopically, brucei sub-group by rodent inoculation and T.congolense by a combination of both methods.

2. The agglutination test was not as efficient as the IFT-B test in detecting serological positives. However, the test is probably of value in ascertaining the importance of brucei sub-group as the infecting organism. Cattle in the Lumino area were probably much more liable to infection with these organisms than cattle in any of the Amudat locations.

3. The IFT-B test showed which of the animals had common antibodies to pathogenic trypanosomes. It was of use as a screening procedure as it eliminated many negative animals. For a more detailed serum examination, the IFT-A test should be used.

4. The 467 cattle examined could be divided into four groups, based on the findings of section 3.27.

Group 1. One cow, Kunyao number 17, which was considered to have an early infection, as although T.congolense was detected it was negative by IFT-B.

Group 2. Nine cattle from Lumino, which were considered to have had the disease for at least 2 months as they were trypanosome positive and had common antibody titres of 1/80 or above to one or more of the antigens.

Group 3. Forty-two cattle, shown on table 3.45, which were considered to be either cryptic infections or recovered animals as although no trypanosomes could be detected their serum common antibody titres were 1/80 or above.

Group 4. The remaining 415 animals, which did not have the disease as they were both STDM and IFT-B negative.

5. By the combination of STDM and serological tests, it was shown that bovine trypanosomiasis was only a problem at Lumino and Kunyao. In both these areas tsetse flies were present. The use of the indirect fluorescent antibody tests would appear to assist in the detection of trypanosomes in individual animals and in the geographical delineation of active trypanosomiasis.

LOCATION	KUNYAO	AMUDAT	KARITA	KACHELIBA	NABILATUK	LUMINO	
No. CATTLE SAMPLED	60	46	244	12	25	80	
% ANIMALS DETECTED POSITIVE	STDM	1.6	—	—	—	11.25	
	FILTER PAPER IFT	congolense antigen	13.3	—	2.4	—	12.5
		vivax antigen	6.6	2.1	4.8	—	7.5
	brucei antigen	1.6	—	3.2	—	3.75	
	PAPER IFT	congolense & vivax antigens	6.6	—	0.8	—	16.25
		brucei & vivax antigen	3.3	—	2.0	—	2.5
	congolense & brucei antigens	3.3	—	0.8	—	5.0	
	congolense brucei & vivax antigens	5.0	2.1	0.4	—	22.5	
	TOTAL	39.7	4.2	14.4	—	—	70.0
	Weak Positives	16.6	—	4.5	—	—	15.0
	AGGLUTINATION	—	—	1.2	—	—	47.5

— NO ANIMALS DETECTED POSITIVE.

Table 3.44. The number of animals, expressed as percentages, detected positive by STDM and the serological tests used in the two areas studied.

	LOCATION BOVINE NUMBERS	GROUP DETECTED BY STDM (if any)	A N T I G E N		
			brucei	congolense	vivax
KUNYAO	9	—	1/160	1/320	1/80
	11	—	1/320	1/320	1/320
	17	congolense	—	—	—
	42	—	1/320	1/80	1/80
	57	—	1/80	1/160	1/40
O N — M D J	1		1/640	1/160	1/160
	2		1/320	1/320	1/320
	3		1/160	1/80	1/320
	4		1/1280	1/320	1/320
	6		1/1280	1/320	1/320
	7		1/160	1/160	1/160
	8		1/160	1/160	1/320
	11		1/640	1/160	1/320
	12	congolense	1/160	1/160	1/160
	13		1/320	1/160	1/320
	15	congolense	1/320	1/640	1/320
	17		1/1280	1/640	1/1280
	18	vivax	1/160	1/160	1/160
	19		1/640	1/160	1/640
	20		1/320	1/320	1/160
	23		1/640	1/160	1/1280
	25		1/640	1/1280	1/640
	27		1/640	1/320	1/1280
	32		1/640	1/640	1/320
	33		1/160	1/80	1/320
	35	congolense brucei	1/160	1/80	1/40
	37		1/640	1/160	1/640
	39		1/160	1/320	1/160
	40	brucei	1/80	1/40	1/40
	41		1/640	1/320	1/640
	43		1/320	1/320	1/320
	44		1/320	1/160	1/320
	45	brucei	1/160	1/320	1/40
	47	brucei	1/80	1/80	1/80
	49		1/640	1/640	1/640
	51	vivax	1/320	1/320	1/1280
	53		1/320	1/320	1/320
	55		1/320	1/320	1/640
	56		1/320	1/640	1/320
	57		1/160	1/160	1/320
58		1/160	1/320	1/160	
59		1/160	1/320	1/160	
61		1/80	1/160	1/640	
62		1/80	1/320	1/80	
63		1/320	1/80	1/320	
64		1/80	1/320	1/80	
65		1/160	1/160	1/160	
66	vivax	1/640	1/640	1/640	
67		1/160	1/80	1/160	
71		1/160	1/320	1/80	
73		1/640	1/160	1/160	
75		1/80	1/160	1/160	

Table 3.45. Titres of common antibody in the sera of the cattle, detected positive by IFT-B, and the group of infecting trypanosome (if any).

LOCATION BOVINE No.		INFECTING TRYPANOSOME GROUP	DETECTION METHODS		
			wet film	thick film	mouse inocul.?
KUNYAO	17	congolense	+	+	+
LUMINO	12	congolense	+	+	-
	15	congolense	+	+	-
	18	vivax	+	+	-
	35	congolense brucei	-	+ *	+ *
	40	brucei	+	+	+
	45	brucei	-	+	+
	47	brucei	-	+	+
	51	vivax	-	+	-
	66	vivax	-	+	-

* thick film detected congolense
 * mouse inoculation detected brucei.

Table 3.46. Comparison of the results of the three standard trypanosome detection methods in the detection of the disease in trypanosome-positive animals.

4. DISCUSSION.

4.1 Identification of Trypanosoma congolense.

Trypanosomes have been classified by morphology, the pattern of development in the insect intermediate host and by host specificity(186,188,372). Thus an isolate, strain or stabilate of trypanosomes may be identified as a member of the salivaria or stercoraria(188), or as belonging to the brucei, congolense, vivax or lewisi groups(186). The accurate identification of species is however more difficult. For example, a brucei sub-group trypanosome population is said to be T.brucei only if it has been proved that it cannot infect man(7). The separation of T.congolense and T.dimorphon by mean length measurements(187) has been questioned by Godfrey(151), who suggested that there was insufficient information available to distinguish these two species. Godfrey considered that T.congolense consisted of a range of different morphological types.

The stabilates EATRO-189 and EATRO-585 were identified as T.congolense, intermediate type using the morphological criteria of Godfrey(see section 3.1). There seems no justification for their separation into either T.congolense or T.dimorphon(187) as, although mean length measurements were not carried out, it is probable that the two stabilates could have been identified as either species. In the derivatives of both stabilates, short forms (photograph 3.1) of approximately 12 to 14 μ in length and long forms(photograph 3.3) of approximately 16 to 18 μ in length were present. It is thus probable that T.congolense does consist of a

range of types and that different isolations cannot be separated by mean length measurements.

It has been suggested that strains of congolense group trypanosomes may be distinguished by pathogenicity(152) and by cross protection tests(227). Binns(23) recorded differences of pathogenicity in seven strains of T.congolense, obtained from cattle in Nyasaland, when they were inoculated into laboratory rodents. Stabilates EATRO-189 and EATRO-585 varied in pathogenicity for zebu cattle(see section 3.20). It is doubtful if this criterion alone will be of value in the species identification of trypanosomes of the congolense group. Hosts of different species and even hosts of the same species(see section 3.25) show different reactions when inoculated with the same stabilate of T.congolense. Distinct antigenic types of T.congolense can be distinguished by the neutralization test (see section 3.20). Similar studies should be conducted using isolations of congolense and dimorphon types of T.congolense. Results may show the development of similar antigenic types indicating that isolations belong to a single species.

There is also the possibility that T.simiae is merely a further type of T.congolense, which is adapted to the pig and the monkey, and which is polymorphic(41). Immunological and further morphological studies on these organisms may elucidate this problem. Although it is known that some common antigens exist in all the pathogenic African trypanosomes(161, section 3.18), it may be that T.simiae has similar common antigens to T.congolense. This possibility could be examined by employing

T.simiae and T.congolense antigens in IFT-A tests using sera from animals known to have been infected with T.congolense only. If the T.simiae antigens gave similar serum titres of common antibody as those produced by the T.congolense antigen, and not weaker titres as would be produced by T.brucei and T.vivax antigens(see sections 3.19 and 3.27), the similarity between the two species may be indicated.

The terms polymorphic, pleomorphic and monomorphic have been used to describe trypanosome populations. Trypanosomes of the brucei sub-group have been termed polymorphic(301,375,376) as a change from long slender to short stumpy forms takes place in the blood of the animal host. The term monomorphic has been used to distinguish T.congolense from the polymorphic trypanosomes (187). This is considered a misnomer by Godfrey(151) as although T.congolense is monomorphic in the sense that it consists of a statistically homogeneous population, a variety of blood forms do exist in this species(see photographs 3.1 to 3.3 in section 3.1). Godfrey considered pleomorphic to be a more accurate description. The meaning of these terms must be made clear as some text books state that all trypanosomes are pleomorphic(57). Also, Ormerod(277) used the term clonal pleomorphism to describe the diversity of forms which occur in brucei sub-group organisms in order to avoid confusion with genetic polymorphism which is also found in members of this group. Genetic polymorphism, a term introduced in 1940, refers to the type of morphological variation, which has presumably taken place as a result of selective adaptation of strains to particular ecological

circumstances.

The study of the morphology of derivatives of T.congolense stabilates EATRO-189 and EATRO-585 indicated that they were pleomorphic as defined by Godfrey(151). Tables 3.3 and 3.4 showed that the ratio of different morphological forms did not change over a 5 day period. This latter period is now considered to be inadequate as antigenic variation was shown to take approximately 10 days(see section 3.20 and 3.21). If morphological changes take place in T.congolense, it is probable that they would occur over the period of antigenic change, as is thought to be the case with the brucei sub-group(80,283) although Luckins(243) considered that antibodies did not play a part. In order to substantiate pleomorphism in T.congolense, a study of the morphological types in populations in infected animals would have to be undertaken over a period of at least 20 days.

4.2. Passage of T.congolense through G.morsitans.

As data on the length of cyclical development of T.congolense in and its ability to infect Glossina are few, the results of the cyclical passage of stabilates EATRO-189 and EATRO-585 through G.morsitans are of interest(see tables 3.1 and 3.2). A development time of 21 days for congolense group trypanosomes in G.morsitans and G.palpalis has been reported(39,150). Infection rates varied from 3 to 35%(150,355).

In section 3.1, the presence of metacyclic trypanosomes in infected flies was judged according to the presence of trypanosomes in the proboscis detected by dissection and stained

probe, and in membrane blood as detected by mouse inoculation. The ability of a fly to infect a susceptible animal is considered to be the only real criterion for ascertaining the presence of metacyclic trypanosomes. The presence of trypanosomes in the proboscis does not necessarily indicate that they are also infective. Thus the mouse inoculation method gives the most accurate measure of the length of cyclical development and the dissection and stained probe methods serve as merely guides. The mouse inoculation method has limitations. Many isolates of T.congolense do not infect mice. The output of infective metacyclics is irregular and varies with the species of tsetse fly(178,382) and thus a negative result does not necessarily indicate that completion of cyclical development has not taken place. The length of cyclical development of the derivatives of EATRO-189 was approximately 34 days and of EATRO-585 approximately 17 days in G.morsitans as judged by mouse inoculation(see tables 3.1 and 3.2). The differences between these times of development may be associated with laboratory adaption, as EATRO-189 had 31 laboratory rodent passages while EATRO-585 had only 10. In the derivatives of both stabilates, the time taken for the trypanosomes to reach the proboscis after the infecting feed was approximately the same, being 22 and 24 days respectively as judged by dissection. However, in the more laboratory adapted EATRO-189, more time was required for the change to metacyclics to take place as they did not become infective until day 34 after the infecting feed. The flies infected with EATRO-189 derivatives, which subsequently became infected with metacyclics, became probe positive for at least 5 days prior to the trypanosomes

becoming infective.

The differences in the ability of the two stabilates to infect G.morsitans may have been due to the concentration of the trypanosomes in the infecting feed or to the host animal from which the trypanosomes were derived. In the infecting feed of EATRO-189, the trypanosomes were present in a 4-plus concentration in mouse blood; in that of EATRO-585, they were present in a 1-plus concentration in bovine blood. As no detailed studies have ever been conducted on the infectivity of T.congolense for Glossina, and to few flies were used in this work to give accurate results, the factors involved cannot be properly discussed. Baker and Robertson(13) considered that the numbers of T.brucei and T.rhodesiense used to infect G.morsitans had no bearing on the resulting infection rate, although Hoof(194) expressed the opposite view. The host in which the trypanosomes were grown was also considered to be unimportant(356) although Corson(66) suggested that a higher than normal infection rate occurred in G.morsitans when they were fed on a reedbuck infected with T.rhodesiense. There remains the possibility that the morphology of organisms of T.congolense is important in resulting fly infection rates as is thought to be the case with the brucei sub-group(361,376). As there is no known morphological cycle of T.congolense in the mammal, this possibility cannot be examined.

In order to compare the ability of many isolates of T.congolense to infect Glossina and their subsequent lengths of developmental cycle, a source of flies of known age and history would be required, hence the importance of the laboratory rearing

of tsetse flies in Bristol and Edinburgh.

4.3. Methods used to count T.congolense.

The inadequacy of the direct methods used to count trypanosomes has been demonstrated by Walker(359). In this study, the haemocytometer method was considered to be the only technique worthy of consideration for counting T.congolense due to the gross inaccuracy of all other methods. For the haemocytometer approach to give accurate results, trypanosomes must be present in concentrations of at least 10^8 per ml(362) and the organisms must be uniformly distributed. The accurate counting of T.congolense in infected cattle presents difficulties as the trypanosomes are normally present in concentrations of between 10^5 and 10^6 per ml, and when present in greater numbers they autoagglutinate, thus biasing the count. The haemocytometer method can thus only be used to estimate numbers of T.congolense.

With the introduction of the indirect counting method by mouse infectivity titration, a considerable advance was made in the study of the parasitaemia in cattle infected with T.congolense. The technique was shown to be reproducible and to detect significant changes in infectivity of T.congolense. It was subsequently used to study changes in the number of infective trypanosomes during infections in a rat and in cattle, in the standardization of antigens for the neutralization test and in the estimation of trypanosomal challenge to cattle. Previously, both the antigens for the neutralization test and trypanosomal challenge employing T.congolense had been standardized in terms of the

numbers of trypanosomes(333,327,332). As both the neutralization test and trypanosomal challenge are judged by the ability of the trypanosomes to infect the inoculated host and as the number of trypanosomes present in a suspension do not necessarily correspond to the number of infective trypanosomes(see sections 3.3 and 3.20), the assessment of numbers by counting is considered to be inadequate.

4.4. Preservation of T.congolense.

The successful preservation of both the metacyclic and blood forms of T.congolense greatly facilitated the subsequent study of these organisms. The conditions required for the preservation of both forms were shown to be similar. They were a slow cooling rate, the addition of glycerol and a volume of not exceeding 1ml of infected blood.

The results of section 3.4 demonstrated that there was a wide margin of safety in the rate of cooling of T.congolense from 0°C to -79°C. However, a marked fall in trypanosome infectivity was noted when they were cooled to -79°C in 27 minutes, which was not noted when they were cooled in over 50 minutes(rates C and D' respectively on figure 3.3). The rates of cooling of C and D' were similar from 0°C to approximately -30°C(about 7 minutes); however from this temperature to -79°C the latter cooled more slowly than the former and it must have been during this period that the trypanosome destruction took place. Cunningham et al(87) demonstrated that between -25°C and -35°C, trypanosomes of the brucei sub-group in capillaries had to be cooled slowly if

efficient preservation was to be achieved. It is probable that this temperature range is also critical for T.congolense.

The results of section 3.5 show that the larger volumes of infected blood in 1ml ampoules required a greater concentration of the freezing protectant than the volumes in capillaries, and that volumes of 5ml could not be preserved by the methods used. This finding may be related to the cooling rate. When trypanosomes are preserved in capillaries, the volume is small enough to ensure a uniform cooling. When 1 and 5ml quantities are preserved, there may be unequal cooling throughout the sample, which could induce injurious effects by too rapid formation of ice crystals.

The studies on the comparison of preservation in capillaries and 1ml ampoules in two infected cattle(see section 3.6) indicated that the stage of infection at which the trypanosomes were isolated was important in their resulting preservation, and that the ampoule method was more efficient. Successful preservation was achieved using both methods when the trypanosomes were isolated during the rising parasitaemia. However, during the period when the number of trypanosomes was decreasing, isolations of trypanosomes were preserved efficiently by the ampoule method only. Statistically significant changes in the infectivity of T.congolense were shown to be related to antigenic change(section 3.20), a change which may have rendered the trypanosomes more sensitive to preservation. The ampoule method has practical advantages over the capillary method in that more parasites can be preserved. Infections of cattle with T.congolense are characterized by low levels of parasitaemia and it is only by the

Preservation of the trypanosomes in ampoules that enough antigen could be obtained for the neutralization test.

The reasons why the ampoule method required the addition of 15% glycerol compared with 7.5% in capillaries, why about 30°C was critical for preservation and why the process of antigenic change was associated with poor preservation in capillaries are obscure. The relationship between trypanosome destruction, cooling rate, volume of suspension and ice crystal formation must be very delicate. In the above circumstances the balance may have been upset leading to more ice crystal formation and subsequent trypanosome destruction.

The results of section 3.7 indicate that T.congolense can be preserved at low temperature for at least a year. This confirmed the findings of Cunningham et al(79). A movement of free radicals still occurs in suspensions at -79°C(171), which could result in the formation of ice crystals with the subsequent destruction of trypanosomes. Preservation at -196°C in liquid nitrogen is thus safer.

That at least 70% of the field isolates of T.congolense, examined in section 3.8, were preserved efficiently gives hope that the methods developed in this study will be of practical use.

4.5. Effect of environment of T.congolense in vitro.

The results of section 3.10 demonstrated that pHs of between 6.8 and 8.0 did not affect the infectivity nor actual numbers of T.congolense, when maintained at 0°C in buffered salts solutions, over a period of 8 hours after removal from the animal host. However pHs of 5.8 or below were inhibitory within 4 hours. This supported the work of Cunningham et al(84) and indicated that

T.congolense was more resistant to pH change than brucei sub-group, whose optimum pH range was shown to be between 7.4 and 8.0(247). Soltys(330) using Ringer's, Tyrode's and Alsever's solutions indicated that pHs of between 7 and 8 were optimum for the maintainance of T.congolense, as judged by changes in trypanosome numbers. The advantages of using infectivity and numbers as criteria to study trypanosome survival have been referred to in section 4.3.

The reason why T.congolense is rather more resistant to pH change than brucei sub-group in the same buffer solutions is not known but may be due to different biochemical properties of these organisms. The loss of infectivity of blood forms of trypanosomes, after their removal from the host, could be due to either inhibitory factors in the diluent used for maintenance, inhibition by metabolic products produced by the trypanosomes during maintainance, or to the change into infect forms which are non-infective to mammals. As trypanosomes of both the brucei sub-group and T.congolense can be maintained for many hours in BS-1 (247, section 3.10), these diluents do not contain substances which are inhibitory to trypanosome infectivity. Differences in the metabolism between brucei sub-group and T.congolense have been reported. Brand and Tobie(26) showed that the oxygen consumption of blood forms of T.brucei at 37°C was greater than that of the blood forms of T.congolense at the same temperature. This was confirmed by Ryley(310). The glycerophosphate system was more active in the blood forms of the brucei sub-group than in those of T.congolense(160). Tseng, quoted in Brand and Tobie(27),

observed that blood forms of T.brucei multiplied faster than those of T.congolense. The rate of development of new antigenic variants of the brucei may be faster than T.congolense(see section 4.8). In wet blood films, brucei sub-group show greater motility than T.congolense. These observations indicate that brucei sub-group organisms have a faster metabolic rate than T.congolense. There would thus be a greater output of metabolic products into the suspension diluent which could affect pH and thus infectivity. The pHs of the buffer solutions were not measured at the end of the 8 hour period of trypanosome maintenance so it is not known whether such a pH change took place. The change from blood into insect forms is thought to occur in vitro(159), but what conditions induce this change or how long it takes has not been studied in detail. Brucei sub-group may change into insect forms at a faster rate.

Populations of T.congolense maintained their infectivity for at least 8 hours at 0-2°C in BS-1. In one of the stabilates examined(KB-1), infective trypanosomes were still present after 24 hours, although in significantly less numbers than in the control(see table 3.12). Similar survival times have been reported for the brucei sub-group(247). The addition of normal serum did not increase the maintenance time of infectivity of T.congolense(see table 3.17) and it is difficult to explain the claim that normal animal sera enhances the maintenance of infectivity (387). Until the precise mode of action of normal sera on trypanosomes is known, their use in maintenance diluents is contraindicated in view of the many reports of the anti-trypanosomal activity of normal sera(190,206,231).

4.6. Serological tests for T.congolense.

In the choice of suitable serological tests for the study of T.congolense, attention had to be paid to the biological properties of the parasite, the reproducibility and sensitivity of the test used and the information required. Tests had to be developed for the study of antigenic variation, of antigenic relationships between T.congolense and other species of African pathogenic trypanosome and of the immune response induced in cattle infected with T.congolense.

The serological tests, which have been used to study antigenic variation and the production of variant specific antibodies in animals infected with the brucei sub-group include agglutination (82,163-165), trypanolysis(238,239,316) and neutralization(77,333). As T.congolense autoagglutinates(72,231), the use of an agglutination test was eliminated. Although the trypanolytic test distinguished between antigenic variants of T.congolense(see section 3.12), it was considered inadequate for general application in studies of field isolates of T.congolense due to difficulties in obtaining stable antigens. To obtain enough antigen, at least 10^8 organisms per ml, the trypanosomes had to be grown for 7-10 days in mice by which time antigenic variation could have occurred. The use of mice for growing antigen is only considered satisfactory when the required concentration of trypanosomes are produced within 2-3 days before the appearance of antibodies can induce antigenic change(333). This never occurred, in my experience, using isolates of T.congolense. The use of culture forms of T.congolense may overcome this problem if culture forms

are antigenically more stable than blood forms, as indicated by Seed(319) for the brucei group. The neutralization test of Cunningham and Van Hove(77) was shown to be applicable to T.congolense(see section 3.15), as only a relatively small number of trypanosomes, a minimum of 10^3 mouse ID_{63s} per ml, were required for the antigen. The test is reproducible and easily carried out using preserved trypanosomes collected directly from cattle, thereby obtaining a stable antigen of known antigenic type. There are disadvantages. As the reading of the test depends on whether mice become infected, it can only be used with strains which infect mice, and at least 40 days are required to obtain a result as T.congolense may have a prepatent period of this length in these animals. Also, the required concentration of 10^3 infective trypanosomes per ml is not always present in the blood of infected cattle, especially late in the infection(see figures 3.8 and 3.9 in section 3.20). Both the stage of the infection and the stage of the disease, at which the trypanosomes are preserved, are very important. Ideally, antigens should be prepared during the rising parasitaemia as it is at this period that the number of infective trypanosomes in the blood is greatest and closely resembles the actual number of trypanosomes(see figures 3.10 and 3.11). This period of rising parasitaemia is difficult to detect as the numbers of trypanosomes remain so constant over a given 30 day period(see figure 3.11). For detailed studies, antigens must therefore be prepared daily. Neutralizing antigens can rarely be prepared when animals have passed into phase 2 of the disease, after approximately 100 days

of infection, as the concentration of infective trypanosomes in the blood is seldom above 10^3 mouse ID_{63s} and is mainly well below (see figures 3.8 and 3.9).

The precipitin, indirect haemagglutination and indirect fluorescent antibody tests have been used to detect common antibodies to trypanosomes in animals. The similarities of common antibodies between antigenic variants within strains and between different species of trypanosome, and the development of common antibodies in infected animals have been examined employing brucei sub-group antigens (147,161,383,82). Application of the precipitin test was not attempted in this study as large numbers of uncontaminated trypanosomes are required for the antigen and no method was developed for the efficient separation of T.congolense from the blood constituents. Williamson and Cover (380) reported the successful separation of these organisms using sucrose gradients. This approach was attempted in this study but with little success. Lanham (224,392) has since reported the successful separation of T.congolense by fractionation on columns through the appropriate anion exchanger using flow solutions buffered at pH 8.0. Preparations of antigens using this approach may be feasible. The development of a standard method to obtain pure T.congolense antigen and (it's) subsequent use in the precipitin test is necessary to confirm the findings of Boreham (24) that the precipitating and fluorescence antibodies may be similar. As attempts to apply the indirect haemagglutination test using a brucei sub-group antibody-antigen system failed (see section 3.14), attempts were not made to use the test

employing a T.congolense antigen. The results of section 3.14 showed that non-specific haemagglutination occurred, which was not a result of an antibody-antigen reaction. It could however have been due to the impurity of the antigen used(295), which would indicate that the method of differential centrifugation to separate the trypanosomes from the blood constituents, followed by freezing and thawing, was inadequate. A more profitable approach may be made using sephadex thin layer chromatography by means of which fractions of trypanosomal protein have been isolated(381). Attempts could then be made to coat the separated proteins on to tanned erythrocytes and if the indirect haemagglutination test then gave definite results, the antigenic reactivity of the different isolations of trypanosomal proteins could then be elucidated. This type of study is urgently required to assess the immunological importance of the protein fractions which have been identified in trypanosomes(33,34,276, 379). The indirect haemagglutination test has been used with success to detect antibodies to T.evansi and T.cruzi(145,147, 272). The indirect fluorescent antibody test was applied with success employing T.congolense antigen(see sections 3.16 to 3.19). The test was shown to be reproducible, easily carried out and to detect common antibodies. The method of the preparation of the antigen was satisfactory and it could be stored at -26°C for at least 6 months without loss of potency(see section 3.17). T.theileri did not interfere with the fluorescence reaction and a serum titre of 1/40 or above was shown to be significant for pathogenic trypanosomiasis(see section 3.16). The importance of

examining unknown samples using antigens obtained from the three main groups of pathogenic trypanosome was demonstrated (sections 3.19 and section 3.27). In a bovine trypanosomiasis survey, carried out by Wain et al(354), the incidence of detectable trypanosomiasis, and serological positives by IFT-B using brucei sub-group antigen only were compared using samples from 960 cattle in an endemic trypanosomiasis area in the same tsetse belt as Lumino(see section 3.28). They detected trypanosomes in 60 of the animals and common antibodies in 253 (26.3%). Of the 60 parasitaemic animals, antibodies were only detected in 16. As brucei sub-group antigen only was used, many of these 44 animals could have had common antibodies to T.vivax and T.congolense which remained undetected. The figure of 26.3% positive by IFT-B is much lower than that of 70% at Lumino(see table 3.44), when all three antigens were used. The main disadvantage of the test is the possible error in the reading of the test by the same person on different occasions and by different people reading the same sample. The correct reading of the test takes constant practice and much experience is required before satisfactory results are obtained. Having gained this experience, it is relatively simple to distinguish between 1,2 and 3-plus fluorescence, as the trypanosomal antigens prepared by the methods described in section 2.10e are not masked by the presence of other cells. The reading of the same samples by different people should not produce erroneous results provided that the criteria for the degrees of fluorescence are strictly adhered to. The IFT-B test only gives a rough indication as to

the presence or absence of common antibodies as it was shown that a 3mm blood spot in 0.025ml PBS gave an approximately 1/50 dilution(10) which is only just above the 1/40 serum titre considered to be significant for trypanosomiasis. Most studies using immunofluorescent techniques have employed a mercury vapour light source. In some recent reviews, the possible use of iodine-quartz light sources was not even mentioned(191,271). In recent years the iodine-quartz lamp has been advocated as a cheap and convenient alternative to mercury vapour for exciting the fluorescence of fluorescein labelled antibody(351,388). The iodine-quartz lamp is an incandescent filament source which has a very small output of ultraviolet light and relies for (it's) efficiency on the blue-violet radiation(wavelengths of between 400 and 500m μ). Antibody conjugated with FITC has a maximum absorption at a wavelength of 495m μ and a peak emission of fluorescent light at 520-525m μ (29,129). An ideal primary filter would, therefore, have a high transmission at wavelengths shorter than 500m μ and a very sharp cut off above this wavelength. The secondary filter must pass light of wavelength 520m μ and longer with a sharp cut off at shorter wavelengths. The filters used in this study(see section 2.10e) fulfilled these exacting requirements.

4.7. Parasitaemic picture of T.congolense disease of cattle.

The detection of patent parasitaemia throughout the disease in only 13 cattle infected with T.congolense, two in section 3.20 and the eleven controls of section 3.25, were examined in this study. Observations are limited as all the animals were inoculated with derivatives from only two stabilates. In previous work on T.congolense infections in cattle(121,131,132), observations were limited to the recording of the presence or absence of trypanosomes. Three main phases of the disease were described, namely, the major crisis when the trypanosomes were detected constantly in the blood, a secondary crisis characterized by the intermittent appearance of the trypanosomes in the blood, and recovery when no trypanosomes could be detected. The position was however complicated by the recording of premunition in recovered animals, in which circulating trypanosomes were continually present in the blood(120,197,281,282). In the present study, the parasitaemic picture of T.congolense in cattle could be divided into three phases(see conclusions of section 3.20) and use was made of the mouse infectivity titration as well as recording the presence or absence of trypanosomes by STDM. Phase 1, when the trypanosomes were detected constantly in the blood was similar to the major crisis; phase 2 to the secondary crisis; and phase 3 to the recovery period. Of the 13 cattle, 7 died during phase 2, 5 remained in phase 2 until at least day 400 postinfection, and only 1 passed into phase 3. The disease may be divided into acute or chronic depending on whether animals die in phase 1. Using this criterion, all 13 animals suffered from chronic trypanosomiasis. The relationship between the numbers of trypanosomes present

and their infectivity to mice was studied in two cattle (see section 3.20) during phases 1 and 2. During phase 1, the numbers of trypanosomes remained relatively constant, between an estimated 10^5 and 10^6 per ml, while the number of infective trypanosomes fluctuated between 10^2 and 10^5 per ml. The fluctuations in infectivity was related to antigenic change. During phase 2, too few trypanosomes were present in the blood to be counted and infectivity fluctuated between $10^{1.4}$ and 10^3 infective organisms per ml.

Two plausible explanations exist as to why the trypanosome numbers remained so constant over the period of antigenic change in phase 1. Either the stimulus for antigenic change, presumably the variant specific antibody, was not lethal to the organisms which then adapted to form a new variant (model 1), or the stimulus was lethal but the rate of multiplication of the new variant was similar to the rate of destruction of the old one (model 2). Due to the lack of fundamental information on the reproductive rate of T.congolense, and on the precise mode of action of the variant specific antibody, it is impossible to state which of these models is correct. That neutralizing antibodies act on trypanosomes within 5 minutes of contact (see section 3.15), but had no effect on trypanosome numbers for at least 360 minutes would fit the first model. However, neutralizing antibodies may sensitise the trypanosomes for phagocytosis (249,254,333), which would fit the second model if the rate of reproduction of the new variant was similar to the rate of removal of the old one.

The apparent disappearance of trypanosomes from the peripheral blood in phase 2 parasitaemia indicates either the inadequacy of the trypanosome detection methods used, or a change of the trypanosome habitat out of the peripheral blood. As the STDM consistently detected trypanosomes during phase 1 parasitaemia (see sections 3.20, 3.21 and 3.27), the possibility that T.congolense migrates out of the peripheral blood into either the smaller blood vessels or the parenchymatous organs is enhanced. Wherever they go, the trypanosomes still remain antigenically active as the common antibody titre does not fall during phase 2 parasitaemia. There have been some reports that trypanosomes do not remain confined to the blood stream of the mammalian host (122, 124, 384). Walker (357) produced evidence that, in a laboratory strain of T.brucei, the stage between divisions was poorly represented in the blood of rodents. Photomicrographs showed that 50-80% of the parasites in the blood had two kinetoplasts. This was compared with a 10% figure for Strigomonas oncopelti. His studies implied that for some period between divisions the trypanosomes did not circulate freely. The solution of this problem may lie in the labelling of trypanosomes with fluorescent brighteners (181) or with radioactive substances in attempts to trace their locations in the host.

When zebu cattle were challenged with trypanosomes of the brucei, congolense and vivax groups, different species predominated in the subsequent infections (see section 3.26). No species consistently appeared to the exclusion of the others. T.congolense and T.vivax appeared in a greater number of animals than brucei

sub-group organisms. This was probably due to the relative infection rates in the flies from which the trypanosomes were isolated. The infection rates of brucei sub-group in the tsetse flies at Lugala is known to be much less than that of either T.congolense or T.vivax(173-175). The predominance of T.congolense over T.vivax, in some circumstances but not in others, is however more difficult to explain. When mixed infections are established in animals with trypanosomes differing in easily recognisable characters, such as the presence or absence of a kinetoplast or drug resistance, one component of the mixture usually diminishes in relative numbers and disappears(27,51,263). The strain with the changed characteristics invariably disappears more rapidly than the unaltered strain. An exception is akinetoplastic T.evansi(263). Sexual reproduction is a possible explanation for the loss of a strain in a mixture(70,112,117) but this explanation is not generally accepted(2,133,344). A more plausible theory is differential reproduction rates(27,51). In section 3.26, it seems unlikely that T.congolense and T.vivax were involved in a sexual reproduction process. The predominance of T.congolense or T.vivax could be explained by differential reproduction rates. However, this would imply that separate isolations of the same species have different rates. It is more likely that the development of infections was influenced by the resistance of the host. When cattle of the same age and breed were inoculated with the same stabilate of T.congolense distinct host responses resulted(see section 3.25). Also, in some of the cattle used in section 3.26 no infections developed, while they did

develop in other cattle inoculated with the same challenge material.

4.8. Antigenic variation of T.congolense in cattle.

The phenomenon of antigenic variation in trypanosomes of the brucei group is well documented(82,163,164,205,239,278,299,300,309,361). It has also been reported from the rat-adapted strain of T.vivax(58). Antigenic variation in the congolense of trypanosomes has however never been demonstrated. That it does occur was demonstrated in sections 3.20 and 3.21. A succession of antigenic variants developed in cattle, during phase 1 of parasitaemia, to which variant specific antibodies were produced. Antigenic change, which occurred at approximately 10 day intervals, was associated with significant falls in the numbers of infective trypanosomes. When cattle were infected with the same antigenic variant, similar antigenic types developed in each of them early in the infections. Twelve distinct antigenic types were isolated. During the period of days 130 to 140 postinfection in zebu 495(see section 3.20), there was a sudden rise in the variant specific antibody to the infecting variant. If neutralizing antibodies are exclusively variant specific, a reversal to the original antigenic type would be indicated. This possible antigenic reversal was associated with an increase in body weight and the subsequent recovery of the animal.

The antigenic lability of T.congolense explains the many field observations on the failure of animals to develop an immunity to this species. Previous reports on the range of different

serological types of T.congolense are limited. Fiennes(121) found that four geographically distinct strains of T.congolense belonged to only two types as judged by the lysin protection test. According to Fiennes, only three distinct types were found by Schilling and Neuman(316) among numerous isolations using the same test. Laveran and Mesnil(230) found only four serologically distinct strains of this trypanosome. As at least twelve distinct types of T.congolense were identified in the cattle infected with the same stabilate(see section 3.21), there is probably many more than this number in nature.

The characteristics of antigenic variation in T.congolense are similar to those described for the brucei group where the process is also related to the production of variant specific antibody(163), infectivity changes(80) and a definite pattern of antigenic variant production(165). However, antigenic variants of the brucei group may be produced at a faster rate than the 10 day period found for T.congolense. Gray(165) showed that they were formed every 2 to 3 days in rabbits and Cunningham and Van Hove(82) demonstrated new variants every 6 to 7 days in the early infection in cattle.

The loss of infectivity in trypanosomes, which are changing their antigenic type, may be associated with changes in their surface soluble antigens. Weitz(368,369) showed that the removal of soluble antigens from trypanosomes of the brucei sub-group resulted in a loss of infectivity for rodents. The development of surface antigens in Paramecium aurelia has been studied using immunofluorescence(20,21) and immunoferritin(260,261) labelling techniques. The ferritin-labelling technique used was similar to that developed by Singer(326). New antigens developed initially

on the pellicle and subsequently on the cilia. A similar process may occur in T.congolense. The loss of infectivity may be an intermediate stage between the 'shedding off' of old surface antigens and the development of new ones. To examine this possibility, similar studies to those carried out on P.aurelia should be conducted using immunoferritin labelling due to its extreme sensitivity(261). Samples of trypanosomes could be obtained, hourly from a host, over a suspected period of antigenic change

Studies on the antigenic variation of P.aurelia have shown that the process is gene controlled(19). The failure to detect sexual reproduction in trypanosomes prevents genetic analysis. Evidence has been put forward that antigenic variation in trypanosomes is due to mutations(52,322,361). These studies were carried out with laboratory-adapted strains and thus the results may not be comparable to those found using freshly isolated strains. In cattle infected with T.congolense EATRO-585, the time taken for antigenic variation to take place must have been less than 24 hours(see table 3.26). When EATRO-585/4 was mixed with its specific antiserum in vitro, antigenic variation did not take place after a maintenance time of 3 hours at 0°C(see section 3.15). As the time taken for antigenic variation is so rapid, between 3 and 24 hours, it is probable that adaptive processes are operating. The maintenance of trypanosome numbers during phase 1 parasitaemia, and the finding that antigenic variants of T.congolense develop in a definite order in cattle infected with the same stabilate, are also indicative of adaption. The adaptation

hypothesis, which has also been put forward by Inoki et al(207) and Gray(166), would also fit model 1 suggested in section 4.7.

The significant rise in the variant specific antibody to the infecting variant in zebu 495, during phase 2 of the disease, may be of great importance. The capacity of T.congolense for antigenic variation may be limited. It may be that, after the development of a definite number of antigenic types, populations of T.congolense revert to antigenic types to which the host has already been sensitized. The selfcure of zebu 495 may be explained using this model. The development of some 13 antigenic types, an antigenic reversal to the type of the infecting population, and a strengthening of the immune response in favour of the host are suggested as the possible sequential order of events. This possibility, which has also been suggested by Fiennes(121), would explain the increase in body weight at about day 140 post-infection, the disappearance of the trypanosomes approximately 50 days later, and the subsequent recovery of the animal. The capacity of the brucei sub-group for antigenic variation is thought to be unlimited and only restricted by the death of the host(164,299,300,309,361). My experiments indicate that the position is not so simple. The many reports of the clinical resistance of cattle to the disease(22,55,56,120,197,281,282,312,315,316), and the ability of cattle to eliminate infections(121,132) indicate a complicated host-parasite relationship in which the antigenic lability of the trypanosomes may play a vital role. This problem is further discussed in section 4.11.

4.9. Effect of passage through G.morsitans on the antigenicity of T.congolense.

When different antigenic variants of a strain of brucei subgroup were passaged through G.morsitans, an antigenic reversion to a 'basic strain antigen' occurred(165). Two antigenic variants of T.congolense, which were passaged through two different groups of G.morsitans, and four first cyclical populations, obtained from an infected fly in each group, were compared serologically using the neutralization test(see section 3.22). Three distinct antigenic types, designated A',B' and C', were identified in the first cyclicals, which were antigenically distinct from both the infecting variants. The populations of types B' and C' were derived from different flies; populations of type A' were derived from both flies(see table 3.36). There is thus no definite evidence for the formation of a 'basic strain antigen' although the presence of first cyclicals derived from both flies in type A' may be significant. In both this work and the work of Gray(165), first cyclicals were used. To obtain enough antigen, populations derived from metacyclics of T.congolense had to be grown for 17 to 20 days in mice(see figure 3.14) in which time antigenic variation could have occurred. The formation of a 'basic strain antigen' could have taken place and been missed by the techniques used. Antigenic type A' may have been present in all 8 of the first cyclical populations, a proportion of which then changed into B' or C'. The use of metacyclic material, as used by Cunningham(73) for studies on T.rhodesiense, would be advantageous. The metacyclic output of flies known to be infected with T.congolense varies between 0 and 500 infective trypanosomes(178,382). These

numbers are too small to be of use as antigens in the neutralization test. Until the development of more sophisticated techniques for the separation of small numbers of T.congolense from blood, first cyclical material has to be used in further studies. Attempts should be made to decrease the prepatent period of T.congolense infections in mice by interfering with the immune response as the latter may suppress infection. Splenectomy followed by the administration of salicylates or cortisone has been used, with some success, to increase the parasitaemia of brucei group trypanosomes in animals(12,134,135,149,179). In addition, the use of methods to increase infections in animals infected with non-pathogenic trypanosomes or non-African pathogenic trypanosomes, could be applied. These methods include reticulo-endothelial system blockade, treatment with mercaptopurines, deprivation of essential nutrients and X-irradiation(9,210).

4.10. Immunological control of animal trypanosomiasis.

Trypanosomiasis in animals can be controlled by either the eradication of the tsetse fly, the elimination of the game animal reservoir, the removal of trypanosomes from hosts by chemotherapeutic or immunological means, or by a combination of all three approaches. Eradication of the fly can be achieved by bush clearance (148,259), the use of insecticides(45,385) and game destruction (49,289,302). Tsetse fly destruction is expensive, requires careful planning and organization, does not necessarily remove the fly(176) and may be impermanent if correct agricultural resettlement of

the cleared areas is not carried out. Wild animal destruction should only be applied where the game feeding tsetse (morsitans group) predominate. The utilization of game animals in parks or game farms(67) may be more profitable. The incorrect or continued use of drugs leads to the development of drug resistance in trypanosomes(374). Thus although effective curative and prophylactic drugs are available, they must be used with great care. Immunological control of the disease is still in it's embryonic stages and still has to be applied practically in the field.

The stimulus for a method for the removal of trypanosomes from hosts by immunological means derives from the knowledge that certain species of game animals(5) and some varieties of cattle (55,56) can survive in a healthy condition in areas where trypanosomiasis is enzootic. While some of this resistance is innate and dependent on the genetic constitution of the hosts, there is some evidence that it may partly depend on factors acquired during life(93). Effective artificial immunization poses many problems. These include, variations in strain virulence, the multiplicity of trypanosome species, antigenic variation of trypanosomes and the extreme variant specificity of the protective antibody. Although it is possible to protect animals against infections by trypanosomes for short periods with hyperimmune serum(100,311), this passive immunization has not been widely applied. Three methods of active immunization have been attempted, namely, the repeated injections of dead trypanosomes(68,225,336) or trypanosomal products(368,369), infection with the virulent

organisms followed by chemotherapy(22,327,332,374), and attempts to produce premunition(315). Which of these methods is the best cannot be correctly assessed until the immunology of the disease is more fully understood. An accurate and rapid method for the serological classification of trypanosome isolates, a general approach for the standardization of trypanosomal challenge, and a study on the strength and duration of the protective antibody response to challenges containing many antigenic types prepared by different methods, are urgently required.

The main problems, concerning an effective serological classification of trypanosomes, are the lack of fundamental information regarding the possible number of antigenic types that are produced during infections in the mammalian host, and the number present in particular areas, and the lack of suitable methods for the study of T.congolense and T.vivax. Using the agglutination test, attempts have been made to type blood forms of brucei sub-group trypanosomes(75,167,168). Although the number of isolates examined was small, it appeared that the host from which the trypanosomes were isolated and the area of their collection may affect the antigenic type. Gray(167,168) used 'basic' and 'predominant' strain antigens in his studies. Immunological studies on T.vivax have only been conducted with the rat-adapted strain(58) and although this study is important, as antigenic variation of this strain was demonstrated, it is limited in application until methods for the study of field isolates of T.vivax have been developed. The development of suitable methods for the study of T.congolense, as described in this work,

have led to a clearer understanding of the immunology of this parasite(see section 4.8). Antigenic variation of T.congolense may be limited and a similar antigenic variant was formed in two cattle, inoculated with strains of T.congolense isolated over 200 miles apart(see table 3.29). The formation of a 'basic strain antigen' after cyclical passage of T.congolense is considered to be a possibility(see section 4.9). The finding that sera, collected from infected animals, agglutinated antigens of the brucei sub-group, which were isolated up to 1,000 miles away(see section 3.23), indicates that antigenic variation of these trypanosomes may also be limited and that similar antigenic variants are formed in animals infected with geographically distinct strains. These results give renewed optimism to the feasibility of serological typing. The following lines of work are thus suggested:-

1. Isolates of the brucei sub-group and T.congolense, which infect laboratory rodents, should be collected systematically from one area. Each isolate should be passaged through Glossina and infected flies used to infect cattle. The development of antigenic variants in separate cattle and their immunological relationships with those in other cattle could then be studied using the neutralization test for T.congolense, as in section 3.20, and the agglutination test for the brucei sub-group, as used by Cunningham and Van Hoeve(82). In this manner, an antigenic variant bank could be developed.
2. A similar study should be carried out in a widely separated area and the antigenic variants of the two areas serologically

compared.

3. Fundamental studies on the antigenic variation of field isolates of the metacyclics of T.vivax should be undertaken using goats as the experimental animal and the cross-protection test as the serological technique. A large herd of goats could be kept at research centres in Africa at relatively little expense. Studies of this nature would demonstrate the feasibility of serological typing and would show whether the production of new antigenic variants was limited and the extent of antigenic variation with reference to locality.

In the development of suitable immunization techniques, the trypanosomal challenge used must be accurately defined and bear a relationship to the strength of challenge that animals are likely to receive in the field. The difficulties in defining trypanosomal challenge were discussed by Smith and Rennison(328). They considered that variables within tsetse populations, in the host, and in the trypanosome made it impossible to measure challenge in statistical terms. Arbitrarily, they defined challenge as the number of infective bites from a tsetse in unit time. Whiteside(373) introduced the index of trypanosomal challenge as the number of non-teneral male and female tsetse per 10,000 yards(the apparent density) multiplied by the infection rate. As the number of infective bites that an animal receives cannot be measured and known infected flies do not eject infective organisms at every feed(76,178,382) and different species of fly do not have the same ability to infect animals(178), these definitions have little value in measuring the real challenge that animals are likely to receive. In previous experimental work,

animals have been challenged with a known number of trypanosomes derived from strains(327) or the animals have been placed in an endemic area(332). In this study, two techniques were used to challenge cattle, namely, the inoculation of stabilates containing a known number of infective trypanosomes or defibrinated bovine blood into which populations of wild tsetse flies had fed. The use of stabilate material is considered to be better than the use of strains. The trypanosomes in stabilates are antigenically stable, those in strains are not. The numbers of infective trypanosomes in isolates or strains are often much less than the actual numbers(see sections 3.8 and 3.20). As one of the criteria in assessing immunity is whether trypanosomes can infect hosts, merely counting trypanosomes is considered inadequate. However, the use of infectivity for mice to standardize challenge is only suitable using isolates known to be infective and is of little use in measuring field challenge as many isolates of T.congolense and all isolates of T.vivax do not infect mice. The use of defibrinated bovine blood, into which wild tsetse flies had fed(see section 3.26), is no advance in the measurement of challenge on the technique of allowing animals to be bitten in the field. However, it sufficed for the purpose for which it was used, namely, to challenge animals repeatedly with metacyclics obtained from the same area. One advantage of this method is that animals can be challenged in areas free from the tsetse fly. Challenge can thus be controlled. There is thus little hope for the development of a method to measure accurately the challenge to cattle by all the pathogenic trypanosomes. Field

challenge can be estimated by using the infection rates of trypanosomes in tsetse as criteria. Detailed studies, such as those of Harley(173-175), on the seasonal and diurnal variation of infection rates with reference to sex and age of the main species of tsetse fly in an area are thus very important.

The precise conditions required to make maximum use of the protective immune response, and the ability of this response to resist challenge of many different antigenic types have not been adequately studied. Cattle have been made resistant to three antigenic types of the brucei sub-group(86,136). The finding that virulent material produced a longer and more durable protective antibody response than the use of dead trypanosomes (35,218,333) must be confirmed. If dead trypanosomes or some of their products were shown to be effective, the danger of drug resistance would be eliminated. The protective properties of the different trypanosomal proteins must be studied in attempts to provide simpler antigenic material. The use of adjuvants to potentiate trypanosomal antigens also should be further examined.

Effective immunological control of trypanosomiasis would be preferable to tsetse eradication methods as a permanent removal of the trypanosomes would be more likely, and to chemotherapy, as the danger of drug resistance would be removed. The development of a control method, solely by immunological means, is not possible in the immediate future due to the problems already discussed. The most promising solution to the problem of immunizing animals against trypanosomiasis probably lies in the maintenance of drug-treated stock in enzootic areas, an opinion also held by Gray(169).

The finding that the administration of berenil to animals, infected with T.congolense, increased the variant specific antibody response (see section 3.25) is thus of great importance and confirms previous observations on the synergistic action of drugs and antibodies(35, 209,335,345). The resistance of stock in enzootic areas can be expected to build up by the strategic use of drugs. The use of berenil is advocated as it is eliminated from the host extremely fast(83), does not appear to cause drug resistance(18,142) and is effective against all three groups of African pathogenic trypanosome(see section 3.26). As its trypanocidal action is of short duration, one would expect a stronger stimulation of the immune response than that caused by other drugs which persist in the body longer. It is suggested that berenil be given to animals 14 days after the first detection of parasites, assuming a prepatent period of 7 days, as the variant specific antibody titre reaches a maximum on day 21 postinfection(see section 3.20).

The reason why berenil was associated with an increase in the titre of the variant specific antibody may lie in the mode of action of the drug on trypanosomes. Recent work by Newton and LePage(273-275) has shown that berenil reacts with the DNA of both the nucleus and the kinetoplast of trypanosomes. However, whether this property is directly concerned with the mode of action of the drug is difficult to establish(LePage, personal communication). The removal of trypanosomes from the host by berenil may lead to an increase of antigenic material thereby raising the antibody response. Alternatively, antibody synthesis may continue for a short period after the removal of the antigenic stimulus.

4.11. Acquired immune mechanisms.

The immune mechanisms, which operate in animals infected with pathogenic blood protozoa are still not completely understood. A feature of pathogenic protozoa is their self-preserving adaptability which in many instances outwits the defences of the host. There seems to be an intricate and extremely sensitive relationship between parasite and host. A recent review of the different disease states and the corresponding acquired immune states caused by protozoa(323) illustrates the complexity of the problem. Basically there are two types of immunity, a sterile immunity which follows acute infection and continues for long periods after the clinical and microbiological cure, and a non-sterile immunity which accompanies the infection and disappears with the infection. The last named state is also known as premunition and occurs in diseases, which after an acute primary attack, show a long stage of chronic latent infection. Both types of immune state have been shown to occur in babesiosis(50,212,296) and trypanosomiasis(120,197,281,282,312,315,316) in cattle. In Theileriasis, a sterile immunity is thought to predominate in cattle recovered from Theileria parva but T.mutans and T.annulata give rise to premunition(16). The type of acquired immune response may thus be associated with the pathogenicity of the parasite as infections with T.parva are often fatal. The concept of premunition is based(324) on observations that animals are clinically resistant to disease only if the infecting parasites are present(17,172,296). However, there is no direct proof that clinical resistance is totally due to the presence of the parasites.

The term parasitic tolerance will thus be used to describe this condition, as suggested by Riek(296).

Autoimmunization may play some part in protozoal immunity (390). Autoimmunity is defined as the production by the body of antibody against one of its own unmodified components or against a foreign antigen whose antibody cross-reacts with a native self-antigen(28). Evidence for the action of autoimmune processes in trypanosomiasis is only circumstantial. Fiennes(121) suggested that the injury to body cells in cattle infected with T.congolense or T.vivax may be due to the adsorption of trypanosomal antigens onto host cells. Such a union may injure the host cell. The development of immunocoagglutinin may be shown to be one of the best examples of autoimmunization(62) as it may be an anti-complimentary antibody. Immunocoagglutinins occur in laboratory animals infected with T.brucei(203,204). Their titre was directly proportional to the severity of the infection.

The relationship between genetic constitution and acquired immunity has not been clearly established in trypanosomiasis. In malaria, it is known that the genes for sickle cell anaemia, persistent foetal haemoglobin and glucose-6-phosphate dehydrogenase-deficiency are associated with resistance(1). There may be such gene relationships in trypanosomiasis.

In this study, both types of acquired immune response were shown to operate in cattle infected with pathogenic trypanosomes. A sterile immunity to one stabilate of T.congolense, which lasted 8 to 24 weeks, developed in five cattle(see section 3.25). This immunity was characterized by the absence of trypanosomes and was

related to the variant specific antibody titre to the infecting trypanosomes. A state of parasitic tolerance to the natural disease developed in four cattle after the sixth challenge (see section 3.26). This immunity was characterized by the presence of circulating trypanosomes and the clinical resistance to the disease. The differences in the characteristics of these two types of resistance may be related to the type of trypanosomal challenge. In the first, the challenge consisted of a single population of trypanosomes of one detectable antigenic type; in the second, a large and immunologically diverse challenge was administered. The protective antibody response successfully eliminated the antigenically simple challenge after two inoculations and berenil treatments (see figure 3.15). However, these antibodies had no therapeutic effect in the cattle which received the immunologically complicated challenge, as berenil administration was essential to keep these animals alive (see figure 3.18). The state of parasitic tolerance, which developed in the animals after challenge 6, may have been due to the action of protective antibodies but must have been caused by a separate mechanism. The host may have developed an anti-trypanosomal factor which rendered the trypanosomes non-pathogenic, or the trypanosomes may have been able to mimic some of the host's antigens so that they were not recognized. However, the circulating trypanosomes in these animals could still infect mice after challenge 6 showing that they were non-pathogenic to the tolerant animals only. Also, common antibodies were still being synthesized after challenge 6 (see section 3.27, results of group 9) demonstrating that the trypanosomes were still antigenically

active. It is possible that the parasitic tolerance mechanism may have been due to premunition. A situation may exist, as suggested by Fiennes(120), in which a population of trypanosomes is maintained in a pre-mune host by the continuous formation of antigenic types. He considered that antigenic variation may be limited and a reversion to types, to which the host had already been sensitized, would occur. The possible antigenic reversion of T.congolense during an infection in a cow has been demonstrated (see section 3.20). In the animal concerned(zebu 495), the trypanosomes were removed from the host, by the immune response, after approximately 100 days of low level parasitaemia. The reported states of premunition in animals suffering from trypanosomiasis(120,197,281,282) may thus merely be a stage in the disease process which may persist for long periods before death or selfcure results. To confirm the definition of premunition it would be necessary to show that the presence of low levels of trypanosomes in hosts caused the elimination of the challenge organisms. The fate of such inoculated organisms could possibly be examined using isotopic or ferritin labelling techniques. The rapid multiplication of trypanosomes in hosts and the lack of fundamental information on their habitat in the host make this approach extremely difficult. The use of optical brighteners may be more useful as daughter cells hold some of the stain(181).

The differentiation of sterile immunity and premunition, in cattle suffering from trypanosomiasis, by the presence or absence of serum antibody(120,122) is considered to be untenable. In this study, the production of antibodies was found to be an integral

part of both mechanisms. The sterile immunity produced in the five test cattle of section 3.25 was associated with an antibody titre to the infecting variant of at least 10^2 . The production of common antibodies was associated with the presence of circulating trypanosomes in the test cattle of both sections 3.25 and 3.26; the titres of these antibodies only fell after the removal of the organisms from the host.

The characteristics of the parasitic tolerance of the four zebu cattle in section 3.26 are similar to those reported in resistant game animals and certain breeds of cattle(8,55,56). The resistance of some game animals and cattle is thought to be principally due to inherent factors. However, it has been shown that some game animals are more resistant than others(5,8) and that game(46,94) and resistant breeds of cattle(93) do produce antibodies against trypanosomes. Parasitic tolerance is thus probably due to both inherent and acquired factors, the combined action of which is critical as to whether pathogenic disease results after infection. The role played by genetic factors in the resistance of N'dama cattle must be greater than that in zebu cattle as, in the former resistance can be relatively easily achieved(55,56), while in the latter artificial means are required(see sections 3.25 and 3.26). In order to elucidate the relationship between innate and acquired resistance in game animals, similar immunological studies to those described in sections 3.20,3.21, 3.25 and 3.26 should be undertaken in as many different species of game as possible. Also, in the search for economically productive breeds of cattle, which can live in Africa, more attention should

be paid to the antibody response. The gene for antibody response should be made use of in breeding programmes.

In potential immunization programmes, it would be more advantageous to produce sterile immunity as opposed to parasitic tolerance, as in the last named state, a focus of the disease would still be present. As a sterile immunity can be produced in cattle challenged with stabilates of T.congolense and the brucei sub-group(see section 3.25), it is to be hoped that this type of immunity can be produced using challenges of many different antigenic types. The development of a practical method for the serological typing of trypanosomes would enable the response of cattle, to such antigenically defined challenges, to be studied.

4.12. The diagnosis of pathogenic bovine trypanosomiasis.

The diagnosis of trypanosomiasis in man and animals is dependent on methods used for the detection of the organism and on those used to detect antibody. A positive diagnosis can only be made when trypanosomes have been isolated and identified from suspect hosts. The detection of antibody is only an indicator to the presence of disease.

In this study, previous observations(81,153) on the effectiveness of the mouse inoculation and the thick and wet film techniques to detect pathogenic trypanosomes, have been confirmed. T.vivax was only detected by wet and thick films, the brucei sub-group mainly by mouse inoculation and T.congolense by all three methods(see sections 3.20,3.25,3.26,and 3.28). Mouse

inoculation was shown to be a more effective method than wet film in the detection of T.congolense(see section 3.20). The use of this method is restricted as many strains and isolates of T.congolense do not infect mice. From the results obtained in section 3.28, it is probable that these methods did not detect trypanosomes in many of the cattle in which they may have been present as judged by common antibody serum titre(see table 3.45 and group 3 in the conclusions of this section). Improved trypanosome detection methods are thus urgently required. More use should be made of the techniques used to separate trypanosomes from blood by sedimentation gradient centrifugation and anion exchangers(380,224,392).

A combination of the precipitin and agglutination(81,167) and the indirect fluorescent antibody and agglutination(354) tests has been used to study the relationship between antibody status and detectable trypanosomiasis. In all these studies, brucei sub-group antigen only was used and the number of serological positives greatly exceeded the number of trypanosome positives. The relationship between antibody titre and patent disease was not studied. The use of brucei sub-group antigen alone is thought to be of limited value as many animals serologically positive to T.congolense and T.vivax will be missed(see section 4.6). The mere detection of antibodies, without quantitative measurement, is also thought to be of little value in view of the long periods that antibodies can be detected in hosts from which trypanosomes have been eliminated(see zebu 495 in section 3.20 and the five test zebras in section 3.25).

Serological tests, which detect common antibodies, are likely

to be of most use in serodiagnosis. The agglutination test, which detects variant specific antibodies(163), was shown to be of limited value in the detection of serological positives(see table 3.44). This finding was to be expected since the antigen contained only six antigenic types of the brucei sub-group. As there are probably many more than this number of antigenic types in nature, some animals serologically positive to the brucei sub-group may have been missed. The limitations of the precipitin test, with reference to the use of T.congolense antigen, have already been discussed(see section 4.6). It is suggested that with the use of the indirect fluorescent antibody tests, A and B, an advance was made in the development of a suitable aid for the diagnosis of bovine trypanosomiasis. The IFT-A test was used to study the pattern of common antibody production in 38 cattle infected with one or all of the three main groups of pathogenic trypanosome(see section 3.27). The findings were correlated with STDM results obtained from the same animals. The results so obtained were used to analyze results from field surveys leading to the separation of cattle into four groups(see conclusions of section 3.28). This separation of animals into groups is of more benefit to the owners than merely recording them trypanosome or serologically positive or negative. The early and well established cases(groups 1 and 2) can be immediately cured with suitable drugs. Animals, which have high antibody levels but no detectable trypanosomes(group 3), must be regarded as suspect and placed under observation, while the animals with no or low antibody levels and no detectable trypanosomes(group 4) can be confidently assumed

to be free of the disease.

A standard approach to all trypanosomiasis surveys could be as follows. Blood from all animals should be examined for the presence of trypanosomes and used to prepare blood spots and sera. The presence or absence of common antibodies to trypanosomes would be ascertained by using the blood spots in the 3 antigen IFT-B. Common antibody serum titres would then be obtained from animals, whose blood spot was positive, using the IFT-A. From the results, the animals would be grouped and treated as described above.

Limitations of the indirect fluorescent antibody test are the possible lack of sensitivity and specificity. The serum titres, obtained by IFT-A, from animals known to have trypanosomiasis were shown to vary from 1/40 to 1/1,280 (see sections 3.20 and 3.27). These titres are low compared to those found by other tests, notably the indirect haemagglutination which may be applicable in the detection of common antibodies to trypanosomes. Using the last named test, serum titres of up to 1/25,600 have been reported in animals infected with T.evansi(145) and in humans infected with malaria parasites(343). The importance and problems associated with the application of indirect haemagglutination to African pathogenic trypanosomiasis has already been discussed(see section 4.6). Increased sensitivity in the detection of common antibodies would be of great benefit in the study of the relationship between the pattern of development of these antibodies and antigenic variation of trypanosomes. The specificity of the IFT-A has not been fully studied. A 2,3 or 4-plus fluorescence reading, to sera at dilutions of 1/40 or above, against antigens obtained from T.congolense, T.vivax

or brucei sub-group, was considered to indicate the presence of common antibodies to pathogenic trypanosomes (see section 3.16). The investigation of the possible antigenic interference by T.theileri in the reading of the test showed that, in sera obtained from cattle infected with this organism, positive fluorescence did not occur at dilutions of 1/40. It was concluded that this trypanosome produced no antibodies, which interfered with serological investigations on pathogenic trypanosomes. This is in agreement with Gray and Nixon (170). There still remains the possibility that common antigens exist between trypanosomes and other pathogenic blood protozoa, which may interfere with the reading of the IFT-A. Thus sera, obtained from cattle infected with Theileria and Babesia spp only, should be tested against trypanosomal antigens.

5. SUMMARY AND CONCLUSIONS.

1. The populations of T.congolense examined consisted of a range of types. There seemed no justification in the separation of T.congolense and T.dimorphon by mean length measurements.

2. The length of cyclical development of T.congolense and the resulting infection rate in G.morsitans varied with the derivatives of two stabilates of the organisms.

3. The mouse infectivity titration technique was a suitable method for counting the number of trypanosomes, in suspensions containing T.congolense, which were infective to mice.

4. The volume of sample, the concentration of glycerol, the cooling rate, and the stage of infection at which the trypanosomes were isolated were important factors in the efficient preservation of T.congolense. Preservation of blood forms in 1ml ampoules with the addition of 15% glycerol was more efficient than that in capillaries to which 7.5% glycerol had been added. Five ml amounts of infected blood could not be preserved efficiently by the methods used. The conditions required for the efficient preservation of metacyclics of T.congolense were similar to those required for blood forms.

5. T.congolense can be maintained for at least 8 hours at 0-2°C, without loss of infectivity or numbers, in buffered salt solutions at pH 6.6, 7.4 and 8.0. At pHs of below 6.6 infectivity was quickly lost. The addition of normal bovine serum did not affect the maintenance of infectivity.

6. The neutralization and indirect fluorescent antibody tests

were suitable serological tests for the examination of T.congolense. The former test distinguished between antigenic variants and detected variant specific antibodies. The latter test detected common antibodies. The trypanolytic and the indirect haemagglutination tests, in the forms described, were unsuitable.

7. In T.congolense disease of cattle, the infection was divided into three phases as judged by the presence or absence of trypanosomes. During phase 1, a succession of antigenic variants were produced to which variant specific antibodies were synthesized. Early antigenic types, in cattle infected with the same stabilate, were similar. The development of distinct antigenic types in a host may be limited. The mechanism of antigenic variation was thought to be adaptive and was associated with a temporary loss of infectivity of the trypanosomes. Variant specific antibodies to the infecting organisms were detected by the second week of infection, rose to a peak during the third week and then fell off in strength but remained in detectable quantities for many subsequent weeks. Common antibodies to the homologous antigen were detected 1 to 3 weeks after infection and rose to a peak titre within the first 100 days. High common antibody levels were only maintained in hosts in which circulating trypanosomes were present; when the trypanosomes were removed by chemotherapy or by self-cure, the common antibody levels steadily declined but remained in detectable quantities for up to 3 subsequent months. When heterologous antigens were used, a weaker antibody response was detected.

8. Brucei sub-group isolates collected up to 1,000 miles

apart have some serological similarities.

9. A sterile immunity to one stabilate of T.congolense developed in a group of five East African shorthorned zebus in response to a regime of challenge with the virulent organisms followed by berenil treatment 21 days later. This immunity, which lasted for between 8 and 24 weeks, was associated with a variant specific antibody titre to the challenge trypanosomes of 10^2 . A non-sterile immunity to the natural disease, termed parasitic tolerance, developed in four zebus which had been challenged with wild metacyclics and subsequently treated with berenil on six separate occasions. This immunity was characterized by the clinical resistance to the disease.

10. Bovine trypanosomiasis surveys should be conducted using mouse inoculation and wet and thick films, in conjunction with the IFT-A and IFT-B tests. From the results, cattle can be divided into four groups based on the absence or presence of trypanosomes or antibodies.

11. The agglutination test is of value in delineating the geographical importance of the brucei sub-group. The IFT-A and IFT-B tests in conjunction with the STDM and known tsetse distribution may be valuable in the accurate delineation of areas of active trypanosomiasis.

6. APPENDICES.

1. Solutions A and B.

a. Solution A.

Chemical	Moles.	gm/litre.	Vol.
NaCl	0.154	9.00	100
KCl	0.154	11.48	4
MgCl ₂ ·6H ₂ O	0.110	22.37	3
CaCl ₂ ·6H ₂ O	0.110	24.10	1

b. Solution B.

Chemical	Moles.	gm/litre.
NaH ₂ PO ₄ ·2H ₂ O	0.154	24.03
Na ₂ HPO ₄ ·2H ₂ O	0.103	18.33

	Volume				
NaH ₂ PO ₄ ·2H ₂ O	8.85	5.27	3.00	1.36	0.36
Na ₂ HPO ₄ ·2H ₂ O	1.15	4.73	7.00	8.64	9.64
pH	5.8	6.6	7.0	7.4	8.0

2. Mouse infectivity tables.

These tables were obtained from Lumsden et al(246). Ratios are expressed as the number of mice infected/6. Useful results are 1/6 to 5/6 and in a given dilution series there may occur one, two or three or no useful points. Series in which there are reversals, or in which four points occur, must be regarded as suspect and discarded. Proceeding from the lower to the higher dilutions the first to yield a useful point is designated x, and series are classified into those with one, two, or three useful points.

a. One point series.

Ratio	ID ₆₃	Standard error.
5/6	x.1	0.5
4/6	x.0	0.3
3/6	(x-1.8)	0.3
2/6	(x-1.6)	0.3
1/6	(x-1.4)	0.5

b. Two point series.

5/6	x.8	0.4
4/6		
5/6	x.7	0.3
3/6		
5/6	x.4	0.3
2/6		
5/6	x.3	0.3
1/6		
4/6	x.5	0.6
4/6		
4/6	x.5	0.3
3/6		

Ratio	ID ₆₃	Standard error.
4/6	x.3	0.2
2/6		
4/6	x.1	0.3
1/6		
3/6	x.3	0.5
3/6		
2/6	x.2	0.4
3/6		
1/6	(x-1.9)	0.5
2/6		
2/6	x.1	0.6
2/6		
1/6	(x-1.8)	0.5

c. Three point series.

4/6		
4/6	(x+1).2	0.6
3/6		
4/6		
4/6	(x+1).0	0.5
2/6		
4/6		
3/6	(x+1).2	0.6
3/6		
4/6		
3/6	x.8	0.4
2/6		

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Addendum.

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