AN INVESTIGATION OF THE SPECIFICITY OF THE INDIRECT FLUORESCENT ANTIBODY TEST FOR THE DIAGNOSIS OF *TRYPANOSOMA CONGOLENSE* INFECTION IN MICE

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SUMMARY

The indirect fluorescent antibody test (I.F.A.T.) has been applied to the diagnosis of *T. congolense* infection in mice, with particular regard to the specificity of the reaction. Three methods of antigen preparation and several methods of fixation were investigated. Problems encountered included damaged trypanosome morphology, loss of parasites after fixation, crystallisation on slides and non-specific fluorescence. Blood smear *T. congolense* antigen fixed in $^{N}/_{250}$ H Cl in normal saline was found to be a sensitive, specific and reliable preparation. Fluorescence was associated with the surface of the parasite and determination of an "end-point" in antiserum titrations was possible. Antigenic quality was related to the level of parasitaemia in the mice from which the antigen was prepared. The limits of reproducibility of the I.F.A.T. using H Cl fixed blood smear antigens were found to be within a four-fold difference in antibody titre and the significance of this is discussed.

Antisera prepared from *T. congolense* infected mice were tested by the I.F.A.T. using the standard *T. congolense* antigen. Antibody was detected at low titre one week post-infection and subsequently developed to high levels. Unexpected features of the pattern of antibody development were attributed to variant specificity and "eclipse" phenomena. Antibody levels in sera from mice infected with *T. brucei* and *T. vivax* were low when tested with *T. congolense* antigen, and it was therefore possible to distinguish between infections caused by different trypanosome species on the basis of higher titres obtained with homologous antisera. Antibodies in antisera from *T. congolense* infected mice which were cured by drug

treatment early in the infection, were still detected 16 weeks after treatment, making it impossible to differentiate between infected and treated animals. Antisera to T. congolense tested with T. brucei and T. vivax antigens reacted at low titres and showed a different character of fluorescence. The significance of this is discussed. Cross reactions were not detected between antisera from mice infected with some non-trypanosomal protozoal infections, and the T. congolense antigen.

INTRODUCTION AND LITERATURE REVIEW

The diagnosis of trypanosomiasis has posed problems to veterinary and medical workers since the causative agents of nagana, surra, dourine, sleeping sickness and Chagas disease were identified.

Weinman (1963), in a review of current diagnostic methods for human sleeping sickness, commented that methods had not changed over the previous fifty years. Such methods, which relied upon the microscopical examination of body fluids and animal inoculation, were estimated to fail to detect at least 20% of infected people. Obviously this situation can be disastrous for the individual patient, and seriously hampers efforts to control the disease.

Killick-Kendrick (1968) enumerated the methods of diagnosis that had been applied to animal trypanosomiasis. Diagnosis was dependent on direct microscopical examination of peripheral blood or gland juice, or animal inoculation. For practical reasons the field veterinarian usually relied totally on blood smear examination. Robson and Ashkar (1972) demonstrated the inadequacy of this approach in a comparative trial of diagnostic methods in the Lambwe Valley of Kenya. In order to detect infections of Trypanosoma congolense, T. vivax and T. brucei it was essential to examine animals by blood smear, gland puncture and mouse inoculation techniques. The time-consuming nature, practical difficulties, and insensitivity of such techniques have prompted workers to investigate serological methods of diagnosis. There has been a great deal of activity in this field over the last fifteen years and of the many tests that have been investigated, the fluorescent antibody test (F.A.T.) has emerged as one of the most promising. However, inherent in any serodiagnostic test is the problem of the delay between infection and the appearance of antibodies in peripheral blood. Also, following successful treatment or self-cure, there is often a period of sterile immunity characterised by high levels

of antibody in the absence of the infecting organism. To these two problems, trypanosomiasis brings further complications in the form of antigenic variation of the infecting organisms, the multiplicity of trypanosome species that are capable of infecting animals and the large host range that is susceptible to infection. Domestic animals can be infected by seven species of pathogenic salivarian trypanosomes, namely, T. congolense, T. simiae, T. vivax, T. brucei, T. evansi, T. equinum and T. equiperdum. In addition they can act as reservoirs of T. rhodesiense and they are also frequently infected by non-pathogenic stercorarian trypanosomes. Multiple infections with more than one species also frequently occur. The host range includes much of the wild game of Africa, and these animals provide a reservoir of infection for domestic stock. In addition to the different species of trypanosome, the possibility of each species being present in a large number of antigenic disguises, each of which stimulate the formation of specific antibodies, poses another diagnostic problem. The detection of variant specific antibodies is generally considered to be of little value for routine diagnostic purposes and workers in this field have therefore concentrated on tests that detect antibodies to more widely distributed antigens. Ideally, the epidemiologist and clinician would like a serological test capable of identifying the species of infecting trypanosome. However, it has been found that species of trypanosomes have some antigens in common which stimulate the formation of antibodies which cannot be differentiated by serological methods. In addition to these common antigens, there does appear to be a species-specific antigenic component which stimulates the formation of species-specific antibodies in the host animal. The detection of such antibodies by a simple, rapid, and reliable diagnostic test would help solve many epidemiological problems.

This project has been concerned with an investigation into the specificity of the indirect fluorescent antibody test for the diagnosis of T. congolense infection in mice. T. congolense was selected because it is probably the most important pathogenic trypanosome of domestic animals in Africa (Hoare, 1970), and is generally believed to be the most virulent trypanosome of cattle in East Africa (Mwambu, 1969).

Before considering the investigations performed, it is relevant to review the work and findings of other workers in this field.

I The application of fluorescent techniques to trypanosomiasis

The original work on the fluorescent labelling of antibody and its subsequent use for the detection of antigen (Coons, Creech and Jones, 1941; Coons, Creech, Jones, and Berliner, 1942) employed a direct reaction between antigen and labelled antibody. This direct F.A.T. was later applied to a protozoan parasite (Goldman, 1953) and in the following year, the indirect fluorescent antibody test (I.F.A.T.) was developed and applied to viruses (Weller and Coons, 1954). The advantages of the indirect over the direct method were rapidly appreciated, and the indirect test became widely used. Coons (1956) assessed the gain in sensitivity of the indirect test over the direct test to be tenfold. Other advantages were that only a single fluorescent labelled antiglobulin was required for each host species, very small amounts of sera could be tested and any loss of antibody titre due to preparation of a conjugate was avoided. Finally, it was feasible to make serial dilutions of the serum until fluorescence disappeared, thus providing a method of titrating antibody. Different fluorescent dyes have been used in the test, but fluorescein isothiocyanate (F.I.T.C.) has probably emerged as the most widely used for diagnostic purposes.

Amongst the trypanosomiases, the I.F.A.T. was first applied to Chagas disease (Fife and Muschel, 1959) in an attempt to overcome the diagnostic problems associated with this chronic and usually latent infection. A suspension of formalin-killed cultured T. cruzi mixed with a sample of the patient's serum was kept for 18 hours at 3°C. The protozoa were then washed and resuspended in a dilution of conjugated anti-human globulin for 30 minutes. Preparations of the deposit were examined microscopically and positive results indicated by fluorescence of the trypanosomes.

Weitz (1963) then applied a direct F.A.T. to T. brucei and T. vivax in an elegant experiment to demonstrate aspects of the specificity of antibodies to the "exo-antigens" and "bound antigens" of these two species.

These observations were extended to work on T. rhodesiense and T. gambiense (Williams, Duxbury, Anderson and Sadun, 1963) and to the development of an I.F.A.T. for the sero-diagnosis of African and American trypanosomiasis in man (Sadun, Duxbury, Williams and Anderson, 1963).

Kimber (1965) further modified the test for the diagnosis of human trypanosomiasis using T. rhodesiense as antigen. In the following year the I.F.A.T. was applied to bovine trypanosomiasis (Wilson, Cunningham and Kimber, 1966) using T. brucei and T. congolense blood smear antigens. The specificity of the test was examined and a modification employing discs of dried blood on filter paper as the source of antiserum was evaluated (Cunningham, Wilson and Kimber, 1966; Bailey, Cunningham and Kimber, 1966). This method had previously been applied to the serodiagnosis of trypanosomiasis by Sadun et al. (1963) after their successful use of the technique for the diagnosis of schistosomiasis (Sadun, Anderson

and Williams, 1961). This modification facilitated the use of the I.F.A.T. in epidemiological surveys of trypanosomiasis in human and animal populations. Two other modifications of the fluorescent antibody technique were also investigated. Kimber and Cunningham (1966) developed a test for antibodies against trypanosomiasis based on specific fluorescent antibody inhibition staining. This simple, one stage test relied on the prevention of staining of an antigen with homologous labelled antibody, by first exposing the antigen to the homologous unlabelled antibody. The technique was applied to human sera tested on a T. brucei antigen, and it was suggested that the test could be used to detect antibody in any animal species. The second modification (Cunningham and Kimber, 1966) employed the technique known as fluorescent staining complement (F.S.C.). The trypanosomal antigen antibody reaction was exposed to Guinea pig complement and then to anti-Guinea pig complement labelled with F.I.T.C. This technique was subsequently applied to the serodiagnosis of toxoplasmosis (Kramar, 1967) and it was found to have equal sensitivity to the I.F.A.T., with the advantage that only a single anti-Guinea pig complement conjugate is needed for any animal species.

II Application of the I.F.A.T. to the field diagnosis of bovine trypanosomiasis.

The first field applications of the I.F.A.T. were made in Kenya (Wain, Burridge, Pullan, Reid and Sutherst, 1966; Mwambu and Omaset, 1967). These early experiences indicated that the test was useful for the screening of herds in epidemiological surveys, but unreliable on an individual animal basis. Other workers in Africa were encouraged to evaluate the test and compare it with other serological methods, and with the traditional methods of diagnosis (Rogers, 1968; Mwambu, 1969; Wilson, 1969; Wiesenhutter, 1969

and 1973; Ashkar and Ochilo, 1972; Zwart, Perie, Keppler and Goedbloed, 1973; Plageman, 1974; Toure, Seydi and Seye, 1975).

The findings of this field experience, together with experimental infections, may be summarised as follows:

1) The I.F.A.T. fails to detect very recent infections. Wilson and Cunningham (1971) working with experimental infections in cattle, showed that most animals had antibodies at a titre considered positive between 8 and 21 days post-infection.

2) In trypanosomiasis endemic areas, the I.F.A.T. detected a high proportion of cattle to be serologically positive. This varied from about 30% of animals (Mwambu and Omaset, 1967, Toure et al., 1975) to about 80% of animals (Zwart et al., 1973). Examination of the same cattle by the standard trypanosome detection methods (Wilson, 1969) revealed much lower percentages of cattle with overt infections. This discrepancy was generally attributed to either a state of chronic infection, or the persistence of antibody after treatment or self-cure. Wie s enhutter (1970) found that "Berenil" treated cattle lost antibodies reacting in the I.F.A.T. after 40 days.

Wilson and Cunningham (1971) found that the period of antibody persistence varied according to the stage of infection at which treatment or self-cure occured. Those treated early in the infection had a mean persistence time of 30 days, those that had been infected for two years, a mean of 58 days, and those which cured themselves later in the infection, had antibody persisting for a mean of 110 days.

Zwart et al. (1973) considered that it should be assumed, unless otherwise shown to the contrary, that an animal with a positive I.F.A.T. is a potential reservoir of trypanosomes.

3) The test was considered to be sensitive in the hands of most workers. Wilson (1969), however, considered that the test lacked

sensitivity when compared with the indirect haemagglutination test (I.H.A.) applied by Gill (1964) to the diagnosis of T. evansi infection. This author found serum titres ranging to 1/25,600 in infected animals, compared with the range of 1/40 to 1/1,280 obtained by Wilson and Cunningham (1971) using the I.F.A.T. on trypanosome infections.

4) In the veterinary field, the test appears to be specific to the genus Trypanosoma. Toure et al (1975) failed to show any cross reactions with sera from cattle infected with Babesia bigemina, Anaplasma marginale or Setarial microfilariae. Medical workers have found cross reactions at low titres with sera from patients suffering from a few non-trypanosmal diseases. However, there is considerable contradiction concerning the specificity of the I.F.A.T. for trypanosome subgenera and species. This aspect of the test will be considered in a later section of this review.

5) In comparison with other diagnostic tests, the I.F.A.T. was found to be more sensitive than, but complimentary to, the microscopical examination of blood smears (Wilson, 1969; Wiesenhutter, 1973), gland juice smears (Zwart et al., 1973), and mouse inoculation (Wilson 1969).

The agglutination test, which detects variant specific antibodies (Gray, 1962), compared with the I.F.A.T. (Wain et al., 1966; Wilson, 1969), detected fewer serological positives.

The complement fixation test (C.F.T.) was found by Schindler (1972) to have comparable specificity but inferior sensitivity to the I.F.A.T. Mehlitz and Deindl (1972) found that the specificity of the C.F.T. varied with the different species of trypanosomes, whilst the I.F.A.T. reacted consistently more strongly with the homologous antibody. Lötzsch and Deindl (1974) found comparable specificity between the C.F.T. and the I.F.A.T. and considered the C.F.T. to be easier to

read, but the antigen was more difficult to prepare.

Wilson (1969) preferred the I.F.A.T. to the agar gel double diffusion test which also detects antibodies to common antigens (Gray, 1960), because the latter test requires large quantities of antigen, there is a delay of 24-48 hours before reading the test, and the technique is more laborious.

Robson (1972) considered the capillary tube agglutination test (C.A.T.) developed by Ross (1971), to be a valuable test for determining the degree of trypanosome challenge experienced by closed and settled herds of cattle. The technique is simpler to apply and less likely to give rise to variations in personal interpretation than the I.F.A.T. 6) Most workers found the I.F.A.T. to be rapid and simple to perform, and useful in epidemiological survey work in conjunction with sera collected by the dried blood on filter paper technique. Two limitations of the test were considered to be the requirement for sophisticated optical apparatus, and the individual variation in reading the test (Wilson, 1969).

Therefore, to conclude this review of the experiences of field and laboratory workers who have examined the I.F.A.T. for the diagnosis of bovine trypanosomiasis, it may be said that they have substantiated the opinions of early workers that the I.F.A.T. is a useful tool in field surveys to detect infected herds, but it is of limited use where information is required about the state of infection in individual animals. Wilson (1969) however had sufficient faith in the technique to recommend a standard approach for all bovine trypanosomiasis surveys. This involves examining every animal in a herd, or a 10% sample where very large numbers are concerned. Blood is examined by the standard trypanosome detection methods, and blood spots on filter paper examined by a "screening" I.F.A.T. using antigens to the three

trypanosome species. Serum titres of antibody would then be obtained by repeating the I.F.A.T. on samples from animals found positive by the screening test. From the results of these tests, cattle could be divided into four groups, namely 1) early infections, 2) established infections, 3) latent or cured infections, and 4) negatives. The differentiation of latent and cured infections (group 3) is dependent on resampling animals after 6-8 weeks. If the antibody titre has remained high, a latent infection is indicated, if the titre has fallen, a cured infection is indicated.

III Application of the I.F.A.T. to the diagnosis of human trypanosomiasis.

Following the work of Sadun et al. (1963) on the application of the I.F.A.T. to African and American trypanosomiasis in man, Lucasse (1964) successfully applied the test to the detection of trypanosomal antibodies in the cerebrospinal fluid (C.S.F.) of patients suffering from Gambian sleeping sickness. Bailey et al. (1966) followed Sadun et al. (1963) by employing the dried blood sample technique in the diagnosis of Rhodesian sleeping sickness, and later (Bailey, 1968) evaluated the I.F.A.T. alongside the diagnostic value of estimations of IgM in human sera. This study led the author to recommend a routine approach for the screening of human populations for trypanosomiasis. This involved performing IgM estimations on every person at risk. Persons with a high IgM level were selected for further clinical and parasitological investigations, whilst persons with an intermediate IgM level were further examined by the I.F.A.T. Cases positive to this test were examined clinically and parasitologically.

Wéry, Van Wettere, Wéry-Paskoff, Van Meirvenne and Mesatewa (1970b) and Frezil and Carrie (1975) found the I.F.A.T. to be more specific

and more sensitive than IgM estimations in the diagnosis of Gambian sleeping sickness. Lucasse (1970) compared the results of the I.F.A.T. after treating blood and serum samples in different ways prior to storage for variable periods. The same author found cross reactions with sera at low dilutions (up to 1/200) from patients suffering from malaria, leprosy and tuberculosis. Patients with trypanosomiasis all had titres in excess of 1/400. Sadun et al. (1963) reported cross reactions with sera from patients with malaria and leishmaniasis. Bailey (1968) reported that antibodies to T. rhodesiense were detected by I.F.A.T. 9-11 days post-infection in volunteer patients. After treatment, antibody titres were found to decay irregularly (Wéry et al., 1970b). Frezil and Carrie (1975) reported that relapsed cases and more especially terminal cases of Gambian sleeping sickness can show normal IgM levels and negative or inconclusive I.F.A.T. results. However, the I.F.A.T. performed on C.S.F. gave a positive result in one such case.

It appears that the I.F.A.T. is considered by medical workers in Africa to be very valuable. Carrie and Frezil (1973) considered it to be the best test available for mass diagnosis in sleeping sickness areas. Wéry and Burke (1972) considered it to be the only way of detecting trypanotolerant individuals who have very high titres of antibodies in the absence of clinical signs or detectable parasitaemia. However, the test was not considered satisfactory for the identification of species or sub-species of trypanosomes.

The role of the I.F.A.T. in the diagnosis of Chagas disease on the American continent appears to be highly significant. Since Fife and Muschel (1959) first applied the I.F.A.T. to the diagnosis of Chagas disease, there has been constant activity in this field

(Voller, 1963; Souza and Camargo, 1966; Camargo, 1966; Cerisola, Alvarez, Lugones and Rebosolan, 1969; Stagno, Knierim and Saavedra, 1971). Most of these workers have compared the I.F.A.T. with some of the other serodiagnostic tests available, namely C.F.T., I.H.A. and Chagas latex reagent agglutination.

Cerisola et al. (1969), comparing I.H.A., C.F.T., and I.F.A.T., found that the I.F.A.T. was the first to become positive in the acute stage of the disease. However, the I.H.A. was considered to be the more simple and rapid test to perform. The sensitivity of the I.H.A. and I.F.A.T. was comparable and superior to that of the C.F.T. Camargo and Rebonato (1969) found some cross reaction between sera from cases of leishmaniasis and Chagas disease, whilst Cerisola, Alvarez, Bock and Wegner (1971) found cross reactions with sera dilutions of 1/20 from patients suffering from syphilis, toxoplasmosis and amoebiasis. Species specificity of the I.F.A.T. for Chagas disease poses little problem as only the one human pathogenic trypanosome is found on the continent.

All authors considered the test to be satisfactory and recommended its application to the routine diagnosis of Chagas disease. Several thought that it should be used in combination with the C.F.T. and I.H.A.

IV The specificity of the I.F.A.T. in respect of trypanosome subgenera.

Mention has been made in earlier sections of this review of the extent of cross reactions of the I.F.A.T. to non-trypanosomal infections. These do not pose any serious problem with the possible exception of the cross-reaction between Chagas disease and leishmaniasis (Camargo and Rebonato, 1969; Araujo and Mayrink, 1968).

However, the question of the specificity of the test to the subgenera of the salivarian trypanosome species has aroused considerable interest since the I.F.A.T. was first applied to this problem.

Williams et al. (1963) and Sadun et al. (1963) found cross reactions between T. rhodesiense, T. gambiense, T. cruzi and T. lewisi. Weitz (1963), using conjugated antisera to the "exo-antigens" and "bound antigens" of T. vivax and T. brucei, demonstrated that the two species have bound antigens in common, but that the exo-antigens of both species reacted with the homologous species antisera only. Similar findings were made by Takayanagi, Kambara, Inoki and Yoshikawa (1971), using the immunofluorescence technique on T. gambiense and T. evansi. Wilson et al. (1966) and Wilson (1967) have shown that animals infected with T. brucei, T. vivax and T. congolense all have antibodies against common antigens, but the species can be differentiated by the I.F.A.T. on the basis of the stronger homologous reaction. Animals infected with the non-pathogenic T. theileri also produce antibodies against antigens which are common to T. theileri and to the salivarian trypanosomes, but the level of antibodies never reached a level considered positive in the I.F.A.T. The stronger reaction of sera with the homologous antigen suggested that specific antibodies must also be detected by the test. However, this specificity did not extend to antigenic variants of T. brucei or T. congolense (Wilson et al., 1966). Schindler and Sachs (1970) were also able to differentiate T. brucei from T. congolense by the titre of sera in the I.F.A.T. Schindler (1972) differentiated T. brucei, T. vivax and T. congolense by the C.F.T. and I.F.A.T. and Mehlitz and Deindl (1972) also recognised stronger homologous reactions with T. vivax, T. brucei and T. congolense.

However, Zwart et al. (1973) found that they were unable to identify pure infections with a single trypanosome species in experimentally infected cattle, sheep and goats. Politzar (1974) examined sera from cattle with mixed infections of T. brucei, T. vivax and T. congolense. The antibody titres to T. brucei and T. vivax were equal at times, whereas titres to T. congolense often remained significantly lower. In infections with either one or two species, homologous antibody titres were generally higher than heterologous titres, but the differences were often small, so that a species specific diagnosis could not always be made from the examination of a single serum sample. The relevance of this work on mixed infections was emphasised by Willett (1972) in a report on the frequency of mixed trypanosome infections in cattle examined in the Lambwe Valley project in Kenya. Observations indicated that the number of multiple infections in cattle were higher than would be expected on the basis of chance. A statistical analysis of the results and comparison with a similar phenomenon in human malarial infections, led the author to suggest that T. brucei infections may make cattle more susceptible to superinfection with T. vivax or T. congolense. If this is so, then T. brucei infections may be of great epidemiological importance in cattle, and increase the demands upon serodiagnostic methods to identify the infecting species.

Turning to the brucei group of trypanosomes, Wéry et al. (1970a) failed to distinguish between T. brucei and T. gambiense, nor could Suter-Kopp and Fricker (1972) distinguish between T. brucei and T. rhodesiense. However Knottenbelt (1971), working with experimental T. brucei infections in laboratory rodents, considered that he was able to detect variant specific antibodies by the I.F.A.T. He

characterised one stabilate and found that his results conformed with those obtained by slide agglutination. Adding to this confusion, Latif and Adam (1973) described a method of performing the I.F.A.T. that enabled T. brucei, T. rhodesiense and T. gambiense to be differentiated by the titre of the sera. If these claims prove to be well founded, the I.F.A.T. will assume a role of supreme importance in diagnostic and epidemiological investigations into human and animal trypanosomiasis in Africa.

In this consideration of the specificity of the I.F.A.T., it has become apparent that results vary from one investigator to another. The reason for this probably lies in the method of preparing the antigen, and of reading and interpreting the test.

V Methods of antigen preparation and performing the I.F.A.T.

A summary has been prepared in the form of a table to illustrate the variety of methods employed by different workers (Table I).

Latif (1972) attempted to develop a sensitive, specific, and reproducible standard technique for antigen preparation. The specificity of his results, compared with those of other workers, emphasise the importance of the method of antigen preparation. Latif (1972) considered that the improved specificity of his method was due to the washing away of antibodies or soluble antigens by phosphate buffered saline (P.B.S.) or normal sera. This eliminated the non-specific reaction and the increased specificity made it possible to differentiate brucei group trypanosomes. Non-specific fluorescence of antigens treated with controls (normal sera, P.B.S. or conjugated antiglobulin alone) has been encountered by several workers. Fife and Muschel (1959) considered that it was associated with dessication of the trypanosome and reduced it by keeping organisms in suspension during the test. Weitz (1963) using the

TABLE I METHODS OF ANTIGEN PREPARATION FOR THE I.F.A.T.

ORGANISM	ANTIGEN PREPARATION	METHOD OF FIXATION	OBSERVATIONS	REFERENCE
T. cruzi	Culture form	0.1% Formalin in saline	Trypanosomes suspended in P.B.S. in tubes to avoid non specific fluorescence of dried smears	Fife and Muschel (1959)
T. brucei T. vivax	Smears of rat or mouse blood diluted with 4-5 volumes of Alsever solution	Methanol for one minute	Direct F.A.T. using antisera to exo-antigens and bound antigens	Weitz (1963)
T. rhodesiense T. gambiense	Rat blood smear	5% Formalin + rhodamine bovine albumin	Rhodamine bovine albumin acts as counterstain	Williams et al. (1963)
T. rhodesiense T. gambiense T. cruzi	Blood smear	"		Sadun et al. (1963)
T. cruzi	Blood smear and culture forms	Acetone	Direct F.A.T., both forms fluoresced strongly in the region of the kinetoplast and around the nucleus	Voller (1963)

TABLE I (Contd.)

ORGANISM	ANTIGEN PREPARATION	METHOD OF FIXATION	OBSERVATIONS	REFERENCE
T. cruzi	Culture form	2% Formalin in buffered saline soln		Camargo (1966)
T. cruzi	Culture form	1% Formalin in P.B.S.		Souza and Camargo (1966) Camargo and Rebonato (1969)
T. rhodesiense	Rodent blood diluted with P.B.S. and centrifuged. Smear made from trypanosomes in supernate	Heat		Kimber (1965)
T. brucei *T. vivax*	As above	Heat		Wilson *et al.* (1966)
T. congolense	Mouse blood smear			
T. congolense	Mouse blood smear	Heat		Wiesenhutter (1969)
T. gambiense	Blood diluted 1:1 P.B.S.	5% Formalin in P.B.S.		Lucasse (1970)

TABLE I (Contd.)

ORGANISM	ANTIGEN PREPARATION	METHOD OF FIXATION	OBSERVATIONS	REFERENCE
T. gambiense	Blood	No fixation		Wéry et al. (1970)
T. cruzi	Culture(Amastigotes forms (Epimastigotes	1% Formalin in P.B.S.	Antigens made from Amastigotes superior	Cerisola et al. (1971)
T. brucei	Rat blood smear	N/100 H Cl or heat		Ashkar and Ochilo (1972)
T. brucei T. vivax T. congolense	Mouse blood smear. /"Triple antigen" smears made by mixing blood containing the three species./	N/100 H Cl for one minute. Distilled water for one minute. P.B.S. rinse.	Other fixation methods unsuitable: /2% Formalin, heat, methanol, acetone, acid alcohol and no fixation/.	Knottenbelt (1971)
T. brucei T. rhodesiense T. gambiense	)Differential)centrifugation of)mouse blood + dilution with normal bovine serum (N.B.S.) + centrifugation	5% Formalin for 15 minutes + P.B.S. rinse		Latif and Adam (1973)
T. congolense	Dilution with N.B.S. + differential centrifugation			

TABLE I (Contd.)

ORGANISM	ANTIGEN PREPARATION	METHOD OF FIXATION	OBSERVATIONS	REFERENCE
T. brucei	1) Differential centrifugation of mouse blood + dilution with: i) normal bovine serum ii) normal horse serum iii) normal mouse serum iv) P.B.S. v) N.B.S. + P.B.S. 1:1. 2) Blood smear. 3) Separation on D.E.A.E.-cellulose.	Heat 50-70°C for 5-15 minutes. Formalin 1,5, 10% for 5-15 minutes. H Cl 0.1, 0.3N for 1,3,5,10 minutes. Methanol for 3,5,10 mins. Acetone for 3,5,10,15 minutes.	Smears prepared from blood treated with N.B.S. and fixed by 5% formalin for 10 minutes found to give best results	Latif (1972)
T. brucei T. congolense	Mouse blood smear	Acetone for 5 mins. Methanol for 10 seconds, 0.01N and 0.03N H Cl	Acetone fixed smears considered to give the best result. H Cl fixed smears also good.	Zwart et al. (1973)
T. vivax	Goat or cow blood smear	0.3% Acetic acid 5% Formalin Heat		

direct F.A.T. considered that any procedure which favoured the proper preservation of the trypanosomes was useful in avoiding non-specific fluorescence. Contrary to the findings of Latif (1972), this author found that centrifugation yeilded non-specifically stained trypanosomes. Best results were obtained when diluted infected blood was smeared without delay, and then the smears fixed immediately after the films were dry. Wilson et al. (1966), using heat fixed blood smear antigens used a baseline serum dilution of 1/40 to overcome non-specific fluorescence and fluorescence associated with T. theileri infection in cattle. Zwart et al. (1973), however, found it necessary to dilute sera to 1/160 to overcome the same problems when testing sera on acetone fixed blood smear antigens.

Latif (1972) also investigated other aspects of the test method including the effect of the reaction time, the period of washing with P.B.S. and the effect of temperature during storage on the properties of the antigen. Knottenbelt (1971) made similar investigations with blood smear antigens which also included a study of the effect of varying the pH of the washing solutions.

From his preliminary investigations, Latif (1972) developed a standard method of performing the test which involved reaction times of 40 minutes with washing times of 10 minutes in 2 changes of P.B.S. Dried preparations were mounted in 90% glycerol in carbonate/hydrogen carbonate buffer pH 9.0. and covered with a coverslip. Slides were examined with blue light excitation, and 4 intensities of fluorescence recognised (+ to ++++). The end point of a titration was the highest dilution that gave 1 plus (+) fluorescence. This classification of fluorescence intensity into 4 or 5 degrees has been used by most authors but the selection of an intensity for the end point of titration, or for a positive result, has varied. For the purpose

of diagnosis, Lucasse (1970) and Wilson and Cunningham (1971) defined a positive serum as one which gives a 2 plus (++) reaction or higher. Wéry et al. (1970a) regarded 3 plus (+++) or 4 plus (++++) reactions as positive.

VI Objectives and outline of current work.

This review has indicated the value of the I.F.A.T. and emphasised the controversy surrounding the specificity of the test. It appears that much of the variation in results can be attributed to the method of antigen preparation and interpretation of the test employed by different workers. In this project it was decided to examine different methods of antigen preparation, to select a standard method, and then to investigate the specificity of the I.F.A.T. using the standard antigen preparation.

The host animal selected for this work was the mouse because of its availability in large numbers, ease of handling, and its susceptibility to infection with most strains of T. congolense. Irfan (1968) has demonstrated the susceptibility of intact mice to T. congolense infection and described the ensuing disease.

Considering their economic importance, the immunological aspects of T. congolense infections have been relatively little studied. The I.F.A.T. has been applied by several workers (Wilson et al., 1966; Wilson and Cunningham, 1972; Wiesenhutter, 1969; Schindler, 1972; Mehlitz and Deindl, 1972; Lötzsch and Deindl, 1974) who have demonstrated that it has antigens in common with other salivarian trypanosomes. Using the neutralisation test, Wilson and Cunningham (1972) have shown that the organism undergoes antigenic variation during the course of infection in cattle. A strain of T. congolense produces a number of antigenic variants which are characteristic of the strain, and variants are formed in a definite sequence. The situation with

T. congolense therefore appears to be similar to that with T. brucei (Gray, 1962, 1965). Wilson (1966) used the I.F.A.T. on different antigenic variants of T. congolense and failed to identify variant specific antibodies. Latif (1972) compared the neutralisation test with the I.F.A.T. and also concluded that variant specificity was not shown by the I.F.A.T.

MATERIALS AND METHODS

I Trypanosomes

1) T. congolense working stabilate

The T. congolense working stabilate used throughout this work was T.R.E.U. 1230 which was derived from T. congolense LUS/N/12/7 whose history is as follows. The original isolation was from a cow at Mtakara, Uganda by inoculation of blood into mice. 32 passages were made in mice before stabilation as E.A.T.R.O. 505 in 1960. In 1972, E.A.T.R.O. 505 was inoculated into a zebu ox and 14 days later, blood was taken and inoculated into a mouse. The mouse was bled 5 days later and stabilate LUS/N/12/7 was made.

For the current work, one capillary of stabilate LUS/N/12/7 was removed from the cryobank of Edinburgh University and the contents drawn into 0.6 ml Locke's solution (see appendix) in a 1 ml graduated syringe fitted with a 25 gauge, 5/8 inch needle. The suspension was mixed and a drop examined for live trypanosomes by phase-contrast microscopy. 5 mice were inoculated intraperitoneally with 0.1 ml doses. Tail wet blood smears were examined daily and on day 6 after inoculation, when one mouse had a teeming parasitaemia, stabilate T.R.E.U. 1230 was made from this animal.

Stabilation procedure

The procedure followed was that described by Cunningham, Lumsden and Webber (1963).

The infected mouse was bled (see section VI) and the volume of heparinised blood measured. Sterile glycerol (Analar quality, B.D.H.) as cryoprotectant, was added to the blood in a Wassermann tube placed in an ice-water bath, to give a final concentration of 7.5% by volume. The suspension was mixed with a Pasteur pipette and used to fill 40 sterile capillary tubes. The capillary tubes were sealed with a

microburner and placed, with an identification label, in a test-tube containing methanol (technical grade, B.D.H.) in an ice-water bath. When all the capillary tubes were filled and sealed, the test-tube was transferred to an insulating jacket which was closed and deposited in the solid CO2 cabinet to cool slowly overnight to -79°C. On the following day, the capillary tubes were transferred to their permanent storage at -79°C and the documentation sheet completed accordingly. The stabilate was designated T. congolense T.R.E.U. 1230 and was used throughout the work for the inoculation of mice for the preparation of antigens and antisera.

2) Other trypanosome stabilates

i) T. congolense T.R.E.U. 1170

This originated from organisms isolated from the proboscis of a male Glossina brevipalpis caught at Lugala, Uganda in 1970. The trypanosomes from the fly were inoculated into a mouse and stabilate E.A.T.R.O. 1487 laid down after 14 days. This was transferred to Edinburgh University and designated T.R.E.U. 920. T.R.E.U. 920 was passaged three times in mice, frozen as T.R.E.U. 1117, passaged again in mice and finally frozen down as T.R.E.U. 1170.

ii) T. brucei T.R.E.U. 667

The history of this stabilate is unknown apart from its origin in Uganda, probably from a zebu ox. It was designated T.R.E.U. 628 on arrival at Edinburgh before being passaged in rats and frozen down as T.R.E.U. 667.

iii) T. vivax T.R.E.U. 1223

This stabilate was obtained from the Lister Institute of Preventive Medicine, London, as (Y486). At Edinburgh it was designated T.R.E.U. 1220 before being passaged in mice and frozen down as T.R.E.U. 1223.

II Mice

The mice used were 6-8 week old, female, Swiss white mice obtained from A. Tuck and Son, The Laboratory Animal Breeding Station, Rayleigh, Essex. Mice were rested for a minimum of two days on arrival, before being inoculated.

Stock mice were caged in groups of 25. Experimental mice were caged in groups of 5 or 6 in wire-topped plastic cages which were placed in free-standing racks in the experimental animal rooms. Cages were lined with litter consisting of wood shavings and "spill dry" which was changed weekly. The mice were fed on a commercially prepared pelleted diet (McGregor and Co., Ltd., Leith, Scotland) on an ad lib basis. Water was always available.

III Mouse inoculation with trypanosome stabilate material

Stabilate capillaries were removed from cryopreservation and placed on a paper tissue. One capillary tube was usually removed for every five mice to be inoculated. Tubes were scored with a diamond marker and both ends were broken off. The contents of the capillary were taken into a syringe containing approximately 0.25 ml sterile Locke's solution. The suspension was mixed and more Locke's solution taken up and mixed to make the volume required. Mice were inoculated intraperitoneally with a 0.1 ml volume of trypanosome suspension in the midline of the ventral abdomen. The inoculated mice were marked with picric acid (4% in 70% methanol) according to the conventional code. When all the mice had been inoculated, cages were labelled and Animal Group Series sheets were completed.

IV Examination of mice for parasitaemia

Inoculated mice were screened daily when the infection was for purposes of antigen preparation, and thrice weekly for mice infected

for antisera preparation.

Mice were removed individually from the cage and allowed to stand on the cage top. Scissors, kept in 70% ethanol in a jar, were wiped dry and used to cut off the tip of the mouse's tail. The drop of blood exuding from the tail tip was picked up on a 6 x 22 mm. coverslip and placed to one end of a clean microscopic slide. The procedure was repeated for the remaining mice in the box, placing the coverslips in order on the slide according to the serial numbers of the mice. Wet films were examined by phase-contrast microscopy at a magnification of 400x. Approximately ten fields were examined, and the trypanosome counts were recorded on Animal Group Series sheets. For the statistical work described in Experimental Chapter A, Section IV, parasitaemia was expressed as log equivalent values (L.E.V.) as described by Walker (1968).

V Treatment of infected mice

Certain groups of mice infected for antisera preparation were treated to cure the trypanosome infection. The drug used was "Berenil" (Hoechst pharmaceuticals; Diminazene aceturate injection, B.Vet.C.) at a dose rate of 25 mg/Kg. The drug granules, containing 44% of active substance, were weighed out and dissolved in sterile distilled water to give a solution containing 5 mg/ml of active substance. 0.1 ml doses of this were injected intraperitoneally into mice of approximately 20 grams body weight, giving a final dose rate of 25 mg/Kg.

VI Bleeding mice

Mice were bled for three purposes.

1) Whole blood for antigen preparation.

2) Whole blood for stabilation of T. congolense T.R.E.U. 1230.

3) Preparation of antisera.

Heparin injection B.P. (Boots, Nottingham) containing 5000 units/ml was used as anticoagulant for whole blood collection. 0.05 ml of heparin was added to 4.95 ml of solution AB (see appendix) in a sterile universal container to give a final concentration of 50 units/ml.

The mice to be bled were deeply anaesthetised in an ether jar whilst a 2 ml syringe and 5/8 inch, 25 gauge needle were prepared. A little heparin solution (50 units/ml) was taken into the syringe and expelled to leave a drop within the needle hub. The anaesthetised mouse was removed from the jar and pinned out, ventral side uppermost, on a cork board. The ventral surface was swabbed with 70% ethanol and the xiphoid process and last left sternal rib were located. The tip of the needle, bevel up, was inserted into the angle between the last rib and the sternum, and advanced forwards and downwards at an angle of about 30^{o}. Slight negative pressure with the plunger of the syringe produced a flow of blood as soon as the needle tip entered the chambers of the heart. When 1.0 to 1.4 ml of blood had been collected by this method, the flow ceased and the needle was withdrawn from the chest. (Mice with chronic trypanosome infections yielded up to 3 ml of blood.) The contents of the syringe were discharged into a graduated conical centrifuge tube containing a drop of the heparin solution (50 units/ml) in an ice-water bath. The blood was mixed and further samples taken as required. Mice bled for antisera were bled in a similar manner except that heparin was not used, and the blood was dispensed into a Wassermann tube at room temperature.

VII Preparation of antisera

162 mice were obtained for the programme of inoculations designed to yield serial samples of antisera over a ten week period. The mice were distributed into ten groups of animals as detailed below.

Animal Series	Group No.	Inoculation	Number of animals	Date of inoculation
1		T. congolense TREU 1230	36	19.2.75
2		" "	36	"
3		not inoculated	12	"
4		" "	6	"
5		T. brucei TREU 667	12	"
6		" "	12	"
7		T. vivax TREU 1223	12	"
8		" "	12	"
9		T. congolense TREU 1170	12	"
10		" "	12	"

Groups 3 and 4 were to provide uninfected control sera.

Inoculations were made by intraperitoneal injection of 0.1 ml doses of the trypanosome stabilate suspensions in Locke's solution. Mice were caged in groups of six. Three labelled mice were selected from each group and their tail blood sampled thrice weekly to monitor the infection until all mice were dead.

A programme of blood sampling for serum collection was commenced seven days after inoculation and was continued throughout the period as shown below.

Animal Group No.	Date	19/2	26/2	5/3	12/3	19/3	24/3	2/4	9/4	16/4	23/4	29/4	7/5	14/5	21/5	4/6	19/6
1	INOC.		2	2	2	2	2	2	2	2	2	2		ALL MICE DEAD			
2(B)			2	2	2	2	2	2	2	2	2	2	2	2	2	3	3
3(B)			2	2	-	2	-	2	-	2	-	2		ALL MICE DEAD			
4			2	2	-	2	ALL MICE DEAD										
5	INOCULATION		2	2	-	2	1	2	ALL MICE DEAD								
6(B)			2	2	-	2	-	2	-	2	-	2		ALL MICE DEAD			
7			2	2	-	2	-	2	ALL MICE DEAD								
8(B)			2	2	-	2	-	2	-	2	-	2		ALL MICE DEAD			
9(B)			2	2	-	2	-	2	-	2	-	2		ALL MICE DEAD			
10			2	2	-	2	-	2	-	2	-	2		ALL MICE DEAD			

(B) = Treated with "Berenil" at day 10 post-infection (28/2/75). The numbers in the boxes refer to the number of mice killed for antiserum preparation on each occasion.

On day 10 of the infection, every mouse was examined by tail blood smear for evidence of parasitaemia. 100% of the inoculated mice were found to be infected and certain groups, as defined in the scheme above, were treated with "Berenil" to cure the infection. Every mouse in the drug treated groups was examined for evidence of infection three days, and again five days after treatment. No parasites were seen, but a weekly check was made thereafter on three mice from each group to detect any possible breakdowns. Mice from those groups that were to be killed for antiserum, were similarly checked for absence of trypanosomes before death. No evidence of a breakdown was found during the 10 week period, but it was found that amongst a few surviving mice from Group 2 (T. congolense TREU 1230 infected and treated with "Berenil"), single mice were detected with heavy infections of T. congolense on 4/6/75 and 19/6/75 respectively.

The technique used for bleeding mice for antiserum has been described in the preceeding section.

VIII Separation and storage of antisera

Blood was collected into labelled Wassermann tubes. Tubes were capped and placed in an incubator at 37^{o}C for 30 minutes. After this period, when a clot had formed, the tubes were removed and the clot ringed with a clean Pasteur pipette. The tubes were placed in the refrigerator at 4^{o}C for approximately one hour before being centrifuged in a refrigerated centrifuge (MSE; "Mistral 2L") at 4^{o}C and 1000g for 10 minutes. The sera were then decanted into another set of labelled, clean Wassermann tubes and centrifugation repeated. The serum was withdrawn from the tubes by an automatic syringe fitted

with a Pasteur pipette, and 0.2 ml aliquots were dispensed into either 1 ml capacity captive-top polythene tubes or 2 ml screw top glass vials (Johnson and Jorganson Ltd., London) according to availability. These were labelled individually according to their contents and date of collection. Vials containing identical samples were fastened together with adhesive tape, placed in labelled cardboard trays and stored at -20°C.

IX Preparation, storage and retrieval of antigen slides

1) Preparation

Standard antigen slides were prepared by the methods described in Experimental Chapter A, Section I.

The table overleaf shows the batches of standard and experimental blood smear antigen slides that were made for use in the experimental work.

2) Antigen storage

Antigen smears were stored at -79°C in a solid CO2 cabinet after fixation (methods of fixation are described in Experimental Chapter A, Section II). Slides were also stored at -20°C in a deep freeze and results were similar to those recorded with slides stored at -79°C. No appreciable deterioration was observed in slides stored by either method over a three month period.

a) Storage procedure

After fixation, smears were left to dry for 2 hours at room temperature before being packed in groups of five in tissue ("Kleenex medical wipes"). Each package was additionally wrapped in aluminium foil ("Alcan foil") and labelled with:

1) name of antigen and method of preparation

2) method of fixation

3) date of preparation.

ANIMAL INOCULATION			ANTIGEN PREPARATION				
Animal Group Series No.	No. of mice inoculated	Inoculum (and population sampled)	Date made/ Ref. No.	No. of mice bled	Days after infection	No. of slides made	Method
13	5	T. congolense TREU 1230	7/7	1 (1)	9	20	Experimental
13	5	" "	9/7	3 (1,2,4)	11	60	"
13	5	" " (First relapse population)	12/7	1 (4)	14	20	"
13	5	T. congolense TREU 1230 (First relapse population)	16/7	3 (1,3,5)	18	50	Standard
16	9	T. congolense TREU 1230	21/7	5	10	100	Standard
17	9	T. congolense TREU 1230	27/7	3	10	50	Standard
17	9	T. congolense TREU 1230 (First relapse population)	1/8	4	15	50	Standard
			7/8	3	9	100	Standard
15	4	T. brucei TREU 667	14/7	3	7	50	Standard
14	3	T. vivax TREU 1223	15/7	3	8	50	Standard

Unless otherwise stated, the population of trypanosomes sampled for antigen preparation, was that present at the first peak of parasitaemia following inoculation with stabilate material. Numbers in brackets e.g. (1,2,4), refer to the identity of mice that were bled individually for the preparation of antigen slides. These slides were labelled according to the mouse from which they were prepared.

Eight packages of five slides were placed in a self-seal polythene bag together with silica gel crystals and stored at -79°C in solid CO2.

b) Retrieval procedure

Packages of five slides were removed from their polythene bags in the solid CO2 chamber. The bag was resealed and replaced, and the packages transferred rapidly to a deep freeze at -20°C for one hour. They were then transferred to a refrigerator at +4°C for approximately 30 minutes and then allowed to equilibrate at room temperature for a further 30 minutes, before being prepared for the I.F.A.T.

X Preparation of "cells" on antigen slides for the I.F.A.T.

a) Whole blood smear antigens (see Experimental Chapter A, Section I(a)).

It was necessary to prepare each antigen slide to provide 12 individual cells to accept test sera and control sera dilutions. The following methods of marking out slides were investigated, using smears fixed in $^{N}/_{250}$ H Cl in normal saline.

1) Wax crayon circles.
2) Nail varnish circles (Weitz, 1963; Kimber, 1965).
3) Circles demarcated by diamond marker (Zwart et al., 1973).
4) "Magic marker" ("Pentel Pen", Japan) circles (Latif, 1972).
5) "Fluoroglide" (TFE Fluorocarbon spray, Chemplast Inc., New Jersey, U.S.A.) over suitable "mask" (Knottenbelt, 1971).

Methods (2), (4) and (5) showed promise on initial examination, but both nail varnish and "magic marker" circles tended to wash off the smear during I.F.A.T. processing. Method (5), spraying over drops of glycerol (Analar quality, BDH) produced an excellent preparation.

5) "Fluoroglide" spray technique (see Plate 1)

Thawed, fixed, blood smear antigens were placed on a black card background (to see the outline of the smear) in batches of three. Twelve drops of glycerol were placed on the smears in two parallel lines of six drops. When three slides had been treated in this way, a card "mask" was laid across the labelled ends of the three slides, and the slides were sprayed with "Fluoroglide" to leave a thin, even film. Slides were allowed to dry at room temperature for 10 minutes before flushing off the glycerol drops with a fine jet of P.B.S. (see appendix) from a wash bottle. The slides were then immersed in P.B.S. for a further 10 minutes to remove all traces of glycerol. Slides, removed from the P.B.S. wash and blotted dry, were then ready for the I.F.A.T.

Before adapting this technique for the standard antigens, it was considered necessary to investigate the possibility that application of glycerol to the smear might affect the reaction of the antigens to the I.F.A.T. A comparison was therefore made between slides with cells prepared in three different ways.

1) "Magic marker" circles.

2) "Fluoroglide" spray over glycerol drops.

3) " " " P.B.S. drops.

Results of I.F.A.T.s using the three methods with a known positive antiserum (AGS 12/17/7) showed no appreciable difference in the intensity of fluorescence or end-point titre.

The technique was found to be equally applicable to formalin fixed smears, but results with acetone and methanol fixed smears were unsatisfactory, as the "Fluoroglide" tended to wash off.

To obviate the use of glycerol drops in preparing slides, attempts were made to design re-usable perspex and brass "masks" (Plate 2).

PLATE 1

Equipment used to make "cells" on antigen slides to receive serum dilutions. The slides shown are at different stages of preparation. From left to right:

1) labelled, fixed blood smear
2) drops of glycerol applied
3) "Fluoroglide" spray applied over drops; label "masked" with card.
4) glycerol washed off to leave prepared slide.

PLATE 2

Prototypes of perspex (left) and brass (right) "masks" designed for "cell" preparation.

1


fluoro
GLIDE
TFE FLUOROCARBON PRODUCT
ALL-PURPOSE DRY-FILM
LUBRICANT and ANTI-STICK AGENT
Film Bonding
Grade
CHEMPLAST
NET WT. 16 OZ.

2


fluoro
GLIDE
TFE FLUOROCARBON PRODUCT
ALL-PURPOSE DRY-FILM
LUBRICANT and ANTI-STICK AGENT
Film Bonding
Grade
CHEMPLAST
NET WT. 16 OZ.

The prototypes were not satisfactory in that particles of "Fluoroglide" were able to penetrate on to the cells, and the cells were not capable of holding drops of serum dilutions satisfactorily. However, it is considered that further efforts to improve these "masks" might be rewarding.

b) Antigen smears of trypanosomes separated by treatment of blood with normal bovine serum and differential centrifugation

and

c) Antigen smears of trypanosomes separated by anion exchange on D.E.A.E.-cellulose (see Experimental Chapter A, Section I(b) and (c))

Slides for trypanosomes separated by these methods were prepared by the "Fluoroglide" spray method over glycerol drops. Particular attention was paid to thorough washing with several changes of double-distilled water after the "Fluoroglide" application. When dry, drops of separated trypanosome suspension were applied to the cells. It was considered that a metal "mask" would be particularly useful for preparing slides for these antigen preparations, as it would avoid the need for prolonged washing of slides after spraying.

XI The Indirect Fluorescent Antibody Test

1) Materials and method

The method adopted closely followed that described by Latif (1972).

Ten antigen slides were brought to room temperature (see Section IX, 2(b)) and for blood smear antigens, cells were prepared (see Section X, (a)). Antigen slides prepared from separated trypanosomes (see Experimental Chapter A, Section I, (b) and (c)) were rinsed in P.B.S. and gently blotted before use. Slides were labelled with a diamond marker according to the test serum being applied, and placed in a moist chamber at room temperature.

Test sera were diluted in a two-fold series from $^{1}/5$ to $^{1}/2560$ in P.B.S. in a W.H.O. perspex tray. Single drops from each dilution, commencing with the highest dilution, were delivered on to separate cells of the antigen slide with a Pasteur pipette. Similarly, single drops of P.B.S. and normal mouse serum, at a dilution of $^{1}/5$, were applied to the control cells. The arrangement is illustrated in Figure 1. In addition, one control slide was included with each test, on which the full series of normal mouse serum dilutions (i.e. $^{1}/5$ to $^{1}/2560$), were placed. When all the drops had been placed, the lid of the moisture chamber was closed and the reaction allowed to continue for 40 minutes. The slides were then removed to a sink and the sera flushed off with a jet of P.B.S. from a wash bottle. Each slide was placed on a rack over the sink and flooded with two changes of P.B.S. at 5 minute intervals, giving a total washing time of 10 minutes. Slides were then gently blotted dry and returned to the moisture chamber. Appropriately diluted swine anti-mouse globulin conjugated with F.I.T.C. (Nordic Pharmaceuticals, Sw.A.M. F.I.T.C.) was applied to each cell, the lid of the chamber closed and the reaction allowed to run for a further 40 minutes. After this, the slides were removed, washed in two changes of P.B.S. for 5 minutes each and blotted dry. Processed slides were placed in a rack in a dark drawer until examined.

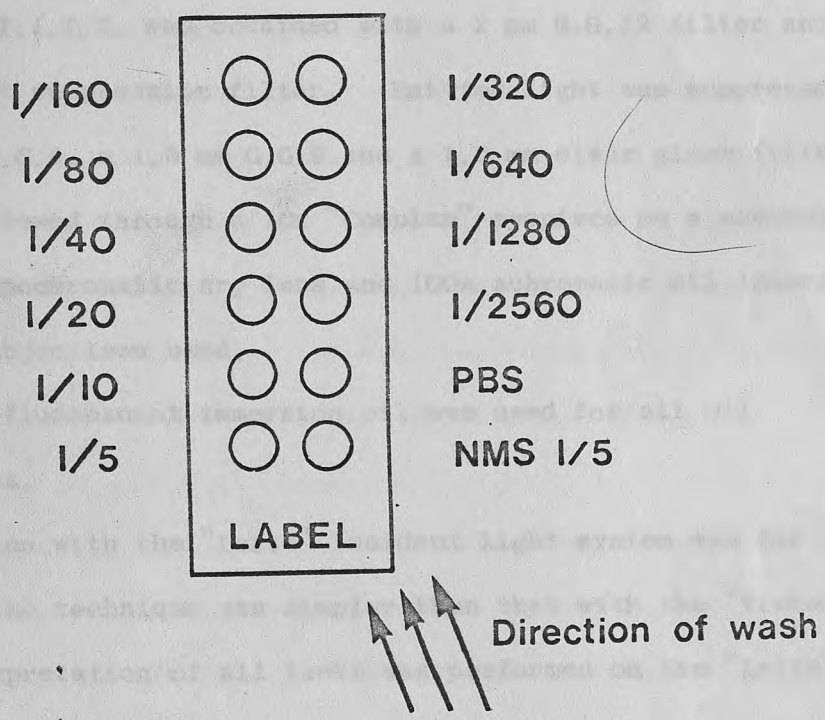
2) Examination by fluorescence microscopy

Fluoresceinisothiocyanate (F.I.T.C.) was used throughout the work as the fluorescent labelling substance. Processed slides were examined as soon as possible* with a "Leitz orthoplan" fluorescence microscope, with incident light fluorescence excitation. This equipment had an H.B.O. 200 (Osram) mercury light source with blue light excitation obtained by using one 1.5 mm B.G.12, and one

*But, see Experiments and Results, Chapter A, VI and VII.

Figure 1

Arrangement of test serum and control dilutions on antigen slides.



3 mm B.G.12 excitation filter. Blue light suitable for F.I.T.C. excitation was obtained by using two K.P.490 interference filters. A 4 mm B.G.38 heat suppression filter was also positioned in the light path. Fluorescent emission was suppressed with a K.510 edge suppression filter. The optics used were "Periplan" 8x eyepieces on a binocular head, and a 54x fluorite, oil immersion objective lens.

For comparative purposes, a few slides were also examined on a "Vickers patholux" fluorescence microscope, using the same light source but by transmitted light through a dark-field condenser. Suitable blue light for F.I.T.C. was obtained with a 2 mm B.G.12 filter and 4 mm B.G.38 heat suppression filter. Emitted light was suppressed with a 1.5 mm O.G.4, a 1.5 mm G.G.9 and a 1.5 mm clear glass filter. The image was viewed through a 10x "Complan" eyepiece on a monocular head. A 20x apochromatic dry lens and 100x achromatic oil immersion lens were the objectives used.

"Leitz" non-fluorescent immersion oil was used for all oil immersion lenses.

Illumination with the "Leitz" incident light system was far superior, and the technique was simpler than that with the "Vickers" system. Interpretation of all tests was performed on the "Leitz".

3) Reading the test

Following the conventions adapted by earlier workers (Kimber, 1965; Wilson, 1969; Latif, 1972), the intensity of fluorescence was graded into five scores from 0 to 4. It was found that the intensity of fluorescence depended largely on the method of antigen preparation and fixation. These findings are described in Experimental Chapter A, Section III, 3. It is relevant at this point to make a few general comments about the interpretation of the I.F.A.T.

Since the fluorescence fades rapidly on exposure to exciting light, it is important to make an assessment as rapidly as possible. Fading occurs more rapidly when the incident light method of illumination is used. However, it has the advantage over the transmitted light system that it is possible to move to a new "field" that has previously not been "excited", since the area of illumination is that covered by the objective lens alone. By comparison, the transmitted light system uses a dark-field condenser which illuminates the entire cell with exciting light.

It was found that it took considerable practice to be able to interpret degrees of fluorescence and end-points. This also applied to an experienced examiner using the microscope after a few days absence, when it was found that it took a little while to "get one's eye in". Absolute darkness and freedom from disturbance are essential for accurate reading. It was also found that examination of more than 10 slides at one "sitting" was tiring, whilst the accuracy of the interpretation decreased.

The procedure adopted when examining slides was to examine the control cells first, and then to move to the highest test sera dilutions, and work up the dilutions until the first "positive" was recognised (bearing in mind the degree of any fluorescence of the controls). This was the end-point dilution and examination of the remaining cells enabled an assessment of fluorescence intensity to be made and recorded.

4) Recording results

Results were recorded on cyclostyled sheets. An example is included overleaf.

The vertical line represents the "end-point" dilution as recognised by fluorescence microscopy.

Titration of serum samples from AGS 1
(T. congolense TREU 1230)

INDIRECT FLUORESCENT ANTIBODY TEST

DATE 30.7.75

MATERIAL

TEST SERA: AGS 1
TEST SERA DILUTIONS: 1/5 → 1/25⁻60
TEST SERA PRESERVATION: -20
CONTROLS: NMS 1/5 → 1/25⁻60
CONJUGATE: Sw. A.M. FITC.
CONJUGATE DILUTION: 1/80
CONJUGATE PRESERVATION: -20
ANTIGEN PREPARATION: Tc. Blood H 21/7
ANTIGEN FIXATION: H
ANTIGEN PRESERVATION: -80

METHOD

Standard.

RESULTS

TEST SERUM	CONTROLS				TEST DILUTIONS												OBSERVATIONS
	NMS 1/5	—	P.Bs.		1/5	10	20	40	80	160	320	640	1280	2560	—	—	
NMS	1			0	1	1	1/0	1/0	1/0	0	0	0	0	0			
AGS 1 1	1			0	2	2	1	0	0	0	0	0	0	0			
2	1			0	4	3	2	2	2	2	2/1	1/0	0	0			
3	1			0	2/3	3	2	1/0	0	0	0	0	0	0			
4	2/1			0	4	4	3	2	2/1	1	1/0	0	0	0			
5	1			0	4	4	3	3½	2	2	2/1	1	1/0	0			
6	2/1			0	4	4	4/3	3	3½	3½	2	1	1/0	0			
7	1			0	4	4/3	3	2	2	2	1	1/0	0	0			
8	1			0	4	4	3	2	2	2	1/0	1/0	0	0			
9	1			0	4	3	3	3	3	2	2	2/1	1	0			

The numbers refer to intensity of fluorescence. It was found that even with practice, it was still sometimes difficult to assign a score to certain degrees of fluorescence when the intensity fell between two scores. These were recorded as such (e.g. 4/3).

5) Storage and titration of swine anti-mouse F.I.T.C. conjugate

The commercial fluorescence conjugate (Sw.A.M. F.I.T.C.) used for the work was reconstituted from its freeze-dried state with 2 ml distilled water. After 15 minutes, 0.05 ml aliquots were drawn off and dispensed into 1.0 ml captive top polythene tubes, labelled and stored at -20°C.

Each batch of conjugate was titrated against a standard antigen and Known positive antiserum and negative controls. Two-fold dilutions of antiserum and appropriate controls were applied to cells on eight antigen slides, allowed to react for 40 minutes and the dilutions washed off. Conjugate dilutions of $^{1}/_{20}$, $^{1}/_{40}$, $^{1}/_{80}$ and $^{1}/_{160}$ were made up in P.B.S. and each dilution was applied to the cells of two slides. After a further 40 minutes and P.B.S. wash, slides were examined by fluorescence microscopy and the highest dilution of conjugate at which non-specific (normal mouse serum and P.B.S. controls) and background fluorescence were minimal, whilst specific (Known positive antiserum) fluorescence was maintained, was determined. This was the conjugate dilution at which all subsequent tests, using this batch of conjugate, were run. For the two batches of conjugate used in this work, the optimum dilution was $^{1}/_{80}$. At this dilution, one ampoule of commercial conjugate will process 400 antigen slides.

XII Photomicroscopy and fluorescence photomicroscopy

All photomicrographs were taken on a "Leitz orthomat" 35 mm camera body mounted on a "Leitz orthoplan" microscope with the 54x fluorite oil immersion objective lens.

Brightfield photomicrographs were taken on Ilford Pan.X film with automatically computed exposure times. The film was developed in "Ilford Developer 11", 1+1, for 7½ minutes at 68°F, intermittent agitation.

Fluorescence photomicrographs were taken on Ilford HP4 film with exposure times automatically computed for a film speed setting of 12 ASA. This gave exposure times of between 1 and 2 minutes. The film was developed in "Ilford Developer" 1+1 for 17 minutes at 68°F, intermittent agitation.

XIII Statistical methods

The statistical methods used for the analysis of results obtained with the I.F.A.T. in investigations of the reproducibility of the test, were as follows.

1) Analysis of variance for one way classification.
2) "t test" to compare means.
3) Calculation of correlation coefficient to detect linear relationships.
4) Linear regression to calculate line of best fit by the method of least squares.

The tables used were from Snedecor (1950).

EXPERIMENTS AND RESULTS

CHAPTER A

Section I Investigations of methods of preparing antigen slides

Three methods of preparing T. congolense antigen slides were investigated.

a) Blood smear preparations.

b) Smears of trypanosomes separated by treatment of infected whole blood with normal bovine serum and differential centrifugation (Latif, 1972; Latif and Adam, 1973).

c) Smears of trypanosomes separated by anion exchange filtration on D.E.A.E.-cellulose (Lanham and Godfrey, 1970).

For all these methods, groups of 4 or 5 mice were inoculated with T. congolense TREU 1230 stabilate material. The infection was followed daily by tail blood smear examination, and attempts at antigen preparation made when the parasitaemia reached 50 trypanosomes per high power field (H.P.F.) (400x magnification) or more.

Antigen smears were made on chemically clean (chromic acid - see appendix) 76 mm x 25 mm microscope slides of 0.8 to 1 mm thickness ("Chance Propper" Ltd.).

a) Blood smear preparations

These were made in the conventional manner using a glass spreader to produce a smear of medium thickness along the whole length of the slide. Mice with a parasitaemia of 100 trypanosomes per H.P.F. were selected, and blood expressed from the cut tail tip directly on to the end of the slide. With practice, it was easy to deposit the correct amount of blood to produce a smear of satisfactory length and thickness. Up to twenty smears could be produced from one mouse without any obvious ill-effect on the animal. If the high parasitaemia persisted, it was possible to repeat the bleeding two

days later.

Trypanosome concentration in heparinised blood

In order to make large batches of antigen slides, it was necessary to pool the blood of several infected mice. It was found that trypanosome numbers on the antigen slides could be improved by concentrating the blood. This was achieved by the following method.

Individual mice were bled from the heart into a heparinised syringe. The blood from several mice was thoroughly mixed in a graduated conical centrifuge tube in an ice-water bath. The tube was transferred to a refrigerated centrifuge at 4°C and centrifuged at 250g for 10 minutes. On removal of the tube from the centrifuge, the approximate volumes of packed red cells and plasma were estimated from the graduated scale on the tube. Using a long Pasteur pipette attached to an automatic syringe, the top half of the plasma was removed from the tube and placed in a clean Wassermann tube. The syringe piston was reset to remove half of the volume of packed red cells. The tip of the pipette was passed to the bottom of the tube and half of the packed cells were removed and added to the extracted plasma in the Wassermann tube. The graduated centrifuge tube was replaced in its ice-water bath and the blood re-mixed. Examination of a wet blood smear by phase-contrast microscopy revealed that the concentration of trypanosomes had been approximately doubled by this procedure. Drops of blood were delivered on to clean slides and smears made in the usual manner.

Following their preparation, all slides were labelled and dried in an incubator at 37°C for 15 minutes.

b) Smears of trypanosomes separated by treatment of infected whole blood with normal bovine serum and differential centrifugation

Heparinised blood from several mice was mixed in a centrifuge tube in an ice-water bath. The blood volume was estimated and mixed with

5 volumes of normal bovine serum. The suspension was allowed to stand for 5 minutes, after which the red cells were sedimented by gentle centrifugation and the supernatant, containing about 90% of the trypanosomes, was removed. This was then centrifuged at 3000g for 10 minutes and about 4/5 of the supernatant removed to leave a concentrated suspension of trypanosomes at the base of the tube. Using a finely drawn out Pasteur pipette, drops of the suspension were placed on slides prepared with cells by the "Fluoroglide" spray technique. Drops were immediately sucked off with the same pipette, to leave a thin film of trypanosomes over the cell. Slides were labelled and dried for 15 minutes in an incubator at 37°C.

c) Smears of trypanosomes separated by anion exchange on D.E.A.E.-cellulose

Equilibration of D.E.A.E.-cellulose

50g D.E.A.E.-cellulose (Whatman D.E.52) was suspended in 200 ml of phosphate glucose saline (P.S.G., see appendix). The pH of the P.S.G. suspension was lowered to pH 8 with 5% orthophosphoric acid (see appendix). After the main bulk of the exchanger had settled, the supernatant fluid containing the fines was removed with a suction pump. The slurry was washed a further three times by decantation with 200 ml volumes of P.S.G. The equilibrated D.E.A.E.-cellulose was left at 4°C if it was to be used within 24 hours. For longer delays the slurry was stored at -20°C, thawed when required, resuspended in 5 volumes of fresh buffer, and any fines then removed.

Preparation of columns

The slurry of equilibrated D.E.A.E.-cellulose was packed in cylindrical sintered glass funnels of porosity size 3, and capacity 30 or 60 ml ("Gallenkamp"). A disc of Whatman No. 1 filter paper was placed on the sintered glass disc beneath the cellulose. The funnel,

which was supported in a retort stand, was packed to within 1 cm of the top. The flow of eluate was controlled by a screw-clip on plastic tubing fitted to the column outlet. When the column was firmly packed, a Whatman No. 1 filter paper disc was placed on top of the cellulose and approximately 1 ml of freshly collected, heparinised, infected mouse blood was carefully run onto the disc with a Pasteur pipette. The clip on the outlet tube was opened, and after all the blood had entered the cellulose, P.S.G. was run through the column and the eluate collected in conical centrifuge tubes. The funnel was kept topped up with P.S.G. and a drop of eluate checked every few minutes for the presence of trypanosomes by phase-contrast microscopy. As soon as trypanosomes appeared, a labelled, empty centrifuge tube in an ice-water bath was positioned to collect the eluate. Approximately 60 ml of eluate containing trypanosomes was collected in four or five centrifuge tubes. The centrifuge tubes were transferred to the refrigerated centrifuge at $4^{o}C$, centrifuged at 3000g for 10 minutes, and the supernatant discarded. The packed trypanosomes were resuspended in the few drops of P.S.G. remaining in the tubes and the suspensions collected together into one tube. The concentration of this suspension was adjusted, so that a drop, placed and then sucked off a cell on a prepared slide, left a thin film of evenly spread trypanosomes. When satisfactory, drops of the suspension were applied to the slides, the slides labelled with a diamond marker, and dried at $37^{o}C$ in an incubator for 15 minutes.

Assessment of antigen preparation methods Table 2

The three methods of preparation described above were assessed according to the five parameters listed in Table 2. Method (C) was considered to produce the best preparation, but this was also the

TABLE 2 ASSESSMENT OF ANTIGEN PREPARATION METHODS

Antigen preparation method	Facility of technique	Trypanosome numbers before fixation and processing ≠	Trypanosome spread ≠	Trypanosome morphology before fixation ≠	State of background
A. Whole blood smears and concentrated whole blood smears.	Simple Rapid	++	Good. Tendency to clump when parasitaemia very high	Good	Cellular elements of whole blood
B. Smears of trypanosomes separated by normal bovine serum and differential centrifugation	Simple Rapid	+++	Clumping	Good	Crystallisation
C. Smears of trypanosomes separated by anion exchange on D.E.A.E.-cellulose	Straightforward but dependent on solutions of critical conductivity and pH. Prolonged	+++	Excellent	Good	Crystallisation

Trypanosome numbers (++ sufficient
(+++ excellent

≠ By phase contrast microscopy and brightfield examination of Giemsa stained preparations.

most laborious technique. Of the simpler techniques, (a) and (b), method (b) was superior when considering these parameters alone.

Section II Investigations of methods of fixing antigen slides for the I.F.A.T.

The methods investigated were as follows.

1) Methanol (Analar quality, BDH) for 1 and 2 minutes.
2) Acetone (BDH) for 15 and 30 seconds, 1, 2 and 5 minutes.
3) 5% Formalin (Formaldelyde solution, 37-41% HCHO $^{w}/_{v}$, BDH) made up in distilled water, or in normal saline (NS., see appendix) for periods of 5, 10 and 15 minutes.
4) $^{N}/_{250}$ Hydrochloric acid (Hydrochloric acid, S.G. 1.180, 35-37% $^{w}/_{w}$, M and B) made up in distilled water or in normal saline, for periods of 2, 5, 10 and 15 minutes.

Antigen smears for fixation were labelled according to the fixative to be used, arranged in slide racks and immersed in the fixing fluid for the period specified. Racks were then removed and the slides were allowed to air dry at room temperature, before being packed in tissue and aluminium foil.

Assessment of antigen fixation methods

Slides were assessed by the parameters listed in tables 3, 4 and 5. This involved examining slides in the following ways.

1) After fixation, by Giemsa staining (see appendix) and brightfield microscopy.
2) After I.F.A.T. processing, by Giemsa staining and brightfield microscopy.
3) After I.F.A.T. processing, by fluorescence microscopy.

The results are summarised in the tables 3, 4 and 5. Plates 3 to 8 are photomicrographs of antigen slides prepared and fixed in different ways, and stained with Giemsa. They illustrate some features of the different methods, as recorded in Tables 2, 3, 4 and 5.

TABLE 3 ASSESSMENT OF ANTIGEN FIXATION METHODS ON WHOLE BLOOD SMEARS

Fixation		Trypanosome numbers		Trypanosome morphology	I.F.A.T. - fluorescence intensity at serum dilutions of 1/10				Other observations	
Method	Time in Mins	After ≠ fixation	After I.F.A.T. processing		Specific antiserum	Control normal mouse serum	Back-ground	Determin-ation of endpoint		
Methanol	1 2	++	++	Good	2	1	3	Impossible	Smear does not dehaemo-globinise. Whole trypanosome fluoresces.	Lymphocytes fluoresce at lower dilutions of specific and control sera
Acetone	2 5	++	Variable	Variable	4	2	2/3	Difficult	Smear dehaemo-globinises after P.B.S. rinse. Whole trypanosome fluoresces	
Formalin 5% in distilled water	2 5 10 15	++	++	Good	2	2	2	Impossible		

TABLE 3 (Contd.)

Fixation		Trypanosome numbers		Trypanosome morphology	I.F.A.T. - fluorescence intensity at serum dilutions of 1/10				Other observations	Lymphocytes fluoresce at lower dilutions of specific and control sera
Method	Time in Mins	After ≠ fixation	After I.F.A.T. processing		Specific antiserum	Control normal mouse serum	Back-ground	Determin-ation of endpoint		
H Cl	2	+	+							
N/250 in distilled water	5 10 15	++	++	Poor	2	1	1	Impossible		
H Cl N/250 in normal saline	5 10 15	++	++	Good	4/3	1	1	Possible	Trypanosomes show fluor-escence concentrated at the perimeter	

Trypanosome numbers	(+	too few	1 Low)	Fluorescence intensity in the I.F.A.T.
	(++	sufficient	2 Moderate)	
	(+++	excellent	3 High)	
			4 Very high)	

≠ By examination of Giemsa stained preparations.

TABLE 4 ASSESSMENT OF ANTIGEN FIXATION METHODS ON SMEARS OF TRYPANOSOMES SEPARATED BY TREATMENT OF BLOOD WITH NORMAL BOVINE SERUM AND DIFFERENTIAL CENTRIFUGATION

Fixation		Trypanosome numbers		Trypanosome morphology	I.F.A.T. - fluorescence intensity at serum dilutions of 1/10				Other observations
Method	Time in Mins	After ≠ fixation	After I.F.A.T. processing		Specific antiserum	Control normal mouse serum	Background	Determination of endpoint	
Acetone	5	+++	Variable	Poor	2	2	-	Impossible	crystallisation on wells at higher dilutions
Formalin 5% in distilled water	15	+++	Variable	Good	4	3	-	Difficult	
H Cl $^{N}/_{250}$ in distilled water	10	+++	Variable	Fair	4	3	-	Difficult	

Key to symbols as TABLE 3

TABLE 5 ASSESSMENT OF ANTIGEN FIXATION METHODS ON SMEARS OF TRYPANOSOMES SEPARATED BY ANION EXCHANGE ON D.E.A.E.-CELLULOSE

Fixation		Trypanosome numbers		Trypanosome morphology	I.F.A.T. - fluorescence intensity at serum dilutions of 1/10				Other observations
Method	Time in Mins	After ≠ fixation	After I.F.A.T. processing		Specific antiserum	Control normal mouse serum	Back-ground	Determin-ation of endpoint	
Acetone	1/4 1/2 1 5	+	Variable	Poor	Variable	2	-	Impossible	Crystall-isation on wells at higher dilutions
Formalin 5% in distilled water	15	Variable	Variable	Fair	Variable	2	-	Difficult	
Formalin 5% in normal saline	15	Variable	Variable	Good	4	2	-	Difficult	
H Cl N/250 in distilled water	10	++	Variable	Fair	2	2	-	Impossible	
H Cl N/250 in normal saline	10	++	Variable	Good	4	2	-	Difficult	

Key to symbols as TABLE 3

PLATES 3 and 4

T. congolense blood smear antigen

PLATE 3

Fixation by 5% formalin for 15 minutes.

Stained by Giemsa.

Trypanosome morphology is good, numbers are adequate, but parasites are tending to clump together.

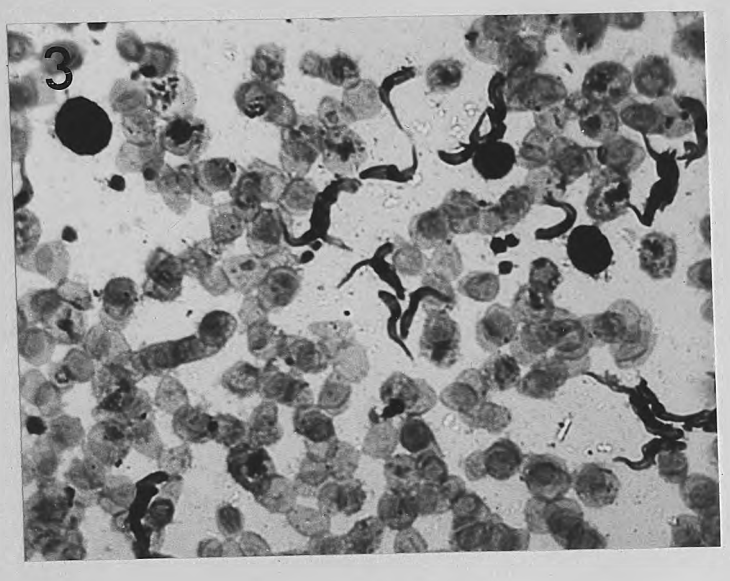
PLATE 4

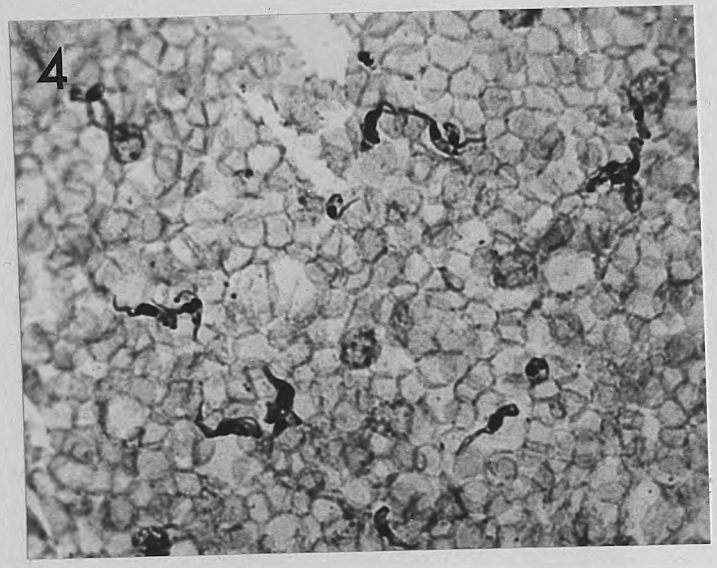
Fixation by $^{N}/_{250}$ HCl in normal saline for 10 minutes.

Stained by Giemsa.

Trypanosome morphology is good, numbers are adequate, but parasites are tending to clump together.

(Dehaemoglobinised red blood cells, platelets and lymphocytes have stained less intensely than in the formalin fixed smear, Plate 3.)


3


4

PLATES 5 and 6

T. congolense antigen prepared from trypanosomes separated by sedimentation of red blood cells with N.B.S., followed by differential centrifugation.

PLATE 5

Fixation by 5% formalin for 15 minutes.

Stained by Giemsa.

Trypanosome morphology and numbers are good, but there is marked clumping.

PLATE 6

Fixation by acetone for 10 minutes.

Stained by Giemsa.

Trypanosomes have clumped together and their morphology is disrupted.

Section III Conclusions from the investigations described in Sections I and II

1) Trypanosome numbers after fixation and I.F.A.T. processing. These were adequate on blood smear antigens, with the exception of acetone fixed slides and two minute fixation with $^{N}/_{250}$ HCl. Numbers of trypanosomes remaining on preparations made from separated trypanosomes, varied from slide to slide and from cell to cell.

2) Trypanosome morphology. The following methods gave trypanosomes of good morphology.

a) Blood smear antigens fixed by methanol, 5% formalin and $^{N}/_{250}$ HCl in N.S.

b) Antigens, prepared from trypanosomes separated by treatment of blood with N.B.S. and differential centrifugation, and fixed by 5% formalin.

c) Antigens, prepared from trypanosomes separated by anion exchange on D.E.A.E.-cellulose, and fixed by 5% formalin in N.S. and $^{N}/_{250}$ HCl in N.S.

3) Performance in the I.F.A.T.

a) Blood smear antigens.

Acetone and $^{N}/_{250}$ HCl in N.S. fixed smears were the only methods giving satisfactory fluorescence with specific antisera. The fluorescence intensity of acetone fixed trypanosomes was excellent, but trypanosome numbers were often low, and background and negative control fluorescence were unacceptably high at the lower dilutions of antisera. $^{N}/_{250}$ HCl in N.S. gave consistently good results, although the intensity of fluorescence was less than that with acetone fixation. The fluorescence was concentrated at the perimeter of the parasite. Negative control and background fluorescence were minimal, and with practice, it became easy to

determine an "end-point" of fluorescence. (See Experimental Section IV, (1)).

All methods of fixation of blood smear antigens investigated, produced smears in which the lymphocytes (which were also concentrated by the trypanosome concentration technique described in Section I (a)) fluoresced at the lower antiserum dilutions. This did not interfere with the reading of the test, and was in fact used to advantage, as the lymphocytes provided excellent points on to which to focus the microscope.

b) Antigens of trypanosomes separated by treatment of blood with N.B.S. and differential centrifugation.

Fixation by 5% formalin and $^{N}/_{250}$ H Cl gave excellent fluorescence but negative control fluorescence was very high and interpretation of the test was therefore difficult. Crystallisation on cells at the higher dilutions of antisera tended to obscure trypanosomes.

c) Antigens of trypanosomes separated by anion exchange on D.E.A.E.-cellulose.

Fixation by 5% formalin in N.S. and $^{N}/_{250}$ H Cl in N.S. gave good specific fluorescence, with a moderate level of negative control fluorescence. Crystallisation at higher dilutions of antisera was a problem, but the main problems were the variability in the numbers of trypanosomes, and the difficulty in determining an "end-point" of fluorescence.

Plates 9 to 12 are fluorescence photomicrographs of different antigen preparations, fixed by various methods. They illustrate some of the features mentioned above.

PLATE 9

T. congolense antigen prepared from trypanosomes separated by anion exchange on D.E.A.E.-cellulose.

Fixation by 5% formalin for 15 minutes

Antigen tested with positive homologous antiserum by I.F.A.T.

Whole body or "somatic" fluorescence of "4 plus" intensity.

Parasites appear "bloated".

PLATE 10

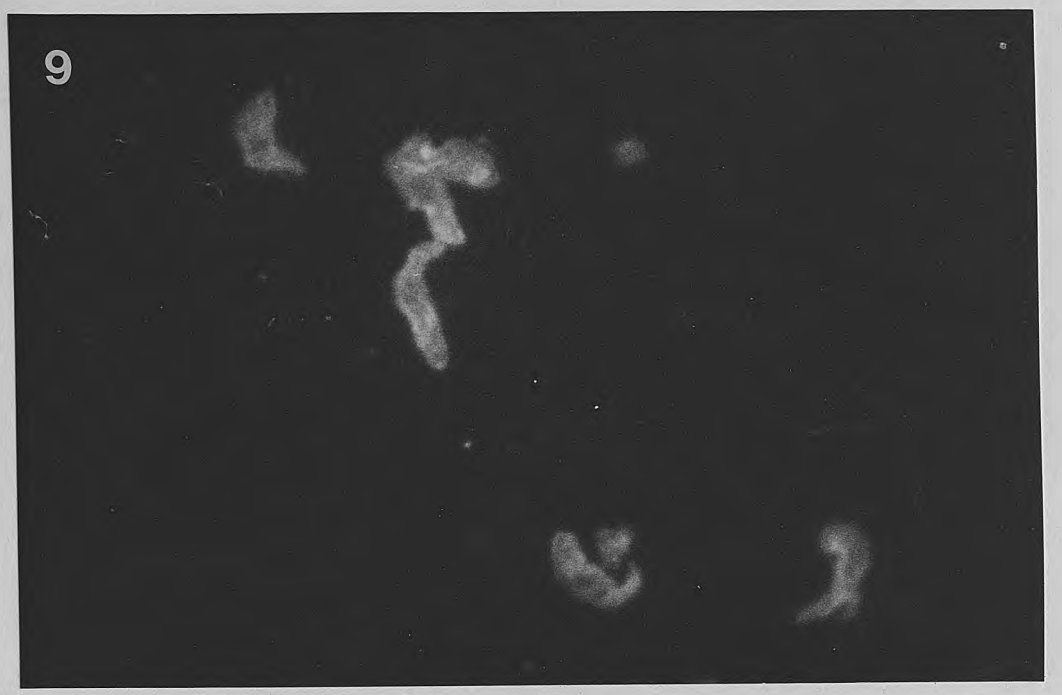
T. congolense blood smear antigen.

Fixation by $^{N}/_{250}$ HCl in normal saline for 10 minutes.

Antigen tested with positive homologous antiserum by I.F.A.T.

Perimeter or "surface" fluorescence of "4 plus" intensity.

Trypanosome morphology good.


9

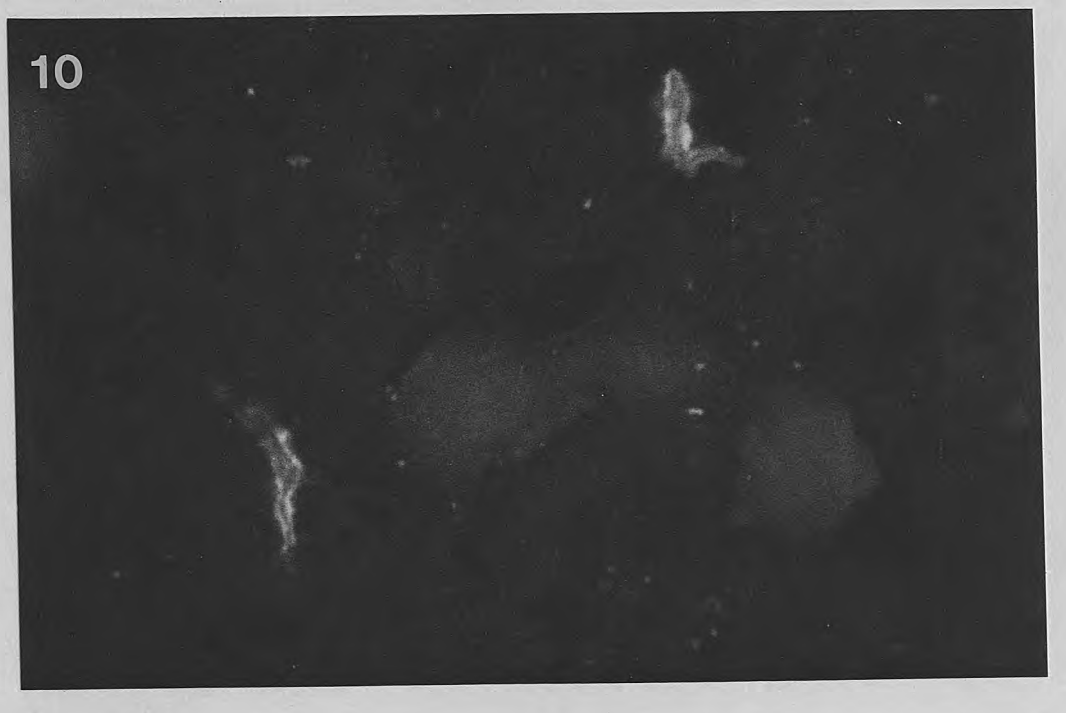

10

PLATE 11

T. congolense blood smear antigen.

Fixation by acetone for 10 minutes.

Antigen tested with positive homologous antiserum by I.F.A.T.

"Somatic" fluorescence of "$^{4}/_{3}$" intensity.

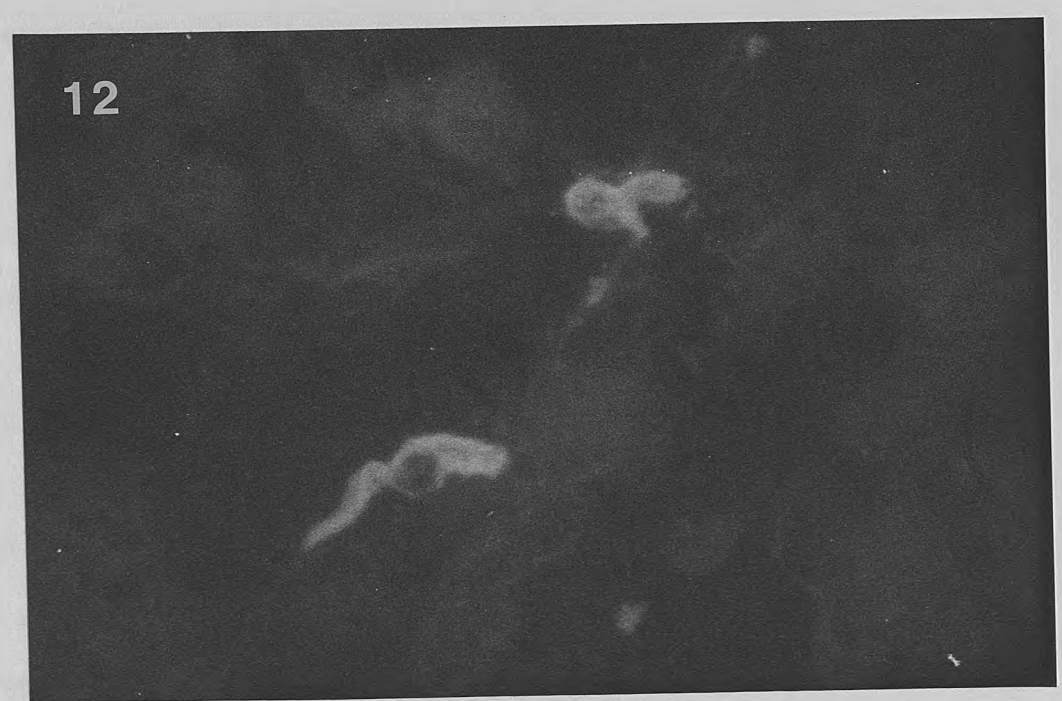
Trypanosome morphology is good.

PLATE 12

T. congolense blood smear antigen.

Treated as above, but showing damaged trypanosomes.


11


12

4) Effect of time of fixation of antigen preparations

The times, recorded in the tables adjacent to the different fixatives, were used. Varying the times had no appreciable effect on the performance of the antigens, with the exception of the 2 minute fix in $^{N}/250$ H Cl of blood smear antigens. These suffered considerable loss of trypanosomes and blood elements.

Selection of the most promising method of antigen preparation and fixation

For the reasons enumerated above, blood smear antigens, fixed in $^{N}/250$ H Cl in N.S., were selected as the most promising antigen preparations.

Section IV Further investigations of the performance of blood smear antigens fixed by $^{N}/250$ H Cl in normal saline

1) Interpretation of the I.F.A.T.

Trypanosomes in blood smears fixed by $^{N}/250$ H Cl in N.S., treated with homologous antiserum and then F.I.T.C. labelled conjugate, characteristically have a fluorescence which is concentrated around the perimeter of the organism. The intensity of the fluorescence was recorded by a system of scoring from 0 to 4 plus.

0 no trypanosomes visible,

1 plus (+) trypanosomes faintly visible after careful scrutiny,

2 plus (++) trypanosomes visible with a lightly fluorescing perimeter,

3 plus (+++) trypanosomes visible with a strongly fluorescing perimeter,

4 plus (++++) intense fluorescence, especially at the perimeter.

It was found that the intensity of fluorescence frequently fell between two scores, and was then recorded as such. Intensities of 3 and 4 plus were often particularly difficult to differentiate.

With practice, it was found to be quite easy to recognise the "end-point" fluorescence intensity. This point was the first dilution (moving up the slide, from high to low dilutions) at which it was possible to see the shape of the trypanosome clearly outlined by its lightly fluorescing perimeter. This intensity was graded 2 plus.

2) Reproducibility of I.F.A.T. results

A batch of antigen slides (Ref. 7/7) was made by the selected method from a single mouse infected with T. congolense TREU 1230. Tail blood was used.

Slides were tested against a known positive antiserum (AGS 12/17/7) prepared from mice with a chronic T. congolense TREU 1230 infection. The test was performed on four different occasions, on a total of seven slides.

The results are illustrated in figure 2. They show a variation from 1/640 to 1/1280 which is statistically insignificant. ($F_3^3 = 2.45$; $P > 0.05$).

3) Investigations of the effect of using different trypanosome populations as the source of antigen material for slide preparations

Five mice were inoculated with stabilate suspensions of T. congolense TREU 1230. The parasitaemia was followed daily and tail blood smear antigen slides were prepared on different occasions from individual mice, as illustrated in Figure 3. Slides were labelled, dried, fixed and stored in the described manner.

Using a known positive antiserum (AGS 12/17/7) prepared against T. congolense TREU 1230 the I.F.A.T. was performed on

a) antigen slides, in duplicate, made from three individual mice on the same day (9/7)

b) antigen slides, in duplicate, made on three separate occasions, from members of the same group of mice (7/7, 9/7 and 12/7).

The results are illustrated in figure 4(a). A similar pattern of results was recorded when the tests were repeated on another occasion. Statistical analysis of both sets of results has shown there to be a significant difference ($F^{4}_{10} = 5.43$ $P < 0.05 > 0.01$) between I.F.A.T. "end-point" titres recorded with the different antigens. It has further been shown that a correlation exists between the parasitaemia of individual mice at the time of antigen preparation and the I.F.A.T. titre recorded with those antigens ($r = +0.6736$). A linear regression has been plotted in Figure 4(b) to show this relationship.

Analysis of the I.F.A.T. titres recorded with antigens made from three individual mice on the same occasion (9/7), failed to show any significant difference ($F^{2}_{6} = 1.7788$; $P > 0.05$).

Section V Development of the standard antigen preparation

Following the observations made above, Sections I, II, III and IV, three factors were considered to be of importance in making a standard antigen preparation.

1) The antigen to be made from a blood smear preparation.
2) " " " " fixed by $^{N}/_{250}$ H Cl in N.S.
3) The antigen preparation to be made from trypanosome populations at a fixed point in the stage of the infection. This was defined as the stage of first peak parasitaemia, when tail wet blood smears showed counts of 100 or more trypanosomes per H.P.F. Using T. congolense TREU 1230 as the inoculum, this stage was reached between 8 and 10 days after inoculation (see Materials and Methods, Section IX).

Explanation of Figures 2,4,5 & 6.

—— Endpoint of titration.

[2] 2 plus intensity of fluorescence.

[3] 3 " " " "

[4] 4 " " " "

Each block on the histogram represents one test slide.

All antigens were prepared from T.congolense TREU 1230 infected mice by the blood smear technique, & fixed by N/250 HCl in N saline.

Figure 2

The intensity of fluorescence shown by blood smear antigens (batch 7/7) tested with positive antiserum (AGS 12/17/7) on 4 occasions.

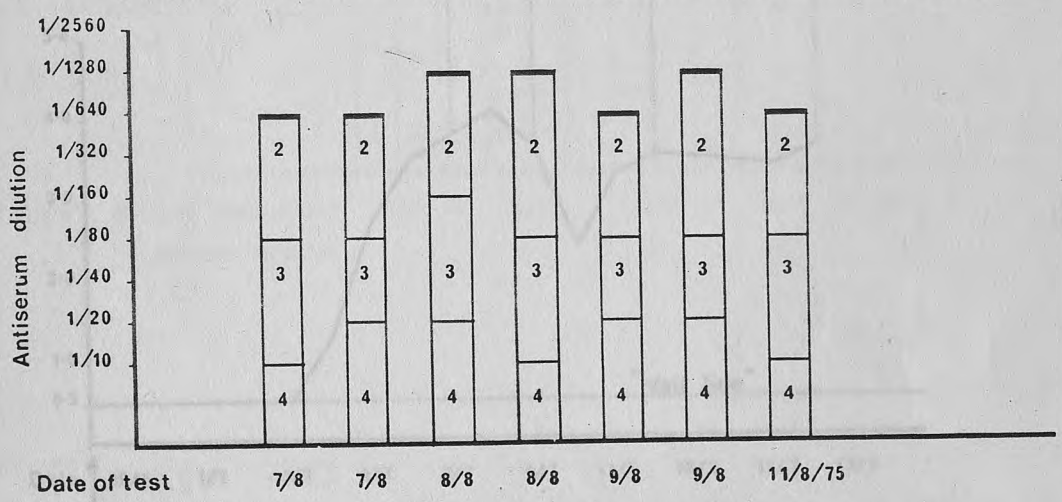


Figure 3 The course of the parasitaemia in mice infected with T.congolense TREU 1230, and the occasions of antigen preparation.

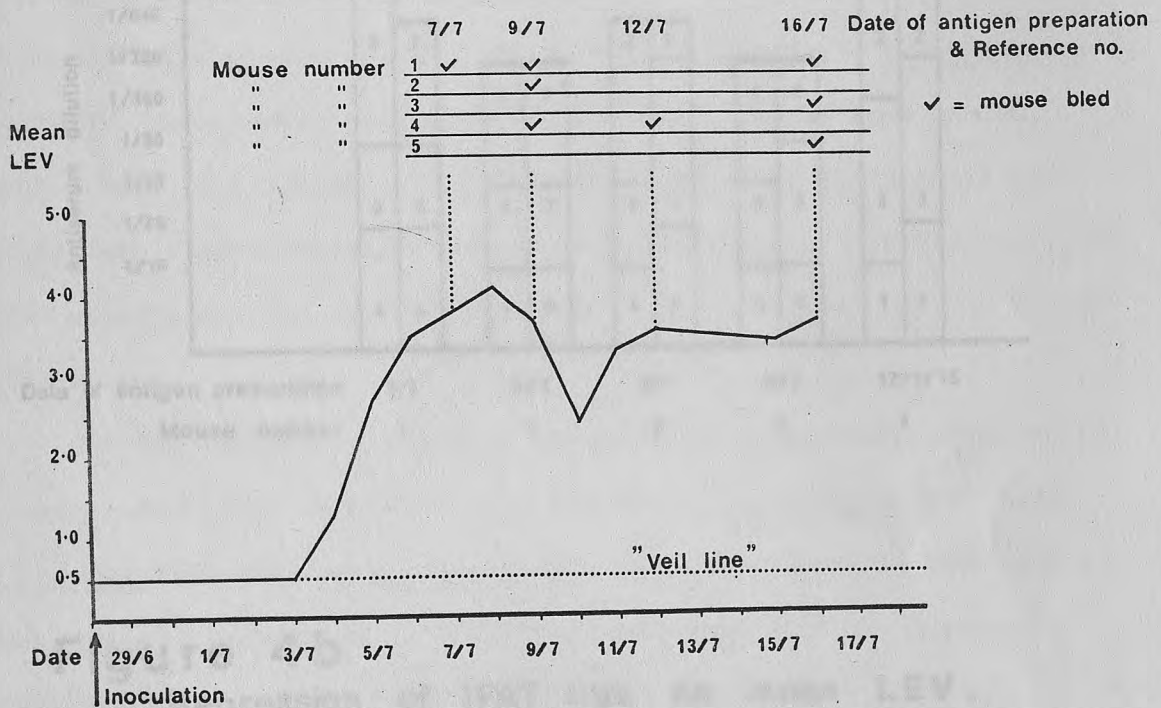


Figure 4a

The intensity of fluorescence shown by blood smear antigens prepared from individual mice at different stages of infection. The test performed with positive antiserum (AGS 12/17/7).

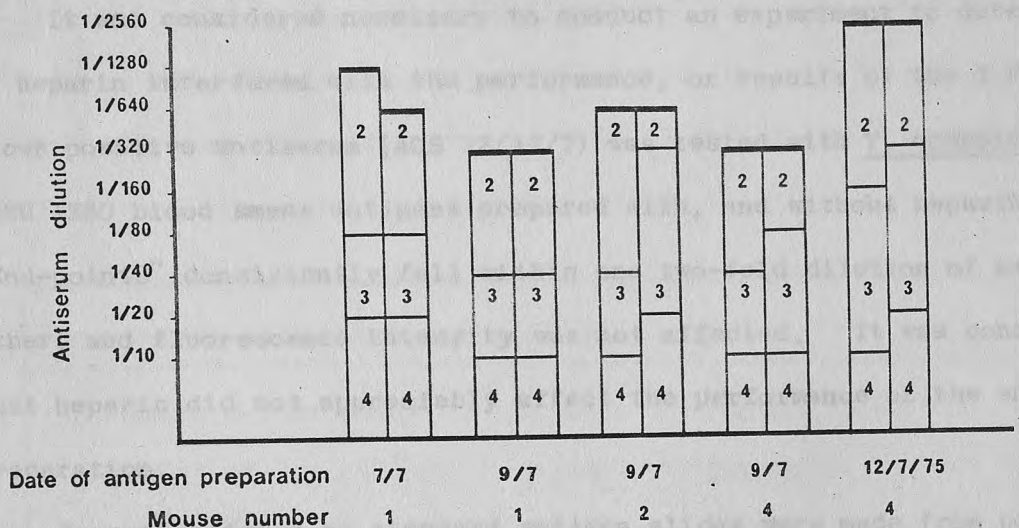
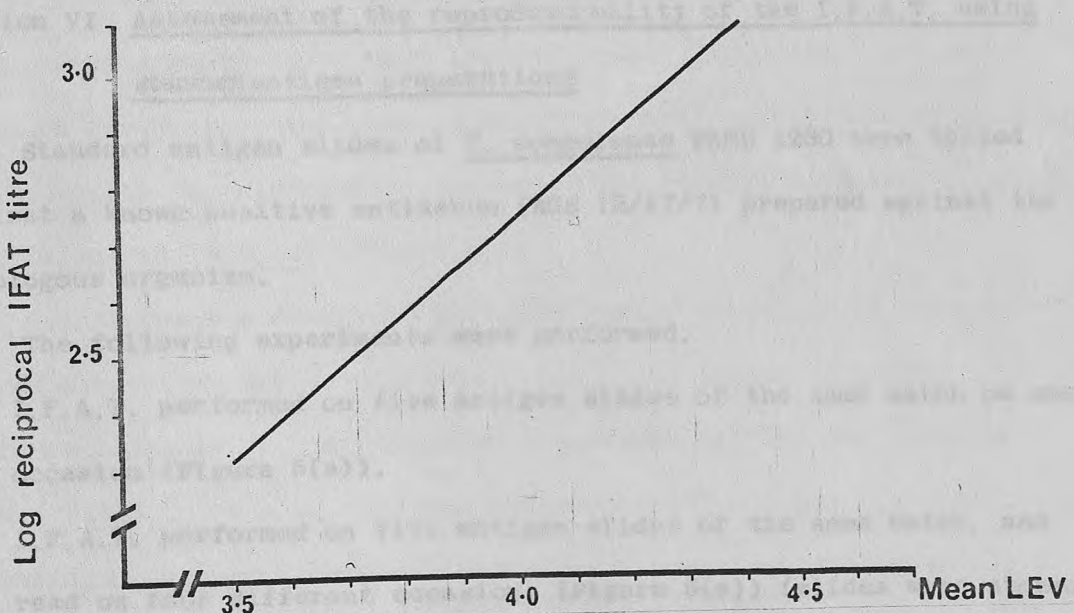


Figure 4b

Regression of IFAT titre on mean LEV.



In order to obtain sufficient blood to make a large batch of antigen slides, it was necessary to pool the blood of several infected mice. The blood was subsequently concentrated as described in Section I (a). Heparin was used as the anticoagulant in minimal quantities when bleeding mice (see Materials and Methods, Section VI).

It was considered necessary to conduct an experiment to determine if heparin interfered with the performance, or results of the I.F.A.T. Known positive antiserum (AGS 12/17/7) was tested with T. congolense TREU 1230 blood smear antigens prepared with, and without heparin. "End-points" consistently fell within one two-fold dilution of each other, and fluorescence intensity was not affected. It was concluded that heparin did not appreciably affect the performance of the antigen preparation.

Several batches of standard antigen slides were made from pooled, concentrated blood from mice infected with T. congolense TREU 1230, T. brucei TREU 667 and T. vivax TREU 1223, as tabulated in Materials and Methods, Section IX. These antigens were used in subsequent experiments.

Section VI Assessment of the reproducibility of the I.F.A.T. using standard antigen preparations

Standard antigen slides of T. congolense TREU 1230 were tested against a known positive antiserum (AGS 12/17/7) prepared against the homologous organism.

The following experiments were performed.

1) I.F.A.T. performed on five antigen slides of the same batch on one occasion (Figure 5(a)).
2) I.F.A.T. performed on five antigen slides of the same batch, and read on four different occasions (Figure 5(a)) (slides were stored in a dark drawer between readings).
3) I.F.A.T. performed on antigen slides of the same batch on different occasions (Figure 6).
4) I.F.A.T. performed on antigen slides of different batches on one occasion (Figure 6).

Results of:

Experiments (1) and (2) (Figure 5(a))

The results show a four-fold dilution factor variation in "end-point" titres between individual slides examined up to 48 hours after performing the test. These differences were statistically significant. ($F_{12}^{2} = 4.51$; $P < 0.05$). A statistical analysis of all four sets of results showed a more significant difference ($F_{16}^{3} = 8.40$ $P < 0.01$) between the four readings. Application of the "t test" to compare pairs of readings gave the following results.

Comparison of readings at	$t_{(8)}$	P
2 hours and 24 hours	3.786	<0.010> 0.001*
2 " " 48 "	1.343	>0.200
2 " " 96 "	2.121	>0.050
24 " " 48 "	1.414	>0.100
24 " " 96 "	4.381	<0.010> 0.001*
48 " " 96 "	2.847	<0.05 > 0.02*

Pairs, corresponding to the probability levels marked*, show a significant difference. Mean log reciprocal "end-point" titres and standard deviations have been represented in histogram form (Figure 5(b)) to illustrate the difference in results recorded at different readings.

Experiments (3) and (4) (Figure 6)

Results show a four-fold dilution factor variation in "end-point" titres between slides of the same batch (6/8) tested on three different occasions. A statistical analysis of this small sample showed there to be a significant difference ($F_{6}^{2} = 5.64$; $P < 0.05$). A four-fold variation between "end-point" titres recorded with different batches of standard antigen slides was also recorded, but

the sample was too small for statistical analysis. There was also a marked difference in the intensity of fluorescence recorded at the lower antiserum dilutions with some antigens (eg. 21/7). However "end-point" titres still fell within the four-fold dilution factor variation shown by other antigens.

Section VII Conclusions

From these limited observations of standard antigen performance, the following tentative conclusions were drawn.

1) There was a significant difference between the results obtained when a serum sample was tested by the I.F.A.T., using standard antigens from the same batch, on different occasions. This finding was considered to reduce the value of the I.F.A.T. for quantitative estimations of serum antibody. It was concluded that in future, it would be necessary to perform comparative examinations of sera by the I.F.A.T. on the same occasion, using antigens of the same batch. The possibility of a four-fold dilution factor variation in "end-point" titre would have to be considered when interpreting and comparing results.

2) The optimal time of reading the test was 24 hours after its performance. Reading after more than 48 hours after testing would significantly increase the error.

Figure 5a

The intensity of fluorescence shown by 5 standard antigens of one batch (6/8), tested with positive antiserum (AGS 12/17/7).
The test read on 4 occasions.

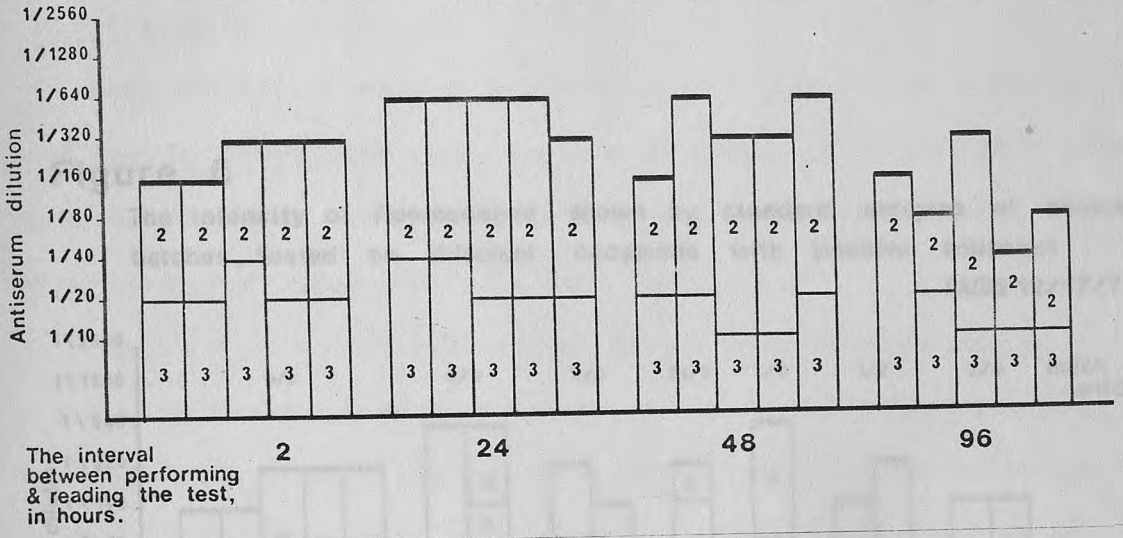


Figure 5b

Means & S.D.s of IFAT titres.

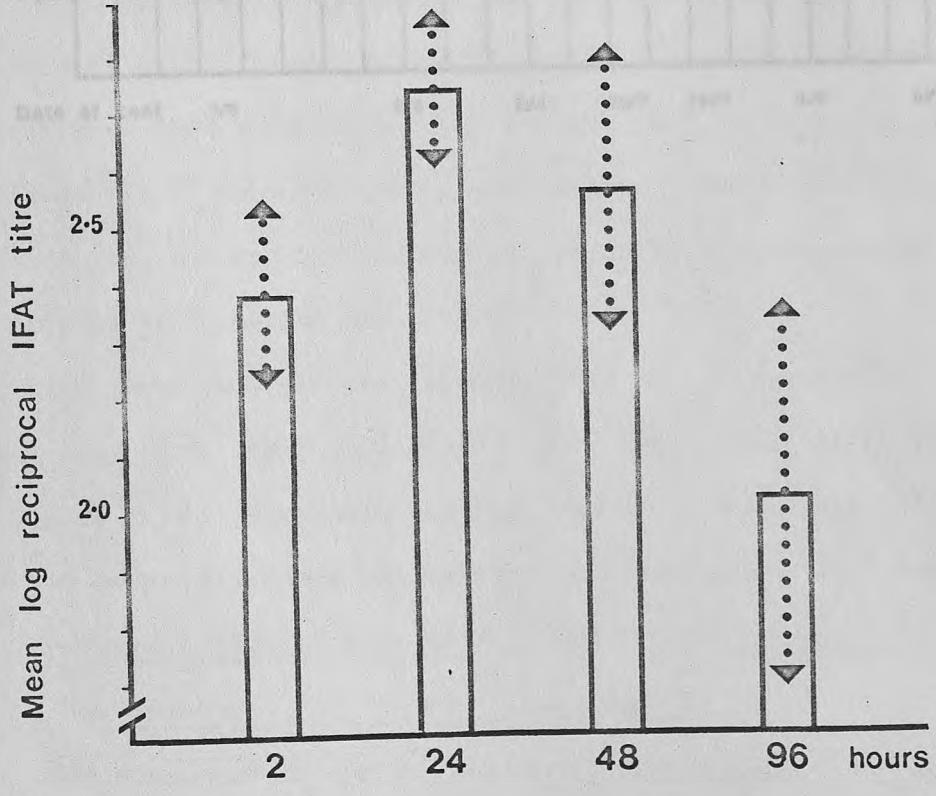
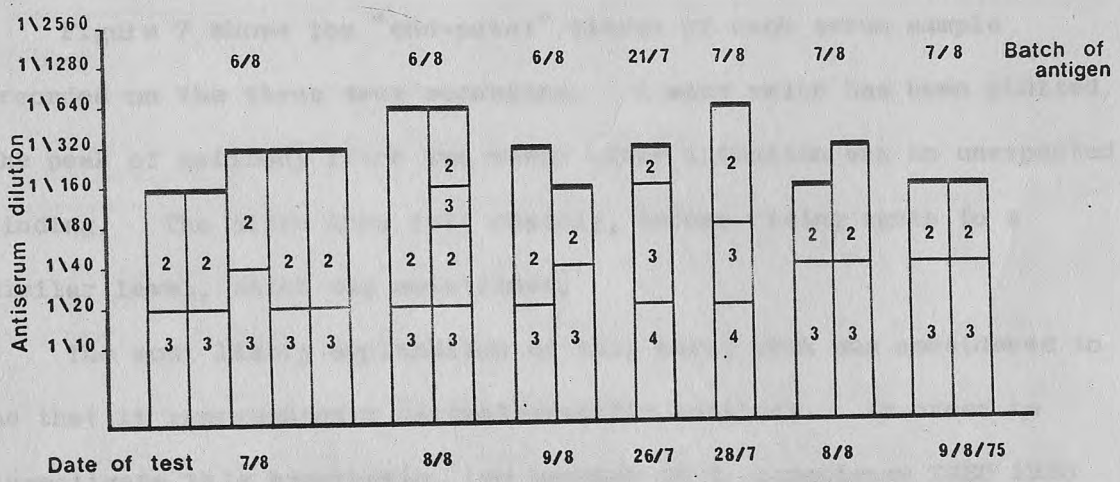


Figure 6

The intensity of fluorescence shown by standard antigens of several batches, tested on different occasions with positive antiserum (AGS 12/17/7).



CHAPTER B

Section I Investigations of the characteristics of the reaction between T. congolense TREU 1230 antigen and homologous antiserum by the I.F.A.T.

a) Antisera collected from untreated infected mice in Animal Group Series 1

Antisera collected over a 9 week period were tested by the I.F.A.T. with T. congolense TREU 1230 standard antigens. The sera were tested on three occasions using antigens from two different batches. Normal mouse serum and P.B.S. controls were included.

Figure 7 shows the "end-point" titres of each serum sample recorded on the three test occasions. A mean value has been plotted. The peak of antibody titre two weeks after infection was an unexpected finding. The titre then fell rapidly, before rising again to a similar level, which was maintained.

The most likely explanation of this early peak was considered to be that it represented a variant-specific antibody. In order to investigate this hypothesis, two batches of T. congolense TREU 1230 antigens (16/7 and 1/8) were prepared (see Material and Methods Section IX), using trypanosomes of the first relapse population developing in infected mice.

The sera were retested against both of these antigens. The results of both tests were almost identical. One series is recorded in Figure 7 and is notable for the absence of the early antibody peak, and the generally lower titres recorded throughout the 9 week period.

b) Antisera collected from mice, treated with "Berenil" 10 days after infection, in Animal Group Series 2

Antisera were tested on standard T. congolense TREU 1230 antigens. Sera from "Berenil" treated normal mice were included as controls.

The results obtained with antisera from the untreated, infected mice of AGS 1 are reproduced for comparison with the results from this group on Figure 8. There is a close agreement between the reactions of sera from mice in the two groups. High titres persisted in the treated group for the duration of the 11 week period. Sera collected after 11 weeks post-infection were tested on the same occasion with the following results.

Date	14/5	21/5	28/5	4/6	12/6	19/6
Weeks after treatment	11	12	13	14	15	16
"End-point" titre	1/320	1/320	-	1/640	-	1/160
"End-point" titre of 2 mice with relapsed infections				1/160		1/640

These results show a persistence of high titres for 16 weeks after treatment. However, it may be relevant to recall that on 4/6 and 19/6/75, two mice were found in this group with relapsed infections. The sera from these mice were tested on the same occasion, with the results recorded above. No other mice in this group showed any sign of uncured infection, and it is considered unlikely, in view of the gross splenomegaly and anaemia of the infected mice, that a chronic infection could have been overlooked.

In Figure 8, mean L.E.V.s are also plotted to show the course of the parasitaemia in the mice.

Section II <u>Investigations of the characteristics of the reaction of antiserum to T. congolense TREU 1230 with T. brucei TREU 667 and T. vivax TREU 1223 standard antigen</u>

The results of these two tests are illustrated in Figure 7. The

antisera were negative at $^{1}/_{5}$ dilution or greater until 4 and 6 weeks post-infection, with the T. brucei and T. vivax antigens respectively. Titres subsequently reached maxima of $^{1}/_{20}$ and $^{1}/_{40}$ respectively.

Observations were also made of the character of the fluorescence. On T. brucei antigens, the area immediately anterior to the nucleus fluoresced more intensely than other parts of the parasite. Fluorescence at the perimeter was weak (cf. the homologous reaction). Using T. vivax antigens, fluorescence was most intense at the anterior end of the parasite, with a "beaded" fluorescence at the perimeter which appeared to run along the free flagellum for a short distance.

Section III Investigations of the characteristics of the reactions of antisera to T. congolense TREU 1170, T. brucei TREU 667 and T. vivax TREU 1223 with T. congolense TREU 1230 standard antigen

a) Preliminary I.F.A.T. to determine the reactions between antisera to T. brucei and T. vivax and their respective standard antigens

Figure 9 shows the results of these tests using antisera from treated and untreated mice of AGS 5, 6, 7 and 8. The sera contained antibody but titres were generally low and slow to rise compared with the equivalent reaction between antiserum to T. congolense and its homologous antigen. There was also no evidence of an early peak in antibody titre.

b) The I.F.A.T. reactions between T. brucei and T. vivax antisera (AGS 5 and 7) and standard T. congolense TREU 1230 antigen

Figure 10 shows the results of these I.F.A.T.s with the reaction between antiserum to T. congolense TREU 1230 and its homologous antigen recorded for comparison. The titres of T. brucei and T. vivax antisera took 2 and 4 weeks to develop respectively, and never rose above $^{1}/_{5}$.

c) The I.F.A.T. reaction between T. congolense TREU 1170 antisera and standard T. congolense TREU 1230 antigen

Results of this test are also illustrated in Figure 10. Antisera from infected and treated (AGS 9) and infected and untreated mice (AGS 10) were tested. The latter showed a slow rise, over the 8 week period, to a titre close to that recorded in the reaction between antiserum to T. congolense TREU 1230 and its homologous antigen. There was no early peak in antibody titre. Results of the I.F.A.T. on sera from the treated mice showed the persistence of a low level of antibody over the 8 week period.

CHAPTER C

Investigations of the I.F.A.T. reaction between antisera from mice infected with other protozoan parasites, and T. congolense TREU 1230 standard antigen

Antisera against *Babesia rodhaini*, *B. microti*, *Nuttalia musculi* and *Plasmodium berghei* were obtained from the serum collection of the Centre for Tropical Veterinary Medicine, Edinburgh University. In the I.F.A.T. all sera, at a dilution of $^{1}/_{5}$ or greater, were negative. It was concluded that cross-reactions between *T. congolense* TREU 1230 antigens and antisera to these non-trypanosomal infections did not occur.

Key to symbols, Figure 7.

	Date of test	Antigen
▲	7-8	T.congolense TREU 1230
■	5-8	" "
●	30-7	" "
———	mean titre	" "
△-----△		T. congolense TREU 1230 "relapse"
□.....□		T. brucei TREU 667
○•-•-•○		T. vivax TREU 1223

NMS = normal mouse serum

Symbols below "veil line" represent sera negative at dilution of 1/5

Figure 7

IFAT titres of antiserum to T.congolense TREU 1230 (AGS 1) with different antigens .

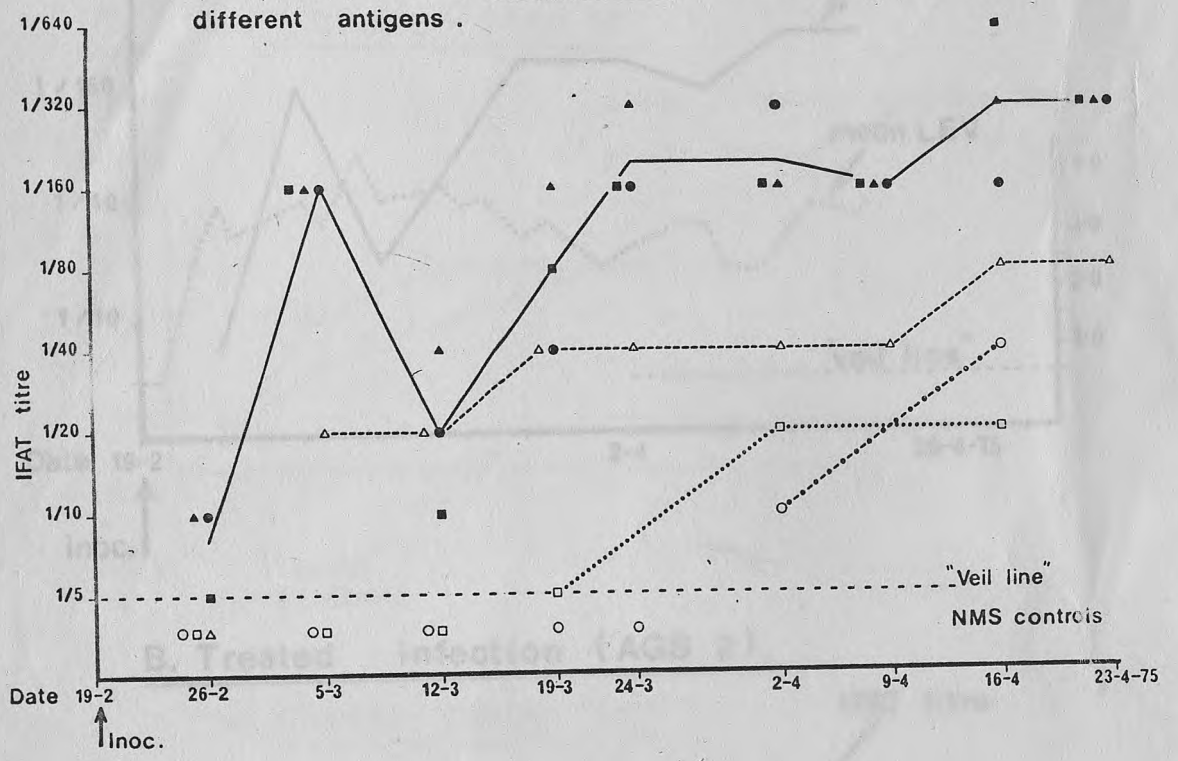
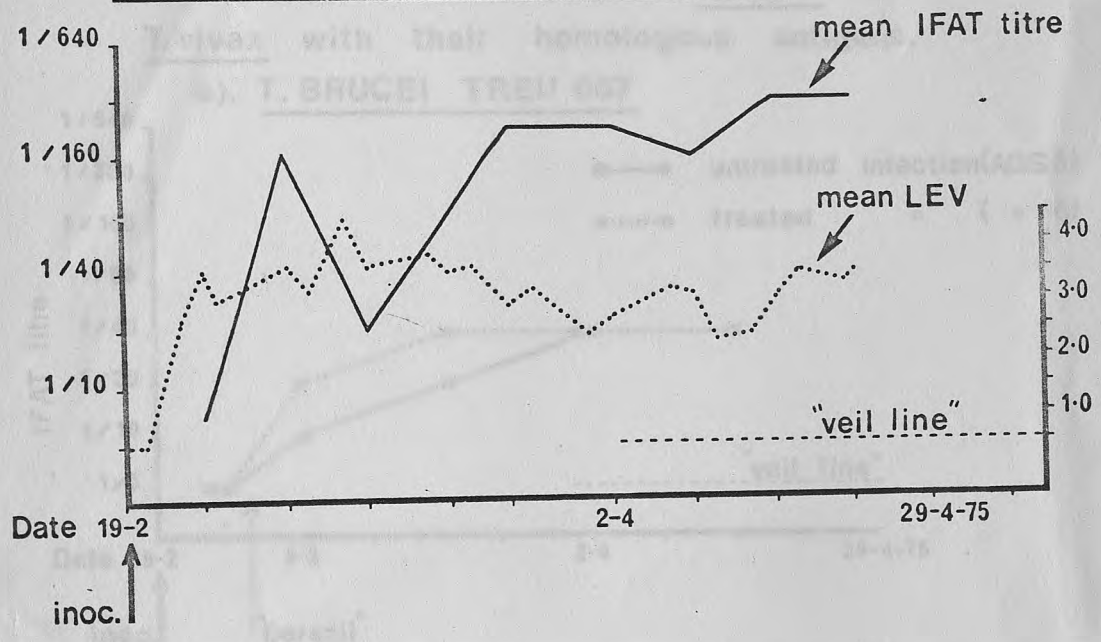


Figure 8

IFAT titres of antisera to T. congolense TREU 1230 with the homologous antigen.

A. Untreated infection (AGS 1).



B. Treated infection (AGS 2).

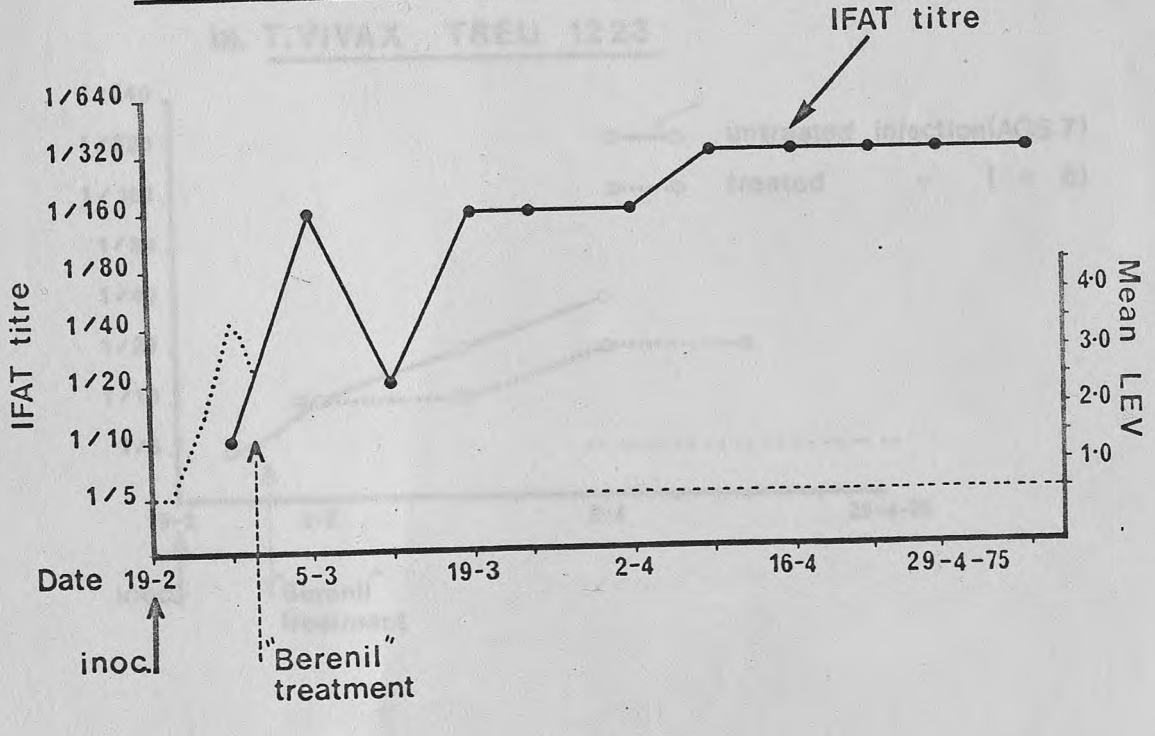
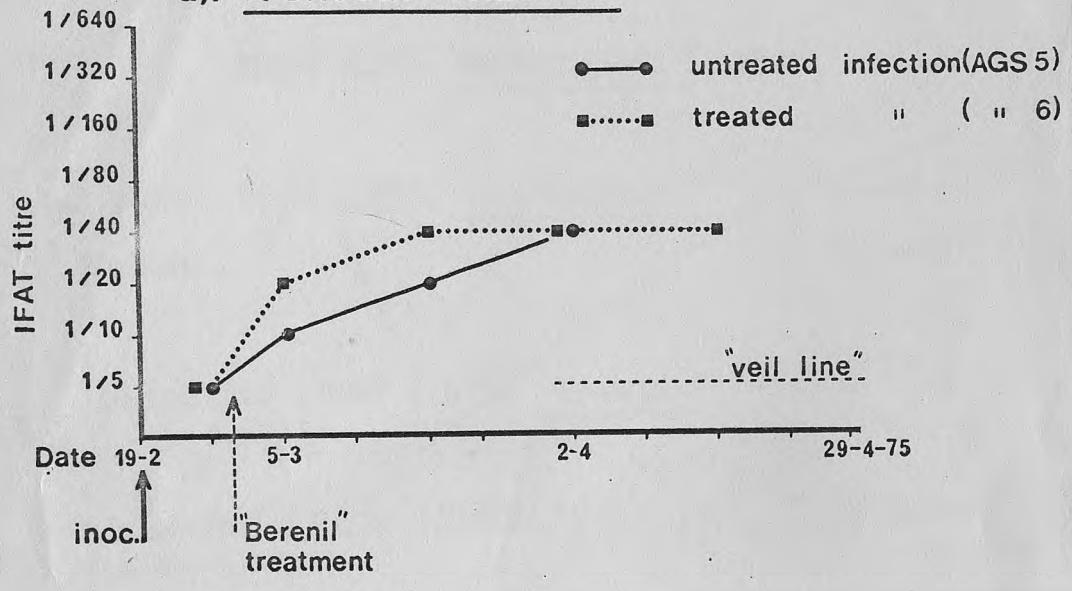


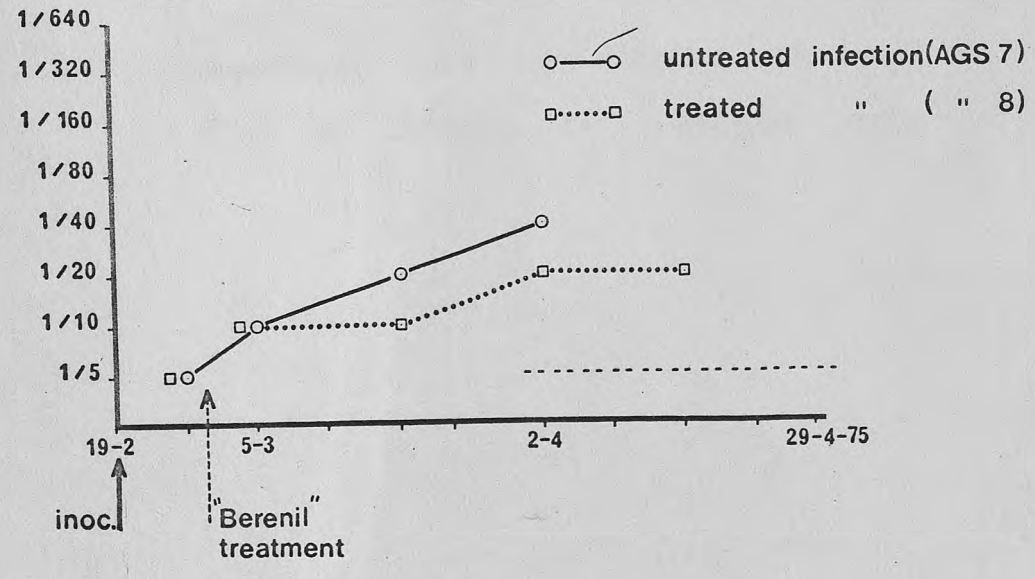
Figure 9

IFAT titres of antisera to T.brucei & T.vivax with their homologous antigens.

a). T. BRUCEI TREU 667



b). T.VIVAX TREU 1223



Key to symbols, Figure 10.

Antiserum

——— mean, T. congolense TREU 1230

c-----c T. congolense TREU 1170, untreated

c^t.....c^t " " , treated

b• • •b T. brucei TREU 667, untreated

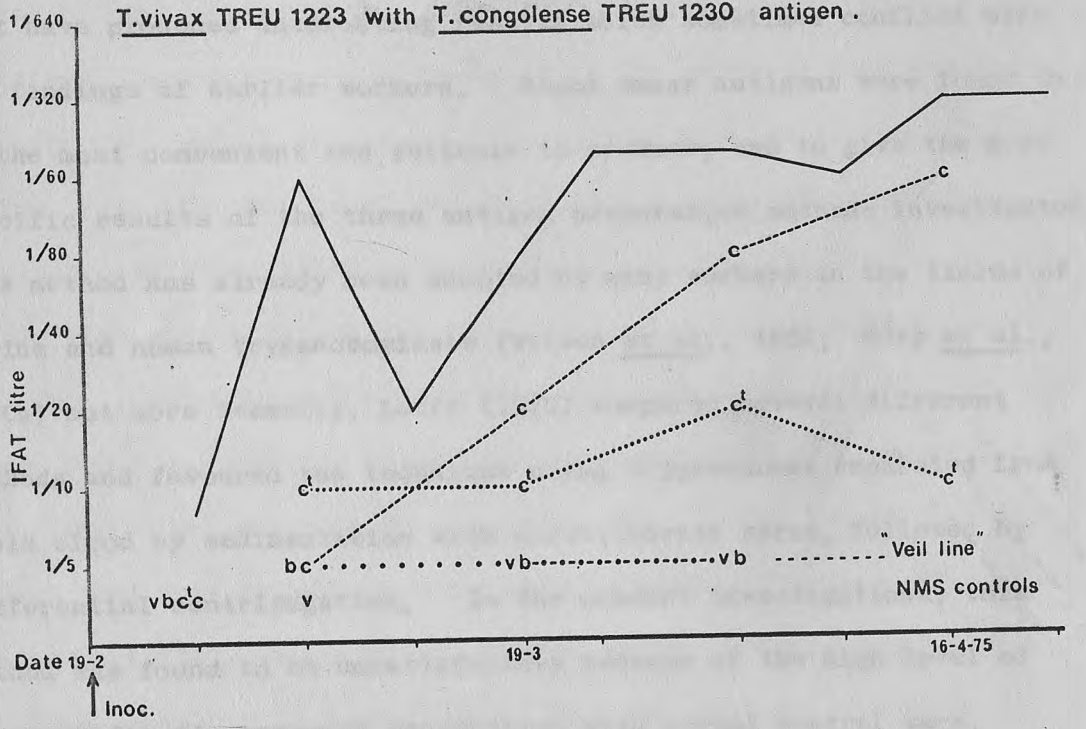
v - - -v T. vivax TREU 1223, untreated

NMS = normal mouse serum

Symbols below "veil line" represent sera negative at dilution of 1/5

Figure 10

IFAT titres of antisera to T.congolense TREU 1170, T.brucei TREU 667 & T.vivax TREU 1223 with T.congolense TREU 1230 antigen.



DISCUSSION

Investigations have been conducted into the application of the I.F.A.T. to the diagnosis of T. congolense infection in mice, with particular reference to the specificity of this immunological reaction.

I Development of test methods

Studies aimed at the development of a suitable antigen for the test have produced interesting results which sometimes conflict with the findings of earlier workers. Blood smear antigens were found to be the most convenient and reliable to produce, and to give the most specific results of the three antigen preparation methods investigated. This method has already been adopted by many workers in the fields of bovine and human trypanosomiasis (Wilson et al., 1966; Wéry et al., 1970a) but more recently, Latif (1972) compared several different methods and favoured the technique using trypanosomes separated from whole blood by sedimentation with normal bovine serum, followed by differential centrifugation. In the present investigations, this method was found to be unsatisfactory because of the high level of non-specific fluorescence encountered with normal control sera, regardless of the method of antigen fixation. Weitz (1963) had similar problems with centrifuged suspensions of trypanosomes. The reason for this is not obvious, but it seems likely that the process of diluting and centrifuging blood has a washing effect upon the trypanosomes which leads to alteration of surface antigenicity. Vickerman (1969) has shown that trypanosomes have a surface coat which can be removed by washing. Latif (1972) found that washing trypanosomes (T. brucei) in P.B.S. for 40 minutes removed the agglutinogens from the parasites and also reduced the I.F.A.T. titre when these organisms were used as antigens to test homologous antiserum. It therefore seems possible that the limited

washing, which occurs in the preparation of antigens by this method, affects the antigenic structure of the surface coat. Since it was the non-specific activity of the reaction that was increased, it is postulated that this process exposes the "host-like" antigens of the surface coat, first described by Ketteridge (1971) on T. vivax. These antigens consist of host serum proteins and have since been detected on T. gambiense by Seed (1974). The same author has observed fluorescence on the surface of the parasite when trypanosome preparations were treated with fluorescein labelled rabbit anti-host serum. If the problem of non-specific fluorescence is due to a similar mechanism, it would be expected that the reaction would only occur when the antiserum was obtained from the same host as the antigen. A conjugated antiserum against globulin of this host would then attach to specific antiserum-antigen complexes if present, and the "host-like" antigens on the trypanosome. The latter reaction would give rise to non-specific fluorescence. This appears to be so, as Latif (1972), using an anti-rabbit serum conjugate and testing antisera prepared in rabbits with antigens prepared from infected mice by his favoured method, did not encounter non-specific fluorescence of any significance.

The preparation of antigens from trypanosomes separated by anion exchange on D.E.A.E.-cellulose was found to be a relatively laborious technique which produced antigen preparations of unreliable quality. However, when trypanosome numbers were adequate, specific fluorescence was generally good and the level of non-specific fluorescence satisfactory, although higher than that seen with blood smear preparations. Seed (1974) has shown that T. gambiense retains its "host-like" antigens after anion exchange on D.E.A.E.-cellulose. It is suggested that the same mechanism of non-specific fluorescence

as described above, may also apply to this method of preparation. The stronger non-specific reaction observed with Latif's separation technique might be partly due to foreign proteins in the bovine serum.

The effect of the non-specific reaction of the antigens discussed above was to make interpretation of the I.F.A.T. very subjective and sometimes impossible.

Methods of fixation of antigens had a profound effect on the character, intensity and specificity of the I.F.A.T. reaction. It was found that the trypanosome morphology was generally better preserved when the osmotic pressure of the fixing solution approximated that of the parasite's normal environment. Thus, blood smear antigens, fixed in $^{N}/250$ H Cl in distilled water, had trypanosomes of disrupted morphology, whilst those fixed in $^{N}/250$ H Cl in normal saline were well preserved. It is thought that the fixative effect of $^{N}/250$ H Cl in normal saline was due to its acidity (approximately pH 2.5) as results with $^{N}/250$ H Cl in P.B.S. (approximately pH 7.0) were unsatisfactory. H Cl and acetone fixed blood smear antigens were found to give the most promising results. Acetone fixation gave brighter fluorescence of a "somatic" character (Plates 11 and 12), but variable trypanosome numbers and some non-specific fluorescence were problems. Zwart et al (1973) also recognised the non-specific fluorescence of smears fixed in this manner. However, smears fixed by $^{N}/250$ H Cl in normal saline showed minimal non-specific fluorescence, satisfactory specific fluorescence involving mainly the trypanosome perimeter ("surface fluorescence", Plate 10), relatively easy "end-point" determination, and always adequate trypanosome numbers. Knottenbelt (1971) also recognised the value of a similar method using immersion in $^{N}/100$ H Cl for one minute followed by rinses in distilled water and P.B.S. for one minute each.

The same author found that fixation after preservation of antigen slides at -79°C gave much higher titres than with slides fixed before preservation. This aspect of antigen preparation was not investigated in this work. Zwart et al (1973) also used $^{N}/_{30}$ and $^{N}/_{100}$ H Cl fixation and reported "good results", but the methods were not further investigated. H Cl fixation of antigens prepared from separated trypanosomes gave similar results to antigens fixed by formalin (Plate 9). Fluorescence was of a "somatic" character and it is considered that the absence of the characteristic "surface" fluorescence of H Cl fixed blood smears (Plate 10), is further evidence for the hypothesis that techniques of separation of trypanosomes interfere with their surface coat.

The minimal non-specific fluorescence obtained with standard antigen preparations allowed antisera to be tested at low dilutions from $^{1}/_{5}$. Knottenbelt (1971), using his method of H Cl fixed mouse blood smears, tested antisera dilutions from $^{1}/_{10}$, whilst workers in the field of bovine trypanosomiasis using heat or acetone fixed rodent blood smears, used antisera dilutions from $^{1}/_{40}$ (Wilson et al., 1966) and $^{1}/_{160}$ (Zwart et al., 1973). These "baseline" dilutions overcame problems of non-specific fluorescence and fluorescence associated with T. theileri infections.

Methods of isolating "cells" on antigen slides to receive the antisera dilutions have varied. In the present work a method using "Fluoroglide" spray was developed which was found to be superior to those already described. However, it was not applicable to all methods of blood smear antigen fixation.

The method of performing the I.F.A.T. closely followed that recommended by Latif (1972). "End-point" determination, which was found to be straightforward with the standard antigen preparation,

relied upon the discrimination by the eye between the presence or absence of a clear fluorescing perimeter to the parasite. Such fluorescence was arbitrarily graded "2 plus". Fluorescence of greater intensities was very often difficult to grade, and it is considered that the reliance on differentiation of such intensities for the determination of an "end-point" introduces an unacceptable degree of subjectivity to the interpretation of the test. This opinion is shared by Latif (1972), but other workers (Wilson et al., (1966) have based their interpretations on such assessments. From limited experience, it is also considered that the quality of the microscope, and particularly the type of illumination used in the test is reflected in the results obtained. Incident light illumination by the "Leitz orthoplan" system gave brighter and clearer images than with the transmitted light illumination of the "Vickers patholux" system.

The application of statistical methods to the preliminary investigations of the reproducibility of the test using blood smear antigens, showed no significant difference in the "end-point" titres of slides from the same batch, tested on different occasions (Figure 2). However, there was a significant disagreement between the results with antigens prepared from mice at different times after inoculation with stabilate material (Figures 3 and 4a). The existence of a linear correlation (Figure 4b) between the size of the parasitaemia at the time of antigen preparation, and the I.F.A.T. titre recorded with those antigens, was an unexpected finding. Subsequent results obtained with the serial T. congolense TREU 1230 serum samples suggested that the I.F.A.T., as performed in this work, was detecting antibodies to variant specific antigens of T. congolense. Consideration of these later findings offered an explanation of the correlation recorded above. It is based on an association between the level of

parasitaemia and the antigenic constitution of the trypanosome population. Wilson and Cunningham (1972) have demonstrated the antigenic lability of T. congolense during infections in cattle by the use of the neutralisation test. Relapsing parasitaemias with T. brucei infections are known to be associated with antigenic variation (Gray, 1962). However, antigenic variation of the T. congolense infections described by Wilson and Cunningham (1972) were associated with small, but statistically insignificant changes in parisitaemia, the fall in infectivity of the trypanosomes for mice being the major manifestation of antigenic variation. This loss of infectivity was considered to occur with the shedding of the old surface coat antigens (Vickerman, 1969; Vickerman and Luckins, 1969) before the new one was formed. It is therefore suggested that the correlation of I.F.A.T. titre with parasitaemia, is probably an expression of the antigenic make-up of the trypanosome population at that time. Animals at peak parasitaemia would be expected to have a majority of trypanosomes of the established antigenic type. Animals with a falling parasitaemia would be expected to contain parasites of changing antigenic type which would react poorly as antigens in a serological test that detected antibodies to variant specific antigens. Antigens prepared from individual mice at the same stage of infection gave I.F.A.T. results that were not statistically different (Figure 4a). This was to be expected, as these animals had similar levels of parasitaemia and probably trypanosome populations of similar antigenic constitution.

Standard antigens, developed from these preliminary investigations, gave I.F.A.T. results which varied by up to four-fold differences in titre when tested and read on different occasions (Figures 5 and 6).

It is considered that these statistically significant differences are probably due in part to human error in reading the test, which is always a subjective assessment. Such differences reduce the value of any serological test, as four-fold differences in serum titres are generally considered to be significant in clinical medicine. Wilson and Cunningham (1971) have suggested that the differentiation of animals with either latent or cured infections could be achieved by comparing the I.F.A.T. titres of paired serum samples taken with a 6-8 week interval. These authors did not indicate the difference in titre that would be considered significant, but in view of the present findings, it is suggested that it should be more than the traditional four-fold difference if the tests are not performed and read on the same occasion. Furthermore, if statistical analysis of larger samples of results obtained by this I.F.A.T. method confirm the limits of reproducibility to be within four-fold differences of titre, the test should be considered unreliable for the quantitative examination of sera. It should possibly be restricted to the qualitative assessment of samples, as in the "screening" methods of Wilson (1969) and Knottenbelt (1971).

II Applicability of the I.F.A.T. to the diagnosis of T. congolense infection in mice

Bearing in mind the limitations of the I.F.A.T. described above, the test was applied to the diagnosis of T. congolense infection in mice. Conflicting reports in the literature had aroused a particular interest in the specificity of the test. Serial serum samples prepared against T. congolense, T. vivax and T. brucei were tested with T. congolense standard antigen. Small numbers of mice were sampled at each occasion so that results are statistically insignificant. However, some interesting features have emerged

which might indicate productive areas of research for the future.

a) The homologous reaction

T. congolense TREU 1230 antigen was tested with antiserum to T. congolense TREU 1230 (hereafter referred to as the "true" homologous reaction) on three occasions using two batches of antigen. A similar pattern of antibody development was recorded at each test (Figure 7). Antibody at low titre was detected one week post-infection and rose to an early peak (^{1}/160) at two weeks post-infection, before falling to a low level (^{1}/20) at three weeks. Knottenbelt (1971) found that sera from 97% of over 200 trypanosome infected mice gave positive results to his I.F.A.T. "screening" method by seven days post-infection. Wilson and Cunningham (1971), working with experimental T. congolense infections in cattle, found antibodies at their "baseline" titre of ^{1}/40 between 8 and 21 days post-infection. Many field workers (Mwambu and Omaset, 1967; Zwart et al., 1973) have commented on the problem of detecting the early infection, which is a feature of any serodiagnostic method. However, the findings of the present work together with those of Knottenbelt (1971), suggest that H Cl fixation of blood smear antigen reduces the non-specific reaction to a minimum and therefore allows very low dilutions of sera to be tested. This appears to improve the sensitivity of the technique for the detection of early infections. However, if this I.F.A.T. method is to be applied to the diagnosis of bovine trypanosomiasis, it will be necessary to quantify the reaction between antiserum to the non-pathogenic T. theileri and T. congolense antigen.

The unexpected peak in antibody titre at two weeks post-infection (Figure 7) prompted further investigations with two batches of "relapse" T. congolense TREU 1230 antigen, which were assumed to contain different antigenic variants from those of the first

parasitaemic population of trypanosomes. The results of I.F.A.T.s with these antigens were conspicuously different from those with the "true" homologous reaction (Figure 7). This was considered to support the hypothesis that the early peak in titre represented an antibody directed against variant specific antigens. Further support for this idea was provided by the reaction of antisera to a different strain of T. congolense (TREU 1170), tested with T. congolense TREU 1230 antigen (Figure 10). This reaction also lacked the two week antibody peak. Knottenbelt (1971) found that he was able to identify variants of T. brucei by his I.F.A.T. method, but Latif (1972), comparing the neutralisation test and I.F.A.T. on T. congolense infections, failed to detect variant specificity with the latter test.

Most workers have recognised that the I.F.A.T. detects antibodies to group specific and species specific antigens. The location and nature of these antigens have not been discovered, but Latif (1972) showed that his I.F.A.T. method detected antibodies directed against both surface and bound, or internal antigens of the parasite. In the present work, the apparent variant specificity and "surface" character of the fluorescence with $^{N}/250$ H C l in normal saline fixed blood smear antigens indicates that variant specific antigens are located on, or close to, the surface membrane of the trypanosome.

The low antibody titre found at three weeks post-infection in the "true" homologous reaction (Figure 7) is thought to represent either a decline in the titre of a short-lived antibody which was at maximum titre two weeks post-infection, or that the antibody was temporarily "eclipsed". The argument for the "eclipse" of a long-lived antibody is based on a suggestion that surface coat antigens which were shed into the plasma on the occasion of the first antigenic change, or an accumulation of "exoantigens" (Weitz, 1960) in the

plasma, effectively "neutralised" the antibody that was detected at high titre two weeks post-infection. Support for this argument was provided by two observations. Firstly, the I.F.A.T. titres to the "relapse" antigen never reached those obtained with the "true" homologous antigen, suggesting that an antibody of similar specificity was still being detected up to nine weeks post-infection. (It might be argued that the antigenic quality of the "relapse" antigen was unproven, as the homologous reaction of this antigen with an antiserum prepared against the same "relapse" population of trypanosomes was not examined.) Secondly, there was extraordinary agreement between the I.F.A.T. results of antisera from the treated and untreated groups of T. congolense TREU 1230 infected mice (Figure 8). The appearance of the two week peak, three week dip and subsequent plateau of antibody titres in the treated group, despite the elimination of infection at 10 days post-infection, is interpreted as representing a long-lived antibody. This antibody can only have been produced in response to the first antigenic population, which Wilson (1967) has shown to be antigenically identical to the original stabilate. It has already been suggested that this antibody is directed against a variant specific antigen.

The second possible explanation of the decline in antibody titre at three weeks post-infection is that the early peak of titre belongs to a very short-lived antibody. The subsequent rise in titre from four weeks post-infection in the treated group of T. congolense TREU 1230 infected mice (Figure 10) must then represent a second, long-lived antibody directed against an antigen of the same variant. In support of this hypothesis, several workers (Seed, 1972; D'Alesandro, 1970) have shown that at least two antibodies develop in sequence in response to trypanosome infections. However, they

belong to the different classes of immunoglobulin, 1gM and 1gG, and Latif (1972) has shown that antibody detected by his I.F.A.T. was confined to the 1gG class. In the present investigations, the commercial conjugated antiglobulin was prepared specifically against mouse 1gG, and would therefore not be expected to react with mouse 1gM. Thus, if two antibodies were detected, they must both be of the 1gG class and show similar specificities. Such antibodies have not been identified with any trypanosome infection and therefore this explanation is not favoured.

Referring to Figure 7, it was observed that the "plateau" stage of the serological response of antisera to T. congolense TREU 1230 with the homologous antigen, was reached five weeks post-infection. Wilson and Cunningham (1971) recorded similar antibody titres with sera from T. congolense infected cattle, but they were not reached until approximately 100 days after infection. High titres persisted in infected cattle until death. Antibody titres in T. congolense infected cattle (Wilson and Cunningham, 1971; Wiesenhutter, 1970) and rabbits (Latif, 1972) fell after treatment. In the present work, high levels were still detected 16 weeks after treatment. The detection, at about that time, of two mice with relapsed infections throws some suspicion on the validity of these results. However, it is considered most unlikely that other chronic, relapsed infections could have been missed because of the symptoms and splenomegaly shown by infected mice.

b) Heterologous reactions

The heterologous reactions between antisera to T. brucei and T. vivax, and T. congolense TREU 1230 antigen, never rose above titres of $^{1}/_{5}$ (Figure 10). At such low titres it was not possible to assess the character of the fluorescence. It was thought that the tests

probably detected antibody which reacted with genus-specific components of the antigen. The very low heterologous titres are associated with low homologous antibody levels, as the reactions between the sera and their respective antigens did not rise above "end-point" titres of $^{1}/40$ (Figure 9).

Antiserum to another strain of T. congolense (TREU 1170) prepared from untreated mice and tested with the T. congolense TREU 1230 antigen, showed a steady rise in titre over an eight week period, but the antibody titres were consistently lower than those found in the "true" homologous reaction (Figure 10). In view of the probable variant specificity of the "true" homologous reation, the similarity between these titres and those recorded when antisera to T. congolense TREU 1230 was tested with T. congolense TREU 1230 "relapse" antigen (Figure 7), it is suggested that antibodies in these antisera (i.e. T. congolense TREU 1170) are reacting with species-specific components of the antigen. The character of the fluorescence appeared the same as that observed with the "true" homologous reaction, which suggests that species-specific antigens are probably associated with the surface of the parasite. It will be recalled that Latif (1972) has demonstrated the activity of surface and bound antigens in his I.F.A.T., which was not variant specific.

On the basis of the results discussed above, it appears that it is possible to distinguish between infections caused by different species of trypanosomes by the I.F.A.T. titres.

c) Further observations

The homologous reactions between antisera to T. brucei and T. vivax and their respective antigens have two interesting features with a bearing on the specificity of the I.F.A.T. Titres were low,

and secondly, there was no evidence of the early peak in titre seen in the "true" homologous T. congolense TREU 1230 reaction, which has been attributed to variant specificity. Knottenbelt (1971) found that he was able to identify variants of T. brucei by his I.F.A.T. method and suggested that antibody titres over $^{1}/_{640}$ in mice, were associated with a variant specific reaction. The two findings in the present work offer slender evidence that this I.F.A.T. is not variant specific when applied to T. brucei and T. vivax. Since all antigens were prepared in a standard manner, this suggests that the location and nature of antigens of T. brucei and T. vivax differ from those of T. congolense.

In further investigations of the specificity of antibodies reacting in the I.F.A.T., antisera to T. congolense TREU 1230 were tested against antigens of T. brucei and T. vivax (Figure 7). Titres rose slowly to a maximum of $^{1}/_{40}$ over an eight week period. Knottenbelt (1971) found similarly low levels. These antibodies were considered to be reacting with genus-specific antigens. This opinion is supported by observations of the localised nature of the fluorescence with these heterologous antigens, compared with the "surface" fluorescence of the homologous reaction.

An interesting feature that was common to all the homologous reactions was the continued development and persistence of antibody after the infections had been cured (Figures 8, 9 and 10). This phenomenom has not been reported by other workers and the only possible explanation that arises is that this feature of the immune response to trypanosomiasis is peculiar to the mouse.

In conclusion, this limited investigation has demonstrated the complex antigenic nature of T. congolense and emphasised the

difficulties of preparing antigens for the I.F.A.T., and of reading and interpreting the test. It has been found that different methods of handling and fixing trypanosomes affect their performance as antigens. It is considered that future efforts should be directed towards understanding the nature of trypanosomal antigens and cytochemical methods should be used to determine their behaviour in response to different fixation methods. Such an approach might provide further information which could serve as a basis for the development of an I.F.A.T. of the required specificity.

CONCLUSIONS

1. The character and specificity of the I.F.A.T. reaction were affected by the method of antigen preparation and fixation. Blood smears fixed by $^{N}/250$ H Cl in normal saline were the most satisfactory antigens. Problems of non-specific fluorescence encountered with antigens prepared from separated trypanosomes might be overcome by obtaining antisera and antigens from different host species.

2. The I.F.A.T. was reproducible within a range of four-fold differences of antibody titre, and this was considered to detract from its value for the quantitative comparison of serum antibody content.

3. High levels of antibody were found in sera from animals infected with T. congolense and the test might therefore have some diagnostic value for infections with this trypanosome. Antibody titres to T. congolense antigens were higher with sera from animals infected with T. congolense than with sera from animals infected with T. brucei and T. vivax, and it might be possible to use the test to distinguish between infections caused by different trypanosome species. There was also evidence that the test detects variant specific antibodies to T. congolense.

4. It was not possible to distinguish between T. congolense infected and cured animals by this I.F.A.T. because antibody titres persisted for at least 16 weeks after drug treatment.

5. No cross reactions were detected between antisera from mice infected with B. rodhaini, B. microti, N. musculi and P. berghei, and T. congolense antigen.

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APPENDIX

COMPOSITION OF SOLUTIONS

Locke's solution

Na Cl	9.0 g
$Ca\ Cl_2$	0.2g
K Cl	0.4g
$Na\ HCO_3$	0.2g
Glucose	2.5g
Distilled water to	1,000 ml

Solution A (After Lumsden, Herbert and McNeillage, 1973)

Stock solutions:

1)	Na Cl	0.154 M, 9.00g/litre
2)	K Cl	0.154 M, 11.48g/litre
3)	$Mg\ Cl_2$	0.103 M, 20.94g/litre
4)	$Ca\ Cl_2$	0.103 M, 22.56g/litre

Mix stock solutions by volume as follows:

Na Cl	100 volumes
K Cl	4 "
$Mg\ Cl_2$	3 "
$Ca\ Cl_2$	1 "

Solution B - phosphate buffer pH 8.0

Stock solutions:

1)	0.154 M, $Na\ H_2PO_4.2H_2O$	24.02g
	Distilled water to	1,000 ml
2)	0.103 M, $Na_2\ HPO_4.12H_2O$	36.89g
	Distilled water to	1,000 ml

Add stock solutions in the following proportions to give pH 8.0

Stock solution 1	0.36 ml

Stock solution 2 9.64 ml

Check pH with pH meter and adjust if necessary.

Solution AB

Mix by volume

Solution A 9 parts

Solution B - phosphate buffer pH 8.0 1 part

Phosphate buffered saline pH 7.2 (P.B.S.)

Stock solutions:

1) $NaH_2PO_4.2H_2O$ 23.4g

Na Cl 8.5g

Distilled water to 1,000 ml

2) Na_2HPO_4, anhydrous 21.3g

Na Cl 8.5g

Distilled water to 1,000 ml

Add stock solutions in the following proportions to give pH 7.2

Stock solution 1 1 part

" " 2 3 parts

Check pH with pH meter and adjust if necessary.

Phosphate glucose saline (P.S.G.)

Na_2HPO_4, anhydrous 13.48g

$NaH_2PO_4.2H_2O$ 0.78g

Na Cl 4.25g

Distilled water to 1,000 ml

Dilute 6:4 with distilled water and add 1% glucose before use.

Normal saline (N.S.)

Na Cl 9g

Distilled water to 1,000 ml

5% orthophosphoric acid

orthophosphoric acid (Analar, BDH, 88% H_3PO_4) 5.68 mls

Distilled water to 100 ml

Chromic acid

Sodium or Potassium dichromate	63g
Distilled water to	35 ml

Dissolve by heating, cool and slowly add concentrated sulphuric acid to 1,000 ml. Keep beaker under running cold water.

Giemsa stain

Concentrated solution

Giemsa powder (BDH)	1g
Glycerol to	66 ml

Heat in water bath at 56°C for two hours.

add methanol	66 ml

Stir for four hours (magnetic stirrer). Mature for seven days on window sill. Filter into bottle.

Working solution

Dilute concentrated solution 1:10 with phosphate buffered saline, pH 7.2, prepared from buffer tablets (Gurrs).

Staining procedure

Apply diluted stain (1:10) for 30 minutes. Wash off thoroughly with P.B.S., pH 7.2 (Gurrs). Air dry and examine.