THE IMMUNOFLUORESCENT REACTION FOR THE DIAGNOSIS OF PROTOZOAL INFECTIONS IN MAN AND ANIMALS

by

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Thesis presented for the Degree of Doctor of Philosophy
of the University of Edinburgh in the Faculty of Science, 1972

SUMMARY

i. Methods for performing the IFA test in the diagnosis of trypanosomiasis were compared. In the method adopted, the trypanosomes are separated from the blood and washed in normal bovine or horse serum. The trypanosomes in serum are dropped onto clean slides and, after drying, are fixed with 5% formalin.

ii. The antigen, prepared as described, retained its properties at room temperature for up to 3 weeks. At -20°C it has retained its antigenicity for at least 18 months.

iii. Blood collected on filter paper had the same degree of fluorescence as serum. The duration of the activity in the dried blood samples depends on the potency of the serum and on the temperature at which the samples were stored.

iv. The IFA test, as described, was used to differentiate between brucei-group trypanosomes. The difference in the titre of a serum when it was reacted with the homologous as compared with the heterologous antigen, was at least 4-fold.

v. Infection by the non-pathogenic T. theileri would not interfere with the diagnosis of trypanosomiasis in cattle. Since the titre of sera from cattle known to be infected with T. theileri was never higher than 1/40 when tested against salivarian trypanosomes.

vi. In trypanosomiasis, a high IFA level, indicates a current infection; a low level may indicate either a cured or new infection.

vii. The IFA test cannot distinguish between variant antigens that appear during the course of infection with trypanosomes.

viii. Agglutination and neutralization tests do differentiate between the variant antigenic types.

ix. There is no change in the antigenicity and infectivity of trypanosomes that had been separated from blood on a DEA-Cellulose column.

x. Absorbing the agglutinins for one antigenic type, had no effect on the agglutinability of second variant. The fluorescent titre was reduced with both variants.

xi. The agglutinating titre of IgM did not differ from that of whole antiserum. Some agglutinins were also present in IgG.

xii. The fluorescent reaction was limited to the IgG fraction.

xiii. Trypanosomes washed in saline for 30-40 minutes, lost their agglutinability and the fluorescent titre was reduced. By adding glucose to the saline, agglutinability was maintained for 40 minutes, although the titre was slightly reduced.

xiv. Antibody to culture or fly form of trypanosomes was present in sera from infected animals. The agglutination reaction was variant and species specific with 3-day old culture forms.

The titre declined and the reaction became non-specific with trypanosomes from 8-day old cultures. The fluorescent titre also declined as the cultures aged, or the longer the trypanosomes had been developing in the fly.

xv. Methods of performing the IFA test with Babesia were tested. Antigen was prepared from a concentrated suspension of washed infected red blood cells. The dried antigen was stored at -70°C and fixed with acetone just before use.

xvi. The IFA test was applied to carry out a serological survey for redwater in cattle on the island of Arran.

xvii. Although only one case of clinical redwater had been reported in recent years, 63 out of a total of 222 sera from beef cattle on 8 farms gave a positive reaction in the IFA test.

xviii. None out of 22 dairy cattle gave a positive reaction in the IFA test.

xix. None of the 55 calves born in the year of the survey had babesial antibody, but 17% of the 2 years old animals had babesial antibody.

xx. The proportion of animals with babesial antibody increased with the age of the animals.

xxi. The farms where clinical cases have been seen in recent years (Dougarie and Auchencar) also have the highest positive rate in adult animals.

xxii. The positive cattle can still infect ticks, and infected ticks can transfer the disease to susceptible cattle. There is therefore still a potential redwater in the island.

xxiii. Titration of 53 positive cattle sera showed that the titres were low, the highest was 1/160.

xxiv. The low IFA titre of the sera from cattle in Arran may indicate a transient infection of cattle by deer species.

xxv. A fluorescein-conjugated antideer serum was prepared.

xxvi. Two samples of blood on filter paper from deer shot on Arran were tested for babesial antibody. One sample fluoresced brightly with B. divergens as the antigen.

xxvii. 6 out of 18 samples of sera collected from deer in West Ross and Invernesshire gave a positive reaction in the IFA test with B. divergens as the antigen.

xxviii. Babesial infection of red deer in Britain has not previously been revealed.

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CHAPTER 1

INTRODUCTION

The diagnosis of diseases in man and animals is carried out by different methods depending on the nature of the causative agents. Diseases can be classified as physiological and infectious. This work is concerned with the diagnosis of diseases caused by infectious agents; and with methods that can be used to detect and identify the causative organisms. Diagnostic methods may be direct, including microscopic and macroscopic examinations or indirect. In macroscopic methods, the samples are examined directly without the aid of any instrument, e.g. helminths, arthropods. In microscopic methods, samples of blood, tissue, or gut contents are examined when fresh or after staining with the aid of a microscope. Indirect methods include culture, animal inoculation and serology.

In recent years, considerable progress has been made in the development of serologic tests for the diagnosis of parasitic infections. In contrast to many other infections, the causative agents of certain blood parasite diseases (e.g. chronic Chagas disease, and malaria in semi-immunes) may be difficult or impossible to demonstrate by the usual laboratory procedures. In such cases, serologic tests provide the only practical means for diagnosis. In other diseases the causative agent has no specific localization (toxoplasmosis) or fluctuates (trypanosomiasis), and so is difficult to diagnose by ordinary

routine laboratory methods. Other advantages of serological methods are that they are specific and can be used for examining a large number of samples (screening tests) and this will help to establish the distribution of the disease.

It is recognized that many of the earlier serodiagnostic procedures were of limited value because of deficiencies in specificity, sensitivity, and reproducibility. In the majority of cases, the antigens were crude and the tests were designed on more or less empirical bases. However, with the introduction of new and more effective methods for the isolation of specific parasite antigen fractions and the improvement of serologic techniques, there are now reliable, specific serodiagnostic tests for many of the parasitic diseases. For practical purposes, the technique must be simple to perform and it should not require expensive apparatus or materials. This study is concerned with serodiagnosis of protozoal infections; methods include: agglutination, neutralization, complement fixation, indirect haemagglutination, precipitation and fluorescent antibody.

Serodiagnostic tests can be grouped in two general categories namely, (1) those that employ the intact organism as antigen e.g. agglutination and fluorescent tests, (2) those that employ soluble antigens e.g. complement fixation, indirect haemagglutination precipitation, and also fluorescent tests. It is the fluorescent antibody test which is the subject of this thesis.

The fluorescent antibody technique consists of labelling antibodies with a fluorescent dye and applying this to a smear which contains specific antigens or antibodies already attached to the antigen. The sites where antibody attaches to antigen could then be seen by fluorescence microscopy.

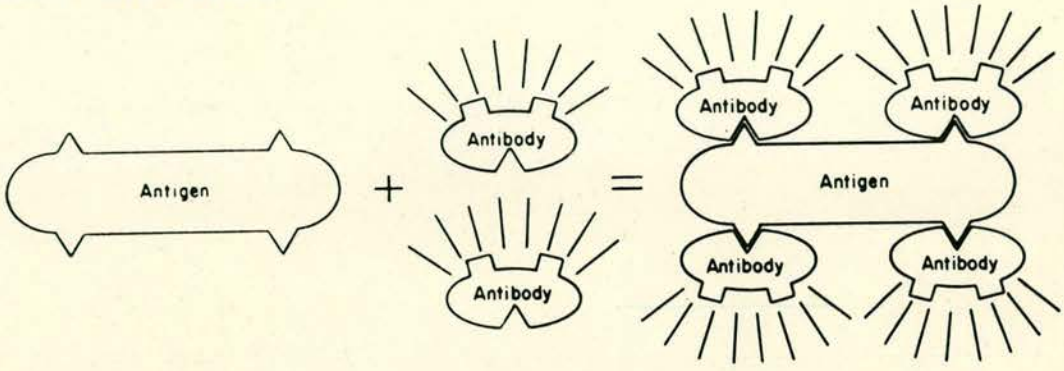
There are two methods of applying the fluorescen technique:-

1. Direct method also called "one layer procedure" (Fig. 1). This was the original method of Coons et al (1941, 1942) and was used first for protozoa by Goldman (1953, 1954).
2. Indirect method (IFA) "double layer" or "Sandwich technique" (Fig. 1). This was first described by Weller and Coons (1954) in studying Viruses.

The IFA test has the following advantages over the direct technique:

a) Only a single fluorescent antiglobulin is needed to test any number of sera for each animal species.
b) Small amounts of sera can be tested and any loss of antibody titre due to preparation of the conjugate is avoided (Nairn et al, 1959).
c) It is four to ten times more sensitive. (Coons, 1956; Nairn, 1964; Voller, 1964).
d) Serial dilution of immune serum until fluorescence disappears, provides a method of titrating antibody of reasonable practical accuracy (Nairn, 1964).

DIRECT METHOD



INDIRECT METHOD

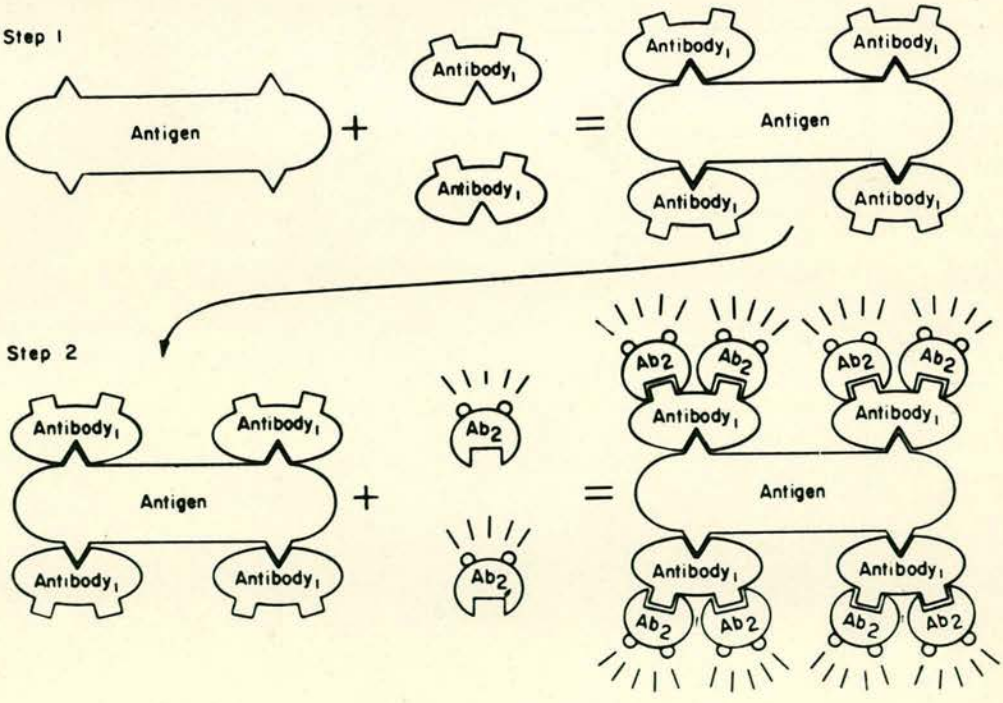


Fig 1

The fluorochromes which have been widely used for labelling proteins are:-

1. Fluorescein isocyanate (FIC): first used by Coons et al (1941, 1942). FIC labelled antisera react with the antigen to give a brilliant apple-green fluorescence which is similar to the autofluorescence of tissues.
   FIC is unstable and fresh synthesis of the dye is always necessary before each conjugation and its use has thus been abandoned.
2. Fluorescein isothiocyanate (FITC): introduced by Riggs et al (1958). It is stable and can be stored for long periods when kept dry. It is the most commonly used fluorochrome. Conjugates give a similar fluorescent emission to that of FIC.
3. 1-dimethylaminonaphthalene-5-sulfonic acid (DANS): first used by Webber (1952). Conjugates give a green fluorescence.
4. Lissamine rhodamine B (RB200): first used by Chadwick et al (1958) to overcome the unsatisfactory contrast between the fluorescent emission of the previous fluorochromes and the autofluorescence of tissues. Conjugates give yellow-orange fluorescence.

The fluorescent antibody test may be carried out with minute amounts of sera or even with eluates of blood collected on filter paper. The former technique allows antibody titration

and very long conservation of the serum at -20°C. The latter allows very fast blood collection for mass examination but, the conservation of these dried blood samples is shortened to a few weeks at -20°C or to a fortnight at room temperature. Sadun et al (1961, 1962) were the first to use a minute amount of dried blood sample for the diagnosis of Schistosomiasis and and trichinosis.

Sadun et al (1963) observed a close agreement of results between fluorescent tests made with sera and with blood smears eluates in trypanosomiasis. Souza and Camargo (1966) showed a close agreement in the results of complement fixation with fluorescent test when dried blood samples were used in the diagnosis of American trypanosomiasis, they also found that keeping dried blood samples at room temperature for 30 days did not affect the reactivity of the samples.

Bailey et al (1966, 1967) used dried blood for a screening test in the diagnosis of human trypanosomiasis in Africa. They showed that dried blood samples stored at a -20°C retain their original activity for up to 2 months. At room temperature, however, all activity is lost after 6 weeks. Lucasse (1970), Wery et al (1970) also used dried blood samples for diagnosis of Gambian sleeping sickness.

Many variations in the actual technique have been described. They concern chiefly the way of preparing the antigen. In earlier work with the test, antigen was prepared as a thin smear of

parasitized blood from an infected donor, but some problems were encountered when the parasitaemias were low. This difficulty was overcome when Sulzer and Wilson (1967) improved the methodology by employing thick smears of washed parasitized erythrocytes as antigen for malaria. Other variations include the fixation of the antigen, the use of a counter stain for decreasing the intensity of the autofluorescence of the specimens, examination of the preparation, i.e. dry or mounted in buffered glycerine and covered, the pH of washing and mounting fluids (pH 7.2 - 9). Every author agrees that antigens, sera and labelled antisera should be kept at -20°C, that the reactions should take place in a moist atmosphere, that the intermediate washing should be made in PBS, that the preparations should be examined under blue or UV light within 24 hours, and the measurement of green fluorescence should be classified in 4 or 5 degrees of intensity.

Application of fluorescent antibody tests to Protozoa

Trypanosomes

Human and animal trypanosomiasis constitute important clinical and public health problems in large regions of the world. An unequivocal diagnosis of trypanosomiasis is often difficult to obtain, since the clinical picture is not always well defined and the organisms frequently cannot be recovered by blood examinations. Consequently, there is a need for reliable, rapid, and inexpensive procedures which could provide the basis for an adequate diagnosis of these infections, especially during the latent and chronic phases of the disease.

The fluorescent antibody test has been used for many years in the diagnosis of trypanosomiasis. The first tests were made in 1959 by Fife and Muschel for the diagnosis of Chaga's disease and culture forms were chosen by these authors as antigens. Since then many workers have studied this system of diagnosis (see Tables) **page 8 & 9**.

For testing the specificity of the test, Williams et al (1963) and Sadun et al (1963) used IFA test to differentiate between T. rhodesiense, T. gambiense and T. cruzi. Thin blood films were made from the tail blood of infected rats. Antisera were derived from rabbits bled at various intervals after being infected by a single I/P inoculation; also sera from patients proved to be trypanosomiasis cases by recognition of the organism.

Infection	Organism	Antigen	Fixative	Serum	Dried sample	Reference
Chagas disease	T. cruzi	Culture form	0.1% formalin in saline	+		Fife & Muschel (1959)
African trypanosomiasis	T. rhodesiense T. gambiense	Blood smear	5% formalin - rhodamine bovine albumin	+		Williams et al (1963)
African & American trypanosomiasis	T. rhodesiense T. gambiense T. cruzi	Blood smear	"	+	+	Sadun et al (1963)
Chagas disease	T. cruzi	Blood & culture forms	Acetone	+		Voller (1963)
Chagas disease	T. cruzi	tissue section	formalin	+		Essenfeld & Fennell (1964)
Chagas disease	T. cruzi	Soluble antigen	fixed to an artificial matrix	+		Toussaint et al (1965)
Chagas disease	T. cruzi	Culture form	2% formalin in buffered saline sol.	+	+	Camargo (1966)

Infection	Organism	Antigen	Fixative	Serum	Dried sample	Reference
Chagas disease	T. cruzi	Culture form	1% formalin in PBS	+	+	Souza & Camargo (1966)
African trypanosomiasis	T. rhodesiense	Blood smear diluted with PBS	Heat		+	Bailey et al (1967)
Bovine trypanosomiasis	T. brucei, T. congolense & T. vivax	Blood smear diluted with PBS	Heat	+		Wilson, Cunningham & Kember (1967)
Bovine trypanosomiasis	T. congolense	Blood smear	Heat	+	+	Wiesenhutter (1969)
Chagas disease	T. cruzi	Culture form	1% formalin in PBS	+		Camargo & Rebonato (1969)
Gambian sleeping sickness	T. gambiense	Blood smear diluted with PBS 1:1	5% formalin in PBS	+	+	Lucasse (1970)
Gambian sleeping sickness	T. gambiense	Blood smear	No fixation	+	+	Wery et al (1970)
African trypanosomiasis	T. rhodesiense	Blood smear	Heat	+	+	Onyango & Woo (1971)

There were extensive cross-reactions between the different antigens; cross-reactivity existed also with T. lewisi and it was suggested that this species might be used for laboratory diagnostic procedures, having the advantage that it is not infective to man. Heterologous conjugates from antisera to plasmodia or helminths did not react with T. cruzi antigen (Voller, 1963). Weitz (1963) prepared conjugated antisera to the "exoantigens" and "bound antigens" of T. brucei and T. vivax. Antisera to the exoantigens of both species of trypanosomes were obtained from white rats after multiple intramuscular injections with serum from rats infected with T. brucei and T. vivax. The infected sera contained soluble antigen "exoantigen" to these trypanosomes. Antisera to the bound antigens were prepared by injecting rats with trypanosome homogenate which had been freed from exoantigen (surface coat) by washing them in Alsever's solution.

Antigens were prepared from blood films that had been diluted with 4-5 volumes of Alsever's solution and fixed with methanol. Conjugated antibodies to the soluble antigens of trypanosomes reacted specifically with the homologous species only; antibodies to the bound antigens reacted with both species. This specificity extended to antigenic variants of the same parent strain; in fact, the results paralleled those obtained

by agglutination tests. The test used to differentiate trypanosome species, Wilson, Cunningham and Kimber (1967) have shown that T. brucei, T. congolense, and T. vivax can be distinguished by the IFA test, but they were not able to distinguish between members of the brucei group (Wilson, 1969; Wilson and Cunningham, 1971). According to Wilson (1969) the IFA titre of bovine sera was higher when the homologous as opposed to the heterologous species was the antigen. Wery et al (1970) failed to distinguish between pleomorphic members of the brucei-group. Schindler and Sachs (1970) were able to distinguish T. congolense from T. brucei by the titre of sera in IFA tests.

In areas where Chagas disease and mucocutaneous leishmaniasis occur, Camargo and Rebonato (1969) showed that positive results were obtained from patients with Chagas disease and with leishmaniasis when the serum was tested against both T. cruzi and Leishmania braziliense. They increased the specificity of the technique by absorbing the heterologous reactivity of the serum and left the homologous reactivity only; in other words, by absorbing serum from patients with Chagas disease with L. braziliensis and by absorbing serum from cases of mucocutaneous leishmaniasis or Kala-azar with T. cruzi, the heterologous reactivity was removed and left the homologous reactivity intact.

In a comparison between the fluorescent and other serological tests, Souza and Camargo (1966) and Camargo (1966) found close agreement in the results of fluorescent and complement fixation tests in serodiagnosis of Chagas disease.

Wain et al (1966) and Wilson (1969) compared agglutination and IFA tests for diagnosis of bovine trypanosomiasis and they showed that the former test was of limited use due to the antigenic variation of the trypanosomes, and this was in contrast to fluorescent tests which did not detect antigenic variants. Wiesenhutter (1969) found that IFA test is more accurate than the microscopical examination and useful as a rapid diagnostic method in bovine trypanosomiasis.

Cerisola (1970) used indirect haemagglutination (IHA), IFA and complement fixation tests for serodiagnosis of Chagas disease, and he stated that IFA test was the first to become positive in the acute phase of the disease, and the IHA test was the most simple and rapid to perform. He further stated that there were certain advantages in performing all three tests for routine diagnosis.

Raised level of IgM has also been used in the diagnosis of trypanosomiasis, Courtois and Bidean (1966); Bailey et al (1967); and Wery et al (1970) showed that the fluorescent antibodies are more specific for trypanosome infections and more constant in the serum of infected people than the IgM level, that a high IgM level of the serum may occur in many

other diseases. Onyango and Woo (1971) and Woo (1971) have shown that sera from people with high IgM level may give a negative reaction with the fluorescent test.

Leishmania

Shaw and Voller (1964) used as slide antigens thin smears made from 10-day-old culture of L. infantum, liver smears of hamsters infected with L. donovani and tissue sections with L. brasiliensis. Slides were air dried, immersed in 0.3 NHCl for 5 minutes. They showed that the test was only group specific, as Trypanosoma cruzi antigen also fluoresced after treatment with L. infantum serum.

Duxbury and Sadun (1964), Bray and Lainson (1965) found that no differentiation of Leishmania species was possible by serial dilution of sera in the fluorescent antibody test; all sera stained all antigen preparations and absorption of sera extinguished the reaction irrespective of the antigen used. As regards use of the test for diagnosis of leishmania infection in the field, Bray and Lainson (1965) conclude that it might be useful for the diagnosis of visceral leishmaniasis, but only where trypanosomiasis was absent, and not for the diagnosis of cutaneous leishmaniasis.

Human sera from patients with leishmaniasis were shown to react once in 7 trials with T. cruzi and T. rhodesiense as antigens (Williams et al 1963; Sadun et al 1963). Camargo

and Rebonato (1969) showed a cross-reaction between sera from patients with Chagas disease and with leishmaniasis when the sera were tested against both T. cruzi and L. braziliensis antigens. These results do not agree with the report by Araujo and Mayrink (1968) who found that serum from patients with Kala-azar or mucocutaneous leishmaniasis reacted only homologously, respectively with L. donovani or L. braziliensis and that there was no cross-reactivity, cross-reactions were observed only when undiluted or slightly diluted serum was used.

In comparison with other tests, Mayrink et al (1967) showed that fluorescent antibody test gives a higher titre than the complement fixation test and is more sensitive in diagnosis of visceral leishmaniasis.

Plasmodium

Tobie and Coatney (1961) attempted to develop fluorescent antibody staining of plasmodium species in man instead of routine staining with Romanowsky stains. Antigen was prepared as thin blood films fixed in 0.1 per cent HCl. Antibody was provided by the globulin fraction of the serum of a long-term P. vivax, patient. Antibody to P. cynomolgi bastianelli yielded fluorescence similar to that elicited by antibody to P. vivax. Ingram

et al (1961) showed that P. cynomolgi bastianellii, and P. gallinaceum could be visualized by fluorescent antibody methods. Voller and Bray (1962) used the IFA test to measure humoral antibody to the parasites P. falciparum, P. malariae and P. ovale.

Test sera were from naturally infected humans and included umbilical cord blood. The antisera failed to stain oocyst or sporozoite material from mosquitoes. Antibody was demonstrated in the sera of naturally infected individuals and in the cord blood of African infants. Kuvin et al (1962) also used the technique for detection of antibody production in human malaria. Unfixed smears stored at -50°C proved most satisfactory showing a minimum of background fluorescence after storage up to 15 weeks.

Immunofluorescent techniques have also been used in the study of other stages of malaria parasites. Corradetti et al (1964) were able to stain the sporozoites of P. gallinaceum from mosquitoes with labelled antibodies prepared in rabbit against the sporozoites and they stated that this may help to identify sporozoites found in mosquitoes captured in nature.

El-Nahal and Bray (1966) used 0.1% Evans blue in PBS - as a counter stain in the reaction. Cox et al (1969) used the test for measuring the antibody level in mice infected with P. vinckei, and P. chabaudi.

The methodology of the technique was improved when Sulzer and Wilson (1967), Sulzer et al (1969) and Wilson et al (1970)

employed thick smears of washed parasitized erythrocytes as antigen. The washing removed host-serum antibodies that interfered to some extent with the reaction, and permitted adjustment of the erythrocyte suspension to provide the optimal number of parasites for the test. Kielmann et al (1970) used Plasmodium gallinaceum as the antigen for the diagnosis of human malaria. Blood smears were prepared from infected chicken when the parasitaemia reached 80-90%. Smears were fixed with acetone for 10 minutes and the antisera were obtained from patients known to be infected with malaria. The specificity of the test was investigated by Ingram and Carver (1963); they found cross reaction between P. berghei, P. vivax and P. bastianellii.

Voller (1965) and El-Nahal (1967) showed that antisera against P. berghei react strongly with the same antigen but less strongly with other species of Plasmodium. Collins et al (1966) titrated antisera from monkeys infected with malaria against 10 homologous and heterologous antigens and he showed that all of the species exhibited some level of cross reaction with the other species examined, but all gave the highest level of heterologous reaction to P. fieldi antigen, suggesting the presence of a common or generic antigen in this species.

Collins et al (1967) tested 498 sera from Nigerians with five Plasmodium antigens by the IFA test. The average fluorescent antibody (FA) response was highest to P. falciparum, followed by P. fieldi, P. brasilianum, P. ovale, and P. vivax. The average

FA endpoint increased with age of the individual with all the antigens, and also was higher in the males than in the females.

Cox and Turner (1970) with their studies on 4 species of Plasmodia and 2 species of Babesia showed that the antiserum of one species of Plasmodium reacts strongly with the same antigen but less strongly with the other three species of Plasmodia and gave a very weak reaction with Babesia species.

In a comparative study Voller (1965) obtained the same results with the haemagglutination and immunofluorescence tests in the diagnosis of malaria, He was of the opinion that the immunofluorescent reaction is considerably more sensitive than complement fixation in serodiagnosis of malaria infection and any small changes in antibody level are more easily detected. Sadun et al (1969) used a soluble antigen fluorescent antibody (SAFA) and indirect haemagglutination (IHA) tests to follow the course of antibody development in human malaria. They showed that these two tests provide a specific and sensitive method for diagnosis.

Babesia

This organism is an intraerythrocytic parasite which causes a disease known as babesiasis or red water. There are 17 distinct species of Babesia recognized by Neitz (1956) from various vertebrate hosts.

Bovine babesiasis is a tick borne disease of cattle widely distributed in both temperate and tropical countries, causing considerable economic loss, yet little is known of the host-parasite interactions involved in cattle or in other animal species If cattle are infected as calves they normally show no symptoms and develop an immunity which may last years. As cattle grow older, however, the infection can be more serious. If cattle are not infected until they are adults the disease can be very acute and invariably fatal unless treated. Any animal which recovers is a "Carrier animal". This means that the causal organism is still present in small numbers in the blood. Ticks feeding on a carrier animal can be infected and later transmit the infection to another animal. Where attempts are being made in the world to eradicate the disease these carrier animals need to be identified and eliminated.

Babesiasis may be diagnosed during the acute stage by finding the parasite in blood films stained with Giemsa or other stains, however, parasites are not readily found in erythrocytes during latent infections. In recent years many serological methods have been developed for diagnosis of babesia infection in animals and the most recent one is the fluorescent test.

Ristic et al (1964) used the direct method for B. caballi and B. equi. The antigen was a thin blood smear fixed with alcohol. Antisera were obtained from horses experimentally infected.

Garnham and Voller (1965) used acetone-fixed B. divergens antigens for measurement of antibody to B. divergens in splenectomized rhesus monkeys. The slide antigen could be stored unfixed at -70°C and did not deteriorate even after storage for 9 months.

Schindler (1965) and Schindler et al (1966, 1970) records the demonstration of antibodies to B. canis by immunofluorescence.

Improvement in the methodology of the technique for serodiagnosis of babesia infection was done by Ross and Lohr (1968). They used IFA test for the detection of B. bigemina antibodies in bovine serum. Infected blood was washed 3 times with PBS and thin smears were made from the packed erythrocytes and air-dried. Antigens were fixed with N/100 HCl for 1 min followed by distilled water and PBS, consecutively. By using this method they were able to detect reactors infected 2 years previously.

For testing the specificity of the test to differentiate between different species of Babesia, Madden et al (1968) used the IFA test to detect antibody in "Carrier" horses experimentally infected with B. caballi. The reactions were found to be specific. There was no cross reaction with sera from carrier horses experimentally infected with B. equi. Sera of B. bigemina or B. major immune animals showed reactions with the heterologous antigens only in the lower dilutions (Zwart et al, 1968); Ludford (1969) found that antisera against B. rodhaini shows very high titre with this parasite but weakly or not at all with B.

argentina, B. bigemina or B. canis. This was supported by the work of Cox and Turner (1970) who demonstrated that two morphologically similar parasites of mice, B. rodhaini and B. microti, were readily distinguishable by the IFA test. Recently (1971) Brocklesby et al, diagnosed B. major infection in British cattle by IFA test and showed that the test can differentiate between B. major and B. bigemina. In comparison between fluorescent test and other serological methods, Johnston and Tammemagi (1969) used both complement fixation and fluorescent antibody tests to measure antibody titre in cattle infected with B. argentina. They found that fluorescent antibody titre lasted longer than complement fixation antibody.

Theileria

Schaeffler (1963), Schindler and Wokatsch (1965), and Schindler and Mehlitz (1968) using a thin blood smear of infected blood as antigen for diagnosis of T. annulata in cattle, Pipano and Cahana (1969) prepared the antigens by grinding the livers of artificially infected calves and then made smears on slides. Lohr and Ross (1969) washed the infected blood with PBS and then prepared the antigen from packed erythrocytes for the diagnosis of T. parva infection in cattle.

Anaplasma

Ristic et al (1957) used the direct method to detect Anaplasma marginale, the antigen was prepared as a thin blood film, it was fixed with absolute alcohol for 10 seconds, and then treated with a drop of fluorescein-labelled anti-Anaplasma globulin.

Madden (1962) applied the technique for study of the structure of A. marginale. The antigen was fixed in 10% formalin in PBS for 10 minutes. He showed that some anaplasma bodies appear as round bodies with a tail connected to them and he stated that the tail is an integral part of the Anaplasma.

Schindler et al (1966) differentially titrated the antisera to A. marginale and A. centrale.

Toxoplasma

In smears prepared from the peritoneal exudate of mice infected with Toxoplasma gondii, Goldman (1957a) readily demonstrated this microorganism by immunofluorescence. Labelled antibody was prepared from both infected human and rabbit sera.

The one-step inhibition procedure was first applied by Goldman (1957b) to T. gondii. The principle of this test is that a mixture of the test serum and known fluorescein-labelled antiserum is added to a dried smear of toxoplasms for one hour at 37°C. The smear is then rinsed and examined with a fluorescence microscope. Reduction in the brightness of fluorescence, as compared to that of a negative control slide,

indicates the presence of antibody in the test serum.

Carver and Goldman (1959) investigating the possibility of staining T. gondii in tissues, found that formalin and other commonly used histological fixatives abolished staining capacity. One to seven days fixation in an absolute ethanol-glacial acetic acid mixture (19:1), however, gave satisfactory results; Ourth (1971) fixed the tissue smears in cold acetone (-20°C) for 10 minutes, rinsed for 1 minute in carbonate-bicarbonate buffer pH 9.0. 1% aqueous solution of Evans blue as counter stain was used.

Fulton and Voller (1964) used the IFA test to detect Toxoplasma antibodies in human sera; Ishizak (1969) in swine sera.

Sulzer and Hall (1967) and Sulzer et al (1971) found that the anterior end or pole of the Toxoplasma organism acquired a brilliant fluorescence when treated with sera negative for specific antibody. They called this factor in the serum as Toxoplasma anterior polar (TAP) factor. When a counter stain, Evans blue, was used to mask non specific staining by the conjugate, the brilliance of polar staining persisted, equal to that of staining by specific antibody. Polar staining, however, was confined to the anterior end, whereas staining by specific antibody extended entirely around the periphery of the Toxoplasma. Polar staining reaction can be titrated in the same manner on specific antibody. The factor is heat stable. It is present in nearly

all sera of humans above the age of 2 years. It apparently does not occur in the sera of infants less than 6 months of age.

In comparison with other tests, Camargo (1964, 1966); Fletcher (1965); Walton et al (1966) and Coutinho et al (1970) used Sabin-Feldman and IFA tests for diagnosis of human toxoplasmosis. The sera tested were from patients with suspected or proved toxoplasmosis. There was a close agreement between the results of both tests, although the fluorescent antibody technique tended to yield higher titres. Shevkunova and Vinogradova (1970) stated that IFA test gives a higher titre and is more sensitive than complement fixation in the diagnosis of toxoplasmosis.

Entamoeba

Goldman (1953, 1954) used fluorescein-labelled antibody to stain cultures forms of Entamoeba histolytica, E. coli, Dientamoeba fragilis, Endolimax nana and E. invadens. Amoebae from cultures were washed, fixed with absolute methyl alcohol, and then suspended in saline. Antisera were prepared in rabbits by the SC inoculation of 10^7 living culture organisms, which had been washed so as to reduce bacterial content. By this method he was able to differentiate E. histolytica, from the closely related E. coli. Later Goldman (1960) and Goldman et al (1962) used Microfluorimeter to detect minute differences in the

intensity of fluorescence between species of Entamoeba. E. hartmannii was found to be serologically different from various strains of E. histolytica. Also they used a series of cross absorption tests to differentiate between E. histolytica, K9 strain, E. histolytica, Huff strain, and E. hartmannii 335 strain. The two strains of E. histolytica were found to be closely related to each other but different antigenically from E. hartmannii. Goldman and Cannon (1967) by using the inhibition reaction and Microfluorimeter were able to distinguish between two groups of E. histolytica strains. Serum from person known to be infected with E. histolytica was labelled with fluorescent-isothiocyanate (FITC). All staining was performed with a mixture of conjugate plus unlabelled normal or antibody containing serum, in order to demonstrate the presence or absence of an inhibitory effect by the latter serum.

Jeanes (1964) and Goldman (1966) used the IFA test for diagnosis of amoebiasis. Sera obtained from patients, some of them were proved to be infected with E. histolytica.

Trichomonas

McEntegart et al (1958) found that Trichomonas vaginalis could be differentiated from T. foetus by immunofluorescence. Organisms from young cultures were deposited on slides, fixed with acetone and dried. Kucera and Kramar (1965) demonstrated antibody in the sera of trichomoniasis patients by the IFA test.

Abarbarchuk (1969) studied antigenic differences in strains of T. vaginalis from 37 women by 3 methods: agglutination, passive haemagglutination and indirect immunofluorescence. The conclusion was reached that strains of Trichomonas could be divided into several antigenic groups, and that the pattern of division of the strains was essentially similar with each of the 3 reactions used. Hosie (1971) used the technique for diagnosis of T. vaginalis and he stated that the higher level of immunofluorescent antibody against T. vaginalis was significant. No immunofluorescent antibody was found in the vaginal exudate of patients with a trichomonal infection. The animal immunofluorescence experiments indicated that different serotype of Trichomonas vaginalis exist.

For other protozoa, Dzbenski (1966) used the test to stain Balantidium coli, the best results were obtained when living Balantidia were used as antigen. Augustine and Lund (1970) showed there is no difference in the fluorescent reaction for both culture and freshly isolated Histomonas meleagridis antigens, and also antisera to H. meleagridis react weakly with H. wenrichi. Among the free-living Protozoa, Beale and Kacser (1957); Beale and Mott (1962) applied the test for Paramecium aurelia.

The review of the literature has shown that the fluorescent antibody test is a practical and useful aid in the diagnosis of protozoal infections. Its value has been proved in human medicine but has not yet been so widely applied in the epizootiology of

domestic animal diseases. A technique to be used routinely for diagnosis should be standardized. The first objective in this study was to compare and standardize the IFA technique for trypanosomiasis, and to find out if it could be used to distinguish between species of trypanosomes. A second point of the work was the application of the IFA test to a field problem, the epizootiology of babesiasis on the island of Arran.

CHAPTER II

MATERIALS AND METHODS

1. Laboratory animals

The following animals were used:

a) Mice, strains Q and B10, weighing approximately 20g, were bred and reared in the animal house of the Zoology Department, Edinburgh.

b) Albino rats of 150-200g body weight were supplied by Centre for laboratory animals - Edinburgh.

c) Half-lop rabbits, weighing 2-3 kg were bred and reared in Zoology Department, Edinburgh. Rats and mice were fed on Edinburgh University Diet, rabbits on diet SG1.

d) Calf, was supplied by the Centre for Tropical Veterinary Medicine (C.T.V.M.), Edinburgh.

2. The organisms

A. Trypanosomes

The trypanosomes were obtained from the low temperature bank of the Centre for Tropical Veterinary Medicine (C.T.V.M.), Edinburgh.

The strains used were as follows:

Trypanosoma brucei, TREU 851 (derived from EATRO 1523), TREU 852 (EATRO 1527), TREU 853 (EATRO 1529), TREU 927 (EATRO 1534), TREU 933 (EATRO 1644), TREU 958 (EATRO 1591), TREU 991 (EATRO 1589), TREU 833 (EATRO 1410), all isolated from Glossina pallidipes

caught in Kenya or Uganda by members of the EATRO/CTVM joint project 1969-71; TREU 900 (EATRO 1691) from wildebeeste in the Serengeti, 1969; TREU 1096 and 1097, from cattle in Nigeria; TREU 667, isolated from a reed buck in East Africa, 1968, and TREU 741 and 1027, old laboratory strains derived from EATRO 38.

T. rhodesiense, TREU 35 (derived from EATRO 173, Kenya), TREU 333 (EATRO 906), TREU 789 (EATRO 165, Tanzania), TREU 788 (EATRO 181, Botswana).

T. gambiense, TREU 390 from Dakar; E1 from Piti and R3 from Aliade, Nigeria (Gray, 1972).

T. evansi, TREU 379 from Colombia.

T. congolense, TREU 732, old laboratory strain, TREU 692, from East Africa, 1968, TREU 1095 from Nigeria, TREU 937, old strain, origin unknown.

T. lewisi, old laboratory strain from England.

B. Babesia

B. rodhaini, old laboratory strain.

B. divergens, Weybridge strain.

3. Methods used to infect laboratory animals

A. Trypanosome

A capillary lymph tube (Stabilate) of the antigen was removed from liquid nitrogen and allowed to warm in water for 30 minutes before opening. The contents of the tube were blown into a watch glass and mixed with citrated saline in a proportion of 1:2. Mice and rats were inoculated intraperitoneally (I/P) rabbits were inoculated into the marginal ear vein (I/V). The number of trypanosomes were estimated by haemocytometer method in which each rabbit inoculated approximately with 5×10^7 T. brucei, and 12×10^7 T. congolense.

B. Babesia

(i) B. rodhaini. The strain of B. rodhaini, obtained from the C.T.V.M., was maintained by weekly subpassage in laboratory mice. Each mouse was inoculated I/P with 0.1 ml of infected blood with citrated saline.

(ii) B. divergens. Calf X2 (6 months old), which served as a source of the antigen, was splenectomized 14 days before being injected subcutaneously and intravenously with 10 ml of blood in which about 7% of the erythrocytes were infected. Heparinized blood was obtained from Weybridge and was sent by rail, chilled. The calf was infected within 24 hours of bleeding.

4. Detection methods

A. Trypanosome

(i) Wet film.

A drop of the blood to be examined was placed on a microscope slide and a cover slip dropped on it. The resulting wet film was examined for motile trypanosomes under the x40 phase contrast objective of a Nikon microscope. If no trypanosomes were seen in 20-30 fields, the blood was recorded as negative.

(ii) Mouse inoculation.

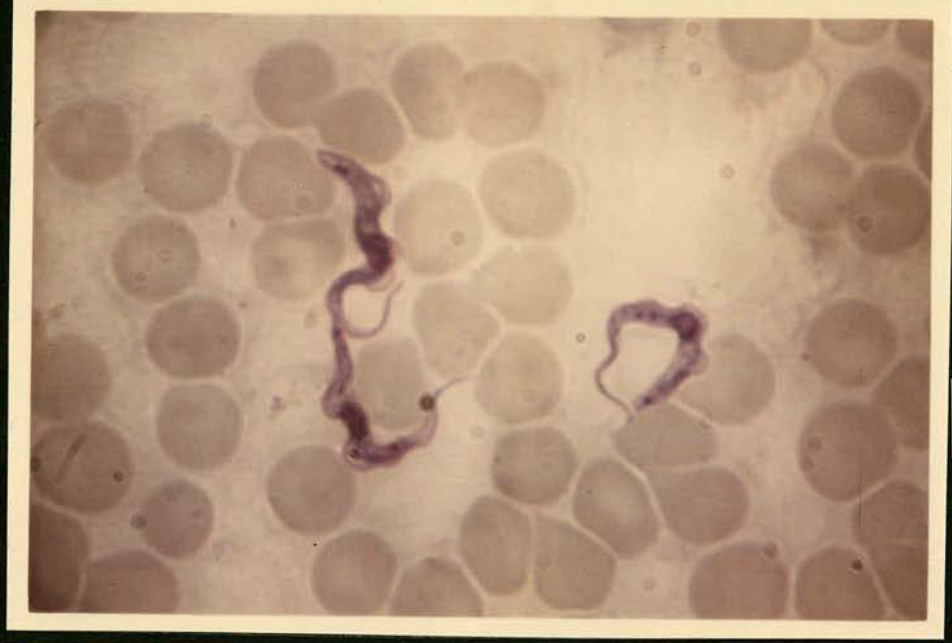
2 ml of the blood to be examined was mixed with 4 ml of citrated saline and inoculated I/P into a group of six mice; each mouse received 1 ml. From day 3 after inoculation, each mouse was examined thrice weekly for trypanosomes by making wet films of tail blood. If trypanosomes were detected, the sample was recorded as positive. Examination was continued for 4 weeks in the case of T. brucei, and for 6 weeks in the case of T. congolense infection, before the sample was recorded as negative.

B. Babesia.

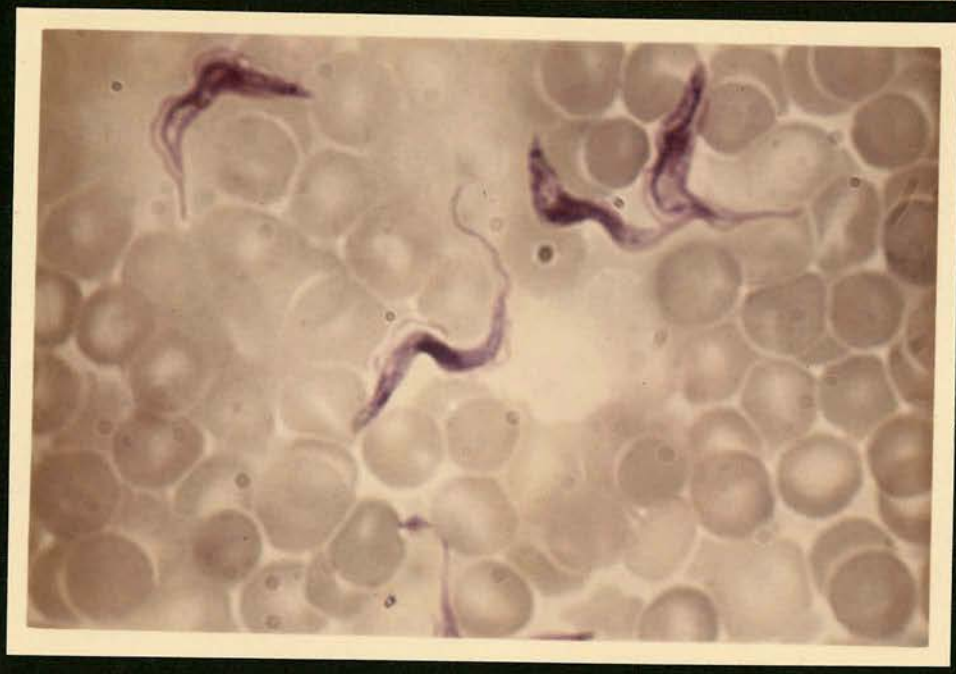
Detection of Babesia species was carried out on stained thin blood films.

Fig. 2 Blood form.

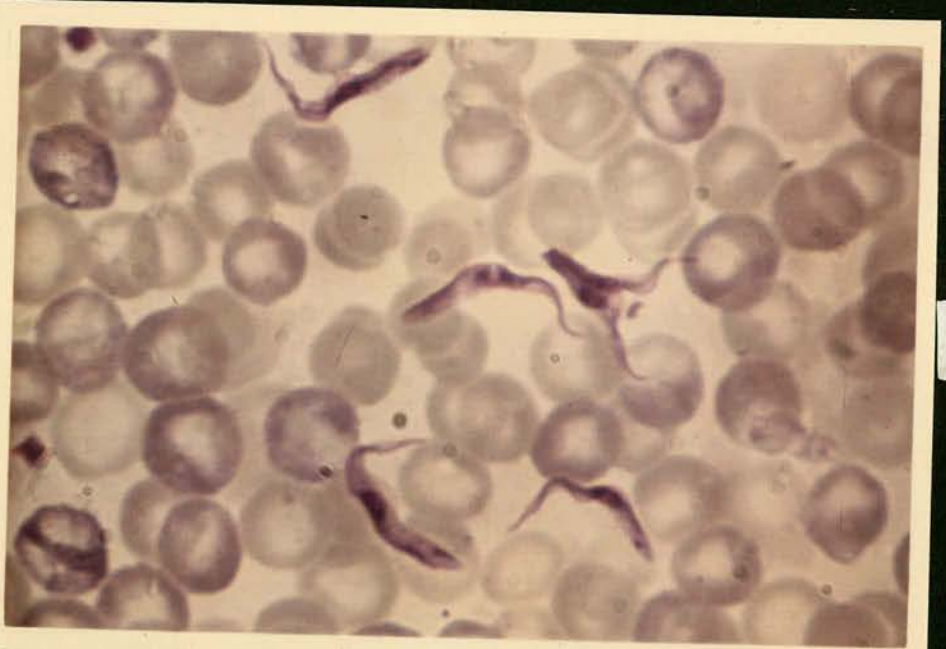
x 2000



T. brucei



T. rhodesiense



T. congolense

5. Counting methods

(i) For counting the percentage of parasitized erythrocytes in the case of Babesia, a thin blood film was fixed in methanol for 2-3 minutes, then stained with Giemsa's stain in buffered distilled water for 30 minutes. The slide was then rinsed in distilled water, dried, then examined under oil immersion, x100 objective. By the aid of ocular grid, the following formulae was used:

$$\frac{\text{Number of parasitized erythrocytes} \times 100}{\text{Number of erythrocytes counted.}}$$

(ii) For counting the number of trypanosomes or erythrocytes per one cubic millimeter; the blood was diluted 1/100 with citrated saline in a clean dry haemocytometer pipette, mixed thoroughly, then one drop of the suspension was placed under the cover glass, on the edge of both counting chambers of an improved neubauer haemocytometer (Hawksley, U.K.). The counting chamber was left in a horizontal position for ten minutes to allow the cells to settle. Some of T. congclense adhere to the cover slip, two levels were focussed, the bottom of the chamber and the cover slip. For the final result to be expressed as the number of cells per cubic millimeter, the following calculation is necessary.

Let N equal the number of cells counted in 80 small squares.

Then $\frac{N}{80}$ equals the number of cells in one small square.

The area of one small square is $\frac{1}{400}$ sq.mm; and the depth is $\frac{1}{10}$ mm.

The volume of one small square is therefore $\frac{1}{400} \times \frac{1}{10} = \frac{1}{4000}$ c.mm.

The result is given as the number of cells in 1 cubic millimeter, therefore $\frac{N}{80} \times 4000$.

The blood was originally diluted 1 in 100.

$\frac{N \times 4000 \times 100}{80}$ = number of cells per c.mm.

6. Preparation of antigen

I. Trypanosomal antigens for:

A. Fluorescent test

B. Agglutination test

A. Most of the strains or species of trypanosomes used in this work were grown in mice. When the first peak of parasitaemia was reached and trypanosomes were about 10^7 - 10^8/ml, the mice were anaesthetized with ether, as much blood as possible was obtained from the heart with a 1 ml heparinized syringe. T. gambiense - Nigerian strains were grown in nursling rats, and T. lewisi, in an adult rat. These animals were bled in the same way.

For separation of the trypanosomes, the blood was centrifuged at 1,500 g for 3 minutes, the supernatant removed and the buffy layer containing the trypanosomes was transferred to another tube. The trypanosomes were resuspended in 5 volumes of normal bovine (or horse) serum. The suspension was then centrifuged, at 12,000 g for 5 minutes, and about 4/5 of the supernatant was removed to leave a concentrated suspension of trypanosomes at the base of the tube. Six circles, about 0.5 cm in diameter were drawn with a pental pen on the under surface of clean microscope slide 0.8/1.0 mm in thickness. A drop of the trypanosome suspension was placed by means of a pasteur pipette on each of the marked circles, and the preparations left to dry in air. After circles had been placed on the slides it was

advisable to check each area under the microscope for the presence of trypanosomes because failure to do this could result in false negative reactions due to the absence of organisms. The dried antigen was fixed by immersing the slide for 15 minutes in a 5% solution of formalin in distilled water. The fixative was washed off with phosphate buffered saline (PBS, pH 7.2). When thoroughly dry, the slides were wrapped in aluminium foil (Alcan, London, U.K.) and placed in a 3 x 5" polythene bag containing a few crystals of silica gel (BDH, Poole, U.K.). The antigen coated slides were stored at -20°C. When required for tests, the slides were brought to room temperature before opening the bag.

B. The source of trypanosomes was blood obtained from the first peak of parasitaemia in infected mice. The procedure was the same as described by Cunningham et al (1963) except that heparin was used in a concentration of 50 units per ml of blood. The filled lymph tubes were placed in tube-shaped containers made of two paper layers strengthened by an intermediate layer of nylon net. The adhesive used in making these containers was Copydex. After loading the tubes into the paper holders, these were inserted into another metal (Dural HE15W) tube surrounded by 1" layer of expanded polystyrene. The mouth of the metal tube was plugged with cotton wool and the insulated containers were placed in a cabinet maintained at -70°C for 24 hours.

The paper containers were then rapidly removed and transferred to a liquid nitrogen for permanent storage. The nitrogen containers were filled each week.

II. Babesial antigen for the fluorescent test.

Antigen was prepared when about 25% of the erythrocytes were parasitized. Whole blood was collected from the jugular vein= of the calf and from the hearts of mice. The blood was mixed immediately with PBS in the proportion of 1 part blood to 9 parts PBS. The mixture was centrifuged at 1500 g for 3 minutes to separate the erythrocytes. After removal of the plasma, the red cells were washed 3 times in PBS. A suspension was then prepared of 1 volume red cells to 19 volumes PBS. Drops of this suspension were placed in marked circles on clean, thin microscope slides. The slides were left to dry in air for 30 minutes, then wrapped in tissue paper and aluminium foil and placed in a 3 x 5" polythene bag containing a few crystals of silica gel and were stored at -70°C until required.

7. Collection and storage of sera and dried blood samples

Sera were collected from animals by the following methods.

(i) Mice, were anaesthetized and bled from the retro-orbital venous plexus.

(ii) Rats were lightly anaesthetized and bled from the tail.

(iii) Rabbits were bled from the marginal ear vein.

(iv) Cattle were bled by jugular puncture using 15 ml vaccutainer (B-D, U.S.A.).

The blood was allowed to stand in the cold room at 4°C overnight; the serum was then separated from the clot. All sera were inactivated by heating at 56°C for 30 minutes in a water bath before being distributed in XLon plastic tube (XT 1530 1 ml). Sera were stored at -20°C until required.

When the animals were bled to obtain sera, a part of the blood was dropped on Whatman No. 4 filter paper. Each drop, when spread, had a diameter of approximately ½". The blood was allowed to dry for at least 10 minutes, it was wrapped in tissue paper, then in aluminium foil and was placed in 3 x 5" polythene bags containing a few crystals of silica gel. The sealed bags were stored at different temperatures.

8. Indirect fluorescent antibody (IFA) test

A. Trypanosome

For the test, the required number of slide antigens were removed from the deep freeze (-20°C), they were allowed to warm slowly by placing in the cold room (4°C) for one hour, then at room temperature for another hour before removing them from the polythene bags. Six circles were painted on the antigen coated surface of the slide with nail varnish. These circles were superimposed on those previously delineated on the under

surface of the slide. The slides were then placed in a plastic sandwich box which had been lined with wet filter paper. Drops of the test sera (0.05 ml) were placed in the marked circles and left for 40 minutes at room temperature. The serum was washed off by placing the slides for 10 minutes in 2 changes of PBS. The slides were dried by gently touching the surface with filter paper. The conjugated antiserum, at a dilution of 1/20, was then applied for 40 minutes and the washing procedure repeated.

The dried preparations were mounted in 90% glycerol in 0.5M carbonate/bicarbonate buffer, pH 9.0, covered with number 0 cover slips and examined under the fluorescent microscope.

B. Babesia

The slide antigens after taking from the -70°C cabinet, were kept over night at -20°C, then one hour at 4°C, and a further hour at room temperature before removal of the wrappings. The slides were fixed by immersing them for 10 minutes in anhydrous acetone, they were then left to dry in air for about 10 minutes. Circles of nail varnish were painted round the antigen, and the slides placed in a moist chamber. Subsequent treatment was as described for trypanosomes, except that the conjugated antiserum was diluted 1/10.

C. Controls

Serial dilutions of the control sera (1/10 up to 1/320) were prepared in PBS.

Control tests included incubation in normal serum or in PBS followed by conjugated serum, and incubation in conjugated serum alone. Controls were treated in the same way as the test sera.

9. Agglutination test

A capillary lymph tube (stabilate) of the antigen was removed from liquid nitrogen and allowed to warm in water for 30 minutes before opening. The contents of the tube were blown into a watch glass. Serial two-fold dilutions of the test sera in PBS from 1/10 to 1/20480 were used in the test. Pre-infection sera were used as controls in dilutions of 1/10 to 1/320. Approximately 0.1 ml of each dilution of the test and control sera was placed within circles marked on clean microscope slides. One drop of the antigen was added to each dilution of the test and control sera through the finely drawn point of a pasteur pipette. The slides were placed in a humid chamber for one hour at room temperature.

The slides were examined microscopically under x10 objective. The scoring system of Cunningham and Vickerman (1962) was used. The degrees of agglutination ranged from 4 to 1 plus according to the size of clump (Fig. 3). The titre of a serum was the highest dilution to give a one plus score.

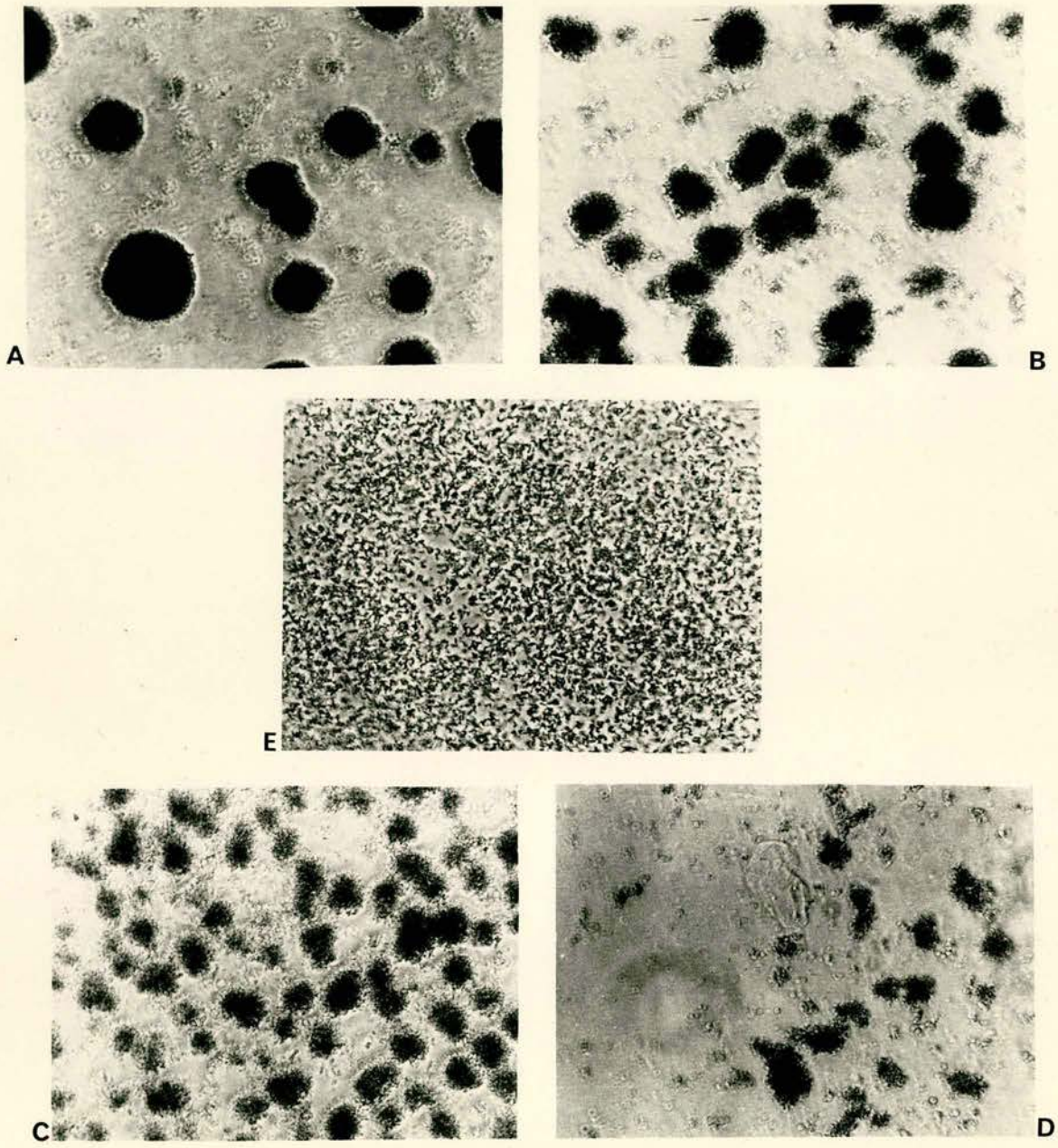


Fig 3 Degree of agglutination X 10

A . 4
B . 3
C . 2
D . 1
E . o

10. Fluorescent microscopy

The microscope was Nikon with mercury super pressure lamp ballast type 2SHC-2203LA (TOkyo Shibaura Electric Co., Ltd., Japan).

In trypanosome the slides were examined with a darkfield condenser at a magnification of 400. The light source was a 200W high pressure mercury vapour lamp in combination with a BV excitation filter. Y51 and Wratten 2B eyepiece filters were used. In Babesia, the unmounted slides were examined under x100 oil objective, and the light source was the same for trypanosome except 054 eyepiece filter used instead of Y51.

Areas of specific fluorescence were a brilliant yellow-green. Examination should be in a dark room and within 24 hours, if carried out later, the fluorescence may fade.

In recording the results of titrations, four intensities of fluorescence have been recognized: Figs. 4, 5, 6, 7

A. 4 = very strongly fluorescent

B. 3 = strongly fluorescent

C. 2 = moderately fluorescent

D. 1 = weakly fluorescent

E. 0 = non fluorescent

TRYPANOSOMA RHODESIENSE

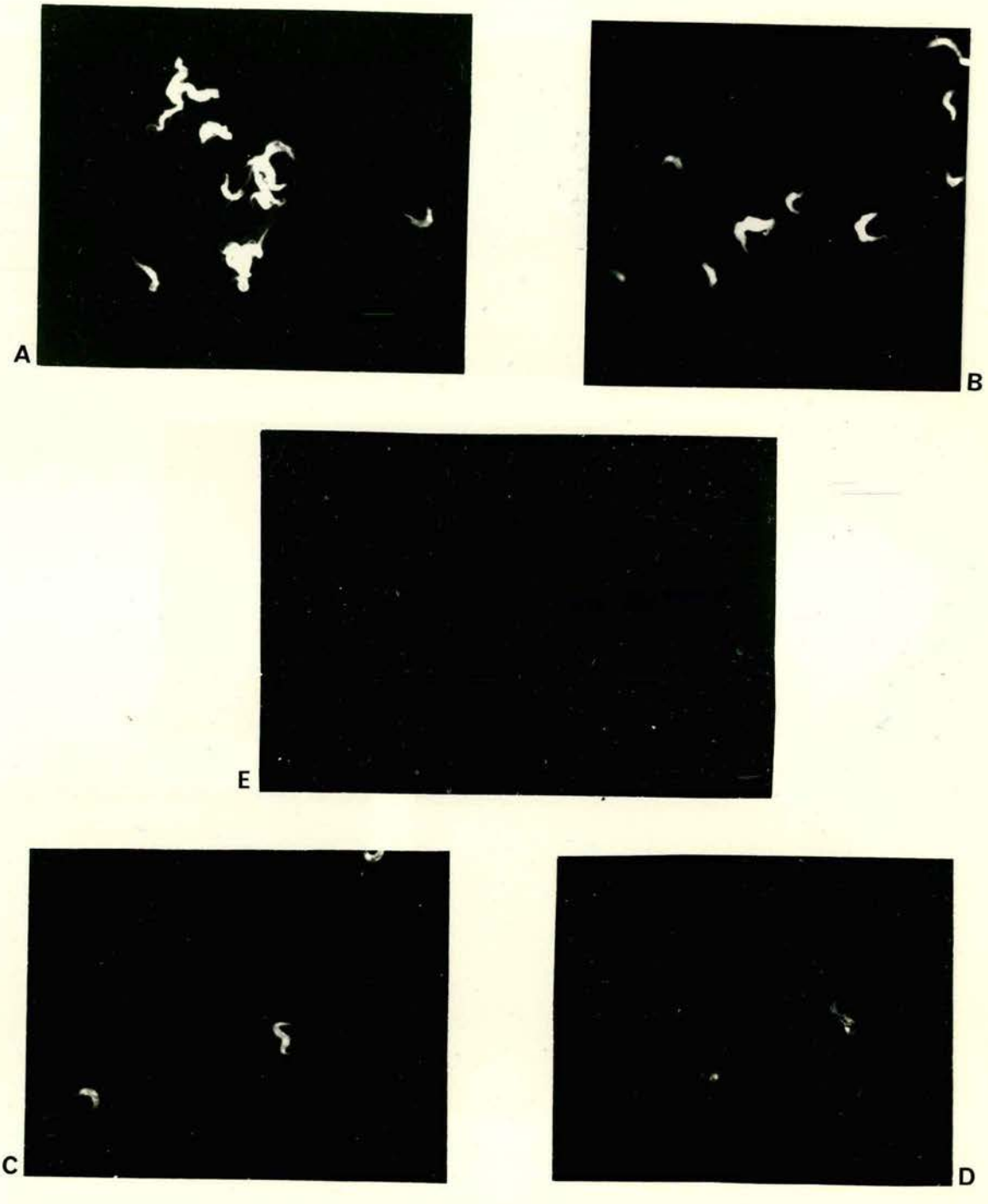


Fig 4

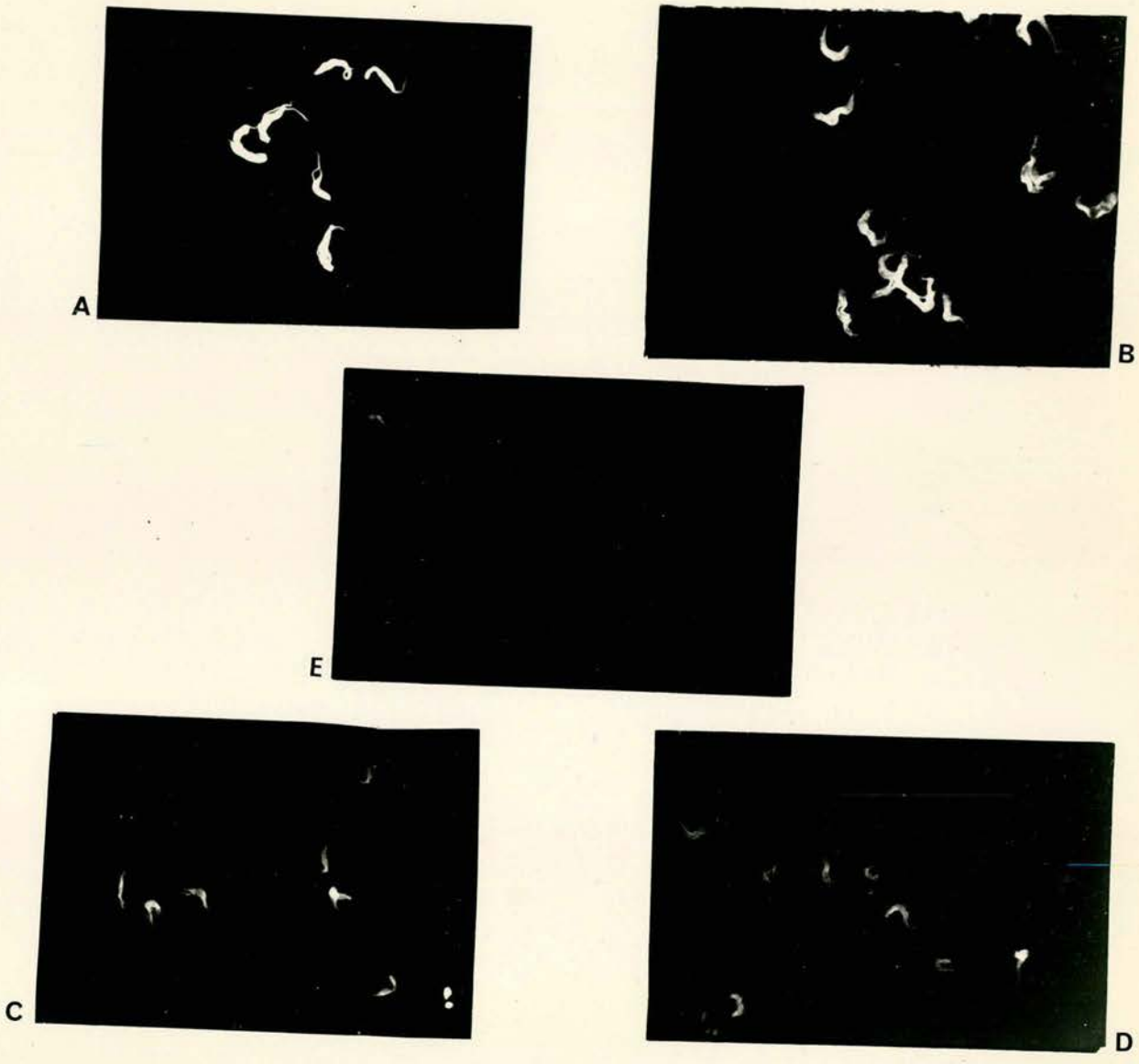


Fig 7

T . brucei . Degree of fluorescence

A . 4

B . 3

C . 2

D . 1

E . o

11. Fluorescent microphotography

The Nikon 35 mm. camera was used throughout the work. Because of the fading image it was necessary to use a high speed black and white film with exposure time one minute (Tri-XPan, Fast Black and White Film - Kodak). The films were developed in Developer (Kodak DPC Developer) diluted with tap water 1/20 for 6 minutes at 20°C, then washed once with running tap water, fixed for 8 minutes in Hypo, followed by washing in running water for 30 minutes.

All negatives were exposed at constant illumination for 5 seconds and developed in 1/10 Developer, for 1½ minutes, washed quickly in water containing few drops of acetic acid, then in Hypo for 10 minutes, followed by washing in running water for 30 minutes. Paper for printing is Bromide paper WSG.3S (6½ x 8½ in. Kodak) for trypanosomes and WSG.4S for Babesia.

12. Chemicals (see appendix)

Chemicals were obtained from the British Drug Houses Ltd. Analar R quality or from Sigma.

13. Reagents

a) Conjugated sera were used and were obtained from Nordic Pharmaceuticals and Diagnostics, Tilburg, Holland: Swine anti-mouse (SwAM/FITC); Swine anti-rabbit (SwAR/FITC); Swine anti-rabbit (SwAR/ielf0); Swine anti-human (SwAHu/FITC); Rabbit

anti-rat (RARa/FITC) and Rabbit anti-bovine (RAB/FITC). Stored at 4°C until use.

To use, 2 ml of sterilized distilled water was added to each ampoule. After 15 minutes, 0.1 ml aliquots were distributed into X-Lon plastic tubes; these were stored at -20°C.

b) Normal bovine and normal horse serum were obtained from Bio-Cult Laboratories Ltd., Glasgow.

c) Berenil: 4,4-Diamidinodiazoaminobenzene diaceturate. Supplied by Forbwerke Hoechst AG, Frankfurt, W. Germany.

d) DE52: "Whatman" Diethylaminoethyl Cellulose, microgranular, grade DE52. Supplied by W. & R. Balston, Ltd., England.

e) G25 - Sephadex, Pharmacia Uppsala, Sweden.

f) Liver acetone powder, rabbit. Sigma.

g) Fluorescein isothiocyanate, Isomer 1. Sigma.

h) Special Agar Noble (Difco).

i) Non-drying immersion oil Type A54 (Vickers Ltd., England).

j) Ponceau S. Michrome No. 725. Edward Vurr Ltd., Michrome Labs. (Berkshire, England.)

14. Glassware

Clean grease free glass microscope slides were used throughout. Slides were cleaned by immersing for 24 hours in Potassium dichromate - sulfuric acid solution, subsequently they were washed for 3 hours in running tap water, and finally twice in distilled water.

Glassware was washed in a solution of Pyroneg detergent followed by at least twelve rinses in tap water, then two in distilled water.

CHAPTER III
TRYPANOSOMES

A. Experimental

Experiment No: 1
Standardization of the IFA test applied to trypanosomes

The methods described previously for applying the IFA test to trypanosomes have varied. The object of the first series of experiments was to develop a technique which was sensitive and reproducible. For these tests T. brucei TREU 667 was used. The antigen was obtained from mice, the antisera from infected rabbits.

I. Preparation of the antigen (slide antigen)

Mice weighing approximately 20 g were infected with T. brucei and bled on the third or fourth day after infection when the parasites had reached their peak and were between 10^7 and 10^8/ml blood. The drawn blood was rapidly transferred to a tube and centrifuged for 3 min at 1500 g when most of the trypanosomes were concentrated in a layer on top of the red cells. The supernatant was removed and the trypanosomes were transferred to another tube and suspended in 5 volumes of:

a) normal bovine serum
b) normal horse serum
c) PBS
d) PBS + bovine serum, 1:1
e) normal mouse serum

The trypanosomes were again sedimented by centrifugation at 12,000 g for 5 min. Most of the supernatant was removed to leave a concentrated suspension of trypanosomes at the base of the tube. Small drops of this suspension were pipetted onto clean microscope slides which were left to dry in air. Antigen was

also prepared by spreading thin films of infected blood directly on to slides.

In case any of the washing procedures had injured the trypanosomes, an aliquot of each suspension was injected into mice. All the mice became infected with similar incubation periods.

The effects of the different washing solutions are shown in Table 1 . The best results were obtained with normal bovine or normal horse serum that is, serum from a different species to that from which the antigen was obtained.

In all subsequent experiments the trypanosomes, after separation from the infected blood, were washed in normal bovine serum unless otherwise stated.

II. Separation of T. congolense from infected mouse blood

It has been shown that only a small proportion of the trypanosomes separate in the buffy layer when blood heavily infected with T. congolense is centrifuged gently (Wilson, 1969). Soltys (1957) used Alsever's solution containing 10% horse serum to separate these trypanosomes from blood. His method was tried, but it was found that a considerable proportion of the trypanosomes was lost and the separation procedure required 1 hr.

Blood was withdrawn from the heart of a heavily infected mouse into a syringe which had been previously rinsed with heparin (1,000 units per ml.). The blood was ejected into a centrifuge tube, mixed with 5 volumes of normal bovine serum

and left for 15 min. The diluted blood was then centrifuged at 500 g for 1 min to sediment the red cells. The supernatant, which was found to contain about 90% of the trypanosomes, was removed and spun for 5 min at 12,000 g. After sedimentation, the trypanosomes were resuspended in a few drops of bovine serum and treated subsequently as described for T. brucei.

III. To test the effect of different fixatives on the quality of the antigen

T. brucei was separated from mouse blood, washed in normal bovine serum and drops of a concentrated suspension of the organisms in bovine serum were placed on slides. When dry, the antigen coated slides were treated with different fixatives as shown below. After fixation, the slides were always rinsed in PBS. The effects of the different fixatives were assessed by titrating with sera taken from a rabbit at 12 and 24 days after infection with T. brucei. An estimate was also made of the proportion of trypanosomes which adhered to the slides and of the degree of non-specific, background fluorescence.

Fixatives

1. Heat

(a) slides were passed through a low bunsen flame

(b) slides were placed on a warm plate at 50°C for 5, 10 & 15 min.

(c) slides were placed on a warm plate at 60°C for 5, 10 & 15 min.

(d) slides were placed on a warm plate at 70°C for 5, 10 & 15 min.

2. Formalin

(a) slides were immersed in 1% formalin for 5, 10 & 15 min

(b) slides were immersed in 5% formalin for 5, 10 & 15 min

(c) slides were immersed in 10% formalin for 5, 10 & 15 min

3. HCl

(a) slides were immersed in 0.1 N HCl for 1, 3, 5 & 10 min

(b) slides were immersed in 0.3 N HCl for 1, 3, 5 & 10 min

4. Methanol

(a) slides were immersed in pure methanol for 3, 5 & 10 min

5. Acetone

(a) slides were immersed in pure acetone for 3, 5, 10 & 15 min

From Table 2 it is evident that fixation with 5% formalin for 10 min. gave the best results. At low dilutions of the antiserum, the fluorescence was very bright and the highest titre was obtained. The trypanosomes adhered well to the slides and non-specific fluorescence was very slight. After fixation in 1% formalin, most of the trypanosomes were lost during washing. After fixation in acetone, methanol or HCl, the adherence of the trypanosomes to the slides was poor, fluorescence was weak and the titre of the serum was low.

The antigenicity of the trypanosomes was retained well after heat fixation, but too many of the organisms came off the slides during washing. Of the different temperatures tested, heating at 60^{o}C for 10 min was the most satisfactory.

IV. Dilution of the conjugated antiserum

To find which concentration of the conjugated antiserum would give the best results, 1/10, 1/20, 1/40, 1/80 and 1/160 dilutions in PBS were prepared. The antigen was incubated for 40 min with:

(a) all the prepared dilutions of conjugated antiserum

(b) PBS followed by 40 min incubation in the different dilutions on conjugated antisera.

(c) test serum followed by washing and then 40 min incubation in the different dilutions of conjugated antisera.

(d) normal serum followed by washing and then 40 min incubation in the different dilutions of conjugated antisera.

Results

No fluorescence was observed when the antigen was incubated with conjugated antisera alone (a) at any of the prepared dilutions. Neither was any fluorescence observed when the antigen was incubated in PBS followed by conjugated antisera (b). At dilutions of 1/20 or higher of the conjugated antisera no fluorescence was seen when the antigen was incubated in normal serum followed by conjugated antiserum (c). However, at dilutions greater than 1/20 the titre of the test serum was reduced. In all subsequent tests the conjugated antisera were used at a dilution of 1/20.

V. Base line for the titrations

In these tests, the conjugated antiserum was applied at a concentration of 1/20. Starting at 1 in 10, serial 2-fold dilutions of sera from mouse, rat, rabbit, ox and man were prepared in PBS and tested against different antigens. In some of the tests, slight fluorescence (±) was observed at a 1 in 10 dilution of normal serum. In none of the tests was any fluorescence seen at a 1 in 20 dilution of the normal serum.

VI. The effect of reaction time

The antigen coated slides were placed on glass rods in a plastic sandwich box lined with damp filter paper. The test and the conjugated antisera were allowed to react for 30, 40 and 60 min. After each serum treatment, the slides were washed in two changes of PBS, 5 min each. The incubation was carried out at room temperature.

After incubation time of 30 min the titre of the serum was less than at incubation times of 40 and 60 min. Since there was no difference between 40 and 60 min incubation times, 40 min was selected as a suitable reaction time for all subsequent experiments.

VII. Removal of sera by washing

Unbound antibody must be removed from the antigen coated slides by washing. After incubation with the sera the slides

were immersed for 5, 10, 15, 30, 60 & 120 min in PBS.

A single wash of 5 min duration was not sufficient to remove unbound serum since slight fluorescence was observed on slides which had been treated with normal serum. Unbound serum was removed effectively when the slides were immersed for 5 min in two changes of PBS; this procedure was adopted.

VIII. The effect of temperature on the properties of the antigen during storage

The antigen was prepared as described and stored in self-seal polythene bags containing a few crystals of silica gel at:

(a) room temperature (18-20°C)

(b) in the refrigerator (4°C)

(c) in the deep freeze (-20°C)

After different periods of storage, the antigen was titrated against the same antiserum obtained from rabbit 1675, 21 days after it had been infected with T. brucei.

As shown in Table 3 , antigen stored for up to 3 weeks at all of the temperatures tested retained its properties since the end point of the titration was the same as when the antigen was freshly prepared. However, after an interval of 3 weeks or more, antigen stored at room temperature or at 4°C had deteriorated. Antigen stored at -20°C has retained its antigenicity for at least 18 months.

Table 1

The effect of washing the trypanosomes in different solutions on the quality of the antigen

In all these tests, the antigen was fixed with 5% formalin for 15 minutes.

Washing solution	Proportion of trypanosomes adhering to slide	Intensity of fluorescent (brightness)	Background	Highest dilution of normal serum giving fluorescence	Shape of cell
Bovine serum	90%	very good	very good	1/10	normal
Horse serum	90%	very good	very good	1/10	normal
PBS	50%	very good	good	1/10	normal
PBS + bovine serum	50%	very good	good	1/10	normal
Normal mouse serum	90%	very good	very good	1/40	normal
Direct serum	90%	good	poor	1/80	normal

Table 2

The effect of different fixatives on the quality of the antigen

Fixative (for 10 minutes)	Reciprocals of antiserum dilution x 10^3												
	.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48	Background
60°C	4	4	3	3	2	1	?	0	0	0	0	0	good
	4	4	4	4	3	3	?	?	1	?	0	0	
0.3N HCl	3	1	1	1	?	?	0	0	0	0	0	0	good
	3	3	2	2	1	1	1	0	0	0	0	0	
Methanol	2	2	1	1	1	0	0	0	0	0	0	0	poor
	3	2	2	1	1	1	0	0	0	0	0	0	
Acetone	?	?	?	?	?	?	1	0	0	0	0	0	poor
	3	3	?	2	2	?	1	1	0	0	0	0	
Formalin 5%	4	4	4	3	3	2	2	1	1	0	0	0	good
	4	4	4	4	3	3	3	2	2	1	1	1	

The upper line of figures are results of titrations with 12 day serum and the lower line, a 24 day serum from a rabbit infected with T. brucei.

The figures indicate the intensity of fluorescence observed.

? = trypanosomes absent, i.e. poor adherence

Table 3

The effect of temperature on the properties of the antigen during storage

Time (weeks)	Reciprocals of serum dilution x 10^3											
	.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
0	4	4	4	3	3	2	2	2	1	1	1	0
1	4	4	4	3	3	3	2	2	1	1	1	0
	4	4	4	3	3	2	2	2	1	1	1	0
	4	4	4	3	3	2	2	2	1	1	1	0
3	4	4	4	4	3	3	2	2	1	1	1	0
	4	4	4	4	3	2	2	2	1	1	1	0
	4	4	4	4	3	3	2	2	1	1	1	0
8	3	3	2	2	1	1	1	0	0	0	0	0
	4	4	3	3	3	2	1	1	0	0	0	0
	4	4	4	4	3	3	2	2	1	1	1	0
14	3	2	2	2	1	1	0	0	0	0	0	0
	4	3	3	2	2	1	0	0	0	0	0	0
	4	4	4	3	3	3	2	2	2	1	1	0

The upper line of figures refer to antigen stored at room temperature, the middle, at 4°C and the lower, at -20°C.

Experiment No: 2

The retention of antibody activity of blood collected on filter paper and stored at different temperatures

It has been shown that blood collected on filter paper can be used in serodiagnosis of parasitic infections.

Method

(1) Collection and storage of blood on filter papers:-

A rabbit was bled at 6, 10, 21 and 24 days after it had been infected with T. brucei. Part of the blood was allowed to clot to obtain serum; the remainder was dropped on Whatman No. 4 filter paper, then treated as described in Materials and Methods, p. 35. Dried blood samples were stored at different temperatures.

(2) The test:-

Discs of 5 mm in diameter were punched from the dried blood samples, with a leather punch. A micropipette was used to place 0.05 ml drops of PBS within marked circles on antigen coated slides. The discs of dried blood were placed in the drops of PBS with the aid of a sharp needle. The slides were left for one hour in a moist chamber. The filter paper discs were removed from the slides with a jet of PBS. The slides were washed by placing them in two changes of 5 minutes duration in a coplin jar containing PBS. The slides were blotted dry and the conjugated serum applied as described (p. 36).

(3) Size of the blood discs:-

The effect of varying size of the dried blood discs from 5 to 10 mm in diameter was studied. No difference in the degree of fluorescence could be detected, hence discs of 5 mm diameter were used. By weighing the filter paper with and without blood, it was calculated that a 5 mm disc in a 0.05 ml PBS was equivalent to approximately 1/50 dilution of serum.

Results

The results of this experiment are shown in Tables 4 & 5. Table 4 shows the IFA titres of sera collected at the same time as the filter paper samples. Table 5 shows that there was no loss of activity in dried blood samples stored at 36°C or room temperature (18-20°C) after 2-3 weeks, at 4°C, after 3 weeks, at -20°C, after 30 days. The duration of the activity in the dried blood depends on the potency of the serum; for example at room temperature, the activity was completely lost in the 6 day sample after 30 days, but some activity remained in the 21 day sample up to 90 days.

Table 4

Titration of sera by IFA test

Serum	Reciprocals of serum dilution x 10^3											
	0.01	0.02	0.04	0.08	0.16	0.32	0.64	1.28	2.56	5.12	10.24	20.48
6 days	3	3	2	2	1	1	0	0	0	0	0	0
10 days	4	4	3	2	2	2	1	1	1	0	0	0
21 days	4	4	4	4	3	3	2	2	1	1	1	0
24 days	4	4	4	4	3	3	3	2	2	2	1	1

Table 5

Measurement of fluorescence in dried blood on filter paper after different periods of storage at different temperatures

Sample	Degree of fluorescence				Storage time (days)
	Room temp.	4°C	-20°C	36°C	
6 days	2	2	2	-	0
	2	2	2	-	15
	1	2	2	-	20
	0	1	2	-	30
	0	0	1	-	40
	0	0	1	-	60
	0	0	0	-	70
10 days	4	4	4	-	0
	4	4	4	-	10
	3	4	4	-	15
	2	3	4	-	25
	1	2	3	-	45
	0	2	2	-	55
	0	0	1	-	90
	0	0	0	-	120
21 days	4	4	4	-	0
	4	4	4	-	21
	2	3	4	-	30
	2	3	3	-	60
	1	1	2	-	90
	0	0	1	-	120
	0	0	0	-	150
24 days	-	-	-	4	0
	-	-	-	4	2
	-	-	-	4	4
	-	-	-	4	6
	-	-	-	4	8
	-	-	-	4	10
	-	-	-	4	14
	-	-	-	3	24
	-	-	-	3	30
	-	-	-	2	38
	-	-	-	2	52
	-	-	-	1	80
	-	-	-	0	90

- = not tested

Experiment No: 3

Differentiation of brucei-group by the indirect fluorescent antibody test

It had been shown that trypanosomes belonging to Hoare's subgenera could be distinguished by the IFA test. But it was not clear whether members of the subgenus Trypanozoon could be separated by this test. In the following experiments the IFA test was applied to differentiate between brucei-group trypanosomes.

Method

(i) Antigen: the various antigens used are described in Materials and Methods (page 27).

(ii) Antisera: rabbit R11 was infected with T. brucei, TREU 667 and the sera prepared from blood obtained at 12 and 24 days after infection, were used. Three rats were infected I/P with 8×10^6 of T. brucei, TREU 667, T. rhodesiense, TREU 788 and 789. They were bled at 6 or 7 day intervals and the infections were cured by treatment with Berenil, 150 mg/Kg, on two consecutive days starting on the fourteenth day after infection. Sera from six patients with Rhodesian sleeping sickness were obtained from East African Trypanosomiasis Research Organization (E.A.T.R.O.). The sera had been preserved with sodium azide. The E.A.T.R.O. code numbers of the sera are: (a) 1438 1474(7); (b) 1716 1740(12); (c) 2150 2201(20); (d) 1880 1933(12); (e) 1870 1872(5); (f) 1881 2209(60), the figure in brackets refers to the time after drug treatment when the second sample of serum was taken from the patient.

Sera of three patients from Zaire with Gambian sleeping sickness were supplied by Institut de Medicine Tropicale Prince Leopold, Antwerp. The sera code numbers M221, M256 and M262, had been preserved by lyophilization. Sera from two rabbits, RH/RRP and BH, infected by fly transmission with two isolates of T. gambiense, from Mayir, Nigeria, were donated by C.T.V.M. Edinburgh. Test procedure was carried out as described before.

Results

The results of the titrations of sera from rabbits with various antigens are shown in Table 6 . Sera from rabbit R11 (Ra.1) were titrated against 14 T. brucei, 4 T. rhodesiense, 3 T. gambiense, 1 T. evansi, 4 T. congolense and 1 T. lewisi antigens. The highest dilution of the 12-day serum to give fluorescence with any of the heterologous antigens was 1/160, whereas the lowest dilution to give fluorescence with any of the homologous antigens was 1/1280.

Similarly with the 24-day serum, the lowest homologous titre was 1/5120 and the highest heterologous titre, 1/640. When the sera from rabbits RH/RRP and BH, which were infected with T. gambiense, were titrated against brucei-group antigens, the heterologous and homologous reactions were distinguished by the titres of the sera. Table 7 gives the results of the titrations of the sera from rats. Although the antibody response of the rat was not as strong as that of the rabbit, it was possible to

to distinguish homologous from heterologous antigens by the titres of the sera. Drug treatment did not destroy the specificity of the sera. The results of the titrations carried out with human sera are shown in Table 8 .

The results show that in a total of 368 cross titrations of antisera to brucei-group trypanosomes, the difference in the titre of a serum when it was reacted with the homologous as compared with the heterologous antigen, was at least 4-fold.

Titres of the sera to the different antigens are given as the geometric mean with range Figs. 10, 11, and 12; the number of titrations is shown in brackets. The end point of the titrations was always three or more dilutions higher in the homologous than in the heterologous reaction.

Table 6

Titrations of sera from rabbits.

Antisera	Reciprocals of serum dilutions							
	Ra.1 12	Ra.1 24	RH/RRP 15	RH/RRP 29	RH/RRP 46	BH 14	BH 30	BH 49
Antigen								
T. rhodesiense								
333	40	160	80	80	40	40	160	80
35	40	320	20	40	160	40	80	80
788	160	640	20	80	160	20	80	80
789	80	640	40	160	160	40	160	160
T. brucei								
667	5120	20840	20	20	40	20	40	80
927	2560	10240	80	80	80	20	20	80
1096	2560	10240	40	80	80	20	40	160
933	1280	5120	20	40	20	20	40	80
991	5120	10240	40	160	160	40	80	160
852	2560	10240						
851	2560	10240						
853	2560	10240						
958	1280	5120						
833	1280	5120						
900	1280	5120						
1097	2560	10240						
741	2560	10240						
1027	2560	10240						
T. gambiense								
390	80	320	320	640	640	160	1280	1280
R 3	80	80	640	1280	2560	320	640	1280
E 1	40	160	320	1280	2560	160	1280	1280
T. evansi								
379	40	80						
T. congolense								
732	40	160						
692	20	160						
1095	40	80						
937	20	160						
T. lewisi	20	80						

Rabbit Ra.1 was infected with *T. brucei*, TREU 667

Rabbits RH/RRP and BH were infected with *T. gambiense*

The figure below the code no. for each serum denotes the day post-infection when the serum was obtained.

The antigens are denoted by their TREU numbers.

Table 7

Titrations of sera from rats.

Antisera	Reciprocals of serum dilutions										
	Rt.1 6	Rt.1 12	Rt.1 18	Rt.2 7	Rt.2 14	Rt.2 21	Rt.2 28	Rt.3 7	Rt.3 14	Rt.3 21	Rt.3 28
Antigen											
T. rhodesiense											
333	20	20	20								
35	20	40	20								
788	10	40	20	80	640	160	160	80	160	320	160
789	10	40	40	80	640	160	160	160	320	320	320
T. brucei											
667	160	320	320	20	20	10	20	10	20	20	20
1096	80	320	320	10	20	40	20	10	40	40	40
958	160	320	320	10	10	20	10	10	10	10	10
T. gambiense											
390	20	20	20	10	40	20	20	10	20	40	20
R 3	20	20	20	10	20	20	20	10	20	20	20
E 1	20	20	20	10	20	20	20	10	20	20	20

Rt.1 was infected with T. brucei TREU 667, Rt.2 with T. rhodesiense TREU 788 and Rt.3 with T. rhodesiense TREU 789

The figure below the code for each serum denotes the day post-infection when the serum was obtained.

The antigens are denoted by their TREU numbers.

Table 8

Titrations of human sera

Antisera	Reciprocals of serum dilutions							
Antigen	1870	1872	1880	1933	1716	1740	2150	2201
T. rhodesiense								
333	640	1280	1280	1280	320	640	2560	640
35	320	1280	2560	1280	320	640	2560	320
788	640	1280	1280	1280	640	640	2560	1280
789	640	1280	2560	640	640	1280	1280	640
T. brucei								
667	40	40	80	40	40	40	20	20
927	40	40	40	20	10	20	10	10
1096	80	160	160	80	40	40	80	40
933	20	40	40	20	20	20	40	20
991	80	160	80	80	40	20	20	20
T. gambiense								
390	10	20	20	20	20	20	40	20
R 3	20	20	20	20	40	40	40	40
E 1	20	20	20	20	20	20	20	20

Table 8 (continued)

Titrations of human sera

Antisera	Reciprocals of serum dilutions						
	1438	1474	1881	2209	M.221	M.256	M.262
Antigen							
T. rhodesiense							
333	640	640	640	640	20	10	10
35	320	320	320	320	40	20	20
788	320	1280	640	640	20	20	20
789	640	640	640	320	80	40	40
T. brucei							
667	20	20	20	20	40	20	20
927	10	10	40	20	20	10	40
1096	40	40	40	40	40	40	40
933	10	20	20	20	20	20	20
991	10	20	80	40	80	40	40
T. gambiense							
390	10	20	10	10	640	160	320
R 3	40	40	20	20	640	320	160
E 1	20	40	20	20	320	320	320

M.221, M.256 and M.262 were from patients with Gambian sleeping sickness, the remaining sera were from patients with Rhodesian sleeping sickness.

The antigens are denoted by their TREU numbers

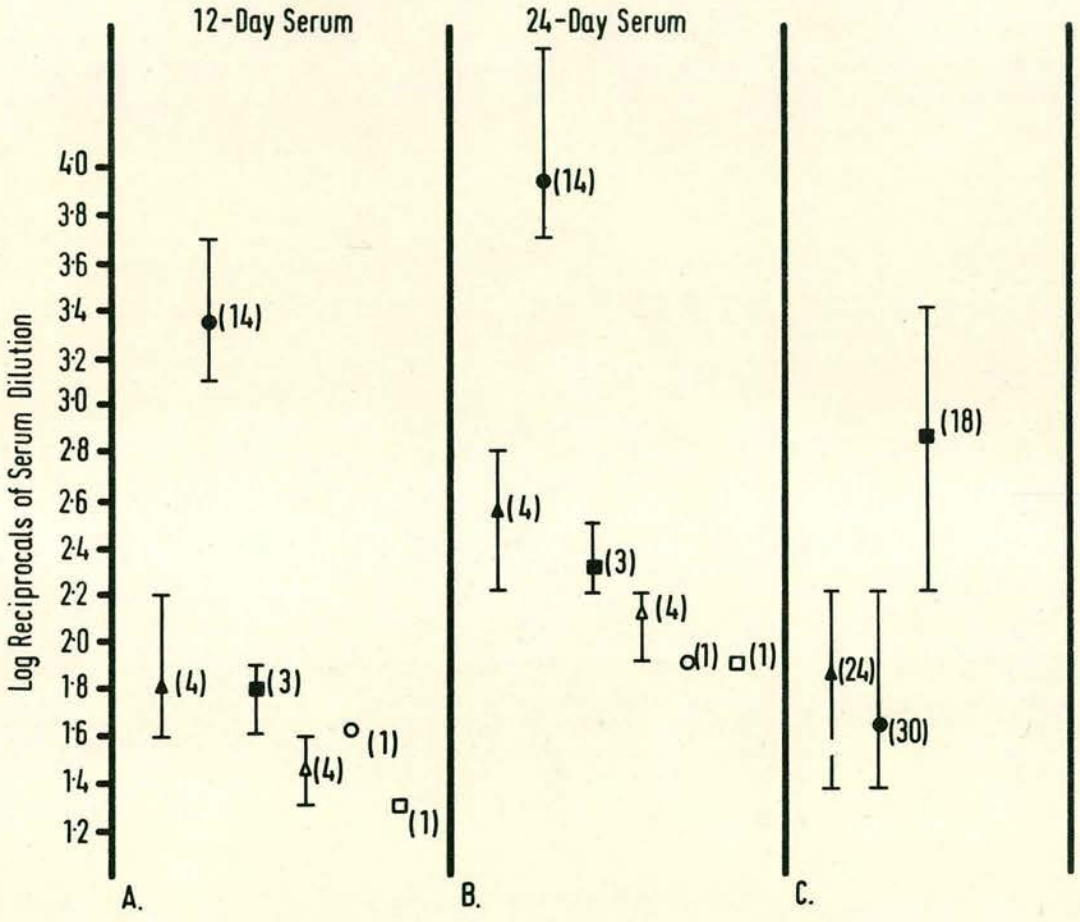

12-Day Serum
24-Day Serum
Log Reciprocals of Serum Dilution
4·0
3·8
3·6
3·4
3·2
3·0
2·8
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
(14)
(4)
(3)
(4)
(1)
(1)
(14)
(4)
(3)
(4)
(1)
(1)
(18)
(24)
(30)
A.
B.
C.

Fig 10

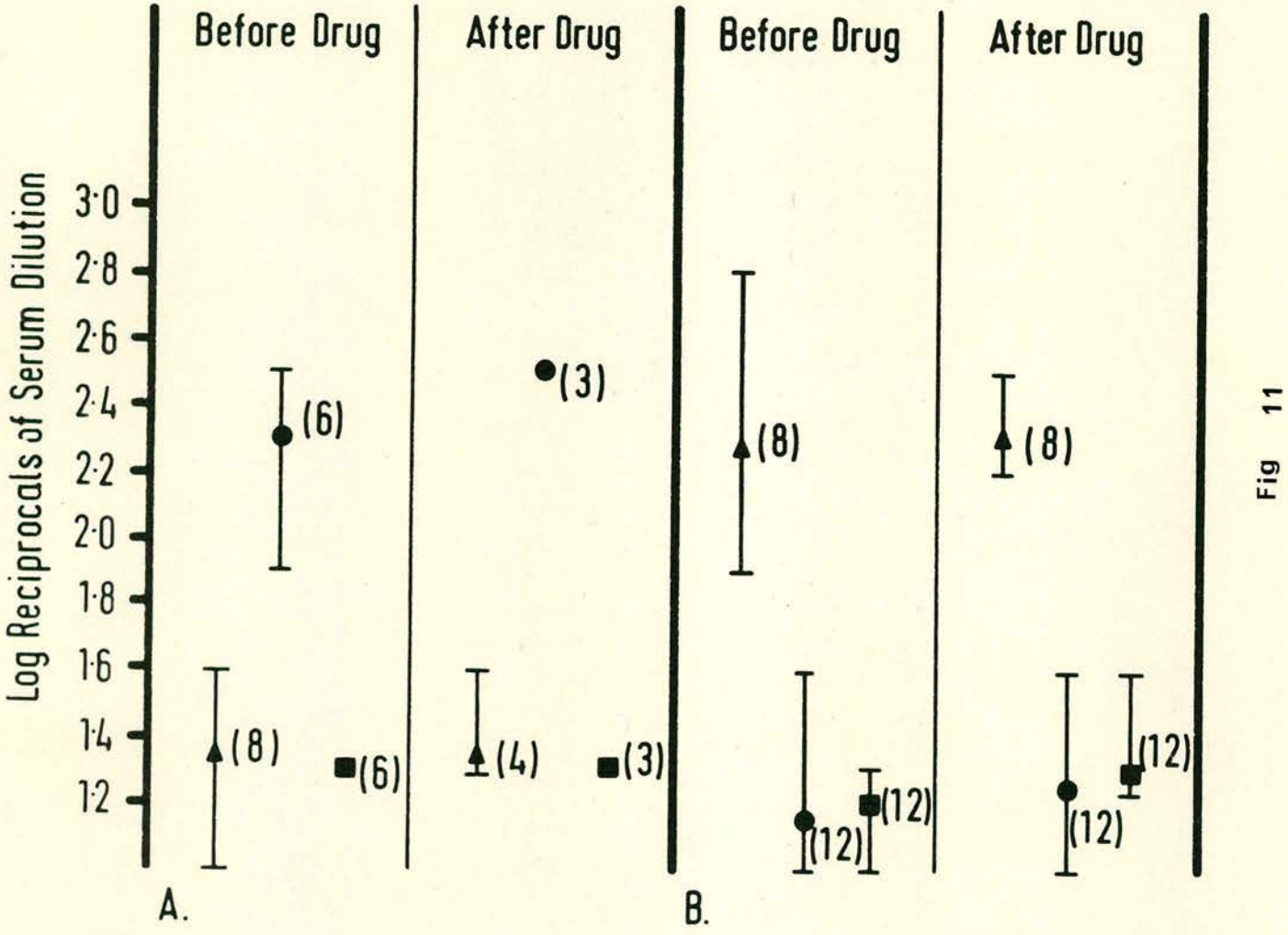

Before Drug
After Drug
Before Drug
After Drug
Log Reciprocals of Serum Dilution
3·0
2·8
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
(6)
(8)
(6)
(3)
(4)
(3)
(8)
(12)
(12)
(8)
(12)
(12)
A.
B.

Fig 11

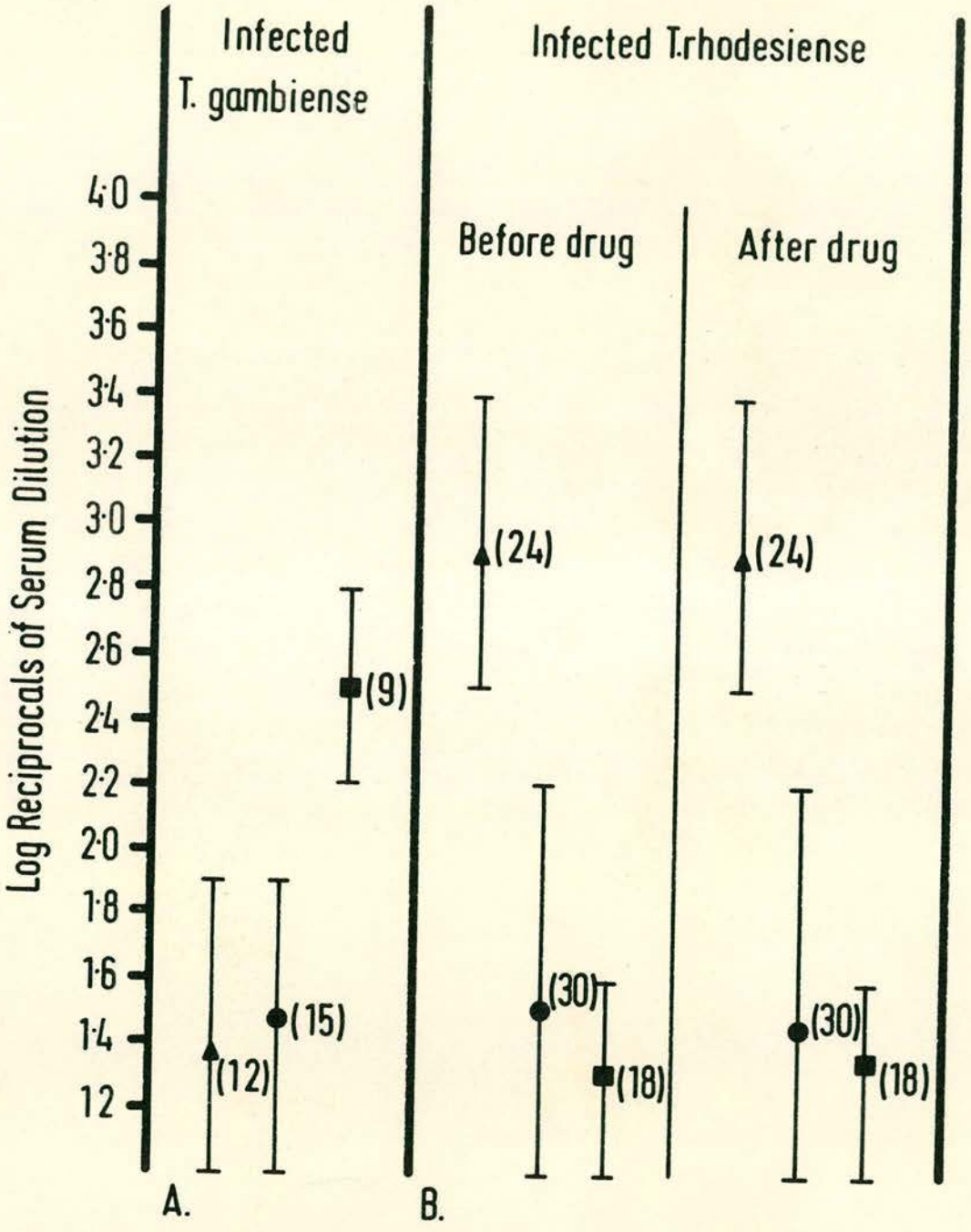

Infected
T. gambiense
Infected T.rhodesiense
Before drug
After drug
Log Reciprocals of Serum Dilution
4·0
3·8
3·6
3·4
3·2
3·0
2·8
2·6
2·4
2·2
2·0
1·8
1·6
1·4
1·2
(9)
(12)
(15)
(24)
(30)
(18)
(24)
(30)
(18)
A.
B.

Fig 12

Titres of the sera to the different antigens are given as the geometric mean with range; the number of titrations is shown in brackets. In all the figures symbols for the antigens are:

▲ T. rhodesiense
● T. brucei
■ T. gambiense
△ T. congolense
O T. evansi
□ T. lewisi

Figure 10. Titre of sera from rabbits infected with T. brucei, TREU 667 (A & B) and T. gambiense (C).

Figure 11. A, titrations of sera from rat infected with T. brucei, TREU 667; B, titration of sera from 2 rats infected with T. rhodesiense, TREU 788 and 784.

Figure 12. Titrations of sera from humans infected with T. gambiense (A) and T. rhodesiense (B).

Experiment No: 4

Differential diagnosis of T. theileri by IFA test.

Trypanosoma theileri Laveran, 1902 has been described as a truly cosmopolitan parasite of cattle and has been reported in many countries (Herbert, 1964). Serological methods have been used to detect T. theileri infection in cattle. In this experiment the IFA test was used to see if it was possible to differentiate T. theileri from other trypanosomes which may infect cattle.

Method

Antigens: T. theileri, TREU 124 was obtained from the low temperature bank of the C.T.V.M. IFA test was carried out on T. theileri, that had been cultured at 28°C in a blood agar medium, and at 37°C in a leucocyte medium as described by Wells (1971). Antigen from the leucocyte cultures was prepared on the 3rd or 4th day after the cultures were set up, by transfer of the medium to a centrifuge tube taking care not to disturb the leucocyte layer. The medium which contained most of the trypanosomes was centrifuged at 12,000 g for 5 minutes, the supernatant was discarded, and antigen was prepared from the sedimented organisms. To obtain antigen from blood agar cultures, the liquid phase of the medium was removed, it was mixed with a few drops of bovine serum, and then the suspension of trypanosomes was pipetted on to slides in the usual way.

In case washing the trypanosomes in bovine serum interfered with the IFA test when the host animal to be examined was the ox, duplicate slide antigens were prepared from trypanosomes which had

been washed and suspended in normal horse serum.

Antisera: Antisera were obtained from adult cattle infected with T. theileri, from which the organism had been isolated by culture. Sera were obtained from cattle from different localities, sera numbers, 159, 162, 167, 190, 191 and 197 were obtained from Scotland, 857 from East Africa, 1634, 1635, 1675, 1677, 1678 and 1745 from Ontario-Canada. Control serum was obtained from a cow at C.T.V.M. which was known to be free from T. theileri.

IFA test was carried out as described for other species of Trypanosomes.

Result

From the results in Table 9, it shows that washing the trypanosomes in bovine serum does not interfere with the fluorescent titre of the serum.

Antisera to T. theileri (Table10) react in a low titre with both theileri antigens, but in most cases the titre was one dilution higher with antigen prepared from leucocyte cultures than with that from blood agar cultures. T. theileri antisera also reacted with antigen prepared from other species of trypanosomes, the highest titre in any heterologous reaction was 1/40. Antisera to these species of trypanosomes also reacted with T. theileri, antigen but the titre in heterologous titrations was low, (Table 11).

Table 9

The effect of washing the antigen in bovine and horse serum on the I.F.A. titre of T. theileri antisera.

Antigen	Reciprocals of serum dilutions x 10^3																	
	Serum No. 190						Serum No. 191						Serum No. 197					
	.01	.02	.04	.08	.16	.32	.01	.02	.04	.08	.16	.32	.01	.02	.04	.08	.16	.32
T. theileri (Leucocyte layer)	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0
	1	1	1	1	0	0	1	1	1	1	0	0	1	1	1	1	0	0
T. theileri (Blood agar)	1	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0
	1	1	1	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0
T. brucei TREU 667	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
	1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0

The upper line of figures gives results of trypanosomes washed with bovine serum, the lower with horse serum.

Table 10

I.F.A. Titration of antisera to T. theileri

Anti theileri serum No	Antigen															
	Theileri (Leucocyte layer)	Theileri (Blood agar)	Brucei					Rhodesiense				Congolense				Evansi
	1 strain	1 strain	5 strains					4 strains				4 strains				1 strain
167	80*	40	10	20	20	10	20	10	10	10	20	10	10	10	10	10
1675	80	40	10	20	20	10	20	10	10	10	10	0	10	10	10	10
1678	80	80	10	10	20	10	20	10	20	10	10	10	20	10	10	10
1635	80	40	20	10	20	20	40	20	20	10	20	10	10	10	20	20
1745	160	80	20	40	20	10	20	10	20	10	20	0	10	0	10	20
1634	80	40	20	20	10	20	10	10	10	0	20	10	10	10	10	20
1677	80	40	10	20	10	20	40	10	10	0	10	10	10	0	0	10

* = Each figure represents the end titre of serum

Table 11

Cross titrations of antisera to species of trypanosome that may occur in cattle

Anti serum to:	Antigen				
	Theileri (Leucocyte layer) TREU 124	Theileri (blood agar) TREU 124	Brucei TREU 667	Rhodesiense TREU 788	Congolense TREU 692
T. theileri	160*	80	20	20	20
T. brucei TREU 667	80	80	20480	640	160
T. rhodesiense TREU 788	20	20	20	640	10
T. congolense TREU 692	40	40	-	-	5120

- = not tested

* = each figure represents the end titre of serum

Experiment No: 5

Measurement of antibody titre in rabbits infected with T. brucei, and T. congolense

Wilson and Cunningham (1971) suggested that when using the IFA test to diagnose trypanosomiasis in cattle, serum with a high titre was indicative of a current infection, but a low titre might indicate either a fresh or a recovered infection. In other words a positive reaction does not necessarily mean that the host carried active trypanosomes at the time of sampling.

Wery et al (1971) found that sera from sleeping sickness patients gave a positive reaction in the IFA test for up to 2 years after recovery. The object of these tests was to follow the level of antibody in rabbits infected with trypanosomes and after the infection had been cured by treatment with a drug.

Method

Six rabbits were used, 4 infected with T. brucei and 2 with T. congolense.

Rabbits were bled from the ear before infection and at various intervals after infection to obtain sera for measurement of antibody level. A drop of blood was examined microscopically for trypanosomes. In addition, 2 ml of rabbit's blood was mixed with 4 ml of citrated saline for injection into mice; each mouse received 1 ml I/P. The mice were examined for trypanosomes thrice weekly for up to 4 weeks in the case of T. brucei and up to 6 weeks in the case of T. congolense.

Details of the treatment for each rabbit are shown in tables, in which results are also summarized.

Rabbit F11 (Table12). Sera collected from this rabbit were titrated against 3 antigens. Antigen made from the same population of trypanosomes that were used to infect the rabbit (0 day antigen) and antigen made from the population of trypanosomes that developed in mice when injected with rabbit blood 6 days (6 day antigen) and 12 days (12 day antigen) after its infection. Twelve days after the infection the rabbit was sick, and was treated with Berenil (at the rate of 7 mg/Kg). Following drug treatment, the condition of the animal improved, and no trypanosomes were seen in the blood. However, the animal died 48 days after drug treatment. Scanty trypanosomes were seen in peripheral blood but there were numerous trypanosomes in the blood of the heart and coronary artery. In this rabbit antibody titre did not fall after drug treatment. The titres of the sera when titrated against the 0, 6 and 12 days antigens were similar.

Rabbit 1660 (Table13), became sick 12 days after infection, was given Berenil on 3 consecutive days. This treatment was apparently effective since the condition of the animal improved, no trypanosomes were detected and the antibody titre fell to a low level. When challenged with the same strain of T. brucei the antibody titre rose rapidly. Although trypanosomes were detected in its blood by mouse inoculation, the rabbit remained in good condition for about 50 days after challenge.

Rabbit 37 (Table 14) was infected with T. brucei, TREU 667 and treated with Berenil on day 12 post-infection. The antibody titre had fallen to a low level 210 days later, when it was challenged with a different strain T. brucei, TREU 1096. At the same time a clean rabbit 46 (Table 15), was infected with an equal number of these trypanosomes since the response of rabbits to this strain of T. brucei had not been tested. Following challenge, the antibody level rose rapidly, the animal lost weight and died within 2 weeks. Rabbit 46 survived for 17 days.

A previous infection by a different strain provided no protection to challenge with a different strain, although the antibody level rose sharply in both cases.

Rabbit F16 (Table 16) was infected with T. congolense, TREU 692. The animal remained in good condition for 90 days, and during this time the antibody titre was also high. Although no trypanosomes were seen in the blood, mice inoculated with blood became infected. After drug treatment, antibody fell sharply and mice no longer became infected. On challenge with the same strain, again the antibody titre rose, but no mice became infected. The response of rabbit 38 (Table 17) infected with T. congolense, TREU 692 was similar to that of rabbit F16.

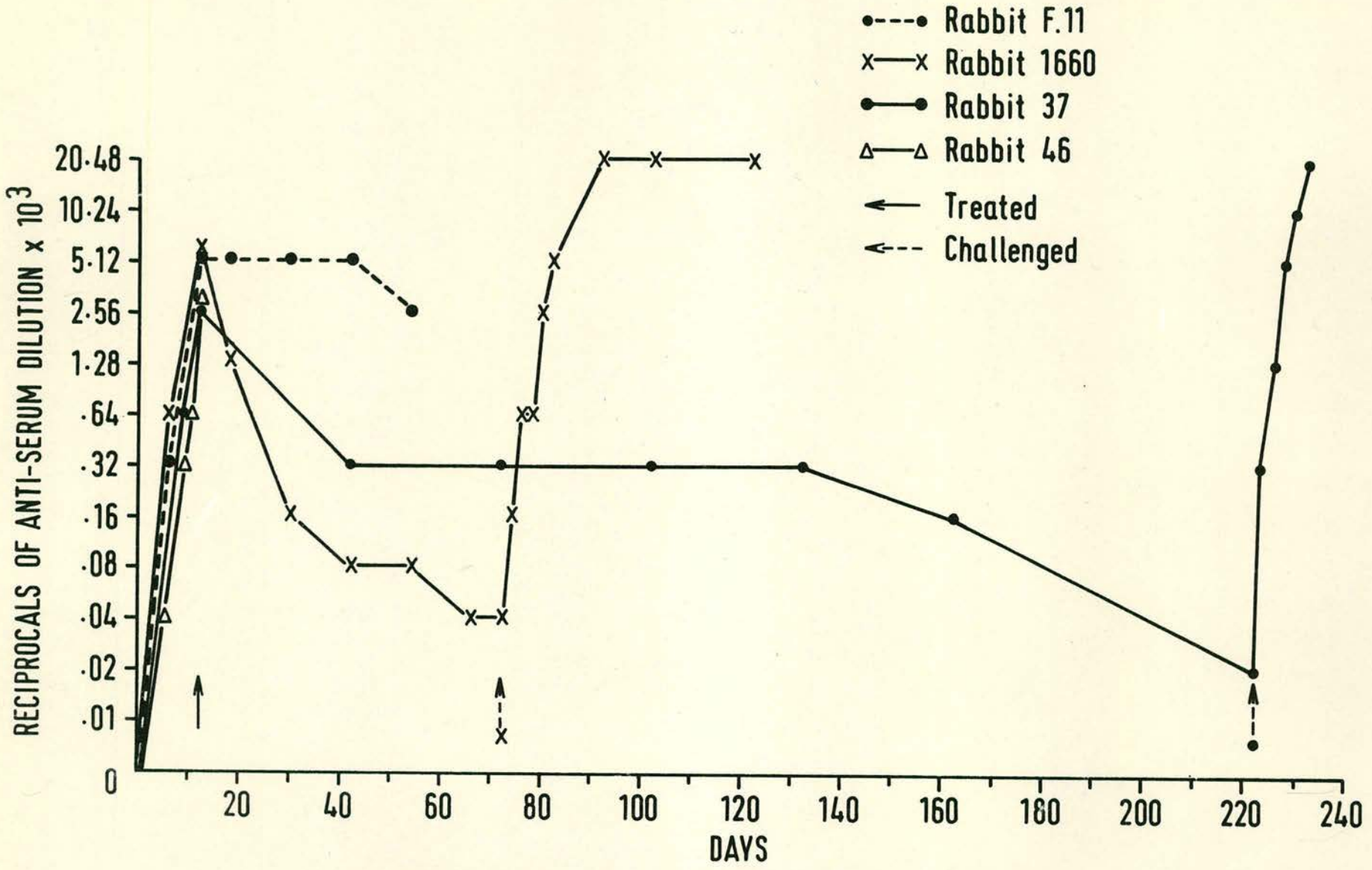


Fig. 8 IFA titre of sera from rabbits infected with T. brucei.

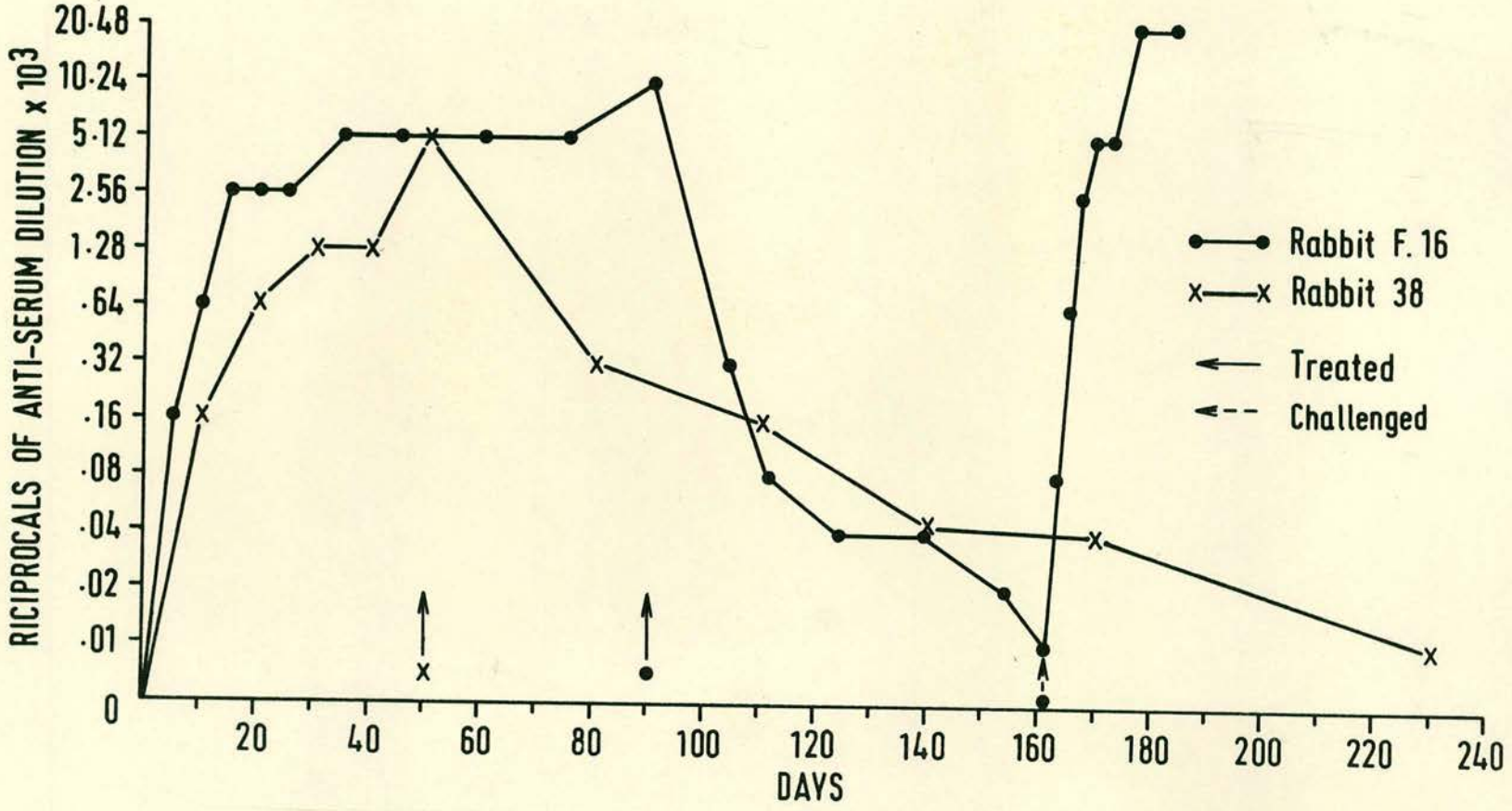


Fig. 9 IFA titre of sera from rabbits infected with T. congolense.

Table 12

The course of infection and antibody level in rabbit F11 infected with T. brucei, TREU 667. The rabbit was given Berenil (7 mg/kg), I.P. on the 12th day after infection.

DAYS		Titre of serum*	Condition of Rabbit	Wet Film	Mice
After Infection	After Drug Treatment				
0		0	good	0	0
6		320 320 320	good	0	+
12		5120 2560 2560	poor	+	+
18	6	5120 5120 5120	moderate	0	0
30	18	5120 2560 2560	good	0	-
42	30	5120 5120 2560	good	0	-
54	42	2560 2560 2560	good	0	-
60	48	-	died	+	+

* = highest dilution of serum giving fluorescence. The upper of the 3 figures gives result for titration against the 0 day antigen, the middle, the 6 day, and the lower, the 12 day antigens.

0 = no trypanosomes seen and mice not infected

- = not examined

Table 13

The course of infection and antibody level in rabbit 1660 infected with T. brucei, TREU 667, and challenged with the same strain. The rabbit was given Berenil (10 mg/Kg), I.P. for 3 days starting at 12th day after infection

DAYS			Titre of* Serum	Condition of Rabbit	Wet Film	Mice
After Infection	After Drug Treatment	After Challenge				
0			0	good	0	0
6			640	good	0	+
12			5120	poor	+	+
18	6		1280	Moderate	0	0
30	18		160	good	0	0
42	30		80	good	0	0
54	42		80	good	0	0
66	54		40	good	0	0
72	60		40	good	0	0
74		2	160	good	0	0
76		4	640	good	0	+
78		6	640	good	0	+
80		8	2560	good	0	+
82		10	5120	good	0	+
92		20	20480	good	0	+
102		30	20480	poor	+	+
122		50	20480	poor†	+	+

* = Highest dilution of the serum giving fluorescence when titrated against the 0-day antigen

0 = No trypanosomes seen and mice not infected

† = Rabbit killed

Table 14

The course of infection and antibody level in rabbit 37 infected with T. brucei, TREU 667, and challenged with the T. brucei, TREU 1096 strain. The rabbit was given Berenil (10 mg/Kg), I.P. for three days starting at 12th day after infection.

DAYS			Titre of* Serum	Condition of Rabbit	Weight of Rabbit Kg	Wet Film	Mice
After Infection	After Drug Treatment	After Challenge					
0			0	good	2.180	0	0
6			640	good	2.180	0	+
12			2560	good	2.180	+	+
42	30		320	good	2.640	0	0
72	60		320	good	3.000	0	0
102	90		320	good	3.160	0	0
132	120		320	good	3.340	0	0
162	150		160	good	3.420	0	0
222	210		20	good	3.470	0	0
224		2	320	good	3.300	0	+
226		4	1280	good	3.190	0	+
228		6	5120	Moderate	2.990	+	+
230		8	10240	poor	2.900	+	+
232		10	20480	poor†	2.690	+	+

* = Highest dilution of serum giving fluorescence. The titration against the 0 day antigen.

0 = No trypanosomes seen and mice not infected.

† = Rabbit died on the following day.

Table 15

The course of infection and antibody level in rabbit 46 infected with *T. brucei*, TREU 1096.

Days after infection	Titre of serum*	Condition of Rabbit	Weight of Rabbit Kg	Wet Film	Mice
0	0	good	3.700	0	0
2	0	good	3.790	0	+
4	40	good	3.660	0	+
6	320	good	3.490	+	+
8	640	good	3.400	+	+
10	2560	good †	3.400	+	+

* = Highest dilution of serum giving fluorescence. The titration against the 0 day antigen.

0 = No trypanosomes seen and mice not infected.

† = Rabbit died, after one week.

Table 16

The course of infection and antibody level in rabbit F.16 infected with T. congolense, TREU 692, and challenged with the same strain. The rabbit was given Berenil (10 mg/Kg), I.P. for three days after 90 days from the infection.

DAYS			Titre of* serum	Condition of Rabbit	Wet Film	Mice
After Infection	After Drug Treatment	After Challenge				
0			0	good	0	0
5			160	good	0	0
10			640	good	0	+
15			2560	good	0	+
20			2560	good	0	+
25			2560	good	0	+
35			5120	good	0	+
45			5120	good	0	+
60			5120	good	0	+
75			5120	good	0	+
90			10240	good	0	+
104	14		320	good	0	0
111	21		80	good	0	0
125	35		40	good	0	0
139	49		40	good	0	0
153	63		20	good	0	0
160	70		10	good	0	0
162		2	80	good	0	0
164		4	640	good	0	0
166		6	2560	good	0	0
168		8	5120	good	0	0
170		10	5120	good	0	0
174		14	20480	good	0	0
180		20	20480	good	0	0

* = Highest dilution of serum giving fluorescence. The titration against the 0-day antigen.

0 = No trypanosomes seen and mice not infected.

Table 17

The course of infection and antibody level in rabbit 38 infected with T. congolense, TREU 692. The rabbit was given Berenil (10 mg/Kg), I.P. for 3 days after 50 days from the infection.

DAYS		Titre of* serum	Condition of Rabbit	Weight of Rabbit Kg	Wet Film	Mice
After Infection	After Drug Treatment					
0		0	good	2.000	0	0
10		160	good	2.200	0	+
20		640	good	2.450	0	+
30		1280	good	2.550	0	+
40		1280	good	2.650	0	+
50		5120	good	2.740	0	+
80	30	320	good	2.950	0	0
110	60	160	good	2.860	0	0
140	90	40	good	3.060	0	0
170	120	40	good	3.020	0	0
230	180	10	good	3.200	0	0

* = Highest dilution of serum giving fluorescence. The titration against the 0-day antigen.

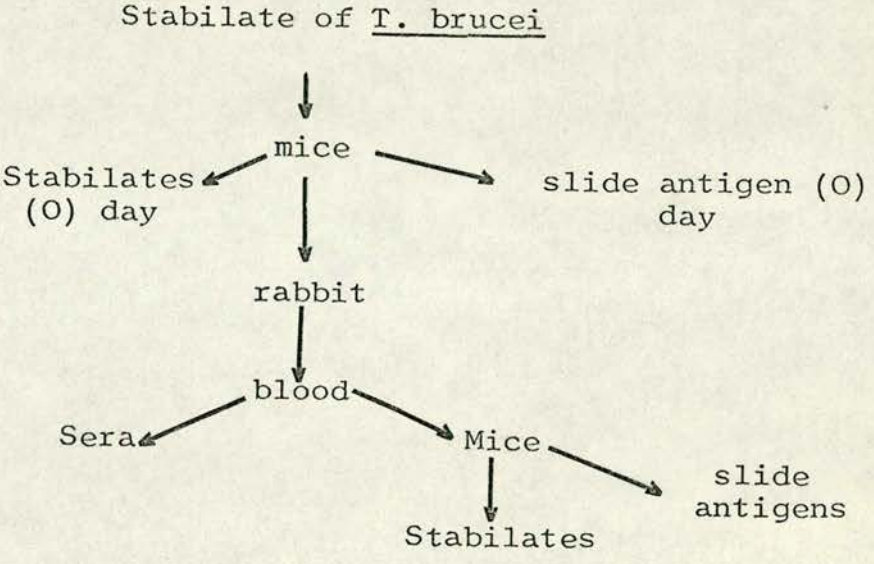
0 = No trypanosomes seen and mice not infected.

Experiment No: 6

A "comparison between the fluorescent and agglutination tests"

The IFA test; thought to detect the "bound" or "common" antigen of trypanosomes, whereas the agglutination test identifies antigenic variants within a species or strain of trypanosomes. Detailed comparisons between the two tests have not been published. The protocol of the experiment is shown below:

Method



When the parasitaemia in the six mice was high (10^8 tryps./ml) they were bled by Cardiac puncture and the blood pooled to provide O-day antigens, O-day stabilates and to infect 2 rabbits R11 and 5-Z.6. At different intervals after infection, about 20 ml blood was taken from the rabbits to obtain sera and for injection into mice for the preparation of further stabilates and slide antigens.

Agglutination test was carried out as described in Material and Methods.

The results of the titrations of the sera of rabbit R11 collected at 0, 12, 18 and 24 days are shown in Table 18

The IFA titre of each serum was the same when titrated against the different antigens. All the sera agglutinated the 0 day antigen at high titre, but sera collected at the same time as the antigen or later, did not agglutinate the trypanosomes or had very low agglutinating titres. Similar results were obtained when titrations were carried out with sera and antigens from rabbit 5-Z.6. (Table19). These experiments confirmed previous observations that the variant specific antigenic types that appear during the course of an infection with T. brucei, cannot be distinguished by IFA reaction.

Table18: Comparison between fluorescent and agglutination tests

Antigen	Reciprocals of antiserum dilation x 10^3											
	12 day serum											
	.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
0 day	4	4	3	3	2	2	2	1	1	1	0	0
	4	4	4	3	3	3	2	2	2	2	1	1
12th day	4	4	3	3	3	2	2	1	1	0	0	0
	1	1	1	0	0	0	0	0	0	0	0	0
18th day	4	4	4	3	3	3	2	2	1	1	0	0
	1	0	0	0	0	0	0	0	0	0	0	0
24th day	4	4	3	3	2	2	2	1	1	1	0	0
	0	0	0	0	0	0	0	0	0	0	0	0

The upper line of figures gives results of IFA test, the lower, the agglutination test.

Antigen	Reciprocals of antiserum dilation x 10^3											
	18 day serum											
	.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
0 day	4	4	4	4	3	3	3	2	2	1	1	0
	4	4	4	3	3	3	3	2	2	2	1	1
12th day	4	4	4	3	3	2	2	1	1	1	0	0
	4	4	3	3	2	2	2	2	1	1	0	0
18th day	4	4	4	4	3	3	2	2	1	1	0	0
	2	2	1	1	0	0	0	0	0	0	0	0
24th day	4	4	4	3	3	3	2	2	1	1	0	0
	0	0	0	0	0	0	0	0	0	0	0	0

The upper line of figures gives results of IFA test, the lower, the agglutination test.

Table 18: continued

Antigen	Reciprocals of antiserum dilation x 10^3											
	24 day serum											
	.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
0 day	4	4	4	4	3	3	2	2	2	1	1	1
	4	4	4	4	4	3	3	3	2	2	2	1
12th day	4	4	4	4	4	3	3	2	2	1	1	1
	4	4	4	4	3	3	3	2	2	2	1	1
18th day	4	4	4	4	3	3	3	2	2	2	1	1
	4	4	3	3	3	2	2	2	1	1	0	0
24th day	4	4	4	3	3	3	3	2	2	1	1	1
	1	1	0	0	0	0	0	0	0	0	0	0

The upper line of figures gives results of IFA test, the lower, the agglutination test.

Table 19: Comparison between fluorescent and agglutination tests

Antigen	Reciprocals of antiserum dilation x 10^3											
	10 day serum											
	.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
(0) day	4	4	4	4	3	2	2	1	1	0	0	0
	4	4	4	3	3	3	2	2	1	1	0	0
10th day	4	4	3	3	3	2	2	1	1	0	0	0
	2	1	1	1	0	0	0	0	0	0	0	0
20th day	4	4	3	3	2	2	1	1	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0

The upper line of figures gives results of IFA test, the lower, the agglutination test.

Antigen	Reciprocals of antiserum dilation x 10^3											
	20 day serum											
	.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
(0) day	4	4	4	4	3	3	3	2	2	1	1	0
	4	4	4	4	3	3	3	2	2	2	1	1
10th day	4	4	4	3	3	3	2	2	1	1	1	0
	4	4	4	3	3	2	2	2	1	1	0	0
20th day	4	4	4	3	2	2	2	1	1	1	0	0
	1	1	0	0	0	0	0	0	0	0	0	0

The upper line of figures gives results of IFA test, the lower, the agglutination test.

Experiment No: 7

A Comparison of the Neutralization and IFA Tests applied to T. congolense

Method

The protocol of this experiment is the same as for experiment No.6 (page 78). Rabbit F16 was infected with T. congolense, TREU 692. The rabbit was bled to obtain preinfection serum and then at 5 day intervals. A part of the blood was inject in to a group of 6 mice, from which the stabilates and slide antigens were prepared.

The neutralization tests were conducted as follows: 0.1 ml of stabilate was added to 0.9 ml of a 10^{-1} dilution of the preinfection serum, and to 0.9 ml of the test serum at dilutions of 10^{-1}, 10^{-2} and 10^{-3}. The diluent used throughout was PBS. After standing for 30 minutes at $0^{o}C$, 0.1 ml aliquots of each suspension were inoculated I.P. into a group of 5 mice. Calculation showed that each mouse received about 10^{6} trypanosomes. All the mice were examined for trypanosomes thrice weekly up to 8 weeks, although the controls usually showed trypanosomes in 6-7 days.

Results

The results of the experiment are shown in Table 20

1) Sera which were collected at 5, 10 and 15 days neutralized the 0 day trypanosomes.
2) Serum of 15 days did not neutralize trypanosomes that were collected on day 35.

3) The IFA reaction of the 15 days serum with the 0 and 35 days antigens was the same.

4) The experiment shows that the neutralizing antibodies are more specific than those detected by IFA test. The variant antigens that appear during the course of an infection cannot be detected by the IFA test.

Table 20

Results of neutralization and IFA tests with serum and stabilates prepared from a rabbit infected with T. congolense.

Neutralization Test

Dilution of Serum		0 day Stabilate			35 days Stabilate	
	5 days serum	10 days serum	15 days serum	Preinfection serum	15 days serum	Preinfection serum
1/10	0/5*	0/5	0/5	5/5	5/5	5/5
1/100	0/5	0/5	0/5	-	5/5	-
1/1000	5/5	0/5	0/5	-	5/5	-

* = Mice infected/mice inoculated
\- = Not tested

IFA Test

Antigen	Reciprocals of serum dilutions x10^3 of 15 days serum											
	.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
0 day	4	4	3	3	3	2	2	1	1	0	0	0
35 days	4	4	3	3	3	2	2	1	1	0	0	0

Experiment No: 8

The effect of separating trypanosomes on a DEAE-Cellulose column on their antigenic properties

Separation of the blood stream forms of trypanosomes from the blood of infected animals, by DEAE-Cellulose column was described by Lanham (1968). The method depends on the surface change of the trypanosomes which is less negative than that of the blood cells of rats and mice in the pH range 6 to 9. On passing infected blood through a column of an anion-exchanger, the blood cells and platelets are completely adsorbed, and motile infective trypanosomes are eluted with the plasma. The object of this experiment was to find out if there is any change in antigenicity of T. brucei, after separation on a DEAE-Cellulose column.

Method

(1) Preparation of the column:

25g of DEAE-Cellulose (Type DE52, Whatman Chromedia) was mixed with about 500 ml Phosphate-saline-glucose buffer (PSG) pH 8.0, and allowed to stand for one hour. The supernatant was decanted and the cellulose was again mixed with PSG and left for one hour to settle. The process was repeated until the pH of the supernatant was within 0.05 units of pH 8.0. The final volume of the slurry was 40 ml. Heparin in normal saline was added to give a concentration of 10 I.U./ml. The slurry was poured into a column 30 cm long and with diameter of 2 cm, and allowed to settle. The flow from the exit was controlled by a screw clip on a rubber

tube. Excess fluid was drained off.

(2) Separation of trypanosomes:

After bleeding the mice by Cardiac puncture, 5 ml of the blood was mixed with heparin to give 10 I.U./ml and then diluted 1:3 with cold PSG. The diluted blood was layered carefully on to the surface of the cellulose column and the control clip released so that eluate escaped in single drops; the flow rate was equivalent to about 5 ml per hour. When all the blood had entered the column, elution was continued with PSG until examination of the eluate showed that most of the trypanosomes had been eluted. The trypanosomes were separated from the eluate by centrifugation at +2°C for 20 minutes at 1,500 g.

The supernatant fluid was discarded and the sediment resuspended in PSG. The elution was carried out at room temperature. Slide antigens for IFA titrations were prepared from the column-separated trypanosomes. For the agglutination test, the trypanosomes were suspended in PSG at approximately the same concentration as those in the stabilates.

IFA and agglutination tests were carried out with trypanosomes prepared by the standard method and with the column-separated organisms. Sera obtained from rabbit 1675 on 10th and 21st day after infection with T. brucei, were used. The viability of the trypanosomes was tested by injecting 6 mice I.P. with a suspension of the separated trypanosomes. At the same time 6 mice were inoculated with whole infected blood. Both groups of mice

received the same number of trypanosomes.

Result

The results were summerised in Table 21

(1) There is no difference in the degree and titre of fluorescent and agglutination reactions of trypanosomes that had been separated by the standard method and those were separated from blood on a DEAE-Cellulose column.

(2) Mice inoculated with trypanosomes had been separated by column, became infected and the incubation period was the same as that of the control group.

Table 21

Titration of sera by IFA and agglutination tests.

Sera	(O) day antigen	Reciprocals of serum dilutions x 10^3											
		.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
10 day serum	IFA	4	4	3	2	2	2	1	1	1	0	0	0
		4	4	4	3	2	2	1	1	1	0	0	0
	Agglutination	4	4	4	3	3	3	2	1	1	0	0	0
		4	4	4	4	3	3	2	1	1	0	0	0
21 day serum	IFA	4	4	4	4	3	3	2	2	1	1	1	0
		4	4	4	4	3	3	3	2	1	1	1	0
	Agglutination	4	4	4	4	3	3	2	2	2	1	1	1
		4	4	4	3	3	3	2	2	2	2	1	1

The upper line of figures gives the results of trypanosomes prepared by the standard method, the lower, of trypanosomes separated on a DEAE-Cellulose column

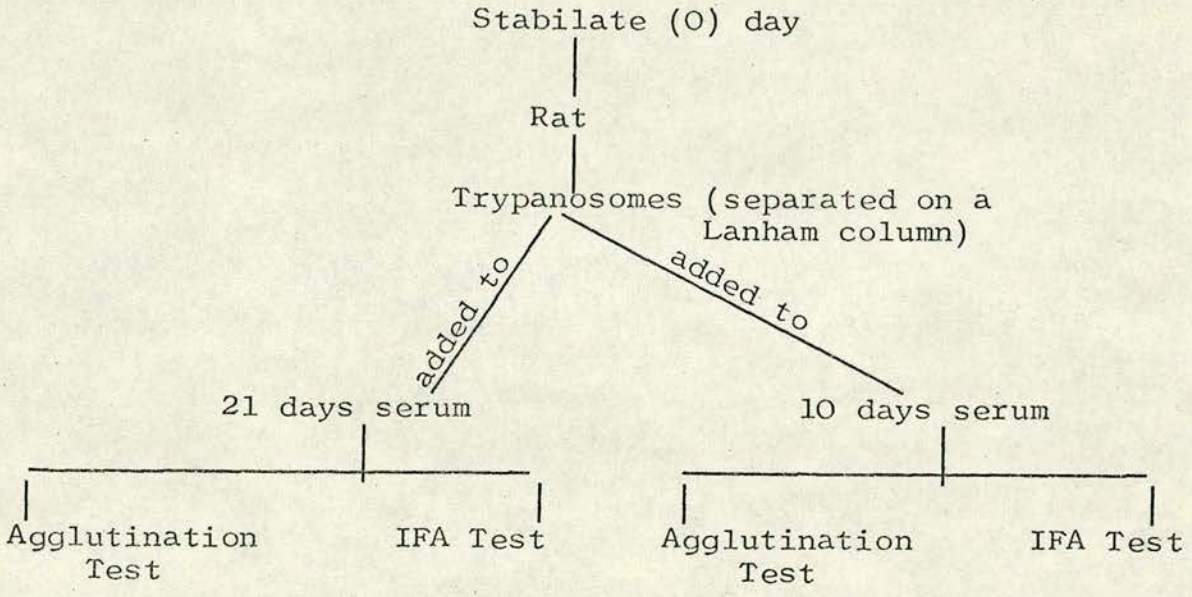
Experiment No: 9

The effect of the absorption of variant specific agglutinins on the IFA titre.

In Experiment No. 6 it was shown that IFA test does not detect antigenic variants of T. brucei. This test was designed to find whether the absorption of agglutinins to one variant would effect the fluorescent titre of the antiserum.

Method

The protocol of the experiment is shown below:-



(1) Trypanosomes suspension:-

Blood was obtained from 2 rats heavily infected with T. brucei and the trypanosomes were separated on a DEAE-Cellulose column as described in Experiment No. 8 (page 86). The concentration of trypanosomes in the final eluate was about 45 x 10^9 tryp./ml of PSG. This suspension was kept in ice.

(2) Antisera and antigens:-

Rabbit 1675 was inoculated with blood from mice infected with T. brucei. At the same time stabilates (0-day) and slide antigens (0-day) were prepared from the infected mice blood. On day 6 after infection, mice were inoculated with blood from rabbit 1675 and 6th day antigens were prepared when the mice became parasitaemic. Sera of 10 and 21 days were obtained from the same infected rabbit.

(3) The test:-

To absorb antibodies for one antigenic type from an antiserum, 1 ml of the trypanosomes suspension was centrifuged at 1,500 g for 3 minutes. The supernatant fluid was discarded and 0.5 ml of the serum was added to the sediment, mixed thoroughly and left on the bench for one hour. The tube was centrifuged again for 10 minutes and the clear supernatant fluid was removed and mixed with a new sample of packed trypanosomes which had been similarly prepared in another centrifuge tube. The procedure was repeated several times until a test showed that agglutination was negligible in a 1/10 dilution of the serum. The final volume of the absorbed serum was 0.3 ml.

Infectivity of trypanosomes suspension that had been kept for 12 hours in an ice bath while the experiment was in progress, was tested by injecting a group of 6 mice with the same dose as was given to a control group of 6 mice which were inoculated when the rats were bled.

Result

Titrations of absorbed and unabsorbed sera with the O-day antigen are shown in Table 22. Agglutinins to this antigenic variant had been completely removed, since there was no agglutination in the absorbed antisera. The IFA titre was also reduced in the absorbed antisera. In Table23 the results are given of titrations with the 6-day antigen. Agglutinins to this variant were still present in the absorbed sera since the titres of the absorbed and unabsorbed sera were about the same. However, again the IFA titre was reduced to the same extent as in the titrations with the O-day antigen.

Mice inoculated with trypanosomes had been kept in ice bath for 12 hours, became infected and the incubation period was the same as that of the control group.

Table 22

Titration of sera by IFA and agglutination tests, before and after absorption of the sera by one antigenic variant of T. brucei.

Serum	(O) day antigen	Reciprocals of serum dilutions x 10^3											
		.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
10 days serum	IFA	4	4	3	2	2	2	1	1	1	0	0	0
		3	2	1	1	1	0	0	0	0	0	0	0
	Agglutination*	4	4	4	3	3	3	2	1	1	0	0	0
		1	0	0	0	0	0	0	0	0	0	0	0
21 days serum	IFA	4	4	4	4	3	3	2	2	1	1	1	0
		3	2	1	1	1	0	0	0	0	0	0	0
	Agglutination*	4	4	4	4	4	4	3	3	2	2	1	1
		1	0	0	0	0	0	0	0	0	0	0	0

The upper line of figures gives results of unabsorbed serum, the lower of absorbed serum.

* Agglutination test was carried out with stabilates.

Table 23

Titration of sera by IFA and agglutination tests.

Serum	6th day antigen	Reciprocals of serum dilutions x 10^3											
		.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
10 days serum	IFA	4	4	3	3	3	2	2	1	1	0	0	0
		3	2	2	1	1	0	0	0	0	0	0	0
	Agglutination*	4	4	3	3	2	2	1	1	0	0	0	0
		3	3	2	2	2	1	0	0	0	0	0	0
21 days serum	IFA	4	4	4	3	3	3	2	2	1	1	1	0
		2	2	1	1	1	0	0	0	0	0	0	0
	Agglutination*	4	4	4	4	4	4	3	3	2	2	2	1
		4	4	4	4	4	3	3	3	2	2	1	1

The upper line of figures gives results of unabsorbed serum, the lower, absorbed serum.

* Agglutination test was carried out with stabilates.

Experiment No: 10

Identification of serum components responsible for the fluorescent reaction

Serum is known to contain many immunologically active components, such as IgM, IgG and others. During infections with trypanosomes, there is an initial increase in the concentration of the serum immunoglobulin, IgM, followed by an increase in IgG. The IgM fraction contains the agglutinins. This experiment was designed to find which fraction was responsible for the fluorescent reaction. For this purpose the activity of whole serum was compared with that of fractions separated by gel filtration.

Method

(i) Preparation of antisera.

Two rabbits were infected with T. brucei, and bled after 20 and 21 days.

(ii) Fractionation of serum.

(a) Preparation of Sephadex Column.

15 g of Sephadex G-200 were added to 1 litre of 0.1M Tris-HCl buffer pH 8.0 and left to swell for 72 hours. The slurry was poured into a glass column 100 cm x 2.5 cm filled with the same buffer. Great care was taken to exclude air bubbles. When the column had settled under gravity it was washed with 500 ml of Tris-HCl buffer before use. The apparatus was set up in a room where the temperature was maintained at approximately 12°C.

(b) Fractionation

3 ml of rabbit serum was layered carefully on to the top of the gel and eluted at a flow rate of 6 ml/hour with Tris-HCl buffer. 2 ml fractions were collected on an LKB fraction Collector. The absorbance of each fraction at 280 mμ was measured in a Spectrophotometer.

The column was washed with 500 ml of Tris-HCl buffer before running the second serum sample. The elution profiles of the 2 sera are shown in Fig. 13.

(c) Collection and concentration of serum fractions.

Three groups of fractions (M , G , A) were collected, one from each absorbance peak as indicated in Fig 13.
Pooled fractions from each peak were concentrated by ultrafiltration through 47 mm PSED type Pellicon membrane (1000 Mol wt Cut off, Millipore, U.K. Ltd.), then dialysed against PBS pH 7.2 for 24 hours at $4^{o}C$ with 3 changes of buffer. Concentration of each fraction was required, so that titrations of the unfractionated antisera could be directly compared with those of the fractions.

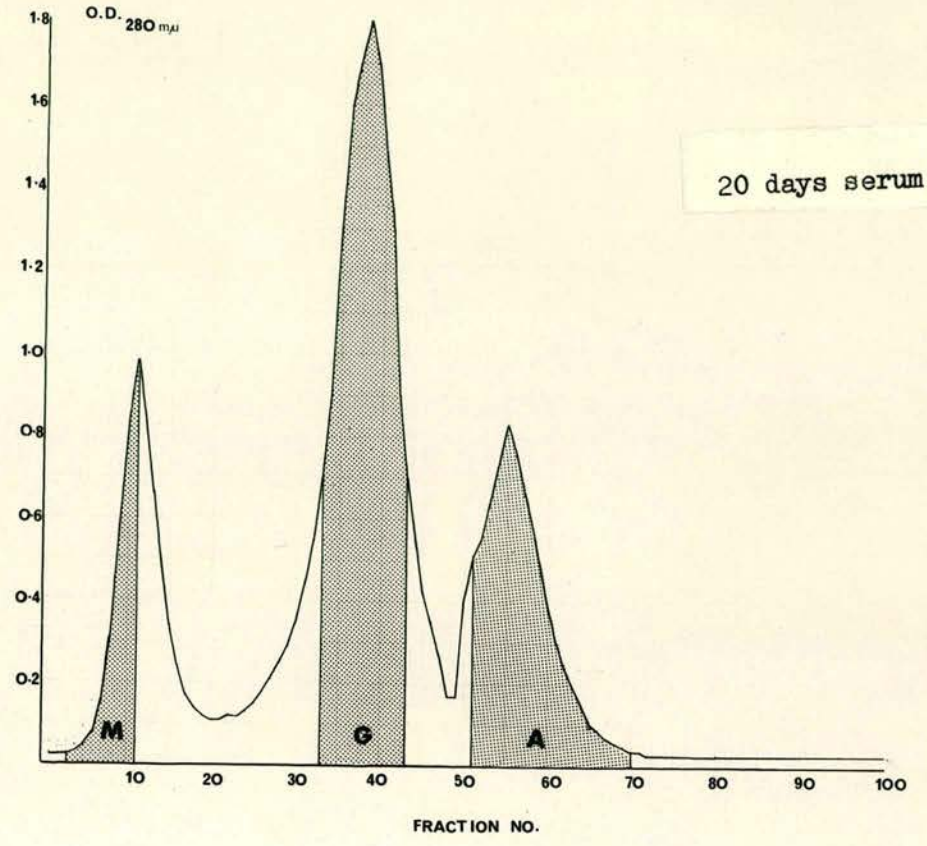
The dilution factor was estimated from the formula:-

$$\frac{\text{Vol. serum}}{\text{Vol. conc. fract.}} \times \frac{O.D_{280} \text{ collected fraction}}{O.D_{280} \text{ peak fraction}} = \text{Diln.}$$

The calculations are shown in Table 24.

(iii) Purity of fractions.

The purity of the three fractions from each anti-serum, was assessed immunoelectrophoretically by the method of Grabar & Burtin



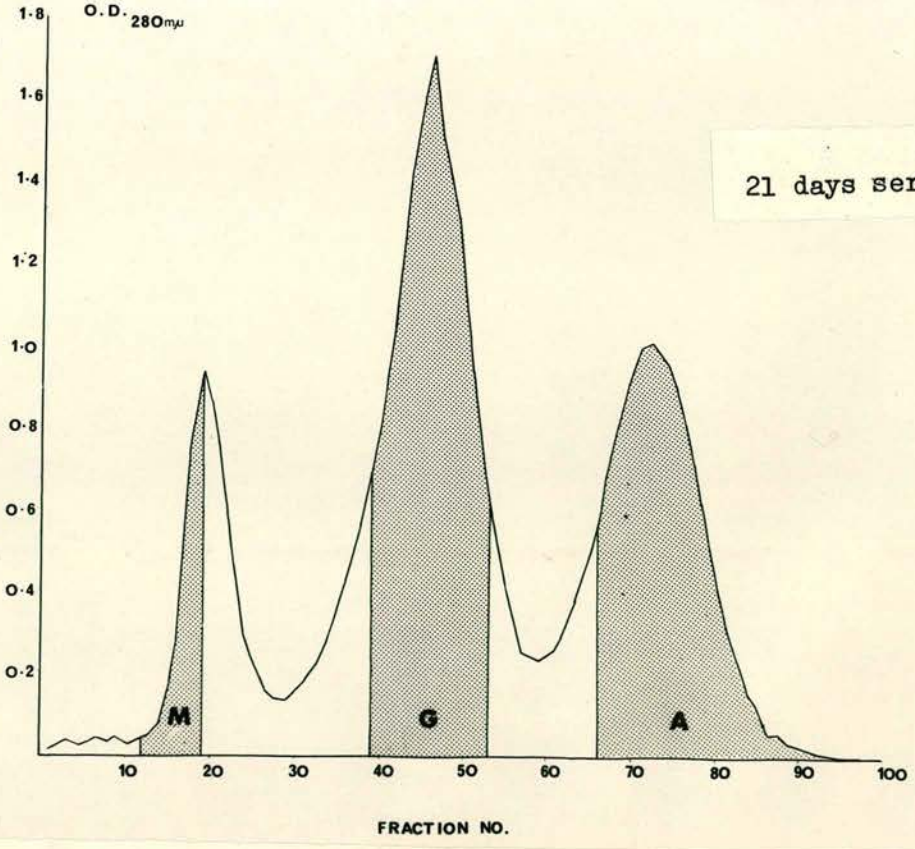


Fig. 13 Fractionation of antisera to T. brucei by Sephadex G-200.

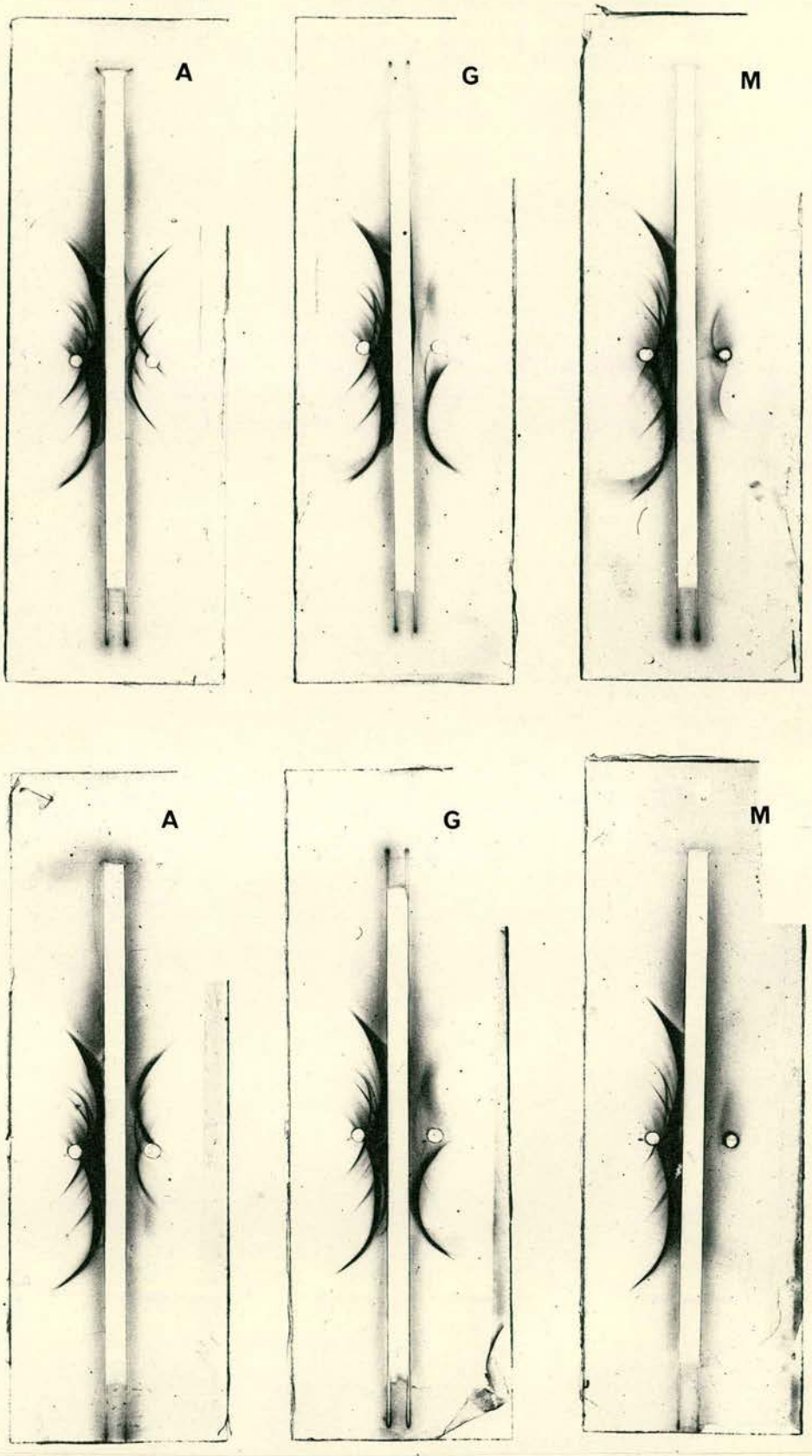


Fig. 14 Immunoelectrophoresis of fractions of T. brucei antisera.

Swine anti-rabbit serum was placed in the trough and samples of whole rabbit serum (left side) and fractionated serum (right side) were pipetted into the wells.

(1964). Microscope slides on a plastic support were covered with a 2 mm thick layer of 1.2% Noble Agar (Difco) in 0.05 M Veronal buffer pH 8.2. Two wells of diameter 1.5 mm were punched in the gel on each slide, and a 3.5 µl sample of either whole serum or fractionated serum was pipetted into each well. The preparation was electrophoresed at 240 volts and 11 ampers per support, for one hour, using an LKB Type 3290B electrophoretic apparatus and power supply. One test sample and one whole serum sample were electrophoresed on each slide. A central trough 2 mm in width was cut and filled with swine anti-rabbit serum (SwAR/ielfo) raised against whole rabbit serum. The slides were incubated at 4°C for 24 hours in a wet box, then washed for 3 days with 3 changes of 0.002M Veronal buffer pH 8.2 in 1.5% saline and one of distilled water. The washed slides were then dried at 37°C and stained for 30 minutes in 0.1% Ponceau Red in acetate buffer. Excess stain was removed by washing in distilled water. Photographs of the slides are shown on Fig. 14 . As expected fraction M contained IgM and α_2 macroglobulin only, uncontaminated with IgG. Fraction G contained mainly IgG and small amounts of non-immune globulins but no IgM. Fraction A contained albumin and many other proteins.

(iii) Activity of serum fractions.

The immunological activity of the 3 fractions against T. brucei antigen was tested by IFA and agglutination tests.

Results Table 25.

1. Immune serum has been separated into 3 fractions, M (IgM + α_2 macroglobulin), G(IgG + some other protein) and A (albumin + many other proteins).

2. Electrophoretic analysis showed that, fraction M contained IgM but no IgG and fraction G contained IgG but no IgM.

3. The agglutinating titre was the same in the M fraction as in the whole serum.

4. Agglutinating antibody was also present in the G fraction but the titre was less than in the whole serum.

5. The fluorescent antibody titre was the same in the G fraction as in the whole serum. Very slight fluorescent antibody activity was present in the M fraction. No activity was present in the A fraction.

Table 24

Calculation of dilution factors of serum fractions

Serum	Fraction	Vol. Serum ml.	Vol.conc. fraction ml.	O.D. collected fraction	O.D. Peak fraction	Effective dilution
20 day serum	M	3	3.16	4.61	7.82	(0.559) ½
	G	3	4.16	14.1	18.650	(0.545) ½
	A	3	4.66	9.320	12.390	(0.484) ½
21 day serum	M	3	1.8	2.760	6.340	(0.725) ⅔
	G	3	4.6	17.160	22.90	(0.488) ½
	A	3	8.6	12.995	15.045	(0.285) ⅓

Table 25

Titrations of fractions of serum by IFA and agglutination tests

Serum	Fraction	Reciprocals of serum dilutions x 10^3											
		0.01	0.02	0.04	0.08	0.16	0.32	0.64	1.28	2.56	5.12	10.24	20.48
20 day serum	Whole serum	4	4	4	4	3	3	2	2	1	1	1	0
		4	4	4	4	4	3	3	3	2	2	1	1
	M	1	1	0	0	0	0	0	0	0	0	0	0
		4	4	4	4	3	3	3	2	2	2	1	1
	G	4	4	4	4	3	2	2	2	1	1	1	0
		3	2	2	2	2	1	1	0	0	0	0	0
	A	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0
21 day serum	Whole serum	4	4	4	4	3	3	2	2	1	1	1	0
		4	4	4	4	3	3	2	2	2	1	1	1
	M	1	1	1	0	0	0	0	0	0	0	0	0
		4	4	4	4	3	3	3	2	2	1	1	1
	G	4	4	4	4	3	3	2	2	2	1	1	1
		2	2	2	2	1	1	0	0	0	0	0	0
	A	0	0	0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0	0	0

The upper line of figures in each fraction gives results of IFA test, the lower, the agglutination test.

Experiment No: 11

The effect of washing on the antigenic properties of T. brucei

It has been shown by electron microscopy that the blood stream forms of trypanosomes possess a layer of material external to the plasma membrane (Vickerman 1969). It has been suggested that the variant specific antigens are located in this surface coat (Vickerman and Luckins, 1969).

The object of this experiment was to remove the surface coat and to test the seriological activity of uncoated trypanosomes.

Method

A group of 12 mice were injected with T. brucei, stabilates which had been prepared at the same time as rabbit R11 was inoculated. The mice were bled after 3-4 days when the parasitaemia reached approximately 5×10^8 per ml. The blood was centrifuged at 1,500g for 3 minutes, and the trypanosomal layer was separated carefully and washed once with bovine serum. The trypanosomes were then separated by centrifugation, the supernatant was discarded, and only the packed trypanosomes were left in the tube.

Trypanosomes were mixed, in the proportion of 1:3, with the following solutions:-

1) Physiological saline.

2) Physiological saline + 1% glucose (w/v)

3) Phosphate buffer saline (PBS) at pH 6, 7 and 8.

The suspensions were left to stand at room temperature for different intervals. A drop of the mixture was examined microscopically

every 10 minutes to observe the motility of the trypanosomes. After the trypanosomes had been standing for the allotted time, they were sedimented by centrifugation and resuspended to the original volume in fresh solution. Serum of 24 days obtained from rabbit R11, was used for testing the agglutinability and fluorescent activity of the trypanosomes. The infectivity of trypanosomes that had been kept in the different solutions was tested by injecting a group of 6 mice with the same dose as was given to a control group of 6 mice which were inoculated when the mice were bled.

Result

The results of the experiment are shown in Table 26. The trypanosomes were no longer agglutinated after suspension for 30 or 40 minutes in saline. By adding glucose to the saline, agglutinability was maintained for 40 minutes, although the agglutination titre was slightly reduced. After 60 minutes in saline-glucose, the trypanosomes were still agglutinated although the titre was now much reduced.

Suspension for 40 minutes in PBS at pH 6.0 completely destroyed the agglutinating properties of the trypanosomes, but when the pH was adjusted to 7 or 8, these properties were not affected since the agglutination titre was the same as in the controls.

Treatments that destroyed or reduced the agglutinating properties also affected the reactivity for the trypanosomes in

the I.F.A. test, since, after these treatments, the fluorescent titre of the sera were reduced.

No change was detected in the pH of the solutions in which the trypanosomes had been suspended. Mice inoculated with trypanosomes that had been kept in saline for 30 minutes and saline-glucose for 30 and 60 minutes, became infected and the incubation period was the same as that of the control group.

Table 26

I.F.A. and agglutination titres of serum tested against T. brucei after the trypanosomes had been kept in different solutions

Solution	Time (minute)	Reciprocals of serum dilutions x 10^3												Motility	Mice
		.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48		
Bovine * serum	0	4 4	4 4	4 4	4 4	3 4	3 3	3 3	2 3	2 2	1 2	1 1	1 1	Strong	+
Saline	0	4 4	4 4	3 4	3 4	3 4	2 3	2 3	2 3	1 2	1 2	0 2	0 1	Strong	+
	30	3 1	2 0	2 0	2 0	1 0	1 0	0 0	0 0	0 0	0 0	0 0	0 0	Weak	+
	40	3 1	2 0	2 0	2 0	1 0	1 0	0 0	0 0	0 0	0 0	0 0	0 0	Weak	-
1% glucose in saline	0	4 4	4 4	4 4	4 4	3 4	3 3	2 3	2 2	2 2	1 2	1 1	1 1	Strong	+
	30	4 4	4 4	4 3	4 3	3 3	3 2	2 2	2 1	2 1	1 0	1 0	0 0	Strong	+
	40	4 4	4 4	4 3	3 3	3 2	3 2	2 2	2 1	1 1	1 0	0 0	0 0	Strong	-
	60	4 2	3 2	3 1	3 1	2 1	2 0	1 0	1 0	0 0	0 0	0 0	0 0	Strong	+
PBS	pH 6.0 40	3 0	3 0	3 0	1 0	1 0	1 0	0 0	0 0	0 0	0 0	0 0	0 0	Weak	-
	pH 7.0 40	4 4	4 4	4 4	3 4	3 4	2 3	2 3	1 3	1 2	1 2	0 1	0 1	Strong	-
	pH 8.0 40	4 4	4 4	4 4	3 4	3 3	2 3	2 3	2 2	1 2	1 2	0 1	0 1	Strong	-

- = not tested

* = the upper line of figures gives results of I.F.A. test, the lower, the agglutination test.

Experiment No: 12

The effect of fixation on the agglutinability of T. brucei

As described before the surface coat plays an important role in seriological properties of trypanosomes. The object of this experiment was to detect the effect of different fixatives on the antigenicity of the trypanosomes.

Method

The same materials that were used in Experiment No.11 were used in this experiment. After washing the trypanosomes in bovine serum, they were resuspended in a fresh bovine serum, then treated with the following fixatives:-

1) 5% formalin in PBS for 15 minutes.

2) Heat at 60°C and 70°C for 5 minutes.

The trypanosomes were mixed with 5 volumes of 5% formalin in PBS and left for 15 minutes. The trypanosomes were then separated by centrifugation, washed once with PBS and resuspended in bovine serum to the original volume.

Fixation with heat was carried out by immersing the tube, which contained the suspension of trypanosomes in bovine serum, in a water bath at 60°C and at 70°C for 5 minutes. A drop of the mixture was examined microscopically after the allotted time in each fixative, to observe the motility of the trypanosomes. The agglutination test was carried out as described before. The controls for the test, were trypanosomes suspended in bovine serum or PBS for 15 minutes without fixation.

Result (Table 27)

The trypanosomes that had been treated with 5% formalin in PBS for 15 minutes or at a temperature of 60°C for 5 minutes, retained slight agglutinability, but those exposed to 70°C for 5 minutes, had completely lost their ability to agglutinate. There was no change in the agglutination titre in tests carried out with trypanosomes taken directly from mouse blood and those left standing in bovine serum or PBS for 15 minutes.

Table 27

The effect of fixation on the agglutination titre

T. brucei in:	Duration of treatment (minute)	Reciprocals of antiserum dilution x 10^3											Motility
		.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48	
Mouse blood	0	4	4	4	4	3	3	2	2	2	1	1	1	Strong
Bovine serum	15	4	4	4	4	4	3	3	2	2	1	1	1	Strong
PBS	15	4	4	4	4	4	3	3	2	2	2	1	1	Strong
5% Formalin in PBS	15	2	1	1	1	0	0	0	0	0	0	0	0	Nil
Water bath 60°C	5	2	1	1	1	1	0	0	0	0	0	0	0	Nil
Water bath 70°C	5	0	0	0	0	0	0	0	0	0	0	0	0	Nil

Experiment No: 13

To compare the serological properties of blood, culture and fly forms of trypanosomes

It is known that trypanosomes in culture or in the fly's midgut, differ morphologically and antigenically from the blood forms. In these experiments, IFA and agglutination tests were applied to follow the antigenic changes that take place when blood forms are transferred into culture or into flies.

Method

The protocol of the experiment is shown on pages 112 and 113.

(i) Antigen:

T. brucei, TREU 667 and T. congolense, TREU 692 were used.

For IFA and agglutination tests, the blood forms were prepared as described before. Culture forms of trypanosomes were obtained from Miss Cunningham and were grown with tsetse fly alimentary tract as described by Cunningham (1972).

To prepare antigen for the IFA test, drops of a concentrated suspension of the trypanosomes in culture medium were placed on clean slides and allowed to dry in air. The trypanosomes were then fixed in formalin and treated as for blood forms. For the agglutination test drops of the suspension of trypanosomes in culture medium were mixed with antisera and left for 30 minutes before examining for agglutination.

Fly forms were obtained from infected G. morsitans. The midgut was dissected out and the trypanosomes separated as far

as possible from debris, were suspended in normal bovine serum. This suspension was used for preparing slide antigen and for agglutination tests.

(ii) Antisera: sera of 12 and 24 days were prepared from rabbit R11, the 5 and 15 day sera were from rabbit M97. Both rabbits were infected with T. brucei TREU 667. Sera of 15 and 50 days were obtained from rabbit 38 that was infected with T. congolense, TREU 692.

T. rhodesiense antiserum was obtained from rat infected for 14 days. T. gambiense antiserum was obtained from C.T.V.M.

IFA and agglutination tests were carried out as described before.

The infectivity of the culture and fly forms was tested by inoculating aliquots of the prepared suspension into mice.

Results

When sera from an infected rabbit were titrated against culture forms of the same strain of T. brucei, the fluorescent antibody titre declined as the cultures aged. The sharpest fall in titre occurred after the trypanosomes had been in culture for about 5 days tables 28, 29.

Titrations against trypanosomes taken from the midgut of tsetse flies showed a similar decline in fluorescent antibody titre.

The results of IFA titrations of sera from rabbits infected with T. congolense against culture and fly's forms of the same

strain were similar to the results obtained with T. brucei, Table 30.

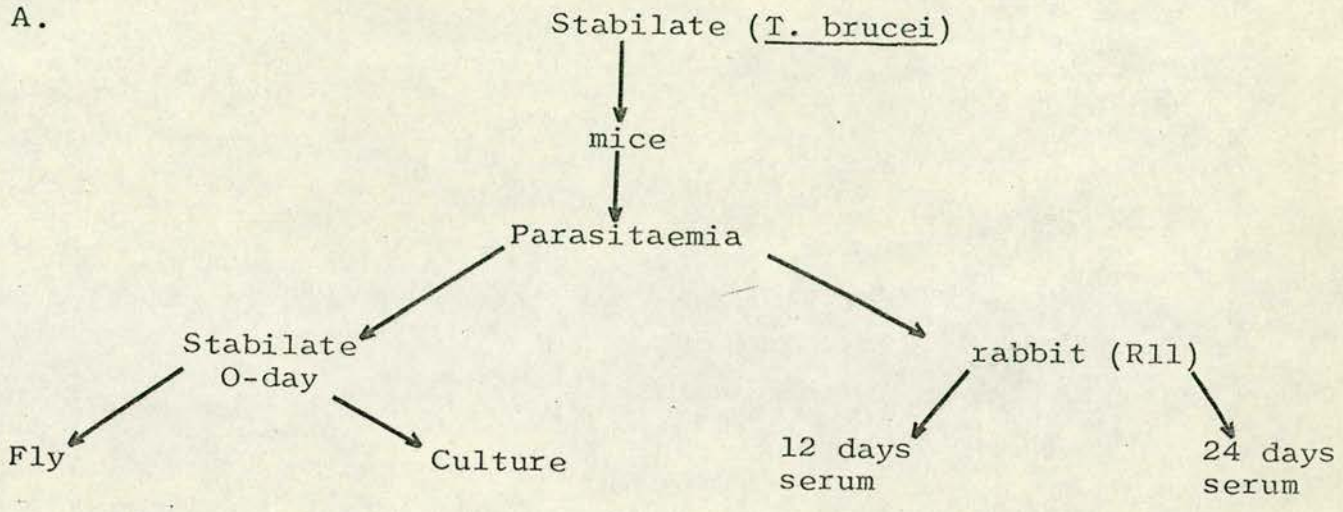
The agglutination titre of the sera to culture forms also dropped when tested against forms harvested from culture after 5 or more days, Table 31.

No agglutination occurred with trypanosomes taken from flies 3 days after infection. This was the earliest time that sufficient trypanosomes could be obtained from flies to do agglutination tests.

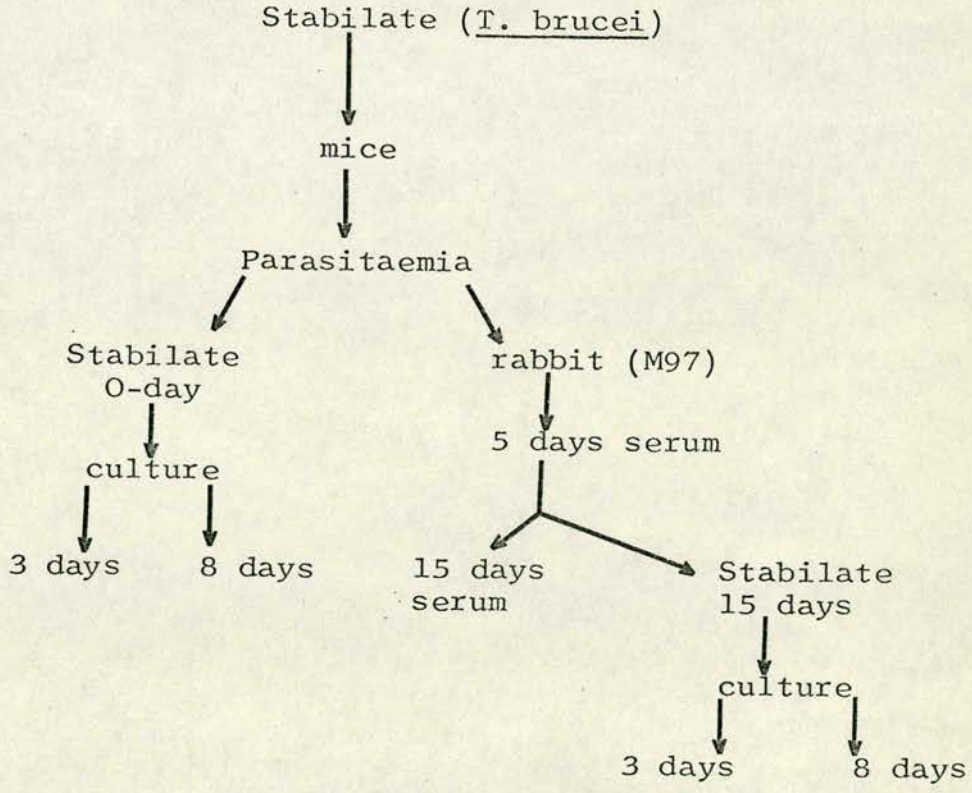
Some tests were carried out to determine the specificity of the agglutination reaction when culture forms were used as antigen. 3 day old culture forms prepared from 0-day stabilates were agglutinated by the 5 and 15 day sera, whereas with 3 day culture forms prepared from 15 day stabilates, the agglutination was negligeable (Table32). 8 day old culture forms prepared from 0-day and 15-day stabilates were agglutinated at a low titre by the 5 and 15 day homologous antisera and also by antisera to T. congolense, T. rhodesiense and T. gambiense (Table 33). Hence, the variant specific agglutinogens were still present in the trypanosomes that had been in culture for 3 days. After 8 days in culture, the variant specific antigens had disappeared and now the agglutinogens reacted with antisera to different species of trypanosomes. When 3 and 7 day culture forms of T. congolense were tested with homologous and heterologous antisera, the titre was higher in the homologous

reaction (Table 34). The 7 day culture form did however agglutinate with T. brucei serum. The lack of agglutination by T. rhodesiense and T. gambiense antisera was probably due to the low antibody level of these sera as was indicated by previous IFA tests. The infectivity of culture forms of T. brucei retained for 4 days. In all the tests, controls were set up in which the trypanosomes were mixed with normal sera and PBS. No agglutination occurred in any of the controls.

A.


Stabilate (T. brucei)
mice
Parasitaemia
Stabilate
O-day
Fly
Culture
rabbit (R11)
12 days
serum
24 days
serum

B.


Stabilate (T. brucei)
mice
Parasitaemia
Stabilate
O-day
culture
3 days
8 days
rabbit (M97)
5 days serum
15 days
serum
Stabilate
15 days
culture
3 days
8 days

C.

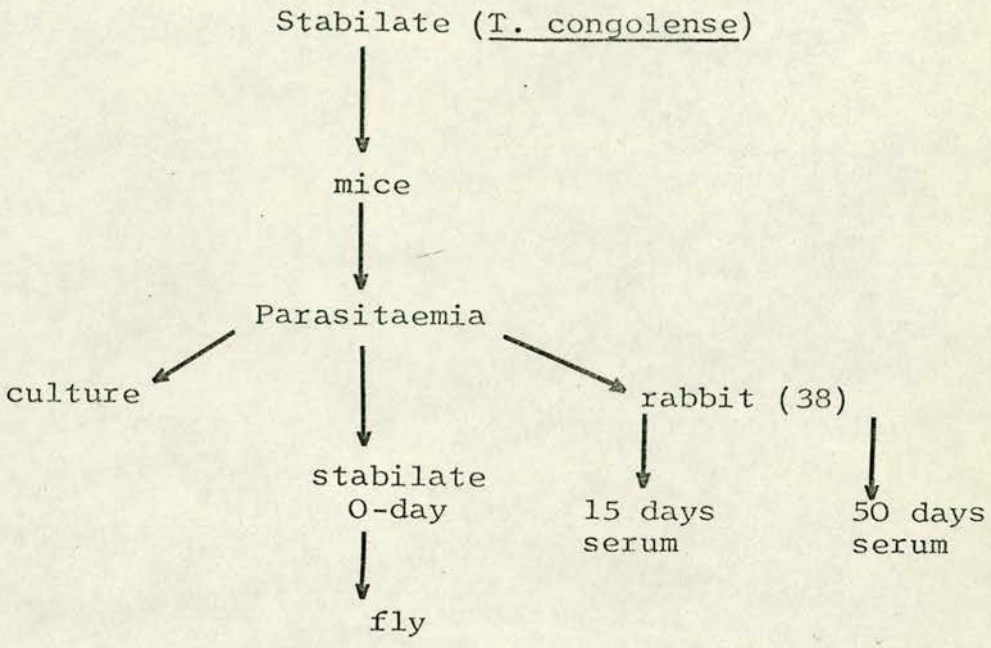

Stabilate (T. congolense)
mice
Parasitaemia
culture
stabilate
0-day
fly
rabbit (38)
15 days
serum
50 days
serum

Table 28

IFA titres of serum from a rabbit infected with T. brucei, TREU 667 against the culture forms of the same strain.

Age of culture (days)	Reciprocals of antiserum dilution x 10^3 - 12 days serum												Mice
	.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48	
0	4	4	3	3	3	2	2	1	1	1	0	0	+
1	4	4	3	3	3	2	2	1	1	0	0	0	+
2	4	4	3	2	2	1	1	1	0	0	0	0	+
3	4	4	3	3	2	1	1	1	0	0	0	0	+
4	3	3	2	2	1	1	1	0	0	0	0	0	+
5	3	3	2	2	1	1	0	0	0	0	0	0	0
6	3	2	1	1	0	0	0	0	0	0	0	0	-
7	2	2	1	1	0	0	0	0	0	0	0	0	-
8	2	2	1	1	0	0	0	0	0	0	0	0	-
10	2	1	1	0	0	0	0	0	0	0	0	0	-
12	2	1	1	0	0	0	0	0	0	0	0	0	-

- = not tested

Table 29

IFA titres of serum from a rabbit infected with T. brucei TREU 667 against culture and fly forms of the same strain.

Antigen	Days	Reciprocals of antiserum dilution x 10^3 - 24 days serum											
		.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48
Culture	0	4	4	4	4	3	3	2	2	2	1	1	1
	3	4	4	4	4	3	3	2	1	1	0	0	0
	4	3	3	2	2	2	1	1	1	0	0	0	0
	5	3	2	2	2	1	1	1	0	0	0	0	0
	6	2	1	1	0	0	0	0	0	0	0	0	0
	7	2	1	1	0	0	0	0	0	0	0	0	0
	8	1	1	0	0	0	0	0	0	0	0	0	0
	13	1	1	0	0	0	0	0	0	0	0	0	0
fly mid-gut	3	4	4	4	4	3	3	2	2	1	1	0	0
	5	4	4	3	3	2	2	2	1	1	1	0	0
	7	4	3	3	2	2	1	1	1	0	0	0	0
	11	4	3	2	2	1	1	0	0	0	0	0	0
	13	3	2	2	1	1	0	0	0	0	0	0	0
	16	2	2	1	1	1	0	0	0	0	0	0	0
	20	2	2	1	1	1	0	0	0	0	0	0	0
	30	1	1	0	0	0	0	0	0	0	0	0	0

Table 30

IFA titres of serum from a rabbit infected with T. congolense, TREU 692 against culture and fly forms of the same strain.

Antigen	Days	Reciprocals of antiserum dilution x 10^3 -50 day serum												Mi
		.01	.02	.04	.08	.16	.32	.64	1.28	2.56	5.12	10.24	20.48	
Culture	0	4	4	4	3	3	3	2	2	1	1	0	0	+
	1	4	4	4	3	3	3	2	2	1	1	0	0	0
	2	4	4	3	3	3	2	1	1	0	0	0	0	0
	3	4	4	3	3	2	1	1	0	0	0	0	0	0
	4	4	4	3	2	2	1	1	0	0	0	0	0	0
	5	4	4	3	3	2	1	0	0	0	0	0	0	0
	7	4	3	3	2	2	1	0	0	0	0	0	0	-
	9	3	2	2	1	1	0	0	0	0	0	0	0	-
	13	2	2	1	1	0	0	0	0	0	0	0	0	-
	16	2	2	1	1	0	0	0	0	0	0	0	0	-
	20	2	2	1	1	0	0	0	0	0	0	0	0	-
Fly Mid-gut	3	4	4	4	3	3	2	1	1	0	0	0	0	0
	5	4	3	3	2	1	1	0	0	0	0	0	0	-
	7	3	3	2	2	1	1	0	0	0	0	0	0	-
	9	3	2	2	2	1	0	0	0	0	0	0	0	-
	14	2	1	1	0	0	0	0	0	0	0	0	0	-
	21	1	1	1	0	0	0	0	0	0	0	0	0	-
	30	2	1	1	0	0	0	0	0	0	0	0	0	-

- = not tested

Table 31

Agglutination titres of serum from rabbit infected with T. brucei, TREU 667 against culture forms of the same strain.

Age of culture (days)	Titration of 12 days serum			
	1:10	1:100	1:1000	1:10000
0	4	3	2	1
1	4	3	2	1
2	4	3	2	1
3	4	3	2	1
4	4	3	2	1
5	3	2	1	0
6	3	2	1	0
7	3	2	1	0
8	2	2	1	0
10	2	2	1	0
12	2	2	1	0

Table 32

Agglutination titres of sera from rabbit M97 against blood and 3 days culture forms of T. brucei

Sera	1/10	1/100	1/1000	1/10000
0 day	0	0	0	-
	0	0	0	-
	0	0	0	-
	0	0	0	-
5 days	3	3	1	0
	3	2	1	0
	1	0	0	0
	1	0	0	0
15 days	4	3	2	1
	3	3	1	0
	2	1	0	0
	1	1	0	0

The first line of figures gives results of blood form and the second line of culture form of 0-day stabilates. The third line of blood form and the fourth line of culture forms of 15-days stabilates.

Table 33

Agglutination test of 8 days old culture forms of T. brucei with homologous and heterologous antisera.

Sera	Serum titration				Normal serum			PBS
	1/10	1/100	1/1000	1/10000	1/10	1/100	1/1000	
Antibrucei (5 days)	2	1	0	0	0	0	0	0
	2	1	0	0	0	0	0	0
Antibrucei (15 days)	2	1	0	0	0	0	0	0
	2	1	0	0	0	0	0	0
Anticongolense (15 days)	2	1	0	0	0	0	0	0
	2	1	0	0	0	0	0	0
Antirhodesiense (14 days)	2	1	0	0	0	0	0	0
	2	1	0	0	0	0	0	0
Antigambiense (15 days)	2	1	0	0	0	0	0	0
	2	1	0	0	0	0	0	0

The upper line of figures gives results of culture forms prepared from 0-day stabilates and the lower line of culture forms prepared from 15-days stabilates.

Table 34

Agglutination titres of homologous and heterologous antisera against blood and culture forms of T.congolense TREU 692.

Sera	Serum titration			
	1/10	1/100	1/1000	1/10000
Anticongolense (15 days)	3	2	1	0
	3	3	1	0
	3	2	1	0
Antibrucei (15 days)	1	0	0	0
	1	0	0	0
	2	1	0	0
Antirhodesiense (14 days)	-	-	-	-
	-	-	-	-
	0	0	0	0
Antigambiense (15 days)	-	-	-	-
	-	-	-	-
	0	0	0	0

The first line of figures gives results of blood form, the second line 3 days culture forms and the third line 7 days culture forms. - = not tested.

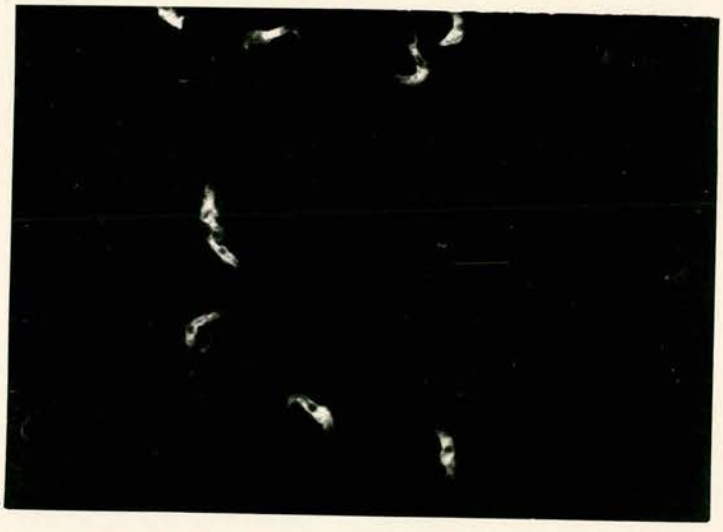
A

B

C

Fig 15

A



B

C

Fig 16

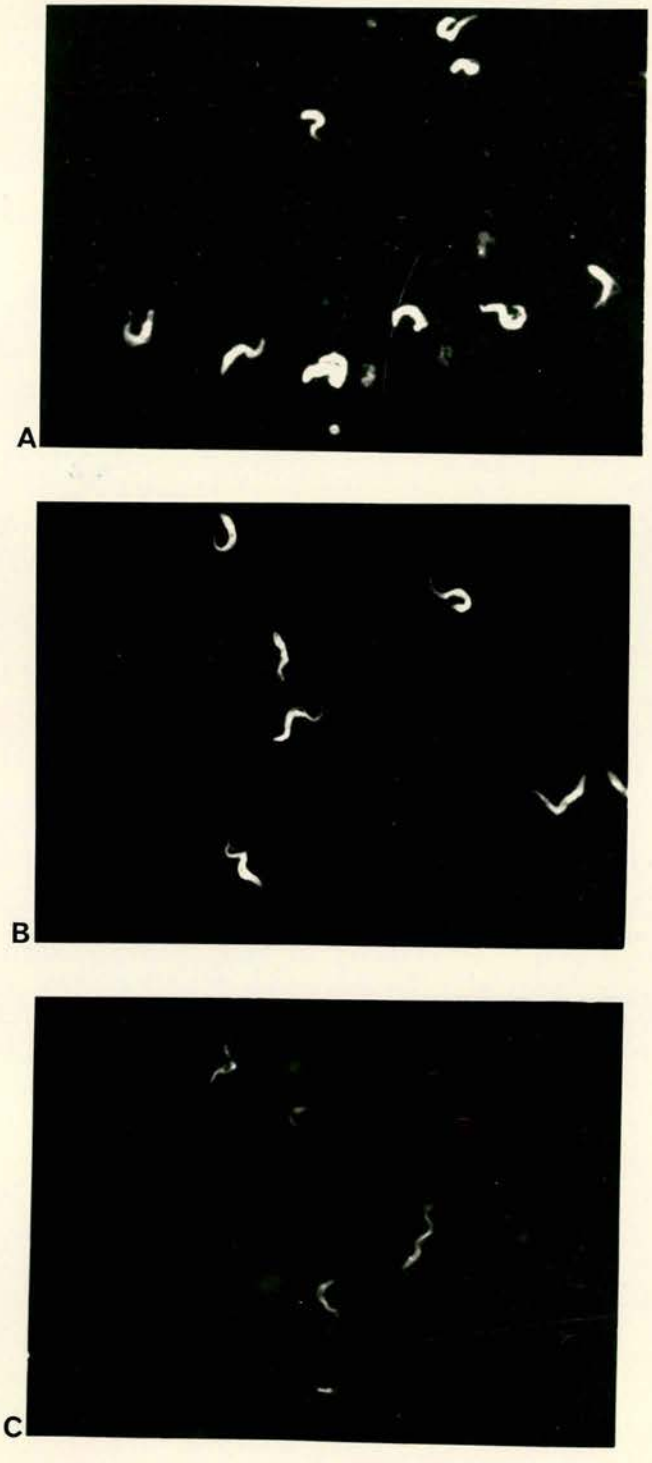


Fig 17

The following photographs were taken at one minute exposure time with x40 objective.

Figure 15. 1/0 dilution of antiserum from rabbit infected with T. brucei, reacted with trypanosomes from tsetse flies

A. 3 days after infection

B. 16 days after infection

C. 30 days after infection

Figure 16. 1/20 dilution of antiserum from rabbit infected with T. brucei, reacted with trypanosomes harvested from culture at:

A. 3 days

B. 6 days

C. 12 days

Figure 17. 1/40 dilution of antiserum from rabbit infected with T. congolense, reacted with trypanosomes harvested from culture at:

A. 3 days

B. 5 days

C. 13 days

CHAPTER IV

BABESIA

Application of the IFA test to study the epizootiology of Babesia

Infection of domestic and wild animals by Babesia species is frequently not manifest and so the disease escapes the attention of investigators.

Serological tests have now been developed which detect antibody to the parasite, and, if applied systematically, the epizootiology of the infection in tick infested areas could be determined.

The fluorescent antibody test was known to be a useful technique for a screening test of many parasitic diseases. In this part of the thesis the IFA test was applied in the field for serodiagnosis of Babesia infection in both cattle and red deer, and to determine if this test is adequate for studying the epidemiology of the disease in a local area.

A. Experimental

(1) Preparation of antigen

Since the methods for applying IFA test to Babesia had varied, tests were first carried out with B. rodhaini to find which method would be most suitable. The parasite was injected into the mice, and antigen and sera were prepared from them.

Antigens were prepared by the following methods:

a) Direct blood smear

b) Thick and thin smears prepared after washing the blood cells with PBS

c) Fixatives:-

i) 5% formalin in distilled water for: 5, 10 and 15 min.

ii) HCl 0.3N for 5 min.

iii) Acetone for 10 and 20 min.

Result

From the tests it appeared that washing the infected blood 3 times with PBS is better than using a direct blood smear in which there was a lot of non specific fluorescence in the background and this will interfere with reading of the test; by using a thick smear there is no need to search for the parasitized cells. Fixation with acetone for 10 minutes gives a satisfactory result. Unfixed antigen stored at -70°C had not deteriorated after 6 months. Subsequent procedures, e.g., the time for washing in PBS, incubation with test and conjugated antisera was exactly as described for trypanosomes except that the conjugated antiserum was used at a dilution of 1/10 and unmounted slides were examined under x100 oil objective.

2. Specificity of the test

Specificity was tested by titrating antisera to B. divergens, B. major and B. rodhaini against B. divergens antigens. The titre of the sera in the heterologous reactions was lower than in the homologous as shown below.

Antiserum to:	B. divergens antigen
B. divergens (CaB7)	1/640
B. divergens (CaB9)	1/640
B. major (B148)	1/40
B. major (C360)	1/20
B. rodhaini (MB3)	1/10
B. rodhaini (MB5)	1/10

3. Serological survey for babesiasis in cattle on the island of Arran

The island of Arran was selected for the survey because the disease in cattle has been reported on the island; the cattle farms are self-contained there being no importation of cattle only export of the new born animals, and finally, the local veterinarian and the farmers were prepared to cooperate in the collection of blood samples from their herds. For these reasons this island seemed to be an appropriate place for an initial serological survey for Babesiasis.

Island of Arran (Fig. 18) is located in the Clyde between the mainland and the Kintyre peninsula. It is estimated to cover an area of 165 square miles. It was part of an elevated plateau which included most of Scotland, and stood in 2-3,000 ft above sea level.

The climate of the island is mild due to the warming incluence of the Gulf Stream. The rainfall in the higher central area of the island must average 80-100 inches per year. At Dougrie, on the west coast the average is 46 inches, and at Brodick on the east it is 65-70 inches. The average daily temperature shows that July is the warmest month, with a maximum of 63-64^{o}F, but June too has high maximum and minimum

ISLE OF ARRAN,
BUTE,
SCOTLAND.

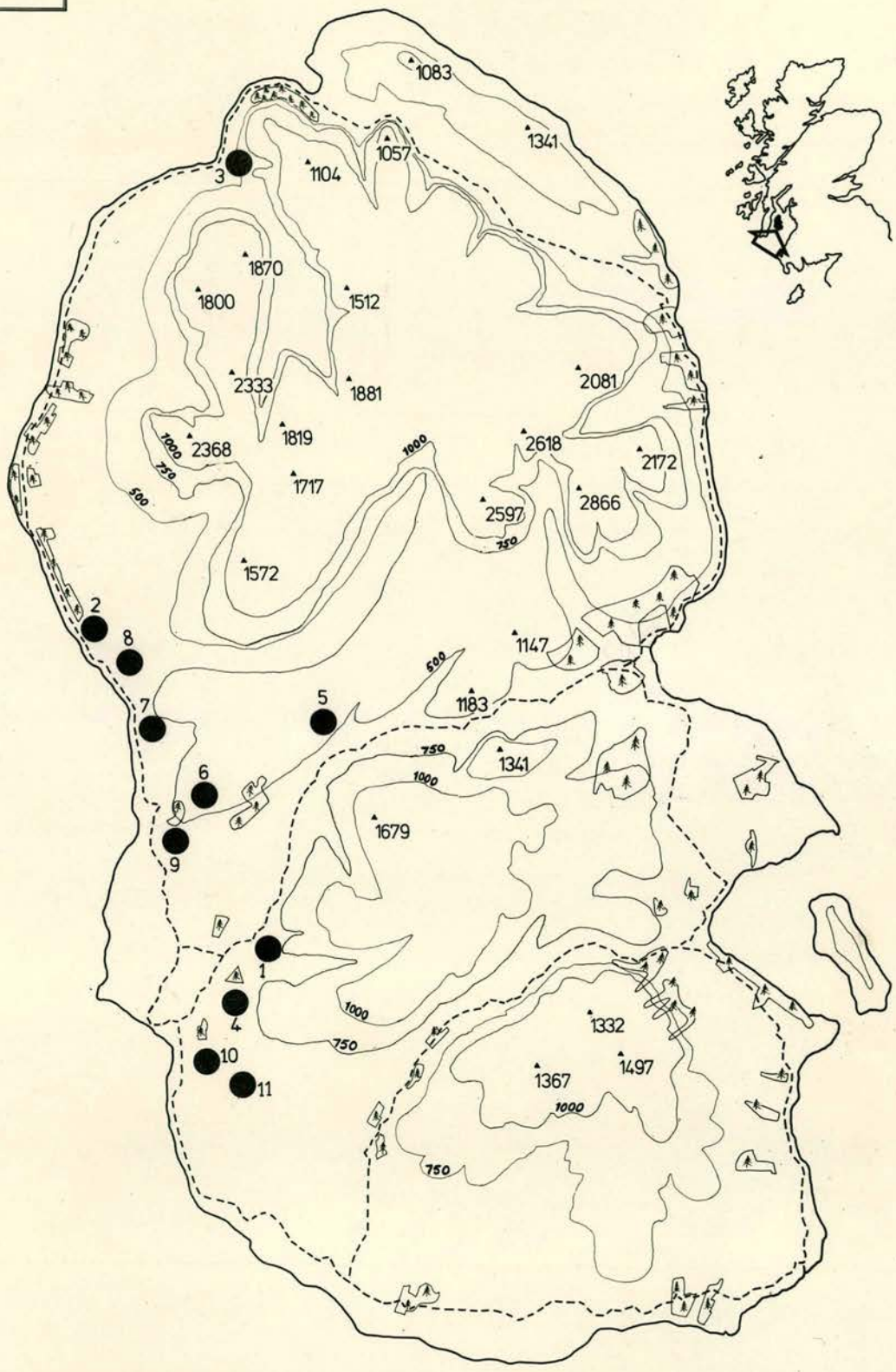


Fig.18 showing location of the farms ● where blood samples were obtained

temperatures, with the lowest rainfall and the most sunshine. January and February are the coldest months, with an average minimum of just under 36°F. The maximum rainfall occurs between November and January and the minimum between May and June. Frost can be expected between the middle of November and the middle of March, but seldom is severe. There is a variety of hill type grasses and plants in the island. Among animals there are cattle, sheep, red deer and hares.

Cattle farming and management

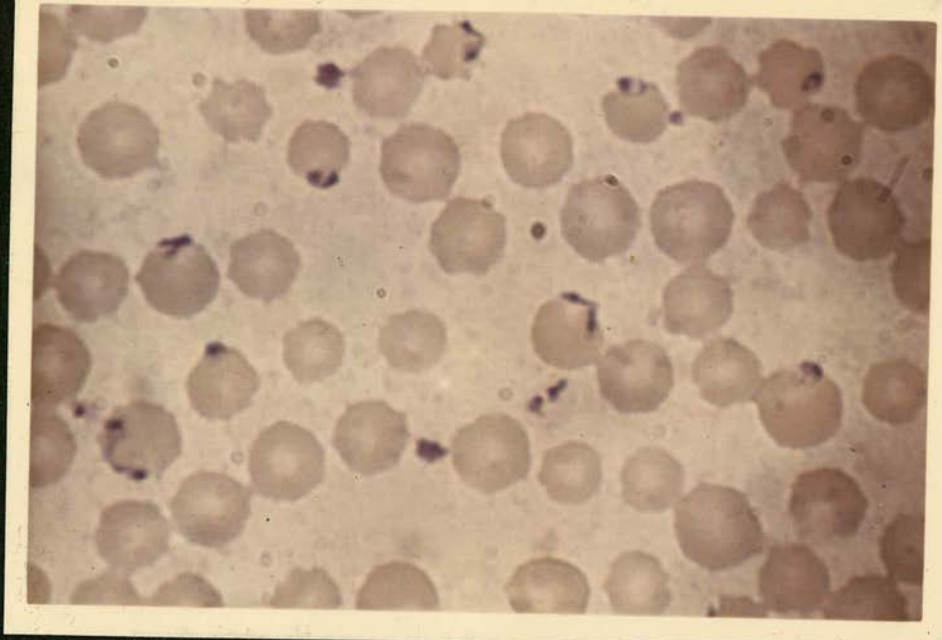
Most of the farms are self-contained with mixed breeds, Highland, Angus, Galloway, Short Horn and a few Pedigree Galloway. The average size of herd is between 100-150. The beef cattle run on unfenced hills from approximately October until May or June. Calves are born in the spring, mostly in March and April. During the summer the cattle are grazed in enclosed low-land fields to fatten the calves for sale in the Autumn. The dairy cattle are reared and grazed on the low-land only.

Ticks

Active ticks are abundant on the hills in March, April and May and again in September and October, although a few may be found on the cattle throughout the summer.

Selection of farms

The farms selected for survey were all on the west coast (Fig. 18) and centred round Dougrie (No. 2) where a case of redwater had occurred in 1970. On the neighbouring farm,



A

B

Fig. 90

A

B

C

Fig 19

Figure 19. A, B & C show the two types of pasture land, the enclosed field in the foreground separated by a wall or a fence from the rough hill land in the background.

Figure 20. A. B. divergens from calf's blood stained with Giemsa stain.

B. Crush used for restraining cattle while obtaining samples

Auchencar (No. 8), some cattle had been imported about six years previously. Shortly after their introduction some of the animals died of redwater; the remainder were quickly returned to the mainland and sold. Cattle of different ages were selected from each farm.

Collection of samples

Blood samples were collected from 222 cattle of varying ages on 8 beef cattle farms during one week spent on the island (31st May - 5th June, 1971). In late September 1971 a second visit was made to collect 22 blood samples from cattle on two dairy farms. About 15 ml of blood was taken from the jugular vein of each animal and the serum was separated by gentle centrifugation after the blood had clotted. Drops of blood on filter paper were also collected.

The test

IFA test was applied using sera and the eluate from filter paper blood against B. divergens antigen. The method was carried out as described in materials and methods (page 37).

Results

The results of the survey are shown in Tables 35, 36. Of the 244 animals examined, 63 had babesial antibody indicating an infection rate of 28.4%. Positive reactors were found on all the farms except the two with dairy cattle only (No. 10 & 11). The proportion of animals infected on the beef cattle farms

BABESIA DIVERGENS

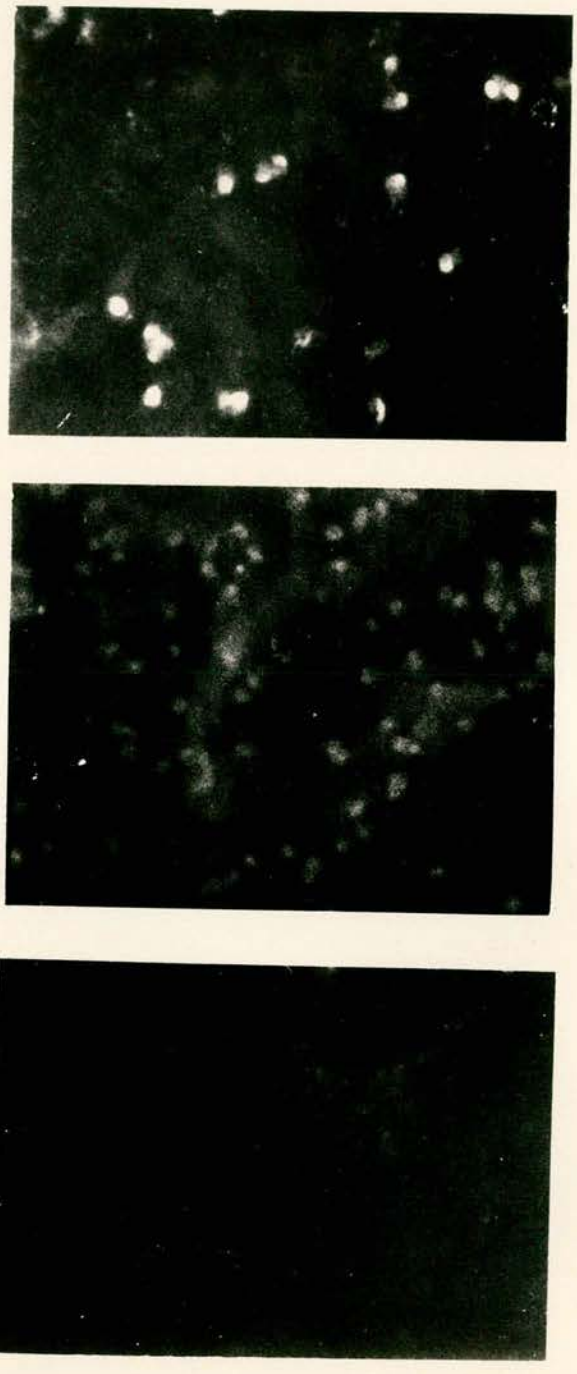


Fig 21 Degree of fluorescence

A. 2

B. 1

C. o

varied from 8 to 40%.

On farms Dougarie (No. 2) and Auchencar (No. 8) where cases of redwater had been reported, the infection rate was 40%.

The proportion of positive animals according to their age is shown in Table 37.

The infection rate in cattle over 4 years was high but this may depend on the number of animals that have been sampled.

In none of the calves born in 1971 was babesial antibody detected. Titration of 53 positive sera (Table 38) showed that the titres were low, the highest was 1/160.

Table 35

Results of the survey for babesiasis on Arran by IFA test

Number of Farm	Farm	Tested/ Positive	Age (years) under 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Unknown age
1	Balnacoole	26/2		26/2																
2	Dougarie	57/23	15/0	1/0	6/2	2/2	5/3		5/4	3/3	6/4	4/3	1/0	2/0						7
3	Catacol	22/6		22/6																
4	Balgowan	20/4	11/0	1/0						2/1		3/1		2/1					1/1	
5	Monyquil	27/3	5/0			3/0	2/0	5/0	1/0		1/1	1/0	2/0	1/0		1/0				5
6	Machrie	22/7	7/0	2/1	4/1	2/0	3/3		1/1	1/1						1/0				1
7	Auchengallon	28/10	9/0	1/0	1/1	1/0	4/2	3/1	4/3		3/2		1/0	1/1						
8	Auchencar	20/8	8/0				2/2		1/0	3/3	1/0		1/1	2/1	2/1					
10	Carmahome (Dairy)	10/0		3/0	2/0	1/0	1/0			2/0	1/0									
11	Tighenmenach (Dairy)	12/0		4/0	2/0	2/0	3/0				1/0									

Table 36

Results of the survey for babesiasis by IFA test

Farm	Total number sampled	Number positive	Number positive per cent
Balnacoole	26	2	7.7
Dougarie	57	23	40.4
Catacol	22	6	27.3
Balgowan	20	4	20.0
Monyquil	27	3	11.0
Machrie	22	7	31.8
Auchengallon	28	10	35.7
Auchencar	20	8	40.0
TOTAL	222	63	28.4%

Table 37

Proportion of animals with positive IFA in age groups

Age of cattle (years)	Total number sampled	Number positive	Number positive per cent
Under 1	55	0	0
2	53	9	17
3	11	4	36
4	8	2	25
5	16	10	62
6	8	1	12.5
7	12	8	75
8	9	8	89
9	11	7	64
10	8	4	50
11	5	1	20
12	8	3	37.5
13	2	1	50
14	2	0	0
15	0	0	0
16	0	0	0
17	1	1	100
UNKNOWN	13	4	30.7
TOTAL	222	63	28.4

Table 38

Titre of Sera

Age in Years	1:40	1:80	1:160
2	0	1	6
3	0	0	3
4	0	1	1
5	0	3	5
6	0	0	1
7	1	2	4
8	0	4	3
9	0	5	1
10	1	0	0
11	1	0	0
12	0	3	0
13	0	0	1
17	0	1	0
UNKNOWN	0	3	1
TOTAL	3	23	27

4. IFA test for diagnosis of Babesia infection of red deer (Cervus elaphus) in Scotland

Preparation of conjugated antideer serum

i. Preparation of deer globulin fraction:

4 ml of normal deer serum was dialysed against 50% saturated ammonium sulphate (SAS) for 12 hours with 3 changes. It was then centrifuged at 12,000g for 10 minutes and the supernatant discarded. The precipitate was resuspended in 50% SAS and centrifuged again. The precipitate was dissolved in 4 ml distilled water and dialysed against 50% SAS as before. Finally it was centrifuged again, and the precipitate was dissolved in, and dialysed against, phosphate buffered saline (PBS) pH 7.2 for 12 hours, with three changes of buffer. All the above procedures were carried out in a cold room or a refrigerated centrifuge at $4^{o}C$. The protein concentration of the final solution was determined by reading the Optical Density of a 1/20 dilution and calculating the concentration from a standard graph.

ii. Preparation of antideer sera:

Two rabbits numbered 56 and 57, aged 6 months and weighing about 3 K.g were each injected subcutaneously at several sites, with 20 mg of deer globulin emulsified in an equal volume of Freund's complete adjuvant. After 7 weeks the rabbits were given a second injection of 20 mg of deer globulin and bled five days later. They were bled twice more at 10 day intervals, 30 mls of blood being taken each time. Before the second injection a test sample of 5 ml of blood was taken from each rabbit. The

sera were tested by immunoelectrophoresis and found to contain a high level of antibodies of the required specificity (Fig. 22).

iii. Preparation of antideer globulin fraction:

5 ml of cold (4°C) SAS was added dropwise to 5 ml of chilled antideer serum, mixed throughly and refrigerated at 4°C for 2 hours. The suspension was centrifuged at 4°C for 20 minutes at 23,000 g and the supernatant was discarded. The precipitate was washed with 50% SAS and centrifuged again. The precipitate was dissolved in 5 ml of 0.15 M saline. An equal volume of SAS was added slowly. After 2 hours at 4°C the suspension was centrifuged, and the supernatant discarded. The precipitate was partly redissolved by adding a minimal volume of PBS, and dialysed against PBS pH 7.2 for 24 hours at 4°C with 3 changes of buffer. After dialysis the solution was concentrated to the original volume (5 ml) by ultrafiltration through a 47 mm PSED type Pellicon membrane (1000 Mol wt Cut off, Millipore, U.K. Ltd.).

The protein concentration was determined by reading the optical density at 280 mμ and calculating the protein concentration from a standard graph.

The concentration was calculated as 25.16 mg protein/ml of serum.

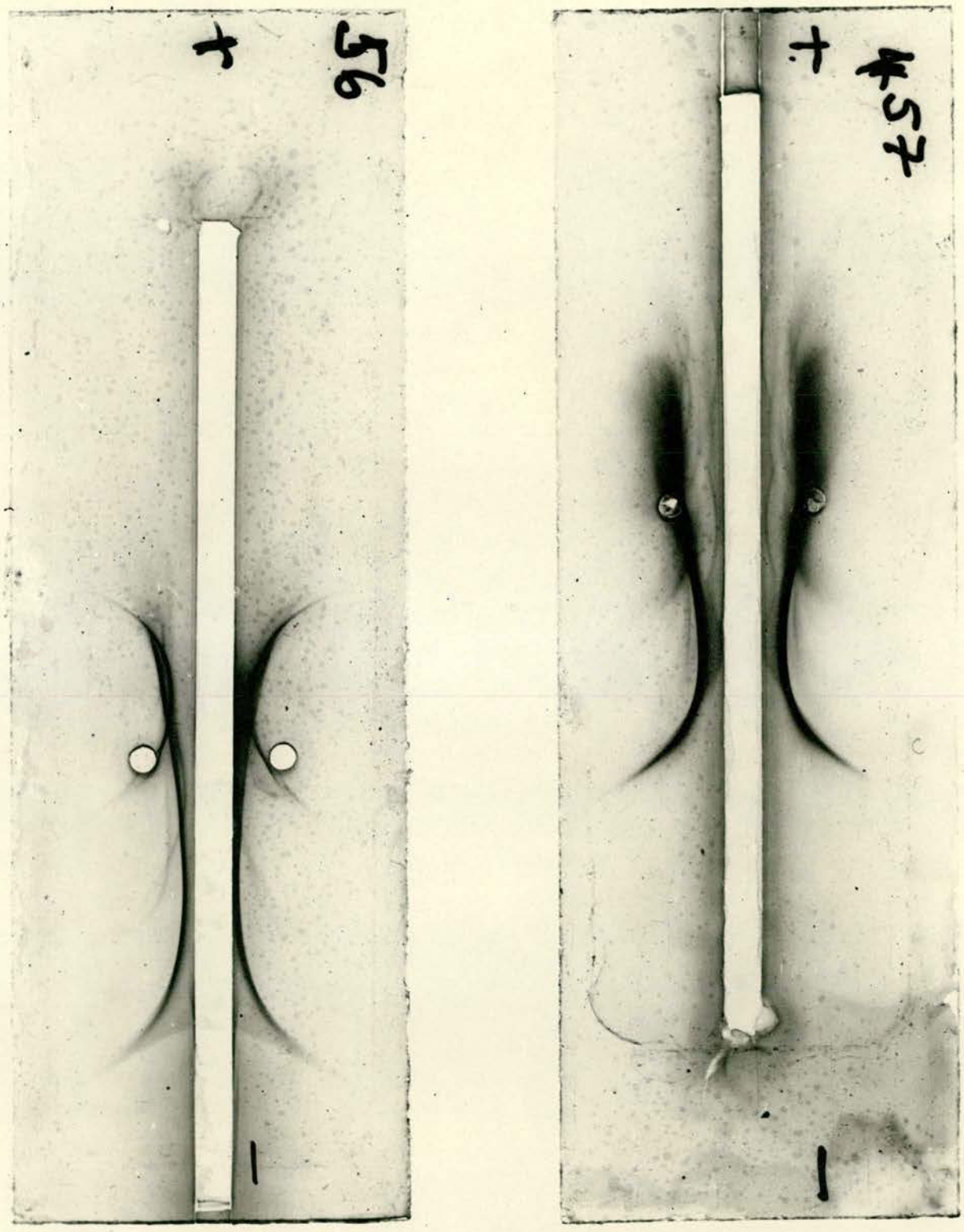


Fig. 22 Immunoelectrophoresis pattern of rabbit antideer globulin.

Rabbit anti-deer globulin was placed in the trough and normal deer serum in the wells.

iv. Conjugation of fluorescein isothiocyanate (FITC) to globulin:

A standard solution of 1 mg fluorescein isothiocyanate isomer 1 per ml in 0.15M $Na_2HPO_4.{}_2H_2O$ (pH 9.0) was made for immediate use. The proportion of FITC/protein required is 10 mg/gm. Fluorescein isothiocyanate solution was added to the globulin solution while stirring at room temperature. The pH was adjusted to 9.5 by addition of 0.1 M $Na_3PO_4.10H_2O$ while stirring. The reaction was allowed to continue for 60 minutes at room temperature, with the pH maintained at 9.5 by the addition of small amounts of 0.1 M $Na_3PO_4.10H_2O$ when necessary.

v. Removal of unconjugated fluorescein

Unconjugated dye molecules must be removed and this was done by gel filtration on Sephadex G-25. About 10 gm of Sephadex G-25 (Pharmacia) swollen in phsophate buffered saline pH 7.2 was packed in a glass column 30 cm x 1.5 cm as described in experiment No. 10 (page 95). The FITC-globulin mixture was layered on top of the column and eluted with PBS. Two well separated yellow coloured peaks were eluted from the column. The first peak absorbed strongly at 280 mμ and was assumed to contain only protein-fluorescein conjugate. The second peak absorbed minimally at 280 mμ and was assumed to contain only unreacted fluorescein molecules.

vi. Calculation of Fluorescein/Protein ratio (F/P ratio):

It is necessary to calculate the average number of fluorescein molecules conjugated to each protein molecule in order to assess the usefulness of the preparation. An average F/P ratio of 2-4 has been found to be the optimum. Below this ratio, preparations are only weakly fluorescent. At higher ratios the risk of non-specific staining increases. Large numbers of fluorescein molecules attached to a single protein molecule may alter its overall charge, so that the molecule may non-specifically attach to many cell surfaces. The F/P ratio was calculated from the O.D. readings at 276 mμ and 493 mμ using the nomogram presented by Goldman (1968). The average ratio was found to be: 5.5 fluorescein molecules/molecule protein.

vii. Removal of non-specific staining:

To remove the risk of non-specific staining by some molecules in the preparation which may have a high F/P ratio (the calculated F/P ratio being only the average), the preparation was absorbed with acetone-dried, rabbit liver powder (Sigma) as described by Goldman (1968). 0.5 gm of rabbit liver powder in the proportion 100 mg of powder per ml of original serum was used. The powder was equilibrated with 2 changes of PBS pH 7.2 and centrifuged at 1,500 g for 5 minutes. The pellet was mixed with the conjugate, and left in a dark place for one hour on a slow shaker. The mixture was then centrifuged at 12,000 g for 20 minutes at 4°C, and the supernatant conjugate

was removed. The procedure was repeated twice.

Finally the conjugated globulin was distributed in 1 ml plastic tubes and kept at -20°C until use.

The test

Sera and dried blood samples on filter papers were collected from 25 deer killed in west Ross and Invernessshire on the estates of Monar, Patt, Struy, Braulen and Fairburn. Two filter papers samples of blood were also obtained from red deer shot on Arran.

IFA test was carried out as described for Babesia of cattle, except that conjugated antideer serum was used in a dilution of 1/40.

Normal deer sera were obtained from the Rowett Research Institute, Bucksburn, Aberdeen.

Results

The result of the test is shown in Table 39 . Of 27 samples 8 gave a positive reaction with Babesia divergens antigen. No fluorescence was detected with normal deer serum in dilution of 1/10.

Table 39

IFA titre of deer sera with B. divergens antigen

Antiserum	Antigen
1	1/320
2	1/160
3	1/40
4	1/80
5	1/40
6	1/40
7 (filter paper)	+*
8 (filter paper)	++

Samples 1-7 from Inverness-shire, 8 from Island of Arran

* = degree of fluorescence

CHAPTER V

DISCUSSION

A. Trypanosomes

Standardization of IFA test

It is already well known that the fluorescent antibody technique can be used in the diagnosis and epidemiology of protozoal infections. When the present work was started the ultimate aim was to apply the IFA test to obtain information about the distribution of protozoal infections in domestic animals in my home country, Iraq. In Iraq, the pathogenic trypanosome *T. evansi* is endemic.

Although the IFA test has been much used for the diagnosis of trypanosomiasis (Sadun *et al*, 1963; Camargo, 1966; Bailey *et al*, 1967; Wery *et al*, 1970a, 1970b), the technique of performing the test had varied, and some of the results were conflicting.

A technique with such wide and important application should be standardized. In the first part of this work, the previously described methods were compared and a modified technique was developed. The advantages of the method adopted are many. Washing the trypanosomes, removes the soluble antigen or antibody that was present in infected blood and this will eliminate non-specific fluorescence and give a very good background. Trypanosomes washed in normal sera adhered more firmly to the slide than when washed in PBS. Another advantage of the method is that the high concentration of trypanosomes in a scribed

circles facilitates the reading of the test, because with prolonged exposure to UV light the trypanosomes will fade and this will interfere with the test.

Fixation of trypanosomes was carried out with different fixatives. 5% formalin in distilled water gave a good result. The antigen had not deteriorated after storage for 3 weeks at room temperature and after 18 months at -20°C. Other fixatives e.g. Hcl, methanol and acetone were tried but the results, as shown in Table 2 showed that the fluorescence was weak and the titre of the test serum was low; this may be due to the effect of these fixatives on the antigenic properties of the organisms. Fixation with heat is not reliable since most of the trypanosomes were washed off after immersing the slide in PBS; this might give a false negative result due to the absence of the organisms.

Separation of T. congolense from the blood is known to be difficult. Soltys (1957) used Alserver's solution containing 10% horse serum, Wilson (1969) used a gentle centrifugation, but in both methods a considerable proportion of the trypanosomes was lost. By mixing infected mouse blood with normal bovine serum in the proportion 1/5, the majority of trypanosomes were separated from agglutinated erythrocytes after 15 minutes.

Dilution of the conjugated antiserum depends exclusively on the nature of the prepared conjugate, in other words, on the ratio of fluorescence molecules to protein e.g. Wery et al (1970a) used two preparations of fluorescein labelled anti-human sera,

one from the Institute Pasteur of Paris at 1/100 dilution and the other from Nordic Pharmaceuticals at 1/15 dilution and both of them gave a satisfactory result.

Another factor concerning the technique is the reaction time; it was varied between 10-45 minutes and the best results were obtained with 40 minutes. Sufficient time must be allowed for the antibodies to bind to the antigens. For removal of unbound antibodies, most of the workers agree that washing for 10 minutes in PBS with two changes is suitable.

End point of titration

Prepared slides were examined under the fluorescent microscope. The intensity of the fluorescence has been classified by most people in 4 or 5 degrees, but the selection of an intensity for the end point of a titration, or for a positive result has varied. For the purpose of diagnosis, Lucasse (1970); Wilson and Cunningham (1971), defined a positive serum as one which gives two Plus (++) or higher; Wery et al (1970a) regard three plus (+++) or four plus as positive. By standardization of the technique, it was found that one plus (+) was the most satisfactory end point of a titration.

Storage of sera and blood on filter paper

It is already known that sera can be stored for long time at -20°C or with chemicals. Dried blood samples on filter papers were used recently in the fluorescent technique and it was found that the globulins retain their original activity for up to 30

days at room temperature (Souzo & Camargo, 1966) and 2 months at -20°C (Bailey et al, 1966, 1967). After the IFA test had been modified it was necessary to confirm that it could be used with sera or with dried blood. There was close agreement in the degree of fluorescence between sera and filter paper blood. Dried blood samples stored at 36°C or room temperature (18-20°C) had not lost their activity after 2-3 weeks, at 4°C after 3 weeks, at -20°C after 30 days. The duration of activity also depends on the potency of the serum. The results indicate that the technique described for the use of minute amounts of dried blood in the fluorescent antibody test is applicable to epidemiological investigations of trypanosomiasis. The degree of sensitivity and specificity observed was at least equivalent to the results obtained from the sera. It is also a relatively simple and satisfactory method of collecting, mailing and testing a large sample in a short time.

Measurement of antibody level by IFA test

Measurement of antibody level in any particular time may indicate the state of infection or immunity. It has been shown by using IFA test for diagnosis of bovine trypanosomiasis, that a high level of antibody indicates a current infection and a low level may indicate either a cured or new infection (Wiesenhutter, 1969; Wilson & Cunningham, 1971). The same pattern was demonstrated in laboratory animals. In 4 rabbits that were infected with T. brucei, the antibody titre remained high so

long as the trypanosomes were present in the blood. The titre started to drop after treatment with Berenil; it reached 1/20 - 1/40 after 6 months but was still higher than in an uninfected animal. The persistance of a low level of antibody after apparent elimination of the organism was shown by Ross and Lohr for Babesia (1968) and Wery *et al* for human sleeping sickness (1970b). In the latter study, antibody was still detected in some patients, 2 years after treatment. Reinfection of the rabbits led to a sharp rise in the titre whether by a homologous or heterologous strain. The animal tolerated the second infection for a longer period when the challenge was with the homologous than with the heterologous strain. The same pattern was demonstrated in rabbits infected with *T. congolense* except that when rabbit F16 was challenged with the same strain, the antibody titre was increased, but no trypanosomes were detected either by wet film or mouse inoculation. This may be due to the fact that the rabbit was resistant to reinfection and the antibody titre might soon have decreased, or the trypanosomes were not present in the peripheral blood but in some other site in the body and so antibody production was stimulated. In serological surveys a low titre will indicate that the animals are newly infected or cured. To distinguish between these conditions, a second test should be made at least one week later, and if the titre is higher than in the first test, it indicates a new infection, if it is the same or less it

indicates a recovered infection. Measurement of antibody level can also be used to assess the success of chemotherapeutic treatment of trypanosomiasis. If the antibody remains at a high level after drug treatment (rabbit F11) this means that trypanosomes are still present which have not been effected by the drug, and may cause a severe reaction later. In cattle trypanosomiasis, it is also necessary to titrate the serum against at least 3 different antigens because a low titre to one species may be higher with another (Wilson, 1969; Wilson and Cunningham, 1971). In the laboratory tests, wet film examination and mouse inoculation were used to confirm that the organisms were eliminated coincident with the fall in antibody level.

Specificity of IFA test in differentiation of trypanosomes species

The standardized method was applied to differentiate between species of brucei-group trypanosomes. Man may be infected either by T. rhodesiense or T. gambiense in Africa. Also domestic animals may carry T. brucei, T. rhodesiense, T. congolense and T. vivax, and wild animals may act as a reservoir, for these trypanosomes. Differentiation between species of brucei-group is very important in epidemiological studies of trypanosomiasis; there may be carriers for the disease in an area but without clinical symptoms, which constitute foci of the infection. If the test is able to differentiate between T. brucei, T. rhodesiense and T. gambiense, the infected animal or the reservoir

can be diagnosed and treated. Sadun et al (1963) and Williams et al (1963) were not able to distinguish between T. rhodesiense, T. gambiense and T. cruzi by IFA test and they found there was also cross-reaction with T. lewisi. Wery et al (1970a), in a series of IFA tests, failed to distinguish between pleomorphic members of the brucei group. These workers used a direct blood smear for the antigen. Antibodies and soluble antigens in the blood will interfere with the test and affect its specificity. Also the duration of the infection in laboratory animals from which the antigen was prepared may also affect the specificity; antigen prepared from guinea pig infected for 80 days with T. gambiense gave a doubtful result (++) with control sera (Wery et al, 1970a). Wilson, Cunningham and Kimber (1967) were able to differentiate T. brucei, T. congolense and T. vivax by the IFA test. Their antigen was prepared from infected blood diluted with PBS hence the non-specific reaction was reduced. These authors, however did not attempt to distinguish between members of the brucei-group. The improvement in the method described in experiment No. 1, is that by washing the trypanosomes in PBS or normal sera they are freed from antibodies or soluble antigens and the non-specific reaction is eliminated. The increased specificity of the test made it possible to differentiate brucei-group trypanosomes. In 368 tests, there was at least a four-fold difference between the titres of the homologous and any heterologous reaction.

The IFA test could be used in conjunction with, or possibly instead of, the blood incubation infectivity test (BIIT, Rickman and Robson, 1970) to distinguish T. brucei from T. rhodesiense. Antigen prepared from a new isolate should be tested against three different T. rhodesiense and three different T. brucei sera and the means of the titrations compared. The present test could be usefully employed in West Africa to search for reservoir hosts of T. gambiense. In endemic areas of Gambian sleeping sickness, sera from these animals could be collected and tested against T. gambiense and T. brucei antigens. A high titre to T. gambiense and a lower titre to T. brucei would indicate that the animal was parasitized by T. gambiense. A systematic search for reservoir hosts could help in the eradication of the disease.

Cattle may be infected with T. theileri; a species which is non-pathogenic and produces a low level of antibodies (Trautmann, 1922; Leach, 1964). It is necessary to differentiate serologically between this species and other bovine pathogenic trypanosomes, especially in an area where T. theileri is known to exist.

Wilson (1968) considered that T. theileri infection of cattle did not interfere with the IFA test, provided that the base line was taken at a dilution of 1/40. In this study, by using sera from cattle known to be infected with T. theileri there was a cross reaction with other species of trypanosomes, but the

titre was never higher than 1/40. These results confirm Wilson's observations, and in the diagnosis of bovine trypanosomiasis only if fluorescence occurs at a dilution higher than 1/40 should the serum be considered as positive. The titres of anti theileri sera were not high whether reacted with forms from leucocyte cultures or with blood agar forms and this was probably due to the nature of the antigen. The blood forms of T. theileri cannot be obtained so culture forms had to be utilised as antigen, and these are likely to differ antigenically from the blood forms.

The nature of trypanosomal antibodies

An attempt was made to determine the nature of antibodies that are involved in the agglutination and fluorescent tests. Antigenic variation in salivarian trypanosomes has been known for a long time (Franke, 1905; Lourie and O'Connor, 1937). Most of the work on antigenic variation has been carried out with brucei-group trypanosomes due to their ability to produce heavy infections in laboratory animals. Different serological methods have been used to detect these variations and it appears that each relapse population of trypanosomes in the blood has a different antigenic character from its predecessor. Agglutination tests were used by Soltys, 1957; Cunningham and Vickerman, 1962; Gray, 1962; McNeillage et al, 1969. It has been suggested that the variant antigens are located in the surface coat of which is present in blood stream forms of trypanosomes

(Vickerman, 1969; Vickerman and Luckins, 1969). Weitz (1960a, 1960b) recognized two antigenic components of trypanosomes, one called soluble or exoantigens, which are located on the surface and released into the serum during the course of an infection, and the second common or bound antigens. These include the components within the cell and they are liberated when the cell was disintegrated. Work of Weitz (1963) and Miller (1965) indicate that the agglutination reaction is at least in part a manifestation of antibodies reacting with the exoantigens of trypanosomes and that antigenic variation largely concerns the exoantigens. These exoantigens may be equivalent to the filopodia as described by Wright and Lumsden, 1969; Macadam and Herbert, 1970; Wright et al, 1970.

Recently the IFA and agglutination tests were compared for the diagnosis of cattle trypanosomiasis (Wilson, 1969). Fewer serological positives were detected by the agglutination than by the IFA test, because the IFA test does not detect different antigenic variants. In this study a comparison between fluorescent and agglutination tests indicated that the agglutination test is variant specific so that antisera prepared in rabbits against T. brucei, agglutinated 0-day antigen, but not, or at very low titre, antigens collected at the same time as, or later than the serum. Sera at least six days older than the antigen will agglutinate that antigen and this may be explained by the development of new antigenic type

every 5 or 6 days. Wilson (1968) showed that the neutralization test detects antigenic variants of T. congolense. In this study the neutralization and IFA tests were compared with T. congolense. Serum of 15 days neutralized antigen of O-day but not of 35 days. Whereas the IFA titre was unchanged when reacted with both antigens. Hence variant specificity is not detected by the fluorescent test in which there is no difference in the brightness or titre of the serum when it is tested against different the antigenic variants of a strain. This result does not agree with that of Weitz (1963). Consequently, it is evident that the fluorescent test is useful for diagnosis of trypanosomes in the field because it is not influenced by antigenic variations that take place through the course of infection.

If the fluorescent test does not detect that variant antigens, what would be the effect on the test, if the antibodies for one antigenic type were absorbed from the serum. In this work, it was shown that when all the agglutinins to one variant were absorbed from an antiserum, there was no effect on the agglutinability of other variants, however the fluorescent titre was reduced with both variants. In other words by absorbing serum with live trypanosomes, antibodies were removed which do react in IFA test and which must represent antigen common to the variants.

Agglutinating antibodies are thought to be in IgM immunoglobulin. An antiserum was fractionated into its components

and the fractions were tested for agglutinating and fluorescent properties. The agglutinating titre was the same in the IgM fraction as in the whole serum. The IgG fraction also had some degree of agglutinability. The same results were obtained by Seed (1969, 1972). The fluorescent reactions were limited to IgG fraction and fluorescence was negligeable in IgM fraction. This shows that the IgM does not react in the IFA test. As well as variant specific antigens on the surface, which react with IgM, there must be another antigen on the surface, which induces the formation of antibodies in the IgG fraction. By separating IgM from the serum, the agglutinins of the IgG became evident; perhaps these antibodies are non-specific. The specificity of IgG agglutinins could be tested by reacting the IgG fraction with different antigenic variants of a species and with different species, and by absorption tests.

Further evidence for the presence of two types of surface antigen comes from the experiments on washing the trypanosomes. When trypanosomes were washed for 40 minutes in saline, the agglutinogens were lost, since no agglutination occurred when they were mixed with specific antiserum. Washing also reduced the IFA titre of the serum. Vickerman (1969) found that washed trypanosomes lost the surface coat. So that antigen which reacts in IFA test was lost from the surface as well as the agglutinogens. From these points we can conclude that the fluorescent antibody reacts with two types of antigens -

1. antigens on the surface of the trypanosomes,
2. bound or internal antigens.

By applying the IFA test to different species of trypanosomes, a cross-reaction between species was demonstrated. This is in agreement with other studies which show that there are antibodies common to the different species (Gray, 1960; Weitz, 1960; Cunningham and van Hoeve, 1964; Wilson, 1968). However, the titre of the sera in IFA tests was higher with the homologous than with the heterologous species, thus indicating that there are species specific antigens which can be detected by the IFA test. Whether these antigens on the surface or inside the cell have not been determined.

Fluorescent antibody test has been used to detect variant specific antigens. This was done by conjugating (FITC) with antisera to individual exoantigens. The results then paralleled those of agglutination tests (Weitz, 1963; Takayanagi et al, 1970).

Antigenic properties of culture and fly forms of T. brucei and T. congolense were studied by fluorescent and agglutination tests. It has been shown that the surface coat contains variant specific antigens. Trypanosomes when transferred from blood to culture or when ingested by the tsetse fly, lose their surface coat (Vickerman, 1969, 1971). Seed (1963)/1964 showed that blood forms of all strains of T. rhodesiense failed to be agglutinated by rabbit-anticulture trypanosome serum and also that antiserum to blood forms failed to agglutinate culture forms. Seed prepared

antisera by injecting rabbit with lyophilized antigen and this may change the antigenic properties of trypanosomes. However, Brown and Vickerman (1970) reported that cultured forms of T. brucei were agglutinated at a high titre by antiserum to the blood forms of the same strain. In this study antisera were prepared against blood forms by injecting rabbits with live trypanosomes, when these antisera were tested against fly and culture forms, the fluorescent and agglutinating titres of both showed a gradual decline with age. The trypanosomes from three days culture showed variant specificity since the titre was at least 1/1000 with the homologous antisera, and 1/100 with heterologous antisera. This specificity was lost later, when it was found that 8 days culture forms reacted at the same low titre with both homologous and heterologous antisera. Further, species specificity was lost in 8 day cultures since antiserum to T. brucei agglutinated culture forms of T. brucei and T. congolense at a titre of 1/100 . This indicates that there are non-specific antigens located on the surface, which are shared by culture and blood forms of different species. It is possibly that the agglutinins in IgG fraction are common between trypanosomes species. Seed (1963)/1964 failed to detect these common agglutinins because he used lyophilized antigen to prepare antisera, whereas the antibodies in this work were induced by living trypanosomes. The fact that culture forms of T. brucei retained their infectivity to mice up to 4 days and that these

retained some variant specific antigens, indicates that the change from blood to culture forms is a gradual one.

B. Babesia

Cattle babesiasis in the United Kingdom was first diagnosed by Stockman and his colleagues more than 50 years ago (Stockman, 1908; M'Fadyean and Stockman, 1911; Stockman and Wragg, 1914).

In 1911 M'Fadyean and Stockman reported the existance of a new species of piroplasm in the U.K., which they called Piroplasma divergens. This parasite is found more frequently in northern Europe and in the past has been confused with B. bovis. Both Wenyon (1926) and Neitz (1956) considered B. divergens to be a synonym of B. bovis, but Davies, Joyner and Kendall (1958) by their studies on B. divergens, which they isolated from a field case of babesiasis, found it differed morphologically from B. bovis.

B. divergens appear as paired, pyriform or club-shaped organisms measuring about 1.5 μ by 0.4 μ and are found most frequently toward the periphery of the erythrocyte (Fig. 20A). The angle between the paired parasites is relatively large and usually more divergent than in B. bovis. Spherical and vacuolated "signet-ring" forms up to 2 μ in diameter are also present in infected corpuscles.

A second species occurring in Britain is B. major; it was recognized recently by Brocklesby and Irvin (1969); Brocklesby and Barnett (1970) and Barnett and Brocklesby (1971) in British cattle. In general, in the erythrocytes it resembles B. bigemina but is smaller measuring about 2.6 μ by 1.5 μ. The

paired pyriform parasites are somewhat elongated, meet at an acute angle, and tend to be located toward the middle of the corpuscle. Spherical forms are usually less than 2 μ in diameter. The vectors of these two species are, in the case of B. divergens, Ixodes ricinus, and of B. major, Haemaphysalis punctata (Arthur, 1961). This was confirmed by Bool et al (1961) and Wilson (1964) in the Netherlands. Joyner, Davies and Kendall (1963) succeeded in transmitting B. divergens cyclically through the larval and nymphal stages of I. ricinus. Recently, Brocklesby and Barnett (1970) transmitted B. major to splenectomized claves by H. punctata that they had collected from the field.

Animals which are infected with Babesia spp. may show clinical symptoms which are characterized by rise of temperature and anaemia which is accompanied by haemoglobinuria. The incubation period varies between 4-10 days depending on the species. The acute stage of the disease is followed by a latent stage which is characterized by the persistence of a small number of parasites in the blood. Rosenbusch and Gonzales (1925) considered that the duration of latent infection in cattle not exposed to reinfection was sometimes as short as 5 months, but more usually between 10 and 12 months. They did not differentiate between B. argentina and B. bigemina in their studies.

Abramov (1940) found that infections with B. bigemina persisted for 15 months. Seddon (1952) stated that the carrier state in cattle is of variable duration, but it does not appear to exceed a year. Neitz (1956) reported that latent infections lasting as long as 12 years have been demonstrated in cattle after recovery from babesiasis due to B. bigemina. Studies by Mahoney (1962) have shown that, although a tick transmitted B. argentina infection may be lost within 66 days, it usually persists for at least 10 months; infections with B. bigemina may persist for at least 9-10 months. Callow (1964, 1967, 1968) showed that cattle became free from infection with B. bigemina after 9-11 weeks and after 21 months with B. argentina. Johnston and Tammemagi (1969), in their work on B. argentina in Australia, showed that cattle inoculated with B. argentina and kept free of ticks remained infected for 13 months. Davies et al (1958) recorded that cattle had lost their infection with B. divergens after 18 months; Joyner et al (1967) failed to detect the parasite after 3-7 years in infected animals, by subinoculation into susceptible splenectomized calves.

It is evident, then, that it is uncertain how long cattle retain the parasites after an infection with Babesia spp.

Animals infected with Babesia will develop a resistance against reinfection. This immunity usually persists only while the animal continues to carry the infecting organism, but there is evidence that in some cattle recovery with elimination of

organism provides a sterile immunity. Legg (1931) observed that cattle lost their protection against babesiasis when kept free of ticks for about 1 year.

Sergent et al (1945) found that immunity may be lost in about 5 weeks after the elimination of sources of infection with B. berbera, but with B. bigemina sometimes not before 14-22 months. Arnold (1948) recorded that immunity to piroplasmosis appears to fade in 3-4 years in the absence of reinfection. Hall (1960) stated that immunity may be lost in a few months after the clinical recovery of cattle infected with B. argentina and not subjected to reinfection. Mahoney (1962) showed that cattle in a tick infested area had almost continuous parasitaemia with Babesia for 2-3 years, and he stated that clinical babesiasis was not observed. This is in contrast to other areas in which there was a much lower incidence of parasitaemia with Babesia (which dropped almost to zero), but here there were clinical cases. These results agree with those obtained by Johnston (1967). Davies et al (1958) showed that two heifers which had lost their infection with B. divergens remained resistant to the effects of reinfection; this was confirmed again by Joyner et al (1967). The duration of this immunity is not well known and the only methods that can be used to test the state of the host is to challenge the animal and then to inoculate the blood of challenged animal into a splenectomized one. This method can not be applied in the field and at the

same time it will be very expensive. Different serological methods have been used in babesiasis and a recent one is the fluorescent test.

The IFA test is considered to be sufficiently sensitive and accurate to detect reactors which were infected with B. bigemina 2 years previously (Ross and Lohr, 1968). The test can also be used to differentiate between the species of Babesia, Madden et al (1968); Zwart et al (1968); Ludford (1969); Cox and Turner (1970); Brocklesby et al (1971). Techniques of applying the IFA test have varied but the most suitable is to use a thick smear of washed red cells for the antigen. This method has a number of advantages, e.g. washing the parasitized red cells removes the soluble serum components (soluble antigens) and also the donor's gamma globulin, which reduces non specific staining (Sulzer and Wilson, 1967; Ross and Lohr, 1968; Sulzer et al, 1969) and so increases the sensitivity and specificity of the test. The other advantage of using a thick smear, is that the searching for parasitized cells is not required. Smears are made in scribed circles which facilitates locating the parasites and reading of the test. Prolonged exposure to UV light reduces fluorescence and so may influence the interpretation of the test.

When the IFA test was applied to sera from cattle on Arran, in 63 out of 244 samples (28%) babesial antibody was detected. Positive reactors were found on all farms except the two with

dairy cattle only. Although the dairy cattle are born and reared on the island, they are never put out to graze on the hill and thus have little contact with ticks. The infection, therefore, only affected the beef cattle which are grazing most of the time on the hill and are in contact with ticks. The view is commonly held that unless cattle are infected with Babesia before they are one year old, the effects of infection are likely to be severe and so redwater would be evident. This view does not seem to accord with the findings on Arran where an average of 28% of the beef cattle were infected or had been infected with Babesia, yet only one clinical case had been seen in recent years. It may be that the strain of B. divergens in this locality is of low pathogenicity or there is a declining prevalence of the infection. An argument against the first reason is that some cattle had been imported about six years previously, shortly after their introduction some of the animals had died of redwater. It is not known whether these cattle got the infection in Arran or whether they were premune and redwater became manifest on Arran because of stress of travelling. This fact is very well known that there is a risk in moving cattle from one area to another and failing to take the appropriate simple precautions; Johnston (1968) reported an outbreak of babesiasis in Queensland, in cattle travelling from tick-free into tick-infested areas. Donnelly et al (1970) reported an outbreak of redwater on a farm in Sussex, in

which the farmer purchased cattle, which spent a few days in the farm. Next summer there was an outbreak of redwater in cattle on his farm which had no previous history of redwater or contact with ticks. Breed susceptibility may be concerned but to a limited extent, according to Daly and Hall (1955); Johnston (1967) showed that there is no difference in susceptibility of different breeds of cattle to B. bigemina infection but there is with B. argentina. For the second possibility, there are many factors involved (a) the immune response in adult cattle is strong enough to destroy parasites more rapidly than they are able to increase through superinfection.

Infected tick feeding on immunized animal ——→ non infected progeny of ticks

(b) annual selling of the calves decreases the percentage of animals susceptible to the infection.

Infection of cattle by species from deer

Babesia may not be highly host specific; Clark (1918) observed Babesia in the brain of deer and he believed that it was B. bigemina. Skrabolo and Deanovic (1957) reported a fatal infection with B. bovis, in a splenectomized man. Garnham and Bray (1959); Garnham and Voller (1965) produced infection of splenectomized rhesus monkeys with B. divergens. Enigk and Friedhoff (1962); Enigk et al (1964) were able to infect splenectomized red deer with B. divergens and B. motasi. Callow (1965) has shown that when ticks infected with B. bigemina

were allowed to feed on sheep, viable parasites appeared in the blood. When blood was passaged from the sheep into susceptible calves, a patent infection with B. bigemina developed. Ristic et al (1971) reported a case of Babesia in an intact woman.

In Scotland, many tick infested pastures are shared by cattle, sheep and red deer. Ixodes ricinus is known to feed on deer, sheep and on cattle, and thus there is a possibility of transient infections in cattle by the species of the deer. Also the low titre of the sera of the Arran cattle to B. divergens antigen may support that possibility. Cross-reaction between different species of Babesia can be detected by fluorescent test.

Zwart et al (1968); Brocklesby et al (1971) by using the IFA test showed that antisera of B. major gives a titre range between 1/40 - 1/160 with B. bigemina antigen. Also by applying the test to differentiate between trypanosomes species (Exp. No. 3) some sera give a titre up to 1/640 with heterologous species. Frerichs and Johnson (1969) showed that sera of splenectomized calves gave a titre of 1:320 in complement fixation test against B. equi antigen 6-9 days after they had been inoculated with that species. The possibility of infecting cattle with deer species may protect them and give them a certain immunity against the bovine species in Arran. Stockman and Wragg (1914) found there is cross immunization between B. bigemina and B. divergens, Legg (1935) found that cattle recovered from an infection with B. bigemina offer considerable resistance to

infection with B. argentina, Zwart et al (1968) showed that animals infected with B. major and then challenged with B. bigemina, will develop a parasitaemia but without any clinical symptoms. Cox and Milar (1968) observed that there is a cross-protection between Plasmodium and Babesia infections of rats and mice.

That cattle in Arran may be infected with B. divergens and developing a parasitaemia but without showing any clinical symptoms could be due to partial protection because of infection with deer species.

Regarding the 55 calves sampled on Arran, none of them had babesial antibody. It is known that infection of young non-splenectomized calves in an enzootic area is generally considered to be symtomless and to be associated with an extremely low parasite density (Smith and Kilborne, 1893). Mahoney (1962), using a stained thick blood film to identify infection showed that in the endemic area of B. bigemina and B. argentina, 45% of the calves, one to six months of age, can be shown to be infected, but they do not have any obvious clinical signs. The 15 calves sampled at Dougarie (No. 2) had been born and reared on the farm that season, contrary to the usual practice, so they would have no opportunity to be in contact with ticks on the hill. Two calves 3-7 days old were too young to have become infected and form antibody. However, most of the remaining 38 would have been exposed to ticks since March and would have had time to produce antibody if they had been infected. If those calves were born from immunized cows, they

will get the immunity through the colostrum (Hall, 1960, 1963, 1968). It is not known if such immunity can be detected by serological methods. Those calves, if they are not exposed to infected ticks to build up an acquired immunity, the natural resistance soon disappears (Riek, 1968).

It is possible that those calves may get the infection later with autumn population of ticks. Sporadic cases would then appear and could appear in the future in animals that remained in the island and had not been in contact with infected ticks when they are young. At any particular time, the cattle population in Arran may be exposed to infected ticks, of these cattle, a proportion will subsequently develop a new parasitaemia (possibly with clinical symptoms). Some of the new infections will fall on immunized cattle, and so will have no effect on the parasite rate, and some will fall on cattle already showing parasitaemia, likewise having no effect on parasite rate. Infected animals may at times show clinical symptoms, if for example, the immune response is depressed by intercurrent infection or pregnancy.

Although the number of samples were obtained from Arran is relatively small, the results so far provide a guide for future work. An indication of the transmission rate could best be obtained by sampling the one and two year old cattle. This is because once an animal is infected, the parasite may persist in the host for several years and hence the antibody would also

persist. It would also be of interest to follow the antibody level in the same animals from year to year. It would seem that in other parts of the West of Scotland the situation is similar to that in Arran. It would therefore be interesting to do a survey in other areas.

The same tick, Ixodes ricinus must be the vector of the Babesia of cattle and of deer. It would be necessary to detect and isolate the Babesia from deer and test its host specificity.

ACKNOWLEDGEMENTS

I would like to express my deepest thanks for Mrs. K.M.G. Adam for her keen supervision and valuable guidance. My thanks also to Dr. E.A. Wells for his supervision and co-operation during the work on babesiasis in the Island of Arran. I am grateful to Dr. J.A. Campbell for useful discussions, especially on ticks, and providing the departmental facilities. The advice and help of Mrs. J. Riddaway in the immunological aspect of this work is greatly appreciated. I also thank Mrs. M.D. Philp, the departmental librarian, for her efficiency and willingness to help, and Mr. D.F. Cremer and Miss C. McKenzie for photography work. I thank all the members of the subdepartment of Parasitology and the technical staff of the animal house for their friendliness, co-operation and help. I thank my family for their general support; their constant communications have always kept me happy. I am grateful to Mrs. S. Polson for her efficiency in typing this manuscript. Finally, I would like to thank the Iraqi Government for providing the opportunity to undertake this work and the Calouste Gulbenkian Foundation for their financial support.

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APPENDIX

Chemicals

a) Phosphate-buffered saline (PBS) at pH 7.2. It is 0.15M Sodium phosphate in physiological saline, consists of:

Solution A

23.4 g $NaH_2PO_4.2H_2O$

8.5 g NaCl

1000 ml distilled water

Solution B

21.3 g Na_2HPO_4

8.5 g NaCl

1000 ml distilled water

They are kept in 4°C. To use one part of solution A + three parts of B.

pH is checked by pH meter W.G. Pye & Co., Ltd., Cambridge.

b) Phsophate-saline-glucose buffer (PSG), pH 8.0. I. 0.22 consisted of:

285 ml of 0.2M Na_2HPO_4

15 ml of 0.2M NaH_2PO_4

300 ml of 0.85 per cent (W/V) NaCl

400 ml of 2.5 per cent (W/V) glucose

c) Citrated saline

0.5 g Sod. citrate

0.9 g Sod. chloride

100 ml distilled water

d) Physiological saline

0.9 g NaCl

100 ml distilled water

e) Tris-HCl pH 8.0

12.11 gm Tris

58.5 gm Nacl

1000 ml distilled water

pH adjacted with 1N HCl

f) Hypo solution

200 g Hypo

12.5 g Pot. metabisulphite

made up to one litre with water (little warm)

g) Potassium dichromate-sulfuric acid solution

100 g Pot. dichromate

200 ml water

2500 ml sulfuric acid

h) Mountant for fluorescent microscopy

90 ml glycerol

10 ml carbonate-bicarbonate

(3.7 g Sod. hydrogen carbonate (NaHCO3)
(
(0.6 g disad. carbonate (Na_2CO_3) anhydrous
(
(up to 100 ml distilled water

i) Giemsa stain

1 ml giemsa (Gurrs improved R66)

9 ml buffered distilled water pH 7.2

(Buffer tablets, G.T. Gurr 7.2 from Hopkin & Williams Ltd., England)

j) Vernnal buffer

4.76 gm Na. barbitone

made up with 4 litres of distilled water

Add 50 ml N/10 HCl. Check pH, adjust to 8.2 with HCl if necessary. When pH is correct make up to 4.3 litres with H_2O.

k) Ponceau Red in acetate buffer

1.0 gm Ponceau Red

500 ml acetic acid 1M

500 ml Na. acetate 0.1M

Trypanosoma theileri media

a) Solution A (diluent)

Constituent	gm./litre	relative volumes
NaCl	9.00	100
KCl	11.48	4
$MgCl_2.6H_2O$	22.36	3
$CaCl_2.6H_2O$	24.10	1

The mixture was buffered to approximately the same pH as the blood agar medium employed (7.4) using 1.36 volumes of $NaH_2PO_4.2H_2O$ (24.03 gm./litre) to 8.64 volumes of $Na_2HPO_4.2H_2O$ (18.33 gm/litre). One ml. of buffer was added to each 10 ml of solution A, the resulting mixture being termed "Solution ABP 7.4".

b) Blood agar medium.

i. blood agar base No. 2 (Oxoid, London): 32.0 gm/litre medium

ii. dextrose (AnalaR; British Drug Houses, Poole, England): 1.6 gm/litre medium.

iii. double glass distilled water: 800 ml/litre medium

iv. sheep blood, defibrinated: 200 ml/litre medium (standard donor).

ABSTRACT OF THESIS

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Degree Ph.D *Date* 8th September 1972

Title of Thesis The immunofluorescent reaction for the diagnosis of protozoal infections in man and animals.

The study is mainly concerned with the fluorescent antibody technique and its use in the diagnosis of trypanosomiasis and babesiasis.

The work on trypanosomes includes standardization of the indirect fluorescent antibody (IFA) test. Different methods of preparing, fixing and storing the antigen were compared. The test was also applied to differentiate between species of trypanosomes. A comparison was made of the IFA and other serological tests for the detection of antigenic variants. The level of antibody in rabbits was measured before and after treatment, to follow the rate of its disappearance. This information is required for the interpretation of field data or to assess the effectiveness of chemotherapy.

Some studies were also carried out on the antigenic properties of blood, fly and culture forms of Trypanosoma brucei and T. congolense. An attempt was made to identify the antibodies that were involved in the agglutination and fluorescent reactions by fractionation and absorption of antisera.

In the second part of the work, the IFA test was applied to study the epizootiology of redwater (babesiasis) in cattle on the island of Arran. Arran was chosen for this survey since redwater was known to occur there and the movement of cattle is only from the island to the mainland. Sixty-three out of 244 sera gave a positive reaction in the IFA test, although the titre was low. This finding indicated a higher infection rate in the cattle than expected, owing to the small number of clinical cases.

Since there are indications that Babesia may not be rigidly host specific, samples of sera from red deer on Arran and on the mainland were tested for babesial antibody. This is the first record of babesial infection of red deer in Britain.

Use other side if necessary.