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Immunological responses of Gallus domesticus to infection with Trypanosoma brucei

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A C K N O W L E D G E M E N T S

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CONTENTS

The following scientific publications have been made from the work reported in this thesis.

1. Joshua, R.A.; Herbert, W.J. and White, R.G. (1978) Acquisition by Trypanosoma brucei brucei of potential infectivity for man by passage through birds. The LANCET, 1, pp 724.

2. Joshua, R.A.; White, R.G. and Herbert, W.J. (1978) Trypanosoma brucei infections in domestic chickens. Transactions of the Royal Society of Tropical Medicine and Hygiene, 72, pp 642. (Abstract)

3. Joshua, R.A.; Herbert, W.J. and White, R.G. (1979) Diagnosis of African Trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene. (In Press). (Letter)

PAPERS READ AT CONFERENCES.

4. Joshua, R.A.; Herbert, W.J. and White, R.G. Experimental T. brucei infections in domestic chickens (Gallus domesticus) Association of Veterinary Teachers and Research workers conference, Scarborough, April, 1979

5. White, R.G.; Joshua, R.A. and Herbert, W.J. B-cell segregation into germinal centres during chronic trypanosomiasis in domestic chickens. Spring meeting; The British Society of Immunology, London, April, 1979

S U M M A R Y

Birds have long been reported to be among the possible hosts of T. brucei (Durham, 1908); however, the use of birds for the elucidation of the immune response to trypanosome infection has had relatively little investigation. This thesis has examined the nature of T. brucei brucei infections in birds as well as the significance of the parasite in the development of the very numerous germinal centres which appear in the spleen during the course of a chronic infection.

Three lines of salivarian trypanosomes were tested for infectivity to birds; only organisms derived from the primary isolation Lugala/55/EATRO/459 were found to produce an infection. This persisted for over one year. A comparison of current diagnostic methods showed that mouse inoculation, DEAE column chromatography and development in the serum of heterophile agglutinins (against rabbit, guinea-pig and rat erythrocytes) were of value for detection of infection in chickens. Parasitaemia was maintained at a low level ($<10^{5.4}$ per ml. of blood) in all of the infected birds; infectivity titration of chicken blood in mice revealed the presence of 3-100 viable organisms per ml. of blood. Quantitative estimation of the number of organisms required to infect chickens showed that trypanosomiasis can follow the inoculation /

inoculation of one hundred mouse infective doses (ID_{63}); the intravenous route was the most efficient route for initiating infection.

Advances and remissions of parasitaemia were detected during the course of the infection in chickens and seven variable antigen types were isolated, thus suggesting that, as in mammals, T. brucei undergoes antigenic variation during a chronic infection.

Starting with a well authenticated stock of T. brucei brucei and after a long passage in chickens a clone of trypanosomes which was resistant to normal human serum and therefore of potential infectivity for man was isolated. This observation implies that T.b. brucei which was devoid of pathogenicity for man could transform by passage in the bird to acquire the ability to infect man.

The infection persisted much longer in cockerels than in pullets. Chronic trypanosomiasis produced no obvious impairment of health in the birds; their growth rate was indistinguishable from normal, but neonatally infected pullets laid fewer eggs than controls.

Histological examination showed an increase of over ten fold in the number of germinal centres in the spleen. Hypotheses are discussed which might account for this increase. It is argued that the development of such a large /

large number of germinal centres reflects the bird's response to the elaboration of a succession of trypanosome antigens.

Injection of an unrelated antigen (Human serum albumin) into infected birds led to the formation of a limited number of germinal centres each containing H.S.A. bearing dendritic cells. The results of these experiments imply that germinal centres of trypanosome infected birds were specific for trypanosome antigen and attempts were made to demonstrate this by the fluorescent antibody technique.

The kinetics of formation of germinal centres during trypanosome infection and the kinetics of their decrease after treatment with a trypanocide were explored.

It was observed that chickens which spontaneously cured themselves of their infection with trypanosomes showed immunity against challenge with variants from the same stock. This demonstration of specifically acquired immunity implies that the use of trypanosome vaccines could lead to the successful induction of immunity.

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I N T R O D U C T I O N

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THE TRYPANOSOMES

Trypanosomes are Protozoan parasites of the blood and sometimes other tissues of animals and some plants. These parasites have been found in every class of vertebrates and usually appear to be innocuous. A few of them, however, produce serious disease (trypanosomiasis) in man and domestic animals and hence the parasites have attained considerable importance and have been the subject of numerous investigations. According to Wenyon (1926) trypanosomes were first observed by Valentin of Berne in the blood of Salmo fario (a small fish popularly called the Trout) in 1841.

Definite knowledge of trypanosomiasis commenced in 1880 when it was first demonstrated that trypanosomes could survive in domesticated animals and cause disease. Griffith Evans, a veterinarian, discovered in 1880 that the disease of horses and camels known in India as 'Surra' was associated with a blood parasite, afterwards given the name Trypanosoma evansi. Steel (1890, quoted by Hornby, 1921) first demonstrated that, when inoculated into an ox, the trypanosomes of surra induce an inflammatory reaction like those of the true equine disease.

Later, Bruce in 1894 (quoted by Wenyon, 1926) discovered that the cause of the tsetse fly disease which at that time was depleting the herds of the Zulus in East Africa was a trypanosome. He sent the first stock of /

TABLE 1. Number of Recorded new cases of human Trypanosomiasis, Africa, 1968-74.

Country	Total Population (in millions)	NUMBER OF NEW CASES 1968	1969	1970	1971	1972	1973	1974
Western Africa								
Dahomey	3	17	18	11	22		157	410
Ghana	10	181	160	166	135		156	69
Guinea	3.7	379	351	357	425	38	55	31
Guinea Bissau	0.5						46	11
Ivory Coast	5	376	176	148		144	136	87
Mali	5.5	356	221	231	190	193	388	188
Niger	4.4	9	0	4	0	1	0	0
Nigeria	79	944	599	393	529	498	331	132
Senegal	4.2	43	16	16		17	10	2
Togo	2.1	36	84	14	57	23	25	21
Upper Volta	5.8	164	262	145	114	69	75	78
Central Africa								
Cameroon	6.2	219	217	229	125	103	297	476
C.A.R.	1.7	19	22	63	175	412	91	79
Chad	4	61	50	47	18	17	15	54
Congo	1	134	73	59	172	49	42	91
Gabon	1	86	49	59	38	413	32	47
Zaire	25	3,357	4,957	5,124	4,126			
Eastern and Southern Africa								
Botswana	0.7	36	37	59	272			
Burundi	4	65	12	78	75	114		
Ethiopia	26	28	173	44	12	1		
Kenya	13	43	20	16	26	7	13	
Malawi	5	1	0	0	2	0		
Rwanda	4	21	20	69	35	13		
Sudan	15			8	2	22	125	287
Tanzania	15	403	530	564	569	612	477	488
Uganda	11	90	56	81	184	160	37	
Zambia	4.5	80	69	127	200	394	391	
TOTAL		7,148	8,172	9,140	8,501	7,126	2,899	2,551

From De Raadt, P, 1976.

of pathogenic trypanosomes to the United Kingdom. The parasite he sent was named Trypanosoma brucei. In 1905 Ziemann (quoted by Hornby, 1921) gave a description of yet another pathogenic trypanosome found, this time, in West Africa. The parasite was characterised by its rapid movement hence Ziemann named it Trypanosoma vivax.

Transmission of salivarian trypanosome infection is achieved either by the bite of infected tsetse flies, by mechanical passage of blood from one host to the other or by contact during coitus.

Bloodstream forms of mammalian trypanosomes are lanceolate in shape with the extremities tapering to a point. A single flagellum arises at the posterior of the body and runs forward along the outer margin of the undulating membrane. This membrane is a fold of the body pellicle.

Trypanosomiasis, the disease resulting from infection of man and his domestic animals by trypanosomes is of great economic and social importance in Africa and South America for it is responsible for numerous deaths and ill-health in both man and animals. The distribution of the disease changes from time to time as efforts are made to control it. The ancient endemic foci in many parts of Africa (Table 1) still remain despite all efforts to get rid of them (De Raadt, 1976). It is uneconomical to keep many breeds of domestic animals i.e. cattle, /

cattle, sheep, goats, horses and pigs in about four million square miles of territory south of the Sahara because of trypanosomiasis (Wilson, Morris, Lewis and Krog, 1963).

General Taxonomy of The Trypanosomes.

Organisms of the genus Trypanosoma can be broadly divided into two sections, the stercoraria and salivaria, which are based on the site of development of metacyclic trypanosomes (the forms of the organism which are infective to mammals) in the insect host and the method of infection of the susceptible host.

Stercoraria - Trypanosomes that develop in the hind-gut of the insect host. The metacyclics are present in the faeces and transmission occurs by contamination.

Salivaria - The developmental cycle is completed in the mouthparts or salivary glands of the insect so that the metacyclics are present in the saliva and transmission is inoculative.

Detailed taxonomic classifications of the salivarian trypanosomes can be found in Hoare (1972) and Baker (1977). A simplified schematic classification is presented in Fig. 1

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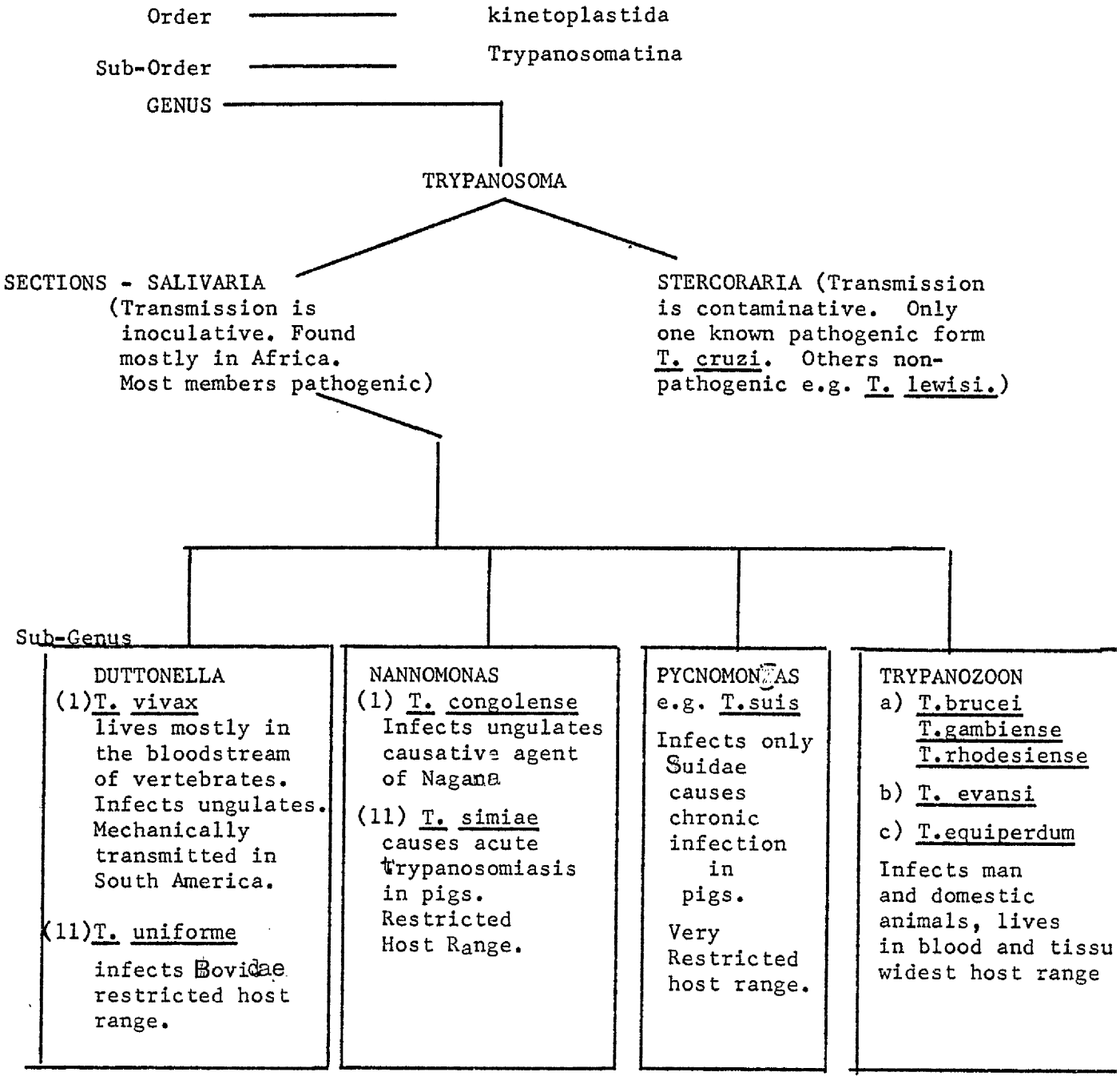
FIGURE 1. TAXONOMIC CLASSIFICATION OF TRYPANOSOMES.



The taxonomic structuring of trypanosomes is not without contradictions. The heterogeneity of the genus Trypanosoma with respect to cell morphology, life cycle, host specificity and antigenic composition defies any consistent logical taxonomic grouping of species. The taxomony of sub-genus Trypanozoon is discussed in detail later in this introduction.

Classification of trypanosomes on clinical evidence.

Trypanosomes may also be grouped into pathogenic and non-pathogenic forms according to their effect on the vertebrate host. Animals harbouring non-pathogenic trypanosomes e.g. T. lewisi, normally show no symptoms of disease, the host remaining a healthy carrier. The classification of trypanosomes into pathogenic and non-pathogenic forms is only germane for practical purposes in medical and veterinary practice. Such a classification does not reflect the true host-parasite relationships in trypanosomiases, since pathogenicity is not an immutable property of even the most virulent trypanosomes. For example, it is known that pathogenic trypanosomes may be parasitic in other animals in which they are apparently harmless, e.g. T. simiae produces an acutely virulent infection in pigs but is apparently harmless in sheep and virtually non-infective to cattle (Stephen, 1970). Also, T. brucei brucei is normally non-infective to man but /

but when there is a hepatic malfunction man has been infected (Culbertson, 1941). Even on clinical evidences, watertight classification of trypanosomes is not practicable.

Host parasite relationship

The host-parasite relationship of pathogenic trypanosomes to a host in which they are perfectly harmless reflects a long term evolutionary association. Trypanosomes transmitted by inoculation are regarded as of more recent origin than those with a contaminative method of transmission (Hoare, 1948).

From the biological point of view, however, pathogenicity indicates a recent and unsatisfactory adaptation between host and parasite. A successful parasite does not kill its host since it requires to transmit its progeny to other hosts. The transmission cycle is not very efficient and depends a great deal on fortuitous circumstances. The long maintenance of the parasite in the reservoir host is therefore essential for the propagation of the parasite.

As shown in the classification diagram (Fig. 1) the genus Trypanosoma embraces many diverse parasites, which are capable of infecting man and his domestic animals. Trypanosomes are classified on the basis of their morphology, life cycle and other biological characteristics.

Mere/

Mere morphological examination does not distinguish the sub-species T.b. rhodesiense and T.b. gambiense which are infective to man from the non-infective T.b. brucei. To minimise risks to laboratory workers, therefore, it is absolutely necessary to establish the true identity of any organisms being worked with, and to handle such carefully.

The experimental work reported in this thesis is concerned entirely with the type species Trypanosoma brucei of the sub-genus Trypanozoon.

The Sub-genus Trypanozoon

The sub-genus Trypanozoon was in the past classified into three species on the basis of their mode of transmission, i.e.

(a) Cyclically by tsetse flies
(T. brucei, T. rhodesiense and T. gambiense).

(b) By blood sucking flies other than tsetse (T. evansi).

(c) By contact during coitus (T. equiperdum).

In the laboratory, however, it is known that all these organisms can be transmitted by syringe inoculation of infected blood and that some stocks of T. brucei and T. rhodesiense lose cyclical transmissibility(i.e. via the tsetse fly) after long syringe passage in the laboratory. Structural and even ultrastructural characteristics have not /

not so far proved infallible in distinguishing the members of the sub-genus Trypanozoon from one another. Some T. equiperdum stocks have been shown to carry T. brucei surface antigens (Van Meirvenne, personal communication). T. brucei brucei is assumed to be infective to animals only. T.b. gambiense to man only and T.b. rhodesiense to both, but these are not absolute properties as T.b. gambiense can be adapted to animal hosts. Inability of T. brucei brucei to infect man is said to reside in high density serum lipoprotein (Rifkin, 1978). Differences in the clinical course of infection in man separate T.b. gambiense from T.b. rhodesiense. The former produces chronic infection with very low parasitaemia and short periods between the peaks (Hoare, 1972). Differences in isoenzymes (Godfrey and Kilgour, 1976) distinguish T.b. gambiense from both T.b. rhodesiense and T.b. brucei. Recently, Parratt and Cobb (1978) used heterophile antibodies to distinguish T.b. gambiense from T.b. rhodesiense infections.

Morphological differences between T.b. evansi and T.b. equiperdum e.g. reduction of the proportion of stumpy trypomastigote have not been found consistent. This led Hoare (1972) to suggest that T.b. evansi and T.b. equiperdum be included with the other species of the brucei family.

Obviously, the validity of taxa of the sub-genus Trypanozoon requires more convincing evidence. Purely mensural /

mensural characterisation of species or infectivity to different hosts or the mode of transmission have not been found immutable characteristics. All members of the Trypanozoon group are therefore now included in the same species e.g. brucei but sub-divided into the following sub-species

T.b. brucei
T.b. gambiense
T.b. evansi
T.b. equiperdum
T.b. rhodesiense

INFECTIVITY OF TRYPANOSOMA BRUCEI TO BIRDS

Infection by trypanosomes of any host is a function of such variables as:

(a) effectiveness of transmission.
(b) Susceptibility of the host,
and (c) the ability of the trypanosomes to elude the hosts' immunological responses.

Of all the pathogenic trypanosomes those of the sub-genus Trypanozoon have the broadest potential host range. The type sub-species T. (Trypanozoon) brucei is able to establish itself in representatives of practically all orders of mammals, birds and even reptiles.

Species of trypanosomes in the other sub-genera (Duttonella, /

(Duttonella, Nannomonas and Pycnomonas) have a somewhat more limited host range than those of the Trypanozoon sub-genus.

Birds have not been reported widely as hosts for Trypanosoma brucei spp. In some circumstances, however, birds can be infected with this organism.

Durham (1908) was the first person to show that intramuscular inoculation of a citrated rat blood, infected with T. brucei, into Falco tinnunculus (the kestrel) resulted in an infection which could only be diagnosed by blood inoculation into rats. The induced infection in the kestrel became self-cured after 116 days.

Much later, Corson (1931) and Duke (1933) both showed that tsetse flies used for transmitting infection to laboratory mammals could equally transmit T. brucei rhodesiense infection to domestic chickens. Extending his previous investigation, Corson (1935) demonstrated that tsetse flies infected with T.b. rhodesiense also transmitted the infection to francolins and guinea fowls. Seager (1944) inoculated mouse blood parasitised with T.b. equiperdum into week-old ducklings and found that while some ducks showed acute fatal trypanosomiasis within 4 days, the majority showed a prolonged sub-patent infection.

Infections have also been initiated after the natural defence mechanisms of the bird have been interfered with by starvation (Sallazo, 1929 quoted by Duke, 1933) or by bursectomy and cytotoxic drugs (Hicks, 1977).

Other /

Other workers (Duke, 1912; Mesnil, Leger and Perard, 1936; Hood, 1949) using direct inoculation into chickens, of parasitaemic blood from infected mammals have, however, been generally unsuccessful in initiating infections. Mistra, Ghosh and Choudhury (1976), on the other hand, reported a patent though transient parasitaemia following inoculation of chickens with T. brucei evansi in rat blood.

It thus seems clear that some stocks of T. brucei ssp can infect domestic chickens, at least for a short time.

THE DISEASE TRYPANOSOMIASIS

Clinical Picture.

Interest in the immunology of trypanosomiasis dates back to the beginning of this century when the principles of the immunology of many newly discovered bacterial and viral diseases were being elucidated. The discovery by Ritz (1916, quoted by Gray, 1967) that a single organism could give rise to at least 22 different antigenic types of trypanosomes showed that the immunology of trypanosomiasis was more complex to study than that of many bacterial and viral diseases.

On the other hand, host environmental factors cannot be excluded in the immune response to trypanosomiasis /

trypanosomiasis. Temperature and hormones are examples of factors which are known to be important in this respect, (Otieno, 1973). There is also some evidence that the immune response may become more rapid or more efficient as the infection progresses (Brown, 1974), and that prolonged infections may generate antibodies that are no longer strictly variant-specific but are cross-reactive.

The mechanisms responsible for antigenic variation are obscure and their elucidation presents a great challenge to protozoologist and immunologists. The infected host mounts an antibody response which is specific and acts to eliminate the antigenically predominant parasite populations from the body (Lourie and O'Connor,1937; Gray, 1965) but antigenic variants then repopulate the blood and in this way successive cycles of infection with different antigenic types of the trypanosomes continue; possibly indefinitely. In addition, the parasites have been shown to cause hypertrophy and change in the structure of the lymphoid system of infected hosts. Numerous review articles have been written on the nature and characteristics of the immune response of man and domestic animals to African trypanosomiasis with emphasis on the antigens of trypanosomes (Weitz, 1963), on antigenic variation (Gray, 1965; Gray and Luckins, 1976), on evasion of immune response (Ogilvie and Wilson, 1976), and on the survival of parasites in the immunized host (Cohen, 1976).

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Hyperglobulinaemia in trypanosome infected Animals.

The primary immune response in most animals is characterised by the sequential synthesis of different classes of immunoglobulins in which, typically, initial IgM synthesis is replaced by the synthesis of IgG antibodies. In trypanosomiasis, however, this sequence of events is apparently modified and infections are characterised by an elevated and protracted production of IgM antibodies. This macro-globulinaemia has been demonstrated in human trypanosomiasis (Mattern, 1964), and in experimentally induced infections and different hostsi.e. T. congolense and T. vivax in cattle (Luckins, 1976).

In mammals the serum IgM level is increased to 8-16 times the normal concentration and the increase commences as soon as two weeks after infection (Clarkson, 1976). Lumsden (1965) showed that increased levels of serum IgM occurred in infection of man with T. rhodesiense and his work was extended by Cunningham, Bailey and Kimber (1967) who used the blood obtained by finger puncture dried onto filter papers. They concluded that tests for elevated IgM levels could be used as a screening method in the diagnosis of sleeping sickness. Onyango, Buttner and Mannweiler (1972) examined the effect of specific antitrypanosome treatment on the serum immunoglobulin levels of 60 patients, 15 of whom were considered to have /

have T. gambiense and the remainder T. rhodesiense. The IgM levels have fallen one month after treatment but the extent of the fall varied considerably.

On the other hand, Binz and Watson (1972) did a survey in various areas of Kenya and found 286 people with increased IgM who did not appear to have trypanosomiasis. They examined the possibility that other diseases such as malaria, leprosy, schistosomiasis and helminthiasis could be responsible but could find no correlation between these diseases and high IgM concentration. It is clear from these studies that increased serum IgM is a useful, but not an infallible, indication of African trypanosomiasis in man. Nonetheless, a normal level virtually excludes trypanosomiasis at least in mammals.

Seed (1969) infected rabbits with T. gambiense, separated the immunoglobulins by chromatography, and examined the fractions for agglutinating antibody. IgM increased rapidly after infection and trypanosome agglutinating antibody first appeared in the IgM fraction. IgM remained high but later, antibody was found in both IgM and IgG. Takayanagi and Enriquez (1973) working with T. gambiense in mice fractionated immune serum into IgM and IgG. They then compared the ability of these fractions to protect mice against challenge and to agglutinate trypanosomes. Whilst both IgM and IgG were active in agglutinating trypanosomes, IgG gave better protection.

The /

The difference observed may be related to the metabolic half-life of each immunoglobulin. IgM being more rapidly degraded than IgG.

Further studies are needed on the role of the increased IgM in the pathogenesis of trypanosomiasis. It seems likely, nonetheless, that such a high level would adversely affect the host. Mackenzie and Boreham (1974) have suggested that the increased IgM level in the body is likely to be responsible for the increased erythrocyte sedimentation rate and increased viscosity of the serum, seen in the disease and which cause circulatory embarrassment. They also suggested that immune complexes may form leading to the release of pharmacologically active substances. In the chicken, however, studies by Hicks (1977) showed that IgM levels remained normal during T. brucei ssp infections.

In mammals the specificity of the IgM antibodies is still in doubt. Houba and Allison (1966) and Mackenzie (1973) indicated that not all of the IgM is directed against the parasites. The cause(s) of the increased IgM is probably due to a combination of factors and has led to considerable discussion (CIBA, 1974). Trypanosomes are very antigenic and their antigenic make up varies frequently. Capbern, Giroud, Baltz and Mattern (1977) isolated 101 VATS from a rabbit infected with T. brucei equiperdum.

Seed /

Seed et al (1969) and Seed (1972) suggested that the raised IgM was caused, at least in part, by the continuous synthesis of new specific antibody to each antigenic relapse. Whilst several workers have failed to absorb the IgM with trypanosome antigens and have concluded that most of the IgM is not specific trypanosome antibody (Houba et al 1969) it would require absorption, with all the antigenic variants to conclusively demonstrate its specificity, which has not yet been attempted.

Antigens of Trypanosomes.

For practical purposes, trypanosome antigens may be classified into two groups on the basis of their immunological specificity. Firstly, there are those variously referred to as bound, internal, homogenate or common antigens. These antigens are only released into the body following dissolution of trypanosomes, probably such antigens are responsible for the antigenic similarities of morphologically unrelated trypanosomes and for the cross-reactions that occur in many serological tests for trypanosome infections. The second group comprises the variant or surface coat antigens (Cross, 1975).

The surface coat antigens are glycoproteins which induce specific immunity to trypanosome infection. Each glycoprotein consists of a single polypeptide chain having a molecular weight of approximately 65,000 and containing about /

about 600 amino acid and 20 monosaccharide residues (Cross, 1975). Vickerman (1969) demonstrated that in bloodstream trypanosomes, the pellicle was uniformly covered with a surface coat 120-150A° thick. This coat is shed when the trypanosomes enter the tsetse fly but reappears in the metatrypomastigote (the infective stage found in the salivary gland). Vickerman and Luckins (1969) using ferritin-labelled antibody, demonstrated that this surface coat represents the variant antigen.

Studies by Weitz (1960) Allsopp et al (1971) have also demonstrated 'exoantigen' in the serum of rats infected with T. brucei and parasitaemic blood respectively. Macadam and Herbert (1970) found a substantial aggregate of free filopodia in the blood of Trypanosoma brucei infected mice and following centrifugation both the filopodia pellet and the supernatent protected against challenge with a homologous trypanosome variant.

The variable antigen type is now believed to be the antigenic identity of a single trypanosome expressed at its surface (Anon, 1978).

Antigenic Variation.

African trypanosomes undergo antigenic variation and thus evade immune destruction by the host. The maximum number of variable antigenic types that a stock of trypanosomes or even a single trypanosome can produce has /

has not yet been determined but Ritz (1916 quoted by Gray, 1967) showed that a single organism could give rise to at least 22 different antigenic types and Capbern, Giroud, Baltz and Mattern (1977) isolated 101 antigenic types from a rabbit infected with T. brucei equiperdum. There is no convincing evidence yet that any antigenic type of a stock is produced twice during an infection and it seems that the total number of antigens produced in one host may be limited only by the length of time the animal lives (Gray, 1967). It is also known that a number of antigenic types may develop at the same time in one host (Barry, Hajduk, Vickerman and Le Ray, 1979). The repeated sequential appearance of the antigens and the antibodies they induce may provide a basis for immunopathology commonly observed in the lymphoid tissues of infected hosts.

A one-time universally accepted hypothesis - proposed by Gray (1965), indicated that trypanosomes revert to a basic antigen type during development in the tsetse fly. Recent work has, however, shown that metacyclics (trypanosomes in the tsetse) are antigenically heterogeneous (Le Ray, Barry and Vickerman, 1978).

Antigenic variation results from pre-existing genetic information, or recombination or mutation. The stimulus to change variants does not derive directly or indirectly from the host immune response, but may be associated with other environmental factors (Anon , 1977).

Antigenic /

Antigenic variation furnishes trypanosomes with a powerful means of circumventing host immune responses.

Application of immunology to the diagnosis of trypanosomiasis

Research in the immunology of trypanosomiasis has led to a better understanding of the disease. Several serological methods have been devised to distinguish between trypanosome variants especially the agglutination test (Cunningham and Vickerman, 1962), the trypanolysis test (Clarkson and Awan, 1969), the neutralization test (Soltys, 1957), and the gel diffusion test (Gray, 1961). While these tests are valuable for distinguishing between populations as a whole, they have the disadvantage that they fail to detect trypanosomes with different surface coats when these are present as a low proportion of the population. More recent developments of the immunofluorescence technique (Sadun, Duxbury, Williams and Anderson, 1963; Van Meirvenne, Janssens and Magnus, 1975) now permit the demonstration of small proportions of individual variant trypanosomes in infected blood smeared on a slide. These studies can also be carried out on trypanosomes in tsetse saliva (Barry et al, 1979) or on preserved suspended organisms (Nantulya and Doyle, 1977).

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Complement fixation Test

The blood-stream form of T. brucei equiperdum shares common antigens (internal antigens) with both T.b. rhodesiense and T.b. gambiense and this antigen provides a source of complement fixation antigen for diagnosis of African sleeping sickness (Baker, 1970). Complement-fixing antibody appears in the serum of patients 7-15 days after infection with trypanosomes and reaches a maximum titre about a week later. Almost 95% of new cases of T. gambiense infection give a positive complement fixation test (Gray, 1967). Host erythrocyte contaminants can render the T.b. equiperdum antigen unsuitable for use. Adoption of the recent method of separating blood cells from trypanosomes on DEAE cellulose has, however, improved the sensitivity of the test.

Even though the antigen is group specific and it occurs in several species of brucei group trypanosomes, the test is still considered by some to be a reliable immunodiagnostic method. The application of immunological techniques in the diagnosis of trypanosomes is fully reviewed by Weinman (1963) and Lumsden (1978).

Immunodepressive effect of trypanosomes on infected hosts.

Trypanosomes have evolved more than one method of avoiding host immune responses. Besides their ability to undergo /

undergo antigenic variation, they have also been shown to cause considerable perturbation in the structure and function of the lymphoid system in infected hosts. Both lymph nodes and spleen are enlarged (Ormerod, 1970; Fiennes, 1970) and it is usual for infected animals to exhibit spleens with abnormally well developed germinal centres, (Moulton and Coleman, 1977; Wallace, 1976). Studies with T. brucei infected mice have shown that the histological picture and the actual cellular content of spleens is marked by an increase in B-cells, macrophages and a lower content of T-cells (Moulton and Coleman, 1977)

One of the common findings of trypanosome infection is a generalised immunodepression accompanying the disease (Goodwin, Green, Guy and Voller, 1972; Greenwood, 1974; Terry, 1976). Antibody responses to variant antigen types appear successively throughout the course of the infection. On the other hand, specific B-cell responses cannot simultaneously be induced to some heterologous antigens (Hudson, Byner, Freeman and Terry, 1976).

Depression of T-cell responses is also manifested in trypanosome infection. One study showed that the symptoms and histopathology of experimental allergic neuritis in the rabbit, an autoimmune disease mediated by T-cells, are suppressed in rabbits infected with T. brucei (Allt, Evans, Evans and Targett, 1971). In human beings, infected with trypanosomes, the delayed type skin test /

test responses to candida and streptococcal antigens were shown to be suppressed (Greenwood, 1974). Urquhart et al, 1973, however, reported no impairment of T-cell responses to oxazolone. Immunodepression to a heterologous antigen is intimately related to the presence of live trypanosomes which multiply, undergo antigenic variation and elicit immunological and inflammatory responses.

Several hypotheses have been advanced to explain the immunodepression:

(a) that macrophages are incompetent because they become overloaded with or 'blocked' by trypanosome antigen and are therefore unable to respond fully to heterologous antigen (Allt et al, 1971). This is commonly called antigenic competition (Terry, 1976)

or (b) that clones of T-suppressor cells, which prevent proper functioning of B-cells are produced (Terry et al, 1973; Greenwood, 1974).

Many workers have shown that animals and men infected with trypanosomes produce antibody to a wide range of antigenic determinants including those of many host tissues (Houba and Allison, 1966; Mackenzie and Boreham, 1974). Arising out of these observations, Urquhart, Murray, Jennings and Bate (1973) and Greenwood (1974) suggested that trypanosomes contain a B-cell mitogen which results in a 'polyclonal' stimulation of the cells, thus giving rise to antibodies of many specificities. /

specificities. Bacterial endotoxin produces a similar effect but no one has been able to detect such a toxin in the salivarian trypanosomes, though Esuruoso (1976) demonstrated that T. brucei extracts are mitogenic in-vitro for normal mouse spleen cells and that the mitogenic effects are directed against B-cells.

More recently, Pearson and his colleagues (1978) showed that lymphocyte stimulation after culture with the mitogen concanavalin A or with histocompatible cells differing at H-2 or minor lymphocyte-stimulating loci was reduced or abolished in spleen cells from T. brucei infected mice when compared with responses of spleen cells from uninfected controls. They therefore concluded that the depressed immune responses in trypanosomiasis were not simply due to low numbers of T-cells in spleens of infected animals but reflected a generalised immune depression which was not antigen-specific.

IMMUNITY TO TRYPANOSOMIASIS.

Natural Resistance to T. brucei ssp.

The existence of any form of natural resistance to members of the T. brucei ssp is a purely theoretical concept. For instance, it is generally accepted that the African baboon is completely resistant to all T. brucei ssp whether administered by most routes or by tsetse fly bite. Nevertheless, /

Nevertheless, Regendanz (1932) showed that infection in these hosts could be successfully initiated if trypanosomes were introduced directly into the cerebrospinal fluid.

Selective natural resistance, however, is the rule. Principally the constitutional factors of a potential host which affect the ability of trypanosomes to survive, metabolize and proliferate in the body all play an important part in the final outcome of the infection. Man is resistant to infection by T.b. brucei because of the cytotoxic effect of his high density serum lipoprotein (Rifkin, 1978). The resistance of cotton rats to T. vivax infection has been attributed to a natural immunity because of the presence of natural antibodies in the serum (Terry, 1957). Another well established example of host species which are completely refractory to infection can be found in T. simiae, a pathogen of domestic pigs, that will not infect cattle,horses, dogs and guinea pigs (Stephen, 1970).

The most widely quoted example of innate resistance is that seemingly shown by wild game animals of Africa. Earlier workers were struck by the contrast in tsetse infested areas between the teeming, apparently healthy, game animals and stricken domestic animals. The resistance of game animals might, of course, be due to trypanosome exposure in early life. It is therefore not clear /

clear whether this resistance is due to innate or acquired factors.

It must, therefore, be said that the mechanisms of innate resistance are still poorly defined.

Acquired Immunity to Trypanosome Infection.

Acquired immunity centres round the fact that the parenteral introduction of antigens into the body is followed by a specific immune response. This can either be cell-mediated or humoral. The antibodies produced by the humoral response are often termed immune antibodies to differentiate them from natural antibodies which sometimes exist in the blood without immunization. Kanthack *et al* (1898, quoted by Taliaferro, 1929) first established that a rat that had recovered from an infection with *T. lewisi* was refractory to a second inoculation. Much later Laveran (1911, quoted by Taliaferro 1929) showed that sheep and goats infected with *T. brucei ssp* recovered after four to twenty-one months and that thereafter an immunity existed which sometimes lasted as long as twenty-eight months. Laveran found that when serum from one of these recovered animals was injected simultaneously with the original passage stock it prevented infection. On the other hand, serum taken from the host during the course of the infection did not protect against the infection. He also found that such /

such an immunity was sharply stock-specific, and that it did not extend to other stocks of trypanosomes of the same species.

Experiments on self-cure and subsequent resistance to rechallenge take time and require diligent observation. Self-cure of trypanosome infection is not always predictable. This may be a reason why few reports have been published. The enthusiasm generated by the early workers was nonetheless extended into practical applications. An observation of self-cure of trypanosome infection in cattle followed by a solid immunity was recorded by Van-Saceghem (1936). He observed that calves infected with T. congolense at two weeks of age self-cured at four to six months. Re-inoculation of the cattle with the original material in guinea pig's blood failed to produce an infection.

Further progress on these lines seem, however, to have been dampened by the finding that the acquired immunity was easily overwhelmed if the animals were moved from one place to the other or exposed to environmental or husbandry stresses. For example, Desowitz (1959) showed that Muturu cattle that have been kept in a tsetse free area suffer from severe infections on syringe challenge. This indicates that the resistance shown by these animals in endemic areas is acquired rather than natural and that husbandry practice can influence the degree of immunity. A similar observation

was /

was reported in East Africa by Hornby (1941). Another example of the influence of husbandry on the outcome of trypanosome infection is that T. brucei infections normally lethal to mice during the first parasitaemic wave may be converted into chronic relapsing infections by maintaining the animals at 37°C (Otieno, 1973).

The phenomenon of self-cure and subsequent resistance to re-infection, though it has stimulated lots of thoughts, has not been successfully exploited in domestic animals.

Immunization by infection with living but attenuated trypanosomes.

After the demonstration of protective antibodies in the serum of animals that recovered from infection, attempts were centred on the immunization of animals by infecting them with attenuated trypanosomes. Attenuation of trypanosomes has been attempted by various methods, e.g. prolonged passage in laboratory rodents Darling (1912); irradiation of trypanosomes (Duxbury, Sadun and Anderson, 1973). Neither method was found to be successful. The length of life of the infected animals did increase considerably but nevertheless death followed eventually.

Although of theoretical interest, this method of immunization has a fundamental drawback for immediate application, /

application it cannot be used on man or valuable animals until a practically perfect method of attenuation is evolved.

Immunization with killed trypanosomes.

Early attempts to immunize laboratory animals with dead trypanosomes or their disintegrated products were almost all either entirely negative or simply prolonged the life of the immunized animal for a short time over that of the controls (Taliaferro, 1929). Injections of dead trypanosomes with an adjuvant such as saponin has been reported to have led to a high level of immunity against T. congolense in mice (Johnson, Neal and Gall, 1963). The success of immunizing animals with dead trypanosomes has been shown to depend on the method of killing the trypanosomes, the frequency of inoculations and the type of organisms used for both immunization and challenge. For example, Soltys (1964) showed that immunity was obtained when trypanosomes were killed by formalin or by freezing and thawing five times but that failure attended attempts to immunize with trypanosomes killed by heating at 56° for half an hour; it was also shown that animals which received ten doses of the mixture produced better results, immunity wise, than those which received the same amount but only in two doses.

The development of methods for the isolation of clones /

clones (Oehler, 1913; Topacio, 1933) of defined antigenic identity, for cryopreservation (Cunningham and Harley, 1962) and for the determination of infectivity of stabilates by titration (Lumsden et al, 1963) have enabled more accurate experimentation to be carried out. Extending the earlier observation of Soltys (1964) and using a more defined trypanosome organism, Herbert and Lumsden (1968) showed convincingly that immunity from a single inoculation with formalinised antigens was long lasting when whole infected blood was used. Only transient immunity was, however, demonstrated when the vaccine was composed of released antigens. It is now known that the method used to prepare the antigen, whether breaking up the organism by freezing and thawing or formalinisation does not appear to affect its antigenicity for immunization of mice (Herbert and Lumsden, 1968).

Prospects of vaccination.

The development of an effective vaccine against trypanosomiasis, however, remains a notion. In seeking to provide effective immunization against trypanosomiasis in both man and his domestic animals, it might be useful to consider the problem as though it consisted of a number of separate disease entities rather than one disease complex. At present chemoprophylaxis seems to offer a more hopeful approach than artificial vaccination.

The /

The repeated development of drug-resistant trypanosomes in Africa (Williamson, 1970), however, casts doubts on the long-term effectiveness of this procedure as a practical means of economic control of the disease.

IMMUNE APPARATUS OF GALLUS DOMESTICUS

The great efficiency of domestic chickens in producing antibodies was demonstrated by Hektoein (1918) and is illustrated by his statement: "The domestic fowl is a prompt, reliable and liberal producer of precipitins, more so than the rabbit".

The avian immune system possesses a number of features different from that of the mammal, the chief being the possession of a lymphoepithelial organ peculiar to birds, the bursa of Fabricius. The immune system can be divided into peripheral and central, or primary, organs. The latter consist of the bursa and the thymus which are associated, respectively, with the development of humoral antibody responses and with cell-mediated immunity. The peripheral organs are almost wholly represented by the spleen, and caecal tonsils. Organised lymph nodes of the mammalian type are not present in the domestic chicken. Lymph ganglia (Jolly, 1909/1910, quoted by Hodges, 1974) or a lymph gland (Dransfield, 1945), however, have been shown to occur /

occur in restricted groups of birds which do not include the Gallinaceous birds. The characteristic features of lymphoid cells are their ability to react to antigens by producing antibodies, to proliferate in response to antigenic stimulation, and to retain specific immunological memory.

In mammals lymph nodes have been shown to react to trypanosome infection earlier than the spleen (Moulton and Coleman, 1977). They indicated that the lymph follicles were large and consisted almost entirely of proliferating germinal centre. Trypanosome infections result in intense antigenic stimulation as evidenced by extraordinarily high IgM responses (Mattern, 1964). The effect of these stimuli on the lymphoid tissues of birds as contrasted to those of mammals may be illuminating.

Central Organs Involved in Immune System:

The bursa of Fabricius (Bursa)

The bursa was originally considered to develop from a proliferation of the endodermal epithelium of the dorsal caudal region. Meyer, Rao and Aspinall, (1959) have since reported that it originates in the four-day old embryo along the ventral caudal contact of the cloaca with the external epithelium. Detailed histology of the bursa is described in Hodges (1974).

At /

At hatching the bursa is well developed and continues to grow rapidly to reach full development in the immature bird and then undergoes regression at the onset of sexual maturity. Glick (1956) showed that the maximum weight of the bursa is reached at different periods in various breeds of birds i.e. 6 weeks in Barred Crosses to 3 weeks in White Leghorns. After ten weeks there is a decline in bursa weight as the organ begins to involute. By 23 weeks of age the bursa is reduced to a remnant (Wolfe, Sheridan, Bilstad and Johnson, 1962). The demand for immunoglobulin synthesis in trypanosome infection might alter the time course of involution of the bursa.

The Bursa in the Immune Response.

The bursa is a source of immunologically competent cells which are seeded out to other tissues. Thus, the bursa and the bursa-derived lymphoid cells found in the spleen and caecal tonsil are needed for immunoglobulin synthesis. In an infection like trypanosomiasis which is characterised by hyperglobulinaemia, at least in mammals, this organ will be expected to play an important role.

The bursa though functioning principally in relation to the humoral immune response also plays a role in local defence of the body. Van Alten and Meuwissen (1971) showed /

showed that introduction of sheep red blood cells into the bursal lumen resulted in the production of 'bursal antibody' without any splenic antibody reaction. T. brucei is both a blood and a tissue parasite, the bursa should, therefore, be essential for the defence of the chicken against some tissue forms of the parasite. The survival of birds and their protection against any haemoparasite, such as a trypanosome, should be influenced by the activities of the bursa.

THE THYMUS.

The thymus of the bird is a paired gland, one half of which is found on either side of the neck. Each half, usually of six or seven lobes, lies in the subdermal connective tissue of the neck. The gland is intimately associated with the jugular vein and vagus nerve which makes experimental ablation difficult.

The thymus in immune responses.

The thymus is usually considered a source of precursor cells that populate other lymphoid tissues, such as the spleen and lymph nodes. The precursor cells mature into immunologically competent cells (Miller, Marshall and White, 1962). This organ is held generally responsible/

responsible for the lymphocytes concerned in cell-mediated immunity.

Isakovic and Jankovic (1967) reported the appearance of germinal centres in the thymus of Rhode Island Red chickens following the intravenous inoculation of human red blood cells. This suggests the involvement of the thymus with blood borne antigens. The thymic cortical lymphocytes are reported to be immunologically incompetent whilst immunocompetent populations of cells are present in the medulla (Warner, 1964). From these considerations, the thymus should probably play a role in the defence of chickens against the tissue forms of T. brucei.

THE AVIAN SPLEEN.

The spleen in chickens is a rounded, reddish-brown organ which lies close to the right side of the junction between the proventriculus and the gizzard. The size varies but in a normal ten-week old bird it is about 0.2% of the body weight. Small accessory spleens may be present in young fowls (Kelly and Abramoff, 1969); they have been observed by the present author. In the embryonic spleen granulocytes are formed in large numbers while erythrocytopoiesis is not very active. Granulocytopoiesis begins in the red pulp at 12-13 days of incubation whilst small lymphocytes do not appear in the white pulp until 16-17 days. By 48 hours after hatching, however, /

however, lymphocytes are the predominant cells of the organ. Histologically the spleen is enclosed in a thin fibrous capsule beneath a flattened layer of peritoneal mesothelium.

Lucas, Denington, Cottral and Burmester (1954) believe that no true trabeculae (such as those of mammals) extend into the splenic substance. However, Taliaferro and Taliaferro (1955) indicated that some trabecular scaffolding existed but that it was much less than in the mammalian spleen.

The underlying framework of the spleen consists of a network of reticular cells and reticulin fibres. Super-imposed on the underlying framework are the two basic types of splenic tissue - the red pulp and the white pulp. Both types of pulp occur in approximately equal amounts (Lucas et al, 1954). The white pulp is composed mainly of lymphoid tissue. The red pulp, on the other hand, is a conglomeration of loose spongy tissue composed of ramifying cellular cords surrounding blood filled spaces. Three types of lymphoid tissues occur in the spleen:

(a) periarteriolar lymphoid tissue of thymic origin

(b) peri-ellipsoidal lymphoid tissue of bursa origin

and (c) germinal centres.

The /

The white pulp is diffusely spread throughout the organ, although predominantly located around the arteries. The sharply distinguished areas of red and white pulp which occur in the mammalian spleen are not seen in the fowl. Lymphoid nodules (germinal centres) have been described as regularly present within the white pulp of normal birds, and have been shown to increase in number during infections with haemoparasites e.g. Plasmodium lophurae (Taliaferro and Taliaferro, 1955), Trypanosoma brucei (Wallace, 1976). Germinal centres are not commonly found in chickens under 4 weeks old and are typically absent from spleens of germ-free chickens of all ages (Hodges, 1974). Plasma cells may be seen occasionally in the normal spleen but tend to become more abundant during infections (Taliaferro and Taliaferro, 1955).

The Avian Spleen in Immune Responses.

After hatching the main function of the avian spleen changes from one of erythrocyte destruction to one of lymphocyte production. The role of the spleen in antibody responses has been authoritatively described by Taliaferro (1956) and White (1969). Prompt production of antibody by chickens was shown by White, French and Stark (1970) in an experiment in which they found the injection of human serum albumin (H.S.A.) resulted in cells containing/

containing anti-H.S.A. appearing in the spleen after about 30 hours. The maximum antibody response is attained at 6-8 days (White, 1969; Kelly and Abramoff, 1969) and this is quickly followed by a precipitous fall to a low level by about 18 days.

The immunological functions of the spleen are chiefly directed towards invading organisms, and other foreign antigens, which are present in the blood because its macrophages are oriented to remove materials from the blood. The liver because of its size is also of great importance in phagocytosis. Although the liver synthesises some serum globulins, it is, however, unimportant in antibody formation. No work has been reported on the role of avian spleen in immunity or suppression of trypanosomiasis in birds. In mammals evidence exists to the effect that a latent T. brucei infection became patent when rats were splenectomised (Kligler, 1929). Splenectomy makes rabbits liable to fatal infections, of T. simiae, to which it is not normally susceptible (Desowitz and Watson, 1953). The mechanism by which splenectomy enhances susceptibility to the infection is not well understood. Nevertheless, the ability to mobilize immunocompetent cells which elaborate humoral antibody is impaired, and this may be an important factor.

The spleen is therefore important in keeping the parasitaemia at a low level.

A /

A parallel look at other reported haemoparasites in birds showed that Herman and Goldfarb (1939) could induce a low grade and short-lived Plasmodium circumflexum infection in birds, normally resistant to the infection, after splenectomy. Phagocytosis of the malaria parasite Plasmodium lophurae and cellular debris is carried out mostly by macrophages of the spleen, liver and bone marrow (Taliaferro, 1956).

The reticular cells of the spleen become very prominent during a blood borne infection. In extraction studies in which the whole organ is ground up and an aliquot portion analysed for antibody content, higher levels of antibody, extractable from the spleen than are present at the same time in the serum,

following intravenous injections of various antigens (Humphrey and White, 1970).

These observations would appear to stress the role of the spleen in forming antibody to antigens which are distributed via the bloodstream. An intense lymphocytopoiesis in the nodules of the spleen during malaria infection in chicken was first described by Taliaferro and Cannon (1936). Taliaferro and his colleagues later (1955) reported an increase in the number of lymphoid nodules (germinal centres) in the spleen of chickens infected with Plasmodium lophurae. In a convincing experiment carried out in chickens, Keilly and Abramoff (1969) showed that if surgical extirpation of the spleen is complete, the serum haemagglutinin titre/

titre against sheep red blood cells was always lower in splenectomised chickens than in controls.

The spleen of the chicken appears to form most of the antibody during the initial rise, but abruptly stops doing so at the time of peak antibody titre, while non-splenic sources (bone marrow etc.) continue the synthesis of additional antibody (Kelly and Abramoff, 1969). A division of chicken antibody into that of splenic and of non-splenic source had earlier been suggested by Taliaferro (1956). Thus, it seems the spleen is critical in the early humoral response to an infection but is soon surpassed by other sites in the maintenance of antibody levels over long periods.

In mammals infected with T. brucei, the spleen was markedly enlarged (Fiennes, 1970). It is common for infected mammals to show spleens enlarged over ten times (Ormerod, 1970) and there is a general disruption of the splenic architecture (Hudson and Byner, 1973). In infected mice, greatly enlarged germinal centres were seen and, the periarteriolar thymus-dependent areas were infiltrated with plasma cells and there was extensive disruption and replacement of lymphocytes (Moulton and Cole, 1977).

In T. brucei infected birds, the spleen retains its normal structure but the number of germinal centres is enormously increased (Wallace, White and Herbert, 1977). Enlargement of bird's spleen to up to twice the normal size has been reported by Hewitt (1940) in birds infected /

infected with the haemoparasite, Plasmodium ssp, but he also warned that this was not a reliable diagnostic criterion for this infection.

Germinal Centres.

Germinal centres or 'lymphocytopoietic centres', are considered the primary location of the central immune mechanism within the organised lymphatic tissue. The name 'Keimzentrum' or germinal centre was coined by Flemming in 1885. The germinal centre (G.C.) is composed predominantly of two cell types, proliferating lymphocytes or lymphoblasts and the unusually large phagocytic cells known as "tingible body macrophages", at least in mammals. The lymphoblasts are believed to be B-cells which can form antibody (Ortega and Mellor, 1957) and/or develop into small, memory cells for subsequent antibody responses. The tingible body macrophages are known to actively phagocytose, at least some of the lymphoblasts, producing dense phagocytic inclusions or tingible bodies (Fliedner, Kesse, Cronkite and Robertson, 1964).

Another cell type, called dendritic reticular cells, are present in the germinal centres (White, 1963 and Wallace, 1976). The dendritic cell differs from typical phagocytic cells in that antigens are retained as immune complexes on the surface of the many fine cytoplasmic processes or dendrites that they possess (Humphrey /

(Humphrey and Frank, 1969; French, Wilkinson and White, 1969). Germinal centres are thus intimately associated with immune responses and not merely concerned with the production of lymphocytes.

In birds, the germinal centre has a circular or compact appearance which makes it very easily recognisable in stained lymphoid tissues. The avian germinal centre (Plate 1) is circular or oval in outline, approximately 50-200 μ in diameter and clearly separated from the adjoining lymphoid tissue by a spongy connective tissue capsule.

The cells of the germinal centre consist of closely packed medium sized, slightly basophilic lymphocytes, including cells in frequent mitosis. The size of the cells and basophilia and even the germinal centre itself are subject to considerable variation, corresponding perhaps to the age of the centres. Staining of the germinal centre with fluorescein-labelled anti 7S antibody (or antibody to injected antigen) reveals the presence of evenly spaced dendritic cells interspersed with the lymphocytes. This appearance is common to all germinal centres and serves as an easy method for identifying these structures in cryostat tissue sections.

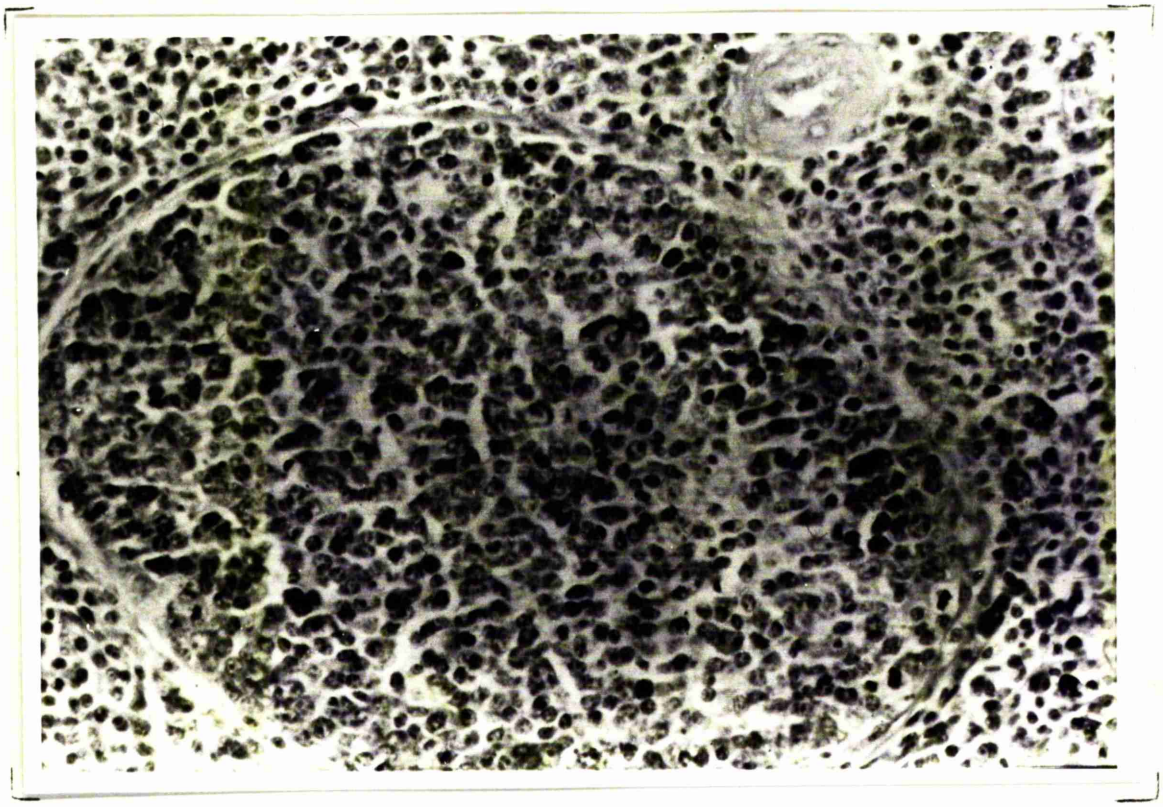
Ultrastructurally, cells in the germinal centre have numerous polyribosomes and their fine structure seems to be dependent on their functional state and location. Plasma cells are usually very rare in germinal/

PLATE 1.

AN ENLARGED GERMINAL CENTRE IN CHICKEN SPLEEN.

THE CHICKEN WAS INFECTED WITH *TRYPANOSOMA BRUCEI BRUCEI*

84 DAYS PREVIOUSLY .



germinal centres, but may appear in greater numbers during the late phase of an immune response (Simar, Betz and Lejeune, 1967).

The presence of 7S immunoglobulin with antigen at the surface of the dendritic cells has been shown (Young and Friedman, 1967; Thorbecke, Cohen, Jacabson and Wakefield, 1967). Further studies of the immunoglobulins on the dendritic cells by French, Wilkinson and White (1969) showed that the presence of 7S does not exclude other immunoglobulins and both the 7S and 19S forms may be present. If high levels of 19S can be found in T. brucei infected chickens (as is common in mammals) then germinal centres from such birds should show many dendritic cells with 19S immunoglobulins.

Germinal centres develop early during the immune response and antigens commonly localise in them as from three days onwards (White, 1963 ; White, French and Stark, 1967).

The localisation of immunoglobulin G, (aggregated or complexed with antigen) within germinal centres of chickens has been shown to be complement dependent by White, Henderson, Eslami and Neilsen (1975). Very recently Klaus and Humphrey (1977) re-echoed the requirement for the level of C3 component of complement for antigen localisation on the dendritic cells. Klaus (1979) pre-formed antigen-antibody complexes (Anti-dinitrophenyl-keyhole limpet haemocyanin) into mice showed that complement /

complement activation is still essential for localization in germinal centres.

In trypanosome infections, large amounts of antigen-antibody complexes are formed (Mackenzie and Boreham, 1974; Herbert and Parratt, 1979), leading to a complement level in the infected host that is generally low (Musoke and Barbet, 1977). The localization of trypanosome antigen in germinal centres in the probable condition of hypocomplementaemia may therefore be affected. The nature of the antigen and antibody has also been shown to affect localisation in germinal centres. Embling and colleagues (Embling et al, 1978) showed that, of various IgG myeloma proteins represented by the four human IgG subclasses, only IgG1 and IgG3 manifest avid germinal centre localisation in mice. It was also observed by Embling and colleagues that while human IgM only manifested weak trapping, no germinal centre localization was observed with human IgA in mice.

A new concept was introduced into the mechanism of antigen localization in the germinal centre by Chen, Adams and Steinman (1978). These workers suggested that the membranes of dendritic cells may have an affinity for colloids in general, since non-immunogens such as thorium dioxide and colloidal carbon were bound to these cells. This hypothesis has, however, yet to be confirmed.

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The Present Investigation.

The initial object of the present work was an investigation of the extent to which individual germinal centres were stimulated by a different antigen. The bird was chosen as a suitable experimental animal because its germinal centres are discreet and easily recognizable.

The work could have been carried out with a set of non-cross-reacting purified antigens, but the observation of Wallace, White and Herbert (1977) suggested that a trypanosome infection could be used for this purpose. Wallace and her colleagues (1977) observed enormous numbers of germinal centres in the spleens of birds infected with salivarian trypanosomes of mammalian origin. They postulated that these centres might result from the periodic challenge of trypanosome antigenic variation.

The experiments of Wallace, White and Herbert had been carried out with an uncloned and probably heterologous population of trypanosomes. It was, therefore, not known whether the infection that they reported in birds had been initiated by an avian trypanosome which was present unbeknown in the stock employed. Alternatively, it might have been that only a 'cocktail' mixture of trypanosomes would result in the numerous splenic germinal centres observed. The stock used might be such a cocktail if the tsetse fly

from /

from which it was obtained had fed on many animals just before capture. Trypanosomes of known identity had been used by Hicks (1977) to infect birds but he could only infect bursectomised birds, and his experiments produced inconsistent germinal centre development.

The construction of a suitable experimental system which avoided the variables of either a heterologous trypanosome or the necessity to use immunosuppressed chickens necessitated that:

(a) an organism of defined antigenic identity that would readily infect domestic chickens be found.

(b) known antigenic types of that trypanosome be isolated so that the antigens could be used to trace the effect of the relapsing infection on the germinal centres.

(c) the spectrum of trypanosomiasis in birds be defined if the significance of the germinal centres was to be clearly established. The present investigation thus falls into two main parts:

(1) a general study of Trypanosoma brucei brucei infections in birds.

(2) the effect of such an infection on germinal centre development.

EXPLANATION OF ABBREVIATIONS AND TERMS USED IN THIS THESIS

The immunological terms used in this thesis are as defined in the "Dictionary of Immunology" (Herbert and Wilkinson, 1977) while the protozoological terms are as described in "Techniques with Trypanosomes" (Lumsden, Herbert and McNeillage, 1973). For convenience some of these terms and abbreviations are explained below.

ABBREVIATIONS.

AnTat	=	Antwerp Trypanozoon antigen type.
^{o}C	=	Temperature in degrees centigrade.
EATRO	=	East African Trypanosomiasis Research Organisation.
ETat	=	Edinburgh Trypanozoon antigen type.
FITC	=	Fluorescein isothiocyanate.
g	=	gramme.
G	=	gravity.
H.S.A.	=	Human Serum Albumin.
I.F.T.	=	Indirect fluorescent antibody technique.
Ig	=	Immunoglobulin.
Kg	=	Kilogramme.
LUMP	=	London University Medical Protozoology.
M	=	Molarity.
m	=	mouse.
mg	=	milligramme.
ml	=	millilitre.
µg	=	microgramme.

/...

µl	=	microlitre.
S.O.	=	Single organism.
Normal Saline	-	This is a 0.85 percent W/V of sodium chloride in distilled water.
Titres	=	Are expressed as the reciprocals of the initial serum dilutions.
U.V.	=	Ultra Violet.
VAT	=	Variable Antigen Type.
WIG	=	Western Infirmary Glasgow.
WITat	=	Western Infirmary Trypanozoon antigen type.
W/V	=	Weight over Volume.

Gallus domesticus has been termed 'chicken', 'fowl' or 'domestic fowl' but as bird only where the context is clear. Where Gallus domesticus of a specific age or state are reported, the details are given.

MATERIALS

AGGLUTINATION PLATES.

World Health Organisation plastic agglutination plates (8 x 10 wells) were obtained from

Prestware Ltd., Raynes Park,
London, S.W.20.

AGGLUTINATION SLIDES.

Teflon coated slides (2 x 8 wells) were obtained from:

/

Fisons Scientific Apparatus Ltd.,
Loughborough, England.

AUTOMATIC GAMMA COUNTER.

Model-Gamma Set 500

Supplied by I.C.N., Tracerlab Division, England.

AUTOMATIC PIPETTES.

Capacity 10 µl - 200 µl for rapid serial dilution of sera. These are supplied by

Gallenkamp, U.K.

BOUIN - HOLLAND. (Fixative).

This is made as shown below:

1500 cc.	distilled water
37.5 gm.	Copper acetate
60.0 gm.	Picric acid (aqueous)
150.0 cc.	40% Formaldehyde.

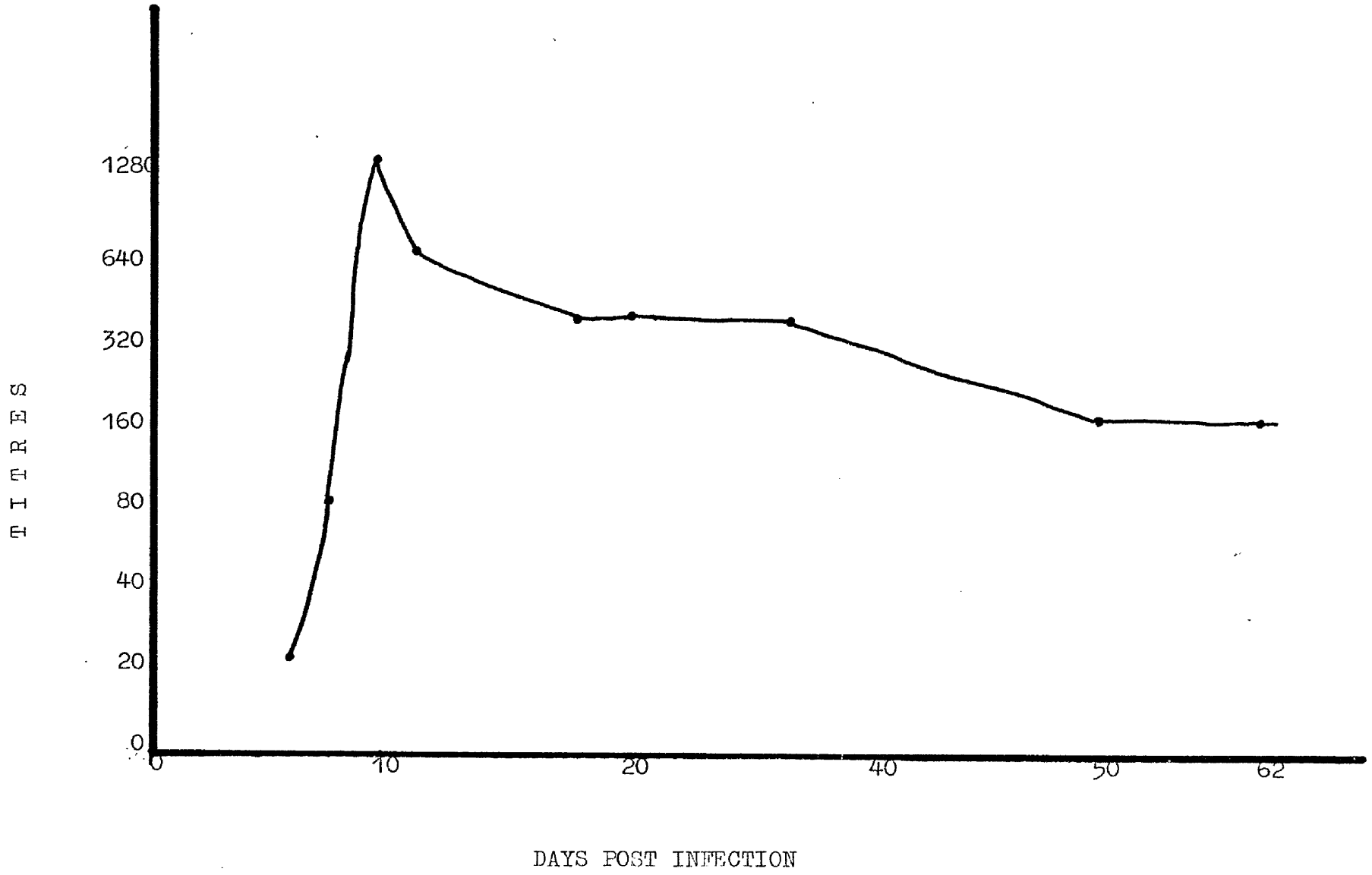
To each 100 ml. of the above is added

1.00 ml.	Acetic acid
0.5 gm.	Trichloroacetic acid just before use.

Berenil, Diminazene aceturate, (Hoechst Pharmaceuticals Ltd., Brentford, Middlesex), was used to cure infections. This drug contains diminazene aceturate, 31 percent and phenyldimethylpyrazolone 69 percent.

For /

FIGURE 2 . Trypanosome agglutinins in the serum of a guinea-pig following infection with 2 x 10^7 T.b. brucei. WIG 73 (WITat 1)



For example, to prepare compound Berenil injection (31% active substance) for treatment of mice (20 gm.) at rate of 25 mg/kg. 27.52 mg. of drug i.e. 27.52 x 0.31 = 8.53 mg. active substance is dissolved in 1.7 ml. of distilled water to give 5mg/ml, 0.1 ml. dose contains 0.5 mg. = 25 mg/kg for a 20 g mouse. For treatment of infections in chickens at 7 mg/kg, 23 mg. of drug is dissolved in 1 ml. of distilled water and 1 ml. is given for each kg. of body weight.

CAPILLARY TUBE HOLDERS.

Plastic (perspex) capillary tube holders made by Dr. W.J. Herbert to hold 25 capillary tubes.

These holders are filled with prepared sterilized tubes immediately before use.

COVER SLIPS.

Two sizes were used and both are rectangular (a) 7 x 22 mm. for rapidly examining wet films of the blood of large numbers of mice. Six separate slips are conveniently placed on one 75 x 25 mm. microscope slide with a frosted end.

(b) 22 x 22 mm. for general use and for examination of organisms in wet film from infected guinea pigs and for fluorescent microscopy.

DISTILLED WATER.

De-ionized or autoclaved water was used.

/

EAR PUNCH.

For marking mice. Supplied by
Animal Suppliers (London) Ltd.,
North Finchley, London, N.12.

END INFECTION SERA.

Six mice were infected with (WIG 72). Three days post inoculation the mice were bled from the retro orbital plexus. Trypanosomes were separated from the mouse blood cells by the method of Lanham and Godfrey (1970). 2×10^8 trypanosomes were inoculated intraperitoneally into a male guinea pig, he became patently parasitaemic on day five. On day sixty-two the infection became terminal and the guinea pig was bled by cardiac puncture. Serum was separated from the blood. The use of trypanosomes devoid of any blood cells plasma or serum ensured that antibodies are produced only against the trypanosomes.

FEEDS FOR EXPERIMENTAL ANIMALS.

All feeds were supplied by
Spillers Farm Feeds Ltd.,
Cannon Street, London, EC4M 6XB.

BIRDS.

Chick Crums: Fed to birds between 0-6 weeks. Salcostat at 125 mg/kg DIMITOLMIDE is added as an aid to control coccidiosis.

/...

Growers Pellets: fed to all birds aged 6 weeks and over. Contained coccidiostat as in above.
Hybrid 99 Layers Pellets: fed to all Breeding stock. It contains no coccidiostat.

All other animals are fed on pellets appropriate to their dietary regimen.

GLASS CAPILLARY TUBES.

Glass capillary tubes, 0.9-1.1 mm. bore, approximately 0.15 mm. wall, 10cm. long of soft glass allowing rapid sealing of ends in a microburner without any danger of heating a contained suspension. These are used for storing stabilates. The capillary tubes are supplied by

Plowden and Thompson Ltd.,
Dial Glass Works,
Stourbridge, U.K.

GLASS MARKING.

Grease pencil was used. This is supplied by
Grants Bookshop, Glasgow.

GIEMSA'S STAIN.

Supplied by Hopkin & William Ltd.,
Chadwell Heath, Essex, U.K.

HEPARIN.

Supplied by Evans Medical Ltd., Speke,
Liverpool

under /

under the Trade name Pularin. It is used at 10 I.U. per ml. of blood. Concentrated solution (1000 IU per ml.) is diluted to 200 I.U. per ml. and stored at -20° when not in use.

'HOLEY BLOWER'.

Used to expel stabilate suspensions from capillary tubes. It consists of an adaptor to which a rubber bulb is attached. The bulb has a hole in it.

HUMIDITY CHAMBER.

For maintaining slides carrying direct agglutination tests or for slides being stained for fluorescent antibody studies at a high humidity during incubation, were improvised from plastic sandwich boxes.

HUMAN SERUM ALBUMIN (H.S.A.)

This was obtained as 'Reinst' from Behringswerke, Marburg, Germany. It was dissolved before injection in 0.15M saline.

METER.

For measuring pH. Pye Model 291 supplied by

PYE Unicam, Cambridge

was used.

MICROHAEMATOCRIT CENTRIFUGE.

Supplied by Gelman-Hawksley Ltd., Sussex.

This /

This is used for rapid sedimentation of blood cells in microhaematocrit capillary tubes at about 1,200 g. using 0.05 ml. of blood.

MICROHAEMATOCRIT CAPILLARY TUBES.

Soft-glass capillary tubes 75 mm. long internal diameter about 1.2 mm, heparinized is used for determining packed cell volume. This is supplied by the same Maker as the Centrifuge.

MICROBURNER.

For sealing glass capillary tubes used to contain stabilates. The microburner is supplied by Flamefast Engineering Ltd.,
Swinton, England.

MICROPIPETTES.

Capacity 100 µl - 200 µl (Shandon, Drummond Microcaps) used for dispensing 0.2 ml. in infectivity titration.

PIPETTES.

Bleeding pipettes: Pieces of glass tubing of about 5.5 mm. external and 3.7 mm. internal diameter, 130 mm. long are cut, care being taken to achieve unchipped ends. They are washed well and dried before having their ends rounded in a flame. Pipettes are drawn from these tubes after they have been softened.

All /

All other pipettes are those generally used in the laboratory.

REFRIGERATED CENTRIFUGE.

Model - Mistral 2L.
Supplied by: M.S.E., England.

SLIDES, MICROSCOPE.

Glass slides 75 x 25 mm, 1.00-1.2 mm. thick with edges finely ground.

(Gallenkamp Ltd., London).

All slides were thoroughly cleaned and freed from grease before use.

SPECTROPHOTOMETER.

Model SP 500, Series 2. Ultra violet and visible, spectrophotometer.
Supplied by Unicam, Cambridge.
This was used for measuring optical density of protein solutions.

SYRINGES.

These are disposable plastic syringes 1 ml, 2 ml, 5 ml, 10 ml, and 50 ml. Supplied by Plastipak.

Each syringe is commonly rinsed three times with sterile normal saline before use in any experimental work.

/...

TRYPANOSOMES.

BUSOGA/60/EATRO/3. (ETat).

These are descendants of a stock of T. brucei rhodesiense isolated from Glossina pallidipes in Busoga, Uganda. (McNeillage et al 1968). The infectivity to man has been demonstrated by an accidental laboratory infection (Robertson and Pickens, 1975).

MUVUBWE/66/EATRO/1125.

This stock was originally isolated from Muvubwe in Uganda.

Full pedigree has been published by Le Ray et al (1977).

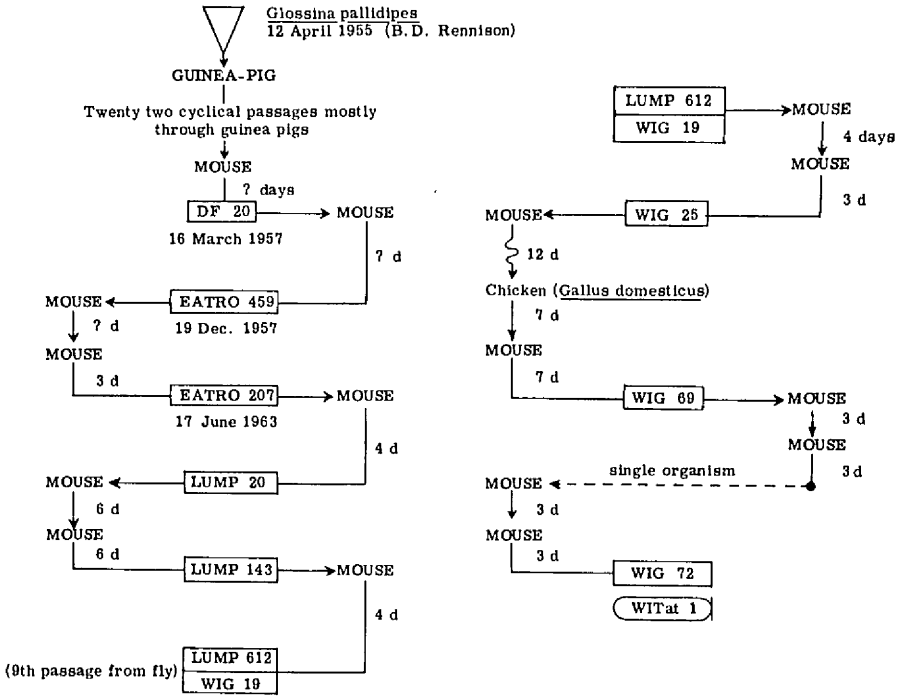
T. brucei brucei Lugala/55/EATRO/459.

This stock was originally isolated from Glossina pallidipes which were allowed to bite guinea pigs. After twenty two cyclical passages, mostly in guinea pigs, the stock was passaged to mouse and syringe passaged thereafter. This stock has also been termed Lugala I and stabilates EATRO 207 and LUMP 612 have been widely distributed. The pedigree to stabilate LUMP 612 (the 9th syringe passage) is given in Plate 2 According to information received from the World Health Organisation stabilate bank at the London School of Hygiene and Tropical Medicine, at the second cyclical passage, the stock was syringe passaged to rats. It was /

PLATE 2.

PEDIGREE OF LUGALA/55/EATRO/459

(After Herbert & Parratt, 1979).



was then tested for infectivity to man by syringe passage to two volunteers on three occasions. It failed to infect at either first challenge or at rechallenge at 9-20 days later.

This stock produces chronic infection in laboratory animals and birds. During the course of the investigation, cloned populations have been prepared from the stock and these have been identified by agglutination reaction (Cunningham and Vickerman, 1962) and by lysis test (Van Meirvenne, et al 1976) as being different variable antigen types. The seven so far identified have been called WITat (Western Infirmary Trypanozoon antigen type) 1-7, Pedigrees showing the derivation of each clone will be found in the results section.

Ultrogel type ACA 22 is supplied pre-swollen in 1 litre bottle by L.K.B., France. This is a gel filtration medium supplied in four different types allowing fractionation of molecules with molecular weights between 5000 and 1,200,000 . The ACA 22 has 2% Acrylamide and 2% Agarose, and the effective range of fractionation is 100,000 - 1,200,000. This gel effectively separates the lipoproteins, macroglobulin, IgM, IgG and albumin, when used in an appropriate column and eluted with normal saline.

The Use of Cloned Populations.

The work described in this thesis was carried out with /

TABLE 2.

CRYOPRESERVED TRYPANOSOMES CLASSIFIED BY PRIMARY ISOLATION NUMBER.

	Primary Isolation Number	Stabilate No.	Antigenic Type
A	Lugala/55/EATRO/459	WIG 25	Uncloned
	Lugala/55/EATRO/459	WIG 69	Uncloned recovered from chicken
	Lugala/55/EATRO/459	WIG 72	WITat 1 (Type-type)
	Lugala/55/EATRO/459	WIG 73	WITat 1
	Lugala/55/EATRO/459	WIG 76	WITat 1
	Lugala/55/EATRO/459	WIG 75	Clone P (Type-type)
	Lugala/55/EATRO/459	WIG 82	WITat 2 (Type-type)
	Lugala/55/EATRO/459	WIG 83	WITat 2
	Lugala/55/EATRO/459	WIG 78	WITat 3 (Type-type)
	Lugala/55/EATRO/459	WIG 87	WITat 4 (Type-type)
	Lugala/55/EATRO/459	WIG 88	WITat 4
	Lugala/55/EATRO/459	WIG 89	WITat 5 (Type-type)
	Lugala/55/EATRO/459	WIG 92	WITat 6 (Type-type)
	Lugala/55/EATRO/459	WIG 100	WITat 7 (Type-type)
B	Muvubwe/66/EATRO/1125	WIG 70	AnTat 1
C	Busoga/60/EATRO/3	WIG 41	ETat 2
		WIG 37	ETat 10

TABLE 3. Cryopreserved trypanosomes classified by stabilate number.

Stabilate Number	Antigen type	Primary Isolation Number
WIG 25	Uncloned (From L.U.M.P.)	Lugala/55/EATRO/459
WIG 37	ETat 10	Busoga/60/EATRO/3
WIG 41	ETat 2	Busoga/60/EATRO/3
WIG 69	Uncloned, recovered from chicken.	Lugala/55/EATRO/459
WIG 70	AnTat 1	Muvubwe/66/EATRO/1125
WIG 72	WITat 1 (Type-type)	Lugala/55/EATRO/459
WIG 73	WITat 1	Lugala/55/EATRO/459
WIG 75	CLONE P	Lugala/55/EATRO/459
WIG 76	WITat 1	Lugala/55/EATRO/459
WIG 78	WITat 3 (Type-type)	Lugala/55/EATRO/459
WIG 82	WITat 2 (type-type)	Lugala/55/EATRO/459
WIG 83	WITat 2	Lugala/55/EATRO/459
WIG 87	WITat 4 (Type-type)	Lugala/55/EATRO/459
WIG 88	WITat 4	Lugala/55/EATRO/459
WIG 89	WITat 5 (Type-type)	Lugala/55/EATRO/459
WIG 92	WITat 6 (Type-type)	Lugala/55/EATRO/459
WIG 100	WITat 7 (Type-type)	Lugala/55/EATRO/459

with trypanosome populations derived from single organisms hereby referred to as clones. Cloning of a stock eliminates some sources of error, e.g. it is known that tsetse flies may harbour more than one species of trypanosome at the same time and vertebrates may thus carry multiple trypanosome infections. The use of uncloned isolates can therefore lead to inconsistency in experimental investigation and even an occasional inability to infect.

In any immunological investigation using uncloned populations, properties defined could be a composite of the features of multiple species which are the actual subject. It is, therefore, essential to be certain that one is working with one and not a mixture of species or diverse antigenic variants.

BUFFERED DISTILLED WATER FOR GIEMSA STAIN.

The following salts were weighed and dissolved in distilled water.

$Na_2 HPO_4$	3.00 gm.
$KH_2 PO_4$	0.6 gm.
Distilled water	1 litre.

The solution is found stable for three months when kept in a brown glass bottle at 4°C.

ISOTONIC BUFFERED SALTS SOLUTION (Solution AB)

This is a comformable solution in which all components /

components are prepared isotonic with rat blood so that any may be altered without affecting the tonicity of the final solution.

Solution B is a buffer component.

Solution A.

Sodium chloride (NaCl)	9.00 gm./litre
Potassium chloride (KCL)	11.48 gm./litre
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	20.94 gm./litre
Calcium chloride ($CaCl_2 \cdot 6H_2O$)	22.56 gm./litre

The last two salts were prepared by first dissolving 25 gm. of each in 1 litre of distilled water. Each sample was titrated against 0.1N Silver nitrate and bulk adjusted to correct concentration by addition of water.

Estimation of chloride ions in Solution A.

1 ml. of the solution to be estimated is diluted to 3 ml. with distilled water and 1 drop of 2% solution of Potassium chromate (K_2CrO_4) was added. This was titrated against 0.1N Silver nitrate with constant stirring. White silver chloride first precipitated then a red precipitate of silver chromate (Ag_2CrO_4) formed locally but disappeared on stirring until the end /

end point - a faint reddish tinge persisting.

Titration and adjustment.

Calcium chloride.

4.815 ml. 0.1N $AgNO_3$ were needed to give the end point with 1.00 ml. $CaCl_2$ solution.

Therefore, $CaCl_2$ solution is 0.4815N, and 0.4066N is needed.

1 ml. 0.4815N $CaCl_2$ = X ml. 0.4066 N $CaCl_2$

$$X = \frac{0.4815}{0.4066} = 1.184$$

Correction: 1.00 ml. of the first solution was made to 1.184 volume.

Accurate records of each stock solution were taken, i.e.

Batch number

Weight of Solute

Volume of Solvent

Date of preparation

Titration result and adjustment (for Mg Cl_2, Ca Cl_2).

The solutions were mixed in the following ratio:

Solutions	Volumes
Solution NaCl	1000
Solution K Cl	40
Solution $MgCl_2$	30
Solution $CaCl_2$	10

The /

The mixture was dispensed in 22 ml. and 50 ml. samples in glass bottles, and autoclaved tightly closed at 121°C for 15 minutes. Bottles were marked with batch number.

SOLUTION B - phosphate.

$NaH_2 PO_4 .2H_2O$	24.00 g/litre.
$Na_2 HPO_4 .12H_2O$	36.89 g/litre.

Each solute was dissolved in one litre of distilled water.

Stock solutions were mixed by adding

3.6 parts	$Na H_2 PO_4$
96.4 parts	$Na HPO_4$.

The pH of the mixture was checked with a pH meter. The homogenised solution was dispensed in 5 ml. and 8 ml. quantities and autoclaved tightly closed at 121°C for 15 minutes. For each batch of solution the following records were taken

Batch Number

Date of preparation.

SOLUTION B - borate.

The stock solution was prepared by dissolving

$Na_2 B_4O_7$ (Sodium borate)	41.95 gm/litre
$H_3 BO_3$ (Boric acid)	19.17 gm/litre

/...

Each solute was dissolved separately in one litre of distilled water. The two solutions were mixed by the following proportions.

Solution	Proportion
$Na_2 B_4O_7$	23.1
$H_3 BO_3$	76.9

Similar records as in Solution B - phosphate were taken.

The final solution was dispensed in about 4 ml. and 2 ml. quantities in bijou bottles. Autoclaved tightly closed at 121°C for 15 minutes.

SOLUTION AB

The complete solution is made in situ before use by mixing nine parts solution A with 1 part of the solution B applicable to the technique being followed.

PHOSPHATE BUFFERED SALINE FOR USE IN IMMUNOFLUORESCENCE.

The stock solution is made as follows:

Dry Powder:

$Na_2 H PO_4 .2H_2O$	60.0 gm.
$Na H_2 PO_4$	13.6 gm.
or	
$Na H_2 PO_4 .2H_2O$	17.67 gm.
Na azide	0.1 gm.
Distilled water	1000 ml.

This solution is stored at 4°C.

/...

When required for use

100 ml. of the stock solution is added to 2400 ml. of Normal Saline.

The pH of this reconstituted solution is 7.2.

Mercuric Chloride Solution.

Ten milligram of dry powder of mercuric chloride is dissolved in 200 ml. of sterile distilled water. This solution is normally prepared just before use.

DE 52 EQUILIBRATING BUFFER.

The buffer was prepared from the salts shown below:

(a)	Disodium hydrogen phosphate	-	13.48 gm.
(b)	Sodium dihydrogen phosphate	-	0.78 gm.
(c)	Sodium chloride	-	4.25 gm.
(d)	Distilled water	to	1000 ml.

The pH of the solution is adjusted to 7.9 with boric acid.

The eluting buffer of NaCl 0.55 M, phosphate 0.055 M and glucose 0.5 M is prepared by diluting the stock phosphate buffer with distilled water as shown below. The dilution being made according to the source of infected blood. Glucose is dissolved as required. The pH of final solution is generally 8.

/...

SPECIMEN SAMPLE OF ELUTING BUFFER. Table 4.

Source of Infected Blood	Ionic Strength	P.B.S.	Distilled Water	Glucose W/V percent
BIRDS	0.145	4	6	1.0
MICE RATS	.217 .217	6	4	1.0

SEPARATION OF TRYPANOSOMA BRUCEI FROM PARASITAEMIC AVIAN BLOOD by DE 52.

It was shown by Brown (1933) that many infective organisms have a lower electronegative charge than mammalian blood cells. On the basis of that Lanham and Godfrey (1970) demonstrated that several species of salivarian trypanosomes can be completely freed from mammalian blood cells by eluting infected blood through columns of DE 52 using buffers of appropriate ionic strength (0.217, pH 7·90.

The DE 52 cellulose column, though, originally described for use in mammals, has been successfully applied to the separation of Trypanosoma brucei from the infected blood of domestic chickens.

DE cellulose (DE 52, Whatman) was equilibrated initially with phosphate buffered saline (NaCl, 0.04M, phosphate 0.055M and glucose 0.5M). It is then freed from /

from fine particles by repeated sedimentation and decantation of supernate and resuspension in P.B.S. The final resuspension was made in phosphate-buffered saline with glucose and heparin. The column was set up by adaptation of normal glass tubing (2 cm. x 60 cm.) with one end drawn out. To the drawn out end was attached a rubber tubing and a pair of clips to regulate the outflow of eluate. A thin layer of absorbent cotton wool was used to support the cellulose inside the column.

The slurry was decanted into the column and was washed down with buffer until it has been adequately packed e.g. no cracks or air bubbles trapped in. The cellulose was covered with a layer of surgical gauze to prevent disturbance of the surface of the cellulose layer when adding blood, or eluting buffer. Three ml. of blood was collected from an infected cockerel from the brachial vein into an equal volume of phosphate-saline-glucose pH8, containing heparin at ten I.U. per ml. of P.S.G. A small volume of slurry was added to the diluted infected blood and this was kept at room temperature for ten minutes. The mixture of diluted infected blood and slurry was gently layered on top of an ion exchange cellulose column.

The flow of eluate was regulated to about twenty drops per minute. Collecting tubes were maintained in an ice-water-bath but the column itself was kept at room temperature. The eluate was centrifuged at 500g. for ten minutes. Supernatant was siphoned off.

/...

EXAMINATION OF SEDIMENT.

The sediment was reconstituted in 0.2 ml. of glucose phosphate buffered saline.

Two drops were deposited on a clean microscope slide a 22 mm. x 22 mm. cover slip was applied to the deposit on the slide. The edges of the cover slip were sealed with vaseline. The wet mount was scanned under the light microscope using a X10 objective and a X10 eyepiece.

Five such preparations were required to accommodate all of the suspension. These were examined under X10 x 10 under ordinary microscope. No avian erythrocytes or leucocytes were seen in the sediment. A total of nine motile trypanosomes were observed from the five preparations. Microscopy was rather laborious and time consuming. All of the whole film in each preparation had to be scanned. On the average it took about eight minutes to examine each wet film preparation.

It can, however, be said that this is an efficient method for confirming trypanosome infections in chickens.

GIEMSA STAINED THIN FILM.

Stock Giemsa Stain Solution (Gurr) was diluted 1:10 in buffered distilled water pH 7.2-7.4. Thin blood film was prepared as described in 'Techniques with Trypanosomes' (Lumsden et al, 1973). It was air dried for 5 minutes and later fixed in methanol for ten minutes. The /...

The slide was stained in Coplin jar, containing diluted Giemsa stain, for 3 hours; it was rinsed for 10 seconds in running tap water and was placed upright to dry.

The film was examined directly with an oil-immersion objective.

Thick Film.

Prepared as described by Baker (1970).

ESTIMATION OF PARASITAEMIA IN MAMMALS.

Wet film from peripheral blood was examined microscopically and the number of trypanosomes expressed as a logarithmic$_{10}$ organisms per ml. after the method of Herbert and Lumsden (1976). Briefly, this method consists of matching the number of organisms seen in a microscope field at magnification of X400 with eight circles drawn on a chart, representing different numbers of organisms in a field. The first circle contains about two organisms and corresponds to $\log_{10}$ 6.9 organisms per ml. and the last circle contains more organisms than blood cells present and corresponds to $\log_{10}^{9}$ organisms per ml. When parasitaemias are low, up to 20 fields corresponds to $\log_{10}^{5.4}$ organisms per ml.

The immunofluorescent method, depends on the detection in tissues of homologous antigen-antibody complexes in which one of the components has been conjugated /

conjugated with a fluorescein dye. These complexes are detected as fluorescent microprecipitates by means of an ultraviolet microscope. There are two basic types of fluorescent antibody technique i.e. the direct and indirect methods. In the direct method, the specific antiserum itself is conjugated to a fluorescein dye and applied to the material tested. In the indirect or sandwich technique, first the unconjugated specific antiserum is applied to a test specimen to allow a combination between antibody and antigen. The specimen is then washed free of excess antiserum and treated with a fluorescein labelled antibody which is directed against the species of antiserum. Thus the central reagent in the technique is the antibody which must be available as a high-titred, highly avid and very specific antiserum.

Ever since Coons, Creech and Jones (1941) used anthracene to achieve fluorescent labelling, modifications have been applied to the methods and chemicals used (Nairn 1969).

The use of immunofluorescent technique in the diagnosis of trypanosome infection was first described by Sadun et al (1963). Improving on the method devised by Sadun et al, McLean and Nakane (1974) suggested that stabilation of the surface antigens on trypanosomes by mild fixation might improve on the immunofluorescent technique to the point where variant specificity could be /

be retained without distortion of the parasites' morphology. The indirect immunofluorescent method described by Van Meirvenne et al (1975) offers yet another improvement as regards the identification of the antigen types present in a population of blood stream trypanosomes. Recently, Nantulya and Doyle (1977) described a method by which living blood stream trypanosomes could be preserved without deterioration of the variant specific surface antigen; thus offering further improvement to the practical application of the method. For a quick, on-the-spot laboratory use, the method of Van Meirvenne et al is adopted in this investigation.

HUMID CHAMBER FOR CLONING.

Two flaps at the top of a cardboard box 45 cm. x 45 cm. x 15 cm. were permanently sealed with sellotape while the other half was left intact so that it can be easily manipulated. A bowl of lukewarm water was kept in the box to keep the inside humidified.

Diluted trypanosome suspension, syringes loaded with 0.1 ml. with guinea pig serum and drawn out tubes are kept in the humid chamber. The flaps of the box are kept closed except when trypanosome dabs are being made on the glass tray.

/...

PLATE 3.

CARDBOARD BOX ADAPTED TO PROVIDE A HUMID CHAMBER

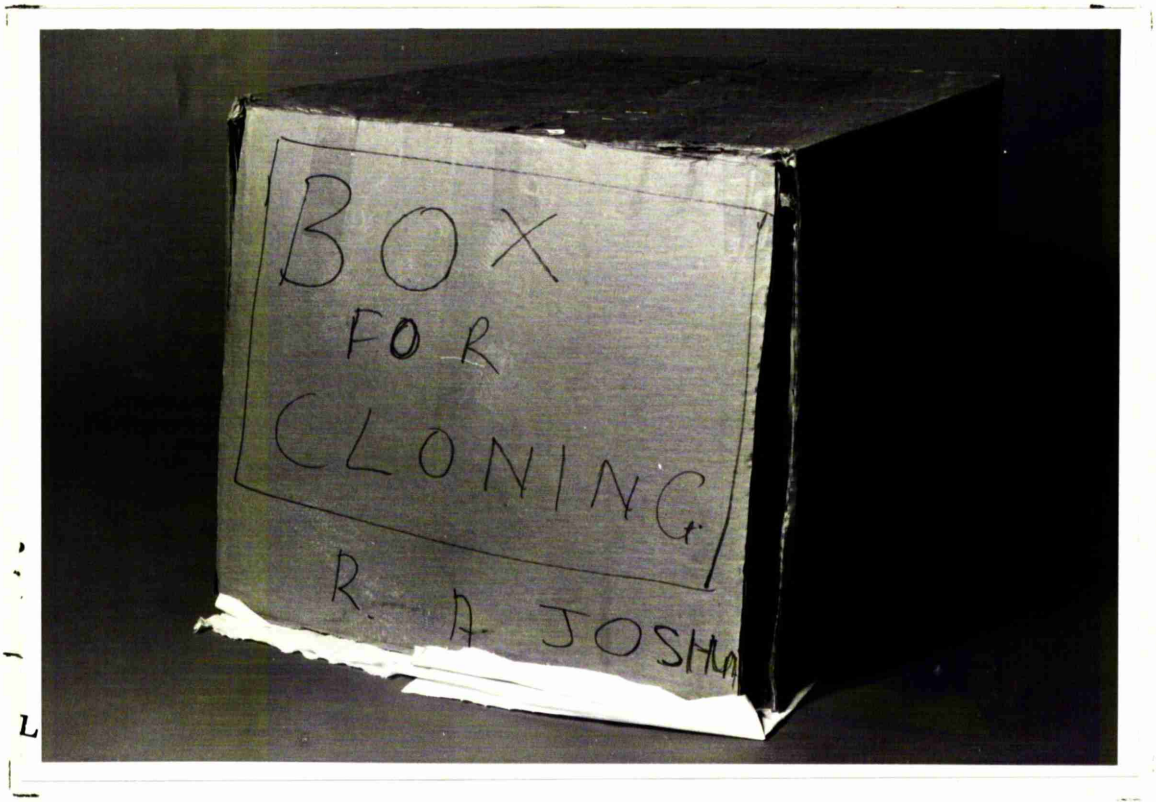


PLATE 3b.

THE HUMID CHAMBER AND CONTENTS

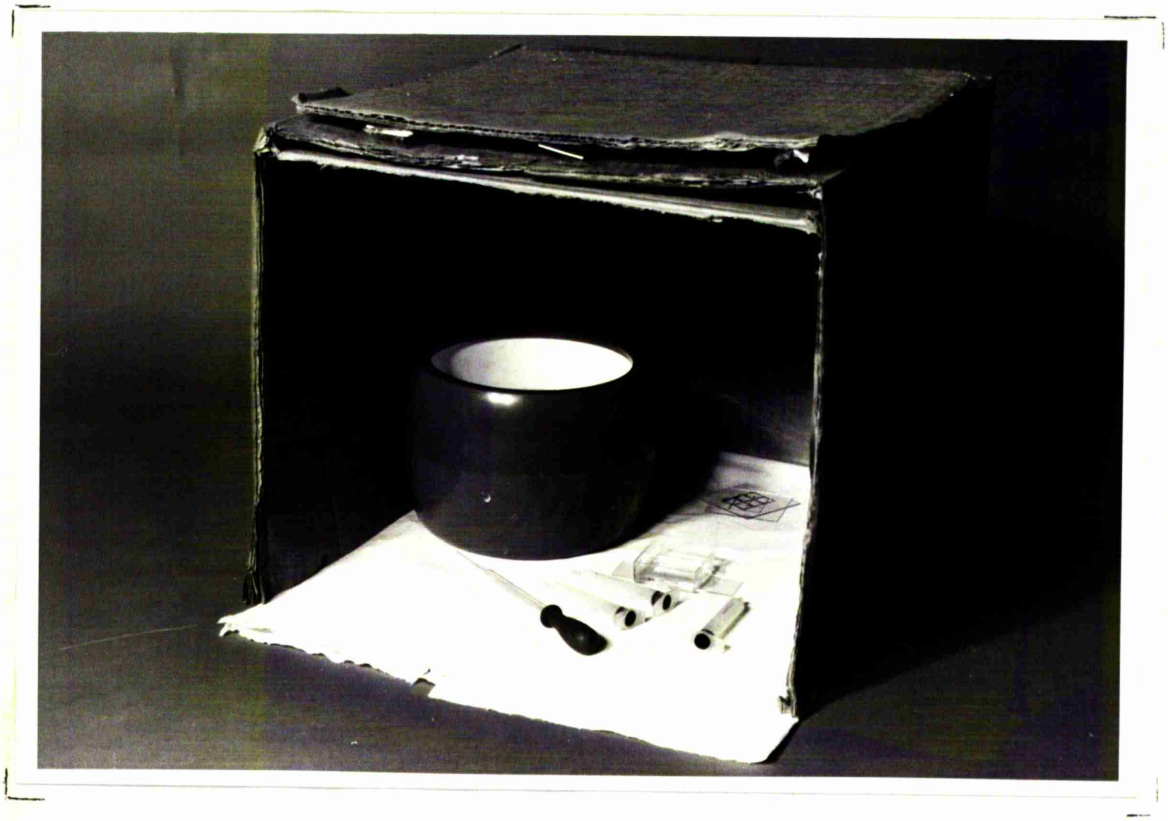


PLATE 4.

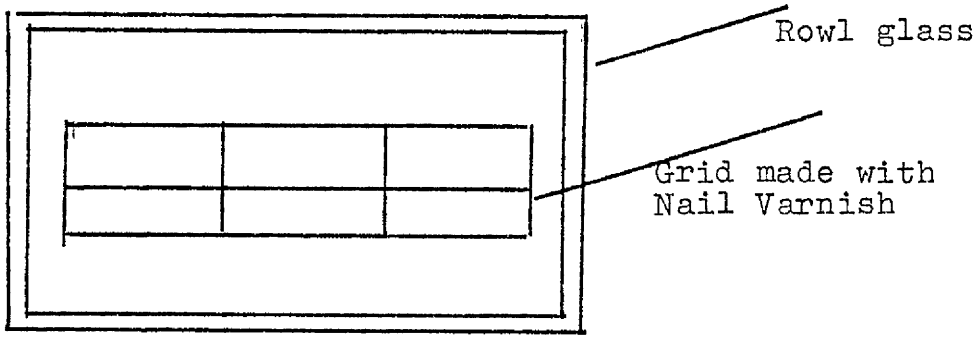
CLONING CHAMBER : CONSISTING OF A WATER CHAMBER, AND A GLASS SLIDE WITH A GRID MADE OF NAIL VARNISH.

DAMP CHAMBER.

This is made by gluing the underside of a transparent coverslip box 3.5 cm. x 3.5 cm. x 1.2 cm. to an ordinary microscope slide with UHU adhesive (a transparent glue).

The chamber is half-filled with lukewarm water (37°C). The insertion of two fingers in the water for two minutes keeps it at a suitable temperature for appropriate condensation. The slide-base is always placed on the microscope stage.

MOUNTING GLASS TRAY.



Rowl transparency cover glass 5 cm. x 5 cm. was modified for use. A grid was made on the glass sheet with a red nail varnish, making out two rows of three cells each. This gave six chances of finding a single organism.

Drawn-out-Tubes. A 50 µl capillary tube was flamed in the middle with a microburner. When it became pliable, it was drawn out. Some fine part was snipped off using the carborundum disc.

/...

CAUTERY.

For debeaking fowl's upper beaks, this was improvised from a flat filing saw flamed red-hot before use. It was mounted on a wooden handle to prevent conduction of heat to the operator's palm.

DOMINICI STAINING TECHNIQUE

Tissues were stained for 6 minutes in 0.5% Eosin, rinsed in water, stained for 1 minute in 0.5% Toluidine blue, rinsed in water and finally dehydrated in alcohol. Stained tissues were cleared in xylene, and mounted in Canada balsam.

DIETHYLAMINOETHYL CELLULOSE (Whatman, DE 52).

This is a microgranular form, fully swollen and supplied wet from:

Whatman Ltd., Springfield Mill, Kent, U.K.

It is used as a bed material for separating trypanosomes from blood cells, using buffer of appropriate molarity and pH.

COLUMNS FOR SEPARATING TRYPANOSOMES.

For experiments with up to 0.20 ml of blood, the slurry of equilibrated DEAE cellulose was packed in 2 ml. plastic syringe barrels. When the blood volume is up to 5 ml., separation was carried out in a 60 ml. syringe barrel.

Columns are set up as shown in (Plate 8). For very large quantities of blood a glass pipette (2.5 x 60 cm.) was used.

/...

LABORATORY ANIMALS.

All animals used in the experiments were bred in the Animal Unit of the Department of Bacteriology and Immunology.

DOMESTIC CHICKENS (GALLUS DOMESTICUS)

Babcock B380 strains were used. These birds were not vaccinated against any infectious diseases.

MICE.

Adult (24+ weeks) Balb/C and CBA strains were used for diagnosis of infection; raising monospecific antisera and isolating clones of trypanosomes. Mice offer several advantages in this study; because T. brucei brucei readily grow in them and mice do not require much space. The difficulty of obtaining a large volume of serum is, however, a limitation.

RATS.

Charles River rats were used for growing trypanosomes when a large quantity of parasitaemic blood was required for separation. Red blood cells for heterophile antibody tests were obtained from this same breed of rats. For reproducibility of results two sets, each of 4 rats, were kept for blood supply.

GUINEA PIGS.

The Dunkin Hartley breed was used for raising the antiserum used in fluorescent antibody investigations.

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RABBIT.

The New Zealand White was used.

DUCK.

Adult male Aylesbury Cross were used. The ducks were about 2 years old by the time they were challenged with trypanosomes. All were kept in deep litter system and were fed on duck pellets.

INOCULATION OF ANIMALS.

BIRDS.

Intravenous route: all intravenous inoculations were made in the brachial vein. For most injections, 25G needle was used.

Intramuscular: The leg muscle was used. The 23G needle was generally employed.

Subcutaneous route: The inoculations are made at the base of the head some 1-2.5 cm. below the occipito-atlantal joint.

Intraperitoneal injections are made, as described by Herbert (1978).

RAISING TRYPANOSOME ANTISERUM.

All mouse anti trypanosome sera were raised by infecting mice or with stabilate material from the 'type-type'.

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MOUSE ANTI-TRYPANOSOME SERUM.

Groups of mice were infected with stabilate material whose variable antigen type is known. Patent parasitaemia was confirmed by visual microscopy of wet blood smear. On day 3 all mice showing parasitaemia of antilog 7.8 and above were treated with Berenil (diminazene aceturate) at 7 mg/kg. Cure of infection was confirmed by complete remission of parasitaemia on day 4-5 post infection. By day 9 post drug treatment all mice are bled for antiserum. All antisera from the mice are pooled and stored at -20°C.

The highest trypanolytic and agglutinating titres for each batch of antiserum were assayed and recorded.

GUINEA PIG ANTI TRYPANOSOME SERUM.

This antiserum was used for the indirect fluorescent technique. To reduce non-specific staining care was always taken not to inject mouse blood cells into guinea pigs, since such erythrocytes may stimulate antibodies against mouse cells.

The source of antigen in this case was motile trypanosomes previously separated from blood cells by the method of Lanham and Godfrey (1970). The guinea pig was infected with 2×10^8 organism and day 3 the infection is terminated by chemotherapeutic treatment with Berenil at 7 mg/kg. The guinea pig was bled for serum on day 9 post treatment.

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A common finding with some moderately virulent trypanosomes is that parasitaemia is not heavy enough (less than $10^{7.8}$ per/ml of guinea pig blood) on day three. In such cases the infection is allowed to go into remission; usually about 11-13, and the guinea pig is bled for antiserum on the next day after the first parasitic remission.

Preparation of Fluorescein labelled Antisera.

The globulin component of hyper immune sheep anti guinea pig antiserum was precipitated by dropwise addition of saturated ammonium sulphate to a final concentration of 50 percent. All operations were carried out at $4^{o}C$. The mixture was mechanically stirred for 1 hour and then centrifuged at 6,000 r.p.m. (750g) for 10 minutes. The precipitated globulin was resuspended in 50% saturated ammonium sulphate, stirred for 15 minutes and centrifuged as before. The precipitate was then dissolved in 0.15 M saline and dialysed against saline to remove ammonium and sulphate ions. The dialysate was changed at least every 8-16 hours. Dialysis was complete when sulphate ions could no longer be demonstrated in the dialysate as indicated by a negative result in the following test:

2 ml. dialysate + 4 drops of 0.1M HCL +

+ 3 drops 2% barium chloride.

White precipitate due to the formation of barium sulphate.

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The globulin solution was removed from the dialysis sac (Visking tubing, Scientific Instrument Centre Ltd., London) and centrifuged at 500 g for 10 minutes to remove insoluble aggregates. The protein content of the solution was measured on an SP 800 Unicam U.V. spectrophotemeter at 280 nm. An optical density of one was assumed to be equivalent to a concentration of 1 mg. globulin per ml.

Conjugation with FITC.

This is based on the method of Cherry, Goldman and Carski (1960). FITC (1.5 mg.) was dissolved in 1.5 ml. of 0.5M carbonate-bicarbonate buffer (pH 9) and added dropwise to 100 mg. of the globulin solution in an ice-water-bath on a magnetic stirrer. After thorough mixing the pH was checked and adjusted to 9 by adding more buffer if required. The final mixture was stirred mechanically for 18 hours at $4^{o}C$.

Removal of Unreacted Fluorescein.

The high pH in which the above reaction was carried out leaves some unreacted fluorescein as well as FITC conjugate globulin. The mixture was passed through a column of 6 gm. Sephadex G25 equilibrated with PBS, to obtain the FITC conjugated globulin (pH 7.2).

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Removal of Non-Specific Fluorescence.

The present conjugate consists mainly of protein linked to fluorochrome. It is usually found, however, that some molecules will be overlabelled with fluorescein and thus have a high negative charge. These molecules tend to be absorbed in a non-specific manner to the tissue and this clouds true specific staining.

These were removed by passage through a column (30 cm. x 5 cm.) of DEAE cellulose (DE22) (1 gm. per ml. of conjugate) equilibrated with P.B.S. (0.15M) and the first peak collected and/or later treated for double absorption with acetone-lyophilized chicken liver powder (100 mg. per ml. conjugate) prepared by the method of Coons & Kaplan (1950). Absorptionswere also done with washed chicken red blood cells.

The final conjugates were stored in either of two ways

(1) at -20°C, no azide added

(11) at 4°C and one drop of 1% azide was added to prevent fungal or bacterial growth.

Just before each use, conjugates were spun at 500 g for 10 minutes.

To demonstrate the presence of chicken Immunoglobulin.

Cryostat sections of spleen were rehydrated in PBS for ten minutes.

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Fluorescein-labelled anti chicken immunoglobulin was applied directly to the tissue section and incubated in a humid chamber for 20 minutes. Unreacted fluorescein was washed off and the section was mounted and examined as previously described.

To demonstrate the presence of Antigens in Chicken Spleen H.S.A.

Fluorescent microscopy methods already described by White, French and Stark (1970) were followed.

The tissues were mounted in phosphate-buffered saline and examined microscopically with Leitz Ortholux fluorescence microscope fitted with an Osram HBO 200 high pressure mercury arc, oil immersion objective (x 54) and a Ploem-type incidence form of illumination. The exciter filters were BG12 (2mm), BG38 (3mm) and Schott interference filter KP490 (the barrier filter was Wratten gelatin number 12 or Schott 091).

Measurement of Serum 19S - Immunoglobulin levels in Chickens.

Serum 19S - globulin levels were measured by the radial diffusion method of Mancini, Carbonara and Heremons (1965). For this one part rabbit anti-chicken 19S - globulin was mixed with nine parts molten 2% Agarose at (60°C) and then poured on well-cleaned microscope /

FIGURE 3.

Quantitative precipitin assay for measurement of chicken serum 19S. Immunoglobulin levels (mg) in relation to the square of the precipitin ring diamter. (The points marked are for standards).

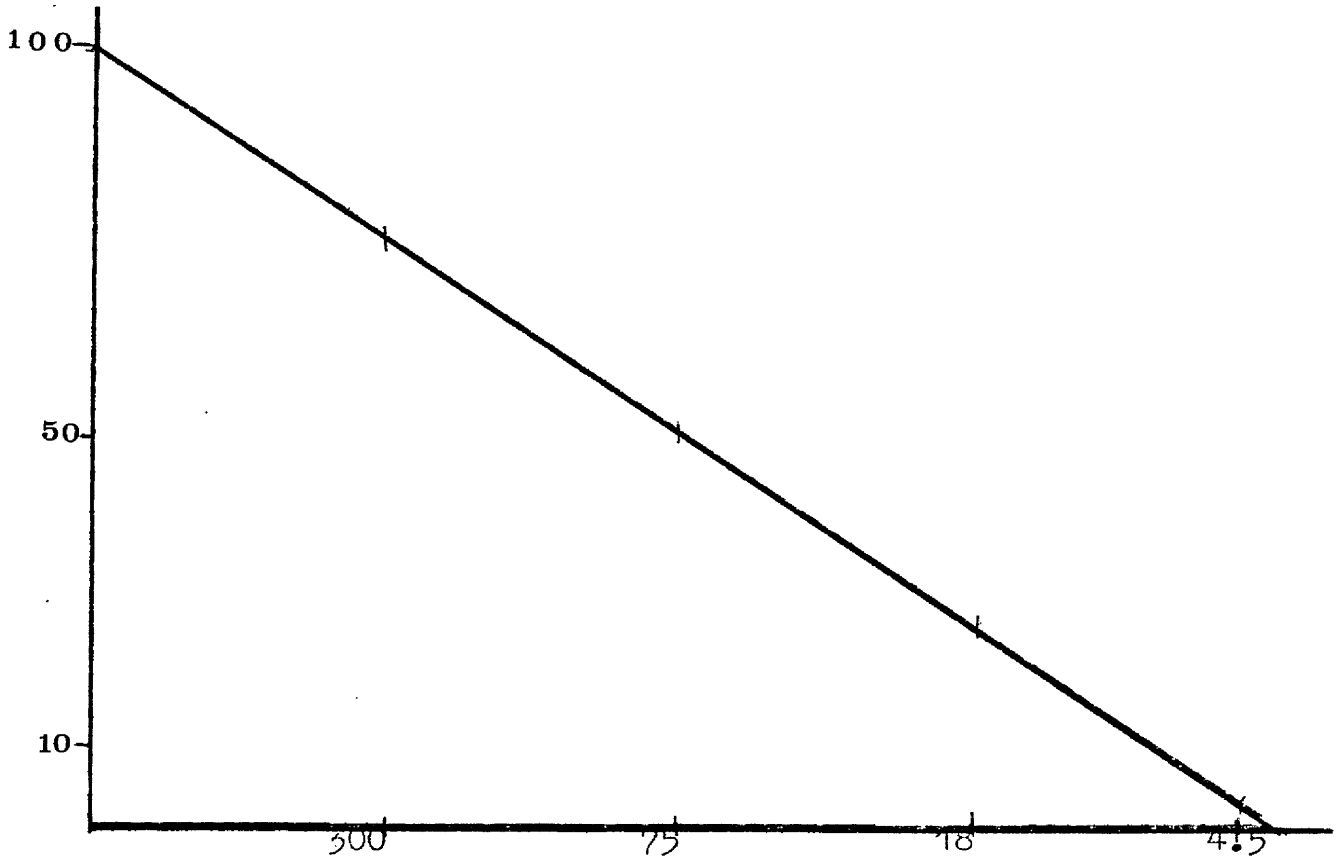
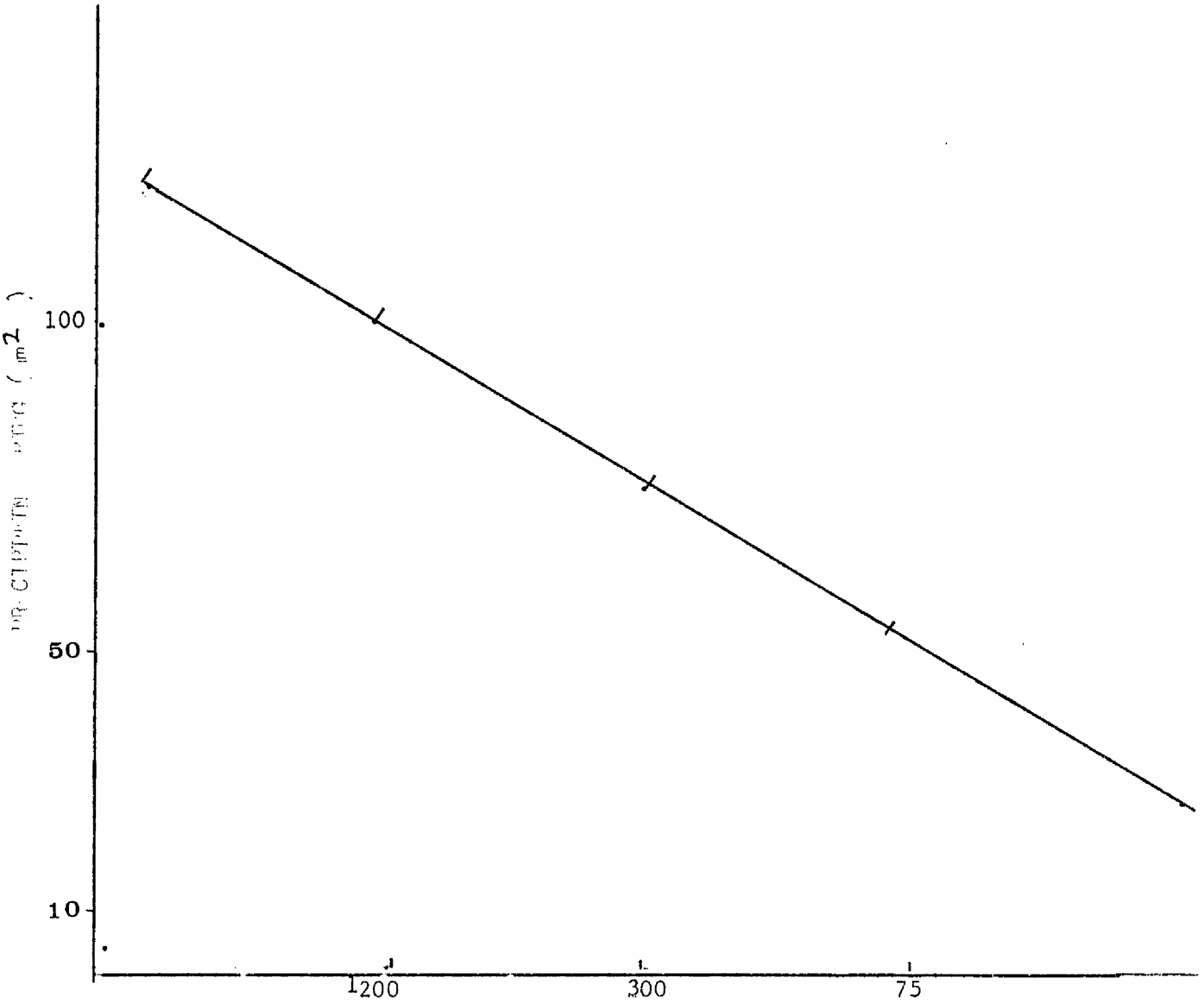


FIGURE 4

Quantitative precipitin assay for measurement of chicken serum 7s Immunoglobulin levels (mg) in relation to the square of the precipitin ring diameter (mm^2).

(The points marked are for standards)



microscope slides. Care being taken to ensure an even distribution of the agarose-anti-immunoglobulin colloid. When set a series of six wells were cut in it and 3 wells were each filled with 5 µl of 3 different known concentrationsof a purified preparation of chicken 19S-globulin. The other wells were each filled with 5µl of test antiserum. After 48 hours incubation at 4°C in a damp chamber (to prevent the agar/drying out) a ring of precipitate was found to have formed around each well. The diameter of this was proportional to the concentration of 19S - globulin in the well The amounts of 19S - globulin actually present in the test antisera were calculated by comparison with the result obtained for the three standard solutions of 19S - globulin, Fig.3

Measurement of Serum 7S - globulin levels in Chickens.

This was carried out as described for 19S - globulin except that Rabbit anti chicken - 7S - globulin was mixed with agarose and that purified preparation of chicken 7S - globulin filled the wells, Fig 4.

Radio-Iodination of Protein.

Proteins were radio-iodinated by the direct oxidation technique of Hunter and Greenwood (1962) using chloramine T and Thiosulphate-free iodine - 125 (Radio chemical Centre, Amersham, England).

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In general, 1 mg. protein in 0.1 ml. PBS was added along with 0.25 mg. chloramine T in 0.25 ml. PBS to 1 mg. iodine 125. The mixture was manually shaken and allowed to interact for 3 minutes before adding 0.12 mg. sodium metabisulphite in 0.05 ml. PBS and 2 mg. potassium iodide in 0.2 ml. PBS.

Free iodide ions were separated from labelled protein by passage through a G25 (coarse) Sephadex column (30 cm. x 1 cm.) in a fume chamber. The concentration of resultant iodine-labelled protein was calculated from optical density at 280 nm wavelength in a Unicam SP800 spectrophotometer, and the percentage of protein-bound radio-label determined by precipitating the protein in 10% trichloro acetic acid in saline and counting the radioactivity of the precipitate and supernate.

Measurement of Serum antibody level to H.S.A.

This was carried out by the Farr Test (Farr 1958).

H.S.A. was trace-labelled with Iodine-125 (Supplied as preparation IBS-30 from the Radio Chemical Centre, Amersham, England) by the direct oxidation technique of Hunter and Greenwood (1962). It was used at a concentration of 0.5 µg per ml.

Tubes containing undiluted test serum and test serum diluted in a doubling series from 1:2 to 1:128 in normal /

normal chicken serum, in 0.1 ml. volumes, were set up. To each sample was added 0.2 ml. of 125 Iodine-H.S.A. solution (0.1 mg.). The tubes were stored overnight at 4°C to allow the antibody to combine with the antigen. By the addition of 0.2 ml. saturated ammonium sulphate to each tube the antigen-antibody complexes were precipitated out from the free antigen.

The tubes were allowed to stand for two hours at 4°C and were subsequently centrifuged at 850 g for 30 minutes. The supernatants were removed and the precipitates were washed once in 0.5 ml. 40% saturated ammonium sulphate then centrifuged. Four tubes containing 0.1 ml. 10% normal chicken serum were included in the test. One pair was treated as were the other sera under test to determine the minimal precipitations of iodine 125 in the absence of antibody. To the other pair of tubes 0.6 ml. 10% trichloroacetic acid (T.C.A.) was added to determine the maximum precipitable radioactivity by I^{125} - H.S.A. The precipitate in this case was washed in 0.5 ml. 10% T.C.A.

The precipitates from all the tubes were counted for radioactivity in the automatic gamma counter.

Calculation of Antigen Binding Capacity.

The method of linear interpolation has two disadvantages. Firstly, it introduces a small systematic error due to the fact that the Farr test

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test binding graph is not linear, and this error can become very significant if it is occasionally necessary to extrapolate rather than interpolate.

Secondly, it makes use of only two experimental points on the graph, and the value obtained for the antigen binding capacity is therefore more sensitive to experimental error than it needs be if all the points on the graph were used.

A method developed by Dr. McKay (Personal Communication) in this department was therefore used. Briefly, for every experimental point on the binding curve, an estimate of the antigen binding capacity was obtained by means of the formula

$$x = \frac{0.3\ CD\ \log F}{\text{Log}\ (1-0.3)}$$

where C = Final concentration of antigen in the reaction mixture.

D = Final dilution factor of serum in the reaction mixture.

F = Proportion of antigen remaining free after equilibration.

From all eight values of x, was calculated the best final estimate of antigen binding capacity ABC-33⅓.

Marking of Animals.

Marking experimental animals for identification is very important in laboratory studies. Such marked

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animals are regularly checked for continuing presence of the mark.

Balb/C mice, rats and guinea pigs were marked with picric acid as described by Lumsden, Herbert and McNeillage (1973).

C.B.A. mice

Are marked by punching their ears as described by Lumsden, Herbert & McNeillage (1973).

Domestic Fowls.

Serially numbered wing tags and coloured leg bands were used. The wing tags were pinned to the left [illegible] of each bird. Suitable leg bands for birds of various ages were used to prevent undue compression by undersized leg bands.

Count of Trypanosome Suspension in Buffer.

Count of trypanosomes separated from blood cells are generally not done by the matching but by the method haemocytometer count.

Generally, the stock suspension is diluted 100 fold or less, depending on the concentration in the final suspension after centrifugation.

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Average count in all low corner squares of the haemocytometer are taken and the final concentration is calculated as described by Lumsden, Herbert & McNeillage (1973).

Trypanosome Infectivity Titration.

The method depends on the serial dilution of the trypanosome suspension whose infectivity is to be measured; a suitable medium is therefore very essential. To minimize metabolism and reproduction of the trypanosomes the suspensions were kept at 0^{o}-2^{o}C. To minimize the possibility of bacterial contamination, the work should be carried out with aseptic precautions. The response of the individual animal is regarded as 'infected or not-infected' or on a basis of examination of tail blood from days 3-30 after inoculation. The end point is estimated on the basis of the distribution of infectivity. The 63 percent is used.

Titration of parasitaemic mouse blood (stabilate).

This is done essentially as described by Lumsden et al (1963).

Infectivity Titration of Infected Chicken Blood.

Necessary precaution of starving mice overnight was taken. . All other steps were

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carried out as before except for the following modifications. Five tenfold dilutions were made in solution AB at 4°C and pH 8.0 in nine ml. volumes (one ml. being transferred at each dilution step). The dilution ranged from neat to 1/5. Mice in six groups of 6 were each inoculated intraperitoneally with 1 ml. of appropriate dilution. Even though large volumes were inoculated no leaks were observed. The mice were checked daily for parasitaemia from day 5; observations were made until day 20 post inoculation.

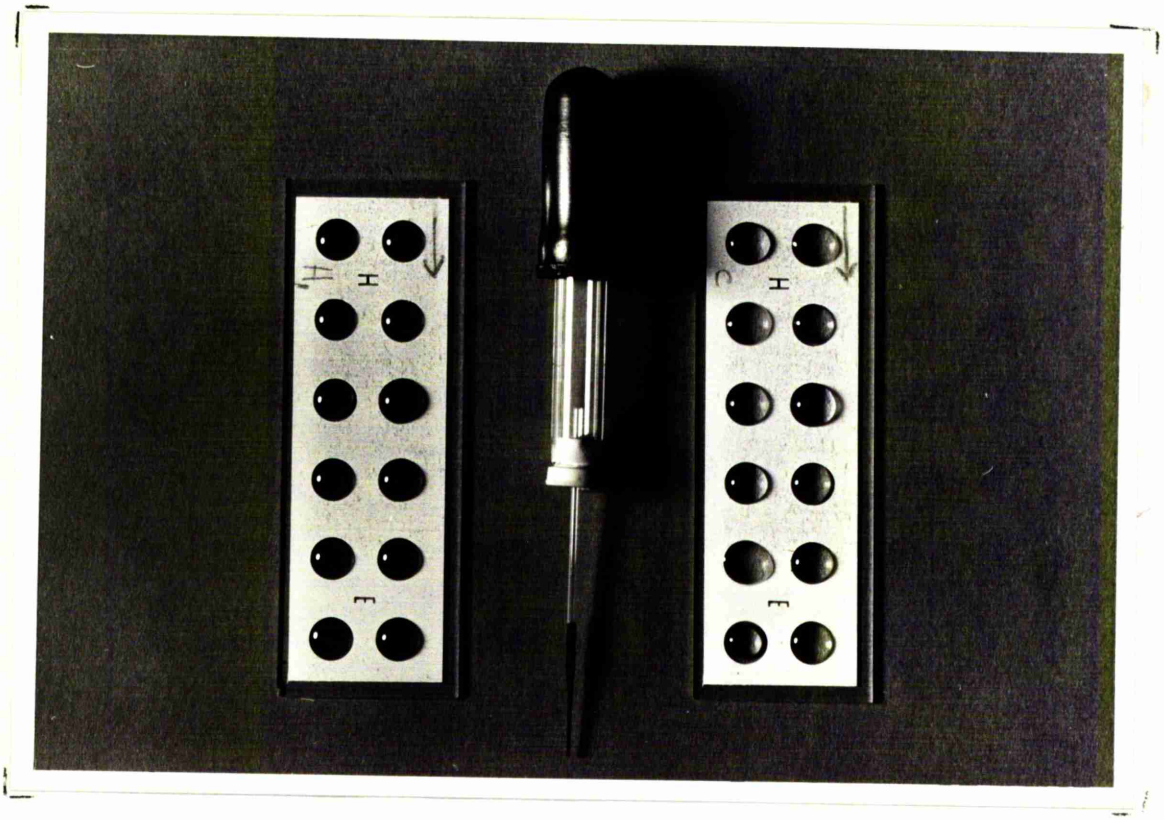
From the tables of Lumsden et al (1963) the number of infective organisms (ID_{63}) per ml. of blood was calculated.

Slide Agglutination Test.

This was carried out essentially as described by Cunningham and Vickerman (1962). With some minor modification, serial two fold dilutions of the antiserum were made in solution AB (pH8) in WHO agglutination plates. Nine dilutions of antiserum (10-5120) were set out at 30 µl drops on Teflon slides in a humidity chamber. Starting with the highest dilution stabilated antigen was added to these drops from a drawn out capillary tube. The antiserum and antigen were incubated for one hour in the humidity chamber Plate 5.

PLATE 5.

TEFLON SLIDE AND HOLEY BLOWER USED FOR AGGLUTINATION TEST.



The resultant reactions, assessed under the ordinary light microscope using X10 objective and X10 eyepiece, were expressed by plus and minus signs. Only agglutinations above two pluses are regarded as diagnostic. This test is very applicable to those trypanosomes which grow in high concentrations (over $10^{7.8}$) and preferably with minimum blood cells at the time of use (stabilated material).

Trypanolysis Test.

This test detects surface variant antigen like the agglutination test. It differs from agglutination test in that it is more specific and unlike agglutination test which is best carried out with a stabilate, trypanolysis uses fresh infected blood. Stabilates normally contain some dead trypanosomes which may confuse the real lysis occurring during the test.

A mouse is infected with stabilate material. On day three post inoculation 54 µl of parasitaemic blood ($10^{8.1}$ organisms per ml. of blood) is collected from the mouse in a heparinized capillary tube and this is diluted to 300 µl in fresh guinea pig serum. The dilution of the mouse infected blood depends largely on the parasitaemia. Using an automatic pipette, 30 µl of fresh guinea pig serum is dispensed into each of eight agglutination wells.

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Two fold serial dilutions of the test antiserum were made such that each well contained equal volumes of diluted antiserum. Dilutions ranged from 10 - 1280. Starting with the highest dilution, each well was seeded with 30 µl of diluted infected blood. The wells covered with sellotape to prevent evaporation were incubated at 26°C for 30 mins. Using an unplugged Pasteur pipette small samples (10-15 µl) were removed and examined under the phase contrast microscope. One hundred trypanosomes are counted and the percentage of lysed among total trypanosomes seen recorded. The lysed trypanosomes were afloat while the viable ones were motile.

The reciprocal of the highest dilution that lysed over 95% of the trypanosomes seen is taken as the titre.

Trypanolysis test on avian Antiserum.

The antibodies of domestic chickens are incapable of fixing guinea pig complement (C1). Preliminary tests were therefore carried out to evolve a standard method of assaying the trypanolytic titre of sera collected from infected chickens.

Serial dilutions of antiserum were carried out in a serum mixture of

3 parts fresh chicken serum

1 part fresh guinea pig serum.

Diluted infected mouse blood in chicken serum was used.

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A duplicate experiment was carried out with antiserum dilutions in guinea pig serum only and in diluents made as shown above. Test suspensions incubated in guinea pig serum alone did not lyse the trypanosomes at high serum dilutions.

Subsequently, all trypanolysis tests with avian antiserum were carried out in dilutions made from a mixture of fresh chicken serum and fresh guinea pig serum. The incubation time is the same as for mouse serum.

Blood Incubation infectivity Test.

This test is performed essentially as described by Rickman and Robson (1970) with the following modifications.

Stabilate material or heparinized blood from trypanosome infected mouse is used. For each stock to be tested paired samples were used; i.e. Sample one, constituting the control was incubated with rat serum. The test medium was normal serum from a man that had never visited a trypanosome endemic area. For the test sample 10^4 mouse infective doses (ID_{63}) of trypanosomes were incubated in 1.5 ml. of human serum. The same quantity of trypanosomes was added to normal rat serum.

Each sample was mixed by gentle rotation and incubated at 37°C for 4 hours; and then separately inoculated intraperitoneally into mice. Each mouse

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received 0.5 ml. of suspension. Wet films were made daily from all inoculated mice as from day three till day 30 and were examined using X40 objective with X10 eyepiece. One hundred microscope fields were examined on each slide. In vitro effect of the sera was checked by direct microscopic examination of samples from each medium after 3 hours of incubation.

Interpretation of Results.

A positive result is obtained where both test and control animals developed a parasitaemia; a negative result where the control animal does but the test animal does not.

Rickman and Robson (1970) made allusion to mortalities in mice in this test. During the period of this investigation over forty mice were used for this test and in no cases did any accidental death occur. It is suggested that a skilful injection of the inocula[illegible] is essential. Any accidental stab on the celiac vessels may result in a serious abdominal haemorrhage culminating in a sudden death.

Aspiration of Bone Marrow from Chicken.

Bone marrow samples in this study were obtained from the femur with femoral tibial joint as the point of incision.

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Bone biopsy required two individuals; one (Assistant) to hold the bird, and the other (Operator) to obtain the bone marrow sample. For ease of manipulation the left leg was preferred to the right. The fowl was placed on its back with the feet directed towards the assistant and neck bent backwards to the assistant's left side. The head, right wing and right leg were held by the assistant's left hand, the left wing was folded beneath the body, and the left leg was secured by the right hand. With a slight twisting motion of the assistant's wrist, the left leg was rotated externally and pushed forward so that the femuro-tibial joint faced the operator. In this position the assistant could easily restrain movements of the bird thus permitting the operator to obtain the marrow without interference.

The area of the body surrounding the femuro-tibial joint was defeathered and disinfected with either 70% alcohol or Hibitane(R). The prominent inner anterior crest of the bone was located by palpation. A sterile needle, eighteen gauge by four inch, was introduced with a steady but firm pressure through the head of the femur into the marrow cavity. The needle when in place, lay in a plane parallel to the long axis of the bone. A heparinised ten ml. syringe was then fitted to the needle. A steady even pull on the syringe plunger aspirated the bone marrow.

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After the bone marrow had been aspirated, the needle was quickly withdrawn, the punctured wound bathed in alcohol and the bird watched for a few minutes for haemorrhage. If no haemorrhage was observed the bird was released.

Identification of Trypanosome Variable Antigenic type by I.F.T.

Trypanosomes, T.b. brucei variant antigen type WITat I, was obtained from a mouse three days post inoculation with WIG72. Thin blood films were prepared on slides with a frosted end. The slides were air dried and were later fixed in acetone for ten minutes.

Antiserum.

Antiserum was raised in guinea pig. Slides were rehydrated in phosphate buffered saline in a Coplin jar for five minutes. Each slide was removed and dried with an absorbent paper except for the area around the blood film.

The slides were then kept in a humid chamber. One drop of the diluted antiserum was added to the tissue. The humid chamber was covered while the antigen and antiserum were being incubated at room temperature. After 30 minutes the slides were immersed in the Coplin jar filled with fresh buffer. The jar was then placed on a Microid flask shaker (Griffin and Tatlock Ltd., London) running at a slow speed so that excess unreacted antibodies

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were gently washed out. Washing continued for 30 minutes following which the excess P.B.S. was removed, except from the blood film itself, as described above. The slides were replaced in the humid chamber.

One drop of fluorescein labelled sheep anti-guinea pig immunoglobulin was placed on each slide. A control slide which had not previously been reacted with anti-serum was also used, this slide was stained with conjugate only.

The slides were kept in the humid chamber for 30 minutes. They were then washed in the buffer for 30 minutes during which time the buffer was changed six times. All the slides were individually mounted in 15% glycerol in buffer.

The above procedure was repeated with the following modifications:

(1) Counter staining with Evans blue.
After the last wash in P.B.S. the slide was dipped in 0.01% Evans blue solution for 15 minutes.

(11) Absorbing both the conjugate and the antiserum with mouse liver powder.

Both were found to increase the specificity of the technique.

Slides were examined under the U/V light using Leitz Ortholux fluorescent microscope.

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ADAPTATION OF ORBITAL BLEEDING TECHNIQUE TO PASSAGE OF TRYPANOSOME CLONES.

During investigations involving growing trypanosome populations from single organisms, it became obvious that tail bleeding would not yield enough blood of mice and would not yield enough infected blood to ensure successful transfer of trypanosomes three days post inoculation of a single organism. From past experience, it was calculated that each trypanosome undergoes binary fission once every six hours. If each mouse carries a blood volume of two ml. by day three, when an infection is to be passaged, one would expect a successful inoculation to have yielded a trypanosome concentration of two thousand and forty eight parasites per ml. of infected mouse blood. It is to be remembered that not all trypanosomes present in the blood are equally infective. Tail bleeding could yield as much as 0.1 ml. of mouse blood if properly carried out whereas, orbital bleeding could yield one ml. of mouse blood. Orbital bleeding, therefore, ensures higher inoculum size and thereby a rapid build up of parasitaemia in subsequent passages.

The technique requires a small glass pipette with a blunt tip for non-traumatic entry into the orbital plexus behind the eyeball. Test-tubes (3 cm. by 4 mm.) were labelled with an appropriate experimental number /

number and were set up in a rack to receive the blood from the pipettes.

The donor mouse is held by the back of the neck with the left hand, and the loose skin of the head is tightened with the thumb and index finger of the operator. With the index finger the eye is made to bulge slightly by further traction of the skin adjacent to the eye. The tip of the pipette is then placed at the lower or inner corner of the eye and gently but firmly alongside of the eyeball to the ophthalmic venous plexus which lines the back of the orbit. If unclotted blood is required the pipette is heparinised before use. The venous capillaries forming the plexus are ruptured upon contact with the tip of the pipette and the resulting haemorrhage filled the orbital cavity which serves as a useful reservoir. A slight withdrawal of the pipette freed from the tip of the pipette so that the accumulated blood immediately entered the bleeding tube due to blood pressure. Residual blood around the eye is swabbed clean with a soft absorbent tissue. Using the above method blood volume as much as 0.5 - 1 ml. were regularly collected from inoculated mice without any hazards to the mice. The method has also been frequently used in bleeding mice for antiserum.

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BLEEDING GUINEA PIG FOR SERUM

Bleeding guinea pigs formed a routine practice in this investigation. A regular blood supply is was obtained from these animals without killing them. Fresh guinea pig serum was used as a rich source of complement in the trypanolysis test.

A sterile five ml. syringe was secured and this was fitted with a twenty-three gauge needle. The guinea pig was anaesthesised in a glass jar of ether vapour for two minutes, the guinea pig was then taken out of the jar. A tent of loose skin was raised in the ventral neck region with the thumb and the index finger. The guinea pig was secured in a supine position by holding the two hind limbs under the left armpit. This posture kept the animal in a secured position and prevented undue twisting and shaking of the animal's body.

The xiphoid process as well as the last sternal rib was located to the left side of the animal. In the animal's position it was the near side of the person bleeding. Holding the syringe parallel to the mid-line and slanted downwards the tip of the needle was inserted caudal to the junction of the xiphoid process and the last rib. The needle was steadily advanced until the pulsation of the heart was felt. The needle was thrust into the cardiac chamber, the

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plunger of the needle was slowly withdrawn until blood started flowing into the syringe. It was found very helpful to synchronize the rate of withdrawing the plunger with the inflow of blood into the syringe.

When blood began to flow into the syringe the needle was maintained in position. Five ml. of blood were regularly collected from a donor guinea pig without any untoward effects. It is essential to immobilise the guinea pig during the whole process of blood collection by cardiac puncture. After bleeding was completed, the needle was withdrawn from the guinea pig and the animal is observed for evidence of shock.

In a few instances when the animals were not properly immobilised laceration to the heart muscle had been caused by twisting of the guinea pig's body when the needle was in the heart chamber. The laceration resulted in massive outflow of blood into the pericardial cavity causing cardiac tamponade and respiratory collapse.

The method for the collection of serum from the blood must ensure minimum destruction of the complement content. Hence such blood samples are never kept at thirty-seven degree centigrade for clotting and retraction. Blood was allowed to stand at room temperature for clot formation while still in the syringe but with the needle removed. Clot was /

separated from the wall of syringe by slightly withdrawing the plunger of the syringe. The syringe containing the clotted blood was stored in the refrigerator for five hours. Serum was poured into a test tube and was centrifuged at 2500 rpm to throw down any sediments that might be present. The serum was later dispensed into aliquots in autopots, each autopot was indexed and samples were later stored at minus twenty or minus seventy degree centigrade until needed for use. Samples for immediate use were stored at minus twenty overnight while samples for future use were stored at minus seventy.

It was found that the yield of serum from guinea pig's blood is much greater than had been found in other animals, e.g. mice, chicken and rats. Following bleeding, serum could be obtained from the blood after thirty minutes, however, for maximum yield the clotted blood was kept in the domestic refrigerator at four degree centigrade for five hours.

ISOLATION OF TRYPANOSOME CLONES.

A small drop of parasitaemic blood (about 10 µl) from an infected mouse was added to 0.5 ml. inactivated guinea pig serum. After centrifugation at 500 g. for two minutes to deposit the red cells, the supernatant suspension of trypanosomes was diluted with more guinea pig serum so that a wet smear contained one

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or two organisms per microscope field (X18 objective X10 occular). The suspension was transferred to the humid chamber where all operations except microscopy were carried out. Six tiny drops were deposited on the grid at a time and the gridded slide was immediately inverted over the damp chamber. The drops are then examined under phase contrast (18 x 10), until a drop was recognised to contain only a single organism.

The presence of a single organism in the sample as well as the absence of any other trypanosomes was confirmed by one other observer. The square containing the selected drop was marked with a grease pencil whilst the plate was still under the microscope. The whole damp chamber was removed from the microscope and transferred to the humid chamber. After the addition of a drop of serum from a 1.0 ml. syringe the whole drop was drawn back into the syringe and inoculated intraperitoneally into one mouse. Blood from each inoculated mice was passed to other mice on day three. The passage was repeated every three days to clean mice. On day six post inoculation of a single organism, all mice inoculated initially were checked for patent parasitaemia by examination of tail blood.

A RAPID METHOD OF RAISING ANTISERUM AGAINST TRYPANOSOME CLONES.

The antigenic identity of a cloned population is sometimes established by the trypanolysis test.

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A single organism is inoculated into one mouse, and parasitaemia is monitored by microscopic examination of mouse blood. The infected mouse is bled for serum one day after the first remission of parasitaemia. Generally, the first day of patent parasitaemia was found to be day six post inoculation of single organism. In the case of some extremely virulent organisms the prepatent period was shorter i.e. five days. No remission of parasitaemia was observed in such virulent organisms. The present method of raising antiserum was therefore not adaptable to use in such cases. Organisms that manifested partial remission of parasitaemia also presented a great obstacle to this method.

In all cases where there was complete remission of parasitaemia such antisera were found specific for lysis tests.

This method of bleeding mice at the first observable parasitaemia proved very time saving and less expensive in raising specific antiserum. The traditional method of infection and chemotherapy involves using many mice and waiting for almost two weeks after the clone has been isolated.

VICES IN CAGED EXPERIMENTAL BIRDS.

Vices like feather picking and cannibalism were occasionally observed in birds aged four weeks and

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and over. Attempts at reducing the light intensity in the poultry houses by either darkening the sources of ventilation or reducing the lighting up time did not completely alleviate the problem.

Effective solution was eventually provided by beak trimming.

The object is to remove about $\frac{1}{8}$-inch from the top beak. Several types of beak trimming machines are in use in commercial poultry production but all rely on an electrically heated element to achieve a cauterizing effect.

The number of birds used for experimental purposes, however, does not justify the purchase of an electrically operated de-beaking machine. Birds were de-beaked by using red-hot metal which cuts and cauterizes simultaneously.

ANAPHYLACTIC SHOCK IN CHICKENS INOCULATED WITH PARASITAEMIC RAT BLOOD.

During the course of the work reported in this thesis over seventy chickens were injected with either parasitaemic mouse or rat blood. The inocula varied from one to three ml.

In the majority of cases no untoward physical signs were observed in inoculated birds.

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On one occasion, two 5-week old cockerels were each inoculated with three ml. of parasitaemic rat blood in order to give a heavy dose of trypanosome challenge. The two birds went into anaphylactic shock immediately. The comb and wattle suddenly became pale and cold to touch and the breathing was markedly depressed.

The affected birds were immediately provided with warmth by wrapping the operator's hands round them, artifical respiration was provided by rapid and successive compression of the breast muscles. Both chickens were then transferred to a warm (37°C) compartment. One of the cockerels recovered while the other died eventually.

R E S U L T S

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CHARACTERIZATION OF TRYPANOSOMES.

Preliminary investigations were carried out to confirm the reputed identity (sub genus, species and sub species) of trypanosomes used in this investigation.

SUB GENUS IDENTITY.

Capillary tubes containing stabilates WIG25, WIG37, and WIG70 were retrieved from the cryobank (-70°C) and rapidly thawed at room temperature. Suspensions taken directly from the capillary tubes were examined microscopically under the X40 objective. Each stabilate was seen to contain actively motile trypanosomes, and one or two dead ones.

Balb/C mice in three groups of six were each inoculated intraperitoneally with each stabilate. Each mouse received about ten thousand motile trypanosomes of one stabilate only.

WIG25 produced uniformly fatal infections in all mice (Table 5). Parasites were detected in peripheral blood by day two and increased rapidly until day six. Parasite level then regressed until day 8 after which they increased steadily and then fluctuated until the infection proved fatal between days 35 and 40.

WIG37 and WIG70, on the other hand, proved 'extremely virulent' and were characterised by fulminating infections that proved fatal without

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remission of parasitaemia.

The ease with which the trypanosomes of the three stabilates infected mice suggests that they are all of the sub-genus Trypanozoon.

TABLE 5.

The course of infection of each of the three stocks of T. brucei in mice.

Primary Isolation Number	Stabilate Number	Host	Duration of Infection
Lugala/55/ EATRO/459	WIG25	Six mice	35 - 40 days
Busoga/60/ EATRO/3	WIG37	Six mice	4 days
Muvubwe/66/ EATRO/1125	WIG70	Six mice	5 days

SPECIES IDENTITY.

Thin blood films were prepared from mice infected with WIG25 and stained by Giemsa technique. Microscopic examination of those films under the oil immersion lens showed that all the trypanosomes possessed the characteristic morphology of the brucei species, i.e. small sub-terminally located kinetoplast, blunt or truncated posterior end and the presence of long free flagellum. There is therefore every reason to assume that trypanosomes of the stock Lugala/55/EATRO/459, i.e. WIG25 are T. brucei, sub species indetermined.

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SUB-SPECIES IDENTITY.

The observation reported in the previous section confirmed that on morphological grounds, the trypanosome population represented by WIG 25 is of the brucei group. Structural and even ultrastructural characters, however, have not so far proved helpful in distinguishing sub-species identity of the Trypanozoon. Further characterization was deemed necessary. More importantly, a recent accidental infection of a laboratory worker by a stock of brucei species underlines that a test of potential infectivity to man be undertaken so as to obtain a precise sub-species identity and indication of the potential hazard to human handlers.

Two mice were each inoculated intraperitoneally with 0.1 ml of stabilate WIG 25 diluted in 0.2 ml. of sterile normal saline. Three days later, infection was confirmed by microscopy, parasitaemia being antilog 7.8 per ml. of mouse blood. Heparinized blood was collected from both mice and subjected to the blood incubation infectivity test. WIG37 (ETat 10) and the Liverpool stock were used as controls. Duplicate experiments were carried out with each of the trypanosomes using rat serum in place of human serum.

Organisms derived from WIG 25 and the Liverpool stock were found non-infective to mice after incubation

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TABLE 6. Sub-species identification of Trypanosoma brucei brucei by blood incubation infectivity test.

Trypanosomes and Treatment	PARASITAEMIA IN RECIPIENT MICE (EXPRESSED IN ANTI-LOGARITHM PER ML. OF BLOOD.									
	Days 2	3	4	5	6	7	8	9	10	15	20
Trypanosomes) + HS derived from) WIG 48) + RS Liverpool stock)		0 0 6.6 7.2	0 0 7.5 7.8	0 0 7.8 7.8	0 0 8.1 7.8	0 0 7.8 8.1	0 0 7.8 7.8	0 0 7.2 8.1	0 0 7.2 8.1	0 0 killed	0 0
Trypanosomes) + HS derived from) WIG 25) + RS		0 0 7.2 6.9	0 0 7.5 6.9	0 0 7.8 7.2	0 0 7.8 7.5	0 0 killed	0 0	0 0	0 0	0 0	0 0
Trypanosomes) + HS derived from) (ETat 10)) + RS WIG 37		7.8 7.2 7.5 7.8	killed								

HS = Human Serum

RS = Rat Serum

with human serum. The control ETat 10 organisms infected all mice as expected (Table 6). In all cases trypanosomes incubated with rat serum infected all inoculated mice.

Trypanosomes derived from stock LUGALA/55/EATRO/459 (WIG 25) therefore have the characteristics of sub-species Trypanosoma brucei brucei and will be expected to pose no serious hazards to laboratory investigators handling them. The reputed identity of the Liverpool stock was not confirmed in this investigation.

INDUCTION OF INFECTIONS

SUSCEPTIBILITY OF DOMESTIC CHICKENS TO THREE UNRELATED STOCKS OF T. BRUCEI SPECIES.

Trypanosomes of the brucei group are generally regarded as parasites of mammals. Nevertheless, reports abound in the literature of infectivity of these trypanosomes to domestic chickens. Previous workers in this laboratory used a sequence of heavy doses of T. brucei derived from various stocks. It was not clear, which stock caused the infection or whether this could only be achieved by the administration of several stocks in sequence. Diagnosis of infection was also found to be difficult as parasitaemia appeared to be very low.

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TABLE 7

Comparison of infections produced in fowls by three different stocks of Trypanosoma brucei. (Groups of 6 chickens).

Stock	Stabilate	Results of blood sub-inoculations in mice. Days after inoculation of trypanosomes						
		3	5	7	14	20	30	90
Lugala/55/EATRO/459	WIG 25	-	+	+	+	+	+	+
Muvumbwe/66/EATRO/1125	WIG 70 (AnTat 1)	-	+	-	-	-	-	-
Busoga/66/EATRO/3	WIG 37 (ETat 10)	ND	-	-	-	-	-	-

+ = at least one, of two, mice inoculated with chicken blood, became parasitaemic

ND = not done

- = all mice remained aparasitaemic

Experiments were therefore designed to investigate the infectivity to chickens of Trypanosoma brucei stocks, available in this laboratory, and to evaluate the various methods of diagnosing the infection. Three unrelated stocks of Trypanosoma brucei were investigated:

(1) LUGALA/55/EATRO/459 represented by stabilate WIG25
(11) MUVUBWE/66/EATRO/1125 represented by stabilate WIG70
(111) BUSOGA/60/EATRO/3 represented by stabilate WIG37

Twelve 11-day old chickens were placed into three groups of four each. Group one was inoculated with parasitaemic mouse blood from mice that had previously been infected with stabilate WIG 25. Group two was inoculated with a three day old passage from mice that had earlier been infected with stabilate WIG 70. Group three was inoculated with a two-day passage population from mice previously infected with stabilate WIG 37. Each chicken was inoculated intravenously with antilog 7.8 organisms.

The course of infection was monitored by inoculation of chicken blood into mice. As shown in Table 7, WIG 25 produced a persistent infection in all inoculated chickens. WIG 70, on the other hand, infected all of the inoculated birds but the infection was very transient lasting only five days. All the infections were microscopically subpatent. WIG 37, on the other hand, did not produce any infection.

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STABILATION OF TRYPANOSOMA BRUCEI RECOVERED FROM CHICKEN FOLLOWING DIAGNOSIS IN MICE.

Since only LUGALA/55/EATRO/459 (WIG 25), out of the three stocks tested, produced a persistent infection in chickens, it was considered expedient that such a population of trypanosomes recovered from mice used for diagnosis should be stabilated as Trypanosoma brucei recovered from chickens.

On day seven after inoculation of one ml. of heparinized chicken blood into each of two mice, both mice exhibited a parasitaemia of antilog 7.8 per ml. of blood. Parasitaemic blood collected from one of the mice was stabilated as WIG 69.

INFECTIVITY TO CHICKENS OF TRYPANOSOMES RECOVERED FROM MICE AFTER DIAGNOSIS.

Observations from previous experiments have shown that the minimum period from inoculation of chickens with T. brucei brucei to recovery of parasites in mice is about two weeks. There is therefore an ample opportunity for antigenic variation to have occurred either in chickens or in mice. Such emerging variants may lead to inconsistent results on further experimentation, if such variants fail to infect chickens.

This experiment was designed to test whether

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trypanosomes recovered from mouse following diagnosis are still infective to chickens or have lost such an ability during passages in both hosts. Stabilate WIG 69 was diluted in 0.2 ml. of sterile normal saline (0.85%). The diluted trypanosomes were inoculated into each of two mice. Both mice became parasitaemic. Blood from both mice was inoculated into each of two cockerels. Each cockerel was estimated to have received thirty million trypanosomes. All the inoculated chickens later proved infected as shown by xenodiagnosis in mice on day seven post inoculation. No trypanosomes were found in chicken blood under both the ordinary light and phase contrast microscopy.

The above experiment thus showed that the trypanosomes originating from WIG 69 have not lost their infectivity to chicken after passage through mouse.

ADJUVANT EFFECT OF RAT SERUM ON THE INFECTIVITY OF AnTat 1 TO DOMESTIC CHICKENS.

It has been reported earlier in this thesis that trypanosomes derived from WIG 70 AnTat 1 do not produce a persistent infection in chickens. This was further investigated in an attempt to ascertain whether the transient infection is due to natural antibody response or to the physiological environment of the

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chicken. In a similar investigation on the infectivity of Trypanosoma vivax in rat the inoculation of susceptible host serum to rats was found to enable a persistent infection to be established (Desowitz, 1954).

Four chickens were each inoculated intravenously with one ml. of rat serum for three consecutive days. On day four all the chickens were each inoculated with one ml. of mouse blood containing one hundred million trypanosomes derived from WIG 70 after a three day passage in mice. Infection was monitored by inoculating one ml. of chicken blood into each of two mice on days 5, 7, 9, 13 and 28. Parasites were recovered on days 5 and 7. All other inoculations into mice failed to produce an infection.

This experiment showed that the infection, in chickens, could be prolonged by two days when chickens are primed with rat serum. This method however failed to produce a persistent infection in chickens. It also leaves room for speculation that a humoral immune mechanism may be responsible for inability of this organism to produce a persistent infection in chickens.

INFLUENCE OF MOUSE SERUM ON THE INFECTIVITY OF T. BRUCEI BRUCEI (WIG 25) TO CHICKENS.

The previous experiment showed that inoculation of rat serum into chicken prolonged the duration of AnTat 1

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infection by two days. Could the persistent infection earlier recorded for trypanosomes derived from WIG 25 be due to an adjuvant effect of mouse serum on the trypanosome invasive mechanism in chickens? If this is so, trypanosomes devoid of mouse serum and blood cells should behave in a way different from those inoculated with parasitaemic mouse blood. Investigations were therefore carried out on the infectivity of stabilate Lugala/55/EATRO/495 (WIG 69) separated from mouse blood cells and plasma to domestic chickens.

Four mice were infected with stabilate WIG 69. On day three post infection all the mice were exsanguinated and trypanosomes were separated from blood cells on a DE 52 column. The trypanosomes were washed two times with glucose phosphate buffered saline and centrifuged after each washing in an attempt to remove mouse protein from the surface of trypanosomes. Concentration of motile trypanosomes was estimated by the haemocytometer method. Finally, the trypanosomes are diluted to make a suspension containing one billion per ml.

Over ninety five percent of the eluted trypanosomes were still motile when viewed under the microscope. No erythrocytes or leukocytes were observed in the eluate. Three 2-week old chickens were each inoculated with 10^9, 10^8, 10^7 organisms respectively. Parasitaemia in all inoculated chickens was monitored by blood

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inoculation into mice.

All three cockerels inoculated with trypanosomes were found infected from day seven. The infection was found to persist for three months until the cockerels were killed for histological investigations.

This investigation thus showed that Trypanosoma brucei devoid of mouse serum, plasma or blood cells are infective to domestic chickens.

ISOLATION OF A PURE POPULATION OF T. BRUCEI BRUCEI.

Trypanosomes derived from stabilate WIG 25 were used to initiate the initial infections in domestic fowls. This stabilate is an uncloned population and therefore probably heterogeneous. It may thus be a mixture of several variants, some of which may be of inconsistent infectivity or even fail to produce infections. Attempts were therefore made to obtain a clone population by inoculating single trypanosomes into mice. The trypanosomes used were obtained from mice that had been inoculated with blood from infected chickens. By using trypanosomes obtained from this source it was hoped that the organisms in any clones would retain their infectivity for birds.

Single organisms were obtained and inoculated intraperitoneally to mice as described in the methods section. Thirty attempts at cloning were made in the first instance, cloning series A and B

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Infections were established in two mice. The prepatent period in both mice was six days.

Trypanosome populations recovered from the infected mice were provisionally identified as clone O and clone P respectively.

Each clone was stabilated in the cryobank at -70°C.

ANTIGENIC RELATIONSHIP BETWEEN CLONE O AND CLONE P.

The two clones isolated in the previous experiment were obtained from a population of parasites growing in infected hosts (chicken and mouse) after at least one week post inoculation. It is possible that antigenic variation might have occurred thus resulting in the presence of many variable antigen types in the blood. It is therefore very essential to characterise the antigenic identity of each clone. The slide agglutination test was used.

Antiserum to each clone was raised in mice by infection and chemotherapy on day three post infection. Three fold serial dilution of each antiserum was prepared in borate buffer pH 8. Dilutions ranged from 10 to 7290.

Antiserum raised against clone O agglutinated both stabilate clone O and clone P. The same was also observed for anti clone P serum.

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TABLE 8.

Identification of clones (Clone 0. Clone P) by means of slide agglutination test.

Antigens	Anti Sera	Dilutions of antisera & agglutination reaction						
		30	90	270	810	2430	Control	Titre
WIG 72 (Clone 0)	Mouse Anti-Clone 0	++	+++	+++	+++	+++	-	2430
WIG 75 (Clone P)	Mouse Anti-Clone 0	++	+++	+++	+++	+++	-	2430
WIG 72 (Clone 0)	Mouse Anti-Clone P	*-	+++	+++	+++	+++	-	2430
WIG 75 (Clone P)	Mouse Anti-Clone P	*-	+++	+++	+++	+++	-	2430

Note that both antisera cross reacted at very high titres thus showing that both trypanosomes represented by WIG 72 (Clone 0) and WIG 74 (Clone P) have the same surface coat.

* Prozone effect.

\- = No agglutination of trypanosomes

++ = indiscrete agglutination of trypanosomes

+++ = clear-cut agglutination of trypanosomes

Both clones were therefore considered to be composed of trypanosomes of the same surface coat i.e. they are antigenically similar. Stabilate WIG 72 of clone O was designated as the type-type of the first antigenic type isolated.

Its variable antigen type was given the identification WITat 1, i.e. Western Infirmary Trypanozoon antigenic type 1.

INFECTIVITY OF CLONED POPULATION OF T. BRUCEI BRUCEI TO CHICKENS.

It is necessary to confirm that the trypanosomes (WITat 1 and clone P) still retain their ability to infect domestic chickens. It is not unlikely that only a minority of the original population was infective to chickens and clones might have come from the non-infective organisms. An experiment was carried out to test the infectivity of the two isolated clones to chickens.

Four male chickens of the Babcock 300 breed were placed into two groups of two each. Group one was inoculated with stabilate (WITat 1) while group two was inoculated with stabilate (clone P). All the trypanosomes were obtained from mice infected three days previously. Infectivity of the clones to chickens was monitored by blood inoculation into mice.

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Trypanosomes were not found on visual examination of the blood of any of these chickens but when one ml. of blood was inoculated into each of two mice both mice became parasitaemic on day six post inoculation. The infection was chronic and it persisted in all chickens for three months until the chickens were killed.

This experiment thus proved that the clones derived from stabilates WIG 72 and WIG 75 (WITat 1 and clone P) both retain their infectivity to chickens. It also indicates that trypanosomes of the same surface coat but of different clones, under this experiment, do not exhibit varying infectivity to chickens.

D I A G N O S I S

SELECTION OF SUITABLE METHODS FOR THE DIAGNOSIS OF INFECTION.

The method of choice for the diagnosis of salivarian trypanosome infection in mammals is the microscopic visualization of the active parasites in either the blood or the lymph gland or the spinal fluid. Organised lymph nodes are not present in chickens and aspiration of spinal fluid is highly traumatic to chickens. All efforts at diagnosing the infection in domestic chickens were therefore concentrated on detecting the parasites in the blood.

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In the present work, however, T. brucei was never detected in the avian blood by visual microscopy. While mouse inoculation sometimes revealed the presence of parasites, the period from chicken infection to confirmed diagnosis has invariably been about two weeks. Attempts were therefore made to evaluate the relative merits of some other techniques of diagnosis in the hope of finding a quicker and more consistent method.

WET BLOOD PREPARATION.

Microscopic examination of wet blood preparation never revealed the presence of trypanosomes in the blood of chickens. The big mass of chicken erythrocytes and leucocytes may obscure the easy visualization of scanty parasitaemia.

GIEMSA STAINED THIN BLOOD FILM.

When the wet blood preparation proved unhelpful in diagnosing the infection the thin blood film was tested since the same blood volume could be spread over a wider surface than was possible with wet film examination. These were prepared as described in the method section. Examination of stained films under oil immersion lens x100 by x 10 eyepiece did not reveal trypanosomes.

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GIEMSA STAINED THICK BLOOD FILM.

Thick blood films were of no diagnostic usefulness.

EXAMINATION OF BUFFY COAT.

When stained thick blood films failed to detect trypanosomes in the blood of chickens the examination of buffy coat was explored since this allowed a greater blood volume to be examined. Fifty microliters of blood was obtained from each of four chickens infected with WIG 69. The tubes containing the blood were sealed at one end with Plasticine and centrifuged in a microhaematocrit centrifuge (Hawking and Sons Ltd.) for five minutes. The red blood cells collected at one end while the plasma remained at the top, the leucocytes (buffy coat) were sqeezed in between them.

Using a carborundum disc the capillary tube was cut at the junction of the buffy coat and the erythrocytes. The buffy coat was then expressed onto a slide with the aid of a holey-blower and squashed out under a coverslip. It was then examined under the ordinary light microscope using x40 objective and x10 eyepiece. Sixty microscope fields were examined but no trypanosomes were seen. This method was tried on five occasions i.e. days 5, 14, 21, 42, and 65 in each of four infected chickens. On no occasion did it reveal the presence of trypanosomes.

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This technique is therefore considered not useful in the diagnosis of T. brucei infections in domestic chickens.

MOUSE INOCULATION.

So far microscopic examination of up to fifty microliter of infected chicken blood has not revealed the presence of trypanosomes. Further experiments were carried out with the hope that examination of a higher blood volume would reveal the presence of the parasites. An attendant problem, however, was that it requires an enormous amount of microscopy to examine thoroughly a series of wet films made from up to one ml. of blood. It was, therefore, decided to inoculate the blood into a susceptible laboratory animal. On day ten post inoculation two ml. heparinized blood was collected from the brachial vein of each of four chickens infected with trypanosomes derived from WIG 69. One ml. of the blood was inoculated into each of two mice. Parasitaemia in the inoculated mice was monitored for twenty days by examination of blood from the tail vein. All mice inoculated became parasitaemic and the prepatent period ranged from six to eight days.

This technique proved useful in the detection of infection in chickens and was subsequently adopted as a standard technique for diagnosing the infection.

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DETECTION OF TRYPANOSOMES BY DE 52 COLUMN-CHROMATOGRAPHY

The method of mouse inoculation only revealed the presence of organisms infective to mice but maybe some of the trypanosomes, present in the chicken blood, are not infective to mice. Besides, a lot of mice are generally committed to this method and it is essential to examine all of them for a fairly long time before concluding that a suspected chicken is not infected.

A search for a more rapid but equally reliable technique suggested that ion-exchange chromatography on DE 52 should be tried. This method is based on work by Brown (1933) which showed that blood cells from infected animals possess a more electronegative charge than the parasites. On this basis, Lanham (1968) was able to separate trypanosomes from mammalian blood cells on DE 52 using a buffer of appropriate molarity. The more negatively charged blood cells are absorbed on the DE 52 while the trypanosomes passed through.

Three and a half ml. of heparinized blood from an infected chicken was passed through a DE 52 column using a glucose phosphate buffered saline, pH 8. The whole eluate was centrifuged at 500 g in a refrigerated centrifuge (4°C) and whole sediment was examined under the ordinary light microscope.

No chicken blood cells were observed in the sediment.

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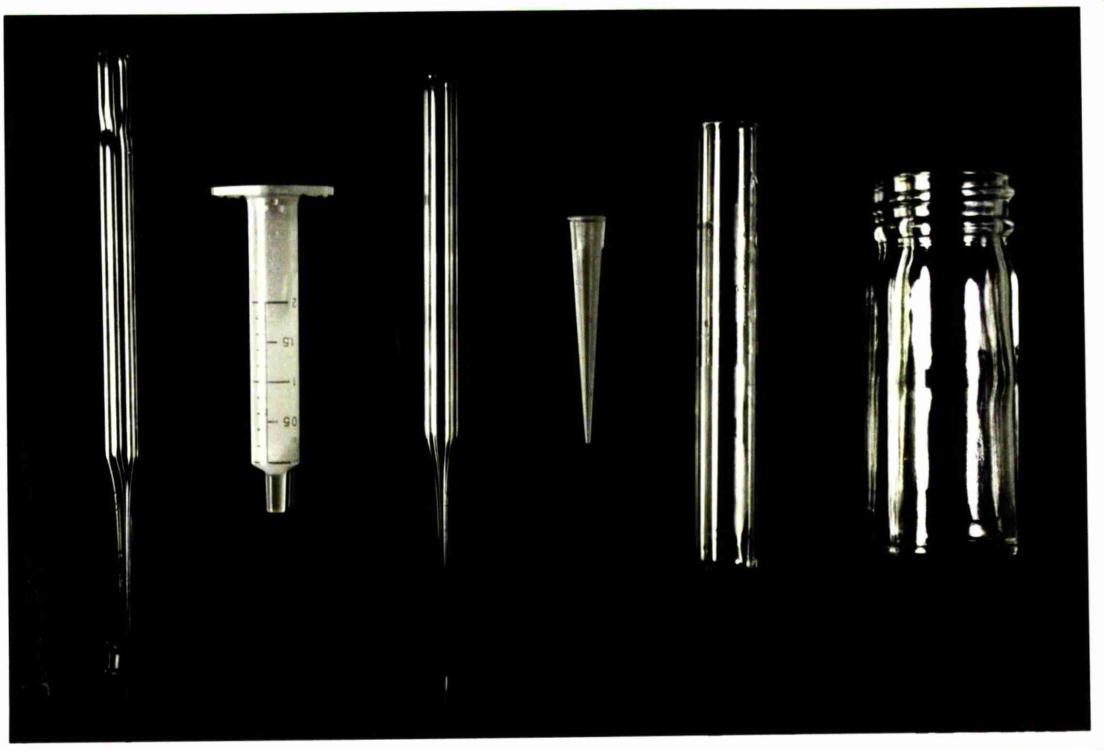
ADAPTATION OF MINI-ANION-EXCHANGER TO THE DETECTION OF T. BRUCEI IN CHICKEN BLOOD.

The miniature version of the chromatographic technique of Lanham (1969), suggested by Lumsden et al (1977, 1979) was used with slight modifications. A small DE 52 column, equilibrated to pH 8.0 with .05M phosphate, .04M NaCl and .055M glucose, is set up within a 2ml. plastic syringe barrel. To this is applied 200 µl. of heparinized chicken blood and any trypanosomes present are eluted through the column with 2.5 ml. of the same buffer. The eluate is collected in an unplugged Pasteur-type pipette which has been drawn out and sealed at its capillary end. After centrifugation at 500g for 5 minutes a brief examination of the tip under the x10 objective shows the presence of any trypanosomes and enables a count to be made. By this method, six chicken blood samples were examined within half an hour, this included the packing of the columns with an equilibrated slurry of DE52, collection of blood, centrifugation and microscopy. The results indicate that the technique increases the sensitivity of detection of T. brucei by direct microscopy by about 10,000 times compared to wet film examination i.e. to about 20 trypanosomes per ml. of blood. Thus, though not as sensitive as mouse inoculation, it is adequate for these purposes and particularly economical of time, materials and microscopy.

The components and the assembly of the mini- anion -exchanger are shown in plates 6 - to - 8 .

PLATE 6.

COMPONENTS OF THE MINI-ANION COLUMN



A B C D E

A = Reservoir

B = Anion-exchange column

C = Centrifuge tube with pipette tip

D)
) = Support for the collecting tube
E)

PLATE 7.

THE RESERVOIR ANION-EXCHANGE COLUMN AND COLLECTING PIPETTE

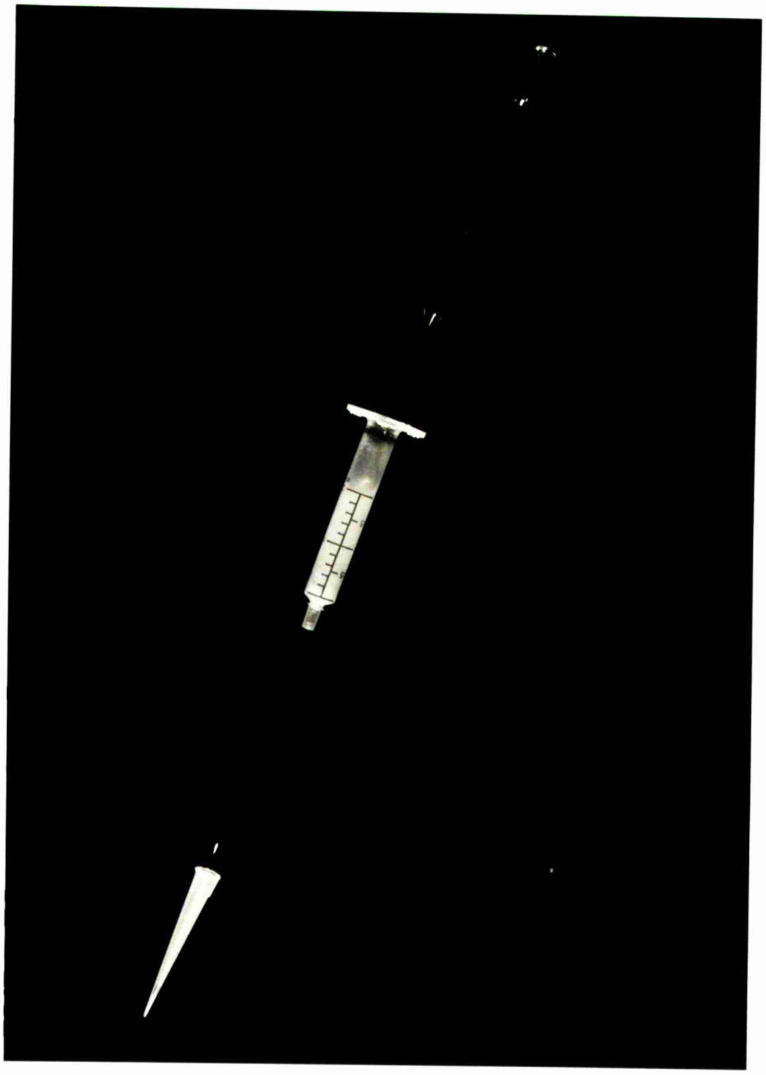
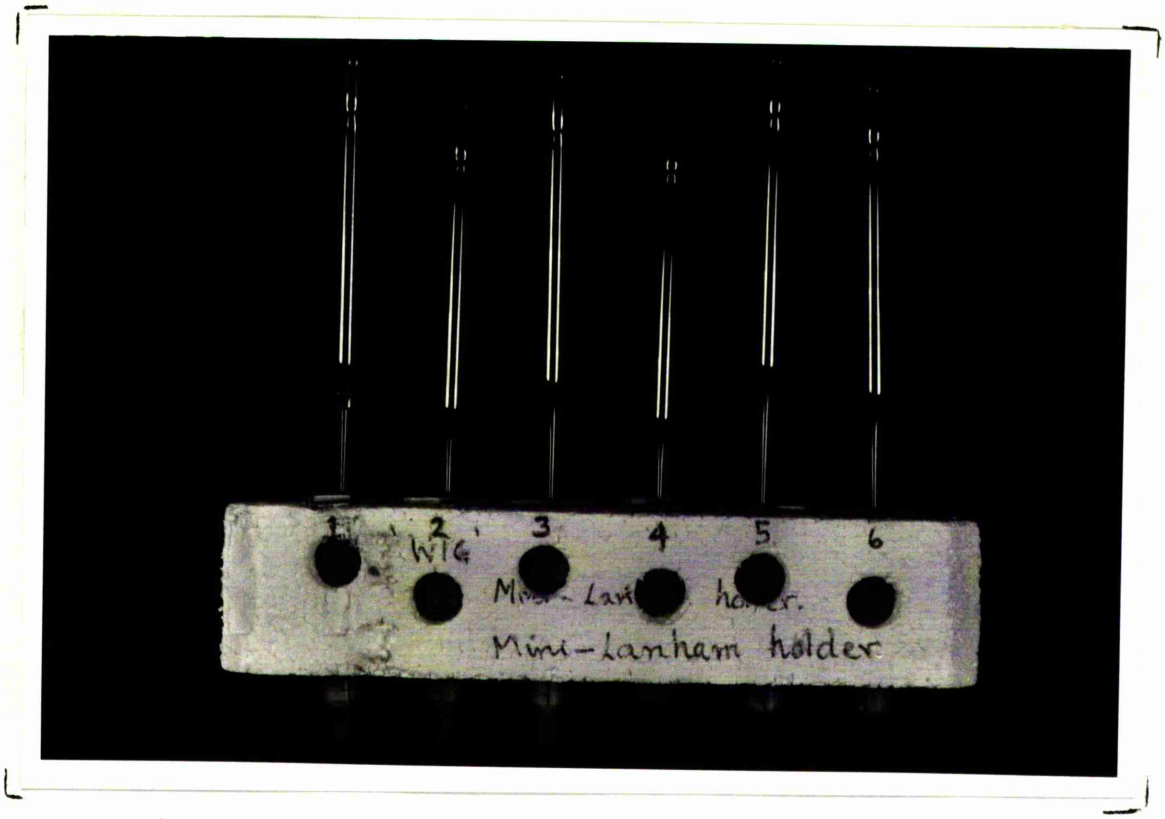


PLATE 8.

ASSEMBLY OF SIX-MINI-ANION EXCHANGERS

BEING USED SIMULTANEOUSLY.



INDIRECT METHODS OF DIAGNOSIS.

The demonstration of T. brucei in the body fluid of infected hosts is the most conclusive evidence of an infection. However, negative microscopic results are often obtained when the parasitaemia is very low. Under such circumstances, immunological tests may be of diagnostic value.

SLIDE AGGLUTINATION TEST.

The direct agglutination test was examined as to its usefulness for demonstrating antibody in the sera of infected chickens. Samples of sera were obtained from six chickens in which trypanosome infection has been confirmed by blood inoculation into mice. The infection had persisted for twenty days in the chickens. Duplicate blood samples were obtained from four uninfected chickens. Serial two fold dilutions of the sera ranging from 1:10 to 1:1280 were made in solution AB pH 8. Using stabilate WIG 76 (WITat 1) as antigen, agglutination tests were performed as described in the method section.

Sera from all infected chickens agglutinated the stabilate material. The agglutinating titre ranged from 640-1280.

Sera from uninfected chickens, on the other hand, did not agglutinate the stabilate.

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The absence of positive reactors among the four controls indicates that agglutination test has potential for the diagnosis of African trypanosomiasis particularly in hosts with latent infections provided the appropriate antigen is available.

NON-SPECIFIC TEST FOR DETECTING INFECTION IN CHICKENS.

Trypanosomiasis is usually associated with a rise in the globulin level in infected mammals. Several tests for detecting trypanosome infection have been based on a detection of this rise.

HETEROPHILE ANTIBODIES IN TRYPANOSOME INFECTION IN CHICKS.

Heterophile antibodies are antibodies acting upon an antigen that is apparently unrelated to the antigen used for immunization. Elevated heterophile agglutinins in the serum of mammals infected with trypanosomes have been reported (Henderson - Begg, 1946; Parratt and Cobb 1978). The main questions appearing to require studies in chickens infected with T. brucei ssp were:

a) Does heterophile agglutinin occur in the sera of chickens infected with these parasites?

b) What is the picture of heterophile antibody reaction?

Sera were obtained from infected chickens and control birds as described in the method section. Each sample of serum was diluted one in four in normal saline

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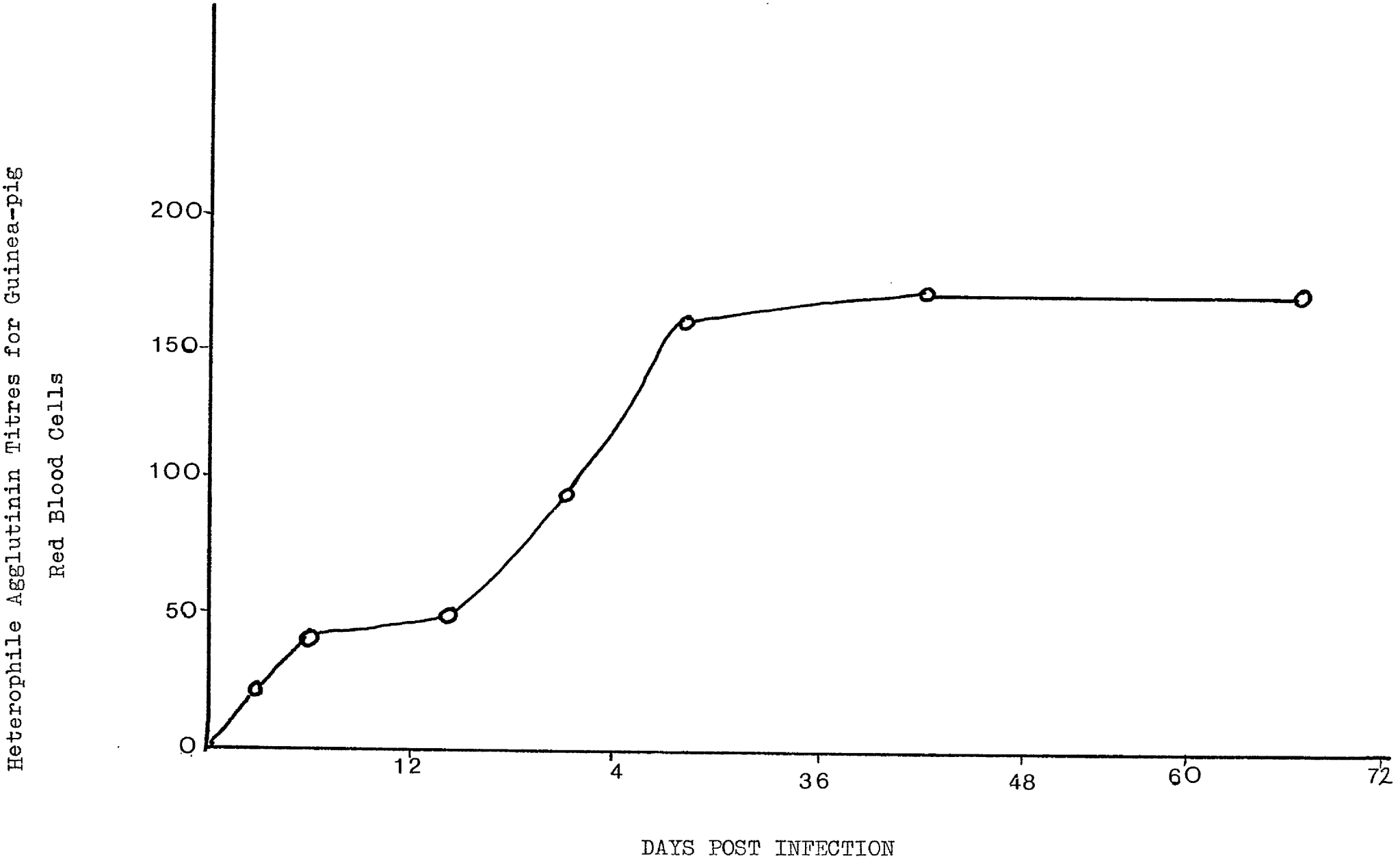
TABLE 10: Chickens infected with T. brucei brucei: heterophile agglutinins against mammalian non-sensitized erythrocytes.

Non-Sensitized Erythrocytes	Highest dilution of Sera that Agglutinated Erythrocytes											
	INFECTED BIRDS							CONTROLS				
	A	B	C	D	E	F	RANGE	G	H	I	J	RANGE
Rat	128	32	256	256	256	128	32-256	8	8	8	8	8
Rabbit	128	64	128	64	256	64	64-256	8	8	16	8	8
Guinea Pig	128	64	16	16	128	16	16-128	8	8	8	8	8
Sheep	8	8	8	8	32	8	8	8	8	8	8	8
Horse	8	8	8	8	8	16	8	8	8	8	8	8
Man	8	8	8	8	8	8	8	8	8	8		8

Haemagglutination reaction of 2% erythrocyte suspension of each mammal in normal saline - the erythrocytes is shown.

FIGURE 5. HETEROPHILE ANTIBODIES IN CHICKENS INFECTED WITH T.B. BRUCEI
(Arithmetic mean of three chickens)



and were subsequently incubated in a water bath at 56°C for thirty minutes to inactivate. Serial dilutions of the sera were then made in normal saline. Four percent suspension of non-sensitized erythrocytes from rat, sheep, horse, mouse, rabbit, guinea-pig and human were made in normal saline. Agglutination tests were performed on each sample of erythrocytes.

Chickens infected with T. brucei ssp had elevated heterophile antibodies for non-sensitised erythrocytes from rat, rabbit and guinea pigs. No elevated agglutinins were observed to blood cells from man, sheep and horses. Sera from control chickens did not show such comparable titres to blood cells from rat, rabbit and guinea pig (Table 10).

This observation thus indicated that heterophile antibody response occur in chickens infected with T. brucei. Further characterization of this heterophile response was carried out in view of the fact that it may be of diagnostic significance. It was found that the heterophile agglutinin level in the sera of infected chickens remained high as long as the infection persisted (Fig.5).

MERCURIC CHLORIDE TEST.

Bennett (1929) used the mercuric chloride test to diagnose T. evansi infection in camels where parasites are not easily detected in the blood. This test is

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carried out by adding one drop of the test serum to one ml. of 1:2500 O aqueous mercuric chloride solution. Positive serum forms a fine precipitate within a few minutes, because of the presence of abnormal quantities of immunoglobulins. Negative samples produced no precipitate after standing for fifteen minutes.

Sera from six chickens known to be infected with WITat 1 and four control pullets were tested. All sera produced turbidity within ten minutes of their addition to mercuric chloride solution.

This test has not proved useful in the diagnosis T. brucei infection in chickens.

QUANTITATIVE ESTIMATION OF PARASITAEMIA BY INFECTIVITY TITRATION METHOD.

Initially all confirmations of Trypanosoma brucei infections in chickens were made by blood inoculation into mice. Attempts at diagnosis by direct blood microscopy were totally unsuccessful. Blood inoculation suffers from two faults:

(a) The period from inoculation to definitive results is about one week.

(b) It gives almost no indication of the number of organisms present. If the inoculated mouse becomes parasitaemic then there must have been at least one infective organism present in the inoculum. In

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TABLE 9.

Mouse infective trypanosomes (ID_{63}) per ml. of chicken blood in four birds infected with T. brucei brucei (WIG 73) for different periods of time.

BIRD	Days Post Infection	Mouse (ID_{63})
A 954	35	3
B 193	84	25
C 141	220	100
D 84	247	4

actual fact there may have been ten thousand. The use of two mice gives a slight improvement in that if only one became infected then the parasitaemia is probably very low.

To overcome these problems the infectivity titration technique as described by Lumsden et al (1963) was tried. This method was adopted with some modifications to cater for the characteristically low parasitaemia in the chicken. Each inoculated mouse received one ml. of inoculum i.e. infected chicken blood or dilutions as indicated. The practicality of the infectivity titration method was tested in each of four fowls that had been infected with T. brucei at different times i.e. 35, 83, 220 and 247 days previously. Eight ml. of heparinized blood was collected from the brachial vein of each chicken just before titration. Each sample of blood was diluted in a tenfold series and each dilution inoculated into mice within 45 minutes of blood aspiration. Full details of the titration procedure are described in the method section.

The number of infective trypanosomes in all the birds were found to show a striking divergence (3-100, Table 9) which could not have been detected when the routine sub-inoculation method was used for diagnosis.

This technique thus affords a quantitative assessment of the parasitaemia in domestic chickens infected with a salivarian trypanosome.

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FACTORS AFFECTING INFECTIVITY.

MINIMUM INFECTIVE DOSE OF T. BRUCEI BRUCEI REQUIRED TO ESTABLISH AN INFECTION IN CHICKENS.

The number of trypanosomes needed to initiate an infection is disputed especially in large animals. Fairbairn and Burtt (1946) reported that 10 organisms of T.b. rhodesiense are required to infect a man, while Willett (1956) showed that man requires up to 80,000 organisms. It seems, however, that some of these organisms may not have been infective as it was shown convincingly by Oehler (1913) that a single organism is enough to infect small rodents like rats. The initial attempts to infect birds with T. brucei had been carried out with a very large number of organisms ($10^{7.8}$). On the concept of a weight ratio, it might be expected that less organisms would be required to infect birds than to infect large animals.

Twelve 11-day old chickens were placed into three groups of six, three and three respectively. T.b. brucei, stabilate WIG 76, which was used to initiate infections, had been found to contain antilog 5.6 ID_{63} (400,000 mouse 63 percent) infective doses per ml. Serial ten fold dilutions, $1:10^2$ to $1:10^5$ of the stabilate were made in solution A_4B_2 pH8·0. Starting with the highest dilution each chicken of the first group of three was inoculated with 0.25 ml. i.e. ten mouse infective doses. Each chicken in the second group of three was inoculated

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with 0.15 ml. of the stabilate diluted 10^3 i.e. sixty mouse infective doses. The group of six chickens were each inoculated with 0.25 ml. of stabilate diluted 10^3 i.e. 100 mouse ID 63. Trypanosome infection in all the chickens was monitored by blood inoculation into mice on days 7, 20, 35 and 60. Blood collected from the chickens inoculated with ten and sixty mouse infective doses failed to produce patent infections in any of the mice. Blood from only one out of the six chickens inoculated with one hundred mouse infective doses was found infective to mice (Table 9). The infection was monitored for sixty days in case the low doses employed might have an unusually long incubation period but no further birds were found infected.

This experiment thus shows that a minimum of one hundred mouse infective doses of stabilate WIG 76 (WITat 1) is required to infect domestic chickens by the subcutaneous route.

INFECTIVITY OF T. BRUCEI INOCULATED INTO CHICKENS BY DIFFERENT ROUTES.

In the preceding experiment inoculation of trypanosomes was carried out by the subcutaneous route and at least one hundred mouse infective doses (ID 63) were required to initiate an infection. However, as trypanosomes are largely blood borne parasites, it seemed possible that direct inoculation into the blood-stream

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might prove a more efficient route. Simmons et al (1963) showed that the intraperitoneal route is more efficient than either IP or IM or Sub C.

An experiment was therefore carried out to examine the effect of different routes of inoculation on experimental infection of domestic chickens with T.b. brucei.

Twenty 13-day old chickens were placed into four groups of five. Food was withdrawn from all the chickens eighteen hours before inoculation, water was however made freely available to them. Stabilate WIG 76 (Variable antigen type, WITat 1) was retrieved from the cryobank. A dilution of the stabilate was made in solution A_4B_2 such that 1 ml. contained 400 mouse infective doses (ID 63). This suspension was kept in an ice-water bath. Each chicken was inoculated with 0.25 ml. of the suspension by one of the following routes:

Group one - intravenous
Group two - intramuscular
Group three - intraperitoneal
Group four - subcutaneous.

All inoculations were made within thirty minutes of the stabilate retrieval from the cryobank. Seven days post-inoculation 2 ml. of heparinized blood was collected from each chicken and one ml. of this was inoculated into each of two mice. Parasitaemia was monitored in the mice by microscopic examination of

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wet blood films. Repeat diagnosis was carried out on days thirty and seventy.

Three out of the five birds inoculated intravenously were found infected on each occasion. None of the chickens inoculated by the other routes was found infected.

It is evident therefore that the intravenous route is the most efficient for initiating T. brucei infections in chickens.

COURSE OF PARASITAEMIA IN A FOWL INFECTED WITH TRYPANOSOMA BRUCEI BRUCEI.

Studies of persistent T. brucei infection in fowls revealed occasional periods during which no organisms could be recovered by mouse inoculation, though the birds were subsequently shown to retain their infection. The test of mouse inoculation is based on an 'all or none' principle. If there is at least one organism per ml. of blood all mice inoculated will be infected, if on the other hand the parasitaemia is less than one organism per ml. the test may be hard put to detect the parasite.

An experiment was therefore carried out to quantify by infectivity titration, the parasitaemia in chickens during the course of an infection.

Four mice were infected with stabilate WIG 73 (WITat 1). On day three post infection blood was

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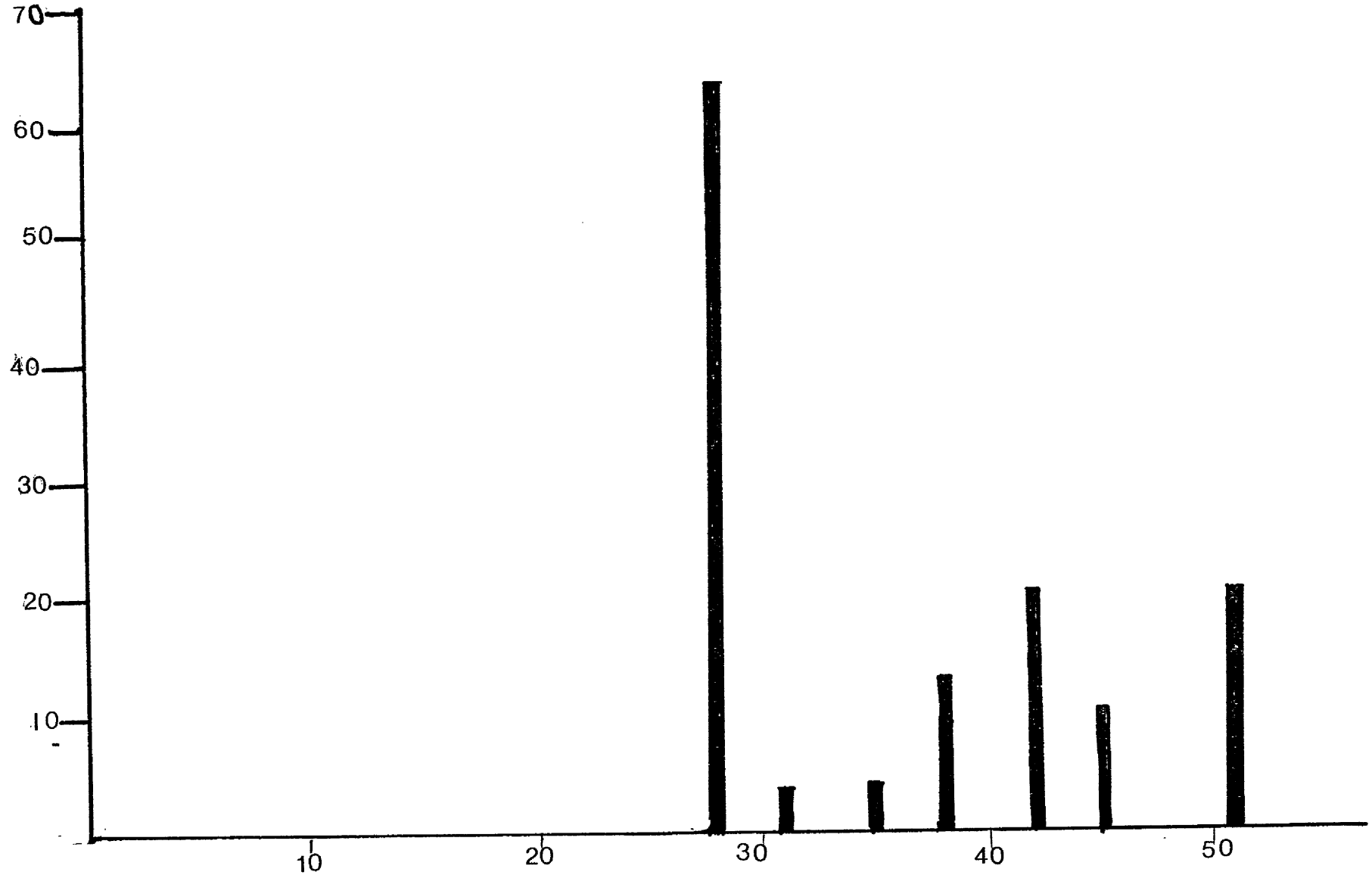
TABLE 11.

Infectivity of T.b. brucei, stabilate suspension, administered by different routes to chickens.

Routes of Inoculation	Chickens Found infected on			
	Day 7	Day 20	Day 35	Day 70
Intramuscular	0/5	0/5	0/5	0/5
Intravenous	3*/5	3*/5	3*/5	3*/5
Intraperitoneal	0/5	0/5	0/5	0/5
Subcutaneous	0/5	0/5	0/5	0/5

* Same chickens.

FIGURE 6 FLUCTUATIONS IN PARASITAEMIA OF A FOWL INFECTED WITH T.B. BRUCEI.



collected from the mice via orbital plexus and the blood was pooled. One ml. of blood was inoculated into a 12-week old cock. Immediately afterwards, the remaining blood was titrated in mice, as described in the materials and method section, so that the number of infective organisms inoculated into the chicken could be determined. From the titration results it was calculated that the chicken had received 10^6 mouse infective trypanosomes.

Infection in the chicken was confirmed on day ten by inoculating one ml. of heparinized blood into each of two mice. A repeat diagnosis was carried out on day twenty to reaffirm that the infection was persistent. On day 28, ten ml. of heparinized blood was collected from the fowl via the brachial vein. Infectivity titration was carried out in mice as described in the materials and method section. Similar titrations were performed with blood collected from the same chicken on days 28, 31, 35, 38, 42, 45, 48 and 51. Parasitaemia in all the inoculated mice was monitored for twenty days. Parasitaemia was found to vary between three and sixty-three mouse infective organisms (mouse ID63), per ml. of chicken blood. On one occasion trypanosomes were not detected in the blood of the bird (day 48). A histogram of the parasitaemia is shown on Fig 6 from which it can be seen that the parasitaemia did show some oscillations as in some mammals.

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PERSISTENCE OF TRYPANOSOMA BRUCEI INFECTION IN DOMESTIC CHICKENS.

Early reports on Trypanosoma brucei infection in in domestic birds (Corson 1931b;Duke 1933) indicated that the infections could persist in avian hosts for only about six weeks. It was unexpected therefore to find in the present work that fowls killed three months after infection retained their infection (Table 7).

It is possible that the infection might have persisted for longer periods if the infected birds, in this investigation, had not been killed. An experiment was therefore set up to investigate how long T. brucei infection can persist in domestic chickens.

Each of twenty 2-week old chickens (eight pullets and twelve cockerels) was inoculated intravenously with one million mouse infective doses of WIG 73 (WITat 1). Infection in all the chickens was confirmed on day five by blood inoculation into mice. Subsequently diagnosis was carried out every two weeks.

A total of twenty-seven diagnoses were carried out on each bird over a period of one year. The infection persisted for one year in the cockerels. The pullets, on the other hand, self-cured between six and seven months post initial infection. Repeated diagnosis carried out in the pullets throughout the remaining months of the investigation failed to reveal the presence of the trypanosomes.

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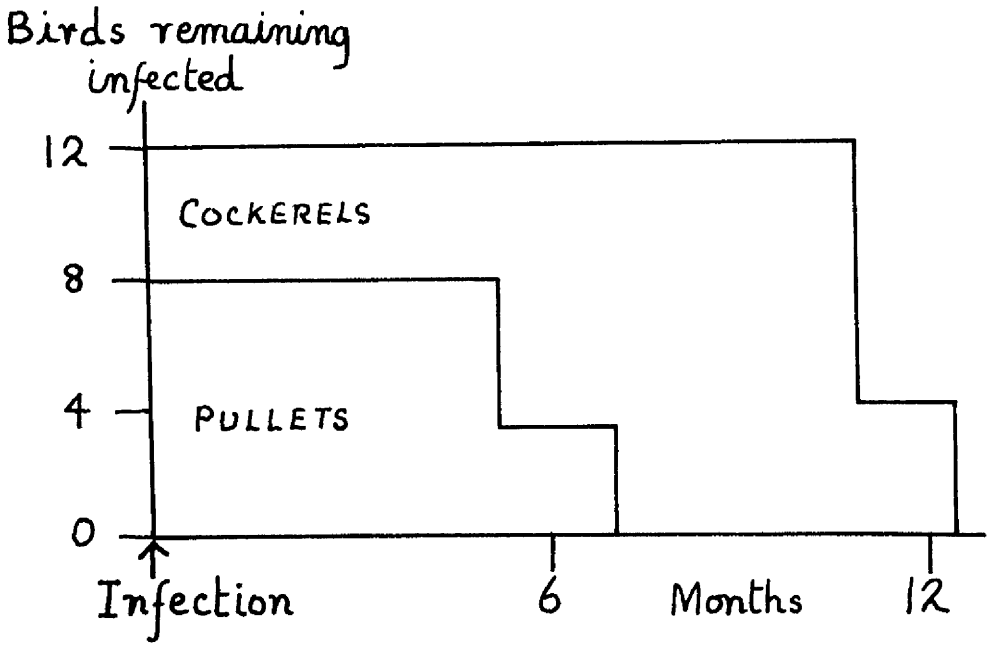
PLATE 9.

PERSISTENCE OF INFECTION IN DOMESTIC CHICKENS



The cockerels were not kept beyond 365 days. These results showed that trypanosomes derived from WIG 73 (WITat 1) can initiate a long lasting infection in chickens. It is also evident that birds can effect a sterile recovery from such a well established infection.

TESTING THE TRYPANOCIDAL ACTION OF CHICKEN SERUM ON TRYPANOSOMA BRUCEI BRUCEI.

It has been shown in this thesis that only one of the three stocks of trypanosomes tested was able to initiate a persistent infection in chickens. Warren and Borsos (1958) demonstrated that normal chicken serum is cytotoxic to the crithidial forms of T. cruzi. The inability of stock Busoga/60/EATRO/3 to infect birds might be related to the cytotoxic effect of avian serum. An experiment was therefore carried out to determine whether normal chicken serum is trypanocidal to the stocks of trypanosomes that failed to infect birds.

Stabilate material from each of the three stocks of trypanosomes:

Lugala/55/EATRO/459 represented by WIG 73.
MUVUBWE/66/EATRO/1125 represented by WIG 70.
BUSOGA/60/EATRO/3 represented by WIG 37

was suspended in 3 ml. of chicken serum. The suspension of trypanosomes in the chicken serum was incubated at

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TABLE 12.

Infectivity for mice of three stocks of *T. brucei* ssp after incubation in normal chicken serum at 37°C for 2 hours. Infectivity of control groups incubated in rat serum under similar conditions is also shown.

Incubation Medium	Trypanosomes	Mice Infected / Mice Inoculated	Survival of Infected mice (Days)
Chick Serum	WIG 70 (AnTat 1)	6/6	4-7
Chick Serum	WIG 37 (ETat 10)	6/6	3-5
Chick Serum	WIG 73 (WITat 1)	6/6	29-35
Rat Serum	WIG 70 (AnTat 1)	4/4	5-7
Rat Serum	WIG 37 (ETat 10)	4/4	3-4
Rat Serum	WIG 73 (WITat 1)	4/4	28-37

37°C for two hours. Duplicate samples were incubated in rat serum.

Microscopic examination of all incubated samples showed actively motile trypanosomes. Each sample was inoculated into six mice such that each mouse received 0.5 ml. of chicken or rat serum. Parasitaemia in recipient mice was monitored as previously described. All mice inoculated became infected. There was no difference in virulence between mice inoculated with either chicken serum or rat serum. (See Table 12).

This investigation thus shows that normal chicken serum is not trypanocidal to any of the three stocks of trypanosomes used. It is therefore probable that the selective infection of birds by these morphologically indistinguishable trypanosomes might be related to antibody response rather than to any naturally occurring serum factors.

EFFECT OF NORMAL CHICKEN SERUM ON THE INFECTIVITY OF T. BRUCEI BRUCEI.

The present studies have shown that successful inoculation of a single organism is enough to produce an infection in a mouse. It is important therefore to establish that the infectivity of trypanosomes is not inhibited by incubation in chicken serum. Two questions came to focus in this regard:

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TABLE 13.

The effect of normal chicken serum fractions on the infectivity of *Trypanosoma brucei* to mice.

Titration Medium	Number of Mouse Infective Trypanosomes (ID63) detected after Incubation for 1 hr. (Antilog)
Borate Buffer	7.1
Chicken Serum	7.6
Chicken IgG	7.4
Chicken IgM	7.1
Chicken Lipoprotein	7.4
Chicken Albumin	7.4
Chicken x-2 Macroglobulin Chicken	7.6

a) Is there any deleterious effect on the infectivity of Trypanosoma brucei after incubation in chicken serum?

b) If so, what are the serum factors responsible for any observed defects?

Sixteen ml. of normal chicken serum was retrieved from serum bank at 20°C, 8 ml. of the serum was fractionated on AcA 22 as described in the method section. The various fractions were concentrated to the original serum volume in Armicon.

Infectivity titrations were carried out in the following serum fractions;

Ig M, Ig G, Lipoprotein, Albumin,
Normal Unfractionated chicken serum,
and Borate buffer,

as described in the method section. Dilutions varied from 10^5 - 10^8. Trypanosomes were incubated in the media for one hour.

The number of mouse infective trypanosomes remaining in each medium after incubation for one hour is shown in Table 15. Infectivity varied from antilog 7.1 to 7.4. Since any difference in infectivity that is less than Antilog 1 .2 is not to be regarded as being significant (Lumsden et al (1963), no fractions can be said to have inhibited the infectivity of the trypanosomes.

This experiment thus shows that normal chicken serum does not inhibit the infectivity of T. brucei. It also

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underlines that the low parasitaemia characteristic of the infection in chickens is not due to any natural trypanosomal growth in inhibitory factor but might be due to the immune reaction of chickens.

BERENIL IN THE TREATMENT OF T. BRUCEI INFECTIONS IN DOMESTIC CHICKENS.

As has been shown in the preceding experiment birds that had self-cured the infection 200 days previously have normal number of germinal centres in their spleens. It becomes necessary therefore to find a drug that will readily cure the infection.

The normal experimental procedure used to cure infections in small animals infected with trypanosomes, especially during the preparation of antisera is drug cure. The drug normally used is Berenil (diminazene aceturate). Makers literature on Berenil gives no instruction for its use in chickens. An experiment was designed to test the effectiveness of this drug in avian hosts.

Ten 12-week old cockerels were inoculated with blood of mice that had been infected three days previously with stabilate WIG 76 (WITat 1). The persistence of infection in chickens was confirmed by mouse inoculation on days 21, 45 and 72. All of the birds were found infected.

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On day eighty four, six of the infected birds were treated with Berenil at the rate of 7 mg. per kg. of body weight. The drug was administered into the leg muscle. The remaining four chickens were not treated and were retained as controls. Parasitaemia was monitored in both the treated and the control groups, for sixty days. All the treated birds became depressed thirty minutes post drug administration. They became recumbent and were dull and huddled together at the corners of the cage. Three hours later, the treated birds appeared to have recovered and were found feeding and drinking. No obvious physical differences in the appearance was noted between treated and control groups on the following day.

Inoculation of 1 ml. of heparinized blood from each chicken into each of two mice on day one post treatment, failed to produce trypanosome infections. The infection never reappeared throughout the sixty days of monitoring for parasitaemia.

Berenil does, as in mammals, cure T. brucei infections in birds and is not highly toxic to these animals.

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MECHANISMS OF BODY DEFENCES TO THE INFECTION

ORGAN AND TISSUE DISTRIBUTION OF TRYPANOSOMA BRUCEI IN DOMESTIC CHICKENS.

The previous experiments have shown that the parasitaemia in T. brucei infected birds is always extremely low. This is very unlike the situation in mammals and the question arises as to whether the number of organisms in the bird are as few as indicated or whether they are distributed in a manner different from that observed in mammals. Even in the latter, T. brucei is known to be a tissue as well as a blood parasite and it could be that in birds the majority of the organisms are in the tissue spaces outwith the bloodstream.

An experiment was therefore carried out to assess the distribution of bloodstream form of trypanosome in the tissues of birds. One cockerel was infected with T. brucei derived from WIG 76 (WITat 1) by a three day passage in mice.

Sixteen days post infection, two ml. of the cockerel's blood was inoculated into two mice and the bird was immediately killed by cervical dislocation.

Between two to three gm. of each of the following organs: liver, spleen, brain, kidney, heart muscle, bone marrow, vitreous humour and lungs were removed and minced in two ml. of solution A.B (pH 8) suspended in an ice-water-bath ($4^{o}C$). In every case the internal

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TABLE 14.

Recovery of Trypanosomes from tissues of an infected chicken.

	ORGANS	MICE INOCULATED	MICE INFECTED
1	Liver	2	0
2	Spleen	2	0
3	Blood	2	2
4	Bone Marrow	2	1
5	Brain	2	0
6	Kidney	2	0
7	Vitreous Humour	2	0
8	Heart Muscle	2	0
9	Pancreas	2	0
10	Lung	2	0

cut surface of each organ was touched lightly to a labelled slide, allowed to dry and stained by the conventional Giemsa technique. Each organ was separately minced in the buffer. The suspensions were allowed to sediment for a few minutes (10-15) and the supernatant from each tube was inoculated intraperitoneally into two mice.

Parasitaemia in all inoculated mice was monitored for twenty days by microscopic examination of wet blood film.

Both mice inoculated with the cockerel's blood became parasitaemic. Of all the mice inoculated with supernatant from the other organs, only those injected with bone marrow became infected with trypanosomes (Table 14). Inoculations from the supernatant of other tissues failed to produce infections. All of the Giemsa stained smears were examined under the ordinary light microscope using the oil immersion lens (100 - x10). Over sixty microscope fields scattered over the film, were scanned on each slide over a period of 8-10 minutes. The impression smears did not reveal the presence of any trypanosomes.

It is suggestive therefore that there are more trypanosomes in the peripheral blood than in the visceral organs. The low parasitaemia in chickens infected with T. brucei might be said to be due to low parasite density and rather than extravascular localization.

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COMPARATIVE EXAMINATIONS OF THE BLOOD AND BONE-MARROW OF CHICKENS INFECTED WITH T. BRUCEI.

The preceding experiment in this thesis has shown that infective trypanosomes could only be demonstrated in the blood and bone marrow. Earlier work by Diamond and Sherman (1954), however, indicated that some trypanosomes of unspecified identity could, most readily, be detected by bone marrow culture rather than microscopic examination of thin blood film. It will therefore be interesting to compare the ease of detecting trypanosomes in the blood of chicken using either blood or bone marrow inoculation into mice.

Six domestic chickens with chronic trypanosome infections were used. The infections had lasted from twenty-four days to one year in these birds. Bone marrow was aspirated as described in the method section. Blood and bone marrow were aspirated concurrently from each bird and were separately inoculated into two mice. Infection in recipient mice was monitored for twenty days in each case.

The procedure of bone marrow biopsy did not seriously incapacitate the fowls, although on one occasion a bird developed a slight lameness which lasted for two days. The lameness disappeared completely later.

Hand pressure exerted on the biopsy needle was adequate to penetrate the head of the femur in young

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TABLE 15 Comparative diagnosis of Trypanosoma brucei ssp infections in chickens by inoculation of either bone marrow or whole blood into mice.

TRYPS.	Days Post Tryps. Infection in birds	HEPARINIZED BONE MARROW			HEPARINIZED WHOLE BLOOD		
		Inoculum	Mice Inoculated	Mice Infected	Inoculum/ mouse	Mice Inoculated	Mice Infected
WIG 25	278	0.5	2	0	1 ml.	2	½
WIG 69	318	0.4	2	0	1 ml.	2	2/2
WIG 88	24	1 ml.	2	½	1 ml.	2	2/2
WIG 88	32	1 ml.	2	½	1 ml.	2	2/2
WIG 88	32	0.8	2	0/2	1 ml.	2	2/2
WIG 25	365	0.8	2	0/2	1 ml.	2	0/2

birds. In older birds (eight months and over) this hand pressure was not effective so greater mechanical force had to be applied.

In general, the volume of marrow samples obtained was very small, especially in old birds. Where over one ml. of marrow sample was obtained, there was a good correlation between success rate of infections resulting from both methods. On occasions when only small volumes of marrow samples were obtained i.e. less than half ml., inoculation of one ml. of chicken blood into mice revealed far more infections than bone marrow. This experiment shows that bone-marrow inoculation is not likely to be of much value as a diagnostic measure, Table 15

AVIAN LYMPHOID TISSUE REACTIONS TO T. BRUCEI INFECTIONS.

The present investigation has shown that a trypanosome infection, once established in birds, persisted for a long time and that the parasitaemia was very low. Immunological responses of the infected birds to suppress the infection might lead to deposition of tissue damaging immune complexes. Histological investigations were therefore carried out to seek for pathological changes in the tissues of infected chickens.

Two groups of 8-week old birds were selected for this study. One group was infected with parasitaemic mouse blood obtained from mice infected three days previously with WIG 76 (WITat 1). The other group was

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used as controls. Infection was confirmed by the standard method. On day fourteen post infection, four infected and an equal number of controls were killed by cervical dislocation and were necropsied.

The carcasses of infected and control birds were grossly indistinguishable. There was no enlargement of the spleen as is commonly found in trypanosome infected mammals. The general appearance of the musculature was grossly normal.

Samples of the liver, spleen, kidney, thymus, bursa of Fabricius, bone marrow and cecal tonsil were fixed in Bouin-Holland, processed and cast in paraffin wax. Embedded tissues were sectioned; 5μ thick, mounted on slides, stained with Dominici stain and examined microscopically.

Histological examination of spleen sections from all infected chickens showed a striking increase in both number and size of germinal centres. While the meridional sections of spleens from control chickens showed 5-8 germinal centres, those from infected chickens were found to contain 85-109 centres. Sections from the other tissues showed no apparent pathological features.

Since trypanosomes show wide antigenic variation in infected hosts, it was thought interesting to investigate the condition of germinal centres in long-term infections. The infections in the remaining

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PLATE 10.

5 x 5 MICROSCOPIC VIEW OF SPLEEN SECTION FROM A TRYPANOSOME INFECTED CHICKEN.

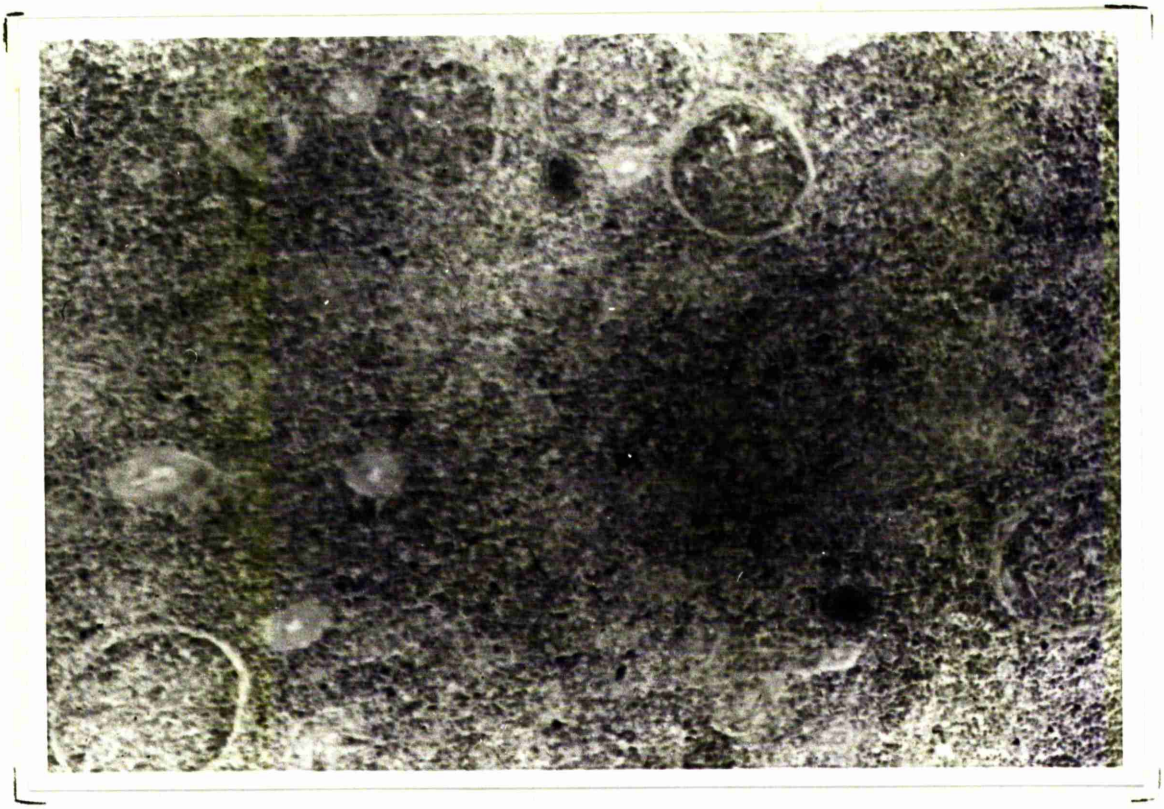
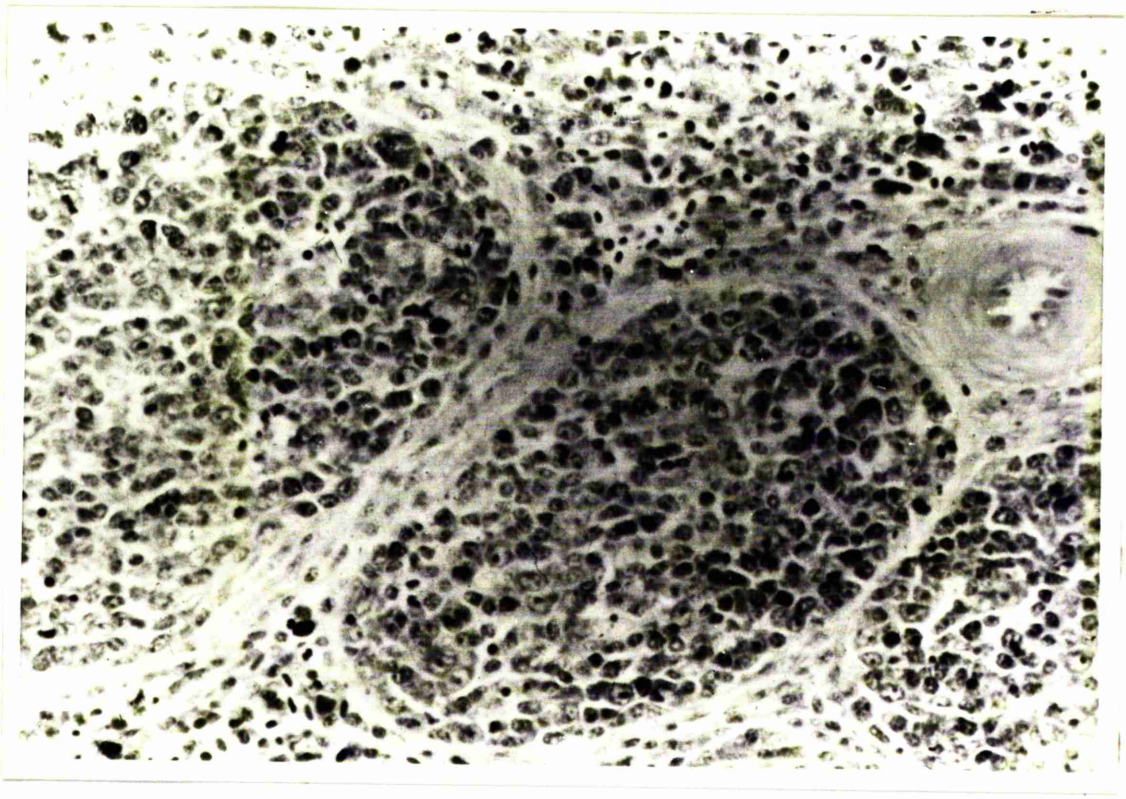


PLATE 11.

GERMINAL CENTRES UNDER 40 x 10 VIEW.



chickens was therefore allowed to run for eighty four days. All chronically infected and control chickens were then killed simultaneously by cervical dislocation. At necropsy the carcasses of infected and control chickens were indistinguishable. The characteristic muscle atrophy and emaciation found in chronically infected mammals were lacking.

Sections of the lymphoid organs were prepared for histology as for the first group of chickens. Microscopic examination of Dominici stained sections of the spleen revealed between 169-269 germinal centres. Spleen sections from control chickens showed a normal number of germinal centres (5-9). The gross architecture of the spleen was not disrupted.

Germinal centres in the infected chickens showed areas of high mitotic activities.

Comparison of the spleen size from infected and control chickens did not show any obvious differences. (See Table 16). Ratio of the spleen to the live body weight of each bird was calculated. This is referred to as Splenic Index (S.I.).

$$S.I. = \frac{\text{Weight of spleen} \times 100}{\text{Total body weight}}$$

Despite the striking increase in number of germinal centres in the spleens of infected birds, this organ did not reveal any abnormal increase in size over those of control birds.

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TABLE 16.

Ratios of spleen and total body weights of *T. brucei brucei* infected birds and those of uninfected controls.

	Chickens	Treatment	Body Wt.	Weight of Spleen	Spleen Index	Mean*
1	34	Control	3260	3.800	0.1160	
2	196	Control	1535	2.480	0.1615	
3	315	Control	2000	2.873	0.1436	0.1502
4	416	Control	2163	3.892	0.1799	
1	140	Infected for 84 days	2632	4.00	0.152	
2	287	"	2220	2.335	0.1380	0.1380
3	398	"	2260	2.743	0.1213	
4	426	"	2164	3.652	0.1687	

*No significant difference was observed as P = 0.522.

The lack of cellular disintegration previously reported in this thesis, may account for lack of splenomegaly commonly found in mammals infected with trypanosomes.

DEVELOPMENT OF GERMINAL CENTRES IN RESPONSE TO INOCULATION OF DEAD TRYPANOSOMES.

In the present work and that of Wallace, White and Herbert (1977) increased numbers of germinal centres have been observed in birds infected with T. brucei undergoing antigenic variation. Germinal centres in mammals are believed to be related to particular antigens (White, Coons and Connolly, 1955). On the other hand, Hellman (1930, quoted by Hanna, Swartzendruber and Congdon, 1967) indicated that germinal centres could be induced by noxious metabolites from bacteria, though this idea has now been proved erroneous (Hanna et al, 1967).

Trypanosomes do produce pyruvate and mitogens and it could be that these are the causes of the increased number of germinal centres rather than the variety of antigens presented to the host by antigenic variation. To test the hypothesis that a mitogen might be responsible, birds were inoculated with a large number of dead trypanosomes and the development of germinal centre was monitored.

Trypanosoma brucei stabilate WIG 88 (WITat 4) was inoculated into six Balb/C mice. On day 3 post

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infection all mice were bled from the orbital plexus. Trypanosomes were separated from mouse blood on a DE-52 column. After centrifugation the parasite concentration was reconstituted to $10^{8.4}$ per ml. of suspension. The trypanosomes were then killed by repeated freezing and thawing. Death of all the trypanosomes was confirmed by the inability of the thawed organisms to produce an infection in inoculated mice.

Two ml. of each dead suspension of trypanosome, which had been stored at -20°C whilst being tested for viability, were inoculated into each of two birds. Duplicate inoculation was carried out with viable organisms derived from the same stabilate (WIG 88).

Both groups of birds were killed on day 32 post inoculation. Meridional sections of spleen were processed for histology by the Dominici technique, as previously described.

Microscopic examination of Meridonial sections showed that while birds inoculated with dead trypanosomes had 28 and 39 germinal centres per section respectively, sections from birds with an active infection showed 68 and 102 germinal centres per section.

$$P = 10.224$$

These results show that germinal centres in chicken spleens can be activated by the inoculation of

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dead trypanosomes. It is, however, evident that birds with active infections developed many more germinal centres in the spleen than the birds inoculated with dead trypanosomes.

SPLENIC GERMINAL CENTRES IN SELF-CURED BIRDS.

The present investigation has shown that some chickens self-cured trypanosome infections after some six months. On the basis of the hypothesis which states that germinal centres are produced to trypanosome variants (Wallace et al, 1977) birds that have self-cured the infection after many months should have fewer germinal centres in their spleen than are present in birds with an active infection.

Six birds that had self-cured T.b. brucei infection, seven months previously, were killed and sections from their spleens were processed for conventional histology. Details of duration between self-cure and slaughter are given in Table 17.

All birds showed normal number of germinal centres.

This experiment shows that an active infection is essential for increased number of germinal centres. It is also evident that germinal centres do not persist indefinitely after cure of trypanosome infection.

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TABLE 17.

Numbers of germinal centres in the meridional section of spleens from birds self-cured of Trypanosoma brucei infections.

Bird	Inoculum	Total duration of Infection (Days)	Day of tissue collection		Number of Germinal Centre
			Post Infection	Post Cure	
78013	WIG 73 (WITat 1)	210	396	186	3
78014	WIG 73 (WITat 1)	210	396	186	5
78015	WIG 70 (AnTat 1)	7	427	420	10
78017	WIG 70 (AnTat 1)	7	428	421	17
78019	WIG 70 (AnTat 1)	5	428	423	2
78018	WIG 70 (AnTat 1)	5	443	438	3

PROGRESSION OF GERMINAL CENTRES DURING THE COURSE OF LONG TERM TRYPANOSOMA BRUCEI INFECTIONS IN DOMESTIC CHICKENS.

Previous experiments have shown that during active T. brucei infections, in chickens, many splenic germinal centres are always formed. Thus an absence of numerous germinal centres in a suspected bird can be regarded as synonymous with an absence of T. brucei infection. It is necessary therefore to establish whether the number of germinal centres bears a lineal relationship to the chronicity of the infection or whether it parallels the pattern of the parasitaemia in the birds (Fig.6) i.e. sudden'ups and downs'.

An experiment was therefore carried out to enumerate splenic germinal centres at various times during the course of a long term T. brucei infections in birds.

Thirty 6-week old chickens were each infected with 10^6 T. brucei organisms derived from WIG 76 (WITat 1) after a three day passage in mice. The infection, monitored by blood inoculation into mice, was found to persist in the birds throughout the duration of the investigation. On days 7,14,21,62, 84,180,225,290,310/birds and/392, were killed, and spleen sections processed for histology as previously described. The number of germinal centres on each section was counted. Ten counts were made for each section and the arithmetic mean of the

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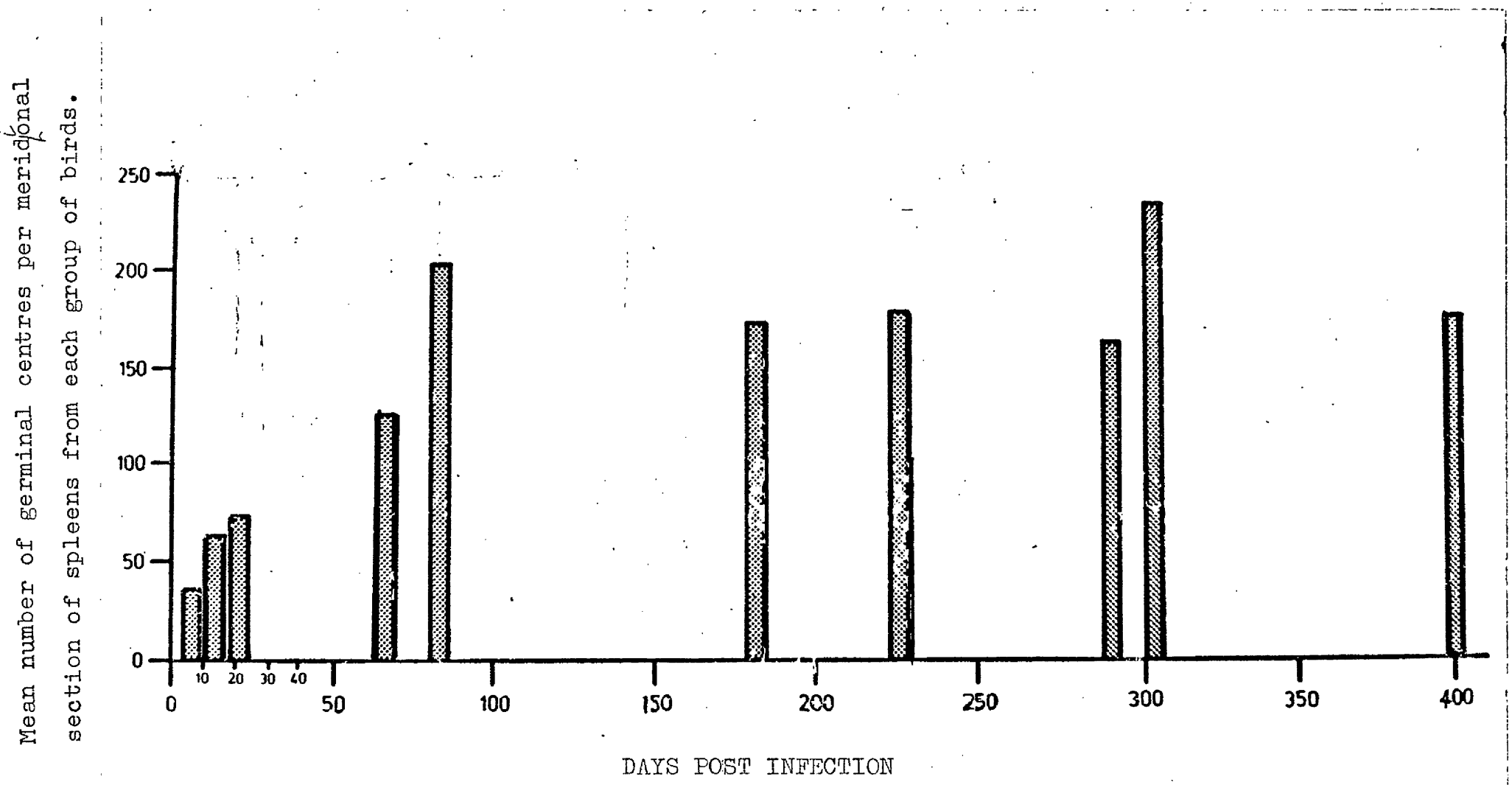
FIGURE 7. PROGRESSIVE DEVELOPMENT OF GERMINAL CENTRES.



counts was taken. Counts were also made from spleen sections from birds killed at the beginning of the investigation as well as from four control birds killed at the end of investigation. The average size of germinal centres from eight control birds was compared with the size of germinal centres in four birds that had been infected for 84 days.

A progressive increase in the number of germinal centres was noticeable in the spleen sections as the infection persisted (Fig. 7). Germinal centres increased to a plateau on day eighty-four post infection. After this day, high numbers of germinal centres were maintained but the numbers did not bear any lineal relationship to the chronicity of the infection. All control uninfected, chickens maintained a normal number (5-13) of germinal centres. A considerable increase in size was also noticed in germinal centres from infected chickens. There was a five fold increase in area.

This experiment showed that germinal centres increased in number during the early stages of the infection in birds, reaching a plateau at about the 84th day after which the numbers were maintained.

PERSISTENCE OF GERMINAL CENTRES IN CHICKENS CHEMOTHERAPEUTICALLY CURED OF TRYPANOSOMA BRUCEI INFECTION

The present investigation has shown that numerous

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Mean number of germinal centres per meridional section of spleens from each group of birds.

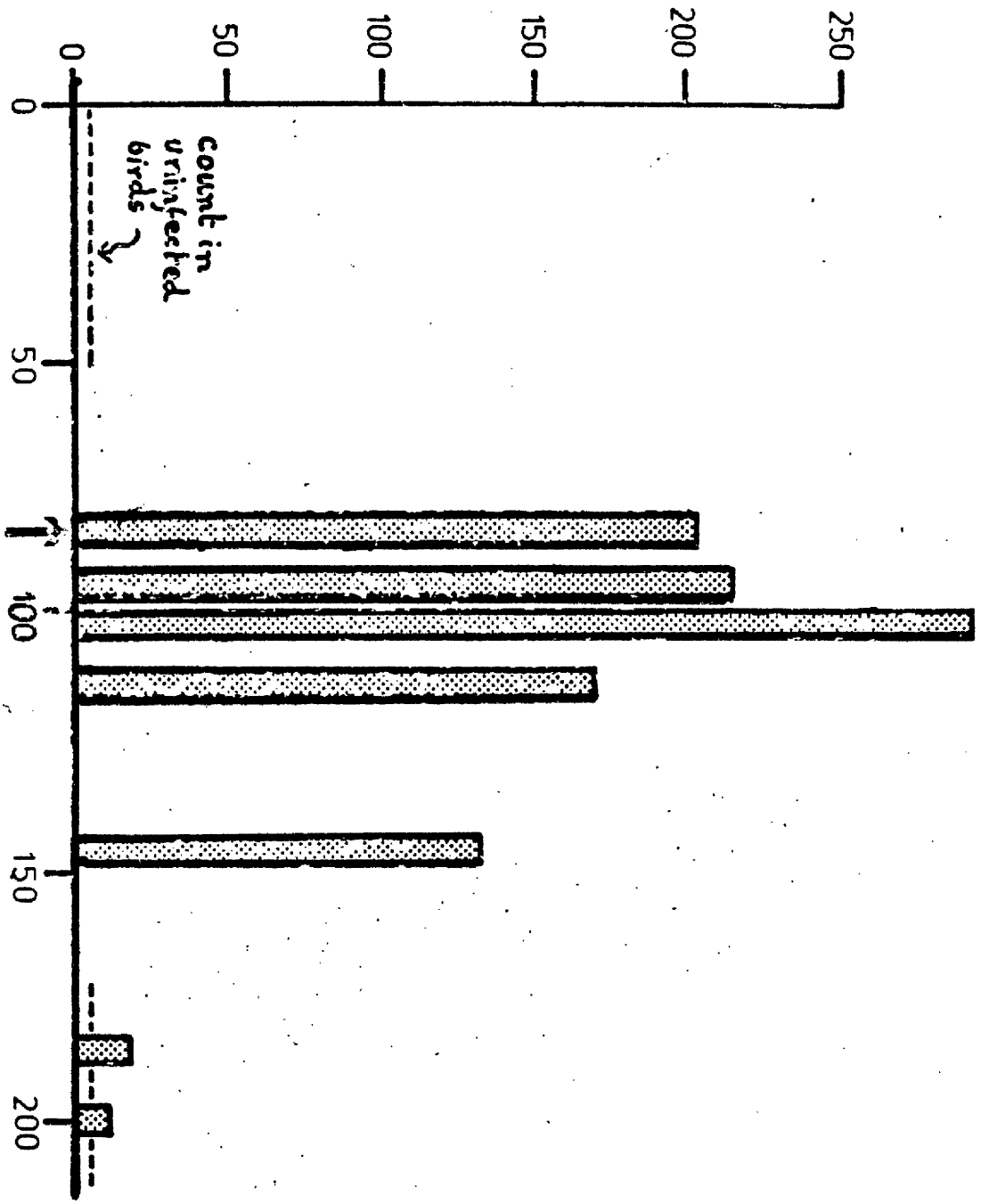

FIGURE 8. TIME COURSE OF REGRESSION OF GERMINAL CENTRES.

splenic germinal centres are highly associated with Trypanosoma brucei infections in domestic birds and that self-cured birds have a normal number of germinal centres after two hundred and forty days. This observation raises the question:
Would the germinal centres regress when the infection is chemotherapeutically eliminated? and if so, when?

An experiment was therefore carried out to study the life span of germinal centres formed during a chronic trypanosome infection.

Eighteen 6-week old chickens were inoculated intravenously with blood from four mice infected three days previously with WIG 75 (WITat 1). Periodic monitoring of infection by the standard method of mouse inoculation showed that a persistent infection had been established in all the birds.

On day 84 post initial infection four birds were killed and spleen sections were removed for conventional histology. Immediately after that the remaining fourteen birds were treated with Berenil at 7 mg. (active principle) per Kg. of live body weight.

On days one, three, six and eleven respectively, one ml. of heparized blood from each bird was inoculated into each of two mice. None of the mice became infected showing that all the birds had been cured of the infection. On days 11, 18, 36, 60, 100 and 120 post treatment of infection, groups of 2-3 birds were killed and spleen sections removed and processed for

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histology as previously described.

An obvious increase in the number of germinal centres was observed in the spleen sections from birds killed on days 11 and 18 post cure of infection. A diminution of number of germinal centres was evident in the spleen sections from birds killed 100 and 120 days post therapeutic cure of infection (Fig.8). In all cases 1 ml. of heparinized blood was inoculated into each of two mice before each bird was killed. None of these mice became infected with trypanosomes.

The results of this experiment indicate that an active infection is required to maintain a plateau number of germinal centres. It is also evident from these results that some germinal centres can persist for three months or more.

IDENTIFICATION OF GERMINAL CENTRES BY IMMUNOFLUCRESCENT TECHNIQUE.

On ordinary microscopic examination, the germinal centres in the spleen of trypanosome infected birds appeared to be morphologically identical to those normally found in antigenically primed birds. It is essential to validate that these germinal centres possess the characteristic dendritic cells found in normal germinal centres.

An experiment was therefore carried out to see if

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immunoglobulin bearing dendritic cells, which are de facto components of germinal centres could be fluorescently stained in the germinal centres from trypanosome infected birds.

Four 5μ cryostat sections of a spleen from a chronically infected chicken were prepared. Two of the slides were stained with fluorescein labelled anti-chicken 7S immunoglobulin by the direct technique and were examined microscopically under the fluorescence microscope to detect immunoglobulin-bearing dendritic cells within the germinal centres. A lacy network of immunoglobulin-bearing dendritic cells was seen in all the germinal centres of the spleen. As a control, for non-specific uptake of immunoglobulin, two control slides were stained with fluorescein labelled sheep anti guinea-pig globulin. No single positive fluorescence was found in any sections examined after staining in this way.

This investigation thus indicates that the germinal centres found in the spleen of trypanosome infected birds are indeed true and normal germinal centres and that chicken immunoglobulin is present on these centres.

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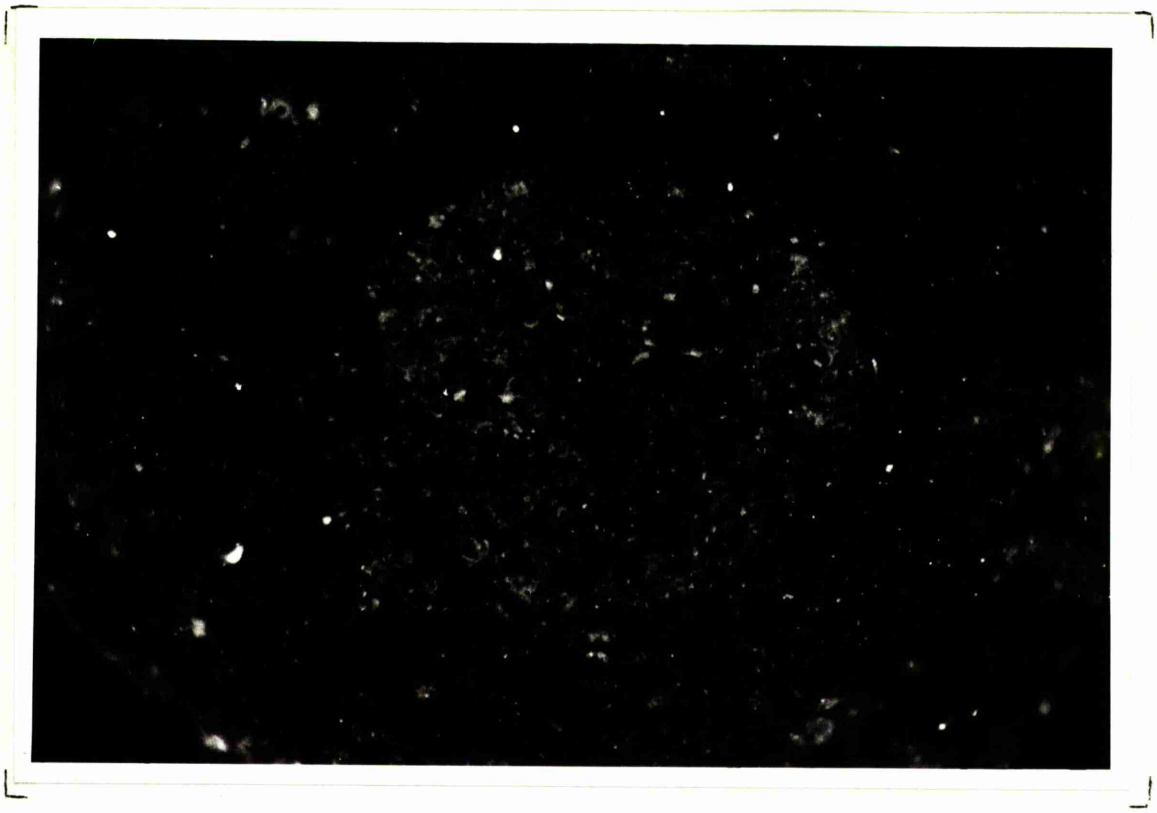
PLATE 13.

PHOTOGRAPH OF TRYPANOSOME ANTIGEN IN CHICKEN SPLEEN



The section shows trypanosome antigen fluorescently stained in the germinal centre of an infected chicken.

LOCALIZATION OF TRYPANOSOME ANTIGEN IN GERMINAL CENTRES OF CHICKENS FOLLOWING AN ACTIVE INFECTION.

Both morphological and immunological evidence have so far shown that the numerous germinal centres formed in the spleen of chickens during a chronic trypanosome infection are indeed true germinal centres formed by the agglutinating growth of lymphocytes and dendritic cells.

An experiment was therefore carried out to see if dendritic cells bearing trypanosome antigen could be fluorescently labelled in these germinal centres thus adding strength to the hypothesis that these centres are produced in response to the sequential release of antigens by the trypanosomes.

Stabilate WIG 88 (WITat 4) was inoculated into six mice. On day three post infection the trypanosomes were separated from mouse blood on a DE52 column. A count of motile organisms was carried out by haemocytometer method. Each of two 6-week old cockerels were infected with $10^{8.1}$ organisms.

Twenty-one days post infection the chickens were killed by cervical dislocation. Spleen sections were removed for cryostat section. Duplicate sections were also removed from uninfected chickens.

Cryostat sections obtained from both infected and

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TABLE 18.

Staining for trypanosome antigen infected chicken spleen.

+ = indicates material used.

- = indicates material not used.

Sources of Spleen	Guinea-pig Anti-WITat 4 Serum. Diluted 1:10	Fluorescein labelled Sheep Anti-guinea pig Conjugate. Diluted 1:10	Normal Guinea Pig Serum	Sheep and Guinea pig conjugate absorbed with Trypanosome	RESULTS
Trypano-some infected chicken	+	+	-	-	Positive Fluorescence
"	+	-	-	-	No Fluorescence
"	-	+	-	-	No Fluorescence
"	-	+	+	-	No Fluorescence
"	-	+	-	+	Very weak Fluorescence
"	-	-	+	-	No Fluorescence
Uninfect-ed Chicken	+	+	-	-	No Fluorescence
"	-	+	-	-	No Fluorescence
"	-	-	+	-	No Fluorescence

control chickens were stained by a 'sandwich' technique using anti WITat 4 serum raised in a guinea-pig. Full details of the technique are given in the method section. Control experiments were set up as shown in Table 18.

Positive apple-green fluorescence was observed in some germinal centres in spleen sections obtained from infected chickens. Spleen sections, obtained from trypanosome infected birds, stained with homologous antiserum, but previously absorbed with homologous trypanosomes, failed to produce the diagnostic brilliant fluorescence. All the other attempted staining schedule (Table 18) failed to show any positive fluorescence when examined under the ultraviolet light.

This experiment showed that trypanosome antigen is present in germinal centres in the spleen of trypanosome infected birds.

IMMUNODEPRESSION TO HUMAN SERUM ALBUMIN IN TRYPANOSOME INFECTED BIRDS.

Experimental infections have been shown to induce immunodepression in mammals (Goodwin, Green, Guy and Voller, 1972). In domestic chickens, the infection is self-limiting and is characterized by a very low parasitaemia.

An experiment was carried out to see whether this low parasitaemia will induce an immunodepression in

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PLATE 12

Immunodepression in chickens:

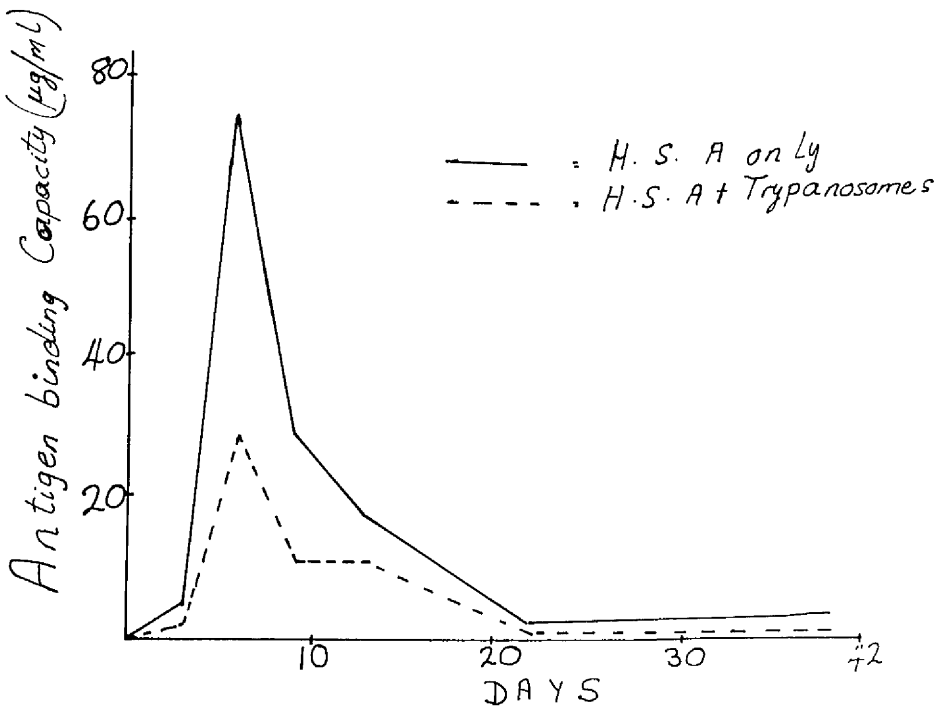
Serum antibody levels to H.S.A. in Trypanosoma brucei brucei infected and control birds.

Each point on the figure is the arithmetic mean of six different samples.

———————— = birds inoculated with H.S.A. only

---------- = Trypanosome infected blood inoculated with H.S.A.

Chicken inoculated intravenously with 10 mg. of H.S.A. in saline.



infected chickens. Twelve 6-week old birds were placed in two groups of six. Both groups of birds were bled for pre-challenge (reference) serum. Viable trypanosomes derived from stabilate WIG 76 (WITat 1) were inoculated into four mice. On day three post infection, trypanosomes were separated from mouse blood on a DE 52 column. All group one birds were each inoculated with $10^{7.5}$ trypanosomes. Infection in all the birds was confirmed by blood inoculation into mice on day five. Each of both control and infected bird was inoculated intravenously with 10 mg. of H.S.A. dissolved in 0.5 ml. normal saline. All the birds were bled for serum on days 3, 6, 10, 15, 22, 30 and 39 post H.S.A. inoculation.

Total antibody level in the sera of both infected and control chickens was assayed by the Farr test. Full details have been given in the method section. The mean levels of total antibody are shown in Plate 12. The rise and fall in serum antibody to H.S.A. followed a similar time course in both infected and control groups. The titre of antibody in the control group was, however, conspicuously higher than in the infected group. Trypanosome infection did not alter the time course of antibody response, it did depress the overall level of serum antibody.

This experiment thus showed that despite the low parasitaemia, immunodepression can indeed be said to be a feature of Trypanosoma brucei infection in birds. It

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is also suggestive that, immunodepression might not be directly related to level of parasitaemia but to the overall functional status of the immune apparatus.

LOCALIZATION OF H.S.A. IN SPLENIC GERMINAL CENTRE OF BIRDS CHRONICALLY INFECTED WITH T. BRUCEI.

The numerous germinal centres in the spleen of chicken infected with T. brucei give an ideal opportunity to test whether an unrelated antigen would indeed localize in germinal centres that had already been formed in response to a non-cross reacting antigen.

Four chickens chronically infected with trypanosomes derived from stabilate WIG 73 (WITat 1) were used. The infection had run from between 78-369 days and by this time numerous germinal centres are present in the spleen. Each chicken was inoculated with 10 mg. H.S.A. dissolved in 0.5 ml. of normal saline. On day seven post inoculation of H.S.A., the four chickens were killed by cervical dislocation and samples of spleen were removed from each chicken. Half of each sample was rapidly frozen for cryostat sections and the other half was fixed in Bouin-Holland for conventional histology.

Cryostat sections of the spleen were stained with fluorescein-labelled rabbit anti H.S.A. to detect H.S.A. antigen. In order to detect antibody to H.S.A.

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TABLE 19

LOCALIZATION OF H.S.A. IN THE SPLEEN OF CHICKENS WITH NUMEROUS GERMINAL CENTRES.

CHICKENS	DAYS POST INFECTION	NUMBER OF GERMINAL CENTRES PRESENT	FLUORESCENTLY STAINED GERMINAL CENTRES
91	98	176	3
186	286	162	12
192	369	220	9
38	78	202	3

The total number of germinal centres in the spleen of chicken infected with T. brucei and the few centres showing H.S.A. localization.

the 'sandwich' technique was applied as described in the Materials and Methods Section. Only very few of the centres 3-12 (see table 19) showed localization of H.S.A. when the sections were observed under the U/V light. Microscopic examination of Dominici-stained meridional sections of the spleen showed the presence of 162-220 germinal centres on each section. Attempts at staining for trypanosome antigen proved unsuccessful. Since only a very small number of the germinal centres showed localization of H.S.A., it is deduced that these represent germinal centres which are newly formed to the injected H.S.A. antigen. This experiment suggests that new antigens do not localize into germinal centres that had already been formed to other antigens. Inability to detect fluorescence corresponding to trypanosome antigen is suspected to be due to a lack of identity between the type of fluorescein-labelled antiserum used and type of trypanosome variable antigen present in the germinal centres at death.

LOCALIZATION OF FLUORESCEIN LABELLED H.S.A. IN THE SPLEEN OF T. BRUCEI INFECTED CHICKENS.

The preceding experiment suggested that H.S.A. does not localize in germinal centres already formed in response to trypanosome antigen. To get a further confirmation of this observation fluorescein-labelled

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TABLE 20.

Numbers of fluorescein labelled germinal centres found in the spleen of trypanosome infected birds intravenously injected with fluorescein-labelled H.S.A.

(All birds were killed six days post H.S.A. inoculation).

Chicken	Trypanosome infection	Days Post Trypanosome infection	No. of Germinal Centres with labelled HSA	No. of Germinal Centres per Section
(33)	Yes	78	4	139
(184)	Yes	192	3	186
(44)	No	-	7	9
(46)	No	-	12	15

H.S.A. was injected into two chronically infected chickens and two uninfected chickens. Both groups of chicken were killed on day six post inoculation. Spleen sections were removed for both conventional histology and cryostat sections. Dominici stained sections of spleen from trypanosome infected birds showed 139, 186 germinal centres respectively. Control uninfected chickens on the other hand only showed 9, 15 germinal centres.

When the cryostat sections were examined under ultraviolet light, there was not much difference between the pattern of fluorescently stained germinal centres (3-12) except that spleen from control group showed more labelled germinal centres. (Table 20).

It is striking to note that in no case was the number of germinal centres with fluorescently-labelled cells equal to the number found under conventional histology.

Spleen sections from trypanosome infected chickens showed a low number of germinal centres with fluorescently labelled H.S.A. This is suggestive that non-cross reacting antigens do not localize in germinal centres that had already formed. The fewer number of fluorescently-labelled germinal centres in the infected group is also an evidence of immunodepression triggered off by the unusually heavy demand on the immune response to keep the trypanosomes in check.

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SERUM ANTIBODY LEVELS IN CHICKENS INFECTED WITH T. BRUCEI SSP.

Observations, from the present studies, that normal chicken serum does not inhibit the infectivity of Trypanosome brucei suggest that chickens infected with T. brucei mount an efficient immune response to the parasite and it is believed that this immunological ability keeps the parasites at a very low level. In contrast, studies by Goedbloed and Southgate (1969) could not demonstrate agglutinating antibodies in the sera of chickens hatched from eggs infected in ovo. The comprehension of the immune phenomenon has so increased since the later observation was made, that a re-evaluation of the observation in adult birds is considered necessary. Goedbloed and Southgate (1969) infected embryos and later checked for antiserum post hatching. The hatched chickens might have developed tolerance to trypanosome antigen.

Experiments were therefore carried out to study serum antibody levels in chickens infected, post hatch, with trypanosomes of defined antigenic types.

Six 8-week old chickens were placed into two groups of three each. Each chicken in the first group was infected with one million mouse infective doses of stabilate WIG 83 (WITat 2) from parasitaemic mouse blood whilst each chicken in the second group was

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FIGURE 10 . Serum agglutinins in chickens infected with WITat 4 (WIG 88)
(Mean of three chickens.

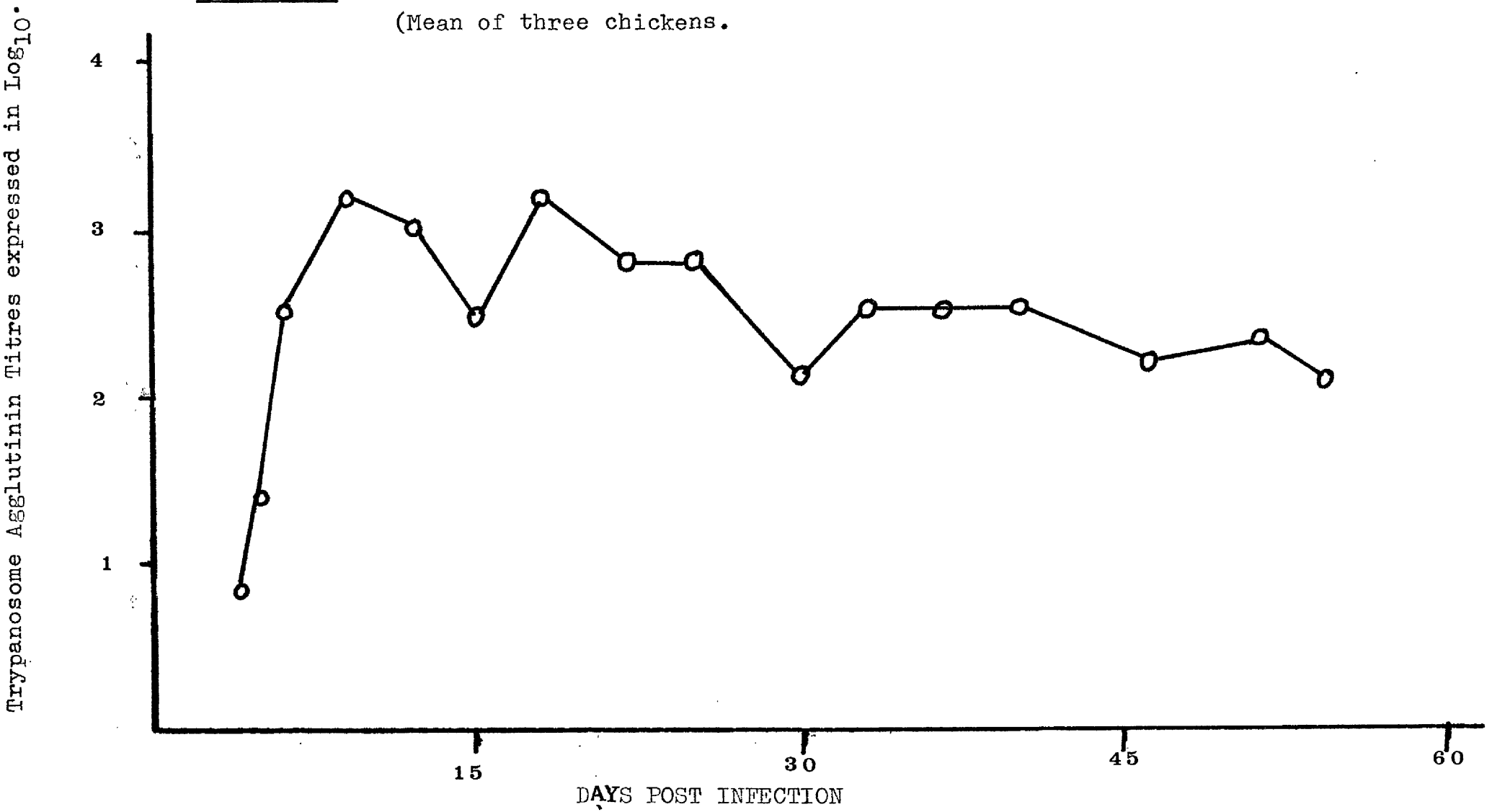
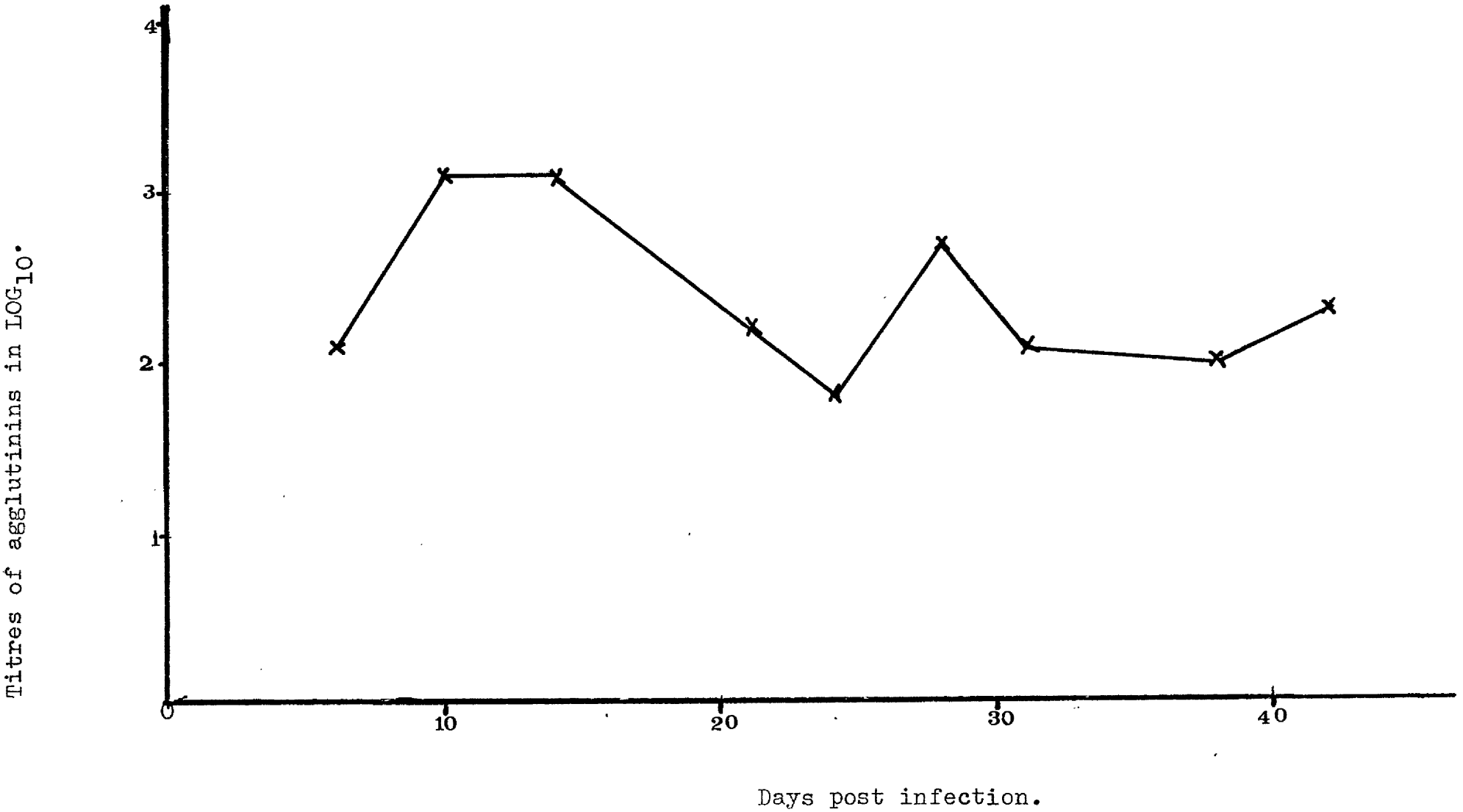


FIGURE 9. Serum agglutinins in chickens infected with Trypanosoma brucei brucei. (Mean titres for 3 pullets) WIG 83 (WITat 2)



infected with 10^8 organisms derived from WIG 88 (WITat 4) Both groups of chickens were found infected by blood inoculation into mice.

Blood samples were collected for antisera from each chicken on days, 3, 4, 5, 6, 10, 12, 15, 18, 22, 25, 30, 33, 36, 40, 46, 51 and 54. Samples were frozen at -20°C until used. The titre of trypanosome agglutinin antibody in the sera was assayed, as described in the method section, using stabilated materials. In both groups of chickens no agglutinating antibodies were detected in sera collected on day 3. Trypanosome agglutinins were first detected in sera collected on day four. Thereafter there was a sharp rise in titre. Over the period of two months, high levels of trypanosome agglutinins were still detectable in all the sera. (Figs. 9 and 10).

The titre of the agglutinins showed undulating waves. It was observed that the highest recorded level of agglutinin was found on days 10-12.

Antiserum collected on day 12 from the group of birds infected with trypanosomes derived from WIG 88 (WITat 4) was fractionated on AcA22 and the titres of trypanosome agglutinin in both the IgG and IgM fractions were assayed using stabilate WIG 88 as antigens.

Fig. 12 shows the distribution of antibody between 19S and 7S fractions. A high titre (1280) was found in the 19S fraction. Thus confirming that IgM is

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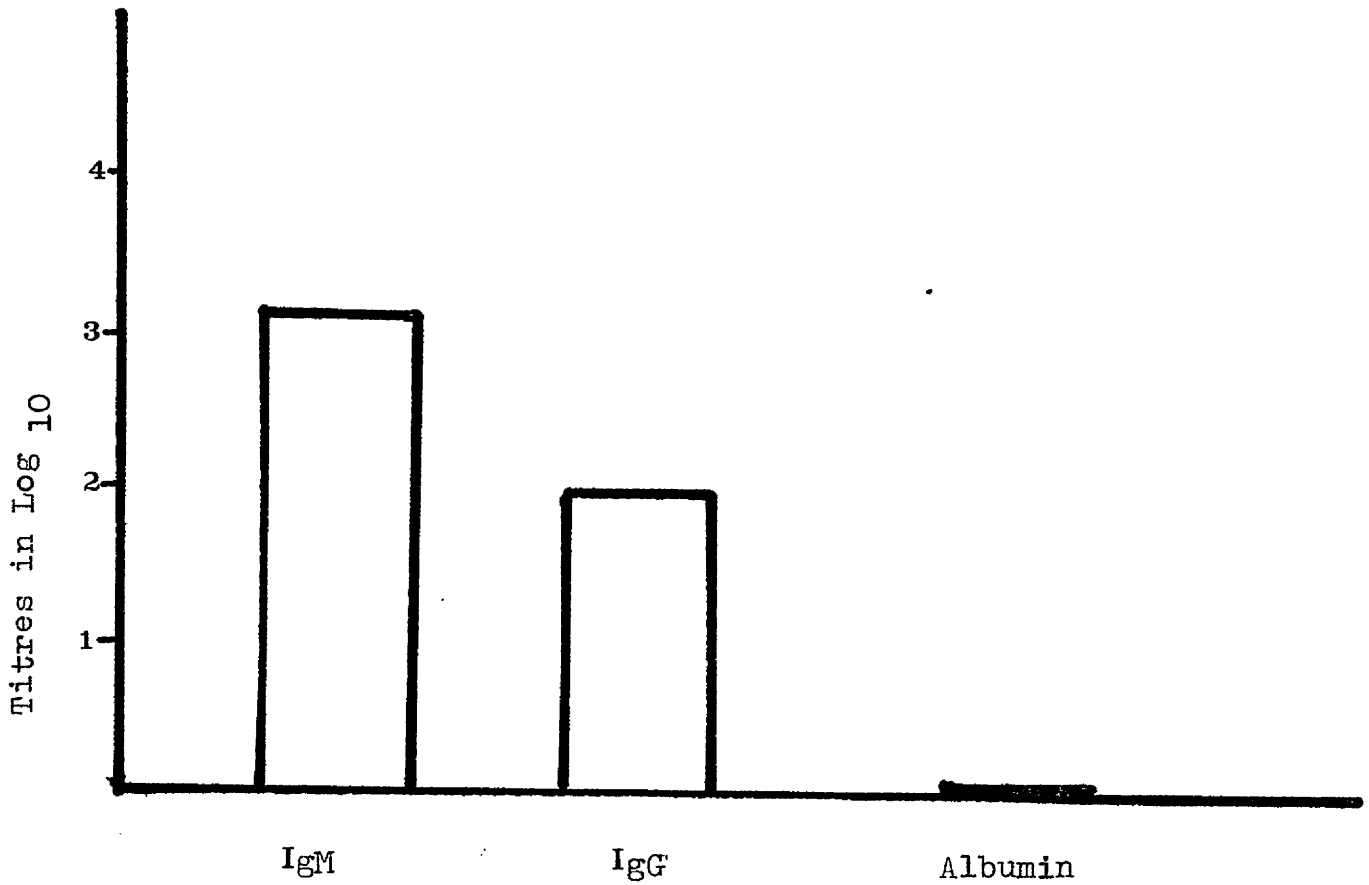
Figure 12 Trypanosome antibody in serum proteins of infected chicken

(Agglutinins)



the most predominant immunoglobulin responsible for agglutination reaction.

The IgG nonetheless, demonstrated some agglutinating ability albeit on a very low scale. The agglutinin titre for the unfractionated serum was higher than that recorded for the IgM alone. Albumin fractions did not agglutinate the trypanosomes.

This experiment shows that serum agglutinin antibody could be detected in chickens as early as day four post infection and that the agglutinin is predominantly an IgM response. The slightly lower titre of IgM agglutinin compared with whole serum suggest that the agglutination reaction is not exclusively an IgM response.

COURSE OF TRYPANOLYTIC ANTIBODY IN BIRDS INFECTED WITH TRYPANOSOMA BRUCEI.

The preceding experiment showed that the course of trypanosome agglutinins in infected domestic chickens is different from that commonly observed in infected mammals in that it exhibited a rhythmic wave. An agglutination reaction is only one of many manifestations of immune responses, attempts were made to study the pattern of another immune reaction. Perhaps the reaction in this case may give a pattern that is different from the oscillating titre observed in agglutination reaction.

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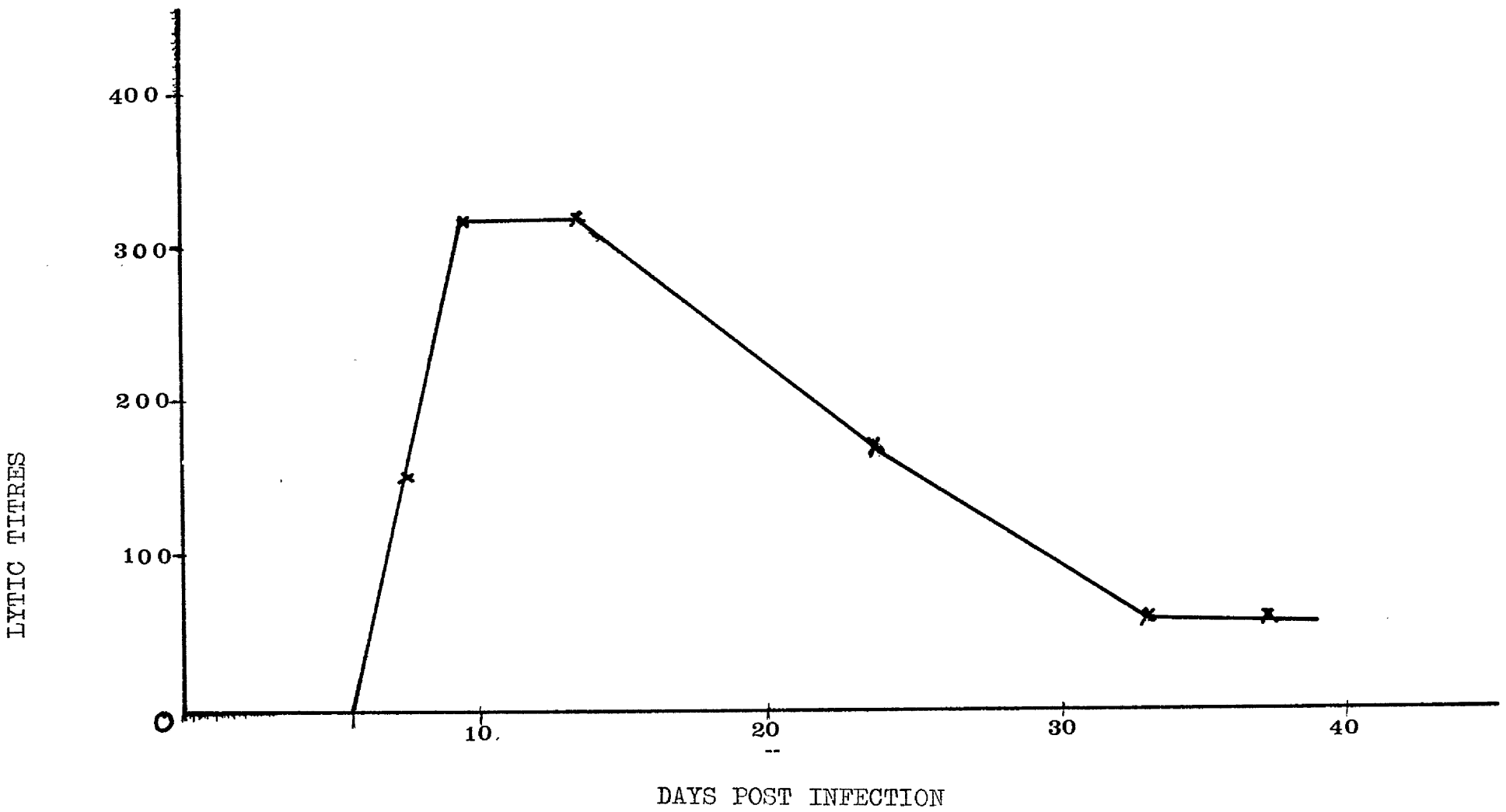
FIGURE 11. LYTIC ANTIBODY TITRES IN CHICKENS INFECTED WITH WIG 83 (WITat 2)



Experiments were therefore carried out to assay the level of trypanolytic antibody in the serum of birds infected with T. brucei.

Each bird in a group of three was inoculated with one ml. of bloodstream T. brucei brucei population derived from WIG 83 (WITat 2) after a three day passage in mice. Infectivity of parasitaemic mouse blood was immediately titrated as previously described, and the results of the titration showed that each chicken had been inoculated with one million mouse infective organisms. All inoculated birds were confirmed infected by the standard method of mouse inoculation. The test birds were bled for antiserum on days 2, 4, 6, 10, 14, 24, 31 and 35; serum samples were kept at -20°C until they were used.

The trypanolytic titre of each serum sample was assayed as described in the method section. No lytic antibody was detected in the samples collected before day 6. Peak trypanolytic titre was detected from sera collected on days 10-14. Thereafter, the titre dropped, though samples collected on day 31, when diluted 1:40, still lysed bloodstream trypanosomes derived from WIG 83 (WITat 2).

This experiment shows that unlike the trypanosome agglutinins the serum trypanolytic antibodies (Fig.11) do not exhibit an undulating wave (Figs. 9 and 10).

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SERUM 7S AND 19S IMMUNOGLOBULINS LEVELS IN TRYPANOSOME INFECTED CHICKENS.

The preceding experiment showed that Trypanosoma brucei infected chickens produced both agglutinating and lytic antibodies. These antibodies are mediated mainly by the serum IgM and IgG.

One of the characteristics of humoral response to salivarian trypanosome infection in mammals is the remarkable rise in IgM response (Mattern, 1964; Lumsden, 1967). The rises in IgM are rather dramatic, amounting sometimes to 16 or more times the normal levels in infected mammals (Lumsden, 1972). The extent to which the immunoglobulin levels are increased in normal chickens, infected with a salivarian trypanosome is not known. An experiment was therefore carried out to compare the relative increase of both IgG and IgM of trypanosome infected chickens during the course of a chronic infection by the parasite.

Anti-chicken 19S Immunoglobulin and Anti 7S and the immunoglobulins were supplied by Dr. Nielsen. Chickens infected with bloodstream trypanosomes, derived from WIG88 (WITat 4) were bled for serum samples on days 0, 10, 15, and 100. Sera were stored at -20°C until used. The levels of IgM and IgG were quantified by the Mancini technique (1965). Using a standard curve, for known chicken 7S and 19S, the concentrations of the two immunoglobulins in the sera were/

TABLE 21.

	19S				7S			
	DAYS POST INFECTION							
	Day 0	10	15	100	0	10	15	100
Infected birds								
61	14	11.34	15.20	16.0	56.75	70	72	91
62	15.20	15.20	15.20	15.20	93.2	95	93	96
63	15.20	15.20	15.90	15.20	50	55	65	82
Controls								
65	19.63	14.90	15.90	15.90	85	86	86.4	86
258	17.35	15.90	15.90	17.3	64	66.4	66.50	66.4

Immunoglobulin levels in chickens infected with T. brucei (WITat 4) - Square of precipitin rings in mm^2.

were calculated. Quite unexpectedly, the increases in agglutinating antibody level, previously reported, were not reflected in both immunoglobulins. There was, however, a slight increase in the IgG level on samples collected on day 100 (Table 21). It is apparent, therefore, that increased IgM is not a feature of T. brucei infections in domestic fowls.

The difference in agglutinin titres between the IgM and the IgG is probably due to the molecular structure of each immunoglobulin. The IgM molecule with at least five antigen-binding sites is particularly effective in bringing about agglutination.

S Y M P T O M A T O L O G Y.

BLOOD PICTURE IN BIRDS INFECTED WITH T. BRUCEI SSP.

Anaemia is a consistent finding in mammals infected with African trypanosomes (Hornby, 1921). The mechanism of the anaemia has not been established. Nothing, however, is known of the effect of Trypanosoma brucei infection on the blood picture of chickens.

Four 2-week old cockerels were each infected with 10^6 mouse infective doses (ID_{63}) of a population of T.b. brucei derived from stabilate WIG 82 (WITat 2). The number of infective doses in the mouse blood was quantified as previously described.

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TABLE 22.

Packed cell volume of a group of four chickens that were infected with trypanosomes derived from WIG 82 (WITat 2), when the birds were two weeks old. The observation was carried out for 31 days. The packed cell volume of a similar control group of two uninfected chickens kept in the same conditions is also shown.

DAYS POST INFECTION	INFECTED CHICKS					CONTROLS		
	A	B	C	D	Mean	E	F	Mean
0	33	36	34.5	35	34.6	34.5	35	34.75
3	33	35	34	35	34.25	34	36	35
5	32.5	35	34	34.5	34	35	35.5	35.25
8	31	29	29	30	29.75	35	36	35.5
11	30	27	27	29	28.25	35.5	35.5	35.5
14	26	24	25	24	24.75	33	35	34.5
17	27	27.5	27	25	26.5	36	34	35
23	30	31	29	28	29.5	35.5	35	35
31	32	32	31.5	32	32	35	36	35.5

The infection in the chickens, monitored by blood inoculation into mice, was found to persist throughout the period of investigation. The packed cell volume of all infected birds as well as those from two control birds were determined on days 0, 5, 8, 14, 17, 23 and 31, by the haematocrit technique. The packed cell volume of all the infected chickens was found to drop from 34% on day five to 24.75% on day fourteen. By days 23 and 31, it was apparently returning to normal. Control chickens on the other hand showed a steady level of packed cell volume, over the same period, compatible with that found by Ross, Christie, Halliday and Jones (1978).

These results thus indicate that:

Trypanosoma brucei infection in birds is accompanied by a transient fall in the packed cell volume (Table 2 2).

THE EFFECT OF NEONATAL T. BRUCEI INFECTIONS ON THE BODY WEIGHT GAINS OF CHICKENS.

None of the T. brucei infected chickens reported in this thesis has presented evidence of ill health.

Most sub-clinical diseases are, however, noted for causing loss of production in animals; for example growth retardation has been reported in goats infected with T. vivax, (Isoun et al, 1976). On the other hand, Linicombe (1963) reported increase growth rate in rats

infected with T. lewisi. It is not known how the growth rate of chickens will be affected by the infection.

Experiments were therefore carried out to elucidate the influence of T. brucei ssp infection on the growth of chickens. Twelve 1-week old pullets were placed in two groups of six. The weights of the various groups were recorded separately and individually.

All of six pullets in one group were inoculated intravenously with one million mouse infective doses (ID_{63}) of trypanosomes derived from WIG 76 (WITat 1) after a three-day passage in mice. Infectivity was determined as previously stated. The infection, monitored by periodic mouse sub-inoculation, persisted throughout the duration of investigation.

Each chicken was weighed separately on days 8, 11, 14, 24, 28, 32, 36, 42, 44, 47, 50 and 57. The overall weight gain, for each group, was calculated as the proportional increase in weight per unit initial body weight. Standard errors and Student's t-test were employed. The initial body weights of the two groups had a very close margin in order to begin the investigation with as little variation as possible.

Body weight increments were studied as proportional gains relative to each unit of beginning weights (plate 14). Statistical examination of the data was limited to body weight gains. Average body weight gains indicated no significant difference between control and

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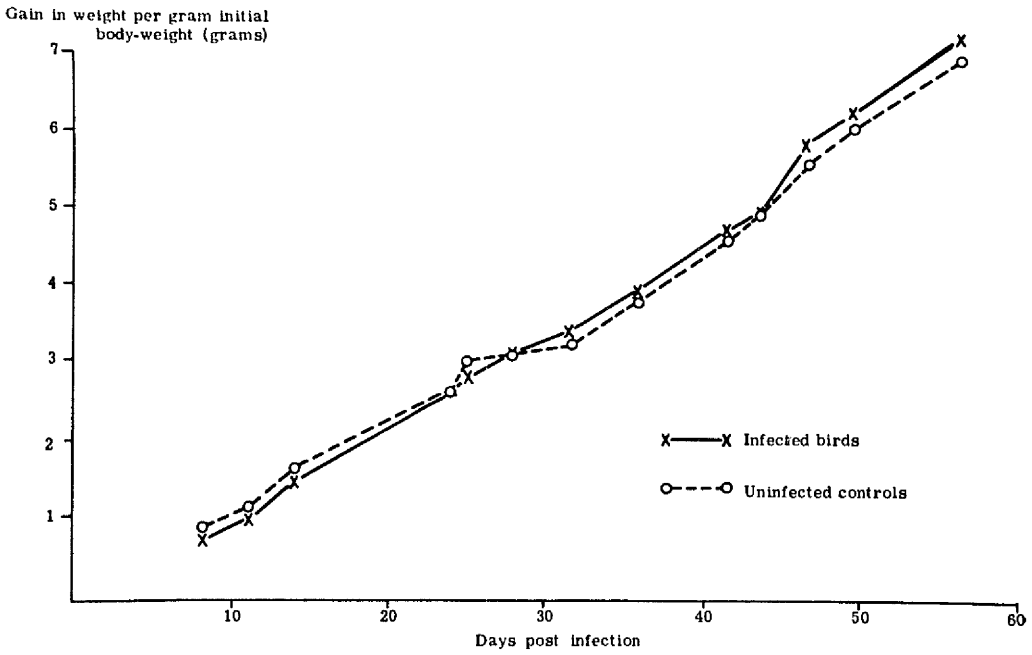
PLATE 14.

GROWTH RATE OF INFECTED AND CONTROL BIRDS



infected pullets (plate 14) standard error of both groups p = 0.05. A mutual adaptation seems to exist between the chickens and the trypanosomes. The effect of trypanosome infection in chicken is contrary to reported cases in mammals, in that the infection neither depressed nor increased the growth rate.

EFFECT OF TRYPANOSOMA BRUCEI INFECTION ON THE EGG PRODUCTION OF PULLETS.

An interest in the relationship between T. brucei ssp infection and egg production has arisen from the observation that this haemoprotozoan does not retard nor depress the growth rate of infected birds. From theoretical consideration, however, it is assumed that no parasite is completely free of any adverse effect on its hosts; hence infected chickens should not grow as fast as the control.

Ten 13-day old pullets were placed into two groups of five. One group was infected with mouse blood containing trypanosome derived from WIG 76 (WITat $1,10^{7.8}$ organisms) whilst the other group acted as a control. Both groups were kept in identical conditions but in separate cages maintained in the same room. Total eggs produced per group were recorded during an observation period of one hundred and thirty five egg production days.

During the observation period deaths of one and

/

TABLE 23.

EGG PRODUCTION OF PULLETS INFECTED WITH T.B. BRUCEI.

BIRDS	EGG PRODUCTION DAYS	AVERAGE/EGG/BIRD/DAY
Neonatally Infected (5)	197	0.20
Control (5)	197	0.45
Infected at 18 weeks (5)	135	0.50
Control (5)	135	0.57

and two pullets were recorded in the infected and control groups respectively. Infected pullets laid as few as half the eggs as did those in the control group, i.e. on the average each infected hen laid 0.2 egg/day while each control laid 0.45 egg/day.

This is the only adverse effect that has so far been found in these hosts. It is evident, therefore, that a parasite may not be without any adverse effect to its host. Reproductive deficiency may thus be one of the cardinal symptoms in chronic parasitic infections.

This investigation was repeated by infecting five pullets aged 18 weeks with trypanosomes derived from stabilate WIG 76 (WITat 1) post 3 day passage in mice. Egg production in these pullets was compared with that from five matched pullets. In contradistinction to the previously reported halving of egg production in hens infected at 2 weeks, post maturity infection with T. brucei was found to have no adverse effect on production. (See Table 23).

On the average, each hen infected post maturity laid 0.54 eggs per day while the controls laid 0.56. The experiment shows that the effect of T. brucei infection in avian hosts may vary under different physiological conditions of birds.

A RARE CASE OF TERMINAL EXHAUSTION IN A T. BRUCEI BRUCEI INFECTED CHICKEN.

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A high degree of biological adaptation has so far been observed between birds and the infecting T. brucei brucei. On only one occasion, however, a chronically infected bird suddenly became lethargic. In view of the rarity of this clinical condition in sixty birds so far studied, it was decided to carry out full clinical pathological investigations on the chickens so as to ascertain whether the lethargy was caused by the trypanosomes or other environmental agents.

The bird was infected with T.b. brucei (WITat 1) when he was eleven days old and persistently showed, by mouse inoculation, to be infected. On day two hundred and eighty the cock suddenly became lethargic.

PHYSICAL APPEARANCE.

No clinical signs of ill-health were observed in the cock until day 280 when it suddenly developed a torticollis-like appearance. The comb and wattles were cold to touch and the bird was oblivious to its surrounding.

The ten-month old cock weighed 1.862 Kg. Ten ml. of blood was collected, in a heparinized syringe, by cardiac puncture. Attempts at collecting the blood from the brachial vein failed, because of circulatory collapse. The blood was immediately titrated in mice, the cock was killed and tissues were removed for histology.

/

BLOOD PARASITE COUNT.

No trypanosomes were found in the blood by microscopic examination of wet and thick films. Estimation of parasitaemia by the infectivity titration method showed that not less than 10^4 mouse infective (ID_{63}) were present in each ml. of the bird's blood (Table 24). This is still below the limit of visual microscopy.

TABLE 24.

TRYPANOSOME INFECTION IN MICE INOCULATED WITH VARIOUS DILUTIONS OF CHICKEN BLOOD.

Group	Dilutions of Chicken blood (-log)	Mice infected/ Mice inoculated
A	Neat	6/6
B	$\bar{1}$	6/6
C	$\bar{2}$	6/6
D	$\bar{3}$	5/6
E	$\bar{4}$	3/6

HISTOLOGICAL EXAMINATION.

The spleen was fibrotic and grossly smaller (1.5 gm.) than what is normally found in a fowl of that age and size (2.5-4 gm).

Germinal centres were strikingly few in the spleen sections stained with Dominici stain. The few germinal

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centres seen (18) were small average diameter 85μ. So far, over sixty domestic chickens have been infected with T.b. brucei during the investigation reported in this thesis. In no case has such a phenomenon been observed.

This case is probably exacerbated by some unknown environmental stress factors. It does show, however, that in extreme cases Trypanosoma brucei infection in chicken could cause immunological exhaustion.

ATTEMPTS TO TRANSMIT T. BRUCEI SSP INFECTION TO CHICKENS BY DIRECT INOCULATION OF INFECTED BIRDS BLOOD.

The ability of stock Lugala/55/EATRO/459 to produce a persistent infection in domestic chickens has been established in the present investigation. It is still necessary, however, to ascertain that parasitised blood from an infected chicken can in turn establish the infection when inoculated into normal birds. This investigation will throw more light on the possibility of mechanical transmission on the field.

Two cockerels were infected with a three day passage population of WIG 76 (WITat 1) in mouse blood. T. brucei infection in both cockerels was confirmed by the standard method of blood inoculation into mice.

On day eighty-three post initial infection, twelve ml. of heparinized blood was collected from one of the

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cockerels. Four ml. was inoculated intravenously into a fourteen-week old cockerel. The remaining 8 ml. was immediately used for infectivity titration test as previously described. This blood was found to contain 25 mouse infective doses (ID_{63})/of trypanosomes/per ml. thus showing that the cockerel inoculated with infected fowl blood received one hundred mouse infective doses.

Parasitaemia in the inoculated cockerel was monitored by the standard method on days 7, 21, 35, 50, 70 and 96. Mice inoculated with the cockerel's blood never showed any patent parasitaemia, when wet blood films were examined under the ordinary microscope. On day 220 post initial infection of the two cockerels, fifteen ml. of heparinized blood was collected from the second cockerel. Four ml. of the blood was inoculated intravenously into each of two sixteen-week old birds i.e. one hen and one cock. The remaining seven ml. was immediately used for infectivity titration. This time the blood was found to contain 75 mouse infective organism per ml. by infectivity titration. Each bird on this occasion was calculated to have received 300 ID_{63}. Parasitaemia was monitored in the inoculated birds for three months by mouse inoculation. On no occasion did the mice become parasitaemic.

This experiment does show that mechanical transmission of T. brucei from one bird to the other would only occur under extreme conditions.

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PLATE 15.

IDENTIFICATION OF VARIABLE ANTIGEN TYPES BY TRYPANOLYSIS TEST

TRYPANOLYSIS

Trypanosomes	Anti WITat1 1:160	2 1:80	3 1:160	4 1:320	5 1:160	6 1:160	7 1:80
WITat 1	+	-	-	-	-	-	-
" 2	-	+	-	-	-	-	-
" 3	-	-	+	-	-	-	-
" 4	-	-	-	+	-	-	-
" 5	-	-	-	-	+	-	-
" 6	-	-	-	-	-	+	-
" 7	-	-	-	-	-	-	+

CHANGES IN VIRULENCE AND ANTIGENICITY

If an attempt was to be made to identify trypanosome variable antigens in splenic germinal centres, it was essential to have antisera to as large a number of different VATS from the WITat serodeme as could be obtained. Several groups of cloned populations were therefore prepared and each examined to see whether it was a previously unidentified variable antigen type. Each attempt at cloning was given an initial A, B, C, etc. and each mouse inoculated with a single organism during that attempt was identified by a letter or number. The first two attempts made at isolating clones were designated A and B series respectively (p 121).

Isolation of new variable antigen types from WITat 1.

Each of twenty mice, designated C1---C20, was inoculated with a single trypanosome derived from WIG 73 (WITat 1) after series of passages in mice (see Figs. 13 and 15). Six populations (CA, CB, CC, CD, CE and CF) grew out amongst which two new variable antigen types (WITat 2 and WITat 3) were identified, Fig. 17 and Table 25.

Isolation of a new antigen type from WITat 2.

Stabilate WIG 83 (WITat 2), Fig. 18, was incubated in mouse anti-WITat 1 serum for two hours at 37°C in an attempt to lyse any WITat 1 organisms that might be present as heterotypes. The suspension was then inoculated into a Balb/C mouse; after three days its blood was passed to a pair of mice. Blood samples from both mice were pooled and 1 ml. ($10^{7.8}$ organisms) was inoculated into a 6-week-old cockerel.

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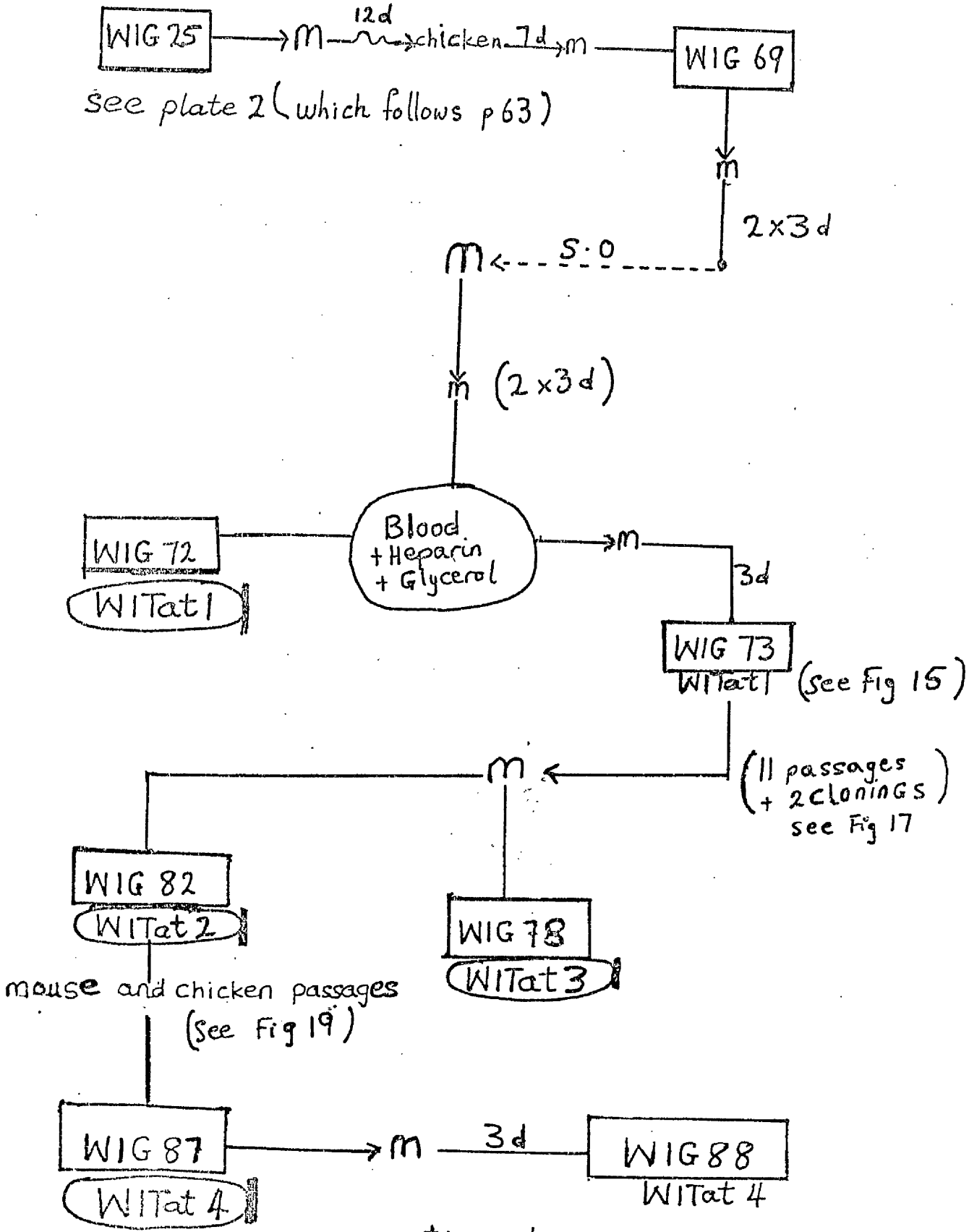
FIGURE 13. Summary of the relationship between SEVEN VATS.



continued on Fig 14

FIGURE 14

CONTINUED FROM FIG. 13

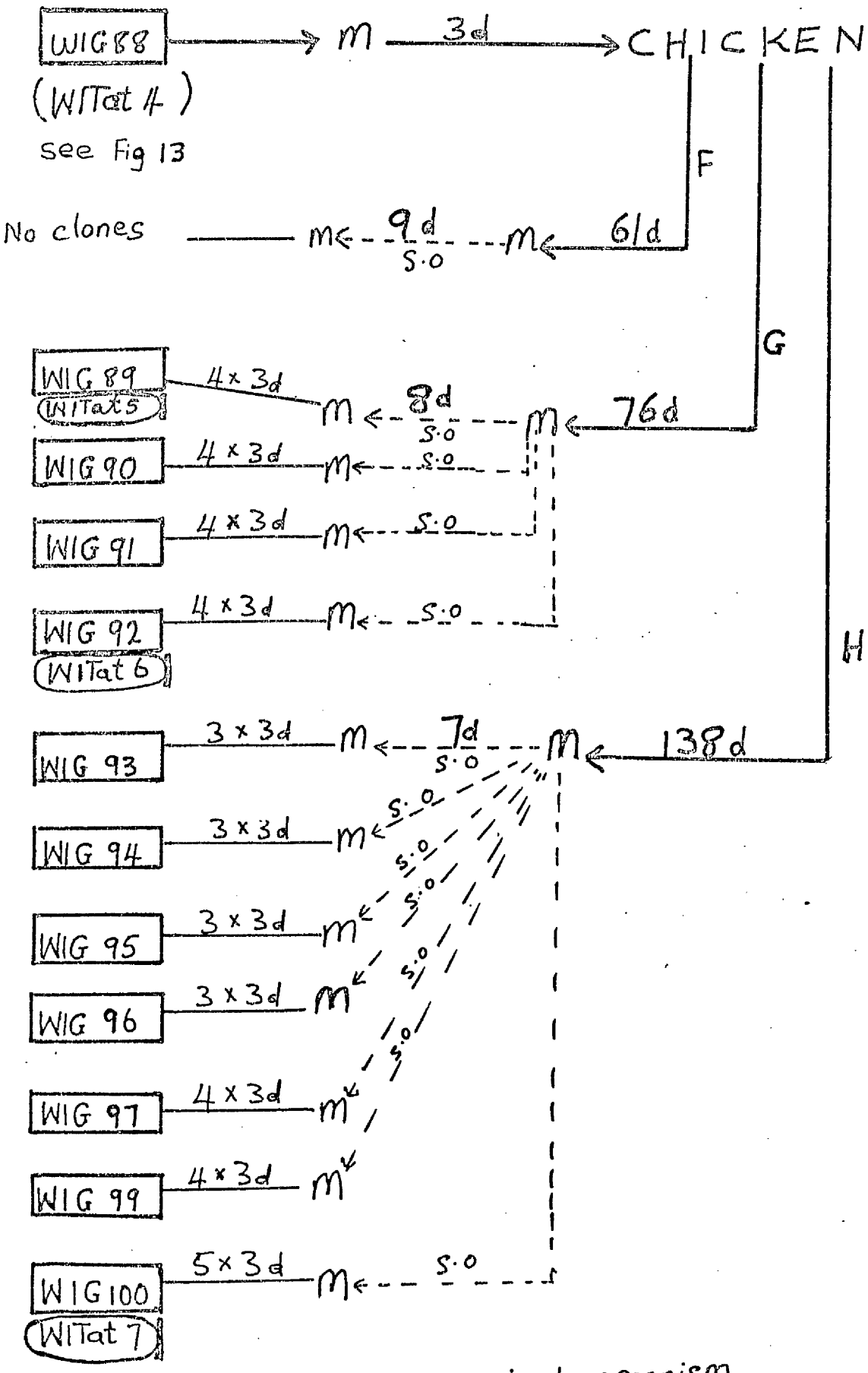
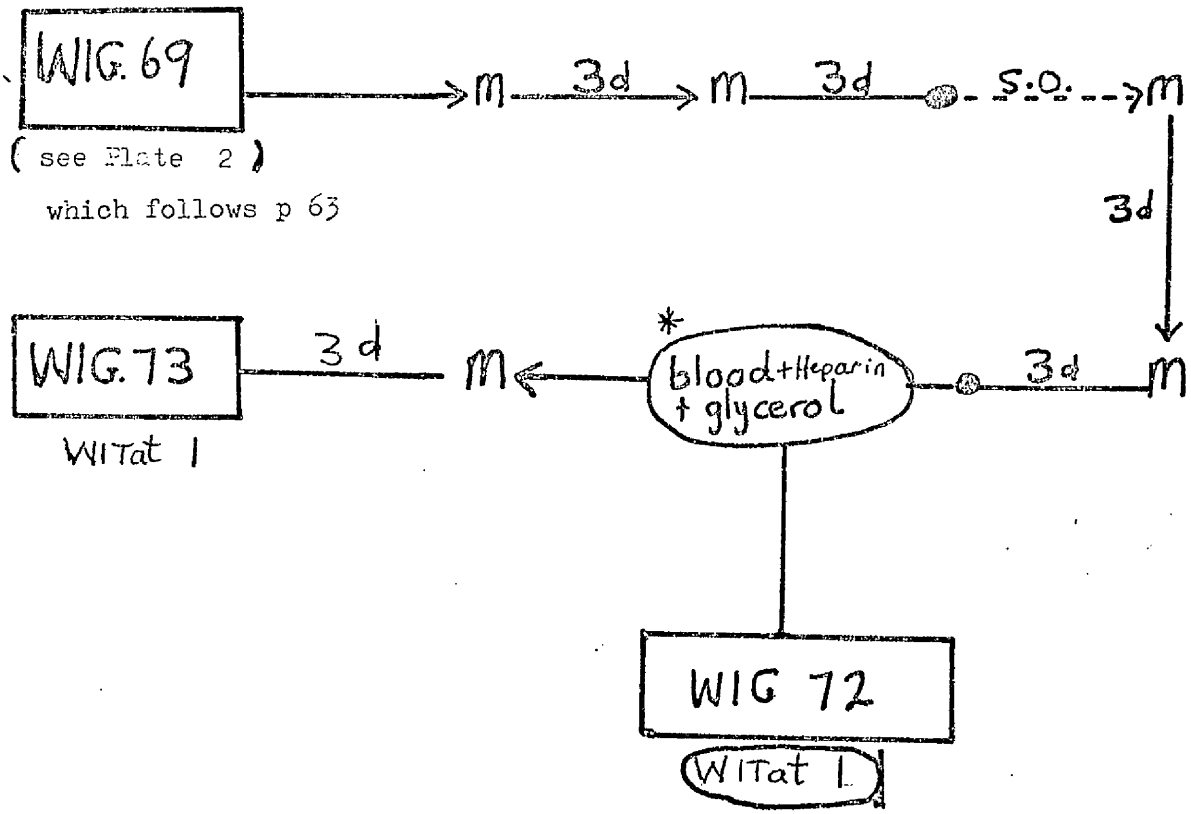


FIGURE 15

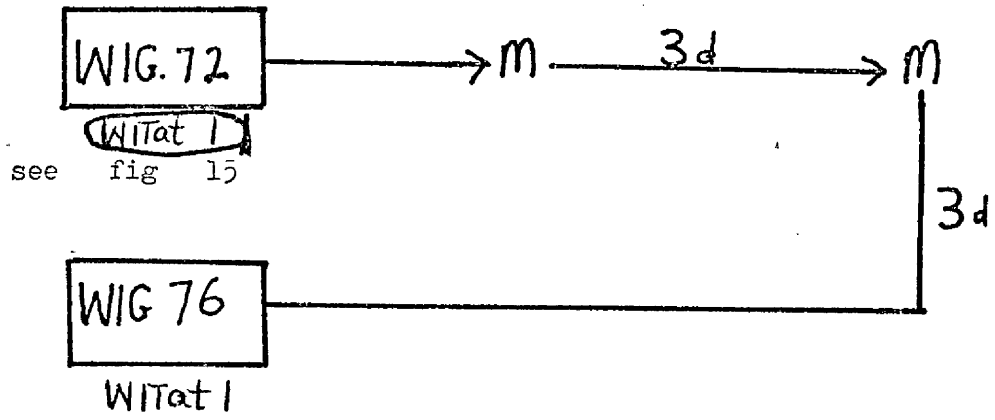
PEDIGREE OF W I G . 73 (WITat 1)



(see Plate 2)
which follows p 63

FIGURE 16

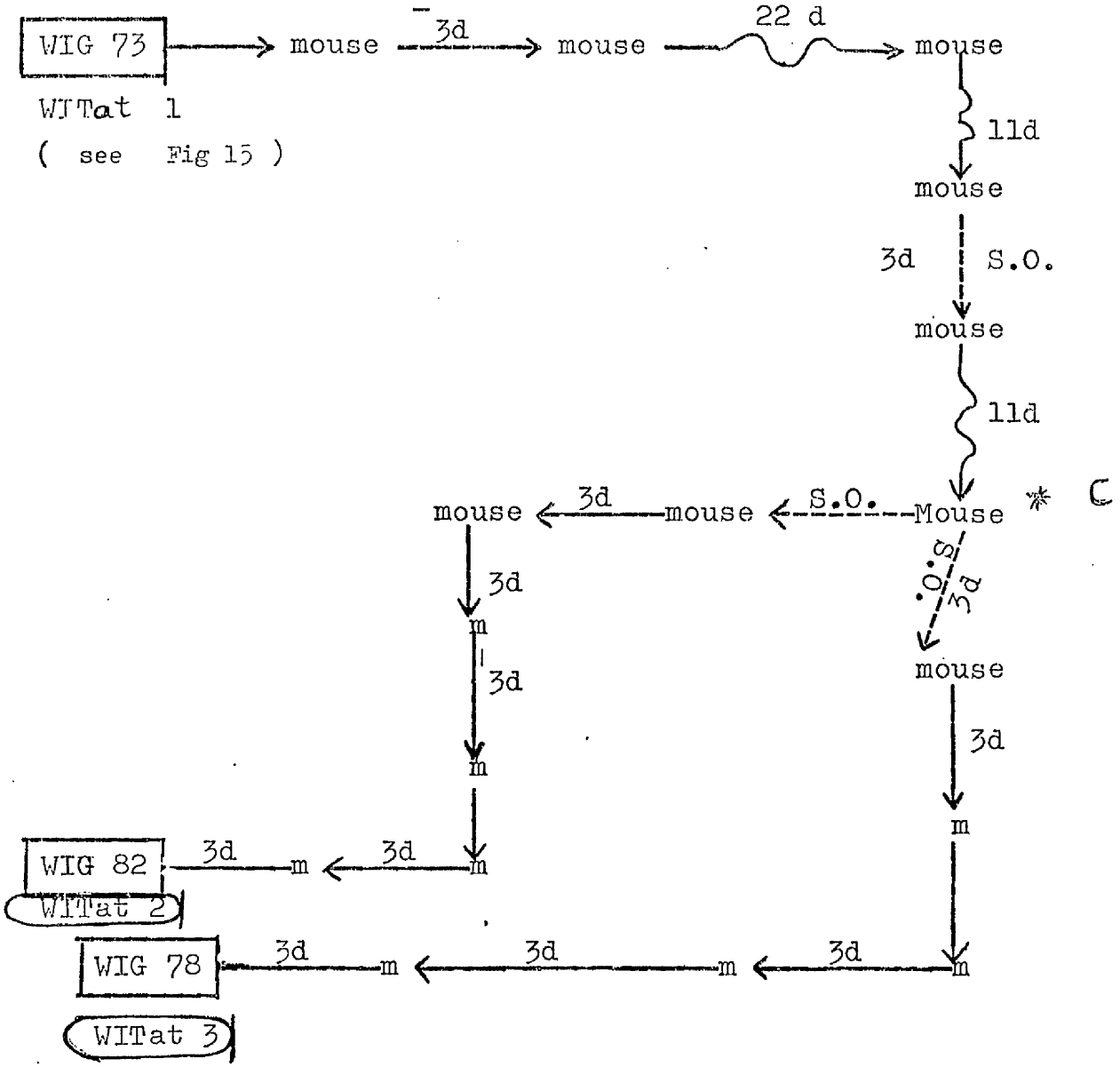
PEDIGREE OF W I G . 76 (WITat 1)



see fig 15

* WIG 72 is the type - type frozen of WITat 1 . WIG 73 is a working population grown up from the excess heparinized and glycerized blood remaining after the freezing .

FIGURE 17 . PEDIGREE OF WIG 82 and WIG 78 , the type -type of WITat 2 and WITat 3 respectively



* Cloning series C

FIGURE 18.

PEDIGREE OF WIG 83. — A WORKING POPULATION OF WITat 2

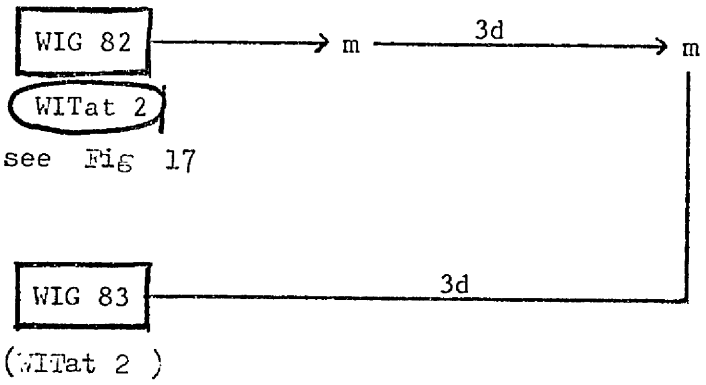
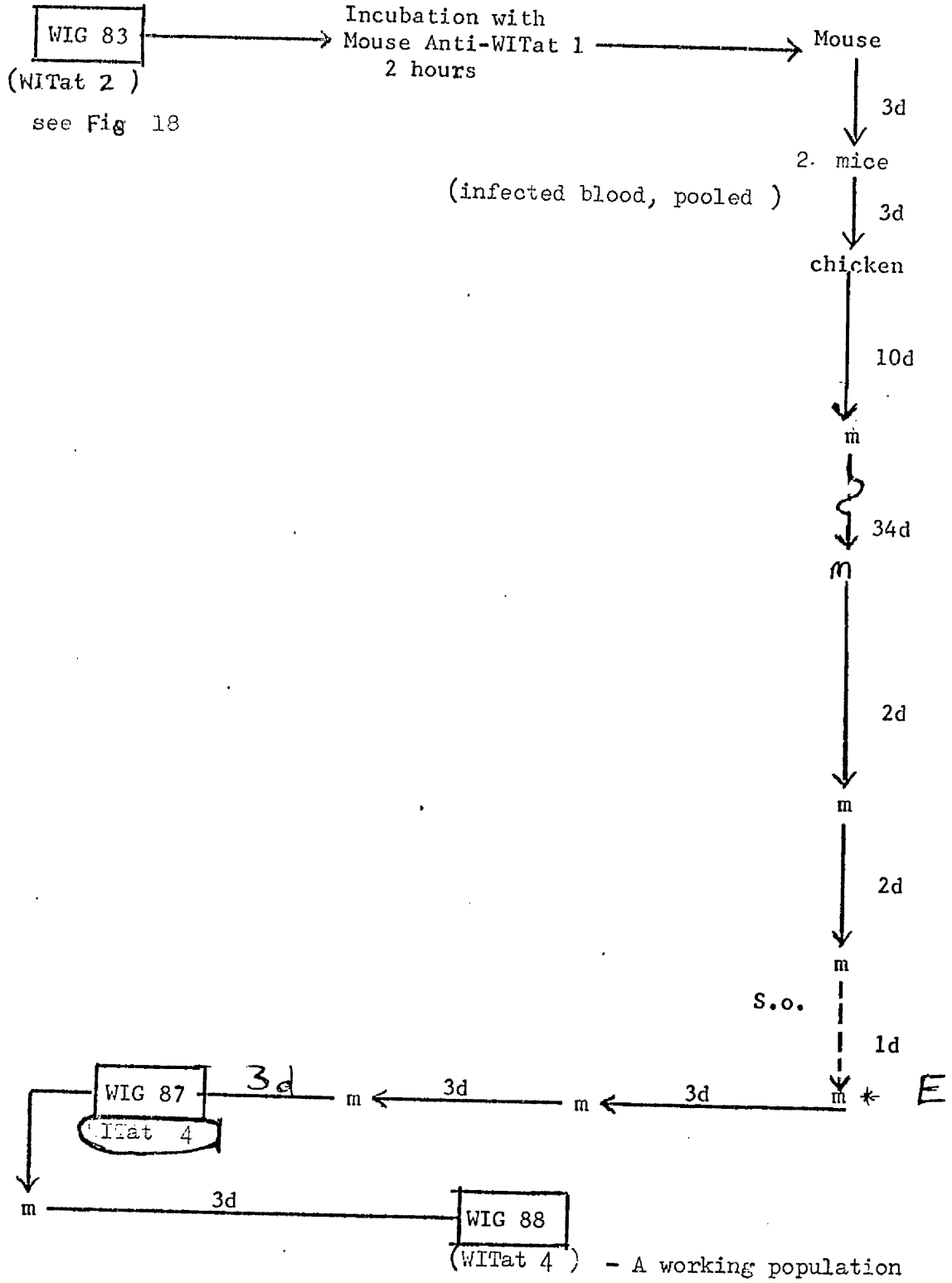


FIGURE 19.

PEDIGREE OF :

WIG 87 -the type-type of WITat 4
WIG 88 - A working population of WITat 4



S . O. = single organism.

~~~> = remission observed , antigenic variation probable .

---> = parasitaemia not followed , occurrence of remission and antigenic variation can only be conjectural .

* Cloning series E .
~~~

TABLE 2.5 a. ANTIGENIC CHARACTERIZATION OF CLONE 'C' SERIES.

AGGLUTINATION TEST

Antigens	Anti-WITat 1 Diluted $\frac{1}{280}$	Anti-Clone CD $\frac{1}{280}$	Remarks
WIG 73 WITat 1	+++	--	WITat 1
WIG 80 Clone CA	-	++	WITat 2
WIG 77 Clone CB	-	++	WITat 2
WIG 79 Clone CC	-	++	WITat 2
82 Clone CD	- -	++	WITat 2
78 Clone CF	- -	--	
WIG 81 Clone CE	- -	--	WITat 3

ANTIGENIC RELATIONSHIP OF CLONES CF and CE.

Antigens	Dilutions of Antiserum against WIG 78 and the corresponding agglutination reactions.							
	$\frac{1}{0}$	$\frac{3}{0}$	$\frac{9}{0}$	$\frac{27}{0}$	$\frac{81}{0}$	$\frac{241}{0}$	$\frac{723}{0}$	Saline
WIG 78 (CF)	+++	+++	+++	+++	+++	+	-	-
WIG 81 (CE)	++	+++	+++	+++	+++	+	-	-

WIG 78 (Clone CF) was codified WITat 3 type-type.

TABLE 26. CLONE E SERIES.

Trypanolytic titre of anti clone E5 Serum.

	Percentage Lysis by Diluted Serum								
Trypanosome	$\bar{1}0$	$\bar{2}0$	$\bar{4}0$	$\bar{8}0$	$1\bar{6}0$	$3\bar{2}0$	$6\bar{4}0$	$1\bar{2}80$	Titre
Clone E_5 P_3 (Day 3)	100	100	100	100	100	100	76	4	320

TABLE 26b

Trypanolytic action of antisera raised against three previously identified variable antigen types on Clone E_5.

Antigen	Anti-WITat 1 ($\bar{1}60$) 1/160	Anti-WITat 2 1/80	Anti-WITat 3 1/80	Anti-Clone E5 ~~1:320~~	Remarks
WIG 87 (After 3-day passage in a mouse)	No lysis	No lysis	No lysis	100% lysis	WITat 4

TABLE 27. ANTIGENIC RELATIONSHIP OF CLONE 'G' SERIES.

This test shows that both Clone G1 and G15 have the same surface coat whilst Clone G2 and G7 have the same surface coat. Two clones G1 and G2 were therefore selected for further tests.

TRYPANOLYSIS TEST

Clones	Anti G1 $\frac{}{40}$	Anti G2 $\frac{}{40}$	Anti G7 $\frac{}{40}$	Anti G 15 $\frac{}{40}$
G1	100%	0%	0%	100%
G2	0%	100%	100%	0%
G7	0%	100%	100%	0%
G15	100%	0%	0%	100%

TABLE 28

Lytic action of antisera to previously identified VATs on Clones G1 and G2.

Antigens	Anti-WITat 1	Anti-WITat 2	Anti-WITat 3	Anti-WITat 4 1/320	Anti-Clone G1	Anti-Clone G2
Clone G1	-	-	-	-	100% Lysis	-
Clone G2	-	-	-	-	-	100% Lysis

Clone G2 was identified WITat 5

Clone G1 was identified WITat 6

TABLE 29

Ease of preparing clones.

Cloning Attempts	Mice Inoculated	Mice Infected	Clone Isolated	VAT Recovered
A	10	0	-	-
B	20	2	2	1
C	20	5	5	2
D	15	0	0	0
E	6	2	1	1
F	15	0	0	0
G	20	4	4	2
H	20	7	7	1

TABLE 30

ANTIGENIC RELATIONSHIP BETWEEN CLONE 'H' SERIES:

(1) Pilot experiment to determine highest agglutin to selected antisera.

Antigen	Antiserum	$\bar{3}0$	$\bar{9}0$	$2\bar{7}0$	$8\bar{1}0$	$2\bar{4}30$	$72\bar{9}0$	Control	Titre
WIG 100 (Clone H_2)	Anti Clone H_2	+++	+++	+++	+	-	-		270
WIG 100 (Clone H_2)	Anti Clone H_6	-	-	-	-	-	-		
WIG 94 (Clone H_6)	Anti Clone H_6	+++	+++	+++	+++	+	-		810
WIG 94 (Clone H_6)	Anti Clone H_2	-	-	-	-	-	-		

TABLE 30b

(11) Analysis of antigenic variants by agglutination test.

Antisera	(STABILATED TRYPANOSOMES - ANTIGENS)					
	WIG 97	WIG 96	WIG 95	WIG 98	WIG 93	WIG 99
	(Clone H_3)	Clone				
Anti Clone H_2 diluted 1:270	-	-	+++	-	+++	-
Anti Clone H_6 diluted 1:810	+++	+++	-	+++	-	+++

+++ = Clear cut agglutination

\- = No agglutination

Two different VATS were isolated , further tests were therefore carried out using antisera raised against previously identified VATS , see Table 31

TABLE 31

Identification of:

Variable antigen types from clone 'H' series.

Antisera used for agglutination reaction	Agglutination Reactions (Antigens)		Antigenic Identity of New Clones
	WIG 100 Clone H_2	WIG 94 Clone H_6	
Anti-WITat 1 1:160	-	-	
Anti-WITat 2 1:80	-	-	
Anti-WITat 3 1:160	-	-	
Anti-WITat 4 1:320	-	+	
Anti-WITat 5 1:160	-	-	
Anti-H_2 1:270	+	-	WITat 7
Anti-H_6 1:870	-	+	WITat 4

TABLE 32

Virulence of trypanosomes: Survival (days) of infected mice after inoculation of a single organism.

CLONES	Antigenic identity	Remission of Parasitaemia (Days)	Duration of Infection (Days)
B A	WITat 1	Yes (12)	30
B B		Yes (11)	30
C A		Yes (12)	35
C B		Yes (15)	29
C C		Yes (14)	39
C D	WITat 2	Yes (13)	40
C E	WITat 3	Yes (12)	39
E	-	Yes (10)	15
E_5	WITat 4	No -	8
G_1	WITat 5	Yes (12)	17
G_2	WITat 6	Yes (8)	12
G_7		Yes (8)	13
G_{15}		Yes (10)	14
H_{10}		Yes (10)	13
H_2	WITat 7	No -	8
H_3	WITat 7	Yes (10)	29
H_4	WITat 4	No -	10
H_5	WITat 7	No -	9
H_6	WITat 4	No -	8
H_8	WITat 4	Yes (10)	12
H_9	WITat 4	No -	8

TABLE 3.3

Doubling time of parasitaemia in trypanosome infection initiated by single organisms.

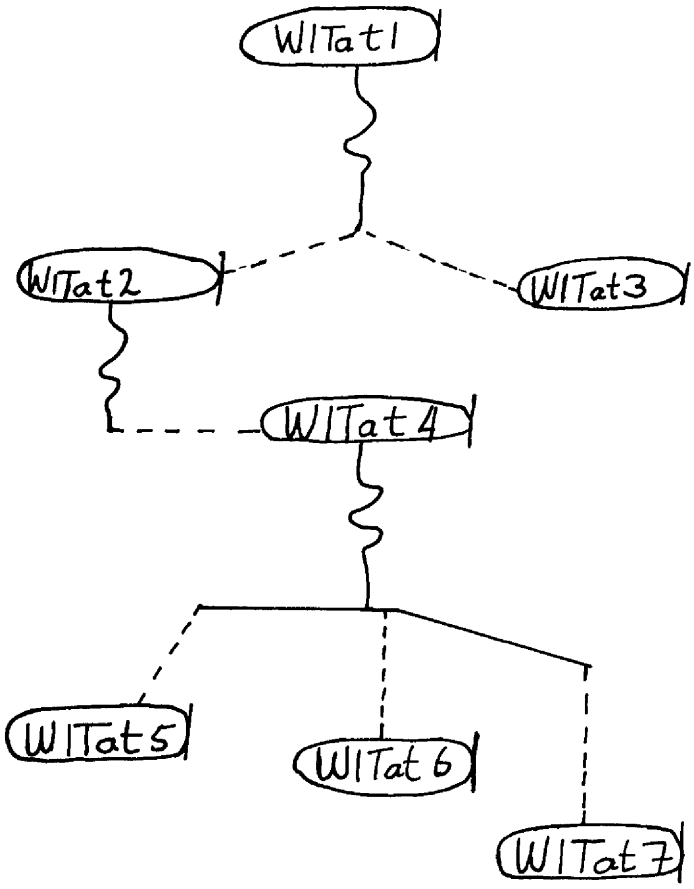
CLONES	Number of hours Post cloning	Parasitaemia (Antilog)	Doubling Time	Number of Divisions
Clone 0	161	7.2	6.73	24
Clone P	160	7.5	6.42	25
C.A.	145	7.5	5.82	25
C.B.	145	7.2	6.06	24
C.C.	145	7.2	6.06	24
C.D.	145	7.5	5.82	25
C.E.	145	7.5	5.82	25
C.F.	143	7.5	5.74	25
ETat 2 (aa)	139	7.8	5.36	26
ETat 2 (ab)	139	7.2	5.81	24
ETat 2 (ac)	139	6.0	6.06	23

The doubling time was calculated by formula -

$$\text{Doubling time} = \left(\frac{\text{Hours Taken}}{\text{log Parasitaemia}}\right) \times 0.3011$$

PLATE 16.

DERIVATION OF CLONES



On day 10 post inoculation, one ml. of heparinized blood from the cockerel was inoculated into each of two mice. Both mice became parasitaemic on day 6 thus showing that the cockerel was infected with the trypanosomes. Further passages from mouse to mouse were carried out as shown in Fig. 19. From the parasite populations that grew in one of these mice single organisms were inoculated into six mice designated E1---E6. Two populations (E_2 and E_5) were obtained one of which (E_5) was identified as a new variable antigen type, WITat 4, (Table 26).

Recovery of three new variable antigen types from one fowl infected with WITat 4.

A mouse was infected with WIG 88, variable antigen type WITat 4, Fig. 14. On day three post mouse inoculation, $10^{7.8}$ viable organisms from the parasitaemic mouse blood were inoculated intravenously into a 6-week-old cockerel. A persistent infection was established in the cockerel as revealed by the standard method of mouse inoculation on days 61, 76 and 138 respectively.

Clones were isolated from the populations of trypanosomes growing in the mice that had been inoculated with the chicken blood (Fig. 14). The groups of mice inoculated with the single organisms were designated the F, G. and H series respectively (Fig. 14). The antigenic identity of the trypanosomes were established as shown in Tables 27, 28, 30 and 31. An analysis of infections initiated by single organisms and the number of isolates recovered are shown in Table 29.

Three /

Three new variable antigen types (WITat 5, WITat 6 and WITat 7) were identified amongst the clones.

Virulence of isolated clones.

A long passage in inappropriate host is often used in reducing the virulence of organisms as in making vaccines. It was possible that long passage in chickens might have resulted in the attenuation of trypanosomes. In the present investigation the results of subinoculation experiments, to the contrary, showed that some trypanosomes taken from chickens have been exalted in virulence. This observation may be related to the number of organisms inoculated.

A careful note was therefore taken, of the pattern of infection, in mice, to which a single organism had been inoculated and the pattern in mice to which sub-passaged on day three. In particular, the occurrence of remission of parasitaemia and the duration of infection were noted.

The pattern of infection of 21 clones in mice is shown in Table 32. Six clones showed 'extreme virulence' (Herbert and Parratt, 1979) in that they each killed a mouse without any remission of parasitaemia.

It is evident from this experiment that long passage of trypanosomes in chickens has resulted in the elevation of their virulance.

ACQUISITION BY T.B. BRUCEI OF POTENTIAL INFECTIVITY TO MAN

The observation that long maintenance of T. brucei in /

in chickens resulted in exaltation of virulence brought many questions into focus. Primary among this is; does the exalted virulence imply change of some other biological characteristics e.g. infectivity to man? or inability to even infect chickens? It is decided to test the first extremely virulent clone WIG 87 (WITat 4) for its potential infectivity to man using the blood incubation infectivity test.

Two control stabilates were included in the test, e.g. WIG 72 (WITat 1) which had earlier been shown to be susceptible to the trypanocidal action of normal human serum and WIG 37 (ETat 10) which has been found to be resistant to normal human serum. Both these trypanosomes reacted as expected to the cytotoxic action of human serum. Surprisingly, however, WIG 87 trypanosomes (variable antigen type WITat 4) were found, not to be inactivated by human serum. This observation was quite unexpected. In order to ensure that no mistake had been made, the population of trypanosomes growing in mice post inoculation of mice with WITat 4 and human serum was tested for lysis by antisera to some selected clones. Only anti-WITat 4 serum lysed this population. This experiment thus shows that:-

(a) A known Trypanosoma brucei brucei can turn out a potentially man infective trypanosomes.

(b) All brucei should be handled with great care since they might possess a few heterotypes of man infective organism even when the great majority are non-infective to man.

Finally, this result thus illuminates the age old problem of the possible common origin of man infective trypanosomes and T. brucei brucei which earlier workers like Yorke et al (1930) and Duke (1935) had long hypothesised.

INACTIVATION REACTION OF NORMAL HUMAN SERUM ON CLONES PRODUCED FROM WITat 4 RELAPSE POPULATION.

The unexpected observation that clones represented by WIG 87 (WITat 4) had acquired potential infectivity to man, necessitated that other clones should be screened for their reaction to human serum.

Stabilate 'type-type' from each clone was used for the blood incubation infectivity test as described previously. The results of these tests are shown in /

TABLE 33b

Trypanosomes			MICE		Remarks
	VATS	Human serum	Inoculated	Infected	
Clone G1 (WIG 92)	WITat 6	0.5ml	2	2	Resistant to human serum
Clone G2 (WIG 89)	WITat 5	0.5ml	2	-	Susceptible to human serum
Clone G7 (WIG 90)	WITat 5	0.5ml	2	1	Resistant
Clone G15 (WIG 91)	WITat 6	0.5ml	2	2	Resistant
WIG 72	WITat 1	0.5ml	2	-	Susceptible
WIG 37	ETat 10	-	2	2	Resistant

The effect of normal human serum on different Trypanosoma brucei ssp clones possessing the same surface coat.

N.B. Both G2 and G7 have the same surface coat as revealed by Trypanolysis test. Note, however, that G2 was lysed by human serum while G7 was not.

Table 33b. These experiments show that a human serum resistant organism does produce human serum susceptible variants in a protracted infection. Also that trypanosomes of the same surface coat do exhibit varying susceptibility to the blood incubation infectivity test.

ACQUISITION OF IMMUNITY TO SEVERAL VARIABLE ANTIGEN TYPES OF TRYPANOSOMA BRUCEI BRUCEI.

It has been shown in the present investigation that some birds self-cured T. brucei infection after about six to seven months. Previous work by Herbert and Lumsden (1968) showed that immunity to a single trypanosome antigen type can be achieved in mice by either infection and chemotherapy or challenge with formalinized antigen. Scott, Holmes, Jennings and Urquhart (1978), on the other hand, failed to achieve vaccination to combined 11 stabilated isolates by infection and chemotherapy in cattle. On theoretical grounds, following self-cure after several months of active infection, antibodies to many variable antigen types within a serodeme should be present since many variable antigen types should have been expressed.

An experiment was therefore carried out to test whether self-cured chickens would be protected against challenge with the original infective organism or whether after several months they had lost the immunity

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that must have initially developed.

Six 8-week old birds were infected with trypanosomes derived from WIG 73 (WITat 1) after a three day passage in mouse. All the birds were found infected when examined by the standard method of mouse inoculation. The birds remained infected until the fifth month when it was found that three of the birds had self-cured. Three consecutive diagnoses within two weeks failed to reveal the presence of trypanosomes in the inoculated mice thus confirming that the birds had self-cured.

On day 147 post initial infection each of the three self-cured fowls was inoculated with $10^{7.8}$ trypanosomes derived from WIG 73 (the stabilate used for the initial infection) after a three-day passage in mouse. None of the three fowls was found infected when they were examined on two consecutive occasions by the standard method of mouse inoculation.

The experiment thus proved that all the self-cured fowls are solidly immune to trypanosomes derived from WIG 73 (WITat 1).

CHALLENGE WITH HETEROLOGOUS ORGANISMS.

The preceding observation showed that all the self-cured birds were immune of the VATs used originally to initiate the infection. Next they were tested for immunity to a different VAT. On day 160 post initial infection the three self-cured chickens were each

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inoculated with $10^{7.8}$ organisms derived from WIG 83 (WITat 2). Repeated inoculation of one ml. of heparinized blood into mice failed to produce trypanosome infection.

This experiment shows that although the original infection was initiated by trypanosomes derived from WIG 73 (WITat 1) they all proved to be immune to organisms derived from WIG 83 (WITat 2).

T. BRUCEI INFECTIONS IN DUCKS.

Conflicting reports abound in the literature about the susceptibility of domestic ducks to salivarian trypanosome infections. Seager (1944) reported a fatal experimental infection by T. brucei equiperdum in ducks following syringe inoculation of rat infected blood. On the other hand, Duke (1912) reported that domestic ducks are insusceptible to T. brucei infection.

Trypanosomes originating from Lugala/55/EATRO/459 were found, in the present work, regularly to produce chronic infections in domestic chickens. An experiment was carried out to test the infectivity of this stock to adult male ducks since ducks and chickens belong to the same taxonomic class, Aves.

(a) In vitro test.

A preliminary experiment was carried out to test the in-vitro trypanocidal action of duck serum on T.b.b.

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WIG 76 (WITat 1).

Stabilate WIG 76 was retrieved from the cryobank and rapidly thawed. The contents of the capillary tube were diluted in 0.6 ml. of normal saline and 0.3 ml. of the suspension was incubated with 2 ml. of duck serum at 37° for 2 hours. A duplicate experiment was carried out with normal rat serum, seeded with 0.3 ml. of the diluted stabilate. Microscopic visualization of the contents of each incubated test-tube showed many motile trypanosomes.

Mice in two groups of five were each inoculated intraperitoneally with 0.4 ml. of one of the two suspensions. The blood of the inoculated mice was monitored for parasitaemia for twenty days by examination of tail blood under the ordinary light microscope. All mice showed patent infections.

This experiment thus showed that duck serum, unlike human serum, is not naturally trypanocidal to T.b. brucei. It was now possible to test the survival in vivo of Trypanosoma brucei in the duck.

(b) In vivo test.

T.b. brucei stabilate WIG 76 (WITat 1) was retrieved from the cryobank and rapidly thawed at room temperature. Four mice were inoculated intraperitoneally with the diluted stabilate. Infection was confirmed in

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all the four mice by microscopic examination of their tail blood. Parasitaemia in each mouse was antilog 7.5 (Herbert & Lumsden 1976).

All of the mice were bled from the orbital plexus and a total of 3 ml. of heparinized blood was collected from the mice. Two ducks, aged 1½ years, were each inoculated intravenously with 1.5 ml. of the parasitaemic blood. The course of the infection was monitored by mouse inoculation on days 7, 14, 28, 42. Visual microscopy of blood was carried out simultaneously. Trypanosomes were found neither in the blood of the mice, nor in the blood of the ducks. This experiment showed that T. brucei brucei, WIG 76 (WITat 1) is not infective to ducks.

INFECTIONS WITH HETEROLOGOUS ANTIGEN TYPES.

It has been shown in the present investigation that trypanosome populations derived from WIG 76 (WITat 1), after a three day passage in mice, are not infective to mature Aylesbury Cross ducks.

In view of the fact that some of the antigen types of the ETat serodeme are infective to man while others are not, it may be necessary to test

the infectivity of some other antigen types of the WITat serodeme to domestic ducks.

Investigations were therefore carried out using populations/

populations derived from WITat 2, WITat 3 and WITat 4 after a three-day-passage in mice. The two ducks were each inoculated with 3 ml. of parasitaemic mouse blood containing $3 \times 10^{7.8}$ trypanosomes of mixed variable antigen types.

Diagnosis was carried out on days 7, 14, 21, 28 and 42 by the standard method of mouse inoculation. Only mice inoculated with blood collected on day 7 exhibited patent parasitaemia. A transient infection may therefore be said to have been initiated. Serum samples were collected from the inoculated ducks on day 0 just before the inoculation of trypanosomes and on days 3, 7 and 18 for indirect diagnosis using the heterophile agglutinin response. Serial dilutions of duck blood were titrated with 2% suspension of washed rat, rabbit and guinea-pig erythrocytes. Sera from all infected ducks exhibited an elevated heterophile antibody response to both rabbit and guinea pig blood cells. Rat blood cells were not of any diagnostic usefulness since normal duck serum was found to possess a naturally occurring agglutinin against rat cells. Both guinea pig and rabbit erythrocytes showed high agglutinin titres from day 3. This reached a peak on day seven and then fell precipitously, on day eighteen. The rapid increase in heterophile agglutinin suggests that the trypanosomes were rapidly cleared from the blood of ducks by immune reactions. The quick fall in elevated agglutinin titre is indicative of characteristic rapid shut-off of antibody response in avian host, White et al (1970).

TABLE 34

Heterophile agglutinins in duck serum during the course of a ransient Trypanosoma brucei ssp infection.

Days Post Inoculation	TITRES		
	Rat blood Cells	Rabbit blood Cells	Guinea-pig blood cells
0	256	0	0
3	388	388	48
7	2048	512	192
18	4096	128	16

D I S C U S S I O N

D I S C U S S I O N

The principal aim of this investigation has been an elucidation of the role of the massive increase in the number of germinal centres observed in the spleen of birds infected with Trypanosoma brucei (Wallace, White and Herbert, 1977). During the course of these experiments it became increasingly clear that the nature of T. brucei infections in domestic chickens simulated trypanosome infections in game animals (Carmichael, 1934; Lumsden, 1965). The number of parasites in the blood is very low and the infected animals show no signs of ill-health. This observation raises the possibility that chickens could act as a reservoir in the cycle of the infection in nature, as apparently happens in many categories of game animals.

The unexpected acquisition, reported in this thesis, of resistance to human serum and therefore potential infectivity for man by a previously confirmed Trypanosoma brucei brucei, following its passage through chickens is a further indication that birds may be important in the epidemiology of the disease.

This discussion will therefore examine the results of the present investigation in three main sections:

infectivity and the course of infection in chickens;

epidemiological implications of these findings; and

immunological responses of birds to the infection.

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Infectivity.

The experiments now reported show that chickens of all ages can be infected readily with at least one stock of Trypanosoma brucei without any need for preliminary immunosuppression or bursectomy. The ease with which the stock Lugala/55/EATRO/459 was found to infect domestic chickens provided confirmation of earlier reports by Corson (1931 a, b, 1935) and Duke (1933). Both Corson and Duke were, however, unable to infect all birds; for example Corson (1931) could infect only two out of five birds inoculated while Duke (1933) could infect only two out of 16 birds. The stock Lugala/55/EATRO/459 was found capable of infecting all the birds into which it was inoculated during this investigation.

Previous workers have reported that different stocks of the same species of trypanosomes have been found to behave differently. For example, Durham (1908) found he could infect Falcon tinnunculus (the kestrel) with T. brucei but he could not infect the pigeon. Corson (1935) on the other hand, reported that he could infect guinea fowls, domestic fowls and francolin with T. brucei rhodesiense, i.e. his own stock infected all birds tested. The present investigation has shown that infectivity cannot be predicted on the basis of the sub-species designation of trypanosomes. Thus it has been found that while the stock Lugala/55/EATRO/459 and all clones derived from it have infected chickens. The same stock failed to produce a long lasting infection in ducks.

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Morphologically indistinguishable trypanosome stocks - Busoga/66/EATRO/3, Muvubwe/60/EATRO/1125 and Lugala/55/EATRO/459 showed wide differences in their ability to infect chickens (Table 7). They also varied in their ability to infect small rodents (Table 5). The stock Lugala/55./EATRO/459 produced a chronic infection with a succession of parasitaemic waves in infected mice. The other two stocks produced an acute infection which killed mice during the first parasitaemia.

Infectivity in general depends on a complex set of inter-actions between the invading organisms and the defensive mechanisms of the host . A possible hypothesis for the ability of the stock Lugala/55/EATRO/459 to infect chickens whilst others do not is that clones derived from this stock might be capable of more rapid onset of variable antigen type heterogeneity than either Busoga/60/EATRO/3 or Muvubwe/66/EATRO/1125.

Experiments by Van Meirvenne, Janssens and Magnus (1975), using fluorescent antibody technique to identify antigenic variants from the stock Muvubwe/66/EATRO/1125, have shown that clones rapidly become antigenically heterogenous and that for every ten thousand organisms about one heterotype appears. These variants are at an advantage when the immune response to the predominant antigen type begins to become effective but are in their turn destroyed as they become dominant and give way to yet other variants.

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In a whole chicken aged eight weeks (weighing 1 Kg) there may be 8000 organisms, since parasitaemia has been shown to be 3-100 organisms per ml. If a stock only produces heterotypes at the rate of one in ten thousand it will obviously be eliminated. On the other hand, any stocks that produce variants at higher rates are likely to be at an advantage against the immune responses of the host.

NON-INFECTIVITY OF SOME STOCKS.

Experiments described in this thesis have shown that both stocks Busoga/60/EATRO/3 and Muvubwe/66/EATRO/1125, which were not infective to chickens, nevertheless would still infect mice after they had been incubated with chicken serum for two hours at 37°C. The natural resistance of man and his domestic animals to infective organisms is often correlated with the cytotoxic activity in the serum of non-susceptible hosts. Normal human serum has been shown to be trypanocidal to T. brucei brucei; hence its inability to infect man (Yorke et al, 1930; Rickman and Robson, 1970 and Rifkin, 1978).

It has been shown in the present investigation that unlike normal human serum, chicken serum was not trypanocidal to T.b. brucei. The inability of the two stocks of trypanosomes to infect chickens, therefore, cannot be attributed to a naturally occurring

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antibody present in normal chicken serum.

Sollazo (1929, quoted by Duke, 1933) found that chickens could be infected with T. brucei after being subjected to a period of starvation. The way in which starvation acts is not readily apparent and could, of course, produce a decreased resistance by affecting non-specific immunity mechanisms. However, the work of Hicks (1977) whereby bursectomy in ovo facilitated infection of chickens points strongly to a defence dependent non-specific antibody production. He used a variable antigen type of the stock Busoga/60/EATRO/3 found in present investigation to be non-infective to chickens.

The transitory nature of the infection produced by the stock Muvubwe/66/EATRO/1125 brings in another facet of argument in this discussion, i.e. Does this stock really infect chickens?

It could be argued that the stock Muvubwe/66/EATRO/1125 (VAT of AnTat 1) did not succeed in producing an infection of the birds inspite of demonstration of recovery of mouse infective organisms after day five of inoculation. Such organisms could be regarded as hardy survivors from the $10^{7.8}$ organisms present in the initial inoculation. The fact that Cunningham (1979) has achieved the survival of T. brucei organisms in culture for over 20 days,

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implies that the viable organisms recovered from the Muvubwe/66/EATRO/1125 infected birds had not necessarily multiplied *in vivo*.

PARASITAEMIA IN INFECTED BIRDS.

It was found that the parasitaemia in birds did not maintain a constant level but was subject to periodic fluctuations as judged by mouse infective units (ID_{63}, Fig. 6). There are rare occasions when inoculated mice have failed to become infected and this may represent "zero parasites in chicken blood", however, this finding has never been reproduced regularly so as to allow the interpretation that each wave of parasitaemia is followed by total absence of parasitaemia. The parasites are probably never decreased to zero, in fact they cannot be else the infection would terminate.

In assessing the concentration of trypanosomes in chicken by infectivity titration (Lumsden *et al* 1963), it is assumed that the trypanosome population possesses a uniform infectivity rate and that any mouse that received one trypanosome would be infected. This assumption may often be wrong. Cunningham, Van Hoeve and Lumsden (1963), pointed out that the infectivity of trypanosomes varies greatly during the course of any infection being highest at the early stages of the infection and then decreases markedly at

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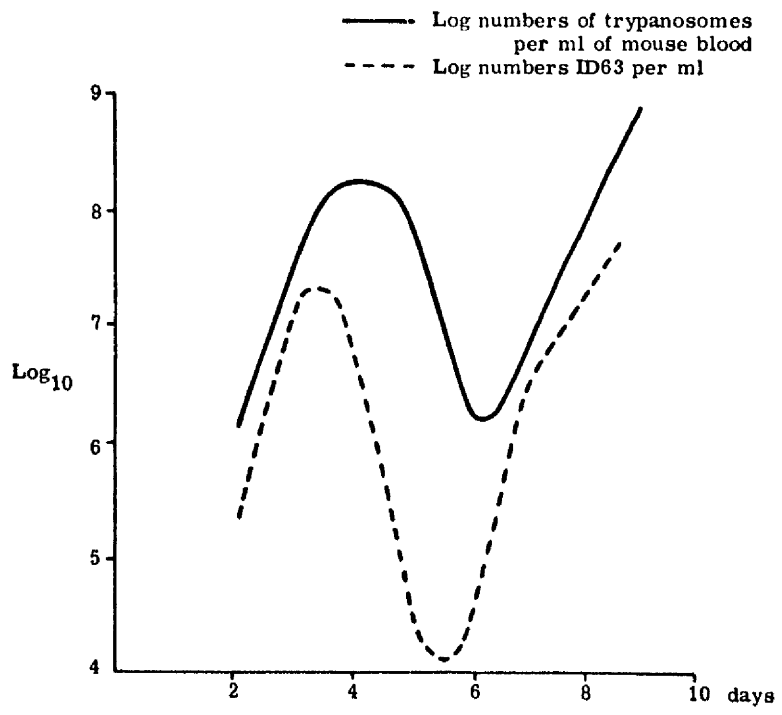
PLATE 17.

RELATIONSHIP OF NUMBERS OF MOTILE TRYPANOSOMES OF T. BRUCEI SSP PRESENT IN THE BLOOD OF RATS, TO THEIR INFECTIVITY, OVER THE COURSE OF A RELAPSING INFECTION. AT THE PEAK PARASITAEMIA ONLY IN ONE HUNDRED OF THE ORGANISMS PRESENT IS INFECTIVE. (FROM HERBERT AND PARRATT, 1979). REDRAWN AFTER CUNNINGHAM et al (1963).



at later stages (Plate 17). It must be emphasised that the technique only measures the numbers of organisms present that are infective to mice.

Parasitaemia estimations in the present studies have revealed that the maximum trypanosome concentration detected in chicken is one hundred organisms per ml. of blood - (Mouse ID_{63}). This is very low and as explained previously many of the methods in current use for the estimation of parasitaemia are unsuitable in this case. Some previous workers who surveyed trypanosome infection in birds employed the Giemsa stained thin film method e.g. Duke, 1912 a.b.c., Bray, Bray and Ashford, (1977), Bennett et al (1977). None of them detected trypanosome infection. In contrast, Novy and MacNeal (1905) detected trypanosome infection in birds by the Giemsa stained thin film method.

The work of Herbert and Lumsden (1976) has shown that the convenient limit of visual microscopy for the detection of parasitaemia is $10^{5 \cdot 4}$ organism/ml. It seems likely, therefore, that the method of thin film or wet blood film microscopic examination will only very rarely detect trypanosome infections in birds. This is probably why reports of T.b. brucei infections in chickens are very scanty. The work of Novy and MacNeal (1905), on the other hand, did not specify the type of trypanosomes detected. Molyneux (1973)

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showed that the avian parasite Trypanosoma bouffardi (a stercorarian trypanosome) could exhibit a parasitaemia of about 4.1×10^5 per ml. of blood, hence trypanosomes can be visualised microscopically in the blood. It is, therefore, not unlikely that what Novy and his colleague found in the blood of birds might be T. bouffardi organisms and not T. brucei. Another test of the inefficiency of wet blood film examination came from the work of Diamond and Herman (1954) who compared success rate of culturing 1 ml. duck blood with conventional Giemsa stained thin films. Only culture method revealed the presence of trypanosomes (identity not specified). This is another confirmation of the low number of parasites in these hosts.

In the present investigation, of the various methods of diagnosis evaluated, only mouse inoculation and DEAE column chromatography were found useful in confirming the infection in chickens. Unfortunately, all field surveys, so far reported, have not used either of these methods but have only used the Giemsa stained thin film. The findings from these field surveys are of great interest but it is clear that a purely parasitological approach i.e. (microscopic examination of blood films) is inadequate as a quantitative assay in epidemiological studies of trypanosomiasis.

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Course of Infection.

As reviewed in the introduction, the general clinical picture of trypanosomiasis in mammals varies from acute to sub-clinical. The outcome is either recovery or death if not treated. In the investigation reported in this thesis all but one of the seventy plus infected chickens showed no clinical signs of illness. The only sign of infection was the sub-clinical anaemia detected in the early stages of the infection (2-3 weeks). Previous reports of T. brucei ssp infections in chickens have also described it as being mild (Corson, 1931; Duke, 1933 and Mistra et al, 1976). Even interference with the immune system by starvation or bursectomy was not found to exalt the virulence of T. brucei ssp in chickens (Sallazo, 1929 and Hicks, 1977).

As shown in Plate 14 , no significant difference was observed between the growth rate of infected and control chickens. This was quite unexpected. Could the trypanosomes be unharmful to the tissues of the chicken or could there have developed a harmonious co-habitation between the trypanosomes and the tissues of the chicken? Neither cachexia nor tissue damage was observed in any of the infected chickens examined. On the other hand, the tissue damage might have been compensated for by the weight of trypanosomes in the chickens. Baker (1959) estimated that the dry mass

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of an individual trypanosome is 3.9-8.6 x 10^{-6}µg. The blood volume of an adult chicken had been calculated to be about two hundred and sixty (260 ml.) (Akester, 1971). Trypanosomaemia in chickens was found to be very low, i.e. 3-100 trypanosomes per ml. Maximum number of trypanosomes in the chicken might therefore be 260 x 100 and their weight 3.3 x 10^{-4} gm. (0.033 µg). This is absurdly low and cannot have added any advantage on the body weight of the infected chickens.

Parasites which produce severe disease are generally regarded as recent associates of their hosts, and the disease as evidence of poor association. As the association evolves, both hosts and parasites adjust by selection that an equilibrium is reached in which the host shows little or no effect from the parasites. Thus, a mutual adaptation might be hypothesized to have existed between T.b. brucei organisms and the chickens.

ANAEMIA

A common presenting symptoms in all T. brucei infected mammals is anaemia. In chicken, however, only a transient anaemia was detected in the early stages of the infection (2-3 weeks). Fiennes (1950) reported that there are two distinct processes in the pathology of bovine trypanosomiasis i.e. the primary disease in

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which the parasite is in complete control of the balance. This is the type of the disease usually suffered by very young calves when infected in early life. This is probably the stage at which anaemia was observed in chickens. The second type of disease is a period of recovery when the body defences are strengthened and the parasite may be overcome. Anaemia in chicken may have resulted from the initial heavy dose ($10^{7.8}$ organisms) used in the challenge. This would have stimulated a strong immune response which resulted in an enormous but transient release of antigen-antibody-complex in the body, or the coating of many erythrocytes with trypanosome antigen and their subsequent lysis when the antibody response to the trypanosomes became elaborated.

The pathogenesis of anaemia in trypanosome infection has frequently been associated with circulating antigen antibody complexes in infected mammals (Goodwin, 1970; Assoku, 1975; Herbert and Parratt, 1979). It has also been shown that normal haematocrit level is promptly restored in trypanosomiasis when the infection, in cattle, is terminated (Holmes and Jennings, 1976). Birds that were found anaemic were not less healthy when compared with those that had normal packed cell volume. It is therefore difficult to reconcile the anaemic changes with the clinical course of the disease. Fiennes (1950) said that injection of massive doses

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of trypanosomes resulted in trypanotoxin which caused erythrolysis in laboratory rodents; this observation has not been supported by workers in this field (Clarkson, 1976). The exact mechanism of the transient anaemia in birds is thus not precisely known but the initial heavy dose of trypanosome used in these experiments may be suspected to have resulted in the release of trypanosome antigens that caused lysis of some erythrocytes. Studies on the course of blood picture in a T. brucei infection initiated with a low dose (100 mouse, ID63), in chickens, would be valuable.

Spontaneous Recovery.

Some fowls infected with trypanosomes, in these studies, self-cured the infection after seven months. It is difficult to answer the question, often asked as to whether a human patient with sleeping sickness can recover in the absence of specific treatment. Yorke (1921) recorded a number of patients diagnosed as suffering from sleeping sickness who received no treatment but who remained in good health and were seemingly free from infection three to eight years later. Also Macfie (1913) described a very mild form of the disease in Eket in Eastern Nigeria and he believed that many of the persons infected recovered in the early stages of the disease. There is,

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therefore, no doubt that instances of self-cure do occur in human trypanosomiasis. How regularly it occurs is not known generally. In the more acute T.b. rhodesience infection, spontaneous recovery, if it ever takes place must be very exceptional. The mild nature of these infections has already been discussed, thus as in the case of mild T.b. gambiense infections, spontaneous recovery without treatment in birds should not be regarded an exception.

Possible effect of sex of host.

A striking feature of T. brucei infections in domestic chickens is that pullets apparently self-cured the infection after 6-7 months, i.e. about four months earlier than the males (Plate 9). The sex of hosts has been shown by Ackert and Dewhirst (1950) to affect resistance for Ascaridia galli infection. There is at present no concensus of opinion as regards the basis of this difference. Some of the explanations advanced are; the immunosuppressive effects of testosterone in male as seen by immune enhancement after castration (Batchelor and Chapman 1965), and the stimulatory effect of estrogen on the reticulo-endothelial system, leading to increased production of phagocytic cells and to the appearance of greater numbers of immunocompetent cells (Feigen, Robin, Peterson and Dandliker 1978). Also, serum IgM concentrations are known to be higher in females than

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in males (Feigen et al 1978). This is believed to be the chief immunoglobulin controlling trypanosome infections generally. The self-cure of the infection that occurred post sexual maturity in 18 week-old pullets is a possible evidence of the role of sex hormones in the reaction. This observation corroborates the finding of Acket and Dewhirst (1950) that injection of diethylstilbesterol increased the resistance of young female chickens to Ascaridia galli, and of Hauschka (1947) that symptoms appeared more severe in men infected with T. cruzi than in women. Also, the bursa is larger in pullets than in cockerels and may therefore be more capable of seeding out immunocompetent cells than in cockerels. Thus, in an infected pullet, the predominant trypanosome variants are more rapidly destroyed and the parasite is able to exhaust its antigenic repertoire more quickly than in males.

Evidence has also been presented that female mice resisted T. cruzi infection more readily than males after both groups had been vaccinated with freeze-thawed cultured epimastigotes of T. cruzi (McHardy, 1978). The observation of a sex effect on the nature of the infection thus seems to extend to several classes of animals and several species of trypanosomes. Difference in the responses of the sexes to trypanosome infection might be important in future when vaccines against trypanosomes are being tested and in drug testing.

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EPIDEMIOLOGICAL SIGNIFICANCE.

Is Trypanosoma brucei infection in chicken a disease?

Pathogenicity has been defined as the capacity of micro-organisms to produce disease in animal hosts (Smith 1977). To cause disease a pathogen must (i) enter the host (ii) multiply in the host tissues (iii) resist and not stimulate host defences and (iv) damage the host tissues. A pathogen must accomplish all the four processes to produce a disease. Lack of any one member of the full complement may result in considerable attenuation of the pathogen concerned (Mims, 1978).

A close look at the above criteria for pathogenicity shows that trypanosomes enter the tissue since subcutaneous inoculation resulted in detection of parasite in chicken blood, and as previously reported by Corson (1931), tsetse flies are capable of cyclical transmission to birds. A very definite and conclusive evidence that T. brucei multiplies in chickens can only be impeccably provided by inoculating a single organism and establishing later that more than one is present in the body. Suffice to note, in the present investigation when 100 mouse infective doses (ID_{63}) were inoculated into each of a total of ten birds four of them became infected. These chickens were then 2 weeks old and weighed 107-113g. If it is

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assumed that about 10% of the body's weight is blood, then each of the chickens had 10-11 ml. of blood volume. All the chickens with confirmed infection remained infected until ten months when they were killed. By this time they each weighed 2470-2614 g. i.e. blood volume at this time would be 240 ml. - 260 ml. Inoculation of 1ml.of their blood into each of two mice resulted in trypanosome infection in both mice. Assuming that only the 100 mouse ID_{63} inoculated remained in the body without multiplying, the distribution of the organisms would be such that only one in three mice should be infected. Since all the inoculated mice were infected it means that at the time of each diagnosis there was at least one organism per ml. i.e. there are at least 240-260 organisms per bird. Since the experiment was started with 100 mouse ID_{63} it means the trypanosomes must have multiplied to reach the number of 240 organism, $(ID_{63})+$, assuming no trypanosomes were lysed by the birds immune response.

Besides this, each time a diagnosis was carried out by mouse inoculation (and this was done 16 times) some trypanosomes were removed from the bird. Thus, if they are not multiplying the number should be decreasing. The trypanosomes resisted the chickens' defence mechanism since variants were isolated and the presence of lytic and agglutinating antibodies were confirmed. The infection failed to produce any obvious

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tissue damage. This is shown by lack of retardation in growth when infected animals were compared with controls. (See Plate 14). On the basis of lack of full complement of criteria for pathogenicity, it can be said that T. brucei infection is not a disease in domestic fowls.

Effect of passage of T. brucei through birds on its virulence and infectivity to mammals.

that/ It was shown (Plate 9)/T. brucei ssp infection could persist for over one year in domestic cockerels and that infected birds showed neither clinical signs of ill health nor retardation of growth as is commonly found in mammals. These observations indicate first of all the relative antiquity of T. brucei as a parasite of birds. They also point clearly to the comparative adaptation of the parasite to the physiological environment in the chicken. It thus seems that the adaptation of the stock Lugala/55/EATRO/459, and clones derived from it, to chickens approaches very nearly the biological ideal i.e. a compromise between the trypanosome and its host without injuring the latter, the parasite becomes peculiarly well equipped to survive in the chicken.

On the other hand, T. brucei organisms recovered from chickens after the infection had persisted for over one year were still readily infective to mice.

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The virulence of some of these organisms was even discovered to have been exalted towards these small rodents.

For instance, during the preparation of clones, six VATs were observed to kill mice during the first parasitaemic wave, whereas all other clones showed at least one remission. These six 'extremely virulent' (Herbert and Parratt, 1979) clones were frozen as stabilates WIG 87 and WIG 100, WIG 94, WIG 96, WIG 98 and WIG 95. On testing, these stabilates were found to be previously unidentified variable antigen types and two of them became the type-types of WITat 4 and WITat 7 respectively. All trypanosome population of the antigen types derived from these six stabilates showed extreme virulence (Table 32).

This observation may be compared with the finding of Wendelstadt and Fellmer (1910, quoted by Herbert and Parratt, 1979) that passage of T.b. brucei through tortoises, lizards, beetles and slugs, increased its virulence to rats. Quite unexpectedly, another clone, stabilated as WIG 97 and also identified as being of VAT WITat 7, was found to be only 'moderately virulent' (on the classification of Herbert and Parratt, 1979).

It is evident therefore that virulence is, at least in this serodeme, not intimately associated with VAT as had been postulated by McNeillage and Herbert (1968).

ACQUISITION OF POTENTIAL INFECTIVITY FOR MAN BY T.B. BRUCEI.

There have been conflicting reports in the literature on the infectivity and virulence of trypanosomes, after a long passage in mammals, to laboratory animals and man. Lester (1932) showed that the virulence of T. brucei to laboratory animals diminished greatly after a series of cyclical transmissions through tsetse flies. In contrast, Yorke Adam and Murgatroyd (1930) indicated that long maintenance of T. rhodesiense in the guinea pig resulted in loss of infectivity to man. Schilling and Schreck (1930) had long postulated that "In the course of chronic infections caused by protozoa, the parasites show changes in their biological qualities".

Experimental results in this thesis show that one of the 'extremely virulent' stabilates, WIG 87 (WITat 4) and some materials derived from it are resistant to inactivation reaction by normal human serum. This same serum inactivated the original stock (stabilate WIG 72) and some clones derived from it after passage through mice and chickens. The serum did not inactivate stabilate WIG 37 (variable antigen type ETat 10) known to be infective to man (Robertson and Pickens, 1975; Van Meirvenne et al, 1976). These findings require a critical analysis when compared with

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the original observations, using human volunteers, that this stock is non-infective to man and can be classified as Trypanosoma brucei brucei.

This population came to attention because on diagnosis of the infection in birds, one of the mice inoculated with chicken blood showed an 'extremely virulent' infection. Trypanosomes from the stock Lugala/55/EATRO/459 (Lumsden, 1977) normally produced 'moderate virulence' (Herbert and Parratt, 1979) in mice, which survive for thirty to forty days. When a clone population was prepared it was found to be a new variable antigen type, and to be of 'extreme virulence', i.e. a single organism caused a mouse to die without showing any remission of parasitaemia. This clone behaved so differently that at first, it was thought that a mistake might have been made in some way, e.g. a stabilate from another stock had been used. A critical check of methodology was therefore made.

The only confirmed man-infective trypanosome in the trypanosome bank were stabilates WIG 37 (ETat 10) of the ETat serodeme (Robertson et al, 1975; Van Meirvenne et al, 1976). Both agglutination and trypanolysis tests conclusively showed that the stabilate representing WITat 4 is a different antigen type and that it does not belong to the ETat serodeme. Moreover, these trypanosomes derived from WIG 87,

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variable antigen type WITat 4, were lysed by end-infection-serum from a guinea pig infected with Lugala/55/EATRO/459. Thus it confirmed that they originate from this primary isolation.

It has long been believed that an animal reservoir of human infective trypanosomes exists (Yorke et al 1930; Heisch et al, 1958; Ascroft, 1958; Onyango, Van Hoeve and de Raadt, 1966). No serious attempts have been made to search for man infective trypanosomes in birds. The idea of searching for man infective trypanosomes in birds may sound exotic but a bird's eye view of the other human diseases may show how important birds can be in their dissemination. For instance, it is known that birds play a role in the zoonosis of Salmonella food poisoning in man. Also, that fowls do spread the fungal disease, favus, caused by Trichophyton meganini, to man (Gordon, 1977). Novy and MacNeal (1905), after sampling different species of birds, indicated that different species of birds might harbour different species of trypanosomes. More importantly, that birds might harbour human trypanosomes. At that time, however, the only technique of testing trypanosomes for their infectivity to man was by inoculation into volunteers, and volunteers were hard to come by. Currently, the blood incubation infectivity test (Rickman and Robson, 1970) as improved by Hawking (1973) affords a more readily

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available technique of testing the infectivity of trypanosomes to man. With the easy methods of testing trypanosomes for potential infectivity to man, large scale survey of birds and even other animals could readily be carried out.

Duke (1923) believed that man infective Trypanosoma brucei rhodesiense and Trypanosoma brucei gambiense arise as 'sports' from stocks of Trypanosoma brucei brucei existing in animal populations. How long a stock of Trypanosoma brucei brucei would remain in a reservoir before transformation to a man-infective form is still speculative. As shown in the results section (Table 33) both organisms derived from clones G2 and G7 (WIG 89 and WIG 90 respectively) possess the same surface coat (as revealed by the trypanolysis test). When both VATs were subjected to the blood incubation infectivity test, it was found that organisms derived from WIG 89 were lysed by human serum whilst WIG 90 were not lysed. An Occam's razor explanation of this observation is that the inactivation reaction of Trypanosoma brucei brucei by human serum is not directly associated with the variable antigen type of trypanosomes. It seems therefore that the current blood incubation infectivity test is not an absolute proof of trypanosomes infectivity for man.

Moreover, an example of experimental infection

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of man with a stock of T. rhodesiense which was susceptible to the inactivation reaction of human serum, was reported by Fairbairn (1933). A possible explanation for this observation came from Van Meirvenne et al, (1975) and Hawking (1977) that only a small proportion of a population of trypanosomes may be resistant to human plasma. It was also shown by Hawking (1978) that some trypanosomes, (T. vivax, T. congolense and T. brucei evansi) that have been hitherto believed to be confined solely to domestic animals, are also resistant to inactivation by normal human plasma.

The finding that some stocks of trypanosomes are resistant to human plasma does not necessarily mean that they are infective to man. The two properties seem separate; T. lewisi is resistant to human serum but does not infect man or any other animal except rats (Culbertson,1934). It does, however, show that trypanosomes of the same surface coat do behave differently. Observations from the present investigation therefore show a need for more incontrovertible tests for determining the infectivity of trypanosomes to man than are currently available.

The present findings corroborate the hypothesis of early workers that man infective trypanosomes are derivable from trypanosomes of the brucei group, and more importantly, that chickens may be important in the

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epidemiology of human African trypanosomiasis since these human serum resistant trypanosomes appeared following passage through chickens. Goodwin (1970) aptly said that Trypanosomiasis, "like Tolkiens Smaug, sometimes slumbers and smoulders quietly for long periods but at any moment it may awaken and cause widespread loss of life to man and domestic animals". Could domestic chickens be one of the miscellaneous allies that allow the embers of trypanosomiasis to glow under the ashes?

The conclusion to be drawn from these results is a warning that it is obviously necessary to handle every Trypanosoma brucei brucei stock held in the laboratory as if it is potentially infective to man. Secondly, there arises the intriguing question from the observation that passage through birds leads to acquisition of an apparent character of human serum resistance. It is important also to consider the possibility that yet other unknown reservoirs in epidemiology of trypanosomiasis may exist.

THE ROLE OF CHICKENS IN THE NATURAL TRANSMISSION OF T. BRUCEI.

It has been shown clearly in the present investigation, that domestic chickens can be infected readily with T. brucei under laboratory conditions. The cardinal question that arises out of this

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observation is, do such infections occur in nature? To answer this question, the natural cycle of trypanosomiasis will have to be considered. The first consideration in this respect would be whether tsetses feed on birds at all. It is in fact known that tsetse flies do feed on birds. Studies on distribution of tsetse flies in the Lado district of Sudan, North Africa, (King, 1912) showed that both birds and lizards are preyed upon by tsetses. Similar studies by Bruce and his colleagues (1914) and Weitz (1963b) in E. Africa showed that tsetse flies obtain about 1.5% of their blood meals from birds. This percentage is of course low, nonetheless, it shows that tsetse flies feed on avian blood.

Another hurdle in this argument is whether these vectors do inject enough organisms to initiate trypanosome infections in birds. Southon and Cunningham (1966), using the infectivity titration method, found that from 5,000 to 63,000 metacyclic trypanosomes infective to mice were extruded at each meal by one tsetse fly. The present investigation has shown that one hundred mouse infective doses (ID_{63}) are enough to initiate trypanosome infection in fowls. It is evident, therefore, that a bite of chicken by an infected tsetse can inject enough organisms to cause trypanosomiasis in them.

It is pertinent to ask whether tsetses may

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become infected from birds. Unless this can be proved the argument, like the infection, comes to a dead-end. The low parasitaemia, 100 mouse ID_{63}, recorded in infected chickens might suggest that onward transmission of infection from chicken to tsetse would be rare if not impossible. Low parasitaemia, nevertheless, does not generally preclude transmission by tsetse flies as it was shown by Bailey (1966) that tsetse flies became infected after feeding on a patient with only 63 T. brucei rhodesiense organisms per ml. of his blood. Thus, birds have all the attributes of being able to sustain a host-vector-host cycle in nature; and tsetses may carry trypanosomes from fowls to other hosts more readily than has previously been considered possible.

IMMUNOLOGICAL RESPONSES.

Mechanisms of Survival of trypanosomes: Immunological basis of the persistent infection.

Noteworthy features of T. brucei ssp infections in birds are the low level of parasitaemia as well as the protracted nature of the infection. These might be due either to the ability of the parasites to evade the immune response of chickens or inability of the parasite to multiply in the tissues of these hosts. It is obvious that the T. brucei infected birds showed

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good and long lasting immune responses, as evidenced by their VAT specific agglutinin and trypanolytic titres (Figs. 9 and 11). What then are the features of trypanosomes that enable them to survive immunological rejection by birds, hence the longevity of the infection?

Parasites in general have evolved numerous adaptations to ensure their survival in susceptible hosts and thus secure their transmission to a new host (reviewed by Culbertson, 1941; Brown, 1974; Ogilvie and Wilson, 1976).

It is characteristic of the salivarian trypanosomes that during the course of an infection, a series of population of different antigenic types appear at intervals. This is reflected by the relapsing nature of the parasitaemia (Fig. 6). Thus, the variability of parasite surface antigens is a mechanism of adaptation which enables the trypanosome to evade the host immune responses, and could permit the longevity of the infection.

Brown (1974) explained the persistence of malaria parasites in monkeys as a dynamic situation in which the variant-specific antibody both induces cycles of antigenic change and at the same time checks successive variant populations. On the other hand, the stimulus for antigenic variation is believed to be in the genome of the trypanosomes and that this

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process is not induced by antibodies (Anson, 1978). This is agreement with the observation of Seed and Effon (1973) showing that different antigenic types occur simultaneously in various tissues in a trypanosome infected animal. This is a further proof that antigenic variation is a means of ensuring survival by the parasites.

In the investigation reported in this thesis, the fluctuations in the level of parasitaemia and the successive isolation of seven different variable antigen types from infected fowls suggest that antigenic variation occurs in infected birds as it does in mammals. As regards the suggestion that the organisms of T. brucei are unable to multiply in chickens, it has been shown quite clearly in this thesis (p. 206) that these trypanosomes multiply in chickens. This suggestion is therefore untenable.

It is equally remarkable to note that the cock with an abnormally high parasitaemia (10^4 per ml.) 2 log above the normal level) had a low number of germinal centres in his spleen whilst similarly infected birds with low parasitaemia had numerous germinal centres. The ability of domestic chickens to control trypanosomal multiplication in the complaisant state may therefore be due to their ability to elaborate numerous germinal centres.

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Possible Physiological Mediators of Low Parasitaemia.

The low parasitaemia in T. brucei infected birds, observed in the present studies, may also be mediated by the internal physiological environment of the domestic chicken. The normal body temperature of birds (41.6°C) is higher than that of most mammals. It is known that temperature affects the course of some diseases in many animals. For example, Hornby (1931) reported lower parasitaemia of T. congolense in peripheral blood of cattle when they were exposed to a high temperature. Otieno (1972) showed that an 'extremely virulent' trypanosome (Herbert and Parratt, 1979) produced a chronic infection in mice kept at 37°C. Also Mathur (1972) demonstrated that mice infected with T. brucei and kept at 35°C developed a lower parasitaemia than similarly infected mice maintained at 26°C.

Experiments performed in this laboratory (Herbert, unpublished) also confirmed that mice infected with T. brucei brucei and kept at 37°C survived much longer than similarly infected mice kept under laboratory conditions at 20°C. High environmental temperature has also been shown to contribute to the attenuation of pathogenicity of Leishmania ssp in mice (Hayatee, 1970). Another example of the importance of temperature in native resistance is found in the resistance of rabbits to the type/Pneumococcus, which loses its /III

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capsule at the height of the fever and becomes susceptible to phagocytosis. It is therefore likely that the high body temperature of chickens may contribute to the low parasitaemia found in infected chickens.

Serum Antibody Levels.

In this investigation, trypanosome agglutinins to the variable antigen type used to initiate the infection were detected in the serum of infected birds as from day 4, and the titre rose gradually reaching its maximum (1:2560) within 10-12 days. (Fig. 10).

The titre subsequently undulated but it never dropped to a low level throughout sixty days of observation.

The only other reported observation of the presence of trypanosome agglutinins in birds is that of Hicks (1977), but he did not follow their course of development. Goedbloed (1971), on the other hand, infected chicken embryo with T. rhodesiense but was unable to detect any agglutinating antibodies. Goedbloed infected some of the chicken embryos at days 5-10, post initiation of egg incubation. This is a period when the developing embryos may be immunologically incompetent (Solomon, 1971). Any antigen presented to the embryos at that time would thus be regarded as self and this might have created

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a state of immunological tolerance.

The undulating nature of the agglutinating antibody against a single antigen type, in the present birds, is peculiar. No such undulations have been reported in mammals. Soltys (1957) investigated agglutination reactions in rabbits infected with T. brucei and found that trypanosome agglutinins were detected from day 5 post infection and that the maximum titre was reached in 12 days. Thereafter, the titre remained high throughout a 28-day observation period. This was confirmed, in the present investigation, in guinea pigs (Fig. 2).

Possible explanations for the peculiar undulating nature of the trypanosome agglutinin production by chickens to a single antigen type are:

Sequestration after antibodies (agglutinins) have been elaborated so as to eliminate the bloodstream form of trypanosomes, organisms having the same surface coat might remain unaffected in some immunologically privileged sites like choroid plexus and vitreous humour. If such homologous organisms were eventually released into the bloodstream a drop in agglutinin titre might occur, initially, followed later by a rise due to a secondary response. However, as shown in page 147 inoculation of supernatant from organs minced in buffer did not produce any infection in mice thus showing that the trypanosomes do not appear to be

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sequestrated in this way in birds and the above reason is not therefore likely to be the case in this situation.

ENDOTOXIN - the production of endotoxin by trypanosomes has been suggested (Taliaferro, 1929) but has not been conclusively proved. The undulating nature of the agglutinin observed in birds may be evidence of periodic release of endotoxin as each different variable antigen type is destroyed. The endotoxin (lipopolysaccharide) acting as mitogen caused multiplication of already primed cells.

IgM COMPLEXES - agglutination is mainly an IgM response. Another possible explanation is that the trypanosome antigen in the various cycles of the immune response was blocked with 19S antibody causing a decline in antibody biosynthesis and as the blocking antibody is subsequently catabolized, antigen again becomes available to stimulate further production of antibody. This point was demonstrated by Nielsen (1974) who found that passively administered 19S antibody from the first peak of the cyclic response suppressed the appearance of the second peak.

INTERNAL ANTIGEN - The internal antigens are used in the complement fixation test (Baker, 1970) in the diagnosis of trypanosomiasis in man and domestic animals. They are therefore immunogenic in mammals and it is probable that they are immunogenic also in

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birds. Repeated release of common antigens plus response to a new VAT may lead to antigenic competition and a temporary decline in the production of antibody to the original VAT.

REAPPEARANCE OF ORIGINAL VAT - It is possible that the homologous trypanosomes re-appear in the chickens blood stream. Hicks (1977) found that organisms of the same surface coat re-appeared during the course of infection of T. brucei ssp in chickens. He, however, used immunologically deficient birds. His work was not confirmed in this study in which intact birds were used. As shown on page 180 no single VAT was isolated twice in any chicken. Nevertheless, this has not been conclusively proved because only two ml. of blood was collected for subinoculation at any one time. There were then still about 260 ml. and hence up to 26×10^3 organism left in the chicken. The statistical distribution is such that the aspiration of such a small volume of blood is not likely to show unequivocally that homologous organisms do not reappear.

As shown in (Fig. 13) seven different variable antigen types were isolated from infected birds. The variable antigens of these trypanosomes may be responsible for antigenic competition and a non-specific inhibition of the immunological responses to antigens of a closely related nature. As indicated previously, in the result section, 7S Immunoglobulin

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was slightly elevated in chronically infected birds, (Table 21). Negative feedback by antibody on antibody biosynthesis involves 7S class Immunoglobulin. The 7S response to new variants may be responsible for the rhythmic wave of agglutinins.

Normal IgM levels in Infected Birds.

One of the most intriguing aspects of T. brucei infections in birds, observed in this investigation, is that the level of IgM in the serum of infected birds remained normal (Table 21). The level of IgG was, however, slightly elevated in serum samples collected on day 100 post infection. Hyperglobulinaemia was never detected either at early or later stages of the infection.

Even though heterophile agglutinins to rat, rabbit and guinea-pig red blood cells appeared throughout the infection (Fig.5) and even though numerous germinal centres developed in the spleen thus showing that polyclonal B-cell activation occurs. The level of IgG was, however, slightly elevated in serum samples collected on day 100 post infection.

As already discussed in the introduction, trypanosome infections in mammals are generally associated with an enormous increase in serum IgM (Mattern, 1964). Many fold increases have been recorded in mammals by various workers, ten times

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Frommel et al, (1970), twenty times (Lumsden, 1972). Chickens differ from mammals in that relatively high levels of antibody can be obtained within a week following a single intravenous injection of soluble protein (B.S.A.) in saline (Wolfe, 1942) and also to H.S.A. (White et al, 1967).

High levels of IgM in trypanosome infected mammals can be detected from about two weeks. In chickens, however, by two weeks the peak antibody response is on the decline. The IgM half-life in chickens is 1-1.7 days which means that even if the IgM level is raised it is rapidly degraded. For example, the normal serum level of chicken IgM is 0.7 mg/ml. The increase IgM levels in mammals has been suggested to be due to cumulative effect of successive antigenic stimulation which occurs at 3-4^{+} day intervals. In an animal in which the metabolic half-life of IgM is longer than 4 days, the increased IgM tends to build up. In domestic chickens, however, the metabolic half-life of IgM is about one day on the average; any elevated level of IgM would be degraded before a new succession of trypanosome variant antigen appears. This is borne out by the fact that the metabolic half-life of 7S immunoglobulin is about 4.0 days in adult birds and a slight increase in 7S immunoglobulin was noticed. It has also been suggested, by Frommel, Perey, Masseyeff

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and Good (1970), that the observed increased IgM levels detected by Mancini method (1965) in the serum of rabbit infected with T. brucei ssp is due to low molecular weight 7S IgM. The monomeric IgM is able to diffuse more readily in the immunoplate thus giving big rings. Low molecular weight 7S IgM has, however, not been reported in chickens. The normal IgM levels found, confirmed the observation of Lajos et al,(1975) that increase level of 7S IgM is only found in pathological conditions; the infected birds remained healthy.

The lack of apparent difference between the levels of IgM and IgG at the initial change of the immune response corroborates the previous observation of Keily and Abramoff (1969) which showed that the amounts of 19S and 7S immunoglobulins are about equal at peak titre in chickens injected with sheep red blood cells. The efficient rapid-shut off mechanism of birds after an antigenic stimulation has been reported by White (1973). It thus seems in this case that the explanation of unregulated synthesis of IgM, commonly adduced for increased IgM in trypanosome infected mammals, does not hold in chickens. Trypanosome variants were isolated from infected birds during the course of the infection; also, the increase in germinal centres (Fig. 7) in the spleen are all further indications of continual stimulation

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by new variants. All these indicate that the infected fowls were being periodically stimulated but no elevated IgM levels were detected in the chickens.

Maintenance of Integrity of Lymphoid Tissues.

Another interesting phenomenon of birds' reaction to T. brucei infection, not characteristic of findings in mammals, is the lymphoid tissue response. Lymphatic tissue reactions in trypanosome infected mammals are extensive. Studies by Hudson and Byner (1973), Urquhart et al, (1973) showed that the spleen and lymph nodes of mice infected with T. brucei became grossly enlarged. Their architecture was profoundly altered. The splenic cords also were thickened due to infiltration of plasmacytes, plasmablasts or both and by small lymphocytes, and there were extensive disruption and replacement of lymphocytes (Moulton and Cole, 1977).

Domestic chickens have no organized lymph nodes. The spleen would therefore be expected to suffer the whole disruptive effect of a trypanosome infection and be even more markedly affected than that of mammals. This assumption was not borne out in this investigation; it was the contrary that was observed. As shown in Table 16, spleens from infected birds were not enlarged when compared with spleens from normal uninfected birds. This was unexpected in view of the

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findings reported in infected mammals.

Microscopic examination of spleens from infected birds showed that the germinal centres in the white pulp were greatly increased in both size and number. Up to 262 germinal centres were found per meridonal spleen section and many of these exceeded 250μ in diameter. By comparison, the normal spleen of an adult fowl contains only 3-8 germinal centres per meridonal section (Fig. 8).

Despite the increased number of germinal centres seen, the architecture of the spleens of infected fowls remained undisrupted. The other lymphoid organs (thymus, bursa, bone marrow and caecal tonsils were grossly and histologically indistinguishable from those of uninfected birds. It could be argued that the compact structure of the chicken spleen may not readily lend it to splenomegaly. This is presumably not so for enlargement of avian spleen is commonly found in diseases such as visceral leucosis, erysipelas (Erysipelothrix insidiosa) and ornithosis. In the latter splenic increase of up to 40 times the normal size has been recorded in pet birds (Blackmore, 1968).

The possible explanations for the maintenance of the integrity of the lymphoid tissue in infected birds are:

The detectable parasitaemia in infected birds is very

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low, being 3-100 organisms per ml. of blood compared with $10^{5.7}$-10^{9} per ml. in mammals. These trypanosomes cause extensive destruction of red blood cells, possibly by releasing antigens which coat the surface of erythrocytes and thus make them susceptible to erythrophagocytosis. The first part of this stage has been shown to occur by Herbert and Inglis (1973) and Woo and Kobayashi (1975).

In both mammals and chickens the macrophages of the red pulp of the spleen remove effete red blood cells. The degree of splenomegaly is always a function of the total erythrocytes phagocytically removed from the bloodstream. With very large numbers of trypanosomes as in some mammalian infections, there will be increased erythrophagocytosis and the spleen will increase the numbers of phagocytic,reticulo-endothelial, cells needed to dispose of fragmented erythrocytes or other waste particule s. There will result an augmentation of the reticulo-endothelium, enlargement of the splenic corpuscles followed by fibrosis, congestion and other changes. The average number of erythrocytes in mammal is of the order 5×10^{7} per ml. of blood while that of birds is 3×10^{6} per ml. of blood. This suggests that for every erythrocyte in a mammal there can be up to twenty trypanosomes thus making destruction of erythrocyte very serious. In chickens, on the other

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hand, there may be as little as one trypanosome per 10^5 erythrocytes.

It follows, therefore, that if there are fewer trypanosomes in chickens fewer erythrocytes will be coated with trypanosome antigen and hence less phagocytosis. Consequently, either nil or negligible enlargement of the spleen will be expected in the infected hosts as is, in fact, shown in these birds.

TRYPANOSOME INFECTION AND GERMINAL CENTRE PROLIFERATION IN FOWL'S SPLEEN.

A striking feature of the lymphatic tissue of a T. brucei infected chicken is the great increase in the number of splenic germinal centres. Spleen sections from all infected birds showed a strikingly great increase in the number of germinal centres. Quantitatively, the number of germinal centres present in the spleens of trypanosome infected fowls was fifteen to twenty times greater than the number found in the spleens of uninfected fowls (Fig. 8).

It has been shown (White, French and Stark, 1970) that germinal centres appear in the white pulp of the spleen following antigenic stimulation. They are formed by the progressive agglutinative growth of antigen bearing cells and B-cells (bursa derived lymphocytes). The increased number of germinal

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centres in spleens of infected birds therefore represents a massive segregation of B-lymphocytes to the germinal centres of the spleen during the chronic trypanosome infection. What are the probable causes of the increased germinal centre development in the spleens of infected chickens? They may be due to the persistence of internal, non-variant trypanosome antigens in the bird during the chronic infection. In addition, new antigens emerging on the coat of the trypanosome could cause repeated bursts of antigenic stimulation.

In mammals infected with salivarian trypanosomes a disproportionate increase in the size of splenic germinal centres has been reported. For example, Mansfield and Wallace (1974) reported great increase in size in splenic germinal centres in T. congolense infected rabbits, Urquhart et al (1973) and Moulton and Cole (1977) reported massive increases in the size of splenic germinal centres but no increase in number. On the investigations of germinal centres in the spleen of trypanosome infected mammals, Greenwood (1974) explained this phenomenon by suggesting that trypanosomes may be exerting a direct mitogenic effect on the B-cells of germinal centres causing the germinal centres to increase in size.

A mitogenic effect on the B-cells of the germinal centres was not confirmed in this study in that

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inoculation of dead trypanosomes (2 x $10^{8.7}$ organisms) into chickens failed to produce an exciting increase in the number of germinal centres (page 153). Also, electronmicrograph studies in the spleen of T. brucei infected birds by Wallace (1976) showed that very few dividing cells could be found within the boundaries of the centres.

The number of germinal centres increased progressively in the present investigation during the first 84 days as shown (Fig. 7). Different variable antigen types were isolated from the infected chickens within this period. This suggests that the germinal centres are formed in response to emerging trypanosome antigens. There are, of course, two types of trypanosome antigens i.e. the common and the variable (see more details in the introduction). If germinal centres are produced to the common antigens, increase at logarithmic rate would have been seen in the spleen sections during the course of an infection, since they are being continually released. On the other hand, germinal centres must have a definite half-life, and so a cumulative increase throughout the course of infection cannot be expected. Germinal centres, on the other hand, represent the 'memory' unit of the central immune mechanism and that they are especially concerned with trapping and retention of antigen by means of dendritic reticular cells (White, 1963).

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The more antigens released by the trypanosomes the greater would be the number of germinal centres in a stimulated host. In the present study, the number of germinal centres increased in number from day 7 to day 84 (Fig. 7). It then plateaued and never showed any strikingly spectacular increase above the highest level on day 84. It seems unlikely that the germinal centres are formed in response to the common antigens since an exponential increase in number was not found in infections over one year. It could therefore be said that the periodic emergence of new surface coat (variable) antigens stimulates new germinal centres and that resolution later occurs in older ones. The regression of germinal centres is discussed later in this thesis.

Another possibility lies in the constitution of the immune apparatus of Gallus domesticus. As reviewed in the introduction, the bursa is not found in mammals. This organ has long been known to be responsible for the ability to expand rapidly the immunological apparatus. Such a response is needed to achieve adequate germinal centre responses to the successive bursts of trypanosome variant antigens.

Perhaps a parallel survey of some other diseases of domestic animals may explain the above more clearly. The diseases bovine leucosis and avian visceral leucosis affect cattle and chicken respectively and in

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both cases metastasis to many organs occur. While increased numbers of germinal centres are not found in the spleen of cattle, large numbers have been reported in chickens, especially in the early stages of leucosis (Cooper, Peterson and Good, 1965). In contrast, the chicken that showed an unusually high parasitaemia of 10^4 (ID_{63}) also showed evidence of immunological exhaustion and a strikingly low number of germinal centres.

The numerous germinal centres found in the spleen of trypanosome infected birds may therefore be due to an adaptive immune response only noticeable in a class of host that is able to elaborate a prompt and liberal antibody response to a battery of antigenic stimuli.

Regression of Germinal Centres Following Cure of Trypanosome Infection.

As shown in the results section, treatment of infected chickens with Berenil completely cured the trypanosome infection and consequently terminated antigenic stimulation. The recovery from trypanosome infection resulted in a gradual diminution in the number of germinal centres in both self-cured and medicated birds. (Table 17 and Fig. 8). In those birds in which the infection was self-cured, it was not possible to determine how long the germinal centres could persist after the infection has been

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terminated since the precise date of cure is not known.

In chemotherapeutically terminated infections, specific information as to the time course of germinal centre regression was investigated. As already alluded to, germinal centres are formed in response to antigenic stimulation. The decline and eventual return to normal number of germinal centres is suggestive of termination of trypanosome antigenic stimulation. The sudden increase in the number of germinal centres (Fig. 8) following the cure of the infection can be explained on the basis of a sudden release of trypanosome antigens into the body. When the antigen had been finally catabolised, there was a switch-off of the stimulus. Lack of a persisting infection is analogous to the absence of antigenic stimulus in a situation such as is found in germ-free animals. In animals that are not exposed to antigenic stimuli germinal centres are often not found in them. The lack of germinal centres, for example, in the foetus and their scarcity in germfree animals have been reported (Silverstein and Lukes, 1962). The gradual return to normal level of germinal centres following the cure of the infections confirms that the numerous germinal centres are due to a persisting trypanosome infection.

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Localization of trypanosome antigen in chicken spleen.

As shown in plate 13, trypanosome antigens were fluorescently labelled in germinal centres in this study. The localization was cytoplasmic in the dendritic cells. This is a further proof that the germinal centres are produced in response to trypanosome antigens. In evaluating this result, the first question is whether trypanosome antigen of any single VAT is present in all the germinal centres, or whether some of the germinal centres have heterologous antigens to the antiserum used.

As reported on page 164 staining spleen sections with guinea-pig anti-WITat 1 serum did not reveal any trypanosome antigen localization in germinal centres from birds that had been infected 286-369 days previously. It is also strikingly clear from both tables that few germinal centres, from the numerous ones in the spleen from T. brucei infected birds showed any evidence of the presence of a single experimental antigen (H.S.A.) that had been introduced to test the localization of a single heterologous antigen.

The fact that less than five percent of total number of germinal centres in the spleen sections from chickens with chronic trypanosomiasis showed H.S.A. localization suggest that two non-cross-reacting-antigens do not commonly localize in the same germinal centre, if the germinal centres had long been

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established before priming with the second antigen.

MECHANISM OF TRYPANOSOME-MEDIATED DEPRESSION OF IMMUNOLOGICAL RESPONSES.

In situations in which pathogenic trypanosomes parasitize a single host for a long period it is obvious that the immunity of the host, though developing has not been able to get rid of them. In the present studies, T. brucei infection persisted for over one year in domestic chickens (Plate 9) without any deleterious effects on the hosts. Many parasites have evolved methods for avoiding the aggressive host immune responses. One of these is to depress the host's ability to make an immunological response (Greenwood, Playfair and Torrigiani, 1971; Goodwin et al, 1972; Mansfield and Wallace, 1974; Ogilvie and Wilson, 1976). Was such an effect seen during this long term infection in birds?

In this investigation, antibody response to H.S.A. by T. brucei ssp infected chickens fell far short of that from the uninfected controls (Plate 12). It appears that one of the likely explanations of T. brucei mediated suppression is a direct action of parasite substances on B-cells or essential assistant cells, e.g. macrophages. It is equally interesting to find that despite the generally low parasitaemia immunodepression could still be demonstrated in these

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birds (Plate 12). Albright, Albright and Dusanic (1978) reported that the addition of more than 10^3 trypanosomes to 1 ml. cultures of spleen cells resulted in marked inhibition whereas less than 10^3 was without any effect. In this study, however, not more than 10^2 trypanosomes (ID_{63}) per ml. of chicken blood were detected. It does seem that the reaction of trypanosomes *in vitro* should be cautiously interpolated to *in vivo* conditions, and more importantly that a latent parasitaemia is not necessarily an infallible index of lack of immunological perturbation.

It is also difficult to ignore the possibility that the greatly increased number of germinal centres in the spleen might be involved in depression of immune responses. Since many B-cells are trapped in the germinal centres. Another evidence of immunodepression is that latterly injected H.S.A. into chronically infected birds showed fewer germinal centre localization than control animals. (Table 20). This could be interpreted as an evidence of antigenic competition in infected hosts. It seems, therefore, that the series of antigenic battery on the immune system resulting in antigenic competition is the basis of the immunodepression observed in this study.

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Immunization Against Trypanosomiasis.

A very promising finding in this investigation is the observation that domestic chickens previously infected with bloodstream trypanosomes derived from WIG 76 (variable antigen type WITat 1) self-cured and were solidly immune to challenges from organisms derived from WIG 76 and WIG 83 (variable antigen types WITat 1 and WITat 2 respectively). This finding kindles some hopes of vaccination against the disease. It is pertinent to ask whether absolute immunity after self-cure of trypanosome is a novelty. The straight forward answer is No, for an earlier occurrence of self-cure and immunity to re-challenge with T. congolense has been reported in cattle by Van Saceghem (1936). Instances of self-cure are in fact not rare in domestic animals, however, in only a few cases has immunity to rechallenge and especially to rechallenge with heterologous organisms, been documented.

There is, nevertheless, considerable field evidence that some degree of protective immunity can develop in cattle maintained over prolonged periods in trypanosome endemic areas (Desowitz, 1959). It has also been shown that following repeated treatment of cattle in such areas with trypanocidal drugs the periods between parasitaemia became extended and a degree of 'biological adaptation' may develop whereby

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the animals show an absence of clinical signs despite low parasitaemia (Fiennes, 1970).

Partial immunization against trypanosomiasis (single antigen types) has been acomplished by the use of trypanosome suspensions inactivated by several means with or without adjuvants and by the use of released trypanosome antigens (Soltys , 1964; ; Weitz 1960; Johnson et al, 1963).

Moreover, most of the successful immunization schedules reported have involved repeated inoculations of inactivated organisms. Only Soltys (1965), Herbert and Lumsden (1968) have reported effective immunity elicited by single inoculations of vaccine. On the other hand, Cross (1975) reported clone specific immunization with purified variant specific surface glycoprotein.

Most published results of early attempts to raise immunity to trypanosome infections in mammals, by means of vaccines, are confused and difficult to interpret because experiments were necessarily carried out with heterologous organisms. Herbert and Lumsden (1968) used stabilated material of known antigenic identity to provide both the antigen and challenge materials so that these were strictly comparable over the period of the experiment. Moreover, as it was possible to titrate each stabilate Herbert and his

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colleague were able to use known numbers of infective organisms (ID_{63}) for the challenges. It is now evident that the size of the dose that they employed for this purpose had an important bearing on the success of their results. As discussed earlier, it is known that any clone population will contain a small number of heterotypes, about 1 in 10,000 organisms. Challenge doses larger than this will inevitably contain a few organisms against which the immunized animals may not be protected. The small numbers of these heterotypes will result in a prolonged time before patency or death compared with unimmunized controls. Herbert and Lumsden (1968) used about 63 mouse infective organisms for each challenge. This number would only on a statistically rare occasion contain a heterotype other than the dominant variable antigen type. In contrast to the very small challenge dose used by Herbert and Lumsden (1968) the challenge, in the present work, was deliberately carried out with large enough dose to contain heterotypes.

As reported on page 185 a variant transcending immunity was observed in birds, that self-cured the infection. What aspect of the chicken immune response may be responsible for this cure? The extent of germinal centre formation coupled with the maintenance of normal architecture of lymphoid tissues found in these /

these chickens has not been equalled in other brucei infected animals. Germinal centres are intimately associated with memory cells and antibody produced during a secondary response appears in the serum more rapidly than in the primary response.

It is also known that most antigen-antibody complexes not trapped in germinal centres would be rapidly degraded by macrophages, and would thus be unavailable to the immune system. An animal that develops numerous germinal centres, therefore, would be at a great advantage in generating antibodies at a fast rate when challenged with a previously experienced antigen. Thus, in a trypanosome infected chicken, the rapid onset of peak antibody response would mean that memory cells to the sequentially expressed surface antigens shall be retained as antigen-antibody complex in the germinal centres. On further contact with the same antigen, antibody can be readily produced at an explosive rate. In addition, there is more antibody produced in a secondary response and the spectrum of the antibody molecule is more efficient in that it is usually enriched for molecules of higher affinity for antigen and for molecules of the IgG class. (Moticka and Streilein, 1978). Protection of mice against T.b. gambiense by IgG from a previously infected host has been reported by Seed (1977). Since the infection was self-curedafter five

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months in the present birds. An Occam's razor explanation of this phenomenon is that the trypanosomes have exhausted their genetic store of antigenic repertoire. Any challenge from the WITat serodeme would therefore be resisted by a rapid response initiated by the information residing in the memory cells. The timing of the rechallenge, within 40 days of self-cure, may also be important as numerous germinal centres are still present at that time (Fig. 8) and their presence will enhance an explosive production of antibodies. This means that slow replicating variants of trypanosomes may have been destroyed before their numbers could induce lots of heterotypes which would keep the infection going, while the fast replicating ones may complete their antigenic repertoire within a short time. Moreover, if it is assumed that one heterotype is present in every 10^4 then these challenges contained $2 \times 10^{3.8}$ heterotypes. A massive dose of heterotypes indeed when compared with eleven trypanosome isolates used by Scott et al (1978). It seems therefore that a possible way of controlling trypanosomiasis might be to hasten antigenic variation in infected host or alternatively to search for trypanosomes of short antigenic repertoires.

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(p. 246 follows)

Finally, an exploitation of immune response and some genetic engineering i.e. selection of animals indigenous to endemic areas may provide the much sought control of the 'dracønic' effect of trypanosomes in domestic animals of Africa.

C O N C L U S I O N S.

The present work has shown that in the areas of immunoprophylaxis and host-parasite relationships, the use of birds as experimental animals in research on trypanosomiasis may be of considerable value.

A salient feature of T.b. brucei infections in the chicken was the lack of ill-health shown by birds despite the protracted nature of the infection. This observation suggests that a harmonious association existed between the trypanosomes and the tissues of the chickens in other words a biological ideal. It may, therefore, be postulated that birds are amongst the natural hosts of T. brucei.

Another general characteristic of the infection in chickens was an absurdly low parasitaemia. The inefficient diagnostic technique (visual microscopy) used in the past may thus have resulted in a gross under-estimation of infections of T. brucei brucei. in birds in general. It is considered that birds may

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be much more important in the epidemiology of trypanosomiasis than has been previously realised.

The acquisition of normal human serum resistance and hence potential infectivity to man by some clones derived from the stock Lugala/55/EATRO/459 confirms the hypothesis of early workers (Duke, 1923, Yorke et al, 1930) that man infective trypanosomes (T.b. gambiense, and T.b. rhodesiense) might arise from T.b. brucei after passage in some reservoir hosts. T.brucei ssp are evidently very labile and this finding emphasized the need to handle stocks of the species as if it was infective for man.

The astonishing increase in splenic germinal centre numbers caused by trypanosome infections in chickens is probably immunologically induced. The immune response to trypanosome infections being intimately involved in control of the parasitaemia. The finding that a test antigen (H.S.A.) when presented to a trypanosome infected bird was restricted to a limited number of germinal centres, indicates that these organelles probably arise in response to each new antigen presented to the individual. Hence the large numbers observed in T. brucei brucei infected birds are probably related to the sequential presentation to them of numerous trypanosome variant antigens.

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The fact that a heterologous antigen when introduced to a chronically infected bird failed to localize in the previously formed germinal centres indicates that immunodepression in trypanosomiasis may be due to antigenic competition.

The demonstration of a variant transcending immunity following self-cure of infection may provide a clue to methods for effective vaccination against trypanosomiasis. At the least, it confirms that immunization against a single stock of trypanosomes is feasible.

There are still many unsolved problems about T. brucei infections in birds. What is the incidence of T. brucei infection in tropical birds? Will a study of bird movement and migration explain instances of resurgence of sleeping sickness in cleared areas? Are there other species and stocks of Salivarian trypanosomes that readily infect birds? Is immunity to reinfection after self-cure so absolute that no amount of challenge will result in infection? If this is so, can knowledge gained in birds be translated to mammals and so lead to the eventual control of African trypanosomiasis by immunoprophylaxis?

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