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A STUDY OF VASCULAR AND EXTRA-VASCULAR FORMS IN AFRICAN TRYPANOSOMIASIS

by

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A thesis submitted to the University of London for the degree of Doctor of Philosophy 1981

London School of Hygiene and Tropical Medicine
ABSTRACT

Patterns of parasitaemia of rodent bloodstream trypanosomes were studied on agar with phase-contrast microscopy (agar technique). The parasites were three strains of *Trypanosoma brucei rhodesiense* isolated from man in Botswana and Zambia. The parasitaemias in the normal and immunodepressed rats were pleomorphic. In all the three strains the patterns of parasitaemia were transiently enhanced by dexamethasone acetate but the enhancement exhibited when total body x-irradiation was applied was sustained. The superiority of the agar technique over the length-measurement methods in assessing pleomorphism is indicated.

Light microscopy showed the possibilities of characterisation of animal and human species of African trypanosomiasis. This was based on the number, location and distribution of the biochemically stable lipoprotein granules.

Scanning electron microscopy illustrated details of the various forms of vascular trypanosomes of medical and veterinary importance. The existence, characteristics and relationship of the cylindrically-shaped long-narrow trypanosome is discussed in contrast to the characteristics of the other major flat-looking forms. The appendages or filaments associated with the different forms are also exhibited. Stages of *in vivo* as well as *in vitro* phagocytosis of *T.b. rhodesiense* by peritoneal macrophages were
also observed by scanning electron microscopy.

Salicylhydroxamic acid in conjunction with glycerol was used to clear the blood of trypanosomes. The new populations were shown to be agranular in form and some of these were long-narrow. Observation on tsetse-transmitted infections and on the bloodstream culture forms showed that the first wave of parasites were agranular but no long-narrow forms could be identified.

Forms from the peritoneal fluid in rats and ascites tumour fluid in mice were found to be pleomorphic. These included the giant forms of multinuclear, multilflagellar and multikinetoplastic types. The round-bodied trypanosomes found in these fluids are related to those found in the vascular system while the multinuclear forms in the fluids are similar to those in the tissues. The tissue forms of Botswana strain of T. b. rhodesiense were studied by electron transmission microscopy. Some parasites located in the choroid plexus of SHAM-treated and untreated rats were discovered to be intracellular. The implication of this discovery in relation to life-cycle, pathology and chemotherapy is outlined.
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To the Creator of all the mysteries.
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CHAPTER 1

INTRODUCTION

1.1 GENERAL

This thesis is about the African trypanosome in the vertebrate host. It is an attempt to study the forms of the parasite in and outside the vascular system and an effort to locate ultrastructurally the sites the organism occupies in the tissue.

The history of the disease in man has been traced as far back as two and a half centuries ago when Atkins (1734), a naval surgeon, recorded his observations aboard a slave vessel; but the parasite itself was not discovered until 1841. That was, however, in the blood of fish (Valentin, 1841). The flagellate was later found in animals of veterinary importance. Evans (1880) recorded it in horses and camels of India and Bruce (1895) in horses and cattle of Africa. Dutton (1902) gave the first account of the parasite in the blood of man in the Gambia and Castellani (1903) in the cerebro-spinal fluid of a case in Uganda.

The epidemiology of the diseases caused by the African trypanosomes depends on the distribution of the vector, the tsetse flies, which occupy about 7 million square kilometres of the continent between latitude 16°N and 20°S.
The geographical distribution of *Glossina* spp. is given in minute detail by Ford (1970) and the analysis of the problem encountered in the study of the fly is available in Ford (1971). It is sufficient to note that *Glossina morsitans* is mainly responsible for transmission in the savannah woodland of central and East Africa while *G. palpalis* is the main vector in the riverine zones of West Africa. The control of the disease has been mainly directed against the fly. There is continuous argument about how much of the scanty resources of each of the countries involved should be spent on the control programmes chiefly carried out in spraying insecticides and bush clearing. Ormerod (1976) warned of possible ecological damages in soil depletion by extensive agricultural activities and overgrazing in newly acquired tsetse-free areas. Yet the same author (Ormerod, 1978a) agreed with a successful example of integration of Mutaru cattle with arable farming in Nigeria as an alternative to uncoordinated eradication campaigns within the individual countries.

The systematics of the African trypanosomes had been sufficiently treated by Hoare (1972). In the genus *Trypanosoma* Gruby, 1843 there are three subgenera of *Salivaria*, characterised by transmission by injection by proboscis from the salivary glands. Subgenus *Duttonella* Chalmers, 1918 is represented by *T. (D.) vivax* Ziemann, 1905; *Nannomonas* Hoare, 1964 by *T. (N.) congolense* Broden 1904 and *Trypanozoon* Luhe, 1906 by *T. (T.) brucei* Plimmer and Bradford, 1899. Hoare (1972), Ormerod (1979a) and Gibson
et al. (1979) agree there is no morphological justification, pathological differences nor isoenzyme variations to justify separations of *T. b. brucei* Bradford, 1899, *T. b. rhodesiense* Stephens and Fantham, 1910 and *T. b. gambiense* Dutton, 1902 into separate species and there is even slight difference of opinion whether they should even be given the status of subspecies (Ormerod, 1979a). The cosmopolitan non-pathogenic rat trypanosome, also studied in this project, belongs to Stercoraria or the group of trypanosomes developed in the posterior station and usually transmitted as contamination of faeces. This is *Trypanosome* (Herpetosoma) *lewisi* Laveran and Mesnil, 1910.

The economic importance of the African trypanosomes has been widely discussed in the literature. Though Urquhart (1980) like Ormerod (1976, 1978a 1979b) has not been as optimistic as others, it has been considered that as many as 120 million cattle (Jasiorowski, 1972) and 150 million sheep and 250 million goats (Griffin, 1978) might be produced in excess of current production if trypanosomiasis in Africa could be controlled. Surra, the trypanosomiasis caused by *T. (T.) evansi* is most fatal in horses, severe in elephants and occasionally acute in cattle (Levine, 1973). Fewer and fewer cases of sleeping sickness may be detected in rapidly developing countries like Nigeria because of reduction of forest and riverine habitats of the vector. But that epidemics can always occur as long as there are foci and stresses caused by
war and famine has been dishearteningly depicted in the recent epidemics in Uganda reported by Gashumba (1981). Goodwin (1974) included human trypanosmiasis on the list of the African exports. While the death toll associated with sleeping sickness may be lower in rate to that of malaria it is now a common occurrence for the Europeans in Africa to pick up the disease and it has been obvious that people or animals that had never been to the tsetse-infected Africa had caught the disease. An example of the former was of a strain from Zambia used in this work, being an isolate in London from an Italian, and of the latter was strain Bida 3, isolated in Nigeria, but infected a laboratory worker in Western Germany (Gibson et al., 1980). T. evansi of Africa had been transported to the Americas and Asia (Hoare, 1972; Gibson et al., 1980). T. vivax formerly restricted to the tsetse belt is known to have spread to other parts of Africa, Central America, South America, the West Indies and Mauritius (Levine, 1973; Ormerod, 1979b).

1.2 THE LIFE CYCLE OF THE TRYPANOSOME

1.2.1 In the Invertebrate Host

Although the terminology used for the forms of the parasite in the tsetse had changed from leishmanial and crithidial of Noble (1955) to amastigote and epimastigote
of Hoare and Wallace (1966), there is no dispute about the identity of these forms. The revelations of Foster (1964), Mshelbwala (1972) and Otieno (1973) about the possibility of the parasite passing through the haemocoel had been revolutionary in changing the orthodox concept as in Noble (1955) to that recently reviewed by Ormerod (1981). The confirmation about a more direct route of the trypanosome to the salivary gland in the fly had been obtained by Evans and Ellis (1975) and Ellis and Evans (1977) who did not only show the passage of the parasite through the peritrophic membrane of tsetse but also demonstrated the penetration of the mid-gut cells of Glossina morsitans. As Ormerod (1981) indicated, the most recent information, still under review before publication, is most advanced.

1.2.2 In the Vertebrate Host

To many parasitologists the life history of the trypanosome in the mammalian host is no more than the longitudinal binary fission of the trypomastigote form developed by growth, in blood, from the metacyclic form injected by the fly. The only detail that may be added is the phenomenon of pleomorphism associated with the difference in lengths of the slender and stumpy forms. Ormerod (1979a) related the history of such concept to the fact that the first laboratory strains of trypanosomes used to study the life history had been passaged several
times through the laboratory rodents and these strains were so easily grown in the blood that their interaction with the host tissues had been least noticeable. He also gave details on the divergence of opinions held by the continental, mostly Italian, workers and the English-speaking counterparts. Recently, Ikede and Losos (1972a,b), Ellis and Ormerod (1973) and van Mark et al. (1980), the extravascular occurrence of the parasite in the vertebrates has to be accepted but with ambiguity about the forms and uncertainty of the sites of occurrence basically assumed to be intercellular.

1.3 THE OBJECTIVES OF THIS WORK

Current controversy about the method and cost of tsetse control has already been noted. The advanced stage the study of the life cycle of the parasite in the vector has also been stressed. Yet trypanosomiasis can occur and continue to be transmitted from one vertebrate host to the other without the tsetse fly. Though the account of Lavelleissier (1976) about sleeping sickness in Upper Volta without tsetse could have been taken with scepticism the recent record of Auregan and Duvallet (1980) about a focus of 99 new cases in north of the same country where there was no fly, is too compelling to ignore. In fact, Wenyon (1926) documented mechanical transmission of T.b. gambiense and T.b. brucei by Mansonia uniformis and
Stomoxys sp. respectively. The mechanical transmission of *T. vivax* is undisputed. Congenital transmission is common in the literature Darre et al., (1937), Capponi (1953), and Pinto (1960). Although it is still to be proved for the African trypanosomes transmission through mammary gland that has been experimentally demonstrated in *T. cruzi* (Cancardo, 1968). Transmission through blood transfusion is also a way of carrying African trypanosomiasis from one patient to another (Hira and Hus ein, 1979). Oral transmission has been shown in *T.b. brucei*, *T. vivax* and *T. congolense* by Clarkson and McCa be (1973). Even through handling of blood trypanosomes the parasite could enter into a new host as assumed in the case of the laboratory worker in Western Germany (Gibson et al., 1980).

There is, therefore, a necessity to know more about the parasite itself in general and its occurrence and distribution in the vertebrate host in particular. In view of this conviction this project was designed to (a) study by an already established simple but efficient method (Ormerod et al., 1963) the blood forms of the parasite and the parasitaemia patterns in selected strains of *T.b. rhodesiense*;
(b) observe the patterns of the lipoprotein granules of blood trypanosomes of selected African trypanosome species and strains of medical and veterinary importance;
(c) study the external morphology of blood forms of the
species and strains by scanning electron microscopy; 
(d) assess the effect of immunodepressants on the vascular forms and the parasitaemia patterns of the same strains as in (a); 
(e) compare the parasite forms in the blood with those in the extravascular fluids such as the peritoneal fluid; 
(f) find a means of increasing the amount of the peritoneal fluid as a form of in vivo cultivation and study the parasite populations; 
(g) study the forms and sequence of occurrence of new populations found in the bloodstream after the former blood parasites have been effectively cleared by chemotherapy (based on Evans and Brightman, 1980); 
(h) compare the forms and sequence of the occurrence of those forms produced by tsetse cyclical transmission with those produced from recrudescence after the chemotherapeutic treatment of (e); 
(i) trace the site or sites in tissues from where the new population in the blood could have been derived (based on Ormerod and Venkatesan, 1971a; Jennings et al., 1979; and Evans and Brightman, 1980); and 
(j) study the ultrastructure of the extravascular forms and compare their morphology with that of the vascular forms.

1.4 DESIGNATION OF STRAINS

The writer is aware of the debate generated by
the differences of opinion in the nomenclature especially the designation of strains of Salivarian trypanosomes (Killick-Kendrick, 1976; Lumsden, 1976; Ormerod, 1978; WHO, 1978). The adoption of the word "strain" in this work is as used by Lumsden (1971) and Ormerod (1978b) - i.e. an isolated parasite material with available documentation on the history of its source, host, age and any characteristics. It is as applicable in other genera of the Protozoa. The word "stock" has not been used because of its other meanings.

1.5 ARRANGEMENT OF CHAPTERS

The materials used and techniques adopted are recorded in Chapter two. In the subsequent six chapters results followed by discussion of each unit are given. Chapter nine relates those chapters to one another and consolidates the units into a whole.
CHAPTER 2

MATERIALS AND METHODS

2.1 LABORATORY ANIMALS: THE HOSTS

Wistar rats and Theilers Original (TO) mice obtained from Messrs A.J. Tuck & Son Ltd of Laboratory Animal Breeders Station, Beeches Road, Battlebridge, Essex, were used. Both rats and mice were specific pathogen free (SPF). They were expected to be free from Myxoplasma arthriditis, M. pulmonis, M. neurolyticum, Pasteurella multocida, Bordetella bronchiseptica, Corynebacterium murium, all species of Coccidia, Hexamita muris, Giardia muris, all nematodes except Syphacia obvelata and Aspiculuris tetraptera, all arthropods. Considering the conditions in the animal house and the absence of potential vectors it was unlikely that they could have become infected with any species of Trypanosoma.

Rats were used when large amounts of pooled blood, necessary for extracting trypanosomes, was required and also for extracting the choroid plexus since this operation is more difficult in mice. Mice were used when transmission experiments demanded several animals for multiple results to minimise costs. They were also used in experiments involving a study of the infection in peritoneal fluid when this involved experiments with mouse ascites tumour cells.
Male animals were usually preferred in order to avoid possibility of breeding during the experiments. But when both sexes should be compared sexes were separated into different cages throughout the experiments.

On one occasion, in the tsetse transmission experiments, Sha-sha mice were used because, with the relatively few hairs, the possibility of transmission was enhanced.

2.2 STRAINS OF TRYPANOSOMES USED: THE PARASITES

Strains of trypanosomes from man (West and East Africa) and from animals were obtained from sources summarised in Table 1. Each strain on receipt was multiplied by infecting donor rats or mice and at appropriate parasitaemia peak several samples for freezing down were made from the harvest. The frozen samples were made by adding equal amount of phosphate buffer of pH 7.2 to the blood. The mixture would then be made into a solution containing 7% of glycerol. This would be dispensed onto 3 ml capsules each containing 0.5 ml of material to be kept at -78°C until required. Each inoculum contained approximately $10^6$ parasites, except where stated otherwise. For clarity some strains are referred to in the text by their origin, thus T.b.rhodesiense (180) is cited as "Botswana", T.b.rhodesiense (83) as Zambia I and T.b.rhodesiense (57) as Zambia II.
Table 1. Summary of strains of trypanosomes used

<table>
<thead>
<tr>
<th>Trypanosome</th>
<th>Code</th>
<th>Origin</th>
<th>Original host</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <strong>T. lewisi</strong></td>
<td>LUMP 814</td>
<td>U.K. (Lincs)</td>
<td>Rat</td>
<td>1972</td>
</tr>
<tr>
<td>2. <strong>T. vivax</strong></td>
<td>Y58</td>
<td>Nigeria (Yakawada)</td>
<td>Cattle (N'dama)</td>
<td>1972</td>
</tr>
<tr>
<td>3. <strong>T. vivax</strong></td>
<td>Y486</td>
<td>Nigeria (Yakawada)</td>
<td>Cattle (Zebu)</td>
<td>1973</td>
</tr>
<tr>
<td>5. <strong>T. congolense</strong></td>
<td>Gamb 19</td>
<td>Gambia (Essau)</td>
<td>Cattle</td>
<td>1977</td>
</tr>
<tr>
<td>6. <strong>T. congolense</strong></td>
<td>TSW 99</td>
<td>Liberia (Ganta)</td>
<td>Pig</td>
<td>1977</td>
</tr>
<tr>
<td>7. <strong>T. congolense</strong></td>
<td>TSW 103</td>
<td>Liberia (Sanni-quele)</td>
<td>Pig</td>
<td>1977</td>
</tr>
<tr>
<td>8. <strong>T. brucei brucei</strong></td>
<td>8/18</td>
<td>Nigeria (Nssuka)</td>
<td>Pig</td>
<td>1962</td>
</tr>
<tr>
<td>9. <strong>T. brucei</strong></td>
<td>S/42</td>
<td>Tanzania</td>
<td>Warthog</td>
<td>1966</td>
</tr>
<tr>
<td>11. <strong>T. brucei</strong></td>
<td>Antat 1</td>
<td>Uganda (Busoga)</td>
<td>Bushbuck</td>
<td>1966</td>
</tr>
<tr>
<td>12. <strong>T. brucei</strong></td>
<td>TSW 3</td>
<td>Liberia (Schwee)</td>
<td>Pig</td>
<td>1975</td>
</tr>
<tr>
<td>13. <strong>T. rhodesiense</strong></td>
<td>Liverpool normal</td>
<td>Not known</td>
<td>Man</td>
<td>1920s</td>
</tr>
<tr>
<td>14. <strong>T. rhodesiense</strong></td>
<td>Liverpool resistant</td>
<td>Not known</td>
<td>Man</td>
<td>1920s</td>
</tr>
<tr>
<td>15. <strong>T. rhodesiense</strong></td>
<td>57</td>
<td>Zambia (II) (Isoka)</td>
<td>Man (David Sinkala)</td>
<td>1974</td>
</tr>
<tr>
<td>16. <strong>T. rhodesiense</strong></td>
<td>58</td>
<td>Zambia (Kasemna)</td>
<td>Man</td>
<td>1974</td>
</tr>
<tr>
<td>17. <strong>T. rhodesiense</strong></td>
<td>83</td>
<td>Zambia (I)</td>
<td>Man (Giorgio Sabbadin)</td>
<td>1977</td>
</tr>
<tr>
<td>18. <strong>T. rhodesiense</strong></td>
<td>180</td>
<td>Botswana</td>
<td>Man (Numelo)</td>
<td>1960</td>
</tr>
<tr>
<td>19. <strong>T. rhodesiense</strong></td>
<td>186</td>
<td>Botswana</td>
<td>Man (Monaxumo)</td>
<td>1960</td>
</tr>
<tr>
<td>20. <strong>T. rhodesiense</strong></td>
<td>LUMP 1198</td>
<td>Uganda (Busoga)</td>
<td>Man Olace Bidonge</td>
<td>1976</td>
</tr>
<tr>
<td>21. <strong>T. gambiense</strong></td>
<td>LUMP 1236</td>
<td>Zaire</td>
<td>Man</td>
<td>1970</td>
</tr>
<tr>
<td>22. <strong>T. gambiense</strong></td>
<td>Bida 3</td>
<td>Nigeria (Bida)</td>
<td>Man</td>
<td>1968</td>
</tr>
</tbody>
</table>
2.3 IMMUNODEPRESSION TECHNIQUES

2.3.1 X-irradiation

Rats placed in well-aerated transparent plastic container made of cellulose acetate were subjected to total body irradiation at the height of 78 cm. The amount of irradiation was either 800 or 1000 rads with electricity power supply at 230 KV. This process was carried out at Middlesex Hospital Medical School. The irradiated animals were kept for 24 hours before they were infected with the trypanosomes. *Trypanosoma brucei rhodesiense* strains, two from Zambia (57 and 83) and one from Botswana (180), were used at each level of irradiation exposure. Animals not irradiated but infected with the same strain of trypanosomes were assessed as controls. These are referred to as "the normal infection".

2.3.2 Dexamethasone Acetate Technique

The stock solution of dexamethasone acetate (DMS) was made by dissolving 30 mg in 1.5 ml of 10% polyvinyl pyrrolidone (PVP). The stock solution was administered by intraperitoneal injection to weighed rats at 3 dose levels:

(a) 8 mg/kg (representing 2 x the usual dose level)

(b) 20 mg/kg (representing 5 x the usual dose level)
(c) 40 mg/kg (representing 10 x the usual dose level).

Two controls were run with

(a) trypanosome-infected rats injected with 10% PVP, and (b) rats infected with trypanosomes but with neither the drug nor the solvent of the drug. Experimental rats were immunodepressed and infected on the same day. The experiments and controls were carried out with the three levels of dose for the three strains of T. b. rhodesiense.

2.4 PERITONEAL FLUID AND ASCITES TUMOUR CELL EXPERIMENTS

2.4.1 Peritoneal Fluid Experiments

Peritoneal fluid from rats was obtained by opening the peritoneum of freshly killed rats and flushing with normal saline solution which was then drawn out by Pasteur pipette or by a 1 ml syringe. The resulting suspension was observed on agar slide and by scanning electron microscopy. The Botswana strain (180) was used in the study.

2.4.2 Ascites Fluid Experiments

Ascites cells known as Sarcoma 180 were obtained from Dr J. Ackers of LSHTM Department of Medical Protozoology. From the history of the tumour cells they were
originally established in Children's Cancer Research
Foundation, Boston, Massachusetts. The cells were later
characterised by the Child Research Center of Michigan.
The tumour cell strain is believed to have been obtained
via the National Institute of Medical Research London
NW7 where they were found not specific to strains of
mice as there were unpublished records that they had
infected ARK, Balb, CBA and TO mice.

The cells were multiplied and maintained by
growing in batches of mice, harvesting with heparinised
syringes and storing in capsules at -78°C until required
for experimental purposes.

2.4.2.1 Infection trials on rats

Two rats were given intraperitoneally 0.5 ml. of
fresh ascites fluid per rat. When no swellings were visible
after four weeks of observation the peritoneum of each rat
was opened up and examined for tumour cells and fluid.
Neither was found.

Then another set of four rats was each given 2 ml.
of fresh ascites fluid. Weekly examination lasting for
four months was followed by monthly observation for swellings
or fluid for another four months before the unsuccessful
experiment was terminated.
2.4.2.2 **Experiments to study the ascites fluid forms of trypanosomes**

Experiments were carried out with mice infected by intraperitoneal inoculation of ascitic fluid. When swellings were sufficiently developed each mouse was infected with Botswana strain of *T.b. rhodesiense*. Samples of peritoneal fluid were taken out and examined for trypanosomes each day until the host died. The forms of trypanosome found were studied by agar technique and by Giemsa staining. Photographs were taken when relevant.

Since during these experiments a raised parasitaemia was observed in blood, further experiments (2.4.2.3) were carried out to investigate the possibility that immunodepression resulted from intraperitoneal growth of the ascites tumour cells. As a part of this study total white cell and differential counts were obtained (see Results).

2.4.2.3 **Observation on parasitaemia of blood forms**

Mice with peritoneum swollen up with ascites fluid were infected with Botswana trypanosomes. Normal mice were also infected for controls. The blood parasitaemia obtained at various stages of infection was noted.
As a complementary observation and in order to see when the ascites fluid would begin to show the immunodepressive effect, mice were infected with the ascites fluid and trypanosomes at the same time. Parasitaemias of parasites were monitored till the hosts died. The control mice had only the trypanosomes injected.

2.5 METHODS ADOPTED TO CONFIRM THE EARLY BLOOD FORMS

2.5.1 Tsetse Transmission Experiments

Trials of infection through flies were studied. In each case a fly was known to be infected with 667 strain of T. brucei brucei. The infection in the fly was ascertained by examining the salivary exudate on a slide by a method (Burtt, 1946; Yondeowei, 1975) modified by Dr C. Letch of London School of Hygiene and Tropical Medicine. The modification essentially was coating the surface of the warm slide with foetal calf serum from which the trypanosomes could be recovered. The infected fly was then made to feed on Sha-sha mice. On finding out, from engorged appearance, that the fly had fed, the mice were kept and their blood examined by wet film and by the modified anion-exchange centrifugation technique (Lumsden et al., 1979) every twelve hours. When either method gave a positive result the agar method was used
to find out the form of parasite. Henceforth the examination on agar was carried out every 24 hours until the granular stumpy forms were noted.

2.5.2 **Culture Observations**

Cultures containing blood form trypanosomes (Brun *et al.*, 1979) were supplied by Dr D. Evans of London School of Hygiene and Tropical Medicine. The trypanosomes were known to be blood form because they successfully infected tsetse flies. They were observed on agar (Ormerod *et al.*, 1963).

2.5.3 **SHAM and Glycerol Chemotherapy Experiments**

Four sets of experiments with salicylhydroxamic acid (SHAM) with glycerol were carried out. In each set the three strains of *T. brucei rhodesiense* were used. In addition to these rhodesian strains *T.b. gambiense* (Bida 3) was also used in the first series of experiments.

Rats were treated intraperitoneally with either 4 or 8 g/kg⁻¹ of glycerol and 400 mg/kg⁻¹ or 500 mg/kg⁻¹ SHAM. Glycerol was injected before SHAM as 50% (v/v) solution in distilled water. SHAM solutions were always freshly prepared by dissolving 500 mg of salicylhydroxamic acid (from Sigma) in 3.25 ml of warm 1.0M NaOH and the volume was adjusted to 5.00 ml with distilled water (Evans and Brightman, 1980).
Each rat was checked by wet film 30 minutes later and by modified anion-exchange centrifugation technique (Lumsden et al., 1979) the following day. If any parasite was detected by either method the treatment was regarded as unsuccessful and had to be repeated.

To monitor the recrudescence in each experiment wet film examination was first carried out. If the result was negative modified anion-exchange centrifugation technique was applied. Whenever either method gave positive results an effort was immediately made to observe the form of the parasite on agar after Ormerod et al. (1963). If the agar technique was negative that day it would be repeated the following day until the form of trypanosome could be registered.

2.5.3.1 Experiments to study recrudescence in different strains

Rats were infected with each strain of trypanosomes and SHAM treatment administered when the infection was well established as determined by the agar technique. After treatment the blood was examined every 6 hours during the day and 12 hours at night. The time of recrudescence, if any, was recorded against each rat in each group and the form of trypanosome, as observed by agar method, was recorded.
2.5.3.2 Experiment to find if recrudescence would take place when treatment was administered before the prepatent period

Rats were infected with each one of the three strains 57, 83 and 180. Sets of rats with the strains were treated on day 1, 2, 3, 4 and 5. Examination of the blood was carried out as explained before until any rat showed a relapse.

2.5.3.3 Experiment to assess the possibility of recrudescence after multiple doses of SHAM and the forms of parasites produced after such doses

Three sets of rats were used. The first set was infected with 57, the second with 83, and the third with 180. If a rat survived the first does of SHAM, it was examined repeatedly until recrudescence occurred; it was then re-treated with SHAM. A maximum number of 3 doses was given thus.

2.5.3.4 Experiment to show the effect on recrudescence of three consecutive daily doses of SHAM

Each group of rats was infected with strains 57, 83 and 180. When the infections were established, three consecutive treatments, one each day, were administered.
2.6 MICROSCOPY AND RECORDS OF WORK BY MICROPHOTOGRAPHY

Examination of blood and the tissues was carried out by the three standard categories of microscopy, namely, the light microscopy (LM), scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Light microscopy was used to study the blood forms of all the strains mentioned in Table 1. Investigation by SEM was concentrated on

(a) T.b. rhodesiense from Zambia (57) and (83) and Botswana (180).

(b) T.b. brucei - Treu 667 and Antat 1.

(c) T. vivax - Y58 and Y486.

(d) T. congoense - Gamb 6 and 19; TSW 99 and 103.

(e) T. lewisi.

TEM was solely confined to T.b. rhodesiense strain from Botswana (180).

For TEM and SEM and sometimes LM it was necessary to separate the parasites from the blood corpuscles and concentrate or dilute to required concentration. This process was also necessary for the Coulter Counter study. Therefore, after the infected rats or mice had been found by the agar technique to have the right quantity and quality of the required infection, they were anaesthetised with ether. The blood was collected from the heart or the jugular vein into ice-cold physiological saline containing 500 IU/ml heparin as the anticoagulant. The blood was diluted 1 : 3 with icecold phosphate-buffered saline glucose pH 8.0 (PSG) (see Lanham, 1968).
The trypanosomes were separated from infected blood through a column of anion-exchanger, DEAE-cellulose (Whatman Chromedia DE 52). Full details of the method could be found in Lanham (1968). The trypanosomes were sedimented from the column eluate by centrifugation for 15 minutes at 2300 g at 4°C. Centrifugation was repeated, when essential, after the supernatant liquid was pipetted out and fresh PSG added for another wash. The number of washes allowed depended on the amount of original parasites in the eluate and for what purpose they were required.

2.6.1 Light Microscopy (LM)

2.6.1.1 Methods by which the blood parasites were examined

(a) Wet film

Wet film examination was generally performed when all that was necessary was to register whether infection was positive or negative and this was recorded as just positive (+), heavy infection (++), or every heavy infection (+++). Exceptionally heavy infection was noted as (++++).

(b) Matching method

A more accurate but fairly quick method of quantifying the parasitaemia was that of Herbert and Lumsden
(1976) popularly known as the 'matching' method.

(c) \textbf{Agar technique}

Most of the LM work was centred on studying the lipid granules in the different species and strains of trypanosomes. The agar technique described by Ormerod et al. (1963) was employed for this. The method was essentially putting a pin-head drop of blood on agar film set on an extra-thin slide (0.6 - 0.8 mm) and studying the phalanx with the phase-contrast, oil-immersion objective.

The field of the count and the number of trypanosomes were recorded on a chart, a sample of which is shown in Figure 1. The data thus obtained were summarised, computed and recorded as histograms and graphs shown in the results.

2.6.1.2 Characterisation by isoenzyme electrophoresis

After some regular patterns of the lipid granules have been observed in some strains of trypanosomes when using the agar technique some comparative studies with isoenzyme patterns were planned. The preparation of the lysates and electrophoresis were carried out by Dr W. Gibson using the methods of Gibson et al. (1978) with modifications given by Gibson et al. (1980).
Counting Forms of Pleomorphic Trypanosomes

Date: 23-4-79.
Strain: 57
Age of infection: 12 hrs

<table>
<thead>
<tr>
<th>Mic. Fields</th>
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<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10+</th>
<th>Expt Code:</th>
<th>Summary</th>
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<td></td>
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<td></td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LN = 89</td>
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<tr>
<td>N/A</td>
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<td>N/A</td>
<td>N/A</td>
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<td>N/A</td>
<td>LF = 50</td>
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<td>N/A</td>
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<td>N/A</td>
<td>N/A</td>
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<td>N/A</td>
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<td>AGR = 26139</td>
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</tr>
<tr>
<td>Fid (N/A)</td>
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<td>N/A</td>
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<td>N/A</td>
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<td>N/A</td>
<td>N/A</td>
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Figure 1. A sample of chart designed for simultaneous counting of Trypanosomes and Granules.
2.6.1.3 Thin slides

Preparation of the thin slides of the different forms of trypanosomes and blood cells and/or ascitic fluid were stained with either Giemsa's stain or Diff-Quik solution. The latter was a recent development of three solutions labelled A, B and C in which blood protozoa are fixed and stained in 15 seconds. Diff-Quik is obtainable through American Hospital Supply (UK) Ltd.

2.6.1.4 Photography

Macrophotography of organs or tissues was done by Minolta T101 camera loaded with Pan F Ilford films. Microphotographic pictures were taken by Nikon Microflex Model AFM of automatic photographic attachment mounted on Nikon microscope Model S-Kell with built-in Koehler Illuminating System. The automatic flash speed was 1/800th second and the setting was at its highest intensity for lighting (setting 4).

2.6.2 Scanning Electron Microscopy (SEM)

2.6.2.1 Preparation of trypanosomes on coverslips and Karnovsky's fixative

9 x 9 mm coverslips were prepared clean and grease-free by heating them in concentrated hydrochloric acid placed in a water-bath for 30 minutes; rinsing them
twice in absolute ethanol and ether. The coverslips were dried in the oven and each was placed in a Leyton tube.

Each coverslip was coated with foetal calf serum (FCS) by soaking it in 20% foetal calf serum in Hank's solution for 1 hour at 37°C. The coverslips were rinsed twice with Hank's solution without the serum. 100 μg/ml concavalin A (ConA) in Hank's was added and the tube incubated at 37°C for 1 hour. The coverslips were subsequently washed with Hank's and 0.5 ml of 10^6/ml, 2 x 10^6/ml or 5 x 10^6/ml of trypanosomes put on the coverslips and left to interact with FCS at 37°C for 30 minutes.

The trypanosomes on the coverslips were then fixed with Karvosky's fixative (2% formaldehyde and 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer). Some preparations of the trypanosomes prepared on the coverslips were also fixed with 2.5% glutaraldehyde only in cacodylate buffer.

2.6.2.2 Preparation of trypanosomes on unipore filters and glutaraldehyde fixation

(a) Single-filter system

Single filter system was initially used to obtain trypanosomes on unipore membranes. 2 ml of 1 x 10^6/ml, 2 x 10^6/ml or 5 x 10^6/ml of trypanosomes in
eluates were filtered onto 1 μm unipore polycarbonate membranes held in 13 mm aerosol holders. 2 ml disposable plastic syringes were found most suitable. Filtration with 5 ml or 10 ml syringes were usually unsuccessful because air bubbles block the pores of the membrane. Both unipore membranes and holders were supplied by Bio-Rad Laboratories.

2.5% glutaraldehyde fixative solution was forced through the membrane by means of a syringe immediately after the trypanosomes had been deposited and before they had had any chance to dry. The membranes were then put into fresh solution of glutaraldehyde and specimens left in the fixative overnight.

(b) Two-filter system

To study the small forms of trypanosomes which escape from 1 μm membrane another holder containing 0.4 μm membrane was plugged to the exit of a holder containing the 1 μm membrane. Thus a few of the small forms were collected and were fixed on the 0.4 μm filter in the same way as those on the 1 μm filter.

2.6.2.3 In vitro study of phagocytosis by SEM

Ascitic fluid and peritoneal fluid with trypanosomes were studied using the single-layer system described above. Observation of some peritoneal fluid preparations
revealed in vivo phagocytosis of T.b. rhodesiense (180) by the macrophages. The mode of entry could not be elucidated from the in vivo observation. Therefore it was decided that in vitro studies with SEM should be undertaken on the interaction between cultivated macrophages and trypanosomes obtained from the DE52 column eluate.

Normal unstimulated peritoneal macrophages were obtained as monolayers on coverslips from Dr Alberto of the Middlesex Hospital, London. They had been obtained from outbred TO mice, prepared by the method of Stuart et al. (1978) and cultivated in complete tissue-culture medium (TCM) consisting of Eagle's MEM, 0.11% NaHCO₃ and 10% foetal calf serum. The preparation was supplemented with antibiotics (500 IU/ml of benzylpenicillin and 2.50 µg/ml of streptomycin sulphate).

Onto each monolayer of macrophages on the coverslips in a bath of fresh TCM was added 2 ml of 10⁶/ml trypanosomes (180) separated from blood cells.

At 5 min, 15 min, 30 min, 1 hour and 2 hours the coverslips were taken out and washed in two baths of TCM to remove any unattached particles and other deposits; they were then put into a bath of fixative of 2.5% glutaraldehyde. All experiments were kept overnight with the specimens in the fixative.

A control of the macrophages only was similarly fixed in glutaraldehyde.
Parallel experiments with killed trypanosomes were set up. Dead trypanosomes were obtained by killing them in glutaraldehyde to preserve the surface coat. The parasites were washed thoroughly in PSG and 2 ml of $10^6$/ml of dead parasites were added to the monolayers of macrophages. At the same intervals of 5 min, 10 min, 15 min, 30 min, 1 hour and 2 hours of interaction between killed parasites and macrophages were fixed in glutaraldehyde.

2.6.2.4 Dehydration and drying

The products of SEM studies whether from Karnovsky's fixative or glutaraldehyde, single- or double-filtered and also those obtained from phagocytosis experiments were dehydrated and dried. They were washed in two or three baths of cacodylate buffer and kept in the buffer for a day with changes every 2 hours. In some experiments "post-fixation" was carried out with 1% osmium tetroxide for 30 minutes but when it was observed that the process made no difference to the subsequent appearance at SEM, it was discontinued.

Each preparation was processed through a graded series of alcohols beginning with 50% (v/v H₂O), in 10% steps, up to absolute ethanol (two baths of absolute alcohol being provided), the specimen resting 5 minutes at each grade.

After dehydration each specimen was impregnated
with 50/50 (v/v) mixture of ethanol and Arcton or Arklon P (supplied by Imperial Chemical Industries Ltd) for 20 minutes before it was finally immersed in pure Arcton for 20 minutes. It was then dried at the critical point in a "Polaron" E300 Critical Point drying apparatus drying with liquid CO$_2$. A few samples air-dried or freeze-dried were compared with those from the critical point drying.

2.6.2.5 Photography

The membrane or the coverslip with the specimens on it was mounted on an aluminium stub and shadowed with gold in an argon atmosphere using a Polaron diode sputter coater E 5000. Examination was done and pictures taken by Jeol JSM 35 scanning electron microscope operating at 18 - 20 KV and at a tilt angle of 20°.

2.6.2.6 Finding the volume of trypanosomes

Phosphate-buffered saline glucose was added to the eluate from the DE52 column. The determination of volumes of T.b. rhodesiense and T. lewisi was done by employing the method of Maxie et al. (1978). Coulter Counter Model B was used in conjunction with Model J plotter. Both machines were supplied by Coulter Electronics Limited. Calibration of the plotter was by latex
particles of known mean diameter. The aperture diameter was 100 μm, the amplification 0.5 μm for T.b. rhodesiense, 16 for T. lewisi; the aperture current was 0.5 for T.b. rhodesiense, 1 for T. lewisi. The switch setting on the plotter was 5 for T.b. rhodesiense, 7 for T. lewisi. The Coulter Counter setting was 16H in both cases.

2.6.3 Transmission Electron Microscopy (TEM)

2.6.3.1 Suspension of trypanosomes in agar blocks

Trypanosomes from the DE52 eluate were concentrated and fixed with 2.5% glutaraldehyde overnight at 4°C. The following day the fixed material was centrifuged at 2300 g for 15 minutes and washed once in cacodylate buffer before final centrifugation was carried out to obtain a pellet. To the pellet was added molten 1.5% agar in distilled water. The mixture was centrifuged for 10 minutes at 4°C. The agar was set so that the pellet was held within the agar. Small blocks of about 2 x 2 mm were cut out and put into cacodylate buffer for washing throughout the day with changes every hour.

2.6.3.2 Mounting trypanosomes onto filters

The trypanosomes were obtained on single layer or double layer systems of unipore membranes as described
in 2.6.2.2(h), held by holders as described for SEM above, in order to study the ultrastructure of the various sizes and forms.

2.6.3.3 Obtaining tissues

Freshly killed rats were dissected for the liver, lung or brain. Minute blocks of approximately 2 x 2 mm of liver and lungs were cut and put into glutaraldehyde fixative immediately. The brain was halved antero-posteriorly with a new surgical scalpel blade. The third and fourth ventricles of each half were flushed with 2.5% glutaraldehyde to fix the choroid plexus in situ. Each ventricle was carefully probed to extract the choroid plexus. When obtained the choroid plexus was immersed in fresh glutaraldehyde fixative and kept at 4°C overnight.

2.6.3.4 Processing the blocks, unipore membranes and tissues

The blocks and membranes were cut into single strips, fixed overnight, and washed in cacodylate buffer for a day and a night with changes every hour of the day. The following day the buffer was changed again and the specimen post-fixed in 1% osmium tetroxide for 1 hour in the fume cupboard. Washed in distilled water for an hour before dehydration successively in 30%, 60%,
70%, 80% 90% and absolute alcohol took place. Each specimen was kept in each grade of alcohol for 10 minutes except for absolute alcohol where two changes each of 10 minutes were undertaken. The specimens went through two changes of propylene oxide (the intermediate solvent) for 30 minutes each change. This process like the post-fixation took place in the fume cupboard. Infiltration with the resin took 24 hours in the case of the blocks and membranes, but 48 hours in the case of tissues. Fluka Aradite was the resin used. Final embedding was done at 60°C for at least 48 hours using the same resin.

The Aradite blocks were cut with Cambridge Huxley Ultramicrotome Model MK II. Thick sections mounted in Canada balsam and stained with methylene blue were previewed with light microscope to estimate the success of the techniques and evaluate the potential of the preparation. The thin sections mounted on New 200 copper grids were double stained in saturated aqueous uranyl acetate for 30 minutes and Renold lead citrate for 5 minutes.

2.6.3.5 Photography

Preliminary examination of materials was often done with Zeiss EM 9 and AEI 6B electron microscopes but pictures were taken not only with these two machines but
also AEI 801 and Jeol 100 CX. The operation voltage for 6B and EM9 was 60 KV. For 801 and 100CX it was 80 KV. The speed was 2 seconds for 100CX but 1 second for the others. The film used was Ilford Technical film, specially made for EM.
CHAPTER 3

PATTERNS OF GRANULAR AND AGRANULAR FORMS IN THE BLOOD

3.1 RESULTS

The study of the patterns of parasitaemia and distribution of the forms was concentrated on three strains of *T. b. rhodesiense*. These were the Botswana, and Zambia I and II strains.

3.1.1 The Forms

In this study the bloodstream forms observed with the agar technique were classified as:

(a) agranular long-narrow form
(b) agranular long-flat form
(c) granular standard form
(d) granular tadpole form
(e) granular moribund form
(f) others.

3.1.1.1 The agranular long-narrow form

The agranular long-narrow form (Plate 1a) was best observed in the blood immediately after the prepatent period which might vary between 48 hours and 96 hours
depending on the amount of inoculum used, the strain and the immunological response of the individual host.

The agranular long-narrow forms were seen in small numbers continuously throughout the infection but their numbers were noticeably increased at the beginning of each relapse. This observation applied to all the three strains.

The long-narrow was refractile and because of its cylindrical or tubular shape, it rolled like a spirochaete when viewed in the phalanx of blood film between the agar and coverslip. It possessed little or no undulating membrane but the free flagellum was conspicuous. No long-narrow form was found to be in dividing stage and the lipid or type II granules were always absent from its cytoplasm. From its posterior end protruded a "plasmaneme" which appeared to flick in and out of vision like the tongue of a snake. While this "organelle" was too faint for light microscopy micrographs to show the scanning electron microscopy confirmed its presence at this stage of the parasite. (See 7.1.1.4b)

3.1.1.2 The agranular long-flat form

The agranular long-flat form (Plate 1b) was seen in blood 24 hours after the appearance of the long-narrow form. It was less refractile and did not roll. The undulating membrane was well developed. Its length was
about the same as that of the long-narrow but was broader. It was less active than the long-narrow and its movement was characteristically "flagellar" rather than "spirochaetal".

3.1.1.3 The standard granular form

This form, in the writer's categorisation, included the so-called "intermediate", "short-stumpy", and "posteronuclear" all of which have been depicted by Ormerod (1979a). However, the number of granules and their quality have been found to be related to the strains and species in the current study. This will be demonstrated and discussed later under the light microscopy in Chapter Six.

3.1.1.4 The granular tadpole form

Tadpole forms (Plate 1c) were few in number and were only occasionally seen in normal infections but they were always common in the immunosuppression experiments and in ascitic fluid. With these granules characteristic of the strain under examination the granules were always present and usually conspicuous.

3.1.1.5 Granular moribund form

This form (Plate 1d) was few. Its moribund
PLATE 1  Trypanosome forms of Botswana strain on agar

(a) Long-narrow form

(b) Long-flat form

(c) Granular form with tadpole form

(d) A moribund form of tadpole nature

(e) A moribund form almost dead

Note: a and b are difficult to distinguish on the photographs but easy in the living preparation because the long-narrow can be seen to be cylindrical when it rolls over.
PLATE 2  Granular form of Botswana strain under different conditions all showing same number of granules per trypanosome.

(a) Normal infection

(b) Cloned infection

(c) X-irradiated infection
a  

b  
c
PLATE 3  Granular forms of Zambia strains

(a) Zambia I (83) strain showing average of 5-6 granules per trypanosome

(b) Zambia II (57) strain showing average of more than ten granules per trypanosome
appearance could vary; while some could hardly be distin­
guished from ordinary active tadpole forms (Plate 1d),
others were so degenerate that although they showed the
granules they might be regarded as being already dead
(Plate 1e).

3.1.1.6 Others

3.1.1.6(a) Sphaeromastigote

On four occasions during the course of study
a form was observed which fitted the stage that Brack
(1968) described as a sphaeromastigote. Two such occasions
were from a normal infection, one was from ascitic tumour
fluid infection and the other from peritoneal population
of trypanosomes. All were observed on agar but photo­
graphy was not possible on these occasions.

3.1.1.6(b) Multiflagellar/multinucleate form

In both phase-contrast microscopy of agar
material and the scanning electron microscopy multiflagellar
form was found. The form was rare except in the immuno­
depression experiments and ascites tumour fluid.
Stained preparation with Giemsa and Diff-quik confirmed
its multinucleate nature as shown in Ormerod (1951).
3.1.2 The Criteria for Comparison

The parasitaemias based on the number of different forms per field on agar slide were transformed into graphs. The average number of granules in the trypanosome was put into the histogram. The agranular forms include both long-narrow and long-flat forms. Similarly all granular forms (standard, tadpole and moribund) are grouped together.

3.1.3 The Standards of Comparison

Instead of describing each infection under a specific condition or treatment it was decided that the following standards would be used for comparison:

(a) general comments on cause of infection;
(b) distribution of peaks and troughs;
(c) height of parasitaemia;
(d) rapidity of rise of parasitaemia;
(e) pattern of development of long-narrow form;
(f) pattern of development of the agranular forms;
(g) pattern of development of the granular forms;
(h) the life-span of the host;
(i) the pattern of granular forms is considered separately (see Section 3.1.7).
3.1.4 The Normal Infection in Rats

3.1.4.1 Botswana strain

The normal prepatent period was 4 days although a few of the inoculated parasites could be detected in the circulation before the first flush of long-narrow form was seen in the blood. There were several peaks (See Fig. 2a). The first was on day 7 and that was over twenty parasites in the field. This was followed by a wide trough with minor peak on day 10. The next substantive peak was between day 14 and 16. Then a sharp drop from a parasitaemia of well over 50 per field to 20 parasites/field. Another major rise occurred on day 19 with a small trough on day 20. Between day 21 and 28 there was a smooth curve, something of a plateau. This was followed by a marked drop in parasitaemia which was below 10 parasites per field until the death of the host.

The height of parasitaemia was never beyond 60 trypanosomes per field. Some hosts died at the height of infection on day 20 and 23. A few died immediately after the fall on days 29 and 30.

The rise of parasitaemia was slow until day 13. The fall of parasitaemia was however drastic particularly after day 28.

Long-narrow form was seen as soon as the infection became patent. There was an average of two in twenty-five fields and the population of that form was never more
than 4-5 per field. This maximum occurred when the agranular forms were rapidly increasing in the blood between days 20 and 23. There was always a slight rise corresponding to any increase in agranular forms - see days 7, 10 and 16 in Figure 2a. These were at relapses.

The total parasitaemia was essentially made up of agranular forms except after peaks when granular forms were predominant and during plateaux when granular forms persisted in the population. The lifespan of the infected rats was about five weeks.

3.1.4.2 Zambia I strain

The prepatent period was three to four days. The strain was more virulent than Botswana. The peaks were fewer but more well defined than in Botswana strain - see Figure 3a. The first was on day 5, two days earlier than in Botswana. The second, third and fourth on days 8, 11 and 13 respectively were progressive before the major trough between days 14 and 18. The last and highest peak of 141 parasites/field was on day 20 just before the hosts died. The rise in parasitaemia was thus regarded as rapid. The development of long-narrow forms was as normal Botswana strain. They were present throughout but never very many, the highest being 4 per field on days 12 and 19. Their increase also coincided with general increase in the number of agranular forms.
The agranular forms appeared to maintain the population except between days 12 and 17: this period was ushered in by a small decline in the overall population at day 13. During this period the high parasitaemia was maintained by a sharp increase in the granular forms. Death of a host occurred on day 14 corresponding to a peak. However the usual lifespan was three weeks.

3.1.4.3 Zambia II strain

The prepatent period was 4 days. The distribution of peaks and troughs was regular and well defined (Figure 4a). The first was small and short-lived and occurred on day 6. The second starting from day 10 progressed into the third which went to over 60 trypanosomes per field. The first real remission occurred between days 20 and 25 when the last and highest recorded peak started. It reached 90 parasites per field on day 31. However it was significant that the parasitaemia peaks and troughs were the reverse to those in Botswana strain. For instance it would be found from Figure 4a that the main relapse was between days 20 and 27. That was the period of highest parasitaemia of Botswana (see Fig. 2a). Also the parasitaemia dropped to about 5 organisms in a field in Botswana strain after day 29. In Zambia II infection the parasitaemia rose dramatically to over 80 per
field during the same period. The rise and fall in parasitaemia was smooth except the sudden rise and fall between days 18 and 20. The pattern of development of long-narrow forms was as in Botswana and Zambia I strains, rising with increase in agranular population but never plentiful. The highest was four or five in a field. Unlike the parasitaemia patterns in Botswana and Zambia I strains, the granular forms were more numerous than the agranulars except between days 25 and 30 when there was an upsurge of new agranular forms which made the parasitaemia reach the highest peak. So in contrast to the other two strains the parasitaemia of Zambia II strain was maintained largely by the granular forms.

The experiment was terminated in the fifth week, in line with others, but two hosts lived up to 44 days.

3.1.5 X-Irradiated Infection

The most obvious observation common to all the strains was that there was sustained and persistent enhancement in parasitaemia when the hosts were irradiated before being inoculated with trypanosomes.

3.1.5.1 Botswana strain

There was only one peak and no relapse or
remission (Fig. 5a). The height of parasitaemia was nearly 100 organisms per field. This was twice the value in normal infection. The rise was rapid. It started almost immediately after the prepatent period and rose most rapidly from days 7 to 12 when the last host died.

The occurrence of long-narrow form was in the usual pattern, i.e. rising with increase in agranular parasitaemia. The population, as in the normal infection, was held up to be essentially agranular with the granular forms taking the second place. The life-span was reduced to one-third that of normal infection.

3.1.5.2 Zambia I strain

There were "spikes" or minor peaks but all these were associated with the major peak (Fig. 6a). There was no remission because the lowest parasitaemia in the peak was still some 90 parasites per field. The highest peak in parasitaemia of 172 organisms in one field was reached on day 20. The rise in parasitaemia was most rapid from about 5 organisms on day to 120 on day 7.

The development of the long-narrow form followed the usual pattern. The population rose rapidly between days 6 and 11. This was when there was a tremendous production of agranular forms. A peak of over 10 long-narrow parasites per field was reached on day 8. The
increase in long-narrow form was repeated between days 14 and 18 also corresponding to the time when the agranulars were increasing.

There was an initial sharp rise in the production of agranular forms but the time appeared to be too short for the elimination of the granular forms. So the early parasitaemia was, as in normal infection, maintained by the agranular forms, and it was also noteworthy to find that both the normal as well as irradiated infection showed a small drop in general parasitaemia between days 9 and 12, and between days 14 and 18.

3.1.5.3 Zambia II strain

Although peaks occurred, these led progressively from one to another without complete remission (Fig. 7a). The first at day 5 with under 5 organisms per field rose to the second on day 6 with parasitaemia of nearly 20. This lingered on until it was over 20 organisms on day 8 before it rose to over 40 on day 9. Two minor troughs occurred on days 11 and 13 but the parasitaemia never went below thirty parasites in one field. The next peak was on day 14 with nearly 80 organisms per field. This rapidly rose to the final peak on day 20 with a record, for the strain, of over 110 parasites per field.

Numbers of long-narrow form were increased whenever the total agranular forms increased as on days 6, 9 and 11 and between days 14 and 20.
As in the normal infection of the strain, the population was largely maintained by the granular forms except when parasitaemia rapidly rose between days 14 and 20. The preponderance of granular forms in the parasitaemia was again in contrast to what was obtained in Botswana and Zambia I strains.

The lifespan was reduced to three weeks (see Fig. 7a).

3.1.5.4 X-irradiation at high dosage level

The above results referred to the experiments where the dose of total body irradiation was 800 rads. When the dose was 1000 rads there was uniformly an extension to the prepatent period. No strain, even the virulent one from Zambia, showed before the fifth day after inoculation. However the general pattern of enhanced parasitaemia was similar to those already described for the irradiation experiments.

3.1.6 Dexamethazone-Treated Infection

Both the ordinary control infection and infection with the solvent PVP gave results similar to that described for normal infections in Figures 2a, 3a and 4a. All strains responded to the treatment with enhanced parasitaemias. But these parasitaemias were
with peaks and troughs in contrast to the general patterns obtained with x-irradiation. The only exception was 40 mg/kg level of dosage with Zambia II strain. The optimum dosage giving the highest peaks in all the strains studied was 20 mg/kg.

### 3.1.6.1 Botswana strain

#### 3.1.6.1(a) Treatment with 8 mg/kg dexamethazone

The major three groups of peaks noted in the normal infection were present (Fig. 8a). But there were two main differences. The first peak still occurring between days 6 - 9 was much enhanced. Instead of 20 organisms per field in the normal infection it was about 90. The second difference was the place of the third peak. In the normal infection it occurred between days 18 and 28. In this experiment this was a time of major remission. The third peak occurred between days 28 and 31. The similarity was that the second peak in both normal and this infection took place between days 11 and 16. The highest peak was well over 110 parasites per field. This was nearly twice the highest peak in the normal infection.

The rise and fall of the peaks were more rapid than in the normal infection. However the pattern of development of long-narrow, agranular and granular forms was as described for the normal and x-irradiated infection.
There was also little difference in the lifespan of hosts, 35 days in the normal infection and 31 in this treatment.

3.1.6.1(b) Treatment with 20 mg/kg

The three major peaks of parasitaemia were present at the same time as in the normal but the second was least enhanced (Fig. 9a). The first and the highest peak occurred with nearly 140 organisms per field in contrast to 20 in the normal. The third was rapidly attained and it was short-lived. It occurred between days 14 and 20 and was over 100 organisms per field. The patterns of development of all the component forms were as found in the normal and x-irradiation experiments. Lifespan was reduced to less than three weeks.

3.1.6.1(c) Treatment with 40 mg/kg

The three main peaks were evidently shown in Figure 10a. Like the treatment with 20 mg/kg the second was depressed but unlike that treatment the first peak was not the most enhanced. It was the third. The peak attained by the 29th day was over 120 parasites in a field. The occurrence of the long-narrow, granular and agranular forms was the same as in the normal and x-irradiation infections. The role each form played was similar. The lifespan of hosts was generally shortened by about 1 week.
3.1.6.2 Zambia I strain

3.1.6.2(a) Treatment with 8 mg/kg

Only two instead of the three major peaks of the normal infection were noticeable in this treatment (Fig. 11a). The first which was enhanced reached 45 organisms per field as compared with under 10 in the normal. The trough was a day more short-lived than in the normal. The second peak reached 120 by the sixteenth day. While it was higher than the second of the normal it did not reach the highest peak of 140 recorded for the third peak of the normal infection. The peaks were, on the whole attained in more rapid succession than in the normal infection. The patterns of development of all the constituent forms were not different from those described for the normal. The lifespan was three or four days shorter.

3.1.6.2(b) Treatment with 20 mg/kg

As in 8 mg/kg treatment there were only two major peaks depicted by Figure 12a. Both were enhanced. The first reached 140 in contrast to 10 of the normal and the second was 150 as against under 110 in the normal. This was even higher than the normal's highest and third peak. That third peak was not displayed in this experiment. The trough was at high parasitaemia of about 80 organisms per field.

The rapidity of rise of parasitaemia was notable.
For instance, a parasitaemia of 10 organisms per field at the fourth day rose to over 75 on the sixth and 140 on the eighth. The occurrence of the different forms and the interdependent roles they played were as noted for the normal and x-irradiated infections. The hosts' lifespan, as in the former treatment, was about 3 days shorter than normal.

3.1.6.2(c) Treatment with 40 mg/kg

There were again only two peaks of total parasitaemia visible (Fig. 13a). The first occurred at the same time as in the normal and in the other two levels of dosage. The second peak was fused with the third. Therefore the only trough which was once again relatively at high parasitaemia occurred between days 10 and 12.

The height of parasitaemia attained in the first peak was not only a record for this experiment, it was for all the experiments. There were over 180 organisms per field, which confirmed this strain not only virulent but fast-growing. That that height was reached on day 8 from a parasitaemia of under 20 organisms per field on day 5 made the rate of infection rapid.

The patterns of development of all the subsidiary forms in the total parasitaemia were similar to former descriptions with one remarkable addition: the second and third peak not separated by total parasitaemia was unmistakingly shown by the production of the agranular forms. In this strain the second major peak would usually
occur by day 14 (see figs. 3a, 6a, 11a, 12a). But in this experiment this was not shown to be a distinct peak. The study of the agranular forms however showed the peak and a trough between days 16 and 18. The third and final peak would occur in the hosts, which lived long enough with the infection, by day 20 (see Figs. 3a, 6a). That peak was demonstrated by the agranular forms at that time in Figure 13a, in spite of the fact that the total parasitaemia was going down. The lifespan was as in the normal infection.

3.1.6.3 Zambia II strain

3.1.6.3(a) Treatment with 8 mg/kg

Three major peaks were featured (Fig. 14a). The first and highest occurred between days 5 and 9, the second between 14 and 20. The third started from day 24 and terminated by day 27. The troughs were much more marked than in the normal infection. The highest peak of 70 parasites per field although enhanced where it appeared was not as high as the highest of the normal infection which reached 90 organisms per field (Fig. 4a).

The interrelationship between the different forms remained the same. The only significant difference was that the peaks of the agranular forms were less clearly delineated and therefore less informative than in the normal infection. The lifespan of the host was little
affected. It was about 3 - 4 days shorter than the normal.

3.1.6.3(b) Treatment with 20 mg/kg

As was shown in the other two strains, the greatest effect in the enhancement of total parasitaemia occurred with this dose. Although there were two other minor peaks occurring at the same time as in the other treatments (3.1.6.3.1) the first peak was the highest in this treatment. It was almost 110 parasites per field. This covered a period of five days starting from day 5. The rise and fall of the parasitaemia peak were most rapid (see Fig. 15a). The relationship between the different forms and their interaction remained as in the normal infection. This included the fact that the parasitaemia was mainly of granular forms although the presence of the agranular forms maintained the dynamic equilibrium of the population. The lifespan was shortened by about ten days.

3.1.6.3(c) Treatment with 40 mg/kg

There was only one peak (Fig. 16a). That corresponded to the first one of the normal and other treatments. Though the height of this peak was only 60 organisms per field all the hosts died before the seventh day. The rise was rapid and the parasitaemia enhanced by the treatment.

The occurrence of the granular forms remained
dominant over the agranular forms. The hosts died too quickly for other comparisons to be made.

3.1.7 Pattern and Number of Granules in the Trypanosome

A uniform number of granules was found throughout the experiments as follows:

(a) 3 - 4 granules per trypanosome as seen in Botswana strain,
(b) 5 - 6 in Zambia I strain, and
(c) more than 10 in Zambia II strain.

They were big and refractile in Botswana but small in both Zambia I and Zambia II strains.

3.1.7.1 Botswana strain

3.1.7.1(a) Normal infection of Botswana strain

Figure 2b and Plate 2a show that throughout the lifespan of the hosts the average number of granules in a trypanosome was either three or four. There were trypanosomes with one or two granules and occasionally some showed up to six or even more, but the mean and majority was always as specified. When an infection in a day contained few trypanosomes with one or two and many had four or more the average might even rise above four as days 12, 18, 25 and 35 in Figure 2b. These days coincided with either the time the infection was becoming
dominant over the agranular forms. The hosts died too quickly for other comparisons to be made.

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more granular (days 12 and 18) or when the granular forms were persistent (days 31 to 35).

The result was consistent with either infection on day 6 of first passage of the strain, day 12 of fourth passage, or day 16 of the ninth passage. The strain was lost in the breakdown of the refrigerator and a new batch which had been tsetse-transmitted was later obtained but the number of granules in the trypanosomes was still characteristic. Both rat infection as well as mice parasitaemia gave the same average number of granules per trypanosome.

A clone of the strain (180 B1) gave the same characteristic pattern and number of granules (Plate 2b).

3.1.7.1(b) X-irradiation infection of Botswana strain

X-irradiation neither increased nor decreased the average number of granules in the trypanosome (Plate 2c). While the mean was four at the early stages this was later three per trypanosome at the later stages when the parasitaemia was increasing and the population was more of the agranular forms (Fig. 5b). There was no difference in the number of granules whether the dose was 800 or 1000 rads.

3.1.7.1(c) Dexamethazone-treated infection of Botswana strain

Not one of the three levels of dosage affected the average number of granules. They remained either three or four (Figs. 8b, 9b, 10b).
3.1.7.2 Zambia I strain

3.1.7.2(a) Normal infection of Zambia I strain

It was possible for wrong conclusion to be drawn if only one or two samples were hastily observed or if a preparation did not spread out all the granules. But for each day of the infection during the study the mean number of all granules was 5 - 6 granules per trypanosome (Fig. 3a and Plate 3a). A trypanosome could have as many as eight or nine granules or as few as four or even two or three, but overall calculation showed the majority to possess an average of 5 or 6. The results obtained from mice infection did not differ from those from rats.

3.1.7.2(b) X-irradiation infection of Zambia I strain

Both 800 and 1000 rads doses gave the same average number of granules per trypanosome (Fig. 6b). It was as in the normal infections.

3.1.7.2(c) Dexamethazone-treated infection of Zambia I strain

All the three levels of dosage (Figs. 11b, 12b, 13b) were as in the normal infection as far as the average number of granules per trypanosome was concerned. Also the observation that when the granular forms persist the higher average of six would be obtained was upheld by Figure 12b at days 5 to 8.
3.1.7.3 Zambia II strain

3.1.7.3(a) Normal infection of Zambia II strain

Except at the initial stage of granulation when all the granules might not have been formed or visible the average number in the trypanosome was always more than ten (Fig. 4b). Of course as in other strains one trypanosome might reveal only 5 or 6 granules the characteristic granular form was a robust stumpy trypanosome with as many granules as fifteen or more (Plate 3b).

As was found for Botswana and Zambia I strains the number and type of granules were not affected whether the trypanosomes were grown in mice or rats.

3.1.7.3(b) X-irradiation infection of Zambia II strain

Similar to those obtained for the other two strains the result from irradiation experiment showed that neither the quantity nor the quality of the granules was affected. The number remained more than 10 granules per trypanosome (Fig. 7b). However the number of granules per trypanosome was below the average on the first day of granulation. The same abnormality was noted in the control experiments earlier described. (Figure 4b).

3.1.7.3(c) Dexamethazone-treated infection of Zambia II strain

The drug at all levels used did not affect the quantity of the granules produced as illustrated in Figures 14b, 15b, 16b.
NOTE (Figs. 2 - 16)
The observation on the parasitaemia of the normal rats infected with Botswana strain was carried out with three experiments. Each experiment was done with two rats. When it was found out that the results on agar were consistent and the method has been mastered so much that even a rat monitored gave similar pattern as another, other experiments were carried out only with two rats in each set.
Fig. 2a  Patterns of parasitemia in Botswana strain (normal rats)

Fig. 2b  Occurrence of granules in Botswana strain (normal rats)
Fig. 3a  Patterns of parasitemia in Zambial strain (normal rats)

- Total parasitemia
- Agranular form
- Granular form
- Long narrow form

Fig. 3b  Occurrence of granules in Zambial strain (normal rats)
Fig. 4a Patterns of parasitemia in Zambia II strain (normal rats)

Fig. 4b Occurrence of granules in Zambia II strain (normal rats)
Fig. 5a  Patterns of parasitemia in Botswana strain (X-irradiated rats)

Fig. 5b  Occurrence of granules in Botswana strain (X-irradiated rats)
Fig. 6a Patterns of parasitemia in Zambia strain (x-irradiated rats)

Fig. 6b Occurrence of granules in Zambia strain (x-irradiated rats)
Fig. 7a Patterns of parasitemia in Zambia II strain (x-irradiated rats)

- ▲ Total parasitaemia
- ○ Granular form
- ● Agranular form
- ■ Long narrow form

Fig. 7b Occurrence of granules in Zambia II strain (x-irradiated rats)
Fig. 8a PATTERN OF PARASITAEMIA IN BOTSWANA STRAIN
(8 mg/kg dexamethasone)

Fig. 8b OCCURRENCE OF GRANULES IN BOTSWANA STRAIN
(8 mg/kg dexamethasone)
Fig. 9a  PATTERNS OF PARASITAEMIA IN BOTSWANA STRAIN
(20 mg/kg dexamethasone)

Fig. 9b  OCCURRENCE OF GRANULES IN BOTSWANA STRAIN
(20 mg/kg dexamethazone)
Fig. 10a PATTERNS OF PARASITAEMIA IN BOTSWANA STRAIN
(40 mg/kg dexamethasone)

Fig. 10b OCCURRENCE OF GRANULES IN BOTSWANA STRAIN (40 mg/kg dexamethasone)
Fig. 11a PATTERNS OF PARASITAEMIA IN ZAMBIA STRAIN
(8 mg/kg dexamethasone)

Fig. 11b OCCURRENCE OF GRANULES IN ZAMBIA STRAIN
(8 mg/kg dexamethasone)
Fig. 12b PATTERNS OF PARASITAEMIA IN ZAMBIA STRAIN
(20 mg/kg dexamethasone)

Fig. 12b OCCURRENCE OF GRANULES IN ZAMBIA STRAIN
(20 mg/kg dexamethasone)
Fig. 13a PATTERNS OF PARASITAEMIA IN ZAMBIA I STRAIN
(40 mg/kg dexamethasone)

- TOTAL PARASITAEMIA
- AGRANULAR FORM
- GRANULAR FORM
- LONG NARROW FORM

Fig. 13b OCCURRENCE OF GRANULES IN ZAMBIA I STRAIN
(40 mg/kg dexamethasone)
Fig. 14a PATTERNS OF PARASITAEMIA IN ZAMBIA II STRAIN
(8 mg/kg dexamethasone)

Fig. 14b OCCURRENCE OF GRANULES ZAMBIA II STRAIN
(8 mg/kg dexamethasone)
Fig. 15a PATTERNS OF PARASITAEMIA IN ZAMBIA II STRAIN
(20 mg/kg dexamethasone)

Fig. 15b OCCURRENCE OF GRANULES IN ZAMBIA II STRAIN (20 mg/kg dexamethasone)
Fig. 16a PATTERNS OF PARASITAEMIA IN ZAMBIAN II STRAIN
(40 mg/kg dexamethasone)

Fig. 16b OCCURRENCE OF GRANULES IN ZAMBIAN II STRAIN
(40 mg/kg dexamethasone)
3.2 DISCUSSION

3.2.1 The Techniques

Several modern parasitological methods detailed in Chapter 2 were used in this study and the up-to-date microscope models employed, but the pivotal technique for the work was the agar method described in Chapter 2. Even when the extravascular forms in fluid and tissue phase were found, it was possible by using the agar technique to classify the trypomastigotes seen into granular or agranular forms. This simple method is greatly superior to the techniques needing fixed and stained preparations for categorising and quantifying populations of blood trypanosomes. The methods used by Ashcroft (1957) and Wijers (1959) were based on measuring length of individual trypanosomes which had been fixed and stained and involved their comparison with the drawings of Lady Bruce (Bruce, 1911); these methods failed to be specific on what intermediate, slender and intermediate stumpy forms were. Ashcroft (1957) was so desperately disappointed in the futility of operating such method that he wrote, "All grades between a typically stumpy and a typically slender form were seen, and it was often extremely difficult to decide to which of the three categories an individual belonged. Even the method used here for assessing the morphology by counting typically slender and stumpy forms is only approximate." It must therefore be asked why
then should scientists continue to use such an approximate method when a much more precise technique was available? It is the opinion of the writer that the agar method is today the best method available for assessing pleomorphism in *T. brucei*.

Opinions may differ about the reliability of the agar method for quantifying parasitaemia. The originators of the technique (Ormerod *et al.*, 1963) specified that the method was only relative. This was to distinguish it from the other methods of counting such as by haemocytometer whereby the numbers of parasites per unit volume could be obtained. The writer of this thesis believes that the agar technique could be standardised and be as absolute as either the matching or haemocytometer method. In fact, during this work a preliminary comparative assessment between agar and matching methods was carried out. The provisional result is given in Table 2. The result of this preliminary work was not included in the major body of results because it cannot be regarded as more than an indication of what is possible. More work by different observers is essential, using the same strain and same type of microscopes at the same time before a thorough statistical analysis could lead to the full standardisation of the method.
Table 2  Possible standardisation of agar method by matching method*

<table>
<thead>
<tr>
<th>Agar technique (no. of tryps/field)</th>
<th>Matching method equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log no. of organism/ml</td>
</tr>
<tr>
<td>Nil</td>
<td>6.9</td>
</tr>
<tr>
<td>1</td>
<td>7.2</td>
</tr>
<tr>
<td>3 - 5</td>
<td>7.5</td>
</tr>
<tr>
<td>10 - 20</td>
<td>7.8</td>
</tr>
<tr>
<td>25 - 30</td>
<td>8.1</td>
</tr>
<tr>
<td>50 - 60</td>
<td>8.4</td>
</tr>
<tr>
<td>70 - 100</td>
<td>8.7</td>
</tr>
<tr>
<td>100+</td>
<td>9.0+</td>
</tr>
</tbody>
</table>

* Zeiss microscope, with X 10 objective and X 8 eyepieces used.

3.2.2 The Immunodepressants Administered

Corticosteroids and x-irradiation are two different agents used for immunodepression. A review of the immunodepressive effects of corticosteroids was given by Claman et al. (1971). In general they inhibit antibody production if given before or at the same time as the antigen but they have little effect once antibody response had been established. That large doses lead to diminished ability to acquire immunity but have little effect on
already acquired immunity, has been demonstrated by Kass and Finland (1953), McMaster (1961) and McMaster and Franz (1961).

Corticosteroids are known to depress immune responses to protozoan infections. In diseases other than trypanosomiasis, Stahl et al. (1966) used corticosteroids in toxoplasmosis; Cox (1968, 1974) in malaria; and Young and Cox (1971) in Babesia infections. In African trypanosomiasis Ashcroft (1957, 1959), Petana (1964), Sherman and Ruble (1967), and Luckins (1972) had used the drugs. In natural state, cortisone used as cortisone acetate by Petana (1964), Cox (1968) and Luckins (1972) is not as effective as its synthetic derivatives such as betamethasone administered by Cox (1968). That explains why dexamethasone in preference to the cortisone acetate was chosen for this work. There is no record known to the author where dexamethasone had been used to induce immunodepression in trypanosomiasis.

Large doses of even the synthetic corticosteroids may fail to inflict total immunodepression. This is because the drug could be metabolised by the host. Young and Cox (1971) proved that the reduction in immunity caused by betamethasone was reversible. Cantrell (1959) found that large doses of cortisone failed to alter the course of infection of T. equiperdum. Herbert and Becker (1961) could not lower resistance of rats sufficiently enough to permit re-infection with T. lewisi.
X-irradiation, usually given by total body exposure, is a more effective agent of immunodepression. It was successfully administered by Balber (1972) and Luckins (1972). High doses of x-irradiation may be disadvantageous in reducing immune response. The work of Dixon and McConakey (1963) supports this assertion in that they obtained enhancement instead of inhibition of antibody formation when they applied large cytotoxic doses. This explains why there was no parasitaemia after three days of infection in all the strains when 1000 rads were administered in the current work.

From the foregoing exposition it is not difficult to understand why it is rare to obtain total suppression of immunity. It is because of the lack of total suppression of immunity by any agent and because the amount suppressed depends on the agent used, the amount administered, and the time it is administered, that it is preferable to designate the immunity-inhibiting agents as immunodepressants rather than immunosuppressants and their effect as immunodepression instead of immunosuppression.

3.2.3 The Patterns of Parasitaemia

3.2.3.1 The patterns of normal infection

In the study of strains of *T. brucei*, Ashcroft (1957) found that while Maswa 1 gave continuous increase
of parasitaemia until the host's death, Sakwa 3 gave relapsing parasitaemia. He also found that there was generally a decrease of the stumpy form as parasitaemia rose in Sakwa 3. He described Maswa 4 as giving parasitaemia of slender form because the amount of slender form increased with increase in total parasitaemia. The same worker in the same year also observed that in Tinde strain of *T. rhodesiense* the total parasitaemia rose sharply before the host died and this sharp rise was associated with a flush of slender form. Wijers (1959) working on *T. gambiense* found that a virulent strain in rat gave an increase in the slender form as the total parasitaemia increased. Similar result was obtained with the same strain in monkey although with less virulence exhibited. Petana (1964), in his work with *T. gambiense* found that his results were similar to those of Ashcroft (1957) and Wijers (1959) in that slender form was predominant throughout the duration of the infection. With *T. rhodesiense* he also recorded that while both the stumpy and slender forms occurred throughout the lifespan of the hosts, the intermittent pattern of parasitaemia was characterised by an increase of slender form during the relapses. His *T. brucei* strain also gave a predominance of slender forms in the virulent infection. He described the virulent strain of *T. congoense* as having a predominance of slender forms in the parasitaemia.

Luckins (1972) working with 181 strain from
Botswana and which was virulent in his laboratory found that slender form was predominant at the start and during increase in parasitaemia. Balber (1972) similarly found a preponderance of slender form during the first 72 hours but the form decreased between 96 and 140 hours when the stumpy form slightly increased with a decrease in total parasitaemia. All these workers adopted measurement method in qualifying the parasitaemia and it is only assumed that in most cases their slender form could by and large be equated to the agranular and the stumpy to the granular.

Ormerod et al. (1963, 1974) adopting the agar technique threw more light on the characteristics of 181 strain. While they agreed with Luckins (1972) on the increase of the agranular forms whenever the total population increased they demonstrated that 181 like the Kenya-Uganda strains (Ormerod et al., 1963) produced parasitaemias essentially maintained by granular form.

The work recorded in this thesis confirms that there is generally an increase in the agranular forms, whenever there is a relapse in a pleomorphic infection, but it also shows that a strain can maintain the parasitaemia either by granular or by agranular forms; thus Botswana (180) and Zambia I (83) strains are designated as having parasitaemias essentially of agranular forms while Zambia II (57) conforms with the other unspecified Kenya-Uganda strains of Ormerod et al. (1963). The work
further differentiates 180 from 181. Though both strains were isolated from patients in the same country, but from different localities, their parasitaemia compositions are different. The former is essentially agranular but the latter granular.

Although the three strains examined in this thesis are all *T. rhodesiense*, the high parasitaemia of Zambia I (83) makes it, on the one hand, as being more virulent than the other two strains (Botswana and Zambia II) which, on the other hand, showed more of a tendency to relapse. Thus the Zambia I strain of this thesis resembles Maswa 1 of Ashcroft, the *T. brucei* of Petana (1964) and 181 in the laboratory of Luckins (1972), while the Botswana and Zambia II strains of this thesis resemble Sakwa 3 of Ashcroft (1957), *T. rhodesiense* strain of Petana (1964) and *T. brucei* of Balber (1972).

Although in the work of this thesis the Zambia I strain is regarded as a virulent strain with progressive increase in total parasitaemia as shown in Figure 3a that does not mean that "peaks" and "troughs" do not occur. But since the peaks are not sharply pointed and the troughs are not deep, the overall appearance suggests a uniformly high parasitaemia rather than a relapsing infection. The identification of remissions and relapses in 181 by Ormerod *et al.* (1974) which Luckins (1972) failed to register might not only be due to the difference in virulence of the same strain but also to difference in techniques in identifying the forms and quantifying parasitaemia.
The work of this thesis shows that the characteristic patterns of individual forms and of their numbers as measured by the agar method were not only demonstrated more clearly but indicated the underlying pleomorphism; how and why the population changes actually occurred, that is, whether they were due to division of trypanosomes in the bloodstream, or to the entry into the blood of new forms. It will be shown later (in Chapter 8) how the pattern given by the Botswana (180) was used to predict the existence and the amount of the occult forms.

3.2.3.2 The effect of immunodepression on the patterns of parasitaemia

Both Balber (1972) and Luckins (1972) applied x-irradiation for enhancing parasitaemias of African trypanosomiasis. The results of this thesis confirm the sustained enhancement of total parasitaemia observed by both workers. But this work goes further by giving greater detail, described in Section 3.1.5 about the quality of the various forms.

Ashcroft (1957, 1959), Petana (1964) and Luckins (1972) administered cortisone for enhancement of the parasitaemia and monitored each parasitaemia throughout the lifespan of the host. But, as has been pointed out in Section 3.2.1, their method of assessing pleomorphism was inadequate for their purpose. Ormerod et al. (1974)
adopted the agar method for their assessment when they used busulphan for trypanosome immunodepression, but they monitored the parasitaemia only for the first 168 hours. The present work is the first record of the pleomorphism of parasitaemias, in normal infections and after immunodepression, assessed from the onset of experiments until the death of the hosts.

Ashcroft (1957) showed an increase in the percentage of slender forms when cortisone was administered. Petana (1964) reported that cortisone decreased both the prepatent period and the lifespan of hosts. Luckins (1972) claimed that low doses of cortisone had transient effect on the enhancement but the enhancement thus produced was prolonged.

Petana (1964) reported that the stumpy form was present up to the third day after inoculum had been introduced but disappeared thereafter. But Luckins (1972) emphasised the continuous existence of the stumpy form throughout infection.

The work discussed in this thesis follows that of Ashcroft (1957) by showing that an increase in the percentage of the agranular form precedes an overall rise in parasitaemias, the percentage being higher at the ascent and lower at the descent of any peak whether in the normal or enhanced parasitaemia, because as shown in Figure 13a even when the total population may fail to show it the different forms in parasitaemia reveal an additional
stage coming from the tissues (see Chapter 8). So it goes further in associating some of the increase to the flushes of the agranular form to a source other than the bloodstream.

The work of this thesis also confirms the transient nature of the effect of corticosteroids noted by Luckins (1972) and explained by Cox (1968), and it goes further to show that the optimum single dose of dexamethasone for inflicting maximum immunodepression is 20 mg/kg. The reduction in the lifespan of hosts due to its toxicity is demonstrated at the highest does of 40 mg/kg in all the strains but most pronounced in the Zambia II strain where the hosts died within six days.

The difference in the observations of Petana (1964) and Luckins (1972) might be due to method adopted for qualifying pleomorphism and if the agar technique had been used in both cases the discrepancy might have been cleared. From the various pictures presented in this work many granular trypanosomes, especially those of Botswana and Zambia I strains, could have been classified, on measurement technique, as slender and on such occasions the populations could have been pronounced as lacking the stumpy forms. Indeed it is more important to be able to classify the physiological state of the trypanosome rather than its length. Not only were such mistakes avoided in this work an understanding of why not all granular forms may be stumpy is gained. From the patterns of
parasitaemia it has been shown on the one hand that Zambia II strain depicts a parasitaemia essentially maintained by the granular form and so there is time for maximum growth of the individual to the stumpy characteristics. On the other hand Botswana and Zambia I strains are essentially parasitaemias of agranular form and possibly the granular forms are removed much earlier than in Zambia II strain. So it could be seen how easy it was possible for Petana to register many granular, but thin-looking, forms as slender in the fast growing population under immunodepression. It is also important to note that as Petana (1964) was able to show many instances in which the stumpy form was seen before the end of the pre-patent period, similarly the present work registers many instances when the granular form was found before the prepatent period was over. It is possible that these forms are part of the inoculum still lingering on in the blood before the first flush of the agranular forms occurred, as suggested by Ormerod (1958).

That the production of antibodies and consequently the availability of the antigenic variations could be affected by immunodepression was established by Dixon and McConahey (1963), Cox (1968) and Luckins (1972). That the amount of immunodepression caused and whether it would be transient or sustained depend upon the type, time and amount of immuno-inhibiting agent used has also been stressed by the same workers. Although this work
does not set out to prove any relationship between antigenic variations and peaks of parasitaemia, the general synchronisation of variation with the occurring peaks both to normal and immunodepressed infections was too clearly defined for the possibility of their being related to be ignored. This relationship, however, indicates the possibility of a link between the tissue phase (later demonstrated in Chapter 8) and the flushes of agranular forms in the vascular system. It thus agrees with the predictions of Seed and Effron (1973) who speculated that new antigenic variations might be related to tissue forms. Indeed the explanations of Hudson and Terry (1979) who implied unusual anti-VAT responses might still be right in spite of the divergence between their hypothesis on the nature of antigenic variation and that which is usually accepted.

Further division of agranular forms into the long-narrow and long-flat forms adds little information to the bulk agranular population. It has been expected that the occurrence of the long-narrow form will be predominant when peaks are rising especially when the parasitaemia is enhanced by immunodepression. This has not been so. Long-narrow forms never exceed ten per cent of total population even during the flushes and the agranular population is mainly composed of the long-flats.

The discussion on the number and location of the granules is deferred until later (Chapter 6).
4.1 Results

4.1.1 The Peritoneal Fluid  (See 2.4.1)

The fluid extracted from the peritoneal cavities of infected rats presented a wide range of forms. The usual forms of long-narrow, long-flat, normal granular, giant and tadpole forms were all represented. But in addition to those were all different shapes and sizes of spherical forms or round bodies. Some difficulty arose as to how all the different shapes and varieties should be classified. Plates 4c-e give some of the types studied with agar techniques. Clearly it was possible that some of the bizarre shapes could have been due to distortion of normal forms squashed between the agar and coverslips. Giemsa-stained and Diff-quick preparations were therefore made and studied carefully (Plate 5 and 6). The presence of the spherical bodies and other variations in forms and sizes was also confirmed by the study of forms from peritoneal fluid by scanning electron microscopy (see Chapter 7).
PLATE 4  Peritoneal and Ascitic fluids

(a) Blood corpuscles of wet preparation demonstrating leucocytosis in blood of mouse

(b) Blood corpuscles on agar demonstrating leucocytosis in blood of mouse

(c) - (e)
   Different forms of trypanosomes in peritoneal fluid on agar
PLATES 5 AND 6  Different forms of trypanosomes in peritoneal fluid - Giensa stained

(5a) Long-narrow form
(5b) Normal granular form
(5c) Normal dividing form
(5d) Trypanosomes in multiple division process

(5c and 5f)
Trypanosomes each with flagella at both ends

(5g) Dividing kinetoplasts and flagella

(5h and 5i)
Trypanosomes difficult to categorise amastigote, promastigote, epimastigote or trypomastigote in dividing stages

(6a - c)
Round-bodied trypanosomes

(6d - f)
Different types of tadpole form

(6g - h)
Giant forms with several kinetoplasts, flagella and dividing nuclei
in flagella
and flagella
to categorise
mastigote in
omes
dpole form
oral kineto-
dividing
4.1.2 Ascitic Tumour Cells in Rats (See 2.4.2.1)

All experiments undertaken to grow the ascitic tumour cells in the peritoneum of rats failed. It could be rightly concluded that these 180 TG tumour cells would not grow in rats; but they were prolific in mice.

4.1.3 Ascitic Fluid in Mice (See 2.4.2

The time taken for a well-extended peritoneum filled with ascitic fluid depended on the number of tumour cells inoculated, whether the fluid was fresh or had been kept at -87°C. When 0.2 ml of fresh supply of ascitic fluid was given to each of the experimental mice the peritoneum became sufficiently swollen by two weeks. When the same amount was taken from the freezer enough fluid was produced in three weeks. When each mouse was given 0.5 ml of fresh fluid 60% of the mice became swollen by day 8. When each of two mice had been given 2 ml of fresh fluid, enough fluid was produced to be harvested by day 6. Further increases in the amount of inoculum of fluid did not produce swellings more quickly. The amount harvested varied from 5 ml to 20 ml per mouse. The average amount harvested after two weeks was 10 ml.

4.1.3.1 The forms of parasite in the fluid

The forms located in the ascitic fluid were
similar to those found in normal peritoneal fluid (Plates 5 and 6). The main difference lay in the quantity of fluid and therefore the number of parasites available. While it was not possible to obtain more than a drop or two of normal peritoneal fluid from a rat up to 20 mls of ascitic fluid with thousands of trypanosomes could be harvested from a single mouse.

4.1.3.2 Parasitaemia of trypanosomes in mice with ascites tumours

It was found that the number of parasites in the blood was more than in normal mice. The first set of experiments gave the following parasitaemia per field on the agar slide (Table 3).

Table 3 showing the parasitaemia in mice infected with ascitic fluid and trypanosomes sixteen days later

<table>
<thead>
<tr>
<th>Forms</th>
<th>Day*4</th>
<th>Day 6</th>
<th>Day 8</th>
<th>Day 10</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-narrow</td>
<td>11</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Agranular</td>
<td>23</td>
<td>42</td>
<td>58</td>
<td>52</td>
<td>8</td>
</tr>
<tr>
<td>Granular</td>
<td>16</td>
<td>37</td>
<td>20</td>
<td>48</td>
<td>136</td>
</tr>
<tr>
<td>Total parasites</td>
<td>39</td>
<td>79</td>
<td>78</td>
<td>100</td>
<td>144</td>
</tr>
<tr>
<td>No. of granules per tryp.</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

N.B. From a mouse dissected at Day 5 all the forms recorded above including giant and spherical forms were found in the ascitic fluid drawn out.

* Day number refers to the day of examination after the parasites had been inoculated.
Table 4 confirmed the initial speculation that the parasitaemia in the ascitic fluid and trypanosome-infected mice was highly enhanced.

Table 4. Blood parasitaemia of ascitic fluid infected and control mice

<table>
<thead>
<tr>
<th></th>
<th>Day 5</th>
<th></th>
<th>Day 6</th>
<th></th>
<th>Day 7</th>
<th></th>
<th>Day 8</th>
<th></th>
<th>Day 9</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Exp. AF</td>
<td>Control</td>
<td>Exp. AF</td>
<td>Control</td>
<td>Exp. AF</td>
<td>Control</td>
<td>Exp. AF</td>
<td>Control</td>
<td>Exp. AF</td>
</tr>
<tr>
<td>Long-narrow</td>
<td>1</td>
<td>NIL</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Agranular</td>
<td>5</td>
<td>NIL</td>
<td>24</td>
<td>6</td>
<td>40</td>
<td>4</td>
<td>18</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Granular</td>
<td>1</td>
<td>NIL</td>
<td>1</td>
<td>1</td>
<td>13</td>
<td>9</td>
<td>57</td>
<td>4</td>
<td>45</td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>NIL</td>
<td>25</td>
<td>7</td>
<td>53</td>
<td>13</td>
<td>75</td>
<td>4</td>
<td>70</td>
</tr>
</tbody>
</table>

Both Tables 3 and 4 indicated that the lifespan of mice infected with ascitic fluid and later inoculated with trypanosomes (Botswana strain) was shorter than two weeks. Parasitaemia was essentially maintained by the agranular forms in the early stages but later the proliferation of granular forms increased to 76%. The average number of granules per trypanosome remained three or four (See Table 3).

4.1.3.3 Forms in the blood

The forms noted were similar in both the normal peritoneal fluid of rats and the ascitic tumour fluid of
mice (see Table 6), but it was significant that although
the tadpole forms were frequently seen, spherical bodies
were rare.

When mice were infected with trypanosomes the
same day as they were inoculated with ascitic fluid the
parasitaemia from both experimental and control animals
was the same as in a normal infection (Fig. 2a in Chapter
3).

4.1.3.4 The white blood corpuscles

It was observed (Plates 4a and b) that mice
with ascites tumour cells produced massive numbers of
white blood cells in the blood whether or not they were
infected with trypanosomes. The normal trypanosome-
infected mice had an average ratio of 650 red cells to
one white cell but a mouse infected with trypanosomes
and ascitic tumour fluid had a ratio of 40 to 1.

Differential white cell counts were in Table 5
with counts for man for comparison. Although the counts
for man differed substantially between the number of
lymphocytes in man and those in mice, there was no sig-
nificant difference between the mice infested with
ascites tumour cells and the normal or control mice.
However the absolute white cell counts showed that
polymorphs, lymphocytes and monocytes were all raised
more than 3-fold by the ascites tumour.
Table 5. Differential counts (% means) of white blood cells in mice with ascitic fluid infection and normal mice

<table>
<thead>
<tr>
<th></th>
<th>Polymorphs</th>
<th>Eosinophils</th>
<th>Basophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascitic fluid-infected mice</td>
<td>48.5</td>
<td>0</td>
<td>0.5</td>
<td>46</td>
<td>5.0</td>
</tr>
<tr>
<td>Control mice</td>
<td>45</td>
<td>0</td>
<td>1</td>
<td>49</td>
<td>4.5</td>
</tr>
<tr>
<td>Human</td>
<td>40-75</td>
<td>1-6</td>
<td>0-1</td>
<td>25-30</td>
<td>5-10</td>
</tr>
</tbody>
</table>

4.1.3.5 Comparison of forms from different locations

Table 5 summarises the layout of the different forms found in blood and peritoneum. All sites contained the normal trypanosomes whether granular or agranular and these form the majority, often as much as 97% of the population. Dividing forms were also universal. But while they were as low as two per cent in parasitaemia dominated by the granular forms they were as high as 9% in the blood of mice infected with ascitic fluid. Peritoneal and ascitic tumour fluids also gave high proportions of dividing forms. In each case they were about 8%.

The occurrence of rarer forms was particularly interesting. Moribund forms could not be seen in blood when agranular forms were abundant, in the blood of mice swollen with ascites tumour cells, the normal peritoneal fluid and the ascitic fluid itself. That did not mean they never occur in these media. In fact they had been
Table 6. Occurrence of forms in the blood and body fluids

<table>
<thead>
<tr>
<th>Media</th>
<th>Forms of Parasites (in percentages)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal trypomastigotes</td>
</tr>
<tr>
<td>Normal mouse blood parasitaemia at 96 hours</td>
<td>93.0</td>
</tr>
<tr>
<td>Normal mouse blood parasitaemia at 2 weeks</td>
<td>97.0</td>
</tr>
<tr>
<td>Peritoneal fluid from rat infected with tryps.</td>
<td>89.0</td>
</tr>
<tr>
<td>Ascitic fluid from mouse infected with tryps.</td>
<td>88.5</td>
</tr>
<tr>
<td>Blood parasitaemia in mouse with ascitic fluid</td>
<td>89.0</td>
</tr>
</tbody>
</table>
registered in the normal infections of mice and rats (see Chapter 3) but they were very rare. In the blood where the agranular forms were common the moribund form was recorded within the two hundred parasites counted in this experiment (Table 6). Giant and tadpole forms were found in all sites except where the population was ageing and granular forms predominated. The spherical forms as noted earlier were many in the ascitic tumour fluid and in normal peritoneal fluid.

4.2 DISCUSSION

4.2.1 Types of Extravascular Forms

The writer recognises two main types of extravascular forms of *T. brucei* and bases his classification on their probable origin. The first type is found in extra-vascular fluid such as the ascitic and peritoneal fluids. The second type comprises the tissue or occult forms, which are situated in the tissues of internal organs. As in any biological categorisation, nature does not often allow watertight compartments. While it is unlikely to be serious argument as to whether the peritoneal forms should be classified as "fluid" forms, it is open to question whether both types can be found in the tissues. These initial comments on the extra-vascular forms will be limited to the fluid type;
the consideration of similarities and differences between the fluid and tissue types will be further treated in Chapter 8.

4.2.2 The Forms Found in the Ascitic and Peritoneal Fluids

Ormerod (1979a) in discussing the lymphatic spread associated with trypanosomiasis stressed the need for further studies on the trypanosomes found in body fluids other than the vascular system. This part of the current study is a response to this call. Although similar forms were also noticed in the pericardium, effort was concentrated on the peritoneal fluid because it was difficult to exclude the possibility of leakage of blood trypanosomes into the pericardial cavity from the pericardium area when one might be sure that the heart itself was not punctured.

The use of the ascites tumour (see Results, section 4.1.3) has been mainly for the enhancement in the production of peritoneal fluid in which the parasites were growing prolifically. Its special features will be discussed separately (4.2.4). Suffice it to note here that all the forms found in the ascitic tumour fluid in mice also tend to be in the peritoneal fluid of normal rats.
4.2.2.1 The giant and dividing forms

Peritoneal fluid, especially at the early stages of infection between 60 and 72 hours, produces a wide range of forms which have been exhibited in this study. The absence of the moribund forms during this phase of growth indicates that the trypanosomes are in active multiplication and there is little degeneration going on. Giant forms of various types with either multiple flagella, nuclei or kinetoplasts are also found. This suggests that a high rate of multiplication is taking place. The giant trypanosomes as demonstrated in the photographs, are similar to the forms seen by Ottolenghi (1910). But while Ottolenghi would like to regard these as macro-gametocytes or products of sexual fusion, this writer does not have any evidence of sexual exchanges, in spite of the variation in sizes between these big forms, the small long-narrow forms and sphaeromastigote which are also present in the fluid.

A higher percentage of the dividing forms in the normal longitudinal binary fission is present in both ascitic and normal peritoneal fluids than in the blood population. This tendency further enhances the belief that the peritoneum is a medium for fast multiplication and growth. This work thus agrees with the finding of Ssenyonga and Adam (1975) who observed, in their study of T. brucei, more dividing forms in the lymph, of the thoracic duct which drains the peritoneal cavity,
than in the blood. It is therefore possible to infer that extravascular fluids promote a greater rate of multiplication and development than the vascular system.

4.2.2.2 The "round" bodies

The occurrence of the round bodies in trypanosomiasis had been reported by several authors. Bradford and Plimmer (1902) reported such forms in *T. brucei* and Walker (1912) in *T. evansi*. The latent bodies of Salvin-Moore and Breinl (1908) located in spleen and lungs were said to be spherical. In human trypanosomiasis other authors who have noted such bodies include Buchanan (1911), Fantham (1911), Laveran (1911), Mott and Stewart (1907), Vianna (1911) and Santana (1913). An up-to-date review on the latent bodies is given by Ormerod (1979a). But it must be pointed out that while some workers believed these spherical bodies to be degenerate forms others would argue that these forms known to have produced new parasitaemia were resistant forms. Laveran (1911) and Santana (1913) belonged to the first school of thought. Salvin-Moore and Breinl (1908) and Fantham (1911) belonged to the second. The work of Soltys and Woo (1969) and Soltys *et al.* (1969) referred to not only the existence of amastigote-promastigote- and epimastigote forms but also showed that trypanosomes as small as 0.8 μ existed and were infective on sub-passage.
In all the above works there was no isolation of trypanosomes from the fluids but the organisms were usually in a mixture of tissues and fluids. Ottolenghi (1910), however, demonstrated some of these spherical bodies from the peritoneal fluid, and the results of the forms shown in the current thesis confirm this. It is also noted in the work of this thesis that some of the spherical bodies are multinucleate and yet much smaller than the normal giant forms and it was considered possible that these forms might get carried into the vascular system, continue to grow and later complete their multiple-division.

These may be the occasional giant forms seen in the blood. The size and shape that the rounded bodies assume in the peritoneal fluid is immaterial to their local development. They may be tadpole form, or amastigote, epimastigote or promastigote in the arrangement of the nucleus and kinetoplasma. It is not certain whether these round bodies are products of budding off from the bigger giant forms or the beginning of budding in multiple division started in the extravascular fluid but continued in the blood. Their significance, however, appears to be the generation of the forms found in the blood but initiated outside the vascular system.

4.2.2.3 The normal granular and agranular forms

The population in the peritoneal and ascitic
fluids is pleomorphic. Type II granules (Ormerod, 1963) shown as punched out holes in the cytoplasm in the Giemsastain preparation and as black dots in the agar films confirm the presence of the granular form. This work thus corrects the less clearly defined observation of Sseyonga and Adam (1975) who reported the absence of stumpy form in the lymph. Barry et al. (1980) in their recent Cambridge demonstration confirm the results reported in this thesis by showing evidence of pleomorphic trypanosomes carried in the lymph. The need for uniform and reliable data for assessing pleomorphism once again needs to be stressed.

Both the long-flat and long-narrow forms of agranular trypanosomes are present in the fluid, the thinnest long-narrow form (Plate 5a) being found here. Because of the occurrence of long-narrow form in the blood as well as in the fluid where multiple-division was going on in each case, makes it possible to associate long-narrow form with multiple-division. Further comment will be reserved until Chapters 8 and 9.

4.2.3 The Role of Peritoneal Fluid

Like any other extravascular fluid such as the lymph, the peritoneal fluid appears to be a medium for fast production of the giant forms which rapidly multiply to give rise to normal trypanosomes. Most of the giant
forms are too big to pass into the blood and their multiplicative phase is restricted to this site. But the presence of the round bodies, many of which are also multinuclear and which can pass easily into the vascular system ensures that some multiple division can continue in the blood. Multiple division forms are observed in the blood to a rate of 2%.

How the normal granular and agranular trypanosomes and the round bodies get into the blood is still uncertain. Barry et al. (1980) implicates the migration of the trypanosomes through the local lymph node as a major route for the systemic dissemination of T. brucei. Whether the trypanosomes pass from the peritoneal fluid into the blood via the lymph or directly into the capillaries or both pathways are followed, future work will tell.

4.2.4 The Special Features of Ascitic Fluid

There is no former record known to the writer where either the trypanosomes have been massively grown in vivo as in the ascitic fluid or where the effect of the tumour cells 180 TG (or any other tumour cells for that matter) on the blood parasitaemia in trypanosomiasis has been registered.

The obvious advantage in using the ascites cells is in the increased production of the peritoneal
fluid in mice. Where a normal infected mouse failed to give up to 0.1 ml of peritoneal fluid one could harvest 20 ml of ascites fluid with trypanosomes. Consequently more parasites were harvested and studied as peritoneal fluid from where number of parasites counted. This study shows that there is no difference in the range of forms and their characteristics whether the parasites are derived from the normal peritoneal fluid or ascites tumour fluid.

As regards the immune competence of the hosts, a significant observation was made by Sartorelli et al. (1966) who showed that Sarcoma 180/TG used in this study to give an ascitic fluid which was rich in antibody and that the quantity of ascitic fluid antibody compared favourably with rabbit serum prepared over a similar brief period. One would have expected that an immune animal should show inhibition of parasitaemia but the opposite happened. As shown in the results, parasitaemia was enhanced. The hyperimmunity and the enhanced parasitaemia were associated with a leucocytosis. The prolific multiplication of trypanosomes may be related to the growth of the tumour and that the immune system appears to have been over-stretched. Hudson and Tetley (1979) showed that the amount of antibodies measured by IgM titres did not constitute a limiting factor in producing new populations of trypanosomes. Barry et al. (1980) also found that "despite the production of antibody
specific for the emergent trypanosome VATs the response lacks the expediency to suppress completely the appearance of these VATs in the peripheral circulation and hence the infection becomes established". This therefore indicates that the factors controlling the production of the new VATs may not be in the blood or the extravascular fluids but in the tissue as discussed in Chapter 8.
CHAPTER 5

PRODUCTION AND STUDY OF THE EARLY FORMS IN THE BLOODSTREAM

5.1 RESULTS

5.1.1 The Forms Derived from Tsetse Transmission Experiments (See 2.5.1)

From the cyclically transmitted parasitaemia no trypanosome with the characteristics of a long-narrow form could be observed in the early population although observation started 24 hours after the inoculum had been introduced. However all the parasites in this first wave of occurrence were agranular. The development of granular forms and sequence of parasitaemia was as in normal infection of Chapter 3.

5.1.2 The Forms from the Cultures (See 2.5.2)

On the two occasions when trypanosomes from the culture media were studied none of the parasites seen could be designated long-narrow. They were all agranular but more long-flat in appearance.

5.1.3 Salicylhydroxamic Acid (SHAM) Experiments and the Forms Derived (See 2.5.3.)

5.1.3.1 Preliminary experiments

The first experiment carried out on three strains
of *T. rhodesiense* from Botswana, and Zambia, and on one of *T. gambiense* (Bida 3) from Nigeria showed that all the three strains of *T. rhodesiense* had a relapse of parasites detected in blood after treatment. *T. gambiense* strain did not show recrudescence. Two repeats of such experiments confirm that Bida 3 was cured by a single dose of SHAM with glycerol. A check on the treated hosts eight months after treatment still gave no parasitaemia in the blood and affirmed that the cure had been permanent.

5.1.3.2 Recrudescence in the three strains of *T. rhodesiense*

Table 7 was the layout of the findings from the series of experiments carried out. Recrudescence occurred in Botswana strain between day 5 and 9 after treatment. It was between day 5 and 12 in Zambia I and day 5 and 17 in Zambia II strain.

In all the strains treatments at both low parasitaemia on day 6 and high on days 12 or 14 resulted in recrudescence. There was generally high mortality with the treatment. The lowest mortality was 400 mg/kg of SHAM and 4 g/kg of glycerol as shown in rats code numbers A1, A2 and C1 in Table 7. The highest mortality was when 500 mg/kg SHAM was combined with 8 g/kg of glycerol - see A4, B4, B8 and C8. It was particularly significant that nearly all hosts died whenever the level of dose of
<table>
<thead>
<tr>
<th>Strain Code</th>
<th>Pre-patent period</th>
<th>Day of treatment (after inoculum introduced)</th>
<th>Parasitaemia (per field) at treatment</th>
<th>Treatment</th>
<th>Glycerol (g kg(^{-1}))</th>
<th>No. of rats survived/No. of rats used</th>
<th>Recrudescence (days after treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>4</td>
<td>6</td>
<td>1.0</td>
<td>400</td>
<td>4</td>
<td>2/3</td>
<td>7, 8</td>
</tr>
<tr>
<td>A2</td>
<td>4</td>
<td>6</td>
<td>1.5</td>
<td>400</td>
<td>8</td>
<td>2/3</td>
<td>6, 9</td>
</tr>
<tr>
<td>A3</td>
<td>4</td>
<td>6</td>
<td>0.5</td>
<td>500</td>
<td>4</td>
<td>1/3</td>
<td>8</td>
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<td>2.0</td>
<td>500</td>
<td>8</td>
<td>0/3</td>
<td>-</td>
</tr>
<tr>
<td>A5</td>
<td>4</td>
<td>14</td>
<td>45.0</td>
<td>400</td>
<td>4</td>
<td>1/3</td>
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<td>30.0</td>
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<td>8</td>
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<td>-</td>
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<td>A7</td>
<td>5</td>
<td>14</td>
<td>42.0</td>
<td>500</td>
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<td>6, 7</td>
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<td>A8</td>
<td>4</td>
<td>14</td>
<td>28.0</td>
<td>500</td>
<td>8</td>
<td>1/3</td>
<td>5</td>
</tr>
<tr>
<td>B1</td>
<td>3</td>
<td>6</td>
<td>9.0</td>
<td>400</td>
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<td>1/3</td>
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<td>1/3</td>
<td>12</td>
</tr>
<tr>
<td>B3</td>
<td>5</td>
<td>6</td>
<td>3.0</td>
<td>500</td>
<td>4</td>
<td>1/3</td>
<td>5</td>
</tr>
<tr>
<td>B4</td>
<td>5</td>
<td>6</td>
<td>8.0</td>
<td>500</td>
<td>8</td>
<td>0/3</td>
<td>-</td>
</tr>
<tr>
<td>B5</td>
<td>4</td>
<td>12</td>
<td>48.5</td>
<td>400</td>
<td>4</td>
<td>0/3</td>
<td>-</td>
</tr>
<tr>
<td>B6</td>
<td>5</td>
<td>12</td>
<td>40.0</td>
<td>400</td>
<td>8</td>
<td>2/3</td>
<td>11, 12</td>
</tr>
<tr>
<td>B7</td>
<td>4</td>
<td>12</td>
<td>32.0</td>
<td>500</td>
<td>4</td>
<td>2/3</td>
<td>8, 10</td>
</tr>
<tr>
<td>B8</td>
<td>4</td>
<td>12</td>
<td>46.0</td>
<td>500</td>
<td>8</td>
<td>0/3</td>
<td>-</td>
</tr>
<tr>
<td>C1</td>
<td>4</td>
<td>6</td>
<td>2.5</td>
<td>400</td>
<td>4</td>
<td>3/3</td>
<td>7, 8, 12</td>
</tr>
<tr>
<td>C2</td>
<td>4</td>
<td>6</td>
<td>4.5</td>
<td>400</td>
<td>8</td>
<td>1/3</td>
<td>7</td>
</tr>
<tr>
<td>C3</td>
<td>4</td>
<td>6</td>
<td>4.0</td>
<td>500</td>
<td>4</td>
<td>2/3</td>
<td>7, 9</td>
</tr>
<tr>
<td>C4</td>
<td>4</td>
<td>6</td>
<td>3.5</td>
<td>500</td>
<td>8</td>
<td>1/3</td>
<td>5</td>
</tr>
<tr>
<td>C5</td>
<td>4</td>
<td>14</td>
<td>32.0</td>
<td>400</td>
<td>4</td>
<td>2/3</td>
<td>7, 8</td>
</tr>
<tr>
<td>C6</td>
<td>4</td>
<td>14</td>
<td>49.0</td>
<td>400</td>
<td>8</td>
<td>1/3</td>
<td>17</td>
</tr>
<tr>
<td>C7</td>
<td>5</td>
<td>14</td>
<td>28.5</td>
<td>500</td>
<td>4</td>
<td>2/3</td>
<td>5, 7</td>
</tr>
<tr>
<td>C8</td>
<td>4</td>
<td>14</td>
<td>41.5</td>
<td>500</td>
<td>8</td>
<td>0/3</td>
<td>-</td>
</tr>
</tbody>
</table>
glycerol was 8 g/kg. A4, A8, B4, B8, C4 and C8 illustrated this point.

Animals with high parasitaemia such as A5, B8, C6 and C8 usually died with either 400 mg/kg or 500 mg/kg of SHAM. In a few cases (like B1) treatment resulting in mortality might not be wholly due to the effect of drug but probably a deficiency in handling and nursing. The amount of SHAM required was high though based on established doses (Oppendoes et al., 1976; Evans and Holland, 1978; Evans and Brightman, 1980).

5.1.3.3 Treatment within- and after prepatent period

The results of treatments were conveyed clearly by Table 8. Treatment given after six hours of infection on the same day cured all hosts infected with Botswana and Zambia II strain. Recrudescence occurred in one animal with Zambia I strain. Treatments on days 2 and 3 were successful in all cases. Zambia I and II strains also responded to treatment given on the fourth day. Botswana showed a relapse. Treatments given two days after the prepatent period of Botswana and Zambia II were, as expected, not successful and recrudescence occurred. But Zambia I failed to show any relapse to treatment given on day 5 which was a day after the prepatent period.
**Table 8. Treatment before and immediately after parasitaemia established**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Prepatent period</th>
<th>Day of treatment (after inoculum introduced)</th>
<th>Treatment</th>
<th>Recrudescence (days after treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SHAM (mg kg(^{-1}))</td>
<td>glycerol (g kg(^{-1}))</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td>Botswana</td>
<td>-</td>
<td>1</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>3</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td>Zambia I</td>
<td>-</td>
<td>1</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>2</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td></td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td>Zambia II</td>
<td>1</td>
<td>1</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>400</td>
<td>4</td>
</tr>
</tbody>
</table>
|               | 5                | 5                                          | 400       | 4                                    | Day 8.                               

5.1.3.4 *Multiple doses applied intermittently (after recrudescence)*

This experiment consumed many animals because hosts that died in the course of experimentation were
ignored and experiment repeated until at least one animal with each strain of parasites survived the three doses of drug, each given after a recrudescence was detected. The final picture was presented in Table 9. All the three strains continued to show recrudescence.

Table 9  Multiple doses of SHAM applied intermittently (after a recrudescence)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Day of treatment (after inoculum introduced)</th>
<th>Treatment</th>
<th>Recrudescence (days after treatment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SHAM (mg kg⁻¹)</td>
<td>Glycerol (g kg⁻¹)</td>
</tr>
<tr>
<td>Botswana</td>
<td>6</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>Zambia I</td>
<td>18</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>Zambia II</td>
<td>22</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>500</td>
<td>4</td>
</tr>
</tbody>
</table>

5.1.3.5  Multiple doses applied consecutively

No animal with established parasitaemia treated thrice in three consecutive days survived. This was common to all the three strains used as shown by Table 10.
Table 10  Multiple doses applied in three consecutive days

<table>
<thead>
<tr>
<th>Strain</th>
<th>Day of treatment (after inoculum introduced)</th>
<th>Treatment</th>
<th>SHAM (mg kg⁻¹)</th>
<th>Glycerol (g kg⁻¹)</th>
<th>No. of host survived/ Recrudescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botswana</td>
<td>6 7 8</td>
<td></td>
<td>500</td>
<td>4</td>
<td>0/4</td>
</tr>
<tr>
<td>Zambia I</td>
<td>6 7 8</td>
<td></td>
<td>500</td>
<td>4</td>
<td>0/4</td>
</tr>
<tr>
<td>Zambia II</td>
<td>6 7 8</td>
<td></td>
<td>500</td>
<td>4</td>
<td>0/4</td>
</tr>
</tbody>
</table>

5.1.3.6 The forms of parasites in the recrudescence and life span of the hosts

The parasites observed direct from blood by agar technique were mixtures of the long-narrow and long-flat forms. It was however noticed that usually a host was positive by modified miniature anion exchange method of Lumsden et al. (1979) two days after and at least a day before there was sufficient parasitaemia for parasites to be detected on agar and the morphology studied. Early effort failed to show any parasite from miniature column broken onto an agar slide. However after some practice two occasions yielded success. At each of such occasions a parasite was obtained on agar from the column. The parasite at one of these times was designated long-narrow but there was some doubt about the identity of the other.

After recrudescence the pattern of parasitaemia
was characteristic of the normal infection of the strain (see Figures 2a, 3a and 4a). Granular forms were present in the blood between forty-eight and sixty hours after the first wave of parasites. Treatment with SHAM did not alter the number, size or position of the granules in each of the strains used. There were three to four big granules for Botswana strain, 5 - 6 small granules for Zambia I and more than ten for Zambia II.

There was a consistent impression that the life span of the hosts with any of the strains was elongated after treatment with SHAM although the treatment failed to clear parasites which eventually killed the hosts. Table 11 was constructed out of the observations made from the experiments recorded above (in Tables 7 - 9).

**Table 11 Comparison of life span of hosts**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Life span with normal infection (mean)</th>
<th>Life span with infection and x-irradiation (mean)</th>
<th>Life span of SHAM-treated hosts (mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botswana</td>
<td>35 days</td>
<td>12 days</td>
<td>54 days</td>
</tr>
<tr>
<td>Zambia I</td>
<td>21 days</td>
<td>20 days</td>
<td>27 days</td>
</tr>
<tr>
<td>Zambia II</td>
<td>31 days</td>
<td>20 days</td>
<td>54 days</td>
</tr>
</tbody>
</table>
5.2 DISCUSSION

5.2.1 The Forms from Tsetse-transmitted Infections and Cultures

No clearly identifiable long-narrow form was found in the infections derived from direct tsetse transmission. This agrees with Ormerod (1971). The absence of long-narrow form together with that of the giant forms was noted. Absence of the two categories was also observed in culture infection bearing in mind the fact that the culture trypanosomes were known to contain metacyclic forms (Brun et al., 1979) and that they were infective to mice (D.E. Evans, personal information). Further comment on the place of the long-narrow form in the life-cycle of T. brucei will be reserved until the general discussion (Chapter 9).

5.2.2 Forms Occuring during the Recrudescence of SHAM-treated Infections

Evans and Brown (1973) and Vickerman and Evans (1974) showed that salicylhydroxamic acid (SHAM) and several aryl hydroxamates inhibited aerobic respiration of bloodstream trypanosomes. But Opperdoes et al. (1976) could not register any effect of SHAM on parasitaemia of T. brucei in rodents because these trypanosomes were able to survive
by anaerobic respiration. Ryley (1962) showed glycerol to be an inhibitor of anaerobic respiration of glucose in *T. brucei* and Hammond and Bowman (1981) explained the mechanism of the glycerol inhibition of the anaerobic metabolism as a reversal of the pyruvic glycerol shunt. Clarkson and Brohn (1976) were therefore able successfully to use a combination of SHAM and glycerol to clear the bloodstream infection of *T. brucei* in rats. Nevertheless, the treatments were not permanent because recrudescence occurred within a week after each treatment. In contrast, Evans *et al.* (1977) and Evans and Holland (1978) recorded complete cure of *T. vivax* in mice when a single dose of SHAM and glycerol was given, and Evans and Brightman (1980) suggested that most monomorphic species and strains of subgenus *Trypanozoon* could be cured in this way. However the pleomorphic strains of *T. brucei* would invariably show recrudescence. In the work of the present thesis the minicolumn of Lumsden *et al.* (1979) was used to show that the bloodstream forms were temporarily cleared as long as the effect of the drugs persisted. The knowledge was employed to ensure total clearance of the vascular forms so that the location and significance of the persisting tissue forms could be investigated.
5.2.2.1 Single dose treatments and Bida 3 strain

The three strains of T. b. rhodesiense conformed to the behaviour expected of pleomorphic T. brucei according to the work of Evans and Brightman (1980). Bida 3 strain of T. b. gambiense did not, although by size variation and by the agar technique it was shown to be pleomorphic. Bida 3 appears to have changed from the characteristic slow growing T. b. gambiense to a fast growing strain with the very high parasitaemia observed in this project as noted previously by Thompson and Robertson (1929). Yet it has not lost apparently its infectivity to man as demonstrated by a recent laboratory accidental infection of a technician in West Germany (Gibson et al., 1980). It must therefore be assumed that Bida 3 is now blood-restricted in rodents and this would explain why it is cured without recrudescence like the monomorphic strains of Evans and Brightman.

This therefore indicates that the first alternative of the two hypotheses (Evans and Brightman, 1980) that suggests recrudescence may be due to the resistant blood form of the pleomorphic strains, may not hold. As shown in Chapter 8, recrudescence may be more related to tissue-dwelling parasites which Bida 3 and the monomorphic strains may have lost.

The new populations from the recrudescence were noted to be agranular with some long-narrow forms. Further
comments (Chapter 9) are reserved on the association of the long-narrow form with the giant forms known to be located in the choroid plexus as outlined in Chapter 8.

5.2.2.2 Multiple doses given intermittently

The same forms of agranular trypanosomes, not easy to designate long-narrow or long-flat, were observed at each recrudescence, each time a treated infection relapsed and irrespective of the number of times the treatment was administered. This observation further strengthens the argument that tissue-invasive strains exhibit recrudescence but blood-restricted strains do not. So long as the tissue forms are not destroyed by the drugs, they will produce new populations of agranular form to start a new parasitaemia in the vascular system but blood restricted infections are totally destroyed. Recrudescence will only occur after the effect of the drugs in bloodstream had been exhausted as there is no residual effect of SHAM which disappears from the blood within an hour (D. Evans, personal information).

5.2.2.3 Toxicity of the drugs

No animal given three consecutive daily doses lived to give any recrudescence. It is also true that the amount of SHAM required either at low dose of 400 mg/kg or high of 500 mg/kg will mean that an unreasonably large dose of SHAM will need to be given intraperitoneally to
man or large domestic mammals similarly. Increasing the amount of glycerol from 4 to 8 kg⁻¹ increases toxicity with no accompanied extra effect. These drugs could not therefore be expected to develop in the foreseeable future into an acceptable trypanocidal medicine for field medical or veterinary practitioners. But it has proved to be a useful research tool since chemotherapeutic value in clearing the blood of all trypanosomal forms is undoubted (see 5.13).

5.2.3 Effect of SHAM and Glycerol on the Prepatent Infections

All infections treated before the end of the prepatent period were permanently cleared except for one instance involving the Zambia I strain. Evans and Brightman (1980) found that the infections of pleomorphic T. brucei failed to become established if the animals were treated one to three days after the inoculum had been given. The results of this thesis generally confirmed their finding and the exceptional case in the first day of infection could be an experimental error. However this would mean that the drugs had failed to kill all blood parasites despite the negative result obtained with the minicolumn. Another way of explaining this anomaly might be that in this case some of the parasites were able to evade the effect of the drugs in the blood by entering the tissue faster than others which were
killed off by the drugs. It is known that in cloning experiments, one single trypanosome is sufficient to start an infection in the vertebrate host. The observed change from 4 to 8 days of prepatent period in the single-dose experiment involving the Zambia I strain would support that view in that there was need for time before a sufficient number, produced in the tissue, multiplied in the blood, would be detectable in the blood.

That the majority of cases were permanently cured can be taken to indicate that SHAM and glycerol might be used prophylactically despite the limitations on these drugs mentioned in 5.2.2.3.

The prolongation of the life-span of hosts could be explained by the hypothesis that the absence of parasites in the blood, albeit temporarily, prevents any pathological effect being produced in the vascular system. This view agrees with the view expressed by Goodwin (1970) who associated pathological effects of trypanosomes in blood to the production of Kinins rather than to the direct damage that the parasites inflict upon the host. This does not contradict that the main pathological destruction is in the brain among other tissues as evident in advanced sleeping sickness cases (Ormerod, 1970) and as more fully discussed in Chapter 8.
CHAPTER 6

LIGHT MICROSCOPY

6.1 RESULTS

The findings in Chapter 3 that the pattern of granules was characteristic of the three strains of a single subspecies of *Trypanosoma* namely *T. brucei rhodesiense* were characteristic with regard to the location, size and number. The emphasis in the present chapter will focus upon the pattern of granules in the different species. Light microscopy in conjunction with the agar technique was again used to study a species of Stercoraria in the subgenus *Herpetosoma* and a range of salivarian trypanosomes from subgenera *Duttonella*, *Nannomonas* and *Trypanozoon*. (See 2.6.1.1.c and table 1)

6.1.1 *Trypanosoma (Herpetosoma) lewisi*

An isolate of the parasite obtained from a wild *Rattus norvegicus* in England gave a wide variety of juvenile forms. These differed in shape and sizes - Plates 7a and b. On one end of the scale were the minute forms in Plate 7a as small as 10.0 μ in length by 1.5 in width. On the other were huge forms of Plate 2 measuring as much as 15.0 μ in width - there were more rounded-up than normal
PLATE 7  

T. lewisi on agar

(a) Variety of juvenile forms
(b) Round juvenile form
(c) Adult form with one granule
(d) Adult form with 2-3 granules
(e) Adult form with 2 granules
PLATE 8  T. vivax on agar

(a) Tadpole form

(b) and (c)
Typical Y58 parasites with broad profiles as distinct from Y486.
The granules are faint to see

(d) Typical Y486 parasites with granules and at high blood parasitaemia

(e) and (f)
Y486 at low parasitaema
PLATE 9 Savannah T. congolense on agar

(a) Trypanosome with seven granules

(b) Trypanosome with five granules

(c) Trypanosome with two or three granules

(d) Variation in sizes demonstrated

(e) Trypanosomes showing the prominent granule posteriorly located

(f) Trypanosome showing mainly the prominent granule

(g) Trypanosome showing another granule posterior to the prominent one

(h) A dividing trypanosome also showing the prominent granule
les
les
the granules
red
prominent
the
granule
one
showing
PLATE 10  Riverine *T. congolense* on agar

(a) TSW 99 trypanosomes, some of which had the free flagella

(b) TSW 99 trypanosomes with the granules shown to be difficult to photograph

(c) TSW 103 trypanosomes demonstrating typical *T. congolense* with no free flagellum. Note the distinct granules

(d) TSW 103 trypanosomes one of which had the free flagellum while the other had not.

(e) "*T. montigomeryi*" form
PLATE 11  *T. brucei* trypanosomes on agar

(a) 8/18 strain with the granules

(b) TSW 3 strain

(c) S/42 strain

(d) and (e)
   Treu 667 strain

(f) Liverpool (LN) strain

(g) Liverpool (LR) strain

(h) 186 strain
PLATE 12  T. brucei on agar continued

(a) 58 strain

(b) 58B strain

(c) - (e)

1198 strain

(f) LUMP 1236 strain

(g) Bida 3 strain
trypanosomes as could be found from Plate 7b. The majority of these juvenile forms were without granules but approximately one in eight had multiple granules. Because of the tubular nature of the parasite these granules were distributed in layers in the posterior end. No accurate counting could be made. Plate 7b was a representative.

Although the parasitaemia rose rapidly and could be detected late on the second or early on the third day after the inoculation, the granules were seldom visible before day 4. The juvenile forms gradually changed into the adult forms - there was no sudden transformation. Mixed populations of juvenile and adult forms were observed between days 8 and 17. When first established the adult forms were few and the parasitaemia was about one parasite per field. In contrast to the juvenile forms they were more regular and constant in shape and size as evident from Plates 7d and e. There were either one or two granules, rarely but not more than three in each trypanosome. These granules were also located in the posterior end of the trypanosome and, as in the juvenile forms, were posterior to the kinetoplast.

6.1.2 Trypanosoma (Duttonella) vivax

Two strains were obtained for the study. These were Y58 and Y486. Y58 was from Ndama cattle while Y486 was isolated from Zebu. Y58 yielded low parasitaemia
and only one or two parasites per field. It was characteristically a broad form, twice as broad as the other strain (Plates 8a - c). Tadpole forms were found (Plate 8a). The granules were numerous but faint and difficult to photograph. They were distributed equally in both sides of the nucleus (Plates 8a - c). The sizes of the granules were also varied (Plates 8a and c).

Y486 was fast-growing and it gave a higher level of parasitaemia. As Y58 it was monomorphic but it was much narrower as evident from Plate 8d compared with Plates 8a - c. The granules were found at both sides of the nucleus and this distribution remained the same whether few granules were shown as in Plate 8e or plentiful as in Plates 8d and f. The pattern for the granules also remained constant at high parasitaemia as in Plate 8d or at low levels as in Plates 8e and f.

Some parasites had two or three granules per trypanosome. The granular pattern was also unusual and resembled that of T.b. rhodesiense. These individuals were few but quite different from the majority of parasites in the population (see Chapter 7).

6.1.3 Trypanosoma (Nannomonas) congolense

Four isolates of T. (N) congolense were studied, Gamb 6 and Gamb 19 were from the African savannah area.
while TSW99 and TSW103 were from forest/riverine zone. The savannah trypanosomes were characterised by a conspicuous posterior granule. The isolates from the forest lacked this prominent characteristic. The number of granules seen in all the four strains varied from three to seven. They were distributed on both sides of the nucleus.

6.1.3.1 Savannah strains

Gamb 6 was characterised by a prominent, posterior granule whether the total number of granules were seven as in Plate 9a, five (Plate 9b) or less than five (Plate 9c) and although the granular parasites were generally of the same shape the variation in size was noticeable (Plate 9d).

In Gamb 19 the conspicuous granule lay anterior to its position in Gamb 6. However its ease of definition depended on the number of other granules present (Plate 9e). Often this prominent granule was the only granule that was defined clearly enough to be photographed (Plates 9f) although other granules could be seen directly through the phase contrast microscope. Another granule might lie posterior to the prominent granule in this strain as seen in Plate 9g and dividing granular forms such as revealed this characteristic granule (Plate 9h). In addition to the variation in size some trypanosomes had
short but free flagellum (see Plates 9g and h).

6.1.3.2 Riverine strains

TSW 99 did not give very clear picture of distinct granules but none of the trypanosomes observed could be associated with the pattern described for Gamb 6 and Gamb 19 (Plates 10a and b). There were no dominant granules. Free flagellum was present in some individual members although most were typical congolense type without any free flagella.

TSW 103 presented a much clearer picture of granules representative of the riverine trypanosomes. The number of granules was, as usual, variable but the mean was three per trypanosome. All were equally conspicuous as shown in Plates 10c and d. Granules were located on both sides of the nucleus. A free flagellum was visible in a few as in Plate 10d. There was also a range of sizes suggestive of "T. montigomeryi" - form as in Plate 10e together with variable club-shaped and tadpole-shaped trypomastigotes.

6.1.4 Trypanosoma (Trypanozoon) brucei

In the subgenus Trypanozoon most of the granules tended to be in the anterior half of the trypanosome.
6.1.4.1 *Trypanosoma (Trypanozoon) brucei brucei*

(a) **Strain 1**: Antat 1 was monomorphic and there was no granule to observe.

(b) **Strain 2**: 8/18 isolated from pig in Nigeria had numerous granules, too many for accurate counting. They were roughly more than twenty per trypanosome. They appeared as a bunch in the cytoplasm and were located at the anterior half of the parasite (Plate 11a).

(c) **Strain 3**: TSW 3 was another strain isolated from pig but from another West African country (Liberia). It also presented a bunch of granules neatly packed in the parasite. They were more than fifteen in each trypanosome and found located in the extreme end of the anterior region of the main body, just before the free flagella (see Plate 11b).

(d) **Strain 4**: S/42 also had several granules but more widespread than TSW 3 and more particulate than 8/18 (Plate 11c). The general morphology of the trypanosome and pattern of granules were as in the Zambia I strain (83). But the number of the granules was more than double that in Zambia I strain.

(e) **Strain 5**: Treu 667 (LUMP 1164) had only 4 or 5 granules per trypanosome by day 6 or 7 as in Plate 11d. The number rose to more than ten after days 10 and 11.
The granules were distinct, refractile and located anteriorly.

6.1.4.2 Trypanosoma (Trypanozoon) brucei rhodesiense

In addition to the three strains from Botswana (180), Zambia I (83) and Zambia II (57) which had been treated in relation to the patterns of parasitaemias and granules other isolates from sleeping sickness patients in the East and Central Africa were studied.

(a) Strain 1: This was the popular Liverpool strain (LN) and its drug resistant (LR). Both sub-strains appeared to be monomorphic although some occasional 3-4 particles per trypanosome were noticed. Sometimes only one of such dense particles was seen in a trypanosome (see Plates 11f and g).

(b) Strain 2: The second strain examined was 186, also from Botswana as 180. Like 180 its granules numbered 3 or 4 per trypanosome and similar in size and location to what had been described for 180 (Plate 11h).

(c) Strain 3: 58 and 58B both presented a pattern similar to that obtained for 57. The number of granules per trypanosome was always more than ten although they were faint and difficult to photograph (Plates 12a and b).
(d) Strain 4: Another isolate studied was 1198 isolated from Mayoga Buying in Uganda. Some of the parasites had the refractile and big granules identical in pattern to what was known for 180 but others had a pattern of several smaller granules characteristic of 57. Plates 12c and d present the two different patterns. More excitingly, an interesting result was obtained when its clone 1198A3 was examined. Both patterns were confirmed to be present (see Plate 12e).

6.1.4.3 Trypanosoma (Trypanozoon) brucei gambiense

(a) Strain 1: This was LUMP 1236 depicted in Plate 12f). It had its granules scattered but mainly within the anterior half of the parasite. The average number of granules was three per trypanosome. These granules were also refractile. Therefore LUMP 1236 was like 180.

(b) Strain 2: Bida 3 had its granules also in the anterior region of the trypanosome (Plate 12g ) and the mean number in a trypanosome was three although some individuals as in 180 had only one or two granules visible. The granules in Bida 3 tend to lump together. Some moribund forms were also found.

6.1.5 Patterns of Granules

The pattern of granules observed in Chapter 3
and these have been summarised into Tables 12-15

Table 12 Patterns of granules in the strains of
*T. (T.) brucei*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pattern of granules</th>
<th>Other comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>8/18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/42</td>
<td>Variable number of granules in the anterior half</td>
<td>T.b. brucei</td>
</tr>
<tr>
<td>TSW 3*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>667</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LUMP 1164</td>
<td></td>
<td></td>
</tr>
<tr>
<td>180*</td>
<td>3-4 large refractile granules per trypanosome</td>
<td></td>
</tr>
<tr>
<td>186</td>
<td>3-4 large refractile granules per trypanosome</td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>5-6 medium sized granules/tryp.</td>
<td>T.b. rhodesiense</td>
</tr>
<tr>
<td>57</td>
<td>10+ small granules/trypanosome</td>
<td></td>
</tr>
<tr>
<td>58*</td>
<td>10+ small granules/trypanosome</td>
<td></td>
</tr>
<tr>
<td>1198*</td>
<td>Mixture of 3 and 10+ granules per trypanosome</td>
<td></td>
</tr>
<tr>
<td>Bida 3</td>
<td>3-4 large-medium sized granules per trypanosome</td>
<td></td>
</tr>
<tr>
<td>LUMP 1236</td>
<td>3-4 large-medium sized granules per trypanosome</td>
<td>T.b. gambiense</td>
</tr>
</tbody>
</table>

* Clones studied.
Table 13 Patterns of granules in the different species of genus Trypanosoma

<table>
<thead>
<tr>
<th>Species</th>
<th>Location and distribution of granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypanosoma (Herpetosome) lewisi</td>
<td>Variable (1-3) granules restricted to kinetoplast end of trypanosome</td>
</tr>
<tr>
<td>T. (Duttonella) vivax</td>
<td>Numerous granules, evenly spread on both sides of the nucleus</td>
</tr>
<tr>
<td>T. (Nannomonas) congolense</td>
<td>Few (3-7) granules spread on both sides of the nucleus</td>
</tr>
<tr>
<td>T. (Trypanozoon) brucei</td>
<td>Variable granules mainly restricted to anterior half of the trypanosome</td>
</tr>
</tbody>
</table>

Table 14 Patterns of granules in the strains of T. (D.) vivax

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pattern of granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y58</td>
<td>Faint granules</td>
</tr>
<tr>
<td>Y486</td>
<td>Conspicuous granules</td>
</tr>
</tbody>
</table>

Table 15 Patterns of granules in the strains of T. (N.) congolense

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pattern of granules</th>
<th>Other comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamb 6</td>
<td>One of the granules more prominent</td>
<td>Savannah</td>
</tr>
<tr>
<td>Gamb 19</td>
<td>One of the granules more prominent</td>
<td></td>
</tr>
<tr>
<td>TSW 99</td>
<td>All granules equally conspicuous</td>
<td>Forest/</td>
</tr>
<tr>
<td>TSW 103</td>
<td>All granules equally conspicuous</td>
<td>Riverine</td>
</tr>
</tbody>
</table>
6.2 DISCUSSION

6.2.1 The Patterns of the Granules Observed Compared with those of other Workers

The works of other authors who used the same method on similar or the same materials is summed up in Table 16 to be compared with Tables 12 - 15. in the Results. Strains from Botswana have been characterised by a few large refractile granules averaging not more than five in the trypanosome. The strains from Zambia especially 57, were and are still found to have smaller but more numerous granules. The inability to register exact number per trypanosome in the previous records (Ormerod, 1958, 1961) was due to the resolution limitations of the old microscopes. The standard of light microscopy has improved over the years and it is now possible to see these granules clearly and count them accurately. Some strains from Zambia (83) and Tanzania are intermediate in size and number of granules per trypanosome. 83 strain from Zambia in this study proved to be intermediate between 180 from Botswana and 57 (Zambia II). The hypothesis of geographical locations, spread and relationships associated with these strains by Ormerod (1961) is, to date, confirmed
6.2.2 Possibility of Characterisation of the Granules

6.2.2.1 Other methods of characterisation in use

Methods already tried for characterisation of trypanosomiasis and other protozoan diseases include the phenotypic characteristics of the cell, genotypic factors, volunteer infectivity tests, blood incubation infectivity tests (BIIT) and enzyme electrophoresis.

6.2.2.1(a) Characterisation by phenotypic characteristics

The characteristics of the phenotype of cells of the organisms have been observed by Cross and Manning (1973) to study the nutritional organisation of *T. brucei* but Newton (1976) had proved the difficulties in determining the nutrients of the kinetoplastids as a whole and so far this method cannot be widely applied.

Metabolic pathways and end products in the different subgenera of *Trypanosoma* has been established by Bowman and Flynn (1976) but the different species cannot be typed by this method.

Cross (1977) suggested morphological differences based on differences between the surface antigens of *T. brucei*, *T. vivax* and *T. congolense* and Vickerman (1974) also showed differences in surface antigens of different species but because of the complications introduced by these being multiple antigen trypanosomes for each clone.
Table 16 Other works that demonstrated granule patterns similar to current study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Pattern of granules depicted</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winches strain of <em>T. lewisi</em></td>
<td>One or two granules in the adult trypanosome, at the posterior end</td>
<td>Ormerod (1958)</td>
</tr>
<tr>
<td>Vom strain of <em>T. vivax</em></td>
<td>Inclusions on either side of the nucleus</td>
<td>Ormerod (1958)</td>
</tr>
<tr>
<td>Unspecified strain of <em>T. congolense</em></td>
<td>Inclusions or sometimes refractile bodies the size of (but in the wrong position for) the nucleus</td>
<td>Ormerod (1958)</td>
</tr>
<tr>
<td>Maun strain of <em>T.b. rhodesiense</em> from Botswana</td>
<td>Large refractile granules, about 4 granules per trypanosome</td>
<td>Ormerod (1958)</td>
</tr>
<tr>
<td>57 strain of <em>T.b. rhodesiense</em></td>
<td>Inclusions more numerous, smaller and less refractile than Maun strain</td>
<td>Ormerod (1958)</td>
</tr>
<tr>
<td>Uncoded strains of <em>T.b. rhodesiense</em> from Tanzania</td>
<td>Inclusions intermediate between those from Botswana and Uganda</td>
<td>Ormerod (1960)</td>
</tr>
<tr>
<td>Maun strain of <em>T.b. rhodesiense</em></td>
<td>Large refractile granules, average ranging from 3-5 granules per trypanosome</td>
<td>Ormerod (1961)</td>
</tr>
<tr>
<td>57 strain of <em>T.b. rhodesiense</em></td>
<td>&quot;Small granules&quot;</td>
<td>Ormerod (1961)</td>
</tr>
<tr>
<td>181 strain from Botswana</td>
<td>Granules are &quot;massive&quot;</td>
<td>Ormerod (1963)</td>
</tr>
<tr>
<td>Kenya-Uganda strain of <em>T.b. rhodesiense</em></td>
<td>Granules are &quot;so small as to be barely visible&quot;</td>
<td>Ormerod (1963)</td>
</tr>
<tr>
<td>Liverpool normal (LN) of <em>T.b. rhodesiense</em></td>
<td>1-3 granules made more visible by drug</td>
<td>Ormerod &amp; Shaw (1963)</td>
</tr>
<tr>
<td>181 strain of <em>T.b. rhodesiense</em> from Botswana</td>
<td>3-5 granules per trypanosome, granules large and conspicuous</td>
<td>Molloy &amp; Ormerod (1965)</td>
</tr>
<tr>
<td>181 of <em>T.b. rhodesiense</em> from Botswana</td>
<td>3-4 granules per trypanosome</td>
<td>Ormerod et al. (1974)</td>
</tr>
</tbody>
</table>
it may be too early to depend on immunological and serological techniques for characterisations.

6.2.2.1(b) Characterisation by genotype characteristics

The relative proportions of the base pairs, guanine-cytosine and adenine-thymine is known to be associated with DNA buoyant density values in subgenera of kinetoplastids but despite the initial report of Newton and Burnett (1972) no consistent results have been recorded for the members of subgenus Trypanozoon (Gibson et al., 1980). DNA hybridisation technique was used by Newton et al. (1973) for differentiating closely related species of kinetoplastids but Newton (1976) showed that unstable hybrids could be formed which would give wrong results. Though T.b. brucei and T.b. rhodesiense were distinguished from each other by Brack et al. (1976) by the analysis of DNA, the method is in its infancy and costly.

6.2.2.1(c) Volunteer infectivity tests

Volunteers have been used to prove if an isolate from wild mammals was T.b. rhodesiense (Onyango et al., 1966) but such method of characterisation will be too dangerous for T.b. gambiense which is slow-growing as it may be too late to cure the volunteer when the parasites are detected.
6.2.2.1(d) Blood incubation infectivity test (BIIT)

Rickman and Robson (1970) devised a technique to differentiate *T.b. brucei* from *T.b. rhodesiense* without using human volunteers by this method. Many strains since then have been grouped either BIIT-positive or negative (Geigy et al., 1971; Robson et al., 1972). However Rickman and Robson (1974) and Geigy et al. (1975) showed that the results could be inconsistent and Gibson et al. (1980) confirmed that claim.

6.2.2.1(e) Enzyme electrophoresis

This is the most widely-used method. It involves both the phenotypic and genotypic characteristics of the cell as the enzymatic reactions can be controlled by either the nucleus, the cytoplasm or both. Isoenzyme electrophoresis has been used in characterising parasitic and non-parasitic protozoa. Tait (1969) applied the method for *Paramecium* and Borden et al. (1973) for *Tetrahymena*. Sargeant et al. (1978) used the method for differentiating invasive and non-invasive *Entamoeba histolytica* and Sargeant and Williams (1979) studied the isoenzyme patterns of pathogenic and non-pathogenic intestinal amoeba of man. *Plasmodium* spp. were studied by Carter (1970) and Carter and Voller (1975); *Eimeria* by Shirley and Rollinson (1979); and *Leishmania* by Chance (1979). In trypanosomiasis enzyme electrophoresis has been
extensively applied for characterisation (Lumsden, 1974; Godfrey and Kilgour, 1976; Miles et al., 1977; Gibson et al., 1980). It would appear that enzyme electrophoresis technique is the most useful technique for characterisation of the protozoa because it mainly measures genetic difference.

However, Gibson et al. (1980) pointed out that the method also has its pitfalls and the results could be misinterpreted. Firstly, two enzymes are not necessarily identical in structure because they have the same electrophoretic mobility and therefore two samples may not be from related organisms although they may have the same pattern for one enzyme. Secondly, electrophoretically distinct enzymes may not arise from the products of different genes where multiple genes are involved in producing a pattern. In fact, Gibson et al. (1980) agreed that some presumably observable enzymatic changes might be artifacts or phenotypic in origin. Finally some VATs can be distinguished by isoenzyme electrophoresis others not, thus suggesting that the difference may be physiological rather than genetic.

6.2.2.2 The Proposal

The proposal for using the lipoprotein granules for characterisation in African trypanosomiasis has, like any other methods for characterisation (reviewed in 6.2.2.1) shortcomings and limitations.

An apparent pitfall of the proposal is in the
observation that the early granular population of any strain usually shows few granules and it takes time before the standard or characteristic number is attained. It may also be said that it takes some training and careful observation before the characteristic number and pattern for a strain could be identified with certainty. As to the first criticism, it should be expected that some time must elapse before development or any growth is completed. The acquisition of lipoprotein granules is a growth phenomenon. Concerning the second, it can only be said that any scientific undertaking takes some training and time and it is minimal in this technique.

There are not enough strains sampled to add anything to the hypothesis of geographical distribution put forward by Ormerod (1961, 1967) and supported by Gibson et al. (1980) but it is noted that there are two types of granular patterns in strain 1198 confirmed by the isoenzyme pattern described by Gibson et al. (1980) as a hybrid between the northern and southern strains.

In conclusion, while accepting there are pitfalls like in the other methods of characterisation and while there is room for improvement of the technique as the standard of microscopy improves with the advanced technology, it is proposed that the granule patterns could be characterised to differentiate the species of the genus Trypanosoma and the strains of T. (Trypanozoon) brucei. It may be too early to include strains of T. (Duttonella) vivax and T. (Nannomonas) congolense because
more work needs to be done on more widely-spread strains. It has been shown that age of up to two decades or more (Ormerod, 1958, 1961), number of passages, cloning, treatment with corticosteroids and x-irradiation failed to change the pattern of the granules in the strains examined. Similarly transmission by tsetse did not change the pattern either. The granules of *T. b. rhodesiense* 180 remained the same whether in normal blood infection of rats and mice, peritoneal fluid of rats, or in the ascites tumour fluid of mice. The lipoprotein granules are therefore biochemically stable enough for consideration for characterisation as proposed.

Since the determination of granule patterns in *T. brucei* as a member for strain differences follows closely the results of isoenzyme electrophoresis (Gibson et al 1980) but is quicker, less expensive of resources and can more easily be preferred in the field, it is further proposed that this method be recommended as an initial field technique to the preliminary study of trypanosomes. Thus Ormerod (1974) was able to note that the granule patterns of Zambia I strain distinguishes it, immediately it had been isolated, as being more virulent than any that had hitherto been isolated in southern Africa.
CHAPTER 7

SCANNING ELECTRON MICROSCOPY

7.1 RESULTS

7.1.1 General Morphology of the Parasite in the Blood

In each of the strains and species examined consideration was given to
a) the variety of forms and general body surface of each;
b) the location and any special features of the flagellar pocket;
c) the flagellum with particular reference to presence or absence of the free portion.

7.1.1.1 Trypanosoma (Herpetosoma) lewisi

The diversity in size of the juvenile forms was as recorded with light microscopy. The total body surface of the parasite was gracefully smooth (Plate 13 a). Except for size variation, there was little to differentiate the adult forms from the juvenile. No dividing form was found. The majority of the adult forms had the characteristic elongated end terminating at a pin-point as shown in Plates 13a and b. However it appeared posterior
PLATE 13  T. lewisi

(A) A parasite showing the surface to be smooth and pin-pointed posterior end.  
(X30,000)  

(B) A parasite showing free flagellum and flagella pocket.  (X20,000)
The surface to be examined should be the posterior end.

The flagellum was photographed at X20,000.
Parasites with shortened posterior ends. (X13,000)
sterior ends.
PLATE 15  *T.vivax* with probable contamination of *T. brucei*.

(A) Profiles of *T.vivax* parasites  
(X6,500)

(B) A dividing *T.vivax* trypanosome  
(X9,000)

(C) Two parasites demonstrating shallow flagellar pockets with a possible *T.brucei* trypanosome.  
(X4,500)
aminination

parasites

trypanosome

trating shallow
th a possible
PLATE 16  *T. congoleses*

(A) Posterior end of a trypanosome  
    (X55,000)

(B) A typical trypanosome without  
    free flagellum.  (X32,500)

(C) A trypanosome with short free  
    flagellum.  (X27,500)
PLATE 17  T.b.brucet,Antat 1 strain

(A) Profiles of trypanosomes showing wide variation in sizes.  
(X6,500)

(B) A trypanosome in multiple division  
(X30,500)

(C) A trypanosome showing two daughter flagella in addition to the parent flagellum.  
(X35,000)
PLATE 18  *T. b. brucei*, Antat 1 strain continued

(A) A trypanosome with free flagella at both ends.  (X23,500)

(B) A trypanosome showing the daughter posterior flagellum just protruding out of the posterior end.  (X40,000)
flagella

The daughter
protruding

continued
PLATE 19  T.b.brucel, Treu 667 strain

(A)  A flat-form showing the anterior half like a leaf being bent.  
     (X15,000)

(B)  Posterior flagellum proving to be the newly-formed flagellum by 
     disappearing half way into the body of the trypanosome.  
     (X20,000)

(C)  A long-narrow form without plasm  
     (X15,000)
anterior
ent.

ing to be
um by
to the
ut
,000)
PLATE 20  T.b.rhodesiense, Zambia II strain

(A)  A long-flat and a long-narrow form with a plasmaneme on the red blood corpuscle.  
     (X15,000)

(B)  Another long-narrow form with plasmaneme.  (X27,500)

(C)  A long-narrow form without a plasmaneme shown.  (X15,000)
PLATE 21  

*T. b. rhodesiense*, Zambia 1 strain

(A) Profiles of different forms.  
(X5,000)

(B) A long-flat form with beads.  
(X10,000)
PLATE 22  T.b.rhodesiense, Zambia I strain continued

(A) Long-flat with beads and rods
   (x18,000)

(B) Stumpy form with popper beads.
   (X16,000)
thin rods

beads.
PLATE 23  

T.b.rhodesiense, Zambia I continued

(A - C)

Cavities which could be the pinocytotic vesicles.

(X30,000)
PLATE 24  T.b.rhodesiense, Zambia I continued

(A) Wrinkles (could be artifacts) on the trypanosome.  (X33,000)

(B) Dividing trypanosome with parent and daughter flagella.  
(X30,000)
PLATE 25  T. b. rhodesiense, Botswana strain

(A)  Trypanosome with "undulating membrane" pulled out.
    (X18,000)

(B)  Trypanosome with rod and popper beads.
    (X30,000)
PLATE 26  In vivo phagocytosis

(A) Normal trypomastigote not affected.  (X30,000)

(B) Engulfment from flagellar end.  
    (X30,000)

(C) and (D)
    Engulfment from posterior ends.  
    (X30,000)

Note: the pitting and the general interaction between the parasite and 
the macrophage as the engulfment started and proceeded.
PLATE 27  In vitro phagocytosis

(A) Trypanosome caught by flagellar end and showing resistance by twisting
Note cap-like protrusion of macrophage.
15 minutes of interaction before fixation.  (X11,200)

(B) Further engulfment of trypanosome
30 minutes of interaction.
(X30,000)

Note: Pitting.
PLATE 28  In vitro phagocytosis continued

(A) Further engulfment via posterior end.  (X15,000)
1 hour of interaction.

(B) Further engulfment via posterior end by cup-like protrusion.
(X15,000)
1 hour of interaction
ued

posterior

posterior

ession.
(A) Higher magnification of Plate 28b showing details of cup-like protrusion of macrophage into which the parasite was drawn. (X30,000)

(B) The macrophage engulfing the parasite. (X22,000) 2 hours of interaction.
Plate 28b

like
e into
drawn.

tag the
2 hours
PLATE 30  In vitro phagocytosis with dead parasites (already fixed with glutaraldehyde)

(A) Posterior end of trypanosome being caught by the macrophage.  
    (X15,000)

(B) Flagellar end being caught.  
    (X45,000)

Note: absence of pitting.
The microsome and lead glutathione were thought to be involved in the process.
PLATE 31  In vitro phagocytosis with dead parasites

(A) Lamellar hold on the parasite.  
(X16,500)

(B) Possible end of phagocytosis with just a bit of flagellum of trypanosome sticking out.  
(X15,000)
end could be rounded up (Plate 14).

The flagellar pocket was in the posterior half in the juvenile but about one-third from the posterior end in the adult. In both cases it was a clear smooth depression with neither secretions nor secretory bodies. In both forms the body, particularly the adult form, was like a barrel with conical ends and the flagellum like a rope glued round the barrel. It was similar to the long-narrow form of *T. brucei* (see Section 7.1.1.4b). The adult form of *T. lewisi* was tubular in its entire length. There was no detectable undulating membrane. Free flagellum was well-defined (Plate 13b).

7.1.1.2 *Trypanosoma* (*Duttonella*) *vivax*

Y486 examined consisted of the monomorphic forms which had the general morphology as detailed by light microscopy (see Plate 15a). The surface smooth in parts was punctuated with folds apparently produced by the twist of the body (Plate 15a). The flagellar pocket was smooth, shallow and with no protrusions. The posterior portion behind the flagellar pocket was about one-eighth of the total length and was tubular. It did not protrude as in *T. lewisi* and instead of being pointed it was blunt at the end. Anterior to the flagellar pocket the body assumed a flat shape until it terminated with a free flagellum (Plates 15a - c).
Dividing forms were common and the separation of the daughter parasites often showed by starting to detach from the flagellar tips (see Plate 15b). As noted in light microscopy in 6.1.2, the possibility of contamination with another parasite, probably *T. brucei*, was suggested by Plate 15c).

7.1.1.3 *Trypanosoma (Nannomonas) congolense*

Gamb 6 from the forest/riverine location and TSW103 of the savannah were two samples examined by SEM. Both had smooth surfaces and both were sturdy or fat-looking.

The posterior portion of Gamb 6 shown in Plate 16a had the usual tubular shape. Though conical it was not as bluntly-pointed at the extreme end as in *T. lewisi* but more pronounced than in *T. vivax*. The flagellum was also observed attached with no visible undulating membrane. The anterior portion was, as in *T. vivax*, flattened out.

TSW103 had forms most of which showed no free flagellum as in Plate 16b but a few had very short free flagella (Plate 16c). The flagellar pocket was deep and broad giving rise to a thick flagellar rod. Although the position of the flagellar pocket in TSW103 appeared to be closer to the posterior end than in Gamb6 no claim of differentiating one strain from another could be made.
7.1.1.4 Trypanosoma (Trypanozoon) brucei

Two strains of *T. brucei* brucei and three of *T. b. rhodesiense* were examined with the following report:

7.1.1.4(a) *T. brucei* brucei

(i) **Antat 1**

As had been recorded with light microscopy in Chapter 6, Antat 1 was a fast growing strain and was monomorphic in that no form possessed the lipid granules though variation in size and shape occurred (Plate 17a). Its high rate of multiplication was shown by observation of as many as 80% of the parasites being in different stages of division. Plate 17b presents details of a multiple division form. From Plate 17c it was observed that the two posterior flagellar rods disappeared into the body whereas the parent and anterior rod continued to the flagellar end. Antat 1 appeared to have flagella, in some instances, at both ends (Plates 18a and b).

No long-narrow form was detected in the strain.

(ii) **Treu 667**

This strain was like any other *T. brucei* trypanosome in possessing smooth surface and in the location of the flagellar pocket. Two different microscopes were used to examine this strain. Both confirmed that the normal trypomastigotes in long-flat and stumpy forms were
not tubular especially between the flagellar end and
the flagellar pocket (see Plate 19a). Though with
some thickness, they were flat. Also the posterior
flagellum, of a dividing parasite, was not present
throughout the whole length of the parasite but the anterior
was (Plate 19b).

Long-narrow forms were few but present in the
population as demonstrated in Plate 19c.

7.1.1.4(b) T. brucei rhodesiense

Zambia I and II and Botswana strains
were studied more intensively than any other of the
strains and species in this section. Yet it was not
possible to differentiate strains on SEM observations
although each presented the three major forms of long-
narrow, long-flat and the stumpy similar to those of
Treu 67. Unlike the other preparations where age of
infections were not specifically considered, these three
strains were related to different times after the inoculum
had been introduced.

(i) Zambia II strain (57)

Infections at 72 hours gave both long-narrow
and long-flat forms (Plates 20a - c). Long-narrow was
found unique in having the plasmaneme (Plates 20a and b)
formerly observed as being associated with this form by
light microscopy. Although not all long-narrow showed
a plasmaneme at the time of fixation as in Plate 20c and as formerly observed from Treu 667, the tubular configuration of the form was unquestionable and each was like the adult form of *T. lewisi*. The flat nature of the majority of trypanosomes was markedly noticeable.

Observations on the divided forms confirmed the results from Antat 1 and Treu 667 in that the anterior flagellum was most developed extending to the anterior extremity but the posterior one was still developing and could not be observed in the anterior end of the organism.

(ii) *Zambia 1* strain (83)

A range of the major forms was presented in infections fixed 72 hours after the inoculum had been given (Plate 21a). A typical long flat was demonstrated in Plate 21b and its flat figure could not be disputed. "Beads" (Ellis *et al.*, 1976), as a group of extruded filaments, were noticed on the flagellar pocket on that parasite and a close look at another long-flat in Plate 22a showed that not only beads were extruded, "rods" were also produced. Two stumpy forms in 9-day infection were shown in Plate 22b. One depicted the standard morphology of the form with only a short visible free flagellum. The other showed beads, "popper beads" and rods arising from the flagellar pocket.

Cavities doubtful to designate pinocytotic vesicles, were located at various parts and in different angles the body turned (Plates 23a - 2 ). Some
wrinkles were also noted as in Plate 24a. Dividing flagella once again indicate that the older flagellar pocket was the anterior (Plate 23b).

(iii) Botswana strain (180)

The strain was like any other T. brucei. Plate 25a reveals the rare occasion when the so-called undulating membrane was detectable. That the occurrence of the filaments was universal in all these three strains and that these filaments originate from the flagellar pocket was shown in Plate 25b.

7.1.2 Peritoneal Fluid Forms and Phagocytosis

One offshoot of the SEM study was the observation of phagocytosis of trypanosomes by peritoneal macrophages in both in vivo and in vitro conditions.

This aspect of the current study was restricted to Botswana strain. In the peritoneal fluid from infected rats normal trypomastigotes conforming to long-narrow, long-flat and stumpy forms were found as in Plate 26a. Tadpole and round forms were also observed. Macrophages were found phagocytosing trypanosomes.
7.1.2.1 In vivo phagocytosis

The first series of the observation portrayed that while some parasites were caught by the flagellar end before engulfment as in Plate 26b others were taken in via the posterior end as in Plates 26c and d. The latter observation was contrary to a transmission electron recording, (Stevens and Moulton, 1978). It was also noted that the cell membranes of the parasites being phagocytosed had a pitted appearance and it was doubtful if some were alive (see Plates 26b - d. A repeat of the in vivo observation gave the same results.

7.1.2.2 In vitro phagocytosis

7.1.2.2(a) Using live parasites

No phagocytosis was observed within the first five minutes. Specimens fixed after 15 minutes showed the flagellar end interacting with the macrophage, as shown in Plate 27a. Materials fixed after 30 minutes of interaction exhibited more advanced stage of phagocytosis with parts of the body of the parasite already inside the macrophages (Plate 27b). After one hour a greater part of the trypanosome had been swallowed as shown in Plates 28a - 29a. Little information was added from observing the materials fixed after 2 hours (see Plate 29b).
In addition, the following general observations were made:

(1) All the phagocytosed parasites had been disfigured or damaged by some external reaction (Plates 26b - 29b). There was strong evidence of resistance by trypanosomes (Plates 26a and 27a).

(2) Interaction between the parasite and the macrophage appeared to be controlled by macrophages which developed envelopes or extensions to engulf the parasites (see Plates 27a, 28b, and 29a and b).

(3) Either end of the parasite could be the point of attachment before being engulfed as in Plates 26b and c but posterior end might be more favoured (Plates 28 and 29). There were parasites which showed the process might start by middle of the body rather than by either end of the parasite.

7.1.2.2(b) Using dead parasites

The stages of phagocytosis were the same as described for the living parasites except that the rate was faster. Greater parts of the parasites had been absorbed into the macrophages within 15 minutes. No phagocytosis was also observed in the first five minutes.

Both ends of the parasite were attacked as shown in Plates 30a and b. Phagocytosis was seen to be by engulfment as demonstrated in Plate 31a when ruffling of the membrane of the macrophage was demonstrated.
No part of the parasite was found to be damaged before, during and after phagocytosis contrary to observations when the living parasites were used (see Plates 26b - 29b). Plate 31b might be the stage when the parasite was almost completely engulfed.

7.2 DISCUSSION

7.2.1 The Technique

As soon as transmission electron microscopy (TEM) became available, efforts were made to obtain a three-dimensional view of specimens by replicating and shadowing methods. However the distortion produced when soft cells were dried prevented any useful advance for this type of material until scanning electron microscopy (SEM) came into use in the mid-1960s. SEM is most suitable for examining the surfaces of cells in detail with a depth of focus, several hundred times that of the light microscope, giving also a three-dimensional appearance.

Until lately most SEM studies were made with hard specimens such as the calcified animal tissues and plant materials. To deal with soft animal tissues and with protozoa, problems of preservation arose due to distortion by fixation and inappropriate dehydration procedures which had not occurred with light and TEM. It
was only after SEM had been developed that a full understanding of what happens to the tissue or cells at each stage of preparation began to be understood.

It has been found in this study that the results obtained for the preparation of trypanosomes on the coverslips using concavalin A and foetal calf serum for adherence did not justify the time taken and materials used. Consequently this procedure had to be abandoned. This was because of the observations that no matter how many parasites were put on the coverslips and no matter how successfully the parasite agglutinated and seemed to have adhered to the glass, the bonding power of Con A was inhibited as soon as the medium was diluted with fixatives and washing reagents. Thus few parasites were found to have adhered by the time the material was examined. The effects that PSG buffer had on the agglutinations were also not consistent and the amount of glucose (or its absence) in the buffer did not show any difference to the adherence of parasites to the glass or coherence to one another.

Despite the findings, firstly, of Zenian et al. (1979) who had successfully caused promastigotes of \textit{Leishmania tropica} to adhere to foetal calf serum on coverslips with con A, and secondly of Vickerman and Tetley (1977) who showed that culture forms of \textit{T. brucei} will bind together with con A, the bloodstream forms of \textit{T. brucei} would not adhere satisfactorily. The probable
was only after SEM had been developed that a full understanding of what happens to the tissue or cells at each stage of preparation began to be understood.

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reason for this phenomenon is that *T. brucei* has a surface coat of glycoprotein consisting of inner layer of carbohydrate and outer surface of protein; when the concentration of con A was high it penetrated deeply enough to cause the inner carbohydrate to agglutinate the trypanosomes together, but as soon as the concentration was diluted with the fixative and cacodylate buffer, the binding effect was removed. Thus the outer protein layer will not bind together in the presence of con A. Similarly as glucose in the PSG buffer is present as free molecules in solution it cannot cause the surface of the trypanosomes to bind to the glass and foetal calf serum.

Zenian et al. (1979) also successfully used Karnovsky's fixative with its high osmolarity for fixing promastigotes, but this was not found to be as good as ordinary glutaraldehyde for fixing the vascular trypanosomes for SEM. No difference was detected between specimens that were fixed only with glutaraldehyde and those which were also post-fixed with osmium tetroxide. Glutaraldehyde is powerful and fast enough for total fixation of the parasite within 30 minutes; yet when the parasites were left overnight in glutaraldehyde, the effect is to harden the specimen (Cohen, 1974). The extra hardening effect of osmium tetroxide is therefore unnecessary in these specimens; however its blackening effect is useful for contrast in TEM. But in SEM the blackened surface is later coated with gold. Consequently there is no advantage
to be set against the high cost, low availability and health hazards which make the use of a fume cupboard essential for processing material.

Strips of body surface cast off from the blood trypanosomes under stress or badly fixed or dried, may be confused with the plasmanemes; a term given by Vickerman and Luckins (1969) and found to be characteristic of the long-narrow form in this work. Since some methods of drying or dehydration may produce these body surface strips as artifacts, a comparison of the results of air- and freeze-drying of Pal et al. (1974) with those of critical point drying of Vickerman and Tetley (1977), Gorenflot et al. (1980) and of this work, was made and on this basis the writer agrees with Cohen (1974) that critical point drying is superior to both air-drying and freeze-drying.

Because of the use of a video image, one observes SEM micrographs with three apparent dimensions assuming these to be real. In fact, the third dimension is illusory being supplied only by the mind (Cohen, 1974). The use of Stereopairs, however, provides more accurate information and this study with some sets of stereopairs observed confirms the observation of Cohen (1974) that a set of stereomicrographs can yield more information than can several conventional micrographs.
7.2.2 The Morphological Features

It is still too early to be confident of the use of SEM for differentiating strains. However members of the subgenus *Trypanozoon* can be defined by their morphological configurations so that their identification by SEM becomes a possibility. In addition, the finding of contamination of a strain of *T. vivax* with *T. brucei* by SEM during this project underlines the possibility that SEM might be used for morphological identification of strains and species of trypanosomes.

Specific features found to be of interest are briefly discussed as follows:

7.2.2.1 The surface of the parasite

Pal et al. (1974) reported that the trypanosome surface was smooth, with some elevations and depressions throughout the length of the body. He used *T. b. gambiense*, *T. b. rhodesiense* and *T. evansi* and these were either air-dried or freeze-dried. Gorenflo et al. (1980) claimed that after fixation in glutaraldehyde *T. b. brucei* trypomastigote was coiled in appearance and pleated parallel to the flagellum. The coiled appearance was, according to these workers, confirmed by the filmed studies of the living parasite but refuted by the examination with SEM. In this study it has been established that when a parasite is fixed undisturbed and very quickly, before it has time
to move, the surface of the body is smooth. But wrinkles, pleats or twists are formed when the parasite moves during fixation. The conclusion agrees with freeze-fracture studies of Vickerman and Tetley (1977) and cytochemical analysis of the pellicle by Wright and Hall (1976) in that neither the plasma membrane nor the surface coat contains any microfilaments that will give permanent ridges visible from the surface.

When trypanosomes are properly fixed and dried by the critical-point drying method and when they are properly dehydrated in suitable medium, the location of either the nucleus or the kinetoplast cannot be identified from the surface. No SEM micrographs of Hoare (1972), Vickerman and Tetley (1977) or Gorenflot et al. (1980) claimed such identification. This study also failed to register such observation. It was therefore possible that the claim of Pal et al. (1972) was due to faulty processing, probably inappropriate drying procedures. This illustrates that the necessity for appropriate technical handling and preparations cannot be overemphasised.

7.2.2.2 The flagellum, flagellar pocket and undulating membrane

The possession of the flagellum is a prerequisite of all flagellates. Composite illustrations of Vickerman (1969, 1970) based on TEM observations gave details of
flagellar origin and its association with the flagellar pocket. The flagellum was also associated with the undulating membrane. The SEM observations in the current work depicted the flagellum stuck to the body whether it had a free extension or not. That there was a free, but very short, flagellum in nearly all the so-called stumpy forms was beyond dispute. The question is to state how short the free flagellum must be before a trypanosome ceases to be slender and becomes stumpy. The unreliability of external morphological characteristics in designating forms is once more shown.

Usually *T. congolense* is characterised by lack of free flagella. The SEM observation showed the presence of the short free flagellum in some individuals in a strain. This agrees with Christina Young (personal information) who also observed free flagella in another strain of *T. congolense* by SEM.

Pal et al. (1974) reported that the flagellum did not arise from any groove in *T. b. gambiense* and *T. b. rhodesiense* but it did so in *T. evansi*. Both the records of Vickerman and Tetley (1977) on *T. b. brucei* and Gorenflot et al. (1977) also on *T. b. brucei* illustrated unmistakably the flagellar pockets. This work also showed the flagellar pocket in each of the strains studied. The failure to register the flagellar pocket in some materials might also be due to technical faults in preparation.

Finally, secretory filaments similar to those
described by Ellis et al. (1976) were all shown to have originated from the flagellar pockets (see 7.2.2.3).

The existence of the undulating membrane was refuted by Gorenflot et al. (1980) though Vickerman and Tetley (1977) reported that the successful preservation of the trypanosome in SEM was to be indicated by the life-like image of the membrane. TEM micrographs do not show undulating membrane as a distinguishable entity. From the current study it is reasonable to conclude that the so-called undulating membrane is nothing more than a condition in which the surface membrane or pellicle was stretched out by the active flagellum when beating. When it stops beating and the pellicle contracts there is no undulating membrane. If a parasite with beating flagellum is fixed and properly processed the fin-like projection is shown. If the parasite is fixed at other times such structures cannot be observed.

7.2.2.3 The filaments

Both the plasmanemes described by Vickerman and Luckins (1969) as well as the various types of secretory filaments of Ellis et al. (1976) were demonstrated. It has been shown that the plasmaneme is not the same as the strips of body surface cast off by trypanosomes in unfavourable conditions and that the long-narrow form is characterised by the plasmaneme although
it may also survive in the long-flat form. While the development of strips of body surface is a degenerating process the existence of the plasmaneme is a property of the young form with the highly elastic developing membrane which can be drawn out at the posterior end but for yet unknown purposes. It is difficult to be sure what previous workers had observed. Filaments arising from the extremities of the trypanosome were seen as far back as the beginning of the century (Schlelipewsky, 1912). Molloy and Ormerod (1965) described projections by phase contrast from posterior end of T. brucei. Both of these would appear to be plasmanemes. Workers who had described what could have been secretory filaments included Babudieri and Tomassini (1967), Wright et al. (1970). None of these observations was by SEM.

There is some dispute about the functional disposition of the filaments as reviewed by Wright et al. (1970) but this work confirmed that the secretory filaments were associated with or probably secreted from the flagellar pockets but the plasmanemes (or filopodia) had a different origin.

7.2.2.4 The shape and volume of the trypanosome

In spite of the clear definition of the vascular trypomastigote as being "basically lanceolate in shape, their body having the form of an elongated flattened
blade, which is elliptic or oval in transverse section" (Hoare, 1972), the general but erroneous impression given by generalised illustrations and selected TEM micrographs is that the blood trypanosome is cylindrical and round in transverse section. This SEM study confirms Hoare's descriptions and disputes the general impression. The various strains of the different subspecies of *T. brucei* studied offered an opportunity to differentiate the three major forms - long-narrow, long-flat and the granular usually referred to as the stumpy. Less than 10% of the vascular trypanosomes even at the peak of long-narrow population is that of the cylindrical form. The rest are flat either as long-flat or as stumpy.

The effort made to determine the volume of *T. b. rhodesiense* and *T. lewisi* could not be completed because of the breakdown of Coulter counter before it could be calibrated. However, it was significant that the three histograms obtained for *T. b. rhodesiense* compared most favourably with the histogram of Williams (1972) for *T. b. brucei*. *T. lewisi* gave two peaks in contrast to one of *T. b. brucei*, indicating the juvenile as well as the adult form populations.

From calculations of Williams (1972) the mean volume of *T. brucei* was 12.75 cubic microns. Maxie *et al.* (1978) who also used Coulter counter also got similarly low values for *T. vivax* and *T. congolense*. Hecker (1980) had 15.75 cubic microns for a strain of *T. b. brucei*. But if all the trypanosomes in blood were tubular or cylindrical
the expected mean volume should be 83.5 cubic microns (Williams, 1972). The wide difference between the expected values given by the various authorities quoted confirms the SEM observation made in this work.

7.2.2.5 The difference between the posterior flagellum and plasmaneme.

Some parasites (Antat 1) appeared to have flagella at both ends. After the SEM observation the apparent double flagellation was found to be due to the high rate of division. It was significant that no long-narrow form was observed from Antat 1 strain which appeared to have been blood-restricted with little possibility of tissue invasion. This will be further commented upon in the general discussion.

The situation in Antat 1 as in T.b. rhodesiense in peritoneal fluid, is an aberrant condition where the rate of division is too fast to allow the daughter flagellum to grow forward (see 7.2.2.6). Instead, the daughter flagellum, still associated with the posterior daughter flagellar pocket, grows out posteriorly. It is the new flagellum of the dividing trypanosome. The new flagellum could be confused with the plasmaneme. With LM it is difficult to see clearly the difference but SEM shows this; the plasmaneme, which is not an integral part of the body, is much finer than the flagellum.
7.2.2.6 Sequence of longitudinal binary fission

From the three strains of *T.b. rhodesiense* intensively studied and Antat 1 strain of *T.b. brucei* which was dividing very fast, the sequence of the binary division was noted. An hypothesis for the sequence observed could be summarised as follows:

1. The posterior flagellar pocket is the new or daughter pocket and the anterior one is the parent.
2. The old or the parent flagellum is complete, extending to the end of the organism as the free flagellum.
3. The new flagellum arises from the posterior pocket and is usually shorter and grows forward until it separates with the daughter trypanosome.

This hypothesis was later tested and found to be supported by TEM (see Chapter 8).

7.2.3 Phagocytosis

Little is known about macrophage phagocytosis of the African trypanosomes. Earliest observation was by Levaditi and Mutermilch (1910), Lumsden and Herbert (1977) compared the effect of normal mouse serum with that of the homologous antiserum on phagocytosis of *T. brucei*. Stevens and Moulton (1978) made a TEM study of phagocytosis of *T. brucei* by activated mouse peritoneal
macrophages. Cook (1978), using LM, as did Lumsden and Herbert, studied the rates of phagocytosis on different surfaces ranging from glass through filter paper to different tissues of rodent. So far the writer has not found any former record of phagocytosis of the African trypanosome by SEM. The nearest material studied by SEM was Leishmania tropica by Zenian et al. (1979) and Muna and Muhammed (1981).

In this study the rate of phagocytosis has been rapid, occurring in approximately 1 hour in vivo, less in vitro. This agrees with Lumsden and Herbert (1977) who recorded that the attachment and ingestion took place in the first "few" minutes. Zenian et al. (1979) also supports the observation of the initial rapid engulfment which in the case of epimastigotes of L. tropica slowed down after 10 minutes but in this work phagocytosis of T. brucei took 30 minutes. No satisfactory conclusion could be reached about whether the engulfing of the parasite was complete or partial. While the indications of possible complete engulfment were given, most parasites observed were only partially covered. Zenian et al. claimed complete phagocytosis of L. tropica and emphasised the relative ease with which up to 6 fixed parasites were engulfed. Stevens and Moulten (1978) recorded incomplete engulfment with T. brucei. It is possible that the main body of trypomastigotes of T.b. brucei and T.b. rhodesiense being longer and fatter than
promastigotes of *L. tropica* does not behave in the same way.

After the incidental observation of phagocytosis *in vivo* from materials being scanned for normal morphological interest, three questions were raised. Firstly, could the attachment of the parasite to the macrophage with either end be repeated *in vitro*? Secondly, could the unhealthy appearance of the parasite be due to technical faults especially the critical point drying procedures or the effect of electron beam on the parasites? Thirdly, could the interaction in phagocytosis be the result of active penetration by the parasite into the macrophage or the engulfment of the parasite by the macrophage? The attempt to answer these questions led to the *in vitro* work whereby live parasites were compared with glutaraldehyde-killed parasites known to retain the surface coat intact (Dwyer, 1977). These specific issues will be considered briefly.

7.2.3.1 Point of attachment

Stevens and Moulten (1978) reported that *T. b. brucei*, in phagocytosis, was attached to the macrophage by the flagellar end. Although some parasites attached by posterior end were noted Zenian et al. (1979) asserted that the promastigotes were predominantly attached by flagellar tips. Muna and Muhammed (1981) repeated the statement about promastigotes of *Leishmania tropica*. 
But literature had not been categorised about the point of attachment by the kinetoplastids as a group. Pulver-taft and Hoyle (1960) saw attachment of *L. donovani* by posterior end. Miller and Twohy (1967) noted that their own *L. donovani* strain was attached by flagellar end. Akiyama and Haight (1971) observed that the same species of *Leishmania* attached by posterior end. Dvorak and Schmunis (1972) recorded posterior end with *T. cruzi*. Nogueira and Cohn (1976) reported that both epimastigotes and trypomastigotes of *T. cruzi* in culture were attached by posterior tips. Liston (1975) and Liston and Baker (1977) indicated entry by posterior tip for epimastigotes and either end for trypomastigotes of *T. dionisii*.

Like the trypomastigotes of *T. dionisii* this work shows vascular trypomastigotes of *T.b. rhodesiense* from Botswana whether *in vivo* or *in vitro*, dead or alive, to be attached to the macrophage by either end although attachment at the flagellar tip is more usual. It is a possibility that the condition in which the trypanosomes were taken in could account for the mode of entry by both ends (see 7.2.3.2).

7.2.3.2 Condition of parasite when taken in

The preliminary observation *in vivo* suggested that even before the entry of the parasite into the macrophage the victim has been degraded was confirmed *in vitro*
and it appeared that some unknown biochemical activity had taken place. The glutaaldehyde-killed parasite was taken in without such degeneration. That the parasitic microorganisms in general and trypanosomes in particular are eliminated by macrophage phagocytosis had been established (Hart and Young, 1975; Stevens and Moulton, 1978). The killing and digestion were explained to be intracellular within the macrophage with activities of phagosomes and lysosomes. It may be extra information to confirm with other organisms the extracellular degradation and partial digestion. Further work is needed.

A fuller discussion on the role of phagocytosis in the peritoneal fluid will be reserved for the overview when the engulfment by macrophages will be contrasted with the penetration of the trypanosomes into the choroid plexus cells. Suffice to note that the extracellular degeneration of the parasite renders it immobile and that may be why either end could be attached as observed.

7.2.3.3 Penetration or engulfing

There were impressions of concavity which appeared to have been created by the flagellar push by the parasite. This could be interpreted as active penetration by the parasite. But careful observation showed that this secondary phenomenon (Zenian et al., 1979) was due to retraction of the lamellar sheath. The active
growth of the lamellar collar which consequently enveloped the parasite partially or completely was consistently demonstrated by SEM micrographs. Therefore the process of phagocytosis could not be voluntary penetration by the parasite but it is essentially engulfment by the macrophage protrusions.
CHAPTER 8

TRANSMISSION ELECTRON MICROSCOPY

8.1 RESULTS

In this section an account is given of the results obtained from transmission electron microscopy (TEM) observation on the bloodstream and tissue forms of normal infections and animals treated with chemotherapy. Botswana strain (180) was the chosen pathogen.

8.1.1 Blood Forms

8.1.1.1 72 hour (4-day) infections

Infections at 72 hours were shown by the agar technique to contain both long-narrow and long-flat forms. On repeating the experiment six times the results were consistent. On two occasions the prepatent period was reduced by 6 and 12 hours respectively. Yet in these two infections the agranular forms available were still a mixture of long-narrow and long-flat forms. The parasitaemia was low represented by one parasite in 50 fields.

Using the double-filter system whereby the bigger parasites were retained on the 1.0 µ unipore membrane
PLATES 32 - 52 are TEM micrographs

PLATE 32  Blood parasites

(a) T.S. of long-narrow form (X80000)

(b) A range of forms in profile including one multiple division form at 96 hour infection (X6,600)

(c) A parasite going through 0.1 μ membrane filter (X 6,600)

(d) A parasite caught on 0.4 μ membrane filter (X20,000)
including at 96 hour
membrane
membrane
(a) T.S. of dividing form with three flagella all within the body of parasite (X60,000)

(b) Oblique section of long-flat form (X80,000)

(c) T.S. of dividing form with two flagella in one membrane and the form also showing early stage of cristae in mitochondria (X60,000)

(d) L.S. of dividing kinetoplasts and flagella (X30000)
PLATE 34 A section of liver sinusoid with trypanosomes in blood (X7,600)

PLATE 35 A pair of choroid plexus (C.P.) fixed in glutaraldehyde and post fixed in osmium tetroxide (X10)
Fixed in osmium
PLATE 36  Histology of C.P.

(a) A section of a "leaf" of choroid plexus  (X4,000)

(b) An epithelial (ependymal) cell of C.P.  (X10,000)

(c) Section of C.P. showing external row of ependymal cells and internal blood vessel  (X6,000)

(d) Section of C.P. showing a gap between the ependymal cells and the blood vessel  (X6,600)
(a) Section of C.P. showing probably an astrocyte between the blood vessel and the ependymal cells  \( \times 13,200 \)

(b) Blood vessel with internal elastic membrane  \( \times 4,000 \)

(c) Section of roof of a ventricle  \( \times 30,000 \)

(d) Section of the brain with nerve cells  \( \times 10,000 \)
PLATE 38  Histology of C.P. continued

(a) Ciliated ependymal cell  (X4,000)

(b) Section of surface of C.P. not deep enough for locating parasites  
    (X10,000)
PLATE 39  Tissue parasites

(a) Intra- and extra-vascular parasites  
(X3,200)

(b) Colonies of parasites in the tissue.  
Note blood vessel did not show any  
parasites  
(X3,200)

(c) C.P. blood vessel infested with parasites  
(X3,200)
PLATE 40  Tissue parasites continued

(a) Higher magnification of Plate (39b) to show details of parasites and the tissue cells (X5,200)

(b) Multiflagellar parasite in tissue (X6,600)

(c) Parasite in a phagocytic cell, probably a macrophage (X15,000)
(a) Parasites in the ependymal cell with enhanced collagen fibres produced and later destroyed by parasites (X6,600)

(b) Parasites deep inside the tissue (X3,200)
1 with
iced and
(X6,600)
tue
PLATE 42  Tissue parasites continued

(a) Profile of section of C.P. showing some pathological effects  (X3,000)

(b) Rbc inside a macrophage.  Note mitochondria being destroyed  (X30,000)

(c) Section of C.P. with internal haemorrhage observed  (X6,000)
PLATE 43  Tissue parasites continued

(a) Profile of a "leaf" of C.P. of 24-day infection showing intra- and extra-vascular parasites  (X2,600)

(b) Profile of a "sinus" with a multiflagellar parasite  (X660)

(c) A colony of intracellular parasites surrounded by a membrane  (X13,200)
PLATE 44  Tissue parasites continued

(a) A colony of parasites some of which were tightly packed and pressed onto the nucleus of host cell  (X6,000)

(b) Colonies of parasites deep inside the tissue  (X6,000)
PLATE 45  Tissue parasites continued

(a) A wider view of Plate 44a, showing the sites with the "nests" of parasites (X3,200)

(b) Details of an isolated intracellular parasite separated from the host cytoplasm by a membrane (X20,000)

(c) Details of a parasite from Plate 44a also showing the membrane surrounding the intracellular parasites (X20,000)
Note destruction of mitochondria
showing the parasites

cellular host (X20,000)

late 44a surrounding (X20,000)

ndria
PLATE 46  Tissue parasites continued

(a) Rbc being destroyed by host's reaction but parasites left intact  (X15,000)

(b) Multiflagellar parasite in tissue similar to that in the "sinus" of Plate 43b  (X40,000)

(c) A parasite tightly surrounded by cytoplasmic organelles and without parastiphorus vacuole.  (X10,000)
PLATE 47  Tissue parasites continued

(a) A trypomastigote form in tissue  
(X16,600)

(b) A trypanosome, possibly a long-narrow, assumed to be going back into the blood circulation  
(X6,600)

(c) Another probable long-narrow form presumed to be entering the blood with cytoplasmic push behind it as arrowed  
(X13,200)
(a) Section of a tissue parasite showing the surface coat (X30,000)

(b) T.S. of trypanosome with double membrane of mitochondria and single unit membrane for the microbody (X52,000)

(c) and (d)
Collagen fibres produced by host but destroyed at the immediate vicinity of the parasites (X10,000) (X6,600)
showing

single membrane

unit

(X52,000)

host but
cinity

(X6,600)
PLATE 49 Tissue parasites and chemotherapy effects

(a) Parasites in the matrix partly obscured by SHAM (X10,000)

(b) Intracellular parasites and infiltrating plasma cell in the C.P. (X6,600)
PLATE 50  Tissue parasites and chemotherapy effects continued

(a) Parasites in the meninges after treatment (X3,200)

(b) Intracellular parasites separated from cytoplasm by membrane and found with partly digested endoplasmic reticulum. Apparently some of these parasites have survived after treatment (X16,500)
PLATE 51  Tissue parasites and chemotherapy effect continued

(a) An intracellular multiflagellar parasite surrounded by cytoplasmic organelles all bound over by cell wall of the host cell  (X13,200)

(b) Details of some parasites at higher magnification. Note that while the cytoplasmic organelles were being destroyed nuclear membrane and nucleus itself were unaffected.  (X52,800)
PLATE 52  Tissue parasites and chemotherapy effect continued

(a)  Another multiflagellar parasite presumably surviving the SHAM treatment  (X40,000)

(b)  C.P. section showing haemorrhage  
     (X4,000)

(c)  A fibroblast with inward-growing single cilium  (X8,000)
Early effect

As a site presumably present (X40,000)

Mark hemorrhage

Growing single
and the smaller ones caught by 0.4 μ membrane placed underneath some separation was possible. On the upper layer were found parasites mainly the dividing or bigger long-flats. Some long-narrow forms were also present. The parasites on the lower membrane were few. About four out of five were long-narrow but occasional long-flat forms managed to squeeze through the upper membrane. Some parasites even went through the 0.4 μ membrane as the filtrate when examined contained trypanosomes. Plate 32a shows a typical parasite from a 72 hour infection and Plate 32c shows some trypanosomes going through 1.0 μ membrane. Plate 32d was a trypanosome caught on 0.4 μ membrane.

8.1.1.2 96 hour (5-day) infections

The predominant form as seen by the agar technique was long-flat although some long-narrow were also found and they were all agranular. The morphology of each of the forms was similar at 96 hours to their morphology at 72 hours. This population in higher parasitaemia was represented by Plate 32b with several dividing long-flat forms and occasional giant (multiflagellar) individuals.

8.1.1.3 8/9 day infections

Parasitaemia observed by the agar technique
showed that between 80 and 90% of the parasites were granular. Processing for TEM was carried out either on day 8 or 9 depending upon when maximum granularity occurred but there was little obvious difference in TEM appearance between parasites harvested at this time and parasites harvested when the majority were agranular. Some long-narrow forms were still available. Dividing forms included some giant ones and long-flat forms could not be easily differentiated from the stumpy forms which agar technique had shown to be granular.

8.1.1.4 14-day infections

The three forms were shown to be present in the parasitaemia by examination with the agar technique. But TEM consistently failed to show any marked difference between the long-flat and stumpy forms.

8.1.1.5 The Organelles

The selected organelles were the surface coat, microtubules, mitochondria, microbody (glycosome), endoplasmic reticulum, golgi apparatus, flagella with the flagellar pockets, and kinetoplast. On one hand, the occurrence, distribution and morphology of these organelles were compared in the long-narrow with the other forms. On the other hand, the characteristics of the
same organelles in the blood forms were contrasted with those of the tissue forms.

In this study all the forms in the blood possessed the surface coat and microtubules separated by the cell membrane. The mitochondria in the long-narrow was without cristae but the same organelle showed some cristae in the long-flat and stumpy forms (Plate 33b). Intermediate developmental stage with beginning of the cristae in the mitochondria could be deduced from the dividing long-flat of Plate 33c which only had evidence of division only internally as the same flagellar membrane contained the two flagellar assemblies (axoplasts). There was no observed difference between the endoplasmic reticulum (smooth and rough) in the long-narrow and other forms. So also there was no difference observed in golgi apparatus and microbodies. Kinetoplasts also looked the same in all forms. Some micrographs showed the flagellar pocket in longitudinal section with endoplasmic reticulum connected to the kinetoplast. Dividing kinetoplasts were also observed (Plate 33d). Plate 33a confirmed the multiflagellar giant forms could be in blood as well as in other parts of the body. Not all the three flagella shown here would have been revealed from the external morphology.

8.1.2 The forms found in tissues

The occurrence of extravascular trypanosomes
in peritoneal and ascitic fluids has been reported in Chapter 4. In this chapter the result of the investigation into the location of trypanosomes in the extravascular tissues will be considered.

Initially lung, liver and choroid plexus were chosen for preliminary TEM undertakings. There was no success from the lungs. Only one incident of the liver infection shown in Plate 34 could be established.

It took time and effort to locate and identify the sites of the parasites in the choroid plexus. An histological undertaking of the tissue was essential. A brief profile is therefore illustrated so that specific sites of the parasites will be easier to explain.

8.1.2.1 Location, Morphology and Histology of Choroid Plexus

The choroid plexuses from the lateral ventricles obtained by bisecting the brain anteroposteriorly and picking out the plexus with forceps through the third ventricle. Plexuses from fourth ventricles were difficult to obtain and only occasionally processed. From evidence of varying amount of tissue only a part of each choroid plexus could be extracted at a time. The bigger the rat the more likely it was to get almost the whole piece from each ventricle. One rat which weighed 959 gm gave the pair of Plate 35. The amount and size were unusual.
Plate 36a shows a general histological section of what Ham (1969) designated as the "leaves" of the choroid plexus consisting of epithelial cells with blood vessels in between. Plate 36b gives further precision of an epithelial cell sometimes referred to in the literature (Rhodin, 1974) as modified ependymal cell. The nucleus is centrally located and conspicuous and each ependymal cell is fringed on the outside with microvilli and may be lined on the inside by a blood vessel as in Plate 36c. There are many mitochondria in each ependymal cell and these mitochondria are enriched with well-developed cristae (Plate 36c). An observation at higher magnification (Plate 36d) of the blood vessel area in a section of the choroid plexus of a clean young rat (the same age as most of the experimentally infected animals) shows a gap surrounding the vessels and is not clear whether it is the basement membrane of the ependymal cell or interstitial matrix of the tissue. However it was noted that this gap was not as wide or clear-cut as it was in infected animals. Two other cells not found in the literature describing the choroid plexus were also encountered. The first is the fibroblast which produces the collagen fibres. The second is the astrocyte. It is not easy to differentiate one from the other (see Plate 37a) as both surround the blood vessels unless when enough portion of the fibroblast is shown, in the section, to contain collagen fibres. These two
cells must not be confused with the endothelial cells which make up the blood vessel wall. Usually the blood vessels in the choroid plexuses as shown in Plates 36a, c and d are small arteries and veins. But occasionally an afferent arteriole thick enough to have the incomplete internal elastic membrane (a term from Rhodin, 1974) was located as depicted in Plate 37b. So far no literature has been found associating this with the choroid plexus.

Pieces of tissues from the roof of the ventricles (Plate 37c) or parts of pia matter of the brain (Plate 37d) could be extracted along with the choroid plexuses. These must not be confused with the choroid plexuses. There is also a particular need to understand that the presence of cilia as in Plate 37c shown in TEM by formula $9 + 2$ as the flagella of parasites would equally show, does not indicate the parasites in the tissue. Though Rhodin (1974) would not associate cilia with the ependymal cells of choroid plexus this study has shown beyond doubt that a few of the ependymal cells in the choroid plexus were ciliated (Plate 38a), although not as massively ciliated as the ependymal cells of the ventricles (Plate 37c).

The preliminary part of this work has shown that the sections examined did not go deep enough into the choroid plexus as shown in Plate 38b. The significance of this observation will be discussed later in this chapter.
8.1.2.2 The Parasites in the Choroid Plexus

Four attempts were made to fix, embed and cut sections from 4-day infections but no parasite could be identified with certainty by TEM observation. Since the parasitaemia patterns of Botswana strain (see Fig. 3a of Chapter 3) suggested that there might be an abundance of tissue forms going into the blood system as agranular forms between days 14 and 16 and between days 22 and 28, it was decided that materials from infections at these periods should be examined. One infected rat sacrificed at day 14 and three out of four at days 24 to 26 produced the following results.

8.1.2.2(a) 14-day infection

Both intra- and extra-vascular forms were found (Plate 39a). Some blood vessels were without parasites or blood corpuscles (Plate 39b) presumably because of draining of most of the blood prior to dissection of the skull for the extraction of the choroid plexus from the brain. Others not affected by drainage (Plate 39c) confirmed the existence of a high parasitaemia previously noted by agar technique and as expected from Figure 3a. Platelets appeared to be particularly numerous (Plate 39a).

A closer observation of some parts of Plate 39b
and Plate 40a showed that parasites were located in the immediate amorphous matrix surrounding the blood vessels and some in the basement membrane and some had moved further into the denser matrix nearer to the modified ependymal cells. Others had even moved into cells (Plate 40b) between the blood vessel and the ependymal cells. These host cells could not be distinguished as to whether they were astrocytes, macrophages or the precursors of macrophages known as microglia in brain tissues. Whatever the nature of these cells, they seemed to be phagocytic and might either be free of parasites as Plate 40a or with their cytoplasm containing parasites as in Plate 40c. The presence of so many flagellar profiles in and around the large parasite shown in Plate 40b suggests that it might be a giant form reminiscent of those found in the peritoneal fluid but this is not certain. That the parasites penetrated far beyond the interstitial matrix or basement membrane was confirmed by Plates 41a and b.

Pathological destructions and reactions were not obvious in some sections, e.g. Plates 40b and c. In others oedema exhibited in the increase of the amorphous space between the vessel and ependymal cells and general inflammation was significant (Plate 42a). Degeneration of mitochondria was common (Plate 42b). Loss of chromatin, excessive vacuolation and formation of myelin figures were visible as in Plate 42a. Haemorrhages were
also observed (Plates 42b and c). Macrophages were sometimes found trying to mop up the internal haemorrhage as in Plate 42b.

From the study of the organelles there were no peculiarities observed from endoplasmic reticulum, microbodies and golgi apparatus. The surface coat was still retained in the tissue and the microtubules were also normal. The mitochondria were without cristae.

8.1.2.2(b) Infections at 24-26 days

The results were similar at 24-26 days to those obtained for 14-day infections although some specific additional information was obtained. Intra- and extravascular forms were repeatedly found in the leaves of the choroid plexus (Plate 43b ) and the "sinuses" also showed infection (Plate 43a ). Niduses of parasites were located both in the immediate vicinity of the blood vessels in the amorphous matrix (Plate 43c ) as well as deep down in the epithelial layer (Plates 44a and b ). In the tissue proper two points need to be emphasised. The first was the overwhelming intensity of infection in that numerous "nests" (Sanabria and Aristimuno, 1969) of parasites were tightly packed in the tissue as shown in Plate 45a. The second was the clear cut evidence of the intracellular existence of the parasites as seen in Plates 45a - c. Higher magnification of some parasites showed that they
were enclosed in a membrane or envelope separating them from the general cytoplasmic contents of the host's cell (see Plates 45b and c). Even where both parasites and red blood corpuscles were found in the tissue the parasites appeared to be in good condition whereas the red blood corpuscles seemed to be undergoing dissolution (Plate 46a). The parasites in some cases were multilagellar (Plate 46b) proving to be similar to those in the peritoneal fluid and evidence of undergoing multiple division, yet surrounded, in the host's cell, by mitochondria, vacuoles and endoplasmic reticulum of the host's cell (Plates 44a - 46c).

While a number of the intracellular dividing forms could be interpreted as multiple division or giant forms, normal trypomastigotes did also occur (Plate 44a - 46c). Morphological details of the organelles did not vary from what were observed from the parasites at day 14. The surface coat and microtubules were maintained as in Plates 48a and b. Microbody with single membrane and mitochondrion with double membrane but with no cristae, were as in the former infection.

From pathological point of view, the production followed by destruction of the collagen fibres were as earlier described (Plates 48c and d). Not only were the usual blood corpuscles found infiltrated into the tissue, plasma cells were also present in the tissue. Some parasites were found located in the meninges.
It has been established in Chapter 5 that Botswana strain trypanosomes could be cleared in the vascular system by SHAM and glycerol but recrudescence later occurred. It has also been established in Section 8.1.2 of this chapter that extravascular tissue forms were located in the choroid plexus. Consequently it was planned to find out if TEM would reveal any parasite still present in the choroid plexus after treatment with SHAM and glycerol. The TEM materials were from infections at 5 days, 12 days, 18 days, 27 days and 32 days after the inoculum had been introduced. Parallel controls of untreated plexuses were also processed and observed. Blood from treated animals and parts of treated and untreated choroid plexus given i.p. to mice were used as indicators of availability of any infective forms.

8.1.3.1 5-day Infections

TEM of choroid plexuses from treated and untreated animals showed parasites that were not clearly recognisable. Parts of cells which might be trypanosomes were found in both but uncertainty as to their identity remained. However both treated and untreated choroid plexus showed inflammation and both showed enhancement in the production of collagen fibres. Platelets in the
blood vessels were also observed. Teased pieces of treated choroid plexus examined by wet slide and agar technique did not show any parasites. There was no tissue left from treated animal but pieces of untreated choroid plexus resulted in an infection in one mouse.

8.1.3.2 12-day Infections

Doubt was still entertained in identifying parasites in the choroid plexus of both treated and untreated animals. However inflammation and enhancement of collagen fibres was clearly present in both treated and untreated choroid plexuses. Pieces of choroid plexus injected into mice produced infection. Two of three mice injected with choroid plexus from treated rats contracted the infection. The two mice infected with untreated choroid plexus both became infected. No infection developed in the two mice injected with blood from the treated animal.

8.1.3.3 18-day Infections

TEM revealed from untreated choroid plexus the same forms of parasites as have been described in normal infections of 8.2. Trypanosomes were found both in the tissue and the blood vessels. In the case of the treated choroid plexus no parasites were found in the blood vessels confirming that the blood clearance was complete. In the tissue, parasites were found in both the matrix (Plate 49a) as well as in the cells of the choroid plexus (Plate 49b).
These parasites were still under the influence of the drug. There was no means in this study to show whether any of these had been killed. Those close to the blood vessel (Plate 49b) seemed to be more badly affected. Destruction of the collagen fibres was evident and lymphocytes were found in the tissue. Plasma cells (Plate 49b) as well as mast cells were also observed in the tissue. Meninges also showed some parasites not destroyed by the treatment (Plate 50a).

Blood from treated animals was not infective to two mice into which it was injected. Both treated and untreated choroid plexuses gave infections to mice. One of the two mice infected with untreated choroid plexus was positive. In the case of the treated choroid plexus one of the three infected mice showed infection six days after the inoculum had been given. The usual period for infections in mice injected with choroid plexuses, to be revealed was 5 to 7 days but one mouse showed a prepatent period of 17 days after being injected with a choroid plexus.

8.1.3.4 27-day Infections

TEM examination of the untreated choroid plexus was as expected in that the parasites were in batches and the forms were as described before. In the treated choroid plexus were also parasites but it was difficult to recognise them because of the effect of the drug still present. High power resolution, however, proved them to
be parasites as in Plates 50b and 51, and some were actively dividing. Intracellular multiple division forms with as many as eight or more flagellar profile as shown in Plates 51a and b were found. There was evidence that some of the parasites close to blood vessels were being lysed.

Pieces of choroid plexus from both treated and untreated animals produced infections in mice. Two mice with choroid plexus from untreated animals became infected. One out of three mice given choroid plexus from treated animal also became infected. Blood from a treated animal failed to produce infection in any of two mice. Some ependymal cells were found ciliated as earlier described. Haemorrhage not known whether caused by the parasites or the drug was also noted (Plate 52b).

Observation of the living forms, teased into saline solution from the treated choroid plexus, with the aid of agar technique, showed these to be agranular.

8.1.3.5 32-day Infection

A large rat weighing 759 g (whose choroid plexuses were demonstrated in Plate 34) was infected and treated at 32 days. No control was used for this experiment. TEM confirmed the presence of parasites which were often dividing or giant forms (Plate 52a). In addition to the usual pathological manifestations internal haemorrhage in the choroid plexus was heavy with many red
blood vessels freely found in the tissue. Note was taken of a fibroblast with single cilium present in the choroid plexus (Plate 52c). The first record of this phenomenon in vivo was made by Brooker et al. (1971) but its significance is unknown.

8.2 DISCUSSION

Despite considerable effort, little additional data on the morphology of bloodstream forms, beyond that already obtained by Vickerman (1965, 1969a, 1969b, 1970, 1974) and by Steiger (1973) and by others, was obtained; neither was the hypothesis of Ormerod (1979a) of a whorl-shaped endoplasmic reticulum being an essential characteristic of the long-narrow form, confirmed or refuted. However, what was achieved is commented upon in Section 8.2.1.

On the other hand, work on the tissue forms has generally been successful in that more was found than had been previously envisaged. The locations of the formerly controversial extravascular forms were clearly depicted and the existence of the intracellular stage was established for T.b. rhodesiense from Botswana. This work entailed the cutting of over three thousand sections, their mounting and observation in four models of electron microscope; more than 600 micrographs were processed. Those exhibited are a representative selection but all pictures helped in
the cumulative understanding and interpretation of the histology of the choroid plexus and the location of the parasites therein. Section 8.2.2 gives the specific comments on the tissue forms.

8.2.1 The Blood Forms

Although not much additional information could be attributed to this project as far as the organisation of the internal organelles of the long-narrow form is concerned, the TEM study confirmed the observations made by LM and SEM. The long-narrow was shown to be circular in transverse cross-section at any part of the body demonstrating that it was cylindrical and not flat like the long-flat and stumpy forms. It should be pointed out at this juncture that other trypomastigote forms are also tubular between the flagellar pocket and posterior extremity, but it is only the long-narrow of *T. brucei* that is found to be tubular all along its length.

The hypothesis that the kinetoplast (with its associated flagellum) which is formed posteriorly, is the daughter, was supported by TEM observation. That it is the kinetoplast and not the basal body that divides first in the dividing trypanosomes conforms with the review of Hoare (1972) but the designation of which is the daughter was absent from available literature.

The mitochondria observed in both the long-narrow as well as the long-flat were without cristae and this
observation agrees with Vickerman (1974), Steiger (1973) and Kilgour (1980). It may be added that the absence of cristae per se is not the significant factor but probably the overall amount of mitochondrial material present and total surface area available for metabolic exchanges. Not only did these mitochondria lack cristae they were single and roundish. On the contrary the mitochondria of the stumpy form were large and extensive in addition to being with the cristae.

The observation on the mitochondria and other organelles especially the surface coat was necessary for logical comparison of the same strain used for both blood and tissue forms (see Section 8.2.2).

8.2.2 The Tissue Forms

The debate on the existence of the tissue forms had been highly controversial and confusing although the search for them started as soon as Shortt and Garnham (1948) had discovered the exoerythrocytic stage of malaria parasites. Different words had been used by different authorities. These in alphabetical order included amastigote phase (Peruzzi, 1928; Deane, 1969; Ormerod and Venkatesan, 1971a) cryptic phase (Ormerod, 1979a), occult visceral form (Ormerod and Venkatesan, 1971a) and oval forms (Gallais et al., 1953), plasmodial stages (Ottolenghi, 1910; Bradford and Plimmer, 1902); spherical forms (Gallais et al., 1953; Ottolenghi, 1910); tissue stages (Ormerod,
1979a), unrolling form (Sant'ana, 1913; Penso, 1934; Peruzzi, 1935; Ormerod, 1979a) and visceral form (Ormerod, 1979a; Lumsden, 1972). Similarly tissues and cells associated with the tissue phase had been widely distributed. These were cerebrospinal fluid (Castellani, 1904), lung (Salvin-Moore and Breinl, 1908; Philippenko, 1929), liver (Soltys and Woo, 1969, 1974; Soltys et al., 1969), heart (Peruzzi, 1928) and even inside the mononuclear cells especially the macrophage (Soltys and Woo, 1969) and red blood cell (Penso, 1934; Peruzzi, 1935).

Because of the uncertainty of its existence the recent emphasis in terminology is noted to have shifted from "tissue phase" to "extravascular form". Thus Ikede and Losos (1972a, b) Losos and Ikede (1972), Ellis and Ormerod (1973) and van Marck (1980) seemed to prefer the latter to the former. Mattern et al. (1972) and Choudhury and Misra (1973) still maintained that the amastigote forms of T. brucei and T. evansi respectively existed in the choroid plexus of the hosts. Similarly Jennings et al. (1979) supported the idea of the existence of the tissue phase in the brain which they upheld, by chemotherapeutic experiments, to be the source of relapsing trypanosomiasis. Although Ormerod (1979a) modified the original idea (Ormerod and Venkatesan, 1971b) about the existence of the amastigote form in the tissue and although he changed his mind about the choroid plexus likely to be the specific or most important site for the tissue stages
he (Ormerod, 1979a) committed himself to the hypothesis that an active multiplicative phase outside the blood circulation is responsible for the production of the non-dividing first stage blood form designated the long-narrow. The results of the current investigation support this hypothesis.

From the foregoing brief review it becomes obvious that the significance of this result lies not in the extravascular occurrence of trypanosomes for this has been demonstrated clearly before (Ikede and Losos, 1972a, b; Losos and Ikede, 1972; Ellis and Ormerod, 1973; van Mark et al., 1980) but in the knowledge about the different locations (8.2.2.1), the illustrations of the morphology of the forms by TEM (8.2.2.2) and particularly in the demonstration of intracellular parasitism, the implications of which will be discussed in 8.2.2.3.

8.2.2.1 Locations of the parasite

(a) Technical factors in locating intracellular forms

Several technical factors appeared to be of importance in the location of intracellular forms in the choroid plexus.

1) Since blocks are cut initially from the outside, no parasites were encountered until the outer layers had been removed. With experience it was found that the surface of the choroid plexus could be removed by thick
sectioning before the parasite level was reached.

2) It was noted that in a normal infection the parasite was established in the choroid plexus as early as 72 hours but enough parasites were not available for successful experiments with SHAM and glycerol until 2 weeks after inoculation. However once the infection had been established parasites could always be found although whether this continuity was due to persistence of the first or subsequent invasions was not clear (arguing from the work of Jennings, and colleagues the increase is likely to be due to subsequent invasions).

3) More than $10^6$ parasites were always given in the inoculum and this probably ensured a substantial initial invasion of the tissue. As Silva and Nussenzweig (1953) and Brener and Chiari (1963) have shown with T. cruzi infection depends on the number of parasites injected. Lima-Pereira (1976) interprets this result as being due to greater tissue penetration and this may also apply to tissue penetration by T. brucei.

4) The strain used in the experiments in which intracellular forms were found in the choroid plexus had recently been passaged through tsetse. This passage had increased its virulence and perhaps also its ability to penetrate tissue.

(b) The sites and cells invaded

Apart from the intercellular spaces and the
basement membrane, parasites were found in cells identified as fibroblasts, astrocytes (or pericytes), microglia, macrophages and ependymal cells. These, as will be shown, are both phagocytic and non-phagocytic cells. As shown in the results the identification of some cells or parts of the cells encountered was inconclusive. The uncertainty in the observation emphasises the need for more ultrastructural studies for histological elucidation of the choroid plexus as the data available in the literature are inadequate. However, from what little is available all the cells specified above appear in other parts of the brain, viz. in the nervous tissues as distinct from the choroid plexus which is essentially a non-nervous tissue (Rhodin, 1974), being a modified extension of the leptomeninges.

i) Astrocytes and Pericytes

Ham (1969) described the blood capillaries in the brain as having a basement membrane around the endothelium with the astrocytes having processes adhering to the capillaries to give rigidity. The astrocytes radiate in various directions extending from capillary to capillary and from capillary to neuron. Since there are very few neurons in the choroid plexus astrocytes are seen to form a network. The major function of the astrocyte is said to be the transfer of nutrients from the blood vessel through its perivascular foot attached to the blood vessel
and the processes (Ham, 1969). According to a theory
given by the same author, astrocytes also form the blood-
brain barrier with their perivascular foot. Bradbury
(1979) has added the occurrence of the ependymal cells
to the effective nature of the blood brain barrier.

Cells similar to the astrocytes on the venules
are called pericytes (Ham, 1969). They have been shown
to be capable of becoming phagocytic. It has also been
suggested by the same authors that pericytes may help in
making the basement membranes and perhaps even collagen.

ii) Microglia

In the body, connective tissue plays both a
role of support and also contains mobile cells with the
ability to phagocytose any foreign material which happens
to be present (Ham, 1969). There is no connective tissue
in the brain, but while the astrocytes play the key role
of support, the microglia play that of defence comparable
to that of macrophages in other organs. Under normal
conditions microglia are small cells evenly scattered
and show little indication of mobility and phagocytosis.
But if there is a local emergency such as a wound or
inflammation, as occurs in trypanosomiasis, dramatic
changes take place. These microglia acquire the ability
to divide and enlarge and they may become mobile, and
filled with phagocytosed material. There is controversy
about the origin of microglia. Most authors as reviewed
by Ham (1969) believe that they come from blood probably by transformation of monocytes. However, some interference to their entry is due to the capillary basement membrane. When they pass across the endothelial cell barrier they become enclosed by an expansion of the basement membrane of the capillary. Therefore they are sometimes described as "perithelial cells". Since these cells under such conditions could be similar to the astrocyte or pericyte, the possibility of confusion in the identity of the cell around the blood vessel, as was experienced in this work, is readily understood.

iii) Fibroblasts

Fibroblasts are characteristic of collagen fibres but since, as has been noted, the pericytes could also produce collagen fibres pieces of the cell could also be confused with the astrocytes and pericytes. It may be significant that until the fibroblasts were incorporated into the culture media pleomorphic bloodstream forms of trypanosomes could not be produced in vitro. It is also noteworthy that Ikede and Losos (1972a, b) concluded that T. brucei were parasites of connective tissue where fibroblasts were abundant.

iv) Ependymal cells

According to Bradbury (1979) the choroid plexuses are infoldings of the lining of the neural tube
and the choroid epithelium is continuous with the rest of the ependyma. That means the modified ependymal cells of the choroid plexus are similar to the internal surface of the brain as well as the surfaces of the meninges.

The ependymal cells as epithelial cells are furthest from the blood vessel through which the parasites invade the tissue. The histology is clear and it is in these cells that the parasites are best observed as intracellular organisms. As far as is known they are non-phagocytic.

8.2.2.2 The parasite forms

Soltys and Woo (1969) claimed to have detected amastigote, promastigote and epimastigote forms from impressions of liver and spleen of mice and rabbit infected with *T. b. brucei*. Ormerod and Venkatesan (1971b) reported amastigote forms from the choroid plexus by light microscopy but electron microscopy work (Ellis and Ormerod, 1973) was unable to confirm this form. Ikede and Losos (1972a, b) identified the extravascular parasites of *T. brucei* as trypomastigotes. Van Mark (1980) demonstrated by TEM trypomastigote forms in the intercellular spaces of choroid plexus infected with *T. b. gambiense*. In this work only the trypomastigote form was identified in both intercellular spaces and cells but the possibility of other forms could not be ruled out. Further study specifically designed to
clarify the forms is necessary.

It is interesting that both extravascular and intracellular parasites have a surface coat and this suggests that they may be able to change and retain different surface antigens at the various sites. This would explain the work of Seed and Effron (1973) who isolated different antigenic variants from different sites of the brain. Similarly the primitive nature of the mitochondria without cristae places the tissue phase more akin to the early agranular stages especially the long-narrow form believed by Ormerod and Venkatesan (1971a) to be the first form to enter the blood from the tissue stage. This, in future work, could be tested with Tritium-labelled trypanosome and TEM autoradiography using the technique of Rechenmann (1967).

The entry of *T. brucei* into the host cell and its continuing existence therein resembles what obtains in *T. cruzi* in four aspects. Firstly, the process is not phagocytic as in *Leishmania* (Zenian, 1979); unlike that parasite, *T. brucei* in the peritoneal fluid shows no lamellar protrusions in any section. The entry of *T. brucei* seems, like *T. cruzi* (Tanowitz et al., 1975), to be by active penetration. Secondly, contrary to the condition of *Leishmania* spp. (Rudzinka et al. 1964; Trager, 1974; Alexander and Vickerman, 1975) where two membranes are found, there is only one membrane observed, as in *T. cruzi* (Sanabria and Aristimuno, 1969) surrounding the
individual or nests of parasites before they break free into cytoplasm; in short, there is no parasitophorous vacuole. Thirdly, as in *T. cruzi* (Sanabria and Aristimuno, 1969), the nucleus of the host cell is unaffected in this strain of *T.b. rhodesiense*. Fourthly, there is no fusion between the phagosomes and lysosomes. *Leishmania* exhibit the fusion (Alexander and Vickerman, 1975), but *T. cruzi* does not (Sanabria and Aristimuno, 1969).

8.2.2.3 The implications of intracellular occurrence

Not all the parasites in the tissue are intracellular. While it is arguable that those in the basement membrane are intracellular, those in the intercellular matrix are definitely outside the cells. But so also are some amastigotes of *T. cruzi* to be found in between the cardiac muscle cells and intercellular spaces of mice colon (Tafuri, 1980). The colonies or groups of *T. brucei* parasites are similar in location to those described for *T. cruzi* as nests by Sanabria and Aristimuno (1969) and as cysts or more strictly as "pseudocysts" by Anselmi and Moliero (1974). Other similarities between *T. brucei* and *T. cruzi* have been considered in Section 8.2.2.2. Intracellular parasites undoubtedly have physiological, biochemical, immunological, life-cycle, pathological and chemotherapeutic implications but no physiological or biochemical data were obtained in this project and the
only relevant observation on the nature of the mitochondria has been noted. So also is it premature to make further comments on the possession of the surface coat and its immunological implications. But these aspects need to be studied further.

a) The life cycle of T. brucei in the vertebrate host

Until the extravascular location of the African trypanosomes was recently accepted the orthodox assertion was that they were all confined to the bloodstream where they multiply by longitudinal binary fission. Losos and Ikede (1972) divided the African trypanosomes of human and veterinary importance into two categories. The haematic group was said to include Trypanosoma (Duttonella) vivax and T. (Nanomonas) congolense because, it was claimed, these species were confined to the plasma and blood vessels. The second category was the humoral group which included all the three subspecies of T. brucei on the understanding that they were extravascular but not intracellular. Thus the latter group was distinguished from T. cruzi. Luckins and Gray (1978) refuted the inclusion of T. congolense in the haematic group by proving with TEM the extravascular occurrence of the parasites in the skin connective tissue of rabbits and recently the extravascular occurrence of even T. vivax in cattle has been proved (van den Ingh and de Neijs-Bakker 1979).

There were also other former TEM records of
extravascular locations. They included Ellis and Ormerod (1973) who demonstrated T.b. rhodesiense in choroid plexus and meninges, Tanner et al. (1980) who sited the parasites of T.b. brucei in the lymph nodes of rats and van Mark et al. (1980) who showed T.b. gambiense in the intercellular matrix of choroid plexus of mice. In showing the intracellular nature of T.b. rhodesiense strain the current record adjusts the life cycle of T. brucei to be similar not only to that of T. cruzi, as has been discussed, but also to that of the malaria parasite (Ormerod, 1981). It is not known yet whether the invasion of the cells of choroid plexus is once and for all and the recurrence of infections shown in the bloodstream is associated with what had been termed "hypnozoites" in Plasmodium vivax or if there is repeated invasion of the tissue by the blood form. Similarly, no claim based on the current work can be made of the choroid plexus being the exclusive tissue for intracellular establishment. Other tissues need to be more thoroughly investigated using not only a strain isolated from human sources but other strains of T. brucei and other species of animal trypanosomiasis.

b) Pathology

Greenwood and Whittle (1980) emphasised that up till now little is known about the causes of the
observable pathological features of sleeping sickness.

It is hoped that with the result of this project the approach to the understanding and interpreting pathological observations of *T. brucei* will alter.

The increased permeability of the capillary vessels with the presence in blood of the pharmacologically active substances reviewed by Boreham (1979) and biologically active products detailed by Tizard et al. (1978) leading to observed oedema of van Mark (1980) are all possibly explained by the invasion of the host cells via the blood vessels as shown in this work. The records of Ormerod and Venkatesan (1971b) and Ellis and Ormerod (1973) would appear to be complementary to the current finding of dead parasites probably killed phagocytotically and intracellularly. These dead parasites might block some blood vessels in the choroid plexus as the host makes an effort to get rid of them. This also agrees with the observation of Tafuri (1979) in that some amastigotes of *T. cruzi* are killed inside the host cells as well as in the interstitial matrix.

The cause of anaemia has been described by Jenkins et al. (1980) as "multifactorial" and one possible factor was said to be increased cell destruction which might be non-specific consequence of the expanded mononuclear phagocytic system (Urquhart, 1980). The current observation of phagocytic activities in the peritoneum and tissue may be relevant to such a factor. Perivascular cuffing usually observed around the blood
capillaries is explicable by the entry and exit of parasites in and out of host cells and consequent leakages similar to what has been envisaged for T. cruzi (Vickerman, 1974).

The cause of tissue degeneration especially the choroid plexus, of the current study, will be obvious. The functional ability of an invaded cell with as many as six or more parasites will not only be impaired but stopped when the parasites are released out to go into the blood circulation. Mott (1906) observed that the main difference between sleeping sickness and general paralysis of the insane was because of absence of the damage to the brain. Ormerod (1970) noted that some damage occurred in advanced cases. The result from the present study makes the observations of these two workers complementary rather than contradictory to each other. Firstly, no nerve cells are destroyed as far as the result shows. This does not only justify the observation of Mott, it also distinguishes T. brucei pathology from that of T. cruzi in that the latter pathogen enters and destroys nerve cells. Secondly, although some parasites are established in tissue as early as the first 72 or 96 hours because both treated and untreated choroid plexuses injected into new hosts produced infections, the number of parasites was so small and the amount of damage negligible that TEM could not
detect any with certainty. But after two weeks onwards, in these laboratory models, the parasites were many and destruction in cells was visible. This may be equivalent to the advanced cases of Ormerod (1970). Detectable degeneration of host tissues and cells could not occur until the parasites have established, grown, multiplied and were leaving the cells for the blood.

c) Chemotherapy

It is not expected that the knowledge about the intracellular nature of *T. brucei* will provide a more effective drug immediately for in spite of such knowledge about *T. cruzi* and *Leishmania* spp. the search for more effective drugs continues. But the approach should change. The common intracellular adaptation in the three groups of parasites should be accepted. It is significant that Peters (1980) could not include *T. brucei* in the recent review of therapy of intracellular parasitic infections. The general consideration in planning chemotherapy against the African trypanosomiasis has been hitherto based on metabolic pathway of the bloodstream forms only (Clarkson and Brohn, 1976; Bowman and Flynn, 1976).

It might be puzzling when Ham (1969) summarised the opinions of authorities as meaning that the choroid plexus as a whole was considered a cerebrospinal-blood brain barrier and yet only gave details of how the cell membranes of the endothelial cells of the vessels performed
such a role. But with the revelation of Bradbury (1979) that the ependymal cells of choroid plexus also contribute significantly to the barrier effect a better perception of the phenomenon of the blood-brain barrier is obtained. In this work it has been shown that SHAM penetrated into the matrix of the choroid plexus and appeared to have killed some extracellular tissue parasites. It also went further into the cells containing some parasites but whether these parasites were killed or not could not be ascertained in the TEM observation. But since successfully treated animals transferred the infection through the injected pieces of choroid plexus it was certain not all the parasites in the cells were killed by the treatment. It would appear that the rate and the amount of inflow of drug into the choroid plexus are significant in the killing of the trypanosomes in the cells of the choroid plexus.

Amole and Clarkson (1980) observed that the percentage of parasites killed by SHAM and glycerol was related to the amount of serum present. The parasitic forms in the blood i.e. those with the greatest amount of serum were successfully eliminated. Those in the intercellular spaces outside the vessels would have less contact with serum constituent and it is probable the effect of the drugs would be less than in the blood. The parasites in the ependymal cells far away from the blood vessels would be least affected. Moreover, the
rate of inflow of glycerol into the tissue and cells is known to be much slower than that of SHAM (D.A. Evans, personal information). So it is possible that the effect of SHAM on aerobic respiration could have been decreased by its removal from the ependyma before enough glycerol filtered in to disturb the anaerobic metabolism. With both possibilities the parasites inside the cells could eventually survive the efficacy of the drugs.

While the possibility of a resistant blood form in the first hypothesis of Evans and Brightman (1980) is inherently unlikely (see Section 5.2.2.1.) the second postulation, that the cryptic parasite might be in tissue, is upheld by this project.

The location of the intracellular parasite in the choroid plexus may also explain why the Berenil used in treating mice affected by cerebral trypanosomiasis by Jennings et al. (1979) gave rise to new infection. This tendency to relapse probably occurs because the drug fails to penetrate in sufficient amount, if at all, into the cells where the parasites lie. Thus the brain in general and choroid plexus in particular is likely to be the source of relapsing T. brucei infections.
CHAPTER 9

GENERAL DISCUSSION

In previous chapters of this thesis a central theme runs through all, binding the different issues discussed, together as an entity. The central theme is the source of parasites occurring in the first wave of infection and at subsequent relapse, and the different issues are as follows:

(a) the forms of parasites found at different sites;
(b) the differentiation between immunosuppression caused by trypanosomiasis and immunodepression induced by external agents during the disease;
(c) comparison and contrast between phagocytosis and cell penetration in the choroid plexus;
(d) origin, identity and role played by the long-narrow form; and
(e) the pathological significance of the invasion of the choroid plexus tissue.

9.1 FORMS OF PARASITES FOUND AT DIFFERENT SITES

It has been shown that the earliest population of the parasites in blood of both normal and immunodepressed infections, has no lipoprotein (type II) granules. Recrudescent population after the initial parasites had
been cleared by SHAM as well as cyclically transmitted population confirm it. The lipoprotein granules develop one or two granules at a time in each trypanosome in the early population as the population turns from agranular to granular forms and thence the granules increase in number, size and density until the characteristic number and pattern for each strain or species are attained. These granular parasites eventually become moribund and Ormerod (1979a) considers that they represent a "dead end" in the cycle (Chapter 3).

The infection in normal peritoneal fluid and the enhanced population in ascitic fluid show the same differentiation of forms, but in addition the big or giant forms which are relatively infrequent in blood, are frequently seen in peritoneal fluid as was first demonstrated by Ottolenghi (1910). These forms were also seen in this study and are illustrated by light microscopy in Chapter 4. They appear to be similar to the huge multiflagellar forms seen by TEM in choroid plexus but these forms have not been seen previously by light microscopy, either by phase contrast or in stained sections, because, being intracellular in location, they have presumably escaped detection through the difficulty of differentiating parasite from host cell. Probably the anatomical metamorphosis from parasites with one or two nuclei and other organelles to "multiple division forms" is the result of biochemical or physiological changes in parasites which can either be in blood, tissue fluid or intracellular
Future work will determine the nature of this change and whether genetical exchange or sexual dimorphism discussed by Noble (1955) and perceived by Tait (1980) could take place in the intracellular stage in mammal rather than in fly.

That SHAM and glycerol cleared the parasites from the blood as well as in the peritoneal fluid but failed to eliminate the intracellular form may not be due solely to intracellular protection but also to lack of a serum factor which would be more readily available in blood for the effective performance of SHAM (Amole and Clarkson, 1980). It may be that the morphological complexity of the giant form is not the important feature as this form was numerous in both extravascular fluid and tissue and also available in the blood. Since the round-bodied multinucleate form in the peritoneal fluid was small enough to pass through the blood capillaries it could be suggested that that form is the origin of the giant form of the vascular system. But the site especially the intracellular condition in the tissue represents a crucial difference (see Section 9.3).

It is significant that no granular forms were detected in the SHAM-treated choroid plexus. Granular as well as agranular parasites were observed in the untreated tissue but it was likely that the granular forms were from the blood vessels. Thus it could be suggested that the acquisition of the granules takes place
in the blood, although this should not prejudge the issue as to whether biochemical changes may also take place before the trypanosome enters the blood.

9.2 IMMUNOSUPPRESSION CAUSED BY TRYpanosomiasis AND IMMUNODEPRESSION INDUCED BY EXTERNAL AGENTS

It is essential to distinguish between the immunosuppression caused by the disease and immunodepression that is induced experimentally. The former was not studied in this project, only the latter.

Low and Castellani (1903) observed that patients with sleeping sickness were more susceptible to secondary infection and Urquhart et al. (1973) described the immunosuppression in T. brucei infection using secondary infection of a nematode in rats and oxazalone hypersensitivity in mice. These two examples differ from the current experiments where rats were x-irradiated and given dexamethazone to induce enhanced parasitaemia of vascular trypanosomes, but stress the relevance of such laboratory procedures.

9.3 PHAGOCYTOSIS AND CELL PENETRATION BY PARASITES IN CHOROID PLEXUS

Both phagocytosis by macrophages observed in the peritoneal fluid and the penetration of host cells by
parasites in the choroid plexus exhibit intracellular location. But while phagocytosis is a phenomenon for getting rid of parasites by host cells and a reaction by the host to parasitism, the parasites in the choroid plexus cells have actively entered the host cell before undergoing a metamorphosis as a phase of the life cycle: the nature of this transformation still remains to be studied.

The parasites in phagocytosis are destroyed by host cells but the intracellular organisms in the choroid plexus are destroying the host cells and actively multiplying by multiple division; many were in colonies. In phagocytosis the parasite is engulfed by outward growth of the macrophage. But it appears that the parasites in choroid plexus have actively penetrated into the cells. Future work may reveal the mechanism of entry and exit of the parasites of the choroid plexus.

9.4 THE ORIGIN, DESIGNATION AND ROLE OF THE LONG-NARROW FORM

The long-narrow form was found in the blood, peritoneal fluid and choroid plexus. In each of the sites there was active multiplication especially the multiple division of the giant form. There appeared to be no long-narrow forms in the earliest population derived from the cyclical transmission where also the giant form was absent. It appears that the long-narrow
form is a product of multiple division of the giant form. Like its progenitor, the giant form, it is not the morphological configuration of the long-narrow that might be significant but the site of production (Section 9.1). Its morphological characteristics had been consistently identical whichever site it was located in. Whether it is produced directly by a sort of budding from the giant form or indirectly through sphaeromastigote as suggested by Ormerod (1979a) its role appeared to be essentially a stage in which binary fission was not involved and that developed into the long-flat which consequently acquired lipoprotein granules to become the granular forms.

From observations on normal parasitaemias, long-narrow forms often occur together with other forms even before the granular forms from the original inoculum have been destroyed. So it still remains to be worked out whether it is the long-narrow proper or its derivatives that are responsible for tsetse infectivity. It also remains to be found out which stage invades and/or re-invades the tissue. With the aid of the agar technique it is easy to type each stage but still difficult to isolate each group exclusively.

9.5 THE PATHOLOGICAL SIGNIFICANCE OF THE INVASION OF THE CHOROID PLEXUS

The search for other tissues where parasites might also be intracellular should continue. In the
meantime it is appropriate to assess the significance of the invasion of the choroid plexus as a tissue, though non-neural, of the central nervous system.

Rhodin (1974) ascribed the production of cerebrospinal fluid as one of the functions of the choroid plexus. From the anamatomical exposition of the tissue with its tufts of capillaries protruding into the two lateral ventricles as well as the third and fourth ventricles the choroid plexus is a major component of the vascular system. The modified ependymal cells with the large central nucleus are not only rich in mitochondria thus signifying its association with efficient respiratory function, but also contain many free ribosomes, granular endoplasmic reticulum and lysosomes. The golgi zone, though not studied in this work, was also reported by Rhodin (1974) to be very prominent. Bradbury (1979) compares the excretory function of choroid plexus to that of the tubules of the kidney, particularly in the exchanges of water and solutes between the blood and cerebrospinal fluid and extraction of organic anions from cerebrospinal fluid and into urine. The choroid plexus is therefore important in the production of cerebrospinal fluid. Its invasion and destruction by the colonies of parasites in its cells will impair the rate of production of cerebrospinal fluid for the central nervous system and will also alter the balance of salt secretion and this may be related to the severe headache associated with sleeping sickness (Ormerod and Segal, 1973).
SUMMARY

1. A comparison of vascular with extravascular forms of the African trypanosome was made with a view to obtaining a better understanding of the cycle of development of the parasite in the vertebrate host.

2. Blood parasitaemia was counted and the pleomorphism of parasites assessed by the agar technique which is considered to be the best method of obtaining quantitative data on the phenomenon of pleomorphism (Section 3.2.1).

3. Parasitaemia was enhanced transiently by dexamethesone acetate and continuously by x-irradiation. The changes produced by these methods in respect of the proportion of the different forms observed was basically the same (Sections 3.1.5, 3.1.6, 3.2.2, 3.2.3).

4. The pattern of the lipoprotein (type II) granules studied in the different trypanosome species of medical and veterinary importance formed the basis of a system of characterization in which these granules in:

   (a) *T. brucei* were concentrated in the anterior half of the body;

   (b) *T. congolense* were few but dense and distributed on both sides of the nucleus;

   (c) *T. vivax* were numerous but diffuse and distributed also on both sides of the nucleus;

   (d) *T. lewisi* were confined to the posterior end of the trypanosome.

(Chapter 6 and Section 3.1.7).
5. The first wave of parasitaemia was studied after the following procedures:
1. infection with blood forms given by syringe;
2. relapse after normal remission;
3. recrudescence after the blood had been cleared with SHAM and glycerol;
4. infection with metacyclic forms from tsetse.

1, 2 and 3 resulted in a wave of agranular trypomastigotes containing a high proportion of long-narrow forms. The results of 4 were inconclusive but there was no obvious initial wave of long-narrow forms.

6. (a) The study, by scanning electron microscopy, of the different species of *Trypanosoma* and strains of subgenus *Trypanozoon* clearly revealed details of external morphology not observed by other methods. It was noted that the majority of the trypanosomes in the blood are flat but the long-narrow form is cylindrical or tubular (Chapter 7, Sections 7.1.1 and 7.2.2).

(b) Phagocytosis studied *in vivo* and *in vitro* with SEM showed that either end of the parasite might be taken in by the macrophage and the phenomenon is by engulfment with the growth of observed lamellar extensions from the macrophage covering the phagocytosed parasite (Chapter 7, Sections 7.1.2 and 7.2.3).

(c) A distinction is made between trypanosomes that
have been phagocytosed and those that have penetrated the cell.

(d) Choroid plexus of untreated infected rats studied ultrastructurally revealed that some parasites, mainly multiple division forms, were intracellular (Chapter 8, Section 8.2.2).

(e) Observation on the hosts treated with SHAM and glycerol confirmed the intracellular location of the parasites (Chapter 8, Section 8.3), that these intracellular forms were able to resist the treatment and were therefore possible sources of recrudescence after treatment.

The different forms of parasites in the blood, peritoneal fluid and choroid plexus were described and discussed (Chapters 3 and 4 and Section, 8.1, 8.2 and 9.1). These forms appear to represent a progression of stages from the long-narrow form (the form that first emerges from the tissues into the blood) through the long-flat (dividing form) to the progressively granular (short stumpy) forms which appear to be a dead end to the progression (Ormerod and Venkatesan, 1971): multiple division and sphaeromastigote forms may represent stages in a continuous cycle of development. There was no intrinsic difference in the stages seen in blood and peritoneal fluid except in the higher proportion of multinucleate stages seen in the peritoneal fluid (Ottolenghi, 1910). It is probable that the proportion of stages in
intracellular fluid is similar to that seen in peritoneal fluid. Intracellular multiple division forms seen in the ependymal cells of the choroid plexus appear to represent a newly discovered stage in the life cycle of T. brucei.

8. The general conclusions of this thesis are as follows:

(a) That an intracellular stage occurs in the life cycle of the sleeping sickness trypanosome. This stage is to be found in the ependymal cells of the choroid plexus although its presence in other cells has not been excluded.

(b) The distribution of pathological manifestations in the later stages of the disease, viz. meningitis and perivascular cuffing can be ascribed to the periodic release of, and tissue reactions against, trypanosomes lying dormant in the ependymal cells of the choroid plexus.

(c) The recrudescence of parasitaemia after remission (natural or induced by chemotherapy) which in the case of gambian trypanosmiasis may last for a number of years, is best explained by the existence of an intracellular stage.

(d) Progressive development of blood forms liberated from an intracellular stage is the best explanation of the phenomenon of pleomorphism.

(e) The design of future chemotherapeutic agents should take into account the existence of an
intracellular stage (as with *Leishmania*, *Plasmodium* and *T. cruzi*) and concentrate upon its destruction.
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