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

by

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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 phago-cytosis 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, multi-flagellar 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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LIST OF ABBREVIATIONS

Ln	Long-narrow form
Lf	Long-flat form
Gt	Giant form
Tp	Tadpole form
Gn	Granular form
Rb	Round-bodied trypanosome
Mb	Moribund form
lg	lipoprotein granule
c	cilium
M	Mitochondrion
K	Kinetoplast
dk	daughter kinetoplast
pk	parent kinetoplast
F	Flagellum
df	daughter flagellum
pf	parent flagellum
af	anterior flagellum
pf	posterior flagellum
E	Endoplasmic reticulum
Er	rough endoplasmic reticulum
Es	smooth endoplasmic reticulum
N	Nucleus
pv	pinocytotic vesicle
rbc	red blood corpuscles
wbc	white blood corpuscles
MEM	minimum essential medium
W	Wall of cell

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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 $16^{o}N$ and $20^{o}S$.

The geographical distribution of <u>Glossina</u> 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 <u>Glossina</u>
<u>morsitans</u> is mainly responsible for transmission in the
savannah woodland of central and East Africa while <u>G.</u>
<u>palpalis</u> 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 acti-
vities 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
<u>Trypanosoma</u> Gruby, 1843 there are three subgenera of
Salivaria, characterised by transmission by injection by
proboscis from the salivary glands. Subgenus <u>Duttonella</u>
Chalmers, 1918 is represented by <u>T.(D.) vivax</u> Ziemann,
1905; <u>Nannomonas</u> Hoare, 1964 by <u>T. (N.) congolense</u> Broden 1904
and <u>Trypanozoon</u> Lühe, 1906 by <u>T. (T.) brucei</u> 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 <u>T.b. brucei</u> Bradford, 1899, <u>T.b. rhodesiense</u> Stephens and Fantham, 1910 and <u>T.b. gambiense</u> 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 <u>Trypanosome (Herpetosoma)</u> <u>lewisi</u> 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 <u>T. (T.) evansi</u> 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 publi-
cation, 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 dif-
ference 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 verte-
brates 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 Laveissiere (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 trans-
mission 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 trypano-
somes 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 (1978 b)
- i.e.an isolated parasite material with available docu-
mentation 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 Syhacia 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 $-78^{\circ}C$ 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 <u>T.b.rhodesiense</u> (180) is cited as "Botswana", <u>T.b.rhodesiense</u> (83) as Zambia I and <u>T.b.rhodesiense</u> (57) as Zambia II.

Table 1. Summary of strains of trypanosomes used

Trypanosome	Code	Origin	Original host	Year of isolation
1. T. lewisi	LUMP 814	U.K. (Lincs)	Rat	1972
2. T. vivax	Y58	Nigeria (Yakawada)	Cattle (N'dama)	1972
3. T. vivax	Y486	Nigeria (Yakawada)	Cattle (Zebu)	1973
4. T. congolense	Gamb 6	Gambia (Keneba)	Cattle	1977
5. T. congolense	Gamb 19	Gambia (Essau)	Cattle	1977
6. T. congolense	TSW 99	Liberia (Ganta)	Pig	1977
7. T. congolense	TSW 103	Liberia (Sanni-quele)	Pig	1977
8. T. brucei brucei	8/18	Nigeria (Nssuka)	Pig	1962
9. T.b. brucei	S/42	Tanzania	Warthog	1966
10. T.b. brucei	Treu 667	Uganda	? Bushbuck	1964
11. T.b. brucei	Antat 1	Uganda (Busoga)	Bushbuck	1966
12. T.b. brucei	TSW 3	Liberia (Sehwee)	Pig	1975
13. T.b. rhodesiense	Liverpool normal	Not known	Man	1920s
14. T.b. rhodesiense	Liverpool resistant	Not known	Man	1920s
15. T.b. rhodesiense	57	Zambia (II) (Isoka)	Man (David Sinkala)	1974
16. T.b. rhodesiense	58	Zambia (Kasempa)	Man	1974
17. T.b. rhodesiense	83	Zambia (I)	Man (Giorgio Sabbadin)	1977
18. T.b. rhodesiense	180	Botswana	Man (Dumelo)	1960
19. T.b. rhodesiense	186	Botswana	Man (Monaxumo)	1960
20. T.b. rhodesiense	LUMP 1198	Uganda (Busoga)	Man (Olase Bidonge)	1976
21. T.b. gambiense	LUMP 1236	Zaire	Man	1970
22. T.b. gambiense	Bida 3	Nigeria (Bida)	Man	1968

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 dexamethesone 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. rho-
desiense.

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 sus-
pension 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 Proto-
zoology.   From the history of the tumour cells they were

originally established in Children's Cancer Research
Foundation, Boston, Massachusetts.   The cells were later
chracterised 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 $-78^{\circ}C$ 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 ascer-
tained 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. congolense - 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 quanti-
fying the parasitaemia was that of Herbert and Lumsden

(1976) popularly known as the 'matching' method.

(c) 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-immer-
sion objective.

The field of the count and the number of trypano-
somes 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 gran-
ules 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: 120 hrs

Mic. Fields	No of granules Agranular L/N (0)	L/F (0)	Granular 1	2	3	4	5	6	7	8	9	10+	Sub Total	Expt Code: ♂ Rat.4/1RR. Summary
1	HHt IIn	HHt I										III		LN = 89
2	HHt III	IIII						I				II		LF = 50
3	HHt II	I-HH										III		AGR = 2̶6̶ 139
4	HHt II	HHt-										II		GR = 26
5	HHHHHH	I III										IIII		Fields (Fld) = 10
6	HHt HH	I HHt										III		LN/fld = 8.9
7	HHt Im	IIII										III		LF/fld = 5.0
8	HH HH	HHt I							I			II I		AGR/fld = 13.9
9	HHt III	HHt										III		GR/fld = 2.6
10	HHt HH	I III												% LN = 53.9
11														% LF = 30.3
12														% AGR = 84.2
13														% GR = 15.8
14														Granules/Tryp.= 10+
15														
16														
17														
18														
19														
20														
21														
22														
23														
24														
25														

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-KeII 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 $37^{\circ}C$. 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 $37^{\circ}C$ for 1 hour. The coverslips were subsequently washed with Hank's and 0.5 ml of 10^{6}/ml, 2×10^{6}/ml or 5×10^{6}/ml of trypanosomes put on the coverslips and left to interact with FCS at $37^{\circ}C$ 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 1×10^{6}/ml, 2×10^{6}/ml or 5×10^{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 trypano-
somes 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 macro-
phages 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%
$NaHCO_3$ 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 cover-
slips in a bath of fresh TCM was added 2 ml of 10^6/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 glutaral-
dehyde.

2.6.2.4 Dehydration and drying

The products of SEM studies whether from
Karnovsky's fixative or glutaraldehyde, single- or double-
filters and also those obtained from phagocytosis experi-
ments 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 H_2O),
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 Elec-
tronics Limited. Calibration of the plotter was by latex

particles of known mean diameter. The aperture dia-
meter 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 concen-
trated and fixed with 2.5% glutaraldehyde overnight at $4^{o}C$.
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 $4^{o}C$. 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(b), 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 $4^{o}C$ 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 $60^{\circ}C$ for at least 48 hours using the same
resin.

The Aradite blocks were cut with Cambridge Huxley
Ultramicrotone Model MK II. Thick sections mounted in
Canada balsam and stained with methylene blue were pre-
viewed with light microscope to estimate the success of
the techniques and evaluate the potential of the prepar-
ation. 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 AEl 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 distri-
bution 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 (1979 a). 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 demon-
strated 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 experi-
ments and in ascitic fluid.   With these granules charac-
teristic 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 photo-
graphs but easy in the living preparation because
the long-narrow can be seen to be cylindrical
when it rolls over.

n on agar

m

ure

n the photo-
ion because
ndrical
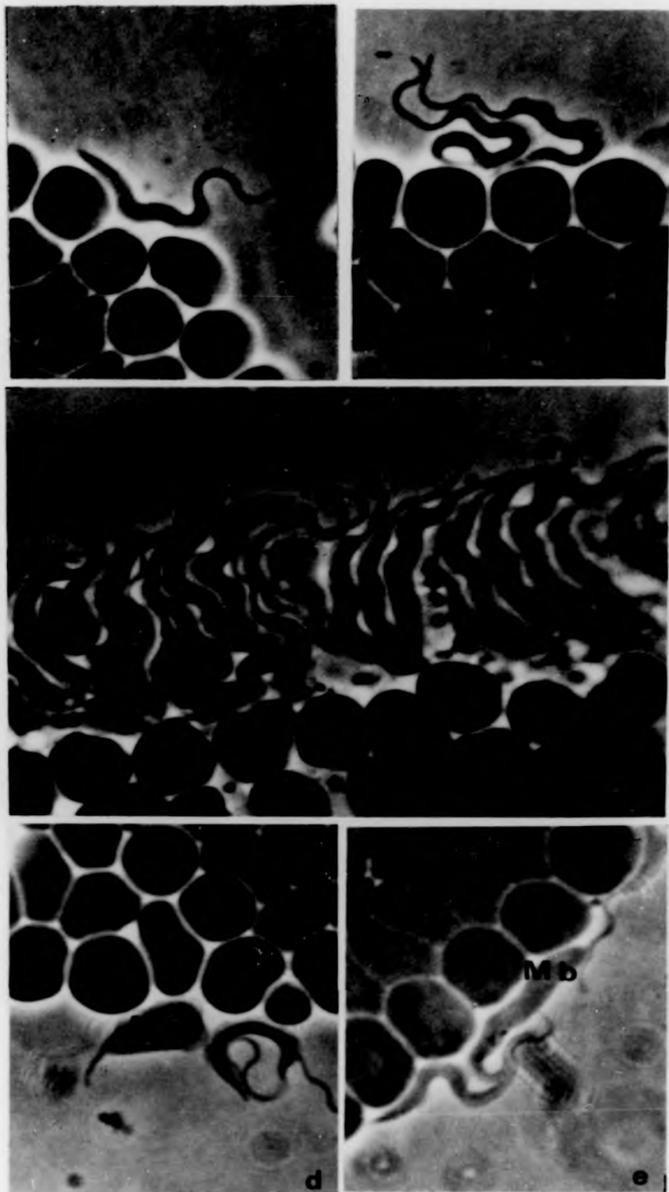
PLATE 2   Granular form of Botswana strain under
different conditions all showing same number
of granules per trypanosome.

(a)   Normal infection

(b)   Cloned infection

(c)   X-irradiated infection
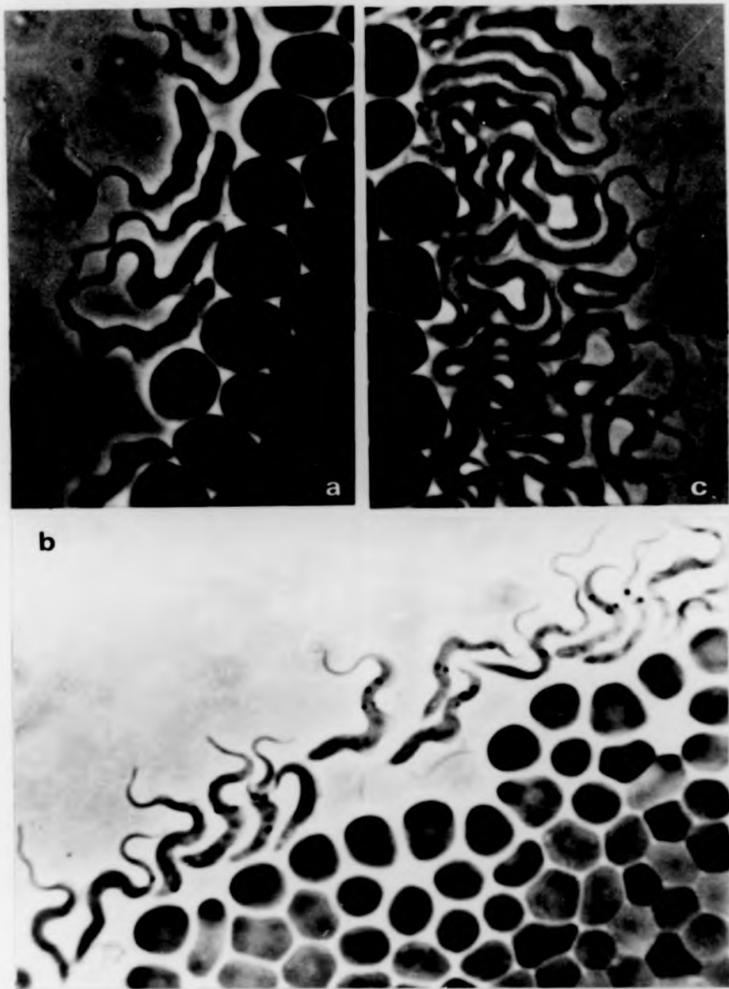
PLATE 3   Granular forms of Zambia strains

      (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
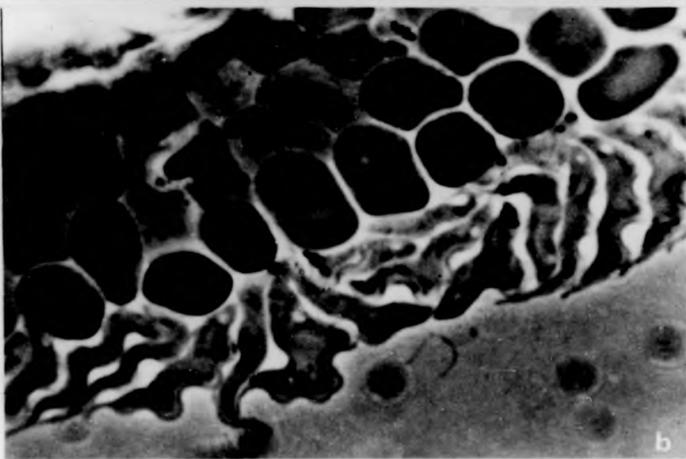
appearance could vary; while some could hardly be distin-
guished from ordinary active tadpole forms (Plate 1d),
others were so degenerate that although they showed the
granules they might be regarded as being already dead
(Plate 1e).

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 popu-
lation at day 13.   During this period the high para-
sitaemia 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 distri-
bution 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 pro-
gressed 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 infec-
tion 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 agra-
nulars were increasing.

There was an initial sharp rise in the produc-
tion 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 when-
ever 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 con-
stituent 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 experi-
ment.   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 para-
sitaemia 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 corres-ponded 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 treat-ment.

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 through-
out 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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3.1.7.1 Botswana strain

3.1.7.1(a) Normal infection of Botswana strain

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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

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 Bl) 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 trypano-
some (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 charac-
teristic 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.

the normal
carried out
nt was done
that the
method has
onitored
experiments
each set.

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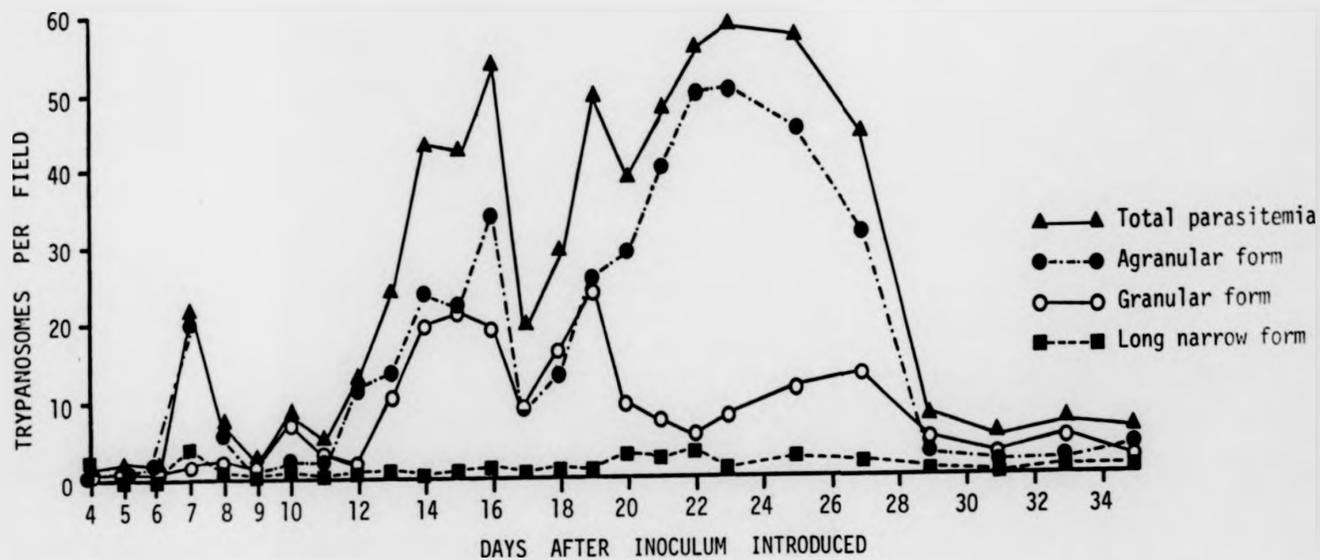
Fig. 2b   Occurrence of granules in Botswana strain (normal rats)
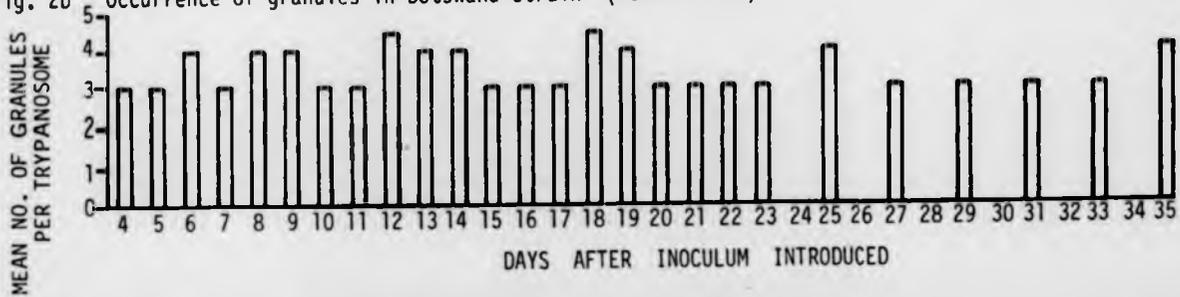
Fig. 3a Patterns of parasitemia in Zambia I strain (normal rats)
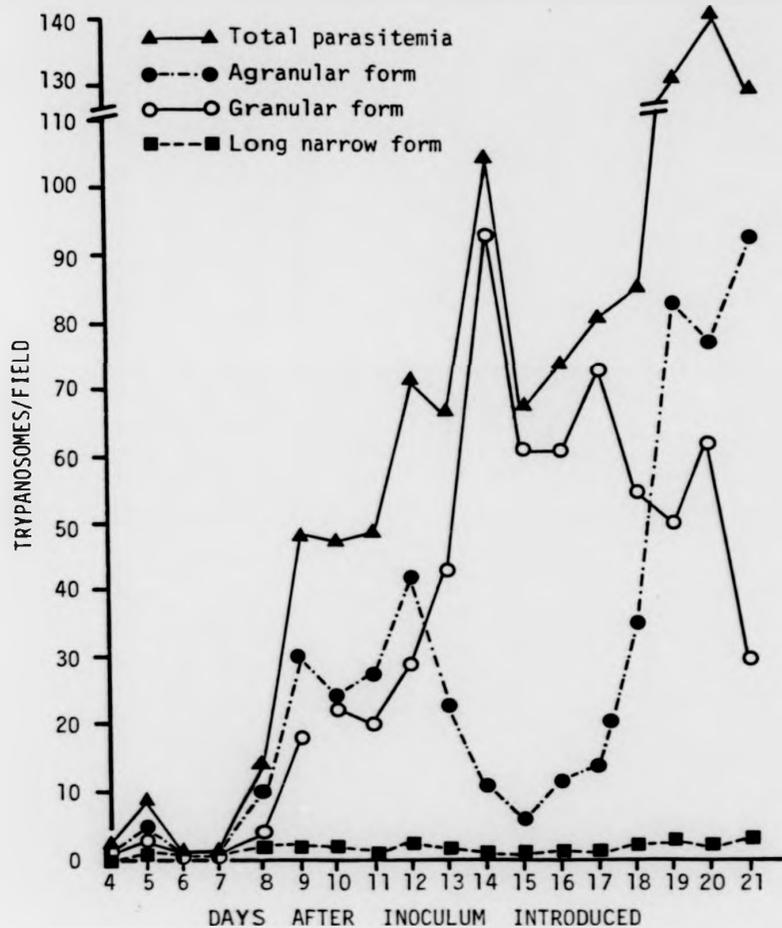
Fig. 3b Occurrence of granules in Zambia I strain (normal rats)

Fig. 4a Patterns of parasitemia in Zambia II strain (normal rats)

Total parasitaemia
Granular form
Agranular form
Long norrow form
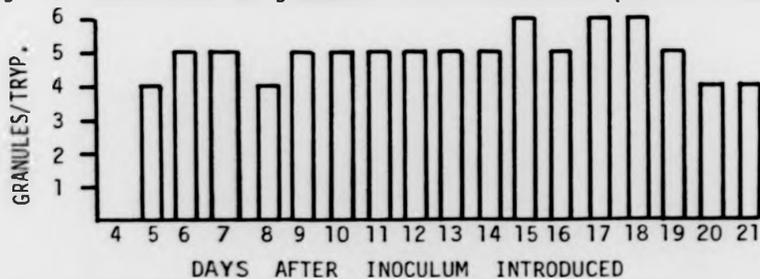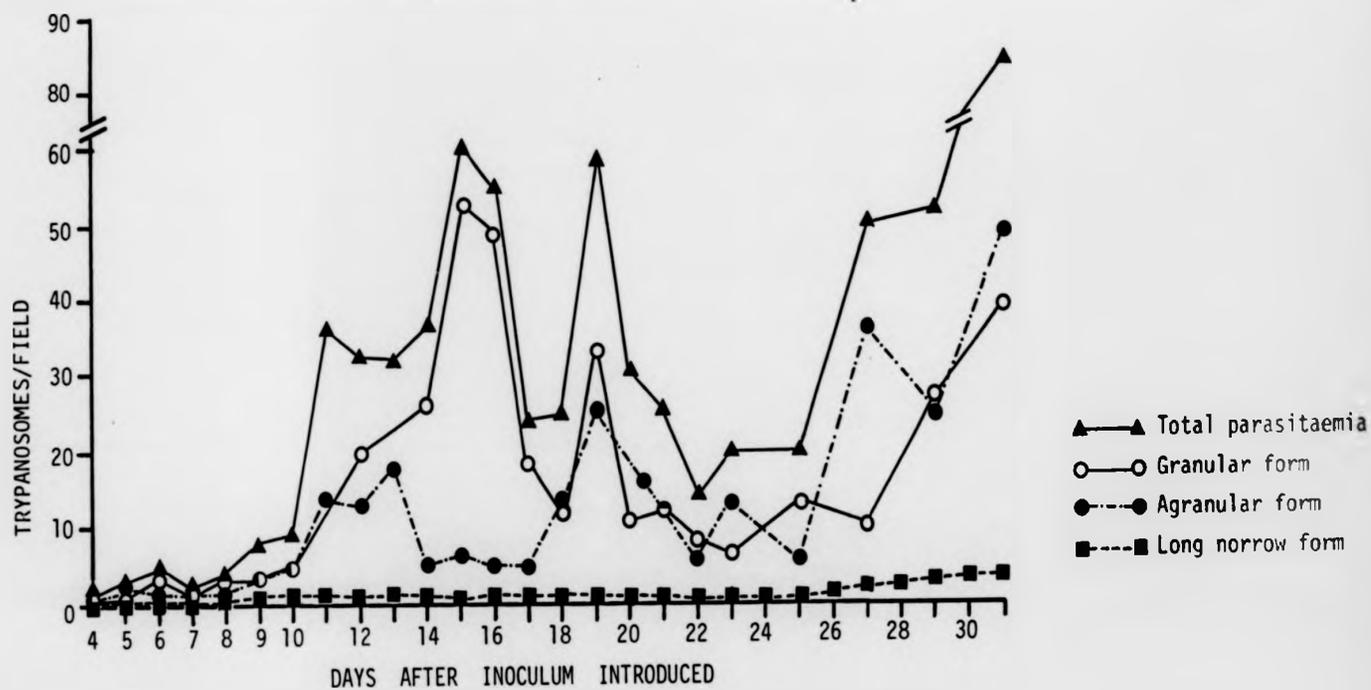
Fig. 4b Occurrence of granules in Zambia II strain (normal rats)

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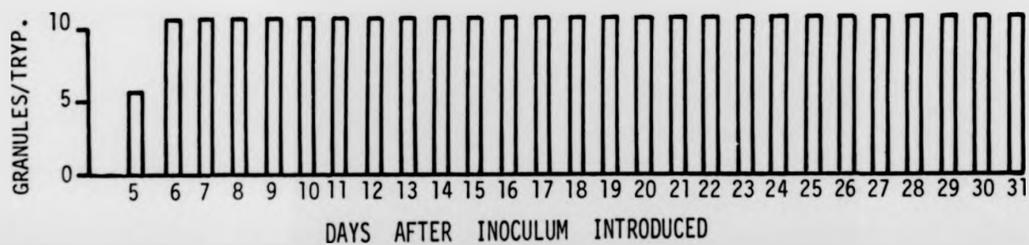
Fig.5a   Patterns of parasitemia in
Botswana strain (X - irradiated rats)
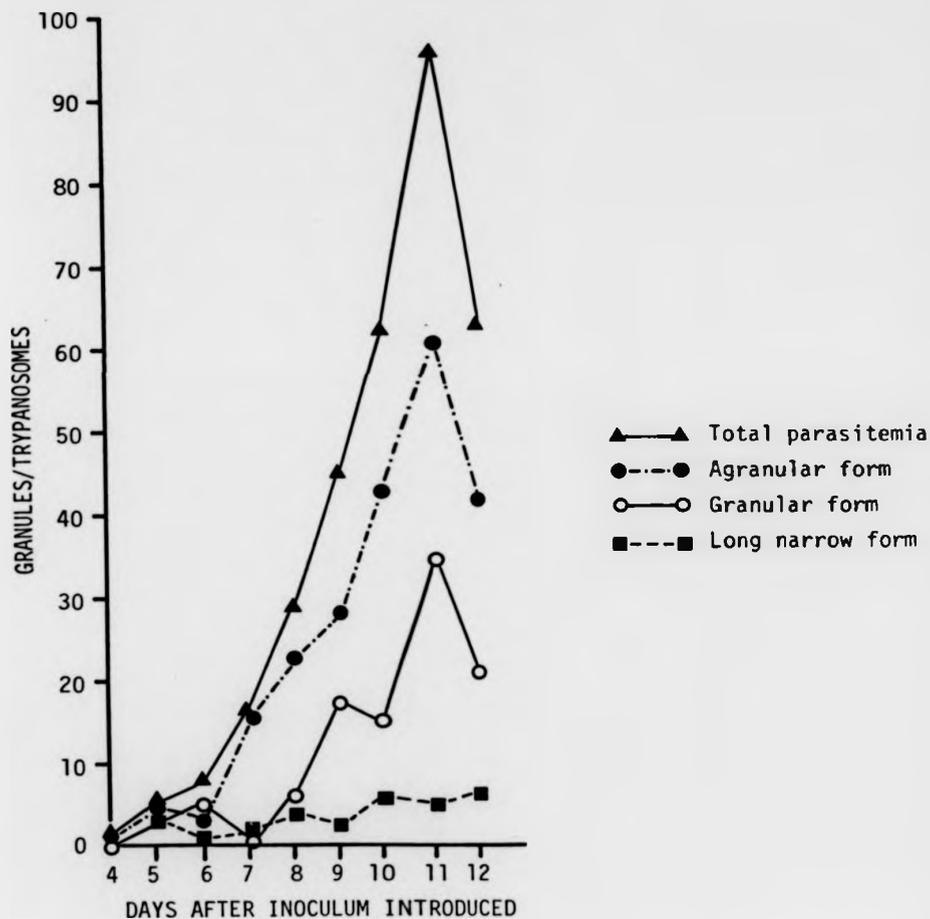
Fig.5b   Occurrence of granules in
Botswana strain (X - irradiated rats)
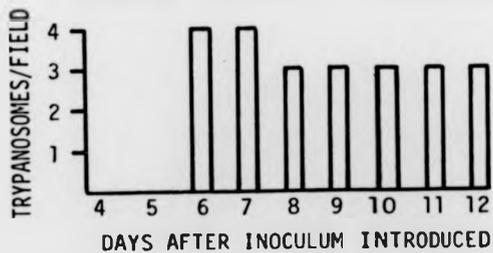
Fig.6a Patterns of parasitemia in Zambia strain (x-irradiated rats)

Fig.6b Occurence of granules in Zambia strain (x-irradiated rats)

Fig.7a   Patterns of parasitemia in Zambia II strain (x-irradiated rats)
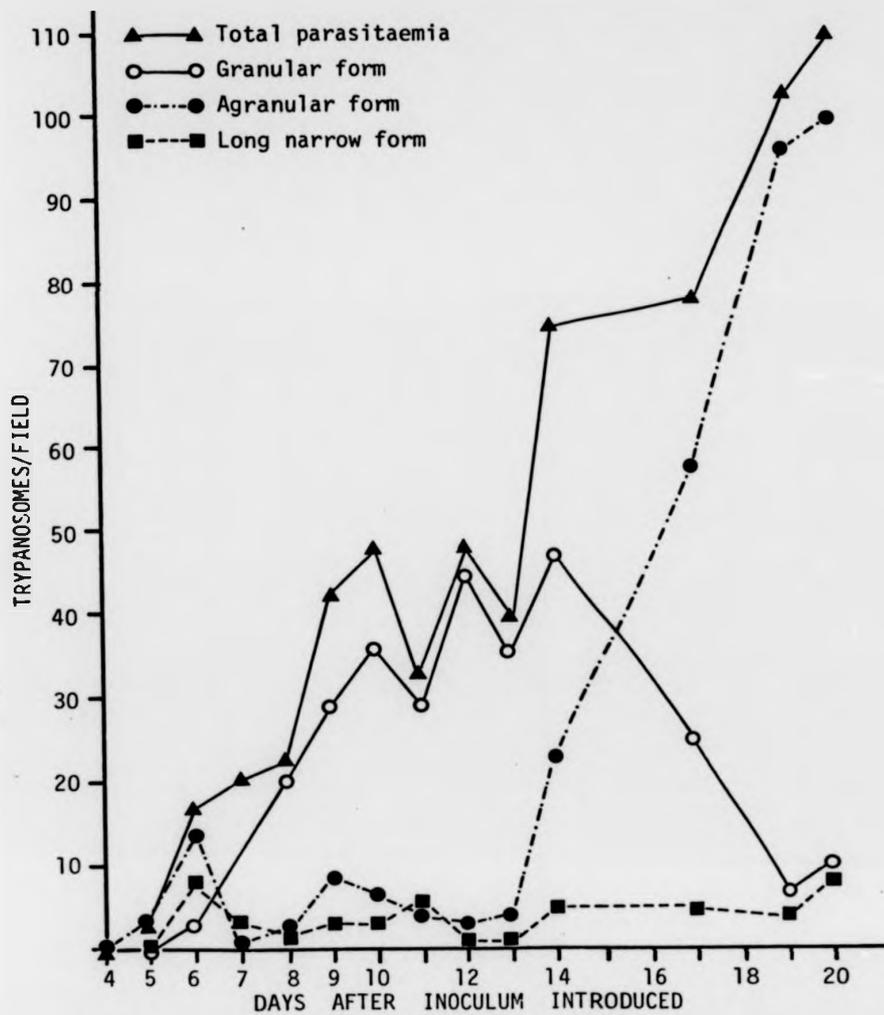
Fig.7b   Occurrence of granules in Zambia II strain (x-irradiated rats)
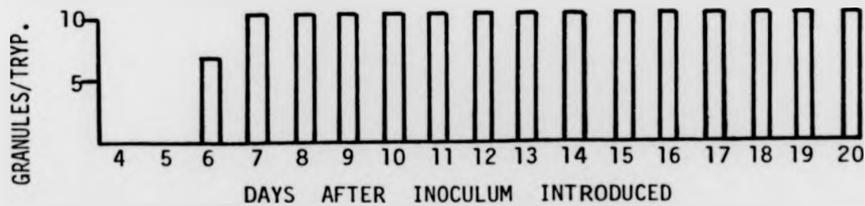
Fig. 8a PATTERNS OF PARASITAEMIA IN BOTSWANA STRAIN
(8 mg/kg dexamethasone)
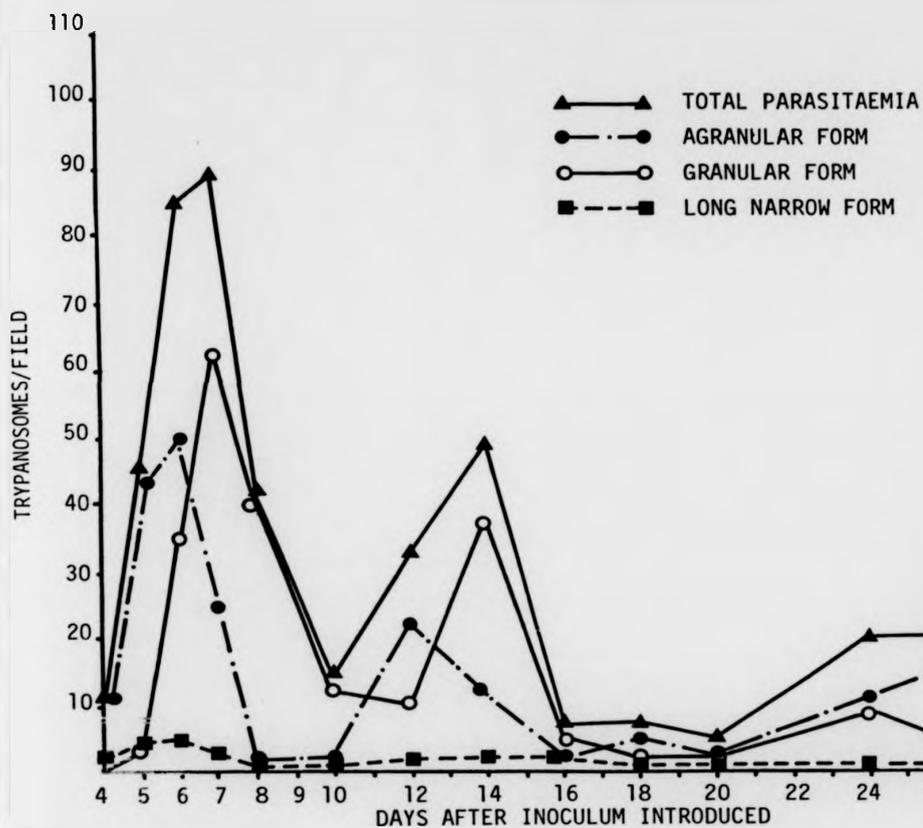
Fig.8b OCCURRENCE OF GRANULES IN BOTSWANA STRAIN
(8 mg/kg dexamethazone)
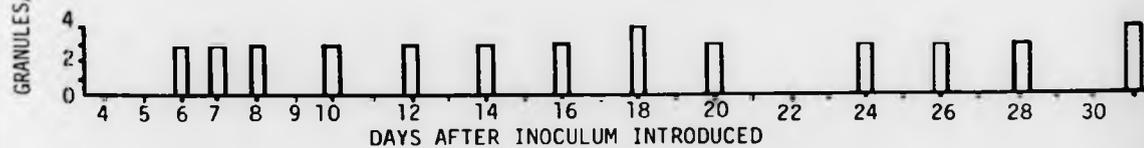
Fig. 9a   PATTERNS OF PARASITAEMIA IN BOTSWANA STRAIN
(20 mg/kg dexamethasone)
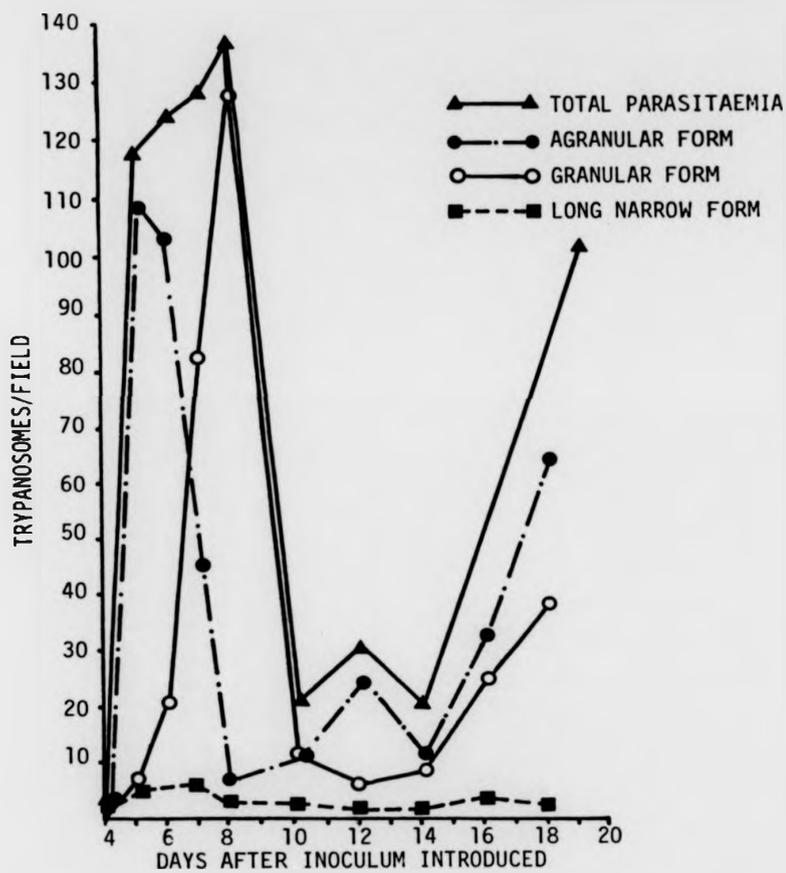
Fig. 9b   OCCURRENCE OF GRANULES IN BOTSWANA STRAIN
(20 mg/kg dexamethazone)
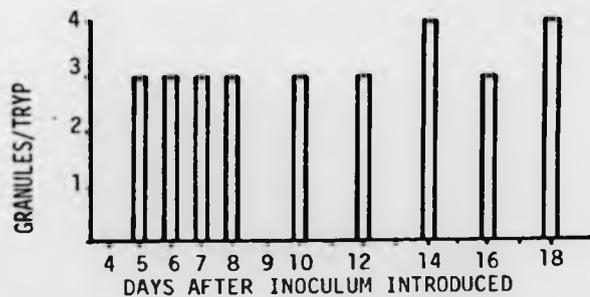
Fig.10a PATTERNS OF PARASITAEMIA IN BOTSWANA STRAIN
(40 mg/kg dexamethasone)
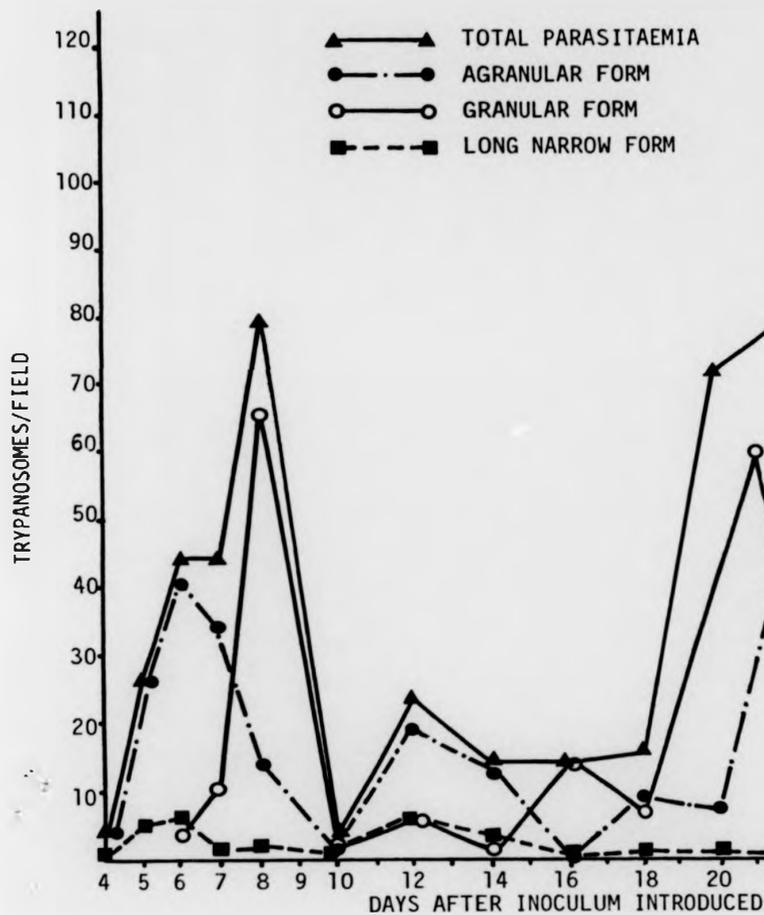
Fig.10b OCCURRENCE OF GRANNULES IN BOTSWANA STRAIN (40 mg/kg dexamethasone)
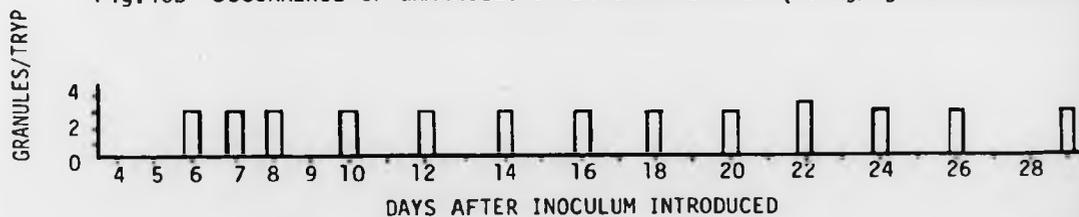
Fig.11a PATTERNS OF PARASITAEMIA IN ZAMBIA-1STRAIN
(8 mg/kg dexamethasone)
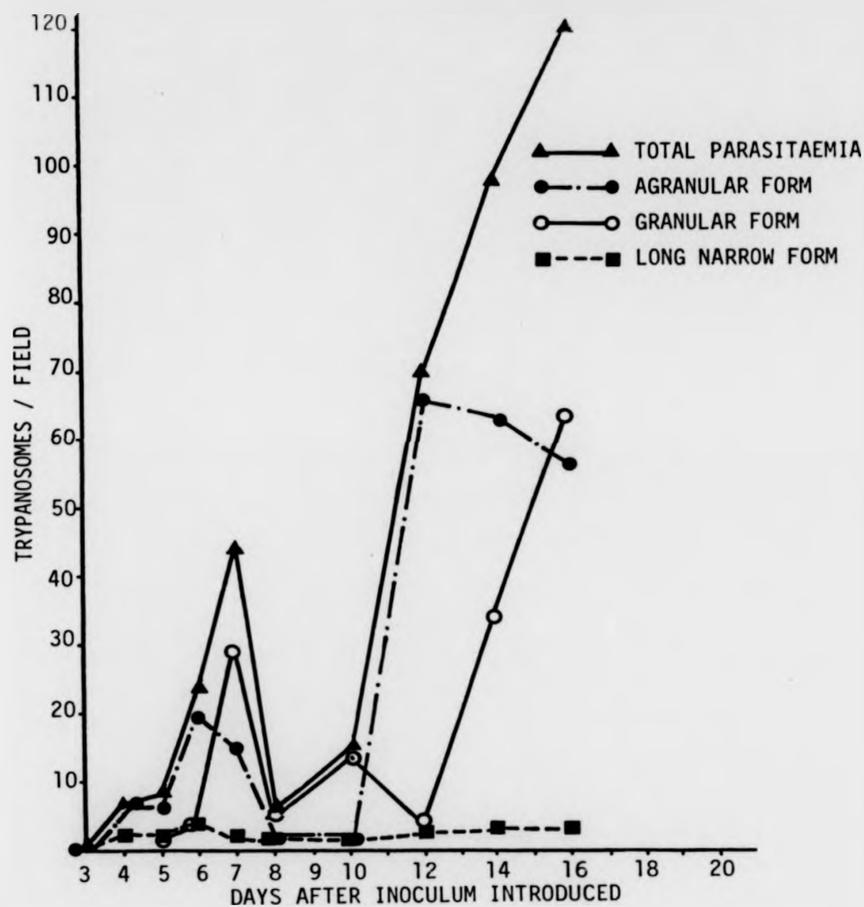
Fig.11b OCCURRENCE OF GRANULES IN ZAMBIA-1STRAIN
(8 mg/kg dexamethasone)
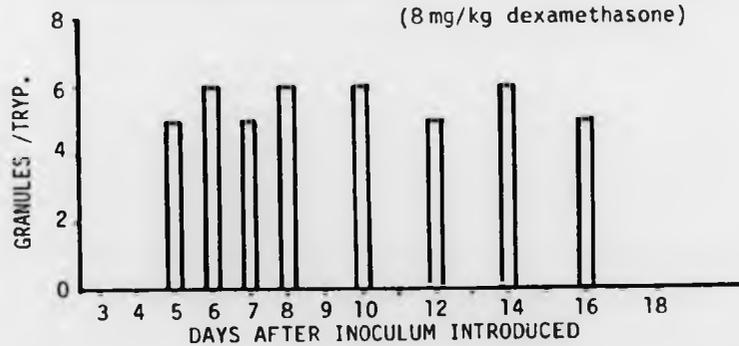
Fig.12b PATTERNS OF PARASITAEMIA IN ZAMBIA STRAIN
(20 mg/kg dexamethasone)

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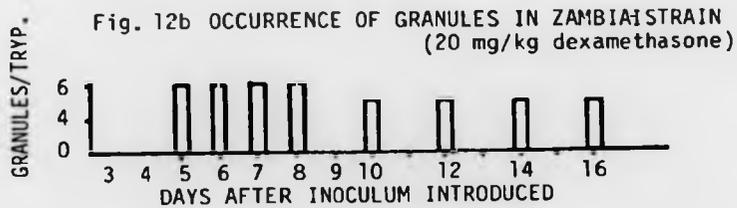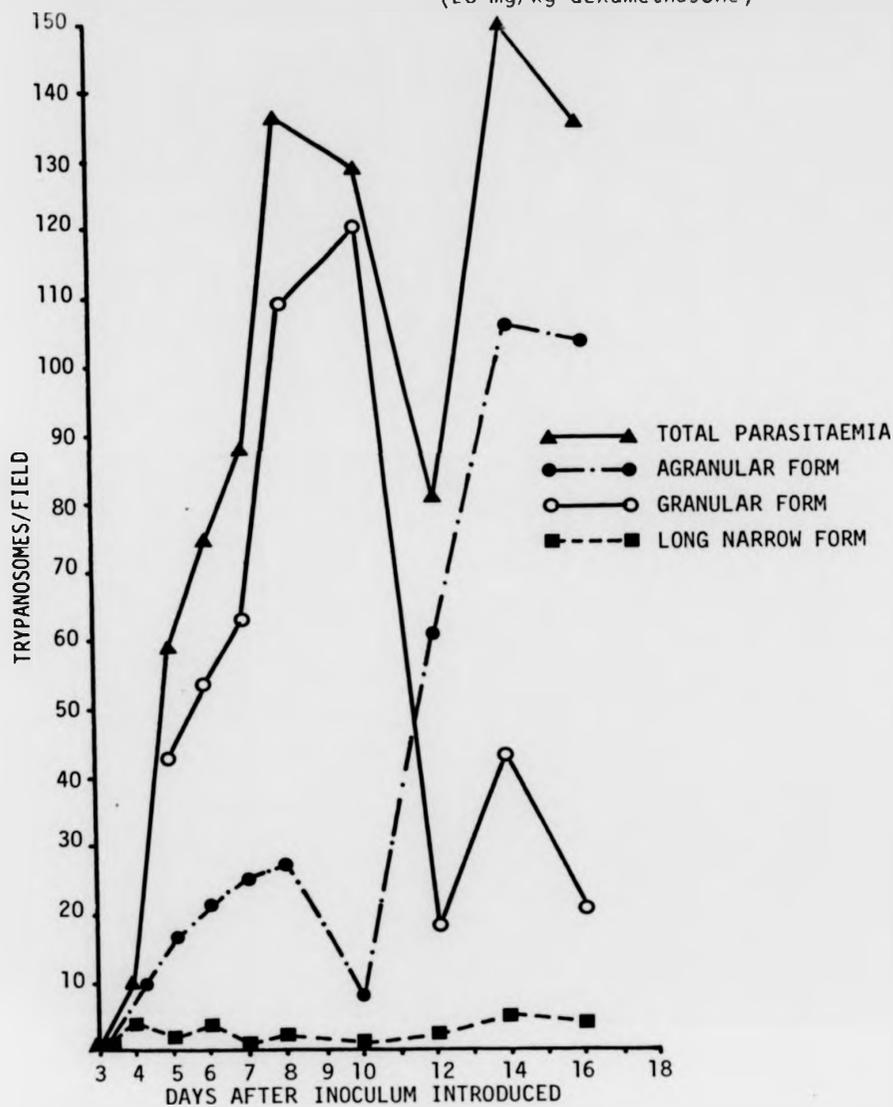
Fig. 13a PATTERNS OF PARASITAEMIA IN ZAMBIA I STRAIN
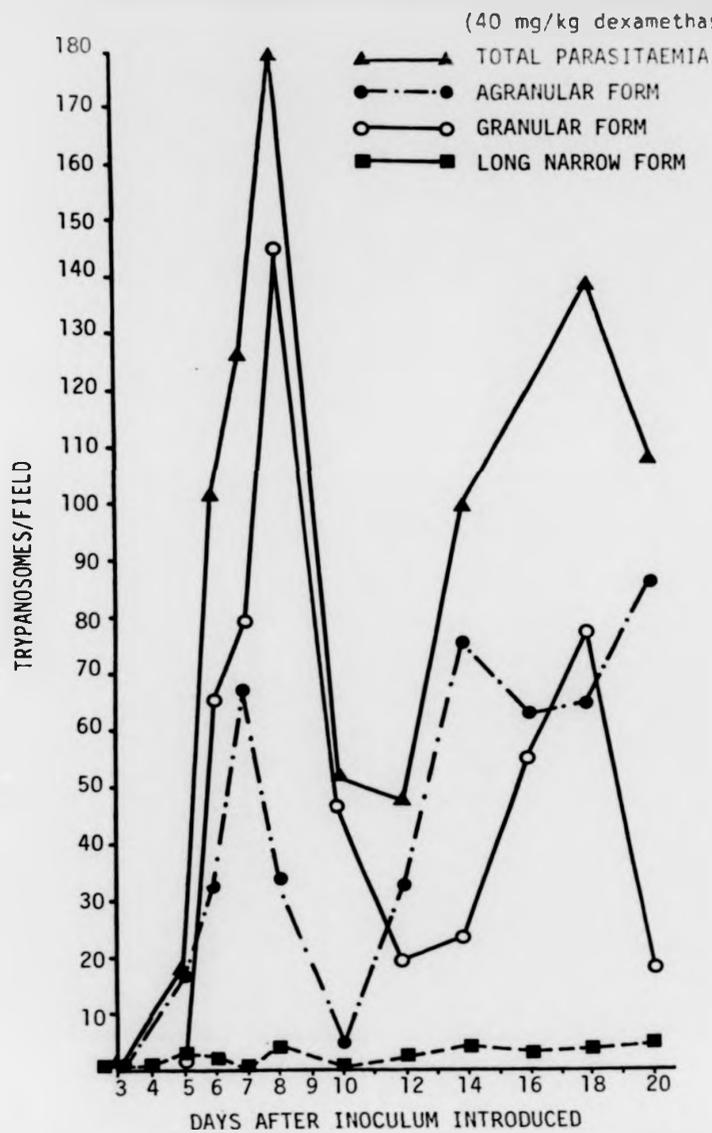
(40 mg/kg dexamethasone)

▲——▲ TOTAL PARASITAEMIA
●—·—·—● AGRANULAR FORM
○———○ GRANULAR FORM
■———■ LONG NARROW FORM

DAYS AFTER INOCULUM INTRODUCED

TRYPANOSOMES/FIELD

Fig. 13b OCCURENCE OF GRANULES IN ZAMBIA I STRAIN

(40 mg/kg dexamethasone)

GRANULES/TRYP.

DAYS AFTER INOCULUM INTRODUCED

Fig. 14a PATTERNS OF PARASITAEMIA IN ZAMBIA II STRAIN
(8 mg/kg dexamethasone)
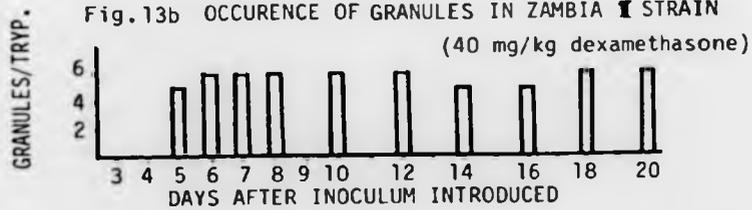
▲——▲ TOTAL PARASITAEMIA
●—·—● AGRANULAR FORM
○——○ GRANULAR FORM
■----■ LONG NARROW FORM
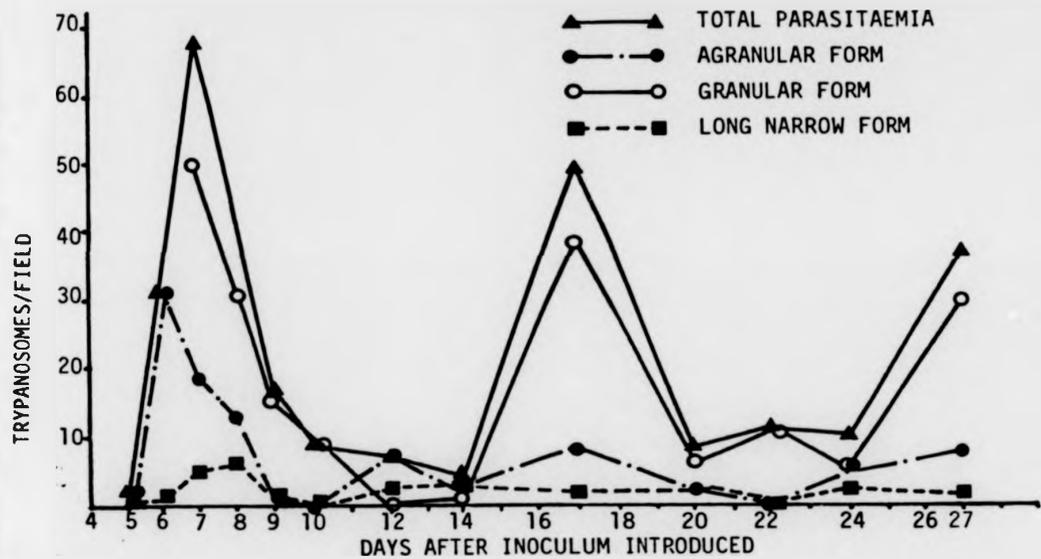
Fig.14b OCCURRENCE OF GRANULES ZAMBIA II STRAIN
(8 mg/kg dexamethazone)
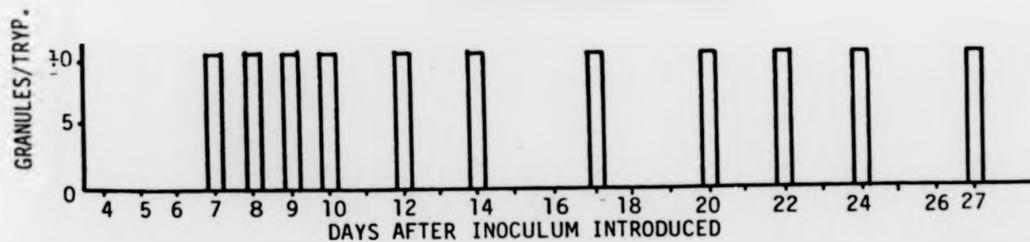
Fig.15a   PATTERNS OF PARASITAEMIA IN ZAMBIA II STRAIN
(20 mg/kg dexamethasone)
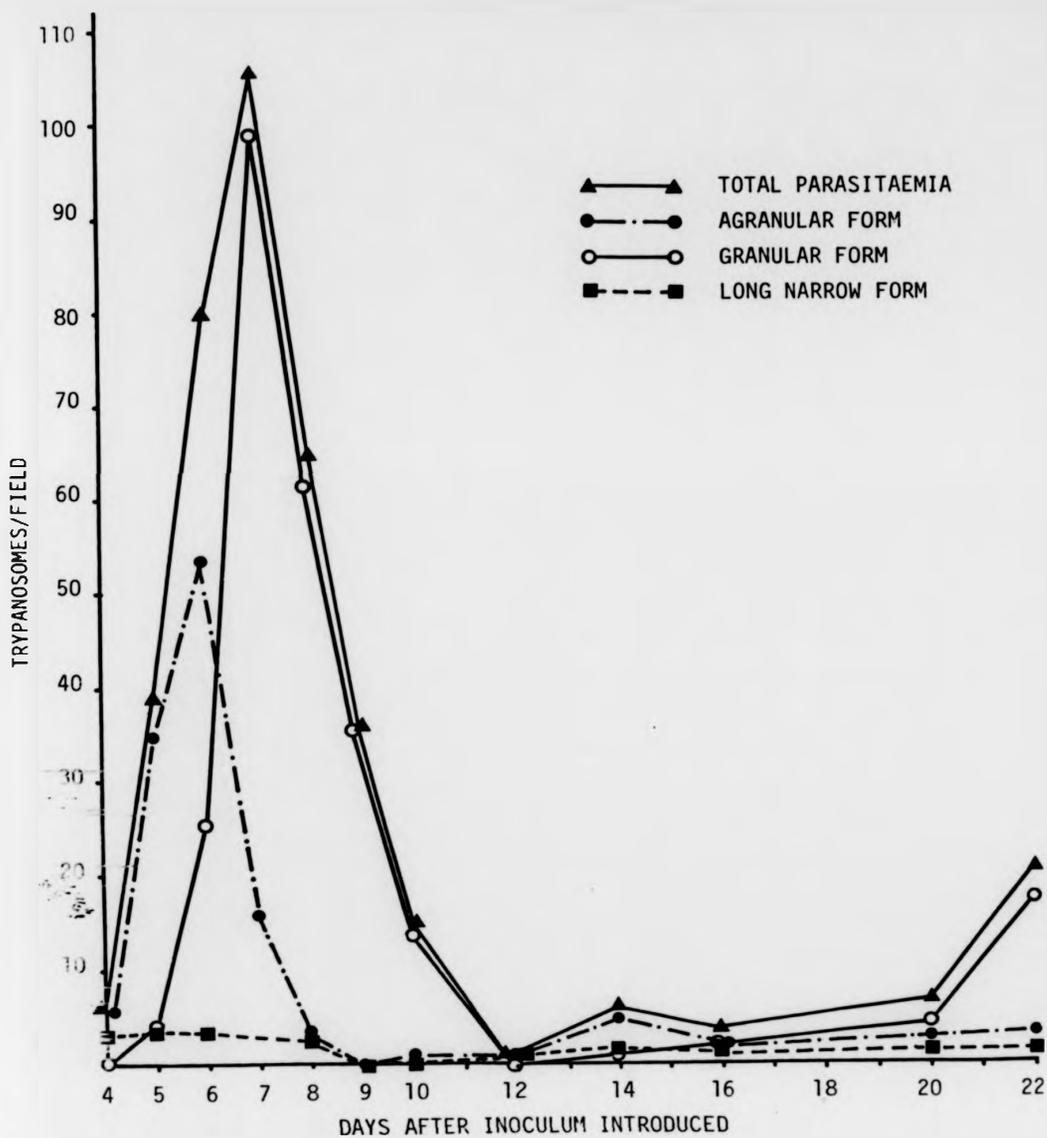
TOTAL PARASITAEMIA
AGRANULAR FORM
GRANULAR FORM
LONG NARROW FORM

TRYPANOSOMES/FIELD

DAYS AFTER INOCULUM INTRODUCED

Fig.15b OCCURRENCE OF GRANULES IN ZAMBIA II STRAIN (20 mg/kg dexamethasone)

GRANULES/TRYP

DAYS AFTER INOCULUM INTRODUCED

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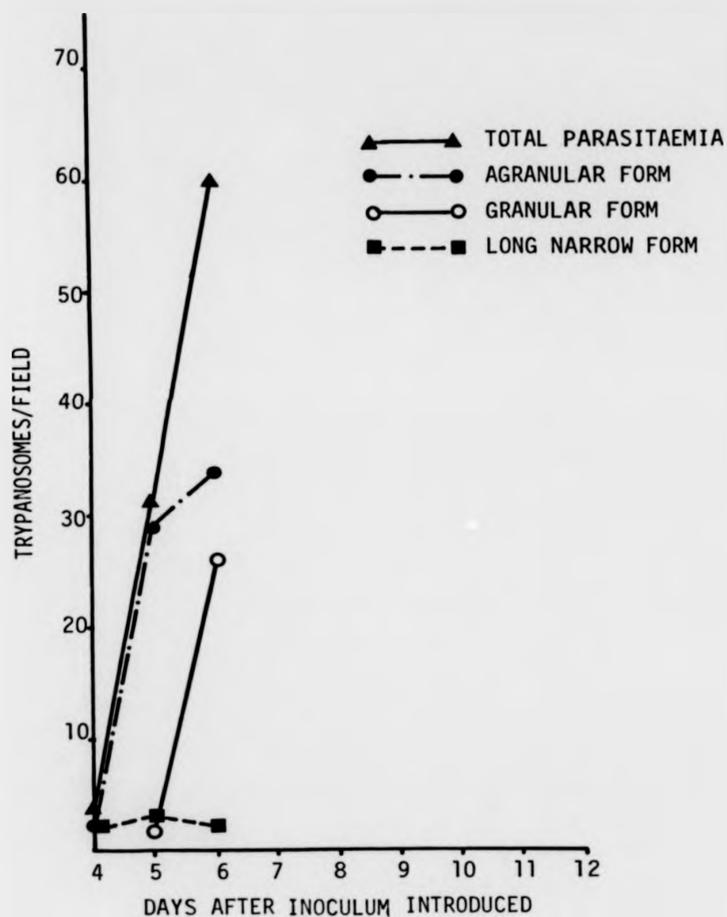
Fig.16b OCCURRENCE OF GRANULES IN ZAMBIA II STRAIN
(40 mg/kg dexamethasone)
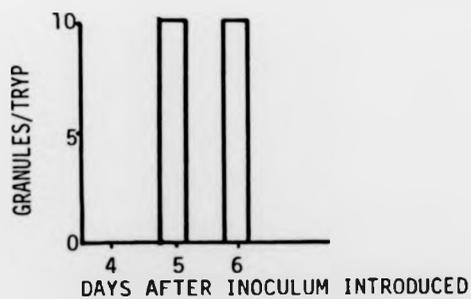
3.2 DISCUSSION

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 tech-
nique to classify the trypomastigotes seen into granular
or agranular forms.   This simple method is greatly
superior to the techniques needing fixed and stained pre-
parations for categorising and quantifying populations of
blood trypanaosomes.   The methods used by Ashcroft (1957)
and Wijers (1959) were based on measuring length of indi-
vidual 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 approxi-
mate 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 provi-
sional 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*

Agar technique (no. of tryps/field	Matching method equivalents	
	Log no. of organism/ml	Actual no/ field
Nil	6.9	2
1	7.2	4
3 - 5	7.5	8
10 - 20	7.8	16
25 - 30	8.1	32
50 - 60	8.4	64
70 - 100	8.7	128
100+	9.0+	256+

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

3.2.2 The Immunodepressants Administered

Corticosteroids and x-irradiation are two dif-
ferent 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 cortico-
steroids 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 corti-
sone 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 dexamethesone in preference to the cortisone
acetate was chosen for this work.   There is no record
known to the author where dexamethesone had been used to
induce immunodepression in trypanosomiasis.

Large doses of even the synthetic cortico-
steroids 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 diffi-
cult to understand why it is rare to obtain total sup-
pression 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 para-
sitaemia 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 viru-
lence 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. congolense as having a predomi-
nance 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 popu-
lation increased they demonstrated that 181 like the Kenya-
Uganda strains (Ormerod et al., 1963) produced parasi-
taemias 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 para-
sitaemia 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 com-
positions 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 viru-
lence of the same strain but also to difference in tech-
niques 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 experi-
ments until the death of the hosts.

Ashcroft (1957) showed an increase in the per-
centage 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 incoulum 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 dexamethesone 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 main-
tained by the granular form and so there is time for
maximum growth of the individual to the stumpy charac-
teristics.   On the other hand Botswana and Zambia I strains
are essentially parasitaemias of agranular form and pos-
sibly 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 conse-
quently 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).

CHAPTER 4

PERITONEAL AND ASCITIC FLUIDS

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 micro-
scopy (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
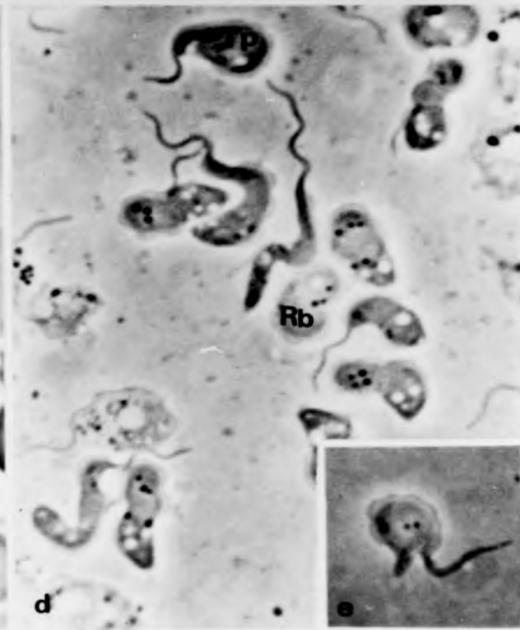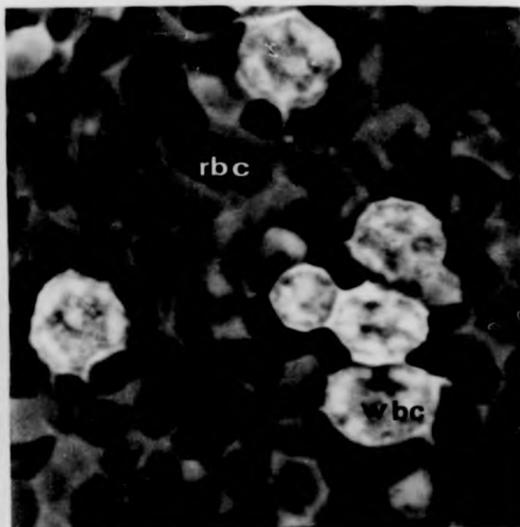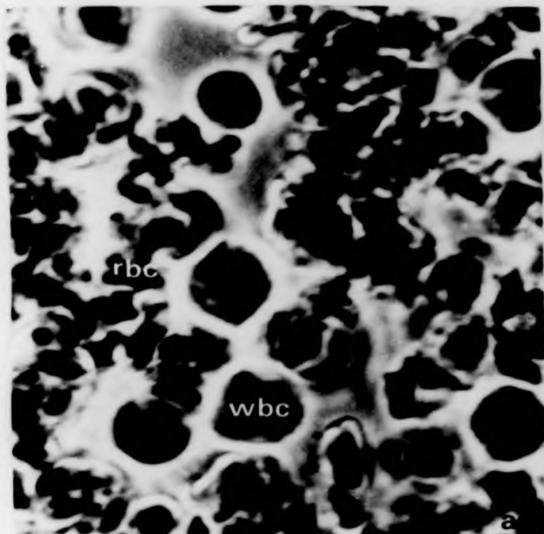
PLATES 5 AND 6   Different forms of trypanosomes in
peritoneal fluid - Giensa stained

(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)
Trpanosomes 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 kineto-
plasts, flagella and dividing
nuclei

les in

ined

ole

flagella

and flagella

to categorise
ote,
mastigote in

omes

dpole form

ral kineto-
dividing
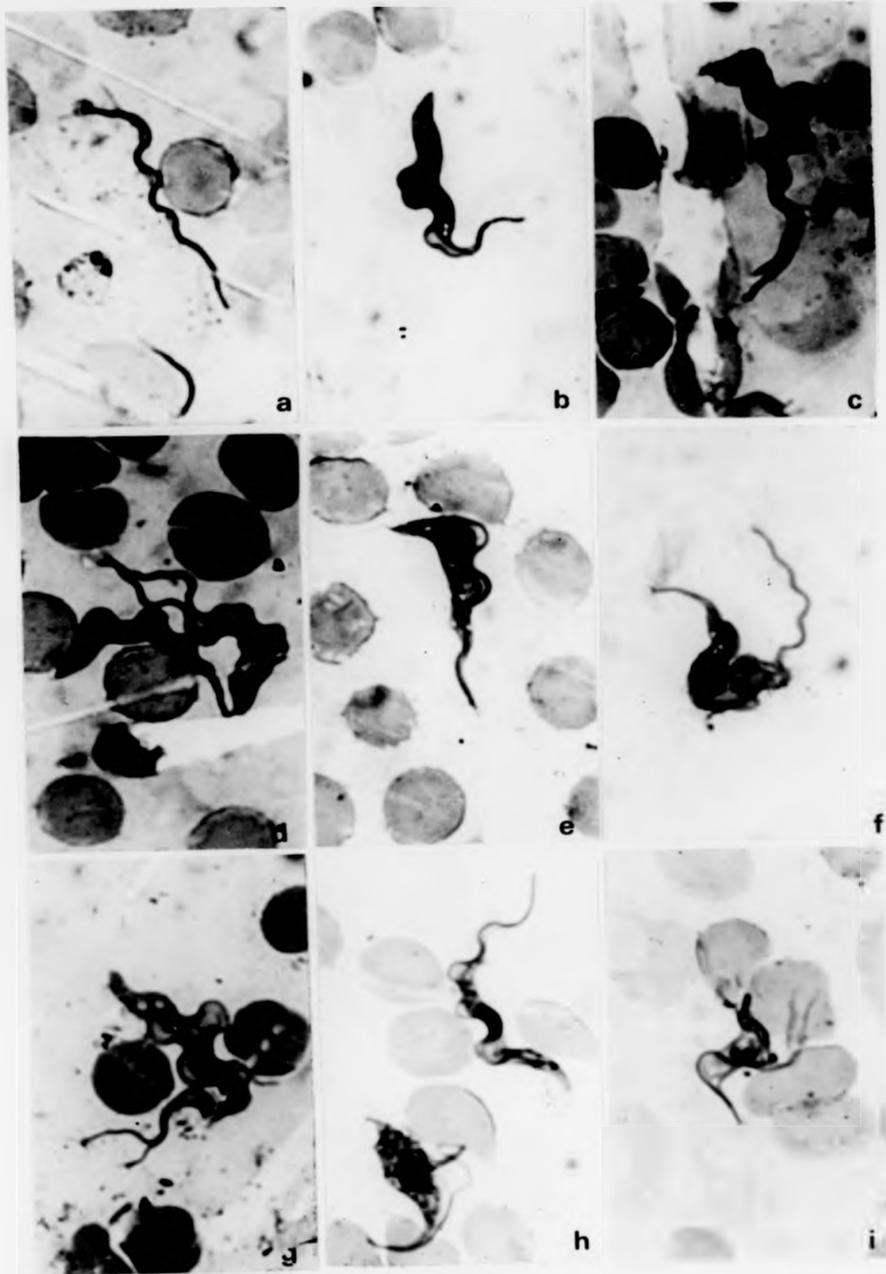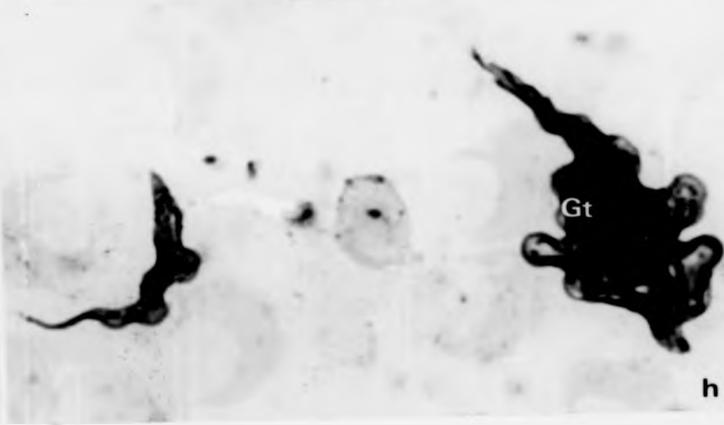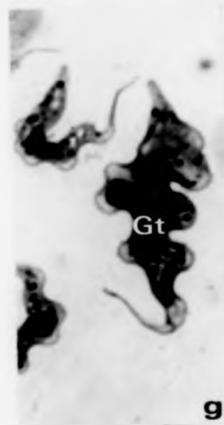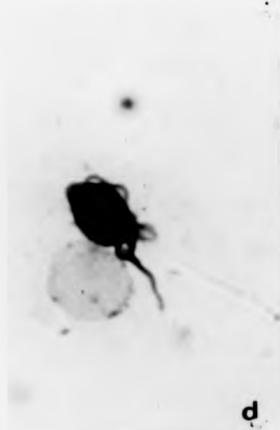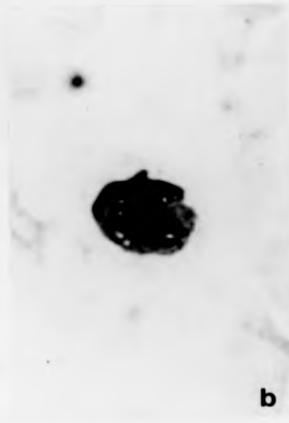
4.1.2 Ascitic Tumour Cells in Rats (See 2.4.2.1)

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 amout 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

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

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

	Day 5		Day 6		Day 7		Day 8		Day 9	
	Exp. AF	Con- trol	Exp. AF	Con- trol	Exp. AF	Con- trol	Exp. AF	Con- trol	Exp. AF	Con- trol
Long-narrow	1	NIL	3	1	4	2	2	0	6	1
Agranular	5	NIL	24	6	40	4	18	0	25	2
Granular	1	NIL	1	1	13	9	57	4	45	14
Total	6	NIL	25	7	53	13	75	4	70	16

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 significant 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

	Poly-morphs	Eosino-phils	Baso-phils	Lympho-cytes	Mono-cytes
Ascitic fluid-infected mice	48.5	0	0.5	46	5.0
Control mice	45	0	1	49	4.5
Human	40-75	1-6	0-1	25-30	5-10

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 popu-
lation. 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

Media	Forms of Parasites (in percentages)				
	Normal trypomastigotes	Dividing form	Giant form	Tadpole form	Moribund form
Normal mouse blood parasitaemia at 96 hours	93.0	4.5	1.5	1.0	NIL
Normal mouse blood parasitaemia at 2 weeks	97.0	2.0	NIL	1.0	0.5
Peritoneal fluid from rat infected with tryps.	89.0	8.5	1.5	1.0	NIL
Ascitic fluid from mouse infected with tryps.	88.5	8.0	1.5	2.0	NIL
Blood parasitaemia in mouse with ascitic fluid	89.0	9.0	1.5	0.5	NIL

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 extra-
vascular 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 trypano-
somiasis 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 San t'ana (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 Sant' ana (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 pos-
sible that these forms might get carried into the vas-
cular 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 amasti-
gote, epimastigote or promastigote in the arrangement of
the nucleus and kinetoplasm.   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
Giemsa-stain 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 pleo-
morphic   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 trypano-
somes 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 capil-
laries 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 perma-
nent.

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 para-
sitaemia 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 7   Recrudescence in the strains of T. rhodesiense

Strain Code	Pre-patent period	Day of treatment (after inoculum introduced)	Parasi-taemia (per field) at treatment	Treatment SHAM (mg kg^{-1})	Glycerol (g kg^{-1})	No. of rats survived/ No. of rats used	Recru-descence (days after treatment)
A1	4	6	1.0	400	4	2/3	7,8
A2	4	6	1.5	400	8	2/3	6,9
A3	4	6	0.5	500	4	1/3	8
A4	4	6	2.0	500	8	0/3	-
A5	4	14	45.0	400	4	1/3	5
A6	4	14	30.0	400	8	0/3	-
A7	5	14	42.0	500	4	2/3	6,7
A8	4	14	28.0	500	8	1/3	5
B1	3	6	9.0	400	4	1/3	11
B2	4	6	5.0	400	8	1/3	12
B3	5	6	3.0	500	4	1/3	5
B4	5	6	8.0	500	8	0/3	-
B5	4	12	48.5	400	4	0/3	-
B6	5	12	40.0	400	8	2/3	11,12
B7	4	12	32.0	500	4	2/3	8,10
B8	4	12	46.0	500	8	0/3	-
C1	4	6	2.5	400	4	3/3	7,8,12
C2	4	6	4.5	400	8	1/3	7
C3	4	6	4.0	500	4	2/3	7,9
C4	4	6	3.5	500	8	1/3	5
C5	4	14	32.0	400	4	2/3	7,8
C6	4	14	49.0	400	8	1/3	17
C7	5	14	28.5	500	4	2/3	5,7
C8	4	14	41.5	500	8	0/3	-

glycerol was 8 g/kg. A4, A8, B4, B8, C4 and C8 illus-
trated 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 (Opperdoes 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

Strain	Prepatent period	Day of treatment (after inoculum introduced)	Treatment SHAM (mg kg^{-1})	glycerol (g kg^{-1})	Recrudescence (days after treatment)
	-	1	400	4	No recrudescence
	-	2	400	4	No recrudescence
Botswana	-	3	400	4	No recrudescence
	4	4	400	4	Day 9
	4	5	400	4	Day 7
	-	1	400	4	Day 8(?)
	-	2	400	4	No recrudescence
Zambia I	-	3	400	4	No recrudescence
	-	4	400	4	No recrudescence
	5	5	400	4	No recrudescence
	1	1	400	4	No recrudescence
	2	2	400	4	No recrudescence
Zambia II	3	3	400	4	No recrudescence
	4	4	400	4	No recrudescence
	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)

Strain	Day of treatment (after inoculum introduced)	Treatment SHAM (mg kg^{-1})	Glycerol (g kg^{-1})	Recrudescence (days after treatment)
	6	500	4	8
Botswana	14	500	4	7
	22	500	4	12
	6	500	4	11
Zambia I	18	500	4	8
	26	500	4	6
	6	500	4	16
Zambia II	22	500	4	5
	27	500	4	7

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

Strain	Day of treatment (after inoculum introduced)			Treatment		No. of host survived/ No. of hosts used	Recru-descence
				SHAM $(mg\ kg^{-1})$	Glycerol $(g\ kg^{-1})$		
Botswana	6	7	8	500	4	0/4	-
Zambia I	6	7	8	500	4	0/4	-
Zambia II	6	7	8	500	4	0/4	-

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

Strain	Life span with normal infection (mean)	Life span with infection and x-irradiation (mean)	Life span of SHAM -treated hosts (mean)
Botswana	35 days	12 days	54 days
Zambia I	21 days	20 days	27 days
Zambia II	31 days	20 days	54 days

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 there-

fore 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 per-

manent 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 per-

sisting 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 recrudes-
cence like the monomorphic strains of Evans and Brightman.

This therefore indicates that the first alter-
native 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 treat-
ment was administered.   This observation further strengthens
the argument that tissue-invasive strains exhibit recrudes-
cence 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 norvegius 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

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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
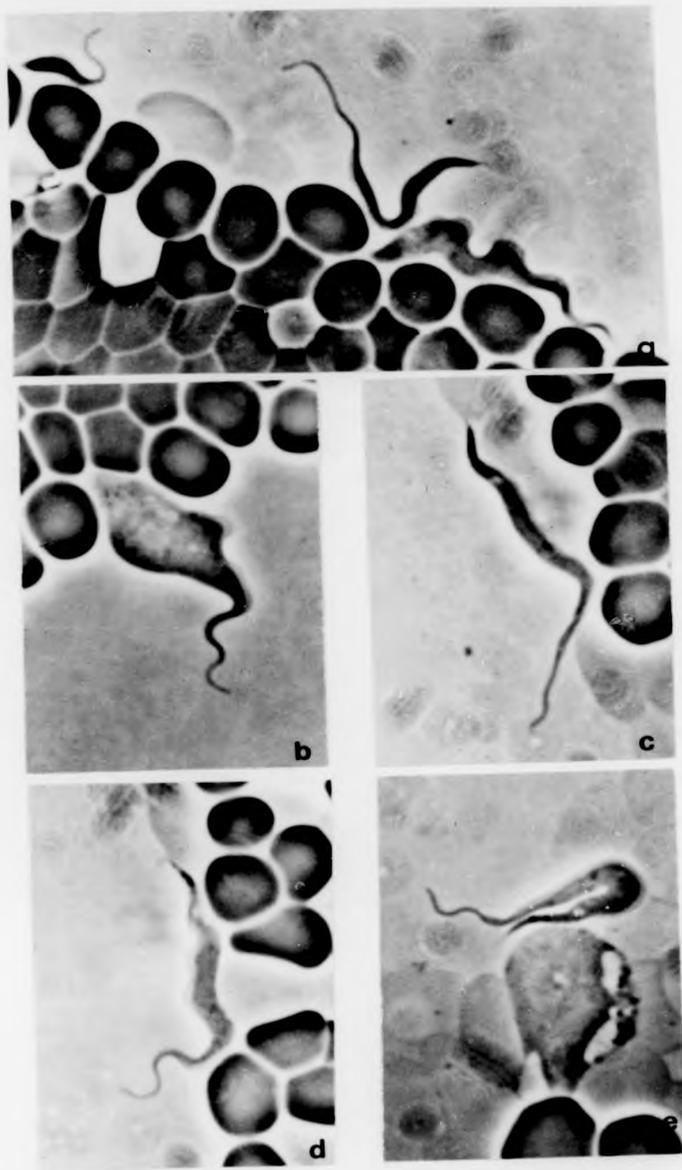
PLATE 8   <u>T. vivax</u> on agar

(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

road

86.

e

granules

mia

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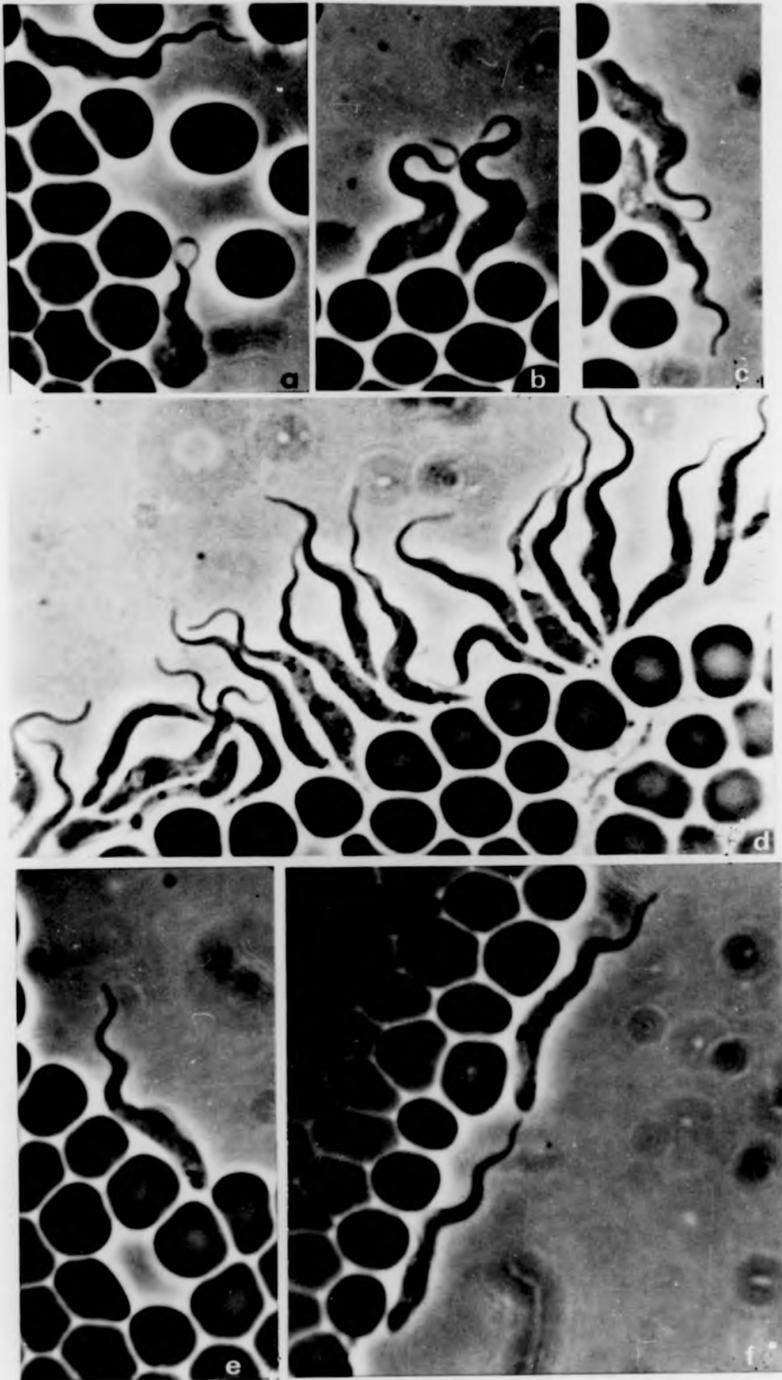
PLATE 9   Savannah <u>T. congolense</u> on agar

        (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

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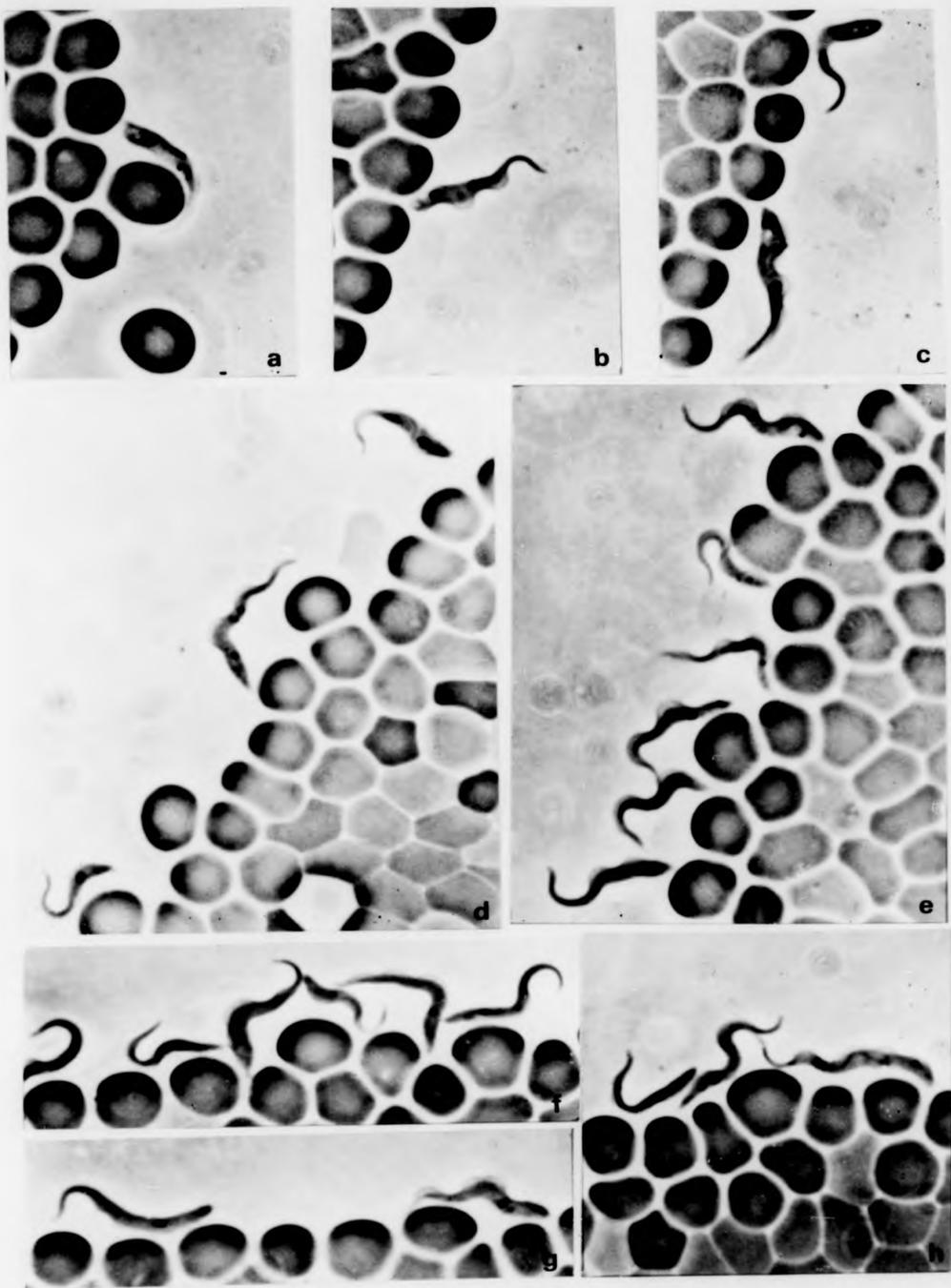
PLATE 10   Riverine T. congolense   on agar

(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

which

granules
tograph

rating
free

which had
other had
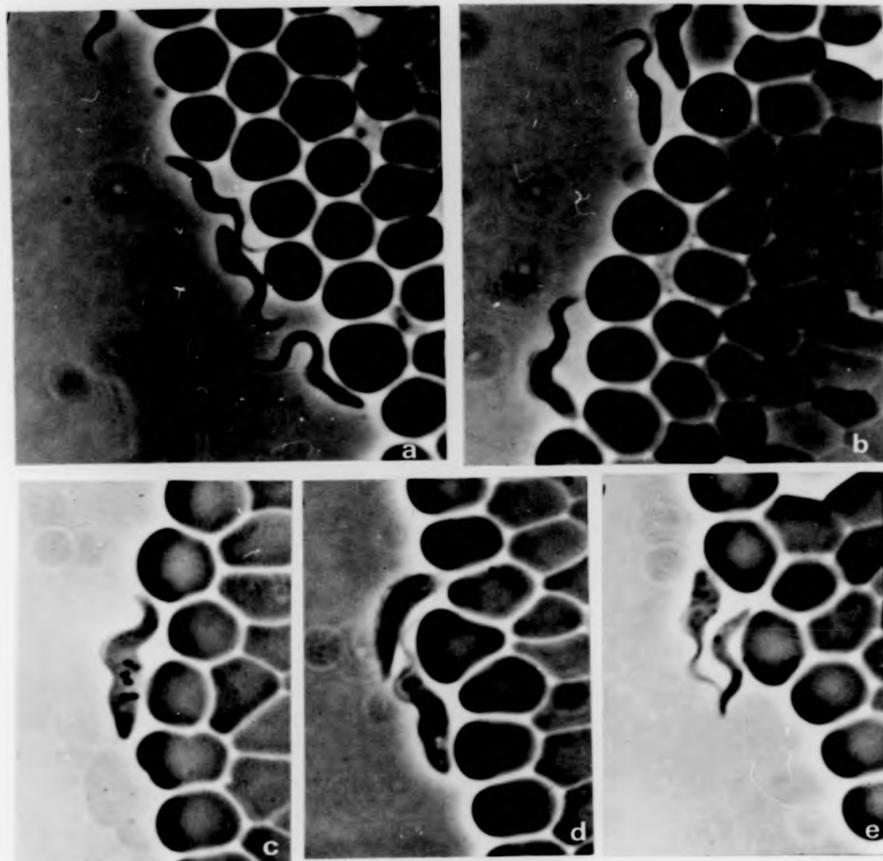
PLATE 11   T.brucei trypanosomes on agar

(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
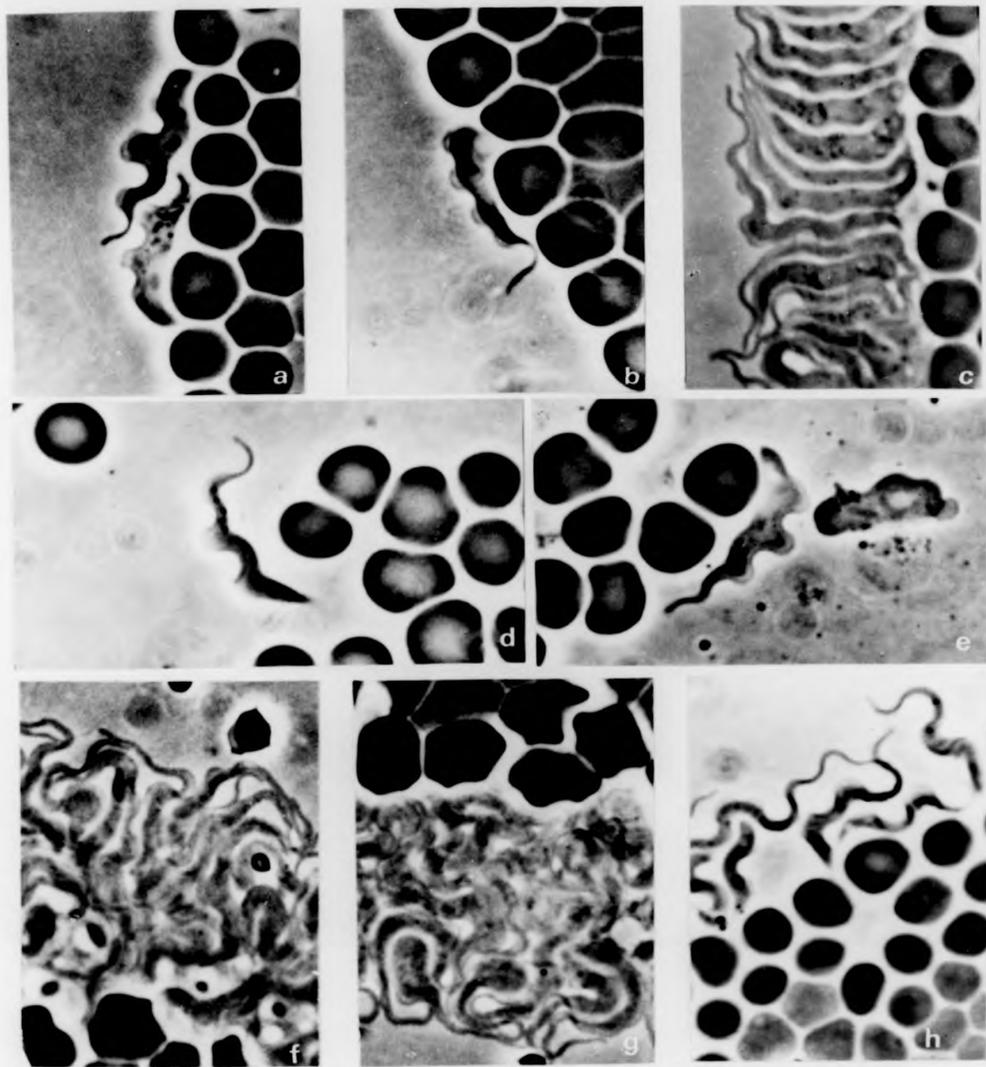
PLATE 12   T. brucei on agar continued

      (a)   58 strain

      (b)   58B strain

      (c) - (e)

          1198 strain

      (f)   LUMP 1236 strain

      (g)   Bida 3 strain
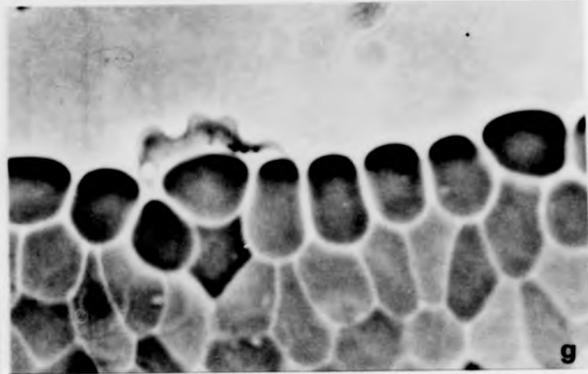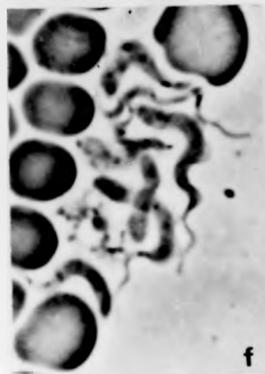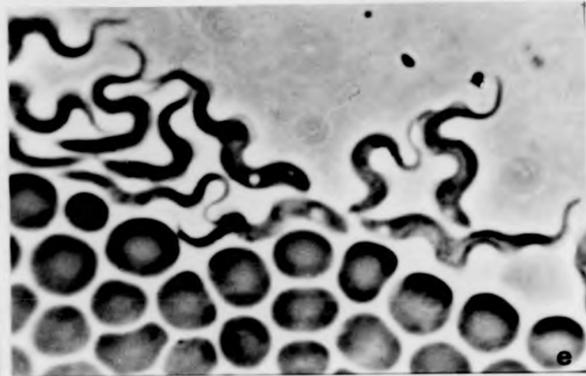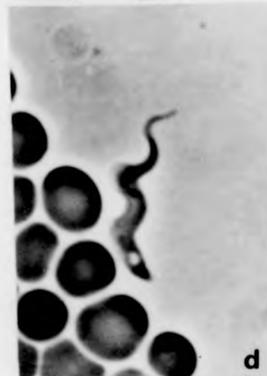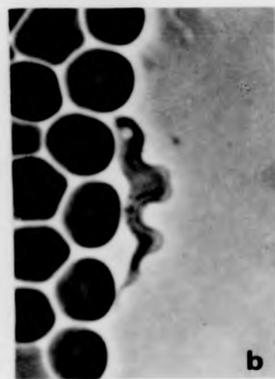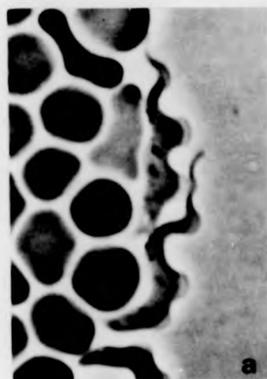
trypanosomes as could be found from Plate 7b. The majority of these juvenile forms were without granules but approximately one in eight had multiple granules. Because of the tubular nature of the parasite these granules were distributed in layers in the posterior end. No accurate counting could be made. 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 8 d and f. The pattern for the granules also remained constant at high parasitaemia as in Plate 8 d or at low levels as in Plates 8 e 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 conspi-
cuous 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, pos-
terior 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

(Plate 11e).   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 tr ypanosome 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) <u>Strain 4</u>: 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 <u>Trypanosoma (Trypanozoon) brucei gambiense</u>

(a) <u>Strain 1</u>: 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) <u>Strain 2</u>: 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 indi- viduals as in 180 had only one or two granules visible. The granules in Bida 3 tend to lump together. Some mori- bund forms were also found.

6.1.5 <u>Patterns of Granules</u>

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

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

* Clones studied.

Table 13 Patterns of granules in the different species
of genus Trypanosoma

Species	Location and distribution of granules
Trypanosoma (Herpe-tosome) lewisi	Variable (1-3) granules restricted to kinetoplast end of trypanosome
T. (Duttonella) vivax	Numerous granules, evenly spread on both sides of the nucleus
T. (Nannomonas) congolense	Few (3-7) granules spread on both sides of the nucleus
T. (Trypanozoon) brucei	Variable granules mainly restricted to anterior half of the trypanosome

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

Strain	Pattern of granules
Y58	Faint granules
Y486	Conspicuous granules

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

Strain	Pattern of granules	Other comment
Gamb 6	One of the granules more prominent)	
		) Savannah
Gamb 19	One of the granules more prominent)	
TSW 99	All granules equally conspicuous)	Forest/
		)
TSW 103	All granules equally conspicuous)	Riverine

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 trypanosomiansis and other protozoon 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

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

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 depsite 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) <u>Blood incubation infectivity test (BIIT)</u>

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

6.2.2.1(e) <u>Enzyme electrophoresis</u>

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 <u>Paramecium</u> and Borden <u>et al.</u> (1973) for <u>Tetrahymena</u>. Sargeant <u>et al.</u> (1978) used the method for differentiating invasive and non-invasive <u>Entamoeba histo-lytica</u> and Sargeant and Williams (1979) studied the iso-enzyme patterns of pathogenic and non-pathogenic intestinal amoeba of man. <u>Plasmodium</u> spp. were studied by Carter (1970) and Carter and Voller (1975); <u>Eimeria</u> by Shirley and Rollinson (1979); and <u>Leishmania</u> 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 pitfal 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 II98 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 pit-
falls 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, treat-
ment 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 pat-
tern 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 charac-
terisation 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 sur-
face of the parasite was gracefully smooth (Plate 13 a).
Except for size variation, there was little to differen-
tiate 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

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PLATE 13   <u>T.lewisi</u>

(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)

surface to be

posterior end.

e flagellum

(X20,000)
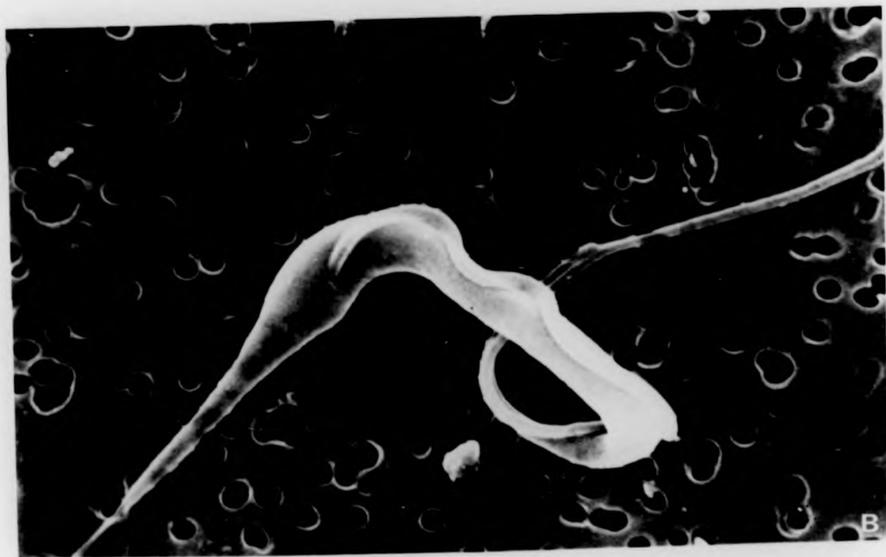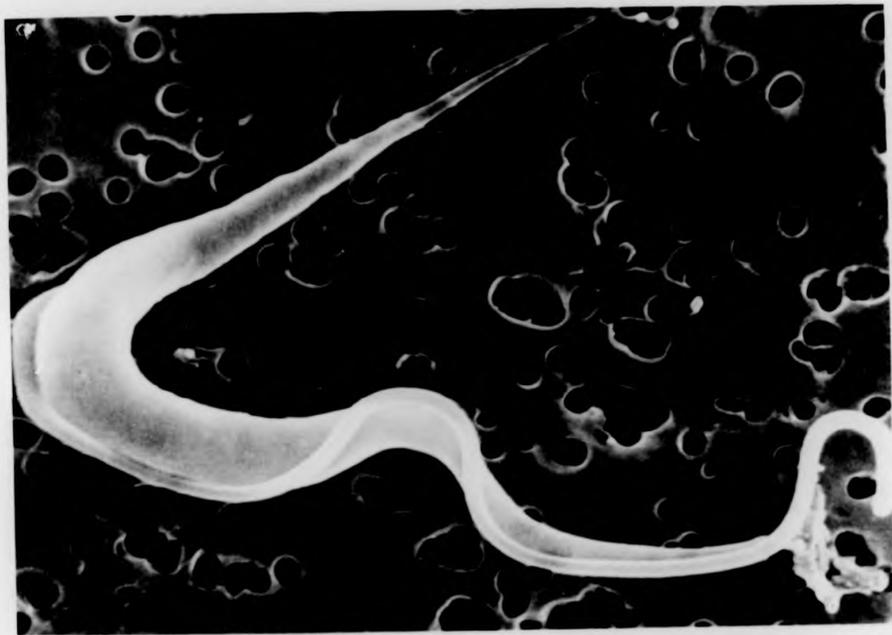
PLATE 14   <u>T.lewisi</u> continued

Parasites with shortened posterior ends.
(X13,000)

sterior ends.

14

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)

amination

parasites

trypanosome

trating shallow
th a possible

149
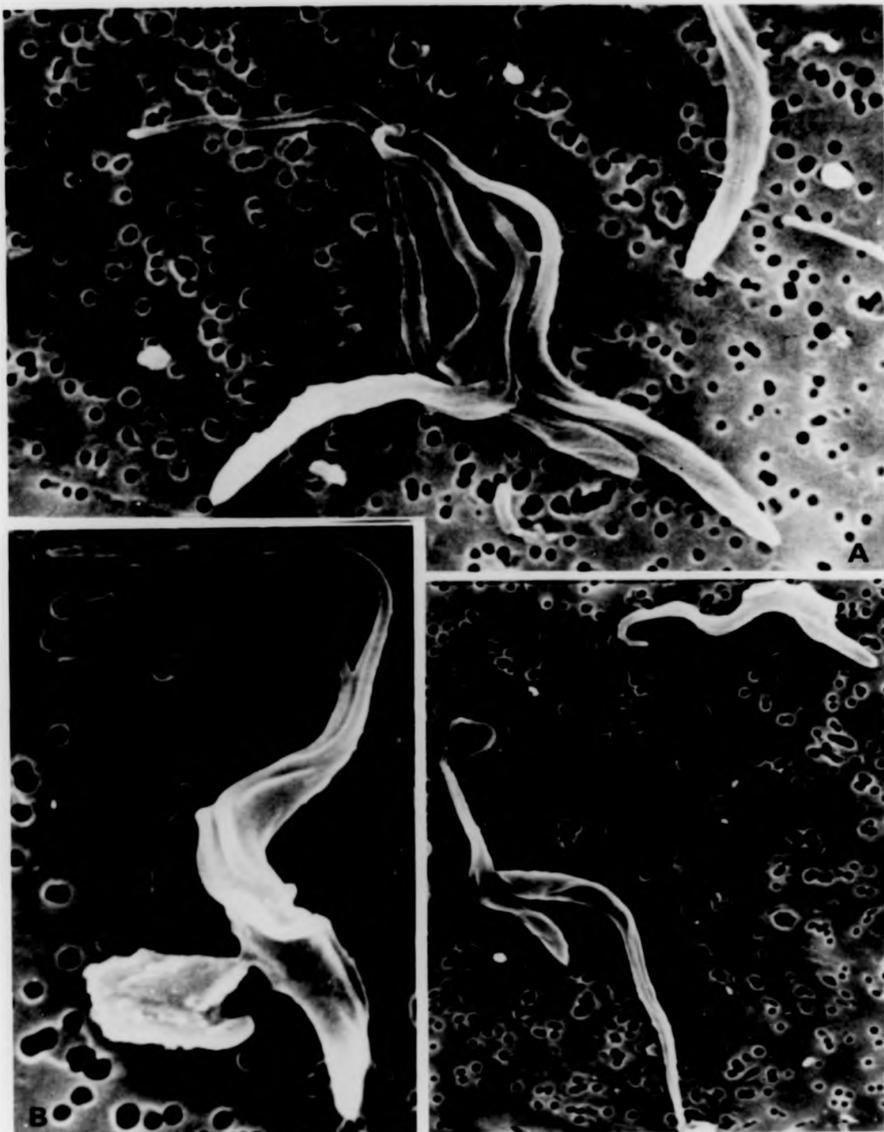
PLATE 16   <u>T.congolese</u>

(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)

trypanosome

me without
X32,500)

short free
00)
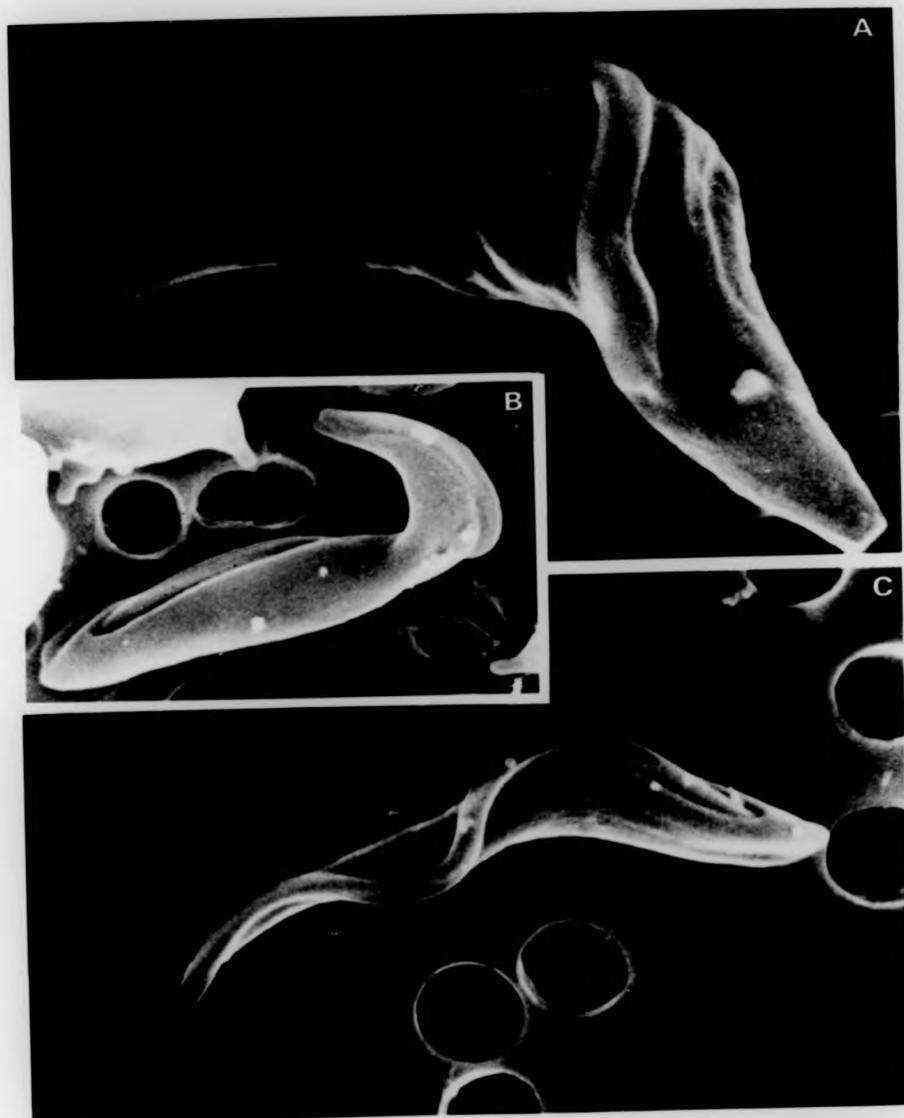
PLATE 17   T.b.brucei,Antat 1 strain

(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)

es showing
s.

ple division

two daughter
the parent

PLATE 18   <u>T.b.brucei</u>, 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)

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PLATE 19   T.b.brucei, 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)

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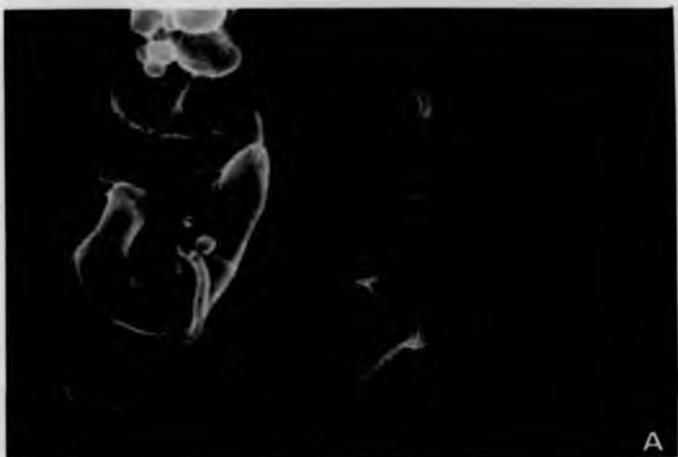
PLATE 20   T.b.rhodesience, Zambia II strain

(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 plas-
       maneme.   (X27,500)

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

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154
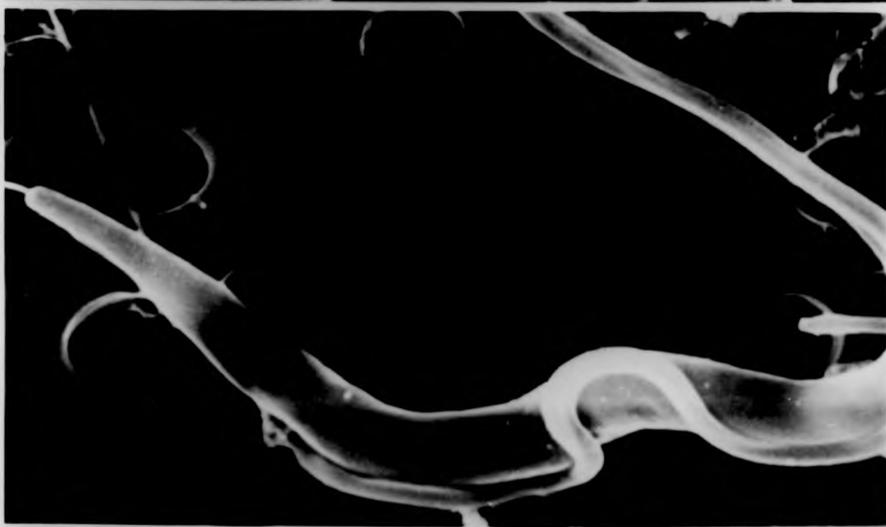
PLATE 21   T.b.rhodesiense,   Zambia I strain

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

(B)   A long-flat form with beads.
(X10,000)

155
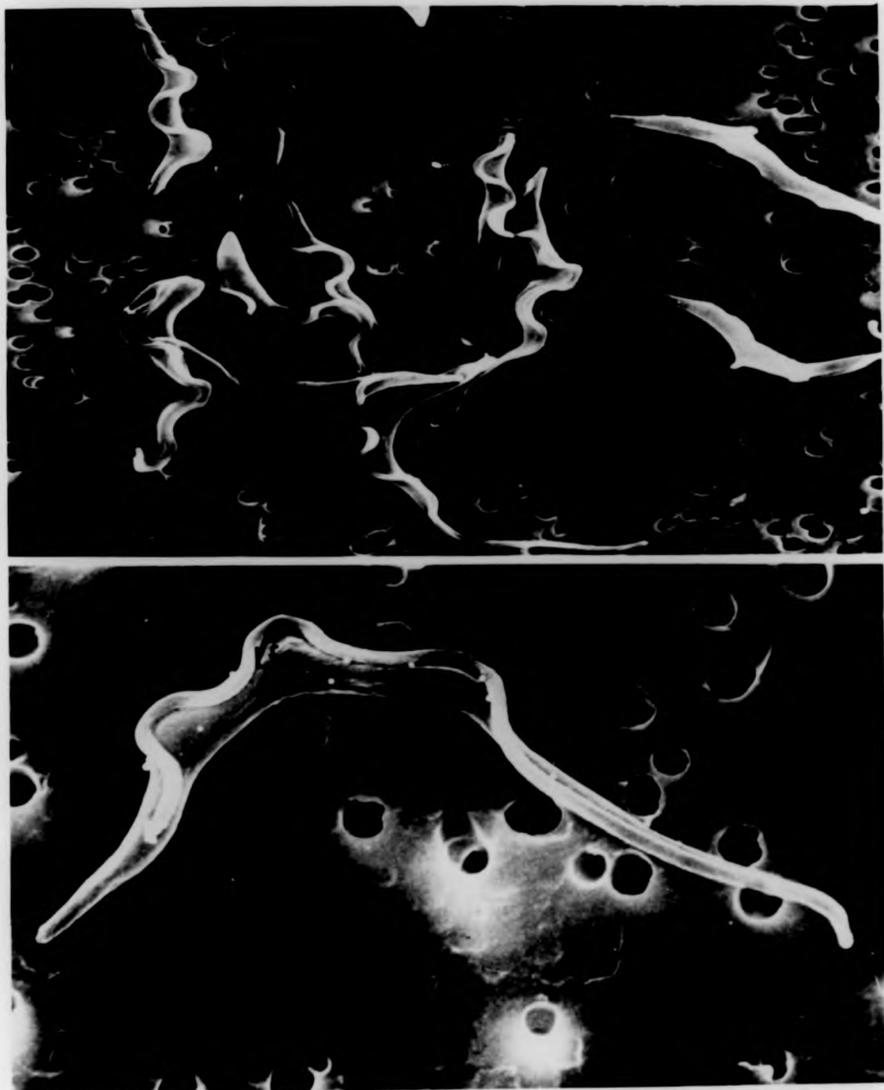
PLATE 22   <u>T.b.rhodesiense</u>, Zambia I strain continued

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

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

ain continued

d rods

beads.

156
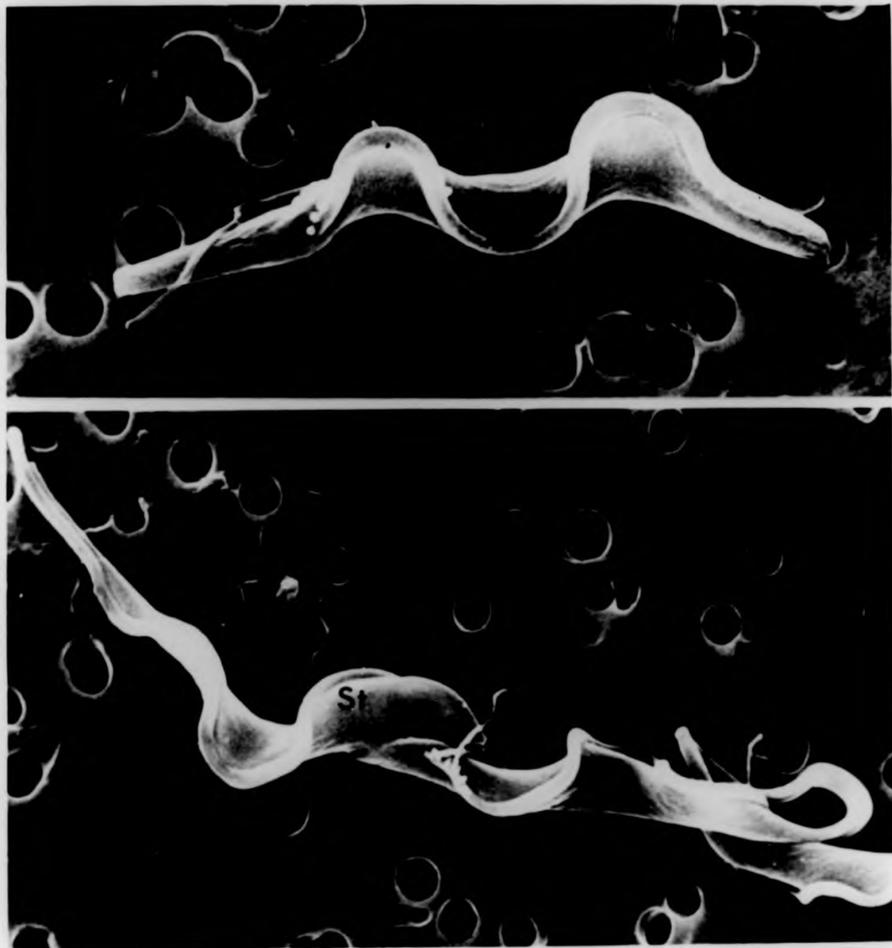
PLATE 23   T.b.rhodesiense,   Zambia I continued

(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)

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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)

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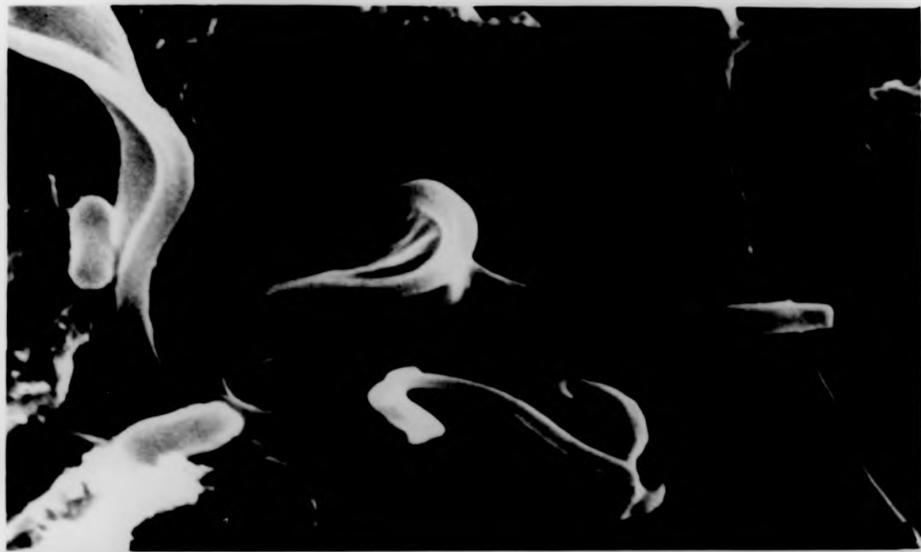
PLATE 26   In vivo phagocytosis

(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 inter-
      action between the parasite and
      the macrophage as the engulfment
      started and proceeded.

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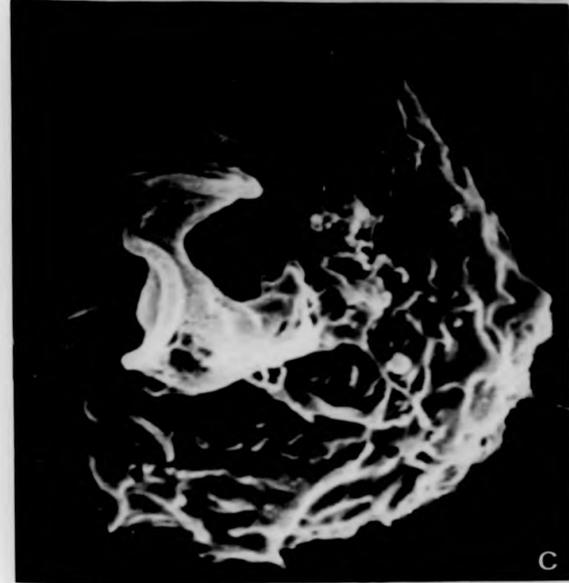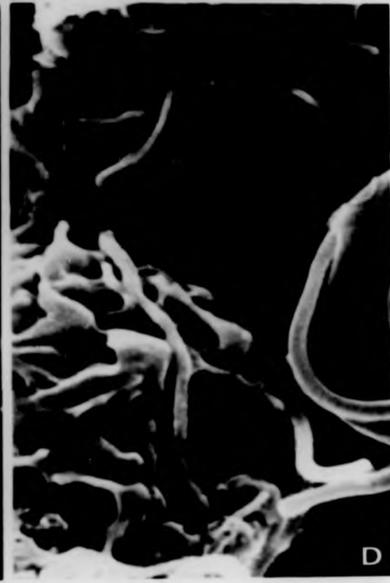
PLATE 27   In vitro phagocytosis

(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.

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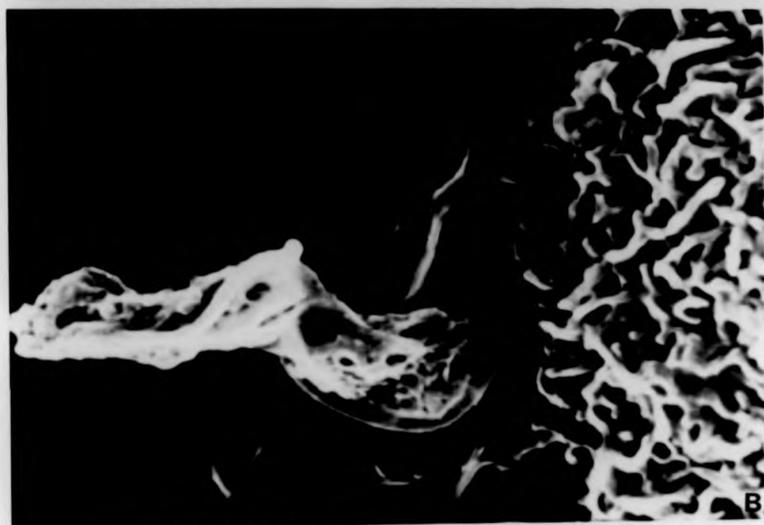
PLATE 28   <u>In vitro</u>   phagocytosis continued

(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

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sion.
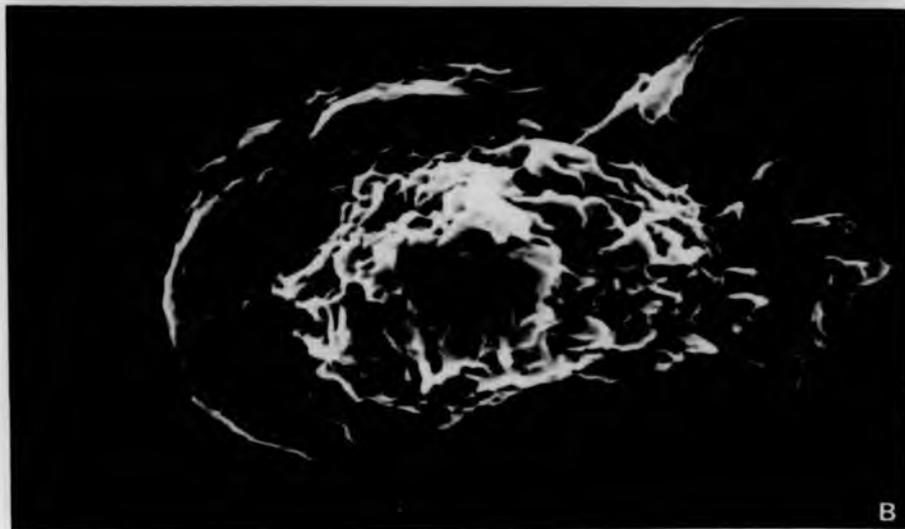
PLATE 29   <u>In vitro</u>   phagocytosis continued

(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.

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Plate 28b
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PLATE 30   <u>In vitro</u> phagocytosis with dead
parasites (already fixed with gluta-
raldehyde)

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

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

    Note: absence of pitting.

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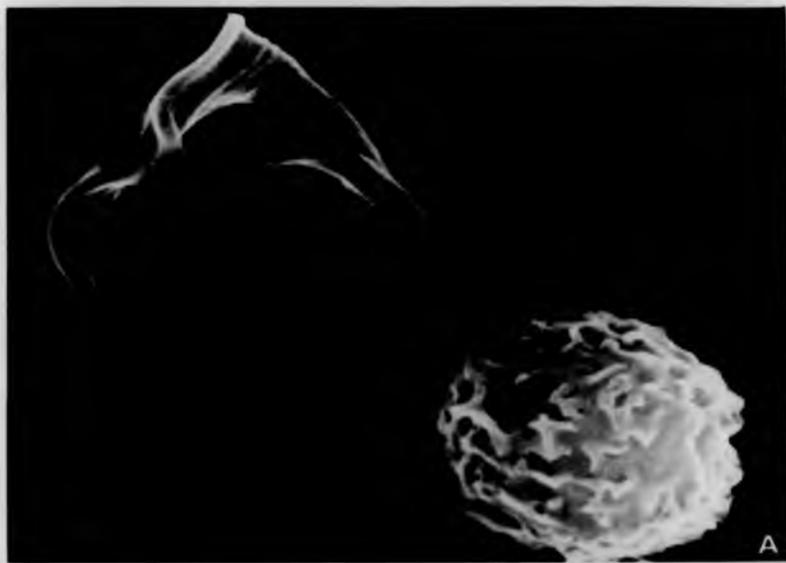
PLATE 31   <u>In vitro</u> phagocytosis with dead parasites

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

(B)   Possible end of phagocytosis with
just a bit of flagellum of try-
panosome   sticking out.
(X15,000)

dead parasites

parasite.

cytosis with
um of try-
t.
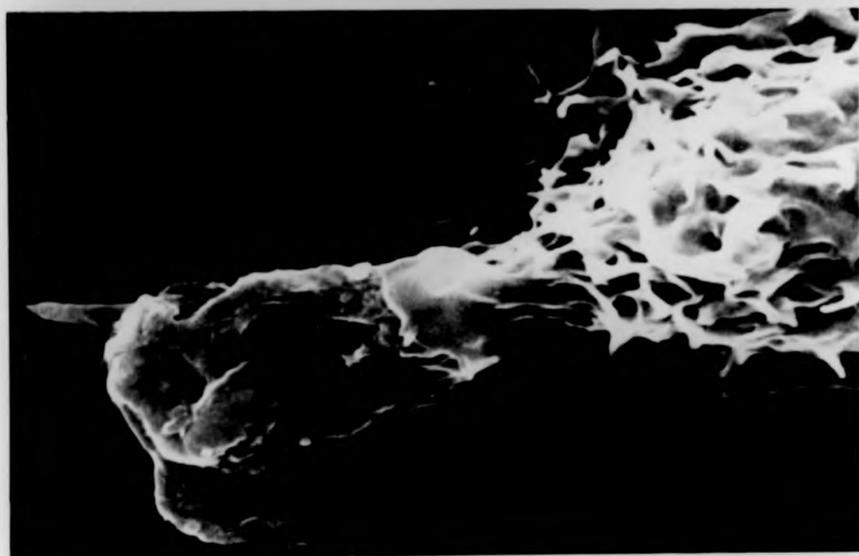
end could be rounded up (Plate 14).

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 mono-morphic 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 trypano-some 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 I 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 mor-
phology of the form with only a short visible free flagel-
lum.   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 miscroscopy (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 under-
standing 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 agglu-
tinations 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
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 T. brucei
will bind together with con A, the bloodstream forms of
T. brucei would not adhere satisfactorily.   The probable

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Tetley (1977) who showed that culture forms of T. brucei
will bind together with con A, the bloodstream forms of
T. brucei would not adhere satisfactorily.   The probable

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. Simi-
larly 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 characteris-
tic 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 steromicrographs 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. Gorenflot et al. (1980) claimed that after fixation in glutaldehyde 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 infor-
mation) 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.(19) also on T.b. brucei illustrated unmistakably
the flagellar pockets.   This work also showed the flagel-
lar 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. 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 expec-
ted values given by the various authorities quoted con-
firms 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 pos-
sibility of tissue invasion.   This will be further com-
mented 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, exten-
ding 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 dif-
ferent 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 satis-
factory 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 <u>L. tropica</u> does not behave in the same way.

After the incidental observation of phagocytosis <u>in vivo</u> 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 <u>in vitro</u>? 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 <u>in vitro</u> 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 <u>T.b. brucei</u>, in phagocytosis, was attached to the macrophage by the flagellar end. Although some parasites attached by posterior end were noted Zenian <u>et al.</u> (1979) asserted that the promastigotes were predominantly attached by flagellar tips. Muna and Muhammed (1981) repeated the statement about promastigotes of <u>Leishmania tropica</u>.

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 macro-
phage the victim has been degraded was confirmed in vitro

and it appeared that some unknown biochemical activity had
taken place.   The glutaldehyde-killed parasite was taken
in without such degeneration.   That the parasitic micro-
organisms 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 pene-
tration 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 u membrane
       filter   (X 6,600)

(d)   A parasite caught on 0.4 u membrane
       filter   (X20,000)

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at 96 hour

l u membrane

membrane

a

b

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PLATE 33   Blood parasites continued

(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)

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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)

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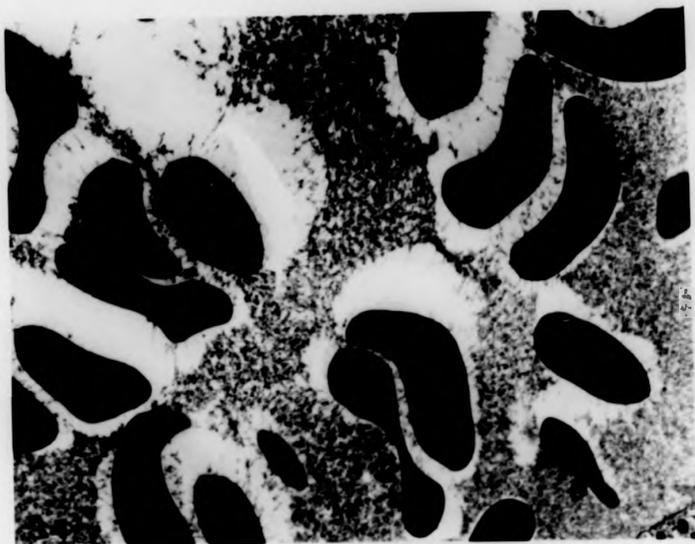
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PLATE 36   Histology of C.P.

(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)

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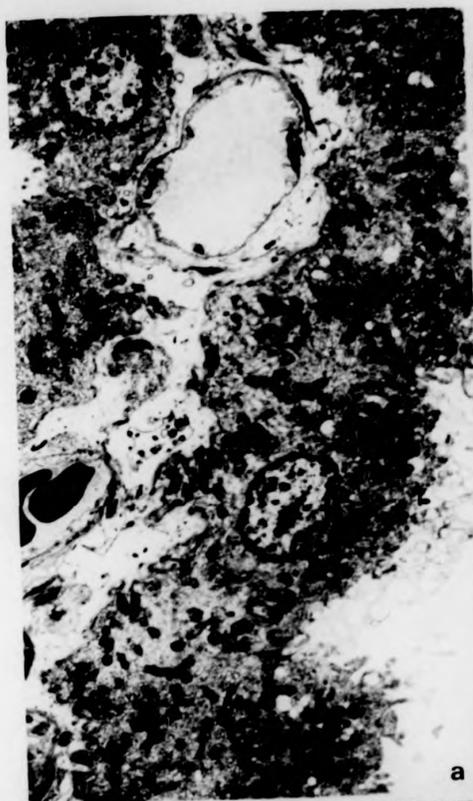
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PLATE 37   Histology of C.P. continued

(a)   Section of C.P. showing probably an
      astrocyte between the blood vessel
      and the ependymal cells   (X13,200)

(b)   Blood vessel with internal elastic
      membrane   (X4,000)

(c)   Section of roof of a ventricle
      (X30,000)

(d)   Section of the brain with nerve cells
      (X10,000)

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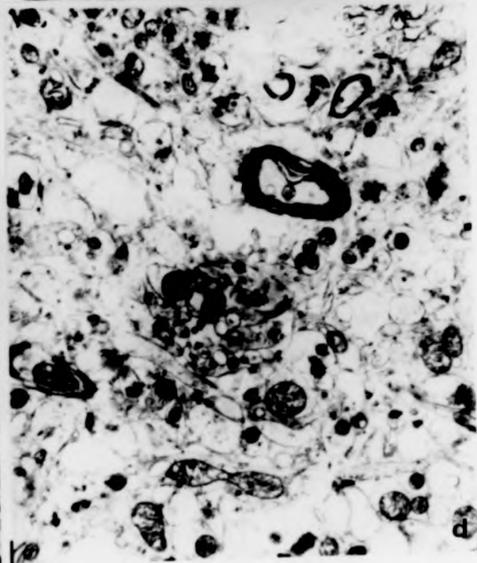
PLATE 38   Histology of C.P. continued

(a)   Ciliated ependymal cell   (X4,000)

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

b

a
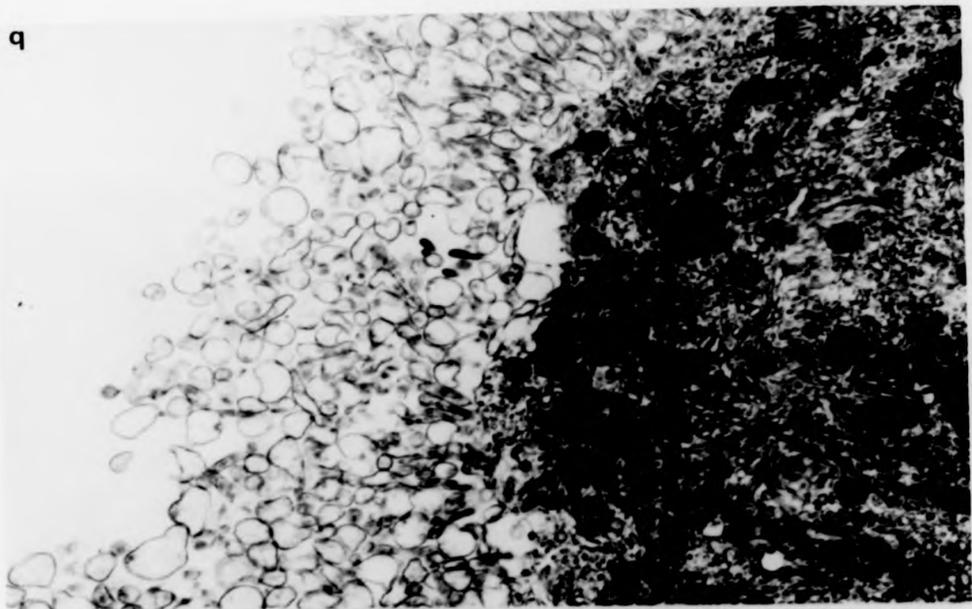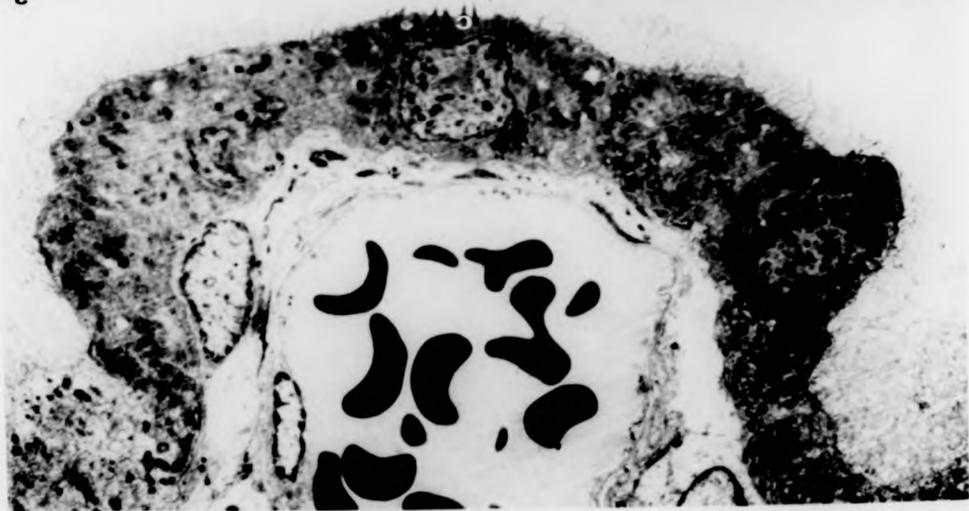
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PLATE 39   Tissue parasites

(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)

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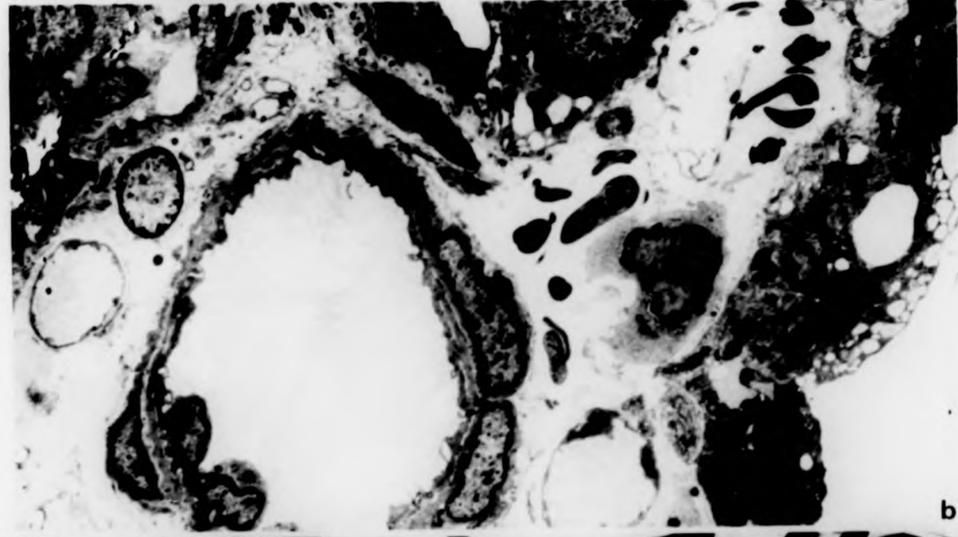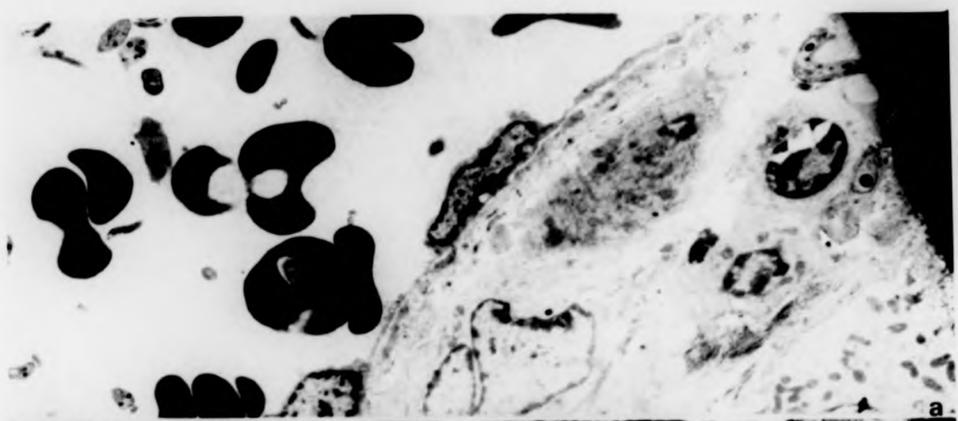
PLATE 40   Tissue parasites continued

(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)

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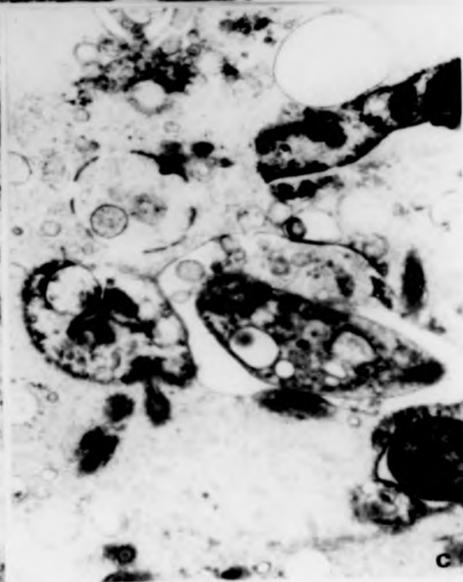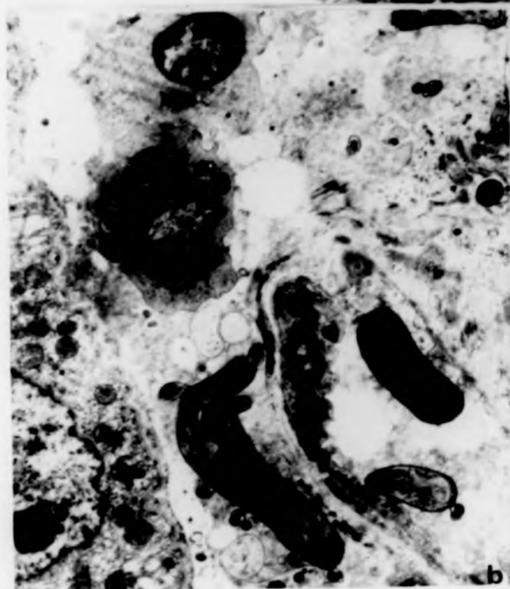
PLATE 41   Tissue parasites continued

(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)

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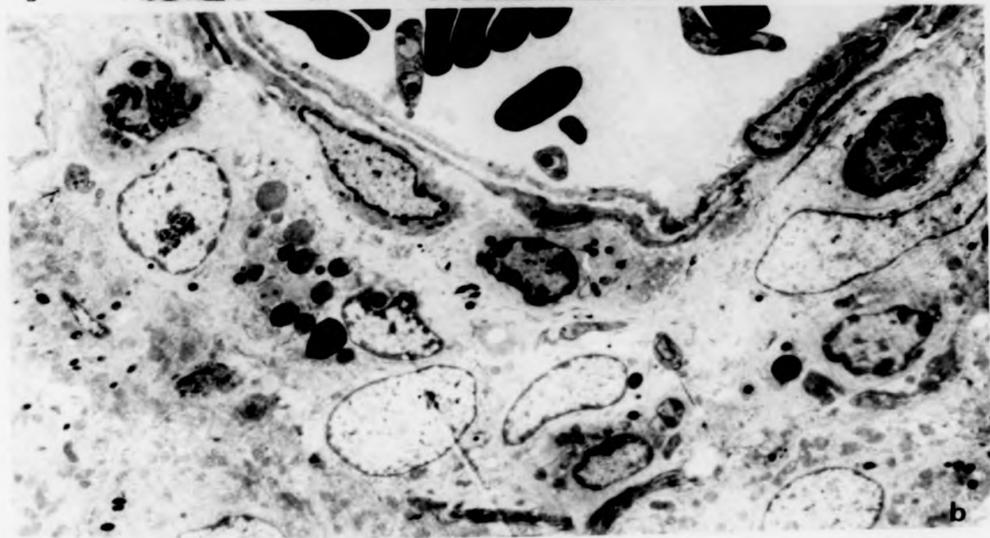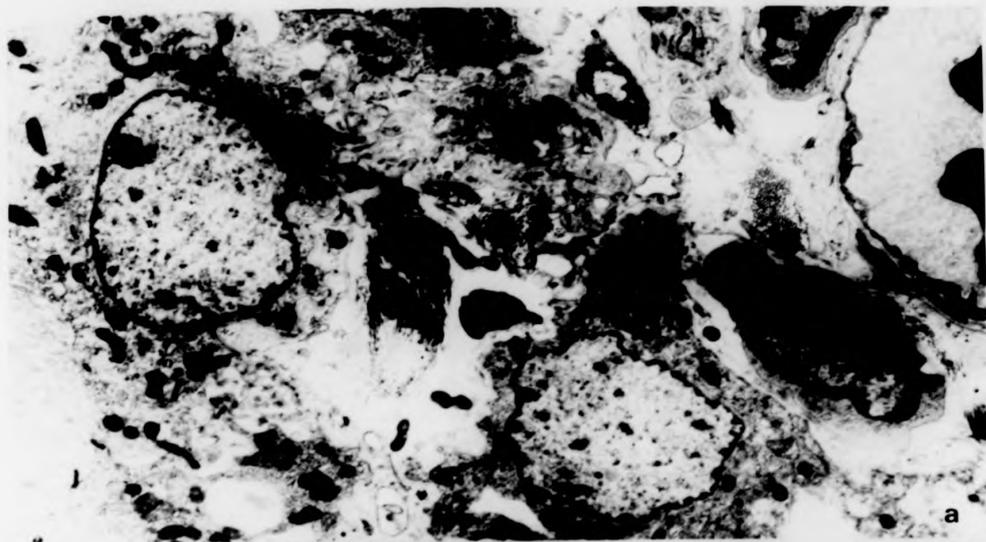
PLATE 42   Tissue parasites continued

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

       (b)   Rbc inside a macrophage.   Note mito-
            chondria being destroyed   (X30,000)

       (c)   Section of C.P. with internal haemorrhage
            observed   (X6,000)

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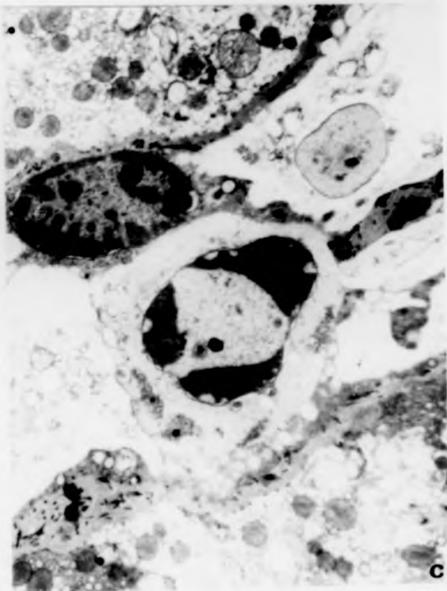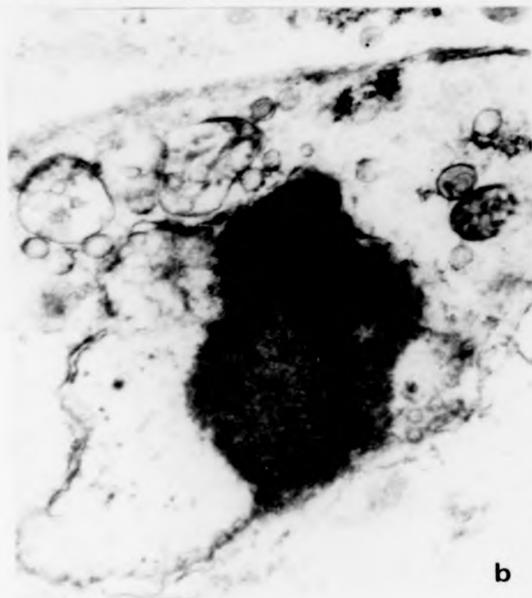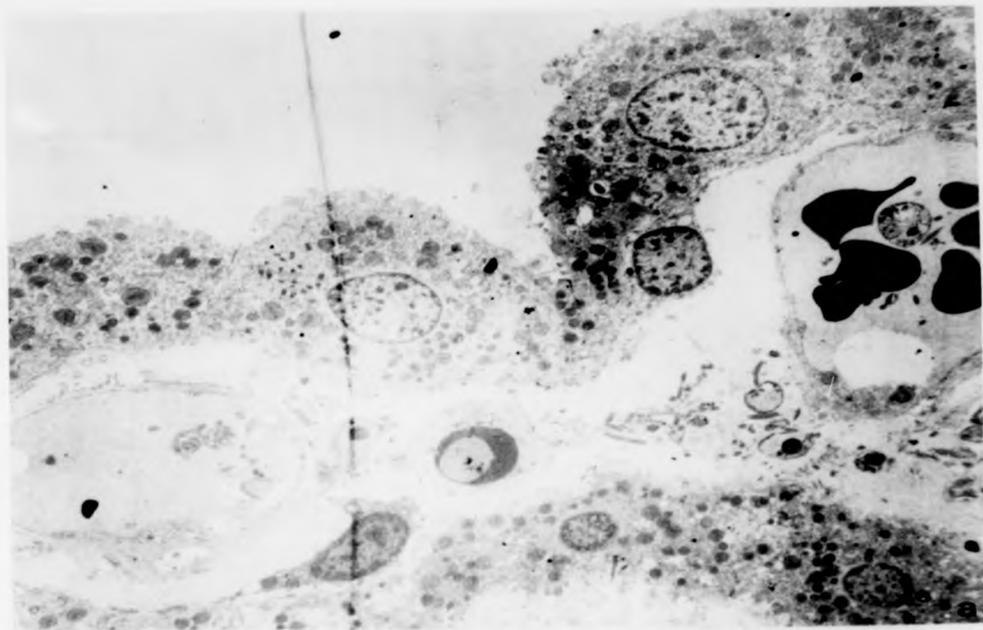
PLATE 43   Tissue parasites continued

(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)

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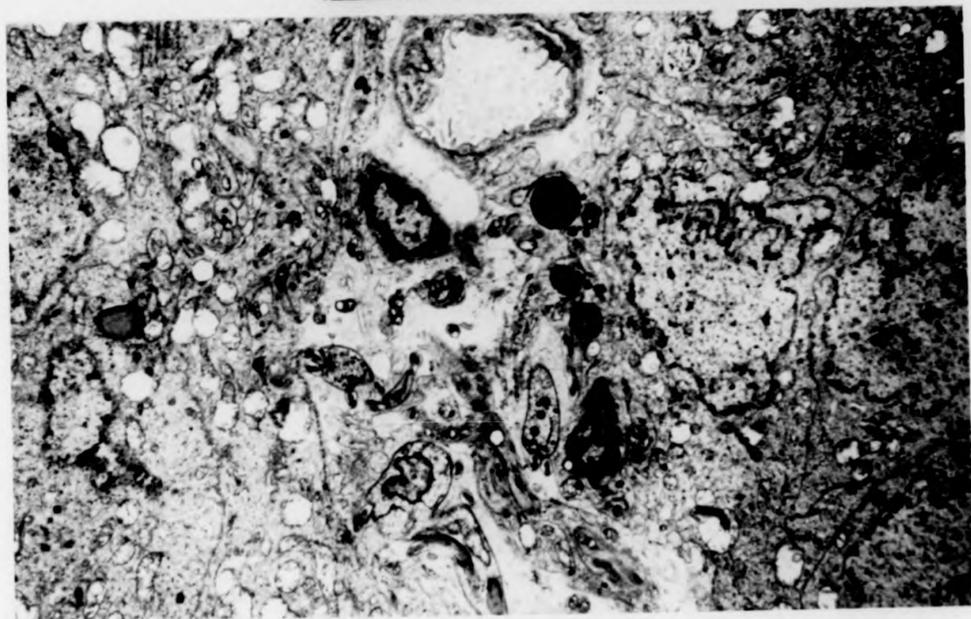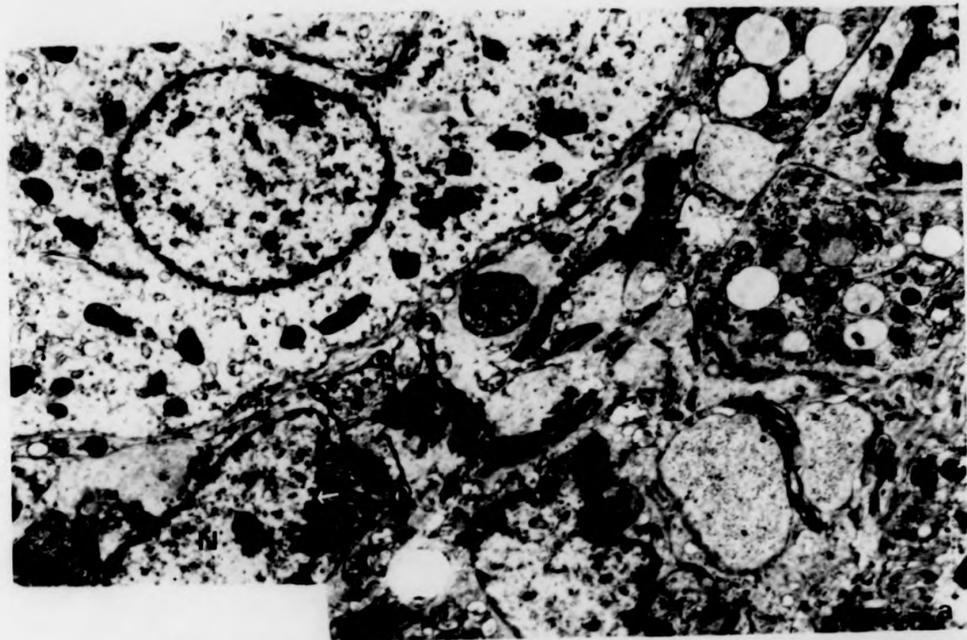
PLATE 45   Tissue parasites continued

(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
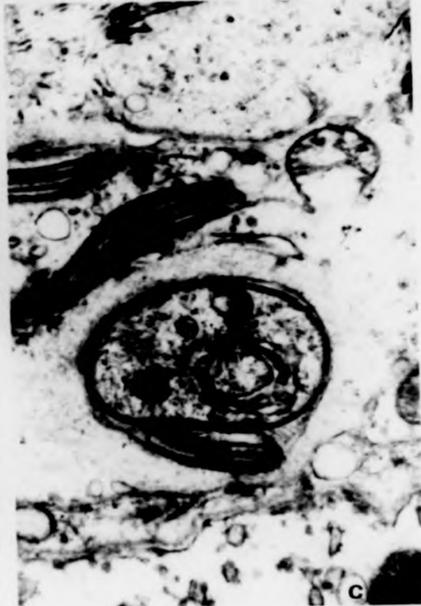
PLATE 46   Tissue parasites continued

(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
       (X40,00)

(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)
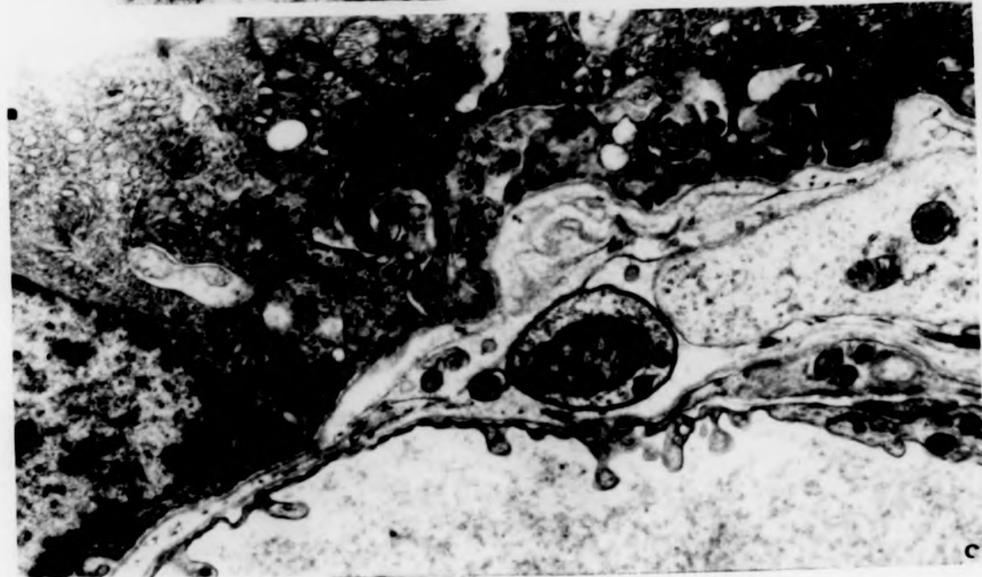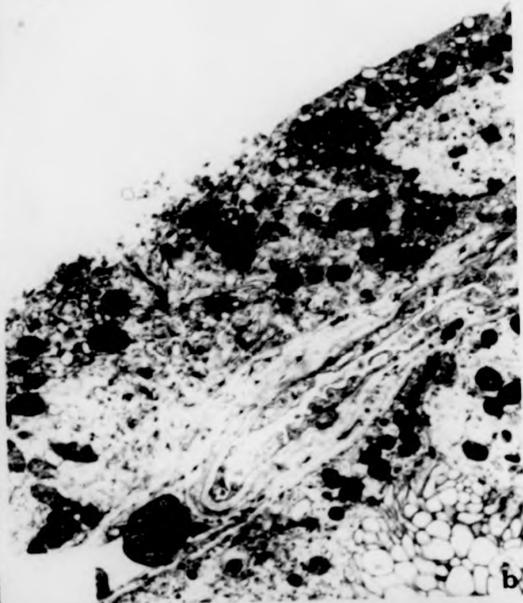
PLATE 48   Tissue parasites continued

(a)   Section of a tissue parasite showing
      the surface coat   (X30,000)

(b)   T.S. of trypanosome with double mem-
      brane 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)

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)
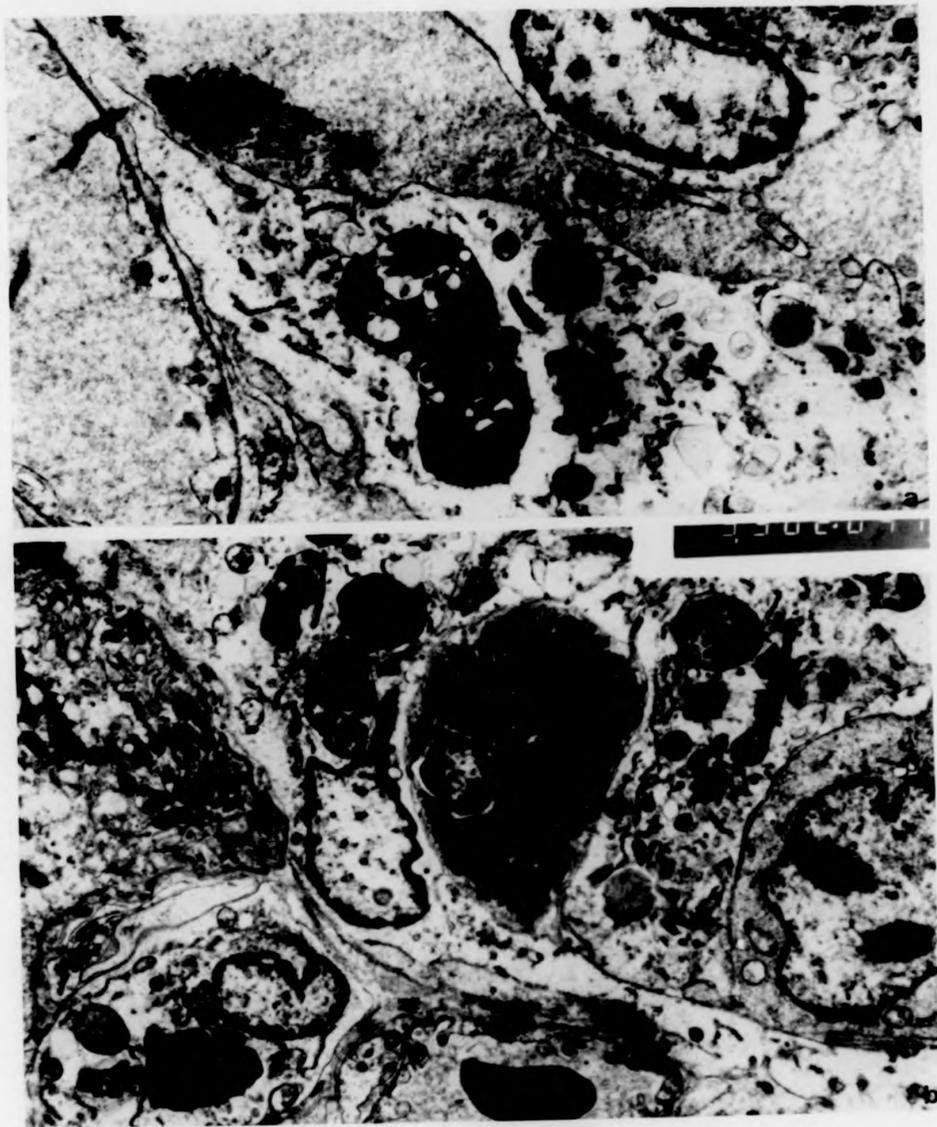
PLATE 50   Tissue   parasites and chemotherapy effects
            continued

            (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)

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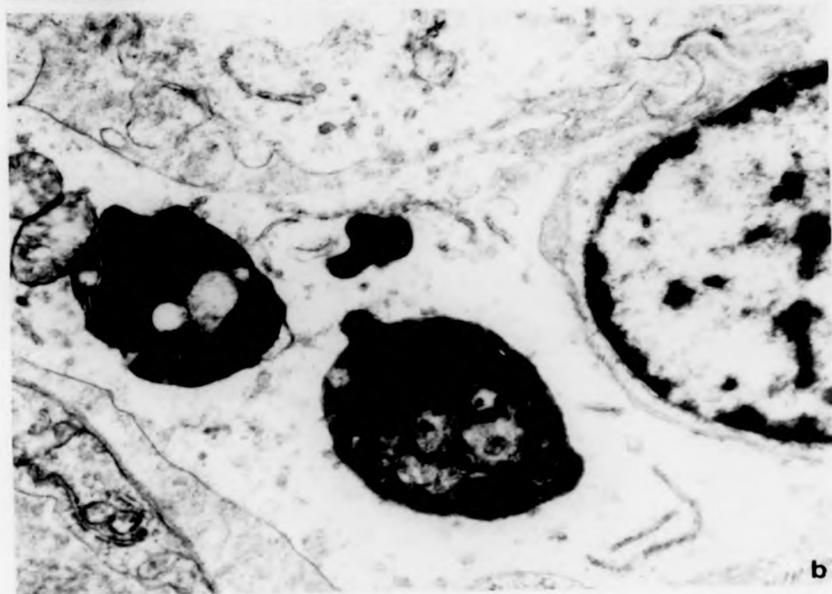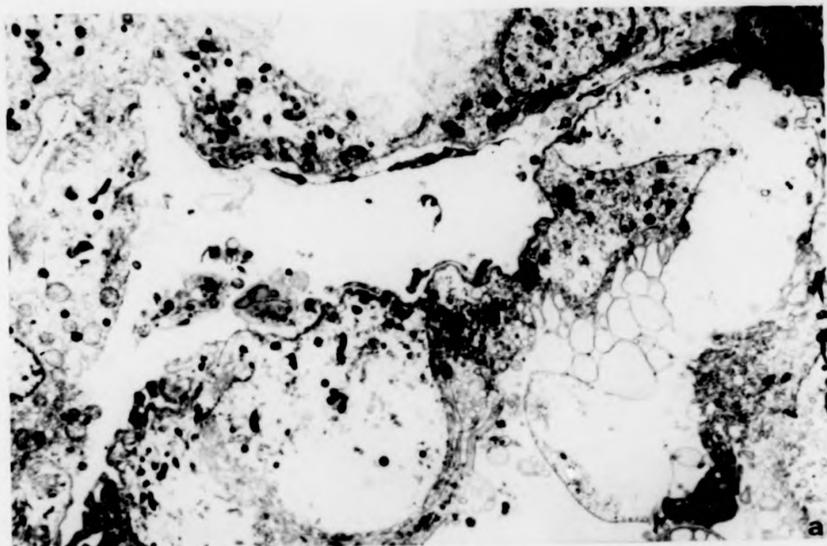
PLATE 51   Tissue parasites and chemotherapy effect
continued

(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)

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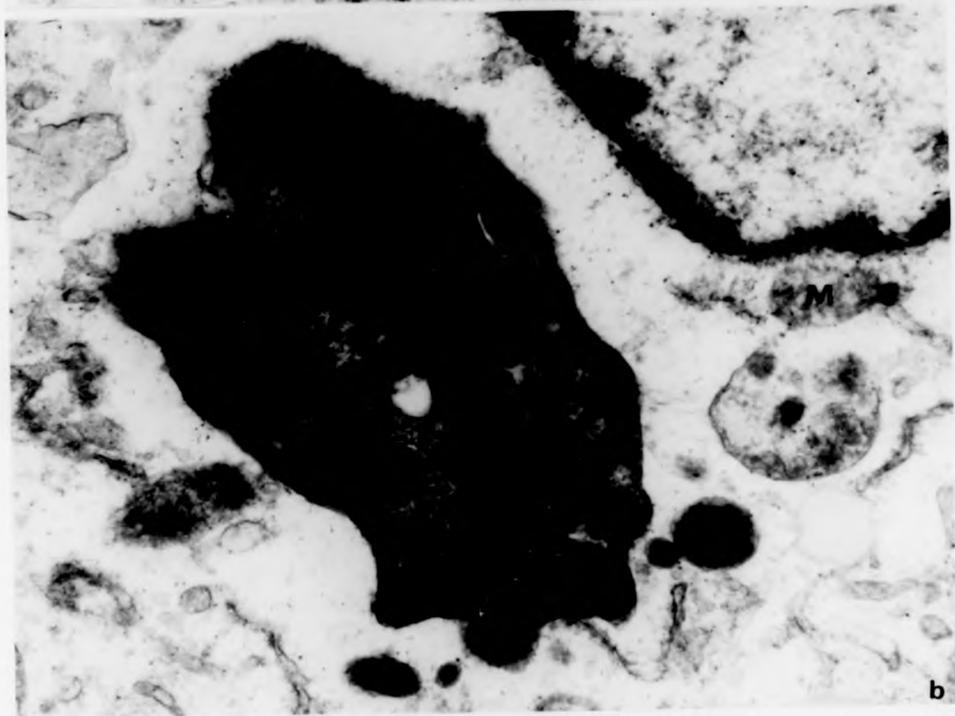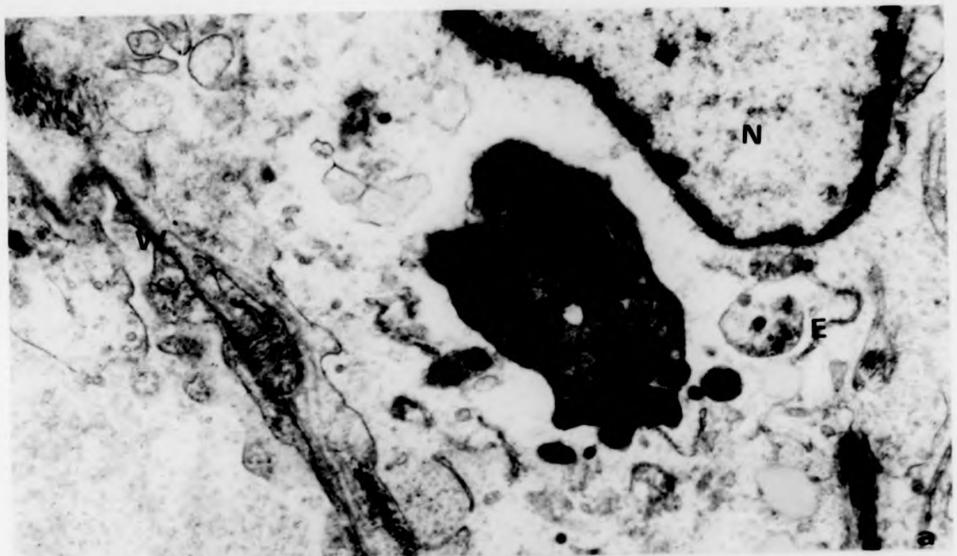
PLATE 52   Tissue parasites and chemotherapy effect
continued

(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)

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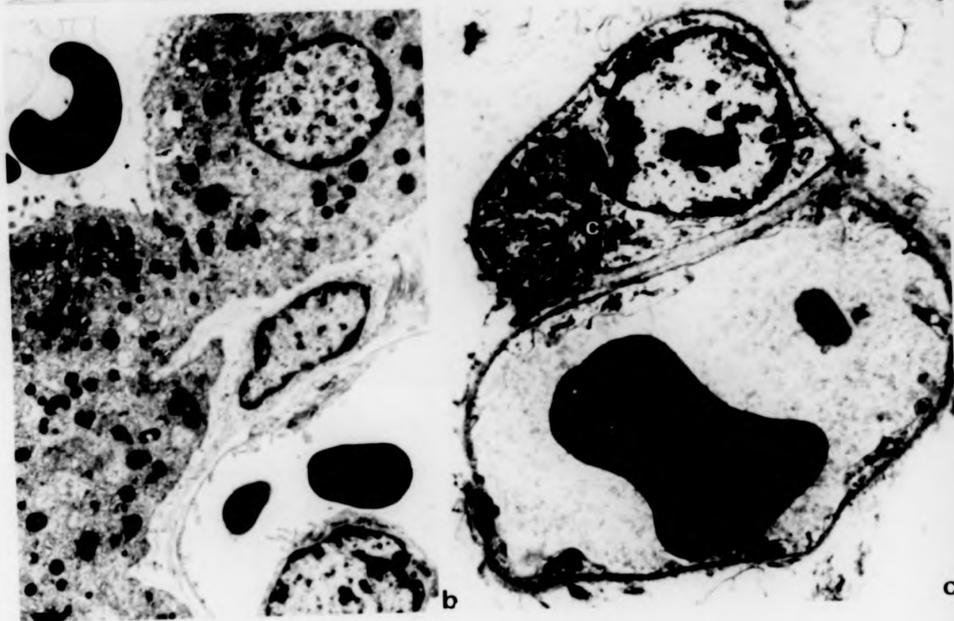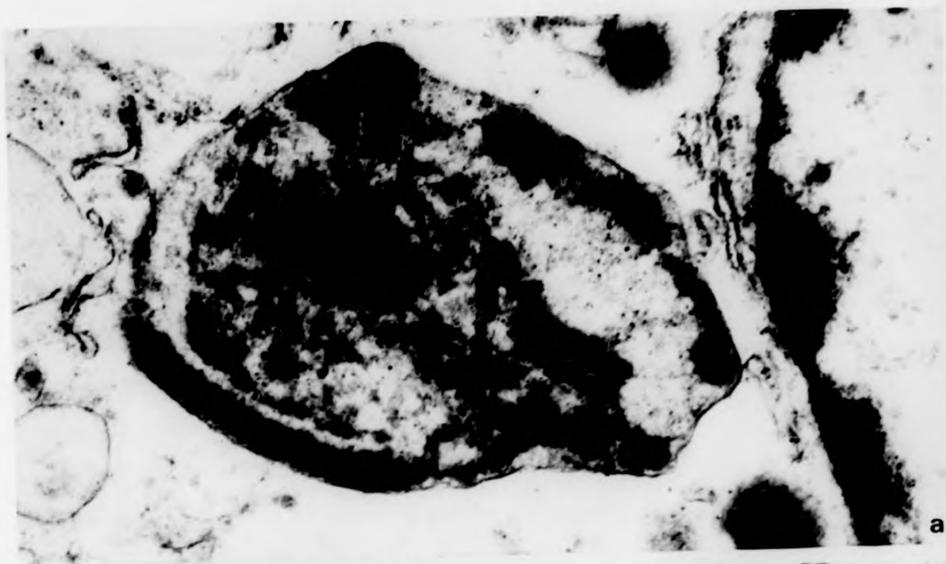
and the smaller ones caught by 0.4 μ membrane placed
underneath some separation was possible.   On the upper
layer were found parasites mainly the dividing or bigger
long-flats.   Some long-narrow forms were also present.
The parasites on the lower membrane were few.   About
four out of five were long-narrow 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 tech-
nique 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 mor-
phology at 72 hours.   This population in higher para-
sitaemia   was represented by Plate 32b with several
dividing long-flat forms and occasional giant (multi-
flagellar) 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), endo-
plasmic reticulum, golgi apparatus, flagella with the
flagellar pockets, and kinetoplast. On one hand, the
occurrence, distribution and morphology of these organ-
elles 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 mem-
brane 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 endo-
plasmic reticulum connected to the kinetoplast.   Divi-
ding 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 p eritoneal and ascitic fluids has been reported in
Chapter 4. In this chapter the result of the investi-
gation into the location of trypanosomes in the extra-
vascular 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 diffi-
cult 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 epi-
dermal cell or interstitial matrix of the tissue.   How-
ever 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 ven-
tricles (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 para-
sites 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 sig-
nificance 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 abun-
dance 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 para-
sites or blood corpuscles (Plate   39b) presumably
because of draining of most of the blood prior to dis-
section 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 parasi-
taemia 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 fur-
there 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 pre-
cursors 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
40 c. 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 extra-vascular 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 multiflagellar (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

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 mito-
condrion 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.

8.1.3   Chemotherapy and the Extravascular Parasites

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 un-
treated 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 lympho-
cytes 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 mito-
chondria 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 ama-
stigote 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,

1979 a),unrolling form (Sant'ana, 1913; Penso, 1934;
Peruzzi, 1935; Ormerod, 1979a)and visceral form (Ormerod,
1979a; Lumsden, 1972).   Similarly tissues and cells asso-
ciated with the tissue phase had been widely distributed.
These were cerebrospinal fluid (Castellani, 1904), lung
(Salvin-Moore and Breinl, 1908; Philiptshenko, 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 chemo-
therapeutic 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 demon-
stration 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 intra-cellular 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 identi-
fied 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 eluci-
dation 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 dis-
tinct 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 endo-
thelium 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 micro-
scopy 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 parasitophrus
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 intra-
cellular. While it is arguable that those in the base-
ment 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 mito-
chondria 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 para-
sites 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 equiva-
lent 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 sig-
nificant that Peters (1980) could not include T. brucei
in the recent review of therapy of intracellular para-
sitic 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 dis-
cussed, 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 fre-
quently seen in peritoneal fluid as was first demon-
strated 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 micro-
scopy, either by phase contrast or in stained sections,
because, being intracellular in location, they have pre-
sumably escaped detection through the difficulty of differ-
entiating 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

(see also section 9.3). 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 espe-
cially the intracellular condition in the tissue repre-
sents 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 under-
going 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 multi-
plying 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 cere-
brospinal 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 ven-
tricles 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 cerebro-
spinal 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 dexamethesome 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 multi-
nucleate 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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