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STUDIES ON THE EFFECT OF VARIATIONS IN AMBIENT TEMPERATURE  
ON PATHOGENICITY AND ON HOST IMMUNE RESPONSE IN TRYPANOSOME  
INFECTIONS; WITH SPECIAL REFERENCE TO TRYPANOSOMA (TRYPANOZOON)  
BRUCEI INFECTIONS IN MICE

A thesis submitted for the Degree of Doctor of  
Philosophy (Faculty of Medicine)

of the

University of London

by

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## ABSTRACT

Studies on the effects of ambient temperature on the course of salivarian trypanosome infections in mice showed that the mortality of infected animals was influenced by the environments in which they were maintained. Virulent strains of T.(N.) congolense and T.(T.) brucei caused mild and chronic infections when infected animals were maintained at high (35°C) ambient temperature; T.(D.) vivax and T.(T.) evansi inoculated mice either failed to become parasitaemic or developed transient infections at this temperature. Infections became rapidly fatal when T.(T.) brucei infected mice were transferred from 35°C to normal room temperature (22-27°C).

Mice kept at the high ambient temperature had significantly higher (39.3°C) mean body temperature than (37.5°C) mice kept at room temperature. They suffered a big weight loss and their spleen weights were comparatively much smaller than mice kept at 22-27°C.

Monomorphic strains of T.(N.) congolense and T.(T.) brucei became pleomorphic when inoculated mice were maintained at 35°C. At this temperature, the infectivity of T.(T.) brucei was not altered, it reacquired its capability to produce variants and was able to develop in salivary glands of Glossina. It became highly pleomorphic when inoculated into chick embryos incubated at 39°C but not at 37°C. The morphological changes were thought to be a direct heat effect on the trypanosome organisms.

Attempts to find tissue phase of T.(T.) brucei and T.(T.) evansi during chronic infections in mice kept at high ambient temperature was unsuccessful, neither was there any evidence that these species could develop in mouse peritoneal macrophages.

The presence of released antigens in T.(T.) brucei infected mouse serum was demonstrated only at certain parasitaemic level ( $\frac{\text{Anti } \&}{\log}$  trypanosomes/ml). Infected serum obtained from mice kept at 35°C was less immunogenic than that obtained from parasitaemic mice kept at room temperature.

Many bizarre forms of T.(T.) brucei appeared when infected mice kept at 35°C had previously been immunosuppressed (x-irradiated or cyclophosphamide treated). These mice had very high parasitaemias. However, treatment with Betamethasone or anti-lymphocyte serum failed to produce the same effect. Fewer antibody producing cells was observed in mice kept at 35°C than in those kept at control conditions. It is suggested that the enhanced resistance to trypanosome infections observed in mice kept at 35°C is due to direct effect of temperature on trypanosomes.



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## I. INTRODUCTION

### A. Importance of African trypanosomiasis

In his introduction to his book, which must be considered a handbook for tsetse fly entomologists, Nash (1969) writes: "Tsetse flies and the trypanosomes which they carry are partners in a widespread crime". Without going into the merits of this statement, it does show the economic importance of trypanosomes. Unfortunately for Africa, she had to and is still paying the price, having been endowed with the optimal conditions necessary for the survival of the vectors of this dreadful disease. The civilization of tropical Africa has consequently been hindered by the terrible scourge of the disease which affects man and his domestic animals.

In man, for example, the disease is characterized by acute, or sometimes irregular and chronic fever. Its main symptom is the involvement of the nervous system which leads to increased debility. The patient wears a dull expressionless look and as time goes on he gradually develops a tendency to fall asleep. The onset of the sleeping phase (which in fact marks the final stages of the disease, to which it owes its name - "sleeping sickness") is characterized by complete lack of control over sleep. At this stage death may intervene either directly from the infection, or some intercurrent infection may be involved.

The disease in domestic animals is varied and depends on the particular strain of the causative agent. But in general, it is characterized by chronic, intermittent fever followed by a period when the animal becomes slowly emaciated. At this time anaemia sets in. During the acute phase of the disease, the animal becomes recumbent,

possibly from the very severe anaemia; the lymphatic glands, liver and the spleen become enlarged and with the more virulent strains death usually supervenes.

In this thesis, attention will be focussed on one important factor which seems to have escaped the notice of many eminent workers on African trypanosomiasis (both of man and of animals) - the effects of the ambient temperature at which hosts are maintained on the course of salivarian trypanosome infections. The enervating temperature of many areas of tropical Africa justifies such a study.

#### B. The causative agent

In 1841 Valentin discovered minute organisms in the blood of Salmo fario (brook-trout). He observed from fresh blood preparations that these organisms moved very actively and presented marked changes in form. The peculiar features of these organisms were: a "tail" at the posterior end of the elongated body, that from one side of the body there appeared to be one to three projections and that the middle part of the body contained dark pigments which he thought were food vacuoles. He gives diagrammatic illustrations of rounded bodies with some outgrowths and probably a nucleus. He classified these organisms with the old genus Proteus Müller (Amoeba Ehrenberg) as a new species of the genus, but he actually realized that these organisms could not fit into any of Ehrenberg's classifications. The organisms were excessively small. He gave their measurements as ranging from "0.0003 to 0.0005". He did not define the units but most probably they were decimetres, if this is so then they would be 30 to 50  $\mu$  which is not unusual for fish trypanosomes. The organisms could not be demonstrated in the peritoneum, kidney, intestines or



brain, but were abundant in blood. A year later Gluge (1842) observed one minute creature in the blood of a frog. This organism appeared to him very much like those described earlier in the blood of a brook-trout by Valentin. The organism he saw was long and drawn out in shape with pointed posterior and anterior ends. It had three projections on one side which he thought aided the organism in its energetic sinuous movement. There is no reference to a "tail" which in all probability would be a continuation of the three projections (undulating membrane). Having only seen one organism, he wisely refrained from giving names to it. But Mayer (1843) felt more confident and gave specific names to Gluge's organism - Amoeba rotatoria, Paramoecium loricatum and P. costatum. It is not clear why he chose to classify this organism under both Amoeba and Paramoecium. These two genera were quite distinct even at that time. According to Laveran and Mesnil (1912), Gruby (1843) classified the organism seen in the frog's blood as Trypanosoma. His reasons for doing so are not mentioned. He transferred Gluge's organism from the genus Amoeba to that of Trypanosoma giving it specific name of sanguinis.

It was later found that trypanosomes occurred also in the blood of birds and of rodents. In all these cases, there was no reference as to their pathogenic effect on the host until Evans (1880) found flagellates in the blood of horses suffering from "surra". Surra was a term used by the local population in India to describe an equine disease characterized by deterioration in condition without visible lesions. Evans describes the organism he saw as having a round body, tapered in front, ending in a blunt end and having extending behind it a long slender lash (flagellum). Evans (1881) succeeded in transmitting the organisms from horse to horse by inoculating infected blood subcutaneously into a horse in whose blood trypanosomes had not

previously been seen. The inoculated horse developed a fulminating infection after a prepatent period of five days. As a further proof that surra was connected with this flagellate, he inoculated blood into a dog and the dog similarly became parasitaemic. This dog had, however, apparently not been checked previously for the absence of parasitaemia. The organisms were called by various names, Spirochaeta evansi Steel, Trichomonas evansi Crookshank, Trypanosoma evansi Steel. Steel (1886) after a series of experiments demonstrated clearly (in mules) that the organism was found only in diseased animals and that blood containing the organism was infectious to healthy animals when inoculated subcutaneously. Healthy monkeys became parasitaemic after being inoculated with parasitaemic blood in a remarkably short time of three days. He, therefore, confirmed Evans' findings (1881) that these flagellates were the causal agents of surra.

Shortly after his arrival in South Africa, Bruce was asked by the Governor of Natal to report on an outbreak of a disease called "Nagana" (Unakane in Zulu, which means a state of depression, sickness and weakness in cattle) (Bruce, 1895). At this time the study of blood had become fashionable as a result of Ehrlich's great discoveries on stains and staining techniques. Bruce prepared blood films from a few infected cattle and stained them with carbol fuchsin. He noticed a curiously shaped object different from anything previously known to him. In order to make sure that the object was not an artifact, he made fresh blood preparations and after a long search, a rapidly moving object was seen lashing its way about among the blood cells. Because of the scarcity of these objects, he injected blood of nagana cattle into horses and dogs, and to his surprise these animals developed fulminating trypanosome infections. From this result he felt convinced that these organisms had some connection



with nagana. However, he failed to make sure that the horses and dogs which he inoculated initially were free from these organisms. Bruce tried, also, to find some connection between "tsetse fly disease" (a disease of cattle transmitted only through the bite of tsetse flies) and nagana. For this he sent two young oxen and several dogs to a lowland tsetse fly-belt for two weeks, after which the animals were returned to the tsetse-free hill area where he was staying. Presumably he had examined the blood of these animals before sending them to the tsetse belt. He found the same organisms in the blood of these animals as were found in that of nagana cattle. He does not state how long after being removed from the tsetse belt it took before the animals showed the infection, nor how many became infected. He nevertheless, concluded that nagana and "fly disease" were one and the same thing. In 1896 Bruce sent a dog, artificially infected with nagana to England; it was from this source of trypanosomes that Plimmer and Bradford (1899) studied the morphology of the blood protozoan. They, eventually, named it Trypanosoma brucei.

Later, Forde, (1902) found "worm-like" bodies, which proved to be trypanosomes (Dutton, 1902), in the blood of a human patient in a Gambian hospital. The trypanosomes occurring in man were named Trypanosoma gambiense Dutton (1902). About the same time Castellani (1903) found trypanosomes by the centrifugation of a specimen of cerebrospinal fluid obtained by lumbar puncture from an advanced case of sleeping sickness in Uganda.

A Glossina sp. (Diptera, Muscidae: tsetse fly) was immediately suspected as the transmitter of the disease (Sambon, 1903). And Christy (1903) regarded Glossina palpalis as the transmitter after



considering the geographical distribution of sleeping sickness. Bruce, Nabarro and Greig (1903) demonstrated that Cercopithecus sp. became infected after the bite of wild G. palpalis. However, nothing is known about the previous history of the monkeys which they used.

In 1910 Stephens and Fantham recorded the presence of yet another trypanosome in man which to them was different from T. gambiense, because of the peculiar position of the nucleus. They named it Trypanosoma rhodesiense. By this time the relationship between trypanosomes, the insect vector and the mammalian host was sufficiently known, and many eminent workers from different disciplines had already been attracted to the problem.

### C. Description and classification of trypanosomes

Wenyon (1926) has given a detailed account of trypanosome morphology and so only a short description of trypanosomes in general will be given in this thesis. Allowing for possible exceptions, trypanosomes as seen for example in Giemsa-stained preparations of blood trypanosomes appear as spindle-shaped bodies, invariably curved, tapering at both ends, with a centrally-placed nucleus, and a kinetoplast placed at the posterior end near to which originates a flagellum. The flagellum passes along the border of an undulating membrane which arises from the curved edge of the body and forms a thin ridge of cytoplasm. The flagellum may terminate at or project as a free locomotory organ beyond the anterior end of the body (see fig. 1).

With the advent of electron-microscopy, much finer details have been revealed. Among the many ultra-structural elements

revealed by electron microscopy (Vickerman, 1970), it has been shown that the main trypanosomal body and the flagellum are composed of a trilaminate membrane, the surface of which is covered by a "surface coat" 12-15 nm thick. The kinetoplast is also shown to be surrounded by a double membranous mitochondrial cover and is distinctly separated from the basal body.

### 1. General classification

The taxonomic position of the genus Trypanosoma among Protozoa is as follows (Honigberg et al., 1964):

Phylum PROTOZOA Goldfuss, 1818, emend. Siebold, 1845.

Subphylum SARCOMASTIGOPHORA Honigberg and Balamuth, 1963.

Superclass MASTIGOPHORA Diesing, 1866.

Class ZOOMASTIGOPHOREA Calkins, 1909.

Order KINETOPLASTIDA Honigberg, 1963.

Suborder TRYPANOSOMATINA Kent, 1880.

Family TRYPANOSOMATIDAE Doflein, 1901; emend. Grobben, 1905.

Genus Trypanosoma Gruby, 1843.

Hoare (1966) has divided the trypanosomes of mammals into two sections; the division is based primarily on the position which the trypanosomes complete their developmental cycle in the insect vectors:

### ■ Section STERCORARIA

These trypanosomes (fig. 1) have a free flagellum, a large kinetoplast placed at some distance from the tip of the pointed posterior end of the body. Multiplication in the mammalian host takes place in the epimastigote or amastigote stages. Most importantly,

they produce infective metacyclic forms in the posterior end of the vector's gut and are transmitted by contaminative route. T. rangeli is an exception since it is transmitted by inoculative route. Representatives of this section are classified in the following subgenera:

i. Subgenus Megatrypanum Hoare, 1964

These are large trypanosomes with kinetoplast situated near the nucleus.

Type species: Trypanosoma (Megatrypanum) theileri Laveran 1902, measures up to 100  $\mu\text{m}$ .

ii. Subgenus Herpetosoma Doflein, 1901

These are medium size trypanosomes with kinetoplast placed at some distance from the sharply-pointed posterior end of the body.

Type species: Trypanosoma (Herpetosoma) levisi (Kent, 1880) Laveran and Mesnil, 1901, has a mean length of 21-36  $\mu\text{m}$ .

iii. Subgenus Schizotrypanum Chagas, 1909; emend. Nöller, 1931.

These are fairly small C-shaped trypanosomes with a large kinetoplast close to the pointed posterior end of the body.

Type species: Trypanosoma (Schizotrypanum) cruzi Chagas, 1909. Mean length 15-24  $\mu\text{m}$ .

b. Section SALIVARIA

These trypanosomes in general have a free flagellum but sometimes a free flagellum is missing. The kinetoplast may be terminal or subterminal and the posterior end of the body is fairly blunt. Multiplication in the host is continuous and so far as is known takes place in the trypomastigote stage. They



complete their development in the vectors in the anterior station of the gut.

i. Subgenus Duttonella (Chalmers, 1918)

Type species: Trypanosoma (Duttonella) vivax Ziemann, 1905.

ii. Subgenus Nannomonas Hoare, 1964

Type species: Trypanosoma (Nannomonas) congolense  
Broden, 1904.

iii. Subgenus Pycnomonas Hoare, 1964

Type species: Trypanosoma (Pycnomonas) suis Ochman, 1905.

iv. Subgenus Trypanozoon Luhe, 1906

Type species: Trypanosoma (Trypanozoon) brucei  
Plimmer and Bradford, 1899.

## 2. Nomenclature

The nomenclature of the various forms assumed by organisms of the Trypanosomatidae during different phases of the life history is based on the relative positions of the nucleus and the kinetoplast and the degree of development of the undulating membrane and flagellum. An old terminology (see below) is being superseded by a new one proposed by Hoare and Wallace (1966). The various forms which may be assumed by the genera of the Trypanosomatidae and their description in the old and new terminology are given below:

<u>Genus</u>	<u>Old terminology</u>	<u>New terminology</u>
<u>Leptomonas</u>	a. leptomonad	promastigote
	b. leishmanial	amastigote
<u>Herpetomonas</u>	a. leptomonad	promastigote
	b. trypanosome	opisthomastigote
	c. leishmanial	promastigote

<u>Blastocrithidia</u>	a. leptomonad	promastigote
	b. crithidial	epimastigote
<u>Crithidia</u>	a. crithidial	choanomastigote
(Syn. <u>Strigomonas</u> )	b. leishmanial	amastigote
<u>Trypanosoma</u>	a. true trypanosome	trypomastigote
	b. crithidial	epimastigote
	c. leishmanial	amastigote

Brack (1968) has introduced the term "sphaeromastigote" to describe rounded forms with flagellum. This is a very convenient addition as it bridges the gap formed by the undefined amastigotes, or the broad forms of promastigotes.

For the sake of consistency and clarity, the subgenus, species and subspecific status of trypanosomes will be included when referring to a particular trypanosome, e.g. T. vivax will be referred to as T.(D.) vivax and the human trypanosomes will be considered under the species T. brucei and will be referred to as T.(T.) brucei gambiense and T.(T.) brucei rhodesiense. T. brucei sensu stricto will be designated as T.(T.) brucei brucei.

### 3. Definition

Isolate. A section of a wild population separated off by transference into artificial conditions of maintenance, usually by inoculation into cultures or into laboratory animals (Lumsden and Wells, 1968).

Strain. A population derived from an isolate, maintained in captivity by inducing it to reproduce continuously by serial passage in cultures or in laboratory animals (Lumsden and Wells, 1968).

Stabilate. A population whose reproduction has been arrested by viable preservation on a unique occasion (Lumsden and Hardy, 1965).

#### Abbreviations

LUMP. Denotes London University Medical Protozoology.

TREU. Denotes Trypanosomiasis Research at Edinburgh University.

ETat. Indicates Edinburgh Trypanozoon antigenic type.

#### D. Motive for the study

As early as 1909, Ronald Ross (Ross and Williams, 1910) had felt that possibilities existed of curing parasitic diseases by such simple treatments as exposure to cold or heat. From his clinical experience in India, he had observed that there was a very high incidence of animal parasites in people living in warm climates. He therefore thought that if the environmental conditions of such people were altered, it was just possible that the resulting effect would be inimical to the parasites. Nevertheless, he realized that temperature of patients was not markedly changed by alterations in environmental temperature. He had particularly noticed that human trypanosomiasis occurred in people living in very hot climates and considered it just possible that the disease could be ameliorated if infected people were moved to colder climates. Giving reasons for his firm belief in this kind of treatment, he cites an example of complete cure from trypanosomiasis. A patient whose history of the disease is not stated was subjected to cold treatment accompanied by healthy exercise in Scotland. He does not indicate what sort of temperature the patient was subjected to; neither does he give the method used to assess complete cure from trypanosomiasis. Van den



Branden (1939) was impressed by an earlier observation of Brumpt (1908) that a mouse infected with T. (T.) brucei gambiense and kept in an ice chest, was found free from trypanosomiasis 24 days later when control mice had died. Van den Branden infected experimentally groups of white rats with T. (T.) brucei. He put one group in an ice box maintained at  $4^{\circ} - 5^{\circ}\text{C}$  and a control group in a warm room in which temperature fluctuated between  $18^{\circ}\text{C}$  to  $20^{\circ}\text{C}$  during the day and  $10 - 12^{\circ}\text{C}$  at night. The number of animals used in these experiments is not given. He found that the infection lasted longer (7 days) in animals kept in the ice box than in control. The controls survived for five days. It is hard to judge whether the increase in survival observed was statically significant unless the number of animals used, their mean survival times and how accurately the trypanosome inocula were administered to each animal is known.

Kligler (1927) used the duration of parasitaemia as a measure of the effect of temperature on host susceptibility to infection. He found no significant differences when groups of rats of the same age (age not specified) and weight were inoculated with 10,000 T. (T.) evansi. One lot of infected rats was kept in the cold room ( $10 - 12^{\circ}\text{C}$ ) and the other in the warm room ( $28 - 30^{\circ}\text{C}$ ). Animals kept in the warm room lived for an average of 17.4 days and those kept in the cold room lived for an average of 18.4 days. On the other hand, Oehler (1914) using a strain of T. (T.) brucei which caused subacute infections in mice, found that the strain became very virulent after the 6th serial passage in mice, but when the passages were carried out in mice maintained at  $35^{\circ}\text{C}$ , it did not acquire the enhanced virulence. Since the original strain was maintained in guinea pigs in which it caused chronic infection,

it is just possible that the effect of heat treatment was delaying the selection process.

In view of these conflicting reports on the influence of environmental temperature on the course of trypanosome infections in laboratory rodents, the present investigations were therefore carried out with the aim of elucidating further the relationship between the hosts' ambient temperature and the course of some pathogenic African trypanosome infections. The idea behind these investigations is to find some explanations for the question, "What is the fate of a parasite, as illustrated by trypanosome, when its host is under stress?" L in this case heat.

These investigations are relevant to the epidemiology of sleeping sickness, particularly with regard to the occurrence of highly parasitaemic healthy human carriers (Blair, 1939) who may not be detected in a population unless a deliberate attempt is made to examine a whole community. Blair (1939) gave examples of two men who had heavy T.(T.) b. rhodesiense infections. These men showed no sign of suffering from sleeping sickness. Incidentally these two people had Plasmodium falciparum infections as well. It is possible that the pathogenic manifestations of trypanosomiasis were kept under control by the malaria fever. Evidence for this will be given later in the text.

Blair, Smith and Gelfand (1968) have offered as an explanation for the healthy human carrier case, that these persons are probably exposed to repeated Glossina bites, which may be inoculating other trypanosome species such as T.(D.) vivax or T.(N.) congolense, as a result of which non-specific resistance to T.(T.) b. rhodesiense may occur. No evidence is available as yet to show that cross protection exists between T.(T.) b. rhodesiense and these trypanosomes



of cattle. Nonetheless, more work is called for to isolate such cases in other parts of Africa apart from Rhodesia, so that their real importance may be fully assessed.

For other human carriers whose infections are below microscopic levels probably due to an early or remitting infection, repeated blood examinations for parasitaemias or serological tests are likely to reveal them. In any case, they are liable to show clinical symptoms with the advancing infection and would therefore be easily recognized. Bentz and Macario (1963) have found hyper-gamma-macroglobulinemia (raised IgM) a good indicator for such low parasitaemia carriers in endemic areas in Ivory Coast Republic.

It is considered that if, indeed, heat as a stressing factor has got any bearing on the distribution of the human and animal trypanosomiasis, then these studies would give some clues to the determination of epidemiology of the two forms of sleeping sickness, and of the cattle disease.

In an attempt to answer some of the questions involved in this broad epidemiological problem, the present study has centred mainly on:

1. The general effects of environmental temperature on the course of trypanosome infection in mice.
  - a. on trypanosomes
  - b. on the host
2. Attempts to see if certain environmental temperatures encouraged the "hidden" phase of trypanosomiasis.
3. Attempts to see if raised ( $35^{\circ}\text{C}$ ) environmental temperature influenced cyclical transmission.
4. Attempts to demonstrate the existence of a tissue phase in a macrophage culture.

5. During the course of these experiments, it was shown that a virulent strain of T.<sub>2</sub>(T.) brucei caused a chronic infection so long as the mice were kept at 35°C, but when the mice were transferred to 22-27°C environment, the infection became acute and the animals died from fulminating parasitaemia. Attempts were therefore made to see if there was any antigenic variation occurring during the chronic infection which occurred in mice kept at 35°C.

6. Having shown that the environmental temperature influenced the course of trypanosome infection, it was interesting to see if environmental temperature had a direct effect on trypanosome growth. This problem was tackled by growing trypanosomes in chick embryos.

7. To determine if the apparently greater resistance to trypanosome infection of mice kept at 35°C was due to an earlier antibody response, the effects of immunosuppressive agents on the course of infection was studied. It was also considered worthwhile to see if subjecting mice to 35°C had any effect on antibody producing cells.

8. The morphology of T.<sub>2</sub>(T.) brucei in immunosuppressed mice kept at 35°C and also in chick embryos incubated at 39°C was different from that seen in animals kept in 22-27°C and embryos incubated at 37°C. An attempt was made to see if these aberrant trypanosomes could be transmitted cyclically.

## II. LITERATURE REVIEW

### A. Morphology of salivarian trypanosomes

#### 1. General appearance

The classification of trypanosomes has been based entirely on the appearance of trypanosomes as seen under light microscopy. Wenyon (1926) has given full description of the main characteristic features which have been used to separate different species. These characters have included: the general shape of the body; variations in the sizes and positions of the nucleus and the kinetoplast; the degree of development of the flagellum; the shape of the posterior end of the body.

These characters have remained the essential diagnostic features of different species. Wenyon (1926), however, pointed out clearly that these characters apply only to blood stages. The stages found in the invertebrate vectors present many more morphological variations during their sojourn in the vector. Some of these characters, such as the position of the kinetoplast, degree of development of the flagellum and the general shape of the body may be used to describe the various developmental stages within the vector. Besides these general morphological structures, phase contrast microscopy (Ormerod, 1958, 1966) has revealed cytoplasmic granules, the number of which varies during the course of infection and with the strain of the parasite. Ormerod (1966) has suggested that these granules are associated with immune response of the host. Phase contrast microscopy has also revealed (Wright, Lumsden and Hales 1970) the presence of long (70  $\mu$ m) filamentous



appendages arising from the posterior end and from the tip of the flagellum of T. (T.) brucei inactivated in vitro in methylcellulose. These workers have suggested that these "filopodia", may be connected with the release of "variant antigen" in the serum of infected hosts.

Ultrastructural studies of blood stream forms of T. (T.) b. rhodesiense, T. (T.) evansi and T. (N.) congolense (Vickerman, 1968) has shown the presence of a thick layer of material outside the surface membrane of the main trypanosomal body. In the midgut of Glossina, this coat cannot be demonstrated but once the metacyclics appear, the coat is reacquired. Vickerman has therefore suggested that the loss and the reacquisition of the surface coat may be intimately connected with the variant antigens. One wonders why all these ultrastructural components are looked upon as being connected with the antigenic nature of the trypanosomes. It seems to me that once the sequence of the trypanosomal variant antigens is settled, these structures will most likely be assigned other functions.

Apart from the general appearance, other criteria have been considered especially in cases where there is close resemblance between species. Bruce, Hamerton and Bateman (1909) used mensural characters to describe a trypanosome obtained from a dying horse in Zanzibar. They inoculated mice, rats, rabbits, guinea pigs and dogs, and at various time intervals made blood films which they stained with Leishman, Giemsa or methyl green stains. They measured 20 trypanosomes from each experimental animal and found wide differences in mean lengths (10.4  $\mu$ m - 17.1  $\mu$ m). The mean lengths of trypanosomes in a particular host over a given regular time interval would probably have given more consistent results. They, however, regarded the organism to be T. (N.) dimorphon. Wide mensural



differences in T.(N.) congolense have also been reported by Godfrey (1960). He (Godfrey, 1961) later showed that these morphological differences were important since he found that they were connected with the nature of the resulting infection. He showed that the shortest forms were less pathogenic than the medium forms, but the longest were very virulent. Hoare and Broom (1938) have also used mensural characters to separate T.(D.) vivax and T.(D.) uniforme. They compared the arithmetic mean lengths of stained trypanosome preparations. The trypanosome specimens had been collected from different natural hosts from 19 different localities. They found clear cut differences when they examined 100 trypanosomes from each host. Mensural differences between these two species could be demonstrated even with as few as 10 randomly selected trypanosomes from any host.

## 2. Pleomorphism in T.(T.) brucei

Exceptionally, the only criterion used to date reliably to separate T.(T.) brucei trypanosomes is their inability or ability to infect man and the type of disease produced. One of the main problems has been and still is that, in natural hosts, these trypanosomes show marked variations in their morphology. The morphological variations shown by these trypanosomes was observed soon after T.(T.) b. gambiense was discovered. Dutton and Todd (1903) observed two morphological forms of T.(T.) b. gambiense from blood smears taken from human patients and experimental animals. These forms were long (30  $\mu$ m) with thin bodies and long flagella, and the stumpy forms with short (16 - 19  $\mu$ m) thick body and very short flagellum. In heavy infections, forms intermediate

between these two extremes were the majority. Minchin (1908) was one of the first people to point out the theory of "trimorphism" in T.(T.) b. gambiense infection, and being influenced by the old school (Schaudinn's), he considered these extreme forms to represent sexual stages. Bruce, Hamerton, Bateman and Mackie (1911) undertook a laborious job of measuring individual T.(T.) b. brucei in blood preparations obtained from ox, monkey, dog, guinea pig and rats in order to confirm the theory of "trimorphism" (or pleomorphism), according to Ormerod (1967). They measured in all some 172 trypanosomes from blood preparations of these animals and found that they could classify them into long forms, 25 - 35  $\mu$ m intermediate 22, 23, 24  $\mu$ m and short forms 13 - 21  $\mu$ m. It must be pointed out that these were not random samples as they measured the trypanosomes as they came in view. Moreover, very few trypanosome specimens were seen in some blood preparations, for example only one trypanosome was seen in the ox blood. Bruce (1911) having failed to observe consistent morphological differences between T.(T.) b. brucei and T.(T.) b. gambiense thought that perhaps mensural differences could be of some diagnostic value. He measured a total of 1000 T.(T.) b. gambiense obtained from Primates (man, chimpanzee, monkey) bovines (ox, reedbuck, bushbuck) and rat and found that the mean body length was about 20  $\mu$ m with wide variations (13 - 33  $\mu$ m). Once again the trypanosomes were measured as they came in view. When the mean body length measurements were compared with those of T.(T.) b. brucei, he found that the mean lengths of T.(T.) b. gambiense were on average shorter than those of T.(T.) b. brucei. But he found more, (38%) non-flagellated forms in T.(T.) b. gambiense than (26%) in T.(T.) b. brucei. The percentage of intermediates were remarkably similar in both cases,



23.1% for the former and 25.5% for the latter. He nevertheless noted that differences in mean lengths occurred also in the same individual. The validity of pleomorphism was thus confirmed but its consistency was still in doubt.

Robertson (1912) related the change in T.(T.) b. gambiense morphology observed in infected monkeys to the periodicity in parasitaemias. She found that as the number of trypanosomes increased, the proportion of long slender trypanosomes increased, but stumpy forms then appeared and their number increased, so that before an absolute drop in trypanosome population, stumpy forms were the only forms present. She regarded these stumpy forms as adult blood forms and constituted the stable forms, since they had the longest duration in circulation. She was lucky in that she did not pick on an infection which could be subpatent for a considerable length of time. Other forms were according to her only transitional. Ogawa (1913) from a series of biometrical studies of some 1,200 T. pecaudi (= T. (T.) brucei brucei) obtained from two guinea pigs found that two forms of this parasite existed: long and slender, and short and stumpy. He noted some relationship between the two during the course of a chronic infection. Slender forms appeared first and short stumpies appeared sometimes later after the long forms, but during the terminal stages of infection, long slender forms predominated. This change in morphology was not apparent during a short and a highly virulent infection.

Oehler (1914) was able to demonstrate these gradations in T.(T.) brucei brucei infection in mice after a series of ingenious experiments using single trypanosome infections. His strains were obtained from chronic and highly pleomorphic strains in guinea pigs. He claimed to have demonstrated pleomorphism even on day one after

infection. He was thus inclined to discount the theory of sexual differentiation or a possibility of mixed infection as the cause of pleomorphism. Fairbairn and Culwick (1946) studied a sample of 8,794 T.(T.) b. rhodesiense trypanosomes in slide preparations prepared over a period of 15 consecutive days. They were able to show that there were three distinct forms of T.(T.) b. rhodesiense and they related the morphological variations to electrical charges which the trypanosomes carried. The writer finds it difficult to follow the complex mathematics involved, but it seems that many factors would have to be put into consideration before the formula given can be adopted. First the method of making the blood smears must be standardized, and probably a given volume of blood must be used. Secondly, the intensity of infection is likely to interfere with the distribution of trypanosomes in a smear, as the larger forms are more likely to be pulled rather than randomly spread.

It is the opinion of the writer that in an established natural T.(T.) brucei infection, the change in morphology is a dynamic process which every individual trypanosome has to undergo. But the rate of change varies with the particular individual and is influenced largely by the chemical changes in the surrounding medium. Some morphological form will be characteristic of early development whereas others will be typical of later or final stages of development before the process is repeated, usually by binary fission. At the same time, some transitional forms (not equal to intermediates) will be more vulnerable to chemical changes. These forms will be removed by lysis or opsonization. Eventually only forms with more survival value will be selected and these forms will become conspicuous during the terminal stages of infection or during serial passages.



Various reasons have been given for the occurrence of short stumpy forms. Bevan and MacGregor (1910) thought that the change in T.(T.) b. gambiense morphology was due to a change in the trypanosome environment. They specifically cited, without any supporting evidence, that the production of antibodies by the host was one of the factors that led to the variation. This view was later revived by Ashcroft (1957), when he considered that periodic relapses were caused by the action of antibodies. He infected some four rats with T.(T.) b. rhodesiense and beginning from the day of infection, subjected the rats to a daily cortisone acetate treatment. He found that 90% of the trypanosomes examined from cortisone-treated rats during the last two weeks of infection were slender, whereas these forms were only seen during the last one or two days before death in control untreated rats. He therefore concluded that the presence of antibodies was essential for the slender forms to change to short stumpy forms. Although plausible, this theory does not explain why variants occur in semi-virulent infections where clear cut morphological variants cannot be demonstrated. Moreover, one does not know what effect is there when the trypanosome environment is flooded with cortisone acetate. I am inclined to think that the animals should have been subjected to cortisone treatment first before inoculating them with the trypanosomes. The host is unlikely to regain its full defence mechanisms for nearly two weeks, during which time one can observe fully the trypanosome morphology.

In attempts to solve the great problem of separating the T.(T.) brucei trypanosomes, Rickman and Robson (1970) have described a method, blood incubation infectivity test (BIIT) which seems to offer much hope for separating the human from cattle trypanosomiasis. The test consists in incubating the strain of trypanosome under test,

for 5 hours at 37°C in vitro in human blood and then observing the effect of the incubation on the infectivity of the strain to rats. For T.(T.) b. rhodesiense strains, the ability to infect rats is retained after incubation, whereas such a treatment destroys the infectivity of T.(T.) b. brucei strains. The method however, has not been applied to T.(T.) b. gambiense yet. The technique appears to be very reliable with recently isolated strains, but strangely enough, T.(T.) b. rhodesiense strains that have undergone many passages in the laboratory, tend to behave more like T.(T.) b. brucei (Dr. G. A. T. Targett, personal communication). It would be interesting to see if such strains have equally lost their infectivity to man.

### 3. Posterionuclear forms

When Stephens and Pantham (1910) isolated a human trypanosome from a patient infected in Rhodesia they noted that this parasite, though similar to T.(T.) b. gambiense, caused infections in guinea pigs, rats and mice in which some stumpy forms with nuclei placed at the extreme posterior end occurred. Therefore T.(T.) b. rhodesiense (Stephens and Pantham, 1910) was given the specific status mainly on this character. Wenyon (1912), studying a trypanosome T. pecaui (= T.(T.) b. brucei) from a Sudanese mule, also noted the existence of postero-nuclear forms. Blacklock (1912) found them in a strain of T.(T.) b. brucei from experimentally infected rats, guinea pigs and rabbits. It was soon realized that the posteronucleated trypanosomes were not of any specific significance, the more so when Lavier (1927) found them in T.(T.) b. gambiense-infected guinea pigs. Lavier's trypanosomes, however, were isolated originally either from recently infected human cases without perceptible glands,



or from very advanced sleeping sickness cases in Uganda. It was therefore not easy to be absolutely certain that the trypanosomes were T.(T.) b. gambiense and not T.(T.) b. rhodesiense. These infections were also virulent in guinea pigs.

Lavier thought that posteronuclear forms were produced by the division of short stumpy forms, a process which he illustrated very impressively. He suggested that when posteronuclear forms divided, forms with the nucleus pushed to the extreme posterior end were produced. Fairbairn and Culwick (1946) showed that posteronuclear forms could be produced in vitro. They found that when a drop of infected ~~rat~~ blood was incubated in a saturated humid atmosphere for five minutes and then smeared, these forms were produced in larger numbers than in control blood films smeared soon after withdrawal from the rat. Hoare (1956) found that posteronuclear forms occurred in T.(T.) evansi infection and noted that on some occasions, the number of posteronuclear forms were as high as 60%. Ashcroft (1957) also observed the high percentage of posteronuclear forms in T.(T.) brucei infection in the rat. On one occasion (2 days before death), during a long infection, he found that 60% of all the trypanosomes examined were posteronucleated. Wijers (1960) thought that these forms were produced by a gradual displacement of the nucleus towards the posterior end of the trypanosome and that the condition was connected with the virulence of the strain. The writer is of the opinion that during a chronic T.(T.) brucei infection, when the host defensive mechanisms are nearly exhausted, the parasites take the advantage and proliferate with immense rapidity. And it is during this exaggerated multiplicative phase that abnormal forms occur, some of which are posteronucleated. These abnormal forms are particularly sensitive to specific host antibody response and are easily removed from the



circulation as soon as the specific immune responses are invoked. Their presence would therefore be expected during peak parasitaemias in a relapsing infection or during the terminal stages of a chronic infection.

#### 4. "Hidden forms"

Salvin-Moore and Breinl (1908) first drew attention to peculiar trypanosomal bodies which they called "latent bodies". These forms occurred in large numbers in the spleen and bone marrow during peak parasitaemias of a relapsing T.(T.) b. gambiense infection in rat. Buchanan (1911) found similar forms in the bone marrow of T.(T.) b. brucei-infected gerbils. Buchanan claimed to have seen some in the red blood corpuscles, an observation which has never been confirmed. Fantham (1911) described them in the lungs, spleens and bone marrow during remissions in parasitaemia.

Recently Goodwin (1971) has drawn attention to tissue phase of T.(T.) brucei and Soltys and Woo (1969) claim to have demonstrated the presence of a tissue phase (amastigotes) of T.(T.) brucei and T.(N.) congolense in macrophages of mouse spleen and liver. They (Soltys and Woo, 1970) have further reported that when homogenates of infected liver and spleen are passed through millipore filters, of pore size 1.2  $\mu\text{m}$  the filtrate proved to be infective to mice and amastigotes were demonstrated when the filtrate was filtered through pores of 0.45  $\mu\text{m}$ . Ormerod and Venkatesan (1970) have demonstrated amastigotes in the choroid plexus of rats infected with T.(T.) brucei. The existence of occult or the hidden forms seem to be settled; what seems to be uncertain is whether they are present all the time during a remitting infection, or whether they

appear in bouts with some regularity. The importance of these forms as a reservoir of blood trypanosomes should be given the highest priority.

5. Morphology of *T.(T.) brucei* in relation to cyclical transmission

Robertson (1912) observed periodicity in *T.(T.) b. gambiense* parasitaemia which concurred with changes in morphology. As the trypanosome population increased, the trypanosomes assumed long and slender shape and were actively dividing, whereas a decrease in numbers was associated with marked reduction in length and the trypanosomes became stumpy. When laboratory-bred *G. palpalis* were fed on a monkey infected with *T.(T.) gambiense* at regular intervals, she observed a phase during the course of infection when the flies could not become infected, although the trypanosomes were numerous in blood. She concluded that only the short stumpy forms which seemed to survive the unfavourable circumstances causing the periodic trypanocidal crises were capable of infecting tsetse-flies. Duke (1935) also observed a phase of development of trypanosomes in the guinea pigs and monkeys during which the trypanosomes are non-infective to *G. morsitans* and *G. palpalis*, but he refrained from giving final opinions in view of the small numbers of tsetse flies used in each experiment. He did not relate this negative phase with the morphology of trypanosomes. Corson (1935) observed that *T.(T.) b. rhodesiense* could be transmitted cyclically to rats during a relapse when most probably the stumpy forms were relatively very few. But van Hoof (1947) in a series of carefully executed experiments showed that batches of laboratory-bred *Glossina* fed

daily on a T.(T.) b. gambiense infected patient, for 21 days and on a monkey for 26 days and on guinea pig for 23 weeks found no relation between trypanosome morphology and the ability to develop in the tsetse flies. Wijers and Willett (1960) have found that the subsequent infection rates of newly emerged G. palpalis to Erythrocebus patas patas (red hussar monkey) was influenced by the absolute numbers of short stumpy forms at the time of the infected feed. Mshelbwala (1967) using skin membrane feeding technique, claim to have succeeded in transmitting slender forms of T.(T.) b. rhodesiense to G. palpalis. He does not state how he excluded the possibility of having an odd stumpy form in the population.

It seems clear that opinions are still divided as to which morphological forms of T.(T.) brucei are infective to Glossina.

#### B. Effect of environmental temperature on the course of microbial infections

This review will not be confined to the effects of environmental temperature on the course of trypanosome infections only, because much more relevant information has been collected in other related microbial infections such as viral and bacterial infections. A review of the important works in these fields has therefore been included. It is hoped that a synthesis of this information will help clarify some of the problems raised in the thesis.



1. Beneficial effect of exposure to cold

a. Viral infections. The beneficial effect of cold treatment has been reported in viral infections. Holtman (1946) acclimated young mice to 13<sup>0</sup>, 22<sup>0</sup>C and 32<sup>0</sup>C environments for three weeks and then inoculated them with suspension of poliomyelitis infected brain after which they were returned to their respective environments. He found that mice held at 13<sup>0</sup>C showed symptoms of paralysis in less than 11 days and 50% of them died after 13 days. Mice held at 22<sup>0</sup>C showed 50% mortality at the end of seven days, but mice held at 32<sup>0</sup>C showed symptoms and started dying as early as the fifth day. He argues that because metabolic rate of the host is increased by heat treatment, this in turn influences the rapid proliferation of the virus. It must be pointed out that metabolic rate is also increased by cold treatment and therefore this argument is not a very convincing one. The effect is more likely to be due to the change in body temperature rather than direct effect of metabolism.

b. Bacterial infections. In a review of past work on the pathologic changes produced by exposure to cold, Foord (1918) refers to Pasteur as having shown experimentally that exposure to cold altered the course of experimental anthrax in hens. He (Pasteur, 1878) subjected a group of anthrax-infected hens to hypothermia by half immersing the fowls into cold water thereby reducing their body temperature from 42<sup>0</sup>C to 38<sup>0</sup>C. He found that the chilled fowls developed the infection while controls survived. The reviewer does not give details as to how long these fowls were chilled, neither does he show whether cold treated fowls ever died from the infection, although this seems to be implied. Filehne (1894) found,

after infecting rabbits ears with Streptococcus pyogenes, that the infection was latent in the rabbit kept at  $0^{\circ}\text{C}$  for 3 days but severe and extensive erysipelas developed throughout the entire ear when the animal was transferred to room temperature. In control rabbits the infection was less extensive.

c. Trypanosome infections. Ross and Williams (1910) were the first people to carry out controlled experiments on the effects of cold on trypanosomiasis. They claimed that rats, mice and guinea pigs inoculated with T. (H.) lewisi, T. (T.) evansi, T. (T.) equiperdum, T. (T.) brucei and kept in a freezing chamber ( $2^{\circ}\text{C}$ ) resisted infection better than controls kept at room temperature. Their claim is not convincing, since it was only one mouse among seven inoculated with T. (T.) equiperdum that failed to become infected. Certainly many reasons can be offered for this exception. The mean survival times in both cold treated and control rats or mice were not very different, for example 14 cold treated mice and 14 controls all infected with T. (T.) brucei had average survival times of 26.8 and 25 days respectively. Moreover, the freezing chamber they used was stopped from time to time for repairs while experiments were going on, and the duration of these intermissions are not indicated. Ross and Thomson (1910) thought they could exploit the above technique (cold-treatment) as a curative measure in human trypanosomiasis. A T. (T.) b. rhodesiense infected patient from Rhodesia was persuaded to visit the freezing chamber ( $2^{\circ}\text{C}$ ). The length of time he was detained in the freezing chamber is not given. However, it is reported that the patient claimed emphatically that he felt much better in the cold chamber. In spite of his claims, his condition worsened and the cold treatment had to be stopped. The patient was not examined for possible intercurrent infections, which could have exacerbated the trypanosome infection.

They, (Ross and Thomson) were not satisfied with their one human case and so they experimented further with guinea pigs and rats. They infected a group of these animals with T.(T.) b. rhodesiense and kept one half in the cold chamber and the other half at room temperature. They observed that the prepatent period was prolonged from about four days to  $13\frac{1}{2}$  days when the animals were kept at approximately  $2^{\circ}\text{C}$ , and the duration of the infection was similarly prolonged from 64 to 94 days as compared with the controls. It is difficult to make any firm conclusions from these impressive figures, since they used only two rats in each environment. Van den Branden (1939) similarly observed that rats infected with T.(T.) brucei and kept in the cold ( $4-5^{\circ}\text{C}$ ) survived for 7 days as compared with controls which survived for 5 days.

## 2. Beneficial effect of high ambient temperature

Wagner von Jauregg (1908) aroused interest in the therapeutic potential of the febrile state when he observed remissions in patients suffering from dementia paralytica (syphilis) following fever due to Plasmodium vivax (tertian malaria). He suggested that the elevation of body temperature as a result of malaria fever was responsible for the favourable outcome of the disease. This remark sparked off a wave of clinical and experimental investigations which eventually led to the widespread acceptance of artificial fever as a therapeutic measure in many diseases.

a. On viral infections. Milzer, Lewin and Levinson (1943) found that when monkeys were immersed in frozen water and infected with poliomyelitis virus, the chilled monkeys developed more severe paralysis than control monkeys held at room temperature. The duration of cold treatment is not indicated. Jungeblut and Kopeloff (1931) noted that the incubation period of poliomyelitis



superimposed on an existing fever ( $38 - 42^{\circ}\text{C}$ ) in monkeys caused by T.(T.) equiperdum was prolonged by 7 days. The fever period was stopped when trypanosome infection was treated with suramin. There was only one experimental animal and so these findings must be viewed with caution. On the other hand, Sarracino and Soule (1941) found that neither the effect of heat nor cold treatment influenced human influenza virus in mice. Their experiments were done in such a way that the body physiology was never allowed to stabilize at some uniform temperature. Mice were first kept at  $37^{\circ}\text{C}$  for one day and then transferred to  $18^{\circ}\text{C}$  environment for another day and then returned to  $37^{\circ}\text{C}$  for five days. For cold treatment, mice were drenched in cold water and then placed in a cold room ( $5^{\circ}\text{C}$ ) and then transferred back to room temperature.

Lillie, Dyer, Armstrong and Pasternack (1937) reported the influence of seasonal variation on the intensity of brain reaction to the virus of St. Louis encephalitis in mice and endemic typhus in guinea pigs. In both instances, animals kept at low ( $10^{\circ}\text{C}$ ) temperature showed more severe reactions than those kept at higher  $35^{\circ}\text{C}$  temperature. These temperatures simulated the winter and summer temperatures. Thomson (1938) found that raising the skin temperature of rabbits to a value approximate to that ( $37^{\circ}\text{C}$ ) of the deeper tissues in the body inhibited the development of lesions caused by fibroma virus and myxoma virus. Thomson and Parker (1941) have also shown that at high external temperature ( $35^{\circ} - 41^{\circ}\text{C}$ ), infection of rabbits with myxoma virus was held completely in abeyance.

Marshall (1959) found that ambient temperature affected the mortality rate and symptomatology in rabbits infected with attenuated strain of myxoma virus. He found that simulated summer temperature

(37<sup>o</sup> C) allowed at least 70% of infected rabbits to recover whereas only 8% recovered when kept at simulated winter temperature (-3 to 20<sup>o</sup> C). He also found that the ambient temperature did not influence the course of infection of a highly virulent strain of rabbit pox virus or myxoma virus. He showed that growth of myxoma virus was inhibited at 39<sup>o</sup> C in chick embryos and this probably accounted for the reduced lethality of the virus in animals kept at high ambient temperature.

McKinley and Acree (1937) tried using fever therapy in the case of myxomatosis and fibroma infections in rabbits. Infected rabbits were subjected to a daily 10 minute heat-treatment during which time the body temperature was elevated to 42<sup>o</sup> C. They found that the disease was not retarded during experimental period and therefore suggested that fever therapy had no effect upon the ultimate development of these two viral infections in rabbits. These workers do not state how soon the body temperature returned to normal after the hyperpyrexia. These short periods of heat treatments were more likely to be ineffective than if the host were allowed to acclimatize to the high temperature.

b. On rickettsial infections. Castaneda (1937) has shown that relatively small (1.5<sup>o</sup> C) reductions in body temperature of rats, guinea pigs and rabbits are associated with increased susceptibility to experimental typhus. Moragues and Pinkerton (1944) have similarly observed that mice infected with murine typhus rickettsiae developed uniformly more fatal rickettsial peritonitis when the animals were kept at 19 - 22<sup>o</sup> C, when a mortality of less than 25% was obtained.

c. On bacterial infections. McDowell (1923) showed that rats exposed for 2 weeks to 28.5<sup>o</sup> C were more resistant to infection with

pneumococcus than rats kept at medium temperatures (18 - 22°C).

Muschenheim, Duerchner, Hardy and Stoll (1943) reported that cold treated rabbits infected with a virulent strain of pneumococcus developed a more severe infection compared to control kept at room temperature. Cold treatment however, did not influence the course of infection of a more virulent strain of the pneumococcus.

Carpenter, Boak and Warren, (1932) found that subjecting rabbits infected with Treponema pallidum to multiple unsustained fever of 41 - 42°C produced by irradiation in a high frequency electrostatic field, destroyed Treponema pallidum. The destruction of the spirochaetes was determined by injecting into normal rabbits extracts prepared from ~~tastes~~ and lymph nodes of infected heat treated rabbits. They further found that one febrile period of 6 hours at 41.5°C - 42°C was sufficient to destroy the spirochaetes. Clinical healing, however, occurred in three to four months after a series of 20 to 30 short period pyrexias. It must be added that in this kind of treatment, it is hard to separate the mechanical effect of heat radiation on the spirochaete from the general physiological involvement. Carpenter, Boak, Mucci and Warren (1933) as a result of this observation therefore tried to see the direct effect of heat on Neisseria gonorrhoea in vitro. They found that 97% of these organisms were destroyed in cultures at temperature of 40°C after 10 hours exposure. They suggested that artificially induced fever was therefore valuable in the treatment of the disease caused by this organism.

d. On trypanosomatid infections. The importance of environmental temperature on the course of protozoal infections seem to have attracted the attention of only a few protozoologists. Oehler (1914) found that when serial passages of a virulent strain



of T.(T.) brucei were carried out in mice kept at 35°C, a chronic and remitting infection occurred. Kolodny (1940) also showed that environmental temperature influenced the course of T.(S.) cruzi in mice. The infection was always fatal when infected mice were kept at about 6.5°C, but at 21°C and 35°C, the course of infections were not significantly different. Trejos, Urquilla and Paredes (1965) found that when mice were inoculated with non-lethal T.(S.) cruzi and kept at 18°C, parasitaemic levels were as high as 4200/mm<sup>3</sup>, but infected mice kept at 37°C had a lower count of 240 trypanosomes per mm<sup>3</sup>. Sections of the myocardium of infected mice kept at 18°C showed numerous parasites while no parasites or signs of myocarditis were found in animals kept at 37°C. Amrein (1967) similarly noted that parasitaemias disappeared from T.(S.) cruzi infected mice within about one month when these animals were kept at 35°C, and no parasites could be seen in tissue sections. Marinkelle and Rodriguez (1968) confirmed these findings and suggested that the disappearance of infection at high temperature (35°C) was due to antibody response.

In cutaneous leishmaniasis the effect of environmental temperature has also been shown to be of importance. Zeledon, De Monge and Blanco (1965) have shown that variations in skin temperature affected which sites were more susceptible to cutaneous leishmaniasis. They infected hamsters subcutaneously with promastigotes of L. brasiliensis in the nose, ear, foot, tail, dorsum and ventral area of the body. Lesions were readily demonstrated in those areas where the skin temperature was below 30°C; nose, ear, foot or tail. But when the infected animals were kept at 37°C, they failed to develop skin lesions and established lesions easily cured themselves. Hayatee (1971) has confirmed these findings. He found that when

albino mice infected with L. brasiliensis pifanoi and showing active lesions were kept at  $36.5^{\circ}\text{C}$ , the parasites disappeared from the infected histiocytes within 39 hours and the lesions healed completely within 35 days.

de Castro and Pinto (1960) have shown that the incubation temperature of culture forms of L. enriettii, L. brasiliensis and L. tropica was very important for normal growth. These cutaneous parasites could not develop in cultures at temperatures higher than  $36^{\circ}\text{C}$  but grew well at  $32 - 34^{\circ}\text{C}$ . They suggested that the failure to develop at a temperature higher than  $36^{\circ}\text{C}$  explained why these parasites could not invade deeper tissues. Bray and Lainson (1966) similarly found that two strains of L. brasiliensis could not develop at  $37.5^{\circ}\text{C}$  but grew well in culture medium consisting of 50% Hank's fluid, 45% rabbit serum and 5% chick embryo extract incubated at  $35^{\circ}\text{C}$ .

Janory and Poorman (1969) have demonstrated that the endogenous and substrate-stimulated respiratory rates of L. mexicana, L. tarentolae and L. donovani grown in blood-agar-Locke's overlay medium rose with temperatures, but dropped above  $35^{\circ}\text{C}$  in L. mexicana and L. tarentolae and above  $37^{\circ}\text{C}$  in L. donovani. They concluded that the metabolism of the cutaneous L. mexicana was adversely affected at a lower temperature than that of the organisms of visceral leishmaniasis and this accounted for the difference in the site of development of the two parasites.

### 3. Morphogenesis

Neva, Malone and Myers (1961) found that there was an increase from 1 - 6.9% of the amnion cell cultures intracellularly infected

with amastigotes of T.(S.) cruzi when the infected cultures were transferred from 33°C to 38°C incubation temperatures; and transfer of the infected cultures from 38°C to 33°C resulted within three days in an increase of (1.7 - 6.7%) trypomastigotes.

Trejos, Godoy, Greenblatt and Cedilos (1963) also found that there was an increase of amastigotes after three to four days when T.(S.) cruzi infected "L" culture tissue were incubated at 37°C, but incubation at 26°C resulted in the appearance of slender trypomastigotes. Marinkelle (1965) has shown that T.(H.) conorrhini cultures grown in Eagle's minimum essential medium and 20% calf serum resulted in trypomastigotes becoming thicker and postero-nucleated. He observed many aberrant forms, some of which were balloon-shaped with as many as 7 flagellae, 6 nuclei and 6 kinetoplasts. Steinert (1965) has observed that besides temperature, T.(H.) conorrhini needs some biochemical factor intimately associated with red blood cells to be able to undergo morphological changes. He has shown that this factor may be urea in the case of T.(M.) mega. And since he had demonstrated that urea interfered with DNA replication in this parasite, he concluded that morphogenesis involved some genetic transcription. While this argument sounds very plausible, the writer believes that it is under the influence of high ambient temperature that the production of a certain biochemical factor, probably urea, is increased in circulation and this increase affects the replication of some essential nucleic acid thereby affecting the normal genetical make up of the parasite.

Lemma and Schiller (1964) have observed that a high proportion (1/10) of promastigotes of L. tropica cultures incubated at 28°C changed to amastigotes within 32 hours when the incubation



temperature was increased to 32°C.

The amastigotes so formed were as infective to hamsters as were the original non-culture forms. Greenblatt and Glasser (1965) noted some morphological changes including a shortening of the flagellum or even complete loss of flagellum when L. enriettii cultures were incubated at a temperature above 30°C.

#### 4. Discussion

These reviews show that authorities are not agreed on the general effect of ambient temperature on trypanosome and related infections. The results are in many ways conflicting. It is also amazing that different results are obtained by different workers when the same infecting agent is used to infect the same species of host. A case in point is that of Ross's and Oehler's findings on T. (T.) brucei infections in rats. Both of these workers failed to make observations on either high or low ambient temperatures respectively. The reason for most of these different results stems from the type of experimentation involved. Most of them lack proper controls. To get clearly reproducible results, one would need to use the same strain of infecting agent, the same species of host and as far as possible the same experimental conditions.

But in spite of the contradictory results, the general consensus of opinion is that high ambient temperature does have ameliorating effect on these infections. What would be of practical importance would be to apply chemotherapeutic trials in such situations, particularly in strains where there is reason to suspect insusceptibility to curative dose levels of the drugs in question.

There also seems to be a direct connection between elevation of body temperature brought about by high ambient temperature and the morphology of the "Trypanosomatidae" whether this is due to the parasite's advantage, I cannot hazard a guess at the moment.

## 5. Effect on immune responses

### a. On antibody production

i. Protective antibodies. Rolly and Meltzer (1908) were the first people to relate subjection to an elevated ambient temperature to immunologic processes. They infected rabbits with repeated sublethal doses of pneumococci, staphylococci, Escherischia coli and Pseudomonas aeruginosa. Each bacterium was injected into two or three rabbits after which one or two rabbits in each group were confined to a heated chamber. The control animals were kept at room temperature. No record of body temperature is given in either case. They found that among the 11 animals subjected to high temperature, only four died whereas all the six control succumbed to infection. They took this to mean that high ambient temperature had helped the animals in their defence against the infection. They do not state how long the rabbits were detained in the hot environment. Ipsen (1952) later showed that mice immunized with tetanus toxoid and kept at 35°C withstood tetanus toxin challenge better than did mice kept at 6° or 25°C. The effect was particularly noticeable with lower dose levels of challenge.

ii. Opsonins. Rolly and Meltzer (1908) tested the phagocytic activity of human leucocytes to the above (pneumococci, staphylococci,

E. coli, P. aeruginosa bacteria. They reported that phagocytosis was enhanced at  $39.5 - 40^{\circ}\text{C}$  as compared to  $37^{\circ}\text{C}$  incubation temperature. They have not indicated the method they used in testing the phagocytic activity and how they harvested the human leucocytes. Clark (1942) found that phagocytic activity of guinea pig leucocytes to Eberthella typhosa in the presence of normal serum increased when temperature was raised to a maximum of  $40^{\circ}\text{C}$ .

iii. Haemolysins. The effect of environmental temperature on the production of haemolysins was studied by Foord (1918). He immunized 8 rabbits over a period of 4 days with increasing doses of washed sheep red blood cells. The animals were then chilled by immersion into ice cold water ( $0 - 8^{\circ}\text{C}$ ) for a period of 7 - 10 minutes twice daily during the period of immunization. The presence of haemolysins was tested for a period of 29 days after immunization. He found that there was no difference in the haemolysin production between control and chilled animals. Ellingson and Clark (1942) thought that by artificially raising the body temperature of animals, the antibody destruction in vivo might be accelerated. They therefore immunized rabbits with sheep red blood cells, typhoid vaccines and egg albumin and induced severe elevation of body temperature ( $41.5^{\circ}\text{C}$ ) in rabbits by subjecting them to short period of heat treatment daily during the period of immunization. They found that antibody responses were impaired in varying degrees in heated animals, but that in rabbits already immunized against Eberthella typhosa, the elevation of body temperature ( $41.6^{\circ}\text{C}$ ), was followed by rapid reduction of antibody titre levels.

The ability of the body temperature of poikilothermic animals



to vary widely without causing any apparent physiologic stress makes the study of the production of antibodies in these animals particularly interesting. Allen and McDaniel (1937) have shown in two species of frogs, Rana pipiens and Rana catesbeiana that haemolysins are not produced in these animals when they are immunized with human red blood cells and maintained at 8 - 10°C. But they were able to demonstrate haemolysin production in frogs kept at 22 - 27°C. Since environmental temperature was the only variable, they concluded that environmental temperature influenced the formation of antibodies. More recently Janssen and Waaler (1957) have demonstrated the presence of antibodies in hedgehogs and fish immunized with paratyphoid B vaccine and sheep red blood cells when these animals were kept at 32 - 33°C, but hibernating hedgehogs having body temperature of 6°C failed to produce antibodies. It would be interesting to see whether protective antibodies against for example, B. typhosa are equally affected by temperature. Kopeloff and Stanton (1942) showed that raising the body temperature by 1.6°C above normal as a result of keeping rats in specially heated boxes raises the haemolysin titre levels in rats from 1:40 to 1:320. Marshall (1959) on the other hand, failed to observe any effect of high (37°C) ambient temperature on the production of haemolysins in rabbits inoculated with washed sheep red blood cells.

iv. Agglutinins. Typhoid fever, (the effect of which simulates artificially induced elevated body temperature) has been studied in relation to antibody production. Tidy (1915) claimed that febrile condition induced by B. typhosus infection in man removed agglutinins against the organism in the blood. But Dreyer and Torrens (1915) after examining some 171 cases of proved paratyphoid fever, (having demonstrated the bacillus in faeces) found that the sera of these people contained agglutinins to B. typhosus with

titres ranging from 1/25 to 1/250. They do not give body temperature of these people. They concluded that febrile conditions caused by paratyphoid does not cause the agglutinin to B. typhosus to disappear from the blood of persons protected against typhoid fever. Tidy (1916) in response gave example of 14 cases with positive agglutinins reaction to B. typhosus and with bacilli present in their stools. Eight of these gave no reaction with B. typhosus in the early stages, and 3 gave slight agglutination reaction. Of the 9 cases with fever ( $40^{\circ}\text{C}$ ), 6 gave no reaction to B. typhosus in serum dilution of 1/20. He consequently observed that marked pyrexia such as  $39^{\circ}\text{C}$  for 5 days was associated with reduced or complete disappearance of the agglutinins resulting from inoculation. He therefore concluded that febrile conditions interfered with antibody response due to infection. Dreyer, Gibson and Walker (1916) could not accept Tidy's claims and so reported an incident of some 108 cases they had examined with febrile conditions other than typhoid fever in persons inoculated against typhoid. They do not show how they separate fever due to typhoid from fever due to other causes. All these cases showed high agglutinin titres. They re-affirmed that fever associated with paratyphoid does not cause the disappearance of typhoid agglutinins. Since both group of workers were using the same test, the only difference seems to be that Dreyer et al. assumed that paratyphoid automatically caused fever, whereas Tidy actually recorded the pyrexia. It is the intensity of hyperpyrexia which really counts rather than the general elevation of body temperature. However, this is an interesting observation which needs confirmation.

Cushing (1942) showed that antibody titre levels of antibody against Strongylocentrotus purpuratus (sea urchin) sperms in two



closely related fish, Cyprenius carpio (carp) and Carassius auratus var. fan tail (gold fish) rose more rapidly in fish kept at 28°C (11 days) than in those kept at 15°C (15 days). He suggested therefore that the ambient temperature influenced the rate of antibody production in poikilothermic vertebrates. Janssen and Waaler (1967) have also demonstrated the presence of agglutinins in hedgehogs immunized with paratyphoid B vaccine and kept at 32 - 33°C; no such antibodies were produced in hibernating hedgehogs (body temperature = 6°C). On the other hand, Ecker and O'Neal (1932) have shown that the effect of fever on antibody production was only a temporary one. They found that when fever was induced for short periods in rabbits one month after immunization with typhoid vaccine, agglutinins were depressed from 1/320 to 1/160, but gradually returned to normal after about one week.

v. Complement fixing Antibodies. Janssen and Waaler (1967) showed that complement fixing antibodies were detected in hedgehogs kept at 32 - 33°C, but not in those hibernating. Ecker and O'Neal (1932) found that induction of fever in rabbits in the course of immunizing them with typhoid vaccine resulted in variable effects on the levels of complement-fixing antibodies. On first heating the titres of complement fixing antibodies were depressed from 1/145 to 1/125 but on second heating, the titres of antibodies were raised from 1/100 to 1/125. On the other hand, Hadjopoulos and Bierman (1934) reported that hyperpyrexia caused a slowing up rather than destroyed complement fixing antibodies in rabbits previously immunized against Staphylococci, Streptococci, Micrococcus catarrhalis and diphtheroid bacilli.



b. On cell-mediated immune reactions

Skin sensitivity. Duerchner, Muschenheim and Hardy (1943) have observed that induced hypothermia (depression of body temperature by  $1.8^{\circ}$  to  $4^{\circ}\text{C}$ ) below normal guinea pig body temperature delayed the development of skin sensitivity and the degree of sensitivity was lessened.

c. Discussion

The effects of environmental temperature on antibody production in poikilothermic animals appear to be more clear cut and consistent. It is not known to what extent these results can be interpreted in terms of antibody production in the homoiothermic animals. The inconsistent results stem from the kind of experimental approach to the problem. Short term exposure to high or low temperatures may have adverse effects on both the host and the infective agent and so only inconsistent results are obtained. It would therefore be advisable to allow experimental animals to adapt to their respective experimental conditions, so that the observed effects are largely accounted for by the temperature effect.

C. Growth of salivarian trypanosomes in chick embryos

In order to observe the direct effect of heat on the course of trypanosome infections, chick embryos were used. Earlier workers have shown that trypanosomes can grow in the chick embryos.

Biocca (1938) was the first to infect chick embryos with trypanosomes. He inoculated T. (T.) brucei into 8 to 14 days old

chick embryos; trypanosomes were established. Embryos developed acute infections and died after 7 or 8 days. He was able to accomplish serial passages from embryo to embryo. It has since been shown (Hood, 1949; Longley, Clausen and Tatum, 1939) that several subspecies of T.(T.) brucei can be maintained in avian embryos. Chabaud (1939) has reported heavy T.(T.) b. rhodesiense infections in chick embryos 7 or 8 days following inoculation when 8-day-old embryos were inoculated, but the infection declined during the last week of incubation. van den Berghe also found that when he infected 10-day-old chick embryos with T.(T.) evansi, the parasites were detected throughout the incubation period and hatched chicks occasionally showed transient parasitaemia. Hood (1949) similarly found parasites of T.(T.) brucei and T. hippicum = (T.(T.) evansi) in chicks which had hatched 48 hours earlier. And more recently Goedbloed and Kinyanjui (1970) have claimed that they were able to detect T.(T.) brucei in chickens up to at least 103 days when 8 - 9 day-old chicks were inoculated.

San Augustin (1952) working with T.(T.) evansi found that after intravenous inoculation of embryos, there was a higher infection rate (96%) in chicks than in ducks (30%). Altire-Webber (1941) found that the route of infection was important in determining the nature of infection. He found that infection by the yolk sac route resulted in higher positive rates than inoculation by the chorio-allantoic route.

The instability of antigenic type of T.(T.) brucei during a chronic infection makes the study of host immune responses to the parasite, a very disturbing problem. The need to overcome this problem has made it desirable to use systems where immunological unresponsiveness is known to occur. Chick embryo has therefore



been used because of its immunological incompetence and because it is known to tolerate trypanosome growth as stated above. Goedbloed and Southgate (1969) have shown that chick embryos do not produce agglutinating and immunofluorescent antibodies. They inoculated 8-day-old chick embryos with T. (T.) brucei stabilates and collected sera between 5 and 13 days after inoculation. They tested the sera for agglutinating and indirect fluorescent antibodies. Brown (1970) has questioned the validity of the system used in these tests. He suggests that trypanosomes adapted to chick embryos may not be antigenically similar to the mouse type used as the source of agglutinating antigen.

Chick embryo experiments are likely to be profitable so long as susceptibility to and, uniformity of infection and pathogenicity of the trypanosome to embryos can always be guaranteed.

#### D. Cultivation of salivarian trypanosomes in culture

Interest in cultivation of trypanosomes in cultures stems from the need to study more closely the development of these organisms in environments which can be easily controlled. This knowledge could lead to greater understanding of their morphogenesis in relation to the biochemical and physiological changes which must occur if they are to survive during transmission from insect to mammalian hosts or vice versa. In this study attention was directed on the possibility that trypanosomes can grow in macrophages.

It is generally accepted that salivarian trypanosomes are more difficult to cultivate in cultures than the stercorarians. Baker (1963) has indicated that the salivarian trypanosomes are the most recently evolved and therefore the suggestion (Taylor and Baker,



1968) that the cultural requirements of different members of Trypanosomatidae becomes more restricted as they advance phylogenetically lends support to reasons for the poor adaptability to cultural growth.

### 1. In vitro growth

Various preparations of blood agar originally described by McNeal and Novy (1903) have formed the main basis of culture medium used to support salivarian trypanosome growth. von Razgha (1929) cultivated T.(T.) b. gambiense in citrated human blood and Ringer's solution (with 0.6 NaCl). Brutsaert and Henrard (1938) used "Liquoide" = <sup>Sodium poly-anethol sulphonate</sup> (heparin) as an anticoagulant instead of sodium citrate. They claim that this anticoagulant allowed them to obtain good trypanosome growths in culture even when infected blood contained very few trypanosomes. They grew T.(T.) b. gambiense, T.(T.) brucei and T.(N.) congolense readily in blood agar cultures. Tobie, von Brand and Mehlman (1950) recommended the use of rabbit blood instead of human blood. They argued that human blood was often difficult to get particularly when large quantities were needed. They preferred a diphasic rabbit blood medium. Weinman (1953) also preferred using inactivated human plasma instead of whole blood, so that he minimized lytic capacity of blood. He reconstituted blood by incorporating washed red blood cells to the medium before cultivating T.(T.) b. gambiense and T.(T.) b. rhodesiense in cultures. He improved his technique further when he used poly-vinyl sulphuric acid as anticoagulant. This compound had anti-complementary activity as well and had the advantage that it could be added directly into infected blood thereby avoiding separating plasma.

On blood agar the trypanosomes grow either in the water of condensation (Novy and MacNeal, 1904; Thomson and Sinton, 1912) or they form colonies on the surface of the agar (Weinman, 1946). But in liquid media (von Razgha, 1929; Reichenow 1932, 1934; Brutsaert and Henrard, 1938) they aggregate usually on the surface of the red blood cell layer. Pittam and Vickerman (1962) have cultivated T.(T.) b. rhodesiense in a liquid medium made up of amino acids, vitamins and bases in buffered physiological saline solution and human blood (lysed blood to 10% v/v). The pH of this medium is not given. They have not indicated whether the trypanosomes are attached on some surface. Weinman (1944) also grew T. (T.) b. gambiense in a semi solid medium, pH 7.4 - 7.5, containing human plasma and haemoglobin. He did not mention where growth occurred.

## 2. In vivo growth

Growth in cell cultures was introduced by Demarchi and Nicoli (1960) who found that T.(T.) b. gambiense and T.(T.) b. rhodesiense could grow in a monolayer culture of Hela and HEP human epithelial cells in Lepine's medium (Lepine et al., 1956) supplemented by casein hydrolysate and 5% calf serum. Demarchi and Nicoli (1960a) found that the supplement was necessary because the trypanosomes could not grow in Lepine's medium alone. They suggested that the cells contained an essential growth factor (haematin). Fromentin (1961) also succeeded in growing T.(T.) b. gambiense in human cells (strain KB) grown in a hydrolysate of casein and chick serum and also in chick embryo fibroblasts. She found that growth occurred only when the inoculum came from blood agar cultures but not when



she used blood stream forms.

### 3. Infectivity of cultured trypanosomes

Thomson and Sinton (1912) noticed that culture forms of T.(T.) b. gambiense and T.(T.) b. rhodesiense grown on blood agar developed into forms resembling those found in the tsetse fly gut and these forms were non-infective to mice. Reichenow (1932) similarly observed that recently isolated blood stream forms of T.(T.) b. gambiense rapidly disappeared when inoculated into von Razgha's medium, instead ~~from~~ resembling midgut trypanosomes appeared. No salivary gland infection was seen.

Trager (1959a) succeeded in growing T.(D.) vivax in culture made with tissues from the alimentary canal or salivary glands of late pupae of Glossina palpalis. He observed various midgut forms, including forms being morphologically like metacyclics. He proved their infectivity to sheep particularly when the cultures had been incubated for a short time (19 hours) to 38°C. But when T.(T.) brucei and T.(N.) congolense were grown in this medium forms like metacyclics were observed, but these failed to become infective (Trager 1959b).

It is considered that further attempts at in vitro cultures of salivarian trypanosomes especially T.(T.) brucei is unlikely to be profitable because these trypanosomes are so exacting in their nutrient requirements. And it is difficult to duplicate the exact nutrients of these trypanosomes. But in vivo cultures hold much hope especially if suitable incubation temperatures could be carefully worked out. It would also be advisable to set up conditions which



simulate day and night tropical temperatures, rather than be tied to some fixed incubation temperatures.

#### E. Trypanosome antigens

Among the many possible trypanosome antigens, two groups stand out quite distinctly. These are "stable antigens" and "variable antigens" (WHO, 1969). The two groups of antigens contain a variety of specific antigens e.g. immunogens, agglutinogens, precipitinogens, etc.

##### 1. Variable antigens

These antigens have been given various names: Exo-antigen (Weitz, 1960), released antigens, sub-cell antigens, (Lumsden, 1965, 1969), metabolic products (Thillet and Chandler, 1957) surface coat antigens (Vickerman and Luckins, 1969).

Immunogenic properties of these antigens was first shown by Thillet and Chandler (1957) when they observed that serum from rats infected with T.(H.) lewisi may contain antigenic material of trypanosomal origin, which was able to protect rats against a challenge from a homologous infection. Weitz (1960) described a soluble trypanosomal antigen which occurred in serum from rats infected with a blood-passaged strain of T.(T.) brucei. He further showed that the antigen preserved the viability of the trypanosomes in vitro, and when used to immunize rats and mice protected them from challenge from a homologous strain of live T.(T.) brucei. He named these antigens as "exo-antigens" and found them to be agglutinogenic

and precipitinogenic.

Antigens with similar properties have been described by Gray (1961) in T.(D.) vivax infection in Zebu cattle and goats and in T.(T.) b. gambiense and T.(T.) brucei brucei in rats. Weitz (1962, 1963) has described them in T.(T.) brucei brucei and T.(T.) b. rhodesiense infections. Seed and Weinman (1963) and Seed (1963) have demonstrated them in T.(T.) b. rhodesiense infections while Johnson, Neal and Gall (1963) have described them in T.(N.) congolense and T.(S.) cruzi infections.

Miller (1965) described specific changes in the variant antigens of T.(T.) brucei in relation to antigenic variation. He showed that specific antibodies were produced against exo-antigen of each variant population and that the immunogenicity of the antigenic components decreased as infection proceeded. Herbert and Lumsden (1968) have shown that immunity lasting about 159 days could be conferred to mice vaccinated with variant antigens absorbed on to aluminium hydroxide.

The part played by the variant antigen/antibody complexes formed during the infinite antigenic variations must be profoundly damaging to the host. Goodwin (1971) has described pathological changes which occur during T.(T.) brucei infections in rabbits. It would be interesting to find out what part is played by the cumulative effect of variant antigens particularly in these pathological changes.

## 2. Stable antigens

Information about these antigens has been collected from the T.(T.) brucei trypanosomes and may possibly not be applicable to other

species of trypanosomes. These antigens have been described under different names, bound antigen (Weitz, 1960), cell antigen (Gray, 1967). They are derived from the disintegrated trypanosome itself. They are found in different strains and are characteristic of a species of trypanosomes. The antigenic nature of stable trypanosome antigens has been amply elucidated by Brown and Williamson (1962). They found four types of antigens; and these were composed of two groups of unconjugated light weight proteins (4S and 1S). They showed that these antigens were composed mainly of precipitinogens. Brown and Williamson (1964) have further showed that the highest concentration of antigens was detectable in the trypanosomal cell sap.

### 3. Antigenic variation

The ability of T.(T.) brucei to produce a series of distinct serological types during the course of an infection was first observed during chemotherapeutic trials (Franke, 1905). The use of trypanocidal agents has therefore been exploited in deliberate attempts to induce this phenomenon in experimental immunological investigations (Browning, Calver and Adamson, 1934). It has been necessary to use chemotherapeutic drugs to control infections particularly in those which become rapidly fatal, so that the infected animals can be kept alive for prolonged periods, thereby allowing time for immune responses to develop. Immune serum was also found (Ehrlich, Roehl and Gulbransen (1909) to have an effect on trypanosomes similar to those of drugs used at subcurative dose levels. Ehrlich et al. (1909) found that when trypanosomes had been in contact with homologous antisera for a given period and then injected into mice,



the trypanosomes from the induced infection could not be agglutinated by the original antisera.

Attempts to estimate the number of relapse variants one particular strain is capable of producing has failed to give clear cut results. Ritz (1916) found that the number of possible antigenic variations a trypanosome was capable of undergoing was at least 22. Lourie and O'Connor (1937) confirmed this finding when they used clone populations. They found 13 variant populations of T.(T.) b. rhodesiense in 22 relapses. And Osaki (1959) found 23 variants in 440 T.(T.) b. gambiense relapses. Gray (1965) found that T.(T.) brucei produced new antigens at intervals of two to four days in infected rodents, sheep and goats. He suggested that the total number of antigens produced in one host was infinite as long as the infected animal was still alive. He produced evidence to show that variations followed a definite sequence and that similar antigens were produced in different hosts by the same strain of trypanosomes.

The instability of the variant populations has been shown (Mesnil and Brimont, 1909; Neumann, 1911) by their tendency to revert to the original antigenic type after prolonged passages in mice. Besides, Lourie and O'Connor (1937) have pointed out that strains are often antigenically heterogeneous. This might explain why basic strains are never entirely lost, although they may be thoroughly diluted with variant strains during the course of infection and so become undetected. Observations on the effects of tsetse fly transmission on the antigenic variation has been particularly informative as regards the stability of trypanosome antigens. Broom and Brown (1940) found that one strain of trypanosomes transmitted by different tsetse flies had one common antigen. Besides,

Cunningham and Vickerman (1962) observed several antigenic types common to ten individual T.(T.) brucei materials which were originally isolated from different sources such as man, domestic and wild animals. Gray (1965a) has shown that when tsetse flies ingest trypanosomes with variant antigens, the trypanosomes tended to acquire the "basic antigen" and after sojourn in the fly the "basic antigen" became predominant but it was not uncommon to find a mixture of basic and variant antigens. The term "predominant" was coined (Gray, 1962) to describe those antigens that developed first when a strain of trypanosomes was introduced into a new host by syringe passage.

Watkins (1964) has offered an explanation for the lability of trypanosome antigens. He calculated the rate of mutation in mice and rats infected with T.(T.) equiperdum and T.(T.) brucei and was able to show that mutation rate was high enough to be responsible for the new antigenic variants. Though the mathematical manipulations may be very impressive, it does not explain why reversals to the original parent strains should occur from time to time. Vickerman (1971) has similarly discounted the mutation theory. He suggests that each trypanosome organism carries a full range of variant antigens in its genotype and that during remissions, some trypanosomes avoid the host antibodies by changing to alternative serotype. This theory does not explain why only some switch to alternative serotype and what proportion of these do switch. The change to basic antigenic type is probably explained this way. The basic antigens are actually not variable antigens but part of the stable antigens. During the course of a chronic infection they are diluted so many times that they cannot be detected by the conventional test methods (agglutination). But when trypanosomes are transmitted after development in the



tsetse fly where they have not been faced with as hostile an environment as the vertebrate hosts, it is the antibodies against the trypanosomal body itself which will be expressed. It is from then onwards that variable antigens are elicited as the organism tries to overcome the host's hostile reaction.

#### F. Immunosuppression

Herbert and Wilkinson (1971) have defined immunosuppression as the artificial suppression of immune response by use of drugs such as antimetabolites or irradiation or antilymphocytic serum which enhances the survival of allografts.

##### 1. The effects of x-irradiation

The first workers to study systematically the effects of x-irradiation on the production of antibodies were Benjamin and Sluka (1908). They observed that rabbits when exposed to whole body x-irradiation before immunization with bovine serum responded more weakly to antigenic stimulation, as shown by lower precipitin titers, than did rabbits irradiated four days after immunization. They emphasized the great importance of timing of the x-ray exposure in relation to the injection of antigen. These observations were later confirmed by Hektoen (1915). Craddock and Lawrence (1948) found in the rabbit that irradiation at 8 hours before antigen injection inhibited antibody production whereas irradiation at 5 days after infection had no effect. In a review of literature on past work on the effects of x-irradiation on immunity Taliaferro and Taliaferro (1951) have concluded that x-rays unquestionably inhibit the production



of antibodies when administered from the beginning of the immunization procedure.

The type of infection caused by some stercorarian trypanosomes is characterized by a mild parasitaemia and a low level reproductive activity during the early phase of infection before acquired immunity becomes manifest. Both cellular and humoral factors may play important roles in this kind of resistance to infection. More attention on the influence of x-irradiation on experimental trypanosomiasis has therefore centred mainly on infections caused by some species belonging to the subgenus Herpetosoma. Naiman (1944) showed that the course of experimental infections of T.(H.) lewisi in rats was altered by exposure to 300 - 500 rads on the day of infection. This treatment caused an increase in parasitaemia when given on the day of infection; but the treatment could not induce a relapse to occur when given after trypanosomes had disappeared. She concluded from her observations that trypanocidal antibodies were markedly diminished by irradiation. Jaroslow (1955) has similarly observed a decreased resistance in mice irradiated with 550 rads from 15 days before to 4 days after infection with T.(H.) duttoni (= T.(H.) musculi). He recorded higher parasitaemias and reproductive activity of trypanosomes in irradiated mice as compared to non-irradiated mice prior to the onset of acquired immunity. He (Jaroslow, 1959) later studied the effects of splenectomy, blockade and x-irradiation in various combinations on T.(H.) duttoni infections in mice. He found that neither splenectomy nor irradiation resulted in an increase in reproductive activity or trypanosomes parasitaemia except when the host was more extensively injured. Tempelis and Lysenko (1965) demonstrated that x-irradiation resulted in an increased severity of T.(H.) lewisi infections in rats; the rats were exposed to 300 rads.

A prolonged presence of reproductive forms and also the delay in the appearance of agglutinins led him to suggest that some radio-sensitive factor important in the control of T.(H.) lewisi early in infection was depressed after irradiation.

Among the salivarian trypanosomes, Walker (1968) failed to show any enhancement of the virulence of T.(T.) b. rhodesiense nor T.(N.) congolense infection in mice when the animals were subjected to 650-800 rads. In the same year Irfan (1968) observed that neither splenectomy nor irradiation separately or jointly had any effect on the susceptibility of animals to T.(N.) congolense infections.

It will be noticed that the results of x-irradiation on the stercorearian trypanosomes mentioned all concur on one thing, and that is, that the trypanosomes meet with much less resistance in irradiated hosts. The effective dose of irradiation is also fairly low (300 rads). Most of these stercorearian trypanosomes have an intracellular phase during their development in the mammalian hosts. It would appear therefore that it is these cells which harbour the intracellular stages of trypanosomes that are most severely affected by irradiation. One would imagine that apart from providing a refuge, these cells play an important role in keeping infection under control.

On the other hand, the scarcity of information on the effects of x-irradiation in the course of salivarian trypanosome infections makes the assessment of the observed negative results uncalled for at the moment.



## 2. Effects of corticosteroids

The importance of cortisone (11-dehydro-17-hydroxy-corticosterone) and pituitary adrenocorticotrophic hormone (ACTH) in medical research was first realized when Hench et al. (1950) showed that these compounds influenced the course of clinical rheumatoid arthritis. A variety of similar studies were therefore extended to other inflammatory disorders. It soon became clear that these hormones altered the course of many diseases.

The effects of cortisone and ACTH on infections have been reviewed by Kass and Finland (1953). They have shown that cortisone and its analogues usually depress resistance of laboratory animals to a wide variety of infections and also activate latent infections. In general, the effects may be anti-inflammatory and excessive doses may upset the normal host-parasite-balance with the outcome being in favour of the parasite. These drugs have therefore been used in situations where there is reason to suspect immunological involvement.

In protozoal infections cortisone and related compounds have found widespread applications although the results have not always been consistent. Wolf et al. (1951) found that administration of 30-100 mg cortisone activated latent unsuspected T. vickersae Brumpt 1909 in rhesus monkeys. They observed no correlation between the dosage administered and the severity of the disease. The severity of T. (S.) cruzi infections in rats has been shown to increase after the administration of cortisone, 12.5 mg/kg (Senecca and Rockenbach, 1952). Sherman and Ruble (1967) reported that the reproductive phase of T. (H.) lewisi was prolonged and that higher levels of parasitaemias resulted in rats treated with 50-100 mg/kg cortisone. They also showed that the greater the amount of cortisone given the more severe were



the resulting parasitaemias. Patton and Clark (1968) have confirmed these findings when they administered a total of 2.5 mg of Dexamethasone to each rat infected with T.(H.) lewisi. On the other hand, Herbert and Becker (1961) found no alteration in the course of T.(H.) lewisi in rats after cortisone treatment.

Cantrell (1955) found that varying amount of cortisone given to infected rats during the entire period of observation exerted little effect on the course of T.(T.) equiperdum infections. Other studies by Cantrell and Betts (1956) have showed that cortisone interfered with the action of ~~ax~~phenarsine hydrochloride at high doses and impaired immunization if the dose of the antigen was near its lethal threshold. Frieble (1952) found that cortisone did not affect T.(T.) evansi infections in mice, and von Brand et al. (1951) failed to influence the course of T.(T.) equiperdum in rats after administering 11 mg/kg cortisone.

Petana (1964) found that 100 mg/kg cortisone had no effect on the course of the infections in rats infected with highly virulent strains of T.(T.) b. gambiense, T.(T.) b. brucei and T.(N.) congolense even when parasites were injected in relatively small numbers. But when cortisone-treated rats were inoculated with less virulent strains of T.(N.) congolense they became highly susceptible. Ashcroft (1957, 1959) failed to find consistent results when he administered varying amounts of cortisone to rats infected with T.(T.) b. rhodesiense. The effects obtained depended on dosage and the time of administration and he concluded that under certain conditions cortisone enhanced trypanosome infection but under other conditions it protected the animals from the ill-effects of parasitaemia.

These results show that there is a difference in the course of infection after cortisone treatment between the salivarian and

stercorarian trypanosome in the rats. Infections due to stercorarian trypanosomes are affected by cortisone treatment while salivarian infections are not. One may ask whether the enhancement of infection here is due to impairment of immune response, and if so why is it that it affects only one type of infection (stercorarian) and not the other (salivarian). Possible explanations for the differences observed may be offered only in the form of speculation. First, it may be that the salivarian trypanosomes need a more severe impairment of the immune mechanisms before the course of their infections are altered. Secondly, because stercorarian trypanosomes are tissue parasites (have an intracellular phase at some time during the mammalian cycle) they have more to gain if these tissues are damaged by the anti-inflammatory effect of cortisone. This is only true if it is accepted as has been suggested in the discussion on x-irradiation that the cells that are invaded by the parasites also play a role in controlling the infection. The salivarian trypanosomes on the other hand, being extracellular parasites are more likely to escape the direct anti-inflammatory effect of the drug, and any effect of cortisone must be an indirect one.

### III. MATERIALS AND METHODS

#### A. Experimental animals

The animals used were all obtained from Messrs A. Tuck and Son Ltd., Essex, England. They were:

Mice. Swiss white Caesarian derived CD-T0 and Parkes (random-bred); CH<sub>1</sub>, CBA and C<sub>3</sub>H (inbred). Both sexes were used at 4-8 weeks of age. Since T0 mice have been used in nearly all experiments, mice as used in this thesis refers to this strain only unless stated otherwise.

Rats. Wistar~~albino~~ albino white rats, 8-10 weeks old.

Rabbits. New Zealand white, 20 months old.

#### B. Trypanosomes

All the trypanosome materials used were stabilates (Lumsden and Hardy, 1965) from the cryobank of the Dept. of Medical Protozoology, London School of Hygiene and Tropical Medicine. They were as follows:

Trypanosoma (Duttonella) vivax, LUMP 45.

The isolation and early history of this material was unknown. It is a rat-adapted strain (Desowitz and Watson, 1952) and was obtained from the Wellcome Laboratories of Tropical Medicine on 27th March 1963. The strain was passaged eight times in Swiss white mice before being stablilated. The infectivity of the stabilate was antilog  $4.4 \pm 0.5$  mouse ID<sub>63</sub> per ml.



Trypanosoma (Nannomonas) congolense, LUMP 52.

The early history of this stabilate was not documented but since its establishment at the Dept. of Medical Protozoology, L. S. H. T. M. in 1969, it has been passaged three times in Swiss white mice before being stabilized as LUMP 52. Its infectivity could not be estimated accurately because only  $\bar{2}$  dilution was infective.

Trypanosoma (Trypanozoon) brucei. The following stabilates were used:

LUMP 36, originally isolated from wild Glossina pallidipes Austen caught at Lugala, Busoga, Uganda in 1960; maintained by serial passage in mice, rats and a cow; stabilized passage 21 (TREU 164); cloned passage 22 and stabilized passage 32 (TREU 255 ETat 2). For details of history up to this point see McNeillage et al. 1969. LUMP 36 was a stabilized passage 33 and contained antilog 7.90 trypanosomes and antilog  $5.8 \pm 0.5$  mouse ID<sub>63</sub> per ml.

LUMP 128, was the first stabilized passage from LUMP 36. Its infectivity was antilog  $6.1 \pm 0.6$  mouse ID<sub>63</sub> per ml.

Trypanosoma (Trypanozoon) brucei brucei, LUMP 43.

This strain was isolated by Dr. J. R. Baker from a female Gorgon taurinus (wildebeest) in Serengeti, Tanzania on the 3rd March 1966 and has been maintained by serial passage in mice and rats. For details of the history, see flow diagram Fig. 2. The infectivity of the stabilate was antilog  $4.4 \pm 0.5$  mouse ID<sub>63</sub> per ml. The strain was tested soon after its isolation on two volunteers at Tabora,

Tanzania and was found to be non infective to man.

Trypanosoma (Trypanozoon) evansi, LUMP 55.

This was a stabilate of infected mouse blood of a dyskinetoplastic strain isolated from a camel in the Sudan in 1937 (Hoare, 1954). It was passaged five times in gerbils and passage 6 was carried out in mice. It was then passaged twice weekly in mice for over 1100 times. A stabilate was finally made which was cloned twice before being stabilized as LUMP 55. The infectivity of the stabilate was antilog  $5.1 \pm 0.5$ . ID<sub>63</sub> per ml.

C. Solutions

1. Buffered salts

a) Buffered salts solution (Lumsden et al., 1965) was the principal diluent for stabilate materials and blood passages. Essentially, this solution was made up from the following components:

Solution A. This solution contained Sodium, Potassium, Magnesium and Calcium salts (AnalaR Reagents, B. D. H., Poole, Dorset, England). Stock solutions of each of these salts were prepared by weighing accurately the following reagents and contained in a litre of double glass distilled water. Because the hygroscopic salts (Magnesium and Calcium salts) cannot be weighed accurately, they were prepared by titration. The component salts were made up in distilled water as follows:

Sodium chloride (NaCl) 0.154M - 9.00 mg/litre

Potassium chloride (KCl) 0.154M - 11.48 mg/litre

Magnesium chloride ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) 0.103M - 20.94 mg/litre

Calcium chloride ( $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ ) 0.103M - 22.56 mg/litre

Magnesium and Calcium salts were first made up to a concentration of 25 mg per litre and then titrated against 0.1N  $\text{AgNO}_3$  (Silver nitrate) and the bulk was adjusted to the correct concentration by addition of distilled water. The stock solutions were stored in plastic bottles at 4°C.

Preparation of Solution A. The above solutions were mixed in the following proportions:

NaCl	100 ml
KCl	4 ml
$\text{MgCl}_2$	3 ml
$\text{CaCl}_2$	1 ml

The mixture was then titrated against 0.1N  $\text{AgNO}_3$ . In this way 5 ml of the solution was found to be equivalent to 7.8 ml of 0.1N  $\text{AgNO}_3$ . A fluctuation between 7.6 and 9.0 ml was acceptable but values below or above these, indicated a wrong procedure, in which case the solution had to be discarded. When acceptable values were obtained the solution was dispensed in 22 ml capped Universal bottles and autoclaved for 15 minutes at 121°C. After cooling the bottles were stored at 4°C until the solution was needed for use.

Solution B. Phosphate buffer. This solution consisted of:

Sodium dihydrogen orthophosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) 0.154M - 24.02 mg/litre

Disodium hydrogen orthophosphate ( $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ) 0.103M - 36.89 mg/litre

The two solutions were mixed in a ratio of:



$\text{NaH}_2\text{PO}_4$	1.36 vols)	} to give a pH of 7.4
$\text{Na}_2\text{HPO}_4$	8.64 vols)	

and dispensed into Universal bottles and autoclaved. The stock solutions were stored in plastic bottles at  $4^\circ\text{C}$ . Solutions A and B were mixed at a ratio of 1:9 respectively to give a final concentration of the buffered salts (BS) to be used for suspending trypanosome stabiliate materials.

b) Buffered water used to prepare Giemsa's stain was made up as follows:

3.0 gm anhydrous $\text{Na}_2\text{HPO}_4$	} in one litre of distilled water
0.6 gm anhydrous $\text{KH}_2\text{PO}_4$	

or

7.5 gm  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  may be used when anhydrous  $\text{Na}_2\text{HPO}_4$  is not available. The pH of this mixture is approximately 7.2.

This buffered water was preferred in making Giemsa's stain because it was simpler to prepare and therefore more convenient for routine usage than the above one.

## 2. Solutions used in suspending spleen cells; Local haemolysis in gel medium (Dresser and Wortis, 1967)

### Dulbecco's PBS

#### Solution A (conc. 5x)

$\text{NaCl}$	8.09 gm
$\text{KCl}$	0.29 gm
$\text{KH}_2\text{PO}_4$	0.29 gm
$\text{Na}_2\text{HPO}_4$	1.159 gm

Distilled water up to 80 ml

Solution B

CaCl<sub>2</sub> 0.19 gm  
Distilled water up to 100 ml

Solution C

MgCl<sub>2</sub>·6H<sub>2</sub>O 0.19 gm  
Distilled water up to 100 ml

The PBS was made up in the following proportions (all solutions having been autoclaved for 10 min at 10 lb/sq in):

Distilled water 720 ml  
Solution A 80 ml  
Solution B 100 ml  
Solution C 100 ml

Gey's solutionSolution A (conc. 5x)

NaCl 35.0 gm  
KCl 1.85 gm  
Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O 1.505 gm  
KH<sub>2</sub>PO<sub>4</sub> 0.1185 gm  
Glucose 5.0 gm  
Phenol red 0.05 gm  
Gelatine 25.0 gm  
Distilled water to 1 litre

Solution B

MgCl<sub>2</sub>·6H<sub>2</sub>O 0.42 gm  
MgSO<sub>4</sub>·7H<sub>2</sub>O 0.14 gm  
CaCl<sub>2</sub> 0.34 gm  
Distilled water to 100 ml

Solution C

NaHCO<sub>3</sub>                      2.25 gm

Distilled water to 100 ml

Gey's solution was made up from autoclaved solutions as follows:

Solution A              20 ml

Solution B              5 ml

Solution C              5 ml

Distilled water 70 ml

The pH of the solution was adjusted by blowing in CO<sub>2</sub> onto the bottle containing the Gey's solution.

D. Antibiotics

a) Penicillin. Benzyl penicillin (sodium) B.P.,  
0.6 gm vials, potency 1670 international units/mg  
(Glaxo; Crystapen).

b) Streptomycin. Streptomycin sulphate, B.P.,  
1.09 gm vials; potency 745 units/mg (Glaxo)

0.1 ml of a mixture of solutions of these antibiotics, (containing 1000 units of penicillin and 74.5 units streptomycin) was injected intramuscularly into chick embryos and irradiated mice to protect them from bacterial infection.

E. Anticoagulants

A quantity of 0.1 ml of heparin, 40 units per ml of phosphate buffer was used as anti-coagulant for whole blood drawn from the heart. This concentration of heparin was adequate for up to 1 ml of mouse blood.



## F. Anaesthesia

Anaesthetic ether was employed for sedation of animals before inoculation. But when complete relaxation for a prolonged interval, for example, narcotizing mice for providing feeds for Glossina spp. "Nembutal" Pentobarbitone sodium, B.P., at a dosage of 0.44 ml/kg body weight of the animal was administered intraperitoneally (IP).

## G. Drugs

### 1. Antitrypanosomal

Pure substance of Diminazene aceturate (Berenil; Hoechst Pharmaceuticals Ltd.) in phosphate buffer pH 7.4 was administered (IP) at a dosage of 10 mg/kg body weight to terminate infections.

### 2. Immunosuppressive

- a) Cyclophosphamide B.P.  $2\text{-}\overline{\text{Di}}\text{-(2-chloroethyl)-amino}\overline{\text{7}}\text{-1-exa-3-aza-2-phospha-cyclohexane-2-oxide}$ ; Endoxana, Ward Blenkinsop and Co. Ltd., London, 50 mg/kg body weight was <sup>injected</sup> intraperitoneally.
- b) Betamethasone disodium phosphate (Glaxo Lab. Ltd.); a total of 400 mg was administered intramuscularly to each mouse, over a period of four days. Immunosuppressive drugs were prepared in pyrogen free distilled water (Glaxo) for injection.

## H. Culture medium

Tissue Culture medium 199, (Morgan, Morton and Parker, 1950) used to grow macrophages consisted of:

Dried medium	10 mg
Deionized water	950 ml
Penicillin	200,000 units/litre of the medium
Streptomycin	100,000 ug/litre of the medium
50 ml, 4.4% Sodium bicarbonate	

The mixture was sterilized by filtration through millipore filter size 0.22  $\mu$ m.

## J. Fixatives (Clayden, 1962)

The fixatives used to fix tissues for histological observations were:

### 1. Formol saline

Sodium chloride	0.9 gm
Water	90 ml
Formalin	10 ml

### 2. Carnoy's fluid

Chloroform	30 ml
Absolute alcohol	60 ml
Glacial acetic acid	10 ml

## K. Staining

1. Giemsa's stain, Revector microscopical stain (Hopkin and Williams Ltd., Chadwell Heath, Essex, England) was used at 10% concentration

for staining blood smears.

2. Amido black

This reagent was used to stain precipitation lines in gel. It was composed of:

Amido black	0.5 gm
Methanol	45 ml
Distilled water	45 ml
Glacial acetic acid	10 ml



#### IV. EXPERIMENTAL PROCEDURES

##### A. General

##### 1. Maintenance of animals

a) General conditions. Animals were maintained in the L. S. H. T. M. animal house where they were fed on standard diet. Mice and rats were fed on mouse cubes, diet 86 (Thomson, 1968) and rabbits were fed on diet RPG (M.O.D. Allington farm specification, 1968). The temperature of the animal house fluctuated between 22-27°C.

b) Special study conditions. When it was necessary to keep animals under controlled environmental temperatures, the animals were maintained in incubators regulated within 0.5°C to 4°C, 28°C, 30°C, 32°C and 35°C. Food and water were supplied ad libitum and sawdust bedding changed every three days. The animals were confined to these environments throughout the observation periods with interruptions only during examinations.

##### 2. Body temperature measurements

Mice were held firmly but gently in hand (see Fig. 3) and a thermistor probe (Grants Instruments, Cambridge, England) inserted 1 cm deep into the rectum. The probe was held in this position for one minute and a reading taken when no more deflections of the temperature indicator occurred.

### 3. Body weight measurements

80

A mouse to be weighed was put in a 250 ml beaker and weighed (C. Stevens and Son Weighing Machines Ltd.). The mouse was removed and the beaker reweighed. The mouse body weight was the difference in two weights rounded to 0.1 gm.

### 4. Infection of animals

a) Trypanosomes used. Stabilates of trypanosome materials suspended in phosphate buffered salts (BS) pH 7.4 were used to inoculate animals. But during direct passages, heparinized infected blood was used.

b) Routes of infection. Mice were routinely inoculated under ether anaesthesia. A standard inoculum of  $\overline{2}$  dilution of stabilate material suspended in (BS) pH 7.4 was inoculated intraperitoneally. Each mouse received 0.1 ml of the inoculum. During subinoculations when larger quantities of the inoculum was necessary, 0.1 ml of heparinized undiluted infected blood was injected to each mouse (IP).

### 5. Bleeding of hosts

The methods generally adopted in obtaining blood from mice were as follows:

a) From retro-orbital plexus (Halpern and Pacaud, 1951).

This method was useful when aseptic conditions were not very essential, and also when small quantities of serum was to be taken from a mouse at regular intervals.

b) From tail. This method was employed for routine examination

of wet preparations of peripheral blood for the presence of trypanosomes.

c) From heart. When sterile blood was required, e.g. for stabilation as for obtaining trypanosomes needed for inoculation into culture medium, the mouse was deeply anaethetized and the skin over the chest opened. Without opening the thoracic cavity, a 0.54 mm diameter needle attached to 1 ml tuberculin syringe was inserted into the heart through the left side of the chest. Blood was then withdrawn into the syringe in one continuous flow.

## 6. Morphology of organisms

Morphology of trypanosomes was studied from Giemsa-stained thin blood films. For the method of fixation and staining see section IV.D.2a.

## 7. Measurement of organisms

In order to detect the existence of mensural differences in trypanosome populations, individual trypanosomes selected at random were measured from stained preparations. A rough estimate of trypanosome lengths was determined using a micrometer eye piece (calibrated in  $\mu$ ).

## 8. Enumeration of organisms

Wet films of tail blood from infected mice were prepared on clean slides and covered with cover-slips (2.2 cm x 0.3 cm). Blood from conveniently marked animals were prepared on one slide and examined at a magnification of 400x under phase contrast microscopy. All the



available blood area on the slide was examined before any preparation was reported as negative. For those smears showing trypanosomes, parasitaemia was scored on a scale, +, ++ and +++; in terms of parasitaemia these symbols represented +, less than 10 parasites seen in 1 minute; ++ intermediate between + and +++; and +++ when parasites were teeming. But when actual numbers of trypanosomes were needed, 0.01 ml of blood drawn from the tail was diluted 200x in buffered salts (BS) and organisms contained in a drop of this solution counted from the four outer squares of a haemocytometer using phase contrast microscopy.

#### 9. Infectivity titration

The method adopted for infectivity testing was that described by Lumsden et al. (1963). One capillary was removed from cold storage, both ends scored off and the capillary weighed on a torsion balance. Its contents were then discharged into 1 ml (BS) maintained at approximately 0°C. The empty capillary was reweighed and knowing the weight of the stabilate, the suspension was adjusted volumetrically so that a  $\bar{2}$  w/v log dilution was obtained. The  $\bar{2}$  log dilution was further diluted in tenfold series volumetrically so that,  $\bar{3}$ ,  $\bar{4}$ ,  $\bar{5}$ ,  $\bar{6}$  and  $\bar{7}$  log dilutions were prepared.

36 mice previously starved overnight but supplied with water only were separated into 6 groups. They were anaesthetized and inoculated (IP), starting from the higher dilution to the lowest, 6 mice in a group were each inoculated in a row with 0.1 ml of the inoculum. The animals were inoculated in one continuous operation so that from the time of removal of capillary from storage to the last inoculation took approximately ten minutes. The concentration

of organisms in the stabilate was estimated from haemocytometer counts of motile trypanosomes made on the  $\bar{2}$  log dilution. The animals were fed and starting on third day after inoculation, they were examined for the presence of trypanosomes using wet films of tail blood. From the number of mice becoming infected at each dilution, the infectivity of the stabilate was estimated as ID<sub>63</sub> the dose required to infect 63% of the test animals.

#### 10. Stabilation of organism suspensions

Sterile blood collected from the heart of a heavily infected mouse was mixed with 0.1 ml heparin and discharged into a chilled (0°C) Wasserman tube. Glycerol was added to give a final concentration of 7.5%. Infected blood was then mixed well with glycerol using a pasteur pipette and the mixture filled into sterile capillary tubes (Plowden and Thompson Ltd., Stourbridge).

The method employed for the low temperature storage of trypanosomes was that used in the Dept. of Medical Protozoology, L. S. H. T. M. This method was originally described by Cunningham et al. (1963). The capillary tubes were held on a horizontal plane in a rack so that they could be filled carefully without overflowing and by slight tilting the contents of the tubes were centred thus leaving some 3 cm at both ends free. The ends were heat sealed in a microburner flame. After sealing the one end of each of the tubes, the rack was tipped to check that all tubes had been properly sealed. The remaining ends were then sealed and the tubes transferred to a test tube containing methanol which had previously been cooled by putting the test tube in an ice bath. A stabilate number was then allocated to the sample and placed inside the test tube together

with the capillary tubes. The test tube was then corked tightly and put in a 25 mm thick-walled insulating jacket made of Onazote (Expanded Rubber Co. Ltd., Croydon). The jacket was plugged tightly with cotton wool and transferred to the solid carbon dioxide ( $\text{CO}_2$ ) cabinet. After 24 hours, the test tube was transferred quickly to methanol maintained at  $-79^\circ\text{C}$  in a perspex bath and the capillary tubes removed from the test tube. A numbered box container from the storage compartment of the storage bank was removed and placed in the perspex bath. The temperature of the perspex bath was maintained at  $-79^\circ\text{C}$  by adding pieces of solid  $\text{CO}_2$ , at which temperature the bath stopped bubbling. The capillary tubes were then very carefully transferred into the box container after which the box filled with methanol and capillary tubes, was at once transferred to the permanent storage bank.

B. Infection of chick embryos

Chicken embryos obtained from the Appleby Farm, Ashford, Kent, England, were incubated at  $39^\circ\text{C}$  (Western Incubators, Ltd.) prior to inoculation. Living embryos were separated from dead ones by placing individual eggs on a viewing box placed in a dark room. Two pencil marks were made, one of the air space side and the other on the side of the egg where the chorioallantois was best developed. The egg shell was drilled with a side to side movement of the rotating carborundum disc along the two pencil marks to perforate the egg shell completely. The openings were brushed with sterile melted paraffin.

Trypanosome stabilate suspended in BS solution to which 0.05 ml of penicillin-streptomycin mixture had been added was deposited on



one exposed side of the shell membrane and the membrane punctured through the drop. A gentle suction force using a rubber teat was applied at the opening into the air sac- the inoculum thereby being drawn in and uniformly distributed over the chorioallantois as the artificial air space forms. A drop of melted paraffin was then spread over the opening in the shell membrane to seal the small opening. After inoculation the embryos were incubated at the desired temperatures.

### C. Infection of cell cultures

#### 1. Preparation of macrophage cultures

Mice were killed by dislocation of the neck. The fur was damped with 70% methanol. The abdominal skin was then lifted upwards from the abdominal wall with a sharp forceps and a small longitudinal incision made (Stuart, 1967). The skin was stripped by pulling the edge. Care was taken not to touch the naked part of the muscle layer. 1.5 ml of Tissue culture medium 199 (Wellcome Research Lab., Beckenham, England) containing heparin was then injected carefully into the peritoneal cavity without puncturing the gut. The fluid was circulated and then withdrawn by gentle aspiration; it was then transferred to a sterile siliconized Universal container. The fluid collected from four mice was pooled and the number of the peritoneal cells made to a given concentration. The fluid was dispersed into sterile Leighton tubes in 0.1 ml samples and incubated for one hour at 37°C. The medium was changed after incubation and sterile calf serum (Burroughs Wellcome No. 1) was added to TC 199 so that a final concentration of 10% was obtained. 1.5 ml of the medium

was estimated to contain  $10^6$  cells. Leighton tubes containing the peritoneal cells were gassed with 5%  $\text{CO}_2$  to maintain the pH at approximately 7.2. The tubes were then incubated at  $37^\circ\text{C}$ . The medium was changed every third day, and during each change 5%  $\text{CO}_2$  was blown in to stabilize the pH (Behbehani, personal communications).

## 2. Inoculation of cultures with trypanosomes

Trypanosomes were harvested from six heavily infected mice by bleeding from the heart, heparin being used as an anticoagulant. The blood from the mice was pooled in a bijou bottle. The blood collected was centrifuged as soon as possible at  $115 \text{ "g"}$  for five minutes, refrigerated ( $0-4^\circ\text{C}$ ) centrifuge being used. The buffy layer was transferred to another bijou bottle and mixed with sterile BS solution and centrifuged further at  $1850 \text{ g}$  for 8 minutes. The final deposit was suspended in 6 ml TC 199 and a trypanosome count made on a haemocytometer. The macrophage culture already prepared was then inoculated with trypanosome suspensions. The medium in the tubes was replaced by an equal volume of trypanosome suspension.

## D. Histological techniques

Portions of tissues from freshly killed animals were fixed in 10% formal saline and Carnoy's fluid. The method of processing the tissues after fixation was adapted from that described by Clayden (1962).

### 1. Tissue processing

The tissues were fixed in Carnoy's fluid for one hour and then

transferred to absolute alcohol. Fixation in formol saline took 24 hours before the tissues were transferred to 70% alcohol. The fixed tissues were processed in automatic tissue processor (Hendrey Automation, Slough, England). The dehydration of the tissues took 24 hours and this involved bathing the tissues in several changes of 70%, 95% and absolute alcohol, xylene and finally embedded in molten paraffin wax. The impregnated tissues were blocked in "paper boats" and allowed to cool. The blocked sections were trimmed to remove excess wax and then firmly attached to block holder by gently warming one end of the wax-impregnated tissue and allowing it to cool on to the block holder. The holder was then fixed to the microtome and sections cut at a thickness of 4 microns with a sharp knife using the Cambridge Rocking microtome (Cambridge Instrument Co. Ltd.).

## 2. Staining

a) Giemsa's stain. Blood smears, impression smears of organs and the gut contents of tsetse flies were dried and fixed in methanol and stained with 10% Giemsa's stain. The slides were stained for 45 minutes, rinsed in the buffered water (pH 7.2) and allowed to dry in an upright position.

b) Giemsa-colophonium. After removing the wax, the sections were washed in tap water and placed in Giemsa stain (10 ml concentrated stain, 10 ml methyl alcohol, 10 ml acetone, 100 ml buffered distilled water pH 7.2). The sections were then allowed to stain for one hour, washed with tap water and drained well. They were then dipped momentarily in tap water containing traces of acetic acid and then bathed in colophonium resin in acetone



(15% resin); the slide was agitated for 15 seconds. The resin was poured off and the sections quickly washed in several changes of 70 ml acetone and 30 ml xylol to remove traces of colophonium. Several changes of xylol was then applied until the sections became clear and also to remove all traces of acetone. The sections were mounted in Euparal under coverslip.

c) Ehrlich's Haematoxylin and Eosin. The wax was removed from the sections by washing with xylol, and xylol washed with alcohol. The sections were then washed in tap water and stained with Ehrlich's haematoxylin for 10-20 minutes, after which they were washed in running tap water until the sections became blue. They were differentiated with 0.5% acid alcohol for 10-20 seconds and then blued as before for five minutes. Counter-staining of the sections was done by applying 1% Eosin for two minutes. The sections were then washed in tap water and dehydrated in three washings of 95% alcohol, cleared in oil of cloves and xylol and finally mounted under coverslip in canada balsam.

## E. Immunological techniques

### 1. Antigens

a) Sources. The antigens for agglutination tests consisted of live trypanosomes preserved as stabulates of infected mouse blood. These were stored at  $-79^{\circ}\text{C}$  in solid  $\text{CO}_2$  cabinet or in liquid nitrogen tank at  $-196^{\circ}\text{C}$ . For immunodiffusion tests the antigen consisted of infected mouse serum or plasma.

b) Preparation of infected mouse serum. Depending on the amount of serum required, infected mice were bled either in small

quantities through the retro-orbital sinus or totally through the heart, at about peak parasitaemia. At this stage parasitaemia was about antilog 8 trypanosomes per ml of blood. In the former case blood was collected in heparinized capillary tubes (Hawksley and Sons Ltd.). One end of the tube was sealed by microburner flame after which the samples centrifuged in a haematocrit centrifuge at 10,500 g' for 10 minutes. The cellular deposit section was discarded. Both ends of the plasma portion were sealed with crista seal (Hawksley and Sons Ltd.) and plasma samples stored at  $-20^{\circ}\text{C}$ .

Blood collected by bleeding mice from the heart was allowed to clot. This was then centrifuged at 1850 g for 10 minutes. To ensure complete removal of trypanosomes and any particulate contaminants, the supernate obtained was filtered through millipore filter, <sup>0</sup>pre size 0.24  $\mu\text{m}$  and then stored at  $-20^{\circ}\text{C}$ . The same procedure was adopted for sera used in immunization experiments.

## 2. Antisera

a) Preparation of antisera for agglutination test. Immune sera containing agglutinins against trypanosome strains and sub-strains were prepared as follows:

Mice were inoculated with trypanosomes strains to be tested and when the infection reached its peak (about antilog 8 organisms per ml) the mice were treated with Diminazene aceturate. Mice were bled from the retro-orbital sinus one week after treatment and plasma collected was pooled and stored at  $-20^{\circ}\text{C}$ .

b) Preparation of antisera for immunodiffusion test. Homologous antisera were prepared in mice by inoculating them (Day 0) with stabilate materials. The mice were treated with Diminazene aceturate

(10 mg/kg) on Day 3 and challenged with a similar inoculum of the same stabilate seven days after treatment. Antisera was harvested 1, 2, 3, 4, 5 and 6 weeks after challenge.

### 3. Gel diffusion test

a) Cleaning of slides. Microscope slides were cleaned in dichromo-sulphuric acid and rinsed in distilled water and stored in methanol. When ready for use, the slides were wiped clean with dry soft cloth and marked with diamond pencil for easy identification.

b) Preparation of agar plates for immuno diffusion.

1 gm/100 ml solution of refined Noble Agar (Difco) was prepared by soaking the Agar in 8 parts of saline 0.85% and 2 parts of phosphate buffer pH 7.4 in a conical flask. The agar was dissolved by immersing the flask in a beaker of boiling water. Five drops of 1:5000 Sodium azide from a pasteur pipette was added to prevent fungal growth. A thin bottom layer of agar was first prepared by smearing one surface of microscope slide with hot agar, before the top gelling layer was added. The hot agar was allowed to cool to 56°C before it was drawn into a 10 ml warmed graduated pipette. 1.8 ml of agar was then allowed carefully to flow into a level slide placed on a level table. The agar layer was 2.5 mm thick when it had cooled. When agar had solidified firmly on the slide, a 3 mm stainless steel gel punch (LKB) was used to cut out wells. The agar discs formed were removed.

c) Conduction of immunodiffusion tests. Immunodiffusion technique as described by Crowle (1961) was carried out on glass slides. After adding the reagents (infected serum and antisera against it) in appropriate wells, the slides were incubated in a



humid chamber at laboratory temperature and reading taken after 48 hours of incubation. The unreacted proteins were washed out by soaking the slides for 2 days in several changes of phosphate buffer BS. The last washing was done in distilled water overnight after which the slides were allowed to dry in air. The precipitation lines in gel were stained for 1 minute in 0.5% amido black, quickly washed under tap water and then transferred to washing solution (same as the solvent for Amido black) until no further colour could be removed. The slides were rinsed in distilled water and dried. The precipitation lines were photographed for permanent record.

#### 4. Agglutination tests

Agglutination tests were carried out on microscope slides, using a slight modification of the method used by Cunningham and Vickerman (1962). Clean siliconed slides were placed in a humid chamber and a drop from each of the serial twofold dilutions ( $1/10 - 1/40,960$ ) of the immune plasma added.

A capillary of stabilate material containing five trypanosome antigen was taken from the cold storage, both ends of the capillary broken off and the contents (undiluted) discharged into a Wasserman tube immersed in an ice bath. Using a very finely drawn pasteur pipette, a tiny drop of the stabilate was mixed with the serially diluted immune sera, starting from the highest and proceeding to the lowest dilution. A drop of the antigen was added to the control normal serum. The slides were incubated in a moist chamber for 40 minutes. The test was read by examining each dilution under microscope at 100x magnification. The last dilution which produced a

few definite clumping of trypanosomes was taken as the end point of the titration.

5. Preparation of rabbit anti-mouse lymphocyte serum (RALMS)

- a) Preparation of thymocyte suspension. The thymus from young mice (3 weeks old) was chosen as the source of antigen (lymphocytes). 3 weeks old T0 mice were killed by dislocation of the neck and the sternal region opened. The thymus was totally removed and suspended in Gey's gelatine solution. Traces of blood was removed from the thymus by washing them in several changes of Gey's solution. The thymocytes were teased out by crushing the thymus over a stainless steel wire mesh 60 gauge. The cells were washed twice in the solution each time spinning the resuspended cells for 5 minutes at 600 g. The final cellular deposit was made up to 10 ml by adding Gey's solution.
- b) Immunization of rabbit. A 5.5 kg rabbit was injected (IV) through the ear-vein with approximately antilog 9 thymocytes suspended in 10 ml Gey's solution. After two weeks, the rabbit was given a booster inoculum, same as the immunizing dose.
- c) Harvesting rabbit antimouse lymphocytic serum. One week after the booster dose, the rabbit was bled from one of the ear lobe veins, using 20 ml plastic syringe. Blood collected was dispensed into 10 ml graduated centrifuge tubes in which the samples were allowed to clot at room temperature. After three hours the tubes were transferred to 4°C where they were kept overnight. The following day the clotted blood was centrifuged and serum collected heated to 56°C for 30 minutes to remove complement. The serum was stored at -20°C. Two days later (when the rabbit was anaemic) the

rabbit was bled totally from the heart and serum collected in the same way as described above.

d) Absorption of serum. 50 five-week old T0 mice were bled from the heart, heparin being used as anticoagulant. Blood collected was pooled and centrifuged for 5 minutes at 600 g. The plasma was discarded and the red blood cells washed twice in normal saline (sterile) by centrifugation and resuspension in the same medium, during each washing the cells were spun at 600 g. The supernate was discarded and cells transferred to 250 ml conical flask. The rabbit serum was then added to the RBC and after one hour the mixture was centrifuged and the agglutinated RBC discarded. The clear serum obtained was stored at  $-20^{\circ}\text{C}$  ready for use.

## 6. Detection of antibody producing cells

Method. The method first described by Jerne, Nordin and Henry (1964) and later modified by Dresser and Wortis (1967) was employed in this investigation.

The reagents used were those recommended by Dresser and Wortis (1967), except that the concentration of Dulbecco's PBS, solution A and Gey's solution A was 5x concentrated (Whitmore, personal communication).

a) Immunization of mice. Sheep red blood cells (SRBC) (Wellcome) used as antigen to immunize mice was prepared as follows: 5 ml (SRBC) in Alsever's solution was centrifuged at 1750 g for 5 minutes and the supernate discarded. The sediment was suspended in Gey's solution and centrifuged for a further 5 minutes. The supernate was once more discarded and the sediment resuspended in 10 ml of Gey's solution. A single (IP) injection of 0.2 ml of the



red blood cell (RBC) suspension was used to immunize each mouse. This volume of cell suspension was estimated to contain about  $5 \times 10^8$  SRBC/ml.

b) Preparation of agarose plates for suspending RBC and spleen cells. Two layers of agarose gel were prepared. A bottom layer of the gel was first prepared to provide smooth non-toxic level surface on which a top layer containing the lymphoid cells and the indicator RBC was evenly spread.

The bottom layer was made of 1.2% w/v agarose (L'Industrie Biologique Gennevilliers Seine France) in Dulbecco's PBS. For 1000 ml quantities of agarose solution, 12 gms agarose was suspended in 720 ml distilled water and dissolved by boiling. The volume of water lost by evaporation was adjusted by adding an equivalent amount of distilled water. The solution was then cooled in a water-bath maintained at 46-48°C. Dulbecco's solutions A, B and C were added in that order to the agarose solution with constant stirring. Using a graduated 25 ml pipette 5.0 ml quantities of the agarose solution was dispensed into each sterile polystyrene petri dish, spread and allowed to set on a level platform after solidification. The petri dishes were stored upside down in polythene bags at 4°C until needed for use.

The top layer was prepared as follows: For 100 ml agarose solution, 0.6 gm agarose was weighed accurately and then suspended in 70 ml distilled water. The suspension was boiled while stirring constantly using a magnetic stirrer. The solution was cooled in water bath and then 20 ml Gey's gelatine solution A was added. 5 ml quantities of solution B and C were then added to bring the total volume to 100 ml. The pH of the mixture was brought to 7.2 - 7.6 by blowing in CO<sub>2</sub> into the bottle containing the mixture. 2 ml

portions of the solution were then dispensed into Wasserman tubes set in a rack in the water bath.

c) Preparation of lymphoid cell suspension. Mice were killed by dislocation of the neck and spleens removed and put in a weighed amount of Gey's solution maintained in ice bath. The spleens and the solution were weighed and the difference in weight was recorded as the spleen weight. Using forceps the cell contents of each spleen were teased out into the Gey's solution. The lymphoid cell suspension was then washed by adding 4 ml Gey's solution and centrifuged at 600 g for 5 minutes. The supernatant was removed and solution added to the desired dilution. The lymphoid cells added to each plate was contained in 0.1 ml of the suspension.

d) Sheep red blood cells (SRBC). Sheep red blood cells in Alsever's solution (Wellcome) was prepared from 20% suspension packed cell volume (PCV), 1750 g for 5 minutes. The sediment was washed twice in Gey's solution and finally made up as 20% suspension in Gey's solution.

e) Plaque tests. Petri dishes containing the bottom agarose layer previously stored at 4°C were brought to room temperature two hours before the test began. For each test 0.1 ml of the lymphoid cell suspension was added to the 2 ml agarose contained in Wasserman tubes set in a rack in a water bath. 0.1 ml SRBC was added and the mixture shaken before pouring it over the bottom layer set on a level platform, so that a thin layer of lymphoid cells spread evenly among the sheet of red cells as the agarose solidified. After the agarose had set the plates were incubated at 37°C for 2 hours after which 1 ml of freshly diluted (10%) complement in the form of guinea pig serum (Wellcome) was added to the plates and spread uniformly over the surface by gentle shaking. The plates were incubated

for a further 45 minutes at 37°C. The plaques formed were counted under a dissecting microscope.

## 7. Skin grafting

The technique of skin grafting in mammals has been described by Billingham and Medawar (1951). The grafting technique employed in this experiment is a much simpler one and needs very little special apparatus, and aseptic conditions are not critical.

Donor and recipient mice were chosen to be of different strains convenient for identification of different coat colours. CBA and T0 strains were therefore used for the test. One adult CBA mouse was deeply anaesthetized so that it remained immobilized for a considerably longer time than the recipient mice. The recipient mouse was also anaesthetized. Using a new razor blade 6 pieces of skin measuring approximately 0.25 cm x 0.5 cm were sliced off from the tail of the donor mouse and 2 from every recipient mouse. Fig. 4 illustrates the technique. One piece of skin from the recipient mouse was discarded and the other piece placed on the spot where the discarded piece had been removed from. In its place was put one piece of skin from the donor mouse. A glass tube measuring about 2.5 cm in length and 0.5 cm diameter was then passed down the recipient mouse-tail to cover the grafts. The tail was then clipped with one <sup>"Michel"</sup>~~"mitchell"~~ clip to keep the glass tubing in position as shown in Fig. 4 so that the mouse could not reach the grafted skin. The tube was removed seven days post grafting.



## F. Entomological techniques

### 1. Maintenance of tsetse flies

Pupae of Glossina austeni Newstead and Glossina morsitans Westwood were obtained from the Tsetse Research Laboratories at Langford, Bristol, England. The pupae and the adult flies which emerged from them were maintained at a relative humidity of 60-90% with an average of 80%. The temperature of the insectary was regulated within  $\pm 1^{\circ}\text{C}$  to  $25^{\circ}\text{C}$  by electric heaters. The jars of pupae and the cages of adult flies were kept on benches about one meter above the floor. Care was taken to protect both pupae and the adult flies from ants. The insectary was lighted for 12 hours daily by fluorescent bulbs as recommended by de Azevedo and Pinhao (1964). Pupae jars were made of perspex measuring 7.6 cm x 6.0 cm and open at both ends. The bottom of the jar was closed with a piece of fine bolting silk which was secured with a strip of sticking plaster. The top was covered with black terylene netting held in place with rubber bands. The bottom of the jar was covered with dry fine sand and the pupae were placed on top of the sand. The jars were kept in enamel basin containing moist sand. About 100 pupae were placed in one jar.

Adult flies. The emerging flies were transferred to a "Geigy" type cages (Geigy, 1948), made by fitting a mesh of black terylene netting sleeve over a frame measuring 14.3 cm x 8.5 cm x 4.5 cm inside dimensions, made from a stainless steel. The open end of the terylene netting sleeves was tied and the cage rested on a support in the basin. About 15 flies were kept in one cage (Willett, 1953). Dirty cages were washed in hot water, rinsed

and dried before use, and in any case the cages were changed once a week.

## 2. Exposure of Glossina to infection

Newly emerged flies were encouraged to feed as soon as they were strong enough to feed within 24 hours. Infected mice were narcotized with "Nembutal" and placed on the Geigy cages containing newly emerged flies (Fig. 5). The mice were left lying in this position as long as they were under sedation to allow as many flies as possible to feed. The same mice were anaesthetized 24 hours later, and the flies that failed to feed the previous day given a second attempt.

## 3. Maintenance of Glossina exposed to infected feed

All flies that fed on the infected animals were subsequently fed on clean anaesthetized mice on alternate days starting from the third day of infecting feed. After every second feed the mice were changed.

## 4. Examination of flies exposed to infection

After 26 days of infected feed, batches of flies surviving were dissected using the method described by Buxton (1955). Flies that died after 10 days after infected feed were also dissected.

## 5. Examination of animals used to feed infected flies

Tail blood of mice used to maintain the infected flies were examined twice a week for one month.

## V. RESULTS

### A. Observations on the effects of ambient temperature on course of trypanosome pathogenicity

#### 1. Influence of ambient temperature on the course of salivarian trypanosome infections in mice

This investigation was designed solely to see whether the ambient temperature of 4°C, 22-27°C and 35°C had any effect on the pathogenicity of some four species and strain of salivarian trypanosomes in mice. A total of five separate experiments were carried out, one for each species or strain examined as shown below:

30 mice were inoculated with T.(T.) brucei stabilate LUMP 36, and 18 inoculated with T.(T.) b. brucei LUMP 43.

18 mice were inoculated with T.(D.) vivax stabilate LUMP 45.

18 mice were inoculated with T.(N.) congolense stabilate LUMP 52.

18 mice were inoculated with T.(T.) evansi stabilate LUMP 55.

Mice were inoculated with standard doses of the inoculum to be tested (Table 1) and separated into three equal groups of 10 or 6 mice. One group was transferred to refrigerator maintained at 4°C, the other group was kept as control in the animal house (22-27°C) and the last group was transferred to an incubator maintained at 35°C. The mice were examined daily for trypanosome infection.

The results of the pathogenic effects of these trypanosomes in mice kept at the respective environments are summarized in Table 1. It may be seen from the table that mortality of infected mice was influenced by the environments in which they were kept.



T. (D.) vivax

Mice maintained at 4°C and 22-27°C developed acute infections and all died during the first peak of infection. The mean survival time (MST) of mice in both environments was found to be  $4.3 \pm 0.21$  days. It was therefore concluded that, lowering the ambient temperature of these mice did not alter the course of T. (D.) vivax infection.

Mice maintained at 35°C became parasitaemic but the course of infection was significantly altered. Five mice had very scanty infection which was detected during the first two days only after inoculation. But in one mouse, the infection lasted for two weeks, most of the time being subpatent. Thereafter no trypanosomes were detected for the rest of the experimental period. All the six mice were removed from the incubator 30 days after inoculation and returned to the animal house (22-27°C) where they were examined twice a week for three weeks. No relapses occurred. It appears therefore that the maintenance of infected mice at 35°C had cured these mice of their infection.

T. (N.) congolense

Mice maintained at 4°C developed acute infection and all died after a mean survival time of  $10.1 \pm 0.16$  days.

Mice maintained at 22-27°C similarly developed acute infection. They died after a mean survival time of  $10.5 \pm 0.22$  days. The mean survival time was found to be significantly longer in this group than in mice kept at 4°C. ( $t = 2.44$ ;  $P < 0.05$ ).

Two among the six mice maintained at 35°C failed completely to develop parasitaemia. The other four became infected, and two of them died from acute infection 13 and 25 days respectively after inoculation.

The remainder showed chronic infection until they were transferred to 22-27°C, 25 days following inoculation. When they were returned to 22-27°C, infection became acute and both died three days after transfer to 22-27°C (Fig. 6). Figure 6 shows the course of infection in the two mice which survived beyond 25 days after inoculation. The courses of infection in the two mice behaved remarkably similar except for the period between days 10 and 16. Although there was no clear cut periodicity in parasitaemias, two infection peaks were observed on days 10 and 18.

The results of keeping T.(N.) congolense infected mice at these ambient temperatures show that lowering the ambient temperature enhanced the infection and raising the ambient temperature decreased it.

T.(T.) brucei LUMP 36 (Table 1)

This strain was highly virulent to mice under normal conditions.

Mice kept at 4°C developed fulminating parasitaemias and died within  $4.4 \pm 0.16$  days after inoculation.

Mice kept at 22-27°C similarly died within  $4.5 \pm 0.16$  days after inoculation. The mean survival times of mice in the two groups (4°C and 22-27°C) were found not to be significantly different ( $t = 0.84$ ;  $P > 0.05$ ).

A chronic and a relapsing infection resulted in mice maintained at 35°C. Figure 7 illustrates the course of infection in four mice among the ten inoculated. The four curves illustrate the courses of infections in two mice which died before and two mice which survived beyond 28 days after inoculation. It may be seen from the figure that there was a definite periodicity in parasitaemia in the four mice. Three peaks came out quite clearly; these occurred between

days 4-7; 13-17 and two animals died during the third peak, between days 22 and 24.

In general the mean survival time for the ten mice was found to be  $28.8 \pm 3.82$  days. The shortest survival time was 12 days, the longest 49 days. It is concluded therefore that high ambient temperature ( $35^{\circ}\text{C}$ ) had materially aided these mice to withstand better the pathogenic effects of this strain of trypanosomes.

T.(T.) b. brucei LUMP 43

This was a less virulent strain; it caused a chronic infection in mice under normal laboratory conditions.

Mice inoculated with this strain and kept at  $4^{\circ}\text{C}$  became parasitaemic and developed chronic infection (Fig. 8). They had a mean survival time (MST) of  $31.1 \pm 2.78$  days.

Mice kept at  $22-27^{\circ}\text{C}$  also showed chronic infection. They survived longer (MST =  $35.6 \pm 4.20$  days) than mice kept at  $4^{\circ}\text{C}$ . These survival times are, however, not statistically significant ( $t = 0.89$ ;  $P > 0.05$ ).

At  $35^{\circ}\text{C}$ , the mice had a much longer mean survival time of  $39.1 \pm 2.16$  days. This MST compared with that observed for mice kept at  $4^{\circ}\text{C}$  was found to be significantly different ( $t = 2.26$ ;  $P < 0.05$ ) and when compared with that at  $22-27^{\circ}\text{C}$ , the differences were not significant ( $t = 0.74$ ;  $P > 0.05$ ).

Figure 8 shows the intensity of parasitaemias in mice kept at the respective ambient temperatures. The depression of parasitaemias which took place in all groups of mice between days 7 and 11, was much more marked in the  $35^{\circ}\text{C}$  group than in the other groups. On the whole, they had a less intense parasitaemia compared to mice kept at  $4^{\circ}\text{C}$  or at  $22-27^{\circ}\text{C}$ . The parasitaemic mean differences were significant



in both cases. ( $t = 3.31$  for the  $4^{\circ}\text{C}$  and  $3.10$  for the  $22-27^{\circ}\text{C}$  group;  $P < 0.05$ ). These observations suggest that the  $35^{\circ}\text{C}$  ambient temperature had definitely interfered with the proliferation of *T.(T.) b. brucei* and rendered it less pathogenic.

#### *T.(T.) evansi*

Mice kept at  $4^{\circ}\text{C}$  and at  $22-27^{\circ}\text{C}$  developed acute infection and died after  $9.0 \pm 0.68$  and  $7.8 \pm 0.54$  days respectively. The MSTs of these groups of mice were not significantly different ( $t = 1.34$ ;  $P > 0.05$ ).

Mice kept at  $35^{\circ}\text{C}$  failed completely to become parasitaemic. They were removed from the environment 20 days after inoculation and transferred to  $22-27^{\circ}\text{C}$ . They were examined for a further ten days. No trypanosomes were seen and the animals were therefore used for the challenge experiments described in section V.A.9.

## 2. Effect of alteration of ambient temperature on the course of *T.(T.) brucei* in mice

Having observed that exposure of mice to an ambient temperature of  $35^{\circ}\text{C}$  reduced the pathogenicity of salivarian trypanosome infections to mice, it was interesting to see if this effect was a permanent one and if return of the experimental animals to lower temperatures would terminate the effect.

18 mice were inoculated each with antilog 2.8 ID<sub>63</sub> stabilate, LUMP 36. 6 mice were kept as controls at  $22-27^{\circ}\text{C}$  and 12 mice were maintained at  $35^{\circ}\text{C}$ . On day 15, 6 of the 12 mice being kept at  $35^{\circ}\text{C}$  were returned to an ambient temperature of  $22-27^{\circ}\text{C}$ .

All the controls died with high parasitaemias on day 4. The courses of the infections in the other experimental animals kept at 35°C are summarized in Table 2. Mice transferred from 35°C to 22-27°C developed high parasitaemias and succumbed to infection after three or four days afterwards. But mice detained at 35°C continued to show chronic infections until they were removed on day 21. The experiment was stopped, and the animals destroyed. It was concluded that the change in pathogenicity was a direct temperature effect and was only effective as long as the mice were detained at 35°C. The effect was reversed when mice were withdrawn from this environment.

3. Influence of ambient temperature on body temperature of <sup>mice</sup> uninfected/and mice infected with *T.(T.) brucei*

It has been shown that the course of the infection of several trypanosome materials in mice is altered by exposure of hosts to 35°C as compared with 22-27°C ambient temperature. It was thought that this effect might be related to consequent alterations of the body temperature of mice. It was therefore decided to study the body temperature of mice, both uninfected and infected maintained at various ambient temperatures (28°C, 30°C and 35°C).

Four groups of 12 mice were set up and maintained respectively at 28°C, 30°C, 32°C and 35°C. From each group of 12, 6 were uninfected and 6 were inoculated with antilog 1.8 ID<sub>63</sub> *T.(T.) brucei* stabilate LUMP 36. The body temperature of the mice were recorded before the animals were transferred to their respective test conditions. The mean body temperature of all 48 mice was found to be  $37.54 \pm 0.05^\circ\text{C}$ .

A daily body temperature record of each animal as well as parasitaemia was kept.

Mean body temperature. No significant differences were found between mean body temperatures of infected and uninfected mice at various ambient temperatures (Table 3a). Table 3b shows a comparison of mean body temperatures of groups of T.(T.) brucei infected mice and maintained at 28°, 30°, 32° and 35°C ambient temperatures.

No significant differences were observed between mean body temperatures of mice kept at 28° and at 30°C, but at 32°C there was a significant elevation of mean body temperature (38.03°C). At 35°C the mean body temperature of mice was very much elevated, 39.34°C. This increase was highly significant compared to mean body temperatures of mice kept at 28°, 30° or 32°C environments.

Table 3c compares the mean body temperatures of non-infected mice similarly kept at 28°, 30°, 32° and 35°C. There was little difference in mean body temperatures of mice kept at 28° and at 30°C, neither was there much difference in the mean body temperatures between mice kept at 28° and at 32°C. But mice kept at 35°C had significantly higher mean body temperatures than mice kept at other temperatures, namely 28°, 30° and at 32°C. Figure 9 compares the mean body temperatures of uninfected mice kept at 28° and at 35°C respectively. It may be seen from the figure that, the mean body temperatures of mice kept at 35°C was constantly higher than that observed for mice kept at 28°C. It is therefore concluded that by maintaining mice at 35°C, an effect amounting to pyrexia was induced in the mice.

Parasitaemia. The mortality rates of infected mice kept at 28°, 30°, 32° and 35°C and whose body temperatures were studied above are recorded in Table 4. It may be seen from the table that the courses



of infections were not changed when mice were kept at 28° or 30°C. Mice in both groups died within five days after inoculation.

Mice kept at 32°C survived much longer (9.40 days) than mice kept at 28° or at 30°C. This increase in MST was highly significant.

At 35°C all except one mouse survived beyond the 16 days before the infected animals were transferred to 22-27°C. The experiment was stopped after 16 days when mice kept at 28°, 30° and 32°C had all died. It appears from these experiments that the change in pathogenicity of T.(T.) brucei LUMP 36 to mice took place at about 32°C ambient temperature. At this environmental temperature, both the mean body temperature and the mean survival time of infected mice were significantly raised or prolonged. It was therefore interesting to see whether mice infected with other species of trypanosomes could be similarly affected when kept at 32°C. The following species of trypanosomes were used to inoculate groups of mice:

12 mice were inoculated with antilog 1.4 ID<sub>63</sub> T.(D.) vivax stabilate LUMP 45

12 mice were inoculated with 0.1 ml of 2 log dilution of T.(N.) congolense suspension (ID<sub>63</sub> not known).

12 mice were inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) evansi stabilate, LUMP 55.

The inoculated mice were divided into two groups of 6. Half the inoculated mice were transferred to 32°C and the rest kept at 28°C. The animals were examined daily for trypanosome infection. The results of the mean survival times of these mice are given in Table 5. The results obtained for T.(T.) brucei, LUMP 36 described in the previous experiment have been included for comparison. It will be

seen from the table that the mean survival times of groups of mice kept at 32°C was greater than that of mice kept at 28°C.

T.(D.) vivax. All mice inoculated with T.(D.) vivax became parasitaemic. Mice kept at 28°C had a mean survival time of 5.50 days, whereas mice kept at 32°C lived for 9.33 days, almost twice as long.

T.(N.) congolense. All mice kept at 28°C became parasitaemic whereas only three out of six kept at 32°C developed parasitaemias. All infected mice, however, succumbed to infection. Mice kept at 32°C lived almost twice as long (18.50 days) as compared to 10.66 for mice kept at 28°C.

T.(T.) evansi. All mice inoculated became parasitaemic. Mice kept at 28°C died after 9.75 days, but only one mouse among the six kept at 32°C developed acute infection and died after 21 days. The other five showed mild chronic infections throughout the observation period. They were transferred to 22-27°C after 25 days and then destroyed.

The results confirm the earlier observation that by maintaining infected mice at 32°C ambient temperature, the mean survival time of infected animals is significantly prolonged as compared to mice kept at 28°C.

#### 4. Morphology of trypanosomes in mice kept at 35°C

T.(T.) brucei. The morphology of T.(T.) brucei LUMP 36 in mice kept at 22-27°C was typically monomorphic (Fig. 10a); composed almost entirely of long forms. 100 randomly chosen organisms had a mean length of  $24.78 \pm 0.51$   $\mu\text{m}$ , ranging from 20.4 to 32.7  $\mu\text{m}$ . The infection



in mice kept at  $35^{\circ}\text{C}$  on the other hand, although predominantly of long forms, also contained short forms (Fig. 10b), having a mean length of  $16.27 \pm 0.48 \text{ }\mu\text{m}$ , in proportion as high as 35% on day 7 after inoculation.

T.(N.) congolense. The infection following inoculation of T.(N.) congolense in mice kept at  $22\text{--}27^{\circ}\text{C}$  was similarly monomorphic, composed exclusively of small thin forms (Fig. 11), a sample of 100 organisms measured  $14.51 \pm 0.16 \text{ }\mu\text{m}$ . At  $35^{\circ}\text{C}$ , besides the small thin forms, two forms (Figs 12ab) measuring  $9.4 \pm 0.34$  and  $11.7 \pm 0.16 \text{ }\mu\text{m}$  appeared. Stumpy forms were first observed on day 8 (Table 6) and appeared in various shapes (Figs 13a, b, c, d, e).

##### 5. Infectivity of different inocula of T.(T.) brucei to mice maintained at $35^{\circ}\text{C}$

It has been noted (section V.A.1) that the course of T.(T.) brucei LUMP 36 infection in mice is modified from a virulent and acute type to a chronic and relapsing one, when mice are maintained at  $35^{\circ}\text{C}$ . It was felt necessary to test whether the infectivity of the same strain of trypanosomes was equally influenced by keeping inoculated animals in this environment. An experiment was therefore set up to test this hypothesis.

Two groups of 24 mice, were inoculated with two different dilutions of LUMP 36 stabilate. Mice in the first group were inoculated with antilog 2.8  $\text{ID}_{63}$ , and those in the second group were inoculated with antilog 0.8  $\text{ID}_{63}$ . Half of the mice in each group were maintained at  $35^{\circ}\text{C}$  and the remainder at room temperature ( $22\text{--}27^{\circ}\text{C}$ ). The animals were examined daily for trypanosome infection.



The resulting infections in both groups of mice maintained at 22-27°C was acute and the animals died 4 or 5 days after inoculation. The effect of giving different dilutions was therefore not apparent.

The course of infection in mice kept at 35°C is shown in Table 7. It may be seen from the table that there was no difference in the infectivity of the inocula to mice whether they were maintained at 22-27°C or at 35°C. All were infected at both inoculum levels as is evidenced by their being parasitaemic by day 9 or earlier. There was, however, wide differences in the degree of parasitaemia. All the mice receiving antilog 2.8 ID<sub>63</sub> were highly parasitaemic at some time or other during days one to five after inoculation and thereafter developed chronic infections. Mice receiving antilog 0.8 ID<sub>63</sub> showed scanty parasitaemia, usually only on single days up to day 9, and thereafter the infection became subpatent.

Both groups were transferred to 22-27°C 17 days after inoculation and blood from all non-parasitaemic mice were sub-inoculated into previously uninfected mice. Soon after removal from 35°C to 22-27°C, mice with patent infections developed acute parasitaemias and died within two or three days after transfer to 22-27°C. But it took some three days for two non-parasitaemic mice to show patent infection and both succumbed to infection after four days.

Subinoculations. It was only the mice that received the smaller inoculum that showed subpatent infection. Tail blood from the six mice was subinoculated into 6 clean mice. Two of the recipients showed infections after four days. Blood from the two donor mice that had showed a relapse, was found to be infective to their respective recipients.

It was therefore concluded that the infectivity of the two inocula was not influenced by keeping inoculated mice at 35°C, but the courses

of infection were vastly different.

6. The effect of ambient temperature on different hosts responses to infection with *T. (T.) brucei*

To test the possibility that different strains of mice under heat stress might react differently to trypanosome infection, four pure lines (strains) of mice (T0, Parkes, CF<sub>1</sub> and C<sub>3</sub>H) were chosen for this experiment.

20 mice of each strain i.e. Parkes, CF<sub>1</sub> and C<sub>3</sub>H and 12 T0 were inoculated with antilog 1.8 ID<sub>63</sub> stabilate LUMP 36. Half of the mice in each group were kept at 35°C and the remainder at 22-27°C. The animals were examined daily for trypanosome infection. Table 8 summarizes mortalities of the different strains of mice.

It was found that all animals kept at 22-27°C died with heavy parasitaemias after 4 to 5 days.

At 35°C environment (Table 8)

T0 strain. All mice became parasitaemic and developed chronic infection. Three died after 17, 18 and 19 days respectively after inoculation. The other three survived for more than 28 days and were destroyed at the end of the experiment.

Parkes strain. There was very high mortality in mice kept at 35°C. Seven out of ten mice inoculated died before becoming parasitaemic, during the first two days after inoculation. The cause of death was most probably heat exhaustion. The other three became parasitaemic 4 days after inoculation and one died from high parasitaemia 12 days later. The remainder (2 mice) had chronic infection for more than 28 days. They were destroyed at the end of the experiment.



CF<sub>1</sub> strain. All the ten inoculated mice became parasitaemic and 8 of them died within  $15.0 \pm 3.74$  days after inoculation. Two survived beyond 28 days.

C<sub>3</sub>H strain. All the ten C<sub>3</sub>H strain mice inoculated developed parasitaemias and died (MST =  $13.80 \pm 1.52$  days) before the experiment was stopped.

At the end of the experiment, 28 days after inoculation, all the C<sub>3</sub>H mice had died whereas 3/6 T0, 2/10 Parkes and 2/10 CF<sub>1</sub> were still surviving. It seems clear from these results that there was no host strain differences in response to infection with T.(T.) brucei LUMP 36, even after exposure to high ambient temperature. The mortalities of Parkes strain of mice when kept at 35°C indicates that the strain was less readily adaptable to hot environment.

#### 7. Influence of high ambient temperature on mouse body weight

Having observed that mice kept at 35°C had raised body temperature it was decided to see whether such mice would grow normally. 24 mice, 4-6 weeks old were randomly separated into 4 equal groups of 6, and the weights of individual mice noted. Two groups were then inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 128. One group of infected and another group of uninfected mice were transferred to 35°C. The other groups were kept at 22-27°C. Mice were examined for trypanosome infection daily for the first four days and thereafter the surviving mice were examined at two or three days intervals. The body weights of all mice were recorded on days 2, 5, 8, 11, 15, 17, 20 and 24, by which time infected mice kept at 35°C had all died.

The results of body weight records are shown in Figure 14. It may be seen from the figure that uninfected mice kept at 22-27°C



grew normally and steadily gained weight. Infected mice kept at this ambient temperature died with heavy infection after four days. Their body weights were therefore recorded on day 2 only.

Infected mice kept at  $35^{\circ}\text{C}$  suffered a big weight loss (8-9%) during the first five days, but thereafter they steadily gained weight, albeit at a much slower pace than uninfected mice.

Uninfected mice similarly lost weight between day 5 and 11 but gained weight thereafter at a much more rapid rate.

The differences between mean body weights of uninfected mice kept at  $22-27^{\circ}\text{C}$  and at  $35^{\circ}\text{C}$  were found to be significant ( $t = 2.58$ ;  $P < 0.05$ ). In spite of these differences, it seems that once the mice have acclimatized to  $35^{\circ}\text{C}$ , they were able to grow normally, although they took sometime to recover the initial weight loss.

#### 8. Adaptation of *T.(T.) brucei* to mice maintained at $35^{\circ}\text{C}$

The observation that at  $35^{\circ}\text{C}$ , the pathogenicity of trypanosomes tested in section V.A.1 was modified made it necessary to try adapting one of the species studied, *T.(T.) brucei* to this environment. The reasoning behind this was that, by maintaining infection in mice constantly kept at  $35^{\circ}\text{C}$ , the trypanosomes would probably lose their virulence completely and thus behave more like the original strain in the larger vertebrate hosts (ungulates).

6 mice were previously maintained at  $35^{\circ}\text{C}$  for two weeks and then inoculated with antilog 3.1 ID<sub>63</sub> *T.(T.) brucei* stabilate LUMP 128. The mice were maintained at  $35^{\circ}\text{C}$  and examined for trypanosome infections once a week. When mice had started dying from chronic trypanosome infection, the surviving mice were examined every two days. And when only two infected animals remained, another 4 mice previously

kept at 22-27°C were transferred to 35°C. These mice were kept in this environment for at least 48 hours before they were subinoculated with infected blood from one of the two surviving infected mice. In this way the trypanosomes were maintained in animals adapted to high ambient temperature. The passages were carried out as shown below:

The 1st passage was carried out after 23 days

The 2nd passage was carried out after 28 days

The 3rd passage was carried out after 10 days

The 4th passage was carried out after 23 days

The 5th passage was carried out after 21 days

The 6th passage was carried out after 12 days

The 7th passage was lost after 21 days' infection

A stabilate was prepared on day 7 of the seventh passage. On examination of the frequency of the passages, it was found that the pathogenicity of this strain of trypanosomes was all the time changing. The 1st, 2nd, 4th and 5th passages were carried out after at least 20 days' infection; the seventh passage was lost after 21 days infection. But the 3rd and the 6th passages were carried out after a short period of infection, 10 and 12 days respectively. It is hard to explain these differences which seem to be occurring at regular intervals, unless it is assumed that the different variants which presumably occur during the course of infection, have different pathogenic effects to mice.

Infectivity test. The infectivity of the stabilate prepared during the seventh passage was tested by calculating the ID<sub>63</sub>. This was found to be antilog  $3.6 \pm 0.5$  ID<sub>63</sub> per ml mouse blood, and the trypanosome count was found to be antilog 7.99 organisms per ml. The infective dose estimate is derived from the titration shown in Table 9.



The infective dose of the original infecting stabilate LUMP 128 was estimated to be antilog  $6.1 \pm 0.6$  ID<sub>63</sub> per ml; and the trypanosome count was found to be antilog 8.92 organisms per ml. Table 9 shows the survival times of groups of mice inoculated with different dose levels of the inoculum used during titration. It was found that so long as the trypanosomes were infective, the pathogenicity of the strain was not altered. All parasitaemic mice died within five days and no infection was detected 6 days post inoculation. The animals were destroyed after 30 days of observation. It is therefore concluded that the maintenance of infection in mice kept at 35°C had not influenced the pathogenicity of the strain to mice kept at 22-27°C. The infectivity, however, was modified.

9. Effect of high ambient temperature on *T.(T.) evansi* infected mice (Table 10)

Because mice infected with *T.(T.) evansi* LUMP 55 and maintained at 35°C failed to become parasitaemic (see Table 1) it was decided to check whether such mice were protected against a challenge from the same stabilate. Mice infected with *T.(T.) evansi* used in experiment described in section V.A.1. were removed from the 35°C environment to 22-27°C 20 days after infection. They were examined for a further 10 days for possible parasitaemias. No trypanosomes were observed during this period. They were then challenged with the same stabilate LUMP 55; they were inoculated with antilog 2.1 ID<sub>63</sub>. A control group of six mice of similar age (9-11 weeks old) were also inoculated with the same inoculum. The animals were examined daily for trypanosome infection. The results are shown in Table 10. All of the control group became parasitaemic within two days and died in less than 8 days. Three of the six challenged mice had high



parasitaemias and died 7, 11 and 19 days respectively after challenge. One mouse showed a transient infection 10 days after challenge and trypanosomes were detected for only 8 days. The other two failed completely to become parasitaemic.

These results indicate that the challenged mice had acquired some protection. This means therefore that the disappearance of parasitaemias as a result of exposing infected mice to 35°C environment was due largely to destruction of trypanosomes.

10. Influence of alterations between 22-27°C and 35°C environments on the course of the infection with *T.(T.) evansi* (Table 11)

18 mice were inoculated with antilog 2.1 ID<sub>63</sub> *T.(T.) evansi* stabilate LUMP 55 and divided into three groups of 6; groups A, B and C. Group A and B were kept at 22-27°C and Group C at 35°C and examined daily for the presence of parasitaemia. On day 4 the Groups B and C were interchanged between the two environments and on day 20 Group B was returned to 22-27°C environment.

Group A. This group was maintained throughout at 22-27°C. All the mice developed heavy infection and died between day 8 and 13 after inoculation (MST = 10.50 ± 1.2 days).

The courses of parasitaemias in the other groups, B and C are shown in Table 11. The periods the two groups spent at each temperature is also shown in the Table.

Group B. Mice kept at 22-27°C were all parasitaemic by day 4 when they were transferred to 35°C environment. The parasitaemias soon disappeared when they were maintained at 35°C. They failed to

show any parasitaemias as long as they were kept in this environment. They were returned to 22-27°C 20 days after inoculation. Infection relapsed in one animal and it died with heavy parasitaemia 33 days after infection. The other five were not parasitaemic throughout the observation period.

Group C. None of the six animals showed parasitaemias when they were kept at 35°C environment for the four days. And when they were transferred to 22-27°C, three out of six became parasitaemic, two succumbed to infection on days 22 and 24 respectively. One developed chronic infection. The other three failed to show parasitaemia throughout the observation period.

At the end of the experiment 70 days later, blood from all the non-parasitaemic mice was sub-inoculated into clean mice previously uninfected. A total of 8 mice were thus sub-inoculated with 0.1 ml of blood from each of the non-parasitaemic mice. No trypanosomes were detected in <sup>either</sup> both the recipient <sup>or</sup> and the donor mice when they were examined for a further 15 days. The experiment was stopped 85 days after inoculation, or rather, 15 days after sub-inoculations.

It appears that the brief period of interchange had interfered with the normal course of T.(T.) evansi infection in these mice. It should have been interesting to check whether there was any difference between the amount of circulating antibodies in parasitaemic and non-parasitaemic mice, for example, on day 20.

B. Attempts to find tissue phase of trypanosome infections  
in mice kept at 35°C

The disappearance of T.(T.) evansi infection when infected mice



were transferred from 22-27°C to 35°C environment and the re-appearance of parasitaemias when the mice were returned to 22-27°C indicated strongly that the trypanosomes either hid somewhere in the internal organs, or they survived in blood in such small numbers that they could not be detected by the conventional methods.

Besides, in one case of T.(T.) brucei LUMP 36 infections described in section V.A.1 a mouse was found to have a prepatent period of 21 days. This was further evidence for suggesting the possibility of existence of a phase of development outside the blood circulation. This possibility was examined as follows:

T.(T.) brucei

6 mice at a time were inoculated with antilog 1.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 36 and kept at 35°C. During remissions which occurred usually between day 6-9 after inoculation, animals which were found to be non-parasitaemic were killed and impression smears from the liver heart, lung, brain, spleen and kidneys prepared and examined. Histological sections of these tissues were also prepared and examined. A total of 3 mice were thus examined.

Histological observations. Examinations of preparations of random serial sections revealed no trypanosomes inside any of the tissues examined. Occasional trypomastigote forms were seen in brain blood vessel (Fig. 16).

Impression smears. Examinations of impression smears from the same tissues similarly failed to show trypanosomes.

Some aberrant forms of trypanosomes, however, were seen in brain smears (Fig. 15a). There was no evidence to suggest that this specimen had been dislodged from the blood vessels. This form is possibly a sphaeromastigote. Ormerod and Venkatesan (1970), later



found similar forms in the choroid plexus of rats infected with T.(T.) brucei. Figure 15b shows another type of aberrant form seen in other smears of the same tissue. It will be noticed that in this case, the aberrant forms are in blood vessel. Some of these forms were seen in association with macrophages. This was the same tissue which revealed typical trypomastigotes in blood vessel shown in Fig. 16.

#### T.(T.) evansi

Attempts to find tissue phase of T.(T.) evansi infection in mice was done by first infection 6 mice with antilog 2.1 ID<sub>63</sub> T.(T.) evansi stabilate LUMP 55. The mice were then kept at 22-27°C to develop parasitaemias, and four days afterwards they were transferred to 35°C. Two days after transfer to 35°C environment, 0.1 ml whole blood from each of the six mice was subinoculated into 6 previously uninfected mice and the subinoculated mice kept at 22-27°C. This procedure was repeated five days later i.e. 7 days after transfer to 35°C. This time, four of the six mice kept at 35°C were killed and impression smears from the liver, heart, lung, spleen, brain, bone marrow and peritoneal exudate were prepared and examined. Histological sections of the same tissues were also prepared and examined.

The last two mice were removed from 35°C after 12 days and transferred to 22-27°C. Their blood was subinoculated into 6 mice. The two animals were examined for possible relapses for a period of 4 weeks.

Blood smears. Trypanosomes were seen in wet blood films prepared from the animals on day 4 when they were transferred to 35°C. But two days after transfer to 35°C, no trypanosomes were seen in all the

six mice. Later examinations revealed no trypanosome throughout the observation period.

Subinoculations. Subinoculations done on day two after the mice had been transferred to 35°C proved to be infective after two days prepatent period. All the recipients developed fulminating infection and died after 8-10 days. Subinoculations done five days later also proved to be infective. Four out of six mice showed parasitaemia after a prepatent period of 4 days. The four infected animals died between 8 and 11 days after inoculation.

Subinoculations done on the 12th day revealed that one of the two donor mice was still harbouring trypanosomes. Two out of six recipients showed parasitaemias 7 and 9 days after subinoculation. They died after a chronic infection lasting 21 and 28 days respectively. It was interesting to note that the infected mice were subinoculated with blood from the same one donor mouse. Infection relapsed in this donor mouse 4 days after being transferred to 22-27°C. The infection, however, was a transient one and lasted for only 8 days. Thereafter the animal remained non-parasitaemic until the experiment was stopped two months later. The animals were destroyed at the end of the experiment.

Histological observations. No trypanosomes were seen in any of the tissues examined.

Impression smears. Occasional trypomastigote forms were seen in heart blood in one of the six mice examined.

Peritoneal exudate. Amastigotes were seen in the peritoneal exudate smears (Figs 17a, b) in one animal. One trypomastigote form was also seen in the same preparation.

Apart from the occasional sphaeromastigote and amastigotes seen in the T.<sub>2</sub>(T.) brucei infected brain smear and the amastigotes in the



peritoneal exudate of T.(T.) evansi infection, it is hard to judge whether these forms constitute a real tissue form of these trypanosomes.

C. Attempts at cyclical transmission of T.(T.) brucei LUMP 36 in mice kept at 22-27°C and at 35°C.

Since the isolation of the original strain from bovine blood, this strain of T.(T.) brucei had not been transmitted cyclically. It was therefore necessary to see if it was still capable of being transmitted by Glossina sp. 10 mice were inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 36 and kept at 22-27°C. On day 3 and 4 when the mice were heavily parasitaemic, newly emerged (less than 24 hours old), Glossina sp. were fed on the infected mice. While it was intended to use both G. morsitans and G. austeni for this work, it became unavoidable to use G. morsitans only, because G. austeni hatched when infected mice had all died. The flies that had actually fed (confirmed by the bulging red stomach) were used for the experiment.

Flies which died before day 10 after infecting feed were discarded, but flies which died between day 11 and 24, were examined for trypanosome infections. All surviving flies after day 25 were killed and examined in batches over a period of 5 days.

The appearance of pleomorphic forms of T.(T.) brucei LUMP 36, when mice were kept at 35°C made it necessary to see whether it was possible to transmit this strain cyclically when maintained in mice kept at 35°C. The experiment was planned in such a way that the emergence of newly hatched flies coincided with remissions in parasitaemia. Remissions in parasitaemia usually occurred in mice



kept at 35°C on days 6, 7, 8 and 9 after inoculation.

Newly emerged G. morsitans and G. austeni were thus fed only on mice showing pleomorphic forms. The flies were examined in the manner described above. The results of these attempts are shown in Table 12.

Source of infection

22-27°C. 105 G. morsitans fed on infected mice kept at 22-27°C. Of these, 89 survived beyond day 10 and were therefore examined for trypanosome infection. 7.8% showed gut infection and none showed salivary gland infection.

35°C. 49 G. morsitans fed on infected mice kept at 35°C. 40 flies survived beyond day 10 after the infected feed. 7.5% showed gut infection but no salivary gland infection was observed.

69 G. austeni fed on the infected mice and 63 managed to live for more than 10 days. 6.3% of these showed gut infection. One fly found dead 13 days after the infecting feed showed salivary gland infection. Two of the clean mice used to feed these flies were found infected 6 days after the infected fly had been isolated. Apart from the two infected mice, no mouse used to feed the flies earlier nor later than 13 days after the infecting feed showed trypanosome infection. It was concluded that the infected mice must have become infected through the bite of the only infected fly observed.

D. Attempts to cultivate T.(T.) brucei and T.(T.) evansi in mouse peritoneal macrophage cultures at 37°C

The observation that amastigote forms of T.(T.) evansi could occur in mouse peritoneal exudate obtained from mice kept at 35°C (see section V.B), made it necessary to try and see if trypanosomes

could grow in mouse macrophages cultured in vitro at 37°C. The tissue culture medium chosen for growing the macrophages was, Tissue Culture medium 199 (TC 199). The macrophage cultures were prepared at least three days before they were inoculated with trypanosomes. By this time a normal growth of macrophages was obtained (Fig. 18a).

8 mice were inoculated; 4 with antilog 3.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 128, and the other 4 inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) evansi stabilate, LUMP 55. When mice were heavily parasitaemic, after 3 days in case of T.(T.) brucei and 7 days for T.(T.) evansi, the animals were bled totally. Blood collected from each infection was pooled and trypanosomes separated from the blood cells. About  $1 \times 10^6$  organisms were inoculated into each of the macrophage culture tubes and incubated at 37°C. Ten culture tubes were thus prepared for each species. The culture tube contents were examined for the presence of trypanosomes and stained macrophage cultures were examined for possible intercellular trypanosome growth. Two tubes for each species were examined at a time daily.

#### Survival of trypanosomes (Table 13)

T.(T.) brucei. Actively dividing trypomastigotes survived in the tissue culture supernate for more than 48 hours. The trypanosomes were, however, decreasing rapidly in numbers. No trypanosomes could be seen in the tissue culture supernate after 72 hours.

T.(T.) evansi. Trypomastigotes survived for more than 24 hours but could not be seen after 48 hours.

#### Intracellular growth

T.(T.) brucei. The entire field covered by the coverslip on which the macrophages grew was examined using x 40 objective. There



were no trypanosomes of any form seen in the macrophages, nor was there any evidence of phagocytosis. Only in one case (one slide), 4 day old culture was found to contain some inclusion bodies (Fig. 18b). It is not easy to determine whether these are of trypanosomal origin.

T.(T.) evansi. No trypanosomes of any form was observed in the cultures inoculated with T.(T.) evansi. Sausage-shaped, spore-like organisms (Figs 19a, b) were seen in macrophages examined on days 2, 3, 4 and 5 after inoculation. One or two such macrophages were observed per slide, and between one and five such organisms were seen in each affected macrophages. Morphologically this organism resembled closely Nosema cuniculi, except that it showed no binary fission, sporulation nor encystment which would be expected during five days' period of growth (Dr. E. Canning assures me that the organisms are not microsporidia).

In order to check whether this organism was peculiar to T.(T.) evansi, the experiment was repeated. 4 mice were inoculated with a similar inoculum as above, and at peak parasitaemia (day 7) the trypanosomes harvested were inoculated into 3 day-old macrophage cultures. Trypanosomes were seen in the culture supernate only on the 1st day after inoculation. No intracellular trypanosomes were seen in the macrophages throughout the five days' observation period.

It seems that the spore-like organisms were only a contamination probably from the peritoneal fluid of one of the experimental mice. It was concluded that macrophages at least under these experimental conditions were not able to support an intracellular growth of T.(T.) brucei nor of T.(T.) evansi.



E. Growth and morphology of *T.(T.) brucei* in chick  
embryos incubated at 37°C and 39°C

To eliminate as far as possible the hosts immune response to trypanosome infections, 8-day-old chick embryos were chosen as systems where trypanosomes could be grown in atmosphere least contaminated by both circulating antibodies and cell mediated immune reactions. It was hoped that by inoculating such embryos with trypanosomes and incubating them at 37°C and 39°C, any differences observed in the course of infection would be accounted for mostly by the temperature effect.

96 eight-day-old chick embryos were divided into two groups; one group of 76 was inoculated with antilog 2.1 ID<sub>63</sub> *T.(T.) brucei* stabilate LUMP 128. The remainder were inoculated with 0.05 ml phosphate buffer solution (BS) pH 7.4 as control. The two groups were divided further into two equal groups 38 and 10 respectively. One group was transferred to 37°C and the other to 39°C incubator. Equal batches of inoculated and control groups were checked for trypanosome infection by examining venous blood from embryos killed on days 4, 5, 7, 8, 9, 10, 11 and 13 after inoculation.

There was no mortality among the controls in both groups incubated at 37°C and 39°C. Table 14 compares the pathogenicity of *T.(T.) brucei* to embryos incubated at 37°C and 39°C.

Infection rates.

Embryos incubated at 37°C. It may be seen from the table that of the 38 embryos inoculated, 31.6% were found dead at the time of examination. No trypanosomes were seen in the dead embryos but half of the surviving embryos were found infected. Table 15 shows

the number of embryos examined on particular days and the number surviving. The table shows that the number of infected embryos observed was always less than 35% of the total examined.

Embryos incubated at 39°C. 36.9% of the embryos inoculated were found dead during examinations (Table 14) and no trypanosomes were seen in them. 44.7% of the total embryos inoculated were found infected. Table 15 shows that there were higher infection rates (up to 87%) in embryos kept at this temperature during the first week of infection. The drop in numbers of infected embryos coincided with the observed increase in mortality of the inoculated embryos. No infected embryos were found alive after day 9 following inoculation.

In spite of the higher infection rates observed in embryos incubated at 39°C comparison of the survival time and susceptibility to infection of embryos incubated at the respective temperatures indicated that these differences were not significant ( $X^2 = 2.54$ ;  $P > 0.05$ ).

#### Parasitaemia (Figure 20)

Figure 20 is based on the number of infected embryos only.

Embryos incubated at 37°C showed very scanty infection which invariably persisted until the embryos hatched.

Embryos incubated at 39°C showed much higher parasitaemias which reached a peak on day 8 after inoculation. It appears that the intensity of parasitaemias in the embryos accounted for their high mortality after the first week of infection.

It is concluded that the direct heat effect, as shown by incubation at 37° and 39°C was not a limiting factor in the infectivity of the trypanosomes to chick embryos, but it greatly influenced the



pathogenicity. Incubation at 39°C encouraged the adaptability of this strain of trypanosomes to chick embryos.

Morphology of trypanosomes in chick embryos

Embryos incubated at 37°C. Because of the very scanty infection in embryos incubated at 37°C, very few trypanosomes were available for examination. But the few observed were all of the slender type (Fig. 21a). All the infected embryos showed monomorphic infection.

Embryos incubated at 39°C. Infected embryos incubated at 39°C showed wide morphological variations, especially on days 7 and 8 after inoculation. This time coincided with the time the highest counts were recorded (Fig. 20). During peak parasitaemias, it appeared as if the trypanosomes had developed other ways of replication apart from the usual binary fission. Table 16 summarizes the major morphological forms observed. Giant and multinucleate forms (Figs 21b, c, d) appeared in relatively large numbers, 3.3%. Many stumpy forms developed vacuolation around the posterior end just behind the kinetoplast (Fig. 22a). The vacuoles became larger and the trypanosomes shrunk in size (Figs 22b, c). Some vacuolated forms were seen to be undergoing binary fission (Fig. 22d). The vacuolated forms finally rounded off completely while still retaining a well formed but not free flagellum (Figs 22e, f). Sphaeromastigotes without vacuoles were also observed (Fig. 23).

The significance of these morphological forms which appear to develop in some predetermined pattern is not known. Since they take the vital stains quite readily, it would appear that they are not degenerating. Similar forms were observed in immunosuppressed mice (see section V.H). It seems that these bizarre forms come about as a result of



direct temperature effect on the trypanosome organisms, since they were not seen in embryos incubated at 37°C.

F. Antigenic variants of *T.(T.) brucei* from mice kept at 35°C

When *T.(T.) brucei* is adapted to small laboratory rodents by syringe passages, it acquires an enhanced pathogenicity for these animals and in case of mice and rats, the animals die in a few days (Murgatroyd and Yorke, 1937). The use of drugs at subcurative dose levels becomes inevitable if the antigenic nature of such trypanosomes is to be studied. By employing the high temperature effect model, the problem of variation in a highly pathogenic strain of *T.(T.) brucei* can be studied without introducing drugs. Besides, it had been observed that infections kept at abeyance under the pressure of high ambient temperature, rose quickly and killed mice in 4-5 days when the pressure was lifted. i.e. when the animals were transferred to 22-27°C (see section V.A.2). It was therefore interesting to see what pattern of variation was underlying the effect exerted by maintenance at 35°C ambient temperature.

6 mice were inoculated with antilog 2.1 ID<sub>63</sub> *T.(T.) brucei* stabilate LUMP 128 and maintained at 35°C. During the first relapse 0.01 ml quantities of blood from two mice showing comparatively (++) higher parasitaemias than the others was diluted 200x. 0.3 ml quantities of the trypanosome suspension from the two animals were subinoculated into 3 clean mice. 2 groups of 3 mice were thus inoculated. On day three after subinoculation, one mouse showing highest parasitaemia from each group was killed and a stabilate made from the infected blood. The first isolation was done 11 days

after infection and subsequent isolations were made from the same two mice which were designated as A and B for convenience. Isolations were made at weekly intervals until the two mice died.

A total of seven substrains of T.(T.) brucei were isolated from the two mice. These substrains and the original strain LUMP 128, were tested against immune sera obtained from homologous infections. The results of agglutination tests carried out for the different isolates are expressed in Tables 17a, b.

#### Homologous reactions

Substrains from Mouse A (Table 17a). Antisera prepared against the original infecting strain LUMP 128, agglutinated none of the three substrains isolated. The antisera against the first isolate cross-reacted only with the second substrain. Antisera prepared against the second substrain isolated after 18 days infection was found to cross-react with all substrains isolated. It is this strain which produced also the highest homologous titres, 40960.

It seems that the first substrain isolated after 11 days' infection was probably the only distinct variant produced during the course of infection in this mouse.

Substrains from mouse B. This mouse survived one week longer than mouse A and so four substrains were isolated from it. There was slight cross-reaction between the antisera to the original LUMP 128 and the first substrain isolated after 11 days' infection. This first isolate cross-reacted also with all the antisera prepared against the other substrains. It is interesting to note that one serotype could stay this long in circulation. This isolate



appears to represent the predominant antigens.

Antisera prepared against the fourth substrain isolated after 32 days contained agglutinins against all the substrains. This strain did not, however, produce the strongest homologous reaction.

#### Heterologous reactions (Table 17b)

When antisera from different substrains isolated from one mouse was incubated with substrains isolated from the other mouse, it was found that some isolates were completely distinct serotypes. The first substrain isolated after 11 days from mouse A, failed to react with antisera prepared against all the other substrains isolated from mouse B. Likewise, the substrain isolated from mouse B after 25 days' infection showed no cross-reaction with any of the antisera prepared from substrains isolated from mouse A. On the other hand, the first substrain isolated from mouse B cross-reacted with antisera from all the substrains isolated from mouse A. This antigen appears to be the only common antigen produced during the course of LUMP 128 infection in the two mice.

These results show in general, that a virulent strain of T.(T.) brucei whose virulence is kept under control by exposing the hosts to 35°C, is capable of undergoing antigenic changes and so able to produce distinct serotypes. It seems therefore that the failure of infected animals to survive beyond the first parasitaemic wave is responsible for the selection of the virulent population and that the capability to vary antigenically is never lost.



G. Influence of ambient temperature on the antigenicity of *T. (T.) brucei* infected mouse serum

The existence of trypanosome antigens in *T. (T.) brucei* infected serum and its specificity is well documented (Weitz, 1960a; Miller, 1965). What is not clear is whether the response of these antigens depends on the intensity of parasitaemia. It was therefore quite opportune to see whether there was a difference in the antigenicity of infected mouse serum obtained from mice infected with *T. (T.) brucei* kept at 22-27°C and at 35°C during the first parasitaemic wave.

1. Production of antisera to infected mouse serum

6 mice were inoculated with antilog 3.1 ID<sub>63</sub> *T. (T.) brucei* stabilate LUMP 128 and treated with diminazene aceturate on day 3 after inoculation. The animals were challenged two weeks after treatment with the same stabilate, using a similar inoculum. The animals were bled through the sinus at weekly intervals for 6 weeks, starting one week after challenge. Strongly precipitating antibodies were detected in all the serum samples collected during the six weeks, when these samples were tested against infected mouse serum from *T. (T.) brucei* LUMP 128 infection (Figs 24a, b, c). Sera collected one week after challenge was used to determine the earliest time released antigens could be detected during acute untreated infections.

2. The relationship between the response of released antigens and parasitaemias

6 mice were inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 128 and examined at 24, 48, 54, 60, 66, 72, 90, 99 and 120 hours after inoculation. Parasitaemia was first determined and then sera collected. The sera collected were assayed for the presence of released antigens using hyper-immune sera described above. The earliest time specific reaction occurred was 66 hours after inoculation (Fig. 25). The intensity of precipitation lines increased with increasing parasitaemias and was strongest when infected sera were obtained from moribund animals. Precipitinogens were observed only from samples collected from animals whose parasitaemia was antilog 8.0 trypanosomes/ml or higher (Fig. 26).

3. Attempts to demonstrate the presence of released antigens in plasma of mice infected with T.(T.) brucei and maintained at 35°C.

Arising from the above observation (released antigens detected only in acutely infected animals), an attempt was made to see if it was possible to detect these antigens in inoculated mice maintained at 35°C. Two groups of six mice were inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 128. One group was kept at 22-27°C and the other group was transferred to 35°C. Both groups were examined for parasitaemias and plasma collected at 24, 45, 54, 66, 72, 78, 90, 99 and 120 hours after inoculation. The plasma collected was assayed for the presence of precipitinogens using the hyper-immune sera.

### Parasitaemia (Table 18)

Animals kept at 22-27°C developed fulminating infections and all died in less than 99 hours after inoculation.

All animals kept at 35°C were parasitaemic by 54 hours after inoculation. But parasitaemias never rose much higher than antilog 7.0 trypanosomes per ml. Infection became subpatent after 90 hours.

### Precipitinogens

Plasma obtained from infected animals kept at 22-27°C showed specific reactions when assayed for the presence of precipitinogens when parasitaemia was of the order antilog 8.0 trypanosomes per ml. Plasma obtained from moribund animals once again showed very strong precipitation reaction.

No reaction was observed when plasma samples collected from mice kept at 35°C was tested for precipitinogens.

To test whether more antigens would be released in vivo as a result of disintegrating trypanosomes, two groups of 6 mice were inoculated with the same stabilate and a similar inoculum. 66 hours after inoculation, one group was subjected to a single dose (10 mg/kg body weight) diminazene aceturate treatment to terminate the infection. The mice were examined for the presence of released antigens one and two days after treatment.

### Parasitaemias

The untreated mice all died with heavy trypanosome infection in less than 120 hours after inoculation.

The course of infection in treated mice is shown in Table 19. It will be seen from the table that parasitaemias dropped after



treatment and no trypanosomes could be detected two days later.

Precipitinogens. Plasma obtained just before treatment showed the presence of precipitinogens and, once again, only mice whose parasitaemia was higher than antilog 8.0 trypanosomes per ml showed this reaction (Fig. 27). Plasma obtained at 30 or 54 hours after treatment failed to show any precipitation reaction, even after 1/8 dilution.

4. Immunizing properties of infected mouse serum obtained from mice kept at 22-27°C and at 35°C.

The failure to show precipitinogens in plasma obtained from infected mice kept at 35°C made it necessary to see whether such plasma was also non-immunogenic. 30 mice were inoculated with antilog 2.1 ID<sub>63</sub> stabilate LUMP 128, and half of them kept at 22-27°C and the remainder transferred to 35°C. On day 3 when all mice were parasitaemic, the two groups were bled separately and serum samples collected. 2.5 ml from the separate serum samples was mixed with Freund's Complete Adjuvant (FCA). An equal amount of normal saline was mixed with (FCA) as control. Groups of previously uninfected mice were immunized as follows:

<u>Group</u>	<u>Number of mice immunized</u>	<u>Immunized with</u>
1	35	Serum from mice kept at 22-27°C
2	35	Serum from mice kept at 35°C
3	35	Normal saline

Another group of 35 non-immunized mice was kept as control. Each animal in the above three groups was inoculated subcutaneously

with 0.05 ml of antigen or saline + FCA mixture. Two weeks after immunization, animals in groups 1, 2 and 3 were each given a booster inoculum of 0.05 ml of the respective antigens or saline without FCA. One week after the booster the inoculations, the immunized and the control animals were divided into 5 subgroups of 7 and then inoculated with T.<sub>9</sub>(T.<sub>9</sub>) brucei LUMP 128. A stabulate of T.<sub>9</sub>(T.<sub>9</sub>) brucei LUMP 128 was serially diluted into 1/100, 1/500, 1/1000, 1/5000 and 1/10000. Mice in the respective subgroups were challenged with 0.1 ml of the above dilutions.

Untreated control mice and mice immunized with saline became parasitaemic when inoculated with all except the 1/10000 dilution. Fig. 28 summarizes the results of challenging immunized mice.

Immunization with infected serum obtained from animals kept at 22-27°C

It may be seen from the figure that three out of seven mice were completely protected after challenge with the lowest dilution 1/100. Mice challenged with 1/500 became parasitaemic after varying prepatent periods ranging from 2 to 15 days. Three out of seven, however, showed complete resistance to infection. The protracted prepatent period was similarly shown in mice challenged with 1/1000 and 1/5000 dilutions.

Immunization with infected serum obtained from animals kept at 35°C

Six out of seven mice challenged with 1/100 or dilution succumbed to infection in 5 days and all died within 8 days after challenge. Protection was first noticed in mice challenged with 1/500 and 1/1000 dilutions. One animal in each group failed to show parasitaemias when challenged with either 1/500 or 1/1000. Here again, the prolonged prepatency was observed. Four animals challenged with 1/500



failed to show parasitaemias.

No infected animal ever recovered nor was there any relapses. 12 out of 35 mice challenged with serum from animals kept at 22-27°C were completely protected whereas only 6 out of 35 were protected when inoculated with infected serum from mice kept at 35°C. These results suggest that protection was better among animals immunized with infected serum obtained from mice kept at 22-27°C. Although not very protective it appears that the infected serum from mice kept at 35°C contained some immunogens.

5. Attempts to destroy the resistance of immunized mice using infected rat serum

Weitz (1960) showed that when a suspension of T.(T.) brucei was incubated with infected rat serum and then inoculated into rats, the trypanosomes became much more infective to their hosts. He thought that infected rat serum (IRS) contained an aggressin-like substance which enabled the trypanosomes to acquire the enhanced pathogenicity. In this experiment, already immunized mice were injected with large quantities of infected rat serum to see whether such a treatment could reduce their resistance on challenge with the same stabilate. If such a system worked, then the IRS could be employed to enhance the pathogenicity of T.(T.) brucei in mice kept at 35°C.

0.1 ml of blood from each of 18 protected mice described in the above experiment (section V.G.4) was diluted 10x and subinoculated into 3 clean mice. None of the mice subinoculated was found parasitaemic when they were examined for 15 days. The 18 protected and 6 previously uninfected mice were inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) brucei



stabilate LUMP 128 and the protected mice immediately separated into the following groups of six:

Group 1, each mouse was injected (IP) with 2 ml (IRS) obtained from infected rats kept at 22-27°C

Group 2, each mouse was injected (IP) with 2 ml normal rat serum (NRS)

Group 3, each mouse was injected with 2 ml normal saline

After IRS administration, the mice suffered a shock but they recovered after one hour. The control mice became parasitaemic and all died after five days. Table 20 summarizes the survival of mice injected simultaneously with infected rat serum and trypanosomes.

#### Mice receiving IRS

Three out of six mice which received IRS became parasitaemic and died on the fifth day after inoculation. The other three remained non-parasitaemic when they were examined for 30 days.

#### Mice receiving NRS

Three out of six mice which were injected with normal rat serum became parasitaemic and died also on the fifth day. The rest failed to show parasitaemias.

#### Mice receiving normal saline

One mouse among the six became parasitaemic and died after five days. A further two became parasitaemic after 13 and 15 days respectively. The two succumbed after four and five days' infection respectively. The other three remained non-parasitaemic.

At the close of the experiment, it was found that 3 mice in each group had not showed any parasitaemias. It was therefore clear that IRS offered no advantage to the infectiousness of T.(T.) brucei when animals had been sufficiently protected against a challenge from

the same stabilate. The delay in the prepatent period in mice injected with saline indicates that either the protection of mice was wearing out quite rapidly or the parasite was changing its antigenic type. The infected serum could therefore not be used as an immunosuppressive agent in mice kept at 35°C.

#### H. Influence of immunosuppressive agents on the course of T.<sub>2</sub>(T.<sub>2</sub>) brucei infection in mice kept at 35°C

##### 1. Effect of x-irradiation

It is known that x-irradiation of animals is strongly anti-phagocytic in its action as well as depressing antibody production (Taliaferro and Taliaferro, 1951). It was therefore expected that mice which had been irradiated would not show the reduction of virulence of T.<sub>2</sub>(T.<sub>2</sub>) brucei infection which was characteristic of normal mice maintained at 35°C. To test this hypothesis, 18 mice were individually exposed to a whole body x-irradiation at 600 rads near to a lethal dose. Four days later these mice and a control group of 18 mice similar except that they had not been irradiated were inoculated with antilog 1.1 ID<sub>63</sub> T.<sub>2</sub>(T.<sub>2</sub>) brucei stabilate LUMP 128. The two groups were then divided, 9 mice of each being placed at 23-27°C and at 35°C. Microscopical examinations of wet films of tail blood was carried out daily from day 2 after inoculation and when trypanosomes were present their numbers were estimated by haemocytometer counts. Stained thin films were also prepared from which morphological observations were made. On the 9th and 12th day after inoculation the surviving mice were bled through the retro-orbital



sinus puncture and plasma stored at  $-20^{\circ}\text{C}$  for test for agglutinating and precipitating antibodies. Mice were watched for ill effects of irradiation throughout the experiment. None died before inoculation. Mice maintained at  $35^{\circ}\text{C}$  were treated with penicillin streptomycin on the 5th day after inoculation to prevent premature death from pathogenic microbial contaminants.

In the mice maintained at  $22-27^{\circ}\text{C}$  irradiation appeared to be without effect on the course of infection. Both irradiated and non irradiated animals died during the first peak of parasitaemias on day 4 or 5 after inoculation. This observation was in sharp contrast to the results in the mice maintained at  $35^{\circ}\text{C}$  (Fig. 29).

#### Maintenance at $35^{\circ}\text{C}$

Non-irradiated mice. The non-irradiated mice kept at  $35^{\circ}\text{C}$  showed low parasitaemias, sometimes subpatent infection; all 9 mice survived to day 18 when they were removed from this environment and transferred to  $22-27^{\circ}\text{C}$  and destroyed.

X-irradiated mice. On the other hand, the irradiated mice showed rapidly rising parasitaemias and three succumbed to infection during the first parasitaemic wave. Animals surviving after day 6 showed fluctuating parasitaemias, but parasitaemia remained at high level until day 14 when they started dying. The experiment was stopped on day 18 when the x-irradiated mice had all died.

#### Morphology of trypanosomes in x-irradiated mice

The morphology of the organisms in the irradiated mice kept at  $22-27^{\circ}\text{C}$  was indistinguishable from that of the organisms in the



non-irradiated animals (Fig. 10a). The trypanosomes were all monomorphic with a very large proportion undergoing binary fission.

In the irradiated animals at 35°C, however, morphological changes were observed between day 6 and 9 after inoculation (Figs 30a, b, c, d, e). Posteroneuclear forms were common and some trypanosomes showed nuclei pushed up to the extreme posterior end past the kinetoplast. Besides, the posterior end assumed a more blunt shape than usually seen in non-irradiated mice in the same environment. Trypanosomes with double or more nuclei and one kinetoplast became numerous.

From the 10th day of infection dividing forms started to increase and the aberrant forms disappeared.

Detection of antibodies in non-irradiated and  
x-irradiated mice kept at 35°C

Irradiated mice. Neither agglutinating nor precipitating antibodies were detected in plasma collected from irradiated animals when they were examined on the 9th and 12th day after inoculation

Non-irradiated mice. Plasma collected from the non-irradiated mice on these occasions (9th and 12th day of infection) contained strongly agglutinating antibodies. Titres of 20480 and 2560 were demonstrated in plasma collected on the 9th and 12th day of infection respectively. Precipitating antibodies were detected only in the sample collected on the 12th but not from that collected 9 days after inoculation.

To exclude the possible effects of the long acting antibiotics

on the trypanosomes, the experiment was repeated, this time omitting antibiotic administration. 14 mice were irradiated with 600 rads and four days later, the irradiated and a control group of 12 were inoculated with the same stabilate and similar inoculum as used previously. The two groups were divided, 8 irradiated and 6 control were kept at 22-27°C and the remainder at 35°C. Stained preparations of thin smears were examined for trypanosome morphology.

Irradiated and non-irradiated mice kept at 22-27°C died within 5 days with high parasitaemias.

35°C (Table 21). Parasitaemias in mice kept at 35°C were recorded from stained preparations.

Non-irradiated. The non-irradiated mice showed low parasitaemias and none of them died during the 12 day observation period.

X-irradiated. The irradiated mice developed fulminating infections and two animals succumbed during the first peak parasitaemias on day 6. The rest died within 12 days after inoculation, and the experiment was stopped.

#### Morphology of trypanosomes in x-irradiated mice (Fig. 31)

Various morphological forms observed in the first experiment were again seen when the experiment was repeated. Large numbers of posteronucleated forms appeared between day 6 and 9 of infection. On one occasion during this period, one animal had as high as 90% of all the trypanosomes counted being posteronucleated. Multinucleated vacuolated and rounding forms were also observed.

It was therefore concluded that the occurrence of the various morphological forms was not influenced by the antibiotic administration.



Antibiotic administration, however, prolonged the survival of x-irradiated mice.

The effects of x-irradiation on the course of T.(T.) brucei infection in mice kept at 35°C was found to lower the resistance of mice to this strain of trypanosomes. Nevertheless, it was interesting to note that in spite of the high parasitaemias, x-irradiated animals kept at this temperature survived the first parasitaemic wave. It appears, therefore, that the effect of heat had reduced markedly, the pathogenicity of trypanosomes to mice, thus suggesting a direct heat effect on the trypanosome organisms.

## 2. Immunosuppressive drugs

a. Effect of cyclophosphamide treatment. Cyclophosphamide a powerful cycostatic agent was employed to see if it could influence the course of trypanosome infection in mice maintained at 35°C. 36 mice were divided into the following groups:

Group 1, consisted of 18 mice; they were treated daily with cyclophosphamide at 50 mg/kg body wt for five days.

Group 2, consisted of 6 mice, they were treated daily with 0.1 ml normal saline for five days.

Group 3, consisted of 12 mice; these mice were kept as control.

After treatment 12 mice in group 1 and mice in group 3 were inoculated with antilog 1.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 128. Half the inoculated mice in each group were kept at 22-27°C and the other half transferred to 35°C. The remaining 6 cyclophosphamide treated but not inoculated mice, and mice in group 2 were transferred



to 35°C as control for cyclophosphamide toxicity. The inoculated mice were examined for trypanosome infection and for ill-effects of the drug for 15 days. No death occurred among the uninoculated mice and so it was taken that cyclophosphamide at the dosage administered was not lethal to the mice.

#### Parasitaemias (Fig. 32)

Cyclophosphamide treated and non-treated mice kept at 22-27°C showed quick rise in parasitaemias, with the consequent intervention of death 4 to 5 days after inoculation.

Non-treated mice kept at 35°C developed low grade, sometimes subpatent infection until they were removed from the environment 15 days after inoculation. Cyclophosphamide treated mice, on the other hand, developed acute infections and death was observed after five days' infection in four treated mice. The remaining two died respectively, 7 and 9 days after inoculation. It appeared therefore that cyclophosphamide at the dosage used reversed the effect of high ambient temperature on the course of T.(T.) brucei in mice.

#### Morphology of trypanosomes

Both treated and non-treated mice kept at 22-27°C showed monomorphic infection. At 35°C, although infections in non-treated mice consisted mainly of slender forms, a few stumpy forms appeared just before the infection became subpatent. On the other hand, infections in cyclophosphamide treated animals showed varying grades of morphological variations particularly in the two animals

which survived beyond day 6. The trypanosomes gradually changed from slender form to a highly pleomorphic one. The number of stumpy forms increased from an occasional one to 34% on the 6th day of infection (Table 22). Many giant or multinucleated forms (Fig. 33a) vacuolated (Fig. 33b) and even rounded forms (Figs 33c, d) appeared at this time. Preceding the emergence of the bizarre forms, many trypanosomes assumed a blunt posterior end and were noticeably larger (2-5  $\mu$ m wide) than usual (Fig. 33c).

Besides pleomorphism, there appeared a tendency for the slender trypanosomes to adhere to leucocytes (Fig. 34). Polymorphs, monocytes and large lymphocytes all showed this tendency. If this tendency to adhere to leucocytes is a normal happening in T.(T.) brucei infections, then this observation is particularly noteworthy, since it suggests that this phenomenon (adhesion) may cause capillary blockage and hence oedema. There was no direct evidence of phagocytosis.

#### b. Effect of Betamethasone treatment

In order to see whether administering large quantities of corticosteroids had any effect on the outcome of trypanosomiasis in mice maintained at 35°C, the following experiment was carried out. 24 mice were divided into two equal groups. One group was treated with Betamethasone disodium phosphate. Each mouse received a total of 400 mg/kg body weight administered in four equal doses over a period of four days. The remainder were inoculated with 0.1 ml sterile normal saline for four days. After the last dose, all mice were inoculated with antilog 1.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 128. 6 mice in each group were kept at 22-27°C and the other 6



transferred to 35°C and examined daily for trypanosome infection.

Both treated and control mice kept at 22-27°C developed acute infections which became fatal in five days.

Mice kept at 35°C (Table 23)

Non-treated animals. Non-treated mice developed very scanty parasitaemias. Only one animal developed slightly more acute parasitaemia, but parasitaemia soon dropped to subpatent levels after the first week of infection.

Treated animals. Betamethasone treated animals similarly developed very scanty parasitaemias, except in one case, when one mouse had slightly higher parasitaemias than the rest. In general Betamethasone treated mice showed lower infections than the controls.

Morphology. Trypanosomes in Betamethasone treated mice did not present any striking morphological features. Trypanosomes from mice kept at 22-27°C were all monomorphic. But trypanosomes observed from mice kept at 35°C were mostly slender forms except in two animals (one from treated and the other untreated) which had slightly higher parasitaemias in which occasional stumpy forms appeared during remission.

3. Effect of anti-mouse lymphocyte serum

X-irradiation and immunosuppressive drugs are known to affect both the humoral and cell mediated immune reactions. In order to eliminate the possibility of there being some cell-mediated immune reactions during trypanosome infections in mice kept at 35°C, anti-mouse lymphocyte serum (ALS) was used. 24 mice were



each injected (IP) with 2 ml ALS for four days. Another group of 24 similar mice were injected with normal rabbit serum (NRS). After serum treatment, the mice were inoculated with antilog 1.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 128. The two groups were then divided into groups of 6. One group of each was detained at 22-27°C and the remainder transferred to 35°C. The animals were examined daily for trypanosome infections for 15 days.

Mice kept at 22-27°C died during the first parasitaemic wave within 5 days after inoculation.

Parasitaemia in mice kept at 35°C (Table 24a)

NRS treated mice. Trypanosomes were first detected from 5 of the 12 NRS treated mice on day 3; and all were parasitaemic by day 4 after inoculation. Three developed high parasitaemias and died between 7 and 9 days after inoculation. The other 9 showed remissions after the first parasitaemic crisis and it became difficult to demonstrate trypanosomes in these animals.

ALS treated mice. Two mice died 2 days after inoculation, before they became parasitaemic. Death was therefore not ascribed to trypanosome infection. Of the 10 survivors, two failed to develop infections throughout the observation period, two showed parasitaemias on one occasion only and the rest developed mild chronic infections until they were removed from the environments and transferred to 22-27°C. At the end of the experiment they were destroyed.

It became clear that anti-mouse lymphocyte serum at the dosage used could not influence the course of T.(T.) brucei in mice kept at 35°C.

The experiment was therefore repeated; this time using higher doses of ALS. 12 mice were treated in the manner described above except that they were injected with 2.4 ml ALS instead of 2.0 ml; another set of 12 mice were injected with 2.4 ml NRS. The mice were then inoculated with antilog 1.1 ID<sub>63</sub> T.(T.) brucei stabilate LUMP 128 and separated into two groups of 6. One group of ALS treated and another of NRS treated mice were kept at 22-27°C and the rest transferred to 35°C. The animals were examined for trypanosome infections for 15 days.

The animals kept at 22-27°C died with heavy parasitaemias within five days after inoculation.

Parasitaemia in mice kept at 35°C (Table 24b)

NRS treated mice. Three out of the six mice showed trypanosome infections after 3 days, and on day 5 all six were parasitaemic. Three mice died 7 days after inoculation and a fourth one died 6 days later. The last two showed light infections until the experiment was stopped.

ALS treated mice. Trypanosomes were first detected from these mice on day 5. Three showed trypanosomes only once during the 15 days. One of them died 12 days after inoculation; the cause of death was not apparent. The other three had light infections until the experiment was stopped. These observations indicate that ALS could not be used to enhance the virulence of T.(T.) brucei in mice kept at 35°C.



4. In vivo test for the potency of the rabbit  
anti-mouse lymphocyte serum

It was important to know whether the rabbit anti-mouse lymphocyte serum used in the above experiment was effective as an immunosuppressant. The serum was tested to see if it could prolong the survival of skin homografts.

Six 5-7 weeks old T0 mice were grafted with CBA skin. The mice were separated into two groups:

1st group was injected IP with 0.5 ml quantities of ALS to be tested

2nd group was injected with 0.5 ml quantities of NRS

3rd group was injected with 0.5 ml quantities of anti-mouse lymphocyte serum whose potency had already been checked

and found to be effective (Behbehani, personal communication)

The dosages were repeated at two days' intervals for 8 days. The mice were checked visually for skin rejection. Mice treated with NRS rejected the skin grafts completely 9 days post grafting. The homologous skin was not rejected.

ALS treated mice. One mouse treated with rabbit anti-mouse lymphocyte serum under test rejected the skin completely in 15 days and the other after 21 days.

Treatment with known ALS. One mouse treated with effective anti-mouse lymphocyte serum rejected the graft in 21 days. Because the glass tubing covering the grafts slipped out one day after grafting from the other mouse, the grafts were lost before any reaction could be observed.

The rabbit anti-mouse lymphocyte serum sample prepared was thus judged potent enough to act as an immunosuppressant. Its



failure to elevate parasitaemia in mice kept at 35°C was an indication that the resistance to trypanosome infection in these mice was not due to cellular immune reactions.

J. Influence of environmental temperature on antibody producing cells

In order to make sure that the change in pathogenicity observed when trypanosome infected mice were maintained at 35°C was not influenced by an early antibody response, the Local Haemolysis in Gel technique was used to demonstrate whether there was any difference in the presence of antibody producing cells (plaque forming cells) in mice kept at 22-27°C and those maintained at 35°C.

40 mice weighing between 20-25 gms were immunized with sheep red blood cells (SRBC) and then divided into two equal groups. The first group was kept at 22-27°C and the second group transferred to 35°C. On day 2 after immunization, 4 animals in each group were killed and spleens removed and weighed. The spleen cells were then teased out and the Local Haemolysis in Gel assay carried out for each spleen sample. The tests were repeated on days 4, 6, 9 and 11 after immunization; 4 animals were examined from each group on these occasions.

Plaque forming cells (Table 25 and Fig. 35)

Mice kept at 22-27°C. It may be seen from Table 25 that direct plaques (PFC) increased from 782 on day 2 to a peak mean count of 32,240 cells on day 4. A gradual decrease in the number of these cells then followed between day 6 and 9. There was a slight elevation

of cell count on day 11 (Fig. 35).

Mice kept at 35°C. These mice had a constantly lower PFC count than mice kept at 22-27°C. A peak count of 20,420 cells was recorded on day 4 and thereafter the mean count steadily dropped. The differences in the mean PFC count in the two groups of mice were statistically significant ( $t = 2.70$ ;  $P < 0.05$ ). It was therefore concluded that maintaining mice at 35°C had influenced the production of antibody producing cells in mice immunized with sheep red blood cells.

#### Spleen weights (Fig. 36)

Mice kept at 22-27°C. The spleen weights of these mice averaged 0.187 gm on day 2 after immunization and thereafter the mean weight fluctuated between 0.136 gm and 0.176 gm. In general there was a tendency towards a decreased mean spleen weight as time went on.

Mice kept at 35°C. A mean spleen weight of 0.164 gm was recorded on day 2 after immunization. The weights then dropped sharply to 0.096 gm on day 4. Thereafter the weights fluctuated between 0.085 gm and 0.144 gm. It was noticeably clear that these weights were constantly lower than those recorded for mice kept at 22-27°C. The mean spleen weights in the two groups of mice were statistically significant ( $t = 6.10$ ;  $P < 0.05$ ).

It was tempting to suggest that the diminution in spleen weights observed in mice kept at 35°C accounted for the fewer number of antibody producing cells in these mice.

If anything, these observations show that the resistance to trypanosome infection in mice kept at 35°C is not due to direct



humoral antibody effect, but most probably to a direct temperature effect.

K. Attempts at cyclical transmission of aberrant *T.(T.) brucei* resulting after immunosuppression (Table 26)

An attempt was made to see if the aberrant *T.(T.) brucei* could be transmitted cyclically. As was pointed out in section V.C the experiment was designed in such a way that the hatching of the tsetse flies coincided with the occurrence of the maximum numbers of aberrant forms. 36 mice were treated with cyclophosphamide 30 mg/kg body weight for four days. Two days after treatment, the animals were inoculated with 2.1 ID<sub>63</sub> *T.(T.) brucei* stabilate LUMP 128. The animals were then transferred to 35°C. When *G. morsitans* and *G. austeni* started to hatch seven days after the mice had been inoculated, mice showing relatively larger proportions of multinucleates, vacuolated or round forms of trypanosomes were selected and used to feed the newly emerged flies. The flies were thus fed on days 7, 8 and 9. Only flies which managed to feed during the first 24 hours after eclosion were used for the experiment. These were examined as described in section V.C. The number of tsetse flies exposed to infecting feed, the number surviving 10+ days and the percentage infection rates are shown in Table 26. It may be seen from the table that nearly equal numbers of 89 *G. morsitans* and 92 *G. austeni* succeeded in taking the infected feed. Of these 56 *G. morsitans* and 70 *G. austeni* survived for 10+ days. Both species showed gut infections, 9% for *G. morsitans* and 10% for *G. austeni*. No salivary gland infections were observed in <sup>either</sup> both species. Although



one does not know what proportions of these aberrant forms were ingested, it appears that their presence in the infecting feed did not influence the salivary gland infection.

## VI. DISCUSSION

The aim of these studies was to see whether there existed any relationship between variations in ambient temperature and the course of induced trypanosome infections in mice, and if so, what was the fate of the parasite under the circumstances.

The results of these investigations fall under the following major divisions and are discussed in that order:

1. The direct effect of high ambient temperature on the host.
2. The effect of temperature on trypanosomes.
3. The effect of temperature on the immune mechanisms of the host.

The results of variations of the ambient temperature on host body temperature show clearly that raising the ambient temperature to  $35^{\circ}\text{C}$  increases significantly the mouse body temperature. It was shown for example that at an ambient temperature of  $35^{\circ}\text{C}$ , the mean mouse body temperature was raised to  $39.3^{\circ}\text{C}$ , an increase of  $1.8^{\circ}\text{C}$  above the mean body temperature ( $37.5^{\circ}\text{C}$ ) at room temperature ( $22 - 27^{\circ}\text{C}$ ). This finding is in keeping with earlier observations (Levine et al., 1940) that mouse body temperature varied with the temperature of the environment. Levine et al. found that at  $21^{\circ}\text{C}$ , mouse body temperature was  $38.1^{\circ}\text{C}$ , but when the ambient temperature was elevated to  $36.7^{\circ}\text{C}$ , the average body temperature was  $40^{\circ}\text{C}$ . It was of further interest to note that the mouse body temperature was not influenced by trypanosome infections. The increase in body temperature was therefore thought to be due to a disturbance in the physiological heat balance, as a result of the failure of the cooling system to keep up with the rate of heat gain. Although

stressing, it was important to note that the mice were able to survive in the hot environment. It was thought that they overcame the stressing effect of the heat as a result of an adjustment in the activities of their cardiovascular and the sweating systems which in turn stimulated the adrenal cortex (an organ of homeostasis) to secrete adrenocortical hormones. The stimulation of the adrenal cortex would ensure that the animal is in a position to resist the stress.

It appears from the experimental evidence given above that this increase in body temperature stimulates some intrinsic factor which in turn regulates the trypanosome pathogenicity. It was found for a number of different salivarian trypanosome species and strains that the ambient temperature in which the hosts were maintained during the course of infection was of utmost importance. This observation was particularly true for the more virulent strains. Strains of T.(D.) vivax, T.(N.) congolense, T.(T.) brucei and T.(T.) evansi which were known to be highly virulent to their hosts when the hosts were maintained at a temperature ranging from 32 - 35°C. Similar reductions in pathogenicity have been observed in many host-virus systems (Thompson, 1938; Parker and Thompson, 1942; Walker and Boring, 1958) when infected hosts were kept at high (35° or 36°C) ambient temperature. None of these authors comes out with any firm conclusions to explain the mechanism for the reduction of virulence in the viral infection. In trypanosome infection, Kolodny (1940) has shown that fatal infections resulted in mice kept at low environmental temperature while much wilder parasitaemia occurred at high ambient temperature. He suggested that the change in trypanosome pathogenicity was due to a modification of hosts' physiologic processes rather than to direct temperature effect on the parasite.



The mechanism by which heat, as induced by the maintenance of hosts at 35°C reduces the virulence of these trypanosomes is as yet not clear. Experimentation with chick embryos revealed that the apparent increase in incubation temperature from 37° to 39°C, in fact, enhanced trypanosome growth. This observation may be taken to mean that the increase in mouse body temperature was not directly inimical to trypanosomes, but triggered off some temperature sensitive factor in the host which controlled trypanosome proliferation. On the other hand, it has been shown that the intracellular growth of amastigote stages of Leishmania spp. and T.(S.) cruzi is encouraged at higher temperatures (Lemma and Schiller, 1964; Neva et al., 1961). It was therefore suggested that it would be sensible to look for the possible existence of intracellular stages of T.(T.) brucei and T.(T.) evansi in mice kept in the hot environment. They were not found and therefore this possibility does not seem to be the explanation for the disappearance of T.(T.) evansi from circulation nor the low T.(T.) brucei parasitaemias in mice kept in the hot environment. They were not found as extracellular amastigotes either, which have been demonstrated in the choroid plexus by Ormerod and Venkatesan (1970) during the prepatent period and Soltys and Woo (1969) at the time of peak parasitaemia.

The failure to demonstrate a tissue phase of T.(T.) brucei and T.(T.) evansi in the histological sections and in the macrophages was an indication that an intracellular phase of development in these trypanosome species was very unlikely.

In the present study, it was shown that monomorphic strains of T.(N.) congolense and T.(T.) brucei were able to become pleomorphic when infections were controlled by high ambient temperature. It appeared that the high temperature disturbed the reproduction of the

parasites. This was evident in normal animals just as a reduction in virulence. When the animals were, however, immunosuppressed the aberrant forms were not removed from the circulation and so large representation of bizarre forms occurred. The appearance of bizarre forms were confirmed by the chick embryo evidence. The occurrence of gross morphological changes in embryos incubated at  $39^{\circ}\text{C}$  but not in those at  $37^{\circ}\text{C}$  was taken as a clear indication that the observed change was a direct temperature effect. Earlier workers (Desowitz, 1960; Ristic and Trager, 1958; Trager, 1959) have shown that the incubation temperature is important in determining which morphological forms of trypanosomes will develop in culture. Marinkelle (1965) has, in addition found that at certain incubation temperatures, various aberrant forms of T.(H.) conorrhini are produced. He demonstrated the presence of balloon-shaped trypanosomes having 7 flagella, 6 nuclei and 6 kinetoplasts when T.(H.) conorrhini infected cultures were incubated at temperatures ranging from  $34^{\circ}$  to  $37^{\circ}\text{C}$ . Such morphological changes are probably a common feature of in vivo growth but are not easy to demonstrate.

It is interesting to speculate on the possible mechanisms underlying the change in morphology. Although trypanosomes are known to withstand big alterations in ambient temperatures especially when they are first taken by the insect vector from the mammalian host, it is not known what is the upper temperature limit that can be tolerated without irreversible damage to the trypanosome organism. It is reasonable to assume that  $39^{\circ}$  or  $40^{\circ}\text{C}$  is very close to the upper limit. The effect of temperature on trypanosome is best visualized if the organism is realized to be a cell composed of a typical biological cell-membrane, trilaminate (Vickerman, 1970). One of the characteristics of such a membrane is to maintain a firm



interface to allow active transport of metabolites and metabolic activity without losing its structure. The cellular lipids which constitute a major ingredient of the cell membrane can only maintain their chemical make up within a certain temperature range (Gaughran, 1947). Johnston and Roots (1964) have shown that these lipids must maintain a certain degree of unsaturation so that a specific liquid-crystalline state is maintained. Near the upper temperature limit, the lipids would be less cohesive and so render the membrane more pliable. This would distort the shape of the cell and would therefore account for the various morphological changes observed at the high ambient temperature. In support of this theory, Greenblatt and Wetzel (1966) have observed that there occurs changes in fatty acids composition in culture forms of L. enriettii incubated at  $36^{\circ}$  to  $37^{\circ}\text{C}$ . They suggested that these changes could affect the cell membrane permeability. The significance of the various morphological forms is considered to be an adaptation to a mode of life which is capable of withstanding the otherwise unfavourable environment created by the high body temperature.

The prompt decline in T.(T.) evansi parasitaemias and their subsequent elimination from circulation when infected mice were kept at  $35^{\circ}\text{C}$  amounted to a curative response; thus suggesting some antitrypanosomal factor involved. But T.(N.) congolense and T.(T.) brucei had controlled parasitaemias when the hosts were kept at the elevated temperature. When the hosts were transferred from the hot environment to  $22-27^{\circ}\text{C}$ , the infection rapidly became acute and the animals succumbed from fulminating infection. It seems that for these species, heat induced a factor which controlled the trypanosome growth but whose action was easily reversed.



It has been suggested (Moner, 1965) that the effect of temperature ( $34^{\circ}\text{C}$ ) on the growth of Tetrahymena pyriformis in cultures may be inhibitory to RNA synthesis, as a result of breakdown of some division associated RNA fraction. In the presence of actinomycin D he found a similar inhibitory effect. And Hunter et al. (1957) have reported increased requirements in growth factors (vitamin  $\text{B}_{12}$ , folic acid, amino acids and trace metals) in free-living flagellate, Ochromonas malhamensis cultivated at temperatures higher than  $35.5^{\circ}\text{C}$ . It would be of interest to see if trypanosomes can be similarly affected by these mechanisms. It would for instance, be interesting to see what trypanosome metabolites are present in mice kept at  $22 - 27^{\circ}\text{C}$  but are lacking in those kept at  $35^{\circ}\text{C}$  or vice versa.

Studies on antigenic variation using a virulent syringe-passaged strain of T.(T.) brucei showed that the strain never lost its capability to produce variant antigens so long as the infection was maintained at  $35^{\circ}\text{C}$ . It seems that the variant antigens did not appear in mice kept at  $22 - 27^{\circ}\text{C}$  because the infection never went beyond the first (fatal) parasitaemic wave. The reversion to a virulent type is therefore indicative perhaps, just of a reduction in reproductive potentiality of the organism so that to continue to survive in the host it must antigenically vary. The observation that mice succumb to infection soon (4 to 5 days) after they are transferred from  $35^{\circ}\text{C}$  to  $22 - 27^{\circ}\text{C}$  means that the first variant appearing after return to  $22 - 27^{\circ}\text{C}$  is lethal and that ends the possibility of variants ever appearing. This observation is suggestive of a direct heat effect on trypanosomal organism rather than an immunological effect, as one would expect that an immunological one would not collapse so quickly. It also shows that the ability to produce variants

during the course of infection cannot be as a result of selection from a mutant population as has been suggested by Watkins (1964).

It has been shown (Cassuto, 1968, 1970) that acclimatization to a hot environment entails to some degree a reduction in metabolic heat production as a result of reduced metabolic activity, but the metabolic activity reverts to normal values 4 days after return to control temperature. It is therefore reasonable to suggest that one of the major limiting factors in determining the loss of virulence or the ability to produce variant populations is the inadequacy of optimal levels of oxygen available in the hosts tissues. In support of this hypothesis, Soule (1925) has shown that oxygen requirements of blood forms of trypanosomes is quite exacting and growth is easily inhibited at reduced oxygen tensions. Jenkins and Grainge (1956) and Fulton and Spooner (1957) have observed that oxygen requirements of an old and a freshly isolated strains of T. (T.) b. rhodesiense differ. They showed that freshly isolated, cyclically-transmitted strains of T. (T.) b. rhodesiense consumed less oxygen than strains which had undergone many serial passages in animals by blood inoculations in the laboratory.

Precipitinogens were not detected in infected mouse serum obtained during the early phase of acute infection, but when infected serum was harvested during peak parasitaemias, the serum contained strongly precipitating antigens. It was shown that the response of these antigens was correlated with the height of parasitaemic level. No precipitinogens could be detected in samples collected when parasitaemia was below antilog 8.0 organisms per ml. Serum collected from mice kept at 35°C during the first parasitaemic wave contained no precipitinogens. This observation is no surprising if it is realized that parasitaemias in mice kept at 35°C never rose beyond antilog 8.0 organisms/ml at peak parasitaemias. It has been suggested



that released antigens (contained in infected mouse serum) may occur as a result of contamination with trypanosomal metabolic products (Thillet and Chandler, 1957), the release of plasmanemes (Wright et al., 1970), shedding of the surface coat (Vickerman, 1968) or the disintegration of organism thus releasing constituents of the cell (Lumsden, 1970). One would expect all these suggested methods to be functional at any time during trypanosome growth in blood, and their concentrations would increase with the increasing parasitaemias. If the presence of these antigens are dependent upon the intensity of infection, then in natural T.(T.) brucei infections, such as in human beings in which the number of trypanosomes present in blood may not be high, it would appear that released antigens may not be important. The significance of these antigens during the course of chronic trypanosomiasis such as T.(T.) b. gambiense is worth investigating.

On the basis of a close positive correlation between the numbers of direct plaque forming cells (PFC) in the spleen and the titre of IgM haemolytic antibody in the serum (Šterzl and Řiha, 1965; Moller and Wigzell, 1965), it has been argued that direct PFC release IgM antibodies. For example, one molecule of rabbit 19S antibody is needed to effect lysis of an erythrocyte, whereas several thousands of 7S molecules are required to cause a similar effect (Borsos and Rapp, 1965). The early demonstration and big increase in IgM antibody levels during trypanosome infection (Mattern et al., 1961; Houba et al., 1969; Rees, 1969) makes it reasonable to assume that IgM antibodies play an important role in trypanosome infection. It was therefore expected that studies on direct plaque forming cells might give some clues as to what part humoral antibodies were playing in the observed resistance to



infection in mice kept in hot environment. From the evidence of PFC experiments and the spleen weights, the number of cells producing IgM in mice kept in the hot environment was less than in normal mice, thus indicating a fall (if anything) in the immune response with the rise in temperature. This observation is a further confirmation that the effect of ambient temperature on trypanosomes pathogenicity was a direct heat effect on trypanosomes rather than an immunological one. The drop in spleen weights with the consequent diminution in the number of antibody producing cells in mice kept at 35°C may be accounted for, if it is recalled that mice kept in this environment are stressed. Dougherty and White (1943) have produced evidence to show that infection of pituitary hormone, ACTH (a stress factor) produces a decrease in the weights of lymphoid tissues (inguinal, axillary, mesenteric nodes and in the thymic mass). The weights of these tissues were reduced by half after the hormone injection. And they (Dougherty and White, 1944) later showed that a single injection of ACTH in mice, rats and rabbits produces within a few hours a severe lymphopenia. The failure of anti-mouse lymphocyte serum to enhance trypanosome pathogenicity to mice kept in the hot environment suggested that cellular immune responses were similarly not directly responsible for the reduced pathogenicity observed in these animals. The loss of virulence could, therefore, not be ascribed to a direct immunological effect, but more likely to a direct effect of temperature on the trypanosome organism. But from the evidence of x-irradiation and cyclophosphamide experiments, there appears to be at least some immunological response. It is necessary to add, here, that the two immunosuppressive agents are very effective in blocking the inductive phase of antibody production. It was, however, shown (at least with x-irradiation) that in spite of the high parasitaemias developed after

immunosuppression, trypanosomes were not as virulent to these as they were to control mice kept at normal room temperature; a further proof of direct effect of temperature on the organisms. It must, nevertheless, be noted that Ipsen (1952) found that specific protective antibody was produced when mice were exposed to high ambient temperature. He found that mice protected with tetanus toxoid withstood better a tetanus toxin challenge when the protected mice were kept at 35°C than controls kept at room temperature. And Stone (1956) found that agglutinin titre levels of a naturally occurring antibody in cattle erythrocyte or serum varied seasonally. During summer months, higher titres were observed but during winter months agglutinin titre levels were minimal.

Finally, it is tempting to suggest that there is some correlation of these findings with the epidemiology of human trypanosomiasis in Africa. Sleeping sickness is attributed to infections caused by T.(T.) b. gambiense and T.(T.) b. rhodesiense, the causative agents of which cannot be distinguished on morphological grounds. The two infections may only be separated from one another by the type of the disease they cause. T.(T.) b. gambiense infection is usually more chronic than the latter, T.(T.) b. rhodesiense which causes a virulent infection. When one looks at the distribution of the two diseases, one finds that T.(T.) b. gambiense is common in tropical West Africa, Central Africa especially the Congo river basin and a few patches around Lake Victoria in East Africa. A close look at the climatic conditions of these areas (Kendrew, 1953) reveals that these areas are plagued by intense monotonous heat and always very humid. Such an environment is more likely to cause an unnecessary heat load and may imperceptibly tip off the physiological heat balance. On the other hand, T.(T.) b. rhodesiense infections are found mostly in

East and Central Africa where climatic conditions are less enervating and certainly not as humid as the above areas. The dry atmosphere in these areas reduces the risk of heat load. It is therefore suggested that besides the many reasons offered for the distribution of the human trypanosomiasis in Africa (Apted et al., 1963; Ashcroft, 1963; Morris, 1963; Ormerod, 1961), the correlation of the heat load and the distribution of the disease is worth giving serious consideration.



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**APPENDICES**

**Table 1.** Mortalities and mean survival times of mice inoculated with stabulates of T.(D.) vivax, T.(N.) congolense, T.(T.) brucei and T.(T.) evansi and maintained at 4°C, 22-27°C and 35°C ambient temperatures. Mice surviving beyond the observation period were transferred to 22-27°C.

Stabilate	Inoculum		Observation period (Days)	Ambient temperature °C	No. para-sitaemic		Mortality at the end of observation period	Mean survival time $\pm$ SE( $\bar{x}$ ) (Days)
	Log no. of trypts/ml	Antilog ID <sub>63</sub>			No. inoculated			
<u>T.(D.) vivax</u> LUMP 45	6.11	2.4	30	4	6/6	6/6	4.3 $\pm$ 0.21	
				22-27	6/6	6/6	4.3 $\pm$ 0.21	
				35	6/6	0/6	, > 30	
<u>T.(N.) congo-lense</u> LUMP 52	4.0	?	25	4	6/6	6/6	10.1 $\pm$ 0.16	
				22-27	6/6	6/6	10.5 $\pm$ 0.22	
				35	4/6	2/6	* 4 > 25	
<u>T.(T.) brucei</u> LUMP 36	6.30	3.8	50	4	10/10	10/10	4.4 $\pm$ 0.16	
				22-27	10/10	10/10	4.5 $\pm$ 0.16	
				35	10/10	10/10	28.8 $\pm$ 3.82	
<u>T.(T.) b. brucei</u> LUMP 43	6.75	2.4	50	4	6/6	6/6	3.1 $\pm$ 2.78	
				22-27	6/6	6/6	35.6 $\pm$ 4.20	
				35	6/6	6/6	39.1 $\pm$ 2.16	
<u>T.(T.) evansi</u> LUMP 55	6.54	3.1	20	4	6/6	6/6	9.0 $\pm$ 0.68	
				22-27	6/6	6/6	7.8 $\pm$ 0.54	
				35	0/6	0/6	> 20	

\* Animals surviving beyond 25 days

**Table 2.** Comparison of the effects of alterations in ambient temperature on the course of infection in mice inoculated with antilog 2.8 ID<sub>50</sub> T.(T.) brucei, LUMP 36. Mice nos. 1-6 were kept at 35°C throughout the 21 days, but mice nos. 7-11 were kept at 35°C for 15 days and then transferred to 22-27°C.

Maintenance Temp. C	Mouse number	Days after inoculation																		
		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
35	1	+	++	+++	+++	-	+	+	+	+	++	++	+++	+++	+++	+++	++	+	+	+
	2	+	+	+	-	-	-	+	+	+	+	+	+	+	++	+++	+++	+++	++	++
	3	+	+	++	-	-	-	+	+	+	+	+	+	++	+++	+++	+++	+	+	-
	4	-	+	-	-	-	-	-	+	-	+	+	+	-	-	-	+	-	+	+
	5	-	-	+	+	++	+++	+++	+	-	-	+	+	++	+++	+++	++	++	+++	+++
	6	+	++	+	-	+	+	+	++	++	+++	+++	+++	D						
35 till day 15 then 22-27	7	+	+	-	-	-	+	+	-	-	+	+	++	++	++	+++	+++	D		
	8	+	+	+	-	-	-	+	+	+	++	+++	+++	D						
	9	+	+	-	-	+	+	+	+	-	-	-	-	+	++	+++	+++	D		
	10	-	+	++	-	-	+	-	-	+	++	+++	+++	+	+	+	++	+++	D	
	11	+	+	++	-	-	-	-	-	-	-	+	+	++	+++	+++	D			
	12	+	++	++	++	+++	++	++	+++	+++	D									

D denotes dead



Table 3a.

Comparison of mean body temperatures of groups of 6 uninfected and 6 T. (T.) brucei infected mice maintained at 28°, 30°, 32° and 35° C ambient temperatures.

Ambient Temp. °C	Mice	Mean body Temp. °C	t-value	Significant at 5%
28	Infected	37.5 ± 0.05	0.25	Not significant
	Uninfected	37.5 ± 0.07		
30	Infected	37.40 ± 0.05	0.77	Not significant
	Uninfected	37.46 ± 0.07		
32	Infected	38.03 ± 0.11	1.89	Not significant
	Uninfected	37.75 ± 0.09		
35	Infected	39.34 ± 0.04	0.30	Not significant
	Uninfected	39.23 ± 0.10		

Table 3c. Uninfected mice.

Comparison of mean body temperatures of groups of mice maintained at various ambient temperatures.

Ambient Temp. °C	Mean body Temp. °C	t - value	Significant at 5% level
28	37.53 ± 0.05	0.65	Not Significant
30	37.46 ± 0.07		
28	37.53	2.00	Not Significant
32	37.75 ± 0.09		
28	37.53	14.36	Significant
35	39.23 ± 0.10		
30	37.46	1.72	Not Significant
32	37.75		
30	37.46	9.54	Significant
35	39.23		
32	37.75	10.64	Significant
35	39.23		

Table 4. Comparison of mortality of groups of mice inoculated with antilog 1.8 ID<sub>63</sub> T.(T.) brucei stabilate, LUMP 36 and maintained at 28, 30, 32 and 35°C ambient temperatures respectively.

Ambient Temp. °C	Inoculum (antilog ID <sub>63</sub> )	No. of mice <u>parasitaemic</u> No. inoculated	Mean survival time $\pm$ S.E.(x) (Days)
28	2.1	6/6	4.16 $\pm$ 0.16
30	2.1	6/6	4.33 $\pm$ 0.21
32	2.1	6/6	9.40 $\pm$ 1.98
35	2.1	6/6	*5 16

\* 5 animals survived beyond day 16



Table 5. Comparison of mortality of groups of mice inoculated with T.(D.) vivax, T.(N.) congolense, T.(T.) brucei and T.(T.) evansi and maintained at 28°C and at 32°C.

Stabilate	Inoculum (antilog ID <sub>63</sub> )	Ambient Temp. °C	No.mice parasi- taemic No.ino- culated	M.S.T. ± S.E. (x) (Days)
<u>T.(D.) vivax</u> LUMP 45	1.4	28	6/6	5.50 ± 0.22
		32	6/6	9.33 ± 1.49
<u>T.(N.) congolense</u>	*	28	6/6	10.66 ± 0.21
		32	3/6	18.50 ± 6.5
<u>T.(T.) brucei</u> LUMP 36	1.8	28	6/6	4.16 ± 0.16
		32	6/6	9.40 ± 1.98
<u>T.(T.) evansi</u> LUMP 55	2.1	28	6/6	9.75 ± 0.62
		32	6/6	5 25

\* Infective dose not known but mice were inoculated with 0.1 ml containing 10<sup>3</sup> trypanosomes in BS.

Table 6. Showing the proportion of stumpy forms of T.(N.) congolense infections in mice kept at 35°C. The observations are taken from two mice among the 6 mice inoculated and kept at 35°C for 25 days before they were transferred to 22-27°C.

Days after inoculation	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Total no. counted per 50 fields	8	27	151	582	18	64	50	39	43	4	32	152	22	19	49	90
Stumpy	-	1	8	-	1	4	6	3	-	-	-	1	-	2	1	-
% stumpy	-	3.7	5.3	-	5.5	6.25	12	7.7	-	-	12.5	0.6	-	10.5	2.04	-

Table 7.

The course of infection following inoculation of mice with antilog 0.8 ID<sub>63</sub> and antilog 2.8 ID<sub>63</sub> T.(T.) brucei stabulate LUMP 36. The mice were maintained at 35°C for 17 days and then transferred to 22-27°C.

Inoculum	antilog ID63	Dilution	Mouse number	Days after inoculation																						
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
2.8	Antilog 2		1	+	++	++	++	-	-	-	+	+	+	++	++	++	+	+	++	++	++	+	+	+	+	+
			2	+	++	++	++	++	+	+	-	-	+	+	+	++	++	+	+	++	++	++	+	+	+	+
			3	+	++	++	++	++	-	-	-	-	-	-	-	-	-	-	+	+	++	++	++	+	+	+
			4	+	++	++	++	++	+	+	+	++	++	++	++	++	++	++	+	+	++	++	++	+	+	+
			5	+	++	+	+	-	-	-	-	++	++	+	-	-	-	+	+	+	++	++	++	+	+	+
			6	+	++	+	+	+	-	-	-	-	-	+	-	-	-	-	-	-	+	+	++	++	++	+
0.8	Antilog 4		1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
			2	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
			3	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	++	D
			4	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			5	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
			6	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	++	++	D

D Denotes dead  
- No trypanosomes seen



Table 8. Comparison of the mortality of T. (T.) brucei LUMP 36 infections in various pure lines (strains) of mice maintained at ambient temperatures of respectively 22-27°C and 35°C.

Strain of mice	Ambient temp. °C	<u>No. parasitaemic</u> <u>No. inoculated</u>	M.S.T. ± S.E. (x) <sup>***</sup> Days
T <sub>0</sub>	22-27	6/6	4.50 ± 0.22
	35	6/6	*3 28 18.0 ± 1.0
** Parkes	22-27	10/10	4.50 ± 0.16
	35	3/10	*2 28
CF <sub>1</sub>	22-27	10/10	4.30 ± 0.15
	35	10/10	*2 28 15.0 ± 3.74
C <sub>3</sub> H	22-27	10/10	4.50 ± 0.16
	35	10/10	13.80 ± 1.52

\* Number of parasitaemic mice surviving beyond 28 days after inoculation.

\*\* Heavy mortality observed during the first two days after inoculation

\*\*\* MST recorded is for mice which died within 28 days after inoculation

**Table 9.** Showing the mortality of 6 groups of mice inoculated with decreasing dose levels of T.(T.) brucei LUMP 128 stabilate. The trypanosome strain was passaged in mice maintained at 35° C.

Group	No. of mice inoculated	Inoculum (antilog ID <sub>63</sub> )	Mortality at Day - 6
1	6	3.1	6/6
2	6	2.1	5/6
3	6	1.1	4/6
4	6	0.1	1/6
5	6	(— 1.1)	0/6
6	6	(— 2.1)	0/6

Table 10. Course of T. (T.) evansi infection in mice kept at 22-27°C. Comparison of courses in mice previously inoculated with L. 201 ID T. (T.) evansi LUMP 55 and maintained subsequently at 35°C for 20 days and then at 22-27°C for 10 days before challenge, with that in previously uninfected mice maintained at 22-27°C.

Initial Temp. °C	Mouse number	Days after challenge															
		1	2	3	4	5	6	7	8	9	10	11	12- 18	19	20- 27	28	29- 70
35	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
	4	-	+	+	+	+	+	D									
	5	-	-	-	-	+	+	+	+	+	+	+	+	D			
	6	-	+	+	+	+	+	+	+	+	+	D					
22-27	1	-	+					↑	D								
	2	-	+				↑	D									
	3	-	+				↑	D									
	4	-	+			↑	D										
	5	-	+			↑	D										
	6	-	+				↑	D									

→ Acute infection

D Death

- No trypanosomes seen.



Table 11. The course of T.(T.) evansi LUMP 55 infection in two groups of 6 mice altered between 22-27°C and 35°C environments. The mice were inoculated with antilog 2.1 ID<sub>63</sub>.

Initial Temp. °C	Mouse number	Days after inoculation																	
		4	5	6	7	8	9	10	11-19	20	21	22	23	24	25	26	27-32	33	34-70
22-27 (Group B)	1	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	D	
	2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35 (Group C)	1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	+	+	+	+	+	+	+	+	+	D					
	3	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	+	+	+	+	D							

Period when mice in the group were kept at 35°C

-

No trypanosomes seen

D

Denotes dead

Table 12. The results of attempts to transmit T. (T.) brucei LUMP 36 cyclically through G. morsitans and G. austeni. The strain of trypanosomes used to infect the flies was maintained in mice kept at 22-27°C in which it caused a monomorphic infection and in mice kept at 35°C in which it caused a pleomorphic infection.

Source of infection		Species	
		<u>G. morsitans</u>	
Mice kept at 22-27°C	No. exposed to infected feed	8	105
	No. dying between 10 and 24 days after infected feed		15
	No. surviving beyond 24 days		74
	Total no. dissected for trypanosome infection		89
	No. with gut infection		7
	% Gut infection		7.8%
	No. with sal. gland infection		-
Mice kept at 35°C		<u>G. morsitans</u>	<u>G. austeni</u>
	No. exposed to infected feed	49	69
	No. dying between 10 and 24 days after infected feed	8	6
	No. surviving beyond 24 days	32	57
	Total number dissected for trypanosome infection	40	63
	No. with gut infection	3	4
	% gut infection	7.5%	6.3%
	No. with sal-gland infection	-	1
	No. with gut + sal. gland	-	1*

\* The same fly showing both gut and sal. gland infection

Table 13. Showing the number of T.(T.) brucei and T.(T.) evansi surviving in TC 199 medium supernate washings of mouse peritoneal macrophage cultures.

Species of trypanosomes	Log number of trypanosomes inoculated	Log Number of trypanosomes surviving after inoculation (Hours)		
		24	48	72
<u>T.(T.) brucei</u>	5.25	5.11	4.97	-
<u>T.(T.) evansi</u>	5.80	5.47	-	-

- denotes, no trypanosomes seen



Table 14. Observations on 8-day-old chick embryos inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) brucei stabilate, LUMP 128, and incubated at 37°C and 39°C and examined between days 4 and 13 after inoculation.

Incubation Temp. °C	Embryos examined	Frequency	%
37	Total number inoculated	38	—
	Number alive during examinations	26	68.4
	Number infected	13	34.2
	Number negative	13	34.2
	Number dead	12	31.6
	Control	10	—
39	Total number inoculated	38	—
	Number alive during examinations	24	63.1
	Number infected	17	44.7
	Number negative	7	18.4
	Number dead	14	36.9
	Control	10	—

No mortality recorded among the control embryos.

Table 15. Compares the infectivity and survival of 8-day-old chick embryos inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) brucei and incubated at 37° and 39°C. The embryos were examined in batches on day 4, 5, 7, 8, 9, 10, 11 and 13 after inoculation.

Incubation Temp. °C	Number examined	Number examined on days after inoculation										Total
		4	5	7	8	9	10	11	13			
37	Total examined	4	8	8	3	3	6	3	3	38		
	Number alive	3	5	5	3	3	5	1	1	26		
	Number infected	0	3	3	3	1	1	1	1	13		
39	Total examined	4	8	8	3	3	6	3	3	38		
	Number alive	4	7	7	1	1	3	1	0	24		
	Number infected	3	5	7	1	1	0	0	0	17		

Table 16. Showing the number of various morphological forms of T.(T.) brucei observed from 50 fields of stained blood preparations from all infected embryos on days 7 and 8 after inoculation. The embryos were incubated at 39°C.

Day after inoculation	Total count per 50 fields	Giant forms		Round forms		Vacuolated forms	
		Count	%	Count	%	Count	%
7	898	30	3.3	11	1.1	21	2.2
8	647	1	0.16	21	3.2	41	6.3



Table 17(a). Agglutination titrations of T.<sub>6</sub>(T<sub>6</sub>) brucei substrains isolated from T.<sub>6</sub>(T<sub>6</sub>) brucei LUMP 128 infected mouse A and B maintained at 35°C.

Homologous reactions

Mouse A

Anti- sera Anti- gen	128	173	179	182
128	10240	-	160	80
173	-	20480	160	-
179	-	40	40960	5120
182	-	-	20480	10240

<u>Antigens from Mouse A</u>	
Antigen	Duration of infection before isolation.
128	0 days
173	11 days
179	18 days
182	25 days

Mouse B

Anti- sera Anti- gen	128	174	183	183	191
128	10240	160	160	-	20
174	80	10240	80	1280	320
180	-	-	20480	10240	10240
183	-	-	-	40960	5120
191	-	-	10240	20480	10240

<u>Antigens from Mouse B</u>	
Antigen	Duration of infection before isolation.
128	0 days
174	11 days
180	18 days
183	25 days
191	32 days

Table 17(b). Agglutination titrations of T.(T.) brucei substrains isolated from T.(T.) brucei LUMP 128 infected mouse A and B maintained at 35° C.

Heterologous reactions

Anti- sera Anti- gen	128	173	179	182
128	10240	-	160	80
174	80	320	320	40
180	-	10	20480	10240
183	-	-	-	-
191	-	-	10240	10240

Antigens isolated from mouse B  
but antisera are those prepared  
against substrains from mouse A.

Anti- sera Anti- gen	128	173	179	182	191
128	10240	160	160	-	20
173	-	-	-	-	-
179	-	-	20480	10240	2560
182	-	-	10240	40960	10240

Antigens isolated from mouse A  
but antisera are those prepared against  
substrains from mouse B.

Table 18. Course of T. (T.) brucei LUMP 128 infection in mice inoculated with antilog 2.1 ID<sub>63</sub> and maintained at 22-27° and at 35° C. The animals were examined at short intervals to see at what stage remissions occur in mice kept at 35° C.

Ambient Temp. °C	Mouse number	Log number of trypanosomes/ml at hours after inoculation								
		24	45	54	66	72	78	90	99	120
22-27	1	+	6.60	7.04	8.04	8.41	8.68	D		
	2	+	6.60	7.11	7.93	8.36	8.72	D		
	3	+	6.07	7.00	7.77	8.23	8.65	9.07	D	
	4	+	6.30	6.84	8.07	8.43	8.66	D		
	5	+	6.97	7.07	7.90	8.14	8.67	9.23	D	
	6	+	6.30	7.07	8.07	8.57	8.74	D		
35	1	-	-	+	5.70	-	-	-	-	-
	2	-	+	+	7.00	-	-	-	-	-
	3	-	+	+	6.78	7.14	+	-	+	+
	4	-	-	+	6.00	+	-	-	+	+
	5	-	-	+	6.00	+	-	-	-	-
	6	-	-	+	+	-	+	-	+	-

+ animal parasitaemic, but the count is too low for haemocytometer count  
 - animal non-parasitaemic  
 D animal dead



Table 19. Showing parasitaemias of mice inoculated with T.(T.) brucei LUMP 128. 6 mice are treated with Diminazene aceturate at 66 hours and the others are not treated.

Mice	Mouse number	Log number of trypanosomes/ml at hours after inoculation					
		24	48	66	75	96	120
Treated after 66 hours	1	-	6.95	8.24	7.68	+	-
	2	+	7.14	8.14	8.04	+	-
	3	-	6.69	8.00	7.50	+	-
	4	-	6.47	7.20	6.60	-	-
	5	-	6.90	8.55	8.20	7.00	-
	6	-	6.84	7.98	7.20	+	-
Untreated	1	-	7.00	8.14	8.66	D	
	2	+	7.20	8.50	8.71	D	
	3	-	6.95	8.20	8.65	D	
	4	+	7.04	7.83	8.79	D	
	5	-	6.30	7.44	8.23	8.68	D
	6	-	6.84	8.20	8.43	8.74	D

D denotes dead

**Table 20.** Showing protection of mice injected simultaneously with T.(T.) brucei LUMP 128 and IRS, NRS and saline. Mice previously shown to be protected against T.(T.) brucei LUMP 128 challenge.

Sample used	<u>No. parasitaemic</u> No. inoculated	Mean survival time $\pm$ S.E. (x)	Protection after 30 days
IRS	3/6	5.00	3/6
NRS	3/6	5.00	3/6
Normal saline	3/6	14.00 $\pm$ 4.5	3/6

**Table 21.** Characteristics of T.(T.) brucei LUMP 128 as observed in 6 non-irradiated and in 8 irradiated mice maintained at 35°C. The number of trypanosomes was recorded from 20 fields at 1000x magnification.

Environment		Days after inoculation									
		3	4	5	6	7	8	9	10	11	12
Control mice at 35°C	No. animals examined	6	6	6	6	6	6	6	6	6	6
	No. trypanosomes counted	4	140	231	205	26	2	5	64	362	475
	No. multiplying	-	22	13	2	-	-	1	5	33	31
	No. posteronuclears	-	-	-	2	-	-	-	-	-	-
	% multiplying	-	15.7	5.6	0.97	-	-	20	7.8	9.1	6.5
X-irradiated mice at 35°C	No. animals examined	8	8	8	6	6	6	4	1	1	D
	No. trypanosomes counted	32	321	1779	2021	2395	1509	1267	563	1383	
	No. multiplying	3	31	76	55	45	5	15	17	59	
	No. posteronuclears	-	-	-	388	347	731	470	37	34	
	% multiplying	9.4	9.6	4.3	2.7	1.9	0.33	1.2	3.0	4.2	
	% posteronucleated	-	-	-	19.2	14.5	48.4	37.1	6.6	2.5	

- No organisms seen  
D Animal dead



**Table 22.** Showing the proportion of the various morphological forms observed in cyclophosphamide treated mice kept at 35°C during the last three days of infection. Trypanosomes were enumerated from 50 microscopic fields, x(1000).

Day after inoculation	Total count	Stumpy		Vacuolated		Round		Giant		Adhering to leucocytes	
		Count	%	Count	%	Count	%	Count	%	Count	%
6	1760	698	34	8	0.45	9	0.51	1	0.05	-	-
7	748	296	39.5	13	1.7	11	1.4	8	1.0	6	0.80
8	1455	392	27	1	0.07	2	0.13	4	0.26	28	0.13



Table 24(a).

Effect of ALS treatment; mice inoculated with  
T.(T.) brucei LUMP 128 and maintained at 35°C.

Treat- ment	Mouse number	Days after inoculation													
		2	3	4	5	6	7	8	9	10	11	12	13	14	15
ALS treated	1	D													
	2	D													
	3	-	-	-	+	++	+++	-	-	-	-	-	-	-	-
	4	-	-	-	+	++	+++	+++	+++	+++	+++	+++	++	++	++
	5	-	-	-	+	+	+	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	7	-	-	-	+	++	+++	-	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	9	-	-	+	++	+++	+++	-	+	-	-	+	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	+	-	-	-	-	-	-	-	-
	12	-	-	+	+	-	+	++	+++	++	+	+	+	+	++
Control	1	-	-	+	++	+++	+++	+	-	+	+	+	+	++	+
	2	-	+	++	+++	+++	D								
	3	-	+	+	+++	+++	D								
	4	-	+	++	++	+	-	-	-	+	-	-	-	-	+
	5	-	-	+	++	+	-	-	-	-	-	-	-	-	-
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	7	-	-	+	++	+++	+++	-	-	+	+	-	-	-	+
	8	-	-	+	+	+	-	-	-	+	-	-	-	+	+
	9	-	-	+	++	+++	+++	+++	D						
	10	-	-	+	-	-	-	-	-	+	-	-	-	-	-
	11	-	+	+	++	+	-	-	-	+	-	-	+	-	-
	12	-	+	+	+++	+++	++	++	-	-	+	+	+	++	++

D denotes dead



Table 24 (b). Effect of ALS treatment, mice infected with T.(T.) brucei LUMP 128 and maintained at 35°C

Treatment	Mouse number	Days after inoculation														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ALS treated	1			-		+		++		-		-		++		++
	2			-		+		+		-		+		+		+
	3			-		+		-		-		-	D			
	4			-		-		-		-		+		-		-
	5			-		-		-		-		-		-		+
	6			-		-		++		+++		-		+		+
Control	1			+		+++		D								
	2			-		+		++		-		+		+		+
	3			+		+++		D								
	4			-		++		+++		+++		++		D		
	5			+		+++		D								
	6			-		++		-		-		-		-		-

D denotes dead

Table 22. Plaque forming cells from and spleen weights of groups of mice immunized with SRBC and kept at 22-27°C and at 35°C.

Mouse number	Day 2		Day 4		Day 6		Day 9		Day 11	
	Heated (H)	Control (C)	H	C	H	C	H	C	H	C
1	100	1600	9100	15500	22800	2000	350	4800	150	2300
2	350	150	29700	11100	15500	38000	750	2250	500	1300
3	3450	100	20700	90700	500	6300	1750	1300	400	3750
4	3100	850	31100	53600	700	9000	1000	150	700	4400
Geom. Mean.	782	386	20420	32240	3335	8100	811	1204	380	2651
H C H C H C H C H C										
1	0.152	0.269	0.081	0.184	0.091	0.159	0.128	0.147	0.152	0.116
2	0.123	0.155	0.106	0.141	0.085	0.166	0.147	0.190	0.091	0.146
3	0.144	0.193	0.073	0.248	0.081	0.133	0.106	0.113	0.090	0.098
4	0.226	0.129	0.125	0.131	0.083	0.125	0.195	0.206	0.065	0.186
Average	0.161	0.187	0.096	0.176	0.085	0.146	0.144	0.164	0.099	0.136

PFC per animal

Spleen weights (gm)

Table 26. Attempts to transmit cyclically aberrant T.(T.) brucei infections in mice using G. morsitans and G. austeni. The mice were previously treated with Cyclophosphamide and then kept at 35°C. The flies were fed on mice during remission in parasitaemia when aberrant trypanosomes were numerous.

Source of infection		Species	
		<u>G. morsitans</u>	<u>G. austeni</u>
Mice kept at 35°C	No. exposed to infected feed	89	92
	No. dying between 10 and 24 days after infected feed	17	11
	No. surviving beyond 24 days	39	59
	Total no. dissected for trypanosome infection	56	70
	No. with gut infection	5	7
	% Gut infection	9%	10%
	Salivary gland infection	-	-



## FIGURES



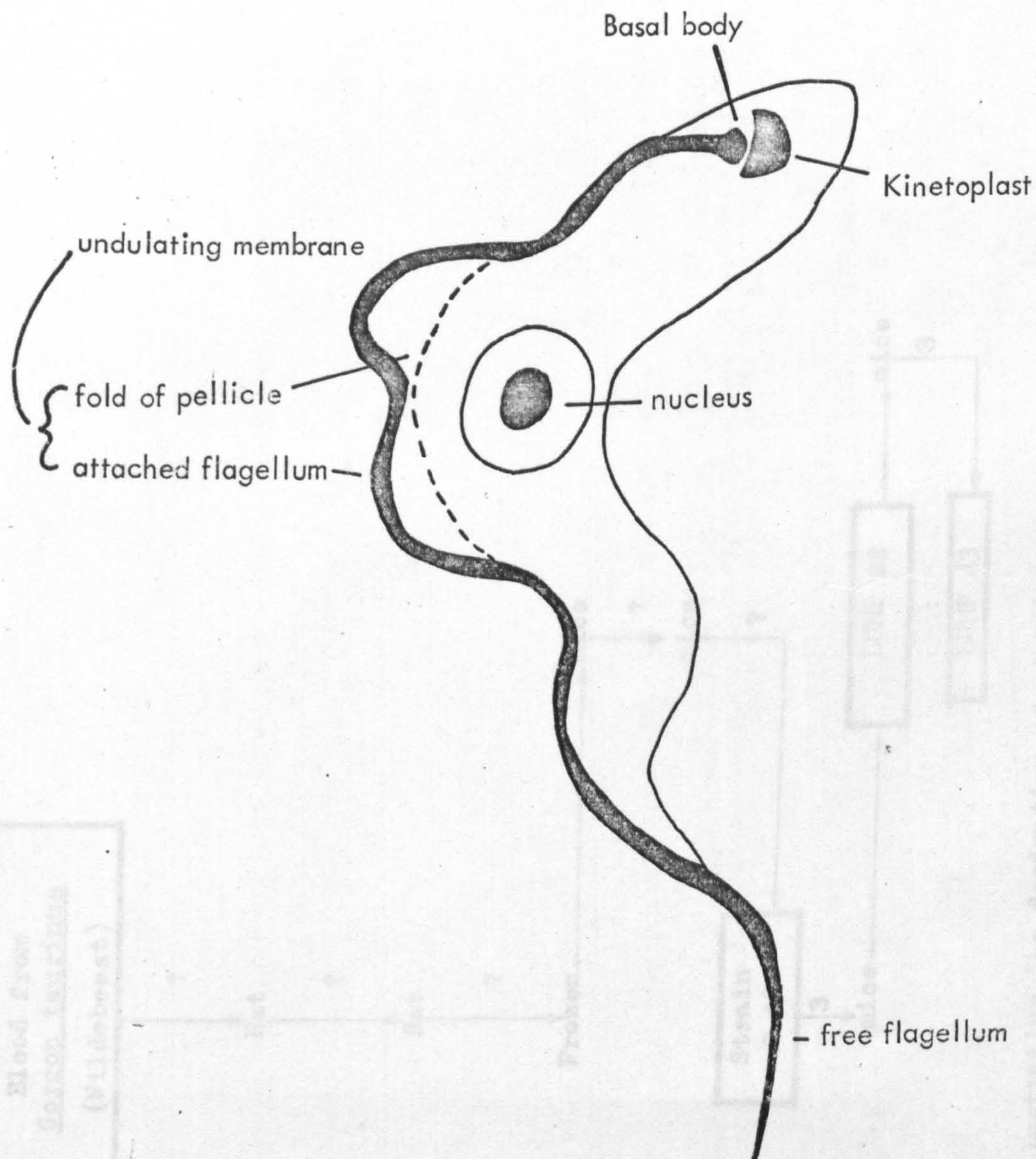


Fig. 1. Diagrammatic illustration of a typical trypomastigote (blood stream form of T. (T.) brucei).



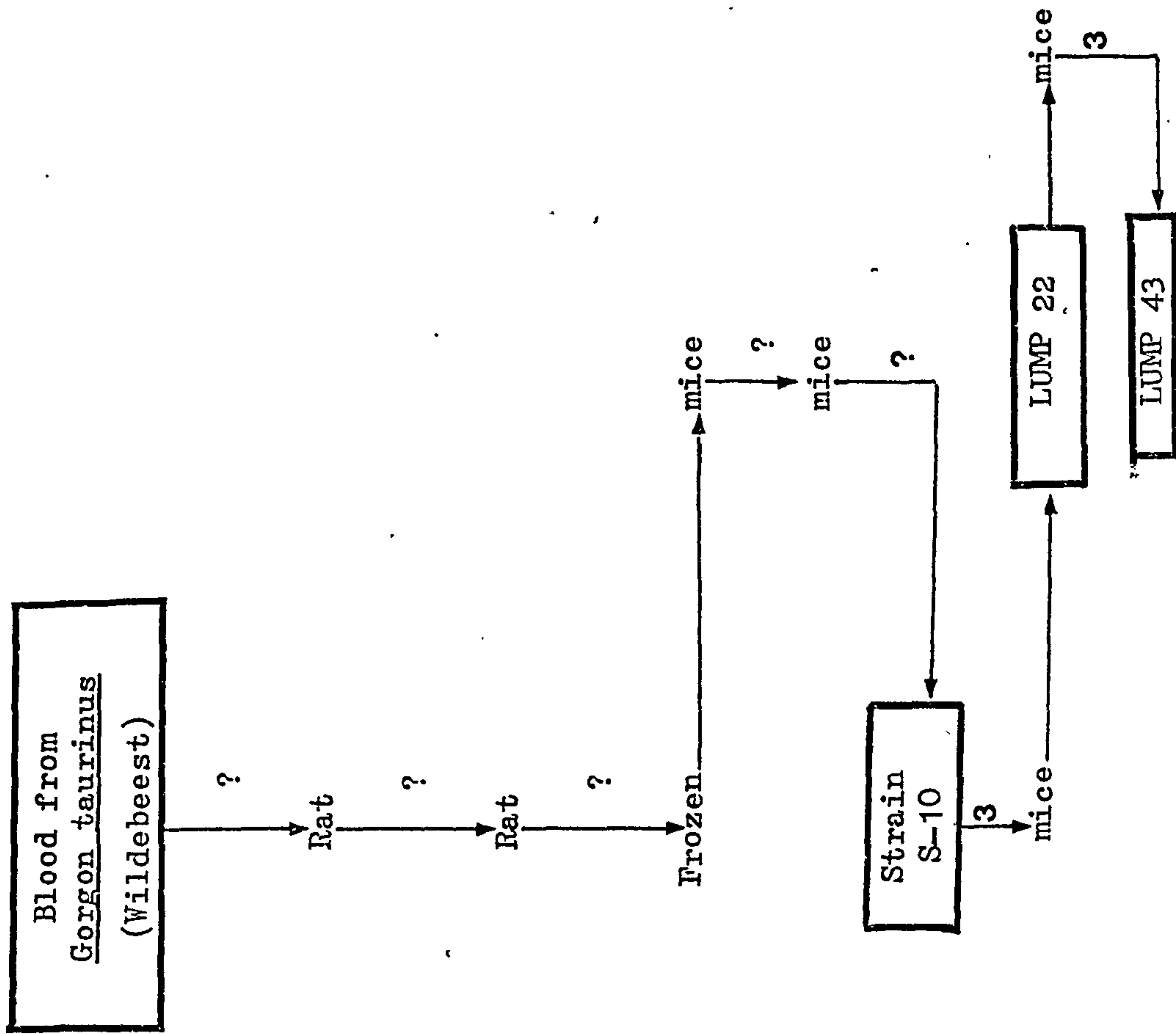


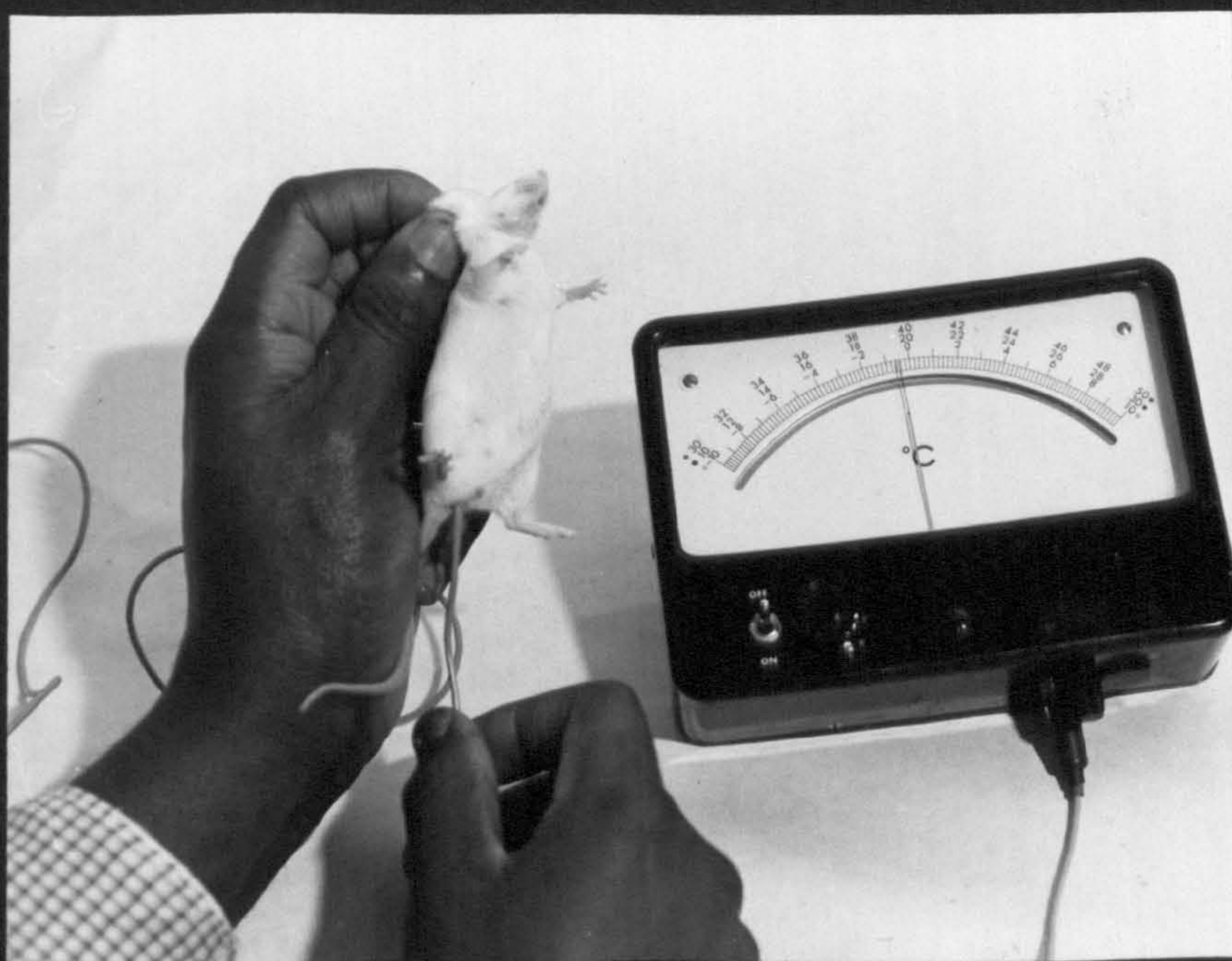
Fig. 2. Flow diagram illustrating the derivation of stablate LUMP 43 from blood of Gorgon taurinus (wildebeest) from Serengeti Tanzania, March 1966. Arrows pointing downwards and associated numerals indicate time elapsed in a mammalian host in days. ? = time elapsed in mammalian host not known.



**Figure 3. Measurement of mouse rectal temperature using a thermistor probe.**



FIG.3.





- a - SKIN GRAFT
- b - SKIN GRAFT
- c - GLASS TUBING FOR PROTECTION
- d - " MITCHELL " CLIP TO PREVENT GLASS TUBING FROM SLIDING OFF .

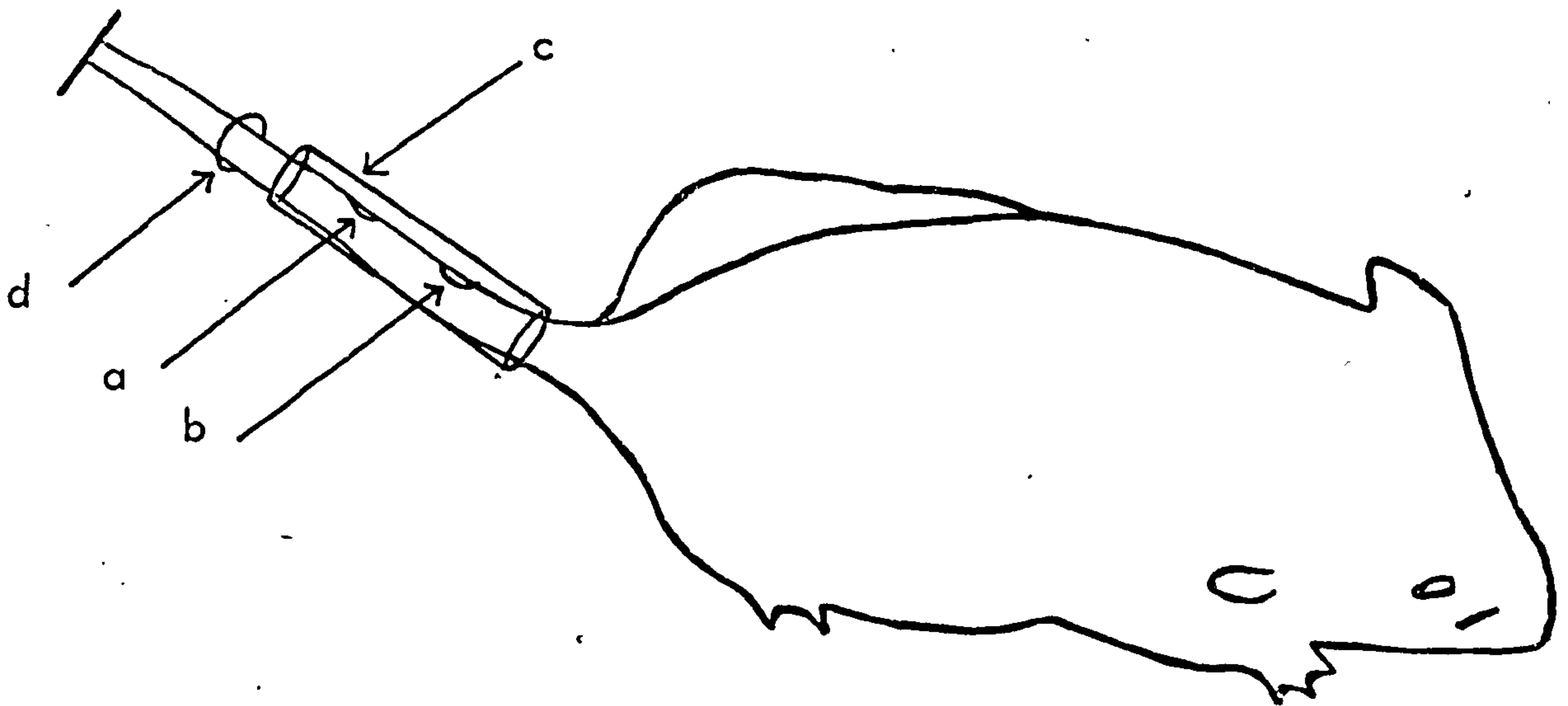


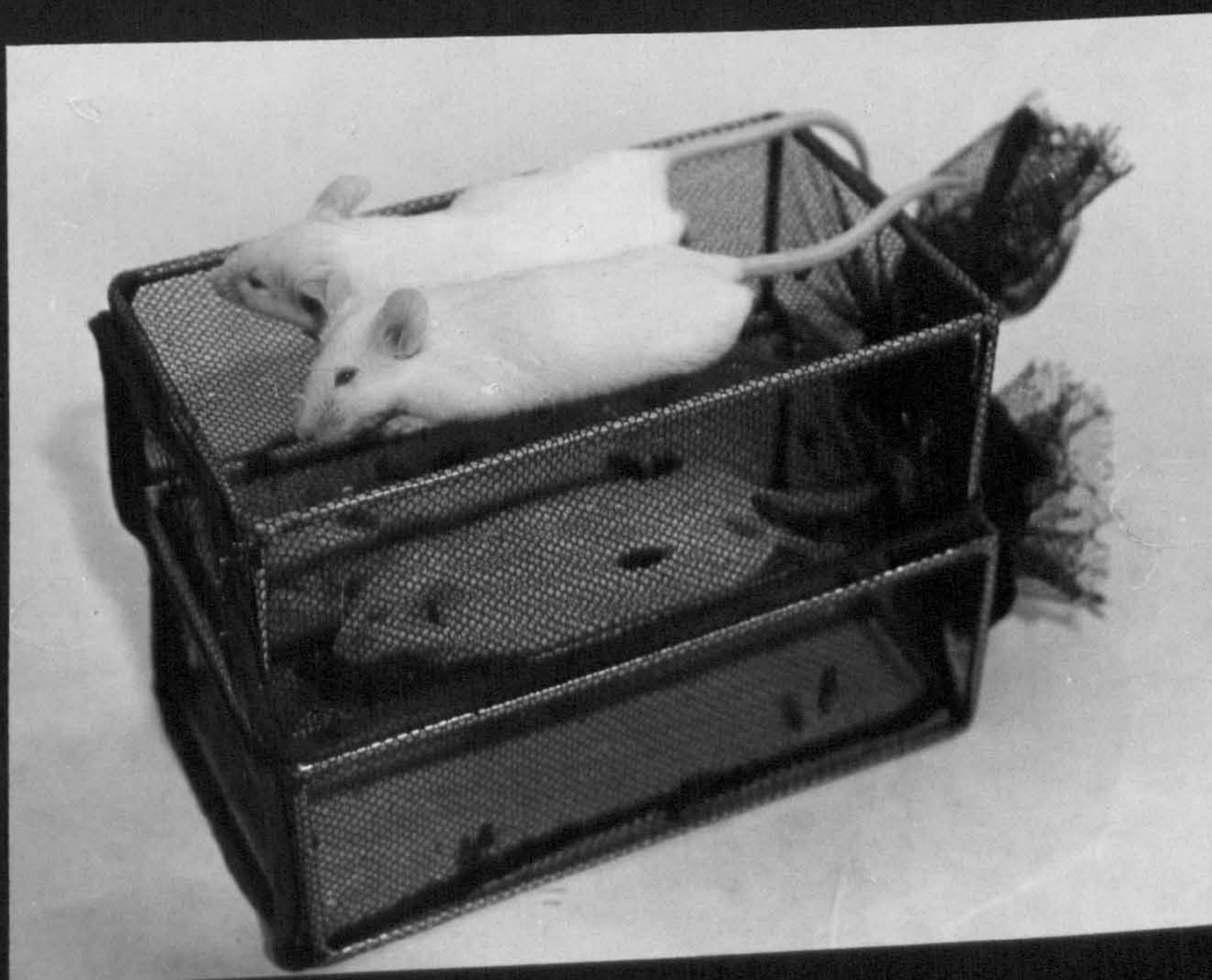
Fig. 4. Diagrammatic illustration of the skin grafting technique.



**Figure 5.      Feeding tsetse flies on anaesthetized mice.**



FIG.5.





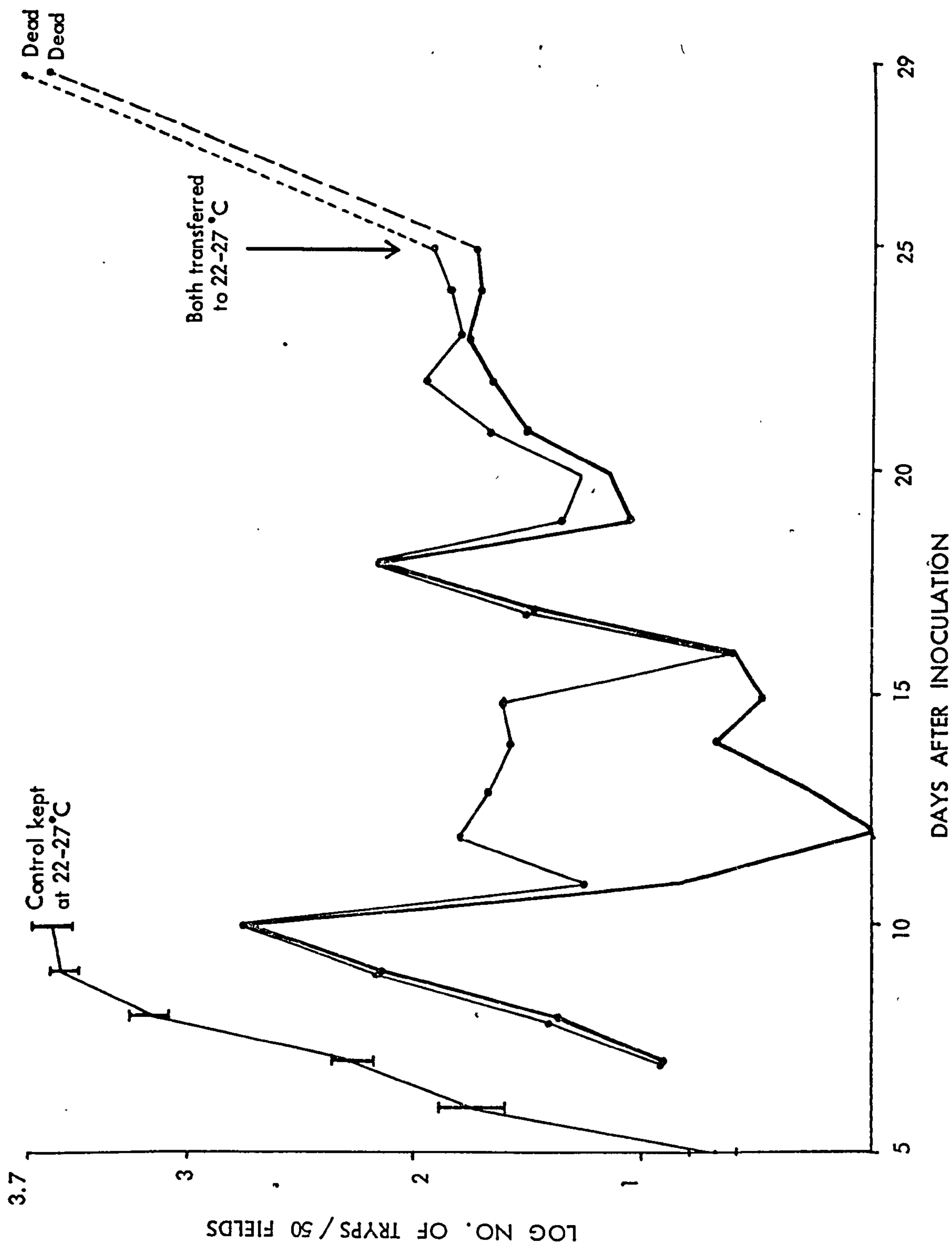


Fig. 6. Showing the parasitaemic curves of T.(N.) congolense infected mice kept at 22-27°C and two mice among the 6 kept at 35°C. The two mice had remarkably similar parasitaemic curves even when they were transferred to 22-27°C.



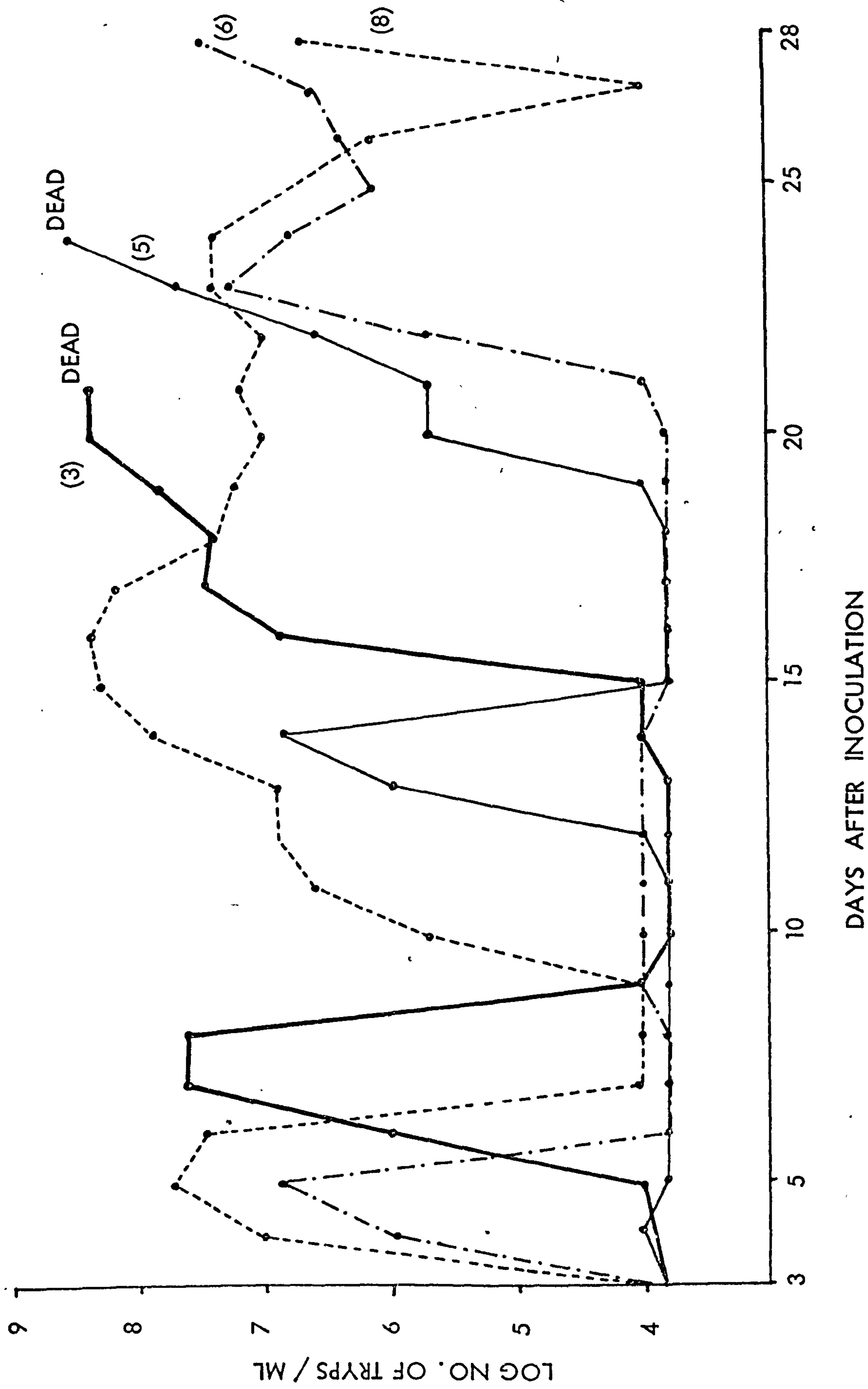


Fig.7. Comparison of parasitaemic curves of 4 among 10 mice inoculated with T.(T.) brucei, LUMP 36 and maintained at 35 C.

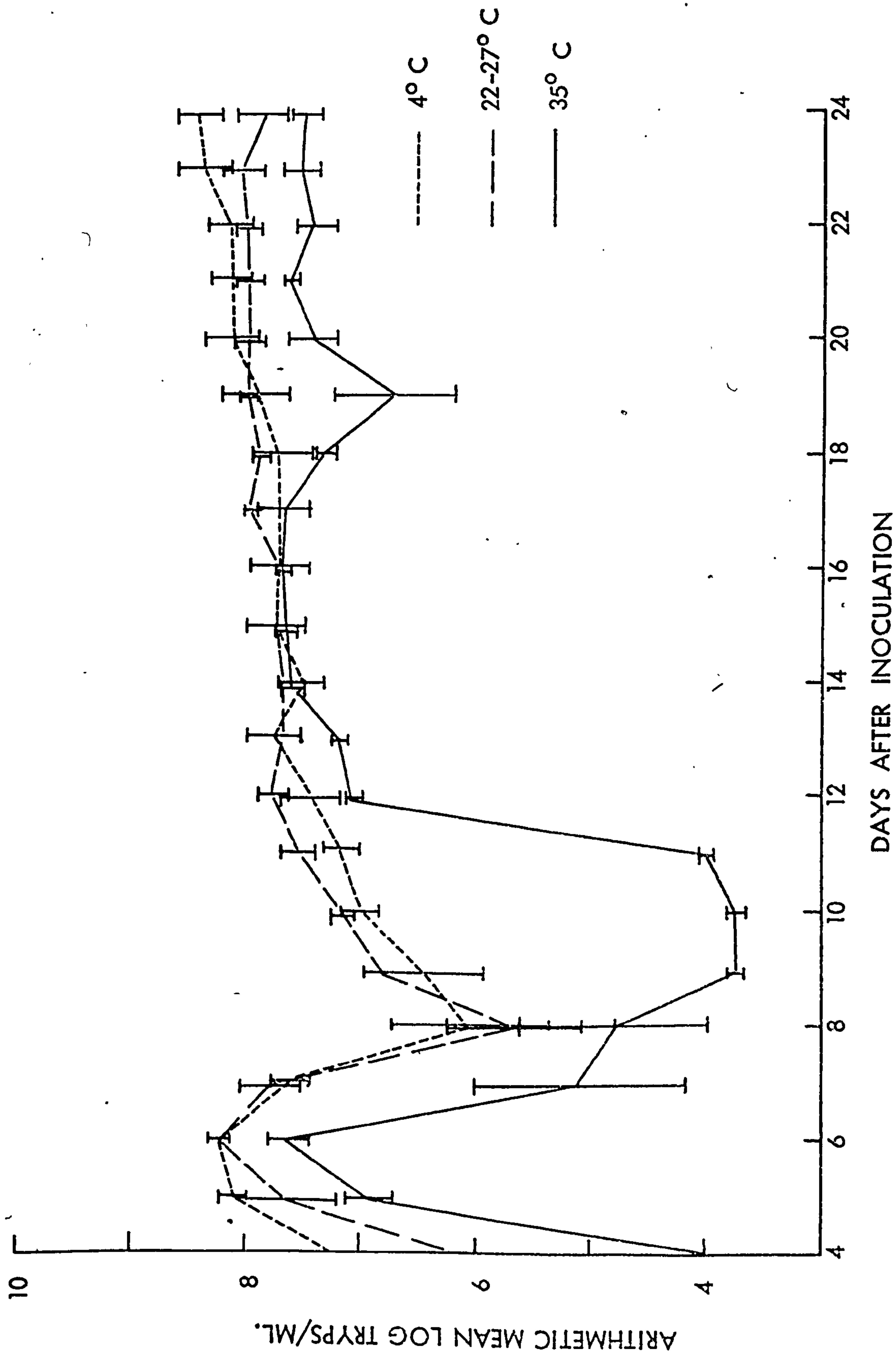


Fig.8. Course of parasitaemia in mice infected with T.(T.) brucei LUMP 43, and maintained at 4, 22-27 and 35 C ambient temperatures. Each curve is based on six mice. Same animals as in Table 1.

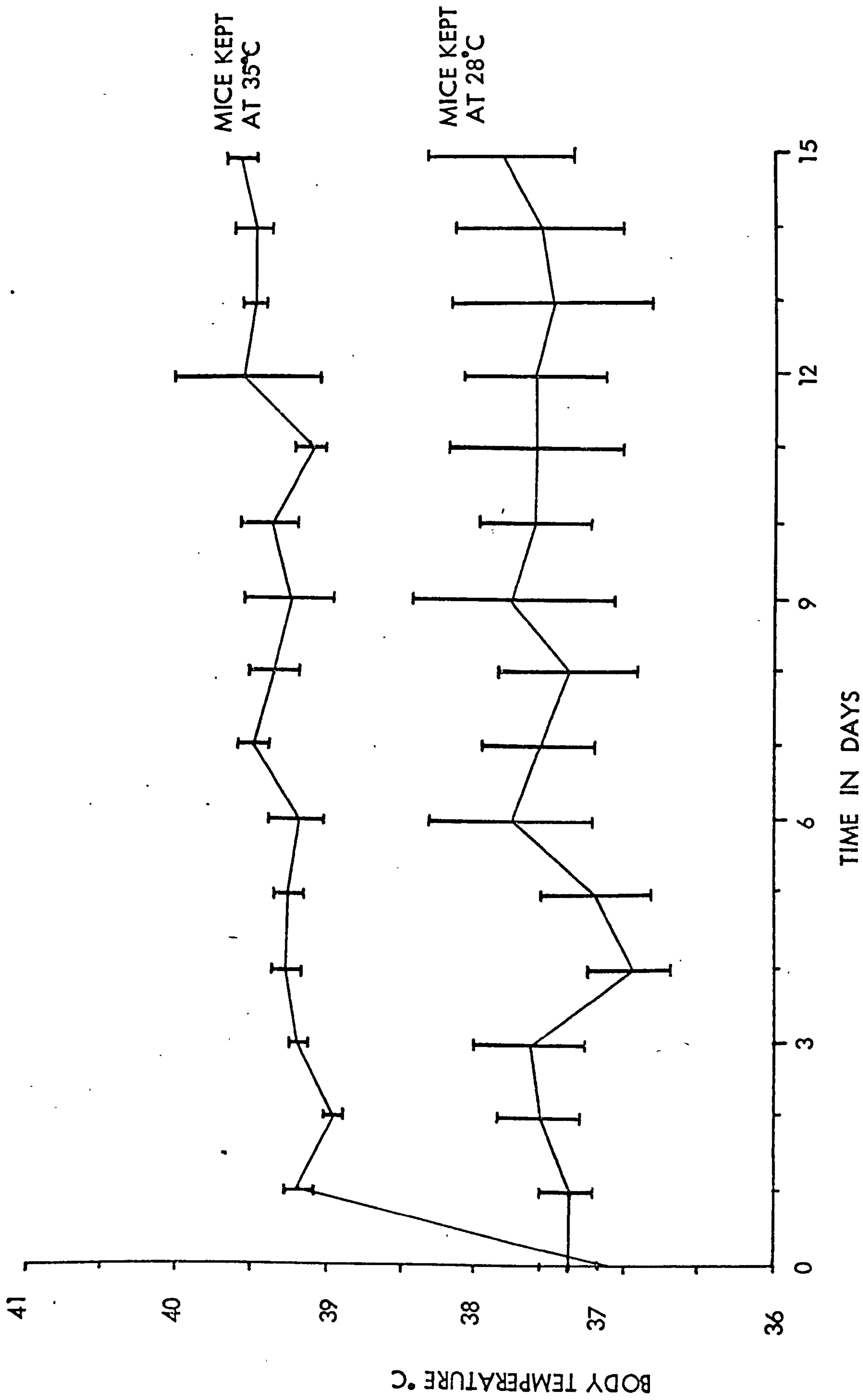


Fig.9. The mean body temperature of groups of non-infected mice kept at 35°C and at 28°C.



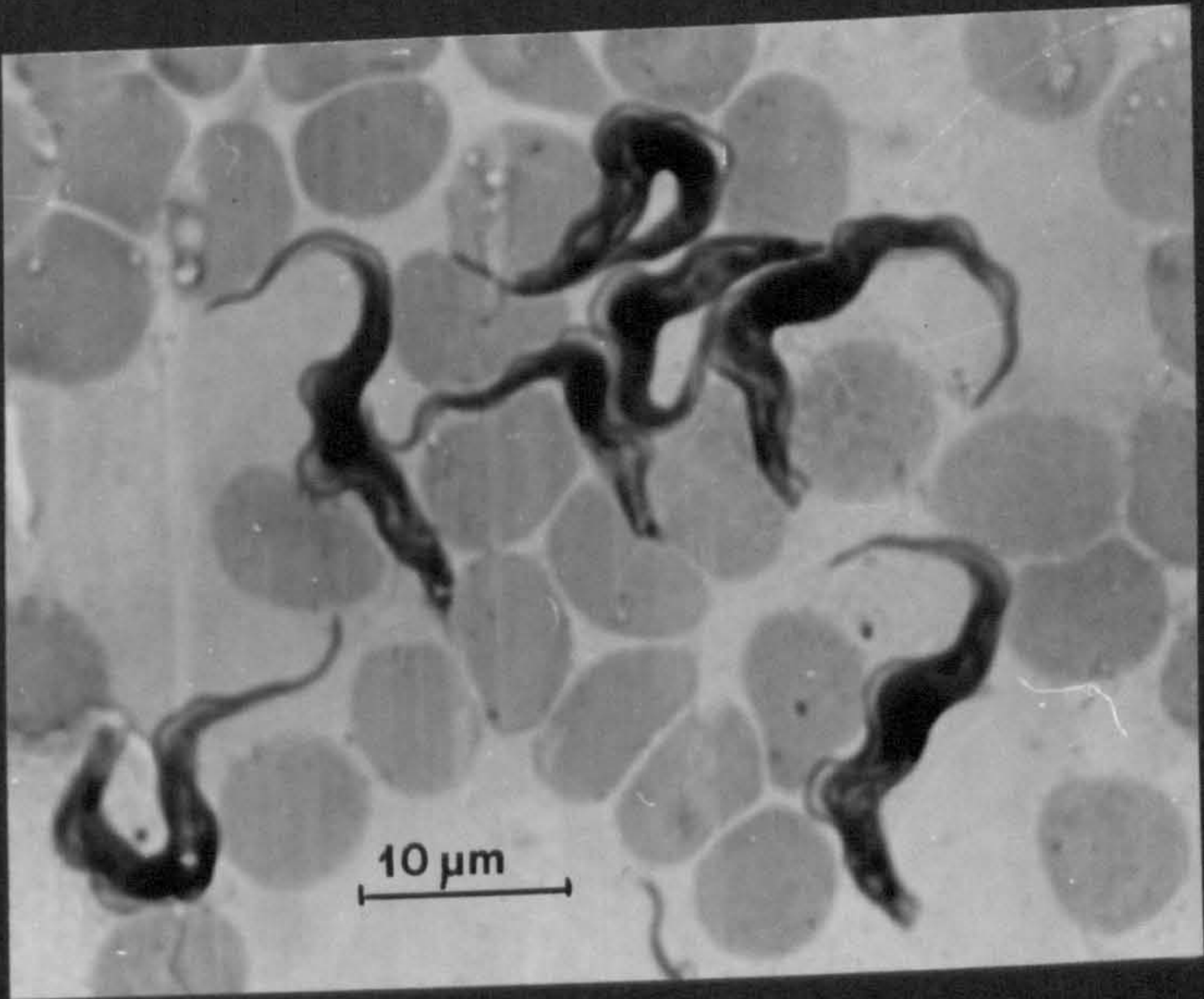
Figure 10. T.(T.) brucei LUMP 36 infection in mice.

- a) Monomorphic forms observed from blood of mice kept at 22-27°C during acute infection (3 days after inoculation)
- b) Typical stumpy form observed in mice kept at 35°C. The stumpy forms were common 7 days after inoculation.



FIG. 10.

a



b

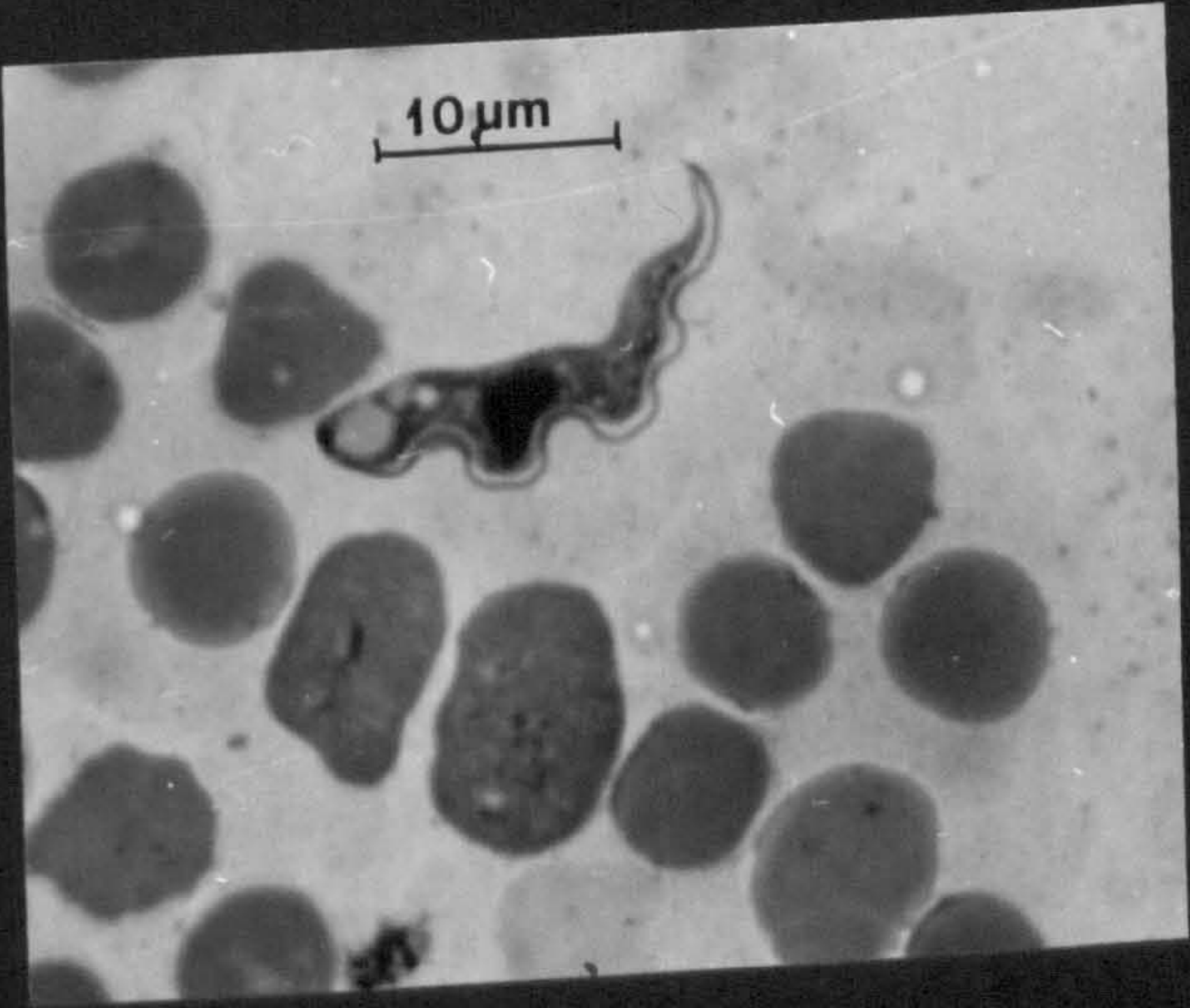




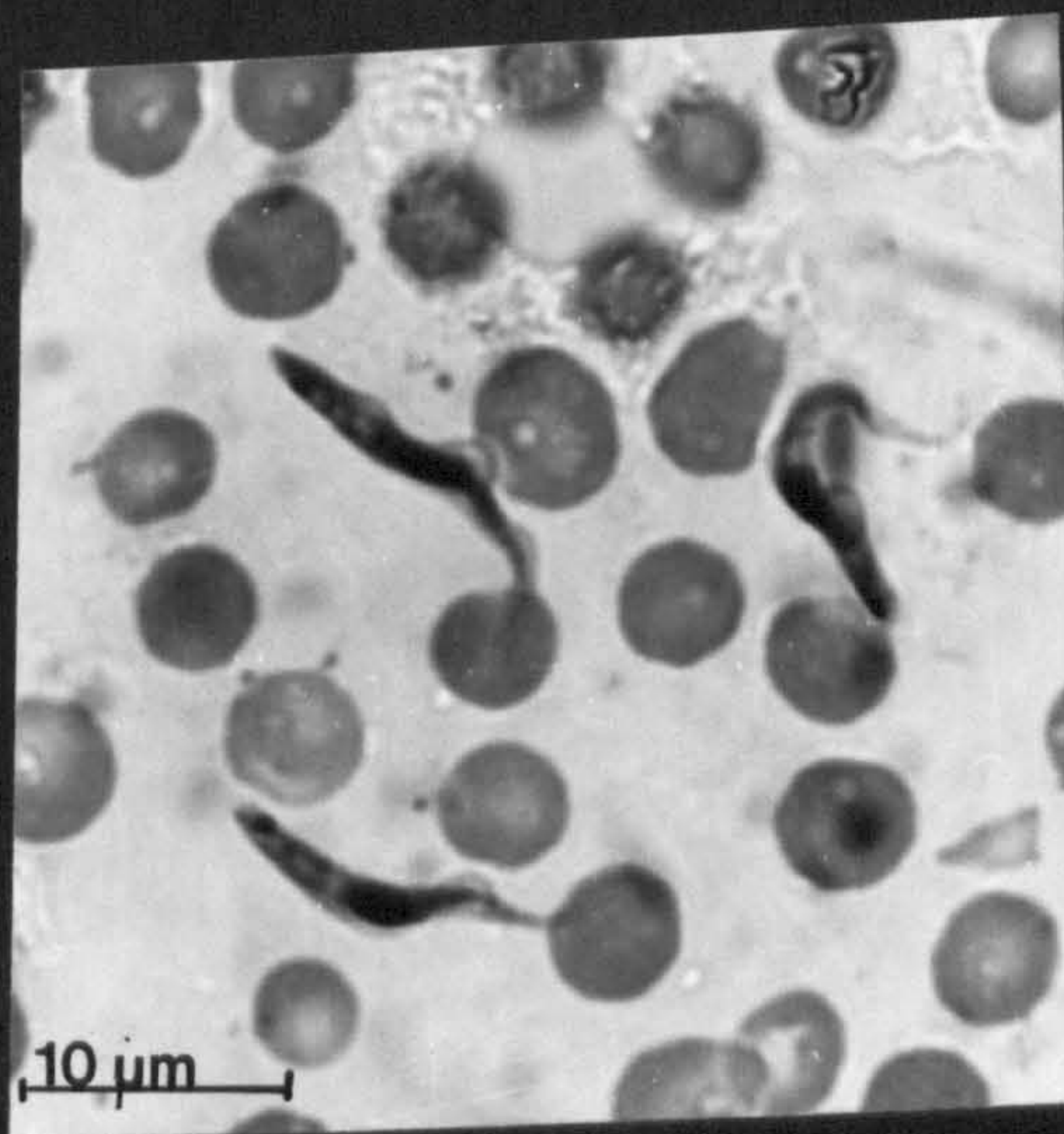
Figure 11. T.(N.) congolense LUMP 52 infection in mice.  
The figure shows monomorphic forms observed  
in mice kept at 22-27°C during peak parasita-  
emia (day 9).

Figure 12a, & b.

T.(N.) congolense LUMP 52 infection in mice  
kept at 35 C. Note the presence of short,  
medium and long forms. These forms were  
first observed on the 7th day of infection.



FIG. 11



12a



12b

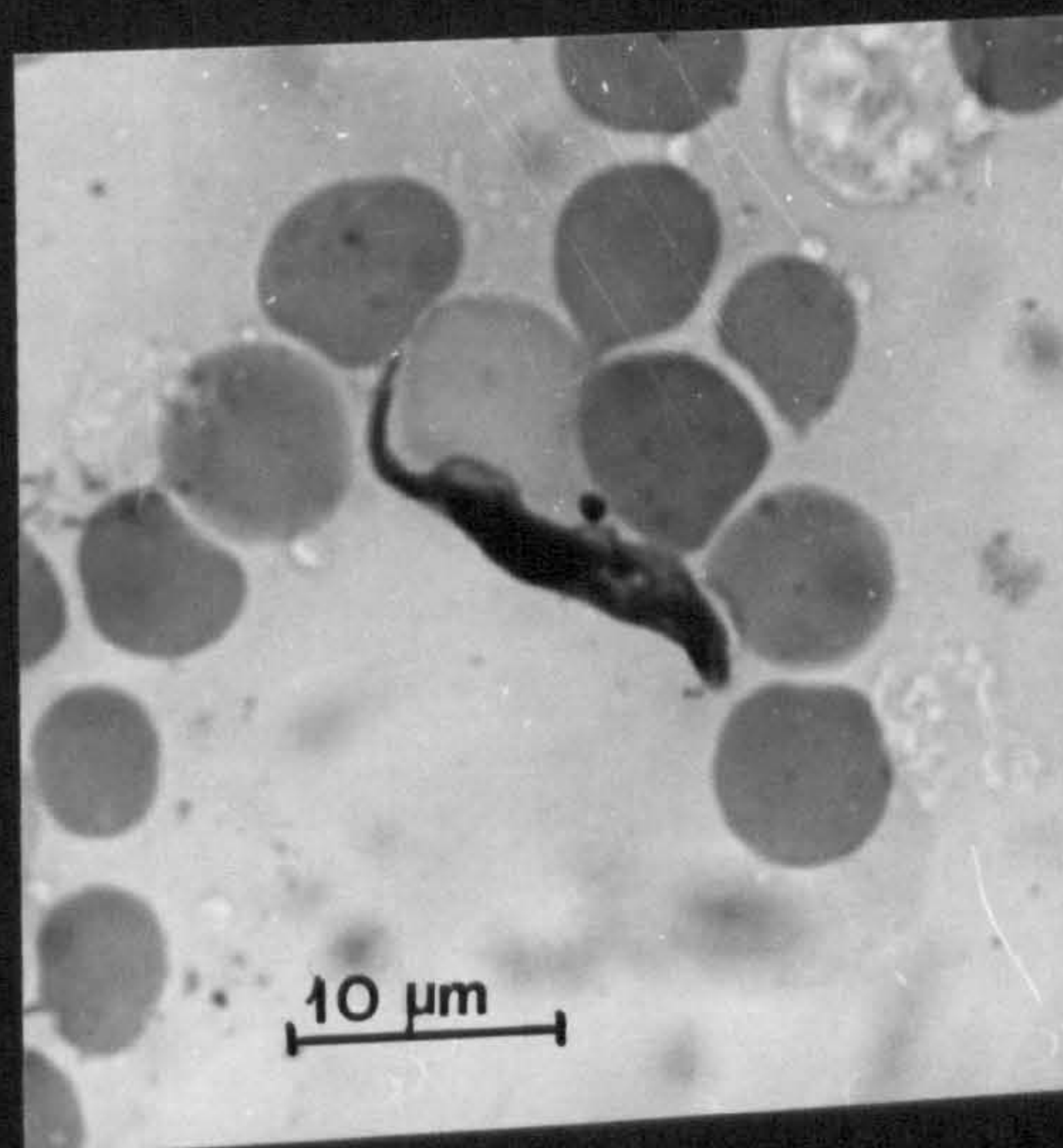
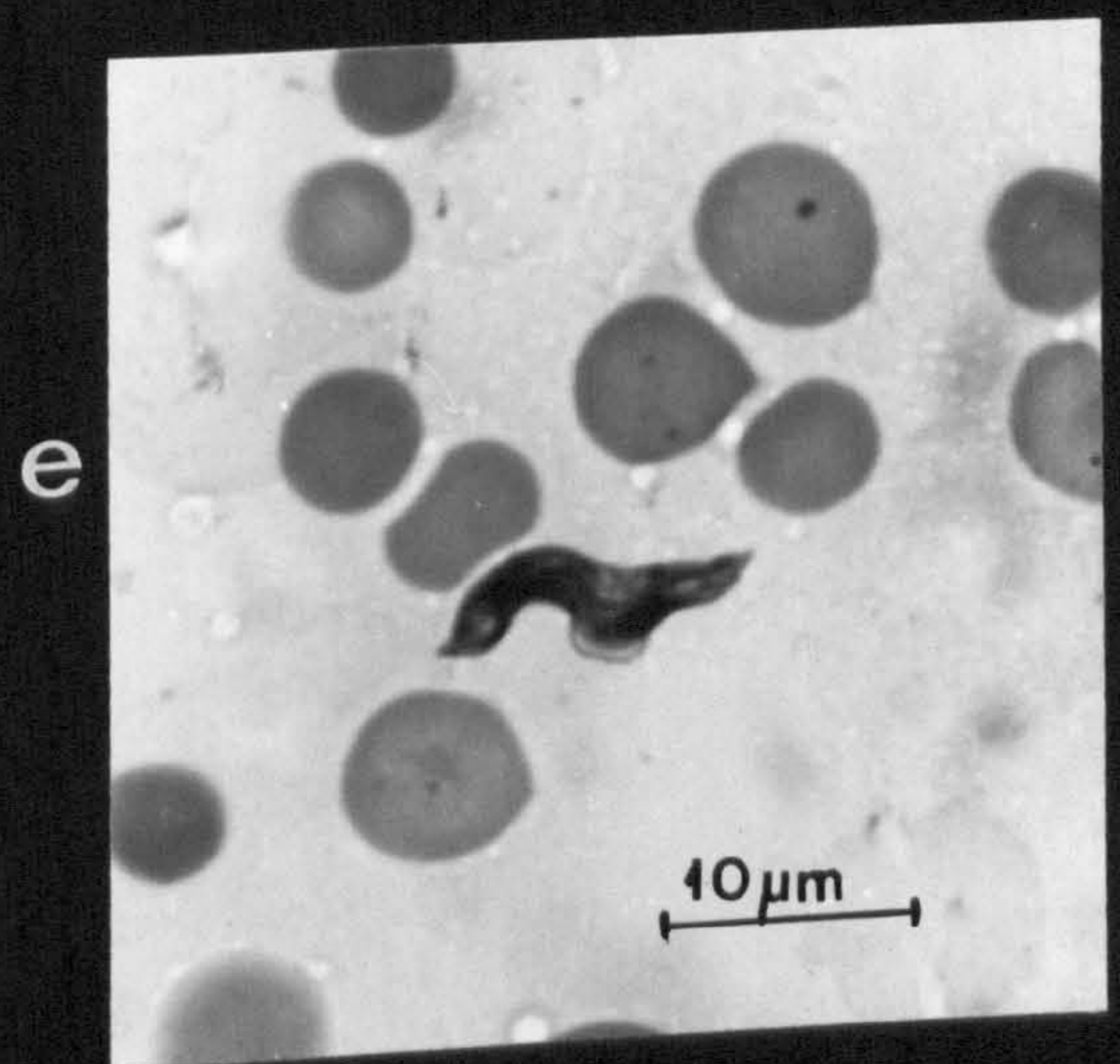
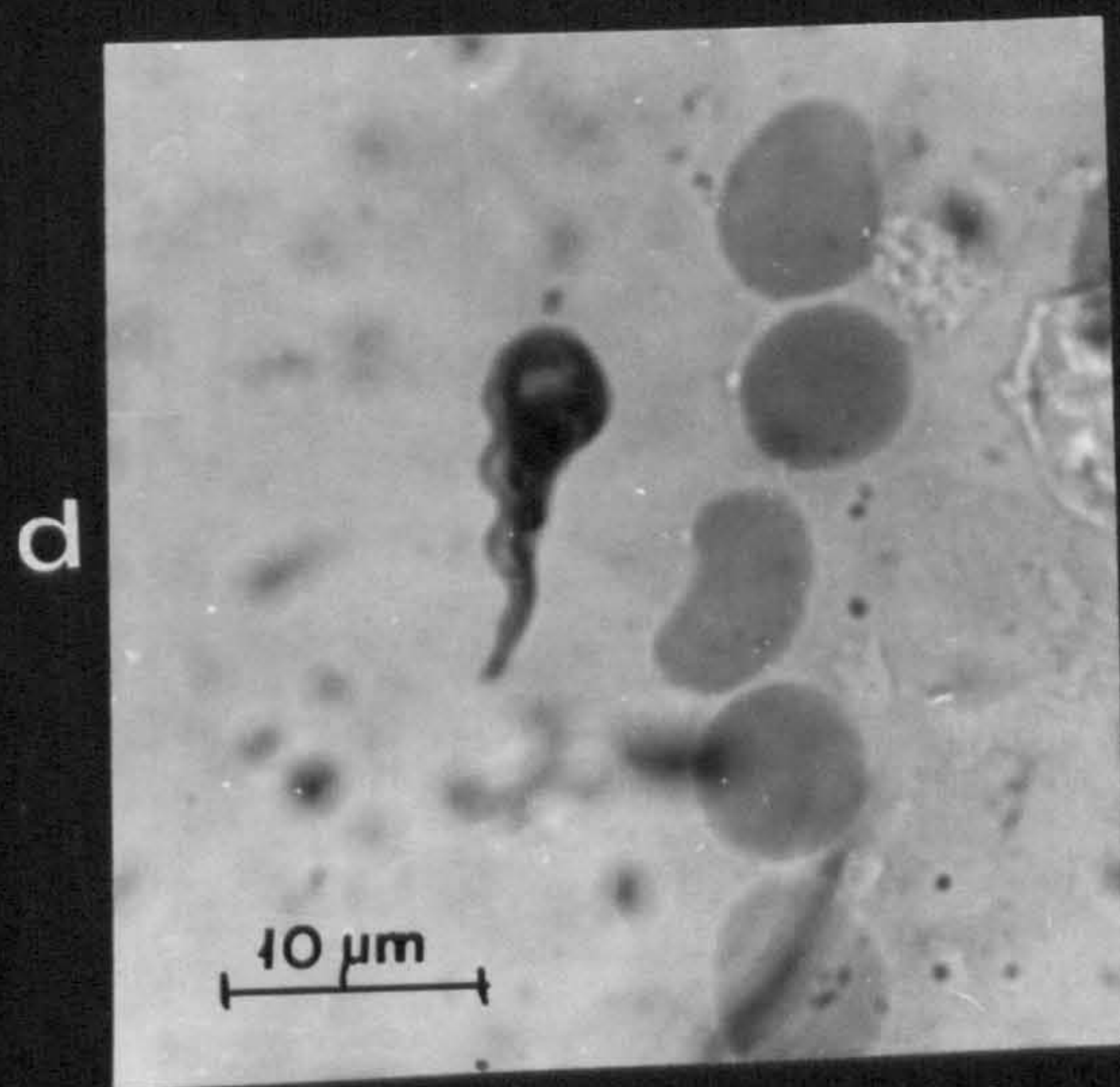
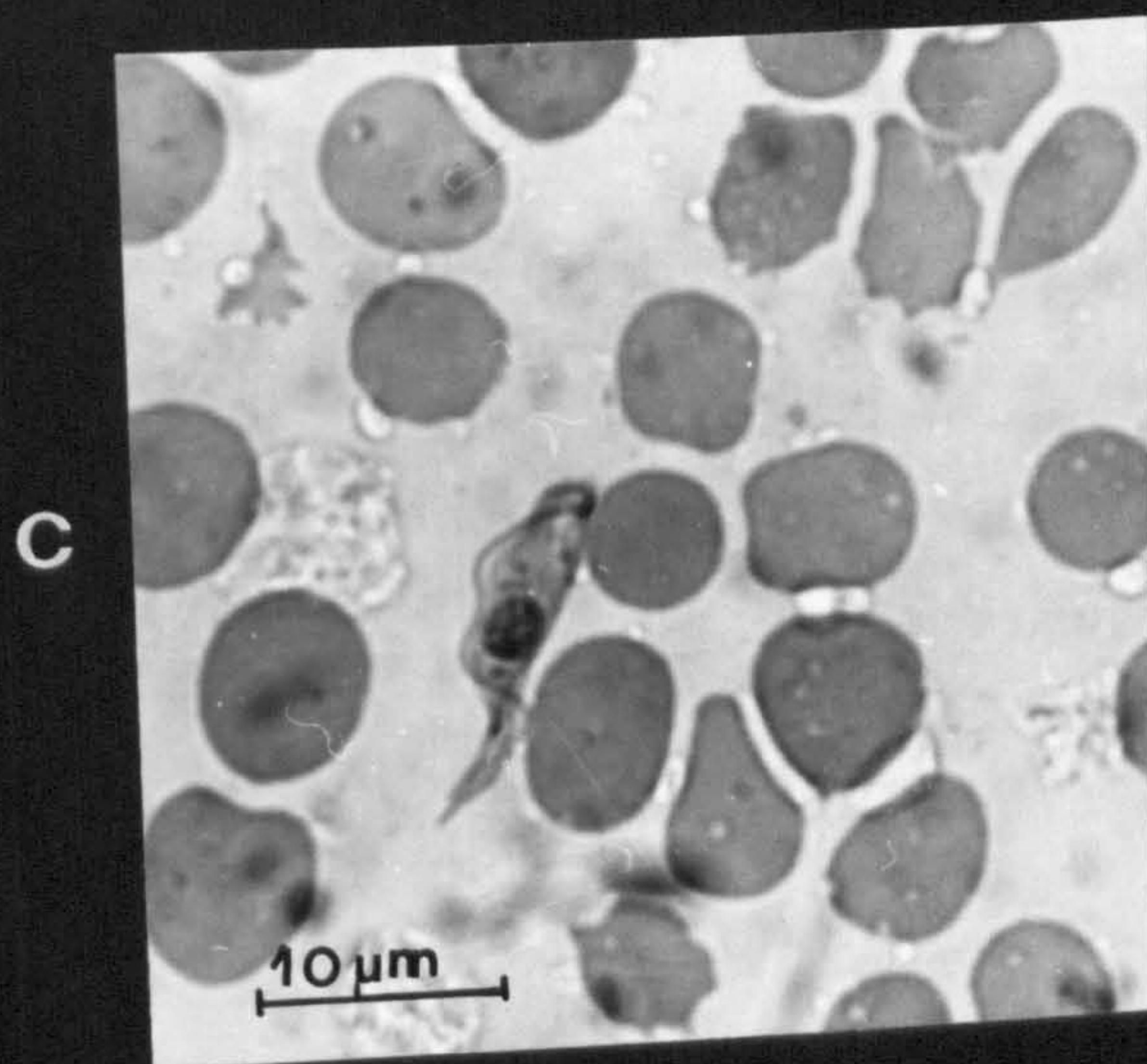
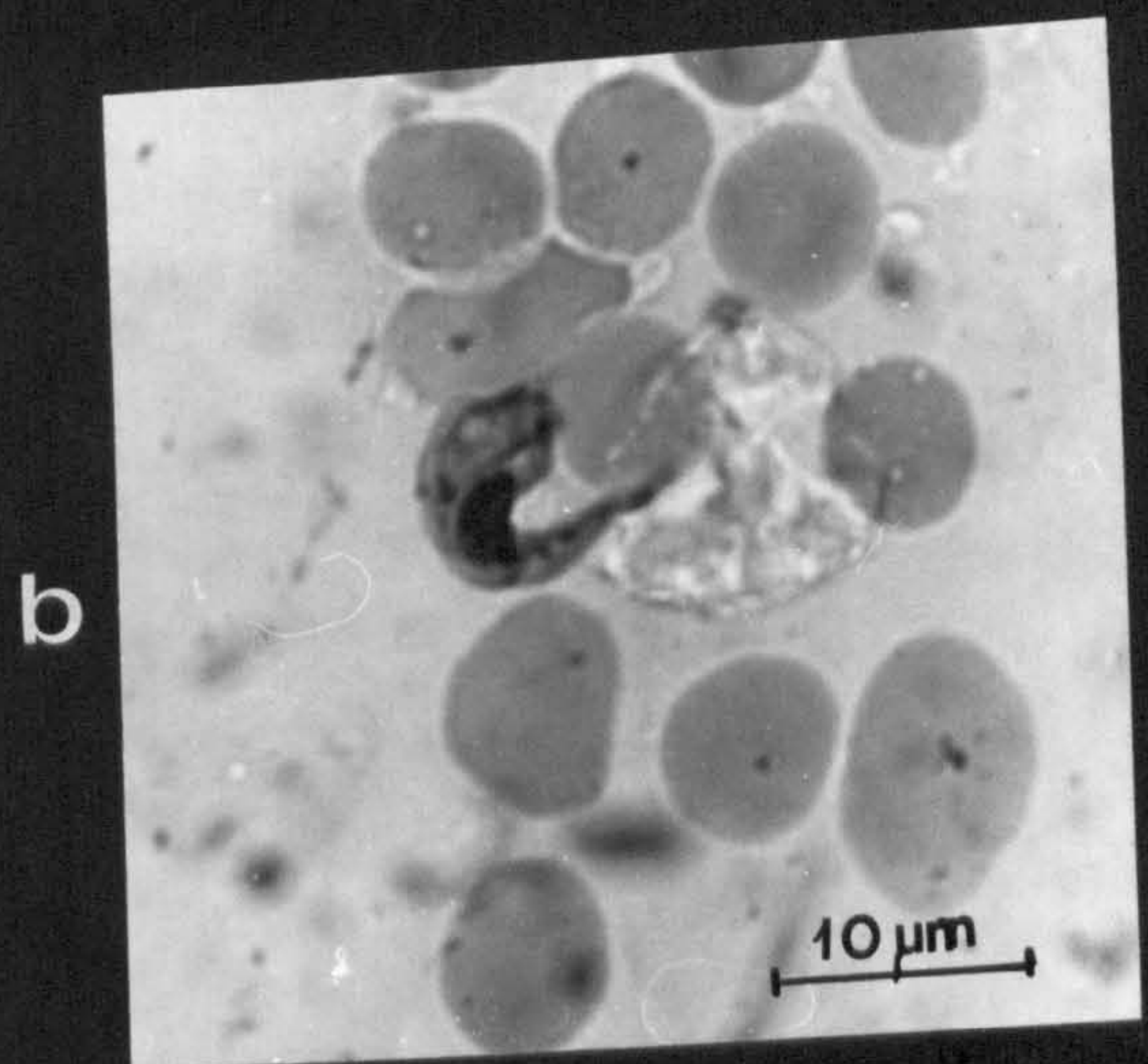
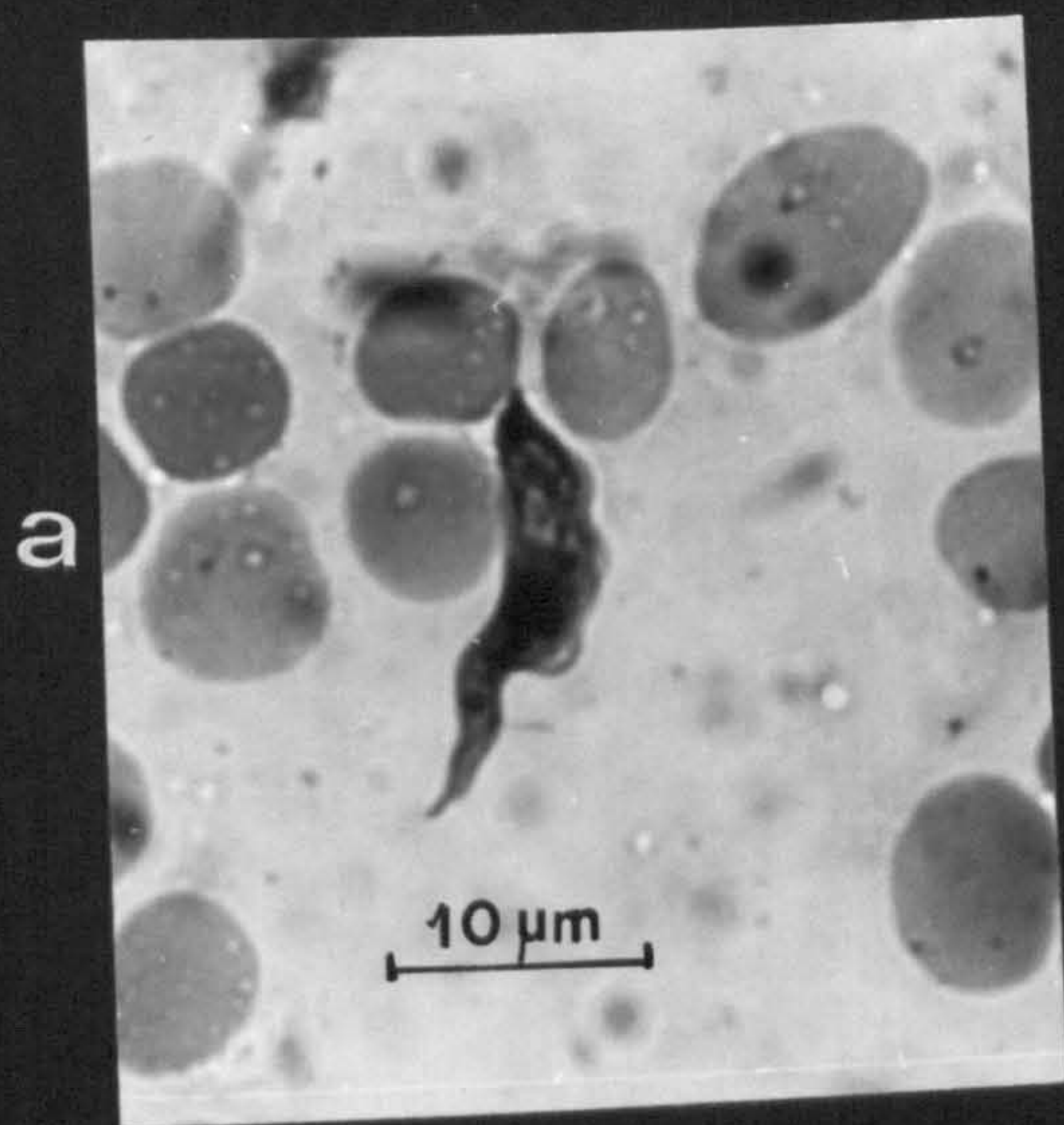




Figure 13. a, b, c, d and e represent the various shapes of stumpy forms of T.(N.) congolense LUMP 52. These forms appeared in the blood of mice kept at 35°C (8 days after inoculation).



fig. 13





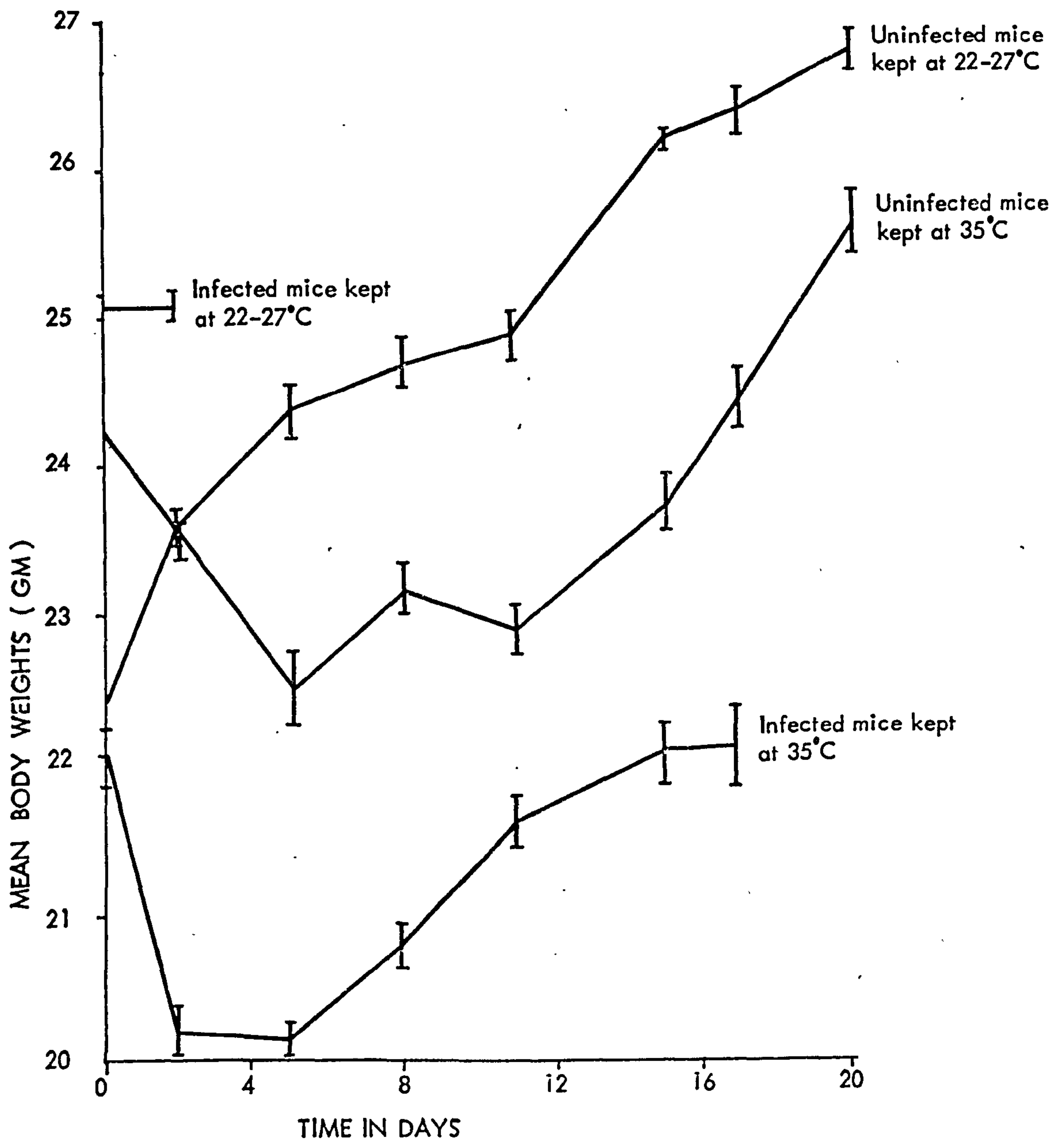


Fig.14. Mean body weights of uninfected and mice inoculated with T. (T.) brucei and maintained at 22-27°C and at 35°C ambient temperatures.

**Figure 15.** T.(T.) brucei LUMP 36 infection in mice kept at 35°C; observations made when infections were subpatent.

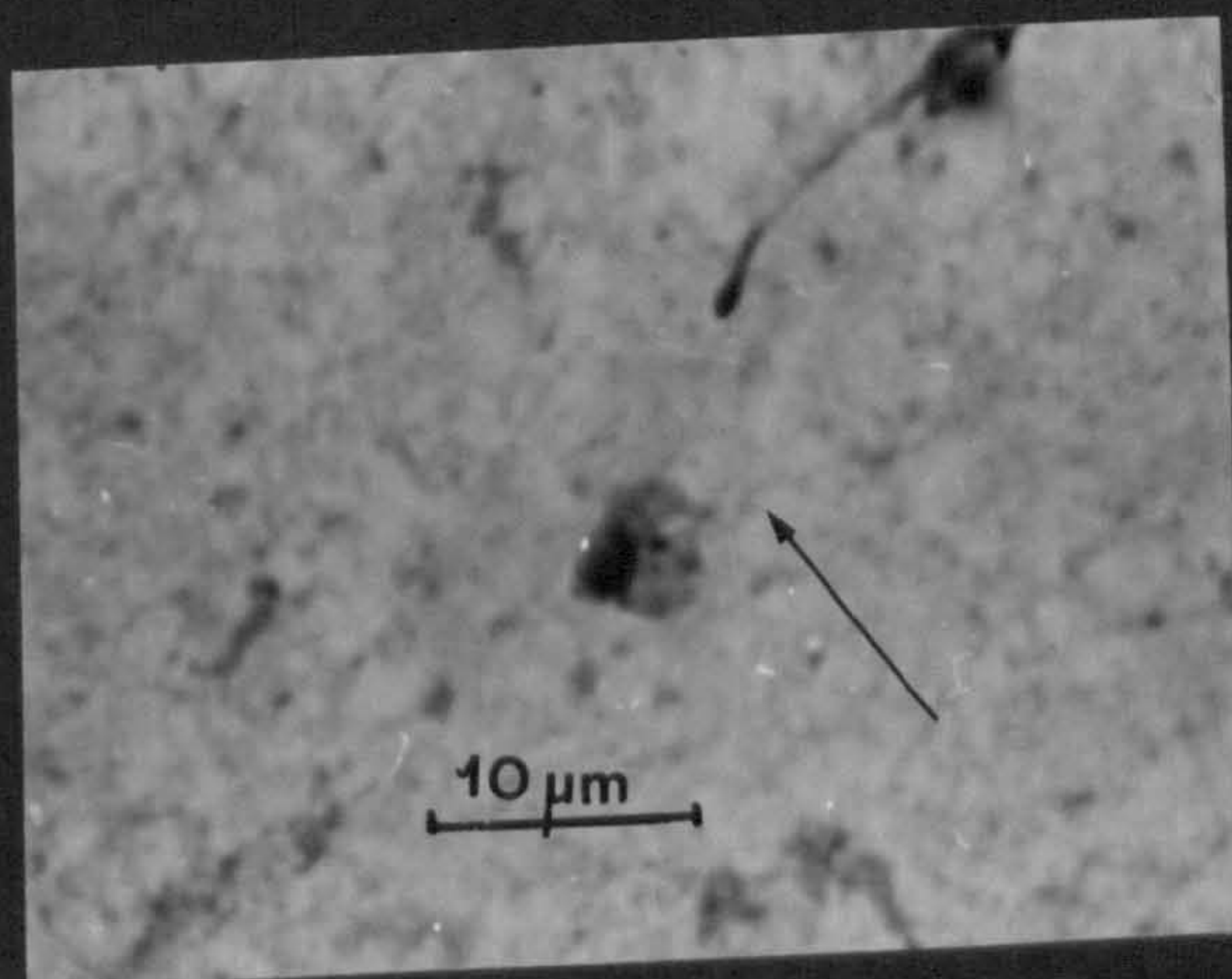
- a) Impression smear of a mouse brain. Note the presence of a sphaeromastigote. The arrow points at the free flagellum.
- b) Impression smear of a mouse brain tissue showing amastigotes in blood vessel.

**Figure 16.** Histological section of the above mouse brain showing a trypomastigote in blood vessel.

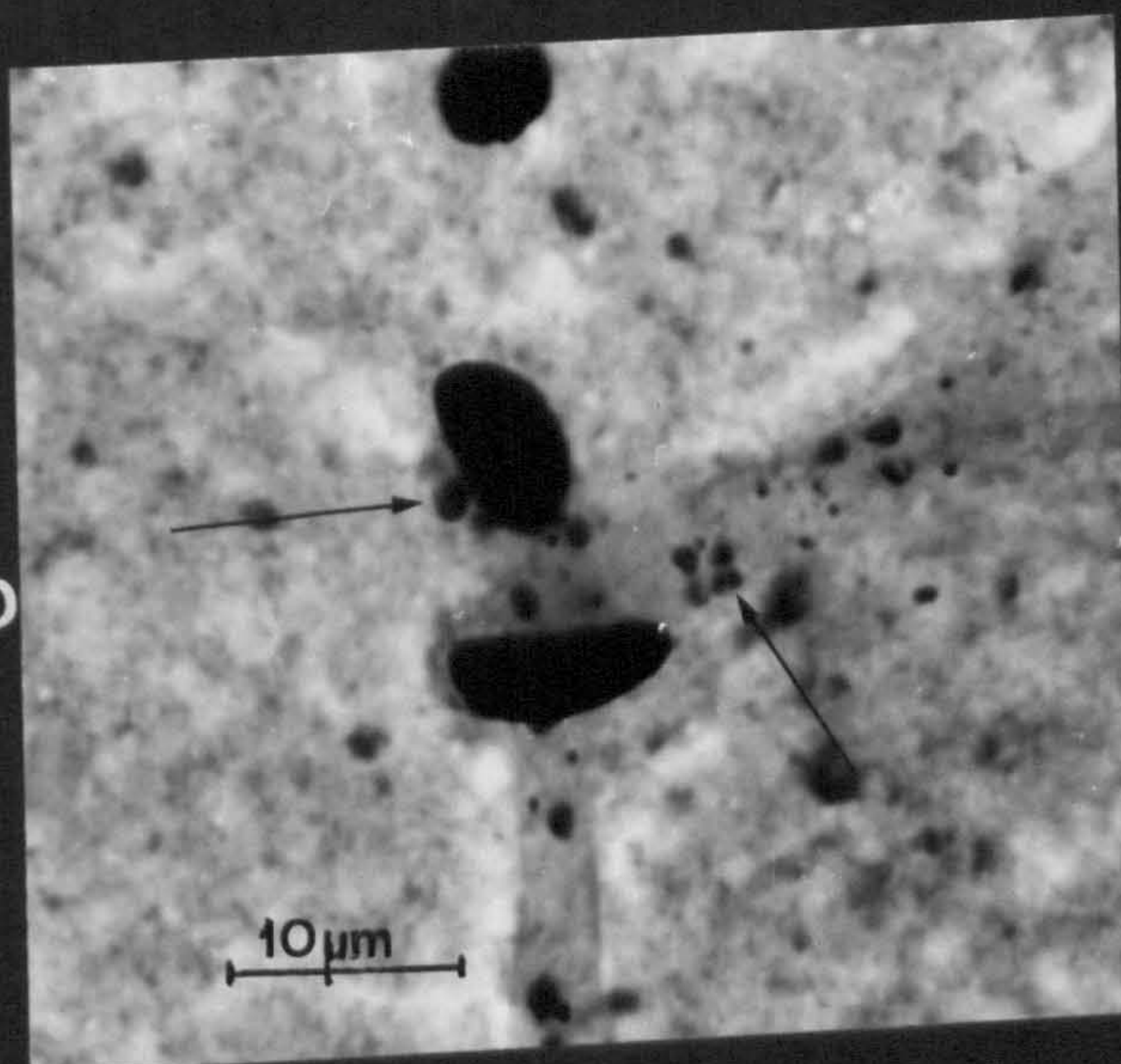


fig.

15a



15b



16

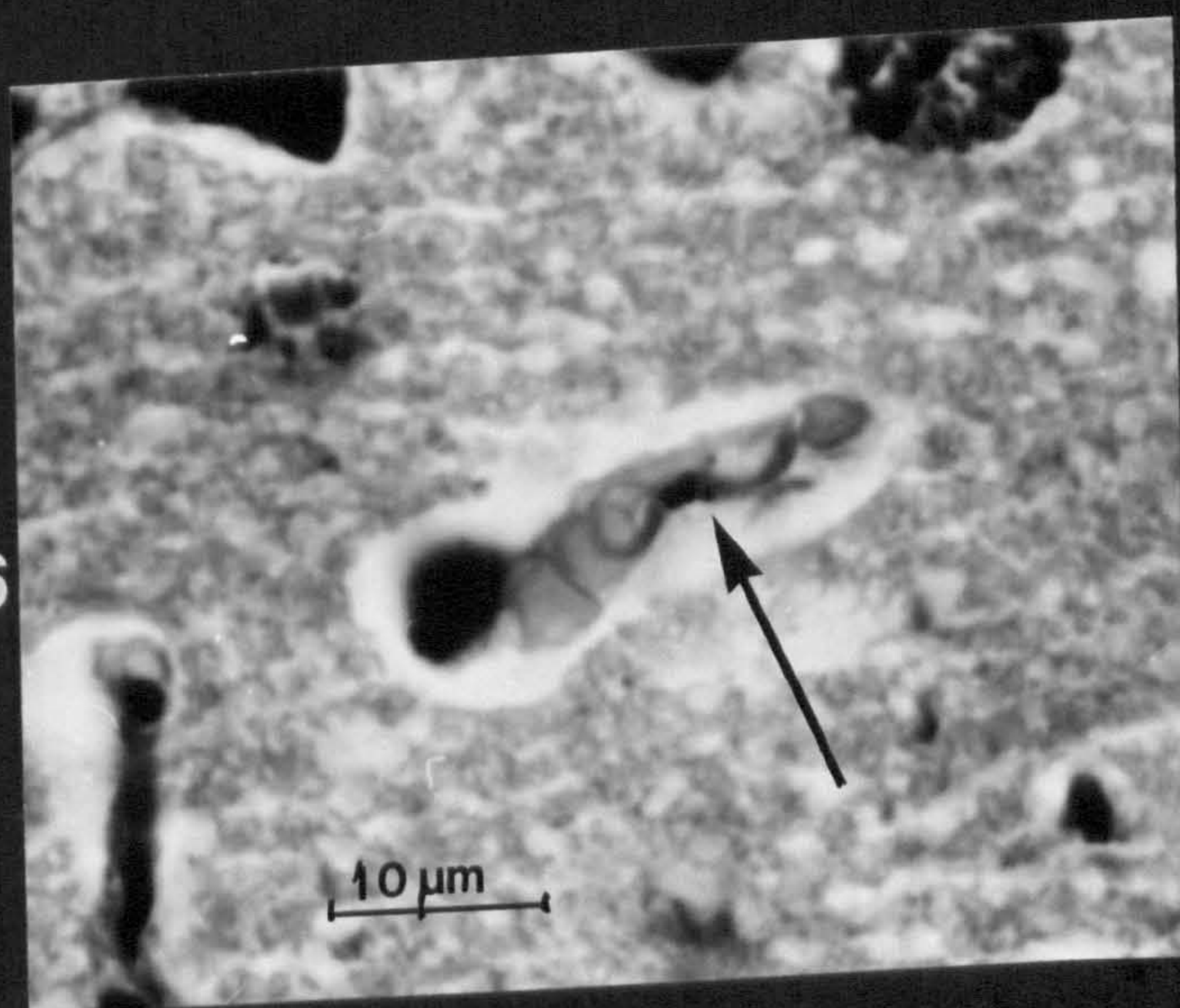


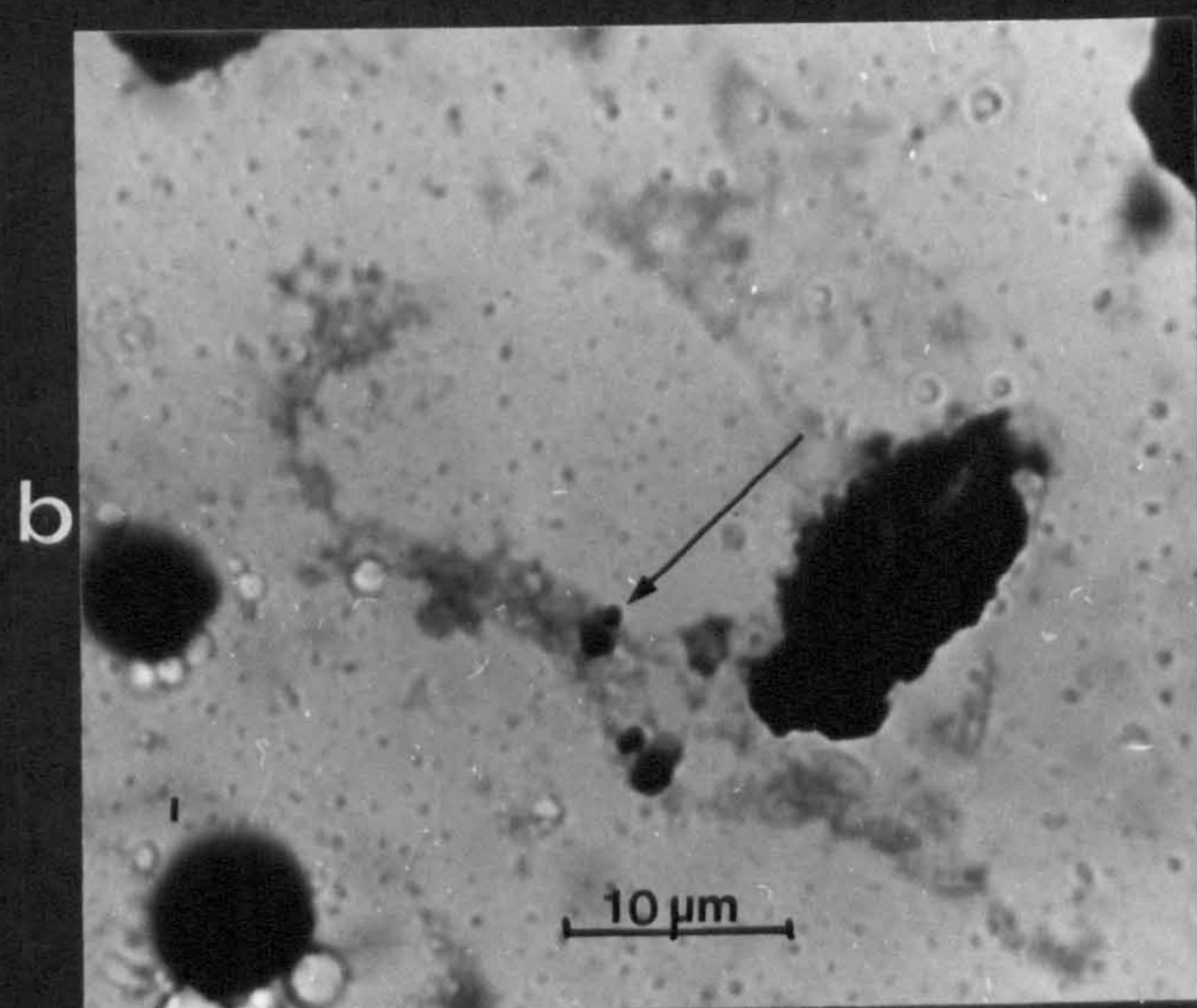
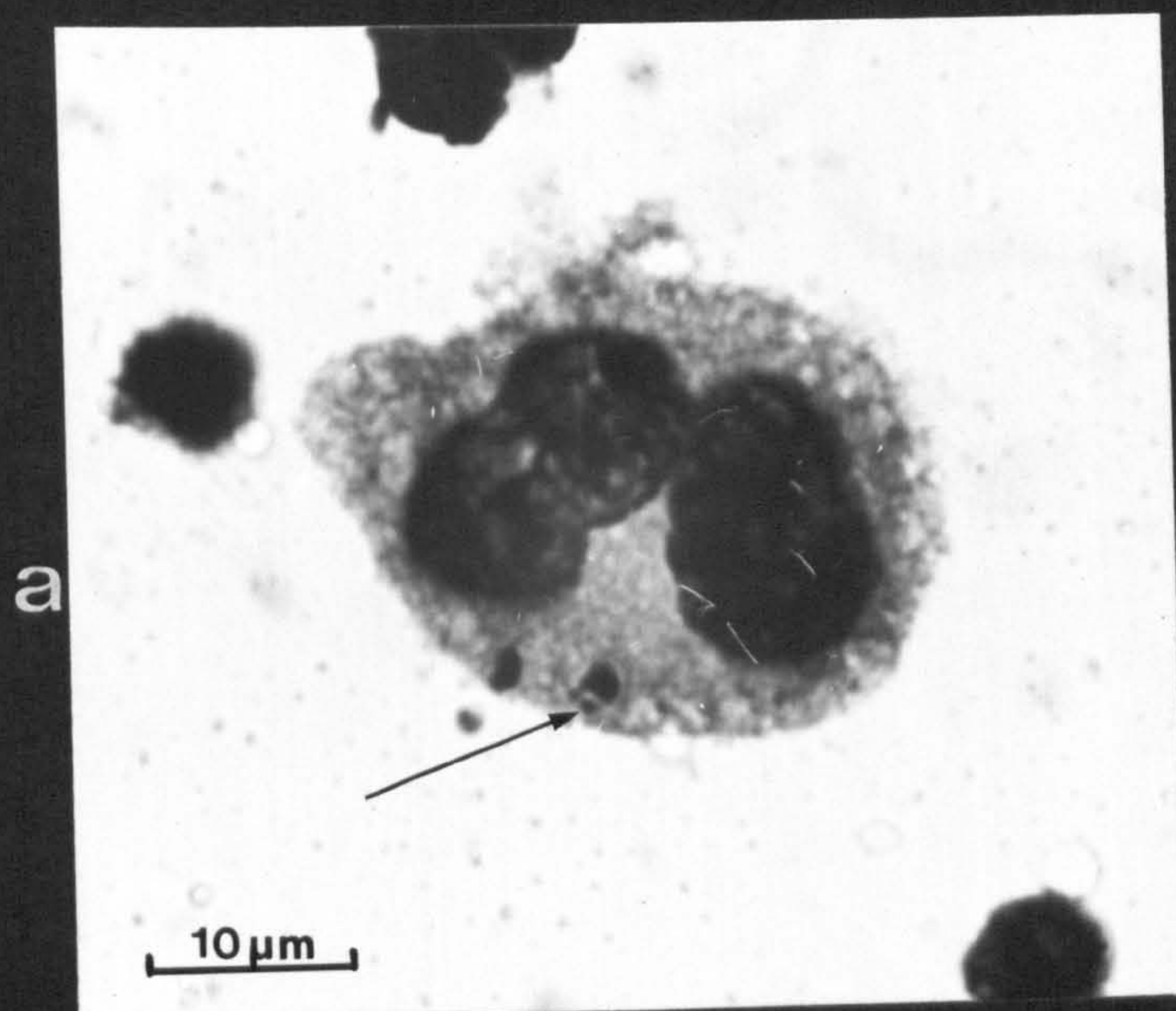


Figure 17. T.(T.) evansi LUMP 55 infection in mice; subpatent infection in mice kept at 35° C. The figure shows amastigotes seen in mouse peritoneal exudate obtained from infected mice five days after transfer to 35° C.

- a) The arrow points at amastigote form in a polymorph.
- b) The arrow points at a free amastigote.



FIG. 17





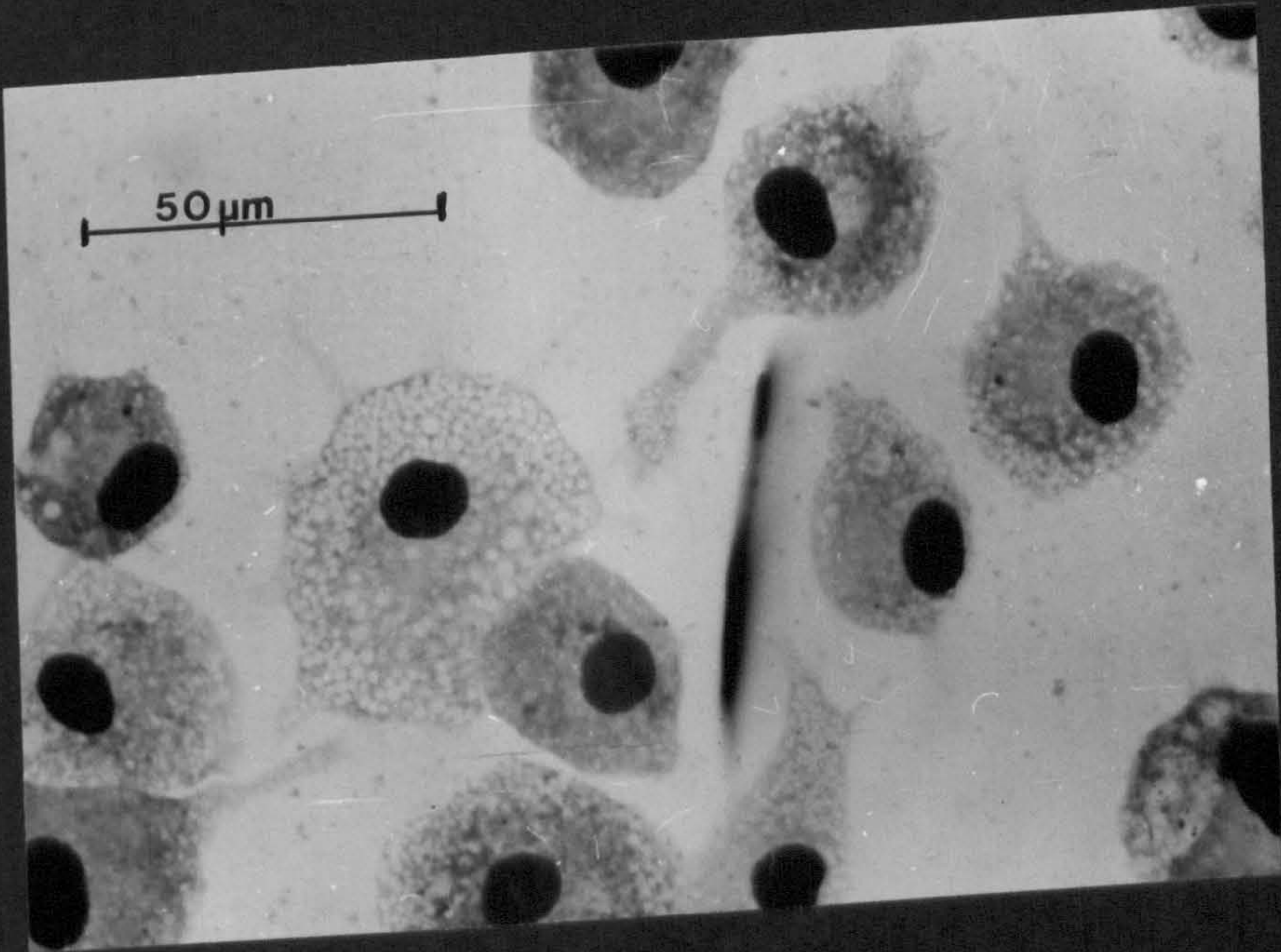
**Figure 18a.** A 3-day-old mouse peritoneal macrophage culture grown in Tissue Culture Medium 199.

**Figure 18b.** Inoculation of the macrophage culture with T.(T.) brucei LUMP 128. The arrows point at inclusion bodies seen in the culture four days after inoculation with trypanosomes.



FIG.18

a



b

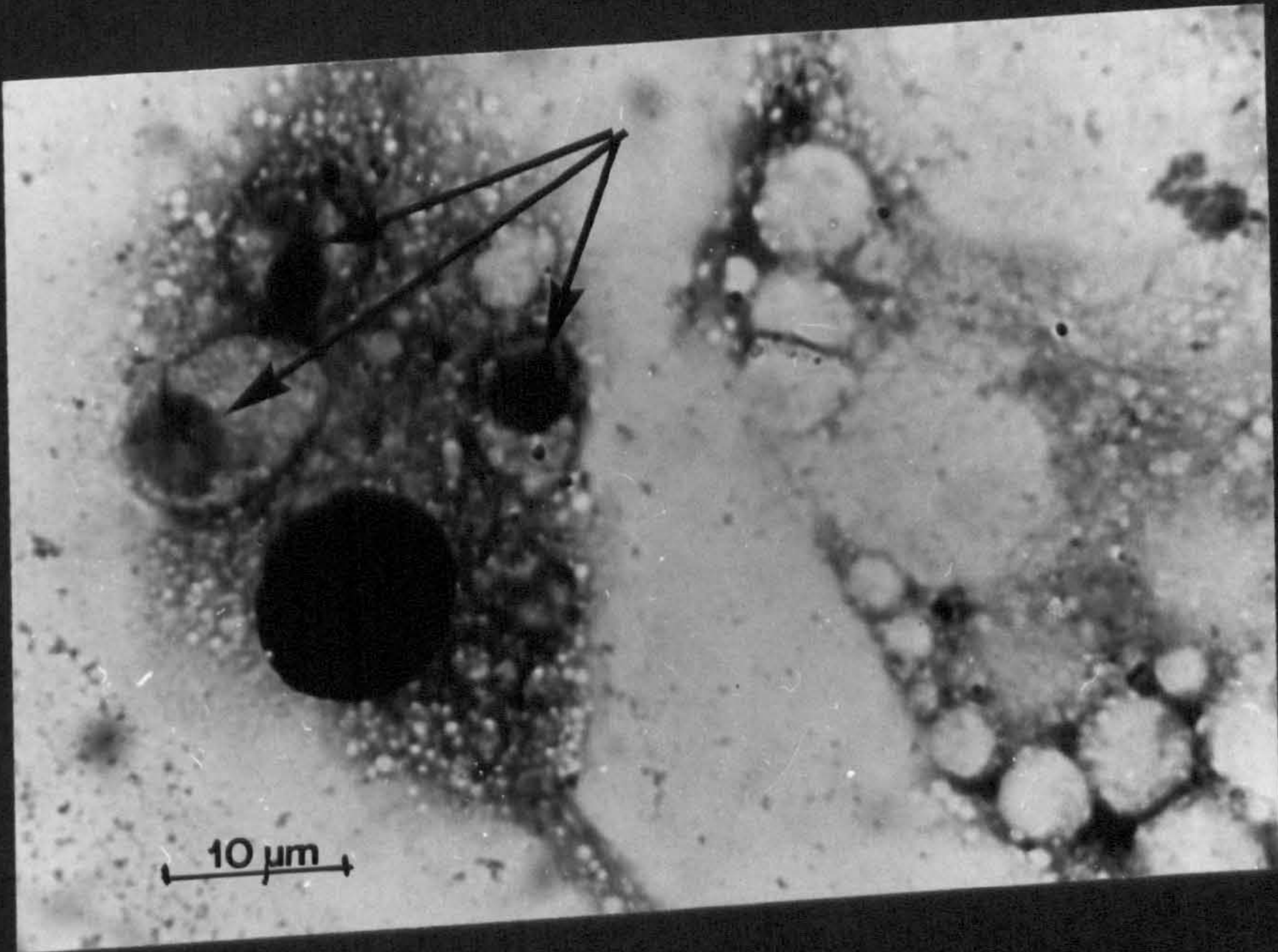
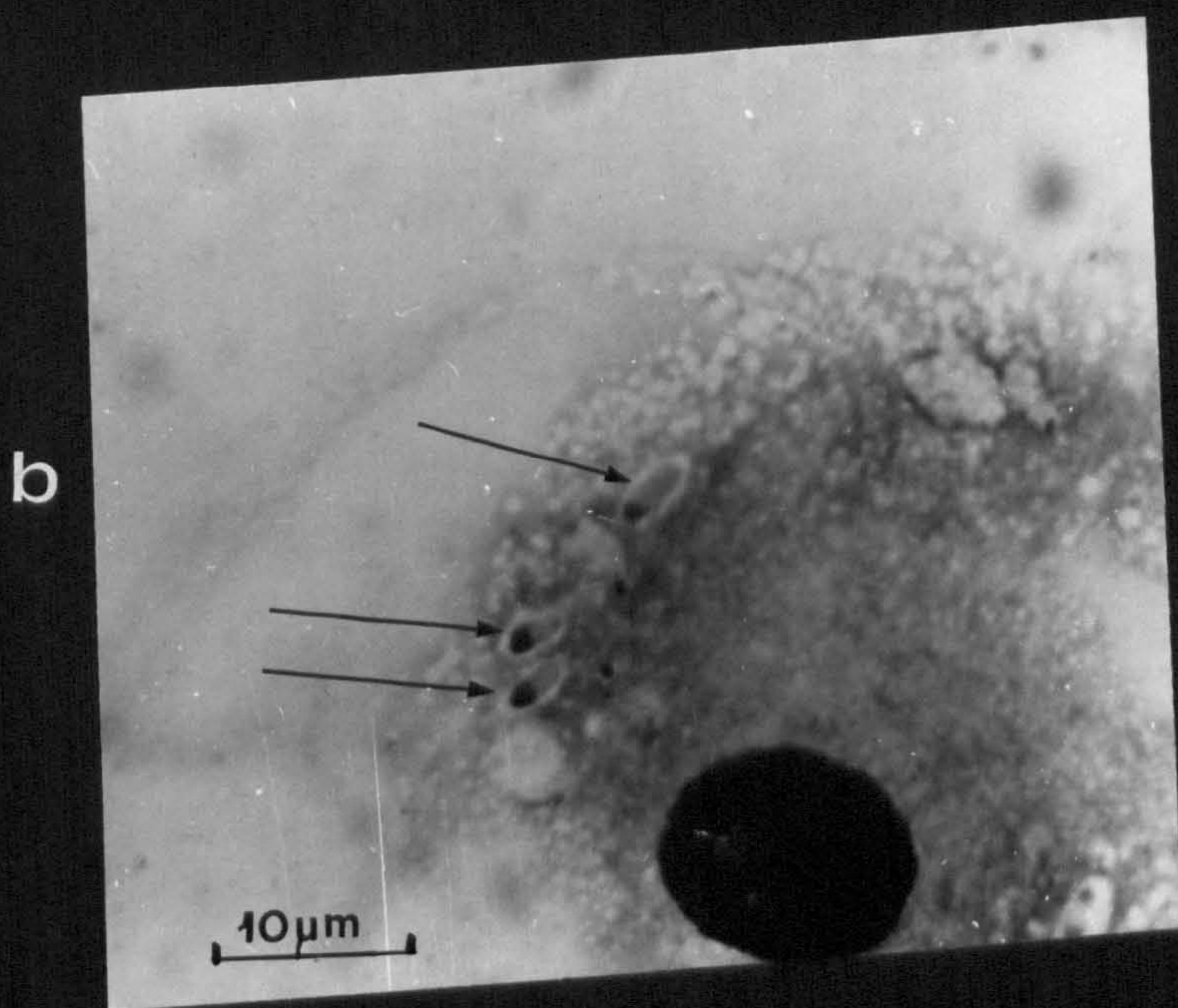
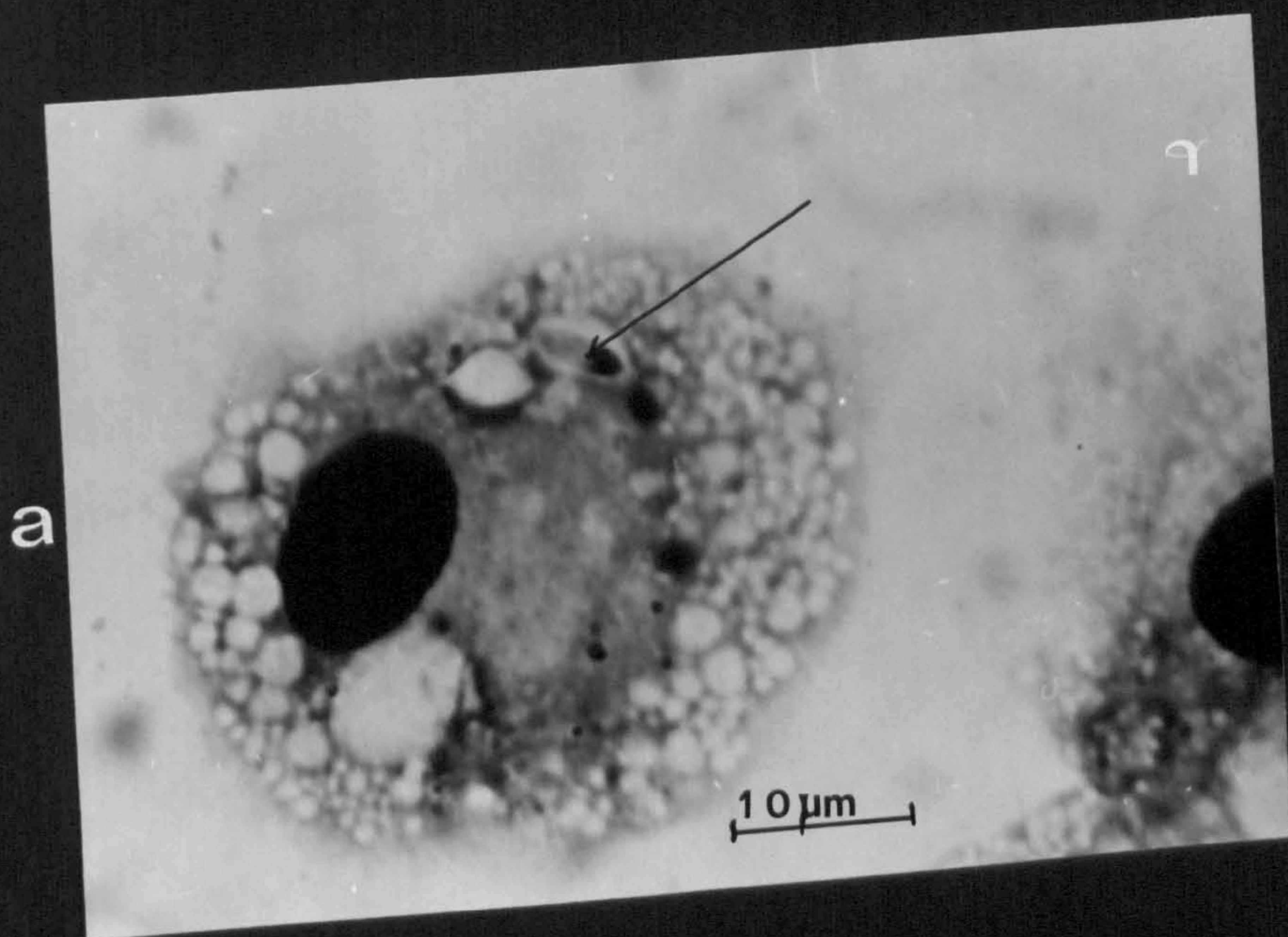




Figure 19. Inoculation of mouse peritoneal macrophage cultures with T.(T.) evansi LUMP 55. The figure shows sausage-shaped, spore-like organisms seen in the macrophages. Their number varied from cell to cell as shown in Figures (a) and (b).



FIG. 19





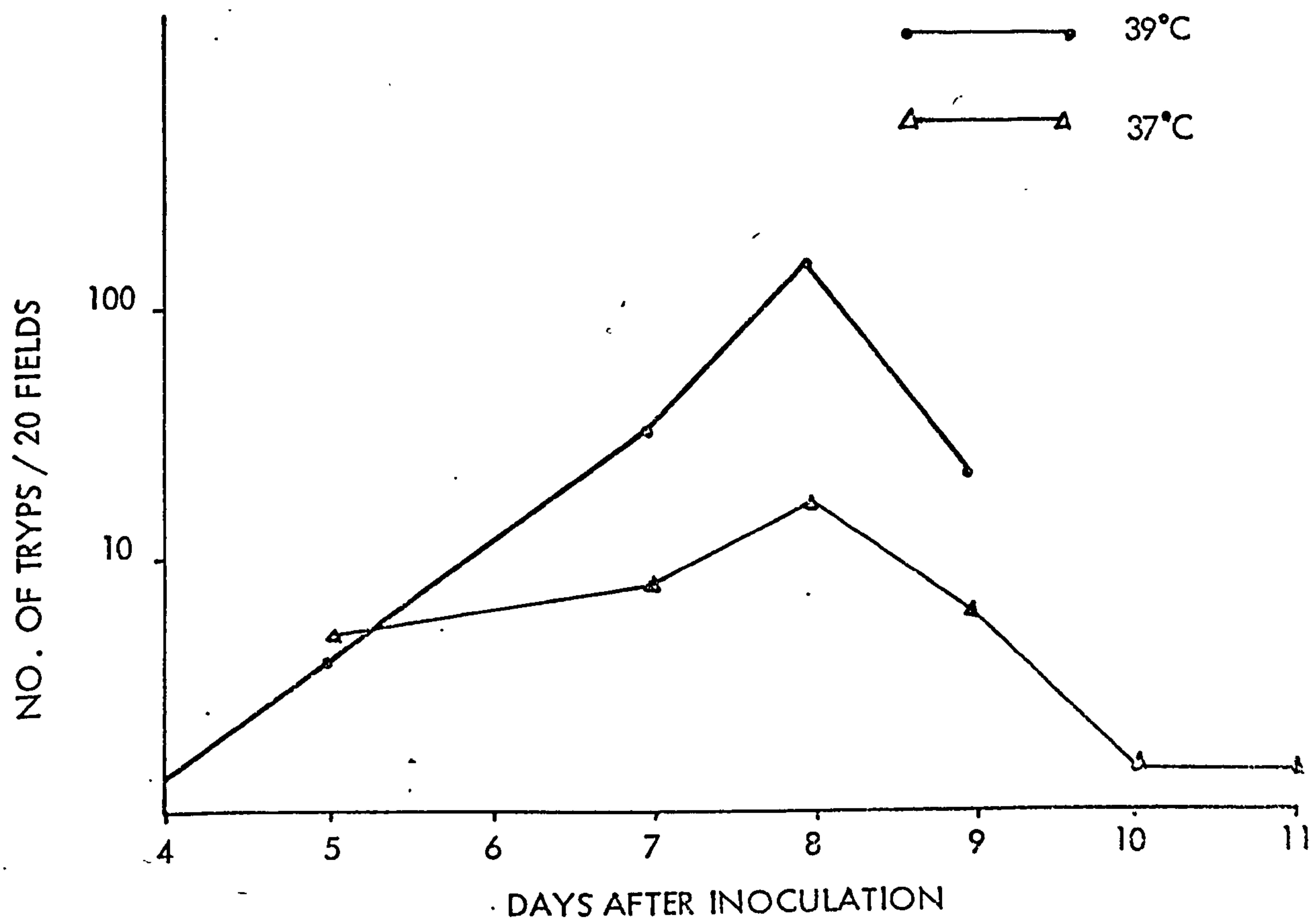


Fig. 20. T.(T.) brucei LUMP 128 infections in chick embryos. The figure shows the course of infections in groups of chick embryos incubated at 37°C and 39°C.

Figure 21. Growth of T.(T.) brucei LUMP 128 in chick embryos.

a) Monomorphic trypomastigotes were the only forms seen in embryos incubated at 37° C.

b, c, d. Giant and multinucleate forms observed during peak parasitaemias in embryos incubated at 39° C.



FIG.21

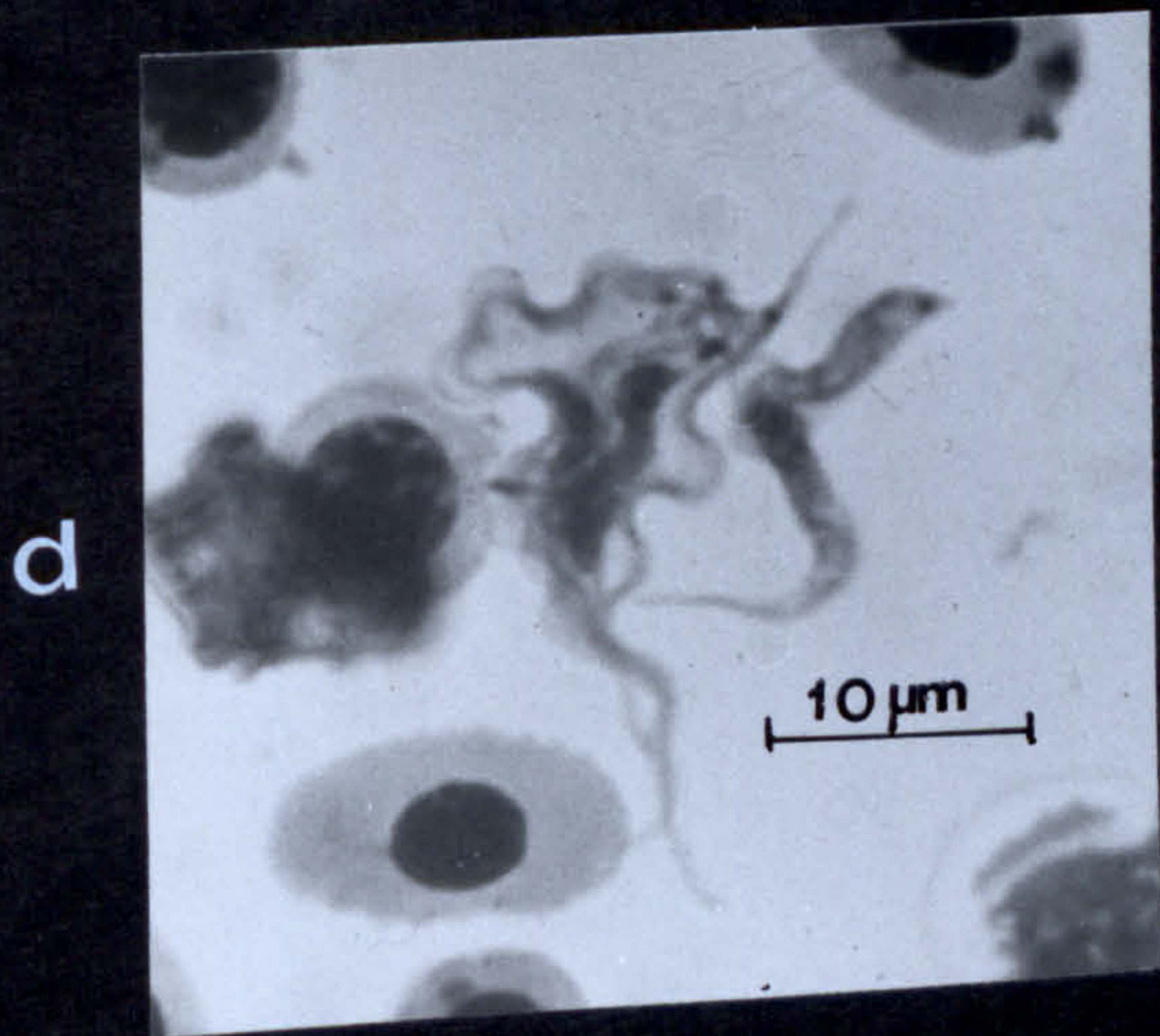
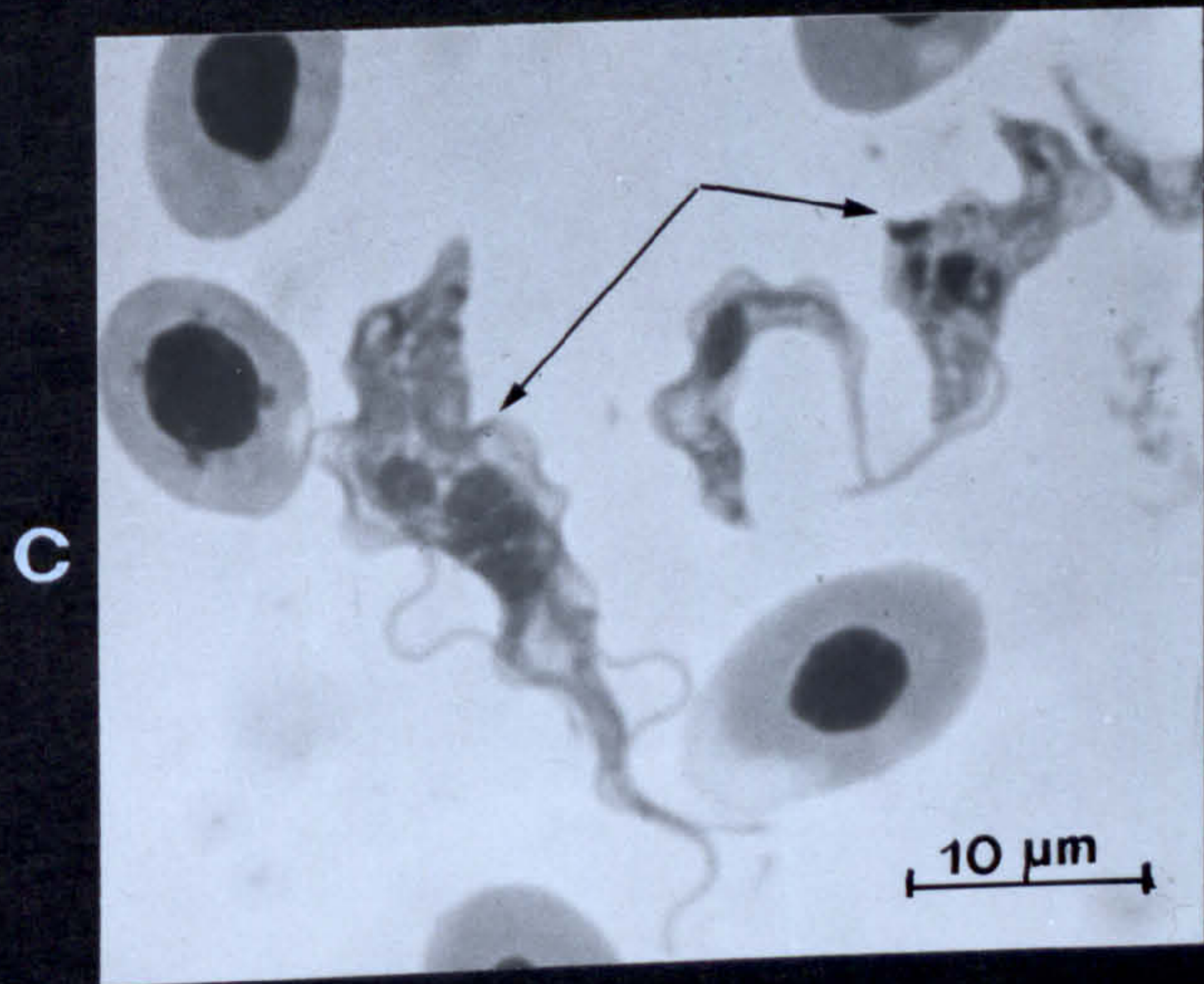
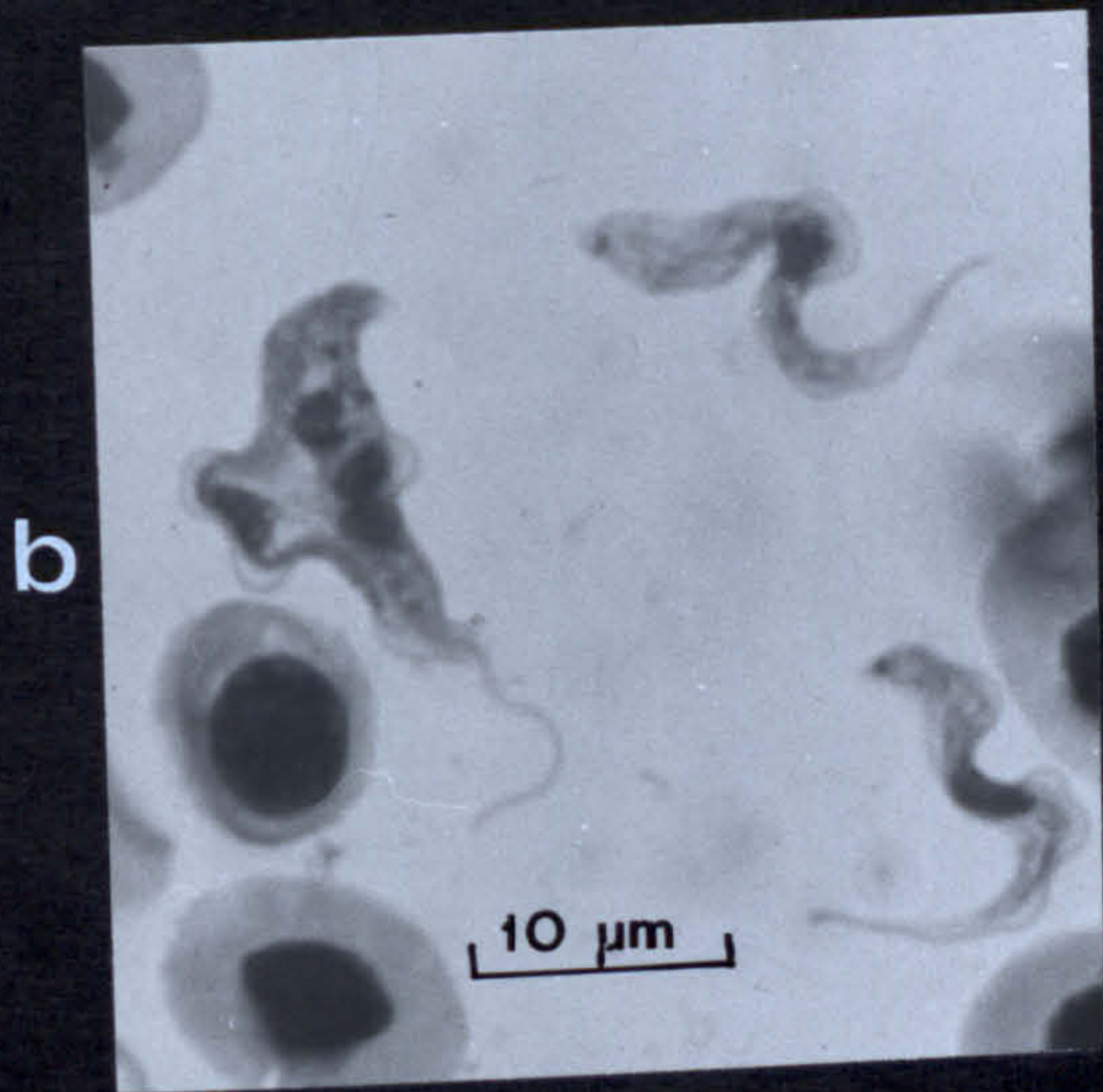
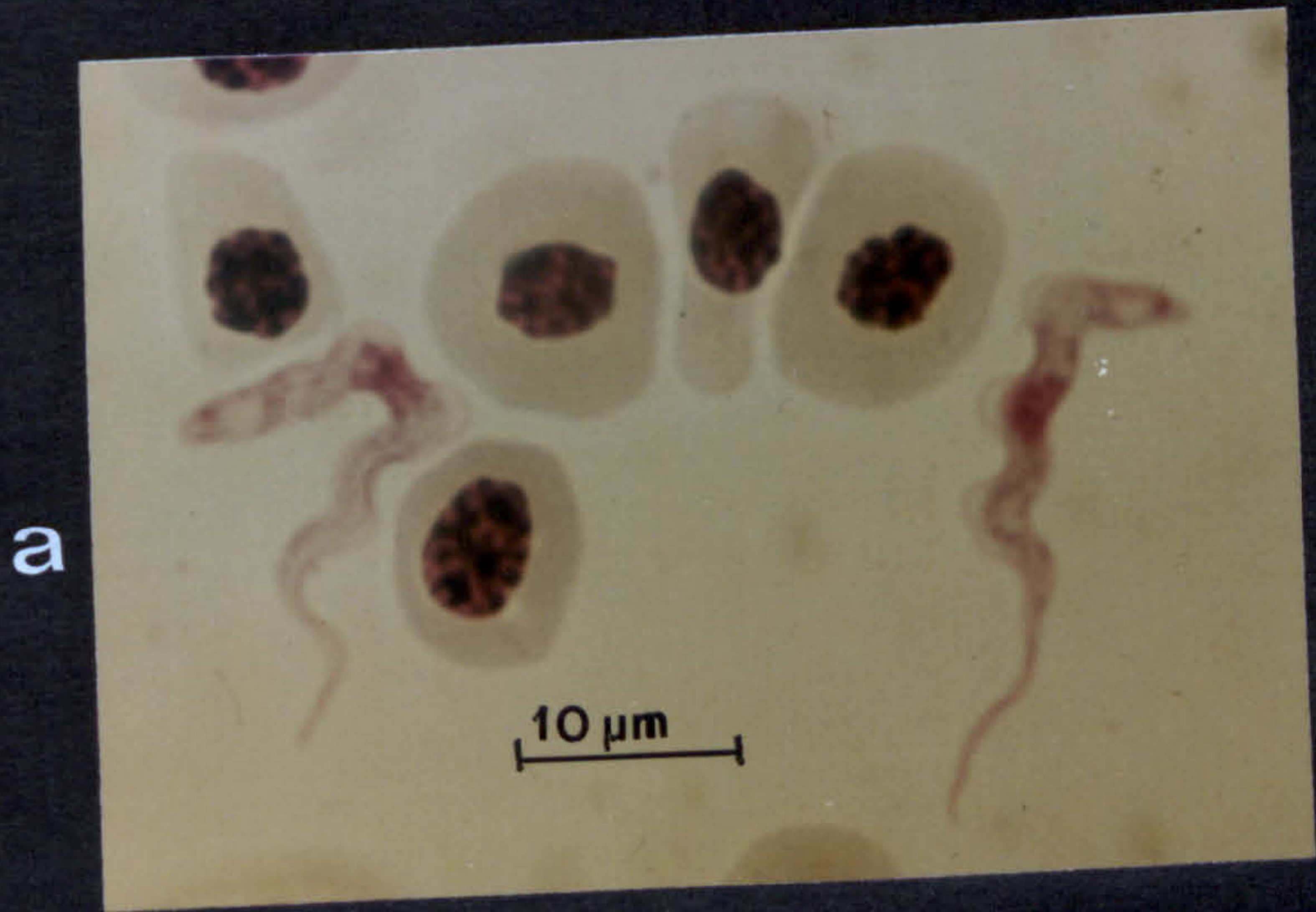




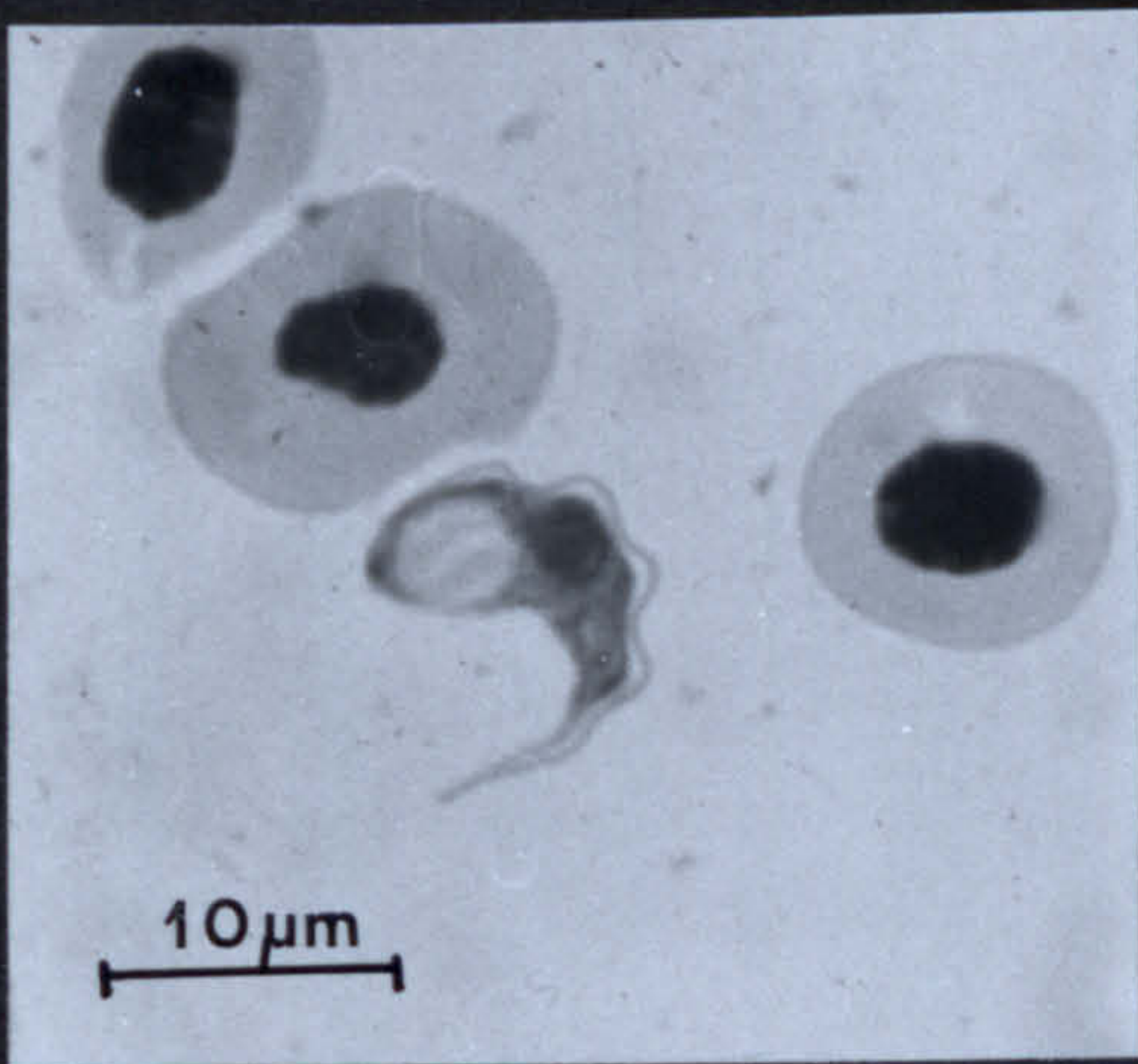
Figure 22. Growth of T.(T.) brucei LUMP 128 in chick embryos incubated at 39°C (8 days after inoculation).

- a) Stumpy and vacuolated trypomastigote
- b) The vacuolated form vacuolated trypomastigote
- c) Rounded trypomastigote
- d) Vacuolated form undergoing binary fission
- e,f) Completely rounded forms. These forms are no longer trypomastigotes (probably sphaeromastigotes). One imagines that if the vacuole breaks, then thin slender forms emerge.

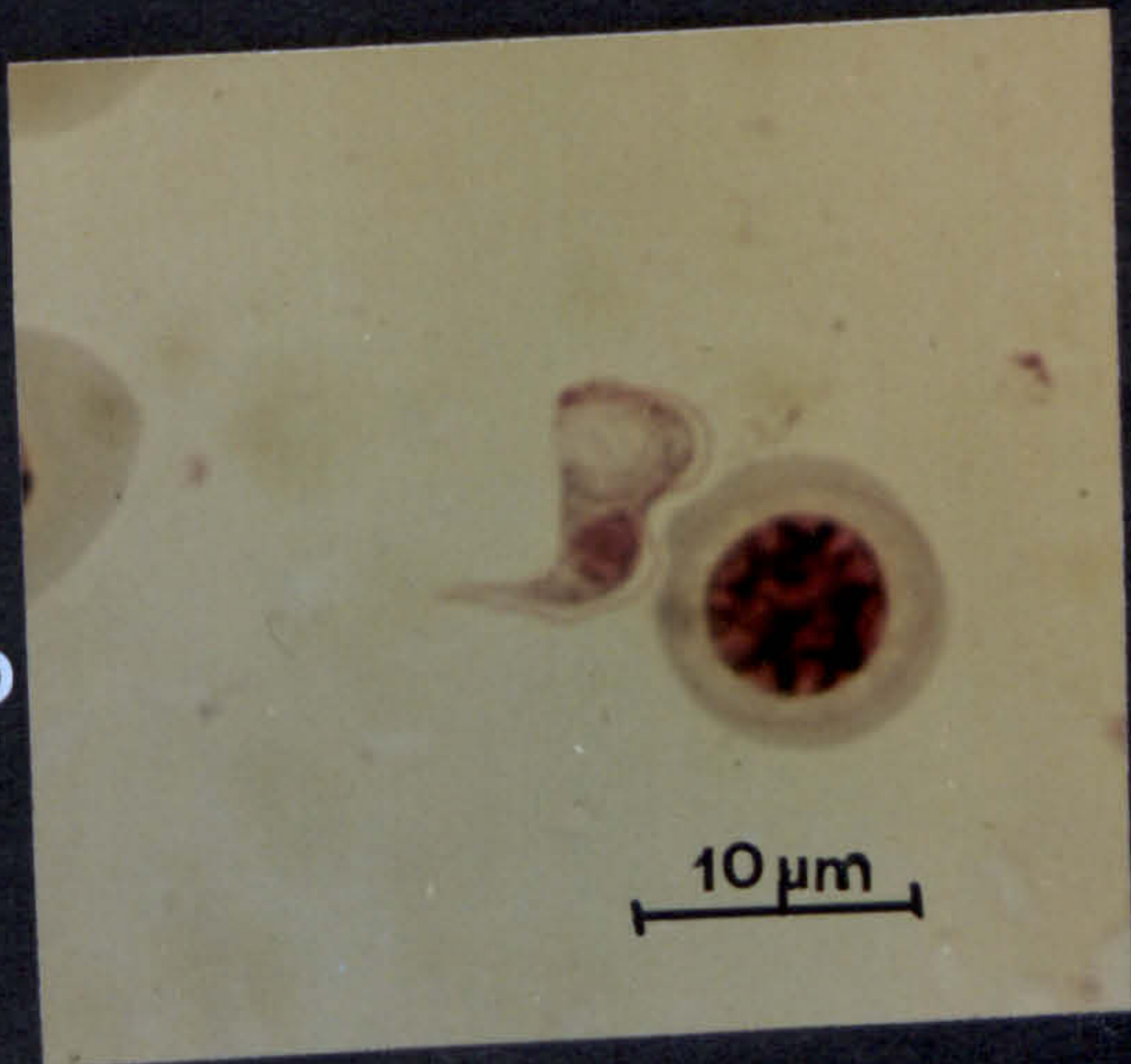


FIG.22

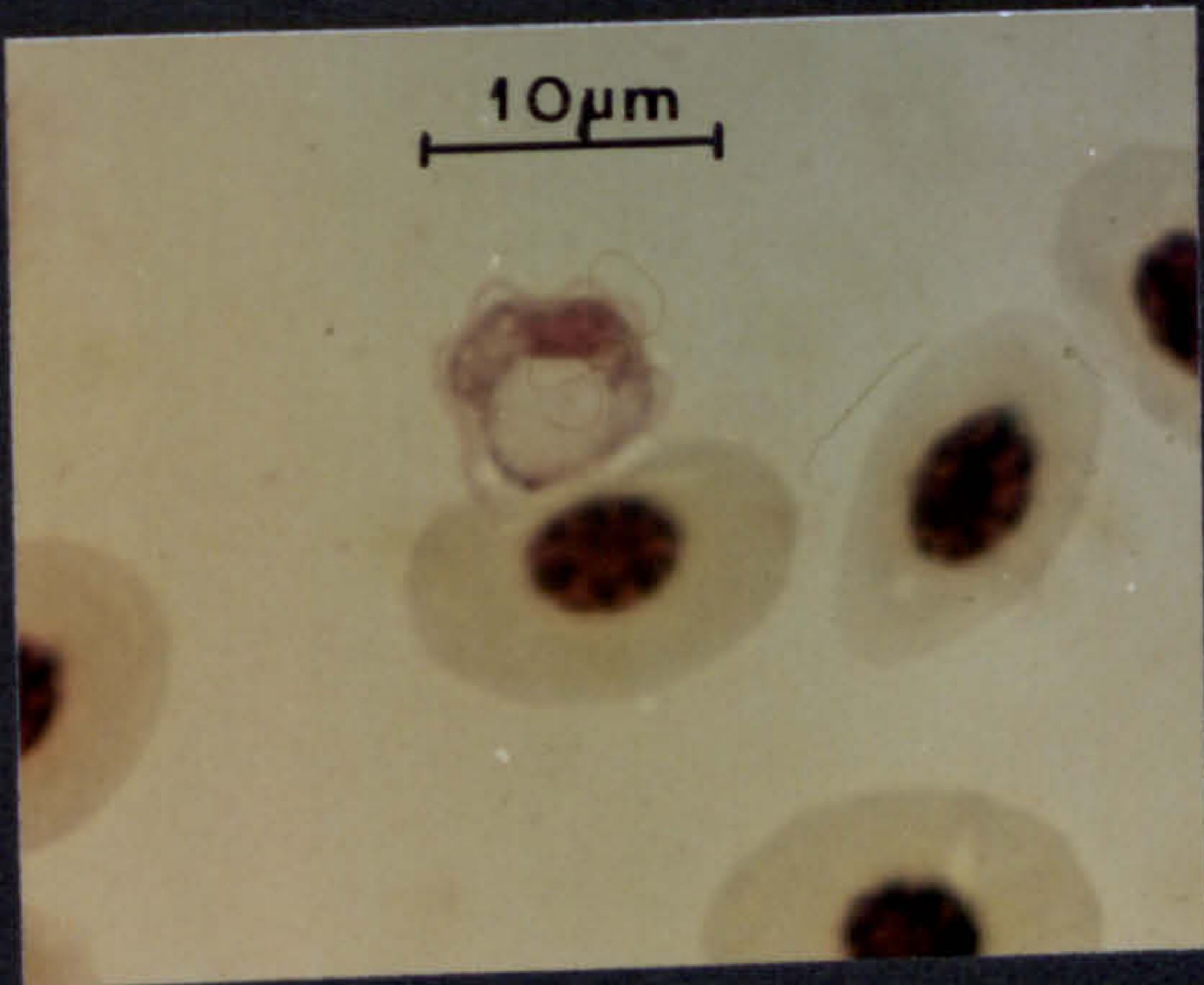
a



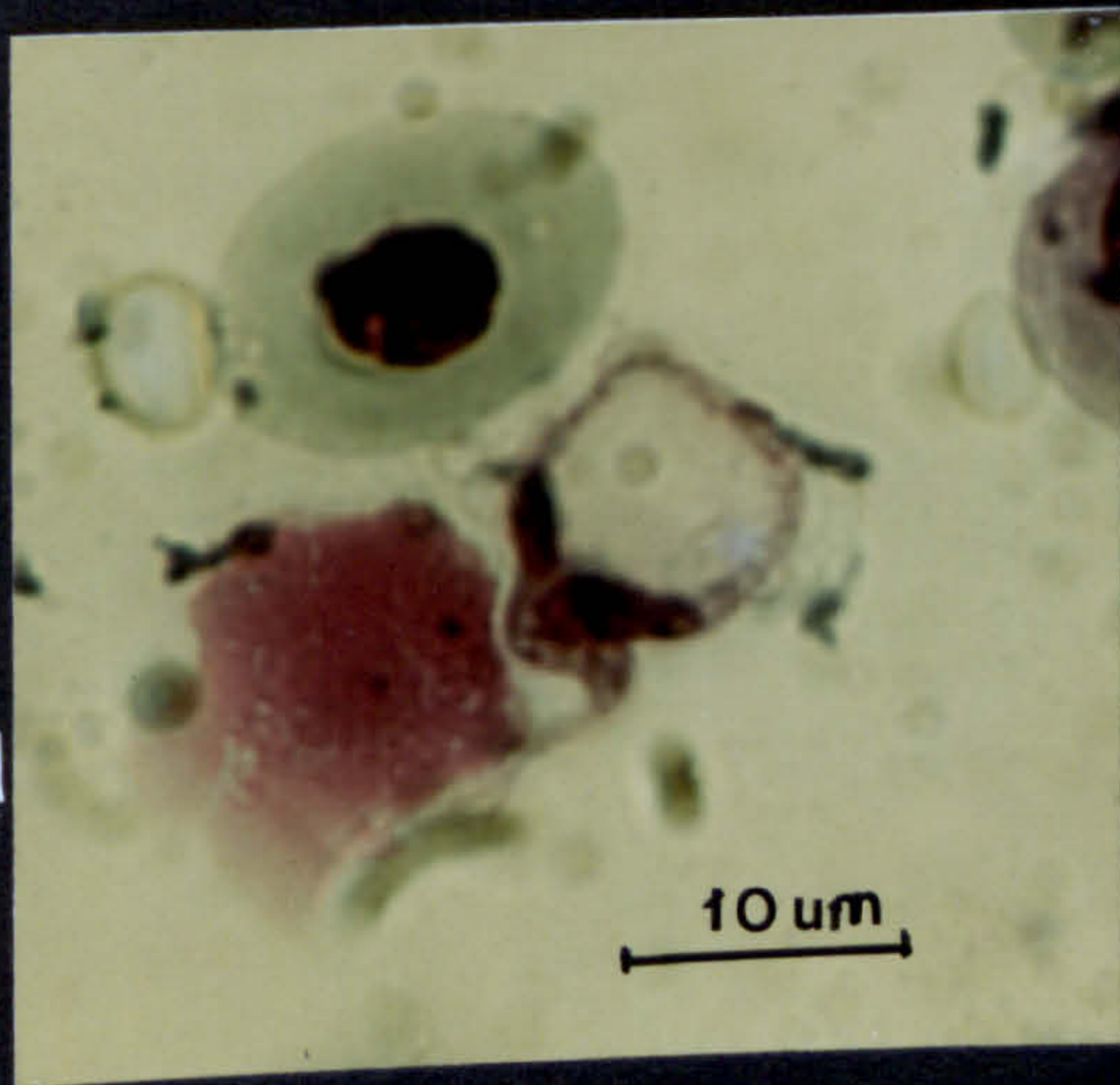
b



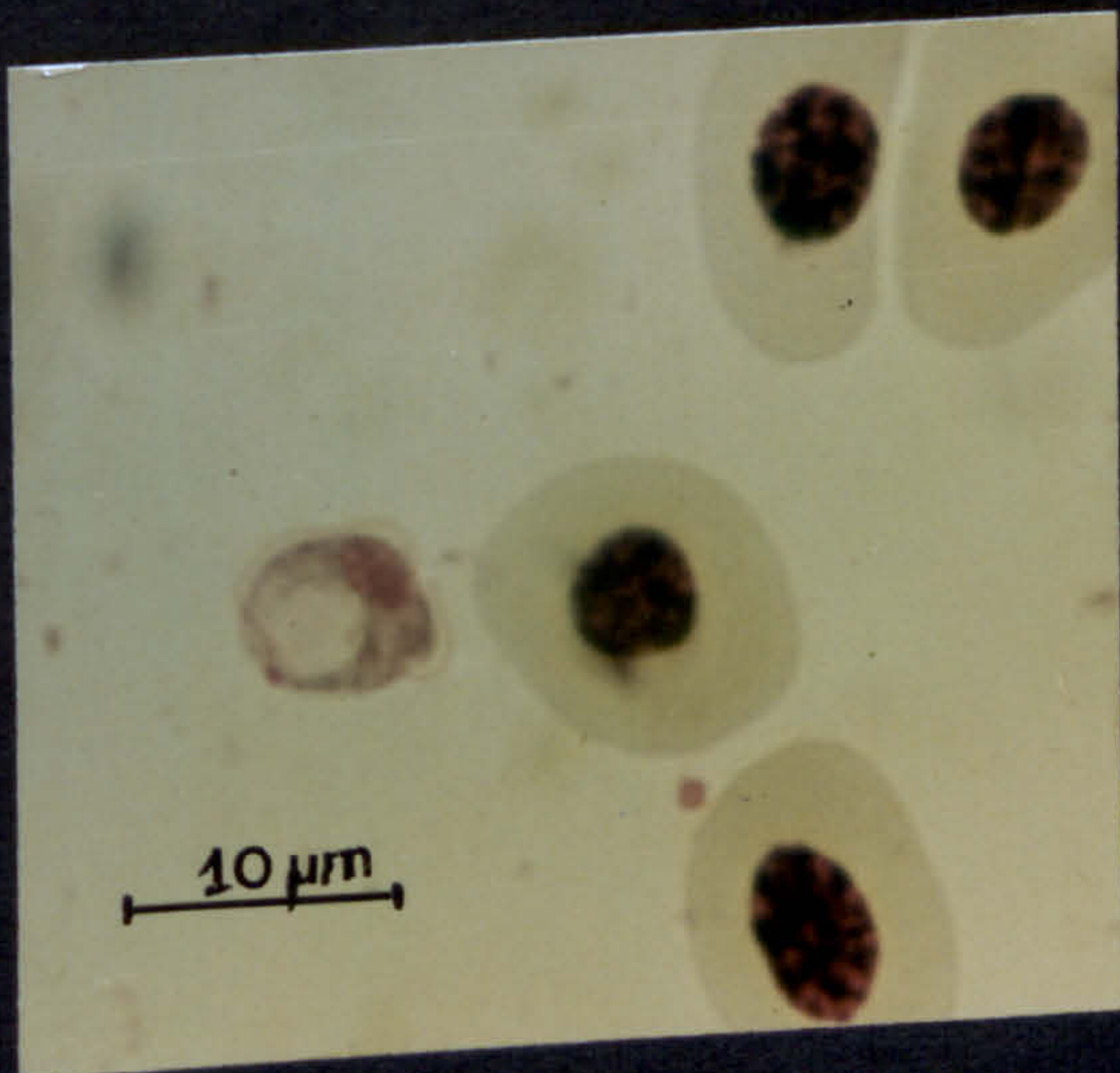
c



d



e



f

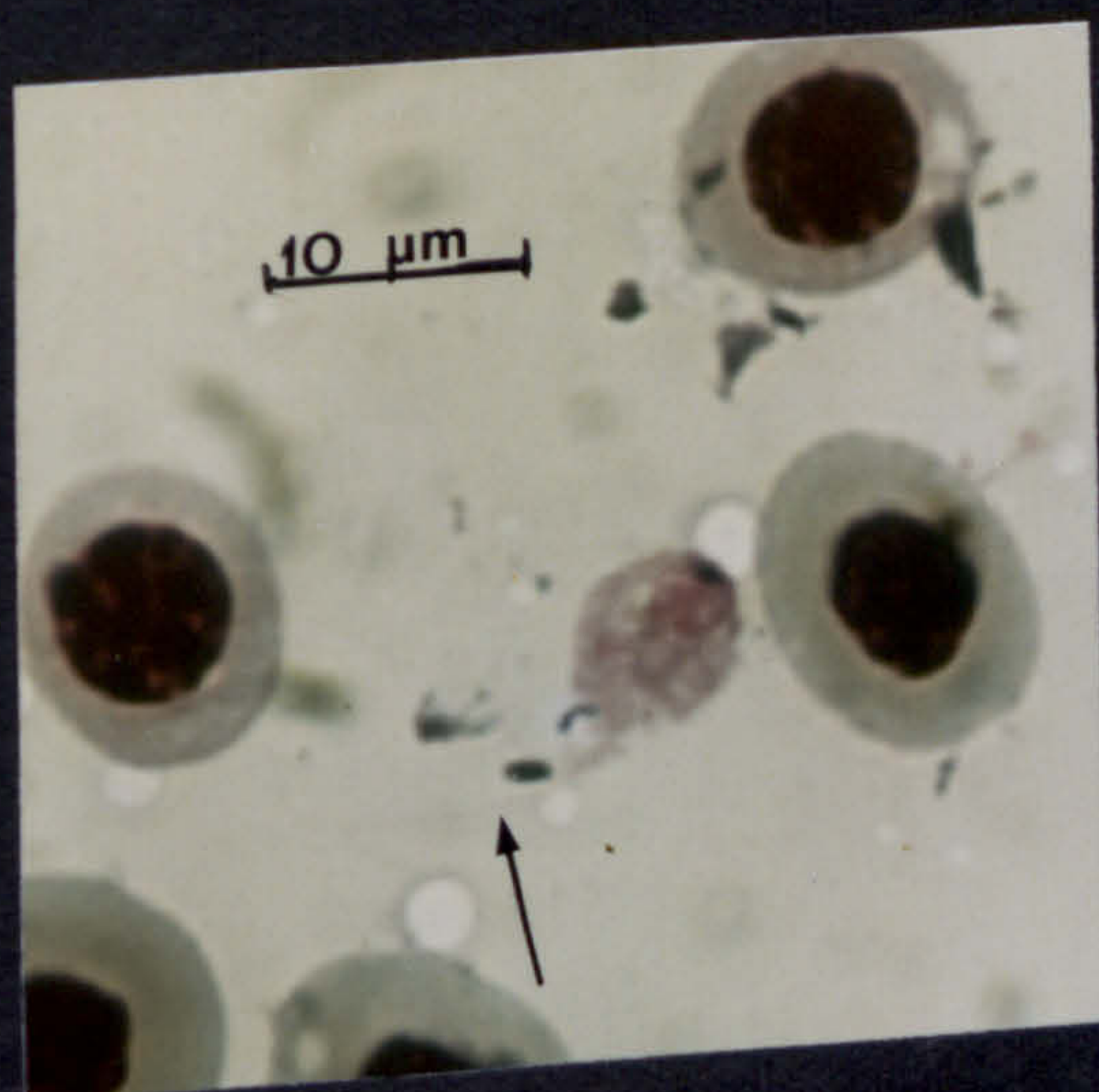




Figure 23. Growth of T.(T.) brucei LUMP 128 in chick embryos incubated at 39°C. The figure shows a flagellated round form.



**FIG.2 3**





Figures 24a & b.

Demonstration of precipitating antibodies  
in plasma obtained from mice inoculated  
with T.(T.) brucei and treated with berenil.  
The plasma samples were collected over a  
period of 6 weeks.

x = antigen in the form of infected  
mouse serum.

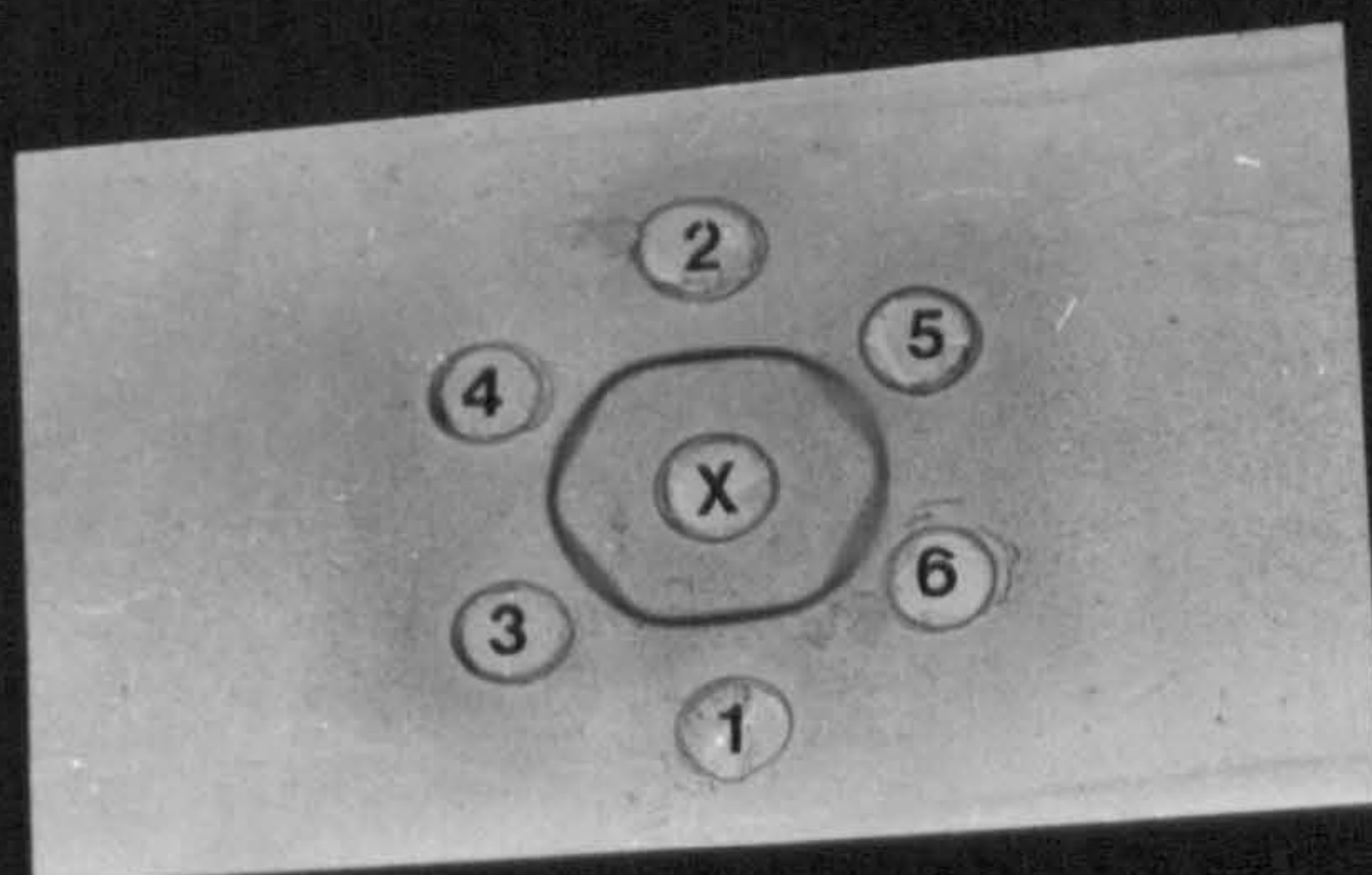
c = control normal serum

1, 2, 3, 4, 5, 6 = (time in weeks when  
antisera was collected).



Fig.24

a



b

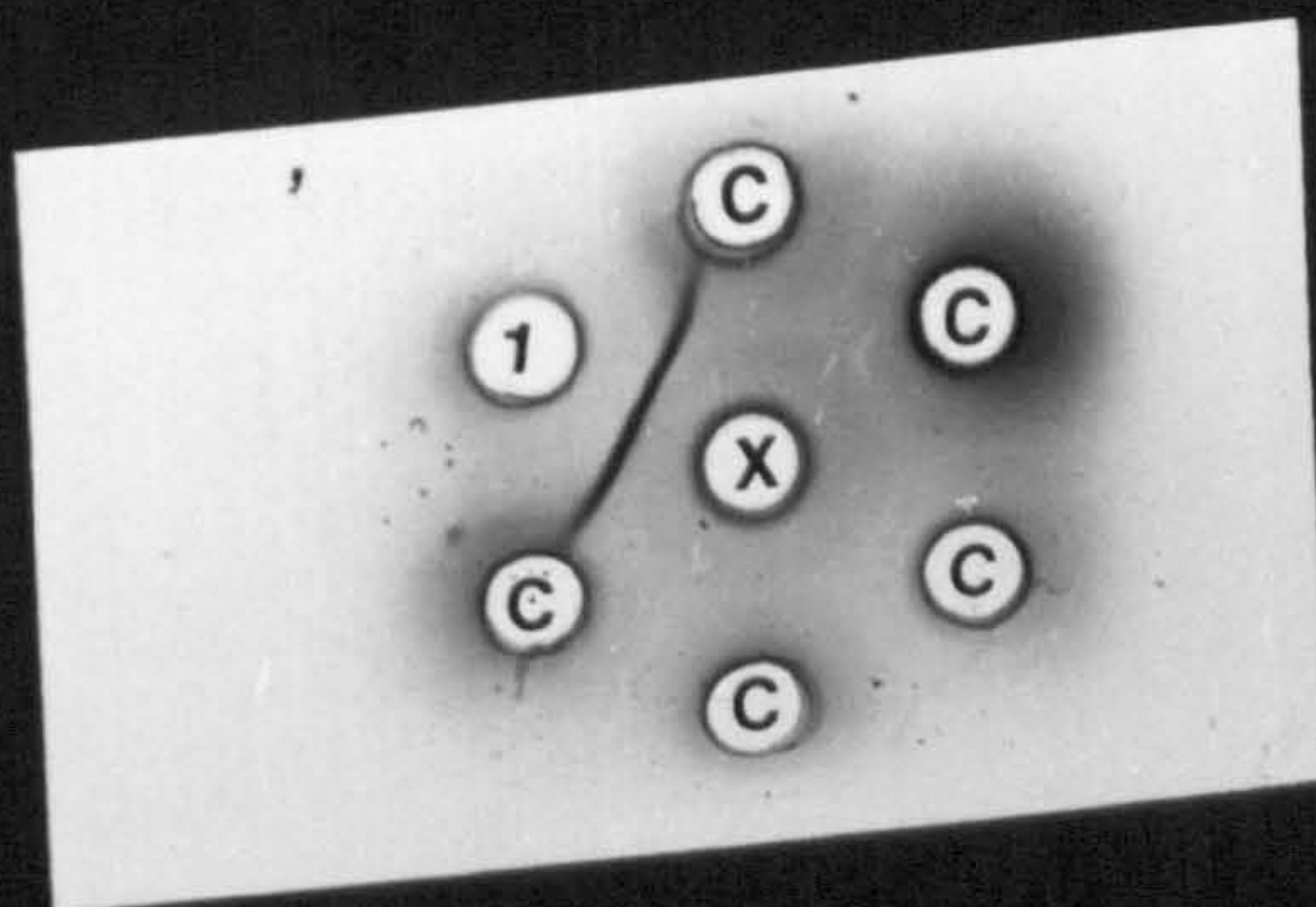




Figure 24c. Titration of antisera (AB) obtained from mice infected with T.(T.) brucei and then treated with berenil.

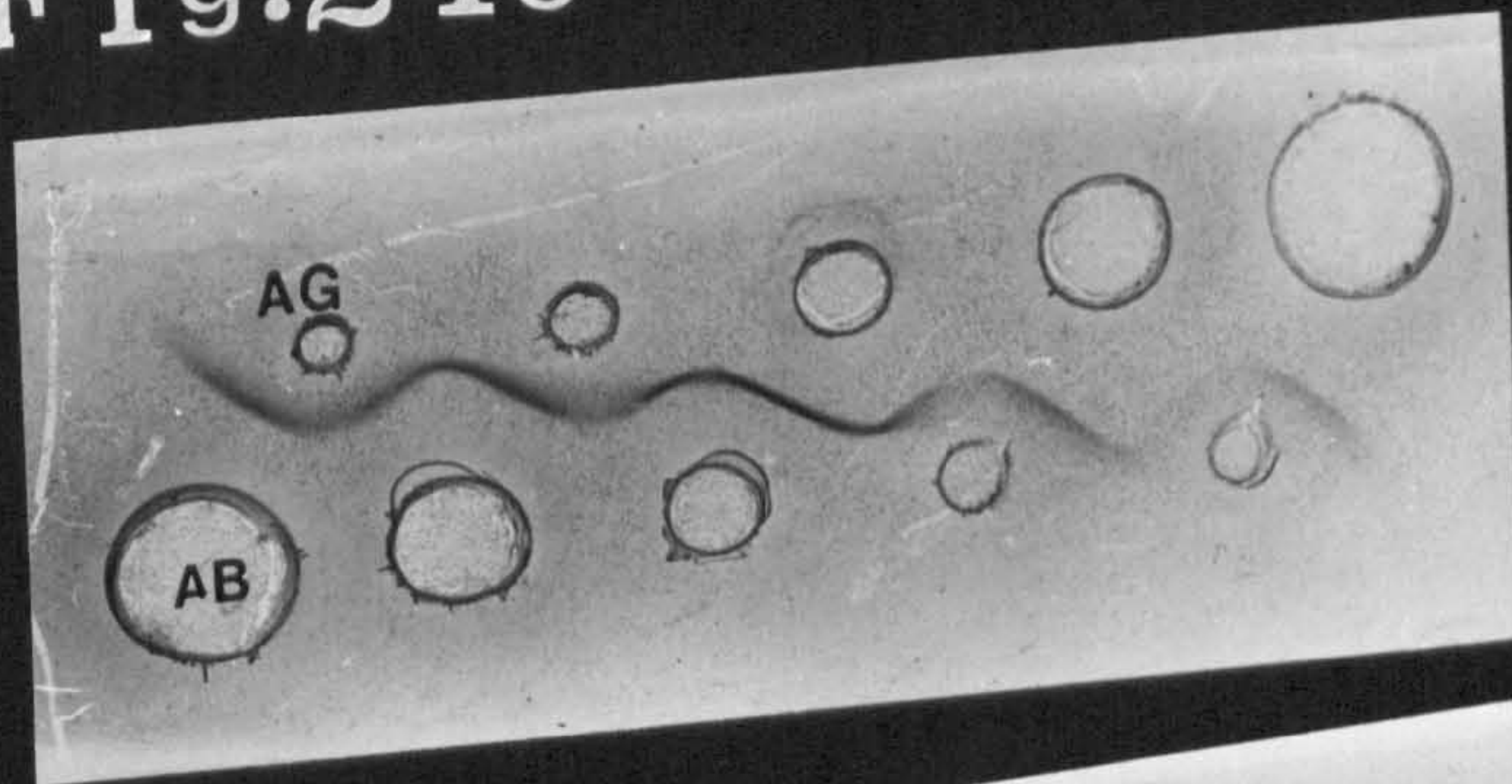
1, 2, 3, 4, 5, 6 = time in weeks when antisera was collected.

AG = infected mouse serum (containing released antigen) obtained from acute T.(T.) brucei in mice kept at 22-27°C.

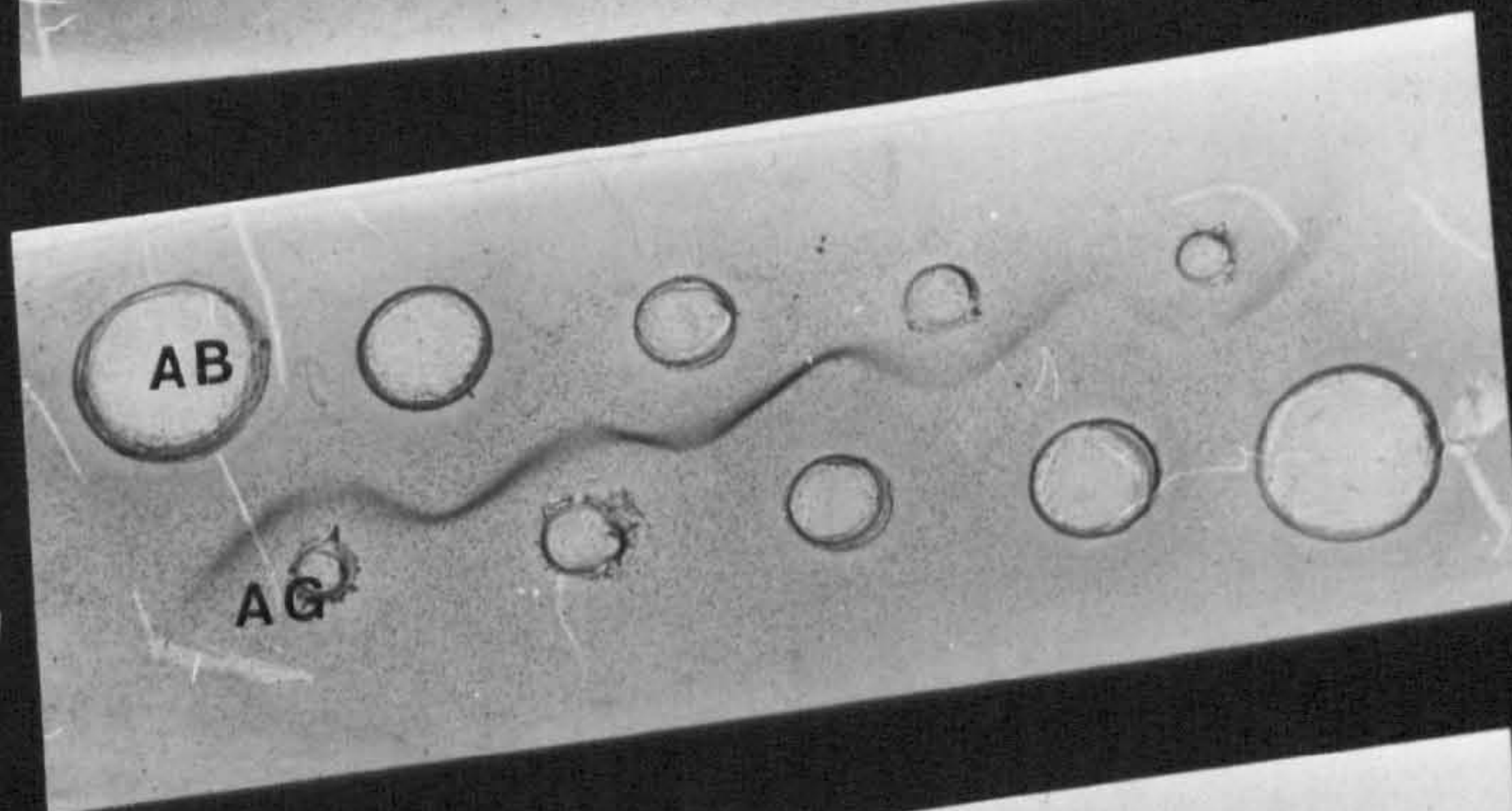


Fig.24c

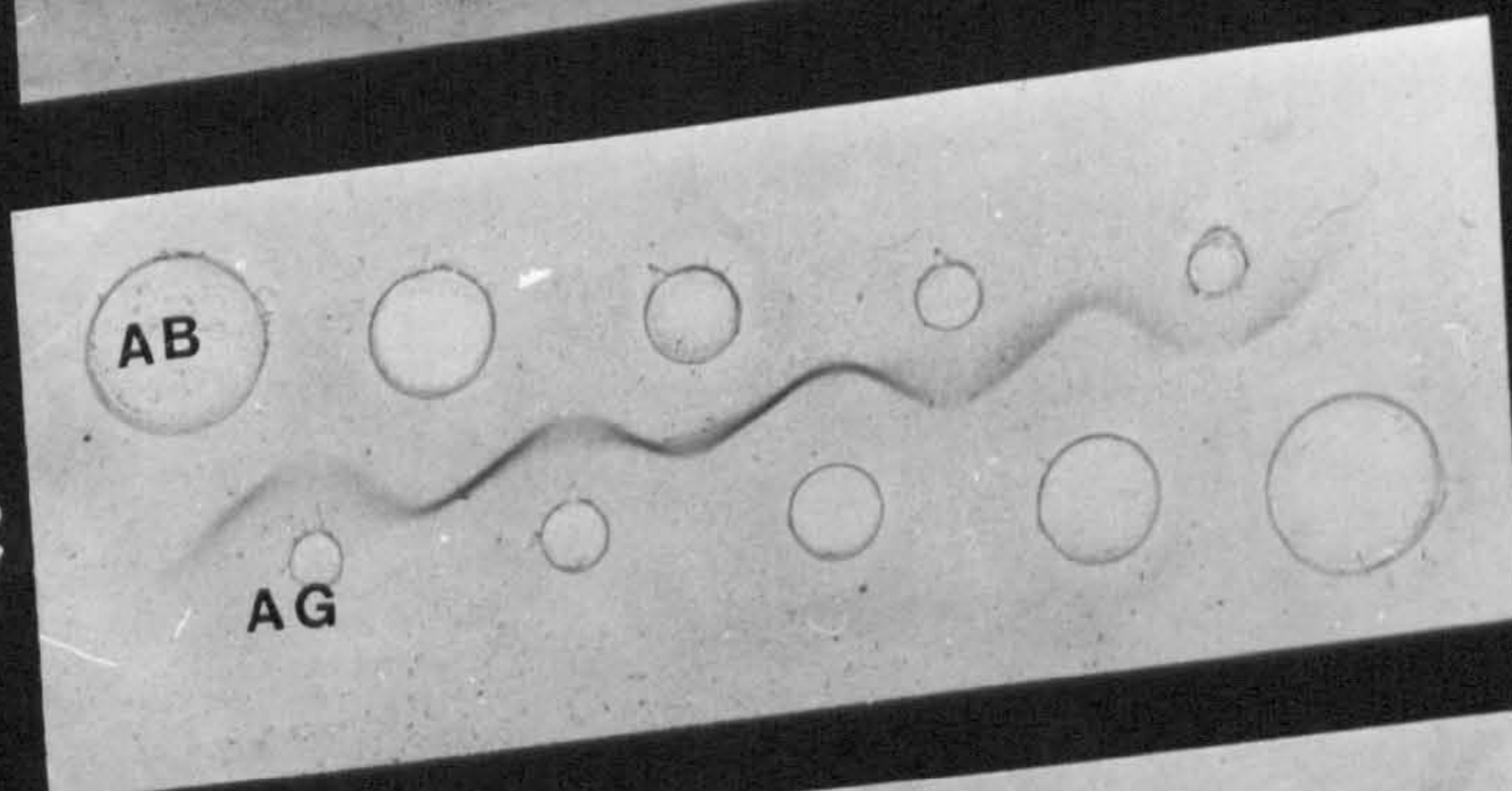
1



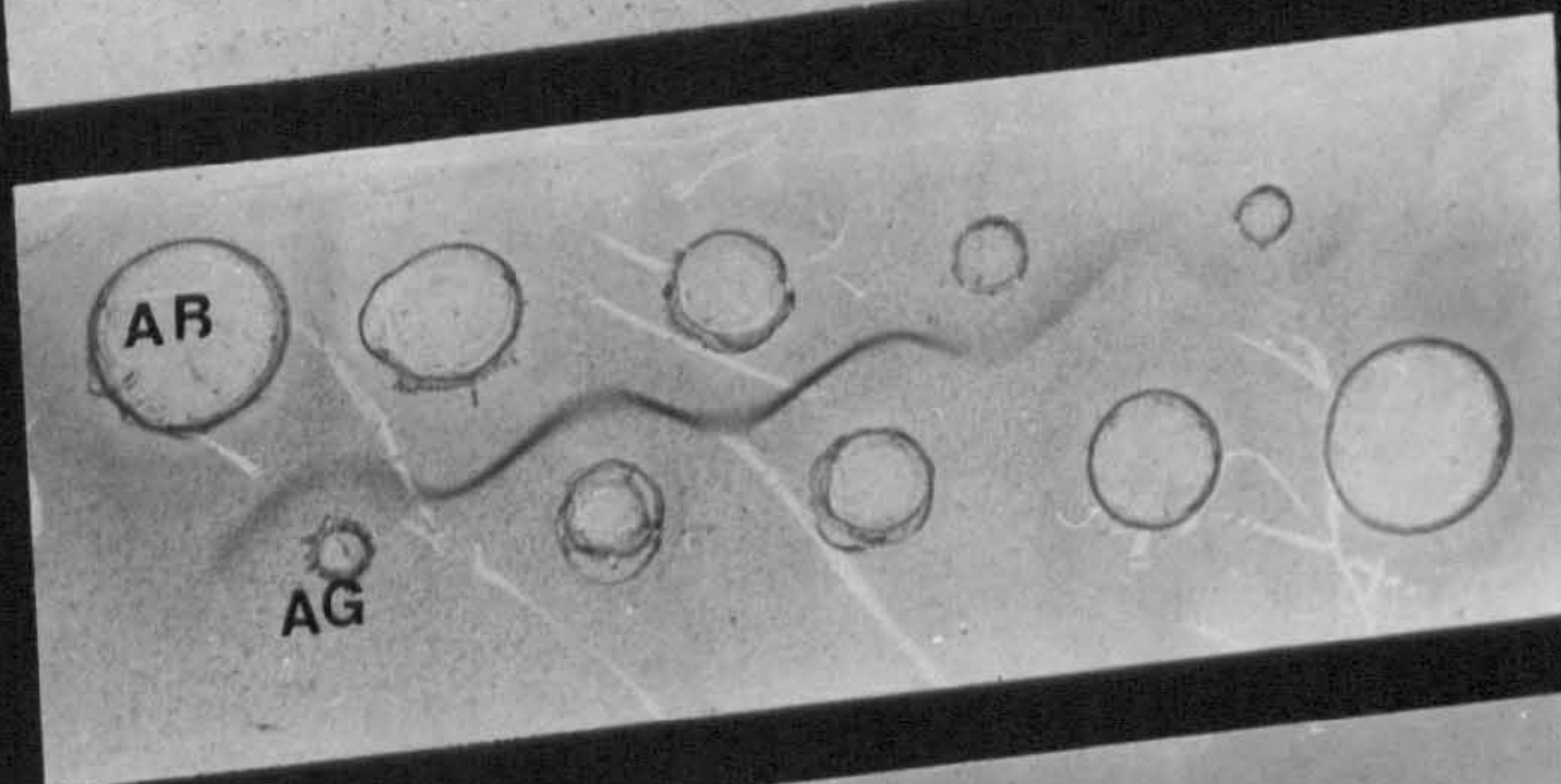
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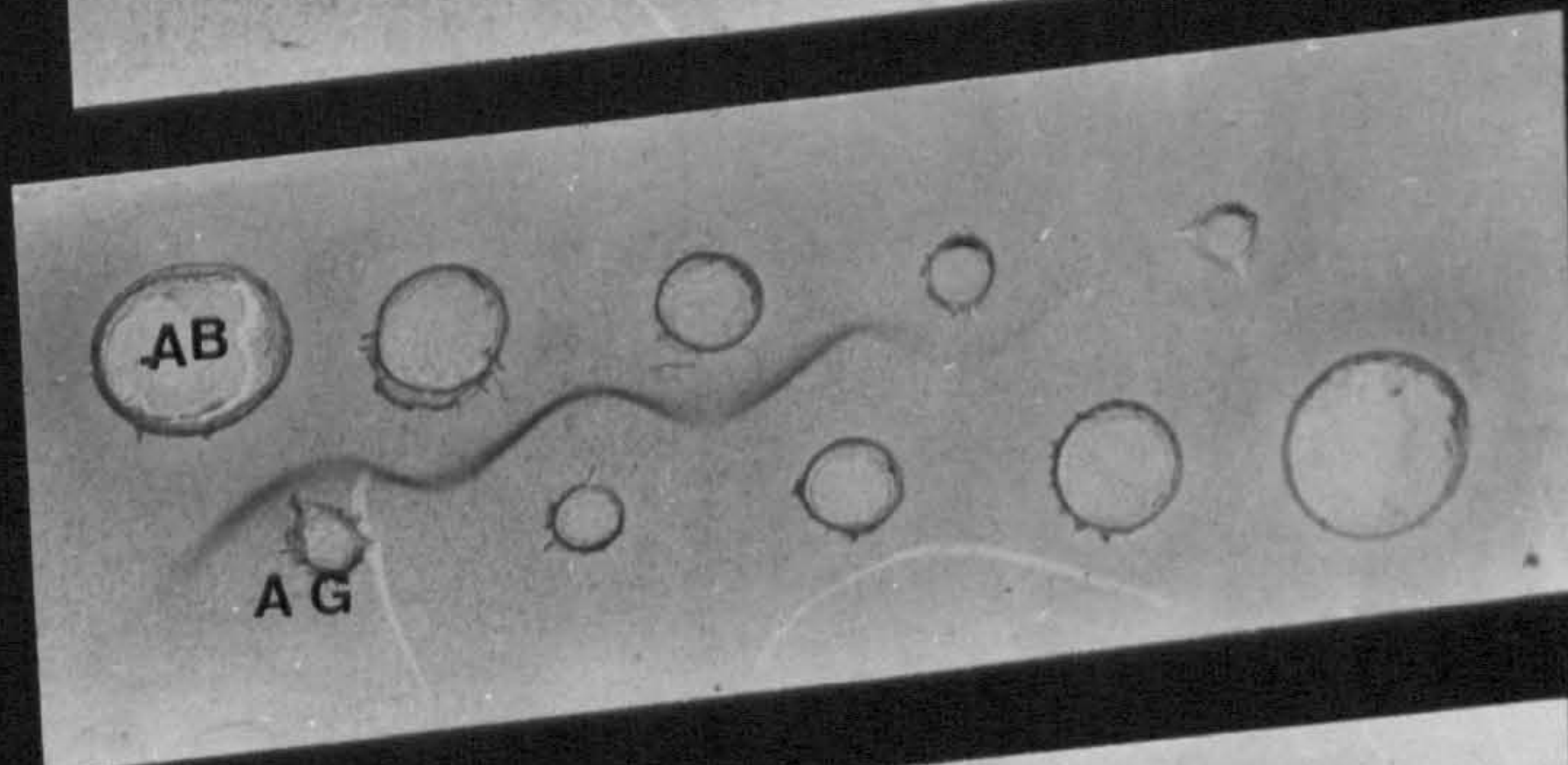
3



4



5



6

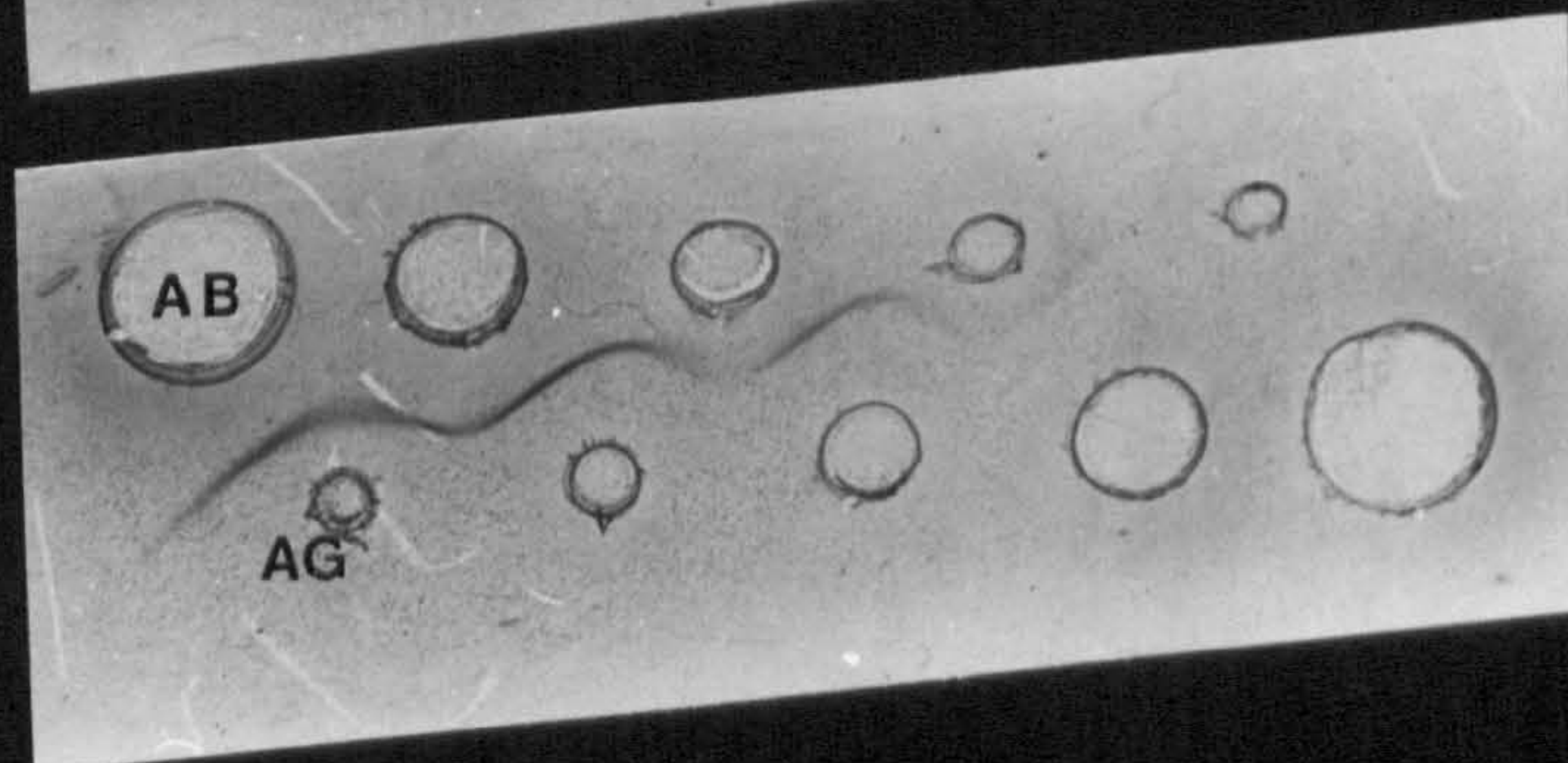




Figure 25. Detection of released antigens from infected mouse serum. The mice were inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) brucei and maintained at 22-27°C. The figure shows the occurrence of released antigen in serum samples collected after:

- a) 66 hours after inoculation
- b) 90 hours after inoculation
- c) 93 hours after inoculation (animals were moribund when the samples were collected)

x = antisera

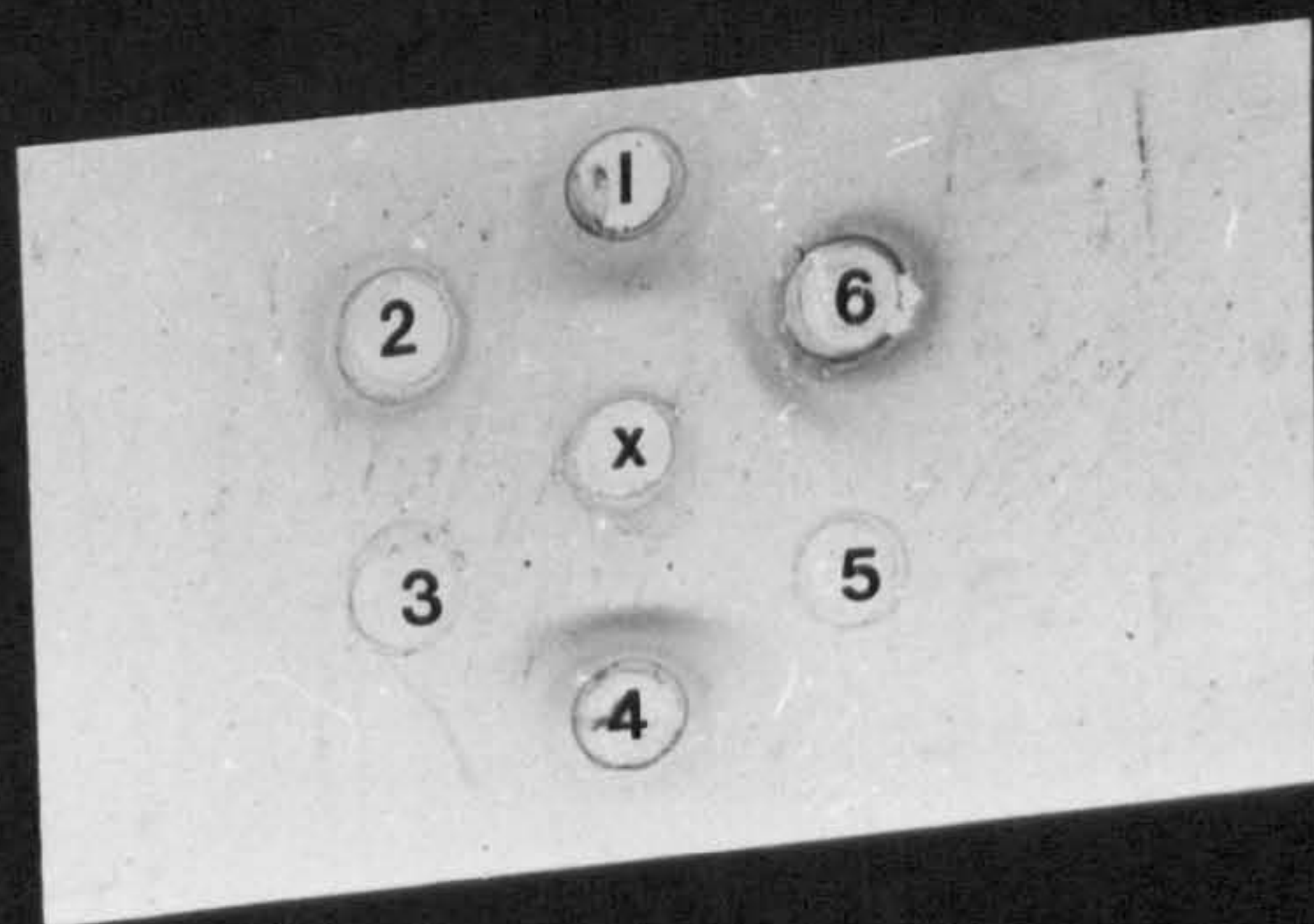
1,2, 3, 4, 5, 6 = mouse no.

c = control normal sera

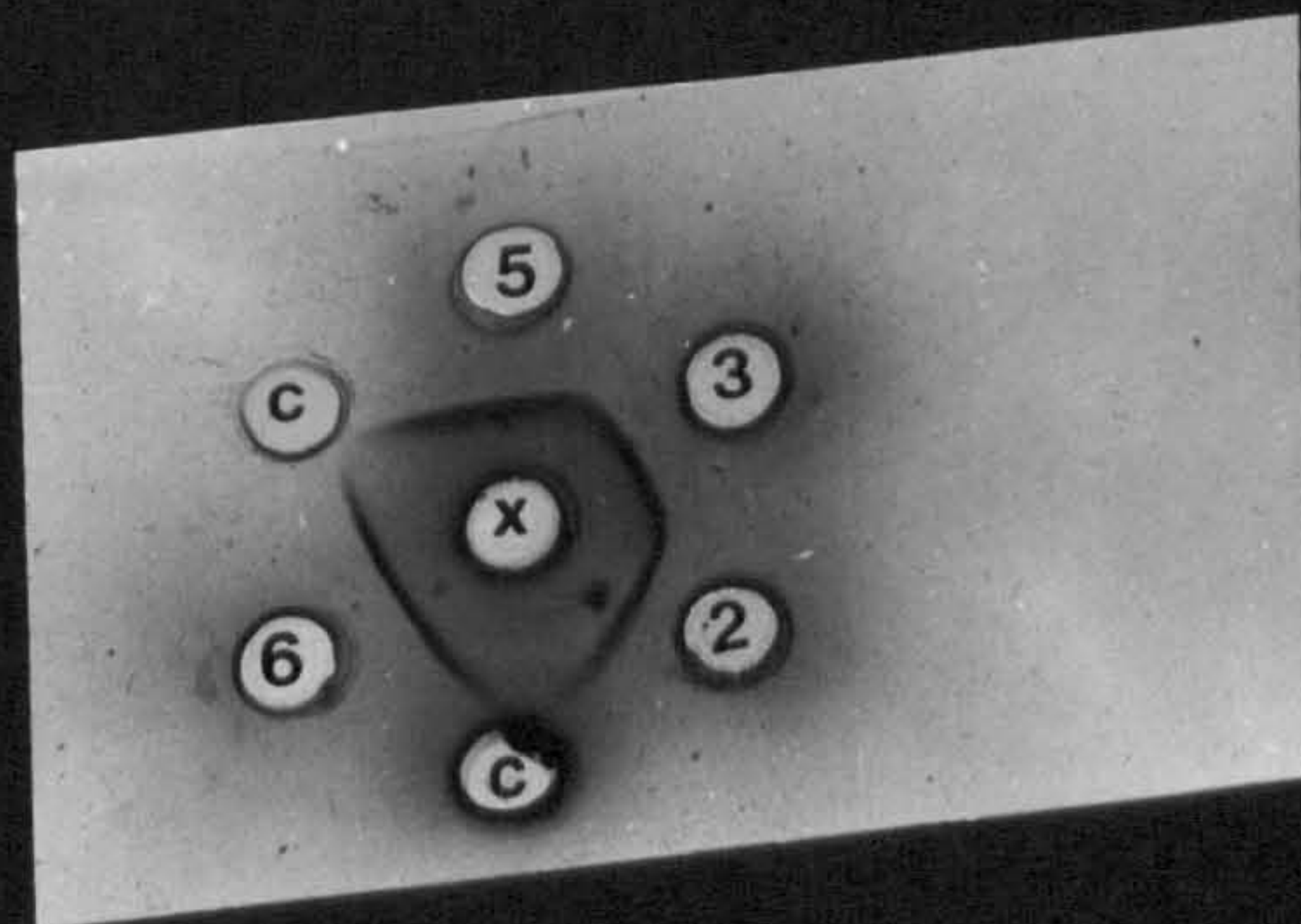


fig.25

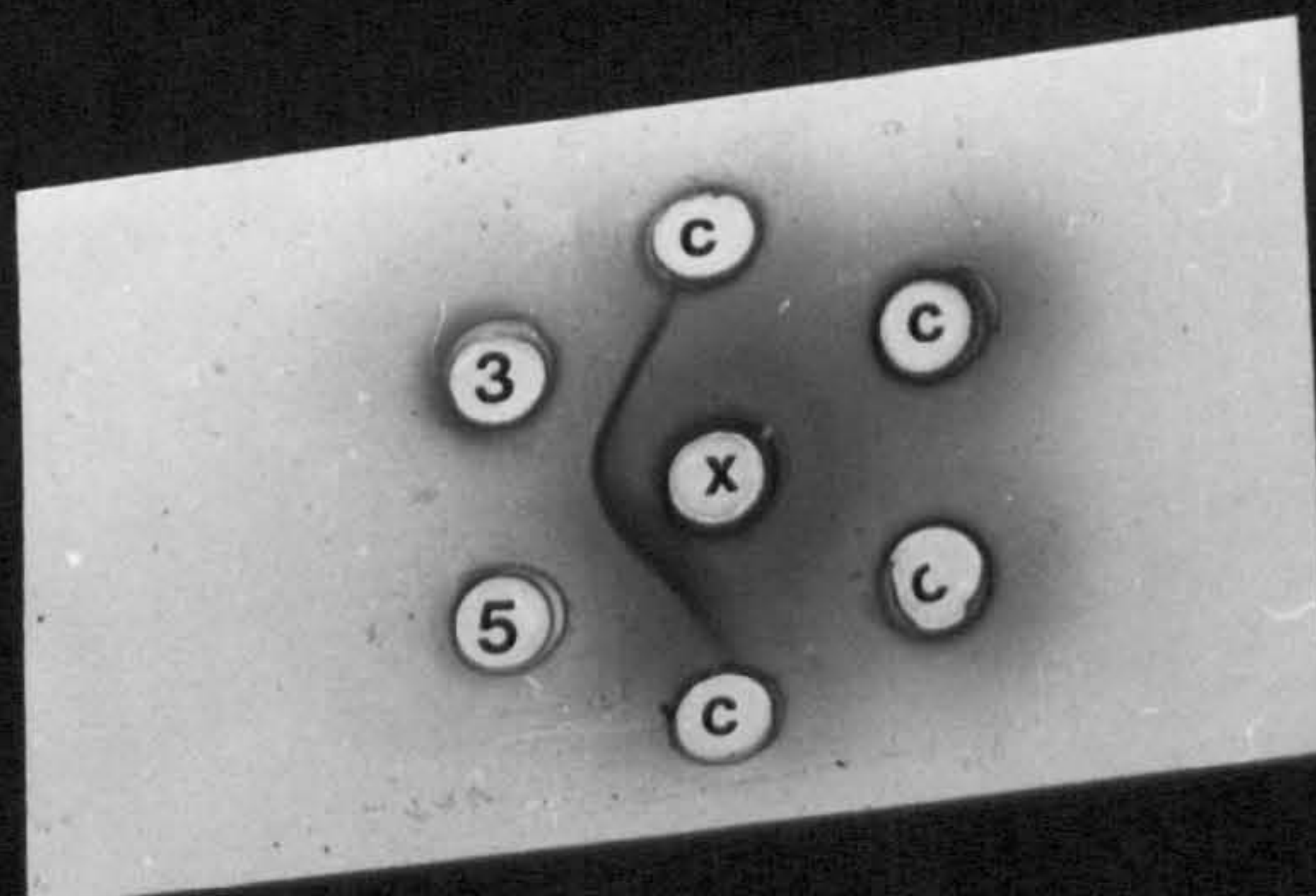
a



b



c





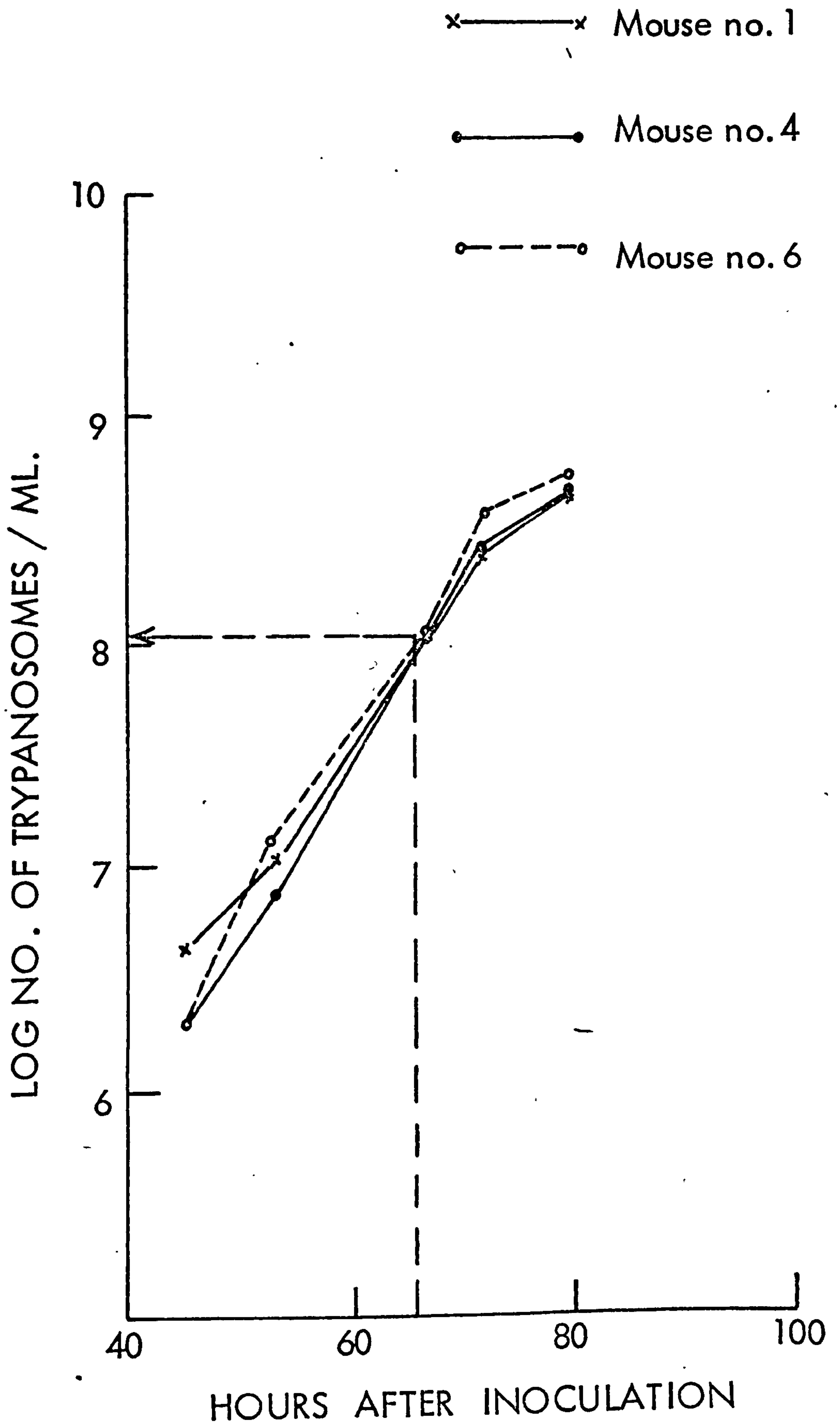


Fig.26. The relationship between the height of parasitaemic level and the appearance of released antigen in infected mouse serum. The figure illustrates parasitaemic curves of three mice in whose serum samples the released antigens were detected at 66 hours after inoculation. The mice were maintained at 22-27°C after inoculation with T. (T.) brucei LUMP 128.



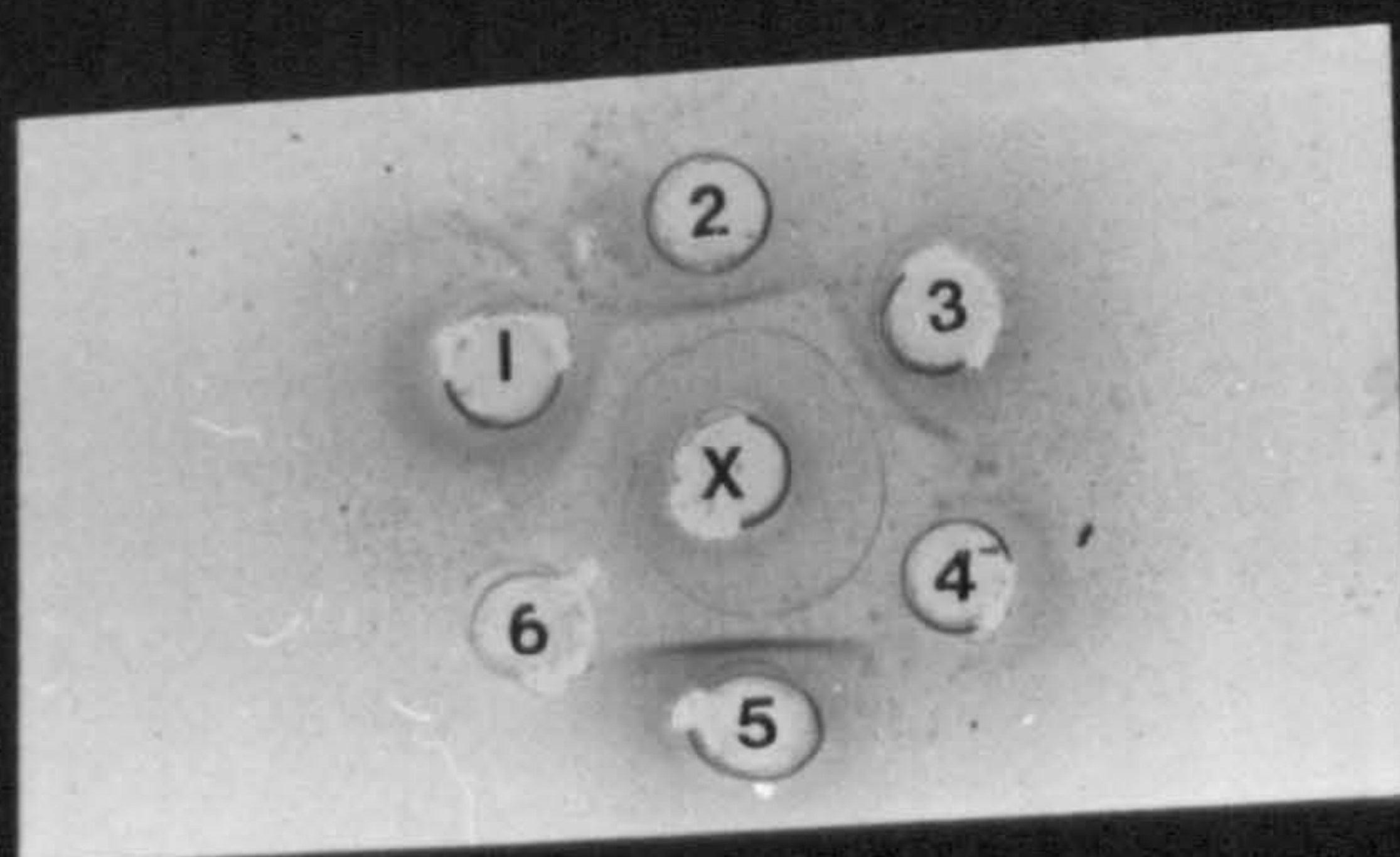
**Figure 27.** Detection of released antigens in infected mouse serum. Mice were inoculated with antilog 2.1 ID<sub>63</sub> T.(T.) brucei, LUMP 128 and treated with Diminazene aceturate 66 hours after inoculation. The figure shows specific precipitating reaction observed just before treatment (see Table 19 for parasitaemic curves recorded for individual mice).

1, 2, 3, 4, 5, 6 = mouse no.

x = antisera.



**Fig.27**





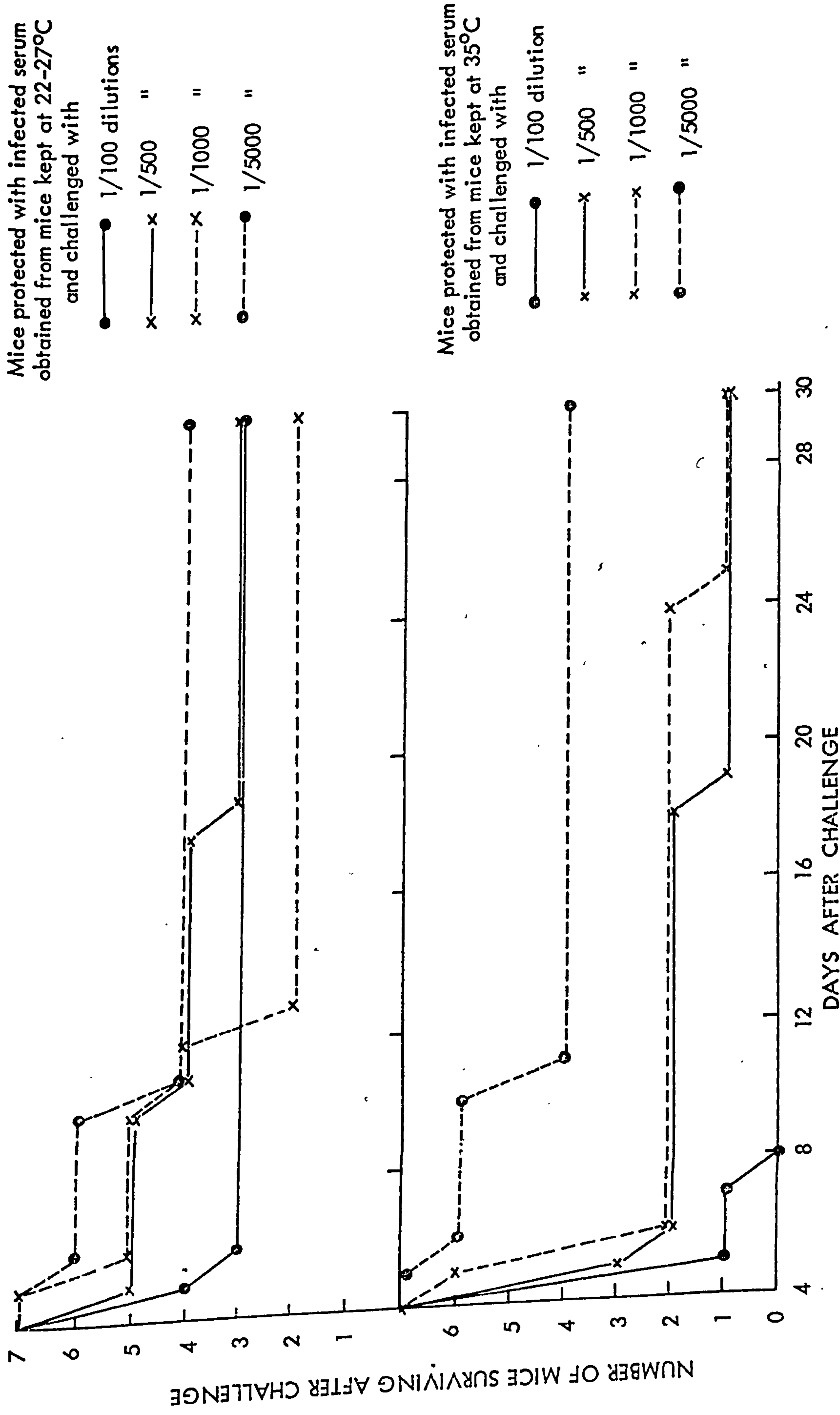


Fig. 28. Results of challenging mice with various dilutions of *T. (T.) brucei*, LUMP 128. Mice were previously protected with infected serum obtained from groups of mice infected with LUMP 128 and kept at 22-27°C and at 35°C.

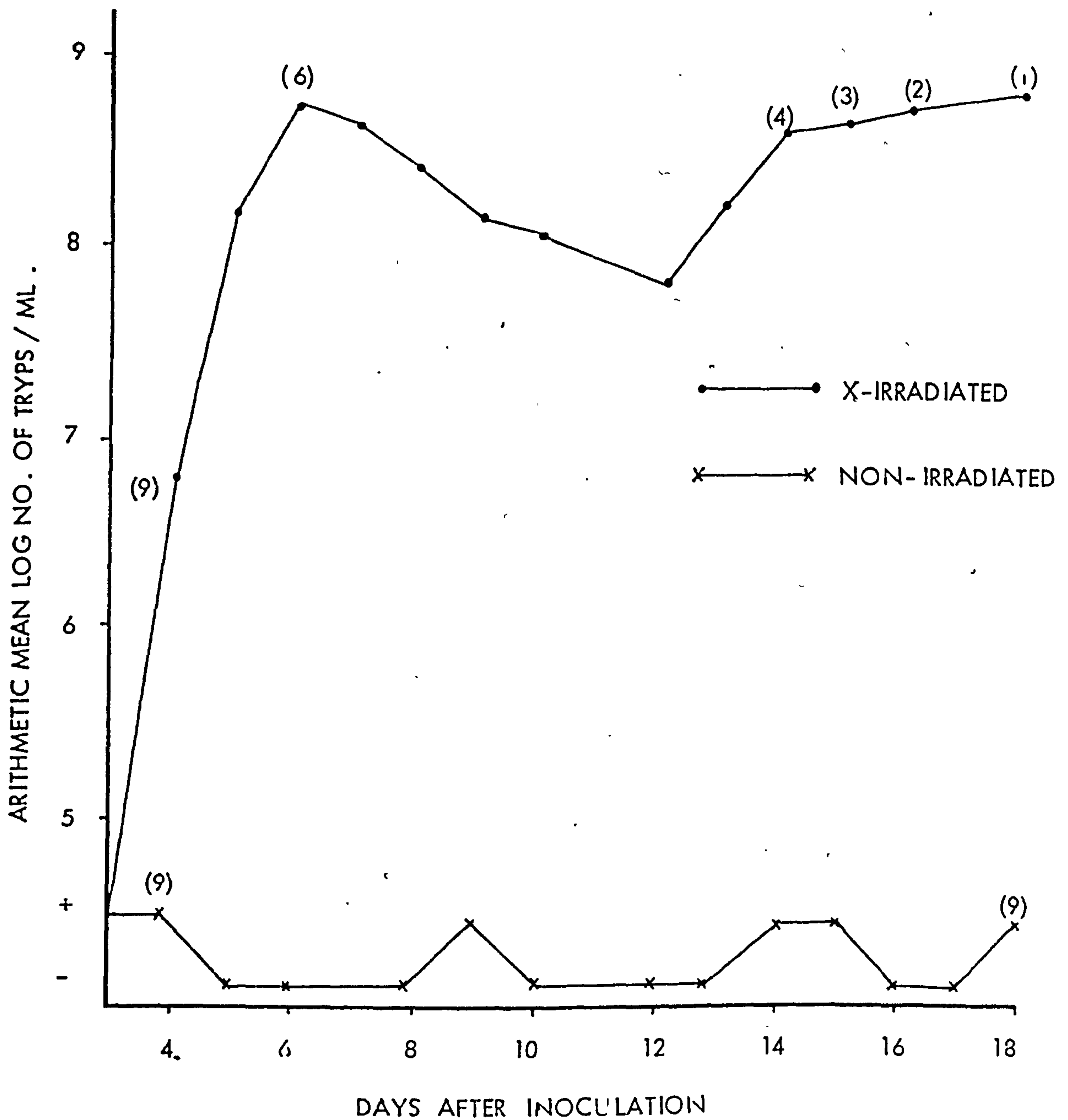


Fig.29. Parasitaemic curves of T.(T.) brucei LUMP 128 infections in groups of x-irradiated and non-irradiated mice kept at 35°C. Figures in parenthesis refer to the number of animals surviving at the time of examination.



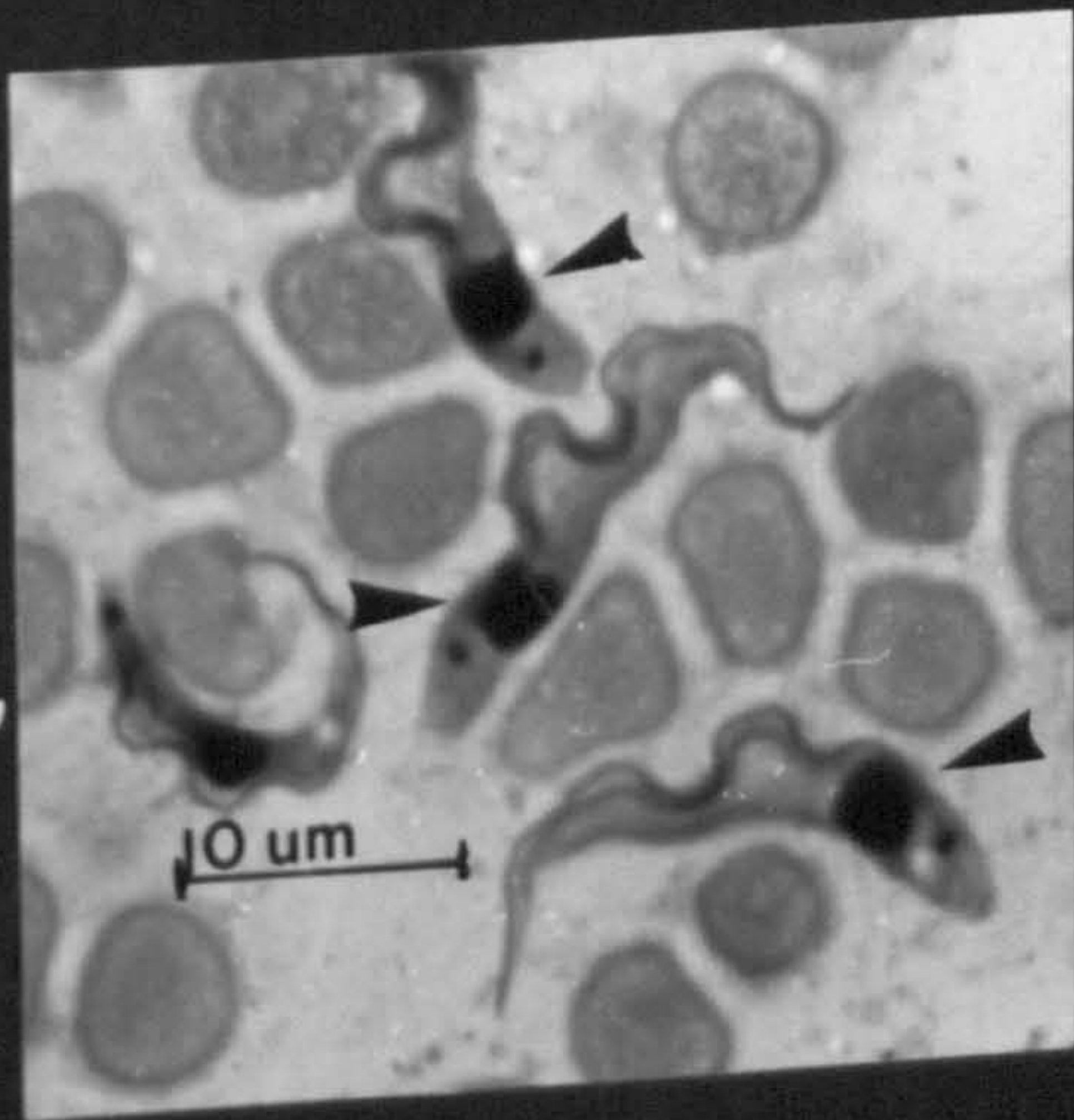
Figure 30. T.(T.) brucei LUMP 128 infection in  
x-irradiated mice maintained at 35°C.

- a) Note the posteronuclear forms observed during peak parasitaemia, during this time forms with:
- b) Nucleus pushed to the extreme posterior end
- c & d) Multinucleated and
- e) Loss of nucleus were observed.

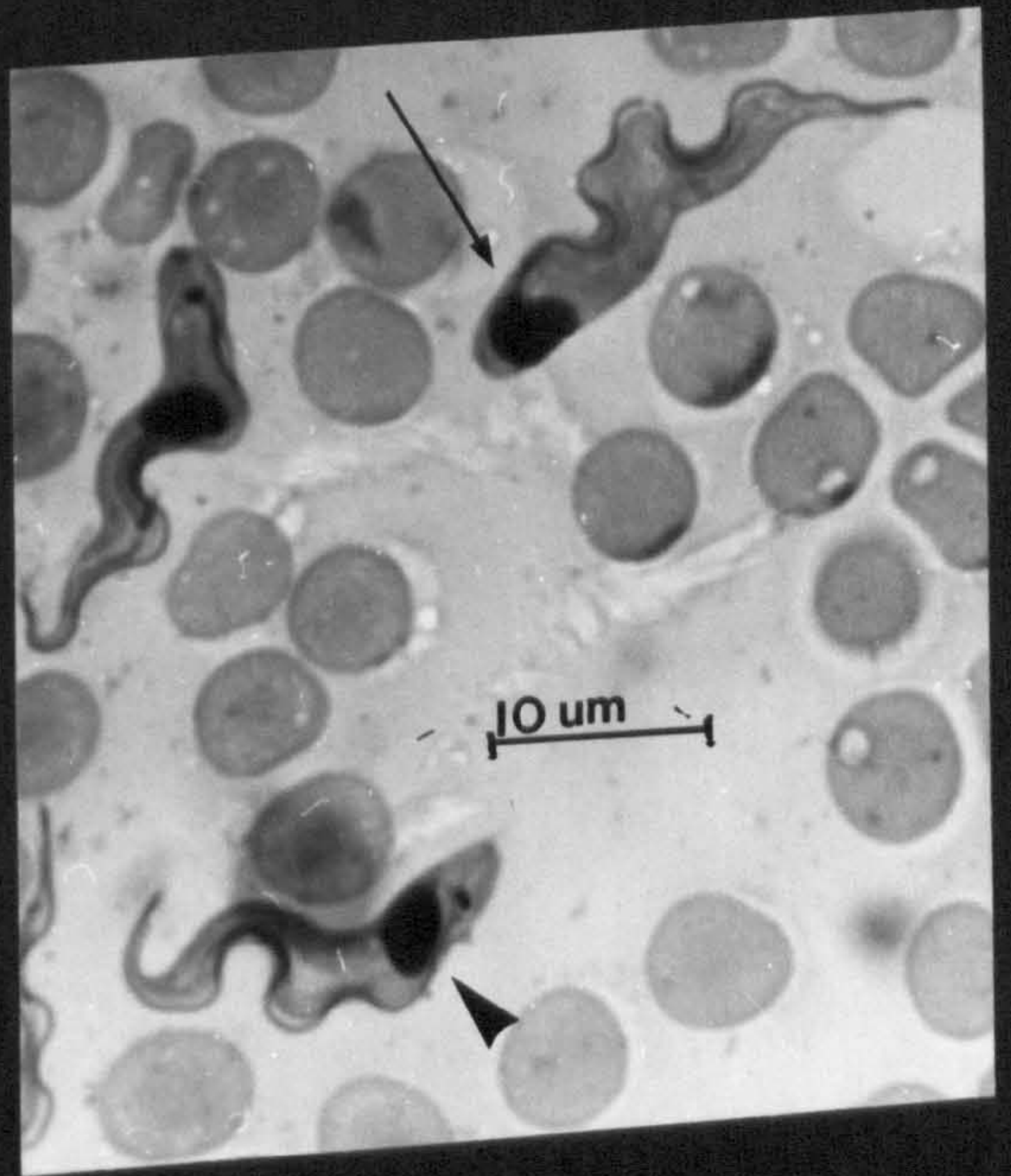


**FIG.30**

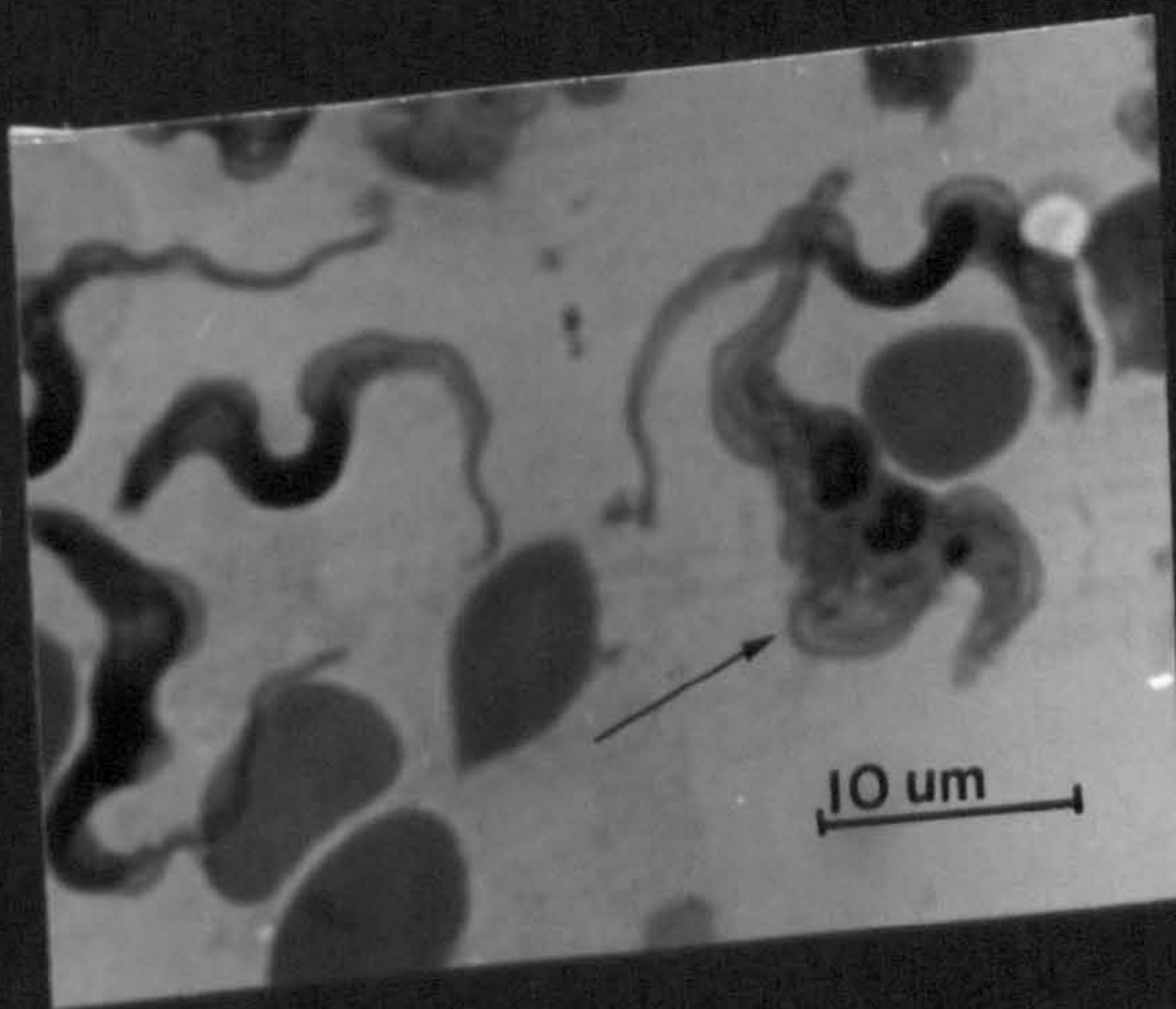
**a**



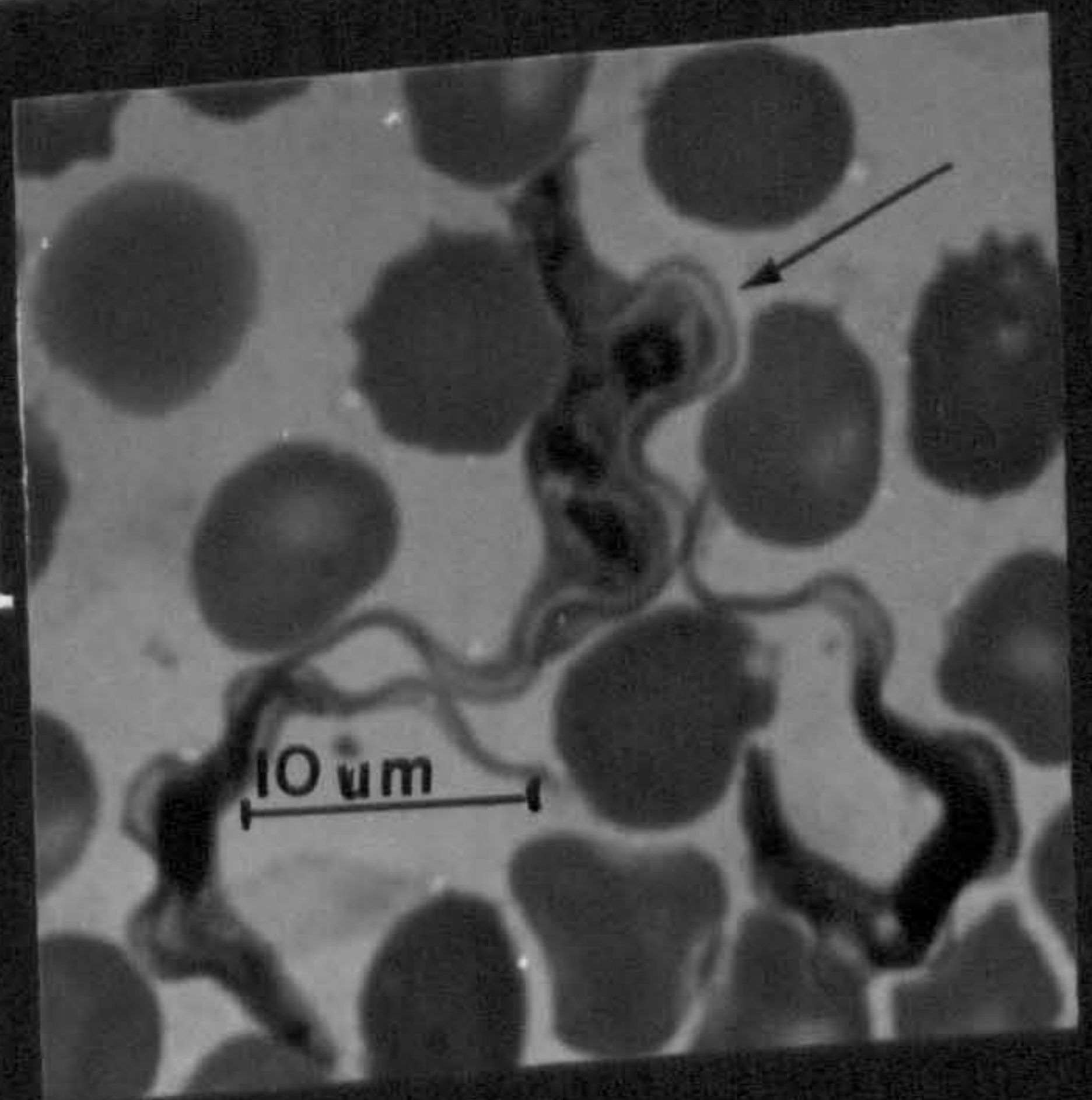
**b**



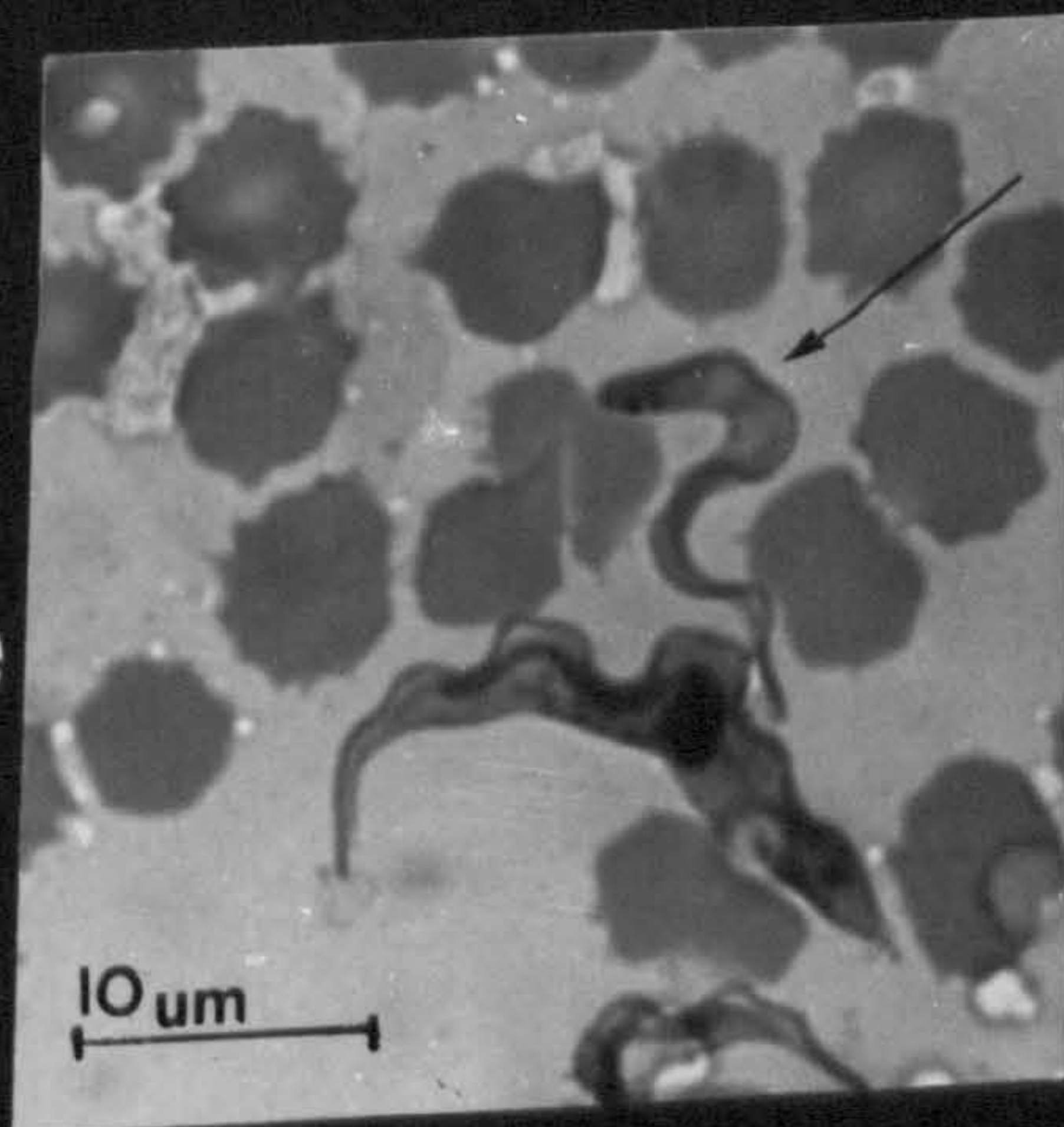
**c**



**d**



**e**





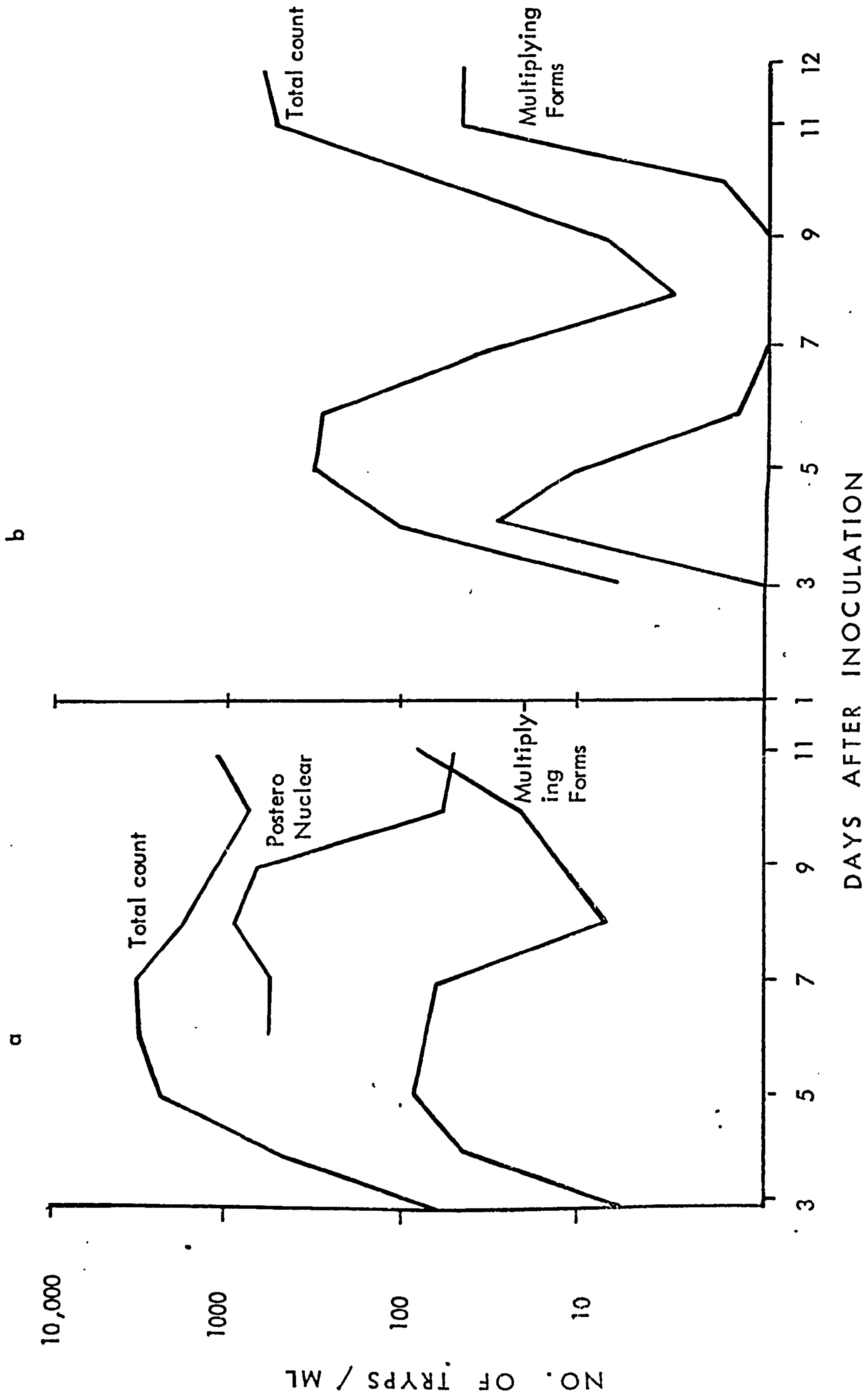


Fig. 31. The relationship between total trypanosomes counts multiplying forms and the posternuclear forms in (a) x-irradiated and (b) non-irradiated T.(T.) brucei infected mice maintained at 35°C.

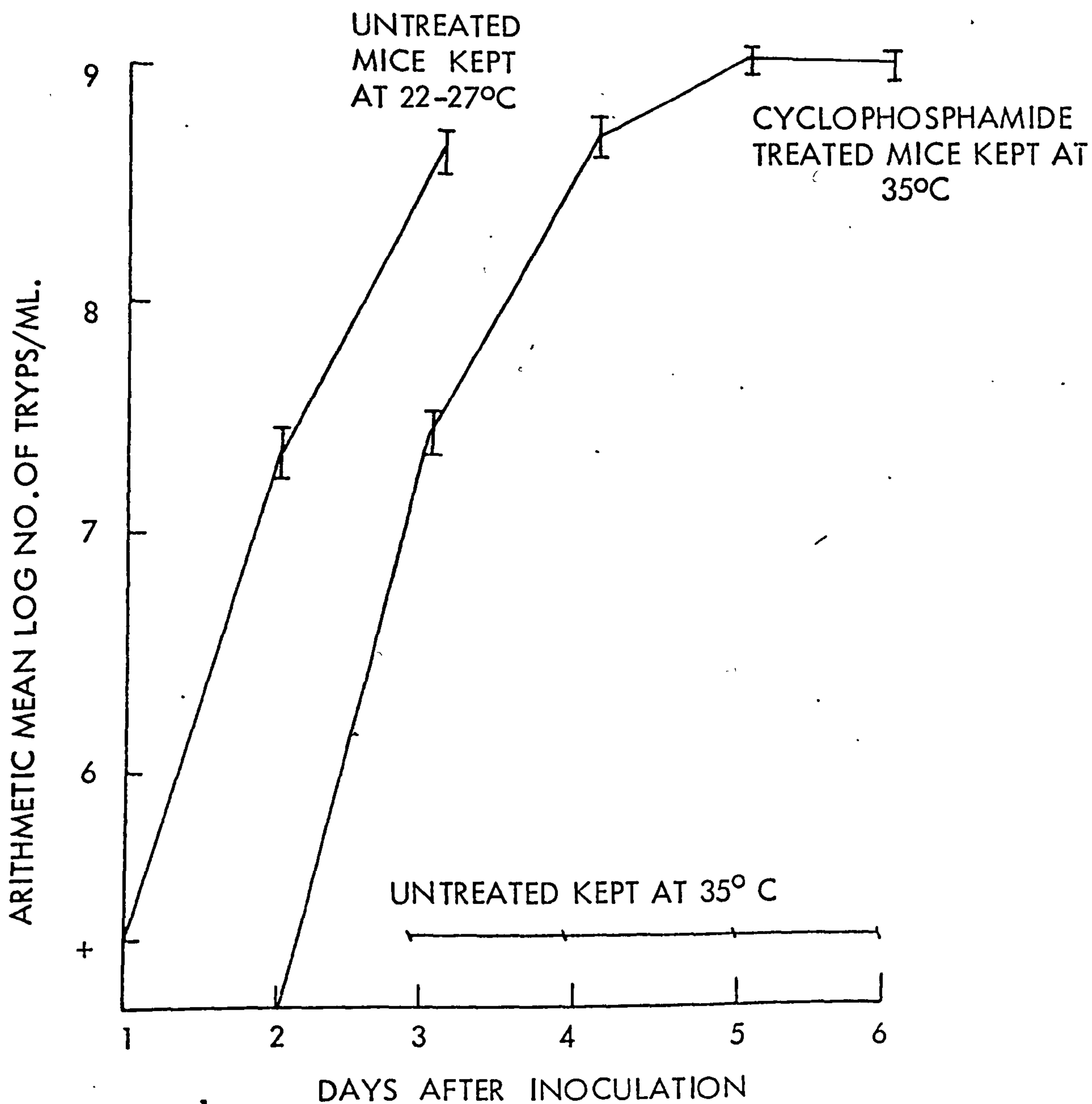


Fig. 32. The course of T.(T.) brucei LUMP 128 infection in cyclophosphamide treated mice kept at 22-27°C and at 35°C. Parasitaemic curve for control untreated mice kept at 22-27°C is included to illustrate the normal course of infection.



Figure 33. T.(T.) brucei LUMP 128 infection in mice treated with cyclophosphamide.

- a) Note the presence of multinucleate forms observed at the height of infection.
- b, c) Vacuolated trypomastigote appeared in mice which survived beyond day 6.
- d) completely rounded forms with free flagellum occurred just before the mice died.
- e) Large trypomastigotes with blunt posterior ends preceded the appearance of the rounded forms, they occurred between day 5 and 6 after inoculation.



**Fig.33**

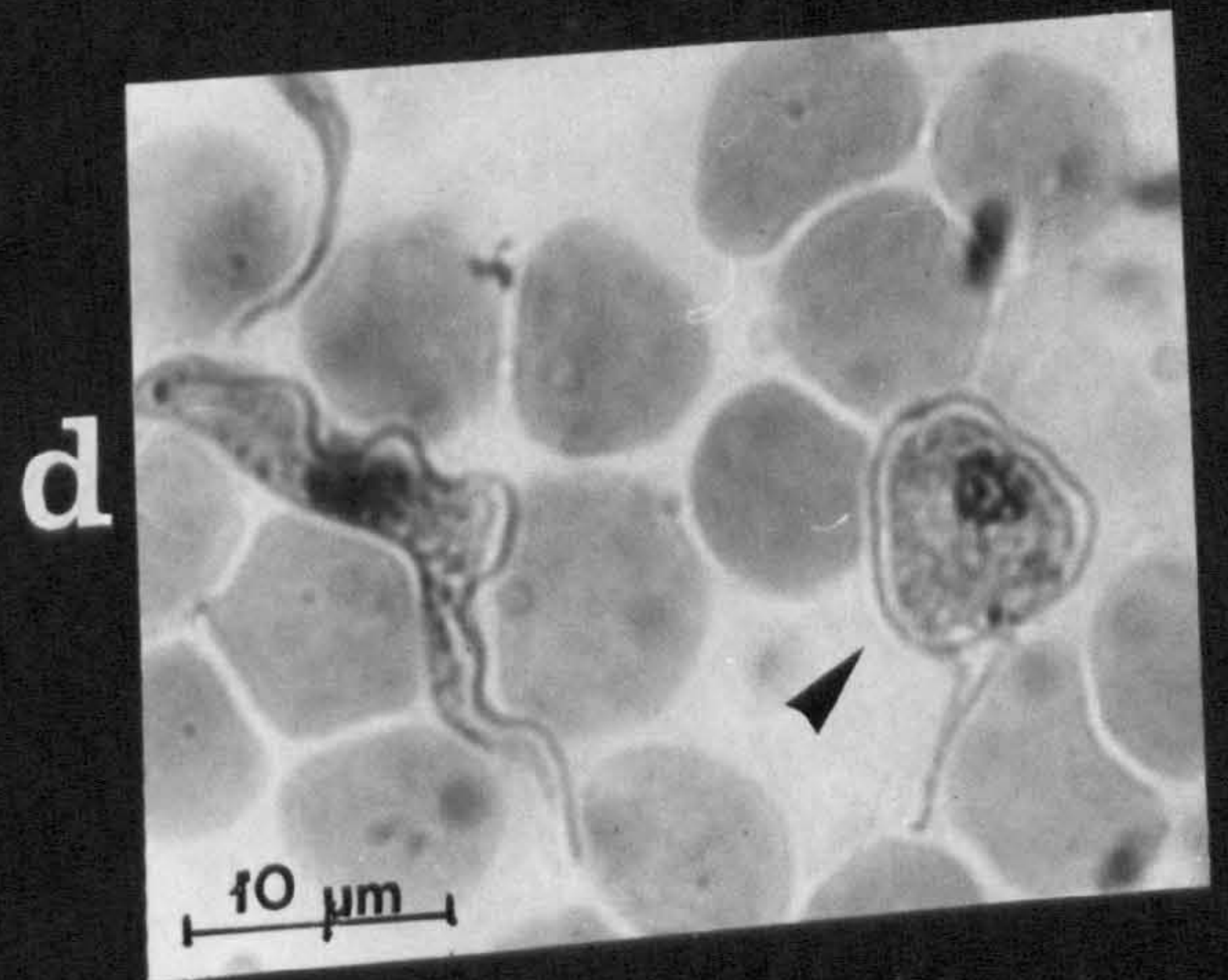
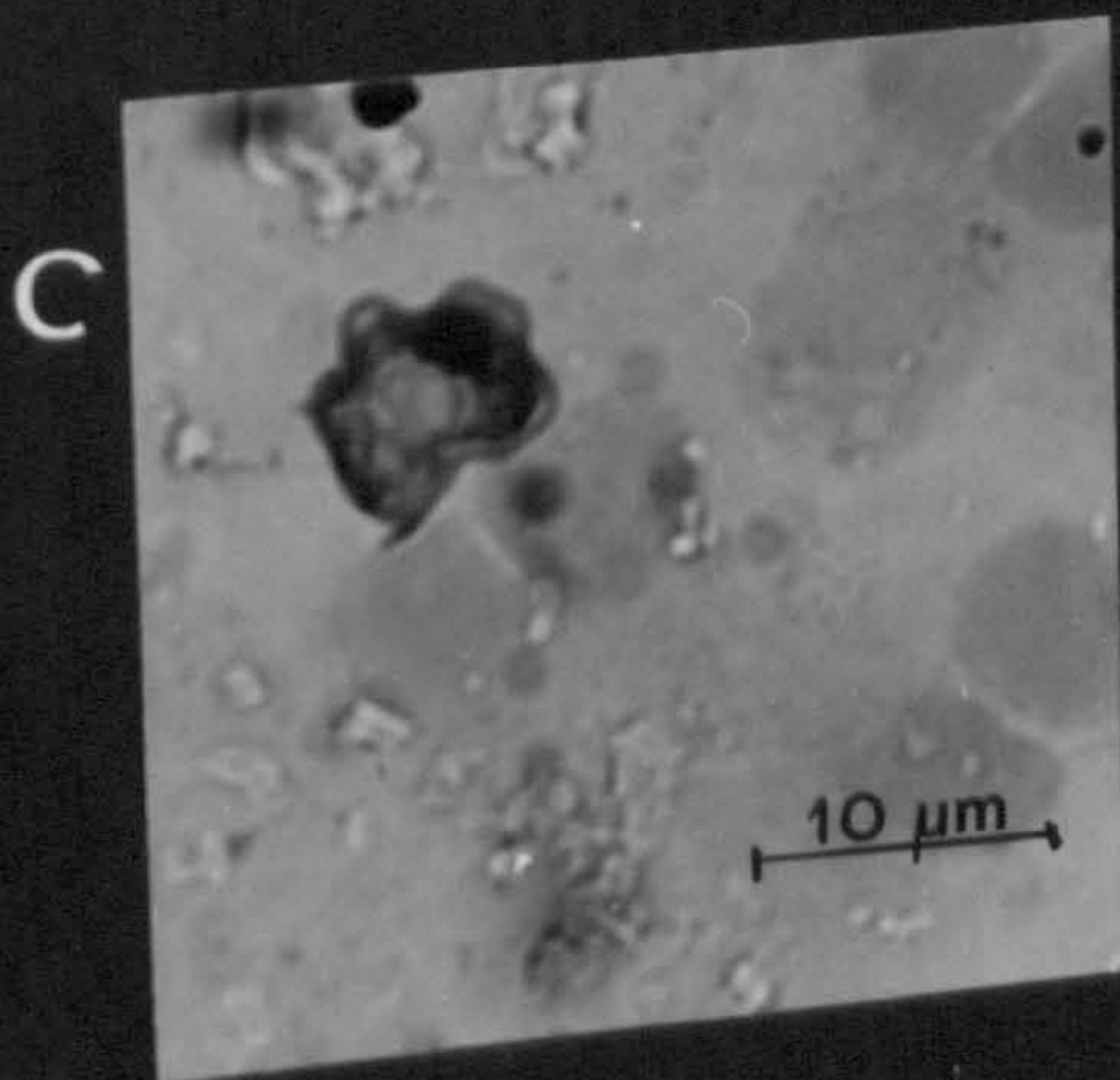
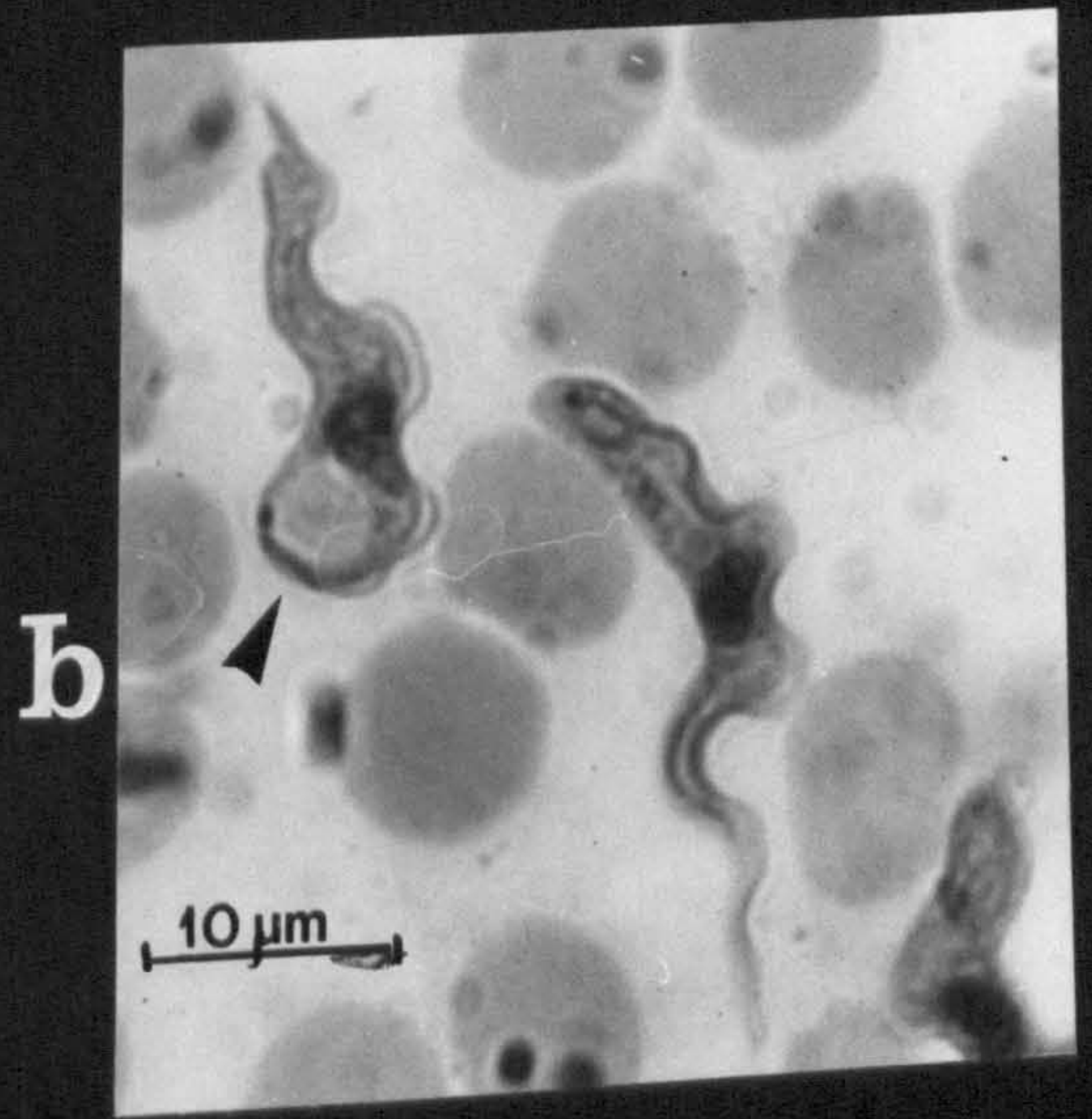
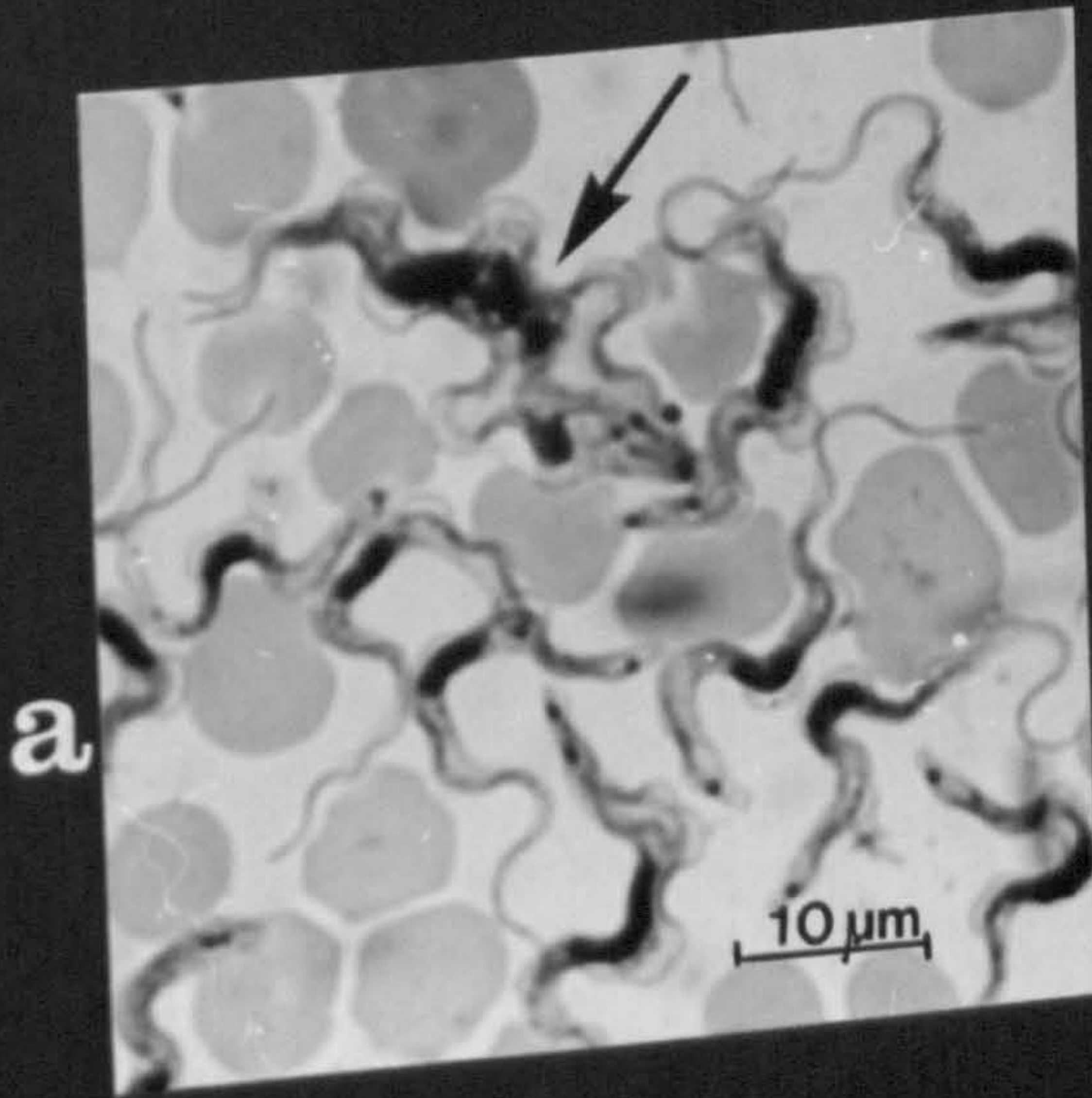




Figure 34. T.(T.) brucei LUMP 128 in mice treated with cyclophosphamide.

- a) Long thin forms adhering to leucocytes observed at the height of infection (day 5).
- b) Bits of flagellum adhering to <sup>monocyte</sup> polymorph. Note a disintegrating trypomastigote adhering to the same polymorph.  
monocyte

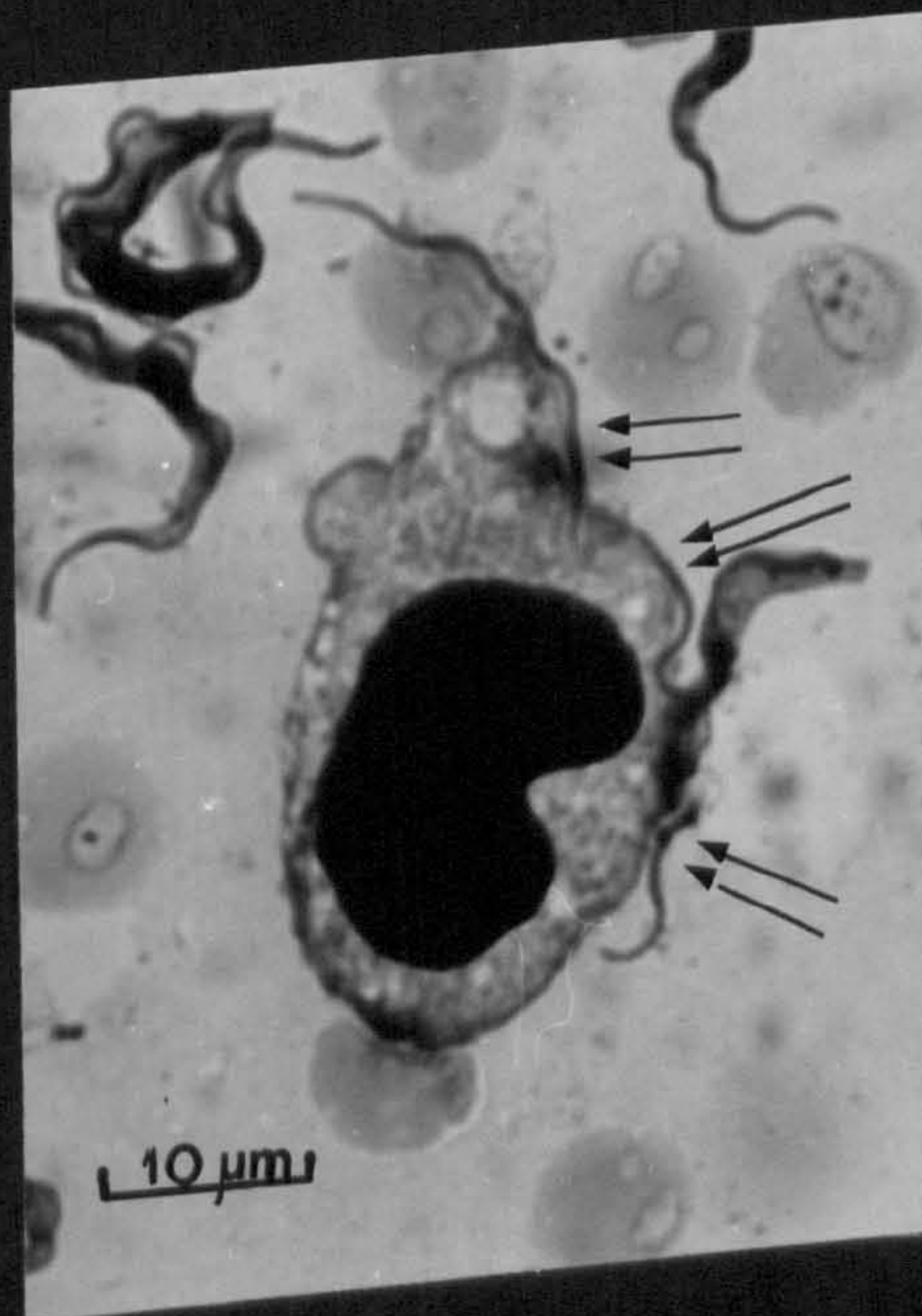


**Fig. 34**

**a**



**b**





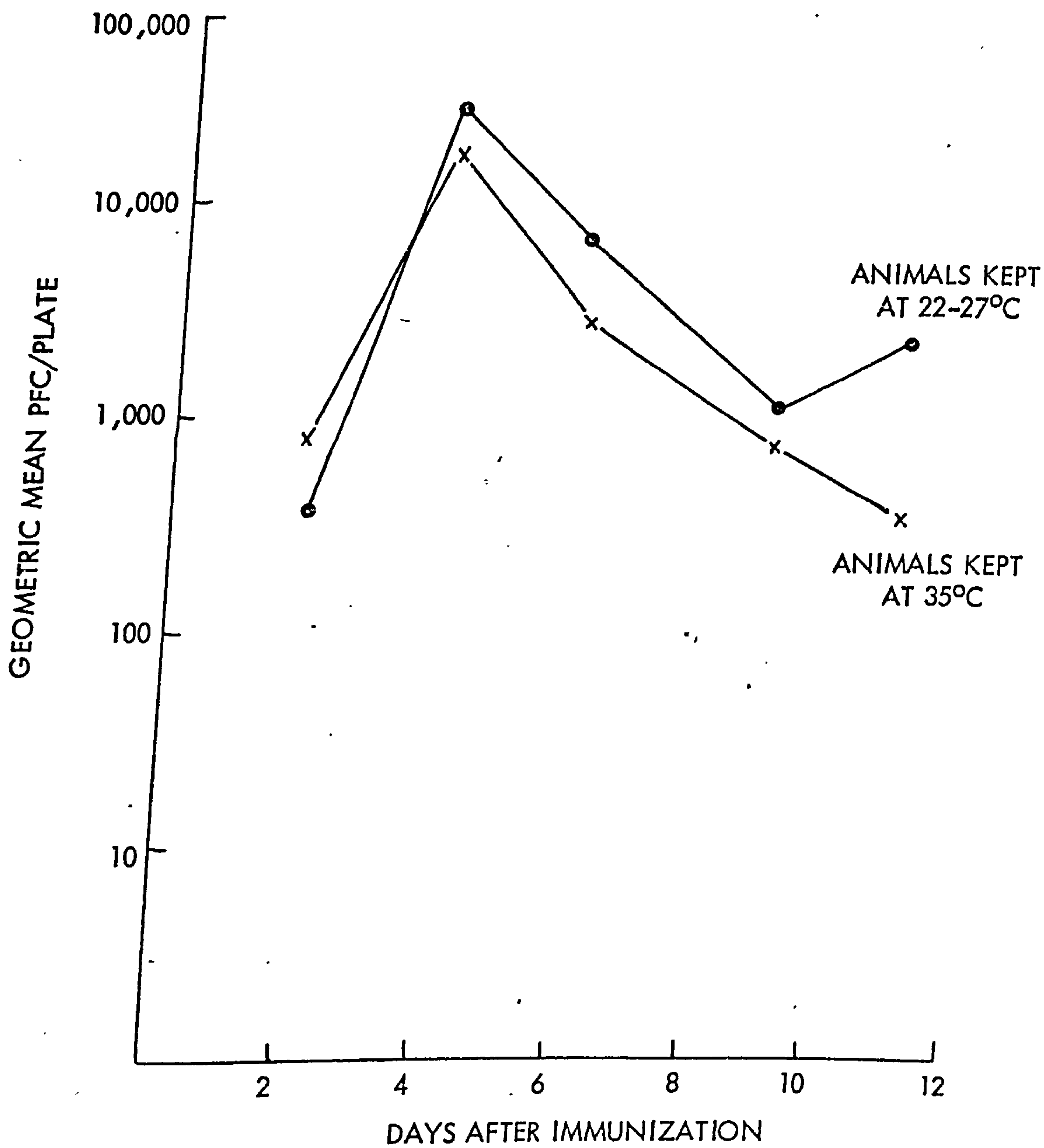


Fig.35. A comparison of the number of direct plaque forming cells detected in groups of mice immunized with sheep red blood cells and kept at 22-27°C and at 35°C.

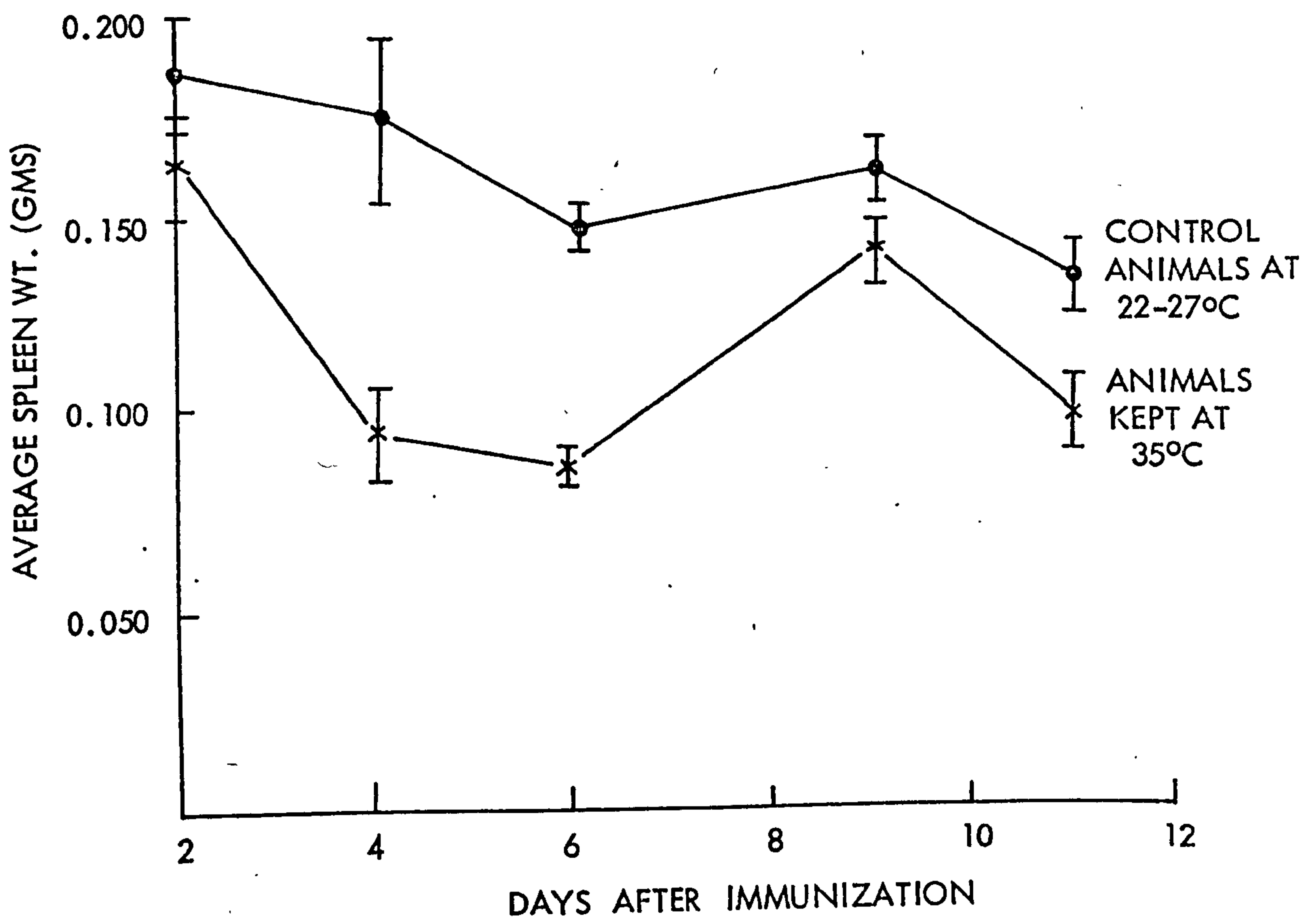


Fig. 36. Spleen weights from which the plaques were detected (see Fig.35 ). The groups of mice were immunized with sheep red blood cells and kept at 22-27°C and at 35°C.



## **PUBLICATIONS**

977. Influence of ambient temperature on the course of experimental African trypanosomiasis.

LEONARD H. OTIENO, London School of Hygiene and Tropical Medicine, England.

The temperatures which affect the growth of trypanosomes are to a large extent determined by the physiological limits of the supporting host animal. In homeothermic animals, homeostatic mechanisms ensure that large fluctuations in the ambient temperatures are adjusted by comparatively small changes in the body temperature, however, these changes may still be sufficient to bring about major alterations in the rate of trypanosome multiplication in the normal host. Experiments are described which show that virulence of *T. brucei* subgroup to Swiss white mice is influenced by the ambient temperature.

A virulent strain of *T. brucei* subgroup was found to cause a mild and relapsing infection when the environmental temperature was raised to 35°C. The change from acute to chronic infection began at 32°C when some of the mice infected died during the first parasitaemic wave. The morphology of trypanosomes also changed from predominantly monomorphic to pleomorphic forms.

The course of infection caused by a strain of a new laboratory strain of *T. brucei brucei* (relapsing type) was not influenced by the environmental temperatures studied, except at 35°C, when parasitaemic levels were depressed.

*T. evansi* akinetoplastic strain was found to be very heat labile and could not develop in mice maintained at 35°C; but reappeared when mice were transferred from this to laboratory temperature.

Measurements of rectal temperatures indicated that mice at 35°C had significantly higher body temperatures than those kept at laboratory environment. In addition, they suffered a big weight loss.

J. Parasit. 57, Abs. No. 977.



142. Temperature effect upon the pathogenicity and morphology of *Trypanosoma (Trypanozoon) brucei* infections in immunosuppressed mice and in chick embryos. LEONARD H. OTIENO, London School of Hygiene and Tropical Medicine, England.

A virulent, monomorphic strain of *T. (T.) brucei* in mice becomes mild when infected mice are maintained at 35°C. In this environment, mice demonstrate slight elevation (1°C) of body temperature (Otieno 1970).

The following experiments were carried out to determine whether this change in pathogenicity was due either to:

1. early formation of antibodies or
2. direct heat effect upon the trypanosomes.

Various immunosuppressive agents were used to treat groups of TO mice prior to inoculation. Mice were inoculated with  $3.8 \times 10^4$  trypanosomes either 4 days after irradiation and cyclophosphamide treatment or 5 days after methotrexate treatment. Half of the inoculated mice were maintained at 35°C and the remainder at 22-27°C. A haemocytometer was used to make daily parasite counts. The morphology of trypanosomes was determined from Giemsa-stained thin blood films.

Immunosuppressive agents had no effect upon infected animals maintained at 22-27°C. However, the virulence of the trypanosomes was enhanced by immunosuppressing heat stressed mice. The cyclophosphamide treated mice died during the first peak of infection. Parasitaemias in irradiated animals reached a peak in 5 days and remained at high level until the animals started dying 14 days later. A remission occurred in methotrexate treated mice.

The morphology of trypanosomes in immunosuppressed mice, kept at 35°C was striking between days 6 and 9 after inoculation. A large proportion of the parasites became stumpy, many of which were posteronucleated and some multinucleate.

Direct heat effect upon the trypanosomes was tested by inoculating 8 day old chick embryos with  $1.9 \times 10^4$  trypanosomes and incubating them at 37°C and 39°C. At 37°C scanty parasitaemia was observed and some infected embryos hatched. At 39°C heavy parasitaemias occurred and no infected embryo survived more than 9 days after inoculation.

Morphological forms similar to those described above, particularly multinucleates appeared between days 7 and 8 in embryos incubated at 39°C. A gradual increase in vacuolation in a large number of trypomastigotes was observed. They rounded off and transformed into vacuolated sphacromastigotes. Degenerating forms were also seen.

Otieno, L. H. (1970). *Proc. 2nd Int. Cong. Parasit.* Washington Part 4, 68.

J. Protozool. 18, Suppl. Abs. No. 142.

## Morphology of *Trypanosoma brucei* in chick embryos

L. H. OTIENO AND P. G. SARGEAUNT

*Department of Medical Protozoology, London School of Hygiene and Tropical Medicine*

*Trypanosoma (Trypanozoon) brucei*, LUMP 128 stabilate used in this investigation was known to be entirely monomorphic and caused a rapidly fatal infection in albino mice T.O. strain. It, however, caused a relapsing infection in heat-stressed mice; when the mouse body temperature was elevated by approximately 1 C. above normal, i.e. 37.5°C. (OTIENO, in press). The question therefore arose, was the change in pathogenicity due to direct heat effect on the trypanosomes? If there were such a direct relationship between the body temperature and trypanosome virulence, it might be possible to detect it if the trypanosomes were cultured in chick embryos and the embryos in turn incubated at a temperature just above that of the normal mouse body temperature.

Examination of this possibility was investigated by inoculating through the chorio-allantoic route 8 day old chick embryos with  $1.9 \times 10^4$  trypanosomes per ml. and incubating them at 37°C. and 39°C. Small numbers of trypanosomes developed in embryos incubated at 37°C. and persisted at low level until some embryos hatched. On the other hand, large numbers of trypanosomes developed in embryos incubated at 39°C. and after day 9, there was no living infected embryo seen.

On day 7 after inoculation, multinucleate trypanosomes were frequently observed in stained blood preparations. On day 8 and 9, at about the peak parasitaemia multinucleate forms had disappeared and were replaced by vacuolated stumpy forms which increased in number. The vacuolated posterior end was increased in size and the rest of the body folded round it. Finally, perfectly rounded flagellated forms with distinct kinetoplasts and elongated well staining nuclei resulted. WIJERS (1966) fails to mention these rounded forms (sphaeromastigotes) in *T. rhodesiense* infections in chick embryos.

The degenerating forms were also seen but these appeared to have lost most of the cytoplasmic contents and only granulated round nuclei remained.

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WIJERS, D. J. B. (1966). *Ann. Rep. Med. Res. Cent., Nairobi*, 115-117.

Trans. R. Soc. trop. Med. Hyg. **65**, 424

In press (1972). Influence of ambient temperature on the course of experimental trypanosomiasis in mice. Ann. trop. Med. Parasit.