

Antigenic variation of Trypanosoma (Trypanozoon) evansi  
(~~Steel, 1905~~) in the mouse host, with related studies  
on culture and on transmission.

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## INTRODUCTION

Trypanosomiasis, whether it affects man or domestic animals, continues to be one of the most baffling problems which has confronted medical and veterinary parasitologists. Surra is the most important of all the animal trypanosomiasis since it has extremely wide geographical distribution and affects a wide variety of mammals. The causative organism Trypanosoma (Trypanozoon) evansi (T. evansi) was the first trypanosome to have been recorded from a mammal, but many fundamental aspects of the parasite such as morphology, taxonomy and phylogeny are not perfectly understood. Hitherto, a cycle of development has not been demonstrated in an invertebrate vector and the parasite is believed to be transmitted non-cyclically. Only a few attempts have been made to culture the parasite in blood-agar medium.

Comparatively little work has been carried out on the immunology of T. evansi. Certain immunological tests, e.g., indirect haemagglutination, agglutination, precipitation and fluorescent antibody tests have been demonstrated in experimental T. evansi infections; but an important immunological aspect, antigenic variation, has not yet been investigated in this species.

The present study is concerned with the establishment of antigenic variation of T. evansi in the mouse host and subsequent comparison of the antigenic types of the first natural relapse populations. The findings on antigenic variation in T. evansi have been compared with similar studies in other species of the subgenus Trypanozoon. Furthermore, the importance of the 'recurrent antigenic

type', which was observed in these investigations, has been evaluated with respect to the immunological classification of salivarian trypanosomes.

Pleomorphism was experimentally induced in an old monomorphic laboratory strain of T. evansi and attempts were made to culture both monomorphic and experimentally developed pleomorphic strains. Subsequently, the transmission of monomorphic and experimentally developed pleomorphic strains of T. evansi employing Glossina moraitans, Stomoxys calcitrans and Ornithodoros moubata was investigated.

In view of the similarity between pleomorphic strains of T. evansi and those of Trypanosoma (Trypanozoon) brucei, the systematic position of T. evansi has been discussed. The current concept on the phylogeny of T. evansi is critically reviewed and a different opinion on the subject is presented.

During these studies, the effect of environmental temperature on T. evansi infection was also studied and clone populations were set up to characterize 3 strains of T. evansi.

The thesis embodies observations on each of these aspects and the relevant literature, together with the present state of knowledge concerning T. evansi, has been reviewed.

## REVIEW OF LITERATURE

The literature review is divided into two sections. The first section deals with the literature relevant to the work carried out on T. evansi and also includes a general section pertaining to the present state of knowledge concerning T. evansi and surra. The second section deals with literature on antigenic variation in salivarian trypanosomes (mainly T. brucei) for comparison with those results obtained during the present investigation of antigenic variation in T. evansi.

### Trypanosoma (Trypanozoon) evansi (Steel, 1885)

#### 1. General

##### 1. 1. History and distribution

T. evansi is the causative organism of surra or surra-like diseases which are characterized by fever, anaemia, oedema, and cachexia and affects a wide variety of wild and domestic animals in the Middle East, Asia, North Africa, Central and South America.

Surra has been known for ages as a dreadful disease of domestic animals in the Old World. T. evansi, the first trypanosome to have been recorded from a mammal, was first described by Griffith Evans in 1880 in Dera Ismail Khan (West Pakistan) from the blood of horses, mules and camels suffering from surra. Steel (1885) later described the organism from transport mules in Burma and called it Spirochaeta evansi. However, Balbiani placed it in the genus Trypanosoma, hence its full zoological name is Trypanosoma (Trypanozoon) evansi (Steel, 1885) Balbiani, 1888.

In the early part of the twentieth century trypanosomes causing surra or diseases like surra were recognized by different names by various workers on the grounds of geographical and clinical differences and host specificity (Bruce et al., 1909; Emelin and Zeiss, 1928; Zeiss, 1937; Mahomed, 1939). They were also differentiated on the basis of cross protection tests (Laveran, 1907; Sergent et al., 1911; Sergent et al., 1915b; Yakinoff et al., 1921; Nattan-Larrier and Noyer, 1931, 1932). A review of the literature concerning this nomenclature is given by Hoare (1956), who found that there was remarkable similarity between 30 strains obtained from different mammalian hosts from Afro-Asian countries and concluded that all the names should be synonymized with T. evansi. Hoare had reached a logical conclusion and only one name Trypanosoma (Trypanozoon) evansi (Steel, 1885) need be retained.

Surra affects a wide variety of domestic animals particularly camels, horses, mules, donkeys, pigs, dogs, cattle, buffaloes and elephants. The first host records of T. evansi infection were established by Evans (1880) in camels, horses and mules; by Lingard in cattle, buffaloes and dogs in 1899 and in goats in 1906; and by Bruce et al. (1909) and Evans (1910) in elephants. T. evansi has also been recorded from tapir (Soetisno, 1933), from a natural outbreak of disease among deer in Mauritius (Adams and Lionett, 1933) and from tigers, jaguars and leopard in India (Sinha et al., 1971). There are no records of natural infections of this species in humans, although an accidental laboratory infection in man has been reported (Anon., 1947).

Surra has the widest distribution of all the trypanosome infections. In Africa the disease occurs southwards from the Atlantic and Mediterranean coasts and extends to 15°N latitude in western Africa and to 13°N latitude in eastern Africa, and in Somaliland surra is found in areas stretching south to the equator (0°) (Hoare, 1956). Since camel trypanosomiasis has been reported from Morocco, Algeria, Libya, Egypt, Sudan, Niger, Upper Volta, Mauritania, Spanish Sahara, Senegal, Gambia, Sierra Leone, Ghana, Ethiopia, French Somaliland, Somalia and Kenya (Rutter, 1967), it is probable that T. evansi occurs in these countries. T. evansi has been recorded from Chad (Cruvel and Balis, 1965) and Nigeria (Godfrey and Killick-Kendrick, 1962). In the Middle East, surra has been reported from Israel, Lebanon, Syria and Iran (Hoare, 1956) and from Jordan and Saudi Arabia (Rutter, 1967). The disease extends from the Middle East into Asia where it is found in Turkey (Argun and Oektem, 1937), Iraq, Afghanistan, Central Asian Republic of U.S.S.R., Pakistan and India (Rutter, 1967). It extends further into the Far East, and infections have occurred in Burma (Hoare, 1956), Indochina (Granauillit and Do Van Vien, 1938), Cambodia (Arrighi, 1949), Vietnam (Broudin et al., 1926), Formosa (Kasai and Akazawa, 1927), Southern China, Malaysia, Indonesia, the Philippines (Hoare, 1956) and Thailand (Kengkrasat, 1929). The disease has also been recorded from the Indian Ocean Islands of Ceylon (Ceylon, 1934) and Mauritius (Hoare, 1956). In Central and South America, surra occurs in Venezuela (Wenyon, 1926) and Colombia (Wells et al., 1968).

## 1. 2. Epizootiology, clinical symptoms and pathology

### Epizootiology

The epizootiology of surra is not perfectly understood. The disease is believed to be transmitted principally by species of Tabanus. This fly breeds extensively in water-logged areas, following the rainy season. Since the incidence of surra assumes its peak from a month or two after onset of rainy season in India (Basu, 1945; Lall, 1947), it is not improbable that the incidences of the disease are correlated with the distribution and abundance of Tabanus. However a severe outbreak of surra has been reported to occur in Mona depot (India) during the dry season when there was complete absence of Tabanus (Allen, 1930). Therefore it would appear that Stomoxys and other haematophagous flies may be equally important in the transmission of this infection. It is speculated that in open field conditions the principal vectors are tabanids while in byres and stables the transmission is effected by Stomoxys (Nieschulz, 1930). This assumption is based on the fact that Tabanus does not enter dark stables whereas Stomoxys is confined mainly to the animal houses (Mitzmain, 1914).

As yet there is no convincing evidence about a reservoir host of T. evansi infection. Cattle and buffaloes, principally the latter, are regarded as reservoir hosts, since the infection in them is generally latent or subclinical (authors cited by Mohan, 1968). On the other hand the incidence of severe outbreak of disease occurs in cattle and buffaloes (Sheather, 1923; Edwards, 1926; Manresa, 1935; Mahajan, 1934, 1939; Iyer and Sarwar, 1935). Similarly, <sup>the</sup> pig is also believed to be a reservoir host since it

runs a very mild infection but nevertheless natural outbreaks of this disease in pigs have been reported (Kraneveld and Mansjoer, 1947; Maa, personal communication). Thus no particular host appears to act as a reservoir of infection and in fact a diseased ox, buffalo or camel, harbouring a chronic infection, provides the source of infection in a specific area.

Oral transmission of infection has been demonstrated in dogs, which have become infected after eating the organs or blood of surra infected animals (Van Dulm and le Coultre, 1932; Angelloti, 1949; De Jesus, 1951). Further evidence to support this method of transmission has been provided by Yakimoff and Nina-kohl (1907), and De Jesus (1962). These workers have shown that T. evansi can survive in tissues of slaughtered animals for up to 48 hours. The experimental work of Terry (1911a) shows that it is possible to infect rats and guinea pigs by placing infected material on the oral mucous membrane.

Congenital infection from mother to offspring has been recorded in camels (Sergent et al., 1919) and in guinea pigs (Pavlov and Guenev, 1941; Kraneveld and Mansjoer, 1954). Milk of infected lactating bitches, however, is not a source of infection for the suckling offspring (Lanfranchi, 1915a, 1916, 1918; Abd-El-Latif, 1964).

Transmission of T. evansi infection through the conjunctiva has been shown experimentally (Ulbrich, 1910; Neiva, 1913) but its significance as a method of transmission is not known.

T. evansi comprises strains differing in their effects upon various hosts present in the same locality (Hoare, 1943). For example a camel strain which proved fatal for dogs, sheep, rabbits



and guinea pigs could only cause chronic infections in horses and subclinical infections in buffaloes and goats (Cross, 1920).

Similarly a goat strain produced a prolonged incubation period in dogs (Sergent and Lheritier, 1915) while in another instance a goat strain caused fatal infection in dogs (Kuppuswami, 1941).

T. evansi is capable of infecting laboratory animals such as mice, rats, guinea pigs and rabbits (Pearce and Brown, 1918; Vincent Scorza and Dagert, 1955; Bellili, 1957). Continuous passage of a strain in one laboratory animal (guinea pig) attenuates its virulence for another (mouse) (Terry, 1910).

T. evansi can infect domestic fowl (Van den Berghe, 1941; Alwar and Ramanujachari, 1953, 1955), day-old chicks, day-old pigeon squabs, ducklings and turkey chicks (Alwar, 1958). However, Manual and Cajita (1967) were unsuccessful in their attempts to infect domestic fowl.

Ray and Harbans (1948) demonstrated that a deficiency of pantothenic acid caused a more intense infection and reduced the length of host survival time. On the other hand Sen et al. (1955a, b) found that the nutritional status of the host did not influence the susceptibility to T. evansi.

Contradictory results have been obtained concerning the effect of splenectomy on the course of infection. Lanfranchi (1910b) reported that in splenectomized dogs the disease was more severe and death more rapid, while Nieschulz et al. (1930) found no difference in the course of the disease in both splenectomized and normal mice, rats, guinea pigs, rabbits, cats and dogs.

### Clinical symptoms

Camels are by far the most susceptible animals to surra. The disease runs either an acute, subacute or chronic course and the infection terminates fatally in untreated cases. Symptoms of camel surra are well documented by Leese (1927) and Curasson (1943).

In horses, the severity of the disease varies from west to east according to Hoare (1956), who states that while in North Africa (Sudan, Somaliland and northern Kenya) the infection usually runs a mild chronic course, in the Middle East (Syria and Palestine) the onset of the disease is subacute, and in Soviet Middle Asia and India it is acute although in both these regions the infection eventually becomes chronic. In areas further east (Indochina, Indonesia and the Philippines) the disease is acute and fatal outbreaks are common. Clinical manifestations of surra in horses in India have been described by Lingard (1893, 1898). In North Africa and the Middle East Velu (1915) and Poursines et al. (1943) described the chronic disease in equines. Stephen (1970) has described the symptoms of an experimentally infected horse which developed oedema and anaemia. Petechial haemorrhages developed in the eyes and nares when the parasitaemia decreased whereas urticarial plaques appeared on the skin when the parasitaemia increased. By the 25th day, muscular weakness was evident, but the horse survived for a further 85 days.

In cattle and buffaloes, T. evansi infection is generally regarded as latent or subclinical in Indonesia (Doeve, 1917; Nieschulz, 1929), Philippines (Manresa, 1935; Manresa and Gonzales, 1935), Indochina (Broudin et al., 1926) and India (Scheather, 1923).

But in India bovine surra sometimes assumes outbreak form in which both acute and chronic infections occur (Mudaliar, 1944; Mudaliar and Ray, 1947; Iyer, 1948). Viswanathan (1947) has described the symptoms of acute, subacute and chronic forms of bovine surra in India. The acute form of the disease is marked by a sudden rise of temperature ( $105^{\circ}\text{F}$ ), a very short course of infection lasting only 1 to 2 hours in which symptoms of meningoencephalitis are exhibited before death. In subacute infection the disease was less severe, and symptoms of meningoencephalitis were not prominent, and the affected animals sometimes recovered. The chronic infection was marked by loss of appetite, a staring coat, and recovery after 1 to 2 days.

In dogs the symptoms of surra are fever, anorexia, oedema of the head and throat, congestion of the conjunctiva, and corneal opacity leading to blindness. In cats, T. evansi infection remains almost symptomless (Launoy et al., 1931; Launoy, 1934a, b).

Manjrekar (1950) reported a natural infection of trypanosomiasis in India in goats in which few goats died. In pigs the disease is reported to be very mild but Maa (personal communication) has described a natural outbreak of T. evansi among pigs in Taiwan, during which some animals died. Kraneveld and Mansjoer (1947) have also reported severe symptoms in pigs.

### Pathology

A variety of pathological changes have been reported in T. evansi infection. The parasite causes lesions such as enlargement of spleen, lymph glands and kidney (Levine, 1961). In experimentally infected animals, the histopathological changes

which have been observed include congestion and erythropoetic activity in the spleen, cloudy swelling in the renal tubules and erythroblastic reaction in the bone marrow (Sen et al., 1959). In the liver, a dilation of blood vessels (Chatterji, 1960) and leucocytic infiltration of parenchyma occurs (Levine, 1961). In two dogs, examined at postmortem, the infection produced a severe meningoencephalitis with marked perivascular infiltration through the brain tissue (Chew, 1968). Skin lesions resembling those of eczema and alopecia have been reported in camels with chronic infection and in experimentally infected rabbits and guinea pigs (Kazanski, 1940). Rodhain and Henry, (1936) found that such lesions were localized in the dermis and contained trypanosomes.

The primary changes which occur in the blood of dogs and camels, naturally infected with T. evansi, and in experimentally infected camels, goats, rabbits and rats, are a reduction in the number of red blood cells and a decrease in the haemoglobin percentage which in turn produce anaemia (Lanfranchi, 1910a; Madin and Soliman, 1967; Samaddar et al., 1962; Samaddar, 1966; Jatkar and Purohit, 1971). The anaemia, in experimentally infected goats, is of microcytic hypochromic type (Samaddar et al., 1962). Tangri et al. (1966) observed that in experimentally infected rats and rabbits the anaemia developed in the late stage of infection and rather suddenly.

There is no uniformity in findings on changes in white blood cells. Kaltenback (1954) reports regenerative leucocytosis and lymphocytosis in natural chronic infections in camels. On the other hand, a decrease in the number of lymphocytes and an increase

in neutrophils have been recorded in experimentally infected camels and dogs (Cabrera and Lui, 1956; Nadim and Soliman, 1967). There is leucopenia in experimentally infected goats (Samaddar et al., 1962). In contrast to the reports of Kaltenback (1954) in which he described regenerative leucocytosis in experimentally infected rats and rabbits and lymphocytosis in rats, Tangri et al. (1966) recorded leucopenia and lymphocytæmia in rats and rabbits.

T. evansi infection causes a gradual reduction of blood sugar level (Krigsman, 1933; Castillo and Joaquín, 1955; Tangri and Sen, 1967; Coel and Singh, 1969). The hypoglycaemia is due to either the consumption of glucose by the trypanosomes (Gaiger et al., 1930; Marshall, 1948) or the depletion of lipids and ascorbic acid (Chatterji and Sengupta, 1962). The level of blood protein decreases in experimentally infected goats and rabbits (Samaddar, 1962; Tangri and Sen, 1967). Coel and Singh (1969) and Samaddar (1966) have shown that there is fall in serum albumin in naturally and experimentally infected dogs and rabbits, respectively, and that there is a concomitant rise in serum globulin which is possibly due to the formation of antibodies.

### 1. 3. Diagnosis and treatment

#### Diagnosis

Although a tentative diagnosis can be made from symptoms presented by the host particulars in the enzootic areas, a definitive diagnosis can only be made by the demonstration of organisms in the blood, cerebrospinal fluid (CSF) or other tissues of the animal.

For the examination of blood or CSF either wet films or thick

or thin smears stained with Giemsa can be examined. But since the chances of trypanosomes being present in the peripheral blood at a given moment, are only 45 to 60% (Antipin et al., 1964), the absence of trypanosomes in the blood does not exclude the possibility of infection.

For the diagnosis of an infection showing only scanty parasitaemia the trypanosomes can be concentrated by centrifuging a volume of blood. More refined techniques have been used recently for the detection of low parasitaemia with other species. These include the centrifugation of blood using a microhaematocrit centrifuge (Woo, 1969), and the separation of trypanosomes from the blood by an anion exchange column followed by centrifugation (Lanham, 1968). These techniques have been successfully operated for detection of salivarian trypanosome infections of man and animals (Lanham and Godfrey, 1970; Godfrey and Lanham, 1971). They could be a valuable method for detection of T. evansi infection as well.

The inoculation of susceptible laboratory animals, e.g., rats, mice, guinea pigs and rabbits with blood or organ suspension is a more sensitive method than the examination of single stained blood films for the detection of low parasitaemias (Godfrey and Killick-Kendrick, 1962; Kraneveld and Djaenoedin, 1941).

#### Chemical tests

A number of chemical tests have been used for the detection of camel trypanosomiasis but none of these methods are specific for T. evansi since they indicate only a rise in serum globulins. One of these, the mercuric chloride test (Bennet and Kenny, 1928; Bennet, 1933) has been widely used for detection of surra in camels.

Various authors have obtained false positive results with this method using serum obtained from animals with infections caused by other organisms (authors cited by Killick-Kendrick, 1968). But more recently Abdel-Latif (1958) and Leach (1961) have confirmed its diagnostic value. Other tests such as the thymol turbidity test (Abd-El Ghaffar, 1962) and formol-gel test (Knowles, 1927) are based on the same principle, but the latter is unreliable (Antipin et al., 1964).

Ray (1950) has found that the stilbamidine test, which is probably based on the same principle as the mercuric chloride test, was useful for the detection of latent bovine trypanosomiasis. The results of this test correlate with those of the complement fixation test (CFT) (Ray and Bhaskaran, 1953; Dhillon, 1953).

Similarly, the mercuric chloride test, although it is not specific, is the most reliable for the detection of horse trypanosomiasis (Poursins and Pigouri, 1949).

The non specific chemical tests, which only detect a rise in serum globulins, should be discarded in favour of immunological tests or laboratory animal inoculations. However, in the absence of these techniques, the chemical tests could have a limited use in the field.

#### Immunological tests

The use of various immunological tests as methods of diagnosis is relatively unexplored. Complement fixation test (CFT) has been in use for a long time (Randall, 1934; Topacio and Acevedo, 1938; Acevedo, 1940) and is reliable, but the disadvantage of the CFT is that it can detect antibody levels only after about 40 days of

infection in experimental animals (Gill, 1965a). Rafalovich (1949) found that by agglutination, he could diagnose latent surra in camels, horses and donkeys. Recently Gill (1965a) has successfully employed other immunological tests such as the fluorescent antibody test (FAT), precipitin reaction, agglutination test and indirect haemagglutination test (IHA) in experimental T. evansi infection. In experimental laboratory animals infected with T. evansi, IHA and agglutination tests detect serum antibody levels from 5 days after inoculation; the precipitin and FAT tests become positive from 20 days onwards; on the other hand, antibodies were detected only after 40 days using the CRT (Gill, 1965a). The FAT, though reliable, sensitive and easy to perform, can detect antibody levels only after about 20 days of infection and thus its utility would be limited for detection of chronic infections only. The precipitin test may not be very sensitive as it may be able to detect only high titres of antibodies. The value of the agglutination test, although it is easy to perform, very sensitive and detects antibodies in sera as early as IHA, is limited as a diagnostic test since it is mediated by variant specific antigens only. Gill (1964a, 1965a, 1966) has shown that IHA, using either tanned or formalized sheep red blood cells, is the most sensitive and reliable method of detecting T. evansi antibodies in experimental animals. Jatkar and Singh (1971) have employed the passive haemagglutination test to study antibody titres in camels suffering from T. evansi. They found titres of 1/40 and above in positive cases and 1/20 and below in camels not having T. evansi infection. A further assessment of the potential value of this test for the detection of natural infections in the field should be



made.

Serum of T. evansi infected guinea pigs has trypanolytic activity (Leger and Rigenbach, 1911). The respiratory activity of T. evansi has been measured by Von Brand et al. (1951) together with the effect of inhibitors on oxygen consumption. The trypanosomes were found to be sensitive to a large number of sulphhydryl inhibitors. Delayed hypersensitivity has been demonstrated by Lanfranchi (1915b). He inoculated dogs and horses with glycerin or alcohol abstracts of T. evansi and produced ophthalmic and intrapalpebral reactions. However, Ray (1946) found that intradermal tests using a suspension of T. evansi in glycerol and saline gave only a slightly positive reaction and such contradictory findings require further investigation.

#### Treatment

The drugs most widely used in the field to cure T. evansi infection are Suramin, Antrycide and Berenil. Tartar emetic is also used in the field in India when above drugs are not available. Before the development of modern drugs, arsenicals were used but these have been discontinued owing to their limited efficacy and toxicity. Earlier studies on chemotherapy have been reviewed by Gill (1961).

Tartar emetic (antimony potassium tartrate) is found to be effective against T. evansi in camels, horses and cattle, but relapses in treated animals do occur. The drug is given as 200 ml. of a freshly prepared 1% solution i/v.

Suramin, an acid naphthylamine compound, which is also marketed

as Antrypol, Haganol and Bayer 205, is the widely used chemotherapeutic agent against surra in camels, horses, cattle and dogs but its prophylactic activity is of short duration (1 to 4 months). A dose of 10 mg/kg. weight is administered i/v and usually 5 to 10 g. is sufficient to cure a camel. Suramin-resistant strains are susceptible to Antrycide.

The trypanocidal drug Antrycide (Quinapyramine B.vet.C.) is normally used either as a curative Antrycide dimethyl sulphate or as a curative and prophylactic Antrycide Prosalt (a mixture of Quinapyramine dimethyl sulphate and sparingly soluble quinapyramine chloride). Antrycide dimethyl sulphate is administered in doses of 3 to 5 mg./kg. s/c to equines and camels. T. evansi infections in camels, even when Suramin resistant, have been successfully treated with Antrycide (Rafyi and Maghami, 1953; Leach, 1961; Novinskaya, 1957, 1961). Antrycide salts though effective in horses, give more reactions than in other species (Castillo, 1962). In cattle, surra infections have been controlled by both Antrycide and its Prosalt, the latter providing up to 12 months protection (Castillo, 1962; Lodha and Singh, 1963; Fernandes et al., 1965). Gill and his co-workers have evaluated the prophylactic and curative effects of Antrycide in experimentally infected mice and rats (Gill, 1961, 1962b; Gill and Malhotra, 1963b; Gill and Sen, 1964; Bhattacharya, Das and Sen, 1962).

The Antrycide-Suramin complex is highly active against infections in rats and the same has marked prophylactic activity (Gill, 1963, 1964b; Gill and Malhotra, 1963a). This complex is capable of inducing protection for up to 7 months in ponies (Gill, cited by Williamson, 1970).

The most recent drug to be used in the field is Berenil, a diminazene compound, diminazene aceturate. Gill (1961) and Gill and Sen (1964) have shown that it is effective against T. evansi infections in rats. Gatapia and Castillo (1968) have treated T. evansi in 175 buffaloes, 15 cattle and 7 horses with a dose of 3.5 mg./kg. i/m but relapses occurred in one of the two cattle (after 90 days) and a buffalo (after 56 days). However, the drug was found to be ineffective in doses of 3.5 mg./kg. in an outbreak of Suramin resistant T. evansi infection in camels and highly toxic at a higher dose (7 mg./kg.) (Leach, 1961). Oppong (1969) has found that the drug is also toxic in dogs. A combination of Antrycide, Suramin and Berenil was found to be effective (Gatapia and Castillo, 1968).

Other drugs which have demonstrated various degrees of protection against experimental and natural T. evansi infections are: Pentamidine, Propamidine, Stilbamidine and Antimosan (Gill, 1961); MSb, MSb B, Mel W and Te 85 (Gill, 1962a); MSb as prophylactic drug (Gill, 1962b; Gill and Malhotra, 1963b); Mel B (Gill, 1964b); Mel W and Samorin (Srivastava and Malhotra, 1967); Nitrofurazone (Gill et al., 1963); Captostibone (Gill, 1971a); Spirotrypan (Fernando and Jayasinghe, 1965) and Ganaseg (diminazene aceturate) (Tongson and Tajon, 1967; Castillo and Mojica, 1968).

## 2. Morphology and pleomorphism

### Morphology

The trypanastigote forms of T. evansi cannot be distinguished from the slender and intermediate forms of T. brucei. Hoare (1956)

has given a precise description of the morphology of T. evansi as follows: 'The slender forms have a long free flagellum and a more or less drawn out narrow posterior extremity, which may be rounded or truncated, with the kinetoplast situated at some distance from the tip. The intermediate forms have a shorter free flagellum and a short, frequently pointed, posterior extremity, with the kinetoplast lying near this end. In addition to these, there are always encountered some individuals showing mixed characters, on account of which they cannot be assigned to either of the thin trypanosomes, and are therefore referred to as transitional forms. The stumpy forms are typically represented by trypanosomes without a free flagellum, but in some a short one may be present. The posterior end of the body is usually rounded and somewhat swollen, being like the head of turtle, to use Bruce's graphic expression, while in some specimens this end may resemble a 'hippopotamus head', to use another simile coined by Bruce, and occasionally the posterior end may terminate in a short point. Normally the nucleus of stumpy forms lies somewhere in the middle of the body, but in many individuals it may be displaced posteriorly, occupying a position near the starting point of the flagellum; such trypanosomes are identical to the postero-nuclear forms encountered in members of the 'Brucei' group. As regards the kinetoplast it is terminal or subterminal in position.'

It is apparent that the characteristics of these pleomorphic trypanomastigotes of T. evansi are identical to those of corresponding forms of T. brucei.

### Biometry

Various authors (cited by Hoare, 1956) have stated that T. evansi varies in length from 17-38  $\mu\text{m}$  and that the mean lengths of different populations vary between 18.4 and 28.4  $\mu\text{m}$ . These observations were recorded from material obtained from camels, dogs, horses, cattle and buffaloes from different countries, e.g., Sudan, India, East Africa, Abyssinia, Morocco and the Philippines. Hoare (1956) recorded the measurements of 22 strains obtained from camels, horses, cattle and other domestic animals from various Afro-Asian countries as well as those of strains maintained in mice and guinea pigs and stated that T. evansi had a range of 15 to 33  $\mu\text{m}$  in length. But, after considering the measurements given by Bruce (1911) he finally described the length of T. evansi as 15 to 34  $\mu\text{m}$  (mean 24  $\mu\text{m}$ ), provided the occurrence of pleomorphism was disregarded. Venkataratnam et al. (1968) recorded a range of 13 to 33  $\mu\text{m}$  on the basis of observations of 5000 trypanosomes from buffaloes in India.

The reported measurements of the pleomorphic strains of this species are:

<u>Strains</u>	<u>Stumpy</u>	<u>Intermediate</u>	<u>Slender</u>	<u>Author</u>
Nb, S.A.K. (Cc)	16.9-19.6 $\mu\text{m}$	19.5-20.7 $\mu\text{m}$	23.0-24.9 $\mu\text{m}$	Hoare (1956)
-	13-20 $\mu\text{m}$	21-26 $\mu\text{m}$	27-33 $\mu\text{m}$	Venkataratnam <u>et al.</u> (1968)

In the present study, the method adopted for categorization of different forms has been based on qualitative morphological characters rather than on quantitative mensural characters, firstly because observations on measurements vary from strain to strain and thereby from author to author and secondly because there is no clear cut

demarcation between the size range of the slender and intermediate forms and that of the stumpy forms, e.g., slender and intermediate forms of T. brucei measure 14 to 39  $\mu\text{m}$  and stumpy forms 11 to 27  $\mu\text{m}$  (Hoare, 1970). It is easier also to make observations of a large number of parasites on the basis of qualitative morphological character. The method of Wijers (1959) provides suitable criteria for differentiation of the 3 morphological forms and was therefore used in this study (see methods).

### Pleomorphism

Pleomorphism has been observed in earlier studies, but only a few attempts have been made to examine the naturally infected hosts for the presence of stumpy forms at each stage of infection. A review of the previous work (Table 1) shows that stumpy forms in T. evansi occasionally occur in different strains. In Table 1, data pertaining to pleomorphic strains only has been given. Strains S.A.K., Nb and N.S. maintained in mice by Hoare (1956) were examined for a number of years and found to be pleomorphic (see Table 1). The S.A.K. strain was isolated 33 years ago and has become essentially monomorphic, but Miles (1970) has been able to demonstrate pleomorphism of this strain in mice. The N.S. strain which had shown pleomorphism on 8 occasions within 2 years of its isolation, has since remained monomorphic. However, Miles (1972) and Mathur (1971) have been able to experimentally induce pleomorphism in this strain which was isolated more than 30 years ago. Morphological studies of T. evansi in the natural host which support the occurrence of pleomorphism in this species have recently been recorded by Venkataratnam et al. (1968) who found up to 42% stumpy forms in a

small number of infected buffaloes in India. The presence of stumpy forms in high proportions e.g., 55.7% (Fiori et al., 1918), and 61% in strain S.A.K., 35.25% in strain Nb (Hoare, 1956) and 42% (Venkataratnam et al., 1968) contradicts the view held until recently that stumpy forms of T. evansi appear only in insignificant proportions (.05%) (see Hoare, 1956). It would appear from these results that highly pleomorphic strains of T. evansi could be confused on morphological grounds with pleomorphic strains of T. brucei. These findings necessitate a more detailed, longitudinal study of the infection of strains in their natural hosts in order to determine the extent of pleomorphism in this species.

Until the precise morphology of T. evansi is determined its correct taxonomic position cannot be ascertained.

#### Dyskinetoplastic strains

In T. evansi, strains lacking a kinetoplast have been found (Laveran, 1915; Lavier, 1928; Wenyon, 1928; Hoare and Bennet, 1937) and such forms have been referred to as akinetoplastic or dyskinetoplastic. The latter term is more appropriate because in such forms the kinetoplast, although not totally absent, assumes the form of a compact body, which lacks functional DNA and consequently loses its ability to take up Romanowsky stains. The dyskinetoplastic condition occurs either naturally within the host (Hoare, 1950) or can be experimentally induced by injecting chemical dyes (e.g. acriflavin, prothidium, Berenil and P. rosaniline) into infected animals (Laveran and Roudsky, 1911; Ray and Malhotra, 1960; Inoki et al., 1961, 1962; Killick-Kendrick, 1964). The dyskinetoplastic condition can be maintained through successive passages to laboratory animals (Hoare,

1954) and such strains multiply at the same rate as the normal strains (Inoki et al., 1960a).

### 3. Taxonomy

According to the taxonomy of Hoare (1970) the systematic position of T. evansi is as follows:

Phylum	Protozoa Goldfuss, 1818; emend. Siebold, 1845.
Subphylum	Sarcomastigophora Honigberg & 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	<u>Trypanosoma</u> Gruby, 1843.
Section	Salivaria
Subgenus	<u>Trypanozoon</u> Luhe, 1906.
Species	<u>evansi</u> (Steel, 1885)

In the above systematic position, T. (T.) evansi has been included under the subgenus Trypanozoon (formerly Brucei group) which consists of the human trypanosomes, T. (T.) brucei rhodesiense and T. (T.) brucei gambiense and T. (T.) brucei brucei which affects animals. T. (T.) evansi and T. (T.) brucei brucei are similar in respect of their host susceptibility and the symptoms and pathology of their infections, but the two species can be distinguished on the following grounds:

1. In T. (T.) brucei brucei (T. brucei) the appearance of pleomorphic forms is a constant feature while in T. (T.) evansi (T. evansi) it is not. It is widely held that stumpy



- forms of T. evansi appear only in insignificant proportions but in some strains up to 42% stumpy forms have been recorded (See literature review on morphology and pleomorphism).
2. T. brucei can develop in Glossina, T. evansi is unable to do so. This aspect is relatively unexplored as few attempts have been made to transmit T. evansi by Glossina. Transmission of T. evansi is regarded as mechanical and in T. brucei it is cyclical.
  3. Geographically, in areas of Africa free from Glossina, the infection in animals is considered to be T. evansi while those trypanosome infections in the tsetse belt are caused by T. brucei. In other continents, where Glossina does not exist, the causative agent of trypanosome infections in animals is considered to be T. evansi.

Thus, the taxonomic position of T. evansi is largely governed by its morphology and by its inability to develop in Glossina. Earlier workers used morphology as the sole criterion for taxonomy with the result that contradictory views were held regarding its inclusion within the 'Brucei' group. Some authors disregarded the rare appearance of small numbers of stumpy forms in some strains and treated it as a monomorphic species which could be separated from the 'Brucei' group (Yorke and Blacklock, 1914; Wenyon, 1926; Hoare and Coutelene, 1933). Other workers considered the occasional appearance of pleomorphic forms and placed it in the 'Brucei' group (Bruce, 1914, 1915; Duke, 1923; Lavier, 1933, 1943). In 1949, Hoare replaced the name 'Brucei' group with 'Brucei-Evansi' group. But, later when the occurrence of stumpy forms of T. evansi was established beyond doubt, the name 'Brucei' group was revived and

evansi formed a subgroup within the 'Brucei' group. Thus, the place of T. evansi was established within 'Brucei group', but because of its inability to develop within Glossina (Hoare, 1948), T. evansi was considered to be a separate species from T. brucei. Hoare (1964) replaced the names 'Brucei group' or Brucei and Evansi subgroups by the subgenus Trypanozoon Luhe, 1906 and the specific name evansi was retained - Trypanosoma (Trypanozoon) evansi (Steel, 1885) with this subgenus. This systematic position of T. evansi is held valid at present (Ormerod, 1967; Hoare, 1970).

However, studies on the morphology and transmission of T. evansi have been inadequate. The presence of stumpy forms in large proportions has only been recently established (Venkataratnam et al., 1968) and if the development of a pleomorphic form in Glossina can be attained, the taxonomic position of T. evansi will undoubtedly have to be reconsidered.

#### 4. Phylogeny

T. evansi and T. brucei are closely related owing to the similarity in host susceptibility, clinical manifestations and the pathology of their infections. It is conceivable that one of these species has evolved from the other but various authors have contradictory opinions on this point.

Leese was probably the first to question the ancestry of T. evansi and he thought that the parasite had originated from T. brucei (Leese, 1911, 1927). However, he did not put forward any argument in support of his hypothesis. Duke (1923) stated that 'since trypanosomes of the T. evansi group do not rely on Glossina for their transmission, valuable light may be thrown on

the ancestry of these forms if they could be persuaded to develop in Glossina. Lavier (1933, 1943) thought that T. brucei originated from T. evansi and Hoare and Coutelene (1933) also held a similar view. But Hoare (1940) later changed his opinion and advanced the hypothesis that T. evansi may have originated from T. brucei and this is held valid today (Hoare, 1971).

The entire concept of the evolution of T. evansi from T. brucei is based on the results of two experiments. Duke (1934, 1935) stated that continuous syringe passage in laboratory mice and rats converted pleomorphic strains of T. brucei to monomorphic strains which were indistinguishable morphologically from monomorphic strains of T. evansi. According to Hoare (1940), such laboratory-adapted monomorphic strains of T. brucei lose their ability to develop in Glossina, and it has been shown that T. evansi also, does not have a developmental cycle within Glossina (Hoare, 1940).

A situation, parallel to these experimental models could have occurred in nature by the introduction of T. brucei into Glossina-free areas where its transmission became noncyclical (i.e. similar to syringe passage) and T. brucei, in the course of time, became monomorphic and eventually lost its ability to develop within the fly. This converted monomorphic race of T. brucei was presumably T. evansi (Hoare, 1956). Hoare (1956) thinks that from this point of view, the occasional appearance of pleomorphism in T. evansi can be regarded as an atavistic trait.

Camels are the chief hosts of T. evansi in those areas of Africa lying to the north of a line which runs approximately from 15°N latitude in the west and almost to the equator in the east, and also marks the northern boundary of the area of distribution

of Glossina. Cameline surra occurs in areas to the north of this line and nagana in those areas lying to the south of it. Hoare (1967) supposed that originally T. brucei infection must have been carried northwards by camels which had been infected in the south. It is possible that in these northern areas it was spread by haematophagous flies and during the subsequent passages from animal to animal T. brucei lost its ability to be transmitted cyclically and became monomorphic i.e. assumed all characteristics of T. evansi.

This hypothesis is based on the assumption of a unilateral situation according to which only the conversion of pleomorphism to monomorphism had been taken into account and the reverse situation whereby monomorphism could be converted to pleomorphism has not been considered.

### 5. Culture

The cultivation of organisms of the subgenus Trypanozoon other than T. evansi has been achieved because they contain stumpy trypanastigotes in the blood of their hosts and they have an invertebrate vector in which a cycle of development occur. The stumpy trypanastigotes are believed to be the forms which infect an invertebrate vector or culture.

On the other hand, cultivation of T. evansi in diphasic blood agar media is relatively unexplored because stumpy forms in this species occur only occasionally and there is no cyclical development in any invertebrate host. Recently the pleomorphic nature of T. evansi has been established and it may be possible for these strains to grow and develop in culture. The past work on culture

of T. evansi regrettably lacks information concerning the morphology of the inoculated organisms which would have enabled further evaluation of the success or failure of the early attempts.

The first attempt to cultivate T. evansi was made by Novy, MacNeal and Hare in 1904. They inoculated 3 tubes of blood agar with the infected blood of a cow in the Philippines and maintained them for 3 days in cold storage before shipping them to Michigan (U.S.A.) where they were first examined 35 days after inoculation. Motile trypanosomes were seen on examination of these cultures.

— Gaiger (1910) tried to cultivate T. evansi in NIN medium in which trypanosomes were seen alive for only 3 days after inoculation.

Balfour (1911) attempted to cultivate a strain of T. evansi which was maintained in gerbils and found that the parasites survived for at least 8 days. Unfortunately these authors did not describe the morphology of the organisms which they had used. The limited but successful survival of these parasites in culture suggests that the strains used in these early experiments were probably pleomorphic.

In recent years the only attempts to culture T. evansi in diphasic blood agar media have been made by Balis (1963, 1964b, 1965). — He was unable to maintain the parasites for more than 12 hours, which suggests that his strain was monomorphic. He made a detailed investigation of biochemical factors affecting the growth of T. evansi in culture and concluded that haemoglobin, glycogen and glucose were requirements for growth in culture. He showed that pyruvic acid, the principal metabolic product of glucose was toxic to T. evansi. But when he attempted to remove the pyruvic acid by absorption with charcoal or by replacing the liquid phase of the medium, an unknown growth factor was lost during these procedures. However, the

addition of 2 compounds, which combine the pyruvic acid, sodium sulphite and bisulphite, were found to enhance the period of trypanosome survival. Balis (1964a) also studied the utilization of carbohydrates and their metabolic products by both T. evansi and T. brucei. He found that each of these species were able to utilize glycerine, glucose, fructose, mannose and glucosamine, but that T. brucei utilized cetoglutaric acid in addition which enabled the biochemical differentiation of this parasite from T. evansi. Certain aminoacids, serine, arginine, histidine, tryptophane, cysteine, glutathione and benzoic acid, stimulated growth of T. evansi in cultures (Anon., 1964). In 1965 Balis reported that glutathione, cysteine and certain compounds containing sulphhydryl group in their molecule, played an active role in the oxidation-reduction process which occurs in cultures. The biochemical changes occurring within the organisms during the process of cultivation are far from being understood and it is difficult to evaluate the necessity of different biochemical factors in the culture media.

Chicken embryo has been used as a method<sup>1</sup> for the growth of T. evansi by a number of workers (Longley et al., 1939; Van den Berghe, 1941, 1943; Rodhain and Van den Berghe, 1943). An extensive

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<sup>1</sup> 'It is not clear if this method of infection constitutes an in vivo or an in vitro procedure. There are several important differences between infections in embryos and those in chickens. It is possible to vary the conditions in the embryo by external influences, e.g. raising the incubator temperature brings about a similar rise in temperature of the embryo since it has no thermoregulation mechanisms. The embryo is also immunologically immature, bacteriologically sterile and the parasites introduced into it grow in an unusual site (chorioallantois)' (Long, 1971).

review of literature has been made by Pipkin (1960), who concluded that organisms of the subgenus Trypanozoon were adaptable for growth in chick embryos. Many of these exhibited blood infections which were capable of embryo to embryo passage, and the infections frequently resulted in death of the embryonic host. Van den Berghe (1943) found that 8 to 14 days embryos were susceptible to these trypanosomes which appeared in the blood 4 days after inoculation, increased in number up to the 7th day and eventually caused death at 8 to 9 days. San Agustin (1952) obtained 96% infection rate in chicken embryos and 90% in duck embryos. Alvar and Ramanujachari (1953) stated that out of 12 chicken embryos inoculated with T. evansi on the 15th day of incubation, only 5 apparently healthy chicks hatched out. Trypanosomes were present in wet blood preparations up to a week after hatching of these chicks but thereafter the infection could be demonstrated only by mouse inoculation up to the 27th day after hatching. Alvar (1958) cultivated T. evansi in pigeon and duck embryos. The infection could be detected within the embryo in 48 to 72 hours, and one third of the hatched birds harboured parasites.

These findings suggest that avian embryos are the most efficient media for cultivation of T. evansi.

## 6. Transmission

So far there is no evidence that T. evansi undergoes any cycle of development in an arthropod intermediate host and it is believed that under natural conditions the transmission of this species may be non-cyclical, either immediate or delayed. Immediate non-cyclical

transmission occurs when a vector ingests an infected blood meal and directly inoculates the contents into a clean animal. For example, a fly bites in a close herd of animals, its meal is interrupted and within a few minutes it travels a few yards and inoculates the organisms left on its mouth parts into a susceptible animal. In this case, the parasites remain confined to external and internal parts of the proboscis.

On the other hand, delayed non-cyclical transmission occurs when haematophagous flies ingest infected blood which is retained in the pharynx and stomach, and presumably regurgitated at the time of inoculation into healthy animals. This is possible in Stomoxys in whose proboscis the trypanosomes survive up to 30 seconds only but are retained in the gut up to 24 hours (Mitzmain, 1912a, 1913). But due to paucity of information on the survival of trypanosomes in the proboscis of Tabanus and other haematophagous flies, it is difficult to imagine whether the transmission by them is immediate or delayed non-cyclical.

The immediate and delayed non-cyclical mode of transmission differ in epizootiological importance. The former can only be effected by interrupted feeding in a closely stocked herd where transmission must occur within a few minutes of the infected feed. Thus, the ability of a fly to transmit the parasite is limited. It is possible, on the other hand, for a delayed non-cyclical transmission to take place 24 hours or even 3 days after an infected blood meal. Consequently the flies could carry T. evansi to other areas and cause a sudden outbreak of disease. However, much depends on the feeding habits of the flies, for example Stomoxys which is able to feed daily could be a potential



disseminator whereas those flies, which do not feed until they ~~oviposit~~ oviposit are unlikely to spread the disease.

Exhaustive investigations carried out at the beginning of the century in the Philippines, Indonesia, Algeria, and India by Mitzmain, Nieschulz, Sergent and Leese respectively have led to the belief that T. evansi is non-cyclically transmitted in nature by haematophagous flies. The genera Tabanus, Haematopota and Stomoxys have been incriminated as transmitters of T. evansi, but it is believed that under field conditions the principal vectors are tabanids while in byres and stables the transmission is effected by Stomoxys (Sergent and Donatien, 1922b; Nieschulz, 1930). This is based on the fact that Tabanus is not found inside dark stables while Stomoxys mostly remains confined to these dwellings (Mitzmain, 1914). Successful experimental transmission has been obtained with other haematophagous flies and ticks.

#### By Tabanus

Experimental work has shown that various species of Tabanus are able to transmit T. evansi. In the Philippines T. striatus, T. rubidus and T. reducens transmit surra (Kelsner, 1927; Yutuc, 1949); in Indonesia, Nieschulz (1928a) has shown that T. striatus, T. rubidus and 11 other species are vectors of the disease; and in India the infection has been transmitted by T. rubidus, T. hilaris, T. albimadius and T. nemocallus (Cross and Patel, 1922).

Tabanus is potentially a more efficient immediate non-cyclical transmitter than Stomoxys, since it has relatively large labella which are capable of carrying greater quantities of infected blood (Oldroyd, 1954). Other factors governing immediate non-cyclical

transmission are intensity of parasitaemia in infected animals, behaviour of the strain and density of flies. The transmission of an infection from an animal with a teeming parasitaemia is greatly facilitated by the large number of trypanosomes which are ingested by the flies, although infections from camels with low parasitaemias, which may not be detected by microscopy have also been transmitted (Cross, 1923). It is possible for a single bite to result in an infection, but the chances of transmission are greater when the fly bites both the infected and uninfected animal a number of times (Mitzmain, 1916), thereby enabling the inoculation of a larger number of trypanosomes.

Sergent and Sergent (1905) have shown that a single fly was able to transmit the infection and it was found by Nieschulz (1928b) that after ingesting an infected blood meal a fly could infect up to 4 animals in succession. Nieschulz and Ponto (1927) fed a number of flies on an infected mouse for 1 to 3 minutes and then fed them on 3 guinea pigs in succession allowing 45 seconds to 1 minute on each guinea pig. Subsequently, the 3 guinea pigs were found to be infected. This suggests that even in a closely stocked herd, a large population of flies is necessary to cause an outbreak of the disease.

Delayed non-cyclical transmission has been brought about experimentally after an interval of 30 minutes to 6 hours (Nieschulz, 1928a, b). Nieschulz (1928a) states that the ability of the fly to infect is retained for up to 3 hours after an infected feed but some positive results have also been obtained after a period of 6 hours. However, the author had earlier demonstrated the transmission of infection at an interval of 1-3 days (Nieschulz, 1926a).

In an earlier study, Sargent and Sargent (1905), were able to transmit the infection 22 hours after an infected feed, but could not demonstrate transmission 24 hours after an infected feed. Trypanosomes which had survived in the gut of Tabanus up to 24 hours after an infected feed were able to infect laboratory guinea pigs (Fraser and Symonds, 1908; Baldrey, 1911). Leclercq (1952) cited some experiments by Nieschulz in which he investigated the ability of Tabanus to transmit T. evansi at varying intervals after the infected feed. He found 1 out of 2 flies were able to transmit 15 minutes after an infected blood meal, 1 in 25 flies after 1 hour, 3 out of 1000 flies after 3 hours and in 1 out of 1000 flies after 24 hours.

Oldroyd (1954) thinks that immediate non-cyclical transmission is the only practical possibility in nature and that transmission after delayed intervals as described by Nieschulz is not possible particularly in view of the feeding habits of the tabanid flies. He cites Cameron (1934) who states that in Haematopota oviposition does not take place until 6 to 12 days after feeding and that it is exceptional for a second meal to be taken during that period. Oldroyd thinks, therefore, that the possibility of transmission after a longer interval is of little practical significance. But, in view of the paucity of information concerning the feeding habits of Tabanus, such opinions should be viewed with caution. Recently, Rogers and Kinyanjui (1969) have observed that T. taeniola and T. biguttatus can feed up to 2 and 3 times a day. If the feeding habits of other species of Tabanus, which have been incriminated in the transmission of surra, are identical then delayed non-cyclical transmission is very much a practical possibility and could be as efficient as immediate non-cyclical transmission.

By Stomoxys

It is believed that Stomoxys is also capable of transmitting surra non-cyclically. The fly inhabits animal dwellings and could be responsible for the transmission of the disease inside byres and stables (Sergent and Donatien, 1922b).

However, the efficacy of Stomoxys as a potential transmitter of the disease has been disputed. Schat (1909) after intensive investigations in Java (Indonesia) concluded that S. calcitrans was the chief carrier of surra there. Bout and Rouband (1912) successfully transmitted T. soudanense (= T. evansi) infection from infected dogs and rats to clean rats, guinea pigs and dogs in 5 out of 6 experiments employing 50 to 100 flies for each experiment. The infection was also transmitted from an infected to a clean dog which was tied together with the former in an area infested with Stomoxys. Similarly, the authors transmitted T. evansi in 10 of the 12 experiments and they believed that the transmission of trypanosomes is easily brought about by Stomoxys. An identical view was expressed by Moutia (1928), who obtained transmission from experimentally infected guinea pigs to clean guinea pig and dogs in 4 out of 8 experiments (using 9 to 20 flies in each experiment) and thought that S. nigra was the principal vector of surra in Mauritius.

On the other hand various authors were able to obtain only limited success in experimental transmission (Sergent and Sergent, 1905; Mitzmain, 1913; Nieschuls, 1940; Chaudhury et al., 1966). Mitzmain (1913) did not obtain any success in 29 experiments involving more than a thousand flies with feeding intervals of 5 minutes to 3 days and in 27 experiments using 38 to 40 flies per experiment with feeding intervals of 25 to 30 seconds. He obtained success in one

experiment only in which 206 interrupted bites were made. Similarly Nieschulz (1940) also could obtain transmission from an infected to a clean guinea pig by 2 out of 500 flies and from an infected guinea pig to clean rat by 4 out of 150 flies only, the interval between the feeds being 4.2 to 5.6 seconds. Chaudhury et al. (1966) also obtained limited success; they could transmit T. evansi from an infected to a healthy guinea pig in 1 out of 13 experiments and from an infected to a healthy dog in 1 out of 6 experiments. They were unable to transmit the infection to ponies in 3 experiments, and concluded that S. calcitrans was not a potential vector of surra.

There are several factors which may affect the ability of Stomoxys to transmit this disease. It has small, thin hard labella which are unable to carry large quantities of blood and the destructive action of saliva prevents the survival of trypanosomes within the proboscis of Stomoxys for more than 30 seconds (Hitzmain, 1913). Furthermore, the transmission may be influenced by the behaviour of the parasite in the infected donor animal and the number of available flies. However, the limited success or failure of non-cyclical transmission experiments does not exclude the possibility of Stomoxys being a potential vector.

Hitzmain (1912a), Nieschulz (1940) and Falana (1970) have shown that T. evansi will survive in the gut for at least 24 hours which suggests that transmission could occur after this interval. However Bout and Rouband (1912) have demonstrated non-cyclical transmission 24, 48 and 72 hours after an infective feed.

Van Saceghen (1922) believes that in nature non-cyclical transmission by Stomoxys may be both immediate or delayed. He suggests

that even when interrupted feeding occurs the trypanosomes are able to survive on the proboscis for less than 30 seconds. On the other hand, those which escape the destructive action of the saliva are rapidly drawn into the stomach and during a subsequent bite are regurgitated through the buccal cavity by the muscular action of the pharynx.

There is some confusing evidence suggesting that there is a developmental cycle of T. evansi (Schat, 1910; Baldrey, 1911). But, various authors have been unable to observe cyclical development (Bout and Rouband, 1912; Sergent and Donatien, 1922b; Mitzmain, 1912a). The latter author fed laboratory bred and wild flies on an infected donor and examined them for 94 and 67 days respectively.

#### By Glossina

Non-cyclical transmission of T. evansi has been reported by G. morsitans by Kunert and Krause (1934). They were able to obtain transmission in one experiment in which they fed 51 G. morsitans on an infected guinea pig for 8 consecutive days, and then starved on 9th day. On day 10 the flies were fed to a clean guinea pig, on day 11 to a second clean guinea pig and to a third clean guinea pig on days 12 to 40. The first and second guinea pigs developed infections on day 19 and 23 respectively and thus transmission was obtained 2 and 3 days after an infective feed. The authors did not describe the morphology of the parasite strain which they used and were unable to demonstrate a cycle of development within the vector. Similarly, Hoare (1940) and Falana (1970) did not find a cycle of development of T. evansi in G. morsitans, but they did show that the parasites survived in the gut for 24 hours. Both of these authors used only monomorphic laboratory adapted strains.

As yet no attempt has been made to investigate the development of pleomorphic strains of T. evansi in Glossina. Stumpy forms of other trypanosomes are considered to be responsible for the developmental cycle in Glossina, so it is possible that pleomorphic strains of T. evansi in which there are 1-2% stumpy forms, could initiate a cycle of development in this fly.

#### By Haematopota and other insects

Haematopota spp. have also been found to transmit surra experimentally (Leese, 1909). Nieschulz (1926b) was able to transmit infection by H. cingulata from an infected to a healthy horse at an interval of half an hour. Later, he (Nieschulz, 1927) also transmitted T. evansi from horse to guinea pig by H. truncata and from an infected dog to guinea pig by H. irritata.

Experimental transmission by Musca (Musgrave and Clegg, 1903; Mitzmain, 1914) and the louse Haematopinus tuberculatus (Mitzmain, 1912b) has also been reported.

#### By ticks

In India, the successful experimental transmission of T. evansi by soft ticks Ornithodoros lahorensis and O. crossi has been reported by Cross and his co-workers (Cross and Patel, 1921, 1922; Cross, 1923; and Singh, 1925). Cross and Patel (1921) first used these ticks to transmit this parasite from an infected camel to clean rabbits after a period of 67, 87 and 101 days. O. crossi could also transmit an infection from infected dogs to clean rabbits and rats at intervals of 17 days and 1 month. 3 out of 32 experimental animals, using groups of 30, 10 and 30 ticks respectively, became positive (Cross,

1923). These observations were confirmed by Singh (1925) who, using O. crossi and O. lahorensis, successfully transmitted T. evansi from infected dogs to clean rats and rabbits, in 11 out of 91 experiments (2 to 30 ticks used for each experiment). The interval between feeds in the successful experiments ranged from 20 to 212 days.

However, these experimental findings could not be confirmed by two other workers (Yorke and Macfie, 1924; Sen, 1947) who also employed the same species of tick viz. O. crossi. Yorke and Macfie used 200 specimens of O. crossi, which had originally been fed by Cross on an infected dog in India and later transported to Liverpool. They were fed during the following 6 months on rabbits and guinea pigs, but no infections were obtained. Sen (1947) also was unable to transmit T. evansi from infected guinea pigs to healthy rabbits by O. tholozani (= O. crossi). He used 333 ticks which were maintained at 20° to 25°C and for which the interval between infective and healthy feeds ranged from 48 hours to 50 days. The early transmission experiments, although convincing, appear to have been omitted from most of the text books, possibly because the findings could not be confirmed. However, the work of Cross et al. does support the possibility of ticks acting as reservoirs of infection. It is suggested that further investigations should examine those factors such as developmental stage, sex and number of ticks used, the frequency of meals, and environmental temperature and humidity which are most likely to influence experimental transmission. The morphology of the strain of T. evansi used to infect ticks in the earlier works was not described but the experiments of Yorke and Macfie, who fed O. crossi on rabbits infected with a pleomorphic strain of T. evansi,



showed that trypanosomes were still present in the stomach of the vectors after 2 months, although they were incapable of infecting a mouse. It is conceivable therefore, that pleomorphic strains of T. evansi might be able to survive in ticks.

## 7. Immunology and antigenic variation

### Immunity in T. evansi

Immunity against T. evansi has been induced by curing the infection (Terry, 1911b; Sergent et al., 1911; Gill, 1971b). The efficacy of different antigens as immunizing agents have been compared, and Kliger et al. (1940) found that dead organisms rendered only partial immunity whereas a cure of the infection resulted in a prolonged immune response. Gill (1965b) obtained partial immunity of mice and guinea pigs by inoculating formalinized or freeze thawed trypanosomes and he recorded that addition of saponin (SPL) as adjuvant enhanced the immune response. Efficacy of different immunizing methods in mice, using T. brucei, has been compared by Herbert and Lumsden, (1968), who found complete immunity in mice when they were inoculated i/v with a vaccine consisting of formalinized trypanosomes. This method has been adopted in the present experiment.

Naturally acquired immunity has been demonstrated in animals which recover naturally or after cure of infection (Sergent et al., 1919). Laveran (1911) found that a goat which recovered from experimental infection remained immune for up to 2½ years.

Transfer of immunity from mother to offspring was not demonstrated in early works by Laveran (1914), Lanfranchi (1915b) and Sergent et al., (1915a). But Favé et al. and Guenev (1941) found immunity in the offspring of infected guinea pigs.

Normal human serum is found to have some protective properties against T. evansi (Laveran and Mesnil, 1907). A similar effect of baboon serum has also been observed (Mesnil and Laboef, 1910).

#### Antigens of T. evansi

Released antigens (Lumsden, 1967a) or exoantigen (Weitz, 1960) have been demonstrated in sera or plasma of infected animals (Gill, 1965c; Pathak and Bansal, 1969). Gill (1965c) found that the released antigens were found to protect mice, but they were not agglutinogenic. He has also detected internal antigens (Lumsden, 1967a) in homogenates which showed 3 bands by gel diffusion, 2 of which were exclusive to the trypanosome whereas a third band was common to both the internal and released antigens. Bigalke (1966) titrated T. rhodesiense antiserum with homogenates of dyskinetoplastic and normal strains of T. evansi by double diffusion precipitation test. He found that the 2 species had a common antigen.

#### Antigenic variation

The only evidence that antigenic variation possibly occurs in T. evansi comes from Gill's experiments (1965a, b). The author had isolated 8 variant populations at weekly intervals from a rabbit which was experimentally infected with the Wellcome strain of T. evansi, but subsequently used only 3 of the populations viz. V1, V2 and V3 together with the parent strain as experimental material for various immunological tests (reviewed above). The agglutination test was used to distinguish antigenic types of the isolated populations. The serum against the parent strain agglutinated the homologous population only in titres of 1/320, the anti-V1 serum agglutinated both the parent and V1 populations showing

1/160 and 1/640 titres respectively. But the agglutinin titres shown by anti-V2 and anti-V3 sera to homologous and heterologous variants ranged from 1/10 to 1/40 only. Further in the author's experiments one of the antigens i.e. V3 did not show agglutination with antisera against either homologous or heterologous populations. Hence the results did not clearly indicate the distinction of various antigenic types.

Apart from this doubtful evidence there is no other work on antigenic variation in T. evansi. Hence the present experiments were designed to establish antigenic variation in 2 old laboratory strains (N.S. and S.A.K.) and a recently isolated Colombian strain of T. evansi in the mouse host. Further, various other aspects of antigenic variation of T. evansi have been investigated in the present study.

#### Antibodies in T. evansi infection

Von Brand (1966) has cited work by Bellilli and Caraffa (1956) who have reported about changes in plasma proteins in guinea pigs during T. evansi infection. The  $\alpha$  globulin fraction increased while the  $\gamma$  globulins and the albumins decreased but the level of  $\beta$  globulins remained unchanged.

## Salivarian trypanosomes

### 1. Antigenic variation

The term 'antigenic variation' has been ascribed to the ability of the trypanosome to produce a series of antigenically different populations in its host during an extended infection. In the course of infection each antigenic type probably corresponds to a particular wave of parasitaemia. The importance of antigenic variation lies in the fact that the variant antigens are the ones which are able to produce protective antibodies.

So far the work on antigenic variation has been done mostly with the trypanosomes belonging to the subgenus Trypanozoon, e.g., T. brucei (Ritz, 1916; Leupold, 1928; Ercos and Brown, 1940; Gray, 1962, 1965a, b; Watkins, 1964; McNeillage, Herbert and Lumsden, 1969); T. rhodesiense (Russell, 1936; Lourie and O'Connor, 1937); and T. gambiense (Inoki et al., 1956; Osaki, 1959; Inoki, 1960; Inoki et al., 1960). However potentiality for antigenic variation of T. vivax (Clarkson and Awan, 1969), T. congolense (Wilson, 1968), T. equinum (Franke, 1905) and T. equiperdum (Raffel, 1934; Cantrell, 1958) has also been explored. Variability of released antigens of T. brucei is reported by Miller (1955). The clone strains are also able to produce antigenic variants (Ritz, 1916; Lourie and O'Connor, 1937; Gray, 1965b; and McNeillage, Herbert and Lumsden, 1969). Literature on antigenic variation in trypanosomes has been reviewed by Taliaferro (1930), Soltys (1963), Lumsden (1967a) and Gray (1967, 1969).

Ever since antigenic variation was first recorded in 1905, work on its various aspects has been carried out. The number of variants

have been recorded in order to find out the extent of antigenic variation occurring in a particular host. The sequence of production of antigenic variants in different hosts have been studied with a view to obtain a comparative picture. When variant antigenic types are inoculated into new hosts, they tend to revert to a former antigenic type, and these findings have led to the study of antigenic reversion. In order to understand the mechanism of antigenic variation, which is possibly the result of an antigen antibody reaction, observations have been made on such aspects as the effect of antibodies on the production of variants, and immune response of the host following an inoculation of variant antigenic types. The literature on these aspects is reviewed.

### 1. 1. History

Franke (1905) was probably the first to have the concept of antigenic variation. He infected a monkey with T. equinum and after treatment its serum was found to have protective properties. But when the monkey was reinoculated, the infection developed. This led him to believe that 'trypanosomes in the immune host had acquired such biological properties' and had become insensitive to the protective substances. Later Ehrlich (1909) showed that a mouse infected by the original strain and subsequently treated, was resistant to the original strain but was fully susceptible to the parasites derived from a relapse in another mouse. Furthermore he found that if the mouse was inoculated with the relapse strain and cured, it was immune to the relapse but not to the original strain.

### 1. 2. Number of antigenic variants

It seems that when a strain is inoculated into a host the first

peak population is the same as that of the infecting stabilate (Wilson, 1968). But, thereafter, new antigenic types develop at 2 to 3 day intervals in organisms of the subgenus Trypanozoon (Gray, 1965a). There is possibly no limit to the number of variants which a strain may produce. Despite painstaking studies by different workers, this problem has not been resolved. The number of antigenic types which various workers have reported merely represents the number that they were able to isolate during the period of their experiments. Ehrlich (1911) recorded 5 antigenic types, whereas Ritz (1914) was able to isolate 9 and 17 variants from 2 mice which were given subcurative treatment at every relapse. A total of 22 antigenic types were represented among these isolates. Lourie and O'Connor (1937) recorded 13 variant antigenic types from 22 relapse populations which were obtained by subcurative treatment. A record number of 24 antigenic types have been reported by Osaki (1959) who induced relapses by inoculating human plasma in mice infected with T. gambiense.

It would appear that most of the past workers have isolated antigenic variants by inducing relapses artificially by subcurative treatment. This does not provide a true picture of the capability of a strain to produce antigenic variants in untreated infection in one individual animal. The only attempts to isolate a number of variants from untreated infections have been made by Gray (1965b) and Wilson (1968). The former isolated 15 variants from a T. brucei-infected rabbit during the entire course of infection of 52 days. The latter isolated variant populations weekly up to 12 weeks from 5 cattle which were experimentally infected with T. congolense. He reported that 12 variant antigenic types were represented among

the 30 isolates.

Therefore, antigenic variation appears to be an endless phenomenon which continues in a host so long as the trypanosomes survive and probably ceases to occur only when the organisms are eliminated by treatment, self cure or death of the host. Thus the ability of a strain to produce antigenic variants is possibly unlimited.

### 1. 3. Mixture of antigenic types in relapse strains

The antigenic character of a variant population results from a combination of characteristics of several different antigenic types. The relapse populations may be composed of 2 or more antigenic types (Ehrlich, 1909; Ritz, 1914, 1916). Lourie and O'Connor (1937) found in 7 out of 21 relapses of T. rhodesiense a mixture of 2 and in 1 a mixture of 3 antigenic types. Gray (1962) working with T. brucei in rabbits and Clarkson and Awan (1969) using T. vivax in sheep, have reported that sera of animals which were infected with some variant populations contained high titres of agglutinins to the infecting variant and low titres of agglutinins to heterologous variants of the same strain and both these authors have suggested that each variant population was a mixture of antigenic types. McNeillage, Herbert and Lumsden (1969), making a more realistic approach by analysing relapse populations by setting up clones, found that a variant population consisted of up to 3 antigenic types. The mixed character of variant populations causes cross reactions due to which distinction of different variant antigenic types become difficult.

### 1. 4. Appearance of variants in different hosts

Although the number of antigenic types a strain is capable of

producing is unlimited, there is some organization in the sequence in which they appear in different individuals of the same species and in different species of hosts. Gray (1965a) has found that in T. brucei infections which were induced by different tsetse flies in a rabbit and two goats, the antigenic types developed in a similar sequence in the early stage of infections; however in the later stages the sequences in which antigenic types developed varied, but many of those produced in different hosts were similar. Identical findings have been reported by Wilson (1968) in 5 cattle which were experimentally inoculated with T. congolense.

#### 1. 5. Recurrence of antigenic types in one infection

Experiments on the recurrence of antigenic types in infection within one host have yielded conflicting results. Ritz (1914) isolating variants from a T. brucei infected mouse found that sometimes the same variant recurred in a later relapse of the same infection. Gray (1965b) on the other hand, could not find evidence of the repetition of a particular antigenic type among isolations which were made from a T. brucei infected rabbit over a period of 52 days. It is unlikely that a particular antigenic type would have appeared in rabbits in Gray's experimental set-up since in these animals the neutralizing and agglutinating antibodies remain for a period of 55 days (Soltys, 1957a, b). Hence the conclusion that an antigenic type will not occur in the same host (Gray, 1967, 1969) may not be correct.

#### 1. 6. Antigenic reversion

It is known that however extensive the antigenic variation might be, the variant antigenic types tend to revert to a former



antigenic type. The antigenic reversion has been brought in many ways.

A variant population usually reverts to a 'parent' antigenic type after prolonged passage in laboratory animals. Although Ehrlich (1911) showed that 'parent' and relapse antigenic types retain their separate character for many years, it was later found that the relapse antigenic types revert to the 'parent' antigenic type after approximately 20 to 50 mouse passages (Mesnil and Brimont, 1909; Neuman, 1911; Braun and Teichman, 1912; Osaki, 1959). Rosenthal (1913) and Ritz (1914) showed that this process was gradual, with the occurrence of a mixture of relapse and 'parent' type organisms initially, and the 'parent' type predominantly later.

Further evidence of reversion to a former antigenic type is observed by antigenic similarity of the first relapse. Trypanosomes constituting the first relapse population, which is obtained after the infected animals have been treated with drugs or serum are of one antigenic type or of a limited number of types. This similarity of the antigenic type of the first relapse populations was first studied by Ehrlich (1911), who thought that the first relapses were of the same antigenic type. Leupold (1928) studying first relapses from over 50 mouse infections of T. brucei found that 96% of the variants were one or other or both of the two main types. Raffel (1934) reported that the first relapse strains of the same original strain of T. equiperdum were the same in 3 rats, but his results were inconclusive. On the other hand, Neuman (1911) found little similarity in first relapses. Lourie and O'Connor (1937), who studied T. rhodesiense first-relapse variants produced

in mice by subcurative treatment, found that certain antigenic types tended to appear more than others in the first relapses; thus one antigenic type appeared in 9 out of 22 populations whereas 8 other types occurred only once. McNeillage, Herbert and Lumsden (1969) studied the antigenic characteristics of the trypanosome populations constituting the first natural relapses of T. brucei infection in mice. They isolated clone populations and found that first relapse antigenic variants were not always of the same antigenic type. However, certain antigenic types were represented more frequently than others, e.g., out of 4 populations analysed ETat 3 was represented in 3, ETat 2 in 2 and ETat 4 in 1 population.

It is apparent from above findings that the opinions of various authors on the similarity of antigenic type of first relapse differ. These inconclusive results are due to the mixed character of relapse populations. The problem should be investigated further by comparing antigenic types of the first natural relapses which are obtained after inoculating different variant antigenic types. In the case of doubtful results, the first relapse populations should be analysed by setting up clones and the frequency of a particular cell antigenic type(s) (clone population) in various populations should be determined.

Further to the work on similarity of the antigenic types of first relapses is the finding that when variant populations are inoculated into new hosts certain antigenic types develop in the early stage of infection which are similar and to which the term 'predominant' antigenic type has been applied (Gray, 1965b). The author had passaged two clones of T. brucei, one derived from

original strain of Shinyanga III strain and another from its antigenic variant, into two different series of rabbits at weekly intervals. He found that although the clones were of different antigenic types, they produced similar antigenic types when passaged in rabbits. He also found that certain antigenic types of both clones tended to develop at an early stage of infection when the new hosts were infected with variants of each clone and has suggested that such antigenic types should be referred to as a 'predominant' antigenic type. Thus, a 'predominant' antigenic type can be differentiated from those antigenic types which appear in the later stages of an infection and to which the term 'less prominent' antigenic type has been applied.

Apparently, the 'predominant' antigenic type includes those that are represented in the first relapse. The term 'predominant' antigenic type is ambiguous since it denotes a number of variant antigenic types appearing in the early stage of infection. Therefore, its utility as a basis for the serological classification of strains may be concealed by its nonspecificity.

The third type of antigenic reversion to a former antigenic type is believed to occur in nature, in cyclically transmitted trypanosomes. Broom and Brown (1940) studied antigenic types of T. brucei before and after cyclical transmission and reported that cyclically transmitted populations closely resembled each other irrespective of the difference in the antigenic types of the strain on which the flies were fed. Gray (1965a) showed that when variant antigenic types were ingested by tsetse flies, they were replaced during cyclical development by trypanosomes with a stable antigenic type called the 'basic' antigenic type. However, those tsetse flies

which ingested trypanosomes of variant antigenic types, transmitted trypanosomes with either the 'basic' antigenic type only or with a mixture of ingested variant antigenic type and the 'basic' antigenic type.

Brown and Vickerman (1970) thought that the antigenic reversion in tsetse fly occurred at the onset of the developmental cycle. This interpretation was based on an experiment in which the original strain and successive variants of T. brucei were cultured in diphasic media. They found that while there was no lag phase in the original or first wave population culture, there was a lag phase in the development of cultures from second and subsequent variants which was followed by clumping of such populations. From this they concluded that in culture, second or subsequent variants reverted to first variant organisms. The interpretation concerning antigenic reversion in the early part of developmental cycle is based on the fact that culture forms are similar to tsetse fly midgut forms.

No one has been able to explain the mechanism of antigenic reversion. However, Inoki et al. (1960) have put forward a genetic explanation. They say that there is a coexistence between original antigenic type strain (O) with relapse (variant) antigenic type strain (R). The antigenic constitution (system) of relapse type strain (R) could be single (R) or compound (represented by R<sub>1</sub>, R<sub>2</sub> ..... R<sub>n</sub>). The reversion from relapse to original occurs by single step in single system (R to O) and 2 or more steps via R in other systems (R<sub>n</sub> to O).

#### 1. 7. Role of antibody in inducing antigenic variation

It is believed that the alteration in the antigenic character

of the organisms within the host is brought about by the antibodies. Massaglia (1907) explained the mechanism underlying relapse. He thought that following the inoculation in a host a particular population rises, host soon produces antibodies against it which results in the suppression of that population. However some trypanosomes which escape the destruction, cause relapse. Levaditi and McIntosh (1910) stated that lytic antibodies were responsible for the suppression of the parasitaemia. They had shown that from guinea pigs infected with T. brucei, sera collected the day after the crisis, contained considerable quantities of lytic antibodies and if mixed in vitro with T. brucei it destroyed them. But the trypanosomes which appeared in the relapse were not destroyed. Similar views have been expressed by Muterailch and Salamon (1928) who thought that 4 to 6 days after appearance of the parasites lytic antibodies appear in the circulation, the amount is at first minimal and suffices to destroy all most sensitive parasites. The fresh quantities of lytic antibody which continue to pour into the circulation destroy progressively the less resistant trypanosomes and finally only those which become resistant, survive. These multiply and give rise to relapses. The authors are of the opinion that the organs do not serve as a place of refuge for the trypanosomes.

From the above work it would appear that the lytic antibodies are responsible for suppression of parasitaemia and those trypanosomes that escape the destruction later multiply and give rise to a second wave. But subsequently Russell (1936) who isolated relapse populations at weekly intervals from rabbits infected with T. rhodesiense, showed that sera collected at the time of isolation

of a particular antigenic type had no lytic effect on that population but contained lysins for the variant which was isolated a week before. Similar findings have been reported by Clarkson and Awan (1969) who isolated variants from peaks of parasitaemia from a sheep infected with T. vivax and collected sera at periodic intervals. Hence the timing of production of antibodies does not coincide with the timing of suppression of parasitaemia, which indicates that at the time of suppression of parasitaemia there may not be a titre of antibodies higher enough to destroy the parasites. Therefore, the relapsing mechanism may not be a sequel to antibody production. This is further supported by observations of Goedbloed and Southgate (1969) who have reported that despite a relapsing parasitaemia in chicken embryos they did not find any evidence of agglutinating or fluorescent positive antibodies. Thus, the role of antibody in inducing antigenic variation becomes questionable.

Other observations which lead to the concept that antigenic variation is caused by antibodies is based on the fact that the antibodies are able to destroy the corresponding antigenic type in vitro. When trypanosomes are incubated with homologous antisera in vitro and the incubated suspension is inoculated into an experimental animal, a new antigenic type of the strain develops (Ehrlich, 1909). Somewhat similar observation was made by Gray (1962) in a different type of experimental set-up in which he inoculated antisera containing antibodies against certain variants of a strain of T. brucei i/v into rabbit and subsequently challenged it with the organisms of the strain by the same route. The organisms that developed in the rabbit were of an antigenic type different from

the one against which the rabbit was protected. He expressed the opinion that the antibodies produced by an infected animal suppress the production of certain variants and thus temporarily alter the relative proportions of antigenic types in that variant and indirectly select a population of trypanosomes of a new antigenic type.

Inoki et al. (1956) claim to have induced antigenic variation in T. gambiense by exposing trypanosomes to immune serum in vitro, but Brown (1963) could not confirm this finding. Luckins (1972) has reported that there was no change in the antigenic type of a T. rhodesiense strain in irradiated rats. The sera of these rats did not contain agglutinins.

#### 1. 8. Experimental systems used to induce antigenic variation

In the past experiments on antigenic variation were performed on virulent (laboratory adapted) strains in which relapses were induced by different methods. Relapses have been obtained by sub-curative treatment (Ritz, 1914; Lourie and O'Connor, 1937; Osaki, 1959; Gray, 1966b), after treatment of infected mice with homologous immune plasma (Osaki, 1959) and in T. rhodesiense infected mice by injecting human plasma (Osaki, 1959; Inoki, 1960). Le

Variants have also been obtained by immunizing mice against one strain and infecting them with another (Neuman, 1911). Osaki (1959) claims to have obtained antigenic variation by keeping infected mice at 45°C to 50°C for 25 to 60 minutes; however, his results were not conclusive.

#### 1. 9. Immunization with antigenic variants

Cunningham (1970) found that in rats immunized with a series of antigenic variants of T. brucei, no significant protection was found

against challenge of the parent antigenic type. Cattle infected with antigenic variants of T. brucei and treated with Berenil 3 days after onset of infection produced homologous agglutinins in high titres; when several antigenic variants were inoculated simultaneously, agglutinins were produced against all inoculated variants (Anon., 1965). Herbert and Lumsden (1968) reported that protection was induced against homologous challenge in mice immunized with formalinized vaccines constituting either of one antigenic type or of several antigenic types. However, there was no protection against challenge by heterologous populations.

#### 1. 10. Phenomenon of antigenic variation

The phenomenon of antigenic variation is not perfectly understood. There are two schools of thought. According to one, antigenic variation is a genetic process and change in antigenic character can be explained in terms of mutation (Cantrell, 1958; Watkins, 1964).

According to the other, change in the antigenic character of the trypanosome cell is brought about by the immune response of the host. In the light of this hypothesis the mechanism underlying a relapsing infection is explained as follows. Following inoculation in a host a particular population rises, host soon produces antibodies against it which results in suppression of that population. However, some trypanosomes which escape the action of antibody gradually become not only resistant to it but change into another antigenic type. These trypanosomes with altered antigenic type multiply and give rise to a second population which is also knocked by a second antibody produced in the host. This process is repeated with the succeeding variants. Thus, variants are selected by the



antibody response of the host. The evidence of this phenomenon comes from the bulk of research work done on antigenic variation (reviewed above under role of antibody in inducing antigenic variation). Thus, a host in which antigenic variation has occurred, has antibodies against the variants which have already appeared but not against the ones which are yet to develop. The suppression of an antigenic variant by its antibody has been demonstrated in vitro. However, there are two bottlenecks to this school of thought. Firstly, relapsing parasitaemia is not necessarily accompanied by production of antibodies (Goedbloed and Southgate, 1969). Secondly the timing of production of antibody, which are detectable by the agglutination and the lytic tests does not coincide with the timing of suppression of parasitaemia. Thus, according to this hypothesis the titre of antibodies should be expected to be very high when the parasitaemia in the host is suppressed which is in fact not so and it is in fact high 8 to 10 days later.

In order to understand the phenomenon of antigenic variation, nature of trypanosome antigens has been studied by many authors but the nomenclature used in literature is often confusing. Recently Lumsden (1969a) has divided all the antigens described in literature into two types, 'cell antigen' and 'subcell antigen'. 'Cell antigen' denotes the antigenic character of the intact trypanosome organism live or inactivated by chemicals while the term 'subcell antigen' is used for antigenic substances released either while the organism is alive or after its death or disruption. Variant antigens are liberated from the surface coat of the trypanosome (Vickerman and Luckins, 1969) and they are either manifested by the 'cell antigen' or are released in the plasma or sera of infected animals wherein they are termed as 'exoantigen' (Weitz, 1960), 'soluble antigen',

'released antigen' (Lumsden, 1967a) or 'metabolic product antigen' (Thillet and Chandler, 1957). Exoantigens are capable of producing agglutinating, precipitating and protective antibodies against homologous systems only (Weitz, 1963; Miller, 1965). They have been fractionated by ammonium sulphate precipitation into PR and AG antigens which are capable of producing precipitating and protective antibodies respectively (Seed, 1963). The 'cell antigens' fall under category of 'specific antigens' since they produce antibodies against a particular variant or species.

The 'subcell antigens' are also known as 'bound antigens' (Weitz, 1960). The bound antigens of one species give cross reactions with other species (Weitz, 1963) and when mice are immunized with bound antigens of one species a degree of protection is induced for other species. By virtue of this fact the term 'common antigen' or shared antigen is often used. The homogenates have been fractionated on starch gel into two main components 4S and 1S which are also variant specific (Brown and Williamson, 1962; Williamson and Brown, 1964; Seed and Weinman, 1969).

#### 1. 11. Application of antigenic variation studies on serological classification of trypanosomes

Of all the aspects of antigenic variation the most important is antigenic reversion of trypanosomes in a new host in the form of a basic antigenic type when the transmission is effected by Glossina and to predominant antigenic type if the transmission is brought about by syringe or by mechanical means. This aspect has mainly led to the possibility of serological classification of trypanosomes, which in turn has the long term objective of

serological diagnosis and immunization (Gray, 1969).

7 With the development of concept of basic antigenic type it was suggested that it might be possible to type organisms of the subgenus Trypanozoon by comparing their basic antigenic type but since cyclically transmitted trypanosomes sometimes possess variant antigenic types as well as basic antigenic type, (Gray, 1965a), attempts to use basic antigenic type for classifying the organisms of the subgenus Trypanozoon, have been frustrated. The same author has reported that the basic antigenic type of a strain behaves as a predominant antigenic type when a strain is inoculated by syringe into a new host.

Gray (1969) thinks that the serological classification on the basis of predominant antigenic type appears to be a practical possibility. This is based on the fact that antiserum from an animal which has been infected for several weeks and in which trypanosomes have undergone antigenic variation contains antibodies to the predominant antigenic type (Gray, 1969). Further, it has been shown that under field conditions the antigenic stability of trypanosomes remains for up to 2 years since two isolates of T. brucei obtained from one area at an interval of 2 years were found to have identical antigenic types. Such long term antigenic stability of trypanosomes could be helpful in discovering predominant antigenic type in a particular area. Thus, attempts could be made to isolate different predominant antigenic types in a particular area. However, the method has limitations since the term predominant antigenic type is ambiguous and denotes an unlimited number of variant antigenic types which appear in the early stages of infection in a host and it becomes difficult to

understand which of the variant antigenic types be treated as predominant antigenic type and which should be left. Hence the system needs a more specific term.

## MATERIALS

### 1. Trypanosoma (Trypanozoon) evansi

#### Colombian strain

This strain was isolated from a horse in Arauca, Colombia (South America). After 3 passages (mouse - horse - mouse) it was stabilised (Lumsden, 1972) on 17.7.1967 and designated TREU (Trypanosomiasis Research Edinburgh University) 381. TREU 381 was passaged into one mouse and was stabilised after 3 days of infection as LUMP (London University Medical Protozoology) 62 at  $-79^{\circ}\text{C}$ . LUMP 62 was further passaged into one mouse and on day 5 post infection was stabilised as LUMP 74.

#### N.S. strain

This strain was isolated on 6.1.1938 from an infected camel in Kordofan, Sudan. Trypanosomes were inoculated into gerbils and at 5th passage a gerbil was sent to London where the strain was designated N.S. (see Hoare, 1954). The strain was stabilised after approximately 5000 passages in mice. It was obtained from Wellcome Research Laboratories and stabilised as LUMP 27. After a further passage in a mouse it was again stabilised as LUMP 46.

#### N.S. strain clone

3 clones were prepared from a population grown up from LUMP 46 in mice by the method of McNeillage et al. (1970) and were designated LUMP 59, 85, and 95.

### S.A.K. strain (dyskinetoplastic)

This strain was isolated by Dr. C. A. Hoare in April, 1937 from an infected camel in Gedaref, Sudan. It was inoculated into gerbils and at the 5th passage a gerbil was sent to London where the strain was designated S.A.K. (Hoare, 1954). Subsequently the strain was passaged from the gerbils into mice and after approximately 1100 passages it was stabilised at Wellcome Research Laboratories. It was obtained from Wellcome Research Laboratories and stabilised LUMP 26. From LUMP 26 after one passage in mice LUMP 33 was prepared and from LUMP 33 after a further passage in mice LUMP 66 was stabilised.

### S.A.K. strain clone

7 clones of this strain were prepared and stabilised as LUMP 106, 107, 108, 109, 110, 111, and 112.

## 2. Experimental animals

### Mice

White T.O. Swiss (Theiler's original strain) mice were obtained from H/s A. Tuck & Sons, Rayleigh, Essex. 4 to 6 week-old mice of either sex were used for all experiments except for preparation of antisera for which 6 to 8 week-old mice were used.

### Rats

Albino (Wistar) rats weighing 150-200 g. were also obtained from H/s A. Tuck & Sons, Rayleigh, Essex.

### 3. Invertebrate vectors

#### Glossina morsitans

Pupae of G. morsitans were obtained through a colleague, Miss J. C. Freeman, from Dr. T.A.M. Nash, Tsetse Research Laboratory, Langford House, Langford, Bristol.

#### Stomoxys calcitrans

Pupae of the stable fly, S. calcitrans were obtained from Cooper Technical Bureau, Berkhamstead, Hertfordshire (England) and Beecham Research Laboratories, Nomansland Farm, Wheathanstead, Hertfordshire.

#### Ornithodoros moubata

Adults and larvae of the soft tick O. moubata were obtained from a colony maintained by the Department of Entomology, London School of Hygiene and Tropical Medicine.

### 4. Culture media

#### 4N nutrient agar-blood medium (Baker, 1966)

4N medium was used for the culture of trypanosomes. This is a diphasic medium having blood agar as solid phase and Locke's solution (Tobie et al., 1950) as liquid overlay. The method of preparation is as follows:

##### Solid phase

40 g. of Oxoid blood agar base No. 2 (Oxoid division, Oxo Ltd., London, S.E.1) was added to 1 litre of distilled water, and thoroughly mixed by shaking. The suspension was dissolved by autoclaving for 20 minutes and later cooled to about 45°C. Fresh

defibrinated rabbit blood was added aseptically to bring the final percentage of blood in the medium to 10%. If the blood had been stored at 4°C, it was brought to room temperature before adding to the agar base in order to minimize the difference in temperature between the agar and the blood. The blood was mixed with the suspension by gently shaking the flask. The suspension was dispensed either in 5 ml. aliquots into screw-capped universal containers or in 2 ml. aliquots into bijou bottles. The base was allowed to set at a slant within the bottles.

#### Liquid phase

Modified Locke's solution (Tobie et al., 1950) was then added as an overlay in aliquots of 1 ml. to each bottle containing 5 ml. of blood-agar, and 0.2 ml. to each bijou containing 2 ml. of blood-agar. The composition of the overlay was as follows:

NaCl	8 g.
KCl	0.2 g.
CaCl <sub>2</sub>	0.2 g.
KH <sub>2</sub> PO <sub>4</sub>	0.3 g.
Glucose	2.5 g.
Distilled water	1 litre.

200 units of benzylpenicillin B.P. and 2 mg. of streptomycin sulphate per ml. of the overlay were added. After the addition of overlay, the bottles were incubated at 28°C for 3-4 days to check sterility and were subsequently stored at 4°C until required.

In the initial experiments designed to test the survival of different strains of trypanosomes in 4N medium either universal containers or bijou bottles were used.



Medium 199

The survival of the Colombian and the S.A.K. strains in medium 199 was studied. A stock solution of medium 199 was prepared by adding 50 ml. of a 4.4% sodium bicarbonate solution to <sup>950 ml. of</sup> 1% T.C. 199 (Wellcome Reagents Ltd., Beckenham, England). Penicillin and streptomycin were added in quantities of 100 units and 2 mg. per ml. of the medium. The pH of the medium was adjusted to 7.2 using CO<sub>2</sub> gas obtained from a piece of solid CO<sub>2</sub>. The stock solution was then filtered through a millipore filter (No. PHW Po 4750 of 0.47 µm size). The stock solution was dispensed in 10 ml. quantities into universal containers under sterile conditions. The medium in universal containers was stored at 4°C.

Before use inactivated calf serum No. 1 (Wellcome Reagents Ltd.) was added to give a final concentration of 10%.

HeLa cell culture

4 bottles of stock HeLa cell cultures were obtained from Dr. C. C. Draper, Ross Institute. Stock HeLa cell cultures were grown in a medium of the following composition:

Eagle's minimum essential culture medium (Eagle, 1959) BSS concentrate X10 (Wellcome Reagents Ltd.)	40 ml.
Calf serum (non-inactivated) Wellcome Laboratories Ltd. No. 2	20 ml.
Sodium bicarbonate (NaHCO <sub>3</sub> ) solution 4.4% in double distilled water	20 ml.
Double glass distilled water	320 ml.
Benzyl penicillin B.P. 500 units and streptomycin sulphate	500 mg. per ml.

A monolayer of HeLa cells was grown in sterile medical flats.

### Macrophage cell culture

Macrophage cell culture in Leighton tubes was kindly supplied by Dr. M. K. Behbehani. The following method was used for the preparation of these cell cultures.

Peritoneal macrophages were obtained by the method described by Stuart (1967) and modified by Behbehani (personal communication). Female Parkes mice, 4 to 6 week-old, were killed by rapid dislocation of neck. After soaking the fur with alcohol, the abdominal skin was lifted upwards and a  $\frac{1}{2}$ " longitudinal incision was made. The skin was stripped towards the anterior and posterior ends with the help of a thumb and forefinger. 3 ml. of medium 199 containing 5 units of Heparin per ml. was then injected into the peritoneal cavity along the mid-anterior line. The injected medium was thoroughly mixed with the peritoneal fluid, the abdominal wall pulled sideways so as to form a pocket into which a pasteur pipette with blunt ends was inserted. The peritoneal fluid was aspirated and transferred to screw-cap test tubes kept in an ice bath. A count of cells was made and the suspension was diluted to contain about  $1 \times 10^6$  cells/ml. About  $1 \times 10^6$  cells were later put into each Leighton tube containing a rectangular glass coverslip. After one hour, the fluid in each Leighton tube was replaced with medium 199 containing 10% calf serum and subsequently a gas mixture (5%  $\text{CO}_2$  and 95% air at 4 lb./in<sup>2</sup>.) was added for 30 seconds. These tubes were used as stock cultures. Stock cultures were usually used when 3 days old. Replacement of the medium and addition of gas mixture was done every 3 days.

## 5. Diluents

### Normal saline

Normal saline was prepared as a 0.85% solution of sodium chloride in distilled water.

### Phosphate buffer (pH 7.4)

For the preparation of phosphate buffer, stock solutions of sodium dihydrogen phosphate and disodium hydrogen phosphate of the following composition were made:

	Moles	g./litre
$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	0.154	24.02
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	0.103	36.84

Both solutions were stored at 4°C and buffer of pH 7.4 was prepared as required by adding 1.36 volumes of  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  stock solution to 8.64 volumes of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  stock solution. It was transferred to universal containers, autoclaved and stored at 4°C.

### Solution ABP

For most of the experimental work solution ABP, a mixture of isotonic salt solution with phosphate buffer, was used. Briefly the method of preparation of the two stock solutions, namely, solution A (metallic chlorides) and solution B (phosphate buffer pH 7.4) was as follows.

#### Solution A (metallic chlorides)

Four separate stock solutions of the following composition were prepared.

	Moles	g./litre
Sodium chloride (NaCl)	0.154	9.00
Potassium chloride (KCl)	0.154	11.48
Magnesium chloride ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ )	0.110	22.37
Calcium chloride ( $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ )	0.110	24.10

All solutions were separately stored in plastic bottles at 4°C. Solution A was prepared by mixing the following volumes of the above solutions.

	Volume
NaCl	100
KCl	4
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	3
$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	1

Solution A was transferred to universal containers which were autoclaved and stored at 4°C.

#### Solution B (phosphate buffer pH 7.4)

The preparation of this solution has been described above.

Solution ABP was prepared by adding 9 parts of Solution A to 1 part of Solution B.

#### Phosphate saline glucose buffer (PSG)

This diluent was used for the separation of trypanosomes from blood by anion exchange method of Lanham (1968). The buffer was prepared from the following reagents of AnalaR grade.

	Moles	g./litre	Volume
$\text{Na}_2\text{HPO}_4$	0.2	28.4	285 ml.
$\text{NaH}_2\text{PO}_4$	0.2	24.0	15 ml.
NaCl	0.145	8.5	300 ml.

The 3 solutions were mixed in the above proportions and stored at 4°C. Before use 400 ml. of freshly prepared 2.5% glucose was added to the mixture. The buffer was used for equilibration of D.E.A.E. cellulose (DE 52, Whatman Ltd.).

## 5. Drugs and chemicals

### Anaesthetics

Anaesthetic ether (May and Baker) was used to anaesthetize mice prior to inoculation or bleeding. A swab soaked in ether was kept in a closed beaker, which served the purpose of an anaesthetizing jar.

Chloroform B.P. (May and Baker) was used to anaesthetize Stomoxys and Glossina prior to dissection. A few drops of chloroform were put on a swab kept in an anaesthetic jar. The cage containing the flies was placed in the jar and left until all the flies were anaesthetized.

Nembutal (Veterinary Nembutal) Pentobarbitone sodium B.P. was used to <sup>sedate</sup> [induce sedation] in mice before splenectomy or providing an infective blood meal to flies. 0.2 ml. of a 10% solution of Nembutal in saline, administered i/p, was sufficient for a mouse.

### Anticoagulants

Heparin (Boots Pure Drug Co. Ltd., Nottingham, England) was used as anticoagulant at a concentration of 5 units per ml. of blood. To 4.95 ml. of diluent 0.05 ml. of Heparin was added and 0.1 ml. of heparinized diluent was used for 1 ml. of blood.

### Antibiotics

Penicillin and streptomycin were used in cultures. The number

of units used is given for each experiment.

#### Chemotherapeutic agents

Berenil (Farbwerke, Hoechst AG, Frankfurt (H), Germany) was used to cure the trypanosome infections. A dose of 25 mg./kg. body weight was used to cure N.S. and Colombian strain infections. 0.1 ml. of a solution of 5 mg. Berenil in 1 ml. distilled water or saline provided 0.5 mg. for a mouse weighing approximately 20 g. The route of administration was i/p. For cure of infection of the dyskinetoplastic S.A.K. strain, this dosage was inadequate. Although a dose of 25 - 50 mg./kg. resulted in disappearance of trypanosomes from the blood within a period of 24 hours after treatment, yet in some treated mice relapses occurred about 10 days after treatment. Hence a dose of 100 mg./kg. was used to cure S.A.K. strain infection. 0.2 ml. of a solution of 10 mg. Berenil in 1 ml. water or saline was appropriate to provide 2 mg. for a mouse weighing 20 - 30 g. In some initial experiments 25 mg./kg. (0.5 mg. per mouse) was used. Dosage used in individual experiments on S.A.K. strain has been mentioned.

#### Stains

Giensa stain 'Revector' brand (Hopkin and Williams, Chadwell Heath, Essex, England) was used for staining blood films, culture forms and forms present in the gut contents of the fly.

## METHODS

### 1., Terminology

The following terms have been used in the thesis for different purposes.

#### Trypanosome populations

Strain refers to 'a population derived from an isolate and maintained in captivity by inducing it to reproduce continuously by serial passage in culture or in laboratory animals' (Lumsden, 1967a).

Original strain refers to the population which was inoculated into animals for the isolation of variant antigenic types.

Stabilate denotes a trypanosome population preserved in viable condition (Lumsden and Hardy, 1965). Stabilate numbers are preceded by LUMP (London University Medical Protozoology) or TREU (Trypanosomiasis Research Edinburgh University).

#### Developmental stages of trypanosomes

The terms trypomastigote, epimastigote, promastigote and amastigote described by Hoare and Wallace (1966) have been used.

#### Antigenic variation

Antigenic type denotes antigenic character of a trypanosome population. The term is used in order to compare antigenic character of different populations.

Variant antigenic type refers to populations which are of different antigenic type than that of the original strain as revealed by the agglutination test. The terms antigenic variant or simply variant have also been used.

Cell antigenic type refers to clone populations passaged at < 3-day intervals in mice and preserved as stabulate (Lumsden, 1969a).

### Infections

Acute infection indicates death of mice in the first wave of parasitaemia.

Chronic or relapsing infection is used for conditions where more than one wave of parasitaemia is observed.

Virulent refers to populations which kill mice in the first wave. The term less virulent has been used to refer to populations which kill mice in subsequent waves of parasitaemia.

## 2. Bleeding of animals

For routine examination of peripheral blood of rats and mice for parasites, blood was taken from the tail and wet preparations were made.

### From heart

In order to bleed from heart the mouse or rat was deeply anaesthetized by ether. The skin was sterilized with ethanol. The heart was exposed by dissection and blood withdrawn using a syringe with a 25 G. ( $\frac{1}{8}$ " ) needle inserted into the left ventricle.

### From the retro-orbital sinus

Blood was withdrawn from the retro-orbital sinus of a mouse either with a capillary tube or a pasteur pipette.

The mouse was held with the left hand, and the skin fold between the ears was gripped between thumb and fore fingers. It



was laid in a lateral position on the bench and its back held with the palm of the left hand. The eye was opened using the tip of the forefinger and usually the eye ball protruded. The capillary or pasteur pipette was held in the right hand, and gently inserted into the medial canthus at an angle of  $45^{\circ}$  to the mid-line, both laterally and superoinferiorly, taking care not to damage the eye ball. By rotating the pipette a little to and fro and pushing gently inwards followed by a slight withdrawal, the blood entered the capillary. Once it started to flow the pipette was kept in the same position.

### 3. Passage

In order to obtain a fulminating parasitaemia from a small inoculum without producing a change in the antigenic type of the trypanosome, blind passages were made at < 3-day intervals. This method was used for the isolation of a clone population and antigenic variants. The initial inoculum which consisted of either a single organism when cloning or 2 to 3 drops of tail blood when isolating a variant, was inoculated i/p into the first mouse on day 0. On day 3, the entire blood of mouse 1, drawn from the heart into 0.1 ml. heparinized ABP, was inoculated into mouse 2. Mouse 2 was similarly exsanguinated on day 3, and the blood inoculated into mouse 3. This process was repeated at 3-day intervals until the development of a fulminating parasitaemia, when the blood of the last mouse was preserved as a stabilate.

Normal syringe passage was used for the routine maintenance of organisms in mice. A drop of tail blood was drawn into a syringe containing 0.1 ml. heparinized ABP and inoculated i/p into a clean mouse.

## Culture

Passaging of 4N medium cultures was performed usually once or twice a week. The exact day of passaging and the score of parasites at the time of passage from parent to subsequent culture varied in individual experiments and the details are given under results. But in all cases, about 0.05 ml. of the liquid phase of the culture was drawn in a sterile pasteur pipette and inoculated into fresh medium.

## 4. Inoculation of animals

In most cases the organisms were inoculated i/p. On the day before inoculation mice were not given any food or water in order to keep the intestines empty at the time of inoculation so as to allow a wide intraperitoneal space. Before inoculation, mice were anaesthetized with ether. A 26 G. ( $\frac{1}{8}$ " ) needle fitted to a syringe, was inserted into the intraperitoneal space, the peritoneum lifted and the inoculum released.

For inoculation of vaccine consisting of formalinized infected blood or for the transfer of immunity by immune sera, the route of inoculation was i/v. In order to make the tail vein more prominent the mice were warmed in a wooden box fitted with electric lamps. The mice were warmed for about 15 minutes until the tail vein dilated and became prominent. The apparatus used for restraining the mouse was an open-ended hair curler, fitted into the clamp of a retort stand. Each mouse was picked up from the box and placed in the hair curler. The tail was held in between the thumb and the first finger and the vaccine was injected gradually into the lateral vein.

### 5. Maintenance of mice at different temperatures

For observing the effect of different temperatures 4°C, 28°C, and 35°C on the course of T. evansi infection, mice were maintained at 4°C by keeping them in the lower chamber of the refrigerator, and at 28°C and 35°C by keeping them in constant temperature rooms. Before inoculation of trypanosomes, mice were adapted to these temperatures for about a week.

### 6. Staining

Blood films were stained by Giemsa stain (Revector brand). Freshly prepared films were fixed for one minute in methanol, air dried and stained with 10% solution of Giemsa stain in phosphate buffer pH 7.2. The buffer was prepared by adding 6.7 g. of disodium hydrogen phosphate and 2.76 g. of potassium dihydrogen phosphate to 1 litre of distilled water.

After staining, the films were air dried and either mounted in Euparal ~~ve~~ or left unmounted.

### Forms recovered from culture and organs of G. moraitans

The method of Lehmann (1964) was used. A drop of sediment from the centrifuged liquid phase of culture was gently spread on a slide in the form of a thick smear. After air drying the smear was fixed in buffered formalin for 1 minute. Buffered formalin had the following composition.

NaH <sub>2</sub> PO <sub>4</sub>	0.4 g.
Anhydrous Na <sub>2</sub> HPO <sub>4</sub>	0.65 g.
10% formalin	100 ml.

The smears were air dried, stained with Giemsa in buffer pH 7.2 for 10 minutes, again air dried and mounted in Euparal vert.

Cap

## 7. Cryopreservation

The trypanosome populations were either preserved in solid CO<sub>2</sub> at -79°C (Cunningham et al., 1963) or at -196°C in liquid nitrogen by LUMP (London University Medical Protozoology) standard method A using 7.5% glycerol and slow cooling.

### Preservation in methanol and solid CO<sub>2</sub>

Infected mice at the height of infection were bled from the heart into 0.1 ml. of heparinized ABP. The blood was transferred to a sterile Wasserman tube at 0°C. Sterile glycerol (AnalaR, The British Drug Houses) was added to give a final concentration of 7.5% by volume and mixed thoroughly.

The special apparatus for filling the capillary tubes (4" long and 0.1 mm diameter) consisted of a tube rack as described by Cunningham et al. (1963). The rack containing capillaries was sterilized previously.

A sterile pasteur pipette was used to dispense the blood into each capillary tube until it was half filled. The contents were brought into the middle of each capillary by tilting the rack in alternate directions. Both ends of the tube were sealed. The capillary tubes were transferred to chilled methanol in a rubber stoppered glass test tube (120 x 15 mm). The test tube was inserted into an insulating jacket (2.5 cm thick walls) which was placed in CO<sub>2</sub> storage overnight for slow cooling.

After cooling the test tube was removed from the CO<sub>2</sub> cabinet to an ice bath containing methanol and brought to -79°C by the addition of solid CO<sub>2</sub>. With the help of a pair of artery forceps, the capillaries were transferred from the tube to the permanent container (M/s E. K. Bowman Ltd. London), which was transferred immediately to permanent storage.

### Preservation in liquid N<sub>2</sub>

For preservation in liquid nitrogen, the sealed capillaries were transferred to a numbered paper container and placed inside a duralumin or brass tube. The duralumin or brass tube was kept in an insulating jacket and left to cool slowly in the CO<sub>2</sub> cabinet overnight.

The following day the brass tube was withdrawn from the insulating jacket with the help of a wire rod. The tube was then slowly lowered into a transport container of liquid nitrogen. A pair of artery forceps was used to withdraw the paper container which was transferred to the permanent storage canister.

### 8. Infectivity titration

The method of Lumsden et al. (1963) was used for infectivity titration of the stabilate populations. Tenfold serial dilutions of the stabilate population were prepared in ABP in 6 Wasserman tubes placed in an ice bath. In order to prepare these dilutions, 1 ml. of ABP was dispensed into the first of the 6 tubes and 1.8 ml. into the remaining 5. The stabilate contained in the capillary was weighed and expelled into tube 1, and the required volume of the ABP was further added to make 1/100 dilution ( $\bar{2}$ ) (for example 20 mg. for 2 ml.). 0.2 ml. of the first dilution was transferred to tube

2 giving a further 1/10 dilution i.e. 1/1000 dilution ( $\bar{3}$ ).

0.2 ml. of  $\bar{3}$  dilution was transferred to tube 3 and so on to give final dilutions of  $\bar{4}$ ,  $\bar{5}$ ,  $\bar{6}$  and  $\bar{7}$  in tubes 3, 4, 5 and 6.

A fresh pipette was used each time.

Each dilution was inoculated into 8 mice starved overnight, each mouse receiving 0.1 ml. i/p. The mice were examined for parasitaemia from the 3rd day after inoculation to the 8th or 10th day. Since some of the animals were not infected the ratio of mice infected to mice inoculated (S) was recorded. The results 0/8 and 8/8 were regarded as weightless data and useful results were 1/8, 2/8, 3/8, 4/8 and 5/8. Proceeding from the lower to the higher dilution, the first which yielded a useful point was designated x and the ID<sub>63</sub> value with one, two or three useful points was recorded from the table given by Lumsden et al. (1963).

#### 9. Preparation of clones

The procedure for isolating a single organism from infected blood was carried out in a room in which the humidity was brought to 75% and with a temperature of approximately 30°C. The infected mouse was bled from the tail into the diluent kept in a Wasserman tube in ice. The suspension was diluted so that some of the single drops laid out on a glass plate might be expected to contain one organism. The suspension was drawn into a capillary tube, having a fine tapered end. Individual drops were placed on a glass plate on each of the marked squares. The glass plate was then kept over a humid chamber with the drops on the ventral side. The drops were examined under the microscope and those containing an individual organism were selected and their contents were drawn up by a syringe

and inoculated into a mouse i/p. Blind passaging was done at <3- day intervals from this mouse, until a high parasitaemia developed. The infected blood was then preserved as a stabilate.

10. Separation of trypanosomes from infected blood by anion exchangers

The trypanosomes were separated from infected blood by anion exchange chromatography (Lanham, 1968). Anion exchanger D.E.A.E. cellulose marketed as DE 52 (Whatman Column chromedia, W & R Balston Ltd., England) equilibrated with phosphate saline glucose buffer (PSG) pH 8.0, was used. Cep/2

About 10 g. of DE 52 was added to 150 ml. of buffer and gently mixed with a glass rod. The adsorbent was allowed to settle for about 20 minutes and the supernatant containing the fines was poured off. Buffer was again added and the process repeated. This process of equilibration was repeated 5-6 times. By addition of the pre-swollen adsorbent to the buffer the pH increased; occasionally it was necessary to lower the pH by addition of 1/20 orthophosphoric acid. After final equilibration, the pH was checked and if the pH at different levels of the adsorbent and the supernatant was within 0.05 units of the buffer pH, further washing was not required. 10 i.u. of Heparin in saline was added to each ml. of slurry. The adsorbent was stored in a refrigerator. The column consisted of glass Buchner funnel 75 mm in diameter and 55 mm in height with a slit sieve plate. Columns of other sizes were also used and once a 20 ml. nylon syringe was used as column. A piece of rubber tubing was attached to the outlet and the flow rate was controlled by attaching a pinchcock clamp to the rubber tubing.

A Whatman No. 41 filter paper was placed on the plate or on the bottom of the column. It was moistened with buffer and the slurry was gently run in. The excess fluid was allowed to run out. The PSG was run through to set the adsorbent with a firm horizontal surface. The outlet was closed.

The infected mouse was bled into the heparinized PSG and the blood was diluted in 2 to 3 volumes of PSG and kept on ice. The separation process was carried out at room temperature. For fractionation the cold blood was gently layered by the side of the funnel. By a gentle suction the flow of blood in the column was maintained. When all the blood had entered the adsorbent, PSG was added to the column. The eluate was collected in a tube kept on ice. When few or no trypanosomes were eluting the collection was stopped. The eluate was centrifuged at 4°C for 20 minutes at 2000 R.P.M. (450 RCF), and the trypanosomes were washed 3 or 4 times to remove plasma proteins.

#### 11. Scoring of parasitaemia

In most of the experiments the degree of parasitaemia was scored from the number of parasites in a wet film (X 10 oculars X 40 objective). The method of Walker (1969) was used for scoring. Routinely the number of parasites per 20 fields were recorded and in cases when no parasites were found 40 fields were examined before declaring a negative. When the number of parasites increased the number per field was recorded.

For representation of data, the parasite count of an individual animal was converted to Logarithmic equivalent values (LEV) from the table of Walker (1969):



Parasite count			LEV
0/20	or	0/20	0.5
1/20	or	1/20	0.7
2/20	or	1/10	1.0
4/20	or	1/5	1.3
10/20	or	1/2	1.7
20/20	or	1/1	2.0
		2/1	2.3
		5/1	2.7
		10/1	3.0
		20/1	3.3
Approximately 60 parasites per field but uncountable		+	3.8
Approximately 120 parasites and above per field but uncountable		++	4.4
		Dead	4.5

The mean of the log equivalent values of all mice of one group on a particular day was calculated. The LEV on days after inoculation has been described in the data and used for plotting the course of parasitaemia.

#### Haemocytometer count

In those experiments in which the level of parasitaemia was sufficiently high, a haemocytometer count was made. The number of organisms was counted in either 2 or all 4 W.B.C. counting chambers of the Improved Neubauer haemocytometer. The mean for one chamber ( $1 \text{ mm}^2$ ) was multiplied by 10 (space between the coverslip and the haemocytometer) to bring it to  $1 \text{ mm}^3$ , the dilution factor ( $10^2$  if diluted in a R.B.C. dilution pipette) and  $10^3$  (for conversion of the figures from  $1 \text{ mm}^3$  to  $1 \text{ cm}^3$ ).

## 12. Isolation of variant populations

Variant trypanosome populations were isolated from successive waves of parasitaemia from an infected mouse. The course of parasitaemia in the mouse was carefully followed and scored as the number of trypanosomes per 20 fields or per field, and later converted to LEV. As far as possible, the trypanosomes were harvested from the peak of a wave, inoculated into a healthy mouse and passed into mice at 3-day intervals until a parasitaemia high enough for the preparation of stabilates developed in the last mouse. The infected blood of the last mouse was preserved as a stabilate. The populations were designated according to the number of waves as W1, W2, W3 and so on, T1, T2 and so on or S1, S2 in different strains. The details of the isolation of variants in different experiments, have been given in the results.

## 13. Induction of relapse parasitaemia in old laboratory strains

When inoculated into mice, both the old laboratory strains N.S. and S.A.K. and their clone populations produce acute infections and kill the mice during the first wave of parasitaemia within a week of inoculation. In order to investigate their potential for antigenic variation in mice, relapse parasitaemias were experimentally induced. Different methods were employed for both the strains which are as follows.

### Maintenance of mice at 35°C.

This method was used for inducing relapse parasitaemias in infections with the N.S. strain. Mice were kept at 35°C for a week prior to inoculation. Water was supplied twice a day. After the mice had undergone adaptation at a higher temperature, a parasite

population was inoculated from a stabilate at room temperature and the mice were returned to 35°C immediately. Many of the mice died from acute infections but some of them showed relapsing infections with a non-parasitaemic stage in between the two waves.

By immunizing mice against S.A.K. strain and challenging with N.S. strain

Relapse parasitaemias in the N.S. strain and its clone were obtained by this method. Mice were immunized against S.A.K. strain or its clone by infection and subsequent Berenil treatment. Their state of immunity was ensured by giving a homologous challenge which they resisted. Subsequently, the mice were challenged with the N.S. strain or an N.S. strain clone and in most of the mice relapsing infections were obtained.

By subcurative treatment

In the S.A.K. strain relapse parasitaemias were obtained by subcurative treatment. The curative dose of Berenil, 25 mg./kg. body weight, proved subcurative for treatment of infection with this strain. Although the trypanosomes disappeared completely within 24 hours of treatment, relapses occurred after about 10 days. The relapse was again treated with a similar dose of Berenil which cured the infection, but a second relapse appeared again 10 days later. Thus a relapse parasitaemia was obtained by administering subcurative treatment at each relapse.

14. Preparation of antisera

Antisera against variant populations were prepared in mice

by infecting and treating them with Berenil (25 mg./kg. body weight) when the parasitaemia was + or ++. In order to avoid any change of antigenic type the infection was always treated on day 3 after inoculation. However, in most cases the mice were negative or only scanty parasitaemia was observed on day 3 after the inoculation of a stabilate population in which case blind passaging was done at < 3-day intervals until an adequate level of parasitaemia was obtained within 3 days of inoculation. In some cases, the blood of the mouse from which variant populations were prepared, was inoculated into 3 or 4 healthy mice and when they developed a parasitaemia on day 3, they were treated. On day 6 or 7 after treatment, the blood of each mouse was collected and pooled. It was stored in a refrigerator for about 2 hours and later the serum was separated by spinning at 2000 R.P.M. (450 RCF) for 10 minutes in a refrigerated centrifuge at 4°C. Before centrifuging, the clot was retracted from the walls of the tube with an orange stick. The sera were stored in polypots at -20°C. No preservative was added.

In some experiments in which antibody levels in the serum of one mouse were estimated at different intervals after treatment, blood for serum separation was drawn from the retro-orbital sinus into capillary tubes. The capillary tube was sealed on one side with Cristaseal (Hawksley & Sons Ltd., Lancing, Sussex, England) and left for about half an hour to allow the blood to clot. All the capillary tubes were then placed in a Microhaematocrit centrifuge (Hawksley, England) with their open ends towards the centre. After spinning at 10,000 R.P.M. (7500 RCF) for 2 minutes, the portion of the capillary containing serum was separated with a

carborundum disc, sealed at both ends with Cristaseal, labelled and stored at  $-20^{\circ}\text{C}$ .

## 15. Immunity tests

### Agglutination test

The agglutination test was employed as the method of establishing the antigenic identity of variant populations and clone populations. The technique of Cunningham and Vickerman (1962) as modified by Cunningham and Grainge (1963) was employed with slight modifications.

Antisera for the test were prepared by the method described earlier. Phosphate buffer pH 7.4 was used as diluent. In most experiments the antigen used was in the form of thawed stabilate material. However, in a few cases in which the stabilate population did not have a high concentration of organisms, fresh trypanosomes were separated from blood by anion exchange column chromatography, and were used as antigen ( $1 \times 10^8$  organisms per ml.). Fresh trypanosomes were harvested either from the first wave population or from a population resulting from 3-day interval passages. The antigenic identity was ensured by testing them against homologous antisera. The method of the test was as follows.

Three fold dilutions of antiserum in phosphate buffer pH 7.4 were prepared in a perspex serum dilution plate. 0.2 ml. of buffer was placed in each of the 8 wells of the dilution plate. In the first well 0.025 ml. antiserum measured by a micropipette or Microcaps (Drummond Scientific Co., U.S.A.) was added to the buffer to make a dilution of  $\log_3^{-2}$  (1 in 9). The antiserum was thoroughly mixed

by drawing into the pipette and expelling 15-20 times. 0.1 ml. of the serum dilution from the first well was added to the second well and thoroughly mixed making a dilution of  $\log_3^{-2}$ . This process continued upto the 7th well. Thus the serum dilutions (on a log scale to base 3) from the 1st to the 7th well were -2, -3, -4, -5, -6, -7 and -8. In the 8th well, 0.025 ml. of normal mouse serum was added making a dilution of -2 and the same was used as a negative control.

The test was performed on siliconed slides (clean glass slides dipped in 1% solution of silica acid for 30 seconds and dried in an oven for 10 minutes). The slides were kept on a disc in a moist chamber. Small drops of each serum dilution were placed on the slide with a pasteur pipette. Normal mouse serum and phosphate buffer pH 7.4 were used for the 2 negative controls.

The stabilate population, which was used as antigen, was thawed and released into a Wasserman tube kept in an ice bath. A drawn out capillary tube was used to add the thawed stabilate to the controls and to each dilution of serum in descending order. Before releasing the antigen into the dilution series of another serum, the pipette was washed in phosphate buffer. This process was repeated when a fresh trypanosome suspension was used as antigen.

The slides were left in a moist chamber for 40 minutes at room temperature and then the degree of agglutination was observed. The degree of agglutination was scored by the method of Cunningham and Vickerman (1962). The different grades were as follows:

- 4+ Large spherical clumps with sharply defined and composed of densely packed organisms.

- 3+ Small spherical clumps with diffuse edges, organisms less densely packed.
- 2+ Clumps much smaller.
- 1+ Clumps as in 2+ but only at the periphery of the drop of antigen.

When stabilate populations were used as antigen, no agglutination was seen in negative controls and the last dilution of serum showing agglutination was treated as the end point. When trypanosomes harvested from fresh blood were used as antigen, 1+ agglutination was sometimes seen in negative controls. Hence, the end point was taken to be the last dilution of serum showing an agglutination of 2+ or above.

#### Immunolysis test

A few attempts were made to detect the antigenic type of a variant population by the immunolysis test devised by Lepage (Lumsden, 1967b) but the results were not uniform. It was impossible to count lysed organisms because they clumped and consequently an assessment of the ratio of lysed organisms to live organisms could not be made. Therefore, further attempts were abandoned.

#### Protection test

Mice were immunized against one antigenic type and challenged with another. Two methods of immunization were used.

- (1) The administration of vaccine consisting of formalinized infected whole blood (Herbert and Lumsden, 1968).
- (2) The infection of mice and subsequent Berenil treatment.

The administration of vaccine consisting of formalinized infected whole blood

This method was used for observing the effect of challenge of homologous and heterologous variant antigenic types in mice vaccinated with W1 antigenic variant of the Colombian strain (see results).

The stabilate population W1 was passaged in mice at 3-day intervals in order to obtain a high parasitaemia without producing a change in the antigenic type. When fulminating parasitaemia developed, mice were bled into heparinized phosphate buffer pH 7.4. The blood was diluted to contain  $1 \times 10^8$  trypanosomes per ml. 0.5% solution of formaldehyde in phosphate buffer pH 7.4 was added to the suspension to bring the final concentration of formalin to 0.05%. Then thiomersalal was added to give a concentration of 1 g. thiomersalal to 10,000 ml. of antigen preparation. The final suspension was allowed to stand on the bench for one hour and it was checked microscopically to ensure that no motile organisms were present.

0.4 ml. of the vaccine was inoculated i/v to each mouse. 15 days after receiving the vaccine, the immunized mice were challenged by homologous and heterologous variant populations. The challenge inocula, which were derived from stabilate populations, were administered i/p.

The protection response was assessed on the basis of the following.

- (1) Number of mice showing sterile immunity.
- (2) Mean prepatent period.



(3) Proportion of mice showing acute infection to those showing relapsing infection. This was recorded in order to compare the virulence of the parasite in the two groups, since the behaviour of the strain was such that some of the infected mice died from an acute infection while others showed a relapsing infection and died after 3 or 4 months.

(4) Level of parasitaemia of the vaccinated and control group was also compared by plotting the mean LEV of the two groups on a graph.

(5) Proportion of mice surviving for a period of 15 days.

#### The infection of mice and subsequent Berenil treatment

This method was used for observing the effect of challenge of N.S. strain clone in mice made immune to S.A.K. strain clone.

#### 16. Induction of pleomorphism in the N.S. strain

In both the N.S. strain and its clone, relapsing parasitaemia was induced by inoculating mice made immune to the S.A.K. strain or its clone. In a few mice, pleomorphic forms appeared in the relapse populations.

#### 17. Categorization of pleomorphic forms

For determining the percentage of slender, intermediate and stumpy forms the method of Wijers (1959) used for T. rhodesiense was applied with some modifications. The morphological characters on the basis of which long slender, long intermediate, intermediate,

short intermediate and stumpy forms have been categorized, are given (Table 2) and drawings of typical forms are made (Figure 1). As stated the intermediate forms according to this method have been split into long intermediate, intermediate and short intermediate forms. In working out the final percentage, half of the long intermediate forms was added to the long slender forms and the other half to intermediate. Similarly, half of the short intermediate forms was added to stumpy forms and the other half to intermediate forms. Finally, the percentage of all 3 forms was calculated.

## 18. Immunosuppression.

### Splenectomy

Mice were anaesthetized with Nembutal and placed on a cork sheet with ventral side uppermost and the legs fixed by pins. After sterilizing the skin with 70% alcohol, a small longitudinal incision was made in the middle of the abdomen, just posterior to sternum and the skin was reflected on either side. Then the muscular and peritoneal layers were incised. The coils of intestines were set aside by hand. The spleen was drawn up, the hilus was ligated and cut and the spleen removed. The intestines were replaced, the muscular and the peritoneal layers were sutured by continuous sutures. The skin was sutured by interrupted sutures. The mice, still under anaesthesia, were left in the cage.

### Irradiation

Mice were irradiated at Middlesex Hospital, London. Each mouse was individually kept in a glass bottle and exposed to an irradiation dose of 850 rads from a  $^{60}\text{Co}$  source.

## 19. Inoculation of culture media

### 4N medium

Prior to inoculation, bottles of stock 4N medium were removed from the refrigerator and kept at 28°C for 1-2 hours in order to avoid wide differences of temperature between the medium and the inoculum. Usually the medium was used within one month of preparation, but in one experiment 2 month-old medium was used and the cultures developed successfully.

For initial experiments on the survival of trypanosomes, the media were inoculated either with stabilate populations or from the tail blood of an infected mouse, but in subsequent experiments on the growth rate and morphology in culture, heart blood was used as the inoculum.

For the inoculation of stabilate populations, the capillary was taken from the deep freeze and after thawing the contents were released into the medium. The inoculation procedure was carried out in a sterile cabinet.

Mouse tail blood was also used for inoculation of the culture. The infected mouse was secured in a hair curler and only its tail was allowed to remain outside through a hole. The tail of the mouse and the outside of the hair curler were sterilized by alcohol. The tip of the tail was removed with sterile scissors and after discarding the first few drops, one or two drops of blood were added to the liquid phase of the medium.

When heart blood was used for inoculation of the medium it was collected by exposing the heart aseptically. The blood was dispensed into sterile universal containers and was diluted to contain antilog 6.0 or 7.0 organisms per 0.1 ml. of suspension,

which was used as the inoculum. In experiments on the growth rate about 40 - 50 bijou bottles were inoculated at a time.

#### Macrophage cell culture and HeLa cell culture

The mice were bled from the heart into heparinized TC 199 (5 units of Heparin per ml. of blood) and the blood was aseptically placed into sterile centrifuge tubes. Trypanosomes were separated from red blood cells by centrifugation and the trypanosome suspension in plasma was diluted with TC 199 to contain about  $10^6$  or  $10^7$  organisms per 0.1 ml., which was used as the inoculum.

#### Medium 199

For inoculation of medium 199, mouse blood drawn from the heart was directly inoculated into the medium. A count of trypanosomes was made before inoculation.

#### 20. Maintenance of cultures

The various culture media were inoculated and maintained at the temperatures noted below:

4N medium	28°C
Medium 199	28°C
HeLa cell culture	28°C and 37°C
Macrophage cell culture	37°C

#### 21. Examination of cultures

##### 4N medium

In the initial experiments cultures were examined once or

twice a week. A drop of liquid phase of the culture was examined under the X 40 objective of a phase-contrast microscope.

The parasite concentration was arbitrarily scored under a 40 objective as +, 1-19 flagellates per 20 fields; ++, 1-5 flagellates per field and +++, 5 flagellates per field. Concentrations of <1 flagellate per 20 fields were specified, e.g., 1 in 50 or 1 in 100 fields. Before declaring a culture negative at least 3 wet films (22 x 22 mm) were examined and found negative.

In experiments on growth, the cultures were checked daily. 2 bijou bottles of cultures were picked at random and 2 ml. of ABP pH 7.4 was added to each bottle to wash off all the organisms growing on the solid phase of the medium. The suspension was removed to a sterile bijou bottle and the clumps of organisms were broken by drawing the suspension into a 2 ml. syringe fitted with a 25 G. ( $\frac{5}{8}$ " ) needle and expelling it 5 times (Wells, 1969). An estimation of the number of trypanosomes in this suspension was made using an improved Neubauer haemocytometer. The organisms were counted in all the 4 W.B.C. counting chambers and the mean was multiplied by  $\times 10^4$ . The average of 2 sample cultures was treated as the final daily count.

All organisms showing movement were treated as live and those organisms which did not show the slightest movement were regarded as dead. Dead organisms either represented by a flagellum or nucleus only or containing debris of other cellular materials were usually found in clumps and were not recorded.

For making stained preparations the suspension was spun at 1000 R.P.M. (110 RCF) for 5 minutes and the flagellates were washed once or twice in saline. After the final wash the supernatant

was discarded and the sediment containing organisms was smeared on to a clean slide. The smears were air dried and stained by the method of Lehmann (1964). In order to observe morphology at least 50 to 100 organisms were observed in stained preparations. Drawings were made from stained preparations of culture with the use of a Wild drawing tube attached to a Wild 20 microscope.

#### Medium 199

Medium 199 culture was checked daily after inoculation. A drop of the medium was removed with the help of a sterile pipette, and examined as a wet preparation (x 40 phase objective). The parasite concentration was estimated as the number of flagellates per field, per 20 fields, per 50 fields and per 100 fields and so on.

For making stained preparations about 0.2 ml. of the suspension was removed to a centrifuge tube. The suspension was spun at 1000 R.P.M. (110 RCF) for 5 minutes and washed once or twice in saline. After washing, smears were prepared from the sediment, fixed in methanol for 60 seconds and stained in 10% Giemsa for 10 minutes.

For observation of the morphology 50 - 100 flagellates in the stained preparations were examined.

Subcultures were made every third day.

#### HeLa cell culture

HeLa cell culture medium was checked on days 3 and 6 after inoculation. The wet preparations of the liquid medium were examined (x 40 phase objective). Cell monolayers in medical flats

were checked directly under the microscope and Giemsa stained smears of cells were also observed for stages of trypanosomes.

#### Macrophage cell culture

Cultures were examined daily for 4 days after inoculation and further checking was suspended because no parasites were found. 2 culture tubes were removed daily from the inoculated stock. The liquid phase was transferred to a Wasserman tube and a count of the organisms in the suspension was made using an improved Neubauer's haemocytometer.

The coverslip with the adherent macrophage cell monolayer was washed three times with sterile saline. Then, it was removed from the Leighton tube, air dried, fixed in methanol for 1 minute and dried again. The coverslip was stained in a Wasserman tube containing 10% Giemsa in buffered distilled water pH 7.2 for 20 minutes, washed in tap water, air dried and mounted in euparal vert. Stained preparations of macrophages were examined for the presence of trypanosomes.

## 22. Rearing and maintenance of Stomoxys, Glossina and Ornithodoros

### S. calcitrans

The flies were hatched and maintained in the insectary at 25°C and 75 to 80% humidity. The temperature was controlled by a thermostat. The humidity was produced by keeping water in about 25 earthenware pots. Artificial lighting only (fluorescent tubes) was provided from 9.30 hours to 19.30 hours daily.

For hatching, the pupae were placed on sand in a petri dish which was placed inside a 12" x 12" x 12" cage. The cage was

made of wire frames with mosquito netting stretched over it. The cage containing the pupae was kept on a stand in a rectangular tray filled with water in order to protect it from ants.

The flies emerged from the 7th day onwards. The flies were collected with the aid of an aspirator and transferred to a 6" x 3" x 2" cage. A total of 10-15 flies were kept in each cage. Glass tubes (3" x 1") with the open end covered by a piece of mosquito netting were also used for the isolation of individual flies.

#### G. morsitans

Pupae of G. morsitans and the flies which emerged were maintained in an insectary at 25°C and 80% relative humidity. The heat was provided by a tubular electric heater placed 6" above the floor and constant temperature was maintained by a thermostat control. The humidity was provided by an electric water heater and controlled by a humidistat. Fresh water taken from a nearby tap through rubber tubes, was constantly circulating within the heater, and was passed out by a siphon system. The room was lit by fluorescent lighting for 12 hours daily. In order to maintain the pupae, perspex cups (3" x 2.4" diameter) open at both ends, were used. The bottom of the cup was closed with bolting silk which was held in position by adhesive plaster. Fine dry sand was kept at the bottom and the pupae, 25-50 in each cup, were placed on the sand. The top end of the tube was then covered with netting or silk which was secured with a plaster lid. In order to avoid ants the cups were placed on a raised platform which was surrounded with water.



On emergence of flies, each cup was opened in a 12" x 12" x 12" cage and the flies were released. They were collected with the aid of an aspirator and were transferred to a rectangular 6" x 3" x 2" cage in which the adult flies were maintained.

Trays and cages were kept on benches at approximately 3 feet above the floor. Considerable care was taken to avoid insecticides and other potentially harmful chemicals within the insectary.

### O. moubata

The ticks were maintained in the insectary of the Department of Entomology at 25°C and 80% relative humidity. Lighting in the insectary was provided by electric lamps from 9 to 17.30 hours daily. The ticks were maintained in 2" x 1½" glass tubes, with open ends. A filter paper disc was placed at the bottom of the tube to absorb moisture and keep the ticks dry. 1 to 5 ticks were kept in each tube. The open end of the tube was then covered with gauze or netting which was held in position with ½" adhesive plaster. The tube containing the ticks was kept in a rectangular tray which was surrounded by water in order to avoid ants.

### 23. Feeding of Stomoxys, Glossina and Ornithodoros

The flies were fed on mice. Each mouse was anaesthetized by 0.1 ml. - 0.2 ml. of 10% Nembutal injected i/p, and was laid on the fly cages (6" x 3" x 2"). Individual flies were kept in glass tubes and the anaesthetized mouse was laid on a piece of cardboard with its four legs secured with adhesive plaster. The open end of the tube was brought close to the skin of the mouse and the flies were able to take a blood meal. The time of feeding was recorded

only when the flies were fed on an infected mouse.

### G. morsitans

G. morsitans were also fed on mice and the method was the same as that used for S. calcitrans. Flies were fed daily or on alternate days.

In two experiments, trypanosomes derived from a stablitate population were fed through fresh mouse skin membrane. For this the technique of Mahelbwala (1968) was used with slight modifications. A mouse was killed by dislocation of neck, a portion of skin was removed for subsequent use as membrane and fresh blood was obtained by cardiac puncture. Trypanosomes from a stablitate were released and mixed with the fresh blood. The blood and the membrane were kept at 37°C (body temperature) in case there was some delay in setting up the apparatus for feeding.

The apparatus (Figure 2) used for feeding consisted of a glass tube 2" x 1" with the bottom depressed inwards. The tube was filled with hot water at 37°C, removed from a water bath and closed with a rubber stopper. The tube was turned up side down and the depression in the bottom of the tube was then filled with blood. The mouse skin membrane was placed over the depression until its inner surface touched the blood, and then it was held in position with an elastic band. The tube was then again turned and placed on the cage of flies with the skin membrane, from which the flies readily fed touching the cage. The water in the tube was frequently replaced by fresh hot water at 37°C, in order to maintain the normal temperature of the blood.

### O. moubata

The ticks were also fed on mice. A mouse was anaesthetized

with Nembutal and fixed on to a piece of cardboard with the aid of  $\frac{1}{2}$ " adhesive plaster. The ticks were removed from the rearing tube with forceps and placed on the abdominal skin of the mouse. A small glass cup was placed over each group of 5-6 ticks in order to prevent them from escaping. The ticks pierced the skin with their tubular stylet and readily fed. The time taken for feeding was half an hour or more. After feeding, the ticks were placed in clean rearing tubes.

#### 24. Transmission of infection by Stomoxys, Glossina and Ornithodoros

##### S. calcitrans

S. calcitrans were used for transmission of the Colombian strain only. Since the experiments were performed to observe the antigenic type of variant populations after experimental transmission by S. calcitrans, in addition to their ability to transmit, the flies were fed on mice infected with one of the variant populations. The transmission of each variant population has been recorded under different experiment numbers. The age of the flies at the time of feeding was 1 to 2 days. In order to provide an infective blood meal for the flies, mice were infected with stabilates of different variant populations. To ensure that the flies ingested trypanosome populations of the antigenic type represented in a certain stabilate population, they were fed on mice 3 days after the inoculation of the stabilate population. It was assumed that the trypanosomes which developed in the mouse remained of the same antigenic type as that of the stabilate population, for at least 3 days after inoculation.

Trituration of *S. calcitrans* and inoculation of fly homogenate to clean mouse

Experiments involving these procedures were performed in order to observe the survival of the trypanosomes in the body of the fly and their ability to infect mice after remaining approximately 18 hours in the fly. Since it was not practical to induce the flies to make a further feed after the initial infective blood meal, the method of transmission described above was impossible. Therefore, homogenized flies were used to transmit the infection to clean mice. The method adopted for homogenizing the flies and subsequent inoculation into an uninfected mouse was as follows.

Flies were collected approximately 18 hours after the ingestion of an infective feed. In order to crush the flies they were first anaesthetized with chloroform by placing the cage or tube containing the flies in a jar in which a swab soaked in chloroform was kept. The flies were removed from the cage or tube, and after cutting their legs and wings, they were transferred to a tissue homogenizer, which was kept in an ice bath. The flies were ground for a minute or two in 0.1 to 0.2 ml. of chilled sterile ABP. The suspension was checked for the presence of trypanosomes and injected i/p into one mouse.

After 3 days the mouse was bled and the entire blood was injected into another clean mouse, and blind passaging at <3-day intervals was continued until the infected mouse showed a high parasitaemia. When this stage was reached, the infected blood was preserved as a stabiate. In the absence of parasites, blind passaging was terminated after 4 passages and the mouse of the

last passage was checked for a period of 15 days.

### G. morsitans

Flies were grouped according to their date of emergence and designated A, B, C, D and so on.

### Feeding on infected mice

The precise details of the feeding schedule are given under results. Each group was allowed to have an infective blood meal either on the day of emergence or on days 1 or 2 after emergence.

The morphology of the trypanosomes in the blood of the mice at the time of fly blood meal, was checked by the observation of wet preparations under phase contrast. Stained smears were also examined. In the LUMP 172 population, stumpy forms were always present in the mouse blood at the time of the infective blood meal, but in the LUMP 315 population, stumpy forms were either scarce or absent.

### Feeding on healthy mouse

After an infective blood meal, each group of flies was allowed to have an uninfected blood meal from a clean mouse which was replaced every 3-4 or more days. Mice providing clean blood meals in succession were numbered in sequence 1, 2, 3 and so on. Thus, the designation of mice 1, 2, 3, providing feeds to group A flies was A1, A2, and so on.

### Probing

In order to detect salivary gland infections in flies the probing technique was performed. Briefly, the technique was as follows.

After about 10 days of feeding on clean mice, the flies were induced to probe on a glass slide. An anaesthetized mouse was placed on the net of the cage and a clean glass slide, which had been gently warmed over a flame, was inserted in between the net and the mouse skin. The hungry flies attempted to feed and projected their proboscis on to the slide and deposited saliva. When the probing was over, the slide was examined against the light to determine the area of salivary tracks which was marked with a grease pencil on the back of the slide. The slide was fixed in methanol, stained in 10% Giemsa and mounted in euparal vert.

#### Examination of clean mice after feeding on infected flies

Infected flies were fed on mice and these animals were checked for trypanosomes from day 2 onwards after feeding.

In all the transmission experiments the enumeration of the day of feeding on infected or clean mice, has been treated as the day after emergence of the fly.

#### O. moubata

Larvae and adults of O. moubata were used for transmission experiments. The ticks were initially fed on an infected mouse at the peak of parasitaemia, and 15 days later they were fed on a clean mouse.

#### 25. Dissection of Glossina and Stomoxys

##### G. morsitans

The flies were anaesthetized and their wings and legs removed

with the help of a pair of scissors. The fly was placed in a drop of 0.85% saline on a slide under a binocular dissection microscope. The head was held with one pair of forceps and the thorax with a second pair. Gentle traction was used to separate the head from the thorax, and the salivary glands were usually drawn out with the head. The salivary glands were examined microscopically (x 10 oculars x 40 objective), for the presence of metacyclic forms. Then the chitinous covering of the thorax was cut transversely on the ventral and dorsal sides with the aid of a scalpel and the proventriculus was exposed and removed for examination. Later, the remaining portion of the thorax of the fly was held with a pair of forceps, the terminal segment of the abdomen was cut, and the gut was pulled out. The midgut was separated from the hind gut and examined for the presence of trypanosomes.

#### S. calcitrans

The flies were anaesthetized and after removal of both wings and legs, the last abdominal segment was cut off and the gut was drawn out posteriorly. The contents of the gut were checked for the presence of trypanosomes.

## RESULTS

### Colombian strain

#### 1. Morphology and behaviour of the parasite (Table 3, Figure 3, Plate 1)

##### In mice

The Colombian strain (Plate 1) was represented by trypanastigotes corresponding to slender and long intermediate forms of T. brucei. The method adopted for categorization of different forms is given in Table 2.

The behaviour of the strain in mice was observed in an experiment on infectivity titration of the stabilate LUMP 74 population (Table 3). The stabilate LUMP 74 contained antilog 7.65 organisms and antilog  $7.1 \pm 0.3$  ID 63 per ml.

The inoculation dose appeared to influence the length of the prepatent period in mice since a decrease in the inoculation dose resulted in an increase in the prepatent period. The behaviour of the strain was not uniform and it is evident from the courses of parasitaemia in different groups of mice (Figure 3). All the mice of the group which was inoculated with  $\bar{2}$  dilution developed acute infections and died within 7 days during the first wave of parasitaemia but only 4 or 5 animals in those groups which were given  $\bar{3}$  to  $\bar{5}$  dilutions behaved in this way. In the group administered  $\bar{3}$  dilution, 2 mice developed relapsing parasitaemia and in groups inoculated with  $\bar{4}$  to  $\bar{5}$  dilutions only one of the infected mice showed relapsing parasitaemia. Thus, the Colombian strain of T. evansi appeared to have a mixture of antigenic types which may demonstrate differing degrees of virulence in mice



(McNeillage and Herbert, 1968).

The survival time of mice with acute infections and that of mice with relapsing infections, differed immensely. Table 3 shows the mean survival time of the mice showing acute infections and individual survival time of the mice showing relapsing infections. Mean survival time of mice showing acute infections increased as the size of inoculum decreased but this was not the case with those mice showing relapsing infections. The level of parasitaemia in the relapsing infections ranged from 1/20 (LEV 0.7) to 20/1 (LEV 3.3). Whenever the level increased beyond 20/1, it continued to rise until a fulminating parasitaemia was reached and the mouse died. The survival time ranged from 20 to 94 days.

#### In rats

3 rats were inoculated, each with 0.1 ml. of  $\bar{2}$  log dilution of stabilate LUMP 74 representing antilog 4.65 organisms or antilog 4.1 ID<sub>50</sub>. The infection was relapsing in all 3 rats, 2 showing 2 waves of parasitaemia and dying on day 15 and one showing 4 waves and dying on day 27.

## 2. Effect of environmental temperature on the course of infection

(Table 4, Figure 4)

A virulent monomorphic strain of T. brucei killed mice in the first wave of parasitaemia but when infected mice were maintained at 35°C relapsing parasitaemia and subsequent pleomorphism developed (Otiño, personal communication). Hence it was decided to investigate the effect of environmental temperatures of 4°C, 28°C, and 35°C on the behaviour of the Colombian strain in mice.

The behaviour of this strain in mice maintained at 28°C and a control group (22°-26°C) was similar with respect to the course of parasitaemia and in the ratio of mice which developed acute infections to those with relapsing infections. In both groups, all mice developed parasitaemia and those with acute infections died within 7 to 10 days after inoculation. However, in groups maintained at 28°C all the mice which developed relapsing infections died within 35 days of inoculation, whereas in the control group 3 of the mice survived for at least 60 days before death occurred. This suggested that 28°C was more favourable than room temperature (22-26°C) for multiplication of the parasites.

In the group maintained at 4°C, the level of parasitaemia was reduced but all other observations were similar to that of the control group.

There was marked difference in the behaviour of the parasite in mice maintained at 35°C. In this group 75% of the mice developed infections. 2 of the 15 infected mice died of acute infections and 1 died after 2 relapses. In the remaining 12 mice, the initial parasitaemia was present for a day or two after which trypanosomes were eliminated from the circulation. No parasites were observed on examination for a further 38 days. A self cure was obviously effected in these mice maintained at 35°C.

### 3. Antigenic variation in mice

#### 3. 1. Variability of the strain in an infected mouse (Table 5,

Figures 5, 6)

In order to isolate variant populations, one mouse was chosen

from a group inoculated with  $\bar{5}$  dilution during an experiment on infectivity titrations (Table 3). The course of parasitaemia in this mouse, based on log equivalent value (LEV) is shown in Figure 5. Details of the isolations of variant populations and the number of passages in mice prior to stabilisation are given (Figure 6).

The mouse was inoculated with the original Colombian strain (stabilate LUMP 74), which has been designated W0 for the purpose of antigenic identity. During the entire course of 56 days, 12 waves of parasitaemia were observed. Variant populations were isolated from each of the 12 waves and designated W1, W2, W3, W4, W5, W6, W7, W8, W9, W10, W11, W12. LUMP numbers for these variants are given (Figure 6). There was insufficient number of parasites in some stabilates of variant populations and therefore the population was passaged at 3 day intervals before a further stabilate was prepared. Therefore there were 2 stabilates of some variant populations and their antigenic similarity was checked.

On the 56th day when the mouse had fulminating parasitaemia, which usually results in death, and was about to die, Berenil treatment was given and 5 days later its serum was collected.

Antisera were prepared against all the variants and their antigenic relationship was established by the agglutination test. A stabilate population was used as antigen in all cases, except in the case of W8 and W9 in which there were insufficient trypanosomes. It was necessary, therefore, to harvest trypanosomes from fresh blood for use as antigen. In the case of W8, a trypanosome population from the first wave of parasitaemia was used and the W9 population was passaged at 3 day intervals in mice until the

development of a fulminating parasitaemia from which trypanosomes were collected. The results of the antigenic relationship of different variant populations are shown in Table 5.

Each antigenic variant appeared to be a mixed population since most of them showed a slight reaction with either the preceding or succeeding population as well as the ability to agglutinate homologous antisera at high titres. The separation of these antigenic types was based only on the difference in agglutinin titres. The table shows that the antigenic type of the first wave, W1, was identical to that of the original strain, W0. W2, W3 and W4 were distinct antigenic types because each reacted with its homologous antiserum at high titres. W5 was a mixture of W5 and W6 since low titres of agglutinins to antigen W8 were demonstrated in antiserum W5. W6 appeared to be a mixed population of W5, W6 and W7 because anti-W5 serum agglutinated antigen W6 and anti-W6 serum agglutinated antigen W7. W7 and W8 were of one antigenic type and they had a mixture of W1 in it. W10 and W11 were of one antigenic type, but W12 which was mixed with W11, was of a different antigenic type to the former variants.

Therefore W2, W3 and W4 were distinct antigenic types but the remaining populations were found to be mixtures:

W0-	W0, W1 and W9
W1-	W0, W1, W7, W8 and W9
W5-	W5 and W6
W6-	W5 and W6
W7-	W1, W8, W7 and W8
W8-	W0, W1, W7 and W8
W9-	W0, W1 and W9
W10-	W10 and W11

W11- W10 and W11

W12- W11 and W12

It is considered that 9 variant antigenic types were represented by W0-W1, W2, W3, W4, W5-8, W7-W8, W9, W10-W11, and W12.

It was of interest to note the reappearance of the W1 antigenic type with the W7-W8 and W9 variants. In order to confirm that W1 had reappeared in the infection mixed with W8, mice which had been previously immunized with W1 were challenged with W8. The results of this experiment are recorded (Table 14, Figure 11).

### 3. 2. Immune response of mice to different variant antigenic types

(Table 6, Figure 7)

Earlier studies (Soltys, 1957a, b; Gray, 1965b; Miller, 1965) have shown that variant antigenic types isolated from the late stages of a trypanosome infection are less able to react with antibody than those variant antigenic types isolated from the early stages of an infection. However, Clarkson and Awan (1969) were unable to confirm their work. Therefore, an attempt was made to compare the ability of variant antigenic types derived from the early, middle and late stage of an infection, to agglutinate homologous antisera obtained from mice in which the infection had been cured. Variant antigenic types W2, W6 and W10 were selected for this purpose. Mice were inoculated with respective stabilates and the trypanosome population was passaged at 3 day intervals until the development of high parasitaemia and these organisms were used for initial inoculum. The 3 variants were inoculated into separate groups of mice, each mouse receiving antilog 7.0 organisms. A high parasitaemia developed on day 2

or 3 after inoculation, and a count of the organisms in each mouse was made in order to assess that number which would produce antigenic stimulus. Subsequently mice were treated with Berenil. Sera were collected on the 6th, 9th, 12th, 15th, 18th, 24th and 30th day after treatment and the titre of agglutinins to homologous variant antigenic types were recorded.

The initial titre of agglutinins was similar in each mouse of a particular group, despite the variability in the number of organisms at the time of treatment. The average titre of W2 (isolated from the early stage of an infection) was higher than that of W10 (isolated from the late stage of infection).

The maximum agglutinin titres were demonstrated on the 6th day after treatment and thereafter there was a gradual fall in titre, the pattern of which was almost similar in each of the 3 variant antigenic types.

### 3. 3. Observations on antigenic reversion

In the light of Gray's (1965b) work, it was investigated which, if any, of the isolated populations of the Colombian strain behaved as predominant antigenic type. Two experiments were performed. In the first, sera of mice with a chronic infection of either the original Colombian strain or one of its variants, were tested for the presence of agglutinins to 6 of the isolated antigenic variants. In the second experiment, antigenic variants W5 and W12 were inoculated into mice and trypanosome populations from the first 3 or 4 parasitaemic waves were subsequently isolated in order to study their antigenic types.

### 3. 3. 1. Variant antigenic types in mice with chronic infection

#### 3. 3. 1. 1. The presence of variant antigenic types in mice with a chronic infection of the original Colombian strain

(W0) (Tables 7, 8)

The experiment was based on the assumption that each infected mouse during the course of a relapsing infection, will necessarily have trypanosomes of the predominant antigenic type. The sera of 12 mice, which had been used for experiments both on infectivity titrations and the effect of temperature, and in which there was evidence of a chronic infection, were utilized. A brief history of infections in these mice is given in Table 7. The duration of infection ranged from 20 to 108 days. All sera were tested for the presence of agglutinins to antigenic variants W1, W2, W3, W4, and W10. The results are shown in Table 8. All antisera contained agglutinins to antigenic variants W1 and W2. Agglutinins to variants W3, W4 and W10 were present in 3, 5, and 4 out of 12 mice, respectively. Antiserum of the mouse from which variant populations were isolated, contained agglutinins against W1, W2, W3 and W10 variant populations. This serum was also tested and found positive for agglutinins to the original population.

Thus 2 variant populations, W1 and W2, were present in all sera and were behaving presumably as predominant antigenic types. The presence of W3, W4 and W10, in addition to W1 in some sera, suggested the development of similar antigenic types in those mice.

#### 3. 3. 1. 2. Variant antigenic types in mice with a chronic infection of variants W5, W6, W8 or W12 (Tables 9, 10)

In order to investigate the development of predominant antigenic types following an infection by a variant population, sera

from mice which had been inoculated with one of the variant populations W5, W6, W8 or W12, were tested for the presence of variant antigenic types. Some of the mice selected for this experiment were originally used for passaging variant populations for the preparation of stabilates or for the preparation of antisera. The brief history of these mice is given in Table 9. All 9 sera were tested for presence of agglutinins against the antigenic variants W1, W2, W3, W4 and W10. The results are shown in Table 10. Agglutinins to these antigenic variant populations with which the mice were inoculated, were present in all sera. All 9 sera had agglutinins to variants W1 and W2. Agglutinins to variants W3, W4 and W10 were also present in 1, 3 and 7 out of 9 sera, respectively.

The results of these 2 experiments were similar. Antigenic variants W1 and W2 developed in each mouse during the course of a chronic infection with either the original Colombian strain or one of its variants.

### 3. 3. 2. Antigenic types of variant populations represented in infections with W5 and W12 variants

A study was made of the antigenic types of variant populations in mice inoculated with either W5 or W12 variant antigenic types.

#### 3. 3. 2. 1. Isolation of variant populations of W5 and W12 antigenic types (Figures 8, 9)

3 pairs of mice were inoculated with antilog 4.85, 3.85 and 2.85 trypanosomes respectively. The organisms were derived from



stabilate population of W5 (LUMP 238) and all 6 mice developed chronic infections. Populations were isolated from 3 waves of parasitaemia in one of the mice inoculated with antilog 2.85 trypanosomes. The course of parasitaemia in this mouse is shown in Figure 8, and further details of these isolations, which were designated W5-1, W5-2, and W5-3, are shown in Figure 9.

3 pairs of mice were inoculated with antilog 4.47, 3.47 and 2.47 trypanosomes respectively in order to isolate variant populations of W12 (LUMP 131). 5 of the inoculated mice developed relapsing infection and one of these was chosen for isolation of variant populations. Parasites of the first wave could not be isolated since one of the mice in which the population was being passaged, died. Subsequently variant populations were isolated from the 2nd, 3rd and 4th wave of parasitaemia and designated W12-2, W12-3 and W12-4 respectively. The course of parasitaemia in that mouse from which the variant populations were isolated is shown in Figure 8 and the details of these isolations are shown in Figure 9.

The antigenic relationship of 10 variant populations is shown in Table 11. A comparison of the antigenic relationship of variants of W5 and W12 was made and their relationship to W1 was also studied in order to determine whether there was any evidence of antigenic reversion.

### 3. 3. 2. 2. Variability of W5 in a mouse (Table 11)

Antigen W5 was able to agglutinate anti W5-1 serum at high titres, but antigen W5-1 was unable to do so because of low number of parasites in the stabilate. Therefore, the first wave population

W5-1 was identical to W5. W5-2 and W5-3 were presumably identical, since the former agglutinated antisera W5-2 and W5-3 at high titres but the latter agglutinated homologous antiserum only at high titres. Both stabilate population and trypanosomes from fresh mouse blood were used as antigen W5-2 and slight differences were observed in the level of their agglutination titres.

### 3. 3. 2. 3. Variability of W12 in a mouse (Table 11)

Stabilate population of W12, W12-3 and W12-4 were used as antigen for agglutination test but the W12-2 antigen consisted of trypanosomes harvested from the first wave of parasitaemia in a mouse which had been inoculated with a stabilate population. All the 3 isolations W12-2, W12-3 and W12-4 were of different antigenic types.

### 3. 3. 2. 4. Antigenic relationship between variants of W5 and W12 (Table 11)

Variants W5 and W12 were of different antigenic types, but those of the second wave populations, W5-2 and W12-2 were identical. The third wave population W5-3, also proved to be identical with the second wave populations W5-2 and W12-2, but because the mouse, from which the W5 variant populations were isolated, died after the third wave of parasitaemia, it was impossible to make a comparison of further antigenic variants.

### 3. 3. 2. 5. Antigenic relationship of variants of W5 and W12 to the variant antigenic types W1 and W2 (Table 11)

It was evident from the previous experiment that the antigenic

type W1 and W2 appeared during the course of infection in all mice, whether the animals had been inoculated with either the original strain or one of its variants (Tables 8, 10). Therefore an attempt was made to compare the antigenic types W1 and W2 with the variants of W5 and W12; in order to observe whether antigenic reversion had occurred during the course of an infection of W5 or W12.

Variants W1, W2, W5, W12 were of different antigenic types, but W5-2 and W12-2 were of similar antigenic type to that of W1 (Table 11). This suggested that when these two variants were inoculated into a new host there was reversion to the former antigenic type W1.

3. 8. 2. 6. The protection of mice immunized with W1 against challenge with W5-2 and W12-2 (Table 12, Figure 10)

In order to confirm the similarity of antigenic type W1 to the first relapse populations of W5 and W12, mice immunized with W1 were challenged 15 days later by W1, W5-2 and W12-2. Vaccination afforded complete protection against challenge with variant antigenic type W1. Protection was also observed in 5 mice challenged with W5-2. Sterile immunity occurred in 2 of these mice and in the remaining mice there was a low grade infection of 1/20 fields except for a single occasion when parasitaemia rose to 1/1. In the control group the parasitaemia was higher (Figure 10b) and 3 of the 5 mice died whereas there were no mortalities in the vaccinated group. Thus an immunity to W1 rendered protection against challenge with W5-2. These observations suggest that both populations were of similar antigenic type. It is assumed that W5-2 was a mixed population, and the development of a mild infection in the vaccinated group must have been due to an additional antigenic type in this population.

Protection was also observed in the group challenged with W12-2 as 4 mice resisted the challenge. Only one mouse which developed infection after an extended prepatent period, later died. It is possible that the cause of development of an infection in this mouse was a breakdown of immunity. There was marked difference in the level of parasitaemia between the vaccinated and the control group (Figure 10c).

### 3. 3. 3. Antigenic types of variant populations after experimental transmission from *S. calitrans* (Table 13)

Antigenic reversion to the basic antigenic type which occurs in cyclically transmitted trypanosomes, is not expected to occur in *T. evansi* which is transmitted non-cyclically and does not undergo cyclical development. But from the work of Brown and Vickerman (1970) it was concluded that in *T. brucei* the antigenic reversion to the basic antigenic type occurs in the early part of the cycle within the vector when the trypanosomes change in morphology from the blood to the midgut forms. Such morphological changes occur from 1 to 3 days after the ingestion of an infective blood meal.

The non-cyclical transmission of *T. evansi* can occur at a delayed interval of 1 to 3 days after its transferrance from an infected animal (Nieschulz, 1926a; Kunert and Krause, 1934; Zumpt, 1949). Furthermore the parasite is known to survive in the gut of *Stomoxys* and *Glossina* for 24 hours (Hoare, 1940; Nieschulz, 1940; Falana, 1970). These factors together with the occurrence of antigenic reversion of *T. brucei* during its early development in the tsetse fly, suggested the study to determine whether

antigenic reversion took place in T. evansi within the arthropod vector S. calcitrans. Antigenic types of certain variant populations before and after experimental transmission by S. calcitrans, were compared by agglutination test. The details of these are given in Table 13. N

The trypanosome populations ingested by flies are referred to as 'ingested populations' and those which developed as a result of inoculation of fly homogenate are termed 'transmitted populations'.

Mice inoculated with the suspensions containing 'ingested trypanosome populations' W3, W12 and W4 developed infection and from the resulting 'transmitted populations' stabilates W3-t, W12-t, and W4-t respectively were prepared. Ingested populations of W1, W2, and W10 did not survive within the fly and were not infective to mice.

The antigenic relationship of the 'ingested populations' to their 'transmitted populations' is shown in Table 13. The ingested and transmitted populations W4 and W4-t were identical and the transmitted population W3-t was similar to W3. However, anti-W12 serum had a lower titre of agglutinins to the transmitted population W12-t when compared with that of the homologous ingested population W12. Anti-W12 serum also had slightly lower titre of agglutinins to ingested population W12 when compared with W12-t. Therefore some antigenic change between ingested W12 and transmitted W12-t population was evident, but it was insufficient to conclude that the antigenic type had changed.

These results suggest that in non cyclically transmitted trypanosomes the variant antigenic types remain unaltered, even when the parasites are transmitted by the fly to a vertebrate after a delayed interval of up to 18 hours.

**3. 4. Effect of challenge of other variants on mice immunized with variant antigenic type W1 (Table 14, Figure 11)**

In an earlier experiment it was observed that when a variant population was inoculated into a healthy host, the parasites in the first natural relapse reverted to the former antigenic type W1. Therefore, the effect of challenging mice rendered immune to W1 with a number of variants was investigated. It was assumed that after inoculation into a new host the variant populations would revert to the former antigenic type W1, and also that in a host immune to W1 they would be subjected to an immunological barrier. This barrier would presumably prevent the further development of parasites or alternatively the trypanosome population could survive within the immune host and change to some other antigenic type.

The results (Table 14) show that immunity to W1 did not prevent infection of other variant antigenic types, and suggest that in W1 immune mice the trypanosome populations did not revert to the former antigenic type W1 but changed to some other antigenic type.

However, immunity to W1 afforded varying degrees of protection against challenge with different variant antigenic types. The mice were completely resistant to a challenge with the homologous population W1, and considerable protection was observed when they were challenged with W0 and W8 which were of identical antigenic type to that of W1 (Table 5). These results confirm the similarity of the 3 antigenic types. In W0 and W8 challenge groups those mice which developed infection had a much increased prepatent period, and the level of parasitaemia was very low (Figure 11 b, c) except

in the case of one mouse in the W8 challenge group which later died. In both W0 and W8 challenge groups all the controls died of acute infection, but only one mouse died in the vaccinated group which had been challenged with W8. The mild infections of the vaccinated groups were probably due to the presence of populations mixed with W0 and W8.

In those groups challenged with heterologous variants W3, W4 and W12 different results were observed. Immunity to W1 rendered considerable protection against challenge with W3; in the vaccinated group 3/5 mice became infected and there was increased prepatent period and reduced level of parasitaemia when compared with that of the control group in which all mice became infected. In the vaccinated group, the parasites appeared 2 or 3 times only (1/1) during the entire observation period and all mice survived for 15 days but only 2 control animals survived this period.

The immunity to W1 rendered considerable protection against challenge with the variant antigenic type W12. In this group, one mouse did not develop an infection and in the remaining mice which developed infections the prepatent period was increased, and the level of parasitaemia was slightly reduced. All mice in the vaccinated group, but only 3 in the control group, survived.

However, in the W4 challenge group there was no evidence of protection since prepatent periods, the levels of parasitaemia and the proportion of survivors were similar in both the vaccinated and control groups.

Thus vaccination by single dose intravenous inoculation of formalinized whole infected blood (Herbert and Lumsden, 1968) was found to be a satisfactory method for the immunization of mice.

Mice which had been immunized against variant antigenic type W1 were protected against a challenge with the original Colombian strain (W0). This suggested that the antigenic type W1 was a major constituent of this strain. In summary, vaccination afforded sizeable protection against variant W8 (which had a mixture of W1), considerable protection against W3 and W12 but there was no protection against W4.

The virulence of the variant antigenic types differed (described below, Table 16, Figure 13). W0, W1 and W8 were more virulent than W3, W4 and W12. In the light of these results, it appears that vaccination with a virulent population W1 rendered considerable protection to the challenge with the less virulent populations W3 and W12 but did not afford protection to a relatively more virulent population W4.

### 3. 5. Passive transfer of immunity (Table 15, Figure 12)

An attempt was made to demonstrate passive transfer of immunity by the administration of antisera. 0.2 ml. of antiserum to variant W1 was inoculated i/v into each of 5 mice, and the controls were given an equal volume of normal mouse serum i/v. One hour later they were challenged (i/p) with antilog 4.7 organisms derived from a stabilate of variant W1. Parasitaemia was observed daily for 20 days and the mice were checked for a further 40 days in order to determine the length of their survival.

As a result of antiserum transfer, there was evidence of almost complete protection, despite a very high challenge dose. 3 of the mice did not develop an infection and in the remaining 2 a very mild infection was observed after an increased prepatent



period. In one of these mice in which parasitaemia developed from the 7th to the 14th day only, the parasites maintained a level of 1 per 20 fields and only rose once to a level of 2 per field (x 10 oculars x 40 objective). In the second mouse, parasites (1 in 20 fields) were seen only on 11th, 13th and 16th days. All the protected mice survived for more than 60 days, but the controls died of an acute infection within 6 days of inoculation.

### 3. 6. Comparison of behaviour of different variant populations in mice (Table 16, Figure 13)

The behaviour of the original Colombian strain was not uniform in mice (see Table 3). A relapsing infection was observed in 1 or 2 of 6 mice, inoculated with antilog 3.65 or fewer organisms, and the remainder died of an acute infection.

A comparison was made of the infective behaviour of the original strain and 5 variant populations in mice in order to determine whether populations of differing virulence appeared amongst different antigenic variants. This study utilized the data on the behaviour of different populations in control mice from the experiment in which mice were vaccinated with W1 and challenged by heterologous variants (see page 117).

The criterion for the assessment of infectivity was based on the ratio of mice dying of an acute infection to the number of mice showing a relapsing infection. The mean prepatent period, level of parasitaemia (expressed as mean LEV), and the number of mice dying within the 15 days observation period were also compared.

The behaviour of the original strain W0, variants W1 and W8, which have a common antigenic type as revealed by agglutination test

(see Table 5), was very similar. These populations were more virulent than W3, W4 and W12 since in each of these groups mice died of an acute infection (during the first wave) within 10 days.

The virulence of W3, W4 and W12 appeared to be lower than that of W0, W1 and W8. Some of the infected mice in these groups died of an acute infection but others developed a relapsing infection. The level of parasitaemia was reduced (Figure 13) and the prepatent period, except in the case of W12, was increased. Only some of the mice died within 15 days.

W4 was more virulent than W3 and W12. 3 of the mice in this group developed an acute infection and 4 died within 15 days. The level of parasitaemia during the major part of observation period was higher than that of W3 and W12. All the mice except one in the group inoculated with W3 developed a relapsing infection. The prepatent period was longer than that of W4 and W12 and there were only 3 deaths during the observation period. In W12 all mice developed a relapsing infection and only 2 died within the observation period.

Thus, W0, W1, and W8 were most virulent; W4 was moderately virulent, and W3 and W12 were least virulent.

These results suggest that the major portion of the original strain consisted of virulent type organisms, which appeared in the first wave of parasitaemia and were probably responsible for the deaths of most of the mice. The remaining variants which appeared later in the infection were less virulent except for W3 which was mixed with the antigenic type W1.

#### 4. Antigenic variation in rats

##### 4. 1. Development of antigenic types in rats (Table 17, Figure 14)

Each of 2 rats was inoculated with antilog 4.6 organisms or antilog 4.1 ID 63 of the original strain WO (LUMP 74). 2 parasitaemic waves were observed before the development of fulminating parasitaemia in each rat. The course of parasitaemia is shown in Figure 14. In order to detect the antigenic types of each wave, serum was collected 3 to 5 days after the peak of each parasitaemic wave. They were tested against antigenic types W1, W2, W3, W4 and W10 (Table 17). Serum collected after the first wave agglutinated W1 and after the second wave agglutinated W1 and W2 suggesting that in rats the antigenic type of the first and second waves were W1 and W2 respectively. Thus, the variant antigenic types W1 and W2 developed in both a mouse and rats in similar sequence.

#### 5. Culture (Table 18)

3 culture media were used to investigate the in vitro culture of this strain.

##### 4N medium

In the first experiment the tail blood of an infected mouse was drawn at the height of infection and inoculated into blood agar medium in a universal container. The culture was checked 6 days later and found negative. In the second experiment, each of 6 bijoux was inoculated with antilog 6.0 organisms in 0.1 ml. of heart blood. Each day a single culture was picked at random and the number of organisms in the liquid phase was counted. The number of organisms fell to antilog 5.7 ml. on the first day and antilog 4.3 per ml. on the second day. No organisms were seen

from the third to sixth day. In the stained smears prepared on the first and second day of cultivation, mostly dead and degenerate forms of trypanosomes were seen. There was no evidence of division which by definition implies multiplication and growth and the organisms could only be maintained for a period of 2 days in this medium.

#### Medium 199 (Plate 2a, b)

A single experiment was done. A mouse was infected with the original strain (LUMP 74) and bled from the heart into heparinized medium 199. The blood was diluted in medium 199 to antilog 6.0 organisms in 0.1 ml., and 0.1 ml. of this suspension was inoculated into a universal containing 5 ml. medium 199. The culture was checked daily. The number of trypanosomes gradually decreased. The count was 2 per field on the first day, 5 in 20 fields on the second day, and only 1 in 20 fields ( $\times 10$  oculars  $\times 40$  objective) on the third and fourth day. On the fifth day, only 1 living trypanosome was seen in the wet preparation (22  $\times$  22 mm ). Thus, the trypanosomes were able to survive for a period of 5 days in this medium.

On the second day, 0.1 ml. of this culture was inoculated into a fresh universal containing medium 199 for a second passage. This subculture was examined for 2 days, but no organisms were seen.

The morphology of organisms from culture was studied in stained smears prepared on days 2 and 3 after inoculation. Trypanosomes which were morphologically similar to peripheral blood forms were present (Plate 2a). Trypomastigotes had a long body, with either a pointed or rounded posterior end, terminal or subterminal kinetoplast and an elongate or oval nucleus. The undulating membrane was

less pronounced than the typical blood forms. Some dividing forms were present (Plate 2b) and some degenerating forms, in which the protoplasm was full of granules, were also seen. The latter were shorter in size, and contained an indistinct nucleus, a terminal kinetoplast, a pointed posterior end, and a free flagellum. A number of dead forms were also observed.

#### Macrophage cell culture

Only one experiment was done. In this experiment 14 Leighton tubes each containing a monolayer of 10 day old  $9.5 \times 10^4$  macrophages were used. Trypanosomes were harvested from the heart blood of a mouse infected with the original strain (LUMP 74). After separation from the blood cells, the organisms were suspended in medium 199, and 1 ml. of this suspension containing antilog 6.3 organisms was inoculated into each tube. The media were checked for 7 days and both liquid phase and Giemsa stained smears were examined for parasites. On the first day, the liquid phase was found to contain antilog 4.6 organisms per ml. But from day 2 onwards no organisms were found. The examination of the culture was discontinued after 7 days. In the stained smears of macrophages, no parasites were seen, and it seems likely that all the organisms were phagocytosed by the macrophages within 2 days of inoculation.

#### 6. Transmission

##### By *S. calcitrans* (Table 19)

These experiments were carried out in order to investigate the survival of the parasite within the fly 18-24 hours after an infective feed and subsequent infectivity to the vertebrate host;

and, also, to determine the antigenic type of variant populations after experimental transmission. The results of experiments are given (Table 19).

In experiments 1 and 2, 1 and 5 flies were fed to mice at the height of infection. They were dissected after 24 hours but no trypanosomes were found in the gut. In the initial experiments no organisms were able to survive in the gut for 24 hours after an infective blood meal, and in subsequent attempts, the time interval between ingestion and killing was reduced to 18 hours or less. Since it was also possible that the parasites were surviving in the other parts of the fly including the haemocoel, the flies were homogenized. The homogenate was examined for organisms and inoculated into mice. The presence of organisms in the homogenate was indicative of their survival within the fly and the development of an infection in the mice was suggestive of their infectivity. The blood of the inoculated mouse was passaged at 3-day intervals until the development of fulminating parasitaemia and in positive cases stabilates were prepared. The designation of these transmitted populations was indicated by adding the letter 't' to the type of ingested variant population, e.g., W3-t, W4-t and W12-t.

In experiments 3 to 10, the flies were homogenized 18 hours after an infective feed except in experiment 6 in which flies were triturated after only 7 hours. In experiments 3 and 4, the parasitaemia at the time of feeding of flies was not very high, and yet organisms were present in the homogenate and resulted in the infection of a healthy mouse. In experiment 5, the parasitaemia was high when the flies fed but no organisms were found in the homogenate. Thus the degree of parasitaemia at the time of feeding was not found to influence the survival rate in the fly. On the contrary, when a smaller number of parasites was ingested, they survived and retained their infectivity.

In experiment 6, the flies were homogenized 7 hours after an

infective feed, and although organisms were present in the suspension they failed to infect mice. At the 4th passage the mouse was examined for 15 days but no parasites were found. It would appear that either the trypanosomes lost their infectivity or that the number of parasites was too small to infect the mouse. In experiment 7, trypanosomes were present in the homogenate and they were found to infect a mouse. In experiments 8, 9 and 10, in which the variant populations W1, W2 and W10 were used the organisms did not survive in the fly for 18 hours.

There was no uniformity in the survival time of different populations in the body of the fly (they survived for 18 hours in experiments 3, 4 and 7 but died in experiments 5, 8, 9 and 10) or in the survival of one variant population (W4 variant population survived until 18 hours in experiment 5 but not in experiment 7). In 3 experiments (experiment 3, 4 and 7) those stages which survived in the fly were infective to mice after remaining 18 hours within the body of the fly, but in experiment 6 their infectivity to mice was lost as early as 7 hours after an infective feed. There was some indication of a relationship between the degrees of parasitaemia in the mouse used for an infective feed and the subsequent infectivity to the mice, of the trypanosomes which survived within the fly. It was found that a lower level of parasitaemia was favourable for survival.

By *O. moubata*

A limited number of adults and larvae were available for this experiment and only 2 adults and 7 larvae were fed on a mouse infected with the original strain (W0) at the height of infection. After a period of 20 days, both adults and larvae were fed on a healthy mouse. The haemolymph which was discharged by the tick after feeding on the mouse was examined and found negative for trypanosomes. The healthy mouse was checked for 15 days but parasitaemia was not detected. Thus the experimental transmission of this strain by *O. moubata* was not achieved.

N.S. strain1. Morphology and behaviour of the parasite (Table 20, Figure 15)

Long intermediate and slender trypomastigote forms of this strain are seen in the blood of infected mice (Plate 3). The behaviour of this strain in mice was studied in an experiment on infectivity titration of the stabilate population LUMP 46 (Table 20). The stabilate contained antilog 7.9 organisms per ml. and its ID 63 was antilog 7.6 S.E.  $\pm$  0.3. As shown in Table 20, the prepatent period increased with a decrease in inoculum size. The behaviour of the strain was typical of that of an old laboratory strain. Even those mice given the lowest dose of trypanosomes developed an acute infection and died within a week of inoculation during the first wave of parasitaemia (Figure 15).

2. Effect of environmental temperature (Table 21, Figure 16)

In the absence of a relapsing infection this strain was not expected to show antigenic variation in mice. However, an old laboratory strain of T. brucei, which was virulent for mice, had developed a relapsing parasitaemia when the mice were maintained at 35°C (Otieno, 1970). Therefore, the effect of ambient temperature 35°C on the N.S. strain in mice was studied in order to obtain a relapsing parasitaemia, and to make subsequent isolations of variant populations from different waves.

As expected, the behaviour of the strain changed (Table 21). In the first experiment, all animals were inoculated with antilog 1.9 organisms (antilog 1.6 ID 63). All the mice of the control group became parasitaemic and died of an acute infection during the first parasitaemic wave. In the test group maintained at 35°C



only 5 mice developed an infection and of these 3 developed a relapsing parasitaemia. The resistance of the 35°C group was further marked by an increase in the mean survival time as compared with that of the control group. In experiments 2 and 3 antilog 2.9 organisms were inoculated and suppression of parasitaemia was evident. Figure 18 compares the course of parasitaemia in the experimental and control groups in experiment 3 and illustrates the reduction in the level of parasitaemia in the group maintained at 35°C. Relapsing parasitaemia was observed in 2 of the 6 mice in experiment 2 and in 5 out of 12 mice in experiment 3. In experiment 2 one of the mice survived for 57 days and showed 5 waves of parasitaemia during the course of infection. Relapse populations from the second, third, fourth and fifth waves were isolated from this mouse in order to compare their antigenic types (see page 131).

The mean survival time of groups maintained at 35°C was significantly increased in all the experiments. In the experimental group even those mice which died in the first wave, survived for a longer period than those in the control group. The suppression of parasitaemia, which was apparent from the relapsing infections and prolonged survival of mice in the experimental group indicated that, either the resistance of the host was increased or the virulence of the strain was decreased in animals maintained at 35°C.

The morphology of the trypanosomes did not alter in those mice kept at 35°C. In experiment 3, the blood of some animals in the test group, which developed relapsing parasitaemias, was examined on agar (Ormerod, 1959). At the peak or in the descending

phase of a parasitaemic wave in these animals, the trypomastigotes appeared to be long-intermediate or intermediate forms, and contained type II granules (Molloy and Ormerod, 1965). These authors described these granules, but in stumpy forms only. In order to investigate whether the presence of these granules in the trypomastigote is related to their subsequent development in the tsetse fly, the blood containing granular trypomastigotes was inoculated into 2 tubes of 4N medium. But the organisms could not be cultured and survived in this medium for 1 to 2 days only.

### 3. Development of pleomorphism (Table 22, Figure 17, Plate 4)

In order to obtain pleomorphism mice were first made immune to the S.A.K. strain and subsequently challenged with the N.S. strain. But the results of the experiments were variable (Table 22).

The course of infection of the N.S. strain, from which mice usually died with a high parasitaemia, was influenced by the immune status of the host. An acute infection was seen only in 2 out of 9 mice; relapsing parasitaemia developed in 4 mice and in 2 of these (mouse 1 and 9) pleomorphism was evident (Figure 17). The only appearance of stumpy forms in mouse 1 was on day 30, when the first wave of parasitaemia was descending. On this day the percentage of slender, intermediate and stumpy forms was 1, 92.5 and 6 respectively.

A further change in the morphology of the trypanosomes was observed. A vacuole appeared between the nucleus and the posterior end of the intermediate and stumpy form trypanosomes (Plate 4). Various stages of its development were evident from the variability

of its size. The smallest vacuole was approximately half the width of the trypanosome and the largest extended to the perimeter of the trypanosome. It is suggested that the gradual progression in vacuole size resulted in the development of the following stages. A gradual increase in vacuole size, from about half the trypanosome width (Plate 4a), was observed until the whole space between the posterior end and the nucleus was occupied (Plates 4b, c). The portion of the organism anterior to the nucleus, together with the free flagellum, gradually rolled around the vacuole so that the trypanosome appeared first as a 'comma' shape (Plates 4d, e, f), then a 'c' shape (Plate 4g), and finally as a globular shape (Plate 4h), identical to the sphaeromastigote form described by Brack (1968). In such forms the large central vacuole was surrounded by the trypanosome body.

In this mouse, a second wave of parasitaemia developed on day 34 and death occurred on day 36. In mouse 3 which was treated twice with Berenil to induce immunity against the S.A.K. strain, pleomorphism of the N.S. strain was observed on day 38. The proportion of stumpy, intermediate and slender forms was 1.5, 98 and 0.5%. The blood of the mouse was stabilised as LUMP 330. On day 39 the stumpy forms were evident but since the parasitaemia was scanty, their percentage in the blood smears could not be calculated. Stabilate LUMP 331 was prepared on this day.

#### 4. Antigenic variation

##### Variability of the strain in an infected mouse kept at 35°C

(Table 23, Figure 18)

Owing to the virulent behaviour of the strain in mice it was not expected to show antigenic variation. However, when

the behaviour of the strain was altered by maintaining mice at 35°C a relapsing infection developed. The antigenic types of these relapse populations which had been isolated from a single mouse (Figure 18) were compared (see page 128). Populations from the second to the fifth waves, designated T2, T3, T4 and T5, were isolated. For the purpose of antigenic variation the original strain was designated T0. As shown in Table 23, T2, T3 and T4 were distinct antigenic types. Anti-T5 serum reacted with T0, T4 and T5, indicating that it was a mixed population. Thus, by maintaining mice at 35°C antigenic variation was induced in this strain.

#### 5. Culture (Table 24)

3 experiments were carried out to determine the viability and morphology of this strain in 4N medium. In the first experiment, 2 drops of tail blood were obtained from a mouse at the height of infection, and inoculated into one tube. It was examined after 6 days and the parasite concentration was found to be 1 in 50 fields (x 10 oculars x 40 objective). No parasites were seen on day 7. On day 6, approximately 0.1 ml. of the culture suspension was subinoculated into another tube but no growth was observed in the subculture.

In the second experiment, each of 3 medium tubes were inoculated with tail blood and checked from day 3 onwards. 2 tubes were found to contain motile organisms. In the first, there were 2 parasites per microscopic field (x 10 oculars x 40 objective) on day 3, and 1 per 20 fields on day 4 and none on day 5. In the second positive culture tube the flagellate population was

scanty and only 1 trypanosome per wet preparation (22 x 22 mm) was found on days 3 and 4. Thereafter, the culture was negative.

In the third experiment,  $1 \times 10^6$  organisms were inoculated into each of the 6 medium tubes. From day 1 onwards, one culture tube was removed daily and the number of organisms in the liquid phase was counted. The number of organisms was  $1.6 \times 10^6$  per ml. on day 1,  $4.3 \times 10^5$  per ml. on day 2 and  $1 \times 10^4$  per ml. on day 3. From day 4 onwards, no flagellates were observed. From day 1 to 3 stained smears of the culture were examined for parasite morphology, but mostly dead and degenerate forms were seen.

The strain survived for a maximum of 6 days in 4N medium.

## 6. Transmission

### By *O. moubata*

2 adult *O. moubata* and 5 larvae were fed on a mouse at the height of parasitaemia and 19 to 20 days later they were fed on a clean mouse. The haemolymph discharged by the tick did not contain any trypanosomes, and the mouse did not develop an infection during the 15 day observation period.

### N.S. strain clone

#### 1. Preparation

3 clones of the N.S. strain were prepared by the method of McNeillage et al. (1970) and were preserved at  $-79^{\circ}\text{C}$ . The clones were designated LUMP 59, 85 and 95. All 3 clones were of one antigenic type (see Table 43).

#### 2. Morphology and behaviour in mice

The clone population was similar in morphology to the original N.S. strain. When inoculated into mice, the clone populations

behaved like the original N.S. strain, producing an acute infection which killed the mice.

### 3. Development of pleomorphism

Attempts to induce pleomorphism in the clone of N.S. strain were made prior to making attempts with the strain. In order to obtain stumpy forms from N.S. clone populations, 4 experiments were carried out and in all of them for inducing immunity against S.A.K. clone, stabilate LUMP 55 was used.

#### Experiment 1 (Table 25)

That part of this experiment involving the immunization (S.A.K. clone) and challenge of animals with N.S. clone, was carried out by Dr. Miles, and the occurrence of pleomorphism has been reported (Miles, 1972).

Following the challenge on day 43, all mice developed an infection on day 47 but only slender forms were seen. All mice were again examined from day 50 onwards. Mouse 1 was negative on day 51 but was positive on day 54 and the parasitaemia continued to rise until day 57. The mouse remained positive until day 77 when it died, but at no stage were stumpy forms seen. In mouse 2, 3, 4, and 5 the parasitaemia was teeming on days 51 and 52, and dropped to a level varying from 1/20 to 10/1 per field (x 40 objective) on day 53. Pleomorphism with predominantly stumpy forms was seen on days 51, 52 and 53. From day 54 onwards, the parasitaemia rose again in the form of a second wave and the population continued to have a mixture of slender and stumpy forms. Pleomorphic populations from 3 of the 4 mice were preserved as stabilates on different days (Table 25). The stabilates

were numbered LUMP 171, 172, 173, 176 and 177 and hereafter these populations have been designated 'N.S. pleomorphic variant', since they were of a different antigenic type to that of the N.S. clone.

#### Experiment 2 (Table 25)

Mice used in this experiment were originally immunized against the S.A.K. clone with mice of experiment 1. Their immunity was confirmed by challenging with the S.A.K. clone on day 120. They were resistant to the S.A.K. infection. On day 136, the N.S. strain clone (LUMP 59) was inoculated in doses identical to that used in experiment 1. All the mice died of an acute infection 5 to 8 days after the challenge and no pleomorphism was found.

#### Experiment 3 (Table 26)

The experimental procedure was similar to that of experiment 1. Acute and relapsing infections were seen in mice irrespective of the inoculation dose. Pleomorphism was observed in 1 mouse only, on day 35 during the third wave of parasitaemia. All mice in the control group died. The data of this experiment have also been utilized elsewhere (Table 44).

#### Experiment 4 (Tables 27, 28; Figure 19a, b; Plate 5)

This experiment differed from previous experiments in 2 ways:  
(1) A subcurative dose of Berenil 0.5 mg. per mouse was administered after which an initial cure of the infection occurred but relapses were observed in some mice. These relapses were treated and the immunity was induced.

(2) In this experiment another stabilate LUMP 85, which was of the same antigenic type as LUMP 59, was used to challenge the mice immunized against S.A.K. clone.

The behaviour of the parasite differed in different mice. While no infection developed in one mouse, 3 of the mice died of acute infection. Only in one mouse (mouse 1) was a relapsing infection with 3 waves of parasitaemia evident before its death. Pleomorphism occurred in this mouse in the second and third waves only (Figure 19a).

The change in morphology from slender to stumpy forms occurred possibly in the following sequence in mouse 1 (Figure 19b). The trypanosomes in the clone were monomorphic slender forms with a kinetoplast in either the subterminal position or towards the posterior end, and a short, free flagellum (Figure 19b.1). The change in morphology started on day 70, at the peak of parasitaemia during the second wave. The kinetoplast migrated towards the posterior end (Figure 19b.2 to 4) but there was no apparent change in the length of the trypanosome. The percentage of different forms is shown in Table 28. On day 71, when the parasitaemia fell, most of the trypanosomes became different forms of the intermediate type i.e. long-intermediate forms with a subterminal kinetoplast and a pointed posterior end (Figure 19b.5); intermediate forms with the kinetoplast nearer to a pointed, posterior end and a short, free flagellum (Figure 19b.6) and short intermediate forms with a very short free flagellum (Figure 19b.7). Stumpy forms were also seen but true slender forms were scanty. On day 77, in the third wave when parasitaemia was at its height, slender forms with a free flagellum of medium length were seen. A few



short, intermediate (Figure 19b.12) and stumpy forms (Figure 19b.14) were also observed. On days 78 and 79, slender forms with a long free flagellum (Figure 19b.6 and 7), short intermediate forms (Figure 19b. 10 and 11) and stumpy forms (Figure 19b. 13) were seen. On days 78 and 79, the blood of this mouse was preserved as stabiliate LUMP 280 and 315 respectively. Photographs showing stumpy forms on days 78 and 79 (19 and 20 days after inoculation of the N.S. strain clone) are shown in Plate 5. The percentage of stumpy, intermediate and slender forms at the time of isolation of LUMP 280 and 315 was 13.5 and 16, 49.5 and 36, 27 and 48 respectively.

It appears that the change in morphology from a monomorphic, slender form with a short, free flagellum was not only directed towards transformation to stumpy forms but also towards a slender form with a long free flagellum. The change towards both the slender and stumpy forms occurred by a backward migration of the kinetoplast. During the transformation to a slender form, the kinetoplast moved towards the posterior end and migrated anteriorly to adopt a subterminal position. The length of the free flagellum gradually increased during this process. It is suggested that the change towards stumpy forms was via intermediate and short intermediate forms. The kinetoplast remained either at the terminal position in the stumpy form or moved anteriorly to take a subterminal position. The shape of the nucleus gradually changed from elongate to oval or round. The free flagellum gradually decreased in length.

#### 4. Antigenic variation (Table 29)

Antigenic types of relapse populations isolated from

experiment 1 were studied in order to assess whether the system could induce antigenic variation in the trypanosomes. All the relapses were antigenic variants because none of them reacted with the original N.S. clone population (Table 29). There were, however, cross reactions among themselves. Antigen LUMP 175 reacted with antisera of all other isolations. Antigen LUMP 172 reacted with antisera to LUMP 171 and LUMP 172 and antigen LUMP 176 reacted with antisera to LUMP 172 and 176. All these variant populations therefore appeared to have an identical antigenic type. Stabilate 171 did not react with any antisera on account of its lower concentration of organisms.

#### N.S. Pleomorphic variant

The pleomorphic populations produced experimentally either from N.S. strain or its clone, have been called 'N.S. pleomorphic variant'. The proportion of stumpy forms in different pleomorphic populations varied. It was 1.5% in LUMP 330 (page 130), 13.5% in LUMP 280 and 16% in LUMP 315 (see page 136). At the time of isolation of LUMP 171, 172, 175, 176 and 177 approximately more than 50% trypanosomes were stumpy forms.

#### 1. Morphology and behaviour after cryopreservation

##### 1. 1. In healthy mice (Figure 20)

The behaviour of these populations was probably affected by the proportion of stumpy forms present at the time of isolation. When LUMP 330 was inoculated in dilutions of log 2 to log 4, 0.1 ml. being inoculated to each mouse, the mice died during the first wave of parasitaemia and did not show any evidence of

pleomorphism. Similar results were obtained with LUMP 280, when it was inoculated in 0.1 ml. volumes of log 2 to log 4 dilutions. With LUMP 315 the picture was slightly different. When antilog 4.17 trypanosomes or less was given the mouse died in the first wave of parasitaemia within approximately 7 days of inoculation. On the last day of survival, pleomorphism usually occurred and a few stumpy forms were present. A typical example of the course of parasitaemia and proportion of stumpy forms is drawn in Figure 20. This figure represents the course of parasitaemia in a mouse which was inoculated with antilog 2.17 trypanosomes. The percentage of stumpy forms on the day of death i.e. 8 days after inoculation, was 4.5%. In another mouse which was given antilog 3.17 trypanosomes, a relapsing infection with 2 parasitaemic waves was seen. Stumpy forms were evident at the descending stage of parasitaemia during the first wave, but the second wave consisted of slender forms only.

LUMP 171, 172, 175, 176 and 177 given in doses of 0.1 ml. of log 2 to log 4 dilutions resulted in a relapsing parasitaemia with 2 waves accompanied by the presence of stumpy forms in proportions of more than 50%. The mice survived for approximately 4 weeks after inoculation. As an example of the behaviour of these populations in mice, the course of infection and morphology following inoculation of LUMP 172 is shown in an experiment on the behaviour of pleomorphic variant in splenectomized and irradiated mice (see text below, Figures 21a, b).

These results suggest that those stabilate populations with a small number of stumpy forms, when inoculated into mice, caused a virulent infection. In the mice the stumpy forms appeared only

occasionally in smaller numbers in the terminal stages of infection. On the other hand, stabilate populations with a large number of stumpy forms, developed a relapsing infection in mice and in these animals stumpy forms always appeared in greater numbers.

1. 2. In splenectomized and irradiated mice (Table 30, Figure 21)

The dominance of stumpy forms in the descending stage of a parasitaemic wave is said to be due to the effect of antibody (Wijers, 1959). Therefore, the morphology of N.S. pleomorphic variant was observed in splenectomized and irradiated mice. 3 groups of mice, 10 in each group, were used. Mice of one group were irradiated with 850 r and mice of another group were splenectomized, and the third group acted as a control. A dose of antilog 3.25 trypanosomes of LUMP 172 was inoculated 4 days after splenectomy and irradiation in the respective groups. The score of parasitaemia is shown in Figure 21.

In all groups the parasitaemia rose from the 5th day of infection. In the control group, the peak parasitaemia was observed on the 7th or 8th day post infection and was followed by scanty parasitaemia or a non-parasitaemic phase on the 10th and 11th day. A second wave of parasitaemia appeared on day 12 and remained until day 20 when the observations were terminated. In the irradiated group, the peak parasitaemia was also observed on the 8th day and the degree of parasitaemia was similar to that of the normal group. However, it was not followed by a non-parasitaemic period. From the 8th day onwards, the level of infection was lower than in the control group. In the splenectomized group, the level of parasitaemia was lower than that of the

control and the irradiated groups. The peak of parasitaemia was on the 10th day and the level of parasitaemia following peak was in between that observed in the irradiated and normal groups.

Proportion of slender and stumpy forms (Table 30, Figure 21)

In the control group, the slender forms were predominant in the ascending phase of the first parasitaemic wave whereas stumpy forms predominated at the descending stage. From day 5 to 7, when the parasitaemia was rising, the slender forms gradually decreased and they were scanty or absent on days 8 and 9, when the parasitaemia decreased. During the second wave, in which the parasitaemia gradually increased from day 12 onwards, there was also a predominance of slender forms.

The percentage of intermediate forms in the first wave appeared to be influenced by the percentage of slender forms and stumpy forms. The intermediate forms increased as slender forms decreased, whereas the percentage of intermediate forms decreased as an increase of stumpy forms occurred. A similar trend was observed in the second wave. This suggested that in the first wave, the slender forms were first transformed to intermediate forms which in turn changed to stumpy forms. The stumpy forms, possibly the terminal stage of the parasite, later disappeared from circulation.

The percentage of stumpy forms in the descending stage of the first wave was higher than that observed in the irradiated group but similar to that in the splenectomized group. However in the second wave, when the parasitaemia rose, the percentage of stumpy forms was lower than that of splenectomized and

irradiated groups during an identical period of infection.

In the irradiated group in which the pattern of parasitaemia was similar to that of control group from days 5 to 8, the number of slender forms declined gradually. However, in the subsequent period when the level of parasitaemia gradually decreased, unlike that in the control group, the slender forms did not disappear from the circulation altogether. These results show that there was a gradual decline of slender forms from day 5 to 12 whereas there was an increase from day 13 to 18; there was a concomitant increase or decrease of the percentage of stumpy forms and decrease or increase in slender forms; and from day 12 onwards the percentage of stumpy forms was much higher than that of the control group.

In the splenectomized group the pattern of change in slender and stumpy forms was similar to that of the irradiated group. However the percentage of different forms was in between that of the control and irradiated groups.

It is considered that the appearance of stumpy forms is not influenced by the immune status of the host because in splenectomized and irradiated animals their appearance was not suppressed. On the other hand, their percentage was more than that of control group in the later stages of infections. Although in the immunosuppressed groups, a count of all the 3 forms in wet preparations did not reveal a wave of parasitaemia. However, a count of different forms in stained smears suggested that there was a descending and ascending phase of slender forms only. This indicates that the rhythm of slender forms was evident in all the 3 groups. However, in the first wave of control group they

decreased abruptly whereas in the immunosuppressed animals they declined gradually. It is suggested that although an impaired immune status of the host delayed the suppression of slender forms it was unable to check the rhythmic pattern of slender forms.

## 2. Culture

### 2. 1. 4N medium

Experiments to determine the in vitro viability and morphology of pleomorphic variant obtained from experiments 1 to 4 were undertaken. Isolations from experiment 1 were LUMP 171, 172, 175, 176, and 177 (see Table 25) and those from experiment 4 were LUMP 280 and 315 (see Table 27).

#### 2. 1. 1. LUMP 172, 171, 177, 175, and 176 (Table 31)

##### 2. 1. 1. 1. Survival in the medium

The mice were inoculated with one of the stabilate populations LUMP 171, 172 or 177. Before inoculation into medium the blood of the mouse was examined for the presence of a sufficient number of stumpy trypomastigotes. The media were inoculated with infected mouse blood taken from the tail or heart. In each of the experiments 1 to 4, all the culture tubes became positive. The majority of these cultures survived for 8 to 12 days in the first passage. However in one culture, the organisms were maintained for 26 days. The mean survival period was 11.4 days. The parasite density ranged from + to +++, the average score being 1 parasite per field (++) . The flagellates were actively motile and on many occasions they were clumped together. The dividing forms had 2 or more active flagella.

## 2. 1. 1. 2. Passages (Table 31)

The parasites were subcultured through a number of passages. The day of subculture and the score at that time is given in Table 31. The parasites survived in 6 out of 7 subcultures from the first passage. The cultures survived for 10 to 12 days but in one experiment they survived until the 19th day. The mean survival period was 8 days but in one culture a minimum survival time of 5 days was observed. In the third passage, only 2 out of 6 subcultures survived, one for 18 days and another for 12 days. Therefore, the mean survival time was 5 days. At the fourth passage, one medium tube was subinoculated in which the culture survived for only 4 days.

## Inoculation of a stabilate population (Table 31)

For the development of cultures initiated by the inoculation of stabilate populations, LUMP 171, 175 and 176 were used. The cultures developed and survived for a period of 27, 9 and 5 days respectively.

A suspension of the culture which had developed from the stabilate population LUMP 171 was inoculated into fresh medium but the subculture was negative on the 5th day after inoculation.

## 2. 1. 2. LUMP 172

Further studies on growth rates, morphology in culture and the infectivity of culture forms to mice were carried out employing LUMP 172 population.

### 2. 1. 2. 1. Growth rate (Table 32, Figure 22)

In order to study the growth rate, 3 experiments were carried



out. The number of live and dead organisms was counted and recorded for each culture. Flagellates showing no movement were treated as dead. The growth curves are plotted in Figure 22.

In the first experiment each of 30 bijou bottles were inoculated with  $1 \times 10^7$  organisms suspended in 0.1 ml. ABP pH 7.4. The infected mouse blood was obtained by cardiac puncture. After the initial lag phase the population reached a peak on the 5th day. All the cultures were found to be contaminated and dead on the 7th day, and further observations were suspended.

In the second experiment,  $1 \times 10^7$  organisms were inoculated into each bijou bottle. Following a lag phase there were peaks on days 3 and 9. From day 10 onwards the observations were made according to an arbitrary system (+ to +++), since the organisms were too few to be counted in a diluted suspension using a haemocytometer. In the undiluted liquid phase the number of flagellates per 20 fields ( $\times 10$  oculars  $\times 40$  objective) was recorded. The culture survived for 12 days.

In the third experiment,  $1 \times 10^6$  organisms were inoculated into each bijou bottle. The number of flagellates gradually decreased from day 0 to day 4. Then the number rose on day 5 in the form of first peak, but fell on day 6. A second peak was observed on day 7 and the number gradually decreased until no parasites were present on day 13. However, there was no corresponding increase or decrease of dead trypanastigote forms.

### 2. 1. 2. 2. Morphology in culture (Figure 23, Plate 6)

The morphology of culture forms was observed from stained smears prepared daily from the experiments on growth rate. Approximately 50 to 100 flagellates were examined. The morphology of organisms started changing on day 2 after inoculation when many trypomastigote forms, with a terminal kinetoplast and less pronounced undulating membrane than the blood trypomastigotes, were seen. On the following days trypomastigotes of varying sizes were observed. The free flagellum was usually present and the kinetoplast was situated at various positions between the posterior end and the nucleus (Plate 6b. d, Figures 23.1, 23.2). Dividing forms were evident (Figure 23.6) and some had two kinetoplasts and one nucleus (Figure 23.4). In some trypomastigotes division occurred by equal binary fission (Figure 23.5). Giant forms having more than 2 nuclei (Plate 6f) and some with up to 8 nuclei (Figure 23.7) suggestive of a multiple fission were also observed. Thin flagellates (Figure 23.3) were also seen and these were probably forms which did not reproduce.

### 2. 1. 2. 3. Inoculation of culture forms to mice

Two experiments were carried out in order to determine infectivity of culture forms to mice. In the first experiment, 0.2 ml. of suspension of a 27 day old first passage culture, having ++ score, was inoculated into a mouse i/p. The mouse was checked for parasitaemia until day 45 but no infection developed. In the second experiment, 0.2 ml. of a 5-day old culture was inoculated i/p into a mouse. Parasitaemia did not develop in the mouse until day 45 of observation.

### 2. 1. 3. LUMP 315 and LUMP 280

LUMP 280 and 315 were obtained as pleomorphic populations from experiment 4 (Table 27). The history of their isolation and their behaviour in mice has already been given.

#### 2. 1. 3. 1. Survival in the medium (Table 33)

Stabilate population LUMP 280 was inoculated into the medium and the growth was seen up to 9 days.

4 experiments were carried out in which the media were inoculated with mouse blood infected with LUMP 315. Prior to inoculation, the blood was checked in a wet film under phase and contained at least a few stumpy forms. In experiment 4 which was made for growth rate studies, stained smears of mouse blood were examined and the blood contained 7.7% stumpy forms, 46% intermediate forms and 46.3% slender forms.

In experiments 1 and 2, 3 cultures survived only for 2 to 4 days. It is probable that the low number of stumpy forms present in the inoculated blood prevented growth. However in one culture the flagellates survived for 10 days. In experiment 3, the parasites survived for 6 days. In experiment 4, when the inoculum contained at least 7.7% stumpy forms, growth occurred for 9 days.

#### 2. 1. 3. 2. Passages (Table 33)

In 2 out of 3 experiments, the culture developed and survived for 5 and 6 days respectively. No growth was observed at the third subculture.

## 2. 1. 4. LUMP 315

### 2. 1. 4. 1. Growth rate (Table 34, Figure 24)

The study of the growth rate was done on the same lines as for LUMP 172. About 30 bijou bottles were inoculated each with  $1 \times 10^7$  organisms. As mentioned above, the percentage of stumpy forms at the time of inoculation was 7.7%. The growth curve is plotted in Figure 24. The number of flagellates declined until day 4 after inoculation but subsequently rose from day 5 onwards. The peak number of parasites was on day 7, and the culture died on day 10. However, there was no corresponding increase or decrease of dead trypanastigote forms.

### 2. 1. 4. 2. Morphology in culture (Figure 25, Plate 6)

Morphology was similar to that of the culture forms seen in the experiment using LUMP 172. On day 1, many slender and stumpy forms (from blood) appeared to be in the process of death (Figures 25.1, 25.2). On day 2, changes in morphology were observed in some trypanastigote forms; the kinetoplast moved to a terminal position (Plate 6a) and the undulating membrane became less pronounced. From day 2 onwards trypanastigotes of variable lengths with kinetoplast in different positions were seen (Plate 6c, Figure 25.3 to 25.8). Probably the change in morphology is produced by a backward migration of the kinetoplast and a narrowing of the trypanosome followed by the elongation of the flagellate and movement of the kinetoplast to a more anterior position. Dividing forms showing equal binary fission (Plate 6e, Figure 25.10) were observed. A few trypanastigotes which were in the process of division, had two nuclei but an intact

kinetoplast (Figure 25.9). Thin trypomastigote forms (Figure 25.11) were also seen. In the terminal stages of culture, short trypomastigote forms with either a terminal or subterminal kinetoplast (Figures 25.12, 25.13, 25.14) were seen. It is probable that these were the young flagellates resulting from division.

#### 2. 1. 5. Sequence of changes in morphology in culture (Figure 26)

After the inoculation of blood forms into culture, the undulating membrane gradually became less prominent and the kinetoplast moved to the posterior end. Later, the trypomastigotes elongated (Figures 26.1, 26.2, 26.3), and the kinetoplast migrated anteriorly. This was followed by either equal binary fission (Figures 26.5, 26.6, 26.7) or multiple fission (Figure 26.8). During reproduction either the nucleus (Figure 26.4) or the kinetoplast (Figure 26.7) divided first followed by division of the trypanosome body (Figure 26.9) into small trypomastigote forms (Figure 26.10). Those trypomastigotes which did not reproduce became thin and elongated.

#### 2. 2. HeLa cell culture (Table 35)

In order to investigate the possibility of a tissue phase, antilog 7.0 organisms were inoculated into each of the 4 HeLa cell cultures, 2 of which were kept at 28°C and the other 2 at 37°C. The monolayers and liquid phase were checked on days 4 and 8 after inoculation, but no organisms or intracellular stages were found.

## 2. 3. Macrophage cell culture (Table 35)

14 Leighton tubes, each containing approximately 950,000 mouse peritoneal macrophages, were used. Each tube was inoculated with antilog 6.07 organisms and 2 tubes were examined daily for a period of 3 days. The liquid phase, medium 199, was checked for trypanosomes and the macrophages were checked for intracellular stages of trypanosomes. No trypanosomes were seen in the stained macrophage preparation or in the liquid phase. It is possible that the organisms were phagocytized immediately after inoculation.

## 3. Transmission

### 3. 1. By G. morsitans

The successful cultivation of pleomorphic variants of T. evansi in 4N medium, suggested the investigation of their development in Glossina.

#### 3. 1. 1. LUMP 171, 175, 176 and 177

In one experiment, 4 mice were infected with 4 different stabilates of N.S. pleomorphic variants, LUMP 171, 175, 176 and 177 respectively, and when stumpy forms were predominant in the circulation of the mice 30-day old G. morsitans were fed on the mice for 5 days. The flies were not fed for 2 days after an infective blood meal. Later the flies were fed daily on 4 clean mice for a period of 20 days and all developed infection on the 20th day of feeding. Trypanosome populations in all the mice were pleomorphic. The blood of one mouse was stabilized as LUMP 220.

### 3. 1. 2. LUMP 172

The stabiliate population LUMP 172 was chosen for experiments involving the more detailed investigation of the morphology of T. evansi in Glossina, infectivity of the flies and transmission of infection. The results of 4 experiments are described under the following headings:

#### Transmission of infection (Table 35)

##### Experiment 1 (Table 35)

75 G. morsitans were divided into 5 groups A, B, C, D and E; the number of flies in each group being 12, 13, 22, 16 and 12. In order to provide infective blood meals for these flies, each of 4 mice was inoculated respectively with antilog 3.25 trypanosomes. When the blood of the mice contained mostly stumpy forms, the flies were allowed to feed on them. Each group of flies was fed on an infected mouse on the day of emergence and on the following 2 days. The flies were subsequently fed daily on clean mice, a different mouse being used for each group. These mice were replaced every 3 to 4 days. All mice were checked for parasitaemia, and in those mice in which an infection developed, the minimum and maximum interval between an infective and a healthy meal was calculated.

4 out of 29 mice became infected. Mouse A3 which had provided a healthy feed to group A flies on days 11 to 15, became infected. The minimum and maximum interval between an infective and a clean feed was 9 days and 15 days respectively. The mouse developed an infection 6 days after it had been used for feeding flies and died on the 14th day. The trypanosome population in the mouse was pleomorphic. Mouse A4 on which the flies were fed on days 16 to 18, also became infected. In this mouse the infection developed on

the fifth day, and the mouse survived until the 18th day after the last feed by the flies. The trypanosomes were pleomorphic.

Flies of group B also infected a mouse, B6, on which they had fed 20 to 25 days after ingesting an infective feed. This mouse developed an infection 4 days after being fed to flies. Flies of group C infected mouse C4 on which they had fed 13 to 17 days after ingesting a blood meal. The mouse became infected 7 days after providing a blood meal for the flies and continued to show pleomorphic populations until day 19 when it died.

In this experiment the minimum and maximum periods after which a fly could transmit an infection were 9 and 25 days respectively. These results have shown that the flies became infective to mice and consequently they were examined 3 to 4 weeks after the infective feed. The observations made during this examination will be described later (Table 37).

#### Experiment 2 (Table 36)

45 flies were divided into 3 groups and fed first on infected and then on clean mice as in experiment 1. Trypanosomes were seen in probes (Table 37, Plates 7a, 7b) on days 11 to 13 after the infective feed; further feeding on clean mice was suspended and the flies were examined. No infections were found to develop in those clean mice on which the flies fed within 14 days after their infective feed. Presumably, the infective forms had not developed by this time.

#### Experiment 3 (Table 36)

In this experiment only 6 flies were used, and after ingesting an infective blood meal they fed on clean mice for 14 days but they failed to infect the mice. It is possible that this was due to the



small number of flies used. On dissection the flies were found to contain trypomastigote forms and the results are incorporated in Table 37.

#### Experiment 4 (Table 36)

29 flies were divided into 2 groups A and B. They were given an infective blood meal on day 1 and subsequently each group of flies fed on a separate series of clean mice numbered A1 to A6 or B1 to B6 according to the group of flies (Table 36).

Flies in group A were unable to transmit the infection but the flies in group B transmitted the infection to mouse B4, B5 and B6. The minimum period at which the flies transmitted the infection was 27 days (mouse B4). The trypanosomes in all 3 mice were pleomorphic. Trypanosomes from mouse B4 were isolated on the 10th day of infection and after a further passage were stabilized as LUMP 607.

#### Examination of flies (Table 37)

##### Experiment 1 (Table 37)

Out of 75 flies used in this experiment, 51 were dissected 22 to 26 days after they had ingested an infective blood meal. Trypomastigote forms were evident in the midgut of 10 flies. The flagellates had a long thin body, a less pronounced undulating membrane, and a free flagellum. The kinetoplast was placed at various positions anterior to the nucleus. These forms were morphologically indistinguishable from proventricular forms of T. brucei as described by Bruce et al. (1914b). As the flies had been starved for a day or two before dissection it is possible that the proventricular forms may have migrated posteriorly to the midgut

(Robertson, 1913). The proventriculus and salivary glands were not examined.

Experiment 2 (Table 37, Plates 7, 8)

45 flies divided into 3 groups were used. Flies of group A were found to be infected since they released trypanastigotes in their salivary secretions in probes (Plate 7a, 7b) made on days 11 and 13. The probes contained only proventricular forms. One dying fly was dissected on day 11, and its midgut proventriculus and salivary glands contained proventricular-type trypanastigotes (Plate 8). 14 flies were dissected on day 14 and one of them had an infection in the midgut, proventriculus, and salivary glands, but again only proventricular forms were seen in the salivary glands. Another fly had an infection in the midgut and proventriculus only.

12 of the 14 flies in group B were dissected and all of them were negative. Some of the flies in group C were positive on day 12. However, the probes contained only proventricular forms. On days 12 and 13, 11 flies were dissected and only one of these was infected. Trypanosomes were found both in the gut and proventriculus of this fly.

Experiment 3 (Table 37)

In this experiment only 6 flies were used. One fly died before it could be dissected, and another was dissected on day 9 and found to contain midgut and proventricular infections. In the midgut, most of the trypanastigotes were proventricular forms, but some were of a comparatively shorter length and resembled midgut-form trypanastigotes. The 4 remaining flies were dissected on day 15

and one of them had infections in the salivary glands, proventriculus and midgut. The salivary glands were examined by phase contrast microscopy, and only a few short flagellates, resembling metatrypanosome forms, were evident. But in the stained smear of the salivary glands no parasites could be seen.

#### Experiment 4 (Table 37)

In this experiment 29 flies, divided into groups A and B, were used. Flies of group B were positive on day 35 and again on day 52 but on both occasions only proventricular forms were extruded by the flies. The experiment was continued until day 58, but unfortunately the flies in both groups died during this time and could not be dissected.

This series of experiments suggests that the pleomorphic variant populations have a cycle of development in G. morsitans similar to that seen in T. brucei (Bruce et al., 1914b).

#### 3. 1. 3. LUMP 315 (Table 38)

Attempts to transmit pleomorphic populations from LUMP 315 which had been successfully cultivated (pages 146-148) were also made. 181 flies (Experiments 1-5) were fed on infected mouse or, through a membrane on blood from a clean mouse mixed with the stabilate population. However infections were not obtained. The possible reason for this is that the parasite population usually killed mice during the first wave of parasitaemia when only a small number (approximately 4.5%) of stumpy forms was present a few hours before death (Figure 20). Therefore, in most of the experiments either a few stumpy forms or none at all were present

in the mouse when the flies were fed. The details of the transmission experiments (Table 28) and the examination of flies (Table 29) are as follows:

#### Experiment 1

28 flies were divided into two groups and fed on a mouse, when a few stumpy forms were present in its blood. On days 2 to 6 they fed on a clean mouse but by the 6th day all the flies had died. It is possible that their deaths were due to heavy bacterial infections found in the gut.

#### Experiment 2

65 flies were divided into 4 groups and fed on an infected mouse. Groups A and B (33 flies) were fed on a mouse which had few stumpy forms in the peripheral blood at the time of feeding and groups C and D (32 flies) were fed on a mouse which had no stumpy forms. All 4 groups were subsequently maintained on a series of clean mice for up to 18 days, but none of these mice developed an infection. 28 flies of group A and B were dissected on day 18, and 26 flies of group C and D were dissected on day 19, but none of these flies had developed even a midgut trypanosome infection. The remaining flies died and were not dissected.

#### Experiment 3

14 flies were fed through fresh mouse skin membrane on stabilate population LUMP 315. The contents of 4 capillaries of this stabilate were expelled into 0.5 ml. mouse blood. The blood of the mouse from which stabilate population had been isolated contained 16% stumpy forms. The trypanosome count in the

suspension, checked before and after feeding to flies, was approximately 15/1 (x 10 oculars x 40 objective). After the feed, the flies were maintained on a series of 4 clean mice for 14 days but none of the mice developed an infection. All flies, including those that died during the experiment, were dissected but were found negative.

#### Experiment 4

The flies used in this experiment were 2 days old and they were fed on mice when the blood contained a few stumpy forms. After feeding on clean mice for 15 days, 10 flies were dissected and found negative. 2 flies which died during the course of the experiment were also found negative.

#### Experiment 5

66 flies were divided into 4 groups and fed on infected mice. Groups A and B were fed when there were few stumpy forms, and groups C and D were fed when there were only slender forms in the blood. All flies, dissected after 4 to 6 days, were found negative.

#### 3. 1. 4. LUMP 330 (Tables 38, 39)

The blood of the mouse from which this stabilate was prepared contained 1.5% stumpy forms (page 130). The contents of 4 capillaries were released into 0.5 ml. of blood from a clean mouse. 19 flies were divided into 2 groups and fed through a membrane on day 1, and subsequently maintained on a series of clean mice for 14 days. None of these flies developed an infection.

### 3. 2. By *O. moubata* (LUMP 172)

2 adult *O. moubata* and 6 larvae were fed to a mouse which contained both slender and stumpy forms in its blood. 22 days later the ticks were fed on a clean mouse. The haemolymph discharged by the ticks and the blood oozing from the mouse skin wound inflicted by the tick bite, contained no trypanosomes. No transmission occurred since the mouse remained negative throughout the observation period of 15 days.

### S.A.K. strain

#### 1. Morphology of the strain and behaviour in mice (Plate 8)

The trypomastigote forms of this strain were of slender or intermediate type and they lacked a kinetoplast (Plate 8). Infections with this strain in mice resulted in death during the first wave of parasitaemia.

#### 2. Effect of environmental temperature on the course of parasitaemia

In one experiment 6 mice, preadapted to 35°C for a week, were inoculated with antilog 5.3 trypanosomes each. An infection did not develop in any of these mice. Similar results have been obtained by Miles (personal communication) and Otieno (1972a). Since the latter author has studied the effect of ambient temperature on this strain in detail, no further attempts were made.

#### 3. Antigenic variation

Antigenic types of relapse populations in a mouse (Tables 40a, b, Figure 27)

Relapsing parasitaemia in this strain was induced by subcurative

Berenil treatment. A first relapse population S1 was isolated on day 17 from one mouse whose initial infection had been treated on day 6; the mouse was also treated with Berenil on day 17. On day 28 population S2 was isolated from a second relapse. The original S.A.K. strain was designated S0. Antiserum to S0 was found to contain agglutinins to S1 and S2, and there was a cross-reaction between S0 and S2. The results of the agglutination test did not indicate distinct antigenic types. Therefore, in order to distinguish between the antigenic types of relapse populations and that of the original strain (S0), mice which had been previously immunized against S0 were subsequently challenged with S1 and S2. The results are shown in Table 40b.

The mice challenged with the homologous population did not develop an infection whereas those mice immunized with S1 and S2 became infected. This suggested that S1 and S2 were antigenic variants of S0.

#### 4. Culture (Table 41)

##### 4N medium

3 experiments were carried out with a clone population (LUMP 55). In experiments 1 and 2, the media were checked 7 days after inoculation and they were found negative. In the third experiment, when a daily check was made, the organisms survived for only 2 days.

##### Medium 199

0.1 ml. of heart blood, from a mouse infected with the S.A.K. strain, was inoculated into medium 199. The medium was checked daily and the number of organisms decreased gradually. The maximum

period of survival was 4 days. The organisms were subcultured on the second day after inoculation, but no growth was observed in the second passage.

Comparison of antigenic types of Colombian strain with other strains of *T. evansi* and 3 strains of *T. brucei* (Table 42)

Gray (1963) tested sera of rabbits which were collected 3 weeks after infection with strains of *T. evansi*, *T. gambiense* and *T. rhodesiense*, against antigens of each strain. He found that a strain of *T. gambiense* and a strain of *T. evansi* were antigenically related. It is possible that serum of an animal having long-standing infection, contains antibodies against variant antigenic types which have appeared in the infection. Sera of mice having chronic infection of Colombian strain (Tables 7 and 9) were tested against 2 other strains of *T. evansi* and 3 strains of *T. brucei*, in order to detect whether there was any antigenic relationship between these strains. The results shown in Table 42 suggest that there was no antigenic relationship.

Characterization of strains of *T. evansi* by cloning (Table 43)

An attempt was made to characterize 3 strains of *T. evansi* by cloning. Out of 16 attempts, 3 clone populations of N.S. strain developed and these were preserved as stabulates LUMP 59, 85 and 90. 7 clone populations, LUMP 106, 107, 108, 109, 110, 111 and 112, were prepared from the S.A.K. (dyskinetoplastic) strain, out of 20 cloning attempts. One more clone of this strain LUMP 55, stabulated by Dr. H. A. Miles of this Department, was available



for comparison. Only one clone population from the Colombian population developed out of 8 cloning attempts and this was stabilized as LUMP 95.

Antigenic relationship of clone populations (Table 43)

Each clone population was tested against the other with the use of the agglutination test. All 3 clone populations derived from the N.S. strain were of one antigenic type and did not react with any of the S.A.K. or the Colombian strain clones. Similarly, all 8 clones of the S.A.K. strain were identical but differed from the N.S. and the Colombian strain clones. Colombian strain clones did not react with either N.S. or S.A.K. strain clones. Therefore, not more than one antigenic type could be isolated from each strain. The similarity of all 3 clones of the N.S. strain and all 8 clones of the S.A.K. strain, suggests that these strains consist of one antigenic type only or that only one antigenic type of these strains is capable of being established from a single organism. Only one clone from the recently isolated Colombian strain was tested, and consequently no conclusion can be drawn.

Cross protection among clone populations derived from 2 different strains (Table 44)

The antigenic character of N.S. strain clones was found to be different, using the agglutination test, from that of the S.A.K. strain clones. Their relationship was also studied by a protection test, and for this purpose data from experiment 3 on the induction of pleomorphism in an N.S. clone (Table 25) was utilized.

Some degree of protection to the N.S. strain clone was induced in mice immune to the S.A.K. clone. In the immunized group, the prepatent period and survival time was prolonged when compared with that of the control group.

## DISCUSSION

### 1. Antigenic variation

#### 1. 1. Colombian strain

T. evansi appears to have a capacity for antigenic variation similar to that of other salivarian trypanosomes. In the present study, a recently isolated Colombian strain was found to exhibit antigenic variation during a relapsing infection in the mouse. The antigenic type of the first parasitaemic wave was identical to that of the original strain inoculated into the vertebrate host. A similar observation has been reported by Wilson (1968) in T. congolense infections in cattle.

#### 1. 1. 1. Mixed character of variant populations

Most of the variants isolated from different parasitaemic waves in the present experiment were mixed populations since they reacted with either the preceding or succeeding population. One reason for this may be that the parasitaemic waves in the mouse from which variant populations were isolated, were not very prominent. However in previous studies, where variant populations were isolated from distinct parasitaemic waves (Clarkson and Awan, 1969) or at periodic intervals (Gray, 1962), the variants were also found to be mixed populations. Hence so far no experimental system is available by which a pure variant population may be isolated.

If a relapse population is analysed by setting up clone populations it may be found to consist of several cell antigenic types (clones). McNeillage, Herbert and Lumsden (1969) have been able to isolate at least 3 cell antigenic types from a single population.

It is difficult to assess the proportion of a particular cell antigenic type within a given population. However, it is conceivable that at least one cell antigenic type constitutes the major portion of a variant population and that other cell antigenic types may be present in minor proportions. The cell antigenic type, which occupies the major portion of a population, agglutinates with the homologous antiserum in high titres whereas those cell antigenic types present in minor proportions are responsible for cross reactions.

Previous work has shown that sera of animals contain antibodies to variant populations which have already appeared but not against those populations which are yet to develop (Russell, 1936; Gray, 1962, 1965a). However, Clarkson and Awan (1969) studying antigenic variation of T. vivax populations by immune lysis showed that certain populations isolated after 40 days were also lysed at a low titre by serum taken before their isolation. Similarly, in the present experiment W7, W8 and W12 populations reacted with antisera to their preceding populations. It would appear that it is the mixed character of each population which causes these cross reactions.

#### 1. 1. 2. Recurrence of an antigenic type in the same infection

In the present experimental study, the antigenic type of the organisms in a mouse which had been inoculated with the Colombian strain, continued to change with the progression of the infection. 9 variant antigenic types were isolated from 12 parasitaemic waves. It is possible that if the infection had been allowed to continue, a greater number of variant antigenic types could have been isolated.

These experiments support the belief that antigenic variation of trypanosomes is an endless process (Gray, 1967). However, the present results indicate that a limit to the number of antigenic types is imposed when there is a decrease in the antibody levels against a particular variant within the host, followed by the reappearance of that variant in the same infection. An example of this is provided by variant antigenic type W1 which was found to recur after an interval of 3-4 weeks in a mouse. The recrudescence of a variant during the same infection in a mouse is not unlikely, because the agglutinin levels against an antigenic type in the mouse fall to low levels after an interval of about 3 weeks (Lumsden, 1969b); and a low agglutinin titre of 1/320 or less against a particular antigenic type fails to prevent the development of infection when that antigenic type is inoculated into mice (Watkins, 1964). Presumably the serum of an animal contains high titres of antibodies against different variants for only a short period after their appearance, but antibody levels against a variant reappearing in a host, may continue to remain high as long as antigenic variation occurs. However, the interval between reappearances of a variant in a host may vary from that in other host species because it may be influenced by the persistency of agglutinin titres in a particular host. For example, in the present experiment, there was an interval of 3-4 weeks in the mouse host in which high agglutinin titres are known to persist for this period. In rabbits, in which high agglutinating and neutralizing antibody titres persist for up to 8 weeks (Soltys, 1957a, b), the interval may be longer than this period.

### 1. 1. 3. Reversion to a former antigenic type

The most important aspect of antigenic variation known so far is the reversion of a strain to a former antigenic type. The concept of antigenic reversion is not new. The antigenic similarity of first relapses has been studied for a long time and first relapse populations obtained after infected animals have been treated by drugs or serum are of either one antigenic type or a limited number of types (Ehrlich, 1911; Leupold, 1928; Raffel, 1934). Those workers who think that there is little similarity in the antigenic type of first relapses admit that certain antigenic types tend to appear more than others in the first relapse (Lourie and O'Connor, 1937). McNeillage, Herbert and Lumsden (1969) found that trypanosomes in the first natural relapses were not always of the same antigenic type, but in the 4 populations which they analysed, ETat 2 and 3 were represented in more populations than ETat 4.

Further evidence of antigenic reversion is obtained when the populations are transmitted to a new host by syringe and reversion to a predominant antigenic type takes place (Gray, 1965b). By definition the term predominant antigenic type refers to certain variant populations which appear in the early stages of infection in different animals and are similar. It is considered that in one strain there may be several predominant antigenic types. Obviously, the antigenic types developed in the early stages of infection include those that are represented in the first relapse. Thus, the concept of predominant antigenic type and similarity of the antigenic type of the first relapse appear to be the same.

The importance of predominant antigenic types lies in the fact that they may be used as a basis for the serological classification of strains (Gray, 1969). This is based on two observations which have shown that the antigenic types of populations remain stable for at least 2 years (Gray, 1969) and that the sera of animals with long standing infections have antibodies to the predominant antigenic type.

In the present studies, attempts were made to determine which of the variant antigenic types of the Colombian strain had a tendency to recrudesce and against which of the antigenic types agglutinins were present in the sera of mice with long standing infections. Antigenic type W1 appeared in first natural relapses in those infections with 2 distinct variants. Agglutinins against W1 were present in the sera of mice with chronic infections of either the original Colombian strain or one of its variants. This antigenic type was also found to reappear in the same infection. Thus, antigenic type W1 was found to recrudesce within one host and also in different hosts. Hence it is appropriate to call it the 'recurrent antigenic type' because of its recurrent nature.

It is assumed that trypanosome strains have at least one recurrent type which may be responsible for the antigenic similarity of first natural relapses. The recurrent antigenic type falls within the definition of predominant antigenic type because of its appearance in the early stages of infection, but it is more specific than the latter. The recurrent antigenic type of a strain may be maintained in nature by virtue of its appearance in every new host which is infected. Antibody levels against the recurrent antigenic type may be maintained in one animal by its recrudescences. The stability

of both antigen-antibody systems suggests, therefore, that the recurrent antigenic type would be a more useful basis for serological classification than the predominant antigenic type.

In order to isolate the recurrent antigenic type from a wild population, that population could be inoculated into a number of laboratory animals and the first relapse populations occurring in these animals may, if similar, represent the recurrent antigenic type. This will occur only when the stem population is a variant, but if the stem population itself contains a recurrent antigenic type, it may be represented in the first wave population in laboratory animals. Hence, the first wave and first relapse in a number of laboratory animals may be compared. Sometimes due to the mixed character of populations either the first relapse populations may not appear similar by the agglutination test or there may be cross reactions with subsequent relapses. Thus a more rational approach would be to analyse the population of the recurrent antigenic type by the establishment of clones and isolation of the 'recurrent cell antigenic type', which would be an even more specific basis for serological classification than the recurrent antigenic type.

It has been shown in other species that there is reversion to a basic antigenic type after cyclical transmission by Glossina but it is impossible to demonstrate this in non-cyclically transmitted T. evansi. The present study shows that the antigenic type remained unaltered even when the transmission of an infection was effected by the vector after a delayed interval of 18 hours.



1. 1. 4. Immune response of mice to different variant antigenic types

There is considerable difference of opinion concerning the dissimilarity of antigenic types isolated from the early stage of an infection and those from later stages of infection. Soltys (1957a, b) had shown that when trypanosomes were passaged at 28-28 day intervals in rabbits, they became insensitive to neutralizing antibody and agglutination. Gray (1965b) tested the antisera of a rabbit, in which a clone was passaged at monthly intervals, for agglutinins. He found that trypanosome populations isolated from rabbits after 4 weeks of infection appeared to be less able to react with agglutinating antibody than trypanosomes isolated after one week. Miller (1965) studied the variation of soluble antigens of T. brucei in rabbits and also stated that the 'immunogenicity of antigenic components decreases as infection proceeds'. On the other hand, Clarkson and Awan (1969) found that in the sera of 2 sheep infected with T. vivax both early and late populations gave equally high titres. In the present study, a different system was used in which the immune response of clean mice to 3 variant antigenic types drawn from an early, middle and late stage of infection was compared. In mice made immune by infection and subsequent Berenil treatment, the agglutinin titres in homologous antiserum, collected on day 8 after treatment, were higher in the case of a variant antigenic type isolated from an early stage of an infection when compared with that of a variant antigenic type isolated from a late stage of infection (Table 6, Figure 7). These findings deserve further investigation employing a greater number of antigenic types.

The agglutinin titres in all the groups gradually fell from

day 6 to 30 after treatment. These findings are similar to those reported by Lumsden (1969b) who found that in mice immunized by treatment of infection, agglutinating antibody titres were highest from about day 6 to 10 after inoculation, and fell off 14-17 days after inoculation.

#### 1. 1. 5. Virulence of different variant populations in mice

The virulence of the original Colombian strain in mice was not uniform because 80% of a group of inoculated mice died of an acute infection during the first parasitaemic wave, but 20% of them showed relapsing infections before death occurred (Table 3). The strain appeared to have a mixture of different cell antigenic types of varying virulence (McNeillage and Herbert, 1968; McNeillage, Herbert and Lumsden, 1969). The variant antigenic types were not expected to exhibit uniform virulence for mice because they were mixed populations. But it seemed likely that the overall behaviour of a population is governed by the behaviour of the cell antigenic type which constitutes its major portion. This conclusion suggested the investigation of the virulence of different variant antigenic types. There was some difference between the behaviours of populations isolated from the first and from the 12th wave since mice inoculated with variant antigenic type W1 died in the first wave whereas those inoculated with W12 developed relapsing infections. The behaviour of other populations was variable. W1, which formed a major portion of W0 by virtue of its higher virulence, appeared in the first wave in mice inoculated with W0 and was responsible for the death of 80% of them in the first wave. Populations with identical antigenic types, e.g., W0, W1 and W8 had a similar virulence for mice.

### 1. 1. 6. Development of variants in different hosts

It has been shown previously that similar variant antigenic types develop in different hosts, and often in similar sequence during the early stages of an infection (Gray, 1965a). In the present study, variant antigenic types of the first and second wave populations from a mouse were also found to develop in the same order in 2 rats.

### 1. 2. Experimental systems used to induce antigenic variation in virulent strains

In the past, several experimental systems have been used to induce antigenic variation in strains which otherwise do not show relapsing parasitaemia. In the present work 3 methods were used to obtain relapsing parasitaemia.

(a) Subcurative treatment was used to obtain relapsing parasitaemia in animals infected with the dyskinetoplastic S.A.K. strain. The usual dose of 25 mg./kg. body weight which cured infections with the N.S. and the Colombian strains proved subcurative for this strain. This suggests that there is a difference in the sensitivity of the kinetoplastic and dyskinetoplastic strains to Berenil.

A mouse, during the first wave of parasitaemia, was treated with Berenil (25 mg./kg.). This eliminated the organisms from the circulation but they reappeared approximately 10 days later in the form of a second wave. The second wave was treated with the same dose of the drug but a third wave appeared, again after approximately 10 days. The occurrence of relapses at 10 day intervals after Berenil treatment suggests that the residual effect of this drug in the above dosage remains only for 10 days. However, Lumsden et al. (1965) have reported that with higher dosage levels of 45

mg./kg., and 91 mg./kg., an extended residual effect of this drug for 33 and 42 days respectively was observed in mice.

The drug is likely to remain in the circulation during the period of its residual effect. Hence the trypanosomes which produced a subsequent relapse would have hidden themselves in certain sites of the body which are cut off from the main blood circulation, and also therefore, the drug. When the drug was excreted, the trypanosomes reentered the circulation in the form of a relapse.

It is unlikely that in the present experiment these relapse populations acquired drug resistance, since the organisms in each relapse were sensitive to the same dose of the drug.

(b) Immunization against a heterologous strain (S.A.K.) or its clone was used to induce relapsing parasitaemia in the N.S. strain or its clone respectively. In one experiment (Table 25), the relapse populations of the N.S. clone were antigenic variants and consisted of pleomorphic long slender and short stumpy forms. The induction of antigenic variation together with concomitant development of pleomorphism in the N.S. strain in this experiment suggests that the two phenomena could be linked. Nevertheless in other experiments (Tables 22, 26) the occurrence of relapsing parasitaemia was not accompanied by development of pleomorphism. This observation together with the occurrence of antigenic variation in the monomorphic Colombian strain and N.S. strain (when infected mice were maintained at 35°C) suggests that the development of pleomorphism is not a necessary accompaniment to antigenic variation. Hence the two phenomena seem to be independent of each other.

(c) The third system which was used to obtain antigenic variation in the N.S. strain was the maintenance of infected mice at an

ambient temperature of 35°C. Osaki (1959) has presented some confusing results and claims to have obtained antigenic variation of T. gambiense in mice after exposing them to a high temperature of 45-50°C for 25 minutes; but it is unlikely that antigenic alteration can be brought about within such a short period. The present results have shown that the exposure of a host to higher temperature (35°C) causes a relapsing parasitaemia which is responsible for antigenic variation and suggest that heat itself does not have any direct bearing on antigenic change. It is suggested that the similar environmental temperatures of tropical countries, may have an identical effect on trypanosome infections in domestic animals and influence the course of parasitaemia and potentiality of a strain for antigenic variation.

The basic mechanism underlying the development of relapses (antigenic variants) either naturally in the Colombian strain, or artificially by drug, heat or immunity against a heterologous population in the S.A.K. and N.S. strains and N.S. clone respectively, appears to be the same. During suppression of parasitaemia, the majority of organisms in a parasitaemic wave are destroyed in the peripheral circulation. It is possible that in the naturally relapsing Colombian strain the elimination of parasites was caused by antibodies, whereas in experimental systems it was brought about by drug administration or the effect of heat. Subsequently, those organisms which escaped the action of these destroying factors might have reached certain unknown sites within the host which are cut off from the main blood circulation and there these organisms acquired an occult phase. During this phase they escaped the residual effect of the drug, heat or antibody. Although no attempts

were made to investigate the possibility of an occult phase in the present study its presence is not unlikely in view of the observations of Ormerod and Venkatesan (1971a). These authors have demonstrated that T. rhodesiense acquired an amastigote form during the occult phase and they have speculated its importance in bringing about antigenic variation (Ormerod and Venkatesan, 1971b). It is conceivable that a change in the morphology of the organism, i.e. from amastigote to trypanastigote, is accompanied by the alteration of its antigenic character. It is assumed that the organisms, after changing their antigenic character, are released into the circulation in the form of a subsequent wave.

The mechanism underlying the development of relapses is far from being perfectly understood and is a subject requiring further intensive investigation for which the present experimental systems would provide a useful basis.

### 1. 3. Antigenic variation of the N.S. strain and the S.A.K. strain

The experimentally-induced relapse populations of N.S. and S.A.K. strains were antigenic variants. The relapse populations of N.S. clone were pleomorphic besides being of a variant antigenic type and it appeared that both changes occurred simultaneously. But, since antigenic variation in the N.S. strain occurred without any changes in morphology, the two phenomena do not appear to be dependent upon each other.

### 1. 4. Phenomenon of antigenic variation

The phenomenon of antigenic variation, though imperfectly understood, is unlikely to be due to mutation, as proposed by

Watkins (1964), but appears to be the result of the adaptation of the trypanosome cell to certain extracellular stimuli present in the host (Gray, 1967). Whether the trypanosome changes its antigenic character as a result of the immune response of the host or whether antigenic variation is caused by the trypanosome cell itself, is yet to be discovered.

It is well established that following the appearance of a variant antigenic type, specific antibodies are produced by the host. Specific antibodies are able to kill the trypanosomes of corresponding antigenic type in vitro and the inoculation of specific antibodies can prevent the appearance of the corresponding variant antigenic type. Antigenic variation does not occur in an immunosuppressed host. These are some of the facts which indicate that it is the host's immune response which causes the trypanosome to alter its antigenic character. But, the main objection to this point of view comes from an observation concerning the time at which antibodies are produced. The time at which antibodies are detectable by agglutination and lysis test does not coincide with the time at which the parasitaemia is suppressed. Levaditi and McIntosh (1910) showed that sera collected the day after the suppression of parasitaemia contained considerable quantities of lytic antibody, but other authors (Russell, 1936; Gray, 1962; Clarkson and Awan, 1969) have shown that the titres of antibodies against a particular antigenic type, in serum collected at the time of its isolation, are very low and that they increase a week later. In view of the direct relationship between agglutinating and protective antibodies (Watkins, 1964), a lower titre of antibodies is not expected to bring about suppression of parasitaemia. Therefore, more evidence is required to support the hypothesis that the immune response of

the host causes antigenic variation.

The second possibility is that changes in the different organelles of the trypanosome cell cause the entire organism to change its antigenic type. This is based on the suggestion that variant antigens are liberated from the surface coat of the trypanosomes (Vickerman and Luckins, 1969) and that the cellular fractions of the trypanosomes are antigenic (Brown and Williamson, 1964). Recently Allsopp et al. (1971) have suggested that the exoantigen occurring in the serum of infected rats and the 4S antigens present in a trypanosome homogenate are identical and that they are a major component of the surface coat of blood stream trypanosomes. More recent studies have established the importance of an occult phase in the life cycle of African trypanosomes within the mammalian host (Ormerod and Venkatesan, 1971b). Further investigations in this direction may reveal the cause of antigenic variation.

#### 1. 5. Application of antigenic variation studies on serological classification of strains

Work on antigenic variation has led mainly to the possibility of a serological classification of trypanosomes. The basic antigenic type or recurrent antigenic type could provide the basis for serological classification. The selection of the recurrent antigenic type has many advantages over a basic antigenic type since the latter can only be used in cyclically transmitted trypanosomes whereas the recurrent antigenic type can be used in both the cyclically and non-cyclically transmitted organisms. Furthermore, the isolation of the basic antigenic type of a particular strain



would be tedious work involving the trapping of wild tsetse flies to be fed on individual laboratory animals. The common antigenic type amongst the transmitted populations would be considered the basic antigenic type. The isolation of recurrent antigenic types, on the other hand, would be relatively easy. But, unfortunately, tsetse flies sometimes transmit populations which are a mixture of basic and variant antigenic types (Gray, 1965a). However, the possibility of this also occurring in the recurrent antigenic type can not be ruled out. Perhaps the analysis of populations with a recurrent antigenic type by cloning, in order to isolate the recurrent cell antigenic type, would overcome this disadvantage. A serological classification on the basis of a predominant antigenic type has already been advocated by Gray (1969). He observed that the sera of animals with long standing infections contained antibodies to the predominant antigenic type. This is further supported by the present experiments in which a recurrent antigenic type kept reappearing in an infection and thereby maintained the antibody levels within the host. Secondly, he found that the antigenic type remains stable in nature. It is suggested that trypanosome populations can be classified on the basis of their recurrent antigenic type.

#### 1. 6. Immunization with variant antigenic types

Active immunity against W1 was induced by the administration of a single dose of formalinized whole infected blood (i/v) as shown by the subsequent resistance of immunized mice to homologous challenge. Partial protection was also obtained against challenge with 2 other heterologous variant antigenic types W3 and W12 which

had a lower virulence for mice than W1. No protection was obtained against heterologous challenge with another variant antigenic type W4 which was more virulent for mice than W3 and W12. These findings indicate that immunization with a variant antigenic type of high virulence could render partial protection against a variant with a lower virulence. In the present experiment the effect of challenge with different antigenic types on mice immunized with the recurrent antigenic type was observed. Cunningham (1970) used a reverse system in which he immunized mice with a series of variant antigenic types of T. brucei and challenged them with the parent antigenic type, but no protection was observed.

#### 1. 7. Passive transfer of immunity

In earlier studies on passive immunity obtained by the transfer of immune serum, only partial protection evidenced by either increased survival time in T. rhodesiense (Seed and Gam, 1966) or increased prepatent period in T. vivax (Clarkson and Awan, 1969) has been reported. However, the present study shows that complete protection can be obtained against W1 by transfer of immune serum (Table 15, Figure 12).

#### 2. Characterization by cloning

In experiments on the cloning of 3 strains of T. evansi, it was observed that clone populations were more readily established from old laboratory strains than from a recently isolated strain. This is probably due to the adaptation of the old strains to mice and their increased virulence for these animals.

### 3. Morphology and pleomorphism

Although T. evansi in the blood usually consists of both slender and intermediate trypomastigotes, inconstant pleomorphism is regarded as an essential feature of the species. The experimental induction of pleomorphism in the N.S. strain of T. evansi confirms that the occasional occurrence of stumpy forms in this species is beyond doubt. Morphologically the slender, intermediate and stumpy forms of an experimentally-developed N.S. pleomorphic variant were indistinguishable from the corresponding forms of T. brucei.

Type II granules have been previously demonstrated in stumpy trypomastigotes only (Molloy and Ormerod, 1965). But, in the present experiment these granules were observed in long intermediate forms of a virulent N.S. strain of T. evansi, when the course of infection was prolonged by maintaining the mice at 35°C. The presence of type II granules may not be a characteristic of the stumpy trypomastigotes only but they do seem to appear in the later stages of an individual trypanosome's life span. This was indicated in the present study by the presence of these granules in trypomastigotes at the peak and in the descending phase of a parasitaemic wave.

In one of the experiments on induction of pleomorphism in the N.S. strain, trypomastigotes with varying sizes of vacuoles were seen in the descending stages of parasitaemia, shortly before the disappearance of trypanosomes from the circulation. Similar vacuolated forms have been reported in tissue cultures of T. cruzi (Miles, 1971) and in chicken embryo cultures of T. brucei (Otieno and Sargeant, 1971). Wenyon (1926) regards trypanosomes with vacuolated cytoplasm to be abnormal or degenerating individuals. Ormerod and Venkatesan (1971a) have recorded 'unrolling' forms with

a central vacuole together with anastigotes, sphaeromastigotes and small trypomastigotes in smears of the choroid plexus and they think that the anastigote forms are converted into trypomastigotes. These authors are of the opinion that in the 'unrolling' forms the unrolling appears to take place from the central vacuole. However, in the present studies, such vacuolated trypomastigotes were found in the terminal stages of a parasitaemic wave, and this observation could offer an explanation of their disappearance from the peripheral circulation. It is possible that in the vacuolated forms, the undulating membrane wraps around the circumference of the vacuole and is unable to induce flagellar undulations with the result that they cannot move against the flow of the blood. Hence they would be forced by the blood current into the capillaries of internal organs.

The system of inducing pleomorphism in the N.S. strain provided an opportunity for the study of certain other aspects of pleomorphism. The phenomenon of pleomorphism in T. brucei, T. rhodesiense and T. gambiense is far from being completely understood, but several hypotheses have been put forward. Wijers (1959) thinks that the transformation of slender trypomastigote forms to stumpy trypomastigotes, is a result of the adaptation of the trypanosome to the changed or changing environment within the host body. Antibody response of the host might be one of the contributory factors in changing this environment, and the preponderance of stumpy forms at the descending stage of parasitaemia has led to this point of view.

In the present study, the relationship between the state of immunity of the host and the development of stumpy forms was studied

in experiments on the conversion of slender, monomorphic forms to stumpy forms and by observing the behaviour of an experimentally developed N.S. pleomorphic variant in normal, splenectomized and irradiated mice.

In experiments on the induction of pleomorphism, the host was immune against a heterologous strain. In each experiment, the immune status of the host was verified by giving a challenge dose, and yet when the N.S. strain or clone was inoculated, uniform results were not obtained. In some mice, the behaviour of the N.S. strain was similar to that in control mice resulting in an acute infection and death; in others only relapsing parasitaemia occurred without any evidence of morphological change and yet in others, relapses were accompanied by the development of pleomorphism. In certain mice, the state of immunity against the S.A.K. strain was so high that they resisted the development of the N.S. strain. Therefore, the state of immunity did not appear to have a direct influence on the development of pleomorphism but some other change in the environment does seem to have caused its occurrence. However, this aspect should be further investigated since this system provides an opportunity for the future study of biochemical, physiological and morphological aspects of pleomorphism.

In experiments on the behaviour of pleomorphic variant in normal mice, the population consisted of predominantly stumpy forms when the level of parasitaemia was descending. Identical results have been reported by earlier workers (cited by Wijers, 1959). In splenectomized and irradiated mice, the proportion of stumpy forms was much higher than that in control group during the later stages of infection. The state of immunity in these mice was not estimated. However, recently Luckins (1972) has reported that irradiated rats

failed to produce agglutinins against T. brucei infection and that in such rats the percentage of stumpy forms was similar to that in control rats. Therefore, the occurrence of pleomorphism does not appear to have a direct relationship to the antibody response of the host.

The first view, concerning changes in the structure of a trypanosome as it undergoes pleomorphism, was put forward by Fairbairn and Culwick (1946) who thought that stumpy forms are produced as a result of syngamy. But Ashcroft (1957) and Wijers (1959) contradicted this idea, and the latter author demonstrated in vitro that stumpy forms were produced by a gradual shrinkage of slender forms through the intermediate stages. Vickerman (1965) thinks that the change from slender to stumpy forms involves the proliferation of the mitochondrion and the development of cristae which causes the trypanosome to preadapt to a metabolic pattern adapted to life in the vector host.

In the present study, the original N.S. strain was composed of slender forms with a short, free flagellum and long intermediate forms. These forms transformed both to slender forms with a long free flagellum and to intermediate, short intermediate and stumpy forms. On the basis of observations of the morphological changes in stained preparations it is assumed that a backward followed by a forward migration of the kinetoplast occurs during the transition from slender to stumpy forms. However, studies on the dyskinetoplastic strain of T. evansi have not revealed the exact role of the kinetoplast in the induction of pleomorphism. A single ultrastructural study has been carried out by Miles (1970) who demonstrated expanded mitochondrion in the stumpy forms of this strain,

but the cytochemical and enzyme activity studies by the same author did not produce any evidence of mitochondrial activity. On the basis of the demonstration of an expanded mitochondrion, he thinks that the synthesis of mitochondrial membrane is independent of kinetoplast function. However, a further investigation is required to explore the possibility of mitochondrial activity in the stumpy forms of dyskinetoplastic strains which may reveal the true role of the kinetoplast in the transformation of slender to stumpy forms.

#### 4. Culture

Culture of the 3 monomorphic strains and of a pleomorphic variant of T. evansi was carried out. Failure of the monomorphic strains to grow on the one hand and successful cultivation of the pleomorphic variant on the other, confirm the earlier belief that it is the stumpy trypanastigotes from the blood which grow in culture. Although the monomorphic strains did not multiply, their survival in 4N medium was for a longer period than that recorded by Balis (1963) for T. evansi in a diphasic medium containing defibrinated horse blood.

The survival of the monomorphic strains in medium 199 containing calf serum, for a longer period than those in 4N medium was probably due to the richness of nutrients in medium 199. The failure of the trypanosomes to grow in a monolayer of macrophages or HeLa cells suggest that this trypanosome species does not have a tissue phase.

The pleomorphic variant populations of N.S. strain were cultivated in 4N medium and their morphology and development was similar to that of other organisms of the subgenus Trypanozoon. Lehmann

(1961b, 1964) using a variety of diphasic blood-agar media, e.g., NNN medium, SN B-9 medium, Tobie's hypotonic medium (TH), and NA-B medium to which citrated non-inactivated rabbit blood was added, could obtain only poor growth of T. brucei in the initial culture. He was unable to obtain subcultures in any of these media except for a single subculture in NA-B medium. But in the present study N.S. pleomorphic variant: grew well in 4N medium, which also contains rabbit blood. Cultures could be maintained for up to 4 passages and up to 26 days in the initial culture. The first appearance of midgut forms in culture was after 2 days which was similar to the observations of Lehmann (1964b). The morphological change from blood to culture form was possibly associated with a backward followed by a forward migration of the kinetoplast. A similar movement of the kinetoplast probably initiated the conversion of slender trypanosomes to stumpy trypanosomes wherein the expansion of the mitochondrion causes the blood form to preadapt to the metabolism required in the vector (Vickerman, 1965). A further movement of the kinetoplast during the process of the conversion to culture forms is probably responsible for the switch over from one metabolic pattern to another.

Lehmann (1961a) observed forms indistinguishable from metacyclic salivary gland forms, in T. rhodesiense cultures which were grown in different media containing Glossina extract, but these forms failed to infect mice. In the present study, although the morphology of culture forms was observed daily from the day of inoculation until the 12th day when the flagellates died, only trypanosomes were seen and they failed to infect mice.



## 5. Transmission

Transmission of monomorphic and pleomorphic strains of T. evansi was attempted employing S. calcitrans for monomorphic strains, G. morsitans for pleomorphic variant and the soft ticks O. moubata for each of these strains.

The experimental results of the present study showed that the Colombian strain of T. evansi survived in the body of S. calcitrans up to 18 hours after ingestion of an infected feed, and was subsequently infective to the vertebrate host. These findings are in agreement with the observations of Mitzmain (1912), Nieschulz (1940) and Falana (1970) who found that T. evansi survived in the gut of Stomoxys for up to 24 hours. These results suggest that delayed non-cyclical transmission of T. evansi by Stomoxys could be possible in nature. Delayed non-cyclical transmission by Stomoxys has been demonstrated for up to 72 hours after the ingestion of an infective feed (Bout and Rouband, 1912). Therefore, it is suggested that in the light of the ability of these trypanosomes to survive in the body of the fly for up to 24 hours and of the Stomoxys to feed daily, this fly could be a potential disseminator of trypanosomiasis in the field.

In the present experiments, T. evansi infections of monomorphic or pleomorphic strains, could not be transmitted by the soft tick O. moubata. The failure of transmission is possibly due to either the small number of ticks employed or to the species of Ornithodoros which was not the one by which transmission had been demonstrated by Cross and his associates (Cross and Patel, 1921; Cross, 1923; Singh, 1925). It is suggested that transmission experiments using a large number of O. tholozani should be carried out in the field.

The most interesting observation in the present transmission experiments was the cyclical development of M.S. pleomorphic variant of T. evansi in G. morsitans. The development was similar to that observed in other species of the subgenus Trypanozoon (Bruce et al., 1914b; Robertson, 1912, 1913). In dissections of tsetse flies, mostly 'ribbon like' proventricular trypanastigotes were recovered from the midgut. This was probably due to the fact that the flies were always dissected at an interval of about 2 weeks or more after ingesting infective feeds. By that time, proventricular forms would have developed. Secondly all the flies were starved for a day before dissection as a result of which proventricular forms would have been pushed back in the gut as has already been speculated by Robertson (1913). In 2 flies, the proventricular form of trypanastigote was also found in the salivary glands on days 11 and 14 after the infective feed. This suggests that from day 11 onwards the proventricular trypanastigotes enter the salivary glands. In all probing attempts from days 11 to 52 after the infective feed(s), only proventricular forms were extruded in the probes. This was observed in flies of those groups which transmitted the infection to healthy mice. The metatrypanosomes are believed to be the forms infective to the mammalian host, and yet they were not detected in the present experiments either in probes or in the salivary glands of those groups of flies which transmitted the infection to mice. On one occasion a few flagellates, which appeared to be of similar morphology to metatrypanosomes, were seen by phase contrast microscopy only. Similar findings have been recorded by Ward and Bell (1971) who observed that when flies from cages containing known infected flies were individually fed on normal mice, the transmission rate was 5 times higher than that

revealed by salivary gland dissections. They postulated that the proventricular trypomastigotes were infective to the vertebrate host. Although Robertson (1912) believes that proventricular trypomastigotes are not infective to the mammalian host, the problem deserves further investigation.

The failure of a population from LUMP 315 to develop in Glossina, was mainly due to the fact that insufficient stumpy forms were present in the blood of mice when the flies were fed. However, this population could be cultivated in 4N medium and the success of cultivation also depended upon the presence of stumpy forms which infect the fly and not the slender forms. However, Mshelbwala (1968), in one experiment, obtained cyclical development in Glossina which ingested a strain containing only slender trypomastigotes. This question should be investigated further to find out whether slender, stumpy or any other morphological form between the two, i.e. long intermediate, intermediate, or short intermediate is infective to fly. This could be done by feeding flies on populations containing predominantly one of the morphological types and comparing the subsequent infectivity to the flies.

## 6. Taxonomy

The morphological characters and the mode of transmission are the factors which separate T. brucei from T. evansi and thus influence the taxonomic position of the latter.

It is already known that a pleomorphic strain of T. brucei is rendered monomorphic by successive syringe passages in laboratory rats and mice (Duke, 1934, 1935). The present experimental findings suggest that in the laboratory a monomorphic strain of T. evansi can

be converted to a pleomorphic strain which is morphologically indistinguishable from T. brucei. Hence the morphological characters of laboratory strains can be altered experimentally; and T. brucei could be converted to T. evansi and vice versa.

In the natural host, T. brucei may be identified morphologically by the presence of stumpy forms but their proportion may fluctuate considerably either in the ascending or descending phase of a parasitaemic wave. A trypanosome population isolated from a T. brucei infected animal at the stage of rising parasitaemia in a parasitaemic wave, may contain only small numbers of stumpy forms and may be considered as T. evansi. On the other hand, T. evansi is either monomorphic slender or contains only small proportions of stumpy forms in its natural hosts, but in certain species of hosts, e.g., buffaloes, it may have a very high percentage of stumpy forms and could be confused with T. brucei. Thus it appears that the morphological characteristics of strains of each of these two species are unstable in both the laboratory and their natural hosts, and do not allow a distinct differentiation.

In the present study, the pleomorphic T. evansi was found to have a cyclical development in Glossina similar to that of T. brucei. The ability of T. evansi and T. brucei to develop in Glossina is similar since monomorphic strains of both species do not develop in this fly, whereas the pleomorphic strains undergo cyclical development.

In nature, the common method of transmission of T. evansi is believed to be non-cyclical by means of Stomoxys, Tabanus and other haematophagous flies, since cyclical development has not been demonstrated. On the other hand, the common method of transmission of T. brucei in nature may be both cyclical by Glossina or non-cyclical by Stomoxys, tabanids or Glossina. The non-cyclical

transmission of T. brucei, T. gambiense and T. rhodesiense has been produced in laboratory mice, rats and monkeys by Stomoxys, tabanids and Glossina on the lines similar to that of T. evansi (authors cited by Duke, 1934). Hence the differentiation of these two species on the basis of their mode of transmission and ability to develop within Glossina is not strictly valid. Therefore, the pursuit of a more detailed study of the morphology and transmission of T. evansi may further throw light on its systematic position. Further studies may reveal that these characteristics of the two species are identical, and may lead to the synonymisation of T. evansi with T. brucei.

## 7. Phylogeny

Our present knowledge concerning the evolution of T. evansi from T. brucei is based on the experimental evidence that a continuous syringe passage of T. brucei in laboratory mice and rats causes the adoption of monomorphism and such forms are incapable of developing in Glossina (Hoare, 1940). But the reverse phenomenon i.e. conversion of monomorphic slender forms to pleomorphic slender and stumpy forms was demonstrated for the first time in the present work, and it has not previously been discussed in relation to the evolution of T. evansi. These results form the basis of the present hypothesis which suggests that T. evansi is of older evolutionary origin when compared with other salivarian trypanosomes.

### 7. 1. Conversion from pleomorphic to monomorphic populations

A comparison cannot be made between syringe passaging of trypanosome strains in laboratory animals vis-a-vis

continuous maintenance of T. evansi in nature by means of non-cyclical transmission. Mostly mice and rats have been employed as laboratory animals in which after prolonged passage the pleomorphic trypanosomes not only become monomorphic, but with the course of time and with the degree of adaptation their virulence to these animals is also increased. The serial passage of the strains in rats and mice is probably not comparable with that situation in which trypanosomes are maintained in nature by non-cyclical transmission. It has been observed that in nature, with the course of time, the virulence of trypanosomes in domestic animals remains unchanged.

Furthermore, the change in morphology appears to differ in various animals which have been used for passaging. For example, in a pleomorphic strain of T. evansi, stumpy forms diminished when initial passages were made in rats (Fiori, Delanoë and Delanoë, 1915) whereas they remained in similar proportions or even increased when passaged through dogs and rabbit or rabbit only. A pleomorphic strain of T. brucei became monomorphic after syringe passages in rats, but when this converted strain was back-passaged through goats, it regained its pleomorphic character (Duke, 1934). The outcome of these two situations, viz., passaging in rats and mice, and non-cyclical transmission through natural hosts, does not appear to be the same. Therefore, the concept that T. evansi has evolved from T. brucei as a result of continuous passage in nature appears to be doubtful.

#### 7. 2. Conversion from monomorphic to pleomorphic population

Present experimental findings suggest that monomorphism could acquire pleomorphism by the unfavourable circumstances created in

a host as the result of an immunological response to the trypanosome parasite. In nature, the partial state of immunity in a host may be established either as a result of natural recovery from an infection or after treatment by chemotherapeutic agents. During the course of infection in an animal in which relapsing parasitaemia occurs, immunity is produced against the antigenic variants which have already appeared in the infection and are suppressed by the host. Hosts with partial immunity are constantly exposed to infection and are liable to be infected either with other strains of the same species or by other variants of the same strain. In such partially immune hosts a situation akin to that found in the present experiment may occur. Therefore, reversion of monomorphism to pleomorphism in nature is not unlikely. Probably this is the explanation of why we get occasionally pleomorphic forms in T. evansi, and they are probably not a sign of atavistic traits as thought earlier by Hoare (1956). This possibility supports the concept that T. brucei originated from T. evansi.

It is possible for a pleomorphic strain which has been developed from a monomorphic strain to develop in the tsetse fly and in the present studies it has been clearly shown that N.S. pleomorphic variants develop in G. morsitans.

### 7. 3. Evidence regarding antiquity of T. evansi

Evidence for the older evolutionary origin of T. evansi when compared with that of other salivarian trypanosomes is provided by the evolutionary trends of the morphology of trypanosomes and by the pattern of development in the vector host.

7. 3. 1. Evidence derived from evolutionary trends of the morphology of trypanosomes

The evidence for the ancient evolutionary origin of T. evansi on the basis of morphology could be derived from the concept of Lavier (1943), who suggested that the phylogenic development of trypanosomes is correlated with the evolution of their vertebrate hosts. He thought that the most ancient trypanosomes were those found in fish, which were long and slender with a well developed undulating membrane. Later, in some amphibians and reptiles, they became broader and subsequently their development underwent regression and they assumed a more uniform appearance in birds and mammals. If one relates the morphological pattern of salivarian trypanosomes to this evolutionary trend, the long slender forms of T. evansi with a prominent undulating membrane should be considered the oldest, followed by the broader forms of T. brucei, the variable sized forms of T. congolense and T. simae and more recently, the more uniform types of T. vivax.

7. 3. 2. Evidence from stages of developmental patterns of salivarian trypanosomes in their invertebrate vector  
(Figure 28)

The evolution of T. evansi involves the concept of the origin of salivarian trypanosomes, and an attempt has been made to review the present concept critically. According to Hoare (1967), salivarian trypanosomes owe their origin to parasites of the wild ruminants which were transmitted by certain insects in which their development was completed in the posterior station. They were simultaneously transmitted, non-cyclically, by Glossina in which they gradually became adapted for cyclical transmission. The various stages of gradual adaptation are evident in the pattern of development



of the various species in the proboscis, gut and proboscis, and gut, proboscis and salivary glands respectively. The degree of adaptation of the representative species T. vivax, T. congolense and T. brucei to the tsetse fly, is demonstrated by the diminishing infection rate of the fly, which is approximately 20%, 10% and 1% respectively.

The same author further believes that the clue to the transition of the stercorarian to salivarian pattern is provided by T. rangeli in which both are evident i.e. development in the hind gut (posterior station) as well as in the salivary glands (anterior station). If this supposition is correct then the pattern of development in the tsetse fly would have been in the following stages; midgut and salivary glands, midgut and proboscis, and only proboscis. This pattern is exactly the opposite of that conceived by Hoare (1967).

Therefore, the oldest species is probably T. evansi, which in unfavourable circumstances became pleomorphic. In the tsetse areas, it initially adapted to development in the gut and salivary glands. Gradually, the degree of adaptation increased and the trypanosomes avoided the tortuous route through the salivary glands and their development became restricted to the gut and proboscis. This was subsequently followed by confinement to the proboscis only. The infectivity of the representative species T. brucei, T. congolense and T. vivax to the tsetse fly appears to be governed by the degree of adaptation and is 1%, 10% and 20%. In this context, it is conceivable that the perfection of the adaptation between trypanosomes and vector is represented by a simple course of development as is evident in the case of stercorarian trypanosomes in which the developing forms are confined to the midgut and hind gut. Therefore, the stage of development in the salivary glands does not appear to

be the terminal stage of adaptation in Glossina, but it is an intermediate stage through which the stercorarian pattern of development evolved to the salivarian pattern. Therefore, T. evansi appears to be the oldest of all the salivarian trypanosomes. The evolutionary trend of the developmental pattern of salivarian trypanosomes can be traced from that in the gut, salivary gland and proboscis, to the gut and proboscis, and finally the proboscis only.

### 8. Effect of temperature

In mice maintained at 35°C there was a remarkable difference in the behaviour of the trypanosomes compared with that of the controls. The failure of the S.A.K. (dyskinetoplastic) strain to develop an infection suggests that the capability of multiplying at higher temperature may be governed by the kinetoplast.

In the old laboratory N.S. strain which killed mice in the first wave, relapsing parasitaemia was observed. Similar findings have been reported by Otieno (1972a) in an old laboratory strain of T. brucei. In the recently isolated Colombian strain which normally developed relapsing parasitaemia, maintenance at 35°C resulted in the elimination of the infection and self cure. Maintenance of mice at 35°C has been found to cure infections of other organisms such as T. cruzi (Amrein, 1967) and cutaneous leishmania diffusa (Hayatee, 1969).

The suppression of parasitaemia at 35°C could be attributed either to an increasingly effective defensive mechanism of the host, reduced virulence of the parasite or to physiological and biochemical changes within the host which would create a less suitable medium for in vivo multiplication of the trypanosomes.

An increase in the defence mechanism of the host, can be brought about either by increased humoral immunity or enhanced cellular response. In the light of evidence from the work of Elligson and Clark (1942), an increase in humoral immunity is less likely. He found that in rabbits exposed to  $40^{\circ}\text{C}$  and immunized with sheep red blood cells, haemolysin titres were similar to those in the controls. In those maintained at  $41.5^{\circ}\text{C}$  and also immunized with either sheep red blood cells, typhoid vaccine or egg albumin, the specific agglutinin and haemolysin titres were impaired in varying degrees. Ipsen (1952) reported an increased protective immunity in mice maintained at  $35^{\circ}\text{C}$  following immunization against tetanus when compared with that in mice maintained at  $25^{\circ}\text{C}$ , but his results indicate that the differences in the experimental and control groups were not significant.

The enhancement of cellular defensive mechanisms at higher temperatures is a possibility. This is indicated by the increased phagocytic activity of leucocytes at higher temperatures in vitro (Elligson and Clark, 1942; Harmon et al., 1946) and by increased activity of the reticuloendothelial cells of bone marrow (Huggins and Noonan, 1936).

The effect of higher temperature on the virulence of trypanosomes has been discussed by Marinkele and Rodriguez (1968) and Otieno (1972b). The former has suggested that by maintenance at  $36^{\circ} \pm 1^{\circ}\text{C}$ , the rectal temperature of mice is increased by  $1.3^{\circ}\text{C}$ , which is insufficient to influence the virulence of T. cruzi. The latter has suggested that the enhanced resistance to trypanosome infection is due to direct effect of temperature on trypanosomes.

It is not known what biochemical and physiological changes occur in the host under the influence of  $35^{\circ}\text{C}$  or what prevents the

in vivo multiplication of trypanosomes. However, in vitro, temperature does affect the multiplication of trypomastigote forms. Optimum growth of salivarian trypanosomes occurs at 28°C and they do not grow at 37°C (see Taylor and Baker, 1968). The possible reason for the arrest of multiplication of protozoan parasites in cultures kept at higher temperatures may be derived from the findings of Kasturi Bai et al. (1968). These authors found that in cultures of Blepharisma intermedium maintained at 38°C, there was a decrease in glycogen reserves, protein and lipids when compared with those of the controls (28°C). These factors, together with a reduction in enzyme activity, change in the pattern of free amino acids and the alteration of metabolism, blocked the cell division of the protozoan and arrested its growth at 38°C. The occurrence of identical changes in vivo is not unlikely.

In the present experiment, a nutritional deficiency was evident in mice maintained at 35°C, since they apparently did not increase in size. A combined effect of temperature and nutritional deficiency may have arrested the growth of trypanosomes.

Since the behaviour of different strains at higher temperatures was varied the capacity for development would seem to be due to innate properties of the trypanosomes. The suppression of parasitaemia at higher temperatures may explain the low summer incidence of surra in India, when compared with that in the winter or rainy season (Basu, 1945). The effect of higher temperatures needs to be further investigated in the natural hosts of T. evansi.

It appears that the behaviour of various species of trypanosome differs at lower temperatures. Amrein (1967) reported that severe parasitaemia and death resulted from an acute infection in mice

which were inoculated with T. cruzi culture forms and maintained at 10°C, but that a chronic infection occurred in the control group (26°C). On the other hand, in the present experiment, the level of parasitaemia of the Colombian strain of T. evansi was reduced in mice maintained at 4°C.

It is difficult to ascribe the change in the behaviour of trypanosomes at different environmental temperatures to a particular factor. It is evident that only a detailed study of all the aspects can reveal the true cause of these changes.

SUMMARY

1. The N.S. and S.A.K. strains of T. evansi are old laboratory strains and produce acute infections in mice. The prepatent period and the survival time of the N.S. strain in mice were inversely proportional to the inoculation dose. The Colombian strain was isolated recently and an inoculum of antilog 3.65 organisms caused an acute infection followed by death in most of the mice, but in others relapsing infections occurred prior to death.
2. The infectivity and virulence of these strains was markedly reduced in mice maintained at an ambient temperature of 35°C when compared with that at room temperature (22-28°C). The N.S. strain failed to infect all the mice and in those that developed infection the survival time was prolonged; in some of these the parasitaemia was suppressed and a relapsing infection resulted. The S.A.K. (dyskinetoplastic) strain failed to infect any of the inoculated mice. The Colombian strain failed to infect all the inoculated mice; in most of those which developed an infection the initial wave was suppressed and trypanosomes were eliminated from the circulation. This resulted in a self cure.

The behaviour of the Colombian strain was not affected by the maintenance of mice at 4°C or 28°C and it was similar to that at room temperature.

3. The present experiments have shown that antigenic variation of T. evansi can occur naturally in the mouse host. The Colombian strain of this parasite produced 12 successive parasitaemic wave populations (W1-W12) in a mouse, and 9

variant antigenic types were represented. The antigenic type of the first wave was identical to that of the original strain which had been used to induce an infection. Most of the populations, especially those in the later stages of the infection, were mixed.

The variant antigenic type W1 was found to recur in a single infection, and the agglutination test revealed that the populations W7, W8 and W9 were mixed with W1. The similarity of W8 to W1 was confirmed by the protection test.

Agglutination and protection tests revealed that it was also represented in the first natural relapses of infections with W5 and W12. Agglutinins to both variant antigenic type W1 and W2 were present in the sera of mice which had chronic infections of either the original strain or one of its variants.

4. In view of the recurrent nature of W1 in a single infection and in first natural relapses of infections with variants W5 and W12, it has been decided to introduce the term 'recurrent antigenic type'. It is suggested that the recurrent antigenic type, which is one of the predominant antigenic types, would be more specific than the latter as reference material for serological classification of trypanosome strains.
5. The variant antigenic types of the Colombian strain are transmitted as such to a new host after non-cyclical transmission and do not revert to the basic antigenic type.
6. There was a difference in virulence of variant antigenic types. W1 was more virulent than W3, W4 and W12. When antilog 3.7 trypanosomes were inoculated, W1 killed mice in the first wave, but mice inoculated with W3 and W4 developed both acute and

relapsing parasitaemias, while mice inoculated with W12 developed only relapsing parasitaemia. W4 was more virulent than W3 and W12.

7. In mice, immunity to W1 gave some protection against W3 and W12 but none was observed against W4. The administration of formalinized, whole infected blood (i/v) induced active immunization and passive immunization was achieved by transfer of immune serum (i/v).
8. A comparison was made of the immune response of clean mice to 3 variant antigenic types drawn from the early, middle and late stages of infection. The mean agglutinin titre of the variant antigenic type isolated from the early stage of an infection was higher when compared with those against the variant antigenic type which had been isolated from the late stage of an infection.
9. Antigenic types of first and second relapse populations in a mouse were similar to those of corresponding populations in a different host, the rat.
10. An antigenic relationship between the Colombian strain and either the N.S. or S.A.K. strains of T. evansi or Lugala-1, S-10, and S-102 strains of T. brucei, could not be demonstrated by agglutination test.
11. 3 experimental systems were used to induce antigenic variation in virulent strains. Relapses of the S.A.K. strain were obtained by subcurative treatment, and those of the N.S. strain were induced by the maintenance of mice at 35°C. Variants of the N.S. clone were obtained by inoculating it into mice made immune to the S.A.K. clone. 4 variant antigenic types were isolated from a mouse infected with the N.S. strain and 2 were isolated from relapse populations of the S.A.K. strain.



12. An attempt was made to characterize 3 strains of T. evansi by cloning. 3 clones of the N.S. strain, 9 clones of the S.A.K. strain and 1 clone of the Colombian strain were established. It was easier to obtain clone populations from the old laboratory strains than from the recently isolated strain. Clone populations derived from one strain were antigenically similar but those of different strains were dissimilar when compared by agglutination test. Immunity against the S.A.K. clone rendered some protection against the N.S. clone.
13. Pleomorphism in the monomorphic N.S. strain was induced by inoculating it into mice already immune to the S.A.K. strain and the morphology of pleomorphic forms was similar to that of T. brucei. It is assumed that the change in morphology from slender to stumpy trypanastigotes occurred by the backward migration of the kinetoplast. The pleomorphic forms were frozen as a stabilate and when the stabilate population was subsequently inoculated into mice, the trypanosomes which developed were also pleomorphic.

When this pleomorphic population was inoculated into clean mice, the proportion of stumpy forms was highest at the descending stage of parasitaemia. If the population was inoculated into splenectomized or irradiated mice, stumpy forms in proportions larger than the control, were observed. These findings are discussed in relation to pleomorphism and antibody response.

14. Trypanosomes with vacuoles were observed during an experiment on the induction of pleomorphism. The possible fate of such vacuolated trypanastigotes within the vertebrate host is discussed.

15. Monomorphic strains of T. evansi could not be cultivated in 4N medium. The Colombian and N.S. strains survived in this medium up to 2 days and the N.S. strain up to 6 days. In medium 199, the Colombian and S.A.K. strains survived for a comparatively longer period of 5 and 4 days respectively. In the liquid phase of a macrophage cell culture, the Colombian strain survived for 2 days but no intracellular stages were observed.
16. The pleomorphic variant of the N.S. strain was grown in 4N medium, and populations derived from either mouse blood or a stabulate were cultivated for up to 4 passages. The average survival of these cultures was 9 to 12 days, but in one sub-culture, survival for 26 days was observed. The growth was marked by one or two peaks which followed an initial lag phase. The parasites transformed from blood to culture trypanostigotes and it is assumed that this was brought about by a backward migration of the kinetoplast. Reproduction of culture trypanostigotes occurred by equal binary fission or by multiple fission. Culture forms failed to infect mice.

Pleomorphic variant of this strain could not be cultivated in HeLa or macrophage cell cultures.

17. This work has shown that cyclical transmission of the pleomorphic variant of the N.S. strain can occur in G. morsitans. The infection was transmitted to clean mice at a minimum interval of 9 days after an infective blood meal. Midgut and proventricular trypanostigotes were recovered from the midgut. Proventricular trypanostigotes were also found in the proventriculus, salivary glands and probes.

18. The monomorphic Colombian strain was able to survive for 18 hours in the body of S. calcitrans and retained its virulence for mice. The soft tick O. moubata was unable to transmit an infection of either of the monomorphic strains or the pleomorphic variants of the N.S. strain.
19. The morphology, cultural behaviour and cyclical development of the N.S. pleomorphic variant and T. brucei are similar and the systematic position of T. evansi is discussed in relation to these aspects.
20. The current hypothesis on the phylogeny of T. evansi suggests that this species originated from T. brucei. However, it can be argued that T. evansi is the ancestral form from which T. brucei has evolved and a discussion of this hypothesis is presented.

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Table 1

The occurrence of stumpy forms in different strains of T. evansi.

Strain	Host(s)	Country	No. of organisms examined	No. of stumpy forms	Author
-	camel, horse, elephant - dog, guinea-pig & rat		820	few	Bruce (1911)
-	camel	Sudan	-	rarely present	Fry (1911)
-	camel	Sudan	-	rarely present	Balfour (1914)
-	horse	Morocco	-	7%	) Fiori et al. (1915)
-	rat (at first passage, isolated from horse)	Morocco	-	lesser than that of above )	
-	rabbit at 3rd passage (isolated from horse)	Morocco	61	34 (55.7%)	Fiori et al. (1916)
-	rabbit at 2nd passage (isolated from horse)	Morocco	-	present	Fiori et al. (1916)
-	camel		-	present	Leckie (1925)
-	-	Mauritius	-	.03 to .05%	) Lavier (1933)
-	horse	Morocco	-	.05%	
U	camel	Sudan	2,000	.05%	)
W	horse	Morocco	1,000	.10%	
X	horse	Morocco	1,000	.10%	) Hoare (1956)
K	horse	India	4,000	.05%	
H	horse	Indochina	1,000	.10%	)
J	horse	Philippines	1,000	.10%	
Z	cattle	Indochina	1,000	.40%	)
R	buffalo	Indochina	1,000	.30%	
SAK* (Cc)	mice (isolated from camel)	Sudan	104,000	0-61%	)
SAK	" "	" "	-	-	
Nb*	mice (isolated from horse)	Bulgaria (imported)	50,000	2-35.25%	)
NS* (AA)	mice (isolated from camel)	Sudan	60,000	0-2%	
"	" "	" "	-	1 specimen	Edristan (1969)
"	" "	" "	-	-	Miles (1972)
"	" "	" "	-	-	Mathur (1971)
-	camel ( <u>Camelus dromedarius</u> )	Nigeria	1,000	2.9%	) Godfrey & Killick-Kendrick (1962)
-	camel	Nigeria	1,000	1%	
-	buffalo	India	5,000	42%	Venkataratnam et al. (1968)

- not given

\* for further details see next page

**\*S.A.K. strain** Blood films examined during the course of 17 years (April 1937 to April 1954) usually at weekly intervals. At each examination, 100 trypanosomes were examined for the presence of stumpy forms and the total number examined in 1035 preparations (=1018 passages) exceeded 104,000. Typical stumpy forms were seen on about 250 occasions in proportions varying from 0 to 61%. During the first 9 years the maximum percentage of stumpy forms recorded was 20 to 61% and during the last 8 years only 2 to 6%. During the 25th passage which was examined for 19 consecutive days, the percentage of stumpy forms varied from day to day as follows: 0, 0, 42, 0, 0, 6, 6, 13, 31, 12, 4, 6, 4, 9, 14, 3, 5, 9, 0.

**\*Nb strain** During the course of 4 years blood films were regularly examined in each passage animal: in some of the earlier passages for 3 to 12 days in succession, in others at intervals of 4 to 5 days, but subsequently once a month. In each slide at least 100 trypanosomes were inspected for the presence of stumpy forms, the total number thus examined in about 500 preparations exceeding 5,000 trypanosomes. Among these, typical stumpy forms were detected on four separate occasions, when 2, 6, 25 and 6% respectively were present. At the 16th mouse passage the percentage of stumpy forms was 35.25%.

**\*N. S. strain**

**Blood films were examined for 5 years: in some passages daily for 3 to 25 days, in most cases at intervals of 4 to 5 days but after 5 years only once a month. 100 trypanosomes were examined at any one time, and the total number of trypanosomes observed in approximately 600 preparations was more than 60,000. Stumpy forms were seen on 8 occasions, their occurrence reaching a maximum of 2%.**

Table 2

**Categorization of different pleomorphic forms**

<b>Form</b>	<b>Nucleus</b>	<b>Kinetoplast</b>	<b>Posterior end</b>	<b>Flagellum</b>	<b>Body</b>
<b>Slender</b>	<b>elongated</b>	<b>subterminal</b>	<b>elongated &amp; often truncated</b>	<b>long free</b>	<b>thinner body</b>
<b>Long intermediate</b>	<b>"</b>	<b>nearer to posterior end</b>	<b>pointed or sometimes elongated</b>	<b>shorter to slender</b>	<b>"</b>
<b>Intermediate</b>	<b>oval</b>	<b>terminal</b>	<b>rounded or slightly pointed</b>	<b>equal or shorter to long intermediate</b>	<b>medium</b>
<b>Short intermediate</b>	<b>oval or round</b>	<b>terminal or subterminal</b>	<b>slightly pointed on one side</b>	<b>very short</b>	<b>thicker</b>
<b>Stumpy</b>	<b>"</b>	<b>"</b>	<b>"</b>	<b>absent</b>	<b>"</b>

**Table 3**

**Titration of the Colombian strain of T. evansi stabilate LUMP 74 in mice.**

**The stabilate contained antilog 7.65 organisms per ml.**

<b>Log Dilution</b>	<b>Inoculum (log numbers of organisms)</b>	<b>Mice infected/ mice inoculated</b>	<b>Mean prepatent period (days)</b>	<b>Survival time (days) of mice showing:</b>	
				<b>Acute infection (mean)</b>	<b>Relapsing infection (individual)</b>
-2	4.65	6/6	3.6	7	-
-3	3.65	6/6	4.1	8	94 & 20
-4	2.65	6/6	4.5	8.8	20
-5	1.65	6/6	5.5	10.2	30
-6	0.65	5/6	6.2*	10.0	56
-7	-1.65	0/6	-	-	-

\* 5 mice

Infectivity from dilution above is antilog  $7.1 \pm 0.3$  ID<sub>63</sub> per ml.

(Lumsden et al., 1963)



Table 4

The effect of different temperatures on the course of the Colombian strain of T. evansi (LUMP 74) in mice. Inoculum antilog 3.65 organisms or antilog 3.1 ID<sub>63</sub>.

Temperature at which animals maintained	4°C	22-26°C	28°C	35°C
Mice infected/ mice inoculated	20/20	20/20	20/20	15/20
Acute infection/ number of deaths	13	14	15	2
Number of mice showing relapsing infection	7	6	5	2
Range of survival time (days)	7 - >60	9 - >60	7 - 35	8 - >60
Number of mice surviving 60 days	4*	3*	0	12**

\*Trypanosomes present in blood

\*\*Trypanosomes eliminated from circulation

Table 5

Colombian strain agglutinin titres in mouse sera, expressed as log<sub>2</sub> of the reciprocal dilution, to homologous and heterologous populations isolated from successive parasitaemic waves.

Antigen (LUMP)	Antisera												
	W0	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
W0(74)	6	6	2	3	3	2	2	2	2	-	-	-	-
W1(113)	6	7	-	-	-	-	3	6	6	5	2	-	-
W2(114)	-	-	7	-	-	-	-	2	2	-	-	2	3
W3(244)	-	-	-	6	-	-	-	-	-	-	-	-	-
W4(124)	-	-	-	-	7	2	-	-	-	-	-	-	-
W5(238)	-	-	-	-	-	5	-	-	-	-	-	-	-
W6(239)	3	3	2	-	3	4	5	3	3	2	3	-	2
W7(409)	3	5	-	-	-	-	5	5	5	2	-	-	-
W8*(134)	5	7	-	-	-	-	3	6	5	-	-	-	-
W9*(426)	5	6	-	-	-	-	-	-	3	5	3	3	-
W10(137)	-	-	-	-	-	-	-	-	-	-	6	7	-
W11(133)	-	-	-	-	-	-	-	-	-	-	7	7	-
W12(131)	-	-	-	-	-	-	-	-	2	-	-	7	7

\*Fresh trypanosomes used as antigen

**Table 6**

Showing the immune response of mice to 3 variant antigenic types expressed as the titre of agglutinins in homologous antisera collected from mice at different periods after cure of infection.

Variant (LUMP)	Mouse No.	Organisms at time of treatment (millions/ml.)	Titre of agglutinins (expressed as log <sub>3</sub> of the reciprocal dilution) in homologous antisera on days after treatment						
			6	9	12	15	18	24	30
W2 (114)	1	28.1	7	6	6	6	6	4	4
	2	26.5	7	7	6	6	6	5	5
	3	36.5	7	7	6	6	6	4	4
	4	20.0	7	7	6	5	5	4	4
	5	6.8	7	6	6	6	6	5	4
	Average			7.0	6.8	6.0	5.8	5.8	4.4
W6 (239)	1	10.1	6	6	5	5	5	4	4
	2	32.6	7	6	6	6	6	4	3
	3	22.3	7	6	6	4	4	4	4
	4	42.5	6	5	5	5	4	4	4
	Average			6.5	5.7	5.5	5.0	4.7	4.0
W10 (137)	1	25.0	6	5	4	4	4	3	3
	2	26.0	6	5	4	4	4	3	3
	3	11.0	5	5	4	4	4	4	3
	4	18.5	5	5	4	4	4	4	3
	5	31.4	5	4	4	3	3	3	3
	6	11.2	5	4	4	4	4	4	3
	Average			5.3	4.7	4.0	3.8	3.8	3.5

Table 7

The history of infection (Colombian strain) in mice whose sera were tested against the variant antigenic types W1, W2, W3, W4 and W10 (Table 8).

No. of mouse	Experiment for which the mouse was used	Duration of infection (days)	Serum No.
1	Effect of the temperature 4°C	64	1
2	"	64	2
3	"	64	3
4	"	58	4
5	"	64	5
6	Infectivity titration	58	6
7	"	108	7
8	"	108	8
9	"	108	9
10	Effect of the temperature (22-26°C)	21	10
11	"	20	11
12	"	27	12
13	Isolation of relapse variants	56	13

**Table 8**

The variant antigenic types of T. evansi (Colombian strain) in sera of mice infected with the original strain (WO),

Serum No. *	Antigen (Agglutinin titres expressed as log <sub>3</sub> of reciprocal dilution)				
	W1	W2	W3	W4	WO
1	5	4	-	-	-
2	4	5	3	3	3
3	3	4	5	-	-
4	3	4	5	5	3
5	4	6	-	4	3
6	4	4	-	4	-
7	4	4	-	-	-
8	4	4	-	-	-
9	3	4	-	-	-
10	6	4	-	-	-
11	4	6	-	-	-
12	5	5	-	3	3
13**	7	5	3	-	7

\* History of infection in mice given in Table 7.

\*\* Serum of mouse from which relapse variants were isolated.

Table 9

History of infection of mice whose sera are tested against variant antigenic types in Table 10.

No. of mouse	Variant antigenic type inoculated	Experiment for which the mouse was used	Duration of infection (days)	Serum No.	
1	W5	) Preparation of antisera	27	14	
2	W5		)	27	15
3	W5	) Isolation of variants	74	16	
4	W5		)	73	17
5	W5		)	35	18
6	W6	) Preparation of antisera	56	19	
7	W6		)	106	20
8	W8	)	70	21	
9	W12	)	13	22	

Table 10

The variant antigenic types in sera of mice infected with variants W5, W6, W8 or W12

Serum No.	Infecting variant (LUMP)	A N T I G E N									
		W5	W6	W8	W12	W1	W2	W3	W4	W7	
14	W5(125)	4				4	4	-	-	-	
15	W5(125)	4				4	4	-	4	3	
16	W5(125)	3				5	5	-	4	3	
17	W5(125)	4				3	4	-	-	3	
18	W5(125)	3				5	4	-	-	-	
19	W6(135)		4			4	4	-	-	-	
20	W6(135)		5			5	6	-	-	-	
21	W8(134)			3		6	4	-	-	-	
22	W12(131)				5	6	4	4	-	-	

- negative  
Blank not tested

Table 11

Antigenic relationship of variants of W5 and W12 populations to W1 and W2 antigenic types.

Antigens (LUXP)	Antigena (Titre of agglutinins expressed as log <sub>3</sub> of reciprocal dilution)										
	W5	W5-1	W5-2	W5-3	W12	W12-2	W12-3	W12-4	W1	W2	
W5(238)	6	7	2	2	2	-	2	2	2	2	
W5-1(416)	3	3	-	-	-	-	-	-	-	-	
W5-2(422)	-	2	5	4	2	5	4	3	5	3	
W5-2*(422)	2	2	6	5	2	6	4	3	6	3	
W5-3(423)	2	2	2	4	-	4	3	-	6	-	
W12(131)	-	-	-	-	7	-	-	-	-	-	
W12-2*(425)	-	-	4	5	-	6	-	-	6	-	
W12-3(480)	-	-	-	-	-	-	4	-	-	-	
W12-4(527)	-	-	-	-	-	2	2	5	2	2	
W1(113)	-	3	5	5	-	6	3	-	7	-	
W2(114)	-	3	4	2	-	-	-	-	-	5	

\* Antigen consisted of trypanosomes from blood, harvested from first parasitaemic wave in mouse, inoculated with stabilate population of the antigenic type.



Table 12

Protection of mice immunised with W1 antigenic type against challenge with W5-2 and W12-2 antigenic types.

Challenge (LUMP)	Group	Prepatent period (days)	Mice infected/ mice inoculated	Mice surviving 20 days
W1 (113)	vaccinated		0/6	6
	control	3.1	6/6	0
W5-2(422)	vaccinated	5.6 (in 3 mice)	3/5	5
	control	4.6	5/5	3
W12-2(425)	vaccinated	9.0 (in 1 mouse)	1/5	4
	control	4.2	5/5	1

Immunization

0.4 ml. of formalinised infected whole blood containing antilog

7.6 non motile organisms of W1 antigenic type was administered i/v.

Controls were given 0.4 ml. of phosphate buffer pH 7.4 i/v.

Challenge - 15 days after vaccination, antilog 3.7 trypanosomes derived from stabilate populations of above antigenic types were administered i/p.

**Table 13**

Showing similarity of antigenic types of an 'ingested variant population' and its corresponding 'transmitted variant population'.\*

Antigen	Agglutinins in antisera expressed as $\log_3$ of reciprocal dilutions	
	W12	W12-t
Population (LUMP)		
W12 (181)	7	5
W12-t (170)	4	6
Population (LUMP)	W4	W4-t
W4 (124)	5	not tested
W4-t (236)	5	not tested
Population (LUMP)	W3	W3-t
W3 (244)	5	5
W3-t (169)	6	5

\* Ingested populations W12, W4, and W3. Corresponding transmitted populations W12-t, W4-t and W3-t.

Table 14

Effect of challenge with different variant antigenic types in mice immunized with antigenic type W1.

Challenge (LUMP)	Group	Prepatent period (days)	Mice infected/ mice inoculated	Mice surviving 15 days
W1* (113)	vaccinated	.	0/6	6
	control	3.2	6/6	0
W0 (74)	vaccinated	5.2 (in 4 mice)	4/5	5
	control	5.4	5/5	0
W8 (134)	vaccinated	7.5 (in 4 mice)	4/5	4
	control	3.8	5/5	0
W3 (119)	vaccinated	7.3 (in 3 mice)	3/5	5
	control	6.4	5/5	2
W4 (124)	vaccinated	4.0	5/5	2
	control	3.4	5/5	1
W12 (131)	vaccinated	4.7	4/5	5
	control	3.6	5/5	3

\*Data of table 10 utilised as both experiments were carried out simultaneously.

Immunization- 0.4 ml. of formalinized infected whole blood containing antilog 7.6 non motile organisms of W1 antigenic type was administered i/v. Controls were given 0.4 ml. of phosphate buffer pH 7.4 i/v.

Challenge - 15 days after vaccination, antilog 3.7 trypanosomes derived from stabilate populations of the above antigenic types were administered i/p.

**Table 15**

**Protection afforded by the administration of antiserum. 0.2 ml. of antiserum to antigenic type W1 (LUMP 113) administered i/v. Challenge of antilog 4.7 organisms of antigenic type W1 administered i/p, one hour later.**

	<b>Test</b>	<b>Control</b>
<b>Mice infected/mice inoculated</b>	<b>2/5</b>	<b>5/5</b>
<b>Prepatent period (days)</b>	<b>9 (in 2 mice)</b>	<b>2</b>
<b>No. surviving 20 days</b>	<b>5</b>	<b>0</b>
<b>Survival time (days)</b>	<b>more than 60 days</b>	<b>5.6 days</b>

Table 16

Behaviour of different variant antigenic types in mice. Antilog 3.7  
organisms of each antigenic type derived from stabilate populations,  
administered i/p.

Population (LUMP)	Mice inoculated	Mean prepatent period (days)	Mice showing		No. died within 15 days
			Acute infection	Relapsing infection	
W0(74)	5	3.4	5	0	5
W1(113)	6	3.1	6	0	6
W8(134)	5	3.8	5	0	5
W3(119)	5	6.4	1	4	3
W4(124)	5	4.0	3	2	4
W12(131)	5	3.6	0	5	2

Table 17

Showing antigenic types of first and second wave populations from 2 rats inoculated with Colombian (original) strain W0 (LUMP 79). Agglutinins in sera expressed as  $\log_3$  of reciprocal dilutions.

Rat No.	Variant antigenic types				
	W1	W2	W3	W4	W10
<b>Sera following first wave</b>					
1	6	-	-	-	-
2	6	-	-	-	-
<b>Sera following second wave</b>					
1	6	4	-	-	-
2	5	4	-	-	-

**Table 18**

**Showing survival of the monomorphic Colombian strain (LUMP 74) in 4N medium, medium 199 and macrophage cell culture.**

Expt.	Medium	Inoculum	Days of survival		Remarks
			1st passage	2nd passage	
1	4N	tail blood - 2 drops	-		checked 6 days after inoculation
2	4N	heart blood - antilog 6.0 organisms	2		
3	Medium 199	heart blood - antilog 6.0 organisms	5	-	
4	Macrophage cell culture	antilog 6.0 organisms	1		organisms in liquid phase only. No intracellular stages observed in macrophages.

Table 19

Showing details of experiments on the transmission of the Colombian strain infection by

3. calciatrans.

Expt.	Trypanosome population		Stomoxys calcitrans			Infection in mice			
	Variant (LUMP)	Lay of infection in mouse	Parasitaemia (LEY)	No. Triturated/Dissected	Interval between feeding & killing (hours)	Organisms in triturated suspension/gut	Passages	Infection	Population frozen (LUMP)
1	W4(124)	4	++(4.4)	4 Dissected	24	Absent			
2	W12(131)	6	++(4.4)	6 Dissected	24	Absent			
3	W3(119)	3	5/1(2.7)	5 Triturated	18	Present	4	Positive	W3-t(169)
4	W12(131)	3	20/1(3.3)	13 Triturated	18	Present	4	Positive	W12-t(170)
5	W4(124)	3	++(4.4)	18 Triturated	18	Absent	4	Negative	
6	W4(124)	4	++(4.4)	8 Triturated	7	Present	4	Negative	
7	W4(124)	3	+(3.7)	15 Triturated	18	Present	4	Positive	W4-t(236)
8	W1(113)	3	+(3.7)	15 Triturated	18	Absent	4	Negative	
9	W2(114)	3	+(3.7)	15 Triturated	18	Absent	4	Negative	
10	W10(137)	3	+(3.7)	15 Triturated	18	Absent	4	Negative	



Table 20

Infectivity titration of N.S. strain of T. evansi stabilate LUMP 46 in mice.

The stabilate contained antilog 7.9 organisms per ml.

Log dilution	Inoculum (log numbers of organisms)	Mice infected/ mice inoculated	Mean prepatent period (days)	Mean survival time (days)
-4	2.9	6/6	3.16	4.3
-5	1.9	6/6	3.5	5.6
-6	0.9	6/6	5.0	6.3
-7	-1.9	2/6	5.0*	6.0*
-8	-2.9	0/6		
-9	-3.9	0/6		

\* 2 mice

Infectivity from dilution above, is antilog  $7.6 \pm 0.3$  ID<sub>63</sub> per ml.

(Lumsden et al., 1963)

Table 21

The effect of ambient temperature 35°C on the course of the N. S. strain of T. evansi (LUMP 46) in mice.

	Experiments					
	1		2		3	
	1.9 (1.6)		2.9 (2.6)		2.9 (2.6)	
Inoculum log number of organisms; (Antilog ID 63)						
Groups	Test	Control	Test	Control	Test	Control
Temperature at which animal maintained	35°C	22-26°C	35°C	22-26°C	35°C	22-26°C
Mice infected/ mice inoculated	5/6	6/6	6/6	6/6	12/12	12/12
Acute infection: number of deaths	2	6	4	6	7	12
Relapsing infection: number of deaths	3	0	2	0	5	0
Mean survival time (days)	12.5	6.3	15.1	5.3	8.0	5.2
(Range)	7-26	6-7	6-57	5-6	5-14	5-6

Table 22

The behaviour of N.S. strain (LUMP 46) in mice made immune<sup>1</sup> to S.A.K. strain. Observations on the development of pleomorphism.

Mouse	Inoculum (log no of organisms)	Type of infection	Number of parasitaemic waves	Pleomorphism*		Survival (days)	Isolations of pleomorphic populations	
				Day	Wave		Day	Stabilate (LUMP)
1	3.9	Relapsing	2	30	1	37	-	-
2	3.9	No infection	-	-	-	-	-	-
3	3.9	Accidental death	-	-	-	-	-	-
4	2.9	No infection	-	-	-	-	-	-
5	2.9	Acute	1	-	-	30	-	-
6	2.9	Acute	1	-	-	32	-	-
7	1.9	Relapsing	2	-	-	67	-	-
8	1.9	Relapsing	2	-	-	39	-	-
9	1.9	Relapsing	4	38-39	2	61	38,39	330,31

\* at the time of falling of parasitaemia

<sup>1</sup>Immunization against S.A.K. strain

Day 0 - Inoculation of antilog 3.87 organisms of the S.A.K. strain (LUMP 66)

Day 16 - First treatment with 2 mg. Berenil per mouse at the stage of fulminating parasitaemia

Day 14 - All mice challenged with antilog 3.87 organisms of S.A.K. strain (LUMP 66). Mouse 9 developed scanty parasitaemia. No infection developed in other mice.

Day 20 - Mouse 9 treated with 2 mg. Berenil.

Challenge with N.S. strain

Day 22 - All mice challenged with N.S. strain (LUMP 46) with the inoculum shown in Table 22.

Table 23

The agglutination of original N.S. strain (LUMP 46) and variant populations by homologous and heterologous antisera.

Antigen (LUMP)	Agglutinin titres in antisera expressed as $\log_3$ of the reciprocal dilution				
	T0	T2	T3	T4	T5
T0 (46)	5	-	-	-	-
T2 (204)	-	5	-	-	-
T5 (212)	-	-	4	-	-
T4 (215) <sup>or</sup>	-	-	-	6	6
T5 (223)	-	-	-	-	6

Table 24

The survival of the monomorphic N. S. strain (LUMP 46) in 4N medium.

Experiment	Inoculum	No. of medium tubes inoculated	No. of surviving cultures	Survival in days	
				First passage	Second passage
1	Tail blood- 2 drops	1	1	6	-
2	Tail blood- 2 drops	3	2	4	-
3	Heart blood: $1 \times 10^6$ organisms	6	3	3	-

Table 25

The behaviour of N.S. strain clone (LUMP 59) in mice made immune<sup>1</sup> to S.A.K. strain clone

Experiment	Mouse	Inoculum (log number of organisms)	Type of infection	Parasitaemic waves	Pleomorphism		Survival (days)	Isolation of pleomorphic populations	
					Day	wave		Day	Stabulate (LUMP)
1	1	3.98	Relapsing	Not recorded	51-77	Not recorded	77	-	-
	2	3.98	Relapsing	"	51-59	"	59*	54	177
	3	3.98	Relapsing	"	50-59	"	59	52,55	171,176
	4	3.98	Relapsing	"	50-83	"	83*	-	-
	5	3.98	Relapsing	"	50-59	2	59	51,53	172,175
2	6-9	3.98	Acute	1	-	-	142	-	-

\* Infected mice treated on this day.  
<sup>1</sup> Immunization against S.A.K. clone (LUMP 55)

Experiment 1

Day 0 - Inoculation of antilog 2.45 trypanosomes of S.A.K. clone (LUMP 55)

Day 7 - First treatment with 2 mg. Berenil per mouse. Parasitaemia ++ (Parasites uncountable but not teeming)

Day 15 - Inoculation of antilog 3.45 trypanosomes.

Day 19 - Second treatment with 2 mg. Berenil per mouse.

Day 29 - Mice challenged with antilog 4.45 organisms per mouse. No infection developed.

Challenge with N.S. clone (LUMP 59)

Day 43 - Mice 1 to 5 challenged with antilog 3.98 trypanosomes.

Observations

Day 47 - Mice 1 to 5 developed infection but only slender forms were seen.

Experiment 2

Immunization against S.A.K. clone (LUMP 55)

Days 0, 7, 15, 19 and 29 - As in experiment 1

Day 120 - Antilog 4.5 organisms of S.A.K. clone (LUMP 59) inoculated. No infection developed.

Challenge with N.S. strain (LUMP 59)

Day 136 - Antilog 3.98 organisms of N.S. clone.

Table 26

The behaviour of N.S. strain clone (LUMP 59) in mice made immune<sup>1</sup> to S.A.K. clone (LUMP 55).

Experiment 3

Mouse	Inoculum (log. no of organisms)	Type of infection	Parasitaemic waves	Pleomorphism		Survival (days)
				Day	Wave	
1	3.98	Acute	1	-	-	31
2	3.98	Acute	1	-	-	32
3	3.98	Relapsing	2	-	-	34
4	3.98	Relapsing	3	-	-	32
5	3.98	Relapsing	4	-	-	60
6	2.98	Acute	1	-	-	33
7	2.98	Acute	1	-	-	33
8	2.98	Relapsing	1	-	-	60
9	2.98	Relapsing	3	35	3	38
10	2.98	No infection	-	-	-	
11	2.98	No infection	-	-	-	
12	3.98	No infection	-	-	-	

<sup>1</sup> Immunization against S.A.K. clone (LUMP 55)

Day 0 - Inoculation with antilog 3.45 trypanosomes

Day 6 - First treatment with 2 mg. Berenil per mouse

Day 14 - Inoculation with antilog 3.45 trypanosomes. Scanty parasitaemia developed in mouse 10, 11 and 12. No infection developed in remaining mice.

Day 20 - Treatment of mouse 10, 11 and 12 with 2 mg. Berenil per mouse

Challenge with N.S. clone (LUMP 59)

Day 22 - Challenge with N.S. clone (LUMP 59) with the inoculum shown in the table

Control group

Mouse 1 to 5 - Each inoculated with antilog 3.98 organisms of N.S. clone LUMP 59

Mouse 6 to 10 - Each inoculated with antilog 2.98 organisms of N.S. clone LUMP 59. These mice were inoculated on the same day of challenge of experimental group by this population. All died within 5-6 days of inoculation.

Table 27

The behaviour of N.S. strain clone (LUMP 85) in mice made immune<sup>1</sup> to S.A.K. strain clone (LUMP 55)  
Experiment 4

Mouse	Inoculum (log number of organisms)	Type of infection	No. of para- sitic waves	Fleomorphism		Survival (days)	Isolations of pleo- morphic populations	
				Day	Wave		Day	Stablate
1	3.95	Relapsing	3	77-79	3	79	78,79	280,315
2	1.95	No infection	-	-	-	-	-	-
3	2.95	Acute	1	-	-	-	-	-
4	2.95	Acute	1	-	-	64	-	-
5	2.95	Acute	1	-	-	67	-	-

<sup>1</sup> Immunization against S.A.K. clone (LUMP 55)

Day 0 - Inoculation of antilog 3.45 trypanosomes.

Day 3 - First treatment with 0.5 mg. Berenil per mouse at the height of infection in mice 1 to 4.

Day 4 - First treatment with 0.5 mg. Berenil to mouse 5.

Day 13 - Treatment of first relapse in mice 2 to 4 with 0.5 mg. Berenil.

Day 24 - Treatment of first relapse in mouse 1 and second relapse in mouse 2 with 0.5 mg. Berenil.

Day 55 - All mice challenged with antilog 3.45 organisms of S.A.K. clone (LUMP 55). No infection developed.

Challenge with N.S. clone (LUMP 85)

Day 59 - All mice challenged with N.S. clone with doses shown in the table.



Table 28

Development of pleomorphism in N.S. strain clone LUMP 85 (Experiment 4). Percentage of slender, intermediate and stumpy trypomastigote forms in the mouse from which LUMP 280 and 315 were isolated. (See Figure 16a)

Day of experiment 4	No. of trypanosomes observed/%	Slender forms	Intermediate forms			Stumpy
			Long	Inter-mediate	Short	
70	200	87*	113**	-	-	
	%	43.5	56.5			
71	100	1	25	38	22	14
	%	13	62			25
77	200	140	26	27	6	1
	%	76.5	21.5			2
78	200	47	54	46	52	1
	%	37	49.5			13.5
79	200	80	128	7	74	11
	%	48	36			16

\* kinetoplast subterminal

\*\* kinetoplast terminal

**Table 29**

The agglutination of N.S. strain clone (LUMP 59) and variant populations by homologous and heterologous antisera.

Antigen (LUMP)	Titre of agglutinins in antisera (expressed as $\log_3$ of the reciprocal dilution)				
	59	172	175	171	176
59	7	-	-	-	-
172	-	6	-	3	-
175	-	6	6	6	6
171	-	-	-	-	-
176	-	4	-	-	4

Table 30

T. evansi N.S. pleomorphic variants LUMP 172. The table shows percentage of slender, intermediate and stumpy forms in splenectomized and irradiated mice. Observations were made from 4 mice, the same mice being used throughout. In each mouse at least 100 organisms were examined each day.

Day	Control group			Splenectomized group			Irradiated group		
	Slender %	Intermediate %	Stumpy %	Slender %	Intermediate %	Stumpy %	Slender %	Intermediate %	Stumpy %
5	89.0	10.0	1.0				89.5	9.5	1.0
6	86.0	13.0	1.0	77.0	16.0	7.0	72.0	25.0	3.0
7	38.0	54.5	7.5	65.0	30.0	5.0	66.0	24.0	10.0
8	0.5	48.0	61.5	39.0	47.0	14.0	50.0	34.0	16.0
9	-	22.0	78.0	14.0	51.0	35.0	36.0	36.0	28.0
10				7.0	34.0	63.0	20.0	23.0	47.0
11				6.0	27.5	66.5	17.5	50.0	32.5
12	76.0	21.0	3.0	4.5	16.0	79.5	11.0	49.0	40.0
13	63.5	34.0	2.5	31.5	37.5	31.0	20.0	15.5	64.5
14	53.0	42.0	5.0	30.5	48.5	21.0	16.0	26.0	58.0
15	45.5	45.0	9.5	26.5	49.5	24.0	21.5	21.0	57.5
16	37.5	56.0	6.5	20.5	35.5	44.0	25.0	29.0	46.0
17	32.5	53.5	14.0	44.0	26.0	30.0	44.5	19.5	36.0
18	37.5	57.0	5.5	31.0	41.0	28.0	60.5	21.0	18.5

Table 31

Cultivation of N.S. pleomorphic variant populations LUMP 171, 172, 175, 176 and 177 in 4N medium

Population (LUMP)	Expt No.	Medium tube inoculated	Inoculum	First passage		Second passage		Third passage		Fourth passage			
				Survival (days)	Day	Score	Survival (days)	Day	Score		Survival (days)	Day	Score
171	1	a)	Mouse tail blood 2 drops	10	7	++	12	7	++	-	-	-	
	1	a)		26	7	++	19	7	+	12	-	-	
	2	a)		10	5	+	5	5	5	+	-	-	-
		b)		10									
		c)		10									
3	d)	Not recorded	5	+	5	5	+	-	-	-			
172	3	e)	Mouse heart blood 1x10 <sup>7</sup> organisms recorded	12	5	+	10	5	+	-	-	-	
		a)		12									
		b)		3.5 x 10 <sup>4</sup>									
		c)		3.5 x 10 <sup>4</sup>									
		d)		Not recorded									
177	1	a)	Mouse tail blood 2 drops	12	5	+	7	7	+	18	10	+	
		b)		5									
		c)		9									
		d)		12									
		a)		9									
2	3	a)	1 capillary containing stabilate	12	4	+++	10	4	++	-	-	-	
		b)		5									
		a)		12									
171	1	a)	Average	27	7	+	-	-	-	5	-	-	
		175		9									
		176		5									
				11.4			8			5			

\*All tubes became positive

- = No growth

Arbitrary scale + = 1-19 flagellates per 20 fields (x 40)

++ = 1-5 flagellates per field (x 40)

+++ = 75 flagellates per field (x 40)

Table 32

Growth rate of LUMP 172 population in 4N medium showing the number of organisms in samples of culture.

Day after inoculation	Live trypomastigotes			Dead trypomastigotes		
	Sample 1	Sample 2	Average ( $\times 10^4$ )	Sample 1	Sample 2	Average ( $\times 10^4$ )
<u>Experiment 1 (Inoculum <math>1 \times 10^7</math> organisms)</u>						
1	80.0	73.2	76.8	-	-	-
2	49.9	48.5	49.2	3.2	4.5	3.8
3	29.2	25.2	27.2	2.2	2.0	2.1
4	16.0	19.9	17.9	4.0	3.0	3.5
5	23.0	28.5	25.7	13.5	15.5	14.5
6	1.0	8.0	4.5	4.2	7.0	5.6
7	Contaminated					
<u>Experiment 2 (Inoculum <math>1 \times 10^7</math> organisms)</u>						
1	89.7	102.5	96.1	-	-	-
2	19.0	23.2	21.1	-	1.7	0.8
3	16.5	33.2	24.8	9.7	2.0	5.8
4	23.7	10.0	16.8	6.7	2.0	4.3
5	5.5	1.5	3.5	1.7	0.2	0.9
6	5.5	0.5	3.0	5.7	-	2.8
7	3.0	0.2	1.6	4.2	3.0	3.6
8	0.5	4.7	2.6	4.5	4.5	4.5
9	8.2	-	4.1	5.5	-	2.7
*10	+(2/20)	+(1/20)				
*11	+(1/20)	+(1/20)				
*12	+(1/20)	+(1/20)				
<u>Experiment 3 (Inoculum <math>1 \times 10^6</math> organisms)</u>						
1	38.5	49.0	43.7	-	-	-
2	13.5	12.2	17.8	1.2	2.0	1.6
3	9.2	8.2	8.7	4.0	2.5	3.2
4	1.7	3.7	2.7	3.7	4.7	4.2
5	8.2	10.7	9.5	3.0	4.0	3.5
6	2.2	2.7	2.5	5.0	3.2	4.1
7	6.0	4.7	5.3	8.2	4.2	6.2
8	3.5	3.2	3.3	3.5	3.7	3.6
9	1.7	3.0	2.3	4.2	4.0	4.1
10	1.7	0.5	1.1	12.0	3.2	7.6
11	0.5	2.0	1.2	7.5	4.7	6.1
*12	+(1/20)	+(1/20)				

\*Score of parasites according to arbitrary scale  
 Figures in the brackets show the number of trypomastigotes per microscopic field ( $\times 10$  oculars  $\times 40$  objective)

Table 33

Cultivation of N.S. pleomorphic variant populations LUMP 315 and 280.

Popu- lation (LUMP)	Experi- ment No.	Media tube inoculated	Inoculum	First passage		Second passage		Third passage	
				Survival (days)	Inoculation to second passage Day	Score	Survival (days)	Inoculation to third passage Day	Score
280	1	a	1 capillary containing stabilate Mouse heart blood 0.5 ml.	9					
	2	a	Mouse tail blood 2 drops	3					
315	1	a	Mouse heart blood 0.2 to 0.5 ml.	4					
	2	b	Mouse heart blood 0.2 to 0.5 ml.	10	6				
	3	a	Mouse tail blood	3					
	4	b	Mouse tail blood	3					
	5	c	2 to 4 drops	2					
	6	a	Mouse heart blood	6					
	7	b	Mouse heart blood	6					
	8	d	Mouse heart blood (1 x 10 <sup>7</sup> organisms	9	5		3.3x10 <sup>4</sup>	5	5
			not recorded	5		3.3x10 <sup>4</sup>	5	5	

- = No growth  
Arbitrary Scale + = 1-19 flagellates per 20 fields (x 40)

Table 34

Growth rate of LUMP 315 population in 4N media. Inoculum  $1 \times 10^7$  organisms

Day after inoculation	Live trypomastigotes			Dead trypomastigotes		
	Sample 1	Sample 2	Average ( $10^4$ )	Sample 1	Sample 2	Average ( $10^4$ )
1	249.7	247.5	248.6	1.7	-	0.8
2	116.0	17.0	66.5	7.2	0.7	3.9
3	4.2	5.0	4.6	3.7	4.0	3.8
4	0.5	1.0	0.7	-	-	-
5	6.2	0.5	3.3	2.7	-	1.3
6	28.5	11.2	18.8	1.5	0.5	1.0
7	22.5	20.2	21.3	8.2	3.0	5.6
8	19.7	17.5	18.6	4.5	1.7	3.1
9	15.5	13.5	14.5	46.2	31.2	38.7

Table 35

Survival of N. S. pleomorphic variants in HeLa or Macrophage cell culture.

Population LUMP	Medium	Inoculum	Incubation temperature	Media tubes inoculated	Media tubes showing survival
172	HeLa cell culture	$1 \times 10^7$	28° C	2	-
172	"	$1 \times 10^7$	37° C	2	-
172	Macro-phage cell culture	$1.2 \times 10^6$	37° C	14	-



Table 36

LUMP 172 - Schedule of feeding G. morsitans and results of transmission experiments

Expt. No.	Group	Flies	On Infected mice	Days of feeding						
				1	2	3	4	5	6	7
1	A	12	0, 1, 2	3-7	8-10	11-15 (+)	16-18 (+)	19-20		
	B	13	0, 1, 2	3-7	8-10	11-15	16-18	19-21	22-25 (+)	
	C	22	0, 1, 2	3-6	7-9	10-14	15-17 (+)	18-20	21-24	
	D	16	0, 1	2-4	5-7	8-12	13-15	16-18	19-22	
	E	12	0	1-3	4-6	7-11	12-14	15-17	18-21	
2	A	18	1	2-10	11-12	13-14				
	B	14	1	2-9	10-11	12-13				
	C	13	1	2-8	9-10	11-12				
3	A	6	1	3-8	9-10	11-12	13-14			
	A	13	1	3-11	13-20	22-26	27-33	35-40	42-49	
4	A	16	1	3-11	13-20	22-26	27-33 (+)	35-40 (+)	42-49 (+)	51-56

(+) Mouse developed infection

Table 37

LUMP 172- The results of dissection of G. morsitans

Expt. No.	Group	No. fed	Probing positive (day)	No. of flies which died	Period of dissection (days after feeding)	No. of flies dissected	Results of dissection		
							No. of flies which developed infection		
							Mid gut	Proventriculus	Salivary glands
1	A	12		4	22	8	1	Not examined	-
	B	13		2	27	11	1	"	-
	C	22		9	26	13	1	"	-
	D	16		5	25	9	3	"	-
	E	12		2	26	10	4	"	-
	Total	75		22		51	10		
2	A	18	11 & 13 <sup>1</sup>	3	11, 14	1, 14	1, 2	1, 2	1, 1 <sup>*</sup>
	B	14		2	12	14	-	-	-
	C	13	12 <sup>*</sup>	2	12 & 13	11	1	1	-
	Total	45		7		40	4	4	2
3	A	6		1	9	1	1	1	1
					15	4	1	1	1
	Total	6		1		5	2	2	1
4	A	13		13	-	-			
	B	16	35 & 52 <sup>*</sup>	16	-	-			
	Total	29		29					

<sup>\*</sup> Only proventricular forms were observed in probes and salivary glands

Table 38

LUMP 315, 330. The Schedule of feeding G. morsitans and results of transmission

Population fed (LUMP)	Expt. No.	Group	No. of flies	Source of feed	On infected mouse	Days of feeding			
						On clean mouse†			
						1	2	3	4
315	1	A	10	Mouse blood	1	2-6			
	2	B	18	"	1	2-6			
		A	15	"	1	2-5	6-9	10-13	14-18
		B	18	"	1	2-5	6-9	10-13	14-18
		C	18	"	1	2-5	6-9	10-13	14-18
		D	14	"	1	2-5	6-9	10-13	14-18
	3	A	14	Stabilate	1	3-8	9-10	11-12	13-14
	4	A	12	Mouse blood	2	3-10	11-15		
	5	A	22	"	1	2-4			
		B	18	"	2	3-5			
C		12	"	1	2-3				
D		14	"	1	2-4				
330	1	A	7	Stabilate	1	3-8	9-10	11-12	13-14
	B	12	"	1	3-8	9-10	11-12	13-14	

\* None of the mice developed infection

Table 39

LUMP 315, 330. The schedule of feeding flies and results of subsequent fly dissections.

Population fed (LUMP)	Expt. No.	No. of flies of source of feed	Age (days)	Flies died	Results of dissection				
					Lay after feeding	No. of flies dissected	No. of flies developing infection		
							Midgut	Proventriculus	Salivary glands
315	1	28	1	28	6	1	-	-	-
	2 A	33	1	5	18	28	-	-	-
	B	32	1	6	19	26	-	-	-
	3	14	1	3	15	11	-	-	-
	4	12	2	2	15	10	-	-	-
	5 A	22	1	-	5	22	-	-	-
	B	18	2	-	6	18	-	-	-
	C	12	1	-	4	12	-	-	-
	D	14	1	-	5	14	-	-	-
	1	19	1	7	7	6	-	-	-
330					13	6	-	-	-

\* Mouse blood contained only slender forms when the flies were fed. In remaining experiments a few stumpy forms were present at the time of feeding.

Table 40a

The agglutination of the original S. A. K. strain (LUMP 66) and relapse populations by homologous and heterologous antisera.

Antigen (LUMP)	Antisera		
	(Titre of agglutinins in antisera to relapse populations expressed as $\log_3$ of the reciprocal dilution)		
	S0	S1	S2
S0 (166)	4	-	4
S1 (251)	5	5	-
S2 (283)	5	6	6

Table 40b

The protection of mice, which had been previously immunized<sup>1</sup> with S0 antigenic type, against challenge with antigenic types S1 and S2.

Challenge (LUMP)	Group	Mice infected/ mice inoculated
S0 (166)	Vaccinated	0/5
	Control	5/5
S1 (251)	Vaccinated	5/5
	Control	5/5
S2 (283)	Vaccinated	5/5
	Control	5/5

<sup>1</sup>Immunization - 0.4 ml. of formalinized infected whole blood containing antilog 7.6 non-motile organisms of S0 population which was derived from mice inoculated with LUMP 66 was administered i/v. Controls were given 0.4 ml. of P. B. pH 7.4.

Challenge 15 days after vaccination, antilog 3.0 trypanosomes derived from stabulate of above populations were administered i/p.

Table 41

**Survival of the monomorphic S. A. K. (dyskinetoplastic)**

**strain in 4N medium and medium 199.**

Popula- tion (LUMP)	Expt.	Medium	Inoculum	No. of media tubes inoculated	No. of media tubes showing survival	Survival in days	
						First passage	Second passage
55	1	4N	Mouse tail blood - 2 drops	1	-	-	
	2	4N	"	1	-	-	
	3	4N	"	2	2	2	
66	1	Medium 199	0.1 ml. of mouse heart blood	1	1	4	-

Table 42

The reaction of N.S. and S. A.K. strains of T. evansi and Lugala-1, S-10 and S102 strains of T. brucei with sera of mice having chronic infection of either original Colombian strain (WO) or one of its variants.

Sera No.	Infecting strain/variant	Duration of infection (days)	Antigens (LUMP)				
			<u>T. evansi</u>		<u>T. brucei</u>		
			NS (46)	S. A.K. (66)	Lugala-1 (143)	S-10 (43)	S-102 (159)
1	WO	64	-	-	-	-	-
2	WO	64	-	-	-	-	-
3	WO	64	-	-	-	-	-
4	WO	58	-	-	-	-	-
5	WO	64	-	-	-	-	-
6	WO	58	-	-	-	-	-
7	WO	108	-	-	-	-	-
8	WO	108	-	-	-	-	-
9	WO	108	-	-	-	-	-
10	WO	21	-	-	-	-	-
11	WO	20	-	-	-	-	-
12	WO	27	-	-	-	-	-
13	WO	56	-	-	-	-	-
14	W5	27	-	-	-	-	-
15	W5	27	-	-	-	-	-
16	W5	74	-	-	-	-	-
17	W5	73	-	-	-	-	-
18	W5	35	-	-	-	-	-
19	W6	56	-	-	-	-	-
20	W6	106	-	-	-	-	-
21	W8	70	-	-	-	-	-
22	W12	13	-	-	-	-	-

\*Source of sera given in Tables 7 and 9.





**Table 44**

The results of the inoculation of N.S. strain clone (LUMP 59) into mice already immunized<sup>1</sup> against S.A.K. strain clone.

Inoculum	Antilog 2.98 organisms		Antilog 3.98 organisms	
	Immune to S.A.K. strain clone	Control	Immune to S.A.K. strain clone	Control
Mice infected/ mice inoculated	4/4	5/5	5/5	5/5
Mean prepatent period*	4	3.8	4	3
Mice showing relapse infection/ Mice died of acute infection	1/3	0/5	3/2	0/5
Mice dead/ mice infected**	3/4	5/5	4/5	5/5
Mean survival time*	17	5.6	30.4	5

\* Days after inoculation of N.S. strain

\*\* 30 days observation period

<sup>1</sup> Immunization against S.A.K. clone (LUMP 55)

Day (0) - Inoculation with antilog 3.45 trypanosomes.

Day (6) - Treatment with 2 mg. Berenil per mouse.

Day (14) - Inoculation with antilog 3.45 trypanosomes. No infection developed.

Challenge with N.S. strain clone (LUMP 59)

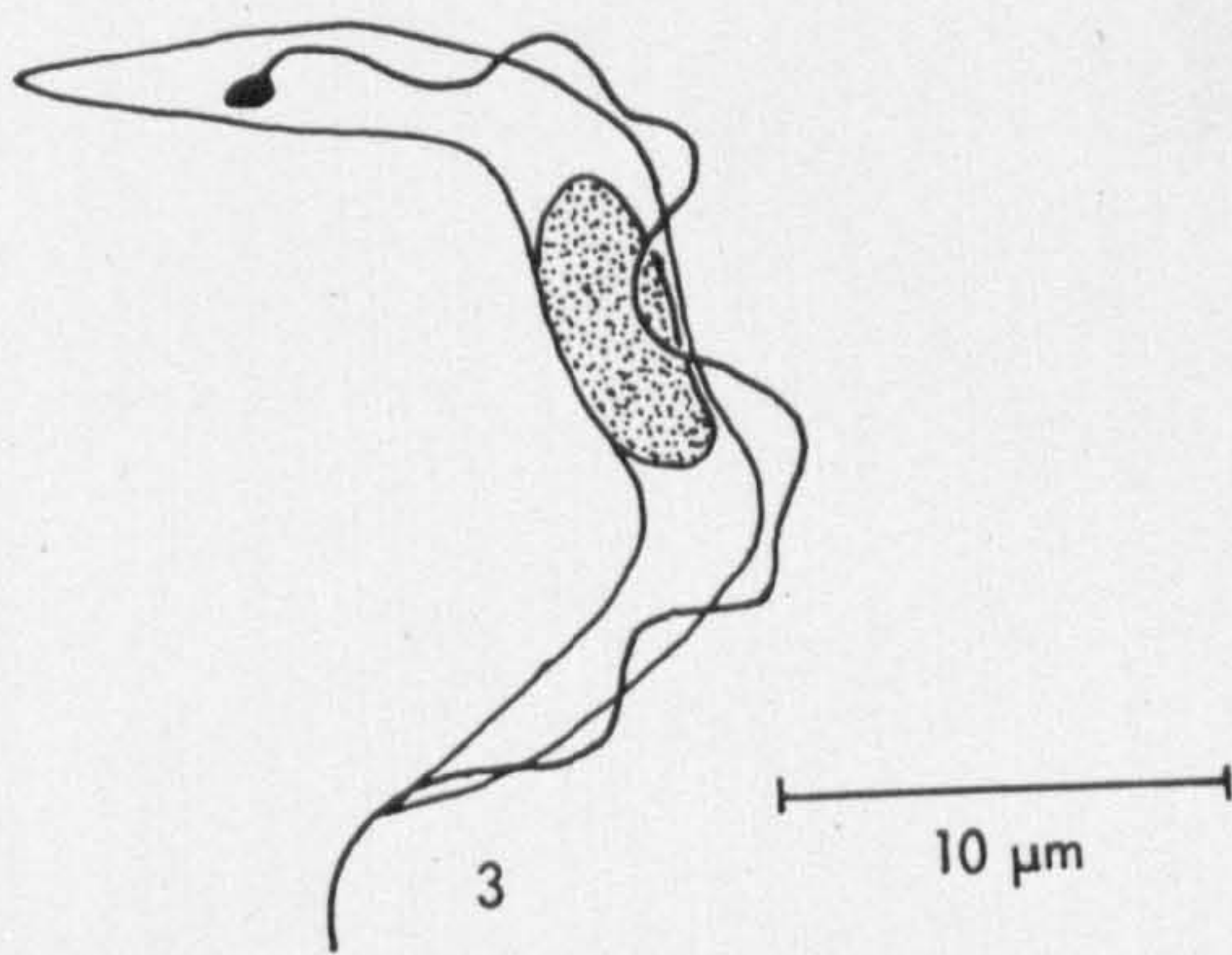
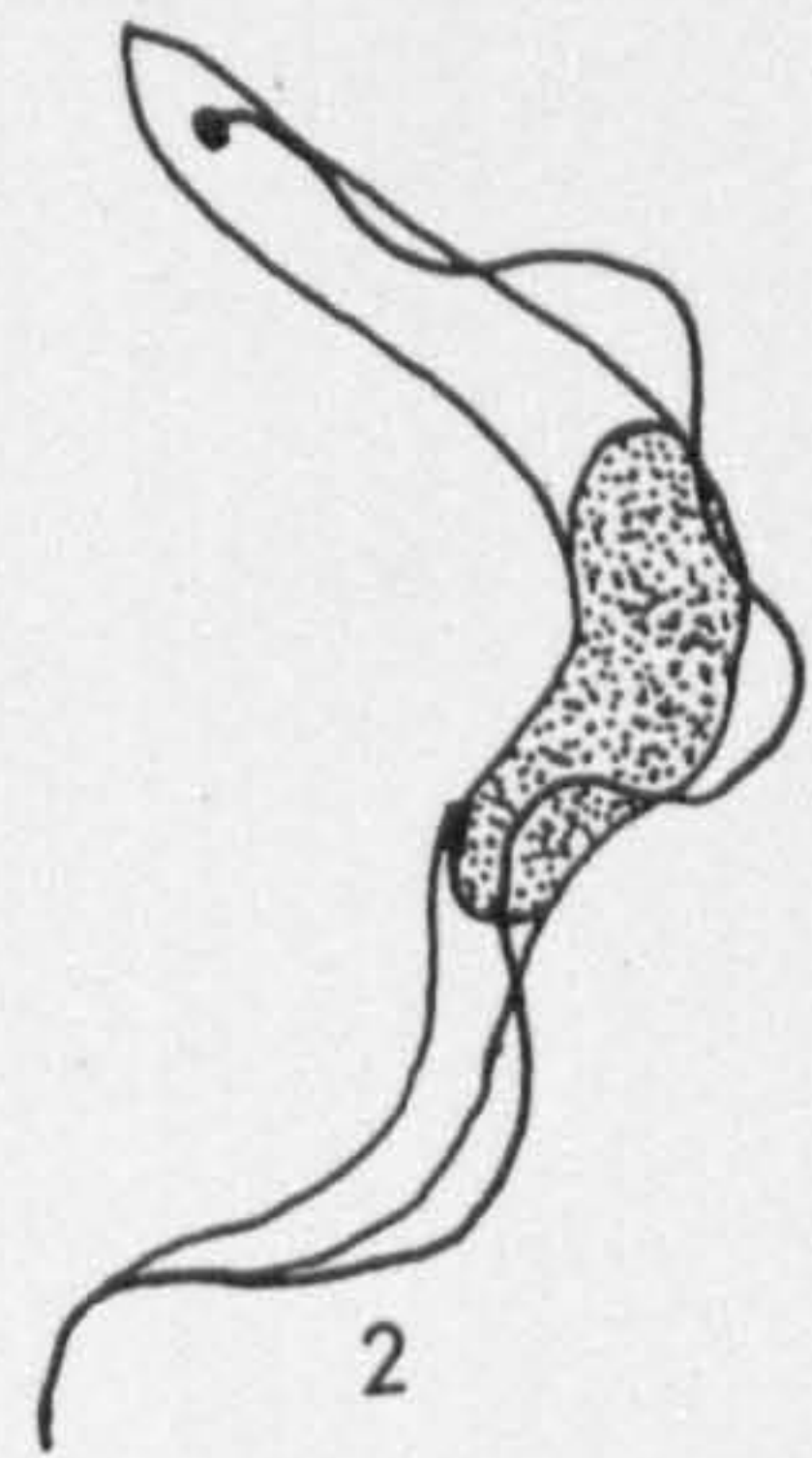
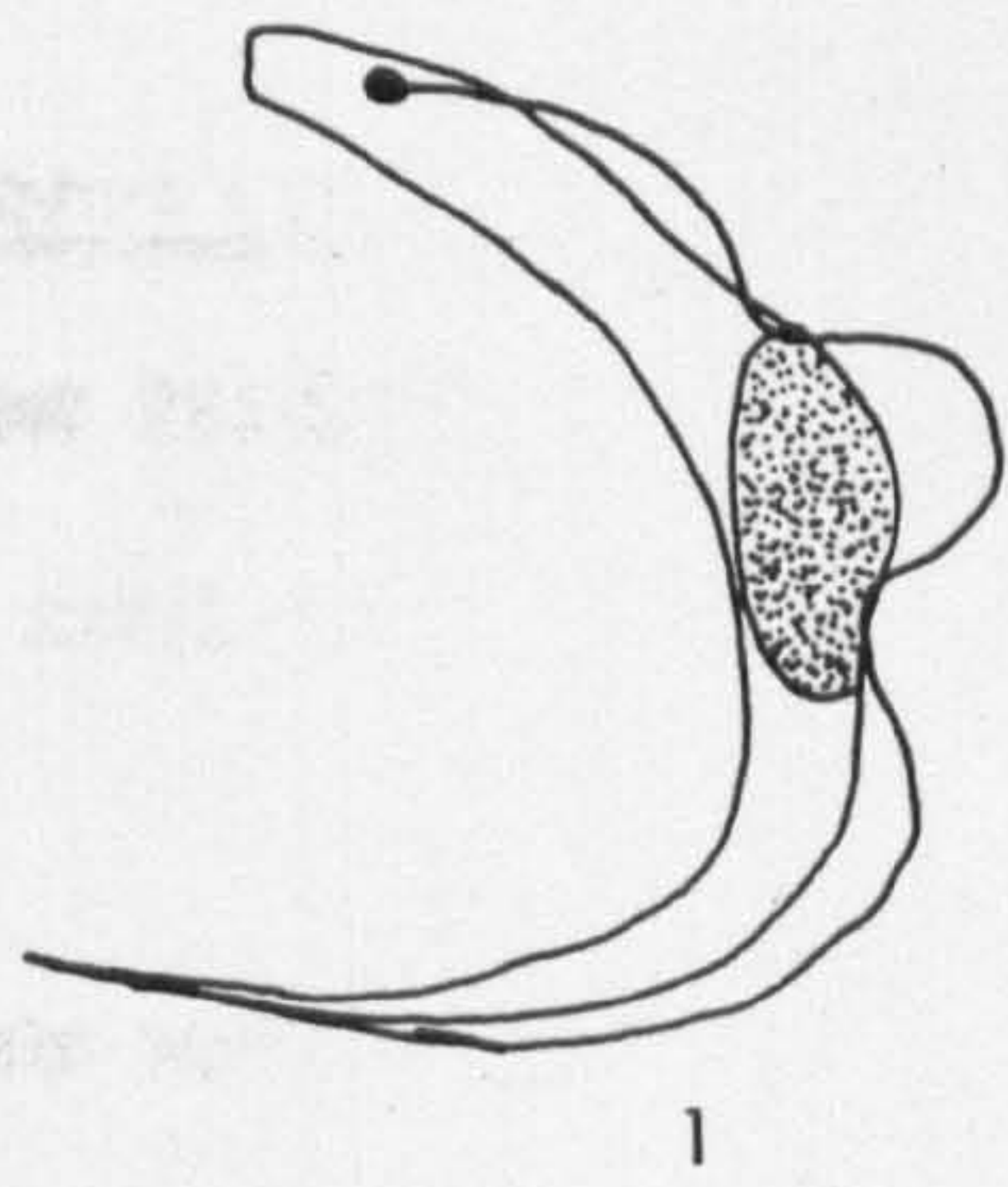
Experimental Group Day 22 - challenged with N.S. clone (LUMP 59) (or Day 0\*) with doses as shown in column 1.

Control group (Day 0\*) - Inoculated N.S. clone (LUMP 59) with doses as shown in column 1.

**Figure 1a. Monomorphic forms of N.S. strain**

**1-3. Slender form and long intermediate form**

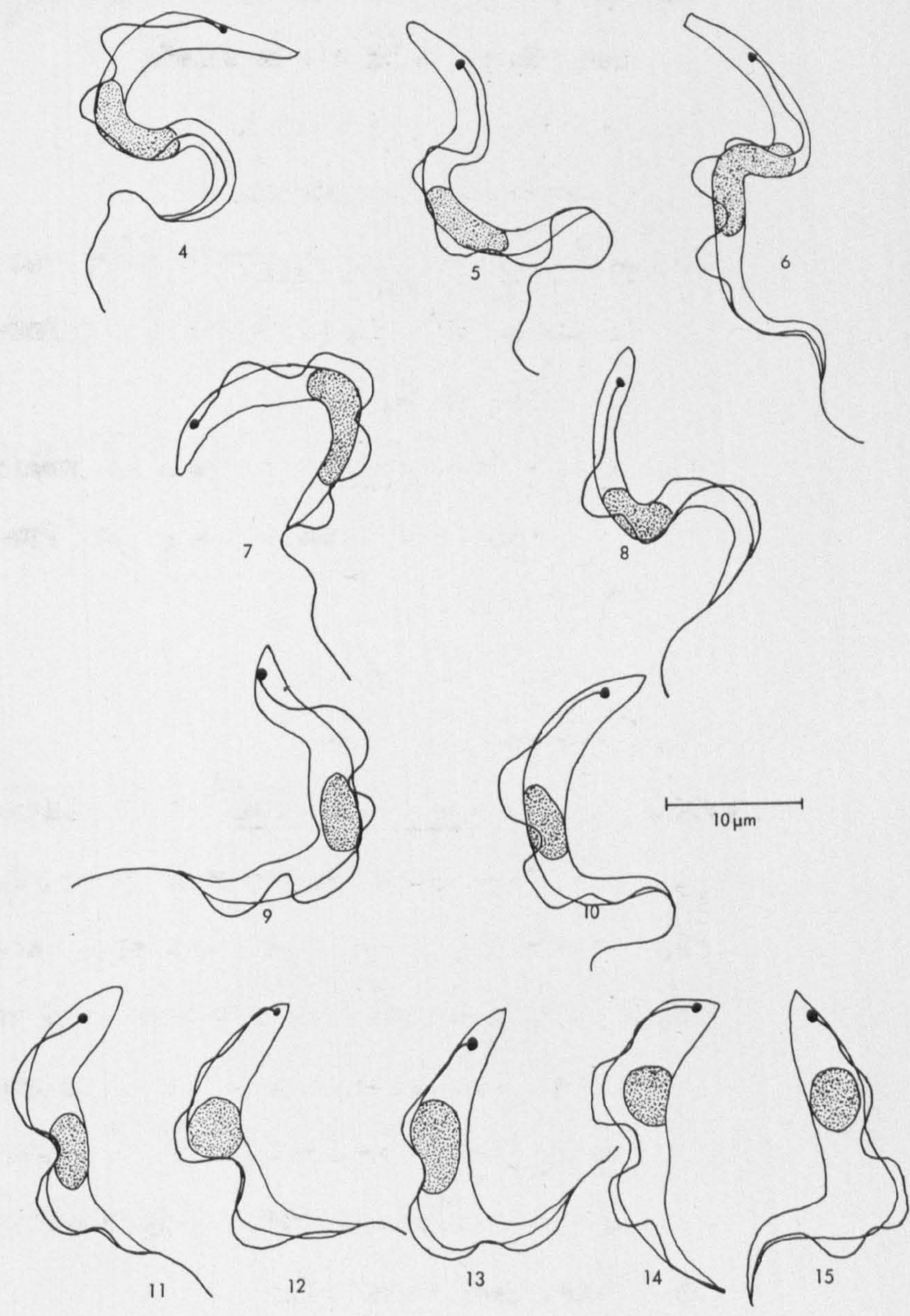
- 1. Long intermediate form - kinetoplast near an elongated posterior end, short free flagellum.**
- 2. Long intermediate form - kinetoplast near posterior end, short free flagellum, posterior end pointed.**
- 3. Slender form - kinetoplast subterminal, posterior end truncated, short free flagellum.**



1 a

Figure 1b. Pleomorphic forms of N.S. pleomorphic variant

- 4-6. Slender forms - body long thin, long free flagellum, nucleus elongated.
- 4,5. posterior end elongated.
6. posterior end truncated.
- 7,8. Long intermediate forms - kinetoplast nearer to posterior end, posterior end pointed, long free flagellum.
- 9,10. Intermediate forms - kinetoplast nearer to posterior end, nucleus oval, long free flagellum.
9. posterior end pointed.
10. posterior end rounded.
- 11-13. Short intermediate forms - body thicker, short free flagellum, nucleus oval or round.
11. kinetoplast subterminal, posterior end rounded.
12. kinetoplast terminal, posterior end pointed.
13. kinetoplast subterminal, posterior end pointed.
- 14,15. Stumpy forms - nucleus rounded or oval, posterior end pointed on one side, free flagellum absent.
14. kinetoplast terminal.
15. kinetoplast nearer to posterior end (subterminal).



1b

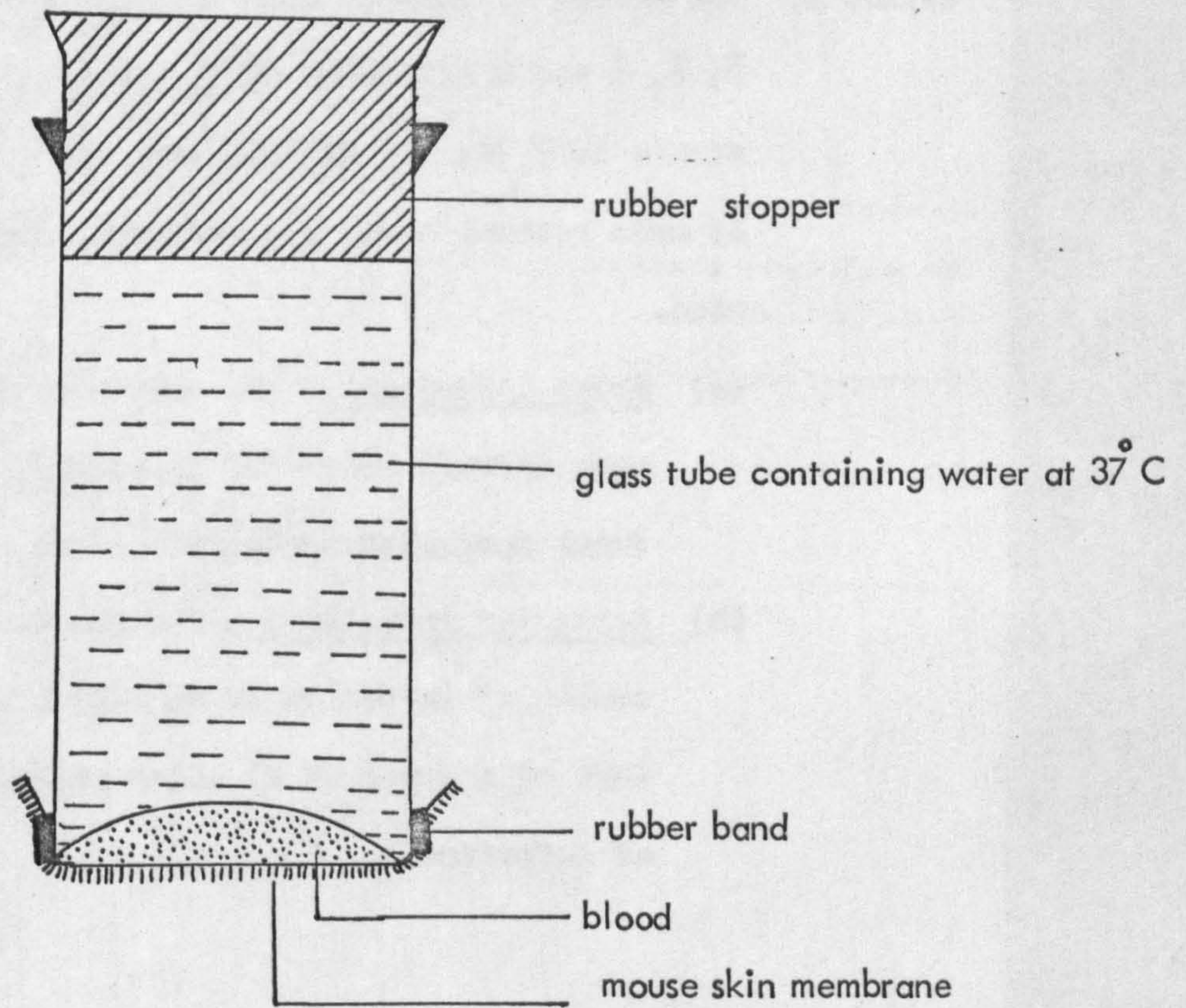
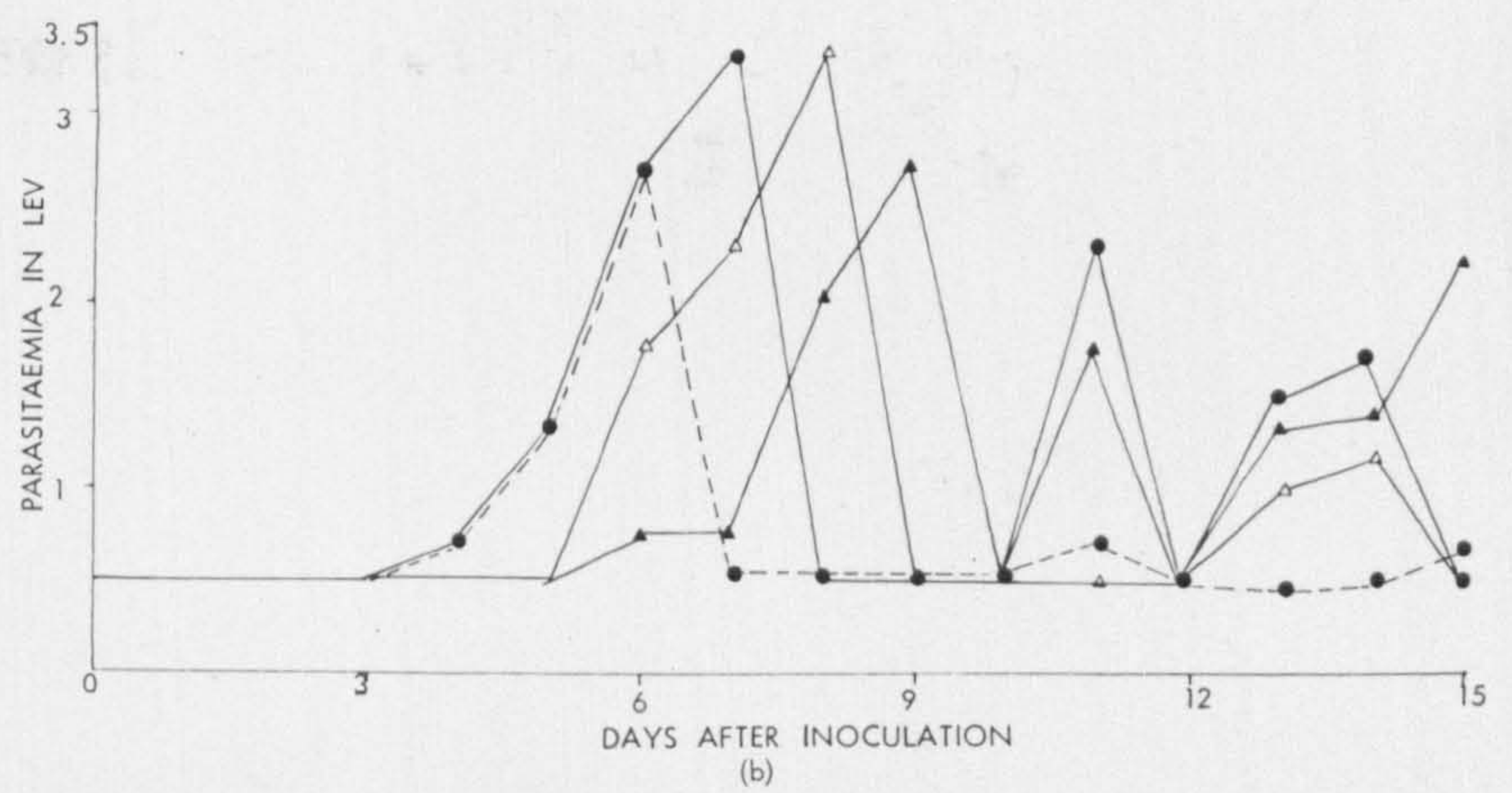
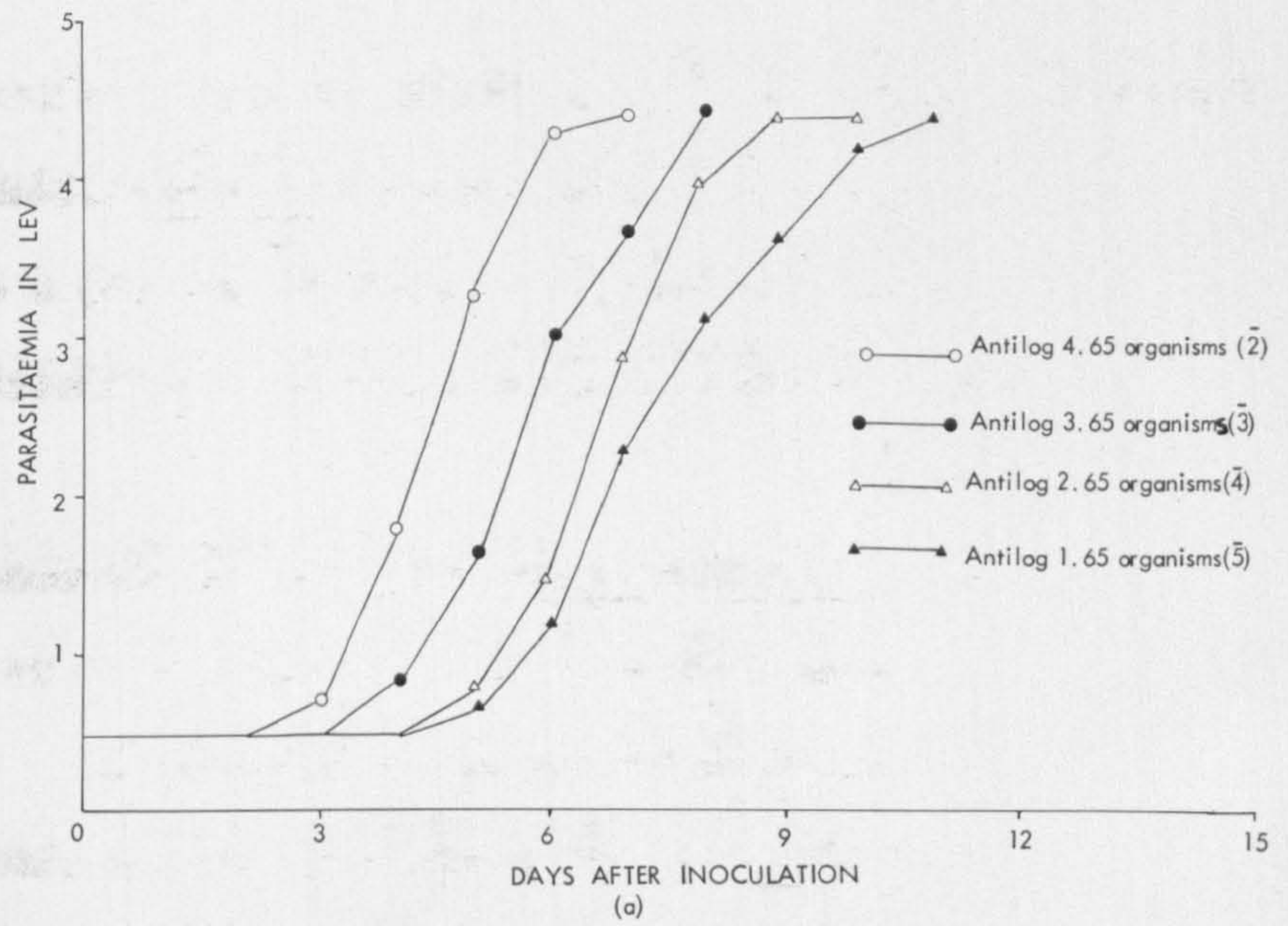


Figure 2. Cross-section diagram of apparatus used for feeding flies through mouse skin membrane

**Figure 3.** The course of parasitaemia in mice inoculated with  $\bar{2}$ ,  $\bar{3}$ ,  $\bar{4}$  and  $\bar{5}$  dilutions of T. evansi Colombian strain LUMP 74. In figures (a) and (b) a comparison is made between acute and relapsing infections in mice.

(a) Acute infections - the curves represent the mean parasitaemias for groups of 4 to 6 mice which developed acute infections.

(b) Relapsing infections - the curves show the course of infection in an individual mouse (out of a group of 6) which showed this type of infection.





**Figure 4. Effect of different environmental temperatures on the course of parasitaemia of the Colombian strain LUMP 74 in mice. Curves represent the mean parasitaemias for groups of 20 mice inoculated with antilog 3.65 organisms or antilog 3.1 ID 63.**

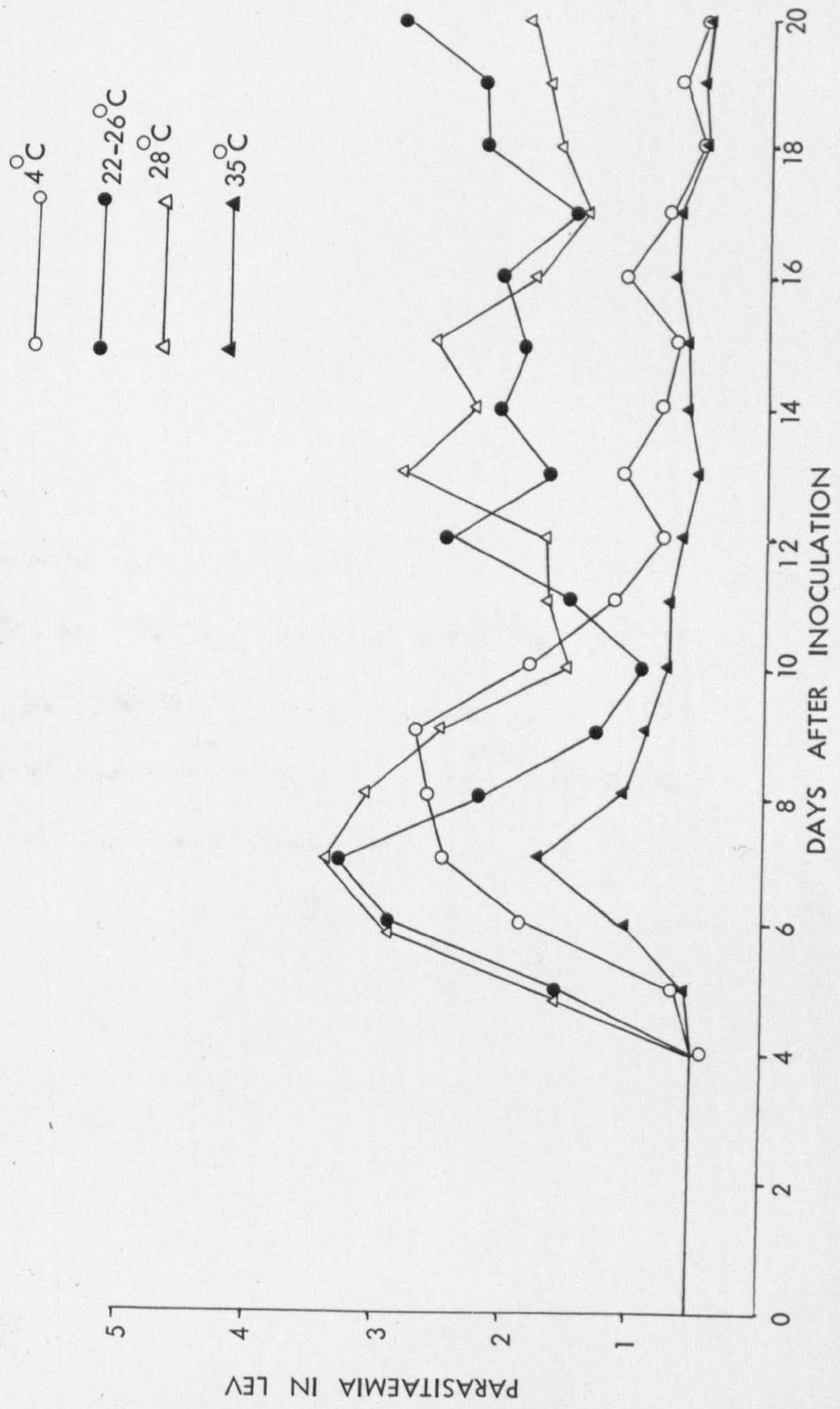
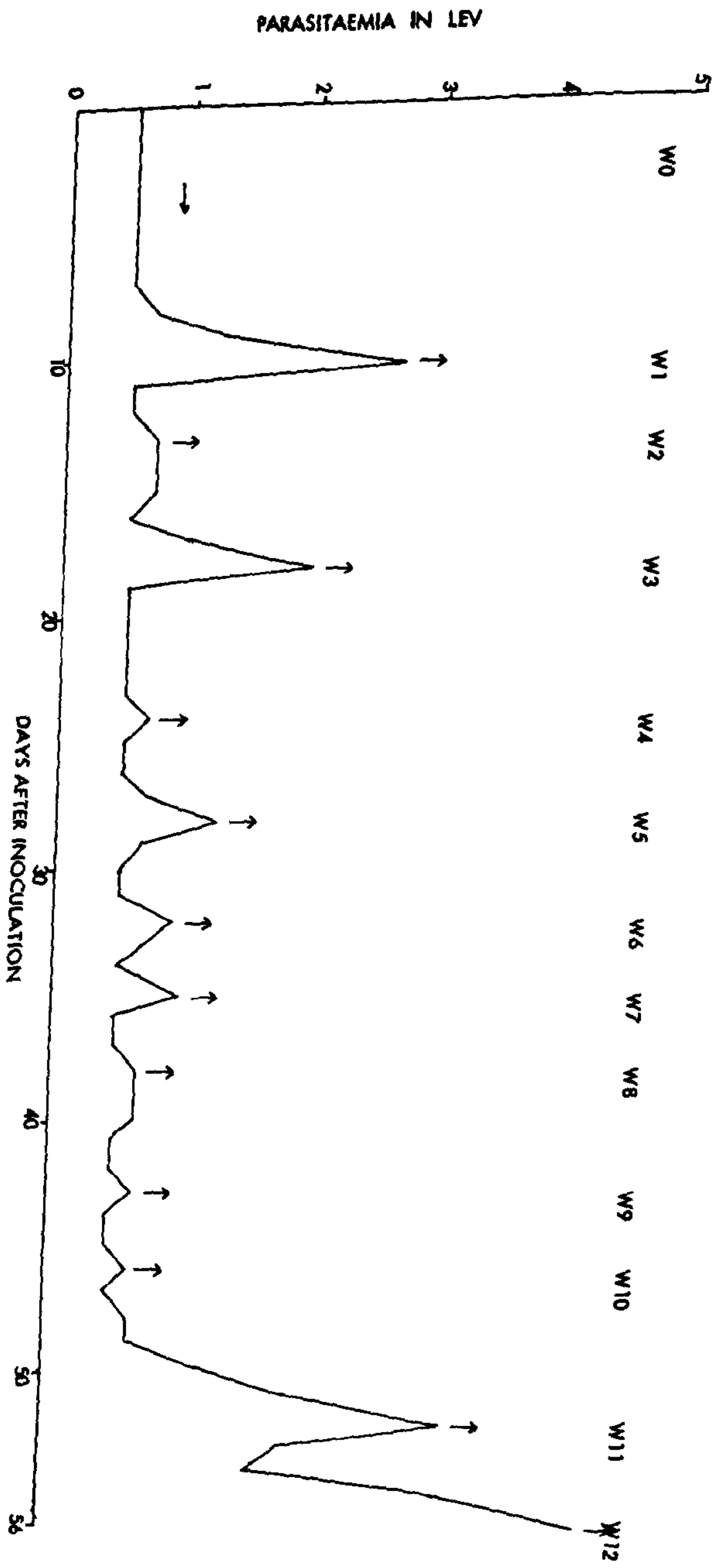


Figure 5. T. evansi Colombian strain LUMP 74.

The course of parasitaemia in a mouse and the times at which the antigenic variants were isolated.



Horse  
(natural infection  
in horse at Arauca  
Bogota, Colombia  
in April, 1967)

1 passage →

Mouse 1 passage Horse

1 passage ↓

Mouse  
(LUMP 74)  
or  
WO  
(inoculated into  
a mouse)

← 1 passage

Mouse (LUMP 62) ← 1 passage Mouse (TREU 381)

No. of passages\*  
in mice

Stabilate No.  
(LUMP)

Passages\*  
in mice

Stabilate Variant No. population  
(LUMP)

Day 10	→ 3	113		W1
Day 13	→ 4	114		W2
Day 18	→ 3	119	→ 4	W3
Day 24	→ 5	124		W4
Day 28	→ 5	125	→ 3	W5
Day 32	→ 6	135	→ 4	W6
Day 35	→ 7	132	→ 7	W7
Day 38	→ 6	134	→ 3	W8
Day 43	→ 10	144	→ 7	W9
Day 46	→ 5	137	→ 7	W10
Day 52	→ 2	133		W11
Day 56		131		W12

\* At < 3 day interval in mice.

Figure 6. T. evansi Colombian strain.

Diagram illustrating the derivation of stabilate LUMP 74 (WO) from a

natural infection in a horse and the subsequent isolation of variant popu-

lations (W1 to W12) from a mouse which was infected with LUMP 74.

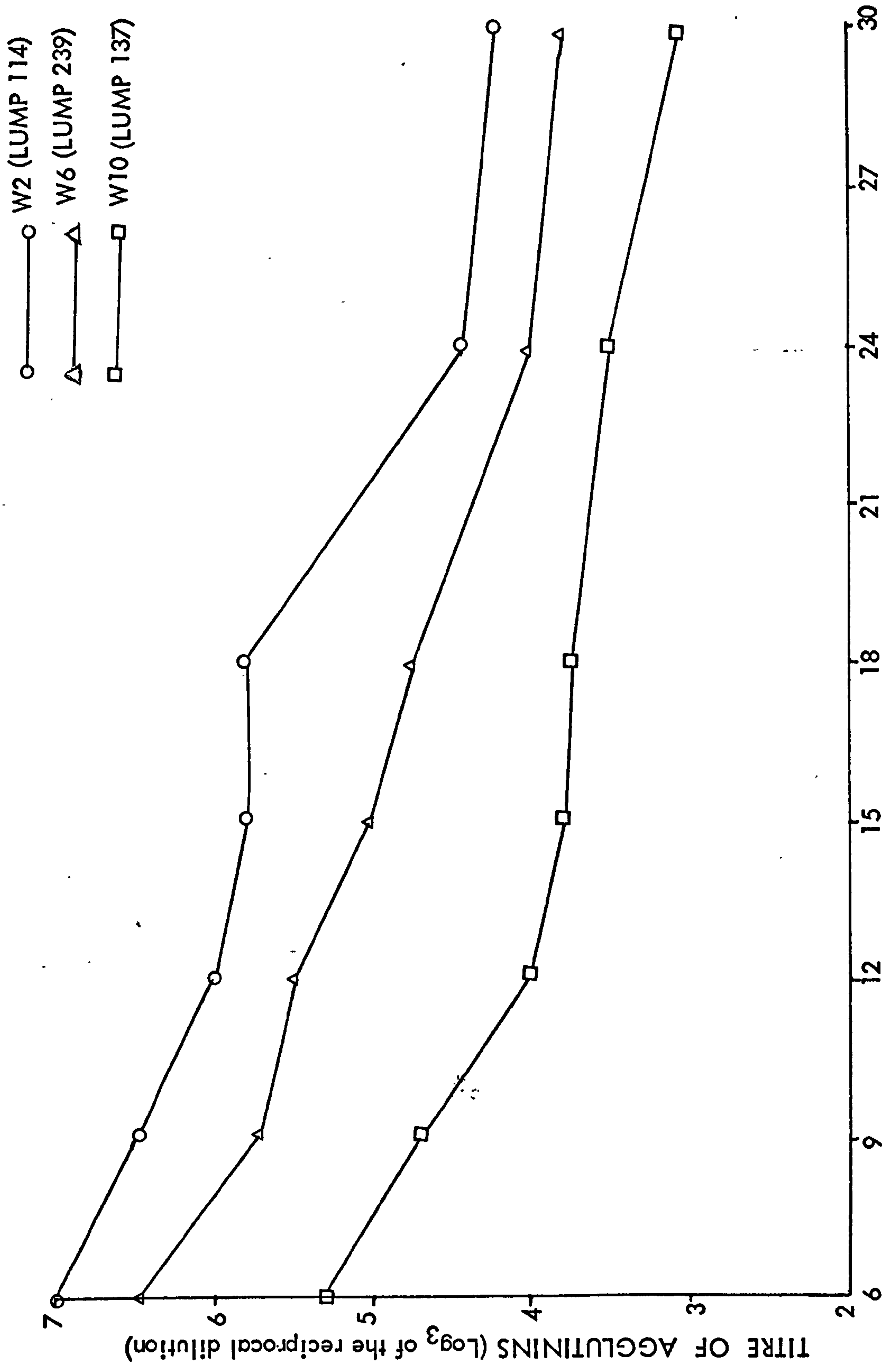
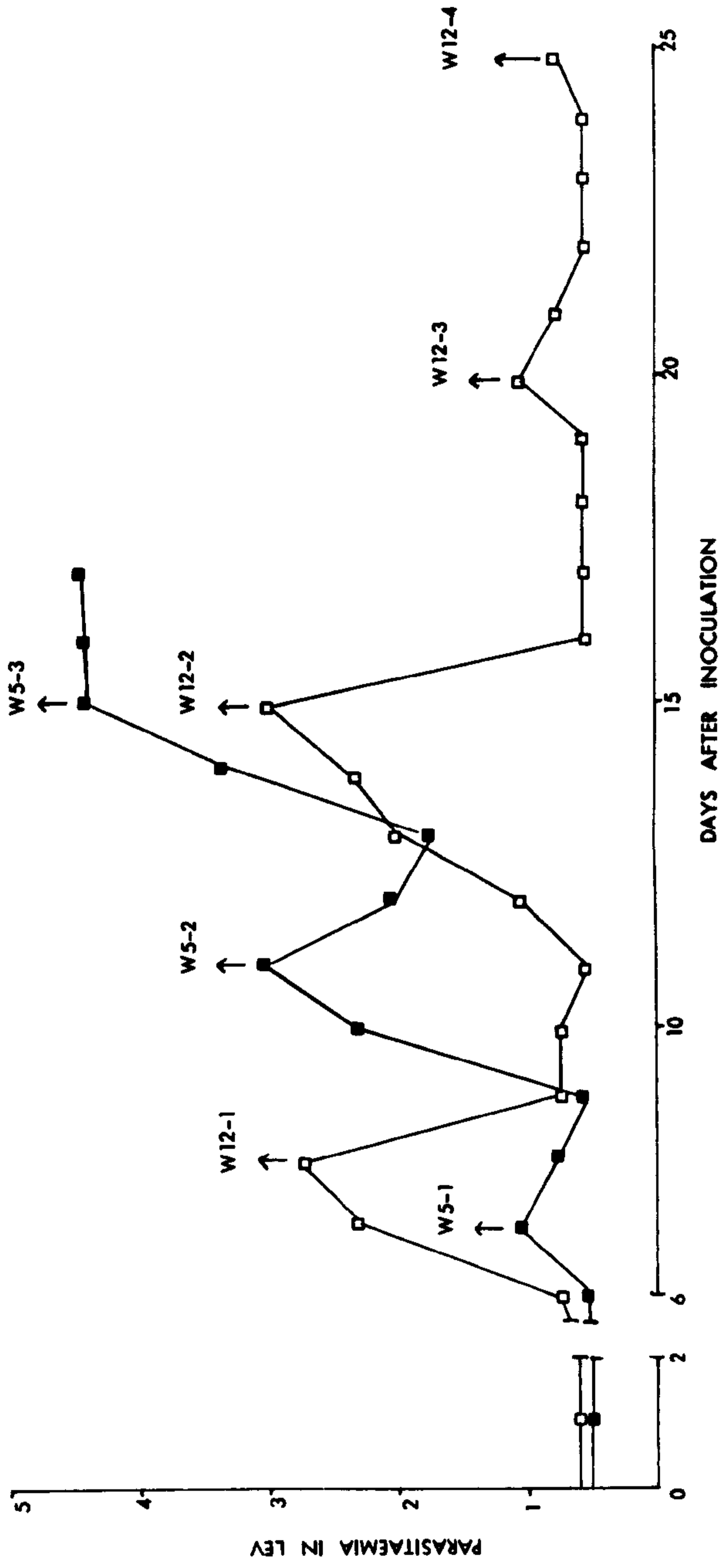


Figure 7. T. evansi Colombian strain: variant antigenic types W2, W6 and W10.

The curves represent the mean of agglutinin titres in homologous anti-sera collected from 4 to 6 mice at different periods after the cure of infections.

**Figure 8. T. evansi Colombian strain variant antigenic types W5 and W12. The course of parasitaemia in 2 mice which were inoculated with W5 (antilog 2.85 organisms derived from LUMP 238) and W12 (antilog 2.42 organisms derived from LUMP 131) respectively, and the times at which subsequent variant populations were isolated.**

■ W5 (LUMP 238)  
 □ W12 (LUMP131)





<b>W<sub>5</sub> (LUMP 238) inoculated into a mouse</b>	<b>No. of Passages in mice</b>	<b>Stabilate No. (LUMP)</b>	<b>Variant population</b>
<b>Day 7 (first wave)</b>	3 —————→	410	W <sub>5</sub> -1
<b>Day 11 (second wave)</b>	2 —————→	422	W <sub>5</sub> -2
<b>Day 15 (third wave)</b>	1 —————→	423	W <sub>5</sub> -3

---

<b>W<sub>12</sub> (LUMP 131) inoculated into a mouse</b>	<b>No. of Passages in mice</b>	<b>Stabilate No. (LUMP)</b>	<b>Variant population</b>
<b>Day 8 (first wave)</b>	2 —————→	Mouse died	
<b>Day 15 (second wave)</b>	2 —————→	425	W <sub>12</sub> -2
<b>Day 20 (third wave)</b>	4 —————→	480	W <sub>12</sub> -3
<b>Day 25 (fourth wave)</b>	8 —————→	527	W <sub>12</sub> -4

**Figure 9. *T. evansi* Colombian strain antigenic types W<sub>5</sub> and W<sub>12</sub>. Diagram illustrating the derivation of subsequent variant populations. Variants W<sub>5</sub>-1 to W<sub>5</sub>-3 were isolated from a mouse inoculated with W<sub>5</sub> antigenic type, and variants W<sub>12</sub>-2 to W<sub>12</sub>-4 were isolated from a mouse which was inoculated with W<sub>12</sub>.**

**Figure 10. T. evansi Colombian strain.**

Graphs illustrating the effect of challenge of W5-2 and W12-2 antigenic types on mice which were immunized against W1 antigenic type (for details of immunization and challenge see Table 12). The graphs show mean parasitaemias for groups of 5-6 mice.

**(a) Effect of challenge with W1 antigenic type**

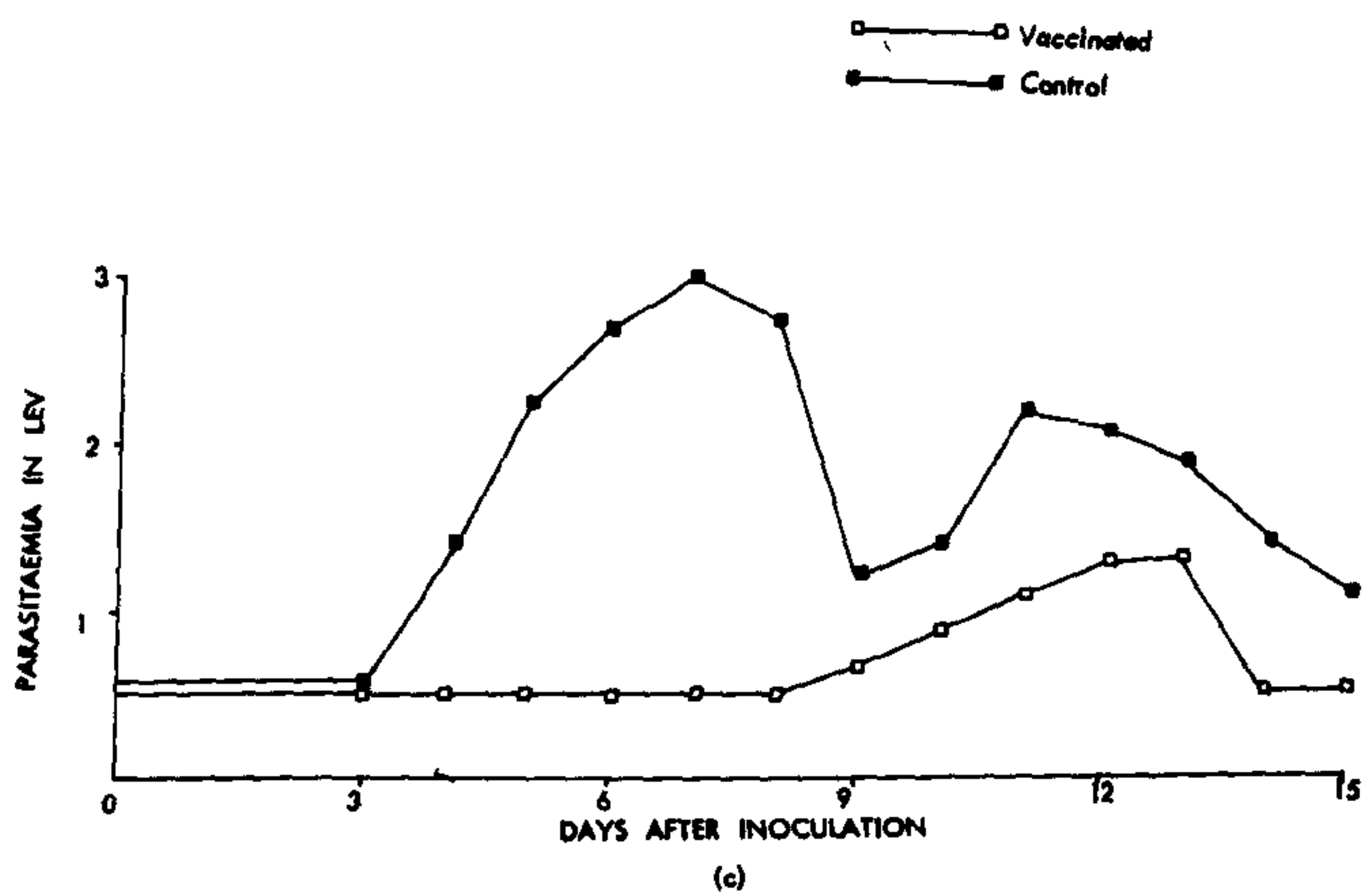
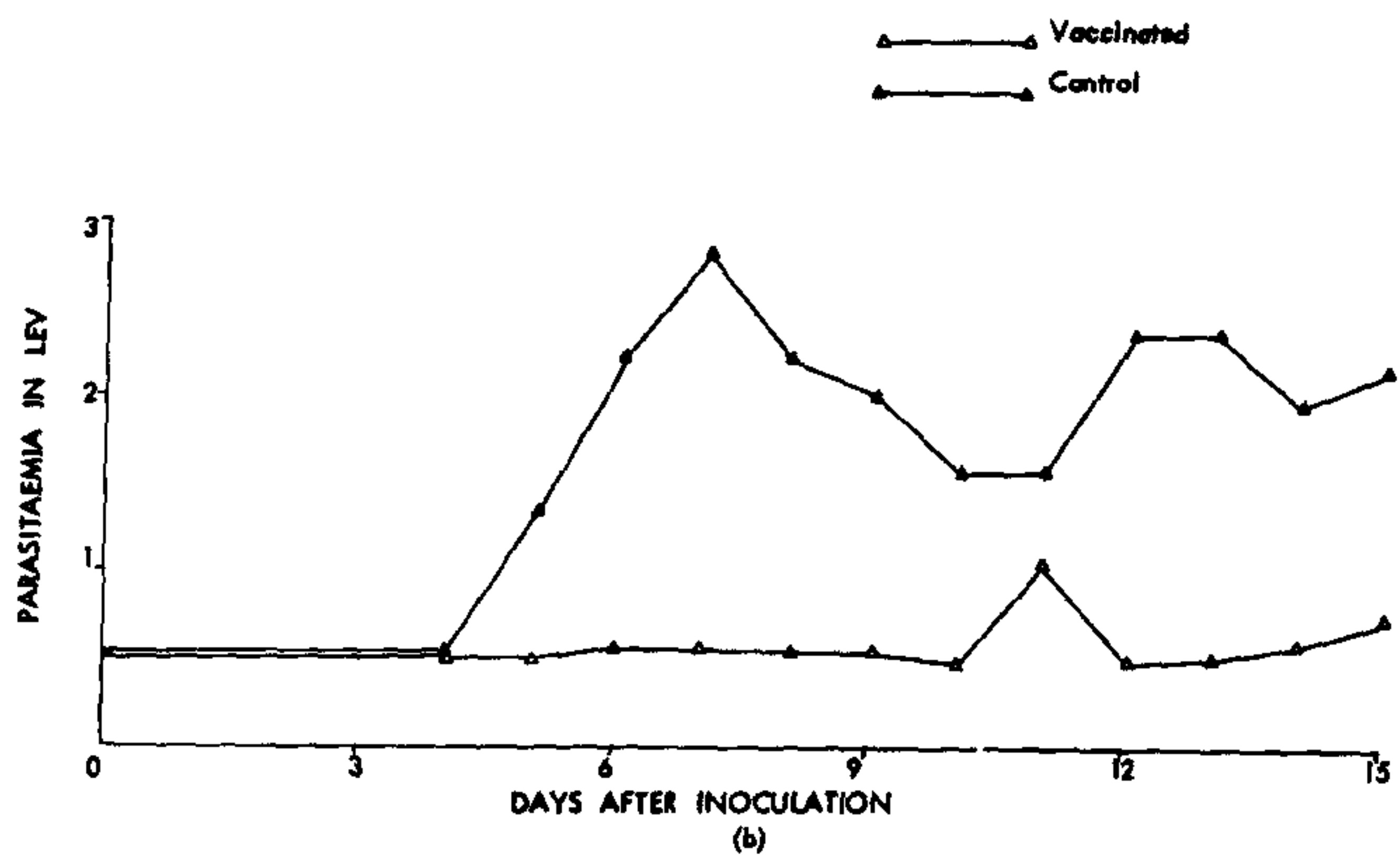
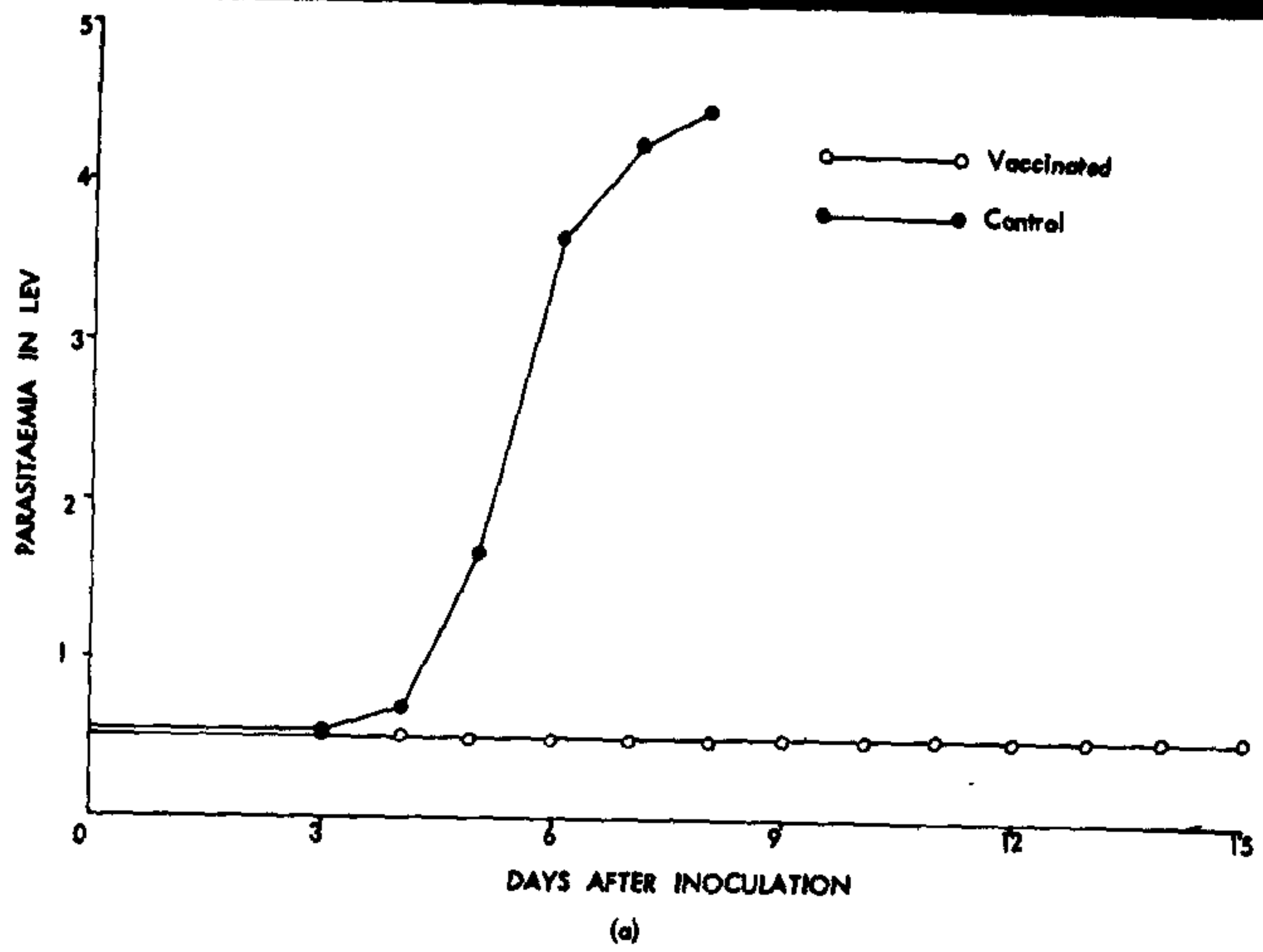
LUMP 113 (homologous population).

**(b) Effect of challenge by W5-2 antigenic type**

LUMP 422.

**(c) Effect of challenge by W12-2 antigenic type**

LUMP 425.



**Figure 11. Y. evansi Colombian strain.**

Graphs showing the effect of challenge with different variant antigenic types on mice immunised with antigenic type W1. Details of immunisation and challenge are given in Table 14. Curves represent the mean parasitaemias for groups of 5 or 6 mice.

(a) Effect of challenge with W1 antigenic type LUMP 113 (homologous population).

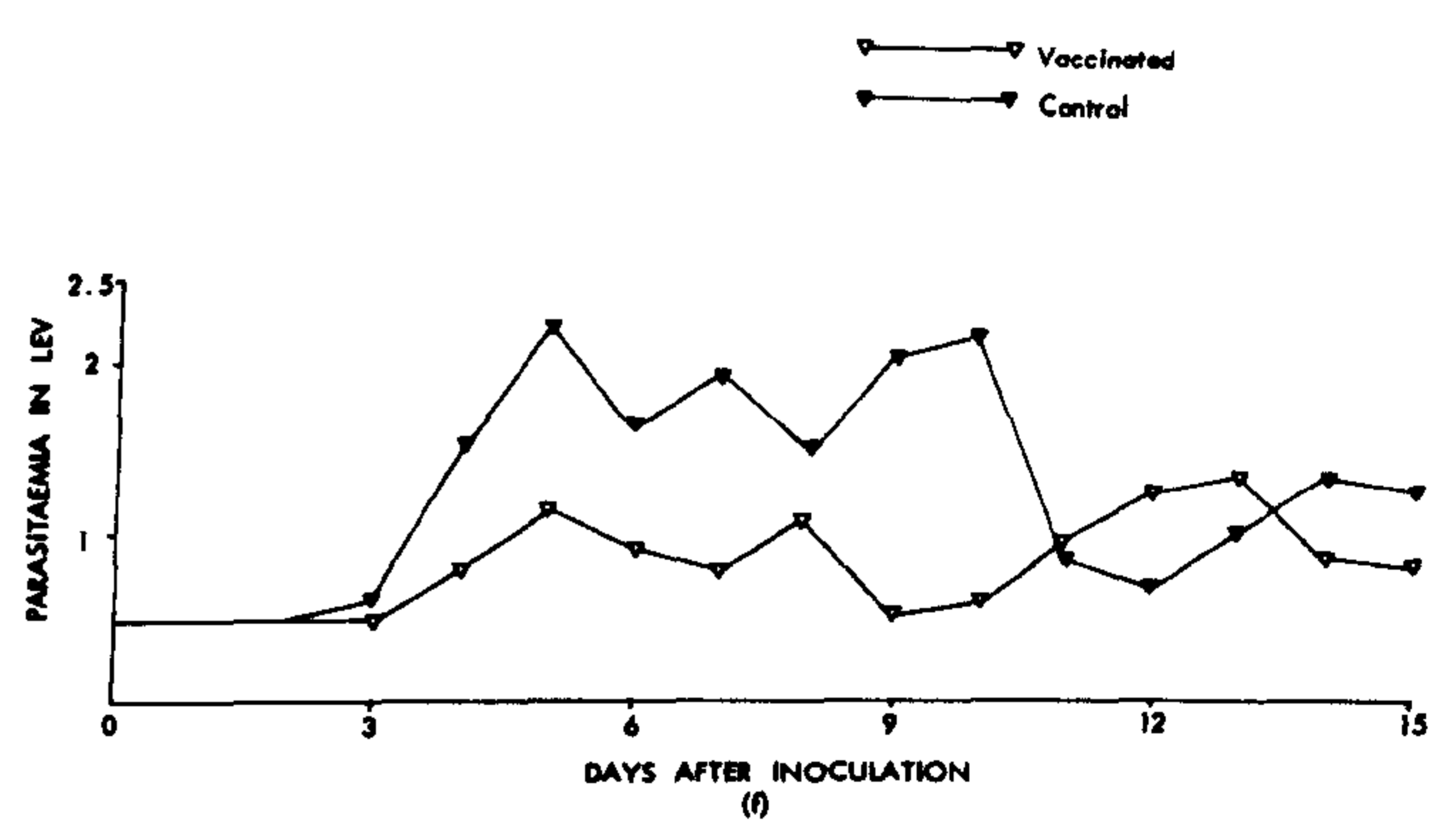
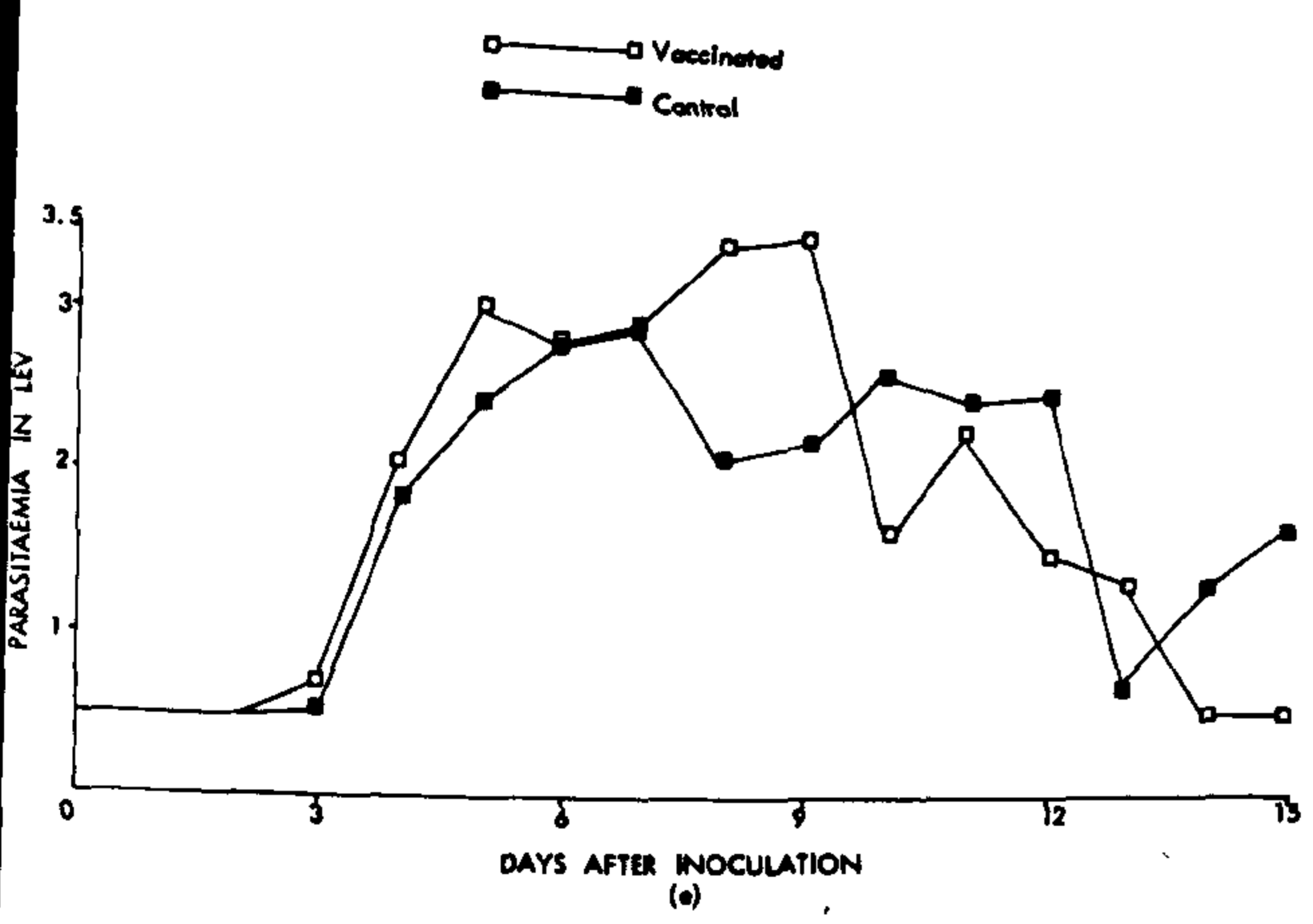
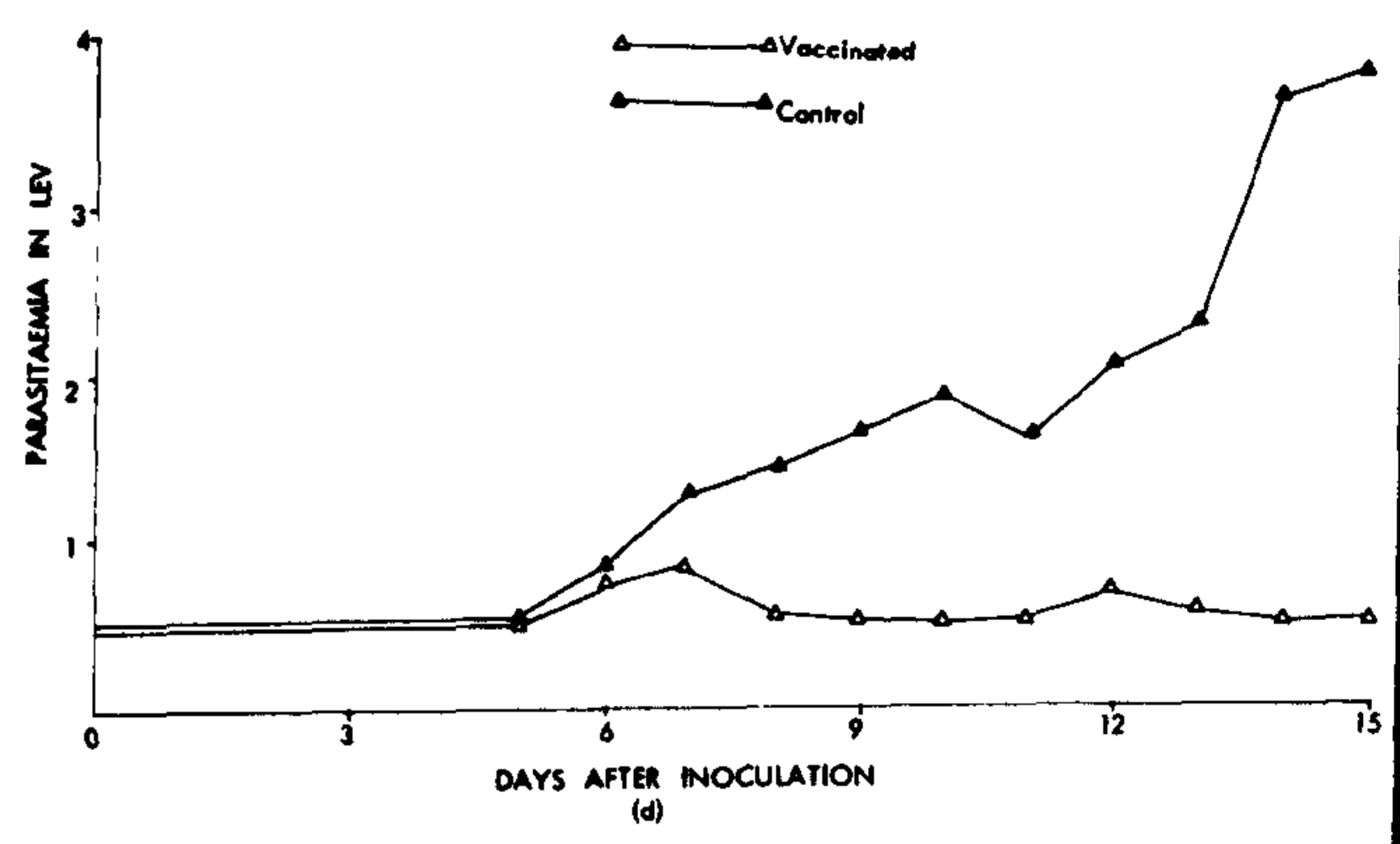
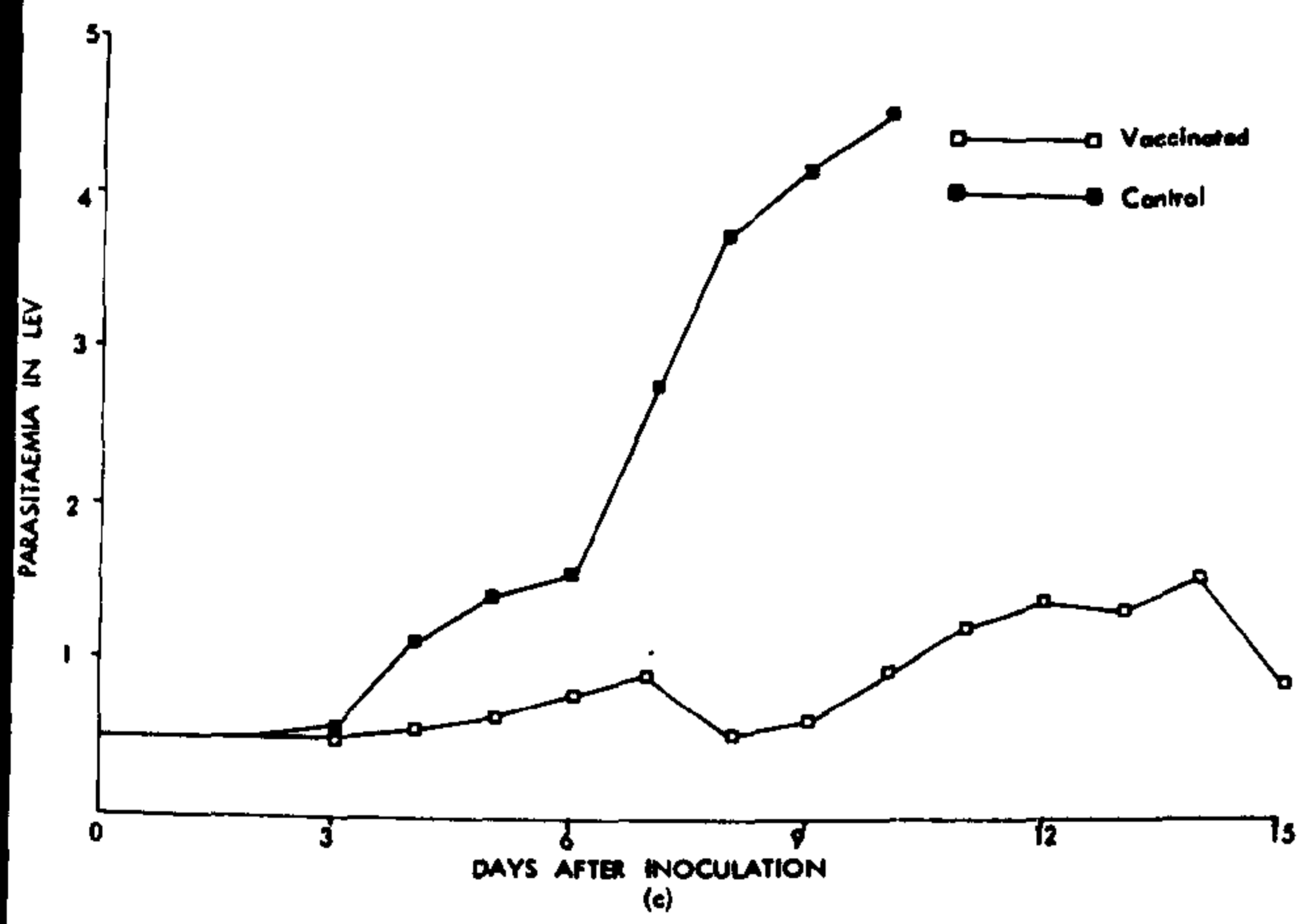
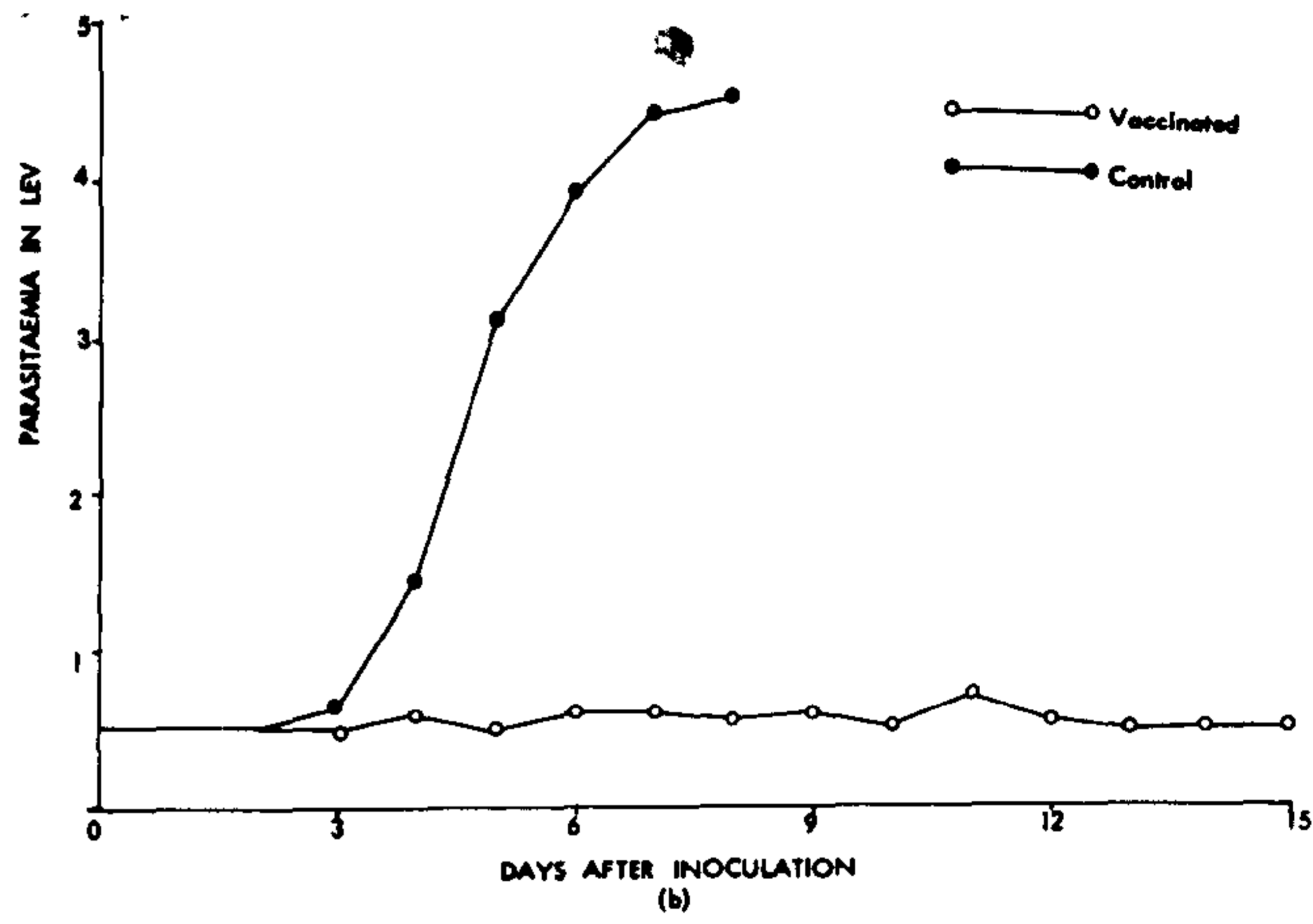
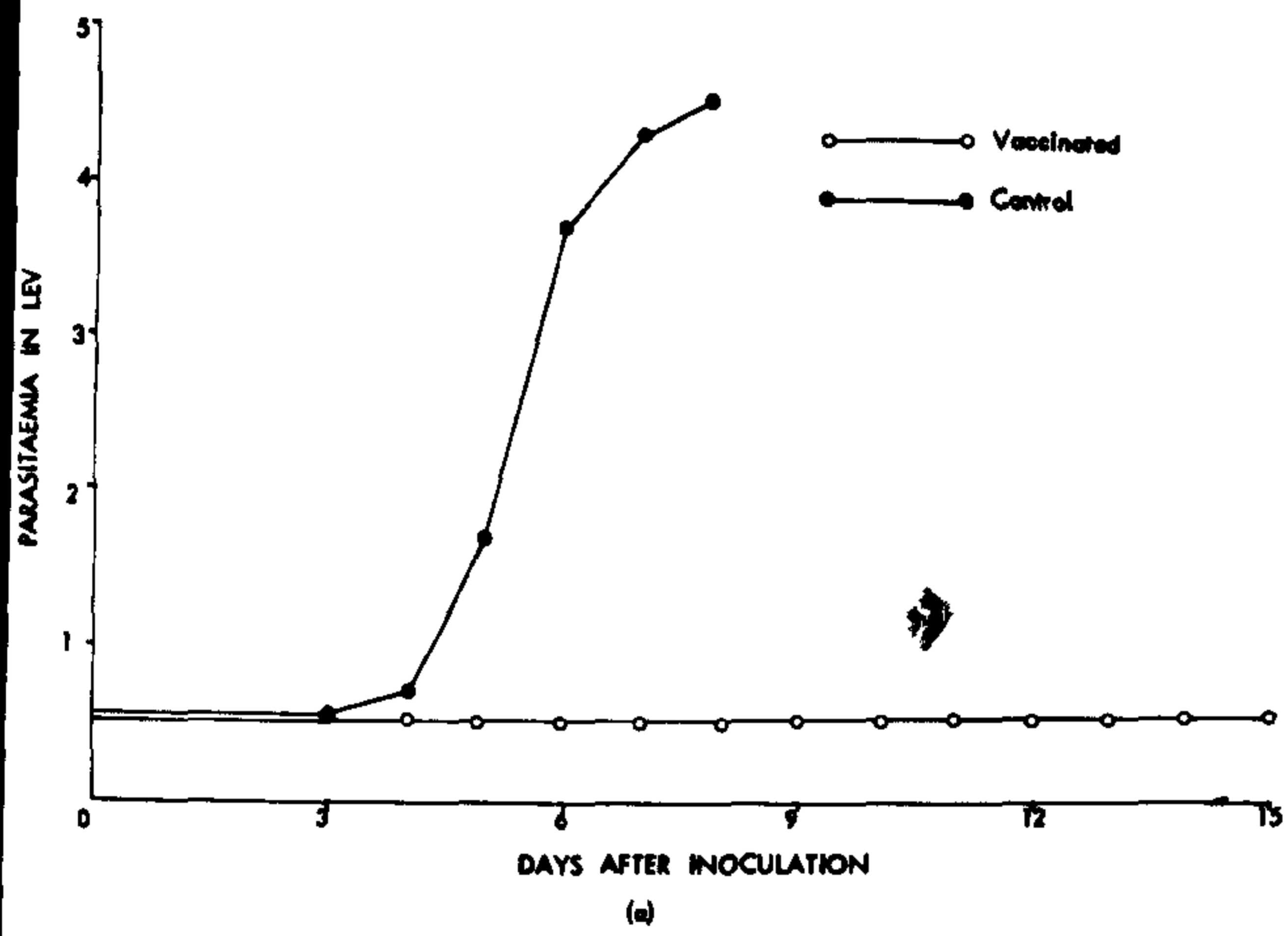
(b) Effect of challenge with W0 antigenic type LUMP 74 (original strain).

(c) Effect of challenge with W8 antigenic type LUMP 134.

(d) Effect of challenge with W3 antigenic type LUMP 119.

(e) Effect of challenge with W4 antigenic type LUMP 124.

(f) Effect of challenge with W12 antigenic type LUMP 131.



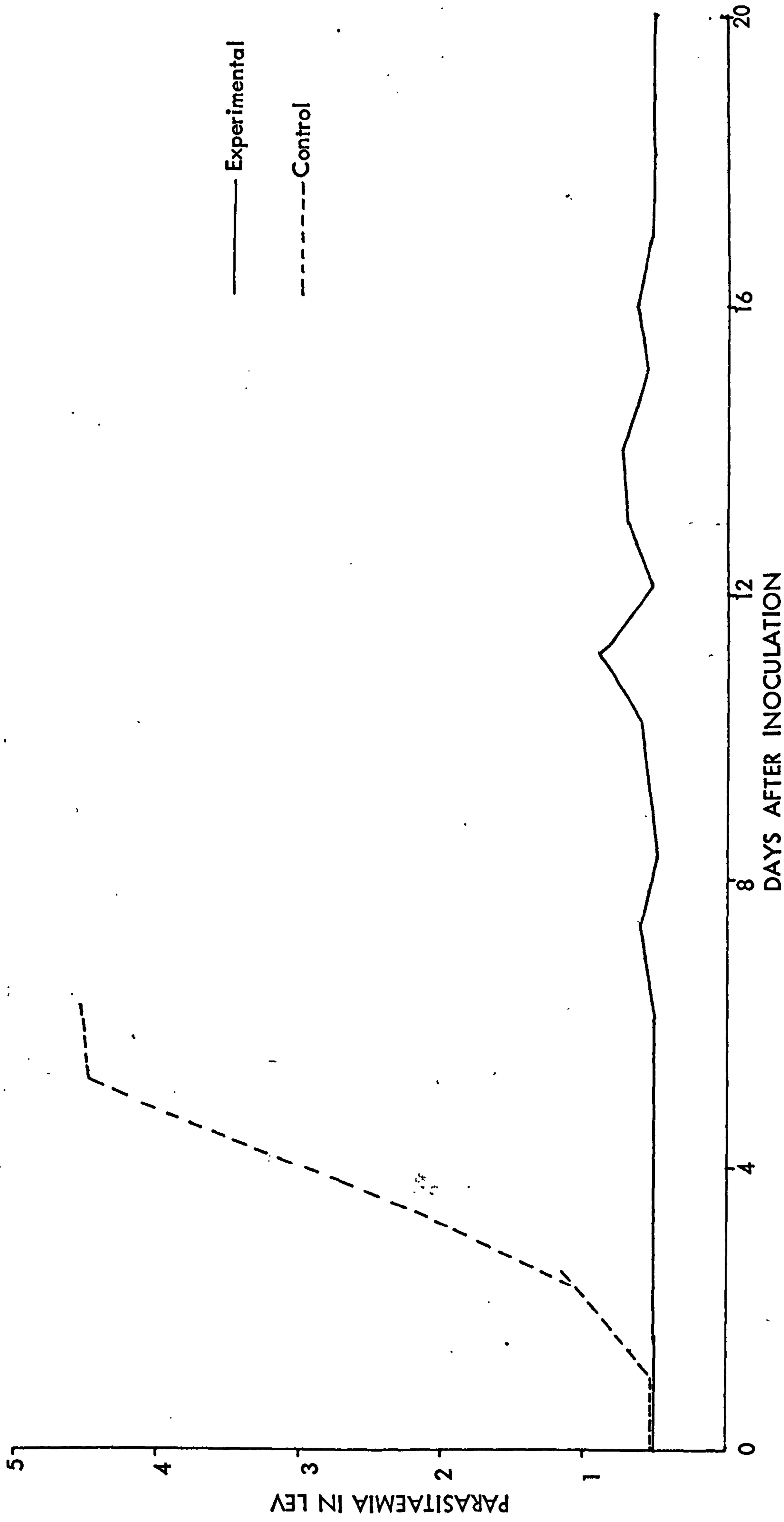
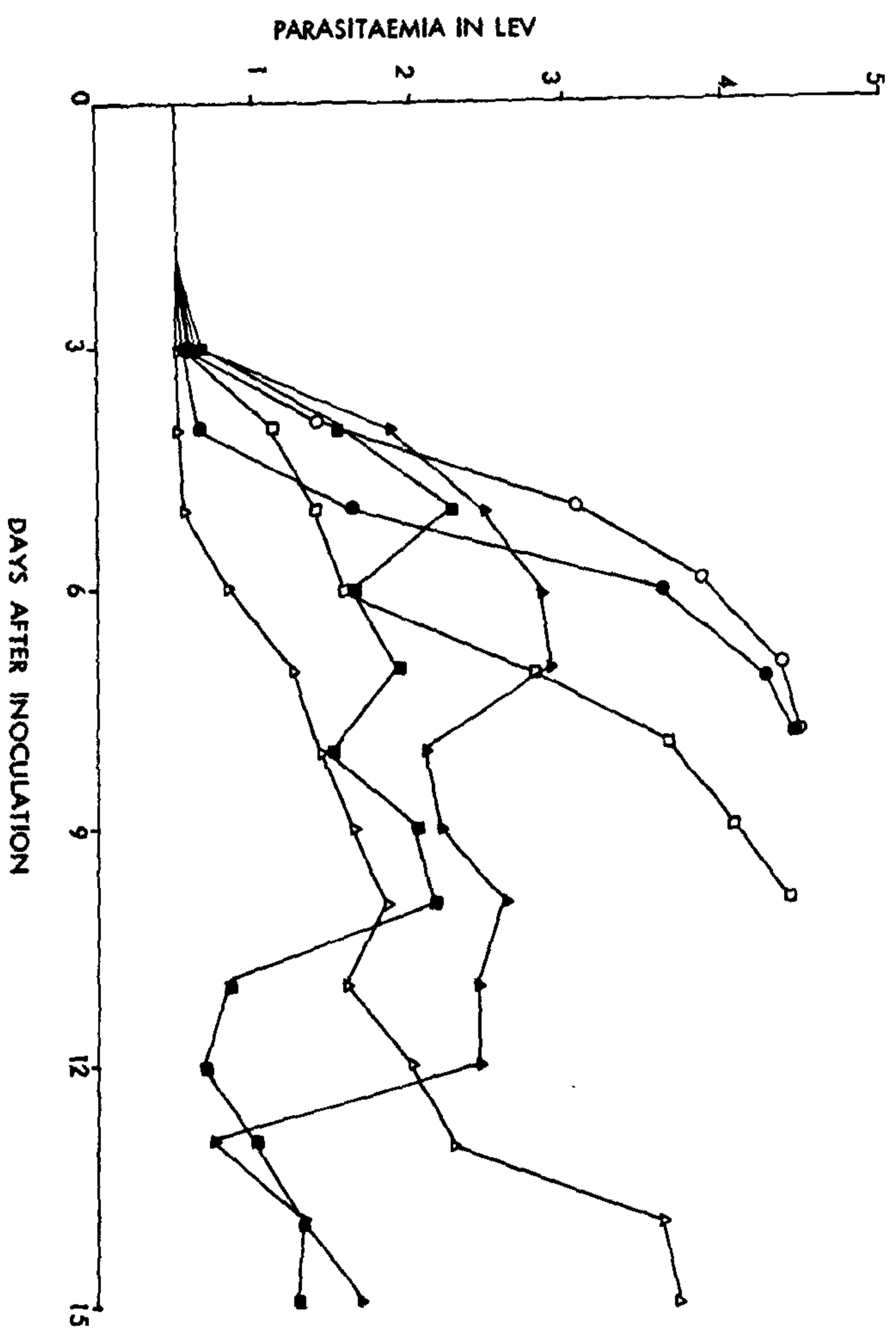


Figure 12. T. evansi Colombian strain, antigenic type W1 (LUMP 113). The passive transfer of immunity by the administration of antiserum. 0.2ml. of antiserum against antigenic type W1 was initially administered (i/v) to each of the 5 mice. One hour later, challenge inocula of antilog 4.7 trypanosomes, derived from stabilate LUMP 113, were administered (i/p). The curves represent mean parasitaemias.

**Figure 13. T. evansi Colombian strain.**

A comparison of the courses of parasitaemia of the original strain (W0) and different variant antigenic types (W1, W3, W4, W8, and W12) in mice. Antilog 3.7 trypanosomes of each antigenic type, derived from stabilate populations were administered i/p. Curves represent the mean parasitaemias for groups of 5 or 6 mice.



- W0 (LUMP 74)
- W1 (LUMP 113)
- △ W3 (LUMP 119)
- ▲ W4 (LUMP 124)
- W8 (LUMP 134)
- W12 (LUMP 131)



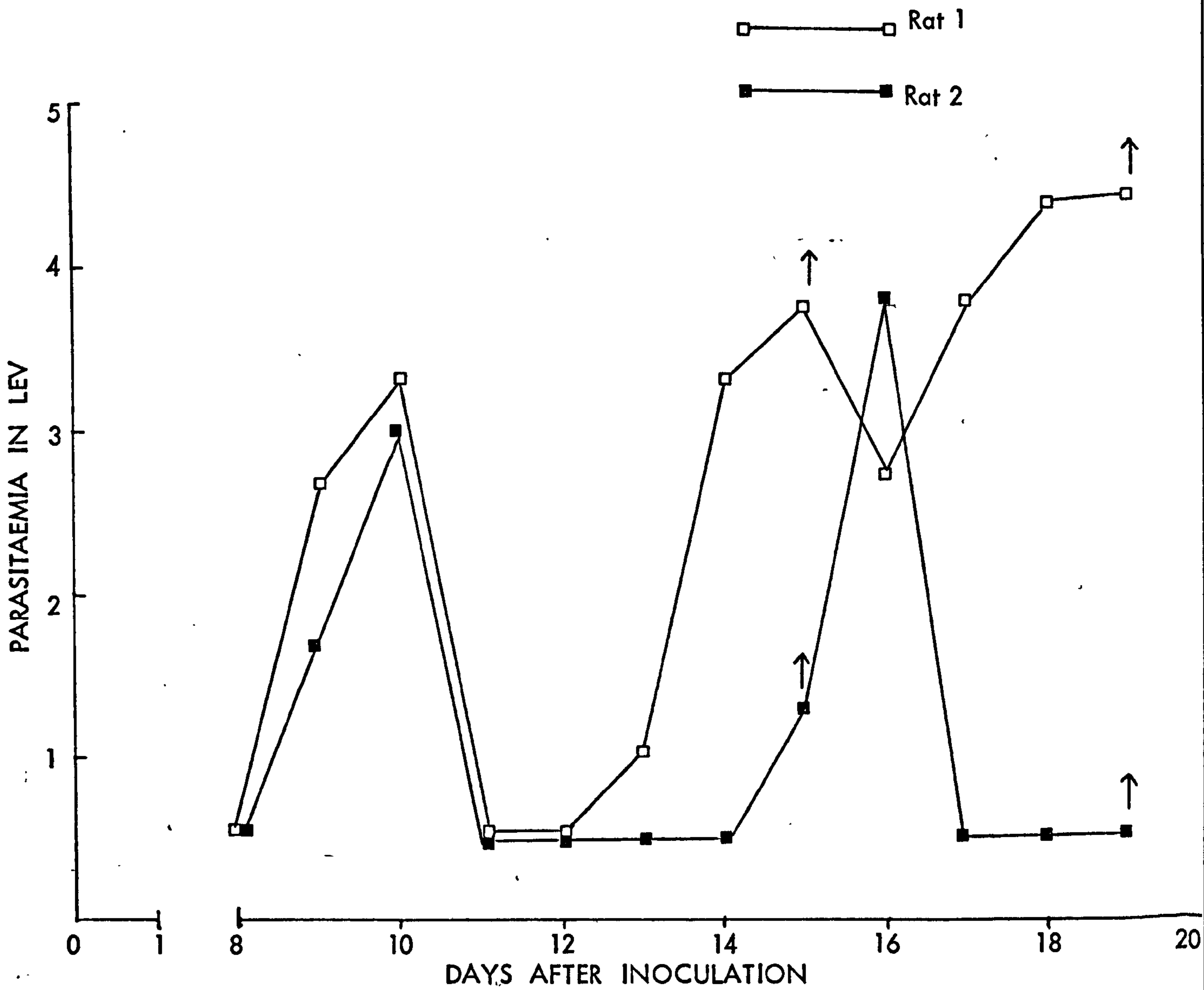


Figure 14. T. evansi Colombian strain. Courses of parasitaemia in 2 rats inoculated with antilog 4.6 organisms or antilog 4.1 ID 63 of Colombian strain LUMP 74 (W0). Arrows pointing upwards indicate the days on which sera were collected.

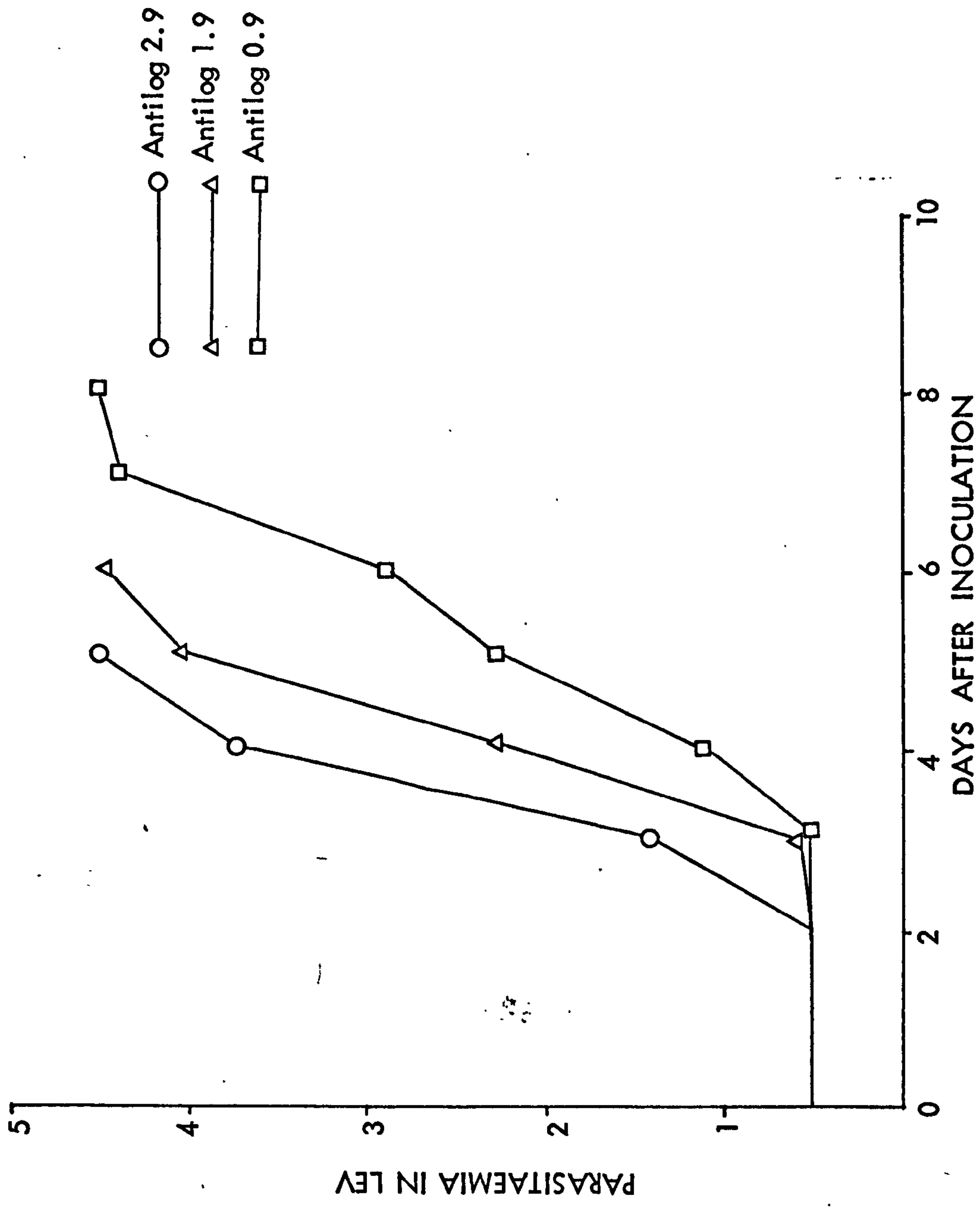
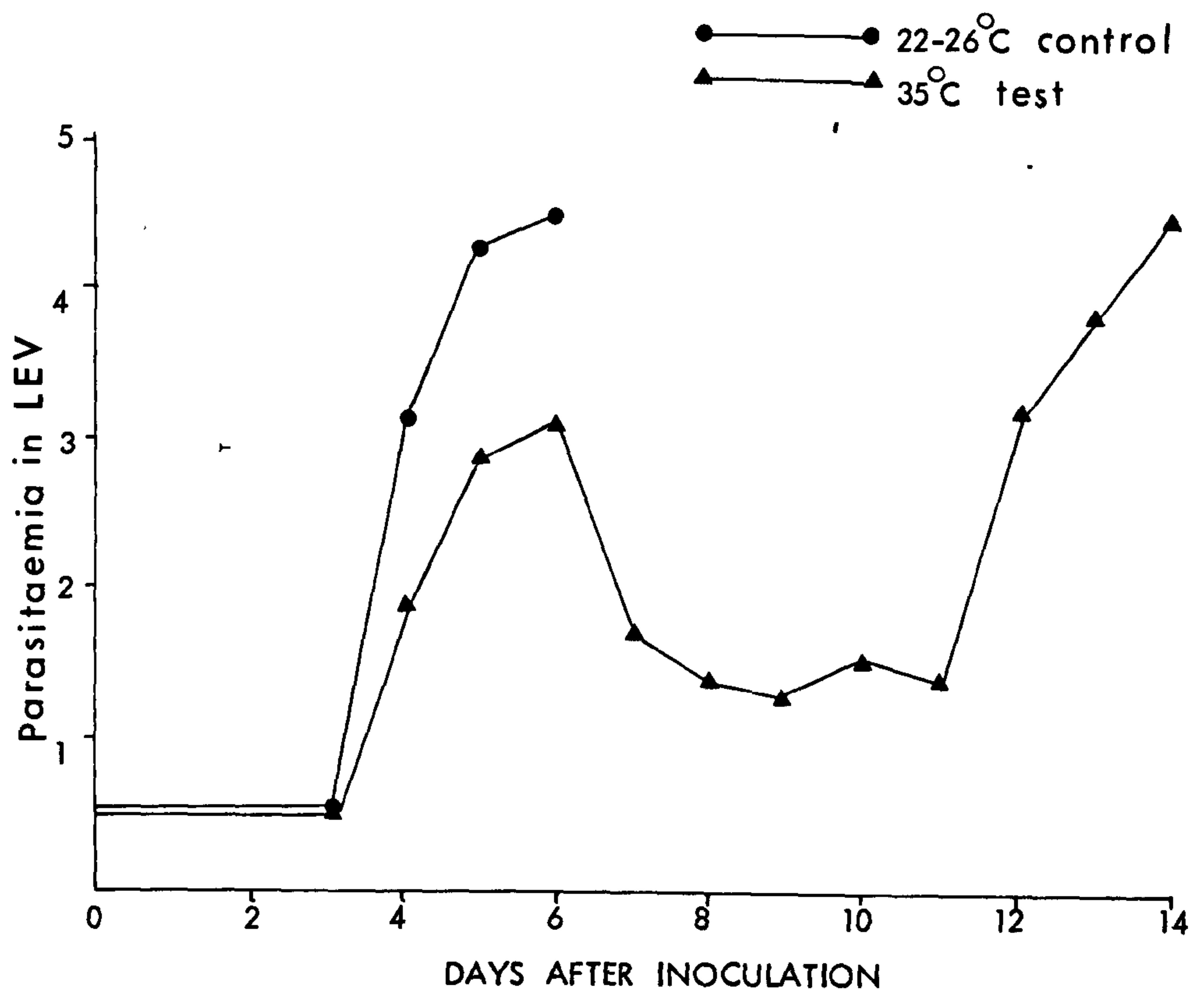


Figure 15. T. evansi N.S. strain. The course of parasitaemia in mice administered different inocula of stabilate LUMP 46. Curves represent the mean parasitaemia for groups of 6 mice.

**Figure 16. Effect of 35°C on the course of parasitaemia of the M.S. strain LUMP 46 in mice. Curves represent the mean parasitaemia for groups of 12 mice inoculated with antilog 2.9 organisms or antilog 2.6 ID 63.**



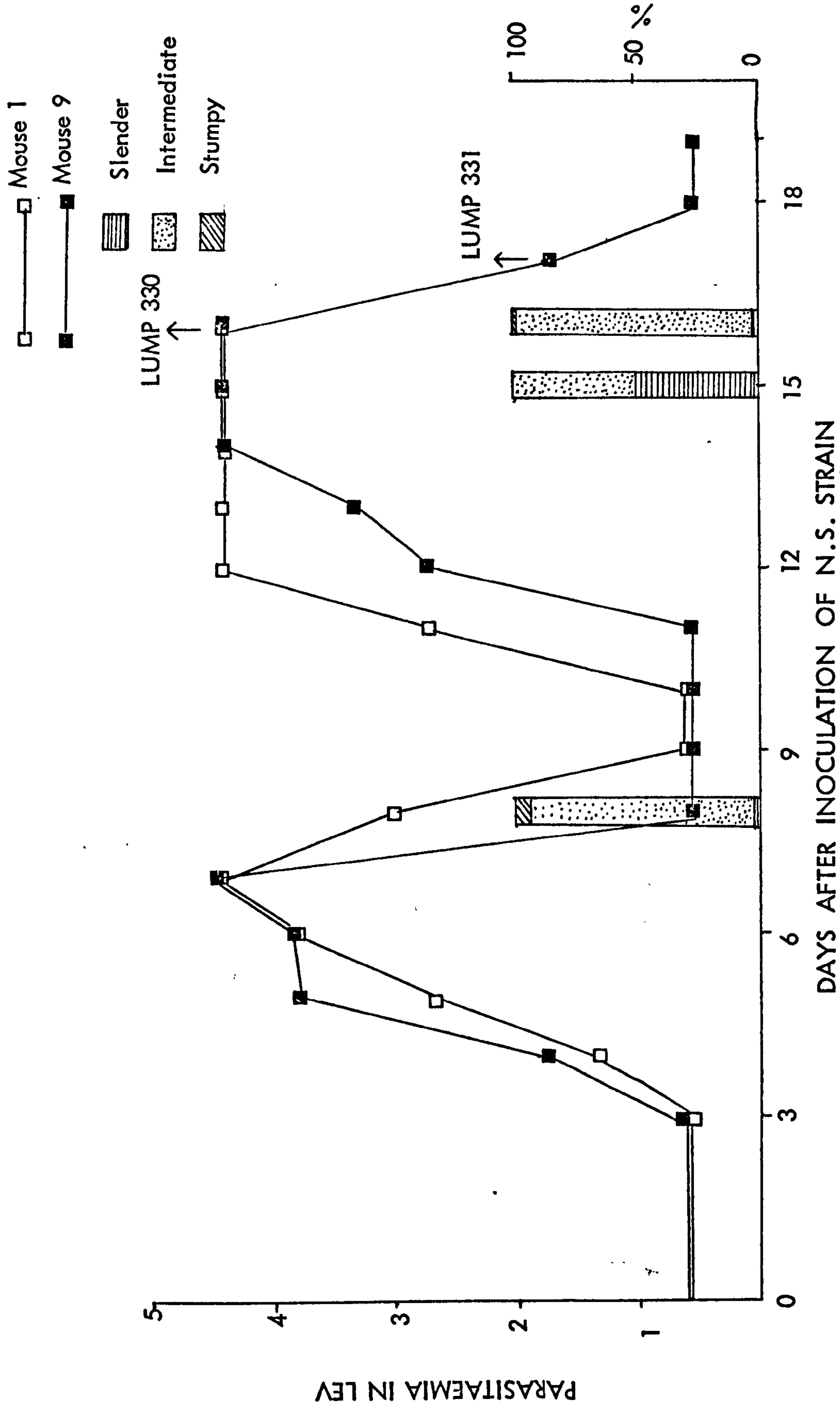


Figure 17. Development of pleomorphism in *T. evansi* N.S. strain LUMP 46 in 2 mice which had been immunized against the S.A.K. strain (LUMP 66). Details of immunization and challenge are given in Table 22. The curves represent the course of parasitaemia in mouse 1 and 9. Histograms show the percentage of different morphological forms. Arrows pointing upwards indicate the times at which pleomorphic populations were isolated.

Wave of parasitaemia	<sup>T</sup> <sub>0</sub> (LUMP 46) inoculated in a mouse	No. of mouse passages	Stabilate No. (LUMP)	Population
1	Day 10			
2	Day 12	→ 2 →	204	T <sub>2</sub>
3	Day 21	→ 1 →	212	T <sub>3</sub>
4	Day 37	→ 1 →	215	T <sub>4</sub>
5	Day 52		223	T <sub>5</sub>

Figure 18. T. evansi N.S. strain LUMP 46. Diagram illustrating the details of isolations of variant populations (T<sub>2</sub> to T<sub>5</sub>) from a mouse which was maintained at 35°C. Antilog 2.9 organisms, representing antilog 2.6 ID 63, were administered to this mouse.

**Figure 19. Development of pleomorphism in T. evansi N.S. strain clone (LUMP 85).**

The figure illustrates the course of parasitaemia in mouse 1 (Table 27) and the morphology of trypanastigotes from the mouse. The mouse was made immune to S.A.K. strain clone (LUMP 55) and subsequently challenged with N.S. strain clone (LUMP 85). Details of immunization and challenge are given in Table 27.

(a) Curves represent the course of parasitaemia in the mouse. Histograms show the percentage of different forms on days when pleomorphism occurred. Arrows indicate the times at which pleomorphic populations (LUMP 280, 315) were isolated.

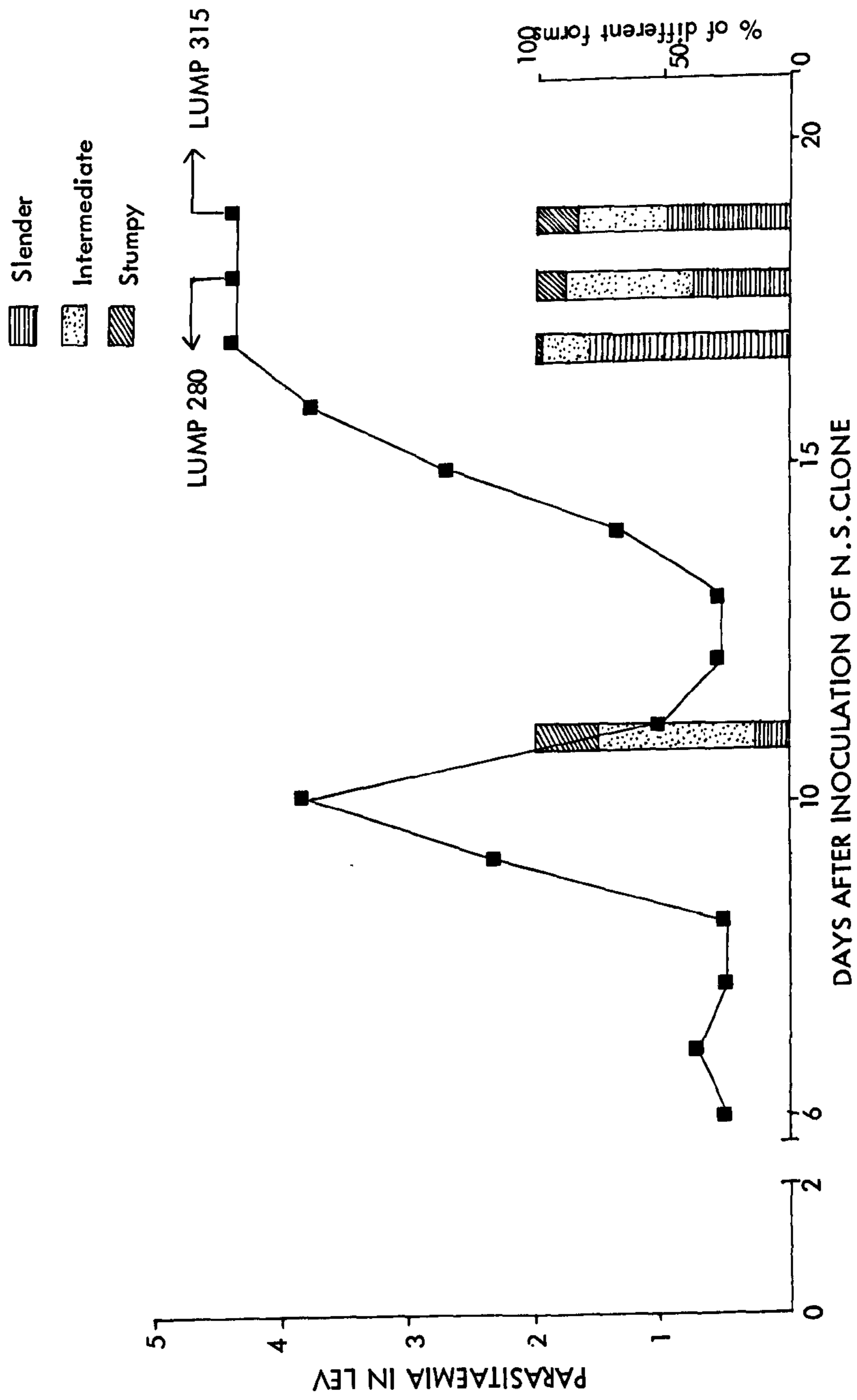


Figure 19 (a)



Figure 19. (b) Drawings of trypomastigotes from the mouse on different days of infection. In the following description the day of the experiment (Table 27) is given. The day after inoculation of N.S. clone is written within brackets. The arrows indicate the possible sequence of morphological changes.

Monomorphic forms before development of pleomorphism

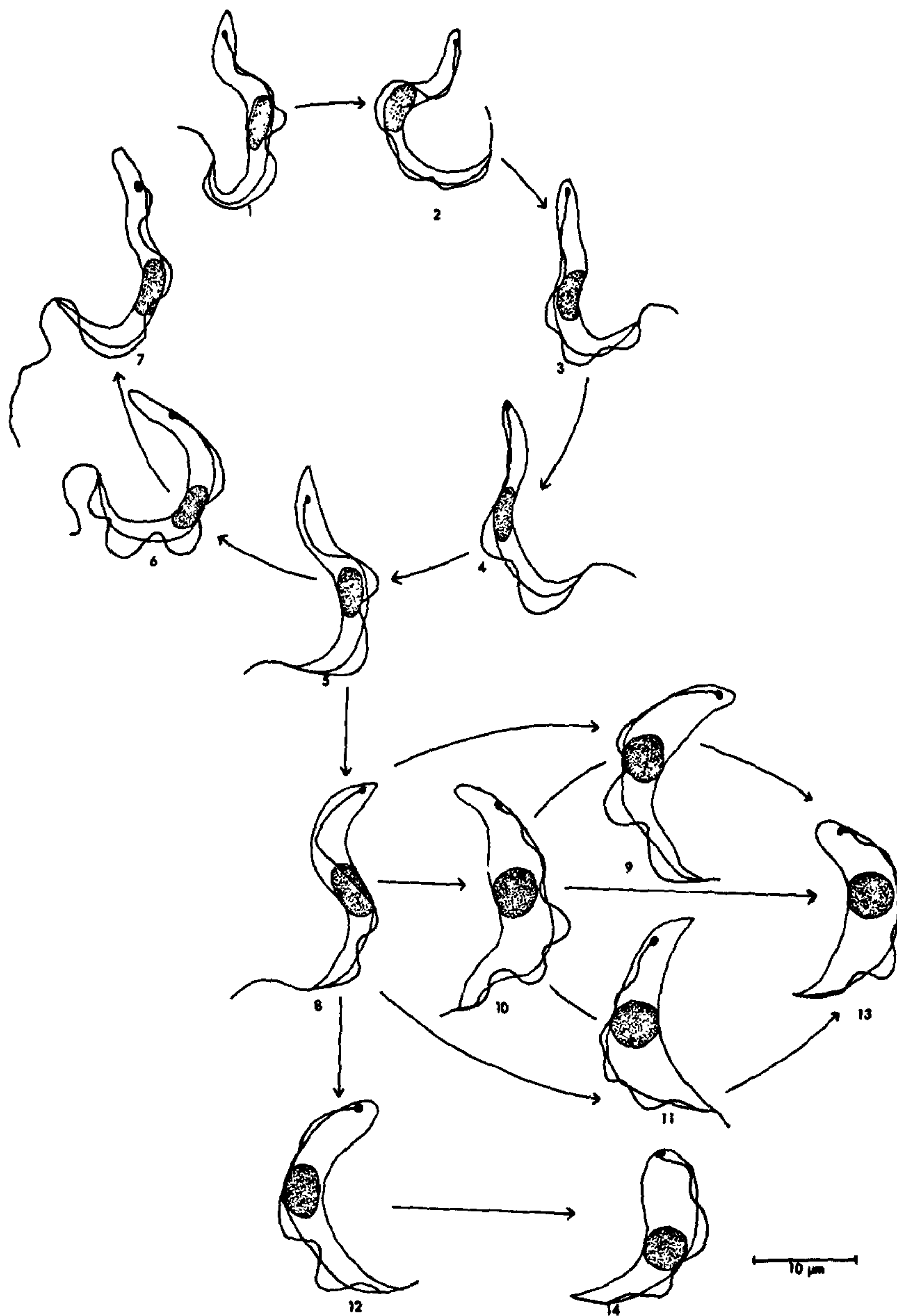
1. Day 70 (11). Slender form - kinetoplast subterminal; short, free flagellum.

Stages of development towards the slender form

- 2-4. Day 70 (11). Stages showing backward migration of kinetoplast.
5. Day 71 (12). Long intermediate forms - posterior end pointed, kinetoplast subterminal.
6. Day 77 (18). Slender form - free flagellum of medium length.
7. Day 79 (20). Slender form - long, free flagellum.

Stages of development towards the stumpy form

8. Day 71 (12). Intermediate form - with kinetoplast nearer to posterior end, posterior end pointed.
9. Day 71 (12). Short intermediate form - nucleus rounded; short, free flagellum.
10. Day 78 (19). Short intermediate form - posterior end rounded, kinetoplast subterminal.
11. Day 79 (20). Short intermediate form - posterior end pointed on one side, kinetoplast subterminal, nucleus rounded.
12. Day 77 (18). Short intermediate form - kinetoplast towards posterior end, nucleus oval, short, free flagellum.
13. Day 79 (20). Stumpy form - kinetoplast nearer to posterior end, free flagellum absent, thick body.
14. Day 78 (19). Stumpy form - posterior end rounded, kinetoplast terminal, nucleus round, thick body.



19b

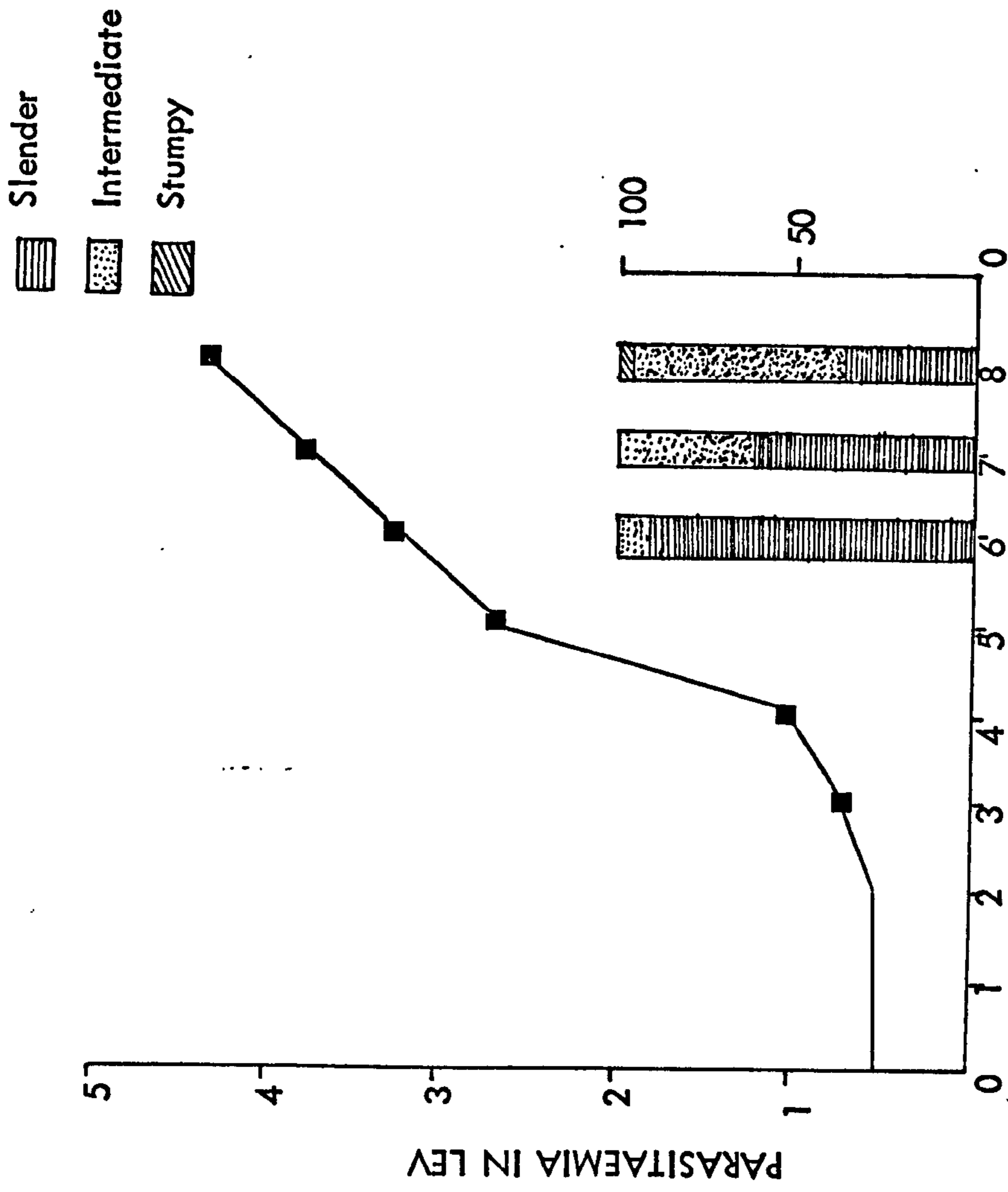


Figure 20. T. evansi N.S. pleomorphic variant LUMP 315. Figure shows the course of parasitaemia in a mouse which was inoculated with antigen 2.17 organisms derived from the stabilate, and the percentage of stumpy forms on the last day of infection.

Figure 21. T. evansi N.S. pleomorphic variant LUMP 172. The course of parasitaemia and percentage of slender, intermediate and stumpy forms in normal, splenectomized and irradiated mice. Each curve represents the mean parasitaemia of 10 mice, inoculated with antilog 3.25 organisms derived from a stabilate population. Histograms represent the mean percentage of slender, intermediate and stumpy forms from 4 of the mice. In each mouse at least 100 organisms were examined each day.

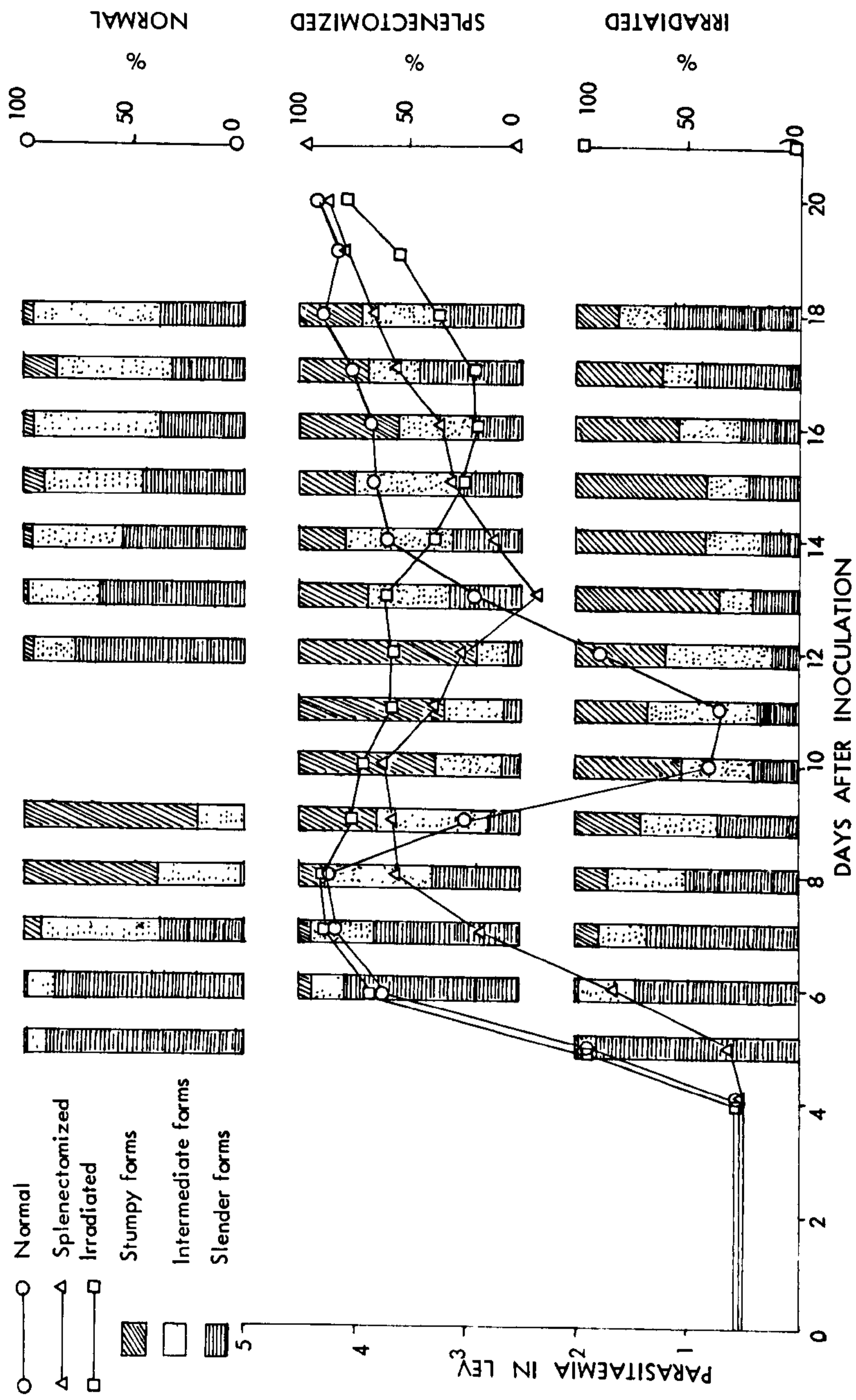
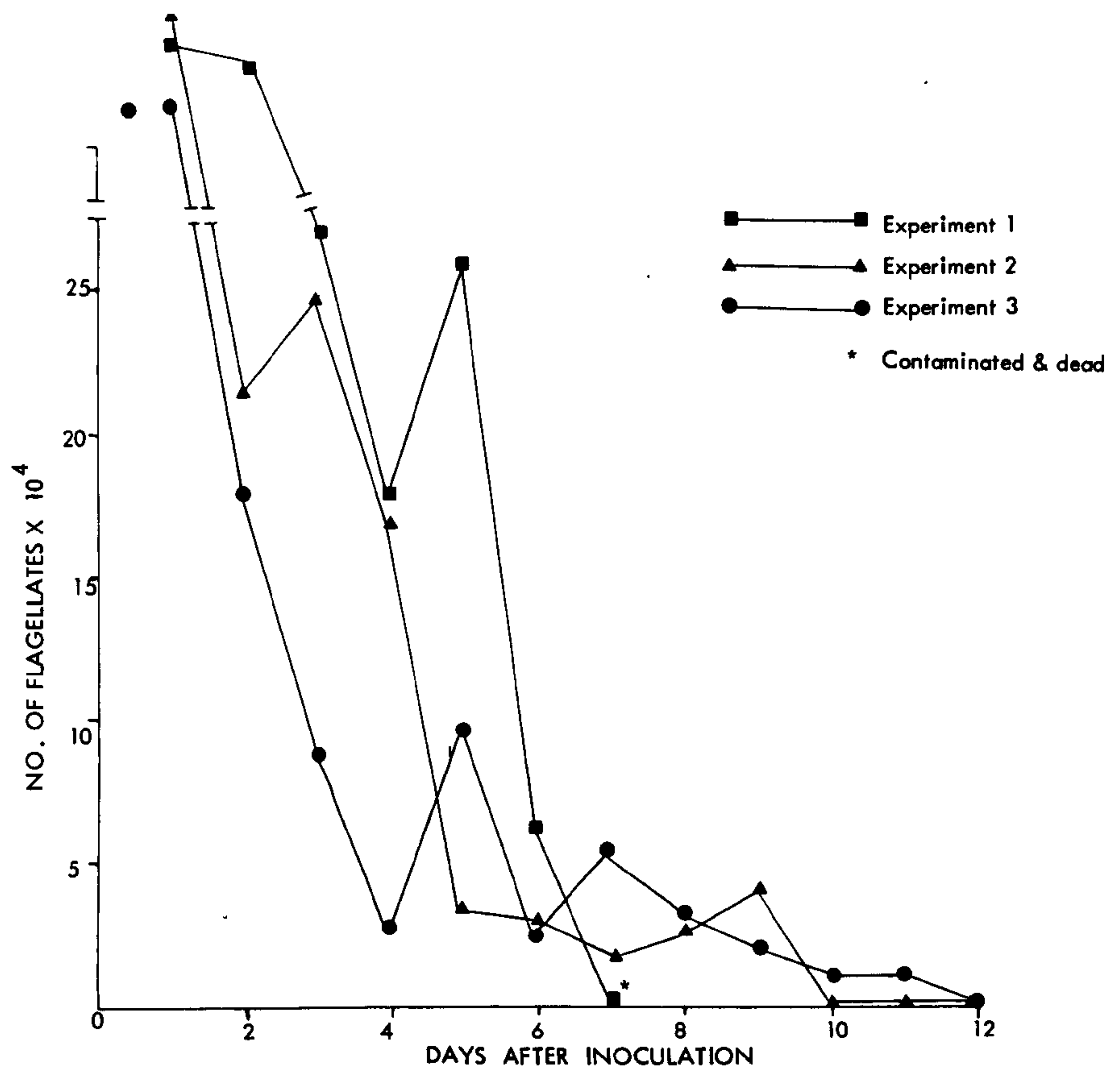


Figure 22. T. evansi N.S. pleomorphic variant LUMP 172.

Graph shows the growth rate of the population in 4N medium. The media were inoculated with infected mouse blood. The curves represent the mean of 2 samples.



**Figure 23. T. evansi N.S. pleomorphic variant LUMP 172.**

**Drawings of trypanastigote forms from 4N medium.**

**1-5. Trypanastigote forms from 8 day old culture.**

**1,2. Trypanastigote forms showing kinetoplast at varying positions between the nucleus and the posterior end.**

**3. Thin trypanastigote form.**

**4. Trypanastigote form showing divided kinetoplast but intact nucleus.**

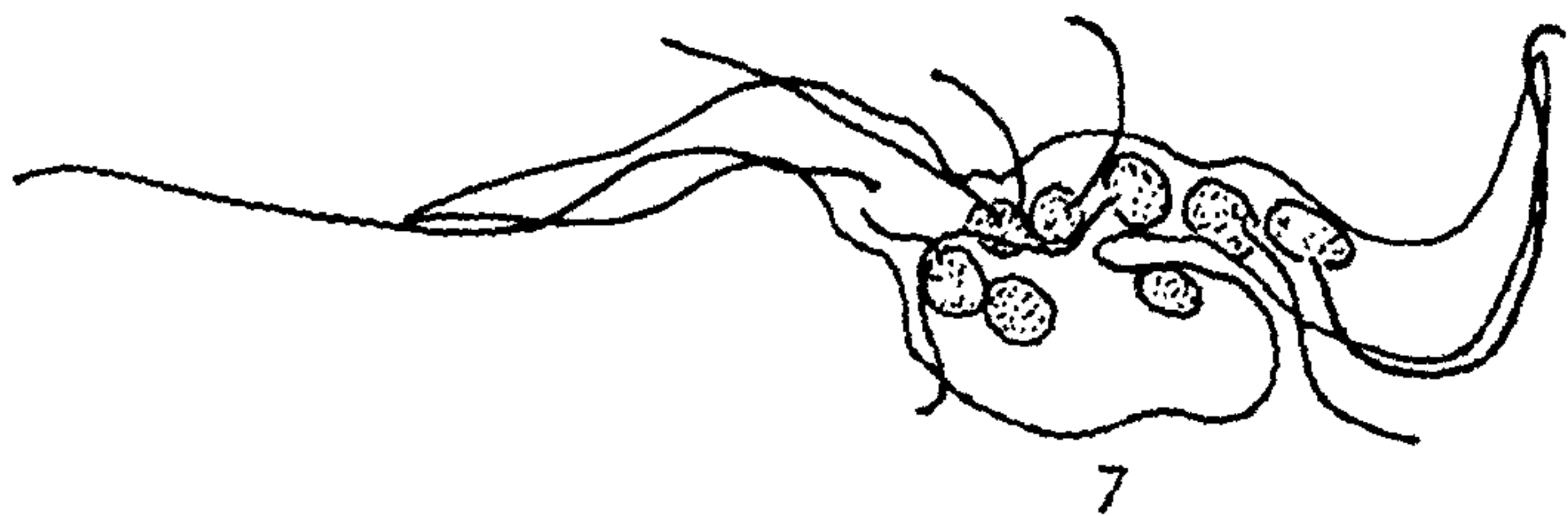
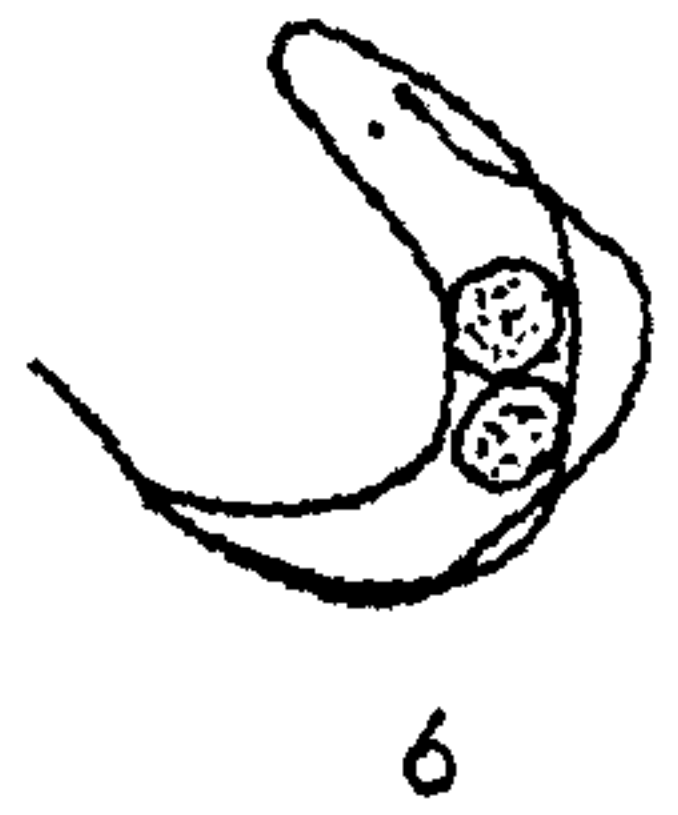
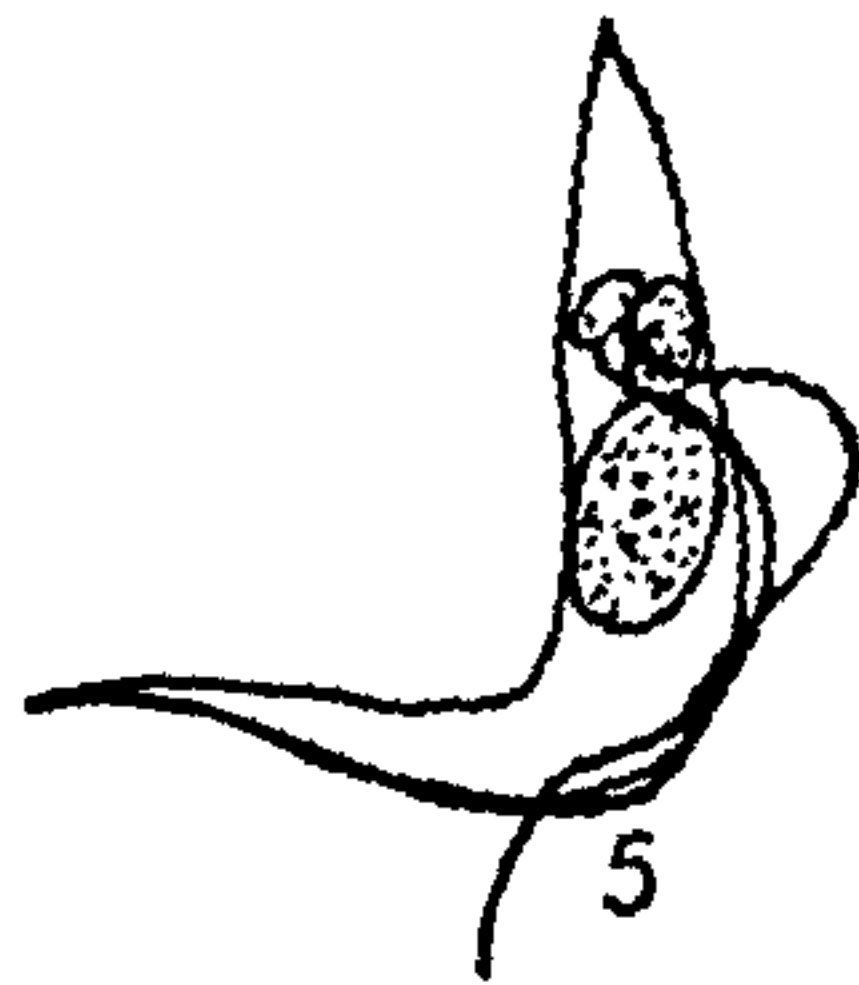
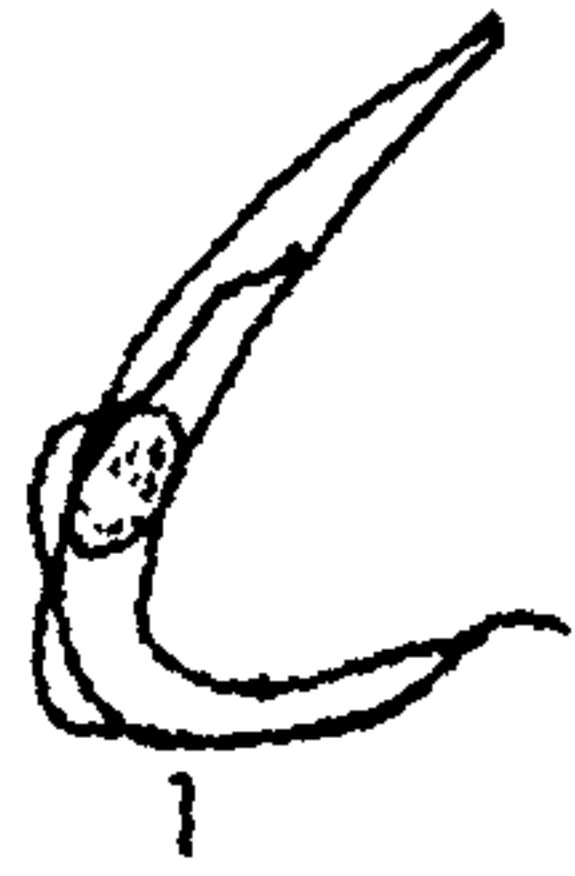
**5. Trypanastigote forms showing unequal division.**

**6,7. Trypanastigote forms from 9 day old culture.**

**6. A dividing trypanastigote.**

**7. Giant form showing multiple fission.**

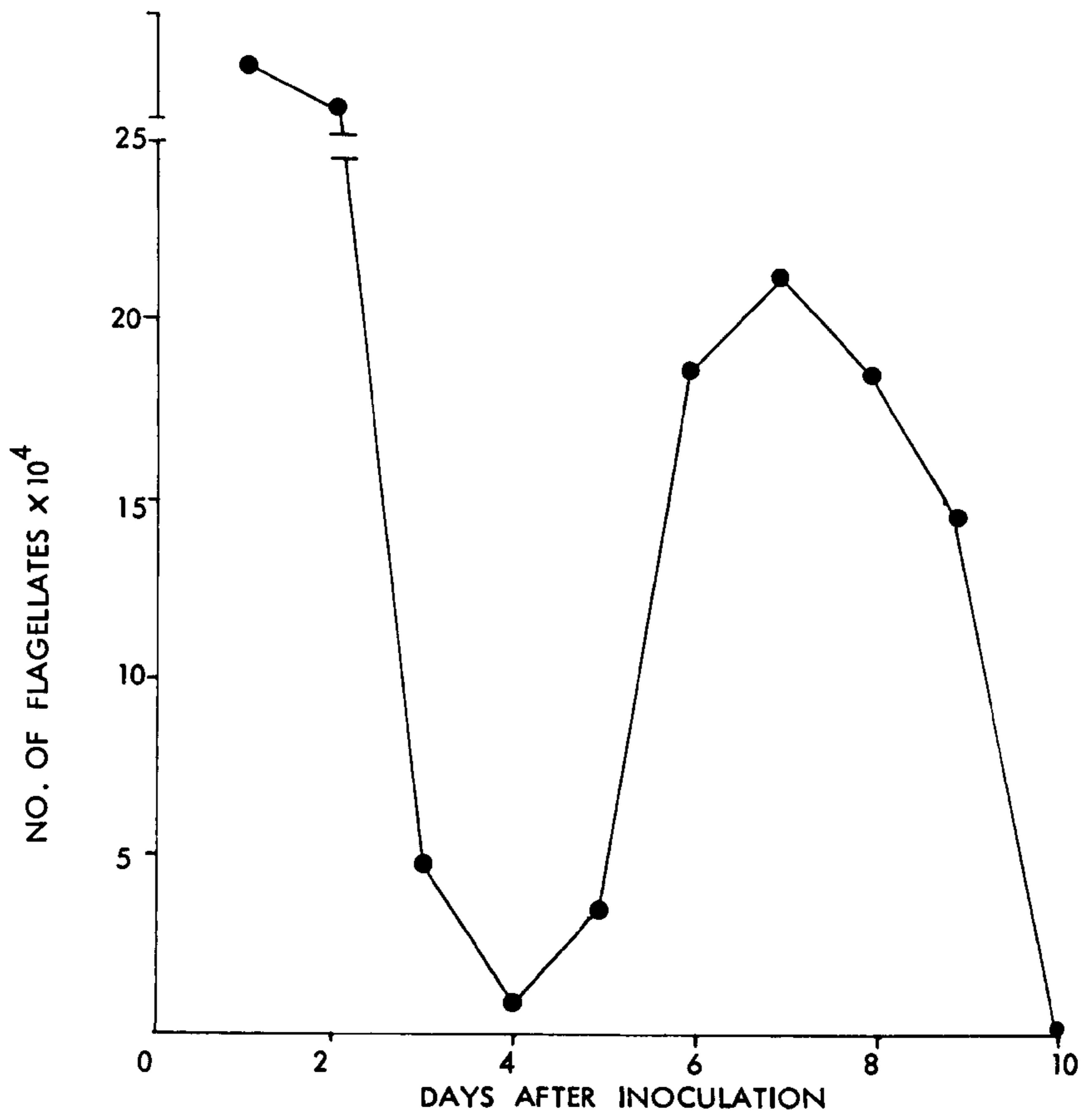




10 $\mu$ m

Figure 24. T. evansi N.S. pleomorphic variant LUMP 315.

Graph shows the growth rate of the population in 4N medium. The media were inoculated with  $1 \times 10^7$  organisms. Curve represents the mean of 2 samples.



**Figure 25. T. evansi N.S. pleomorphic variant LUMP 315.**

**Drawings of trypanastigotes from 4M medium.**

**1-2. From 1-day old culture.**

**1. Slender form contracted before degenerative changes.**

**2. Vacuolated stumpy form in the process of death.**

**3-5. From 2 to 4-day old culture. Trypanastigotes increasing in length and showing kinetoplast placed at various positions between the nucleus and posterior end.**

**6-7. From 6 and 7-day old cultures. Trypanastigotes increasing in length, kinetoplast situated in subterminal position.**

**8-11. From 8-day old culture.**

**8. Elongated trypanastigote form showing kinetoplast midway between the nucleus and the posterior end.**

**9. Dividing form showing 2 nuclei and an intact kinetoplast.**

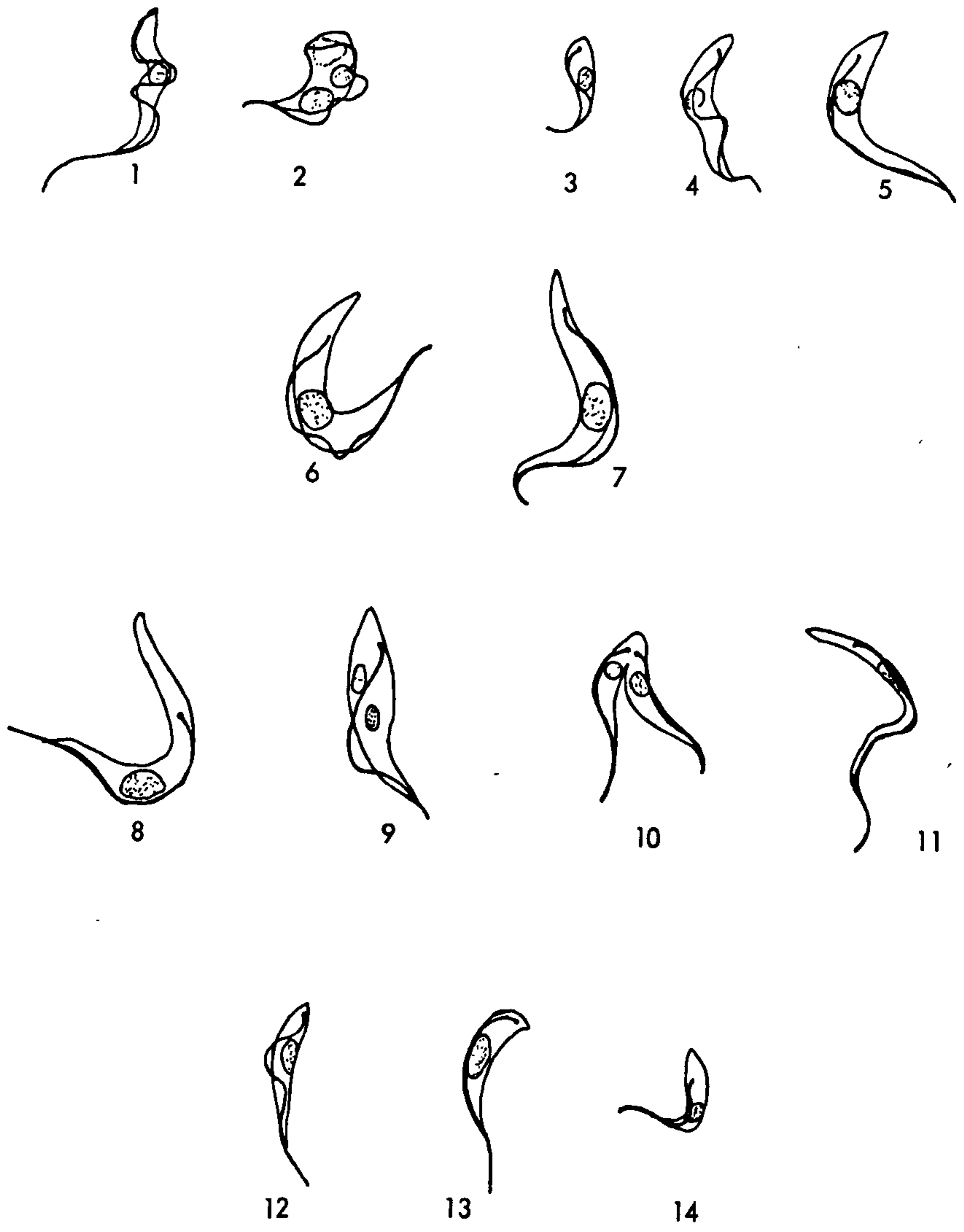
**10. Trypanastigote body in which division is almost complete.**

**11. Thin, ribbon-like trypanastigote.**

**12-14. From 9-day old culture.**

**12,13. Young trypanastigote forms after division. Kinetoplast is terminal.**

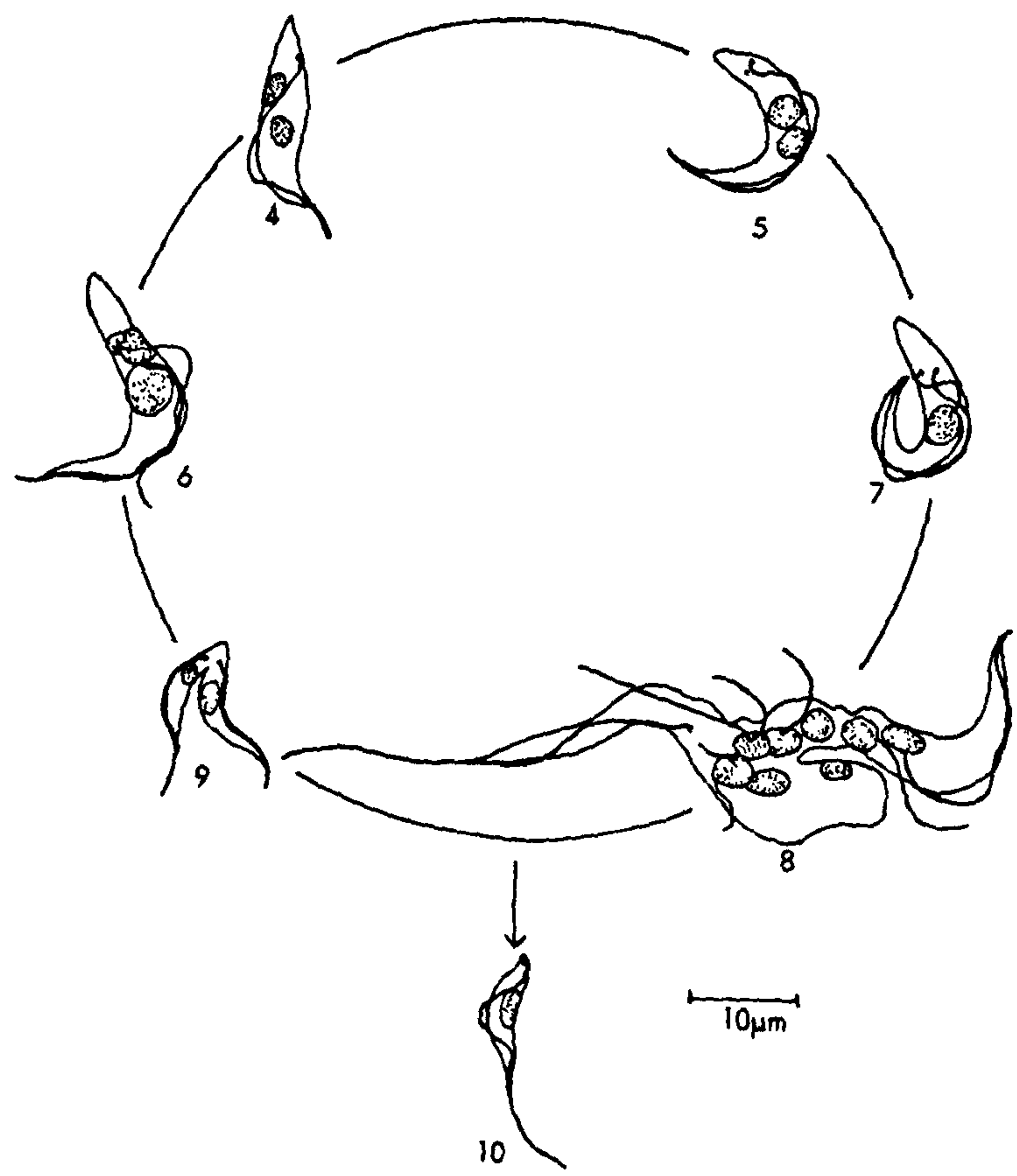
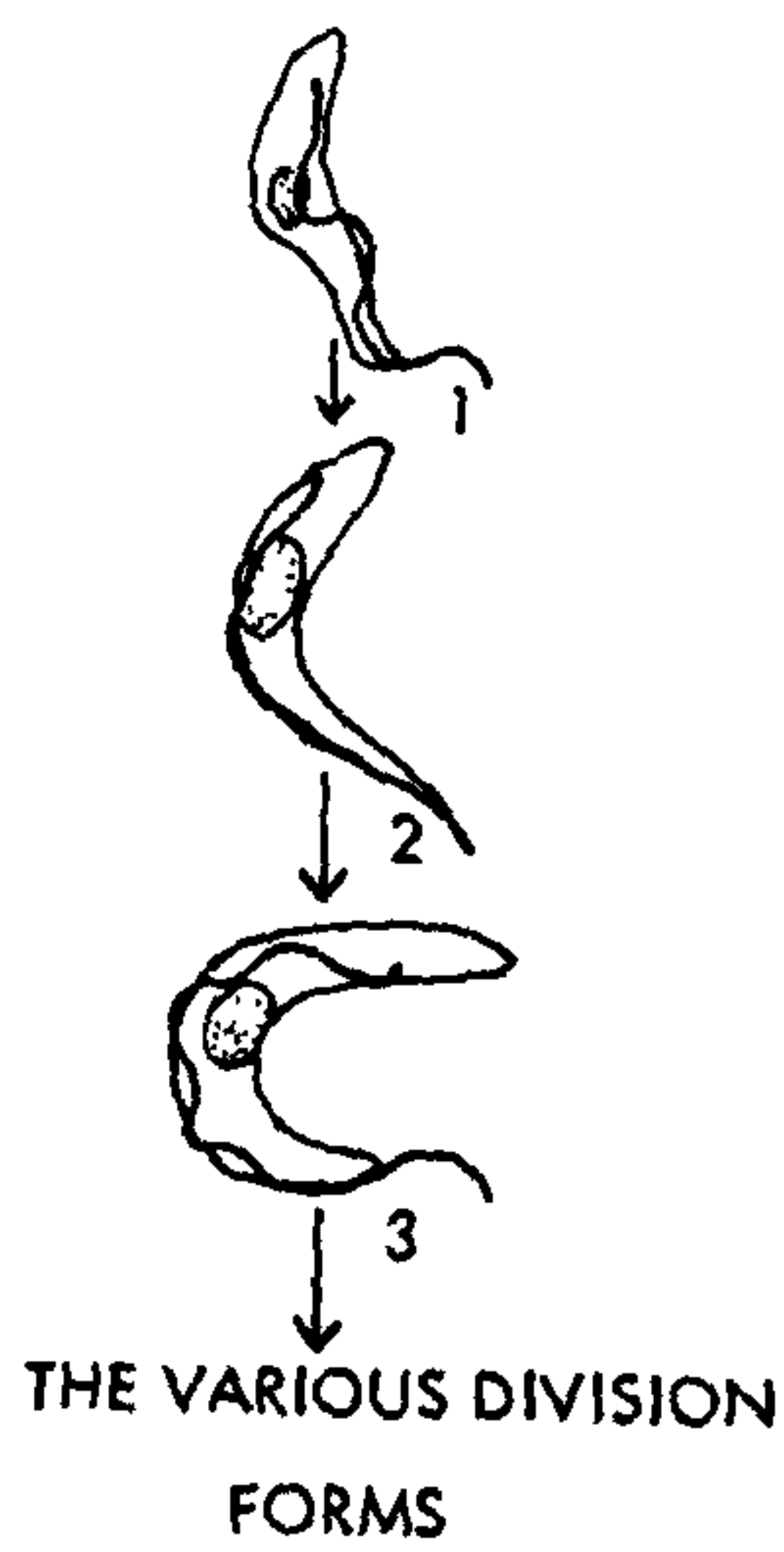
**14. Young trypanastigote form in which the kinetoplast has moved to a subterminal position.**



10μm

**Figure 26. T. evansi N.S. pleomorphic variant. Life cycle diagram showing sequence of changes.**

- 1. Initial stage of a culture form trypomastigote showing kinetoplast close to the posterior end.**
- 2-3. Stages of elongation of the trypomastigote forms.**
- 4-7. Stages of binary fission in the trypomastigote forms.**
- 4. Trypomastigote showing intact kinetoplast and a divided nucleus.**
- 5. Trypomastigote showing both kinetoplast and nucleus divided.**
- 6. Trypomastigote form showing intact kinetoplast, dividing nucleus and 2 flagella.**
- 7. Trypomastigote showing divided kinetoplast but intact nucleus.**
- 8. Giant form showing multiple fission.**
- 9. Trypomastigote body in which division is almost completed.**
- 10. Young trypomastigote formed after division.**



**The details of isolations of relapse  
populations of S. A. K. strain**

<b>Parasitaemic wave</b>	<b>SO (LUMP 66) (Inoculated into a mouse)</b>	<b>Stabilate No. (LUMP)</b>	<b>Population</b>
<b>1</b>	<b>Day 6  (infection treated by Berenil)</b>		
<b>2</b>	<b>Day 17  (infection treated by Berenil)</b>	<b>251</b>	<b>S<sub>1</sub></b>
<b>3</b>	<b>Day 28</b>	<b>283</b>	<b>S<sub>2</sub></b>

**Figure 27. T. evansi S. A. K. strain LUMP 66. Diagram illustrating the isolations of variant populations from a mouse which was inoculated with antilog 4.0 trypanosomes and treated at each relapse.**



Changes in stages of developmental patterns in vector

Stercorarian

Salivarian (Glossina)

Infectivity to Glossina

Species

Stages of changes in morphology

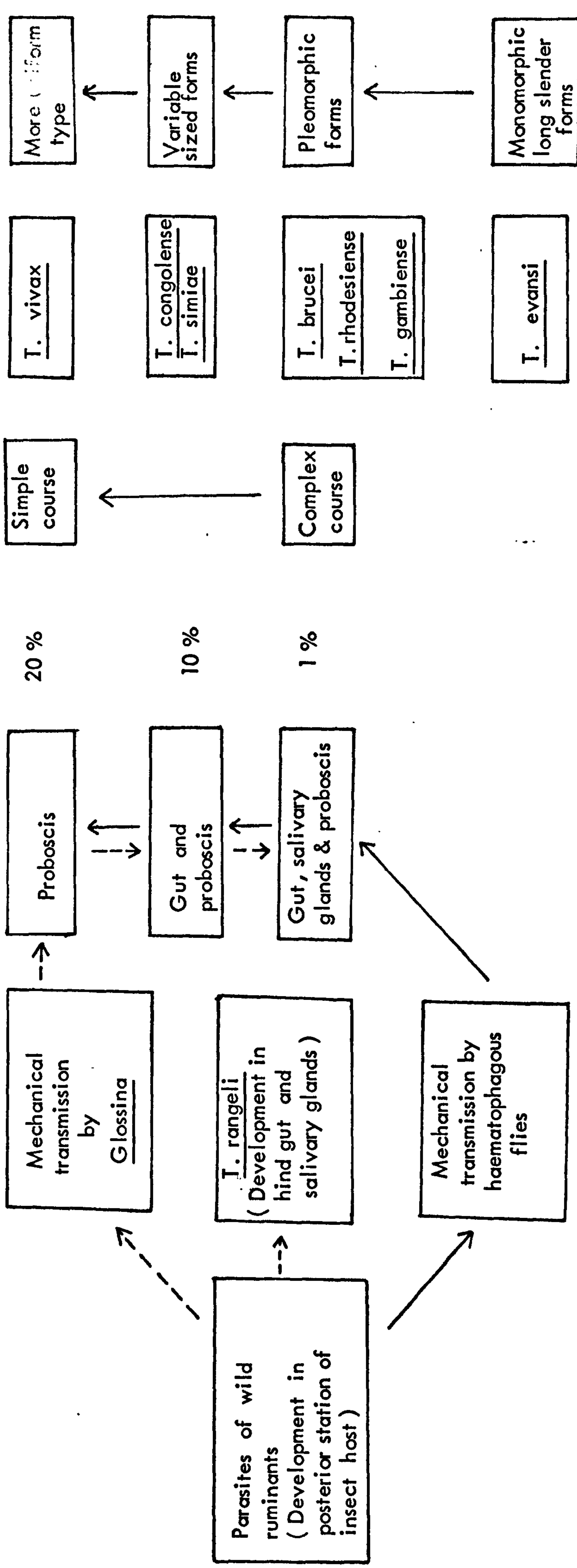
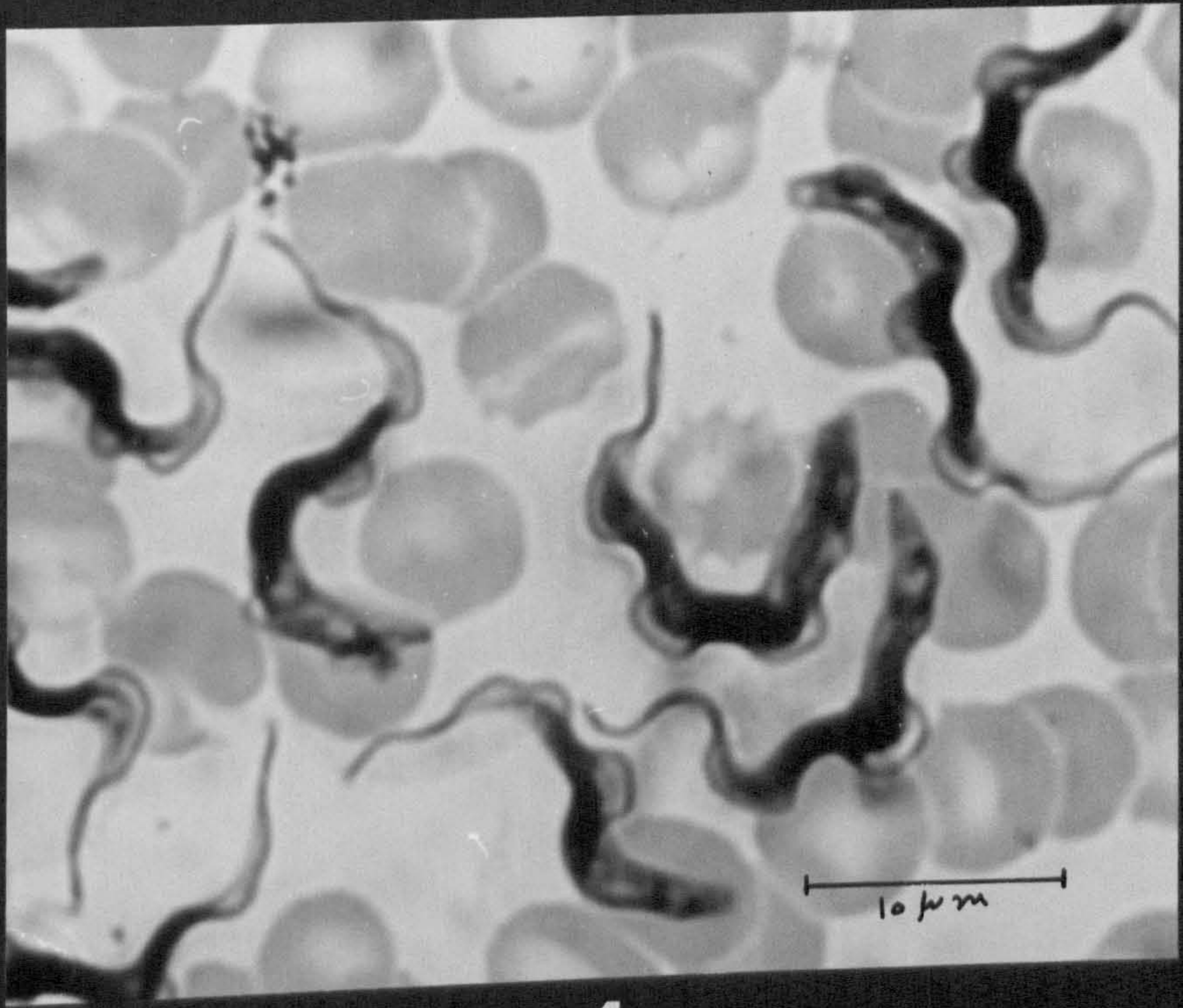


Figure 28. Stages in the evolution of salivarian trypanosomes

Plate 1. T. evansi Colombian strain infection in mice  
derived from stabilate LUMP 74 at day 7 of  
infection, showing trypanastigotes corresponding  
to slender and long intermediate forms of  
T. brucei (X 2500)



1

**Plate 2. T. evansi Colombian strain.**

**Trypomastigotes in medium 193 on day 3 after inoculation. The medium was inoculated with the blood of a mouse infected with LUMP 74 (X 2500).**

**(a) A trypomastigote.**

**(b) A dividing trypomastigote. Note 2 nuclei and 2 flagella.**

a

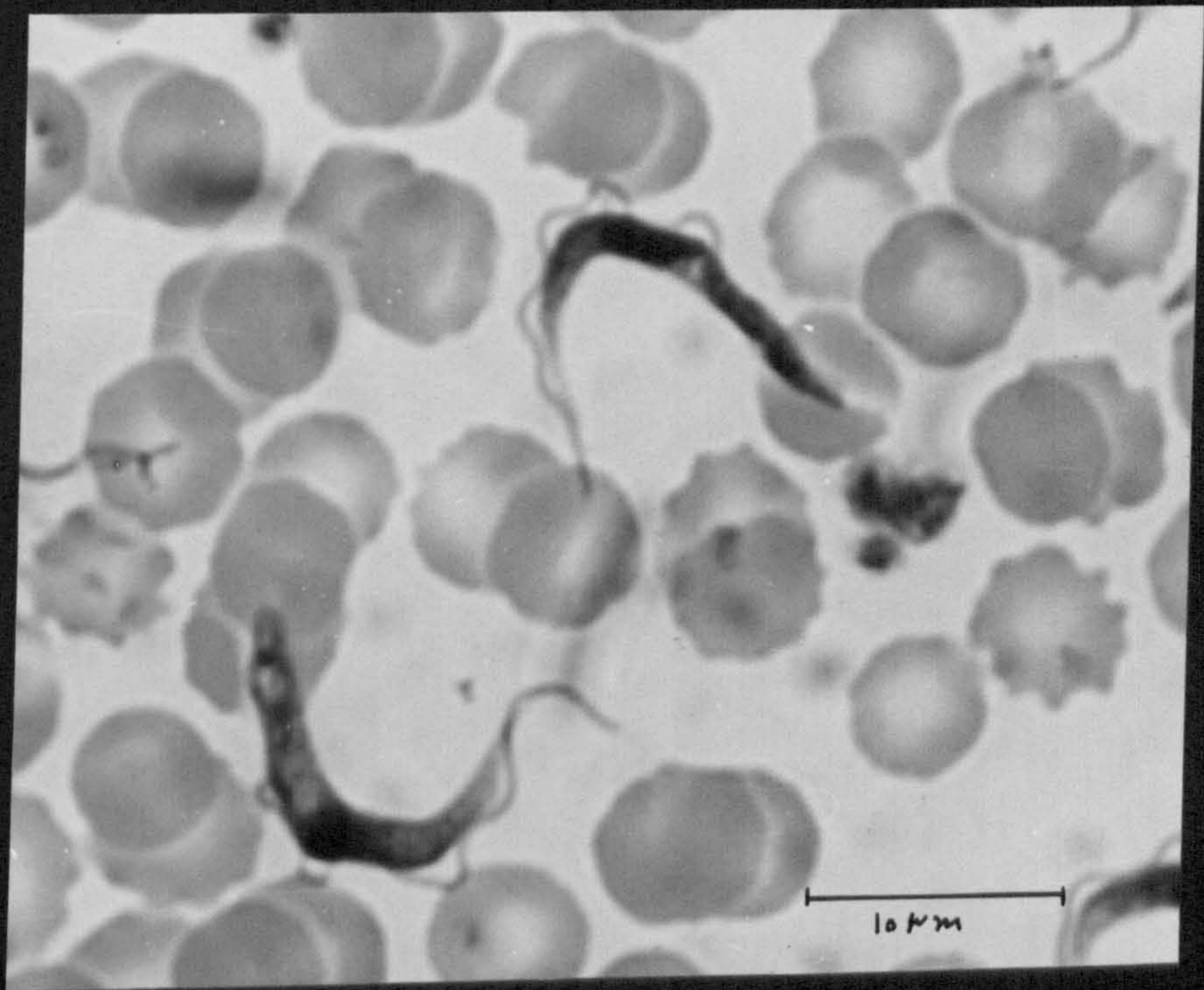


b



2

Plate 3. T. evansi N.S. strain infection, derived from stabilate LUMP 46 at day 5 of infection in a mouse. The photograph illustrates trypomastigotes corresponding to the slender and long intermediate forms of T. brucei (X 2500).

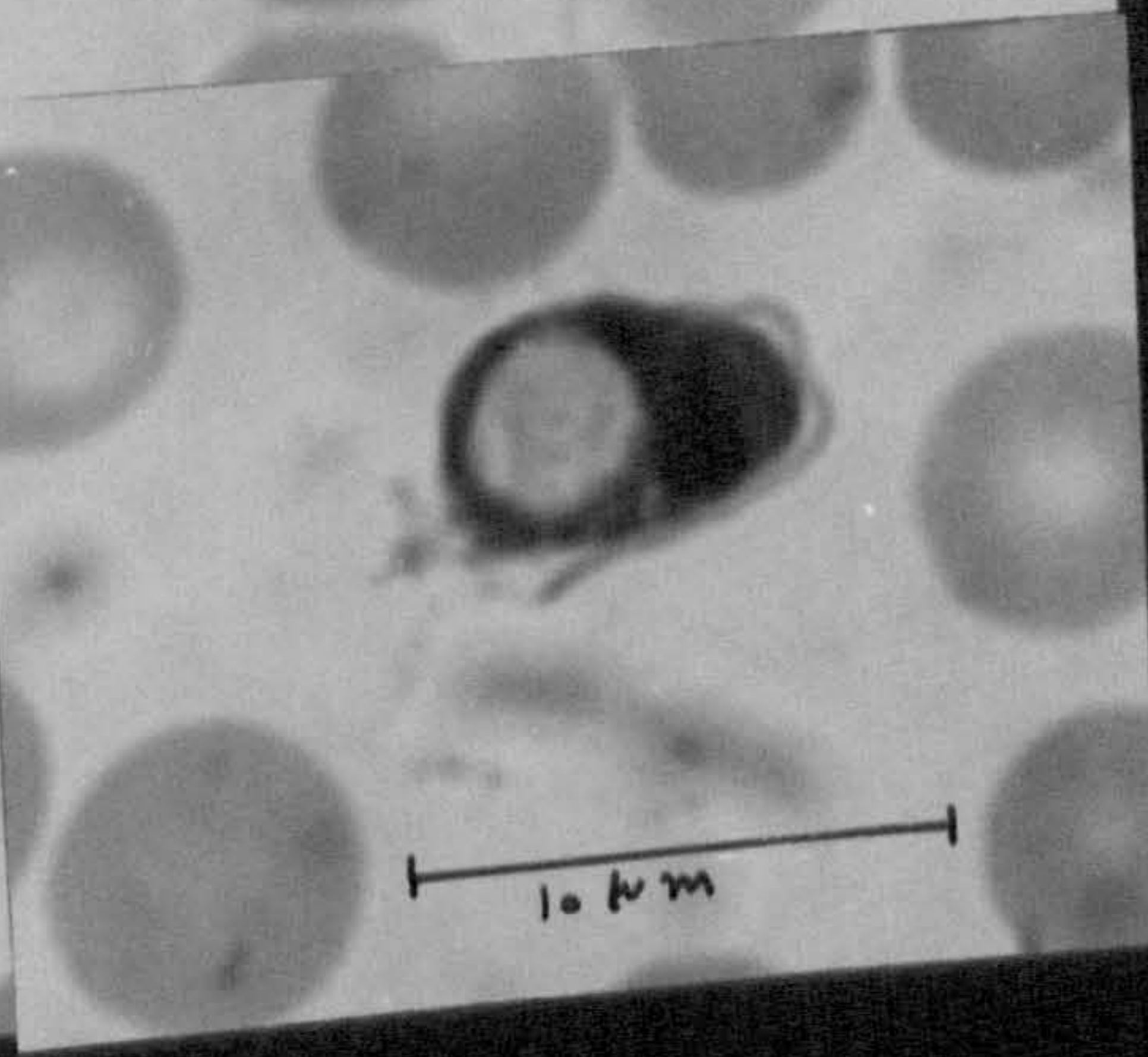
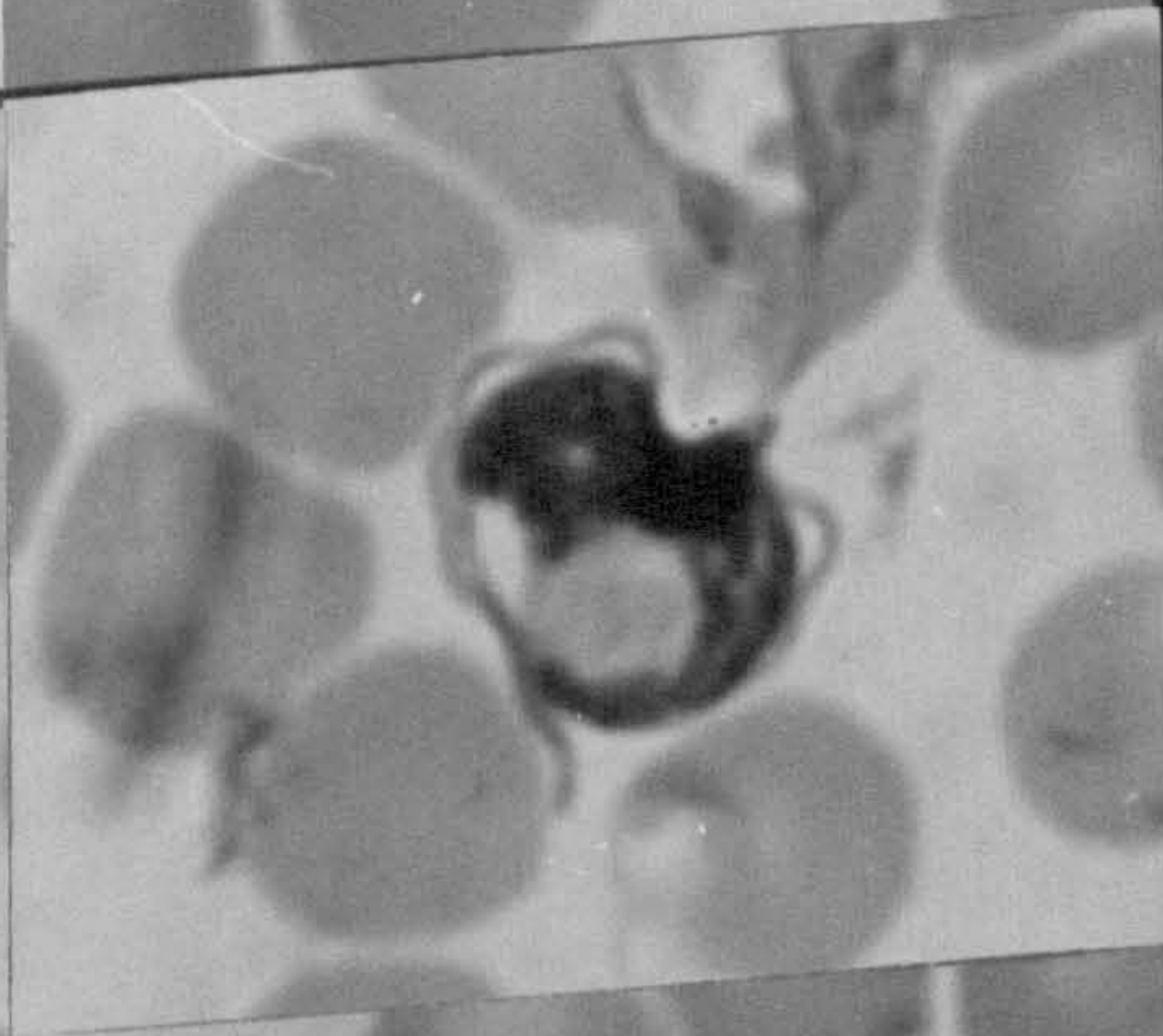
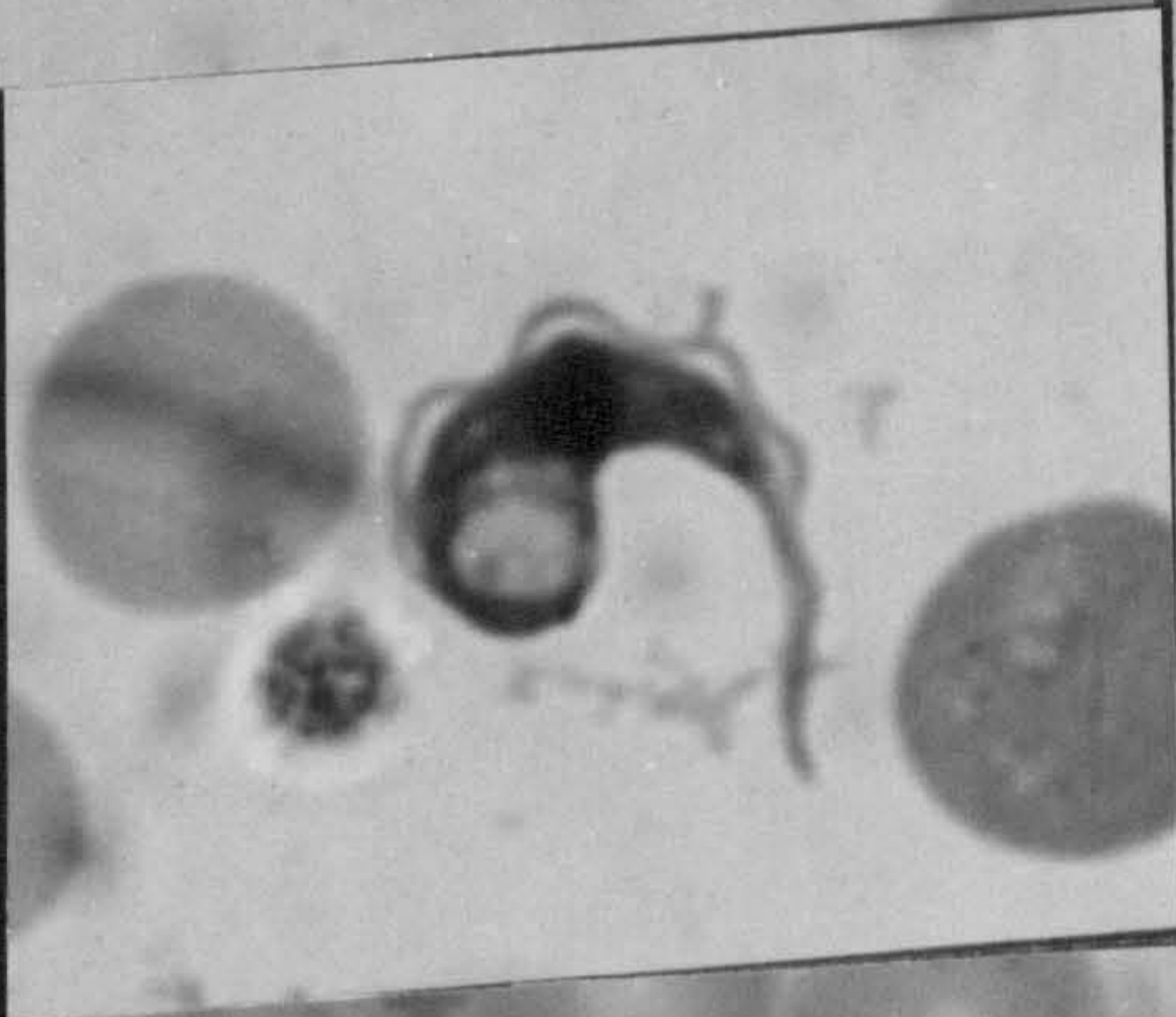
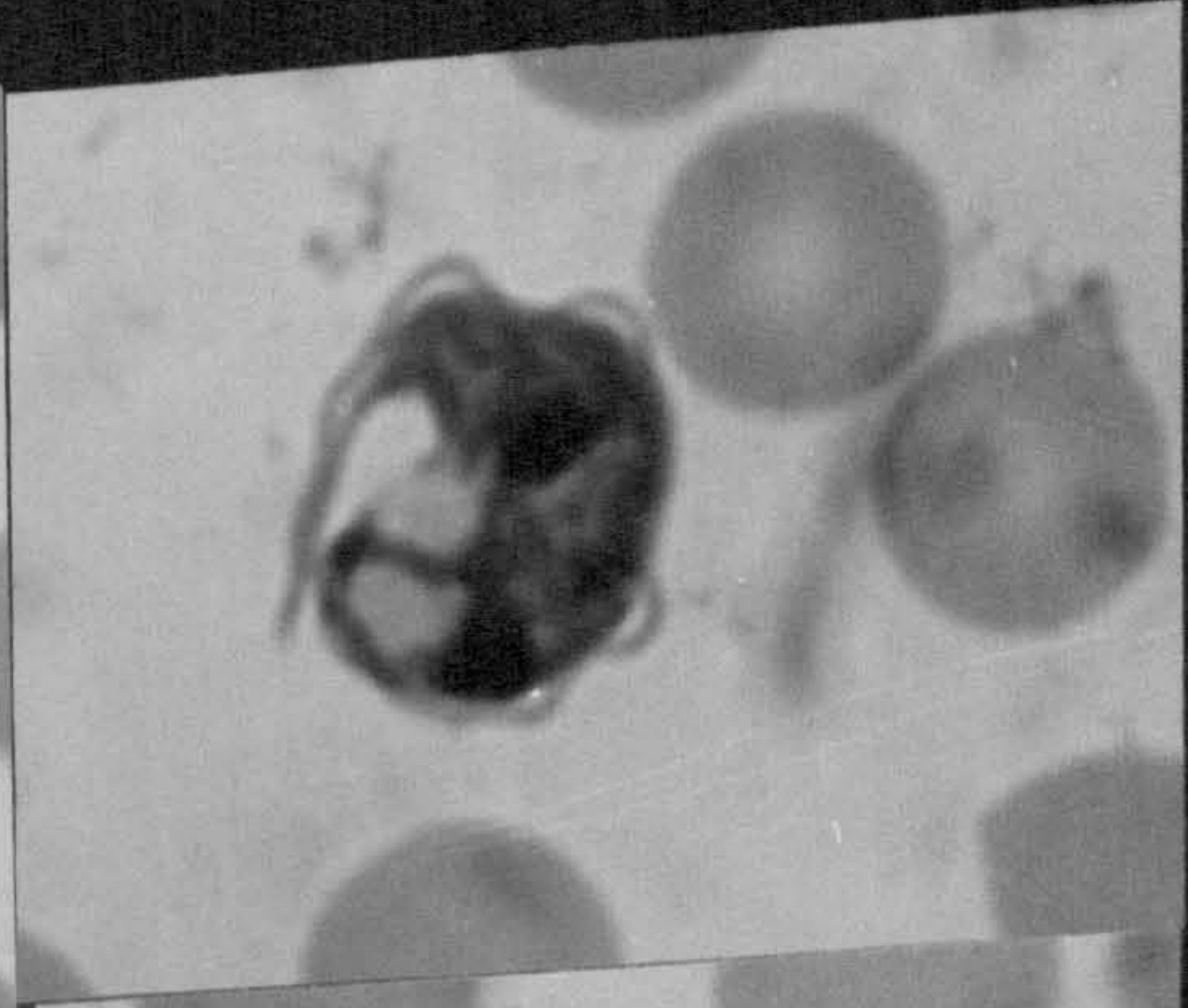
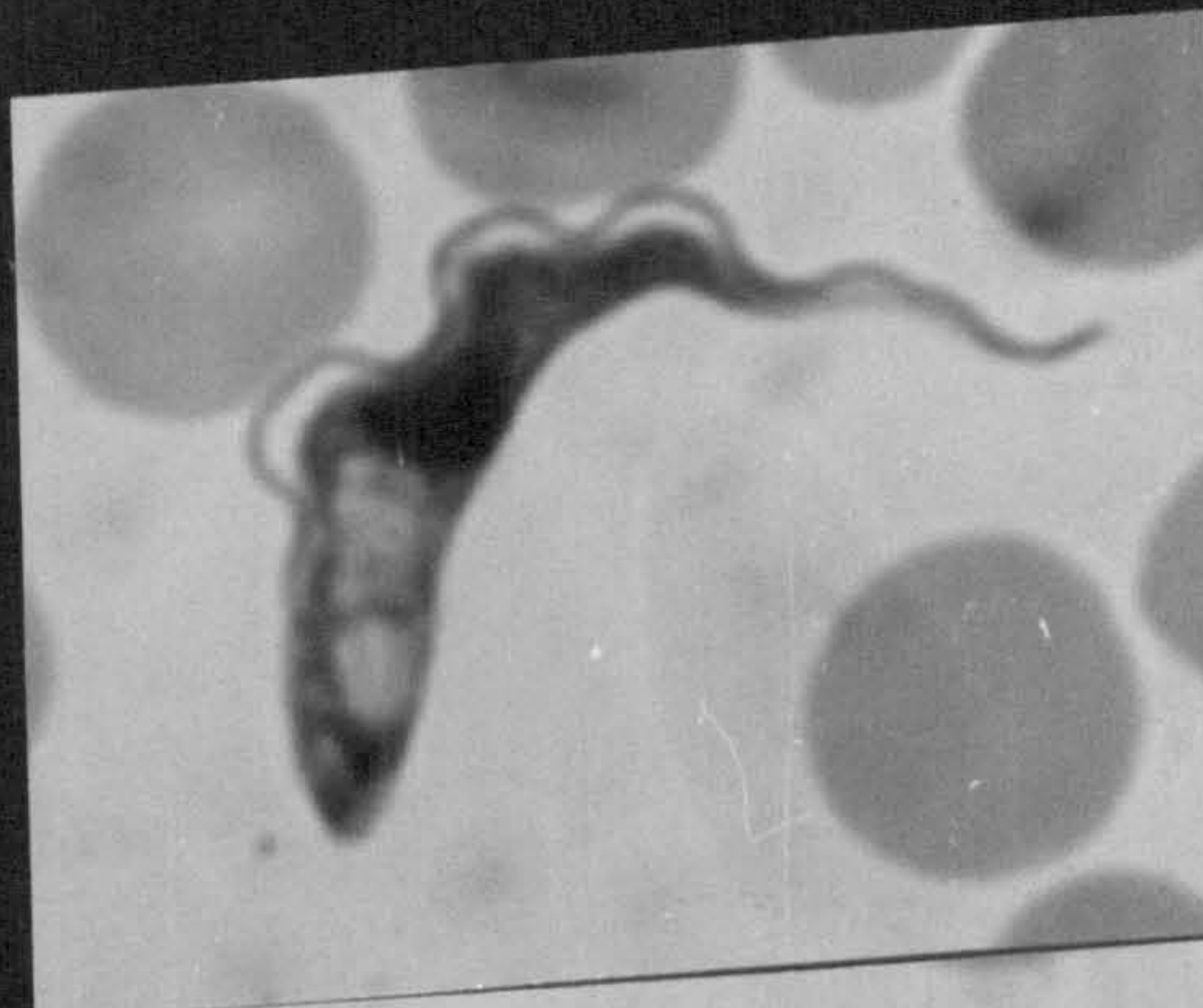


3

**Plate 4. T. evansi N.S. strain LUMP 46 infection in a mouse (mouse 1) which was immune to S.A.K. strain (LUMP 66). The photographs illustrate trypomastigotes (day 30) showing various stages of the development of a vacuole within them (X 3000).**

- (a) Initial stage of vacuole formation.**
- (b) 2 vacuoles in a dividing trypomastigote.**
- (c) Enlarged vacuole occupying the portion between the posterior end and the nucleus.**
- (d, e and f) Developmental stages of the vacuole resulting in the transformation of the blood trypomastigote to a comma-shaped form.**
- (g) 'C' shaped blood trypomastigote.**
- (h) Globular-shaped blood trypomastigote.**





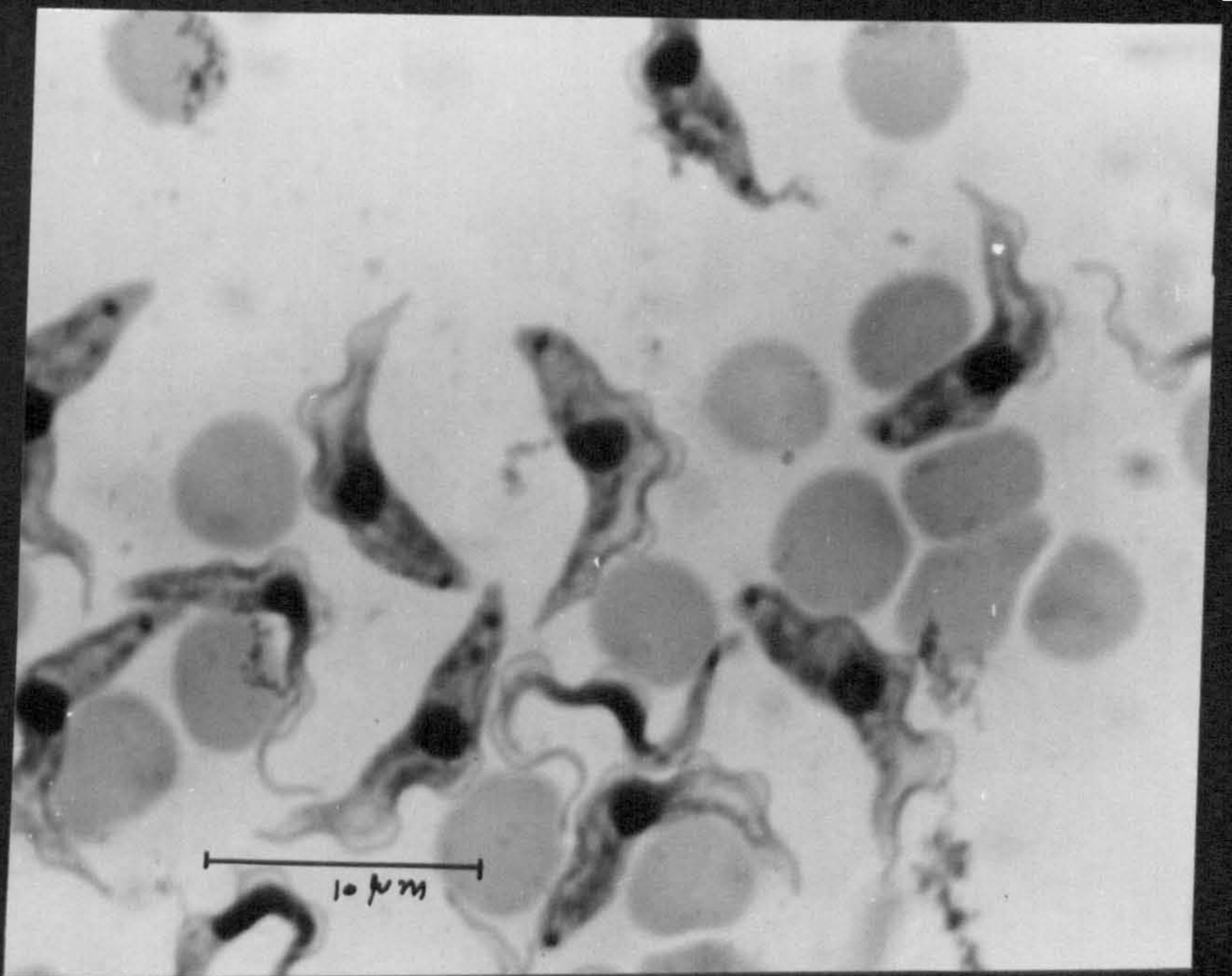
**Plate 5. T. evansi N.S. strain clone LUMP 85 infection in a mouse (mouse 1) which was immune to S.A.K. strain clone (LUMP 55). The photographs illustrate stumpy trypanastigotes on day 78 of the experiment (29 days after inoculation of LUMP 85).**

**(a) Stumpy trypanastigote (X 2500).**

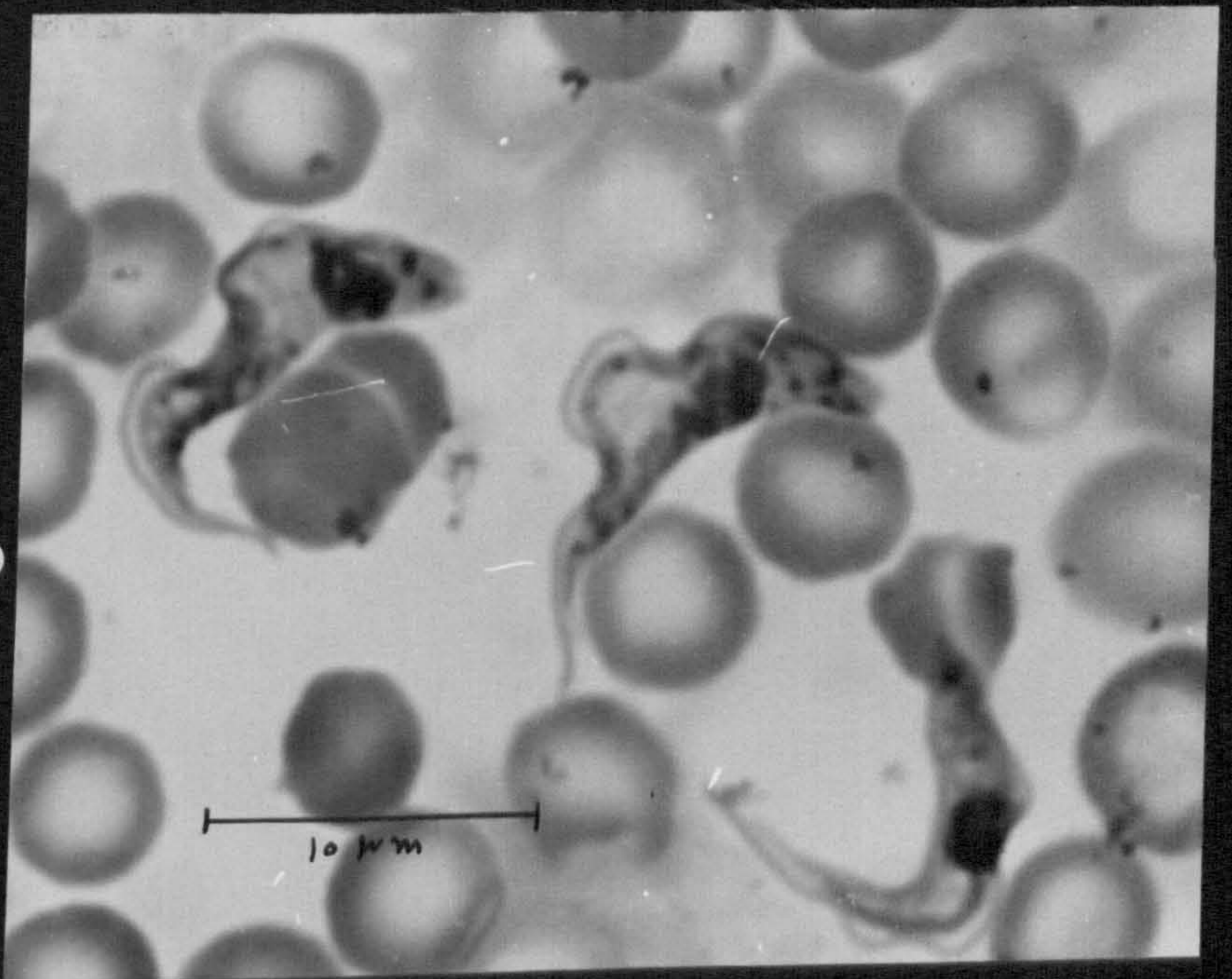
**(L) Posteronuclear form (X 3000).**

**(c) Stumpy trypanastigote showing granules (X 2500).**

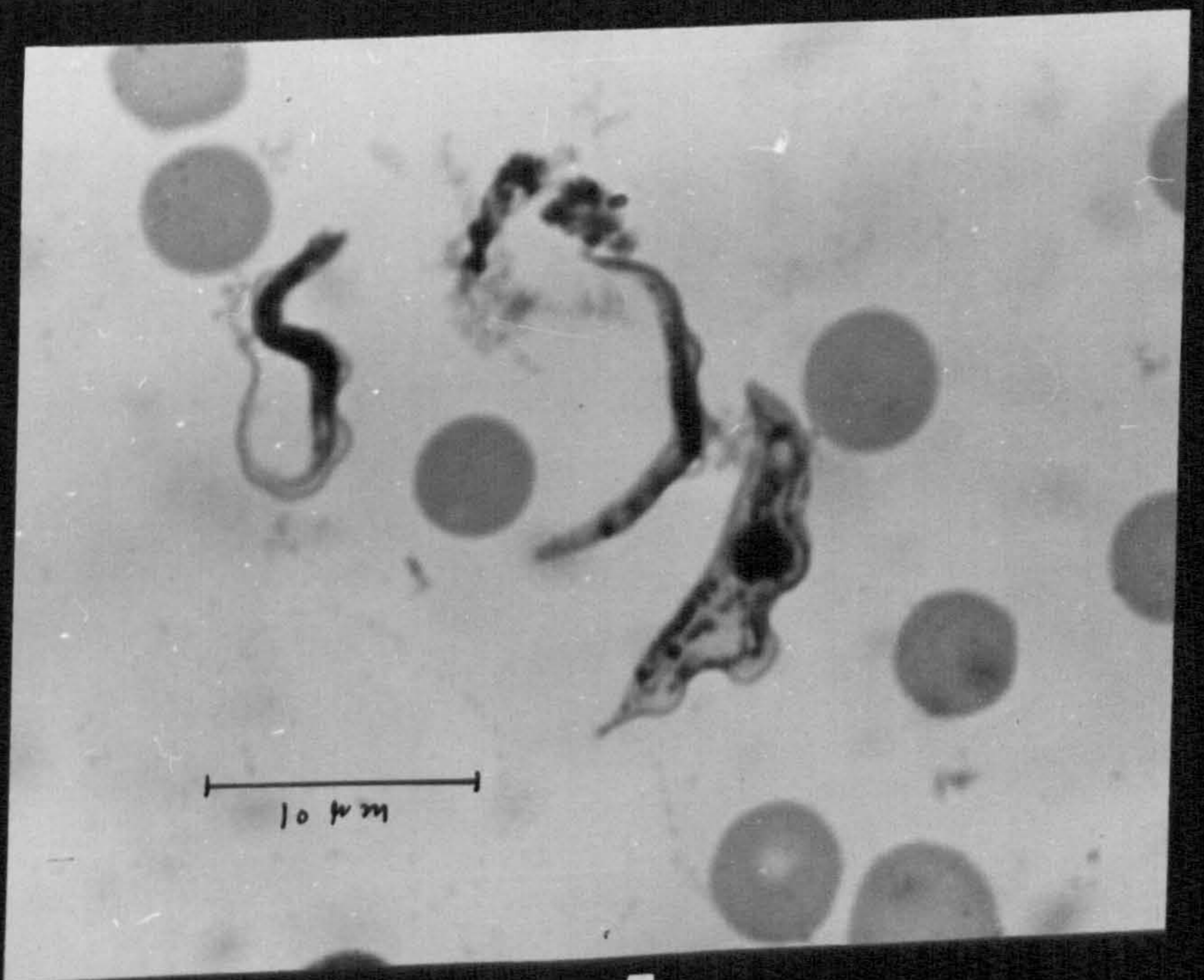
a



b



c



**Plate 6. T. evansi N.S. pleomorphic variants.**

Photographs show trypanastigotes derived from 4N culture medium (X 2500). The cultures were inoculated with blood of mice which were infected with either stabilate LUMP 315 or 172.

**Trypanastigotes from 2-day old culture**

(a) LUMP 315 - The kinetoplast is situated in the terminal or subterminal position.

**Trypanastigotes from 8-day old cultures**

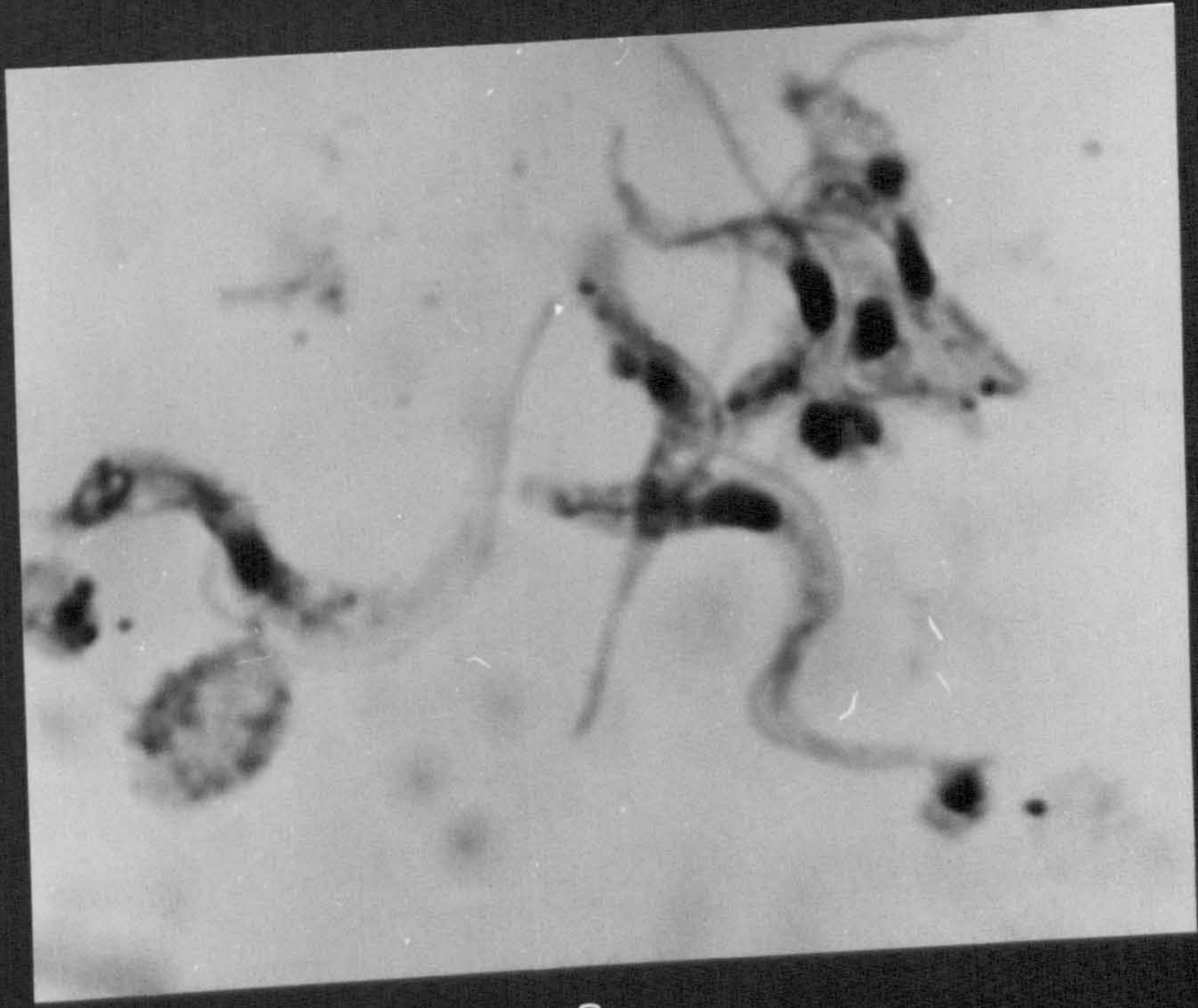
(b) LUMP 172 - Trypanastigote showing kinetoplast in between the nucleus and the posterior end.

(c) LUMP 315 - Trypanastigotes showing kinetoplast posteriorly adjacent to the nucleus.

(d) LUMP 172 - Trypanastigotes showing kinetoplast posteriorly adjacent to the nucleus.

(e) LUMP 315 - Dividing trypanastigote illustrating equal division.

(f) LUMP 172 - Dividing trypanastigote illustrating multiple fission.



a



b

c

d



e



f

10 μm

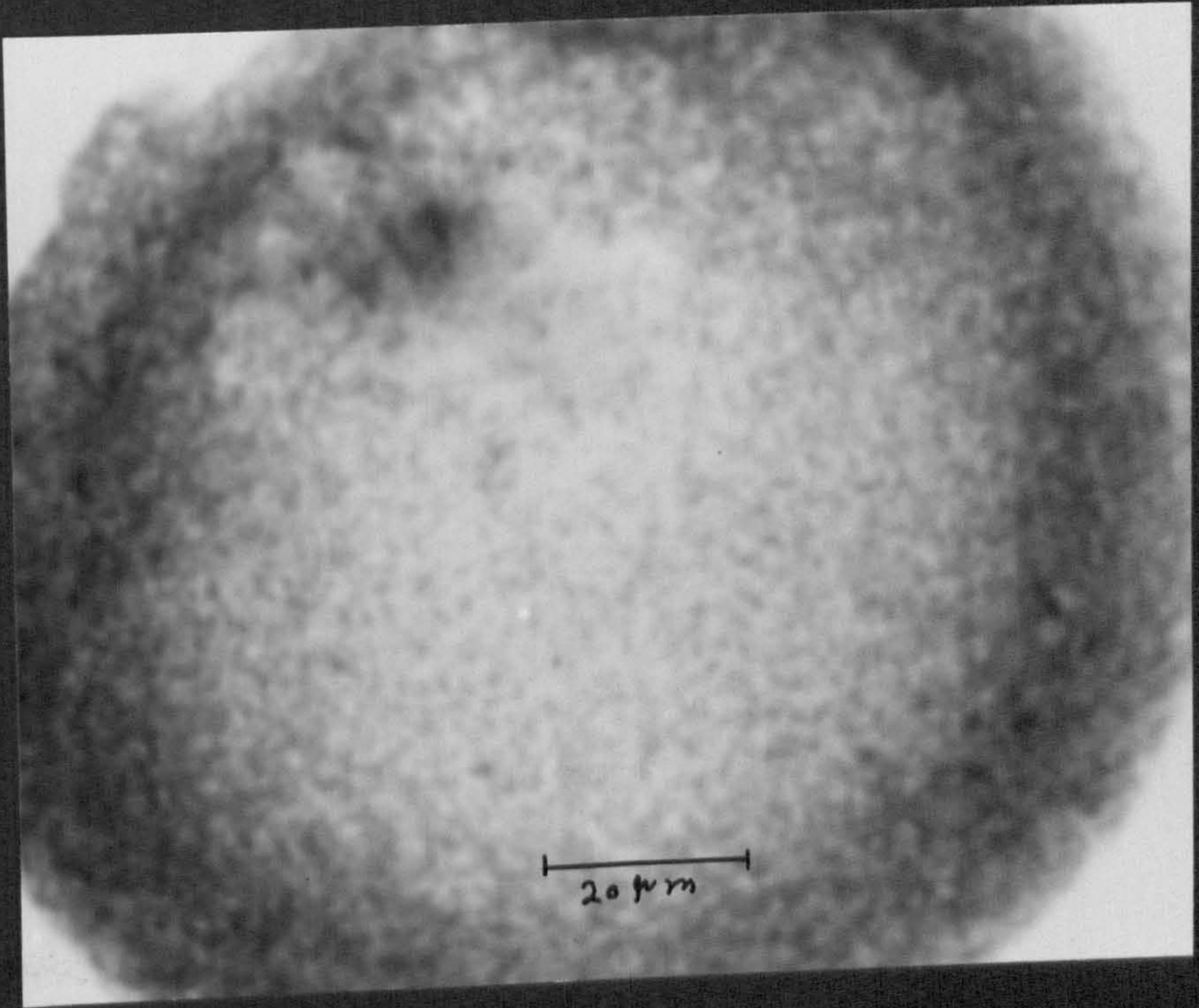
Plate 7. T. evansi N.S. pleomorphic variant LUMP 172.

Photographs showing organisms in a probe, extruded by an infected G. morsitans, on the 11th day after an infective feed on a mouse which was infected with LUMP 172.

(a) An entire probe, packed with organisms (X 1000).

(b) Enlargement of a portion of (a) showing proventricular trypanastigotes (X 2500).

a



b

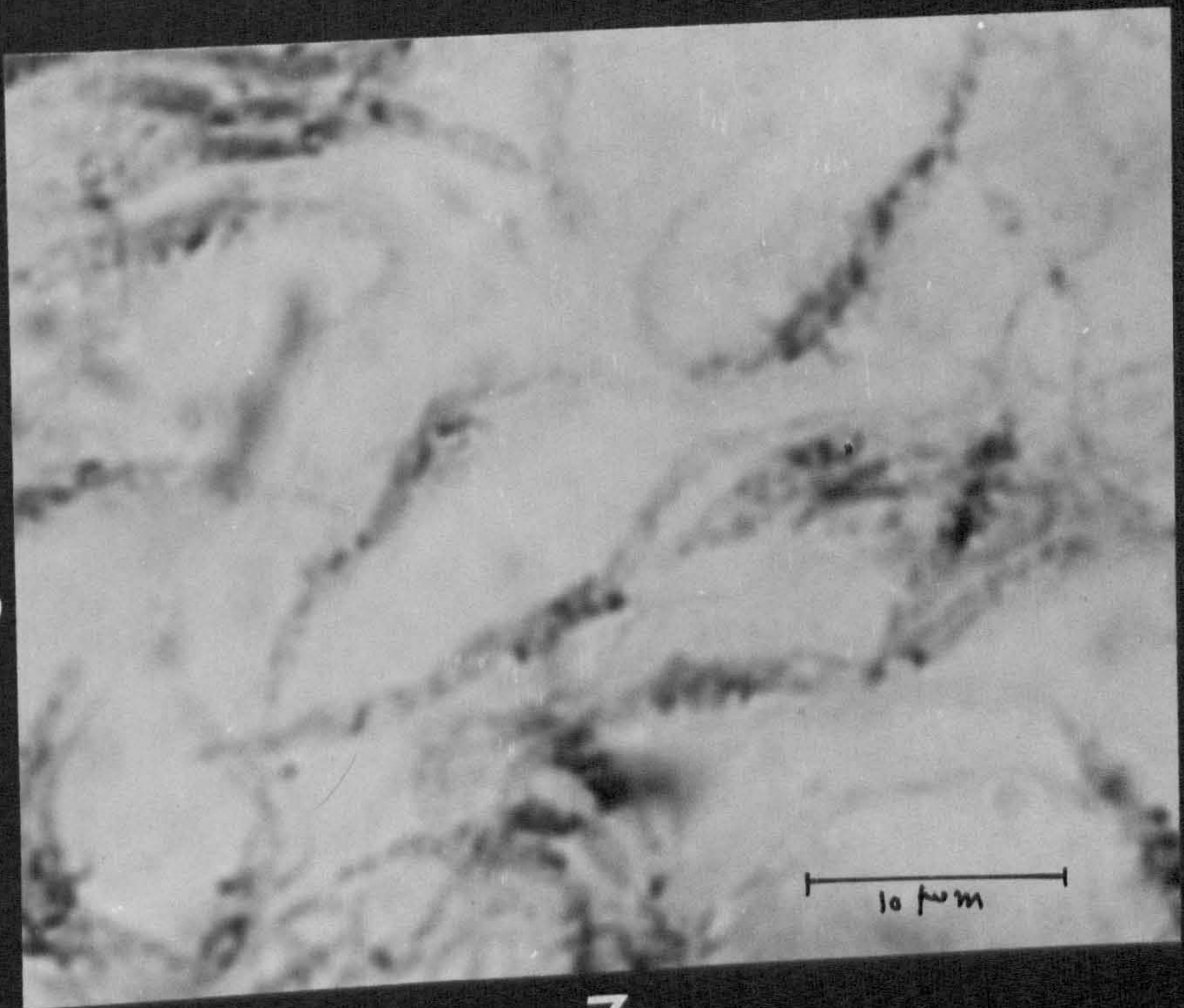
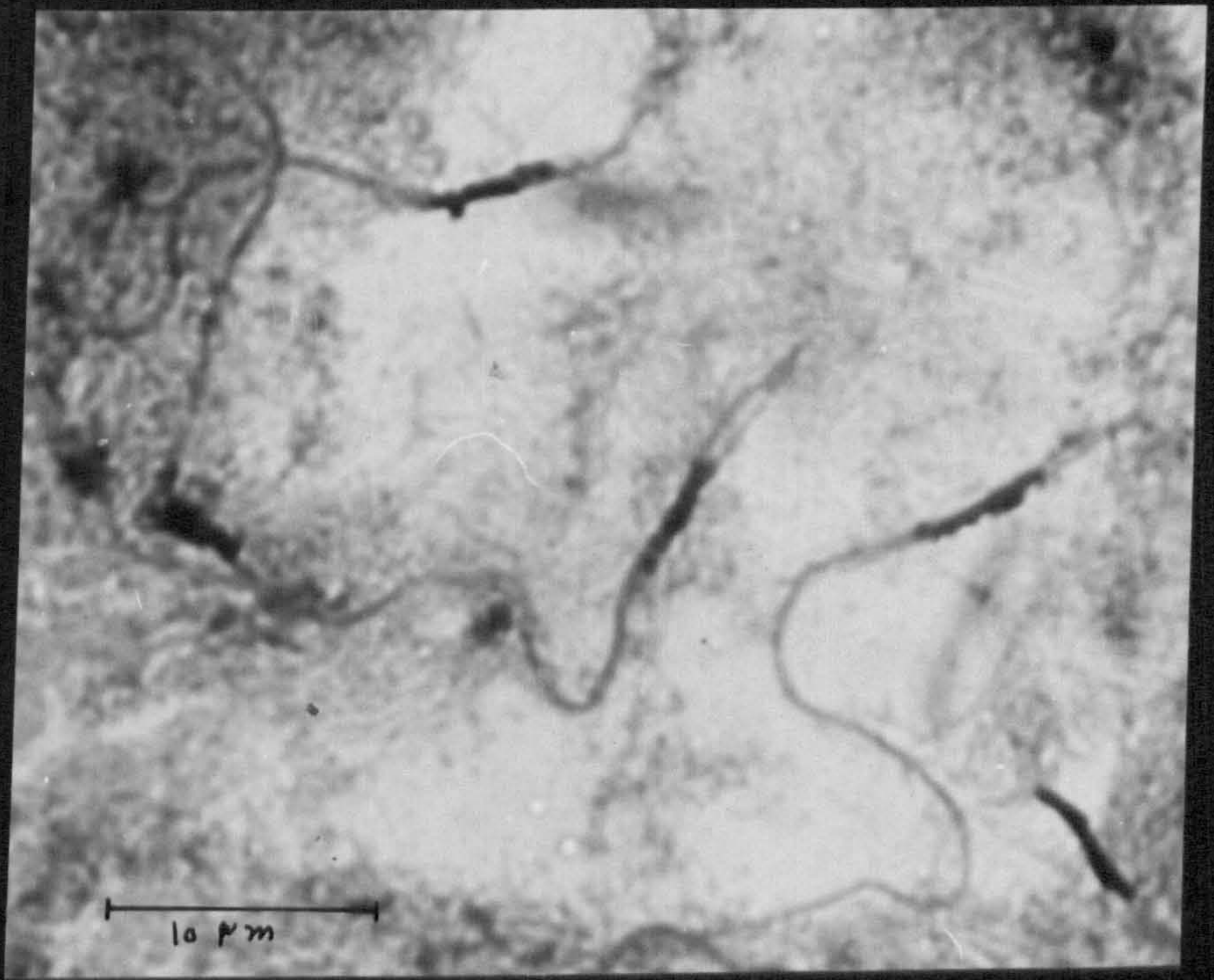


Plate 8. T. evansi N.S. pleomorphic variant LUMP 172.

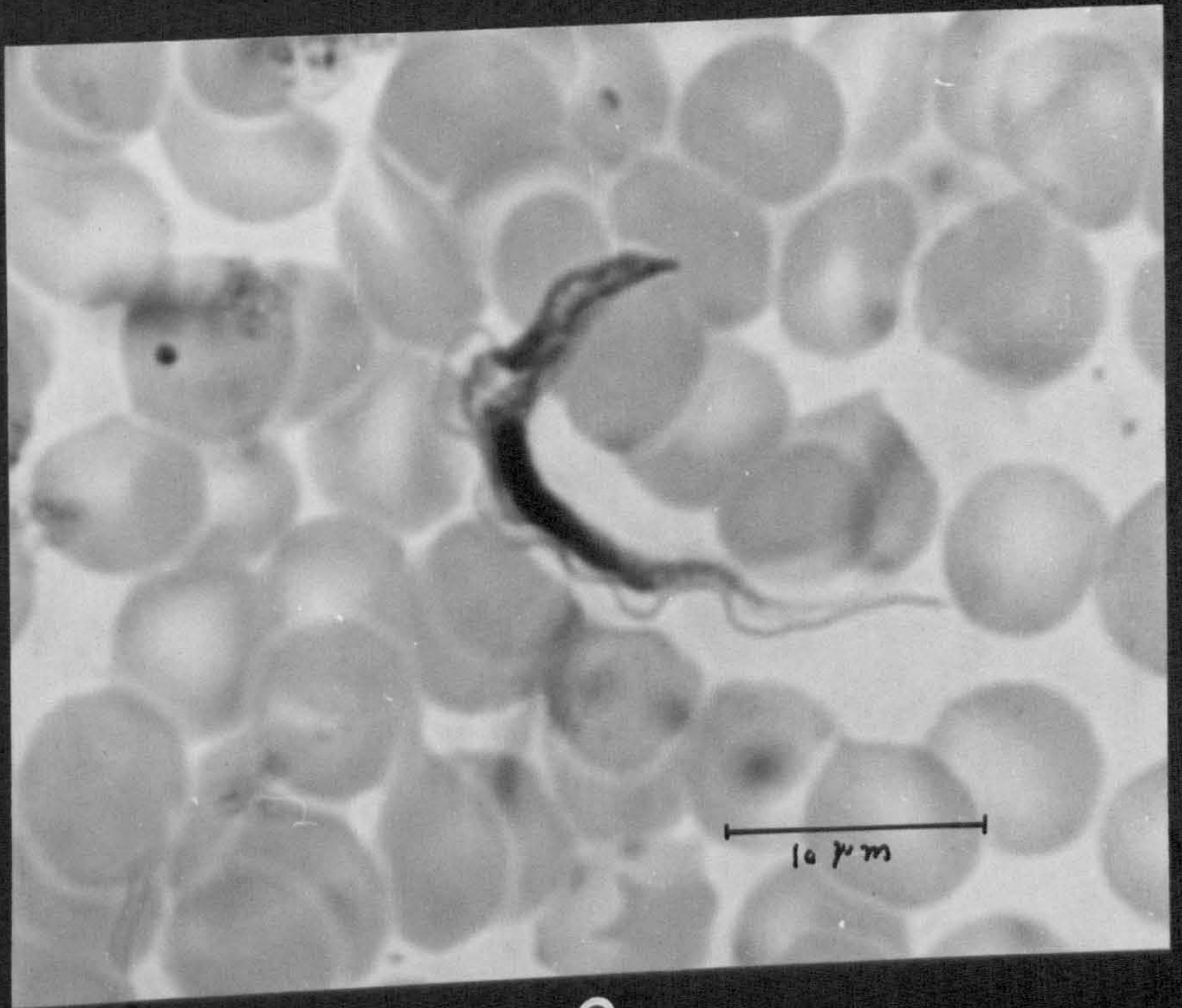
Proventricular trypomastigote forms recovered from the salivary glands of a G. morsitans, which was dying on the 11th day after the ingestion of an infective blood meal. The mouse was infected with LUMP 172 (X 2500).





8

Plate 9. T. evansi S.A.K. strain (dyskinetoplastic) infection  
in mice derived from stabilate LUMP 66 on day 6 of  
infection, showing trypanastigotes corresponding to  
long intermediate forms of T. brucei (X 2500).



9

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**Cultivation of a pleomorphic variant of *Trypanosoma (Trypanozoon) evansi* and its transmission by *Glossina morsitans***

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

Pleomorphic variants of a monomorphic N.S. strain of *T. evansi* (HOARE, 1954) were obtained by the inoculation of this strain into mice made hyper-immune to the akinetoplastic S.A.K. strain (HOARE, 1954) by infection and subsequent Berenil treatment (MILES, in press). The pleomorphic trypanosomes were stabilated. Inoculation of this stabilate into clean mice, resulted in a trypanosome population which was also pleomorphic.

When inoculated into 4N medium (BAKER, 1966) the pleomorphic trypanosomes, derived from either stabilate or mouse blood, were readily maintained in culture and some growth was observed. The cultures survived for an average of 9-10 days and flagellates were seen for a period of 26 days in one culture. Subcultures were made at 3-4 day intervals and the organisms could be successfully maintained through 4 such passages. Only trypomastigote forms were seen. The undulating membrane was less pronounced and the kinetoplast was either subterminal or at varying positions between the nucleus and posterior end. A free flagellum was usually present. Reproduction was by equal and unequal binary fission and many dividing forms were seen. Also forms containing 2-8 nuclei were seen. Flagellates from culture were not infective to mice.

75 *Glossina morsitans*, divided in 6 groups, were initially fed on infected mice on days 0-2 after emergence. They were subsequently fed on healthy mice, which were replaced every 4-5 days. 4 mice, on which different groups of flies were fed on days 11-15, 15-17, 16-18 and 22-25 after emergence, were found to be infected. On dissection of 51 flies, 3-4 weeks after an infective feed, trypomastigote forms were recovered from the midgut of 10 flies. No salivary gland infection was found. These results suggest that pleomorphic variants of *T. evansi* can have a cycle of development in *G. morsitans*.

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HOARE, C. A. (1954). *J. Protozool.*, **1**, 28.  
MILES, M. A. *Ann. trop. Med. Parasit.* (in press).

**Antigenic variation of a strain of *Trypanosoma (Trypanozoon) evansi* in the mouse host**

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A Colombian strain of *Trypanosoma evansi* (isolated in 1967 from a horse in Arauca, Colombia, South America), when inoculated in doses of antilog 3.65 organisms or less results in the development of relapsing parasitaemia in approximately 20% of mice. From a mouse which was inoculated with original Colombian strain (W0) and in which the course of parasitaemia was expressed as log equivalent values (WALKER, 1969), isolations were made and those corresponding to parasitaemic waves, 12 in all (W1 to W12), were studied. The method of determining antigenic identity was the agglutination test (CUNNINGHAM and GRAINGE, 1963). From the pattern of agglutination obtained by testing each isolate against antisera prepared against each isolate, 3 antigenic types W2, W3 and W4 appeared to be unique and all the other populations appeared to be mixtures as follows: W0-W0, W1 and W9; W1-W0, W1, W7, W8 and W9; W6-W5 and W6; W7-W1, W6, W7 and W8; W8-W0, W1, W7 and W8; W9-W0, W1 and W9; W10-W10 and W11; W11-W10 and W11; W12-W11 and W12; Thus in the 12 parasitaemic wave populations possibly 9 variant antigenic types were represented by W0-W1, W2, W3, W4, W5-W6, W7-W8, W9, W10-W11 and W12. The antigenic type W1 was found to reappear in the same infection mixed with W7-W8 and W9 variant antigenic types.

Variant antigenic types W5 and W12 were inoculated into mice and trypanosome populations from the successive parasitaemic waves were subsequently isolated. Although W5 and W12 were of different antigenic types, trypanosome populations in their first natural relapses (W5-2 and W12-2) were of identical antigenic types and were similar to W1.

In order to confirm the similarity of antigenic types of W1 to the first relapse populations of W5 and W12, mice immunized by intravenous inoculation of formalinized infected whole blood (HERBERT and LUMSDEN, 1968) of antigenic type W1, were challenged 15 days later by W1, W5-2 and W12-2. The results suggest that mice immunized with W1 had considerably resisted the challenge of W5-2 and W12-2.

The findings demonstrate that antigenic variation occurs in the Colombian strain of *T. evansi* and suggest that variant antigenic types, when inoculated into a new host, revert to a former antigenic type in the first relapse population.

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HERBERT, W. J. & LUMSDEN, W. H. R. (1968). *J. comp. Path.*, 78, 341.  
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infection in mice. Trans. R. Soc. trop. Med. Hyg. (in press)