I

THE INFLUENCE OF MODE OF TRANSMISSION OF *TRYPANOSOMA CONGOLENSE* ON THE STABILITY AND INDUCTION OF RESISTANCE TO SAMORIN.

by

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DECLARATION

I, J.H.P. Nyeko declare that this thesis is my original work and it has never been submitted for a degree award in any other University.

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ABSTRACT

A series of experiments were performed to determine if the level of drug susceptibility of Trypanosoma congolense strains changed during cyclic or syringe passages. It was interesting to note that when a drug-resistant trypanosome strain was maintained through 12 cyclical transmissions in the tsetse flies (in the absence of drug), no significant reduction in the level of resistance was observed. When the same trypanosome strain was maintained in mice through 20 syringe passages (in the absence of drug), a significant reduction in the level of resistance was noted after the 15th syringe passage.

In another experimental series, tsetse flies infected with a drug sensitive T. congolense strain were maintained on rabbits that were previously treated with Samorin. It was observed that the infections arising from flies maintained for over 60 days on the drug-treated rabbits needed higher doses to cure. Attempts to induce resistance in the same trypanosome strain by syringe passages of the relapsed infections rapidly produced high levels of resistance.

The course of parasitaemia was studied in both the treated and untreated animals. It was observed that parasitaemia persisted for 2 to 3 days in the treated animals before disappearing. Tsetse flies were fed on parasitaemic but recently treated animals.

Some of the flies were dissected within 6 to 48 hours after ingestion, and the gut contents inoculated into mammalian hosts. It was observed that the ingested trypanosomes (previously exposed to samorin) lost their infectivity to the mammalian hosts shortly after 6 hours of ingestion. Trypanosomes from tsetse flies fed on untreated animals were still infective 24 hours after ingestion. The infectivity of trypanosomes (previously exposed to drug) to tsetse flies was found to be greatly reduced.

Tsetse flies, infected with the drug sensitive strain, were allowed to feed on previously treated animals (within 1-2 hours treatment). It was interesting to note that although the same dose of samorin could cure infections in the mammalian hosts, it did not destroy all the parasites in the tsetse flies. The drug however caused serious morphological damages to a large percentage of the vector forms of trypanosomes. The degree of parasite damage was higher in the flies fed by artificial membrane technique on bloodmeal containing Samorin.

It was thought that Samorin might exert some chemoprophylactic effects in tsetse flies by preventing the establishment of trypanosome infections as it does in livestock. Tsetse flies were allowed to feed on bloodmeal containing Samorin before being infected. No significant difference in the fly infectivity was observed in the flies following the

prophylactic bloodmeal. In another investigation, flies having immature unestablished infections were maintained on rabbits that were regularly treated with samorin. The drug did not have any significant effects on the maturation of infection in the treated flies.

CHAPTER 1 INTRODUCTION

Tsetse flies and the trypanosomes they transmit are partners which have contributed tremendously to the economic and social backwardness in tropical Africa. African trypanosomiasis causes sleeping sickness in man and Nagana in cattle. The diseases are responsible for the death of tens of thousands of people and hundreds of thousands of livestock annually. In many African countries, agricultural potential and fertile land capable of supporting thousands of human beings and millions of livestock is left unproductive due to tsetse infestation (Fulton, 1959; Bloom, 1976 and Trail et al., 1985). The specific association of trypanosomes with tsetse flies has made the study of these flies very important in applied biology unlike other haematophagus flies such as Stomoxys or Tabanus.

From the time tsetse flies were implicated in the maintainance and transmision of trypanosomes, several methods of control have been described and practised. The control of trypanosomiasis is aimed at either eliminating the insect vectors or the disease. Tsetse control has for a long time depended on; environmental manipulation and insecticide application. The use of sterile insect technique has met with little success and biological control is still at the experimental stage. Detailed description of the various methods of control have been reviewed by Buxton, (1955); Laird, (1977); Jordan, (1979) and Pollock, (1980).

Chemotherapy is the only method in current use in controlling clinical cases and chemoprophylaxis is used for preventing the disease outbreak. Vaccination, a method of disease control which has been successfully used against viral and bacterial diseases such as polio, Newcastle disease, Yellow fever and Tuberculosis has not been as successful in parasitic diseases. The appearance of frequent antigenic variants is a major setback in the development of vaccine against trypanosomiasis (Weitz, 1958, 1970; Johnson et al., 1963; Bloom, 1976 and Le Ray et al., 1978).

Before the advent of chemotherapy, epidemics of human sleeping sickness occurred on a vast scale. Over one half million people were estimated to have died of the disease in Zaire between 1895 and 1902 and in the epidemic around Lake Victoria between 1902 and 1905, about 200,000 people died of sleeping sickness (Williamson, 1962). Grazeable land capable of supporting 120 million cattle remained largely unreproductive and over 3 million cattle died of trypanosomiasis annually (Bloom, 1976 and Trail et al., 1985).

With the introduction of chemotherapy, large scale epidemics have subsided and it is now partially possible to keep cattle economically in areas that are infested with tsetse flies (Whiteside, 1958, 1962; Stephen, 1960, 1962; Boyt et al., 1962 and Fairclough, 1963). No new drug has been introduced during the past 30 years and the few existing drugs are not readily available in all countries in which they are needed (de Raadt, 1976 and Njogu et al., 1985).

Prominent among the trypanocides which have played part in the treatment and control of animal trypanosomiasis are the phenanthridinium compounds (prothidium, ethidium, novidium and isometamidium), quinapyramine, and diminazene aceturate (Berenil). Isometamidium chloride (Samorin) was introduced in 1960 and it is the latest veterinary trypanocide in the market today.

The most outstanding problems in the use of trypanocides are toxicity and the development of resistance by the trypanosomes. The drugs were developed from only one or two basic chemical structures, such that a trypanosome strain resistant to one drug will also fail to respond to several other drugs having similar chemical structure (Williamson, 1962 and Hawking, 1963).

In the light of these facts, a thorough knowledge of the trypanosome biochemistry in relation to modes of actions of trypanocides are required in order to screen newer drugs of diversified chemical structures. Research into the mechanisms through which trypanosomes develop resistance to drugs should also be intensified to control and prevent the spread of drug resistant trypanosome strains in the field.

CHAPTER 2 LITERATURE REVIEW

2.1. TSETSE FLIES

2.1.1. General introduction

Tsetse flies are placed in the genus Glossina Wiedemann 1830, with 22 known species and subspecies. The flies measure between 7.5 and 14 mm in length and have a mottled brown-grey thorax. A technical description of the genus has been given by Newstead et al., (1924) and Buxton, (1955). These authors stressed the importance of the branched hairs on the antennal arista and the hatchet-shaped distal cell between the fourth and fifth longitudinal wing veins as being diagnostic for the genus.

According to Potts, (1970); Glasgow, (1970) and Buxton, (1955), tsetse flies are in the:

Order: Diptera
Family: Glossinidae
Subfamily: Stomoxyiane or Glossinae and
Genus: Glossina.

The genus is divided into three, viz fusca, palpalis and morsitans groups. The identification of these groups is based on the taxonomic characters of the genital armatures of both sexes (Newstead et al., 1924 and Buxton, 1955). The number of species in each group is still far from clear. According to Newstead et al., (1924) and Buxton, (1955) the fusca group has 10, palpalis 6 and morsitans 6 species. But Glasgow, (1963) reported that fusca has 15, palpalis 9 and morsitans 7 species.

Swynnerton, (1936)., Buxton, (1955)., Ford, (1971) and Katondo, (1984) described the cartographical distribution of the tsetse flies in detail. Generally, they observed that palpalis group is riverine and lacustrine inhabitors. The group is found along the Central African rivers, lakes and the West African forests and is responsible for transmitting trypanosomiasis to cattle and T. gambiense sleeping sickness to humans in this region of Africa.

The fusca group occupies the Central African forests spreading from Congo Basin eastwards and southwards. The group transmits both the T. rhodesiense and T. gambiense types of sleeping sickness and animal trypanosomiasis. The third group, the morsitans group, is widely distributed and inhabits the savannah areas throughout West Africa, Southern and Central Africa and the evergreen thickets along the East African coasts and lake shores. The tsetse species in this group are efficient transmitters of a wide range of both human and animal trypanosomes. The complete lists of the Glossina species and subspecies in the three groups has been authoritatively discribed by Pollock, (1980). Glossina morsitans morsitans, the species under the present study is in the morsitans group.

2.1.2. Distribution and feeding of tsetse flies

The study of tsetse flies in relation to climate, vegetation and the mammalian hosts is important for the knowledge of their

distribution and disease incidence. According to Buxton, (1955) and Ford, (1971) the suitable climate, vegetation and the abundance of large mammals (ungulata) in tropical Africa have made the continent an excellent habitat for tsetse flies. And indeed it has been estimated that about 10 million square kilometers of tropical Africa is under tsetse infestation (Fulton, 1959; Nash, 1969 and Bloom, 1976).

The limit of tsetse distribution in the north is the Senegal river at 15°N to the Nile river at about 12°N. This corresponds to an area with a mean rainfall of 750 mm and above. The southern limit is Coastal Angola at about 29°S (Horsfall, 1962). High altitude, low temperature, bush clearance and proper land utilization by the white settlers may account for the absence of these flies further South of the continent.

Tsetse flies are known to feed on wild game, livestock and human beings depending on their availability. The most preferred hosts are suids and bovids, but zebra, impala, gazelles and wildebeest are hardly attacked (Pollock, 1980).

2.1.3. Morphology and classification of the Morsitans group

According to Newstead et al., (1924); Buxton, (1955) and Pollock, (1980) the morsitans group has hard interrupted abdominal bands with the inner lateral margin rounded or tapering. The superior claspers of the male genital armature are united by a

membrane and fused medially at the distal extremity. Most evident in G. morsitans group is a blunt tooth-like extension of the distal margin of the superior claspers. The external armature of the female consists of a pair of fused anal plates and a median sternal plate.

According to Ford, (1971)., Laird, (1977) and Pollock, (1980) the morsitans group consists of five species with morsitans species having three subspecies:

G. morsitans a) G. morsitans morsitans West. 1950
b) G. m. submorsitans Newst. 1910
c) G. m. centralis Machado 1970

G. swynnertoni Aust. 1923

G. longipalpis Wied. 1930

G. pallidipes Aust. 1903

G. austeni Newst. 1912.

2.2. TRYPANOSOMES

2.2.1. General introduction

The systematic position of the trypanosomes and their relation with allied flagellates, their taxonomy and nomenclature have been reviewed in detail by Hoare, (1966, 1970, 1972). According to Hoare, (1972) and Vickerman, (1976), and Molyneux and Ashford (1983) trypanosomes are in the:

Phylum:	Sarcomastigophora
Class:	Zoomastigophorea
Order:	Kinetoplastida
Suborder:	Trypanosomatina
Family:	Trypanosomatidae and
Genus:	Trypanosoma Gruby, 1843.

Many reports appear in literature on the morphology of trypanosomes and a comprehensive account on this subject has been reported by Hoare, (1972) and Vickerman, (1976). They described trypanosomes as being lanceolate in shape with the anterior end tapering to a point while the posterior end may be rounded or pointed. The parasites may be C- or S-shaped or convoluted in different ways while the flagellum and the undulating membrane follow the bands. The apparent shape and size of the kinetoplast, as well as its position in the body; the presence or absence of a free flagellum and the shape of the posterior end are of taxonomic importance for the species of trypanosomes (Hoare, 1972 and Vickerman, 1976).

The genus Trypanosoma is divided into two major sections or groups namely the Stercoraria and Salivaria. This division is based on the site of production of metacyclic trypanosomes in the insect hosts and the subsequent method of infection of the mammalian hosts (Vickerman, 1976). Stercoraria group complete their developmental cycle in the hind-gut of the insect host, the metacyclic trypanosomes are present in the faeces and transmission is by contamination. The group Stercoraria has three subgenera namely

Megatrypanum, Herpetosoma and Schizotrypanum. The species of trypanosomes in this group are basically non-pathogenic except T. cruzi (subgenus Schizotrypanum) which is the causative agent of Chagas' disease in man. It is transmitted by triatomine bugs in Central and South America.

The Salivaria group complete their developmental cycle in the mouth parts of the insect host, the metacyclics are present in the salivary secretions and transmission is mostly by the infective vector bites but mechanical contact transmissions have also been reported (Hoare 1972). The Salivaria has four subgenera namely Duttonella, Nannomonas, Trypanozoon and Pycnomonas. The Salivaria trypansomes are pathogenic to man and domestic animals. Trypanosoma congolense, the trypanasome species used in the present study is in the Nannomonas subgenus.

2.2.2. Morphology and Classification of Nannomonas

The trypanosome species in the subgenus Nannomonas are relatively small parasites as compared to Trypanozoon and Duttonella and the detailed biometrical and morphological description of this subgenus have been outlined by Hoare, (1972). The most characteristic feature of the Nannomonas is the lack of a free flagellum in both the mammalian and insect vector hosts, though some individual parasites may have short free flagellum. The kinetoplast is positioned subterminal and marginal in a great

majority of the parasites. The Nannomonas are typically monomorphic although a certain degree of polymorphism has been observed in T. simiae (Hoare 1972).

The Nannomonas subgenus is composed of two highly pathogenic species to livestock (Hoare, 1972 and Vickerman, 1976):-

Trypanosoma (Nannomonas) congolense Broden, 1904

Trypanosoma (Nannomonas) simiae Bruce et al., 1912

and perhaps Trypanosoma (Nannomonas) montgomeryi Laveran, 1909 but this spcies is believed to be the same as T. (N.) congolense (Stephen, 1963).

Trypanosoma congolense is comprised of diverse strains which differ in their dimensions and certain morphological features and virulence to the mammalian hosts. In the light of this morphological diversification, earlier workers (reviewed by Hoare, 1972) described several forms and divided them into species or subspecies such as T.dimorphon, T. c. urundiense, T.c. berghei, T.c. mossoense and T. montgemeryi.

2.2.3. Life Cycle - in the mammalian hosts

It is of interest to note that our knowledge of the development of salivarian trypanosomes in the mammalian hosts is still incomplete. In T. brucei group of trypanosomes it was

observed that when the metacyclic trypanosomes are inoculated by tsetse flies into the mammalian hosts, they cause the development of nodular swelling the "chancre" at the site of bite (Fairbairn and Godfrey, 1957; Willett and Gordon, 1957 and Gordon and Willett, 1958). The formation of local skin reactions has also been reported in mammals following T. congolense infections (Roberts et al., 1969, Luckins and Gray, 1978, 1979; Gray and Luckins, 1980; Akol et al., 1981; Akol and Murray, 1982 and Luckins et al., 1983). These workers recovered trypanosomes in the fluids from the nodules at the site of infections.

When the metacyclic trypanosomes are inoculated into the mammalian host, they undergo morphological changes characterized by drastic reduction in volume and cell membrane areas of the mitochondria. The trypanosomes are transformed into long slender bloodstream forms (Harmsen, 1973; Vickerman 1970; Brun et al., 1984). The mitochodrial changes are reported to be the expression of changes in respiration (Langley, 1975; Baker, 1977 and Ghiotto et al., 1979). The respiratory metabolism of the slender bloodstream forms are wholly glycolytic involving Glucose-6-phosphate oxidase enzymes but the metacyclic and stumpy bloodstream forms show evidence of mitochondrial oxidative decarboxylation (Bowman et al., 1972; Harmsen, 1973; Langley, 1975; Bowman and Flynn, 1976; Gutteridge and Coombs, 1977; Opperdoes and Borst, 1977; Ghiotto et al., 1979 and Brun et al., 1984).

T. congolense was thought to be strictly a plasma parasite (Losos and Ikede, 1972) but Roberts et al., (1969) Ssenyonga and

Adam (1975); Luckins and Gray, (1978, 1979) and Luckins et al., (1983) obtained evidence to show that T. congolense is capable of developing outside the circulatory system at least during the early stages of the infection. These workers recovered dividing trypanosomes in the fluids at the sites of infection, in association with collagen fibres and in the lymphatic system. The isolation of T. congolense parasites from the cerebrospinal fluid (CSF) of cattle reported by Masake et al., (1984) gives further evidence that T. congolense may not have a strictly haematic distribution.

Knowledge about the distribution of trypanosomes in the mammalian hosts is important in the chemotherapy of the disease. Jennings et al., (1979), and Jennings and Gray (1983) reported that early treatment of T. brucei with berenil gave complete cure whereas delaying treatment invariably resulted in relapse infections and they believed that brain infections were responsible for relapses following treatment. The importance of cryptic foci of T. congolense in the relapse infection following chemotherapy has also been reviewed by Fiennes, (1950). While studying the comparative morphology of bloodstream and lymph forms of T. brucei, Hecker and Brun, (1982) noted that the lymph forms closely resemble the culture forms of trypanosomes.

2.2.4. Life Cycle - in the invertebrate hosts

Knowledge of the internal anatomy and physiology of the digestive system of the tsetse is important in understanding the life cycle of the trypanosomes in the flies. The importance of peritrophic membrane and the salivary glands as barriers to successful development of trypanosomes in the flies has been reported by many workers (Wijers, 1958; Willett, 1966; Moloo *et al.*, 1970; Hoare, 1972; Freeman, 1973; Vickerman, 1974; and Evans and Ellis, 1975).

Although only a few tsetse species are implicated as vectors of trypanosomes in Africa, there is no evidence that any species is incapable of cyclical transmission of trypanosomes. Several of the factors that are known to affect fly infection would be expected to affect all fly species equally, however, Maudlin, (1982) reported that fly infection by *T. congolense* has some genetic controlling aspects.

The *G. morsitans* is the most important vector of cattle trypanosomiasis and it is also a major vector for *T.b. rhodesiense* in East and Central Africa (Hoare, 1972). This species is readily infected with several *Trypanosoma* spp. and it is easily reared in the laboratory. These points do account for the vast literature on the *G. morsitans*/parasite interactions as opposed to those of the other *morsitans* group.

The general course of development of the salivarian trypanosomes in their vector, Glossina spp., has been known for many years. The Nannomonas subgenus develops in the midgut and the proboscis of the tsetse flies while the Duttonella subgenus completes its development in the proboscis only. The Trypanozoon subgenus undergoes the most complicated route of development passing through the midgut, salivary glands and the proboscis (Hoare, 1972) and their presence in the haemolymph of the tsetse flies had been reported by Mshelbwala, (1972) and Otieno, (1973). Some Trypanozoon subspecies such as T. evansi, T. equiperdum are mechanically and sexually transmitted respectively and found generally outside the tsetse fly belt (Hoare, 1972). T. evansi also occurs in the tsetse fly belt where it is transmitted mechanically and in some areas like Kenya it is a serious problem in camels.

Under natural conditions, T. congolense has a very low (0.6 - 10%) infection rate in the tsetse flies but in experimental situations, the infection rate can be as high as 35% (Hoare, 1972). Factors that determine the trypanosome infection rates in the tsetse flies have been reported by many workers amongst which the age and species of the fly, nature of the first bloodmeal and temperature are important (Duke, 1933; Desowitz and Fairbairn, 1955; Buxton, 1955; Wijers, 1958; Wijers and Willett, 1960; Harley, 1971 and Baker, 1974). Recently Maudlin et al., (1984) reduced infection in G.m. morsitans by maintaining the flies on animals that received antiserum to procyclic T. congolense.

If tsetse flies ingest trypanosomes in the blood while feeding, the trypanosomes must adapt themselves to life inside the tsetse flies. The processses involved are thought to be more pronounced in the case of T. (T.) brucei subgroup (Baker, 1977). According to Harmsen, (1973) and Langley, (1975) the process of adaptation in T. brucei is effected by an enzyme transformation (l-glycerol-3-phosphate oxidase) while the parasites are still in the crops of the flies. They believed that the age of the flies at the time of infection was important and in young flies, the crops would still be small thus retaining blood longer allowing time for the enzyme transformation to act on the trypanosomes. Similar observations stressing the importance of age and low temperature as major factors on T. brucei infection rate in tsetse flies had been reported by Jenni, (1977) and Otieno et al., (1983). In T. congolense and T. vivax little is known about the bloodstream forms pre-adapting to life in the vector (Baker, 1977).

During the developmental cycles of trypanosomes especially in the Trypanozoon subgenus there is an alternating proliferation and regression of the mitochondrial structure. This complex process of physiological alterations undertaken by trypanosomes during their developmental cycles is believed to be controlled by Kinetoplast DNA (kDNA) (Bowman et al., 1972; Newton, 1976; Newton and Burnett, 1972 and Vickerman and Preston, 1976). The bloodstream stumpy forms of T. brucei spp. which are reported to be infective to the tsetse flies show evidence of mitochondrial oxidation decarboxylation and

they have greatly increased mitochondrial volume (Langley, 1975; Bowman and Flynn, 1976; Ghiotto et al., 1979 and Brun et al., 1984).

After transformation there is an initial multiplication of procyclic trypomastigotes in the midgut. The promastigotes later invade the ectoperitrophic space and reach the proventriculus where they continue to multiply. The proventricular forms finally migrate to the proboscis where they transform into the epimastigotes and multiply further to give rise to the infective metacyclic trypanosomes (Hoare, 1972 and Baker, 1974). During the long process of development the trypanosomes are reported to cross three barriers before reaching the metacyclic stage viz. establishment in the midgut, penetration of the peritrophic membrane and migration to the proboscis or salivary glands (Fairbairn, 1958; Harmsen, 1973; Freeman, 1973; Baker, 1974; Dipeulu and Adam, 1974 and Evans and Ellis, 1975).

The reports on the duration from the time the fly ingests trypanosomes to the time the fly becomes infective have been conflicting. The prepatent period of T. congolense in the fly is reported to be 19-53 days (Buxton, 1955 and Hoare, 1972). It is important to note that Jenni, (1977) obtained mature infection of T. brucei in G. morsitans in 9 days. This is in sharp contrast to the popular belief that T. congolense takes a shorter time to develop in the fly than the brucei group (Buxton, 1955 and Hoare, 1972). This point stresses the importance of temperature, age and species of the tsetse flies and the trypanosome species in determining the duration and rate of infection in tsetse flies.

It has been reported by Borst and Fairlamb (1976) Opperdoes et al., (1976) and Hajduk and Cosgrove, (1979) that loss of the kDNA leads to an inability of the trypanosomes to synthesize functional mitochondria and they fail to develop in the insect vector as found naturally in dyskinetoplastic forms of T. evansi. Under natural situations, loss of kinetoplast in T. evansi and T. equiperdum was reported to reach 100% in certain strains, whereas in T. congolense and T. vivax dyskinetoplastic forms do not exceed 0.8% (Hoare 1954).

According to Newton, (1972) the kDNA is the target for some trypanocide inhibition leading to the appearance of dyskinetoplastic trypanosomes (Hajduk, 1979). But the importance of the kDNA in the transformation process of T. brucei for life inside the tsetse flies has been reported by many workers. As the trypanocides in the phenanthridium group (e.g. Samorin and ethidium) affect the normal functions of the kDNA and cause a high percentage of dyskinetoplastic forms in a trypanosome population (Riou, 1976; Riou et al., 1980), they are likely to be important as factors determining the infection rate in tsetse flies in field situations of host/drug/parasite/vector interactions. Indeed, Agu (1984) observed drastic reduction in the fly infection rate with T. vivax when the flies were given prophylactic treatment of Samorin.

2.3. CHEMOTHERAPY OF TRYPANOSOMIASIS

The exploitation of organic chemistry of new synthetic dyes as cellular stains by Ehrlich contributed much to the success of modern chemotherapy. The cell fixing properties of these dyes were used for establishing the principles of selective dye uptake by bacteria and protozoa for chemotherapeutic purposes. Detailed accounts of the historical development of chemotherapy and chemoprophylaxis of trypanosomes with special emphasis to the work of Ehrlich and other investigators on testing various synthetic dyes for therapeutic purposes have been reviewed by many scholars (Finlay, 1950; Davey, 1958; Williamson, 1962, 1970 and Hawking, 1963). Recent advances in the chemotherapy of trypanosomiasis in relation to the classification and mechanisms of action of the current trypanocides have been discussed by Williamson, (1962,1976, 1979); Newton, (1974, 1976); Leach and Roberts, (1981).

Trypanosomes were the first parasites for early experimental chemotherapy because they were found to infect most of the laboratory animals and were also easily visible under light microscopy. The first successful treatment of experimental infection of T. brucei in rats and mice was achieved in 1902 with human serum and sodium arsenite (Williamson, 1970).

Following this success, progress in chemotherapy was made along two main lines; testing of synthetic dyes from which Suramin with activity against T. brucei, arose from Congo Red, - and the

testing of organic arsenicals which gave rise to Tryparsamide. Both of these drugs received clinical trials in Zaire against T. rhodesiense and T. gambiense with encouraging results (Apted, 1970).

It was later realised that glucose was a very important substrate for the bloodstream trypanosomes and the application of insulin, to induce hypoglycemia in the infected mammalian hosts was suggested and trials with a synthetic insulin, Synthalin, an aliphatic biguamide were shown to be of therapeutic benefit (Williamson, 1970). Improved work along this line led to the discovery of three aromatic diamidines, Stilbamidine, Propamidine and Pentamidine all with activities against human trypanosomiasis (Williamson, 1962, 1970 and Waddy, 1970). Pentamidine became widely used in mass prophylactic treatment in West Africa and Zaire. Further modification of the chemistry of diamidine compound gave rise to diminazene aceturate (Berenil) the first drug with therapeutic activities on both human and animal trypanosomiasis (Davey, 1958 and Waddy, 1970).

Tartar emetic, the first animal trypanocide, was shown to be active against Nagana and Surra in rats as early as 1908 (Williamson, 1970). Several attempts to find synthetic dyes with trypanocidal activities against animal trypanosomes were made in the course of which Surfen C (congasin) was found to be active against T. congolense, but the dye was highly toxic. By modifying its chemical structure, the toxicity was reduced and a new compound, antrycide prosalt (Quinapyramine), with activities against T. vivax,

T. congolense, T. equinum, T. brucei and T. equiperdum was obtained (Marshall, 1958 and Williamson, 1962, 1970). The drug was found to have a prophylactic activity lasting two months.

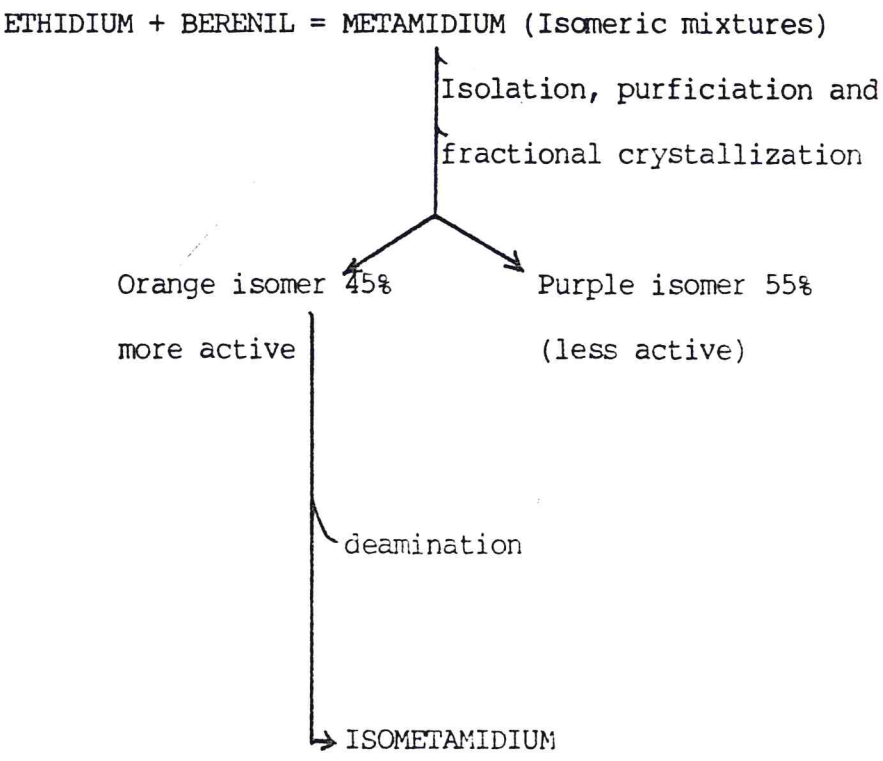
There were extensive trials with the acridine dye nucleus which led to the first phenanthridium compounds - Phenidium and Dimidium both active against T. congolense, but their usage was limited because of toxicity and the development of drug resistance. . Dimidium bromide was modified structurally and Ethidium bromide (Homidium) was obtained with therapeutic and prophylactic activities against T. congolense (Woolfe, 1956 and Davey, 1958).

In another chemical hybridization reaction between Berenil and ethidium, a new trypanocide named metamidium chloride was obtained (Wragg et al., 1958 and Williamson, 1962). This compound was found to be a mixture of orange and purple isomers and it was realised that the orange isomer was more active therapeutically. Therefore, in a further process of chemical isolation, purification, fractional crystallization and deamination of the orange isomer, a new potent trypanocide Isometamidium chloride was obtained (Wragg et al., 1958; Berg, 1960, 1963 and Berg et al., 1961).

2.4. ISOMETAMIDIUM CHLORIDE

2.4.1. Origin, chemical and physical properties

Isometamidium chloride, a phenanthridium drug, owes its orgin to metamidium (which is a product of ethidium bromide and diminazene aceturate). The chemical synthetic procedure from the isomeric mixture, metamidium, with the subsequent isolation and purification by fractional crystallization and deamination of the orange isomer to obtain isometamidium chloride has been described by Wragg et al., (1958); and Berg, (1960, 1963). The process can be illustrated by the following equation:-



The pure orange isomer was designated ISOMETAMIDIUM CHLORIDE HYDROCHLORIDE (7-m-Amidinophenyl-diazoamino-2-amino-10-ethyl-9 phenyl-phenanthridium chloride hydrochloride) or ($CH_{28}H_{25}ClN_7HCl$), with a molecular weight of 531.5 (Berg et al., 1961 and Williamson, 1970).

Isometamidium is a dark reddish-brown powder. It has a solubility of 2% in water at 25°C and a 1% solution has a pH of 6.0 (Berg et al., 1961). The drug is marketed in East Africa under the trade name 'SAMORIN'.

2.4.2. Modes of action, Laboratory and field trials

The studies of the biochemical modes of action of antimicrobial drugs over many years has led to the identification of six key areas of metabolisms as targets for drug action. According to Gutteridge and Coombs, (1977) these targets are energy metabolism, membrane function, cofactor synthesis, nucleic acid synthesis, protein synthesis and cell wall synthesis.

The phenanthridinium drugs (ethidium, prothidium, isometamidium etc) interact with Deoxyribonucleic Acid (DNA) primer and inhibit the synthesis of nucleic acid (Riou, 1976). Delain et al., (1972) and Newton, (1972, 1976) observed that ethidium interacts with the trypanosome DNA causing a local unwinding and lengthening of the DNA double helix and thus interfering with its

function as a primer in nucleic acid synthesis. These drugs have particular predilection for the trypanosome kinetoplast DNA resulting into the loss of kDNA. And indeed dyskinetoplastic trypanosomes have been induced by the use of these drugs (Riou, 1976; Hajduck 1979, and Riou et al., 1980).

The first laboratory investigations with samorin was by Berg et al (1961) who reported that when administered at the rate of 0.025 mg/kg the drug protected 30 out of 32 mice from T. congolense infection. Further work on the prophylactic properties of samorin were studied by Hill and MacFadzean, (1963) who reported that the drug when administered at 0.0006mg/kg into the tail of mice, accorded protection against T. congolense infection for 112 days and fluorescence due to the drug was detected at the site of injection for over 210 days. They observed that protection was reduced to one week only if the tail was amputated within 24 hours after injection of the drug. From these results, the workers concluded that the prophylaxis of samorin is due to its ability to adhere to protein and form a deposit at the site of injection. The deposit slowly releases small quantities of the active compounds which circulate in the blood and exert chemoprophylactic activity and this is maintained until the deposit is exhausted.

Using the samorin parent compound metamidium chloride in Kenya, Fairclough, (1958) observed that metamidium injected at the rate of 0.1mg/kg and 0.2mg/kg body weight cured the local strains of T. congolense and T. vivax, respectively. The curative and

prophylactic action of metamidium was reported by Kirkby (1960) who protected cattle against T. congolense infection for 111 days with 0.5mg/kg but the same dose protected cattle against T. vivax for only 41 days.

The prophylactic activities of metamidium have been tested in East, Central and West Africa on various breeds of cattle exposed to the risk of different Trypanosoma species and the results were encouraging (Whiteside 1958, 1960; Marshall, 1958; Fiennes, 1960 and Fairclough, 1963). Kirkby, (1961, 1964) and Finelle, (1962) in reports of their studies on Nigerian cattle demonstrated that curative doses of isometamidium against T. congolense and T. vivax was between 0.25 and 1.0mg/kg bw. And in a later study, Kirkby, (1963) reported obtaining a cure at 0.1mg/kg bw and protecting cattle for 120 days against T. congolense infection using the same dose.

Recently Fluck, (1985) obtained longer prophylactic period using the slow release formulation of samorin with liposomes as carriers for the drug. The liposomal samorin preparation was found to have a gradual plasma level reduction and was less irritant at the site of injection. This point is important as higher doses than the recommended 2 mg/kg could be given to cure resistant strain with little danger of dermal toxicity.

The use of low doses of trypanocides has been cited as one of the factors causing the development of drug resistance (Leach and

Roberts, 1981). Indeed it has been demonstrated by Whiteside, (1960); Stephen, (1963a); Folkers, (1966) and Stephen, (1970) that the repeated use of drugs at a low dose of 0.25mg/kg resulted into the development of drug resistance.

The efficacy of various trypanocides in the cure and protection of mammalian hosts from trypanosomiasis has been reviewed by several authors, but knowledge about the action of these drugs on the vector forms of trypanosomes is very scanty. Hawking, (1963) tested the effects of various trypanocides on the vector and culture forms of trypanosomes and observed that berenil killed all the parasites in the fly and in culture but samorin neither killed all the parasites in the fly nor in culture. The author worked with tsetse flies collected from the field and he did not prove whether each fly was actually infected before the start of experiment neither did he perform cyclic transmission experiments to confirm whether the infections from the flies were completely destroyed following the blood-meal containing drugs.

Van Hoof et al., (1937) allowed infected flies to feed on animals that were treated with tryparsamide and reported that the flies either lost their infections or transmitted a very mild infection as a result of treatment. The prophylactic action of samorin on the infectivity of flies was tested by Agu, (1984) who reported drastic reduction in the fly infection by T. vivax when a bloodmeal containing Samorin was offered to the flies.

2.5. MECHANISMS OF DRUG RESISTANCE

Knowledge of the biochemistry and physiology of the mechanisms of development of drug resistance in protozoa is still not clear. The basic mechanisms thought to be involved in the development of drug resistance are altered drug uptake, altered metabolism and inactivation of the drugs.

According to Zakrzewski, (1973), these alterations may be due to changes on the cell wall or enzymes responsible for active transport of the drug into or outside the cell resulting in reduced amount of the drug in the resistant parasites. Changes in enzyme proteins (amino acid substitutions) or development of new enzymatic pathways that bypass the steps sensitive to the drugs and the production of enzymes that degrade the drugs into less active products, as observed in the insecticide DDT and antibiotic penicillin (Elliott, 1973 and Oppenoorth and Welling, 1976), are also reported. Whatever the actual mechanisms involved, there is a growing speculation that drug resistance owes its origin to some genetic regulatory factors.

A dominant theme in biology is that Nature favours selective rather than instructive modes of development and control is not only true in evolution and embryogenesis, but also in antibody synthesis and the development of drug resistance. Jacob and Monod (quoted by Miller, 1978) believed that rather than instructing cells to acquire the capacity to synthesize a specific antibody molecules, the cells

do recognise the antigens, and those cells that had pre-existing capacity to synthesize the antibodies are the ones that are selected and proliferate. And indeed the importance of the presence of pre-existing genetic capacity of the cells as being the predisposing requirements for the cells to develop drug resistance had been reviewed by many workers (Zakrzewski, 1973; Elliot, 1973; Oppenoorth and Welling, 1976).

In describing the lactose operon gene regulatory systems in bacteria Escherichia coli, Miller, (1978) stated in simple terms that a substrate does not dictate to cells how to synthesize an enzyme with the appropriate stereospecific properties against itself, but instead, the recognition of the substrate by the cell at the molecular level permits the selection and enhancement of expression of several genes that had the pre-existing capacity to metabolise the substrate. In the specific case of the substrate lactose, he reported that in the absence of lactose, there is a repressor gene which binds to a small region of the DNA, termed the operator gene, thus blocking the synthesis of the enzyme B-galactosidase. When lactose and its analogs are introduced to the bacteria, the cells recognise them and the repressor gene changes (loses) its affinity for the operator gene and the pre-existing genes with the capacity to synthesize enzymes for lactose are activated (Zubay et al., 1976).

The analysis of the lactose operon principles and their regulatory activities on genes in the bacteria E. coli may well

offer the principles of genetic mechanisms involved in the development of drug resistance in protozoa. Likewise, the introduction of trypanocides may lead to the recognition of these chemicals at molecular level thus the selection of those trypanosomes that had the pre-existing genes and the capacity to synthesize enzymes which can metabolise the drugs into less active substances. And indeed it has been reported by Elliott, (1973) that the enzyme penicillinase is usually absent in the bacteria Bacillus spp., but its production is induced by the introduction of penicillin to the bacteria and this leads to the development of drug resistance.

Most drugs that inhibit bacterial growth also inhibit biogenesis and metabolism of mitochondria and therefore, changes on the mitochondrial membrane or on the mitochondrial protein synthesis may lead to the development of drug resistance (Elliott, 1973 and Beale and Knowles, 1979). Knowledge that mitochondrial DNA may be important in the development of drug resistance had been gained from the study of yeast Saccharomyces cerevisiae and the protozoa Paramecium aurelia. Linnane et al., (1968) and Thomas and Wilkie, (1968) reported that the inheritance of erythromycin resistant mutants was non-mendelian and non-genetic in nature and that the erythromycin resistance factor was located in the mitochondrial DNA.

Further proof that mitochondrial DNA has resistance factor was demonstrated by Knowles, (1974) when he injected the mitochondrial preparations from drug resistant paramecia into the

drug sensitive strain and the recipient became permanently resistant. The hypothesis of mitochondrial drug resistance may be important in drug resistance development by trypanosomes as most trypanocides act on the kinetoplast/mitochondrial DNA (Newton, 1972, 1974).

Resistance in bacteria can either be acquired by mutation in their own genome or by accepting from another cell extranuclear plasmid DNA containing resistance genes (Elliott, 1973). Drug inactivation by biochemical alteration such as addition of substituents involving acetylation or adenylation as in streptomycin and chloramphemicol is a common mechanism of drug resistance expressed by plasmid DNA (Shaw, 1967; Harwood and Smith, 1969). In depth studies on the genetics of plasmid DNA and their importance in bacterial drug resistance have been treated in detail by Mitsuhashi, Rosival and Krcmery, (1977).

In bacteria, the extranuclear plasmid DNA which contains the resistance transfer factor (RTF) together with genes that can confer resistance to several drugs are responsible for the development of drug resistance by the sensitive strains in a mixed infection with a resistant strain (Beale and Knowles, 1979). The acquisition of drug resistance by a drug sensitive plasmodia parasites during a mixed infection with a drug resistant strain has been reported by Yoeli et al., (1969) and Peters, (1970). Attempts to demonstrate transfer of drug resistance by allowing two strains of drug sensitive and

resistant Trypanosoma to multiply in a common host were unsuccessful (Amrein, 1957; Amrein and Fulton, 1959). However Inoki and Matsushiro (quoted by Zakrzewski, 1973) were able to produce resistance to p-rosaniline in T. gambiense by direct transfer of DNA from a resistant strain to the sensitive strain in mice. Successful transformation of drug resistance to arsenical trypanocides by the introduction of DNA extracted from a drug-resistant strain of T. rhodisiense into cultures of a non-resistant strain was also demonstrated by Fulton, (1960).

In trypanosomes, drug resistance is usually accompanied by morphological changes on the kinetoplast expressed as loss of the kinetoplast (Schnitzer and Grunberg, 1957; Williamson and Rollo, 1959; Riou, 1976; Hajduk, 1979 and Riou et al., 1980). Earlier workers observed that T. brucei or T. equiperdum resistant to acridine and p-rosaniline dyes lose their kinetoplast when exposed to these drugs. Ormerod, (1952) studied drug absorption of resistant and sensitive T. equiperdum using fluorescence microscopy. He observed that following treatment with antrycide the kinetoplast and cytoplasmic granules still showed strong fluorescent twenty four hours post-treatment, but in the resistant strain most of the fluorescence was lost, except in the kinetoplast. He believed that the drug penetrated the resistant and sensitive cells equally well, but the sensitive strain retained the drug much longer.

Decreased drug uptake as a mechanism of drug resistance has been cited by many scholars (Yorke et al., 1931, 1932; Fulton and Grant, 1955). Using the fluorescing drug stilbamidine, Fulton and Grant, (1955) were able to demonstrate that whereas T. rhodesiense sensitive to this drug took up enough drug to show the whole shape of the parasites, in the resistant parasites, only a few granules were fluorescing. According to Gutteridge and Coombs, (1977) decreased drug penetration is a major cause of resistance in trypanosomes particularly to phenanthridium drugs which are taken by the parasites through differential permeability mechanism.

The capacity of trypanosome population arising from a single parasite (clone) to have varying degrees of drug resistant levels has been reported by Zakrzewski, (1973). This capacity for the population from a clone to have variation in their response may be the same as the production of variable antigen types observed from a single trypanosome (Doyle et al., 1980).

The hypothesis of clonal drug resistance variation in bacteria has been discussed in detail by Yudkin, (1953). He argued that a bacterial cell of given resistance may divide producing two daughter cells with different and unequal resistance as the distribution of the genetic or enzymatic materials responsible for the development of resistance would not be completely identical in the two cells.

2.5.1. Stability of drug resistance

The stability of drug resistance in trypanosomes in the absence of the drug has been reported with conflicting results. Some workers observed that drug resistance in T. vivax and T. congolense tends to disappear during cyclic transmissions by tsetse flies in 6 - 9 months provided the use of the drug was suspended (Whiteside, 1960); but Whiteside, (1962) and Jones-Davies, (1968) reported that an homidium resistant strain was maintained and cyclically transmitted by tsetse flies for over 2 years in the absence of the drug. Gray and Roberts, (1971) observed no progressive loss of drug resistance when they maintained T. vivax and T. congolense resistant to various trypanocides through 8 cyclic transmissions in the absence of drugs.

It must be noted however that, these workers did not determine the actual degree of resistance, in quantitative terms, before the start of cyclic and syringe passages. This is important because a decrease from 20-fold to say 10-fold resistance could be quite significant though the parasites would still be resistant to the normal therapeutic doses. Again the authors investigated samorin at the doses 0.25 - 0.5mg/kg bw and concluded that the trypanosome strains were resistant. But according to Leach and Roberts, (1981), the normal curative doses of

Samorin for drug sensitive trypanosomes are 0.5 - 1.0mg/kg bw. It would appear from this that Gray and Roberts worked with Samorin at lower doses on a trypanosome strain that may have been sensitive to 1.0mg/kg bw, such that cyclical or syringe passages in the absence of drug could not influence the parasites' degree of drug sensitivity any more. Indeed the importance of the degree of resistance and its impact on the stability of drug resistance during cyclic and syringe passages have been discussed (Hawking, 1963b and Peters, 1970, 1980).

Van Hoeve and Grainge, (1966) reported loss of drug resistance in T. congolense after two cyclic transmissions by tsetse flies. Willett, (1966a) described a marked increase in sensitivity of a T. brucei isolate to a range of trypanocides at the 32nd syringe passages in the absence of drugs. While reviewing the status of Plasmodium parasites to antimalarial drugs, Peters, (1970) and Rosario et al., (1978) stated that except in Quinine and Mepacrine, resistance has proved to be a stable character during cyclic transmissions through mosquito vectors.

However, it should be stressed that all the conclusions drawn above were based on success or failure to respond to the normal recommended doses of the drugs concerned. No attempts were made by any of the earlier workers to determine the actual curative doses of these drugs on trypanosome strains before the start of the experiment and to use them to compare with the doses obtained after the cyclic or syringe passages. In view of this fact, the reports on the status of the stability of drug resistance during cyclic and

syringe passages in the absence of drugs remain debatable.

During his studies on the effects of drugs on trypanosomes at cellular level, Hajduk, (1979) observed that T. equiperdum exposed to drugs divide producing one daughter cell lacking kDNA and this is rapidly destroyed by the host defence mechanism. The other daughter cell contains normal amount of kDNA but in a 'clumped' configuration. As the drug concentration decreases in the host's blood, the kDNA replication resumes and viable dyskinetoplastic cells containing normal amount of kDNA appear, their dyskinetoplastic phenotype is the result of fragmentation and dispersion of the kDNA throughout the mitochondria. The apparent loss of kinetoplast in the resistant trypanosome strains could be one way of evading the lethal effects of the drug by the parasites.

CHAPTER 3 THE OBJECTIVES OF THE STUDY

The curative and prophylactic values of samorin against livestock trypanosomes have been reported and reviewed by many workers, (Stephen, 1970, 1960; Whiteside, 1960; Kirkby, 1961; Boyt et al., 1962; Williamson, 1962, 1970, 1979; Leach and Roberts, 1981). Knowledge about the effects of the drugs on the vector forms of trypanosomes is very scanty (Van Hoof et al., 1937 and Hawking, 1963a). According to Agu, (1984) some trypanocides like samorin have prophylactic activity on the infectivity of tsetse flies.

The frequent appearance of drug resistant trypanosomes particularly to the prophylactic drugs is a major source of frustration in the use of trypanocides. The actual biochemical mechanisms of development of drug resistance are not fully understood. Several factors amongst which underdosing, high incidence of trypanosomes in conjunction with the use of trypanocides and irregular treatment with prophylactic drugs have been reported by many workers (Whiteside, 1960; Williamson, 1962, 1970; Leach and Roberts, 1981). However, effects of continuous feeding of the infected flies on treated animals (thereby exposing the vector forms of trypanosomes to a sublethal drug environment within the flies) as a possible factor leading to the development of drug resistance has not been investigated.

Both persistance and disappearance of resistance following cyclic or syringe passages have been reported in the past (Van Hoeve

and Grainge, 1966; Willett, 1966; and Gray and Roberts, 1971). However, none of these workers determined the actual curative doses of the trypanosome strains before the start of their experiments in order to compare with doses obtained following cyclic or syringe passages. This was necessary before any valid conclusions could be drawn.

In view of the above considerations, the present investigations deal with drug resistant and drug sensitive Trypanosoma congolense strains with the following main objectives:

1. to determine the doses required to cure 50 and 90 per cent (CD_{50} and CD_{90}) of the infected animals before the start of the cyclic or syringe passages. These doses would form the baseline data.

2. to determine the stability of the drug resistance in T. congolense (Maruma strain) after several cyclic or syringe passages in the absence of drug.

3. to assess the infectivity to tsetse flies of trypanosomes which persist in recently treated animals.

4. to determine how long fly ingested trypanosomes (unestablished infections) take before losing their infectivity to mammalian hosts.

5. to determine whether continuous exposure of a drug sensitive trypanosome strain to samorin during cyclic or syringe passages would lead to the induction of drug resistance.

6. to observe whether tsetse flies can transmit trypanosomes exposed to curative doses of samorin and to note any effects of drug on the morphology of the vector forms of trypanosomes.

7. to observe whether offering drug-treated bloodmeal to tsetse flies before or immediately after ingesting trypanosomes would prevent the establishment of the parasites in the flies.

CHAPTER 4 MATERIALS AND METHODS

4.1. Experimental Animals, Flies and Trypanosomes

White (albino) mice, random bred (BALB) over 6 weeks old weighing between 20-25 g were used in the experiments. The mice were bred at the International Centre of Insect Physiology and Ecology (ICIPE) animal house. They were fed on mice pellets and water ad libitum. Both sexes were used.

New Zealand white rabbits (both sexes) weighing 2.0 to 2.5 kg were obtained from the ICIPE animal house. They were kept in individual cages and maintained in the animal house at ambient temperature. They fed on cabbages, carrots, rabbit pellets and allowed plenty of water.

Tsetse flies Glossina morsitans morsitans (both sexes) were used in the investigations. The flies were bred and reared at the ICIPE insectary maintained at 25 $\pm$ 0.5°C and 70 - 80 percent relative humidity (RH). They were used in cyclic transmissions.

Two strains of Trypanosoma congolense were used in the experiments:

T. congolense (Maruma strain), isolated from Maruma farm in the Coast Province of Kenya, and reported to be resistant to samorin was obtained from the Chemotherapy of Trypanosomiasis Project, Kabete. It was designated as NYK 223 strain.

T. congolense (IL 1180 strain) known to be sensitive to samorin was obtained from Kenya Trypanosomiasis Research Institute (KETRI) Muguga. It was designated as NYK 222 strain.

4.2. Preparation of reagents and chemicals

Isometamidium chloride hydrochloride (May and Baker), marketed in E. Africa under the trade name 'Samorin' was the drug used in the investigations. It was purchased from the Veterinary Research Laboratories, Kabete. The appropriate weights of the drug required to obtain doses of 8 mg/kg body weight (bw) were determined and the drug was weighed using Millibalance (model 75550, CAHN Electrobalance DTL). Doses of 4, 2, 1, 0.5, 0.25 and 0.125 mg/kg bw were obtained by serial dilutions of the 8 mg/kg bw stock with distilled water. These doses were used for the treatment of the drug sensitive strain.

For drug resistant strain, the drug was weighed and constituted as described above to obtain doses of 25, 20, 17.5, 15, 12.5 and 10 mg/kg bw. The doses chosen were arrived at empirically in trial experiments in order to get enough points for a reliable regression line.

Sodium chloride, phosphate-buffer solutions (PBS) and PBS containing glucose and heparin (PBSGH) pH 8.0 were the media used for dilution of trypanosomes (stabilates, blood or gut contents). The method for preparation of these reagents were those described by Lumsden et al., (1973).

The preswollen gel of diethylaminoethyl cellulose (DEAE-52 Whatman Ltd.) was used for the separation of trypanosomes from the blood cells. The gel was suspended in PBS and PBSGH and adjusted to pH 8.0. It was packed in 10 ml plastic syringe column using drops of PBSGH as described by Lumsden et al., (1973) and Lanham and Godfrey, (1970).

The stock solution of Giemsa stain was obtained by heating pure Giemsa stain powder to which glycerin and methyl alcohol were added. The prepared stock solution was diluted with PBS or distilled water to give 1 to 10 concentration before using it for staining the thin or thick blood smears.

4.3. Examination of infection in experimental mice

Animals were examined for infections by the following parasitological methods; wet blood film, thick and thin blood smears, micro-haematocrit centrifuge technique (MHCT) and animal inoculation as described by Murray et al., (1977).

The tip of the tail of each mouse was pricked with a needle and a small drop of blood was squeezed on a clean microscope slide and a coverslip placed gently over it. The wet blood film so obtained was examined for infection (at x 400 magnification). Over 50 microscope fields were searched before a negative result was recorded.

Thin blood smears were fixed in methanol for about 30 seconds and then the dry thin and thick smears were stained for 30-60 minutes in Giemsa stain. The stain was flushed with distilled water, the slide dried and examined (at x 400 or x 1000 magnifications under oil immersion) for infection. At least 50 fields were examined.

Where the above methods failed to detect the infection, the MHCT was employed as described by Woo and Kauffmann, (1971). A small amount of blood from the tip of the tail was collected into the heparinized capillary tube. One end of the tube was sealed with plasticine. They were centrifuged for 5 minutes at 12,000 rpm. (14890 x g). Trypanosomes were examined at the interface of plasma and buffy coat layer directly in the capillary tube (Woo, 1970).

Blood from mice found to be negative to infection by all the above methods were inoculated into clean mice. The recipient mice were examined for infection as described earlier for 30 days after which they were destroyed.

4.4. Preparation of Trypanosome Clones and Stabilates

It was necessary to clone the trypanosomes in order to start the experiments using a homogeneous parasite population. The detailed method of cloning was that described by Lumsden et al., (1973) and Peters, (1980). Blood from infected mice was diluted in PBS to give antilog 6 (3.1×10^6) parasites per/ml. By further

serial dilutions, a concentration of one parasite per 0.1 ml of the diluent was obtained, such that if a drop of the diluent was examined under the microscope at x 10 x 40 magnification, only one parasite would be found. Mice were then given an intraperitoneal injections of 0.1 ml of the inoculum. Blood from these mice was passaged into other mice at 5 days intervals until infected mice with high parasitaemia were obtained.

Mice infected by the trypanosome clones were bled for stabilate preparation by intracardiac method using heparinized syringe. A few drops of glycerol were added to the blood, shaken properly and allowed to stand for 30 minutes in ice-water bath. The blood was then filled to $^{1}/3$ into the heparinized capillary tubes and sealed at both ends by heating using the microburner. The sealed capillary tubes were kept in ice-water bath before transfering them into large tube jackets containing the labels of the stabilate and the tube jackets placed into the solid carbon dioxide cabinet at $-79^{\circ}C$ as described by Lumsden et al., (1973).

4.5. Counting Trypanosomes

The number of trypanosomes in the blood sample or those in the stabilates to be inoculated into mice were counted using the modified Neubauer Haemacytometer. A small amount of blood was filled into Thomas pipette to the 0.5 point mark and PBS added to fill the pipette to the 101 point mark and thoroughly shaken to give a dilution of 1:200. The haemacytometer chambers were filled with

diluted sample and the trypanosomes counted were only those found in the four corner and centre square millimeters (Lumsden et al., 1973). The calculations of the number of trypanosomes from the counts were based on those described by Murray et al., (1983). In all the syringe passage inoculation experiments, the number of trypanosomes were adjusted to 3.1×10^6 parasites per ml of the sample.

Trypanosomes were also counted from the Giemsa stained preparations as a means of following the course of parasitaemia and assessing the effects of drugs on the morphology of the parasites. The course of parasitaemia was followed by counting trypanosomes from the thin smear Giemsa stained preparations taken daily over a period of two weeks from the time of first appearance of infection. Trypanosomes were counted from 50 microscope fields and expressed as the (number of parasites) "x /50 microscope fields".

To avoid errors due to differences in thickness of the smears, counts were done only in those fields showing uniform thickness as judged by the concentration of red blood cells. The number of normal and aberrant trypanosomes in Giemsa stained preparations following treatment were counted as above and the percentage destruction expressed as the number of aberrant (a) parasites over normal (n) plus aberrant parasites (a) multiply by 100 = $(\frac{a}{n + a} \times 100)$.

CHAPTER 5 EXPERIMENTATIONS AND RESULTS

5.1. EXPERIMENT 1

5.1.1. Drug sensitivity test

The doses required to cure 50 and 90 per cent (CD_{50} and CD_{90} of the infected animals were determined before the start of cyclic or syringe transmissions for each trypanosome strain. They were again determined at known intervals of cyclic or syringe passages during the experiments as described in Experiments 4 and 5.

For both trypanosome strains, between 25 - 35 mice were each inoculated with 0.1 ml by intraperitoneal (i/p) injection of the inoculum. Beginning on day 2 post inoculation, the mice were examined for infection using the wet blood film method. The infected mice having 1-20 parasites per 50 microscope fields at x 10 x 40 maginification were weighed, divided into groups of 5 or 6 and injected i/p with the appropriate volumes of freshly prepared samorin solution at the following doses 25.0, 20.0, 17.5, 15.0, 12.5 and 10.0 mg/kg bw for the resistant strain and 8.0, 4.0, 2.0, 1.0, 0.5, 0.25 0.125 mg/kg bw for the sensitive strain. The control infected mice each received 0.1 ml of saline injections.

The mice were examined on days 1, 2, 3 and two times a week for 30 days following treatment to determine cure. The blood from mice that had no relapse infection by day 30 were inoculated into clean mice and these mice examined for infections for another 30 days by the methods described by Murray et al., (1977) to confirm

cure. The number of mice confirmed cured by the animal inoculation method were used to obtain the dose/response relationship. The CD_{50} and CD_{90} were obtained after transforming the dose/response relationship into the probit/log concentration relationship and the regression analysis calculated by the method of Finney (1952).

5.1.2. Results

The prepatent periods for both drug sensitive and resistant strains of trypanosomes were determined before the start of cyclic or syringe passages. It was shown that the drug resistant strain has a prepatent period of 5.9 $\pm$ 0.4 days and the sensitive strain has 4.8 $\pm$ 0.47 days before the start of passages. There was no significant difference in the prepatent period between the two strains at 95 percent confidence limits. Changes in the prepatent periods following cyclic or syringe passage are presented in Tables 9 and 13.

The results in Table 1 show the response of mice infected with T. congolense (resistant strain) to different dose levels of samorin. A dose of 10 mg/kg bw cured only 20% of the infected mice and 25 mg/kg bw cured all the mice. When the dose/response relationship was converted to log concentration/probit of cure, the regression analysis, Fig. 3 shows that the calculated doses required to cure 50 and 90 percent of the infected mice (CD_{50} and CD_{90}) were 13.9 $\pm$ 1.02 and 20.3 $\pm$ 1.13 mg/kg bw respectively.

Table 2 shows that the response of the drug sensitive strain to the lowest dose of 0.125 mg/kg bw was 46.6%, and a dose of 2 mg/kg bw cured all the animals. The CD_{50} and CD_{90} of this strain was 0.15 $\pm$ 0.03 and 0.64 $\pm$ 0.103 mg/kg bw respectively (Fig.4).

To determine the degree of resistance (index of resistance) the mean CD_{50} of the resistant strain (13.9 mg/kg) was divided by the mean CD_{50} of the sensitive strain (0.15 mg/kg). The Maruma strain was 92.6 times resistant. Changes in the index of resistance following syringe or cyclic passages are presented later (Tables 8 and 12).

Table 1. Number and percentage of mice cured over treated against T. congolense (Maruma strain) following treatment with Samorin at different dose rates.

Samorin mg/kg body weight	Number of mice cured/treated and percentage cured cured per replicate (R)				
	R1	R2	R3	Total	% cured
25	5/5	5/5	5/5	15/15	100
20	5/5	4/5	5/5	14/15	93.3
17.5	4/5	3/5	4/5	11/15	73.3
15	3/5	2/5	3/5	8/15	53.3
12.5	2/5	2/5	1/5	5/15	33.3
10	1/5	2/5	0/5	3/15	20
Control 0.0	0/5	0/5	0/5	0/15	00

Fig. 3: The probit regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (Maruma strain) before passages.

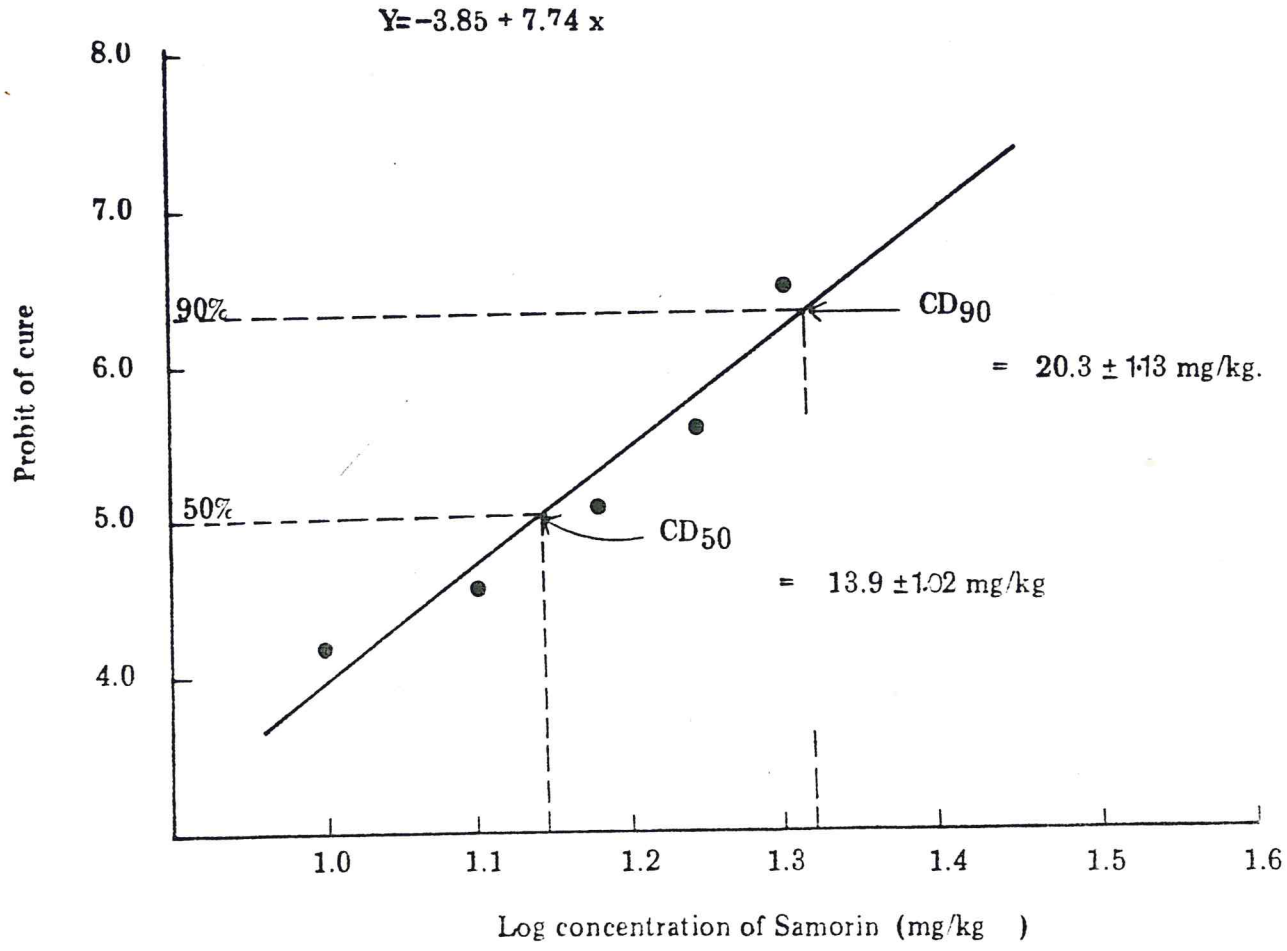


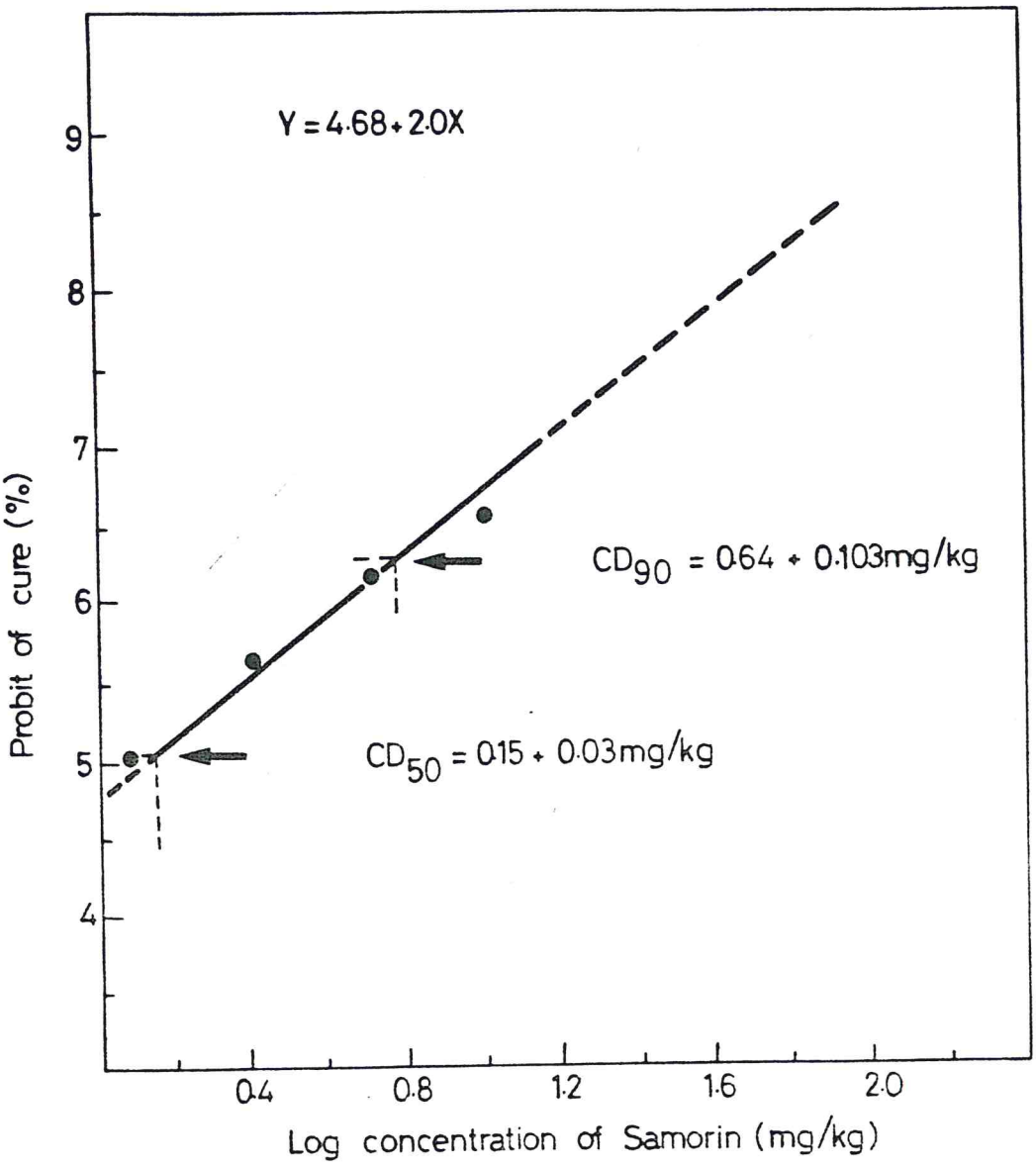
Table 2. Number and percentage of mice cured following treatment with Samorin at different levels (*T. congolense* IL 1180 drug sensitive)

Samorin mg/kg body weight	Number and percentage of mice cured per replicate (R)				
	R1	R2	R3	Total	% cured
4.0	5/5	5/5	5/5	15/15	100
2.0	4/4	5/5	5/5	14/14	100
1.0	5/5	4/5	5/5	14/15	93.3
0.5	3/5	5/5	5/5	13/15	86.6
0.25	3/5	3/5	4/5	10/15	71.4
0.125	2/5	3/5	2/5	7/15	46.6
Control 0.0	0/5	0/5	0/5	0/15	0/0

Numerator denotes number of mice cured

Denominator denotes number of mice treated

Fig. 4: The relationship between log concentration of Samorin and the probit of cure of mice treated against T. congolense (IL 1180 strain) showing the regression line.



5.2. EXPERIMENT 2

5.2.1. Course of parasitaemia and infecting flies with trypanosomes previously treated with samorin.

Infected mice, at first parasitaemic peak (1×10^8 to 1×10^9 parasites/ml blood), were weighed and treated with appropriate volumes of Samorin at 20 mg/kg bw. The course of parasitaemia was studied daily in both the treated and untreated mice for 12 days. Thin blood smears were made and stained with Giemsa's stain and parasite counts was performed as described earlier.

Three groups of 50-60 teneral flies were allowed to feed on the mice 3, 24 and 48 hours after treatment and the control flies were fed on infected but untreated mice. Between 8-12 flies from each group were dissected 24 and 48 hours after the infective bloodmeal and the gut smears examined for the presence and motility or vitality of the parasites. Thin smears of the gut contents were stained with Giemsa stain, the parasites examined for any aberrant morphology.

The remaining flies from each group were maintained on separate rabbits. From day 20, their infectivity was examined by salivary probe and cyclic transmission methods. On day 30, they were dissected and the midguts and the proboscis examined for the presence of trypanosomes. Chi-squared test was used to analyse any differences in the infection rates.

5.2.2. Results

The general course of parasitaemia in the treated and untreated mice is presented in Fig. 5. Irrespective of the dose administered the parasitaemia went up on day 1, dropped slightly on day 2 and sharply declined or completely disappeared on day 3 after treatment.

The effects of Samorin on the morphology of the bloodstream forms of trypanosomes taken 24 hours after treatment as observed in the Giemsa stained preparations were characterized by generalized swelling, vacuolisation of the parasites, granulation of kinetoplast and the nuclei as shown in Plates 1,2 and 3. Complete loss of the kinetoplast (dyskinetoplasty) was observed in what otherwise appeared normal trypanosomes. The abnormality and dyskinetoplasty went up to 94.5% following treatment and from the control animals, only 7.8% of the parasites were aberrant, Plate 1.

Over 175 flies were fed on infected mice which had been treated with Samorin at 20 mg/kg bw. The flies were examined for trypanosome infection 30 days later. Table 3 shows that only 1 (0.97%) fly had established gut infection but no parasites in the proboscis. In the control group, 14 flies (43.7%) had established gut infection out of these 8 (25%) flies had mature infections in the proboscis. This showed a significant reduction in the infection rate following treatment ($P < 0.05$).

Some flies were dissected within 24 and 48 hours after ingesting blood from treated parasitaemic animals. It was interesting to note that the trypanosomes in the gut of these flies were morpholigically normal, but they were all immobile, inactive and appeared dead. The parasites from the guts of the control flies were mobile and active (Table 3). The Giemsa stained gut preparations, Plates 4 and 5, revealed numerous rounded, comma-shaped and granulated aberrant trypanosomes with some parasites appearing normal as shown in Plates 4a and 5a.

FIG. 5 COURSE OF PARASITAEMIA BEFORE AND AFTER TREATMENT WITH SAMORIN AT DIFFERENT DOSES

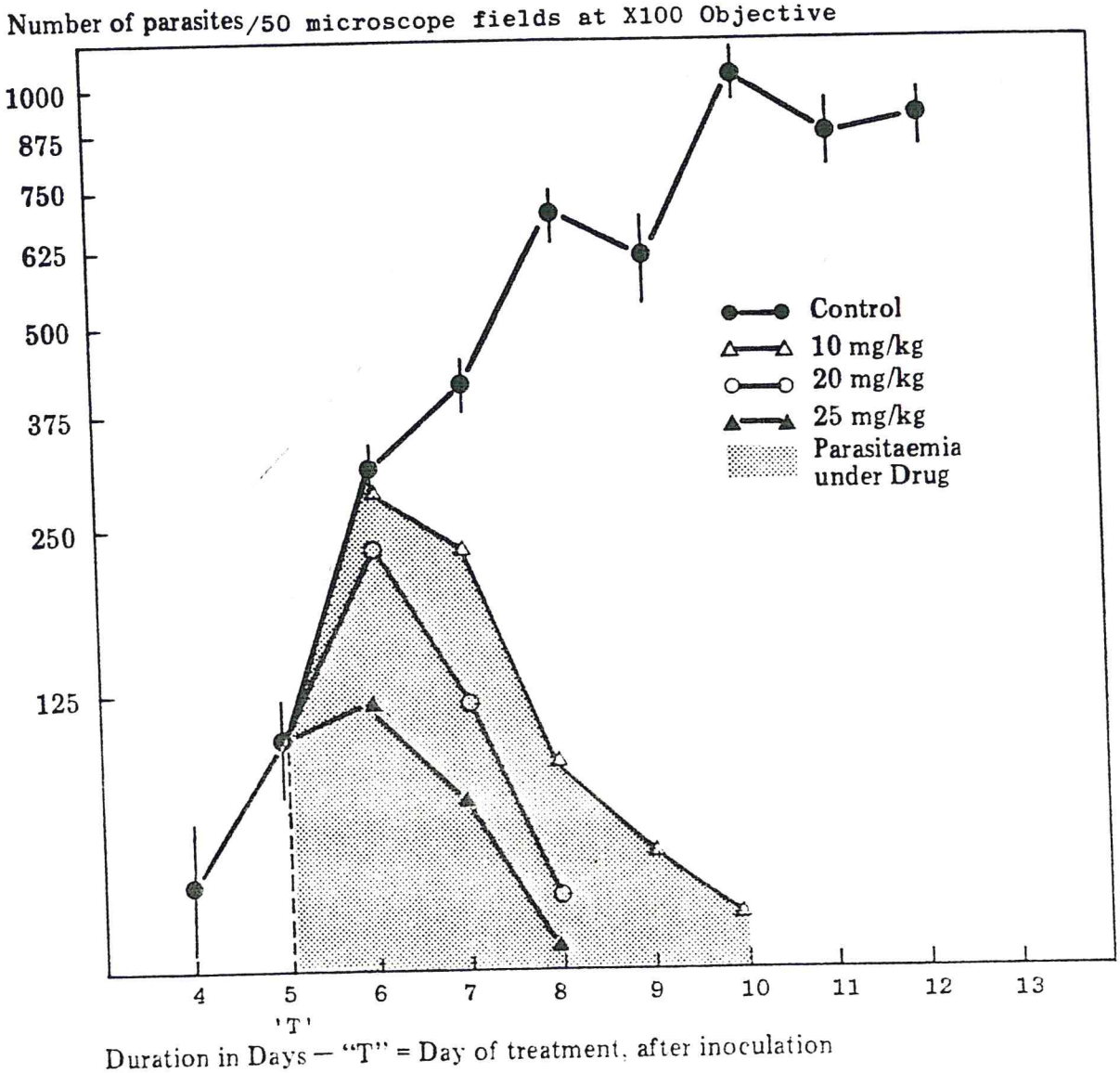


Table 3. Number of flies fed on mice hours after treatment. Number with active gut infection over number dissected hours following ingestion of treated bloodmeal and number with mature infection examined by probing dissection on day 30.

No. flies fed on mice treated with 20 mg/kg samorin hours after treatment	No. flies infested and having active parasites over the number dissected or probed within hours or days after feeding on treated mice.							
	No. infected/dissected flies				No. infected flies on day 30.			
	24 hours		48 hours		Salivary Probe	Dissected		% infected
Time and No. flies	Infected	Active	Infected	Active		Proboscis	Gut	
3 hours No. 55	10/10	0/10	0/10	0/10	0/32	0/32	1/32	3.1
24 hours No. 60	7/12	0/12	4/12	0/12	0/31	0/31	0/31	00
48 hours No. 60	0/8	0/8	0/10	0/10	0/40	0/40	0/40	00
Control Untreated No. 65	15/15	15/15	9/15	9/15	3/32	8/32	14/32	43.7

5.3. EXPERIMENT 3

5.3.1. Infectivity of fly ingested trypanosomes previously exposed to samorin

Group 1. Teneral flies were fed on infected but untreated mice and between 5 - 10 flies were dissected 3, 6, 24, 48 and 72 hours after feeding. Gut contents were diluted in PBS and inoculated into groups of 5 mice each. The mice were examined for infection over a period of 30 days.

Group 2. In another experiment, teneral flies were fed on infected mice treated 24 hours earlier with samorin at 20 mg/kg bw. The flies were dissected and the gut contents inoculated into mice as described above. The recipient mice were examined for infection.

Group 3. Over 10 infected mice at the first parasitaemic peak were treated with samorin at 20 mg/kg bw. Two mice at a time were bled 3, 6, 24, 48 and 72 hours after treatment. Trypanosomes were separated from the blood using DEAE-52, centrifuged and washed three times to remove any traces of samorin. The parasite pellets were diluted in PBS and inoculated into groups of 5 mice each. The animals were examined for infections as described above.

5.3.2. Results

Table 4 shows that the parasites from the guts of flies (group 2) fed on infected treated mice lost their infectivity within 24 hours after ingestion. In the control flies (group 1) fed on untreated animals, the parasites remained infective for over 24 hours but had lost their infectivity 48 hours after ingestion. Table 4 also shows that the blood from treated mice (group 3) remained infective as long as the parasites still persisted in the mammalian blood following treatment.

Table 4. Infectivity of persisting parasitaemia following treatment and of the gut contents of flies dissected within 3 to 72 hours after ingesting bloodmeal from infected treated or untreated animals.

Source of trypanosomes	No. of mice infected over no. inoculated				
	3hr	6hr	24hr	48hr	72hr
Group 3 treated mice	10/10	10/10	10/10	10/10	8/10
Group 2 treated flies	10/10	10/10	0/10	0/10	0/10
Group 1 untreated flies	10/10	10/10	7/10	0/10	0/10

5.4. EXPERIMENT 4a

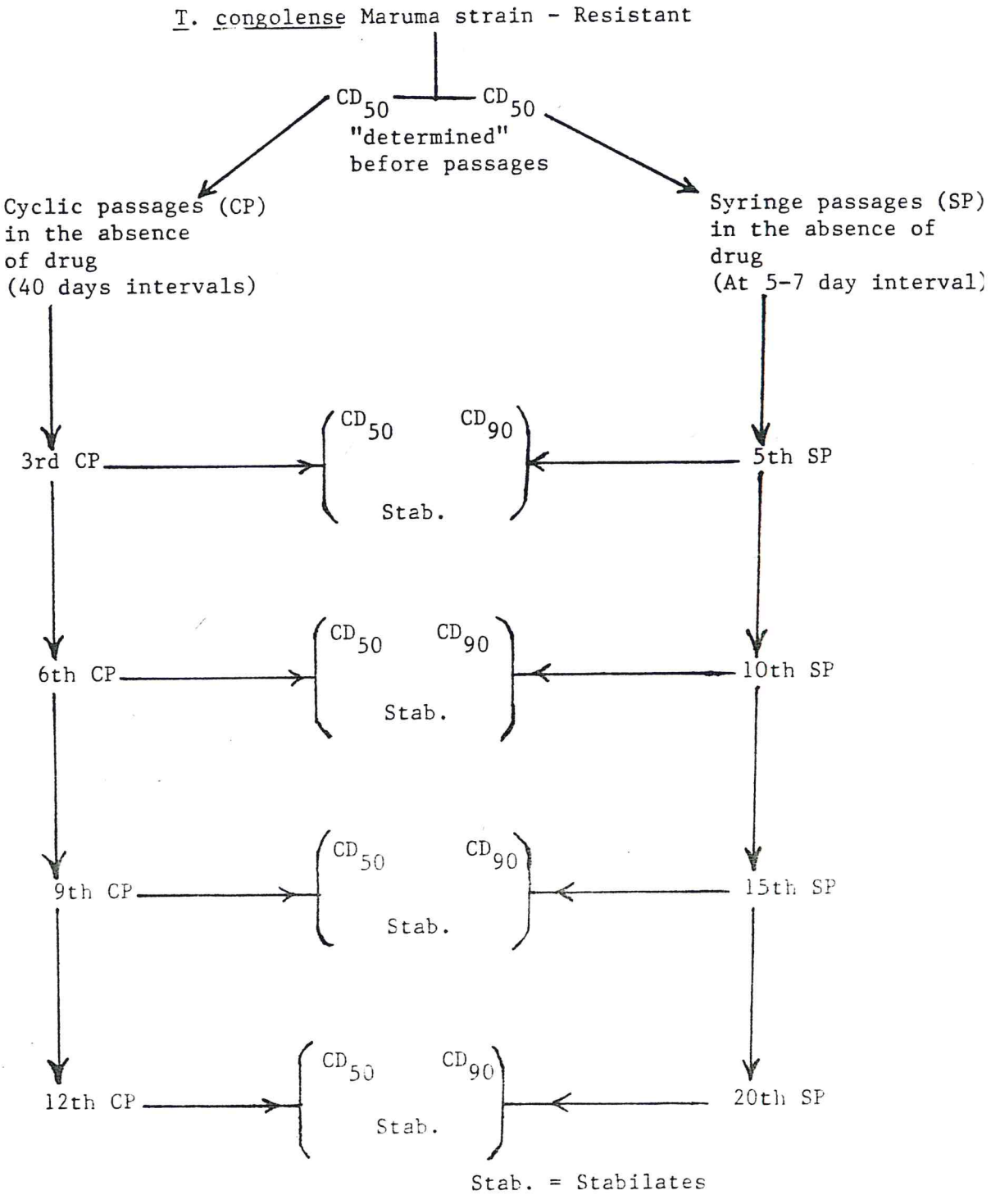
5.4.0. TRYPANOSOMA CONGOLENSE (MARUMA) STRAIN.

5.4.1. Stability of drug resistance through cyclic transmissions:

Trypanosomes with a known CD_{50} and CD_{90} were cyclically transmitted through tsetse flies. Parasites were maintained in the vectors for 40 days in the absence of drug before they were transmitted to susceptible mice. The infected mice were used to infect another batch of flies which were allowed to maintain the parasites for another 40 days in the absence of drug. This procedure was repeated through 12 cyclic transmissions.

Drug sensitivity tests and stabilate preparations were performed on the infections arising from the 3rd, 6th, 9th, and 12th cyclic transmissions, as shown in Fig. 1. The CD_{50} and CD_{90} obtained following cyclic passages in the absence of drug were compared to the standard doses and the 't' test at the 95% confidence limits was used to assess any significant differences.

Fig. 1: Experimental procedure for testing the stability of drug resistance in Trypanosoma congolense (Maruma strain) during cyclic and syringe passages.



5.4.2. Results

Table 5 shows that the drug resistant T. congolense strain has an average infection rate of 21.1% in the fly. Changes in the response of mice to treatment after several cyclic passages of this strain are shown in Fig. 6. It was observed that after 12 cyclic passages, 10 mg/kg bw cured 33.3% of the infected mice compared to 20% in the standard. The percentage of cure of infections arising from the 3rd and 6th cyclic passages were slightly lower than the standard. Notable increase in the percentage cure was observed at the 9th and 12th cyclic passages.

Figs. 7, 8, 9 and 10 show that the curative doses after cyclic passages ranged between 14.3 $\pm$ 1.10 to 11.9 $\pm$ 1.06 mg/kg bw for CD_{50} and 21.4 $\pm$1.17 to 18.0 $\pm$ 1.08 mg/kg bw for CD_{90}. Although there was an apparent rise in the response to treatment after 12 cyclic passages, data in Table 6 show no significant difference (P = 0.05) in the curative dose requirements through 12 cyclic passages in the absence of drug.

Table 5. Number and percentage of flies found infected by salivary probe and dissection on day 35 after feeding on mice infected with T. congolense (Resistant strain) at each cyclic passage.

No. flies fed on infected mice per cycle	No. and % of flies infected by probing and dissection			
	Salivary probe	Dissection Proboscis	Gut	% Infected proboscis
Cycle 1				
No. 65	7/58	11/58	23/58	18.9
Cycle 2				
No. 2	3/24	5/24	5/24	20.8
Cycle 3				
No. 45	5/42	8/42	13/42	19.0
Cycle 4				
No. 25	6/25	7/23*	11/23	30.4
160	22/149	31/147*	52/147	(21.1%)

* Some flies died

Fig. 6: Histogram of the percentage response of mice to different doses of Samorin before and after several cyclic or syringe passages of T. congolense (Maruma strain) in the absence of the drug.

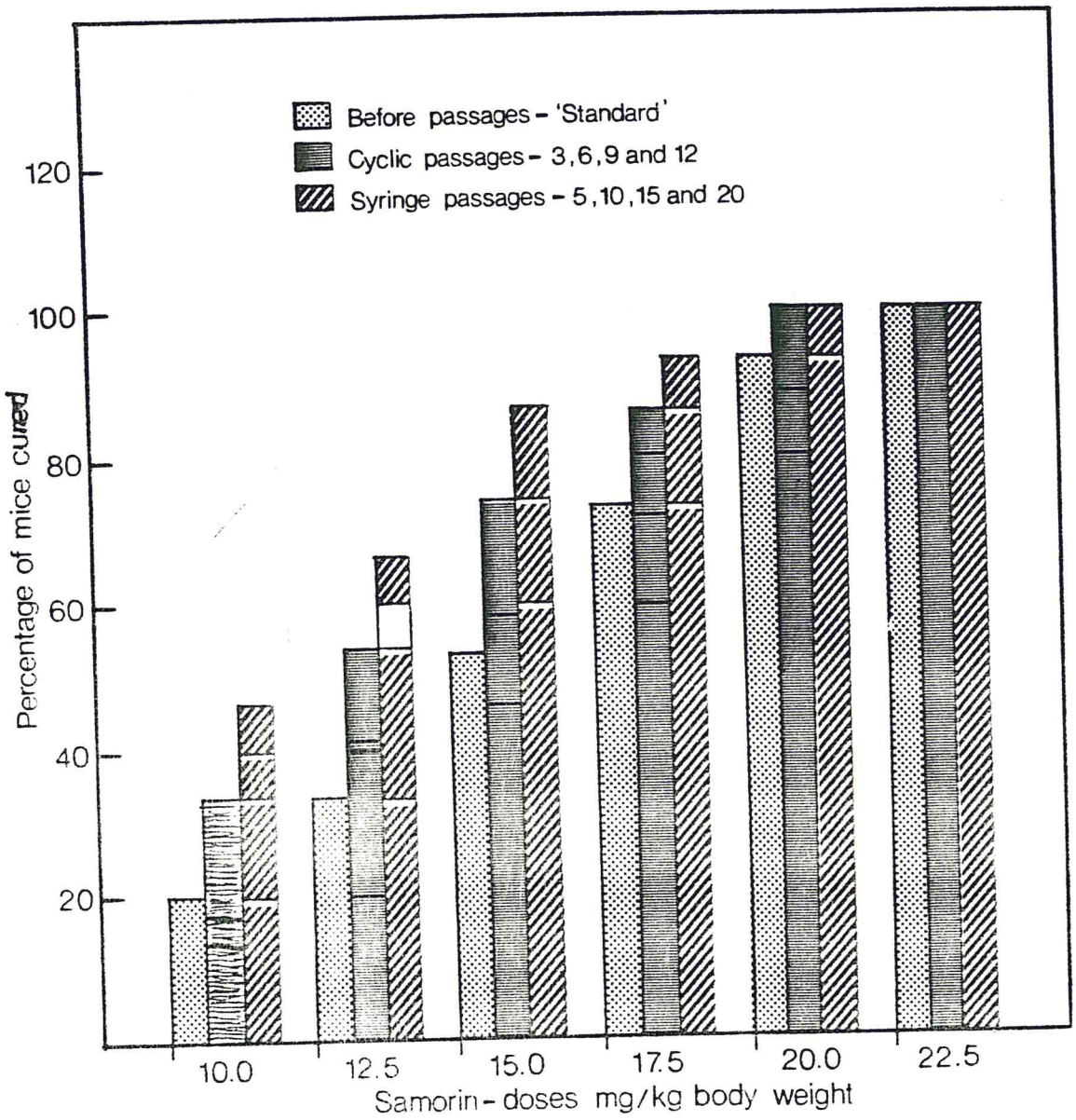


Fig. 7 The probit regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (Maruma strain) 3rd cyclic passage

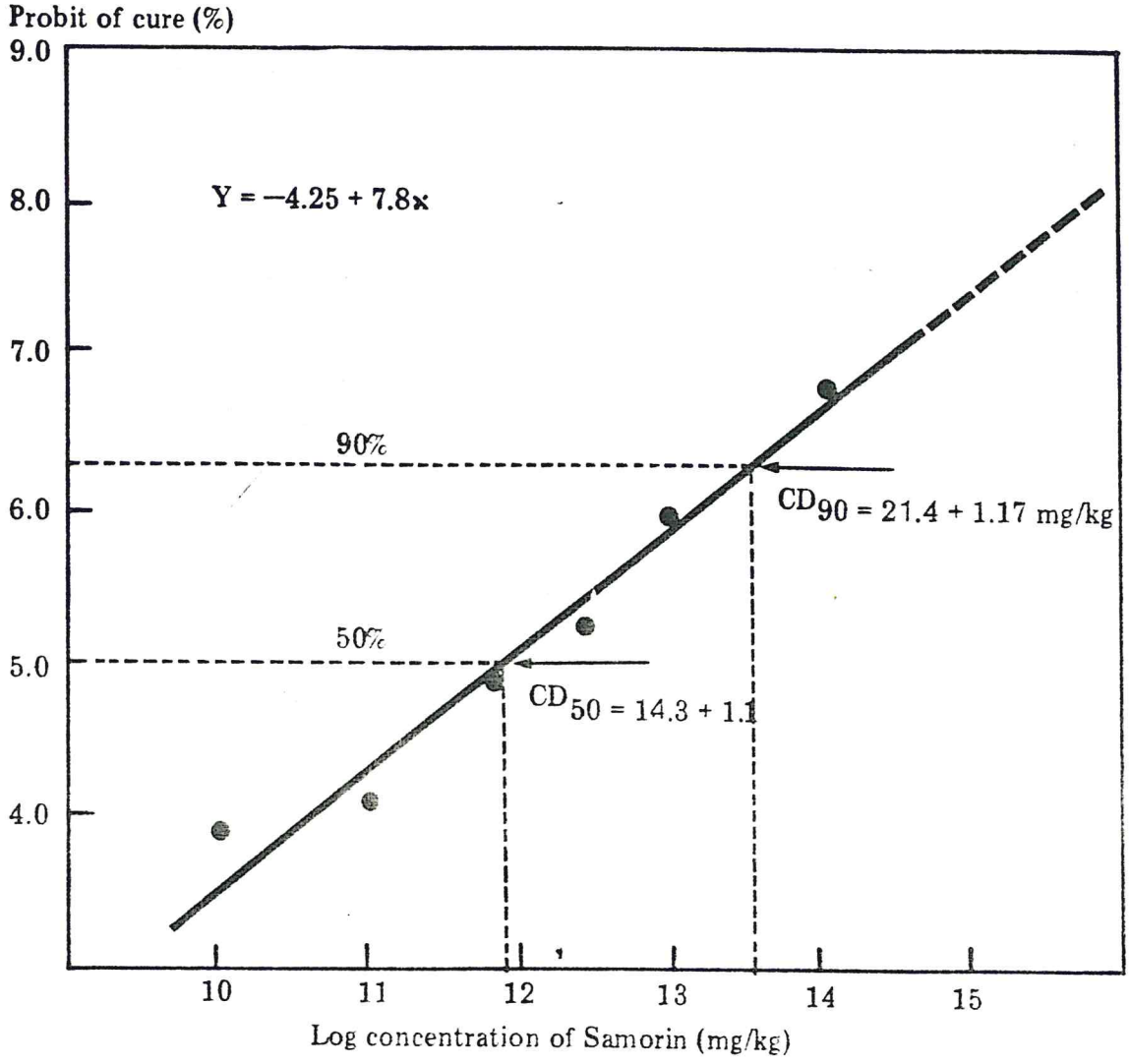


Fig. 8. The probit regression line showing the relationship between log concentration of mice following treatment against T. congolense (Maruma strain) 6th cyclic passage

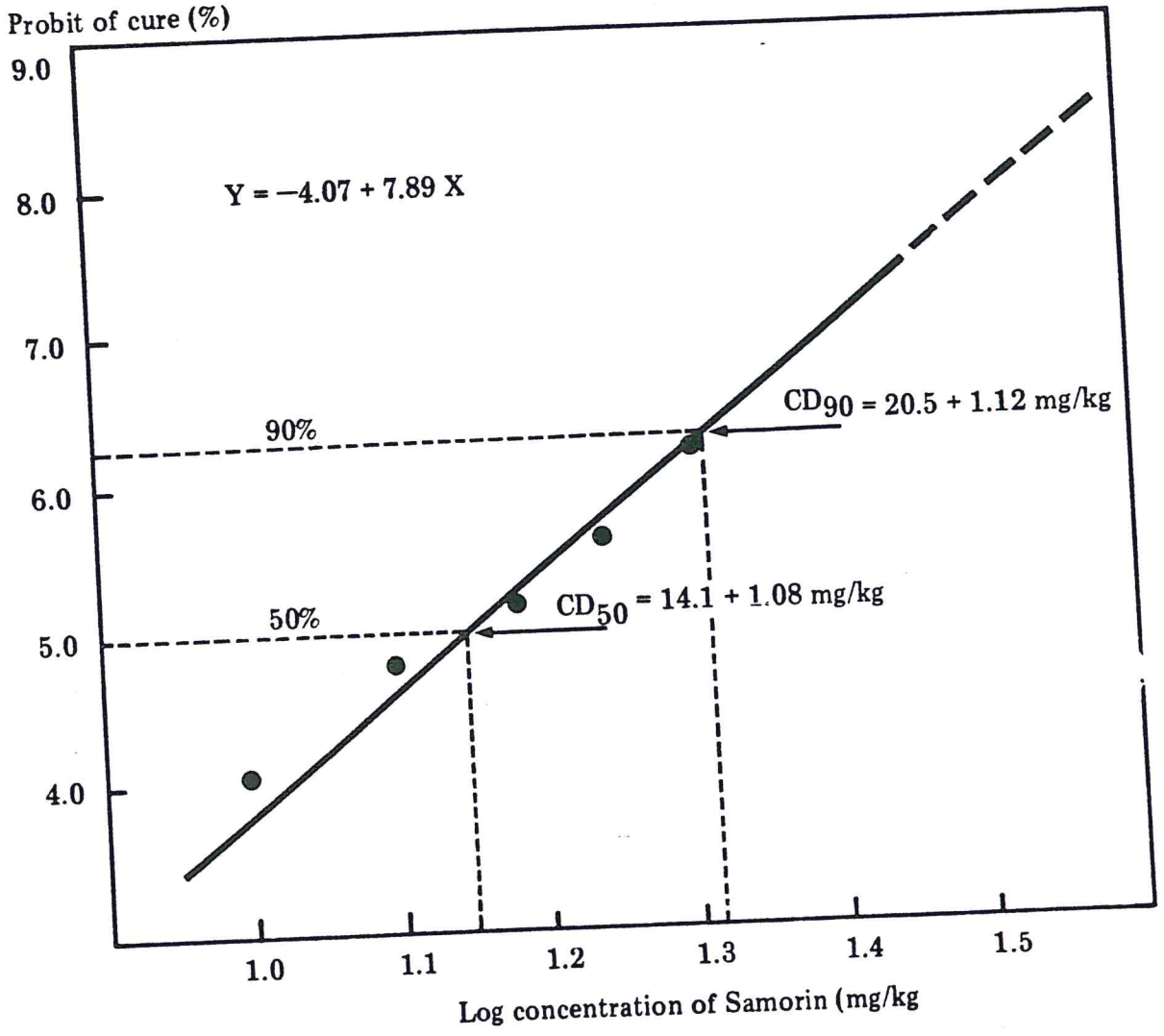


Fig. 9: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (Maruma strain) after 9 cyclic passages in the absence of drug.

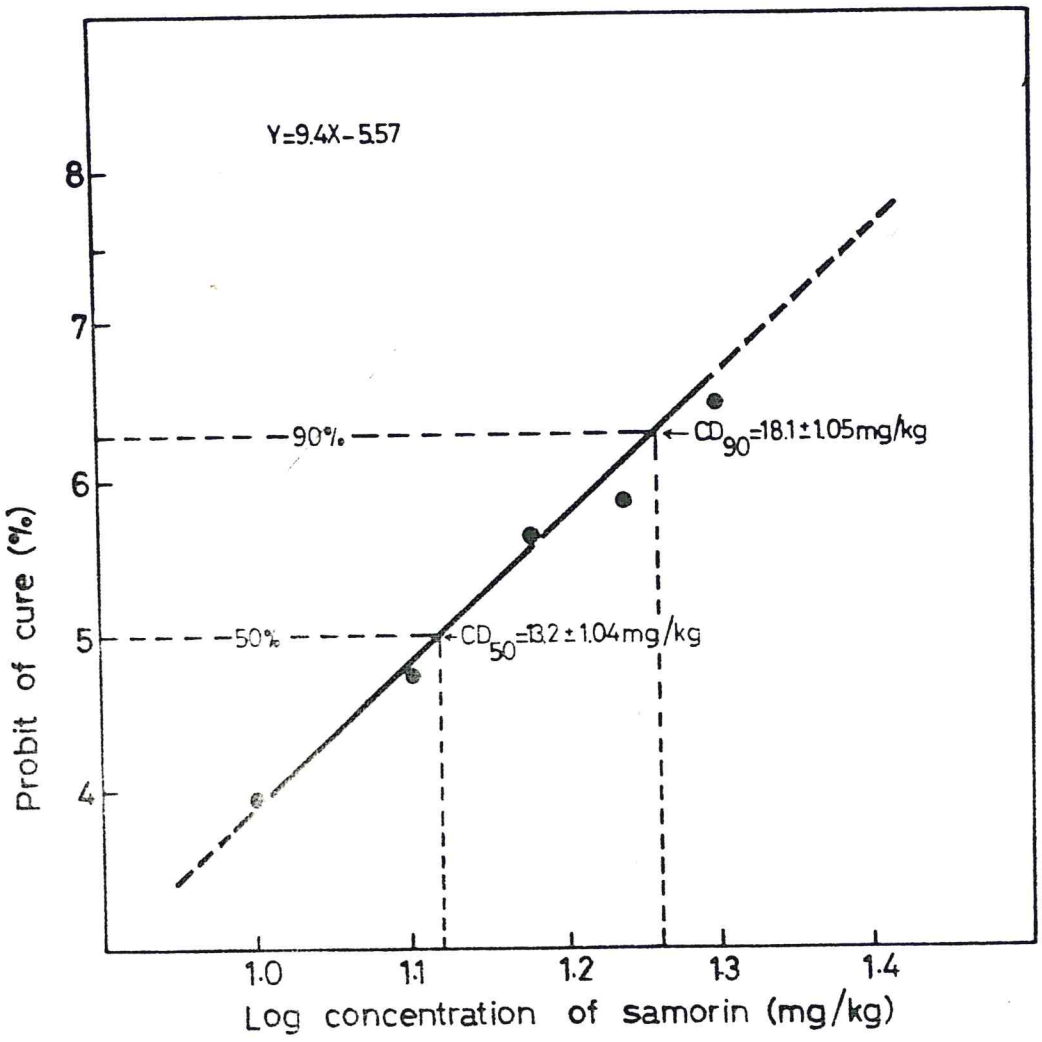


Fig. 10: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (Maruma strain) after 12 cyclic passages in the absence of drug.

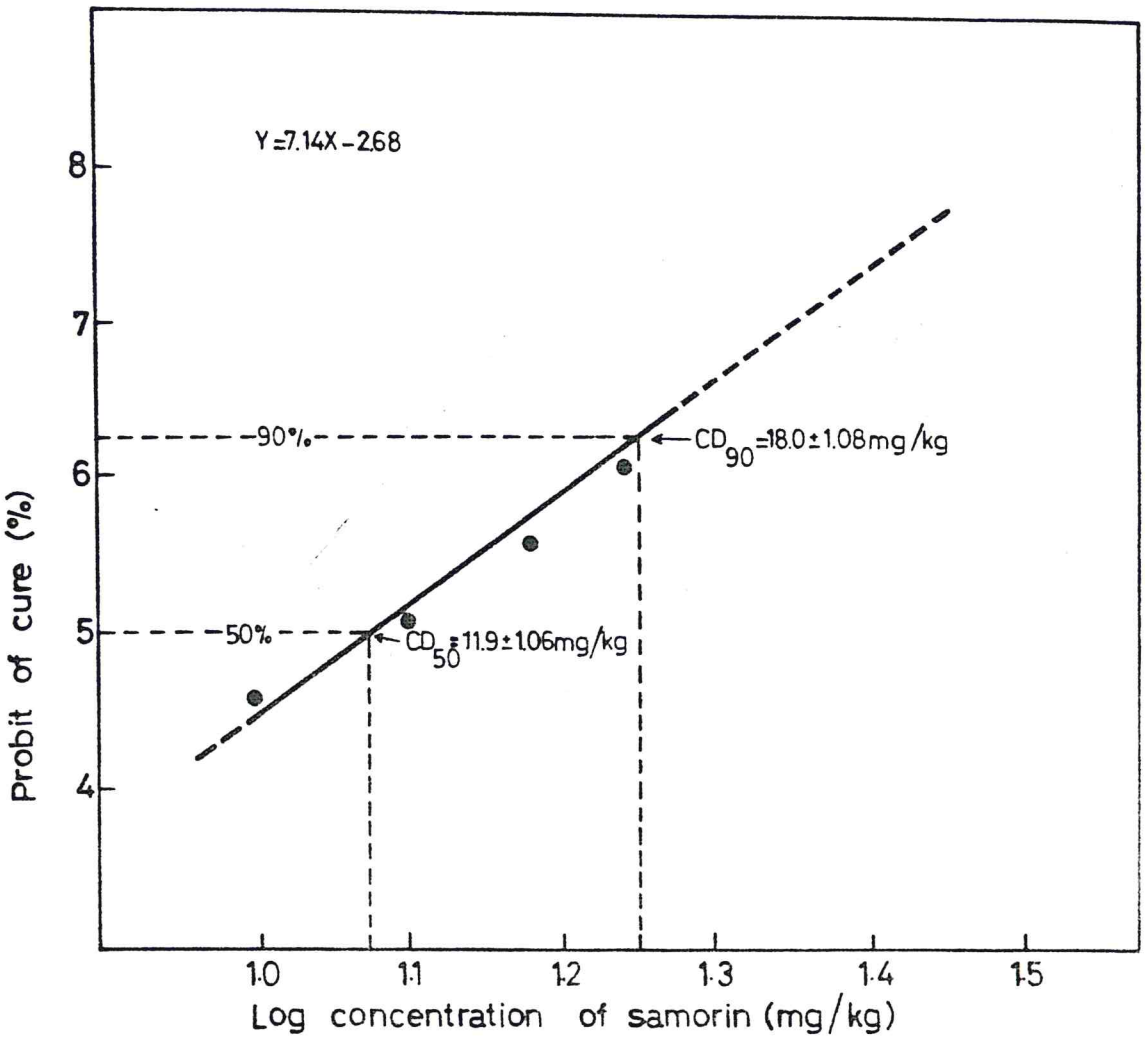


Table 6. CD_{50} and CD_{90} of T. congolense (Maruma strain) after cyclic passages in the absence of drug- showing the mean CD ± S.E., and the 95% Confidence limits

Cyclic CP		Mean CD ± SE mg/kg bw	95% Confidence limits		* Significant difference (sd)
			lower	upper	
Before passages	CD_{50}	13.9±1.02	12.86	14.94	
	CD_{90}	20.3±1.13	19.03	21.57	
CP no drug	CD_{50}	14.3±1.10	13.1	15.5	No sd
3rd CP	CD_{90}	21.4±1.17	20.04	22.76	No sd
6th CP	CD_{50}	14.1±1.08	12.94	15.26	No sd
	CD_{90}	20.5±1.12	19.25	21.75	No sd
9th CP	CD_{50}	13.2±1.04	12.12	14.28	No sd
	CD_{90}	18.1±1.05	17.0	19.2	No sd
12th	CD_{50}	11.9±1.06	10.78	13.02	No sd
	CD_{90}	18.0±1.08	16.84	19.16	No sd

* CD_{50} and CD_{90} after cyclic passages compared to those before passages.

No sd = $P > 0.05$

sd = $P < 0.05$

EXPERIMENT 4b

5.4.3. Stability of drug resistance through syringe passages:-

Three mice were each inoculated with 0.1 ml of inoculum having 3.1×10^6 parasites/ml. After every 5 - 7 days post infection, blind passage of the pooled blood from the tails of the mice were subinoculated into three other mice and blood from these mice was again blindly pooled and subinoculated into another group three mice. The procedure of inoculation and subinoculation at every 5 - 7 day intervals continued through 20 syringe passages as shown in Fig. 1. Drug sensitivity tests and stabilate preparations were done at the 5th, 10th, 15th and 20th syringe passages. The CD_{50} and CD_{90} values obtained following syringe passages, in the absence of the drug, were compared to the standard CD_{50} and CD_{90} and the data analysed using the 't' test at the 95 percent confidence limit.

5.4.4. Results

Fig. 6 shows that the drug/response of infections arising after 5 syringe passages was closely related to that of the standard. Significant increase in the percentage response was recorded when parasites were passaged over 10 times. Data in Fig. 6 show that after 20 syringe passages, 10 ml/kg bw cured 46.6% of the infected animals compared to 20% in the standard. The curative doses decreased steadily from 13.9 $\pm$ 1.02 to 10.6 $\pm$ 1.03 mg/kg for CD_{50} and from 20.3 $\pm$ 1.13 to 15.8 $\pm$ 1.07 mg/kg for CD_{90} as shown in Fig. 11, 12, 13 and 14. Table 7 shows statistical significant decrease ($P < 0.05$) in the CD_{50} and CD_{90} after 15 and 20 syringe passages.

Table 8 shows the changes in the degree of resistance following cyclic or syringe passages in the absence of drug. After 12 cyclic passages the resistant strain has lost some level of resistance from 92.6 to 70.6 times. The degree of resistance did not change significantly during cyclic passages, but syringe passages show a significant decrease in the level of resistance with the increasing number of passages.

The prepatent periods of the infections arising from the 12th cyclic and 20th syringe passages were assessed. The results Table 9 show that the prepatent periods were 5.5 $\pm$ 0.3 and 5.3 $\pm$ 0.3 days for cyclic and syringe passages respectively as compared to 5.9 $\pm$ 0.4 days for the standard. There was no significant difference in the prepatent period at 95% confidence limits.

Fig. 11: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (Maruma strain) after 5 syringe passages in the absence of drug.

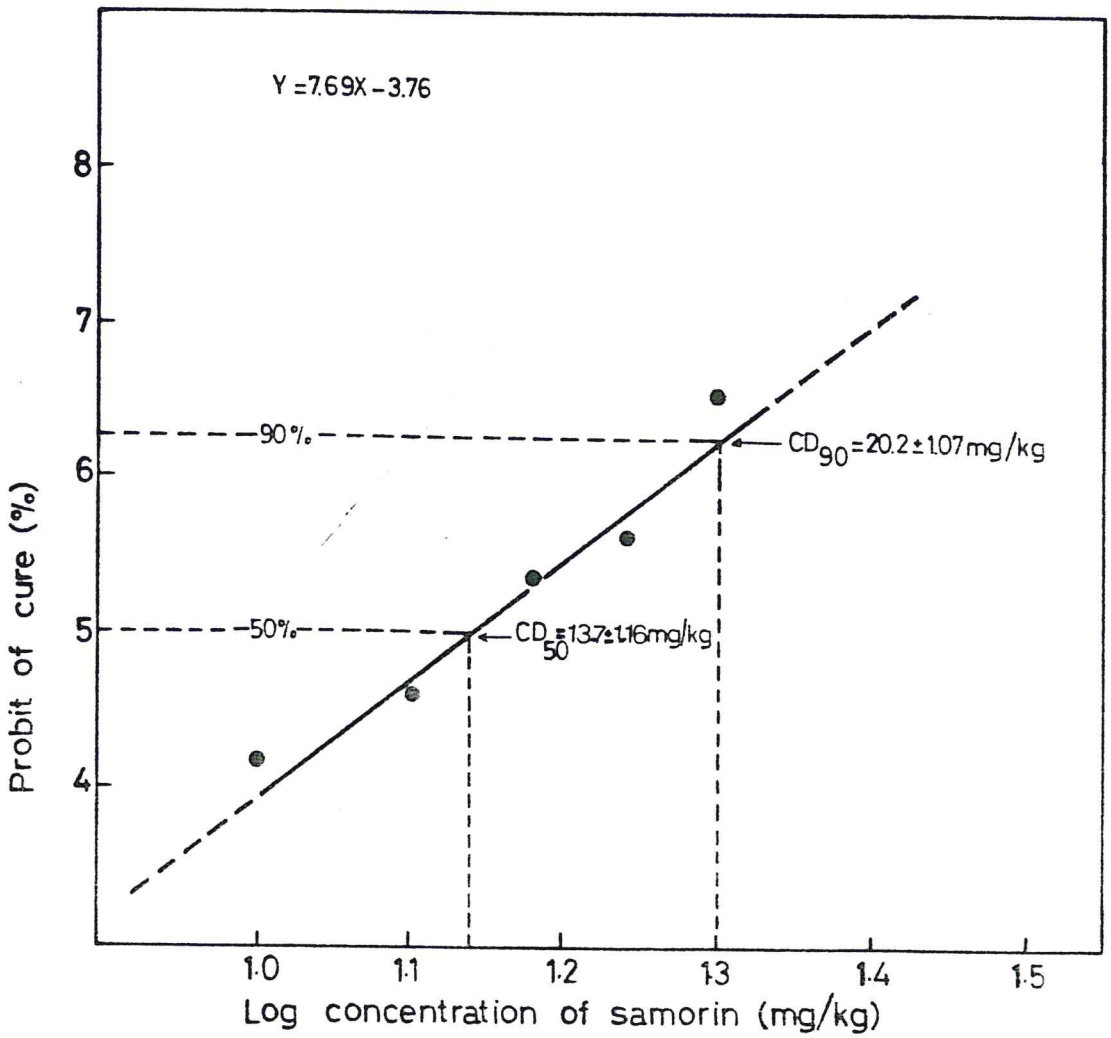


Fig. 12: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (Maruma strain) after 10 syringe passages in the absence of drug.

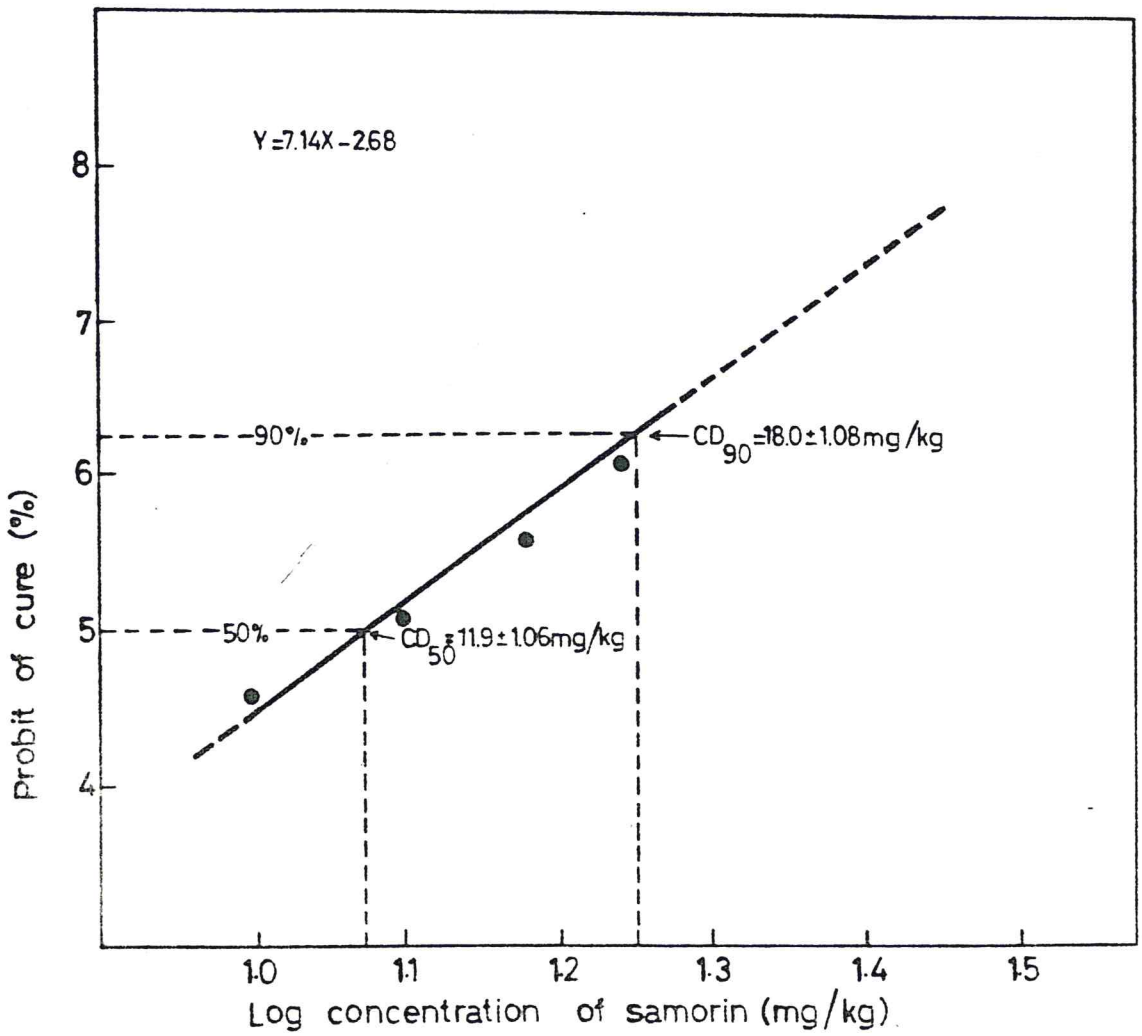


Fig. 13: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (Maruma strain) after 15 syringe passages in the absence of drug.

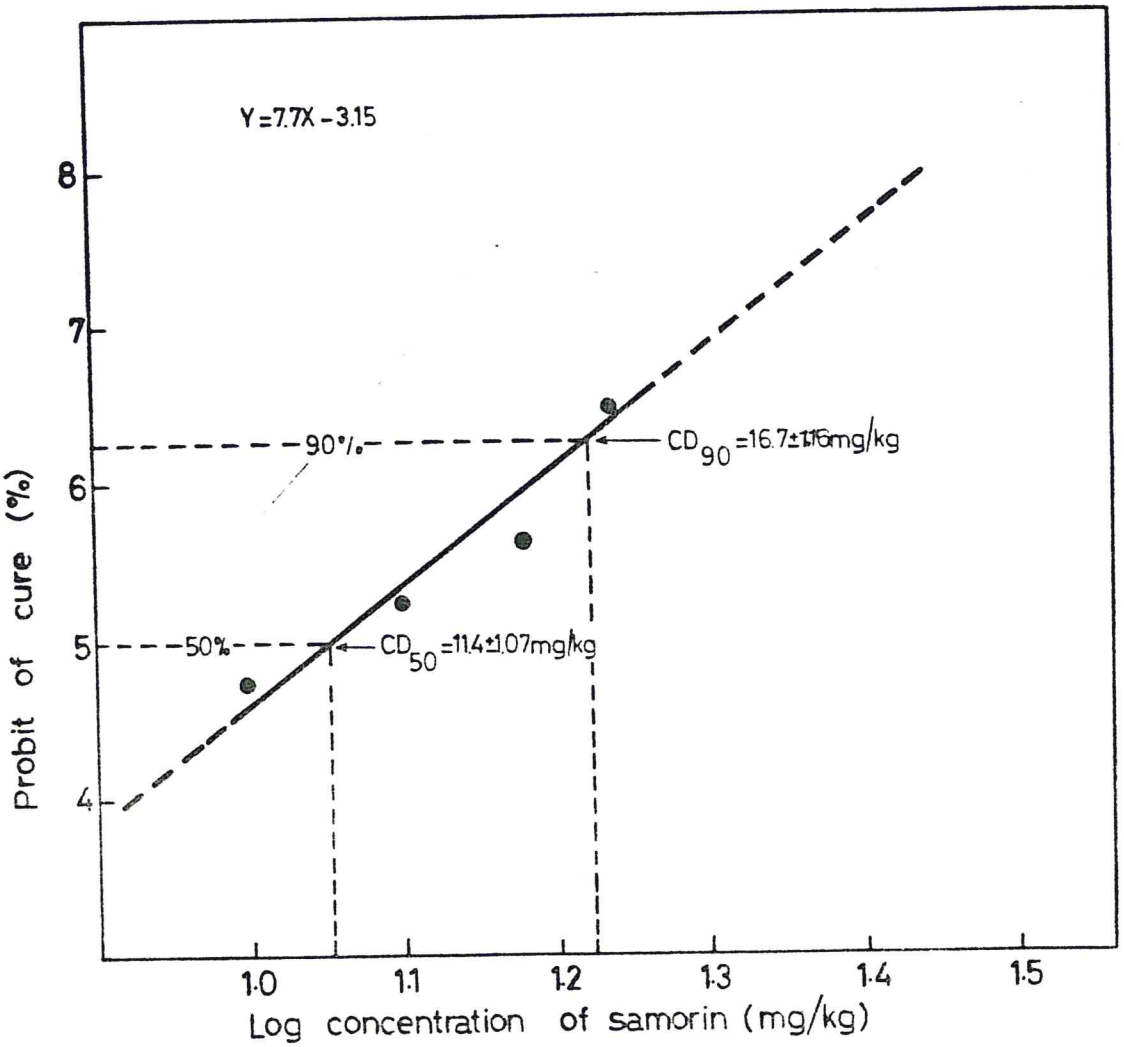


Fig. 14: The regression line showing the relationship between the log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (Maruma strain) after 20 syringe passages in the absence of drug.

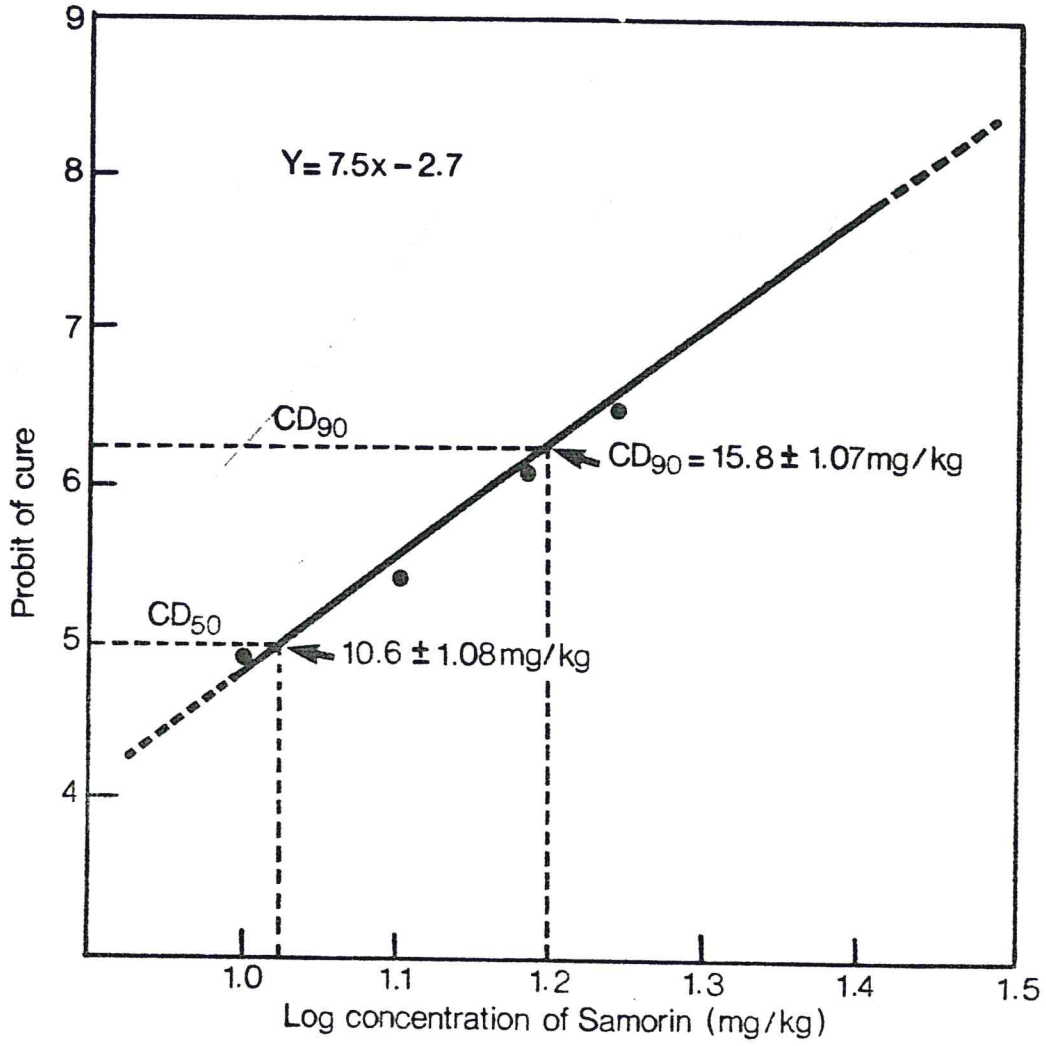


Table 7. CD_{50} and CD_{90} of T. congolense (Maruma strain) after syringe passages in the absence of drug- showing the mean CS ± SE, and the 95% Confidence limits.

Syringe passages (SP) no drug	Mean CD+SE mg/kg bw	95% Confidence limits lower	upper	* Significant difference (sd)
Before passages CD_{50}	13.9±1.02	12.86	14.94	
CD_{90}	20.3±1.13	19.03	21.57	
Syringe passages No drug 5SP CD_{50}	13.7±1.16	12.4	15.0	no sd
CD_{90}	20.2±1.07	19.06	21.36	no sd
10 SP CD_{50}	11.9±1.06	10.78	13.02	no sd
CD_{90}	18.0±1.08	16.84	19.16	no sd
15 SP CD_{50}	11.4±1.07	10.26	12.54	sd
CD_{90}	16.7±1.16	15.72	17.92	sd
20 SP CD_{50}	10.6±1.08	9.44	11.76	sd
CD_{90}	15.8±1.07	14.66	16.94	sd

* CD_{50} and CD_{90} after syringe passages compared to those before passages

No sd = $P > 0.05$

sd = $P < 0.05$

Table 8. The changing degrees of resistance (Index of resistance) following cyclic and syringe passages of drug resistant T. congolense (Maruma strain) in the absence of drug.

DRUG RESISTANT

Before and after passages in the absence of drug	Mean CD_{50} mg/kg	Index of resistance $CD_{50}/0.15$
Before Passages Standard	13.9	92.6
Cyclic Passages No drug 3rd CP	14.3	95.3
6th CP	14.1	94.0
9th CP	13.2	88.0
12th CP	11.9	79.3
Syringe Passages No drug 5th SP	13.7	91.3
10th SP	11.9	79.3
15th SP	11.4	76.0
20th SP	10.6	70.6

Table 9. Mean prepatent periods and the standard errors, the 95 percent confidence limits and the significant levels for T. congolense (Maruma strain) before and after cyclic or syringe passages in the absence of drug.

Before/After cyclic or syringe passages (CP/SP)	Mean prepatent period ± S.E. (in days)	95% confidence limits		Statistical significant difference (sd)
		lower	upper	
Before passages	5.9 ± 0.4	5.1	6.7	
12th CP	5.5 ± 0.3	4.85	6.15	No sd
20th SP	5.3 ± 0.3	4.64	5.96	No sd

No sd = $P > 0.05$
sd = $P < 0.05$

5.5. EXPERIMENT 5a.

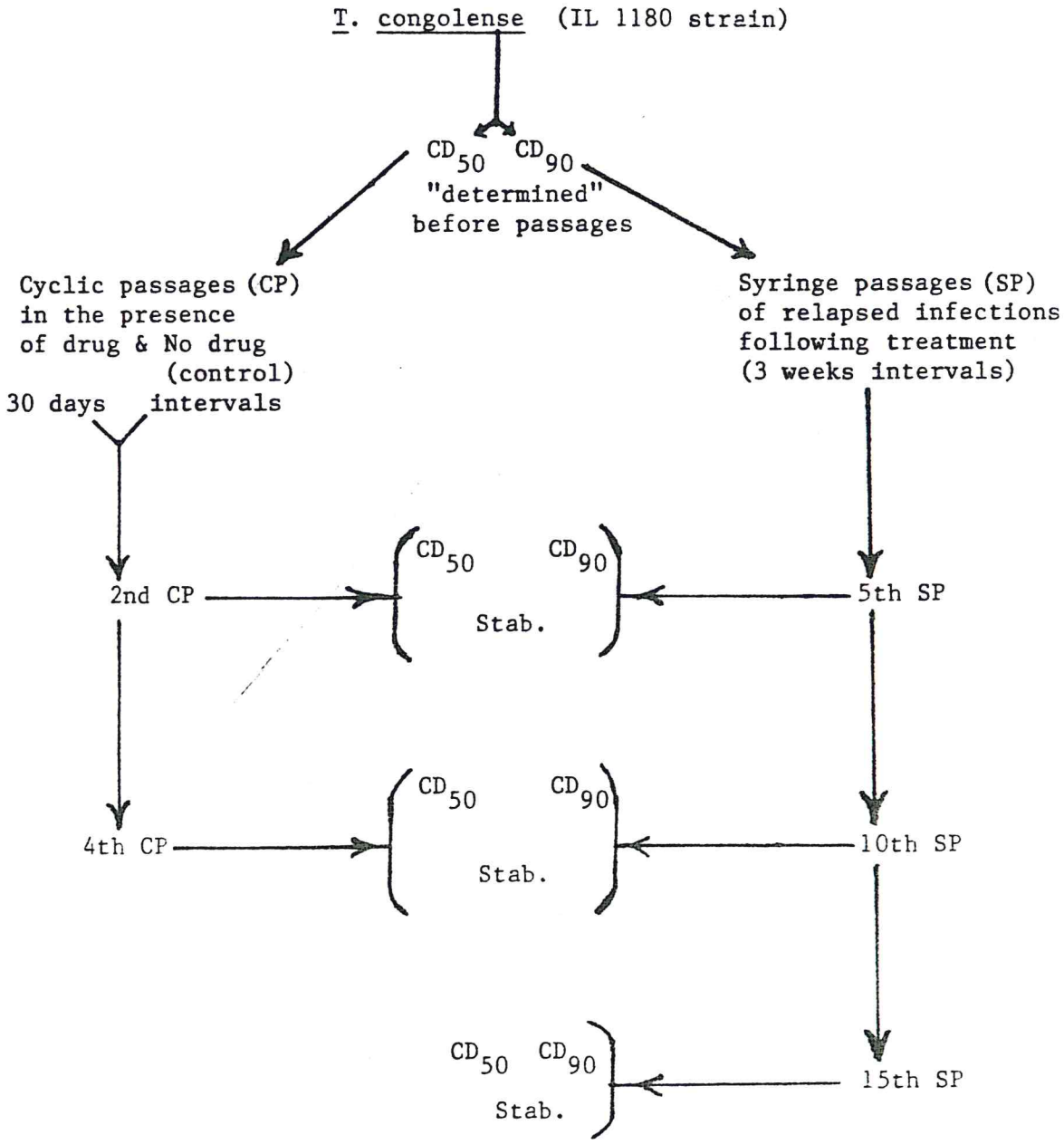
5.5.0. TRYPANOSOMA CONGOLENSE (IL 1180 strain)

5.5.1. Inducing drug resistance by cyclic passages:

Between 120 and 150 teneral flies were fed twice on infected mice at the first parasitaemic peak. The flies were maintained on clean untreated rabbits for 15 days after which they were divided into two groups. The first group of 80 - 100 flies was maintained for 30 days on clean rabbits that received a weekly prophylactic treatment of Samorin at 2 mg/kg bw. Flies were not fed on rabbits within 3 hours from the time of treatment. The other group of 40 - 50 flies was maintained for 30 days on rabbits that were injected with normal saline. Both groups of flies were allowed to transmit the infections to separate groups of clean mice. The mice that became infected by cyclic transmission were used to infect a second batch of two groups of teneral flies.

These second groups of infected flies underwent the same experimental procedures described above and illustrated diagramatically in Fig. 2. After every 2nd and 4th cyclic transmission (giving a total of 60 and 120 days of continuous exposure of the infected tsetse flies to the drug or saline treated rabbits) drug sensitivity tests were performed to determine any

Fig. 2: Experimental procedure for inducing drug resistance to Trypanosoma congolense (IL 1180 strain) during cyclic and syringe passages in the presence of drug.



Stab = Stabilates

changes in the CD_{50} and CD_{90}. The curative doses were compared with the standard CD_{50} and CD_{90} and the 95% confidence limits attached to show any significant differences (Finney, 1952). This method of inducing drug resistance in the vector forms of trypanosomes is similar to the constant exposure (diet-drug) method for malaria described by Peters, (1970, 1980).

5.5.2. Results

After 4 cyclic passages, the results (Fig. 15) show that 0.125 mg/kg bw cured only 6.6% of the mice that were infected by flies maintained on treated animals compared to 40% and 46.6% in the control and the standard respectively. Figs. 16 and 17 show that the curative doses had risen to over 0.43 $\pm$ 0.014 mg/kg for CD_{50} and 1.07 $\pm$ 0.02 mg/kg for CD_{90}. In the control the CD_{50} and CD_{90} were under 0.22 $\pm$ 0.02 and 0.8 $\pm$ 0.13 mg/kg bw as shown in Figs. 18 and 19. Applying the 95% confidence limits, data in Table 10 show significant increase in the CD_{50} and CD_{90} following continuous exposure of the infected flies to drug. No significant difference was observed in the control group.

Fig. 15: Histogram of the percentage response of mice to different doses of Samorin before and after several cyclic or syringe passages of T. congolense (IL 1180 strain) with maintenance on drug treated or untreated rabbits.

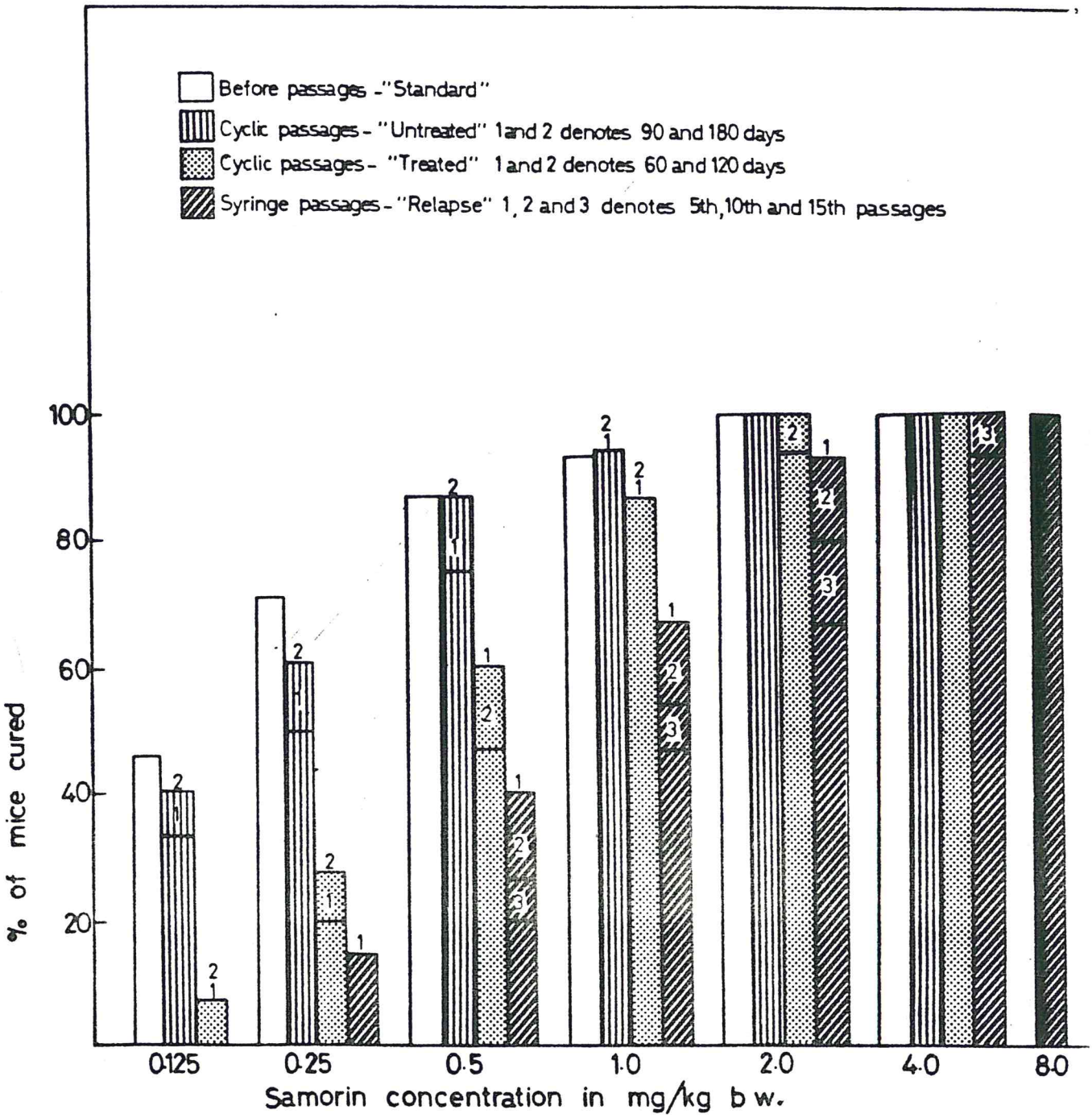


Fig. 16: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (IL 1180 strain) after 2 cyclic passages and maintenance for 60 days on drug treated rabbits.

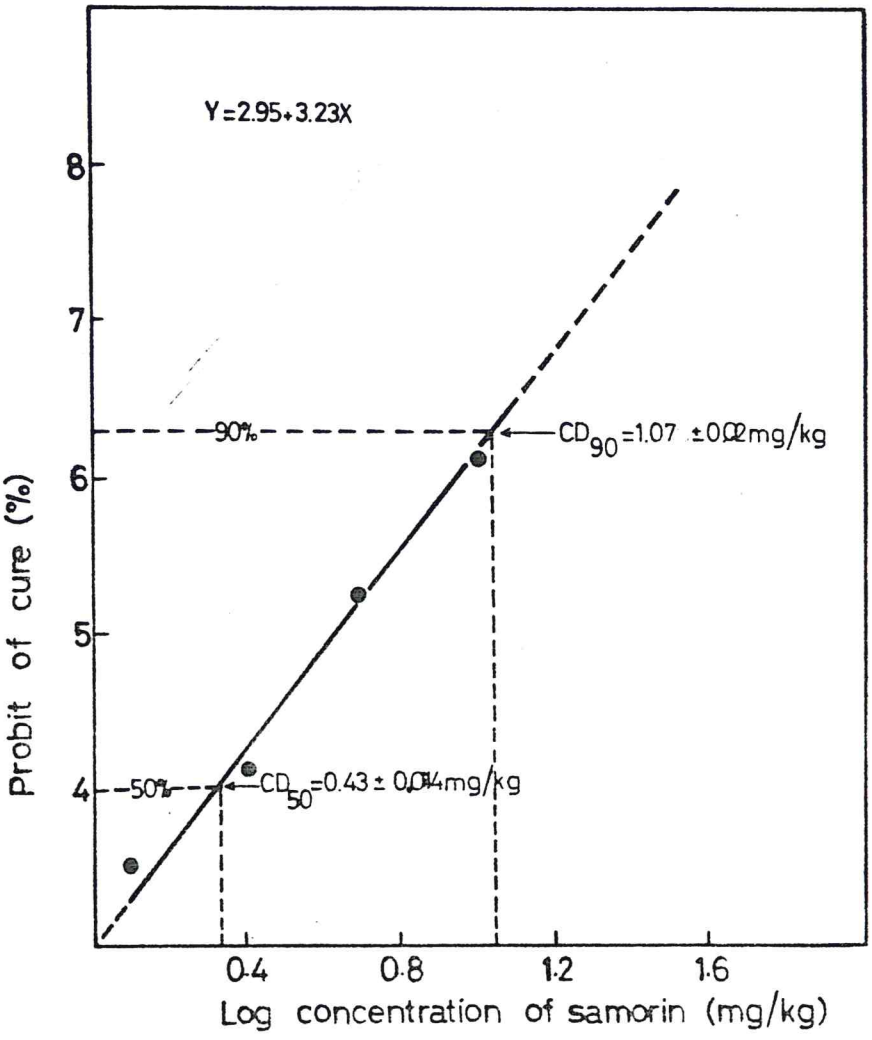


Fig. 17: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (IL 1180 strain) after 4 cyclic passages and maintenance for 120 days on drug treated rabbits.

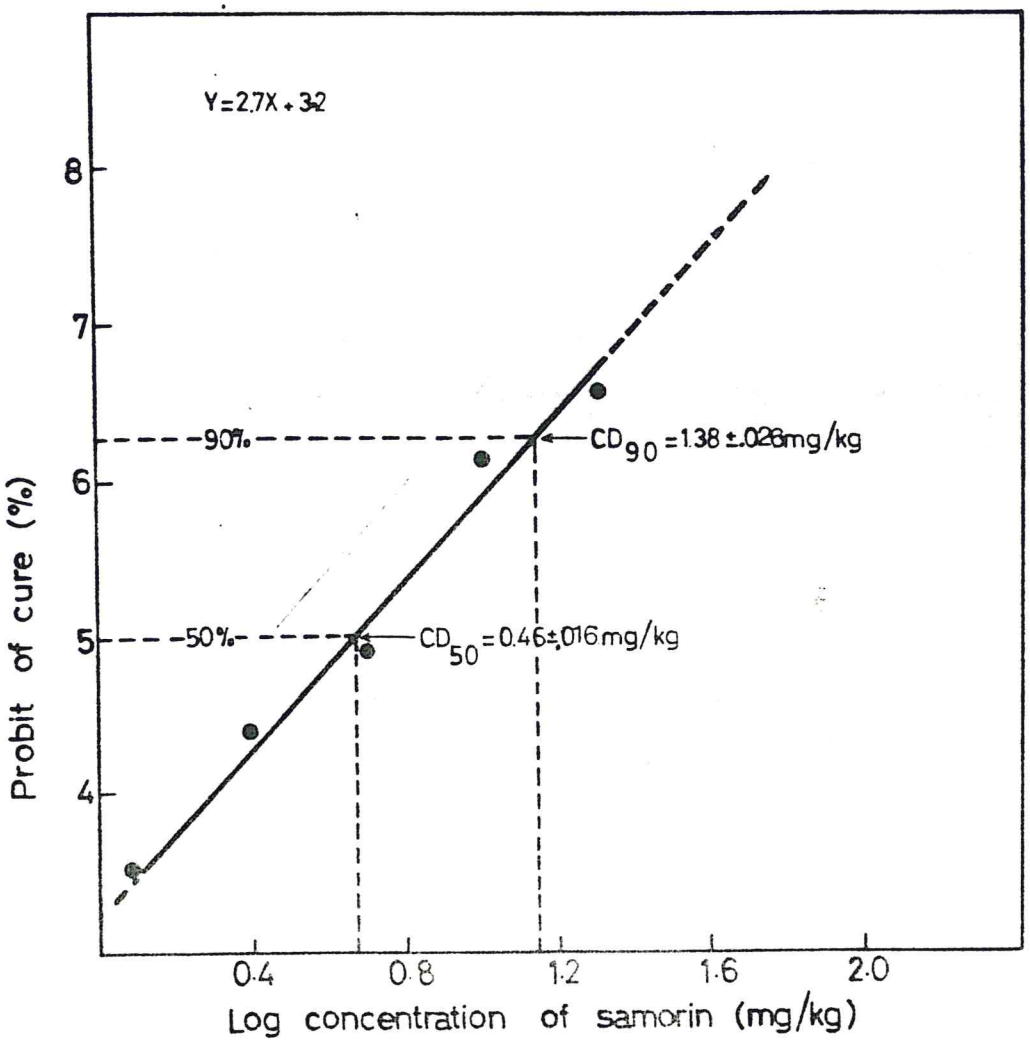


Fig. 18: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (IL 1180 strain) after 2 cyclic passages and maintenance for 60 days on untreated rabbits.

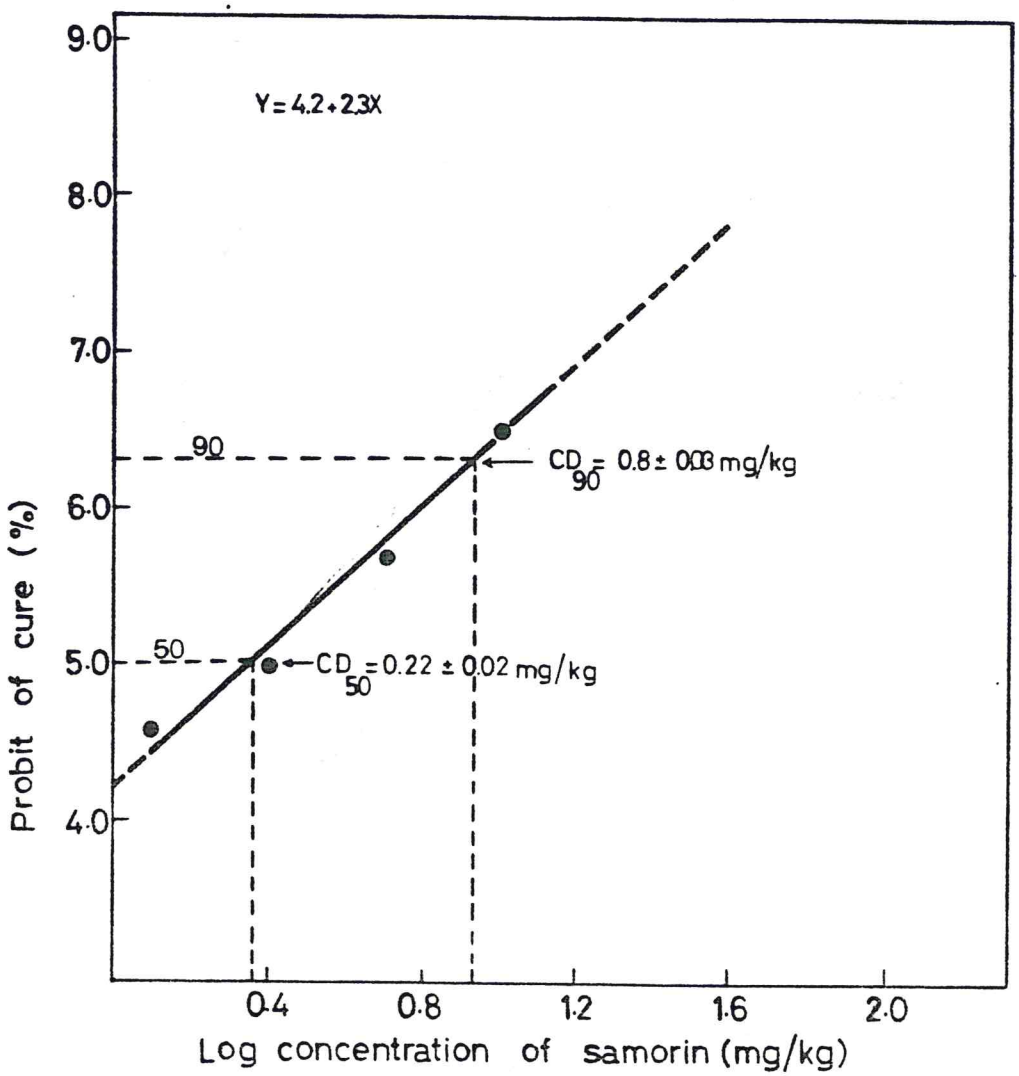


Fig. 19: The relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (IL 1180 strain) after 4 cyclic passages and maintenance for 120 days on untreated rabbits.

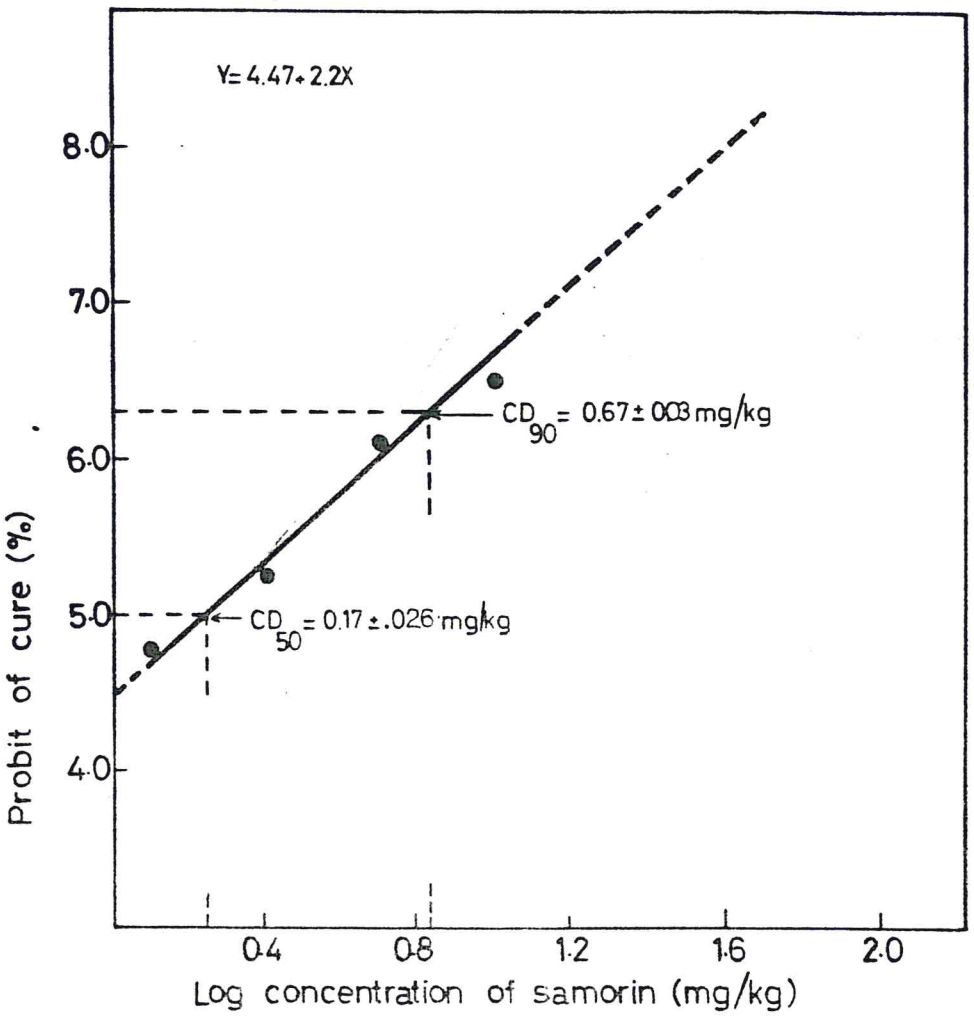


Table 10. CD_{50} and CD_{90} of T. congolense (IL 1180 strain) after cyclic passages in the presence of drug - showing the mean CD ± SE, and the 95% confidence limits.

Cyclic passages (CP)		Mean CD ± SE mg/kg bw	95% confidence limits lower	upper	Significant* difference (Sd)
Before passages	CD_{50}	0.15 ± 0.03	0.07	0.23	
	CD_{90}	0.64 ± 0.103	0.524	0.746	
CP+Samorin for 60 days	CD_{50}	0.43 ± 0.014	0.40	0.46	sd
	CD_{90}	1.07 ± 0.02	1.025	1.115	sd
CP+Samorin for 120 days	CD_{50}	0.46 ± 0.016	0.426	0.494	sd
	CD_{90}	1.38 ± 0.026	1.354	1.406	sd
CP-No Drug 60 days	CD_{50}	0.22 ± 0.022	0.198	0.242	No sd
	CD_{90}	0.80 ± 0.03	0.73	0.87	No sd
CP-No Drug 120 days	CD_{50}	0.17 ± 0.026	0.11	0.23	No sd
	CD_{90}	0.67 ± 0.03	0.60	0.74	No sd

* The significant difference is in CD_{50} and CD_{90} after cyclic passages compared to those before passages.

No sd = $P > 0.05$

sd = $P < 0.05$

EXPERIMENT 5b.

5.5.3. Inducing drug resistance by syringe passages:-

Infected mice, at the first parasitaemic peak, were divided into groups of five. The groups received Samorin at the following doses; 0.25, 0.5, 1.0, 2.0 and 4.0 mg/kg bw. The mice were examined for any relapse infections starting two weeks after treatment. Blood from mice that showed relapse infections (from the highest dosage group only) was passaged during the peak parasitaemia to another group of mice. These were again divided into groups of five and treated as described above during the first parasitaemic peak. The procedure of treatment-relapse-passage-treatment was repeated and drug sensitivity tests done to determine CD_{50} and CD_{90} were performed at the 5th, 10th and 15th syringe passages. Stabilates were prepared during these treatments (Fig.2).

5.5.4. Results

Fig. 15 shows that a dose of 0.125 mg/kg bw could not cure any infections arising after 5 syringe passages of the relapsed infections and 4 mg/kg bw also failed to cure all the infected animals. Data in Figs. 20, 21 and 22 show that the curative doses had risen to over 0.64 ± 0.14 mg/kg for CD_{50} and 1.76 ± 0.16 mg/kg for CD_{90}. The results in Table 11 show significant increases in the CD_{50} and CD_{90} following syringe passages of the relapsed infections ($P < 0.05$).

Table 12 shows that the sensitive strain had become 2.86 times more resistant after 60 days of continuous exposure to drug while the control was below 1.46 times, falling within the range of the standard sensitivity. Following syringe passages, the strain developed a level of drug resistance to over 7 times (Table 12). In both the cyclic and syringe passages, there was no significant difference in the prepatent periods at 95% confidence limits as shown in Table 13.

Fig. 20: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (IL 1180 strain) after 5 syringe passages of the relapsed infection.

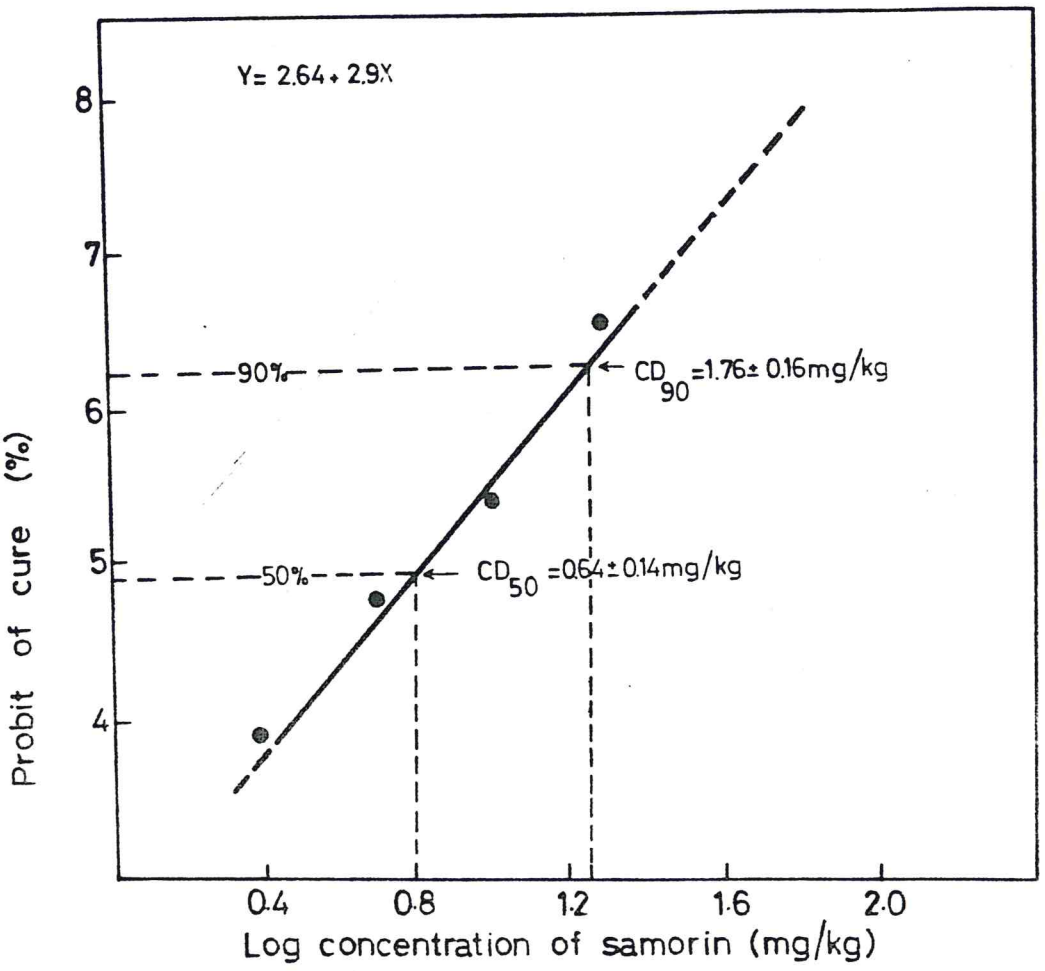


Fig. 21: The regression line showing the relationship between log concentration of Samorin and the probit of cure of mice following treatment against T. congolense (IL 1180 strain) after 10 syringe passages of the relapsed infections.

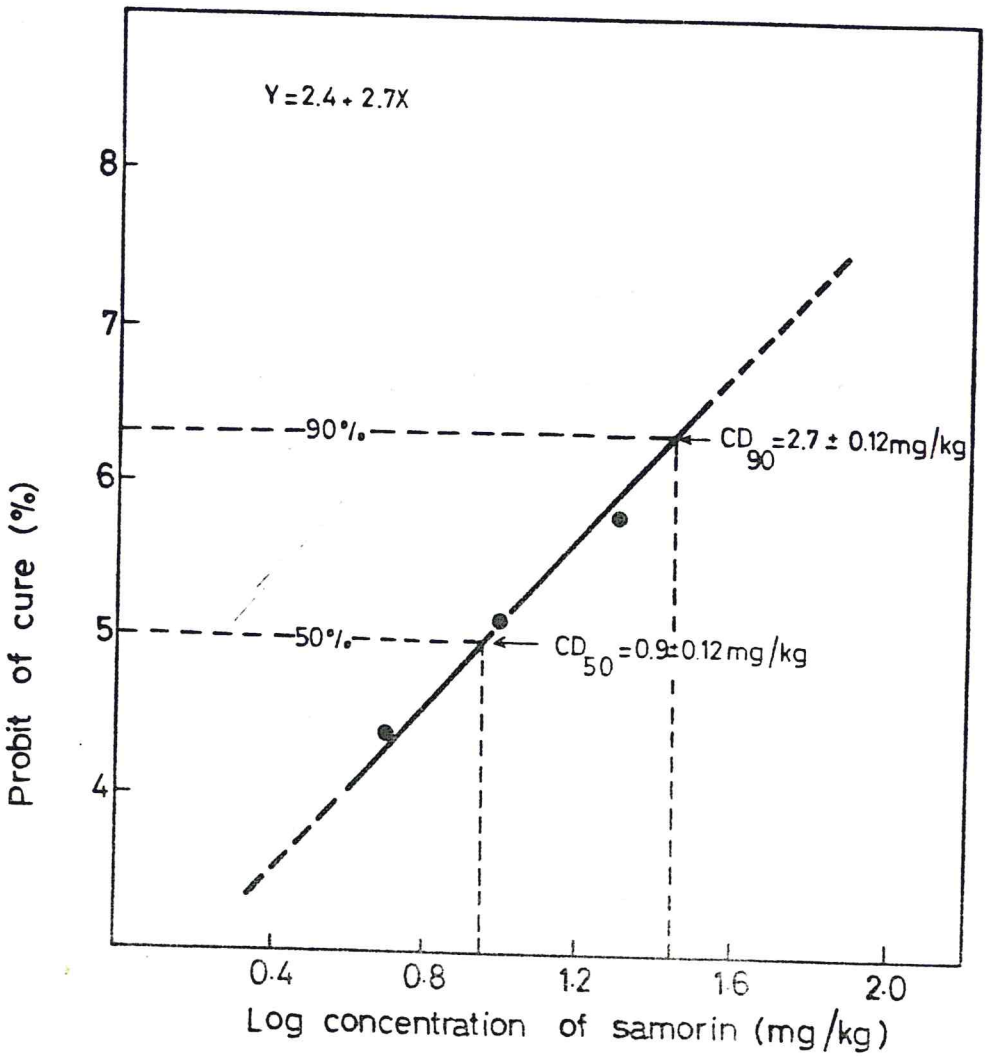


Fig. 22: The relationship between log concentrations of Samorin and the probit of cure of mice following treatment against T. congolense (IL 1180 strain) after 15 syringe passages of the relapsed infections.

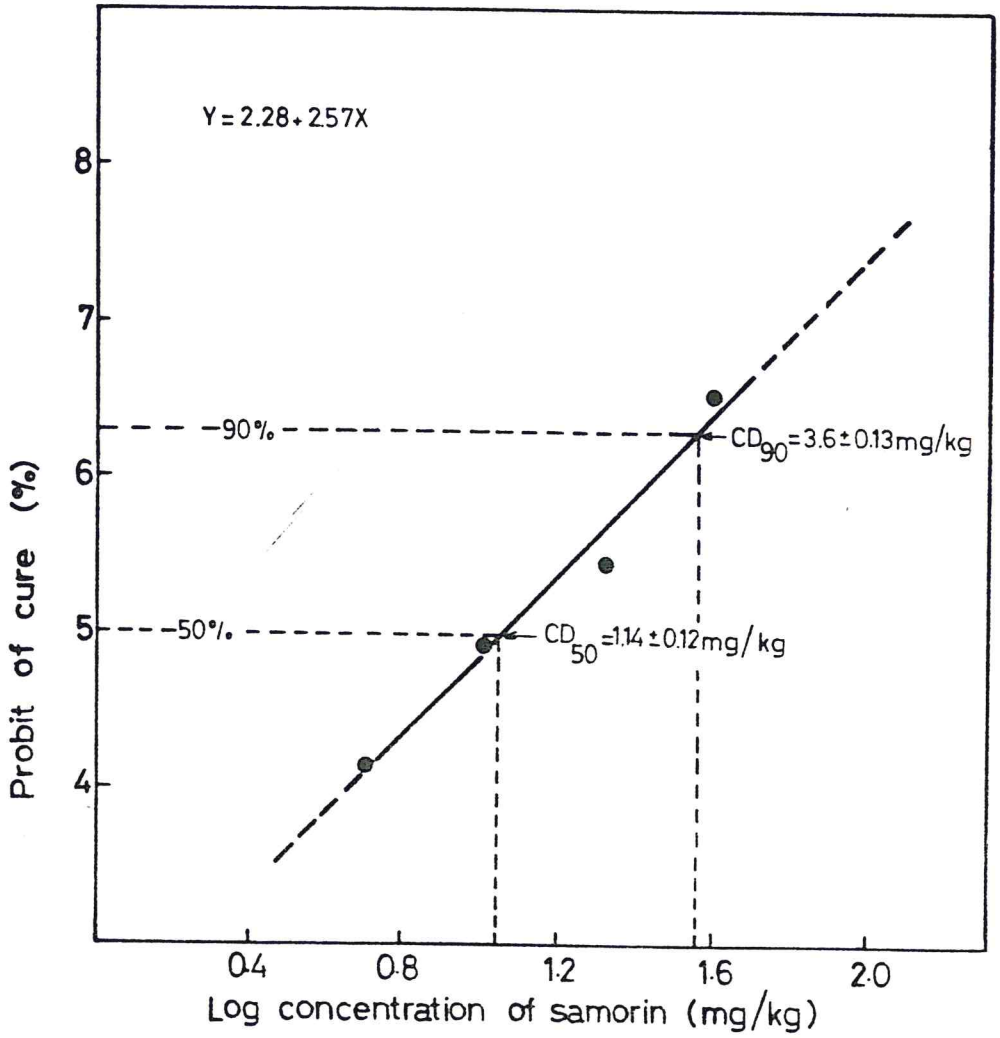


Table 11. CD_{50} and CD_{90} of T. congolense (IL 1180 strain) after syringe passages of relapse infections following treatment-showing the mean CD ± SE, and the 95% confidence limits.

Syringe SP and drug		Mean CD ± SE mg/kg bw	95% Confidence limits lower	upper	* Significant difference (sd)
Before passages	CD_{50}	0.15 ± 0.03	0.07	0.23	
	CD_{90}	0.64 ± 0.103	0.534	0.746	
Syringe passage and drug					
5th SP	CD_{50}	0.64 ± 0.14	0.45	0.83	sd
	CD_{90}	1.76 ± 0.16	1.50	2.0	sd
10th SP	CD_{50}	0.9 ± 0.12	0.76	1.04	sd
	CD_{90}	2.7 ± 0.12	2.55	2.85	sd
15th SP	CD_{50}	1.14 ± 0.12	1.00	1.28	sd
	CD_{90}	3.6 ± 0.13	3.43	3.77	sd

* CD_{50} and CD_{90} after syringe passages compared to those before passages.

sd = $P < 0.05$

Table 12. The changing degrees of resistance (Index of resistance) following cyclic and syringe passages of drug sensitive T. congolense (IL 1180 strain) in the presence or absence of drug.

DRUG SENSITIVE Before and after passages with or no drug	Mean CD_{50} mg/kg	Index of Resistance $CD_{50}/0.15$
Before Passages		
Standard	0.15	1.0
Cyclic Passages		
CP with drug		
60 days	0.43	2.86
120 days	0.46	3.06
Cyclic Passages		
CP no drug		
60 days	0.22	1.46
120 days	0.17	1.13
Syringe Passages (SP)		
Relapsed infections		
5th SP	0.67	4.26
10th SP	0.9	6.0
15th SP	1.14	7.6

Table 13. Mean prepatent periods and their standard errors, the 95 percent confidence limits and the significance levels of T. congolense (IL 1180 strain) before and after cyclic or syringe passages in the presence of drug.

Sensitive strain Before/After cyclic/syringe passages	Mean prepatent period ± S.E.	95% confidence limits lower	upper	Statistical significant difference (sd)
Before passages	4.8 ± 0.47	3.9	5.7	
4th CP + drug	4.9 ± 0.5	4.0	5.8	No sd
15th SP + drug	5.04± 0.47	4.1	5.9	No sd

No sd = ($P > 0.05$)
sd = ($P < 0.05$)

5.6. EXPERIMENT 6

5.6.1. Treating infected flies with samorin

Tsetse flies that were found to be infected were divided into three groups. One group of 35 flies was allowed to feed on clean mice treated with Samorin at 2 mg/kg bw (within 1 - 2 hours of treatment). The second group of 24 flies was fed by membrane feeding method on defibrinated blood to which Samorin at the rate of 2 mg/ml of blood had been added. The third group of 11 flies (control) was fed on clean mice injected with saline. Between 3 - 14 flies from each group were dissected 24 and 48 hours after feeding on drug or saline treated bloodmeals. The parasites in the gut or proboscis wet films were examined for motility. Thin smears from the gut and proboscis were stained with Giemsa's stain and the parasites examined for any aberrant or abnormal morphology.

The remaining flies from each group were allowed two to three more drug treated bloodmeals as mentioned above for each group. On day 7 after their first treated bloodmeals, the ability of the flies to transmit the infections was assessed by allowing them to feed on clean mice. These mice were later examined for trypanosome infection for a period of 30 days. The flies were then dissected and the proboscis and gut were examined for infections. Thin smears were prepared, stained with Giemsa's stain and the parasites examined for any morphological change.

5.6.2. Results

A total of 173 infected flies were fed either on clean animals treated with samorin or artificially on bloodmeal containing samorin. Table 14 shows that all the flies fed on drug-treated animals transmitted the infections. Three flies fed artificially on blood containing drug failed to transmit the infections and the others transmitted mild infections with a longer prepatent period (11.7 $\pm$ 0.84 days), lower parasitaemia and longer survival time of over 30 days.

Table 14 also shows that two of the three flies that failed to transmit the infections had no parasites in the proboscis, but all the three had scanty trypanosomes in the gut. Between 8 - 10 infected flies were dissected within 24 to 48 hours after ingesting drug-treated bloodmeal. A large number of gut and proboscis forms of trypanosomes were seen floating, immobile and appeared moribund. The population of dead trypanosomes was more pronounced from flies that fed artificially on blood containing drug.

Some of the gut and proboscis smears were stained with Giemsa's stain and examined for any aberrant morphology. Plates 7 and 8 show that the effects of the drug on the vector forms of trypanosomes were characterized by swelling, vacuolisation, granulation and fragmentation of the kinetoplast and the nuclei as compared to the control in Plate 6. Complete loss of the

Table 14. Number of infected flies fed on mice treated with samorin, those fed artificially by membrane feeding on blood containing samorin, the controls fed on saline treated mice and the infections observed in flies dissected 24 and 48 hours after treatment and the number of flies that transmitted the infections 7 days after the drug treated bloodmeal.

SAMORIN OR SALINE TREATMENT	Number of infected flies treated	Number of flies found infected by cyclic transmission/dissection following treatment and in the control							
		Dissected 24 hours		Dissected 48 hours		Transmission/dissection day 7			
		Gut	Proboscis	Gut	Proboscis	Cyclic transmission	Gut	Proboscis	% transmission
Samorin treated Mice 2mg/kg bw feeding within 2 hrs	35	10/10	10/10	*4d 14/14	14/14	11/11	11/11	11/11	100
Samorin treated blood membrane feeding 2mg/ml blood	24	6/6	6/6	*2d 8/8	8/8	7/10	10/10	8/10	70
Saline treated mice feeding within 2 hrs	11	*1d 4/4	4/4	3/3	3/3	4/4	4/4	4/4	100

Numerator denotes number of flies infected or transmitted
Denominator denotes number of flies dissected or allowed to transmit
*d - number of flies dead within 48 hours of feedin on treated bloodmeal.

kinetoplast was also common. In some cases the flagella remnants were seen persisting (Plate 9) as the skeleton of the lysed parasites. Plates 10 and 11 show similar aberrant morphology on the metacyclic trypanosomes from the proboscis of the treated flies as described above. These abnormalities went up to 94.5% in the treated flies and the control untreated flies had 7.8% defective parasites.

5.7 EXPERIMENT 7

5.7.1. Prophylaxis of samorin in tsetse flies

Teneral tsetse flies were divided into two groups. One group of 210 flies were fed twice on clean mice (within 1-2 hours) of injection with Samorin at 2 mg/kg bw. The other group of 91 flies fed on clean mice injected with normal saline. Both groups were then fed two times on trypanosome infected mice. Each group was thereafter fed (daily except Sundays) on separate clean rabbits and on day 30 after infection, they were dissected and the parasites examined from the midgut and proboscis wet films.

In another experiment, teneral flies were fed twice on infected mice during peak parasitaemia and then divided into two groups. One group of 125 flies was maintained for 30 days on clean rabbits that received a weekly prophylactic treatment of Samorin at 2 mg/kg bw. The other group of 71 flies (control) was maintained on clean rabbits that were injected every week with normal saline. No flies were fed on the rabbits within three hours of treatment. On

day 30 after infection, the flies were dissected and examined for trypanosome infection in the gut and proboscis. Chi-squared test was used to analyse any statistical differences in the infection rates.

5.7.2. Results

Over 210 teneral flies were first fed on clean mice previously treated with samorin and then fed on infected mice. Data in Table 15 show that 11 (5.2%) flies developed mature infections and 4 (4.4%) from the control were infected. There was no significant difference in the infection rates between the treated and untreated flies ($P \geq 0.05$).

In another experiment, over 125 teneral flies were fed on infected mice and then maintained on rabbits that were treated with samorin. Table 16 shows that out of 125 flies treated, 33 (26.4%) became infected and 17 out of 69 (24.6%) flies from the control were found infected. Again there was no significant difference in the infection rate ($P \geq 0.05$).

Table 15. Infection rates in flies that had two bloodmeals on mice injected with samorin then another two bloodmeals on infected mice and followed by maintenance on clean untreated rabbits for 30 days then examined by salivary probe and dissection methods for infection (T. congolense IL 1180).

SAMORIN treated/ untreated bloodmeal	Number infected over probed/dissected and % infection (proboscis)				
		No. found infected by:			
	No. of flies infected	Salivary probe	Gut	Proboscis	% Infection proboscis
SAMORIN treated mg/kg	226	7/210	17/210	11/210	5.2
SALINE injected (control)	97	4/91	6/91	4/91	4.4

Table 16. Infection rates in flies that had two bloodmeals on infected mice and then maintained for 30 days on rabbits that were injected weekly with samorin at 2 mg/kg bw after which they were examined for infections by salivary probe and dissection methods (T. congolense IL 1180 strain).

Infected then maintained on SAMORIN treated/ untreated rabbits	Number infected over probed/dissected and % infection (proboscis)				
	Original No. of flies infected	Salivary probe	Dissection Gut	Proboscis	% Infection Proboscis
SAMORIN treated 2 mg/kg	133	21/125	58/125	33/125	26.4
SALINE control untreated	77	11/69	21/69	17/69	24.6

CHAPTER 6 DISCUSSION AND CONCLUSION

6.1. T. congolense (Maruma strain)

It is of interest to know the precise level of drug resistance a particular strain of trypanosomes has developed in order to plan a control or drug treatment strategy. By determining the curative doses of T. congolense (Maruma and IL 1180 strains), it was shown that the Maruma strain had developed resistance to such high level that drug treatment with samorin was almost impracticable. The recommended doses of Samorin are between 0.5 - 1 mg/kg bw and this is raised to 2 mg/kg bw when drug resistance is encountered (Leach and Roberts, 1981). Doses above 2 mg/kg is reported to be toxic (Fairclough, 1963).

Quantification of the degree of resistance before the start of passages allowed several lines of investigations to determine if and how the levels of drug resistance change during cyclic or syringe passages in the absence or presence of drug.

It was observed that the parasites persisted in the blood for 2 - 3 days after treatment before disappearing temporarily or completely. Similar observation had been reported by Pinder and Authie, (1984) in T. congolense from West Africa. The trypanosomes that persisted briefly after treatment appeared normal with respect to morphology and motility in the wet blood film. Careful examination of the Giemsa stained preparations revealed varying degrees of morphological defects such as swelling, vacuolisation of

the parasites and granulation and fragmentation of the kinetoplast and the nuclei. These observations confirm previous reports by Raether and Fink, (1979); Peters, (1980); Minelli et al., (1981) and Pinder and Authie, (1984).

It is not known why some parasites persisted in the blood for so long after treatment. Hecker and Brun, (1982) studied the morphology of trypanosomes from the lymph nodes and reported that they were very similar to the culture forms. According to Hawkings, (1963a) culture forms of trypanosomes respond very poorly to trypanocides. The persisting parasites could be due to the lymph node forms of trypanosomes entering the bloodstream after treatment.

The high percentage of dyskinetoplastic trypanosomes following treatment reported here agrees with previous observations where 40-100% dyskinetoplasty were induced using various trypanocides and polycyclic dyes (Newton, 1974; Vickerman and Preston 1976; and Riou et al., 1980;). The low percentage of dyskinetoplasty from the untreated trypanosomes is comparable to those reported by Hoare, (1954) on T. congolense and T. vivax. In some Trypanozoon groups dyskinetoplastic forms survive and multiply normally in the bloodstream (Hoare, 1954; Opperdoes et al., 1976 and Hajduk and Cosgrove, 1979).

The infectivity of the persisting parasites to the fly was very low. The only infection obtained was from the group of flies that fed on the animals within three hours of treatment. Probably in this short time, not all the parasites had absorbed enough drug,

therefore, some of the ingested trypanosomes may still have had enough functional kDNA necessary to initiate transformation for life inside the vector (Langley, 1975). The high percentage of the aberrant and dyskinetoplastic trypanosomes 24 hours after treatment probably explains the reduction in the fly infectivity. And indeed it has been reported by Hoare, (1954, 1972); Opperdoes et al., (1976) and Englund, (1981) that loss of kinetoplast leads to failure of the trypanosomes developing in the insect vector as found naturally in T. evansi and T. equiperdum.

It was interesting to note that a few of the persisting parasites had a normal looking kinetoplast and these would be expected to initiate infections in the fly, as the number of trypanosomes ingested by the fly is not a major factor on the infectivity (Otieno et al., 1983). Borst and Hoeijmakers (quoted by Englund, 1981) failed to infect tsetse flies with T. brucei having normal kDNA network but deficient in oligomycin-sensitive ATPase. They speculated that although this strain had kDNA, it might have had a mutation in a key maxicircle gene. Drug effects on the maxicircle would render the trypanosomes uninfective to the flies although the minicircle network of the kDNA may not be destroyed (Simpson, 1968). This point probably explains why the trypanosomes with normal kinetoplast after treatment may still fail to infect tsetse flies.

In contrast to the above observation, results of this study show that some of the parasites from treated animals remained infective throughout the period while in the mammalian blood. Studying the effects of acriflavine on T. equiperdum, Hajduk, (1979)

reported that the drug caused clumping of the kDNA. As the drug concentration decreases in the blood, the kDNA replication resumes and viable dyskinetoplastic cells appear. Working with Leishmania tarentolae, Simpson, (1968) observed that acriflavine at a low dose did not stop nuclear synthesis and that both cell and kinetoplast division proceeded normally for several generations. The ability of the Samorin treated trypanosomes to maintain their infectivity to the new host reported in the study confirms the recovery nature of the parasites observed by previous workers (Hajduk, 1979).

The trypanosomes from the gut of flies fed on infected and recently treated animals had lost their infectivity by 24 hours after ingestion. While the parasites from the flies fed on infected untreated animals were still infective 24 hours after ingestion. Ghiotto, et al., 1979 and Otieno et al., (personal communication) recently noted that T. brucei ingested by tsetse flies remained infective for 72 hours. Indeed T. congolense is reported to be monomorphic and the process of transformation to the insect forms in a population would be expected to be completed within the same time and according to the present observation the process was accomplished within 48 hours of ingestion.

Whereas in T. brucei, transformation is believed to take place on the short stumpy forms (Langley, 1975; Gutteridge & Coombs, 1977). The long slender forms which do not transform readily would be expected to maintain their infectivity to the mammalian host until they are destroyed by the unfavourable condition in the gut. In this study the fact that all the ingested parasites have lost infectivity to mammalian hosts within 24 hours of ingestion by the

flies agrees with the previous suggestion that the persisting trypanosomes in the blood of recently treated animals may be due to the lymphatic forms or those from a cryptic source (Fiennes, 1950 and Hecker and Brun, 1982).

It was noted that recently treated animals remained infective to tsetse flies for a short time; nevertheless, there was a great reduction in their infectivity to the flies. This lowered potential could in certain cattle/tsetse circumstances contribute to a reduction in the infection rates in the fly population. Thus regular treatment with samorin has several effects; curing the infection in cattle, prevention of infection in cattle and lowering the probability of infection in the flies.

The impact of this last effect could warrant the integration of this knowledge in tsetse control programmes employing insecticides and sterile insect release technique. It would be recommended that mass prophylactic treatment of livestock be carried out at the same time or one to two days before insecticides are sprayed or the sterile insects are released. This approach would ensure that the sterile flies released in the field or any newly emerged flies will have very low chances of getting infected.

The drug resistant strain was maintained through 12 cyclic transmissions in the tsetse flies and no significant progressive loss of resistance was observed. Gray and Roberts, (1971) also failed to record any loss of drug resistance to various trypanocides during 8 cyclic transmissions of T. congolense and T. vivax through

tsetse flies. Hoeve and Grainge, (1966) obtained drug sensitive T. rhodesiense after maintaining the original resistant strain through 2 cyclic passages in the flies. Based on field observations, Whiteside, (1960) and Finelle and Yvore, 1962 (quoted by Gray and Roberts, 1971) reported that resistance in T. congolense and T. vivax undergoing cyclical transmissions tend to disappear after 6 - 9 months provided the use of drug was suspended.

Indeed the previous observations are conflicting and debatable possibly because the workers did not determine the actual degree of resistance of the trypanosome isolates before cyclic passages in order to compare with those obtained following cyclic transmission. Several workers (quoted by Peters, 1970), reported that with the exception of Quinine and Mepacrine, drug resistance in malaria parasites has invariably proved to be a stable character after cyclic passages through mosquito vectors. The present observation showed that drug resistance in the Maruma strain of T. congolense is a stable character following 12 cyclic transmissions through tsetse flies.

In experiment 4b, a significant progressive loss of drug resistance was observed when the same strain was maintained by syringe passages. This finding agrees with those reported by Hawking, (1963b); Willett, (1966) and Riou, (1976) on various trypanosome species losing their drug resistant characters during syringe passages in the absence of the drugs concerned. Hawking, (1963b) stressed that the stability of drug resistance was a function of the degree of resistance a trypanosome strain has developed. And indeed Fussganger and Bauer, (1960) and Riou,

(1976) induced drug resistance of 4 - 5 folds and later reported complete loss of the resistance after several syringe passages in the absence of drug.

It was of interest to note that the degree of resistance fell progressively during syringe but not in the cyclic passages. Under field situation, Hawking, (1963b) proposed that loss of resistance could be due to overgrowth of the less resistant individuals in the population which multiply more rapidly than the resistant ones. Working with T. equiperdum, Cantrell, (1956) observed that the drug sensitive strain was highly virulent and outgrows the less virulent drug-resistant organisms. Similar observations have been reported to account for loss of drug resistance in the malaria parasites (Peters, 1970).

Altered drug uptake, altered metabolism and inactivation of the drug are some of the basic mechanisms believed to be responsible for the development of drug resistance in any living organism (Zakrzewski, 1973). Indeed an alteration in the plasma membrane or enzymes affecting active transport of the drug may also affect the uptake of several other vital substrates needed by the parasite.

Altered metabolism leading to alternative enzyme pathways may be in favour of a less efficient utilization of certain substrates and this in turn leads to reduced growth rate in the resistant parasites. In fact Williamson, (1953) observed that drug resistant T. rhodesiense differed markedly from the normal parasite

strain with respect to utilization of glycerol, pyruvate, glutamate or succinate but has increased ability to utilize citrate and lactate. The appearance of enzyme systems that inactivate the drug may also degrade some vital protein or substrates required by the parasites leading to slow growth of the parasites concerned. In natural infection, the host's immune system would be expected to destroy quite a number of parasites and the slow dividing resistant parasites are targets for such antibody destruction (Hajduk, 1979).

Based on the observations of the above workers, syringe passages performed at short intervals would favour the selection and concentration of less resistant trypanosomes. Whereas for cyclic transmissions, the process of infecting flies is usually delayed until the infection in the animals has reached a parasitaemic peak. It would be expected that during this peak parasitaemia, even the less virulent, resistant, population will have multiplied several folds. The flies therefore will maintain and transmit infections of the original level of drug resistant trait.

6.2. T. congolense (IL 1180 Strain)

The Trypanosoma congolense strain used in these investigations was found to be highly sensitive to samorin. This strain would be controlled by drug treatment in the field using samorin at the recommended doses of 0.5 - 1 mg/kg bw.

There was no significant change in the infection rate when attempts were made to infect flies after a prophylactic bloodmeal or after maintaining flies with unestablished infection on drug-treated rabbits. Whereas samorin has both curative and prophylactic value on the vertebrate host against trypanosomiasis (Whiteside, 1962; Kirkby, 1964 and Stephen, 1970), it failed to protect the flies from the infection.

The prophylactic potential of samorin is reported to be in its ability to form depots at the site of injection and binding to protein and organ tissues (Hill and McFadzean, 1963 and Williamson, 1976). The depot is slowly and continuously released into the circulation where it exerts chemoprophylactic activity and this process is reported to last 4 - 6 months (Hill and McFadzean, 1963).

In the insect gut, the drug is possibly degraded such that by the time the fly ingests an infective bloodmeal (24 hours later) the concentration or the active ingredient of the drug in the gut would be too low to interfere with the trypanosome metabolism. The drug also failed to arrest the development of the recently ingested or unestablished infection. Indeed, following an infective feed, the fly had to wait for another 24 hours before feeding on a drug-treated bloodmeal. By this time most of the parasites will have transformed into the insect forms. The drug is less effective on the trypanosomes once they have transformed into vector forms (Hawking, 1963a).

Infected flies fed on regularly treated animals did not lose their infectivity but they all transmitted less virulent infections with a longer prepatent periods. It was however observed that a larger number of trypanosomes in the gut or proboscis wet films were immobile and virtually dead. In the Giemsa stained preparation the morphological defects observed were similar to those described in the bloodstream trypanosomes.

The degree of parasite damage was more severe on the trypanosomes from the flies fed through membrane on blood containing drug. Three flies fed through membrane failed to transmit the infection and when dissected two of them had no parasites in the proboscis but all had scanty trypanosomes in the gut. During artificial feeding, the original drug concentration in the blood would wholly be available to the flies. But, in the living animal much of the drug would be retained at the site of injection and tissues (Hill and McFadzean, 1963). This point explains why drug effects on trypanosomes from flies fed through membrane was more pronounced than on those from flies fed on drug-treated animals.

According to Buxton (1955), when tsetse flies infected with T. brucei feed on animals treated with arsenical drugs such as tryparsamide, the trypanosomes in the midgut were killed, but those already in the salivary glands were not destroyed. Van Hoof et al., (1937) reported reduced virulence of the parasites and or loss of ability to transmit the infection by tsetse flies fed on animals

treated with various trypanocides. Hawking, (1963a) fed tsetse flies artificially on bloodmeals containing drugs and observed that Berenil and Samorin destroyed most of the vector forms of trypanosomes. The results of this work agree with previous observations where bloodmeals containing drug never completely destroyed the trypanosome infections in the flies.

The reported reduction of virulence by the parasites from flies after the ingestion of drugs (van Hoof et al., 1937) and failure of the infected flies to exude the parasites at certain times during feeding (Buxton, 1955 and Otieno and Darji, 1979) may explain why some flies failed to transmit the infection after treatment.

This work however has shown that the drug exerts serious detrimental effects on the vector forms of trypanosomes. For the complete destruction of the infection in the flies higher doses may be required. In clinical use, Samorin at doses higher than 2 mg/kg bw is reported to be toxic to cattle (Fairclough, 1963). The results of this work suggest that further investigations should be done using membrane feeding to determine the right doses of samorin required to destroy all the infections in the tsetse fly. This knowledge would enable the wild caught flies to be used in the sterile insect technique after sterilisation and treatment with the correct dose of trypanocides.

It was interesting to note that infections arising from flies maintained for over 60 days on rabbits which received regular prophylactic treatment of Samorin required higher doses to achieve 50 and 90 percent cure. Following syringe passages in the presence of drug, it was observed that the degree of drug resistance developed faster and higher. Because of the frequent exposure of vector forms of trypanosomes to the drug in the flies, they acquired mechanisms needed for survival as shown by the higher doses they required.

To my knowledge, the induction of drug resistance to the vector forms of trypanosomes by continuous exposure of the flies to drug-treated bloodmeals has not been previously reported. However, Riou (1976) obtained drug-resistant T. cruzi by continuously subculturing them in progressively higher concentration of ethidium bromide. Recently, Jenni (personal communication) reported inducing drug resistance in T. brucei in culture forms. Indeed vector forms of trypanosomes are morphologically and biochemically closely related to the culture forms (Hoare, 1972 and Gutteridge and Coombs, 1977). The results of this work show that continuous exposure of vector forms of trypanosomes to Samorin leads to the development of drug resistance.

The actual mechanisms of development of drug resistance is not known. The administration of sublethal doses has been cited as an important factor for the development of drug resistance in the

field (Leach and Roberts, 1981). Glew et al., (1978) obtained P. falciparum resistant to quinine after seven serial syringe passages. Following a single large dose administration of pyrimethamine, Diggins, (1970) induced drug resistance of over 164 times in P. berghei and the strain remained resistant through 20 blood passages. Whiteside, (1963) produced a fourfold drug resistance in T. congolense by repeated treatment with sublethal doses of Berenil. In a separate experiment, Fussganger and Baur, (1960) administered a three course treatment of Berenil to cattle infected with T. congolense and obtained a 4-5 times increase in drug resistance.

According to Peters, (1970) the method of inducing drug resistance by serial syringe passages of the relapse infections after treatment favour the selection of parasite mutants having pre-existing drug resistant traits; but that continuous exposure to drug will favour parasite adaptation and or selection. Indeed the presence of drug resistant mutants in a drug sensitive trypanosome population has been reported by Hawking and Walker, (1966) after exposing a mixture of drug sensitive and resistant trypanosomes to different concentrations of Tryparsamide and obtained one drug resistant mutant in every 10^9 sensitive trypanosomes. Stephen, (1963a) observed that under field conditions, every trypanosome in a population has varying susceptibility to trypanocidal drugs. Homologous bacteria population arising from a clone was reported to display differences in their level of drug sensitivity (Yudkin, 1953).

Therefore, in the selection of the rare drug resistant mutants and propagating them, the chances of success are proportional to the size of the parasite population that are exposed to drug selection pressure. Indeed Ramakrishnan et al., (cited by Peters, 1970) and Martin and Arnold, (1968) induced resistance in Plasmodium parasites more rapidly when they treated mice at a higher parasitaemic peak than during early and low parasitaemic phase.

The importance of late treatment in the frequent appearance of relapse infections in T. brucei has been discussed by Jennings et al., (1980) and Jennings and Gray, (1983). In this investigation, treatments were delayed until the first parasitaemic peak and subinoculation was performed when the relapse infection had reached a high peak. The highly resistant parasites in a population are believed to grow more slowly than the less resistant ones (Hawking, 1963b and Cantrell, 1956) and late treatment and subinoculation would allow time for them to multiply several folds. The results of this work further support the idea that delayed treatment and subinoculation favour rapid selection and propagation of drug resistant trypanosomes.

It should be mentioned that the degree of drug resistance obtained following continuous exposure of the infected flies to the drug may be of practical importance under field conditions, as the curative dose of 1.38 mg/kg bw obtained could not be cured by the

recommended doses of 0.5 - 1 mg/kg bw, except when increased to 2 mg/kg bw (Leach and Roberts, 1981). In nature, the flies would not be expected to feed continuously on treated animals, since the game animals would offer alternative untreated bloodmeals. However, considering that a single exposure to drug may cause the developments of drug resistance of several folds (Diggins, 1970), the importance of this observation cannot be under-estimated in the field.

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Plate 1a. Drug resistant Trypanosoma congolense (Maruma Strain), before treatment. Showing normal trypanosomes (N) having nucleus (n) and kinetoplast (k).

N
8μm

Plate 1b. Drug sensitive Trypanosome congolense (1L 1180 strain), before treatment. Showing normal trypanosomes (N) having nucleus (n) and Kinetoplast (k).

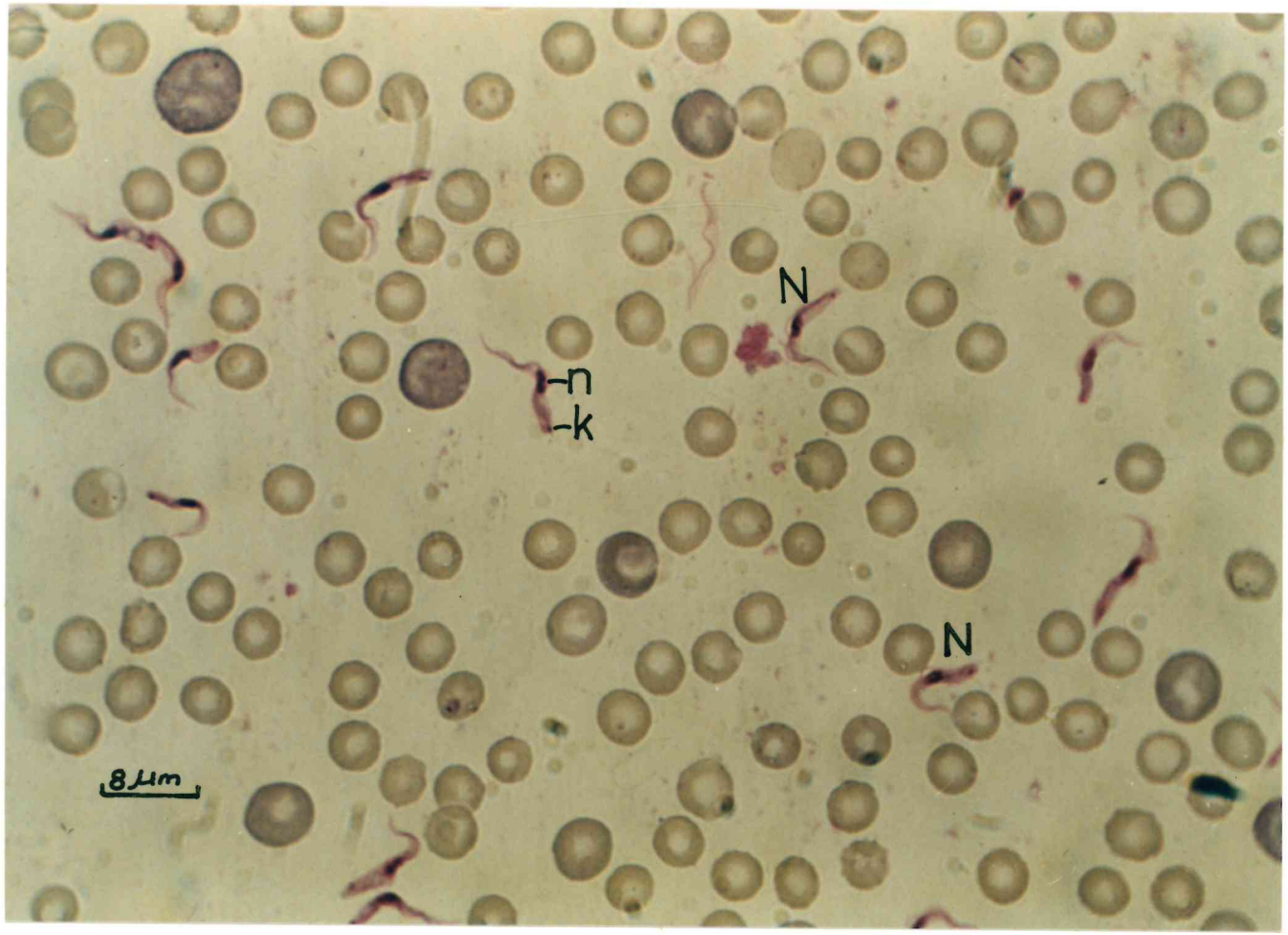

N
n
k
N
8 µm

Plate 2. Drug resistant trypanosomes 24 hours after treatment, showing aberrant trypanosomes (AB) which are swollen, granulated or completely lysed leaving a skeleton flagellum (fr). Several dyskinetoplastic (dk) and occasional normal (N) trypanosomes are also observed.

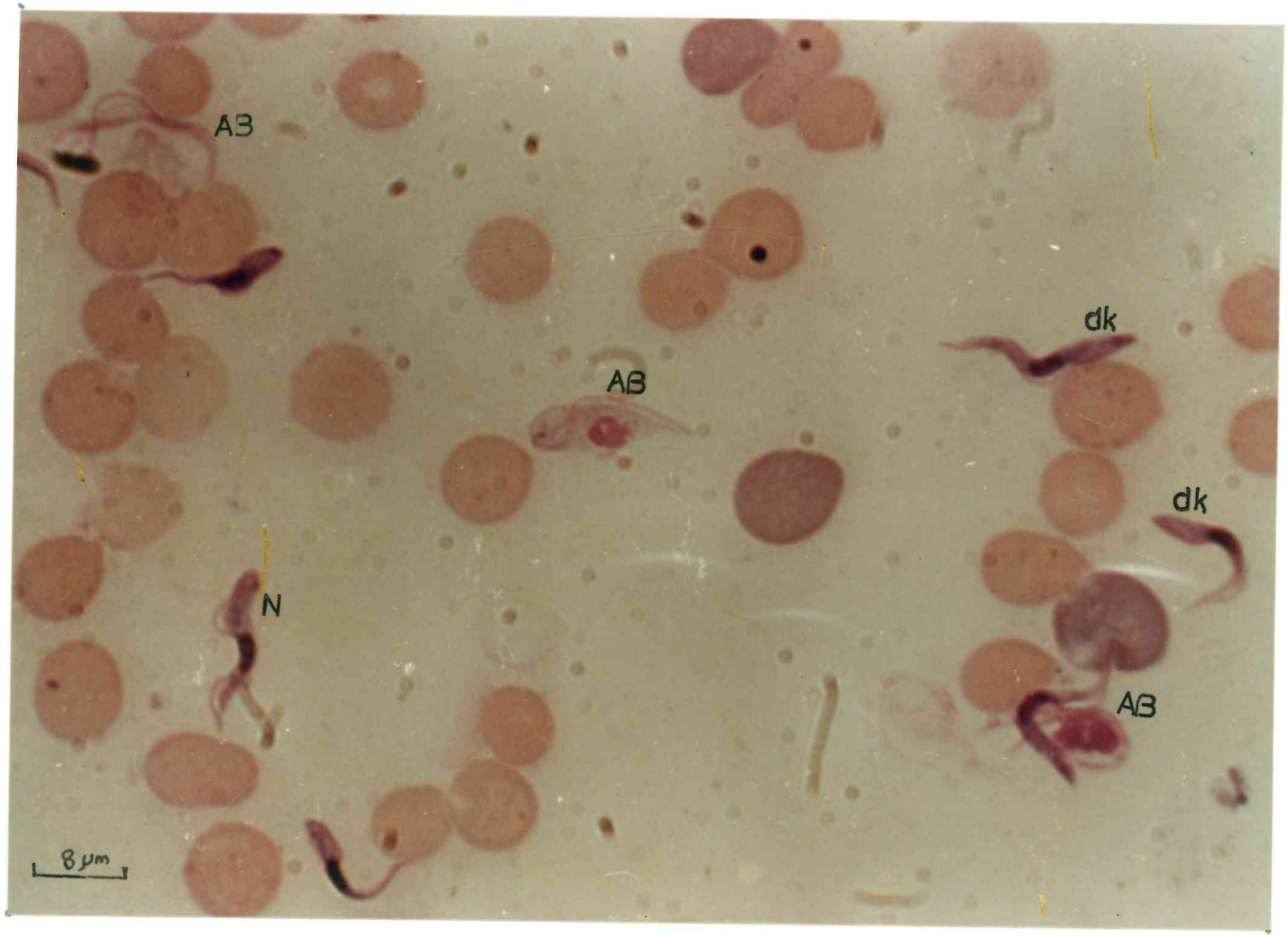

AB
AB
dk
dk
N
AB
8 µm

Plate 3. Drug sensitive trypanosomes 24 hours after treatment, showing aberrant trypanosomes (AB) which are swollen, and granulated. Several dyskinetoplastic (dk) and occasional normal (N) trypanomes are also observed.

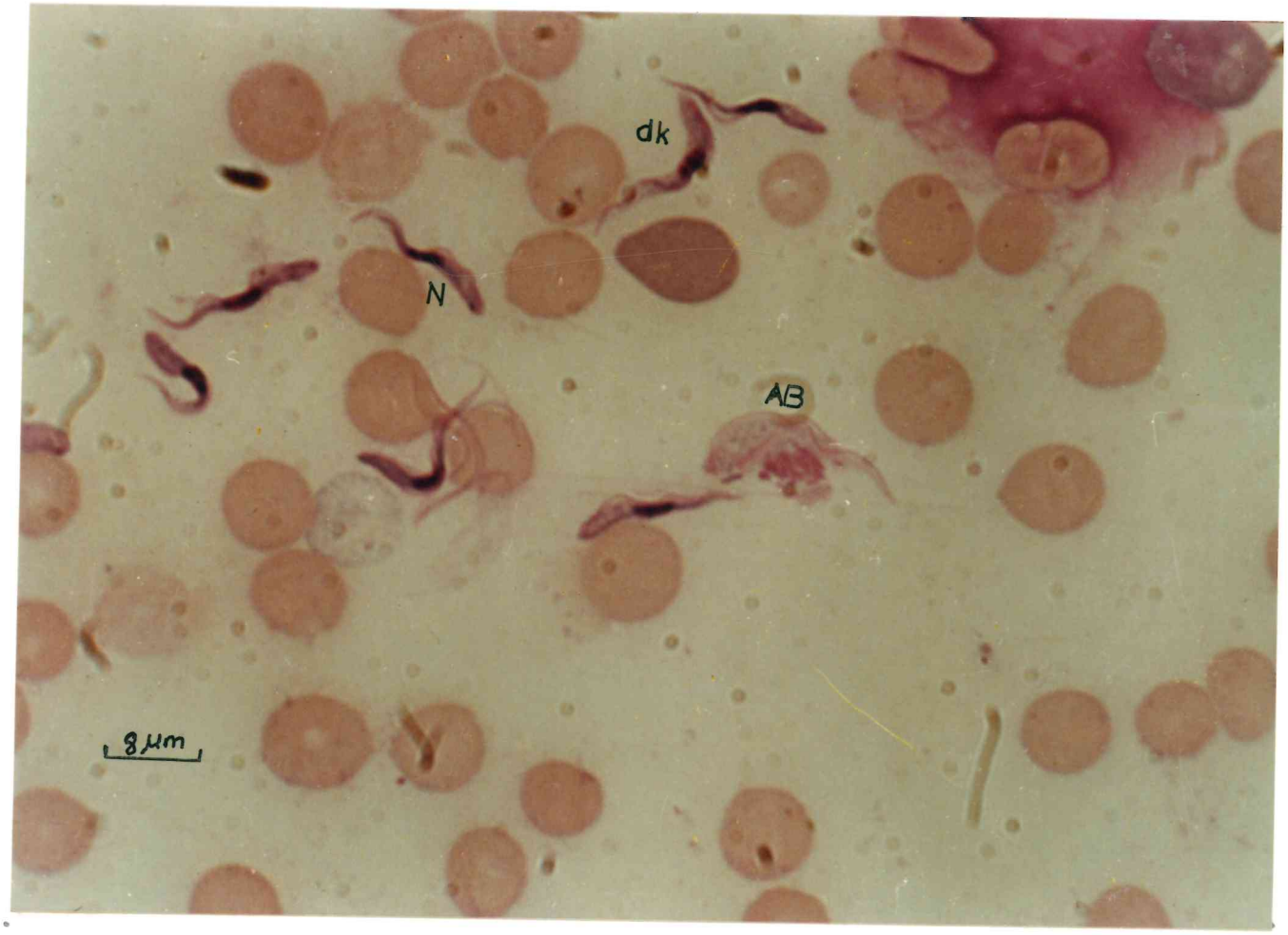

dk
N
AB
8 µm

Plate 4. Trypanomes from gut of flies, 24 hours after ingesting blood from a recently treated infected animal. Note the aberrant (AB) rounded comashaped and some normal (N) trypanosomes.

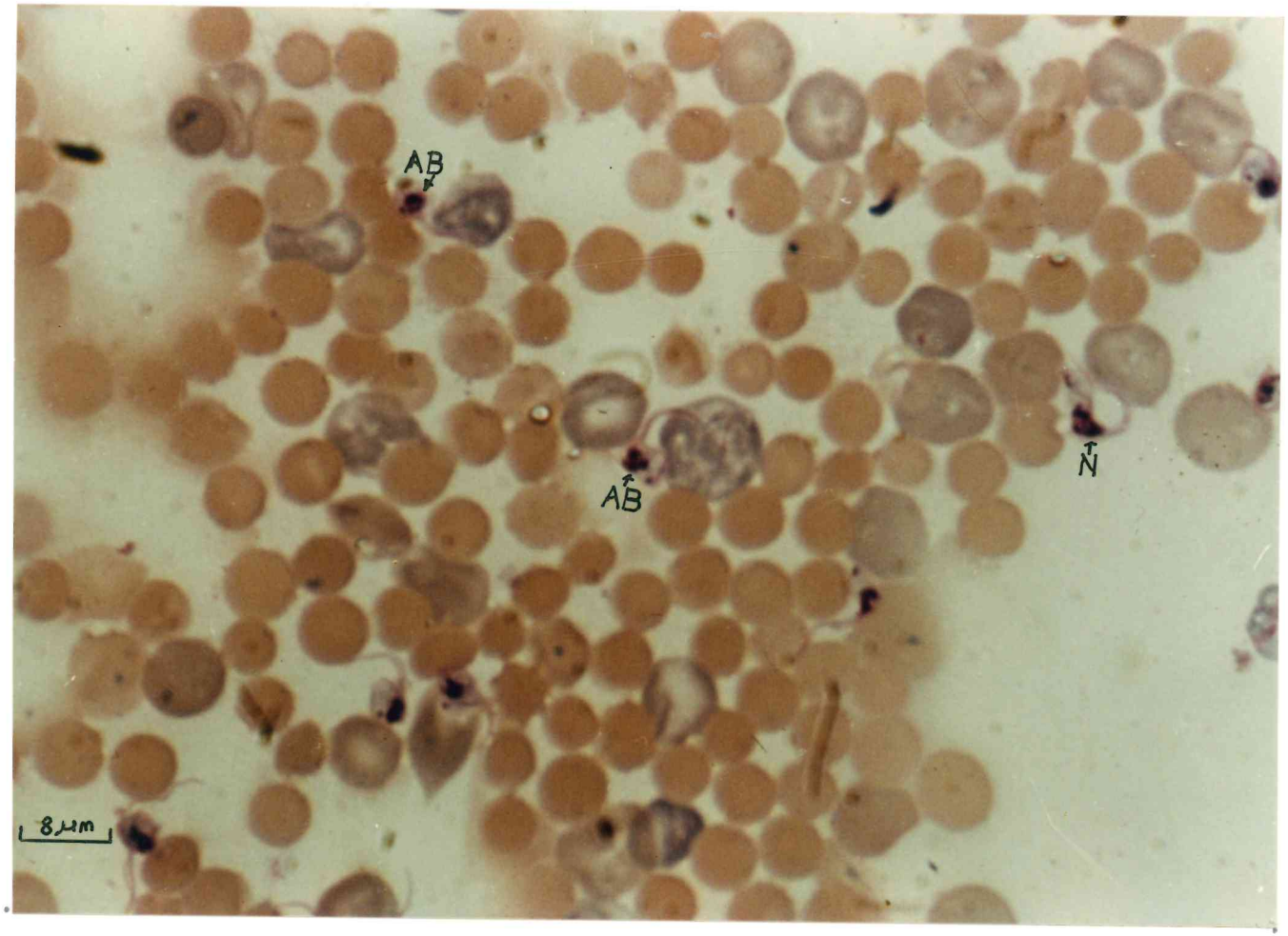

AB
AB
N
8 µm

Plate 4a. Trypanosomes from gut of flies - 24 hours after ingesting blood from infected untreated animal. Note the transformed (Tft), aberrant (AB) degenerating (dg) and some normal untransformed (Untf) trypanosomes.

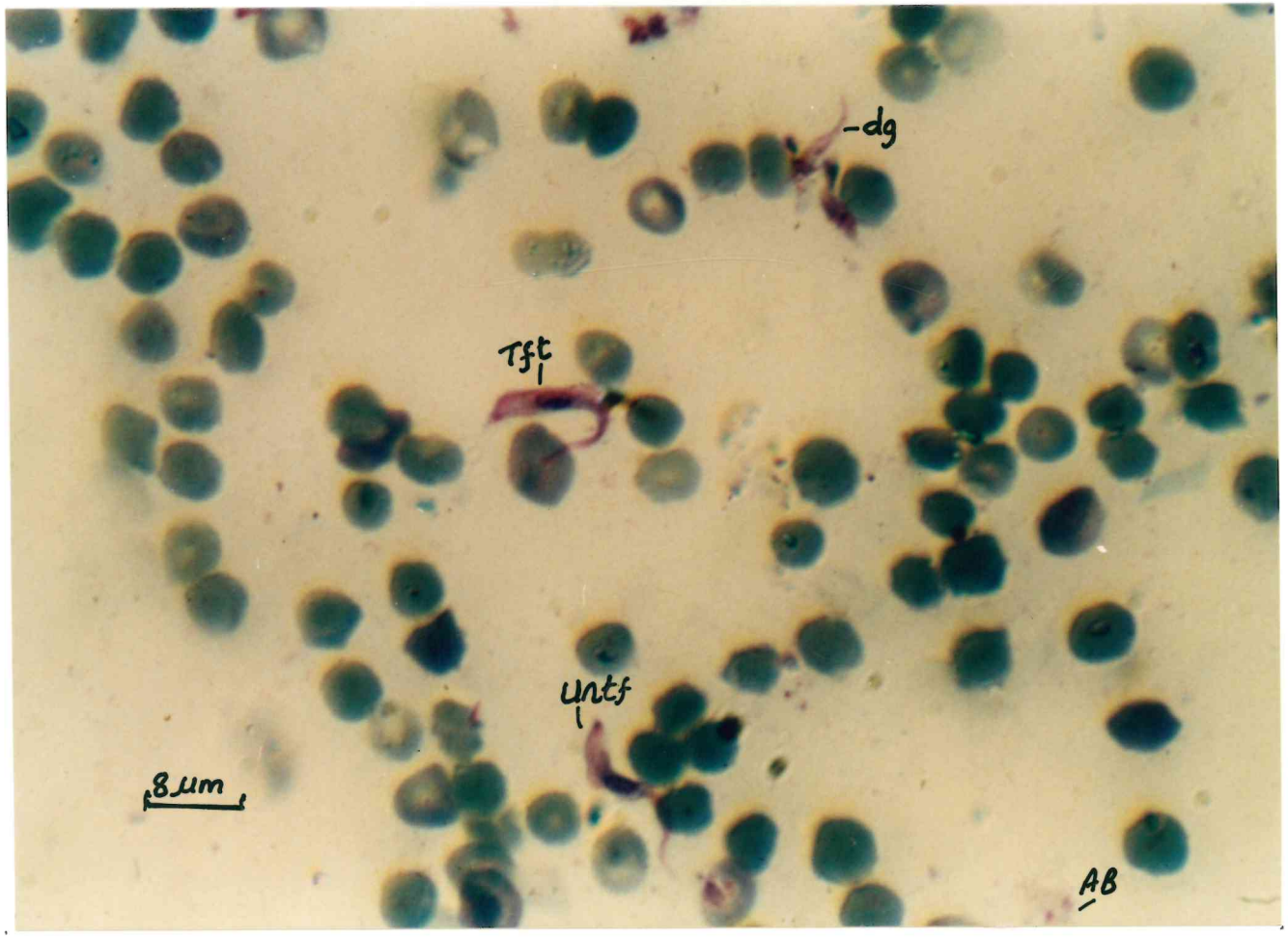

-dg
Tft
Untf
8 µm
AB

Plate 5a. Trypanosomes from gut of flies - 48 hours after ingesting blood from infected untreated animals - showing transformed (Tft), untransformed (Untf) and aberrant (AB) trypanosomes.

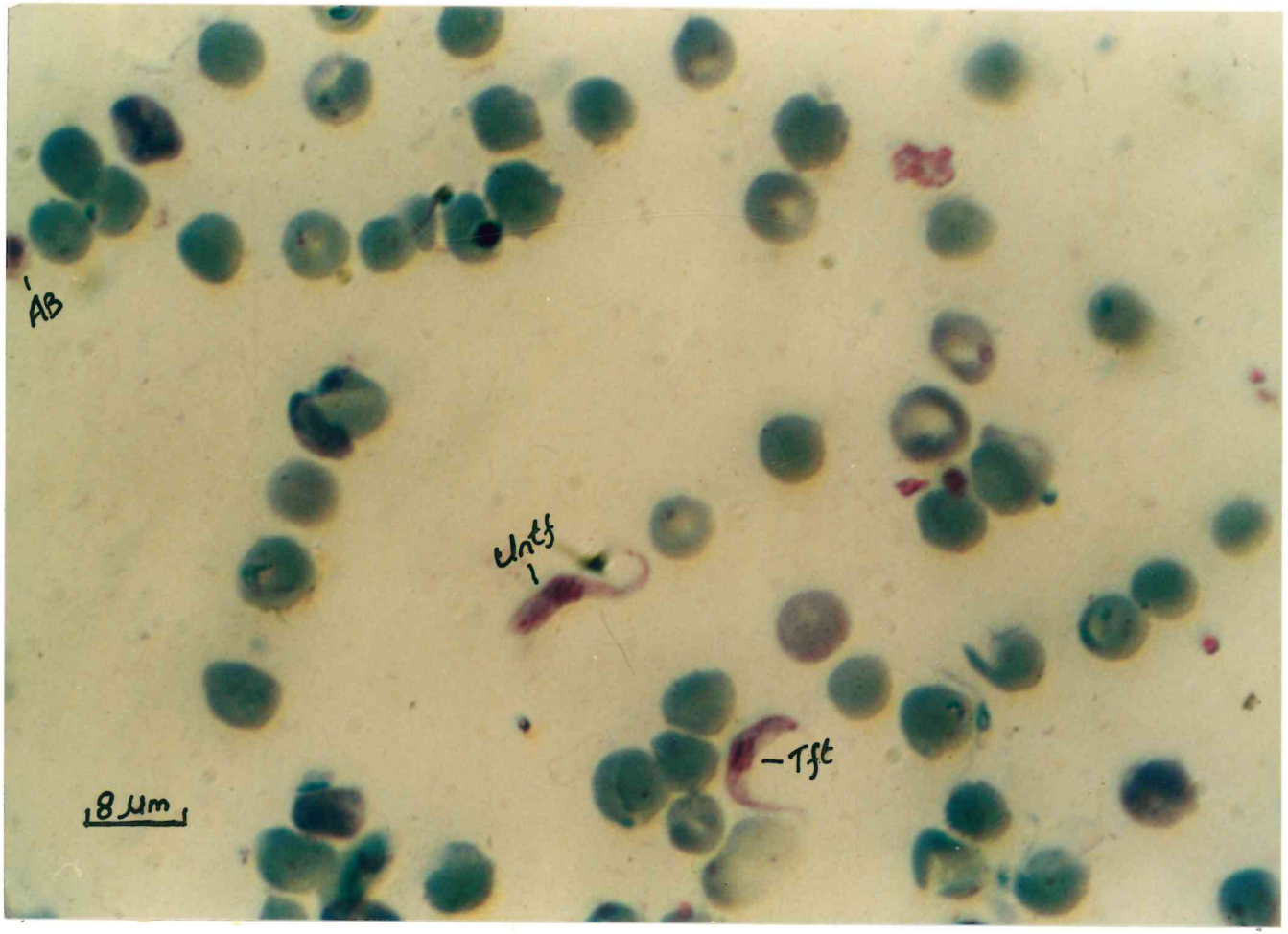

AB
Untf
Tft
8 µm

Plate 5. Trypanosomes from gut of flies 48 hours after ingesting blood from a recently treated infected animal. Note the presence of aberrant (AB) rounded, coma-shaped and some normal (N) trypanosomes.

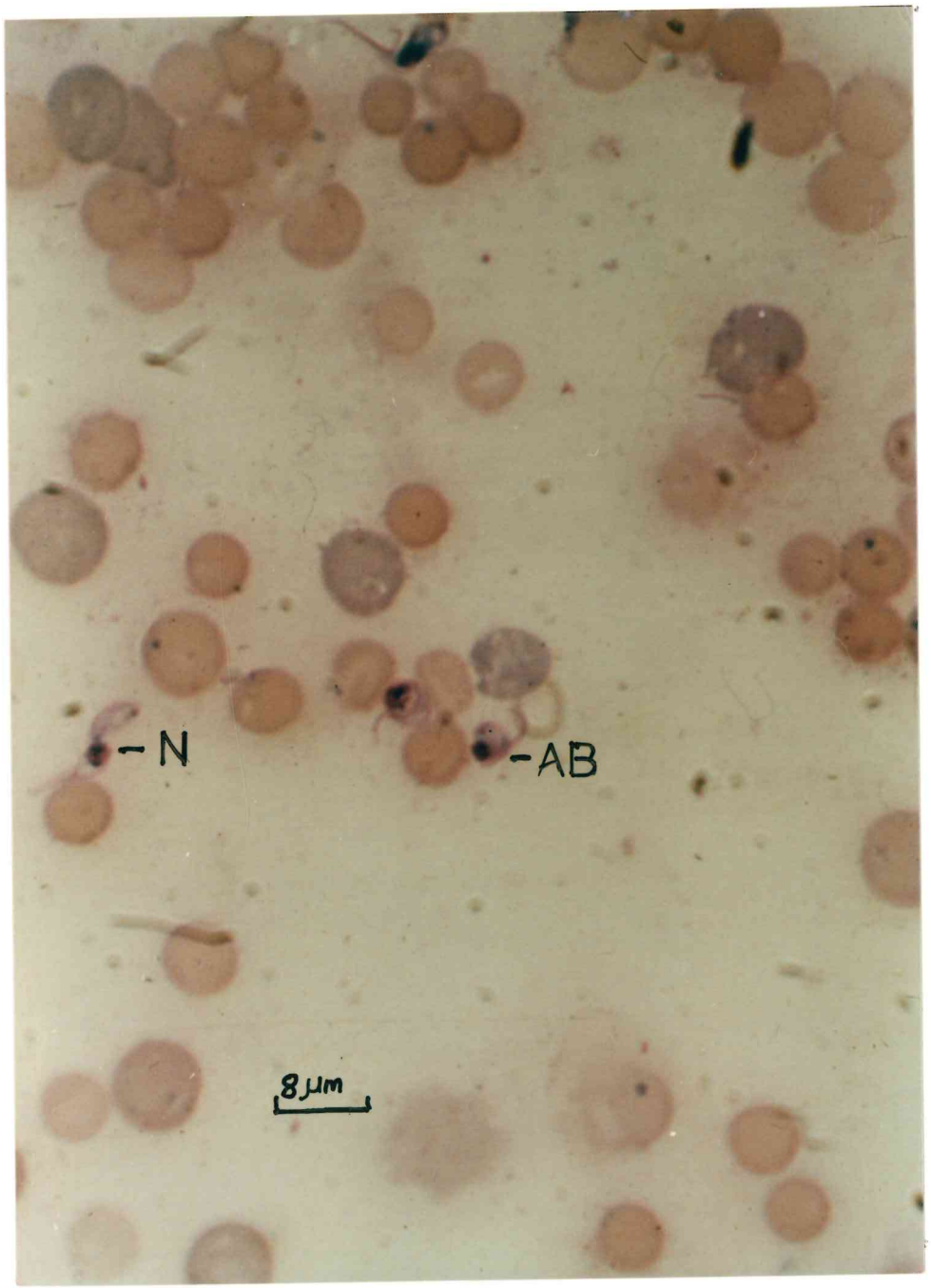

N
AB
8 µm

Plate 6. Mature gut infection showing normal trypanosomes (N) having prominent nucleus (n) and kinetoplast (k).

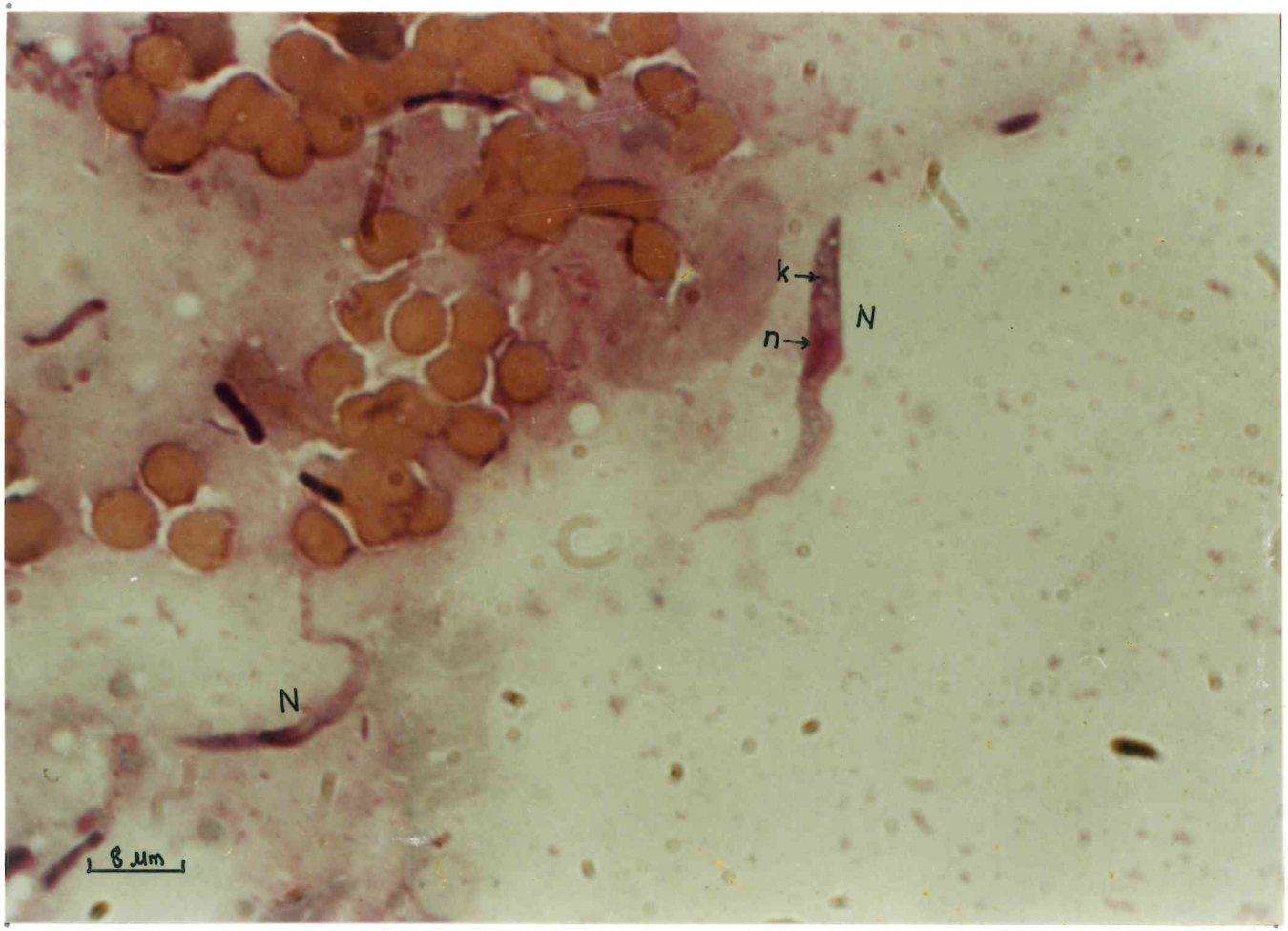

k
N
n
N
8 µm

Plate 7. Drug sensitive trypanosomes from the gut of infected flies 24 hours after artificial feeding on bloodmeal containing drug. Note the aberrant (AB) swollen and granulated trypanosomes, most parasites have no kinetoplast and their total number reduced.

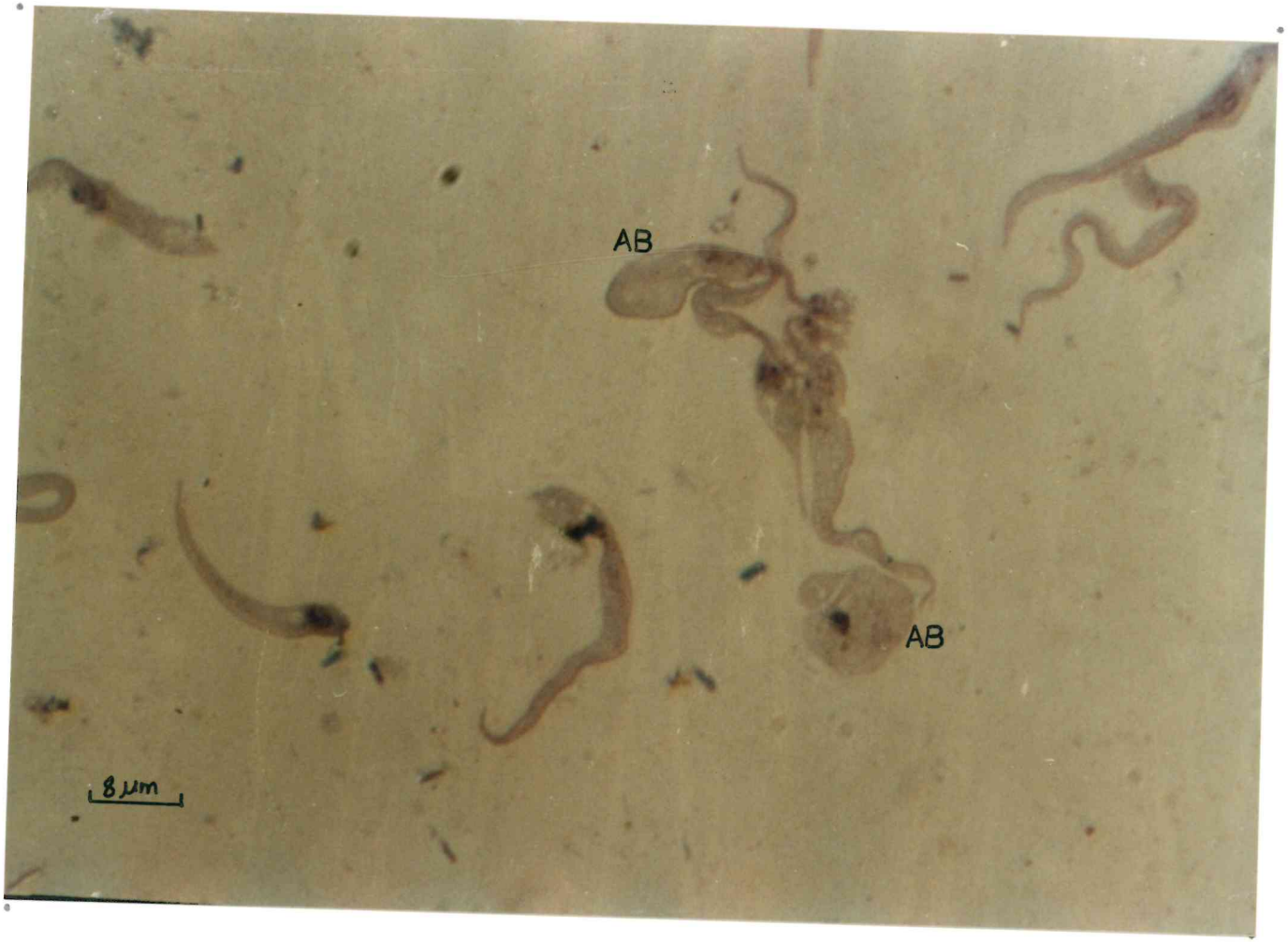

AB
AB
8 µm

Plate 8. Mature gut injection 24 hours after the infected fly fed on a recently treated animal. Note the numerous aberrant (AB) swollen, granulated and dyskinetoplastic trypanosomes. Some normal (N) trypanosomes are also observed.

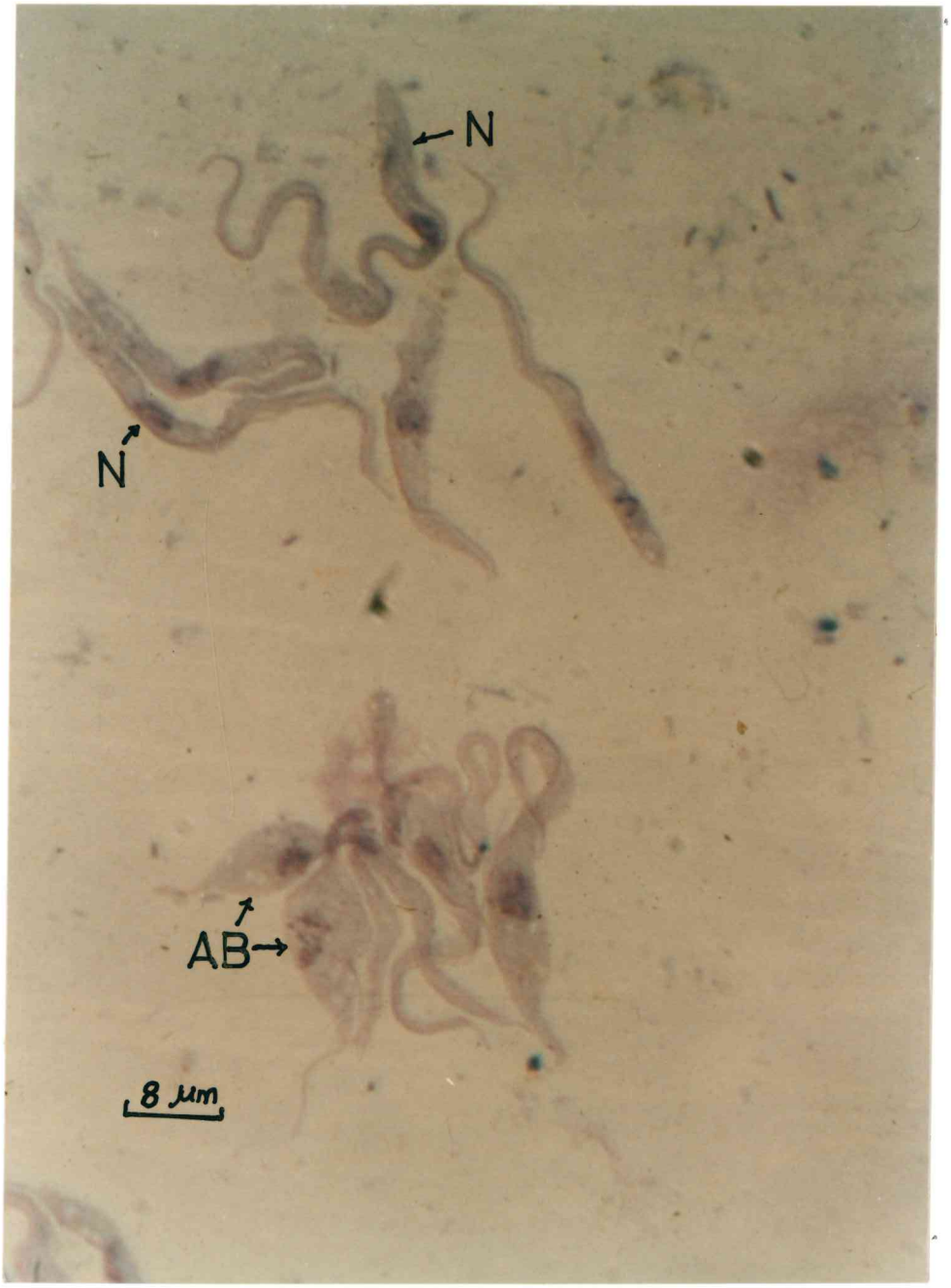
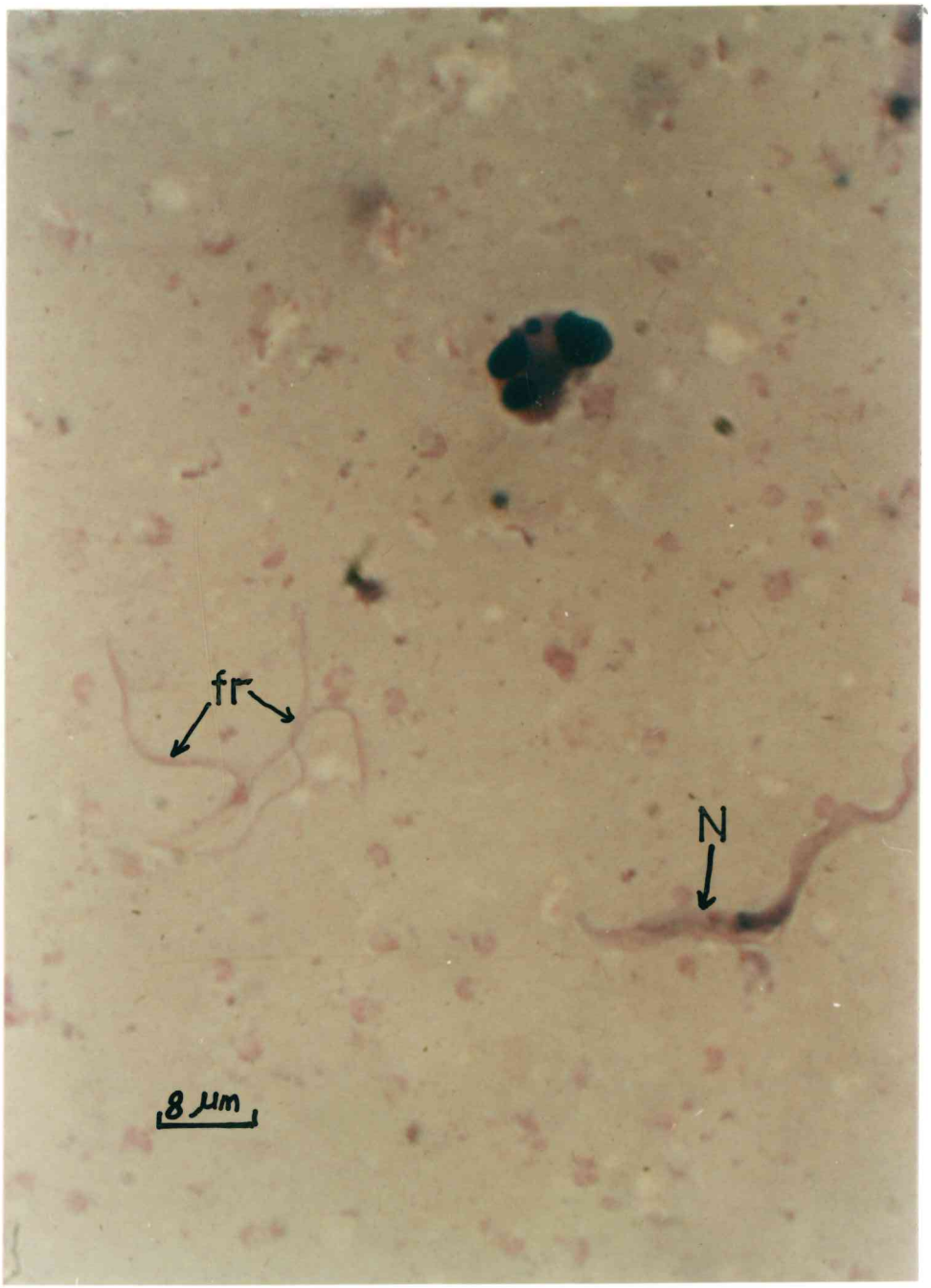

N
N
AB
8 µm

Plate 9. Mature gut infection 48 hours after the infected fly ingested bloodmeal containing drug, showing remnants of flagella (fr) from dead parasites, some normal (N) trypanosome and reduced number of trypanosomes in the gut in general.


fr
N
8 µm

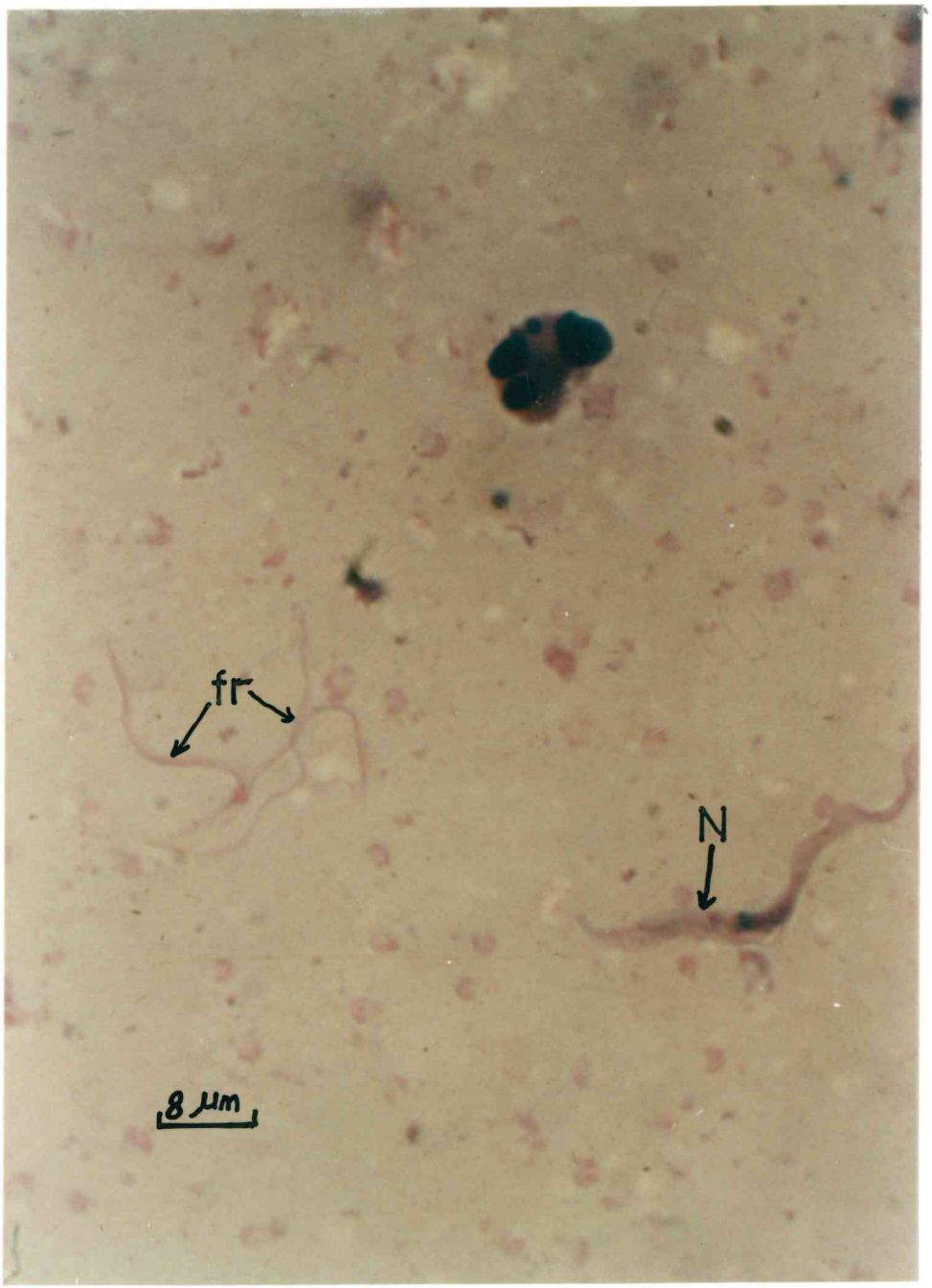

fr
N
8 µm

Plate 10. Salivary smear of an infected fly showing the normal metacyclic trypanosomes (NM).

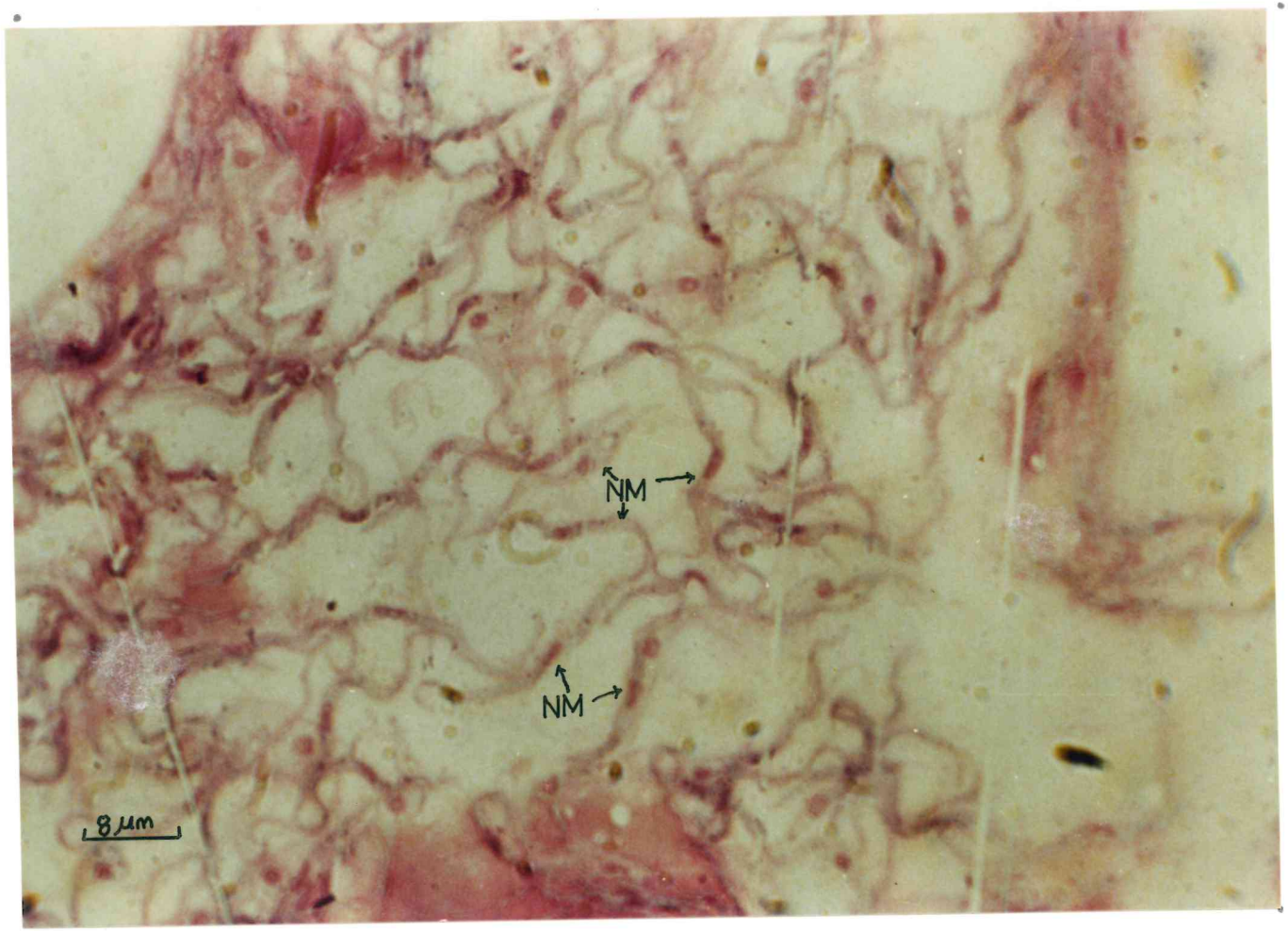

NM
NM
8 µm

Plate 11. Proboscis of an infected fly dissected 24 hours after the fly ingested blood from recently treated animal, showing swollen, vacuolated, aberrant metacyclic trypanosome (AB). Trypanosomes were very scanty indeed.


AB
8 µm